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Comprehensive structure-activity-relationship studies of sensory active compounds in licorice (*Glycyrrhiza glabra*)

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ABSTRACT

Licorice saponins, the main constituents of *Glycyrrhiza glabra* L. roots, are highly appreciated by the consumer for their pleasant sweet and long lasting licorice taste. The objective of the present study was to understand the molecular features that contribute to bitter, sweet and licorice sensation of licorice roots, and whether individual compounds elicit more than one of these sensations. Therefore, a sensomics approach was conducted, followed by purification of the compounds with highest sensory impact, and by synthesis as well as full characterization via HRESIMS, ESIMS/MS and 1D/2D-NMR experiments. This led to the discovery and structure determination of 28 sweet, bitter and licorice tasting key phytochemicals, including two unknown compounds. A combination of sensorial, cell-based and computational analysis revealed distinct structural features, such as spatial arrangement of functional groups in the triterpenoid E-ring, driving to different taste sensations and sweet receptor hTAS1R2/R3 stimulation.

1. Introduction

Licorice roots (generally *Glycyrrhiza glabra* L., Leguminosae) are main ingredients of diverse licorice sweets which are highly appreciated by consumers all over the world due to their sweet and typical long-lasting licorice impression (Kitagawa, 2002). Licorice triterpenoid saponins elicit a high sweetness, which is utilized in various products, e.g., licorice confectioneries, food additives, cosmetics and flavor additives for tobacco. The quantitative predominating phytochemical in *Glycyrrhiza* spp. is the monodesmosidic saponin glycyrrhizin with an 18β-glycyrrhetinic acid skeletal structure linked with a disaccharide, consisting of two β-D-glucuronic acids (Fenwick, Lutomski, & Nieman, 1990; Kitagawa, 2002). First sensory experiments showed that glycyrrhizin is 150 times sweeter than sucrose. Further triterpenoid saponins were isolated from licorice roots and semi-synthesized based on glycyrrhetinic acid with a relative sweetness of 70 for LS-A3 and of 941 for the β-D-glucuronic acid monosaccharide of 3-*O*-glycyrrhetinic acid (Kitagawa, 2002; Kitagawa, Hori, Sakagami, Hashiuchi, Yoshikawa, & Ren, 1993). Conversely, hydrolysis of the saponin revealed a complete elimination of the sweet taste (Esaki, Konishi, & Kamiya, 1978; Mizutani, Kuramoto, Tamura, Ohtake, Doi, Nakaura, et al., 1994).

Gustatory sense, especially sweet and bitter taste, plays a key role in food selection. The attractive sweet taste promises a carbohydrate-rich food, whereas unsavory bitter taste may cause a cautionary effect of possible poisonous food (Chandrashekar, Mueller, Hoon, Adler, Feng, Guo, et al., 2000; Nissim, Dagan-Wiener, & Niv, 2017). The hTAS1R2/hTAS1R3 sweet taste receptor heterodimer possesses a broad receptive range, enabled by several different active sites, to detect diverse sweet tasting compounds, such as mono-, disaccharides, sweet D-amino acids, sweet proteins, glucosides as steviol glucosides and synthetic non-nutritive sweeteners (Behrens, Meyerhof, Hellfritsch, & Hofmann, 2011; Hellfritsch, Brockhoff, Stähler, Meyerhof, & Hofmann, 2012; Morini, Bassoli, & Temussi, 2005). In contrast to sweet taste, bitter taste in human is mediated by about 25 GPCRs, (G protein-coupled

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receptors), namely the hTAS2R receptor family. Members of this family often detect a wide range of bitter molecules, in order to recognize many bitter compounds with a small number of receptors (Chandrashekar, et al., 2000; Matsunami, Montmayeur, & Buck, 2000; Meyerhof, Batram, Kuhn, Brockhoff, Chudoba, Bufe, et al., 2010), as summarized in BitterDB database (Dagan-Wiener, Di Pizio, Nissim, Bahia, Dubovski, Margulis, et al., 2018). Some compounds such as certain artificial sweeteners can interact with both the sweet receptor heterodimer hTAS1R2/hTAS1R3 and with bitter taste receptors, inducing a sweet taste with a bitter off-taste (Hellfritsch, Brockhoff, Stähler, Meyerhof, & Hofmann, 2012; Kuhn, Bufe, Winnig, Hofmann, Frank, Behrens, et al., 2004). Investigations of the signaling pathway of the human sweet taste receptor after stimulation with different artificial sweeteners and the licorice saponin glycyrrhizin showed an activation of different patterns of intracellular signals (Nakagawa, Nagasawa, Mogami, Lohse, Ninomiya, & Kojima, 2013; Ohtsu, Nakagawa, Nagasawa, Takeda, Arakawa, & Kojima, 2014). However, nothing is known about molecular mechanisms of licorice impression at receptor level.

As most publications are dealing with the pharmacological impact of the licorice root, systematic studies on the non-volatile sensometabolome of *G. glabra* are lacking. In particular, all investigations of sensory active compounds correspond to sweet taste, whereas the characteristic licorice impression has not been much of a topic in research so far (Fenwick, Lutomski, & Nieman, 1990; Kitagawa, 2002). For decoding the key compounds responsible for the typical taste signature of a specific food or food product (e.g., thermally generated bitter compounds, bitter off-tastants of carrots, sensory active compounds in roasted cocoa nibs and asparagus) the so-called taste dilution analysis (TDA) was designed (Dawid & Hofmann, 2012a, 2012b; Frank, Ottinger, & Hofmann, 2001; Stark & Hofmann, 2005).

The object of the present investigation is the isolation and structure determination by means of UV, ESIMS/MS, HRESIMS, 1D/2D-NMR as well as sensory evaluation of the compounds isolated from European licorice root (*Glycyrrhiza glabra* L.). Molecular features that contribute to sweet, bitter and licorice sensation are elucidated using a combination of instrumental analytics, psychophysical studies, cell-based receptor assays and computational docking experiments.

2. Materials and methods

2.1. Chemicals

The following compounds were purchased from listed sources given in parenthesis: dimethylformamide, formic acid, and potassium hydroxide (Merck, Darmstadt, Germany), glycyrrhizin ammoniacal (Extrasynthése, Genay, France), methyl iodide and trifluoracetic acid (Sigma-Aldrich, Steinheim, Germany). Deuterated solvent (pyridine-d_5) for NMR-spectroscopy was obtained from Euriso-Top (Gif-Sur-Yvette, France). Solvents (acetonitrile and methanol) were of HPLC grade (Mallinckrodt Baker, Griesheim, Germany). Ultrapure water for HPLC separation was purified by means of a Milli-Q® Water Advantage A 10 water system (18 MΩ; Millipore, Molsheim, France). For sensory analyses bottled water (Evian, Évian-les-Bains, France) was adjusted to pH 5.5 with diluted formic acid (1% formic acid in Evian). All further investigated compounds were isolated from dried peeled licorice roots Radix Liquiritiae mundat conc PH. EUR. 6.0 (Caesar & Loretz GmbH, Hilden, Germany). Glycyrrhizin derivatives glycyrrhizin-30-methylester (**20**), glycyrrhizin-6́-methylester (**21**), glycyrrhizin-6́-methylester (**22**), glycyrrhizin-6́,6́-dimethyl ester (**23**) and 11-hydroxy-glycyrrhizin (**27**) were synthesized similar to the procedures of Saito, Furumoto, Ochiai, Hosono, Hoshino, Haraguchi, et al. (1996). Synthetic procedures as well as spectroscopic data are listed in the **Supplementary Material**.

2.2. Instruments

Optical rotations were measured on a P-2000 polarimeter (Jasco, Groß-Umstadt, Germany) at 25 °C. One and two dimensional nuclear magnetic resonance (NMR) spectra were acquired on a Bruker Avance III^{TM} 500 MHz spectrometer (Bruker, Rheinstetten, Germany). Chemical shifts are reported in parts per million, relative to the solvent signals. Electrospray ionization tandem mass spectrometry (ESIMS/MS) in positive mode was carried out by acquisition of mass and product ion spectra on an API 4000^{TM} QTrap mass spectrometer (Sciex, Darmstadt, Germany). Accurate mass determination by high-resolution electrospray ionization mass spectrometry (HRESIMS) was achieved by an Acquity UPLC® core system (Waters UK Ltd., Manchester, UK) connected to a SYNAPT^{TM} G2 HDMS spectrometer (Waters) operating in positive and negative electrospray ionization (ESI) mode. Sugar identity determination was carried out after hydrolysis of saponins (**Supplementary Material**) by ion chromatography using an ICS-2500 system (Dionex, Idstein, Germany). For isolation of saponins in higher yields (upscale for receptor studies) a preparative MPLC Sepacore® system (Büchi, Flawil, Switzerland) was used. Preparative separation of MPLC fractions was carried out on an SD-1 purification system (Varian, Middelburg, Netherlands). Preparative and semi-preparative separations as well as analytical HPLC analysis were conducted on an LC-2000Plus system (Jasco, Groß-Umstadt, Germany) using Nucleodur® C18 Pyramid, 5 µm (Macherey Nagel, Düren, Germany) columns with dimensions of 250 × 21 mm, 250 × 10 mm and 250 × 4.6 mm.

2.3. Pre-separation of licorice root extract by means of solid phase extraction

Lyophilized licorice root extract (39 g) as stated previously was portioned in aliquots (800 mg) (Schmid, Dawid, Peters, & Hofmann, 2018), dissolved in water (10 mL) and applied on top of Strata C18-E SPE cartridges (10 g/60 mL; Phenomenex, Aschaffenburg, Germany), which were preconditioned with methanol (100 mL) followed by water (100 mL) using a vacuum extraction box (J. T. Baker, Philipsburg, NJ). For sequential solvent extraction the cartridges were flushed with a sequence of methanol/water mixtures (60 mL, each) to give fraction A (0/100, v/v; 17.8 g yield), fraction B (10/90, v/v; 1.3 g), fraction C (20/80, v/v; 1.4 g), fraction D (30/70, v/v; 2.6 g), fraction E (40/60, v/v; 2.8 g), fraction F (50/50, v/v, 3.1 g), fraction G (60/40, v/v; 3.4 g), fraction H (70/30, v/v; 4.1 g), fraction I (80/20, v/v; 4.6 g), fraction J (90/10, v/v; 0.2 g) and fraction K (100/0, v/v; 0.6 g). Each fraction was collected individually, separated from solvent in vacuum and freeze-dried afterwards, yielding 11 fractions, which were analyzed by comparative profile sensory and analytical HPLC. (Figure S 1).

2.4. Isolation of key taste compounds in fraction G/H for TDA

Fraction G and H were combined due to their similar content and taste impact (Fig. 1), dissolved in acetonitrile/water (30/70, v/v; 900 mg/14 mL) and fractionated by preparative HPLC (Nucleodur® C18 Pyramid, Macherey-Nagel) operated with a flow rate of 24 mL/min. Chromatography was performed by using 0.1% formic acid in water (v/v) as solvent A and 0.1% formic acid in acetonitrile (v/v) as solvent B with the following gradient: 0 min, 30% B; 3 min, 30% B; 5 min, 35% B; 40 min, 38% B; 45 min, 38% B; 50 min, 100% B; 55 min, 100% B; 58 min, 30% B; 65 min, 30% B. The effluent was monitored by means of an evaporative light scattering detector (ELSD) and individually collected in several runs into 32 subfractions. The fractions were freed from solvent, lyophilized and evaluated by TDA (Fig. 1, A). Subsequent isolation of pure compounds for structure elucidation of tasting fractions was carried out by semipreparative HPLC. Chromatographic conditions are stated as **Supplementary Material**. Pure compounds were analyzed by analytical HPLC-ELSD, ESIMS, ESI-MS/MS, HRESIMS and 1D/2D-NMR spectroscopy as well compared with spectroscopic data recently published (Schmid, Dawid, Peters, & Hofmann, 2018). The following compounds could be isolated and characterized in fractions shown in brackets: glycyrrhizin (**8**, G/H-19, 1.88 g), glucoglycyrrhizin (**9**, G/H-

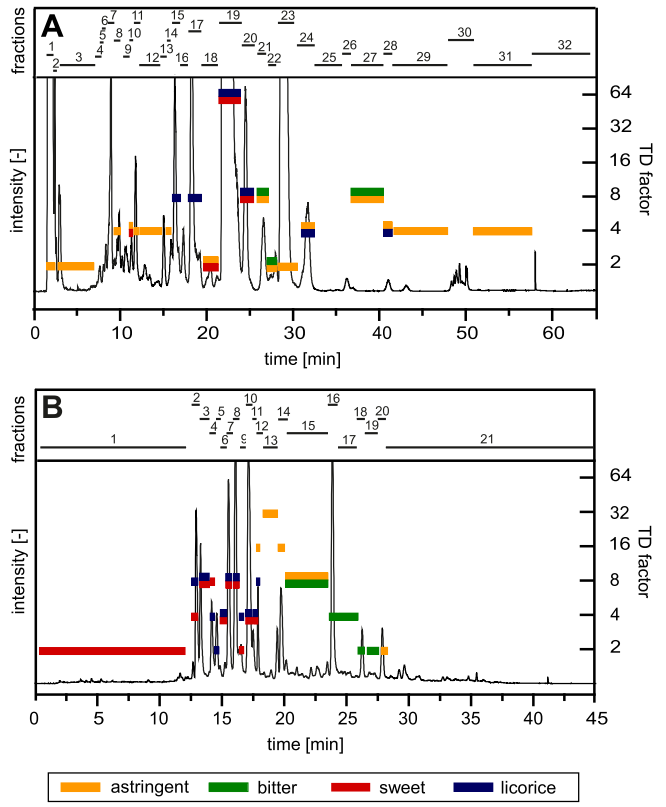


Fig. 1. Preparative RP-HPLC-UV chromatograms of SPE-fractions (A) G/H and (B) I prepared from methanolic licorice root extract and taste dilution (TD)-factors of 32 fractions collected from G/H (A) and 21 fractions collected from I (B).

20, 13 mg), rhaoglycyrrhizin (**12**, G/H-17, 32 mg), rhaoglucoglycyrrhizin (**13**, G/H-17, 10.6 mg), rhaogalactoglycyrrhizin (**14**, G/H-16, 3.7 mg), 24-hydroxy-glycyrrhizin (**16**, G/H-15, 36 mg), 24-hydroxy-glucoglycyrrhizin (**17**, G/H-15, 4.3 mg), 30-hydroxy-glycyrrhizin (**18**, G/H-21, 10.1 mg), glycyrrhizin-30-methanoate (**19**, G/H-21, 2.2 mg), 20α-glycyrrhizin (**3**, G/H-22, 178 mg), 20α-galacturonicacidglycyrrhizin (**4**, G/H-24, 26 mg), 20α-rhaoglycyrrhizin (**5**, G/H-21, 3.8 mg), 24-hydroxy-20α-glycyrrhizin (**6**, G/H-18, 10.6 mg). An unknown compound (yield: 12 mg) in fraction G/H-16 was characterized as the following:

(3β,20α)-20-carboxy-21-hydroxy-11-oxo-30-norolean-12-en-3-yl 2-*O*-β-D-apiofuranosyl-β-D-glucopyranosiduronic acid (21-hydroxy-20α-apioglycyrrhizin, **1**). White amorphous solid; $[\alpha]^{25}_{D}$ −17 (*c* 0.1, MeOH); ^{1}H and ^{13}C NMR (pyridine-d_5), see Table S 1; HRESIMS *m/z* 793.4041 $[M-H]^-$ (calcd for $C_{41}H_{61}O_{15}$, 793.4010, mass error: 3.9 ppm); ESIMS *m/z* 795 $[M+H]^+$; ESI-MS/MS (DP 40 V, CE 14 V) *m/z* 795 (7), 663 (34), 627 (5), 487 (12), 469 (100), 369 (3), 261 (7), 229 (2), 175 (5), 133 (8).

Sugar identity of the β-D-apiofuranoside moiety was confirmed after acidic hydrolysis by ion chromatography. Detailed information of hydrolysis and chromatographic conditions are given as **Supplementary Material**.

2.5. Isolation of key taste compounds from fraction I

A solution of fraction I (1.4 g/18 mL) in acetonitrile/water (30/70, v/v) was separated by preparative HPLC (Nucleodur® C18 Pyramid) operated at 21 mL/min with a gradient of 0 min, 30% B; 30 min, 70% B; 33 min, 100% B; 38 min, 100% B, 40 min, 30% B and 45 min and 30% B. For solvent A 0.1% formic acid in water (v/v) and for solvent B acetonitrile was used. The effluent was detected by a DAD detector at 200/250 nm and separated into 21 subfractions, I-1 to I-21, which were collected individually in several runs (Fig. 1). After evaporation of the solvent in a vacuum and freeze-drying, fractions I-1 to I-21 were dissolved in natural concentrations using sensory water and were subjected to TDA. Sweet and licorice tasting fraction I-5, I-7, I-8, I-10, I-12 and I-16 were further separated by means of (semi)-preparative HPLC. The following compounds could be isolated and characterized in fractions shown in brackets: araboglycyrrhizin (**10,** I-7–1, 7.2 mg), apioglycyrrhizin (**11,** I-8–1, 7.2 mg), glycyrrhetinic acid mono-glucuronide (**15,** I-8–4, 9.7 mg), 11-deoxo-glycyrrhizin (**24,** I-12–1, 78 mg), 11-deoxo-rhaoglycyrrhizin (**25,** I-8–3, 6.1 mg), 11-deoxo-24-hydroxy-glycyrrhizin (**26,** I-8–2, 8.6 mg), 11-deoxo-11,13-glycyrrhizindiene (**28,** I-10–3, 3.6 mg) and (**7,** I-16–3, 12 mg) as well as an unknown compound in fraction I-5–2 (**2**, yield: 8.3 mg). Chromatographic conditions used for the isolation of target compounds are given as **Supplementary Material**.

Fraction I-5–2: (3β,20α)-20-carboxy-11-oxo-30-norolean-12-en-3-yl 2-*O*-β-D-glucopyranosyl-β-D-glucopyranosiduronic acid (20α-glucoglycyrrhizin, **2**). White amorphous powder; $[\alpha]^{25}_{D}$ 52.5 (*c* 0.02, MeOH); ^{1}H and ^{13}C NMR (pyridine-d_5), see Table S2; HRESIMS *m/z* 807.4188 $[M-H]^-$ (calcd. for $C_{42}H_{63}O_{15}$, 807.4167, mass error: 2.6 ppm); ESIMS *m/z* 809 $[M+H]^+$; ESI-MS/MS (DP 20 V, CE 8 V) *m/z* 809 (6), 647 (19), 629 (3), 471 (18), 464 (12), 453 (100), 440 (7), 384 (4).

2.6. Sensory analyses

Eight female and six male panelists (24–34 years in age), who had given their consent to attend in the sensory tests of the current investigation and had additionally no known taste disorders, participated in this study. Each panelist was trained in weekly training sessions for at least one year in order to get familiarized with the taste language and methodologies used and to evaluate aqueous reference solutions of tastants (Dawid & Hofmann, 2012a; Scharbert, Holzmann, & Hofmann, 2004). Additionally, glycyrrhizin (0.2 mmol/L) was used for the licorice flavor impression. The sensory sessions were performed at 22–25 °C in an air-conditioned room as reported earlier (Stark & Hofmann, 2005). The pH-value of all samples was adjusted to 5.5 using trace amounts of formic acid. For precautionary reasons all fractions or isolated compounds were confirmed to be free of solvents, buffer or other impurities by analytical HPLC and 1D/2D-NMR spectroscopy (Dawid & Hofmann, 2012a). Detailed information of conducted psychophysical studies are summarized in **Supplementary Material**.

2.6.1. Taste profile analysis

In order to judge the intensity of sweet, sour, umami, salty, bitter, astringent and licorice taste quality on a linear scale from 0 (not detectable) up to 5 (very intense) for all comparative taste profile analyses, a glycyrrhizin solution (0.2 mM) in sensory water as a control, was handed to the trained sensory panel.

2.6.2. Comparative taste profile analysis

Licorice root extract (3 mg/mL) and aliquots of SPE fractions were dissolved in “natural” concentration ratios in sensory water and were rated regarding to the taste qualities sweet, sour, umami, salty, bitter, astringent and licorice on a linear scale from 0 to 5 with glycyrrhizin (0.5 mg/mL) as a reference.

2.6.3. Taste dilution analysis (TDA)

Preparative HPLC fractions of fraction G/H and I were aliquoted and dissolved in their “natural” ratios in sensory water (40 mL), then sequentially 1:2 diluted and presented to the trained sensory panel in order of increasing concentration. The panel was asked to evaluate the taste differences between the diluted fraction and blank (control, Evian, pH 5.5) as well as to assess the taste quality of each fraction. TD factor was defined, as reported earlier, as the dilution at which a taste difference between the serial dilution and the blank could just be detected (Frank, Ottinger, & Hofmann, 2001).

2.6.4. Taste recognition threshold Concentrations

Taste threshold concentrations of individual compounds were determined by means of three-alternative forced choice tests in increasing concentrations (5, 10, 20, 30, 40, 50, 60 and 70 μM for **1**, **2**, **4**–**28** as well as 15, 30, 60, 120, 240 and 480 μM for **3**). The individual taste recognition threshold was calculated as the geometric mean of the first missed and the last correctly identified concentration. The overall taste threshold was approximated by averaging of individual taste thresholds.

2.6.5. Psychometric Concentration–Response curves

Concentration range of test compounds presented to the sensory panel for concentration–response recordings was defined by its taste threshold values on the lower end and its maximum solubility (800 μM) on the higher end. For sweet and licorice impression independent sessions were performed. In a first set of experiments, panelists evaluated the sweet or licorice taste intensity of a series of 10 dilutions (10–600 μM) of the reference compound glycyrrhizin (**8**) on a free scale. Further individual sweet or licorice taste intensities were normalized to the highest sweet or licorice intensity, which was set to 10, and average taste intensities for each concentration were calculated over the whole panel as relative sweet or licorice intensities. In a second set of experiments, panelists were asked to separately judge the sweet or licorice taste intensity of increasing test compound concentration (5–800 μM) by cross-checking to glycyrrhizin (**8**, 40 μM) for compound concentrations 5–150 μM and glycyrrhizin (**8**, 400 μM) for compound concentrations 200–800 μM. Concentration–response curves with preliminary sweet suppression were carried out by pre-administration of *Gymnema sylvestre* extract (2.5 mL, extracted with 70/30 MeOH/H_2O, freed from solvent, lyophilized and afterwards a saturated solution of gymnema extract was diluted 1:25, v/v). Testing compounds were tasted as stated above. Subsequent concentration–response functions for each compound and taste modality were half-logarithmically plotted against saponin concentration by extrapolating the progression of the curve with nonlinear regression to the sigmoidal function $f(x) = \min+(\max-\min)/(1 + [x/EC_{50}]^{Hillslope})$ (OriginPro 2016G b9.3.226; OriginLab Corporation, Northampton, MA).

2.6.6. Crossmodal perception profile sensory analysis

For investigation of crossmodal interaction of sweet and licorice taste, three profile sensory analyses of sucralose (50 μM), 24-hydroxy-20α-glycyrrhizin (**6**, 100 μM) and a mixture of both were presented to a trained sensory panel. The three solutions were independently rated regarding the taste qualities sweet and licorice on a linear scale from 0 (not detectable) up to 5 (very intense).

2.7. Characterization of in vitro taste receptor responses to licorice saponins

To analyze the genuine taste properties, sweet/licorice and pure licorice acting licorice saponins were applied on a cell-based heterologous expression system. While human taste receptor heteromer hTAS1R2/hTAS1R3 was used for sweet taste, functional expression of human bitter taste receptors was carried out with hTAS2R gene variants previously used (Brockhoff, Behrens, Massarotti, Appendino, & Meyerhof, 2007; Brockhoff, Behrens, Roudnitzky, Appendino, Avonto, & Meyerhof, 2011; Slack, Brockhoff, Batram, Menzel, Sonnabend, Born, et al., 2010). Detailed information about cell transfection, induction and growth conditions is described in the **Supplementary Material**.

2.8. Modeling

A model of the human sweet taste receptor Venus Fly Trap (VFT) ligand binding domain was generated for the Uniprot sequence - Q8TE23 (The UniProt Consortium, 2016). The I-Tasser server was used to create models of the hT1R2 VFT using the specified templates PDB 5K5T and 1EWT. The model was prepared for docking with Maestro and Glide Grid Generation (Schrodinger tools 2020–1), 12 Å around a glutamate of a superimposed crystal-structure (PDB 1EWK). The docking protocol included Maestro Schrodinger 2020–1, Glide Extra Precision mode (XP), flexible ligand sampling, and Glide XP docking scores and was defined similarly to previous protocol for docking of large compounds to sweet taste receptor (Ben Shoshan-Galeczki & Niv, 2020). GRAS is a dataset of compounds Generally Recognized as Safe by FDA. The data set includes 98 compounds over 460 g/mol, and thus similar to the saponins studied here. Taste and odor descriptions of the GRAS compounds were obtained by data mining (annotations of taste thresholds of GRAS compounds by FEMA ID (https://www.femaflavor.org/flavor-library)) of Fenaroli's handbook of Flavor Ingredients (fifth edition) (Burdock, 2016).

The ligands were prepared for docking using LigPrep, retaining the specified chiral centers (Schrodinger tools 2020–1). The 3D figures of the ligand-receptor complexes were prepared with ChimeraX (Goddard, Huang, Meng, Pettersen, Couch, Morris, et al., 2018). Structure–Activity radial scope plot was prepared with the substrate-scope-plot git (https://github.com/duerrsimon/substrate-scope-plot).

3. Results and discussion

In order to get a comprehensive insight into the sweet, licorice and bitter acting compounds of licorice roots, the molecular sensory science (SENSOMICS) approach was applied on a methanol as well as methanol/water extract of licorice roots. Consequently, the following investigations were focused on the preparative isolation, structure elucidation of taste active compounds and subsequent sensory evaluations by means of taste thresholds, dose–response as well as receptor-based *in vitro* studies.

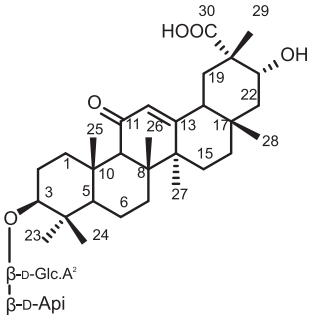
3.1. Taste profile analysis of licorice roots (Glycyrrhiza glabra)

First, a glycyrrhizin solution (0.5 mg/mL) was presented as reference substance to a trained sensory panel, who were asked to rate the intensity of the taste modalities sweet, licorice, bitter, salty, sour, umami and astringent on a linear 5-point intensity scale. Glycyrrhizin, which was described as 150 times sweeter than sucrose (Kitagawa, 2002; Kitagawa, Hori, Sakagami, Hashiuchi, Yoshikawa, & Ren, 1993), exhibited a strong sweet taste with a score of 2.9. The typical licorice-like impression was rated with the second highest score of 2.5, which was judged as a lingering long-lasting orosensation, followed by bitterness (0.6), astringency (0.3), sour (0.1), salty (0.1) and umami (0). To elucidate the licorice root taste, licorice root extract (3 mg/mL) was solubilized in sensory water (pH 5.5) to match the natural concentration of glycyrrhizin. Licorice root extract was sensorially evaluated by given taste descriptors on a linear 5-point scale using glycyrrhizin as reference compound. The extract showed a lower sweet (-0.7) intensity but a higher bitter (+1.0) taste, in contrast to the licorice sensation, which showed almost the same intensity (–0.1) as the glycyrrhizin reference. The higher bitter taste intensity could be explained by bitter tasting phenolic compounds in licorice, and might be a reason for the lower sweet intensity compared to glycyrrhizin (Green, Lim, Osterhoff, Blacher, & Nachtigal, 2010; Jakob, Katharina, & Gerhard, 2011). Therefore, the following experiments were focused on the identification of the compounds imparting the sweet and the typical licorice-like orosensation.

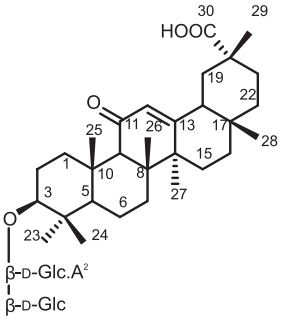
3.2. Solid-Phase extraction (SPE) of licorice root extract

In order to gain a first insight into the hydrophobicity of the taste-active components, licorice root extract was separated in 11 fractions (A–K) by reversed-phase SPE. Highest yields were found for the polar fraction A (45.8% yield) and more hydrophobic fractions F to H accounting for yields of 8.1 to 10.5%. Fractions B to E and I to K were

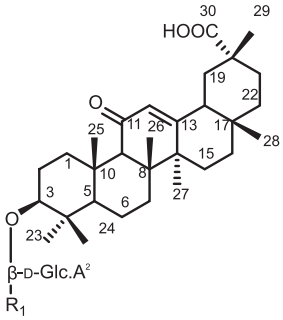
obtained in comparatively low yields of 0.4 to 7.1%. To evaluate their taste impact, all isolated fractions (A–K) were comparatively analyzed in their natural concentration by means of taste profile analysis with given taste descriptors using glycyrrhizin as reference. Fractions G and H showed the highest sweet (1.8/0.8) and licorice intensities (2.0/1.0) followed by fraction F (sweet: 0.8, licorice: 0.8) and fraction I (sweet: 0.4, licorice: 0.5). Analytical HPLC at typical licorice saponin absorption wavelength of 250 nm of the fractions (A–K) revealed fractions G and H to be substantially equivalent bearing high amounts of saponins (retention time (RT) 40–50 min, glycyrrhizin RT 42.8 min). Thus, the following investigations were focused on combined fractions G and H (G/H) as well as fraction I, fitting to the highest sweet and licorice taste



21-hydroxy-20α-apioglycyrrhizin (**1**)



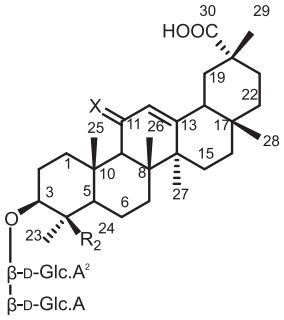
20α-gluco-glycyrrhizin (**2**)



20α-glycyrrhizin (**3**) **R**$_1$ = ß-D-Glc.A

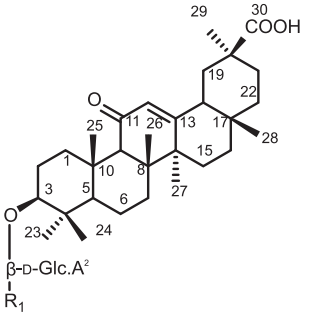
20α-galacturonicacid-glycyrrhizin (**4**) **R**$_1$ = ß-D-Gal.A

20α-rhao-glycyrrhizin (**5**) **R**$_1$ = ß-D-Glc.A-α-L-Rha



24-hydroxy-20α-glycyrrhizin (**6**) **R**$_2$ = CH_2OH **X** = O

11-deoxo-20α-glycyrrhizin (**7**) **R**$_2$ = CH_3 **X** = H, H



glycyrrhizin (**8**) **R**$_1$ = ß-D-Glc.A

glucoglycyrrhizin (**9**) **R**$_1$ = ß-D-Glc

araboglycyrrhizin (**10**) **R**$_1$ = ß-D-Ara

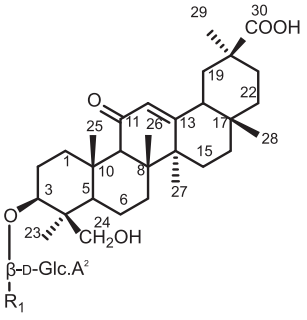
apioglycyrrhizin (**11**) **R**$_1$ = ß-D-Api

rhaoglycyrrhizin (**12**) **R**$_1$ = ß-D-Glc.A²-α-L-Rha

rhaoglucoglycyrrhizin (**13**) **R**$_1$ = ß-D-Glc²-α-L-Rha

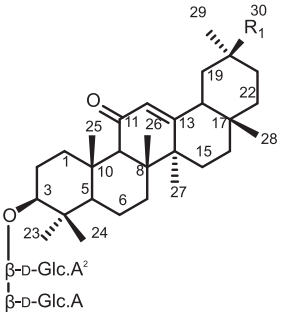
rhaogalactoglycyrrhizin (**14**) **R**$_1$ = ß-D-Gal²-α-L-Rha

glycyrrhetinic acid mono-glucuronide (**15**) **R**$_1$ = H



24-hydroxy-glycyrrhizin (**16**) **R**$_1$ = ß-D-Glc.A

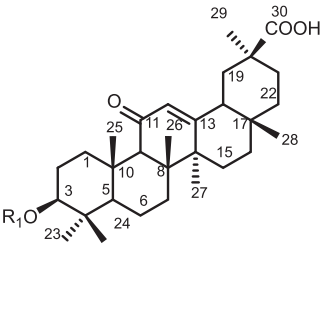
24-hydroxy-gluco-glycyrrhizin (**17**) **R**$_1$ = ß-D-Glc



30-hydroxy-glycyrrhizin (**18**) **R**$_1$ = CH_2OH

glycyrrhizin-30-methanoate (**19**) **R**$_1$ = CH_2OCOH

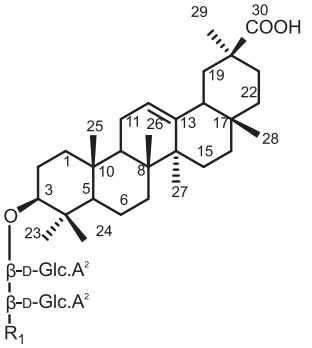
glycyrrhizin-30-methylester* (**20**) **R**$_1$ = COOMe



glycyrrhizin-6´-methylester* (**21**) **R**$_1$ = ß-D-Glc.A(Me)²-ß-D-Glc.A

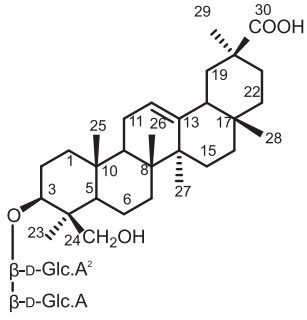
glycyrrhizin-6´´-methylester* (**22**) **R**$_1$ = ß-D-Glc.A²-ß-D-Glc.A(Me)

glycyrrhizin-6´,6´´-dimethylester* (**23**) **R**$_1$ = ß-D-Glc.A(Me)²-ß-D-Glc.A(Me)

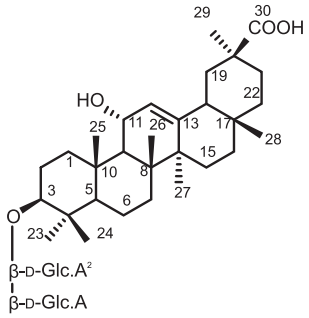


11-deoxo-glycyrrhizin (**24**) **R**$_1$ = H

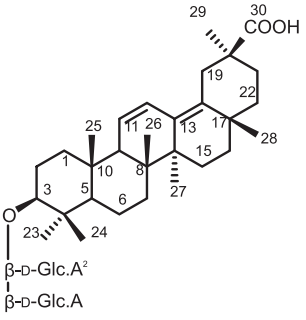
11-deoxo-rhao-glycyrrhizin (**25**) **R**$_1$ = α-L-Rha



11-deoxo-24-hydroxy-glycyrrhizin (**26**)



11-hydroxy-glycyrrhizin* (**27**)



11-deoxo-11,13-glycyrrhizindiene (**28**)

Fig. 2. Chemical structures and trivial names of isolated licorice root (1–19, 24–26, 28) and synthesized* (20–23, 27) saponins.

impressions.

3.3. Taste dilution analysis of fraction G/H

To trace the compounds responsible for sweet and licorice impression, the taste dilution analysis was applied on fraction G/H. Therefore, separation was achieved by preparative RP-HPLC coupled to an ELSD to give a total of 32 fractions (Fig. 1, A). After lyophilization the residues of each fraction were dissolved in equal amounts of water (pH 5.5), stepwise diluted 1:1 and then presented in order of ascending concentrations to a trained sensory panel, who were asked to determine taste dilution (TD)-factors and to evaluate the taste of each dilution step. Fraction G/H-19 was rated with the highest TD-factor of 64. This was followed by fractions G/H-15, G/H-17 and G/H-20, which showed the second highest TD-factor of 8, bearing a pure licorice impression (G/H-15, 17), respectively a sweet and licorice taste (G/H-20). Further, fractions G/H-15/17 and G/H-24 also exhibited a pure licorice taste with a TD-factor of 4. It is interesting to mention that fraction G/H-23, which owns a three times higher yield as fractions G/H-15/17, is not taste active. In order to gain pure substances an optimized HPLC separation protocol was used (**Supplementary Material**), utilizing preparative and semipreparative HPLC to isolate the following pure substances from fractions G/H-15 to G/H-24: G/H-15 (**16**, **17**), G/H-16 (**1**, **14**), G/H-17 (**12**, **13**), G/H-18 (**6**), G/H-19 (**8**), G/H-20 (**9**), G/H-21(**18**, **19**, **5**), G/H-23 (**3**) and G/H-24 (**4**).

3.4. Taste dilution analysis of fraction I

To localize sweet and licorice like tasting compounds of fraction I, preparative RP-HPLC/UV separation was carried out yielding a total of 21 subfractions, which were applied to taste dilution analysis (Fig. 1, B). Sweet and licorice acting fractions I-3, I-7 and I-8 offered TD-factors of 8. Further, high TD-values were detected at fractions I-2 (sweet: 4; licorice: 8), *I*-4 (sweet: 8; licorice 4) and I-12 (licorice: 8). Moreover, sweet and licorice impression of fractions I-6, I-10 and I-11 could be tasted until a dilution up to 1:4. Fraction I-5 exhibited a pure licorice impression with a TD-factor of 2. More hydrophobic fractions I-13 to I-21 featured mainly a bitter taste and an oral astringency sensation with TD values up to of 16. After screening sweet/licorice-appearing fractions by means of analytical HPLC, fractions I-2 to *I*-4 consisted of same compounds already described in G/H-19–21 and G/H-24. Thus, aimed at discovering the compounds imparting typical sweet and licorice impression of licorice roots, rechromatography of fractions I-5, I-7, I-8, I-10 and I-12 yielded eight pure compounds I-5 (**2**), I-7 (**10**), I-8 (**11**, **15**, **25**, **26**), I-10 (**28**) and I-12 (**24**) (**Supplementary Material**).

3.5. Structure determination of isolated and synthesized compounds

Preparative and semipreparative HPLC of licorice root extract, esterification and reduction syntheses of glycyrrhizin yielded 28 compounds (Fig. 2). In order to identify these substances, the structure elucidation protocol referring to Schmid, Dawid, Peters, and Hofmann (2018) was used. By comparison of spectrometric as well as spectroscopic data recently published, compounds **3**–**19**, **24**–**26** and **28** could be identified as shown in Fig. 2.

HRESIMS analysis of unknown compound **1** revealed a molecular formula of $C_{41}H_{62}O_{15}$, based on the deprotonated compound $[M-H]^-$ at *m/z* 793.4041, calcd. for $C_{41}H_{61}O_{15}$ (*m/z* 793.4010). ESI-MS/MS fragmentation of **1**, based on the molecular ion peak $[M+H]^+$ at *m/z* 795 showed fragments of *m/z* 663 and 469, indicating a pentose as terminal sugar moiety and a uronic acid as aglycone-bound sugar moiety. The aglycone of compound **1** demonstrated a similar backbone and configuration as the aglycone of **3**–**5** as previously stated by Schmid, Dawid, Peters, and Hofmann (2018). ROE correlations of H-28 (δ_H 0.94)/H-18 (δ_H 2.38) and H-18 (δ_H 2.38)/H-29 (δ_H 1.47) highlighted an α-configuration of carboxyl group C-30 concluding a 20α-glycyrrhizin aglycone of **1** with the exception of an additional hydroxyl group at position 21 in α-configuration, which could be substantiated by HMBC and ROESY correlations: H-29 (δ_H 1.47)/C-21 (δ_C 30.1), H-21 (δ_H 4.52)/C-30 (δ_C 180.2) and H-29 (δ_H 1.47)/H-21 (δ_H 4.52). The carbohydrate identification of **1** highlighted a similar aglycone bound glucuronic acid as observed in **3**–**5**. Moreover, an HMBC correlation of H-1́ (δ_H 6.52)/C-2́ (δ_C 79.5) indicated a 1́→2́ linkage of the glucuronic acid and the second carbohydrate moiety. COSY and HMBC NMR experiments demonstrated the following correlations: H-1́ (δ_H 6.52)/H-2́ (δ_H 5.00), H-1́ (δ_H 6.52)/C-2́ (δ_C 78.4), H-1́ (δ_H 6.52)/C-3́ (δ_C 81.0), H-1́(δ_H 6.52)/C-4́(δ_C 76.0), H-2́ (δ_H 5.00)/C-3́ (δ_C 81.0), H-2́ (δ_H 5.00)/C-5́, H-4́ (δ_H 4.77)/C-3́ (δ_C 81.0) and H-4́ (δ_H 4.77)/C-5́ (δ_C 66.4). Altogether, the determination of two CH_2 groups (4́ and 5́) and one quaternary carbon atom (3́) and coupling constants of $^3J_{1',2'}$ = 2.1 Hz (2́) and $^2J_{4',4}$=9.2 Hz (4́), substantiated the presence of a 1́→2́ linked β-D-apiofuranoside moiety, which was confirmed by Ion chromatography (Rinaldo, Rodrigues, Rodrigues, Sannomiya, Santos, & Vilegas, 2007). Consequently, for the first time **1** could be identified as (3β,20α)-20-carboxy-21-hydroxy-11-oxo-30-norolean-12-en-3-yl 2-*O*-β-D-apiofuranosyl-β-D-glucopyranosiduronic acid (**1**, 21-hydroxy-20α-apioglycyrrhizin).

Saponin **2** showed in the HRESIMS analysis the molecular formula $C_{42}H_{64}O_{15}$ based on the ion $[M-H]^-$ at *m/z* 807.4188, calcd for $C_{42}H_{63}O_{15}$ (*m/z* 807.4167). For further structure elucidation ESIMS/MS experiments were used to identify the saccharide pattern, in which protonated **2** $[M+H]^+$ in the ESIMS/MS resulted in fragments of *m/z* 647 and *m/z* 453, indicating the presence of a uronic acid and a hexose. ^1H and ^{13}C NMR signal assignment of aglycone **2** revealed similar COSY and HMBC correlations to previously identified 20α saponin derivatives (**3**–**5**) (Schmid, Dawid, Peters, & Hofmann, 2018). ROE correlations of H-28 (δ_H 0.88)/H-18 (δ_H 2.25) and H-18 (δ_H 2.25)/H-29 (δ_H 1.43) highlighted an α-configuration of carboxyl group C-30 concluding a 20α-glycyrrhizin aglycone of **2**. Further, the determination of the carbohydrate moiety as a 1́→3 linked β-D-glucuronic acid and a 1́→2́ linked hexose was indicated by the HMBC and COSY correlations of H-1́ (δ_H 5.02)/C-3 (δ_C 89.3), H-1́ (δ_H 5.02)/H-2́ (δ_H 4.38), H-1́ (δ_H 5.44)/C-2́ (δ_C 83.3), H-1́ (δ_H 5.44)/H-2́ (δ_H 4.17), H-1́ (δ_H 5.44)/C-5́ (δ_C 78.7), H-2́ (δ_H 4.17)/C-3́ (δ_C 78.3), H-3́ (δ_H 4.30)/C-4́ (δ_C 72.0), H-4́ (δ_H 4.38)/C-5́ (δ_C 78.7) and H-5́ (δ_H 3.97)/H-6́ (δ_H 4.53). Characterization of the sugar protons coupling constants ($^3J_{2',3'}$= 9.2 Hz, $^3J_{3',4'}$= 9.5 Hz, $^3J_{4',5'}$= 9.5 Hz) revealed the terminal carbohydrate moiety of **2** and could be identified as β-D-glucose. Consequently, the new compound could be assigned to (3β,20α)-20-carboxy-11-oxo-30-norolean-12-en-3-yl 2-*O*-β-D-glucopyranosyl-β-D-glucopyranosiduronic acid (**2**, 20α-glucoglycyrrhizin).

For comprehensive taste investigation, carboxylic groups of glycyrrhizin were modified by methylation and, then, sensorially characterized. However, ESIMS exhibited identical protonated molecular ions $[M+H]^+$ at *m/z* 837, but ESIMS/MS offered distinct fragment ions for compound discrimination at *m/z* 661 (**20**), *m/z* 467 (**20**), *m/z* 661 (**21**), *m/z* 453 (**21**), *m/z* 647 (**22**) and *m/z* 453 (**22**). Consequently, esterification of glycyrrhizin could be located at the carboxylic group of the aglycone (**20**), aglycone bound β-D-glucuronic acid moiety (**21**) and terminal β-D-glucuronic acid moiety (**22**). With a molecular ion signal $[M+H]^+$ at *m/z* 851 and a fragmentation pattern of *m/z* 661 and *m/z* 453 esterification of both β-D-glucuronic acid moieties could be evidenced in **23**. Unequivocal identification was achieved by means of NMR spectroscopy with observed HMBC correlations H-31 (δ_H 3.68, **20**)/C-30 (δ_C 177.2, **20**); H-7́ (δ_H 3.72, **21**, **23**)/C-6́ (δ_C 170.7, **21**, **23**) and H-7́ (δ_H 3.86, **22**, **23**)/C-6́ (δ_C 170.6, **22**, **23**). Consequently, synthetic products **20–23** were assigned as (3β,20β)-20-carboxy-11-oxo-30-norolean-12-en-3-yl 2-*O*-β-D-glucopyranuronosyl-β-D-glucopyranosiduronic acid-30-methylester (**20**), (3β,20β)-20-carboxy-11-oxo-30-norolean-12-en-3-yl 2-*O*-β-D-glucopyranuronosyl-β-D-glucopyranosiduronic acid-6′-methylester (**21**), (3β,20β)-20-carboxy-11-oxo-30-norolean-12-en-3-yl 2-*O*-β-D-glucopyranuronosyl-β-D-glucopyranosiduronic acid-6′′-methylester (**22**) and (3β,20β)-20-carboxy-11-oxo-30-norolean-12-en-3-yl 2-*O*-β-D-glucopyranuronosyl-β-D-glucopyranosiduronic acid-6′,6′′-dimethylester (**23**). In order to check the influence of the carbonyl

moiety at position 11 on the taste activity, glycyrrhizin was reduced by sodium borohydride. Synthesized glycyrrhizin derivative **27** afforded a molecular formula of $C_{42}H_{64}O_{16}$ based on the $[M - H]^-$ peak at *m/z* 823.4133 in the HRESIMS, calcd for $C_{42}H_{63}O_{16}$ (*m/z* 823.4116), revealing a 2 Da increased saponin mass compared to glycyrrhizin (**8**). Moreover, the ESIMS/MS of **27** showed an aglycone fragment at *m/z* 455, bearing an OH group at C-11 instead of a carbonyl group. The ^{1}H NMR resonance at δ_H 3.96 (1H, d, $J = 7.7$ Hz, H-11) and COSY correlations of H-9 (δ_H 1.47)/H-11 (δ_H 3.96) and H-11 (δ_H 3.96)/H-12 (δ_H 5.11) revealed the presence of a hydroxyl group at position 11. Thus, compound **27** was identified as (3*β*,20*β*)-20-carboxy-11-hydroxy-30-norolean-12-en-3-yl 2-*O*-*β*-D-glucopyranuronosyl-*β*-D-glucopyranosiduronic acid.

3.6. Taste and Structure–Activity relation

Taste recognition thresholds of isolated and synthesized saponins **1–28** ranged between 6 and 40 μmol/*L* for sweet, 2 and 60 μmol/*L* for licorice and 23 and 210 μmol/*L* for bitter (Table 1). The test substances could be divided in three groups, saponins **8**–**19**, **22**, **25**–**27**, exhibiting a sweet and licorice taste, compounds **3**, **7**, **20**, **21** and **23** owning a bitter taste, and saponins **1**, **2**, **4**–**6**, **24** and **28**, exhibiting a pure licorice sensation. All saponins shared a triterpenoid aglycone with different functional groups and at least one glucuronic acid moiety, which separately resulted in different characteristic taste qualities. Consequently, diverse substructures can be brought into account with correspondent compounds' taste qualities and thresholds. Naturally occurring saponins **8**–**17** exhibited a *β*-configured carboxyl group at the triterpenoid E-ring and a carbonyl function (C-11) at the triterpenoid C-ring resulting in a sweet and licorice taste. In contrast to these sweet and licorice tasting saponins, compounds **1**–**6** owned an *α*-configured carboxyl group (C-30) at the E-ring, which resulted in the case of (20*α*)-glycyrrhizin (**3**) in a bitter taste. In contrast to the literature, this saponin was described as 120 times sweeter than sucrose (Kitagawa, 2002). In contrast, saponins **1**, **2**, **4**–**6**, which also offered an *α*-configured carboxyl group (C-30), showed a pure licorice taste. Differences in the chemical structure of bitter tasting (20*α*)-glycyrrhizin (**3**) and other 20*α*-glycyrrhizin derivatives consist of an additional hydroxyl group at position C-24 or another terminal carbohydrate moiety like *β*-D-glucose, *α*-L-rhamnose or *β*-D-apiofuranose. Further pure licorice tasting compounds could be identified as 11-deoxo-glycyrrhizin (**24**) and 11-deoxo-11,13-glycyrrhizindiene (**28**) with a missing carbonyl function at position C-11 or two conjugated double bonds at the C/D-ring. In contrast, an additional hydroxyl group at position C-24 or a terminal *α*-L-rhamnose moiety rendered the compound sweet. Accordingly, a carbonyl group at position C-11 or an additional polar group, in detail *α*-L-rhamnose or a hydroxyl group at position 24 are essential for sweet taste. Compound **7**, which featured a combination of *α*-configured carboxyl group (**30**) and 11-deoxo function, exhibited a bitter taste (taste threshold: 51.2 μmol/L). Reduction of the carbonyl group at position C-11 to a hydroxyl group (**27**) showed no effect with regard to taste quality and thresholds compared to glycyrrhizin. Methylation of glycyrrhizin's carboxyl groups revealed a correlation of taste and free carboxyl groups. Glycyrrhizin-30-methylester (**20**) offered a sole bitter taste with a threshold of 38.9 μmol/L, indicating that the carboxyl group at position C-30 is important for sweet and licorice taste. 30-Hydroxy-glycyrrhizin (**18**) exhibited similar taste thresholds as glycyrrhizin (**8**). In conclusion, at least one proton at position C-30 is necessary to obtain licorice and sweet taste, which was in line with sweet/licorice tasting glycyrrhizin-30-methanoate (**19**). Generation of the aglycone bound glucuronic acid-methylester represented by glycyrrhizin-6′-methylester (**21**) and glycyrrhizin-6′,6′′-dimethylester (**23**) highlighted a sweet, licorice and bitter taste with similar thresholds (Table 1). Contrary to **21** and **23**, glycyrrhizin-6′′-methylester (**22**) showed a sweet and licorice but no bitter taste, revealing the importance of a free carboxyl group at aglycone bound glucuronic acid for a native sweet and licorice taste perception (Fig. 3, A). In context with the results of Esaki, Konishi, and Kamiya (1978) and Mizutani, et al. (1994) to the taste of synthesized glycyrrhetinic acid glucosides, hydroxyl groups at position C-4́ for pyranose or a free carboxyl group at position C-6́ are inevitable for the saponin to evoke a natural taste without a bitter off-taste.

Table 1
Human taste recognition thresholds and Human TAS1R2 VFT model Docking scores of isolated licorice root saponins and synthesized* saponins.

comp. no.	compound name	taste threshold (μmol/L)			docking scores
		sweet	licorice	bitter	
1	21-hydroxy-20*α*-apioglycyrrhizin		9.4		–6.877
2	20*α*-glucoglycyrrhizin		15.9		–5.572
3	20*α*-glycyrrhizin			210.0	–3.958
4	20*α*-galacturonicacidglycyrrhizin		35.4		–5.608
5	20*α*-rhaoglycyrrhizin		48.8		–5.241
6	24-hydroxy-20*α*-glycyrrhizin		34.6		–3.067
7	11-deoxo-20*α*-glycyrrhizin			51.2	–5.079
8	glycyrrhizin	35.1	32.6		–8.93
9	glucoglycyrrhizin	12.3	11.7		–6.504
10	araboglycyrrhizin	21.1	15.4		–5.906
11	apioglycyrrhizin	5.7	2.3		–6.304
12	rhaoglycyrrhizin	31.0	17.9		–8.521
13	rhaoglucoglycyrrhizin	15.4	12.3		–7.457
14	rhaogalactoglycyrrhizin	11.5	7.1		–7.032
15	glycyrrhetinic acid mono-glucuronide	26.8	14.0		–3.948
16	24-hydroxy-glycyrrhizin	25.4	22.5		–5.861
17	24-hydroxy-glucoglycyrrhizin	20.9	8.0		–5.399
18	30-hydroxy-glycyrrhizin	27.0	24.0		–5.738
19	glycyrrhizin-30-methanoate	37.7	33.4		–5.284
20	glycyrrhizin-30-methylester*			38.9	–5.78
21	glycyrrhizin-6'-methylester*	29.1	60.2	27.0	–4.604
22	glycyrrhizin-6''-methylester*	21.4	22.0		–9.995
23	glycyrrhizin-6',6''-dimethylester*	42.0	31.1	23.0	–6.141
24	11-deoxo-glycyrrhizin		28.1		–6.681
25	11-deoxo-rhaoglycyrrhizin	12.2	9.1		–8.573
26	11-deoxo-24-hydroxy-glycyrrhizin	12.0	9.1		–6.146
27	11-hydroxy-glycyrrhizin*	28.0	28.0		–7.777
28	11-deoxo-11,13-glycyrrhizindiene		26.1		–6.115

3.7. Human concentration–response functions

To evaluate the sensory activity of taste-active compounds, suprathreshold measurements are more suitable in comparison to threshold values (Bartoshuk, 2000; Galindo-Cuspinera, Waeber, Antille, Hartmann, Stead, & Martin, 2009). Due to the need of higher yields, six representative compounds were chosen, three of them eliciting a sweet and licorice impression (compounds: **8**, **12** and **16**) and three evoking a sole licorice impression (compounds: **1**, **5** and **6**). Details on the isolation process are stated in the **Supplementary Material**. Concentration–response functions of saponins **1**, **5**, **6**, **8**, **12** and **16** were recorded using 800, 600, 400, 300, 200, 150, 100, 50, 30, 10 and 5 μM compound concentration. Due to the lack of solubility, a maximum applicable concentration of 800 μM was used. Preliminary defined glycyrrhizin concentrations of 30 and 400 μM acted as control. With maximum intensities of 9.2–9.3 for sweet and 9.4–9.5 for licorice, concentration–response functions of compounds **8**, **12** and **16** showed similar sigmoidal progressions (Fig. 3, B), whereas licorice concentration–response functions exhibited negligible higher intensities at corresponding concentrations. Differences of sweet/licorice curve progressions (**8**, **12** and **16**) revealed saturation effects at high glycyrrhizin (**8**) concentrations, whereas for rhaoglycyrrhizin (**12**) and 24-hydroxy-glycyrrhizin (**16**) this effect could not be detected. In contrast to

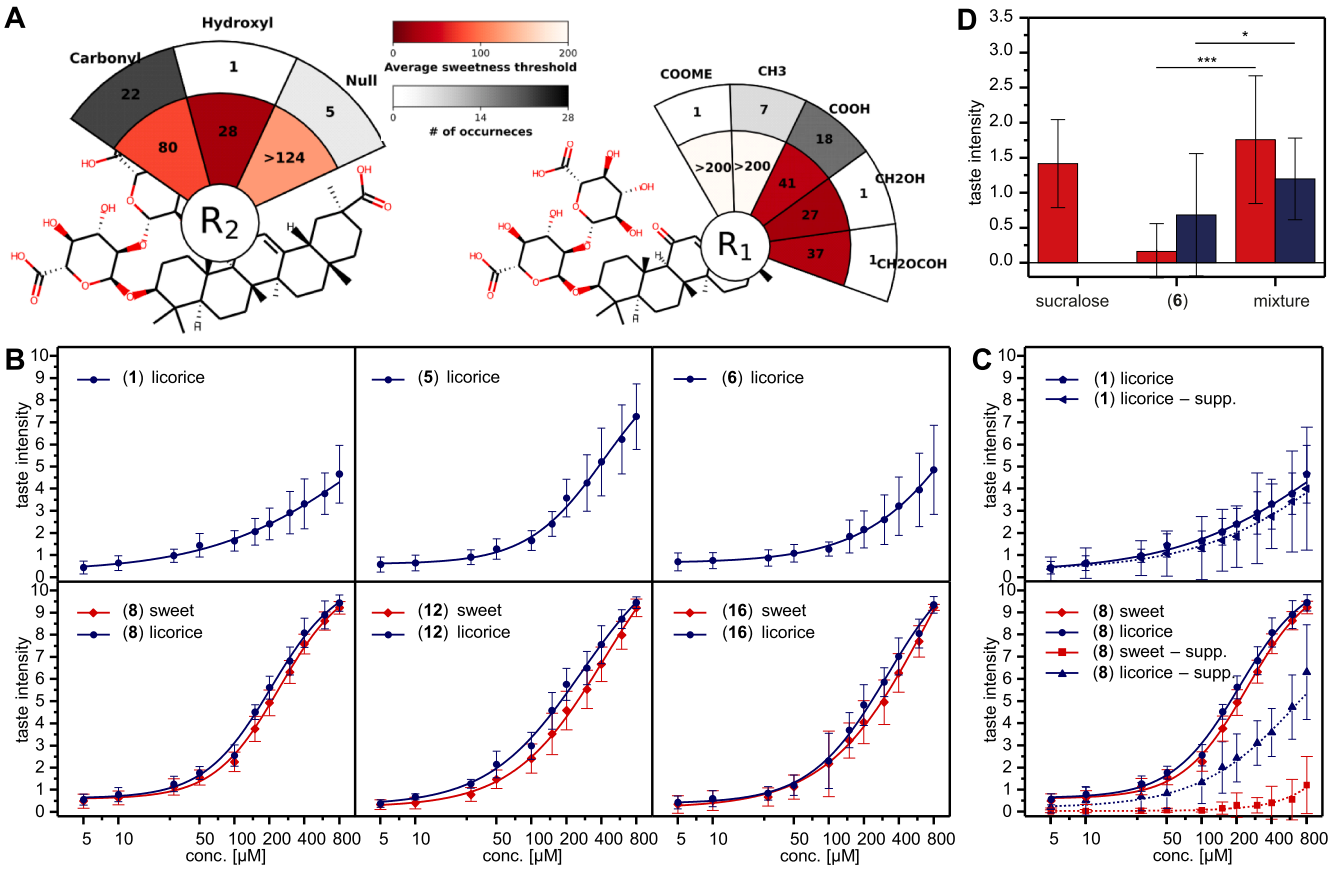


Fig. 3. (A) SAR models of the main differences between the compounds as represented by different R-groups in selected locations. (B) Concentration-response functions of licorice saponins on perceived sweet/licorice taste intensity and concentration of corresponding saponin (5–800 µM). Error bars represent the confidence interval ($p < 0.05$). (C) Concentration-response functions of **1** and **8** with and without sweet suppression. (D) Synergistic effect of sucralose (50 µM) and licorice acting 24-hydroxy-20α-glycyrrhizin (100 µM) to licorice impression compared to the sensory profile of the corresponding single compounds. by a one sided *t*-test, demonstrating significant ($p \leq 0.05$) with *, very significant ($p \leq 0.01$) with ** and highly significant ($p \leq 0.001$) differences with ***.

saponins with a *β*-configured carboxyl group C-30, compounds **1**, **5** and **6**, exhibiting a corresponding carboxyl group in *α* configuration, revealed completely different licorice curves (Fig. 3, B and C). Maximum licorice intensities of compounds **1**, **5** and **6** were clearly lowered to values of 4.7 (**1**), 7.3 (**5**) and 4.9 (**6**) compared to 9.4 (**8**), 9.5 (**12**) and 9.4 (**16**). Compound **5** exhibited the highest maximum intensity of the sole licorice tasting compounds. However, the taste threshold (48.8 µM) is about five times higher than that of **1** (9.4 µM). At concentrations of 5–150 µM compounds **1**, **5** and **6** showed only a marginal increase of licorice perception, contrary to **8**, **12** and **16** which indicated the same slope at concentrations of 5–50 µM. A more detailed view at 150 µM saponin concentration revealed for the compounds **8**, **12** and **16** two times higher licorice impression intensities in contrast to saponins **1**, **5** and **6** (Fig. 3, B), resulting in completely different curve progressions. Moreover, compounds **12** and **16**, as well as **1**, **5** and **6** behaved similarly and showed no saturation. In summary, the triterpenoid E-ring is highly important for sweet and licorice perception, highlighting different concentration–response curve progressions of compounds with *α*- (**1**, **5** and **6**) and *β*-configured (**8**, **12** and **16**) carboxyl group C-30. Similar sweet and licorice curves of **8**, **12** and **16,** together with varying licorice curves of **1**, **5** and **6** suggest that the licorice impression is mediated by a receptor or receptor family differing from sweet hTAS1R2/hTAS1R3 receptor, whereupon a crossmodal interaction of sweet and licorice taste could be indicated.

In order to get more information about the interaction of sweet and licorice taste, sweet-suppressed dose–response sensory experiments of **1** and **8** were comparably performed using the above mentioned set-up, but with pre-administration of *Gymnema silvestre* R. Br. extract. The licorice concentration–response curve of **8** showed a clearly lowered maximum taste intensity of 6.3, relative to not suppressed concentration–response sensory at correspondent concentration (taste intensity 9.4). Furthermore, the curve slope and progression were similarly altered to concentration–response curve of pure licorice tasting compounds **1**, **5** and **6** (Fig. 3, C). Concentration–response sensory experiment of sweet-suppressed, pure-licorice-acting saponin **1** revealed similar curve slope and progression. Altogether, these results propose independent interaction of sweet taste and licorice impression. In order to get a clearer picture about a possible interaction of these two impressions, comparative profile sensory investigations of sweet tasting sucralose (50 µM), licorice-acting 24-hydroxy-(20*α*)-glycyrrhizin (100 µM, **6**) as well as the binary mixture of sucralose (50 µM) and **6** (100 µM) were conducted. In comparison to **6**, a significant ($\alpha = 0.04$) increase of licorice sensation intensity could be observed for the mixture of sucralose and **6**, supporting a cross-modal effect of sweet tastants on licorice sensation (Fig. 3, D). To get a deeper insight into the complex structure–activity relationships of licorice root saponins, they were tested on sweet hTAS1R2/R3 and bitter hTAS2R receptors.

3.8. *Investigation of licorice root Saponins' action on functionally expressed human sweet and bitter receptors*

In a first step, stereoisomers **8**/**3**, **12**/**5**, **16**/**6** and compound **1** were applied on human HEK293 FlpIn *T*-Rex $G\alpha_{15}G_{i3}$/hTAS1R2 sweet receptor cells. In the second step, licorice saponins were assayed for activation of 25 different bitter receptors in order to examine if the typical licorice-like taste could be mediated by one or several bitter

receptors. These functional calcium imaging experiments were performed with a stable expressed chimeric G protein $G\alpha_{15}G_{i3}$, which couples in the case of the sweet receptor heterodimer to cytosolic calcium levels monitored *via* a calcium-sensitive fluorescence dye (Galindo-Cuspinera, Winnig, Bufe, Meyerhof, & Breslin, 2006). Sweet-receptor-expressing cells, challenged with licorice saponins, exhibited an immediate transient increase of calcium fluorescence for all applied saponins (**1, 5**, **6**, **8**, **12** and **16**). Application of different saponin concentrations on hTAS1R2/R3-expressing cells revealed similar concentration–response functions for sweet/licorice acting saponins **8** and **12** (Fig. 4, A) as well as similar to psychophysical tests (Fig. 3, C). Psychometric dose–response functions and cell assay fluorescence excitations of compounds **8** and **12** highlighted comparable curve progressions and, therefore, evidenced a clear activation of hTAS1R2/R3 sweet receptor. To examine relative sweet receptor agonist activity of sweet/licorice acting saponins **8**, **12** and **16**, the fluorescence intensity at 100 μM was compared. Rhaoglycyrrhizin (**12**) exhibited the highest fluorescence value with 0.40 ± 0.07, followed by glycyrrhizin (**8**) and 24-hydroxy-glycyrrhizin (**16**) with 0.27 ± 0.04 and 0.12 ± 0.07, respectively. These observations were not comparable with respective taste intensities of human concentration–response experiments with 2.2 ± 0.4 (**8**), 2.4 ± 0.7 (**12**) and 2.1 ± 1.4 (**16**). Further, even bitter tasting saponin (20*α*)-glycyrrhizin (**3**) and sole licorice tasting compounds **1, 5** and **6** induced fluorescence at concentrations of 300 μM (**1**, 0.37), (**3**, 0.13), (**5**, 0.20) and 100 μM (**6**, 0.18) (Fig. 4, A). Examination of cell vitality and integrity of cellular signal transduction by application of the β-adrenergic receptor agonist isoproterenol revealed no diminished fluorescence signal. So, fluorescence excitation of 20*α* isomers might be explained by interaction of these saponins with other naturally occurring receptors on HEK293 FlpIn *T*-Rex $G\alpha_{15}G_{i3}$/hTAS1R2 cells. In order to bypass this problem, HEK293T cells were used which transiently express the sweet receptor heterodimer hTAS1R2/R3 (stable expression of chimeric G-protein subunit Gα16gust44 and co-transfected with plasmids coding for human sweet receptor subunit hT1R2 and hT1R3). Application of **1, 5, 6** and **8** with varying concentrations (100, 50, 25 and 10 μM) as well as with and without 1 mM lactisole on HEK293T cells, as mentioned by Galindo-Cuspinera, Winnig, Bufe, Meyerhof, and Breslin (2006), indicated an explicit fluorescence excitement exclusively at 100 and 50 μM glycyrrhizin (**8**) without lactisole (Fig. 4, C). Saponins **1**, **5** and **6** (50 μM; w/ and w/o lactisole) with *α*-configured carboxyl group at C-30 exhibited contrary to glycrrhizin (**1**) (*β*-configuration) no fluorescence signal. Compared to licorice taste thresholds of 9.4 μM (**1**), 48.8 μM (**5**) and 34.6 μM (**6**), the cell assay concentration was much higher, but no fluorescence signal in the sweet receptor cell assay was detected. However, sweet and licorice tasting glycyrrhizin (**8**) showed a distinct receptor signal. In conclusion, licorice taste is not mediated by the sweet receptor. The licorice sensation might consequently be independent from sweet taste.

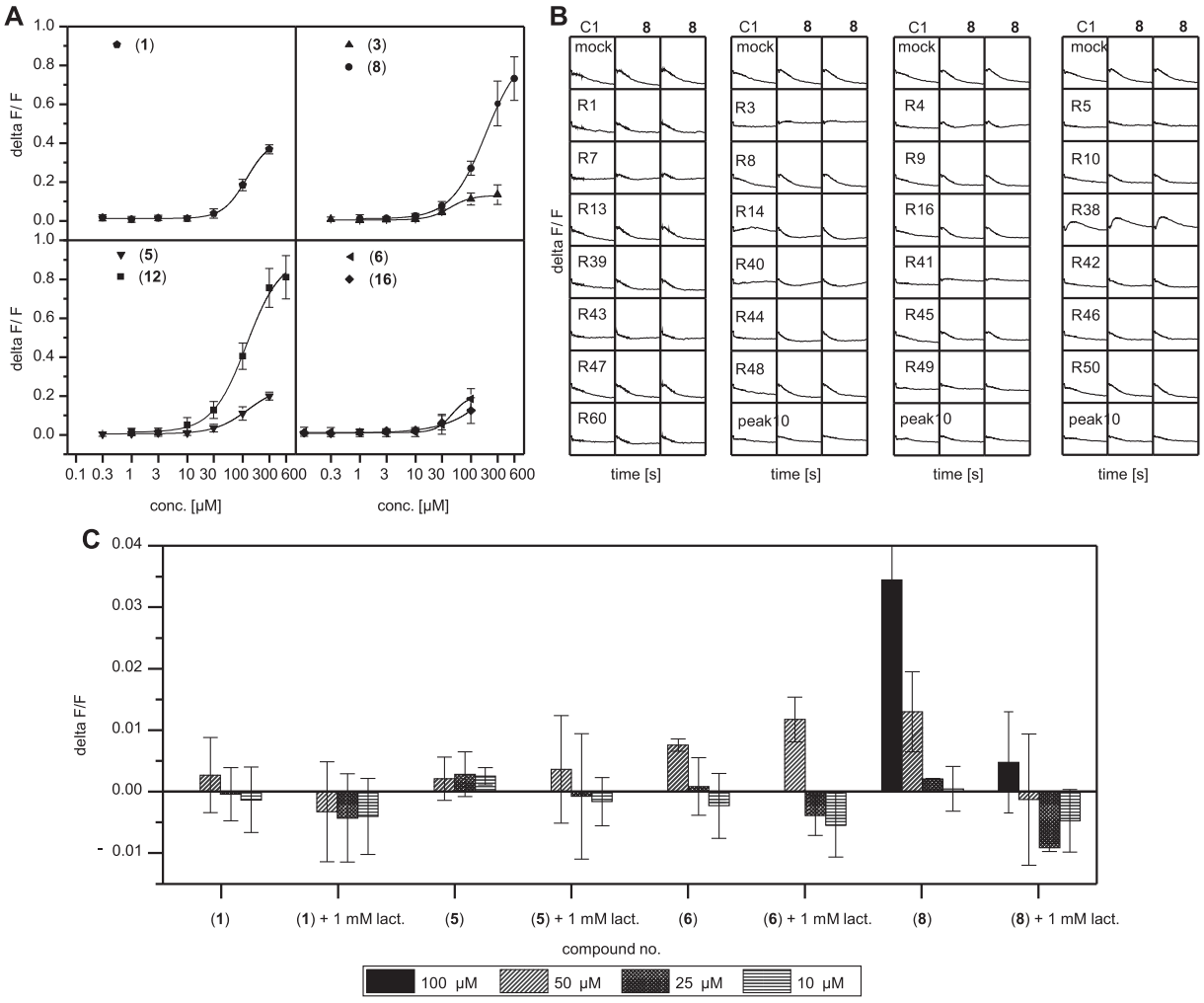


Fig. 4. (A) Concentration-responses of human sweet taste receptor-expressing cells (HEK293 FlpIn *T*-Rex cells stable expressing hTAS1R2 and inducible hTAS1R3) to saponins 1, 3/8, 6/12 and 6/16 with different substitution pattern of triterpenoid E-ring. (B) Calcium traces of human bitter taste receptor-expressing cells and mock to 300 μM 8. (C) Calcium responses of HEK293T Gα15gust44-cells expressing hTAS1R2/*R*3 administered with different concentrations of 1, 5, 6 and 8 as well as with sweet inhibiting lactisole.

To assess a possible interaction of licorice saponins with bitter receptors, glycyrrhizin (**8**) (300 μM) was adjusted on Gα16gust44 cells transiently expressing 25 hTAS2R receptors individually. Receptor function can be influenced by numerous single nucleotide polymorphisms (SNP) in human TAS2R genes (Roudnitzky, Bufe, Thalmann, Kuhn, Gunn, Xing, et al., 2011). For the following experiments receptor variants of previous publications were used (Brockhoff, Behrens, Roudnitzky, Appendino, Avonto, & Meyerhof, 2011). At a concentration of 300 μM, compound **8** exhibited a strong sweet and licorice-like taste in the preceding human psychophysical studies, but in the cell-based bitter receptor experiments only an unspecific receptor signal at R38 could be detected (Fig. 4, B). For 20*α*-glycyrrhizin (**3**) and 21-hydroxy-20α-apioglycyrrhizin (**1**), which possessed a sole bitter taste with a human taste threshold of 210 μM and a sole licorice taste at a taste threshold of 9 μM, at concentrations above 3 μM and 10 μM (**Supplementary Material**), respectively, artefact signals overlapped with possible bitter signals. In conclusion, neither an interaction of sole licorice-tasting compounds **1**, **5** and **6** with sweet receptor nor a reaction of sweet/licorice tasting glycyrrhizin (**8**) with bitter receptors could be detected. Therefore, we propose that the licorice taste is mediated by another unknown receptor family.

3.9. Licorice root saponins computationally Predicted interactions with the 3D-model of sweet taste receptor

In order to evaluate further the activation by licorice root saponins, computational experiments were carried out. We hypothesized that similar to the *in vitro results*, licorice or bitter tasting compounds that had not evoked sweetness *in vivo* nor *in vitro*, would yield ligand–sweet receptor complexes that are ranked lower than sweet tasting compounds complexes with that receptor. The compounds were docked to a sweet taste receptor model (Ben Shoshan-Galeczki & Niv, 2020), and docked to the orthosteric binding site in the Venus Fly Trap (VFT) ligand binding domain of hTAS1R2. We used an open form model of hT1R2 and a GRAS (Generally Recognized As Safe) dataset of compounds that are bigger than 460 g/mol. Overall, docking scores were lower on average for sweet compounds, and only sweet-tasting compounds (according to their reported annotation) received a docking score better (lower) than –7. Compounds isolated from Licorice Root Saponins followed the same pattern and only sweet compounds had docking scores better than the –7 score (Fig. 5, A). Thus, a docking score below –7 is likely to indicate a sweet compound. Sweet compounds dock well into the sweet-taste receptor model and interact with residues in the upper lobe of the VFT binding site, known to interact with sweeteners such as sucrose, sucralose and larger sweeteners such as stevia and superaspartame (Zhang, Klebansky, Fine, Liu, Xu, Servant, et al., 2010). All the sweet compounds listed in Fig. 2 interact with residues Y103, D142, S144 and S165, which were shown by mutagenesis to affect binding of sweet compounds to the VFT binding site (Chéron, Golebiowski, Antonczak, & Fiorucci, 2017). The saccharide moiety and the functional group at position C-30 were common among the sweet compounds. The main sweet compounds studied here are presented in Fig. 5. R1 functional group formed hydrogen bonds with N44 (Fig. 5, C). Compounds with carbonyl or carboxylic groups on C-11 or R2 (Fig. 3A), formed a hydrogen bond with Y103 (Fig. 5, C–D). These interactions appear important for the orientation of compounds to the upper lobe of the binding site typical for the sweet compounds, while non-sweet compounds (such as sole licorice-tasting compounds 4–6) had lower docking scores, and were oriented towards the lower lobe of the binding site. The poorer docking scores for some of the sweet compounds may be due to lack of hydrogen bonds with residues N44 or Y103, a hypothesis that could be tested *via* mutagenesis.

4. Conclusion

With application of a multi-dimensional HPLC approach, 23 saponins were isolated from *Glycyrrhiza glabra* L., whereupon two saponins were characterized for the first time. Moreover, selective methylation and carbonyl reduction yielded four different glycyrrhizin-methyl esters and 11-hydroxy-glycyrrhizin. Psychophysical studies of the isolated and

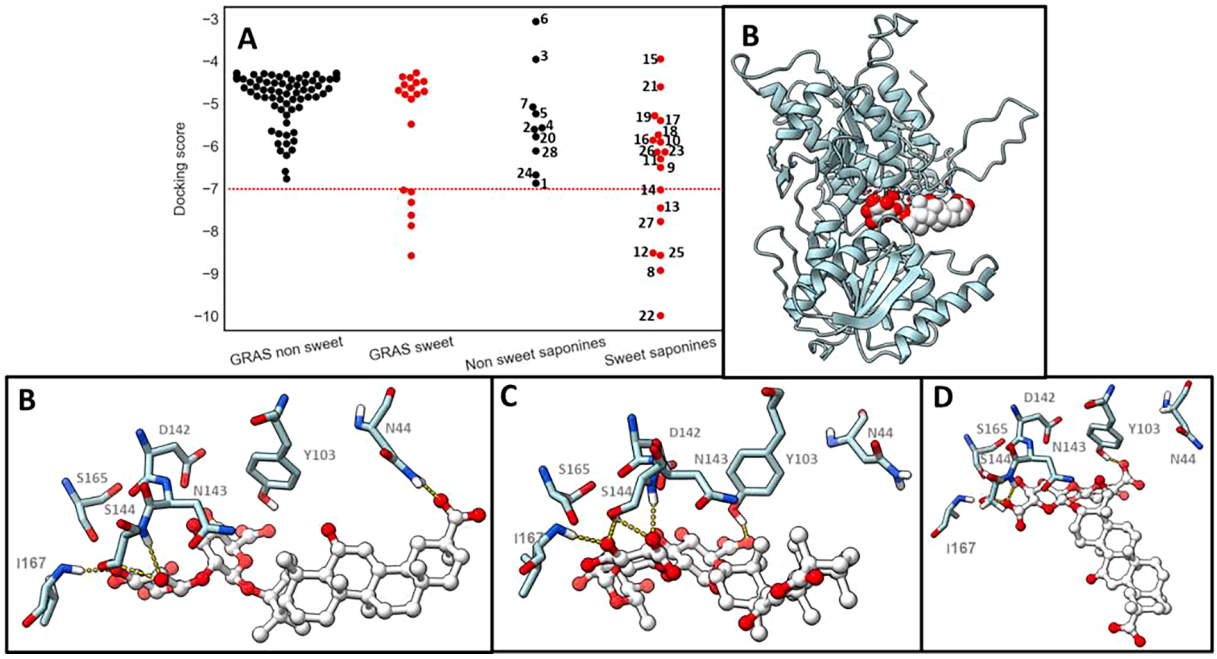


Fig. 5. (A) Docking scores for GRAS compounds, and for saponins identified in this work, shown in red for sweet and in black for non-sweet. The –7 score is marked with red dashed line. (B) Model of hT1R2 VFT domain with docked Glycyrrhizin, (C–E) Predicted binding pose of main compounds: C (compound 8), D (compound 12) and E (compound 16) in complex with the hT1R2 model. Saponine carbons are colored in white, main interacting residues are colored in light blue sticks, and hydrogen bonds are represented by yellow dashed lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

synthesized saponins resulted in human taste thresholds of 6 to 43 μmol/L for sweet, 2 to 56 μmol/L for licorice and 23 to 210 μmol/L for bitter sensation. Interestingly, besides the compounds **4**–**6**, **24** and **28,** the newly characterized saponins **1** and **2** both have a 20α substitution pattern, evoking a pure licorice sensation. Besides the stereochemistry and the substitution pattern of the triterpenoid C- and E-ring, the saccharide moiety and the functional group at position C-30 are essential motifs for the sweet, licorice and the bitter sensation of the licorice saponins. Further sensory experiments including dose–response analyses revealed different curve progressions of sweet/licorice and sole licorice-acting compounds. Combination of sweet suppression dose–response sensory experiments of sweet/licorice and pure licorice-acting compounds suggested that the licorice sensation might not be mediated by the known sweet receptor. Further, the artificial sweetener sucralose exhibited a cross-modal interaction with the pure licorice-acting compound **6**, resulting in a synergistic increase of licorice taste. Application of sweet/licorice and sole licorice acting compounds on sweet and bitter receptor expressing cells lead to sweet receptor excitation for the sweet/licorice-acting glycyrrhizin only. Sweet or bitter receptor activation did not occur for sole licorice-tasting compounds with a 20α substitution pattern. Together with the psychophysical findings and computational analysis of ligand–receptor complexes, this observation strongly suggests that licorice sensation is not mediated by hTAS1R2/R3 sweet or hTAS2R bitter receptors. In comparison to sweet compounds, licorice-tasting compounds did not surpass the docking score of the known sweet compounds. Analysis of docked sweet saponins indicated that for this scaffold it is important to form hydrogen bonds with residues N44 or Y103.

CRediT authorship contribution statement

Christian Schmid: Methodology, Formal analysis, Visualization, Investigation, Writing - original draft. **Anne Brockhoff:** Methodology, Formal analysis, Writing - review & editing. **Yaron Ben Shoshan-Galeczki:** Methodology, Formal analysis, Visualization. **Maximilian Kranz:** Formal analysis. **Timo D. Stark:** Methodology, Writing - review & editing. **Rukiye Erkaya:** Formal analysis. **Wolfgang Meyerhof:** Resources, Supervision, Funding acquisition, Writing - review & editing. **Masha Y. Niv:** Resources, Supervision, Funding acquisition, Writing - review & editing. **Corinna Dawid:** Supervision, Project administration, Writing - review & editing. **Thomas Hofmann:** Supervision, Project administration, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.130420.

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