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The Odorant (*R*)-Citronellal Attenuates Caffeine Bitterness by Inhibiting the Bitter Receptors TAS2R43 and TAS2R46

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ABSTRACT

Sensory studies showed the volatile fraction of lemon grass and its main constituent, the odor-active citronellal, to significantly decrease the perceived bitterness of a black tea infusion as well as caffeine solutions. Seven citronellal-related derivatives were synthesized and shown to inhibit the perceived bitterness of caffeine in a structure-dependent manner. The aldehyde function at carbon 1, the (*R*)-configuration of the methyl-branched carbon 3, and a hydrophobic carbon chain was found to favor the bitter inhibitory activity of citronellal, e.g. even low concentrations of 25 ppm were observed to reduce bitterness perception of caffeine solution (6 mmol/L) by 32%, whereas (*R*)-citronellic acid (100 pm) showed a reduction of only 21%, and (*R*)-citronellol (100 pm) was completely inactive. Cell-based functional experiments, conducted with the human bitter taste receptors TAS2R7, TAS2R10, TAS2R14, TAS2R43, and TAS2R46 reported to be sensitive to caffeine, revealed (*R*)-citronellal to completely block caffeine-induced calcium signals in TAS2R43-expressing cells, and to a lesser extent in TAS2R46-expressing cells. Stimulation of TAS2R43-expressing cells with structurally different bitter agonists identified (*R*)-citronellal as a general allosteric inhibitor of TAS2R43. Further structure/activity studies indicated 3-methyl-branched aliphatic aldehydes with a carbon chain of ≥4 C-atoms as best TAS2R43 antagonists. While odor-taste interactions have been mainly interpreted in literature to be caused by a central neuronal integration of odors and tastes, rather than by peripheral events at the level of reception, the findings of this study open up a new dimension regarding the interaction of the two chemical senses.

Key words: bitterness inhibition, citronellal, caffeine, monoterpenes, taste modulation, TAS2R

INTRODUCTION

Whereas in humans sweet and umami tastes are mediated by only one receptor of the taste receptor family 1,[1] perception of bitterness involves ~25 members of the taste family 2 (TAS2R) of G protein-coupled receptors.[2-6] Different subsets of the entire TAS2R repertoire are expressed by the oral taste receptor cells that are specifically dedicated to detect bitter compounds.[7] The TAS2Rs differ in their tuning breadth; so called generalists recognize numerous chemically diverse compounds with substantial overlap,[2,4-6] whereas specialist receptors detect only single or a very few bitter agonists.[2,8,9] The bitter receptors hTAS2R14 and hTAS2R39 were found to be activated by a series of structurally similar (iso)flavonoids, like homoeriodictyol and eriodictyol.[10] Moreover, two TAS2Rs recognize molecules that share common structural properties, e.g. TAS2R16 was found to respond specifically to bitter tasting ß-glucopyranosides such as salicin and amygdalin, while molecules with a N-C═S moiety as found in phenylthiocarbamide and propylthiouracil can activate TAS2R38.[11,12] It is well accepted that the combined agonist spectra of the entire TAS2R repertoire determine the bitter perceptual space of humans.[13] Clearly, the facts that a single TAS2R can recognize multiple bitter compounds and that *vice versa* a single bitter compound can activate several TAS2Rs makes it a challenging task to identify receptor selective bitter antagonists.

Human sensory experiments has led to a substantial progress in the discovery of bitter taste inhibitors, both of synthetic and natural origin. Systematic studies on the bitter inhibiting potential of a series of hydroxylated benzoic acid amides revealed 2,4-dihydroxybenzoic acid *N*-(4-hydroxy-3-methoxy-benzyl) amide (**1**, **Figure 1**) as a potent target molecule reducing the perceived bitterness of caffeine, quinine, salicin, and *N*-L-leucyl-L-tryptophan.[14] Moreover, synthetic vanillyl derivatives revealed that

[2]-gingerdione (**2**, **Figure 1**), [2]-dehydroisogingerdione (**3**), and [3]-gingerdione (**4**) decreases the perceived bitterness of caffeine by 35, 23, and 27%, respectively.[15] Apart from synthetic bitter receptor antagonists, some naturally occurring bitter inhibitors have been reported, all belonging to the group of flavonoids. Already in 1976, neodiosmin (**5**) was observed to increase the bitter taste threshold concentrations of naringin and limonin solutions.[16] Several bitter inhibiting flavonones, namely homoeriodictyol (**6**), sterubin (**7**), and eriodictyol (**8**), have been isolated from the medicinal plant Herba Santa, *Eriodictyon californicum* (H. & A.) Torr., Hydrophyllaceae. While tasteless on their own, all three flavonones were shown to decrease bitterness when added to caffeine solutions. For the sodium salt of **8**, bitter inhibiting potential was reported for various bitter taste compounds, namely guaifenesin, paracetamol, quinine, denatonium benzoate, salicin and amarogentin.[17] Further evaluation of structural homologues revealed that phloretin (**9**), even though exhibiting intrinsic bitter taste at higher concentrations, can reduce the caffeine bitterness when applied in subthreshold concentrations.[18] Moreover, the Maillard reaction product 1-carboxymethyl-5-hydroxy-2-hydroxymethylpyridinium inner salt (**10**),[19] structurally related to the sweetness enhancer alapyridaine formed upon beef meat processing,[20] as well as the ribonucleotide adenosine 5‘-monophosphate (**11**) have been described to show bitterness reducing activity.[21] However, in all of these cases the mode of action of how the observed reduction in bitterness is achieved remained unknown. Particularly, the involvement of specific bitter taste receptors has not been investigated.

Yet, only few bitter inhibitors have been discovered that block bitter substance-evoked TAS2R-activation in heterologous receptor expression assays. The first attempt aimed at identifying antagonists of the bitter taste receptor TAS2R31, which mediates the bitter off-taste of sulfonamide sweeteners.[22] This study successfully

identified 4-(2,2,3-trimethylcyclopentyl) butanoic acid (**12**, **Figure 1**) as reversible insurmountable inhibitor of TAS2R31 and demonstrated that the inhibitor effectively reduced the perceived bitterness but not the sweetness of sulfonamide sweeteners. This inhibitor did not only block TAS2R31 responses but also reduced the bitter agonist-mediated responses of five additional TAS2Rs, i.e., TAS2R4, TAS2R7, TAS2R40, TAS2R43, and TAS2R49.[23] Similar observations have been made in the case of two natural sesquiterpene lactones occurring in various edible plants; these also blocked the activation of not only one but several TAS2Rs.[24] Moreover, 6-methoxyflavones have been identified as reversible insurmountable blockers of TAS2R39.[25] Another group of flavonoids, namely sakuranetin (**13**), 6-methoxysakuranetin (**14**) and jacoesidin (**15**) were isolated from *Eriodictyon californicum* and demonstrated to decrease the responses of the bitter receptor TAS2R31.[26] The sulfonamide probenecid (**16**) was described as negative allosteric modulator of TAS2R16.[27] Guided by molecular docking experiments, the amino acid derivatives γ-aminobutyric acid, *N*α,*N*α-bis(carboxymethyl)-L-lysine, and abscisic acid were recently revealed as inhibitors of quinine-induced responses from TAS2R4-expressing cells.[28,29] These previous studies clearly indicate that bitter antagonists share the ability of bitter agonists to bind to more than one type of TAS2R, and the set of TAS2Rs activated or inhibited is strictly compound specific.[23,24,30]

In the present study, we report on the identification of natural compounds which reduce the bitterness of caffeine by attenuating the caffeine-mediated activation of cognate TAS2Rs. Such compounds may be promising agents to optimize the taste of foods and beverages.

MATERIALS AND METHODS

Chemicals. The following chemicals were obtained commercially: (*R*)-(+)-citronellal ((*R*)-**17**; 95% ee by chiral GC), (*S*)-(-)-citronellal ((*S*)-**17**; 96% ee by chiral GC), (*R*)-(+)-citronellol ((*R*)-**18**), (*R*)-(+)-citronellic acid ((*R*)-**19**), Dess-Martin periodinane, hydrogen peroxide, and *p*-toluensulfonic acid monohydrate from Sigma-Aldrich (Steinheim, Germany), acetone, Amberlyst 15, caffeine, citronellal (**17**), dichloromethane, ethyl acetate, formic acid, monosodium L-glutamate monohydrate, sodium chloride, and sulfuric acid (95-97%) from Merck KGaA (Darmstadt, Germany), diethyl ether and ethanol absolute from J.T.Baker (Deventer, Netherlands), sodium sulfate from Grüssing (Filsum, Germany). HPLC grade solvents were purchased from J.T.Baker (Deventer, Netherlands), deionized water used for the chromatography was purified by means of Milli-QGradient A10 system (Millipore, Molsheim, France). Deuterated solvents were supplied by Euriso-Top (Gif-Sur-Yvette, France). For medium pressure liquid chromatography LiChroprep RP-18, 25–40 µm, bulk material was used (Merck KGaA, Darmstadt, Germany). Sensory analyses were performed with bottled water (Evian). Samples of black tea (Assam), sugar, organic lemon balm, and organic lemon grass were purchased in a local supermarket.

Preparation of Tea Infusions. A portion (2 g) of black tea was infused with boiling tap water (100 mL) for 4 min, followed by filtration using a cellulose filter. To evaluate the impact of additives on the bitter taste of the fresh tea infusion, organic lemon balm (4 g) or organic lemon grass (4 g), respectively, were added to the black tea drug and the infusion process was carried out as described above.

Isolation of Volatile Fraction from Lemon Grass. A lemon grass infusion was prepared as follows: 4 g lemon grass were infused with 100mL boiling tap water for 4 min, followed by filtration using a cellulose filter. Thereafter, the volatile fraction

was carefully isolated from a freshly prepared, aqueous lemon grass infusion by means of solvent-assisted flavor evaporation using a SAFE apparatus.[31]

Sensory Experiments. *Precautions taken for Sensory Analysis.* Prior to sensory analysis, the test (**20**-**26**) were suspended in water, and, after removing the solvent in high vacuum (< 5mPa), were freeze dried three times and analytically confirmed to be essentially free of solvents and buffer compounds used. The more volatile compounds (**20**-**22, 25**, **26**) were dissolved in ethanol and the solvent was carefully removed by flushing with nitrogen. This procedure was repeated three times and analytically confirmed to be essentially free of solvents and buffer compounds used. Formic acid, which is considered as ‘‘Generally Recognized as Safe” (GRAS) as a flavoring agent for food and feed applications, was used to adjust the pH value of solutions which should be sensorially analysed to pH 6.0, because trace amounts of this acid do not influence the sensory profile of the test solution. In order to minimize the ingesting of test compounds, the “sip and spit” method was applied, meaning that the test solutions were expectorated. To prevent the impact of olfactory cues, each sensory experiment was performed using nose-clips. During the sensory analyses, the oral cavity was frequently rinsed with water.

Training of the Sensory Panel. Thirteen subjects (8 women and 5 men, aged 22-30 years), who gave informed consent to participate in the sensory tests of the present investigation and have no history of known taste disorders, were trained to evaluate the taste of aqueous solutions of the following standard taste compounds in bottled water (pH 6.0):[32] sucrose (50 mmol/L) for sweet taste, lactic acid (20 mmol/L) for sour taste, NaCl (20 mmol/L) for salty taste, caffeine (1 mmol/L) for bitter taste, monosodium L-glutamate (3 mmol/L) for umami taste. The assessors had participated earlier at regular intervals for at least 18 months in sensory experiments (triangle tests, iso-intensity testings, scale training) and were, therefore, familiar with

the techniques applied. To prevent any cross-modal interactions with olfactory inputs, the panelists wore nose clips.

Sensory Evaluation of Black Tea Infusions. (i) Fresh infusions prepared from black tea (2.0 g/100 mL) mixed with lemon balm (4.0 g/100 mL) or lemon grass (4.0 g/100 mL), respectively, (ii) fresh black tea infusions (50 mL, 4 g/100 mL) spiked with the volatile SAFE distillate (50 mL) obtained from a fresh lemon grass infusion (4 g/100 mL), and (iii) fresh black tea infusions (100 mL, 2 g/100 mL) spiked with citronellal (**17**; 25, 50, and 100 ppm) were presented to panellists at room temperature in 3-alternative forced choice (3-AFC) tests in comparison to the standard tea infusion (control, without any additive) in randomized order. First, the panellists were asked to identify the odd sample and, then, to describe the sensory difference subsequently.

Sensory Evaluation of the Bitterness Inhibiting Potential of Oxygenated Monoterpenes. Following a protocol reported earlier,[32-35] the target monoterpenes were dissolved in an aqueous caffeine solution (6 mmol/L; pH 6.0) containing 5% ethanol. Using duo tests and randomly coded cups, these solutions were compared to a series of 5% hydroethanolic reference solutions containing increasing caffeine concentrations (2.0, 3.5, 5.0, 6.5, and 8 mmol/L). Assessors were asked to identify the solution with the weaker bitter taste, sensory data were transferred into the percentage of answers considering the monoterpene containing solution to have a less intense bitter taste. After Probit transformation, the caffeine concentration at which 50% panelists consider the test solution to be less bitter is calculated and considered to be the isointensity concentration of the monoterpene/caffeine solution in comparison to the pure caffeine reference solution. To minimize carry-over effects, panelists rinsed their mouth with a 5% hydroethanolic solution between the tests.

Synthesis of (*R*)-7-Hydroxycitronellol ((*R*)-20) and (*R*)-7-Hydroxycitronellic acid ((*R*)-21). For the synthesis of *(R)*-**20** and *(R)*-**21**, (*R*)-citronellol *(R)*-**18** (1.6 mmol) or (*R*)-citronellic acid *(R)*-**19** (1.6 mmol), respectively, were added drop wise to a solution of aqueous sulfuric acid (50/50, v/v) at 0°C. After stirring for 1 h, the ice bath was removed and stirring continued for additional 2 h. The reaction mixture was poured into ice, neutralized with sodium hydroxide solution (10%, w/v) and the reaction products isolated by means of MPLC on RP-18 material. After removing the solvent under reduced pressure and lyophilisation, the structure of the target compounds *(R)*-**20** and *(R)*-**21** (purity >98%, HPLC-DAD/ELSD) were confirmed by UPLC-TOF-MS and NMR experiments. While the (*R*)-configuration of the carbon atom 3 of the educt is expected to be retained, the non-enantioselective synthetic procedure used is expected to lead to a racemic (*R*/*S*)-mixture at carbon atom 7 of the products.

(3R)-7-Hydroxycitronellol, (R)-**20**, **Figure 2**: UPLC-TOF-MS (ESI^-): *m/z* 173.1553 ($[M-H]^-$, measured), *m/z* 173.1547 ($[M-H]^-$, calculated for $C_{10}H_{21}O_2$). ^1H NMR (400 MHz, $CDCl_3$, COSY): δ [ppm] 0.90 [d, 3H, J = 6.6 Hz, H-C(10)], 1.16-1.19 [m, 1H, H-C(4a)], 1.22 [s, 6H, H-C(8), H-C(9)], 1.26-1.41 [m, 4H, H-C(2a), H-C(4b), H-C(5)], 1.41-1.48 [m, 2H, H-C(6)], 1.52-1.67 [m, 2H, H-C(2b), H-C(3)], 3.61-3.75 [m, 2H, H-C(1)]. ^{13}C NMR (100 MHz, $CDCl_3$, HSQC, HMBC): δ [ppm] 19.7 [CH_3, C(10)], 21.6 [CH_2, C(5)], 29.1, 29.2 [CH_3, C(8), C(9)], 29.4 [CH, C(3)], 37.5 [CH_2, C(4)], 39.7 [CH_2, C(2)], 43.9 [CH_2, C(6)], 61.0 [CH_2, C(1)], 71.4 [C, C(7)].

(R)-7-Hydroxycitronellic acid, (R)-**21**, **Figure 2**: UPLC-TOF-MS (ESI^-): *m/z* 187.1336 ($[M-H]^-$, measured), *m/z* 187.134 ($[M-H]^-$, calculated for $C_{10}H_{19}O_3$). ^1H NMR (400 MHz, CD_3OD, COSY): δ [ppm] 0.96 [d, 3H, J = 6.6 Hz, H-C(10)], 1.17 [s, 6H, H-C(8), H-C(9)], 1.18-1.26 [m, 1H, H-C(4a)], 1.27-1.39 [m, 2H, H-C(4b), H-C(5a)], 1.39-1.49 [m, 3H, H-C(5b), H-C(6)], 1.87-2.00 [m, 1H, H-C(3)], 2.08 [dd, 1H, J = 8.0, 14.7

Hz, H-C(2a)], 2.29 [dd, 1H, J = 6.1, 14.7 Hz, H-C(2b)]. ^{13}C NMR (100 MHz, CD_3OD, HSQC, HMBC): δ [ppm] 20.1 [CH_3, C(10)], 22.8 [CH_2, C(5)], 29.18, 29.22 [CH_3, C(8), C(9)], 31.5 [CH, C(3)], 38.5 [CH_2, C(4)], 42.7 [CH_2, C(2)], 44.9 [CH_2, C(6)], 71.4 [C, C(7)], 177.2 [C, C(1)].

Synthesis of (3*R*)-3,7-Dimethyloctane-1,6,7-triol ((3*R*)-22) and (3*R*)-6,7-Dihydroxycitronellic Acid ((3*R*)-23). (*R*)-Citronellol (*(R)*-**18**, 1 mmol) or (*R*)-citronellic acid *(R)*-**19** (1 mmol), respectively, was added drop wise to a solution of hydrogen peroxide (1.2 mmol) in formic acid (10 mL) and, then stirred at 70°C for 2 h. After separating the solvent under reduced pressure, sodium hydroxide solution (1 mL; 20%, w/w) was added. The reaction products were isolated by means of RP-18 MPLC. After removing the solvent under reduced pressure and lyophilisation, the reaction product (*3R*)-6,7-dihydroxycitronellic acid (*(3R)*-**23**) was obtained in a purity of >98% (HPLC-DAD/ELSD) and its structure confirmed by UPLC-TOF-MS and NMR. To isolate (*3R*)-3,7-dimethyloctane-1,6,7-triol (*(3R)*-**22**), methanol was removed under reduced pressure and the aqueous extract extracted with ethyl acetate (3x100 mL). The organic layer was dried over sodium sulfate and the solvent evaporated in vacuum to afford the target product *(3R)*-**22** in high purity of >98% (HPLC-DAD/ELSD) and its structure confirmed by UPLC-TOF-MS and NMR. While the (*R*)-configuration of the carbon atom 3 of the educt is expected to be retained, the non-enantioselective synthetic procedure used is expected to lead to a racemic (*R*/*S*)-mixture at carbon atoms 6 and 7 of the products.

(3R)-3,7-Dimethyloctane-1,6,7-triol ((3R)-6,7-dihydroxycitronellol), *(3R)*-**22**, **Figure 3**: LC- MS (ESI$^+$): *m/z* (%) 124 (50, [M+Na]$^+$), 191.0 (100, [M+H]$^+$), 175 (80, [M-OH]$^+$), 155 (80, [M-H_3O_2]$^+$). ^{1}H NMR (400 MHz, $CDCl_3$, COSY): δ [ppm] 0.90 [d, 3H, J = 6.2 Hz, H-C(10a)], 0.92 [d, 3H, J = 6.2 Hz, H-C(10b)], 1.15 [s, 6H, H-C(8)], 1.20 [s, 6H, H-C(9)], 1.22-1.28 [m, 1H, H-C(5a)], 1.28-1.49 [m, 4H, H-C(2a), H-C(5b),

H-C(5c)], 1.50-1.62 [m, 5H, H-C(2b), H-C(3a), H-C(5d)], 1.62-1.73 [m, 2H, H-C(3b)], 3.31 [dd, 1H, J = 1.7, 10.2 Hz, H-C(6a)], 3.35 [dd, 1H, J = 1.7, 10.2 Hz, H-C(6b)], 3.58-3.78 [m, 4H, H-C(1)]. ^{13}C NMR (100 MHz, $CDCl_3$, HSQC, HMBC): δ [ppm] 19.6 [CH_3, C(10a)], 19.9 [CH_3, C(10b)], 23.1 [CH_3, C(9a)], 23.2 [CH_3, C(9b)], 26.4 [CH_3, C(8)], 28.4 [CH_2, C(5a)], 28.7 [CH, C(3a)], 29.2 [CH_2, C(5b)], 29.7 [CH, C(3b)], 33.2 [CH_2, C(4a)], 33.9 [CH_2, C(4b)], 39.5 [CH_2, C(2a)], 39.8 [CH_2, C(2b)], 60.6 [CH_2, C(1a)], 60.6 [CH_2, C(1b)], 73.25 [C, C(7a)], 73.26 [C, C(7b)], 78.2 [CH, C(6a)], 79.0 [CH, C(6b)].

(3R)-6,7-Dihydroxycitronellic acid, (3R)-**23**, **Figure 3**: UPLC-TOF-MS (ESI^-): *m/z* 203.1283 ([M-H]-, measured), *m/z* 203.1291 ($[M-H]^-$, calculated for $C_{10}H_{19}O_4$). ^{1}H NMR (500 MHz, DMSO-d_6, COSY): δ [ppm] 0.86 [d, 3H, J = 7.0 Hz, H-C(10a)], 0.88 [d, 3H, J = 7.0 Hz,. H-C(10b)], 0.98 [s, 6H, H-C(8)], 1.03 [s, 6H, H-C(9)], 1.04-1.11 [m, 2H, H-C(4a), H-C(5a)], 1.11-1.18 [m, 1H, H-C(5b)], 1.21-1.29 [m, 1H, H-C(4b)], 1.31-1.40 [m, 1H, H-C(4c)], 1.43-1.50 [m, 1H, H-C(5c)], 1.50-1.59 [m, 2H, H-C(4d), H-C(5d)], 1.75-1.86 [m, 2H, H-C(3)], 1.93 [dd, 1H, J = 8.3, 14.5 Hz, H-C(2a)], 1.96 [dd, 1H, J = 8.3, 14.5 Hz, H-C(2b)], 2.15 [dd, 1H, J = 5.7, 11.4 Hz, H-C(2c)], 2.18 [dd, 1H, J = 5.8, 11.4 Hz, H-C(2d)], 2.95-3.08 [m, 2H, H-C(6)]. ^{13}C NMR (125 MHz, DMSO-d_6, HSQC, HMBC): δ [ppm] 19.6 [CH_3, C(10a)], 20.0 [CH_3, C(10b)], 24.49 [CH_3, C(9a)], 24.51 [CH_3, C(9b)], 26.3 [CH_3, C(8)], 28.23 [CH_2, C(5a)], 28.24 [CH_2, C(5b)], 29.9 [CH, C(3a)], 30.1 [CH, C(3b)], 33.7 [CH_2, C(4a)], 33.9 [CH_2, C(4b)], 42.0 [CH_2, C(2a)], 42.5 [CH_2, C(2b)], 71.61 [C, C(7a)], 71.64 [C, C(7b)], 77.5 [CH, C(6a)], 77.7 [CH, C(6b)], 174.5 [C, C(1)].

Synthesis of (3*R*)-3-Methyl-5-(2,2,5,5-tetramethyl-1,3-dioxolan-4-yl) pentan-1-ol ((3*R*)-24). Using a literature procedure with some modifications,[36] (*3R*)-3,7-dimethyloctane-1,6,7-triol (*(3R)*-**22**, 0.7 mmol) and *p*-toluensulfonic acid (0.7 mmol) are dissolved in dried acetone (5 mL). After stirring at room temperature for 24

h, the crude reaction mixture was added to an aqueous ammonium hydroxide solution (25%, v/v; 20 mL), and the product was isolated by extraction with diethyl ether (3x50 mL). The combined organic layers were washed with water (2x150 mL) and brine (1x150 mL) and, after drying over sodium sulfate, the solvent was removed under reduced pressure to afford the target compound in a purity of >98% (HPLC-DAD/ELSD) and the structure confirmed by NMR experiments.

*(3R)-3-Methyl-5-(2,2,5,5-tetramethyl-1,3-dioxolan-4-yl)pentan-1-ol, (3R)-***24***,* **Figure 3**: ^{1}H NMR (400 MHz, benzene-d_6, COSY): δ [ppm]. 0.82 [d, 3H, J = 6.6 Hz, H-C(10a)], 0.82 [d, 3H, J = 6.6 Hz, H-C(10b)], 1.079 [s, 3H, H-C(8/9)], 1.082 [s, 3H, H-C(8/9)], 1.156 [s, 3H, H-C(8/9)], 1.163 [s, 3H, H-C(8/9)], 1.14-1.27 [m, 4H, H-C(5a), H-C(2a)], 1.27-1.35 [m, 3H, H-C(5b)], 1.365 [s, 3H, H-C(12/13)], 1.367 [s, 3H, H-C(12/13)], 1.40-1.47 [m, 3H, H-C(5c), H-C2b)], 1.48 [s, 6H, H-C(12/13)], 1.47-1.70 [m, 9H, H-C(5d), H-C(3), H-C(4)], 3.33-3.47 [m, 4H, H-C(1)], 3.62 [dd, 1H, J = 1.9, 3.4 Hz, H-C(6a)], 3.64 [dd, 1H, J = 2.1, 3.3 Hz, H-C(6b)]. ^{13}C NMR (101 MHz, benzene-d_6, HSQC, HMBC): δ [ppm] 19.8 [CH_3, C(10)], 23.2 [CH_3, C(8/9)], 26.3 [CH_3, C(8/9)], 26.4 [CH_3, C(8/9)], 27.2 [CH_2, C(4)], 27.4 [CH_3, C(12)], 29.0 [CH_3, C(13)], 29.7 [CH, C(3)], 30.0, 34.7 [CH_2, C(5a)], 34.8 [CH_2, C(5b)], 40.0 [CH_2, C(2a)], 40.1 [CH_2, C(2b)], 60.7 [CH_2, C(1a)], 60.6 [CH_2, C(1b)], 80.1 [C, C(7)], 84.0 [CH, C(6a)], 84.2 [CH, C(6b)], 106.6 [C, C(11a)], 106.7 [C, C(11b)].

Synthesis of (*R*)-7-Hydroxycitronellal ((*R*)-25) and (3R)-6,7-Hydroxycitronellal ((3*R*)-26). Using a literature procedure with some modifications,[37] a solution of (*R*)-**20** and (3*R*)-**24** (1 mmol), respectively, in dried dichloromethane (0.8 mL) was added dropwise to a solution of Dess-Martin periodinane (1.2 mmol) in dried dichloromethane (4 mL). After 1 h, the solution was diluted with diethyl ether (50 mL) and extracted with an aqueous sodium thiosulfate solution (50 mL; 2 M, saturated with sodium hydrogencarbonate). The aqueous

phase was extracted twice with diethylether (50 mL), the combined organic phases were extracted with of aqueous sodium hydrogencarbonate solution (200 mL), followed by water (200 mL). After drying the organic phase over sodium sulfate, the solvent was separated in vacuum and (*R*)-**25** (purity: >98%, HPLC-ELSD) isolated by means of RP-18 MPLC. To obtain *(3R)*-**26**, the acetonide was hydrolyzed as reported earlier.[38] The product was dissolved in a mixture of THF and water (4+1, v/v) in presence of Amberlyst-15 (300 mg) and is stirred at room temperature for 16 h. After filtration, the reaction product (3*R*)-**26** was isolated by RP-18 MPLC and its structure confirmed by means of UPLC-TOF-MS and NMR experiments. While the (*R*)-configuration of the carbon atom 3 of the educt is expected to be retained, the non-enantioselective synthetic procedure used is expected to lead to a racemic (*R*/*S*)-mixture at carbon atoms 6 and 7 of the product.

*(3R)-7-Hydroxycitronellal, (R)-***25***,* **Figure 3***:* UPLC-TOF-MS (ESI^+): *m/z* 173.1520 ($[M+H]^+$, measured), *m/z* 173.1536 ($[M+H]^+$, calculated for $C_{10}H_{21}O_2$). ^1H NMR (400 MHz, DMSO-d_6, COSY): δ [ppm] 0.89 [d, 3H, J = 6.7 Hz, H-C(10)], 1.06 [s, 6H, H-C(8), H-C(9)], 1.17-1.26 [m, 1H, H-C(4a)], 1.27-1.36 [m, 5H, H-C(4b), H-C(5), H-C(6)], 1.91-2.04 [m, 1H, H-C(3)], 2.21 [ddd, 1H, J = 2.5, 7.8, 16.3 Hz, H-C(2a], 2.39 [ddd, 1H, J = 2.3, 5.7, 16.2 Hz, H-C(2b)], 9.66 [t, J = 2.3 Hz, 1H, H-C(1)]. ^{13}C NMR (100MHz, DMSO-d_6, HSQC, HMBC): δ [ppm] 19.0 [CH_3, C(10)], 21.7 [CH_2, C(5)], 27.9 [CH, C(3)], 29.7, 29.8 [CH_3, C(8), C(9)], 37.5 [CH_2, C(4)], 44.2 [CH_2, C(6)], 50.8 [CH_2, C(2)], 69.2 [C, C(7)], 204.2[C, C(1)].

*(3R)-6,7-Hydroxycitronellal, (3R)-***26**, **Figure 3**: UPLC-TOF-MS (ESI^+): *m/z* 189.1502 ($[M+H]^+$, measured), *m/z* 189.1485 ($[M+H]^+$, calculated for $C_{10}H_{21}O_3$). ^1H NMR (500 MHz, $CDCl_3$, COSY): δ [ppm] 0.88 [d, 3H, J = 6.5 Hz, H-C(10a)], 0.92 [d, 3H, J = 6.5 Hz,. H-C(10b)], 1.16 [m, 14H, H-C(8), H-C(9), H-C(5a), H-C(5b)], 1.35-1.58 [m, 1H, H-C(4a)], 1.39-1.51 [m, 1H, H-C(4b)], 1.40-1.58 [m, 2H, H-C(4c), H-

C(5c)], 1.59-1.68 [m, 3H, H-C(5c), H-C(3)], 2.23-2.39 [m, 2H, H-C(2a)], 2.40-2.48 [m, 2H, H-C(2b)], 3.33-3.40 [m, 2H, H-C(6)], 9.77 [t, 2H, J = 2.0 Hz, H-C(1)]. ^{13}C NMR (126 MHz, $CDCl_3$, HSQC, HMBC) δ [ppm] 19.6 [CH_3, C(10a)], 19.9 [CH_3, C(10b)], 23.19 [CH_3, C(9a)], 23.24[CH_3, C(9b)], 26.5 [CH_3, C(8)], 28.70 [CH_2, C(4a)], 29.1 [CH_2, C(4b)], 29.77 [CH, C(3a)], 29.81 [CH, C(3b)], 33.7 [CH_2, C(5a)], 34.1 [CH_2, C(5b)], 39.56 [CH_2, C(2a)], 39.83 [CH_2, C(2b)], 73.06 [C, C(7a)], 73.11 [C, C(7b)], 78.57 [CH, C(6b)], 79.10 [CH, C(6b)], 202.85 [CH, C(1)].

Cell-based Bitter Taste Receptor Experiments. To assess whether oxygenated monoterpenes directly target human bitter taste receptors to reduce caffeine bitterness, we implemented functional expression of TAS2Rs in HEK293T Gα16gust44 cells as described previously.[8,39] As five members of the family of human bitter taste receptors, namely TAS2R7, TAS2R10, TAS2R14, TAS2R43, and TAS2R46, have been reported to be sensitive to caffeine,[2] cells were transfected with expression constructs for TAS2R7, TAS2R10, TAS2R14, TAS2R43, and TAS2R46 using Lipofectamine2000 (Invitrogen). After 22 h, cells were loaded with the calcium-sensitive fluorescent dye Fluo4-AM and incubated for 1.5 h at 37°C. Excess dye was removed by subsequent washing with bath solution (130 mM NaCl, 5 mM KCl, 10 mM Hepes, 2 mM $CaCl_2$, and 10 mM glucose (pH 7.4)). Test compounds were dissolved in bath solution, and pre-mixed for co-application. Changes in cytosolic calcium levels to automated application of test compounds were monitored with a FLIPR Tetra ICCD (Molecular Devices). Fluorescence signals were corrected for responses of mock-transfected cells and normalized to background fluorescence. Concentration-response relations and values for half-maximal receptor activation (EC_{50}) were calculated in SigmaPlot (SPSS) by nonlinear regression using the function: $f(y) = min + (max-min)/(1 + (x/EC_{50})^{-Hillslope})$. Data were collected from at least three independent experiments carried out in duplicates.

Medium Pressure Liquid Chromatography (MPLC). Medium pressure liquid chromatography was performed on a Sepacore chromatography system (Buechi, Flawil, Switzerland) consisting of two C-605 pumps with a C-615 pump manager, a manual rheodyne injection port (20 mL loop), a C-635 UV/Vis detector, and a C-660 fraction collector. Before the eluent was directed into the fraction collector, an aliquot (1 mL) of the eluent was split into a Sedex LT-ELSD Model 85 detector (Sedere, Alfortville, France). Büchi Sepacore Record 1.0 (Büchi Labortechnik AG, Flawil, Switzerland) software was used for data acquisition. Chromatography was performed with a flow rate of 40 mL/min on a self-packed 150 x 40 mm i.d. polypropylene cartridge filled with 25-40 µm LiChroprep RP-18 Material (Merck KGaA, Darmstadt, Germany), monitoring the eluent on the ELS detector and using 0.1% aqueous formic acid (solvent A) and methanol (solvent B) as eluents. After sample injection (5 mL), isocratic elution with 40% solvent A was performed for 3 min. Subsequently, the solvent B was increased linearly to 75% within 20 min, then to 100% within 3min and maintained at 100% for 5 min. After separating the organic solvent under reduced pressure, fractions containing the product were extracted three times with ethyl acetate (100 mL) and, after drying over sodium sulfate, the solvent was removed under reduced pressure.

High-Performance Liquid Chromatography (HPLC). Analytical HPLC was carried out with an HPLC apparatus (Jasco, Groß-Umstadt, Germany), consisting of two PU-2087 Plus Pumps, a M800 gradient mixer, a DG-2080-53 degasser, an auto sampler (AS-2055 Plus), a diode array detector (MD-2010 Plus) and a Sedex LT-ELSD Model 85 (Sedere, Alfortville, France). Chrompass Software was used for data acquisition. With a flow rate of 0.8 mL/min, chromatography was performed on a 250 x 4.6mm i.d., 5 µm, Microsorb-MW 100-5 C18 column (Varian, Darmstadt, Germany), monitoring the eluent at 220 and 260 nm and using 1% aqueous formic acid (solvent

A) and methanol (solvent B) as eluents. After sample injection (10 µL), isocratic elution with 100% solvent A was performed for 5 min. Subsequently, the content of organic solvent was linearly increased to 100% within 25 min, maintaining 100% solvent B for additional 5 min.

High-Performance Liquid Chromatography/Mass Spectroscopy (HPLC-MS/MS). For HPLC-ESI-MS/MS analysis, a Dionex UHPLC UltiMate® 3000 HPLC (Dionex, Idstein), consisting of a binary pump (HPG-3400SD), a degasser (SRD-3400), an autosampler (WSP-3000TSL), a column compartment (TCC-3000SD), and operating with the DC MS Link 2.8.0.2633 software, was linked to an API 4000 QTRAP mass spectrometer (AB Sciex Instruments, Darmstadt, Germany), which was equipped with an electrospray ionization (ESI) source with direct flow infusion. Nitrogen served as nebulizer gas (3.4 bar), and, heated at 400°C, as turbo gas for solvent drying (4.1 bar). Nitrogen also served as curtain (1.3 bar) and collision gas (0.06 bar). The ion spray voltage was set at -4500 V in the negative mode and at 5500 V in the positive mode. The mass spectrometer was operated in the full-scan mode detecting positive or negative ions.

UPLC-Time-of-Flight Mass Spectrometry (UPLC/TOF-MS). Mass spectra of the compounds were measured on a Waters Synapt G2 HDMS mass spectrometer (Waters, Manchester, UK) coupled to an Acquity UPLC core system (Waters) consisting of a binary solvent manager, sample manager and column oven. The compounds were dissolved in methanol (1 mL) and aliquots (1-5 µL) were injected into the UPLC-TOF-MS system equipped with a 150 x 2 mm i.d., 1.7µm, BEH C18 column (Waters). Operated with a flow rate of 0.3 mL/min at a temperature of 40°C, the following gradient was used for chromatography: starting with a mixture (5/95, v/v) of acetonitrile and aqueous formic acid (0.1%, pH 2.5), the acetonitrile content was increased to 95% within 3 min and held at 95% for 1 min. Calibration of the

Synapt G2 was performed using a solution of sodium formate (0.5 mmol/L) in 2-propanol/water (9/1, v/v). Data was processed using MassLynx 4.1 (Waters) and the tool elemental composition. All data was lock mass (leucine enkephalin) corrected ($[M+H]^+$, m/z 556.2771, $[M-H]^-$, m/z 554.2615). Measurements were performed using negative ESI and the resolution modus consisting of the following parameters: capillary voltage (+2.5 or -3.0 kV), sampling cone (30 V), extraction cone (4.0 V), source temperature (150°C), desolvation temperature (450°C), cone gas (30 L/h), and desolvation gas (850 L/h).

Nuclear Magnetic Resonance Spectroscopy (NMR). The ^{1}H, ^{13}C, and 2D-NMR-spectroscopic experiments were performed on a 500 MHz Avance III NMR spectrometer from Bruker (Rheinstetten, Germany), equipped with a a triple res. cryo probe (TCI) and and a DRX or a Avance-III-400 (400 MHz) spectrometer equipped with a QNP or a Broadband Observe BBFOplus probe (Bruker, Rheinstetten, Germany). Unless stated otherwise, all experiments were performed at 300 K. Samples were dissolved in deuterated chloroform ($CDCl_3$), deuterated dimethylsulfoxide (d_6-DMSO), deuterated benzene (benzene-d_6) or deuterated methanol (CD_3OD). For accurate NMR signal assignment, COSY, HSQC, and HMBC experiments were carried out using pulse sequences of the Bruker software library. While data processing was performed using Topspin Version 1.3 (Bruker, Rheinstetten, Germany), the individual data interpretation was done with MestReNova 5.1.0-2940 (Mestrelab Research S.L., Santiago de Compostela, Spain).

RESULTS AND DISCUSSION

To reduce the typical bitter taste of black tea infusions, many consumers add milk thus decreasing the concentration of "free" bitter compounds available for taste receptor activation by non-covalent binding to milk proteins.[40] In our search for food-derived molecules that inhibit the bitter taste of foods and beverages like tea, preliminary sensory studies recently gave indication that lemon grass extract is able to reduce the perceived bitter taste of tea (*data not published*). To further substantiate this observation and to identify the active principle in lemon grass, first, more detailed sensory studies were performed in the following.

Lemon Balm and Lemon Grass Attenuate Perceived Bitterness of a Black Tea Infusion. Black tea beverages were spiked with lemon balm or lemon grass extract, respectively, and were sensorially compared to a beverage without any additive (control) by means of a trained panel wearing nose clips to exclude any olfactory cues. Using a three-alternative forced choice (3-AFC) test, the panelists were asked to identify the least bitter tasting sample. Both tea infusions containing either lemon balm, or lemon grass were identified as significantly ($\alpha = 0.001$) less bitter when compared to the standard tea infusion (**Table 1**). To study the potential impact of volatiles in inducing the perceived bitterness reduction, the volatile fraction was isolated from the lemon grass extract by means of SAFE distillation. Spiking this volatile fraction to the standard tea infusion still induced a decrease in bitterness ($\alpha = 0.05$). Confirming literature knowledge,[41] GC/MS analysis revealed (*R*)-citronellal (**17**; **Figure 2**) as a major constituent in the volatile fraction isolated from lemon grass (*data not shown*). Therefore, another sensory experiment aimed at evaluating the bitterness inhibitory potential of this monoterpene aldehyde. Indeed, the perceived bitterness of the tea infusion was significantly decreased in the presence of racemic citronellal (**17**) and (*R*)-citronellal ((*R*)-**17**) even at 50 ppm ($\alpha = 0.01$), whereas the

corresponding (*S*)-citronellal ((*S*)-**17**) did not show any significant effects (α = 0.2; **Table 1**).

Synthesis of Citronellal-Related Compounds. To gain some more insight into the structural requirements of citronellal (**17**) to affect bitterness perception of caffeine, a series of mono- and dihydroxylated monoterpenes was synthesized (**Figure 2**). Since sensory experiments revealed bitterness inhibitory potential for (*R*)-citronellal (**Figure 2A**) only but not for (*S*)-citronellal (**Table 1**), the following syntheses were limited to the (*R*)-stereoisomer. To introduce an additional hydroxyl function, water was added to the double bond at position 6 and 7 of (*R*)-citronellol ((*R*)-**18**) and (*R*)-citronellic acid ((*R*)-**19**) in strongly acidic reaction medium to result in 7-hydroxycitronellol ((*R*)-**20**) and 7-hydroxycitronellic acid ((*R*)-**21**), respectively (**Figure 2B**) with a yield of 46 and 66%, respectively. Dihydroxylation of the double bond of (*R*)-**18** and (*R*)-**19**, respectively, was accomplished by epoxidation with hydrogen peroxide and formic acid and subsequent hydrolysis to give (3*R*)-6,7-dihydroxycitronellol ((3*R*)-**22**)) in a yield of 44% and (3*R*)-6,7-dihydroxycitronellic acid ((3*R*)-**23**)) in a yield of 44% (**Figure 2C**). Furthermore, (*R*)-**18** was oxidized to (*R*)-7-hydroxycitronellal ((*R*)-**25**) in a yield of 34% (**Figure 2D**). To oxidize (3*R*)-**22** to the corresponding aldehyde, the vicinal hydroxyl functions were protected as 1,2-acetonide, followed by Dess Martin periodinane oxidation, and subsequent hydrolytic deprotection, thus affording (3*R*)-6,7-dihydroxycitronellal ((3*R*)-**26**) in a yield of 12%, (**Figure 2E**). In total, a series of nine monoterpene derivatives (**17**-**22**, **25**, **26**), differing both in the degree of hydroxylation at position 6 and 7, as well as in the oxidation status at position 1 were prepared and purified to evaluate their caffeine bitterness inhibitory activity.

Human Sensory Evaluation of Citronellal and Related Monoterpenes. To investigate the bitter inhibitory potential of the monoterpenoids **17**-**22**, **25**, and **26**, an

appropriate sensory assay was developed. Adapting the principles of sensory tests allowing for the evaluation of umami enhancers,[32-35] aqueous solutions containing caffeine (6 mmol/L) and a constant concentration of the test compound were compared to five solutions of increasing caffeine concentration in duo tests applying forced choice conditions. Panelists were asked to name the sample showing the less intense bitter taste. The sensory responses were transformed into the percentage of panelists rating the monoterpene containing sample as less bitter compared to the caffeine sample. After *Probit* transformation, the value corresponding to 50% of positive answers was calculated and considered to be the iso-intensity concentration at which the bitterness of the reference and the test solution could not be differentiated.

In order to test the accuracy of this experimental design, a series of pure caffeine solutions was offered to the sensory panel as pseudo-test solutions. In three independent sessions, test solutions containing 4.0, 5.0 and 6.0 mmol/L caffeine, respectively, were assessed with an isointense caffeine concentration of 3.8, 5.0, and 5.9 mmol/L, thus demonstrating the high precision of the sensory test. Therefore, the sensory test was considered fit for purpose to evaluate the bitter inhibiting potential of the monoterpenes **17**-**22**, **25**, and **26**, respectively.

In a first set of experiments, the impact of the stereochemical configuration in position 3 of citronellal (**17**) was evaluated by comparing racemic **17** against the corresponding (*R*)- and (*S*)-stereoisomers. The bitterness perception of caffeine was reduced by 18% by 100 ppm racemic **17** and 28% by 100 ppm (*R*)-**17**, whereas the same concentration of (*S*)-**17** did only show a marginal effect, e.g. the bitterness of the 6 mmol/L caffeine solution was perceived to be isointense to a 5.4 mmol/L caffeine solution (**Table 2**). A further decrease of the concentration of (*R*)-**17** from 100 ppm to 50 and 25 ppm did not result in an increase in caffeine's perceived bitter

intensity, thus suggesting that the bitter inhibiting potential of (*R*)-**17** was fully exploited even at the lowest concentration tested.

Modification of the oxidation status at carbon atom 1 had a fundamental impact on the bitter inhibitory activity, e.g. oxidation of the aldehyde (*R*)-**17** (28% bitterness reduction) to the carboxylic acid (*R*)-**19** showed only somewhat lower bitterness inhibitory activity of 21%, whereas reduction to the alcohol (*R*)-**18** completely diminished its inhibitor activity (**Table 2**). Similarly, all other monoterpene alcohols tested, namely (*R*)-**18**, (*R*)-**20**, and (3*R*)-**22**, did not affect caffeine bitterness perception. In the group of citronellic acids, the bitter inhibiting activity decreased with increasing hydrophilicity, e.g. (*R*)-**19** lacking any hydroxyl group in the carbon chain showed a 21% bitterness reduction, while introduction of one hydroxyl group ((*R*)-**20**) induced a decreased inhibitory effect with a 15% reduction and the dihydroxylated (3*R*)-**23** did not affect caffeine bitterness at all (**Table 2**). In comparison, within the series of aldehydes, 100 ppm of citronellal ((*R*)-**17**) as well as the mono- ((*R*)-**25**) and dihydroxylated aldehydes ((*3R*)-**26**) showed similar bitter inhibitory activity of 28, 25, and 28%, respectively (**Table 2**). Decreasing the concentration of (*R*)-**17** and (*R*)-**25** to 50 and 25 ppm revealed a difference between both compounds only at 25 ppm. While (*R*)-**17** still showed a high bitter inhibition of 32%, the perceived caffeine bitterness was reduced by (*R*)-**25** (25 ppm) only by 17%, thus indicating lower activity with increasing hydroxylation degree as observed for the citronellic acids.

Cell-based Bitter Taste Receptor Experiments. In order to identify the molecular target responsible for the citronellal-mediated reduction of the bitterness of caffeine, cell-based functional experiments were conducted with human bitter taste receptors. As TAS2R7, TAS2R10, TAS2R14, TAS2R43, and TAS2R46 have been reported to be sensitive to caffeine,[5] these five TAS2Rs were functionally expressed in heterologous cells, challenged with caffeine (3.0 mmol/L), and the calcium

responses were monitored in the absence and presence of (*R*)-**17** and (*S*)-**17**. At a concentration of 0.50 mmol/L, stereoisomer (*S*)-**17**, which lacked any significant bitter inhibitory potential (**Table 2**), induced strong calcium responses in mock-transfected cells preventing the assessment of its potential to block activation of the five bitter taste receptors (*data not shown*). In comparison, (*R*)-**17** did not generate any fluorescence signals at 0.5 mmol/L in mock-transfected cells, thus enabling the examination of its bitter inhibitory activity. Cells transfected with cDNAs for TAS2R7, -10, -14, -43, or -46 responded to 3.0 mmol/L caffeine by increasing their intracellular calcium concentrations, albeit with greatly different signal amplitudes (**Figure 3A**). The comparatively high signal amplitudes of TAS2R43 and TAS2R46 to 3.0 mmol/L caffeine might indicate a major role of these two receptors for the perceptual sensitivity to caffeine bitterness. Co-application of (*R*)-**17** with caffeine completely blocked the caffeine-induced calcium signals in TAS2R43-expressing cells, and to a lesser extent in TAS2R46-expressing cells (**Figures 3 A, B**). Surprisingly, two receptors, namely TAS2R7 and TAS2R14, showed a robust calcium increase upon sole application of (*R*)-**17** (**Figures 3 A, C**). However, only cells transfected with TAS2R14 elicited the typical immediate and transient fluorescence signal, suggesting that (*R*)-**17** acts as an agonist on this bitter taste receptor. Indeed, a similar ambivalent nature in terms of selective suppression of few TAS2Rs, but contrariwise activation of other members of this family, has also been revealed for natural sesquiterpene lactones and flavonones.[10,24,25] Since other studies on bitter taste receptor antagonists lack analyses on their potential agonistic properties, it still remains to be determined, if such ambivalent effects concern a unique feature of few compounds or were inherent to all TAS2R inhibitors.

To examine its inhibitory potential in more detail, inhibition responses to (*R*)-**17** were recorded next. Cells expressing TAS2R43 or TAS2R46 were challenged with

(*R*)-**17** up to 0.60 mmol/L, and a fixed concentration of 3.0 mmol/L caffeine (**Figure 3D**). Caffeine exhibits similar potency at both receptors, indicated by EC_{50} values of about 0.94 ± 0.14 mmol/L (TAS2R43; n=4; **Figure 4A**) and 1.35 ± 0.32 mmol/L (TAS2R46; n=3; data not shown). Intriguingly, the cellular calcium responses to caffeine mediated by TAS2R43 started to be significantly reduced in the presence of 0.03 mmol/L (*R*)-**17**, and were entirely blocked at 0.3 mmol/L (*R*)-**17** (**Figure 3D**). The half-maximal inhibitory concentration (IC_{50}) amounted to 0.084 ± 0.012 mmol/L (*R*)-**17** (n=4). The caffeine response of TAS2R46-expressing cells was significantly attenuated only by co-application of 0.6 mmol/L (*R*)-**17** (**Figure 3D**), indicating different sensitivities of the two sequence-conserved bitter taste receptors for the monoterpene antagonist. These findings are consistent with previous studies which demonstrated a partial overlap of the TAS2R43 and TAS2R46 bitter agonist and antagonist repertoires.[2,24,42]

Next, we recorded concentration responses from TAS2R43-expressing cells to caffeine in the absence and in the presence of increasing concentrations of (*R*)-**17** in order to determine the type of antagonism on hand (**Figure 4A**). Co-application of the monoterpene caused parallel right-shifts of the response function and diminished the maximum response amplitude R_{max} of the agonist caffeine, suggesting that (*R*)-**17** acts as an allosteric inhibitor on TAS2R43. The changed potency of caffeine can be explained by cooperative effects, which have been described to occur with allosteric interactions.[43]

In order to assess whether the inhibitory effect of (*R*)-**17** on TAS2R43 is specific for caffeine, cells were stimulated with EC_{90}-concentrations of four additional bitter agonists of distinct chemical classes. Like responses to caffeine, those to the glucopyranoside helicin, the quaternary ammonium compound denatonium benzoate, and the benzothiazole saccharin were completely blocked by co-application of 0.3

mmol/L (*R*)-**17** (**Figure 4B**). Calcium transients elicited by the most potent agonist, the phenanthrene aristolochic acid I, were only partially attenuated at this antagonist concentration, but completely suppressed when inhibitor concentration was raised to the highest applicable concentration of 0.6 mmol/L (**Figure 4B**). Therefore, the odorant (*R*)-citronellal ((*R*)-**17**) can be concluded to represent a general blocker of the TAS2R43 bitter taste receptor.

In order to investigate the TAS2R43 inhibitory activity of some structurally related odor-active aliphatic aldehyde, the following experiments were expanded to include the malty smelling propionaldehyde (**27**), isobutyraldehyde (**28**), isovaleraldehyde (**29**), and 2-methylbutyraldehyde (**30**), as well as the green, grassy smelling hexanal (**31**), all of which has been recently reported to be among the 230 key food odorants constructing the characteristic scent of foods and beverages (**Figure 5**).[44] In addition, racemic 3-methylhexanal (**32**) was tested as it contains the 3-methylalkylcarbaldehyde motif as found in citronellal. These test compounds were co-applied with caffeine (3.0 mmol/L) up to their highest applicable concentration (**Figure 6A**). Propionaldehyde, isobutyraldehyde, and hexanal showed no effect on the caffeine-induced TAS2R43-responses. Sole application of these compounds did not trigger calcium transients suggesting that aldehydes *per se* are incapable of interacting with TAS2R43 (**Figure 6B**). In contrast, co-application of caffeine with the two methyl-branched C4 aldehydes, isovaleraldehyde and 2-methylbutyraldehyde, led to significantly increased calcium responses (**Figure 6A**). This effect can be attributed to the ability of isovaleraldehyde (**29**) and, in particular, of 2-methylbutyraldehyde (**30**), to elicit transient calcium signals from TAS2R43-expressing cells, and hence, to act as agonists at this bitter receptor (**Figure 6B**). While hexanal (**31**) did not show any significant antagonistic effects, intriguingly, co-application of caffeine with 3-methylhexanal (**32**) efficiently and comprehensively

blocked caffeine-induced fluorescence (**Figure 6A**). With a threshold of 0.03 mmol/L and an IC_{50} value of 0.074 ± 0.026 mmol/L (n=3), 3-methylhexanal (**32**) turned out to be an equally potent antagonist of TAS2R43 compared to (*R*)-**17** (threshold: 0.03 mmol/L; IC_{50}: 0.084 ± 0.012 mmol/L, n=4). These data clearly indicate the 3-methyl-branched aliphatic aldehydes 3-methylhexanal and *(R)*-citronellal as best TAS2R43 antagonists, while isovaleraldehyde (**29**) and, in particular, of 2-methylbutyraldehyde (**30**) were found to function as TAS2R43 receptor agonists.

Taken together, our results support the role of TAS2R43, and, to a lesser extent, TAS2R46, as the molecular target of the natural monoterpene *(R)*-citronellal for attenuating the bitterness of caffeine. To the best of our knowledge, this study is the first to disclose the influence of odorants on bitter taste perception at the level of taste receptors. Previous research on the sensory interaction between odors and tastes though provided indication of specific mutual interference, mainly by compounds which are commonly encountered together in food or beverages. Most previous studies focussed on the taste-odor combination of sucrose and fruity odors like strawberry odor, caramel-like (e.g. 4-hydroxy-2,4-dimethyl-3(2*H*)-furanone) and vanilla-like odors (e.g. vaniline).[30,45-48] Sensory evaluation of such congruent mixtures suggested that the perceived intensity of the odor can be enhanced by sucrose, and to a lesser extent, the perceived sweetness of sucrose is selectively enhanced by some odors.[30,45-48] In comparison, suppression of taste by an odor component of a mixture has rarely been reported.[48] However, the observed effects have been interpreted as the consequence of a central neuronal integration of odors and tastes to construct the perceived flavor,[46] and have not been considered as peripheral events at the level of reception. In this context, the findings of this study open up a new dimension regarding the interaction of the two chemical senses.

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Table 1. Impact of Lemon Balm Extract, Lemon Grass Extract, Lemon Grass Volatile Fraction, and Citronellal (**17**) on the Bitter Taste of a Black Tea Infusion.

Ingredient added to tea infusion[a]	**result of tringle test[f]**
lemon balm[b]	significantly less bitter (α = 0.001)
lemon grass[c]	significantly less bitter (α = 0.001)
lemon grass volatile fraction[d]	significantly less bitter (α = 0.05)
rac-citronellal (**17**, 50 ppm)[e]	significantly less bitter (α = 0.01)
(*R*)-citronellal ((*R*)-**17**, 50 ppm)[e]	significantly less bitter (α = 0.01)
(*S*)-citronellal ((*S*)-**17**, 50 ppm)[e]	no significant difference (α = 0.2)

[a] Black tea (2.0 g) was infused with boiling tap water (100 mL) for 4 min prior to filtration [b] Black tea (2.0 g) was mixed with lemon balm (4.0 g) and infused with boiling water (100 mL, 4 min) prior to tasting. [c] Black tea (2.0 g) was mixed with lemon grass (4.0 g) and infused with boiling water (100 mL, 4 min) prior to tasting. [d] An aliquot (50 mL) of a double strength fresh tea infusion (4 g/100 mL) was mixed with the aqueous, volatile SAFE[31] distillate (50 mL) obtained from a fresh lemon grass infusion (4g/100mL). [e] An aliquot (50 mL) of standard tea infusion (2 g/100 mL) was spiked with 50 ppm of racemic, (*R*)- and (*S*)-configured citronellal (**17**), respectively, prior to tasting. [f] For sensory analysis, the samples were presented to panellists at room temperature in a 3-alternative forced choice setting in comparison to the standard tea infusion (2.0 g/100 mL) in randomized order. First, the panellists were asked to identify the odd sample and, then, to describe the sensory difference.

Table 2. Impact of Oxygenated Monoterpenes on Perceived Caffeine Bitterness

monoterpene added (applied concentration)	isointense caffeine conc. [mmol/L][a]	rel. bitter reduction [%]
without addition	5.9	2
rac-Citronellal (100 ppm), **17**	4.9	19
(*S*)-Citronellal (100 ppm), (*S*)-**17**	5.4	10
(*R*)-Citronellal (100 ppm), (*R*)-**17**	4.3	28
(*R*)-Citronellal (50 ppm), (*R*)-**17**	4.1	32
(*R*)-Citronellal (25 ppm), (*R*)-**17**	4.1	32
(*R*)-Citronellol (100 ppm), (*R*)-**18**	5.9	2
(*R*)-Citronellic acid (100 ppm), (*R*)-**19**	4.7	21
(*R*)-7-Hydroxycitronellol (100 ppm), (*R*)-**20**	5.8	3
(*R*)-7-Hydroxycitronellic acid (100 ppm), (*R*)-**21**	5.1	15
(*3R*)-6,7-Diydroxycitronellol (100 ppm), (3*R*)-**22**	6.1	0
(*3R*)-6,7-Dihydroxycitronellic acid (100 ppm), (3*R*)-**23**	5.6	7
(*R*)-7-Hydroxycitronellal (100 ppm), (*R*)-**25**	4.1	32
(*R*)-7-Hydroxycitronellal (50 ppm), (*R*)-**25**	4.2	31
(*R*)-7-Hydroxycitronellal (25 ppm), (*R*)-**25**	5.0	17
(*3R*)-6,7-Dihydroxycitronellal (100 ppm), (3*R*)-**26**	4.3	28

[a]The target monoterpenes were dissolved in an aqueous caffeine solution (6 mmol/L; pH 6.0) containing 5% ethanol. Using duo tests and randomly coded cups, these solutions were compared to a series of 5% hydroethanolic reference solutions containing increasing caffeine concentrations (30% intervals). Assessors were asked to identify the solution with the weaker bitter taste, sensory data were transferred into the percentage of answers considering the monoterpene containing solution to have a less intense bitter taste. After *Probit* transformation, the caffeine concentration at which 50% panelists consider the test solution to be less bitter is calculated and considered to be the isointensity concentration of the monoterpene/caffeine solution in comparison to the pure caffeine reference solution.

1 **FIGURE LEGENDS**

2

Figure 1. Chemical structures of bitter inhibitors reported in literature: 2,4-dihydroxybenzoic acid *N*-(4-hydroxy-3-methoxy-benzyl) amide (**1**), [2]-gingerdione (**2**), [2]-dehydroisogingerdione (**3**), [3]-gingerdione (**4**), 4-(2,2,3-trimethylcyclopentyl)butanoic acid (**5**), probenecid (**6**), neodiosmin (**7**), homoeriodictyol (**8**), sterubin (**9**), eriodictyol (**10**), phloretin (**11**), sakuranetin (**12**), 6-methoxysakuranetin (**13**), jacoesidin (**14**), alapyridaine (**15**), and adenosine 5'-monophosphate (**16**).

Figure 2. (**A**) Chemical structure of (*R*)-citronellal ((*R*)-**17**) and synthesis of (**B**) the monohydroxylated derivatives (*R*)-**20** and (*R*)-**21**, (**C**) the dihydroxylated derivatives (3*R*)-**22** and (3*R*)-**23**, (**D**) the monohydroxy-monoterpene aldehyde (*R*)-**25**, and (**E**) the dihydroxy-monoterpene aldehyde (3*R*)-**26**.

Figure 3. Effect of *(R)*-**17** on functionally expressed human bitter taste receptors. (**A**) Calcium fluorescence ratio (ΔF/F) of HEK293T Gα16gust44-cells expressing TAS2R7, TAS2R10, TAS2R14, TAS2R43, or TAS2R46 upon stimulation with caffeine (3.0 mmol/L, white bars), *(R)*-**17** (0.5 mmol/L, black bars), or both (striped bars). *, $P < 0.05$ (t-test). (**B**) Calcium fluorescence traces of cells transfected with TAS2R43, TAS2R46, or empty vector upon bath application (↑) of caffeine, and co-application with *(R)*-**17** (0.5 mmol/L). Scale: y, 1000 counts; x, 150 sec. (**C**) Calcium fluorescence traces of cells transfected with cDNAs for TAS2R7, TAS2R10, TAS2R14, TAS2R43, TAS2R46, or empty vector upon bath application (↑) of *(R)*-**17** (0.5 mmol/L). Scale: y, 1500 counts; x, 150 sec. (**D**) Inhibition response curve of cells expressing TAS2R43 (black circles,

solid line; n=4), or TAS2R46 (black squares, dashed line; n=5) to caffeine (3.0 mmol/L) in the presence of *(R)*-**17** (0.001 – 0.6 mmol/L). *, P < 0.05 (one-way ANOVA [0.001-0.003 mM vs. 0.01/0.03/0.1/0.3/0.6 mM *(R)*-**17**], Bonferroni posthoc means comparison test with 5% α-risk level).

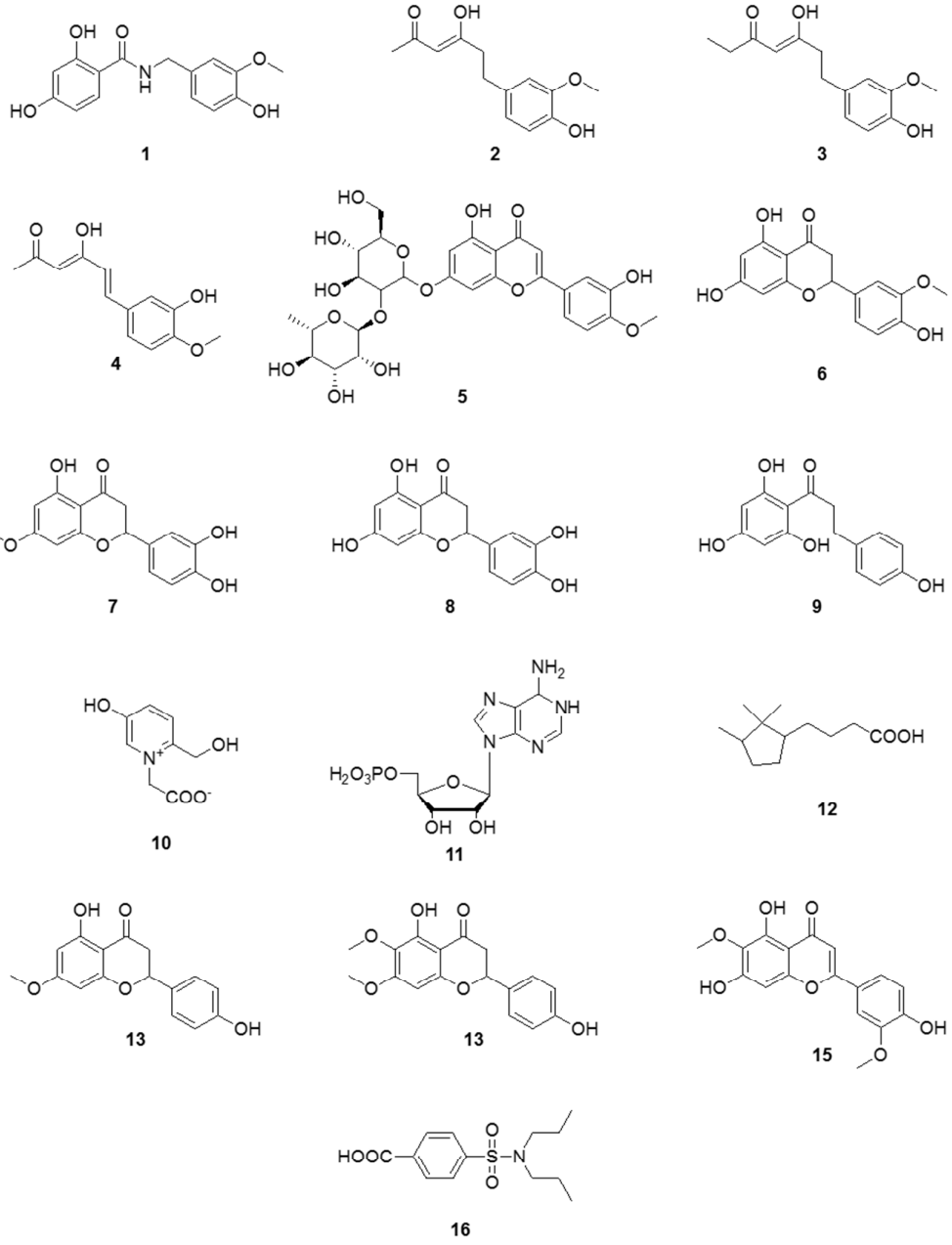
Figure 4. Characteristics of TAS2R43 inhibition by *(R)*-**17**. (**A**) Left, concentration response curves of TAS2R43-expressing cells to caffeine in the absence (white circles, dashed line) and in the presence of *(R)*-**17** (0.025 mmol/L *(R)*-**17**: light grey squares, light grey line. 0.075 mmol/L *(R)*-**17**: dark grey diamonds, dark grey line. 0.150 mmol/L *(R)*-**17**: black triangles, black line). N = 4. Right, EC_{50} values and maximum receptor responses (R_{max}) to caffeine in the absence and in the presence of *(R)*-**17**. *, P < 0.05 (one-way ANOVA [0 µM *(R)*-**17** vs. 25/75/150 µM *(R)*-**17**], Bonferroni posthoc means comparison test with 5% α-risk level. Repeated measures one-way ANOVA for R_{max}). >, value extrapolated. (**B**) Representative calcium fluorescence traces of TAS2R43-expressing cells upon sole application (↑) of cognate agonists (black lines. denat, denatonium benzoate. sacch, saccharin), and co-application with *(R)*-**17** (0.3 mmol/L, 2nd column: 0.6 mmol/L; grey lines). Scale: y, 2000 counts. x, 150 sec.

Figure 5. Chemical structures of odor-active aldehydes structurally related to citronellal (**17**): propionaldehyde (**27**), isobutyraldehyde (**28**), isovaleraldehyde (**29**), 2-methylbutyraldehyde (**30**), hexanal (**31**), and 3-methylhexanal (**32**).

Figure 6. Inhibitory potential of aldehydes structurally related to citronellal. (**A**) Inhibition response curves of TAS2R43-expressing cells stimulated with

3.0 mmol/L caffeine and propionaldehyde (**27**), isobutyraldehyde (**28**), isovaleraldehyde (**29**), 2-methylbutyraldehyde (**30**), hexanal (**31**), and 3-methylhexanal (**32**). *, P value < 0.05 (One-way ANOVA [caffeine 3 mM *vs*. co-application of aldehyde], Bonferroni posthoc means comparison test with 5% α-risk level). (**B**) Representative calcium fluorescence traces of TAS2R43-expressing cells upon sole application (↑) of aldehydes. Scale: y, 4000 counts. x, 200 sec.

Suess et al. (Figure 1)



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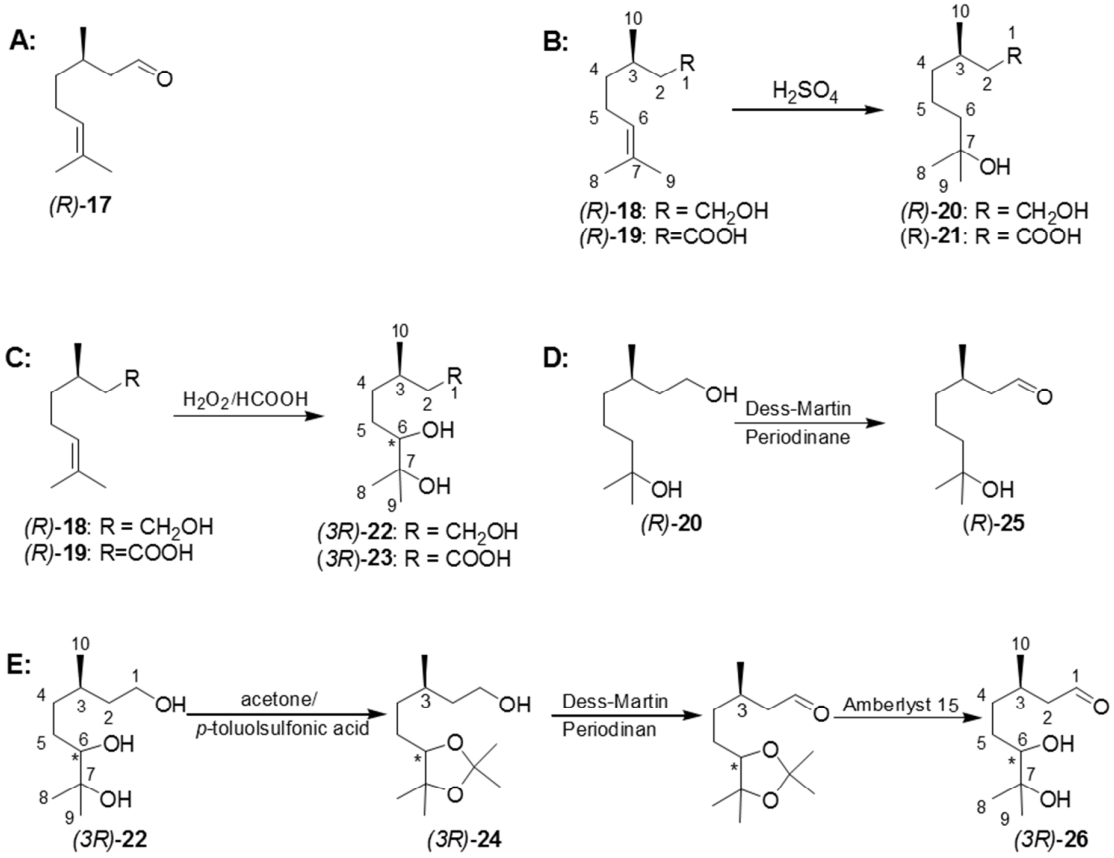

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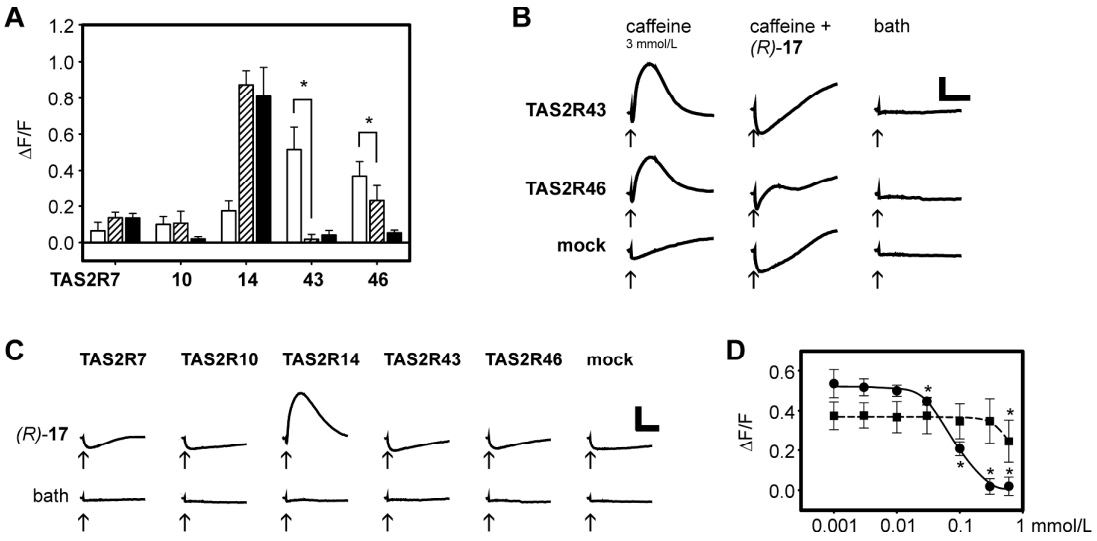

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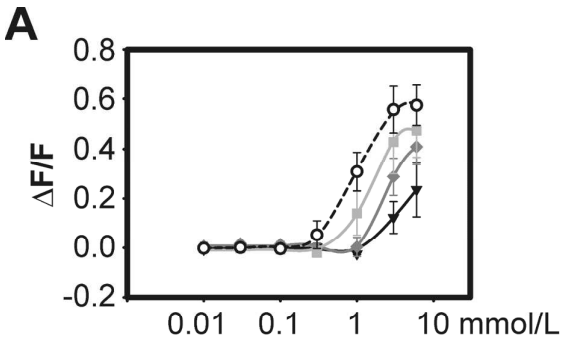
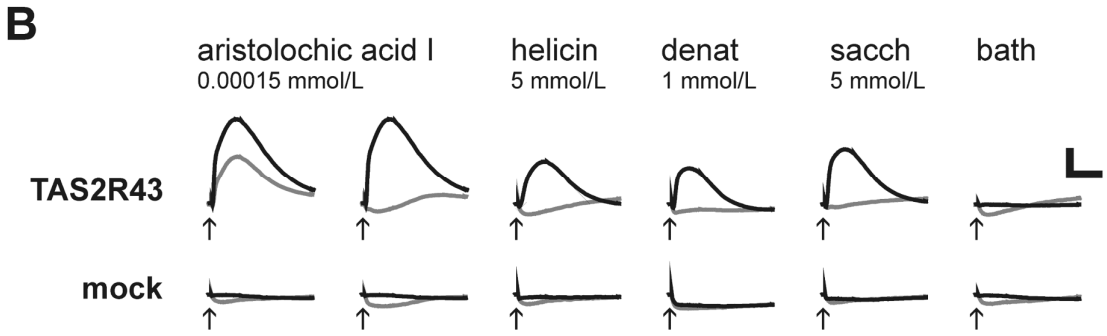


(R)-17 (mmol/L)	EC_{50} caffeine (mmol/L)	R_{max} caffeine
0	0.94 ± 0.14	0.58 ± 0.08
0.025	1.46 ± 0.43	0.47 ± 0.11 *
0.075	2.65 ± 0.50 *	0.41 ± 0.07 *
0.150	> 2.9 *	(0.24 ± 0.11) no saturation



1 Suess et al. (Figure 5)

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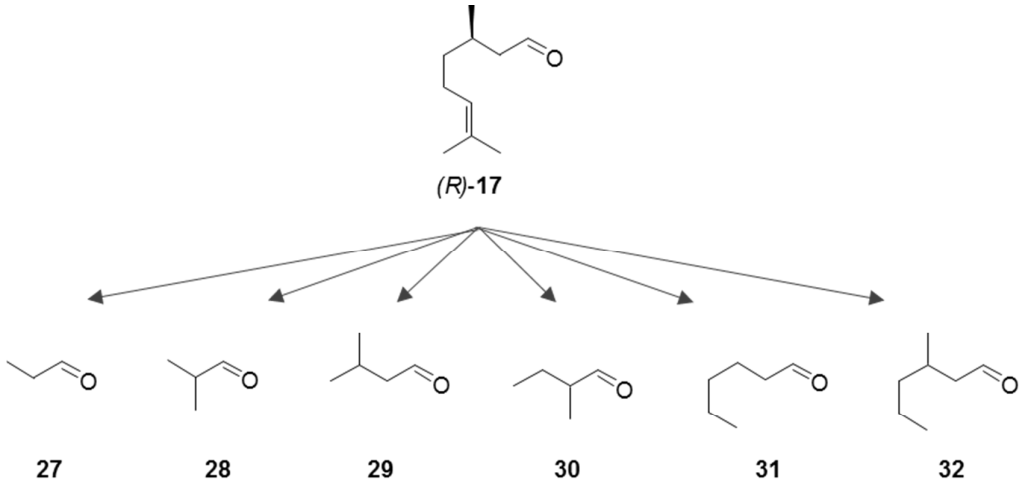

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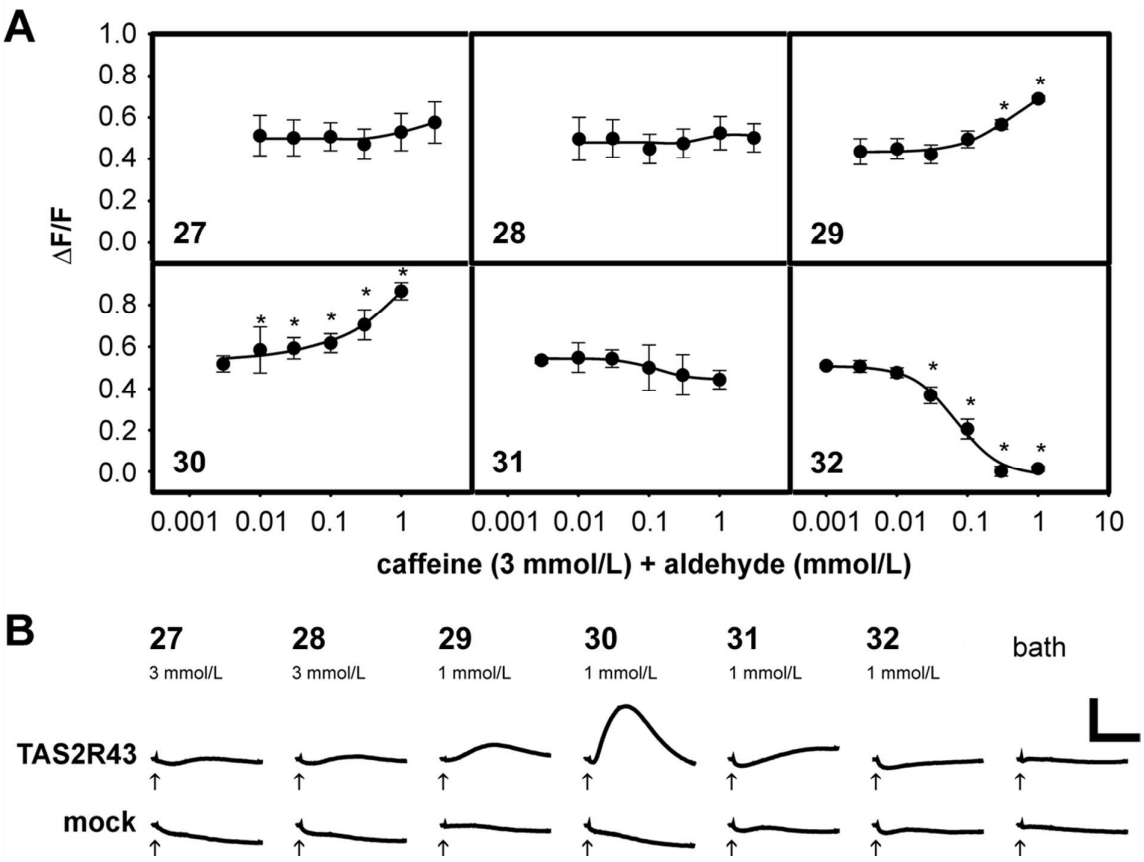
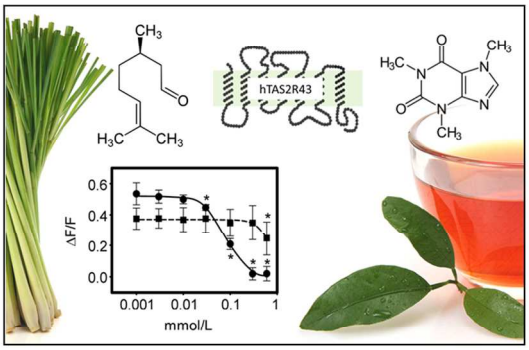
Suess et al. (Figure 6)





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