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Article

Key Food Furanones Furaneol and Sotolone Specifically Activate Distinct Odorant Receptors

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ABSTRACT: Furanones formed during the Maillard reaction often are natural aroma-determining compounds found in numerous foods. Prominent economically relevant representatives are the structural homologues Furaneol and sotolone, which are important natural flavoring compounds because of their distinct caramel- and seasoning-like odor qualities. These, however, cannot be predicted by the odorants' molecular shape, rather their receptors' activation parameters help to decipher the encoding of odor quality. Here, the distinct odor qualities of Furaneol and sotolone suggested an activation of at least two out of our ca. 400 different odorant receptor types, which are the molecular biosensors of our chemical sense of olfaction. While an odorant receptor has been identified for sotolone, a receptor specific for Furaneol has been elusive. Using a bidirectional screening approach employing 616 receptor variants and 187 key food odorants in a HEK-293 cell-based luminescence assay, we newly identified OR5M3 as a receptor specifically activated by Furaneol and homofuraneol.

KEYWORDS: *GPCRs, narrowly tuned, high-throughput screening, HDMF, high-impact aroma compound*

INTRODUCTION

Furaneol (4-hydroxy-2,5-dimethyl-3(2*H*)-furanone) is probably one of the most important aroma compounds for the food industry. The annual global market consumption of Furaneol is about 7 tons.[1] With its caramel-like odor quality and an odor threshold in water of 87 μg/kg,[2] Furaneol is an indispensable component for a variety of consumer products, such as soft candy, puddings, frozen dairy, nonalcoholic beverages, sweet sauces, baked goods, and chewing gum.[1] Furaneol is a key food odorant (KFO),[3] naturally found, for example, in strawberry,[4] blackberry,[5] raspberry,[6] kiwi,[7] tomato,[8] coffee,[9] and bread.[10] It is synthesized by enzymatic steps and is also a product of the Maillard reaction.[11]

Another economically relevant but completely different smelling furanone is sotolone (3-hydroxy-4,5-dimethyl-2(5*H*)-furanone). The annual global market consumption of sotolone is about 0.75 kg.[1] Sotolone is characteristic for lovage.[12] Its seasoning- and lovage-like odor quality with the typical smell of fenugreek and an odor threshold in water of 1.7 μg/kg[2] make sotolone an important natural KFO in savory foods.[3] It is used as an aroma compound in the food industry for products like baked goods, condiments and relishes, gravies, soups, meat products, and poultry.[1] Sotolone has been reported to enhance saltiness, which may become important as one measure alongside reducing sodium levels in certain food products to help reducing cardiovascular diseases and incidences of hypertension in the population.[13]

The aroma-characteristic combinations and concentration ratios of volatiles decisively determine the perception, recognition, and hedonic experience of food and consumer products. Our perception of volatiles typically starts with chemoreception of odorants by their odorant receptors (ORs)[14] located in the cilia of olfactory sensory neurons of the nose. ORs are seven transmembrane domain G protein-coupled receptors (GPCRs) encoded by about 400 functional genes within the human genome. The identification of "best hit" odorants for ORs, as well as compound-specific receptor activity patterns, in bioassay-based screening experiments, therefore, is a prerequisite for an understanding of the encoding of odor quality at the molecular level. From such screenings, typically, chemoreception-based compound-specific receptor activity patterns are obtained, as well as receptor activation parameters, e.g., the EC_{50} value as a measure of the potency of a compound. Until now, Furaneol could not be functionally assigned to an OR. In 2012, Adipietro et al.,[15] and recently other groups[16−18] reported sotolone to be the sole agonist of OR8D1 so far.

Here, we set out to identify the best responding OR for Furaneol and sotolone and to characterize the identified receptors in a bidirectional screening approach, employing our KFO and IL-6-HaloTag-OR libraries in a HEK-293 cell-based luminescence assay, equipped with GloSensor technology.[2,18,19]

MATERIALS AND METHODS

Chemicals. The following chemicals were used: Dulbecco's MEM medium (#F0435), FBS superior (#S0615), L-glutamine (#K0282), penicillin (10 000 U/mL)/streptomycin (10 000 U/mL) (#A2212), and trypsin/EDTA solution (#L2143) (Biochrom, Berlin, Germany);

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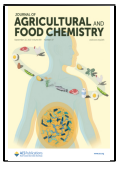

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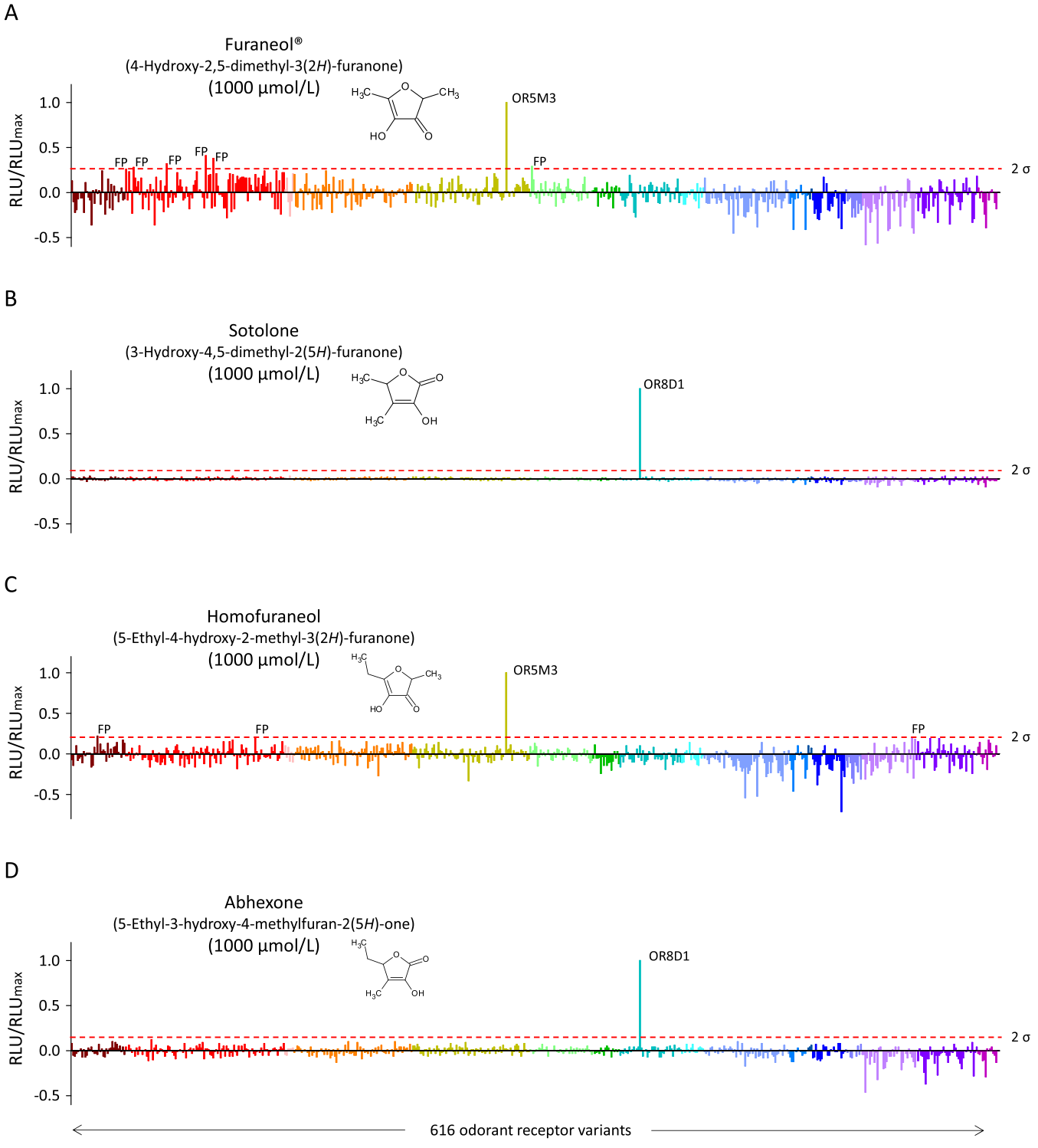

Figure 1. Furaneol and sotolone and their respective structural homologues exclusively activate OR5M3 and OR8D1, respectively. Screening of 616 human OR variants with either 1000 μmol/L Furaneol (A), sotolone (B), homofuraneol (C), and abhexon (D). Shown is the mean (n = 1 measured in duplicate). Data were normalized to the maximum responding OR. Note that OR8D1 has a better signal-to-noise ratio due to much higher signal amplitudes (see Figure 2A,B). The different OR families are color coded. The red dashed line indicates the 2σ threshold. RLU = relative luminescence units; FP = false positive (for concentration−response relations, see Figure S1).

MEM nonessential amino acid solution (100×) (#M7145, Sigma-Aldrich, Steinheim, Germany); calcium chloride dihydrate (#22322.295), D-glucose (#101174Y), dimethyl sulfoxide (DMSO) (#83673.230), HEPES (#441476L), potassium chloride (#26764.230), and sodium hydroxide (#28244.295) (VWR Chemicals BDH Prolabo, Leuven, Belgium); sodium chloride (#1064041000, Merck, Darmstadt, Germany); D-luciferin (beetle) monosodium salt (#E464X, Promega, Madison); and 4-hydroxy-2,5-dimethyl-3(2H)-furanone (Furaneol) (#40703), 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone (homofuraneol) (#W362301), 3-hydroxy-4,5-dimethyl-2(5H)-furanone (sotolone) (#W363405), and 5-ethyl-3-hydroxy-4-methylfuran-2(5H)-one (abhexone) (#W315303, Sigma-Aldrich, Steinheim, Germany).

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The KFOs used in the present study were as previously published[20,21] (Table S3). Further structurally related substances were selected using the "Structure Search" tool from Sigma-Aldrich. These are 2,2-dimethyl-3(2*H*)-furanone (#33527), 3-methyl-2(5*H*)-furanone (#393509), and 2,5-dimethylfuran-3-one (#W410100, Sigma-Aldrich, Steinheim, Germany).

Molecular Cloning of Odorant Receptors. The protein-coding regions of human OR5M3 (NCBI reference sequence: NM_001004742.2) and human OR8D1 (NCBI reference sequence: NM_001002917.1) were amplified from human genomic DNA by polymerase chain reaction (PCR) using gene-specific primers (Table S1), ligated with T4-DNA ligase (#M1804, Promega, Madison) *Eco*RI/*Not*I (#R6017/#R6435, Promega, Madison) into the expression plasmid (#pFN210A SS-HaloTag CMV-neo Flexi-Vector, Promega, Madison), and verified by Sanger sequencing (Eurofins Genomics, Ebersberg, Germany) using vector internal primers (Table S2).

In the same way, all human ORs used (for names and accession numbers, see Table S4) were cloned and ligated into the expression plasmid. One part of the plasmids was purified in-house and the other part by Eurofins Genomics, Ebersberg, Germany, using a PureYield Plasmid Midiprep System (#A2495, Promega, Madison).

Cell Culture. We used HEK-293 cells,[22] a human embryonic kidney cell line, as a test cell system for the functional expression of recombinant ORs.[19,23] HEK-293 cells were cultivated at 37 °C, 5% CO_2, and 100% humidity in 4.5 g/L D-glucose-containing DMEM with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin.

Luminescence Assay. For luminescence experiments, cells were plated in a 96-well format (Thermo Scientific Nunc F96 MicroWell, white, #137103, Thermo Fisher Scientific Inc., Waltham) with a density of 12 000 cells per well. On the next day, the transfection was performed using the lipofection method with 100 ng/well of the corresponding plasmid-DNA as well as with 50 ng/well of the transport protein RTP1S,[24] G protein subunit Gαolf,[25,26] and pGloSensor-22F[27] (Promega, Madison), each, using ViaFect (#E4982, Promega, Madison). pGloSensor-22F is a genetically engineered luciferase with a cAMP-binding pocket, which allows measurement of an immediate, cAMP-dependent luminescence signal. As a control, the transfection was performed with the vector plasmid pFN210A, which lacks the coding information of an OR, together with plasmid-DNA for Gαolf, RTP1S, and cAMP-luciferase pGloSensor-22F (mock). The amount of transfected plasmid-DNA was equal in OR-transfected and mock-transfected cells. Luminescence assays were performed 42 h after transfection as reported previously.[19,23] For experiments, the cells were loaded with a physiological salt buffer (pH 7.5) containing 140 mmol/L NaCl, 10 mmol/L HEPES, 5 mmol/L KCl, 1 mmol/L $CaCl_2$, 10 mmol/L glucose, and 2% of beetle luciferin sodium salt (Promega, Madison). For the luminescence measurements, a Glomax MULTI+ detection system (Promega, Madison) was used. After an incubation of the cells for 1 h in the dark, the basal luminescence signal of each well was recorded. Afterward, the odorant, serially diluted in the physiological salt buffer, was applied to the cells. Odorant stock solutions were prepared in DMSO and diluted 1:1000 in the physiological salt buffer to obtain a final DMSO concentration of 0.1% DMSO on the cells. Real-time luminescence signals for each well were measured 4 min after odorant application.

The automated screen of a cDNA expression library,[18] comprising 616 cDNAs, coding for 391 human OR types (NCBI reference sequences) and 225 of their most frequent variants (Table S4), was carried out using a Fluent Automation Workstation base for liquid handling (Tecan, Männedorf, Switzerland), including transfection, with an integrated Spark multimode microplate reader (Tecan, Männedorf, Switzerland) for luminescence readout. We used a 2σ-threshold (2-fold the standard deviation above the average of all signaling amplitudes in a screening experiment) as an empirical rule to identify amplitudes beyond that threshold as potential "hits" for subsequent experimental attempts to validate a concentration-dependent receptor activation. The data sets of all screening experiments have been published with MENDELEY (doi: 10.17632/zwtb2k6929.1).

Data Analysis of the cAMP Luminescence Measurements of Odorant Concentration−Response Relations. The raw luminescence data obtained from the Glomax MULTI+ detection system was analyzed using Instinct Software (Promega). Data points of basal level and data points after odorant application were each averaged. From each luminescence signal, the corresponding basal level was subtracted.

For concentration−response relations the baseline-corrected data set was normalized to the maximum amplitude of the reference odorant−receptor pair. The data set for the mock control was subtracted, and EC_{50} values and curves were derived from fitting the function

$$f(x) = \left(\frac{(\min - \max)}{\left(1 + \left(\frac{x}{EC_{50}}\right)^{\text{hillslope}}\right)} \right) + \max$$

to the data by nonlinear regression (SigmaPlot 14.0, Systat Software). All data are presented as mean ± SD.[28]

RESULTS AND DISCUSSION

OR5M3 and OR8D1 Exclusively Responded to Furaneol and Sotolone, Respectively. The distinct odor qualities of Furaneol and sotolone suggested at least one OR that would respond selectively or even specifically to either of the two odorants. Indeed, the KFO sotolone has been identified previously as the sole agonist for human OR8D1 so far.[15,17] A receptor for Furaneol is unknown. We, therefore, screened both compounds at 1000 μmol/L each, against 616 human OR variants (Figure 1). Notably, both Furaneol and sotolone each exclusively activated a single OR over a 2σ threshold (Figure 1). These are OR5M3 for Furaneol (Figure 1A) and OR8D1 for sotolone (Figure 1B).

We then investigated two other furanones that are structurally related to Furaneol and sotolone. Homofuraneol is a structural homologue to Furaneol, with a similar caramel-like odor quality and a reported odor threshold of 20 ppb.[29] Homofuraneol is a KFO, for example, in coffee.[30] A structural homologue to sotolone is the KFO abhexone, which has a similar seasoning-like odor quality, has an odor threshold of 1.1 μg/kg (in water),[2] and is also found, e.g., in coffee.[31] We screened both structural homologues, homofuraneol and abhexone, each at 1000 μmol/L, against our human OR library (Figure 1C,D). Here, too, homofuraneol and abhexone exclusively activated OR5M3 (Figure 1C) and OR8D1 (Figure 1D), respectively.

The largely different signaling efficacies of OR8D1 and OR5M3 do not correlate with their surface expression—in fact, OR5M3, which has the smaller signaling amplitudes, has a better surface expression (Figure S3). Other reasons for individually different signaling amplitudes of ORs could be, e.g., their individual equipment with specific C-terminal amino acid motifs or deviation from highly conserved motifs, which might affect receptor signaling.[16]

Some downward deflections in signals (Figure 1) may suggest an inhibition of some receptors' constitutive activities. Thus, an additional functional role of Furaneol as an inverse agonist on receptors other than OR5M3 cannot be excluded. However, even with a sequential and largely equal treatment and measurement schedule, slight decreases of the lumines-

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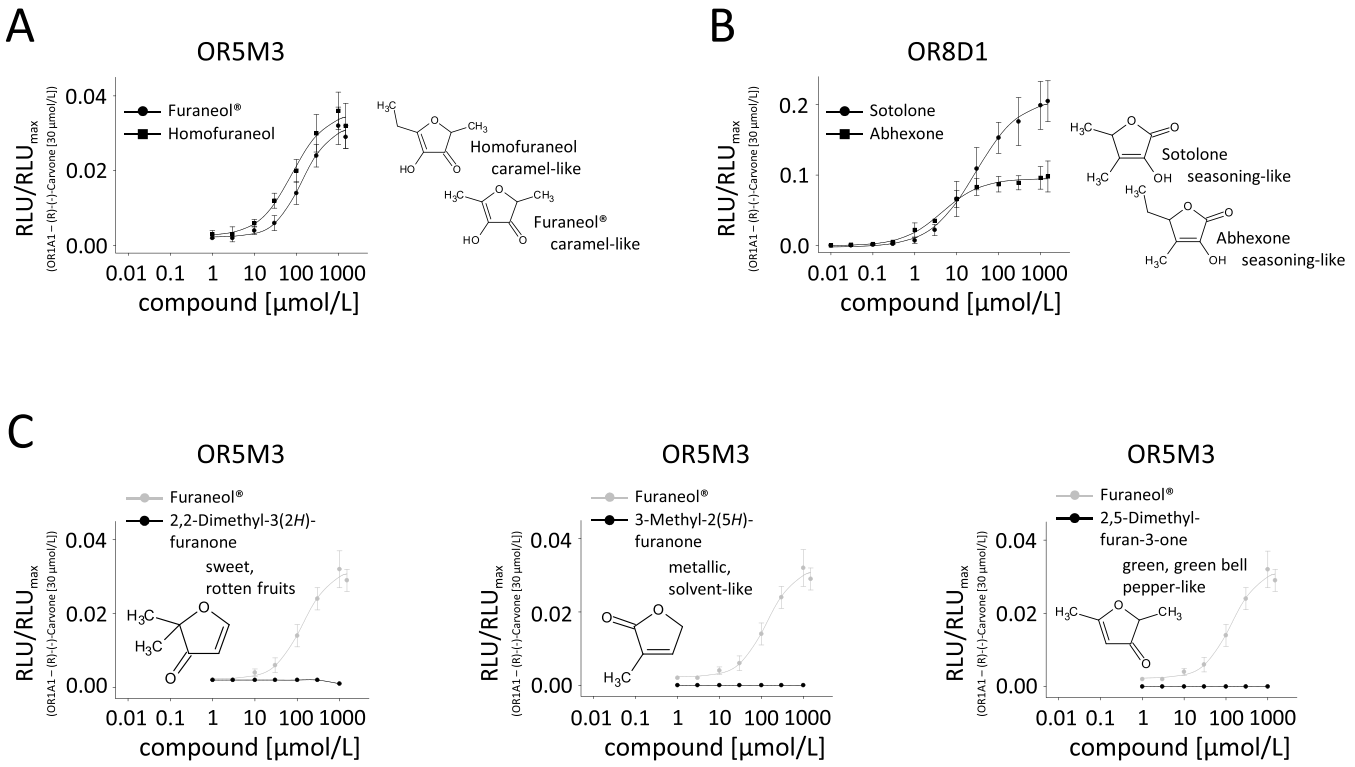

Figure 2. OR5M3 and OR8D1 concentration-dependently responded to their respective furanone KFO agonists. Concentration−response relations of Furaneol and homofuraneol on OR5M3 (A); sotolone and abhexone on OR8D1 (B); and 2,2-dimethyl-3(2*H*)-furanone, 3-methyl-2(5*H*)-furanone, and 2,5-dimethylfuran-3-one on OR5M3 (C). Note that the Furaneol curve from panel (A) is shown in gray in all subpanels in (C) for didactic reasons. Data were mock control-subtracted, normalized to the response of OR1A1 to (*R*)-(−)-carvone (30 μmol/L) and displayed as mean ± SD (n = 3−6). RLU = relative luminescence units. For individual odor qualities derived from a 3-AFC test with 8 subjects, see Table S5.

cence signals may not be completely prevented in complex screening experiments.

We then validated OR5M3 and OR8D1 as receptors for the compounds tested in the screening experiments by establishing concentration−response relationships. Indeed, Furaneol and homofuraneol concentration-dependently activated OR5M3, with homofuraneol being the more potent agonist, with an EC_{50} value of 78.94 ± 18.83 μmol/L, as compared to Furaneol, with an EC_{50} value of 135.33 ± 24.01 μmol/L (Figure 2A). This bears analogy to vanillin (4-hydroxy-3-methoxybenzaldehyde) and ethyl vanillin (3-ethoxy-4-hydroxybenzaldehyde), two structurally related substances of similar odor quality that are widely used in the food industry.[1] Here, the flavoring power of ethyl vanillin is two to four times stronger than that of vanillin.[1] However, these two compounds have been reported to activate several different OR types, albeit using different experimental approaches and test cell systems. For example, vanillin has been described as an agonist of the receptors OR1G1,[32] OR1L3, OR2G2, OR2M4, OR2T10, OR2T34, OR5AC2,[33] and OR10G3,[17] whereas ethyl vanillin has been reported to activate OR1G1,[32] OR2J2,[15,17] and OR10G3.[15]

Similar to the case with Furaneol and homofuraneol, we then validated sotolone and abhexone as agonists for OR8D1. In our hands, sotolone activated OR8D1 with an EC_{50} value of 27.77 ± 2.61 μmol/L (Figure 2B). Notably, abhexone appeared to be a partial agonist of OR8D1, with a lower efficacy compared to sotolone but with an EC_{50} value of 4.49 ± 0.54 μmol/L, which was significantly different from the EC_{50} value for sotolone (paired t-test, $p < 0.05$), suggesting a higher potency, at least at near-threshold concentrations (Figure 2B).

To better characterize the newly identified Furaneol receptor OR5M3, we searched for further structurally related furanones via the "Structure Search" tool from Sigma-Aldrich and identified three compounds, 2,2-dimethyl-3(2*H*)-furanone (Figure 2C), 3-methyl-2(5*H*)-furanone (Figure 2C), and 2,5-dimethylfuran-3-one (Figure 2C), which we tested against OR5M3. However, all of these Furaneol-related non-KFOs had odor qualities deviating from Furaneol, and none of these activated OR5M3 (Figure 2C). This supports the notion that OR5M3 may be an OR that is a rather narrowly tuned receptor for the selective detection of the KFOs Furaneol and homofuraneol.

OR5M3 and OR8D1 Are Highly Selective and Narrowly Tuned Receptors. KFOs have been suggested as best natural agonists for the majority of our ORs.[3,34] We, therefore, used those food aroma-relevant odorants published in the work by Dunkel et al.[3] to characterize the KFO agonist spectra of OR5M3 and OR8D1. We screened both ORs against 187 KFO compounds, which according to the ClassyFire classification[35] are distributed as follows: 2 allyl sulfur compounds, 1 azoline, 14 benzenes and substituted derivatives, 1 benzodioxole, 15 carboxylic acids and derivatives, 2 cinnamic acids and derivatives, 1 cinnamic alcohol, 2 coumarines and derivatives, 6 diazines, 5 dihydrofurans, 25 fatty acyls, 4 heteroaromatic compounds, 1 hydroxy acid, 1 indole, 8 lactones, 2 organic disulfides, 3 organic trisulfides, 48 organooxygen compounds, 2 oxanes, 2 phenol ethers, 16 phenols, 1 phenylpropanoic acid, 17 prenol lipids, 1 pyran, 1

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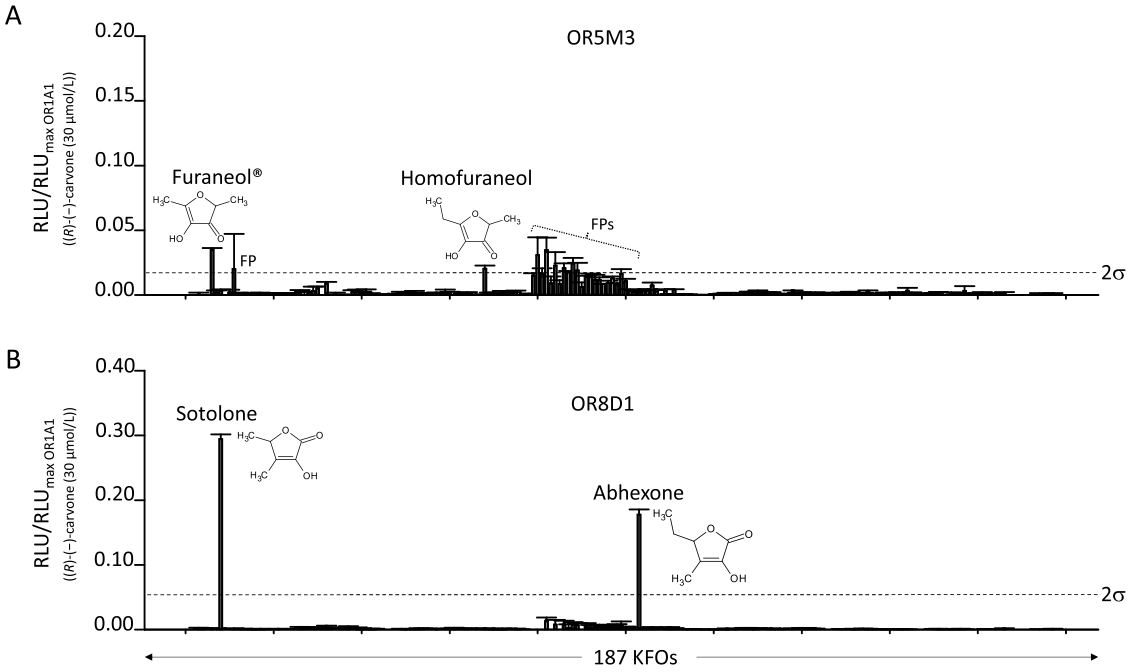

Figure 3. OR5M3 and OR8D1 are narrowly tuned receptors, each selective for two highly related KFO structures. Screening of 187 KFOs against the human odorant receptors OR5M3 (A) and OR8D1 (B). Shown are mean ± SD (n = 1 measured in triplicate). Mock control was subtracted. Data were normalized to the response of OR1A1 to (*R*)-(−)-carvone (30 μmol/L). The concentration of each KFO was 300 μmol/L. RLU = relative luminescence units. Dashed line depicts the 2σ threshold. FP = false positive (for concentration−response relations, see Figure S2).

pyridine, 1 pyrroline, 1 thioacetal, 2 thioethers, and 2 thiols. Each KFO was tested at a concentration of 300 μmol/L in triplicate against OR5M3 and OR8D1, and all receptor response amplitudes were normalized to the response of another receptor, OR1A1, responding to its agonist (*R*)-(−)-carvone (Figure 3).

Figure 3 shows the KFO activation patterns of OR5M3 and OR8D1. OR5M3 selectively responded to Furaneol and homofuraneol (Figure 3A). We could not verify other KFOs as agonists, which also reached the 2σ threshold, i.e., 2-pentylpyridine, 3-mercapto-2-pentanone, (*E*)-2-hexenal, octanoic acid, 2,3,5-trimethylpyrazine, δ-dodecalactone, estragole, or ethyl-2-methylbutanoate. In follow-up experiments, these compounds did not show a concentration-dependent activation of OR5M3 (Figure S2), suggesting 4% false positives in our screening approach.

Previously, OR8D1 emerged as a sotolone-specific receptor out of the cell-based screening experiments with 42 and 73 odorants by Adipietro et al.[15] and Mainland et al.,[17] respectively. These experiments already suggested that OR8D1 is a rather narrowly tuned receptor. However, in these early studies, the odorant collections used for screening experiments were rather randomly selected compounds mainly comprising non-KFOs and did not represent ecologically relevant groups of natural volatiles, such as KFOs[3] and semiochemicals.[36] In the present study, out of 187 KFOs, OR8D1 selectively responded to sotolone and, shown for the first time, to its structural homolog KFO abhexone (Figure 3B). Both receptors, OR5M3 and OR8D1, in our hands emerged as bona fide narrowly tuned receptors from screenings with 187 KFOs. Regarding KFOs, so far, there is only one other narrowly tuned human OR reported in the literature, OR2M3, which emerged from a screen against a comparable number of natural odorants.[20] However, other receptors, e.g., OR7D4,[37] OR10J5,[15,17,38] and OR51L1,[15,17,38] have also been tested against a large number of compounds (>65), mainly comprising non-KFOs, and emerged as narrowly tuned receptors.[15,17,37,38]

Since bioassay-based evidence suggested that KFOs are among the best agonists for the human ORs,[3,20,21,34,36,39] together with the fact that they have accompanied human and thus OR evolution,[39] the specificity of narrowly tuned ORs for only one or a few structurally related odorants may hint to their biological relevance. Indeed, both KFOs, Furaneol and sotolone, are generalists, that is, they are involved in shaping the aroma of many different foods.[3] Moreover, Saraiva et al. reported that transcript levels in the olfactory mucosa of human ORs detecting at least one KFO are 2.4-fold higher than those of ORs detecting non-KFOs.[39] Indeed, with regard to their transcript levels, OR5M3 and OR8D1 ranked among the top 26 and 4% of human ORs, respectively.[39] Similar numbers were reported by Verbeurgt et al.[40]

Here, we show that the two important furanones, Furaneol and sotolone, each specifically activated a distinct single receptor out of all human ORs. This compound-specific receptor activation may serve as a quality control parameter in the future. For example, sotolone has been described in the literature as a Madeira off-flavor to aged beers[41] and also as an off-flavor in stored citrus soft drinks.[42] The odor threshold of sotolone is quite low, i.e., in the lower μg/L range in different matrices,[2] and our testings revealed OR8D1 as a highly specific, narrowly tuned receptor. OR8D1 may, thus, be suitable as a sensor to detect untypical/unwanted concentrations of sotolone in a complex mixture of odorants, as they naturally occur in foods.

A cautionary note: We may have missed other Furaneol- or sotolone-responsive ORs or responsive genetic variants (i) since our receptor screening experiments did not include all known genetic human OR variants and (ii) some receptors of our OR library may not work with the assay used in our study.

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Future studies on the ORs investigated in the present study and their genetic OR variants in the population may reveal their roles as genuine sotolone or Furaneol receptors in vivo. Some odorants have not been considered as KFOs, even if they participate in shaping a food's aroma, since they may not meet the standard of being a KFO as set by Dunkel et al., because of, for example, a lack of quantitation in the food(s) or a lack of aroma recombinates with omission experiments.[3] The 2014 list of KFOs, however, will likely have to be extended in the future.

In summary, our comprehensive, dual-screening strategy, testing highly relevant, food-related compounds against an entire set of human ORs, and, vice versa, testing an entire set of KFOs against the best responding ORs, revealed KFO-enriched, highly specific odorant profiles for narrowly tuned receptors OR5M3 and OR8D1. Beyond the assignment of new cognate KFO/OR combinations, our results demonstrate that different odor qualities are likely to be represented by distinct receptor activation patterns, adding to an understanding of odor quality coding of the biologically relevant group of food-typical and aroma-determining KFOs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.1c03314.

> Oligonucleotides for molecular cloning of odorant receptors (Table S1); vector internal oligonucleotides (Table S2); investigated key food odorants according to Dunkel et al. (2014) (Table S3); cDNA expression plasmid OR library (Table S4); individual odor qualities of selected furanones (Table S5); validation of false-positive OR hits of the OR-library screening (Figure S1); validation of false-positive KFO hits on OR5M3 (Figure S2); cell-surface expression of OR5M3 and OR8D1 (Figure S3) (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

GPCR, G protein-coupled receptor; KFO, key food odorant; OR, odorant receptor

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