The Broadly Tuned Odorant Receptor OR1A1 is Highly Selective for 3-Methyl-2,4-nonanedione, a Key Food Odorant in Aged Wines, Tea, and Other Foods

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Abstract

Key food odorants are the most relevant determinants by which we detect, recognize, and hedonically evaluate the aroma of foods and beverages. Odorants are detected by our chemical sense of olfaction, comprising a set of approximately 400 different odorant receptor types. However, the specific receptor activity patterns representing the aroma percepts of foods or beverages, as well as the key food odorant agonist profiles of single-odorant receptors, are largely unknown. We aimed to establish comprehensive key food odorant agonist profiles of 2 unrelated, broadly tuned receptors, OR1A1 and OR2W1, that had been associated thus far with mostly non-key food odorants and shared some of these agonists. By screening both receptors against 190 key food odorants in a cell-based luminescence assay, we identified 14 and 18 new key food odorant agonists for OR1A1 and OR2W1, respectively, with 3-methyl-2,4-nonanedione emerging as the most potent agonist for OR1A1 by 3 orders of magnitude, with a submicromolar half maximal effective concentration. 3-Methyl-2,4-nonanedione has been associated with a prune note in oxidized wine and is an aroma determinant in tea and apricots. Further screening against the entire set of 391 human odorant receptors revealed that 30 or 300 μmol/L 3-methyl-2,4-nonanedione activated only 1 receptor, OR1A1, suggesting a unique role of OR1A1 for the most sensitive detection of this key food odorant in wine, tea, and other food matrices.

Key words: chemosensory, key food odorants, luminescence, olfaction, receptors, screening

Introduction

Food- and beverage-specific chemosensory percepts are significantly shaped by characteristic proportions of just a few compounds from a group of approximately 230 key food odorants (KFOs) of circa 10,000 food volatiles (Dunkel et al. 2014). KFOs appear in food at concentrations above their odor thresholds, contributing to the aroma of a large variety of foods (Grosch 2001; Dunkel et al. 2014). Odorants are detected by the odorant receptors (ORs) (Buck and Axel 1991), residing in the cilia of the olfactory sensory neurons of the nose (Menco et al. 1997), by means of combinatorial odorant coding at the receptor level (Malnic et al. 1999; Nara et al. 2011), suggesting narrowly tuned “specialist” ORs being specific for single odorants (e.g., Noe et al. 2015), and broadly tuned “generalist” ORs, which nevertheless may have a selectivity for certain odorants over others as a function of concentration (Sanz et al. 2005; Schmiedeberg et al. 2007; Saito et al. 2009; Adipietro et al. 2012; Mainland et al. 2014).
Indeed, KFOs, rather than non-KFOs, appear to be the better OR agonists (Krautwurst and Korthoff 2013; Dunkel et al. 2014) within a yet unknown adequate stimulus space for human olfactory perception. However, so far, there are few comprehensive studies in which the entire set of aroma-relevant KFOs of a food, for example, butter aroma (Geithe et al. 2015), have been tested against a significant collection or even the entire set of recombinant human ORs in a test cell system. Such a bioassay would enable the identification of food aroma-specific OR activity patterns or to characterize the KFO agonist profile of ORs for which only a single-odorant agonist is currently known.

In wine, a wide variety of KFOs have been reported to be aroma relevant, such as terpenoids and other compounds (Guth 1997, 1998; Dziadas and Jelen 2010; Frank et al. 2011; Benkwitz et al. 2012; Ugliano 2013; Mayr et al. 2014; Frank et al. 2013; Schwab and Wust 2015; Shi et al. 2015). For instance, the β-diketone 3-methyl-2,4-nonanedione has been associated with the appearance of the characteristic prune note in oxidized wine (Pons et al. 2008, 2013; Ugliano 2013).

Here, we report on an analytical bioassay capable of bidirectional screening to establish the KFO agonist profiles of 2 broadly tuned receptors from different OR families, OR1A1 and OR2W1, whose known agonists are mostly not KFOs, but have been reported with a partially overlapping agonist spectrum, including terpenoids, ketones, and acetates (Schmiedeburg et al. 2007; Saito et al. 2009; Geithe and Krautwurst 2015a). In a cell-based, fast luminescence assay for odorant-induced changes in intracellular cyclic adenosine monophosphate (cAMP), we tested both receptors against 190 of the 226 known KFOs (Dunkel et al. 2014). Moreover, we then screened the best emerging KFO agonist against an entire set of 391 human ORs to demonstrate that an OR previously believed to be broadly tuned actually has high specificity for a single KFO.

### Material and methods

#### Chemicals

The following chemicals were used: Dulbecco’s modified Eagle’s medium (DMEM) (#F0435), fetal bovine serum (FBS) superior (80615), L-glutamine (#K0282), penicillin (10 000 U/mL)/ streptomycin (10 000 U/mL) (#A2212), trypsin/ethylenediaminetetraacetic acid solution (#L2143) (Biochrom).

The following chemicals were further used: calcium chloride dihydrate (CaCl₂*2H₂O; #22322.295), d-glucose (#1011744), dimethyl sulfoxide (DMSO) (#83673.230), HEPES (#441476L), potassium chloride (KCl; #26764.232), potassium hydroxide (KOH; #26668.296), sodium hydroxide (#28244.295) (VWR Chemicals BDH Prolabo); potassium dihydrogen phosphate (KH₂PO₄; #1048735000), sodium chloride (NaCl; #1064041000) (Merck); and d-luciferin (beetle) monosodium salt (#E464X, Promega).

All odorants were purchased from Sigma–Aldrich, Alfa Aesar, Chemos GmbH, and TCI Europe GmbH. The nuclear magnetic resonance (NMR) solvents deuterated DMSO (DMSO-d₆; #D310B), trideuteracetionitrile (acetonitrile-d₃; #ACN-d₃; #D021B), and deuterium oxide (D₂O; #D214H) were obtained from Euroiso-top.

Odorant stock solutions were prepared in DMSO and diluted 1:1000 in physiological salt buffer (140 mmol/L NaCl, 10 mmol/L HEPES, 5 mmol/L KCl, 1 mmol/L CaCl₂, 10 mmol/L d-glucose, pH 7.5) for luminescence measurements. Final DMSO concentration was always 0.1%. To keep all measurement conditions the same, the water-soluble substances were also dissolved in DMSO. For hardly soluble odorants, we added Pluronic PE-10500 (#50053867, BASF) to the buffer. The final Pluronic PE-10500 concentration on the cells was 0.05%.

#### Molecular cloning of human OR1A1 and OR2W1

The protein-coding regions of human OR1A1 (NCBI reference sequence: NM_014565.2) and OR2W1 (NCBI reference sequence: NM_030903.3) were amplified from human genomic DNA by polymerase chain reaction as previously described (Geithe and Krautwurst 2015a), ligated with T4 DNA ligase (#M1804, Promega) either MfeI/Not1 for OR1A1 (#R05895/#R0189S, New England Biolabs) or EcoRI/Not1 for OR2W1 (#R6017/#R6435, Promega) into the expression plasmid p12-dk39aa rho-tag (aa, amino acids) (Krautwurst et al. 1998; Shirokova et al. 2005) and verified by Sanger sequencing (Eurofins Genomics). By this procedure, we also cloned the protein-coding regions of 391 different human ORs (Supplemental Table 1).

#### Cell culture, transfection, and cAMP luminescence assay

HEK-293 cells, human embryonic kidney cell line (Graham et al. 1977), were cultivated at 37 °C, 5% carbon dioxide and 100% humidity in 4.5 g/L d-glucose containing DMEM with 10% FBS, 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 U/mL streptomycin. The HEK-293 cells were seeded with a density of 12000 cells per well in white 96-well plates (#136102, Nunc). The transfection was done by lipofection (Lipofectamine 2000, #11668-027, Life Technologies) using 100 ng OR1A1 or OR2W1 plasmid DNA, 50 ng olfactory G protein subunit Gtolf (Jones and Reed 1989; Shirokova et al. 2005), 50 ng RTP1S (Saito et al. 2004), 50 ng olfactory G protein subunit Gy13 (Li et al. 2013), and 50 ng genetically modified cAMP-luciferase pGloSensor-22F (Promega) (Binkowski et al. 2009) each. A transfection of the vector plasmid p12-dk39aa rho-tag (Krautwurst et al. 1998; Shirokova et al. 2005) lacking the coding information for an OR together with Gtolf, RTP1S, Gy13 and cAMP-luciferase pGloSensor-22F, was performed as a control (mock). The amount of transfected plasmid DNA was equal in OR-transfected and mock-transfected cells. The transfected cells were taken into cAMP luminescence experiments 42 h post transfection. The cells were loaded with luciferin-supplemented (2%) physiological salt buffer, and the CAMP luminescence assay using GloMax-MULTI+ detection system (Promega) was performed as previously described (Geithe et al. 2015; Geithe and Krautwurst 2015a).

#### Data analysis of cAMP luminescence measurements

The raw luminescence data obtained from the GloMax-MULTI+ detection system was analyzed using Instinct Software (Promega). Three data points before (baseline) and after odorant addition (signal) were averaged, and the respective baseline was subtracted from each signal. Data for the KFO screening were normalized to the signal of OR1A1 with (R)+-(−)-carvone, and the respective data from vector-transfected cells (mock control) were subtracted. Concentration–response relations were obtained by normalizing the baseline-corrected data to maximum. The mock controls were subtracted. Half maximal effective concentration (EC₅₀) values were derived from fitting the function $f(x) = 1/(1+(EC_{50}/x)^{h})$ to the data by nonlinear regression (SigmaPlot 10.0, Systat Software), where $h = \text{hill coefficient}$. For 3-methyl-2,4-nonanedione and 2,4-nonanedione, we additionally performed a 2-site fit using the function $f(x) = (E_{\text{max1}} \times x/(EC_{50,1} + x))+(E_{\text{max2}} \times x/(EC_{50,2} + x))$, where $E_{\text{max1}}$ and $E_{\text{max2}}$ are the maximum responses at $x = 0$, and $EC_{50,1}$ and $EC_{50,2}$ are the half-maximal effective concentrations.
where $E_{\text{max}}$ is the maximum effect to obtain the EC$_{\text{50}}$ values for the 2 individual curves. All data are presented as mean ± SD. The data for the OR screening experiment were normalized to the maximum responding receptor. All OR signals that exceeded the 2-sigma threshold (mean ± 2 SDs over all signals) were monitored as hits.

**Purification of 3-methyl-2,4-nonanedione (compound 23)**

To get rid of any possible impurities dissolved in 3-methyl-2,4-nonanedione, firstly, we distilled it under vacuum using a spinning band distillation column (Normag). Secondly, we finally purified the freshly distilled compound by means of column chromatography. For this purpose, compound 23 (0.5 mL) was placed on the top of a glass column (2.5 × 2 cm) filled with a slurry of LiChroprep DIOL (40–63 μm, Merck) in n-pentane. After the column has been flushed with n-pentane (150 mL), the target compound was eluted with n-pentane/diethyl ether (150 mL, 95:5, v/v).

**Gas chromatography–olfactometry**

Gas chromatography–olfactometry (GC-O)/flame ionization detector (FID) was performed by means of a gas chromatograph type GC 2000 (Thermo Fisher Scientific), equipped with detectors FID and sniffing port. The following fused silica capillary columns and temperature programs were used: DB-FFAP (30 m × 0.32 mm inner diameter [i.d.]), 0.25 μm film thickness, J&W Scientific), initial temperature of 40 °C was held for 2 min, raised at 8 °C/min up to 230 °C and held for 5 min; DB-5 (30 m × 0.32 mm i.d.; 0.25 μm film thickness; J&W Scientific), initial temperature of 40 °C was held for 2 min, raised at 8 °C/min up to 240 °C and held for 5 min. The samples were injected at 40 °C by means of the “cold on column” technique.

For GC-O/FID, the effluent was evenly split at the end of the column between an FID (250 °C) and a heated sniffing port (230 °C) using a Y-shaped glass splitter (Chrompack) and 2 deactivated fused silica capillaries (50 cm × 0.2 mm i.d.). Helium adjusted to a flow rate of 2.0 mL/min served as the carrier gas.

**NMR spectroscopy for 3-methyl-2,4-nonanedione**

All NMR (1H, 13C, COSY, HSQC, HMBC, NOESY, and 1H quantitative NMR [qNMR]) experiments were performed using an Avance III 400 MHz spectrometer equipped with a BBI probe (Bruker). Topspin software (version 3.2) was used for data acquisition. For structure elucidation, we dissolved 3-methyl-2,4-nonanedione in ACN-d$_6$. However, in order to achieve similar conditions as in the luminescence assay, the compound was dissolved in DMSO-d$_6$ (stock solution) and diluted in a buffer containing 130 mM KH$_2$PO$_4$, 100 mM KOH solved in D$_2$O and adjusted with KOH to pH 7.5. Chemical shifts were referenced against tetramethylsilane (TMS), trimethylsilyl propionate (TSP), or the solvent signal.

$^1$H qNMR was carried out according to (Frank et al. 2014). For this, an aliquot (600 μL) of the dissolved solutions was analyzed in NMR tubes (5 × 178 mm, Bruker) to determine the precise concentrations.

**Results**

Screening of 190 KFOs against the human odorant receptors OR1A1 and OR2W1

The human odorant receptors OR1A1 and OR2W1 were reported to respond to several odorants (Schmiedberg et al. 2007; Saito et al. 2009; Adipietro et al. 2012; Audouze et al. 2014; Geith and Krautwurst 2015a), but most of those tested odorants were non-KFOs, and thus by themselves may have little impact on food aroma. So far, 30 agonists have been identified for OR1A1, but of those only 5 are KFOs. For OR2W1, 39 agonists have been identified, so far, including only 10 KFOs (for details, see Supplemental Tables 2 and 3).

KFOs have been suggested as best agonists for the majority of our ORs (Krautwurst and Korthoff 2013; Dunkel et al. 2014). The basis of our current study are those food aroma-relevant odorants that were published in the work by Dunkel et al. (2014). This meta-analysis is based on several criteria and represents the most comprehensive and stringent, concentration-based definition of KFOs found up-to-date.

Therefore, we screened both ORs against 190 of the 226 KFOs that were described by Dunkel et al. (2014). We tested in total 20 terpenes, 15 phenols, 6 pyrazines, 26 sulfur-containing compounds, 25 aldehydes, 7 benzaldehydes, 18 esters, 9 acetates, 13 alcohols, 15 acids, 5 furanones, 9 lactones, 11 ketones, and 11 other compounds (Figure 1). These different KFO groups were illustrated and labeled in a color scale on the x-axis of Figure 1. A detailed list of all investigated KFOs is given in Supplemental Table 4. Each KFO was tested in a concentration of 300 μM/L against OR1A1 and OR2W1, and all KFO–receptor response amplitudes were normalized to the response of OR1A1 to (R)-(−)-carvone. Figure 1 shows the partially overlapping activation patterns for both receptors that preferably responded to esters, acetates, ketones, terpenes, sulfur-containing compounds, aldehydes, alcohols, and lactones, but not to phenols, pyrazines, benzoaldehydes, acids, and furanones.

**OR1A1 and OR2W1 have overlapping agonist spectra and 3-methyl-2,4-nonanedione is the most potent OR1A1 agonist tested**

To validate the cognate OR/KFO hits from the screening, we established concentration–response relations. OR1A1 and OR2W1 responded to the same terpene- and sulfur-containing compounds, albeit with a different rank order of potency (Figure 2A, Table 1). Of these, (R)-(−)-carvone was the strongest KFO agonist for OR1A1, followed by (R)-(+)−limonene, 2-phenylethanol, (S)-(+)−limonene and 3-mercaptotetethyl acetate. OR2W1 preferentially responded to the thiols 2-phenylethanol and 3-mercaptotetethyl acetate, followed by the terpenes (S)-(−)-limonene, (R)-(+)−limonene, and (R)-(−)-carvone. With the exception of (R/S)-1-octen-3-ol, only OR2W1 was activated by the tested aldehydes and alcohols, and in the following rank order (EC$_{50}$): octanal < nonanal ~ 1-nonal < (R/S)-1-octen-3-ol, (E)-2-heptenal < 2-heptanol (Figure 2B, Table 1). The concentration–response curves of esters and acetates showed 2-phenylethyl acetate, followed by ethylphenyl acetate, ethyl hexanoate, and ethyl cyclohexancarboxylate as KFO agonists for OR1A1 (Figure 2C, Table 1). For OR2W1, we observed concentration-dependent effects for methyl cinnamate, cinnamyl acetate, and 2-phenylethyl acetate as strongest KFO agonists, followed by ethyl cyclohexancarboxylate, hexyl acetate, and 3-methylbutyl acetate. Figure 2D shows the concentration–response curves for the ketones, lactones, and some other compounds. The β-diketone...
3-methyl-2,4-nonanedione by far was the best KFO agonist for OR1A1, with the lowest EC$_{50}$ (Figure 2D, Table 1).

The concentration–response relation of 3-methyl-2,4-nonanedione was best described by a 2-site fit (Figure 3A, left panel), ranging from nanomolar to millimolar, suggesting either different binding sites with different affinities for 3-methyl-2,4-nonanedione or that more than 1 structural form of 3-methyl-2,4-nonanedione was involved in a receptor activation. The 2 separate curves, with EC$_{50}$ values of 0.28 ± 0.05 μmol/L for the lower concentration range (i), and 368.33 ± 57.87 μmol/L for the higher concentration range (ii), are given in Figure 2D and Figure 3A right panel. It was known that 3-methyl-2,4-nonanedione displays keto enol equilibrium...
Figure 3A (Guth and Grosch 1989; Pons et al. 2008). However, NMR spectroscopy revealed that predominantly (>90%) the keto form of 3-methyl-2,4-nonanedione was present at pH 7.5 assay conditions.

Further, trans-anethole, 2-pentylpyridine, estragole, (R/S)-γ-nonalactone, and 2-nonanone had concentration-dependent effects on OR1A1. Of the ketone/lactone/others group, 2-pentylpyridine was the strongest KFO agonist for OR2W1, followed by 4-methyl...
Table 1. EC$_{50}$ values of KFO concentration–response relations for OR1A1 and OR2W1

<table>
<thead>
<tr>
<th>No.</th>
<th>KFOs</th>
<th>Odor quality (Guth and Grosch 1989; Ohloff 1994; Czerny et al. 2008)</th>
<th>OR1A1 EC$_{50}$ (µmol/L)</th>
<th>OR2W1 EC$_{50}$ (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>(R)-(−)-Carvone</td>
<td>Mint-like</td>
<td>116.69 ± 18.79</td>
<td>227.25 ± 25.50</td>
</tr>
<tr>
<td>(2)</td>
<td>(S)-(−)-Limonene</td>
<td>Turpentine-like</td>
<td>242.70 ± 37.42</td>
<td>100.34 ± 7.86</td>
</tr>
<tr>
<td>(3)</td>
<td>(R)-(−)-Limonene</td>
<td>Orange-like</td>
<td>185.06 ± 13.09</td>
<td>111.00 ± 6.76</td>
</tr>
<tr>
<td>(4)</td>
<td>2-Phenylethanethiol</td>
<td>Saltury, rubber-like</td>
<td>191.69 ± 35.84</td>
<td>35.16 ± 2.60</td>
</tr>
<tr>
<td>(5)</td>
<td>3-Mercaptohexyl acetate</td>
<td>Black currant-like</td>
<td>379.94 ± 17.46</td>
<td>72.04 ± 4.49</td>
</tr>
<tr>
<td>(6)</td>
<td>(E)-2-Heptenal</td>
<td>Fatty, green</td>
<td>—</td>
<td>271.02 ± 23.73</td>
</tr>
<tr>
<td>(7)</td>
<td>Octanal</td>
<td>Citrus-like, green</td>
<td>—</td>
<td>35.16 ± 1.32</td>
</tr>
<tr>
<td>(8)</td>
<td>Nonanal</td>
<td>Citrus-like, soapy</td>
<td>—</td>
<td>61.56 ± 4.13</td>
</tr>
<tr>
<td>(9)</td>
<td>Methyl cinnamate</td>
<td>Fruity</td>
<td>—</td>
<td>41.85 ± 2.16</td>
</tr>
<tr>
<td>(10)</td>
<td>Ethyl hexanoate</td>
<td>Fruity</td>
<td>337.62 ± 28.55</td>
<td>—</td>
</tr>
<tr>
<td>(11)</td>
<td>Ethyl cyclohexanecarboxylane</td>
<td>Fruity</td>
<td>603.64 ± 70.35</td>
<td>365.85 ± 43.18</td>
</tr>
<tr>
<td>(12)</td>
<td>3-Methylbutyl acetate</td>
<td>Fruity</td>
<td>—</td>
<td>615.27 ± 90.18</td>
</tr>
<tr>
<td>(13)</td>
<td>Ethylphenyl acetate</td>
<td>Fruity, sweet</td>
<td>289.46 ± 30.65</td>
<td>—</td>
</tr>
<tr>
<td>(14)</td>
<td>2-Phenylethyl acetate</td>
<td>Flowery, fruity</td>
<td>273.01 ± 18.10</td>
<td>78.72 ± 13.54</td>
</tr>
<tr>
<td>(15)</td>
<td>Hexyl acetate</td>
<td>Fruity</td>
<td>—</td>
<td>474.45 ± 88.89</td>
</tr>
<tr>
<td>(16)</td>
<td>Cinnamyl acetate</td>
<td>Cinnamon-like, spicy</td>
<td>—</td>
<td>63.74 ± 5.34</td>
</tr>
<tr>
<td>(17)</td>
<td>1-Nonanol</td>
<td>Citrus-like</td>
<td>—</td>
<td>89.59 ± 8.59</td>
</tr>
<tr>
<td>(18)</td>
<td>2-Heptanol</td>
<td>Lemon-like, fruity</td>
<td>—</td>
<td>433.99 ± 18.45</td>
</tr>
<tr>
<td>(19)</td>
<td>(R/S)-1-Octen-3-ol</td>
<td>Mushroom-like</td>
<td>580.20 ± 68.89</td>
<td>223.07 ± 13.19</td>
</tr>
<tr>
<td>(20)</td>
<td>(R/S)-γ-Octalactone</td>
<td>Coconut-like</td>
<td>—</td>
<td>483.63 ± 23.15</td>
</tr>
<tr>
<td>(21)</td>
<td>(R/S)-γ-Nonalactone</td>
<td>Coconut-like</td>
<td>345.75 ± 32.46</td>
<td>—</td>
</tr>
<tr>
<td>(22)</td>
<td>2-Nonanone</td>
<td>Fruity, soapy</td>
<td>470.20 ± 43.43</td>
<td>278.05 ± 23.31</td>
</tr>
<tr>
<td>(23)</td>
<td>3-Methyl-2,4-nonanedione (e)</td>
<td>Lard-like, strawy, fruity</td>
<td>68.80 ± 20.69</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>3-Methyl-2,4-nonanedione (1)</td>
<td></td>
<td>0.28 ± 0.05</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>3-Methyl-2,4-nonanedione (2)</td>
<td></td>
<td>368.33 ± 57.87</td>
<td>—</td>
</tr>
<tr>
<td>(24)</td>
<td>4-Methyl acetoephone</td>
<td>Sweet, almond-like</td>
<td>—</td>
<td>151.28 ± 15.15</td>
</tr>
<tr>
<td>(25)</td>
<td>trans-Anethole</td>
<td>Aniseed-like</td>
<td>201.24 ± 6.70</td>
<td>198.48 ± 7.42</td>
</tr>
<tr>
<td>(26)</td>
<td>Estragole</td>
<td>Aniseed-like, liquorice-like</td>
<td>239.84 ± 4.89</td>
<td>162.97 ± 9.83</td>
</tr>
<tr>
<td>(27)</td>
<td>p,α-Dimethylstyrine</td>
<td>Terpeny</td>
<td>—</td>
<td>204.10 ± 9.07</td>
</tr>
<tr>
<td>(28)</td>
<td>2-Pentylpyridine</td>
<td>Fatty</td>
<td>215.23 ± 55.30</td>
<td>30.00 ± 6.71</td>
</tr>
</tbody>
</table>

(−−) No response in KFO-screening. The entire concentration–response relation (e) of 3-methyl-2,4-nonanedione was divided into the two data sets (1) and (2), representing different concentration ranges and fitted accordingly.

(Krautwurst and Korthoff 2013). Should quantitative data become available in the future, further compounds may emerge as genuine KFOs. The non-KFO allylphenyl acetate was reported to activate OR1A1 and OR2W1 (Saito et al. 2009). In this study, both OR1A1 and OR2W1 were activated by allylphenyl acetate (Figure 4).

Figure 4 summarizes the EC$_{50}$-based KFO agonist spectra for OR1A1 and OR2W1, including some related non-KFOs which we tested, and orders them by circular categories. Within the inner circles, best KFO agonists with EC$_{50}$ values <100 µmol/L are displayed (Figure 4). Within this category, only 3-methyl-2,4-nonanedione turned out to be a strong KFO agonist for OR1A1 (Figure 4A). In contrast, 9 of the 190 tested KFOs activated OR2W1 with an EC$_{50}$ <100 µmol/L (Figure 4B).

We additionally tested related terpenoid structures that had been reported before as agonists for OR1A1: (S)-(−)-carvone (Saito et al. 2009; Mainland et al. 2014; Geithe and Krautwurst 2015a), (−)-dihydrocarvone (Saito et al. 2009), (−)-carvone (Schmiedeberg et al. 2007), (−)-menthone (Geithe and Krautwurst 2015a), and (−)-menthone (Geithe and Krautwurst 2015b) (Figure 4A). The (S)-(−)-carvone with its characteristic caraway odor has not been listed as KFO here, because we applied the stringent KFO classification criteria by Dunkel et al. (2014). Nevertheless, (S)-(−)-carvone may be considered as KFO under less stringent classification criteria (Krautwurst and Korthoff 2013). Should quantitative data become available in the future, further compounds may emerge as genuine KFOs. The non-KFO allylphenyl acetate was reported to activate OR1A1 and OR2W1 (Saito et al. 2009). In this study, both OR1A1 and OR2W1 were activated by allylphenyl acetate (Figure 4).

We identified the related KFO ethylphenyl acetate as agonist only for OR1A1 (Figure 4A). Concentration–response curves and detailed EC$_{50}$ values for the non-KFO agonists are shown in Supplemental Figure 1 and Supplemental Table 5.

The 16 new, EC$_{50}$-ranked OR1A1 agonists, including non-KFOs, are 3-methyl-2,4-nonanedione, (+)-menthone, (R)/(+)-limonene, 2-phenylethanethiol, trans-anethole, 2-pentylpyridine, estragole, (S)-(−)-limonene, 2-phenylethyl acetate, ethyl phenylacetate, (−)-menthone, (R/S)-γ-nonalactone, ethyl hexanoate, 3-mercaptohexyl acetate, (R/S)-1-octen-3-ol, and ethyl cyclohexanecarboxylate. The 6 confirmed OR1A1 agonists comprise (R)-(−)-carvone (Saito et al. 2009), (−)-carvone (Schmiedeberg et al. 2007), allylphenyl acetate (Saito et al. 2009), (S)-(−)-carvone (Saito et al. 2009; Adipietro et al. 2012; Mainland et al. 2014), (−)-dihydrocarvone (Saito et al. 2009), and 2-nonanone (Saito et al. 2009). The 18 new, EC$_{50}$-ranked OR2W1 agonists are 2-pentylpyridine, 2-phenylethanethiol, methyl cinnamate, cinnamyl acetate, 3-mercaptohexyl acetate, 2-phenylethyl acetate, (S)-(−)-limonene, (R)-(−)-limonene, 4-methyl acetoephone, estragole, trans-anethole, p,α-dimethylstyrine, (R/S)-1-octen-3-ol, (E)-2-heptenal, ethyl cyclohexanecarboxylate, 2-heptanol, (R/S)-γ-octalactone, and 3-methylbutyl acetate. The 7 confirmed OR2W1 agonists comprise octanal, ethyl hexanoate, and 2-phenylethyl acetate.
Allylphenyl acetate (Saito et al. 2009; Adipietro et al. 2012), nonanal (Saito et al. 2009), 1-nonanol (Saito et al. 2009), (R)-(−)-carvone (Saito et al. 2009), 2-nonanone (Saito et al. 2009), and hexyl acetate (Saito et al. 2009).

**Long-chain β-diketones related to 3-methyl-2,4-nonanedione activate OR1A1**

We selected the diketones 2,3-butanedione (KFO), 2,3-pentanedione (KFO), 2,3-hexanone (non-KFO), 2,3-heptanone (non-KFO), 3-methyl-2,4-pentanone (non-natural), 6-methyl-2,4-heptanone (non-KFO), 2,4-octanone (non-natural), and 2,4-nonanone (non-KFO) as structurally related compounds to 3-methyl-2,4-nonanedione to investigate whether they are able to induce receptor responses on OR1A1 and OR2W1. As shown in Figure 5A, neither OR1A1 nor OR2W1 were activated by 300 μmol/L of α-diketones. Only β-diketones elicited responses but with different efficacies between both receptors. OR1A1 was more efficiently activated by increasing the carbon chain lengths of the β-diketones from C7 to C9. In contrast, increasing the carbon chain lengths of β-diketones from C7 to C9 did not improve the receptor performance of OR2W1. Although OR2W1 did not respond to 3-methyl-2,4-nonanedione (see Figure 1), interestingly, it responded to the non-methylated homolog 2,4-nonanone. In summary, the OR1A1 receptor response, however, depended on the chain length, as well as on the position of the keto group. Here, the methylation at C3 played a minor role. In contrast, OR2W1...
The receptor response did not depend on chain length, but the methylation of C3 or the position of the keto group had a major impact on the receptor response.

To validate the screening results, we established concentration–response relations for β-diketones on OR1A1 and OR2W1 and determined the EC$_{50}$ values by nonlinear regression.

Figure 4. KFO agonist spectra of OR1A1 and OR2W1. Shown are EC$_{50}$-ranked categories of agonists with <100 μmol/L (inner circle), 100–250 μmol/L (middle circle), and >250 μmol/L (outer circle) for (A) OR1A1 and (B) OR2W1. KFO agonists: (1) (R)-(−)-carvone, (2) (S)-(−)-limonene, (3) (R)/(+)-limonene, (4) 2-phenylethanol, (5) 3-mercaptohexyl acetate, (6) (E)-2-heptenal, (7) octanal, (8) nonanal, (9) methyl cinnamate, (10) ethyl hexanoate, (11) ethyl cyclohexanecarboxylate, (12) 3-methylbutyl acetate, (13) ethylphenyl acetate, (14) 2-phenylethyl acetate, (15) hexyl acetate, (16) cinnamyl acetate, (17) 1-nonanol, (18) 2-heptanol, (19) (R/S)-1-octen-3-ol, (20) (R/S)-γ-octalactone, (21) (R/S)-γ-nonalactone, (22) 2-nonanone, (23) 3-methyl-2,4-nonanediol, (24) 4-methyl acetophenone, (25) trans-anethole, (26) estragole, (27) p,p′-dimethylstyrene, (28) 2-pentylpyridine. Non-KFO agonists: (I) (+)-menthone, (II) (−)-carveol (III) (−)-menthone, (IV*) (S)-(+)-carvone, (V) (+)-dihydrocarvone, (VI) allylphenyl acetate, (VII) 2,4-nonanediol, (VIII) 2,4-octanediol, and (IX) 6-methyl-2,4-heptanediol. Here (S)-(+)-carvone has been listed as a non-KFO using stringent classification criteria (Dunkel et al. 2014). Nevertheless, (S)-(+)–carvone may be considered as KFO under less stringent classification criteria (Krautwurst and Kotthoff 2013).
OR1A1 was activated by 3-methyl-2,4-nonanedione over a wide concentration range (0.003 to 1500 \(\mu\)mol/L), so we tested all \(\beta\)-diketones in this range. OR1A1 could not be activated by any concentration of 3-methyl-2,4-pentanedione, but showed concentration-dependent effects that depended on the \(\beta\)-diketone chain length (Figure 5B). 6-Methyl-2,4-pentanedione and 2,4-octanedione activated OR1A1 only at higher concentrations. The concentration–response relations of the C\(_9\) \(\beta\)-diketones on OR1A1, however, were best described by a 2-site fit (Figure 5B, Supplemental Figure 2, for statistics see Supplemental Table 6). The \(EC_{50}\) value derived from a 1-site fit of the entire data set was better for 3-methyl-2,4-nonanedione (\(EC_{50}\): 68.80 \(\pm\) 20.69 \(\mu\)mol/L) than for 2,4-nonanedione (\(EC_{50}\): 126.61 \(\pm\) 22.96 \(\mu\)mol/L). Applying a 2-site fit to the data revealed a higher potency of 3-methyl-2,4-nonanedione (\(EC_{50}\): 0.28 \(\pm\) 0.05 \(\mu\)mol/L) over 2,4-nonanedione (\(EC_{50}\): 1.46 \(\pm\) 0.48 \(\mu\)mol/L), at least in the lower concentration range. In contrast, in the higher concentration range, the \(EC_{50}\) value for 2,4-nonanedione (\(EC_{50}\): 265.73 \(\pm\) 15.76 \(\mu\)mol/L) was lower than for 3-methyl-2,4-nonanedione (\(EC_{50}\): 368.33 \(\pm\) 57.87 \(\mu\)mol/L). OR2W1 responded to 6-methyl-2,4-heptanedione, 2,4-octanedione, and 2,4-nonanedione only in the high concentration range but not to the short-chain 3-methyl-2,4-pentanedione or the methylated long-chain 3-methyl-2,4-nonanedione (Figure 5C). The \(EC_{50}\) values for 6-methyl-2,4-heptanedione and 2,4-octanedione were equal but lower for the long-chain 2,4-nonanedione (Table 2).

GC-O experiments with 3-methyl-2,4-nonanedione showed at least (after purification) no contamination with other odor active compounds that could also activate the receptor, neither using a polar (DB-FFAP) nor an unpolar (DB-5) capillary column. Using the latter, a separation (2 discrete peaks) into keto and enol tautomers of...
compound 23 was detectable (peaks A and B, Figure 3B). 3-Methyl-2,4-nonanedione was attributed with a lard-like and strawy odor quality by Guth and Grosch (1989). Both identified peaks had the same lard-like and strawy odor quality as well as very similar mass spectra (Figure 3C). This appearance was in good agreement with the data reported by Guth and Grosch (1989).

For an unambiguous characterization of 3-methyl-2,4-nonanedione (present as keto enol tautomers) by NMR spectroscopy, it was dissolved in ACN-d$_3$ ($c = 7.69$ mmol/L, Supplemental Figure 3). However, for further cell-based luminescence experiments, the compound was solved in DMSO-d$_6$ (stock solution) and the correct concentration of 3-methyl-2,4-nonanedione (and its tautomer) was determined via qNMR ($c = 16.60$ mmol/L, Supplemental Figure 4).

To get an insight into the ratio of 3-methyl-2,4-nonanedione and 2-hydroxy-3-methylnon-2-en-4-one ($c = 3.46$ mmol/L) present at pH 7.5 in buffer solution, a further qNMR experiment was performed. These experiments revealed that the ratio was shifted to the diketone (>90%) toward a greater extent.

Of 391 human ORs, 3-methyl-2,4-nonanedione activates OR1A1

In our study, of the 190 KFOs, the β-diketone 3-methyl-2,4-nonanedione emerged as the best KFO agonist for OR1A1. To investigate the receptor specificity of 3-methyl-2,4-nonanedione, we then screened this compound at 30 μmol/L, which elicited maximum responses in the high-potency part of the concentration–response relation (cf. Figure 3), against 391 human ORs (Figure 6). Notably, 3-methyl-2,4-nonanedione exclusively activated OR1A1 of all the 391 receptors at 30 μmol/L screening concentration. Screening this compound with a higher concentration (300 μmol/L) revealed the same receptor activity pattern (Supplemental Figure 5).

We then compared the relative distances between in vitro EC$_{50}$ values with those between in-house behaviorally determined odor threshold values for OR1A1 best KFO agonist 3-methyl-2,4-nonanedione, the second-best KFO agonist (R)-(−)-carvone, and its enantiomer (S)-(+) -carvone. On a logarithmic scale, both EC$_{50}$ and odor threshold values of 3-methyl-2,4-nonanedione differ by 2–3 orders of magnitude from those of the carvones (Figure 7). In contrast, the odor threshold distance of 1.5 orders of magnitude between the carvones is not mirrored by the distance between their EC$_{50}$ values (Figure 7).

### Table 2. EC$_{50}$ values of β-diketone concentration–response relations for OR1A1 and OR2W1

<table>
<thead>
<tr>
<th>β-Diketone</th>
<th>OR1A1 EC$_{50}$ (μmol/L)</th>
<th>OR2W1 EC$_{50}$ (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Methyl-2,4-pentanedione</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>6-Methyl-2,4-heptanedione</td>
<td>405.43 ± 69.31</td>
<td>632.26 ± 18.65</td>
</tr>
<tr>
<td>2,4-Octanone</td>
<td>210.98 ± 31.73</td>
<td>647.63 ± 29.24</td>
</tr>
<tr>
<td>2,4-Nonanediene (c)</td>
<td>126.61 ± 22.96</td>
<td>494.68 ± 20.08</td>
</tr>
<tr>
<td>2,4-Nonanediene (1)</td>
<td>1.46 ± 0.48</td>
<td>—</td>
</tr>
<tr>
<td>2,4-Nonanediene (2)</td>
<td>265.73 ± 15.76</td>
<td>—</td>
</tr>
<tr>
<td>3-Methyl-2,4-nonanediene (c)</td>
<td>68.80 ± 20.69</td>
<td>—</td>
</tr>
<tr>
<td>3-Methyl-2,4-nonanediene (1)</td>
<td>0.28 ± 0.05</td>
<td>—</td>
</tr>
<tr>
<td>3-Methyl-2,4-nonanediene (2)</td>
<td>368.33 ± 57.87</td>
<td>—</td>
</tr>
</tbody>
</table>

The entire concentration–response relation (c) of the respective β-diketone was divided into the two data sets (1) and (2), representing different concentration ranges, and fitted accordingly. (−) A 2-site fit of the data was not possible. (n.d.) no response detected up to 1.5 mmol/L.

Figure 6. Screening of 391 human ORs with 30 μmol/L 3-methyl-2,4-nonanedione. Shown are mean ± SD ($n = 3$). Data were normalized to the maximum responding OR. The numbers below the color scale refer to the different OR families. M = mock. Dashed line = 2 sigma threshold. RLU = relative luminescence unit.
which could explain the wide concentration range of OR1A1 activation by this β-diketone.

ORs displaying a broad responsiveness to a diverse set of odorants and over a wide concentration range have been speculated to serve as general odorant detectors and/or sensors for the total odorant concentration, at least in sensory neurons of the septal organ of mice (Grosmaire et al. 2009). Several theoretical and data-based models, however, predicted that in olfaction as a multidimensional stimulus detection system, OR populations with broadly tuned receptors, having different or partly overlapping receptive ranges, or even mixed arrays of broadly and narrowly tuned ORs, have higher coding capacities and estimation performance toward stimuli than just receptors with single-odorant specificities (Alkasab et al. 2002; Sánchez-Montañés and Pearce 2002; Reisert and Restrepo 2009; Fonollosa et al. 2012). An outstanding specificity for a particular odorant of an OR, broadly or narrowly tuned, may point to a certain biological relevance of that odorant (see Noe et al., this issue). In the case of KFOs, which have accompanied human evolution, and thus OR evolution, there is bioassay-based evidence that the nearly 230 KFOs found in most human foods (Krautwurst and Korthoff 2013; Dunkel et al. 2014) are among the best agonists for the approximately 400 human ORs (Olender et al. 2012; Verbeurgt et al. 2014).

The outstanding selectivity of OR1A1 for 3-methyl-2,4-nonanediol may thus point to an at least hedonic relevance of this compound. 3-Methyl-2,4-nonanediol has been attributed a strawy or lard-like odor of reversed soybean oil (Guth and Grosch 1989) and has been identified as a KFO in black and green tea, as well as in apricots (Naef et al. 2006; Schuh and Schieberle 2006; Greger and Schieberle 2007). 3-Methyl-2,4-nonanediol plays a key role for the prune note of aged red wines and other oxidized wines (Pons et al. 2008; Pons et al. 2013; Ugliano 2013). The odor threshold of this compound is rather low in the pg/L-ng/L range in different matrices (Schuh and Schieberle 2006). In wine model solutions, 3-methyl-2,4-nonanediol has been concentration dependently associated with a minty, anise-, or prune-like odor (Pons et al. 2008). Indeed, sensory experiments with nonoxidized wines spiked with 3-methyl-2,4-nonanediol revealed a loss of “fresh fruit”-like flavor and an increase in “dried-fruit,” “prune-like” notes, characteristic for oxidized wines, for example, aged red wines (Pons et al. 2013). Other compounds, such as β-damascenone and γ-nonalactone, have been suggested to participate to a prune note in aged red wines (Pons et al. 2008). In a complex mixture like aged wine, several KFOs at their wine-specific concentration ratios may participate in eliciting an “aged wine/prune note”-like OR activity pattern, even though the odor characters of the single compounds deviate from “aged wine/prune note.” In our hands, OR1A1 responded best to 3-methyl-2,4-nonanediol but also responded to γ-nonalactone, emphasizing its role as an important receptor participating in an aged wine/prune note-specific OR activation pattern. Such a pattern may be identified by testing a wine-specific aroma recombine, including 3-methyl-2,4-nonanediol, against the entire set of human ORs in a cell-based assay.

Our testing of 3-methyl-2,4-nonanediol against 391 different human ORs repeatedly revealed OR1A1 as the exclusive responder both at 30 µmol/L and at 300 µmol/L. However, single nucleotide polymorphisms (SNPs), which frequently occur in OR genes (Menashe et al. 2003; Olender et al. 2012; Mainland et al. 2014), may affect certain ORs in our library, such that their function may be impaired. SNP-derived loss-of-function ORs have been described in several cases (Keller et al. 2007; Jaeger et al. 2013; Mainland et al. 2014; Geithe and Krautwurst 2015b). Moreover, ORs may not be
equally well expressed at the plasma membrane level (McClintock and Sammetta 2003; Lu et al. 2004; Bush and Hall 2008; Matsunami et al. 2009) (Supplemental Figure 6). Therefore, we cannot entirely exclude the existence of other ORs responding to low concentrations of 3-methyl-2,4-nonanedione. An exclusive selectivity of OR1A1 for nanomolar concentrations of 3-methyl-2,4-nonanedione, however, would open the possibility to design an analytical biosensor to sensitively detect this compound, for instance as a marker in aged, oxidized wines. Further studies including spiking experiments with 3-methyl-2,4-nonanedione in red wines according to Pons et al. (2013), combined with OR1A1 genotyping of the panelists, are conceivable and will provide information about the impact of OR1A1 and its high frequent SNP-affected variants on the 3-methyl-2,4-nonanedione detection.

The logarithmic distance of the odor thresholds in water of 3-methyl-2,4-nonanedione to those of the carvone enantiomers is mirrored by their EC50 values as determined in our in vitro assay. This, together with the observation of OR1A1 being the only human OR responding to 3-methyl-2,4-nonanedione, supports the notion of a unique function of OR1A1 for the near-threshold detection of this KFO. In contrast, the differences in EC50 values for the (R)-(−) and (S)-(+) carvone enantiomers on OR1A1 are not congruent with their odor threshold distances, suggesting that OR1A1 is not the most sensitive carvone receptor. Indeed, several carvone-responding ORs have been identified in a screening approach, testing both carvone enantiomers against 391 human ORs, with OR1A1, however, being the only receptor that emerged as a responder for both carvones (Geithe and Krautwurst 2015b).

In summary, our comprehensive, dual-screening strategy, testing an entire set of food-relevant compounds against single receptors, and, vice versa, testing the single best compound against an entire set of human ORs, revealed KFO-enriched odorant profiles for broadly tuned receptors OR1A1 and OR2W1. We identified (i) 17 new agonists (14 KFOs and 3 non-KFOs) for the human odorant receptor OR1A1, (ii) confirmed 6 (2 KFOs and 4 non-KFOs) of its previously reported agonists, (iii) revealed 21 new agonists (18 KFOs and 3 non-KFOs) for OR2W1, and (iv) confirmed 7 (6 KFOs and 1 non-KFO) of its previously reported agonists. Our findings support the notion of a key role of KFOs as OR agonists. Importantly, and despite its broad odorant tuning, OR1A1 appears to be highly selective for 3-methyl-2,4-nonanedione, suggesting a unique role of OR1A1 for the near-threshold detection of this KFO in wine, green tea, and other food matrices.

Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/

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References


