

BIOCHEMISTRY

Functional odor classification through a medicinal chemistry approach

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Crucial for any hypothesis about odor coding is the classification and prediction of sensory qualities in chemical compounds. The relationship between perceptual quality and molecular structure has occupied olfactory scientists throughout the 20th century, but details of the mechanism remain elusive. Odor molecules are typically organic compounds of low molecular weight that may be aliphatic or aromatic, may be saturated or unsaturated, and may have diverse functional polar groups. However, many molecules conforming to these characteristics are odorless. One approach recently used to solve this problem was to apply machine learning strategies to a large set of odors and human classifiers in an attempt to find common and unique chemical features that would predict a chemical's odor. We use an alternative method that relies more on the biological responses of olfactory sensory neurons and then applies the principles of medicinal chemistry, a technique widely used in drug discovery. We demonstrate the effectiveness of this strategy through a classification for esters, an important odorant for the creation of flavor in wine. Our findings indicate that computational approaches that do not account for biological responses will be plagued by both false positives and false negatives and fail to provide meaningful mechanistic data. However, the two approaches used in tandem could resolve many of the paradoxes in odor perception.

INTRODUCTION

It is nearly impossible to predict whether a given molecule will be odorous and what its odor quality might be from the chemical structure alone. Although all odor molecules are typically organic compounds of low molecular weight, they may be aliphatic or aromatic, may be saturated or unsaturated, and may have any of several polar functional groups. However, there are many molecules that conform to those characteristics, which are nonetheless odorless, to humans and other animals. A crucial difficulty lies in the definition of the categories of smell. Without unambiguous and measurable categories, it is virtually impossible to build any set of odor-structure relationships. The existing categories are constructed from consensus among observers, but these descriptors tend to be mostly subjective and not universal. In 1988, Chastrette and colleagues (1) studied a collection of 2500 odor descriptions (2) and concluded that only 3% of the descriptors led to a fruitful odor-structure relationship. A 1985 attempt by Dravnieks (3) ended up with more than 100 descriptors to describe 144 odorants. Some of those descriptors were used in a recent attempt to apply machine learning algorithms in a contest among programmers to find correlations between the physicochemical properties of more than 400 odorants and their odor quality, as judged by human subjects (4). However, this was performed with a limited number of percept descriptors, including ambiguous labels, such as “bakery” or “chemical,” and other very specific types, such as “garlic” or “fishy,” which the subjects were forced to use for classification. The particular chemical structural characteristics were obtained from a set of more than 4000 chemical features provided by the chemical database DRAGON. As might be imagined with such a large database, many of the chemical classifiers in DRAGON are obscure chemical properties that relate to physical and organic chemistry. Although the winning programs revealed mixed fortunes in predicting the odor quality on a hidden data set, even the correct correlations

did not provide any rational scheme for classification or any biological mechanism for detection and discrimination. This is perhaps not surprising because the chemical systems tend to heavily weigh structures and qualities that would be important to a synthetic chemist designing or analyzing a reaction pathway. However, there is no reason to believe, a priori, that these same features would be of any interest to a biological receptor. In a previous study, we have already shown that biological responses are a better predictor of chemical detection than chemical descriptors (5). As an alternative to using subjective descriptors from human subjects, we have turned to mouse olfactory sensory neurons (OSNs) where we can read out receptor activity through a calcium signal. We were also able to extend and confirm our results in mouse behavior experiments and, finally, to a human panel without requiring the use of descriptors.

In addition, rather than attempting to choose a “diverse” panel of odorants based solely on the diversity of their physicochemical structures, which may have little biological relevance and may not be biologically diverse at all, our study used well-established techniques from the pharmaceutical chemistry. Dunkel *et al.* (6) suggested that, although the olfactory system is able to respond to a wide variety of chemicals, the best ligands will be those with behavioral and evolutionary significance. Thus, they focused on food odorants and revealed that 230 “key food odorants” out of 10,000 identified flavor compounds were necessary and sufficient to reconstruct most food and beverage percepts. These results simplify the odor landscape by two orders of magnitude (6). Taking advantage of this simplified landscape, Krautwurst and colleagues (7) succeeded in identifying highly sensitive receptors for two key food odorants found in red wine and onions that activate the broadly tuned OR1A1 and narrowly tuned OR2M3 human receptors, respectively. These studies provide further rationale for using a smaller set of odorants chosen for their ecological or other relevant properties (4, 7).

For this study, therefore, we have chosen to investigate esters. Applying a medicinal chemistry-based approach, we have made use of a series of related medial and terminal ester compounds to seek a rational set of parameters for understanding why related chemical compounds may be perceived as different or similar based on their biological activation

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properties. Medicinal chemistry emphasizes the biological functions over chemical properties by investigating the effect of subtle changes of the chemical structures in molecules on their targets (that is, the receptors). In the case of the esters used here, the position of the ether oxygen and the lengths of the carbon chain on either side of the ether oxygen or the carbonyl group were varied, and we investigated the reverse esters of each compound—esters in which the ether and carbonyl oxygens are transposed compared to the original compound (Fig. 1). Knowledge of esters and their reactivity is central to food science today. Since the official term for esters was coined in 1850 by L. Gmelin (who is also responsible for introducing the term for ketones in 1848), esters have become the most widely used compounds in fragrance and flavor chemistry. In particular, esters play a major role in the production of wine, where they are not only responsible for its fruity notes but also mitigate its acidity (8, 9). Esters also represent the core of the sex pheromones of numerous crop pest insects (10, 11). Because these sex pheromones can often vary very subtly between different species, understanding what

features are actually relevant for their discrimination may lead to a better understanding of these insects' speciation and pave the way for crop protection strategies based on sex pheromone antagonists or agonists (12). We have therefore chosen the ester molecule as a model ligand for its widespread detection and discrimination in olfactory systems across evolutionary distant species. Our results show that, although a classical chemistry-centric approach fails to predict odorant similarity, a medicinal chemistry-based biology-centric approach offers a more accurate strategy to comprehend odor discrimination through molecular structures.

RESULTS

Dissociated OSN responses to esters

We used OSNs dissociated from mice engineered to express GCaMP6f under the olfactory marker protein (OMP) promoter to monitor cell activity when exposed to odor compounds. In this case, we were

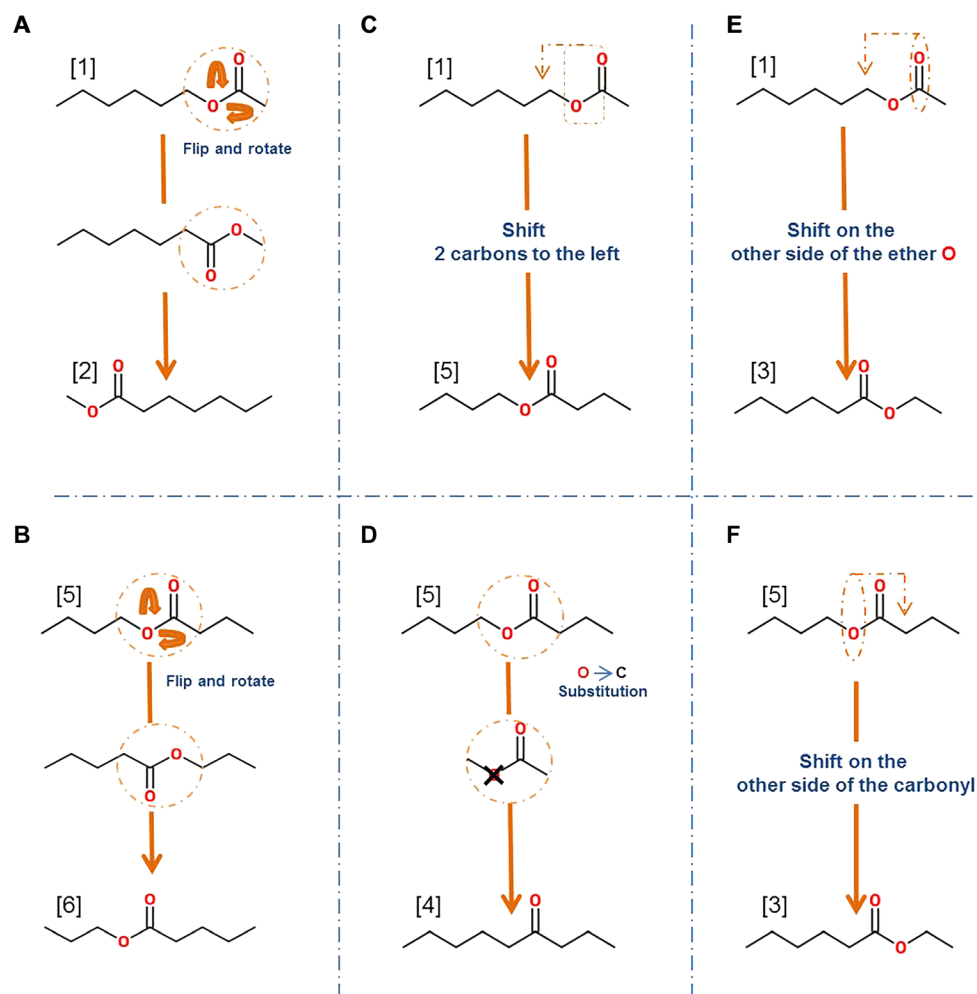


Fig. 1. Ester group manipulations. These structures show the relations between the odorants of our panel. All the odorants of this panel have a nine-atom-long backbone chain. (A) [1] and [2] are terminal reverse esters. Their ether oxygen and the carbonyl are reciprocally transposed so that the carbonyl is now situated where the ether oxygen used to be. They both have one six-carbon nonpolar “arm” and a terminal polar group. (B) [5] and [6] are medial reverse esters. Their ether oxygen and the carbonyl are reciprocally transposed. They both have two nonpolar carbon chain “arms” and a central polar group. (C) [3] represents a two-carbon terminal-to-medial shift of the ester group compared to [1]. (D) The ketone [4] represents an O→C substitution compared to both [5] and [3]. (E) [3] and [1] have their ether oxygen located at the same relative position of their backbone chain but represent a symmetrical displacement of the carbonyl around this ether oxygen. (F) [5] and [3] have their carbonyl located at the same relative position along their backbone chain but represent a symmetrical displacement of their ether oxygen around this carbonyl.

interested in understanding the particular features of closely related ester compounds that allowed them to be detected and discriminated by odorant receptors (ORs). Because mature OSNs expressing OMP only express a single OR (13), we can use the activity of individual cells as a proxy for specific receptors activation (Fig. 2A).

Our panel consisted of hexyl acetate [1] and the following derivatives: methyl heptanoate [2], ethyl hexanoate [3], butyl butyrate [5], and propyl pentanoate [6]. Each compound represents a displacement along the molecule's carbon chain of the ether oxygen, the carbonyl group, or the entire ester functional group (Fig. 1). A ketone, 4-nonanone [4], was

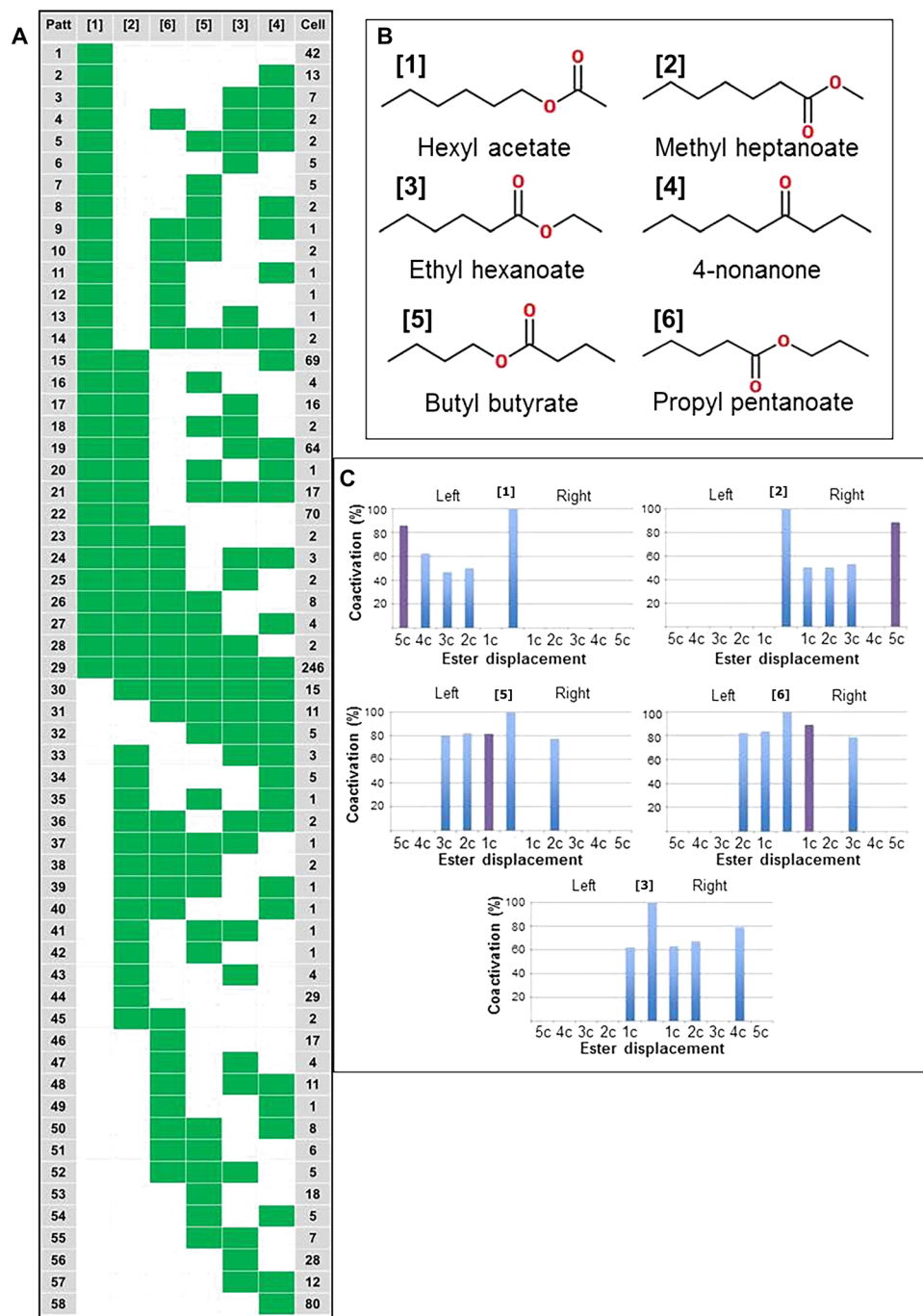


Fig. 2. Responses of dissociated OSNs to ester odors. (A) A total of 872 of 4523 viable OSNs responded to at least one odorant, leading to 58 distinct binary response patterns (enumerated on the left). The numbers in the rightmost column indicate how often a particular response pattern was observed. A green box denotes activation of the OSN by the corresponding odorant. (B) Two-dimensional (2D) representations of the tested odors. (C) Coactivation of OSNs responding to each of the five esters plotted according to ester group displacement along the carbon chain of the primary odorant (numbers above each graph). Reverse esters, denoted by the purple bar, revealed the highest levels of coactivation for both medial and terminal esters, although the terminal esters represent a four-carbon displacement. Pairwise coactivation values are provided in fig. S1. Examples of OSNs' Ca^{2+} response traces are provided in fig. S4.

added to this panel as an outsider chemical. A total of 872 OSNs exhibited a calcium response to at least one odorant of this panel among the approximately 4500 OSNs tested. Fifty-eight distinct patterns of activity were observed when responses were conservatively scored in a binary fashion, indicating that a minimum of 58 different ORs were involved in the detection of this panel (Fig. 2A). Of these, 31 patterns were represented by fewer cells apiece, including 13 patterns observed in only one cell (fig. S4).

Of the 872 responding OSNs, 68.3% responded to [1], 66.3% to [2], 54% to [3], 44.2% to [5], and 40.6% to [6]. The higher levels of activation seen in [1] and [2] indicated that terminal esters, regardless of their orientation, are the preferred ligands across the OR repertoire. A total of 246 of the 872 OSNs (28.2%) responded to all of the odorants in the panel, including the ketone [4], suggesting a significant amount of crossover activation among these odorants (Figs. 2 and 3). Surprisingly, the ketone odorant [4] was able to activate nearly 74% of the OSNs responding to [1], although it represents a terminal-to-medial two-carbon displacement of the carbonyl and an oxygen/carbon substitution. Moreover, [4] activated more than 82% of the OSNs that responded to the medial esters [3], [5], or [6] (Fig. 2 and fig. S1). Ester [5], which can be viewed as a C→O analog of the ketone [4], activated 44.2% of OSNs, on par with the ester [6], but decidedly less than [4], which activates 67.5% of the OSNs. This was a first indication that the ether oxygen, and in particular, its placement relative to the carbonyl, had an impact on odorant recognition. Further support came from the finding that in [1] and [3], which preserve the location of the ether oxygen but where the carbonyl swaps sides, there was only 62.2% co-recognition for this now more medial ester. This leads to the hypothesis that the ether oxygen, although only a poor contributor to hydrogen bonding, nevertheless imparts a type of “reading orientation” to the ester when the ester is terminally located. Accordingly, we focused on a number of additional chemical manipulations, including the medicinal chemistry tactic of reversing the ester (compounds [1] and [2], and [5] and [6]), which creates a pronounced change in the identity of the alcohol and the acid arm of the compound but preserves the location of the polar group (Fig. 1). Although the ether oxygen does change position in this manipulation, the carbonyl also shifts, and the combination of changes may thus compensate for one another.

As expected from medicinal chemistry, OSNs responding to a given ester appear to better tolerate the reverse ester than any other substitution. Indeed, a total of 85.6% of the OSNs responding to the terminal ester [1] were coactivated by its reverse ester [2] (Fig. 2C and fig. S2). This is also true for the OSNs responding to the medial ester [5], which shows 81.5% of coactivation when challenged with its reverse ester [6] (Figs. 2C and 3). As a comparison, [2] represents a medial-to-terminal one-carbon displacement of the ester group along the “backbone chain” of [3] (Fig. 1 and fig. S2B). This one-carbon shift is similar to the shift induced by the ester-to-reverse ester substitution. However, [2] shows a reduction of coactivation of the OSNs responding to [3] to 61.9% (fig. S1). Next, we looked at “translating” the ester along the backbone chain of the odorants. Although our panel limits the possible pairings, we noted that there was a consistent trend for terminal esters to be better activators than medial esters (Fig. 2). Only 50% of the OSNs responding to [1] were coactivated by [5] (fig. S2A), which represents a terminal-to-medial two-carbon displacement of the ester group compared to [1]. Furthermore, when OSNs responding to [1] were challenged with [6], which represented a terminal-to-medial three-carbon displacement of the ester group, the level of coactivation was only 46.5% (Figs. 1 and 2C). The carbonyls of the odorants [3] and

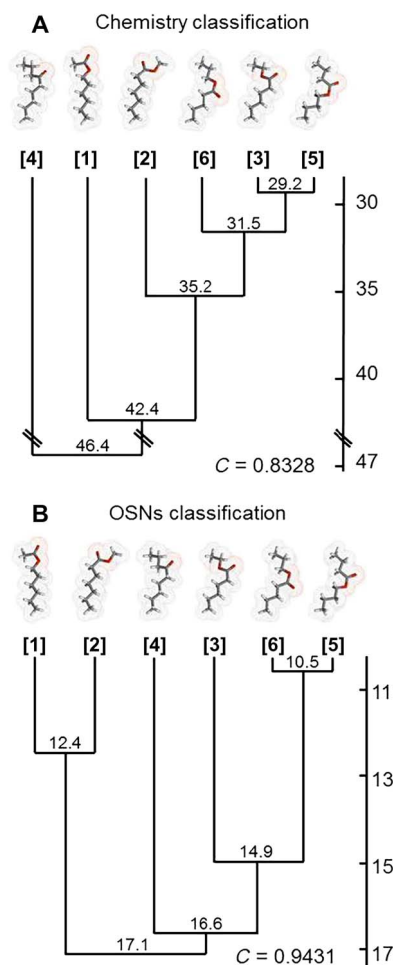


Fig. 3. Hierarchical clustering analysis of tested esters. (A) Odorants clustered according to chemical similarity as evaluated by 1666 molecular descriptors downloaded through the e-Dragon applet. Note that in this chemical-based clustering, the major division is the functional group (that is, ester or ketone). C denotes the cophenetic correlation coefficient. (B) Odorants clustered according to biological response similarity based on calcium imaging of dissociated OSNs (form the data shown in Fig. 2). In this biology-centric classification, the relative positions of the functional group (that is, medial or terminal) emerge as the main organizational feature of the classification. The closest odorants appear to be reverse esters in both medial and terminal clusters. All distances in the dendrograms are Euclidean. See Materials and Methods for the details of dendrogram generation. The top 20 e-Dragon descriptors that best recapitulate the OSN responses to the esters ([1], [2], [3], [5], and [6]) are provided in table S1.

[5] are located at the same distance from their terminus, but their ether oxygen is placed on the opposite sides of this carbonyl. Nonetheless, 82% of the OSNs responding to [5] also showed a response to [3]. These results surprisingly indicate that the ether oxygen is probably not a key feature for the binding of medial esters to their receptor and suggests instead that the relative location of the carbonyl from the terminus may be the key feature for esters binding to their cognate ORs. Reciprocal activity was also observed within the pairs of odorants, and those levels are reported in fig. S1.

Odorant classification by chemistry or by OSN responses

We next compared the classification of our ester odorants using both a traditional chemistry-centric and a medicinal chemistry-based

biology-centric approach. For the chemistry-centric approach, we used the e-Dragon software to obtain 1666 molecular descriptors for each odorant and generated a dendrogram of similarity (Fig. 3A). With this method, the segregation of our panel of odorants was first driven by their functional group, with the ketone [4] expectedly forming one cluster and the ester odorant grouping together in a second cluster. In the latter group, the segregation level appeared to be based on the comparative length of the arms, leading to more asymmetrical or symmetrical esters (although they all span the same end-to-end breadth). The “asymmetric” terminal ester [1] segregated from the rest of the molecules that formed a group inside a “symmetric-armed ester” cluster in which [5] and [3] appear to be the closest molecules.

For the biology-centric classification, the response patterns of the 872 responsive OSNs were used as the basis for a hierarchical cluster analysis that led to a very different dendrogram of similarity (Fig. 3B). Notably, the terminal ester [1] and its reverse ester [2] were grouped within the same cluster and segregated from the other odorants of the panel. More surprisingly, [4], with its ketone group, does not represent an outside group but is incorporated inside a cluster of “symmetric-armed odorants” including [3], [5], and [6]. Inside this cluster, contrary to what is observed with traditional chemistry, [5] appeared to be closer to its reverse ester [6] than to [3]. These results suggest that the relative position of the carbonyl group is the key component that leads to the recognition of the odorant by its receptor, rather than the functional group (that is, ketone versus ester), as predicted by traditional chemistry.

Finally, in an attempt to reverse-engineer the biology data into the chemical descriptor database, we calculated and compared the distance matrix of every molecular descriptor provided by e-Dragon with our biology-centric classification using their Spearman's correlation factor (table S1). Surprisingly, although this method allowed for the identification of several molecular descriptors simulating the biology-centric classification of the five esters of our panel, no descriptors were found to recapitulate the clustering of the ketone [4] with the medial esters, as observed with the classification obtained from the OSNs' calcium responses. We thus decided to exclude [4] from this “reverse” analysis, and only then were we able to identify the top 20 molecular descriptors that best qualitatively simulated the biology-centric classification of the medial esters [3], [5], and [6] and terminal esters [1] and [2] (table S1).

Behavioral response of mice to esters

Although the evidence from OSNs strongly implicates an alternative biology-based classification system for odor stimuli, it is pertinent to ask whether this classification is reflected in behavioral responses to the odors. To determine this, we used the habituation-dishabituation test on several cohorts of mice with the same panel of odors, presented in pairs. This test reveals whether two odors are perceived as having a similar or dissimilar quality (Fig. 4). First, a single odor is presented to an animal for several consecutive trials, until the animal no longer responds to the odor, indicating that it has habituated. Then, a second odor is presented. If that odor has the same or nearly the same percept as the habituated odor, then the mouse will ignore it. If it has a dissimilar quality (that is, new perceptual quality), then the mouse will attend to it. From these data, we can estimate the “perceptual similarity” between our odorants and compare it with the chemistry- or biology-derived dendrograms above.

Notably, several results were obtained from these behavioral experiments. First, the ketone [4] was discriminated from both the

medial ester [5] and the terminal ester [1] (Fig. 4, C and F). This result indicates that [4] represents a true outsider compound among our panel. Mice also easily discriminated [1] from [5] (Fig. 4B). These results could have been predicted from both the chemistry and biology classifications. However, according to the traditional chemistry-centric classification, [5] should be perceived as more similar to [3] than to [6], but our test mice appeared to be unable to discriminate [5] from its reverse ester [6] (Fig. 4D), although they discriminated it from [3] (Fig. 4E). Similarly, and contrary to the chemistry-centric classification prediction, mice did not discriminate [1] from its reverse ester [2] (Fig. 4A). However, these results are consistent with the biology-centric classification using OSN responses.

Human discrimination of esters

These results suggested that it would be interesting to see whether humans would classify these odorants in a chemistry- or biology-centered way. To compare the human results with the mouse olfactory discrimination test made at 30 μM , we challenged a group of 11 human subjects (eight females and three males) with seven solutions consisting of 30 μM solutions of the odorants [1], [2], [3], [5], and [6], a blank (no odorant), and a second solution of [1]. We asked them to group the odor samples into however many groups they perceived as similar (Fig. 5A). Note that we did not ask for any verbal descriptor of the odors nor did we provide any. Subjects were asked only to discriminate whether odors were similar or different. Not unexpectedly, there were important differences in classification between subjects, but each subject showed considerable consistency in their groupings over three iterations of the experiment (fig. S3). From the clustered data, we constructed dendrograms based on the perceived similarity of the odors. Our human subjects, like mice, perceive the medial esters [5] and [6] to be the most similar but, unlike mice, classified them as closer to [2] than to [3]. Humans also clustered [1] with [3] rather than [2]. The 30 μM odorant concentration seemed too faint to allow a clear discrimination from the blank solution by human subjects. Nonetheless, the blank solution was correctly identified between 27 and 36% of the time over the three repetitions while the chance level is 15% (fig. S3).

To confirm these results, we challenged the subjects with an even simpler olfactory test—a same versus different discrimination. We also decided to increase the odorant concentration to 3 mM for this second experiment to see whether increasing the concentration would change the percepts of the odorant for humans. In this case, each subject was given three vials of solution containing two identical 3 mM odorant solutions (of either [5] or [1]) and a 3 mM odorant solution of a different odorant ([2], [3], [6], or a blank) and was asked to identify the different odorant. As in the first test, the subjects were not asked for descriptors nor were given any. Notably, this 100 \times difference in concentration did not change the overall classification of the odorants by the human subjects, with the exception of a better blank discrimination. The discriminated odorants at 30 μM were still discriminated at 3 mM, whereas the nondiscriminated odorant at 30 μM remained nondiscriminated at 3 mM. Subjects discriminated the blank vial from those containing either [5] or [1] in more than 70% of the cases and those containing [2] from the [1] duplicates in 66% of trials (Fig. 5C). When challenged with [5] as the duplicate, subjects were able to discriminate it from the sample of [2] or [3] at around a 55 to 60% level but could only identify odor [6] as different 28% of the time, lower than even the chance level of 33% (Fig. 5D). The results are interesting in that humans discriminate these odors differently from the predictions made by a chemistry-centric classification but also differ from the

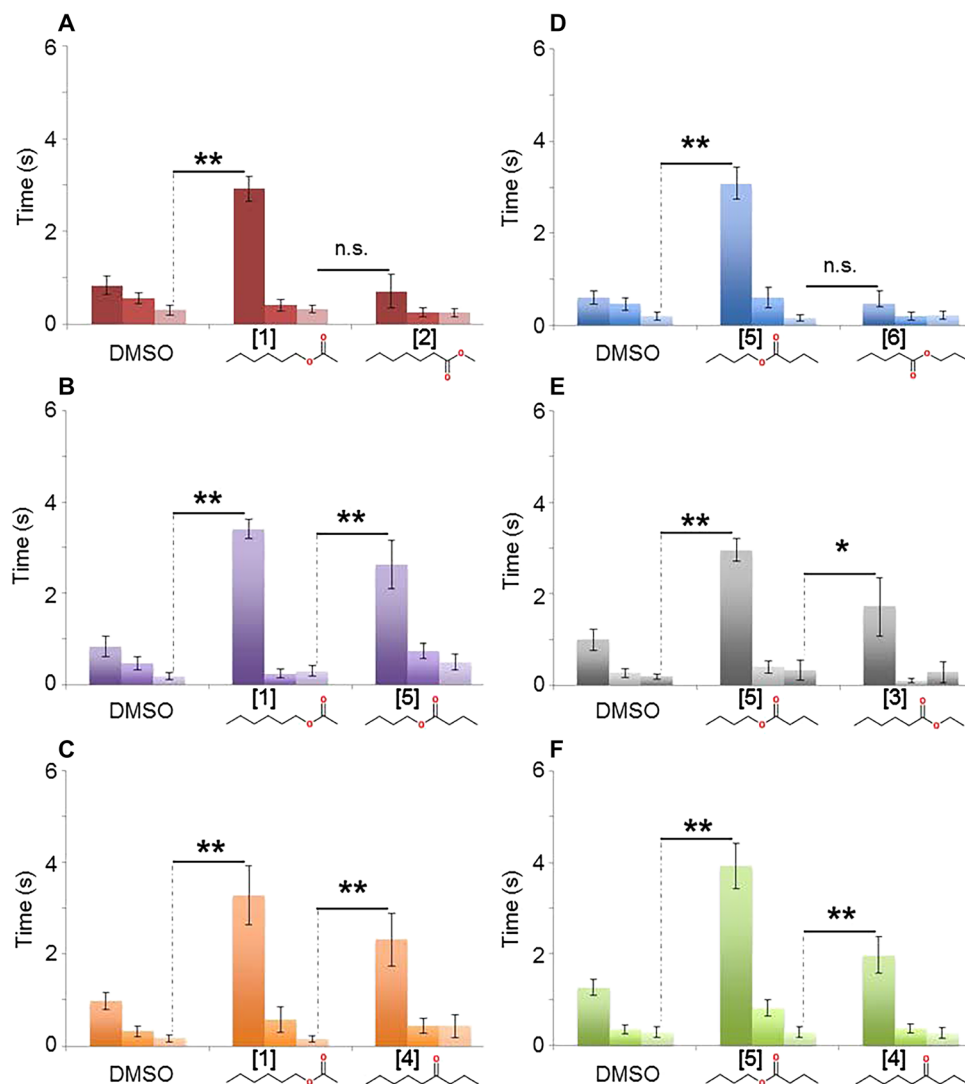


Fig. 4. Habituation-dishabituation olfactory test. Histograms indicate the average olfactory investigation time (in seconds) by mice during repetitive 2-min exposures to odorant pairs or dimethyl sulfoxide (DMSO) (solvent). Mice that habituated to the terminal ester [1] remained habituated to its reverse ester [2] (A) but dishabituated to the medial ester [5] (B) and the ketone [4] (C). Similarly, mice that habituated to the medial ester [5] remained habituated to its reverse ester [6] (D) but dishabituated to the medial ester [3] (E) and the ketone [4] (F). Note that these behaviors recapitulate the ester classification obtained from the dissociated OSN response in Fig. 3. Behavioral data were analyzed using the analysis of variance (ANOVA) test, followed by a post hoc paired *t* test (* $P < 0.05$ and ** $P < 0.005$, paired post hoc *t* test). n.s., not significant. Error bars indicate SEM. Between 9 and 12 animals were tested for each pair of esters.

mouse discriminations. This lends further support to the model that odor quality is determined as much by receptors as by chemistry and will differ depending on the repertoire of receptors possessed by the discriminating organism.

DISCUSSION

It is widely accepted that peripheral olfactory discrimination works through a reciprocal combinatorial code in which one chemical can be detected by different ORs and one OR can detect a group of different, presumably related, chemicals (14, 15). In addition, the axons of all OSNs expressing a particular OR project to the same glomerulus in the olfactory bulb, suggesting a labeled line-style “odor map” (16, 17) that relies on the molecular receptive range of each receptor (18). Commonly, what is meant by the molecular receptive range of a

receptor is defined in terms of chemical categories—a receptor is sensitive to aldehydes or ketones, to aromatic rings or chain length, etc. However, these receptive ranges frequently extend beyond obviously defined chemical categories. Thus, understanding the olfactory code requires a correct set of assumptions about the relation between chemical structure and odor quality. Currently, the field primarily makes use of chemical data and psychophysical descriptions of odor qualities (4). The crucial role of biology, the odor receptors, is either discounted or assumed to be neutral. Thus, there have been numerous attempts, historically and currently, to classify odors by chemical structure and perceptual quality and to then search for relations that will describe various odor sets (19). Nonetheless, there remain numerous paradoxes in the field because of these schemes. For example, the perception of musk odor may be created by any one of several molecules that have little shared chemical structure (20, 21).

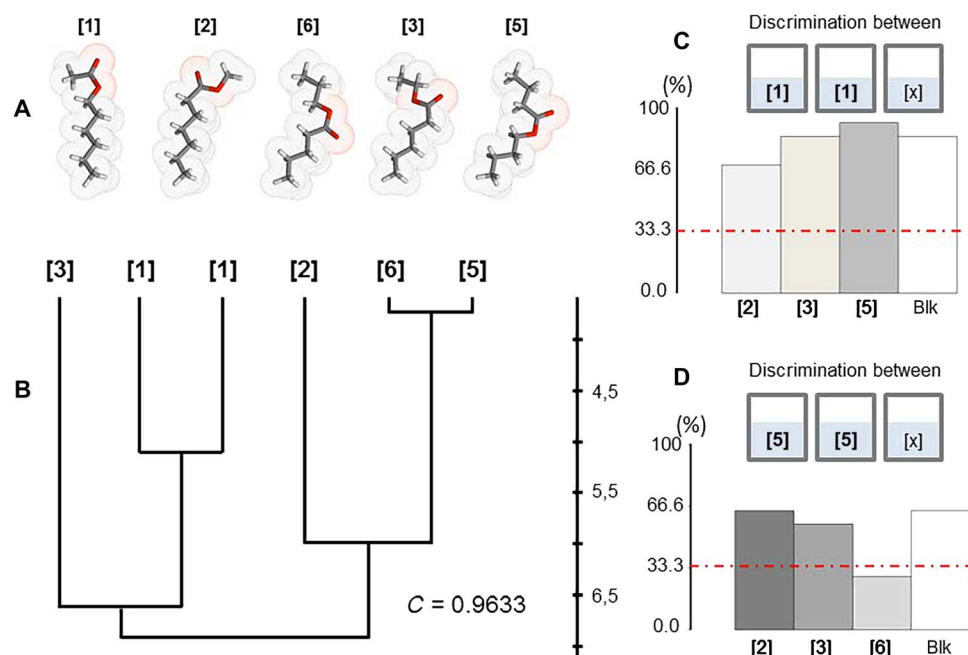


Fig. 5. Human olfactory discrimination tests. (A) 3D representations of the tested odorants. (B) Odorants clustered according to similarity based on 11 human subjects' perception of 30 μM odorant solutions over three iterations. All distances in the dendrograms are Euclidean. See Materials and Methods for the details of dendrogram generation. The two reverse medial esters [5] and [6] were grouped together more frequently than the two odorant solutions containing [1] (iteration results are reported in fig. S3). (C) Histograms represent the percentage of correct identification by human subjects of the different 3 mM odorant solution [1] (that is, [2], [3], [5] or blank (Blk)) from the two identical 3 mM odorant solutions containing [1]; chance level (33%) is shown by the red dashed line. $n = 13$ volunteers. (D) Histograms represent the percentage of correct identification made by the human subjects of the different 3 mM odorant solution [x] (that is, [2], [3], [5] or blank) from the two identical 3 mM odorant solutions containing [1]; chance level (33%) is shown by the red dashed line. $n = 14$ subjects.

There are thousands of chemical descriptors that have been developed through decades of analytical chemistry research (22). However, all of those descriptors can describe molecules that are equally likely to have an odor or not. There are, to be certain, coarse rules of thumb—aliphatic aldehydes generally have fruity smells if they are also of a certain minimum chain length—but none of these are consistently reliable. Chemical features do not lead to any biological mechanism that suggests why aldehydes are fruity sometimes. It would seem that the only reliable definition of an odor is an operational one: Odors are molecules that bind to and activate odor receptors. Although this may, at first, seem a trivial definition, it can be used to devise a method of classifying odors that is much more empirical and less theoretical than the more common schemes pairing chemistry-derived descriptors with subjective judgments.

Just such an exercise was recently published as a contest between programming groups who were given a large database containing psychophysical data from many subjects tested on a large array of odors. Applying machine learning algorithms to the data, one or more groups arrived at programs that could predict chemical attributes for 8 of the 19 descriptors. In some cases, more than 100 chemical features (out of a database of more than 4000) were required to predict some odor qualities (4). Most of these features would be unknown to all but a specialized organic or physical chemist and fail to provide any intuition into why or how these would be important to receptor activation. Notably, human subjects were required to classify odors according to a predetermined set of descriptors that all have unknown and perhaps idiosyncratic psychological meanings. Although this is an impressive accomplishment for screening large sets of molecules for potential odor qualities, it provides little or no mechanistic insight into odor

detection or discrimination. It also resulted in a large number of both false positives and false negatives.

An additional problem arises from the unsupported assumptions made when constructing odor panels intended to reflect diversity. Although they may reflect chemical diversity, they may not actually be sampling a biologically diverse odor space. For example, including aldehydes, alcohols, and acids in a panel, as is often done, does not necessarily assure diversity because there are numerous receptors that do not discriminate between those chemical functional groups (23). A further consequence of this reliance on chemical structure is that it will easily lead to incorrect models of how odor quality or perception may arise in the brain. As an example, the classical chemistry classification failed to predict our compound [1] and its reverse ester [2] to have these similar percepts. Reciprocally, the medial esters [5] and [3], although being the closest molecules of our panel according to a classical chemistry classification, were easily discriminated by both mice and humans in behavioral tests. Notably, our psychophysical tests did not require humans (or mice, of course) to make a perceptual classification—simply a discrimination of sameness or difference.

Therefore, we suggest that understanding the rules of odor classification in biological systems requires an approach that combines chemical features with biological function. In the pharmaceutical industry, this is often achieved by the application of medicinal chemistry. Medicinal chemistry makes various changes in known molecules and assays their biological effects on cells, systems, or organisms. It searches for bioisosteres, molecules that have similar biological functions regardless of their chemical similarity or dissimilarity (that is, stereoisomers) (24). This approach notably revealed interesting results in designing parapheromones to perturb insect communication for crop protection

purposes (12, 25). Combining chemistry with a biological focus classifies odors in a physiologically meaningful way that is a reflection of the operation of the biological system. It allows us to identify the crucial chemical characteristics of biological relevance, which may not always be the most salient chemical features.

In the current instance of ester chemistry presented here, we find that there is a strong relation between esters and ketones that is not predicted by chemical analyses. This unexpected result led us to theorize that the critical feature of ester molecule discrimination is not the ester group but rather the position of the carbonyl group. Instead, the ether oxygen of the ester group seems to provide a directional reading frame to the molecule. Furthermore, we find that an ester and its reverse ester were well tolerated by ORs. This result is surprising because the transformation of an ester to its reverse ester represents a marked alteration by standard chemical classification schema. In a famous example in the 1980s, the use of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), the reverse ester of meperidine, instead of meperidine by drug addicts, led to the development of Parkinson's-like disease in consumers (26, 27). This was because the reverse ester could not be bound and properly metabolized by a crucial enzyme. In this case, and in numerous other pharmacological cases, the ester and reverse ester are discriminated. However, it is not true in all cases and is certainly not the case in olfaction.

Especially gratifying for the use of this approach is that the mouse and human behavioral data, which do not rely on subjective odor descriptors, corroborate the cell data, thus suggesting that perception can be linked to molecular features so long as one accounts for these through a biological assay. This study and previous works (5, 28–30) have underscored that relying too strongly on chemical analyses will result in sometimes serious miscalculations about discrimination and perception in olfaction. These miscalculations will not be overcome by the simple application of machine learning to large but idiosyncratically constructed databases. They are more likely to exacerbate the problems. On the other hand, by including biological data in the data sets, the value of using big data approaches is considerably strengthened.

MATERIALS AND METHODS

Chemicals

The odorants of the panel were derived around a lead odorant, hexyl acetate [1], and consisted of hexyl acetate [1], methyl heptanoate [2], ethyl hexanoate [3], 4-nonanone [4], butyl butyrate [5], and propyl pentanoate [6]. Odorants were all purchased from Sigma-Aldrich, except for [6], which was purchased from Chem Service Inc. Odorant stocks were made in >99% DMSO (Sigma-Aldrich) and diluted in freshly prepared Ringer's solution to a final concentration of 30 μ M just before experiments. Dimensional representations (2D and 3D) of the molecule were obtained using Molinspiration Cheminformatics free software (www.molinspiration.com).

Animal and tissue collection

All animal procedures conformed to the guidelines for care and use of animals of Columbia University and were reviewed and approved by the Institutional Animal Care and Use Committee. OMP-Cre-driven GCaMP6f mice used in this work were generated by crossing the OMP-Cre line (#006668, the Jackson Laboratory) with the line B6;129S-Gt(ROSA)26Sortm95.1(CAG-GCaMP6f)Hze/J (#024105, the Jackson Laboratory). In these compound mutant mice, the expression of the genetically encoded calcium sensor GCaMP6f was restricted to the mature OSNs. All

mice were reared and maintained in the department animal facility. OSNs were isolated from 5- to 8-week-old OMP-Cre-driven GCaMP6f male mice with a genotype of OMP-Cre^{+/+} GCaMP6f^{+/+}. The mice were overdosed with anesthetics [ketamine (90 mg kg⁻¹, intraperitoneally) and xylazine (10 mg kg⁻¹, intraperitoneally)] and decapitated. The head was cut open sagittally, and the septum was removed to expose the medial surface of the olfactory epithelium and turbinates. The olfactory epithelium and turbinates were dissected and collected in divalent-free Ringer's solution [145 mM NaCl, 5.6 mM KCl, 10 mM Hepes, 10 mM glucose, and 4 mM EGTA (pH 7.4)]. The tissue was incubated at 37°C for 45 min in 5 ml of divalent-free Ringer's solution containing collagenase (0.5 mg ml⁻¹), bovine serum albumin (5 mg ml⁻¹; Sigma-Aldrich), dispase (5 U ml⁻¹; Roche), and deoxyribonuclease II (50 mg ml⁻¹; Sigma-Aldrich). The tissue was then transferred to a clean tube of culture medium and washed. The OSNs were dissociated by tapping the tube containing the tissue. The OSNs (50- μ l volume) were split onto four concanavalin-coated glass coverslips (10 mg ml⁻¹; Sigma-Aldrich) and placed in 35-mm petri dishes. After allowing the cells to settle for 20 min, 2 ml of culture medium was added to each dish, and the dishes were placed at 37°C for at least 1 hour. Culture medium consisted of DMEM (Dulbecco's modified Eagle's medium)/F12 (Gibco BRL) supplemented with 10% fetal bovine serum, 1 \times insulin-transferrin-selenium (Gibco BRL), penicillin (100 U ml⁻¹) and streptomycin (100 mg ml⁻¹; Gibco BRL), and 100 mM ascorbic acid (Sigma-Aldrich).

Calcium-imaging recording

After being washed with fresh Ringer's solution, the coverslips were mounted on a recording chamber. Imaging was carried out at room temperature on an inverted fluorescence microscope (IMT-Olympus) equipped with an SIT camera (C10600, Hamamatsu Photonics), a Lambda XL light source (Sutter Instrument), and Lambda-10B optical filter changer (Sutter Instrument). Using a 1260 Infinity HPLC system (Agilent Technologies), the dissociated OSNs were stimulated with the odorants in random order. A final stimulation with a 10 μ M forskolin (Sigma-Aldrich) solution was made to assess the viability of the OSNs. Recordings were made at 490-nm excitation and 520-nm emission. Images were taken every 4 s, and there was a 4-min delay between stimulations. The images were then computed using MetaMorph Premier software (Molecular Devices LLC), and the cells were manually counted. Cells exhibiting an intensity increase of at least 10% $\Delta F/F_0$ amplitude between 8 and 12 frames after the odorant injection were considered responsive cells.

Data analysis of calcium imaging recording

A total of 1666 molecular descriptors for the panel odorants were downloaded through e-Dragon free applet (www.vclab.org/) (31). Normalized descriptors were used to calculate Euclidean distances and generate dendrograms based on the shortest Euclidean distance using MATLAB (MathWorks). Parameters of all 1666 descriptors were z-scored before clustering. We did not manually exclude any values nor did not treat categorical/continuous values differentially. Neuron responses to odorants in calcium imaging were transformed to an m \times n bool matrix, where "m" is the number of neurons responding to at least one chemical, and "n" is the number of chemicals used; "1" means "response," and "0" means "no response." This matrix was used to calculate Euclidean distances and generate dendrograms of the odorants using MATLAB. A Cochran's Q test comparison, followed by post hoc McNemar tests, was performed to compare the odorant "Response" and "No Response" heat maps using StatView (SAS Institute). The Spearman's rank correlation

between the distance matrix of every molecular descriptor and the distance matrix of OSN activity was then calculated to identify the descriptors that better recapitulate the OSN response-based classification.

Habituation-dishabituation behavioral test

Similarities in perceptual odor quality among the panel of odorants were evaluated by a habituation-dishabituation olfactory test in the mouse. Thirty minutes before experimentation, 5- to 8-week-old OMP-Cre^{+/-} GCaMP6f^{-/-} male mice were placed individually into a hood in an empty mouse cage containing a cotton swab soaked in DMSO/Ringer's solution (1:1000). Each animal was then stimulated three consecutive times over 2 min with the DMSO/Ringer's solution soaked in a cotton swab as a negative control. Then, they received three consecutive presentations of a cotton swab soaked in the first odorant solution at 30 μM. Each presentation lasted 2 min, with a 1-min interval between presentations. Following a 1-min rest, animals were then given three presentations of the second odor in a similar manner. Following a final 1-min break, a 30 μM solution of 2-acetylthiazole was given in a 2-min single stimulation as a positive control. The cumulative sniffing time of the cotton swab was recorded using a silent clock. An ANOVA statistic comparison, followed by post hoc paired *t* test, was performed on the results using StatView. Each mouse was used only once with the same odorant. Mice that were unable to detect the first odorant stimulation or that responded to the negative control were removed from further analysis.

Human olfactory perception of esters

Human experiments were performed at Cornell University under the supervision of T.A. and reviewed and approved by the Institutional Review Board. In a first experiment, 11 human subjects were presented three iterations of seven solutions consisting of 30 μM solutions of the odorants [1], [2], [3], [5], and [6], a blank (no odorant), and a second solution of [1], [4], the "outside" ketone, was not used in these tests because of possible adverse effects on humans. The seven solutions were presented in a double-blind routine. Subjects were asked to group these solutions according to their perceived similarity/differences. The subjects were not asked for descriptors nor were they given any. No limits were placed on the number of times or the length of time subjects could smell the odors. Responses to the odorants were transformed to an *m***n* bool matrix, where "*m*" is the number of groups perceived by the subjects, and "*n*" is the number of chemicals used; "1" means "similar," and "0" means "different." This matrix was used to calculate Euclidean distances and generate dendrograms of the odorants using MATLAB. The subject group consisted of eight females and three males aged between 18 and 52 years (average age, 29.4 years).

In a second experiment—a same versus different task—subjects were given three vials containing two identical 3 mM odorant solutions (of either [5] or [1]) and a 3 mM odorant solution of a different odorant ([2], [3], [6], or a blank), again presented in a double-blind routine. They were asked to identify the different odorant. As in the first test, the subjects were not asked for descriptors nor were given any. The human subject group consisted of eight females and six males aged 18 to 68 years (average age, 32.3 years). The percentage of correct odorant identification for each odorant pair was calculated and compared to the chance level.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/4/2/eaao6086/DC1>

fig. S1. OSN coactivation table.

fig. S2. Venn diagram representation of the overlapping activation of OSNs by esters.

fig. S3. Human olfactory discrimination test repetitions.

fig. S4. Examples of OSNs' Ca²⁺ responses to the odorant panel.

table S1. Top 20 e-Dragon descriptors describing distances between the esters.

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Functional odor classification through a medicinal chemistry approach

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