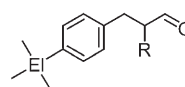


Prediction of Perception: Probing the hOR17-4 Olfactory Receptor Model with Silicon Analogues of Bourgeonal and Lilial**

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The award of the Nobel prize to Axel^[1a] and Buck^[1b] for their pioneering studies on the mechanism of odor perception generated high expectations concerning the prediction of olfactory properties. This was perhaps unjustified, as Buck et al.^[2a] also discovered that each odorant triggers a combination of different receptors, that odors consequently correspond to complex activation patterns of glomeruli, and that mixtures can even stimulate cortical neurons that are not stimulated by their individual components.^[2b] Thus, it seems almost impossible to predict the olfactory properties of a new odorant that has a different affinity to some estimated 347 different human olfactory receptors.^[3] Consequently, the design of new odorants relies heavily on structural similarities to the reference compound rather than on the complementarity to the receptor binding site.^[4,5] To what extent, however, can we predict the odor and intensity of a new compound on the basis of an olfactory receptor model?

To put our understanding of the odorant–receptor interactions to the test we decided to predict the effect of the substitution of a carbon atom for a silicon atom^[6] in the lily-of-the-valley odorants lilial (**1a**) and bourgeonal (**2a**). This C/Si exchange (sila substitution) would affect their molecular shape only subtly, which according to the latest lily-of-the-valley olfactophore^[5] should not affect the main character of



1a: Et = C, R = Me
1b: Et = Si, R = Me
2a: Et = C, R = H
2b: Et = Si, R = H

the odor, only the thresholds and nuances. Lyrall^[7a] and lilial (**1a**)^[7b] were the first two ligands for which odorant–receptor complexes were computed by employing the OR5 receptor of rats (= O1r1469) expressed in the baculovirus-Sf9 cell system.^[8] In the human olfactory epithelium, the hOR17-4 receptor (= OR1D2) responds to lilial (**1a**) and bourgeonal (**2a**). It can also be used for quantitative evaluations as it is also expressed in human sperm cells.^[9] Furthermore, the hOR17-40 receptor (= OR3A1) in the human olfactory epithelium responds to lilial (**1a**),^[10] but still the hOR17-4 receptor seemed ideal for a comparison of the in vivo, in vitro, and in silico data.

A homology model of the hOR17-4 receptor, based on the crystal structure of bovine rhodopsin (1U19), was generated with the MOLOC software.^[11] The sequence alignment of hOR17-4 to 1U19 was based on the published alignment of OR1E1 to bovine rhodopsin,^[12] which has a high homology to hOR17-4. In the resulting model, the ligand binding pocket (LBP) is lined with 21 amino acids of the transmembrane (TM) helices plus two of the extracellular loop EL2, thereby forming an almost closed cavity (see the Supporting Information).

As for rhodopsin, it is difficult to imagine how a ligand should enter or leave the LBP. Possibly, the long, double-folded EL2 chain, which seals the extracellular side, unfolds and opens the entrance to the LBP. It has also been suggested^[13] that small lipophilic ligands could enter the LBP sideways between TM5 and TM6 when the phenyl ring of F212 (TM5) and the sidechain of I269 (TM6) move to generate another rotamer.

Random, but stereochemically reasonable, conformations of the respective odorants are flexibly fitted into the LBP, whose 23 amino acids have flexible sidechains in an otherwise rigid receptor, by using the MOLOC docking procedure (Figure 1). The docked ligands were energetically optimized to convergence, and ranked according to their enthalpic binding energy. The best binding energies $\Delta E_{\text{inter,corr}}$ for lilial (**1a**) and bourgeonal (**2a**) and their silicon analogues **1b** and **2b** are summarized in Table 1. Lilial (**1a**) and its silicon analogue **1b** possess stereogenic centers, but racemize readily, possibly also under physiological conditions. Therefore, we studied the racemic mixtures and averaged the energy values of the enantiomeric forms of **1a** and **1b**. There are contradictory reports about the odor differences of the enantiomers

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Supporting information for this article is available on the WWW under <http://www.angewandte.org> or from the authors.

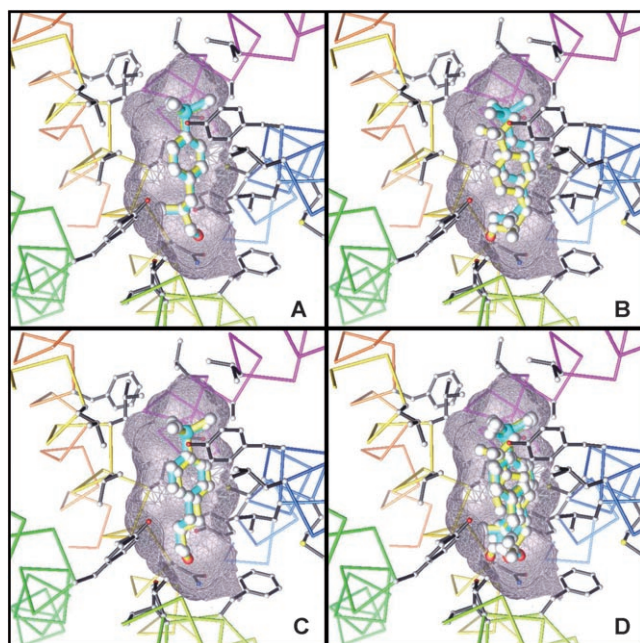


Figure 1. Docking of A) (S)-lilial [(S)-1a], B) (R)-lilial [(R)-1a], C) bourgeonal (2a) (all shown in yellow) and their respective silicon analogues (S)-1b, (R)-1b, and 2b (cyan) on the calculated hOR17-4 receptor model, and D) superposition of all ligands.

Table 1: Comparison of calculated binding energies $\Delta E_{\text{inter,corr}}$ measured GC odor thresholds, and EC_{50} values for sperm cells.

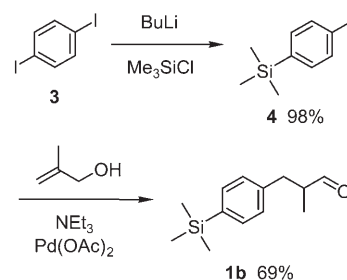
hOR17-4 ligand	$\Delta E_{\text{inter,corr}}$ [kcal mol ⁻¹]	GC threshold [ng L ⁻¹ air]	EC_{50} (sperm cells) [μM]
lilial (1a)	-10.4	0.10	10
sila-lilial (1b)	-5.5	3.30	20
bourgeonal (2a)	-12.1	0.16	10
sila-bourgeonal (2b)	-7.5	0.55	15

of lilial (1a), but the *R* enantiomer has always been found to be the more intense one.^[14] The differences were not too pronounced in our calculations, but it is also the *R* enantiomer that binds more strongly in both cases (see the Supporting Information).

Figure 1 shows that all the ligands are bound to the hOR17-4 model receptor in very similar positions. The formyl groups of all ligands form two hydrogen bonds, although of different strength, to both the NH₂ moiety of N125 (TM3) and the OH group of Y261 (TM6). Also, the strong van der Waals interactions of their aromatic rings with the hydrophobic clamp consisting of Y268 (TM6) and V121 (TM3) varies distinctly in intensity (see the Supporting Information). The silicon analogues 1b and 2b show consistently lower binding energies than the parent carbon compounds because of the higher van der Waals repulsion of the sterically larger trimethylsilyl moiety. The difference in the binding energies of very similar compounds can to a first approximation be equated with the difference in the free energies of binding (since entropic effects should approximately cancel out), and the calculated differences in the binding energy $\Delta E_{\text{inter,corr}}$

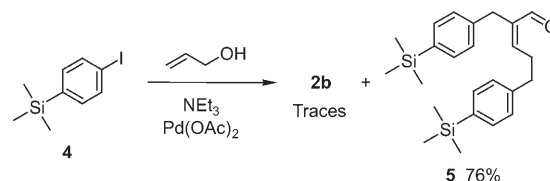
indeed reflect the trends of the biological data obtained (Table 1).

The synthesis of racemic sila-lilial (1b; Scheme 1) started from 1,4-diiodobenzene (3), which upon monolithiation and



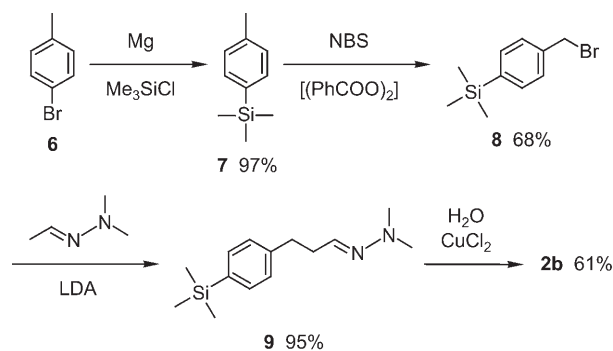
Scheme 1. Synthesis of sila-lilial (1b).

treatment with chlorotrimethylsilane afforded (4-iodophenyl)trimethylsilane (4). This was then converted into 1b by a Heck reaction^[15] with 2-methylprop-2-en-1-ol. An analogous Heck reaction of 4 with prop-2-en-1-ol however furnished only traces of the expected sila-bourgeonal (2b). Under the reaction conditions applied, 2b immediately gave the aldol condensation product 5 (Scheme 2, see the Supporting Information). Thus, an alternative route via a dimethylhydrazone^[16] was chosen.



Scheme 2. Reaction of 4 with prop-2-en-1-ol.

(4-Methylphenyl)trimethylsilane (7) was prepared from 4-bromotoluene (6) according to Ref. [17] (Scheme 3). Radical bromination of 7 with *N*-bromosuccinimide (NBS)^[18] furnished [4-(bromomethyl)phenyl]trimethylsilane (8), which was treated with lithiated ethanal *N,N*-dimethylhydrazone to provide the corresponding hydrazone 9, which upon



Scheme 3. Synthesis of sila-bourgeonal (2b).

treatment with water and copper(II) chloride^[19] then afforded sila-bourgeonal (**2b**).

For comparison, sila-lilial (**1b**) was also prepared by this route, and the synthesis of sila-bourgeonal (**2b**) was performed on a multigram scale without purification of the hydrazone **9** and by employing oxalic acid^[20] instead of copper(II) chloride (see the Supporting Information).

As predicted, all the odorants synthesized have typical floral-aldehydic lily-of-the-valley odors. Sila-lilial (**1b**), however, is somewhat more rosy and fatty in tonality and less fresh, sparkling, and watery than lilial (**1a**). The floral, green-aldehydic fresh-watery lily-of-the-valley note of sila-bourgeonal (**2b**) lies between that of **1a** and **2a**, being softer, and less green-aldehydic than the latter. Therefore, different odor receptors are certainly involved in their differentiation. Their floral-aldehydic notes can, however, no longer be discriminated around their threshold levels. We thus assumed that at this concentration only the most sensitive lily-of-the-valley receptor is addressed. The measured odor thresholds (Table 1) for lilial (**1a**), bourgeonal (**2a**), and their silicon analogues **1b** and **2b** correlate quite well with the calculated $\Delta E_{\text{inter,corr}}$ values obtained with the hOR17-4 receptor model. The more negative the enthalpic binding energy $\Delta E_{\text{inter,corr}}$, the stronger the odorant is bound and the lower the threshold concentrations required to fire the same amount of receptors.^[21] While lilial (**1a**) and bourgeonal (**2a**) are comparable in odor threshold and binding energy, the threshold of sila-lilial (**1b**) is about 30 times higher than that of the corresponding carbon compound **1a**, which is in good agreement with the difference of -4.9 kcal in the free binding energy. The threshold value of sila-bourgeonal (**2b**) is about four times higher than that of the carbon compound **2a**, which corresponds to the calculated energy difference of -4.6 kcal.

Even though the correlation is astonishingly good, we could not take it for granted that the most sensitive lily-of-the-valley receptor is indeed hOR17-4. It was necessary to compare the threshold and modeling results with data from the recombinant and native hOR17-4 receptor. The functional expression of recombinant OR proteins of different species has already been reported in detail.^[22–24] Here we first expressed hOR17-4 recombinantly in human embryonic kidney (HEK293) cells. Receptor activation leads singularly to a transient increase in the cytosolic concentration of Ca^{2+} ions which can be detected by ratiometric fluorescence imaging techniques.^[23] The odorants lilial (**1a**), sila-lilial (**1b**), bourgeonal (**2a**), and sila-bourgeonal (**2b**), each at $500\ \mu\text{M}$ concentration, induced transient signals for Ca^{2+} ions in about 1% of all the cells tested, as is typical for transient olfactory receptor transfection.^[23] In nontransfected HEK cells, signals for Ca^{2+} ions were never observed even with a tenfold increase in the concentration of the agonist. ATP (at a concentration of $10\ \mu\text{M}$) served as a control for the excitability of HEK cells (Figure 2). All four ligands tested gave Ca^{2+} signals of nearly the same response amplitude. The threshold concentration for lilial (**1a**), sila-lilial (**1b**), bourgeonal (**2a**), and sila-bourgeonal (**2b**) was found to be in the 5 – $10\ \mu\text{M}$ range.

Only a more qualitative description was possible by using the HEK293 system. However, the hOR17-4 receptor is also

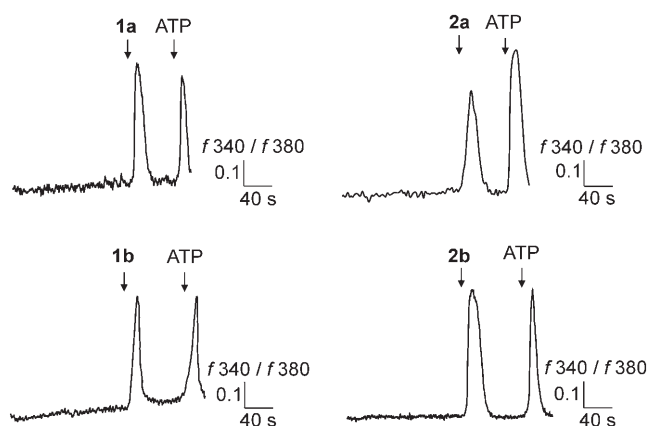


Figure 2. Representative Ca^{2+} imaging recordings of hOR17-4-transfected HEK293 cells. The cytosolic Ca^{2+} level of different fura-2-AM-loaded cells is depicted as the ratio of the integrated fluorescence (f_{340}/f_{380}) and viewed as a function of time. Compounds **1a**, **1b**, **2a**, and **2b** were applied at a concentration of $500\ \mu\text{M}$ for 10 s. ATP ($10\ \mu\text{M}$) served as a control for the excitability of the HEK cells.

functionally expressed in human spermatozoa and the spectrum of the effective ligands of human sperms matched fairly well with the receptive field of the recombinantly expressed receptors.^[9] The spermatozoa are much more sensitive and exhibited a detection threshold for lilial (**1a**) and bourgeonal (**2a**) at concentrations two orders of magnitude lower than those recorded in a HEK cell expression system.^[9] In contrast to HEK293 cells, brief stimulation of spermatozoa with lilial (**1a**) and bourgeonal (**2a**) induced reproducible and stable Ca^{2+} signals in a concentration-dependent manner. Therefore, we compared the potency of the four ligands **1a**, **1b**, **2a**, and **2b** on fura-2-AM-loaded spermatozoa.

As shown in Figure 3A, all four agonists induced Ca^{2+} transients in one particular spermatozoon at a concentration of $50\ \mu\text{M}$, which guarantees an excellent relative comparability. The amplitudes and signal kinetics of the responses were similar in magnitude. Coapplication of undecanal, an aliphatic aldehyde which was demonstrated to be a specific competitive antagonist for hOR17-4,^[9] blocked completely the response to sila-bourgeonal (**2b**; Figure 3B) and sila-lilial (**1b**), so even if other receptors are expressed in spermatozoa they would not interfere with this measurement. All four odorants stimulated spermatozoa in a dose-dependent manner. The EC_{50} values for lilial (**1a**), sila-lilial (**1b**), bourgeonal (**2a**), and sila-bourgeonal (**2b**; Table 1) were in the 10 – $20\ \mu\text{M}$ range and correspond well with the order of threshold intensities measured by GC. The threshold concentration for the four agonists of hOR17-4 were in the $100\ \text{nM}$ (**1a**, **2a**, **2b**) to $1\ \mu\text{M}$ (**1b**) range.

Just like there are exceptions in the grammar of a language, there are many examples of the unpredictability of odor; some impressive ones were compiled in a recent minireview by Sell.^[25] Just as a native speaker is guided by his feeling for language, the fragrance chemist will also in the foreseeable future mainly be led by experience, structural intuition, imagination, and instinct. In the example of lilial (**1a**) and bourgeonal (**2a**), it was however possible to predict

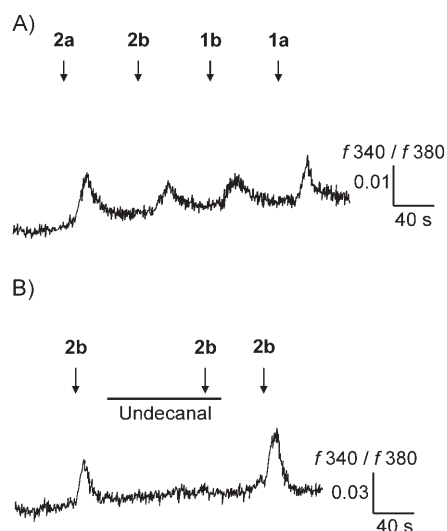


Figure 3. Single-cell Ca^{2+} imaging measurements. A representative ratiometric fluorescence recording of an individual fura-2-AM-loaded human spermatozoon. The cytosolic Ca^{2+} level is depicted as the ratio of the integrated fluorescence (f_{340}/f_{380}) and viewed as a function of time. A) Induction of transient signals of similar amplitude after pulses (10 s) of **1a**, **1b**, **2a**, and **2b** at a concentration of 50 μM . B) After preincubation of undecanal for 100 s, the response of silabourgeonal (**2b**, 50 μM) was completely suppressed by coapplication (10 s) with undecanal (50 μM).

the relative odor intensities of the silicon analogues **1b** and **2b** quite accurately on the basis of their stereoelectronic properties alone from a computational homology model of the hOR17-4 receptor. At the threshold level, only the receptor(s) of highest affinity should be addressed, and the good correlation indicates that the hOR17-4 receptor is an important factor in perception at this threshold concentration. While the four lily-of-the-valley odorants **1a**, **1b**, **2a**, and **2b** can easily be distinguished at higher concentrations by their additional nuances, they all possess the same floral-aldehydic note at the threshold concentration. The complex lily-of-the-valley odor of these odorants above the threshold level is certainly a result of the activation of different odor receptors and the mental processing of this information. However, since the hOR17-4 receptor is activated by these lily-of-the-valley odorants qualitatively in transfected HEK293 cells, and quantitatively in the more sensitive single-spermatozoon model, a corresponding dose–response relationship could be determined. The different cellular systems means that the single-cell Ca^{2+} imaging measurements cannot be compared with the measured GC thresholds on an absolute basis, but the ranking order is again in complete agreement. These results taken together clearly demonstrate that it is indeed the electronic surface structure that determines the interaction of an odorant with its olfactory receptors. Thus, the C/Si switching strategy^[6] can even provide insight into the mechanism of receptor activation in olfaction.

Experimental Section

The synthesis of **4**, the attempt to synthesize **2b** by Heck coupling, the synthesis of **1b** by the hydrazone methodology, and the multigram-scale synthesis of **2b** can be found together with general information in the Supporting Information.

Lilial (1a): Odor: Typical powerful and diffusive aldehydic odor reminiscent of lily-of-the-valley and linden blossom, mild floral, and natural. Odor threshold: 0.10 ng L^{-1} air (standard deviation (SD): 0.08).

Bourgeonal (2a): Odor: Powerful and diffusive, watery-floral lily-of-the-valley note with a green-aldehydic character and hints of melons and hyacinth. Odor threshold: 0.16 ng L^{-1} air (SD: 0.17).

1b: $\text{Pd}(\text{OAc})_2$ (113 mg, 503 μmol) was added to a mixture of **4** (2.77 g, 10.0 mmol), 2-methylprop-2-en-1-ol (865 mg, 12.0 mmol), and NEt_3 (1.21 g, 12.0 mmol). After stirring the reaction mixture for 12 h at 80–90 °C, it was allowed to cool to 20 °C, and the product was isolated by column chromatography on silica gel (63–200 μm ; pentane/ Et_2O (9/1)) to afford **1b** (1.51 g, 69%) as a colorless liquid. ^1H NMR (400.1 MHz): δ = 9.71 (d, 3J = 1.5 Hz, 1H; CHO), 7.43 ($\delta_{\text{XX'}}$, 2H; SiCCH) and 7.14 ($\delta_{\text{AA'}}$, 2H; CHCCH_2 , AA'XX' system, $^4J_{\text{XX'}} = 1.5$, $^3J_{\text{AX}} = ^3J_{\text{AX'}} = 7.6$, $^5J_{\text{AX}} = ^5J_{\text{AX'}} = 0.6$, $^4J_{\text{AA'}} = 1.9$ Hz), 3.07 (dd, 2J = 13.5, 3J = 5.8 Hz, 1H; CH_2), 2.72–2.61 (m, 1H; CH), 2.57 (dd, 2J = 13.5, 3J = 8.3 Hz, 1H; CH_2), 1.08 (d, 3J = 6.9 Hz, 3H; CHCH_3), 0.24 ppm (s, 9H; $\text{Si}(\text{CH}_3)_3$); ^{13}C NMR (100.6 MHz): δ = 204.4 (CO), 139.4 (CHCCH_2), 138.2 ($\text{CSi}(\text{CH}_3)_3$), 133.6 (2C, SiCCH), 128.4 (2C, CHCCH_2), 48.0 (CHCH_3), 36.6 (CH_2), 13.3 (CHCH_3), –1.1 ppm (3C, $\text{Si}(\text{CH}_3)_3$); ^{29}Si NMR (79.5 MHz): δ = –4.2 ppm; elemental analysis (%) calcd for $\text{C}_{13}\text{H}_{20}\text{OSi}$ (220.39 g mol^{-1}): C 70.85, H 9.15; found: C 70.71, H 9.39. Odor: Lilial-like, typical aldehydic lily-of-the-valley smell, more rosy and fatty with a slightly spicy connotation, less fresh, sparkling, and watery than **1a**. Odor threshold: 3.30 ng L^{-1} air (SD: 1.77).

2b: A 1.6M solution of BuLi in hexanes (13.8 mL, 22.0 mmol BuLi) was added dropwise at –78 °C to a stirred solution of $i\text{Pr}_2\text{NH}$ (2.43 g, 24.0 mmol) in THF (20 mL). The cooling bath was removed and the mixture allowed to warm to 20 °C, followed by dropwise addition of ethanal N,N -dimethylhydrazone^[16] (2.07 g, 24.0 mmol) at –78 °C. The reaction mixture was again allowed to warm to 20 °C (white precipitate), followed by dropwise addition of **8**^[18] (4.86 g, 20.0 mmol) at –78 °C with stirring. The reaction mixture was stirred at 20 °C for 12 h. Subsequently, a saturated aqueous NaCl solution (20 mL) and pentane (20 mL) were added. The organic layer was separated and the aqueous layer extracted with pentane (3 $\times$ 20 mL). The combined organic extracts were dried (MgSO_4), the solvent was removed under reduced pressure, and the residue purified by column chromatography on silica gel (63–200 μm ; pentane/ Et_2O (1/1)) to afford **9** (4.73 g, 95%) as a colorless liquid (^{13}C NMR (75.5 MHz): δ = 142.1 (CHCCH_2), 138.0 (CHN), 137.5 ($\text{CSi}(\text{CH}_3)_3$), 133.5 (2C, SiCCH), 127.9 (2C, CHCCH_2), 43.3 ($\text{N}(\text{CH}_3)_2$), 34.6 and 34.1 ($\text{CH}_2\text{CH}_2\text{CHN}$), –1.1 ppm ($\text{Si}(\text{CH}_3)_3$)), which was hydrolyzed. Thus, a solution of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (7.37 g, 43.2 mmol) in water (36 mL) was added at 20 °C to a solution of **9** (4.47 g, 18.0 mmol) in pentane (180 mL). A few drops of conc. hydrochloric acid were added, and the mixture was stirred vigorously at 20 °C for 2 h until the starting material had been consumed. The layers were separated, the organic layer was dried (MgSO_4) and concentrated under reduced pressure, and the residue was purified by column chromatography on silica gel (63–200 μm ; pentane/ Et_2O (5/1)) to afford **2b** (2.26 g, 61%) as a colorless liquid. ^1H NMR (300.1 MHz): δ = 9.81 (t, 3J = 1.4 Hz, 1H; CHO), 7.44 ($\delta_{\text{XX'}}$, 2H; SiCCH) and 7.18 ($\delta_{\text{AA'}}$, 2H; CHCCH_2 , AA'XX' system, $^4J_{\text{XX'}} = 1.5$, $^3J_{\text{AX}} = ^3J_{\text{AX'}} = 7.6$, $^5J_{\text{AX}} = ^5J_{\text{AX'}} = 0.6$, $^4J_{\text{AA'}} = 1.9$ Hz), 2.99–2.89 (m, 2H; CHCCH_2), 2.82–2.73 (m, 2H; CH_2CHO), 0.24 ppm (s, 9H; $\text{Si}(\text{CH}_3)_3$); ^{13}C NMR (75.5 MHz): δ = 201.5 (CO), 140.9 (CHCCH_2), 138.2 ($\text{CSi}(\text{CH}_3)_3$), 133.7 (2C, SiCCH), 127.7 (2C, CHCCH_2), 45.1 (CH_2CHO), 28.1 (CCH_2), –1.1 ppm ($\text{Si}(\text{CH}_3)_3$); ^{29}Si NMR (59.6 MHz): δ = –4.0 ppm; elemental analysis (%) calcd for $\text{C}_{12}\text{H}_{18}\text{OSi}$ (206.36 g mol^{-1}): C 69.84, H 8.79; found: C

69.35, H 8.29. Odor: Floral, green-aldehydic, fresh-watery lily-of-the-valley note, softer and less green-aldehydic than **2a**, in its floralcy between **1a** and **2a**. Odor threshold: 0.55 ng L⁻¹ air (SD: 0.99).

Cell culture and transfection of HEK293 cells: HEK293 cells were maintained under standard conditions in Dulbecco Modified Eagle Medium (D-MEM) supplemented with 10 % fetal bovine serum (FBS), penicillin, and streptomycin (100 units mL⁻¹ each). For transient transfection of HEK293 cells, the cells were plated (5×10^4 /dish) two days before transfection. Cells were transfected with the olfactory receptor hOR17-4 construct^[24] by using a calcium phosphate precipitation technique.

HEK293 imaging: Two days after transfection, the growth media were removed and replaced with loading buffer (pH 7.4) containing Ringer solution and fura-2-AM (3 μ M, molecular probes). The dishes were incubated in the dark at room temperature (30 min) and thereafter washed once with a fura-2-AM-free solution and replaced with standard Ringer solution (140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (Hepes), 10 mM glucose, pH 7.4) to perform Ca²⁺ imaging. Ca²⁺ images were acquired from up to 50 HEK293 cells in a randomly selected field of view and the ratios of the integrated fluorescence (f_{340}/f_{380} ratio) were viewed as a function of time. Ca²⁺ imaging was performed using a Zeiss inverted microscope equipped for ratiometric imaging.^[24]

Sperm preparation: Human sperm was freshly obtained from young and healthy donors. For Ca²⁺ imaging, a Percoll (Amersham Biosciences) density gradient centrifugation was performed after liquefaction (30 min at 35.5 °C) to isolate mature and motile sperm. In brief, liquefied semen was overlaid on a two-layer Percoll density gradient consisting of 80 and 55 % isotonic Percoll in Ham's F-10 medium (Invitrogen). After centrifugation (40 min, 500g, RT), the pellet was collected, washed in standard Ringer solution (140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM Hepes, 10 mM glucose), and centrifuged again (15 min).

Sperm imaging: The cell density was photometrically adjusted to an absorption of $E_{260\text{nm}} = 0.035$. Sperm were incubated (45 min at 35.5 °C, in the dark) in loading buffer (pH 7.4) containing Ringer solution and fura-2-AM (7.5 μ M) (Molecular Probes), and Pluronic F-127 (0.1 %; Sigma). Next, sperm were centrifuged (15 min, 500g), and the pellet was resuspended in fura-2-AM-free buffer solution. The suspension of mature motile fura-2-AM-loaded spermatozoa (150 μ L) was transferred to 35-mm dishes coated with concanavalin A (Sigma; 30 min, 35.5 °C). Ca²⁺ imaging was performed using a Zeiss inverted microscope equipped for ratiometric imaging.^[23] Ca²⁺ images were acquired from up to 30 spermatozoa in a randomly selected field of view, and ratios of the integrated fluorescence (f_{340}/f_{380} ratio) were viewed as a function of time. Exposure to lilial (**1a**; Givaudan SA, Vernier, Switzerland), sila-lilial (**1b**), bourgeonal (**2a**; Quest Intl., Naarden, Netherlands), and sila-bourgeonal (**2b**) was accomplished by using a specialized microcapillary application system.^[26] Only spermatozoa with their heads and midpiece attached and their tails beating were included in the analysis.

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