Specificity Determinants of the Silkworm Moth Sex Pheromone

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Abstract

The insect olfactory system, particularly the peripheral sensory system for sex pheromone reception in male moths, is highly selective, but specificity determinants at the receptor level are hitherto unknown. Using the Xenopus oocyte recording system, we conducted a thorough structure-activity relationship study with the sex pheromone receptor of the silkworm moth, Bombyx mori, BmorOR1. When co-expressed with the obligatory odorant receptor co-receptor (BmorOrco), BmorOR1 responded in a dose-dependent fashion to both bombykol and its related aldehyde, bombykal, but the threshold of the latter was about one order of magnitude higher. Solubilizing these ligands with a pheromone-binding protein (BmorPBP1) did not enhance selectivity. By contrast, both ligands were trapped by BmorPBP1 leading to dramatically reduced responses. The silkworm moth pheromone receptor was highly selective towards the stereocchemistry of the conjugated diene, with robust response to the natural (10E,12Z)-isomer and very little or no response to the other three isomers. Shifting the conjugated diene towards the functional group or elongating the carbon chain rendered these molecules completely inactive. In contrast, an analogue shortened by two omega carbons elicited the same or slightly higher responses than bombykol. Flexibility of the saturated C1–C9 moiety is important for function as addition of a double or triple bond in position 4 led to reduced responses. The ligand is hypothesized to be accommodated by a large hydrophobic cavity within the helical bundle of transmembrane domains.

Introduction

The identification of bombykol, (10E,12Z)-hexadecadien-1-ol (1), the sex pheromone for the silkworm moth, Bombyx mori [1], more than five decades ago triggered physiologists’ interest in insect olfaction, and paved the way for current molecular studies. Probing the system with earlier techniques such as electroantennogram (EAG) and single-sensillum recordings (SSR), pioneers in the field unraveled an inordinate sensitivity and selectivity of the insect’s olfactory system [2]. These earlier studies clearly demonstrated that structural modifications dramatically reduce neuronal responses or render the molecules completely inactive [3], but it remains mostly unknown how pheromone molecules interact with odorant receptors (ORs) housed in these neurons, although various moth sex pheromone receptors have been orphanized to date [4–11]. To identify pheromone specificity determinants, we challenged with a panel of bombykol analogs the silkworm moth sex pheromone receptor, BmorOR1, co-expressed with its obligatory co-receptor, BmorOrco [4] in the Xenopus oocyte system. As the BmorOR1+BmorOrco-expressing oocytes showed robust and moderate responses to bombykol and bombykal, respectively, we investigated whether a functional recombinant pheromone-binding protein, BmorPBP1 [12], would enhance selectivity. Here, we provide strong evidence that bombykol does not require BmorPBP1 to activate BmorOR1. Additionally, we show that the stereochemistry of the double bonds, flexibility of saturated moiety, the functional group, and the number of carbons atoms after the unsaturations are specificity determinants of the pheromone molecule.

Results and Discussion

Selectivity of the Functional Group

First, we examined the response of BmorOR1+BmorOrco-expressing oocytes to bombykol. The silkworm moth receptor responded to the sex pheromone in a dose-dependent fashion (EC50 4.54×10−8M) and with a remarkable low threshold (<0.1 nM) (Figure 1). Then, we compared the OR responses elicited by bombykol and bombykal. The literature is dichotomous regarding the selectivity of BmorOR1 towards these two components of the silkworm moth’s sex pheromone system [3]. Using the Xenopus oocyte recording system, it has been shown that BmorOR1+BmorOrco is narrowly tuned to bombykol [4]. By contrast, it has been reported that BmorOR1-expressing HEK 293 cells responded almost equally to bombykol and bombykal [5]. In our hands, BmorOR1+BmorOrco-expressing oocytes were indeed more sensitive to bombykol, but responded to bombykal with about one order of magnitude higher threshold (Figure 2). After
activation stimulus was applied, oocytes were thoroughly washed until a steady baseline was reached. To save odorant samples and expedite these recovery times, all comparative studies were made by injecting test odorants rather than by perfusion, and comparative EC50s were calculated on the basis of source doses. Therefore, they are underestimation of the actual EC50s. The comparative EC50 for bombykol and bombykal were 9.9×10⁻²M and 9.6×10⁻²M, respectively (Figure 2). We analyzed our synthetic samples just prior to electrophysiological recordings to avoid possible misinterpretation derived from sample quality. There are two potential problems to consider, i.e., aldehydes are prone to degradation through auto-oxidation leading to lower than nominal concentrations and the bombykal sample may contain considerable amounts of unreacted bombykol (used as starting material). Our chemical analysis indicated that the two samples had the same concentration and that bombykol contamination in bombykal samples is very low (<0.9%) (Figure 3). If the response would be elicited by residues of bombykol in the bombykal samples, one would expect at least 2 orders of magnitude differences. Interestingly, the responses of the “naked receptor” differ from the neuronal activity of the olfactory system of the silkworm, which showed no cross-over whatsoever, with the bombykol and bombykal neurons responding specifically to the alcohol and aldehyde, respectively [2,3]. It has been suggested that addition of a pheromone-binding protein, BmorPBP1, to the HEK 293 cell system restores selectivity [5].

**Bombykol and Bombykal are “Trapped” by BmorPBP1**

In an attempt to reconcile the data in the literature we investigated whether addition of PBP would enhance selectivity of the BmorOR1•BmorOrco receptor complex when expressed in *Xenopus* oocytes. We compared the receptor responses to bombykol and bombykal solubilized either by DMSO or BmorPBP1. Interestingly, receptor activity was dramatically reduced when the ligands were solubilized by BmorPBP1 (Figure 4). Bombykol (1 μM) dissolved in DMSO elicited robust receptor response, but very weak response when solubilized by BmorPBP1. Here, the ratio of BmorPBP1 to bombykol was 10:1. Bombykal (10 μM) elicited strong response when dissolved in DMSO and weak response when solubilized by BmorPBP1. The receptor response to bombykol solubilized by BmorPBP1 was on average ca. 34% of the response to the same ligand in DMSO, whereas for bombykol the ratio was 13%. This relatively higher response to bombykal in PBP might be merely because of the ratio of PBP:ligand. Given that bombykol requires a 10x higher dose, we prepared samples at a 1:1 ratio, whereas bombykol samples had a 10:1 protein/ligand ratio. These findings suggest that in *Xenopus* oocyte there are no negatively-charged surfaces in the vicinity of the receptors or the vitelline membrane surrounding the oocytes prevents the PBP-pheromone complexes from interacting with regions of localized low pH, which are necessary to trigger a conformational change that “ejects” ligands from PBP-pheromone complexes [12–14]. Regardless, the robust responses recorded without PBPs (Figures 1 and 2) strongly suggest that, unlike what has been
unknown if specificity is determined by pheromone receptors alone or in combination with other olfactory proteins. We tested the four possible isomers of bombykol (compounds 1, 9, 10, Figure 5) and found that BmorOR1-BmorOrco-expressing oocytes respond with high intensity only to the natural stereoisomer of bombykol, (10E,12Z)-hexadecadien-1-ol, with very low responses to the (10Z,12E)- and (10Z,12Z)-isomers, and no response to the (10E,12E)-isomer (Figure 6). These findings suggest that stereochemistry selectivity is mediated entirely by the receptor. This is in line with the experimental observation that, albeit with different affinities, all four geometric isomers of bombykol bind to the pheromone-binding protein, BmorPBP1 [17]. We also tested whether these double bonds could be replaced by triple bonds, but the receptor was not activated by 10,12-hexadecadiyn-1-ol (6) (Figure 7). Next, we compared the effect of the alkyl moiety distal to the unsaturation. Elongating the bombykol molecule by adding two omega carbons renders (10E,12Z)-octadecadien-1-ol (7) completely inactive (Figure 8). However, truncating two omega carbons led to a molecule with apparent higher affinity for the odorant receptor. Indeed, BmorOR1-BmorOrco receptor complex responded to (10E,12Z)-tetradecadien-1-ol (8) with nearly the same or even slightly higher intensity than that elicited by the native ligand, bombykol (Figure 9). Contrary to the stringent requirement for unsaturation with the proper stereochemistry, our findings suggest that the binding pocket in BmorOR1-BmorOrco can accommodate a shorter ligand thus begging questions about the length and flexibility of the moiety between the functional group and unsaturation.

**Flexibility and Length of the C1–C9 Saturated Moiety**

To evaluate the positional effect of the unsaturation, we tested another ligand with the double bonds shifted towards the functional group, i.e., (6E,10Z)-hexadecadien-1-ol (9). This ligand showed minimal activation of the BmorOR1-BmorOrco receptor complex (Figure 7) thus implying that the length between the unsaturation and functional group is critical for receptor activation. To determine if the flexibility generated by an unsaturated moiety is important, we tested two bombykol-related compounds each with an additional unsaturation between the functional group and the conjugated double bond moiety. The moderate and low responses elicited by (10E,12Z)-hexadecatrien-1-ol (10) and (12Z,10E,12Z)-hexadecatrien-1-ol (11), respectively (Figure 9), strongly suggest that flexibility of the unsaturated moiety is essential for fitting into the binding pocket, particularly given the stronger effect of the double than the triple bond.

**Conclusions**

Structure activity analysis showed that the most important features of the sex pheromone of the silkworm moth are the stereochemistry of a conjugated diene, and the length and flexibility of the hydrocarbon moiety between the diene and the hydroxyl functional group. The length of the hydrocarbon chain distal from the diene moiety is limited to two carbons as in the natural pheromone, but a shorter version elicited as high activity in the receptor as bombykol. BmorOR1-BmorOrco-expressing oocytes responded not only to bombykol, but also to bombykal. Addition of BmorPBP1 did not enhance selectivity, but dramatically reduced current responses thus suggesting that ligands are trapped. The requirements for a large hydrophobic cavity strongly suggest that the yet-to-be-identified binding site in BmorOR1 might be buried in the transmembrane domain.
Materials and Methods

Chemicals

Bombykol and bombykal were purchased from Plant Research International (Wageningen, The Netherlands) and kept sealed under helium at \(-80^\circ C\) until use. For synthesis, solvents were dried by distillation over CaH2 (benzene, dichloromethane) or sodium wire (tetrahydrofuran) or over dry potassium hydroxide (piperidine, pyrrolidine).

Chemical Analysis

Nuclear magnetic resonance spectroscopy was performed using a Bruker Avance 500 MHz instrument and deuteriochloroform as solvent. Mass spectra were recorded on a Mat95 XP magnetic sector mass spectrometer (Thermo Finnigan). Ionization was by electron impact at 70eV in positive ion mode with a source temperature of 220°C. Column chromatography was performed on silica gel (220–400 mesh, Fluka) and silica gel Merck 60 F254 plates were used for TLC.

Scheme A (Figure 10). Examples Demonstrating the General Synthesis of the \((10E,12Z)\)-Moieity for the Preparation of Compounds 7, 8, 9, 10, and 11

\((10E,12Z)\)-Octadecadien-1-ol (7) was prepared from 10-undecyn-1-ol starting material and coupling with 1-heptyne using the following procedures. \((10E,12Z)\)-Tetradecadien-1-ol (8) was prepared from 10-undecyn-1-ol starting material and coupling with 1-propyne using the following procedures. \((8E,10Z)\)-Hexadecadien-1-ol (9) and \((8E,10Z)\)-Hexadecadien-4-yn-1-ol (10) were also prepared as described. (4Z,10E,12Z)-Hexadecatrien-1-ol (11) was prepared from 10-undecyn-1-ol and coupling with 1-propyne using the following procedures.

Figure 5. Chemical structures. Structures of the silkworm moth sex pheromone (1) and bombykol-related compounds, which were used to challenge BmorOR1\_BmorOrco-expressing oocytes.

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Figure 6. Stereochemical selectivity. (A) Traces and (B) quantification of current responses from BmorOR1\_BmorOrco-expressing oocytes perfused with four isomers of bombykol at 0.1 \(\mu M\). \(n = 5\). Bars with the same letter were not significantly different (One-way ANOVA, \(P < 0.01\)).

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Figure 7. Effect of altering unsaturation on receptor response. (A) Traces and (B) quantification of current responses elicited by \((8E,10Z)\)-hexadecadien-1-ol (9) and 10,12-hexadecadiyn-1-ol (6) presented at 1 mM. \(n = 3\). Bars with the same letter are not significantly different (One-way ANOVA, \(P < 0.01\)).

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1-ol (9) was prepared from 8-nonyn-1-ol starting material and coupling with 1-heptyne using the following procedures. (5E,7Z)-Undecadien-1-ol for synthesis of 10 and 11 was prepared using 5-hexyn-1-ol as starting material.

(E)-1-Iodohex-1-en-6-ol. To a solution of Schwartz reagent (8.95 g, 30.6 mmol) in dry THF (40 mL) at room temperature and covered in foil to exclude light, was added super-hydride (30.6 mL, 1 M, 30.6 mmol). The solution was stirred for 1 h after which a lithium salt of 5-hexyn-1-ol, generated from the alcohol (1.5 g, 15.3 mmol) in dry THF (20 mL) at room temperature with super-hydride (15.3 mL, 1 M, 15.3 mmol), was added via a canula. After 10 minutes, a solution of iodine (11.7 g, 45.9 mmol) in dry THF (20 mL) was added and the reaction stirred overnight, quenched with sat. NaHCO₃ solution, extracted into ethyl ether and the combined organic fractions washed with brine, dried (MgSO₄), filtered and concentrated in vacuo. Purification by flash column chromatography (30% ethyl acetate in petroleum ether) afforded a brown oil (3.24 g, 94%).

dH (500 MHz, CDCl₃) 6.52 (1H, dt, J 15.8, 7.1 H-2), 6.01 (1H, d, J 15.8, H-1), 3.78 (2H, t, J 6.5, H-6), 2.09 (1H, q, J 7.0, H-3), 1.60–1.40 (4H, m, H-4,5); dc (125 MHz, CDCl₃) 146.3, 74.7, 62.5, 35.8, 31.9, 24.6.

(5E)-Undecen-7-yn-1-ol. To a solution of the iodoalkene (3.24 g, 14.4 mmol) and an excess of 1-pentyne (4.9 g, 72.0 mmol) in dry piperidine (60 mL), was added copper (I) iodide (273 mg, 1.44 mmol) and PdCl₂(PhCN)₂ (275 mg, 0.72 mmol). The reaction was stirred for 3 days, quenched with aqueous ammonium chloride, extracted into dichloromethane and the combined organic extracts washed with 1M HCl and brine, dried (MgSO₄) and concentrated in vacuo. Purification by flash column chromatography (30% ethyl acetate in petroleum ether) gave a pale brown oil (1.46 g, 61%). δH (500 MHz, CDCl₃) 6.04 (1H, dt, J 15.8, 7.1 H-5), 5.47 (1H, d, J 15.8, H-6), 3.63 (2H, t, J 6.5, H-1), 2.26 (1H, t, J 6.9, H-9), 2.12 (2H, q, J 7.2, H-4), 1.72 (1H, br s, OH), 1.59-1.52 (4H, m, 2CH₂), 1.29-1.44 (2H, m, CH₂), 0.99 (3H, t, J 7.4, H-11); δc (125 MHz, CDCl₃) 147.2, 110.3, 88.8, 79.2, 62.6, 32.6, 22.1, 25.0, 22.3, 21.3, 13.6.

(5E,7Z)-Undecadien-1-ol. Fresh zinc dust (8.50 g, 130 mmol) was suspended in water (80 mL) and a solution of Cu(OAc)₂·2H₂O (832 mg, 4.58 mmol) in hot water (40 mL) added. After stirring for 15 minutes a solution of AgNO₃ (961 mg, 5.06 mmol)
in hot water (40 mL) was added and the mixture stirred in the dark for 15 minutes. The resulting solid was filtered and washed with water, methanol and diethyl ether before it was dried in vacuo and transferred to a solution of (5E)-undec-7-en-1-ol (800 mg, 4.82 mmol) in water (40 mL) and methanol (60 mL). The reaction was heated at 65°C until starting material was consumed. The reaction was filtered and the filtrate concentrated in vacuo before purification by flash column chromatography (20% ethyl acetate in petroleum ether) yielded the diene as a clear, colorless oil (490 mg, 61%).

Figure 10. Schemes A–C. Synthetic sequence for preparation of analogues 7–11 containing the (E,Z)-dienyl moiety. doi:10.1371/journal.pone.0044190.g010
m. H-4, H-9), 1.61 (2H, qu, δ 6.5, H-2), 1.52-1.48 (2H, m, H-3), 1.47-1.39 (2H, m, H-10), 0.93 (3H, t, δ 7.4, H-11); δ (125 MHz, CDCl3) 134.0, 130.2, 128.6, 126.1, 62.9, 32.6, 32.3, 29.9, 25.5, 22.9, 13.8. 

Scheme B (Figure 10). Synthesis of Ligand 10

(5E,7Z)-1-Bromoundecadiene. To a solution of (5E,7Z)-undecadien-1-ol (450 mg, 2.68 mmol) in dry dichloromethane (20 mL) at 0°C was added triphenylphosphine oxide (0.77 g, 2.94 mmol). Over 5 minutes carbon tetrabromide (0.89 g, 2.68 mmol) was added in portions and the reaction subsequently stirred until complete by TLC. The crude reaction was concentrated in vacuo and passed through a short chromato graphic column with petroleum ether to yield the bromide as a clear, colourless oil (530 mg, 86%). δH (500 MHz, CDCl3) 4.68 (1H, d, δ 7.1, 15.1, H-6), 5.96 (1H, t, δ 7.1, H-10), 5.65 (1H, dt, δ 7.0, 15.1, H-5), 5.35 (1H, dt, δ 7.1, 15.3, H-4), 5.45-5.38 (2H, m, H-5), 5.34 (1H, d, δ 7.5, 15.0, H-5), 1.77 (2H, qu, δ 7.6, H-2), 1.30 (3H, d, δ 7.5, CH3), 1.21 (3H, t, δ 7.1, CH2); δ (125 MHz, CDCl3) 99.6, 88.5, 63.2, 60.8, 28.7, 19.8, 15.3, 15.3.

Protected 4-pentyn-1-ol. To a solution of 4-pentyn-1-ol (1.00 g, 11.9 mmol) and vinyl ethyl ether (2 mL) in dichloromethane (20 mL) was added a small spatula of PPTS catalyst. The reaction was stirred overnight, washed with bicarbonate and brine, dried (MgSO4), filtered and washed with water, methanol and diethyl ether before being dried in vacuo. The product of the reaction was compound 10 as a clear, colorless oil (9 mg, 8%). The major product of the reaction was compound 10 from incomplete reduction. δH (500 MHz, CDCl3) 3.34 (2H, m, CH2O), 2.89-2.85 (2H, m, CH2O), 2.34-2.29 (2H, m, CH2O), 1.75-1.70 (6H, m, 3CH3), 1.51-1.48 (4H, m, 2CH2), 1.41 (2H, s, δ 6.6, CH3), 1.32 (3H, d, δ 5.3, CH3), 1.22 (3H, t, δ 7.1, CH3), 0.91 (3H, t, δ 7.4, CH3); δ (125 MHz, CDCl3) 134.1, 130.0, 128.7, 125.9, 99.6, 80.4, 79.5, 63.6, 60.7, 32.4, 29.8, 29.3, 28.6, 28.5, 22.9, 19.8, 18.6, 15.6, 15.3, 13.8. 

Scheme D (Figure 11). General Synthesis of the Dienemoiety for the Preparation of Compound 6

1-Iodopent-1-ynyl. 1-Pentyne (1.00 g, 14.7 mmol) was dissolved in dry Et2O (15 mL) at −78°C under nitrogen. n-Butyllithium (5.87 mL, 2.5 M, 14.68 mmol) was added drop wise and the reaction stirred for 1 h. Iodine (4.10 g, 16.2 mmol) was added in dry Et2O and the mixture was stirred overnight. The reaction was quenched (aqueous ammonium chloride), extracted into Et2O, and the combined organic extracts washed with sodium thiosulfate, brine, dried (MgSO4) and concentrated in vacuo to yield the crude product as a clear colorless oil (2.70 g, 95%). δH (500 MHz, CDCl3) 7.45-7.38 (2H, m, H-4, H-5), 3.54-3.46 (2H, m, CH2O), 2.29-2.25 (2H, m, CH2O), 1.75-1.70 (6H, m, 3CH3), 1.51-1.48 (4H, m, 2CH2), 1.41 (2H, s, δ 6.6, CH3), 1.32 (3H, d, δ 5.3, CH3), 1.22 (3H, t, δ 7.1, CH3), 0.91 (3H, t, δ 7.4, CH3); δ (125 MHz, CDCl3) 134.1, 130.0, 128.7, 125.9, 99.6, 80.4, 79.5, 63.6, 60.7, 32.4, 29.8, 29.3, 28.6, 28.5, 22.9, 19.8, 18.6, 15.6, 15.3, 13.8.
stirred overnight before separation between water and dichloromethane. The combined organic fractions were washed with brine and concentrated in vacuo before purification by flash column chromatography (25% ethyl acetate in petroleum ether) to yield the product (6) as a white, waxy solid (276 mg, 99%); δH (500 MHz, CDCl3) 3.64 (2H, t, J 6.7, H-1), 2.26-2.22 (4H, m, H-9, H-11), 1.58-1.50 (6H, m, 3CH2), 1.39-1.25 (10H, m, 5CH2), 1.00 (3H, t, J 7.4, H-16); δc (125 MHz, CDCl3) 77.7, 77.6, 65.4, 65.2, 63.0, 32.0, 29.4, 29.4, 29.0, 28.8, 28.3, 25.7, 21.2, 21.1, 19.2, 13.5.

![Synthetic Scheme D](image1)

Synthetic Scheme D

1. BuLi
2. I2

10-undecyn-1-ol Cu(I)I

![Synthetic Scheme E](image2)

Synthetic Scheme E

(Z)-1-bromopent-1-ene
PdCl2(PhCN)2 Cu(I)I

![Synthetic Scheme F](image3)

Synthetic Scheme F

(E)-1-iodopent-1-ene
NaOEt Pd(0)

![Synthetic Scheme G](image4)

Synthetic Scheme G

(Z)-Hexadecen-10-yn-1-ol. To a solution of 10-undecyn-1-ol (271 mg, 1.61 mmol) in piperidine (7 mL) was added 1-bromopent-1-ene (240 mg, 1.61 mmol). Copper(I) iodide (31 mg, 0.16 mmol) and PdCl2(PhCN)2 (31 mg, 0.08 mmol) were added and the mixture was stirred until the starting material was consumed, then quenched with aqueous ammonium chloride and extracted with dichloromethane. The combined organic extracts were washed with 1 M HCl and brine, dried (MgSO4), filtered and vacuum-dried before use.

Figure 11. Schemes D–G. Synthetic sequence for preparation of analogues 3–6 differing in unsaturation. doi:10.1371/journal.pone.0044190.g011

Scheme E (Figure 11). Synthesis of (10Z,12Z)-hexadecadien-1-ol (5)

(12Z)-Hexadecen-10-yn-1-ol. To a solution of 10-undecyn-1-ol (271 mg, 1.61 mmol) in piperidine (7 mL) was added 1-bromopent-1-ene (240 mg, 1.61 mmol). Copper(I) iodide (31 mg, 0.16 mmol) and PdCl2(PhCN)2 (31 mg, 0.08 mmol) were added and the mixture was stirred until the starting material was consumed, then quenched with aqueous ammonium chloride and extracted with dichloromethane. The combined organic extracts were washed with 1 M HCl and brine, dried (MgSO4), filtered and vacuum-dried before use.
concentrated in vacuo. Purification by flash column chromatography (15% ethyl acetate in petroleum ether) yielded the enyne as a clear, colorless oil (336 mg, 88%).

\(12\)-hexadecadien-10-yn-1-ol (3). To a solution of the boron compound (400 mg, 1.96 mmol) and Pd(Ph3)3 (107 mg, 0.33 Ca(NO3)2, 0.41 CaCl2, 10 HEPES, pH 7.4) supplemented with 10 μg/ml of gentamycin, 10 μg/ml of streptomycin and 1.8 mM sodium pyruvate.

Scheme F (Figure 11). Synthesis of \((10E,12E)\)-hexadecadien-1-ol (3).

(10E,12E)-hexadecadien-1-ol (3). To a solution of the boron compound (140 mg, 1.96 mmol) and Pd(PPh3)3 (107 mg, 0.99 mmol) in benzene was added vinyl iodide (400 mg, 2.04 mmol) and sodium ethoxide in ethanol (21% in 2 mL). The mixture was refluxed for 2 hours, then pre-absorbed onto silica gel for flash column chromatography (10% ethyl acetate in petroleum ether) to yield the diene as a clear, colorless oil (240 mg, 54%).

In vitro Transcription Oocyte and Microinjection

In vitro transcription of cRNAs (BmorOr1 and BmorOrco) was performed by using a mMESSAGE mMACHINE T7 Kit (Ambion) according to the manufacturer's protocol. Plasmids were linearized with Nhe I, and capped cRNA was transcribed using T7 RNA polymerase. The cRNAs were purified with LiCl precipitation solution and re-suspended in nuclease-free water at a concentration of 200 μg/ml and stored at −80°C in aliquots. RNA concentrations were determined by UV spectrophotometry.

cRNA were microinjected (2 ng of a receptor cRNA and 2 ng of an Orco cRNA) into Xenopus laevis oocytes on stage V or VI (EcoCyte Bioscience, Austin TX). The oocytes were then incubated at 18°C for 3–7 days in modified Barth’s solution [in mM: 88 NaCl, 1 KCl, 2.4 NaHCO3, 0.92 MgSO4, 0.33 Ca(NO3)2, 0.41 CaCl2, 10 HEPES, pH 7.4] supplemented with 10 μg/ml of gentamycin, 10 μg/ml of streptomycin and 1.8 mM sodium pyruvate.

Scheme G (Figure 11). Synthesis of \((10Z,12E)\)-hexadecadien-1-ol (4).

(10Z,12E)-hexadecadien-10-yn-1-ol (935 mg, 3.95 mmol), was reduced using the Zn/Ag/Cu amalgam described above to yield 5 as a clear, colorless oil (400 mg, 1.96 mmol) and Pd(Ph3)3 (107 mg, 0.99 mmol) in benzene was added vinyl iodide (400 mg, 2.04 mmol) and sodium ethoxide in ethanol (21% in 2 mL). The mixture was refluxed for 2 hours, then pre-absorbed onto silica gel for flash column chromatography (10% ethyl acetate in petroleum ether) to yield the diene as a clear, colorless oil (240 mg, 54%).

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Author Contributions

Conceived and designed the experiments: PX AMH WSL. Performed the experiments: PX AMH WSL. Analyzed the data: PX AMH WSL. Contributed reagents/materials/analysis tools: PX AMH JAP WSL. Wrote the paper: AMH PX WSL.
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