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New Analogues of (*E*)-β-Farnesene with Insecticidal Activity and Binding Affinity to Aphid Odorant-Binding Proteins

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ABSTRACT: (*E*)-β-Farnesene is a strong and efficient alarm pheromone in most aphid species. However, applications in agriculture are prevented by its relatively high volatility, its susceptibility to oxidation and its complex and expensive synthesis. To develop novel compounds for aphid control, we have designed and synthesized analogues of (*E*)-β-farnesene, containing a pyrazole moiety present in several insecticides. Their structures have been confirmed by ^{1}H NMR, elemental analysis, high-resolution mass spectroscopy and IR. Binding activities to three odorant-binding proteins (OBPs) of the pea aphid *Acythosiphon pisum* have been evaluated and correlated with their structures with reference to (*E*)-β-farnesene. Several derivatives were shown both to bind to *A. pisum* OBPs with a specificity similar to that of (E)-β-farnesene and to have aphicidal activity comparable to that of thiacloprid, a commercial insecticide. The compounds synthesized in this work represent new potential agents for aphid population control and provide guidelines to design analogues of (*E*)-β-farnesene endowed with both insecticidal and repellent activity for aphids.

KEYWORDS: (*E*)-β-farnesene, aphids, insecticides, odorant-binding proteins, OBP, ligand-binding assay

1. INTRODUCTION

Aphids are one of the major pests in agriculture, responsible for spreading plant virus infections, apart from their direct damage to the plant. Mildew is nourished by their excreted honeydew that causes fulvia leaf mold. The great number of aphid species, their fast breeding and their ability to develop resistance to chemical insecticides make their population control challenging. The major component of alarm pheromone, (*E*)-β-farnesene (Figure 1, EBF), that is released by most aphid species when disturbed,[1−5] has been shown to be endowed with multiple biological functions. Recently, this compound has been reported to be involved in pea aphid (*Acyrthosiphon pisum*) wing induction,[6] while its insecticidal activity at high doses has also been demonstrated.[7] However, the use of this pheromone in aphid population control presents some disadvantages. In fact, (*E*)-β-farnesene is relatively volatile and unstable in the environment, due to the easy oxidation of its several double bonds.[8] Therefore it is necessary to find analogues that are more stable and efficient.

Recent studies have indicated that odorant-binding proteins (OBPs), water-soluble polypeptides that mediate olfactory detection in the sensillar lymph of insects,[9,10] are required for correct functioning of the olfactory system and are responsible for recognition and discrimination of different olfactory messages.[11−13] A recent study on the functional characterization of three OBPs of the pea aphid *A. pisum* has suggested that OBP3 could be specifically involved in the detection of the alarm pheromone (*E*)-β-farnesene.[14] Under such hypothesis, that however still needs further support, simple and fast binding assays with OBPs could predict if a synthetic compound might be perceived as an alarm message by the aphids, while modeling studies could also help designing new semiochemicals for aphid population control.[14]

Based on such working hypothesis, we have designed and synthesized analogues of (*E*)-β-farnesene endowed with insecticidal activity and investigated their biological activity, as well as their affinity to aphid OBPs.

2. MATERIALS AND METHODS

2.1. Apparatus and Chemicals. Melting points of compounds were determined on a Yanagimoto microscope, with an uncorrected thermometer. ^{1}H NMR spectra were recorded on a Bruker Avance DPX300 spectrometer. Chemical shifts are reported in δ (ppm) relative to the signal of tetramethylsilane (TMS) as internal standard, using deuterochloroform as solvent. Coupling constants are given in Hz. Elemental analysis was performed with a ST-Carlo Erba Co. elemental analyzer. High-resolution mass spectra were recorded under electron impact (150 eV) condition using a Bruker APEX IV instrument. Liquid chromatography−mass spectrometry (LC/MS) was performed on a Waters Alliance 2695/ZQ4000 spectrometer. IR spectra were recorded on neat samples on a Shimadzu IR-435 spectrophotometer using a KBr pellets. All reagents and solvents were of analytical reagent and when necessary were purified and dried before use.

2.2. Synthesis of (*E*)-β-Farnesene Analogues. *2.2.1. General Synthetic Procedure for Intermediates* ***2a*****−*****2j*****.** Metal sodium (5.0 g,

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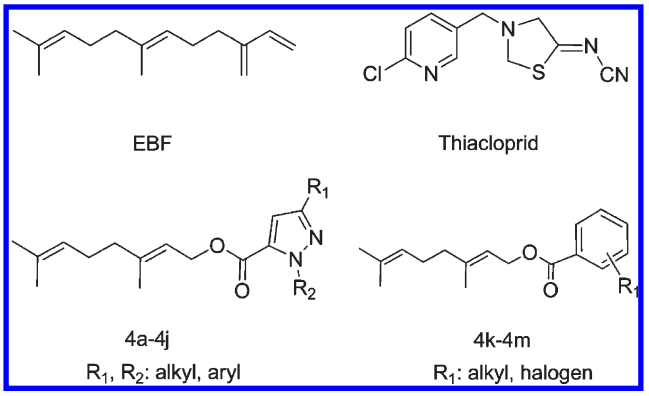


Figure 1. Structure of (*E*)-*β*-farnesene (EBF), the alarm pheromone for most aphid species, thiacloprid, the insecticide used as reference in the mortality experiments, and the general structures of the two classes of synthetic compounds described in this work and containing a region structurally similar to EBF and one reproducing part of an insecticide molecule.

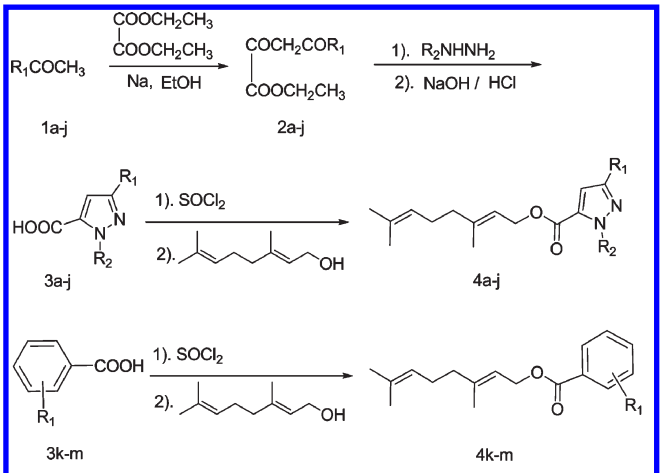
217 mmol) was slowly added to ethanol (125 mL) in a 250 mL round-bottom flask equipped with a condenser. After the sodium was completely dissolved, the mixture was heated at 50 °C under stirring for 1 h and then cooled to 10 °C. A mixture of the appropriate methyl ketone (**1a**−**1j**, 200 mmol) and diethyl oxalate (29.2 g, 200 mmol) was then added dropwise. The reaction mixture was stirred for 4 h while the temperature was kept below 10 °C. A solid precipitate formed, which was filtered and dissolved in 100 mL of iced water. The solution was then brought to pH 3.0 with 20% sulfuric acid and extracted with dichloromethane (2 × 50 mL). The organic layer was dried with anhydrous Na_2SO_4, and the solvent was removed under reduced pressure to yield intermediates **2a**−**2j** (see refs 15−20 and Scheme 1) all as sticky oils of pale pink color. Yields were as follows: **2a** or **2b**, 22.8 g (72%); **2c** or **2d**, 26.0 g, (70%); **2e**, **2f** or **2g**, 28.0 g, (69%); **2h**, **2i** or **2j**, 13.96 g (32%).

*2.2.2. General Synthetic Procedure for Intermediates **3a**−**3j**.* A solution containing the intermediate **2a**−**2j** (25 mmol) in methanol (10 mL) was slowly added to a solution of hydrazine (25 mmol) in methanol at 0 °C (8.0 mL). The mixture was adjusted to neutrality with a saturated solution of sodium bicarbonate and stirred at 60 °C for 2 h. After concentration under reduced pressure, the residue was added into 7 mL of 6 M aq NaOH and stirred at 80 °C for 2 h. After cooling to room temperature, the resulting mixture was poured into 50 mL of iced water and adjusted to pH 3.0 with concentrated hydrochloric acid. The solid thus formed was filtered and recrystallized from methanol to give white to yellow crystals (see refs 16 and 20 and Scheme 1). Yields were as follows: **3a**, 2.6 g (57%); **3b**, 3.5 g (70%); **3c**, 4.23 g (74%); **3d**, 2.84 g (49%); **3e**, 4.13 g (79%); **3f**, 1.73 g (30%); **3g**, 5.96 g (91%); **3h**, 3.16 g (52%); **3i**, 4.47 g (68%); **3j**, 5.53 g (73%).

*2.2.3. General Preparation for Geranyl Pyrazole-5-formate Derivatives **4a**−**4j**.* A mixture containing intermediate **3a**−**3j** (7 mmol) and thionyl chloride (11.9 g, 100 mmol) was added into a 50 mL flask equipped with a condenser and refluxed for 8 h. After cooling to room temperature, the excess thionyl chloride was distilled under reduced pressure. The residue was refluxed with geraniol (7 mmol) in 20 mL of acetonitrile for 8 h in the presence of pyridine as an acid scavenger. After filtration, the solvent was removed under reduced pressure to give crude products **4a**−**4j**. The products were purified by column chromatography on silica gel using ethyl acetate−petroleum (60−90 °C) at a ratio of 1:4−1:7 as the eluent (see Scheme 1).

*Data for **4a**:* 1.6 g of light yellow oil. Yield: 71.9%. ^{1}H NMR ($CDCl_3$): 1.60 (s, 3H, CH_3−C), 1.66 (s, 3H, CH_3−C), 1.67 (s, 9H, $(CH_3)_3$-C), 1.74 (d, 3H, *J* = 1.06 Hz, CH_3−C), 2.04−2.11 (m, 4H, C−CH_2−CH_2−C), 2.46 (t, 3H, *J* = 2.43 Hz, CH_3−C═N), 4.82 (d, 2H, *J* = 6.66 Hz, CH_2−O), 5.09 (t, 1H, *J* = 1.41 Hz, C=CH), 5.43−5.48 (m, 1H, C=CH), 6.55 (d, 1H, *J* = 0.68 Hz, pyrazole-H). Anal. Calcd for $C_{19}H_{30}N_2O_2$: C (71.66), H (9.50), N (8.80); found, C (71.75), H (9.42), N (8.87). IR (KBr pellet press, cm^{-1}): 2972, 2924, 1718, 1671, 1376, 1217, 1011.

Scheme 1. General Synthetic Routes for the Target Compounds



Compd.	R_1	R_2	Compd.	R_1	R_2
4a	CH_3	$C(CH_3)_3$	**4h**	C_6H_5	$C(CH_3)_3$
4b	CH_3	C_6H_5	**4i**	C_6H_5	C_6H_5
4c	$CH_2CH_2CH_3$	$C(CH_3)_3$	**4j**	C_6H_5	4-$NO_2C_6H_4$
4d	$CH_2CH_2CH_3$	C_6H_5	**4k**	4-$C(CH_3)_3$	
4e	$CH(CH_3)_2$	$C(CH_3)_3$	**4l**	4-Br	
4f	$CH(CH_3)_2$	C_6H_5	**4m**	2,6-F_2	
4g	$CH(CH_3)_2$	4-$NO_2C_6H_4$			

*Data for **4b**:* 1.69 g of yellow oil. Yield: 71.5%. ^{1}H NMR ($CDCl_3$): 1.60 (s, 3H, CH_3−C), 1.68 (d, 6H, *J* = 0.85 Hz, $(CH_3)_2$−C), 1.99−2.10 (m, 4H, C−CH_2−CH_2−C), 2.33 (t, 3H, *J* = 5.58 Hz, CH_3−C═N), 4.68 (d, 2H, *J* = 7.14 Hz, CH_2−O), 5.04−5.09 (m, 1H, C=CH), 5.26−5.31 (m, 1H, C=CH), 6.81 (s, 1H, pyrazole-H), 7.37−7.47 (m, 5H, ArH). Anal. Calcd for $C_{21}H_{26}N_2O_2$: C (74.52), H (7.74), N (8.28); found, C (74.04), H (7.74), N (8.17). IR (KBr pellet press, cm^{-1}): 2967, 2924, 1729, 1670, 1598, 1502, 1377, 1222, 1029.

*Data for **4c**:* 1.13 g of yellow oil. Yield: 47.6%. ^{1}H NMR ($CDCl_3$): 1.04 (t, 3H, *J* = 7.33 Hz, CH_3−CH_2), 1.60 (s, 3H, CH_3−C), 1.66 (s, 3H, CH_3−C), 1.67 (s, 9H, $(CH_3)_3$−C), 1.70−1.78 (m, 5H, CH_3−C, CH_3−CH_2), 2.05−2.11 (m, 4H, C−CH_2−CH_2−C), 2.77 (t, 2H, *J* = 7.77 Hz, $CH_2CH_2CH_3$), 4.82 (d, 2H, *J* = 6.99 Hz, CH_2−O), 5.09 (t, 1H, *J* = 1.38 Hz, C=CH), 5.46 (d, 1H, *J* = 1.20 Hz, C═CH), 6.62 (s, 1H, pyrazole-H). HRMS: calcd for $C_{21}H_{34}N_2O_2$ (M + H), 347.2693; found, 347.2699. MS *m*/*z* (%): 347 (M + H, 20), 211 (100), 155 (20), 137 (35). IR (KBr pellet press, cm^{-1}): 2967, 2932, 1714, 1671, 1371, 1215, 1006.

*Data for **4d**:* 1.37 g of yellow oil. Yield: 53.6%. ^{1}H NMR ($CDCl_3$): 0.99 (t, 3H, *J* = 7.35 Hz, CH_3−CH_2), 1.60 (s, 3H, CH_3−C), 1.67 (d, 6H, *J* = 2.09 Hz, $(CH_3)_2$−C), 1.71−1.76 (m, 2H, CH_3−CH_2), 2.06 (m, 4H, C−CH_2−CH_2−C), 2.67 (t, 2H, *J* = 7.60 Hz, $CH_2CH_2CH_3$), 4.68 (d, 2H, *J* = 7.11 Hz, CH_2−O), 5.07 (s, 1H, C═CH), 5.29 (d, 1H, *J* = 1.07 Hz, C═CH), 6.83 (s, 1H, pyrazole-H), 7.38−7.43 (m, 5H, ArH). HRMS: calcd for $C_{23}H_{30}N_2O_2$ (M + H), 367.2380; found, 367.2385. MS *m*/*z* (%): 367 (M + H, 100), 265 (2), 232 (9), 231 (44). IR (KBr pellet press, cm^{-1}): 2962, 2930, 1718, 1671, 1598, 1502, 1378, 1223, 1012.

Data for ***4e***: 2.0 g of light yellow oil. Yield: 58.3%. ^{1}H NMR ($CDCl_3$): 1.28 (d, 6H, J = 6.78 Hz, $(CH_3)_2CH$), 1.60 (s, 3H, CH_3−C), 1.66−1.68 (m, 12H, $(CH_3)_3C$, CH_3−C), 1.74 (s, 3H, CH_3−C), 2.03−2.12 (m, 4H, C−CH_2−CH_2−C), 3.36 (q, 1H, J = 6.78 Hz, $(CH_3)_2CH$), 4.82 (d, 2H, J = 7.35 Hz, CH_2−O), 5.07−5.11 (m, 1H, C═CH), 5.44−5.49 (m, 1H, C═CH), 6.67 (s, 1H, pyrazole-H). Anal. Calcd for $C_{21}H_{34}N_2O_2$: C (72.79), H (9.89), N (8.08); found, C (73.02), H (9.91), N (8.00). IR (KBr pellet press, cm^{-1}): 2976, 2930, 1715, 1671, 1371, 1214, 1007.

Data for ***4f***: 1.49 g of light yellow oil. Yield: 58.3%. ^{1}H NMR ($CDCl_3$): 1.18 (d, 6H, J = 6.84 Hz, $(CH_3)_2CH$), 1.60 (d, 3H, J = 0.3 Hz, CH_3−C), 1.67 (d, 3H, J = 0.93 Hz, CH_3−C), 1.75 (d, 3H, J = 0.99 Hz, CH_3−C), 2.04−2.11 (m, 4H, C−CH_2−CH_2−C), 2.99 (t, 1H, J = 6.78 Hz, $(CH_3)_2CH$), 4.87 (d, 2H, J = 7.11 Hz, CH_2−O), 5.06−5.11 (m, 1H, C═CH), 5.46−5.51 (m, 1H, C═CH), 6.77 (d, 1H, J = 0.45 Hz, pyrazole-H), 7.40−7.51 (m, 5H, ArH). HRMS: calcd for $C_{23}H_{30}N_2O_2$ (M + H), 367.2380; found, 367.2381. MS m/z (%): 367 (M + H, 14), 232 (15), 231 (100), 213 (3), 137 (25). IR (KBr pellet press, cm^{-1}): 2968, 2929, 1717, 1670, 1597, 1501, 1381, 1222, 1009.

Data for ***4g***: 0.98 g, yellow oil. Yield. 35.3%. ^{1}H NMR ($CDCl_3$): 1.23 (d, 6H, J = 6.81 Hz, $(CH_3)_2CH$), 1.60 (d, 3H, J = 0.42 Hz, CH_3−C), 1.67 (d, 3H, J = 0.96 Hz, CH_3−C), 1.76 (d, 3H, J = 1.11 Hz, CH_3−C), 2.04−2.13 (m, 4H, C−CH_2−CH_2−C), 3.08 (t, 1H, J = 6.77 Hz, $(CH_3)_2CH$), 4.89 (d, 2H, J = 6.99 Hz, CH_2−O), 5.07−5.11 (m, 1H, C═CH), 5.45−5.51 (m, 1H, C═CH), 6.83 (d, 1H, J = 0.57 Hz, pyrazole-H), 7.66−7.71(m, 2H, ArH), 8.35−8.40 (m, 2H, ArH). HRMS: calcd for $C_{23}H_{29}N_3O_4$ (M + H), 412.2230; found, 412.2231. MS m/z (%): 412 (M + H, 6), 277 (10), 276 (78), 258 (3), 138 (8), 137 (100). IR (KBr pellet press, cm^{-1}): 2969, 2929, 1720, 1671, 1597, 1526, 1501, 1379, 1345, 1224, 1005, 856.

Data for ***4h***: 1.33 g, yellow oil. Yield. 59.8%. ^{1}H NMR ($CDCl_3$): 1.49 (t, 9H, J = 6.20 Hz, $(CH_3)_3$−C), 1.60 (s, 3H, CH_3−C), 1.67 (d, 3H, J = 0.87 Hz, CH_3−C), 1.75 (d, 3H, J = 0.96 Hz, CH_3−C), 2.02−2.12 (m, 4H, C−CH_2−CH_2−C), 4.85 (d, 2H, J = 7.00 Hz, CH_2−O), 5.06−5.11 (m, 1H, C═CH), 5.44−5.49 (m, 1H, C═CH), 6.67 (s, 1H, pyrazole-H), 7.31−7.42 (m, 5H, ArH). HRMS: calcd for $C_{24}H_{32}N_2O_2$ (M + H), 381.2536; found, 381.2540. MS m/z (%): 381 (M + H, 10), 245 (100), 187 (90), 137 (50). IR (KBr pellet press, cm^{-1}): 2976, 2930, 1716, 1671, 1606, 1577, 1462, 1397, 1217, 1179, 1004.

Data for ***4i***: 2.10 g of yellow oil. Yield: 75.0%. ^{1}HNMR($CDCl_3$): 1.60 (s, 3H, CH_3−C), 1.68 (d, 3H, J = 0.84 Hz CH_3−C), 1.77 (d, 3H, J = 0.90 Hz, CH_3−C), 2.02−2.13 (m, 4H, C−CH_2−CH_2−C), 4.92 (d, 2H, J = 6.99 Hz, CH_2−O), 5.07−5.11 (m, 1H, C═CH), 5.48−5.53 (m, 1H, C═CH), 7.05 (s, 1H, pyrazole-H), 7.19−7.39 (m, 10H, ArH). HRMS: calcd for $C_{26}H_{28}N_2O_2$ (M + Na), 423.2043; found, 423.2048. MS m/z (%): 401 (M + H, 20), 265 (100), 137 (31). IR (KBr pellet press, cm^{-1}): 2966, 2922, 1720, 1670, 1597, 1498, 1372, 1222, 1001.

Data for ***4j***: 0.42 g of yellow crystal. Yield: 13.5%. mp 60−61 °C. ^{1}H NMR ($CDCl_3$): 1.60 (d, 3H, J = 5.66 Hz, CH_3−C), 1.08 (s, 3H, CH_3−C), 1.78 (d, 3H, J = 0.89 Hz, CH_3−C), 2.04−2.15 (m, 4H, C−CH_2−CH_2−C), 4.93 (d, 2H, J = 7.16 Hz, CH_2−O), 5.07−5.12 (m, 1H, C═CH), 5.48−5.53 (m, 1H, C═CH), 7.06 (s, 1H, pyrazole-H), 7.21−7.42 (m, 5H, ArH), 7.52−7.56 (m, 2H, ArH), 8.18−8.22 (m, 2H, ArH). Calcd for $C_{26}H_{27}N_3O_4$: C (70.09), H (6.11), N (9.43); found, C (69.73), H (6.16), N (9.28). IR (KBr pellet press, cm^{-1}): 2966, 2918, 1732, 1660, 1596, 1518, 1495, 1368, 1339, 1221, 1002.

2.2.4. General Preparation for Geranyl Benzoate ***4k***−***4m***. A mixture of **3k**−**3m** (7 mmol) and thionyl chloride (11.9 g, 100 mmol) was refluxed in a 50 mL flask for 8 h. The excess thionyl chloride removed under reduced pressure. The residue was dissolved in 20 mL of acetonitrile. Then geraniol (7 mmol) was added batchwise and the mixture was refluxed for 8 h in the presence of pyridine as an acid scavenger. After filtration, the solvent was evaporated and the residues (**4k**−**4m**) were purified by column chromatography on silica gel with ethyl acetate and petroleum (60−90 °C) at a ratio of 1:4−1:10 as the eluent (see Scheme 1).

Data for ***4k***: 1.876 g of colorless oil. Yield: 85.4%. ^{1}H NMR ($CDCl_3$): 1.33 (d, 9H, J = 1.86 Hz, $(CH_3)_3$-C), 1.60 (d, 3H, J = 5.19 Hz, CH_3−C), 1.68 (d, 3H, J = 0.90 Hz, CH_3−C), 1.76 (d, 3H, J = 0.99 Hz, CH_3−C), 2.05−2.13 (m, 4H, C−CH_2−CH_2−C), 4.83 (d, 2H, J = 7.05 Hz, CH_2−O), 5.07−5.12 (m, 1H, C═CH), 5.44−5.49 (m, 1H, C═CH), 7.42−7.47 (m, 2H, ArH), 7.95−8.00 (m, 2H, ArH). Anal. Calcd for $C_{21}H_{30}O_2$: C (80.21), H (9.62); found, C (80.01), H (9.60). IR (KBr pellet press, cm^{-1}): 2966, 2927, 1718, 1672, 1610, 1572, 1378, 1272, 1017.

Data for ***4l***: 1.65 g of colorless oil. Yield: 70.0%. ^{1}H NMR ($CDCl_3$): 1.60 (s, 3H, CH_3−C), 1.67 (s, 3H, CH_3−C), 1.76 (s, 3H, CH_3−C), 2.04−2.15 (m, 4H, C−CH_2−CH_2−C), 4.83 (d, 2H, J = 7.08 Hz, CH_2−O), 5.08 (d, 1H, J = 6.93 Hz, C═CH), 5.45 (t, 1H, J = 7.08 Hz, C═CH), 7.58 (t, 2H, J = 5.28 Hz, ArH), 7.92 (t, 2H, J = 5.33 Hz, ArH). Anal. Calcd for $C_{17}H_{21}B_rO_2$: C (60.54), H (6.28); found, C (60.95), H (6.36). IR (KBr pellet press, cm^{-1}): 2967, 2922, 1721, 1672, 1591, 1378, 1268, 1012.

Data for ***4m***: 1.26 g of colorless oil. Yield: 61.2%. ^{1}H NMR ($CDCl_3$): 1.60 (s, 3H, CH_3−C), 1.68 (d, 3H, J = 0.78 Hz, CH_3−C), 1.76 (d, 3H, J = 1.02 Hz, CH_3−C), 2.04−2.17 (m, 4H, C−CH_2−CH_2−C), 4.88 (d, 2H, J = 7.20 Hz, CH_2−O), 5.06−5.12 (m, 1H, C═CH), 5.43−5.49 (m, 1H, C═CH), 6.90−6.98 (m, 2H, ArH), 7.34−7.44 (m, 2H, ArH). Anal. Calcd for $C_{17}H_{20}F_2O_2$: C (69.37), H (6.89); found, C (69.23), H (6.91). IR (KBr pellet press, cm^{-1}): 2968, 2920, 1735, 1670, 1593, 1470, 1378, 1289, 1014.

2.3. Fluorescence Binding Assays. OBP1, OBP3 and OBP8 of *A. pisum* were prepared and purified following the reported procedures.[14] To measure the affinity of the fluorescent probes *N*-phenyl-1-naphthylamine (1-NPN) to OBPs, a 2 μM solution of the protein in 50 mM Tris-HCl was titrated with aliquots of 1 mM ligand in methanol to final concentrations of 2−16 μM. The probe was excited at 337 nm, and emission spectra were recorded between 380 and 450 nm. For determining binding constants of 1-NPN, the intensity values corresponding to the maximum of fluorescence emission (406−409 nm) were plotted against free ligand concentrations. Bound ligand was then evaluated from the values of fluorescence intensity assuming that the protein was 100% active, with a stoichiometry of 1:1 protein:ligand at saturation. The curves were linearized using Scatchard plots. The affinity of each (*E*)-β-farnesene analogue was measured in competitive binding assays, using both 1-NPN and protein at 2 μM concentration and each analogue as the competitor at 2−16 μM. Dissociation constants of (*E*)-β-farnesene analogues were calculated from the corresponding IC_{50} values, using the equation $K_i = [IC_{50}]/(1 + [1 - NPN]/K_{1\text{-}NPN})$, with [1-NPN] being the free concentration of 1-NPN and $K_{1\text{-}NPN}$ being the dissociation constant of the protein/1-NPN complex.

2.4. Molecular Modeling. A three-dimensional model of *A. pisum* OBP3 was generated using the online program SWISS MODEL,[21−23] using the crystal structure of the PBP of *Leucophaea maderae* (PDB: 1ORG_A; ref 24) as a template. Amino acid identity between the two proteins is 23% based on the models was displayed using the SwissPdb Viewer program "Deep-View"[22] (http://www.expasy.org/spdbv/).

2.5. Insecticidal Activity. The insecticidal activity of (*E*)-β-farnesene analogues against *Aphis gossypii* was evaluated using the following procedure.

The compounds were dissolved in acetone/methanol/water (1:1:18) at the concentration of 600 μg/mL. Cabbage leaves of about 5 cm diameter with 3 day old aphids on them were immersed in the solution for 5 s, air-dried and kept in Petri dishes for 24 h at 24−26 °C, and then the number of dead aphids was counted. Experiments were performed three times and the results statistically analyzed.

3. RESULTS AND DISCUSSION

3.1. Aim of the Work. It is known that the efficacy of an insecticide increases if insects move around, thus coming in contact with higher amounts of the chemical than if they remain

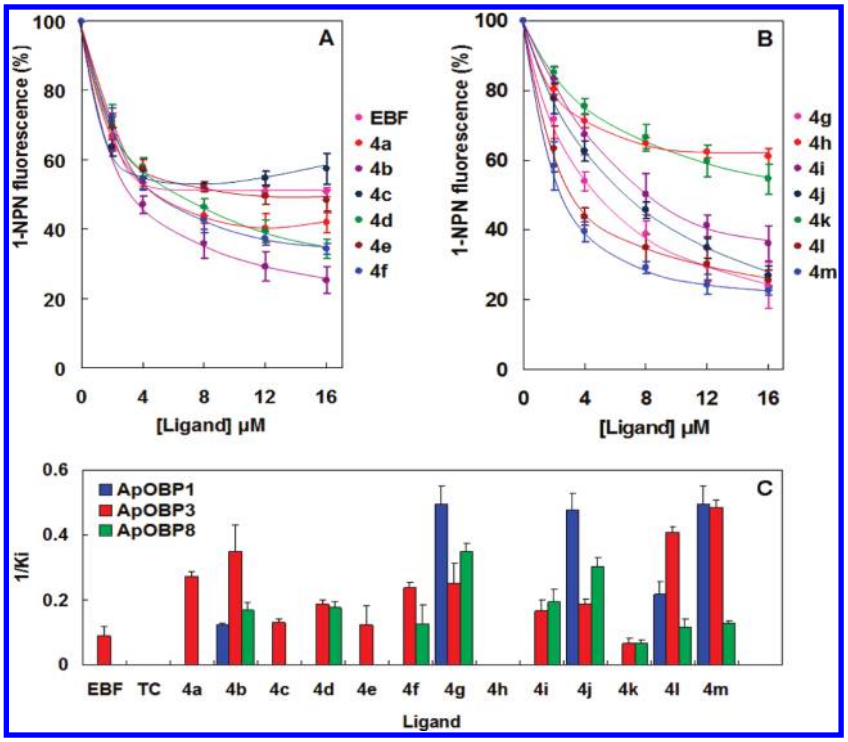


Figure 2. Competitive binding of the compounds prepared to OBP3 of *A. pisum*. (A, B) Displacement curves of the fluorescent probe 1-NPN from the complex with OBP3 by each synthetic compound. A solution of 2 μM protein and 2 μM 1-NPN in Tris was titrated with aliquots of 1 μM solution of the ligand up to the final concentration of 16 μM. Fluorescence intensities are reported as percent of the values in the absence of competitor. Parallel experiments were also performed with OBP1 and OBP8 (data not shown). (C) Comparison of the affinity of each ligand to the three OBPs used. Reciprocal of dissociation constants (divided by 10^6) are reported, as calculated from the concentration values of each ligand halving the initial fluorescence value. EBF: (*E*)-β-farnesene. TC: thiacloprid. The structures of all the ligands are reported in Figure 1.

in the same place.[25] Aphids usually stay on a leaf for a long time feeding on the same spot. However, in response to their specific alarm pheromone, they become very active, eventually abandoning the place.[26,27] For most aphid species, the alarm pheromone is (*E*)-β-farnesene, either alone or in combination with minor components.[5] We therefore decided to combine the insecticidal effect and the alarm activity in the same molecule, designing structural analogues of (*E*)-β-farnesene, that also contained a pyrazole moiety based on the structures of several insecticides.[28]

Accordingly, the synthesized compounds contain a geranyl group, mimicking the terpene structure of (*E*)-β-farnesene, linked to heteroaromatic rings, that have been employed in some commercial insecticides. In particular, the ethylidene group of (*E*)-β-farnesene is replaced by the carbonyl of the ester group, while the vinyl group is part of the aromatic ring in the analogues. The reduced number of the double bonds in the designed analogues should also increase the stability of the parent (*E*)-β-farnesene.

3.2. Preparation and Characterization of the Ligands. The key intermediates **3a**−**3j** were prepared by the Claisen condensation reaction (Scheme 1). The target compounds **4a**−**4j** and **4k**−**4m** were synthesized from geraniol and the corresponding acyl chloride in dry acetonitrile under refluxing with pyridine as an acid scavenger. Pyridine·HCl was found to be the main byproduct, and it was easily removed by column chromatography. The identity of the final compounds was confirmed by ^{1}H NMR, elemental analysis, high-resolution mass spectrometry and IR. Purity was evaluated by LC/MS.

The syntheses followed well-described routes, starting from inexpensive commercial chemicals, and did not present particular problems. The compounds were easily purified and were found to be stable at room temperature. In particular, we checked the stability of compounds **4a** and **4e** by mass spectrometry after leaving them at room temperature and in contact with air for periods up to 4 days. In such conditions we could not detect any degradation products of the compounds examined.

3.3. Binding Studies. A previous study showed that (*E*)-β-farnesene and several other compounds bound selectively to *A. pisum* OBP3, but bound at lower or undetectable levels to OBP1 and OBP8 of the same species, suggesting that OBP3 may be involved in the detection of the alarm pheromone. Therefore, we decided to measure the binding specificity of our new ligands to these three recombinant *A. pisum* OBPs, as a first step to study their olfactory properties with reference to (*E*)-β-farnesene.

Following the approach previously described,[14] we evaluated the affinity of the ligands to the protein in competitive binding assays, where the strength of interaction between ligand and protein is measured by the capacity of the ligand to displace the fluorescent probe *N*-phenyl-1-naphthylamine (1-NPN) from its complex with the protein. The three proteins used, OBP1, OBP3 and OBP8, bind 1-NPN with dissociation constants of 6.9 μM, 5.9 μM and 5.5 μM respectively, as previously reported.[14]

Figures 2A and 2B show the results of the competitive binding assays obtained with OBP3. Parallel experiments were performed with the other two proteins. The calculated binding constants of the ligands for the three proteins are compared in Figure 2C. We also included in our binding assay thiacloprid, a commercial insecticide, that we have used as a positive control when evaluating the aphicidal activity of our compounds. Thiacloprid did not show affinity to any of the three OBPs tested. On the

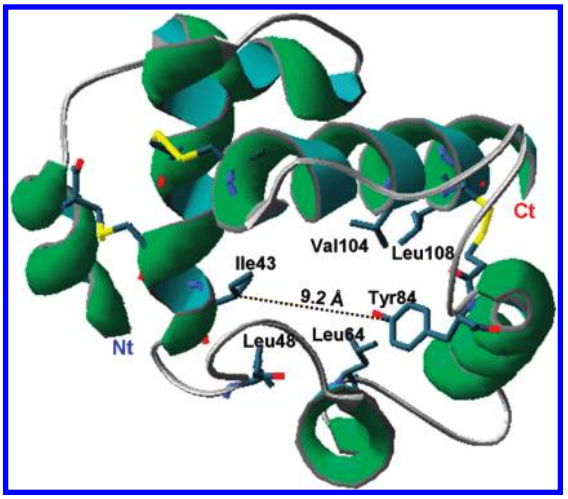


Figure 3. Molecular model of *A. pisum* OBP3. The model was generated on line using the Swiss-Model software,[21−23] using the PBP of *L. maderae*[24] as a template. The binding cavity is lined with branched aliphatic residues (Ile43, Leu48, Leu64, Val104, Leu108), while a tyrosine (Tyr84) is found at the entrance of the pocket. Binding of several of the synthesized compounds can be rationalized assuming that the aromatic part of the ligand establishes p−p bonds with the benzene ring of Tyr84, while the branched hydrocarbon chain can efficiently interact with the branched chains of the other amino acids. The distance between Tyr84 and Ile43 is 9.2 Å, compatible with the length of the aliphatic chain in the synthesized compounds. N-terminus (Nt) and C-terminus (Ct) are marked.

contrary, the competition binding curves exhibit an increasing trend with the concentration of thiacloprid, due to nonspecific effects. The displacement curve of thiacloprid has not been included, but only the lack of binding is reported in the bar graph of Figure 2C.

Some of the chemicals tested exhibited good affinity to all three proteins, whereas others were more selective. In particular, **4a**, **4c** and **4e** exhibited marked selectivity to OBP3. These three chemicals all bear aliphatic chains on the pyrazole ring. The size and branching of such groups does not seem to affect the selectivity of binding, but the affinity is highest for the derivative with shortest chains (**4a**). An additional aromatic ring, on the contrary, seems to produce compounds with no selectivity and variable affinities for the three proteins. The presence of electron-withdrawing groups, such as halogens or a nitro group (as in **4g**, **4j**, **4l** and **4m**), increases, as expected, the affinity for the three proteins, without any selectivity to each of them. It has been suggested that (*E*)-β-farnesene might bind to *A. pisum* OBP3 by establishing hydrophobic interactions between the terpene branched chain and the side groups of several hydrophobic amino acids lining the binding pocket (Ile43, Leu48, Leu64, Val104 and Leu108), while the two conjugated double bonds could establish π interactions with the benzene ring of Tyr84, located at the entrance of the binding cavity. In the model of OBP3, reported in Figure 3, the positions of these key residues are indicated. Accordingly, substituting the vinyl group with an aromatic ring would still allow the same type of interactions between ligand and protein. We can also observe that the distance between the benzene ring (at the carbon bearing the phenolic group) of Tyr84 and the CH of the side chain of Ile43 is 9.2 Å, as measured in the model. This value is very close to the length of the branched chain in the derivatives here described,

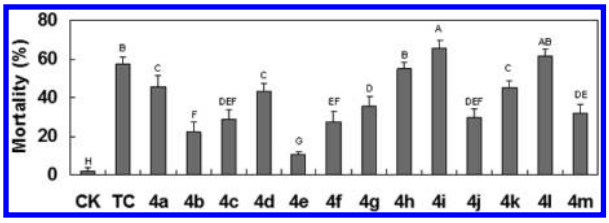


Figure 4. Aphicidal activity of (*E*)-β-farnesene analogues. The aphicidal activity of the synthesized compounds with reference to that of thiacloprid was assessed using solutions of 0.6 mg/mL, as described in Materials and Methods. Some derivatives showed insecticidal activity comparable to or even better than that of thiacloprid. The statistical analysis was performed with "Statistical Program for Social Sciences".

allowing an efficient interaction of the aromatic ring with Tyr84 and of the terminal isopropylidene group with Ile43.

Once the role of OBP3 in the detection of (*E*)-β-farnesene by the aphids is established, molecular modeling studies could provide a more detailed understanding of the interactions of OBP3 with (*E*)-β-farnesene and its analogues. Such information would then help in designing new compounds mimicking alarm pheromones to be used as alteratives to or in combination with traditional insecticides to control aphid populations in the field.

3.4. Insecticidal Activity. We then evaluated the insecticidal activity of the synthesized compounds against the aphids *in vivo*, using the pesticide standard operation practice (SOP). In such a test, the solvent, a mixture of acetone, methanol and water (1:1:18), was used as a negative control, while a commercial aphicide, thiacloprid (TC), was used as a positive reference. The results are summarized in Figure 4. Some derivatives, such as **4h**, **4i** and **4l**, showed comparable or even better aphicidal activity than the commercial insecticide, when applied at the same concentration of 600 μg/mL.

This work demonstrates that the structure of (*E*)-β-farnesene can be modified with the addition of a pyrazole moiety without losing the affinity of (*E*)-β-farnesene to aphids OBPs and the specificity to OBP3, supposed to be responsible for perceiving the alarm message. On the other hand, the molecules of some insecticides can be modified by adding a region structurally similar to (*E*)-β-farnesene without losing their aphicidal properties. These results provide guidelines for designing and synthesizing compounds endowed with both repellent properties and aphicidal activities. Such compounds would represent more efficient insecticides, as the aphids, stimulated by the alarm message, will become more active, thus increasing their chances of coming in contact with the compounds. Therefore, we expect that in the field the same level of efficacy could be reached with lower doses of these new insecticides. In the end, such compounds, or others designed following the same strategy, could prove more environmentally friendly than thiacloprid and other classical pesticides.

Moreover, the region of the molecule structurally similar to (*E*)-β-farnesene results as more stable to oxidation when coupled to the aromatic moiety, and all the molecules are less volatile than (*E*)-β-farnesene. In fact, in our compounds the two terminal double bonds of (*E*)-β-farnesene, which greatly contribute to the instability of this molecule,[8] are replaced by a carbonyl group and an aromatic ring. The synthesized derivatives are also much less volatile than (*E*)-β-farnesene, being both larger and more polar. Therefore, our compounds are likely to remain in the aphids' environment for longer times than (*E*)-β-farnesene, thus increasing their efficacy.

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