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Synthesis of Haptens and Development of an Immunoassay for the Olive Fruit Fly Pheromone

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An enzyme-linked immunosorbent assay (ELISA) for the olive fruit fly pheromone, *Bactrocera oleae* Gmelin, was developed. The assay uses polyclonal antibodies, raised in rabbits, against (±)-β-[3-(1,7-dioxaspiro[5.5]undecane)]propionic acid, **2** (hapten I), conjugated to the KLH (keyhole limpet hemocyanin) by the carbodiimide method. A second hapten, (±)-δ-[3-(1,7-dioxaspiro[5.5]undecane)]-butylamine, **3** (hapten II), after conjugation to a biotin moiety, was used for indirect immobilization onto ELISA microwells precoated with the glycoprotein avidin. The developed ELISA method measures the synthetic olive fruit fly pheromone in concentrations ranging between 0.08 and 10 μg/mL and shows great promise for practical applications for pheromone detection in environmental and biological samples. The results obtained strongly indicate that this technique, to our knowledge the first insect pheromone enzyme-linked immunosorbent assay so far reported, is a fast, sensitive, inexpensive, and highly convenient method for the analysis of a volatile and low molecular weight compound such as 1,7-dioxaspiro[5.5]undecane, **1**.

KEYWORDS: Olive fruit fly; *Bactrocera oleae* (Gmelin); pheromones; hapten; antipheromone polyclonal antibodies; enzyme-linked immunosorbent assay (ELISA); avidin−biotin system

INTRODUCTION

The olive fruit fly, *Bactrocera oleae* Gmelin, is the major pest of olive cultivation in the Mediterranean area. The overuse of chemical insecticides for the control of this insect and the consequent damage to the environment and to public health is a serious problem. Recently, considerable efforts have been made worldwide for the development of alternative control methods which exploit biological factors (natural enemies, predators, bacteria, viruses, etc.) or biochemical factors (hormones, pheromones, etc.) to keep the insect population under control. From all these methods the most promising results are obtained by use of the insect's own pheromone by either the mass-trapping technique (*1*, *2*), using pheromone traps, or diffusion in the environment of enough pheromone to induce mating disruption (*3*). Successful application of the above methods requires a fast, convenient, and reliable analytical method for monitoring the quantity of pheromone in the dispersing systems and also in the environment. The extremely low application rate, as well as the volatility of this pheromone, requires a highly sensitive analytical method for quantitative determination in natural samples. The early applied instrumental methods of pheromone analysis (*4*) are accurate, but they are expensive, time-consuming, and need considerable effort for sample preparation before analysis. In contrast, immunoassays generally are rapid, sensitive, specific, and cost-effective.

Our efforts were directed toward the development of a competitive enzyme-linked immunosorbent assay (*5*) (ELISA) for monitoring and efficiently quantifying the pheromone of the olive fruit fly in biological and environmental samples using polyclonal antibodies raised against an immunogen mimicking the analyte in rabbits. For this reason, we were interested in the synthesis of derivatives of the pheromone of olive fruit fly, 1,7-dioxaspiro[5.5]undecane **1** (Figure 1), not only as part of a total synthesis but also as haptens to raise specific antibodies that can recognize natural pheromone and to prepare other reagents that are necessary for the development of a pheromone ELISA. Despite the large number of published syntheses of specific derivatives (*6*, *7*), there is not, to our knowledge, a report of the synthesis of monosubstituted 1,7-dioxaspiro[5.5]undecanes suitable for the development of antibodies or for the immobilization of a pheromone-mimicking antigen onto the ELISA microwells. The natural, female-generated sex pheromone of the olive fruit fly is (±)-1,7-dioxaspiro[5.5]undecane in its racemic form (*8*), identical to the synthetic pheromone that is actually in use for all applications for the control of populations

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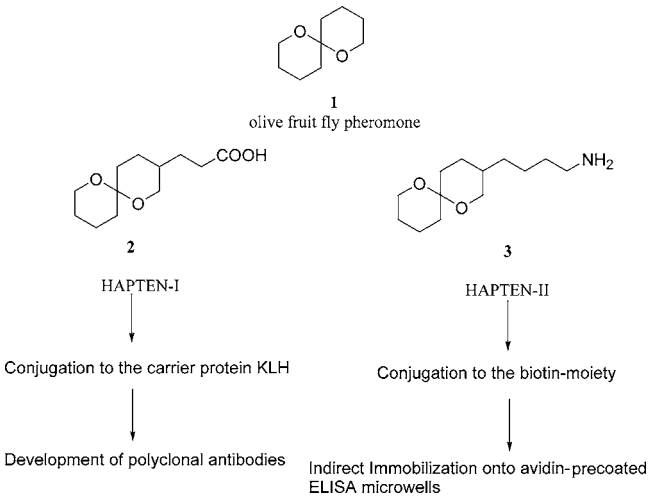

Figure 1. Racemic olive fruit fly pheromone 1,7-dioxaspiro[5.5]undecane 1, the haptens I and II, bearing a carboxyl or amino group, respectively, and their schematic use in the development of an ELISA-immunoassay for the olive fruit fly pheromone.

of this insect pest (*1*). However, it has been reported that the biological activity of each enantiomer is different. Synthetic (*R*)-(−)-1,7-dioxaspiro[5.5]undecane attracts significantly more males in laboratory and field tests, and this response coincided with the mating period of the insect, showing that this enantiomer functions as a sex attractant. (*S*)−(+)-1,7-dioxaspiro-[5.5]undecane is less potent as an attractant, with only a slight preference to females (*8*), acting rather as a tranquilizer and an aphrodisiac in the process of mating. It was then decided that the candidate derivatives should be in racemic form so that the antibodies that will be developed will recognize both enantiomers of the natural or synthetic pheromone in biological and environmental samples.

In this investigation we developed a total synthesis of two monosubstituted derivatives, **2** and **3**, of the olive fruit fly pheromone haptens I and II (Figure 1) and their conjugation to a carrier protein or to a biotin moiety, respectively. The protein conjugate of hapten I was used to raise polyclonal antibodies by immunization of rabbits. The biotinylated hapten II was used for indirect immobilization onto avidin-precoated ELISA plates. Finally, a competitive ELISA was developed and successfully applied to measurement of the synthetic pheromone. This technique will be useful for monitoring and quantifying the olive fruit fly pheromone in biological and environmental samples. To our knowledge, the reported technique is the first insect pheromone enzyme-linked immunosorbent assay so far reported.

MATERIALS AND METHODS

All reagents for hapten synthesis were of reagent grade and used as supplied. Solvents for synthesis were distilled and dried before use. TLC was performed on 0.25 mm precoated silica gel 60 F_{254} aluminum sheets and column chromatography on silica gel 60 (0.063−0.2 mm), products of Merck & Co. (Darmstadt, Germany). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and avidin were products of Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany), while sulfo-NHS-LC-biotin was a product of Pierce (Rockford, IL). The horseradish peroxidase-labeled second (goat antirabbit γ-immunoglobulin, whole molecule, peroxidase conjugate) antibody was a product of Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). (±)-1,7-Dioxaspiro[5.5]undecane, of analytical grade, was a gift from Vioryl S.A. (Athens, Greece). IR spectra were obtained on a Perkin-Elmer 7200 spectrophotometer (Perkin-Elmer, Boston, MA) in 5% CCl_4 solutions. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian Mercury 300 MHz spectrometer (Varian Inc., Palo Alto, CA) in $CDCl_3$ with TMS as the internal standard. Mass spectra were obtained on a Hewlett-Packard 5890−5970 GC-MS system 5050 (Hewlett-Packard Inc., Palo Alto, CA) equipped with a nonpolar HP-1, 12 m × 0.2 mm i.d. fused silica capillary column: Carrier gas, helium 1 mL/min; injector temperature, 230 °C; oven temperature, 50 °C (5 min isothermal) raised at 4 °C/min up to 250 °C; ion source temperature, 220 °C; interface temperature, 250 °C; mass range 40−500, amu; EI, 70 eV. ESI mass spectral analysis was performed at the "Mass Spectrometry and Dioxin Analysis Lab", NCSR "Demokritos". Test solution in 50% aqueous acetonitrile containing 1% acetic acid was infused into an electrospray interface mass spectrometer AQA Navigator, Finnigan (Bremen, Germany) at a flow rate of 0.1 mL/min using a Harvant Syringe pump. Hot nitrogen gas (Dominic-Hunter UH-PLCMS-10, U.K.) was used for desolvation. GC analyses were performed on a Varian 3600 apparatus (Varian Chromatography Systems, Walnut Creek, CA) equipped with a 30 m × 0.25 mm i.d. Carbowax 20M capillary column: carrier gas, nitrogen 3 mL/min; injector temperature, 200 °C; detector temperature FID, 300 °C; oven temperature, 50 °C (5 min isothermal) raised at 5 °C/min up to 220 °C. AEG Precision incubator (Chicago, IL), Tricontinent (Suffolk, U.K.) Multiwash II ELISA-washer, and Dynatech (Denkendorf, Germany) MR 5000 ELISA-reader were used in the ELISA experiments, which were performed on Corning 96-well microtiter plates.

Hapten Synthesis and Verification. The procedure for the synthesis of haptens I and II is shown in Figures 2 and 3. For hapten I, compounds **11** and **12** were synthesized first. For hapten II, compounds **18** and **19** were first synthesized. Detailed procedures are given below.

*Methyl (±)-β-[3-(1,7-Dioxaspiro[5.5]undec-2-ene)]propionate (**11**).* A mixture of methyl-2-methylene-5-oxopentanoate **10** (9.1 g, 0.064 mol), enol ether **6** (16.6 g, 0.169 mol), and triethylamine (1.67 g, 16.5 mmol) was heated for 3 days at 80 °C in a base-washed (KOH), oven-dried round-bottom flask. Diethyl ether (50 mL) was added to the reaction mixture; the organic phase was washed with dilute HCl (5%), saturated aqueous $NaHCO_3$, and water and finally dried over anhydrous Na_2SO_4. Removal of the solvent under reduced pressure gave a viscous residue, which was purified by column chromatography on silica gel

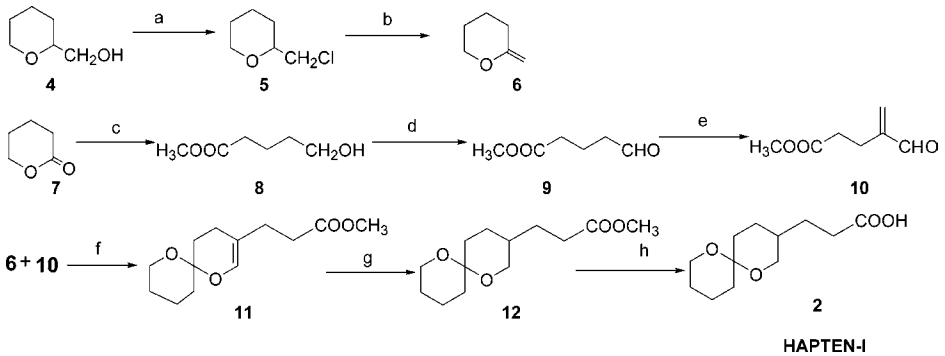

Figure 2. Synthesis of Hapten I: (a) $SOCl_2$, py, 45 °C, 8 h, 68%; (b) KOH, reflux 3.5 h, 93%; (c) conc. H_2SO_4, CH_3OH, reflux 10 h, 62%; (d) PCC in CH_2Cl_2, rt, 2 h, 81%; (e) $(CH_3)_2NH{\cdot}HCl$, HCHO 47%, 80 °C, 3 h, 45%; (f) NEt_3, 80 °C, 3 days, 20%; (g) H_2, PtO_2 in dry diethyl ether, rt, 5 h, 60%; (h) KOH in H_2O, rt, 4.5 h, 96%.

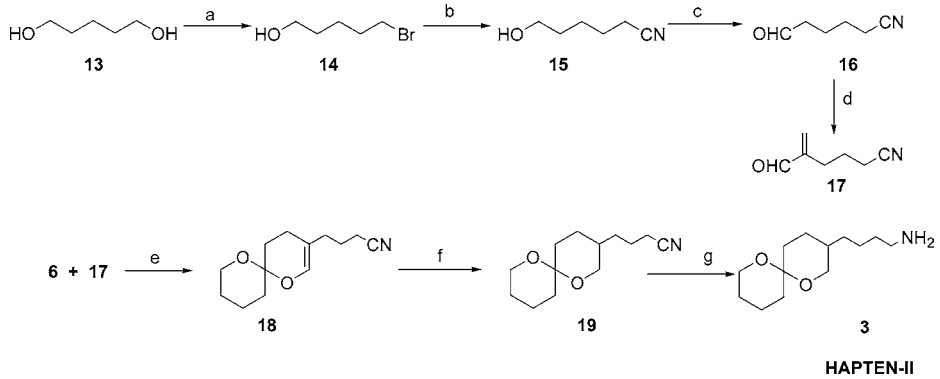

Figure 3. Synthesis of Hapten II: (a) HBr 47%, conc. H_2SO_4, reflux 4 h, 41%; (b) KCN, $Bu_4N(HSO_4)$, NaOH, H_2O, 60 °C, 4.5 h, 97%; (c) PCC in CH_2Cl_2, rt, 2.5 h, 73%; (d) $(CH_3)_2NH{\cdot}HCl$, HCHO 47%, 80 °C, 3 h, 49%; (e) NEt_3, 80 °C, 3 days, 40%; (f) H_2, Pd/C, $CHCl_3$, EtOH, rt, 2 h, 1 atm, 38%; (g) $LiAlH_4$ in diethyl ether, 0 °C, 15 min, 99%.

60 (petroleum ether:ether, 10:1) to give compound **11** (1.82 g, 20%); IR (cm^{-1}) 2946, 2853, 1742, 1670; ^1H NMR δ 1.40−1.93 (8H, m), 2.04−2.29 (4H, m), 2.33−2.43 (1H, m), 3.65 (3H, s), 3.56−3.81 (2H, m), 6.10 (1H, s); ^{13}C NMR δ 18.38, 19.74, 25.19, 28.31, 31.89, 33.14, 34.25, 51.48, 61.52, 94.83, 111.46, 135.68, 173.75; MS *m*/*z* 240 (5, M^+), 167 (8), 149 (11), 98 (100), 83 (16), 43 (29). Anal. Calcd for $C_{13}H_{20}O_4$: C, 88.57; H, 11.43. Found: C, 88.89; H, 11.46.

Methyl (±)-β-[3-(1,7-Dioxaspiro[5.5]undecane)]propionate ***(12)***. Into a stirred solution of the olefin **11** (1.82 g, 7.6 mmol) in dry ether, PtO_2 (10 mg) was added. Hydrogen was introduced by using a hydrogen-filled balloon, and the mixture was stirred for 5 h at room temperature. When the starting material was exhausted (monitored by TLC), the reaction mixture was filtered through Celite and washed with ether (2 × 5 mL), and the filtrate was concentrated under reduced pressure. The viscous residue was purified by column chromatography on silica gel 60 (petroleum ether:ether, 10:1) to afford the reduced product **12** (1.09 g, 60%); IR (cm^{-1}) 2933, 2865, 1741; ^1H NMR δ 1.3−1.95 (13H, m), 2.2−2.37 (2H, m), 3.2−3.7 (4H, m), 3.65 (3H, s); ^{13}C NMR δ 18.50, 24.81, 24.91, 27.38, 31.23, 34.75, 35.27, 35.47, 51.48, 60.27, 64.64, 94.67, 173.86; MS *m*/*z* 242 (5, M^+), 187 (11), 155 (5), 142 (45), 98 (100), 83 (29), 55 (89). Anal. Calcd for $C_{13}H_{22}O_4$: C, 64.44; H, 9.15. Found: C, 64.60; H, 9.13.

(±)-β-[3-(1,7-Dioxaspiro[5.5]undecane)]propionic Acid ***(2)***. The methyl ester **12** (1.09 g, 4.5 mmol) was added to a solution of potassium hydroxide (1.26 g, 22.5 mmol) in water (35 mL), and the mixture was stirred for 4.5 h at room temperature. This was then extracted by diethyl ether (2 × 25 mL) in order to remove any unreacted ester **12** and neutral impurities. The aqueous phase was cooled in an ice bath and acidified with HCl (5%) to pH ≈ 2. The mixture was extracted with diethyl ether and dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure to give pure compound **2** (1.05 g, 96%); IR (cm^{-1}) 2873, 1711; ^1H NMR δ 1.19−2.01 (13H, m), 2.25−2.41 (2H, m), 3.25−3.43 (2H, m), 3.52−3.71 (2H, m), 9.0 (1H, s); ^{13}C NMR δ 18.47, 24.67, 25.10, 27.12, 31.20, 34.62, 34.93, 35.41, 60.35, 64.64, 94.91, 179.08; MS *m*/*z* 228 (5, M^+), 155 (16), 128 (47), 98 (100), 83 (34), 55 (76). Anal. Calcd for $C_{12}H_{20}O_4$: C, 64.14; H, 8.83. Found: C, 63.07; H, 8.81.

(±)-δ-[3-(1,7-Dioxaspiro[5.5]undec-2-ene)]butyronitrile ***(18)***. A mixture of freshly purified compound **17** (2.62 g, 0.021 mol) and enol ether **6** (7.22 g, 0.0735 mol) and triethylamine (0.80 mg, 7.9 mmol) was heated for 3 days at 80 °C in a base-washed (KOH), oven-dried flask. The solution was diluted with ether, washed with dilute HCl (5%), saturated aqueous $NaHCO_3$, and water, and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure, and the viscous residue was purified by column chromatography (petroleum ether 40−60 °C:ether, 2:1) to give compound **18** (1.05 g, 40%); IR (cm^{-1}) 3070, 2945, 2877, 2848, 2247, 1670; ^1H NMR δ 1.47−1.91 (10H, m), 2.02−2.14 (4H, m), 2.23−2.32 (2H, m), 3.6−3.63 (1H, m), 3.72−3.77 (1H, m), 6.14 (1H, s); ^{13}C NMR δ 15.79, 18.28, 19.41, 23.10, 25.09, 31.24, 31.71, 34.23, 61.41, 94.80, 110.36, 119.62, 136.29. Anal. Calcd for $C_{13}H_{19}NO_2$: C, 70.56; H, 8.65; N, 6.33. Found: C, 70.81; H, 8.63; N, 6.30.

(±)-δ-[3-(1,7-Dioxaspiro[5.5]undecane)]butyronitrile ***(19)***. Into a solution of pure compound **18** (1.05 g, 4.75 mmol) in EtOH (50 mL), Pd/C (10 mg) and $CHCl_3$ (2 mL) were added. The mixture was hydrogenated for 2 h at atmospheric pressure and room temperature. The catalyst was filtered off and washed with diethyl ether (2 × 5 mL). The combined filtrates were concentrated at room temperature under reduced pressure. The crude product thus obtained was purified by column chromatography (petroleum ether 40−60 °C:ether, 1:1) to give compound **19** (400 mg, 38%); IR (cm^{-1}) 2946, 2872, 2250; ^1H NMR δ 1.19−1.75 (18H, m), 2.32 (2H, t, J = 8.6 Hz), 3.26−3.37 (1H, m), 3.52 (2H, m), 3.5−3.66 (2H, m). Anal. Calcd for $C_{13}H_{21}NO_2$: C, 69.92; H, 9.48; N, 6.27. Found: C, 70.16; H, 9.46; N, 6.25.

(±)-δ-[3-(1,7-Dioxaspiro[5.5]undecane)]butylamine ***(3)***. Into a three-necked flask containing anhydrous diethyl ether (40 mL), cooled by an ice−water bath, $LiAlH_4$ (500 mg, 13.12 mmol) was added slowly under nitrogen, and the mixture was stirred for 15 min. Through a dropping funnel a solution of pure saturated nitrile **19** (400 mg, 1.79 mmol) in ether (10 mL) was added dropwise. When the addition was finished, the ice−water bath was removed and the reaction mixture was stirred 15 min at room temperature. Then H_2O (1 mL) was added followed by NaOH (15%) (4 mL) and H_2O (1 mL). The white precipitate was filtered off, the organic phase was washed once with water and dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure. Pure product was collected (1.05 g, 99%): IR (film, cm^{-1}) 3366, 2935, 2871; ^1H NMR δ 1.1−1.5 (16H, m), 1.5−1.6 (1H, m), 1.6−1.7 (1H, m), 1.8−1.9 (1H, m), 2.73 (2H, s), 3.25−3.60 (2H, m), 3.63 (2H, t, J = 13.6); ESI-MS *m*/*z*: 228.2 $[M + H]^+$; Anal. Calcd for $C_{13}H_{25}NO_2$: C, 68.68; H, 11.08; N, 6.16. Found: C, 68.53; H, 11.10; N, 6.14.

Conjugation of Hapten I to the Carrier Protein KLH. The protein (10 mg) was dissolved in 0.1 N HCl (1 mL). The carboxyl derivative (±)-β-[3-(1,7-dioxaspiro[5.5]undecane)]propionic acid, **2** (hapten I) (3 mg), in water (100 μL) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) (25 mg) in water (100 μL) were added to the above solution, and the pH was immediately adjusted to 5.0. The solution was left to react under stirring for 5−6 h at room temperature and then overnight at 4 °C. The next morning the solution was dialyzed (molecular weight cut off 6000−8000 Da) against water for 48 h. After the dialysis, the solution was transferred from the dialysis membrane to a tube, appropriately diluted with saline water to a concentration calculated as 200 μg of KLH/mL, divided into 0.5 mL aliquots, and kept at −35 °C.

Immunization. Female New Zealand, 2-month old white rabbits were used for raising polyclonal antibodies. Routinely, the conjugate of hapten I to KLH dissolved in saline (quantity of conjugate corresponding to 100 μg of KLH/0.5 mL) was thoroughly emulsified with an equal volume of Freund's complete adjuvant. The emulsion was subcutaneously injected on the back of the rabbit. The first boost was given 7 weeks after the first immunization, with the following ones every 4 weeks in the same manner. On the 13th day after each boost, a blood sample was drawn from the marginal ear vein of the rabbit to check the titer of the polyclonal antibody. Boosts were given

7 times. The blood sample was centrifuged (2000*g*) and then left to stand in order to separate the antiserum from the blood cells. The antiserum was divided into 0.5 mL aliquots, an equal volume of glycerol was added to each aliquot, and the aliquots were stored at −35 °C.

Conjugation of Hapten II to Biotin. The amine derivative (±)-δ-[3-(1,7-dioxaspiro[5.5]undecane)]butylamine, **3** (hapten II) (5 mg, 0.022 mmol), in DMF (100 μL) was added to a solution of the long-chain succinimidyl ester of biotin (12.24 mg, 0.022 mmol) in DMF (100 μL). The mixture was stirred overnight at room temperature. The end of the reaction was confirmed by determination of pH (pH changed from alkaline to neutral). The crude product was dissolved in chloroform and washed twice successively with $NaHCO_3$ 5%, 0.1 N HCl, and H_2O. The solvent was then removed under reduced pressure, and the pure product was dissolved in a mixture of H_2O:EtOH, 8:2 (v/v) and lyophilized.

ELISA Titer Curve for the Antipheromone Antisera. ELISA microwells were coated with 100 μL/well of avidin 10 μg/mL in a pH 9.6 carbonate buffer and allowed to stand overnight at 4 °C. On the following day, the plate was washed twice with pH 7.4 phosphate-buffered saline (PBS) and thoroughly tapped dry. Afterward, 200 μL/well of blocking buffer [0.05% (v/v) Tween 20 in pH 7.4 PBS, which contained 2% (w/v) bovine serum albumin (BSA)] was added to each microwell, and the plate was incubated for 1 h at room temperature. The wells were then washed three times with washing buffer [0.05% (v/v) Tween 20 in pH 7.4 PBS]. Afterward, 100 μL of biotinylated pheromone (conjugate hapten II−biotin), 1 μg/mL in pH 7.4 PBS, was added to the wells. After incubation for 1 h at room temperature, the plates were washed as described above. Then 100 μL of each antipheromone antiserum diluted (1:20 000−1:1000) with dilution buffer (0.05% (v/v) Tween 20 in pH 7.4 PBS that contained 0.2% (w/v) BSA) was added to the wells, and the plate was incubated for 2 h at 37 °C. The plate was washed 3 times with washing buffer, and then 100 μL of a second antibody, enzyme-labeled (i.e., with the enzyme horseradish peroxidase, HRP), was added, and the plate was incubated for 2 h at 37 °C. Afterward, the plate was washed 3 times with washing buffer, and 100 μL of a suitable enzyme substrate [solution of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (1 mg/mL)/H_2O_2 (0.003%), in pH 4.4 citric/phosphate buffer] was added to each well. The plate was kept for 30 min at room temperature for color development, and the optical absorption was measured at 405 nm. Various blank microwells were included in each run of the ELISA titer curve in which either the avidin coating solution, or the biotinylated pheromone (conjugate hapten II−biotin), or the specific antipheromone antiserum was omitted.

ELISA Displacement Curve Using Pooled Antipheromone Antiserum. ELISA microwells were coated with 100 μL/well of 10 μg/mL avidin in a pH 9.6 carbonate buffer and kept overnight at 4 °C. The next day the plate was washed twice with pH 7.4 PBS and thoroughly tapped dry. Afterward, 200 μL/well of blocking buffer [0.05% (v/v) Tween 20 in pH 7.4 PBS which contained 2% (w/v) BSA] was added to each microwell, and the plate was incubated for 1 h at room temperature. The wells were then washed 3 times with washing buffer [0.05% (v/v) Tween 20 in pH 7.4 PBS]. Afterward, 100 μL of biotinylated pheromone (conjugate hapten II−biotin), 1 μg/mL in pH 7.4 PBS, was added to the wells, and the plate was incubated for 1 h at room temperature. The wells were then washed 3 times with washing buffer. A 50 μL amount of standard pheromone solutions, in concentrations ranging from 10 μg/mL to 20 ng/mL (or 50 μL of unknown samples for analysis) and 50 μL of specific antipheromone antiserum (pool of the antisera with best titers), diluted 1:5000 with dilution buffer [0.05% (v/v) Tween 20 in pH 7.4 PBS which contained 0.2% (w/v) BSA], was added to each microwell in triplicate, and they were incubated for 2 h at 37 °C. After washing the plate 3 times with washing buffer, 100 μL of a second antibody, enzyme-labeled (i.e., with the enzyme horseradish peroxidase), was added, and the plate was incubated for 2 h at 37 °C. The plate was washed 3 times with washing buffer, and 100 μL of a suitable enzyme substrate [solution of 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (1 mg/mL)/H_2O_2 (0.003%), in pH 4.4 citric/phosphate buffer] was added to each well. The plate was kept for 30 min at room temperature for color development, and then the optical absorption was measured at 405 nm. Various blank microwells were included in each run of the ELISA displacement curve in which either the avidin coating solution, or the biotinylated pheromone (conjugate hapten II−biotin), or the specific antipheromone antiserum was omitted. The concentration of the pheromone, which was present in the unknown samples, was determined using the standard curve of the immunoassay method.

Cross-Reactivity Studies. ELISA microwells were coated with 100 μL/well of 10 μg/mL avidin in a pH 9.6 carbonate buffer and kept overnight at 4 °C. The next day the plate was washed twice with pH 7.4 PBS and thoroughly tapped dry. Afterward, 200 μL/well of blocking buffer [0.05% (v/v) Tween 20 in pH 7.4 PBS which contained 2% (w/v) BSA] was added to each microwell, and the plate was incubated for 1 h at room temperature. The wells were then washed 3 times with washing buffer [0.05% (v/v) Tween 20 in pH 7.4 PBS]. Afterward, 100 μL of biotinylated pheromone (conjugate hapten II−biotin), 1 μg/mL in pH 7.4 PBS, was added to the wells, and the plate was incubated for 1 h at room temperature. The wells were then washed 3 times with washing buffer. Solutions of the putative cross-reacting molecules (namely, lysine, tetrahydropyran, tetrahydropyranyl-butyl ether, and 1-methyl-2-pyrrolidone) were prepared in the same way as the standard synthetic pheromone, in concentrations ranging from 10 μg/mL to 20 ng/mL. A 50 μL amount of the above solutions and 50 μL of specific antipheromone antiserum (pool of the antisera with best titers), diluted 1:5000 with dilution buffer [0.05% (v/v) Tween 20 in pH 7.4 PBS which contained 0.2% (w/v) BSA], were added to each microwell in triplicate, and they were incubated for 2 h at 37 °C. After washing the plate 3 times with washing buffer, 100 μL of a second antibody, enzyme-labeled (i.e., with the enzyme horseradish peroxidase), was added, and the plate was incubated for 2 h at 37 °C. The plate was washed 3 times with washing buffer, and 100 μL of a suitable enzyme substrate [solution of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (1 mg/mL)/H_2O_2 (0.003%), in pH 4.4 citric/phosphate buffer] was added to each well. The plate was kept for 30 min at room temperature for color development, and then the optical absorption was measured at 405 nm.

RESULTS AND DISCUSSION

Design and Preparation of Haptens. The molecule to be assayed is a low molecular weight compound (M_w 156), completely symmetrical and without a reactive group for conjugation to other molecules (e.g., immunogenic proteins), and cannot, by itself, elicit an antibody response. To elicit antibodies against this molecule, it is necessary to synthesize a derivative with a flexible arm through which it can be linked to an immunogenic carrier protein and thus used as a hapten conjugate, capable of stimulating the mammalian immune system. Design of the most suitable hapten has been considered to be the crucial step in the development of an immunochemical technique for low molecular weight analytes. In the present case the derivatives that should be ideal include the 1,7-dioxaspiro-[5.5]ketal unit, in which a linear side chain of carbons is attached opposite to the characteristic spiroketal center of the molecule. This side chain should end with a suitable functionality, such as a free carboxyl or amino group. These groups are convenient for the binding of the hapten to a protein, so that the final conjugate could be used as immunogen. On the other hand, these functional groups are also suitable for the relevant conjugation of the corresponding hapten to an activated biotin moiety, so that the final biotinylated hapten could be indirectly immobilized onto avidin precoated ELISA plates. It is worth noting that the carboxyl or amino group should be situated on the free end of the chain so as to avoid stereochemical hindrance that may interfere with the development of specific antibodies against the pheromone part or with avidin recognition, respectively. Two such haptens have been designed and prepared: (±)-β-[3-(1,7-dioxaspiro-[5.5]undecane)]propionic acid, **2** (hapten I), to be used for the development of an immunogen, and (±)-δ-[3-(1,7-

dioxaspiro-[5.5]undecane)]butylamine, **3** (hapten II), to be used as a plate-coating antigen (Figure 1).

The key intermediate for the synthesis of derivative **2** is the 5.5-spiroketal **11**, which derives from a hetero-Diels−Alder reaction (*9*) between the α-methylene-aldehyde **10** and the enolether **6**. Synthesis of the reactants **6** and **10** is depicted in Figure 2. 2-Methylenetetrahydropyran **6** was prepared according to the literature (*9, 10*), with a yield of 64% from tetrahydropyran-2-methanol **4**. Methyl 4-methylene-5-oxopentanoate **10** was prepared from δ-valerolactone by refluxing the δ-valerolactone in methanol in the presence of sulfuric acid to give the methyl 5-hydroxypentanoate **8** in 62% yield (*11*). Oxidation of **8** by pyridinium chlorochromate in methylene chloride gave methyl 5-oxopentanoate **9** in 81% yield. Mannich reaction (*12*) of methyl 5-oxopentanoate **9** with formaldehyde and dimethylammonium chloride gave **10** in 45% yield, after purification by column chromatography. Under these conditions, no methylenation to the α-position of the ester has been observed (*13*). During the hetero-Diels−Alder reaction (*9*) between compounds **6** and **10** to the product **11** (Figure 2), the propensity of the exo double bond of the enol ether **6** to undergo facile isomerization to the endo position (*14*) is a major problem. It has been found (*9*) that this isomerization was suppressed if the cycloaddition occurred at 80 °C in the presence of triethylamine. Under these conditions, in 3 days the unsaturated spiroketal **11** was obtained in 20% yield. Hydrogenation of **11** in dry ether and PtO_2 as catalyst led to the saturated spiroketal ester **12,** and subsequent saponification gave the desired acid derivative **2** (hapten I) in good yield and excellent purity.

Hapten II was prepared in a similar way from the key 5.5-spiroketal **18**, which was obtained by a hetero-Diels−Alder reaction (*9*) between enolether **6** and α-methylene aldehyde **17**. The latter was prepared from 1,5-pentanediol **13** by refluxing 1,5-pentanediol with hydrobromic acid in toluene in the presence of sulfuric acid to give 5-bromopentanol **14** in 41% yield (*15*). Reasonably pure bromopentanol was obtained by simple Kugelrohr distillation of the crude reaction mixture. Reaction of the bromopentanol **14** with KCN in water in the presence of $Bu_4N(HSO_4)$ as a phase-transfer catalyst gave the corresponding hydroxynitrile **15** in 97% yield after purification by flash chromatography. Oxidation (*11*) of **15** by pyridinium chlorochromate in methylene chloride gave 5-cyanopentanal **16** in 73% yield. A Mannich reaction (*12*) on **16** gave 2-methylene-5-cyanopentanal **17** in 49% yield. Diels−Alder reaction (*9*) of **17** with **6** in the presence of triethylamine for 3 days at 80 °C led to the unsaturated nitrile derivative of spiroketal **18** in 40% yield. Reduction of the double bond of the unsaturated spiroketal **18**, under the same conditions as previously (PtO_2 in diethyl ether), gave a complex mixture of products, none of them having the desired structure. This is due to the presence of the nitrile group, which can be reduced to imine (*16*), giving many byproducts. Several catalysts were tested under various conditions, and the best results were obtained when hydrogenation was carried out in ethanol in the presence of Pd/C as catalyst and a small amount of $CHCl_3$ (*17, 18*) at room temperature. Under these conditions, reduction of the double bond left the nitrile and ketal groups intact. Thus, the saturated nitrile **19** was obtained, after column chromatography, in 38% yield. Finally, reduction of the cyano group of the nitrile **19** by $LiAlH_4$ (*19*) in diethyl ether gave the amine **3** (hapten II) in 99% yield.

Conjugation of Hapten I to the Carrier Protein KLH for Preparation of the Immunogen. The spiroketal carboxyl derivative **2** was used as a specific hapten for the preparation of a suitable immunogen by conjugation with the carrier protein KLH (keyhole limpet hemocyanin). The conjugation reaction was performed according to the well-established and widely used carbodiimide method (*20*). The conjugation protocol followed is based on a similar one described by Chard (*21*) which uses hapten and carbodiimide in great excess (10 times, 0.013: 0.13mol) compared to the carrier protein. The extent of conjugation was estimated using the conjugate formed in the developed ELISA and found to be over 1000 molecules of haptens per molecule of KLH (M_w 2 500 000). For comparison reasons, the extent of conjugation to another conjugate of the same hapten with a different carrier protein as the ovalbumin, prepared under the same conditions, was analyzed and found to be also very high, about 50 molecules of hapten per molecule of ovalbumin (M_w 42 000). The KLH conjugate was subsequently used for immunizing (*22*) New Zealand white rabbits, affording specific antipheromone polyclonal antibodies.

Conjugation of Hapten II to Biotin for Preparation of the Coating Antigen. In this work the "avidin−biotin technology" was used for the indirect immobilization of the pheromone hapten II onto ELISA plates so as to eliminate any nonspecific binding of the antibodies due to the well-known "bridge recognition" effect (*23*). The amine derivative **3** was appropriately biotinylated using an active biotin ester as biotinylating reagent (*24, 25*) and then indirectly immobilized onto ELISA microwells that had been precoated with the glycoprotein avidin. Avidin recognizes and binds biotin and biotinylated molecules with high specificity and great chemical affinity (*26*). This derivative has a different active group than hapten I, which was used for the antibody production, so as to avoid interference of any similarity in the subsequent conjugation chemistry, which might influence the assay characteristics. The longer arm of hapten II, compared to the arm of hapten I, was necessary in order to keep the pheromone moiety adequately away and free from the biotin moiety and, thus, available for antibody binding.

Evaluation of the Antipheromone Antisera Using ELISA Titer Curve. Seven different antisera, corresponding to seven consecutive bleedings, were tested as described in the Materials and Methods section. According to the obtained results, the titers ranged from 1:3000 to 1:15 000. A pool of selected antisera, showing the highest titer values, was used in the ELISA displacement curve experiments (titer of the pooled antiserum, evaluated in the same ELISA system = 1:10 000).

ELISA Displacement Curve. The parameters that might affect the ELISA displacement curve were investigated, including concentration of the avidin coating solution, concentration of the biotinylated hapten II, temperature, and incubation time with the antipheromone antiserum. The optimal conditions found are as follows: Avidin coating concentration, 10 μg/mL; pH of coating buffer, 9.6; hapten II−biotin conjugate concentration, 1 μg/mL; incubation of avidin-coated ELISA microwells with hapten II−biotin conjugate, 1 h, rt; maximum % DMF present in the standard solutions, 0.5; incubation with specific antibody, 2 h, 37 °C, 1:10 000; incubation with second antibody (HRP-labeled), 2 h, 37 °C, 1:3000. A typical ELISA displacement curve using standard solutions of pure synthetic olive fly pheromone **1** is shown in Figure 4.

Cross-Reactivity Studies. Various substances that might cross-react with the developed antibodies were tested as putative cross-reacting molecules including amino acids, such as lysine (which is the main amino acid of the protein−immunogenic carrier, through which hapten I is conjugated to the protein), and simple heterocyclic compounds, such as tetrahydropyran, tetrahydropyranyl−butyl ether, and 1-methyl-2-pyrrolidone, which are structurally related to the natural pheromone. Ac-

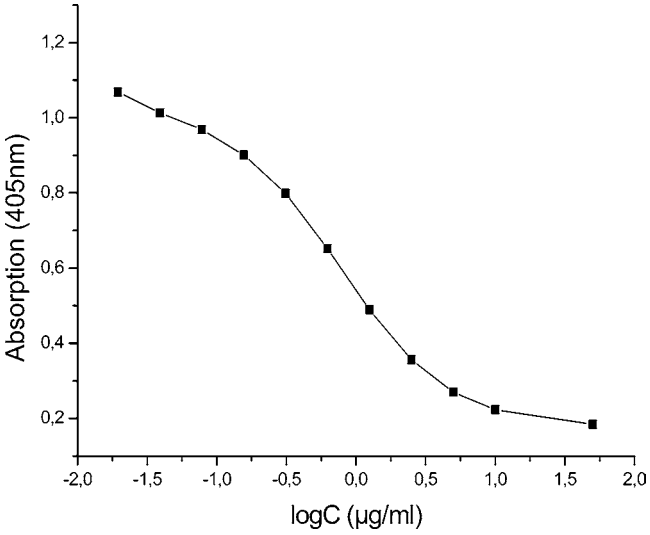

Figure 4. Typical displacement curve of the ELISA-immunoassay developed for the olive fruit fly pheromone. The curve was obtained, after indirect immobilization of the biotinylated hapten II onto ELISA microwells, precoated with avidin, and subsequent incubation with antipheromone antiserum and standard solutions of pure synthetic olive fruit fly pheromone **1** at increasing concentrations.

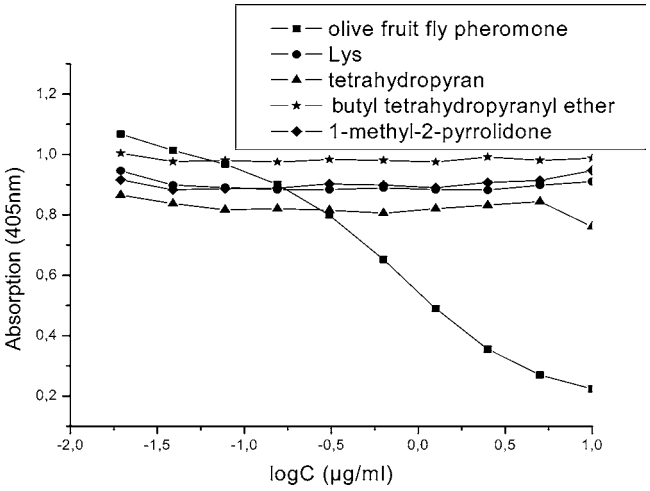

Figure 5. Cross reactivity studies with Lys (●), tetrahydropyran (▲), butyl tetrahydropyranyl ether (★), and 1-methyl-2-pyrrolidone (◆). Olive fruit fly pheromone (■) was used as control material.

cording to the results obtained, none of the above substances cross-reacts with the antibodies developed (Figure 5).

Finally, to evaluate the immunoassay accuracy of the developed ELISA, blind spiked samples were measured using pure synthetic olive fruit fly pheromone **1** in a range of concentrations from 150 to 1500 ng/mL. The results indicate good accuracy of the method, since the measured values match the spiked concentrations very well and the recovery values were close to 100%.

In conclusion, the synthesis of two derivatives of the olive fruit fly pheromone, the carboxyl derivative **2** (hapten I) and the amine derivative **3** (hapten II), has been achieved. Hapten I was conjugated to a carrier protein for the preparation of a suitable immunogen and successfully used to raise polyclonal antibodies by immunization of rabbits. Hapten II, after conjugation to a biotin moiety, was used for indirect immobilization onto ELISA microwells precoated with the glucoprotein avidin. The antibodies, developed against hapten I−KLH and the biotinylated hapten II, were used for the development of the olive fruit fly pheromone ELISA. The presented ELISA constitutes a simple, fast, and accurate analytical tool which specifically recognizes the pheromone of the olive fruit fly. Further research is under way for the determination of the olive fruit fly in biological and environmental samples. To our knowledge this is the first insect pheromone enzyme-linked immunosorbent assay so far reported, which will be of valuable help to the application of alternative control methods against the olive fruit fly, *B. oleae* Gmelin.

ABBREVIATIONS USED

DMF, *N*,*N*-dimethylformamide; EDC, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride; ELISA, enzyme-linked immunosorbent assay; ESI-MS, electron spray ionization-mass spectrometry; HRP, horseradish peroxidase; KLH, keyhole limpet hemocyanin; PBS, phosphate-buffered saline; rt, room temperature; TMS, trimethylsilane.

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Supporting Information Available: Simple experimental procedures for compounds **5**, **6**, **8**−**10**, and **14**−**17**, spectra of compounds **10**, **11**, **2**, **17**, **18**, **19**, and **3**, and a table with the main features of the ELISA displacement curve are included. This material is available free of charge via the Internet at http://pubs.acs.org.

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