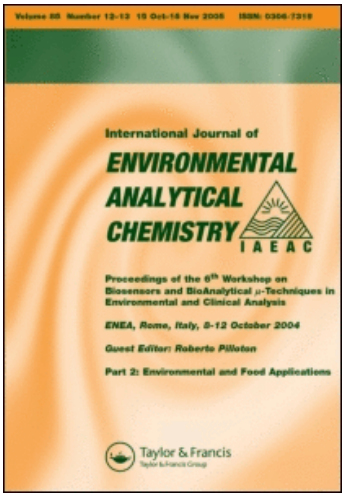

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Production of antibodies from chicken egg yolk to phenyl-*N*-methylcarbamate insecticides for analytical purposes

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With the aim of developing polyclonal antibodies binding as many phenyl-*N*-methylcarbamate insecticides (PNMCs) as possible, IgY antibodies were produced in laying hens. Two haptens (3-(2,6-dimethyl-4-(methylcarbamoyl)phenylaminocarbonyl)propanoic acid and 4-((2,6-dimethyl-4-(methylcarbamoyl)phenylaminocarbonyl)-3,3-dimethyl)butanoic acid) were synthesized preserving the major structural features of PNMCs, by a novel synthetic pathway. These haptens differed only in the spacer arm incorporated. Immunizing antigen and coating antigen were prepared by coupling the first hapten with bovine serum albumin and the second with thyroglobulin, from porcine thyroid glands, respectively. The titre and maturation increase in the developed antibodies, in the egg yolk, were assessed by non-competitive ELISA. Avidity and cross-reactivity of the antibodies with selected pesticides were estimated by means of competitive ELISA. The produced IgYs exhibited a high binding capacity to carbaryl, trimethacarb, metolcarb, aminocarb, and promecarb. These antibodies can be used for immunosorbent preparation for analytical purposes.

Keywords: IgY; Antibodies; Pesticides; Phenyl-*N*-methylcarbamates; 3-(2,6-Dimethyl-4-(methylcarbamoyl)phenylaminocarbonyl)propanoic acid; 4-((2,6-Dimethyl-4-(methylcarbamoyl)phenylaminocarbonyl)-3,3-dimethyl)butanoic acid

1. Introduction

Phenyl-*N*-methylcarbamates (PNMCs) comprise a group of about 20 compounds. They are the most important and largest subgroup of methylcarbamate insecticides, which were introduced to the market in the 1950s. Most of these substances have wide-range insecticidal activity, and they are used as sprays inside houses, as much as in gardens and fields for crop protection. Their insecticidal activity is attributed to reversible carbamoylation, thus inhibition, of the enzyme acetycholinesterase, resulting in acetylcholine accumulation across the synapse. This effect prevents efficient nervous coordination, causing convulsions and finally death [1–3]. Modern widely used PNMCs

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are fairly selective for insects' acetylcholinestarase, having a relatively low toxicity to mammals.

Their chemical structure can be generally described as a benzene aromatic ring substituted with the *N*-methylcarbamate group (–O–CO–NH–CH_3, ring position 1) and several other groups in *ortho*, *para*, and *meta* positions (figure 1 and table 1). Their *N*-methyl group can be easily hydrolysed in alkaline media or temperatures over 40°C, so their persistence in the environment is relatively short. PNMCs and many of their metabolites, derived from chemical and biological degradation, have become targets for analytical chemists in water, soil, plants, and air [4, 5]. They are scarcely directly determined using gas chromatography (GC), because of their thermal lability, and there are very few GC methods, involving hydrolysis and derivatization [6, 7]. On the contrary, there are numerous analytical methods, based on high-performance liquid chromatography (HPLC), using either direct UV detection [8, 9] or transformation to appropriate derivatives, following fluorescence detection [10, 11]. HPLC coupled with mass detection is an especially efficient technique, applied in more recent methods [12, 13]. Moreover, other techniques such as immunoassays [14] and biosensors [15] have been developed for their determination.

In the early 1990s, the isolation of antibodies from the egg yolk of previously immunized chickens gained exceptional scientific interest. Since then, they have been used for diagnostic and analytical purposes. This type of antibody (avian IgY, corresponding to mammals IgG) exhibits several remarkable features: it is more resistant to temperature, ionic strength, and pH of the environment than IgG; it does not bind to the rheumatoid factor or proteins A and G; and it does not activate mammalian complement. It has a molecular mass of about 180 kDa and an isoelectric point of 5.7–7.6, and its isolation from egg yolk is fairly easy. Moreover, production of antibodies from laying hens has several particular advantages: a single hen can substitute up to 12 rabbits in antibody production over 1 year, since IgY quantities

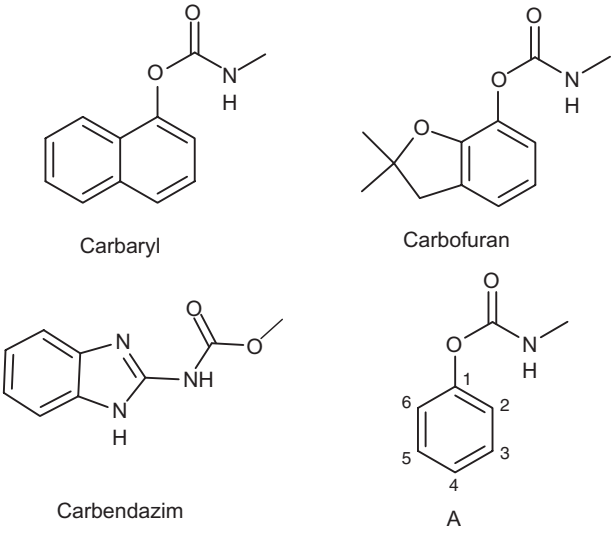

Figure 1. Structures of carbamate pesticides mentioned in table 1; carbofuran and carbaryl are *N*-methyl carabamate acaricides of great commercial interest; carbendazim is a benzimidazolylcarbamate fungicide.

Table 1. Chemical structures of common carbamate pestcides and percentage cross-reactivity values.

Chemical name	Substituents	Cross-reactivity (%)
Carbaryl	–	300
Carbofuran	–	IN[a]
Aminocarb	4-Dimethylamino-3-methyl-[b]	100
Bufencarb	3-(Pentan-3-yl)- or 3-(pentan-2-yl)-[b]	NA[c]
Butacarb	3,5-Di-tert-butyl-[b]	NA
Carbanolate	2-Chloro-4,5-dimethyl-[b]	NA
Dioxacarb	2-(1,3-Dioxolane-2-yl)-[b]	IN
Ethiofencarb	2-(Ethylthio)methyl-*	IN
Fenobucarb	2-Sec-butyl-[b]	IN
Isoprocarb	2-Isopropyl-[b]	NA
Methiocarb	3,5-Dimethyl-4-methylthio-[b]	NA
Metolcarb	3-Methyl-[b]	115
Mexacarbate	4-Dimethylamino-3,5-dimethyl-[b]	NA
Promecarb	3-Isopropyl-5-methyl-[b]	75
Propoxur	2-Isopropoxy-[b]	IN
Trimethacarb	2,3,5-Trimethyl- or 3,4,5-trimethyl-[b]	167
XMC	3,5-Dimethyl-[b]	NA
Xylylcarb	3,4-Dimethyl-[b]	NA
Compound **4** (figure 2)	4-Amino-3,5-dimethyl-[b]	1670
Carbendazim	–	IN

[a]IN: insignificant.
[b]Substituents of structure **A** presented in figure 1, common in all PNMCs.
[c]NA: not assessed.

isolated from the yolk are huge, compared with IgG amounts in serum. The unpleasant and painful bleeding procedure of the immunized animal is replaced by harmless egg collection and antibody extraction from their yolks. Besides, keeping laying hens indoors or outdoors for antibody production does not incur any particular difficulty, compared with other types of laboratory animal maintenance [16].

Our study involves the production of IgY polyclonal antibodies binding to phenyl-*N*-methylcarbamate insecticides. The antigen used for immunization was produced by coupling a properly designed hapten (figure 2, **hapten 1**) to bovine serum albumin (BSA). Moreover, cross-reactivity of the antibodies to several PNMCs was measured, in order to assess their applicability in pesticide analytical procedures.

2. Experimental

2.1 *Reagents, buffers, equipment, and animals*

All standards of PNMCs were from Riedel-de-Haën (PESTANAL, Sigma-Aldrich). Stock solutions of the pesticides and compound **4** were prepared in dry *N*,*N*-dimethylformamide (DMF) at a concentration of 0.02 mmol mL^{-1} and stored at −20°C. Thyroglobulin, from porcine thyroid glands (THR), and albumin bovine Fraction V (BSA) were obtained from Fluka and Sigma, respectively. All inorganic reagents used were of pro-analysis grade and purchased from Merck. Triphosgene (99%), 3,5-dimethylphenol, sodium dithionite, methylamine hydrochloride, succinic anhydride, 3,3-dimethylglutaric anhydride, sulfanilic acid, hydrogen peroxide

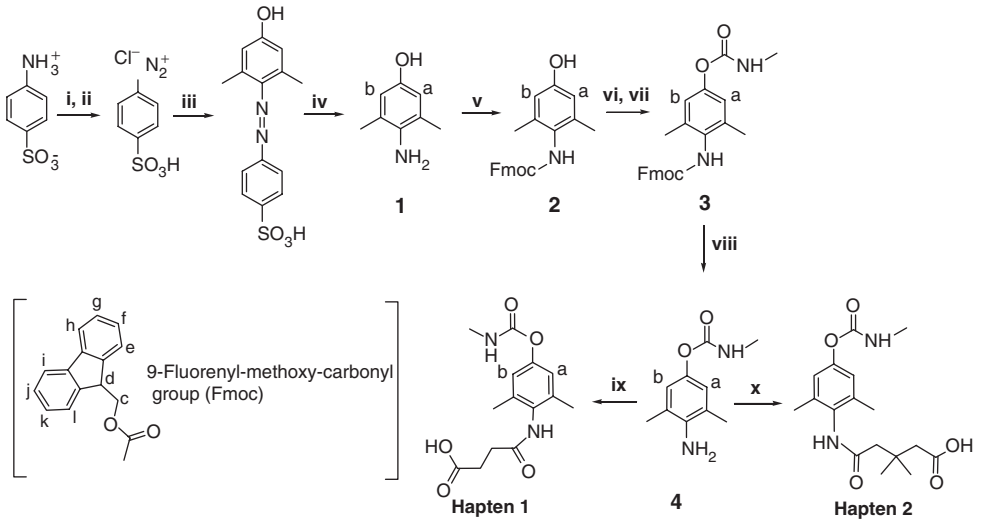

Figure 2. Reaction scheme of the synthesis of haptens and antigens: (i) Na_2CO_3, (ii) $NaNO_2$, HCl, (iii) 3,5-dimethylphenol in 2.5 M NaOH, (iv) $Na_2S_2O_4$, (v) Fmoc-*N*-hydroxysuccinimide, anhydrous CH_3CN, (vi) triphosgene, DIPEA, anhydrous DMF, (vii) methylamine hydrochloride, DIPEA, (viii) 20% (v/v) piperidine in anhydrous CH_2Cl_2, (ix) succinic anhydride, DMAP, anhydrous CH_2Cl_2, and (x) 3,3-dimethylglutaric anhydride, anhydrous CH_2Cl_2.

30% (w/w), and *o*-phenylenediamine (OPD) tablets were obtained from Acrõs. Dichloromethane, benzene, acetonitrile, ethyl acetate, dimethylsulfoxide (DMSO), propanone, DMF, diisopropylethylamine (DIPEA), piperidine, trifluoroacetic acid (TFA), triethylamine, isobutylchloroformate, PD-10 columns, trinitrobenzene sulfonic acid (TNBS, 5% (w/w) solution in water), 1-ethyl-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), Freund's complete and incomplete adjuvants, Tween-20, and 4-dimethylaminopyridine (DMAP) were from Sigma (Sigma-Aldrich). Fmoc-*N*-hydroxysuccinimide was from CBL (Greece). Tetrahydrofuran (THF) was purchased from Sigma-Aldrich and always distilled prior to use to remove the stabilizer. Solvents were dried over A4 molecular sieves except THF, which was dried by distillation over sodium.

Thin-layer chromatography (TLC) was run on commercially available plates of silica on aluminium sheets (Merck). Silica gel 60 (Merck) was used for column chromatography. ^{1}H NMR were recorded with a Bruker DPX Avance spectrometer (400 MHz for ^{1}H). Chemical shifts (δ) are expressed in ppm downfield from tetramethylsilane. Splitting patterns were designated as s, singlet; d, doublet; t, triplet; q, quartet; and m, multiplet. A Waters Micromass ZQ LC-MS system, with Electospray ionization operated in positive mode, was used for mass-spectra acquisition.

Phosphate-buffer saline (PBS), pH 7.3 (without adjustment), contains 0.1 M NaCl, 0.008 M Na_2HPO_4 and 0.002 M NaH_2PO_4. Citrate buffer, pH 5 (adjustment with NaOH), contains 0.1 M citric acid. Carbonate buffer, pH 8.5 (adjustment with HCl), contains 0.1 M $NaCO_3$ and 0.15 M NaCl. PBS-Tween contains 0.1% Tween-20. Glycine buffer pH 2.5 (adjustment with HCl) contains 0.1 M glycine and 2% Tween-20. Polysterene microtitre plates (high binding) were obtained from Greiner (Bioline, Greece). Affigel-10 was from BioRad. Water was deionized and double-distilled

before use. Absorbances of ELISA plates were read in an Emax Precision microplate reader (Molecular Devices).

Lowman brown laying hens were purchased from a local breeder. They were kept individually to properly constructed cages with nest boxes and were fed a laying-hen diet (antibiotic- and pesticide-free), crushed oyster shells, and water *ad libitum*.

2.2 *Hapten synthesis*

2.2.1 4-Amino-3,5-dimethylphenol (1). Sulfanilic acid (5.19 g, 30 mmol) and 1.8 g (17 mmol) of sodium carbonate were dissolved in 50 mL of hot water. Then, the mixture was cooled at 15°C, and 2.1 g (30 mmol) of sodium nitrite was slowly added while stirring. The mixture was transferred to a flask, containing 6 mL of 37% (w/w) hydrochloric acid and 30 g of ice. The mixture was left in an ice bath for 20 min, and precipitation of a slightly red solid was observed. 3,5-Dimethylphenol (3.6 g, 30 mmol) was dissolved in 20 mL of hot 2.5 M sodium hydroxide, and the solution was slowly added to the above cold mixture. The reaction mixture was stirred at room temperature (RT) for 1 h. During the addition, a vivid red solid was formed. The solid was dissolved by heating at 55°C, and 22.8 g (130 mmol) of sodium dithionite was added, in small portions, until the solution's colour turned to yellow. The mixture was cooled at RT, and the precipitated product was isolated by filtration to yield 3.5 g of crude chestnut solid. Recrystallization from benzene yielded 1.1 g of 4-amino-3,5-dimethylphenol. ^{1}H NMR (DMSO-d_6) $\delta = 1.98$ (s, 6 H, Ar-CH_3, figure 2), 3.85 (s, 2 H, Ar-NH_2), 6.26 (s, 2 H, H_a, H_b), and 8.16 (s, 1 H, Ar-OH). ESI-MS (CH_3CN) $m/z = 138$ [$M + H^+$].

2.2.2 Fmoc-4-amino-3,5-dimethylphenol (2). **1** (0.1 g, 0.7 mmol) and 0.25 g (0.7 mmol) of Fmoc-*N*-hydroxysuccinimide were dissolved in 5 mL of anhydrous acetonitrile. The reaction mixture was stirred at RT for 5 days, and the product (white solid) was isolated by centrifugation (2800 *g*, 10 min). The crude product was washed with acetonitrile, dried under vacuum, over P_2O_5, to yield 0.14 g of Fmoc-4-amino-3,5-dimethylphenol. ^{1}H NMR (CH_3OH-d_4) $\delta = 2.04$ (s, 6 H, Ar-CH_3, figure 2), 4.13–4.19 (m, 1 H, H_d), 4.44 (d, 2 H, H_c), 6.39 (s, 2 H, H_a, H_b), 7.23 (t, 2 H, H_f, H_k), 7.31 (t, 2 H, H_g, H_j), 7.62 (d, 2 H, H_e, H_l), 7.72 (d, 2 H, H_h, H_i). ESI-MS (CH_3CN) $m/z = 360$ [$M + H^+$].

2.2.3 Fmoc-4-amino-3,5-dimethylphenyl methylcarbamate (3). Triphosgene (0.04 g, 0.1 mmol) was dissolved in 1 mL of anhydrous THF under argon, and the solution was stirred in an ice bath. About **2** (0.08 g, 0.2 mmol) was dissolved in 2 mL of anhydrous THF, and 0.043 mL (0.2 mmol) of DIPEA was added. This solution was slowly added to the cold triphosgene solution, and the reaction mixture was stirred for 30 min at 0°C, then for 2 h at RT. The mixture was cooled again at 0°C, then 0.075 g (1.1 mmol) of methylamine hydrochloride and 0.31 mL (1.8 mmol) of DIPEA were added, and the reaction mixture was stirred for 30 min at 0°C and overnight at RT. The white solid ($DIPEAH^+Cl^-$) was removed by centrifugation (2800 *g*, 10 min) and washed with THF. All washings were collected together with the supernatant and concentrated under vacuum. The residual solid was dissolved in the minimum volume of propanone

and recrystallized from water. The product was further purified by column chromatography (silica) eluted with dichloromethane : ethyl acetate (7 : 1 v/v). Yield: 0.05 g of Fmoc-4-amino-3,5-dimethylphenyl methylcarbamate. ^{1}H NMR (CH_3OH-d_4) $\delta = 2.11$ (s, 6 H, Ar-CH_3, figure 2), 2.73 (s, 3 H, NH-CH_3), 4.19 (t, 1 H, H_d), 4.45 (d, 2 H, H_c), 6.71 (s, 2 H, H_a, H_b), 7.24 (t, 2 H, H_f, H_k), 7.32 (t, 2 H, H_g, H_j), 7.64 (d, 2 H, H_e, H_l), 7.73 (d, 2 H, H_h, H_i). ESI-MS (CH_3CN) $m/z = 417$ [M + H$^+$], 833 [2M + H$^+$].

2.2.4 4-Amino-3,5-dimethylphenyl methylcarbamate (4). About **3** (0.07 g, 0.2 mmol) was dissolved in 2 mL of 20% (v/v) piperidine (large excess) in anhydrous dichloromethane. The reaction was stirred for 1 h at RT. The mixture was concentrated under vacuum, and the crude product was further purified by column chromatography (silica) eluted with dichloromethane : acetonitrile (3 : 1 v/v). Yield: 0.0021 g of 4-amino-3,5-dimethylphenyl methylcarbamate. ^{1}H NMR ($CHCl_3$-d_1) $\delta = 2.16$ (s, 6 H, Ar-CH_3, figure 2), 2.87 (d, 3 H, NH-CH_3), 3.48 (s, 2 H, Ar-NH_2), 4.86 (s, 1 H, NH-CH_3), 6.71 (s, 2 H, H_a, H_b). ESI-MS (CH_3CN) $m/z = 195$ [M + H$^+$], 389 [2 M + H$^+$], 138 [M-57 + H$^+$].

2.2.5 3-(2,6-Dimethyl-4-(methylcarbamoyl)phenylaminocarbonyl)propanoic acid (hapten 1). About **4** (0.018 g, 0.09 mmol), 0.010 g (0.1 mmol) of succinic anhydride, and 0.001 g (0.01 mmol) of DMAP were dissolved in 1 mL of anhydrous dichloromethane. The reaction mixture was stirred under reflux for 3 h. The precipitated white solid (product) was isolated by filtration and washed with dichloromethane. It was then dried under vacuum, over P_2O_5, yielding 0.024 g of product. ^{1}H NMR (DMSO-d_6) $\delta = 2.09$ (s, 6 H, Ar-CH_3, figure 2), 2.54 (t, 4 H, HOOC–CH_2–CH_2–CO), 2.64 (d, 3 H, NH–CH_3), 6.77 (s, 2 H, H_a, H_b), 7.58 (q, 1 H, NH–CH_3), 9.20 (s, 1 H, Ar-NH–CO), 12.16 (s, 1 H, COOH). ESI-MS (CH_3CN) $m/z = 295$ [M + H$^+$], 317 [M + Na$^+$], 277 [M-18 + H$^+$].

2.2.6 4-((2,6-Dimethyl-4-(methylcarbamoyl)phenylaminocarbonyl)-3,3-dimethyl) butanoic acid (hapten 2). Two milligrams (0.01 mmol) of **4**, 1.6 mg of 3,3-dimethylglutaric anhydride (0.011 mmol), and 0.1 g (0.001 mmol) of DMAP were dissolved in 0.5 mL of anhydrous dichloromethane. The reaction mixture was stirred under reflux for 3 h. Then, the reaction mixture was concentrated under vacuum and redissolved in 1 mL of methanol. As was found by LC/MS, **hapten 2** was the only product present, contaminated by small amounts of unreacted starting materials and the catalytic amount of DMAP. A small volume (50 µL) of the solution in methanol of the crude compound was injected to ESI-LC/MS. The product was eluted at 11.2 min from the column (Waters Symmetry C18, particle size: 5 µm, internal diameter: 4.6 mm, length: 150 mm) by a linear gradient (5–100% acetonitrile in 35 min), at a flow rate of 1 mL min^{-1}. Water and acetonitrile, which made up the mobile phase, contained 0.08% TFA. ESI-MS m/z 338 (M + H$^+$), 673 (2M + H$^+$). Thus, the crude compound was used for the preparation of the coating conjugate, without any further purification.

2.3 *Preparation of immunizing and coating conjugates (antigens)*

2.3.1 Immunizing conjugate. Hapten 1 was covalently attached to BSA using the mixed anhydride method [17]. Hapten 1 (1.6 mg, 5.4×10^{-3} mmol) was dissolved in

anhydrous DMF. Triethylamine (1.5 μL, 10.8×10^{-3} mmol) and 1.4 μL (10.8×10^{-3} mmol) of isobutylchloroformate were added, and the reaction mixture was agitated for 5 min at RT. Then, the mixture was slowly added to 15 mg of BSA, dissolved in 0.3 mL of carbonate buffer, pH 8.5. The new mixture was stirred for 30 min at RT. The conjugate was purified by gel filtration on PD-10 columns using PBS as eluent. Conjugation was confirmed, and substitution was measured by the TNBS method [18]. Fifty-three per cent of the lysine ε-amino groups of BSA were covalently attached to **hapten 1**. The concentration of the protein in the conjugate solution was assessed using the Folin–Lowry method, 1700 μg mL^{-1}.

2.3.2 Coating conjugate. **Hapten 2** was covalently attached to THR, using the active ester method [19] and the water-soluble EDC. The solution of **hapten 2** of methanol (see section 2.2.6) was concentrated under vacuum and redissolved in 2 mL of DMSO. A small amount, 0.1 mL, of this solution was slowly added to 5 mg of THR, dissolved in 0.5 mL of PBS. Then, 0.13 mL of a 0.15 mmol mL^{-1} solution of EDC (0.02 mmol) in PBS was added, and the reaction mixture was stirred for 2.5 h at RT. The conjugate was purified by gel filtration on PD-10 columns, using PBS as eluent. Conjugation was confirmed, and substitution was measured by the TNBS method. Sixty-one per cent of the lysine ε-amino groups of THR were covalently attached with **hapten 2**. The concentration of the protein in the conjugate solution was assessed with the Folin–Lowry method, 2150 μg mL^{-1}.

2.4 *IgY antibody generation*

Immunizations were carried out intramuscularly, to two Lowman Brown Laying hens, about 1 year old. In particular, each hen was initially injected with 1 mL of 1 : 1 (v/v) emulsion of the conjugate solution with Freund's complete adjuvant. Approximately 500 μg of the antigen was injected into multiple sites of the pectoral muscle and the muscles of the legs of each hen. Subsequently, three boosts were given after 21, 86, and 117 days, respectively. In each boost, about 300 μg of the antigen in 0.5 mL PBS solution was emulsified with the same volume of Freund's incomplete adjuvant and administered to the animal.

Eggs were collected, marked, and cracked daily. The egg yolk was separated and washed thoroughly with deionized water. Then, the yolk was transferred to an appropriate vial, diluted fourfold with double-distilled water and left at 4°C overnight. Afterwards, each yolk mixture was centrifuged (8900 *g*, 25 min, 4°C) and the supernatant was collected, split into 2 mL aliquots, and stored at −20°C until use. IgY solutions derived from aliquots thawed and diluted thousand-fold were applied for monitoring the antibody titre increase and changes in IgY binding activity. The titre assessment was performed according to the non-competitive ELISA protocol described below. Moreover, the IgY solution corresponding to the egg collected 126 days after the initial immunization (highest titre) was used in competitive ELISA (cELISA), in order to estimate antibody avidity and cross-reactivity with selected phenyl-*N*-methylcarbamate insecticides.

2.5 *ELISA protocols*

2.5.1 Non-competitive ELISA for titre assesement. Every ELISA plate was coated with 100 μL/well of a 1 μg mL^{-1} coating conjugate solution in PBS and incubated overnight at 4°C. Then, the plate was emptied and washed three times with PBS-Tween, and blocking was performed by the addition of 4% (w/v) gelatine solution in PBS-Tween (100 μL/well) and 1 h incubation at 37°C. Afterwards, the plate was washed three times with PBS-Tween, and the IgY samples were added (100 μL/well), following 1 h of incubation at 37°C. Each IgY sample was derived from a thousand-fold dilution of the appropriate thawed aliquot, with PBS-Tween. Washing of the plate was repeated, and the peroxidase-conjugated anti-chichen IgG (anti-IgY) solution was added (100 μL/well). This solution was derived from a 4000-fold dilution of the initial solution (commercial available) with PBS-Tween. After 1 h incubation at 4°C, the plate was washed twice with PBS-Tween and twice with citrate buffer pH 5. *O*-phenylene diamine (OPD) solution was added (100 μL/well). This solution was made by dissolving 2.5 mg mL^{-1} of OPD and 1 μL mL^{-1} of 30% (w/w) hydrogen peroxide, in citrate buffer pH 5. After 30 min incubation in the dark at RT, the reaction was stopped by the addition of 2 M H_2SO_4 (100 μL/well). The colour developed was read at 490 nm in the ELISA-plate reader. IgY solutions from eggs collected before the immunization were used as negative control (blank) samples. Each sample was applied in triplicate. The antibody titre development is depicted in figure 3.

2.5.2 cELISA for avidity and cross-reactivity evaluation. Every ELISA plate was coated with 100 μL/well of a 1 μg mL^{-1} coating conjugate solution in PBS and incubated overnight at 4°C. Then, the plate was emptied and washed three times with PBS-Tween, and blocking was performed by the addition of 4% (w/v) gelatine solution

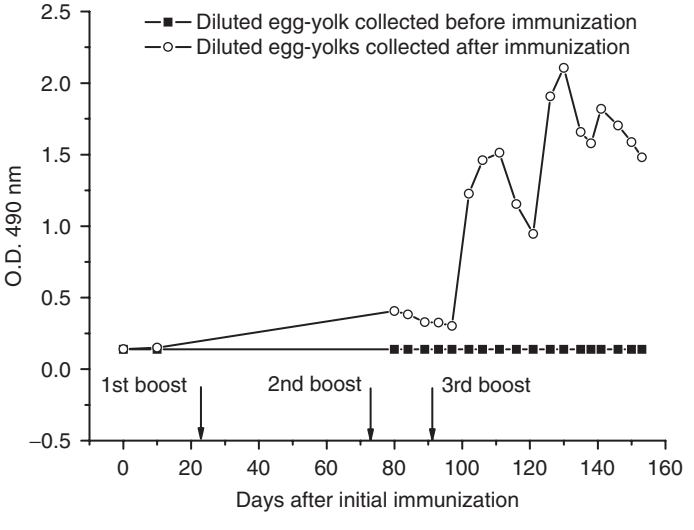

Figure 3. Titre and maturation increase for the specific IgYs. Assessment of the specific IgY titre was achieved by means of non-competitive ELISA using diluted egg yolks before (blank sample) and after immunization of a hen.

in PBS-Tween (100 μL/well) and 1 h of incubation at 37°C. Afterwards, the plate was washed three times with PBS-Tween, and the samples were added (100 μL/well), following 1 h of incubation at 37°C. Each sample was the product of 3 h of incubation of the IgY solution with an equal volume of every inhibitor's solution. IgY solution was derived from a thousand-fold dilution with PBS of the thawed aliquot corresponding to the 126th day egg. Solutions of the inhibitors were prepared by diluting the stocks solutions with PBS at concentrations: 0, 5, 50, 500, 5000, 50,000, 500,000 and 1,000,000 pmol mL^{-1}. Washings of the plate were repeated, and the anti-IgY solution was added (100 μL/well). After 1 h of incubation at 4°C, the plate was washed twice with PBS-Tween and twice with citrate buffer, pH 5. OPD solution was added (100 μL/well), and after 30 min incubation in the dark at RT, the reaction was stopped by the addition of 2 M H_2SO_4 (100 μL/well). The colour developed was read at 490 nm. IgY solutions from eggs collected before the immunization were used as negative control (blank) samples. Both specific IgY solutions and blank sample solutions were incubated with all the inhibitors' dilutions. Each sample was applied in triplicate.

The percentage inhibition (I) was calculated as $(A - A_{blk}/A_{max} - A_{blk\,max}) \times 100$, where: A is the mean optical density at 490 nm, corresponding to the specific concentration of an inhibitor incubated with specific IgYs; A_{blk} is the mean optical density at 490 nm, corresponding to specific concentration of an inhibitor incubated with blank sample; A_{max} is the mean optical density at 490 nm, corresponding to the 0 pmol mL^{-1} concentration of an inhibitor incubated with specific IgYs; $A_{blk\,max}$ is the mean optical density at 490 nm, corresponding to the 0 pmol mL^{-1} concentration of an inhibitor incubated with blank sample; and Aminocarb (table 1) was chosen as the reference analyte, for cross-reactivity estimation. The percentage cross-reactivity was calculated as $(I_{50\,aminocarb}/I_{50\,inhibitor}) \times 100$, where I_{50} is the concentration of a compound producing 50% inhibition of the antibody binding.

3. Results and discussion

3.1 *Hapten synthesis and antigen preparation*

The initial and most crucial step in the production of polyclonal antibodies, binding small, not antigenic molecules (such as PNMCs), is the synthesis of suitable haptens, which will be coupled to antigenic carrier proteins, in order to elicit immunity. These haptens should preserve the chemical and physical properties of PNMCs, and at the same time, they should provide a functional group, which allows their conjugation to proteins. Moreover, a suitable spacer arm, placed between the protein and the hapten, provides a higher recognition for the small molecule, located farthest from the carrier, and may result in more specific antibody generation. Most of the relative published works concern antibody generation (usually monoclonal) to a single N-methylcarbamate compound and the application in immunoassays [14, 20, 21–24]. On the contrary, the scope of this work was to produce antibodies binding as many PNMCs as possible. Thus, the characteristic *N*-methylcarbamate functional group and the aromatic ring were exposed to the immune system. The hapten was coupled to BSA via an amine group placed at the *para* position of the ring, as far as possible from the carbamate group. Methyl substitution in ring positions 3 and 5 made the hapten resemble

meta-substituted PNMCs. The space of four C atoms between the hapten and the carrier was provided by succinic anhydride (figure 2, **hapten 1**).

Albumins (e.g. BSA) isolated from the serum of mammals were considered efficient immunogens for hens, because of the great phyllogenetic distance of the two species. Therefore, the common carrier protein BSA was used for the preparation of immunizing conjugate. Regarding the coating antigen, this should be coupled to a different carrier via a different spacer because a large number of antibodies raised against the protein and/or the spacer of the immunizing hapten will generate false-positive results in ELISA. Therefore, THR was selected as the coating antigen's protein, and 3,3-dimethylglutaric anhydride was used as spacer (figure 2, **hapten 2**).

A special point that should be discussed is the method of hapten synthesis. Two common synthetic pathways for the production of PNMC insecticides are cited in the literature. The first involves the use of the highly toxic phosgene gas [20] and the second the use of methylisocyanate, a labile and toxic liquid (which may be lethal if inhaled, as stated on its packaging) [25, 26]. These two reagents beside dangerous, are difficult to handle, and only experienced personnel should deal with their applications in synthesis. In this work, a new efficient and easy way to synthesize PNMCs is introduced. This involves a phenol reaction with triphosgene, following methylamine hydrochloride addition. The first is an odourless, non-volatile, crystalline white solid that is easy to handle and becomes dangerous (may convert into phosgene) only in contact with water. It reacts readily with alcohols and phenols to give very active intermediates, which can react with amines, forming carbamates. Methylamine hydrochloride converts to the reactive methylamine, in the presence of a base (DIPEA). Preparation of the carbamate function of PNMCs derivatives though this new reaction is rapid and easy, while the non-optimized yield exceeds 60%. Optimization of the reaction conditions is expected to improve this yield further.

3.2 *Titre development*

Generally, Lowman brown hens, lay one egg per day, from the age of 6 months to almost 3 years old. However, both immunized hens stopped laying approximately 10 days after the initial immunization and started again after the 70th day. For this reason, the second boost was delayed (86th day). The antibody titre and maturation were tested by non-competitive ELISA (figure 3). A rapid increase was observed just after the second boost, reaching a maximum point about 10 days after the third boost. IgYs, contained in the 126th-day egg yolk, had the highest antibody titre, so they were used for the assessment of the avidity and cross-reactivity.

3.3 *Non-competitive and competitive ELISA optimization*

Initial experiments revealed that a titre and avidity assessment could be performed, without any problem in the ELISA, on solutions derived from treated egg yolks, as described at section 2.4. The coating antigen differed, from the immunizing one, in both the spacer and the carrier protein. In this way, an effort was made to minimize its interactions with polyclonal antibodies, raised against BSA or the spacer. Only specific IgYs (for the carbamate compound **4**) are considered to bind to the coating antigen. For the determination of the optimum coating antigen concentration and

antibody solution's dilution, several experiments were carried out, in both non-competitive and competitive ELISA. Combinations of 1, 2, 5, 10, and 25 $\mu g\,mL^{-1}$ of antigen with 1/250, 1/500, 1/1000, 1/2000, 1/5000, and 1/10,000 antibody dilutions were tested. The combination of 1 $\mu g\,mL^{-1}$ and 1/1000 for the antigen and the antibody, respectively, were found to be the best, giving optical density values between 0.4 and 1.5 (cELISA), after the reaction with OPD.

Moreover, the use of 0.1% (v/v) Tween-20 in non-competitive ELISA proved to have no effect in antibody binding strength to the antigen. Thus, this detergent was used in order to avoid non-specific interactions, mostly between contents remaining in the egg yolk (e.g. irrelevant proteins or lipids) and the coating conjugate. However, no detergent was added when specific IgYs were incubated with several concentrations of the inhibitors, in cELISA. In this case, non-specific interactions, such as those described above, are unlikely to occur, but the use of the detergent could weaken the interactions between the antibodies and the inhibitors. The conditions for blocking with gelatin and OPD reaction are standard and have been used successfully in our laboratory, in several ELISA protocols.

3.4 *Assessment of inhibition capacity and cross-reactivity*

Compound **4**, aminocarb, carbaryl, carbendazim, carbofuran, dioxacarb, ethiofencarb, fenobucarb, metolcarb, promecarb, propoxur, and trimethacarb (figure 4) were tested for their inhibition capacity and cross-reactivity. Aminocarb was chosen as the reference analyte because of its high structural resemblance to the immunizing hapten. As we expected, compound **4** exhibited the highest avidity towards the polyclonal IgYs (the lowest $I_{50} = 0.9$ nmol mL^{-1}). Aminocarb, carbaryl, metolcarb, promecarb, and trimethacarb (figure 4a) showed intense inhibition and significant cross-reactivity to IgYs produced ($I_{50} = 15$, 5, 13, 20 and 9 nmol mL^{-1}, respectively). We consider as 'cross-reacting' compounds those which can effectively inhibit the binding of the IgYs to the coating conjugate, in cELISA. The shape of the inhibition curve corresponding to a 'cross-reacting' inhibitor resembles the shape of the curve corresponding to compound **4** (hapten without spacer). Furthermore, the I_{50} value of every compound tested implies the effectiveness of the inhibition. An inhibitor with a low I_{50} value, close to the I_{50} of compound **4**, can bind strongly to the specific IgYs, inhibiting their binding to the coating antigen. Moreover, the inhibition curves of the compounds in figure 4(b) differ significantly from the curve corresponding to compound **4**. This indicates poor inhibition capacity and a very high I_{50} value. All these compounds (except carbaryl) are PNMCs substituted in positions 3 and/or 4 and/or 5, of the aromatic ring, like the hapten. Taking this into account, their potent inhibition capacity is easy to explain. This cannot be said for carbaryl. The fact that this compound exhibited the highest inhibition capacity, among all tested insecticides, is rather surprising, because it is very different (structurally) from the hapten. However, it seems that the carbon atom (of the second aromatic ring) at the *ortho* position is a rather small substituent, unable to rotate, and so it cannot hinder the inhibitor–antibody interaction.

All the other PNMCs tested (carbofuran, dioxacarb, ethiofencarb, fenobucarb, and propoxur) bear a bulky substituent at the *ortho* position (table 1). This prominent structural difference is considered to be the cause of their low cross-reactivity (figure 4b). Taking into consideration the high inhibition capacity of carbaryl,

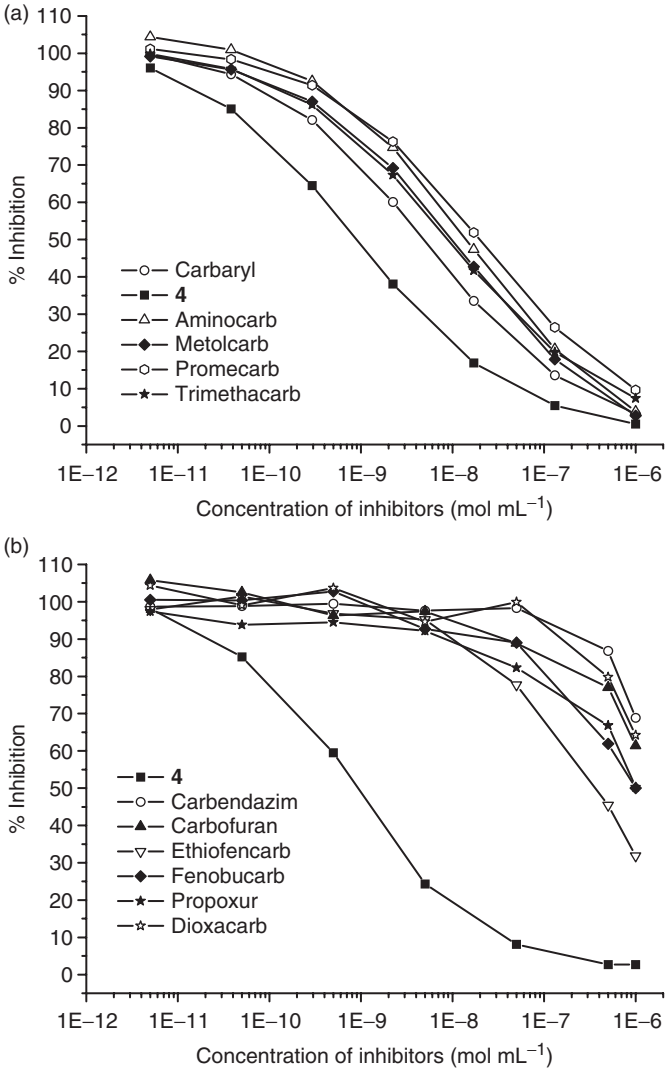

Figure 4. Inhibition of IgY binding accomplished by compound **4** and several PNMCs. Assessment of the inhibition capacity was achieved by means of competitive ELISA, using the diluted egg yolk, containing IgYs with the highest binding capacity. Similarly diluted egg yolk, from the same hen before immunization, was used as blank sample. The concentration range of the inhibitors was 0–1,000,000 pmol mL^{-1}. (a) Cross-reacting inhibitors with produced IgYs; (b) non-cross-reacting inhibitors with produced IgYs.

we expected similar results for carbofuran. However, in this case, the bulky oxygen atom at the *ortho* position seems to hinder its interaction with the IgYs. Finally, the purpose of testing the structurally completely different fungicide carbendazim was to examine IgYs binding to another class of carbamate compounds (not PNMCs). Judging from its extremely low inhibition capacity, we can claim that the produced IgYs are specific for PNMCs. Pilot experiments on the purification of the IgYs by immunoaffinity chromatography showed that the conjugate produced by coupling of Affigel-10 (activated agarose) with compound **4** adsorbs the specific IgYs selectively.

The adsorbed antibodies were eluted after washing with glycine buffer, pH 2.5 [16], and the inhibition capacity and cross-reactivity of the purified IgYs to aforementioned PNMCs were assessed by competitive ELISA. The results were in agreement with those depicted (figure 4). Unfortunately, we could not find any more analytical standards belonging to this class of carbamates, commercially available, in order to carry out a more thorough study.

3.5 *Conclusions*

We produced IgYs able to bind PNMCs, substituted at positions 3, 4, and 5 (figure 1 and table 1). Moreover, they interacted strongly with the widely used pesticide carbaryl. These antibodies can be isolated purified and used for the determination of these insecticides by developing appropriate cELISA methods. Besides, they could be covalently attached to appropriate solid materials (e.g. shepharose or agarose), aimed at the production of immunosorbent material. The latter could be applicable for the extraction of the aforementioned compounds from liquid samples (e.g. water) or from extracts derived from complex matrices, such as soil, fruits, and vegetables. In this way, most interfering co-extractives are withdrawn, and samples containing PNMCs residues can be analysed using a common liquid-chromatographic method.

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