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Hapten and Antibody Production for a Sensitive Immunoassay Determining a Human Urinary Metabolite of the Pyrethroid Insecticide Permethrin

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Permethrin is the most popular synthetic pyrethroid insecticide in agriculture and public health. For the development of the enzyme-linked immunosorbent assay (ELISA) to evaluate human exposure to permethrin, the glycine conjugate (DCCA-glycine) of a major metabolite, *cis*/*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (DCCA), of permethrin was established as the target analyte. Four different types of the *cis*- and *trans*-isomers of immunizing haptens were synthesized as follows: *N*-(*cis*/*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carbonyl)glycine (hapten **3**), *N*-(*cis*/*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carbonyl)-4-amino-L-phenylalanine (hapten **5**), *N*-(*N*-(*cis*/*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carbonyl)glycine)-amino-6-(2,4-dinitrophenyl)aminohexanoic acid (hapten **9**), and *N*-(*cis*/*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carbonyl)glycine-4-oxobutanoic acid (hapten **24**). Sixteen polyclonal antibodies produced against each *cis*- or *trans*-hapten−thyroglobulin conjugate as immunogens were screened against numerous hapten−bovine serum albumin conjugates as coating antigens. Six ELISAs with both a heterologous hapten structure and a heterologous hapten configuration (*cis*/*trans* or *trans*/*cis)* between antibody and coating antigen showed a high sensitivity for the target analyte. The IC_{50} was 1.3, 2.1, and 2.2 μg/L for the *trans*-target analyte and 0.4, 2.3, and 2.8 μg/L for the *cis*-target analyte. The immunizing haptens, except for hapten **5**, provided the target specific antibodies. Molecular modeling of the haptens supported the selection of reasonable immunizing haptens that best mimicked the target analyte. Hapten **5** was suitable as a coating antigen rather than as an immunogen since it had a different geometry. Very low cross-reactivities were measured to permethrin, its free metabolite (DCCA), PBA-glycine conjugate, and glycine. The ELISA will be optimized for the detection of total *cis*/*trans*-DCCA-glycine in human urine samples.

KEYWORDS: ELISA; permethrin; glycine conjugate; human exposure; hapten

INTRODUCTION

The pyrethroid insecticide permethrin acts as a neurotoxin to control a wide range of insects in agriculture, forestry, homes, horticulture, and public health around the world (*1*−*5*). Permethrin is very nontoxic to mammals, whereas it is highly toxic to some nontarget insects such as honeybees and other beneficial insects (*6*). This compound has apparent toxic effects to some aquatic species such as fish, aquatic insects, crayfish, and shrimp at parts per billion levels (*7*−*9*). Its high octanol−water partition coefficient suggests that it may have a tendency to bioaccumulate in living organisms (*10*). In the study on the neonatal effect of the exposure, Cantalamessa (*11*) suggested that permethrin is more acutely toxic to children than to adults. Because permethrin causes lung and liver tumors in mice, the U.S. Environmental Protection Agency has classified permethrin as a potential carcinogen (*12*). It also belongs to categories of endocrine disrupting compounds (*13*), priority pollutants (*14*, *15*), and environmental contaminants.

Permethrin is the most popular insecticide among the pyrethroid insecticides in the United States as the active ingredient in personal care products, such as shampoos and lotions for lice. Permethrin residues have been found in ground and surface waters throughout the United States. It is also detected in agricultural products, particularly spinach, tomatoes, celery, lettuce, and peaches, and peach and plum baby food (*16*). The most common route of exposure to pyrethroid insecticides for the general population is through drinking water and the ingestion of foods such as vegetables and fruits onto which the insecticide has been applied. Farmers, pesticide applicators, manufacturers, and military service personnel may also receive additional occupational exposure through dermal contact and

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inhalation. For humans, pyrethroid insecticides are much less toxic than many other insecticides. The ADI values (acceptable daily intake for man) range from 0.01 (deltamethrin) to 0.05 (permethrin and cypermethrin) mg/kg body weight per day (*17*, *18*). However, overexposure causes reversible symptoms such as headache, dizziness, nausea, irritation of the skin and nose, and paraesthesia (*19*, *20*).

In mammals, permethrin, like other pyrethroid insecticides, is rapidly metabolized by ester cleavage to its constituent acids (*cis*- and *trans*-DCCA) and alcohol (PBOH) and subsequent oxidation of the alcohol to PBA. There is little tendency for it to accumulate in tissues. These metabolites might be partially conjugated to glycine and/or glucuronic acid and then finally excreted in the urine (*21*−*23*), which is a common pathway for xenobiotics.

In humans, the major metabolic pathway is ester hydrolysis followed by conjugation. Ring hydroxylation, oxidation at the *gem*-dimethyl group, and other oxidation pathways result in minor metabolites (*24*). The major permethrin metabolites, *cis*- and *trans*-DCCA, and PBA were determined to serve as biomarkers in the urine of exposed people (*25*). While little is known about the conjugates of pyrethroid metabolites in humans, it has been well-established that glycine is the most common amino acid used in conjugation reactions with free carboxylic acids of xenobiotics (*26*). The glucuronide and glycine conjugates of DCCA in rat and mouse and the glucuronide, glycine, serine, glutamine, and glutamic acid conjugates of DCCA in cockroach, housefly, and cabbage looper have been found (*27*).

Analytical methods for detection of pyrethroid metabolites in biological samples such as urine and blood include some sample preparation steps such as acid hydrolysis, liquid−liquid or solid phase extraction, and derivatization with high-performance liquid chromatography or gas chromatography with mass spectrometry (*25*, *28*−*30*).

Although the instrumental methods are very sensitive for these metabolites, they are time-consuming and expensive and not suitable for a routine and rapid analysis. Immunoassay techniques are widely used in clinical diagnostics, environmental monitoring, food quality, agriculture, and field or on-site test of personnel. An example of the latter is deployed soldiers exposed to toxic chemicals. The assays are rapid, sensitive, and selective analytical tools to determine trace chemicals such as agrochemicals and their metabolites as key urinary biomarkers of exposure (*27*).

A sensitive ELISA with an IC_{50} value of 0.4 μg/L and a detection range of 0.04−10 μg/L in buffer was developed for the glycine conjugate of the pyrethroid metabolite PBA (PBA-glycine) (*32*). The limit of quantitation was 1 μg/L in real human urine.

It is likely that the relative amount of DCCA-glycine conjugate is higher than that of the PBA-glycine, because the PBOH that is directly hydrolyzed from the parent can enter several metabolic pathways including direct conversion to the glucuronide conjugate prior to further oxidation to PBA. Therefore, DCCA-glycine can exist in high abundance and then be a biomarker as an indicator of exposure to this insecticide and a good target analyte for biomonitoring in human urine.

The preparation of haptens as immunogens and coating antigens is one of the major stages in the development of immunoassays for small molecules (*31*, *33*). The synthesis of haptens can sometimes require extensive investment of time even before Ab production. Therefore, the hapten chemistry should be thoroughly considered in order to develop sensitive and selective immunoassays. In this study, the authors report the syntheses of *cis*/*trans*-haptens and their use for Ab production and ELISA development. Because the target analyte possesses separate *cis*- and *trans*-isomers of DCCA-glycine, optimal haptens would produce antibodies specific to each isomer of the target analyte as well as for use as coating antigens. Various haptens with different *cis*- or *trans*-configurations were synthesized and used to produce polyclonal antibodies in rabbits. The screening of the antibodies against the various coating antigens and characterization of the antibodies are reported.

MATERIALS AND METHODS

Chemicals. The *cis*- and *trans*-DCCA, as the starting materials for hapten syntheses, were prepared, according to published synthetic procedures (*34*−*36*). Organic materials for the synthesis were purchased from Aldrich Chemical Co. (Milwaukee, WI) and Fisher Scientific (Pittsburgh, PA). Thin-layer chromatography (TLC) utilized 0.2 mm precoated silica gel 60 F254 on glass plates from E. Merck (Darmstadt, Germany), and detection was by ultraviolet light or iodine vapor stain. Column chromatographic separations were carried out using Baker silica gel (40 μm average particle size) using the indicated solvents where the → notation denotes a stepwise concentration gradient. The coupling reagents were purchased from Aldrich. BSA, Thyr, GAR-HRP as the second Ab, Tween 20, and 3,3′,5,5′-TMB were purchased from Sigma Chemical Co. (St. Louis, MO).

Instruments. ^{1}H nuclear magnetic resonance (NMR) spectra of compounds synthesized were obtained on a General Electric QE-300 spectrometer (Bruker NMR, Billerica, ME) using tetramethylsilane as an internal standard. Electrospray mass spectra in the positive (MS-ESI$^+$) or negative (MS-ESI$^-$) mode were recorded by a Micromass Quattro Ultima triple quadrupole tandem mass spectrometer (Micromass, Manchester, United Kingdom). Melting points were determined on a Thomas-Hoover Uni-Melt apparatus (Thomas Scientific, Swedesboro, NJ) and are uncorrected. R_f values refer to TLC on silica gel 60 F254, precoated plates (Merck) with visualization under exposure to either ultraviolet light (254 nm) or iodine vapor. ELISA was performed on 96 well microtiter plates (Nunc-Immuno plate, MaxiSorp surface, Roskilde, Denmark) and read spectrophotometrically with a microplate reader (Molecular Devices, Menlo Park, CA) in the dual wavelength mode (450−650 nm).

Hapten Synthesis and Verification. The proposed target haptens are shown in **Figure 1**. Because the target DCCA-glycine is of a small molecular weight (MW 265) and requires conjugation to carrier proteins in order to be immunogenic, various haptens with the carboxylic group or amine group were synthesized. The reactions followed the procedures used in previous publications (*32*, *37*).

***N*-(*cis*/*trans*-3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane-1-carbonyl)glycine (*cis*/*trans*-DCCA-glycine, *cis*/*trans*-Hapten 3; Figure 2).** Thionyl chloride ($SOCl_2$, 3 mL) was added to *cis*-DCCA (1500 mg, 7.17 mmol) in a 10 mL round bottomed flask. The mixture was stirred under N_2 at 65 °C for 1.5 h. The solution was concentrated under reduced pressure to remove excess $SOCl_2$. Hexane was added, and then the solution was concentrated again. The crude acid chloride, *cis*-**2**, was obtained as a pale yellow liquid. It was added dropwise to a vigorously stirred ice-cooled solution of glycine in 10 mL of 2 N KOH. A 1 N solution of KOH was added to keep the mixture slightly basic. After 3 h, the odor of the acid chloride was no longer detectable. The mixture was acidified with 6 N HCl and extracted twice with ethyl acetate (30 mL). The combined organic phase was dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure. The residue was recrystallized from the mixture of ethyl acetate and *tert*-butyl chloride to give 1084 mg (56%) of *cis*-DCCA-glycine, *cis*-**3**, as a white solid; mp 141−143 °C. TLC [ethyl acetate/hexane/acetic acid (1:1:0.1, v/v/v)] R_f, 0.85. ^{1}H NMR (DMSO-d_6): δ 1.15 (s, 1H, CH_3), 1.32 (s, 3H, CH_3), 1.85 (d, J = 8.5 Hz, 1H, CHCO), 2.03 (dd, J = 8.7, 8.7 Hz, C=C−CH), 3.78 (t, J = 5.8 Hz, 2H, NCH_2), 6.06 (d, J = 8.9 Hz, 1H, C=CH), 8.40 (t, J = 5.8 Hz, 1H, NH). MS-ESI$^-$ m/z calcd for $[M - H]^- = C_{10}H_{13}Cl_2NO_3$, 264.03; observed, 264.09.

The *trans*-isomer of compound **3** (1023 mg, 53%) was prepared as a white solid from the *trans*-DCCA by the same method as described

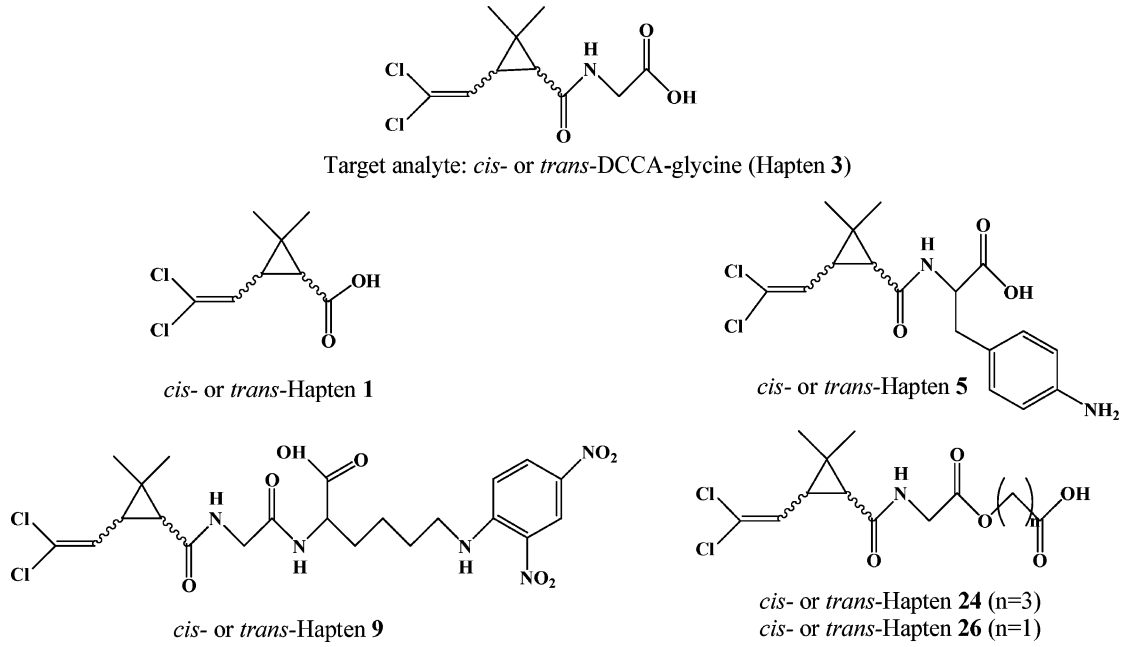

Figure 1. Target analyte and haptens designed.

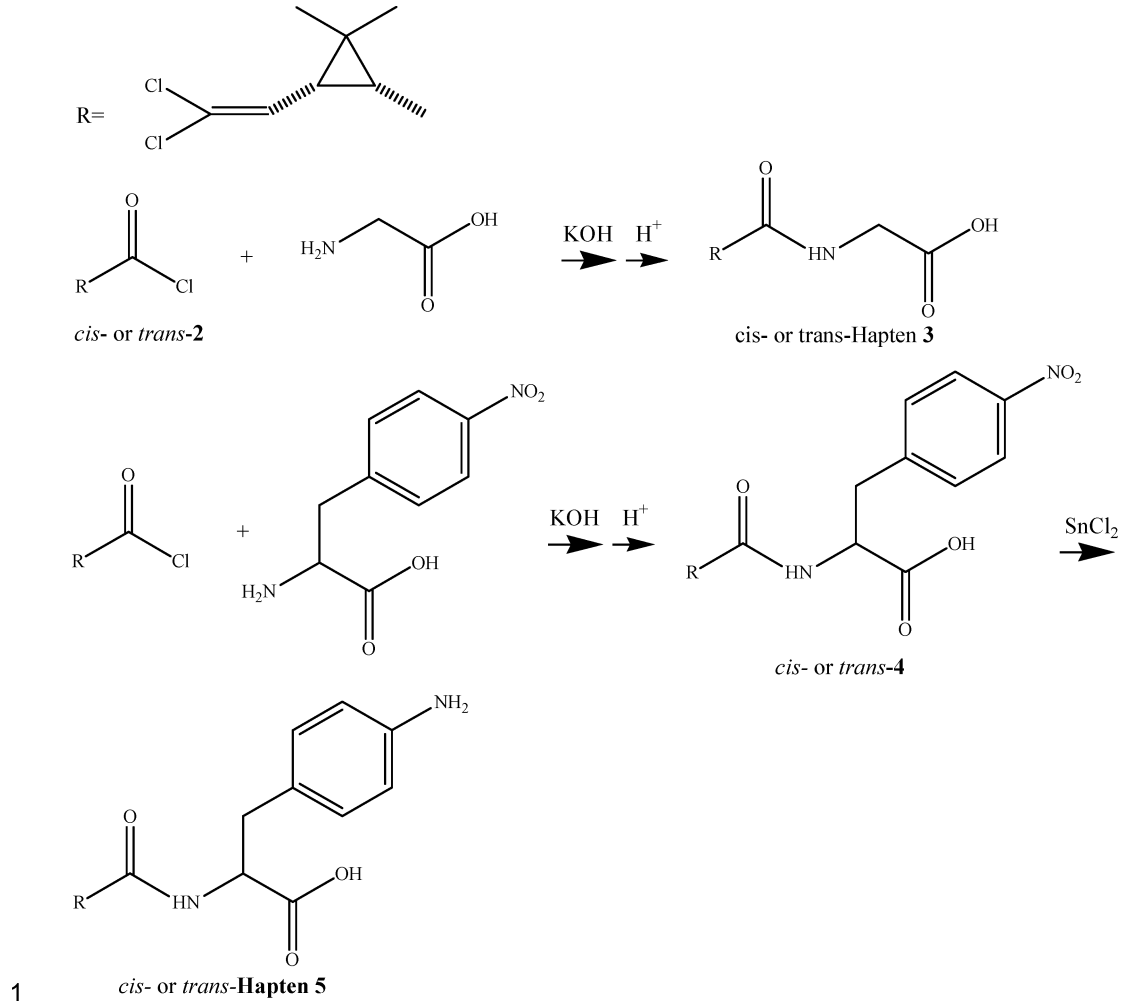

Figure 2. Scheme for the synthesis of the target analyte (*cis*/*trans*-DCCA-glycine, hapten **3**) and *cis*/*trans*-hapten **5**.

above for the *cis*-isomer of compound **3**; mp 178−179 °C. TLC (ethyl acetate/hexane/acetic acid [1:1:0.1, v/v/v)] R_f, 0.75. ^{1}H NMR (DMSO-d_6): δ 1.14 (s, 3H, CH_3), 1.15 (s, 3H, CH_3), 1.83 (d, J = 5.3 Hz, 1H, CHCO), 2.04 (dd, J = 5.3 Hz, 8.2 Hz, 1H, C=C−CH), 3.78 (t, 5.6 Hz, 2H, NCH_2), 6.04 (d, J = 8.3 Hz, 1H, C=CH), 8.36 (t, J = 5.8 Hz, 1H, NH). MS-ESI$^-$ m/z calcd for $[M - H]^- = C_{10}H_{13}Cl_2NO_3$, 264.03; observed, 263.95.

***cis*/*trans*-Compound 4 (Figure 2).** Crude acid chloride, *cis*-**2** (0.06 mmol), was added dropwise to a vigorously stirred ice-cooled solution of 4-nitro-L-phenylalanine (139 mg, 0.66 mmol) in 10 mL of 2 N KOH.

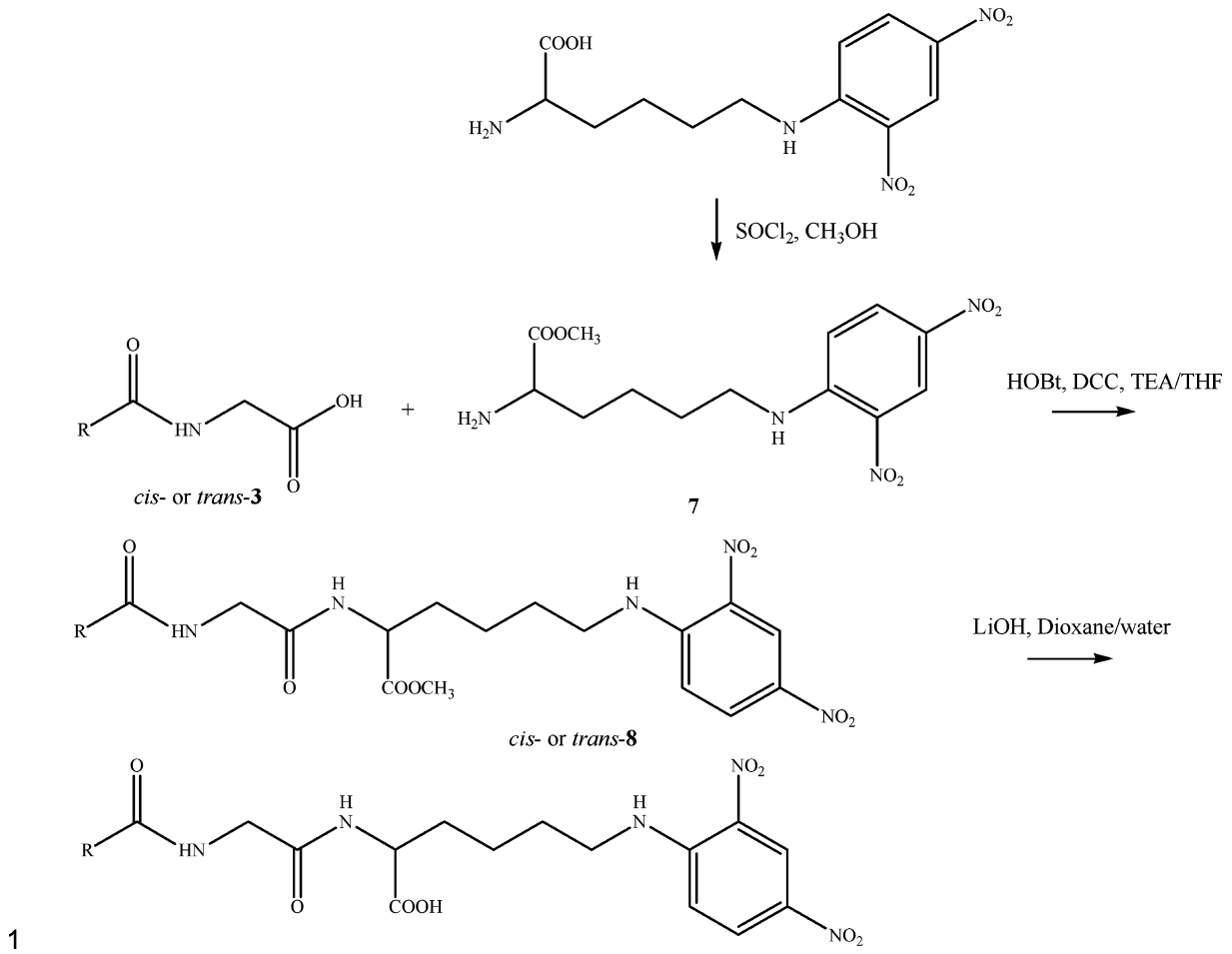

Figure 3. Scheme for the synthesis of *cis*/*trans*-hapten **9**.

Over the course of 30 min, 1 N KOH was added to keep the mixture slightly basic. After 3 h, the odor of the acid chloride was no longer detectable. The mixture was acidified with 6 N HCl and extracted twice with ethyl acetate (30 mL). The combined organic phase was dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure. The residue was purified by using preparative TLC on silica gel with the mixture of ethyl acetate/hexane/acetic acid (1:1:0.01, v/v/v) as an eluent to give 180 mg of *cis*-compound **4** as a yellow oil. TLC [ethyl acetate/hexane/acetic acid (1:1:0.1, v/v/v)] R_f, 0.19. ^{1}H NMR ($CDCl_3$): δ 1.22 (s, 3H, CH_3), 1.33 (s, 3H, CH_3), 1.63 (d, J = 8.5 Hz, 1H, CHCO), 2.27 (dd, J = 8.7, 8.8 Hz, 1H, C═C−CH), 3.22−3.26 (m, 2H, NCHCH_2Ar), 4.95 (dt, J = 7.1, 5.8 Hz, 1H, NCHCO), 5.63 (d, J = 8.9 Hz, 1H, C═CH), 7.26−7.40 (m, 4H, Ar), 8.20 (d, J = 7.1 Hz, 1H, NH). MS-ESI$^-$ m/z calcd for $[M - H]^-$ = $C_{17}H_{18}Cl_2N_2O_5$, 399.06; observed, 399.24.

The *trans*-isomer of compound **4** (150 mg) was prepared as a yellow oil from the *trans*-acid chloride by the same method as described above for the *cis*-isomer of compound **4**. TLC [ethyl acetate/hexane/acetic acid (1:1:0.1, v/v/v)] R_f, 0.20. ^{1}H NMR ($CDCl_3$): δ 1.23 (s, 3H, CH_3), 1.35 (s, 3H, CH_3), 1.63 (d, J = 5.4 Hz, 1H, CHCO), 2.26 (dd, J = 5.4, 8.4 Hz, 1H, C═C−CH), 3.21−3.25 (m, 2H, NCHCH_2Ar), 4.91 (dt, J = 7.3, 5.5 Hz, 1H, NCHCO), 5.63 (d, J = 8.4 Hz, 1H, C═CH), 7.28−7.42 (m, 4H, Ar), 8.23 (d, J = 7.2 Hz, 1H, NH). MS-ESI$^-$ m/z calcd for $[M - H]^-$ = $C_{17}H_{18}Cl_2N_2O_5$, 399.06; observed, 399.24.

***N*-(*cis*/*trans*-3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane-1-carbonyl)-4-amino-L-phenylalanine (*cis*/*trans*-Hapten 5; Figure 2).** The mixture of *cis*-compound **4** (261 mg, 0.65 mmol) and stannous chloride dihydrate (733 mg, 3.25 mmol) in 2 mL of ethanol was stirred at 70 °C in an oil bath for 30 min. The mixture was cooled and poured into the slurry of water, ethyl acetate, and Celite. $NaHCO_3$ was added in portions. The neutral mixture was filtered, and the solids on the filter and in the flask were washed with water and ethyl acetate. The ethyl acetate phase was separated and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure. The oil residue was purified by using preparative TLC on silica gel with the mixture of methanol/methylene chloride/acetic acid (1:9:0.2, v/v/v) as an eluent to give 112 mg (47%) of *cis*-hapten **5** as a yellow solid; mp 93−97 °C. TLC [methanol/methylene chloride/acetic acid (1:9:0.2, v/v/v)] R_f, 0.59. ^{1}H NMR (DMSO-d_6): δ 1.15 (s, 3H, CH_3), 1.32 (s, 3H, CH_3), 1.86 (d, J = 8.6 Hz, 1H, CHCO), 2.03 (dd, J = 8.7, 8.7 Hz, 1H, C═C−CH), 3.32−3.48 (m, 2H, NCHCH_2Ar), 4.28 (dt, J = 7.0, 5.6 Hz, 1H, NCHCO), 6.05 (d, J = 8.8 Hz, 1H, C═CH), 6.99−8.13 (m, 4H, Ar), 8.40 (d, J = 7.1 Hz, 1H, NH). MS-ESI$^-$ m/z calcd for $[M - H]^-$ = $C_{17}H_{20}Cl_2N_2O_3$, 369.09; observed, 369.10.

By the same method as described above for the *cis*-isomer of hapten **5**, 98 mg (41%) of *trans*-hapten **5** was prepared as a yellow solid from the *trans*-compound **4**; mp 102−105 °C. TLC [methanol/methylene chloride/acetic acid (1:9:0.2, v/v/v)] R_f, 0.59. ^{1}H NMR (DMSO-d_6): δ 1.20 (s, 3H, CH_3), 1.40 (s, 3H, CH_3), 1.75 (d, J = 5.0 Hz, 1H, CHCO), 2.11 (dd, J = 5.5, 8.4 Hz, 1H, C═C−CH), 3.34−3.50 (m, 2H, NCHCH_2Ar), 4.52 (dt, J = 7.3, 5.5 Hz, 1H, NCHCO), 6.07 (d, J = 8.5 Hz, C═CH), 6.99−8.13 (m, 4H, Ar), 8.23 (d, J = 7.2 Hz, 1H, NH). MS-ESI$^-$ m/z calcd for $[M - H]^-$ = $C_{17}H_{20}Cl_2N_2O_3$, 369.09; observed, 369.04.

***cis*/*trans*-Compound 8 (Figure 3).** Compound **7** as a yellow solid was synthesized according to Watanabe et al. (*37*). TLC [methanol/methylene chloride (1:9, v/v)] R_f, 0.63.

In a 25 mL flask, *cis*-DCCA-glycine (79.84 mg, 0.3 mmol), 1-hydroxy-1H-benzotriazole monohydrate (81.08 mg, 0.2 mmol), and compound **7** (97.89 mg, 0.3 mmol) were dissolved in 2 mL of dry tetrahydrofuran (THF), and then, 41.8 μL of triethylamine (0.3 mmol) and *N*,*N*′-dicyclohexylcarbodiimide (68.09 mg, 0.33 mmol) dissolved in 500 μL of dry THF were added to the mixture. The reaction mixture was stirred for 1 h in an ice bath and for 3 h at room temperature and then was filtered to remove the dicyclohexylurea. Ethyl acetate (50 mL) was added and was washed with saturated sodium bicarbonate

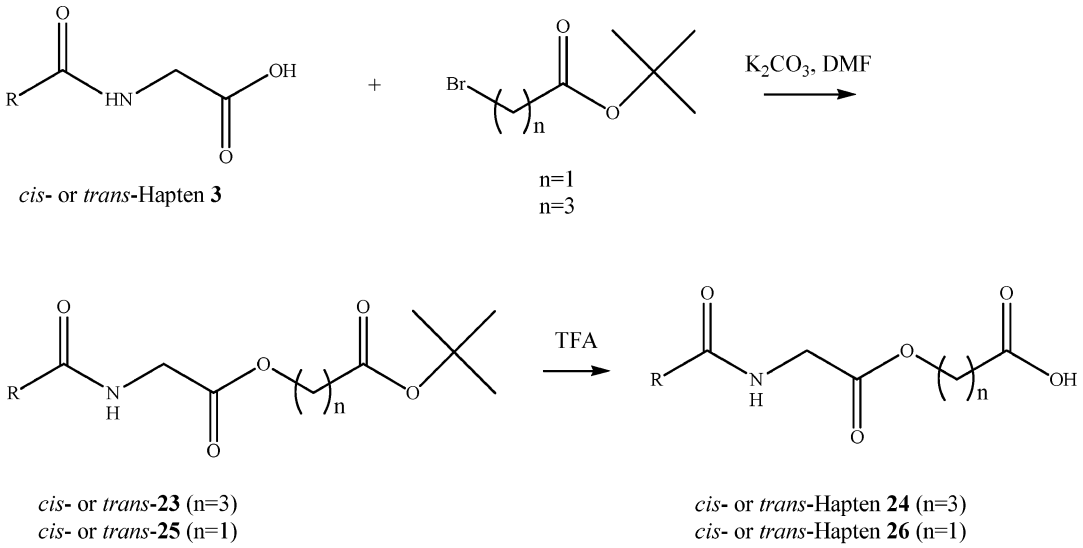

Figure 4. Scheme for the synthesis of *cis*/*trans*-haptens **24** and **26**.

solution, saturated ammonium chloride solution, saturated sodium bicarbonate solution, and water (50 mL of each). After the organic phase was dried over anhydrous sodium sulfate, ethyl acetate was removed under reduced pressure. The resultant residue was recrystallized from ether to give 140 mg of *cis*-compound **8** as a yellow solid. TLC [methanol/methylene chloride (0.5:9.5, v/v)] R_f, 0.60. ^{1}H NMR ($CDCl_3$): δ 1.21 (s, 3H, CH_3), 1.27 (s, 3H, CH_3), 1.51−1.96 (m, 8H, $NHCH_2CH_2CH_2$ and CHCO), 2.27 (dd, J = 8.6 Hz, 8.7 Hz, 1H, C═C−CH), 3.80 (s, 3H, OCH_3), 4.01 (t, 5.8 Hz, 2H, NCH_2CO), 4.08−4.18 (m, 1H, CHCO), 4.65 (q, J = 14.7 Hz, 2H, CH_2CH), 5.64 (d, J = 8.9 Hz, 1H, C═CH), 6.82 (t, J = 5.8 Hz, 1H, CONHCH$_2$), 6.92−8.58 (m, 3H, Ar). MS-ESI$^+$ m/z calcd for $[M + H]^+ = C_{23}H_{29}Cl_2N_5O_8$, 574.14; observed, 574.12.

By the same method as described above for the *cis*-isomer of compound **8**, 158 mg of the *trans*-isomer of compound **8** was prepared as a yellow solid from the *trans*-DCCA-glycine, *trans*-**3**. TLC [methanol/methylene chloride (0.5:9.5, v/v)] R_f, 0.60. ^{1}H NMR ($CDCl_3$): δ 1.21 (s, 3H, CH_3), 1.24 (s, 3H, CH_3), 1.51−1.94 (m, 8H, $NHCH_2CH_2CH_2$ and CHCO), 2.25 (dd, J = 5.3 Hz, 8.2 Hz, 1H, C═C−CH), 3.78 (s, 3H, OCH_3), 4.05 (t, 5.7 Hz, 2H, NCH_2CO), 4.08−4.18 (m, 1H, CHCO), 4.64 (q, J = 14.5 Hz, 2H, CH_2CH), 5.63 (d, J = 8.3 Hz, 1H, C═CH), 6.80 (t, J = 5.7 Hz, 1H, CONHCH$_2$), 6.91−8.57 (m, 3H, Ar). MS-ESI$^+$ m/z calcd for $[M + H]^+ = C_{23}H_{29}Cl_2N_5O_8$, 574.14; observed, 574.12.

***N*-(*N*-(*cis*/*trans*-3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane-1-carbonyl)glycine)amino-6-(2,4-dinitrophenyl)aminohexanoic Acid (*cis*/*trans*-Hapten 9; Figure 3).** The hydrolysis reaction of *cis*-compound **8** (100 mg, 0.17 mmol) was conducted in 10 mL of 1,4-dioxane/water (1:1, v/v) with 10 equiv of lithium hydroxide monohydrate. After 4.5 h, the resulting mixture was acidified to pH 4 with 6 N HCl and extracted twice with ethyl acetate (30 mL). The combined organic phase was washed with distilled water (30 mL) and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure. The residue was purified by using preparative TLC on silica gel with the mixture of methanol/methylene chloride (1:9, v/v) as an eluent to give 80 mg (84%) of *cis*-hapten-**9** as a yellow solid; mp 50−55 °C. TLC [methanol/methylene chloride (1:9, v/v)] R_f, 0.59. ^{1}H NMR ($CDCl_3$): δ 1.18 (s, 3H, CH_3), 1.25 (s, 3H, CH_3), 1.53−1.95 (m, 8H, $NHCH_2CH_2CH_2$ and CHCO), 2.25 (dd, J = 8.6 Hz, 8.7 Hz, 1H, C═C−CH), 3.97 (t, 5.8 Hz, 2H, NCH_2CO), 4.08−4.15 (m, 1H, CHCO), 4.63 (q, J = 14.7 Hz, 2H, CH_2CH), 5.63 (d, J = 8.9 Hz, 1H, C═CH), 6.78 (t, J = 5.8 Hz, 1H, CONHCH$_2$), 6.90−8.55 (m, 3H, Ar). MS-ESI$^-$ m/z calcd for $[M - H]^- = C_{22}H_{27}Cl_2N_5O_8$, 558.12; observed, 558.29.

By the same method as described above for the *cis*-isomer of hapten **9**, 75 mg (79%) of *trans*-hapten **9** was prepared as a yellow solid from the *trans*-isomer of compound **8**; mp 65−70 °C. TLC [methanol/methylene chloride (1:9, v/v)] R_f, 0.59. ^{1}H NMR ($CDCl_3$): δ 1.25 (s, 3H, CH_3), 1.27 (s, 3H, CH_3), 1.50−1.91 (m, 8H, $NHCH_2CH_2CH_2$ and CHCO), 2.23 (dd, J = 5.3 Hz, 8.2 Hz, 1H, C═C−CH), 4.01 (t, 5.7 Hz, 2H, NCH_2CO), 4.03−4.16 (m, 1H, CHCO), 4.61 (q, J = 14.5 Hz, 2H, CH_2CH), 5.61 (d, J = 8.3 Hz, 1H, C═CH), 6.83 (t, J = 5.7 Hz, 1H, CONHCH$_2$), 6.93−8.55 (m, 3H, Ar). MS-ESI$^-$ m/z calcd for $[M - H]^- = C_{22}H_{27}Cl_2N_5O_8$, 558.12; observed, 558.35.

***cis*/*trans*-Compound 23 (Figure 4).** The mixture of the *cis*-DCCA-glycine, *cis*-**3** (150 mg, 0.56 mmol), 4-bromo-butyric acid *tert*-butyl ester (249.88 mg, 1.12 mmol), and potassium carbonate (111.63 mg, 0.616 mmol) in 1 mL of anhydrous DMF was reacted at 100 °C for 3 h. The resulting mixture was filtered to remove excess K_2CO_3 and HBr produced in the reaction. The filtrate diluted with 20 mL of ethyl acetate was washed twice with 20 mL of distilled water. The organic layer was dried over anhydrous sodium sulfate, and the solvent was removed by evaporation. The residue was chromatographed on silica gel eluting with the mixture of ethyl acetate/hexane (1:2, v/v). Fractions containing pure product by TLC were stripped under high vacuum to obtain 179 mg of *cis*-compound **23** as a transparent oil. TLC [ethyl acetate/hexane (1:2, v/v)] R_f, 0.67. ^{1}H NMR ($CDCl_3$): δ 1.25 (s, 3H, CH_3), 1.35 (s, 3H, CH_3), 1.45 (s, 9H, $3CH_3$), 1.50 (d, J = 8.5 Hz, 1H, CHCO), 2.03 (dd, J = 8.7, 8.7 Hz, 1H, C═C−CH), 1.90−2.05 (m, 2H, $CH_2CH_2CH_2$), 2.31 (t, 2H, $CH_2CH_2CH_2$), 4.03 (t, 2H, C$H_2CH_2CH_2$), 4.20 (d, J = 5.7 Hz, 2H, NCH_2COO), 6.01 (bs, 1H, NH), 6.41 (d, J = 8.8 Hz, 1H, C═CH).

By the same method as described above for the *cis*-isomer of compound **23**, the *trans*-isomer of compound **23** (179 mg) was prepared as a transparent oil from the *trans*-isomer of DCCA-glycine, *trans*-**3**. TLC [ethyl acetate/hexane (1:2, v/v)] R_f, 0.64. ^{1}H NMR ($CDCl_3$): δ 1.20 (s, 3H, CH_3), 1.33 (s, 3H, CH_3), 1.44 (s, 9H, $3CH_3$), 1.61 (d, J = 5.4 Hz, 1H, CHCO), 2.05 (dd, J = 5.4, 8.7 Hz, 1H, C═C−CH), 1.92−2.07 (m, 2H, $CH_2CH_2CH_2$), 2.35 (t, 2H, $CH_2CH_2CH_2$), 4.10 (t, 2H, C$H_2CH_2CH_2$), 4.18 (d, J = 5.7 Hz, 2H, NCH_2COO), 6.10 (bs, 1H, NH), 6.42 (d, J = 8.3 Hz, 1H, C═CH).

***N*-(*cis*/*trans*-3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane-1-carbonyl)glycine-4-oxobutanoic Acid (*cis*/*trans*-Hapten 24; Figure 4).** Trifluoroacetic acid (TFA) (0.5 mL) was added to the *cis*-ester **23**, and the mixture was allowed to stand at ambient temperature for 15 min. The TFA was stripped off under vacuum, and ethyl acetate was added twice and stripped to remove residual TFA. The residue was chromatographed on silica gel eluting with the mixture of ethyl acetate/hexane (1:1, v/v). Fractions containing pure product by TLC were stripped under high vacuum, and the residue was recrystallized from ethyl acetate and *tert*-butyl chloride to give 37 mg (19%) of *cis*-hapten-**24** as a white solid; mp 85−88 °C. TLC [ethyl acetate/hexane/acetic acid (1:1:0.1, v/v/v)] R_f, 0.55. ^{1}H NMR ($CDCl_3$): δ 1.25 (s, 3H, CH_3),

1.27 (s, 3H, CH_3), 1.71 (d, J = 8.5 Hz, 1H, CHCO), 1.96−2.02 (m, 3H, C═C−CH and $CH_2CH_2CH_2$), 2.40 (t, 2H, $CH_2CH_2CH_2$), 3.99 (t, 2H, $CH_2CH_2CH_2$), 4.23 (d, J = 5.7 Hz, 2H, NCH_2COO), 4.72 (bs, 1H, NH), 6.38 (d, J = 8.8 Hz, 1H, C═CH). MS-ESI$^+$ m/z calcd for $[M + H]^+ = C_{14}H_{18}Cl_2NO_5$, 352.06; observed, 352.09.

By the same method as described above for the *cis*-isomer of hapten **24**, 150 mg (76%) of *trans*-hapten **24** from the *trans*-isomer of ester **23** was prepared as a white solid; mp 103−104 °C. TLC [ethyl acetate/hexane/acetic acid (1:1:0.1, v/v/v)] R_f, 0.55. ^{1}H NMR ($CDCl_3$): δ 1.25 (s, 3H, CH_3), 1.27 (s, 3H, CH_3), 1.52 (d, J = 5.4 Hz, 1H, CHCO), 2.09−2.17 (m, 3H, C═C−CH and $CH_2CH_2CH_2$), 2.57 (t, 2H, $CH_2CH_2CH_2$), 4.12 (t, 2H, $CH_2CH_2CH_2$), 4.30 (d, J = 5.7 Hz, 2H, NCH_2COO), 5.70 (d, J = 8.8 Hz, 1H, C═CH), 6.21 (bs, 1H, NH). MS-ESI$^+$ m/z calcd for $[M + H]^+ = C_{14}H_{18}Cl_2NO_5$, 352.06; observed, 352.19.

***cis*/*trans*-Compound 25 (Figure 4).** The mixture of *cis*-DCCA-glycine, *cis*-**3** (100 mg, 0.38 mmol), *tert*-butyl bromoacetate (148.25 mg, 0.76 mmol), and potassium carbonate (76.11 mg, 0.418 mmol) in 1 mL of anhydrous DMF was reacted at 100 °C for 3 h. The resulting mixture was further prepared by the same method as described above for hapten **23**. The residue was chromatographed on silica gel eluting with the ethyl acetate/hexane (1:3, v/v) mixture. The fractions containing pure product identified by TLC were stripped under high vacuum. After toluene was added, the solvent was removed under reduced pressure to give 126 mg of *cis*-compound **25** as a white solid. TLC [ethyl acetate/hexane (1:2, v/v)] R_f, 0.64. ^{1}H NMR ($CDCl_3$): δ 1.24 (s, 3H, CH_3), 1.27 (s, 3H, CH_3), 1.48 (s, 9H, $3CH_3$), 1.61 (d, J = 8.5 Hz, 1H, CHCO), 2.02 (dd, J = 8.7, 8.7 Hz, 1H, C═C−CH), 4.16 (d, J = 5.7 Hz, 2H, NCH_2COO), 4.58 (s, 2H, $COOCH_2COO$), 6.07 (bs, 1H, NH), 6.40 (d, J = 8.7 Hz, 1H, C═CH).

By the same method as described above for the *cis*-isomer of compound **25**, 138 mg of the *trans*-isomer of compound **25** was prepared as a white solid from the *trans*-DCCA-glycine. TLC [ethyl acetate/hexane (1:2, v/v)] R_f, 0.53. ^{1}H NMR ($CDCl_3$): δ 1.20 (s, 3H, CH_3), 1.23 (s, 3H, CH_3), 1.50 (s, 9H, $3CH_3$), 1.53 (d, J = 5.4 Hz, 1H, CHCO), 2.03 (dd, J = 5.3, 8.3 Hz, 1H, C═C−CH), 4.12 (d, J = 5.7 Hz, 2H, NCH_2COO), 4.70 (s, 2H, $COOCH_2COO$), 6.23 (bs, 1H, NH), 6.41 (d, J = 8.7 Hz, 1H, C═CH).

***N*-(*cis*/*trans*-3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane-1-carbonyl)glycine-oxoacetic Acid (*cis*/*trans*-Hapten 26; Figure 4).** TFA (0.5 mL) was added to the *cis*-ester **25**, and the mixture was allowed to stand at ambient temperature for 15 min. By the same method as described above for hapten **24**, the mixture was further prepared, chromatographed on silica gel eluting with the mixture of ethyl acetate/hexane (1:3 → 1:1, v/v), and recrystallized to give 65 mg (53%) of *cis*-hapten **26** as a white solid; mp 120−123 °C. TLC [ethyl acetate/hexane/acetic acid (1:1:0.1, v/v/v)] R_f, 0.38. ^{1}H NMR ($CDCl_3$): δ 1.20 (s, 3H, CH_3), 1.27 (s, 3H, CH_3), 1.61 (d, J = 8.5 Hz, 1H, CHCO), 2.04 (dd, J = 8.7, 8.7 Hz, 1H, C═C−CH), 4.18 (d, J = 5.7 Hz, 2H, NCH_2COO), 4.58 (s, 2H, $COOCH_2COO$), 6.17 (bs, 1H, NH), 6.42 (d, J = 8.7 Hz, 1H, C═CH). MS-ESI$^+$ m/z calcd for $[M + H]^+ = C_{12}H_{15}Cl_2NO_5$, 324.03; observed, 324.03.

By the same method as described above for *cis*-hapten **26**, 75 mg (61%) of the *trans*-isomer of hapten **26** was prepared as a white solid from the *trans*-compound **25**; mp 123−125 °C. TLC [ethyl acetate/hexane/acetic acid (1:1:0.1, v/v/v)] R_f, 0.15. ^{1}H NMR ($CDCl_3$): δ 1.24 (s, 3H, CH_3), 1.27 (s, 3H, CH_3), 1.43 (d, J = 5.4 Hz, 1H, CHCO), 2.13 (dd, J = 5.3, 8.3 Hz, 1H, C═C−CH), 4.13 (d, J = 5.7 Hz, 2H, NCH_2COO), 4.77 (s, 2H, $COOCH_2COO$), 6.25 (bs, 1H, NH), 6.38 (d, J = 8.7 Hz, 1H, C═CH). MS-ESI$^+$ m/z calcd for $[M + H]^+ = C_{12}H_{15}Cl_2NO_5$, 324.03; observed, 324.03.

Hapten Conjugation. Hapten−protein conjugates were synthesized using the water soluble carbodiimide method (*38*) for haptens with a carboxylic acid and the diazotization method (*39*) for haptens with an amine group. For immunogens, *cis/trans*-DCCA-glycine (*cis/trans*-hapten **3**), *cis/trans*-hapten **5**, *cis/trans*-hapten **9**, and *cis/trans*-hapten **24** were conjugated to Thyr. For coating antigens, *cis/trans*-DCCA and *cis/trans*-hapten **26** as well as the above haptens were conjugated to BSA.

*Diazotization Method for Conjugation of cis/trans-Hapten **5** with Amine Group to Proteins. cis/trans*-Hapten **5** (0.10 mmol) was dissolved in 4 drops of ethanol and treated with 1 mL of 1 N HCl. The resulting solution was stirred in an ice bath as 0.5 mL of 0.20 M sodium nitrite was added. DMF (0.4 mL) was then added dropwise to give a homogeneous solution, which was divided into two equal aliquots. Twenty-five milligrams of Thyr or BSA was dissolved in 5.5 mL of 0.05 M borate buffer (pH 9.6) and 1.0 mL of DMF. Aliquots of the activated hapten solution were added dropwise to the two stirred protein solutions. The reaction mixture was stirred at 4 °C overnight, and the resulting yellow conjugates were purified by exhaustive dialysis in normal strength phosphate-buffered saline (1 × PBS), which was changed for a new buffer twice a day for 4 days. Finally, the conjugates were dispensed into the 2 mL cryogenic vials and stored at −80 °C.

NHS Method for Conjugation of Haptens with Carboxylic Acid to Proteins. cis/trans-DCCA-glycine and *cis/trans*-haptens **9**, **24**, and **26** (0.04 mmol) were dissolved in 1 mL of dry DMF with sulfo-NHS (0.06 mmol) and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (0.05 mmol). After the mixture was stirred at room temperature overnight, the activated hapten was added slowly to the protein solution (25 mg of protein in 6 mL of 0.05 M borate buffer at pH 8) with vigorous stirring. The reaction mixture was purified as described above.

Immunization and Antiserum Preparation. The immunization was made by following the protocol reported previously (*40*, *41*). Two female New Zealand white rabbits were immunized for each isomer of immunogen (rabbits 3700 and 3701 for *cis*-hapten **3**−Thyr, rabbits 3702 and 3703 for *trans*-hapten **3**−Thyr, rabbits 3704 and 3705 for *cis*-hapten **5**−Thyr, rabbits 3706 and 3707 for *trans*-hapten **5**−Thyr, rabbits 3708 and 3709 for *cis*-hapten **9**−Thyr, rabbits 3710 and 3711 for *trans*-hapten **9**−Thyr, rabbits 369 and 3696 for *cis*-hapten **24**−Thyr, and rabbits 3697 and 3698 for *trans*-hapten **24**−Thyr). Each immunogen (100 μg) in 0.5 mL of 0.85% saline was emulsified with an equal volume of Freund's complete adjuvant, and then, the emulsion was injected subcutaneously. After 3 weeks, the animals were boosted with an additional 100 μg of immunogen that was emulsified with Freund's incomplete adjuvant (1:1, v/v). The boosts were given every 3 weeks, and blood samples were drawn 7 days after each boost to check the titer of antibodies. The final serum was collected 5 months following the first immunization. The blood was collected into the Vacutainer tube with a serum separation gel. The antiserum was obtained by centrifugation and stored at −80 °C. The antiserum was used without any purification.

ELISA. *Buffer Solutions.* Normal strength PBS [1 × PBS; 8 g/L of sodium chloride (NaCl), 0.2 g/L of potassium phosphate, monobasic (KH_2PO_4), 1.2 g/L of sodium phosphate dibasic anhydrous (Na_2HPO_4), and 0.2 g/L of potassium chloride (KCl), pH 7.5], PBST [1 × PBS containing 0.05% (v/v) Tween 20, pH 7.5], 0.1 × PBST (PBS diluted 1:10 with distilled water and containing 0.05% Tween 20), carbonate buffer [1.59 g/L sodium carbonate (Na_2CO_3), 2.93 g/L sodium hydrogen carbonate ($NaHCO_3$), pH 9.6], 0.05 M borate buffer (19.1 g/L $Na_2B_4O_7 \cdot 10H_2O$, pH 8), and 0.05 M acetate buffer (14.71 g/L $Na_3C_6H_5O_7 \cdot 2H_2O$) were used for immunoassay.

ELISA. Indirect ELISA and competitive indirect ELISA were performed according to the method of Voller et al. (*42*). The 96 well microtiter plates were coated overnight at 4 °C with 100 μL/well of the appropriate coating antigen concentration in 0.1 M carbonate−bicarbonate buffer (pH 9.6). After it was washed five times with PBST, the plate was incubated with 200 μL/well of a 0.5% BSA solution in PBS for 1 h at room temperature. After another washing step, 100 μL/well of antiserum diluted in PBST per well (for titration experiment) or 50 μL/well of antiserum diluted in PBST and 50 μL/well of standard analyte and sample solution were added, mixed for 30 s in the reader, and incubated for 1 h at room temperature. The standard analyte concentrations ranged from 0.003 μg/L to 5 mg/L. After the plate was washed, 100 μL/well of the secondary Ab GAR-HRP (1:5000 in PBST) was added and incubated for 1 h at room temperature. The plate was washed, and 100 μL/well of a substrate solution (0.1 mL of 1% hydrogen peroxide and 0.4 mL of 0.6% 3,3′,5,5′-TMB in DMSO added to 25 mL of citrate−acetate buffer, pH 5.5) was added to each well. After 15 min at room temperature, the reaction was stopped by adding 50 μL/well of 2 N sulfuric acid. The absorbance was measured using a dual wavelength mode at 450 minus 650 nm. Standard curves were obtained by plotting absorbance against the logarithm of analyte

concentration, which were fitted to a four parameter logistic equation: $y = \{(A - D)/[1 + (x/C)^B]\} + D$ where A is the maximum absorbance at no analyte, B is the curve slope at the inflection point, C is the IC_{50}, and D is the minimum absorbance at infinite concentration. The IC_{50} value was expressed as the sensitivity of the immunoassay.

Molecular Modeling. Molecular modeling was done using the CS Chemoffice 6.0 software package (CambridgeSoft Corporation, Cambridge, MA). The geometries optimized of the *cis*-target analyte and *cis*-haptens at their minimum energy levels with the minimum RMS gradient of 0.1 were calculated by semiempirical quantum mechanics MNDO model (*43*) with a wave function of close shell (restricted). The best immunizing and coating haptens on the target analyte are evaluated with both real data (**Table 2**) and modeling.

Cross-Reactivity (CR). The CR studies were evaluated by using the standard solution of the permethrin metabolites and other structurally related compounds. The test compounds are listed in **Table 3**. The CR was calculated as (IC_{50} of the target analyte/IC_{50} of the tested compound) $\times$ 100.

RESULTS AND DISCUSSION

Hapten Design and Synthesis. Possessing two chiral centers in the cyclopropane ring of the permethrin molecule, permethrin has a mixture of four different optical and geometrical isomers. It is mainly classified into two pairs of isomers with different geometries, referred to as the *cis*- and the *trans*-isomers. The target analyte, *cis*/*trans*-DCCA-glycine, in itself cannot be used as an immunogen because of its low molecular weight (MW 265.1). Therefore, haptens mimicking the target analyte and containing reactive groups such as a −COOH or $-NH_2$ group for conjugation to carrier proteins were synthesized to develop an immunoassay. One of the strategies for designing the immunizing hapten was to link the spacer through the −COOH group of the target molecule and leave the two Cl atoms and the amide bond (−RCONH−) and carbonyl group (−RCO−) in the molecule to improve Ab specificity. The target analyte (DCCA-glycine) molecule also possesses a cyclopropane ring that is also divided into the *cis*- and *trans*-isomers such as the parent permethrin. As another strategy, the haptens were designed so that each individual *cis*- and *trans*-isomer was obtained. This is both for the production of Ab specific to each isomer of the target and for the enhancement of sensitivity with heterologous *cis*/*trans* or *trans*/*cis* configurational immunoassay format that for example uses the *cis*-isomer for coating antigen with Ab raised against the *trans*-isomer of the immunogen.

For the production of a specific Ab capable of recognizing *N*-(*cis*/*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carbonyl)glycine, four different types of haptens as immunizing antigens were prepared. The target analyte (*cis*/*trans*-DCCA-glycine) with the carboxylic acid also served as *cis*/*trans*-hapten **3** with no spacer. It was synthesized via the Schotten−Boumann reaction with a synthetic procedure similar to that used for the PBA-glycine conjugate (*32*) (**Figure 2**). That is, the acid chloride of DCCA was reacted with glycine in alkaline solution and then acidified to obtain *cis*/*trans*-DCCA-glycine. *N*-(*cis*/*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carbonyl)-4-amino-L-phenylalanine (hapten **5**) was synthesized to preserve the carboxylic acid group of the target acid. Briefly, the acid chloride of *cis*/*trans*-DCCA was also reacted via the Schotten−Boumann reaction with 4-nitro-L-phenylalanine to give the *N*-acylated nitro intermediate **4**. Subsequent reduction of the nitro group with stannous chloride gave hapten **5**. To get high titer of Ab more rapidly (*37*), *N*-(*N*-(*cis*/*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carbonyl)glycine)amino-6-(2,4-dinitrophenyl)-aminohexanoic acid (hapten **9**) was synthesized by coupling *N*ϵ-2,4-dinitrophenyl-L-lysine to the target analyte according to the synthetic method by Watanabe et al. (*37*) (**Figure 3**). Finally, as a common modification of the acid moiety on the target, *cis*/*trans*-DCCA-glycine was reacted with bromo-butyric acid *tert*-butyl ester to give ester intermediate **23**. Subsequent alkaline hydrolysis with lithium hydroxide and acidification gave the four carbon spacer linker of *N*-(*cis*/*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carbonyl)glycine-4-oxobutanoic acid (hapten **24**) (**Figure 4**).

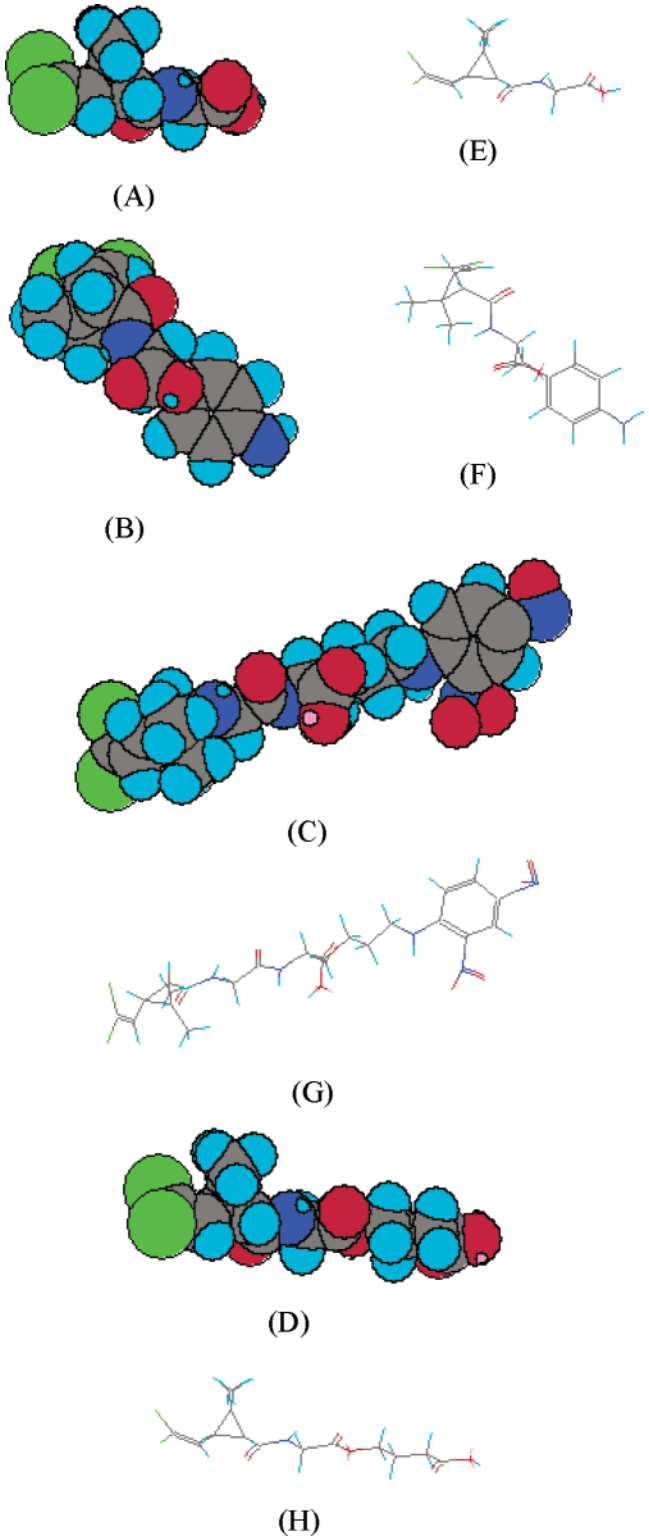

Figure 5. Optimized molecular model of (**A**,**E**) DCCA-glycine, (**B**,**F**) hapten **5**, (**C**,**G**) hapten **9**, and (**D**,**H**) hapten **24**; **A**–**D**, space filling models, and **E**–**H**, stick models.

Table 1. Antiserum Titer Response of Rabbits against Various Coating Antigens[a]

		coating antigen											
immunogen	antiserum	*cis*-**1**–BSA	*trans*-**1**–BSA	*cis*-**3**–BSA	*trans*-**3**–BSA	*cis*-**5**–BSA	*trans*-**5**–BSA	*cis*-**9**–BSA	*trans*-**9**–BSA	*cis*-**24**–BSA	*trans*-**24**–BSA	*cis*-**26**–BSA	*trans*-**26**–BSA
cis-hapten **3**–Thyr	3700	+++	+++	+++[b]	+++	+++	+++	+++	+++	+++	+++	+++	+++
	3701	+++	+++	+++[b]	+++	+++	++	+++	+++	+++	+++	+++	+++
trans-hapten **3**–Thyr	3702	+++	+++	+++	+++[b]	++	+++	+++	+++	+++	+++	+++	+++
	3703	+++	+++	+++	+++[b]	+++	+++	+++	+++	+++	+++	+++	+++
cis-hapten **5**–Thyr	3704	++	++	++	++	+++[b]	+++	++	++	+++	+++	+++	+++
	3705	+++	+++	+++	+++	+++[b]	+++	+++	+++	+++	+++	+++	+++
trans-hapten **5**–Thyr	3706	+++	+++	+++	+++	+++	+++[b]	+++	+++	+++	+++	+++	+++
	3707	+++	+++	+++	+++	+++	+++[b]	+++	+++	+++	+++	+++	+++
cis-hapten **9**–Thyr	3708	+	++	+++	+++	–	–	+++[b]	+++	+++	+	+	+++
	3709	++	+++	+++	+++	+	–	+++[b]	+++	+++	++	+++	+++
trans-hapten **9**–Thyr	3710	–	++	+	+	–	–	+++	+++[b]	++	+++	+	+++
	3711	+	++	++	+++	–	+	+++	+++[b]	+	++	+	+++
cis-hapten **24**–Thyr	3696	+++	+++	+++	+++	+++	+++	+++	+++	+++[b]	+++	+++	+++
	369	+++	+++	+++	+++	+++	+++	+++	+++	+++[b]	+++	+++	+++
trans-hapten **24**–Thyr	3698	++	+++	+++	+++	–	+	++	+++	+++	+++[b]	+++	+++
	3699	+++	+++	+++	+++	–	++	+++	+++	+++	+++[b]	+++	+++

[a] The data shown are at a coating antigen concentration of 1 μg/mL and an Ab dilution of 1:32 000; –, absorbance < 0.3; +, absorbance 0.3–0.6; ++, absorbance 0.6–0.9; and +++, absorbance > 0.9. [b] Homologous systems.

Additionally *cis*/*trans*-hapten **26** with a carboxylic acid was synthesized according to the same method as hapten **24**. *cis*/*trans*-DCCA-glycine was reacted with bromo-acetic acid *tert*-butyl ester to give ester intermediate **25**. The alkaline hydrolysis and acidification gave *N*-(*cis*/*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carbonyl)glycine-2-oxoacetic acid (hapten **26**) with a shorter two carbon space linker to serve as a coating antigen.

Molecular Modeling of Haptens. Although the planar chemical structures of haptens are shown to completely mimic the target analyte, their geometries can be different. The geometry study of the molecules is useful to select an immunizing hapten to produce Ab against the target analyte (*44*−*46*). The immunizing haptens synthesized were characterized by computing the molecules at their minimum energy levels. Observing the molecular models (**Figure 5**), the geometries of hapten **24** and the target analyte were completely matched, and the geometry of hapten **9** showed a small difference in the cyclopropane ring moiety of the molecules of the target and hapten **9**. On the contrary, that of hapten **5** did not exhibit any similar geometry to the model of the target analyte.

Hapten Conjugation. For immunogens, haptens with a carboxylic acid group were conjugated to Thyr by the sulfo-NHS ester method and with an amino group by the diazotization method. The sulfo-NHS conjugation method was used to obtain the more stable sulfo-NHS ester intermediate as compared to an NHS ester and thus more increased coupling yield (*47*). In this study, the carrier protein Thyr was chosen as an immunizing protein (*48*). For coating antigens, haptens were conjugated to BSA by the NHS ester and diazotization methods. This avoids recognition of protein−urea adducts that form with the use of water soluble diimides. *trans*-Hapten **9**−protein conjugates were confirmed by the UV/vis spectrophotometry. *trans*-Hapten **9** showed a characteristic peak at 360 nm distinguishing it from that of each protein. The hapten−protein conjugates also showed a peak at 360 nm. These results indicate that hapten was successfully conjugated to the protein by the sulfo-NHS method. In addition, the yellow color of the hapten **5**− and **9**−protein conjugates was an indicator of a successful coupling reaction, whereas haptens **1**, **3**, and **24** as white solids did not show any characteristic peak distinguished from that of protein. From the available amino groups according to the TNBS method of Habeeb (*49*), 20−61% of the *cis*/*trans*-haptens **1**, **3**, and **24** were conjugated in the hapten−Thyr or −BSA conjugates, suggesting successful hapten loading on the proteins.

Titers and Screening of the Antisera. For the production of Ab specific to the *cis*/*trans*-target analyte, the *cis*/*trans*-hapten−Thyr conjugates were injected a total of seven times into each rabbit. The antisera collected after each boost were subjected to titration by the homologous indirect ELISA. All of the antisera showed relatively constant high titers after the fifth immunization (data not shown). These results indicate that specific antibodies in the rabbit antiserum were produced against each immunogen. The final serum was collected 5 months following the first immunization and was used for the subsequent screening in search of antibodies specific to the target compound. Despite showing high titers of antisera after the fifth immunization, rabbits were boosted twice more to raise more sensitive and specific antibodies. None of the antisera demonstrated any significant affinity for BSA itself as a coating antigen.

The checkerboard titration was used for the screening of Ab and antigen combinations. All antisera had high titers in homologous systems, but in the heterologous systems, antisera produced against hapten **9**−Thyr and *trans*-hapten **24**−Thyr conjugates showed especially low titers (absorbance < 0.3) against the coating antigens, hapten **5**−BSA and *cis*-hapten **5**−BSA, respectively (**Table 1**).

All of the 16 antisera were screened against 12 coating antigens via the simple inhibition test at the two concentrations (10 and 1000 μg/L) of each individual *cis*- or *trans*-target analyte. There was no or low inhibition in homologous systems, whereas there were much higher inhibitions in heterologous systems (data not shown). The combinations of Ab and coating antigen with over 80% inhibition at the concentration of 1000 μg/L of the target analyte and with over 20% inhibition at the concentration of 10 μg/L were again screened at the 10 different concentrations of each target analyte.

As shown in **Table 2**, none of the antibodies generated against hapten **5**−Thyr had IC_{50} values below 3 μg/L for either the *trans*- or the *cis*-target analyte. The antisera specific to the target *trans*-DCCA-glycine were mainly raised against *trans*-haptens **3**−, **9**−, and **24**−Thyr except for *trans*-hapten **5**−Thyr. This result suggests that because the spacer arm of hapten **5** possessed a bulky aminophenyl functional group that may serve as an antigenic determinant, the hapten **5**−Thyr conjugate appeared

Table 2. Combination Data for Screening with Competitive Indirect ELISA against *cis*- or *trans*-DCCA-glycine[a]

analyte	immunogen	combination: antiserum/coating antigen	IC_{50} (μg/L)
trans-DCCA-glycine	*trans*-hapten **3**–Thyr	Ab 3702/*cis*-hapten **5**–BSA	26
		Ab 3703/*cis*-hapten **5**–BSA	1.2
	trans-hapten **5**–Thyr	Ab 3706/*cis*-hapten **9**–BSA	42
		Ab 3706/*cis*-hapten **26**–BSA	50
		Ab 3707/*cis*-hapten **1**–BSA	52
		Ab 3707/*cis*-hapten **9**–BSA	7.6
		Ab 3707/*cis*-hapten **26**–BSA	16
	trans-hapten **9**–Thyr	Ab 3710/*cis*-hapten **5**–BSA	1.2
		Ab 3710/*trans*-hapten **5**–BSA	5.8
		Ab 3711/*cis*-hapten **1**–BSA	81
		Ab 3711/*trans*-hapten **5**–BSA	25
	trans-hapten **24**–Thyr	Ab 3698/*cis*-hapten **3**–BSA	93
		Ab 3698/*cis*-hapten **5**–BSA	2.0
		Ab 3698/*trans*-hapten **5**–BSA	14
		Ab 3699/*cis*-hapten **1**–BSA	15
		Ab 3699/*cis*-hapten **5**–BSA	2.0
		Ab 3699/*trans*-hapten **5**–BSA	4.1
		Ab 3699/*cis*-hapten **9**–BSA	8.7
cis-DCCA-glycine	*cis*-hapten **3**–Thyr	Ab 3700/*cis*-hapten **5**–BSA	18
		Ab 3700/*trans*-hapten **5**–BSA	5.8
		Ab 3701/*cis*-hapten **5**–BSA	39
		Ab 3701/*trans*-hapten **5**–BSA	3.5
	cis-hapten **5**–Thyr	Ab 3704/*cis*-hapten **1**–BSA	84
	cis-hapten **9**–Thyr	Ab 3709/*cis*-haptne **1**–BSA	10
		Ab 3709/*trans*-hapten **1**–BSA	3.5
		Ab 3709/*trans*-hapten **3**–BSA	92
		Ab 3709/*cis*-hapten **5**–BSA	2.9
		Ab 3709/*trans*-hapten **5**–BSA	0.7
		Ab 3709/*trans*-hapten **26**–BSA	23
	cis-hapten **24**–Thyr	Ab 369/*cis*-hapten **5**–BSA	35
		Ab 369/*trans*-hapten **5**–BSA	3.7

[a] The analytes were prepared in the PBS solution.

to produce spacer specific antibodies, which resulted in lower specificity to the target analyte as compared to other Ab groups. Showing low IC_{50} values (<2 μg/L) for the *trans*-target analyte, the Ab coating antigen combinations of Ab 3703/*cis*-hapten **5**−BSA, Ab 3710/*cis*-hapten **5**−BSA, and Ab 3698/*cis*-hapten **5**−BSA were chosen for more study. The antisera specific to *cis*-DCCA-glycine were also generated against the *cis*-haptens **3**−, **9**−, and **24**−Thyr. Showing low IC_{50} values (<3 μg/L) for the *cis*-target analyte, the combinations of Ab 3701/*trans*-hapten **5**−BSA, Ab 3709/*trans*-hapten **5**−BSA, and Ab 369/*trans*-hapten **5**−BSA were also chosen. These results suggest that the structure of hapten **5** was not suitable to produce the Ab specific to the target but was suitable as a coating antigen in a heterologous system. With respect to the isomeric configuration of the molecule, the heterologous *cis*/*trans* or *trans*/*cis* configuration systems between Ab and coating antigen were much more sensitive than homologous configuration systems. This result was consistent with studies of immunoassays for the pyrethroids permethrin and cypermethrin (*36*, *50*). These assays also used the heterologous configuration. For an example, the combination of the Ab raised against the *trans*-permethrin hapten was used with a *cis*-coating antigen for a sensitive immunoassay.

Inhibition curves of the target analyte with six combinations of the antiserum and the coating antigen (**Figure 6**) resulted in IC_{50} values of 1.3, 2.1, and 2.2 μg/L for *trans*-DCCA-glycine and 0.4, 2.3, and 2.8 μg/L for *cis*-DCCA-glycine under unoptimized conditions, indicating that the sensitive immunoassay for the analysis of the target analyte can be developed.

CR. As seen in **Table 3**, the antibodies generated against the immunizing haptens of the *trans*-configuration had a higher

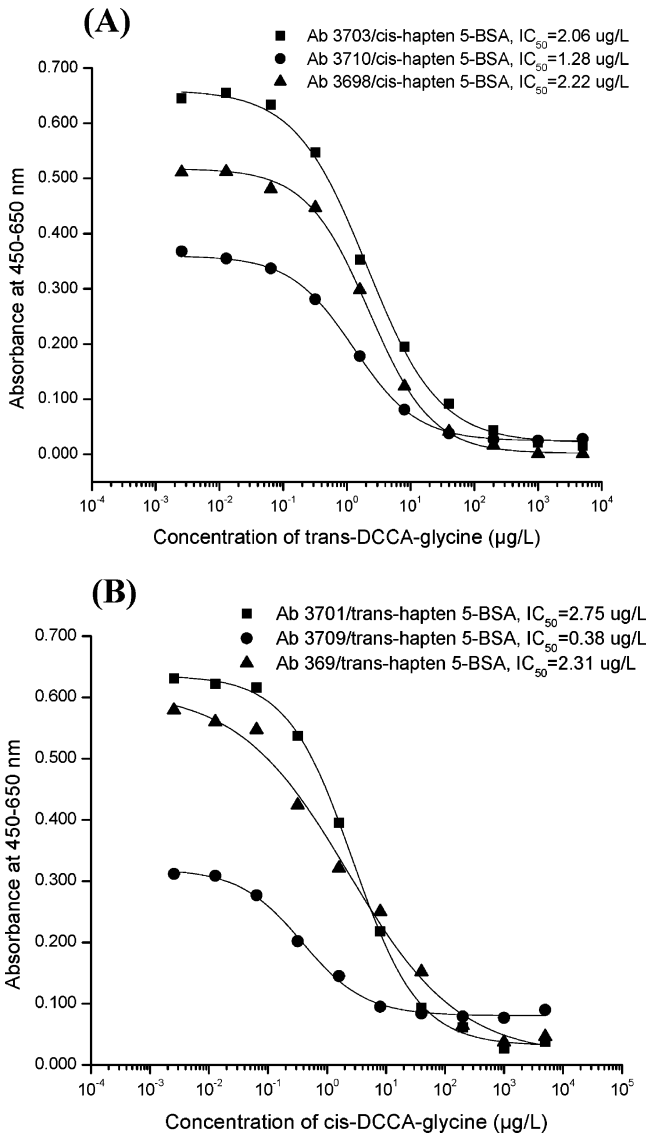

Figure 6. Six ELISA inhibition curves for *trans*-DCCA-glycine (**A**) and *cis*-DCCA-glycine (**B**) under unoptimized conditions. The reagent concentrations were as follows: the assay for *trans*-DCCA-glycine using Ab 3703, 1:32 000; Ab 3710, 1:8000; and Ab 3698, 1:16 000; and coating antigen *cis*-hapten **5**–BSA, 1, 5, and 2 μg/mL, respectively. The assay for *cis*-DCCA-glycine using Ab 3701, 1:16 000; Ab 3709, 1:8000; and Ab 369, 1:16 000; and coating antigen *trans*-hapten **5**–BSA, 1 μg/mL. The dilution ratios of the Ab are the final concentrations in the wells.

affinity for the *trans*-configuration of target analyte than the *cis*-configuration, whereas the antibodies generated against the *cis*-configuration had a higher affinity for the *cis*-target analyte. The CRs of the tested *trans*-antibodies to *cis*-DCCA-glycine were below 28%. The Ab 3703 showed relatively higher CR to *cis*-DCCA-glycine than that of other testes antibodies, Ab 3710 and Ab 3698, indicating the mixture of *cis*/*trans*-DCCA-glycine can be analyzed using this Ab. Additionally, the shorter linker of hapten presents the hapten relatively close to the protein carrier surface; thus, the Ab produced against the hapten can provide broader selectivity to the structurally related compounds (*47*). This theory was applicable to Ab 3703. Antibodies produced against the *trans*-isomer of haptens **9** and **24** with longer linkers showed a lower CR to the *cis*-target analyte. The Ab (Ab 3703) raised against *trans*-hapten **3** (*trans*-DCCA-glycine) with no spacer displayed a better recognition to the *cis*-isomer of the target analyte than that of other antibodies

Table 3. CR (%) of the Selected Antibodies to Permethrin Metabolites and Other Structurally Related Compounds[a]

	IC_{50} (CR [%])		
compound	Ab 3703/*cis*-**5**–BSA	Ab 3710/*cis*-**5**–BSA	Ab 3698/*cis*-**5**–BSA
trans-DCCA-glycine	1.9 (100)	2.6 (100)	0.8 (100)
cis-DCCA-glycine	6.7 (28)	18 (15)	37 (2)
trans-DCCA	430 (0.40)	808 (0.30)	>8200 (<0.01)
permethrin	4475 (0.04)	2357 (0.11)	2357 (0.04)
glycine	>19 000 (<0.01)	>26 000 (<0.01)	>8200 (<0.01)
PBA-glycine	>19 000 (<0.01)	>26 000 (<0.01)	>8200 (<0.01)

	IC_{50} (CR [%])		
	Ab 3701/*trans*-**5**–BSA	Ab 3709/*trans*-**5**–BSA	Ab 369/*trans*-**5**–BSA
cis-DCCA-glycine	3.1 (100)	0.7 (100)	6.2 (100)
trans-DCCA-glycine	194 (1.61)	179 (0.38)	322 (1.93)
cis-DCCA	590 (0.53)	22 (3.00)	2947 (0.21)
permethrin	>31 400 (<0.01)	>6700 (<0.01)	>61 900 (<0.01)
glycine	>31 400 (<0.01)	>6700 (<0.01)	>61 900 (<0.01)
PBA-glycine	>31 400 (<0.01)	>6700 (<0.01)	>61 900 (<0.01)

[a] The antibodies, Ab 3703, Ab 3710, and Ab 3698, were produced against the *trans* configuration of immunogens, and the antibodies, Ab 3701, Ab 3709, and Ab 369, were produced against the *cis* configuration of immunogens. The compounds were prepared in the PBS solution.

(Ab 3710 and 3698). The ELISA combined with Ab 3703 and coating antigen *cis*-hapten **5**−BSA can be good for the detection of total *cis*/*trans*-DCCA-glycine. All of the antibodies showed no or very little CRs to free permethrin metabolite (DCCA), permethrin, PBA-glycine conjugate, and glycine, suggesting that the antibodies are very selective for the target structure.

Unlike the above antibodies produced against the *trans*-immunogens, the antibodies, Ab 3701, Ab 3709, and Ab 369, generated against the *cis*-configuration, had a low CR to the *trans*-configuration. The antibodies showed a high selectivity for the *cis*-target analyte. These antibodies also showed very low CRs to other structurally related compounds. On the basis of these results, it is thought that the most antigenic determinants of the target analyte for the Ab production are the glycine moiety (−$NHCH_2COO$−), and the *cis*- or *trans*-isomeric configuration of the structure.

Discussion. The toxicity of permethrin is dependent on its three-dimensional configuration. The *cis*-isomer is more toxic than the *trans*-isomer. However, *trans*-permethrin is metabolized more rapidly than *cis*-permethrin in mammals (*2*, *51*). The *trans*-permethrin predominates (60−75%) in the commercial product. The amount of *trans*-DCCA metabolite not conjugated and/or after acid hydrolysis of conjugates of permethrin in human urine ranged from 65 to 87% (*52*, *53*). The ratios of conjugates of *cis*- and *trans*-DCCA might be similar to that of free *trans*-DCCA. No information is reported about the ratios of the free conjugates of the metabolites. The immunoassay with *cis* or *trans* specific Ab will be developed for separate analysis of each *cis*- and *trans*-isomer as well as the analysis of total isomers, depending upon CR studies of different ratios of the *cis*- and *trans*-mixtures.

On the basis of the optimized model of the hapten molecules, all of the immunizing haptens except for hapten **5** were fairly good matches to the geometry of the target analyte. As a result, they generated specific antibodies to the target, which resulted in a sensitive and selective immunoassay. Therefore, using a molecular modeling technique can help with reasonable hapten designs to develop a sensitive immunoassay. Finally, a very sensitive ELISA has been obtained by using both heterologous hapten structure and configuration systems between antibodies and coating antigen. The IC_{50} values are as low as 1.3−2.2 μg/L for *trans*-DCCA-glycine and 0.4−2.8 μg/L for *cis*-DCCA-glycine. Depending in part on the application, the optimum Ab for the assay will be selected. The Ab 3703 generated against the *trans*-immunogen provided broad selectivity for the *cis*-target analyte. The ELISA with the combination of Ab 3703 and coating antigen, *cis*-hapten **5**−BSA, can be good for detection of the *cis*- and *trans*-isomers of DCCA-glycine in the sample. The immunoassay will be optimized with the parameters such as detergent, solvent content, pH, and ionic strength in the assay buffer for the best sensitivity and reproducibility, providing a powerful tool for human monitoring for pyrethroid exposure.

ABBREVIATIONS USED

Ab, antibody; Thyr, thyroglobulin; BSA, bovine serum albumin; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; FAB-MS, fast atom bombardment mass spectrum; GAR-HRP, goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase; IC_{50}, concentration of analyte giving 50% inhibition; sulfo-NHS, *N*-hydroxysulfosuccinimide sodium salt; OD, optical density; PBST, phosphate-buffered saline with 0.05% of Tween 20; TMB, tetramethylbenzidine; DCCA, 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid; DCCA-glycine, *N*-(3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carbonyl)-glycine; PBOH, 3-phenoxybenzyl alcohol; PBA, 3-phenoxybenzoic acid.

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