# Development of an Enzyme-Linked Immunosorbent Assay for Azadirachtins†

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An enzyme-linked immunosorbent assay for azadirachtin A, a bioactive tetranor−triterpenoid from the neem tree (*Azadirachta indica* A. Juss), was developed. The conjugates were synthesized by employing the hemisuccinate method and an ester activation method, which were previously developed. Four rabbits were immunized with either 7-hemisuccinylazadirachtin−keyhole limpet hemocyanin (KLH) or 3-hemisuccinylazadirachtin−KLH. All sera were tested against the coating antigens 7-hemisuccinylazadirachtin−bovine serum albumin (BSA) and 3-hemisuccinylazadirachtin−BSA. From this screening procedure, an assay was developed using antiserum 7056 . The assay showed an $IC_{50}$ value of about 100 ppb, with a range of detection from 10 to 1000 ppb for azadirachtin A and related compounds. Thus, the method presented is a step forward in terms of inexpensive, fast, and sensitive (improvement by a factor of 100) analysis of azadirachtin and should provide a method that can easily be used more widely.

**Keywords:** *Azadirachtin; immunoassay; neem*

## INTRODUCTION

Azadirachtin is a secondary plant product of the neem tree (*Azadirachta indica* A. Juss). It is the main active component of neem seeds and a prime example of biorational insecticides that disturb an insect's development rather than the biochemical or metabolic activity of enzymes that are found more ubiquitously in nature (Schmutterer and Rembold, 1980). Because of its selective action against target pest insects (Schmutterer, 1988) and low mammalian toxicity (Schmutterer, 1990), it is an insecticide of considerable environmental safety (Schmutterer, 1995). Nevertheless, both ecotoxicological studies and quality control of this natural product were hindered by difficulties in setting up sufficiently sensitive and inexpensive methods for analytical evaluation. Current methods utilizing high-performance liquid chromatography−ultraviolet spectrophotometry (Schneider and Ermel, 1987) are expensive and not acceptably sensitive. Widely variable amounts of azadirachtin in the biological raw material (neem kernels), depending on origin and storage conditions, pose serious problems regarding quality assurance of biorational pesticides based on azadirachtin. However, quick monitoring of large numbers of samples for product quality control and environmental fate studies demands more cost-effective screening methods with the least amount of sample pretreatment. Immunochemical assays offer such a desired screening method, because they are very sensitive, often need little or no sample cleanup or preparation, and can be applied to numerous samples simultaneously (Karu *et al.*, 1992). The synthetic strategy for the design of antigens, the subsequent development of antibodies, and the main characteristics of the immunoassay for azadirachtin A are presented in this paper.

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## MATERIALS AND METHODS

**Chemicals.** Azadirachtin A (compound **1**; Figure 1) was extracted from neem kernels (Schmutterer and Rembold, 1980) and purified according to a procedure similar to a multilayer countercurrent chromatography (MLCCC) method for marrangin (Hein and Hummel, 1995) to a purity of approximately 90%. Immunochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Other chemical reagents were obtained from Aldrich Chemical Co. (Milwaukee, WI). Solvents for synthesis were distilled and dried; solids were recrystallized and dried prior to reaction. The borate buffer for protein conjugation consisted of 1.9 g of $Na_2B_4O_7{\cdot}10H_2O$, 0.9 g of NaCl, and 0.02 g of $NaN_3$ in 100 mL of $H_2O$, pH adjusted to 7.8 with 1 M HCl; 0.1 M PBS solution consisted of 8 g of NaCl, 1.15 g of $Na_2HPO_4$, 0.2 g of $KH_2PO_4$, and 0.2 g of KCl in 100 mL of $H_2O$ adjusted with HCl to pH 7.5. PBST consisted of 0.01 M PBS with 0.05% Tween 20.

**Instruments.** Normal phase thin-layer chromatograms (TLC) were performed on 0.25 mm precoated silica gel F254 plastic sheets from Merck (Darmstadt, Germany) and evaluated by iodine vapor staining. $^1$H nuclear magnetic resonance ($^1$H-NMR) spectra were obtained with a Bruker QE-300 spectrometer (Billerica, MA) operating at 300.1 MHz. Chemical shifts ($\delta$) are given relative to tetramethylsilane (TMS) as an internal reference. Mass spectra were obtained with a quadrapole mass spectrometer VG Trio-2 (VG Masslab, Altrincham, U.K.) by electron impact ionization (EI). Immunoassay absorbances were read with a Spectra Max 250 microplate reader (Molecular Devices, Menlo Park, CA) in dual-wavelength mode (450−650 nm). The binding and competition curves were analyzed with a commercial software package (Softmax Pro 1.20, Molecular Devices) using a four-parameter logistic equation. Unless otherwise mentioned, all data presented from ELISA experiments correspond to the average of at least three well replicates on one plate. Polystyrene microtiter plates were purchased from Nunc (Maxisorb, Roskilde, Denmark, catalog no. 442404).

**Preparation of Haptens.** *Preparation of 7-Hemisuccinylazadirachtin (**2a**).* Freshly distilled dry pyridine (8 mL) and

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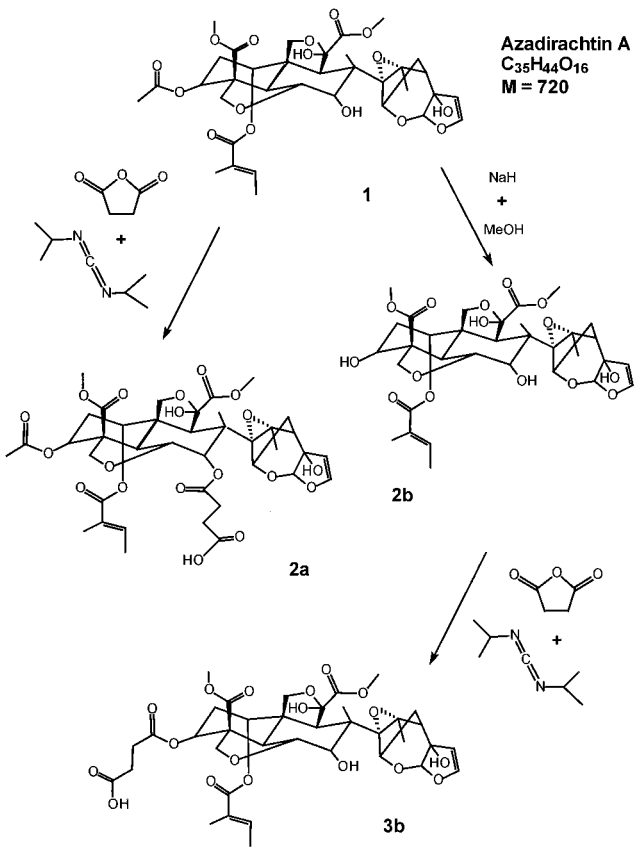


**Figure 1.** Synthesis of haptens **2a** and **3b** used for immunogens and for coating antigens.

60 mg of dry **1** (5 h, 0.03 Torr) were mixed under nitrogen flushing. Dry (5 h, 0.03 Torr) succinic anhydride (100 mg), followed by 50 $\mu$L of diisopropylcarbodiimide (99%) and a few crystals of 4-(dimethylamino)pyridine (DMAP), was transferred with the aid of a nitrogen hood into the nitrogen-flushed solution. The mixture was stirred overnight at room temperature in the dark. The pyridine solvent was separated from the precipitated diisopropylurea and was evaporated (4 h, 0.03 Torr) to yield 173 mg of a yellow oil. Analytical TLC (solvent system 1, toluene/ethyl acetate/methanol, 5:1:2) showed only one additional spot at $R_f = 0.08$; preparative TLC yielded 30 mg (44% with respect to **1**) of a white glassy product, which was identified by NMR as 3-hemisuccinylazadirachtin (**2a**). This compound was rather unstable and, therefore, was used immediately in further synthesis (15 mg) and for identification (15 mg). The degradation half-life time in solvents such as $CDCl_3$ and DMSO-$d_6$ at 20 °C was about 30 min and at −20 °C about 2 h (kinetics determined by TLC). Therefore, only $^1$H-NMR (DMSO-$d_6$) spectra of mixtures of the parent compound and the degradation products could be obtained. The esterification of the free 7-OH moiety was concluded from the disappearance of the broad peak in $^1$H-NMR (DMSO-$d_6$) at 3.0 ppm (br s, 1 H, 7-OH) and the observation of a new peak at 2.65 ppm [br s, 4 H, $(CH_2)_2$] and of a peak shift from 4.75 ppm (s, 1 H, 7) to 5.26 ppm (s, 1 H, 7).

*Preparation of 3-Deacetylazadirachtin (**2b**).* A three-neck, 25-mL round-bottom flask with a magnetic stirring bar, two rubber septa, and one glass stopper was flushed with nitrogen. Anhydrous methanol (10 mL) and 200 mg of dry (5 h, 0.03 Torr) azadirachtin (**1**) were mixed under a stream of nitrogen. Sodium hydride (120 mg; 60% in mineral oil) was transferred with the aid of a nitrogen hood into the nitrogen-flushed azadirachtin solution. The mixture was allowed to stir for 30 min in the dark. Thereafter, excess of $NH_4Cl$ was added and the solvent was evaporated in a stream of nitrogen. The residue was resuspended in dry $CH_2Cl_2$ and filtered. Evaporation of the filtrate yielded 220 mg of a yellow-orange oil showing four spots on analytical TLC. Preparative TLC ($R_f = 0.22$, solvent system 1) yielded 63 mg (32% with respect to **1**) of a white glassy product, which was characterized by NMR and MS as 3-deacetylazadirachtin (**2b**). The bis(hemisuccinates) from succinylation of both the 3- and 7-hydroxy groups had a much lower $R_f$ on TLC. Thus, we were able to monitor its appearance. Using the reaction conditions described, both TLC and NMR indicate that none of the bis derivative was formed. In solution, **2b** was rather unstable and, therefore, was used immediately in further synthesis (50 mg) and for identification (13 mg). $^1$H NMR ($CDCl_3$) $\delta$ 6.85 (dq, $Jd = 1.0$ Hz, $Jq = 7.0$ Hz, 1 H, 3′), 6.44 (d, $J = 2.9$ Hz, 1 H, 23), 5.65 (s, 1 H, 21), 5.04 (d, $J = 2.9$ Hz, 1 H, 22), 5.02 (s, 1 H, 11-OH), 4.74 (s, 1 H, 7), 4.65 (d, $J = 3.3$ Hz, 1 H, 15), 4.59 (dd, $J = 2.6$, 12.4 Hz, 1 H, 6), 4.21 (d, $J = 10.0$ Hz, 1 H, 19a), 4.05 (d, $J = 8.7$ Hz, 1 H, 28b), 3.76 (s, 3 H, methyl ester), 3.69 (s, 3 H, methyl ester), 3.60 (d, $J = 9.9$ Hz, 1 H, 19b), 3.20 (d, $J = 12.4$ Hz, 1 H, 5), 3.00 (br, 1 H, 7-OH), 2.75 (s, 1 H, 20-OH), 2.37 (d, $J = 5.2$ Hz, 1 H, 17), 2.1 (br, 1 H, 3-OH), 2.00 (s, 3 H, 18), 1.85 (s, 3 H, 5′), 1.78 (d, $J = 7.0$ Hz, 3 H, 4′), 1.75 (s, 3 H, 30); EI-MS (70 eV), *m/e* (rel intensity) 95 (100), 151 (54), 195 (4.5), 229 (4.5), 277 (3.3), 347 (3.7), 403 (2.1), 475 (1.7), 507 (1.3), 517 (11.7), 521 (1.3), 535 (0.7), 546 (1.9), 561 (1.5), 579 (1.7), 601 (0.6), 619 (0.2) (no parent ion at 677).

*Preparation of 3-Hemisuccinylazadirachtin (**3b**).* The preparation of this compound was done in analogy to the preparation of compound **2a**. Freshly distilled dry pyridine, 8 mL, and 40 mg of dry (5 h, 0.03 Torr) **2b** were mixed under nitrogen flushing. Dry (5 h, 0.03 Torr) succinic anhydride (100 mg), followed by 50 $\mu$L of diisopropylcarbodiimide and a few crystals of DMAP, was transferred into the nitrogen-flushed solution. The mixture was stirred overnight at room temperature in the dark, yielding 197 mg of a light yellow solid after separation of the precipitate and evaporation of the solvent. Analytical TLC showed only one additional spot at $R_f = 0.06$ (solvent system 1); preparative TLC yielded 31 mg (68% with respect to **2b**) of a white glassy product, which was identified by NMR as 3-hemisuccinylazadirachtin **3b**. The bis(hemisuccinate) from succinylation of both the 3- and 7-hydroxy groups had a much lower $R_f$ on TLC. Thus, we were able to monitor its appearance. Using the reaction conditions described, both TLC and NMR indicate that none of the bis derivative was formed. The formation of detectable amounts of **2a** was avoided by optimization of reaction time. In TLC solvent and NMR solvent systems, **3b** was rather unstable and, therefore, was used immediately for further synthesis (16 mg) and characterization (15 mg). The degradation half-life time in DMSO-$d_6$ solvent at 20 °C was about 20 min and at −20 °C about 2 h (kinetics determined by TLC). Therefore, only $^1$H-NMR (DMSO-$d_6$) spectra of mixtures of the parent compound and the degradation products could be obtained. The esterification of the free 3-OH moiety was concluded from the disappearance of the broad peak in $^1$H-NMR (DMSO-$d_6$) at 2.07 ppm (br s, 1 H, 3-OH) and the observation of a new peak at 2.65 ppm (br s, 4 H, $(CH_2)_2$) and of a peak shift from 4.84 (s, 1 H, 7) to 5.36 ppm (s, 1 H, 7).

**Preparation of Hapten−Protein Conjugates.** Haptens **2a** and **3b** were conjugated to bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), and tyramine. Tyramine conjugate formation was monitored by TLC to optimize the conjugation conditions. Spectrophotometric examination of the conjugation products was not possible because of the weak absorbance of the haptens in the UV−vis range.

*Preparation of the Activated 7-Hemisuccinylazadirachtin (**3a**).* A three-neck, 25-mL round-bottom flask with a magnetic stirring bar, two rubber septa, and one glass stopper was flushed with nitrogen. Anhydrous tetrahydrofuran (THF, 5 mL) and 15 mg of dry (5 h, 0.03 Torr) **2a** were mixed under nitrogen flushing. Dry (5 h, 0.03 Torr) *N*-hydroxysuccinimide (10 mg) was transferred with the aid of a nitrogen hood into the nitrogen-flushed solution. The mixture was stirred overnight on ice in the dark. The solvent was evaporated in a stream of nitrogen to yield 24 mg of a pale yellow solid that showed one additional spot in TLC with $R_f = 0.67$ (solvent system 1). Preparative TLC yielded 10 mg (59% with respect **2a**) of **3a**, as a white solid. The unstable and highly moisture sensitive compound was used immediately for further synthesis.

*Preparation of the Activated 3-Hemisuccinylazadirachtin (**4b**).* Dry (5 h, 0.03 Torr) **3b** (16 mg) was treated analogously to the preparation procedure of **3a**. The solvent was evaporated in a stream of nitrogen to yield 27 mg of a pale yellow solid that showed one additional spot in the analytical TLC with $R_f = 0.72$ (solvent system 1). Preparative TLC yielded 10 mg (56% with respect to **3b**) of **4b**, a white solid. The unstable and highly moisture sensitive compound was used immediately for further synthesis.

*Preparation of the BSA Conjugates.* These reactions were conducted in 5-mL glass flasks containing magnetic stirring bars. BSA (fraction V, 96−99%) was dissolved (10 mg) in 2 mL of 0.2 M borate buffer, and 0.2 mL of dimethylformamide (DMF) was added very slowly (ca. 15 min) with rigorous stirring at room temperature, causing a slight precipitation. A solution of 5 mg of active ester **(3a** or **4b)** in 0.2 mL of anhydrous DMF was added slowly (ca. 20 min). A small amount of precipitate developed during this addition. The mixture was stirred gently overnight on ice and then dialyzed versus 0.2 M PBS (five changes of 2 L each, 5 days, at room temperature) in a SpectraPor membrane (6000−8000 MW cutoff) to desorb and separate hapten molecules that were bound to the protein by physisorption only. The dialyzed solution was transferred into polypropylene vials and stored at −30 °C.

*Preparation of the KLH Conjugates.* The reactions were conducted analogous to the preparation of BSA conjugates. Precipitate developed during the addition of the active ester **(3a** or **4b)**. The dialyzed solution was transferred into polypropylene vials and stored at −30 °C.

*Preparation of Tyramine Derivatives.* The reaction was conducted in a 5-mL glass flask containing a magnetic Teflon stirring bar. Tyramine hydrochloride (99%), 10 mg, was dissolved in 2 mL of 0.2 M borate buffer. DMF (0.2 mL) was added very slowly (ca. 15 min) during rigorous stirring at room temperature, causing a slight precipitation. The active ester **(3a** or **4b)** was dissolved (5 mg) in 0.2 mL of anhydrous DMF and added very slowly (ca. 20 min). A slight precipitate developed during this addition. The solution was stirred gently overnight on ice, and the solvent was subsequently evaporated (24 h, 0.03 Torr). The residue was redissolved in 2-propanol and subjected to preparative TLC with $R_f = 0.22$ (solvent system 2, toluene/1-propanol, 5:1), yielding 1.4 mg (25% with respect to the active ester) of the 7-hemisuccinylazadirachtin−tyramine derivative. The yield of the 3-hemisuccinylazadirachtin−tyramine derivative was only 0.8 mg (14% with respect to the active ester). Therefore, the similar $R_f$ value on TLC examination with $R_f = 0.23$ (solvent system 2) was considered as tentative identification; 7-hemisuccinylazadirachtin−tyramine was characterized by NMR and MS: $^1$H NMR ($CDCl_3$) $\delta$ 7.05 (m, 4H, aromatic), 6.90 (dq, $Jd = 1.0$ Hz, $Jq = 7.0$ Hz, 1H, 3′), 6.82 (dd, $J = 2.1$, 9 Hz, 4H, 7′), 6.60 (br, 1H, NH), 6.46 (d, $J = 2.9$ Hz, 1H, 23), 5.65 (s, 1H, 21), 5.05 (d, $J = 2.9$ Hz, 1H, 22), 5.02 (s, 1H, 11-OH), 4.76 (s, 1H, 7), 4.65 (d, $J = 3.3$ Hz, 1H, 15), 4.61 (dd, $J = 2.4$, 12.6 Hz, 1H, 6), 4.17 (d, $J = 9.5$ Hz, 1H, 19a), 4.05 (d, $J = 8.9$ Hz, 1H, 28b), 3.76 (s, 3H, methyl ester), 3.68 (s, 3H, methyl ester), 3.64 (d, $J = 9.8$ Hz, 1H, 19b), 3.30 (d, $J = 12.4$ Hz, 2H, 5, 9), 3.2 (br, 1H, tyramine-OH) 2.9 (br, 1H, 20-OH), 2.37 (d, $J = 5.2$ Hz, 1H, 17), 2.03 (s, 3H, 18), 1.85 (s, 3H, 5′), 1.78 (d, $J = 7.0$ Hz, 3H, 4′), 1.75 (s, 3H, 30); EI-MS (70 eV), *m/e* (rel intensity) 95 (90), 105 (100), 120 (65), 143 (23), 151 (54), 195 (4.5), 229 (4.0), 277 (3.1), 347 (3.4), 403 (2.1), 475 (1.8), 507 (1.3), 521 (1.2), 535 (1.4), 545 (1.3), 559 (3.7), 595 (9.5), 588 (1.1), 620 (0.8), 661 (0.3), 704 (0.4), 719 (0.1) (no parent ion at 955).

**Immunization of Rabbits.** Rabbits (female New Zealand, white, 3−5 kg) 7052 and 7054 were immunized with **2a**−KLH, and rabbits 7053 and 7056 were immunized with **3b**−KLH. Rabbits were injected intradermally (5−20 sites per animal) with 100 $\mu$g of the corresponding hapten−protein conjugate. For injection, the immunogens were dissolved in 0.9% sterile NaCl solution and mixed 1:1 with Freund's complete adjuvant to a final volume of 500 $\mu$L/injection. For the boost immunization, the immunogens were prepared in the same way except Freund's incomplete adjuvant was used. The boosting was started 1 month after the initial immunization and was repeated every 4 weeks. Animals were bled (2−5 mL) 10 days after each boost from the central ear artery under light anesthesia. All procedures, except for the preparation of the immunogens, were carried out by personnel of the Laboratory Animal Health Clinic at the University of California at Davis. The blood was kept overnight at 4 °C to allow clotting. After centrifugation (500*g*, 10 min), removal of the clot, and a second centrifugation (500*g*, 10 min), the clear serum was carefully removed with a Pasteur pipet, aliquoted, and stored in cryovials at −20 °C.

**Screening of Antisera with Two-Dimensional Titration.** For the determination of initial titers of each serum, a microtiter plate (Nunc) was coated with 100 $\mu$L/well of a dilution series of coating antigen (0.016−2 $\mu$g/mL) and an additional row with an indifferent fenvalerate-conjugated BSA antigen (2 $\mu$g/mL) in 0.5 M carbonate buffer (pH 8.5). Plates were sealed with adhesive plate sealers and incubated overnight at 4 °C. The following day, the coated plates were washed three times with PBST. Diluted serum (100 $\mu$L/well) was added to each well and incubated for 2 h at room temperature. The sera were checked in a two-dimensional titration dilution series (1:500 to 1:256000 and two controls containing a fenvalerate antiserum 1:20000 in 0.2 M PBS, pH 7.5) against the dilution series of coating antigens. After another washing step, 100 $\mu$L/well of goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP; GAR/HRP), diluted 1:8000 in 0.2 M PBS, pH 7.5, was added and incubated for 2 h at room temperature. The plates were washed again, and 100 $\mu$L/well of substrate solution [800 $\mu$L of a 1.2% 3,3′,5,5′-tetramethylbenzidine (TMB) in DMSO and 13.2 $\mu$L of 30% $H_2O_2$ in 100 mL of a 0.1 M acetate buffer, pH 5.5] was added. After 10−20 min, the enzyme reaction was stopped with 50 $\mu$L/well of 4 M $H_2SO_4$. The absorbance was read at 450−650 nm.

**ELISA Coating Antigen Format. Competition.** To determine the analytical characteristics of the ELISA coating antigen format for each serum, one microtiter plate was divided into two equal (12 columns, 3 rows) parts and one unequal (12 columns, 2 rows) part. The equal parts were coated with 100 $\mu$L/well of the two coating antigens **2a**−BSA and **3b**−BSA, and the unequal part was coated with a fenvalerate−BSA conjugate as a protein control, with the optimized concentrations (0.03 $\mu$g/mL **2a**−BSA, 1 $\mu$g/mL **3b**−BSA, 1 $\mu$g/mL fenvalerate−BSA) overnight at 4 °C. The next day, the coated plates were washed three times with PBST and the analyte (0.0013−100 $\mu$g/mL in 0.2 M PBS, pH 7.5, 50 $\mu$L/well) and 50 $\mu$L/well of the optimized dilution of the antiserum (no. 7053 or 7056, 1:20000; no. 7052 or 7054, 1:100000 in 0.2 M PBS, pH 7.5) were added and incubated for 2 h at room temperature. After another washing step, 100 $\mu$L/well of GAR/HRP, diluted 1:8000 in 0.2 M PBS, pH 7.5, was added and incubated for 1 h at room temperature. The plates were washed again, and 100 $\mu$L/well of substrate solution was added; after 10−20 min, the enzyme reaction was stopped with 50 $\mu$L/well 4 M $H_2SO_4$. The absorbance was read at 450−650 nm.

## RESULTS AND DISCUSSION

**Hapten Synthesis.** Since antibodies recognize best the part of the hapten that is most distant from the conjugate linkage, a carboxylic group opposite from the most characteristic groups of the molecule was desirable. However, the complex structure of azadirachtin with a densely packed array of oxygen functionalities of many different types made it difficult to determine which portions of the molecule are most unique. In the course of studies on azadirachtin, it was determined (Simmonds *et al.*, 1995a) that there is not much change in the biological activity of azadirachtin when the acyl groups attached to the 1- and 3-hydroxys are modified or removed. It is known from extensive synthetic work (Grossmann and Ley, 1994) on azadirachtin that the 3-hydroxy group of azadirachtin is readily saponified

and re-esterified in good yield without concomitant rearrangement of the azadirachtin skeleton. Therefore, the 3-position was chosen for attachment of the handle to provide optimization of recognition and the 7-position for reason of simplicity because no saponification prior to derivatization was necessary at the free 7-hydroxy group.

For reasons of literature precedence (Wing *et al.*, 1978) and commercial availability, succinic acid was chosen as a linker to join azadirachtin to the carrier protein. Since azadirachtin is a comparably large molecule, the succinate derivatization should not present a serious handle recognition problem. Ester formation was used as a means of linking because it is a mimic of the natural derivatives, especially the 3′-acyl compound. Because of the short linker group, the attaching ester bond should be shielded by the large azadirachtin molecule from ester hydrolase-catalyzed hydrolysis.

Purification problems due to insufficient separation of azadirachtin−hemisuccinate from succinic acid by TLC could be solved by switching the solvent from THF (high solubility of succinic acid) to pyridine (low solubility for succinic acid). When the deacetylated **2b** was reacted with hemisuccinate, we initially expected a mixture of succinyl groups at both the 3- and 7-positions. However, these monosuccinylated derivatives were easily distinguished by their $R_f$ on TLC. TLC thus indicated that under the reaction conditions used, no disuccinate was formed and no detectable 7-hemisuccinylazadirachtin was present in the 3-hemisuccinylazadirachtin preparation. This chromatographic evidence was supported by NMR data. Although this observation was initially surprising, the ease with which the 3-position of azadirachtin is deacylated and selectively reacylated (Grossmann and Ley, 1994) indicates that this position is sufficiently reactive to allow a selective reaction to occur without acetylating the other secondary hydroxy group. The stability of the hapten molecule is low, because of the destabilizing effect of the free carboxylic group. The intrinsic acidity of the compound may be causing rapid degradation of the hapten molecule. The short chain length of the spacer was chosen to reduce intramolecular reactions. An approach using an $\omega$-amino acid as linker (Grossmann and Ley, 1994) might yield more stable haptens.

**Conjugation of Haptens to Proteins and Confirmation of Conjugation.** Activation of the carboxylic acid by *N*-hydroxysuccinimide was utilized to suppress de- and re-esterifications within the azadirachtin molecule. Using a low reaction temperature significantly decreased the amount of byproducts. Solubility problems of either azadirachtin or protein were solved by employing a DMF−water mixture as a solvent for performing hapten−protein conjugation (Karu *et al.*, 1992). An estimate of hapten density could not be achieved by spectrophotometric methods, since the absorption of the azadirachtin molecule and the handle is weak within the UV−vis range. Therefore, we used tyramine as a mimic of protein $NH_2$ moieties. This led to an estimate of 0.6 mg of hapten (20% yield)/10 mg of protein. Using a molecular mass of 68 000 amu for BSA, a hapten density of 4 per BSA-molecule was calculated. The same quantity of tyramine hydrochloride (10 mg) was used. Because of the lower molecular mass of tyramine−HCl, however, tyramine was in a molar excess compared to BSA. Thus, tyramine conjugate yields may be elevated. Moreover, not every amino acid of BSA or KLH is capable of binding haptens. Therefore, the estimate of four hapten molecules attached to a protein molecule might be too high. An approach using a handle with dye properties (Sashidar *et al.*, 1994) would facilitate an estimate of the success of conjugation.

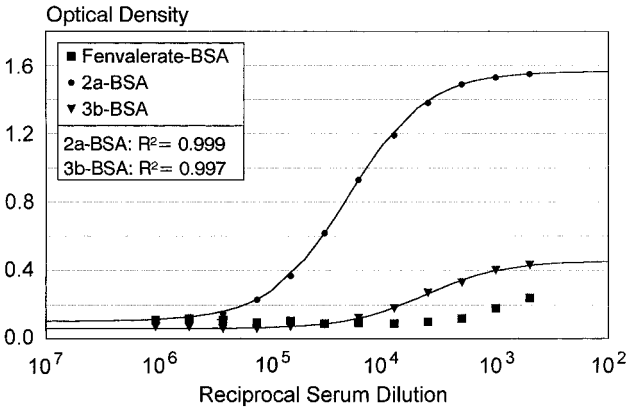


**Figure 2.** Antibody binding of conjugated azadirachtin. ELISA was used to evaluate the binding of antibodies raised against **2a**−KLH to 96-well microplates coated with fenvalerate-conjugated BSA or the conjugates **2a**−BSA or **3b**−BSA. Microplates coated with the conjugates were exposed to various dilutions of antiserum (concentration × 1000). Similar experiments were repeated for other antigen−antibody combinations to determine effective antibody titers.

**ELISA Results: Recognition of Conjugated Azadirachtin.** Antibodies raised against KLH conjugates of haptens **2a** and **3b** were tested by ELISA for binding to conjugates of the same haptens to BSA. Figure 2 shows the binding of one selected antiserum (anti-**2a**−KLH) to **2a**−BSA, **3b**−BSA and a fenvalerate-conjugated BSA as a negative control, confirming the covalent attachment of **2a** and **3b** to BSA and demonstrating the ability of this antiserum to recognize the azadirachtin structure. The superior binding of **2a**−BSA (in preference to **3b**−BSA) shown in Figure 2 was typical of the antisera from rabbits immunized with conjugates of hapten **2a**, suggesting significantly better binding to homologous conjugates (spacer attached at the same point on the azadirachtin molecule as the immunizing hapten) than to heterologous conjugates (spacer attached at a different site). The above data suggest that these antisera can discriminate between positions of conjugation to the azadirachtin skeleton and that homology of spacer attachment may be an important determinant of binding strength. However, because of the uncertainty of the exact nature of hapten−protein conjugates, specificity studies are most conclusive when conducted with free haptens in a competitive immunoassay, as described in the next section.

**Indirect Competitive ELISA Results: Recognition of Free Azadirachtin.** Antibodies were tested for binding of several azadirachtin-related compounds (Figure 3), including azadirachtin A, deacetylazadirachtin, and several methanol hydrolysates of azadirachtin by using indirect competitive ELISA. However, only the combination of **2a**−BSA as coating antigen and antibody 7056 yielded good competition curves with azadirachtin A. In Table 1, azadirachtin A and methanol-hydrolyzed azadirachtin are shown to compete for the binding to the antibody with conjugated 7-hemisuccinylazadirachtin. Table 1 data indicate that this antibody recognizes the target compound azadirachtin and related compounds (3-deacetylazadirachtin, 1-detigloylazadirachtin, 1-detigloyl-3-deacetylazadirachtin, azadirachtinin), not just their hemisuccinyl derivatives. This illustrates the

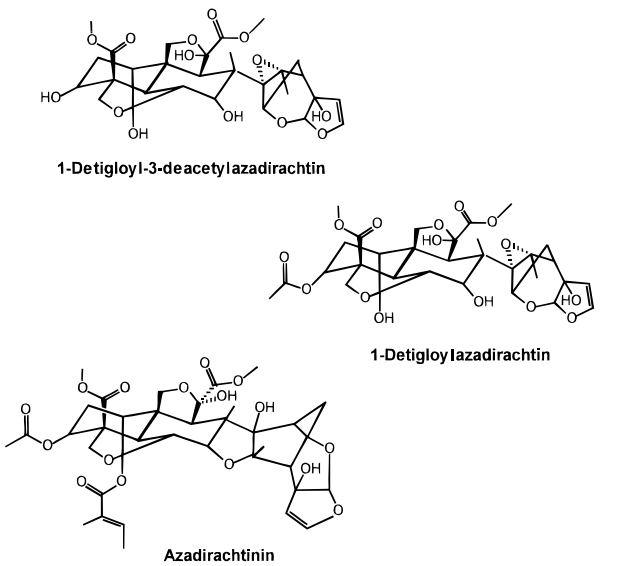


**Figure 3.** Structures of azadirachtin derivatives tested for cross-reactivity.

**Table 1. Standard Curve for Free Azadirachtin A and Hydrolyzed Azadirachtin Using 7056 (1:40000) Antiserum and 2a−BSA Conjugate (0.2 mg/L) as Coating Antigen**

| compound | no. | $IC_{50}$ ($\mu$g/L) | SD | CR[a] (%) |
|---|---|---|---|---|
| azadirachtin A | **1** | 100 | 41 | 100 |
| 3-deacetylazadirachtin | **2b** | 60 | 35 | 63 |
| 1-detigloylazadirachtin[b] | | 200 | 50 | 50 |
| 1-detigloyl-3-deacetylazadirachtin[b] | | 220 | 45 | 45 |
| azadirachtin[b] | | 250 | 42 | 40 |

[a] CR = ($IC_{50}$ for azadirachtin A/$IC_{50}$ for compound) × 100. [b] Shown in Figure 3.

potential usefulness of these antibodies for detection and measurement of azadirachtin-related compounds at levels commonly found in environmental samples. The difference in $IC_{50}$ confirms the ability of this serum to distinguish among closely related structures on the basis of minor changes (i.e. in an acyl group).

**Assay Characteristics.** Under optimized conditions (**3b**−KLH antiserum 7056 dilution 1:40000 final dilution in the well, **2a**−BSA 0.2 $\mu$g/mL) the assay for azadirachtin A showed a standard curve that allowed the measurement of the analyte from 10 to 1000 $\mu$g/L with an average $IC_{50}$ value of 100 ± 41 $\mu$g/L (Table 1). The assay showed comparable sensitivity toward azadirachtin-related compounds such as deacetylazadirachtin and other early products of azadirachtin hydrolysis.

The analyte concentration in this study is the original concentration pipetted into the wells of the microtiter plates. This was preferred because of better comparison with conventional methods which also refer to the original concentration in the sample.

## CONCLUSION

Ecotoxicological studies and quality control of azadirachtin have been hindered by difficulties in setting up sufficiently sensitive and inexpensive analytical evaluation methods. Widely variable amounts of azadirachtin in the biological raw material (neem kernels), depending on origin and storage conditions, pose serious problems regarding quality assurance of biorational insecticides based on azadirachtin. Conventional HPLC methods using UV detection methods are able to detect azadirachtin A down to 0.2 $\mu$g/g (Hull *et al.*, 1993), but they fail in detecting bioactive degradation products of azadirachtin A because of missing UV-absorbing moieties such as the tigloyl or the vinyl ether group. The ELISA for azadirachtin A and related compounds has detection limits of about 10 ng/g and is able to detect these early products of azadirachtin A hydrolysis. The bioactivity of the identified hydrolysis products of azadirachtin was examined recently (Simmonds *et al.*, 1995b).

The usefulness of this immunoassay depends on its ability to solve two different problems. One problem is to discriminate compounds of different biological activity to facilitate quality assurance of biorational insecticides based on azadirachtins. Evidence is presented in this study that the ELISA can discriminate among the tested compounds. Extended screening of bioactive limonoids will be a subject of following papers using both chromatographic separation (Schneider and Ermel, 1987; Hein and Hummel, 1995; Krämer *et al.*, 1994) and multianalyte approaches (Wortberg *et al.*, 1996). The relative importance of structural changes in recognition of azadirachtin-related compounds, and practical utilization of the developed assays will be investigated further.

The second problem is the need for quick monitoring of large numbers of samples for environmental fate studies of azadirachtin that demand cost-effective screening methods with the least amount of sample pretreatment. This ELISA based on antibodies against azadirachtin-related compounds will be a useful tool for screening requirements, especially because of its unrivaled sensitivity. Levels of azadirachtin detection in environmental samples such as soil (Stark and Walter, 1995) or tree foliage (Sundaram and Curry, 1994) have been in the parts per million ($\mu$g/g) range, whereas the presented ELISA method allows detection in the parts per billion (ng/g) range. Thus, the method presented is a step forward in terms of inexpensive, fast, and sensitive analysis of azadirachtins and could therefore have wide application.

## ABBREVIATIONS USED

BSA, bovine serum albumin; CR, cross-reactivity = ($IC_{50}$ for azadirachtin A/$IC_{50}$ for compound) × 100; DMAP, 4-(dimethylamino)pyridine; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; GC, gas chromatography; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; $IC_{50}$, concentration of the analyte, where 50% of the antibody is inhibited by the analyte, midpoint of the assay; KLH, keyhole limpet hemocyanin; OD, optical density; OVA, ovalbumin; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline with 0.5% Tween 20 (polyoxyethylene sorbitan monolaurate); TLC, thin-layer chromatography; TMB, 3,3′,5,5′-tetramethylbenzidine.

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