Hapten Synthesis, Antibody Development, and Competitive Inhibition Enzyme Immunoassay for *s*-Triazine Herbicides

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Two families of carboxylic acid derivatives of the herbicides atrazine and simazine were synthesized for use as haptens in the development of immunoassays. One family was made by using monosubstituted (alkylamino)cyanuric chlorides and a variety of ω-amino acids, giving spacers of varying lengths attached to one secondary amino group. The second family resulted from the replacement of the 2-chloro group of atrazine or simazine with 3-mercaptopropanoic acid. These haptens were conjugated to carrier proteins via *N*-hydroxysuccinimide active esters to make immunogens and ELISA antigens. Mono(alkylamino)cyanuric chlorides were also conjugated directly to bovine serum albumin and thyroglobulin under physiological conditions. ELISA and competitive inhibition ELISA results demonstrated that antibodies from immunized rabbits bound all of the conjugated and free *s*-triazines tested, including the parent compounds atrazine and simazine. The degree of binding was dependent on the *N*-alkyl groups present and on the length and position of attachment of the spacer arm. These results illustrate the usefulness of this synthetic approach in easily producing immunogenic haptens for a class of compounds that are difficult to work with because of their low solubility.

The *s*-triazine herbicides are among the most commonly detected pesticides in water (Muir et al., 1978; Glotfelty et al., 1984; Ervin and Kittleson, 1988; LeMasters and Doyle, 1989). This observation is due to a combination of factors, including widespread and heavy use, chemical and biological stability, and mobility in water. From 1983 through 1987 over 3 000 000 pounds of simazine and atrazine (Figure 1; **3a** and **3b**) were used in California alone (California Department of Food and Agriculture, 1983–1987). For these reasons, the State of California considers them as prime indicator compounds of groundwater contamination. Of additional concern is the poorly understood potential of the *s*-triazines and their nitroso and other derivatives for teratogenic, mutagenic, and carcinogenic effects (Janzowski et al., 1980; Waters et al., 1981). Thus, a rapid and inexpensive assay is needed to allow rapid screening of numerous surface and subsurface water samples, as well as for a variety of other analytical applications. The value of immunoassays for the analysis of pesticide residues has been well established (Hammock and Mumma, 1980; Newsome, 1986; Harrison et al., 1988; Van Emon et al., 1989; Jung et al., 1989). Recent literature includes descriptions of immunoassays for three specific *s*-triazine herbicides, atrazine (Huber, 1985; Bushway et al., 1988; Dunbar et al., 1985; Schlaeppi et al., 1989), cyanazine (Robotti et al., 1986), and terbutryne (Huber and Hock, 1985).

Since the commercially important *s*-triazine herbicides constitute a large class of structurally related compounds that should be amenable to immunoassay as a group, we chose a synthetic approach that allows for the development of a wider range of antibody specificities and ultimate assay designs than previously possible. The scientific rationale for the selection of haptens to be synthesized is discussed in Hammock and Mumma (1980) and illustrated nicely with the development of immunoassays for the acylurea insecticides (Wie and Hammock, 1984). We will focus here on two basic principles of hapten design. First, antibodies generally are most specific for the part of a molecule distal from the point of attachment to a carrier protein. This property allows one to influence the specificity of an antibody population by careful hapten design. Second, the library of triazine haptens we describe here provides expanded possibilities for the tuning of assay sensitivity and specificity through the selection of optimum haptens in the preparation of immunogens, plate-coating antigens, and/or enzyme–hapten conjugates. This approach follows the earlier work of Vallejo et al. (1982), Wie and Hammock (1984), and Harrison et al. (1989b) in the exploration of multiple positions of derivatization on the parent molecule for the purpose of influencing assay sensitivity and specificity. These studies showed that while not all positions of spacer attachment will lead to the desired antibody specificities, even haptens that are not acceptably immunogenic may prove useful in the design of immunoassays.

MATERIALS AND METHODS

Chemicals. 3-Mercaptopropanoic acid was purchased from Evans Chemetics. Amines, amino acids, and other chemical reagents were supplied by Aldrich Chemical Co. (Milwaukee, WI). Simazine and atrazine were gifts from Ciba-Geigy (Greensboro, NC), and atrazine was donated by Shell Agricultural Chemical Co. (Modesto, CA). TLC was performed on 0.25-mm precoated silica gel 60 F254 plastic sheets (Aldrich Chemical Co.). Compounds were detected first by viewing under ultraviolet light (254 nm) and then by exposure to iodine vapor; eluent systems are described in the individual experiments. Immunochemicals were obtained from Sigma Chemical Co. (St. Louis, MO) and ICN ImmunoBiologicals (Lisle, IL). Analytical standards of simazine and atrazine were prepared as 50 mM solutions in DMSO, diluted to 1 mM working stocks in DMSO, and then diluted 20-fold into PBST for competitive inhibition ELISA.

Apparatus. Melting points were determined with a Thomas-Hoover apparatus and are uncorrected. Infrared spectra (IR) were recorded on an IBM IR/32 FTIR spectrophotometer. ^{1}H NMR spectra were measured with a Varian EM-390 90-MHz

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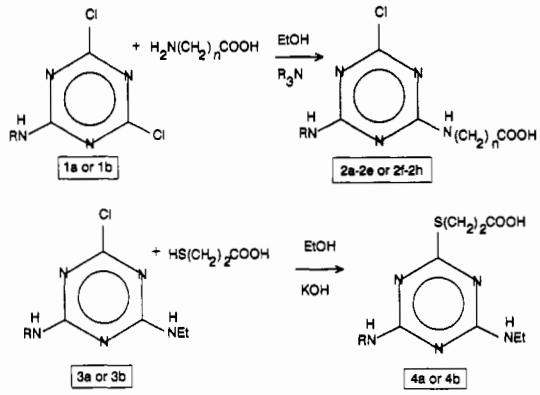

Compound	R	n
1a	Et	na
1b	iPr	na
2a	Et	1
2b	Et	2
2c	Et	3
2d	Et	4
2e	Et	5
2f	iPr	1
2g	iPr	2
2h	iPr	5
3a (simazine)	Et	na
3b (atrazine)	iPr	na
4a	Et	na
4b	iPr	na

na = not applicable

Figure 1. Synthesis and structure of triazine haptens for conjugation to carrier proteins.

spectrometer (Varian Associates, Palo Alto, CA) using tetramethylsilane as an internal standard. Low-resolution mass spectra (MS) were obtained on a Trio-2 mass spectrometer (VG Masslab, Altrincham, UK) using 70-eV EI; high-resolution mass spectra (HRMS) were obtained on a ZAB-HS-2F mass spectrometer (VG Analytical, Wythenshawe, UK) also at 70-eV EI. Ultraviolet–visible spectra (UV) were obtained as 25–100 μg/mL solutions in PBST (unless noted otherwise) on a Beckman DU-6 spectrophotometer (Beckman Instruments, Palo Alto, CA). ELISA performed in 96-well microplates (NUNC, no. 442404) were read with a Vmax microplate reader (Molecular Devices, Menlo Park, CA).

Synthesis of Haptens. Haptens were synthesized by the routes shown in Figure 1. When possible the purity of compounds was confirmed by TLC. Severe streaking of the carboxylic acid derivatives prevented such analysis for compounds **2a–h** and **4a,b**. Intermediate compounds were confirmed by IR and/or ^{1}H NMR analysis. Final haptens were characterized more definitively by IR and ^{1}H NMR analysis as well as low- and sometimes high-resolution mass spectroscopy.

4,6-Dichloro-*N*-ethyl-1,3,5-triazin-2-amine (1a). To a well-stirred solution of 9.22 g (50.0 mmol) of cyanuric chloride in 400 mL of ether cooled to −20 °C was added, over 45 min, a solution of 3.35 g (52.0 mmol) of ethylamine (70 wt % in water) and 6.72 g (52.0 mmol) of *N,N*-diisopropylethylamine in 50 mL of ether. The mixture was filtered, and the filtrate was washed sequentially with 1 M HCl (25 mL), 5% $NaHCO_3$ (25 mL), and saturated NaCl (2 × 25 mL) and then dried (Na_2SO_4). Removal of the ether under reduced pressure to a 25-mL volume followed by the addition of 25 mL of hexane and cooling provided 3.97 g (82%) of crystalline **1a**, mp 107.0–108.5 °C [lit. (Diels, 1899) mp 107 °C]: ^{1}H NMR ($CDCl_3$) δ 6.7 (br, 1 H, NH), 3.53, 3.47 (two q, J = 7.1 Hz, 2 H, CH_2), 1.24 (t, J = 7.1 Hz, 3 H, CH_3); TLC R_f 0.72 (THF–ethyl acetate–hexane, 2:13:35).

4,6-Dichloro-*N*-(1-methylethyl)-1,3,5-triazin-2-amine (1b). Compound **1b** was obtained in 83% yield by the same procedure and molar quantities as for **1a**, but utilizing anhydrous isopropylamine. After removal of the insoluble salt, the filtrate was washed and dried in the same manner as for **1a**. The ether was removed under reduced pressure, leaving a pale yellow oil, which could not be crystallized despite demonstrated purity by TLC: ^{1}H NMR ($CDCl_3$) δ 5.9 (br, 1 H, NH), 4.2 (m, 1 H, CH), 1.25 (d, J = 7.0 Hz, 6 H, 2 CH_3); TLC R_f 0.75 (THF–ethyl acetate–hexane, 2:13:35).

***N*-[4-Chloro-6-(ethylamino)-1,3,5-triazin-2-yl]glycine (2a).** This procedure was typical for the preparation of haptens **2a–h** (Figure 1). 4,6-Dichloro-*N*-ethyl-1,3,5-triazin-2-amine (**1a**) (1.45 g (7.51 mmol)), 0.600 g (8.0 mmol) of glycine, 2.02 g (16 mmol) of *N,N*-diisopropylethylamine, and 30 mL of absolute ethanol were stirred and heated 9 h at 60–65 °C until the absence of **1a** was determined by TLC. After the mixture cooled, solvent was removed at reduced pressure. The residue was dissolved in 75 mL of 5% aqueous $NaHCO_3$ and washed with CH_2Cl_2 (2 × 20 mL), and the aqueous layer was acidified to pH 1 to precipitate the acid (**2a**), 1.41 g (81%), mp 170 °C (dec): IR (KBr) 3260 (m, N–H), 3114 (m, N–H), 1724 (s, C=O), 1561 (vs, C=N) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 7.8 (m, 2 H, 2 NH), 3.83 (d, J = 6.3 Hz, 2 H, CH_2CO_2), 3.2 (m, 2 H, CH_2N), 1.03 (t, J = 7.4 Hz, 3 H, CH_3); MS m/z (relative intensity) 233 (7, M + 2), 232 (5, M + 1), 231 (22, M$^+$), 216 (14, M − CH_3), 203 (5, M − C_2H_4), 187 (100, M − CO_2), 186 (68, M − H − CO_2); UV ϵ_M (263 nm) 2650.

***N*-[4-Chloro-6-(ethylamino)-1,3,5-triazin-2-yl]-β-alanine (2b).** β-Alanine (7.80 mmol) and **1a** (7.50 mmol) gave 1.39 g (76%) of **2b**, mp 168 °C (dec): IR (KBr) 3335 (m, N–H), 3269 (m, N–H), 1732 (m, C=O), 1590 (vs, C=N); ^{1}H NMR (DMSO-d_6) δ 7.7 (m, 2 H, 2 NH, disappeared with D_2O), 3.3 (m, 4 H, 2 CH_2N), 2.48 (t, J = 6.6 Hz, 2 H, CH_2CO_2), 1.18, 1.15 (two t, J = 7.0 Hz, 3 H, CH_3); MS m/z (relative intensity) 247 (19, M + 2), 246 (6, M + 1), 245 (58, M$^+$), 230 (8, M − CH_3), 217 (8, M − C_2H_4), 200 (93, M − H − CO_2), 68 (100, $C_2H_2N_3$); UV ϵ_M (263 nm) 2825.

4-{[4-Chloro-6-(ethylamino)-1,3,5-triazin-2-yl]amino}butanoic Acid (2c). 4-Aminobutanoic acid (7.80 mmol) and **1a** (7.81 mmol) gave 1.59 g (82%) of **2c**, mp 180.0–181.0 °C: IR (KBr) 3268 (m, N–H), 3123 (w, N–H), 1701 (s, C=O), 1566 (vs, C=N) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 13.1 (br, 1 H, CO_2H), 7.7 (m, 2 H, 2 NH), 3.3 (m, 4 H, 2 CH_2N), 2.28 (t, J = 6.9 Hz, 2 H, CH_2CO_2), 1.72 (quin, J = 6.9 Hz, 2 H, CH_2), 1.10 (t, J = 6.9 Hz, 3 H, CH_3) (the 13.1 and 7.7 ppm peaks disappeared with D_2O); MS m/z (relative intensity) 261 (3, M + 2), 260 (2, M + 1), 259 (8, M$^+$), 244 (2, M − CH_3), 241 (10, M − H_2O), 214 (14, M − H − CO_2), 200 (87, M − CH_3 − CO_2), 186 (65, M − H − C_2H_4 − CO_2), 173 (24, M − C_3H_6 − CO_2), 158 (12, M − CH_3 − C_3H_6 − CO_2), 44 (100, CO_2); HRMS m/z calcd for $C_9H_{14}ClN_5O_2$ 259.0836, obsd 259.0832; UV ϵ_M (263 nm) 3025.

5-{[4-Chloro-6-(ethylamino)-1,3,5-triazin-2-yl]amino}pentanoic Acid (2d). 5-Aminopentanoic acid (7.51 mmol) and **1a** (7.51 mmol) gave 1.25 g (61%) of **2d**, mp 166.0–167.5 °C: IR (KBr) 3265 (s, N–H), 3122 (m, N–H), 1701 (s, C=O), 1597 (vs, C=N) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 7.7 (br, 2 H, 2 NH, disappeared with D_2O), 3.2 (m, 4 H, 2 CH_2N), 2.23 (t, J = 6.0 Hz, 2 H, CH_2CO_2), 1.5 (m, 4 H, CH_2CH_2), 1.08 (two t, J = 7.0 Hz, 3 H, CH_3); MS m/z (relative intensity) 275 (2, M + 2), 274 (2, M + 1), 273 (6, M$^+$), 258 (1, M − CH_3), 228 (6, M − H − CO_2), 214 (54, M − CH_3 − CO_2), 200 (18, M − H − C_2H_4 − CO_2), 186 (100, M − H − C_3H_6 − CO_2), 173 (17, M − C_4H_8 − CO_2), 159 (14, M − C_5H_{10} − CO_2), 44 (60, CO_2); HRMS m/z calcd for $C_{10}H_{16}ClN_5O_2$ 273.0993, obsd 273.0988; UV ϵ_M (263 nm) 2650.

6-{[4-Chloro-6-(ethylamino)-1,3,5-triazin-2-yl]amino}hexanoic Acid (2e). The crude product from 6-aminohexanoic acid (8.0 mmol) and **1a** (7.5 mmol) required recrystallization from acetone to provide 1.85 g (86%) of **2e**, mp 140.5–142.5 °C: IR (KBr) 3254 (s, N–H), 3104 (m, N–H), 1708 (s, C=O), 1559 (vs, C=N) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 11.8 (br, 1 H, CO_2H), 7.6 (br, 2 H, 2 NH), 3.2 (m, 4 H, 2 CH_2N), 2.18 (t, J = 6.6 Hz, 2 H, CH_2CO_2), 1.4 (m, 6 H, $CH_2CH_2CH_2$), 1.07 (two t, J = 7.0 Hz, 3 H, CH_3); MS m/z (relative intensity) 289 (5, M + 2), 288 (4, M + 1), 287 (14, M$^+$), 271 (5, M − H − CH_3), 242 (4, M − H − CO_2), 228 (78, M − CH_3 − CO_2), 214 (27, M − H − C_2H_4 − CO_2), 200 (39, M − H − C_3H_6 − CO_2), 187 (34, M − C_4H_8 − CO_2), 186 (100, M − H − C_4H_8 − CO_2), 173 (32, M − C_5H_{10} − CO_2); UV ϵ_M (263 nm) 2500.

***N*-{4-Chloro-6-[(1-methylethyl)amino]-1,3,5-triazin-2-yl}glycine (2f).** Glycine (7.5 mmol) and **1b** (8.0 mmol), heated under reflux for 7 h, provided 0.978 g (53%) of **2f**, mp 160 °C (dec): IR (KBr) 3263 (s, N–H), 3111 (m, N–H), 1707 (s, C=O), 1594 (vs, C=N) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 7.8 (m, 2 H, 2 NH, disappeared with D_2O), 4.0 (m, 1 H, CH), 3.88 (d, J = 6.0 Hz, 2 H, CH_2CO_2, became a singlet with D_2O), 1.13 (d, J = 6.6

Hz, 6 H, 2 CH_3); MS m/z (relative intensity) 247 (7, M + 2), 246 (4, M + 1), 245 (21, M^+), 230 (56, M - CH_3), 203 (19, M - C_3H_6), 201 (23, M - CO_2), 200 (21, M - H - CO_2), 184 (79, M - H_2 - CH_3 - CO_2), 58 (100, C_3H_8N); HRMS m/z calcd for $C_8H_{12}ClN_5O_2$ 245.0680, obsd 245.0671.

N-{4-Chloro-6-[(1-methylethyl)amino]-1,3,5-triazin-2-yl}-β-alanine (2g). β-Alanine (8.0 mmol) and **1b** (7.5 mmol), heated at 65 °C for 9 h, gave 1.24 g (64%) of **2g**, mp 169 °C (dec): IR (KBr) 3262 (s, N-H), 3118 (m, N-H), 1698 (s, C=O), 1594 (vs, C=N) cm^{-1}; ^1H NMR (DMSO-d_6) δ 7.8 (m, 2 H, 2 NH, disappeared with D_2O), 4.1 (m, 1 H, CH), 3.4 (m, 2 H, CH_2NH), 2.5 (m, 2 H, CH_2CO_2), 1.10, 1.13 (two d, J = 6.6 Hz, 6 H, 2 CH_3); MS m/z (relative intensity) 261 (13, M + 2), 260 (10, M + 1), 259 (39, M^+), 244 (33, M - CH_3), 217 (15, M - C_3H_6), 214 (14, M - H - CO_2), 186 (22, M - H - C_2H_4 - CO_2), 184 (62, M - H_2 - CH_2CH_3 - CO_2), 172 (43, M - H - C_3H_6 - CO_2), 58 (100, C_3H_8N); HRMS m/z calcd for $C_9H_{14}ClN_5O_2$ 259.0836, obsd 259.0822.

6-{[4-Chloro-6-[(1-methylethyl)amino]-1,3,5-triazin-2-yl]amino}hexanoic Acid (2h). 6-Aminohexanoic acid (20.0 mmol) and **1b** (20.0 mmol) gave 3.97 g (66%) of **2h**, mp 161.0–162.0 °C [lit. (Dunbar et al., 1985) mp 165–166 °C]: IR (KBr) 3264 (m, N-H), 3128 (m, N-H), 1692 (s, C=O), 1596 (vs, C=N) cm^{-1}; ^1H NMR (DMSO-d_6) δ 11.6 (br, 1 H, CO_2H), 7.4 (m, 2 H, 2 NH), 3.9 (m, 1 H, CH), 3.1 (m, 2 H, CH_2N), 2.18 (t, J = 6.6 Hz, 2 H, CH_2CO_2), 1.4 (m, 6 H, $CH_2CH_2CH_2$), 1.10 (two d, J = 6.5 Hz, 6 H, 2 CH_3); UV ϵ_M (263 nm) 2775.

3-{[4,6-Bis(ethylamino)-1,3,5-triazin-2-yl]thio}propanoic Acid (4a). To a stirred, heterogeneous mixture of 1.01 g (5.01 mmol) of technical grade 6-chloro-*N*,*N*'-diethyl-1,3,5-triazine-2,4-diamine (**3a**, simazine) and 100 mL of absolute ethanol was added a solution of 0.574 g (5.40 mmol) of 3-mercaptopropanoic acid and 0.714 g (10.8 mmol) of 85% KOH in 10 mL of absolute ethanol under N_2. At reflux the mixture became homogeneous and soon a precipitate (KCl) began to form. Reflux was continued (4 h) until **3a** was no longer detectable by TLC. The hot mixture was filtered, and the filtrate was concentrated to a white solid. The solid was triturated with 25 mL of 5% $NaHCO_3$ and filtered. A substantial quantity, 0.750 g, of presumed (IR) 2-hydroxy compound, mp >290 °C, was obtained. Acidification of the filtrate to pH 3 with 6 N HCl and cooling provided 0.157 g (12%) of **4a**, mp 177.0–179.0 °C: IR (KBr) 3404 (m, N-H), 3294 (m, N-H), 1698 (m, C=O), 1564 (vs, C=N), 1251 (s, C-O) cm^{-1}; ^1H NMR (DMSO-d_6) δ 12.2 (br, 1 H, CO_2H), 7.1 (br, 2 H, 2 NH), 3.2 (m, 6 H, CH_2S and 2 CH_2N), 2.60 (t, J = 6.6 Hz, 2 H, CH_2CO_2), 1.06 (t, J = 7.0 Hz, 6 H, 2 CH_3) (the 12.2 and 7.1 ppm peaks disappeared with added D_2O); MS m/z (relative intensity) 273 (6, M + 2), 272 (17, M + 1), 271 (100, M^+), 256 (5, M - CH_3), 226 (98, M - H - CO_2), 212 (12, M - CH_3 - CO_2), 199 (65, M - C_2H_4 - CO_2), 184 (21, M - H - C_3H_6 - CO_2), 167 (46, M - $SCH_2CH_2CO_2H$); UV ϵ_M (CH_3OH, 263 nm) 4450.

3-{[4-(Ethylamino)-6-[(1-methylethyl)amino]-1,3,5-triazin-2-yl]thio}propanoic Acid (4b). The reaction procedure and molar quantities used were the same as for **4a**, with two exceptions. The starting material was technical grade 6-chloro-*N*-ethyl-*N*'-(1-methylethyl)-1,3,5-triazine-2,4-diamine (**3b**, atrazine) and the reaction mixture was heated under reflux for 5 h. The workup was altered slightly in that the solvent was distilled to leave a white solid. The residue was taken up in 25 mL of 5% $NaHCO_3$. The basic solution was washed with $CHCl_3$ (3 × 10 mL) and acidified to pH 2 with 6 N HCl. The resultant white solid was collected, washed with water, and dried to obtain 0.430 g (30%) of **4b**, mp 165.0–166.0 °C: IR (KBr) 3381 (m, N-H), 3288 (m, N-H), 1700 (m, C=O), 1557 (vs, C=N), 1249 (m, C-O) cm^{-1}; ^1H NMR (DMSO-d_6) δ 12.1 (br, 1 H, CO_2H), 7.0 (br, 2 H, 2 NH), 4.0 (m, 1 H, CH), 3.1 (m, 4 H, CH_2S and CH_2N), 2.57 (t, J = 6.6 Hz, 2 H, CH_2CO_2), 1.1 (m, 9 H, 3 CH_3) (the 12.1 and 7.0 ppm peaks disappeared with added D_2O); MS m/z (relative intensity) 287 (6, M + 2), 286 (16, M + 1), 285 (100, M^+), 270 (30, M - CH_3), 240 (74, M - H - CO_2), 226 (9, M - CH_3 - CO_2), 212 (21, M - H - C_2H_4 - CO_2), 198 (61, M - H - C_3H_6 - CO_2), 181 (33, M - $SCH_2CH_2CO_2$); UV ϵ_M (CH_3OH, 263 nm) 4160. Additional **4b**, 0.227 g, mp 165.5–166.5 °C, was obtained from the filtrate on evaporation and crystallization of the residue from methanol.

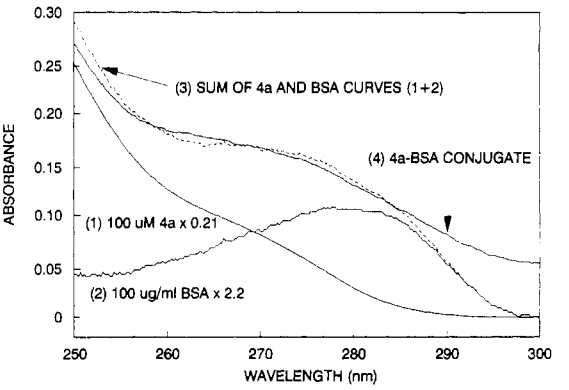

Figure 2. Verification of conjugation for typical hapten–protein conjugate. Curves 1 and 2 are spectra from 250 to 300 nm of compound **4a** and BSA, multiplied by the constants noted. Curve 3 is the sum of curves 1 and 2. Curve 4 is the spectrum from 250 to 300 nm of the conjugate **4a**–BSA.

Direct Conjugation of Monosubstituted Cyanuric Chlorides to Carrier Proteins. Compounds **1a** and **1b** were coupled covalently to carrier proteins by incubation in aqueous solution with BSA or THY. Individual reactions consisted of 1.00 mL of DMF containing 0.10 mmol of **1a** (19.3 mg) or **1b** (20.7 mg) added slowly to a solution of 68 mg of protein in 4.0 mL of H_2O (1.0 μmol of BSA). The reaction mixtures were stirred gently at 22 °C for 1 h and then dialyzed exhaustively against water at 4 °C. Slight precipitates in the THY conjugates were dissolved by the addition of a small amount of 4 N NaOH to a final pH of 8.5–9.0.

Conjugation of Carboxylic Acid Haptens to Carrier Proteins via NHS Esters. Compounds **2e**, **2h**, **4a**, and **4b** were coupled covalently to carrier proteins by the *N*-hydroxysuccinimide ester method of Langone and Van Vunakis (1975). The carboxylic acid hapten (0.20 mmol) was dissolved in 1.0 mL of dry DMF with equimolar *N*-hydroxysuccinimide and a 10% molar excess of dicyclohexylcarbodiimide. After 3.5 h of stirring at 22 °C, the precipitated dicyclohexylurea was removed by centrifugation, and the DMF supernatant was added to protein solutions. Proteins (50 mg each of BSA, CONA, KLH, and THY) were dissolved in 5.00 mL of H_2O, and 1.05 mL of DMF was added slowly to each tube with vigorous stirring. One-quarter of the DMF supernatant was added to each protein solution (0.050 mmol per tube), bringing the final DMF concentration to 20%. The reaction mixtures were stirred gently at 4 °C for 22 h to complete the conjugation and then dialyzed exhaustively against PBS. Precipitates in the conjugates of **2e** and **2h** were partially redissolved by adjusting the pH to 10–11 while mixing, followed by a gradual return to pH 7.3. Significant precipitate remained in all conjugate stock preparations (2.5–7.0 mg/mL), although less was present in the BSA and CONA conjugates. All conjugates dissolved easily at the lower concentrations and higher pH used for ELISA plate coating (<10 μg/mL, pH 9.6) and for measurement of hapten density (25–100 μg/mL).

Determination of Hapten Density of Conjugates. Hapten densities of BSA conjugates were determined spectrophotometrically by measuring ratios of absorbance at 260 and 280 nm (corresponding approximately to the respective peaks of the *s*-triazines and BSA) for free haptens and BSA. A pair of simultaneous equations in two unknowns was set up for each hapten–conjugate pair using the measured ratios. These equations were solved to give the absorbances due to hapten and protein at 260 and 280 nm. Graphic verification was obtained by combining the spectra of free hapten and free BSA (after multiplication of each by a constant) and comparing to the conjugate spectrum. An example of this verification for **4a**–BSA is given in Figure 2.

Immunization. Female New Zealand white rabbits weighing 2–4 kg were used for raising antibodies. Routinely, 100 μg of immunizing antigen dissolved in PBS was emulsified with Freund's complete adjuvant (1:2 volume ratio) and injected intradermally at multiple sites on the back. After 1 month, the animal was boosted with an additional 100 μg of antigen and bled 7–10 days later. If the antibody obtained was useful, boosting

and bleeding as above continued on a monthly basis. Serum was isolated by centrifugation, and sodium azide was added as a preservative at a final concentration of 0.02%. Serum was then aliquotted and stored at −20 °C or used without further treatment.

ELISA and Competitive Inhibition ELISA. Enzyme immunoassay and competitive inhibition enzyme immunoassay were performed according to Voller et al. (1976) as modified by Harrison et al. (1989a). Microplate reader performance was verified by the reversed plate wet test procedure of Harrison and Hammock (1988). Hapten–protein conjugates (plate-coating antigens) were immobilized in the wells of 96-well microplates, and the wells were treated sequentially with rabbit anti-hapten antibody, enzyme-labeled goat anti-rabbit antibody, and enzyme substrate. The amount of enzyme bound, as indicated by the conversion of colorless substrate to colored product, is directly related to the amount of rabbit anti-hapten antibody bound to the plate-coating antigen. Thus ELISA is used for the measurement of the amount of specific antibody in a sample or for the comparison of the relative binding of an antibody to different conjugates immobilized on the solid phase. Competitive inhibition ELISA is used for the measurement of free analyte, such as atrazine, or for the comparison of the relative binding of soluble inhibitors to an antibody for the determination of antibody specificity.

RESULTS AND DISCUSSION

Hapten Synthesis. The simplicity of the *s*-triazine molecules, bearing only three attachments to the ring system, leaves few choices for the placement of spacer groups. Hence to maximize exposure of haptenic determinant groups, we retained two of these three attachments in each hapten, while attempting to choose a spacer arm that was as similar as possible to the third attachment. In the first series of haptens, one *N*-alkyl group and the chloro group were retained unchanged, while the other *N*-alkyl group was replaced by varying length *N*-alkyl chains terminated with a carboxyl group for conjugation to proteins. Thus haptens were prepared by the replacement of one chlorine of cyanuric chloride with ethylamine or isopropylamine at low temperature, followed by the replacement of the second chlorine at 50–60 °C with an appropriate ω-amino acid, leading to the *N*-alkyl acid haptens **2a–h** of Figure 1.

The second series of haptens utilized the 2-position for spacer arm attachment and retained both *N*-alkyl groups unchanged. Since retention of the monovalent chlorine would preclude spacer attachment at this position, a multivalent replacement atom was required, preferably one resembling chlorine. Our selection of sulfur was based on several factors: (1) sulfur bears two pairs of nonbonded electrons, resembling chlorine better than other possible atoms, except perhaps oxygen, (2) there is limited resonance interaction between sulfur and the triazine ring, (3) the atomic size of sulfur is closer to that of chlorine than other possible atoms, (4) sulfur will not allow hydrogen bonding to it, as will nitrogen and oxygen, (5) the nucleophilicity of the thiolate anion facilitates chloride substitution, and (6) starting materials are readily available. We thus prepared the 2-mercaptopropanoic acid derivatives of simazine and atrazine (**4a,b**) by heating them with β-mercaptopropanoic acid in alcoholic KOH solution. These thioethers should provide good haptens not only for the detection of *s*-triazines containing a sulfur in the 2-position but also for metabolites containing sulfur in the same position.

The triazine haptens are an excellent example of compounds that initially appeared simple to synthesize, but in fact some were difficult or impossible to obtain by conventional means. A case in point was our attempt to prepare triazine haptens with a 6-[*N*-(4-aminophenethyl)] spacer, one that had proved particularly useful in our molinate immunoassay (Gee et al., 1988) and our thiobencarb immunoassay (unpublished data). Although the simazine and atrazine analogues bearing the 6-[*N*-(4-nitrophenethyl)] group were successfully prepared from **1a** or **1b**, respectively, application of the selective dodecacarbonyltriiron-catalyzed nitro reduction reaction (Landesberg et al., 1972) failed due to the insolubility of nitro compounds in the requisite solvents. Attempts at reduction using sodium borohydride–cobalt chloride and methanol as solvent (Satoh and Suzuki, 1969) failed to provide detectable quantities of the aminoaryl haptens among the multicomponent product mixtures. The successfully synthesized haptens were poorly soluble in many solvents, often precluding extensive purification of useful quantities of the materials by column chromatography. Thus it was important in most cases that reactions gave high yields. A striking feature noted during synthesis and conjugation was the significant difference in solubility in pairs of compounds differing only in alkyl substitution, the ethyl being consistently less soluble than the isopropyl in both organic and aqueous systems. This property led to differences in the solubilities of the active esters when added to aqueous solutions of proteins for conjugation, possibly contributing to the observed differences in hapten density (next section).

Conjugation. Quantitative changes in the 260–280-nm spectral region, after exhaustive dialysis, indicated covalent attachment. Spectrophotometrically determined hapten densities were 3.9 for **2e**–BSA, 2.9 for **2h**–BSA, 6.5 for **4a**–BSA, and 13.7 for **4b**–BSA. Addition of the spectra of free hapten and free BSA adjusted to the proper concentrations exhibited good agreement with conjugate spectra as shown in Figure 2. Thus the ratio of 0.21 × 100 μM hapten **4a** (curve 1) to 2.2 × 1.47 μM BSA (100 μg/mL ÷ 68 μg/nmol; curve 2) is 21/3.2 or 6.5 mol of hapten per mol of BSA. Compounds **2a** and **2f** yielded pinkish colored supernatants during the NHS active ester reaction in DMF, in contrast to the clear supernatants of all of the other haptens.

ELISA Results: Recognition of Conjugated Triazines. All results of antibody characterization are for serum from terminal bleeds, which yielded 50–75 mL of serum per rabbit. Antibodies raised against KLH, THY, and CONA conjugates of haptens **2e**, **2h**, **4a**, and **4b** were tested by ELISA for binding to conjugates of the same haptens to BSA. These antibodies were also tested by ELISA for binding to conjugates of **1a** and **1b** to BSA. Figure 3 shows the binding of one selected antiserum (anti-**4b**–KLH) to BSA, **1a**–BSA, and **1b**–BSA, confirming the covalent attachment of **1a** and **1b** to BSA and demonstrating the ability of this antiserum to recognize the *s*-triazine structure without a spacer arm. The superior binding of **1b**–BSA (in preference to **1a**–BSA) shown in Figure 3 was typical of the antisera from rabbits immunized with conjugates of haptens **2h** and **4b**, suggesting significant discrimination between the isopropyl group of **1b** and the ethyl group of **1a**. Further data supporting this observation will be given in the next section on recognition of free *s*-triazines.

Figure 4 shows the binding of another selected antiserum (anti-**2h**–CONA) to BSA and BSA conjugates of **2e**, **2h**, **4a**, and **4b**. The superior binding of **2e**–BSA and **2h**–BSA shown in Figure 4 was typical of the antisera from rabbits immunized with conjugates of haptens **2e** and **2h**. The reverse was true for the antisera of rabbits immunized with conjugates of **4a** and **4b**, which typically bound **4a**–BSA and **4b**–BSA better than BSA con-

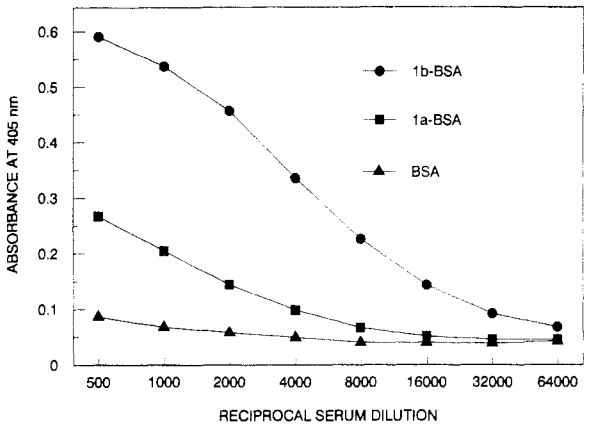

Figure 3. Antibody binding of conjugated *s*-triazines. ELISA was used to evaluate the binding of antibodies raised against compound **4b** (anti-**4b**–KLH serum) to 96-well microplates coated with unconjugated BSA or the conjugates **1a**–BSA or **1b**–BSA. Miocroplates coated with the conjugates were exposed to varying dilutions of antiserum. Similar experiments were repeated for other antigen–antibody combinations to determine effective antibody titers.

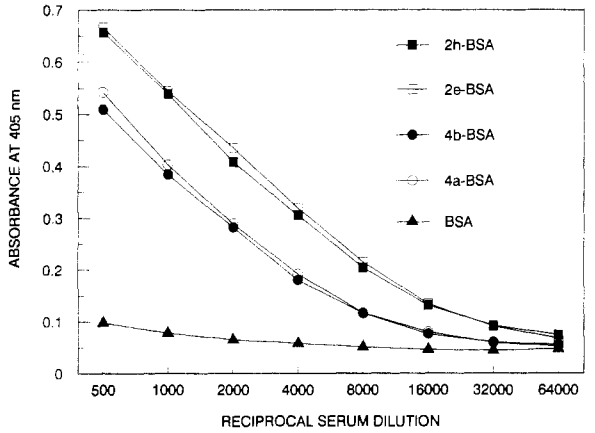

Figure 4. Antibody binding of conjugated *s*-triazines. ELISA was used to evaluate the binding of antibodies raised against compound **2h** (anti-**2h**–CONA serum) to 96-well microplates coated with unconjugated BSA or the conjugates **2e**–BSA, **2h**–BSA, **4a**–BSA, or **4b**–BSA. Microplates coated with the conjugates were exposed to varying dilutions of antiserum. Similar experiments were repeated for other antigen–antibody combinations to determine effective antibody titers.

jugates of **2a–h** (data not shown). Thus these sera each bind better to homologous conjugates (spacer attached at the same point on the triazine ring as the immunizing hapten) than to heterologous conjugates (spacers attached at a different point). The above data suggest that these antisera can discriminate between positions of conjugation to the *s*-triazine ring and that homology of spacer attachment may be an important determinant of binding strength.

The titration curves of Figures 3 and 4 using conjugated *s*-triazines demonstrate the recognition of the *s*-triazine structure by these sera despite major changes in spacer arm length and conjugation position. 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 inhibition immunoassay, as described in the next section.

Competitive Inhibition ELISA Results: Recognition of Free Triazines. Antibodies were tested for binding of several *s*-triazines, including simazine, atrazine, and the haptens shown in Figure 1, by using the compounds

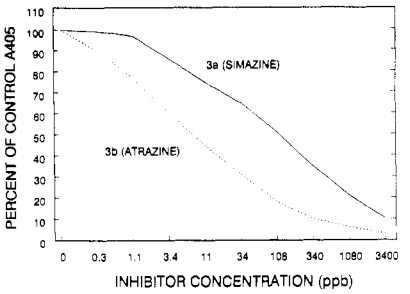

Figure 5. Antibody binding of atrazine and simazine. Competitive inhibition ELISA was used to evaluate the binding of antibodies raised against compound **2h** (anti-**2h**–CONA serum) to free atrazine and simazine. Microplates coated with the conjugate **4a**–BSA were exposed to a fixed amount of diluted antiserum preincubated with varying concentrations of the inhibitors atrazine and simazine.

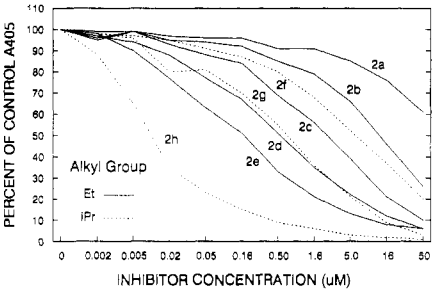

Figure 6. Antibody binding of haptens as a function of spacer arm length and alkyl substituent group. Competitive inhibition ELISA was used to evaluate the binding of antibodies raised against compound **2h** (anti-**2h**–CONA serum) to the haptens **2a–h**. Microplates coated with the conjugate **4a**–BSA were exposed to a fixed amount of diluted antiserum preincubated with varying concentrations of the haptens **2a–h**. Substituent groups on the second nitrogen are indicated by solid lines (ethyl) or dashed lines (isopropyl). The respective number of CH_2 groups in the spacer arm is 1–5 for compounds **2a–e** and 1, 2, and 5 for compounds **2f–h**.

as inhibitors in the competitive inhibition ELISA. In Figure 5, free atrazine and simazine are shown to inhibit the binding to conjugated triazines shown in Figure 4. Figure 5 indicates that this antibody recognizes the target compounds atrazine and simazine, not just their derivatives. This illustrates the potential usefulness of these antibodies for detection and measurement of *s*-triazine herbicides at levels commonly found in environmental samples. The difference between the curves confirms the ability of this serum to distinguish between two closely related structures based on a minor change in alkyl group.

Figure 6 shows the relative binding of one antibody (anti-**2h**–CONA) for a series of haptens varying only in the length of their spacer arms and the alkyl group on their second nitrogen. The results clearly demonstrate that this serum is capable of discrimination based on both spacer length and alkyl group. While the curves of **2a–h** in Figure 6 span over three decades of concentration, such range was seen only for antibodies against conjugates of the second series of haptens. The curves for the same eight haptens using the same serum as in Figure 3 (anti-**4b**–KLH) spanned only one decade of concentration, although in the same relative order (data not shown). Thus spacer arm length is logically much more important for recognition by antibodies when the spacer position is homologous. The data illustrate the importance of both spacer length and *N*-alkyl group in antibody binding, confirming the observations on specificity in the previous section. The range of binding strengths demonstrated here creates the possibility of tuning of assay sen-

sitivity or specificity through the selection of haptens of the appropriate spacer length or position of attachment.

Conclusions. The syntheses described here have formed the basis for further work leading to the characterization of the crossreactivity of a variety of antibodies, the development of highly sensitive assays, and studies detailing the relative importance of triazine structure in binding by antibodies. In all of these studies the availability of a variety of hapten structures was invaluable. As will be discussed in subsequent papers, the two series of haptens described herein have produced very different specificities of both polyclonal and monoclonal antibodies. The first series (**2a–h**) has resulted largely in antibodies more specific for atrazine and simazine, while the second series (**4a,b**) has resulted in antibodies that also recognize the *S*-methyl *s*-triazines, including prometryne, ametryne, and simetryne. Subsequent papers continuing this work will include more complete characterization of several antibodies, optimization of assay sensitivity and ruggedness using the library of haptens described here, characterization of serum crossreactivity using optimized assays, the relative importance of structural changes in recognition of *s*-triazines, and practical utilization of the assays developed.

Because of the widespread use of *s*-triazines throughout most of the world and because they are used as indicator compounds for pollution by agricultural chemicals, there is a great need for rapid and sensitive assays that are resistant to matrix effects. Often the development of such assays is attempted by the production of monoclonal antibodies to a limited number of haptens. Our experience with the *s*-triazines has indicated that an investment in hapten chemistry for a series of compounds is very cost effective in the development of assays of varying and predictable properties. The resulting assays have proven to be very sensitive and are presently in use with a variety of matrices, including water, soil, and human body fluids. Some of these assays recently have been transferred to the California Department of Food and Agriculture for the analysis of *s*-triazines in environmental water samples. We also expect to use some of these assays for analysis of some of the major triazine metabolites, especially those of the *S*-methyl *s*-triazines, including glutathione conjugates and protein adducts via sulfoxide or sulfone intermediates.

ABBREVIATIONS

BSA, bovine serum albumin; CONA, conalbumin; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; KLH, keyhole limpet hemocyanin; NHS, *N*-hydroxysuccinimide; PBS(T), phosphate-buffered saline (plus Tween 20); THF, tetrahydrofuran; THY, thyroglobulin; TLC, thin-layer chromatography.

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A New Method for the Analysis of Granular Pesticide Formulations

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A new method is described for examining the chemical nature of a pesticide as a granular formulation. A screening program revealed that β-nitrostyrene was highly active against soybean root knot nematode but a granular formulation was completely inactive. Solid-state (CPMAS) ^{13}C NMR spectroscopy of a granular formulation prepared with ^{13}C-labeled active ingredient led to identification of the inactive form as a polymer of the active ingredient. Initiation of polymerization by the clay granule carrier was confirmed.

β-Nitrostyrene (1) was shown to be highly effective in the control of soybean root knot nematode and soybean cyst nematode in preliminary greenhouse tests when applied as a soil drench in acetone-water solution. In preparation for field testing, a granular formulation of 1 was prepared and applied in a greenhouse test. No control of nematode infection could be detected at any of the test rates with the granular formulation. Furthermore, no appreciable amount of 1 could be extracted from the granular formulation.

EXPERIMENTAL SECTION

Labeled starting materials were purchased from MSD Isotopes and were of 99% isotopic purity at the labeled carbon. Solution NMR spectra were obtained in chloroform-*d* on a Bruker WM-360 spectrometer (^{13}C resonance frequence 90.6 MHz) operating in the pulsed Fourier transform mode. Chemical shifts are reported (ppm) downfield from the internal standard tetramethylsilane. Infrared spectra were obtained on a Perkin-Elmer Model 781 infrared spectrometer as solid dispersions in potassium bromide.

Solid-State NMR. Cross-polarization magic-angle spinning (CPMAS) (Schaefer and Stejskal, 1976) ^{13}C NMR spectra were collected on a home-built spectrometer operating at a proton Larmor frequency of 60 MHz corresponding to a ^{13}C resonance frequency of 15.1 MHz. Samples were spun at the magic angle (54.7°) with respect to the static magnetic field at a spinning speed of 1859 Hz. The spinner on the magic-angle probe had an internal volume of 600 μL, and a typical sample weighed 400-450 mg. A more detailed description of the magic-angle spinner can be found elsewhere (Schaefer et al., 1987). Spectra were collected following 500-μs matched ^{13}C-^{1}H spin-lock contacts with high-power proton dipolar decoupling. The repetition rate on the experiments was 500 ms.

Dipolar Rotational Spin-Echo ^{13}C NMR. Dipolar rotational spin-echo (DRSE) ^{13}C NMR is a two-dimensional NMR technique that allows the strength of ^{13}C-^{1}H dipolar interactions to be measured (Munowitz and Griffin, 1982; Schaefer et al., 1983). With ^{1}H-^{1}H couplings suppressed by homonuclear multiple-pulse decoupling (Waugh et al., 1968; Mansfield et al., 1973; Rhim et al., 1973; Burum et al., 1981), magic-angle spinning caused the ^{13}C-^{1}H dipolar line shape associated with a particular carbon spin to break up into spinning sidebands. Because the characteristic dipolar spinning-sideband patterns for ^{13}CH and $^{13}CH_2$ groups are quite different, the experiment can be used to count the number of protons attached to a particular carbon (Webb and Zilm, 1989).

DRSE ^{13}C NMR experiments were performed on the same

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