Radioimmunoassay for Psychotropic Drugs III: Synthesis and Properties of Haptens for Trifluoperazine and Fluphenazine

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Received July 27, 1982, from the *College of Pharmacy, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 0W0.* Accepted for publication January 10, 1983.

Abstract □ For the development of radioimmunoassay procedures for trifluoperazine and fluphenazine, three haptens, *N*-(2-carboxyethyl)desmethyltrifluoperazine, *N*-(4-carboxybutyl)desmethyltrifluoperazine, and 10-[3-(4-carboxyethylpiperazinyl)-3-oxopropyl]-2-trifluoromethyl-10*H*-phenothiazine, were synthesized and characterized. Each hapten was coupled to bovine serum albumin, and the number of hapten residues per mole of bovine serum albumin was calculated by UV spectrophotometric methods. Antibodies to each hapten–protein conjugate were developed in rabbits, and titers of the antisera were checked by evaluating their binding characteristics to the tritiated drug.

Keyphrases □ Trifluoperazine—hapten synthesis, conjugation with bovine serum albumin, antisera production in rabbits, RIA □ Fluphenazine—hapten synthesis, conjugation with bovine serum albumin, antisera production in rabbits, RIA □ Psychotropic drugs—trifluoperazine, fluphenazine, hapten synthesis, conjugation with bovine serum albumin, antisera production in rabbits, RIA

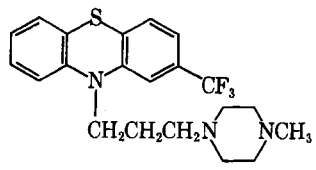
Trifluoperazine (I), a piperazine-type phenothiazine, is widely used orally for the treatment of certain psychotic disorders. However, until recently there were no suitable sensitive and specific analytical methods available to quantitate this drug in the plasma of patients under treatment. This slow method development was primarily due to the reputed instability and adsorptive loss of phenothiazines in all stages of handling for analysis, the low plasma levels encountered (arising from such reasons as the low doses used), the probable significant first-pass metabolism, and extensive metabolism to numerous metabolites. The available reported chemical methods of analysis include GC–NPD (1–3) procedures sensitive to 0.5 ng/mL using a 2-mL plasma sample (1, 3) and a GC–MS method involving the use of a single-ion monitoring technique, which permitted the determination of 78 pg of trifluoperazine/mL of plasma using a 2-mL sample (4). Although of sufficient sensitivity to monitor plasma levels of patients under chronic treatment with normal therapeutic doses of trifluoperazine, these procedures are tedious, requiring extraction of the drug from the biological media, and are thus not easily amenable for routine clinical monitoring.

Radioimmunoassay (RIA) procedures, on the other hand, are simple, sensitive, require a small amount of plasma sample generally with no extraction, and are readily applicable to routine analysis with their ability to handle large numbers of samples with a short turnaround. In fact, an RIA procedure for trifluoperazine was recently reported from these laboratories, which can quantitate 0.25 ng/mL with a 200-μL plasma sample (5). This method is based on an antiserum developed to a protein conjugate (IIa), where the protein is linked to the side chain of the piperazine ring. This site was chosen since from previous experience, as with antibodies raised to chlorpromazine (6, 7), the antiserum raised to conjugates where the protein is coupled to carboxylic acid groups attached to the *N*-10 side-chain sites cross-reacted significantly less with phenothiazine ring metabolites (such as the sulfoxide and 7-hydroxy derivatives) than the antiserum raised to conjugates where the protein is coupled through carboxylic acid groups attached through other positions of the phenothiazine ring. Furthermore, antibodies have now been raised to the protein conjugates (IIb, III) of two other haptens, where the protein is also coupled through the side chain.

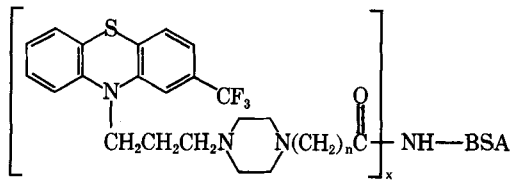
This paper describes the syntheses and characterizations of three drug–protein conjugates, the production of the antibodies to these conjugates in rabbits, and comparison of the titers of the antisera. Since fluphenazine only differs structurally from trifluoperazine in having a *N*-hydroxyethyl substituent on the piperazine ring in place of the *N*-methyl group shown in I, these antisera should be suitable for developing an RIA for fluphenazine as well as trifluoperazine.

RESULTS AND DISCUSSION

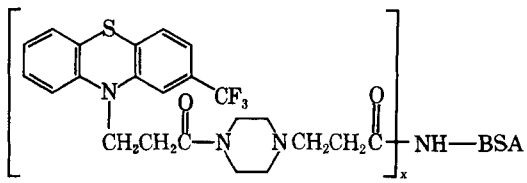
To modify the structure of trifluoperazine so as to contain an acid moiety in the side chain (*i.e.*, VII), 10-(3-chloropropyl)-2-trifluoromethylphenothiazine (V) was treated with carbomethoxyalkyl-substituted piperazines (IV), and the resultant esters (VI) subsequently hydrolyzed with base. The desired substituted piperazines were readily



I



IIa: n = 2
IIb: n = 4



III

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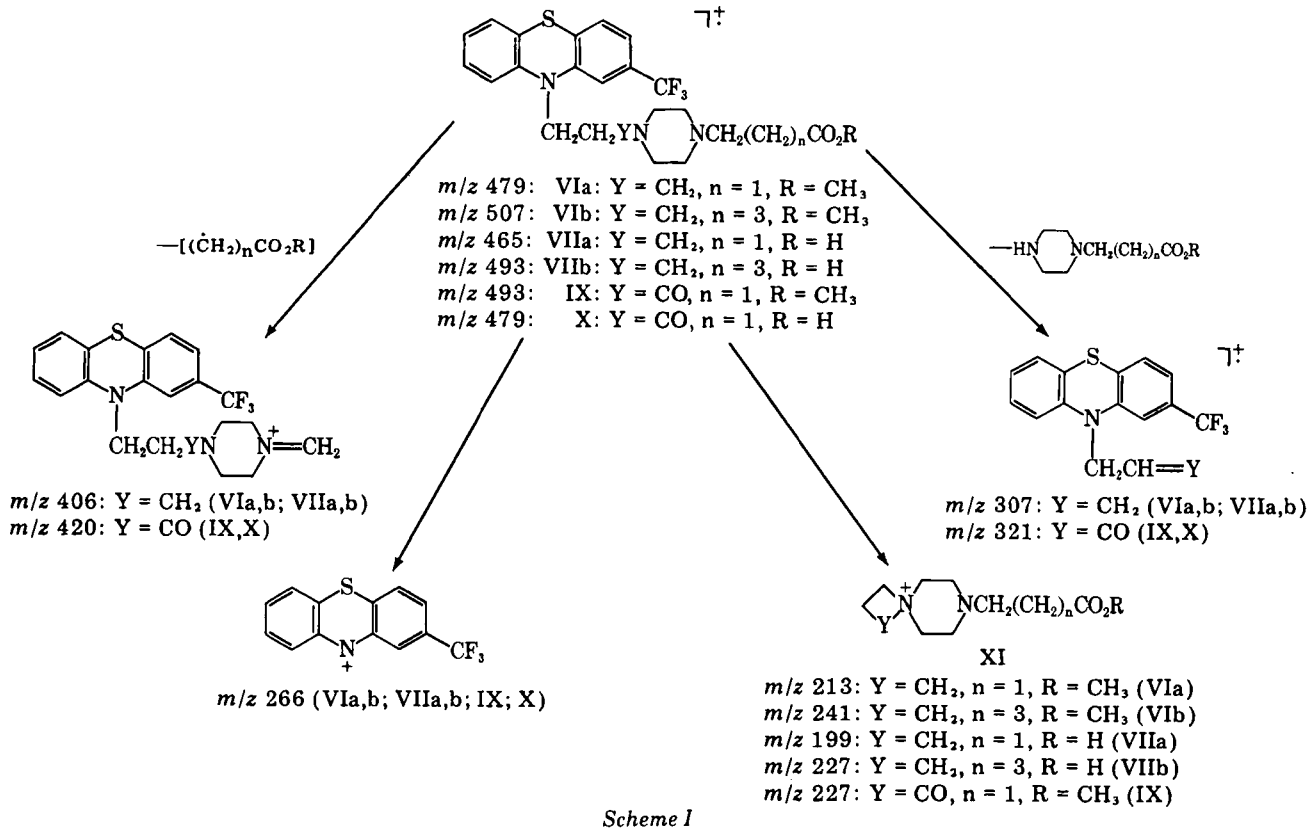
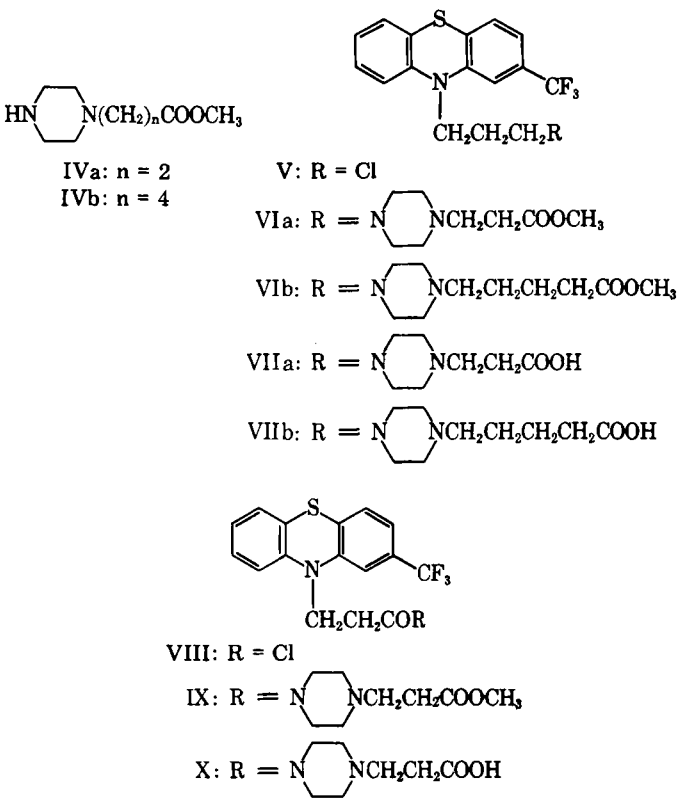


Scheme I

synthesized by treatment of piperazine with methyl acrylate or 5-bromovalerate. This approach to the synthesis of carboxyalkyl-substituted trifluoperazine differs from that initially reported for the synthesis of the analogous hapten of chlorpromazine (8), where *N*-desmethylchlorpromazine was reacted with methyl acrylate and the resultant ester of the desired hapten subsequently hydrolyzed (5). The lack of availability of *N*-desmethyltrifluoperazine and the simple synthetic accessibility of the chloropropyl starting material (V) prompted the approach reported here. Additionally, to investigate the effect that a carbonyl group in the side chain of the hapten has on the sensitivity and specificity of the antiserum raised to the protein conjugate, X was obtained by treatment of 3-[10-(2-trifluoromethylphenothiazinyl)]propionyl chloride (VIII) with 1-(2-methoxycarbonylethyl)piperazine (IVa) to yield the ester IX which was subsequently hydrolyzed to the desired acid (X).



All three haptens and their methyl esters gave NMR and MS entirely consistent with the given structures. The mass spectra of the three esters (VIa, VIb, and IX) and their corresponding acids (VIIa, VIIb, and X) gave molecular ions in each case. The fragmentations of these esters and acids can be rationalized in terms of the structures of the compounds. Some of the major diagnostic ions of the mass spectra of all six compounds are shown in Scheme I, the origin of the various ions being indicated in parentheses. In each case, the loss of $(\dot{C}H_2)_nCO_2CH_3$ from the esters and the loss of $(\dot{C}H_2)_nCO_2H$ from the acids leads to the diagnostic ions at *m/z* 406 (VIa, VIb, VIIa, and VIIb) or *m/z* 420 (IX and X). Also, the loss of $NH(CH_2CH_2)NCH_2(CH_2)_nCO_2CH_3$ from the esters or $HN(CH_2CH_2)$-$NCH_2(CH_2)_nCO_2H$ from the acids, as a neutral molecule from the parent molecular ions, results in diagnostic ions at *m/z* 307 for VIa, VIb, VIIa, and VIIb and at *m/z* 321 for IX and X. The presence of the ion at *m/z* 266 in the mass spectra of all six compounds is due to the cleavage of the N—C bond between the ring and the side chain, with the charge being accommodated at the phenothiazine ring system (8). On the other hand, when the charge is accommodated in the side chain, then the diagnostic ions at *m/z* 213 (VIa), 241 (VIb), and 227 (IX) for esters and at *m/z* 199 (VIIa) and 227 (VIIb) for acids can be rationalized by the proposed structure for XI. Also, the ions due to XI can further fragment by loss of one or more of the neutral molecules, $CH{\equiv}CH$, $CH_2{=}CH_2$, $CH_2{=}C{=}O$, $CO(CH_2)_2$, $RN(CH_2)_2$, and $CH_3{-}N{=}CH_2$. The absence of the corresponding ion XI in the mass spectrum of X may be due to the fact that the charge is completely retained by the phenothiazine ring system (*m/z* 266, 100% relative abundance). The β-aminocarboxylic acids, VIIa and X, lose a neutral molecule of acrylic acid with a transfer of hydrogen as reported earlier (9). This results in the ions at *m/z* 393 and 407 for VIIa and X, respectively. However, in the mass spectrum of the γ-aminocarboxylic acid VIIb, the ion at *m/z* 393 was of low abundance, while the abundant ion at *m/z* 406 probably formed from the loss of $\dot{C}H_2CH_2CH_2CO_2H$ due to the bulkiness of the chain.

All attempts to find a suitable recrystallization solvent for the haptens failed and, thus, analytical figures were obtained for the hydrochloride salts of their methyl esters. Further confirmation of the integrity of the hapten was obtained by conversion of the acid group back to the methyl

ester by reaction with an ethereal solution of diazomethane (8). GC analysis of the crude methylated residue showed in each case only one peak, which gave on GC–MS the same electron-impact mass spectrum as the appropriate authentic hapten methyl ester.

The haptens were successfully coupled to bovine serum albumin by a modified mixed anhydride method (8, 10, 11). To investigate whether the number of moles of hapten coupled to one mole of the protein influences the quality of the antiserum in terms of specificity and sensitivity, both IIa and IIb were obtained with variation in the number of moles coupled to bovine serum albumin. Thus, while all three haptens were coupled with bovine serum albumin to produce an immunizing antigen with 15–30 hapten residues/mol of bovine serum albumin, IIa and IIb were also obtained with ~70 hapten residues/mol of bovine serum albumin.

Rabbits were immunized with each hapten–protein conjugate as described in the *Experimental* section. The preliminary assessment of the antisera so produced, together with the number of intravenous booster shots required, is presented in Table I. In the first experiments using at least five rabbits, operational titers were produced from all five conjugates. However, only the antisera produced from the conjugate with two carbon atoms between the piperazine ring and the amide linkage in structurally modified trifluoperazine (*i.e.*, IIa) gave titer values greater than 1:500. Thus, by comparison of the antisera produced from IIa and IIb it can be observed that on increasing the chain length between the piperazine ring and the amide linkage in the hapten–protein conjugate, the titer value did not increase. To check this important observation, two more rabbits each were immunized with IIa (X = 70) and IIb (X = 71) in a second experiment. Apart from a 1:500 titer in two of the rabbits, a 1:8000 titer was produced in one of the IIa rabbits, thus confirming the initial observation. It can also be deduced from the first experiments that since III only produced 1:500 titers, the insertion of a carbonyl group in the side chain of the hapten had a detrimental effect on the quality of the antisera raised to the resultant hapten–protein conjugate. Finally, from the initial experiments it was also observed that increasing the number of hapten residues per mole of bovine serum albumin did not have a dramatic effect on titer values. In the case of the two types of IIb (X = 27 and X = 71), there were virtually no differences with respect to the titer produced; however, in the case of IIa higher titers were produced from the antibodies raised to the high moles-coupled hapten–protein conjugate (*i.e.*, IIa, X = 70) than the low moles-coupled hapten–protein conjugate (*i.e.*, IIa, X = 27).

RIAs sensitive to the nanogram range have been developed for trifluoperazine using antibodies to IIa. The assays were not influenced by plasma and endogenous substances. A procedure using antibodies to the IIa (X = 27) conjugate has been previously published (5), where the antisera cross-reacted with the presumed major metabolites of trifluoperazine (the *N*-desmethyl, 7-hydroxy, and sulfoxide compounds) to the extent of 26, 24, and <1%, respectively. By comparison, the three 1:10,000 titer antisera raised to the IIa (X = 70) conjugate cross-reacted (12) with these same metabolites 12–14, <1–14, and <1%, respectively. These improved RIA procedures using the IIa (X = 70) conjugate are described in depth elsewhere (13). Also, these antisera raised to the IIa conjugates recognize especially well (100–143%, cross-reactivity) the side-chain-modified analogue of trifluoperazine, fluphenazine; therefore, RIA procedures are also being developed for fluphenazine using these same antisera.

Table I—Rabbit Antiserum Titers of Trifluoperazine Hapten–Protein Conjugates [a]

Immunizing Antigen[b]	No. of Positives/ No. Tested	No. of Intravenous Boosters	Titers[c]
IIa, X = 70	3/5	1	1:10,000 (3)
	2/2	4	1:500 (1)
		4	1:8000 (1)
IIa, X = 27	2/8	1	1:2000 (1)
		1	1:4000 (1)
IIb, X = 71	3/5	2–4	1:500 (3)
	1/2	4	1:500 (1)
IIb, X = 16	3/5	3–4	1:500 (3)
III, X = 24	6/8	3	1:500 (6)

[a] Titer values 1:250 or less are not recorded. [b] X = number of hapten residues per mole of bovine serum albumin. [c] Number of rabbits giving this titer in parentheses.

EXPERIMENTAL[1]

1-(4-Methoxycarbonylbutyl)piperazine (IVb) and 1,4-Bis(4-methoxycarbonylbutyl)piperazine—A stirred mixture of methyl 5-bromovalerate (7.80 g, 40 mmol) and anhydrous piperazine (6.88 g, 80 mmol) in 50 mL of dry benzene was heated under reflux for 4 h. The resulting piperazine hydrobromide was removed by filtration and washed with dry benzene (20 mL). The organic phase was evaporated, and the residue was distilled *in vacuo* to obtain 4.5 g (56%) of IVb as a colorless oil, bp 72–74°C/0.08 mm Hg; ^{1}H-NMR: δ 1.44–1.74 (m, 5, butyl central CH_2CH_2 and NH which exchanges with D_2O), 2.14–2.58 (m, 8, piperazine CH_2), 2.78–3.06 (m, 4, $CH_2CO_2CH_3$ and CH_2-piperazine), and 3.70 ppm (s, 3, CO_2CH_3); MS: m/z 200 (M^+, 21%), 169(34), 159(11), 158(81), 115(33), 100(13), 99(100), 98(13), 85(12), 84(23), and 83(15).

Anal.—Calc. for $C_{10}H_{20}N_2O_4$: C, 59.97; H, 10.07; N, 13.99. Found: C, 60.57; H, 10.40; N, 13.55.

The vacuum distillation also gave 1.5 g (12%) of the bis compound as a colorless oil, bp 134–136°C/0.08 mm Hg; ^{1}H-NMR: δ 1.30–1.80 (m, 8, butyl central CH_2CH_2), 2.17–2.57 (m, 16, piperazine CH_2, $CH_2CO_2CH_3$, and CH_2-piperazine), and 3.68 ppm (s, 6, CO_2CH_3); MS: m/z 314 (M^+, 11%), 283(17), 214(15), 213(100), 185(12), 158(16), 144(37), 115(19), 99(32), 98(35), 84(17), and 83(15).

***N*-(2-Methoxycarbonylethyl)desmethyltrifluoperazine (VIa)**—A mixture of 10-(3-chloropropyl)-2-trifluoromethylphenothiazine (V) (1.38 g, 4 mmol), mp 69–70°C [lit. (14) mp 70–71°C] synthesized by modification of a literature procedure (15), IVa (1.03 g, 6 mmol) (16), and sodium iodide (0.60 g, 4 mmol) in 20 mL of methyl ethyl ketone was refluxed with stirring for 20 h. The solvent was removed *in vacuo*, and the residue was extracted with 5% HCl (5 × 4 mL). The acidic extract was brought to pH 7.0 with sodium carbonate and extracted with ether (3 × 20 mL). The combined ether extract was dried (magnesium sulfate) and treated with dry hydrogen chloride in ether to afford 1.61 g (73%) of the dihydrochloride salt of the ester VIa as pale-yellow crystals from methanol–ether, mp 194–196°C; ^{1}H-NMR: δ 1.83–2.14 (m 2, propyl central CH_2), 2.34–2.81 (m, 14, piperazine CH_2, CH_2-piperazine, and $CH_2CH_2CO_2CH_3$), 3.74 (s, 3, CO_2CH_3), 4.04 (t, 2, J = 6.2 Hz, CH_2-phenothiazine ring), and 6.79–7.26 ppm (m, 7, aromatic); MS: m/z 479 (M^+, 37%), 406(10), 307(10), 280(28), 267(11), 266(15), 258(12), 248(14), 213(24), 199(42), 186(15), 185(100), 157(12), 142(14), 130(13), 116(28), 113(14), 111(16), 99(15), 98(35), 97(13), and 84(21).

Anal.—Calc. for $C_{24}H_{28}F_3N_3O_2S$·2HCl: C, 52.17; H, 5.11; N, 7.61. Found: C, 52.29; H, 5.29; N, 7.70.

***N*-(4-Methoxycarbonylbutyl)desmethyltrifluoperazine (VIb)**—This compound was synthesized from IVb (1.00 g, 5 mmol), V (1.38 g, 4 mmol), and sodium iodide (0.60 g, 4 mmol) in 20 mL of methyl ethyl ketone in the same manner as described for VIa. The yield was 65% of the dihydrochloride salt of VIb. recrystallized from methanol–ether, mp 235–236°C; ^{1}H-NMR: δ 1.36–2.57 (m, 20, side-chain CH_2 except CH_2-phenothiazine ring), 3.60 (s, 3, CO_2CH_3), 3.88 (t, 2, J = 6.2 Hz, CH_2-phenothiazine ring), and 6.80–7.23 ppm (m, 7, aromatic); MS: m/z 507 (M^+, 67%), 476(17), 406(28), 307(14), 306(11), 295(13), 281(21), 280(100), 267(26), 266(25), 248(27), 241(28), 228(15), 227(90), 214(18), 213(68), 203(21), 185(12), 170(11), 144(33), 115(12), 113(40), 99(17), 98(30), and 97(18).

Anal.—Calc. for $C_{26}H_{32}F_3N_3O_2S$·2HCl: C, 53.79; H, 5.90; N, 7.24. Found: C, 53.60; H, 6.04; N, 6.91.

***N*-(2-Carboxyethyl)desmethyltrifluoperazine (VIIa)**—A mixture of VIa as the dihydrochloride salt (0.55 g, 1 mmol), sodium hydroxide (0.16 g, 4 mmol), methanol (15 mL), and water (15 mL) was heated under gentle reflux for 1 h. The methanol was removed under reduced pressure, and a further 15 mL of water was added. After washing with ether (2 × 10 mL), the pH of the aqueous phase was adjusted to neutral with 1% HCl to yield an oily solid. The product was extracted with methylene chloride (3 × 10 mL), dried (magnesium sulfate), and concentrated under reduced pressure to give 0.41 g (88%) of pale-yellow scales; ^{1}H-NMR: δ 1.72–2.17 (m, 2, propyl central CH_2), 2.47–2.94 (m, 14, piperazine CH_2, $CH_2CH_2CO_2H$, and CH_2-piperazine), 4.00 (t, 2, J = 6.0 Hz, CH_2-phenothiazine ring), 6.94–7.43 (m, 7, aromatic), and 10.03 ppm (s, 1, acid H

[1] Melting points were determined in open-glass capillaries with a Gallenkamp melting point apparatus and are uncorrected. ^{1}H-NMR spectra were determined, with the free base in each case, on a Varian T-60 instrument in deuterochloroform with tetramethylsilane as internal reference. Low-resolution electron-impact mass spectra were recorded on a VG Micromass MM16F instrument at 70 eV, and except for base peak, diagnostic ion and parent ion peaks <m/z 75 or relative intensity <10% of the base peak were omitted. UV determinations were carried out using a Unicam SP 1700 instrument. Microanalyses for samples dried over phosphorus pentoxide at 60°C under reduced pressure were carried out by Mr. R. E. Teed, Department of Chemistry and Chemical Engineering, University of Saskatchewan.

which exchanges with D_2O); MS: *m/z* 465 ($M^{+\cdot}$, 23%), 406(2), 394(13), 393(49), 307(16), 306(17), 280(24), 267(22), 266(36), 248(28), 199(12), 185(12), 171(23), 127(36), 99(99), 97(17), 84(29), and 58(100).

***N*-(4-Carboxybutyl)desmethyltrifluoperazine (VIIb)**—This compound was synthesized from VIb by alkaline hydrolysis in the same manner as described for VIIa to yield 94% of pale-yellow scales; ^{1}H-NMR: 1.44–3.03 (m, 20, side-chain CH_2 except CH_2-phenothiazine ring), 3.97 (t, 2, J = 6.0 Hz, CH_2-phenothiazine ring), 6.78–7.41 (m, 7, aromatic), and 12.09 ppm (s, 1, acid H which exchanges with D_2O); MS: *m/z* 493 ($M^{+\cdot}$, 74%), 406(22), 393(2), 350(10), 307(17), 306(12), 294(16), 281(20), 280(100), 267(38), 266(36), 248(33), 227(37), 214(12), 213(78), 203(22), 200(17), 199(100), 197(10), 171(14), 156(17), 143(11), 139(10), 130(27), 113(45), 105(12), 99(29), 98(36), 97(22), 96(11), 85(12), 84(35), 83(18), 82(13), and 77(10).

10-[3-(4-Methoxycarbonylethylpiperazinyl)-3-oxopropyl]-2-trifluoromethyl-10*H*-phenothiazine (IX)—The propionyl acid chloride (VIII) (0.90 g, 2.5 mmol), prepared as previously reported (17), in 5 mL of dry benzene was added in a dropwise manner over a period of 1 h to a stirred solution of IVa (0.47 g, 2.75 mmol) and triethylamine (0.5 mL) in 10 mL of benzene at 5°C. After stirring at room temperature for an additional 2 h, the benzene layer was washed twice with water, dried over anhydrous magnesium sulfate, filtered, and evaporated *in vacuo* to leave an oily residue. Column chromatography with alumina as the adsorbent and benzene as the solvent gave, after evaporation of the solvent, 1 g (81%) of a colorless oil which yielded the hydrochloride salt as fine white crystals from methanol, mp 141–142°C; ^{1}H-NMR: δ 2.18–2.94 (m, 10, piperazine CH_2 and CH_2-piperazine), 3.17–3.73 (m, 7, CH_2CON, $CH_2CO_2CH_3$, and CO_2CH_3), 4.30 (t, 2, J = 7.0 Hz, CH_2-phenothiazine ring), and 6.77–7.40 ppm (m, 7, aromatic); MS: *m/z* 493 ($M^{+\cdot}$, 100%), 420(21), 321(13), 320(15), 280(66), 267(16), 266(42), 248(27), 227(55), 213(10), 210(17), 199(4), 185(3), 171(11), 142(49), 130(11), 127(16), 125(11), 116(13), 99(16), 98(10), and 97(11).

Anal.—Calc. for $C_{24}H_{26}F_3N_3O_3S$·HCl: C, 54.38; H, 5.14; N, 7.93. Found: C, 54.74; H, 5.12; N, 7.96.

10 - [3-(4-Carboxyethylpiperazinyl)-3-oxopropyl]-2-trifluoromethyl-10*H*-phenothiazine (X)—This compound was obtained from IX by alkaline hydrolysis in the same manner as described for VIIa to yield 86% of white scales; ^{1}H-NMR: δ 2.38–2.94 (m, 10, piperazine CH_2 and CH_2-piperazine), 3.22–3.87 (m, 4, CH_2CON and $CH_2CO_2H_3$), 4.31 (t, 2, J = 7.0 Hz, CH_2-phenothiazine ring), 6.91–7.37 (m, 7, aromatic), and 9.60 ppm (s, 1, acid H which exchanges with D_2O); MS: *m/z* 479 ($M^{+\cdot}$, 2%), 420(1), 408(10), 407(40), 340(12), 339(61), 321(2), 281(15), 280(78), 268(11), 267(50), 266(100), 248(39), 247(10), 141(46), 85(13), 79(10), 78(100), and 77(31).

Coupling of VIIa to Bovine Serum Albumin—Compound VIIa was coupled to bovine serum by a modified mixed anhydride method (8, 10, 11). Compound VIIa (0.20 g, 0.43 mmol) and isobutyl choroformate (56 μL, 0.43 mmol) were dissolved in freshly distilled dioxane (3 mL), and the solution was cooled to 15°C and allowed to stand for 20 min. The mixture was then slowly added in 0.5-mL aliquots to a stirred, cooled (4°C) solution of bovine serum albumin[2] (0.30 g, 0.0043 mmol) in a mixture of 20 mL of water and 6 mL of dioxane, which had been adjusted to pH 9.3 with 2 M NaOH. Throughout the addition, the pH was maintained at pH 9.3 with 2 M NaOH. After the addition was complete, the clear solution was allowed to stand at 4°C for 12 h with continuous stirring.

The solution was initially dialyzed[3] alternately every 15 min at room temperature against bicarbonate buffer (0.2 M, pH 8.0, 3 × 600 mL) and acetate buffer (0.2 M, pH 5.0, 3 × 600 mL), and then finally against distilled water continuously for 4 h. After lyophilization, the conjugate IIa was obtained as a white crystalline solid (0.48 g). A blank was prepared in the same manner, but without VIIa. The number of hapten residues per mole of bovine serum albumin was calculated by the UV method (10, 11) to be 70 (IIa, X = 70).

The procedure was repeated, decreasing by half the relative amount of VIIa (0.20 g, 0.43 mmol) used with bovine serum albumin (0.60 g, 0.0086 mmol). The conjugate (0.71 g) was determined to have 27 mol of hapten residues/mol of bovine serum albumin (IIa, X = 27).

Coupling of VIIb to Bovine Serum Albumin—Compound VIIb was coupled to bovine serum albumin by a modified mixed anhydride method (8, 10, 11) in the same manner as described for the coupling of VIIa. VIIb (0.20 g, 0.41 mmol) and bovine serum albumin[2] (0.29 g, 0.0041 mmol) gave 0.47 g of conjugate IIb, where the number of hapten residues per mole of bovine serum albumin was calculated by the UV method (10, 11) to be 71 (IIb, X = 71).

The procedure was repeated, decreasing by half the relative amount of VIIb (0.050 g, 0.10 mmol) used with bovine serum albumin (0.15 g, 0.00215 mmol). The conjugate (0.17 g) was determined to have 16 mol of hapten residues/mol of bovine serum albumin (IIb, X = 16).

Coupling of X to Bovine Serum Albumin—Compound X was coupled to bovine serum albumin by a modified mixed anhydride method (8, 10, 11) in the same manner as described for the coupling of VIIa. Compound X (0.080 g, 0.167 mmol) and bovine serum albumin (0.117 g, 0.00167 mmol) gave 0.15 g of conjugate III, where the number of hapten residues per mole of bovine albumin was calculated by the UV method (10, 11) to be 24 (III, X = 24).

Immunization—Each hapten–protein conjugate (4 mg) was dissolved in normal saline (2.0 mL) and emulsified with Freund's complete adjuvant[4] (2.0 mL). Each conjugate was administered to at least five New Zealand White female rabbits[5] by injection of 0.5 mL of the emulsion into each shoulder. At 2-week intervals thereafter, the injections were repeated with an emulsion prepared as described above, except that Freund's complete adjuvant was replaced by Freund's incomplete adjuvant. The antiserum titer of each rabbit was checked 1 week after the fifth injection, when the route of administration was changed to intravenous. In the experience of the authors (5, 7), selectivity does not change after five immunizations, whereas intravenous booster shots at this stage lead to improved sensitivity with maintained selectivity.

Serum was obtained from the marginal ear vein at 2-week intervals after the fifth injection. The antiserum titer of each rabbit was checked by evaluating the binding characteristics of tritiated trifluperazine[6] to each antiserum. After optimal titer values were achieved, blood was obtained from each rabbit by cardiac puncture. The harvested serum was immediately lyophilized in 1.0-mL samples and stored at −20°C.

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ACKNOWLEDGMENTS

The study was financially supported by the Medical Research Council of Canada (Development Grant 222). The authors are greatly indebted to K. Hall who measured the mass spectra and Mrs. Carol Constable for typing the manuscript.

[2] RIA Grade, Fraction V Powder; Sigma Chemical Co., St. Louis, Mo.

[3] Fisher dialyzer tubing (Size C); Fisher Scientific Co., Edmonton, Alberta, Canada.

[4] Grand Island Biological Co., Grand Island, N.Y.

[5] Mr. Avaldermeer, Sherwood Park, Alberta, Canada.

[6] Radioactive trifluoperazine, generally labeled with tritium, having a specific activity of 12.8 Ci/mmol; Nuclear Research Centre–Negev, Beer-Sheva, Israel. Radiochemical purity was checked by analytical TLC and HPLC.