Production and Characterization of Polyclonal Anti-S 20499 Antibodies: Influence of the Hapten Structure on Stereospecificity

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Received January 10, 1997, from the *Department de Bio-Analyse, Technologie SERVIER, 27 rue Eugène Vignat, F-45000 Orléans, France, [‡]Institut de CHIMIE ORGANIQUE et ANALYTIQUE associé au CNRS, Université d'Orléans, BP 6759, 45067 Orléans Cedex 2, France, and [§]INSERM Unité 26, 200 rue du Faubourg St. Denis, 75010 Paris, France. Accepted for publication February 19, 1997[⊗].

Abstract □ Immunoassays were studied as an alternative to HPLC methods for the stereoselective determination of a chiral drug, S 20499, a new anxiolytic compound that is chemically related to buspirone. The production of highly stereospecific polylclonal antibodies was sought following the construction of appropriately optimized hapten–protein conjugates. This process involved the selection of the structure and the length of the spacer arm used to couple S 20499 to the carrier protein as well as deciding on the location of the coupling site with respect to the chiral center. Two haptens were prepared: one a derivative resembling the original structure of S 20499, with the effective addition of a carboxylic acid group, and a second with the effective addition of a butanoic acid moiety that is supposed to favor stereorecognition. Six stereospecific polyclonal antisera were obtained in rabbits with two groups of antibody families defined in terms of specificity. Both approaches gave high levels of stereospecificity (cross-reactivity towards the optical antipode of S 20499 ranged from 4.1% to <0.1%). Although it did not decrease the mean apparent affinity constant, the longer spacer improved antibody specificity by decreasing cross-reactions towards dealkylated S 20499 derivatives. Hence, the addition of a four carbon atom bridge should be a valuable tool for increasing antibody stereospecificity with no drawbacks in terms of specificity and affinity. It was also shown that long immunization periods appear to have no effect on the stereospecificity of the antibodies obtained.

Introduction

Despite the possible beneficial effects from the administration of drug substances containing various proportions of stereoisomers,[1] the development of single enantiomers, as opposed to racemates, has been viewed preferential in the light of possible differences in the pharmacokinetic and/or pharmacodynamic properties of different configurations.[2−4] In all cases, stereospecific analytical methods must be developed to measure all of the possible enantiomers present after racemate dosing or to study chiral inversion, racemization, or epimerization after the administration of single enantiomers. For these reasons, chiral chromatographic methods are widely used.[5−8] However, because the use of very potent drugs leads to lowered doses, analytical methods have to be sensitive enough to quantify extremely low concentrations of drugs in biological fluids. Stereospecific immunoassays, which combine sensitivity and selectivity, have recently been developed and applied in the domains of pharmacology[9−11] and toxicology.[12] Several techniques allow the modulation of the antibody−drug specificity.[13] In terms of stereospecificity, the preparation of an appropriate hapten conjugate appears to be of major importance. The influence of the immunization period on the stereospecificity could also be an important factor to consider and study. The aim of the present work was to examine the influence of the chemical structure of the hapten on the specificity of the elicited antibodies, especially in terms of asymmetric center recognition. To develop this approach, we describe herein the production and characterization of polyclonal anti-S 20499 (Figure 1), the eutomer of S 20244 and a potent agonist of $5HT1_a$ receptors related to buspirone.[14] We also conducted a study to determine the influence of the immunization period on the titre, the mean apparent affinity, and the stereospecificity. The same procedure was applied for the production of anti-S 20500 antibodies (S 20500 being the distomer of S 20244). The question of preferential recognition of the active configuration was addressed by comparing the stereospecificity obtained for anti-S 20499 and anti-S 20500 antibodies.

Materials and Methods

Reagents—[^{3}H]S 20499 (specific activity 49.6 Ci/mmol) was obtained from CEA (Gif sur Yvette, France). S 20499 and all related compounds, including compounds used for hapten synthesis and ethyl-7-bromoheptanoate, were provided by Technologie Servier (Orléans, France). Triethylamine, dimethylformamide (DMF), methanol, *N*-methylmorpholine, isobutylchloroformate, ethyl-4-bromobutanoate, potassium iodide, potassium carbonate, Freund's complete and incomplete adjuvants, and bovine serum albumin (BSA) were purchased from Sigma Chemicals (St. Louis, MO). Ammonium sulfate, sodium phosphate monobasic and dibasic, and citric acid were purchased from Merck (Darmstadt, Germany).

Preparation of the Hapten—The procedure for the hapten synthesis is shown in Figure 2.

(*S*)-8-[4-[*N*-(5-Methoxy-3,4-dihydro-2*H*-1-benzopyran-5-yl)-amino]butyl]-8-azaspiro[4.5]-decane-7,9-dione (2)—5-Methoxy-3,4-dihydro-3-amino-2*H*-1-benzopyran **1** (1.00 g, 5.59 mmol), 8-(4-bromobutyl)-8-azaspiro[4.5]decane-7,9-dione (1.86 g, 6.15 mmol), triethylamine (1.70 g, 16.76 mmol), and potassium iodide (catalytic amount) were mixed in anhydrous DMF (15 mL). The solution was stirred for 24 h at 70 °C under argon, then cooled to room temperature. The solvent was removed under reduced pressure, and the residue was taken up in 20 mL of methylene chloride and washed three times with water. The organic layer was dried over anhydrous magnesium sulfate, and the solvent was evaporated. The crude product was then chromatographed on silica gel (eluent diethylether:methylene chloride 50:50) to give 1.52 g (68%) of pure **2** as a clear oil. IR (neat): 1720 and 1670 cm^{-1} (ν C=O); ^{1}H NMR ($CDCl_3$): δ 1.45−1.85 (m, 12H, **CH_2**), 2.42 (dd, J_1 = 16.0 Hz, J_2 = 7.1 Hz, 1H, **CH**Ar), 2.56 (s, 4H, **CH_2**CO), 2.72 (t, J = 7.0 Hz, 2H, **CH_2**N), 2.8−3.0 (m, 1H, **CH**Ar), 3.0−3.15 (m, 1H, **CH**N), 3.76 (t, J = 7.0 Hz, 2H, **CH_2**NCO), 3.80 (s, 3H, **CH_3**O), 3.80−3.90 (m, 1H, **CH**O), 4.10−4.20 (m, 1H, **CH**O), 6.41 and 6.46 (2d, J = 8.0 Hz, 2H, arom.), 7.04 (t, J = 8.0 Hz, 1H, arom.).

(*S*)-Ethyl(4-{[(4-{7,9-dioxo-8-azaspiro[4,5]decan-8-yl}butyl)-(5-methoxy-3,4-dihydro-2*H*-1-benzopyran-5-yl)]amino}butanoate (3) and (*S*)-Ethyl(4-{[(4-{7,9-dioxo-8-azaspiro[4,5]decan-8-yl}butyl)(5-methoxy-3,4-dihydro-2*H*-1-benzopyran-5-yl)]-amino}heptanoate (4)—Compound **2** (1.00 g, 2.50 mmol), ethyl-4-bromobutanoate (1.95 g, 10.00 mmol), or ethyl-7-bromoheptanoate (2.37 g, 10.00 mmol) for **3** or **4**, respectively, triethylamine (0.76 g, 7.50 mmol), and potassium iodide (catalytic amount) were mixed in anhydrous DMF (15 mL). The solution was stirred for 24 h at 70 °C under argon, then cooled to room temperature. The solvent was removed under reduced pressure, and the residue was taken up in

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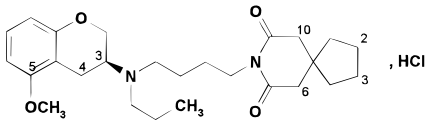


Figure 1—Structure of S 20499.

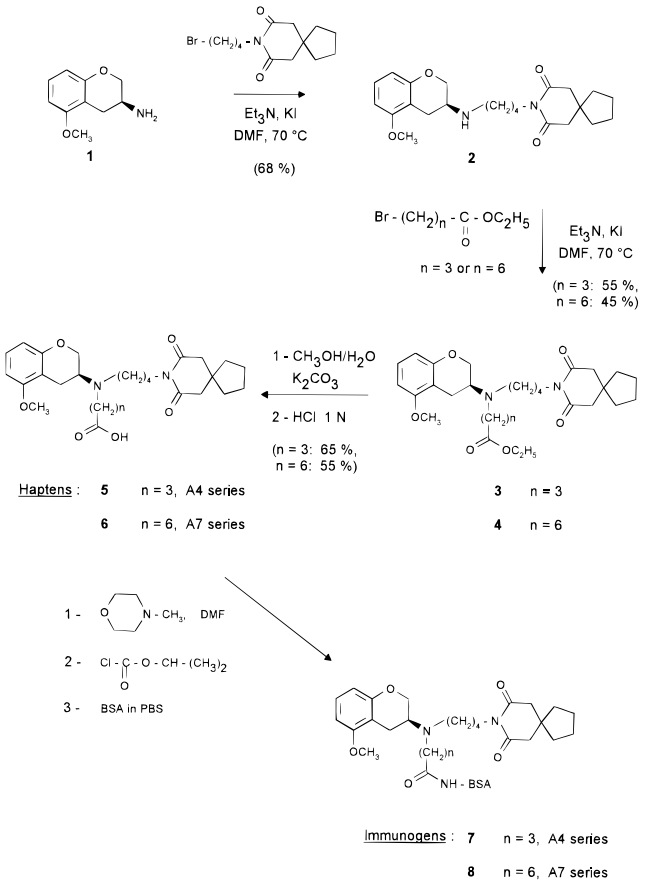


Figure 2—Hapten synthesis and immunogen preparation.

20 mL of methylene chloride and washed three times with water. The organic layer was dried over anhydrous magnesium sulfate, and the solvent was evaporated. The crude products were then chromatographed on silica gel column (eluent, ether:ethylacetate, 60:40) to give 0.70 g (55%) or 0.63 g (45%) of pure **3** or **4**, respectively, as clear oils.

Compound **3**: IR (neat): 1730 and 1670 cm^{-1} (ν C═O); ^1H NMR ($CDCl_3$): δ 1.26 (t, J = 7.0 Hz, 3H, $CH_2\mathbf{CH_3}$) 1.40−1.80 (m, 14H, $\mathbf{CH_2}$), 2.33 (t, J = 7.0 Hz, 2H, $\mathbf{CH_2}$COOEt), 2.40−2.50 (m, 1H, **CH**Ar), 2.50−2.70 (m, 4H, $\mathbf{CH_2}$N), 2.59 (s, 4H, $\mathbf{CH_2}$CO), 2.80−3.00 (m, 1H, **CH**Ar), 3.00−3.20 (m, 1H, **CH**N), 3.70−3.80 (m, 1H, **CH**O), 3.70−3.80 (m, 2H, $\mathbf{CH_2}$NCO), 3.80 (s, 3H, $\mathbf{CH_3}$O), 4.12 (q, J = 7.0 Hz, 2H, $\mathbf{CH_2}$-CH_3), 4.20−4.30 (m, 1H, **CH**O), 6.41 and 6.45 (2d, J = 8.0 Hz, 2H, aromatic), 7.03 (t,. J = 8.0 Hz, 1H, aromatic).

Compound **4**: IR (neat): 1730 and 1670 cm^{-1} (ν C═O); ^1H NMR ($CDCl_3$): δ 1.25 (t, J = 7.0 Hz, 3H, $CH_2\mathbf{CH_3}$) 1.30−1.80 (m, 20H, $\mathbf{CH_2}$), 2.28 (t, J = 7.0 Hz, 2H, $\mathbf{CH_2}$COOEt), 2.40−2.80 (2m, 5H, **CH**Ar and $\mathbf{CH_2}$N), 2.57 (s, 4H, $\mathbf{CH_2}$CO), 2.70−2.90 (m, 1H, **CH**Ar), 3.00−3.20 (m, 1H, **CH**N), 3.60−3.80 (m, 1H, **CH**O), 3.60−3.80 (m, 2H, $\mathbf{CH_2}$NCO), 3.82 (s, 3H, $\mathbf{CH_3}$O), 4.11 (q, J = 8.0 Hz, 2H, $\mathbf{CH_2}$Et), 4.20−4.30 (m, 1H, **CH**O), 6.40 and 6.45 (2d, J = 8.0 Hz, 2H, aromatic), 7.03 (t, J = 8.0 Hz, 1H, aromatic).

(*S*)-4-{[(4-{7,9-Dioxo-8-azaspiro[4,5]decan-8-yl}butyl)(5-methoxy-3,4-dihydro-2*H*-1-benzopyran-5-yl)]amino}butanoic acid (5) and (*S*)-4-{[(4-{7,9-Dioxo-8-azaspiro[4,5]decan-8-yl}butyl)-(5-methoxy-3,4-dihydro-2*H*-1-benzopyran-5-yl)]amino}heptanoic acid (6)—Compound **3** (0.50 g, 0.97 mmol) or **4** (0.50 g, 0.90 mmol) was dissolved in 35 mL of methanol. A solution of K_2CO_3 (0.41 g, 3.00 mmol) dissolved in 15 mL of water was then added, and the resulting solution was stirred for 3 days at room temperature to liberate acidic function. The methanol was removed under reduced pressure, and the aqueous phase was washed twice with 28 mL of ethyl ether. The aqueous phase was then adjusted to pH 6 with 1 M hydrochloric acid. After three extractions with methylene chloride (v/v), the organic layer was dried over anhydrous magnesium sulfate, and the solvent was evaporated. The crude products were chromatographed on silica gel (eluent, methylene chloride:methanol, 92:8) to give 0.31 g (65%) or 0.26 g (55%) of pure **5** and **6**, respectively, as colorless oils. Compounds **5** and **6** correspond to the haptens of the A_4 and A_7 series, respectively.

Compound **5**: IR (neat): 1725 and 1670 cm^{-1} (ν C═O); ^1H NMR ($CDCl_3$): δ 1.40−1.80 (m, 12H, $\mathbf{CH_2}$), 1.80−1.90 (m, 2H, $\mathbf{CH_2}CH_2$-COOH), 2.56 (t, J = 7.0 Hz, 2H, $\mathbf{CH_2}$COOH), 2.58 (s, 4H, $\mathbf{CH_2}$CO), 2.60−2.70 (m, 1H, **CH**Ar), 2.70−2.90 (m, 4H, $\mathbf{CH_2}$N), 2.90−3.10 (m, 1H, **CH**Ar), 3.30−3.50 (m, 1H, **CH**N), 3.74 (t, J = 7.0 Hz, 2H, $\mathbf{CH_2}$-NCO), 3.78 (s, 3H, $\mathbf{CH_3}$O), 4.00 (t, J = 9.2 Hz, 1H, **CH**O), 4.25−4.40 (m, 1H, **CH**O), 6.41 and 6.46 (d, J = 8.0 Hz, 2H, aromatic), 7.06 (t, J = 8.0 Hz, 1H, arom.).

Compound **6**: IR (neat): 1725 and 1670 cm^{-1} (ν C═O); ^1H NMR ($CDCl_3$): δ 1.20−1.80 (m, 20H, $\mathbf{CH_2}$), 2.30 (t, J = 7.0 Hz, 2H, $\mathbf{CH_2}$-COOH), 2.50−2.80 (m, 5H, **CH**Ar and $\mathbf{CH_2}$N), 2.58 (s, 4H, $\mathbf{CH_2}$CO), 2.80−2.95 (m, 1H, **CH**Ar), 3.10−3.30 (m, 1H, **CH**N), 3.70−4.00 (m, 3H, **CH**O and $\mathbf{CH_2}$NCO), 3.83 (s, 3H, $\mathbf{CH_3}$O), 4.20−4.35 (m, 1H, **CH**O), 6.41 and 6.47 (2d, J = 8.0 Hz, 2H, aromatic), 7.04 (t, J = 8.0 Hz, 1H, aromatic).

Preparation of Immunogens—The reaction pathways used for the synthesis of conjugates are shown in Figure 2. Haptens **5** and **6** (A4 and A7 series respectively) were coupled to BSA according to the mixed anhydride method.[15] A solution of each hapten (75 mg, 0.15 mmol) in 2 mL of DMF cooled to −15 °C was prepared, neutralized with *N*-methylmorpholine (23 mg, 0.23 mmol in 1.5 mL of DMF), and stirred for 10 min. Isobutyl chloroformate (25 mg, 0.18 mmol) in 1.5 mL of DMF was then added. After 10 min of stirring, the reaction mixture was allowed to warm up to 0 °C, and a solution of BSA (100 mg, 0.15 μmol) in 5 mL of 50 mM phosphate buffer (pH 7.4) was added in a dropwise manner. After filtration, the conjugates were extensively dialysed against a buffer/DMF mixture followed by buffer alone for 48 h. Following this procedure, partially soluble conjugates (**7** and **8**) were obtained and used for immunizations.

Characterization of the Coupling by Mass Spectrometry—Low-resolution electron-impact mass spectra (EI-MS, 70 eV) were recorded on a VG Analytica 70 VS mass spectrometer. Samples (1−10-μg immunogen aliquots) were introduced *via* the direct insertion probe that was heated to 430 °C. The source was maintained at 180 °C, and an accelerating voltage of 8 kV was applied. Scanning spectra were acquired over the range m/z 45−800. High-resolution measurements were made on the same instrument by peak matching at a resolution of 10 000 (M/ΔM).

Immunization Procedure—Two groups of three white New Zealand rabbits (IFFA CREDO, France) were immunized by the administration of 10 intradermal dorsal injections (100 μL for each injection). Primary immunizations were composed of 500 μL of 0.9% NaCl containing 100 μg of immunogen emulsified in 500 μL of Freund's complete adjuvant. Subsequent immunizations, at 3−8-week intervals, were of the same volume, with complete adjuvant replaced by incomplete adjuvant. After the fourth immunization, rabbits were bled from the ear vein 7−10 days after each injection. The last bleedings were performed ∼10 months after the first immunization.

Assay Procedure for the Characterization of the Polyclonal Antibodies—A liquid-phase radioimmunoassay (RIA) was used to follow the antisera titre and to characterize the elicited antibodies. For titration experiments, 100 μL of rabbit antiserum were incubated at various dilutions in citrate buffer (0.03 M, pH 6.4) with 200 μL of 0.6 nM [^{3}H]S 20499, 50 μL of human plasma, and 150 μL of a 5-mg/mL BSA solution in citrate buffer. The titre is determined as the antiserum dilution factor that corresponds to the binding of 50% of [^{3}H]S 20499.

In competitive experiments, increasing amounts of cold drugs in BSA-citrate buffer (100 μL, up to 2000 ng/mL) or BSA-citrate buffer (100 μL) were added to the incubation medium, while maintaining a total reaction volume of 500 μL. Nonspecific binding was determined by replacing antiserum with citrate buffer. In both experiments, reactions were allowed to proceed for 1.5 h at room temperature. Proteins were then precipitated with 500 μL of a saturated ammonium sulfate solution. Bound [^{3}H]S 20499 was separated from the free ligand by centrifugation. A 500-μL aliquot of the supernatant was

added to 4 mL of Picofluor (Packard, France) and the radioactivity was counted in a Packard Tricarb counter (Packard, France).

Displacement curves and binding parameters were obtained and calculated with the GraphPad InPlot program (GraphPad, San Diego, California). The 50% inhibitory concentration (IC_{50}) was calculated as the ligand concentration required to displaced 50% of the labeled ligand. In accordance with the criteria of Abraham,[16] cross-reactivity was expressed as the ratio (percent) of the IC_{50} for S 20499 with respect to the IC_{50} for the test ligand. Mean apparent affinity constants were estimated according to the method of Müller.[17]

Results and Discussion

Construction of the Hapten—The structurally important features of S 20499 are an azaspiro moiety, attached to the amine function of an aminochroman moiety through a four-carbon-atom methylene bridge. An asymmetric center is located in the 3-position of the chroman moiety, and a propyl side chain is coupled to the amine nitrogen atom (Figure 1). The objectives of the study were first to produce anti-S 20499 stereospecific antibodies and, second to make them as specific as possible towards potential metabolites. As the molecular weight of S 20499 is too low to induce an immunogenic response in rabbits, a coupling between S 20499 and a carrier protein was undertaken to produce an immunogen. To carry out this coupling, the introduction of a functional group on S 20499 to yield the desired hapten was required due to the absence of derivatizable functionalities in this compound. S 20499, like other structurally related molecules (buspirone, tiospirone), can be hydroxylated on the azaspiro moiety during metabolism.[18,19] Dealkylation of the methoxy group and hydroxylation(s) of the chroman moiety were also considered as potential biotransformations, as was the loss of the propyl side chain.

The pioneering studies of Landsteiner[20] showed that antibodies resulting from conjugates are generally more selective for the part of the molecule not involved in protein linkage. With this in mind, a coupling site on the propyl side-chain was selected (Figure 2). In underlining this selection, it should be pointed out that several studies concerning stereospecific immunoassays have demonstrated that stereorecognition may be increased when the asymmetric center is farther away from the coupling site.[21] Having chosen an appropriate coupling site, two hapten series were selected for synthesis. In the first of these series, a structure close to that of S 20499 was chosen, with the effective introduction of a carboxylic function at the end of the propyl chain (Figure 2, A4 series). Again, in justifying this choice, some studies have previously shown that the presence of long spacer arms, or the use of haptens not closely related to the free drug, appears to decrease the emergence of high-affinity antibodies towards the unchanged (i.e., nonderivatized drug).[22,23] Thus, in the second series, to study the influence of the distancing of the coupling site from the asymmetric center, a heptanoic acid group was added in place of the propyl chain of S 20499 (Figure 2, A7 series). In both series, methylene spacer groups were chosen because of their low immunogenic reactivity. In effect, highly immunogenic groups, sometimes used as a spacer arm for the coupling of haptens to carriers, may interact with the paratope of elicited antibodies. For low molecular weight drugs, this increases the risk of not obtaining the three minimum interactions between haptens and antibodies necessary for stereospecificity. Finally, because enantiomerically pure immunogens favor the formation of enantioselective antibodies,[24] the chemical structures of the haptens were fully identified and the enantiomeric purities for each of the hapten series were found to be >99%.

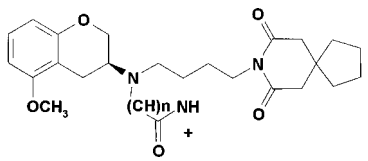


Figure 3—Structure of molecular ions including a peptidic link, characteristic of the coupling between haptens and BSA ($n = 3$, m/z 484 for A4 series; $n = 6$, m/z 526 for A7 series).

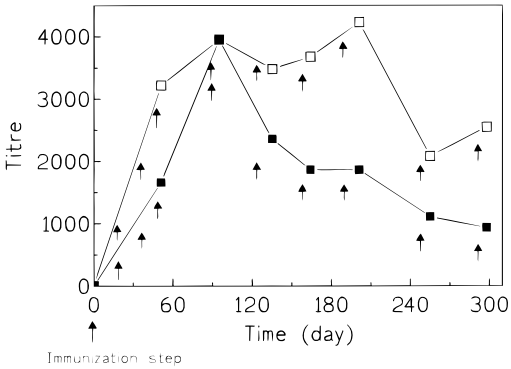


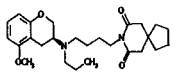
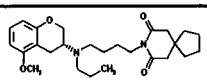
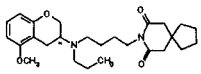
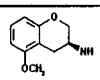
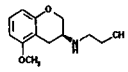
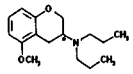
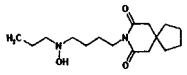
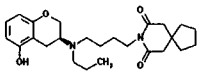
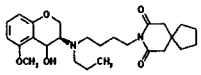
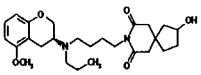
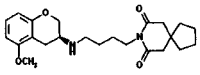
Figure 4—Anti-S 20499 antibody titre from rabbits nos. 2 and 6 as a function of time.

Immunogen Preparation—The hapten–BSA ratio could not be easily determined because radiolabeled hapten was not available and the conjugates were not totally soluble. The immunization procedure is time consuming, so it was important to have at least some qualitative information relating to the coupling. An original indirect characterization of the coupling was performed by mass spectrometry in the electronic ionization mode. The analytical conditions did not allow us to record the full spectrum of the hapten–BSA complex, nevertheless ion fragments compatible with a modified hapten structure, including the peptidic link, were found at m/z 484 (A4 series) and 526 (A7 series), (Figure 3). These fragments were not found in the control in the presence of BSA and all other reagents used in the formation of the conjugates. Significantly, high-resolution analyses indicated that the difference between the theoretical masses and the masses found were as little as 1 ppm. The immunological response obtained in all rabbits confirmed that the coupling was efficient and the hapten–BSA ratio was sufficient.

Antibody production—Six rabbits, three from each hapten series (A4 and A7, Figure 2), were immunized against S 20499, the pharmacologically active enantiomer, and the elicited antibodies were checked as described. The six animals showed significant antibody response from the first bleeding, taken after the fourth conjugate administration. The production of anti-S 20499 antibodies followed the pattern shown in Figure 4, which describes the response kinetics for two rabbits representative of both series of immunogens. The highest titres were observed after the first and the third bleedings, with a marked decrease for rabbit no. 2 after the second bleeding and a slight decrease for rabbit no. 6 up until the last bleeding. The third bleeding yielded a high titre for all rabbits, so the characterization of antibodies (in particular their specificity) was performed following this bleeding in all six cases.

The IC_{50} values, obtained from S 20499 displacement curves ranging from 4.0 to 15.5 nM (Table 1), led to a mean apparent affinity constant (calculated according to the Müller method[17]) of 1×10^8 to 5×10^9 L/mol, showing no clear-cut differences between the immunized rabbits from both series. The evolution of the IC_{50} throughout the immunization process was

Table 1—Cross-Reactivity with Anti-S 20499 Antisera

	Drug		A4 series			A7 series		
			L1***	L2	L3	L4	L5	L6
	S 20499 	IC_{50}* (nM)	14.7	4.0	14.0	15.5	8.3	6.2
Modification:								
on the chiral center	**S 20500** 	CR** (%)	4.1	< 0.1	< 0.2	< 0.2	1.4	< 0.1
	S 20244 	CR (%)	51.3	43.9	51.3	54.9	53.0	47.6
cleavage compounds	**Y 395** 	CR (%)	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
	Y 607 	CR (%)	< 0.1	0.7	< 0.1	< 0.1	< 0.1	< 0.1
	Y 412 	CR (%)	< 0.1	7.6	< 0.1	< 0.1	< 0.1	29.3
	Y 528 	CR (%)	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
on the chroman moiety	**S 20580** 	CR (%)	55.7	3.7	16.3	7.8	50.5	12.8
	S 21556 	CR (%)	22.8	14.2	10.3	6.1	7.0	4.9
on the azaspiro moiety	**S 21555** 	CR (%)	6.1	68.9	7.2	40.2	9.8	24.7
on the propyl side chain	**Y 464** 	CR (%)	34.4	23.9	19.9	11.0	12.0	14.2

* IC_{50}, Concentration required to displace 50% of [^{3}H]S 20499. **CR, Cross-reactivity expressed as the ratio of the IC_{50} for S 20499 to that of the test compound. ***L, denotes rabbits nos. 1 to 6.

followed for rabbits nos. 2 and 6. No apparent differences were seen from the first to the last bleedings.

Serum Antibody Specificity—Stereospecificity—Cross-reactivity was expressed as the concentration of the compound required to inhibit 50% of the binding of the antibody to tritiated S 20499 (IC_{50}), according to Abraham's index.[16] Clear-cut stereoselectivity was found for both antibody series, as shown in Table 1, with cross-reactivity ranging from 4.1 to <0.1% for S 20500. From these results, the methylene spacer groups appear to be good enough to allow stereospecific recognition. Moreover, a high level of stereospecificity had already been obtained with the shorter spacer arm, and no apparent difference was found in the two series. In this case, even if no conclusion could be drawn about the value of distancing the coupling site from the asymmetric center, we were at least able to confirm that the methylene spacer allows this distancing without decreasing affinity and could favorably be used for the construction of other immunogens. Concerning the evolution of the antibody stereospecificity throughout the immunization process, Abraham's index remained <1% for the two fully studied antisera (rabbits nos. 2 and 6, Figure 5). This result indicates that a short immunization period is

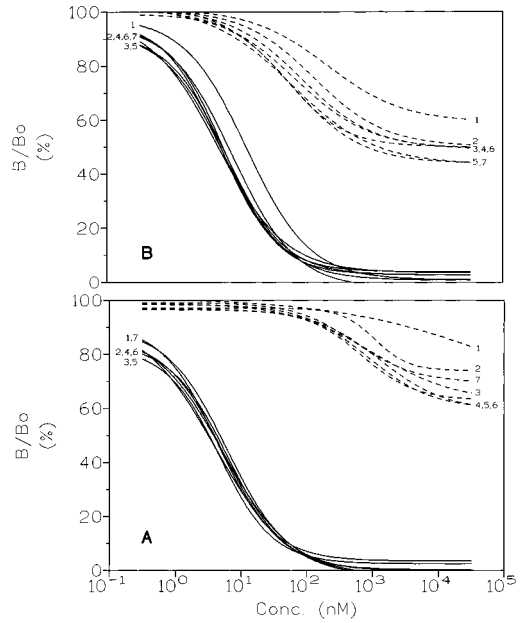


Figure 5—Evolution of the antibody stereospecificity from the first (1) to the last (7) bleedings: (A) rabbit no. 2, A4 series; (B) rabbit no. 6, A7 series. Displacement curves obtained with S 20499 and S 20500 are represented by solid and dashed lines, respectively.

consistent with the induction of highly stereospecific antibodies. Further improvement was not found after subsequent immunization steps.

Lastly, four out of the six anti-S 20499 antibodies did not cross-react with S 20500 (the distomer of S 20244) to any significant extent, whereas the two others showed only slight cross-reactivities. Antibodies raised in response to distomer−conjugates (four rabbits) all cross-reacted in the range of 0.7 to 7.4% with S 20499 (data not published), indicating the possible preferential recognition of S 20499, the more active configuration. These results may be compared with earlier studies concerning β-adrenergic antagonists, where the antibodies produced recognized the pharmacologically active configurations preferentially.[25] Moreover, other work performed with a nonchiral hapten led to the production of antibodies that recognized an active configuration preferentially when the ligand studied was chiral.[26] All of these results suggest that antibodies could exhibit higher affinities for pharmacologically active enantiomers.

Specificity Towards Structural Analogs—Potential metabolites and analogs of S 20499 were studied for concentrations up to at least 2000 ng/mL to determine their cross-reactivities. The results from the different compounds tested (namely, cleavage compounds and those with modifications to the chiral center, to the propyl side-chain, and to both the chroman and azaspiro moieties) are given in Table 1. The compounds not recognized by antibodies were those that had lost the chroman or the azaspiro moieties. Clearly, these two parts of S 20499 belong to the epitope recognized by the antibodies. Hydroxylation or dealkylation led to various decreases in cross-reactivity depending on the antibodies, and none of the six antisera were highly specific of S 20499. Overall, two antibody families can be defined that are unrelated to the immunogen series. One family is directed towards the chroman moiety (rabbits 2, 4, and 6), with a higher level of cross-reactivity against S 21555 (the 2-hydroxyazaspiro derivative) and a lower cross-reactivity against S 20580 (the 5-hydroxychroman derivative), whereas the other family shows opposing cross-reactivity results for the same potential metabolites (rabbits 1, 3, and 5) and is predominantly directed towards the azaspiro moiety. Profitable differences in cross-reactivities were observed as a consequence of the immunogen series. Cross-reactivities towards the *N*-depropylated S 20499 (Y 464) and S 20499 hydroxylated in the 4-position of the chroman moiety (S 21556), two other potential metabolites, were appreciably decreased with antibodies obtained from the longer of the two spacer groups (cross-reactivities towards Y 464 of 26.1 ± 7.5% compared with 12.4 ± 1.7% for A4 and A7 series, respectively; cross-reactivities towards S 21556 of 15.8 ± 6.3% compared with 6.0 ± 1.1% for A4 and A7 series, respectively; Table 1). As the ligands tested (Y 464 and S 21556) were chemically modified near the coupling site, a higher specificity of antibodies was expected as a consequence of the longer spacer arm between the hapten and BSA.

Conclusion

Highly stereospecific antibodies were sought against S 20499, notably a $5HT1_a$ agonist chemically related to buspirone. The choice of a hapten structurally resembling that of the enantiomer proved to be a good means of obtaining stereospecific antibodies with high affinities, because the asymmetric center is located farther away from the coupling site. The distancing of the asymmetric center by effectively lengthening the spacer arm with a four carbon atom methylene bridge was carried out as a means of increasing stereospecificity. Both approaches gave high levels of stereospecificity. Although it did not decrease the mean apparent affinity constant, the longer spacer improved antibody specificity. Hence, the additional four carbon atom bridge should be a valuable tool for increasing antibody stereospecificity, with no drawbacks in terms of specificity and affinity. Although the coupling site was relatively distant from the chroman and the azaspiro moieties, which are vulnerable to hydroxylations, none of the antibodies exhibited narrow specificities. This result may be explained by the folding up of the molecule onto BSA due to the flexibility of the spacer arm. To avoid this hypothetical folding up in future studies, or at least limit it, one may consider integrating a more rigid spacer arm, such as the crotonate structure described for anti-pentobarbital antibodies.[27]

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