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The Synthesis of Haptens and Their Use for the Development of Monoclonal Antibodies for Treating Methamphetamine Abuse

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In addition to addiction, the repeated use of (+)-methamphetamine [(+)-METH], (+)-amphetamine [(+)-AMP], or (±)-3,4-methylenedioxymethamphetamine ((±)-MDMA, commonly called ecstasy) can lead to life-threatening medical problems including cardiovascular injury, severe depression, and psychosis. Currently, there are no specific pharmacotherapies to treat these medical problems. In this study, we report the design and synthesis of two haptens, (*S*)-(+)-3-(9-carboxynonyloxy)methamphetamine (**3a**, (+)-METH MO10) and (*S*)-(+)-3-(5-carboxypentyloxy)methamphetamine (**3b**, (+)-METH MO6), and their use in generating high affinity (low K_D value) monoclonal antibodies (mAbs) against (+)-METH, (+)-AMP, and/or (+)-MDMA. On the basis of results from the determination of mAb K_D values and ligand specificity, the mAbs generated from hapten **3a** showed the greatest promise for generating active and passive immunotherapies for treating overdose or addiction from (+)-METH-like stimulants.

The abuse of (*S*)-(+)-methamphetamine [**1a**-(+)-METH][a] and other amphetamine-like stimulants continues to be a major health problem worldwide.[1−3] Indeed, a study by RAND Corporation estimates the economic cost of (+)-METH use in the United States in 2005 was $623.4 billion.[4] This comprehensive estimate includes the economic burden of addiction, premature death, drug treatment, and many other aspects of the drug's impact on Americans. The 2008 National Survey on Drug Use and Health estimates that over 12 million individuals, aged 12 and older, had used (+)-METH in their lifetime, that 850000 had used (+)-METH during the past year, and that 314000 individuals had used (+)-METH during the last month, which defines them as current users.[5] According to the 2005 Drug Abuse Warning Network (DAWN) report, there were 108905 methamphetamine-related emergency departments (ED) visits in 2005.[6] If (+)-(*S*)-amphetamine [**1b**, (+)-AMP] was included, there were 138950 ED visits.[6]

At present, there are no specific pharmacotherapies for managing adverse (+)-METH-induced effects like acute overdose and chronic addiction. Preclinical studies in rats show that systemic administration of anti-(+)-METH monoclonal antibodies (mAbs) can rapidly remove the drug from its sites of action in critical tissues like the brain and heart, suggesting that immunotherapy could provide an important new medical strategy for addressing (+)-METH-induced adverse health effects in humans while others suggest antibody catalyzed inactivation of METH could be a possible therapeutic approach.[7] (*S*)-(+)-Amphetamine [**1b**, (+)-AMP], which is a major metabolite of (+)-METH and a drug of abuse, and (+)-methylenedioxymethamphetamine, the stimulant-inducing chemical in the racemic mixture of (±)-methylenedioxymethamphetamine (**2**, (±)-MDMA, commonly referred to as ecstasy) are two other widely abused and dangerous stimulants. The potency and stimulant effects of these (+)-3,4-METH-like compounds are influenced by the drug's stereochemistry, with the (+)- or (*S*)-isomers producing significantly more psychomimetic effects, stereotyped behavior, locomotor activity,[8] and increased production of reactive oxygen species in mice.[9] Given the medical importance of all three of these structurally related (+)- or (*S*)-isomers, it could be medically advantageous to have a single mAb that could be used in the treatment of medical problems resulting from (+)-METH, (+)-AMP, and/or (+)-MDMA. Indeed, we recently reported the immunological design strategy, discovery, and development of an mAb antibody named anti-(+)-METH mAb4G9 that exhibited a novel optimal combination of high affinity and drug-class specificity for (+)-METH, (+)-AMP, and (+)-MDMA without significant cross-reactivity against other METH-like ligands, over-the-counter medications, or endogenous neurotransmitters.[10]

In this study, we report the synthesis of the hapten (*S*)-(+)-3-(9-carboxynonyloxy)methamphetamine (**3a**, designated (+)-METH MO10) used to prepare the mAb4G9 along with

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[a] Abbreviations: (+)-METH, (+)-methamphetamine; mAb, monoclonal antibody(ies); (+)-AMP, (+)-amphetamine; (+)-MDMA, (+)-3,4-methylenedioxymethamphetamine; (−)-METH, (−)-methamphetamine; (−)-AMP, (−)-amphetamine; (−)-MDMA, (−)-3,4-methylenedioxymethamphetamine; (±)-MDMA, (±)-3,4-methylenedioxymethamphetamine; BSA, bovine serum albumin; c-BSA, cationized bovine serum albumin; RIA, radioimmunoassay; DAWN, Drug Abuse Warning Network; ED, emergency department; OVA, ovalbumin; ELISA, enzyme-linked immunosorbent assay; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry.

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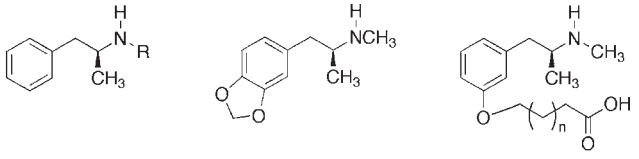
comparisons with the synthesis of (*S*)-(+)-3-(5-carboxypentyloxy)methamphetamine (**3b**, designated (+)-METH MO6), an earlier prototypic hapten. We also present the development and characterization of new anti-(+)-METH mAbs resulting from immunizations with these haptens covalently bound to three different carrier proteins. The mAbs generated from immunization with (+)-METH MO10 hapten had significantly lower K_D values than the mAbs generated from the (+)-METH MO6 hapten. Furthermore, the mAbs generated against the (+)-METH MO10 hapten coupled to ovalbumin showed the richest diversity of specificity and higher affinity for all three medically important (+)-METH-like stimulants, (+)-METH, (+)-AMP, and (+)-MDMA (see below).



1a, (+)-METH, R = CH_3
b, (+)-AMP, R = H

2, (+)-MDMA

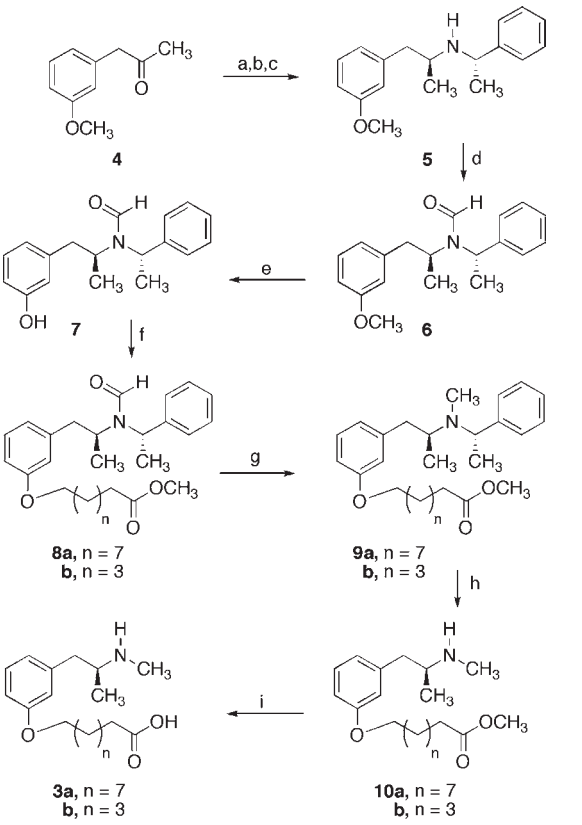
3a, n = 7
b, n = 3

Chemistry

The synthesis of the haptens **3a** and **3b** is shown in Scheme 1. (*S*,*S*)-3-Methoxyphenyl-2-propyl-α-methylbenzylamine (**5**) was synthesized by modification of a method reported for other similar compounds.[11] Thus, reductive alkylation of *m*-methoxyphenylacetone (**4**) with (*S*)-(−)-α-methylbenzylamine using triacetoxyborohydride in ethylene dichloride gave a mixture of (*R*,*S*)- and (*S*,*S*)-**5**. Recrystallization of the hydrochloride salts of the mixture from an isopropyl alcohol and ethyl ether mixture gave pure (*S*,*S*)-**5**, which was converted to the *N*-formyl intermediate **6** using a formic acid/acetic anhydride mixture. *O*-Demethylation of **6** using bromine tribromide in methylene chloride afforded the phenol **7**. The sodium salt of the phenol prepared using sodium hydride in dimethylformamide was alkylated with methyl 10-bromodecanoate or methyl 6-bromohexanoate to give **8a** and **8b**, respectively. Reduction of **8a** and **8b** using diborane in tetrahydrofuran provided the *N*-methyl compounds **9a** and **9b**. Subjection of **9a** and **9b** to transfer hydrogenation using ammonium formate and 5% palladium on carbon catalyst in methanol yielded **10a** and **10b**. Hydrolysis of **10a** and **10b** using 6N hydrochloride acid afforded the desired haptens **3a** and **3b**.

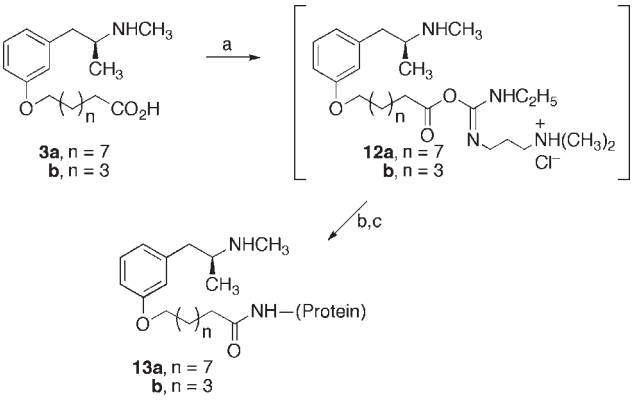
The hapten/protein antigens were synthesized as shown in Scheme 2 using a carbodiimide coupling procedure.[12] Briefly, a solution of the hapten **3a** or **3b** was combined with a solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) hydrochloride to give the activated intermediate **12a** or **12b**, which was not isolated. Addition of a solution of bovine serum albumin (BSA), cationized BSA (c-BSA), or ovalbumin (OVA) to the solution of **12a** or **12b** provided the desired hapten−protein antigen **13a** or **13b**, which was separated from unconjugated haptens and other starting materials by dialysis against multiple changes of distilled water followed by phosphate-buffered saline (pH 7.35). The degree of incorporation of the haptens onto the OVA and BSA carrier proteins was determined by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) analysis. The degree of incorporation of haptens onto the c-BSA carrier proteins was not determined because these antigens were generated before MS analysis of proteins was

Scheme 1[a]



[a] Reagents: (a) $Na(OAc)_3BH_3$, $(CH_2Cl)_2$; (b) ethenal HCl; (c) recrystallization from $(CH_3)_2CHOH$ and Et_2O; (d) HCO_2H, $(CH_3CO)_2O$, toluene; (e) BBr_3, CH_2Cl_2; (f) NaH, $Br(CH_2)_9CO_2CH_3$ or $Br(CH_2)_5CO_2CH_3$, DMF; (g) BH_3, THF; (h) HCO_2NH_4, 5% Pd/C, CH_3OH; (i) 6N HCl.

Scheme 2[a]



[a] Reagents: (a) EDCI, DMF of 0.1 M buffer; (b), BSA, OVA, or cationized BSA in buffer; (c) dialysis against water then PBS buffer.

available to this project. Parts A and B of Figure 1 show the MALDI mass spectra obtained from BSA and MO10-modified BSA, respectively. The observed mass for BSA by MALDI MS was in excellent agreement with the value derived from the known amino acid sequence. The mass difference between the two spectra divided by the mass added by each hapten (317 Da) indicated an average incorporation of 5.6 haptens. A similar MALDI MS analysis of OVA and the MO10-modified OVA indicated an incorporation of 4−5 haptens.

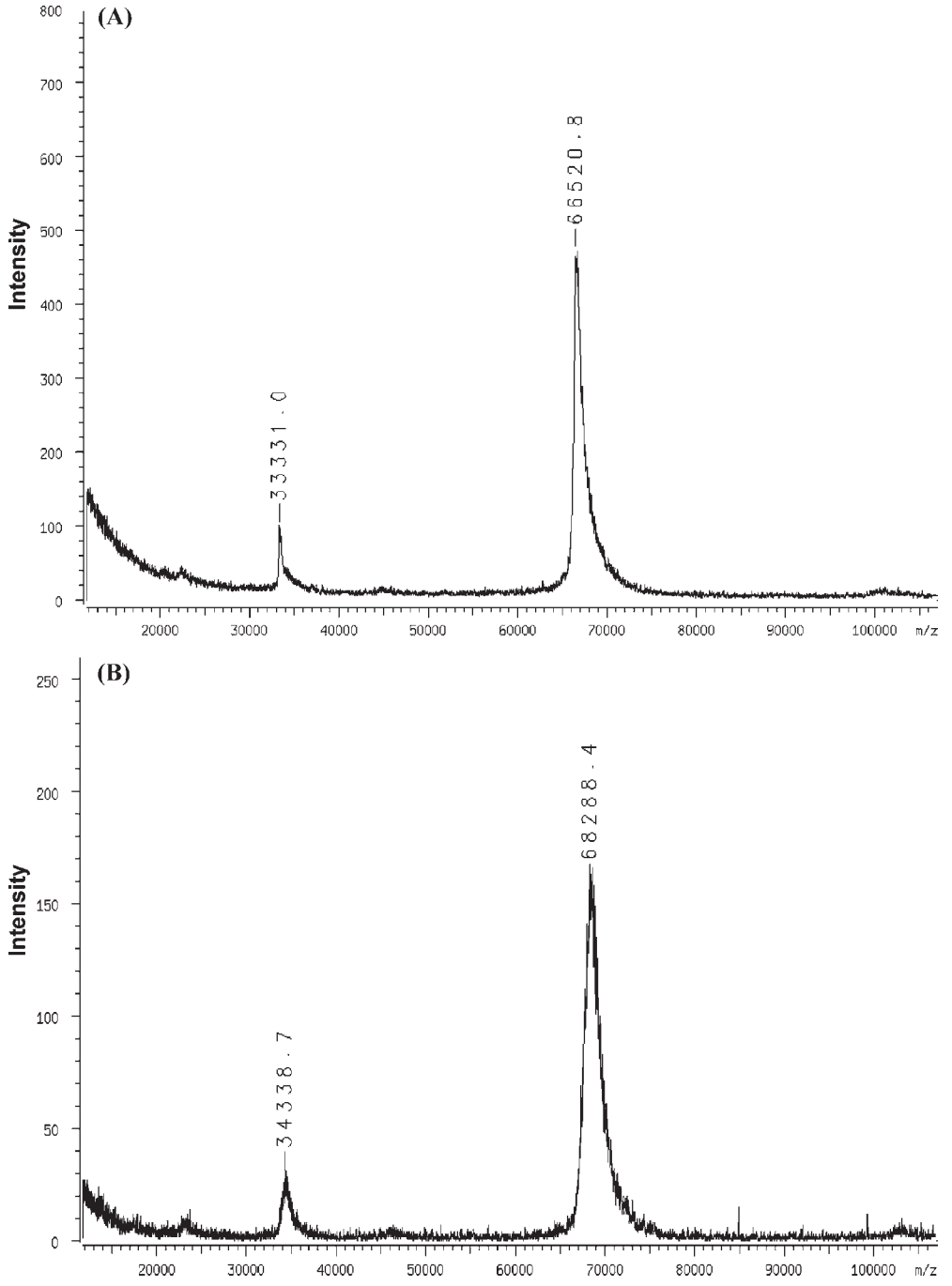


Figure 1. (A) MALDI mass spectrum of BSA. (The measured m/z is taken as the correct mass to minimize error in mass assignment). (B) MALDI mass spectrum of METH MO10-modified BSA. The number of modifying groups (5.6) was determined by dividing the mass increase upon modification by the mass added by each modifying group (317 Da).

Results and Discussion

The overall goal of this study was to design and develop a hapten that would stimulate immune cell production of mAbs with high affinity for (+)-METH as well as (+)-AMP and/or (+)-MDMA. Because all three structures have in common an (*S*)-aminopropyl substituent on an aromatic ring that helps make the molecules pharmacologically and immunologically distinct, it was decided to leave this part of the structure alone and to attach the linker moiety of the haptens to the aromatic ring at a site that was distal to the (*S*)-aminopropyl structures. The linker was connected to the aromatic ring through an oxygen atom at the meta position to improve the probability of MDMA recognition, which has oxygen atoms at both the meta and para positions.

Because (+)-AMP does not possess the *N*-methyl group present in (+)-METH and (+)-MDMA, we hypothesized that a longer linker group between the hapten (like MO10) and backbone structure would lead to improved immune recognition and higher affinity antibodies for (+)-METH while maintaining cross-reactivity and affinity with (+)-MDMA as well as (+)-AMP. While our primary goal was to discover haptens that would generate mAbs with high affinity for (+)-METH, we also hoped to discover mAbs with a broader specificity for all three of these drugs of abuse. We hypothesized that this broader specificity for (+)-METH-like drugs would improve the likelihood of producing a single therapeutic antibody that could be used to treat medical problems

Table 1. Haptens, Antigens, and Immunochemical Specifications of Anti-(+)-METH Monoclonal Antibodies

(+)-METH-like hapten and antigen	monoclonal antibody	IgG isotype, light chain	(+)-METH K_D (nM)	(+)-AMP K_D (nM) or K_I (nM)[a]	(+)-MDMA K_I (nM)[b]
MO6 bound to c-BSA	mAb9B11	IgG1, λ	110	> 1000	24
	mAb10E1	IgG1, λ	91	> 5000	24
	mAb7H11	IgG1, κ	52	> 5000	30
	mAb5C2	IgG1, κ	39	> 5000	28
	mAb9C4	IgG2a, κ	77	> 5000	45
	mAb1C1	IgG1, κ	58	> 5000	22
MO10 bound to OVA	mAb4G9	IgG2b, κ	16	50	68
	mAb2F11	IgG2a, κ	13	49	65
	mAb1A12	IgG2a, κ	13	> 1000	5
	mAb6C1	IgG1, κ	47	47	52
	mAb10D1	IgG1, κ	34	51	69
MO10 bound to BSA	mAb7F9	IgG1, κ	9	> 1000	14
	mAb9C10	IgG1, κ	38	> 1000	27

[a] Only four mAbs (all from immunization with a MO10-OVA antigen) had high affinity for (+)-AMP, and these K_D values were determined in a homologous immunoassay with [^{3}H]-AMP as the radioligand and inhibition with unlabeled (+)-AMP. If we determined in screening assays that the mAb would have a very high K_D value > 1000−5000 nM, we conducted a heterologous immunoassay with [^{3}H]-METH as the radioligand and inhibition with a high range of unlabeled (+)-AMP doses to obtain an approximate K_I value for (+)-AMP binding. [b] [^{3}H]-(+)-MDMA was unavailable, so we determined a K_I value for binding to each mAb using [^{3}H]-METH as the radioligand and inhibition with unlabeled (+)-MDMA.

(e.g., addiction, overdose) resulting from any of these drugs. By analogy, we attempted to produce a "designer antibody" to treat the medical problems caused by these "designer drugs." We also reasoned that the future medical applications for a broader specificity antibody would be greater because hospital pharmacies would only have to stock one medication for the treatment of medical problems resulting from (+)-METH, (+)-MDMA, and (+)-AMP.

Our hypothesis was supported by the finding that immunizations with antigens containing an MO10 hapten epitope produced significantly greater affinities for (+)-METH (as judged by lower K_D values for (+)-METH) than did immunization with the MO6-containing hapten epitope ($p < 0.05$ using a Student's t test; Table 1 and Figures 2 and 3). It should be noted that we designed our immunization schedules to include the minimum antigen dose and long periods between boost (up to 2 months) to favor the likelihood that we would generate high affinity anti-(+)-METH mAbs. We also screened for anti-(+)-METH mAbs with a minimum amount of hapten protein conjugate to favor the discovery of high affinity antibodies. In practice, only antibodies of the highest affinity can stay bound when the hapten dose is minimal. However, on many occasions, we also discovered low affinity antibodies but only kept the mAbs with K_D values for (+)-METH of approximately 100 nM or less. We chose this cutoff point after considering the outcomes of a wide range of pharmacological and behavioral studies in rats from our laboratory using various anti-(+)-METH mAbs. From these observations, we hypothesize that mAbs with K_D values of ≥100 nM will not be clinically useful, and K_D values of at least ≤10−30 nM will be needed for the treatment of medical problems caused by addiction.[13,14]

Another critical factor in the discovery process was the number of haptens bound to the antigen. In our earlier studies with the MO6 haptens attached to c-BSA carrier proteins, we did not have the analytical capabilities to directly determine the number of haptens per c-BSA molecule. Furthermore, we later found MALDI MS analysis (or any other method) was not dependable with hapten-c-BSA, which is slightly larger in molecular size than BSA. More importantly, the c-BSA protein has less uniform properties than the other carrier proteins, with varying amounts of activation within lots or batches. However, by the time synthesis of the MO10 antigens was started, the mass spectrometry technology to directly determine the number of haptens per protein molecule became available to us. These mass spectrometry studies showed that about five haptens per protein molecule (either OVA or BSA) were sufficient to produce high affinity anti-(+)-METH antibodies. We also found that hapten incorporation rates on OVA and BSA of less than five per protein molecule led to inconsistent results or no antigenic response (results not shown). While the c-BSA proved a good antigenic protein, we chose to use OVA and BSA antigens because these proteins were small enough to allow direct mass spectrometric analysis and direct determination of hapten epitope densities. This proved valuable in optimizing various outcomes. In general, this allows the user to optimize the hapten to protein ratios (i.e., number of (+)-METH-like antigenic epitopes) in synthetic reactions and will also prove valuable in future studies of the relationship between epitope density and antidrug response in vaccinated animals.

The current studies provide a comprehensive analysis of the importance of hapten length and its relationship to affinity and specificity for very small haptens. Previous studies from our laboratory have shown that five unique mAbs generated against five different METH-like haptens (including MO6 and MO10) can produce mAbs against (+)-METH-like haptens that range in K_D value from 250−10 nM.[10] These five mAbs resulted from immunizing mice with (+)-METH coupled to protein antigens through progressively longer linker groups of 4, 6, and 10 atoms connected to the aromatic ring at the para or meta positions.

For the current studies, we redetermined the (+)-METH K_D values for mAb9B11 and mAb4G9 (from the previous studies), along with 11 other never before reported anti-(+)-METH mAbs, using a significantly improved radioimmunoassay (RIA) for determination of K_D and K_I values. This improved RIA method does not require a second dilution or incubation step to separate the drug (+)-METH mAb complex from the unbound drug. These procedural steps in an

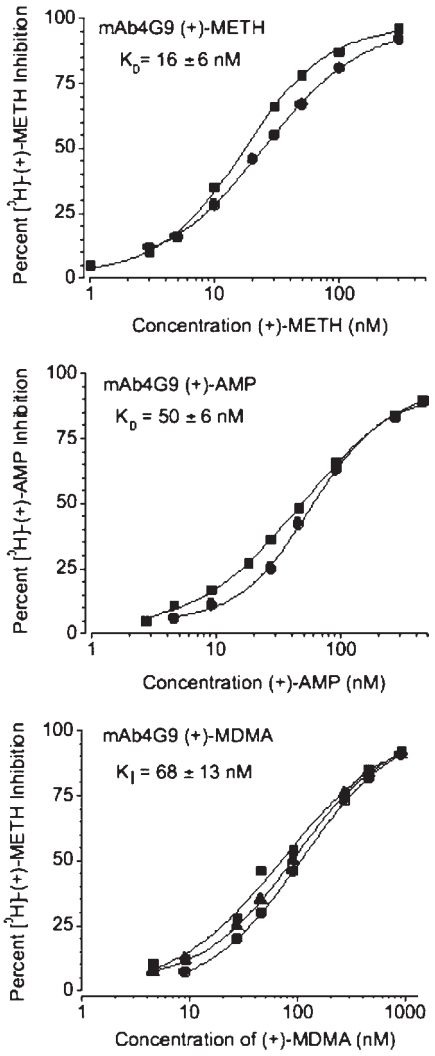


Figure 2. Representative RIA plots for the determination of anti--(+)-METH mAb4G9 K_D values for (+)-METH (upper) and (+)-AMP (middle), and K_I values for (+)-MDMA (lower). Similar RIA inhibition curves were determined in duplicate or triplicate for all 13 mAbs listed in Table 1. After a mathematical correction for the contribution of [^{3}H]-METH or [^{3}H]-AMP binding, the final average K_D and K_I value was calculated.

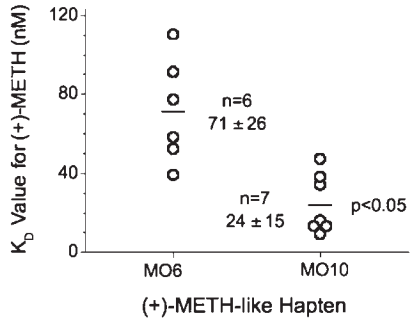


Figure 3. Individual (open circles) and average (solid bar) K_D values for (+)-METH binding to all 13 monoclonal antibodies generated for these studies. Side-by-side circles indicate that two different antibodies had the same apparent K_D value. The K_D values for (+)-METH binding to antibodies generated against (+)-METH MO10 haptens ($n = 7$) were significantly lower ($p < 0.05$; t test) than the K_D values for the antibodies generated against (+)-METH M06 haptens ($n = 6$).

RIA often result in a less than optimal estimation of the K_D values for ligand binding. Indeed, mAb9B11 and mAb4G9 K_D values for (+)-METH in our previously reported RIA were 41 and 34 nM,[10] respectively, but in the current study, they are 110 and 16 nM with improved reproducibility.

Importantly, four mAbs generated from MO10, bound to the OVA, have K_D or K_i values of 13−47, 47−51, and 52−69 nM for (+)-METH, (+)-AMP, and (+)-MDMA, respectively. In contrast, six mAbs generated from (+)-METH MO6 bound to c-BSA antigen, and two mAbs generated from MO10, bound to BSA antigen, sometimes had very low K_D values for (+)-METH and (+)-MDMA binding but always possessed K_D values of >1000 nM for (+)-AMP. One mAb (mAb1A12) generated from the MO10, bound to OVA antigen, also had a K_D value of >1000 nM for (+)-AMP. We are now in the process of testing the best of these antibodies as medications in preclinical studies in rodents. It will be of particular interest to determine if the mAb4G9, with its broad specificity for all three (+)-METH-like drugs, is actually therapeutically better than mAb7F9 (generated from the (+)-METH MO10-BSA antigen). In our experience, therapeutic efficacy of mAbs is best judged by the ability of the mAbs to (1) block and/or reduce brain concentration of (+)METH, (2) provide prolonged redistribution of (+)METH out of the brain and into the bloodstream, and (3) the ability of the mAbs to block or significantly reduce (+)METH-induced behavioral effects in preclinical animal models like the rat. MAb7F9 had the highest affinity for (+)-METH of any of the antibodies ($K_D = 9$ nM) in this report but no significant binding to (+)-AMP. Also worthy of noting is the very high affinity binding of mAb1A12 for (+)-MDMA ($K_I = 5$ nM).

Over the course of more than 10 years of studies of anti-(+)-METH mAbs, we have used a range of protein antigens including OVA, c-BSA, BSA, keyhole limpet hemocyanin, thymoglobulin, and immunoglobulin. We have also used five different adjuvants: Freund's Complete, Freund's Incomplete, RIBI, TiterMax, and Alum. The adjuvant does not appear to be the major factor in generating a broad specificity mAbs (i.e., cross-reactivity with (+)-METH, (+)-AMP, and (+)-MDMA), but it does significantly affect the ability to generate high affinity antibodies. Freund's Complete adjuvant consistently proved to be the best of the five adjuvants. In addition, the use of haptens derived from (+)-isomers has never generated mAbs that significantly cross-react with (−)-isomers of METH-like ligands (results not shown). Our studies also suggest that attachment of the hapten spacer arm linkage at the meta portion of the (+)-METH aromatic ring structure appears superior to a para position linkage because it is the only hapten to ever generate mAbs with significant cross reactivity with (+)-AMP (Table 1 and ref 7). Another important factor to emerge from this research program is the suggestion that the combination of MO10 linked to OVA is best for generating mAbs with simultaneously high affinity (i.e., low K_D values) for (+)-METH, (+)-AMP, and (+)-MDMA (Table 1). On the basis of the results of these studies, the ability of a single (+)-METH-like hapten to generate high affinity for (+)-METH and (+)-MDMA appears less dependent on choosing the optimal combination of hapten and carrier protein.

In summary, haptens were designed and developed to maximize the potential for a single mAb to specifically recognize the common molecular features of (+)-METH, (+)-AMP, and (+)-MDMA. Four mAbs have been

developed from the MO10 hapten bound to OVA, which have simultaneously low K_D and K_I values for (+)-METH, (+)-AMP, and (+)-MDMA. The high affinity Abs generated in this study have the potential to block or reduce the stimulant-induced medical effects in patients addicted to (or abusing) these drugs. In addition, the mAbs derived from hapten **3a** have the potential to provide an acute treatment for life-threatening stimulant overdose resulting from (+)-AMP, (+)-METH, or (+)-MDMA by removing the drugs from their sites of action in the central nervous, heart, and other critical organs, thereby reducing METH-induced psychosis, neurotoxicity, and cardiovascular effects. Finally, these studies also suggest MO10 or a similar hapten will be the best for use in developing active vaccines. These studies are currently in progress in our laboratories.

Experimental Section

Nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) spectra were recorded on a 300 MHz (Bruker AVANCE 300) spectrometer. Chemical shift data for the proton resonances were reported in parts per million (ppm) relative to internal standard. Optical rotations were measured on an AutoPol III polarimeter, purchased from Rudolf Research. Elemental analyses were performed by Atlantic Microlab, Norcross, GA. Purity of compounds (>95%) was established by elemental analyses. Analytical thin-layer chromatography (TLC) was carried out on plates precoated with silica gel GHLF (250 μM thickness). TLC visualization was accomplished with a UV lamp or in an iodine chamber. All moisture-sensitive reactions were performed under a positive pressure of nitrogen maintained by a direct line from a nitrogen source. Anhydrous solvents were purchased from Aldrich Chemical Co.

(*S*)-3-(9-Carboxynonyloxy)methamphetamine (3a) Hydrochloride. Compound **10a** (420 mg, 1.1 mmol) was heated under reflux in 9 mL of 6N HCl for 8 h. The cooled solution was concentrated to a tan residue that was recrystallized 3× from a CH_3OH and Et_2O mixture to yield 260 mg (65%) of **3a** as its white powdery HCl salt: mp 124−127 °C; $[\alpha]^{25}_D$ +0.30 (c 1.0, CH_3OH). ^{1}H NMR (CD_3OD) δ 7.25 (t, J = 7.9 Hz, 1H), 6.75−6.90 (m, 3H), 3.97 (t, J = 6.4 Hz, 3H), 3.40−3.55 (m, 1H), 3.11 (dd, J = 13.2, 4.9 Hz, 1H), 2.73 (dd, J = 13.2, 9.0 Hz, 1H), 2.72 (s, 3H), 2.28 (t, J = 7.2 Hz, 2H), 1.77 (ap, J = 7.2 Hz, 2H), 1.60 (ap, J = 7.2 Hz, 2H), 1.42−1.54 (m, 2H), 1.28−1.43 (m, 9H), 1.25 (d, J = 6.8 Hz, 3H). ^{13}C NMR (300 MHz, CD_3OD) δ 161.5, 138.8, 131.4, 122.8, 117.2, 114.7, 69.3, 58.1, 40.3, 35.0, 30.9, 30.5, 30.4, 30.3, 30.2, 27.1, 26.1, 15.9. LCMS (APCI) m/z 336.7 $(M + 1)^+$. Anal. ($C_{20}H_{34}ClNO_3 \cdot 0.25H_2O$) C, H, N.

(*S*)-3-(5-carboxypentyloxy)methamphetamine Hydrochloride (3b). Compound **3b** was prepared by a procedure analogous to that described for **3a** to afford 59% of **3b** as the hydrochloride salt: mp 73−76 °C; $[\alpha]^{25}_D$ +0.64 (c 1.10, CH_3OH). ^{1}H NMR (CD_3OD) δ 7.24 (t, J = 7.9 Hz, 1H), 6.76−6.91 (m, 3H), 3.97 (t, J = 6.4 Hz, 3H), 3.39−3.59 (m, 1H), 3.35 (s, 3H), 3.16 (dd, J = 13.2, 4.9 Hz, 1H), 2.75 (dd, J = 13.2, 9.0 Hz, 1H), 2.72 (s, 3H), 2.32 (t, J = 7.2 Hz, 2H), 1.78 (ap, J = 7.2 Hz, 2H), 1.67 (ap, J = 7.2 Hz, 2H), 1.42−1.58 (m, 2H), 1.25 (d, J = 6.8 Hz, 3H). ^{13}C NMR (300 MHz, CD_3OD) δ 131.4, 123.0, 117.1, 114.8, 69.2, 58.2, 40.6, 35.3, 31.4, 30.5, 27.2, 26.3, 16.2. LCMS (ESI) m/z 280.3 $(M + 1)^+$, m/z 278.6 $(M - 1)^+$. Anal. ($C_{16}H_{26}ClNO_3$) C, H, N.

(*S,S*)-*N*-α-Methylbenzyl-3-methoxyamphetamine (5) Hydrochloride. To a solution of *m*-methoxyphenylacetone (5.0 g, 0.030 mol) and (*S*)-(−)-α-methylbenzylamine (3.81 g, 0.031 mol) in ethylene dichloride (200 mL) at room temperature was added sodium triacetoxyborohydride (10.0 g, 0.047 mol) in four equal portions over a 10 min interval. After stirring overnight at ambient temperature, the reaction mixture was quenched with 15% NH_4OH (200 mL). The organic layer was separated, and the aqueous layer was extracted with CH_2Cl_2 (3 × 100 mL). The combined organic layers were washed with saturated brine solution (150 mL), dried (Na_2SO_4), and concentrated to give 9.24 g (99%) of **5** as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 7.27−7.44 (m, 4H), 7.20−7.27 (m, 1H), 7.16 (t, J = 7.9 Hz, 1H), 6.72 (dd, J = 7.9, 2.3 Hz, 1H), 6.68 (d, J = 7.5 Hz, 1H), 6.62 (at, J = 2.3 Hz, 1H), 3.92 (q, J = 6.4 Hz, 1H), 3.76 (s, 3H), 2.85 (dd, J = 12.8, 5.3 Hz, 1H), 2.78 (dq, J = 6.4, 5.3 Hz, 1H), 2.46 (dd, J = 12.8, 7.2 Hz, 1H), 1.42 (bs, 1H, NH), 1.30 (d, J = 6.4 Hz, 3H), 0.92 (d, J = 6.4 Hz, 3H). ^{13}C NMR (300 MHz, $CDCl_3$) δ 159.5, 146.2, 141.2, 129.1, 128.4, 126.8, 126.5, 121.8, 115.0, 111.3, 55.3, 55.1, 51.8, 42.7, 24.5, 21.2.

The free base dissolved in Et_2O (100 mL) was treated with HCl solution in Et_2O (2.0 M, 16.0 mL, 10.0 equiv) at ice-cold conditions. Addition of Et_2O (200 mL) yielded 8.48 g (92%) of a white, amorphous solid. Recrystallization of the solid using hot isopropyl alcohol (725 mL) followed by the slow addition of Et_2O (600 mL) at room temperature yielded 5.5 g (65%) of **5**·HCl as a white crystalline solid: mp 219−221 °C. ^{1}H NMR ($CDCl_3$) δ 10.32 (bs, 1H), 9.84 (bs, 1H), 7.69 (ad, J = 7.2 Hz, 2H), 7.48 (tt, J = 7.2, 1.9 Hz, 2H), 7.41 (tt, J = 7.2, 1.5 Hz, 1H), 7.15 (t, J = 7.9 Hz, 1H), 6.75 (dd, J = 7.9, 1.9 Hz, 1H), 6.59 (d, J = 7.5 Hz, 1H), 6.51 (at, J = 1.9 Hz, 1H), 4.39 (ap, J = 6.8 Hz, 1H), 3.73 (s, 3H), 3.40 (dd, J = 12.8, 4.1 Hz, 1H), 3.05 (bs, 1H), 2.85 (dd, J = 12.8, 9.8 Hz, 1H), 1.96 (d, J = 6.8 Hz, 3H), 1.43 (d, J = 6.8 Hz, 3H). ^{13}C NMR ($CDCl_3$) δ 159.8, 138.1, 136.5, 129.7, 129.5, 129.3, 127.9, 121.4, 114.6, 112.7, 55.9, 55.2, 53.9, 38.1, 21.2, 17.9. Anal. ($C_{18}H_{24}ClNO$) C, H.

(*S,S*)-*N*-Formyl-*N*-α-methylbenzyl-3-methoxyamphetamine (6). To a stirred solution of 24 mL of formic acid (36.6 g, 0.795 mol) at 0 °C, 40 mL of acetic anhydride (58.3 g, 0.572 mol) in toluene (100 mL) was added dropwise. After 30 min, the amine **5** (10.2 g, 0.038 mol, obtained from the hydrochloride salt) in a minimum volume of formic acid was added. The reaction mixture was heated under reflux for 16 h and allowed to stir at RT for 8 h. The acidic solution was basified with 40 mL of 30% NH_4OH (aq) solution and extracted with 3 × 100 mL of CH_2Cl_2. The extracts were washed with brine (100 mL) and dried ($MgSO_4$). Concentration of the extracts gave an oil. The oil was purified by flash chromatography (silica gel, 200 g ISCO column) to give 10.3 g (92%) of **6** as a clear, colorless thick oil. ^{1}H NMR ($CDCl_3$) δ 8.41 (s, 1H), 8.40 (s, 1H), 7.27− 7.50 (m, 5H), 7.07 (t, J = 7.9 Hz, 1H), 7.07 (t, J = 7.9 Hz, 1H), 6.69 (d, J = 1.9 Hz, 1H), 6.66 (d, J = 1.9 Hz, 1H), 6.41 (d, J = 7.5 Hz, 1H), 6.30 (at, J = 1.9 Hz, 1H), 6.26 (d, J = 7.5 Hz, 1H), 6.09 (at, J = 1.9 Hz, 1H), 5.94 (q, J = 7.2 Hz, 1H), 4.65 (q, J = 7.2 Hz, 1H), 3.70 (s, 3H), 3.68 (s, 3H), 3.34−3.53 (m, 1H), 3.19−3.36 (m, 1H), 3.00 (dd, J = 13.2, 9.8 Hz, 1H), 2.61 (dd, J = 13.2, 4.9 Hz, 1H), 2.38−2.54 (m, 2H), 1.58 (d, J = 7.2 Hz, 3H), 1.54 (d, J = 7.2 Hz, 3H), 1.31 (d, J = 6.8 Hz, 3H), 1.25 (d, J = 6.8 Hz, 3H). ^{13}C NMR ($CDCl_3$) δ 162.5, 161.4, 159.55, 159.48, 140.9, 140.2, 139.8, 139.6, 129.3, 129.1, 128.6, 128.4, 128.1, 127.9, 127.7, 127.2, 121.5, 121.3, 114.3, 114.2, 112.1, 111.9, 56.9, 55.1, 53.3, 51.1, 49.7, 44.7, 40.4, 21.8, 19.7, 17.3, 16.4. LCMS (APCI) m/z 298 (M + 1).

(*S,S*)-*N*-Formyl-*N*-α-methylbenzyl-3-hydroxyamphetamine (7). To a stirred cold (0 °C) solution of **6** (2.10 g, 0.007 mol) in 60 mL of CH_2Cl_2 under N_2 atmosphere was added dropwise 1.33 mL (3.54 g, 0.014 mol) of BBr_3. The reaction mixture was allowed to stir at 25 °C for 8 h, 100 mL of water was carefully added to quench excess BBr_3, and the resulting mixture was extracted with 3 × 100 mL of CH_2Cl_2 and dried (Na_2SO_4). The resulting oil was subjected to silica gel chromatography using hexanes/CH_2Cl_2/MeOH (4:8:1) as the eluent to give 1.96 g (98%) of pure **7** as a white powder: mp 195−198 °C; R_f 0.43 (1:1, hexanes/EtOAc). ^{1}H NMR ($CDCl_3$) δ 8.36 (s, 1H), 8.32 (s, 1H), 7.29−7.46 (m, 4H), 7.26 (at, J = 6.8 Hz, 1H), 7.01 (t, J = 7.2 Hz, 1H), 6.99 (t, J = 7.2 Hz, 1H), 6.72 (d, J = 1.9 Hz, 1H), 6.69 (d, J = 1.9 Hz, 1H), 6.55 (at, J = 1.9 Hz, 1H), 6.26 (at, J = 1.9 Hz, 1H), 6.24 (d, J = 7.5 Hz, 1H), 6.11 (d, J = 6.8 Hz, 1H), 5.89 (q, J = 7.2

Hz, 1H), 4.56 (q, J = 7.2 Hz, 1H), 3.42−3.63 (m, 1H), 3.23−3.42 (m, 1H), 2.93 (dd, J = 13.2, 9.4 Hz, 1H), 2.61 (dd, J = 13.2, 5.3 Hz, 1H), 2.25−2.46 (m, 2H), 1.54 (d, J = 6.4 Hz, 3H), 1.52 (d, J = 6.4 Hz, 3H), 1.30 (d, J = 6.8 Hz, 1H), 1.21 (d, J = 6.8 Hz, 1H). ^{13}C NMR (300 MHz, $CDCl_3$) δ 162.9, 162.1, 156.61, 156.58, 140.5, 139.8, 139.3, 139.2, 129.4, 129.3, 128.65, 128.57, 128.5, 128.0, 127.91, 127.86, 127.0, 120.4, 116.2, 116.1, 113.7, 113.4, 57.5, 53.8, 51.2, 50.2, 44.2, 40.1, 21.6, 19.8, 17.2, 16.3. Anal. ($C_{18}H_{21}NO_2 \cdot 0.25H_2O$) C, H, N.

(*S*,*S*)-*N*-Formyl-*N*-α-methylbenzyl-3-(9′-carbomethoxynonyloxy)amphetamine (8a). A solution of 1.97 (0.007 mol) of phenol **7** in 3 mL of DMF was carefully added to a suspension of 300 mg of 60% NaH (washed with hexanes in 5 mL of DMF) followed by the addition of 2 g of methyl-10-bromodecanoate in 4 mL of DMF. After 16 h of stirring, the reaction mixture was quenched with water and extracted with 4 × 200 mL of CH_2Cl_2 and dried (Na_2SO_4). The oil obtained on concentration was purified using an 80 g silica gel ISCO column with gradient 0−10% of polar B: hexanes/CH_2Cl_2/MeOH (4:8:1) in a polar CH_2Cl_2 collecting 25 mL fraction. Concentration of the product fraction gave 1.98 g (63%) of pure **8a** as an oil. ^{1}H NMR ($CDCl_3$) δ 8.40 (s, 1H), 8.37 (s, 1H), 7.25−7.47 (m, 5H), 7.054 (t, J = 7.9 Hz, 1H), 7.045 (t, J = 7.9 Hz, 1H), 6.68 (d, J = 1.9 Hz, 1H), 6.65 (d, J = 1.9 Hz, 1H), 6.39 (d, J = 7.5 Hz, 1H), 6.29 (at, J = 1.9 Hz, 1H), 6.23 (d, J = 7.5 Hz, 1H), 6.08 (at, J = 1.9 Hz, 1H), 5.93 (q, J = 7.2 Hz, 1H), 4.64 (q, J = 7.2 Hz, 1H), 3.80 (qd, J = 13.2, 6.4 Hz, 2H), 3.66 (s, 3H), 3.36−3.55 (m, 1H), 3.18−3.36 (m, 1H), 2.98 (dd, J = 13.2, 9.8 Hz, 1H), 2.61 (dd, J = 13.2, 5.3 Hz, 1H), 2.36−2.53 (m, 2H), 2.31 (t, J = 7.5 Hz, 2H), 1.68−1.82 (m, 2H), 1.50−1.68 (m, 2H), 1.57 (d, J = 6.8 Hz, 3H), 1.54 (d, J = 7.2 Hz, 3H), 1.39−1.50 (m, 2H), 1.28−1.39 (m, 13H), 1.25 (d, J = 7.2 Hz, 3H). ^{13}C NMR ($CDCl_3$) δ 174.1, 162.4, 161.4, 159.05, 158.98, 140.8, 140.1, 139.7, 139.5, 129.2, 129.0, 128.5, 128.4, 128.0, 127.9, 127.8, 127.7, 127.6, 127.15, 127.07, 121.2, 121.0, 114.9, 114.7, 112.7, 112.5, 67.73, 67.7, 56.9, 53.3, 51.3, 51.1, 49.7, 44.7, 40.4, 34.0, 29.23, 29.21, 29.18, 29.16, 29.1, 29.0, 25.9, 24.8, 21.7, 19.7, 17.2, 16.3. Anal. ($C_{29}H_{41}NO_4 \cdot H_2O$) C, H, N.

(*S*,*S*)-*N*-Methyl-*N*-α-methylbenzyl-3-(9′-carbomethoxynonyloxy)-amphetamine (9a). To 1.90 g (0.004 mol) of **8a** in 5 mL of anhydrous THF was added 10 mL of 1 M BH_3 in THF solution. The reaction mixture was stirred for 1 h, carefully quenched with 1 mL MeOH, 10 mL of 1 M HCl, and basified with 20 mL of 15% NH_4OH (aq) solution. The aqueous layer was extracted with 3 × 50 mL of CH_2Cl_2. The oil obtained on concentration was subjected to chromatography using a 120 g silica gel ISCO column with hexanes/Et_2O/TEA (10:9:1) as the eluent collecting 25 mL fractions. The fractions containing the first major product were concentrated to give 489 mg (26%) of **9a** as an oil. ^{1}H NMR ($CDCl_3$) δ 7.18−7.34 (m, 4H), 7.09−7.18 (m, 1H), 7.03 (t, J = 7.9 Hz, 1H), 6.60 (dd, J = 7.5, 1.9 Hz, 1H), 6.52 (d, J = 7.5 Hz, 1H), 6.45 (s, 1H), 3.69−3.87 (m, 2H), 3.56 (s, 3H), 3.50−3.68 (m, 1H), 2.89−3.07 (m, 1H), 2.82 (dd, J = 12.8, 4.5 Hz, 1H), 2.17 (s, 3H), 2.15−2.30 (m, 2H), 1.44−1.74 (m, 5H), 1.09−1.44 (m, 13H), 0.82 (d, J = 6.4 Hz, 3H). ^{13}C NMR ($CDCl_3$) δ 174.3, 159.0, 146.4, 142.6, 128.9, 128.3, 127.3, 126.6, 121.4, 115.1, 111.8, 67.8, 62.7, 62.1, 55.8, 51.4, 38.0, 34.1, 32.3, 29.8, 29.3, 29.14, 29.08, 26.0, 24.9, 21.9, 15.5.

(*S*)-3-(9-Carbomethoxynonyloxy)methamphetamine (10a). A mixture of 489 mg (1.08 mmol) of **9a** in 40 mL of CH_3OH containing 2.7 g of HCO_2NH_4 and 350 mg of 5% Pd/C was refluxed for 2 h. The cooled reaction solution was filtered through a Celite pad and concentrated. The resulting residue was basified in 100 mL of Et_2O using 1 mL of TEA. This mixture was gravity filtered (to remove excess formate salts), and the filtrate was concentrated to an oil. The oil was dissolved in a minimum amount of CH_2Cl_2 (about 1−2 mL) and was subjected to column chromatography using a 120-g silica gel ISCO column using 5% B/A to 100% B/A gradient elution over 30 min (A = hexanes/Et_2O/TEA (10:9:1); B = 10% MeOH/CH_2Cl_2). Product fractions were concentrated to give 378 mg (91%) of **10a** as an oil. ^{1}H NMR ($CDCl_3$) δ 7.16 (dt, J = 7.2, 1.5 Hz, 1H), 6.62−6.83 (m, 3H), 3.90 (t, J = 6.4 Hz, 2H), 3.63 (s, 3H), 2.76 (sixtet, J = 6.4 Hz, 1H), 2.60 (dq, J = 13.2, 6.4 Hz, 2H), 2.35 (s, 3H), 2.27 (t, J = 7.2 Hz, 2H), 1.74 (p, J = 6.4 Hz, 2H), 1.59 (p, J = 7.2 Hz, 2H), 1.18−1.50 (m, 10H), 1.03 (d, J = 6.4 Hz, 3H). ^{13}C NMR ($CDCl_3$) δ 174.1, 159.1, 140.9, 129.2, 121.3, 121.4, 115.5, 112.0, 67.7, 56.2, 51.3, 43.4, 34.0, 33.8, 29.21, 29.20, 29.04, 28.99, 25.9, 25.8, 19.6.

(*S*,*S*)-*N*-Formyl-*N*-α-methylbenzyl-3-(5′-carbomethoxypentyloxy)amphetamine (8b). Compound **8b** was prepared by a procedure analogous to that described for **8a** to afford 76% of **8b**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.41 (s, 1H), 8.40 (s, 1H), 7.22−7.50 (m, 5H), 7.06 (t, J = 7.9 Hz, 1H), 7.05 (t, J = 7.9 Hz, 1H), 6.67 (d, J = 1.9 Hz, 1H), 6.64 (d, J = 1.9 Hz, 1H), 6.39 (d, J = 7.5 Hz, 1H), 6.28 (bs, 1H), 6.24 (d, J = 7.5 Hz, 1H), 6.08 (bs, 1H), 5.93 (q, J = 7.2 Hz, 1H), 4.64 (q, J = 7.2 Hz, 1H), 3.82 (q, J = 6.4 Hz, 1H), 3.68 (s, 3H), 3.35−3.54 (m, 1H), 3.19−3.35 (m, 1H), 2.98 (dd, J = 12.8, 9.8 Hz, 1H), 2.60 (dd, J = 12.8, 4.9 Hz, 1H), 2.42−2.50 (m, 2H), 2.33 (t, J = 7.2 Hz, 3H), 1.64−1.83 (m, 4H), 1.58 (d, J = 6.8 Hz, 3H), 1.54 (d, J = 6.8 Hz, 3H), 1.41−1.52 (m, 2H), 1.31 (d, J = 6.8 Hz, 3H), 1.25 (d, J = 6.8 Hz, 3H). ^{13}C NMR (300 MHz, $CDCl_3$) δ 173.5, 162.4, 161.4, 158.94, 158.88, 140.8, 140.2, 139.7, 139.5, 129.2, 129.0, 128.5, 128.4, 128.0, 127.8, 127.6, 127.1, 121.3, 121.0, 114.8, 114.7, 112.6, 112.4, 67.9, 60.1, 56.8, 53.2, 51.1, 49.7, 44.6, 40.4, 34.1, 28.9, 28.8, 25.5, 24.6, 21.7, 19.7, 17.2, 16.3.

(*S*,*S*)-*N*-Methyl-*N*-α-methylbenzyl-3-(5′-carbomethoxypentyloxy)amphetamine (9b). Compound **9b** was prepared by a procedure analogous to that described for **9a** to afford 58% of **9b**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.24−7.44 (m, 4H), 7.15 (m, 1H), 7.10 (t, J = 7.9 Hz, 1H), 6.67 (d, J = 1.9 Hz, 1H), 6.60 (d, J = 7.9 Hz, 1H), 6.52 (at, J = 1.9 Hz, 1H), 3.78−3.96 (m, 2H), 3.55−3.75 (m, 3H), 2.96−3.12 (m, 1H), 2.58 (dd, J = 12.8, 4.5 Hz, 1H), 2.31 (dd, J = 12.8, 9.8 Hz, 1H), 2.24 (s, 3H), 1.69−1.84 (m, 2H), 1.54−1.69 (m, 2H), 1.37−1.54 (m, 4H), 1.32 (d, J = 6.8 Hz, 3H), 0.90 (d, J = 6.8 Hz, 3H). ^{13}C NMR (300 MHz, $CDCl_3$) δ 129.35, 128.72, 127.68, 127.07, 121.95, 115.52, 112.22, 67.83, 62.56, 56.22, 51.90, 38.36, 34.41, 32.77, 29.42, 26.11, 25.14, 22.41, 16.02.

(*S*)-3-(5-Carbomethoxypentyloxy)methamphetamine Hydrochloride (10b). Compound **10b** was prepared by a procedure analogous to that described for **10a** to afford 67% of **10b**. ^{1}H NMR ($CDCl_3$) δ 7.20 (t, J = 7.5 Hz, 1H), 6.67−6.81 (m, 3H), 3.94 (t, J = 6.4 Hz, 3H), 3.67 (s, 3H), 2.73−2.87 (m, 1H), 2.68 (dd, J = 13.2, 6.8 Hz, 1H), 2.58 (dd, J = 13.2, 6.4 Hz, 1H), 2.39 (s, 3H), 2.35 (t, J = 7.2 Hz, 2H), 1.80 (ap, J = 7.2 Hz, 2H), 1.71 (ap, J = 7.2 Hz, 2H), 1.40−1.62 (m, 3H), 1.06 (d, J = 6.0 Hz, 3H). ^{13}C NMR (300 MHz, $CDCl_3$) δ 174.4, 159.7, 138.0, 129.7, 121.4, 115.4, 113.0, 67.6, 56.8, 51.4, 39.9, 33.9, 30.5, 28.9, 25.6, 24.6, 16.1.

General Synthesis of Hapten−Protein Antigens. In preliminary studies, each hapten, (+)-METH MO6 or (+)-METH MO10, was covalently bound to at least 2−3 different protein antigens, cationized bovine serum albumin (c-BSA), ovalbumin (OVA), or bovine serum albumin (BSA), and then used to immunize groups of mice (see below) to test for the serum anti-(+)-METH immune response. The individual mouse and hapten−protein antigen combination that yielded the highest anti-(+)-METH IgG titers from each group of immunizations was chosen for production of mAbs.

A. From c-BSA. To generate the antigens for immunization with the c-BSA carrier protein, each hapten was conjugated to Imject SuperCarrier Immune Modulator (c-BSA; Thermo Fisher, Rockford, IL), following the manufacturer's protocol. To separate the hapten/c-BSA conjugates from uncoupled hapten and other synthesis reagents, the antigens were passed through a PD-10 Sephadex G-25 column (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) equilibrated with phosphate-buffered saline. Absorbance of the protein eluent was monitored at 280 nM to determine when to collect the conjugated product as it eluted from the column. Once collected, the final antigen

concentrations were determined by adding 8.3 μL of antigen solution to 250 μL of Coomassie Plus protein assay reagent (Pierce Chemical Company, Rockford, IL) and comparing the color development against a BSA standard curve. A microtiter plate reader with detection at 590 nM was used to detect the color change. The final products were stored at −20 °C until needed.

B. From BSA and OVA. To generate the antigens for immunization with OVA or BSA carrier proteins, we used a carbodiimide procedure similar to the method of Owens et al.[15] All reagents used in hapten−protein conjugation were obtained from Sigma Chemical Company (St. Louis, MO) unless otherwise specified. For the synthesis of MO10 antigens using OVA or BSA, the starting hapten/carrier protein molar ratio was 60:1. For comparison the synthesis of MO6 antigens using c-BSA, the starting hapten/carrier protein molar ratio was 90:1. These ratios of hapten to carrier protein were optimized in preliminary experiments. Each hapten was dissolved in 1 mL of DMF or 0.1 M 2-(*N*-morphalino)ethanesulfonic acid buffer (pH 4.5) to conduct the synthesis. The reaction mixture was allowed to stir overnight at 25 °C protected from light. The final product was dialyzed against 4 L of deionized water (with at least 2−3 changes of water) for 24 h at 4 °C then against 4 L of pH 7.35 phosphate-buffered saline (with at least 2−3 changes) for 24 h at 4 °C to yield the hapten/protein antigens. Because of the protein modification resulting from the addition of haptens to the protein surface, the antigens often partially precipitated. After dialysis, the soluble and insoluble fractions of the antigenic preparations were stored at −20 °C until needed for immunization.

Mass Spectrometry Analysis of (+)-MO6-BSA, (+)-MO6-OVA, (+)-METH MO10-BSA, and (+)-METH MO10-OVA. Because the analysis of (+)-MO6 and (+)-MO10 antigens were similar, we will only discuss (+)-MO10 antigens. Hapten to carrier protein incorporation rates for the (+)-MO6-cBSA or (+)-M010-cBSA antigens were not determined, as discussed earlier. Mass spectra were obtained of (+)-METH MO10-OVA and (+)-METH MO10-BSA using a Bruker Reflex III MALDI TOF mass spectrometer. The mass spectrometer was operated in the linear mode with an acceleration voltage of approximately 25 kV. Pulsed ion extraction with a delay of 700 ns was used. The instrument was calibrated using a commercial reference material from the manufacturer (Bruker Protein Standard II) for high mass (10−100 kDa) range calibration. Ion suppression was used up to *m*/*z* 10 KDa to enhance the high mass ions. After synthesis of the (+)-METH MO10-OVA or (+)-METH MO10-BSA conjugate, a small aliquot of the soluble antigen in the supernatant fraction, along with the native OVA or BSA protein, was analyzed using dihydroxy benzoic acid (approximately 10 mg/mL in 0.1% TFA/ACN 2:1) as the MALDI matrix. Small volumes of dialyzed samples were mixed 1:1 with the matrix solution, and 1 μL was spotted on the target. For samples that had not previously been dialyzed, a standard desalting Zip Tip procedure was used to cleanup and concentrate the samples. However, the protein dialysis procedure against deionized H_2O consistently produced better results.

Immunization, Screening, and Hybridoma Generation. Female BALB/c mice (Charles River Laboratories, Wilmington, MA) were used for all immunizations, as previously described by Peterson et al.[10] For production of the (+)-METH MO6 and (+)-METH MO10 mAbs, all mice were initially immunized subcutaneously in the hindquarters with 10−100 μg of antigen emulsified in either Hunter's TiterMax adjuvant (for mAb9B11 and mAb10E1) or Freund's Complete Adjuvant (for all others). This initial immunization was followed by a boost with 10−50 μg of antigen emulsified in TiterMax or Freund's Incomplete Adjuvant (or Freund's Complete Adjuvant for the mice used to produce mAb1A2, mAb1C1, mAb10D1, mAb7F9, and mAb9C10; see Table 1) 3−6 weeks later. Subsequent boost followed at 6 week intervals until a high anti-(+)-METH IgG titer was reached. Serum samples were taken via tail bleed periodically to measure anti-(+)-METH IgG response by a enzyme-linked immunosorbant assay (ELISA) similar to the method described by Peterson et al.[10]

After sufficient anti-(+)-METH IgG titers were achieved (typically after 2−4 months of immunization), hybridomas were produced using previously reported methods.[16] The hybridoma fusion partner for mouse B cells was cell line P3 × 63Ag8.653 (American Type Culture Collection, Manassas, VA). Once hybridomas were produced, initial screening for potential anti--(+)-METH mAbs was conducted by an ELISA using 96-well microtiter plates coated with the original hapten (either MO6 or MO10) conjugated to a different protein carrier (thyroglobulin, Sigma Chemical Company). This procedure avoided selecting carrier protein-reactive antibodies (e.g., anti-OVA antibodies for mice immunized with a MO10-OVA antigen). To ensure that only mouse IgG isotypes were selected during these processes, anti-IgG constant region antibodies were used for the ELISA. The IgG isotype and light chain identity were determined using a mouse antibody isotyping kit (Boehringer Mannheim, Indianapolis, IN). Once potential anti-(+)-METH secreting hybridoma cell lines were discovered in a specific well of a microtiter plate, the cells from this location were repeatedly subcloned to ensure the monoclonality of the cell line. The supernatant from these cell lines were also rescreened by a [^{3}H]-(+)-METH radioimmunoassay (RIA) with (+)-METH, (+)-AMP, and (+)-MDMA as the inhibitors to confirm that the cell line was secreting IgG antibodies against (+)-METH-like compounds. This RIA method is described in an upcoming section.

Production and Purification. Monoclonal antibodies were produced in either a Cell-Pharm System 2500 hollow fiber bioreactor[16,17] (Unisyn Technologies, Inc., Hopkinton, MA) or in a Biostat B 10-L bioreactor (Sartorius Corp., Edgewood, NY).[10] All antibodies were harvested and stored at −80 °C until purification. mAb were purified either by affinity chromatography using Protein-G Sepharose (Amersham Biosciences, Piscataway, NJ) as described by Peterson et al.,[10] ion exchange chromatography using SP Sepharose (Amersham Biosciences, Piscataway, NJ) as described in Hardin et al.,[18] or a combination of the two methods. Following purification, mAbs were concentrated and buffer exchanged into 15 mM sodium phosphate containing 150 mM sodium chloride (pH 6.5−7.5), as described in McMillan et al.[19]

Determination of Monoclonal Antibody K_D and K_I Values for (+)-METH, (+)-AMP, and (+)-MDMA. A 100 μL aliquot of (+)-[2′,6′-^{3}H(*n*)]methamphetamine ([^{3}H]-(+)-METH; specific activity = 18 Ci/mmol; ∼50000 decays/min) in RIA buffer (0.05 M Tris; 0.9% NaCl; 2% BSA; 0.2% NaN_3; 0.05% Tween 20, adjusted to pH 7.6) was added to 50 mm × 14 mm polypropylene test tubes. The [^{3}H]-(+)-METH radioligand was synthesized at the Research Triangle Institute (Research Triangle Park, NC) and was a gift from the National Institute on Drug Abuse (Bethesda, MD). The mAbs (in 100 μL) were diluted in RIA buffer to a concentration that would bind ∼15−22% of the ∼50000 decays/min of the [^{3}H]-(+)-METH in each tube. Each tube also received 10 μL of unlabeled (+)-METH (or (+)-MDMA for determining the K_I value for (+)-MDMA) at an appropriate range of (+)-METH concentrations above and below the expected K_D (or K_I) value for each mAb. [^{3}H]-(+)-MDMA was unavailable, so we determined a K_I value for binding to each mAb using [^{3}H]-METH as the radioligand and inhibition with unlabeled (+)-MDMA. Next, 20 μL of Pierce MagnaBind Protein G Magnetic Beads was added to each tube to allow easy separation of bound (to mAb) and free [^{3}H]-(+)-METH at the end of the assay. The nonspecific binding tubes had all of the same reagents except that a saturating dose (10 μM) of (+)-METH was added to inhibit all specific antibody binding. The tubes were vortexed, centrifuged at 1000 rpm for 30 s, and incubated overnight with gentle shaking at 4 °C. The next day tubes were centrifuged at 1000 rpm for 30 s and placed

in a magnetic separating rack for 6 min. The supernatant fluid was slowly aspirated, and the magnetic bead pellet containing mAb was resuspended in the same test tube with 2 mL scintillation fluid (Ecoscint A, National Diagnostics, Atlanta, GA). The tubes were placed into an empty 20 mL scintillation vial carrier and the mAb bound concentration of [^{3}H]-METH in each tube was determined by liquid scintillation spectrometry. An IC_{50} value for inhibition of [^{3}H]-(+)-METH for each mAb was determined after fitting a logistic curve to the data points using Origin graphing and data analysis software (OriginLab Corporation, Northampton, MA). K_D (for (+)-METH or K_I (+)-MDMA values for (+)-METH binding to each mAb) were determined after correction for the binding of [^{3}H]-(+)-METH by the method of Akera and Cheng.[20]

A similar homogeneous assay was conducted using [^{3}H]-(+)-AMP with inhibition using (+)-AMP for determination of K_D values for (+)-AMP binding to the mAbs. If we determined in initial screening assays that the mAb would have a very high K_D value (> 1000−5000 nM) for (+)-AMP, we conducted a heterologous immunoassay with [^{3}H]-METH as the radioligand and inhibition with a high range of unlabeled (+)-AMP doses to obtain an approximate K_I value for (+)-AMP binding.

Representative binding curves for mAb4G9 for (+)-METH, (+)-AMP, and (+)-MDMA are shown in Figure 2. Each of the determinations was made with duplicate (for (+)-METH and (+)-AMP) or triplicate (for (+)-MDMA) RIA dose−response curves, and the average value was reported in Table 1.

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Supporting Information Available: Elemental analysis of compounds **5**, **7**, **8a**, **3a**, and **3b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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