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Synthesis of Mercapto (+) methamphetamine Haptens and Their Use for Obtaining Improved Epitope Density on (+) Methamphetamine Conjugate Vaccines

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Abstract

This study reports the synthesis of the mercapto hapten (*S*)-*N*-(2-(mercaptoethyl)-6-(3-(2-(methylamino)propyl)phenoxy)hexanamide [**3**, (+)-METH HSMO9] and its use to prepare METH-conjugated vaccines (MCV) from maleimide activated proteins. MALDI-TOF mass spectrometry analysis of the MCV synthesized using **3** showed there was a high and controllable epitope density on two different carrier proteins. In addition, the MCV produced a substantially greater immunological response in mice than previous METH haptens, and a monoclonal antibody generated from this MCV in mice showed a very high affinity for (+)-METH ($K_D = 6.8$ nM). The efficient covalent coupling of (+)-METH HSMO9 to the activated carrier proteins suggests this approach could be cost effective for large-scale production of MCV. In addition, the general methods described for the synthesis of (+) METH HSMO9 (**3**) and its use to synthesize MCV will be applicable for conjugated vaccines of small molecules and other substances of abuse such as morphine, nicotine, and cocaine.

Keywords

Amphetamines; antigen; drug abuse; hapten; immunochemistry; methamphetamine; vaccines

(+)-Methamphetamine's (METH)¹ ease of illegal synthesis, low cost, long duration of action, and powerful rewarding effects lead to destructive individual and societal health in the United States.¹ Chronic METH use causes addiction, neurological damage, and altered

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SUPPORTING INFORMATION AVAILABLE: Elemental analysis of compounds **3**, **6**, **9**, and **10**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

¹ABBREVIATIONS. (+) AMP, (+) amphetamine; (-)-AMP, (-)-amphetamine; BSA, bovine serum albumin; c-BSA, cationized bovine serum albumin; CNS, central nervous system; DAWN, Drug Abuse Warning Network; ED, emergency department; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; ELISA, enzyme-linked immunosorbent assay; HOAT, 1-hydroxy-7-azabenzotriazole; mAb, monoclonal antibody (both singular and plural); MALDI, Matrix Assisted Laser Desorption/Ionization; (+)-METH HSMO9, mercapto-hapten (*S*)-*N*-(2-(mercaptoethyl)-6-(3-(2-(methylamino)propyl)phenoxy)-hexanamide; MCV, METH-conjugate vaccine; (+)-MDMA, (+)-3,4-methylenedioxymethamphetamine; (-)-MDMA, (-)-3,4-methylenedioxymethamphetamine; (±)-MDMA, (±)-3,4-methylenedioxymethamphetamine; (+)-METH, (+)-methamphetamine; (-)-METH, (-)-methamphetamine; MS, mass spectrometry; OVA, ovalbumin; RIA, radioimmunoassay; Sulfo SMCC, sulfosuccinimidyl 4-[*N* maleimidomethyl]cyclohexane-1-carboxylate.

behavioral and cognitive functions.²⁻⁵ Acutely, METH can produce renal and liver failure, cardiac arrhythmias, heart attack, stroke, and seizures.^{2,6,7} At present there are no specific pharmacotherapies for treating these adverse medical effects.

Since METH's psychostimulant and addictive properties are mediated through sites of action in the central nervous system (CNS), METH must first cross the blood-brain barrier to produce its neurological effects. A novel treatment strategy is to use high affinity anti-METH antibody medications as pharmacokinetic antagonists to prevent or slow METH's access to these CNS sites of action.⁸⁻¹⁰ Anti-METH immunotherapy includes high affinity, preformed anti-METH monoclonal antibodies (mAb) for immediate protection and anti-METH antibodies generated over time through active immunization with METH conjugate vaccines (MCV).¹¹ With both types of immunotherapy, preclinical studies suggest the two major determinants of therapeutic success are the affinity of the antibodies for METH and the ability to maintain adequate levels of anti-METH antibodies in the vascular circulation.⁸⁻¹⁰

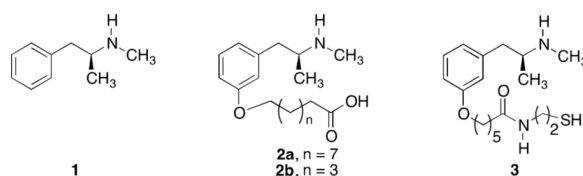
The important process of creating high affinity anti-METH antibodies starts with immunization of animals with a MCV consisting of a METH-like hapten attached (conjugated) to an antigenic carrier protein (*e.g.*, albumin, Keyhole limpet hemocyanin) in a specific molecular orientation. The METH-like hapten is composed of a METH backbone molecular structure linked to a spacer group at a site that is distal to the key molecular features to be recognized by the immune system. The other end of the linker terminates in a functional group that can readily form covalent attachments to the antigenic carrier protein. The linker arm also serves to separate the hapten from the carrier protein at an optimal distance to maximize affinity and specificity for METH.^{12,13} The chemical bonds formed at both ends of the linker and the length of this spacer arm (*e.g.*, nine carbon atoms) are critical to the quality (*e.g.*, affinity and specificity for METH) and quantity (*i.e.*, amount of antibody) of the immune response.

We previously described the synthesis of METH haptens **2a** [designated (+)-METH MO10] and **2b** [designated (+)-METH MO6] and their use for the development of highly refined mAb medications for treating METH abuse.¹² These METH haptens proved extremely valuable for producing anti-METH mAb and for understanding immune system structure activity requirements for control of the specificity and affinity of anti-METH antibodies. However, the anti-METH serum antibody titers to these MCVs were relatively low, and the immune response was inconsistent. These factors made it an unsatisfactory prototype MCV for use in humans. We also knew from our longer term studies of phencyclidine-like haptens that the quality and quantity of immune response are greatly improved with greater phencyclidine hapten epitope densities (*i.e.*, 8–12 haptens per albumin molecule).¹⁴

Thus, we reasoned that the low immune response to these previous MCV was primarily due to the low METH hapten epitope density (*i.e.*, moles of hapten per mole of antigenic carrier protein) on the MCV. Hapten epitope density on the carrier protein is important because if there are too few haptens on the carrier protein the immune system will generate low levels of anti-METH antibodies or even no immune response. For example, when we covalently bound (+)-METH MO10 haptens to prototype ovalbumin (OVA) and bovine serum albumin (BSA) carrier proteins using a carbodiimide procedure, we could only achieve a maximum METH hapten epitope density of approximately 5:1 with both OVA and BSA.^{12,13,15} This relatively low epitope density from the MCV synthesis occurred even when the starting hapten/carrier protein ratio was $\geq 60:1$. Studies of the anti-METH immunological response to these antigens and antigens with $<5:1$ epitope densities in mice revealed that MCV with a epitope density of 5:1 produced a modest to weak immune response, and epitope densities of $<5:1$ failed to stimulate an anti-METH immune response. These data suggested that

procedures used for conjugation of hapten to protein carriers were both inefficient and inadequate for our needs.

We hypothesized that an improved hapten to carrier protein conjugation synthesis procedure could lead to increased MCV epitope densities that utilized lower excesses of hapten to carrier protein in the MVC synthesis. Based on over a decade of preclinical testing of active vaccines and monoclonal antibodies produced from METH conjugate vaccines, we think immunotherapy for the treatment of human METH addiction will require high affinity antibodies with K_D values for METH in the range of $<10\text{--}20\text{ nM}$.⁹ Previous METH hapten structure activity studies also showed that the best chance for achieving these high affinity antibodies would be through immunization with MCV containing haptens with long linker chains connected to the *meta* position on the aromatic ring of METH.^{8,9,12} In this report, we present the design and synthesis of the METH hapten **3** [designated (+)-METH HSMO9], which has an 11 atom linker chain, and report that conjugation of **3** to antigenic carrier proteins leads to MCV with significantly higher hapten to carrier protein ratios than **2a**, lower excesses of hapten to carrier protein, and improved immune responses.



Chemistry

The synthesis of the METH hapten **3** is shown in Scheme 1. Reduction of the *N*-formyl compound of **4**¹² using diborane in tetrahydrofuran provided the *N*-methyl compound **5**. Subjection of **5** to transfer hydrogenation using ammonium formate and 5% palladium on carbon catalyst in methanol yielded **6**. O-Demethylation of **6** using boron tribromide in methylene chloride afforded the phenol **7**. Treatment of **7** with di-*tert*-butyl dicarbonate in methanol containing triethylamine gave the *N*-Boc protected **8**. The sodium salt of the phenol **8** prepared by using sodium hydride in dimethylformamide was alkylated with methyl 6-bromohexanoate to give **9**. Hydrolysis of **9** using lithium hydroxide in a water methanol tetrahydrofuran mixture afforded the acid **10**. Coupling **10** with 2-(trilylmercapto)ethylamine using 1-ethyl-3-(3-diethylaminopropyl)carbodiimide (EDCI) and 1-hydroxy-7-azabenzotriazole (HOAt) in dimethylformamide followed by removal of *N*-Boc with trifluoroacetic acid in methylene chloride yielded **11**. Compound **11** was detritylated by treatment with mercuric acetate in ethanol to give the mercuric acetate sulfhydryl derivative which was treated with hydrogen sulfide to give the free thiol **3** isolated as the tartrate salt. The free thiol must be handled in non oxidative conditions to avoid dimer formation.

(+)-METH HSMO9 was coupled to commercially available maleimide activated BSA **12** by combining a solution of **3** in de gassed conjugation buffer with a reconstituted solution of the maleimide activated protein in buffer as shown in Scheme 2. The commercially available lyophilized maleimide activated BSA contains buffer, and the solution is reconstituted with distilled water. A portion of the reconstituted maleimide activated protein was set aside as an analytical control. The optimum conditions for haptens incorporation were determined by assessing various molar ratios of haptens to protein. Ratios of 10:1, 15:1, 20:1, and 25:1 were evaluated by adding various amounts of (+)-METH HSMO9 stock solution to a stock solution of maleimide activated protein. The protein solutions became cloudy after addition of the hapten, suggesting changes in solubility due to hapten incorporation. The optimum conditions from this experiment were used to scale up production of (+)-METH HSMO9 MCV **13** for studies in mice to determine immunological response.

(+)-METH HSMO9 was also coupled to commercially available maleimide activated OVA in a similar fashion. Several molar ratios of hapten to protein were explored including 20:1, 30:1, 40:1, and 50:1. As with BSA, protein solutions became cloudy after hapten addition, suggesting incorporation.

Analytical

The extent of hapten incorporation was determined by MALDI-TOF using procedures developed for high mass analysis.¹⁶ Figure 1 shows a representative MALDI-TOF mass spectrum of a maleimide activated OVA protein before (control) and after conjugation with (+)-METH HSMO9. Before conducting the MALDI-TOF analysis, the salts from each protein solution were removed by equilibrium dialysis with multiple changes of distilled water. As little as 20 μ L of the MCV was needed for this procedure. Previous experiments showed that multiple steps of dialysis-based desalting lowered the measured m/z value as alkali ions (usually Na^+) were replaced with protons until the solution was essentially salt free. These salts could not be adequately eliminated using a ZipTip procedure nor did it improve upon the results obtained from repeated dialysis. Based on these observations and the potential loss of sample using ZipTips for purification, desalting by equilibrium dialysis alone was judged the best approach.

The MALDI measured mass value of the maleimide activated OVA was about $47,268 \pm 100$ Da. A peak at m/z 23,572 showed the doubly charged species. Because of the low resolution at the high m/z values for the MCV, the center of mass (centroid) of the roughly Gaussian shaped peaks (see Figure 1) represents the overlapping masses of an ensemble of components rather than a single molecular species.

After desalting (+)-METH HSMO9, hapten epitope density on the MCV could be calculated by the mass difference between the maleimide activated OVA (before hapten conjugation, Figure 1a) and the (+)-METH HSMO9 OVA final product (Figure 1b). In this example the measured mass of the final product was $49,951 \pm 100$ Da. Because we used an extensive (but simple) desalting step before MALDI-TOF analysis, the mass difference of 2,683 Da could be attributed exclusively to (+)-METH HSMO9 hapten incorporation. Dividing the 2,683 Da mass by the hapten mass (339 Da) yielded an average hapten epitope density of 7.9 moles of (+)-METH HSMO9 per mole of maleimide activated OVA in this experiment. The non-integer value resulting from this calculation (and the increased peak width) in Figure 1b compared to Figure 1a occurred because the data in Figure 1b reflect a distribution (or family) of haptenated species (*e.g.*, 7–9 haptens per mole of protein), rather than incorporation of a single specific integer value of hapten per protein molecule.

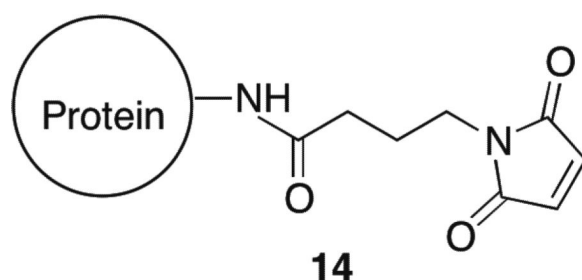
Results and Discussion

The relationship between the degree of hapten incorporation (*i.e.*, epitope density) and the immune response is not known for most haptenated antigens because it is usually very difficult to accurately measure, yet it is a critical factor in immune response optimization. A recent trend in vaccine development is to couple haptens to proteins via the Michael addition of a free thiol to a maleimide activated protein. These reactions are high yielding and efficient. They have been primarily used to couple peptides to large antigenic proteins where an efficient chemical coupling reaction is beneficial to overcome any steric issues of linking two relatively large moieties. For example, the Danishefsky laboratory has applied this linking approach to the synthesis of pentavalent and hexavalent antigenic constructs in which multiple carbohydrate molecules are attached at each member of a peptide chain, then linked to Keyhole limpet hemocyanin.¹⁷

Most clinical vaccines are based on neutralizing the disease processes of proteins, peptides, or carbohydrates. Not until recently have clinically useful vaccines against small pharmacological active molecules been extensively explored, and most of the studies involved drugs of abuse such as cocaine and nicotine.¹⁸ Conjugation of small molecules to large antigenic proteins using carboxylic acid coupling reactions can effectively generate very high affinity monoclonal antibodies in mice, as was observed for our MCV.^{10,12} However, greater and more reproducible immune responses will be needed for human active vaccines.⁸

An important problem with the current generation of nicotine and cocaine conjugate vaccines for treating addiction is the poor and unsustained immune response over time in human subjects. Indeed, only about one-third of human subjects mounted even a modest immune response.^{19,20} Therefore, anti-addiction vaccines that produce immune responses in 100% of the subjects should be an important criteria for all anti-addiction vaccines. To achieve this goal, improvements in epitope density along with other factors are needed.

Toward the goal of developing a better MCV, a study was conducted on the use of the Michael addition approach with our methamphetamine based haptens, (+)-METH HSM09 using OVA and BSA as antigenic proteins. In previous studies, we reported that the METH antibody binding function of monoclonal antibodies generated from haptens with short linkers attached at the *para* position of the aromatic ring is significantly inactivated *in vivo* in rats within 24 h of antibody treatment. However, this does not happen with monoclonal antibodies generated from active immunization of mice with longer chain METH-like haptens coupled to linker groups at the *meta* position of the aromatic ring.¹⁰ Based on these discoveries, we designed (+)-METH HSMO9 with a relatively long spacer group connected to the *meta* position on the aromatic ring. In addition, the linker in the maleimide activated BSA and OVA (see structure **12**) used in our studies has a cyclohexylmethylene in the spacer arm which is reported to decrease the rate of hydrolysis of the maleimide group compared to similar reagents.²¹ The only other example of the use of a Michael addition in METH vaccine development using METH is very recent report by Moreno et al.²² In this study the spacer arm of the maleimide activated protein had three methylene groups (see structure **14**).



OVA and BSA are not suitable for human vaccines because these proteins are part of the diet of many humans. They can, however, be used in animal studies as a proof of principle and more importantly are small enough (<100,000 Da) to be directly analyzed by MALDI-TOF for determination of hapten incorporation. Moreover, maleimide-activated OVA and BSA are commercially available making the need to activate native protein unnecessary.

Initial probe experiments suggested haptens additions were significantly greater than 5:1 for both BSA and OVA; therefore, in order to optimize epitope densities using this approach, a range of molar equivalents were studied. Haptens to protein molar ratios of 10:1, 15:1, 20:1, and 25:1 were evaluated for additions to maleimide activated BSA, using BSA with 14–16 available functional maleimides (as reported by Pierce). The number of available

maleimide-active sites on each carrier protein varies between commercial batches and was provided by the vendor (ThermoFisher Scientific). The optimum molar ratio for maximum hapten incorporation was 20:1, which provided an epitope density of 10:1 (Figure 2). This finding was double the epitope density of our methamphetamine haptens on BSA compared to our earlier efforts using the carboxylic acid coupling methods.¹² Importantly we found a linear relationship between final epitope density (y-axis) and the starting molar ratios of (+)-METH HSMO9 hapten/protein (x-axis) when using hapten/protein ratios up to 20:1. Less than a two-fold excess of hapten to the number of maleimide active sites was needed to achieve maximum covalent coupling with (+)-METH HSMO9 and BSA in this experiment. In a later scale-up experiment using a new batch of commercial maleimide-activated BSA with 18 functionally active maleimide sites, we used a molar ratio of 22:1 and found 12 (+)-METH HSMO9 haptens per BSA. This was a 140% increase in hapten incorporation compared to coupling (+)-METH MO10 to BSA using carbodiimide chemistry material.¹² This MCV was used for immunization experiments (see below). Both experiments with BSA suggested maximum incorporation of METH haptens requires only an approximate 1.8–2.0-fold excess of hapten to the number functional maleimide sites. This suggests fairly precise epitope densities could be easily achieved for refined studies of the effect of epitope density on immune response. Furthermore, this appeared to be a very efficient synthesis process, which would likely be cost effective for large-scale production of MCV.

A similar set of experiments were conducted using maleimide activated OVA with 12 functional maleimides, using 20:1, 30:1, 40:1, and 50:1. Western blot analysis of the MCV (data not shown) suggested significant hapten incorporation. Mass spectral analysis of the 30:1 experiment showed a hapten incorporation rate of eight (+)-METH HSMO9 haptens per maleimide-activated OVA. This is a 60% increase in efficiency of coupling compared to the carbodiimide coupling of (+)-METH MO10 to OVA (+)-METH MO10 haptens per OVA (results not shown).¹²

The importance of increased epitope density was assessed in two types of immunotherapeutic applications: active immunization and production of monoclonal antibodies. For studies of active immunization, mice that were immunized with the (+)-METH HSMO9 BSA MCV (hapten epitope density of 12) showed a substantial increase in their anti-METH immune response after the second and subsequent MCV booster immunization. The second immunological boost is typically the point at which the most substantial anti-METH IgG immune response occurs in animals. This significant increase occurred in 90% of the mice, and continued booster injections maintained these high anti-METH titers. By comparison, no more than 10% of the mice responded with anti-METH antibodies when immunized with (+)-METH MO10 BSA (hapten epitope density of 5). Weak immune responses and a low percentage of immune responders has also been a significant problem for early clinical trials of active vaccines for nicotine and cocaine in humans.^{19,20}

In a second series of immunological studies, we produced a monoclonal antibody using mice immunized with HSMO9 BSA MCV. From one of these mice, we generated a very high affinity mAb with a (+)-METH K_D = 6.8 nM and (+)-MDMA K_I of 1.1 nM. Our previous highest affinity mAb against a *meta* position hapten linker was 9 nM.¹³ This is the highest affinity mAb against (+)-METH and (+)-MDMA that we have ever produced against a METH conjugate vaccine.

In studies by Moreno et al.,²² a series of constrained and unrestrained METH-like haptens were synthesized and then linked to antigenic carrier proteins like BSA through a sulfhydryl linker. The best three of these METH conjugate vaccines showed high epitope densities (24–29 METH haptens per BSA) and produced high levels of circulating antibodies (45–108 μ g/

ml of antibody) in mice after immunization. However, the anti-(+)-METH immune response after their third immunization decreased. The anti-METH polyclonal antibodies in the mice were only in the range of moderate to poor affinity (82–169 nM). When we used similar short chain linkers coupled at the *para* position, we generated similar low affinity antibodies in mice.¹⁰ However, very high affinity (9–16 nM) anti-METH monoclonal antibodies that were produced from active immunization of mice with longer chain METH-like haptens coupled to linker groups at the *meta* position of the aromatic ring.^{8,9,12}

Conclusions

(+)-METH HSMO9 proved significantly better than previous METH haptens by several measures. The major advantages include the ability to achieve a high and easy to control epitope densities on at least two different carrier proteins, and the substantially greater immunological response to this MCV in mice following active immunization. We also showed the MCV's broader utility by generating a very high affinity mAb with a K_D for (+)-METH of 6.8 nM and a K_I for (+)-MDMA of 1.1 nM. Very efficient covalent coupling to the carrier proteins suggests this hapten could be cost effective for large-scale production of a MCV. This is the second report of the synthesis of a mercapto-hapten from a drug of abuse and its use to prepare conjugates for antigenic proteins. The general methods reported for methamphetamine will be applicable to other small molecules and substances of abuse such as morphine, nicotine, and cocaine.

EXPERIMENTAL SECTION

Nuclear magnetic resonance (^1H 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. Matrix assisted laser desorption ionization (MALDI) mass spectra were acquired using a Bruker (Billerica MA) Reflex III time-of-flight (TOF) mass spectrometer (MS) in the linear, positive ion mode. Samples were prepared for MALDI MS by using both equilibrium dialysis and a ZipTipC₁₈ (Millipore, Billerica, MA) to remove salts prior to analysis. Anhydrous solvents were purchased from Aldrich Chemical Co. CMA is a mixture of 80% chloroform, 18% methanol, and 2% concentrated ammonium hydroxide. [^3H]-METH [(+)-[2',6'- ^3H (n)] methamphetamine; 28.3 Ci/mmol; 0.521 mCi/mL] for radioimmunoassay was synthesized with the radiolabel at the 2 and 6 positions of the aromatic ring by the Research Triangle Institute (Research Triangle Park, NC) for the National Institute on Drug Abuse.

(S)-N-(2-(Mercaptoethyl)-6-(3-(2-(methylamino)propyl)phenoxy)hexanamide (3) Tartarate

Compound **11** (0.630 g, 0.001 mol) was dissolved in ice cold EtOH (12 mL), mercuric acetate (0.775 g, 0.002 mol) was then added, and the mixture was slowly brought to room temperature and stirred overnight. The residue resulting from concentration of the solution was washed with diethyl ether (5 \times 10 mL). After decanting the ethereal layer, the resulting residue containing mercuric salts was suspended in EtOH (20 mL) and treated with hydrogen sulfide gas for 2 h under moderate pressure. The black orange mercuric salt precipitate was removed using a syringe filter under N_2 atmosphere to isolate the free mercaptan into a round bottom flask containing (L) tartaric acid (150 mg, 0.994 mmol). The

mixture was purged with positive flow of N₂ gas to remove the solvent from the mercaptan tartarate salts. This oily residue was redissolved in minimum amount of degassed MeOH, filtered, and the solvent removed under a stream of N₂ gas. Washing the oily substance with (CH₃)₂CHOH pentanes, followed by drying under high vacuum for a few days, gave a white hygroscopic foam that was amenable for transferring to a vial (under N₂) to yield 0.565 g (87%) of **3** as a hygroscopic solid. $[\alpha]^{20}_D +5.90$ (c 0.290, MeOH). ¹H NMR (300 MHz, CDCl₃) δ 7.18 (t, *J* = 8.0 Hz, 1H), 6.67–6.82 (m, 3H), 6.57 (t, *J* = 5.4 Hz, 1H), 3.93 (t, *J* = 6.3 Hz, 2H), 3.55 (q, *J* = 6.0 Hz, 2H), 2.76–2.92 (m, 3H), 2.71 (dd, *J* = 13.1, 7.0 Hz, 1H), 2.60 (dd, *J* = 13.1, 6.3 Hz, 1H), 2.40 (s, 3H), 2.07–2.33 (m, 4H), 1.64–1.87 (m, 4H), 1.44–1.60 (m, 2H), 1.07 (d, *J* = 6.1 Hz, 3H). ¹³C NMR (300 MHz, CDCl₃) δ 130.9 (CH), 129.8 (CH), 121.9 (CH), 116.0 (CH), 112.5 (CH), 67.9 (CH₂), 56.7, 43.7 (CH₂), 38.8 (CH₂), 38.2 (CH₂), 36.8 (CH₂), 34.2, 29.4 (CH₂), 26.2 (CH₂), 25.8 (CH₂), 19.9; IR (CH₂Cl₂): 1666 cm⁻¹ (NC=O); LCMS (ESI) *m/z* 339.9 (M+1)⁺.

(S)-1-(3-Methoxyphenyl)-*N*-methyl-*N*-((S)-1-phenylethyl)propan-2-amine (**5**)

To an ice cooled stirring solution of **4** (4.87 g, 0.016 mol) in THF (50 mL) was added BH₃ THF solution (1.0 M, 57 mL, 0.057 mol) drop wise over 15 min. After the addition, the reaction mixture was brought to room temperature and stirred overnight. The reaction mixture was carefully quenched with MeOH to provide a colorless oil. The oil was purified by flash chromatography (silica gel, 80.0 g Analogix column) using hexane Et₂O NEt₃ (10:1:0.1) as eluent to yield 4.56 g (94%) of **5** as a clear, colorless oil. This compound was used in the next step without further purification.

(S)-1-(3-Methoxyphenyl)-*N*-methylpropan-2-amine (**6**)

To a methanol solution (50 mL) of **5** (2.45 g, 0.0082 mol) was added ammonium formate (2.70 g, 0.042 mol) and 5% Pd/C (0.245 g). The solution was gently warmed ca. 50 °C for a period of 4 h. The cooled reaction mixture was filtered through a pad of celite under moderate pressure. The celite pad was washed with methanol (3 × 10 mL), and the collected organics were concentrated to yield an oily substance, which was dissolved in diethyl ether (100 mL) containing small amount of NEt₃ (10 mol%). The mixture was vacuum filtered (to remove excess formate salts), and the filtrate was concentrated to give an oil. The oil was subjected to flash chromatography (silica gel, 40.0 g ISCO column) using hexane Et₂O NEt₃ (10:1:0.1) as the eluent to yield 1.37 g (93%) of **6** as a clear, colorless oil. $[\alpha]^{20}_D +5.83$ (c 1.15, MeOH). ¹H NMR (300 MHz, CDCl₃) δ 7.21 (t, *J* = 7.9 Hz, 1H, H-5), 6.70–6.83 (m, 3H, H-2, 4, 6), 3.80 (s, 3H, H-11), 2.80 (hextet, *J* = 6.4 Hz, 1H, H-8), 2.65 (dd, *J* = 13.6, 6.4 Hz, 1H, H-7), 2.63 (dd, *J* = 13.6, 6.4 Hz, 1H, H-7), 2.39 (s, 3H, H-10), 1.07 (d, *J* = 6.4 Hz, 3H, H-9). ¹³C NMR (300 MHz, CDCl₃) δ 159.6 (C-3), 141.1 (C-1), 129.3 (C-5), 121.6 (C-6), 115.0 (C-2), 111.4 (C-4), 56.2 (C-8), 55.1 (C-11), 43.5 (C-7), 33.9 (C-10), 10.7 (C-9).

Compound **6** was converted into its HCl salt by slow addition of HCl (2.0 M in Et₂O) to the ice cold ethereal solution: mp 132–134 °C. $[\alpha]^{20}_D +2.251$ (c 1.435, MeOH). ¹H NMR (300 MHz, CDCl₃) δ 9.65 (bs, 2H), 7.22 (t, *J* = 7.5 Hz, 1H, H-5), 6.69–6.88 (m, 3H, H-2, 4, 6), 3.79 (s, 3H, H-11), 3.46 (dd, *J* = 12.8, 3.8 Hz, 1H, H-8), 3.28–3.41 (bm, 1H, H-8), 2.82 (dd, *J* = 12.8, 10.6 Hz, 1H, H-7), 2.72 (bs, 3H, H-10), 1.35 (d, *J* = 6.4 Hz, 3H, H-9). ¹³C NMR (500 MHz, CDCl₃) δ 159.8 (C-3), 137.5 (C-1), 129.8 (C-5), 121.5 (C-6), 114.8 (C-2), 112.6 (C-4), 57.0 (C-8), 55.2 (C-11), 39.4 (C-7), 30.1 (C-10), 15.5 (C-9). Anal. (C₁₁H₁₈ClNO): C, H.

(S)-3-(2-(Methylamino)propyl)phenol Hydrobromide (**7**)

To a stirred solution of **6** (2.90 g, 0.016 mol) in CH₂Cl₂ (50 mL) at –78 °C, boron tribromide solution (13.25 g, 0.053 mol) was added drop wise over a period of 15 min. After the

addition, the mixture was brought to room temperature and allowed to stir at 25 °C for 8 h. The reaction mixture was carefully quenched with methanol at ice-cold condition and concentrated under vacuum. Addition of methanol followed by concentration was carried out at least five more times to ensure that the boron compounds were converted into its volatile. The resulting brown oily residue was purified by flash chromatography (silica gel, 220 g ISCO column) using CH₂Cl₂-CMA (1:1) as the eluent to yield **7** (2.92 g, 73%) as a brown colored oily substance. $[\alpha]_D^{20} +7.59$ (c 1.305, MeOH). ¹H NMR (300 MHz, CD₃OD) δ 7.17 (dt, *J* = 8.3, 2.3 Hz, 1H, H-5), 6.57–6.86 (m, 3H, H-2, 4, 6), 3.39–3.54 (m, 1H, H-8), 3.07 (dd, *J* = 13.6, 5.3 Hz, 1H, H-7), 2.71 (s, 3H, H-10), 2.69 (dd, *J* = 13.6, 9.0 Hz, 1H, H-7), 1.25 (d, *J* = 6.8 Hz, 3H, H-9). ¹³C NMR (300 MHz, CD₃OD) δ 159.0 (C), 138.4 (C), 131.0 (CH), 121.4 (CH), 117.2 (CH), 115.3 (CH), 57.8 (CH), 40.2 (CH₂), 31.0 (CH₃), 15.8 (CH₃).

(S)-*tert*-Butyl-1-(3-hydroxyphenyl)propan-2-yl(methyl)carbamate (**8**)

A stirred solution of **7** (2.75 g, 0.011 mol) in MeOH (20 mL), triethylamine (6.17 g, 0.056 mol), and di-*tert*-butyl dicarbonate (2.0 g, 0.009 mol) was heated at reflux for 30 min. The reaction mixture was concentrated and the oil residue dissolved in EtOAc (100 mL). The EtOAc solution was successively washed with H₂O (50 mL), saturated NaHCO₃ (50 mL), and brine (50 mL), and the organics were dried (MgSO₄) and concentrated. The resulting oil was purified by column chromatography (silica gel, 40.0 g ISCO column) using CMA (100%) as eluent to provide 2.30 g (95%) of **8** as a brown oil. $[\alpha]_D^{20} +31.8$ (c 1.650, MeOH). ¹H NMR (300 MHz, CDCl₃) δ: major rotomer: 7.11 (t, *J* = 7.2 Hz, 1H), 6.50–6.85 (m, 3H), 4.31 (bs, 1H), 2.75 (s, 3H), 2.50–2.72 (m, 2H), 1.23–1.60 (m, 9H), 1.13 (bs, 3H); minor rotomer: (diagnostic peaks) 4.56 (bs, 1H). ¹³C NMR (500 MHz, CDCl₃) δ 156.3 (C), 156.1 (C), 140.7 (C), 129.4 (CH), 120.9 (CH), 116.0 (CH), 113.4 (CH), 79.6 (C), 52.8, 40.3 (CH₂), 28.4, 28.2, 27.5, 18.5; minor rotomer: (diagnostic peaks) 140.2 (C), 115.8 (C), 50.6, 27.9, 17.6; rotomer ratio: 1.3:1.0 (based on ¹H NMR).

(S)-Methyl 6-(3-(2-(*tert*-Butoxycarbonyl(methyl)amino)propyl)phenoxy)hexanoate (**9**)

A solution of 1.04 g (0.004 mol) of phenol **8** in 5 mL of DMF was carefully added to a suspension of 0.267 g of 60% NaH (washed with hexanes in 5 mL of DMF) followed by the addition of 0.920 g (0.004 mol) of methyl 6 bromohexanoate in 2 mL of DMF. After 16 h stirring, the reaction mixture was quenched with water and extracted with CH₂Cl₂ (3 × 75 mL). The combined organic layers were washed with H₂O (2 × 75 mL), dried (MgSO₄), and concentrated to yield an oil. The oil was purified by flash chromatography using hexane-Et₂O (4:1) as the eluent to yield 0.798 g (66%) of **9** as an oil. $[\alpha]_D^{24} +32.5$ (c 2.340, MeOH). ¹H NMR (300 MHz, CDCl₃) δ: major rotomer: 7.14 (t, *J* = 7.9 Hz, 1H), 6.51–6.86 (m, 3H), 4.30 (bs, 1H), 3.91 (t, *J* = 6.4 Hz, 2H), 3.66 (s, 3H), 2.47–2.86 (m, 5H), 2.34 (t, *J* = 7.5 Hz, 2H), 1.56–1.89 (m, 4H), 1.44–1.56 (m, 2H), 1.19–1.41 (m, 9H), 1.11 (bs, 3H); minor rotomer: (diagnostic peaks) 4.40 (bs, 1H). ¹³C NMR (300 MHz, CDCl₃) δ 174.0 (C), 159.1 (C), 155.5 (C), 140.6 (C), 129.2 (CH), 121.3 (CH), 115.3 (CH), 112.1 (CH), 79.1 (C), 67.5 (CH₂), 52.6, 51.4, 40.6 (CH₂), 34.0 (CH₂), 29.0 (CH₂), 28.3, 27.7, 25.7 (CH₂), 24.7 (CH₂), 18.3, 17.6. MS (EI) *m/e* 416.5 (MNa)⁺, 394.6 (MH)⁺, 338.6 (MH OtBu)⁺, 294.2 (MH-C₅H₁₀O₂)⁺; rotomer ratio: 1.4:1.0 (major to minor, based on ¹H NMR). Anal. (C₂₂H₃₅NO₅): C, H, N.

(S)-6-(3-(2-(*tert*-Butoxycarbonyl(methyl)amino)propyl)phenoxy)hexanoic Acid (**10**)

A mixture of **9** (0.657 g, 0.0017 mol), LiOH•H₂O (0.258 g, 0.006 mol) in THF-MeOH H₂O (1:1:1, 10 mL each) was refluxed for 8 h. The reaction mixture was concentrated and the resulting residue dissolved in CH₂Cl₂ and 3.0 N HCl (1:1, 75 mL). The organic layer was separated, and the aqueous layer was extracted with CH₂Cl₂ (3 × 25 mL). The combined organic layers were washed with H₂O (2 × 50 mL), dried (MgSO₄), and concentrated to

yield an oil. The oil was used in the next step without any further purification. $[\alpha]^{24}_D +33.57$ (c 0.855, MeOH). ^1H NMR (300 MHz, CDCl_3) δ : major rotomer: 7.16 (t, $J = 7.9$ Hz, 1H), 6.61–6.83 (m, 3H), 4.33 (bs, 1H), 3.94 (t, $J = 6.3$ Hz, 2H), 2.54–2.83 (m, 5H), 2.39 (t, $J = 7.3$ Hz, 2H), 1.66–1.85 (m, 4H), 1.46–1.60 (m, 2H), 1.25–1.44 (m, 10H), 1.14 (bs, 3H); minor rotomer: (diagnostic peaks) 4.53 (bs, 1H). ^{13}C NMR (300 MHz, CDCl_3) δ 178.4 (C), 159.0 (C), 155.6 (C), 140.6 (C), 129.2 (CH), 121.4 (CH), 115.4 (CH), 112.1 (CH), 79.3 (C), 67.5 (CH_2), 52.7, 51.2, 40.6 (CH_2), 33.8 (CH_2), 31.6, 28.9 (CH_2), 28.3, 27.8, 25.6 (CH_2), 24.4 (CH_2), 22.6, 20.6, 18.4, 17.6, 14.1. MS (EI) m/e 402.2 (MNa^+), 380.5 (M^+), 378.4 (M-H^+), 280.3 (MH-DeBoc^+); rotomer ratio: 1.3:1.0 (based on ^1H NMR). Anal. ($\text{C}_{21}\text{H}_{33}\text{NO}_5 \cdot 0.75 \text{H}_2\text{O}$): C, H, N.

(S)-6-(3-(2-Methylamino)propyl)phenoxy)-N-(2-(tritylthio)ethyl)hexanamide (11)

To a solution of **10** (1.0 g, 0.0026 mol) and 2-(tritylmercapto)ethylamine (1.07 g, 0.003 mol) in 15 mL DMF at 0 °C was added HOAt in DMF (0.5 M, 0.0065 mol), EDCI·HCl (1.82 g, 0.0095 mol), and NEt_3 (1.33 mL) under a N_2 atmosphere. The reaction mixture was slowly brought to room temperature and stirred for 24 h. The residue obtained on concentration was quenched with saturated NaHCO_3 and extracted with CH_2Cl_2 (3×150 mL). The combined organics were washed with brine, dried (MgSO_4), and concentrated to an oil which was purified by flash chromatography using EtOAc-hexane (1:1) to give 1.52 g (85%) of an oil. $[\alpha]^{21}_D +13.9$ (c 1.00, MeOH). ^1H NMR (300 MHz, CDCl_3) δ : major rotomer: 7.41 (ad, $J = 7.41$ Hz, 6H), 7.28 (dt, $J = 6.8, 1.0$ Hz, 6H), 7.20 (dt, $J = 7.3, 1.0$ Hz, 3H), 7.14 (d, $J = 7.9$ Hz, 1H), 6.63–6.79 (m, 3H), 5.60 (bs, 1H), 4.32 (bs, 1H), 3.92 (t, $J = 6.4$ Hz, 2H), 3.09 (q, $J = 6.1$ Hz, 2H), 2.55–2.79 (m, 5H), 2.33–2.48 (m, 2H), 2.12 (t, $J = 7.6$ Hz, 2H), 1.70–1.84 (m, 2H), 1.60–1.70 (m, 2H), 1.43–1.58 (m, 2H), 1.30–1.58 (m, 9H), 1.13 (bs, 3H); minor rotomer: (diagnostic peaks) 4.52 (bs, 1H). ^{13}C NMR (300 MHz, CDCl_3) δ major rotomer: 172.7 (C), 159.0 (C), 144.7 (C), 137.8 (C), 129.5 (CH), 129.2 (CH), 128.0 (CH), 126.8 (CH), 121.3 (CH), 115.3 (CH), 112.2 (CH), 79.3 (C), 67.5 (CH_2), 52.5, 40.6 (CH_2), 38.1 (CH_2), 36.6 (CH_2), 32.1, 29.0 (CH_2), 28.4 (CH_2), 25.8 (CH_2), 25.4 (CH_2), 14.2; minor rotomer: (diagnostic peaks) 155.5 (C), 140.6 (C), 79.1 (C), 66.8, 60.4, 33.7, 28.8, 25.5, 24.5, 21.0. rotomer ratio: 1.4:1.0 (based on ^1H NMR).

The above oil was dissolved in CH_2Cl_2 (20 mL) at room temperature, and trifluoroacetic acid (5.0 mL) was added drop wise over a period of ~5 min and stirred for an additional 10 min. The oily residue obtained on concentration was co-evaporated with MeOH (3×10 mL) followed by purification using flash chromatography with CH_2Cl_2 -CMA (1:1) as eluting to yield 1.06 g (81%) of **11** as an oil. $[\alpha]^{22}_D +0.561$ (c 0.535, MeOH). ^1H NMR (300 MHz, CDCl_3) δ 9.31 (bs, 2H), 7.41 (ad, $J = 7.3$ Hz, 6H), 7.28 (at, $J = 7.3$, 6H), 7.14–7.24 (m, 4H), 6.67–6.80 (m, 3H), 5.60 (t, $J = 5.3$ Hz, 1H), 3.91 (t, $J = 6.3$ Hz, 2H), 3.31 (bs, 1H), 3.20 (dd, $J = 13.2, 4.4$ Hz, 1H), 3.08 (q, $J = 6.1$ Hz, 2H), 2.58–2.75 (m, 5H), 2.41 (t, $J = 6.3$ Hz, 2H), 1.71–1.85 (m, 2H), 1.58–1.71 (m, 2H), 1.42–1.54 (m, 2H), 1.26 (d, $J = 6.4$ Hz, 3H). ^{13}C NMR (300 MHz, CDCl_3) δ 159.5 (C), 144.6 (C), 137.8 (C), 129.9 (CH), 129.5 (CH), 128.0 (CH), 126.8 (CH), 121.3 (CH), 115.5 (CH), 113.3 (CH), 67.6 (CH_2), 56.7, 50.8, 39.5 (CH_2), 38.1 (CH_2), 36.5 (CH_2), 32.0 (CH_2), 30.2, 28.9 (CH_2), 25.7 (CH_2), 25.3 (CH_2), 15.5; MS (EI) m/e 581.9 (MH^+), 307.7 ($\text{M-C}_{21}\text{H}_{20}\text{S}^+$).

Conjugation of Maleimide activated OVA and Maleimide activated BSA to (+)-METH HSMO9 Hapten

Representative conditions for synthesis of MCV using maleimide activated OVA or maleimide activated BSA are as follows. Two mg of maleimide-activated OVA or maleimide activated BSA (ThermoFisher Scientific) were reconstituted in 1.0 mL of distilled H_2O for a 2 mg/mL solution, which contains 44.4 μmol or 27.8 μmol of protein, respectively. At the same time, (+)-METH HSMO9 hapten (**3**) stock solutions (11.38 mM or

20.8 mM, respectively) were prepared by dissolving (+)-METH HSMO9 hapten (22 mg or 42.0 μ mol for OVA; 7 mg or 11.64 μ mol for BSA) in de-gassed reaction buffer (2.0 mL, supplied by the vendor). A portion of the maleimide activated protein stock solution (0.5 mL) was kept aside for later analysis as a control representing the starting conditions prior to conjugation reactions. The remaining activated protein solution (1.5 mL) was transferred into a sterile cryogenic vial and added to the (+)-METH HSMO9 hapten stock solution. The reaction mixture was purged with N₂ and incubated for 2 h under N₂ at ambient temperature with gentle stirring. After the synthesis was complete at 2 h, aliquots of the OVA-MCV and BSA-MCV reaction mixture were sealed in vials and shipped under ice-cold conditions to laboratories at the University of Arkansas at Fayetteville (Fayetteville, AR) for MALDI-TOF determination of the (+)-METH HSMO9 epitope density, and to the University of Arkansas for Medical Sciences (Little Rock, AR) for immunological studies.

Mass Spectrometry Analysis

Mass spectra were obtained of (+)-METH, (+)-METH HSMO9, and (+)-METH HSMO9 using a Bruker Reflex III MALDI-TOF mass spectrometer 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.

While developing the methods for MALDI-TOF analysis of the MCV, we realized the protein solution salt content from buffers adversely affected the method. To optimize analytical conditions, we compared several methods for removal of interfering salts using the OVA-MCV. For the experiment, we compared results from the MALDI-TOF analysis of proteins without desalting (control), after dialysis against distilled H₂O (model HTD96b; HT Dialysis LLC, Gales Ferry, CT), and after desalting on a ZipTipC₁₈ (Millipore Corporation, Bedford, MA).

Immune Response to (+)-METH HSMO9 BSA MCV in Mice

Female BALB/C mice were housed in an animal care facility with a 12 hour light/dark cycle (6:00 AM–6:00 PM) and an average 22 °C ambient temperature. They received food and water *ad libitum*. Animal protocols were in accordance with the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by the National Institutes of Health. They were approved by the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences (Little Rock, AR) prior to beginning the experiments.

For studies of active immunization, female BALB/c mice (10 per group; Charles River Laboratories, Wilmington, MA) were subcutaneously immunized with either 20 μ g of (+)-METH HSMO9 BSA (with a (+)-METH HSMO9 hapten epitope density of 12) or 20 μ g of (+)-METH MO10 BSA (with a (+)-METH MO10 hapten epitope density of 5). The antigens were emulsified in Freund's Complete Adjuvant (EMD Chemicals, Gibbstown, NJ) near each front and hindquarter per the adjuvant manufacturer's instructions. This initial immunization was followed by 20 μ g booster immunizations at three to six week intervals with Freund's Complete Adjuvant. Immune serum was collected 10–14 days after each boost and serum antibody titers were determined by examining binding to [³H]-(+)-METH using radioimmunoassay.¹⁵

Production of a Monoclonal Antibody against (+)-METH HSMO9 BSA in Mice

For production of monoclonal antibodies, female BALB/c mice (five per group) were immunized by the subcutaneous and intraperitoneal routes with 20 μ g of (+)-METH HSMO9 BSA. The (+)-METH HSMO9 hapten epitope density was 10 (see Figure 3). This

MCV was emulsified in Sigma Adjuvant System (Sigma Chemical Co., St Louis, MO) according to the manufacturer's instructions. This initial immunization was followed by 20 µg booster immunizations at three weeks followed by two booster injections at two six week intervals. Immune serum was collected 10–14 days after each boost and serum antibody titers were determined by examining binding to [³H]-(+)-METH using radioimmunoassay.¹⁵ The generation, production and characterization of the monoclonal antibodies was as described by Peterson et al.¹³

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. SAMHSA. (Substance Abuse and Mental Health Services Administration) Results from the 2009 National Survey on Drug Use and Health. Department of Health and Human Services; Washington, DC: 2010.
2. Albertson TE, Derlet RW, Van Hoozen BE. Methamphetamine and the expanding complications of amphetamines. *West. J. Med.* 1999; 170:214–219. [PubMed: 10344175]
3. Barr AM, Panenka WJ, MacEwan GW, Thornton AE, Lang DJ, Honer WG, Lecomte T. The need for speed: an update on methamphetamine addiction. *J. Psychiatry Neurosci.* 2006; 31:301–313. [PubMed: 16951733]
4. Murray JB. Psychophysiological aspects of amphetamine methamphetamine abuse. *J. Psychol.* 1998; 132:227–237. [PubMed: 9529666]
5. Scott JC, Woods SP, Matt GE, Meyer RA, Heaton RK, Atkinson JH, Grant I. Neurocognitive effects of methamphetamine: a critical review and meta analysis. *Neuropsychol. Rev.* 2007; 17:275–297. [PubMed: 17694436]
6. Darke S, Kaye S, McKetin R, Duflou J. Major physical and psychological harms of methamphetamine use. *Drug Alcohol Rev.* 2008; 27:253–262. [PubMed: 18368606]
7. Perez JA Jr, Arsura EL, Strategos S. Methamphetamine-related stroke: four cases. *J. Emerg. Med.* 1999; 17:469–471. [PubMed: 10338241]
8. Gentry WB, Ruedi-Bettschen D, Owens SM. Development of active and passive human vaccines to treat methamphetamine addiction. *Hum. Vaccin.* 2009; 5:206–213. [PubMed: 19276653]
9. Gentry WB, Ruedi-Bettschen D, Owens SM. Anti-(+)-methamphetamine monoclonal antibody antagonists designed to prevent the progression of human diseases of addiction. *Clin. Pharmacol. Ther.* 2010; 88:390–393. [PubMed: 20668443]
10. Laurenzana EM, Hendrickson HP, Carpenter D, Peterson EC, Gentry WB, West M, Che Y, Carroll FI, Owens SM. Functional and biological determinants affecting the duration of action and efficacy of anti-(+)-methamphetamine monoclonal antibodies in rats. *Vaccine.* 2009; 27:7011–7020. [PubMed: 19800446]
11. Byrnes-Blake KA, Carroll FI, Abraham P, Owens SM. Generation of anti-(+)methamphetamine antibodies is not impeded by (+)methamphetamine administration during active immunization of rats. *Int. Immunopharmacol.* 2001; 1:329–338. [PubMed: 11360933]
12. Carroll FI, Abraham P, Gong PK, Pidaparathi RR, Blough BE, Che Y, Hampton A, Gunnell M, Lay JO Jr, Peterson EC, Owens SM. The synthesis of haptens and their use for the development of monoclonal antibodies for treating methamphetamine abuse. *J. Med. Chem.* 2009; 52:7301–7309. [PubMed: 19877685]

13. Peterson EC, Gunnell M, Che Y, Goforth RL, Carroll FI, Henry R, Liu H, Owens SM. Using hapten design to discover therapeutic monoclonal antibodies for treating methamphetamine abuse. *J. Pharmacol. Exp. Ther.* 2007; 322:30–39. [PubMed: 17452421]
14. Owens SM, Zorbas M, Lattin DL, Gunnell M, Polk M. Antibodies against arylcyclohexylamines and their similarities in binding specificity with the phencyclidine receptor. *J. Pharmacol. Exp. Ther.* 1988; 246:472–478. [PubMed: 2457075]
15. Davis MT, Preston JF. A simple modified carbodiimide method for conjugation of small-molecular-weight compounds to immunoglobulin G with minimal protein crosslinking. *Anal. Biochem.* 1981; 116:402–407. [PubMed: 7316174]
16. Karas M, Hillenkamp F. Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. *Anal. Chem.* 1988; 60:2299–2301. [PubMed: 3239801]
17. Ragupathi G, Koide F, Livingston PO, Cho YS, Endo A, Wan Q, Spassova MK, Keding SJ, Allen J, Ouerfelli O, Wilson RM, Danishefsky SJ. Preparation and evaluation of unimolecular pentavalent and hexavalent antigenic constructs targeting prostate and breast cancer: a synthetic route to anticancer vaccine candidates. *J. Am. Chem. Soc.* 2006; 128:2715–2725. [PubMed: 16492059]
18. Kinsey BM, Jackson DC, Orson FM. Anti-drug vaccines to treat substance abuse. *Immunol. Cell. Biol.* 2009; 87:309–314. [PubMed: 19333250]
19. Hatsukami DK, Rennard S, Jorenby D, Fiore M, Koopmeiners J, de Vos A, Horwith G, Pentel PR. Safety and immunogenicity of a nicotine conjugate vaccine in current smokers. *Clin. Pharmacol. Ther.* 2005; 78:456–467. [PubMed: 16321612]
20. Martell BA, Orson FM, Poling J, Mitchell E, Rossen RD, Gardner T, Kosten TR. Cocaine vaccine for the treatment of cocaine dependence in methadone-maintained patients: a randomized, double-blind, placebo-controlled efficacy trial. *Arch. Gen. Psychiatry.* 2009; 66:1116–1123. [PubMed: 19805702]
21. Ishikawa E, Imagawa M, Hashida S, Yoshitake S, Hamaguchi Y, Ueno T. Enzyme-labeling of antibodies and their fragments for enzyme immunoassay and immunohistochemical staining. *J. Immunoassay.* 1983; 4:209–327. [PubMed: 6413534]
22. Moreno AY, Mayorov AV, Janda KD. Impact of Distinct Chemical Structures for the Development of a Methamphetamine Vaccine. *J. Am. Chem. Soc.* 2011; 133:6587–6595. [PubMed: 21473576]

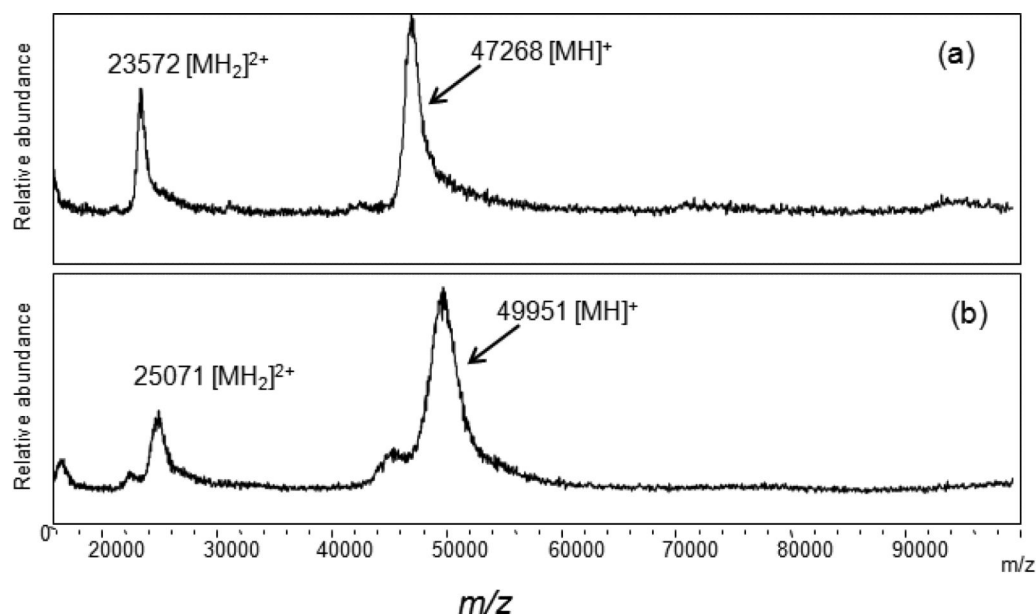


Figure 1.

The MALDI-TOF mass spectra of maleimide activated OVA protein (a) before and (b) after reaction with (+)-METH HSMO9 hapten (3). Before conducting the MALDI-TOF analysis, the salts from protein solutions were removed by equilibrium dialysis with multiple changes of distilled water. The left axis of 1a and 1b is signal intensity in arbitrary units.

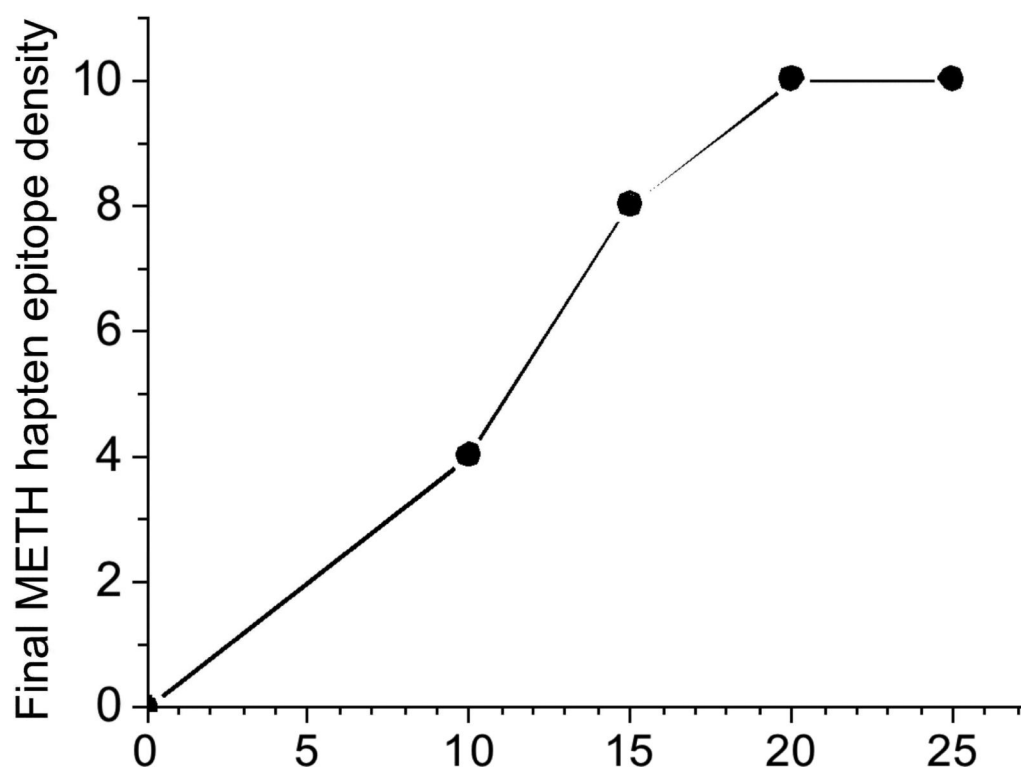
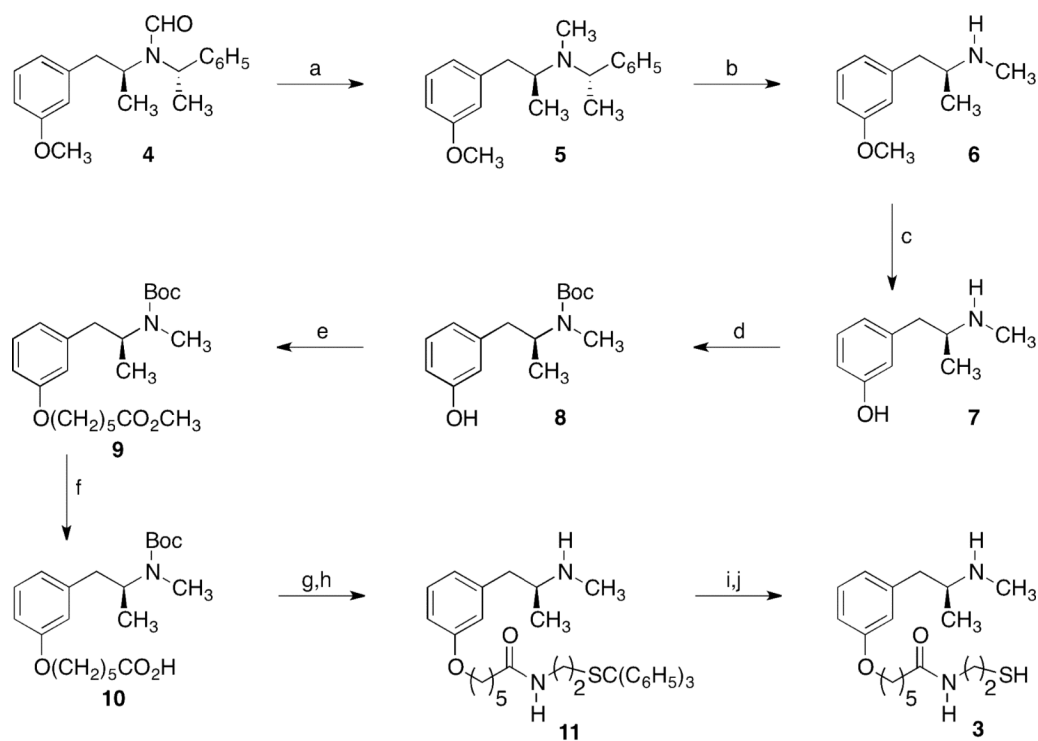
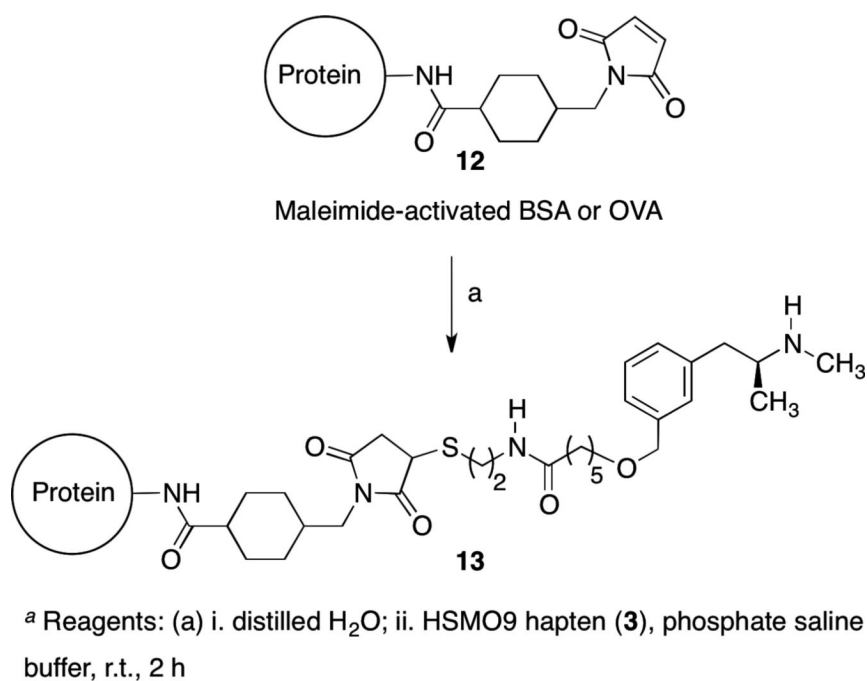


Figure 2. Relationship between the molar ratio of METH hapten to maleimide activated BSA at the start of the synthesis versus the final METH hapten epitope density at the end of reaction. Epitope density of BSA was determined by MALDI-TOF MS.



^a Reagents: (a) THF•BF₃, THF, 0°; (b) HCO₂NH₄, 5% Pd/C, CH₃OH, 50 °C; (c) BBr₃, CH₂Cl₂, -78 °C; (d) (Boc)₂O, CH₂Cl₂, Et₃N; (e) Br(CH₂)₅CO₂CH₃, NaH, DMF; (f) LiOH, H₂O, CH₃OH, THF; (g) (C₆H₅)₃CS(CH₂)₂NH₂, EDCI, HOAt, DMF; (h) TFA, CH₂Cl₂; (i) Hg(OAc)₂, C₂H₅OH; (j) H₂S, C₂H₅OH

Scheme 1.



Scheme 2.