Antibody-catalyzed anaerobic destruction of methamphetamine

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Methamphetamine [(+)-2] abuse has emerged as a fast-rising global epidemic, with immunopharmacotherapeutic approaches being sought for its treatment. Herein, we report the generation and characterization of a monoclonal antibody, YX1-40H10, that catalyzes the photooxidation of (+)-2 into the nonpsychoactive compound benzaldehyde (14) under anaerobic conditions in the presence of riboflavin (6). Studies have revealed that the antibody facilitates the conversion of (+)-2 into 14 by binding the triplet photoexcited state of 6 in proximity to (+)-2. The antibody binds riboflavin (Kd = 180 μM), although this was not programmed into hapten design, and the YX1-40H10-catalyzed reaction is inhibited by molecular oxygen via the presumed quenching of the photoexcited triplet state of 6. Given that this reaction is another highlight in the processing of reactive intermediates by antibodies, we speculate that this process may have future significance in vivo with programmed immunoglobulins that use flavins as cofactors to destroy selectable molecular targets under hypoxic or even anoxic conditions.

A buse of drugs such as amphetamine (1) and its congeners, methamphetamine (2), ephedrine (3), methylenedioxymethamphetamine (4), and methylenedioxymethamphetamine (5) (Fig. 1), is a serious global problem (1–3). Immunopharmacotherapy that utilizes antibody-based therapeutics is being studied as an innovative approach to controlling the abuse of these psychoactive agents (4, 5). In this regard, antibody catalysts that can convert drugs of abuse into inactive forms are viewed potentially as more efficient immunotherapeutic agents than immunoglobulins that simply bind and either sequester or eliminate the drug. Our group and others have developed antibodies with cocaine esterase activity (6–8). More recently, we have disclosed catalytic antibodies that, in conjunction with the naturally occurring photosensitizer riboflavin, can oxidatively degrade nicotine into putatively nonpsychoactive substances (9). Here we report a viable, cofactor-based catalytic antibody system for the destruction of psychoactive substances. To aid in developing efficient systems for decomposing drugs of abuse, we have generated a murine monoclonal antibody (mAb), YX1-40H10, which, in the presence of white light and riboflavin (6), is able to photodegrade the psychoactive enantiomer of methamphetamine [(+)-2] into a nonpsychoactive product via a type I photooxidation process.

Results and Discussion

Hapten 7 was designed to elicit antibodies that recognize the panel of amphetamine (1) and amphetamine-like psychoactive agents 2–5, and it contains a number of key structural features (Fig. 1). First, it possesses the substituted β-phenethylamine pharmacophore, which is common to this family of drugs. Second, hapten 7 was prepared without imposition of stereocontrol at C-3 of the tetrahydroisoquinoline nucleus. Agents 2–5 all have a chiral center at the analogous carbon in their structure; however, they are not all abused in enantiomerically pure form. The use of rac-7 is not simply a product of synthetic expediency, rather we have shown previously that immunization with racemic hapten such as rac-7 elicits panels of mAbs that contain members with exquisite enantioselectivity against one of the two enantiomers of their corresponding substrate (10). Finally, the hapten 7 contains a constrained tetrahydroisoquinoline moiety, whereas the substrates 1–5 all have a conformationally free substituted aminoethyl aromatic side chain. This apparent departure from structural simile between hapten and substrate, wherein the conformationally free component of a target molecule is mimicked by locking the isostructural locus within the hapten into a constrained conformation, has previously been shown by us to increase the immune response against the substrate (11).

Hapten 7 was synthesized in seven steps from 3,4-(dimethylenedioxy)phenylacetic acid (8) (Scheme 1). In brief, acid 8 was converted into methyl ketone 10 by addition of a

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The authors declare no conflict of interest.

Abbreviation: DNPH, dinitrophenylhydrazine.

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methyl Gringard reagent to Weinreb amide 9 in an overall yield of 60% (for two steps). Subsequent reductive amination of 10 afforded amine 11 in low yield (32%). A Pictet–Spengler reaction between 11 and formaldehyde installed the tetrahydroisoquinoline core of 12 in 64% yield. The spacer was then extended by coupling of an additional β-alanine unit to give amide 13, which was then converted into hapten 7 by hydrogenolysis (78% yield for two steps).

Hapten 7 was conjugated to carrier proteins [BSA and keyhole limpet hemocyanin (KLH)], and the KLH-7 conjugate was used to immunize mice, using standard protocols. After hybridoma production, 12 murine mAbs were generated, and ELISA studies with the BSA-7 conjugate revealed that each antibody bound hapten 7. These mAbs were then further examined for their ability to recognize substrates 2–5. Competition ELISA between BSA-7 and the panel of substrates (2–5) revealed that a single mAb, YX1-40H10, could bind to (+)-2 with an apparent binding constant, \( K_m \), of 75 \( \mu M \).

In a preliminary screen for catalysis, the 12 mAbs with affinity for BSA-7 (see above) were tested for their ability to degrade the amphetamines 1–5 in the presence of riboflavin (6) and visible light in aqueous buffer. Thus, 6 (60 \( \mu M \)), the mAb (20 \( \mu M \)), and 1–5 (1 mM) in PBS (pH 7.4) were incubated in the presence of white light (3.4 mW cm\(^{-2} \)) under ambient aerobic conditions at 4°C. Activity was initially assessed by simply monitoring the loss of 1–5 by HPLC. From this screen, only mAb YX1-40H10, which binds (+)-2, was found to accelerate the photochemical degradation of (+)-2. No other antibody-mediated photodegradation of 1–5 by the 12 mAbs was detected.

### YX1-40H10-Mediated Photodegradation of (+)-2

**Scheme 1.** Synthesis of hapten 7: Reagents and conditions. a, HNMe(OMe), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and triethylamine, 74% yield; b, MeMgCl and THF, 0°C, 80% yield; c, H-β-Ala-OBn (Bn, benzyl), NaBH(OAc)\(_2\), and AcOH, 32% yield; d, \( \text{CH}_2\text{O}, \text{HCO}_2\text{H}, \text{reflux}, 64% \) yield; e(1), \( \text{H}_2\), \( \text{Pd(OH)}_2\), and \( \text{MeOH} \), e(2), \( \text{H}_-\), \( \text{H}_2\)-Ala-OBn, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyuronium hexafluorophosphate (HBTU), and triethylamine, 78% yield in two steps; f, \( \text{H}_2 \) and \( \text{Pd(OH)}_2 \), 48% yield.

**Fig. 2.** RP-HPLC analysis of the photooxidation of (+)-2 by riboflavin (60 \( \mu M \)). The analytical HPLC was performed on a Hitachi L-7400 instrument using a Grace Vydac reversed-phase C18 column. The column was eluted with an isocratic solvent system, 20% MeCN (0.1% TFA). All of the HPLC runs were monitored at 254 nm. See Results for a description of runs A-D.

**Results**

was formed as a major product in this reaction [see supporting information (SI) Materials and Methods]. This conclusion was further strengthened when a cojunction of the YX1-40H10-catalyzed reaction with 14 (20 \( \mu M \)) revealed that the unknown peak 2 has the same retention time as authentic benzaldehyde \( (R_T \approx 13.0 \) min; Fig. 2C). Further support for 14 as being a major photoproduct in the YX1-40H10-catalyzed process came from chemical derivitization of the assay mixture with dinitrophenylhydrazine (DNPH). When DNPH was added to the diethyl ether extract of the YX1-40H10-catalyzed reaction, a yellow precipitate formed. Analysis of this precipitate by \(^1\)H NMR also clearly established its identity as the DNPH hydrazone of benzaldehyde (see SI Materials and Methods).

Given that peak 1 formation occurs independently of YX1-40H10 and that this same peak is observed upon photoradiation of riboflavin (6) alone in PBS (pH 7.4) (data not shown), it was surmised that the unknown peak was originating as a result of the known photochemical decomposition of riboflavin. This supposition was confirmed by coinjection of the YX1-40H10-catalyzed photodecomposition assay with an authentic sample of lumichrome (15), a major photodecomposition product of riboflavin (12, 13). This coinjection with 15 resulted in an increase in the area of peak 1, confirming its constitution (Fig. 2D).

**Characterization of YX1-40H10-Catalyzed Photodegradation of (+)-2**

Given that benzaldehyde (14) is a known product of the YX1-40H10-mediated process, kinetic studies were undertaken to measure the catalytic parameters, \( K_m \) (Michaelis–Menten constant) and \( k_{cat} \) (catalytic rate constant), for production of 14. Conditions of saturating riboflavin (60 \( \mu M \)) were determined, and the initial rates of benzaldehyde formation were obtained at varying (+)-2 concentrations in the presence of YX1-40H10 (10 \( \mu M \)). Under such conditions, saturation kinetics with respect to (+)-2 are observed (Fig. 3A).

**Materials and Methods**

Due to the limitations of the image in the document, the full text content cannot be accurately transcribed and translated. However, the text content includes discussions on the synthesis and characterization of hapten 7, the screening process for antibody-mediated photodegradation, and the analysis of photodegradation products using HPLC. The text also references a supporting information section that provides additional experimental details and methods.
The high concentration of YX1-40H10, relative to substrate concentration, used in these kinetic studies invalidates the usual Michaelis–Menten approximation that free and total substrate should be essentially equal. Therefore, the reaction velocity (v) for the YX1-40H10-catalyzed formation of 14 was derived from Eq. 1, wherein the free concentration of (+)-2 is explicitly solved for. Fitting of the experimental kinetic data of initial rate v vs. (+)-2 (under conditions of saturating riboflavin) to Eq. 1 yields a $k_{\text{cat}}$ of $3.7 \times 10^{-3}$ min$^{-1}$ and a $K_{m,\text{fit}}$ of $27 \pm 3$ μM. The combination of these two parameters gives an apparent second-order specificity constant for YX1-40H10 ($k_{\text{cat}}/K_{m,\text{fit}}$) of $23.3 \pm 2$ M$^{-1}$s$^{-1}$.

$$v = \frac{k_{\text{cat}} S_{T} + A_{T} + K_{\text{m}}}{2} - \frac{\sqrt{(S_{T} + A_{T} + K_{\text{m}})^{2} - 4S_{T}A_{T}}}{2},$$

where $S_{T}$ is the total (+)-2 concentration and $A_{T}$ is the total YX1-40H10 binding site concentration (40 μM).

Initial rates of benzaldehyde (14) formation were also measured under conditions of saturating (+)-2 (300 μM) as a function of varying riboflavin 6 concentration (Fig. 3B). Saturation of the initial rate v with increasing 6 was again observed, and unexpectedly the $K_{m,\text{fit}}$ determined from fitting the experimental data to Eq. 1 was found to be 10 μM. This value is substoichiometric with respect to antibody concentration. We next examined the dependence of riboflavin saturation on YX1-40H10 kinetic studies at several concentrations of antibody (10, 20, and 40 μM) (Fig. 2B). The experimental initial rate data were then fit to Eq. 2, wherein the critical parameter $K_{m}^{\text{opt}}$ represents the ratio of riboflavin to antibody concentration at 0.5 $V_{\text{max}}$. This system then reveals a best-fit solution of $k_{\text{cat}} = 5.4 \pm 0.2 \times 10^{-2}$ min$^{-1}$ and a $K_{m}^{\text{opt}}$ of 0.26 ± 0.03.

$$v = \frac{k_{\text{cat}}A_{T}[R]}{[R] + A_{T}K_{m}^{\text{opt}}},$$

where [R] is the riboflavin concentration; $A_{T}$ is the antibody concentration; and $K_{m}^{\text{opt}}$ is the ratio of [6] to [40H10] that produces one-half the velocity at saturation.

The YX1-40H10 $k_{\text{cat}}$ value of $5.4 \times 10^{-2}$ min$^{-1}$ calculated under conditions of saturating (+)-2 and varying photosensitizer 6 is almost identical to the $k_{\text{cat}}$ determined under conditions of riboflavin saturation with varying concentrations of (+)-2 ($3.7 \times 10^{-2}$ min$^{-1}$). Importantly, the equivalency in the $k_{\text{cat}}$ values in both cases offers strong support for the formation of a kinetically viable ternary complex between YX1-40H10 and (+)-2 and 6.

After administration of a typical dose of 2 (20 mg), the plasma level of 2 has been shown to be $\approx 0.5$ mM, with a clearance half-life of $\approx 12$ h ($k_{\text{cl}} = 0.0096$ min$^{-1}$). Such a systemic concentration is below the $K_{m}$ of YX1-40H10, and therefore the antibody would be functioning in vivo under substrate-limiting conditions. Under such a scenario, the rate, v, of destruction of 2 by YX1-40H10 at a plasma concentration ($E_{\text{total}}$) of 0.5 mM is given by Eq. 3 and is $\approx 0.00070$ min$^{-1}$.

$$v = \frac{k_{\text{cat}} E_{\text{total}} \times S}{K_{m} + S}.$$
presence of various oxygen concentrations (Fig. 4). The initial rate data reveal that the YX1-40H10-catalyzed process is quenched by oxygen and that the oxygen atom in 14 does not originate from molecular oxygen, because benzaldehyde formation occurs facilely under anaerobic conditions. The oxygen quenching of the YX1-40H10-mediated process is typical for a process that involves triplet excited riboflavin (36) (14) and suggests that this overall process is a type I photooxidation process with the origin of the oxygen atom in 14 being a water molecule.

Given that 36 was seen as a critical intermediate in this photooxidation process, hematoporphyrin-IX and methylene blue were studied as alternative triplet dye sources for the YX1-40H10-mediated process (15). However, when these dyes were used as photosensitizers, there was no enhancement in the rate of benzaldehyde formation above that observed in buffer alone (data not shown). These findings suggest that recognition of riboflavin by YX1-40H10 may be a key aspect in this overall process.

No specific antibody recognition of riboflavin had been programmed during hapten design; however, it is known that a fraction of immunoglobulins bind flavins in vivo (16–18). Therefore, the dissociation constant, Kd, of 6 for YX1-40H10 was measured by equilibrium dialysis with [3H]riboflavin. These binding studies reveal that riboflavin is a weak ligand for YX1-40H10 (Kd = 180 µM). Interestingly, the Bmax value (14.9 µM for 13.4 µM antibody concentrations) reveals that the stoichiometry of riboflavin binding is one molecule per antibody molecule. Although the location of riboflavin binding on YX1-40H10 is unknown, given that this process is a photooxidation process, the substrate 2 and riboflavin (6) would have to be in relatively close proximity for efficient energy/electron transfer.

**Reaction Mechanism Studies.** GC-MS analyses of organic extracts of the riboflavin-mediated photodegradation reaction of (+)-2 in aqueous buffer under anaerobic conditions revealed unreacted (+)-2 with trace amounts of benzaldehyde and the previously undetected component phenylacetone (16) (Fig. 5). In the YX1-40H10-mediated process, 14 is the major component observed in the GC-MS analysis, but trace amounts of 16 are also observed (Fig. 5).

To investigate whether phenylacetone (16) observed in the GC-MS arises as an intermediate or byproduct of the photooxidation of (+)-2, phenylacetone (16, 1 mM) was photoirradiated (400–700 nm, 3.4 mW/cm²) with 6 (60 µM) in PBS, pH 7.43, at 4°C in the presence or absence of YX1-40H10 (20 µM) under anaerobic conditions. At times during the photoirradiation, aliquots of the reaction were removed and analyzed for benzaldehyde content by RP-HPLC. Benzaldehyde was indeed formed in the aqueous buffer background reaction (2.6 ± 0.5 nM-min⁻¹), suggesting that 16 could be an intermediate (rather than a byproduct) in this process. Interestingly, in the YX1-40H10-mediated process, the rate of benzaldehyde formation was significantly enhanced (17.4 ± 0.2 nM-min⁻¹) over the reaction in aqueous buffer (Fig. 6A). This ability to process 16 into 14 by YX1-40H10 constrains the mechanism for the process (Scheme 2).

Although the mechanism is still far from resolved, guided by the GC-MS data and the fact that the oxygen in 14 originates from water and not molecular oxygen (see above), it seems reasonable to propose a process that proceeds via a stepwise photooxidation of (+)-2 by 6, leading to the putative N-methyliminium species 17. Although there are no reports of this specific process, there is clear precedent for photooxidation of amines by riboflavin that proceeds via radical cations on nitrogen (19, 20). The iminium species 17 has two clear fates. (i) It may be trapped by water, with subsequent elimination of methylene that yields phenylacetone (16) and from there onto 14. (ii) Alternatively, the N-methyliminium 17 may isomerize into the benzyl cation 18, which can then be trapped by a water molecule, yielding ephedrine (3), and can then be further photooxidized to 14.

The potential involvement of 3 in the YX1-40H10-mediated process was investigated by anaerobic photooxidation (400–700 nm, 3.4 mW/cm²) of a solution of (+)-3 (1 mM) and 6 (60 µM) in PBS, pH 7.43, at 4°C in the presence or absence of YX1-40H10 (20 µM). At times during the photoirradiation, aliquots of the reaction were analyzed by RP-HPLC. These studies reveal that 3 is rapidly converted into 14 in aqueous buffer, suggesting that if this β-aminoalcohol is generated as an intermediate in the aqueous background reaction, it would not accumulate and probably would not be detected (Fig. 6A). In fact, we do not detect, by either GC-MS or RP-HPLC, the formation of 3 in either the YX1-40H10-catalyzed or buffer-only photooxidation of (+)-2. Importantly, there is no rate enhancement in the photooxidation of 3 above the aqueous buffer background in the presence of YX1-40H10, suggesting that the antibody cannot
utilize 3 as an intermediate (Fig. 6B) and strengthening the potential for the YX1-40H10-mediated pathway to proceed via phenylacetone (16).

Methamphetamine abuse continues to spread across the United States at an ever-increasing rate. This fact, coupled with the potent neurotoxicity of (H11001)-2, leads to a clear and urgent need for development of effective therapies to treat the addiction associated with (H11001)-2. Although catalytic antibody approaches have proven effective in the hydrolysis of the benzoyl ester of cocaine to generate nonpsychoactive metabolites, examination of the methamphetamine structure yields no such sites for catalytic-antibody development. Consequently, we have turned to a cofactor-based approach predicated on the intrinsic ability of all antibodies to oxidize their cognate antigen when presented with singlet dioxygen (1O2) (21–25). Analogous to our previous studies into antibody-catalyzed nicotine oxidation (9, 11), methamphetamine oxidation is indeed a facile process in the presence of specific antibodies and the innate photosensitizer riboflavin. However, this work has revealed that under appropriate conditions of light flux, efficient photochemical destruction of antigens can be antibody-catalyzed, even in the absence of oxygen, providing alternative chemical routes to those reported previously. In addition, the discovery of this photooxidation process has additional implications for immune defense, autoimmune disease, and the treatment of cancer in cases where one is dealing with sites of inflammation that are hypoxic and even anoxic. In total, this study hints that, under appropriate conditions of riboflavin, light, and antibody, antigen photooxidation, and hence destruction, is a real possibility.

**Materials and Methods**

Experimental procedures and spectroscopic data for the preparation of hapten 7 are available in SI Materials and Methods. Hyperimmunization of a KLH-7 conjugate was performed as described previously (26). All HPLC analyses were performed on a Hitachi (San Jose, CA) L-7400 instrument equipped with a Vydac reversed-phase C18 column and an isocratic mobile phase of 20% MeCN/80% H2O (0.1% TFA) at 1 ml/min with UV detection at 254 nm.

**Standard Method of Photooxidation of (+)-2.** A solution of methamphetamine (+)-2 (1 mM) and riboflavin (6) (60 μM) in PBS (pH 7.4) was irradiated on a white light transilluminator (400–700 nm, 3.4 mW cm−2 Fischer-Biotech transilluminator) at 4°C. Throughout the irradiation, aliquots (25 μl) were removed and analyzed by RP-HPLC for formation of benzaldehyde (14) as described above. Data were collected from at least duplicate samples and are reported as mean ± SEM.

**Oxygen Dependence of the YX1-40H10-Mediated Photooxidation of (+)-2.** Solutions of both (+)-2 (1 mM), YX1-40H10 (20 μM) in PBS (pH 7.4), and riboflavin (60 μM) in water were lyophilized (Labconco) in duplicate. The lyophilized riboflavin was then dissolved in rigorously degassed water (200 μl) under ambient air, oxygen, or argon, and the resultant solution was transferred into the lyophilized solid containing (+)-2 and YX1-40H10 under argon. This mixture was then photoirradiated on a white light transilluminator (400–700 nm, 3.4 mW cm−2) at 4°C. Aliquots were then subjected to RP-HPLC analysis to measure the formation of 14.

Scheme 2. Proposed mechanism for the YX1-40H10-catalyzed conversion of (+)-2 into benzaldehyde (14) via either phenylacetone (16) or ephedrine (3). RF, riboflavin; B, general base.
Measurement of the YX1-40H10–Riboflavin Dissociation Constant, \( K_d \).
All experiments were performed in a Spectrum equilibrium dialysis apparatus with samples (1 ml) introduced into both the ligand and receptor chambers. In all cases, the receptor chambers were filled with YX1-40H10 in PBS (2 mg/ml). The ligand chambers were filled with solutions of 6 (5–160 \( \mu M \)) containing a fixed concentration of tritiated 6 (0.04 nmol/ml) in PBS in duplicate. The receptor and ligand were equilibrated overnight in the dark. Samples (150 \( \mu l \)) from the receptor/ligand chamber were added into scintillation fluid (5 ml) and analyzed (Beckman scintillation counter). The membranes were also analyzed by the same method to allow a calculation of total ligand in counts per minute. Analysis was carried out by calculating free and bound ligand concentrations and fitting to a single binding saturation model, using Prism 4 software (GraphPad, San Diego, CA).

Inhibition of YX1-40H10 with Hapten 7.
In a typical experiment, YX1-40H10 (20 \( \mu M \)) was preincubated at room temperature for 5 min with 7 (10 \( \mu M \) or 40 \( \mu M \), final concentration) in PBS (pH 7.4). Then, (+)-2 (1 mM) and 6 (60 \( \mu M \)) were added to this mixture, and the resultant solution was irradiated on a white light transilluminator at 4°C. Aliquots were taken from the reaction mixture and analyzed by RP-HPLC, as detailed above. Reactions were run in duplicate and were reported as the mean ± SEM of benzaldehyde (14) formation.

Kinetic Analysis of the YX1-40H10-Mediated Photooxidation of (+)-2.
Initial rate of benzaldehyde (14) formation vs. (+)-2 concentration. All of the reaction mixtures contained antibody YX1-40H10 (20 \( \mu M \)), 6 (60 \( \mu M \)), and (+)-2 (at various concentrations ranging from 25 to 300 \( \mu M \)). The reaction was performed with photoirradiation at 4°C, and three time points were taken in the first 30 min of the reaction. It was confirmed that <5% of the (+)-2 was consumed during this period. The initial rate was obtained from the linear part (\( r^2 > 0.985 \)) of a plot of 14 formation vs. time. Data were corrected for background. Data analysis was performed using KaleidaGraph for the PC (Synergy Software, Reading, PA). This kinetic characterization was repeated at least duplicate, with interassay data showing a low variance (<15%).

Initial rate of benzaldehyde (14) formation vs. concentration of riboflavin (6).
Initial rates of formation of 14 were measured with assay mixtures consisting of (+)-2 (300 \( \mu M \)) and 6 (at various concentrations ranging from 5 to 60 \( \mu M \)). The initial rates, \( v \), of 14 formation were then plotted against the concentration of 6. Data were corrected for background, and data analysis was performed using KaleidaGraph for the PC.

Photooxidation of Ephedrine (3) or Phenylaceton (16) with Riboflavin Under Anaerobic Conditions.
A solution of racemic ephedrine (3) (1 mM; Sigma–Aldrich) or phenylaceton (16) plus or minus antibody YX1-40H10 (20 mM) and 6 (60 \( \mu M \)) in PBS (pH 7.43) was lyophilized. To the resulting mixture was added deoxygenated water under argon. The reaction solution was then photoreacted with white light at 4°C. Aliquots of the reaction were then taken and analyzed by RP-HPLC for benzaldehyde (14) formation over a period of 24 h. The same assay was then repeated in the presence of YX1-40H10 (20 \( \mu M \)). Photodegradation of phenylaceton (16) was performed under the same conditions, replacing 3 with 16.

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**SI Materials and Methods:**

**General Procedures** The $^1$H NMR and $^{13}$C NMR spectra were recorded on Bruker AMX-400 or Varian Inova-400 instrument. The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, b = broad. High-resolution mass spectra (HRMS) were recorded at The Scripps Research Institute on a VG ZAB-ZSE mass spectrometer using MALDI. All reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25 mm E. Merck silica gel plates (60F-254), with fractions being visualized by UV light. Column chromatography was carried out with Mallinckrodt SilicAR 60 silica gel (40-60 µM). Reagent grade solvents for chromatography were obtained from Fisher Scientific. Reagents were purchased at the highest commercial quality and used without further purification. All reactions were carried out under an argon atmosphere, unless otherwise noted. Reported yields were determined after purification for a homogenous material.

**2-(benzo[d][1,3]dioxol-5-yl)-N-methoxy-N-methylacetamide**

![Chemical Structure](image)

To a solution of 3,4-(methylenedioxy)phenyl acetic acid (1.0 g, 5.55 mmol, 1 eq) in THF was added N,O-dimethylhydroxylamine hydrochloride (544 mg, 5.55 mmol, 1 eq), EDCI (1.06 g, 5.55 mmol, 1 eq), HOBt (0.75 g, 5.55 mmol, 1 eq) and NEt$_3$ (1.55 ml, 11.1 mmol, 2 eq). The reaction was allowed to stir at room temperature for 2 h. A solution of saturated aqueous NaHCO$_3$ was then added and the reaction mixture extracted with EtOAc. Combined organic layers were washed with aqueous saturated NH$_4$Cl and brine then dried (MgSO$_4$) and concentrated *in vacuo*. The crude product mixture was purified by flash chromatography on silica gel (eluting with 50 % EtOAc/Hx) to give 2-(benzo[d][1,3]dioxol-5-yl)-N-methoxy-N-methylacetamide (0.911g, 4.08 mmol, 74 %) as a yellow oil.
\(^1\)H NMR (400, MHz, CDCl\(_3\)) \(\delta \) 6.73 (m, 3H), 5.93 (s, 2H), 3.65 (s, 2H), 3.63 (s, 3H), 3.19 (s, 3H)

\(^{13}\)C NMR (100, MHz, CDCl\(_3\)) \(\delta \) 174.3, 149.1, 147.9, 129.8, 123.5, 110.8, 109.1, 102.3, 61.9, 39.4, 32.5, 26.6

MS (ESI-TOF): [M+H], \(C_{11}H_{14}NO_4\) requires 224.0917, found 224.0931

1-(benzo[d][1,3]dioxol-5-yl)propan-2-one

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\begin{align*}
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\text{O} & \quad \text{O}
\end{align*}
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To a solution of 2-(benzo[d][1,3]dioxol-5-yl)-N-methoxy-N-methylacetamide (110 mg, 0.49 mmol, 1 eq) in THF (5 ml) at 0\(^\circ\)C was added a solution of MeMgCl (0.25ml of 3 M in THF, 0.74 mmol, 1.5 eq). The reaction was allowed to stir at 0\(^\circ\)C for 2 h. A solution of aq. Sat. NH\(_4\)Cl was added and the reaction mixture extracted with Et\(_2\)O. Combined organic layers were washed with brine, dried (MgSO\(_4\)) and concentrated \textit{in vacuo}. The crude product mixture was purified by flash chromatography on silica gel (20% EtOAc/Hx) to give 1-(benzo[d][1,3]dioxol-5-yl)propan-2-one (69.9 mg, 0.39 mmol, 80%) as a yellow oil.

\(^1\)H NMR (500, MHz, CDCl\(_3\)) \(\delta \) 6.74 (d, \(J = 8.0 \) Hz, 1H), 6.65 (d, \(J = 1.5 \) Hz, 1H), 6.62 (d, \(J = 8.0 \) Hz, 1H), 5.92 (s, 2H), 3.58 (s, 2H), 2.12 (s, 3H)

\(^{13}\)C NMR (125, MHz, CDCl\(_3\)) \(\delta \) 206.4, 147.9, 146.7, 127.7, 122.5, 109.8, 109.7, 101.1, 50.5, 29.1

MS (ESI-TOF): [M+H], \(C_{10}H_{10}O_3\) requires 179.0703, found 179.0699

benzyl 3-(1-(benzo[d][1,3]dioxol-5-yl)propan-2-ylamino)propanoate

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\begin{align*}
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{HN} \quad \text{O} \\
\text{O} \quad \text{HN} & \quad \text{OBn}
\end{align*}
\]
To a solution of 1-(benzo[d][1,3]dioxol-5-yl)propan-2-one (32.6 mg, 0.18 mmol, 1 eq) in CH$_2$Cl$_2$ (3.6 ml) was added benzyl 3-aminopropanoate (131 mg, 0.37 mmol, 2 eq), NaBH(OAc)$_3$ (116 mg, 0.54 mmol, 3 eq), AcOH (105 µl, 1.83 mmol, 10 eq) and the reaction allowed to stir at rt for 18 h.

$^1$H NMR (400, MHz, CDCl$_3$) $\delta$ 7.23 (m, 5H), 6.61 (d, $J$ = 8.0 Hz, 1H), 6.58 (d, $J$ = 1.6 Hz, 1H), 6.50 (dd, $J$ = 7.6, 1.6 Hz, 1H), 5.80 (s, 2H), 4.99 (s, 2H), 2.84 (m, 1H), 2.74 (m, 2H), 2.53 (dd, $J$ = 13.6, 6.8 Hz, 1H), 2.40 (m, 3H), 0.92 (d, $J$ = 6.4 Hz, 3H)

MS (MALDI-FTMS), [M+H]: C$_{20}$H$_{23}$NO$_4$ requires 342.1705, found 342.1703.

benzyl 3-(7-methyl-7,8-dihydro-[1,3]dioxolo[4,5-g]isoquinolin-6(5H)-yl)propanoate

Benzyl 3-(1-(benzo[d][1,3]dioxol-5-yl)propan-2-ylamino)propanoate (30 mg, 0.09 mmol, 1 eq) was dissolved in formic acid (1 ml) and formaldehyde (1 ml) and the mixture heated at reflux for 1.5 h. A solution of saturated aqueous NaHCO$_3$ was added until the mixture became basic. The mixture was then extracted with CH$_2$Cl$_2$ and the combined organic layers were concentrated in vacuo. The crude product mixture was purified by flash chromatography on silica gel (80 % EtOAc/Hx) to give benzyl 3-(7-methyl-7,8-dihydro-[1,3]dioxolo[4,5-g]isoquinolin-6(5H)-yl)propanoate (20 mg, 0.057 mmol, 64 %).

$^1$H NMR (400, MHz, CDCl$_3$) $\delta$ 7.23 (m, 5H), 6.42 (s, 1H), 6.36 (s, 1H), 5.77 (s, 2H), 5.03 (s, 2H), 3.57 (d, $J$ = 15.6 Hz, 1H), 3.52 (d, $J$ = 15.6 Hz, 1H), 2.82 (m, 4H), 2.50 (t, $J$ = 7.2 Hz, 2H), 2.35 (dd, $J$ = 16.4, 6.4 Hz, 1H), 0.97 (d, $J$ = 6.4 Hz, 3H)

$^{13}$C NMR (100, MHz, CDCl$_3$) $\delta$ 172.4, 146.0, 145.6, 135.9, 128.5 (x 2), 128.2 (x 2), 126.7, 126.6, 108.6, 106.3, 100.5, 66.2, 52.2, 51.8, 47.9, 35.4, 32.9, 15.5

MS (MALDI-FTMS), [M+H]: C$_{21}$H$_{24}$NO$_4$ requires 354.1705, found 354.1689.
benzyl 3-(3-(7-methyl-7,8-dihydro-[1,3]dioxolo[4,5-g]isoquinolin-6(5H)-yl)propanamido)propanoate

A solution of benzyl 3-(7-methyl-7,8-dihydro-[1,3]dioxolo[4,5-g]isoquinolin-6(5H)-yl)propanoate (20 mg, 0.056 mmol, 1 eq) in THF/H$_2$O (5ml/0.5 ml) was placed under an atmosphere of argon. 10% Pd/C (2 mg) was added and the reaction mixture placed under an atmosphere of hydrogen at balloon pressure. After 1.5 h the hydrogen atmosphere was removed and the reaction mixture was filtered through Celite and concentrated in vacuo to give benzyl 3-(7-methyl-7,8-dihydro-[1,3]dioxolo[4,5-g]isoquinolin-6(5H)-yl)propanoate a white solid which was used without further purification. Benzyl 3-(7-methyl-7,8-dihydro-[1,3]dioxolo[4,5-g]isoquinolin-6(5H)-yl)propanoate was dissolved in DMF (5 ml) then β-alanine benzyl ester (29 mg, 0.083 mmol, 1.5 eq), HBTU (42 mg, 0.11 mmol, 2 eq) and NEt$_3$ (31 µl, 0.22 mmol, 4 eq) were added and the reaction was allowed to stir at room temperature for 2 h. EtOAc was added to the reaction and the mixture was washed with brine, dried (MgSO$_4$) and concentrated in vacuo. The crude product mixture was purified by flash chromatography on silica gel (10% MeOH/EtOAc) to give benzyl 3-(3-(7-methyl-7,8-dihydro-[1,3]dioxolo[4,5-g]isoquinolin-6(5H)-yl)propanamido)propanoate (18.5 mg, 0.044 mmol, 78 %) as a white solid.

$^1$H NMR (400, MHz, CDCl$_3$) δ 7.25 (m, 5H), 6.47 (s, 1H), 6.41 (s, 1H), 5.79 (d, J = 1.6 Hz, 1H), 5.78 (d, J = 1.6 Hz, 1H), 4.91 (s, 2H), 3.54 (d, J = 14.0 Hz, 1H), 3.53 (d, J = 14.0 Hz, 1H), 2.96 (m, 1H), 2.85 (dd, J = 16.0, 4.8 Hz, 1H), 2.74 (m, 1H), 2.61 (m, 1H), 2.46 (t, J = 6.0 Hz, 2H), 2.40 (dd, J = 16.4, 4.8 Hz, 1H), 2.31 (t, J = 6.4 Hz, 2H), 0.98 (d, J = 6.4 Hz, 3H).

$^{13}$C NMR (100, MHz, CDCl$_3$) δ 172.6, 171.9, 146.2, 145.8, 135.8, 128.5, 128.2, 128.0, 126.2, 125.9, 108.7, 106.3, 66.3, 51.9, 50.1, 48.6, 34.9, 34.4, 34.3, 32.5, 14.6

MS (MALDI-FTMS), [M+H]: C$_{24}$H$_{25}$N$_2$O$_5$ requires 425.2071, found 425.2073.
3-(3-(7-methyl-7,8-dihydro-[1,3]dioxolo[4,5-g]isoquinolin-6(5H)-yl)propanamido)propanoic acid

\[
\begin{align*}
\text{Benzyl } & 3-(3-(7-methyl-7,8-dihydro-[1,3]dioxolo[4,5-g]isoquinolin-6(5H)-yl)propanamido)propanoate (18.5 \text{ mg, 0.044 mmol, 1 eq}) \text{ was dissolved in THF/H}_2\text{O (5 ml/0.5 ml) and placed under an atmosphere of argon. 10\% Pd/C (6 mg) was added and the reaction mixture placed under an atmosphere of hydrogen at balloon pressure. After 1 h the hydrogen atmosphere was removed and the reaction mixture was filtered through Celite and concentrated in vacuo to give 3-(3-(7-methyl-7,8-dihydro-[1,3]dioxolo[4,5-g]isoquinolin-6(5H)-yl)propanamido)propanoic acid (7.0 mg, 0.021 mmol, 48 \%).}
\end{align*}
\]

\[\begin{align*}
^1H \text{ NMR (400 MHz, CDCl}_3) & \delta 6.63 (s, 1H), 6.62 (s, 1H), 5.89 (s, 2H), 4.06 (s, 2H), 3.52 (m, 1H), 3.41 (m, 2H), 3.21 (m, 2H), 3.06 (dd, J = 17.2, 4.8 Hz, 1H), 2.69 (dd J = 16.8, 6.8 Hz, 1H), 2.60 (t, J = 6.8 Hz, 2H), 2.39 (t, J = 6.6 Hz, 2H), 1.29 (d, J = 6.8 Hz, 3H). \\
^{13}C \text{ NMR (100 MHz, CDCl}_3) & \delta 172.8, 148.9, 148.1, 126.1, 123.0, 109.5, 107.5, 102.5, 55.8, 51.6, 37.4, 34.0, 32.0, 15.6
\end{align*}\]

MS (MALDI-FTMS), [M+H]: C\text{\textsubscript{17}}H\text{\textsubscript{23}}N\textsubscript{2}O\textsubscript{5} requires 335.1601, found 335.1596.

**Immunization with hapten 7.** Hapten 7 was coupled to the carrier proteins BSA and KLH as described. The hapten-KLH conjugate were mixed with an MPL + TDM adjuvant system, and used to immunize mice (strain 129 GIX+). The first injection (200 \(\mu\)l) contained the immunoconjugate (100 \(\mu\)g based on KLH) and adjuvant (50 \(\mu\)g) reconstituted in PBS. The injection was administered i.p. A booster injection was administered 2 weeks later in a similar fashion. An orbital bleed of anesthetized mice 1 week later was used to assess the titer. A final i.v. injection of hapten-KLH (50 \(\mu\)g) in PBS (150 \(\mu\)l) was administered about 6 months after the final i.p. boost. Three days later the spleen cells were harvested and fused with a myeloma cell line (X63-Ag8.653) to
produce hybridomas according to standard techniques. All the hybridomas were cloned into 96-well plates and screened with the corresponding hapten-BSA conjugate by immunosorbant assay (ELISA) during the cloning process. Each member of the final respective panels was produced:

(a) in vitro via large-scale cell culture. Supernatant clarification by centrifugation. Concentration of culture supernatant, and a single step affinity purification using Protein G.

(b) in vivo via passaging stable hybridoma clones into mice (strain BALB/c x 129 GIX+) for ascites production. mAbs were precipitated from ascites fluid using saturated ammonium sulfate, and purified via ion exchange chromatography on DEAE-Sephacel. A subsequent affinity purification step using Protein G is required to achieve desired purity.

Each member of the final panel was then assessed for binding to MDMA and (+)-methamphetamine (+)-2 using indirect competition immunosorbant assay (ELISA). Here a pre-titrated indirect assay with optimal Ag1 (hapten-BSA) and mAb is competed for by Ag2 (MDMA or (+)-2), as a dilution range, in the liquid phase.

Spectral Characterization of Benzaldehyde as the Main Product of Photosensitized Degradation of (+)-Methamphetamine [(+)-2]. A solution of (+)-2 (1 mM), riboflavin (60 μM) and antibody 40H10 (20 μM) in PBS (pH = 7.4) was irradiated on a transilluminator plate (white light, 3.4 mW cm⁻² Fischer-Biotech transilluminator) at 4°C for over 12 days. The reaction mixture was divided into three portions. To two of the portions, hydrogen chloride (2 mM) and sodium hydroxide (2 mM) were added, respectively, till the pH of the two solutions reached 2 and 10, which was monitored by pH paper. Diethyl ether was added to the solution, and resulted mixtures were vortexed for 30 min vigorously. The samples were then centrifuged till the organic layer was separated completely from the aqueous layer. 10 ul of ether extract from each sample was taken for GC-MS analysis. The same reaction was performed and the entire reaction mixture was extracted by ether. The combined ether layer was place under a beam of argon to remove all the solvent and the final reaction extract was subjected to the NMR analysis. The aqueous layer was analyzed by HPLC before and after ether extraction and
we found that nearly 100% of the product was partitioned into the organic layer. We note that the diethyl ether extraction of the reaction under basic conditions was performed; however, we were not able to obtain its GC-MS analysis data to date.

\[ ^1 \text{H NMR (400, MHz, CDCl}_3 \delta^a 9.98 (s, 1H), 7.86 (d, J = 8.95Hz, 2H), 7.63 (t, J = 7.41Hz, 1H), 7.54 (t, J = 7.65, 2H), ^b 7.26 (t, J = 7.24Hz, 6H), 7.17 (t, J = 8.12Hz, 9H), 2.76 (m, 3H), 2.69 (dd, J_1 = 6.76Hz, J_2 = 13.18Hz, 3H), 2.56 (dd, J_1 = 6.35Hz, J_2 = 13.17Hz, 3H), 0.98 (d, J = 6.18Hz, 9H) \]

\(^a\) Peaks corresponding to benzaldehyde are in bold

\(^b\) Peaks corresponding to un-reacted (+)-2 are in italic

**DNPH Hydrazine Test.** A 2 ml reaction of (+)-2 (1 mM), riboflavin (60 µM) and antibody 40H10 (20 µM) in PBS (pH = 7.4) was extracted with diethyl ether (2 ml x 3) after 24-hour incubation. To the ether extract, 50 µl of DNPH reagent was added. Yellow precipitate formed instantly. The extract was then centrifuged and the supernatant was decanted. The yellow solid obtained was then suspended in 2 ml of ethanol and centrifuged again. The supernatant ethanol was removed. This washing step was repeated in a total three times. The final yellow solid was then dried and characterized by NMR.

\[ ^1 \text{H NMR (400, MHz, CDCl}_3 \delta 9.16 (d, J = 2.48 Hz, 1H), 8.37 (dd, J_1 = 8, J_2 = 9.52Hz, 1H), 8.14 (s, 1H), 8.12 (d, J = 9.54Hz, 1H), 7.79 (dd, J_1 = 2.83Hz, J_2 = 6.47Hz, 2H), 7.48 (m, 3H). \]

**MS (MALDI-FTMS), [M-H]: C_{13}H_9N_4O_4 requires 285.0629, found 285.0630.**

(+)-Methamphetamine (+)-2 Oxidation using Ultraviolet Light. (+)-2 (5 mg) was dissolved in de-oxygenated, de-gassed water (1 ml). The reaction was place on the top of a UV transilluminator (312 nm, 0.8 mW cm\(^{-2}\) Fischer-Biotech transilluminator) at 4°C. Aliquots were taken throughout the reaction and subjected to reverse-phase HPLC analysis.