Anti-Cocaine Catalytic Antibodies: A Synthetic Approach to Improved Antibody Diversity

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Abstract: Catalytic antibodies are potential therapeutic agents for drug overdose and addiction, and we previously reported the first such artificial enzymes to degrade cocaine. However, as described herein, these catalytic monoclonal antibodies (Mab's) were found to have nearly identical complementarity-determining regions (CDR's). Such limited diversity among catalytic antibodies of similar specificity has been reported previously and poses a problem since the capacity of any single group of homologous catalytic antibodies to yield one of high activity, whether through repetitive screening of hybridomas or through antibody mutagenesis, is unpredictable. One strategy to increase the diversity of the immune response to an analog would be to vary the tether site of the immunogenic conjugate thereby exposing unique epitopes for immunorecognition. We now report the syntheses of three immunogenic conjugates of a transition-state analog (TSA) of cocaine benzoyl ester hydrolysis which have identical phosphonate monoester core structures but varying tether sites for attachment to carrier protein: TSA **1** at the methyl ester, TSA **2** at the 4′-phenyl position, and TSA **3** at the tropane nitrogen. Mixed phosphonate diester precursors were obtained from phosphonic dichlorides and ecgonine alkyl esters through our 1*H*-tetrazole catalysis method. We found that all three analogs provided catalytic antibodies that hydrolyze cocaine at the benzoyl ester; the most active catalytic antibody, Mab 15A10, displayed a rate acceleration ($k_{cat}/k_{uncat} = 2.3 \times 10^4$) sufficient to commence preclinical studies. On competitive ELISA, all nine catalytic antibodies, regardless of the eliciting antigen, bound TSA **1** with high affinity but four bound TSA **3** poorly and five failed to bind TSA **2** despite the inhibition of all antibodies by free TSA (TSA **4**). A comparison of heavy and of light chain CDR's showed four discrete groups with TSA **1** and **3** each yielding two non-overlapping families of catalytic antibodies; TSA **2** yielded one antibody with CDR's nearly identical to those of the largest group of catalytic antibodies elicited by TSA **1**. The failure of TSA **2** and TSA **3** to bind to catalytic antibodies derived from alternative immunogenic conjugates demonstrates that the tether site does limit the catalytic antibodies produced and supports the general strategy of varying the attachment to carrier protein.

Introduction

Catalytic antibodies have unique potential for the treatment of cocaine addiction and overdose. Cocaine reinforces self-administration by inhibiting a dopamine re-uptake transporter[1] in the mesolimbocortical "reward pathway". No antagonist to cocaine is known,[2] perhaps reflecting the difficulties inherent in blocking a blocker. As an alternative to receptor-based therapeutics, a circulating agent could interrupt the delivery of cocaine to its binding site in the brain.[3] An agent such as an antibody that merely bound the drug could be depleted stoichiometrically by complex formation, but an enzyme that bound drug, transformed it, and released product would be available for additional binding. Catalytic antibodies, a novel class of artificial enzyme, are inducible for a wide array of reactions, and their substrate specificity is programmable to small molecules such as cocaine.[4]

Cocaine detoxification is particularly well suited for a catalytic antibody approach. First, hydrolysis of the benzoyl ester of cocaine yields the biologically inactive products[5] ecgonine methyl ester and benzoic acid (Scheme 1). The plasma enzyme butyrylcholinesterase deactivates cocaine in humans[6] by means of this reaction. Second, acyl hydrolysis is the best studied of

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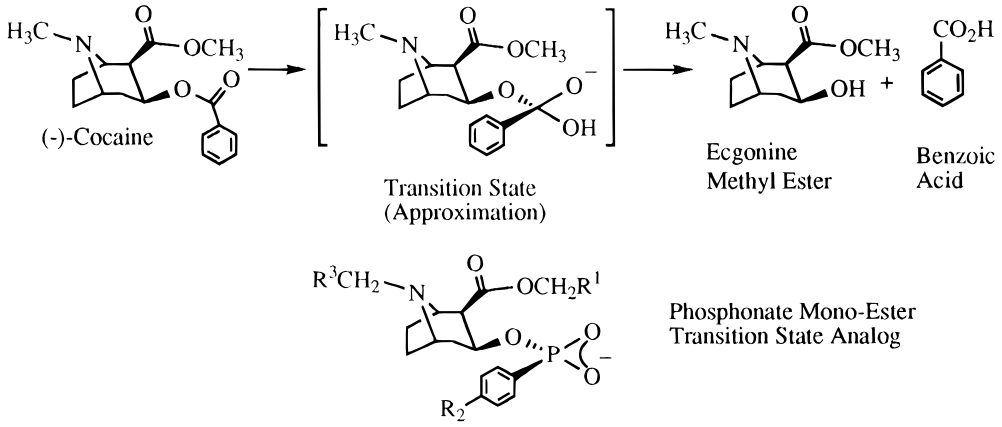
Scheme 1



TSA **1** $R^1 = (CH_2)_3NH^{14}CO(CH_2)_2{}^{14}CONH$–carrier protein; $R^2 = R^3 = H$
TSA **2** $R^2 = (CH_2)_3NH^{14}CO(CH_2)_2{}^{14}CONH$–carrier protein; $R^1 = R^3 = H$
TSA **3** $R^3 = (CH_2)_2NH^{14}CO(CH_2)_2{}^{14}CONH$–carrier protein; $R^2 = R^1 = H$
Free TSA **4** $R^1 = R^2 = R^3 = H$

all antibody-catalyzed transformations.[7,8] Esterase activity approaching that of natural enzymes has been reported[7] for catalytic antibodies, and the large hydrophobic surface of the benzoyl ester is well adapted to elicit antibodies with strong binding and catalysis.

We have previously described[9] the first catalytic antibodies to degrade cocaine, Mab's (monoclonal antibodies) 3B9 and 6A12. The antibodies were elicited by an immunogenic conjugate (TSA **1**) (transition-state analog **1**) of a phosphonate monoester TSA (Scheme 1). The rate acceleration of these first artificial cocaine esterases (10^2-10^3) corresponded in magnitude to their relative stabilization of the ground state to the transition state ($\sim K_m/K_i$). Catalytic antibodies with more potent catalytic mechanisms and with turnover rates $>10^5$ are possible and, we estimated, necessary for clinical applications. Increased activity can be pursued either through repeated hybridoma generation or through mutagenesis of catalytic antibodies in hand. However, sequencing of the variable domains of Mab's 3B9 and 6A12 revealed 93% homology at the complementarity-determining regions (CDR's) (see below). Such a lack of diversity has been noted previously for catalytic antibodies[10] and limits the opportunities for improving activity since a particular class of homologous catalytic antibodies may fail to optimize to the desired activity. A potential solution to this problem that would not compromise the core structure of the analog would be to vary the surfaces of the analog rendered inaccessible by attachment to carrier protein and thereby present distinct epitopes for immunorecognition.

We now report the syntheses of three analogs of cocaine hydrolysis with identical phosphonate replacements but differing constructions for the immunoconjugates. We characterize both the kinetics and the structural diversity of the catalytic antibodies elicited by these analogs. We identify the preferred catalytic antibodies for mutagenesis studies.

Results

Synthesis of Transition-State Analogs. Phosphonate monoesters, which stably mimic the geometry and charge distribution of the transition state for second-order ester hydrolysis by hydroxide, have yielded, in some instances, catalytic antibodies of high activity.[8] However, such analogs are also known to idiosyncratically fail to elicit any catalytic antibodies, and so the rules for analog construction must be defined empirically.[11] Strategies to improve analog efficiency have been devised, including "bait and switch" [11] and substrate attenuation,[12] but the cost of such expedients is a divergence between analog and substrate structure which results on average in catalytic antibodies with higher values for K_m. Inhalation of vaporized cocaine yields a peak pulmonary vein concentration[13] of $10-30$ μM, and this is less than the K_m of most catalytic antibodies with esterase activity. At a subsaturating concentration of cocaine, a higher K_m would result in a lower turnover rate and increase the already limiting requirement for a high k_{cat}. Thus, we chose to construct a high-fidelity analog that differed from cocaine only by a phosphonate replacement at the acyl group and by the incorporation of a tether for construction of an immunogenic conjugate. On the basis of their distances from the locus of reaction and their separation from each other, three tether sites were chosen: at the methyl ester for analog **1**, the 4′-position of the phenyl group for analog **2**, and the tropane nitrogen for analog **3** (Scheme 1). The "free TSA" corresponded to the untethered structure **4**.

The synthesis of TSA **1** began with the commercially available starting material (−)-ecgonine (Scheme 2). Selective alkylation of the carboxylate salt of (−)-ecgonine with 4-azido-1-iodobutane yielded ester **5** in 78% yield. The absence of epimerization at C-2 was confirmed by ^{1}H-NMR spectroscopy. The base labile and sterically hindered alcohol of alkyl ecgonine **5** reacted smoothly with phenylphosphonic dichloride using our procedure for 1*H*-tetrazole catalysis,[14] and addition of methanol provided the phosphonate diester **6** in 89% yield. The tether was elaborated at the azido moiety by reduction to the unstable amine with $P(CH_3)_3$ and acylation with [1,4-^{14}C]succinic anhydride. The hemisuccinate was purified and characterized as the benzyl ester, obtained in 70% yield from **6**, and the acid was quantitatively regenerated by catalytic hydrogenolysis. Acid

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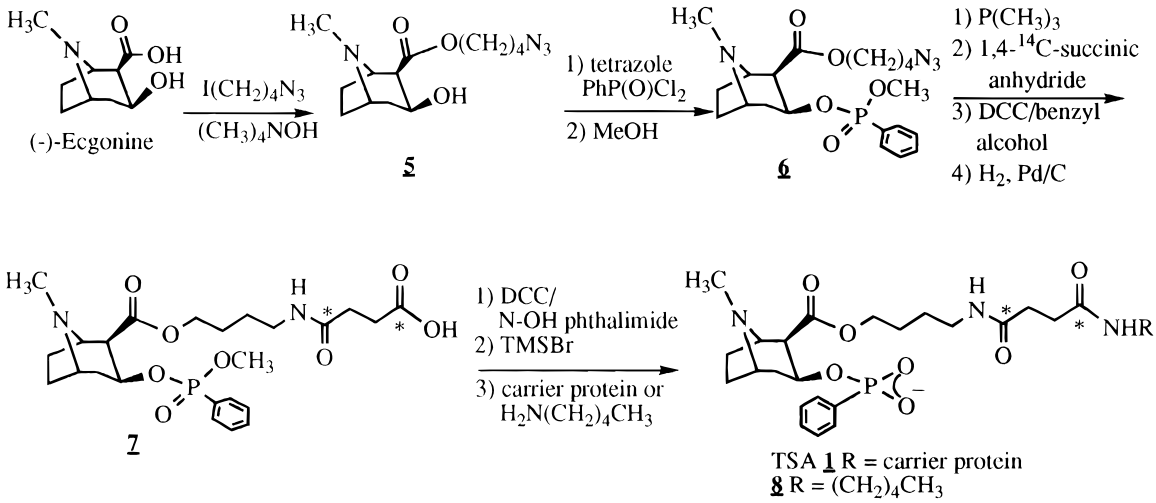
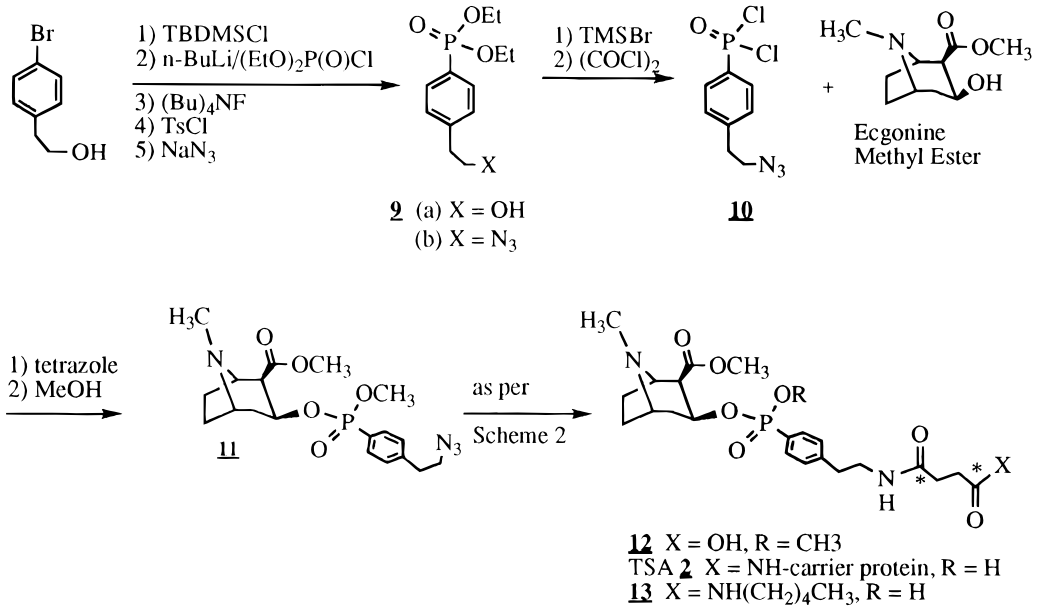
Scheme 2



Scheme 3



7 was activated as the *N*-hydroxyphthalimide ester and selectively deesterified at the phosphonate methyl ester with trimethylsilyl bromide.[15] The unstable monophosphonate product was immediately coupled to carrier protein to yield TSA **1**. The analog:carrier coupling ratio was 6:1 for bovine serum albumin (BSA) and 15:1 for ovalbumin based on the incorporation of radiolabel into protein. In support of our assignment of structure to the carrier-bound analog, an aliquot of the monophosphonate was coupled to *n*-amylamine to yield the expected amide **8**.

Synthesis of TSA **2** required a phenylphosphonic dichloride appropriately substituted at the 4′-position for elaboration of a tether (Scheme 3). Silylation of 2-(*p*-bromophenyl)ethanol followed by transmetalation with *n*-butyllithium, quenching with diethyl phosphorochloridate, and desilylation provided alcohol **9a** in 23% yield. The tosylate of **9a** was displaced by azide, and transesterification with trimethylsilyl bromide, followed by reaction with oxalyl chloride,[16] provided the required phenylphosphonic dichloride **10**. Using the tetrazole catalysis method described above, chloride **10** was coupled with ecgonine methyl ester and, after the addition of methanol, the mixed diester **11** was obtained in 25% yield. The tether was elaborated from the azide by a sequence of reactions identical to that employed for TSA **1**.

For the synthesis of TSA **3** (Scheme 4), *N*-norcocaine was monoalkylated in 75% yield and acid hydrolysis followed by reesterification with acidic methanol provided alcohol **15** in 72% yield. Tetrazole-catalyzed synthesis of mixed phosphonate diester **16** proceeded in 48% yield, and the tether was elaborated from the azido moiety as described above.

Generation of Anti-Cocaine Catalytic Antibodies. Balb/C mice were immunized with individual analogs conjugated to BSA, and high-titer antisera were elicited by each antigen. Monoclonal antibodies were prepared by standard protocols,[9,17] and we selected hybridomas secreting analog-specific antibodies as determined by an enzyme-linked immunosorbent assay (ELISA). All IgG antianalog antibodies were subcloned, propagated in ascites or cell culture flasks, and purified by protein A affinity column chromatography. Catalytic antibodies were identified by their capacity to release [^{3}H]benzoic acid from [$^3H_{phenyl}$]cocaine. The radiolabeled benzoic acid was

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Scheme 4

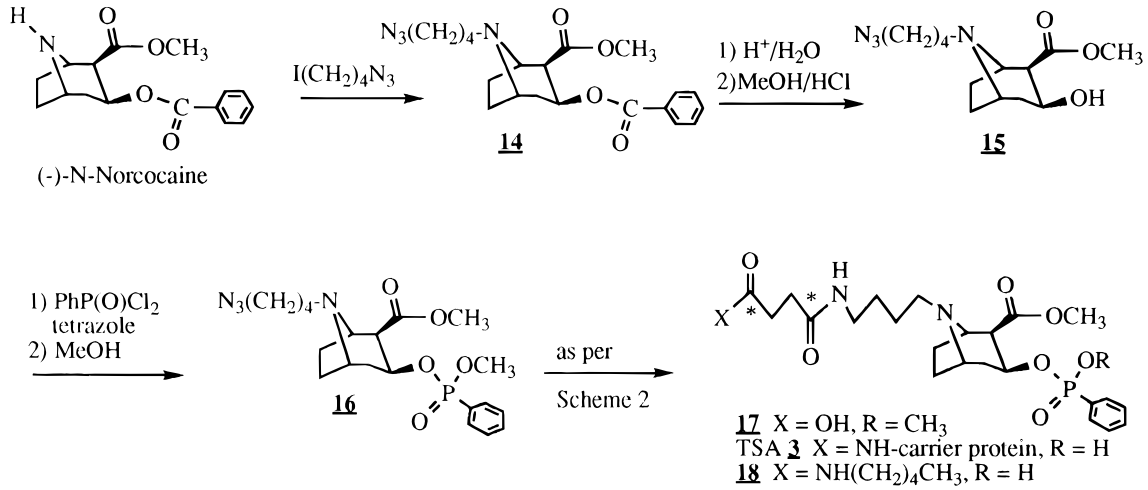

Table 1. Kinetic Parameters for the Hydrolysis of [^{3}H]Cocaine by Mab's[a]

Mab	TSA	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/k_o
3B9	**1**	490	0.11	1100
6A12	**1**	1020	0.072	880
2A10	**1**	3000	0.011	420
9A3	**1**	270	0.015	140
19G8	**1**	900	0.091	830
15A10	**1**	220	2.3	23000
12H1	**2**	150	0.16	1500
8G4G	**3**	530	0.60	5500
8G4E	**3**	1200	0.12	1100

[a] Michaelis constant, K_m; catalytic rate constant, k_{cat}; spontaneous rate, k_0. Assays were performed at the pH that optimized k_{cat}/k_0: in general, pH 7.8; for 6A12, pH 7.4; for 2A10, pH 7.0.

Table 2. Competitive Inhibition Enzyme Immunoassay of Catalytic Mab's[a]

Mab (TSA)	K_4 (μM)	K_8 (μM)	K_{13} (μM)	K_{18} (μM)
3B9 (**1**)	0.01	0.02	3	100
6A12 (**1**)	0.01	0.01	4	90
2A10 (**1**)	0.5	3	20	150
12H1 (**2**)	0.001	0.01	2	60
9A3 (**1**)	0.05	0.02		0.003
19G8 (**1**)	0.008	0.001		0.001
15A10 (**1**)	0.009	0.003		0.0005
8G4G (**3**)	0.003	0.001		0.001
8G4E (**3**)	0.003	0.0005		0.003

[a] Dissociation constants for free TSA **4** and TSA-related amides **8**, **13**, or **18** were determined for each catalytic Mab by CIEIA through competitive inhibition of Mab binding to the TSA (**1**, **2**, or **3** tethered to ovalbumin) that elicited the Mab.

conveniently partitioned from [^{3}H]cocaine by extraction of the acidified reaction mixture into organic solvent. Hydrolysis of cocaine with commercially available carboxyl esterase provided a positive control, and the production of benzoic acid was confirmed by high-performance liquid chromatography. A total of nine catalytic antibodies out of 107 antianalog antibodies were identified from nine fusions with TSA **1** yielding six out of 50 and TSA **3** yielding two out of 49. TSA **2** generated eight antianalog antibodies of which one was catalytic. Catalytic antibodies were further purified by DEAE anion exchange chromatography, and they retained activity. All enzymes were inhibited completely by 50 μM free TSA **4** (see below), and the Fab portion of each antibody tested retained catalytic activity; the potent inhibitor of serum esterases, eserine[18] at 1 mM, did not inhibit the activity of any catalytic Mab, and 150 μM free TSA **4** did not inhibit the cocaine esterase activity present in serum (results not shown).

Characterization of Catalytic Antibodies. We determined the rate of hydrolysis of [$^3H_{phenyl}$]cocaine in the presence and absence of each monoclonal antibody as a function of substrate concentration. Production of radiolabeled benzoic acid at time points corresponding to <5% reaction provided initial rates. We observed saturation kinetics and obtained a linear Lineweaver−Burk plot for each artificial enzyme. The first-order rate constants (k_{cat}) and Michaelis constants (K_m) of the nine catalytic antibodies ranged from 0.011 to 2.3 min^{-1} and from 150 to 3000 μM, respectively, as shown in Table 1.

The rate acceleration of the most active catalytic antibody, Mab 15A10, was higher and the Michaelis constant lower than those we previously reported[9] for Mab 3B9; this corresponds to almost 2 orders of magnitude improvement in activity at subsaturating concentrations of cocaine. We also reported previously that Mab 3B9 displayed a rate acceleration commensurate with the ratio of K_m to the K_i for free TSA **4**. This ratio approximates the affinity of antibody for the ground state relative to the transition state and in the case of Mab 3B9 suggested that the rate acceleration resulted primarily from transition-state stabilization.[19] We determined the inhibition constant (K_i) of free TSA **4** for Mab 15A10 to be 0.23 μM; the rate acceleration of this catalytic antibody ($k_{cat}/k_{uncat} = 2.3 \times 10^4$) significantly exceeded K_m/K_i (9.6×10^2).

We obtained the dissociation constant K_{TSA} for all the catalytic antibodies by competitive inhibition enzyme immunoassay[20] (CIEIA) as shown in Table 2. K_{TSA} determined by CIEIA provides a relative measure of K_i and permits assay at very low concentrations of antibody. As shown in Figure 1, a log−log plot of k_{cat}/k_{uncat} vs K_m/K_{TSA} displayed a linear relationship ($r = 0.85$) for seven of the nine catalytic antibodies; since K_{TSA} is proportional to K_i, the relationship $k_{cat}/k_{uncat} \cong K_m/K_i$ for Mab 3B9 is likely true for all seven antibodies. Mab 15A10 deviated from this line, as expected since k_{cat}/k_{uncat} exceeded K_m/K_i as described above; Mab 8G4G also apparently deviated as shown. Thus, the rate acceleration for 15A10, and perhaps 8G4G, appears too great to be solely attributed to transition-state stabilization, and the participation of chemical catalysis, such as acid−base or nucleophilic catalysis, is likely.

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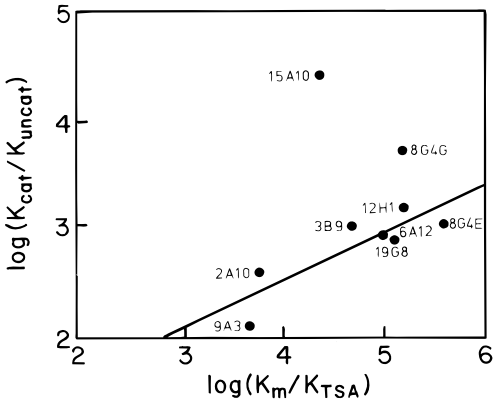

Figure 1. Plot of $\log(K_m/K_{TSA4})$ versus $\log(k_{cat}/k_{uncat})$ for catalytic antibodies generated by TSA **1**, **2**, and **3**. Data represented in this figure are from Tables 1 and 2. Linear relationship by least squares method; $r = 0.85$ excluding Mab's 15A10 and 8G4G.

Mab 15A10 was not inhibited by the product of cocaine hydrolysis, ecgonine methyl ester, at a concentration of 1 mM. Benzoic acid did inhibit with a K_i of 250 μM. However, in humans, benzoic acid plasma levels are markedly suppressed by a rapid and nearly complete conversion to hippuric acid.[21] We found that 1 mM hippuric acid did not inhibit Mab 15A10. Also, there was no inhibition from 1 mM benzoyl ecgonine, a prominent metabolite of cocaine in humans.[22] Inactivation of Mab 15A10 by repetitive turnover was not observed; after 6 h, and >200 turnovers, the k_{cat} remained >95% of base line. The presence of minimal product inhibition by ecgonine methyl ester was fortuitous; heterologous immunization[23] with TSA **1**, **2**, and **3** and the corresponding 1,2-amino alcohol analogs of cocaine is planned both for its potential to minimize product inhibition and its capacity to increase the yield of active enzymes.

The rationale for varying the tether sites of TSA to carrier protein (BSA) was to expose unique epitopes and elicit catalytic antibodies specific to each immunogen. In order to assess binding specificity, the catalytic antibodies were examined by ELISA with TSA **1**, **2**, and **3** bound to ovalbumin. Unexpectedly, we identified two groups with broad affinities, a "3B9 group" (Mab's 3B9, 6A12, 2A10, 12H1), which bound all three conjugates, and a "9A3 group" (Mab's 9A3, 19G8, 15A10, 8G4G, 8G4E), which bound only TSA **1** and **3**.

To estimate the affinities for TSA **1**, **2**, and **3** within these groups we determined the relative K_d's of the corresponding amides **8**, **13**, and **18** by CIEIA. As shown in Table 2, CIEIA confirmed the ELISA result, identifying the same two broad groups of catalytic antibodies. The 3B9 group displayed the rank order of affinities: **8** > **13** > **18**. The relative K_d for the amide of the TSA that elicited each antibody ranged from 0.01 μM for Mab 3B9 and 6A12 to 3 μM for Mab 2A10. Mab 12H1 derived from TSA **2** showed a greater affinity for the TSA **1** related amide **8** (0.01 uM) than for the TSA 2 related amide **13** (2 uM). TSA **1** could have elicited Mab 12H1, and the affinities of Mab's 3B9, 6A12, and 2A10 for **13** are also probably sufficient for TSA **2** to have elicited them. The very low affinities of the 3B9 group for the TSA **3** related amide **18** suggest that TSA **3** could not have elicited this group.

The 9A3 group showed a distinctly different pattern with very high affinity for TSA **1** related amide **8** and TSA 3 related amide **18** but virtually none for TSA **2** related amide **13**. Apparently, TSA **1** or TSA **3** could have elicited every member of this group; TSA **2** could not have elicited any.

Table 3. Deduced Amino Acid Sequences

A. Catalytic Antibody Light Chain CDR's

Mab	CDR1	CDR2	CDR3
3B9	RSSRSLLYRDGKTYLN	LMSTRSS	QHFVDYPFT
6A12	RSSKSLLYEDGKTYLN	LMSTRAS	QHFEDYPFT
2A10	RSSKSLLYEDGKTYLN	LMSTRAS	QQFVEYPFT
12H1	RSSRSLLYRDGKTYLN	LMSTRAS	QHFEDYPFT
9A3	RSSTGTI−TTSN−YAN	INNNRPP	ALWYSNHWV
19G8	RSSAGTI−TTSN−YAN	VNNNRPP	ALWYSNHWV
15A10	RSSTGTI−TSDN−YAN	VNNYRPP	ALWYSNHWV
8G4G	RSSSGTI−TANN−YGS	VSNNRGP	ALWNSNHFV
8G4E	KSSQSLLYSDGKTYLN	LVSKLDS	VQGYTFPLT

B. Catalytic Antibody Heavy Chain CDR's

Mab	CDR1	CDR2	CDR3
3B9	SDYAWT	YIR−HIYGTRYNPSLIS	YHYYGS−AY
6A12	SDYAWT	YIR−HIYGTRYNPSLIS	YHYYGS−AY
2A10	SDYAWN	YIR−YSGITRYNPSLKS	IHYYG−YGN
12H1	SDYAWT	YIR−HIYGTRYNPSLIS	YHYYGS−AY
9A3	−DYNMY	YIDPSNGGIFYNQKFKG	−G−GGLFAY
19G8	−DYNMY	YIDPHNGGIFYNQKFKG	−G−GGLFAY
15A10	−DYNMY	YIDPSNGDTFYNQKFQG	−G−GGLFAF
8G4G	T−YYIY	GMNPGNGVTYFNEKFKN	− −VGNLFAY
8G4E	−DHWMH	TIDLSDTYTGYNQNFKG	−R−G− −

To assess the structural diversity of the catalytic Mab's, we pcr-cloned and sequenced the variable regions of the heavy and light chains of each antibody. Primers were generally derived from published consensus sequences.[24] The 600−700 bp pcr fragment from each reaction was cloned into *p*Bluescript, and independently prepared clones were sequenced in both directions. The deduced primary amino acid structures contained the N-terminal amino acid sequences derived from authentic catalytic antibody samples. Amino acid sequencing also provided primers for pcr-cloning of Mab's 2A10 and 15A10. The CDR's were aligned for comparison (Table 3), and several discrete families of anti-cocaine catalytic antibodies were identified.

TSA **1** yielded two structural families, 3B9-6A12-2A10 and 9A3-19G8-15A10. The light chain CDR homology for pairings within the 3B9 family averaged 96%; within the 9A3 family the average was 93%; whereas between these families the average was 14%. The heavy chain CDR homology within the 3B9 family was high, with 3B9 and 6A12 identical and 2A10 67% homologous; within the 9A3 family the average heavy chain CDR homology was 88%; but between the 3B9 and 9A3 families the average was 32%. TSA **3** yielded two single-membered families, 8G4G and 8G4E. The light chain CDR homology for 8G4G showed 68% homology to the 9A3 group and ≤20% homology to the others; 8G4E showed 56% homology with the 3B9 group and ≤20% to all others. The heavy chain CDR homology between 8G4G and 8G4E was 24%; for each, to the 9A3 group, 48%, and <20% to all others. Mab 12H1, derived from TSA **2**, showed high homology (96%) to the light chain CDR's of the 3B9-6A12-2A10 group and was identical to the heavy chain CDR's of 3B9 and 6A12.

Discussion

The clinical application of a catalytic antibody against cocaine relies on a kinetic argument since a 100 mg dose of cocaine if antagonized solely by antibody binding would require 25 g of

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antibody (assuming an antibody molecular mass of 150 kDa and 2:1 cocaine:antibody stoichiometry). Active immunization with cocaine tethered to an immunoconjugate would be unlikely to provide more than a few percent of this requirement.[25] Polyclonal gamma globulin can be administered in doses of this magnitude, but clearly only enzymatic turnover reduces the antibody requirement to a practical magnitude and, most importantly, allows for the burden of repetitive self-administration, the hallmark of addiction.

To estimate the kinetic characteristics of a clinically useful catalytic antibody we can assume the following parameters: a dose of catalytic antibody at 1 g or less; a dose of cocaine of 100 mg; a cocaine concentration in the pulmonary veins of 30 μM; and a duration of reaction of approximately 15−20 s, the transit time from pulmonary capillaries to central nervous system (CNS) capillaries. Under these constraints a catalytic antibody should ideally have a turnover number >2 s^{-1} and a K_m <50 μM in order to deactivate cocaine before it partitions significantly into the CNS. Within limits, an increased concentration of catalytic antibody can compensate for a lower activity. Thus Mab 15A10 ($k_{cat} = 2.3$ min^{-1}, $K_m = 220$ μM) is of sufficient activity for preclinical trials in animal models of addiction and overdose. However, the need to improve on the activity of Mab 15A10 prior to clinical use is anticipated. Mab's 15A10 and 8G4G are the preferred candidates for optimization since they are the most active catalytic antibodies; they are structurally distinct (see below); and Mab 15A10, and possibly 8G4G, could already manifest some element of chemical catalysis.

The optimization of an anti-cocaine catalytic antibody to meet the kinetic parameters desired for human therapeutic use can be approached through improved analog design, large-scale antibody selection,[26] and antibody mutagenesis.[27] The failure of decades of effort to identify classical receptor blockers of cocaine, together with the magnitude of the cocaine problem, justifies an exhaustive strategy employing all three approaches. One impediment to this effort is the limited diversity of the antibodies elicited by a given analog. Clearly, antibody diversity is not necessary if, by chance, a single class of antibodies ultimately yields a member with the desired kinetic parameters. However, the capacity of a given antibody to be optimized to specification cannot be predicted due to the scarcity of structural data on catalytic antibodies. The generation of a diverse group of anti-cocaine catalytic antibodies should improve the prospects for successful optimization whether through repetitive large-scale hydridoma preparation or through mutagenesis.

Using our tetrazole catalysis method for phosphonate ester synthesis, three transition-state analogs of cocaine hydrolysis were synthesized. The core phosphonate monoester structure was identical in each, and only the tether sites varied. All three elicited catalytic antibodies, and we used a competitive ELISA and CDR sequencing to define functional and structural groupings, respectively.

A comparison of the CDR's of the active antibodies delineated four discrete non-overlapping families that were elicited specifically by TSA **1** (3B9-6A12-2A10 and 9A3-19G8-15A10) and TSA **3** (8G4G and 8G4E). TSA **2** yielded one antibody highly homologous to the 3B9-6A12-2A10 family from TSA **1** and without homology to the antibodies derived from TSA **3**. These structural families overlapped in part with two broad groups defined by a CIEIA method in which amides **8**, **13**, and **18** (representing TSA **1**, **2**, and **3**, respectively) inhibited the binding of each catalytic antibody to its eliciting TSA.

One group defined by CIEIA consisted of Mab's 3B9, 6A12, 2A10, and 12H1. This group displayed high affinity for **8**, moderate affinity for **13**, and very low affinity for **18**. All of the highly homologous members of this group could have been elicited by TSA **1**; the one antibody derived from TSA **2**, Mab 12H1, bound TSA **1** related amide **8** with even greater affinity than TSA **2** related amide **13**. Nonetheless it is possible that most if not all of the group could have been elicited by TSA **2** since the range of affinities for **13** in this group overlapped with the range of affinities for the amides of the TSA's that elicited each antibody. In contrast, the very low affinity of **18** for every member of this group suggests that TSA **3** could not yield any member of the group. A strategy to obtain catalytic antibodies against cocaine based only on a TSA tethered at the tropane nitrogen[28] would fail to identify this group of antibodies.

The second group defined by CIEIA consisted of five catalytic antibodies from three structural families: 9A3-19G8-15A10 derived from TSA **1**; 8G4G and 8G4E from TSA **3**. These five antibodies displayed equally high affinity for amides **8** and **18**, and in principle either TSA **1** or **3** could have elicited every catalytic antibody in this group. That TSA **1** and **3** did not yield members of a common structural family may reflect the inadequacy of a sample size averaging three fusions per analog. None of the five antibodies could have been obtained with TSA **2**, and thus three of the four structural families would not have been identified with this conjugate.

TSA **1** elicited the most active catalytic antibody, Mab 15A10. Moreover, on the basis of the high affinity of amide **8** for all nine catalytic antibodies, TSA **1** could plausibly have elicited every antibody described. This result was unexpected but not a definitive endorsement of TSA **1** as the preferred analog. With more aggressive screening, TSA **2** or **3** may ultimately yield a more active antibody not recognized by TSA **1**.

The failure of a TSA (e.g., TSA **2**) to bind to a catalytic antibody (e.g., 15A10) derived from an alternate immunogenic conjugate confirms that the location of the tether limits the catalytic antibodies produced and supports varying the site of attachment to carrier protein. Exhaustive screening of hybridomas from TSA **1**, **2**, and **3** and detailed structural studies of the catalytic antibodies elicited may clarify the rules for analog construction. The pursuit of high-activity anti-cocaine catalytic antibodies provides a compelling justification for this effort.

Experimental Section

General Methods. Unless otherwise noted, reactions were carried out in oven-dried glassware under an atmosphere of argon. Reagent and solvent transfers were made with oven-dried syringes and needles. Dichloromethane, tetrahydrofuran (THF), and benzene were continuously distilled from calcium hydride; a fume hood was used for procedures requiring benzene or chloroform. [$^3H_{phenyl}$]Cocaine was prepared as previously reported;[8] radiolabeled materials were handled with appropriate caution. All reagents were purchased from Aldrich Chemical Co. All chromatography solvents were obtained commercially and used as received. Reactions were monitored by analytical thin-layer chromatographic methods (TLC) with the use of E. Merck silica gel 60F glass plates (0.25 mm). Flash chromatography was carried out with the use of E. Merck silica gel 60 (230−400 mesh) as described by Still.[29] High-pressure liquid chromatography (HPLC) was performed on a system of Waters 590 using a Dynamax-C_8 (21.4 ×

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250 mm) column and a detector set at 220 nm. The solvent system was acetonitrile/water (0.1% trifluoroacetic acid).

All carbon NMR spectra were obtained at ambient temperature on either a Bruker AMX-500 (500MHz) spectrometer equipped with a 5 mm broad-band inverse probe, a Varian VXR-300 (300 MHz), or a Varian Gemini (50 MHz). All proton NMR spectra (400 MHz) were obtained at ambient temperature on a Bruker AM-400 spectrometer; chemical shifts (δ) are reported in parts per million relative to internal tetramethylsilane (0.00 ppm).

FAB high-resolution mass spectrometric analyses were performed at Michigan State University, Mass Spectrometry Facility. EI mass spectrometric analyses were performed at Columbia University, Mass Spectrometry Facility, on a JEOL DX303 HF instrument. All results were within 5 ppm of calculated values.

Free TSA 4. Ecgonine methyl ester free base was generated by passing a MeOH solution of ecgonine methyl ester hydrochloride through an Amerlite IRN methoxide-exchange column (Polyscience, Inc). To ecgonine methyl ester (0.049 g, 0.25 mmol) in CH_2Cl_2 (10 mL) at 0 °C were added phenylphosphonic dichloride (0.042 mL, 0.30 mmol), 1*H*-tetrazole (catalytic), and *N*,*N*-diisopropylethylamine (0.11 mL, 3.4 mmol). The reaction mixture was allowed to warm to room temperature. After stirring for 12 h, MeOH (0.150 mL) was added, and after 4 h, the reaction mixture was concentrated *in vacuo*. Chromatographic purification (SiO_2, 99:1 $CHCl_3$/MeOH) afforded the mixed diester **4** (0.042 g, 52%) as an oil. To the methyl ester of **4** (0.030 g, 0.095 mmol) dissolved in CH_2Cl_2 (3 mL) was added trimethylsilyl bromide (0.05 mL, 0.38 mmol) at room temperature for 2 h. The reaction mixture was concentrated *in vacuo*. Water (5 mL) was added, and the reaction mixture was extracted with $CHCl_3$ (5 mL × 2). The organic portions were extracted with another 5 mL of water. The combined aqueous fractions were concentrated *in vacuo*. The residue was taken up in MeOH (5 mL), and propylene oxide (excess) was added. After concentration *in vacuo*, the free TSA **4** (29 mg, 90%) was precipitated as a white solid from a solution of the crude product in $CHCl_3$: ^{1}H NMR (400 MHz, D_2O) δ 7.51 (m, 2H), 7.32 (m, 3H), 4.37 (m, 1H), 3.83 (m, 1H), 3.67 (m, 1H), 3.54 (s, 3H), 2.95 (m, 1H), 2.54 (s, 3H), 2.14−1.92 (m, 3H), 1.91−1.74 (m, 3H); ^{13}C NMR (300 MHz, D_2O) δ 179.21, 139.31, 136.92, 136.43, 136.30. 134.00, 133.81, 69.24, 69.04, 68.57, 58.45, 53.49, 43.96, 40.17, 28.95, 27.83; high-resolution mass spectrum (FAB) for $C_{16}H_{23}NO_5$ P calcd 340.1314 (M + 1), found 340.1319.

Compound 5. To ecgonine hydrochloride (0.35 g, 1.6 mmol) in MeOH (4 mL) were added DMF (40 mL), Me_4NOH (2.7 mL, 6.4 mmol), and 1-azido-4-iodobutane (1.8 g, 8 mmol). The reaction mixture was stirred at 50 °C for 12 h and then concentrated *in vacuo*. Chromatographic purification (SiO_2, 9:0.9:0.1 EtOAc/MeOH/NH_4OH) afforded the ester (0.35 g, 78%) as an oil: ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.23 (m, 1H), 4.12 (m, 1H), 3.81 (m, 1H), 3.58 (m, 1H), 3.26 (t, 2H, J = 7.0 Hz), 3.18 (m, 1H), 2.74 (t, 1H, J = 4.7 Hz), 2.19 (s, 3H), 2.03 (m, 2H), 1.98−1.63 (m, 6H), 1.61−1.47 (m, 2H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 173.73, 64.37, 64.29, 63.56, 61.58, 51.74, 50.94, 41.23, 40.26, 25.92, 25.61, 25.51, 24.82; high-resolution mass spectrum (FAB) for $C_{13}H_{23}N_4O_3$ calcd 283.1770 (M + 1), found 283.1783.

Compound 6. To alcohol **5** (0.43 g, 1.5 mmol) in benzene (10 mL) at 0 °C were added phenylphosphonic dichloride (0.27 mL, 1.7 mmol), 1*H*-tetrazole (8 mg), and *N*,*N*-diisopropylethylamine (0.6 mL, 3.4 mmol). The reaction mixture was allowed to warm to room temperature, and a precipitate was observed after 15 min. After stirring for 12 h, MeOH (0.1 mL) was added, and after 4 h, the reaction mixture was concentrated *in vacuo*. Chromatographic purification (SiO_2, 9.5:0.5:0.02 $CHCl_3$/MeOH/NH_4OH), afforded the mixed diester as a mixture of diastereomers (0.53 g, 89%) as an oil: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.73 (m, 2H), 7.60 (m, 1H), 7.49 (m, 2H), 5.09 (m, $^1/_2$H), 4.98 (m, $^1/_2$H), 4.24 (m, 2H), 4.15−3.96 (m, 2H), 3.71 (d, $^3/_2$H, J = 14.6 Hz), 3.68 (d, 2H, J = 14.6 Hz), 3.35−3.15 (m, 3H), 2.91 (s, $^3/_2$H), 2.89 (s, $^3/_2$H), 2.87 (t, $^1/_2$H, J = 7.5 Hz), 2.59 (t, $^1/_2$H, J = 7.5 Hz), 2.43−2.22 (m, $^5/_2$H), 2.17−1.95 (m, $^5/_2$H), 1.71−1.57 (m, 2H), 1.39 (m, 2H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 161.55, 149.12, 134.32, 132.55, 129.80, 129.66, 66.72, 66.54, 66.45, 66.28, 64.80, 63.90, 63.81, 53.81, 51.60, 51.50, 49.58, 49.15, 40.30, 35.60, 35.27, 26.35, 26,06, 26.02, 25.82, 25.10, 23.98; high-resolution mass spectrum (FAB) for $C_{20}H_{30}N_4O_5$ calcd 437.1954 (M + 1), found 437.1953.

Compound 7. Me_3P (1.1 mL, 1 M in THF, 1.1 mmol) was added to azide **6** (0.217 g, 0.5 mmol) in 6 mL of THF/MeOH/H_2O (9:9:2), and the reaction mixture was stirred at room temperature for 5 h. After concentration *in vacuo*, the crude unstable amine (36 mg, 0.084 mmol) was taken up in dry CH_2Cl_2 (5 mL) and [1,4-^{14}C]succinic anhydride (9 mg, 0.093 mmol) was added. The reaction mixture was stirred under Ar for 12 h and then concentrated. For purification, the crude acid **7** (44 mg, 0.087 mmol) was esterified in CH_2Cl_2 (10 mL) with DCC (36 mg, 0.17 mmol), benzyl alcohol (36 μL, 0.35 mmol), and DMAP (catalytic). The reaction mixture was stirred for 12 h and concentrated. Chromatographic purification (SiO_2, 0.5:99.5 MeOH/$CHCl_3$ and 2:98 MeOH/$CHCl_3$) afforded the benzyl ester of **7** as a mixture of diastereomers (32 mg, 59%) as an oil: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.73 (m, 2H), 7.62 (m, 1H), 7.49 (m, 2H), 7.33 (m, 5H), 6.64 (br s, $^1/_2$H), 6.56 (br s, $^1/_2$H), 5.10 (s, 2H), 4.96 (m, $^1/_2$H), 4.89 (m, $^1/_2$H), 4.38−3.85 (m, 4H), 3.74 (d, $^3/_2$H, J = 15.2 Hz), 3.68 (d, $^3/_2$H, J = 15.2 Hz), 3.32−3.12 (m, 3H), 2.89 (s, $^3/_2$H), 2.87 (s, $^3/_2$H), 2.70−2.59 (m, 3H), 2.52−2.26 (m, 4H), 2.10−1.97 (m, 2H), 1.68 (m, 1H), 1.55 (m, 1H), 1.38 (m, 2H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 173.55, 172.66, 171.37, 161.62, 161.28, 136.59, 134.17, 132.37, 129.56, 129.24, 128.88, 128.71, 67.04, 66.81, 66.64, 66.25, 64.66, 63.75, 53.74, 49.37, 49.00, 40.11, 39.42, 35.55, 35.26, 31.35, 30.31, 26.19, 26.06, 24.89, 23.91; high-resolution mass spectrum (FAB) for $C_{31}H_{42}N_2O_8P$ calcd 601.2679 (M + 1), found 601.2682.

The benzyl ester of **7** (17 mg, 0.028 mmol) in methanol (10 mL) was stirred with a catalytic amount of Pd on C (10%) under H_2 (1 atm) for 4 h. The reaction mixture was filtered and concentrated *in vacuo* to provide acid **7** quantitatively: ^{1}H NMR (400 MHz, CD_3OD) δ 7.69 (m, 2H), 7.60 (m, 1H), 7.51 (m, 2H), 4.99 (m, 1H), 4.20−4.08 (m, 2H), 3.89 (m, 1H), 3.73 (d, $^3/_2$H, J = 21.5 Hz), 3.66 (d, $^3/_2$H, J = 21.5 Hz), 3.62 (m, 1H), 3.22 (m, 1H), 3.10 (m, 1H), 3.01 (m, 1H), 2.76 (s, $^3/_2$H), 2.75 (s, $^3/_2$H), 2.50 (m, 2H), 2.38−2.28 (m, 5H), 2.04 (m, 2H), 1.61 (m, 1H), 1.50 (m, 1H), 1.34 (m, 3H); ^{13}C NMR (500 MHz, CD_3OD) δ 176.22, 174.52, 173.47, 162.22, 134.97, 132.79, 130.18, 67.66, 67.53, 66.99, 65.47, 64.44, 53.89, 39.63, 39.33, 35.99, 31.50, 30.23, 26.71, 24.65, 23.67; high-resolution mass spectrum (EI) for $C_{24}H_{36}N_2O_8P$ calcd 511.2209 (M + 1), found 511.2218.

Compound 8. To the acid **7** (40 mg, 0.078 mmol) dissolved in acetonitrile (5 mL) were added *N*-hydroxyphthalimide (14 mg, 0.086 mmol) and DCC (32 mg, 0.16 mmol). After 1 h at room temperature, a white precipitate formed. The reaction mixture was concentrated *in vacuo*. The crude activated ester was taken up in CH_2Cl_2 (5 mL), and trimethylsilyl bromide (100 μL, 0.78 mmol) was added. The reaction mixture was stirred for 1 h and concentrated *in vacuo*. The crude reaction mixture was taken up in acetonitrile (5 mL), and amylamine (100 μL, 0.78 mmol) was added. A bright orange color developed immediately and faded to light yellow in 1 h. Another portion of amylamine (100 μL) was added. The reaction mixture was stirred for 12 h at room temperature and concentrated *in vacuo*. Water (3 mL) was added, and the reaction mixture was extracted with $CHCl_3$ (5 mL × 2). The organic portions were extracted with another 5 mL of water. The combined aqueous fractions were concentrated *in vacuo*. High-pressure liquid chromatography on a Dynamax 300 Å, 12 μ, C-8 (10 × 250 mm) column eluting with a 4−40% CH_3CN/H_2O gradient (0.1% trifluoroacetic acid) provided the amide **8** (16 mg, 36% yield): ^{1}H NMR (400 MHz, CD_3OD) δ 7.72 (m, 2H), 7.56 (m, 1H), 7.47 (m, 2H), 4.12 (m, 3H), 3.87 (m, 1H), 3.23 (m, 2H), 3.14 (m, 3H), 2.77 (m, 4H), 2.58 (m, 4H), 2.34 (m, 3H), 2.16 (m, 1H), 1.97 (m, 2H), 1.55−1.48 (m, 6H), 1.26 (m, 4H), 0.846 (t, 3H, J = 6.3 Hz); ^{13}C NMR (500 MHz, CD_3OD) δ 175.76, 173.62, 133.83, 132.23, 131.01, 129.07, 66.56, 66.52, 65.26, 64.33, 41.13, 40.36, 39.33, 35.93, 31.13, 29.91, 29.48, 28.95, 26.57, 26.28, 24.73, 23.66, 23.22; high-resolution mass spectrum (FAB) for $C_{28}H_{45}N_3O_7P$ calcd 566.2995 (M + 1), found 566.2997.

TSA 1. Acid **7** (14 mg, 0.027 mmol) in CH_3CN (5 mL) was stirred at room temperature with *N*-hydroxyphthalimide (4.8 mg, 0.029 mmol) and DCC (11 mg, 0.053 mmol). A red color developed immediately. After 2.5 h, the reaction mixture was partially concentrated *in vacuo*, filtered through a small cotton plug, and then fully concentrated. The crude, unstable activated ester (0.027 mmol assumed) was taken up in CH_2Cl_2 (5 mL), and trimethylsilyl bromide (20 μL, 0.15 mmol) was added. The reaction mixture was stirred for 1 h and concentrated *in vacuo*. BSA (5 mg) or ovalbumin (5 mg) in $NaHCO_3$ (5 mL, 1 N, pH

8.0) at 0 °C was added and the mixture vigorously stirred. The reaction mixture was allowed to warm to room temperature and after 1 h, the reaction was terminated by gel filtration chromatography (Sephadex G-25 M, pH 7.4 PBS). Protein-containing fractions were combined and dialyzed against PBS at 4 °C overnight (pH = 7.4, 3 × 1000 mL). The coupling efficiency was estimated to be 6:1 for BSA and 15:1 for ovalbumin based on incorporation of radiolabel.

Compound 9a. To 2-(*p*-bromophenyl)ethanol (1.3 g, 6.5 mmol) were added methylene chloride (20 mL), *tert*-butyldimethylsilyl chloride (1.07 g, 7.1 mmol), and imidazole (660 mg, 9.7 mmol). The reaction mixture was stirred at room temperature for 12 h, filtered, and concentrated *in vacuo*. Chromatographic purification (SiO_2, 95:5 hexane/$CHCl_3$) afforded the silyl ether (1.28 g, 66%). To the ether (792 mg, 2.51 mmol) in THF (25 mL) under Ar at −78 °C was added *n*-BuLi (1.2 mL, 2.3 M hexanes, 2.76 mmol) dropwise. The reaction mixture was stirred for 30 min, and a solution of diethyl phosphorochloridate (370 μL, 2.5 M THF, 0.93 mmol) was added. The reaction mixture was stirred at −78 °C for an additional 5 min and allowed to warm to room temperature. Aqueous NH_4Cl (20 mL) was added, and the reaction mixture was extracted with EtOAc (3 × 10 mL). The combined organic layers were washed with brine, dried with anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. THF (10 mL) and aqueous Bu_4NF (2.5 mL, 1 M, 2.5 mmol) were added to the residue. This solution was stirred at room temperature for 30 min and concentrated *in vacuo*. Chromatographic purification (SiO_2, 9:1 EtOAc/MeOH) provided the alcohol **9a** (229 mg, 35%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 (dd, 2H, J = 12.5, 7.1 Hz), 7.33 (dd, 2H, J = 12.5, 4.5 Hz), 4.11 (m, 4H), 2.92 (t, 2H, J = 6.5 Hz), 2.89 (t, 2H, J = 6.5 Hz), 1.32 (t, 6H, J = 7.8 Hz); ^{13}C NMR (50 MHz, $CDCL_3$) δ 144.32, 132.51, 129.78, 129.47, 63.61, 62.69, 39.74, 16.98; high-resolution mass spectrum (El) for $C_{12}H_{20}O_4P$ calcd 259.1099 (M + 1), found 259.1092.

Compound 9b. To alcohol **9a** (193 mg, 0.75 mmol) were added CH_2Cl_2 (7.5 mL), Et_3N (115 μL, 0.83 mmol), TsCl (145 mg, 0.75 mmol), and DMAP (catalytic). The reaction mixture was stirred at room temperature for 12 h. Concentration and purification (SiO_2, 3:1 EtOAc/hexane) provided the tosylate (251 mg, 81.5%), and to a portion of this product (232 mg, 0.56 mmol) were added benzene (3 mL), water (3 mL), tricaprylmethylammonium chloride (catalytic), and NaN_3 (150 mg, 2.25 mmol). The reaction mixture was refluxed at 65 °C for 12 h. Saturated aqueous NH_4Cl (5 mL) was added, and the reaction mixture was extracted with EtOAc. The combined organic layers were treated with $MgSO_4$, filtered, and dried *in vacuo*. Chromatography (SiO_2, 1:1 hexane/EtOAc) afforded the azide **9b** (137 mg, 86%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 (dd, 2H, J = 12.5, 7.1 Hz), 7.32 (dd, 2H, J = 12.5, 4.5 Hz), 4.09 (m, 4H), 3.86 (t, 2H, J = 7.5 Hz), 2.92 (t, 2H, J = 7.5 Hz), 1.32 (t, 6H, J = 7.3 Hz); ^{13}C NMR (50 MHz, $CDCl_3$) δ 143.31, 132.65, 129.50, 129.20, 125.31, 62.58, 52.47, 35.89, 16.94; high-resolution mass spectrum (EI) for $C_{12}H_{19}N_3O_3$ P calcd 284.1164 (M + 1), found 284.1168.

Compound 10. Diethyl phosphonate ester **8b** (600 mg, 2.12 mmol) in CH_2Cl_2 (5 mL) was stirred with trimethylsilyl bromide (1 mL, 11 mmol) and warmed to 45 °C. After 20 min, the mixture was concentrated *in vacuo*. The residue was dissolved in CH_2Cl_2 (3.2 mL), and oxalyl chloride (3.2 mL, 2 M in CH_2Cl_2, 6.36 mmol) and one drop of DMF were added. After stirring for 20 min at room temperature, the volatiles were removed *in vacuo*. The unstable phosphonic dichloride was used directly.

Compound 11. Ecgonine methyl ester free base was generated as described for compound **4**. To ecgonine methyl ester (170 mg, 0.854 mmol) in benzene (20 mL) at 0 °C were added *N*,*N*-diisopropylethylamine (0.74 mL, 4.26 mmol), 1*H*-tetrazole (catalytic), and the phosphonic dichloride **10** (225 mg, 0.854 mmol). The reaction mixture was allowed to warm to room temperature and stirred for 12 h. Methanol (3 mL) was added, and after 20 min the reaction mixture was concentrated *in vacuo*. Chromatographic purification (SiO_2, 1:9 MeOH/$CHCl_3$) afforded the mixed diester as a mixture of diastereomers (108 mg, 30%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71 (m, 2H), 7.29 (m, 2H), 4.63 (m, 1H), 3.73 (s, $^3/_2$H), 3.70 (s, $^3/_2$H), 3.63 (d, $^3/_2$H, J = 11.4 Hz), 3.62 (d, $^3/_2$H, J = 11.4 Hz), 3.51 (t, 2H, J = 7.2 Hz), 3.48−3.39 (m, 1H), 3.23−3.15 (m, 1H), 3.05 (m, $^1/_2$H), 2.91 (t, 2H, J = 7.2 Hz), 2.75 (m, $^1/_2$H), 2.57−2.26 (m, 1H), 2.14 (s, 3H), 2.09−1.52 (m, 5H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 170.91, 170.65, 143.27, 132.80, 132.61, 129.45, 129.11, 125.08, 78.22, 77.73, 76.95, 70.15, 65.31, 62.14, 52.50, 52.84, 52.15, 41.56, 37.84, 35.97, 25.70, 25.58; high-resolution mass spectrum (EI) for $C_{19}H_{27}N_4O_5P$ calcd 422.1719 (M^+), found 422.1714.

Compound 12. To azide **11** (370 mg, 0.877 mmol) were added THF (9 mL) and triphenylphosphine (400 mg, 1.75 mmol). After stirring at room temperature for 12 h, water (1 mL) was added. The mixture was stirred for 3 h and concentrated *in vacuo*. To the crude amine (200 mg, 0.51 mmol) were added CH_2Cl_2 (7.5 mL) and succinic anhydride (3.5 mg, 0.35 mmol). The reaction mixture was stirred for 12 h and concentrated *in vacuo*. The crude acid **12** (290 mg, 0.51 mmol) was dissolved in CH_2Cl_2 (10 mL), and DCC (200 mg, 0.97 mmol), DMAP (catalytic), and benzyl alcohol (0.2 mL, 1.9 mmol) were added. The reaction mixture was stirred at room temperature for 12 h and concentrated *in vacuo*. Chromatography (SiO_2, 10:10:0.4 $CHCl_3$/EtOAc/NH_4OH) afforded the benzyl ester of **12** (197 mg, 65%) as a mixture of diastereomers: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79−7.61 (m, 4H), 7.33−7.25 (m, 5H), 5.11 (s, 2H), 4.69−4.58 (m, 1H), 3.73 (s, $^3/_2$H), 3.69 (d, $^3/_2$H, J = 18.1 Hz), 3.62 (d, $^3/_2$H, J = 18.1 Hz), 3.59 (s, $^3/_2$H), 3.46 (m, 2H), 3.27−3.03 (m, 3H), 2.81 (t, 2H, J = 7.2 Hz), 2.69 (t, 2H, J = 6.8 Hz), 2.42 (t, 2H, J = 6.8 Hz), 2.15 (s, 3H), 2.08−1.80 (m, 3H), 1.69−1.51 (m, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 173.35, 171.42, 132.38, 132.11, 129.99, 129.93, 129.80, 129.67, 129.61, 129.56, 129.48, 129.94, 128.66, 128.49, 67.07, 66.16, 66.43, 63.40, 53.28, 50.49, 50.18, 50.06, 49.64, 49.36, 49.21, 48.79, 39.58, 36.14, 31.14, 30.07, 24.73; high-resolution mass spectrum (EI) for $C_{30}H_{39}N_2O_8P$ calcd 586.2444 (M^+), found 586.2428.

Acid **12** was quantitatively regenerated from the benzyl ester as described for acid **7** as a mixture of diastereomers: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 (m, 2H), 7.60 (m, 1H), 7.49 (m, 2H), 5.02 (m, $^1/_2$H), 4.92 (m, $^1/_2$H), 4.24 (m, 2H), 3.83 (s, $^3/_2$H), 3.74 (d, $^3/_2$H, J = 12 Hz), 3.67 (d, $^3/_2$H, J = 12 Hz), 3.51 (s, $^3/_2$H), 2.79 (m, 1H), 2.75 (s, $^3/_2$H), 2.74 (s, $^3/_2$H), 2.45 (m, 1H), 2.35 (m, 6H), 2.02 (m, 2H), 1.20 (m, 4H); ^{13}C NMR (300 MHz, CD_3 OD) δ 175.92, 174.33, 173.72, 147.06, 132.85, 132.72, 130.62, 130.41, 129.56, 129.29, 67.31, 65.28, 64.37, 53.69, 53.43, 53.24, 41.25, 39.21, 36.42, 35.83, 35.70, 31.35, 30.58, 30.07, 24.52, 23.50; high-resolution mass spectrum (EI) for $C_{23}H_{34}N_2O_8P$ calcd 497.2053 (M + 1), found 497.2064.

Compound 13. To the acid **12** (23 mg, 0.049 mmol) dissolved in acetonitrile (5 mL) were added *N*-hydroxyphthalimide (9 mg, 0.054 mmol) and DCC (20 mg, 0.097 mmol). Reaction with trimethylsilyl bromide (0.65 mL, 0.49 mmol) and amylamine (0.57 mL, 0.47 mmol) proceeded by the protocols developed for compound **8** to yield amide **13** (8 mg, 30% yield): ^{1}H NMR (400 MHz, CD_3OD) 7.69 (m, 2H), 7.32 (m, 2H), 4.75 (m, 1H), 4.08 (m, 1H), 3.86 (m, 1H), 3.71 (s, 3H), 3.39 (m, 3H), 3.14 (m, 2H), 2.82 (m, 5H), 2.42 (s, 3H), 2.38−2.22 (m, 4H), 2.13−2.00 (m, 3H), 1.49 (m, 2H), 1.32 (m, 4H), 0.91 (t, 3H, J = 1.5 Hz); ^{13}C NMR (500 MHz, CD_3OD) δ 173.39, 159.53, 159.22, 144.10, 132.23, 130.95, 129.61, 117.04, 64.83, 64.62, 64.12, 63.92, 62.53, 40.89, 39.54, 36.83, 36.23, 34.31, 31.21, 30.52, 30.14, 29.24, 27.94, 23.95, 21.47; high-resolution mass spectrum (EI) for $C_{27}H_{43}N_3O_7P$ calcd 552.2839 (M + 1), found 552.2863.

TSA 2. To acid **12** (70 mg, 0.14 mmol) were added DMF (4 mL), DCC (116 mg, 0.57 mmol), and *N*-hydroxyphthalimide (92 mg, 0.57 mmol) at room temperature. The reaction mixture was stirred for 12 h at 4 °C, concentrated *in vacuo*, and filtered through a small cotton plug with rinsing with $CHCl_3$ (10 mL). To an aliquot of this solution (2 mL) was added bromotrimethylsilane (0.1 mL, 0.76 mmol). Workup and coupling proceeded by the protocol developed for TSA **1**. The coupling efficiency to BSA was 15:1; to ovalbumin, 10:1.

Compound 14. To *N*-norcocaine (206 mg, 0.713 mmol) and *N*,*N*-diisopropyethylamine (186 μL, 1.07 mmol) in THF (30 mL) was added 1-azido-4-iodobutane (160 mg, 0.713 mmol) at room temperature. The reaction mixture was heated to 60 °C for 2 days. Concentration *in vacuo* and chromatographic purification (SiO_2, 1:9 EtOAc/hexane) yielded the ecgonine ester **14** (205 mg, 75%) as a colorless oil: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.02 (d, 2H, J = 6.0 Hz), 7.58 (t, 1H, J = 6.1 Hz), 7.41 (t, 2H, J = 7.0 Hz), 5.25 (m, 1H), 3.70 (s, 3H), 3.68 (m, 1H), 3.50 (m, 1H), 3.28 (t, 2H, J = 7.4 Hz), 3.03 (m, 2H), 2.43 (m, 1H), 2.26 (m, 2H), 2.04−2.00 (m, 2H), 1.86 (m, 1H), 1.73−1.65 (m, 4H), 1.47 (m, 2H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 171.47, 166.96, 133.77, 131.24, 130.59, 129.16, 68.10, 63.55, 61.24, 52.89, 52.21, 52.05,

53.13, 36.49, 27.29, 26.95, 26.86, 26.34; high-resolution mass spectrum (FAB) for $C_{20}H_{27}N_4O_4$ calcd 387.2032 (M + 1), found 387.2041.

Compound 15. N-Substituted cocaine **14** (205 mg, 0.53 mmol) was hydrolyzed with aqueous HCl (10 mL, 0.7 N) at 90 °C for 4 h. The mixture was extracted with ether, concentrated, and dissolved in MeOH (25 mL) saturated with HCl(g). After 2 h at 60 °C, solvent was removed under vacuum, and the residue was dissolved in MeOH and passed through an Amberlite IRN methoxide-exchange column (Polysciences, Inc.) (1 mL) to generate the crude free base. Chromatographic purification (SiO_2, 5:95 MeOH/$CHCl_3$) afforded alcohol **15** (102 mg, 72%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.80 (m, 1H), 3.69 (s, 3H), 3.03 (m, 1H), 3.66 (m, 2H), 3.24 (t, 2H, J = 7.2 Hz), 3.18 (m, 1H), 2.75 (t, 1H, J = 5.1 Hz), 2.21 (m, 1H), 1.95−1.78 (m, 4H), 1.61−1.38 (m, 6H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 169.58, 65.55, 62.89, 61.27, 53.10, 52.61, 52.26, 52.18, 41.20, 27.36, 27.08, 27.02, 25.83; high-resolution mass spectrum (FAB) for $C_{13}H_{23}N_4O_3$ calcd 283.1770 (M + 1), found 283.1779.

Compound 16. To the ecgonine derivative **15** (102 mg, 0.37 mmol) in benzene (15 mL) at 0 °C were added 1*H*-tetrazole (catalytic), *N*,*N*-diisopropylethyl amine (0.163 mL, 0.94 mmol), and phenylphosphonic dichloride (0.67 mL, 0.47 mmol). The reaction mixture was allowed to warm to room temperature overnight. Excess MeOH was added, and the mixture was stirred at room temperature for 3 h. Chromatographic purification (SiO_2, 5:95 4% NH_4OH/MeOH and 1:1 hexane/$CHCl_3$) and preparative TLC (2.5:97.5 MeOH/CH_2Cl_2) afforded the mixed diester **16** as a mixture of diastereomers (78 mg, 49%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.66 (m, 2H), 7.62 (m, 1H), 7.49 (m, 2H), 5.08 (m, $^1/_2$H), 4.97 (m, $^1/_2$H), 4.32 (m, 1H), 4.18 (m, 1H), 3.88 (s, $^3/_2$H), 3.75 (d, $^3/_2$H, J = 16.4 Hz), 3.71 (d, $^3/_2$H, J = 16.4 Hz), 3.49 (s, $^3/_2$H), 3.45−3.25 (m, 4H), 2.98 (m, 1H), 2.63−2.22 (m, 4H), 2.19−2.01 (m, 2H), 1.92−1.63 (m, 4H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 160.10, 159.72, 133.37, 133.23, 131.61, 131.53, 131.46, 130.29, 128.86, 128.76, 128.64, 66.76, 63.74, 63.58, 62.55, 62.43, 54.46, 54.17, 52.64, 51.67, 49.11, 48.79, 36.57, 36.28, 26.91, 25.58, 25.18, 24.18; high-resolution mass spectrum (FAB) for $C_{20}H_{30}N_4O_5P$ calcd 437.1954 (M + 1), found 437.1928.

Compound 17. Me_3P (0.156 mL, 1 M, in THF, 0.157 mmol) was added to azide **16** (12 mg, 0.026 mmol) in MeOH (5 mL), and the reaction mixture was stirred at room temperature for 2 h. After concentration *in vacuo*, the crude amine was taken up in CH_2Cl_2 (5 mL), and succinic anhydride (2.6 mg, 0.026 mmol) was added. The reaction mixture was stirred at room temperature overnight and concentrated. The crude acid **17** was dissolved in CH_2Cl_2 (10 mL), and benzyl alcohol (0.05 mL, 0.048 mmol), DCC (10 mg, 0.048 mmol), and DMAP (catalytic) were added. The reaction mixture was stirred overnight at room temperature and concentrated. Column chromatography (SiO_2, 5:95 MeOH/CH_2Cl_2) and preparative TLC (5:95 MeOH/CH_2Cl_2) afforded the benzyl ester as a mixture of diastereomers (11 mg, 70% from **13**): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (m, 2H), 7.63 (m, 1H), 7.51 (m, 2H), 7.32 (m, 5H), 7.01 (br s, 1H), 5.09 (s, 2H), 5.03 (m, $^1/_2$H), 4.94 (m, $^1/_2$H), 4.29−4.09 (m, 2H), 3.83 (s, $^3/_2$H), 3.77 (d, $^3/_2$H, J = 17.1 Hz), 3.69 (d, $^3/_2$H, J = 17.1 Hz), 3.49 (s, $^3/_2$H), 3.38−3.22 (m, 4H), 3.01 (m, 2H), 2.69−2.33 (m, 8H), 2.04−1.60 (m, 6H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 172.94, 172.68, 172.09, 135.86, 133.30, 131.64, 128.90, 128.78, 128.65, 128.54, 128.17, 128.82, 66.24, 65.81, 62.71, 62.54, 61.16, 61.03, 52.95, 51.49, 47.69, 37.64, 35.18, 30.41, 29.39, 25.67, 24.00, 23.54, 21.95; high-resolution mass spectrum (FAB) for $C_{31}H_{42}N_2O_8P$ calcd 601.2679 (M + 1), found 601.2676.

Acid **17** was quantitatively regenerated from the benzyl ester as described for acid **7**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 (m, 2H), 7.60 (m, 1H), 7.48 (m, 2H), 5.02 (m, $^1/_2$H), 4.92 (m, $^1/_2$H), 4.33−4.09 (m, 2H), 3.83 (s, $^3/_2$H), 3.74 (d, $^3/_2$H, J = 23 Hz), 3.67 (d, $^3/_2$H, J = 23 Hz), 3.51(s, $^3/_2$H), 3.33−3.19 (m, 6H), 2.98 (m, 1H), 2.63 (m, 2H), 2.49 (m, 4H), 2.34 (m, 2H), 2.06−1.96 (m, 2H), 1.81−1.76 (m, 2H), 1.57 (m, 2H); ^{13}C NMR (300 MHz, $CDCl_3$) δ 175.23, 173.41, 172.06, 133.21, 131.65, 128.90, 128.58, 65.87, 62.75, 60.89, 53.30, 52.98, 51.54, 48.16, 47.75, 37.61, 31.02, 30.33, 25.76, 24.15, 23.54, 21.92; high-resolution mass spectrum (EI) for $C_{28}H_{36}N_2O_8P$ calcd 511.2209 (M + 1), found 511.2213.

Compound 18. To acid **17** (6 mg, 0.012 mmol) dissolved in CH_3CN (3 mL) were added *N*-hydroxyphthalimide (2.2 mg, 0.013 mmol) and DCC (5 mg, 0.024 mmol). Reaction with trimethylsilyl bromide (0.016 mL, 0.12 mmol) and the amylamine (0.14 mL, 0.012 mmol) proceeded by the protocols developed for compound **8** to yield amide **4** (4.4 mg, 65%): ^{1}H NMR (400 MHz, CD_3OD) δ 7.81 (m, 2H), 7.56−7.38 (m, 3H), 5.95 (m, 1H), 5.39 (m, 1H), 5.05 (m, 1H), 4.79 (s, 3H), 4.29−4.12 (m, 6H), 3.61−3.04 (m, 10H), 2.83−2.34 (m, 11H), 0.94 (t, 3H, J = 7.2 Hz); ^{13}C NMR (300 MHz) δ 175.12, 174.98, 174.39, 132.49, 129.36, 129.21, 65.79, 64.72, 62.26, 53.33, 52.52, 40.44, 39.01, 36.78, 32.17, 31.91, 30.23, 30.14, 27.39, 24.69, 24.32, 23.45, 23.22, 14.36; high-resolution mass spectrum (FAB) for $C_{28}H_{45}N_3O_7P$ calcd 566.2995 (M + 1), found 566.2997.

TSA 3. To the acid **17** (12 mg, 0.023 mmol) and *N*-hydroxyphthalimide (16 mg, 0.096 mmol) in DMF (2 mL) was added DCC (19 mg, 0.096 mmol). The reaction mixture was stirred at 4 °C overnight, concentrated *in vacuo*, and filtered with $CHCl_3$ (10 mL) The activated ester was kept as a $CHCl_3$ solution (10 mL) at −20 °C and used without purification. Trimethylsilyl bromide (0.050 mL, 0.379 mmol) was added to a 5 mL aliquot of the activated ester at room temperature. Workup and coupling proceeded by the protocol developed for TSA **1**. The coupling ratio to BSA was 11:1; to ovalbumin, 12:1.

Hybridoma Generation. As previously described,[9] BALB/c mice were immunized with the analog carriers and the immune response was followed by ELISA. Hybridomas were prepared by standard methods.[9,17]

Hybridoma cells ($\sim 2 \times 10^6$) were placed either into a mouse peritoneum that had been pretreated with pristane or into T-150 flask cell culture. The harvested ascites or cell culture supernatants were subjected to affinity chromatography on a preparative protein A HPLC column (Bio-Rad) (purity > 90% by SDS−polyacrylamide gel electrophoresis). Samples of catalytically active antibodies were purified by anion-exchange HPLC with an analytic DEAE column (TOSOH HASS TSK-gel) using 0.02 M Tris and a linear gradient pH 8.8/0.0 M NaCl to pH 7.0/0.3 M NaCl without loss of cocaine esterase activity.

Protocol for Binding Studies (CIEIA). Plates were coated with the TSA (tethered to ovalbumin) that elicited the catalytic antibody intended for CIEIA. Free TSA **4** or the TSA-related amides **8**, **13**, or **14** were tested for inhibition of antibody binding to the eliciting TSA by published protocols.[20b]

Protocol for Kinetic Measurements. Catalytic antibody in 50 mM phosphate-buffered saline pH 8.0 (except 2A10 and 6A12 at pH 7.0) was incubated with [^{3}H]cocaine typically at five concentrations. At three time intervals, aliquots were acidified with cold HCl (aqueous) to a final pH of 2 and partitioned with hexane/diethyl ether (1:1), and the organic phase was assayed by scintillation counting. Background hydrolysis was determined in otherwise identical reactions without antibody, and observed rates were corrected. Assays were performed in triplicate with standard error <10%. As a control, the release of benzoic acid was confirmed by HPLC (Perkin-Elmer) using an analytical reverse-phase C_{18} column (VYDAC) with an acetonitrile/water (0.1% trifluoroacetic acid) gradient and the detector set at 220 nm.

HPLC analysis of a reaction mixture without antibody showed that the methyl ester of cocaine spontaneously hydrolyzes to benzoyl ecgonine with $t_{1/2}$ = 20 h (pH 7). Thus, benzoyl ecognine is not available as a benzoyl esterase substrate at the early reaction times of the [^{3}H]cocaine hydrolysis assay, and the release of benzoic acid is attributed solely to cocaine hydrolysis.

Amino Acid Sequencing. Light and heavy chains were separated by SDS−polyacrylamide gel electrophoresis and then electroblotted to a poly(vinylidene difluoride) membrane[30] for direct NH_2-terminal sequencing by automated Edman degradation on an Applied Biosystems 470A or 477A sequencer. To obtain internal sequence, separated bands from 2A10, 19G7, 9A3, and 15A10 were reduced with dithiothreitol, alkylated with iodoacetamide, and cleaved with trypsin[31] in 1 M urea, 0.05 M NH_4HCO_3, pH 8.0. The peptide fragments were extracted from the membrane, separated by HPLC (Hewlett-Packard) on a reverse-phase C4 column (VYDAC) using an acetonitrile/water (0.07% trifluoroacetic acid) gradient, and sequenced.

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Pcr Cloning of Variable Domains. Mouse hybridoma cell lines producing catalytic antibodies were grown to 1×10^8 cells, and total RNA was prepared using a microadaptation of the guanidine thiocyanate/phenol procedure[32] and selection on a oligo(dT) cellulose column.

Degenerate and non-degenerate oligonucleotide PCR primers were designed using amino acid sequences (2A10, 15A10) or the data base of Kabat et al.[24] Restriction endonuclease sites were incorporated into the primers at their 5′ prime end to facilitate cloning. The restriction sites utilized were *Eco*RI, *Spe*I, *Xba*I, or *Xho*I. The sense and antisense oligonucleotide primers for light chain (LC) and heavy chain (HC) of each hybridoma line were as follows: for 9A3, 19G8, 15A10, 8G4E, and 8G4G LC, 5′-GGAATTCCACIA/TC/GICCIGGIGAA/GACIG-3′ and 5′-GCTCGAGCC/TTCA/GTGIGTIACITGA/GCA-3′; for 3B9, 6A12, and 12H1 LC, 5′-CCAGTTCCGAGCTCCAGATGACCCAGTCTCCA-3′ and 5′-GCGCCGTCTAGAATTAACACTCATTCCTGTTGAA-3′; for 2A10 LC, 5′-GCTCTAGAGCGAT/CATIGTIATGACICAA/GGAT/CGA-3′ and 5′-GGAATTCCA/GTTA/GTGICT/CT/CTCA/GTAT/CTCA/GTC-3′; for 3B9, 6A12, 12H1, 9A3, 19G8, 8G4E, and 84G4G HC, 5′-AGGTCCAGCTGCTCGAGTCTGG-3′ and 5′-AGGCTTACTAGTACAATCCCTGGGCACAAT-3′; for 2A10 HC, 5′-TCCCAGGTCCAACTGCAGCAGCC-3′ and 5′-ATAACCCTTGACCAGGCATCC-3′; for 15A10 HC, 5′-CCAGTTCCGAGCTCGTGATGACACAGTCTCC-3′ and 5′-AGCGCCGTCTAGAATTAACACTCATTCCTGTTGAA-3′.

cDNA templates were synthesized using 0.5 μg of hybridoma mRNA and Moloney murine leukemia virus reverse transcriptase. Amplifications were carried out in a Perkin-Elmer/Cetus thermal cycler for 30 cycles of denaturation (96 °C, 1 min), annealing (50 °C, 1 min), and extension (72 °C, 3 min). The PCR products were purified by electrophoresis in 1.5% agarose gel. Isolated PCR products from each reaction were subcloned into *p*Bluescript plasmid and analyzed by DNA sequence analysis for the presence of open reading frame. Nucleotide sequences were assembled using the IBI MacVector 3.0 program.

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