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Substrate-assisted antibody catalysis

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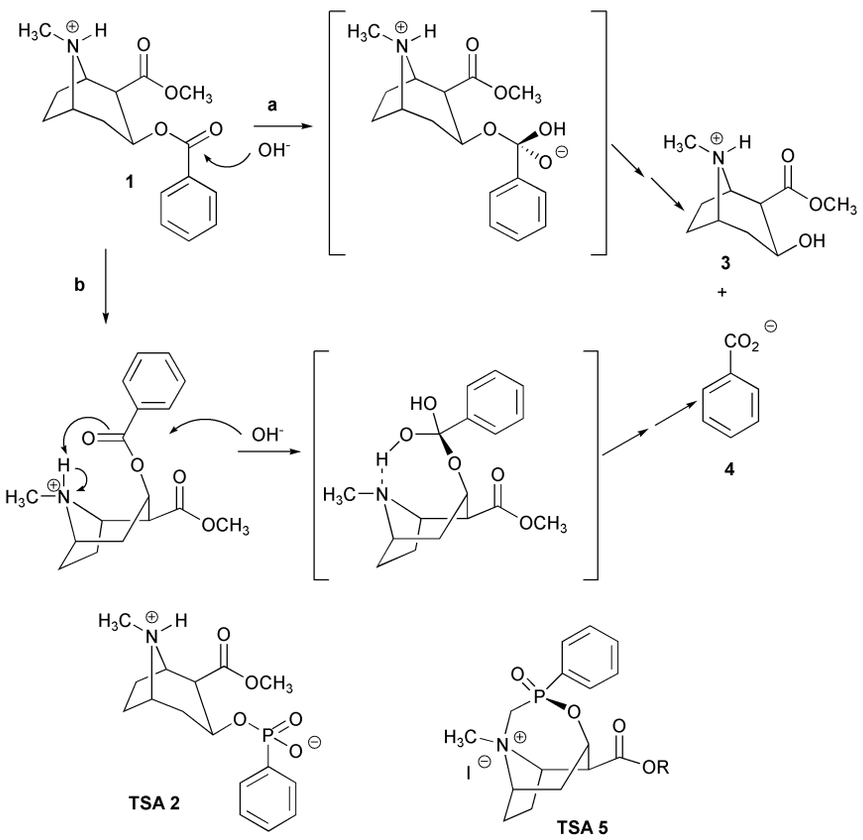
A new strategy in transition-state analog design is demonstrated to elicit catalytic antibodies. The strategy is based on substrate-assisted antibody catalysis and utilizes analogs designed to mimic the transition-state for intramolecular catalysis and thereby favor antibodies that can recruit catalytic groups from substrate. The hydrolysis of the benzoyl ester of cocaine provides an illustration. The benzoyl ester of cocaine is distant from the protonated nitrogen in the stable chair conformer but proximate in the strained boat form. An antibody stabilizing the boat form and approximating ester and amine could catalyze ester hydrolysis. To mimic the transition-state for the intramolecular catalysis, we synthesized a cocaine analog that replaces this ester with a methylenephenylphosphinate bridge to the tropane nitrogen. This bridged analog elicited 85 cocaine esterases out of 450 anti-analog antibodies–a performance markedly superior to that of a simple phosphonate ester-based analog with an identical tether. The correspondence of the analog to a "high energy" conformer eliminated product inhibition. For certain polyfunctional targets, substrate assistance can be an effective strategy for eliciting catalytic antibodies.

Catalytic antibodies[1] have unique potential as therapeutic artificial enzymes based on their biocompatibility and extended plasma half-life. As a therapeutic approach to cocaine (**1**) abuse,[2] we prepared the phosphonate monoester transition-state analog (TSA) **2** and obtained antibodies that cleave cocaine into the non-addicting, non-toxic products ecgonine methyl ester (**3**) and benzoic acid (**4**) (Scheme 1, path a).[3,4] However, this analog of simple alkaline hydrolysis yielded relatively few catalysts. New strategies favoring more complex, and potentially more efficient, hydrolytic mechanisms have focused on eliciting catalytic groups in the antibody.[5–7] A complementary strategy to recruit catalytic groups from substrate was suggested by our observation that the protonated tertiary amine of cocaine internally promotes hydrolysis of its methyl ester but not its benzoyl ester.[8] We hypothesized that for polyfunctional substrates a properly designed analog could elicit antibodies that favor participation of one or more functional groups from the substrate in the stabilization of an otherwise unfavored transition state. We now report the synthesis of **TSA 5**, a transition-state analog designed to promote substrate assistance in the hydrolysis of the benzoyl ester of cocaine (Scheme 1, path b), and the development of the first catalytic antibodies using this strategy.



Scheme 1 Alkaline hydrolysis of cocaine (**1**) to ecgonine methyl ester (**3**) and benzoic acid (**4**). Path a: hydrolysis mimicked by **TSA 2**; Path b: intramolecular catalysis mimicked by **TSA 5**.

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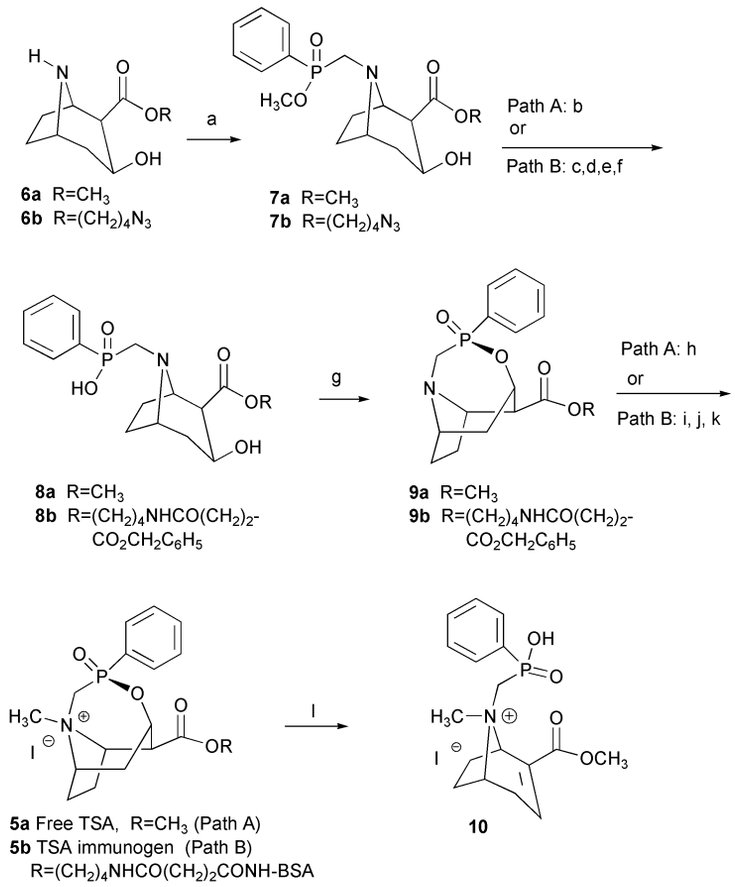

Scheme 2 Synthesis of **TSA 5**: a) (Ph)(TfOCH$_2$)PO(OMe), Et$_3$N, DMF; b) TMSBr; c) PMe$_3$; d) succinic anhydride; e) DCC–DMAP, PhCH$_2$OH; f) TMSBr; g) DCC, pyridine, reflux; h) CH$_3$I; i) CH$_3$I; j) H$_2$–Pd-C; k) EDC–NHS, BSA; l) H$_2$O, 14 days.

Bridged analog **5** was designed to elicit antibodies that would stabilize the boat conformation of cocaine, bring the tropane nitrogen, protonated under physiological conditions, into proximity of the benzoyl ester group and favor stabilization of the transition-state for alkaline hydrolysis through protonation of the incipient oxy-anion. Also, the tetrahedral phenylphosphinate group would mimic the geometry of the transition state for benzoyl hydrolysis.[1] Because a single analog can yield a multi-step catalyst,[1c] we did not consider further the proton transfer steps required to yield product. Finally, the present strategy is distinct from the "bait-and-switch" strategy because there is no "switch", *i.e.*, the positive charge in the analog is also present in the substrate.[6] Nonetheless, the quarternized nitrogen could elicit a negatively charged group for participation in the hydrolysis. A preferred attack of hydroxide *anti* to the carbomethoxy group (Scheme 1, path b) might be expected but either phosphinate diasteromer would be useful. As small molecules do not elicit an immune response, we required both free **TSA 5a** and the immunogenic conjugate to carrier protein **5b**.

In our route to **5a**, norecgonine methyl ester (**6a**) was *N*-alkylated with the triflate of methyl (hydroxymethyl)phenylphosphinate at room temperature to provide phosphinate ester **7a** in 81% yield (Scheme 2, Path A). ^{1}H and ^{13}C NMR revealed a 1 : 1 mixture of diastereomers at phosphorus. Selective demethylation at the phosphinate methyl ester of **7a** with TMSBr afforded phosphinic acid **8a** in 99% yield. Reflux in pyridine with excess DCC provided cyclic phosphinate ester **9a**, in 68% yield, as a single diastereomer by ^{1}H, ^{13}C and ^{31}P NMR spectroscopy. ^{1}H NMR COSY and ^{1}H–^{13}C correlation spectra did not permit a definitive structure assignment and we ultimately confirmed formation of the desired diastereomer by X-ray crystallographic analysis of the HCl salt.[9a] The fortuitous formation of the desired diasteromer could be rationalized by the interaction of the dipoles of the two esters.[9b] *N*-methylation of **9a** with excess CH$_3$**I** in DMF at 40–45 °C provided free **TSA 5a** in 85% yield. Quarternization of the nitrogen rendered the tropane mildly unstable at room temperature to decomposition *via* a ring opening elimination to yield 10 ($t_{1/2} \approx 2$ weeks in H$_2$O, pH 7.4). The synthesis of **TSA 5b** with bovine serum albumin as carrier protein was similarly executed (Scheme 2, Path B).

Balb/C mice were immunized with **TSA 5b**, and high-titer antisera were elicited. Monoclonal antibodies were prepared by standard protocols.[4] Anti-analog antibodies were identified by enzyme-linked immunosorbent assay (ELISA) against immobilized **5b** (ovalbumin conjugate). Protein A affinity-purified antibodies were screened for the capacity to release [^{3}H]-benzoic acid from [^{3}H$_{phenyl}$]-cocaine. Catalytic antibodies further purified by DEAE anion exchange chromatography retained activity. Catalysis of cocaine hydrolysis was confirmed by HPLC detection of benzoic acid; the absence of anhydroecgonine methyl ester by ^{1}H NMR excluded production of benzoic acid by elimination. The antibodies derived from **TSA 5b** were inhibited completely by 10 μM free **TSA 5a** and not inhibited by elimination product **10** at 100 μM, confirming **5b** as the true immunogen. Kinetic and binding (Biacore surface plasmon resonance) parameters were measured for several antibodies (Table 1).

Table 1 Parameters for some antibodies elicited by **TSA** 5 at pH 7.4

Mab	$k_{cat/min^{-1}}$	$K_{m/\mu M}$	k_{cat}/k_0	K_i (**5a**)/nM
13F12	0.046	500	573	29
105G7	0.030	615	370	93
8H1C11	0.037	263	460	65
19E2	0.029	108	362	100

Several lines of evidence support substrate assistance. First, the antibodies showed a decline in activity at pH > 8 (p*K*a for cocaine is 8.6) as illustrated in Fig. 1 and failed to accept *N*-acetyl norcocaine as a substrate. These results support intramolecular catalysis by the protonated tropane nitrogen. However, we cannot exclude the possibility that the positively charged TSA elicits antibodies with a complementary carboxylate group that participates in catalysis. None of the antibodies elicited by **TSA 5b** were inhibited by benzoic acid on competitive ELISA (Fig. 2). In contrast, product inhibition was found for antibodies from **TSA 2**. This observation is consistent with the reaction of **TSA 5b**-antibodies through a more highly strained conformer.[10] Lastly, catalytic antibodies elicited by **TSA 5b** were potently inhibited by **TSA 2**. The boat conformation of **TSA 2** is more easily accessible then that of cocaine due to internal stabilization of the zwitterions and X-ray crystallographic analysis of an antibody-**TSA 2** complex will be of interest.

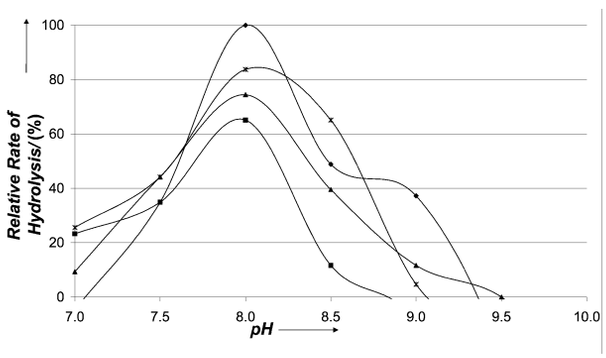

Fig. 1 The effect of pH on relative rate of hydrolysis of cocaine by illustrative catalytic antibodies: 8H1C11(◆), 13F12(■), 19E2(▲) and 105G7(*). Rate is corrected for background hydrolysis at each pH.

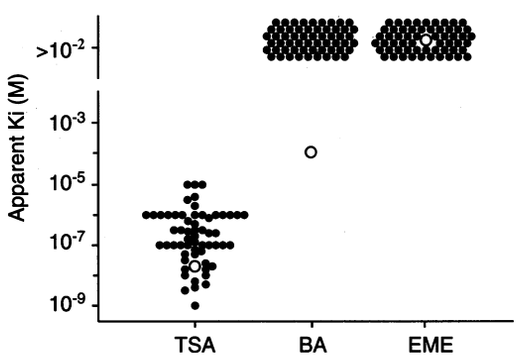

Fig. 2 Competitive ELISA for catalytic antibodies against the eliciting TSA and products benzoic acid (BA) and ecgonine methyl ester (EME). Antibodies from **TSA 5** (●; 58 most productive clones) and illustrative antibody mAb 15A10 from **TSA 2** (○).

Based on a preliminary screen of unpurified supernatants, the efficiency of **TSA 5** (85 catalysts among 450 anti-analog antibodies) was much greater than that of **TSA 2** (9/670). The tether length, a major determinant of analog efficiency,[11] was identical for each. The superior effectiveness of **TSA 5** is consistent with the premise that the structural requirements for antibody catalysts are reduced by substrate assistance. This observation is important because if shape complementarity by antibodies is more easily achieved than complex functional group arrangements, then analogs targeting the former would be more successful generally.

In conclusion we have successfully implemented a new strategy in transition-state analog design applicable to substrates with potentially catalytic, conformationally accessible, functional groups. The antibodies from these initial screenings were not more active than the most potent antibodies elicited by **TSA 2** ($k_{cat}/k_0 > 10^3$),[4] but analog **5** favors only substrate-mediated stabilization of the developing oxy-anion and additional antibody stabilization through mutagenesis is achievable. Tertiary amines are only modest intramolecular catalysts for acyl hydrolysis,[12] but substrate-assistance could be more potently applied to targets containing a consensus sequence that would correspond to elements of a catalytic triad.[13] Thus, histidine and serine could be predictably recruited for a serine protease-like mechanism by antibody binding. We are now testing this hypothesis.

Acknowledgements

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References

1 (*a*) A. Tramontano, K. D. Janda and R. A. Lerner, *Science*, 1986, **234**, 1566; (*b*) S. J. Pollack, J. W. Jacobs and P. G. Schultz, *Science*, 1986, **234**, 1570; (*c*) S. J. Benkovic, J. A. Adams, C. L. Borders, K. D. Janda and R. A. Lerner, *Science*, 1990, **250**(4984), 1135; (*d*) G. M. Blackburn and A. Garcon, *Biotechnology*, 2000, **8b**, 403; (*e*) T. Fujie, *Chem. Rev.*, 2002, **102**(12), 4885.
2 (*a*) B. Mets, G. Winger, C. Cabrera, S. Seo, S. Jamdar, G. Yang, K. Zhao, R. J. Briscoe, R. Almonte, J. H. Woods and D. W. Landry, *Proc. Natl. Acad. Sci. USA*, 1998, **95**, 10176; (*b*) T. J. Baird, S. X. Deng, D. W. Landry, G. Winger and J. H. Woods, *J. Pharmacol. Exp. Ther.*, 2000, **295**(3), 1127; (*c*) R. J. Briscoe, P. M. Jeanville, C. Cabrera, T. J. Baird, J. H. Woods and D. W. Landry, *Int. Immunopharmacol.*, 2001, **1**(6), 1189.
3 D. W. Landry, K. Zhao, G. X. Q. Yang, M. Glickman and M. Georgiadis, *Science*, 1993, **259**, 1899.
4 G. Yang, J. Chun, U. H. Arakawa, X. Wang, M. A. Gawinowicz, K. Zhao and D. W. Landry, *J. Am. Chem. Soc.*, 1996, **118**, 5881.
5 H. Suga, O. Ersoy, S. F. Williams, T. Tsumuraya, M. N. Margolies, A. J. Sinskey and S. Masamune, *J. Am. Chem. Soc.*, 1994, **116**(13), 6025.
6 K. D. Janda, M. I. Weinhouse, D. M. Schloeder, R. A. Lerner and S. J. Benkovic, *J. Am. Chem. Soc.*, 1990, **112**(3), 1274.
7 P. Wirsching, J. A. Ashley, C. H. Lo, K. D. Janda and R. A. Lerner, *Science*, 1995, **270**(5243), 1775.
8 P. Li, K. Zhao, S. Deng and D. W. Landry, *Helvetica Chim. Acta*, 1999, **82**, 85.
9 (*a*) S. X. Deng, D. W. Landry, C. G. Zhan, manuscript in preparation; (*b*) R. Y. Chen and S. C. Dan, *Phosphorus, Sulfur Silicon Relat. Elem.*, 1991, **56**(1–4), 39.
10 A similar absence of product inhibition was observed previously for antibodies elicited by an analog that mimicked a high-energy conformer of a chloramphenicol ester. H. Miyashita, Y. Karaki, M. Kikuchi and I. Fujii, *Proc. Natl. Acad. Sci. USA*, 1993, **90**(11), 5337.
11 M. Matsushita, T. Z. Hoffman, J. A. Ashley, B. Zhou, P. Wirsching and K. D. Janda, *Bioorg. Med. Chem. Lett.*, 2001, **11**(2), 87.
12 (*a*) M. L. Bender, E. J. Pollock and M. C. Neveu, *J. Am. Chem. Soc.*, 1962, **84**, 595; (*b*) R. S. Brown and A. Aman, *J. Org. Chem.*, 1997, **62**(14), 4816.
13 P. Carter and J. A. Wells, *Science*, 1987, **237**(4813), 394.