Promiscuity in Antibody Catalysis: Esterolytic Activity of the Decarboxylase 21D8

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Dedicated to *Jack D. Dunitz* on the occasion of his 80th birthday

The high structural similarity of decarboxylase antibody 21D8 and esterase antibody 48G7 suggests that 21D8 might also possess hydrolytic activity. Kinetic investigations show that 21D8 does promote the selective hydrolysis of methyl 4-nitrophenyl carbonate and sodium 4-(acetoxy)benzenesulfonate with catalytic proficiencies $(k_{cat}/K_m)/k_{un}$ of *ca.* $10^5\,M^{-1}$. The ability of 21D8 to accelerate a reaction for which it was not developed suggests that certain antibody scaffolds are intrinsically predisposed toward catalysis, a property that can be enhanced and refined during affinity maturation in response to a transition-state analog. At the same time, however, the restricted structural diversity of the immune system may ultimately limit the catalytic efficiency that can be achieved.

1. Introduction. – The immune system provides the largest combinatorial library for molecular recognition in nature. It has been estimated that the primary immunoglobulin repertoire contains *ca.* 10^8 different antibodies [1], and that this diversity is further expanded by several orders of magnitude through the process of somatic hypermutation [2], allowing high affinity recognition of virtually any antigen.

The selectivity and diversity of the immune system have been exploited for the generation of tailored antibody receptors for many applications, including catalysis [3][4]. The latter is achieved when a small molecule carrying chemical information about a particular reaction mechanism, such as a stable analog of a transition state, is used to induce an immune response. Catalytic antibodies, with their programmable activities, have proved to be valuable model systems for analyzing the mechanism and evolution of catalysis in proteins.

In light of the many different reactions that have been catalyzed by antibodies [5], the immunoglobulin scaffold is clearly a versatile starting point for enzyme design. Nevertheless, rather than providing many distinct structural solutions to a given recognition problem, as might have been expected from the enormous sequence diversity of the primary repertoire, antibodies appear to use a relatively limited number of strategies for binding certain classes of ligand. The high degree of structural similarity in hydrolytic antibodies generated in response to aryl phosphonates and phosphonamides is a case in point [6]. Even haptens that are less obviously related can induce homologous active sites. For example, 2-(acetamido)naphthalene-1,5-disulfonate (**1**) and 4-nitrophenyl phosphonate **2**, which both contain tetrahedral anions and hydrophobic aryl groups but are otherwise dissimilar, gave rise to antibodies that bear a striking resemblance to one another [7] (*Fig. 1*).

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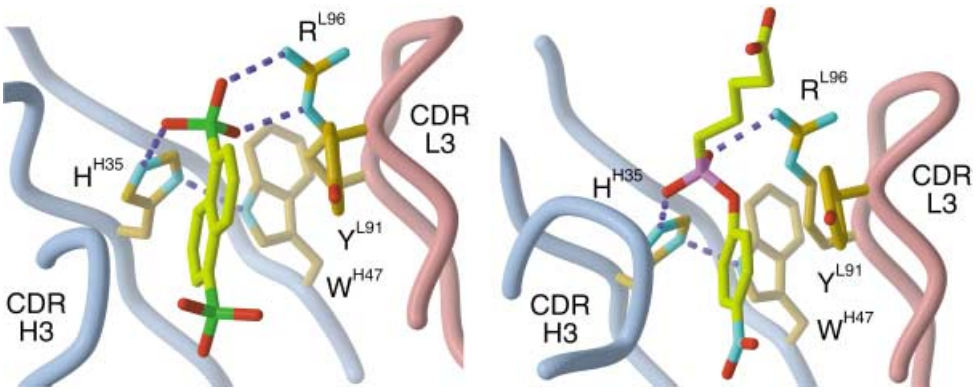
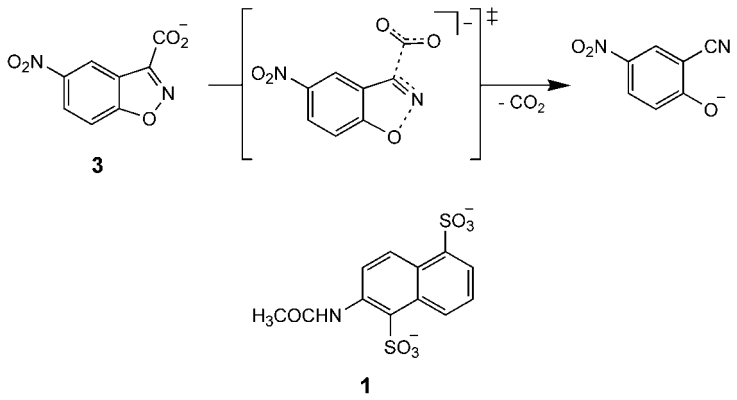


Fig. 1. *Comparison of 21D8* (left) [7] *and 48G7* (right) [8] *shows remarkable structural convergence for recognition of tetrahedral anions by independently derived antibodies.* C_α Traces of the light (L) and the heavy chains (H) are colored pink and blue, respectively; the segments corresponding to the CDR (*complementary-determining region*) loops are slightly darker and labeled. C-Atoms are yellow, O-atoms are red, N-atoms are blue, and S-atoms are green. Only the residues that significantly contribute to ligand recognition are displayed. The linker of the hapten for 21D8 is omitted for clarity. Figures were created with the programs Molscript [9], Raster3D [10], and Adobe Photoshop 5.0 (*Adobe Systems Inc.*).

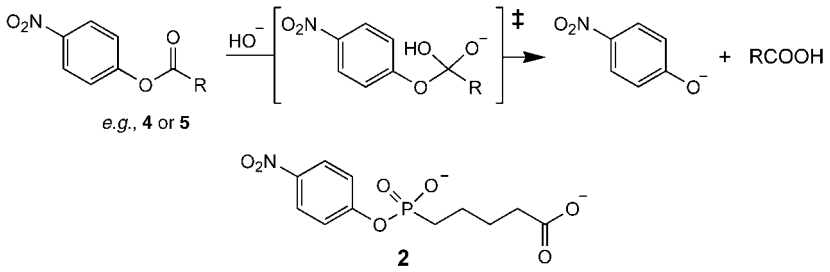
Antibody 21D8, which was elicited with **1**, was originally characterized as a catalyst for the medium-sensitive decarboxylation of 3-carboxy-5-nitro-1,2-benzisoxazole (**3**; *Scheme 1*). It accelerates this reaction 23,000-fold over background [11]. Detailed experimental [7][11–13] and theoretical [7][11] investigations have shown how this antibody exploits a mixture of polar and nonpolar sites for ligand binding and catalysis of a reaction normally facilitated by aprotic dipolar solvents. Specific subsite interactions between the transition state and polar residues like Arg^{L96} and His^{H35}, rather than the general hydrophobic nature of the active site, appear to be crucial [12].

Scheme 1



Aryl phosphonate **2** (*Scheme 2*) was designed as a transition-state analog for ester hydrolysis, and its cognate antibody 48G7 catalyzes the hydrolysis of a series of activated aryl esters **4** and aryl carbonates **5** with rate accelerations ranging from 2,000

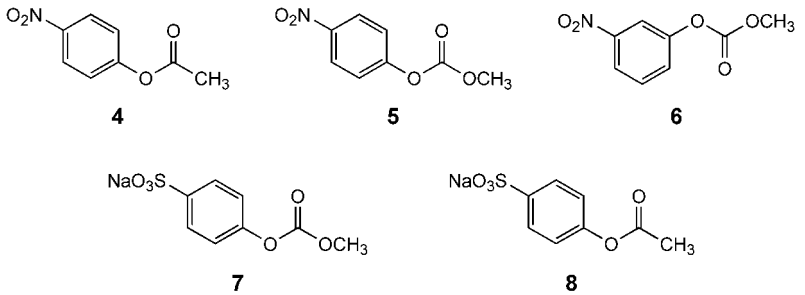
Scheme 2



to 40,000-fold over background [8][15]. Mature 48G7 has a 14,000-fold higher affinity for its hapten and a 20-fold higher activity than its germ-line precursor as a consequence of nine somatic mutations that lie outside the binding site [16]. These changes are responsible for subtle remodeling of the active site and positioning of residues, such as Tyr^{H33}, which contribute to transition-state stabilization [8].

Although 21D8 and 48G7 derive in part from different germ-line sequences and catalyze very different chemical reactions, they obviously share many features. Ten of the fourteen amino acid residues in close proximity to bound hapten are conserved [7], including Arg^{L96} and His^{H35}, which contribute to oxyanion stabilization. Furthermore, both antibodies bind the aryl group of their respective haptens in a similar way in a well-defined hydrophobic slot [7]. Based on these structural similarities, it was predicted that 21D8 might possess unanticipated hydrolytic activity in addition to its normal decarboxylase activity [7]. This conjecture is supported by the results of computational docking of 4-nitrophenyl acetate **4** into the binding site of 21D8, which indicate that the ester can bind in an orientation suitable for catalysis [17]. To test these predictions, we have examined the hydrolytic activity of 21D8 experimentally. Here, we report its ability to catalyze the hydrolysis of nitro- and sulfonate-substituted aryl esters and carbonates.

2. Results. – In analogy to 48G7, the hydrolysis of the aryl ester 4-nitrophenyl acetate (**4**), as well as the aryl carbonates methyl 4-nitrophenyl carbonate (**5**), and methyl 3-nitrophenyl carbonate (**6**) were investigated in the presence and absence of 21D8.



A comparison of the initial rates of product formation shows that the hydrolysis of all three compounds is accelerated approximately two- to four-fold over the spontaneous background reaction in the presence of 4.5 μM of IgG binding site. However, the rates of the hydrolytic reaction for substrates **4** and **6** with 21D8 do not differ significantly in the presence or absence of excess hapten **1**, indicating that the observed rate enhancement is the result of nonspecific catalysis to which the binding pocket makes no contribution. Instead, amino acid residues on the protein surface are presumably responsible [18]. In agreement with this view, unrelated antibodies accelerate these reactions to a similar extent and the rates are independent of added hapten (data not shown).

In contrast to substrates **4** and **6**, the 21D8-accelerated hydrolysis of methyl 4-nitrophenyl carbonate (**5**) is inhibited by increasing concentrations of hapten **1** (*Fig. 2*). When more than one hapten per antibody binding site is present in the reaction mixture, hydrolysis is reduced to the nonspecific background level.

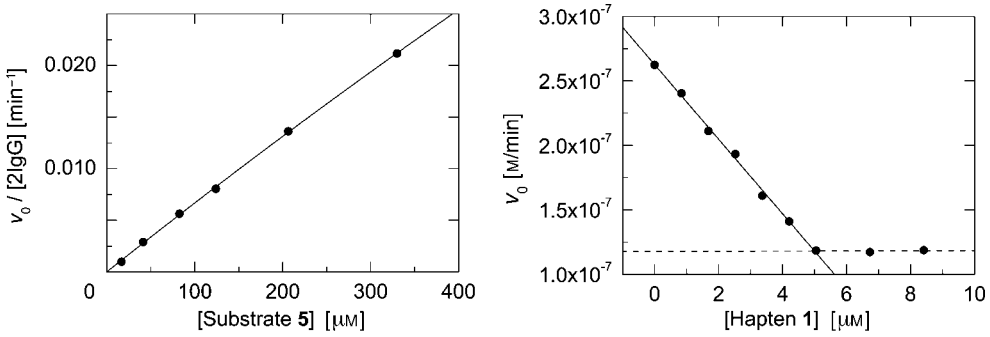


Fig. 2. *Initial rate* (left) *and hapten* **1** *inhibition* (right) *plots for the hydrolysis of methyl* 4*-nitrophenyl carbonate* (**5**) *by catalytic antibody 21D8*. Initial rate data obtained in the presence of 4.5 μM 21D8 (binding site) are plotted against increasing concentrations of substrate **5** (*left*) and hapten **1** (*right*), respectively.

Since the binding pocket of 21D8 is configured to bind an aromatic disulfonate dianion, the NO_2-substituted carbonate **5** is unlikely to be an optimal hydrolytic substrate. Replacement of the NO_2 substituent in the leaving group with a sulfonate should increase substrate affinity by harnessing extra binding energy from one of the two tetrahedral anion recognition sites and enhance water solubility, increasing the chances of being able to saturate the antibody with substrate. We, therefore, synthesized sodium 4-[(methoxycarbonyl)oxy]benzenesulfonate (**7**) and sodium 4-(acetoxy)benzenesulfonate (**8**), and investigated the kinetics of their hydrolysis.

Since the UV/VIS spectra for substrates **7** and **8**, and the hydrolysis product sodium 4-hydroxybenzenesulfonate (**9**) are very similar, direct spectroscopic monitoring of the reaction is not possible. Instead, the initial rates of reaction were determined after separation of substrate and product by RP-HPLC, both in the presence and in the absence of the antibody. Although the hydrolysis of sodium 4-[(methoxycarbonyl)-oxy]benzenesulfonate (**7**) was unaffected by 21D8, even at substrate concentrations as high as 6 mM, the hydrolysis of sodium 4-(acetoxy)benzenesulfonate (**8**) was accelerated moderately (*Fig. 3*). Further analysis demonstrated that the reaction is inhibited completely with 1 equiv. of hapten **1** per binding site (*Fig. 3*). Although the

hydrolysis product **9** could bind to at least one of the two anionic recognition sites in 21D8, product inhibition was not detected.

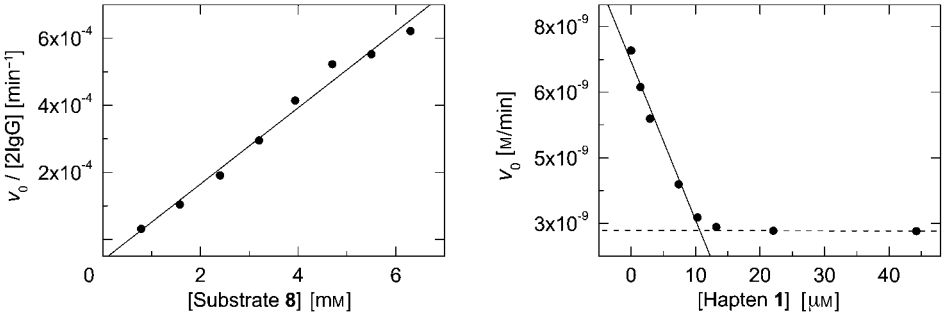


Fig. 3. *Initial rate* (left) *and hapten* **1** *inhibition* (right) *plots for the hydrolysis of sodium* 4*-(acetoxy)benzenesulfonate* (**8**) *by catalytic antibody 21D8*. Initial rate data obtained in the presence of 12.6 μM 21D8 (binding site) are plotted against increasing concentrations of substrate **8** (*left*) and hapten **1** (*right*), respectively.

For neither active substrate was it possible to determine the steady state kinetic parameters k_{cat} and K_m, as saturation was not observed at the solubility limit of **5** (*ca.* 300 μM) or up to 7 mM of **8**. These aryl esters apparently bind very weakly to the antibody. Nevertheless, the apparent bimolecular rate constants for the 21D8-catalyzed hydrolyses, k_{cat}/K_m, were estimated from the initial rates (*i.e.*, $[S] \ll K_m$) to be 66 M^{-1} min^{-1} and 0.11 M^{-1} min^{-1} for **5** and **8**, respectively. Dividing k_{cat}/K_m by k_{uncat}, the second-order rate constant for the corresponding uncatalyzed reaction provides a measure of catalytic proficiency [19], which was on the order of 10^5 M^{-1} for both substrates (*Table*). For comparison, mature 48G7 has a catalytic proficiency of 1.1×10^8 M^{-1} for the hydrolysis of 4-nitrophenyl esters, whereas the value for its germ-line precursor is 1.3×10^6 M^{-1}.

Table. *Kinetic Parameters for the 21D8-Catalyzed Hydrolysis of Substrates* **5** *and* **8**[a]), *and for the 21D8-Catalyzed Decarboxylation of 5-Nitro-1,2-benzisoxazole-3-carboxylate* (**3**) [31].

	k_{cat}/K_m [M^{-1} min^{-1}]	$(k_{cat}/K_m)/k_{uncat}$ [M^{-1}]
5	66	1.7×10^5
8	0.11	1.8×10^5
3	8.25×10^4	8.25×10^7

[a]) Assays were performed in 10 mM *Tris* · HCl (pH 8.0) at 20°.

3. Discussion. – Our results demonstrate that antibody 21D8 can catalyze the selective hydrolysis of *para*- but not *meta*-substituted esters and carbonates, a completely different reaction than the decarboxylation for which it was originally developed [11]. Nevertheless, its hydrolytic activity is rather weak. Judging from the respective chemical proficiencies (*Table*), the oxyanionic transition state for ester hydrolysis is stabilized *ca.* 500-times less effectively than the anionic but charge-delocalized transition state for decarboxylation of **3**.

Antibody 21D8 is also 760-fold less proficient in catalyzing hydrolytic reactions than the structurally related esterase 48G7, which was elicited with an aryl phosphonate transition-state analog [15]. It more closely resembles the germ-line precursor of 48G7 [8], which has a catalytic proficiency only eightfold higher than 21D8 and also displays inaccessibly high K_m values. Mutagenesis studies on 48G7 showed that the primitive active site was fine-tuned during the course of affinity maturation by independently optimizing the light and heavy chains with a small number of mutations [8]. Although 21D8 and 48G7 have very different V_H genes, their germ-line V_L genes are closely related. Conceivably, introduction of the $Asp^{L55}His$ and $Ser^{L34}Gly$ somatic mutations, which significantly enhanced ligand affinity and catalytic efficiency in 48G7 [8], might similarly augment the hydrolytic properties of the decarboxylase active site.

Control experiments show that the hydrolytic activity of 21D8 is not a general feature of antibodies but appears to result from the fortuitous constellation of residues present in this class of aryl oxyanion binders. Nevertheless, surprisingly diverse reaction profiles have been seen in other antibodies. For example, 14D9, which was generated against a substituted piperidinium hapten, catalyzes the hydrolysis of enol ethers [20], glycosides [21], ketals [22], and epoxides [23]. Another example is the aldolase 38C2, which accelerates a broad range of aldol and *retro*-aldol reactions [24][25], *Robinson* annulations [26], *retro-Michael* reactions [27], and β-keto acid decarboxylations [28]. In contrast to 21D8, however, the observed catalytic versatility in these cases depends on an unusually reactive residue, a carboxylic acid in 14D9 [29] and a lysine amine in 38C2 [24], located in a generic hydrophobic binding pocket.

Catalytic promiscuity is a hallmark of other proteins as well [30]. Bovine serum albumin (BSA) is a notable example. It binds a large variety of aromatic anions and catalyzes many different transformations [31]. For example, both the hydrolysis of ester **4** [32] and the decarboxylation of **3** [33] are accelerated by BSA, albeit with rate accelerations (k_{cat}/k_{uncat}) that are 30- and 150-times lower, respectively, than with 48G7 and 21D8. The chemical proficiencies for both reactions are also two orders of magnitude lower than for the corresponding antibody-catalyzed processes.

It has been argued that the ability of an enzyme to catalyze reactions unrelated to its normal biological function, even at a low level, may provide an evolutionary head start in the creation of new activities [30][34]. That antibody scaffolds, like the oxyanion binders discussed here, possess low but intrinsic catalytic activities may similarly account for the success in generating tailored antibody catalysts for so many different reactions [4]: immunization with a transition-state analog may simply enhance features already extant in certain classes of immunoglobulin. Given the relatively modest activities of even the best antibody catalysts (compared to natural enzymes), however, it is also possible that the limited structural diversity of the immune system imposes inherent limits on catalytic efficiency. New strategies for further increasing transition-state-analog affinity [35] or genetically selecting for catalytic activity directly [36] will be needed to determine whether the antibody scaffolds that have been characterized to date are evolutionary dead ends or local minima that can be further improved.

Experimental Part

General. Reagent-grade solvents and reagents were purchased from commercial suppliers and used without further purification. *4-Nitrophenyl acetate* (**4**) was obtained from *Aldrich*. *Methyl 4-nitrophenyl carbonate* (**5**) [37], *methyl 3-nitrophenyl carbonate* (**6**) [38], and *sodium 4-(acetoxy)benzenesulfonate* (**8**) [39] were synthesized according to published procedures and gave satisfactory spectroscopic and elemental data. HPLC Analyses were performed with a *Macherey & Nagel Nucleosil* (4.6 mm × 250 mm, 100–5 μm; *C-18*) stationary phase on a *Waters* HPLC system, eluting with a linear gradient from 95% H_2O (containing 0.1% CF_3COOH) to 95% MeCN (containing 0.05% CF_3COOH) over 45 min at an elution rate of 1.2 ml/min and an UV/VIS detector set to an observation wavelength of 256 nm. The identities of the observed peaks were verified by injecting authentic samples under the same conditions. ^{1}H-NMR Spectra (300 MHz) were recorded on *Varian XL-300* instruments at 298 K; chemical shifts (δ) in ppm downfield from TMS ($\delta = 0$ ppm).

Sodium 4-[(Methoxycarbonyl)oxy]benzenesulfonate (**7**). Compound **7** was prepared according to modified published procedures [40]. Sodium 4-hydroxybenzenesulfonate dihydrate (7.5 g, 32.3 mmol) was dissolved in a soln. of NaOH (1.3 g, 32.5 mmol) in a mixture of H_2O (15 ml) and THF (11 ml). Methyl chloroformate (7.5 ml, 97.5 mmol) was added dropwise at 0°. After completion of the addition, the mixture was removed from the ice bath and stirred for an additional 1 h at r.t. Removal of the solvents under reduced pressure gave a white solid, which was recrystallized from aq. EtOH to give 6.4 g of **7** (78%). ^{1}H-NMR (CD_3OD, 300 MHz): 7.77 (*m*, 2 H); 7.13 (*m*, 2 H); 3.79 (*s*, 3 H).

Antibody Generation and Purification. Murine IgG 21D8 was produced from the corresponding hybridoma cell line [11] in a hollow-fiber bioreactor (*Cell-Pharm 100, Unisyn*), with *Iscove's Modified Dulbecco's Medium* (*IMDM*) supplemented with 5% fetal bovine serum (FBS). Cell suspension was harvested continually, centrifuged, and concentrated with an *Amicon YM 30k* membrane. The concentrate was purified first by affinity chromatography on a *Protein G Sepharose* column with 100 mM glycine · HCl, pH 2.7, as elution buffer, and subsequently by ion-exchange chromatography, with a prepacked *Mono Q* anion-exchange column *HR 10/10* on a FPLC system (*Pharmacia*) with PBS as elution buffer. The purified antibody was concentrated with *Macrosep 30k* centrifuge filters to a final concentration of 5–10 mg/ml. The purity of the sample was confirmed by SDS-PAGE. Antibody concentrations were determined by absorbance at 280 nm (1 mg/ml = 1.37 OD, assuming a molecular weight of 160,000 for IgG). The protein solns. were stored in PBS buffer at 4° containing 0.01% NaN_3. Catalytic activity was tested by monitoring the decarboxylation of *5-nitro-1,2-benzisoxazole-3-carboxylate* (**3**) as described in [8]. The observed data are in good agreement with the published values.

Kinetic Analyses. All assays were performed in aq. buffer (10 mM *Tris* · HCl buffer (pH 8.0)) at 20.0 ± 0.1°. Initial rates of **4** and **5** were determined spectroscopically at 405 nm ($\Delta\varepsilon = 15300$ M^{-1} cm^{-1}). Initial rates of hydrolysis of **6** were determined spectroscopically at 340 nm ($\Delta\varepsilon = 1580$ M^{-1} cm^{-1}). The antibody concentration [2IgG] was 4.5 μM. In the case of the sulfonates **7** and **8**, the initial rates of hydrolysis were determined after separation by anal. RP-HPLC. In a typical experiment, increasing concentrations of the substrates in a total sample volume of 100 μl each, prepared by dissolving the substrates in a buffer soln. of the antibody, were allowed to stand for at least 3 h at 20°. After addition of 2 μl of acetophenone (51 mM) as an internal standard, 50 μl of each sample soln. were injected onto the HPLC column at increasing time intervals and constant temp. (20°). The antibody concentration [2IgG] was 12.6 μM. The observed peak areas were related to a calibration curve, generated by injecting increasing concentrations of sodium 4-hydroxybenzenesulfonate (**9**) (0–5 mM) in buffer at 20°.

All data were corrected for the corresponding spontaneous background reaction, which was assessed under the same conditions. The values of k_{cat}/K_m were derived from the slope of the plot of initial rate (v_0) *vs.* substrate concentration [S]: $k_{cat}/K_m = v_0/([S][2IgG])$.

Hapten Inhibition Assays. Initial rates of hydrolysis of **5** were determined spectroscopically at 405 nm ($\Delta\varepsilon = 15300$ M^{-1} cm^{-1}) in the presence of 4.5 μM 21D8 (binding site) and increasing concentrations of hapten **1**. Initial rates of hydrolysis of **8** were determined by anal. RP-HPLC in the presence of 12.6 μM 21D8 (binding site) and increasing concentrations of hapten **1**. Initial rates were plotted against hapten concentration.

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