# Catalysis of 3-Carboxy-1,2-benzisoxazole Decarboxylation by Hydrophobic Antibody Binding Pockets

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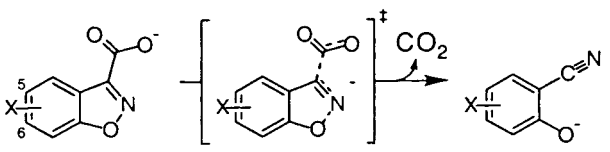
Dedicated to *Albert Eschenmoser* on the occasion of his 75th birthday

Monoclonal antibodies were generated against a 3-phenyl-1,2-benzisoxazole derivative and shown to catalyze the solvent-sensitive decarboxylation of 3-carboxy-1,2-benzisoxazoles. In addition to rate accelerations up to 2300-fold over background, the antibodies exhibit distinctive selectivities for substrates bearing 5- or 6-$NO_2$ substituents, with preferential decarboxylation of the less reactive substrate in one case. These effects are the likely consequence of substrate destabilization, achieved by forcing the carboxylate group into a relatively apolar binding pocket and stabilization of the charge-delocalized transition state through dispersive interactions. Comparison with a more active antibody decarboxylase previously raised against 2-acetamidonaphthalene-1,5-disulfonate suggests, however, that a judicious mix of polar and apolar interactions may ultimately be more effective for achieving high decarboxylase activity.

**1. Introduction.** – The decarboxylation of 3-carboxy-1,2-benzisoxazoles (**1**) to give salicylonitriles (2-hydroxybenzonitriles; **2**) is remarkably sensitive to solvent effects [1]. Upon transfer of the reaction from aqueous solution to aprotic dipolar solvents, rate increases of up to $10^8$-fold can be observed. Protic solvents apparently inhibit the reaction by stabilizing the localized charge on the ground-state carboxylate through H-bonding, whereas aprotic dipolar solvents are believed to accelerate the reaction by stabilizing the charge-delocalized transition state through dispersion interactions and by breaking up H-bonded ion pairs. These properties have made this a valuable model system for probing the microenvironment of micelles, vesicles, and synthetic polymers [2].

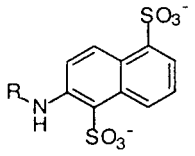
Recently, antibody catalysis of this reaction has also been used to explore the contribution of medium effects to the overall efficiency of enzymes. Antibodies raised against a derivative of 2-acetamidonaphthalene-1,5-disulfonate (**4**) have been shown to accelerate the decarboxylation of **1a** by as much as 23,000-fold over the spontaneous reaction in $H_2O$ [3][4]. The crystal structure of 21D8 [5], one of the most efficient antibody decarboxylases, has revealed how a melange of polar and apolar interactions induced in response to the hapten are used to achieve both substrate binding and transition-state stabilization. Nevertheless, hapten **4** does not mimic the transition state for decarboxylation of 3-carboxy-1,2-benzisoxazoles particularly well. The rate accelerations of the best catalysts elicited by it are four orders of magnitude lower than those achieved by transferring the reaction to a dipolar aprotic solvent such as hexamethylphosphoramide [1], raising the question of whether better catalysts might be designed.



**1a** X = 5-$NO_2$
**1b** X = 6-$NO_2$

**2**

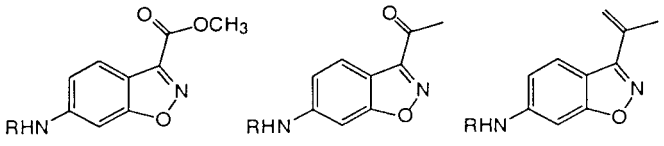
**3**



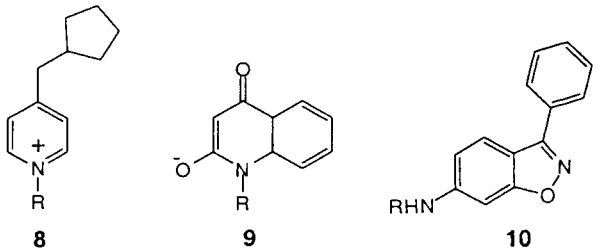
**4** R = Ac

Sulfonate groups were originally included in hapten **4** to induce cationic counterions for binding the anionic species along the reaction coordinate [3], but residual H-bonding interactions with the bound substrate are likely to limit the efficiency of catalysis in this system [5]. A potentially more effective strategy might involve creating an aprotic binding pocket for the charged carboxylate. Although preliminary experiments with haptens like **5**, **6**, and **7**, which have neutral surrogates for the substrate carboxylate group, failed to yield catalysts for the decarboxylation of **1** [3][6], antibodies raised against compounds **8** [7] and **9** [8], which have larger hydrophobic groups, reportedly accelerate the decarboxylation of pyridine-4-acetic acid and orotate, respectively. To see whether an analogous approach can be applied to the decarboxylation of 3-carboxy-1,2-benzisoxazoles, we have raised antibodies against the 3-phenyl-1,2-benzisoxazole derivative **10**. Here, we report the characterization of the resulting catalysts and a comparison of their properties with those of anti-**4** antibodies.

**2. Results.** – 3-Phenyl-1,2-benzisoxazol-5-amine **11** was prepared by reduction of the corresponding nitro compound [9], and conjugated to carrier proteins *via* a

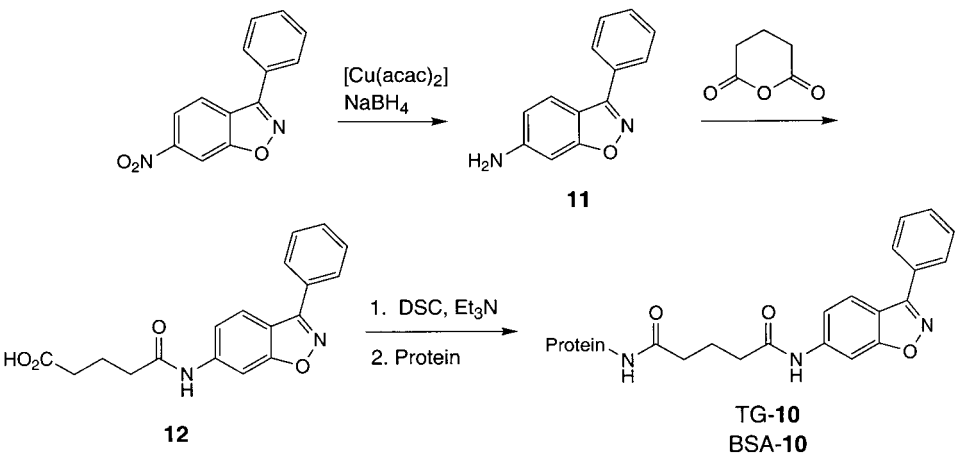


**5** **6** **7**



**8** **9** **10**

*Scheme*




glutaric-acid linker (*Scheme*). The thyroglobulin-**10** conjugate was used to generate an immune response, and monoclonal antibodies were prepared by standard methods [10]. In a single fusion, 400 hybridomas were obtained that secreted antibodies specific for the hapten. Tissue culture supernatants were screened directly for decarboxylase activity in sets of 96 with a kinetic microplate reader using both 5- and 6-nitro-substituted 3-carboxy-1,2-benzisoxazoles as substrates. In contrast to our previous experience with antibodies raised against hapten **4** [3], little or no activity over background was detected for any of the clones under these conditions. For this reason, 20 monoclonal antibodies with high affnity for the hapten were selected at random, subcloned, and propagated in mouse ascites.

The antibodies were purified by affinity and ion-exchange chromatography and then tested individually for decarboxylation activity with both **1a** and **1b**. Three catalysts were identified (23E10, 36A7, and 43A10) and subjected to further characterization. In each case, the rate of decarboxylation was found to depend linearly on antibody concentration. Moreover, catalysis decreased with increasing concentration of hapten **12** and was completely abolished in the presence of 1 equiv. of hapten per binding site. All three antibodies bind the hapten with nM affinity (*Fig. 1* and *Table 1*), as determined by tryptophan fluorescence titration [11].

Table 1. *Dissociation Constants for Antibody·***12** *Complexes*

| | 23E10 | 36A7 | 43A10 | 21D8 |
|---|---|---|---|---|
| $K_d$ [nM] | $2.6 \pm 1.5$ | $4.9 \pm 0.85$ | $2.6 \pm 1.5$ | $6.8 \pm 0.2$ |

Saturation kinetics were observed for the antibody-catalyzed decarboxylation of both substrates **1a** and **1b** (*Fig. 2*). Clones 23E10 and 43A10 have similar activity and selectivity, whereas 36A7 appears distinct. Representative steady-state kinetic parameters for 23E10 and 36A7 are summarized in *Table 2*. For comparison, data are also

presented for the previously described decarboxylase antibody 21D8 [3] and for bovine serum albumin (BSA), which catalyzes a variety of solvent-sensitive reactions [12], including the decarboxylation of **1**. The new antibodies are substantially better catalysts than BSA, but less active than 21D8. Interestingly, 36A7 achieves a greater rate acceleration for the less reactive 6-substituted substrate ($k_{cat}/k_{uncat} = 2300$ *vs.* 750 for **1b** and **1a**, resp.). The spontaneous decarboxylation of 3-carboxy-6-nitro-1,2-benzisoxazole is 16-times slower than that of the 5-$NO_2$ derivative [1], but, in the antibody binding pocket, it is only six-times slower. Moreover, **1b** binds roughly five-times more tightly to the antibody than **1a** (as judged by the relative $K_m$ values), in accord with the structure of the hapten that was coupled to carrier proteins through a linker attached at C(6) of the 1,2-benzisoxazole ring. As a result, these two substrates have comparable specificity constants ($k_{cat}/K_m$). In contrast, the specificity of 23E10 and BSA matches the intrinsic reactivity of **1a** and **1b**, with greater rate accelerations for the more reactive 5-$NO_2$ compound.

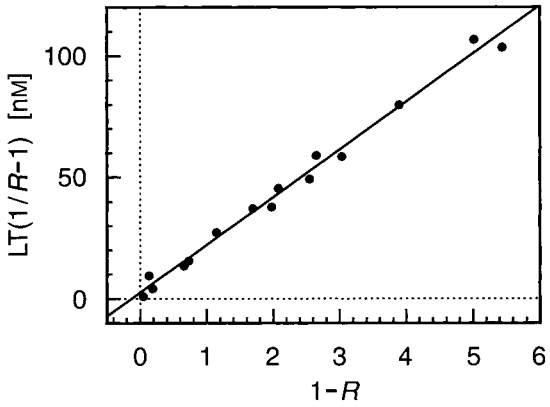



Fig. 1. *Representative* Scatchard *plot for the binding of derivatized hapten* **12** *by antibody 23E10*. Quenching of the fluorescence emission was plotted against increasing concentrations of **12**. The measurements were performed in the presence of an initial concentration of 170 nM 23E10 (binding site). Linear regression analysis of the data yielded a straight line with a correlation coefficient of 0.995.

**3. Discussion.** – Medium effects are believed to contribute significantly to the efficiency of natural decarboxylase enzymes [13]. For example, studies on histidine decarboxylase, which is potently inhibited by L-histidine methyl ester [14], have revealed a predominantly hydrophobic binding site for the substrate carboxylate [15]. As pointed out by *Jencks* [16], if sufficient binding energy is available to force the charged substrate into the destabilizing hydrophobic environment and if the destabilization is relieved at the transition state, very large rate accelerations ($k_{cat}/k_{uncat}$) should be accessible.

Nevertheless, initial attempts to elicit antibody catalysts using methyl ester **5** as a hapten were unsuccessful [3]. Haptens with similarly sized ketone and isopropenyl groups in place of the ester (*i.e.*, **6** and **7**) also failed [6]. The binding pockets elicited by these carboxylate surrogates may simply have been too spatially constrained to accommodate the charged substrate, since haptens like **8** and **9**, in which relatively large

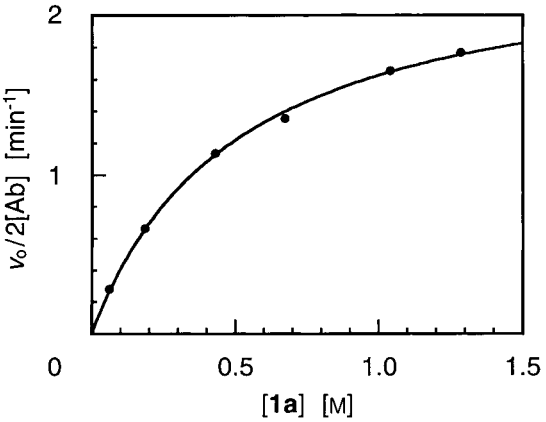



Fig. 2. *Representative* Michaelis-Menten *plot for the decarboxylation of 3-carboxy-5-nitro-1,2-benzisoxazole by catalytic antibody 23E10*. Initial rate data obtained in the presence of 1.17 μM 23E10 (binding site) are plotted against increasing concentrations of **1a**. The data were fit as described in the *Exper. Part*.

Table 2. *Kinetic Parameters for the Decarboxylation of Substrates* **1a** *and* **1b**[a])

| | $k_{cat}$ [$min^{-1}$] | $K_m$ [μM] | $k_{cat}/K_m$ [$M^{-1}$ $min^{-1}$] | $k_{cat}/k_{uncat}$ |
|---|---|---|---|---|
| *3-Carboxy-5-nitro-1,2-benzisoxazole* (**1a**) | | | | |
| 23E10 | 2.4 ± 0.07 | 500 ± 33 | 4900 | 2600 |
| 36A7 | 0.78 ± 0.02 | 600 ± 41 | 1300 | 850 |
| 21D8[b]) | 17 ± 0.4 | 210 ± 12 | 82500 | 17000 |
| BSA | 0.10 ± 0.002 | 130 ± 9 | 770 | 110 |
| *3-Carboxy-6-nitro-1,2-benzisoxazole* (**1b**) | | | | |
| 23E10 | 0.042 ± 0.003 | 300 ± 33 | 140 | 750 |
| 36A7 | 0.13 ± 0.004 | 120 ± 41 | 1100 | 2300 |
| 21D8[c]) | n.d. | n.d. | n.d. | n.d. |
| BSA | 0.0029 ± 0.0001 | 160 ± 13 | 19 | 52 |

[a]) Assays were performed in 10 mM *Tris* · HCl, 100 mM NaCl (pH 8.0) at 20°. [b]) Data are from [3]. [c]) n.d.: Not determined.

hydrophobic groups were used to elicit more spacious pockets, yielded antibodies with significant decarboxylase activity for other substrates [7][8]. Our studies with **10**, containing a Ph ring in place of the substrate carboxylate, strengthen this conclusion.

The success of this strategy depends on marshaling sufficient binding interactions with the rigid 1,2-benzisoxazole ring to force the negatively charged carboxylate of **1** into the induced apolar pocket. Although we cannot be certain about the precise microenvironment of the bound substrate, insofar as $K_m$ values provide information about substrate affinity, we see that carboxylates **1** are well-recognized by the anti-**10** antibodies (*Table 2*). They bind roughly $10^4$- to $10^5$-fold less tightly than hapten (*Table 1*), however, as would be expected if the carboxylate occupied the relatively apolar pocket induced by the Ph group of **10**. Moreover, the modest (2- to 5-fold) preference for the 6-$NO_2$ over the 5-$NO_2$ substrate, evident for both 23E10 and 36A7,

reflects the way in which the hapten was linked to the carrier proteins. Because the edge of the ring bearing the 5- and 6-substituents presumably binds near the mouth of the combining site, discrimination is not high, however.

In the case of 36A7, the higher affinity for the 6-$NO_2$ substrate is directly manifest in greater catalytic efficiency ($k_{cat}/k_{uncat}$). Despite the 16-fold higher reactivity of **1a** over **1b**, the two substrates are processed equally well by this antibody as judged by their respective specificity constants $k_{cat}/K_m$. The less reactive compound is a comparatively better substrate, perhaps because its 6-substituent more effectively positions the carboxylate within the desolvating environment of the active site. In contrast, 23E10 (and BSA) achieve the largest effects with the more reactive **1a** (*Table 2*). Apparently, the $NO_2$ group has little directing influence on the orientation of the bound substrate in these catalysts. Its electronic role in stabilizing the incipient phenolate appears to be much more important, leading to enhanced specificity for **1a** ($(k_{cat}/K_m)_{\mathbf{1a}}/(k_{cat}/K_m)_{\mathbf{1b}} = 35$ for 23E10) compared to the uncatalyzed decarboxylation in $H_2O$. This increase in specificity provides some evidence for an aprotic reaction environment, since it is known that transfer of the substrate from $H_2O$ to aprotic dipolar solvents is accompanied by an increase in *Hammett* $\rho$ value [1].

Although significant rate accelerations have been achieved in these experiments, the anti-**10** antibodies are substantially less efficient than the best catalysts generated in response to the naphthalene-1,5-disulfonate **4**. Their low activity suggests that the bound substrate is only partially desolvated, and screening larger populations of antibodies might yield catalysts that are more effective in this regard. It is also possible that the relatively weak, non-directional *van der Waals* interactions available for binding the small 1,2-benzisoxazole ring will always be insufficient to drive its appended carboxylate into a completely aprotic environment. In 21D8, the structurally characterized anti-**4** decarboxylase [5], electrostatic interactions appear to provide the primary driving force for binding all the anionic species along the reaction coordinate. Model studies by *Kemp* [17] have shown that such interactions are not necessarily deleterious for catalysis, so long as strong H-bonding with the carboxylate group is avoided. In addition, transition-state docking experiments indicate that specific H-bonding interactions between H-bond donors in the combining site of 21D8 and the heteroatoms of the breaking N–O bond are crucial for lowering the barrier to reaction [5], in agreement with previous theoretical considerations [18]. Analogous interactions are not programmed by hapten **10** and are likely absent in the active sites of 23E10 and 36A7.

While natural decarboxylases often have reasonably hydrophobic pockets, they (like 21D8) typically contain at least one ionizable group in the vicinity of the substrate carboxylate that may facilitate substrate binding in a relatively low dielectric environment and/or serve as a proton source following decarboxylation [15][19]. They also exploit extensive non-covalent or even covalent interactions to hold their substrates in a destabilizing environment. For example, recent studies on orotidine 5′-monophosphate (OMP) decarboxylase, one of the most proficient enzymes known, have revealed numerous interactions with the phosphoribosyl group that anchor the pyrimidine of OMP within the active site [20]. An eight order of magnitude decrease in efficiency upon removal of the substrate phosphate group underscores the importance of such interactions for catalysis [21]. Interestingly, substrate destabilization in OMP

decarboxylase has been proposed to arise not from a low dielectric environment, but from unfavorable electrostatic interactions between the substrate carboxylate and an active-site aspartate residue [20].

Antibody catalysis provides us with an opportunity to systematically explore multiple strategies for generating protein catalysts. The results of the present study highlight the critical balance between substrate-destabilizing and transition-state-stabilizing effects in the design of decarboxylases. Structural comparisons of the apolar active sites generated here and the previously characterized 21D8 are likely to supply additional valuable insights into the requirements for efficient catalysis and, at the same time, suggest approaches for improving these first-generation catalysts.

## Experimental Part

*General*. Reagent-grade solvents and reagents were purchased from commercial suppliers and used without further purification unless otherwise stated. *3-Carboxy-5-nitro-1,2-benzisoxazole* (**1a**) and *3-carboxy-6-nitro-1,2-benzisoxazole* (**1b**) were synthesized according to literature procedures [1]. M.p.: uncorrected. HPLC Analyses were performed with a *Rainin Microsorb-MV 86-203* (*C-18*) stationary phase with a 1:1 mixture of MeCN and $H_2O$ without $CF_3COOH$ at an elution rate of 1.0 ml/min. The identity of the observed peaks was verified by injecting authentic samples. $^1$H- and $^{13}$C-NMR spectra were recorded on *Bruker AC 250* and *500* instruments at 298 K; chemical shifts $\delta$ in ppm are referenced to the solvent peak. Mass spectra were obtained with an *IonSpec Ultima FT-ICR* mass spectrometer using 2,5-dihydroxibenzoic acid as matrix and detection in the positive-ion mode.

*6-Amino-3-phenyl-1,2-benzisoxazole* (**11**). A soln. of $NaBH_4$ (3 mg, 0.083 mmol) in EtOH was added to a stirred suspension of copper(II) acetylacetonate (4.4 mg, 0.017 mmol) in i-PrOH (200 µl) under $N_2$ at r.t. [22]. 6-Nitro-3-phenyl-1,2-benzisoxazole (20 mg, 0.083 mmol) was added to this soln., followed by 2 equiv. of $NaBH_4$ (6.5 mg, 0.17 mmol) in EtOH. The reaction was monitored by TLC, and, upon total consumption of the starting material, solvent was removed under reduced pressure. The residue was extracted with $CHCl_3$ and purified by silica-gel chromatography (AcOEt/hexanes 3:7) to give 15.6 mg of **11** (89%). $R_f$ (AcOEt/hexane 3:7) 0.22. $^1$H-NMR ($CDCl_3$, 250 MHz): 7.88–7.96 (*m*, 2 H); 7.64 (*d*, *J* = 6.9, 1 H); 7.48–7.58 (*m*, 3 H); 6.81 (*d*, *J* = 1.6, 1 H); 6.71 (*dd*, *J* = 6.9, 1.6, 1 H); 4.13 (*s*, 2 H).

*5-Oxo-5-[(3-phenyl-1,2-benzisoxazol-6-yl)amino]pentanoic Acid* (**12**). 6-Amino-3-phenyl-1,2-benzisoxazole (14.3 mg, 0.068 mmol) and glutaric anhydride (10 mg, 0.088 mmol) were dissolved in 5 ml of dry acetone, and the mixture was refluxed for 5 h. Removal of solvent *in vacuo*, followed by crystallization with AcOEt/hexane, afforded **12** in quant. yield (22 mg, 0.068 mmol). M.p. 203–204°. $R_f$ (MeOH/$CH_2Cl_2$ 3:7) 0.43. $^1$H-NMR ((D$_6$)DMSO, 500 MHz): 10.57 (*s*, 1 H); 8.29 (*d*, *J* = 1.3, 1 H); 8.03 (*d*, *J* = 8.7, 2 H); 7.99–8.01 (*m*, 2 H); 7.61–7.65 (*m*, 3 H); 7.48 (*dd*, *J* = 8.7, 1.3, 1 H); 2.44 (*d*, *J* = 7.4, 2 H); 2.28 (*d*, *J* = 7.3, 2 H); 1.87–1.81 (*m*, 2 H). $^{13}$C-NMR ((D$_6$)DMSO, 125 MHz): 174.11; 171.66; 164.05; 156.17; 141.49; 130.44; 129.27; 128.09; 127.66; 122.56; 116.71; 114.49; 98.52; 35.60; 33.24; 20.34. HR-MS (MALDI): 325.119 ($[M+H]^+$, $C_{18}H_{17}N_2O_4$; calc. 325.1188).

*Protein Conjugates*. The hapten was activated as an *N*-hydroxysuccinimide ester immediately prior to coupling. Compound **12** (10 mg, 0.031 mmol) was dissolved in 500 µl of dry $CHCl_3$ under $N_2$. $Et_3N$ (3.5 mg, 0.034 mmol) was added, followed by a soln. of *N*,*N*′-disuccinimidyl carbonate (8.5 mg, 0.033 mmol) in 500 µl of $CHCl_3$ and 50 µl DMF, and the mixture was allowed to stir until the carboxylate was completely consumed as judged by TLC. Solvent was removed *in vacuo*, and the residue was redissolved in AcOEt and filtered through silica gel to give 13 mg of the desired product (79%). A 20 mM soln. of the *N*-hydroxysuccinimide ester in dioxane was added dropwise to a vortexed soln. of either bovine serum albumin (BSA, 1 mg/ml) or thyroglobulin (TG, 2 mg/ml) in 100 mM borate buffer (pH 10.0). Typically, a 100–1000-fold molar excess of hapten was used. After incubating for 90 min at r.t., the protein-hapten conjugates were separated from unreacted hapten by gel filtration on a *Sephadex G-25* column eluted with 100 mM borate buffer (pH 10.0). Protein concentration was determined by the BCA method [23], while the residual amine groups were titrated with 2,4,6-trinitrobenzenesulfonic acid [24]. For BSA, 22 of 61 lysine residues were modified, while 42 of 170 lysine residues were modified in TG.

*Antibody Preparation*. Mice (129 GIX$^+$ strain) were immunized with the TG conjugates of **10**. Serum titer was checked with the corresponding BSA conjugates by enzyme-linked immunosorbent assay (ELISA) [25]. Hybridomas were obtained by fusion of SP2/0$^+$ myeloma cells with spleen cells from the immunized mice according to standard protocols [10]. Tissue-culture supernatants were screened for high affinity anti-**10**

antibodies by ELISA and assayed directly for decarboxylase activity in sets of 96 with a kinetic microtiter plate reader as described in [3]. Selected hybridomas were subcloned twice and propagated as ascites in (BALB/c × 129GIX$^+$)F1 mice. Monoclonal antibodies were obtained from ascites fluid by $(NH_4)_2SO_4$ precipitation, followed by chromatography on *DEAE-Sephacel* with a NaCl step gradient (0, 50, 100, 200, 500 mM) in 10 mM *Tris* (pH 8.0). Antibodies were further purified by FPLC on *Protein G Sepharose* followed by *Mono Q HR 10/10*. Antibody concentrations were determined by absorbance at 280 nm (1 mg ml$^{-1}$ = 1.37 OD, assuming a molecular weight of 160,000 for IgG).

*Kinetic Analyses.* All assays were performed in 10 mM *Tris* · HCl, 100 mM NaCl buffer (pH 8.0) at 20°, unless indicated otherwise. Initial rates of decarboxylation were determined spectroscopically by measuring the absorbance increase at 380 nm ($\Delta\varepsilon = 10800$ M$^{-1}$ cm$^{-1}$) for 3-carboxy-5-nitro-1,2-benzisoxazole (**1a**) and at 398 nm ($\Delta\varepsilon = 3026$ M$^{-1}$ cm$^{-1}$) for the 6-$NO_2$ substrate **1b**. The product of the reaction was confirmed by HPLC and comparison with an authentic sample. The data were corrected for the spontaneous background reaction, which was measured under the same conditions and in good agreement with previously reported values [1]. Steady state kinetic parameters $k_{cat}$ and $K_m$ were calculated from initial rates using the *Michaelis-Menten* equation: $v_0/[E] = k_{cat}[S]/(K_m + [S])$, where $v_0$ is the initial rate, [E] is the antibody binding-site concentration, and [S] is the substrate concentration.

*Fluorescence Spectroscopy.* Fluorescence measurements were performed with an *Aminco-Bowman* series *2* luminescence spectrometer at r.t. For fluorescence titration experiments [11], tryptophan emission was monitored at 333 nm following excitation at 285 nm. Fluorescence intensity was recored in the absence of hapten ($F_E$), in excess of hapten ($F_{EL}$), and over the increasing concentration of hapten at a fixed concentration of antibody ($F$). All emission spectra were corrected by subtracting appropriate background spectra. Dissociation constants ($K_d$) were obtained by *Scatchard* analysis from the equation $(L_T)(1/R - 1) = K_d + nE_T(1 - R)$, where $L_T$ is the total hapten concentration, $n$ is the number of ligand binding sites, $E_T$ is the total antibody concentration, and $R$ is the ratio of the antibody-hapten complex to total antibody concentration ($E_L/E_T$). The ratio $R$ was quantified by measuring $(F_E - F)/(F_E - F_{EL})$ at a given hapten concentration $L_T$.

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