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Toward bifunctional antibody catalysis

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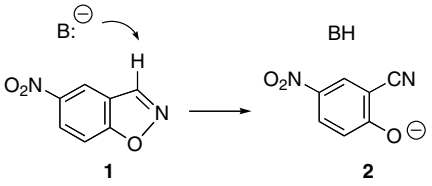
Abstract—Antibodies that catalyze the deprotonation of unactivated benzisoxazoles to give the corresponding salicylonitriles were prepared using as antigen a 2-aminobenzimidazolium derivative coupled to a carrier protein via its benzene ring. The hapten was designed to induce an antibody binding site with both a base and an acid, in position to initiate proton transfer and stabilize developing negative charge at the phenoxide leaving group, respectively. Consistent with this design, the catalysts exhibit bell-shaped pH-rate profiles, while chemical modification identified several functional groups that could participate in bifunctional catalysis. One of the antibodies, 13G5, is particularly notable in catalyzing the elimination of 6-glutaramidebenzisoxazole with a $>10^5$-fold rate acceleration over background and an effective molarity of $>10^4$ M for its catalytic base. These properties compare favorably to the efficiencies achieved by the best previously characterized antibodies with substantially more reactive substrates.

1. Introduction

Highly evolved enzymes often depend on the cooperative interaction of multiple functional groups. Although much progress has been made toward constructing synthetic receptors containing arrays of acids and bases capable of concerted catalysis,[1] introduction of such ordered arrays into protein pockets still poses a significant experimental challenge.

Charge complementarity between antibodies and antigen represents one strategy that has been successfully implemented to create immunoglobulin binding sites possessing a single catalytic acid or base. Positively charged amidinium[2,3] and guanidinium haptens,[4–7] for example, have yielded antibodies that exploit negatively charged carboxylate residues to deprotonate benzisoxazoles to give salicylonitriles (e.g., **1** → **2**; Scheme 1), a well-studied model system for proton transfer.[8,9]

Antibody 34E4, which was generated in response to the 2-aminobenzimidazolium derivative **3** (Scheme 2), is particularly notable for catalyzing the decomposition of 5-nitrobenzisoxazole with large rate accelerations and multiple turnovers.[4] The crystal structure of the catalyst complexed with its hapten shows that a combination of hydrogen bonding, π stacking, and van der Waals interactions is utilized to position both the induced base, Glu^{H50}, and the substrate for efficient proton transfer.[5] Although mutagenesis experiments suggest that Glu^{H50} is as effective as carboxylate bases



Scheme 1. Base-promoted Kemp elimination of 5-nitrobenzisoxazole.

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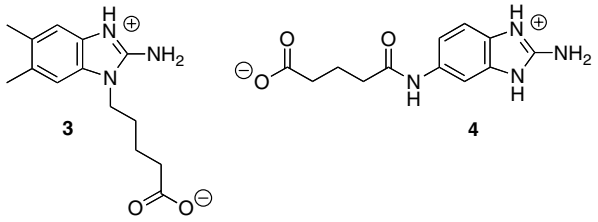


Scheme 2. Haptens for eliciting antibody catalysts for the Kemp elimination. Compound **3** previously afforded antibody 34E4, whereas compound **4** was used in the current study.

in typical enzymes,[10] the overall efficiency of 34E4 is many orders of magnitude lower than that of a catalyst like triose phosphate isomerase,[11] which also promotes proton transfers, probably because the antibody lacks a general acid that could stabilize developing negative charge at the leaving group phenoxide (Scheme 1).

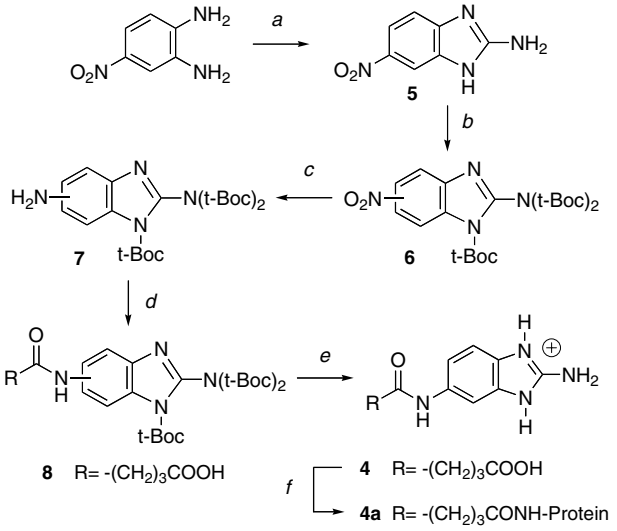
The absence of a general acid in 34E4 was not unexpected since hapten **3** (Scheme 2) was coupled to a carrier protein for immunization via N1,[4] the surrogate for the substrate's leaving group oxygen. The hydrophobic pentanoic acid linker at this position presumably disfavored induction of potentially beneficial polar interactions in this region of the binding site.[5] In principle, by simply altering the coupling strategy and linking the hapten through its benzene ring rather than through the imidazolium moiety, both N1 and N3 would be made available to elicit an acid–base pair preorganized for bifunctional catalysis of benzisoxazole decomposition. Such a coupling mode would have the additional advantage that the induced catalytic functionality would reside at the bottom of the binding pocket, rather than near the entrance of the cavity where potential electrostatic effects might be attenuated.

To test this idea, we synthesized the 2-aminobenzimidazolium derivative **4** and used it to elicit an immune response. The 2-amino group, which lacks a counterpart in the substrate, was included to increase the basicity of the benzimidazole ring. The properties of two monoclonal antibodies that bind this compound tightly and also promote the decomposition of substituted benzisoxazoles are described in this report.

2. Results

2.1. Antibody generation and screening

Hapten **4** was prepared as shown in Scheme 3. The pK_a of the benzimidazolium moiety was determined to be 7.22, so a significant fraction of the molecule will be protonated under physiological conditions. The hapten was coupled to the carrier protein thyroglobulin via its free carboxylate, and the resulting conjugate was used to generate monoclonal antibodies by standard methods.[12] Hybridoma supernatants were screened directly for catalytic activity with the chromogenic substrate 5-nitrobenzisoxazole. Eleven antibodies from a population of ca. 1200 hapten binders were identified as promising



Scheme 3. Synthesis of hapten **4** and its coupling to carrier proteins. Reagents and conditions: (a) BrCN, 100%; (b) $(t\text{-BocO})_2CO$, 77%; (c) H_2, Pd/C, 98%; (d) glutaric anhydride, 85%; (e) HCl, 86%; (f) sulfo-NHS, EDC, followed by addition to the carrier protein thyroglobulin.

candidates for further study and, after subcloning, the corresponding hybridomas were propagated in mouse ascites. After purification, two antibodies, 13G5 and 47C4, showed significant catalytic effects and were characterized in detail.

2.2. Kinetic analysis

Hapten **4** is a potent inhibitor of antibodies 13G5 and 47C4. Catalytic activity is completely blocked upon addition of 1 equiv of the 2-aminobenzimidazolium derivative per binding site, indicating that the elimination reaction occurs exclusively in the induced binding pocket. Although the preliminary assays were performed with 5-nitrobenzisoxazole, 13G5 and 47C4 accept a variety of substituted benzisoxazoles as substrates (Fig. 1). In general, saturation kinetics were not observed at accessible substrate concentrations, implying K_m values in excess of 1 mM. Consequently, the apparent second-order rate constants k_{cat}/K_m were estimated directly from the initial rate data.

As shown in Figure 1, 13G5 is very tolerant to substitution at the benzene ring of the substrate. With the exception of 5,6-dinitrobenzisoxazole and 6-glutaramidebenzisoxazole, which are much better substrates than expected based on their intrinsic reactivity, the benzisoxazoles follow a linear free energy relationship, with the value of k_{cat}/K_m correlating with the pK_a of the product phenol. The Brønsted coefficient β_{lg} is -0.50 ($r^2 = 0.980$). In contrast, antibody 47C4 is more discriminating and its Brønsted plot shows considerable scatter (Fig. 1). For example, 6-nitrobenzisoxazole is a substantially better substrate for this catalyst than the intrinsically more reactive 5-nitro or 5-cyano derivatives. Notably, 5-glutaramidebenzisoxazole is not a substrate for either antibody.

The carboxylate side chain increases the solubility of 6-glutaramidebenzisoxazole (**9**) sufficiently to allow deter-

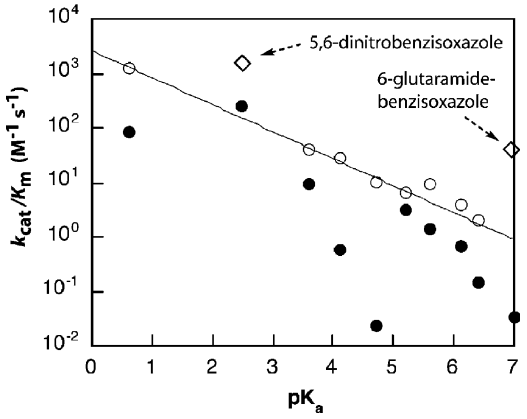


Figure 1. Brønsted (leaving group) plot for the decomposition of substituted benzisoxazoles catalyzed by antibodies 13G5 (○) and 47C4 (●). Initial velocities were determined at 20 °C with substituted benzisoxazoles in the presence of the antibodies in phosphate buffer (40 mM, containing 100 mM NaCl, pH 7.4), and corrected for the rate of the background reaction under the same buffer conditions. Substrates (substituents/pK_a of product phenol): 5,7-$(NO_2)_2$, 0.6; 5,6-$(NO_2)_2$, 2.5; 5-NO_2, 6-Cl, 3.6; 5-NO_2, 4.1; 5-CN, 4.7; 6-NO_2, 5.2; 5,6-Cl_2, 5.6; 6-Cl, 6.1; 5-Cl, 6.4; 6-glutaramide-, 7.0.

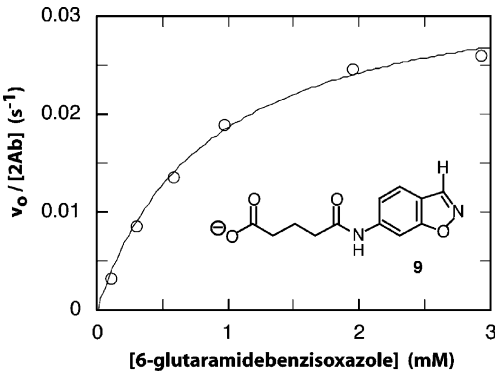


Figure 2. Michaelis–Menten plot for the conversion of 6-glutaramidebenzisoxazole **9** with 13G5 (pH 7.4, 40 mM phosphate buffer, 100 mM NaCl, 20 °C). Initial rates were determined spectrophotometrically as a function of time. The data were corrected for the buffer-catalyzed background reaction which was measured under the same conditions ($k_{uncat} = 2 \times 10^{-7}$ s^{-1}) and fitted to the equation $v/[2Ab] = k_{cat}[S]/(K_m + [S])$.

mination of the steady-state parameters of the 13G5-catalyzed elimination reaction. At pH 7.4 and 20 °C, saturation kinetics were observed (Fig. 2), consistent with a kinetic mechanism in which substrate and antibody form a Michaelis–Menten complex before proton abstraction. The values of k_{cat} and K_m are 0.034 ± 0.001 s^{-1} and 840 ± 80 μM, respectively. For comparison, the rate constant for the spontaneous hydrolysis of this substrate in the absence of catalyst is 2.0×10^{-7} s^{-1}. The rate acceleration over background (k_{cat}/k_{uncat}) is therefore 1.7×10^5, which is similar in magnitude to that seen for the 34E4-catalyzed decomposition of the ca. 100-fold more reactive 5-nitrobenzisoxazole.[4]

2.3. pH-rate profiles

The pH dependence of 13G5 and 47C4 was examined with 5-nitro- and 6-nitrobenzisoxazoles, respectively. In contrast to previously described antibody catalysts for the Kemp elimination which show sigmoidal pH-rate profiles,[2,4] bell-shaped curves were obtained in both cases, albeit with different pH optima (Fig. 3).

These results are what would be expected if catalysis required the participation of two ionizable groups, one in its acidic form and one as a base. Fitting the data to a kinetic scheme for a doubly ionizing system gives apparent ionization constants of 6.3 and 9.0 for 13G5 and 4.9 and 7.4 for 47C4 (Fig. 3). The limiting values of k_{cat}/K_m are 29 and 6.2 M^{-1} s^{-1}, respectively.

A similar bell-shaped curve was observed in the 13G5-catalyzed decomposition of 6-glutaramidebenzisoxazole, with apparent ionization constants of 6.1 and 8.4, and a limiting value of k_{cat}/K_m of 36 ± 4 M^{-1} s^{-1}. Because of this substrate's favorable solubility properties, the pH dependence of k_{cat} could also be determined. As shown in Figure 4, the k_{cat} parameter increases sigmoidally as

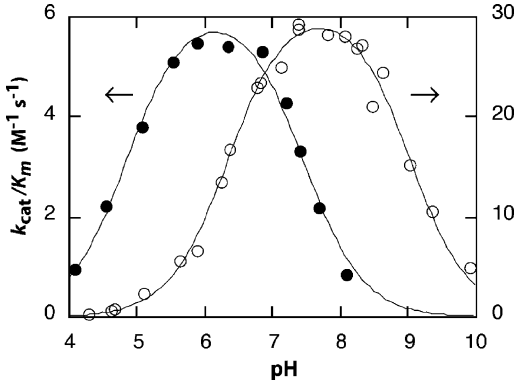


Figure 3. Plots of k_{cat}/K_m versus pH for the 47C4-catalyzed elimination of 6-nitrobenzisoxazole (●) and the 13G5-catalyzed elimination of 5-nitrobenzisoxazole (○). Kinetics were performed at 20 °C in the following buffers (40 mM, containing 100 mM NaCl): sodium acetate (pH <6); sodium phosphate (6 < pH < 8); and sodium carbonate (pH >8). Initial rates were determined spectrophotometrically as a function of time. The data were fitted to the equation $k_{cat}/K_m = (k_{cat}/K_m)^{max}/(1 + 10^{pK_{a1}-pH} + 10^{pH-pK_{a2}})$.

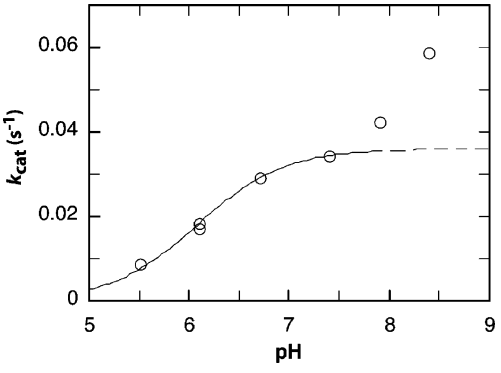


Figure 4. Plot of k_{cat} versus pH for the 13G5-catalyzed elimination of 6-glutaramidebenzisoxazole **9**. Kinetics were performed at 20 °C in the following buffers (40 mM, containing 100 mM NaCl): sodium acetate (pH <6); sodium phosphate (6 < pH < 8); and sodium carbonate (pH >8). Initial rates were determined spectrophotometrically as a function of time. The acidic limb were fitted to the equation $k_{cat} = (k_{cat})^{max}/(1 + 10^{pK_a-pH})$.

the medium becomes more basic, reaching a plateau at around pH 7.5 before rising steeply above pH 8. The large increase in K_m values above pH 8.5 precluded determination of reliable k_{cat} values for the basic limb of the pH-rate profile, so the acidic limb was fit to a model for a single ionizing group. This analysis afforded an apparent pK_a of 6.1 and a limiting k_{cat} value of $0.036 \pm 0.004\ s^{-1}$.

2.4. Chemical modification

The identity of ionizing residues possibly involved in substrate binding and catalysis was explored by chemical modification.[13] Carbodiimide-mediated derivatization of carboxylate groups with glycine ethyl ester had no effect on antibody 13G5, but incorporation of 1 equiv of fluorescein isothiocyanate (FITC, which preferentially reacts with lysines) led to nearly complete inactivation of the catalyst. Preincubation of the antibody with one equivalent of hapten **4** per binding site blocked the reaction with FITC. Treatment of 13G5 with up to 2000-fold molar excess of diethyl pyrocarbonate (DEPC, a histidine-specific reagent) similarly resulted in nearly complete loss of catalytic activity. Antibody 47C4 is also inactivated by reaction with FITC. However, in contrast to 13G5, it retains more than 90% of its activity when treated with a large excess of DEPC, but loses 50% activity upon treatment with GlyOEt/EDC. These findings suggest that 13G5 contains a histidine and a lysine at or near the active site, whereas 47C4 apparently possesses a carboxylate residue, either aspartate or glutamate, in addition to a reactive lysine.

2.5. Catalytic efficiency

The results of the chemical modification experiments raise the possibility that 13G5 may exploit the imidazole side chain of a histidine residue, rather than the carboxylate group of an asparate or glutamate, as its catalytic base. We consequently examined the decomposition of 6-glutaramidebenzisoxazole (**9**) in the presence of imidazole (Fig. 5). The second-order rate constant in water ($k_{im,water}$) was found to be $2.11 \times 10^{-6}\ M^{-1}\ s^{-1}$. In acetonitrile, this value increases roughly 10-fold to $2.6 \times 10^{-5}\ M^{-1}\ s^{-1}$.

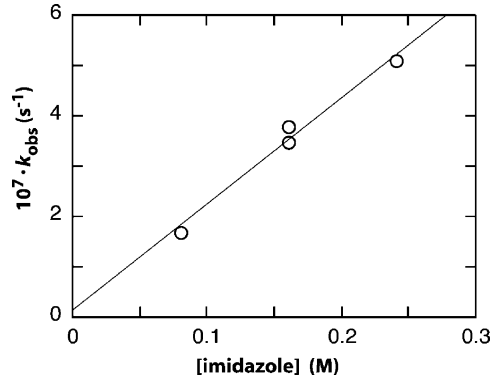


Figure 5. Variation of the pseudo-first order rate constant for the elimination reaction of 6-glutaramidebenzisoxazole (**9**) as function of imidazole concentration. The k_{obsd} values were obtained by the method of initial rates in water (pH 7.5, 20 °C).

If antibody 13G5 exploits a histidine residue as a general base, then the efficiency of this catalytic group can be estimated to be $>10^7$ from the ratio $(k_{cat}/K_m)/k_{im,water}$. The effectiveness of the catalytic base at the antibody active site is further underscored by an effective molarity[14] (EM = $k_{cat}/k_{im,water}$) greater than 10^4 M.

3. Discussion

Because the conversion of benzisoxazoles (**1**) to salicylonitriles (**2**) is sensitive to base strength and solvent environment,[8,9] this reaction has served as a valuable model system for developing proton transfer catalysts. Over the last decade, catalytic antibodies,[2,4,7] albumins,[15,16] polyethyleneimine synzymes,[17] organic hosts,[18] cationic vesicles,[19] and even natural coals[20] have all been shown to promote this reaction with rate accelerations between 10^2 and 10^6 over background.

In designing catalysts for the Kemp elimination, provision of a base in an appropriate microenvironment has generally been the paramount consideration. Relatively little attention has been paid to exploiting a second functional group to stabilize developing charge on the phenolate leaving group, although calculations have suggested that hydrogen bonding to the ether oxygen could contribute substantially to overall catalytic efficiency.[21] Indeed, some of the more efficient synzymes may benefit serendipitously from such interactions.[17]

In the current study, we explored the possibility of using compound **4** as a template for generating antibodies capable of bifunctional catalysis. This hapten is similar in shape to a benzisoxazole, and its guanidinium group could conceivably induce complementary base and acid groups, arrayed on opposite edges of the molecule, which would, respectively, initiate proton transfer from the substrate and stabilize partial negative charge at the phenoxide leaving group in the transitions state.

In the event, hapten **4** was successful in eliciting two antibodies, 13G5 and 47C4, that accelerate benzisoxazole decomposition. These catalysts display bell-shaped pH-rate profiles and, based on chemical modification experiments, contain several reactive groups that could plausibly assume the roles of base and acid. For comparison, previously described antibody catalysts for the Kemp elimination that exploit a carboxylate base for proton abstraction exhibit sigmoidal pH-rate behavior;[2–5,7] BSA, which uses a more basic amine (p$K_a \approx 10$) for catalysis, also has a sigmoidal pH-rate profile.[15] These findings are thus consistent with the involvement of two functional groups in catalysis by antibodies 13G5 and 47C4, although alternative interpretations of the pH-rate data[22] cannot be excluded as yet. A Brønsted analysis correlating reaction rate with the pK_a of the leaving group was performed to gain additional information about the structure and solvation of the transition state. Although 47C4 failed to give a meaningful

correlation, the β_{lg} of −0.50 obtained with 13G5 is substantially lower than the values seen for antibody 34E4 (−1.48)[23] or BSA (−0.84),[23] indicating that the new antibody is substantially less sensitive to the stability of the leaving group than these previously examined systems. The large β_{lg} for 34E4 has been attributed to a relatively nonpolar environment that destabilizes the anionic product,[5] whereas 13G5 presumably provides a more hospitable environment for the developing negative charge.

The preferential cleavage of an unactivated substrate, namely 6-glutaramidebenzisoxazole (**9**), which closely resembles the immunizing hapten **4**, also sets 13G5 apart from BSA and other antibody catalysts. Interestingly, the corresponding 5-glutaramide derivative is not recognized by the immunoglobulin, implying that specific binding interactions with the linker help to position the 6-glutaramide substrate optimally within the active site. Indeed, the rate acceleration for the cleavage of this substrate rivals that achieved by 34E4, the most active of the previously described catalytic antibodies,[4,10] with the 100-fold more reactive 5-nitrobenzisoxazole. Although some uncertainty exists regarding the identity of the catalytic base in 13G5, if we assume participation of an imidazole as suggested by the chemical modification experiments, an effective molarity $>10^4$ M is obtained. This large value is comparable to the effective molarity deduced for the carboxylate base in 34E4[10] and suggests that a high degree of positional ordering may also be operative in the new catalyst.

Whether 13G5 exploits bifunctional acid-base catalysis to cleave the unactivated 6-glutaramidebenzisoxazole clearly requires further investigation. Both the k_{cat}/K_m and k_{cat} parameters for this substrate show biphasic pH dependencies, but only the former is bell-shaped. Although this observation does not rule out participation of a general acid in catalysis, the continuous rise in k_{cat} across the experimentally accessible pH range argues against an assignment of the apparent ionization at pH 9 to the deprotonation of such a group. Detailed structural and mutagenesis data will be needed to clarify just how the antibody stabilizes the phenoxide leaving group.

In summary, the first tailored protein catalysts capable of deprotonating unactivated benzisoxazoles with reasonable efficacy have been prepared. Although the absolute efficiency of these antibodies still lags far behind that of natural enzymes that promote proton transfers, their properties raise the intriguing possibility that they utilize a more sophisticated mechanism than the original antibody catalysts for this reaction. They may consequently represent good starting points for the evolution of even more active proton transfer catalysts.

4. Materials and methods

4.1. General methods

^{1}H NMR spectra were recorded on Bruker AC 250, AMX 300 and 500 instruments. Chemical shifts in parts per million are referenced to the internal standard tetramethyl silane. Mass spectra were obtained on a VG ZAB-2VSe double focusing high-resolution mass spectrometer. UV spectra and spectroscopic kinetic assays were recorded on Perkin-Elmer Lambda 40 UV–vis spectrometers. Stopped flow techniques were used for measurements at high pH (>8.5) and for the most reactive substrates.

4.2. 2-Amino-5-nitrobenzimidazole (5)

Cyanogen bromide (28.4 g, 0.268 mol) was added to a solution of 4-nitro-1,2-phenylenediamine (40.5 g, 0.265 mol) in dioxane/water (630 ml/160 ml), and the mixture was shaken at room temperature overnight. The clear, deep red solution was evaporated to dryness and 500 ml of 1.2 N NaOH was added to the residue. The mixture was warmed to 60–70 °C on a water bath and mixed with 10 g charcoal. The mixture was kept at 60–70 °C for a few minutes prior to filtering off the charcoal. A solution of NH_4Cl (17 g in 100 ml water) was added slowly to the hot filtrate while stirring. The mixture was cooled to room temperature, filtered, and the precipitate was dried in air to give 48.4 g (100%) of crude **5**. ^{1}H NMR (DMSO-d_6): δ 7.95 (d, 1H, J = 2.3 Hz), 7.87 (dd, 1H, J_{AB} = 8.7 Hz, J_m = 2.3 Hz), 7.18 (d, 1H, J = 8.7 Hz), 6.85 (s, 2H, exchanges with D_2O). ^{13}C NMR (DMSO-d_6): δ 159.225, 147.252, 139.876, 136.887, 116.885, 111.633, 106.156.

4.3. 1-*tert*-Butoxycarbonyl-2-(*N*,*N*-bis-*tert*-butoxycarbonyl)-5-nitrobenzimidazole (6a) and 1-*tert*-butoxycarbonyl-2-(*N*,*N*-bis-*tert*-butoxycarbonyl)-6-nitrobenzimidazole (6b)

Di-*tert*-butyl dicarbonate (6 ml) was added to a suspension of **5** (3.6 g) in 200 ml dry THF. The mixture was stirred at room temperature for 5 h to give an almost clear solution. Small amounts of insoluble impurities were filtered off, and a further 6 ml of di-*tert*-butyldicarbonate was added to the filtrate, followed by 0.25 g DMAP. After stirring at room temperature overnight, solvent was evaporated and the residue purified by silica chromatography (eluted with an ethyl acetate/hexane gradient, 1:10 → 1:5) to give the triply protected 2-aminobenzimidazole **6** (7.4 g, 76.8%) as a mixture of two isomers. ^{1}H NMR ($CDCl_3$): isomer I: δ 8.94 (d, 1H, J = 2.4 Hz), 8.28 (dd, 1H, J_{AB} = 8.8 Hz, J_m = 2.4 Hz), 7.83 (d, 1H, J_{AB} = 8.8 Hz), 1.75 (s, 9H), 1.41 (s, 18H). Isomer II: δ 8.62 (d, 1H, J = 2.2 Hz), 8.32 (dd, 1H, J_{AB} = 9.2 Hz, J_m = 2.2 Hz), 8.14 (d, 1H, J_{AB} = 9.2 Hz), 1.68 (s, 9H), 1.41 (s, 18H).

4.4. 1-*tert*-Butoxycarbonyl-2-(*N*,*N*-bis-*tert*-butoxycarbonyl)-5-aminobenzimidazole (7a) and 1-*tert*-butoxycarbonyl-2-(*N*,*N*-bis-*tert*-butoxycarbonyl)-6-aminobenzimidazole (7b)

A mixture of **6a** and **6b** (7.9 g, 16.5 mmol) was suspended with 1 g of 10% Pd/C in 400 ml methanol and stirred overnight under hydrogen. The catalyst was filtered off and the solvent evaporated to give a mixture of **7a** and **7b** (7.3 g, 98.2%). The isomers were separated on silica eluted with ethyl acetate/hexane (1:4 → 1:2 → 2:1). ^{1}H

NMR ($CDCl_3$): isomer I: δ 7.84 (d, 1H, J_{AB} = 8.5 Hz), 7.33 (d, 1H, J = 2.2 Hz), 6.70 (dd, 1H, J_{AB} = 8.5 Hz, J_m = 2.2 Hz), 3.7 (br, 2H, exchanges with D_2O), 1.63 (s, 9H), 1.39 (s, 18H). Isomer II: δ 7.74 (d, 1H, J_{AB} = 8.55 Hz), 7.00 (d, 1H, J = 2.4 Hz), 6.78 (dd, 1H, J_{AB} = 8.55 Hz, J_m = 2.4 Hz), 3.7 (b, 2H), 1.63 (s, 9H), 1.39 (s, 18H).

4.5. 1-*tert*-Butoxycarbonyl-2-(*N*,*N*-bis-*tert*-butoxycarbonyl)-5-glutaramidebenzimidazole (8a) and 1-*tert*-butoxycarbonyl-2-(*N*,*N*-bis-*tert*-butoxycarbonyl)-6-glutaramidebenzimidazole (8b)

Glutaric anhydride (1 g) was added to a mixture of **7a** and **7b** (2.74 g, 6.1 mmol) dissolved in 80 ml dry dichloromethane, and the resulting solution was stirred at room temperature overnight. The clear solution became cloudy after a few minutes as a precipitate began to form. The precipitate was collected by filtration, washed with dichloromethane, and dried under vacuum to give the glutaric acid half-amide as a mixture of two isomers (2.9 g, 84.5%). ^{1}H NMR (DMSO-d_6): δ 12.2 (bs, 1H, exchanges with D_2O), 10.1 (s, 1H, exchanges with D_2O), 8.64 (d, 0.5H), 8.11 (d, 0.5H), 7.86 (d, 0.5H), 7.65 (d, 0.5H), 7.56 (dd, 0.5H), 7.42 (dd, 0.5H), 2.40 (t, 2H, J = 7.3 Hz), 2.30 (t, 2H, J = 7.3 Hz), 1.84 (quintet, 2H), 1.6 (s, 9H), 1.35 (s, 18H). MS-FAB −ve: 346.1 (30%), 461.3 (50%), 561.4 (100%, M−1).

4.6. 2-Amino-5-glutaramidebenzimidazole (4)

Boc-protected hapten (0.4 g) was suspended in concentrated HCl (10 ml, diluted with 5 ml water and 5 ml THF). The solution became clear after 5 min. After stirring at room temperature for about 2 h, a precipitate appeared. After stirring for a further 5 h, the solvent was evaporated. The residue was washed with ether and dried in vacuo to give hapten **4** (0.18 g, 85.7%). ^{1}H NMR (DMSO-d_6): δ 12.45 (br, 2H), 12.2 (br, 1H), 10.22 (s, 1H), 8.51 (s, 2H), 7.91 (d, 1H, J = 1.6 Hz), 7.32 (dd, 1H, J_{AB} = 8.6 Hz, J_m = 1.6 Hz), 7.27 (d, 1H, J_{AB} = 8.6 Hz), 2.37 (t, 2H, J = 7.5 Hz), 2.28 (t, 2H, J = 7.5 Hz), 1.80 (quintet, 2H, J = 7.5 Hz); the protons at 12.45, 12.2, 10.22, and 8.51 all exchange with D_2O. ^{13}C NMR (DMSO-d_6): δ 174.264, 170.849, 150.713, 135.327, 129.800, 125.268, 114.660, 111.355, 102.608, 35.536, 33.183, 20.672. MS-FAB +ve: 263.1 (100%), (M−Cl+H). Elemental analysis $C_{12}H_{14}N_4O_3$·HCl, calcd: C, 48.25; H, 5.06; Cl, 11.87; N, 18.75. Found: C, 47.44; H, 5.04; N, 18.97. The pK_a value of the protonated benzimidazolium ring was determined by standard pH titration.

4.7. Hapten conjugates

Compound **4** was coupled to thyroglobulin (TG) via its carboxyl group as described by Lauer et al.[24] The epitope density of the resulting protein conjugate (42 haptens per carrier) was determined by the method of Habeeb.[25] A hapten with bovine serum albumin (BSA) was prepared analogously for binding studies. It had an epitope density of 10 haptens per BSA.

4.8. Substrates

5-Nitro, 6-nitro-, 5,7-dinitro-, 5-chloro-, and 6-chlorobenzisoxazole were prepared according to the literature.[8,26] 4-Cyano- and 4-fluorosalicylaldehyde were prepared from the corresponding phenols by a Duff reaction.[27] 4,5-Dichlorosalicylaldehyde was prepared by a Reimer–Tiemann reaction.[28] The general literature was followed to prepare benzisoxazoles from the corresponding salicylaldehydes.[8]

4.9. 5-Cyanobenzisoxazole

^{1}H NMR (300 MHz, $CDCl_3$): δ 8.83 (d, J = 0.9, 1H), 8.16 (dd, J_1 = 0.9, J_2 = 1.8, 1H), 7.84 (dd, J_1 = 1.8, J_2 = 8.6, 1H), 7.76 (dd, J_1 = 1.1, J_2 = 8.6, 1H); MS (FAB-NBA/NaI) 145 $(M+H)^+$; mp 245 °C (dec).

4.10. 5,6-Dichlorobenzisoxazole

^{1}H NMR (300 MHz, $CDCl_3$): δ 8.68 (d, J = 1.0, 1H), 7.85 (s, 1H), 7.80 (d, J = 1.0, 1H); MS (FAB-NBA/NaI) 189 $(M+H)^+$, 211 $(M+Na)^+$; mp 92 °C (sub).

4.11. 5-Fluorobenzisoxazole

^{1}H NMR (300 MHz, $CDCl_3$): δ 8.7 (d, J = 0.8, 1H), 7.59 (m, 1H), 7.38 (m, 1H), 7.32 (m, 1H); MS (FAB-NBA/NaI) 139 $(M+H)^+$; mp 68 °C (sub).

4.12. 5,6-Dinitrobenzisoxazole

6-Nitrobenzisoxazole (200 mg, 1.22 mmol) was dissolved in 1.6 ml ice-cooled H_2SO_4. Two hundred microliters of fuming HNO_3 (ρ 1.5) was added dropwise. The reaction mixture was heated at 80 °C for 30 min. The starting material was completely consumed. The reaction mixture was poured on ice and then extracted immediately with CH_2Cl_2. The organic layer was dried over $MgSO_4$ and purified by column chromatography (silica gel 60, CH_2Cl_2) to give 142 mg (679 mmol) product (56%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.15 (d, 1H), 8.52 (s, 1H), 8.17 (d, 1H); MS (FAB-NBA/NaI) 210 $(M+H)^+$; mp 112 °C.

4.13. 5-Nitro-6-chlorobenzisoxazole

6-Chlorobenzisoxazole was nitrated at position 5 by the same method used to prepare 5,6-dinitrobenzisoxazole in 61.9% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.83 (d, J = 1.1, 1H), 8.36 (dd, J_1 = 0.9, J_2 = 1.8, 1H), 7.88 (d, J = 1.1, 1H); MS (FAB-NBA/NaI) 199 $(M+H)^+$; mp 106 °C.

4.14. 5-Aminobenzisoxazole[29] (with modifications)

5-Nitrobenzisoxazole (70 mg, 0.43 mmol) was mixed with 914 mg (0.55 μmol) $SnCl_4$ in 400 μl of 12 M HCl and ice cooled. $SnCl_2$ (291 mg, 1.29 μmol) in 12 M HCl was added dropwise. The ice bath was removed and the reaction mixture was stirred for 5 h at rt with an additional 2.5 ml of 12 M HCl. The mixture was

extracted with Et_2O, the aqueous layer treated with saturated $NaHCO_3$ until the solution was basic, and extracted with EtOAc. The organic solvent was removed and the residue dried under high vacuum for 2 h yielding 43 mg (0.32 mmol, 74%) product. ^{1}H NMR (250 MHz, $CDCl_3$): δ 8.52 (s, 1H), 7.40 (d, $J = 8.9$, 1H), 6.96 (dd, $J_1 = 2.9$, $J_2 = 9.1$, 1H), 6.89 (dd, $J_1 = 1.5$, $J_2 = 2.6$, 1H).

4.15. 5-Glutaramidobenzisoxazole

5-Aminobenzisoxazole (40 mg, 0.30 mmol) was dissolved in 2 ml acetone and stirred with glutaric anhydride (44.5 mg, 0.39 mmol) at rt overnight. The solvent was removed and the residue crystallized from EtOAc/hexanes yielding pure product (53.8 mg, 0.217 mmol, 72%). ^{1}H NMR (250 MHz, DMSO): δ 10.13 (s, 1H), 9.17 (s, 1H), 8.26 (s, 1H), 7.72–7.63 (m, 2H), 2.39 (m, 2H), 2.28 (m, 2H), 1.82 (m, 2H).

4.16. 6-Aminobenzisoxazole

6-Aminobenzisoxazole was synthesized from 6-nitrobenzisoxazole by the same method as 5-aminobenzisoxazole in 55% yield. The product was recovered in 36% yield from the ether extract. ^{1}H NMR (250 MHz, $CDCl_3$): δ 8.46 (s, 1H), 7.43 (d, $J = 8.4$, 1H), 6.76 (s, 1H), 6.63 (d, $J = 8.5$, 1H), 4.07 (br, 2H).

4.17. 6-Glutaramidebenzisoxazole (9)

6-Aminobenzisoxazole (62 mg, 0.46 mmol) was dissolved in 2 ml acetone and stirred with 69 mg (0.601 mmol) glutaric anhydride at rt overnight. Another portion of anhydride was added and stirring was continued for one day. The acetone was removed, and the residue was purified by column chromatography (silica gel 60, CH_3OH/CH_2Cl_2/AcOH 1:8:0.1). Seventy-seven milligrams of pure product (0.31 mmol, 67%) was isolated. ^{1}H NMR (500 MHz, DMSO): δ 10.39 (s, 1H), 9.08 (s, 1H), 8.23 (s, 1H), 7.77 (d, $J = 8.4$, 1H), 7.38 (d, $J = 8.4$, 1H), 2.42 (m, 2H), 2.26 (m, 2H), 1.80 (m, 2H); MS (FAB-NBA/NaI) 249 $(M+H)^+$.

4.18. Antibody purification

Standard protocols were used to generate and characterize hybridomas produced by fusion of mouse spleen cells with SP2/0$^+$ myeloma cells.[12] About 1200 hybridomas were obtained from the spleen of the host mouse. In a total volume of 120 μl, 80 μl of tissue culture supernatant was placed in a 96-well ELISA plate. The assay was started by the addition of 850 μM of substrates in 200 mM MES buffer (pH 6.0). Product formation was monitored at 380 nm for 30 min. Subcloning, propagation in (BALB/c X 129 GUX$^+$)F_1 mouse ascites, and purification by ammonium sulfate precipitation and DEAE ion-exchange chromatography were performed as previously described.[30] Monoclonal antibodies were further purified by FPLC affinity chromatography on Protein G Sepharose, followed by FPLC ion-exchange chromatography (Mono Q HR 10/10).

4.19. Kinetic analysis

All kinetic experiments were performed at 20.0 ± 0.2 °C. Product formation was monitored by UV–vis spectroscopy. The reactions were initiated by addition of substrate to a solution of the antibody, except in the case of 5- and 6-glutaramidebenzisoxazoles when the antibody was added to a solution of the substrate. The apparent extinction coefficients ($M^{-1}cm^{-1}$) of the substituted salicylonitriles at pH 7.4 are 15,200 (352 nm) for 5,7-diNO_2–, 7690 (380 nm) for 5,6-diNO_2–, 8050 (380 nm) for 5-NO_2, 6-Cl–, 15,800 (380 nm) for 5-NO_2–, 5500 (324 nm) for 5-CN–, 2870 (404 nm) for 6-NO_2–, 7350 (324 nm) for 5,6-diCl–, 6630 (329 nm) for 6-Cl–, 5100 (339 nm) for 5-Cl–, 4650 (338 nm) for 5-F–, 6820 (329 nm) for 6-GluNH–, 3360 (335 nm) for 5-GluNH–, and 4050 (325 nm) for unsubstituted benzisoxazole. Apparent ε values at other pHs were measured individually for compounds that were incompletely ionized under the reaction conditions. Kinetic assays were performed in 40 mM phosphate buffer containing 100 mM NaCl (pH 7.4) with 0–10 μM protein and 50 μM–5.5 mM substrate. Acetonitrile (2% of final volume) was used to dissolve the substrate, where the substrate was used to initialize the reaction. For the pH-rate profile, reactions were performed in the following buffers (40 mM, containing 100 mM NaCl): sodium acetate (pH <6), sodium phosphate (6 < pH < 8), and sodium carbonate (pH >8) with 0–10 μM protein and 50 μM (140 μM for 6-GluNH-) substrate. The second-order rate constants were obtained by the initial velocities with the lower substrate condition compared to K_m values (>1 mM). The data were fitted to the equation $k_{cat}/K_m = (k_{cat}/K_m)^{max}/(1 + 10^{pK_{a1}-pH} + 10^{pH-pK_{a2}})$.

4.20. Chemical modification of the antibody

All modifications were performed according to the literature.[13] Carboxyl functional groups were modified via carbodiimide-mediated reaction. Glycine ethyl ester (1 M) and 100 mM 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide were added to 25 μM solution of protein based on the antibody binding site in pH 5.0 acetate buffer for 24 h at 4 °C. Control experiments were performed by the addition of weaker binder: 50-fold excess of benzimidazole. The other control experiments were performed by the addition of respective reagents. The reaction mixture was thrice dialyzed for 24 h and concentrated before use. Histidine residues were modified by adding diethylpyrocarbonate. A diethylpyrocarbonate solution in ethanol (1- to 2000-fold excess of antibody binding site) was added directly to the kinetic cuvettes 2 h before the kinetic run at rt. To modify lysine residues, fluorescein isothiocyanate (FITC) as added (0.015- to 15-fold excess of antibody binding site) in DMF was added to the protein solution at pH 8.5 for 2 h at rt. Then the reaction mixture was separated by gel filtration (Sephadex G-25) eluted with 50 mM citrate buffer, pH 3.0, to stop the reaction and to remove the unreacted FITC. The protein fraction was neutralized to pH 7.4 by passing through the Sephadex G-25 column eluted with PBS. The protein concentration was

determined by the BCA method.[31] The FITC residue was titrated by measuring the OD at 491 nm (ε 75,900) in 1 N NaOH.[32]

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