A Nitroxyl Synthase Catalytic Antibody

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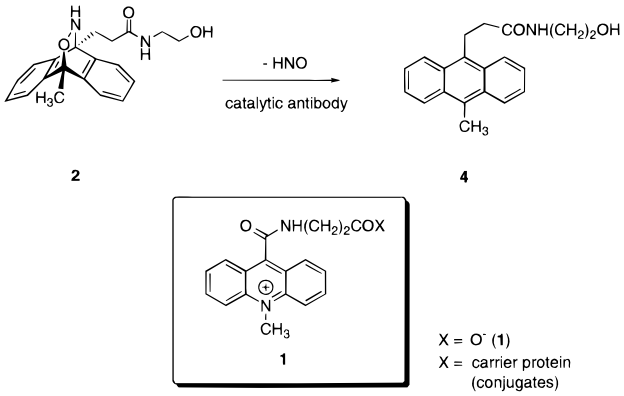
Abstract: An antibody raised against acridinium hapten **1** is shown to catalyze the retro Diels−Alder reaction of the anthracene−HNO cycloadduct **2** to release anthracene **4** and nitroxyl (HNO). Nitroxyl is oxidized to nitric oxide (NO) in the presence of superoxide dismutase. Since the enzyme superoxide dismutase is ubiquitous in vivo, this catalytic antibody system may be equivalent to NO-synthase. Antibody catalysis is triggered by recognition of the phenyl rings in hapten **1** at an angle near that of the transition state of the retro Diels−Alder reaction of **2**. Acridinium hapten **1** is in chemical equilibrium with its conjugate Lewis base 9-hydroxyacridane **1**-OH (p$K \sim 8.2$). Catalytic antibody 9D9 (K_M(**2**) = 100 μM, k_{cat} = 0.07 min^{-1}, k_{uncat} = 3 × 10^{-4} min^{-1}) is the result of an heterologous immunization and binds both **1** (K_i = 16.6 μM at pH 6.1) and **1**-OH (K_i = 0.9 μM at pH 9.0). The more tightly bound neutral conjugate base **1**-OH probably represents a more accurate mimic of the transition state of the retro Diels−Alder process.

Introduction

In the last years a considerable variety of organic transformations have been catalyzed by monoclonal antibodies, covering most reaction types from hydrolytic reactions to redox and pericyclic processes.[1] Catalytic antibodies for Diels−Alder cycloadditions[2] represent one of the most striking accomplishments in this field not only because of their ability to control the enantio- and stereoselectivity of the bond formation but also because no other protein catalysts were known for this reaction until recently.[3] Antibodies catalyzing such "abiological" processes fundamentally broaden the scope of biocatalysis beyond classical enzymology and offer new opportunities for organic synthesis.[4] They are also very attractive candidates for prodrug activation *in vivo* since interferences by naturally occurring enzymes are ruled out.[5] Herein we report the generation of a catalytic antibody obtained by immunization against hapten **1** that promotes a retro Diels−Alder reaction releasing nitroxyl (HNO) as a heterodienophile from the inert prodrug **2** (Scheme 1). Nitroxyl can in turn be oxidized to nitric oxide (NO) by the ubiquitous enzyme superoxide dismutase (SOD).[6]

This prodrug release system is of particular biological significance since nitric oxide acts as a chemical messenger for a number of fundamental bioregulatory processes, including blood pressure regulation and memory.[7] Originally NO was identified in biological systems as the active principle of EDRF (endothelium-derived relaxing factor).[8] NO is produced in neurons by oxidation of L-arginine to L-citrulline by the enzyme NO-synthase in response to transient increases in intracellular Ca^{2+} and exerts its action by binding to the iron of the prosthetic heme group in soluble guanylate cyclase, which triggers cGMP production as a second messenger. The biological effect of nitric oxide can be mimicked by NO-releasing drugs like nitroglycerine, which are commonly used to treat angina and hypertension. However, these drugs decompose rapidly, must be administered in large doses, and eventually induce tolerance. The nitroxyl synthase catalytic antibody/SOD couple described here offers a more controlled and selective route to nitric oxide.

Scheme 1. Retro Diels−Alder Reaction and Hapten Used for Production of Catalytic Antibodies



Results and Discussion

Substrate Synthesis. Anthracene **4** was prepared starting from commercially available 10-methylanthracene-9-carboxaldehyde (Scheme 2). Wittig reaction with (carbethoxymethylene)-triphenylphosphorane yielded 93% of *trans*-ene **5**. Hydrogenation of the α,β-unsaturation in ester **5** proved difficult due to the concurrent reduction of the anthracene moiety. Selective reduction was achieved employing $NaBH_4/NiCl_2$ as described

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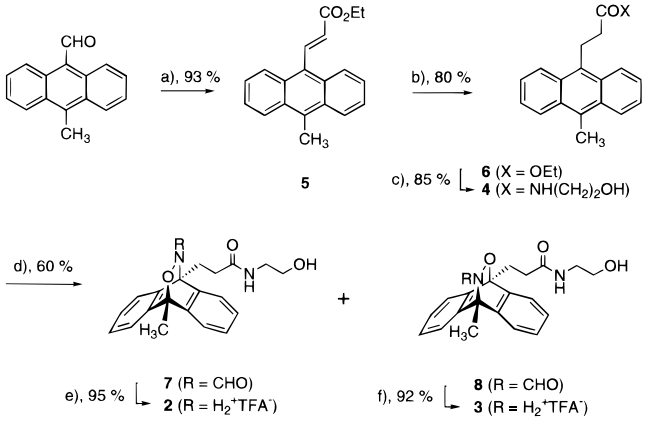
Scheme 2. Synthesis of Substrates **2** and **3**[a]



[a] Conditions: (a) $Ph_3P{=}CHCO_2Et$, CH_2Cl_2, reflux, 36 h; (b) 0.3 equiv of $NiCl_2$, 1 equiv of $NaBH_4$, EtOH, 20 °C, 6 h; (c) 25 equiv of ethanolamine, 0.1 equiv of NaCN, EtOH, 20 °C, 36 h; (d) 2 equiv of $Me_3N^+Bn\ IO_4^-$, 2 equiv of HCONHOH, dry DMF, 20 °C, 1.5 h, **7**: 30%, **8**: 30%, recovered **4**: 20%; (e) 0.1% TFA in MeOH, 0 °C, 24 h; (f) 0.1% TFA in MeOH, 20 °C, 12 h.

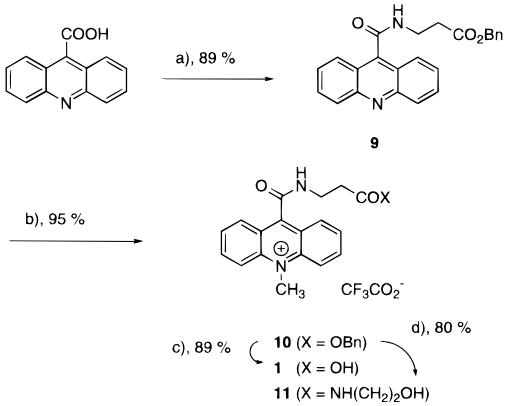
by Suzuki et al.[9] giving an 80% yield of **6** in gram quantities. Cyanide-catalyzed aminolysis[10] of ester **6** gave light yellow, crystalline **4**. Anthracene **4** is stable to air and can be stored at −18 °C for months without decomposition.

Diels−Alder reaction of **4** with nitrosoformate, generated *in situ* by periodate oxidation of hydroxamic acid, gave a 1:1 mixture of regioisomeric cycloadducts **7** and **8** in moderate yield.[11] The isomers were separated by preparative RP-HPLC, and their structure unambiguously resolved by NOE ^{1}H-NMR experiments. Thus, a strong NOE was observed between the C*H*$_3$ and *H*CON signals in **8** only. Acid-catalyzed removal of the formyl group with trifluoroacetic acid in methanol gave **2** or **3** quantitatively. The products were purified by RP-HPLC and stored at −78 °C as their trifluoroacetate salts as solids or as 5 mM stock solutions in H_2O.

Hapten Design and Synthesis. Catalysis of Diels−Alder reactions is often effected by Lewis acids in aprotic solvents. In aqueous environment cycloadditions can be promoted using catalytic antibodies.[2] Catalysis is achieved by binding both diene and dienophile, leading to a rate enhancement by an increase in effective molarity of the substrates. Clearly this effect would not be operative for the targeted unimolecular retro Diels−Alder reaction. We concentrated instead on the geometrical parameters affected by the reaction. In particular the dihedral angle (α) between the two phenyl rings changes from approximately α = 110° in **2** and **3** to α = 180° in **4** (Scheme 1). A compound presenting these two phenyl rings with an angle between these two values would represent a geometrically correct transition state mimic. Since immune recognition of aromatic groups is usually very strong, it was expected that this design would be suitable for the production of catalytic antibodies.

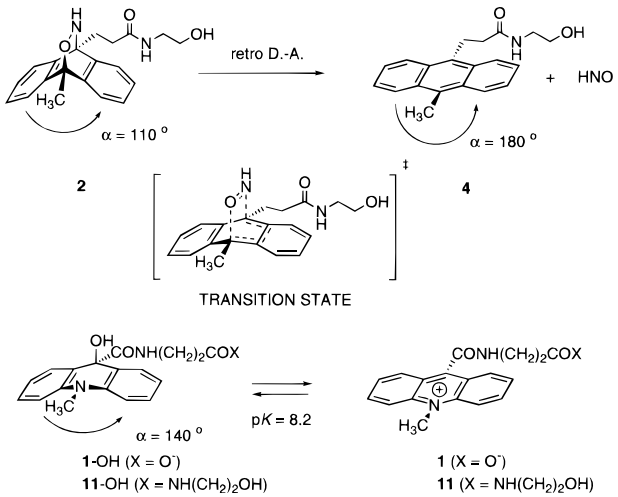
Acridinium **1** was used as a transition state analog for immunization. For its synthesis, acridine-10-carboxylic acid served as starting material (Scheme 3). Condensation with *β*-alanine benzyl ester gave **9**.[12] Quaternization of **9** to **10**

Scheme 3. Synthesis of Hapten **1**[a]



[a] Conditions: (a) $SOCl_2$, reflux, 6 h, then 1.3 equiv of *β*-alanine benzyl ester, 3 equiv of Et_3N, CH_2Cl_2, 20 °C, 14 h; (b) 1.2 equiv of Me_2SO_4, toluene, reflux, 2.5 h; (c) 2:1 0.1 N KOH/MeOH, reflux, 20 min; (d) 50 equiv of ethanolamine, EtOH, 20 °C, 36 h.

Scheme 4. Acridinium **1** and Its Pseudo-Base **1**-OH as Transition State Analogs for the Retro Diels−Alder Reaction of **2**



proceeded smoothly using dimethyl sulfate. Saponification of benzyl ester **10** and acidification furnished hapten **1** as a bright yellow, crystalline substance in an overall yield of 75%. Hapten **1** was purified by preparative RP-HPLC and stored as the trifluoroacetate (TFA) salt. The carboxyl function in **1** was activated to the sulfo-*N*-hydroxysuccinimide ester for conjugation to the carrier proteins BSA (bovine serum albumin) and KLH (keyhole limpet hemocyanin).[13] Alternatively, reaction with ethanolamine yielded **11**, which was used for competitive inhibition studies.[14]

The bright yellow acridinium compounds **1** and **11** are classical Lewis acids.[15] In aqueous medium these compounds are in rapid chemical equilibrium with their conjugate pseudobases **1**-OH and **11**-OH, which are colorless (Scheme 4). The apparent pK_a values of **1** and **11** were determined by UV spectroscopy to be approximately 8.2. Consequently, the acid−

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base equilibrium at physiological pH ≈ 7.5 allowed a heterologous immunization experiment[16] with two compounds (**1**: 84%, **1**-OH: 16%) presenting two different orientations of the phenyl rings either in a completely product-like orientation (**1**: α = 180°) or in bent orientation possibly quite close to the actual transition state of the reaction (**1**-OH: α = 140°, puckered boat form).[17] The presence of a positive charge in the more product-like acidic form **1** was also expected to avoid the induction of product-inhibited catalysts.

Antibody Catalysis. Twenty-three monoclonal *anti*-**1** antibodies were produced following standard protocols by fusion and hybridoma selection from spleen cells of Balb/C mice immunized with the KLH conjugate of **1** (Scheme 1).[18] **1**-BSA was used in the screening of hybridoma cell supernatants in an enzyme-linked immunosorbent assay (ELISA). The retro Diels−Alder reactions of **2** and **3** to give anthracene **4** take place spontaneously in aqueous buffer above pH = 4.0 at the rate of approximately 2% per hour at 20 °C ($t_{1/2}$(**2**) = 36 h). Catalysis of the reaction was assayed on ELISA plates by following the appearance of the anthracene fluorescence. Upon excitation with UV light the anthracene product **4** showed strong fluorescence in the visible range (≈460 nm). Individual wells on a 96-well half-area tissue culture plate were prepared with each 50 μL containing 1.5 mg/mL of purified antibody and 200 μM **2** or **3** in aqueous 10 mM phosphate, 160 mM NaCl, pH 7.4 with 10% v/v DMF to ensure product solubility. Within 2 h at 20 °C, a bright blue fluorescence of characteristic intensity developed that was visually detectable for concentrations of **4** ≥2 μM by illuminating the ELISA plate in a dark room with a standard UV lamp (TLC analysis; excitation at 254 nm or 366 nm). Three *anti*-**1** antibodies catalyzed the retro Diels−Alder reaction of cycloadduct **2**. As a control, none of the 120 unrelated monoclonal antibodies catalyzed the cycloreversion. Catalysis was inhibited by addition of hapten **1** or **11** (Scheme 3), confirming that the reactions were taking place in the antibody combining site. One antibody, 9D9, was characterized in detail.

Antibody 9D9 chemoselectively catalyzed the formation of anthracene **4** from isomer **2**. No activity was detected with **3** (Scheme 2). The identity of anthracene **4**, which was already evident from its fluorescence, was further confirmed by RP-HPLC analysis. The release of nitroxyl (HNO) by antibody 9D9 was then investigated. The reaction was carried out in argon-saturated buffer at pH 7.4, under which conditions nitroxyl (pK_a = 4.7) was released as its conjugate base NO^- and trapped with ferric (Fe^{III}) hemoglobin.[19] The reaction was monitored by recording UV spectra of the reaction mixture at 30-min intervals. An increase in absorbance was observed between 530 and 590 nm with two maxima at 545 and 575 nm, a characteristic signature for nitrosyl-hemoglobin. In the presence of 400 μM substrate **2**, 4 μM catalytic antibody 9D9, and [Met-Hb] = 6.25 μM ([heme] = 25 μM), nitrosyl-hemoglobin was formed at an apparent rate of 0.06 μM min^{-1} (Figure 1). Since the rate of cycloreversion amounts to 0.33 μM min^{-1} under these conditions, the efficiency of the NO^- transfer was approximately 20%. The loss of nitroxyl is probably due to concurrent oxidation by remaining oxygen. By comparison, a transfer efficiency of 70% has been reported for the titration of Met-Hb

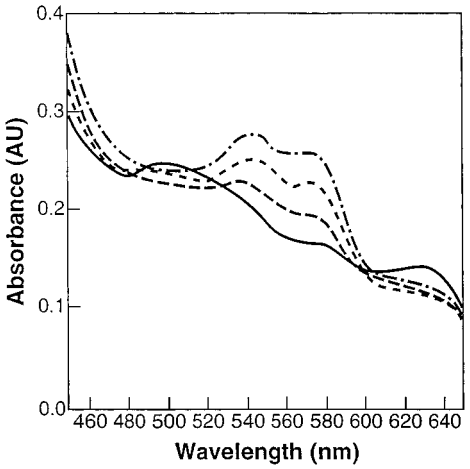

Figure 1. Nitroxyl release revealed by the conversion of Methemoglobin to nitrosyl-hemoglobin. Measured at 20 °C in 10 mM phosphate, 160 mM NaCl, pH 7.4. (—) Initial conditions: [**5**] = 400 μM, [catalytic antibody 9D9] = 4 μM, [Met-Hb] = 6.25 μM. (— —) t = 55 min. (- - -) t = 180 min. (— · —) t = 270 min.

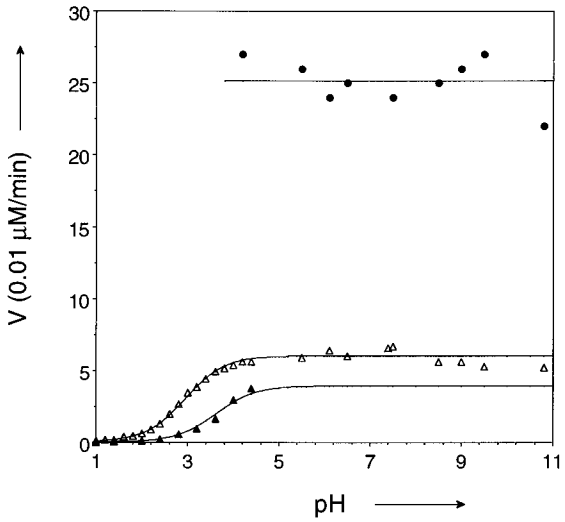

Figure 2. pH−rate profile for the retro Diels−Alder reaction. Apparent rate V_{app} with [**2** or **3**] = 200 μM substrate at 20 °C, 50 mM NaCl, 50 mM buffer (see Experimental Section): (●) apparent rate under catalysis by 4 μM Ab 9D9, the horizontal line represents the average values of all points; (△) background reaction of **2**, apparent pK = 3.0; (▲) background reaction of **3**, apparent pK = 3.60. The lines were calculated from the best linear fit of the experimental points as $1/V_{app} = 1/V_{uncat} + (K/V_{uncat})[H^+]$.

by nitroxyl generated from Angeli's salt ($Na_2N_2O_3$) at 20 °C, pH 7.0, with [heme] = 50 μM under rigorously oxygen free conditions.[20] In our experiment there was no decrease of catalytic activity of the antibody over a period of several hours, showing that the released nitroxyl did not react with the antibody itself.

Catalytic antibody 9D9 was further characterized by kinetic analysis. Initial rates were determined by following the increase of anthracene product **4** by HPLC. Catalysis followed Michaelis−Menten kinetics (K_M(**2**) = 100 μM, k_{cat} = 0.07 min^{-1}, k_{uncat} = 3 × 10^{-4} min^{-1}). Antibody 9D9 showed multiple turnover, and no product inhibition was observed for [**4**] up to 60 μM.

A pH−rate profile analysis showed that the antibody catalysis was constant from pH 4.2 to 10.8 (Figure 2). The catalytic parameters K_M, k_{cat}, and k_{cat}/k_{uncat} were essentially identical at either pH 6.1, 7.4, or 9.0, suggesting that no general acid/base

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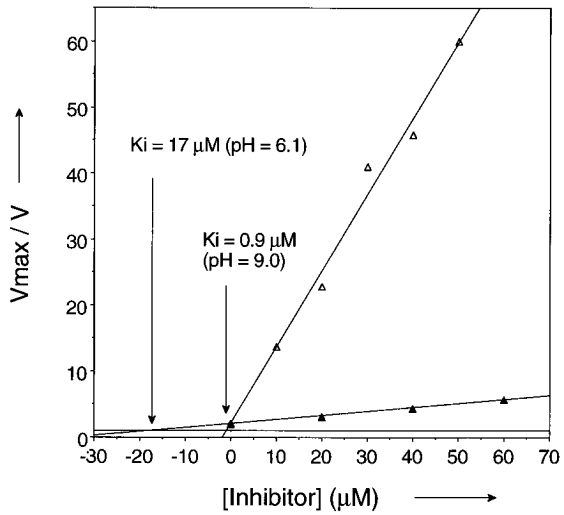

Figure 3. Dixon plot for competitive inhibition of antibody 9D9 catalyzed retro Diels−Alder reaction of **2** by acridinium **11**. Measured at 20 °C with [**2**] = K_M in 50 mM buffer, 50 mM NaCl, 10% v/v DMF: (▲) 50 mM MES pH 6.1, K_M(**2**) = 114 μM, K_i(**1**) = 17 μM; (△) 50 mM bis-Tris propane, pH 9.0, K_M(**2**) = 71 μM, K_i(**1**-OH) = 0.9 μM. The lines are the best linear fit of the experimental points. The *x*-coordinate of their intersection point with the horizontal line at $V = V_{max}$ gives $-K_i$.

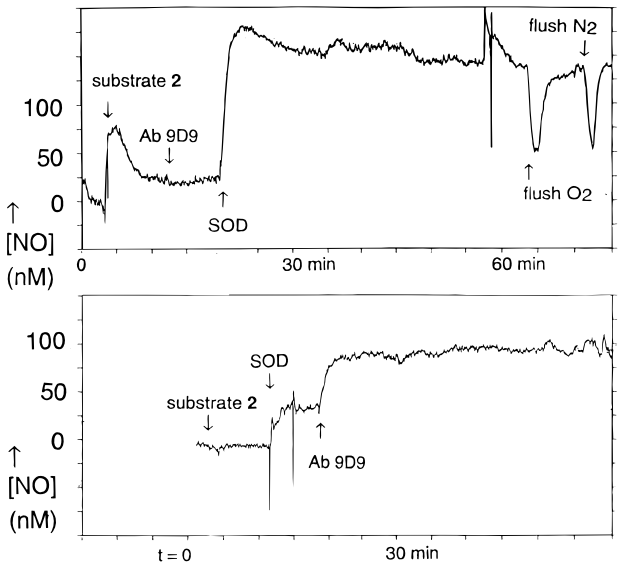

Figure 4. Nitric oxide (NO) released from substrate **2** by Ab9D9/SOD couple. Amperometric reading from an NO-meter electrode immersed in 1 mL of 10 mM phosphate, 160 mM NaCl, pH 7.4, stirred open to air at 20 °C. Upper trace: reaction of 400 μM **2** with 8 μM Ab 9D9 (*anti*-**1**) and 32 μM Cu−Zn SOD. A steady NO level is quickly restored after flushing with either oxygen or nitrogen. Lower trace: reaction of 125 μM **2** with 8 μM Ab 9D9 (*anti*-**1**) and 32 μM Cu−Zn SOD.

or ionizable group influenced either substrate binding or catalysis, in accordance with the hypothesis that the catalyzed reaction was a concerted cycloreversion triggered by binding interactions to the two phenyl rings of the substrate.

The rate for the spontaneous, uncatalyzed reaction was equally constant throughout the pH range and completely insensitive to catalysis by buffers. A decrease in the rate of spontaneous cleavage was observed when the pH was lowered below pH 4.0. In fact the trifluoroacetate salts of **2** and **3** were both stable, which greatly facilitated their preparation, handling, and storage. Thus only the free base forms of either **2** or **3** were reactive for cycloreversion. The apparent p*K* values determined from the pH−rate profile are pK(**2**) = 3.0 and pK(**3**) = 3.6. The lower p*K* for **2** *vs* **3** is correlated with the easier deprotection of formyl derivative **7** vs **8** (Scheme 2), and probably reflects a stronger inductive effect of the propanamide *vs* the methyl substituent.

The inhibition of catalytic antibody 9D9 by **11** (Scheme 4) was found to be strongly pH dependent. At pH 9.0 where the neutral base **11**-OH (Scheme 4) is predominant (86%), tight binding was observed with K_i = 0.9 μM. However, when the pH was lowered to pH 6.1, where the acidic form **11** is predominant (99.2%), a much weaker inhibition was observed, with K_i = 16.6 μM. Thus catalytic antibody 9D9 binds to the neutral pseudo-base **11**-OH much more tightly than to the acidic acridinium form **11**, which suggests that the pseudo-base is a much better transition state analog of the reaction. Interestingly, the dissociation constant of the antibody 9D9−transition state complex, given by $K_{TS} = K_M/(k_{cat}/k_{uncat})$, is comparable to the K_i of pseudo-base **11**-OH with K_{TS} = 0.3 μM at pH 9.0 and K_{TS} = 0.5 μM at pH = 6.1, suggesting that almost all the energy available in hapten binding has been converted to catalysis.[21]

The observed affinity of catalytic antibody 9D9 for the hapten, K_i = 16.6 μM at pH 6.1, is too strong to be attributed to the fraction of pseudo-base **11**-OH at that pH (0.8%), and must therefore be attributed to binding by the acridinium form itself. The UV spectrum of acridinium **11** (10 μM in 10 mM phosphate, 160 mM NaCl, pH 7.4, λ_{max} (ϵ) = 366 nm (1700)) is unaffected by addition of up to 100 μM (7.6 mg/mL) of antibody 9D9, which implies that the hapten is bound in its acidic form **11** and is not hydrated to the conjugate base upon binding to the antibody. It appears that antibody 9D9 is genuinely the result of a heterologous immunization with both immunogens being partially recognized. Although the weaker binding of acridinium **11** might be related to its positive charge, the absence of significant product inhibition (K_i(**4**) > 60 μM) suggests that its product-like geometry is also not well recognized by antibody 9D9.

Nitric Oxide Release. NO^- is rapidly converted to nitric oxide by oxidation with Cu(II)−Zn superoxide dismutase [at pH 7.4: $E°(NO/NO^-)$ = +0.255 V; $E°$(Cu(II)−Zn SOD/Cu(I)-Zn SOD) = +0.42 V]. Nitric oxide release was followed using an ISO-NO meter. This instrument measures NO concentrations by selective amperometric oxidation of nitric oxide to nitrate. The electrode is shielded from solution by a gas-permeable membrane, and there is no interference from dissolved nitroxyl (NO^-). The experiment was carried out in a stirred solution open to air. Under these conditions a steady source of nitric oxide must be present to obtain a durable concentration since NO is rapidly removed by air oxidation and by diffusion. As shown in Figure 4, a steady concentration of NO above 100 nM was obtained in the presence of substrate **2**, catalytic antibody 9D9, and Cu(II)−Zn SOD. This concentration is sufficient to mediate the biological functions of nitric oxide. While the order of addition did not matter, each of the three components was necessary to trigger significant NO production. Steady NO production was also illustrated by the fact that the NO level was quickly restored after flushing with either nitrogen or oxygen.

Conclusion

The first catalytic antibody for a retro Diels−Alder reaction has been reported. Catalytic antibody 9D9 (*anti*-**1**) catalyzes the release of anthracene derivative **4** and nitroxyl from prodrug **2** with multiple turnovers. The catalytic antibody binds the neutral pseudo-base **1**-OH, suggesting that this form of the

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hapten, and not the product-like conjugate acid **1**, is a better transition state mimic for the reaction. As a consequence, there is no measurable product inhibition of catalysis. Catalysis is pH-independent and is probably mediated by interactions between the two phenyl rings of the substrate and the antibody. Nevertheless, the observed chemoselectivity for substrate **2** suggests that additional factors also influence catalysis, for example, electrostatic interactions between the antibody and the transition state if the reaction proceeds via an asynchronous transition state with partial negative charge on oxygen, or hydrogen bonding interactions between an antibody residue and the HNO bridge.

The biological relevance of the reaction has been demonstrated by the production of physiological concentrations of nitric oxide in the presence of the enzyme superoxide dismutase. Releasing nitroxyl by antibody catalysis at a controlled rate under physiological conditions also offers a unique opportunity to study its direct biological effect. Most importantly, both the rate and location of nitroxyl release from prodrug **2** can be controlled by the localization of the catalytic antibody. Preliminary studies have shown that either **2** or anthracene **4** has no measurable cytotoxicity at the concentration used for nitric oxide production, which suggest that the antibody might be a useful source of nitric oxide *in vivo*.

While the rate accelerations we observe are modest, the importance of the reaction is prompting us to explore the production of catalytic antibodies with higher efficiencies of catalysis. Due to the ease of detection of the anthracene product by fluorescence, large-scale screening is the obvious route toward catalysis improvement. Since antibody 9D9 appears to bind tightly to the pseudo-base **1**-OH, this form is probably the most relevant for inducing catalysis. Immunizations with haptens featuring stabilized forms of pseudo-base **1**-OH have been initiated to generate libraries of catalytic antibodies.

Experimental Section

General Remarks. Reagents were purchased from Aldrich or Fluka. Solvents were A.C.S. grade from Fisher. All chromatographies (flash) were performed with Merck Silicagel 60 (0.040−0.063 mm). Preparative HPLC was done with Fisher Optima grade acetonitrile and ordinary deionized water using a Waters prepak cartridge 500 g installed on a Waters Prep LC 4000 system from Millipore, flow rate 100 mL/min, gradient + 0.5% min^{-1} CH_3CN, detection by UV at 254 nm. TLC was performed with fluorescent Merck F254 glass plates. NMR spectra were recorded on a Brucker AM-300 MHz or AM-500 MHz instrument. Chemical shift δ are given in ppm and coupling constant ^3J in hertz. UV spectra were recorded on a HP-8452A instrument. MS, HRMS (high-resolution mass spectra) were provided by the Scripps Research Institute facility (Gary Siuzdak). NO measurements were performed with an ISO-NO meter from World Precision Instruments Inc.

(A) Synthesis. (a) 9-[2′-(Ethoxycarbonyl)vinyl]-10-methylanthracene (5). 10-Methylanthracene-9-carboxaldehyde (2.50 g, 11.4 mmol) and (carbethoxymethylene)triphenylphosphorane (6.38 g, 18.3 mmol) were dissolved in 50 mL of CH_2Cl_2. The mixture was heated to reflux for 36 h. Removal of the solvent and flash chromatography (hexane/ethyl acetate, 30:1) yielded **5** (3.08 g, 10.6 mmol, 93%). Yellow crystals: mp 89 °C (hexane). ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.67 (d, J = 16.5 Hz, 1H), 8.30 (m_c, 4H), 7.52 (m_c, 4H), 6.40 (d, J = 16.5 Hz, 1H), 4.42 (q, J = 7.3 Hz, 2H), 3.08 (s, 3H), 1.45 (t, J = 7.3 Hz, 3H). FABLRMS (3-NBA−NaI): *m/e* 313 $[M + Na]^+$, 290 $[M]^+$.

(b) 9-[2′-(Ethoxycarbonyl)ethyl]-10-methylanthracene (6). **5** (1550 mg, 5.35 mmol) and $NiCl_2 \cdot H_2O$ (425 mg, 1.79 mmol) were dissolved in 60 mL of ethanol. $NaBH_4$ (202 mg, 5.35 mmol) was added at ambient temperature portionwise over a period of 2 h. The reaction mixture turned black. It was stirred for an additional 4 h and then acidified with 2 M HCl. The aqueous phase was extracted (CH_2Cl_2), and the organic phase was washed ($NaHCO_3$, H_2O) and dried ($MgSO_4$). Evaporation of the solvent and flash chromatography (hexane/ethyl acetate, 30:1) gave **6** (1249 mg, 4.28 mmol, 80%). Colorless needles: mp 67 °C (methanol). ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.36 (m_c, 4H), 7.58 (m_c, 4H), 4.25 (q, J = 7.2 Hz, 2H), 4.01 (t, J = 8.5 Hz, 2H), 3.18 (s, 3H), 2.82 (t, J = 8.5 Hz, 2H), 1.34 (t, J = 7.2 Hz, 3H). FABLRMS (3-NBA−NaI): *m/e* 315 $[M + Na]^+$, 292 $[M]^+$.

(c) 9-[2′-((2″-Hydroxyethyl)carbamyl)ethyl]-10-methylanthracene (4). The solution of **6** (1000 mg, 3.42 mmol), ethanolamine (5230 mg, 85.6 mmol), and NaCN (15 mg, 0.30 mmol) in 30 mL of ethanol was stirred at room temperature for 36 h. CH_2Cl_2 and aqueous KH_2PO_4 solution were then added until a clear two-phase system was obtained. The pH of the aqueous phase was adjusted to ≈7 (2 M HCl). The aqueous phase was extracted with CH_2Cl_2, the organic phase was washed (H_2O) and dried ($MgSO_4$), and the solvent was evaporated. Flash chromatography (CH_2Cl_2/methanol, 20:1) and crystallization ($CHCl_3$) afforded **4** (893 mg, 2.91 mmol, 85%). Slightly yellow needles: mp 186 °C ($CHCl_3$). ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.32 (m_c, 4H), 7.49 (m_c, 4H), 5.60 (s br, 1H), 3.95 (t, J = 7.9 Hz, 2H), 3.48 (t, J = 4.9 Hz, 2H), 3.23 (q, J = 4.9 Hz, 2H), 3.04 (s, 3H), 2.63 (t, J = 7.9 Hz, 2H). FABLRMS (3-NBA−NaI): *m/e* 330 $[M + Na]^+$, 307 $[M]^+$. UV (PBS, pH 7.4): λ_{max} (ϵ) 260 (1.1×10^5), 360 (568), 378 (928), 400 (905).

(d) 9,10-Epoxyimino-12-formyl-9-methyl-10-[2′-((2″-hydroxyethyl)carbamyl)ethyl]anthracene (7) and 9,10-Epoxyimino-12-formyl-9-[2′-((2″-hydroxyethyl)carbamyl)ethyl]-10-methylanthracene (8). To the clear solution of **4** (300 mg, 0.98 mmol) and $[Me_3N^+Bn]IO_4^-$ (665 mg, 1.95 mmol) in 2 mL of absolute DMF was added the solution of HCONHOH (119 mg, 1.95 mmol) in 2 mL of absolute DMF over a period of 30 min at ambient temperature. The mixture turned yellow. It was stirred for an additional 1 h and then poured on ice. CH_2Cl_2 and 0.1 M aqueous $Na_2S_2O_5$ solution were added until a clear two-phase system was present. The aqueous phase was extracted (CH_2Cl_2), and the combined organic phases were washed ($NaHCO_3$, H_2O) and dried ($CaCO_3$). The solvent was removed *in vacuo* at room temperature. The oily, slightly yellow residue was taken up in H_2O/CH_3CN/TFA 80/20/0.1 and subjected to preparative RP-HPLC. Besides recovered **4** (60 mg, 0.20 mmol, 20%), **7** (108 mg, 0.29 mmol, 30%) and **8** (105 mg, 0.29 mmol, 30%) were isolated as colorless, crystalline substances. **7**: mp 95 °C dec. ^{1}H-NMR (CD_3OD, 500 MHz): δ 8.38 (s, 1H), 8.20 (s br, 1H), 7.52 (m_c, 2H), 7.47 (m_c, 2H), 7.30 (m_c, 4H), 3.66 (t, J = 5.7 Hz, 2H), 3.42−3.35 (m, 4H), 2.82 (t, J = 7.8 Hz, 2H), 2.24 (s, 3H). ^{13}C-NMR (CD_3OD, 125 MHz): δ 175.0, 152.2, 141.7, 140.3, 129.0, 122.6, 122.5, 121.8, 81.2, 67.1, 61.6, 43.3, 31.7, 23.9, 14.9. FABHRMS (3-NBA−NaI): *m/e* calcd for $[C_{21}H_{22}N_2O_4 + H]^+$ 367.1658, found 367.1641. **8**: mp 97 °C dec. ^{1}H-NMR (CD_3OD, 500 MHz): δ 8.27 (s, 1H), 8.15 (s br, 1H), 7.49 (m_c, 4H), 7.33 (m_c, 4H), 3.65 (t, J = 5.7 Hz, 2H), 3.37 (q, J = 5.7 Hz, 2H), 3.11 (m_c, 2H), 2.82 (m_c, 2H), 2.47 (s, 3H). ^{13}C-NMR (CD_3OD, 125 MHz): δ 175.8, 153.7, 141.7, 141.6, 129.0, 122.7, 122.6, 121.8, 83.0, 63.5, 61.5, 43.3, 31.0, 25.8, 14.5. FABHRMS (3-NBA−NaI): *m/e* calcd for $[C_{21}H_{22}N_2O_4 + H]^+$ 367.1658, found 367.1642.

(e) 9,10-Epoxyimino-9-methyl-10-[2′-((2″-hydroxyethyl)carbamyl)ethyl]anthracene (2). The solution of **7** (100 mg, 0.27 mmol) in 5 mL of methanol and 0.5 mL of TFA was stirred at 0 °C for 24 h. The reaction was stopped by addition of 30 mL of H_2O, and the crude product was purified by RP-HPLC. **2** (117 mg, 0.26 mmol, 95%) was isolated as its TFA salt and storred at −78 °C. Colorless needles: mp 64 °C dec. ^{1}H-NMR (CD_3OD, 300 MHz): δ 7.61 (m_c, 4H), 7.42 (m_c, 4H), 3.62 (t, J = 5.6 Hz, 2H), 3.33 (t, J = 5.6 Hz, 2H), 3.23 (t, J = 6.9 Hz, 2H), 2.91 (t, J = 6.9 Hz, 2H), 2.28 (s, 3H). FABHRMS (3-NBA): *m/e* calcd for $[C_{20}H_{22}N_2O_3^+ + H]^+$ 339.1709, found 339.1719.

(f) 9,10-Epoxyimino-9-[2′-((2″-hydroxyethyl)carbamyl)ethyl]-10-methylanthracene (3). The solution of **8** (100 mg, 0.27 mmol) in 5 mL of methanol and 0.5 mL of TFA was stirred at 20 °C for 12 h. The reaction was stopped by addition of 30 mL of H_2O, and the crude product was purified by RP-HPLC. **3** (115 mg, 0.25 mmol, 93%) was isolated as its TFA salt and stored at −78 °C. Colorless needles: mp 63 °C dec. ^{1}H-NMR (CD_3OD, 300 MHz): δ 7.61 (m_c, 4H), 7.45 (m_c, 4H), 3.61 (t, J = 5.7 Hz, 2H), 3.36 (t, J = 5.7 Hz, 2H), 3.20 (m_c, 2H), 2.76 (m_c, 2H), 2.36 (s, 3H). FABHRMS (3-NBA): *m/e* calcd for $[C_{20}H_{22}N_2O_3 + H]^+$ 339.1709, found 339.1715.

(g) *N*-[2′-(Benzyloxycarbonyl)ethyl]-9-acridinecarboxamide (9). 9-Acridinecarboxylic acid hydrate (97%, 1000 mg, 4.34 mmol) was

dissolved in 10 mL of refluxing $SOCl_2$. After 6 h a clear solution was obtained, and all volatiles were removed *in vacuo*. The slightly yellow, crude acid chloride was suspended in 15 mL of CH_2Cl_2. A solution of β-alanine benzyl ester *p*-toluenesulfonate (1980 mg, 5.64 mmol) and NEt_3 (1317 mg, 13.02 mmol) in 15 mL of CH_2Cl_2 was added at room temperature, and the mixture was stirred for 14 h. The organic phase was washed ($NaHCO_3$, NaCl) and dried ($MgSO_4$). Removal of the solvent and flash chromatography (hexane/ethyl acetate, 1:1) yielded **9** (1487 mg, 3.86 mmol, 89%). Light yellow crystals: mp 153 °C (hexane/$CHCl_3$). ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 8.15 (d, J = 8.4 Hz, 2H), 7.91 (d, J = 8.5 Hz, 2H), 7.71 (m_c, 2H), 7.49 (m_c, 2H), 7.21 (m_c, 5H), 6.91 (s br, 1H), 5.11 (s, 2H), 3.98 (q, J = 6.0, 2H), 2.88 (t, J = 6.0 Hz, 2H). FABLRMS (3-NBA−NaI): *m/e* 407 $[M + Na]^+$, 384 $[M]^+$.

(h) 9-(*N*-[2′-(Benzyloxycarbonyl)ethyl]carbamyl)-10-methylacridinium Sulfate (10). **9** (50 mg, 0.13 mmol) and freshly neutralized (K_2CO_3) $(CH_3)_2SO_4$ (20 mg, 0.16 mmol) were heated in 1 mL of toluene to reflux for 2.5 h. Removal of all volatiles and flash chromatography (CH_2Cl_2/methanol, 5:1) afforded **10** (55 mg, 0.12 mmol, 95%, calcd as sulfate salt). Bright yellow crystals: mp 158 °C (ether). ^{1}H-NMR (CD_3OD, 300 MHz): δ 8.70 (m_c, 2H), 8.42−8.35 (m, 4H), 7.88 (m_c, 2H), 7.42−7.25 (m, 5H), 5.18 (s, 2H), 4.88 (s, 3H), 3.98 (t, J = 6.3, 2H), 2.90 (t, J = 6.3 Hz, 2H). FABLRMS (3-NBA): *m/e* 399 $[M]^+$.

(i) 9-(*N*-[2′-carboxylethyl]carbamyl)-10-methylacridinium Trifluoroacetate (1). A solution of **10** (50 mg, 0.11 mmol, sulfate salt) in 3 mL of 0.1 M aqueous KOH/methanol 2:1 was heated to reflux for 20 min. The colorless mixture was acidified with 2 M HCl (pH 2). The bright yellow color and fluorescence reappeared. The crude product was subjected to preparative RP-HPLC. **1** (42 mg, 0.10 mmol, 89%) was isolated as its TFA salt. Yellow crystals: mp 203 °C dec. **1:** ^{1}H-NMR (CD_3OD, 300 MHz): δ 8.82 (d, J = 9.3 Hz, 2H), 8.51 (m_c, 4H), 8.10 (m_c, 2H), 5.01 (s, 3H), 4.00 (t, J = 6.3, 2H), 2.88 (t, J = 6.3 Hz, 2H). ^{1}H-NMR (D_2O, 300 MHz): δ 8.40 (d, J = 9.3 Hz, 2H), 8.15 (m_c, 4H), 7.78 (t, J = 7.6 Hz, 2H), 4.68 (s, 3H), 3.78 (t, J = 6.4, 2H), 2.68 (t, J = 6.4 Hz, 2H). ^{13}C-NMR (CD_3OD, 125 MHz): δ 175.0, 165.9, 155.5, 143.4, 140.5, 140.4, 129.9, 124.0, 120.0, 39.6, 37.4, 34.3. FABLRMS (3-NBA): *m/e* 309 $[M]^+$. FABHRMS (3-NBA): *m/e* calcd for $[C_{18}H_{17}N_2O_3]^+$ 309.1239, found 309.1247. **1**-OH: ^{1}H-NMR (D_2O/K_2CO_3, 300 MHz): δ 7.10 (m_c, 4H), 6.89 (d, J = 8.9 Hz, 2H), 6.75 (t, J = 7.2 Hz, 2H), 3.28 (t, J = 6.8, 2H), 3.12 (s, 3H), 2.20 (t, J = 6.8 Hz, 2H).

(j) 9-(*N*-[2′-((2″-Hydroxyethyl)carbamyl)ethyl]carbamoyl)-10-methylacridinium Trifluoroacetate (11). A solution of **10** (45 mg, 0.10 mmol, sulfate salt) and ethanolamine (305 mg, 5.0 mmol) in 3 mL of ethanol was stirred at room temperature for 36 h. All volatiles were removed *in vacuo*. The oily residue was dissolved in H_2O/0.1% TFA, and the crude product was purified by RP-HPLC. **11** (37 mg, 0.08 mmol, 80%, TFA salt), yellow crystals: mp 184 °C. **11:** ^{1}H-NMR (CD_3OD, 300 MHz): δ 8.80 (d, J = 9.2 Hz, 2H), 8.51−8.38 (m, 4H), 8.02 (t, J = 7.7 Hz, 2H), 4.92 (s, 3H), 3.97 (t, J = 6.4, 2H), 3.60 (t, J = 5.7, 2H), 3.34 (t, J = 5.7, 2H), 2.73 (t, J = 6.4 Hz, 2H). FABLRMS (3-NBA): *m/e* 352 $[M]^+$. FABHRMS (3-NBA−NaI): *m/e* calcd for $[C_{20}H_{22}N_3O_3]^+$ 352.1661, found 352.1668. UV (0.1% TFA/H_2O, pH 2): λ_{max} (ϵ) 260 (8.4×10^4), 366 (1700). **11**-OH: UV (CAPS buffer, pH 10.8): λ_{max} (ϵ) 285 (1790).

(k) Conjugation of Hapten 1 with Carrier Proteins BSA and KLH. 1-(3-(Dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC) (5.4 mg, 0.028 mmol) in 10 μL of H_2O and *N*-hydroxysulfosuccinimide (Sulfo-NHS) (6.1 mg, 0.028 mmol) in 10 μL of H_2O were added successively to a solution of **1** (8.0 mg, 0.019 mmol) in 400 μL of DMF. After a 24-h incubation at ambient temperature, 200 μL of the solution of the activated hapten was added to 1800 μL of a solution of keyhole limpet hemocyanin (KLH, 4.4 mg/mL) in 50 mM phosphate buffer (pH 7.4), and 200 μL of the solution of the activated hapten was added to 1800 μL of a solution of bovine serum albumin (BSA, 4.4 mg/mL) in 50 mM phosphate buffer (pH 7.4). The mixtures were kept at 4 °C for 24 h and were then stored at −18 °C.

B. Antibody Assays. (a) Antibodies. Monoclonal antibodies against the KLH conjugate of **1** were produced using standard procedures and were obtained from ascites fluid grown from the individual hybridoma cell lines.[17] Each antibody was purified to homogeneity by ammonium sulfate precipitation followed by anion exchange and protein G chromatography, dialyzed into 10 mM phosphate, 160 mM NaCl, pH 7.4, and its concentration estimated by UV at 280 nm as c (mg/mL) = Abs/1.4.

(b) Stock Solutions. Substrate **2** and **3**, obtained from HPLC purification, were stored at −78 °C as 5 mM stock solution in 0.1% v/v CF_3COOH in water. **1** and **11** were used as 5 mM stock solutions of the HPLC purified salt in water.

(c) Kinetic Measurements. Each reaction (200 μL) was initiated by adding 10 μL of a prediluted substrate solution to 190 μL of the appropriate buffer (50 or 100 mM buffer, 50 mM NaCl) containing 10% v/v DMF and, when used, the antibody at 0.3−1.5 mg/mL concentration. The following buffers were used: KCl/HCl for $1.0 \le$ pH ≤ 2.0; glycine/HCl for $2.2 \le$ pH ≤ 3.6; citrate/phosphate for $2.6 \le$ pH ≤ 4.4; citrate for $3.5 \le$ pH ≤ 6.0; phosphate (PBS: phosphate buffer saline) for $6.0 \le$ pH ≤ 8.0; bis-Tris propane (1,3-bis[tris-(hydroxymethyl)methylamino]propane) for $6.5 \le$ pH ≤ 9.5; CAPS (3-(cyclohexylamino)-1-propanesulfonic acid) for $10.0 \le$ pH ≤ 11.0; MES (morpholinoethanesulfonic acid) for pH 6.1; HEPES (4-(2-hydroxyethyl)-1-piperazinylethanesulfonic acid) for pH 7.5; Tris (tris(hydroxymethyl)aminomethane) for pH 8.0. None of these buffers affected either the background or the antibody-catalyzed retro Diels−Alder reaction with **2** or **3**.

For activity screening, the reaction progress was followed visually by running the reactions in individual wells of a 96 well half-area tissue culture plate and illuminating the plate in a dark room with a standard laboratory lamp for TLC at either 254 or 366 nm.

Kinetic measurements were done by following the formation of anthracene product **4** over a period of 2 to 3 h by RP-HPLC (Microsorb MV C-18, 300 Å poresize, 0.45 × 22 cm, isocratic elution at 1.5 mL/min with 35.5% CH_3CN, 64.4% H_2O, 0.07% CF_3COOH, detection by UV 254 nm) against propyl-4 hydroxybenzoate as internal standard (t_R(**4**) = 8.8 min, t_R(propyl 4-hydroxybenzoate) = 6.2 min). The net catalytic rate V_{cat} was obtained by subtracting the apparent rate in a sample without antibody V_{uncat} from the apparent rate in an identical sample with antibody. Lineweaver−Burk analyses were done at [Ab 9D9] = 0.3 mg/mL, using five different substrate concentrations [**2**] = 50, 100, 150, 200, 400 μM. The uncatalyzed rate V_{uncat} was directly proportional to the substrate concentration within that range. Plots of $1/V_{cat}$ vs 1/[**2**] were linear (5 points, $r^2 > 0.990$). The results were as follows: at pH 6.1 (MES buffer) $k_{cat} = 7.2 \times 10^{-2}$ min^{-1}, $k_{uncat} = 3.2 \times 10^{-4}$ min^{-1}, K_M = 114 μM; at pH 7.4 (PBS buffer) $k_{cat} = 7.3 \times 10^{-2}$ min^{-1}, $k_{uncat} = 3.3 \times 10^{-4}$ min^{-1}, K_M = 125 μM; at pH 9.0 (bis-Tris propane buffer) $k_{cat} = 7.0 \times 10^{-2}$ min^{-1}, $k_{uncat} = 2.8 \times 10^{-4}$ min^{-1}, K_M = 71 μM. Quantitative inhibition studies with hapten **1**-OH at pH 9.0 showed that the antibody sample had two catalytic sites per antibody molecule, with [Ab 9D9] = 0.3 mg/mL corresponding to 4 μM active sites. The catalytic activity k_{cat} is reported per active site.

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