Catalytic Antibodies by Fluorescence Screening

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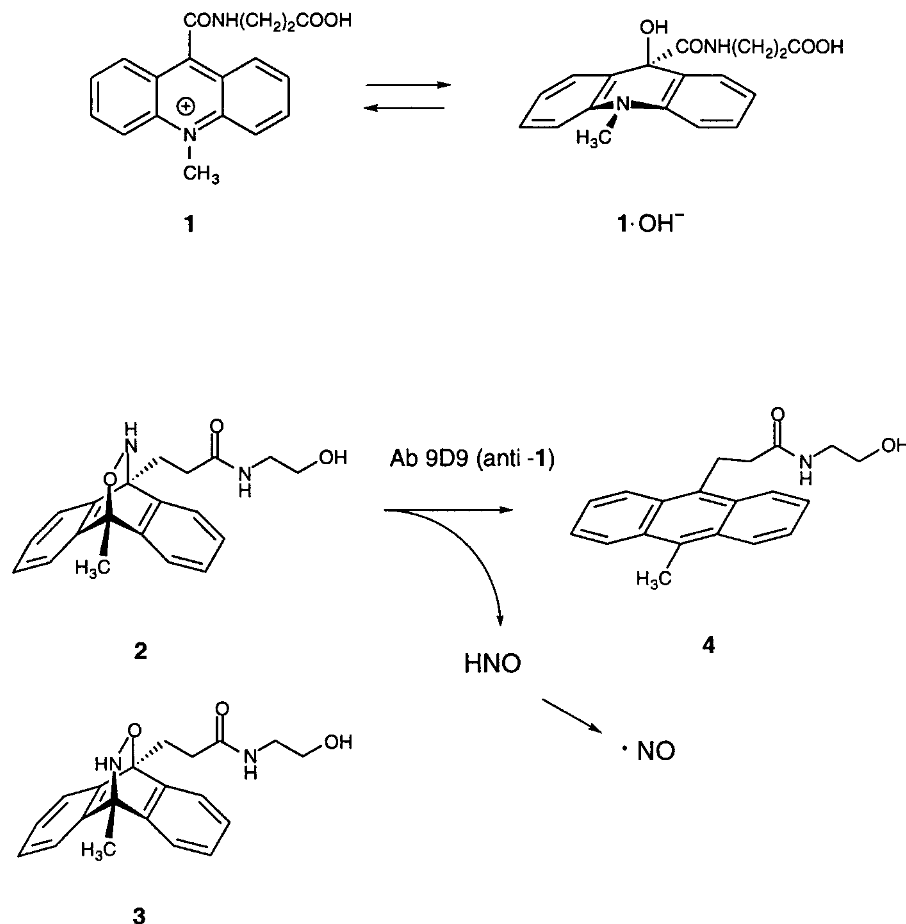
A series of eleven immunizations against transition-state analogs were carried out to improve the catalytic properties of Ab 9D9, a catalytic antibody that catalyzes a fluorogenic *retro-Diels-Alder* reaction liberating nitroxyl. By a direct fluorescence assay of cell-culture supernatant, eight new hybridoma cell lines producing catalytic antibodies for the reaction were readily identified among more than 14000 individual samples. Our results demonstrate that early catalysis screening by fluorescence allows an efficient survey of large antibody libraries, and may lead to rapid and significant improvement in catalysis.

Introduction. – Catalytic antibodies can be obtained from immunizations against stable transition-state analogs of chemical reactions used as haptens. In the past decade, this method has established itself as a powerful approach to generate novel biocatalysts [1]. The success of this method depends heavily on the ability to screen a large number of antibodies binding to a transition-state analog and identify those antibodies, usually only a few of them, that also display catalytic properties. This approach requires an assay for catalysis applicable repetitively and reliably on a very small scale. In recent years, a number of such assays have been reported that rely on reagents on a solid support [2], or on substrates labeled with the fluorescent tag acridone [3]. However, the most straightforward method consists in following product formation spectroscopically in real time using a fluorogenic or chromogenic substrate [4]. Herein, we show that the latter approach, which is by far the simplest, succeeds surprisingly well in identifying catalytic antibodies directly in cell culture. We also show that the systematic screening for catalysis thus made possible allows one to survey large numbers of antibodies within very short time periods.

Results and Discussion. – Catalytic antibody 9D9 (Ab 9D9), an antibody against hapten **1**, catalyzes the *retro-Diels-Alder* reaction of prodrug **2** to release anthracene **4** and nitroxyl (HNO) [5]. Nitroxyl can be converted *in situ* to nitric oxide ($NO^{\bullet}$) [6], a reactive gas implicated in a number of biological processes [7]. Catalytic antibody 9D9 binds selectively $\mathbf{1} \cdot OH^-$, the conjugate base of **1**, which suggests that $\mathbf{1} \cdot OH^-$ is the actual transition-state analog of the reaction in hapten **1** (*Scheme 1*). The *retro-Diels-Alder* reaction of prodrug **2** or of its isomer **3** can be measured directly in cell culture by following the release of anthracene **4** by fluorescence. We set out to apply this simple fluorescence assay to investigate large numbers of antibodies against hapten **1** for catalysis. These antibodies would be generated in the form of hybridoma cell lines derived from hapten immunizations. Instead of subcloning all of these hybridoma cell lines, fluorescence screening would be applied from the beginning to identify cells producing catalytic antibodies, and only those would be followed. Our goal was to see if

Scheme 1. retro-Diels-Alder *Reaction of Prodrug* **2** *or* **3** *Releasing Nitroxyl and Anthracene* **4**, *with Antibody 9D9 against Hapten* **1** *Catalyzing the Reaction with Prodrug* **2**

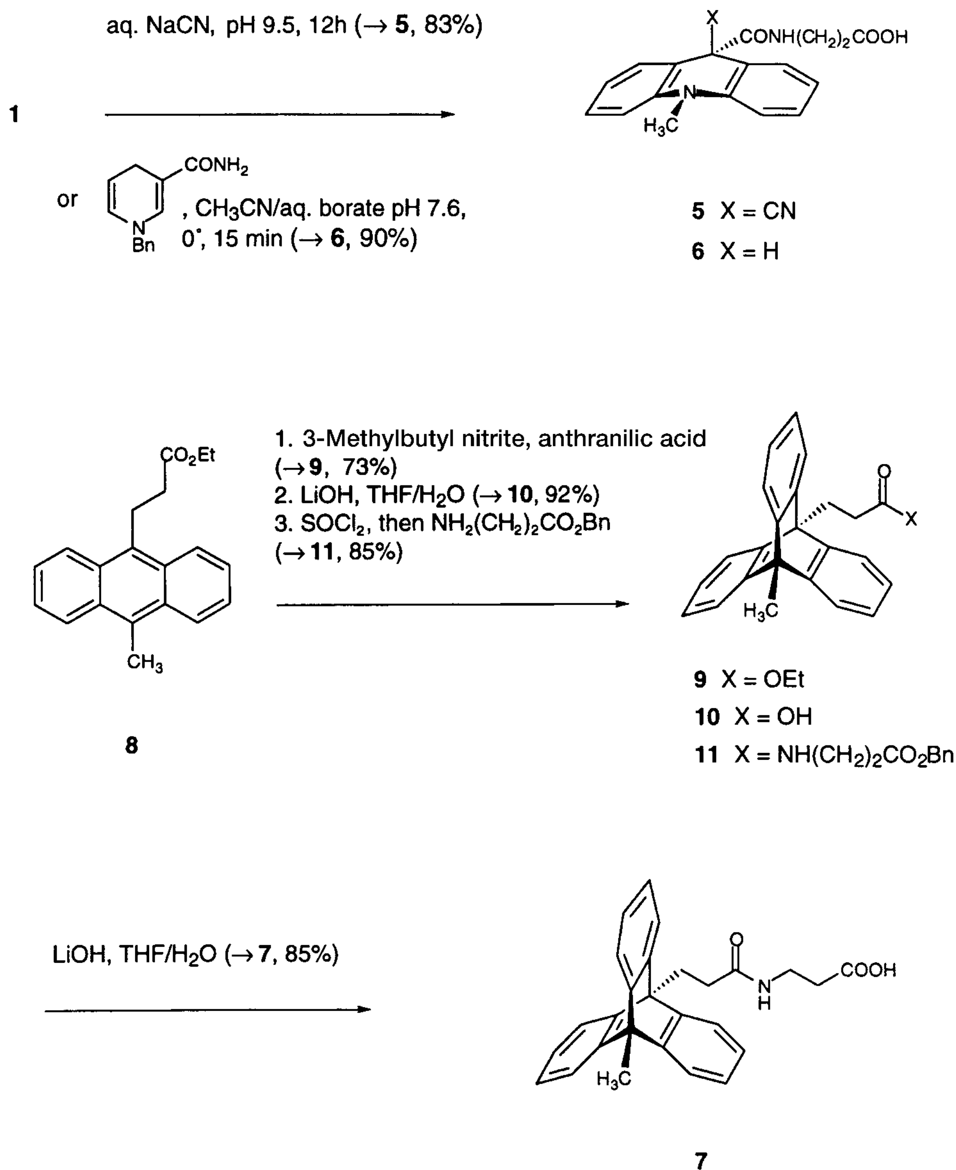


such an early screening approach with selection based on fluorescence could succeed in practice as a method to improve upon the catalytic properties of Ab 9D9.

To broaden the spectrum of antibodies generated by immunization, we prepared cyano derivative **5** and dihydroacridine **6** (*Scheme 2*). These close variants of the original hapten **1** represent stabilized forms of transition-state analog **1** · OH^-, which was found to bind preferentially to catalytic antibody 9D9. In addition, trypticene hapten **7** was prepared to check if a simple substrate analog might also lead to catalytic antibodies (*Scheme 3*). Haptens were conjugated to the carrier proteins KLH (*k*eyhole-*l*impet *h*emocyanin) and BSA (*b*ovine-*s*erum *a*lbumin) by amide-bond formation involving the terminal carboxy group.

One immunization was carried out for each hapten **1**, **5**, **6**, and **7**. In addition, we conducted two series of heterologous immunizations [8] using either hapten **1** followed by hapten **5** and finally **6**, or hapten **5** followed by hapten **1**. Hybridoma were generated

Scheme 2. *Synthesis of Haptens* **5** *and* **6**, *Two Stabilized Forms of* **1** · OH^- *and of Triptycene Hapten* **7**, *a Stable Analog of Substrates* **2** *and* **3**



from each immunization following standard protocols [9], and the cells plated out in 96-wells cell-culture plates. After ten to fifteen days of cell growth, each well was assayed for the presence of hapten-binding antibodies by ELISA (*e*nzyme-*l*inked *i*mmuno*s*orbent *a*ssay). Each well was also tested for catalysis of the *retro-Diels-Alder* reaction with both substrates **2** and **3**. In each case, a subset of approximately 48 wells

containing hapten-binding antibodies, including all wells that also tested positive for catalysis, were kept and cultured for another 10–15 days. Selection was repeated on the same criteria, which reduced the total number of samples to be considered to eighteen. Further cell culture of these eighteen samples finally yielded eight stable monoclonal hybridoma cell lines, each of which produced a catalytic antibody. Cells were propagated either in ascites fluid or in cell culture, and the antibodies produced by these cells purified to homogeneity by ammonium sulfate precipitation, ion exchange, and protein-G chromatography.

Overall, each of the eleven immunizations gave a fair number of wells testing positive for binding to its respective hapten (1.5 to 15%, see *Table 1*). Five immunizations did not give any catalysis, including that against hapten **1**, which had yielded Ab 9D9 in a previous immunization, and that against trypticene **7**, which was the most immunogenic hapten. Only six immunizations using either **1**, **5**, **6**, or mixtures of these actually yielded catalytic antibodies for the reaction. Interestingly, none of the immunizations showed catalysis for substrate **3**.

Table 1. *Immunization Results for Binding and Catalysis*

Entry	Hapten[a])	Wells[b])	Binders[c])	Catalytic[d])	Monoclonal[e])
1	**1/1/1**	1536	202	0	–
2	**5/5**	1440	228	1	8E5
3	**6/6**	1536	54	3	10F11, 10F4, 8B8
4	**7/7**	1728	270	0	–
5	**1/5/6**	1152	170	1	6C6
6	**1/5/6**	1248	49	0	–
7	**1/5/6**	1440	23	0	–
8	**5/1**	1056	34	5	42B5
9	**5/1**	1152	17	6	27C5
10	**5/1**	1152	22	0	–
11	**5/1**	1248	74	2	12E8
Total		14668	1143	18	8

[a]) Successive immunizations were carried out at 15 days interval using the indicated haptens as KLH conjugates. [b]) Number of cell-culture wells tested. [c]) Binding to the respective hapten-BSA conjugates as tested by ELISA. Mixed immunizations (*Entries 5–11*) were tested against an equal mixture of **1**-BSA, **5**-BSA, and **6**-BSA. [d]) Apparent catalysis of the *retro-Diels-Alder* reaction $\mathbf{2} \rightarrow \mathbf{4}$. [e]) Antibodies isolated as stable hybridoma cells lines. Code indicates initial 96-well cell-culture plate number followed by well position (A1 through H12).

The activity of all catalytic antibodies was completely inhibited in the presence of hapten **5** (15 μM). Cross-reactivity to hapten **5** could be expected from the close structural similarity between **1**, **5**, and **6**, and was confirmed by ELISA. Inhibition of catalysis established that all catalytic reactions were taking place specifically in the antibody combining sites. All antibodies catalyzed the *retro-Diels-Alder* reaction of substrate **2** in aqueous buffer. As can be appreciated from *Table 2*, catalytic antibodies with very different kinetic parameters were isolated (see also *Fig.*). Two antibodies (Ab), Ab 10F11 (anti **6**) and Ab 27C5 (anti **5**/**1**), were found to be five to ten times more efficient than the original Ab 9D9 (anti **1**). Thus, early selection had allowed us to

Table 2. *Kinetic Parameters of* retro-Diels-Alderase *Catalytic Antibodies with Substrate* **2**

Antibody	K_M [μM]	$k_{cat} \cdot 10^{-3}$ [s^{-1}]	k_{cat}/k_{uncat}	K_{TS} [μM]
9D9[a])	165	4.0	**406**	**0.41**
8E5	120	1.44	148	0.81
10F11[a])	256	24.5	**2505**	**0.102**
10F4	91	1.0	103	0.89
8B8	48	0.6	62	0.78
6C6	–	0.07	ca 8	–
42B5	85	0.96	98	0.87
27C5[a])	85	18.9	**1930**	**0.044**
12E8	112	1.4	146	0.77

[a]) Measured in aq. 10 mM phosphate and 160 mM NaCl, at pH 7.4 and 31°. Under these conditions, the uncatalyzed reaction rate was k_{uncat} (**2**) = $9.8 \cdot 10^{-6}$ s^{-1}. Measured with 10% (*v*/*v*) DMF.

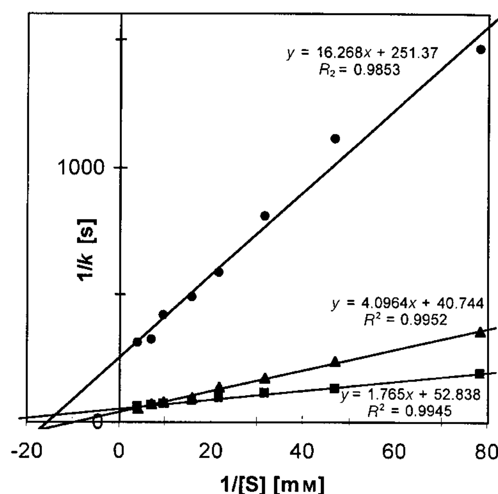


Figure. *Double reciprocal plot for catalysis of the* retro-Diels-Alder *reaction of* **2** *by catalytic antibodies 9D9* (●), *10F11* (△), *and 27C5* (■). Measured in aq. 10 mM phosphate (pH 7.4), 160 mM NaCl, and 10% (*v*/*v*) DMF at 31°.

reach our primary goal to improve on the properties of Ab 9D9 (anti **1**). This was all the more remarkable since we had invested only very limited resources in cell cultures.

In addition to improved catalytic antibodies, we also found several antibodies displaying only poor catalytic properties. This was quite surprising because we expected that only efficient catalytic antibodies would yield measurable catalysis signals in the cell-culture supernatant. The fact that such poor catalysts give an observable catalysis signal implies that the concentration of antibodies in the cell-culture supernatant is sometimes much higher than the standard values of 5–50 μg · ml^{-1}. It also illustrates the fact that selecting for observable catalysis in the cell-culture supernatant puts pressure not only on catalytic efficiency of the antibodies, but also on productivity of the hydridoma cell line. If one considers that expression in hybridoma is currently by far the most efficient method to produce antibodies, selecting for productivity is

basically a sound choice, particularly if large amounts of the antibodies are eventually needed. Direct selection such as that demonstrated here could, however, in principle miss a highly efficient catalytic antibody expressed in vanishingly small amounts.

Conclusion. – The above experiments show that selecting for catalysis in the hybridoma cell-culture supernatant using a fluorogenic reaction allows one to identify catalytic antibodies efficiently among a large number of samples. Fluorescence screening in cell culture detects product formation in the 10^{-6} M concentration range for the substrate being present in 10^{-4} M concentration. Our results clearly demonstrate that this sensitivity is more than sufficient to detect any useful catalytic antibody present in the medium. Of equal importance is the impact of early screening on the overall extent of the experiment. As is also possible with other catalysis assay procedures [2], selection by fluorescence screening was applied as early as fifteen days after hybridoma generation and reduced the number of hybridoma cell lines to be followed dramatically. Taking advantage of this reduced load of cell culture, we had the opportunity to cover a significant number of immunizations within a short time window.

Having covered a series of immunizations, we feel confident that the catalytic potential available in antibodies raised against hapten **1** and close derivatives has been fairly well surveyed, and amounts to approximately 10^3 in rate enhancement for the *retro-Diels-Alder* reaction of substrate **2**. We have surveyed more than one hundred purified monoclonal antibodies against unrelated haptens for this reaction and failed to observe any catalysis, as was the case using substrate analog **7** as hapten. This observation is very reliable given the high sensitivity of our fluorescence assay, and stresses the importance of hapten design in raising catalytic antibodies. The mechanism of action of our antibodies is currently being investigated. We are also developing fluorogenic assays for other reactions to make them amenable to similar investigations of catalysis by antibodies [4c–e].

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Experimental Part

General. All reagents and enzymes were purchased from *Aldrich* or *Fluka*. Flash chromatography (FC): *Merck* silica gel *60* (0.040–0.063 mm). Prep. HPLC: HPLC-grade MeCN and *MilliQ*-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 MeCN, detection by UV at 254 nm.

3-{[(9-Cyano-9,10-dihydro-10-methylacridin-9-yl)carbonyl]amino}propanoic Acid Trifluoroacetate (**5**·CF_3COOH). Hapten **1** (40 mg, 0.09 mmol) and NaCN (10 mg, 0.20 mmol) were dissolved in H_2O/CH_2Cl_2 1:1 (2 ml). The pH of the aq. phase was adjusted to pH 9.5 by addition of 0.05M HCl, and the mixture was stirred for 12 h at r.t. The aq. phase was acidified (pH 2) and extracted (CH_2Cl_2), the org. phase dried ($MgSO_4$) and evaporated, and the crude product purified by prep. reversed-phase HPLC: **5**·CF_3COOH (35 mg, 83%). Colorless crystals. M.p. 70° (EtOH). IR (KBr): 3020*m*, 2490*w*, 1752*m*, 1654*m*, 1592*m*, 1508*m*, 1474*m*, 1386*m*, 1348*m*, 1216*s*, 772*s*, 748*s*. ^{1}H-NMR ($CDCl_3/CD_3OD$ 1:1, 300 MHz): 7.70 (*m*, 2 H); 7.45 (*m*, 2 H); 7.15–7.05 (*m*, 4 H); 6.72 (br. *s*, 1 H); 3.45 (*s*, 3 H); 3.27 (*q*, $J = 6.2$, 2 H); 2.30 (*t*, $J = 6.2$, 2 H). ^{13}C-NMR ($CDCl_3/CD_3OD$ 1:1, 50 MHz): 173.79; 140.37; 129.98; 127.90; 121.75; 118.15; 35.72; 35.59; 33.20; 32.91. ESI-MS: 336 ([M+H]$^+$). HR-FAB-MS (3-nitrobenzyl alcohol/CsI): 443.0385 ([$C_{18}H_{18}N_2O_3$ + Cs]$^+$, calc. 443.0372).

3-{[(9,10-Dihydro-10-methylacridin-9-yl)carbonyl]amino}propanoic Acid Trifluoroacetate (**6** · CF_3COOH). *N*-Benzyl-1,4-dihydronicotinamide (24 mg, 0.11 mmol) was added to a soln. of **1** (40 mg, 0.09 mmol) in MeCN/borate buffer pH 7.6 1 : 1 (8 ml) at 0°. The mixture was stirred until the yellow color of **1** had vanished (20 min). It was then directly subjected to prep. reversed-phase HPLC: **6** · CF_3COOH (36 mg, 90%). Colorless, crystalline solid. M.p. 143° (dec.). IR (KBr): 3210*m*, 2360*w*, 1714*m*, 1662*m*, 1522*m*, 1474*m*, 1346*m*, 1216*s*, 758*s*. ^{1}H-NMR ($CDCl_3/CD_3OD$ 1 : 1, 300 MHz): 8.0 – 7.15 (*m*, 4 H); 6.87 (*m*, 4 H); 6.25 (br. *s*, 1 H); 4.61 (*s*, 1 H); 3.26 (*s*, 3 H); 3.15 (*q*, $J = 6.1$, 2 H); 2.15 (*t*, $J = 6.1$, 2 H). ^{13}C-NMR ($CDCl_3/CD_3OD$ 1 : 1, 50 MHz): 174.65; 172.32; 141.96; 129.27; 128.42; 121.85; 121.25; 112.80; 51.48; 35.01; 33.45; 32.95. ESI-MS: 311 ($[M + H]^+$). HR-FAB-MS (3-nitrobenzyl alcohol/CsI): 336.1359 ($[C_{19}H_{17}N_3O_3 + H]^+$, calc. 336.1348).

Conjugation of Haptens **5** *and* **6** *with Carrier Proteins BSA and KLH. N*-[3-(Dimethylamino)propyl)-*N'*-ethylcarbodiimide hydrochloride (EDC; 5.4 mg, 0.028 mmol) in H_2O (10 µl) and *N*-hydroxysulfosuccinimide sodium salt (= sodium 1-hydroxy-2,5-dioxopyrrolidine-3-sulfonate; Sulfo-NHS) (6 mg, 0.028 mmol) in H_2O (10 µl) were added successively to a soln. of **6** (8.0 mg, 0.019 mmol) or **5** (8.5 mg, 0.019 mmol) in DMF (400 µl). After a 24 h incubation at r.t., the soln. of the activated hapten (200 µl) was added to 1800 µl of a soln. of keyhole-limpet hemocyanin (KLH, 4.4 mg/ml) in 50 mM phosphate buffer (pH 7.4), and the soln. of the activated hapten (200 µl) was added to 1800 µl of a soln. of bovine-serum albumin (BSA, 4.4 mg/ml) in 50 mM phosphate buffer (pH 7.4). The mixtures were kept at 4° for 24 h and were then stored at – 18°. These conjugates were used without further purification.

Ethyl 9,10-Dihydro-10-methyl-9,10[1',2']-benzenoanthracene-9-propanoate (**9**). To a refluxing soln. of ethyl 10-methylanthracene-9-propanoate (**8**; 300 mg, 1.0 mmol) and 3-methylbutyl nitrite (481 mg, 4.1 mmol) in 1,2-dichloroethane (5 ml) a soln. of anthranilic acid (562 mg, 4 mmol) in bis(2-methoxyethyl) ether (3 ml) was added within 30 min. The mixture was stirred for additional 30 min, cooled, and diluted with AcOEt. The org. phase was washed (H_2O), dried ($MgSO_4$), and evaporated. FC (hexane/AcOEt 40 : 1) yielded **9** (276 mg, 73%). Light-yellow crystals. M.p. 227° (hexane). ^{1}H-NMR ($CDCl_3$, 300 MHz): 7.40 (*m*, 6 H); 7.05 (*m*, 6 H); 4.39 (*q*, $J = 7.2$, 2 H); 3.42 (*t*, $J = 7.0$, 2 H); 3.22 (*t*, $J = 7.0$, 2 H); 2.41 (*s*, 3 H); 1.42 (*t*, $J = 7.2$, 3 H). ESI-MS: 391 ($[M + Na]^+$), 369 ($[M + H]^+$).

9,10-Dihydro-10-methyl-9,10[1',2']-benzenoanthracene-9-propanoic Acid (**10**). A soln. of **9** (100 mg, 0.27 mmol) and LiOH · H_2O (57 mg, 1.35 mmol) in H_2O/THF 1 : 1 (3 ml) was stirred at r.t. for 12 h. The aq. phase was acidified (0.1M HCl) and extracted with CH_2Cl_2. The org. phase was dried ($MgSO_4$) and evaporated and the crude product subjected to FC (hexane/AcOEt 1 : 1): **10** (85 mg, 92%). Light-yellow crystals. M.p. 281° ($CHCl_3$). ^{1}H-NMR ($CDCl_3$, 300 MHz): 7.42 (*m*, 6 H); 7.05 (*m*, 6 H); 3.45 (*m*, 2 H); 3.35 (*m*, 2 H); 2.42 (*s*, 3 H). FAB-MS (3-nitrobenzyl alcohol/NaI): 363 ($[M + Na]^+$), 341 ($[M + H]^+$).

Benzyl 3-{[3-(9,10-Dihydro-10-methyl-9,10[1',2']-benzenoanthracen-9-yl)-1-oxopropyl]amino}propanoate (**11**). Acid **10** (100 mg, 0.29 mmol) was dissolved in refluxing $SOCl_2$ (3 ml). After 2 h, a clear soln. was obtained which was evaporated. The yellow, crude acid chloride was suspended in CH_2Cl_2 (5 ml), a soln. of β-alanine benzyl ester *p*-toluenesulfonate (206 mg, 0.59 mmol) and Et_3N (290 mg, 2.9 mmol) in CH_2Cl_2 (5 ml) added at r.t. and the mixture stirred for 14 h. The org. phase was washed ($NaHCO_3$ and NaCl soln.), dried ($MgSO_4$), and evaporated. FC (hexane/AcOEt 5 : 1) furnished **11** (125 mg, 85%). Light-yellow crystals. M.p. 198° (hexane). ^{1}H-NMR ($CDCl_3$, 300 MHz): 7.65 – 7.35 (*m*, 11 H); 7.20 (*m*, 6 H); 6.50 (br. *s*, 1 H); 5.35 (*s*, 2 H); 3.89 (*q*, $J = 6.0$, 2 H); 3.60 (*t*, $J = 7.4$, 2 H); 3.15 (*t*, $J = 7.4$, 2 H); 2.88 (*t*, $J = 6.0$, 2 H); 2.60 (*s*, 3 H). FAB-MS (3-nitrobenzyl alcohol): 502 ($[M + H]^+$).

3-{[3-(9,10-Dihydro-10-methyl-9,10[1',2']-benzenoanthracen-9-yl)-1-oxopropyl]amino}propanoic Acid (**7**). A soln. of **11** (100 mg, 0.20 mmol) and LiOH · H_2O (42 mg, 1.0 mmol) in H_2O/THF 1 : 1 (3 ml) was stirred at r.t. for 12 h. The aq. phase was acidified (0.1M HCl) and extracted with CH_2Cl_2. The org. phase was dried ($MgSO_4$) and evaporated and the crude product subjected to FC (hexane/AcOEt 1 : 1): **7** (70 mg, 85%). Light-yellow crystals. M.p. 258° ($CHCl_3$). IR (KBr): 3025*w*, 2360*w*, 1734*m*, 1718*m*, 1684*m*, 1616*m*, 1560*m*, 1448*m*, 1216*s*, 750*s*, 668*m*. ^{1}H-NMR ($CDCl_3$, 300 MHz): 7.35 (*m*, 6 H); 6.95 (*m*, 6 H); 6.35 (br. *s*, 1 H); 3.61 (*q*, $J = 5.8$, 2 H); 3.35 (*t*, $J = 7.4$, 2 H); 2.99 (*t*, $J = 7.4$, 2 H); 2.65 (*t*, $J = 5.8$, 2 H); 2.37 (*s*, 3 H). ^{13}C-NMR ($CDCl_3$, 50 MHz): 176.15; 173.27; 148.78; 121.68; 120.67; 52.85; 48.59; 35.06; 33.93; 32.64; 22.30; 13.74. HR-FAB-MS (3-nitrobenzyl alcohol/CsI): 544.0868 ($[C_{27}H_{25}NO_3 + Cs]^+$, calc. 544.0889).

Conjugation of Hapten **7** *with Carrier Proteins BSA and KLH*. As described above for the conjugation of **5** and **6**, with EDC (5.4 mg, 0.028 mmol) in H_2O (10 µl), Sulfo-NHS (6.1 mg, 0.028 mmol) in H_2O (10 µl) and **7** (8.0 mg, 0.019 mmol) in DMF (400 µl). Then with the soln. of the activated hapten (200 µl) and 7800 µl of KLH (2.6 mg/ml) in 50 mM phosphate buffer (pH 7.4), and with the soln. of the activated hapten (200 µl) and 3800 µl of BSA (2.6 mg/ml) in 50 mM phosphate buffer (pH 7.4).

Preparation of Antibodies. 129 GIX + /boy mice were immunized with the KLH conjugates of haptens **1**, or **5**–**7** by two intraperitoneal injections of these conjugates (100 μg) in 200 μl *RIBI* adjuvant (*RIBI ImmunoChem Research Inc.*, USA) at 15 days interval. After at least 30 days, a final tail-vein injection of 50 μg of hapten-KLH in 150 μl of PBS (aq. 10 mM phosphate, 160 mM NaCl, pH 7.4) was carried out, and spleen cells were collected four days later. Spleen cells were fused with P3X63.Ag8.653 myeloma using 1500-polyethylene glycol (*Boehringer Mannheim*) according to standard protocols [9] at a ratio of approximately five spleen cells to one myeloma cell. Fused cells were centrifuged, re-suspended in cell-culture media (*Sigma*) containing either 10% FCS (fetal-calf serum, *Sigma*) plus 4% mouse serum (*Gemini*), or 20% FCS, and plated out in 96-well tissue culture plates (*Costar*).

Screening of supernatant for binding and catalysis was carried out after 10–15 days of incubation at 37°. Cell-culture supernatant (115 μl) was taken from each well and used for testing as follows: 25 μl for ELISA against the respective hapten-BSA conjugates; 2 × 45 μl for testing catalysis with substrates **2** and **3**. Hybridoma from selected wells were further subcloned twice following standard cell-culture procedures. The monoclonal nature of the cloned cell lines was assessed by determination of the isotype. Antibodies were produced either in ascites or in cell cultures (*Table 3*). Antibodies produced in ascites were purified by sequential ammonium sulfate precipitation, chromatography on weak-anion-exchange and finally protein-G columns. Antibodies produced in cell cultures were purified by protein-G chromatography. After final concentration by dyalysis centrifugation, all antibodies were stored at −20° as 5–10 mg/ml soln. in PBS (aq. 10 mM phosphate, 160 mM NaCl, pH 7.4).

Table 3. *Data on Purified Catalytic Antibodies*

Antibody	Hapten	Isotype	Production	Crude	Purified
9D9	**1**	$\kappa\gamma$2a	ascites [a])	714 mg	455 mg
8E5	**5**	$\kappa\gamma$1	ascites [a])	492 mg	150 mg
10F11	**6**	$\kappa\gamma$2a	ascites [a])	800 mg	327 mg
10F4	**6**	$\kappa\gamma$3	cell culture	0.9 l	7.6 mg
8B8	**6**	$\kappa\gamma$2a	cell culture	0.85 l	20.5 mg
6C6	**1/5/6**	$\kappa\gamma$3	cell culture	0.6 l	3.5 mg
42B5	**5/1**	$\kappa\gamma$2a	cell culture	1.6 l	28 mg
27C5	**5/1**	$\kappa\gamma$2b	cell culture	3.6 l	31 mg
12E8	**5/1**	$\kappa\gamma$1	ascites [a])	540 mg	320 mg

[a]) Ascites production was carried out at *The Scripps Research Institute*, La Jolla, CA, USA.

Kinetics. Reactions were followed by fluorescence in round-bottom polypropylene 96-well plates (*Costar*) using a *Cytofluor II Fluorescence Plate Reader* (*Perceptive Biosystems*, filters $\lambda_{ex} = 360 \pm 20$ nm, $\lambda_{em} = 440 \pm 20$ nm). Solns. (45 μl for activity screening and 90 μl for kinetics on purified antibodies) containing antibody were thermostated at 31°. The reactions were initiated by addition of 5 or 10 μl of a properly pre-diluted soln. of substrate **2** or **3**, resp., as trifluoroacetate salt in H_2O. Activity-screening concentration was 50 μM, kinetic measurements concentrations were 20, 30, 50, 70, 100, 150, 200, and 300 μM. The fluorescence increase at 440 nm was linear over 60 min.

Fluorescence was converted to product concentration according to a calibration curve with pure **4**. The net reaction rate of background was used to calculate the kinetic parameters according to the *Michaelis-Menten* model as described before [10]. The cat. constants k_{cat} are reported for one active site, assuming a molar mass of 150 kDalton and two cat. sites for each antibody. Exact active-site titrations were possible with the more active antibodies 9D9, 10F11, and 27C5 using hapten **5** as tight binding ligand. These titrations showed the expected active-site concentration within 10%.

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