Pivalase Catalytic Antibodies: Towards Abzymatic Activation of Prodrugs

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Abstract: Screening of monoclonal-antibody libraries generated against the *tert*-butyl phosphonate hapten **2** and the chloromethyl phosphonate hapten **3** with pivaloyloxymethyl-umbelliferone **1** as a fluorogenic substrate led to the isolation of eleven catalytic antibodies with rate accelerations around $k_{cat}/k_{uncat} = 10^3$. The antibodies are not inhibited by the product and accept different acyloxymethyl derivatives of acidic phenols as substrates. The highest activity was found for the bulky, chemically less-reactive pivaloyloxymethyl group; there is no activity with acetoxymethyl or acetyl esters. This difference might reflect the preference of the immune system for hydrophobic interactions in binding and catalysis. Pivalase catalytic antibodies might be useful for activating orally available pivaloyloxymethyl prodrugs.

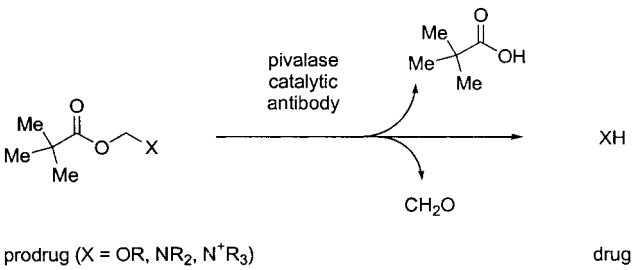
Keywords: catalytic antibodies · fluorogenic substrates · hydrolysis · immunochemistry · prodrugs

Introduction

Catalytic antibodies are obtained by immunization against stable transition-state analogues of chemical reactions. This technique allows, in principle, the installation of any catalytic activity in the combining site of immunoglobulins.[1] On account of their excellent long-term stability in human blood and their ability to escape immune rejection, immunoglobulins are also an outstanding framework for designing whole-protein drugs. Several monoclonal-antibody drugs have reached the market recently; in particular, antibodies that bind to tumor antigens and that are thus effective as antitumor agents.[2] Similarly, one of the best applications envisioned for catalytic antibodies lies in the medicinal area, particularly for drug detoxification[3] and prodrug activation.[4] Provided that a useful catalytic activity is available in an antibody binding pocket, its adaptation to a tool for prodrug activation follows a straightforward sequence that has been well studied owing to work with antibody–enzyme conjugates[5] and therapeutic antibodies.[2] In particular, the "humanization" of the starting antibody, which is essential to ensure the immunocompatibility of an antibody, can be done by CDR (complementarity-determining regions) grafting without altering the specificity of the antibody's binding pocket.[6] One of the critical factors that remains today is the identification of prodrug-activating reactions that are both selective and suitable for antibody catalysis.[7] Herein we report on the activation of pivalate prodrugs by an esterolytic reaction that makes use of catalytic antibodies.

Results and Discussion

Design: The reaction catalyzed most often by catalytic antibodies is ester hydrolysis. Catalytic antibodies, however, most often hydrolyze reactive esters, which are chemically too unstable to be suitable for prodrug-masking chemistry. We reasoned that a useful catalytic antibody for prodrug activation should be able to cleave less-reactive esters, for example, sterically hindered esters such as pivalate esters. The pivaloyloxymethyl group seemed interesting since it can be used to protect a variety of functional groups found in drugs, such as phenols, amides, amines, pyridines, or phosphates, and is frequently used in prodrug formulations to make drugs orally available.[8] We therefore set out to investigate the preparation of "pivalase" catalytic antibodies (Scheme 1).



prodrug (X = OR, NR_2, N^+R_3) drug

Scheme 1. Principle of pivalase catalytic antibodies. Pivaloyloxymethyl-protected drugs (XH) become selectively activated by pivalase catalytic antibodies.

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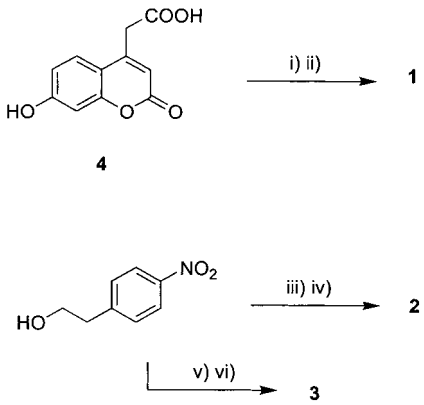
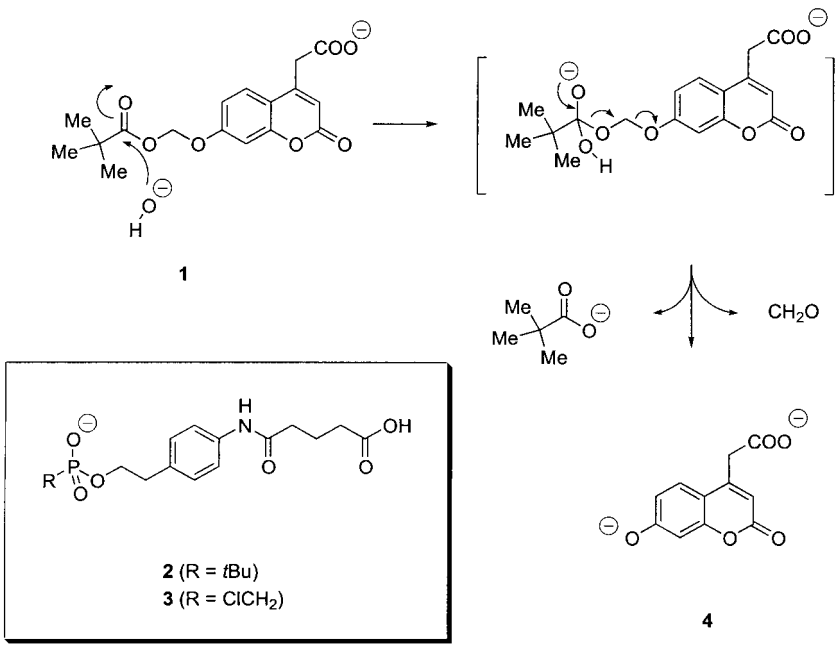
The pivaloyloxymethyl derivative of 4-carboxymethyl umbelliferone **1** was selected as a convenient fluorogenic prodrug model (Scheme 2). Fluorescence screening with substrate **1** would allow us to test catalysis early in the process of cell culture that leads from immunized mice to the stable hybridoma cell lines that produce catalytic antibodies.[9] Phosphonate-type haptens **2** and **3** were chosen to elicit a catalytic immune response. *tert*-Butyl phosphonate **2** has a phosphonate group that corresponds to the hydrolyzing pivalate and also the correct spacing to the anilide aromatic group. We selected this anilide group and its adjacent glutaramide linker because this immunogenic combination is often found in haptens that lead to catalytic antibodies. The discrepancy between the anilide group in the hapten and the coumarin nucleus in the assay substrate was intended to enforce the selection of catalytic antibodies with a broad substrate tolerance. In addition to the *tert*-butyl phosphonate **2**, we also prepared the chloromethyl phosphonate hapten **3**, which might trigger a process of reactive immunization, whereby a nucleophilic residue in the antibody binding pocket would displace the reactive chloride and become covalently attached to hapten **3**.[10] This nucleophilic residue would then assist the hydrolysis of the substrate.



Scheme 3. Synthesis of assay substrate **1** and haptens **2** and **3**. The haptens were conjugated to carrier proteins KLH for immunization and to BSA for ELISA assays. i) NaH, ICH_2O_2CtBu, DMF, $-60\,^{\circ}C$ to RT, 10 h; ii) NH_3/NH_4Cl in MeOH, 20 °C, 6 h (5 %). iii) NaH, dioxane, $tBuPOCl_2$, 50 °C, 1 h (35 %); iv) a) 1 atm H_2, Pd/C, MeOH, 25 °C, 2 h; b) glutaric anhydride, CH_2Cl_2, 25 °C, 3 h (80 %). v) $ClCH_2POCl_2$, $iPrEt_2N$, cat. tetrazole, 25 °C, 8 h (20 %); vi) a) 1 atm H_2, Pd/C, MeOH, 25 °C, 3 h; b) glutaric anhydride, DMF/CH_2Cl_2, 25 °C, 3 h (27 %).

Synthesis and immunization: Alkylation of 4-carboxymethyl umbelliferone **4** with iodomethyl pivalate under optimized conditions gave an equal mixture of the desired pivaloyloxymethyl derivate **1** together with the corresponding pivalate derivative. Selective cleavage of this more reactive pivalate ester with buffered ammonia in methanol and subsequent purification by preparative reverse-phase HPLC allowed the isolation of substrate **1** in a pure (>99 %) form, as required for high-throughput screeening (HTS) (Scheme 3). Hapten **2** was prepared by esterification of *tert*-butyldichloro phosphoridate[11] with the sodium salt of 4-nitrophenethyl alcohol,[12] followed by hydrogenation of the nitro group and amidation with glutaric anhydride. Hapten **3** was obtained by a similar sequence by means of the tetrazole-catalyzed phosphonate synthesis described by Landry et al.[13] The haptens were conjugated to carrier proteins KLH (keyhole limpet hemocyanin) and BSA (bovine serum albumin) through their *N*-hydroxysuccinimide esters.

Two series of four 129GIX+ mice were immunized with the KLH conjugate of either hapten **2** or hapten **3**, according to standard procedures.[14] The spleen cells of the immunized mice were fused with NS1 myeloma cells, and the resulting hybridoma plated out in 96-well microtiter cell-culture plates. Wells containing cells that were producing hapten-specific antibodies against the hapten–BSA conjugates, as judged by ELISA (enzyme-linked immuno-sorbens assay),[15] were propagated to a volume of 5 mL. At that stage, the antibodies present in the supernatant of each cell-culture sample were isolated by a short protein-G-affinity column and assayed for catalysis. Catalysis was assayed in each antibody sample by measuring the rate of hydrolysis of fluorogenic substrate **1**, by taking the identical sample with added hapten **2** or **3** as inhibitor as reference for the uncatalyzed reaction. This



Scheme 2. Hapten design and HTS assay for pivalase catalytic antibodies. Antibodies raised against hapten **2** (exact match) or **3** (reactive immunization) might catalyze the hydrolysis of fluorogenic prodrug model **1** to release pivalate, formaldehyde, and the strongly fluorescent umbelliferone **4**.

procedure allowed the unequivocal identification of any catalysis occurring specifically in the combining site of the antibodies. This procedure was used systematically to confirm all ELISA data for hapten binding, and proved indispensable in the further steps of subcloning for distinguishing cell colonies producing catalytic antibodies from those producing only hapten-binding antibodies.

The overall experiment lead to the isolation of 24 hybridoma (Table 1). The monoclonal antibody expressed by each cell line was produced in 5 to 50 mg amounts by cell culture, and purified to homogeneity by ammonium sulfate precipitation and protein-G-affinity chromatography. Immunization with *tert*-butylphosphonate hapten **2** did not produce any clones that tested positive for catalysis. Nevertheless, seven clones were isolated as controls. None of these showed any catalytic activity in the purified form; this showed that the HTS fluorescence assay did not produce false negatives. The immunization with chloromethylphosphonate hapten **3**, by contrast, showed a number of "hits". Seventeen of the initial hits were successfully subcloned twice to stable hybridoma. Eleven of these showed confirmed catalytic activity after two subclonings and large-scale production and purification. All catalytic antibodies were obtained from a single mouse (Table 1).

Table 1. Data for immunization with haptens **2** and **3** and catalysis HTS with substrate **1**.

hapten		mouse L[a]	mouse R[a]	mouse LL[a]	mouse LR[a]
2 (*t*Bu)	serum titer[b]	12800	6400	25600	25600
	no. of binders[c]	13	47	55	75
	monoclonals[d]	1	1	3	2
	catalytic[e]	0	0	0	0
3 ($ClCH_2$)	serum titer[b]	12800	19200	25600	25600
	no. of binders[c]	36	45	160	50
	monoclonals[d]	0	0	16	1
	catalytic[e]	0	0	11	0

[a] Mouse code according to ear marks. [b] Dilution factor of blood serum (after immunization with hapten–KLH conjugates) for 50% reduction of ELISA signal against the hapten–BSA conjugate. A high number indicates strong immune response. [c] Number of hybridoma cell lines that test positive for binding by ELISA against the hapten–BSA conjugate after fusion and that were grown up to 5 mL for catalysis testing. [d] Number of fully subcloned and stabilized hybridoma. [e] Number of fully subcloned hybridoma producing catalytic antibodies.

Kinetic analysis: All eleven catalytic antibodies showed Michaelis–Menten kinetic behavior for the hydrolysis of **1** (Table 2). All the antibodies also bound to hapten **3** tightly, and their activity could be titrated quantitatively to two equivalent binding sites per antibody molecule. The rate accelerations k_{cat}/k_{uncat} observed with substrate **1** are in the range of three orders of magnitude, a typical number for esterolytic catalytic antibodies. The transition-state dissociation constants $K_{TS} = K_M/(k_{cat}/k_{uncat})$ are in the range of 10^{-7} M, as found for most known catalytic antibodies. Remarkably, the catalytic antibodies were not susceptible to any product inhibition by pivalic acid or umbelliferone.

Table 2. Catalytic anti-**3** antibodies: immunological data and kinetic parameters with substrate **1**.

Antibody[a]	Amount [mg][b]	Isotype[c]	k_{cat} [10^{-3} s^{-1}]	K_M [μM][d]	k_{cat}/k_{uncat}[d]	K_{TS} [μM][d]
8D5	18.5	κγ2a	0.12	43	1700	0.025
8A9	14.8	κγ1	0.17	270	2450	0.11
7H11	15.5	κγ1	0.08	57	1120	0.05
8A11	53.7	κγ1	0.11	47	1900	0.02
2G8	15.3	κγ1	0.07	69	1020	0.07
8E1	11.5	κγ1	0.12	83	2100	0.04
8C5	18	κγ1	0.09	185	1280	0.14
9D12	15.5	κγ1	0.09	130	1260	0.10
8F10	39	κγ2a	0.07	185	990	0.19
6F4	23	κγ1	0.05	60	700	0.09
1C12	15.8	κγ2a	0.07	150	980	0.15

[a] The code refers to the numbering of the original fusion cell-culture plate followed by the well code (A1–H12). [b] Amount of pure antibody isolated from 1 L of culture of the corresponding hybridoma cell line. [c] Isotype, as determined by immunological isotyping. All isotypes were cleanly unique and establish the monoclonal nature of the cell lines. [d] Measured at 30 °C in aq. bis-tris buffer (20 mM), NaCl (150 mM), pH 7.55, 5% *v*/*v* DMF, with antibody (0.2 mg mL^{-1}). The uncatalyzed reaction is 7×10^{-8} s^{-1} under these conditions. See also Experimental Section. Error margins are ±10%.

The hydrolysis of **1** was proportional to the hydroxide ion concentration between pH 6.5 and 9.5, in agreement with the generally accepted mechanism of hydrolysis of acyloxymethyl esters, as shown in Scheme 2.[16] A pH-profile analysis with catalytic antibody 8A11 showed that its catalytic efficiency was also proportional to the concentration of hydroxide between pH 6.5 and 9.5. This suggests that the antibody-catalyzed process also used the classical esterolytic mechanism, in agreement with the phosphonate transition-state analogues used for immunization. This suggested that no protein sidechain ionizable in that range participated in the catalysis, and that reactive immunization had not occurred with chloromethyl phosphonate hapten **3**. Indeed, while all the antibodies lost their catalytic activity in the presence of hapten **3**, the activity returned in all cases by repurifying the antibodies by protein-G-affinity chromatography. This procedure included an extensive washing step in which the antibody was bound to the column (at neutral pH) to remove the hapten before acidic elution. The inhibition by hapten **3** was thus reversible and most likely noncovalent. The catalytic activity of these antibodies probably reflects simple transition-state stabilization. The absence of catalytic antibodies in the immunization with hapten **2** is unclear, especially in view of the fact that all the anti-**3** catalytic antibodies are also inhibited by hapten **2**.

Substrate specificity: The substrate specificity of the catalytic antibodies was then investigated. The simple fluorogenic umbelliferone derivative **5** and the chromogenic nitrophenyl derivative **6** were both accepted as substrates, with kinetic parameters almost equivalent to those of substrate **1** for all catalytic antibodies. Derivatives of substrate **5**, whose synthesis was less problematic than that of substrate **1**, were thus prepared (Scheme 4). Gradual removal of the pivalate's methyl substituents to give isobutyryloxymethyl umbelliferone **7** and propionyloxymethyl umbelliferone **8**, resulted in a

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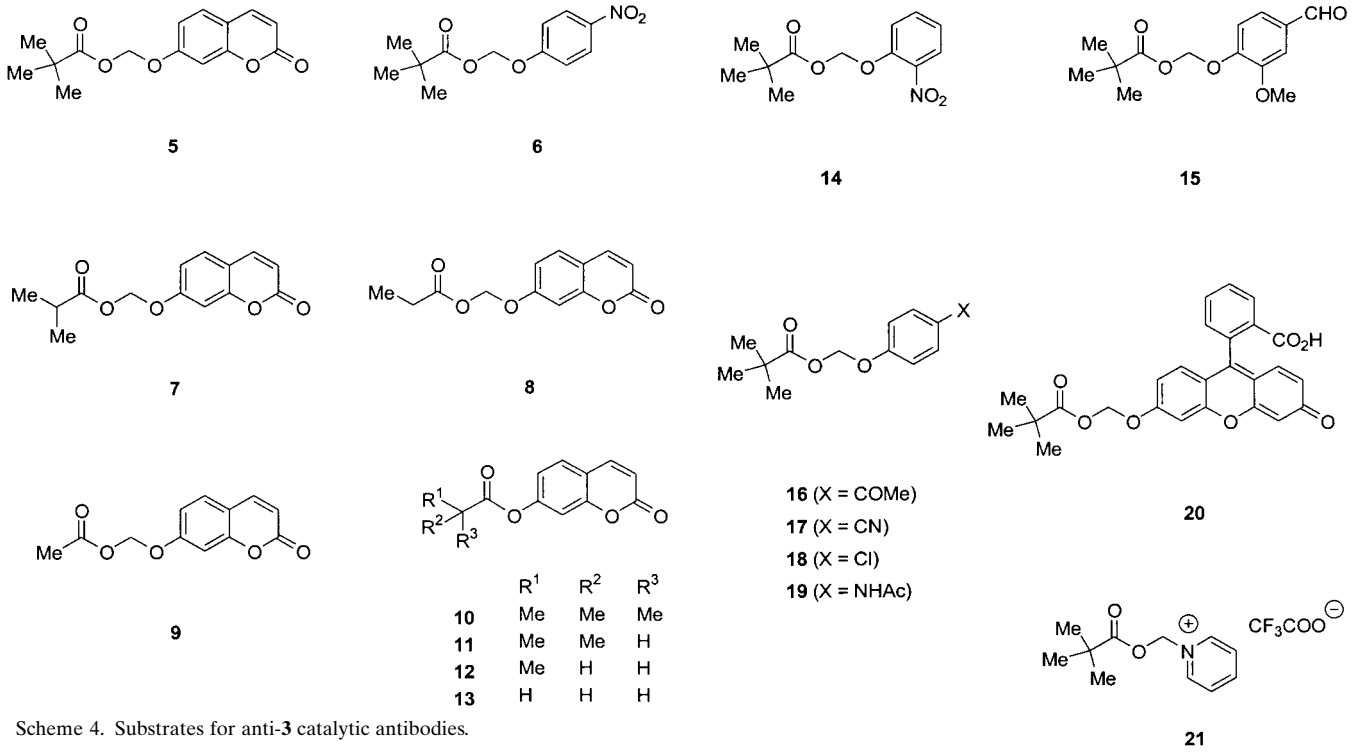
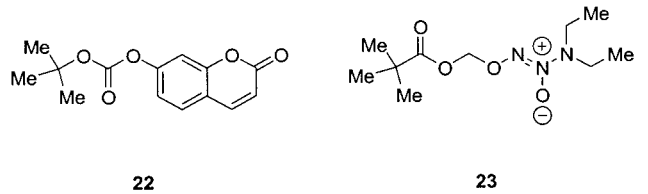


Scheme 4. Substrates for anti-**3** catalytic antibodies.



Scheme 5. Some POM derivatives tested with anti-**3** catalytic antibodies.

decrease in the catalytic activity, as measured by k_{cat}/k_{uncat} with all catalytic antibodies (Table 3). Only two of the antibodies catalyzed the hydrolysis of acetoxymethyl substrate **9** (8A9, $k_{cat}/k_{uncat} = 200$; 8E1, $k_{cat}/k_{uncat} = 300$). The antibodies also hydrolyzed the umbelliferyl esters **10**–**12**, but showed no activity with umbelliferyl acetate **13**. The highest turnover numbers k_{cat} were observed with propionate derivative **12**, but these reflect its high spontaneous reactivity. Overall, the pivaloyloxymethyl (POM) derivatives **5** and **6** were the best substrates in terms of rate acceleration k_{cat}/k_{uncat} and transition-state binding K_{TS}; this reflects the antibody-specific contributions to catalysis.

With the POM group providing the optimal functional group for our antibodies, we next investigated whether POM-protected derivatives other than umbelliferone would react with our antibodies. While the chromogenic POM derivative **6** of 4-nitrophenol was a good substrate with all antibodies, the corresponding POM-protected 2-nitrophenol **14** (Scheme 5) was not accepted as a substrate by any of the catalytic antibodies. Competitive inhibition experiments with this and other POM derivatives of *ortho*-substituted phenols, such as the vanilline derivative **15** (Scheme 5), showed that such derivatives were not recognized by the catalytic antibodies; this points to strict steric requirements for access to the antibody's binding pocket. Among the sterically less-demanding *para*-substituted phenols, only 4-acetylphenol **16** and 4-cyanophenol **17** showed marginal activity with one of the antibodies, while the others, such as the 4-chlorophenol derivative **18**, paracetamol derivative **19**, or fluoresceine derivative **20**, all acted as competitive inhibitors of the catalytic antibodies but did not undergo any reaction. Pivaloyloxymethyl pyridinium trifluoroacetate (**21**) was accepted weakly by one of the antibodies. These observations showed that a strongly acidic leaving group was required for the antibodies to hydrolyze the pivaloyloxymethyl prodrugs. It should be noted that the Boc-protected umbelliferone **22** was not accepted as a substrate by any of the catalytic antibodies, despite its high spontaneous reactivity.

Interestingly, the difficulty encountered with our catalytic antibodies for hydrolyzing the bulky and unreactive POM derivatives when attached to nonacidic leaving groups was also observed with esterolytic enzymes. Thus the caged NO prodrug **23**, which we had synthesized from DEA NONOate (2-(*N*,*N*-diethylamino)diazenolate-2-oxide sodium salt) by reaction with chloromethyl pivalate, was not hydrolyzed by the antibodies; it was also resistant to a range of esterases, lipases, and to exposure to mouse serum. This is in contrast to the corresponding acetoxymethyl derivative.[17] Similarly, while esterolytic enzymes cleaved our fluorogenic umbelliferone substrates (horse liver esterase and *Pseudomonas fluorescens* lipase were used as representative cases), they were not capable of cleaving the POM derivate of the less acidic fluorescein **20**. This showed that a bulky pivalate group combined with an unreactive leaving group poses particular problems for enzymes and catalytic antibodies alike.

Table 3. Kinetic parameters for anti-**3** catalytic antibodies.[a]

	Substrate	**5**	**6**	**7**	**8**	**10**	**11**	**12**
background	k_{uncat} [10^{-5} s^{-1}]	0.007	0.04	0.3	0.4	0.06	0.2	0.6
Ab 8D5	k_{cat} [10^{-5} s^{-1}]	12	60	180	115	26	22	255
	k_{cat}/k_{uncat}	1770	1580	610	285	440	110	430
	K_M [μM]	65	120	245	100	100	1040	225
	K_{TS} [μM]	0.037	0.07	0.4	0.36	0.23	0.93	0.52
Ab 8A9	k_{cat} [10^{-5} s^{-1}]	17	37	154	280	5	22	195
	k_{cat}/k_{uncat}	2450	940	515	710	80	110	325
	K_M [μM]	265	200	70	100	35	1040	155
	K_{TS} [μM]	0.11	0.21	0.14	0.14	0.42	0.93	0.48
Ab 7H1	k_{cat} [10^{-5} s^{-1}]	8	50	133	106	10	14	485
	k_{cat}/k_{uncat}	1125	1240	445	265	165	70	810
	K_M [μM]	60	265	70	80	70	120	350
	K_{TS} [μM]	0.05	0.2	0.16	0.3	0.42	1.72	0.43
Ab 8A11	k_{cat} [10^{-5} s^{-1}]	11	50	125	83	27	15	266
	k_{cat}/k_{uncat}	1590	1250	415	210	445	80	445
	K_M [μM]	95	135	95	45	282	220	165
	K_{TS} [μM]	0.06	0.11	0.2	0.22	0.63	2.87	0.37
Ab 2G8	k_{cat} [10^{-5} s^{-1}]	7	37	110	180	5	40	300
	k_{cat}/k_{uncat}	1015	940	370	460	80	200	500
	K_M [μM]	70	200	70	240	35	310	265
	K_{TS} [μM]	0.07	0.21	0.19	0.52	0.41	1.54	0.52
Ab 8E1	k_{cat} [10^{-5} s^{-1}]	12	37	70	135	13	40	170
	k_{cat}/k_{uncat}	1740	940	225	340	215	200	280
	K_M [μM]	95	90	55	135	70	310	415
	K_{TS} [μM]	0.055	0.09	0.25	0.39	0.33	1.54	1.48
Ab 8C5	k_{cat} [10^{-5} s^{-1}]	9	12	65	95	5	70	275
	k_{cat}/k_{uncat}	1280	310	215	240	90	355	460
	K_M [μM]	185	65	90	120	60	1440	420
	K_{TS} [μM]	0.14	0.21	0.42	0.49	0.65	4	0.91
Ab 3E2	k_{cat} [10^{-5} s^{-1}]	9	19	57	56	6	21	224
	k_{cat}/k_{uncat}	1260	470	190	140	100	105	375
	K_M [μM]	130	100	255	65	65	165	240
	K_{TS} [μM]	0.1	0.21	1.33	0.46	0.65	1.52	0.64
Ab 8F10	k_{cat} [10^{-5} s^{-1}]	7	19	45	175	7	14	415
	k_{cat}/k_{uncat}	990	470	150	440	125	70	695
	K_M [μM]	185	150	52	130	130	255	310
	K_{TS} [μM]	0.19	0.32	0.35	0.3	1	3.55	0.45
Ab 6F4	k_{cat} [10^{-5} s^{-1}]	5	20	45	175	5	34	162
	k_{cat}/k_{uncat}	700	470	145	435	90	170	270
	K_M [μM]	60	100	50	175	30	215	165
	K_{TS} [μM]	0.09	0.21	0.34	0.4	0.34	1.25	0.62
Ab 1C12	k_{cat} [10^{-5} s^{-1}]	7	19	30	150	5	180	440
	k_{cat}/k_{uncat}	985	470	100	380	90	890	735
	K_M [μM]	150	200	50	70	30	600	485
	K_{TS} [μM]	0.15	0.43	0.5	0.18	0.34	0.67	0.65

[a] Measured at 31 °C in 20 mM bis-tris pH 7.55, see Experimental Section for details. The background reaction was measured in the presence of catalytic antibody with excess (20 μM) hapten **3**. Error margins are ± 10 %.

Conclusion

Immunization with chloromethyl phosphonate hapten **2** has yielded the first catalytic antibodies capable of hydrolyzing bulky pivalate esters, in particular the rather stable POM-protected derivatives, which can be incorporated as elements of orally available prodrugs. The catalytic antibodies obtained appear to be limited to POM derivatives of acidic phenols and do not cleave POM derivatives with less acidic phenol leaving groups. The limitation to activated leaving groups is often encountered with esterolytic catalytic antibodies. In the present case, the reactivity pattern also parallels that observed with esterases and lipases—with the enzyme-resistant POM derivatives, which are the most interesting in prodrug applications, being presently left untouched. The preference of our catalytic antibodies for pivalate esters over less bulky analogues might reflect the strong preference of the immune response for exploiting hydrophobic interactions as a means of achieving high binding affinity to antigens. Further immunizations or manipulation of the current set of catalysts by recombinant methods might lead to pivalase catalytic antibodies with improved kinetic properties.

Experimental Section

Synthesis: All reagents were either purchased from Aldrich or Fluka or synthesized following literature procedures. Chromatography (flash) was performed with Merck silica gel 60 (0.040 – 0.063 mm). Preparative HPLC was performed with HPLC-grade acetonitrile and MilliQ deionized water in a Waters prepak cartridge 500 g (RP-C18 20 μm, 300 Å pore size) installed on a Waters Prep LC 4000 system from Millipore (flow rate 100 mL min^{-1}, gradient + 0.5 % min^{-1} CH_3CN) following the conditions

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given in Table 4. TLC was performed with fluorescent F254 glass plates. MS was provided by Dr. Thomas Schneeberger (University of Bern, Switzerland).

Table 4. Reverse-phase HPLC conditions for analysis and purification.[a]

Compounds	A [%]	B [%]	t_R [min]	Preparative conditions[b]
1	30	70	18.3	50–70% B in 20 min
2	40	60	6	[c]
3	70	30	4.8	30–50% B in 20 min
5	40	60	24.9	[c]
6	15	85	13.5	[c]
7	50	50	26.9	45–75% B in 30 min
8	50	50	13.5	40–60% B in 20 min
9	75	25	15.6	10–40% B in 30 min
10	40	60	27	[c]
12	50	50	10.5	40–60% B in 20 min
13	65	35	10	40–60% B in 20 min
21	90	10	14.9	0–20% B in 20 min
24	20	80	8.3	60–80% B in 20 min
25	60	40	3.8	20–40% B in 30 min

[a] Isocratic conditions for analytical RP-HPLC on a Vydac 218-TP54 C18 RP, particle size 5 μm, pore size 300 Å, 0.45 × 22 cm, flow 1.5 mL min^{-1}, A = 0.1% CF_3COOH in water, B = acetonitrile/water (1:1). [b] Preparative HPLC was performed by gradient elution on a Waters prepak cartridge (500 g), flow rate 100 mL min^{-1}, with the same eluents as indicated. [c] This compound was purified by flash chromatography on silica gel.

4-Nitrophenethyl-*tert*-butylphosphonate (24): A solution of nitrophenethyl alcohol (100 mg, 0.57 mmol) in dry dioxane (2 mL) at −20 °C was treated with NaH (23 mg, 55% in oil, 1.1 equiv). After 5 minutes at 25 °C, *tert*-butyl phosphonyl dichloride[11] (100 mg, 0.57 mmol) was added, and the reaction was heated to 50 °C for 1 h. Water (1 mL) was added, and the reaction mixture was heated for 45 minutes at 50 °C. The reaction was then acidified with aq. 1N HCl (50 mL) and extracted twice with ethyl acetate. The organic phase was dried (Na_2SO_4) and concentrated, and the residue purified by preparative reverse-phase HPLC (gradient 30–40% acetonitrile in 20 minutes). The main fraction was lyophilized to give compound **24** as a yellow solid. Yield: 55 mg (0.19 mmol, 35%); ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 8.20$ (d, $J = 8.5$ Hz, 2H), 7.42 (d, $J = 8.5$ Hz, 1H), 4.45 (m, 2H), 3.17 (t, 2H), 1.23 (d, $J = 21$ Hz, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 144.7$, 141.2, 129.8, 123.7, 65.8 (d, $J = 36.4$ Hz), 37.8 (d, $J = 466$ Hz), 36.1 (d, $J = 26.7$ Hz), 24.2 (d, $J = 4.8$ Hz); ^{31}P NMR (81 MHz, $CDCl_3$): $\delta = 57.2$.

4-{4-[2-(*tert*-Butylphosphonyloxy)ethyl]phenylcarbamoyl} butyric acid (2): A solution of *tert*-butylphosphonate **24** (90 mg, 0.31 mmol) in methanol (2 mL) was stirred with Pd/C (9.5 mg) under 1 atm H_2 for 2 h, filtered over celite, and concentrated. The residue (80 mg of a colorless oil) was dissolved in anhydrous CH_2Cl_2 (3 mL), and glutaric anhydride (53 mg, 1.5 equiv) was added at 0 °C. After 3 h at 25 °C, the solution was washed with 1N HCl, dried with Na_2SO_4, and concentrated. The residue was purified by flash chromatography [i) hexane/ethyl acetate 3:7, ii) ethyl acetate, iii) CH_2Cl_2/MeOH 7:3] to give hapten **2** as a yellow oil. Yield: 92 mg (0.25 mmol, 80%); $R_f = 0.15$ (CH_2Cl_2/MeOH 7:3); ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 8.28$ (s, 1H), 7.49 (d, $J = 8.4$ Hz, 2H), 7.13 (d, $J = 8.4$ Hz, 2H), 4.36 (m, 2H), 2.98 (t, 2H), 2.42 (m, 4H), 2.04 (qu, 2H), 1.25 (d, $J = 20.7$ Hz, 9H); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 177.3$, 171.2, 136.8, 132.5, 129.4, 120.2, 67.1 (d, $J = 9.4$ Hz), 37.1 (d, $J = 704$ Hz), 33.1, 24.3, 20.7; ^{31}P NMR (81 MHz, $CDCl_3$): $\delta = 55.8$; IR ($CHCl_3$): $\tilde{\nu} = 3021$, 1711, 1604, 1519, 1414, 1216, 760 cm^{-1}; MS (SIMS): 371 $[M]^+$, 297 $[M - t\text{BuOH}]^+$, 234 $[M - t\text{BuPO}_3\text{H}]^+$, 120.

Carrier-protein conjugates of hapten 2: The following four solutions were prepared:

A: EDCI (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, 44 mg) in DMF (0.125 mL) and water (0.025 mL), $c = 1.5$ M.

B: *N*-Hydroxysuccinimide (25 mg) in DMF (0.09 mL) and water (0.04 mL), $c = 1.6$ M.

C: KLH (5 mg mL^{-1}) in phosphate buffer saline (PBS: Na_2HPO_4 (10 mM), NaCl (160 mM), pH 7.4).

D: BSA (5 mg mL^{-1}) in PBS.

A solution of hapten **2** (10 mg, 0.027 mmol) in DMF (0.15 mL) was treated with EDCI (soln A, 0.045 mL, 2.5 equiv) and NHS (soln B, 0.042 mL, 2.5 equiv). After 24 h, HPLC analysis (Table 4) showed that **2** ($t_R = 6.0$ min) was >90% converted to the active ester ($t_R = 12.9$ min). 0.075 mL of the active solution was then added to KLH (soln C, 0.5 mL) and 0.15 mL to BSA (soln D, 1 mL). Aq. sat. $NaHCO_3$ (0.07 mL) was added to each solution. After 24 h at 4 °C, HPLC analysis indicated that the active ester had been consumed. No free acid was detected. The carrier-protein solutions were used without further purification.

4-Nitrophenethyl chloromethyl phosphonate (25): A solution of nitrophenethyl alcohol (100 mg, 0.6 mmol) in toluene (3 mL) and anhydrous DMF (0.5 mL) at 0 °C was treated with ^{1}H-tetrazole (4 mg, 0.1 equiv), *N*,*N*-diisopropylethylamine (0.15 mL, 2.2 equiv), and chloromethyl dichlorophosphoridate (100 mg, 0.062 mL, 1 equiv). The temperature was slowly raised to 25 °C. After 8 hours, water (1 mL) was added. After 45 minutes, the reaction was acidified (50 mL HCl, 1N) and extracted twice with CH_2Cl_2. The organic phase was dried over Na_2SO_4 and concentrated, and the residue was purified by preparative reverse-phase HPLC (gradient 10–20% acetonitrile in 30 minutes). The main fraction was lyophilyzed to give the phosphonate **25** as a colorless solid. Yield: 50 mg (0.18 mmol, 30%); m.p. 101 °C; ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD): $\delta = 8.14$ (d, $J = 6.6$ Hz, 2H), 7.45 (d, $J = 6.6$ Hz, 2H), 4.32 (q, $J = 6.3$ Hz, 2H), 3.47 (d, $J = 10.7$ Hz, 2H), 3.09 (t, $J = 6.3$ Hz, 2H); ^{13}C NMR (75 MHz, CD_3OD/$CDCl_3$): $\delta =$ 147.5, 146.2, 130.6, 124.1, 66.9 (d, $J = 6.1$ Hz), 37.2 (d, $J = 6.6$ Hz), 34.0 (d, $J = 156$ Hz); IR ($CHCl_3$): $\tilde{\nu} = 2956$, 2322, 1598, 1511, 1394, 1341, 1270, 1046, 1035, 852 cm^{-1}; MS (SIMS): 281 $[M+H]^+$, 267, 221, 207, 154, 147, 136, 120; elemental analysis calcd (%) for $C_9H_{11}ClNO_5P$ (279.01): C 38.71, H 3.97; found: C 38.78, H 3.94.

Chloromethylphosphonic acid 2-[4-(5-hydroxy-1,5-dioxopentalamino)phenyl]ethyl ester (3): A solution of chloromethyl phosphonate **24** (100 mg, 0.36 mmol) in methanol (3 mL) was stirred with Pd/C (8 mg) under 1 atm H_2 for 3 h, filtered over celite, and concentrated. The residue (86 mg of a pale yellow oil) was dissolved in anhydrous CH_2Cl_2 (3 mL) and dry DMF (3 mL); then glutaric anhydride (80 mg, 2 equiv) was added. After 3 h at 25 °C, the solution was concentrated under vacuum, and the residue purified by preparative reverse-phase HPLC. Lyophilization of the main fraction yielded hapten **3** as a yellow oil. Yield: 37 mg (0.1 mmol, 27%); ^{1}H NMR (200 MHz, CD_3OD): $\delta = 7.43$ (d, $J = 8.2$ Hz, 2H), 7.14 (d, $J = 8.2$ Hz, 1H), 4.21 (q, $J = 6.9$ Hz, 2H), 3.43 (d, $J = 10.2$ Hz, 2H), 2.92 (t, $J = 6.9$ Hz, 2H), 2.37(m, 4H), 1.98 (q, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta =$ 176.2, 172.5, 137.3, 133.3, 129.7, 120.6, 67.6 (d, $J = 6.1$ Hz), 36.7 (d, $J = 5.5$ Hz), 36.2, 34.0 (d, $J = 119.5$ Hz), 33.5, 21.2; IR ($CHCl_3$): $\tilde{\nu} = 3002$, 1712, 1673, 1518, 1388, 1093 cm^{-1}.

Carrier protein conjugates of hapten 3: The same procedure as for hapten **2** was applied starting with hapten **3** (10 mg, 0.028 mmol).

7-[(Pivalyloxymethyl)oxy]-4-(carboxymethyl)-2*H*-benzopiran-2-one (1): 7-hydroxy-4-carboxymethyl-2*H*-benzopiran-2-one **4** (150 mg, 0.68 mmol) was dissolved in anhydrous DMF (3 mL), and NaH (35 mg , 55% in oil, 2.1 éq.) was added at −20 °C. The temperature was raised to 25 °C for 30 min, then cooled again to −60 °C, and a solution of iodomethyl pivalate (250 mg, 1.5 equiv; prepared from the chloride by reaction with NaI and purified by distillation) in anhydrous THF (1 mL) was added dropwise. The temperature was maintained at −60 °C for 2 h, then the mixture was stirred for a further 10 h up to 25 °C. The reaction was poured into aq. 0.1N HCl and extracted twice with ethyl acetate. The organic phase was dried (Na_2SO_4) and concentrated, and the residue dissolved in MeOH (1 mL) buffered with NH_3/NH_4Cl. After 6 h at 25 °C, the solution was evaporated and the residue purified by preparative reverse-phase HPLC (gradient 25–35% acetonitrile in 20 minutes). After lyophylization, the main fraction gave the desired product **1**. Yield: 6.5 mg (5%); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta =$ 7.55 (d, $J = 8.8$ Hz, 1H), 7.01 (dd, $J = 8.8$ Hz, $J = 2.2$ Hz, 1H), 6.97 (d, $J = 2.2$ Hz, 1H), 6.20 (s, 1H), 5.82 (s, 2H), 2.43 (s, 2H), 1.22 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 174.2$, 173.4, 160.9, 159.7, 155.0, 152.2, 125.8, 115.2, 113.3, 113.0, 103.5, 85.0, 39.0, 26.9, 18.7; IR ($CHCl_3$): $\tilde{\nu} = 3265$, 2985, 1754, 1724, 1619, 1243, 1109, 997 cm^{-1}; MS (EI): 290 $[M - CO_2]^+$, 259, 174, 147, 57.

General procedure for the synthesis of oxymethyl esters of 7-hydroxy-2*H*-1-benzopiran-2-one or 4-nitrophenol: A solution of 7-hydroxy-2*H*-1-benzopiran-2-one (300 mg, 1.85 mmol) in dry DMF (3 mL) was treated with NaH (118 mg, 55 % suspension in oil, 1.5 equiv). After 30 min at 25 °C, the reaction was cooled to −60 °C, and the alkylating agent was added as a solution in dry THF (1 mL). The temperature was maintained at −60 °C for 2 h, then slowly raised to 25 °C overnight. Subsequently, the reaction mixture was poured into aq. 1N HCl (50 mL) and extracted twice with CH_2Cl_2. After evaporation of the organic phase, the residue was purified by preparative reverse-phase HPLC.

The alkylating agents bromomethyl acetate (distilled at 80 °C, 180 mbar), bromomethyl propanoate (distilled at 70 °C, 190 mbar), and chloromethyl isobutyrate (distilled at 85 °C, 170 mbar) were obtained as pure compounds in 30–50 % yield by adding the acyl chloride (20 g) dropwise over 1 h to an ice-cold mixture of paraformaldehyde (1.0 equiv) and $ZnCl_2$ (0.02 equiv), then heating the reaction at 55 °C for 15 h, and distilling the product under reduced pressure.[18] Iodomethyl isobutyrate was obtained from chloromethyl isobutyrate by reaction with NaI in acetonitrile (24 h).

7-[(Pivalyloxymethyl)oxy]-2*H*-benzopiran-2-one (5): Colorless solid; yield: 228 mg (0.82 mmol, 44 %); $R_f = 0.5$ (hexane/AcOEt 7:3); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.66$ (d, $J = 9.6$ Hz, 1H), 7.42 (d, $J = 8.4$ Hz, 1H), 7.02 (d, $^4J = 2.6$ Hz, 1H), 6.95 (dd, $J = 2.6$, 8.4 Hz, 1H), 6.31 (d, $J = 9.6$ Hz, 1H), 5.82 (s, 2H), 1.20 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 177.0$, 160.8, 159.8, 155.5, 143.1, 128.9, 114.3, 114.0, 113.6, 103.4, 84.9, 38.9, 26.9; IR ($CHCl_3$): $\tilde{\nu} = 3020$, 1736, 1618, 1216, 762 cm^{-1}; MS (EI): 276 $[M]^+$; 175; 162; 134; 85; 57.

7-(*iso*-Propylcarbonyloxymethyl)oxy-2*H*-1-benzopiran-2-one (7): Colorless solid; yield: 107 mg (0.41 mmol, 30 %); m.p. 54 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.68$ (d, $J = 9.6$ Hz, 1H), 7.42 (d, $J = 8.5$ Hz, 1H), 7.02 (d, $J = 2.2$ Hz, 1H), 6.95 (dd, $J = 8.5$, 2.2 Hz, 1H), 6.31 (d, $J = 9.6$ Hz, 1H), 5.82 (s, 2H), 2.62 (hept, $J = 7.0$ Hz, 1H), 1.19 (d, $J = 7.0$ Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 175.6$, 160.8, 159.7, 155.5, 143.1, 128.9, 114.3, 114.0, 113.5, 103.4, 84.7, 33.9, 18.7; IR ($CHCl_3$): $\tilde{\nu} = 2977$, 1732, 1616, 1506, 1397, 1343, 1278, 976, 759 cm^{-1}; MS (EI): 262 $[M]^+$, 232, 175, 163, 134, 71, 43; elemental analysis calcd (%) for $C_{14}H_{14}O_5$ (262.26): C 64.12, H 5.38; found: C 64.02, H 5.32.

7-[(Ethylcarbonyloxymethyl)oxy]-2*H*-1-benzopiran-2-one (8): Colorless solid; yield: 110 mg (0.44 mmol, 24 %); m.p. 83 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.67$ (d, $J = 9.6$ Hz, 1H), 7.42 (d, $J = 8.4$ Hz, 1H), 7.03 (d, $J = 2.6$ Hz, 1H), 6.95 (dd, $J = 8.4$, 2.6 Hz, 1H), 6.31 (d, $J = 9.6$ Hz, 1H), 5.82 (s, 2H), 2.42 (q, $J = 7.4$ Hz, 2H), 1.19 (t, $J = 7.4$ Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 173.1$, 160.8, 159.7, 155.5, 143.1, 128.9, 114.2, 113.5, 113.9, 103.4, 84.7, 27.4, 8.7; IR ($CHCl_3$): $\tilde{\nu} = 3080$, 1759, 1712, 1621, 1507, 1413, 1345, 1280, 1162, 1044, 959 cm^{-1}; MS (EI): 248 $[M]^+$, 218, 175, 162, 134, 105, 57.

7-[(Acetoxymethyl)oxy]-2*H*-1-benzopiran-2-one (9): Colorless solid; yield: 110 mg (0.43 mmol, 25 %); m.p. 122 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.67$ (d, $J = 9.6$ Hz, 1H), 7.42 (d, $J = 8.4$ Hz, 1H), 7.03 (d, $J = 2.6$ Hz, 1H), 6.95 (dd, $J = 8.4$, 2.6 Hz, 1H), 6.31 (d, $J = 9.6$ Hz, 1H), 5.82 (s, 2H), 2.14 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 169.6$, 160.8, 159.7, 155.5, 143.1, 129.0, 114.3, 114.0, 113.5, 103.5, 84.7, 27.4; IR ($CHCl_3$): $\tilde{\nu} = 3084$, 1763, 1720, 1621, 1508, 1430, 1349, 1221, 1169, 1052, 970 cm^{-1}; MS (EI): 234 $[M]^+$, 204, 175, 162, 134, 105, 43.

General procedure for the synthesis of 7-hydroxy-2*H*-1-benzopiran-2-one esters: A solution of 7-hydroxy-2*H*-1-benzopiran-2-one (200 mg, 1.23 mmol) in dry DMF (3 mL) was treated with NaH (118 mg, 55 % suspension in oil, 2.3 equiv). After 30 minutes at 25 °C, the reaction was cooled to 0 °C, and the acyl chloride (1.5 equiv) as a solution in dry THF (1 mL) was added dropwise. After 2 hours at 25 °C, the reaction was poured into aq. 1N HCl (50 mL) and extracted twice with CH_2Cl_2. The organic phase was dried over Na_2SO_4, and the residue purified either by flash chromatography or by reverse-phase HPLC.

7-Pivaloyloxy-2*H*-benzopiran-2-one (10): Colorless solid; yield: 228 mg (0.97 mmol, 79 %); m.p. 139 °C; $R_f = 0.5$ (hexane/AcOEt 7:3); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.70$ (d, $J = 9.6$ Hz, 1H), 7.49 (d, $J = 8.4$ Hz, 1H), 7.10 (d, $J = 2.6$ Hz, 1H), 7.02 (dd, $J = 8.4$, 2.6 Hz, 1H), 6.40 (d, $J = 9.6$ Hz, 1H), 1.38 (s, 9H); ^{13}C NMR (50 MHz, CD_3OD): $\delta = 176.4$, 160.3, 154.7, 153.7, 142.8, 128.4, 118.3, 116.2, 115.9, 110.3, 39.2, 27.0; IR ($CHCl_3$): $\tilde{\nu} = 2977$, 1744, 1723, 1621, 1264, 1232, 989 cm^{-1}; MS (EI): 246 $[M]^+$, 162, 134, 105, 85, 77, 57; elemental analysis calcd (%) for $C_{14}H_{14}O_4$ (246.26): C 68.28, H 5.73; found: C 68.28, H 5.69.

7-(Isobutyroyl)oxy-2*H*-1-benzopiran-2-one (11): Colorless solid; yield: 180 mg (0.77 mmol, 63 %); m.p. 102–103 °C; $R_f = 0.3$ (hexane/AcOEt 8:2); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.69$ (d, $J = 9.6$ Hz, 1H), 7.48 (d, $J = 8.4$ Hz, 1H), 7.08 (d, $J = 2.2$ Hz, 1H), 7.04 (dd, $J = 8.4$, 2.2 Hz, 1H), 6.40 (d, $J = 9.6$ Hz, 1H), 2.84 (hept, $J = 7.0$ H, 1Hz), 1.34 (s, $J = 7.0$ Hz, 6H); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 174.6$, 160.8, 159.7, 155.5, 143.1, 129.0, 114.3, 114.0, 113.5, 103.5, 33.6, 20.4; IR ($CHCl_3$): $\tilde{\nu} = 2976$, 1754, 1716, 1616, 1399, 1266, 1145, 1130, 1095, 990, 832 cm^{-1}.

7-(Propanoyl)oxy-2*H*-1-benzopiran-2-one (12): Colorless solid; yield: 226 mg (1.0 mmol, 84 %); m.p. 94 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.70$ (d, $J = 9.6$ Hz, 1H), 7.49 (d, $J = 8.4$ Hz, 1H), 7.12 (d, $J = 2.2$ Hz, 1H), 7.05 (dd, $J = 8.4$, 2.2 Hz, 1H), 6.40 (d, $J = 9.6$ Hz, 1H), 2.64 (q, $J = 7.4$ Hz, 2H), 1.29 (t, $J = 7.4$ Hz, 3H); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 172.2$, 160.4, 154.7, 153.3, 142.8, 128.5, 118.4, 116.5, 116.0, 110.4, 27.7, 8.9; IR ($CHCl_3$): $\tilde{\nu} = 2976$, 1754, 1716, 1616, 1399, 1266, 1145, 1130, 1095, 990, 832 cm^{-1}; MS (EI): 218 $[M]^+$, 162, 134, 105, 77, 57.

7-Acetoxy-2*H*-1-benzopiran-2-one (13): Colorless solid; yield: 195 mg (0.95 mmol, 79 %); m.p. 140 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.69$ (d, $J = 9.6$ Hz, 1H), 7.48 (d, $J = 8.4$ Hz, 1H), 7.12 (d, $J = 2.2$ Hz, 1H), 7.05 (dd, $J = 8.4$, 2.2 Hz, 1H), 6.39 (d, $J = 9.6$ Hz, 1H), 2.34 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 169.4$, 161.0, 155.3, 153.8, 143.5, 129.2, 119.7, 117.3, 116.7, 111.1, 21.7; IR ($CHCl_3$): $\tilde{\nu} = 3079$, 1740, 1620, 1565, 1506, 1427, 1400, 1372, 1198, 1121, 1011, 988, 906 cm^{-1}; MS (EI): 204 $[M]^+$, 162, 134, 105, 78, 43.

Synthesis of pivaloyloxymethyl derivatives of phenols: A solution of phenol (300 mg) in dry DMF (4 mL) was treated with NaH (55 % suspension in oil, 1.5 equiv) at 0 °C. After 10 min, the temperature was lowered to −60 °C, and iodomethyl pivalate (1.5 equiv) as a solution in dry THF (4 mL) was slowly added. The temperature was allowed to rise to 25 °C over 2 hours. The mixture was poured into aq. 1N NaOH and extracted twice with ethyl acetate. The organic phase was dried over Na_2SO_4 and concentrated, and the residue purified by flash chromatography.

2,2-Dimethylpropionic acid (4-nitrophenoxy)methyl ester (6): Colorless solid; yield: 450 mg (1.78 mmol, 82 %); m.p. 135–137 °C; $R_f = 0.8$ (hexane/AcOEt 1:1); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.23$ (d, $J = 9.2$ Hz, 2H), 7.13 (d, $J = 9.2$ Hz, 2H), 5.85 (s, 2H), 1.21 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 176.9$, 161.5, 142.9, 125.8, 115.9, 84.6, 38.9, 26.8; IR ($CHCl_3$): $\tilde{\nu} = 3120$, 2979, 1744, 1594, 1348, 1135, 850 cm^{-1}; MS (EI): 253 $[M]^+$, 223, 180, 152, 85, 57.

2,2-Dimethylpropionic acid (2-nitro-phenoxy)methyl ester (14): Colorless solid; yield: 431 mg (1.78 mmol, 79 %), $R_f = 0.7$ (hexane/AcOEt 1:1); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.84$ (dd, $J = 8.1$, 1.5 Hz, 1H), 7.55 (ddd $J = 8.5$, 8.4, 1.5 Hz, 1H,), 7.28 (dd, $J = 8.5$, 1.1 Hz, 1H), 7.16 (ddd, $J = 8.4$, 8.1, 1.1 Hz, 1H), 5.85 (s, 2H), 1.21 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 173.4$ (C_3); 149.9, 140.7, 133.9, 125.5, 122.9, 117.8, 86.3, 38.8, 26.8; IR ($CHCl_3$): $\tilde{\nu} = 3028$, 2979, 1752, 1609, 1532, 1482, 1356, 1244, 1218, 1147, 1122, 1092, 1023, 983, 858, 772, 747 cm^{-1}; MS (EI): 253 $[M]^+$, 223, 152, 139, 122, 85, 57.

2,2-Dimethylpropionic acid (2-methoxy-4-formylphenoxy)methyl ester (15): Prepared from vanillin (200 mg, 1.3 mmol). Colorless solid; yield: 220 mg (0.83 mmol, 63 %); $R_f = 0.45$ (hexane/AcOEt 9:1); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 9.70$ (s, 1H), 7.27 (m, 2H), 7.03 (d, $J = 8.8$ Hz, 1H), 5.70 (s, 2H), 3.75 (s, 3H), 1.02 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 190.6$, 176.8, 151.1, 150.2, 131.9, 125.5, 115.1, 110.0, 85.5, 55.8, 38.7, 26.7; IR ($CHCl_3$): $\tilde{\nu} = 3026$, 2978, 1749, 1687, 1594, 1509, 1466, 1425, 1395, 1268, 1227, 1112, 1025, 980, 768, 752, 668 cm^{-1}; MS (EI): 266 $[M]^+$, 236, 165, 152, 85, 57.

2,2-Dimethylpropionic acid (4-methyl-phenoxy)methyl ester (16): Colorless oil; yield: 358 mg (1.43 mmol, 65 %); $R_f = 0.4$ (hexane/AcOEt 95:5); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.95$ (d, $J = 8.8$ Hz, 2H), 7.07 (d, $J = 8.8$ Hz, 2H), 5.82 (s, 2H), 1.20 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 196.6$, 177.1, 160.5, 131.8, 130.5, 115.5, 84.7, 38.9, 26.8, 26.3; IR ($CHCl_3$): $\tilde{\nu} = 2981$, 1749, 1679, 1603, 1510, 1273, 1236, 1123, 1028, 757 cm^{-1}; MS (FAB$^+$): 251 $[M+H]^+$, 221, 165, 137.

2,2-Dimethylpropionic acid (4-cyanophenoxy)methyl ester (17): Colorless solid; yield: 387 mg (1.66 mmol, 66 %); $R_f = 0.6$ (hexane/AcOEt 4:1); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.62$ (d, $J = 8.8$ Hz, 2H), 7.10 (d, $J = 8.8$ Hz, 2H), 5.81 (s, 2H), 1.20 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 177.0$, 159.9, 134.1, 118.7, 116.5, 106.1, 84.5, 38.9, 26.9; IR ($CHCl_3$): $\tilde{\nu} = 3024$, 2979, 2230, 1751, 1608, 1510, 1241, 1119, 1027, 983, 839, 765, 756 cm^{-1}; MS (FAB$^+$): 234 $[M+H]^+$, 149, 135, 123, 119.

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2,2-Dimethylpropionic acid (4-chlorophenoxy)methyl ester (18): Colorless solid; yield: 387 mg (1.66 mmol, 58%); $R_f = 0.7$ (hexane/AcOEt 95:5); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.27$ (d, $J = 8.8$ Hz, 2H), 6.96 (d, $J = 8.8$ Hz, 2H), 5.75 (s, 2H); 1.21 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta =$ 177.2, 155.5, 129.5, 127.7, 122.8, 85.7, 38.9, 26.9; IR ($CHCl_3$): $\tilde{\nu} = 3034$, 2978, 1748, 1493, 1281, 1231, 1156, 1157, 1122, 1055, 1029, 973, 829 cm^{-1}; MS (EI$^+$): 242 $[M]^+$, 212, 141, 128, 111, 85, 57.

2,2-Dimethylpropionic acid (4-(*N*-acetylamino)phenoxy)methyl ester (19): Colorless solid; yield: 187 mg (0.70 mmol, 71%); $R_f = 0.3$ (hexane/AcOEt 9:1); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.43$ (d, $J = 8.8$ Hz, 2H), 7.00 (d, $J =$ 8.8 Hz, 2H), 5.74 (s, 2H), 2.17 (s, 3H), 1.21 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 177.4$, 168.5, 153.5, 133.0, 121.6, 116.6, 86.1, 38.8, 26.9, 24.2; IR ($CHCl_3$): $\tilde{\nu} = 2979$, 1744, 1659, 1608, 1505, 1412, 1318, 1216, 1125, 1057, 975, 824 cm^{-1}; MS (EI$^+$): 265 $[M]^+$, 235, 164, 151, 122, 109, 85, 57.

(Pivaloyloxymethyl)fluorescein (20): A solution of fluorescein (200 mg, 0.45 mmol) in dry DMF (2 mL) and dry THF (2 mL) at 0 °C was treated with NaH (29 mg, 55% suspension in oil, 1.5 equiv). After 10 min, the temperature was lowered to −60 °C, and a solution of iodomethylpivalate (174 mg, 1.2 equiv) in dry THF (4 mL) was added dropwise. The temperature was raised to 25 °C over 2 hours, after which the solution was poured into aq. 1N HCl (50 mL) and extracted twice with ethyl acetate. The organic phase was dried over Na_2SO_4, and the residue purified by flash chromatography to give **20** as a yellow solid. Yield: 80 mg (0.18 mmol, 30%); $R_f = 0.7$ (hexane/AcOEt 1:9); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.03$ (m, 1H), 7.66 (m, 2H), 7.26 (m, 1H), 6.72 (m, 3H), 6.55 (m, 1H), 5.78 (s, 2H), 1.22 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 177.5$, 176.5, 170.0, 158.5, 158.2, 153.0, 152.4, 135.2, 129.8, 129.3, 126.6, 125.1, 124.0, 113.2, 110.7, 103.7, 103.1, 85.3, 38.9, 26.9; MS (FAB$^+$): 447 $[M+H]^+$, 417, 333, 287, 271, 119.

(*N*-Pivaloyloxymethyl) pyridinium trifluoroacetate (21): Pyridine (300 mg) was treated dropwise under stirring with a solution of iodomethyl pivalate (1.5 equiv) in hexane (5 mL). After 4 hours at 25 °C, the precipitate was filtered off and purified by preparative RP-HPLC to give **21** as a pale yellow solid. Yield: 580 mg (1.89 mmol, 50%); m.p. 64 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 9.15$ (d, $J = 7.5$ Hz, 2H), 8.58 (t, $J = 7.5$ Hz, 1H), 8.13 (t, $J = 7.5$ Hz, 2H), 6.53 (s, 2H); 1.21 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 175.7$, 148.1, 145.8, 128.9, 80.8, 39.6, 27.3; IR ($CHCl_3$): $\tilde{\nu} = 3096$, 2990, 1754, 1634, 1504, 1484, 1410, 1200, 1137, 1111, 1049 cm^{-1}; HRMS calcd for $C_{11}H_{16}NO_2$: 194.118104, found: 194.117460

7-(*tert*-Butoxycarbonyl)oxy-2*H*-1-benzopiran-2-one (22): A solution of umbelliferone (215 mg, 1.34 mmol) in dry CH_2Cl_2 (1 mL) and dry DMF (4 mL) at 0 °C was treated with di-*tert*-butyldicarbonate (440mg, 1.5 equiv) and pyridine (0.16 mL, 1.5 equiv). After 2 h at 25 °C, the reaction mixture was directly purified by flash chromatography (hexane/ethyl acetate 6:4) to give **22** as a white solid. Yield: 190 mg (0.725 mmol, 55%); m.p. = 109 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.69$ (d, $J = 9.6$ Hz, 1H), 7.48 (d, $J =$ 8.5 Hz, 1H), 7.21 (d, $J = 2.6$ Hz, 1H), 7.14 (dd, $J = 9.6$, 2.6 Hz, 1H), 6.40 (d, $J = 9.6$ Hz, 1H), 1.58 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 160.2$, 154.6, 153.4, 150.7, 142.7, 128.4, 117.8, 116.4, 115.9, 109.9, 84.4, 27.5; IR ($CHCl_3$): $\tilde{\nu} = 3021$, 1741, 1623, 1285, 1250, 1218, 1144, 1125, 775, 744, 670 cm^{-1}; MS (EI$^+$): 262 $[M]^+$, 247, 203, 162, 145, 134, 117, 57.

1-(Pivalyloxymethyl)-2-oxo-3,3-diethyltriazene (23): A suspension of 1-(*N*,*N*-diethylamino)diazen-1-ium-1,2-diolate (sodium salt; DEA/NO; 100 mg, 0.64 mmol) in DMF (2 mL) at 0 °C was treated with *N*,*N*-diisopropylethylamine (0.05 mL) and chloromethyl pivalate (0.1 mL, 1.1 equiv). The solution was stirred for 30 min at 0 °C and for 12 h at 25 °C, then directly deposited onto silica gel. Elution with hexane/ethyl acetate (95:5) gave product **23** as a colorless solid. Yield: 30 mg (0.12 mmol, 18%); $R_f = 0.3$ (hexane/AcOEt 6:4); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 5.82$ (s, 2H), 3.18 (q, 4H, $J = 7.35$ Hz), 1.19 (s, 9H), 1.1 (t, 6H, $J = 7.35$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 176.7$, 87.5, 48.3, 38.7, 26.8, 11.4; IR ($CHCl_3$): $\tilde{\nu} = 2983$, 1752, 1519, 1481, 1386, 1281, 1238, 1168, 1131, 1054, 1033, 963, 759 cm^{-1}; MS (SIMS): 248 $[M+H]^+$, 187.

Immunization:[19] Two sets of four mice (129 GIX/boy +) were immunized with the KLH conjugates of either hapten **2** or **3**. Each mouse received two intraperitoneal injections of hapten–KLH conjugate (100 μg in 200 μL PBS emulsified with MPL + TDM adjuvent (Sigma M6536)) one on day 1 and one on day 14. Samples of serum were taken by means of a tail bleed on day 21 to estimate the titer.

Fusion: Myelomas (NS1) were grown for a week in RPMI-1640 (Sigma R0883) with 10% FCS (Fetal Calf Serum) (Sigma F2442) and 8-azaguanine (Sigma A5284) as a selective agent. A minimum of 10^7 myeloma cells were used to fuse one spleen. Three days before the scheduled fusion, and at least 40 days after the second hapten–KLH injection, four mice were given a final intravenous injection of 50 μg KLH–conjugate in PBS. On the fusion day, the spleen cells of each mouse were collected and washed with no-serum media, together with the myelomas. The spleen cells and the myelomas were then fused with PEG 1500 (Roche 783 641). The fused cells were diluted in 150 mL media containing HAT (H = hypoxanthine 9×10^{-3} M, A = aminopterin 2×10^{-5} M, T = thymidine 8×10^{-4} M) (Sigma H0262) and plated out in (10) 96-well cell-culture plates (Corning Costar 3598).

Cell culture: The fused cells were fed twice a week with media containing HT (H = hypoxanthine 9×10^{-3} M, T = thymidine 8×10^{-4} M) (Sigma H0137) for two weeks. After two weeks, the plates were tested by ELISA to identify those hybridomas that were producing antigen binding. Preclones were also selected for their ability to catalyze the hydrolysis of **1** (see below). Positive preclones were cultured further and cloned twice by limited dilution, and finally produced in 1 L cultures or with Integra CL 1000 (Integra Biosciences 90005). After removal of the cells by centrifugation, antibodies were purified from the cell-culture media by ammonium sulfate precipitation and protein-G-affinity chromatography (Amersham Pharmacia Biotech 27-0886-01). The antibodies were finally dialyzed into PBS and concentrated to 3–7 mg mL^{-1}. Although repeated freeze–thaw cycles may be damaging, the antibodies can be stored in this form at −20 °C without loss of activity for at least several months. Isotypes were determined by means of an isotyping kit (Pierce no. 37501).

Screening for catalysis: A fluorescence assay for catalysis with substrate **1** was performed on all preclones when the cell culture reached a volume of 5 mL, before the first subcloning dilution. Thus 5 mL of cell-culture supernatant were passed on 100 mg of protein-G gel (Gammabind Plus sepharose, Pharmacia Biotech) in a cotton-plugged Pasteur pipette. The gel was washed with PBS (2 × 1 mL) and 0.1M aq. NaCl (2 × 1 mL), and finally eluted with citrate (50 mM, 2 × 150 μL, pH 2.7). The eluted acidic buffer containing the antibody, was neutralized with Tris-base (tris(hydroxymethyl)aminomethane; 1M, 40 μL). 90 μL of this antibody-containing solution were transferred to a well of a 96-well microtiter plate and mixed with borate buffer (0.5 M, 5 μL, pH 8.8) and substrate **1** (5 μL of a 2 mM stock solution in DMF). 50 μL of the solution was immediately transferred to another well containing a solution of hapten **2** or **3** (0.5 μL, 2 mM in PBS). The time dependence of fluorescence increase was then followed over the next 3 h. The difference in apparent rate between the antibody and the antibody + inhibitor samples was used as a criterion for specific catalysis. The purification/assay procedure was performed easily in parallel with up to 40 different cell-culture samples, and was performed repeatedly with each individual hybridoma at all stages of cell culture.

Kinetics: Reactions were followed either by fluorescence in round-bottom polypropylene 96-well plates (Costar) with a Cytofluor II Fluorescence Plate Reader (Perceptive Biosystems, filters $\lambda_{ex} = 360 \pm 20$ nm, $\lambda_{em} = 460 \pm$ 20 nm), or by UV/Vis spectroscopy at $\lambda = 405$ nm (for nitrophenyl substrate **6**) in half-area flat-bottom clear polystyrene cell-culture plates (Corning–Costar) on a UV Spectramax 250 instrument from Molecular Devices. Solutions containing antibodies (90 μL at 0.2 mg mL^{-1} in either 20 mM bis-tris [bis(hydroxyethyl)-tris(hydroxymethyl)aminomethane] pH 7.55 or 20 mM borate pH 8.8) were thermostated at 31 °C. The reactions were initiated by addition of DMF (5 μL) and of a properly prediluted solution (5 μL) of substrate in DMF/water (1:1). The activity screening concentration was 100 μM, kinetic measurement concentrations were 17, 26, 39, 59, 89, 133, 200, and 300 μM (as obtained by a ⅔ serial dilution of substrate in the 20X stock starting with 6 mM). The fluorescence or UV signals were converted to product concentration according to a calibration curve with either **4**, umbelliferone, or 4-nitrophenol. The net reaction rate of background was used to calculate the kinetic parameters according to the Michaelis–Menten model, as already reported.[20] The lines obtained from 8 datapoints had correlation factors $R^2 > 0.995$. Systematic errors of ±10% must be assumed as a result of uncertainty in the antibody concentrations and pipetting inaccuracy of small volumes.

The catalytic constants k_{cat} are reported for one active site, assuming a molecular weight of 150 kDa and two catalytic sites for each antibody. Exact active-site titrations were carried out with all antibodies by using hapten **3** as a tight binding ligand. These titrations showed the expected active-site concentration within 10%.

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