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Characterization of a Catalytic Antibody for Stereoselective Ester Hydrolysis—A Catalytic Residue and Mode of Product Inhibition†

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Abstract—A catalytic antibody which catalyzes stereoselective ester hydrolysis was characterized, and the role of a catalytic Arg residue is discussed in terms of product inhibition. A monoclonal antibody 1C7 generated against the phosphonate **1** was highly stereoselective for (*R*)-isomer in hydrolyzing racemic ester **2**. However, the reaction was almost stoichiometric due to strong inhibition by the product acid **3**. One Arg residue in the antibody combining site was essential to the catalysis, and the same Arg was expected to play a dominant role in product inhibition by charge interaction with the negatively charged product acid. Indeed, the antibody experienced much less product inhibition with the hydrolysis of a carbonate ester **7**, which yields a neutral alcohol **8** devoid of a negative charge, and exhibited at least 100 turnovers without any loss of activity. In addition, high stereoselectivity for (*R*)-isomer was still retained. The amino acid sequence and computer modeling of the variable domain of 1C7 suggested that Arg^{97} in the complementarity-determining region (CDR) of heavy chain was the putative catalytic residue.

Introduction

Since 1986 when antibody-mediated catalysis was first demonstrated, the number and diversity of reactions catalyzed by antibodies have been increasing rapidly.[1] Catalytic antibodies have attracted much attention due not only to their potential as novel protein catalysts with tailored or predetermined selectivity,[2,3] but also to their similarities to enzymes in their catalytic behavior.[4–7] In fact, some antibodies have been shown to catalyze the reaction with very similar mechanisms and pathways to those of highly evolved enzymes, such as covalent acyl intermediate[4,6,7] or induced fit.[7]

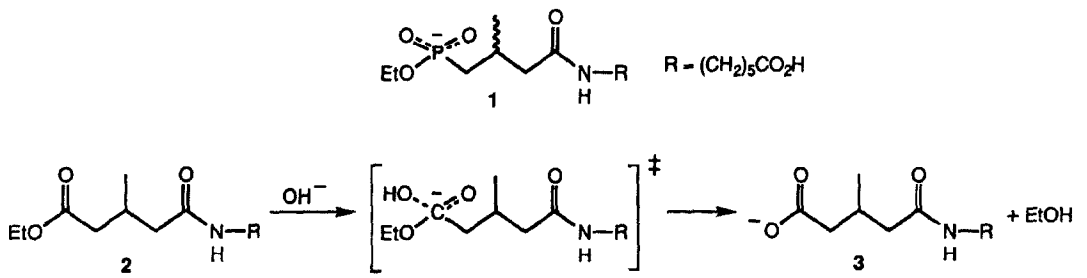
Although the diversity and selectivity of antibody-catalyzed reactions can rival or even exceed those of enzymatic reactions,[2,8–10] many problems still remain to be solved in the catalytic efficiency of antibodies. Antibodies in general do not catalyze chemical reactions as efficiently as most enzymes.[11] One reason is that catalytic antibodies are subject to product inhibition and hence the number of turnover is limited.[5,10,12] So far several attempts have been directed towards alleviating product inhibition,[9,13,14] but little is known about the mode and mechanism of product inhibition. This paper describes the details of our previous report[15] and deals with the characterization of an esterolytic antibody in terms of stereoselectivity, catalytic residue and a mode of product inhibition.

Results

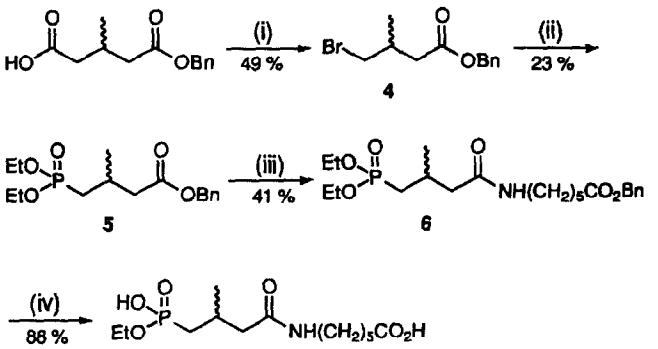
Induction of catalytic antibodies

Monoclonal antibodies were generated against racemic hapten **1**, which is a transition state analogue for the hydrolysis of the ester **2** under basic conditions (Scheme I). The hapten **1** was synthesized in seven steps from racemic 3-methylglutaric acid monobenzyl ester (Scheme II) and was attached to keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) with the aid of a water-soluble carbodiimide. Mice were immunized with the KLH conjugate, and monoclonal antibodies were obtained by a standard protocol.[16] The antibodies were first screened with enzyme-linked immunosorbent assay (ELISA) for binding to the BSA conjugate of **1**. Twenty-one hybridomas producing extrapositive antibodies were cloned and propagated in a synthetic culture medium. Monoclonal antibodies secreted were purified by protein A affinity chromatography to homogeneity as judged by SDS-polyacrylamide gel electrophoresis.[17] The purified antibodies were subjected to further screening for their ability to catalyze the hydrolysis of ester **2** by monitoring ethanol formation with a coupled alcohol dehydrogenase enzyme assay (see Experimental). As a result of the second screening, three monoclonal antibodies were found to be catalytic.[18] In the preliminary assays, one antibody 1C7 significantly accelerated the hydrolysis of **2** and was characterized further.

†Dedicated to Professor J. Bryan Jones on the occasion of his 60th birthday.



Scheme I.



Scheme II. Synthesis of hapten **1**. (i) (a) $AgNO_3$, KOH in H_2O, room temperature, 2 h (b) Br_2 in dry CCl_4, reflux, 5 h (ii) $P(OEt)_3$, 155–160 °C, 8 h (iii) (a) H_2, 5 % Pd/C, EtOH (b) benzyl 6-aminohexanoate *p*-toluenesulfonate salt, DCC in $CHCl_3$, 48 h (iv) (a) H_2, 5 % Pd/C, EtOH (b) KOH in 80 % EtOH, 90 °C, 6 h.

Kinetic behavior and stereoselectivity of 1C7-catalyzed hydrolysis of ester 2

The antibody 1C7 exhibited Michaelis–Menten kinetics for the hydrolysis of the ester **2**. The steady-state kinetic parameters were determined by a nonlinear least square fit of the initial rates,[19] and a K_m of 285 μM and a k_0 of 1.97 min^{-1} were obtained for racemic **2**. No background hydrolysis was observed without 1C7 in at least 1 h under the same reaction conditions. As expected, the reaction was competitively and strongly inhibited by the hapten **1** with a K_i of 3.95 μM. A typical reaction profile is shown in Figure 1. Although the initial rate of hydrolysis came up to 29 μM min^{-1}, the catalyzed reaction rate decreased gradually as the reaction proceeded, and the reaction reached almost a plateau when two equivalent moles of the substrate **2** were consumed. This suggested that the reaction was strongly inhibited by the product, as well as by the hapten **1**. Indeed, the product **3** was found to be a rather strong competitive inhibitor with a K_i of 74.7 μM. Thus the reaction was almost stoichiometric rather than catalytic with the ester **2** due to strong product inhibition.

The stereoselectivity of the 1C7-catalyzed hydrolysis of **2** was examined. Since direct kinetic resolution of racemic **2** was not successful due to difficulty in measuring the *e.e.* of the hydrolyzed product **3**,[20] kinetic parameters were determined separately for optically active (*R*)-**2** (96 % *e.e.*), (*S*)-**2** (88 % *e.e.*) and racemic **2**, and these parameters were compared (Table 1). The hydrolysis of (*R*)-**2** (96 % *e.e.*) was catalyzed by 1C7 more efficiently than racemic **2**, with a K_m^{app} of 124 μM and a k_0^{app} of 1.80 min^{-1}, while the hydrolysis of (*S*)-**2** (88 % *e.e.*) was extremely slow, a K_m^{app} and a k_0^{app} being 1340 μM and 1.93 min^{-1}, respectively. It should be noted that there was a large difference in the K_m^{app} values for these samples, whereas the k_0^{app} values were almost the same.

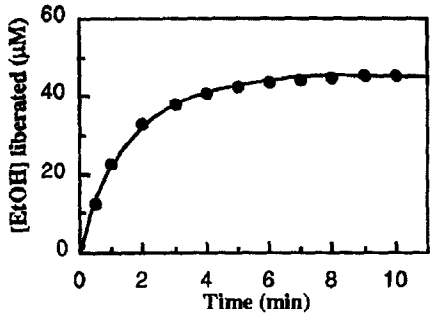


Figure 1. Reaction profile for the antibody (1C7)-catalyzed hydrolysis of racemic ester **2**. 1C7 (20 μM), ester **2** (20 mM) in 0.1 M Tris–HCl (pH 8.0), 30 °C. The liberation of EtOH was monitored by a coupled alcohol dehydrogenase enzyme assay (see Experimental).

The pH dependence of the 1C7-catalyzed hydrolysis of the (*R*)-**2** was examined. As shown in Figure 2, the catalyzed rate (k_0) of hydrolysis exhibited a first-order dependence on hydroxide ion concentration between pH 7 and 10, while K_m did not vary appreciably in this pH range.

Table 1. Kinetic parameters for hydrolysis of ester **2** by 1C7[a]

	K_m^{app} [μM]	k_0^{app} [min^{-1}]	$(k_0/K_m)^{app}$ [$\times 10^{-4}$ $\mu M^{-1}min^{-1}$]
(*R*)-**2** (96 % ee)	124	1.80	145
racemic **2**	285	1.97	69
(*S*)-**2** (88 % ee)	1340	1.93	14

[a]Hydrolysis was done at 1C7 (20 μM), ester **2** (12.5 μM - 5.0 mM) in 0.1 M Tris-HCl (pH 8.0), 30 °C. The release of ethanol upon hydrolysis was monitored with a coupled alcohol dehydrogenase enzyme assay.

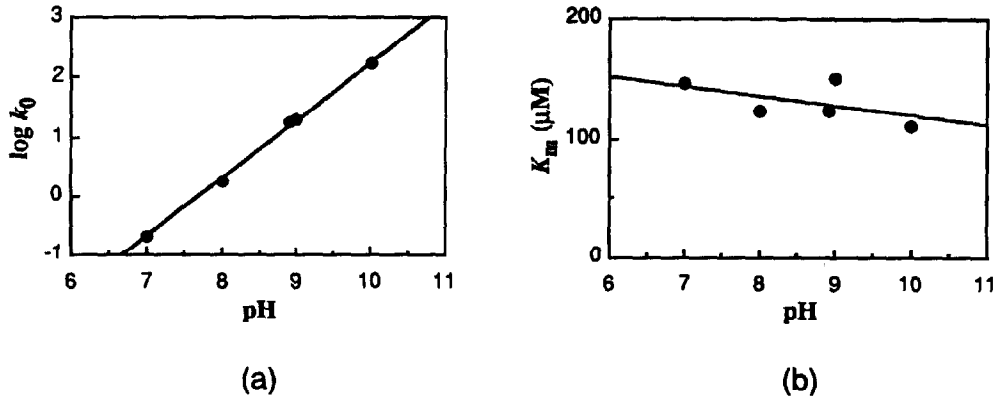


Figure 2. pH Dependence of the kinetic parameters. Kinetic parameters for the hydrolysis of (*R*)-**2** (96 % *e.e.*) were determined under the following reaction conditions: 1C7 (0.2–20 μM), (*R*)-**2** (12.5–400 μM) in 0.1 M Tris–HCl (pH 7–8.9) or 0.1 M Na_2CO_3–$NaHCO_3$ (pH 9–10).

Chemical modification

The identity of the amino acid side chains responsible for the catalysis was probed by a series of chemical modification targeted Arg, Lys, Trp, and His. As shown in Table 2, treatment of 1C7 with phenylglyoxal resulted in complete inactivation, while none of the other amino acid residues tested were necessary for the catalysis. Interestingly, treatment with diethyl pyrocarbonate (DEP) resulted in a slight increase in the apparent catalytic activity, and the activity was more than tripled by increasing the concentration of DEP to 20 mM (data not shown). The increased activity, however, returned to the original value by treating the DEP-modified antibody with hydroxylamine.

Table 2. Chemical modification of antibody 1C7

Amino acid	Relative remaining activity[a] (%)
Arg[b]	0.9
Lys[c]	94
Trp[d]	78
His[e]	134 (103)[f]

[a]The remaining hydrolytic activity for racemic **2** was measured after removing the unreacted modification reagent..
[b]Phenylglyoxal 20 mM antibody 31 μM in 0.2 M Na_2CO_3-$NaHCO_3$ (pH 8.2), 25 °C, 1 h.
[c]Maleic anhydride 100 mM, antibody 31 μM in 0.1 M borate buffer (pH 9.0), 0 °C, 20 min.
[d]*N*-Bromosuccinimide 100 μM, antibody 20 μM in 0.1 M acetate buffer (pH 4.5), 25°C, 1 h.
[e]Diethyl pyrocarbonate 300 μM, antibody 31 μM in 20 mM Mes-NaOH (pH 6.0) and CH_3CN 1 % (v/v), 25 °C, 1 h.
[f]After the treatment of DEP-modified 1C7 with 0.5 M NH_2OH·HCl (pH 9.0), 0 °C, 24 h.

The hapten **1** and product **3** exhibited a protective effect on Arg modification (Table 3). As expected, the hapten **1** was more effective in Arg protection than the product **3**, exhibiting complete protection in Arg modification at the concentration of 10 mM. In addition, protective effect by the hapten **1** was also observed in His modification.

The putative catalytic Arg was titrated with *p*-hydroxyphenylglyoxal (HPG).[21] The remaining activity was plotted against the incubation time (Figure 3a) or against the number of modified Arg residues (Figure 3b). The inactivation was first-order, and the catalytic activity decreased linearly according to the extent of Arg modification and disappeared when just 2 mol of Arg per mol of 1C7 were modified (Figure 3b). Considering that one immunoglobulin molecule has two identical binding sites, it was most likely that one Arg residue per antibody combining site was essential to catalysis.

Since the catalytic activity of an antibody is closely related to its specific binding to the hapten or the transition-state analogue, the remaining binding ability of 1C7 to the hapten **1** was examined after Arg modification. 1C7 was partially modified with HPG and its binding to the BSA conjugate of **1** was measured by ELISA. As shown in Figure 4, the apparent binding of the modified 1C7 dropped sharply according to the extent of Arg modification.

1C7-Catalyzed hydrolysis of carbonate ester 7

To investigate the role of Arg in product inhibition, the hydrolysis of carbonate ester **7** was examined. The structure of carbonate ester **7** was similar to that of ester **2**, but was so designed as to undergo decarboxylation upon hydrolysis to yield a neutral alcohol **8**, rather than a negatively charged acid like **3**, as the final product (Scheme III). The antibody 1C7 exhibited the saturation kinetics with a K_m of 898 μM and a k_0 of 0.80 min^{-1} for racemic **7**, the initial rate being a little smaller than that with ester **2**. However, 1C7 experienced much less product inhibition with this substrate, and the reaction was catalytic rather than stoichiometric (Figure 5). The hydrolysis of **7** proceeded linearly until at least 100 turnovers per antibody combining site was achieved without any loss of activity over 72 h. As expected, the product alcohol **8** was found to be a very poor competitive inhibitor with a K_i of 44.2 mM.

Table 3. Protective effect of hapten **1** and product **3** on the chemical modification of 1C7[a]

Amino acid	Additive	conc. (mM)	Relative remaining activity (%)
Arg	(none)	–	0.9
	hapten **1**	0.1	24
		1	65
		10	98
	product **3**	10	32
		100	74
His	(none)	–	134 (103)[b]
	hapten **1**	1	101 (96)[b]

[a]Chemical modification was carried out in the presence of hapten **1** or product **3** using the same reagent under the same reaction conditions as in Table 2.
[b]After the treatment of the DEP-modified 1C7 with 0.5 M NH_2OH·HCl (pH 9.0), 0 °C, 24 h.

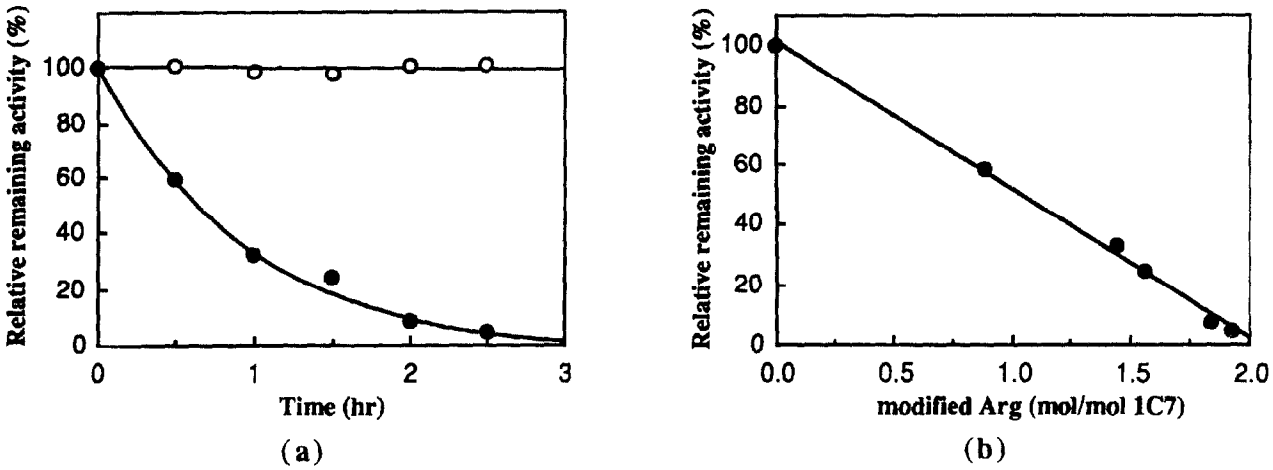


Figure 3. Chemical modification of antibody 1C7 with *p*-hydroxyphenylglyoxal HPG. (a) Time-dependent inactivation of antibody 1C7 by HPG. Antibody 1C7 (20 μM) was treated with HPG (200 μM) in 0.2 M Na_2CO_3–$NaHCO_3$ buffer pH 9.0. at 25 °C in the dark (•). Control experiment without HPG (o). (b) Titration of catalytic Arg with HPG. The extent of modification was determined photometrically at 340 nm using the molecular absorption coefficient of 1.83×10^4 M^{-1} cm^{-1} (pH 9.0, 25 °C) for the modification product.[21]

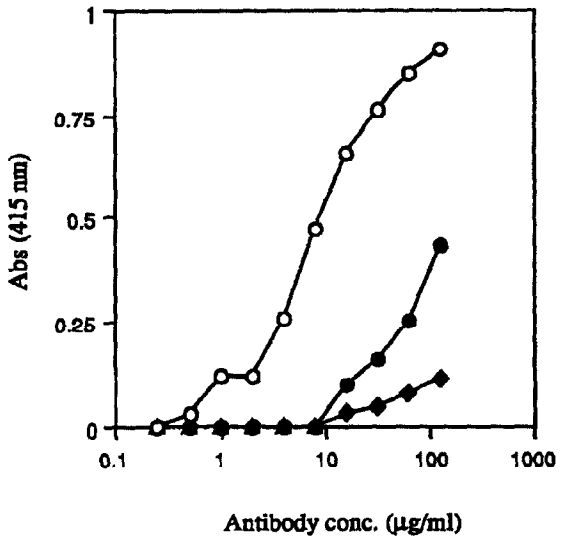


Figure 4. Relative binding ability of Arg-modified antibody 1C7 to hapten **1**. Antibody 1C7 (20 μM) was treated with HPG (200 μM) in 0.2 M Na_2CO_3–$NaHCO_3$ buffer (pH 9.0) at 25 °C for 1 h (•) or 2.5 h (♦) in the dark. The relative remaining activity and the number of modified Arg per antibody were: (•) (39 %, 1.3 mol mol^{-1} antibody), (♦) 18 %, 1.8 mol mol^{-1} antibody). Control experiment without HPG(o). The remaining binding ability was measured by ELISA using BSA-hapten **1** conjugate (see Experimental).

The stereoselectivity of 1C7-catalyzed hydrolysis of **7** was examined by the same method as **2**. The apparent kinetic parameters were K_m^{app} = 441 μM, k_0^{app} = 0.80 min^{-1} for (*R*)-**7** (94 % *e.e.*) and K_m^{app} = 3650 μM, k_0^{app} = 0.81 min^{-1} for (*S*)-**7** (80 % *e.e.*) (Table 4). 1C7 was also found to be specific for (*R*)-isomer with K_m^{app} varying considerably according to the mole fraction of (*R*)-isomer, and k_0^{app} being almost constant.

Sequence analysis of antibody 1C7 Fv fragment

To obtain more information about the combining site of 1C7, the genes encoding the variable region of heavy and light chains (V_H and V_L) were cloned into *E. coli*, and their nucleotide sequences were determined. The amino acid sequences of V_H and V_L deduced from their nucleotide sequences are depicted in Figure 6. Three complementarity-determining regions (CDRs) for each V_H and V_L, were determined by amino acid sequence alignment according to the reported procedure.[22] One Arg residue (Arg^{97}) was found in the third CDR of the heavy chain (H3), and this Arg is the only one Arg found in all six CDRs. In addition, His^{93} found in the third CDR of the light chain (L3) is the only one His throughout the whole sequence of both heavy and light chain variable regions.

Discussion

We have utilized racemic tetrahedral phosphonate hapten **1** to mimic the putative tetrahedral intermediate or transition state in ester hydrolysis of **2**. One antibody (1C7) performed the hydrolysis of **2** with a fairly good initial velocity and very high enantiomeric preference for (*R*)-**2**. It has been observed that immunization with racemic mixtures of hapten usually give catalytic antibodies specific to either of the enantiomeric hapten and hence show enantiomeric preference.[2,3,5,12,23–25] Comparison of kinetic parameters determined with different enantiomeric mixtures of **2** revealed a large difference in K_m, but no difference in k_0. This result suggests that the antibody 1C7 discriminates the enantiomer in substrate binding. Presumably the true hapten was the (*R*)-isomer of **1** and

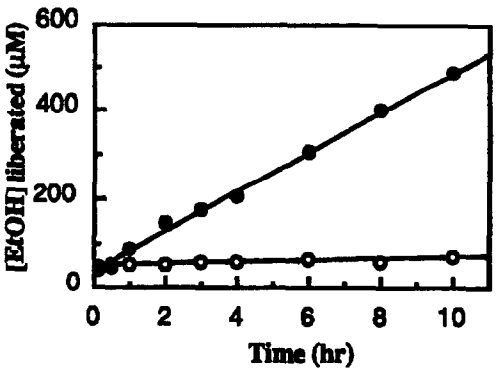
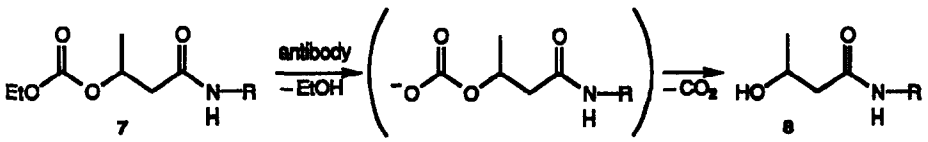


Figure 5. Reaction profile for the antibody (1C7)-catalyzed hydrolysis of racemic **7** (•). 1C7 (20 μM), carbonate ester **7** (20 mM) in 0.1 M Tris–HCl (pH 8.0), 25 °C. Reaction profile for racemic **2** (o) is also shown for comparison.



Scheme III.

Table 4. Kinetic parameters for hydrolysis of carbonate ester **7** by 1C7[a]

	K_m^{app} [μM]	k_0^{app} [min^{-1}]	$(k_0/K_m)^{app}$ [×10^{-4} μM^{-1}min^{-1}]
(*R*)-**7** (94 % ee)	441	0.80	18
racemic **7**	898	0.80	8.9
(*S*)-**7** (80 % ee)	3650	0.81	2.2

[a]Hydrolyis was done at 1C7 (20 μM), carbonate ester **7** (94 μM - 20 mM) in 0.1 M Tris-HCl (pH 8.0), 30 °C. The release of ethanol upon hydrolysis was moitored with a coupled alcohol dehydrogenase enzyme assay.

Heavy chain

10 20 30 40 50 60
---LQESGPGLVKPSQSLSLTCSVTGYSITSGYYWNWIRQFPGNKLEWMGYISYDGSKNYN
CDR-H1 CDR-H2

70 80 90 100 110
PSLKNRISITRDTSKNQFFLKLNSVTTEDTATYYCARSARATKFAYWGQGTTVTVSS
CDR-H3

Light chain

10 20 30 40 50
DVLMTQTPLTLSVTIGQPASISCKSSQSLLDSDGQTYLNWLLQRPGQSPKRLIYLVSKLDS
CDR-L1 CDR-L2

60 70 80 90 100
GVPDRFTGSGSGTDFTLKISRVEAEDLGVYYCWQSTHFTLTFGAGTKLEIKR
CDR-L3

Figure 6. Amino acid sequences of antibody 1C7 variable region (V_H and V_L). Complementarity-determining regions (CDRs) are underlined. Amino acid numbering is according to Kabat *et al.*[22].

the antibody only accepts the (*R*)-isomer of **2** as the substrate. This kinetic behavior stands in contrast to that of previously reported antibodies catalyzing stereoselective aminoacylation[12] or imine formation,[26] where a small difference in K_m and a much larger difference in k_{cat} implied that the antibodies discriminated the diastereoselective transition states rather than the ground state enantiomeric substrate.

Although 1C7 exhibited very high stereoselectivity and fairly good initial velocity for the hydrolysis of an unactivated ester,[5,25,27] 1C7 suffered from a severe product inhibition by the product **3**. The K_i value (74.7 μM) of racemic **3** was 19-fold larger than that of racemic hapten **1**, but was 3.8-fold smaller than the K_m of racemic substrate **2**. This magnitude of inhibition was strong enough to block the antibody, because the accumulation of this inhibitory product **3** of as much concentration as the antibody combining site resulted in near-complete depletion of the esterolytic activity (Figure 1).

Chemical modification had great diagnostic significance in probing the catalytic residue of 1C7. We confirmed by the following evidence that one Arg residue in the antibody combining site plays a key role in catalysis. First, an Arg modification led to complete inactivation of 1C7, and the hapten **1** exhibited a protective effect on the inactivation. Secondly, the titration of this putative catalytic Arg was first order and one essential Arg per combining site was detected. Thirdly, the Arg modification also resulted in depletion of the specific binding of 1C7 to the hapten **1**. In addition, a positively charged Lys or Arg is expected to be induced in antibody 1C7 combining site, because charge complementarity is commonly observed in antibody–hapten binding[28,29] and negatively charged phosphate moiety is a prominent recognition element in the hapten **1**.

The pH dependence of 1C7-catalyzed hydrolysis showed that the rate constant was first order with respect to hydroxide ion concentration between pH 7 and 10, arguing for rate-determining attack of hydroxide ion in this pH range. Although the possibility of general base or nucleophilic catalysis cannot be ruled out, it is most likely that a positively charged Arg residue in the antibody combining site stabilizes the negatively charged transition state or intermediate by electrostatic interaction. The participation of an Arg residue in ester hydrolysis was also noted in phosphorylcholine-binding antibodies, in which an active site Arg and Tyr residues were shown to stabilize the negatively charged tetrahedral transition state.[30,31]

The fact that the product **3** is a fairly strong competitive inhibitor and exhibited a protective effect on Arg modification suggests that the binding mode of product **3** is similar to that of hapten **1** and substrate **2**. If the catalytic Arg is positioned in such a way as to stabilize the anionic transition state or intermediate by electrostatic interaction, then it should also 'stabilize' the anionic product acid **3**, thereby hindering the product release. To test this notion, we employed the carbonate ester **7** as a substrate. Since the carbonate ester **7** yields a neutral product **8** rather than a negatively charged acid such as **3**, the product should be devoid of electrostatic interaction with the catalytic Arg and hence alleviate the product inhibition. Indeed, the antibody 1C7 experienced much less product inhibition with this substrate, and the reaction was catalytic rather than stoichiometric (Figure 5). More than 100 turnovers observed per antibody combining site is a good indication of negligible product binding; in fact, the K_i of **8** was 44 mM. These results thus confirmed that the electrostatic interaction involving the catalytic Arg plays a dominant role not only in catalysis, but also in product inhibition as illustrated in Figure 7. Since negatively charged phosphate moiety has been commonly used as hapten for generating esterolytic antibodies by transition state stabilization,[1] the product inhibition observed so far with those antibodies may be due, in part, to a similar electrostatic interaction involving a positively charged amino acid residue induced in the antibody active site.

The antibody 1C7 was also specific to the (*R*)-isomer of **7**. The observed high enantiomeric preference for (*R*)-**7** was due to a large difference in substrate binding (K_m) between both enantiomers. This result suggests that the same mode of enantiomer recognition is operative in the substrate binding of both esters **2** and **7**.

Cloning and sequence analysis of the genes encoding V_H and V_L of 1C7 revealed that one Arg residue (Arg^{97}) was found in the third CDR (H3) of the heavy chain. Although we have no direct evidence that this Arg is the catalytic one, it is most likely that this Arg is responsible for the catalysis, because (1) this is the only one Arg found in all six CDRs of 1C7, (2) the Arg^{97} is positioned in the central part of H3 which is the most variable region of six CDRs of an antibody and hence is important in antigen recognition,[32] (3) the Arg^{97} is sandwiched between relatively small amino acid residues such as Ala and Ser, reminiscent of a small consensus motif found in the active site of Ser hydrolases (Gly–X–Ser–X–Gly).[33] In addition, computer modeling based on a structurally defined antibody having a high sequence homology with 1C7 variable domain suggested that the Arg^{97} is located in the central region of the combining site and its side chain sticks out in solvent contact.

It is an interesting observation that one His was found in the third CDR of the light chain (His^{93} in L3) and that this is the only one His in the whole sequence of V_H and V_L of 1C7. The chemical modification of His unexpectedly resulted in a slight, but reversible increase in the catalytic activity. The computer modeling suggested that L3 is also positioned in the central region of the antibody combining site, indicative of a possible participation of this His in the catalysis. Now that the antibody-coding genes are in hand, this His as well as the Arg^{97} can be a promising target of mutagenesis studies.

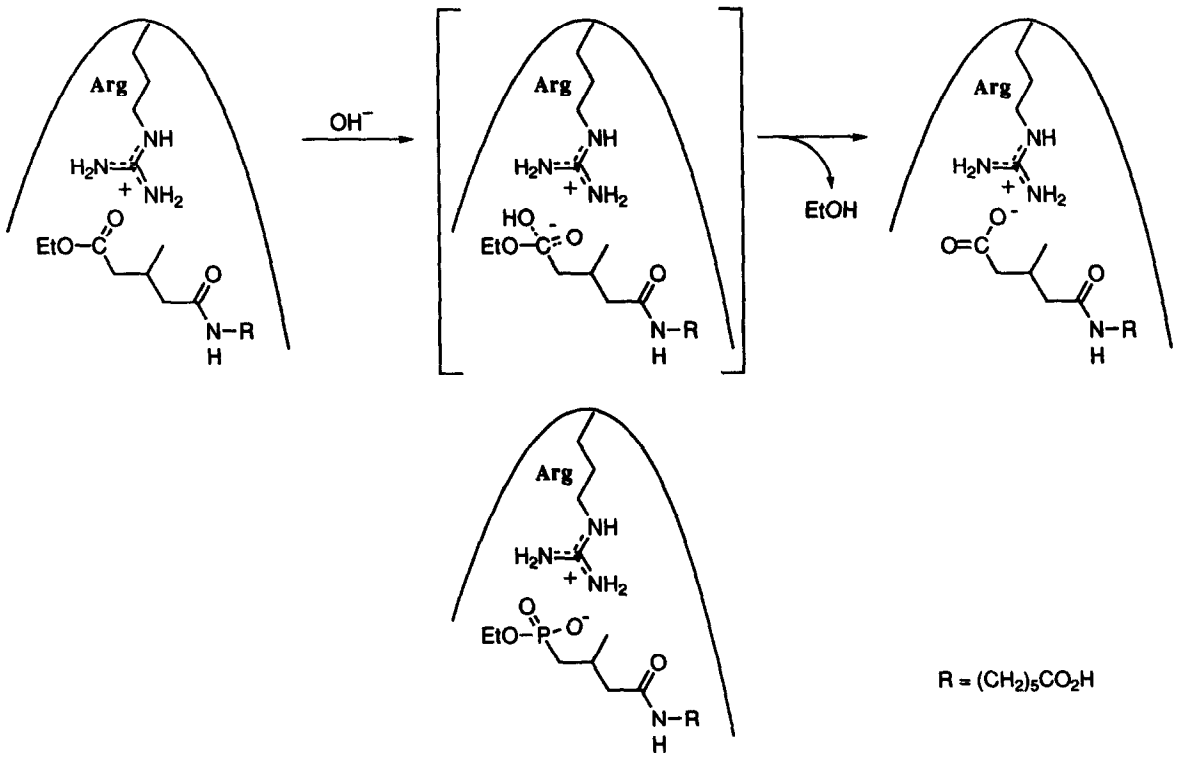


Figure 7. A possible reaction mechanism for 1C7-catalyzed hydrolysis of **2**.

Experimental

General procedure

The spectroscopic and analytical instruments were: ^1H, ^{13}C and ^{31}P NMR, Varian VXR-200 (200 MHz); MS, JEOL JMX-DX-300; IR, Hitachi 215; UV, Hitachi 340; Optical Rotation, Perkin-Elmer 241 polarimeter; Elemental analysis, Yanaco MT-5; HPLC, Jasco BIP-I equipped with silica gel NUCLEOSIL 50-5, 4.6 × 250 mm. DNA sequences were analyzed on A.L.F. DNA Sequencer (Pharmacia). ^1H NMR spectra were measured in $CDCl_3$ or D_2O with TMS or 3-(trimethylsilyl)propanesulfonic acid sodium salt as an internal standard, respectively. An external standard for ^{31}P NMR was 85 % H_3PO_4 and chemical shifts downfield of the reference are indicated as positive. The products were isolated by flash column chromatography on silica gel or by distillation. CH_2Cl_2 and CCl_4 were distilled over P_2O_5 and stored over molecular sieves 4 Å. ELISA was performed with the following materials: enzyme-labeled second antibody, horseradish peroxidase-conjugated goat anti-mouse IgG; substrate, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) and hydrogen peroxide. The binding activity was determined from the absorbance at 415 nm.

Synthesis of hapten 1 and preparation of hapten–protein conjugates

Benzyl 4-bromo-3-methylbutyrate (4). Racemic 3-methylglutaric acid monobenzyl ester (40.9 g, 173 mmol) was suspended in distilled water (200 mL) and mixed successively with an aqueous solution (50 mL) of KOH (9.72 g, 173 mmol) and $AgNO_3$ (28.8 g, 173 mmol) at 0 °C. The resulting mixture was stirred at room temperature for 2 h to liberate the silver salt as colorless precipitates. The precipitates were collected and dried thoroughly over P_2O_5 under reduced pressure at 80 °C for 3 days (50.9 g, 86 %).

Bromine (5.9 g, 36.9 mmol) dissolved in dry CCl_4 (15 mL) was added dropwise to a suspension of the silver salt (12.7 g, 36.9 mmol) in dry CCl_4 (50 mL) with ice-cooling.[34] After the addition was complete, the mixture was stirred at room temperature for 1 h and then refluxed for 5 h with exclusion of moisture. The resulting suspension was filtered, and the filtrate was washed with 10 % Na_2CO_3 and dried (Na_2SO_4). Solvent was removed *in vacuo* and the residual oil was distilled to afford benzyl 4-bromo-3-methylbutyrate (**4**) as a colorless oil (5.7 g, 57 %): bp 115–125 °C (0.3–0.5 mmHg); IR ν_{max} 1730 cm^{-1} (C=O); ^1H NMR ($CDCl_3$) δ 1.08 (3H, d, J = 6.4 Hz, CH_3), 2.26–2.65 [3H, m, $CH(CH_3)CH_2CO$], 3.35–3.50 (2H, m, $BrCH_2$), 5.13 (2H, s, OCH_2Ph), 7.36 (5 H, m, aromatic); anal. calcd. for $C_{12}H_{15}O_2Br$: C, 53.15; H, 5.58. Found: C, 52.91; H, 5.64.

Benzyl 4-diethylphosphono-3-methylbutyrate (5). A mixture of the bromide **4** (6.38 g, 23.5 mmol) and triethyl phosphite (7.75 g, 46.7 mmol) was refluxed at 155–160 °C for 8 h. The reaction mixture was subjected to fractional

distillation to afford the phosphonate **5** as a colorless oil (1.8 g, 23 %): bp 155–162 °C (0.3–0.5 mmHg); ^{1}H NMR ($CDCl_3$) δ 1.11 (3H, d, J = 6.4 Hz, CH_3CH), 1.31 (6H, t, J = 7.1 Hz, 2 × CH_3CH_2O), 1.58–1.98 (2H, m, PCH_2), 2.27–2.63 (3H, m, $CHCH_2CO$), 4.0–4.2 (4H, m, 2 × CH_3CH_2OP), 5.12 (2H, s, OCH_2Ph), 7.35 (5H, m, aromatic); anal. calcd. for $C_{16}H_{25}O_5P$: C, 58.53; H, 7.67. Found: C, 58.44; H, 7.75.

Benzyl 6-(4-diethylphosphono-3-methylbutanoyl)aminohexanoate (6). The benzyl ester of the phosphonate **5** (6.0 g, 18.3 mmol) was cleaved by hydrogenolysis [5 % palladium carbon (2.0 g), in EtOH (25 mL), room temperature, 99 %]. The resulting carboxylic acid (4.10 g, 17.2 mmol) was coupled with benzyl 6-aminohexanoate *p*-toluenesulfonate salt (6.77 g, 17.2 mmol) in the presence of *N*, *N'*-dicyclohexylcarbodiimide (DCC) (3.55 g, 17.2 mmol) and triethylamine (1.74 g, 17.2 mmol) in $CHCl_3$ (50 mL) [0 °C for 1 h, then at room temperature for 48 h]. After the reaction was complete, the solvent was replaced with AcOEt and the mixture was filtered. The filtrate was washed successively with 10 % citric acid, water and 4 % $NaHCO_3$, and dried (Na_2SO_4). The solvent was evaporated to give a colorless oil. Purification by flash column chromatography on silica gel ($CHCl_3$: EtOH = 15 : 1) gave the phosphonate **6** as a colorless oil (3.1 g, 41 %): ^{1}H NMR ($CDCl_3$) δ 1.13 (3H, d, J = 6.0 Hz, CH_3CH), 1.32 (6H, t, J = 7.2 Hz, 2 × CH_3CH_2O), 2.36 (2H, t, J = 7.4 Hz, CH_2CO_2Bn), 1.3–2.4 [11H, m, $(CH_2)_3$ and $CH_2CH(CH_3)CH_2$], 3.22 (2H, dt, J = 6.5 and 6.6 Hz, $NHCH_2$), 4.10 (4H, m, 2 × CH_3CH_2O), 5.11 (2H, s, OCH_2Ph), 6.43 (1H, br t, NH), 7.35 (5H, m, aromatic); anal. calcd. for $C_{22}H_{36}O_6NP$: C, 59.85; H, 8.22; N, 3.17. Found: C, 59.66; H, 8.46; N, 2.91.

Ethyl hydrogen 3-[N-(5-carboxypentyl)carbamoyl]-2-methylpropylphosphonate (1). The fully protected compound **6** (1.17 g, 2.65 mmol) was subjected to hydrogenolysis by the same procedure as **5** to give the corresponding carboxylic acid (800 mg, 86 %). The resulting product (1.63 g, 4.64 mmol) was treated with 1.7 N KOH in 80 % EtOH (6.8 mL) at 90 °C for 6 h. EtOH was evaporated and the residual aqueous solution was acidified (pH < 1) with 12 N HCl. The acidic aqueous solution was washed with AcOEt to remove a trace of unreacted material. The aqueous solution was neutralized with 2 N KOH and was evaporated to leave a colorless oil. The resulting oil was dissolved in water (40 mL) and was mixed and stirred with cation-exchange resin Amberlite IR-120B (H^+ form, 20 mL) at room temperature for 2 h. The mixture was filtered and the filtrate was evaporated to give the hapten **1** as a slightly yellow syrup (1.10 g, 88 %): IR ν_{max} 3400 (br, COOH, P-OH), 1730 (C=O, carboxylic acid), 1630 (C=O, amide), 1040 (P-O) cm^{-1}; ^{1}H NMR (D_2O) δ 0.96 (3H, d, J = 5.8 Hz, CH_3CH), 1.20 (3H, t, J = 7.0 Hz, CH_3CH_2O), 2.29 (2H, t, J = 7.3 Hz, CH_2CO_2H), 1.2–2.3 [11H, m, $(CH_2)_3$, $CH_2CH(CH_3)CH_2$], 3.10 (2H, t, J = 6.4 Hz, $NHCH_2$), 3.97 (2H, m, CH_3CH_2O); ^{13}C NMR (D_2O) δ 15.71 (d, $^3J_{CP}$ = 5.75 Hz, CH_3CH), 19.86 (d, $^3J_{CP}$ = 6.65 Hz, CH_3CH_2O), 23.89, 25.54 and 27.91 [$(CH_2)_3$], 26.35 (d, $^2J_{CP}$ = 3.38 Hz, CH), 31.61 (d, $^1J_{CP}$ = 135.8 Hz, CH_2P), 33.65 and 39.11 ($NHCH_2$, CH_2CO_2H), 43.91 (d, $^3J_{CP}$ = 14.52 Hz, CH_2CONH), 62.16 (d, $^2J_{CP}$ = 6.10 Hz, CH_3CH_2O), 174.8 and 178.9 (CONH, CO_2H); ^{31}P NMR (D_2O) δ +30.3; MS (high resolution) calcd. for $C_{13}H_{26}O_6NP$: m/z 323.150. Found: m/z 323.147.

The hapten **1** was attached to keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) by 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC). Hapten **1** (6.5 mg, 20 μmol) dissolved in distilled water (0.5 mL) was mixed with KLH (6.3 mg) solution in sodium phosphate buffer (25 mM, pH 6.0, 2.0 mL). After 5 min, EDC (7.6 mg, 40 μmol) was added to the solution, and the mixture was stirred at room temperature overnight and was dialyzed against distilled water (4 × 1000 mL). The hapten–BSA conjugate was prepared by the same procedure. Epitope density of the hapten–protein conjugates was determined by measuring the number of free amino groups of the proteins with 2,4,6-trinitrobenzenesulfonic acid[35] before and after the conjugation. Hapten **1** (2 mol) was found to be attached to 1 mol of BSA (M.W. 69,000), and 23.5 mol of the hapten to 1 mol of KLH (M.W. 450,000).

Synthesis of the substrate 2

6-(4-ethoxycarbonyl-3-methylbutanoyl)aminohexanoic acid (2). 3-Methylglutaric anhydride (10.0 g, 78.0 mmol) and benzyl 6-aminohexanoate *p*-toluenesulfonate salt (33.8 g, 85.8 mmol) were dissolved in dry CH_2Cl_2 (100 mL). Triethylamine (19.7 g, 195 mmol) was added to the solution with ice-cooling. The mixture was stirred at 0 °C for 1 h and at room temperature for 2 h. The solution was washed with 2 N HCl and sat. NaCl successively, and dried over Na_2SO_4. Purification by flash column chromatography (AcOEt : hexane : AcOH = 2 : 1 : 0.15) gave the monoamide as an oil (24.5 g, 90 %): ^{1}H NMR ($CDCl_3$) δ 1.04 (3H, d, J = 6.2 Hz, CH_3CH), 1.25–1.75 [6H, m, $(CH_2)_3$], 2.12–2.52 [5H, m, $CH_2CH(CH_3)CH_2$], 2.37 (2H, t, J = 7.3 Hz, CH_2CO_2Bn), 3.24 (2H, dt, J = 6.9 and 6.5 Hz, $NHCH_2$), 5.11 (2H, s, OCH_2Ph), 6.0 (1H, br t, NH), 7.35 (5H, m, aromatic), 9.0 (1H, br s, CO_2H).

The monoamide (7.0 g, 20.0 mmol) was dissolved in dry ether (70 mL) and the solution was cooled to −10 °C. Thionyl chloride (3.58 g, 30 mmol) was added slowly to the solution, and the mixture was stirred at −10 °C for 30 min. A solution of EtOH (1.9 g, 40 mmol) and dry pyridine (3.16 g, 40 mmol) in dry ether (40 mL) was added dropwise to the mixture at −10 °C. The mixture was stirred at −10 °C for 1 h, and at room temperature for 2 h. The precipitate was removed by filtration, and the filtrate was washed with 2 N HCl and sat. NaCl successively. Purification by flash column chromatography (AcOEt : hexane = 1 : 1) gave benzyl 6-(4-ethoxycarbonyl-3-methylbutanoyl)aminohexanoate (4.40 g, 58 %): ^{1}H NMR ($CDCl_3$) δ 1.02 (3H, d, J = 6.4 Hz, CH_3CH), 1.26 (3H, t, J = 7.1 Hz, CH_3CH_2O), 1.22–1.70 [6H, m, $(CH_2)_3$], 2.35 (2H, t, J = 7.6 Hz, CH_2CO_2Bn), 2.00–2.50 [5H, m, $CH_2CH(CH_3)CH_2$], 3.24 (2H, dt, J = 7.0 and 6.4 Hz,

NHCH_2), 4.13 (2H, q, J = 7.2 Hz, CH_3CH_2O), 5.11 (2H, s, OCH_2Ph), 5.85 (1H, br s, NH), 7.35 (5H, m, aromatic).

The benzyl group of this compound (4.0 g, 10.6 mmol) was subjected to hydrogenolysis [5 % palladium carbon (3.0 g) in THF (50 ml)–AcOH (10 ml), room temperature, 8 h] to give the racemic ester **2** as a slightly yellow syrup (2.8 g, 92 %): IR ν_{max} 3300 (br, CO_2H), 1760 and 1730 (C=O, CO_2H and ester), 1630 cm^{-1} (C=O, amide); ^1H NMR ($CDCl_3$) δ 1.02 (3H, d, J = 6.4 Hz, CH_3CH), 1.26 (3H, t, J = 7.1 Hz, CH_3CH_2O), 1.23–1.69 [6H, m, $(CH_2)_3$], 2.05–2.50 [5H, m, $CH_2CH(CH_3)CH_2$], 2.37 (2H, t, J = 7.3 Hz, CH_2CO_2H), 3.26 (2H, dt, J = 6.1 and 6.1 Hz, NHCH_2), 4.14 (2H, q, J = 7.2 Hz, CH_3CH_2O), 6.2 (1H, br t, NH), 9.0 (1H, br s, CO_2H); ^{13}C NMR ($CDCl_3$) δ 14.20 (CH_3CH), 19.78 (CH_3CH_2O), 24.23, 26.21 and 29.09 [$(CH_2)_3$], 28.31 (CH_3CH), 33.81, 39.25, 40.63 and 43.03 [$CH_2CH(CH_3)CH_2$, CH_2CO_2H, $NHCH_2$], 60.44 (CH_3CH_2O), 172.23, 172.91 and 178.05 (C=O); anal. calcd. for $C_{14}H_{25}O_5N$: C, 58.52; H, 8.77; N, 4.87. Found: C, 58.41; H, 8.89; N, 4.68.

(R)- or (S)-2. Optically active esters [(*R*)- or (*S*)-**2**] were prepared from (*R*)- or (*S*)-ethyl hydrogen 3-methylglutarate.[36]

(*R*)-Ethyl hydrogen 3-methylglutarate [4.2 g, 24.1 mmol, $[\alpha]^{25}_D$ -4.86 (*c* 1.11, AcOEt), 96 % *e.e.*[37]] was converted to its dicyclohexylamine (DCHA) salt. The DCHA salt (5.66 g, 16.0 mmol) was coupled with benzyl 6-aminohexanoate *p*-toluenesulfonate salt (6.30 g, 16.0 mmol) in $CHCl_3$ (80 mL) by using DCC (3.30 g, 16.0 mmol) (0 °C, 1 h then at room temperature, 10 h). Solvent was removed by evaporation, and the residue was allowed to stand in AcOEt (80 mL) at –20 °C for 1 day. The precipitate was removed by filtration and the filtrate was evaporated to give a colorless oil. Purification by flash column chromatography (AcOEt : hexane = 1 : 1) gave (*R*)-benzyl 6-(4-ethoxycarbonyl-3-methylbutanoyl)amino-hexanoate (2.6 g, 43 %). The benzyl group was removed by hydrogenolysis [5 % palladium carbon, 10 % AcOH in THF, 8 h] to give (*R*)-**2** as a colorless oil (74 % yield): $[\alpha]^{25}_D$ +1.78 (*c* 1.12, MeOH); anal. found: C, 58.26; H, 8.92; N, 4.65.

(*S*)-**2** was prepared from (*S*)-ethyl hydrogen 3-methylglutarate ($[\alpha]^{25}_D$ +4.00 (*c* 1.15, AcOEt), 88 % *e.e.*[37]) by the same procedure as (*R*)-**2**: $[\alpha]^{25}_D$ –1.04 (*c* 1.15, MeOH); anal. found: C, 58.37; H, 8.89; N, 4.68.

Synthesis of the substrate 7

Racemic, (*R*)- or (*S*)-**7** were prepared from commercially available racemic 3-hydroxybutyric acid and (*R*)- or (*S*)-methyl 3-hydroxybutyrate [(*R*), 94 % *e.e.*; (*S*), 80 % *e.e.*][38], respectively. A typical procedure is as follows. Racemic 3-hydroxybutyric acid (15.7 g, 150 mmol) was converted to its DCHA salt, and the DCHA salt (8.0 g, 28.1 mmol) was coupled with benzyl 6-aminohexanoate *p*-toluenesulfonate salt (11.1 g, 28.1 mmol) in CH_2Cl_2 (100 mL) by using DCC (5.80 g, 28.1 mmol) (0 °C, 3 h then room temperature overnight). The reaction mixture was worked up as **2** gave a colorless oil. Purification by flash column chromatography (CH_2Cl_2 : EtOH = 15 : 1) gave benzyl 6-(3-hydroxybutanoyl)aminohexanoate (3.67 g, 43 %): ^1H NMR ($CDCl_3$) δ 1.21 (3H, d, J = 6.2 Hz, CH_3CH), 1.2–1.8 [6H, m, $(CH_2)_3$], 2.31 (2H, 2 × dd, J = 5.6 and 14.2 Hz, J = 7.8 and 15.2 Hz, CH_2CONH), 2.36 (2H, t, J = 7.2 Hz, CH_2CO_2Bn), 3.23 (2H, dt, J = 6.8 and 6.4 Hz, NHCH_2), 3.62 (1H, br s, OH), 4.1–4.2 (1H, m, CH), 5.11 (2H, s, OCH_2Ph), 6.3 (1H, br t, NH), 7.35 (5H, m, aromatic).

The resulting hydroxy amide (2.55 g, 8.32 mmol), pyridine (7.65 mL, 94.6 mmol) and a catalytic amount of 4-dimethylaminopyridine (DMAP) were dissolved in dry CH_2Cl_2 (60 mL). Ethyl chloroformate (1.35 g, 12.5 mmol) was added to the solution at 0 °C and the mixture was stirred for 2 h at room temperature. Solvent was removed and the residue was dissolved in AcOEt. The solution was washed successively with 2 N HCl, sat. $NaHCO_3$ and sat. NaCl, and dried (Na_2SO_4). Purification by flash column chromatography (AcOEt : hexane = 1 : 1) gave the benzyl 6-(3-ethoxycarbonyloxybutanoyl)amino hexanoate as a colorless syrup (3.32 g, 95 %): ^1H NMR ($CDCl_3$) δ 1.28 (3H, t, J = 7.2 Hz, CH_3CH_2O), 1.35 (3H, d, J = 6.4 Hz, CH_3CH), 1.2–1.7 [6H, m, $(CH_2)_3$], 2.36 (2H, t, J = 7.7 Hz, CH_2CO_2Bn), 2.46 (2H, 2 × dd, J = 5.6 and 14.4 Hz, J = 7.2 and 14.4 Hz, CH_2CONH), 3.23 (2H, dt, J = 7.2 and 6.4 Hz, NHCH_2), 4.17 (2H, 2 × q, J = 7.2 Hz, CH_3CH_2O), 5.11 (2H, s, OCH_2Ph), 5.0–5.2 (1H, m, CH), 5.9 (1H, br t, NH), 7.35 (5H, m, aromatic).

The benzyl group of the ethyl carbonate (2.24 g, 5.93 mmol) was cleaved by hydrogenolysis [5 % palladium carbon (1.1 g) in THF (36 mL)–AcOH (5 mL), room temperature, 5 h] to give the racemic carbonate ester **7** as a colorless syrup (1.61 g, 95 %): IR ν_{max} 3300 (br, COOH), 1740 (C=O, CO_2H, carbonate), 1640 (C=O, amide), 1260 cm^{-1} (C-O); ^1H NMR ($CDCl_3$) δ 1.30 (3H, t, J = 7.2 Hz, CH_3CH_2O), 1.36 (3H, d, J = 6.2 Hz, CH_3CH), 1.3–1.7 [6H, m, $(CH_2)_3$], 2.35 (2H, t, J = 7.3 Hz, CH_2CO_2H), 2.49 (2H, 2 × dd, J = 5.4 and 14.4 Hz, J = 7.2 and 14.4 Hz, CH_2CONH), 3.26 (2H, dt, J = 6.7 and 6.4 Hz, $NHCH_2$), 4.19 (2H, q, J = 7.2 Hz, CH_3CH_2O), 5.0–5.2 (1H, m, CH), 6.04 (1H, br t, NH), 7.8 (1H, br s, CO_2H); ^{13}C NMR ($CDCl_3$) δ 14.17 (CH_3CH_2O), 19.81 [$CH(CH_3)$], 24.25, 26.14 and 28.94 [$(CH_2)_3$], 33.92, 39.30 and 42.88 [CH_2CO_2H, $NHCH_2$, CH_2CONH], 63.93 (CH_3CH_2O), 72.06 (CH), 154.30, 169.83 and 177.95 (OCO_2, CONH, CO_2H); anal. calcd. for $C_{13}H_{23}O_6N$: C, 53.97 ; H, 8.01; N, 4.84. Found: C, 53.75; H, 8.24; N, 4.72.

(*R*)-**7** (94 % *e.e.*): $[\alpha]^{25}_D$ +8.06 (*c* 1.06, AcOEt); anal. found: C, 53.73; H, 8.19; N, 4.73.
(*S*)-**7** (80 % *e.e.*): $[\alpha]^{25}_D$ –7.33 (*c* 1.05, AcOEt); anal. found: C, 53.70; H, 8.26; N, 4.78.

Preparation of monoclonal antibodies

Mice (BALB/c) were immunized with the hapten **1**–KLH conjugate emulsified in Freund's adjuvant.[39] Spleen cells

from these mice were subjected to cell fusion with myeloma cells (P3.X63.NS-1) as the fusion partner by standard procedures.[16] Hybridomas that secreted IgG antibodies specific to the hapten **1**–BSA conjugate were selected by ELISA and cloned.

The selected hybridomas were propagated in GIT culture medium supplemented with 1 % calf serum (CS), penicillin G, streptomycin, and L-glutamine. After 3 weeks incubation, the culture medium was centrifuged. The supernatant was added to the same volume of 1.5 M glycine (pH 8.9) containing 3 M NaCl and was applied to AUTO MASS 1000 antibody-purification apparatus equipped with a MASS γ-Protein A column device [NYGene Corp. (Yonkers, NY)]. The column was washed with the same buffer to remove unbound protein, and the bound antibody was eluted with 0.1 M citrate buffer (pH 3.0). The eluted antibody fraction was immediately neutralized and the solution was concentrated and dialyzed exhaustively against 0.1 M Tris–HCl (pH 8.0). The protein concentration of purified antibody was calculated from the absorbance ($E_{0.1\ \%}$ = 1.4 at 280 nm) or by CBB protein assay kit (Pierce) with BSA as standard.

Assay conditions

The rate of ester hydrolysis of **2** and **7** was determined by monitoring EtOH formation with a coupled alcohol dehydrogenase enzyme assay. A typical assay method is as follows. A mixture of 1.93 mM NAD^+, yeast alcohol dehydrogenase (from baker's yeast, Sigma, 0.64 mg mL^{-1}, 45 unit), and antibody (4.11 mg mL^{-1}, 25.7 μM) in 0.1 M Tris–HCl (pH 8.0, 350 μL) was preincubated at 30 °C for 5 min. The reaction was started by adding 100 μL of substrate solution (13.5 mM) in 0.1 M Tris–HCl (pH 8.0). The final assay mixture (450 μL) contained 1.5 mM NAD^+, alcohol dehydrogenase (0.5 mg mL^{-1}, 45 unit), antibody (3.2 mg mL^{-1}, 20 μM) and 3 mM substrate. Initial velocities were determined spectrophotometrically by measuring the initial linear increase in absorbance at 340 nm,[40] and kinetic parameters were determined by a nonlinear least square fit of the initial rates.[19]

In case of long-term reaction (e.g. 12 h) where the inactivation of the alcohol dehydrogenase was a problem, another assay system was employed. A solution of antibody (4.11 mg mL^{-1}, 25.7 μM) in 0.1 M Tris–HCl (pH 8.0) was preincubated at 25 °C for 10 min. The reaction was initiated by adding 750 μL of the substrate stock solution (90 mM). The final reaction mixture (3.38 mL) contained the antibody (3.2 mg mL^{-1}, 20 μM) and the substrate (20 mM). An aliquot (80 μL) of the reaction mixture was withdrawn after a certain period of incubation and was added to 370 μL of an assay solution containing alcohol dehydrogenase (1.2 mg mL^{-1}) and NAD^+ (3.6 mM). The final assay mixture (450 μL) consisted of NAD^+ (3 mM) and alcohol dehydrogenase (1.0 mg mL^{-1}, 90 unit). The ethanol concentration in the assay mixture was proportional to the initial dehydrogenase reaction rate and was determined by the initial linear absorbance increase at 340 nm.

Chemical modification of 1C7

Typical procedure is as follows. Antibody 1C7 (31 μM) was incubated with phenylglyoxal[41] (20 mM) in Na_2CO_3–$NaHCO_3$ buffer (0.2 M, pH 8.2) for 1 h at 25 °C. The mixture was then exhaustively dialyzed against Tris–HCl (0.1 M, pH 8.0) at 4 °C and the remaining activity was measured under the typical assay conditions. Protein modification reactions targeted for tryptophan[42] [*N*-bromosuccinimide 100 μM, 1C7 20 μM, acetate buffer (0.1 M, pH 4.5)], histidine[43,44] [diethyl pyrocarbonate 300 μM, Mes–NaOH (20 mM, pH 6.0)], and lysine[45] [maleic anhydride 100 mM, borate buffer (0.1 M, pH 9.0), at 0 °C] were carried out by a similar procedure. Part of the His-modified sample was dialyzed against NH_2OH•HCl (0.5 M, pH 9.0) at 4 °C for 1 day for deprotection. Protective effect by the hapten **1** or product carboxylic acid **3** on the modification of Arg and His was measured under the same reaction conditions in the presence of hapten **1** (0.1–10 mM) or product **3** (1–100 mM).

The essential arginine was titrated with *p*-hydroxyphenylglyoxal (HPG).[21] Antibody 1C7 (3.2 mg mL^{-1}, 20 μM) was treated with HPG (200 μM) in 0.2 M Na_2CO_3–$NaHCO_3$ buffer (pH 9.0) at 25 °C in the dark. A sample of the solution was withdrawn after a certain period of incubation, and the unreacted reagent was removed by gel filtration on Sephadex G-25 spun column,[46] and the remaining activity was measured under the typical reaction condition. The number of modified Arg was determined photometrically at 340 nm [molecular absorption coefficient of $1.83 \times 10^4\ M^{-1}\ cm^{-1}$ (pH 9.0, 25 °C)].

Cloning and sequence analysis of antibody V_H and V_L genes

Poly(A)$^+$ mRNA was isolated from 6.3×10^6 1C7 cells by using Quick Prep mRNA Purification Kit (Pharmacia). cDNA encoding the V_H and V_L regions was separately amplified with PCR by using Mouse scFv Module/Recombinant Phage Antibody System (Pharmacia). The amplified V_H and V_L cDNAs were purified with 5 % polyacrylamide gel electrophoresis and were cloned into the Hinc II site of pUC 18 vector. The resulting plasmids transformed *E. coli* HB101. The sequence of plasmids carrying V_H and V_L fragments were determined by dideoxynucleotide method.[47]

Homology search and model building of antibody 1C7 combining site was performed as follows. Protein Data Bank (PDB)[48] was searched for immunoglobulin homologous sequences to the V_H and V_L domain of antibody 1C7 by using the FASTA program.[49] Two antibodies [anti-dinitrophenyl antibody[50] (PDB entry, 1BAF), a human IgG[51] (PDB entry, 3FAB)] and one antibody [anti-myohemerythrin peptide homolog antibody[52] (PDB entry, 2IGF)] were found to have similar sequences with the V_H and V_L of 1C7, respectively. The model of the 1C7 V_H and V_L domains was generated using the crystal structures of these three antibodies. All

calculations were done on an IRIS workstation 4D/35TG (Silicon Graphics) running the QUANTA program (Polygen Corp.). CHARMm program[53] (Polygen Corp.) coupled to QUANTA was used for energy minimizations of substituted residues.

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17. The present method in which monoclonal antibodies were obtained from a synthetic medium rather than from mouse ascites minimized the contamination of the antibody sample with unknown hydrolases, thereby simplifying the purification method.

18. Evidence that the ester hydrolytic entity was an antibody, not a contaminating hydrolase, is summarized as follows: 1. An anti-calpain antibody, which was produced and purified by the same method, had no hydrolytic activity. 2. The catalytic antibodies from several batches of hybridoma culture exhibited the same catalytic activity. 3. The $F(ab')_2$ and Fab' fragments prepared from the catalytic antibody by pepsin digestion followed by purification with gel filtration exhibited the same catalytic activity as the parent antibody. 4. The esterolytic activity observed was not affected by the treatment of the antibody solution with diisopropyl fluorophosphate (DFP), a strong inhibitor of serine hydrolases.

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20. A reasonable degree of conversion was achieved for the hydrolysis of **2** (46 %), but all our attempts to measure the *e.e.* of the hydrolyzed product **3** with chiral chromatography on HPLC and GC were unsuccessful even after appropriate derivatization of **3** [methyl, *p*-bromophenacyl, *p*-nitrophenacyl, 4-(7-methoxycoumaryl) methyl, 9-anthrylmethyl esters, and (*S*)-1-(1-naphthyl)ethylamide]. None of the peaks of these derivatives was separated with chiral chromatography, and this is probably because the stereochemical difference of the substituents on the chiral center is rather small (CH_3 vs H).

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38. The *e.e.* of the starting materials for (*R*)-**7** or (*S*)-**7** were determined as follows. (*R*)- or (*S*)-methyl 3-hydroxybutyrate was converted to the corresponding diastereomeric MTPA esters. The *d.e.* of the MTPA ester was determined by ^{1}H NMR (δ 1.34 and 1.42 ppm, CH_3CH) or HPLC (hexane : EtOH = 25 : 1, flow rate 1 mL min^{-1}, t_R 47.2 and 56.1 min, 254 nm).

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