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Comparison of phosphonate transition state analogs for inducing catalytic antibodies and evaluation of key structural factors by an *ab initio* study

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Abstract: The relation between the structure of haptens and the esterolytic activities of antibodies was investigated. We synthesized two phenylalanine analogs, the negatively charged phosphonate derivative **1** and the neutral phosphonamidate derivative **2**. Seventeen out of 41 monoclonal antibodies generated against the hapten **1** hydrolyzed the relevant phenylalanine ester *R*-**12**. On the contrary, none of 27 monoclonal antibodies generated against the hapten **2** had catalytic activity. An *ab initio* study of the structural and electronic properties of the modeled haptens showed that the value of the negative electrostatic potential around the phosphonyl oxygen was an important factor affecting the induction of esterolytic antibodies.

Keywords: Antibodies; Phosphonic acids and derivatives; Transition states

Introduction

A technology has been established for producing catalytic antibodies, which can catalyze desired chemical transformations, through the use of natural immune systems. In this technology, the putative transition state analog (TSA), which has geometric and electronic properties similar to those of the expected transition state, is required for immunization. Since Lerner and Schultz succeeded in obtaining antibodies that catalyze acyl-transfer reactions,[1] phosphonate derivatives have been frequently used as haptens for eliciting esterolytic antibodies.[2] The phosphonates could be good approximations of the transition states because of their tetrahedral geometry and anionic character. It is believed that a negative charge distribution is necessary for haptens to elicit esterolytic antibodies.[3] However, not all of the haptens have a negative charge. Neutral secondary alcohols[4] as well as ethyl phosphonamidate derivatives,[5] which appear to have only tetrahedral geometry of the transition states, also can generate hydrolytic catalytic antibodies. An L-lysine derivative was also shown to elicit antibodies that had pyridoxal 5'-phosphate (PLP)-dependent

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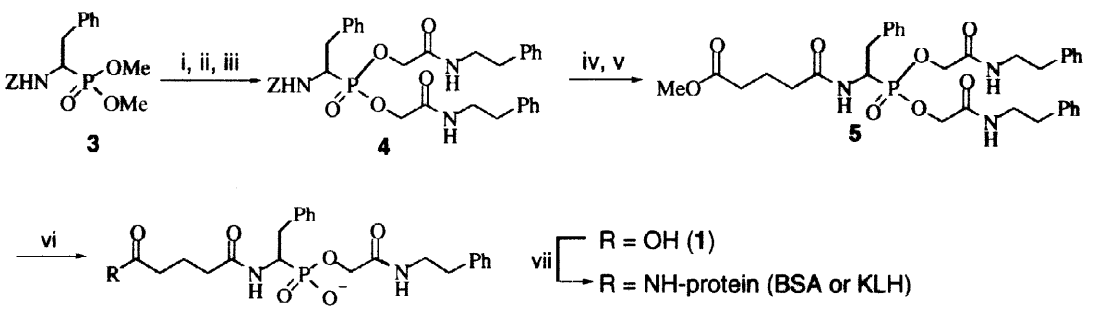
transaminase activity, although the reaction proceeds via the planar aldimine intermediate formed by PLP-lysine and a deprotonation step.[6] Sigmatropic rearrangement is most likely caused by significant orbital overlap. Thus, it is easy to understand how an antibody that catalyzes sigmatropic rearrangement is elicited by a TSA that has only geometrical similarity to the transition state.[7] However, some electrically neutral TSAs have elicited catalytic antibodies that are capable of hydrolysis. The discrepancy between the catalytic activities of antibodies and the structures of the TSAs remains to be resolved. Resolution of this question is important because development of catalytic antibodies is labor intensive and time consuming. The ability to predict the capabilities of a TSA by a computational study would considerably speed up research in this field. Thus, we synthesized the negatively charged phosphonate derivative **1** and the neutral phosphonamidate derivative **2** for phenylalanylglycine analogs. TSAs **1** and **2** were investigated by an *ab initio* study to determine their structural properties, and were used for immunization. Taking the properties of TSAs and the elicited antibodies into account, we discuss the factors that are required for haptens to elicit catalytic antibodies.

Results and discussion

Hapten synthesis

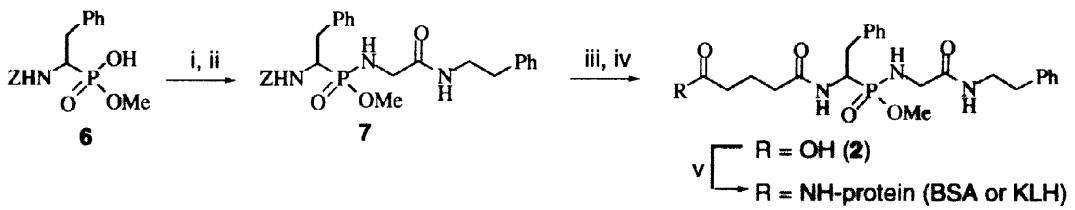
Dimethyl phosphonate **3** and methyl phosphonate **6** of a phenylalanine analog were prepared via the Arbuzov reaction.[8] After removal of two methylesters from compound **3**, chlorination of the phosphonate with $SOCl_2$ followed by addition of the alcohol led to the desired diester **4** in good yield. Compound **4** was hydrogenolized to give the free amine. Subsequent coupling with *mono*-methyl glutarate gave **5**. The phosphonate diester **5** was then hydrolyzed with LiOH to afford the hapten of phosphonate derivative **1** (Scheme 1).



Scheme 1. *Reagent and yields:* i, TMSBr; ii, $SOCl_2$; iii, $PhCH_2CH_2NHCOCH_2OH$ (81%); iv, 10% Pd-C / H_2; v, *mono*-methyl glutarate, EDC (74%); vi, LiOH (44%); vii, BSA or KLH, EDC.

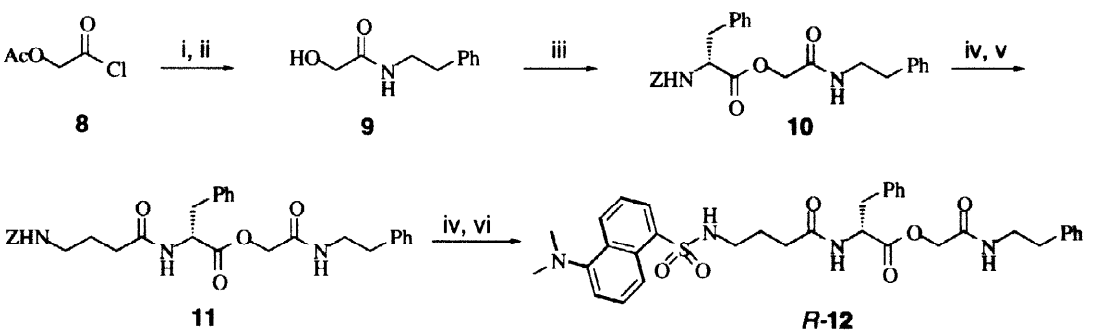
The phosphonamidate **7** was prepared by the coupling of methyl phosphonate **6**[9] with a glycine derivative via phosphonyl chloride. Removal of benzyl carbamate from **7** followed by introduction of glutaric

acid afforded methyl phosphonamidate derivative **2** (Scheme 2). These haptens were combined with carrier proteins, *i.e.* keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA).[10] The number of hapten molecules linked to each molecule of BSA was determined to be 10–20 by the trinitrophenylation method.[11] The haptens conjugated with KLH (KLH-**1**, **2**) were used for immunization and the BSA-haptens were used for an ELISA. In order to increase the sensitivity, the dansylated ester ***R*-12** was prepared for screening esterolytic activity (Scheme 3). Acylation of 2-phenylethylamine with compound **8** followed by deprotection of the *O*-acetyl group afforded the alcohol **9**. The compound **9** was condensed with Z-D-Phe to give **10**. Removal of the benzyl carbamate group and introduction of Z-γ-amino-*n*-butyric acid, and a dansyl group in the usual manner gave the desired substrate *R*-**12**. The *S*-enantiomer of ***R*-12** was also synthesized by the same means as described in Scheme 3.



6 **7**

Scheme 2. *Reagent and yields:* i, $SOCl_2$; ii, $PhCH_2CH_2NHCOCH_2NH{\cdot}HCl$ (75%); iii, 10% Pd-C / H_2; iv, glutaric anhydride, Et_3N (44%); v, BSA or KLH, EDC.



8 **9** **10** **11** ***R*-12**

Scheme 3. *Reagents and yields:* i, 2-phenylethylamine, Et_3N; ii, NaOMe (60%); iii, Z-D-Phe, EDC (85%); iv, 10% Pd-C / H_2; v, Z-γ-amino-*n*-butyric acid, EDC (55%); vi, dansylchloride (78%).

Antibody production and activity

Four MRL/*lpr* mice received a subcutaneous injection of KLH-**1** emulsified in complete Freund's adjuvant. In these experiments, two or three injections of KLH-haptens were tried in order to determine the difference in production of catalytic antibodies (Table 1). Three days after the last injection, the spleens were removed and the splenocytes were prepared and fused with X63.653 myeloma cells.[12] The hybridomas

having binding affinity to BSA-**1** were screened by an ELISA. These hybridomas were selected and cloned. Nineteen monoclonal antibodies were obtained from one experiment (Table 1, entry 1) and 22 monoclonal antibodies were obtained from the other (entry 2).

Table 1. Conditions used for immunizations and production of monoclonal antibodies

Entry	Hapten	Mouse	Immunization Injection; Frequency[a]		Number of monoclonal antibodies Binding for hapten;	Catalytic
1	**1**	MRL/*lpr*	s.c.[b]	2[d]	19	11
2	**1**	MRL/*lpr*	s.c.[b]	3[d]	22	6
3	**2**	MRL/*lpr*	s.c.[b]	3[d]	1	0
4	**2**	MRL/*lpr*	s.c.[c]	2[e]	7	0
5	**2**	MRL/*lpr*	s.c.[c]	3[e]	1	0
6	**2**	Balb/c	s.c.[c]	2[e]	3	0
7	**2**	Balb/c	s.c.[c]	2[e]	3	0
8	**2**	MRL/*lpr*	foot-pad[b]	2[d]	5	0
9	**2**	MRL/*lpr*	foot-pad[b]	3[d]	1	0

[a]Number of injection times of KLH-hapten conjugate. [b]Initial injections were given with complete Freund's adjuvant. Second injections were given with incomplete Freund's adjuvant. [c]Injections were given with Gerbu adjuvant. [d]Spleen cells were fused with myeloma of X63.653 after the last injection. [e]Spleen cells were fused with myeloma of PAI after the last injection.

After purification of antibodies from the hybridoma supernatants via column chromatography, 17 out of 41 antibodies showed hydrolytic activities toward *R*-**12**. These activities were strongly inhibited by adding an amount of the hapten **1** equivalent to that of the antibody. All of the antibodies had no hydrolytic activity toward *S*-**12**. Although the racemate **1** was used for all immunizations, the enantioselectivities of the antibodies for *R*-**12** were consistent with the result of a previous study.[13] The kinetic constants of the 17 catalytic antibodies were measured by fitting the data to the Michaelis-Menten equation. The rate accelerations (k_{cat}/k_{un}) of the 17 catalytic antibodies were found to be moderate values (10^2–10^3) at pH 7.0. The kinetic data of several antibodies are shown in Table 2. Antibodies with various binding affinities for the hapten **1** were obtained. The most effective antibody, MS 6-164, had a k_{cat} of 0.43 min^{-1} and a K_m of 2.8 mM, and a rate acceleration of 4300. The antibody MS 6-164 exhibited 13 turnovers per hour, and no product inhibition even when more than 70% of the *R*-**12** was consumed.

Table 2. Kinetic constants of hydrolysis of *R*-**12**[a] and the dissociation constants of the hapten **1**

Antibody	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($min^{-1}\mu M^{-1}$)	k_{cat}/k_{un}[b]	K_d (nM)
MS 6-164	0.43	2.8	0.15	4300	0.61
MS 6-191	0.28	5.4	0.052	2800	1.11
MS 5-393	0.062	6.7	0.0093	620	4.5
MS 5-298	0.062	2.5	0.025	620	600
MS 5-252	0.034	5.7	0.0060	340	0.49

[a]Reaction conditions: 100 mM MES, 0.01% NaN_3, pH 7.0, 5% DMSO, 30°C. [b]The first-order kinetic constant of the background reaction (k_{un}) was 1.0×10^{-4} min^{-1}.

Immunization with KLH-**2** was carried out as described above (Table 1, entry 3). The experiments raised only one monoclonal antibody that is capable of binding to BSA-**2**, raising the possibility that the hapten **2** may have poor immunogenicity. Additionally, we used Gerbu adjuvant with KLH-**2**, and spleen cells were harvested to fuse with PAI myeloma cells (entries 4, 5). The experiments were repeated with Balb/c mice (entries 6,7). Further, the foot-pad injection method was tried (entries 8, 9),[2e] because it could shorten the period for immunization. As a result, a total of 27 monoclonal antibodies were obtained from the hapten **2**, although none of the monoclonal antibodies could hydrolyze *R*-**12**. Taken together, these results show that the hapten **1** elicited esterolytic antibodies much more readily than did the hapten **2**.

Binding constants of antibodies

The dissociation constants (K_d) of all obtained antibodies for the respective haptens were determined by a competitive ELISA.[14] The K_ds for **2** of the antibodies generated from **2** were found to be in the range $5.4 \times 10^{-8} - 6.5 \times 10^{-6}$ M , which were comparable to the K_ds for **1** ($0.5 \times 10^{-9} - 1.5 \times 10^{-5}$ M) of the antibodies generated from **1**. The lack of catalytic antibody generated from **2** was not due to poor binding. Thus, activity from an antibody repertoire clearly depends on the structure of the haptens.

In addition, we measured the K_ds for the modeled hapten **13** (Fig. 1) which mimicked only the phosphonate moiety of **1**. The K_ds for **13** of 41 antibodies were in the range $0.3 \times 10^{-3} - 19 \times 10^{-3}$ M. The K_ds for the compound **13** with each antibody correlated well to that for the hapten **1** ($r = 0.904$),[15] indicating that the compound **13** is appropriate for a model of the hapten **1** for an *ab initio* study of its structure. It is easier to do the computational study with the modeled hapten **13**.

Stereoelectronic properties of haptens

N-Methyl phosphonamidate **14**, as well as **13**, was used for a model of the hapten **2**. Major conformers of **13** and **14** were optimized by the HF/6-31G* basis set of Gaussian 94 (Rev.D.4).[16] The electrostatic interaction between haptens and antibodies was expected to be an important factor affecting the induction of catalytic antibodies. Knowledge of electrostatic potential can be a valuable tool for predicting the reactivity of molecules in biological interactions. The region where the lone-pair electrons on nitrogen or oxygen are

localized can be evaluated from the negative electrostatic potential surface (EPS). Regions with large negative EPS values have high electron densities. It is energetically favorable for the lone-pair electrons to be localized in these regions. The global minima of **13** and **14** are shown in Fig. 1. The figure also shows that the region of the negative EPS of –157~ –120 kcal/mol around the phosphonyl oxygens in **13** (Fig. 1, left panel) and the region of the negative EPS of –63~ –20 kcal/mol in **14** (right panel). We found that the bond lengths and the angles of optimized conformers of **13** and **14** were similar, but the torsion angles and the values of the negative EPS around the phosphonyl oxygens were different (Table 3, Fig. 1). Model **13** had a *–gauche* conformation in which two lone-pairs of the methoxy-oxygen were located *synclinal* to the P-C bond. The conformations around the methoxy moiety in all stable conformers of **13** were identical to the global minimum as shown in Fig. 1. On the contrary, model **14** had a *+gauche* conformation in which the *N*-methyl group exists between the phosphonyl oxygen and the phenylethyl group. Consequently, the lone-pair on the nitrogen was located *antiperiplanar* to the P-C bond. The total population of the conformers having the antiperiplanar conformation was over 90%. The difference in conformation between **13** and **14** is probably caused by an electronic repulsion. That is the large electronic repulsion between the phosphonyl oxygens and the methoxy oxygen in **13**; by the weak electronic repulsion between the phosphonyl oxygens and the nitrogen in **14**.

Table 3. Geometrical and electronic parameters for major conformers of model compounds **13** and **14**.

distance (Å)	**13**	**14**	torsion angle (deg)	**13**	**14**
P-O^1	1.485	1.464	C^4-O^3(N^3)-P-C^5	176.9	114.7
P-O^2	1.480	1.595	O^3(N^3)-P-O^1-O^2	–127.5	–120.9
P-O^3 or N^3	1.643	1.651	O^1-P-O^3(N^3)-C^4	65.4	–16.7
P-C^5	1.857	1.834	O^2-P-O^3(N^3)-C^4	–69.2	–142.7
C^4-O^3 or N^3	1.397	1.456	O^1-P-C^5-N^6	19.4	74.9
angle (deg)	**13**	**14**	O^2-P-C^5-N^6	154.1	–162.0
O^1-P-O^2	121.8	114.9	O^3(N^3)-P-C^5-N^6	–92.8	–52.7
O^1-P-O^3 or N^3	108.5	112.2			
O^2-P-O^3 or N^3	108.9	105.9			
O^1-P-C^5	107.8	117.6			
O^2-P-C^5	109.8	105.9			
P-O^3(N^3)-C^4	117.4	123.0			

The numbers on the elements correspond to the structures shown in Fig. 1.

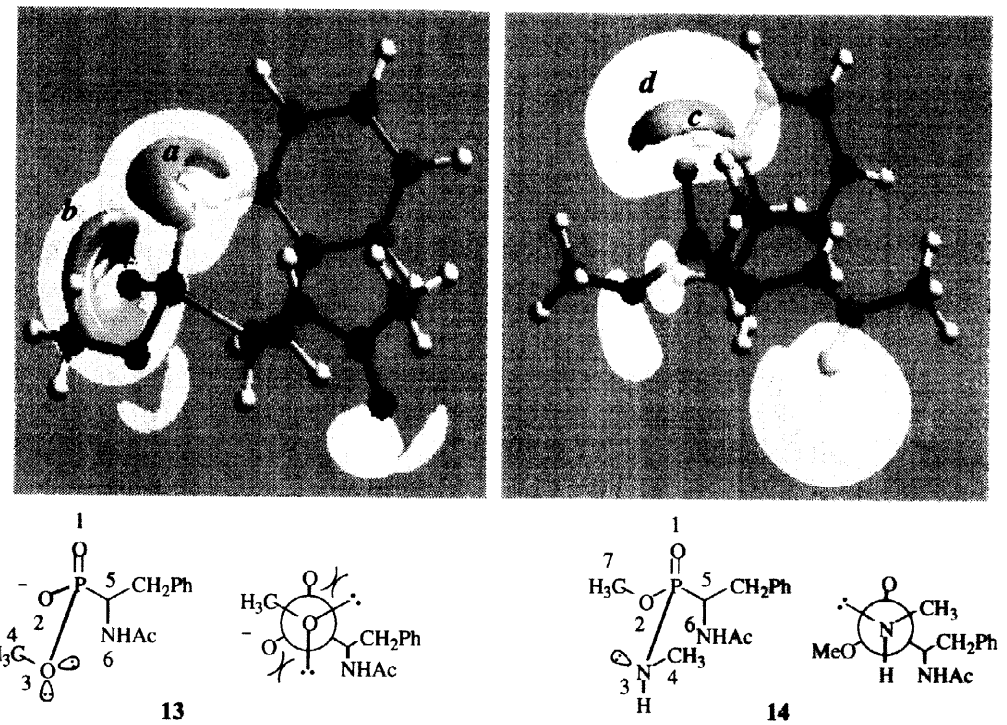


Fig. 1. Structure of modeled haptens **13** [left panel] and **14** [right panel] optimized by an *ab initio* study with the HF/6-31G* basis set. Gray area (labeled *a*) and white area (labeled *b*) in **13** indicate negative electronic potential surfaces of −157 and −120 kcal/mol, respectively. Gray area (labeled *c*) and white area (labeled *d*) in **14** indicate negative electronic potential surfaces of −63 and −20 kcal/mol , respectively.

Evaluation of catalytic activities based on the stereoelectronic properties of haptens

Our results suggest that the ability to elicit catalytic antibodies by TSAs depends on the torsion angle around the phosphorus and/or the value of the negative EPS around the phosphonyl oxygens. We discuss key factors determining whether the haptens can elicit catalytic antibodies from a mechanical perspective. Teraishi *et al.* conducted an *ab initio* study of the difference in the mechanism for hydrolysis of methyl acetate and methyl acetamide. From a comparison of the energy barrier heights toward the reactant and the product from each anionic tetrahedral intermediate, the authors suggested that it was easier for an ester to go toward the product from the intermediate, but easier for an amide to go back to the reactant. If the leaving amino group is protonated, in which case the intermediate is zwitterion, the potential barrier for the cleavage of the C-N bond is reduced.[17] This supports the importance of the protonation of the leaving nitrogen for the amide hydrolysis to proceed. Daggett *et al.* found that the rotation about C-N bonds was also important. The rotation gives the lone-pair on nitrogen the appropriate orientation to abstract a proton.[18] According to our results, this conformation has an advantage in that the leaving group can easily receive a proton from the nucleophile. This conformation is like that of **14**, in which the lone-pair on the nitrogen is located *synclinal* to

the incoming OMe group (Fig. 1). This conformation is also similar to that of the substrate-enzyme intermediate of amide cleavage in the serine protease model.[18] Based on the above reasoning, the hapten **2** was expected to elicit catalytic antibodies. However, only the phosphonate **1** elicited catalytic antibodies. This suggests that in our case, the negative electrostatic potential around the phosphonyl oxygens is more important than the geometry and the orientation of the lone-pair on the leaving group. In the process of hydrolysis, the first step should be an attack on the carbonyl carbon by a water molecule, and then the resulting charged intermediate would be stabilized by the amino acids residues in an antibody. The effect of the stabilization could be make up an oxyanion hole in an active site. When the induced antigen-combining site electronically complements the hapten, a hapten possessing a large negative EPS (–157~–120 kcal/mol) on the phosphonyl-oxygens would be more effective than a hapten with a small negative EPS (–63~–20 kcal/mol) in eliciting an appropriate oxyanion hole in the active site of the catalytic antibodies. A compound possessing both a large negative EPS and the appropriate orientation of the lone-pair on the leaving group may be a good hapten for generating antibodies that can hydrolyze amides. While we were able to synthesize the negatively charged phosphonamide derivative of **2**, it was not stable enough for immunization ($t_{1/2}$= *c.a.* 5h at pH 7.0). In the case of neutral TSAs, it seems that hydrolytic antibodies result from the enormous diversity of immune systems by chance. The successful examples are most likely caused by effective selection systems for the catalytic antibodies, such as a combination of an ELISA and a competition ELISA.[5,6]

In conclusion, we were able to determine the geometrical and electronic properties of two phosphonate haptens by an *ab initio* study and also to evaluate the relationship between these properties and the catalytic activities of antibodies. The negative electrostatic potential surface (EPS) around the phosphonyl oxygens is more important than the geometry and the orientation of the lone-pair on the leaving group with respect to eliciting esterolytic antibodies. Recently, both the mechanism and the transition state of some proteases have been determined using advanced computational chemistry techniques.[19] Theoretical calculations would enable us to evaluate the capability of TSAs, or to design novel TSAs, to elicit catalytic antibodies with high rates of acceleration.

Experimental

Melting points were measured with a Yanagimoto melting point aperture and were uncorrected. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. ^{1}H NMR spectra were recorded with Bruker AC-300P for solutions in $CDCl_3$ or MeOH-d_4 with Me_4Si as an internal standard. Chemical shifts of ^{31}P NMR were refereed to H_3PO_4 as an external standard. Flush column chromatography was performed with silica gel 60 (Merck). Preparative HPLC was performed with a YMC packed column (ODS, S-5, 120-A, 20 x 2 cm). FAB mass spectra were obtained on a Kratos Mass Spectrometer CONCEPT IIH using 800V of electron acceleration. All computations were performed on an Alpha Server (Digital Equipment, Corp.), and the modeled haptens were calculated by the HF/6-31G* basis set of Gaussian 94.[16]

O,O'-*Bis*[N-*(2-phenylethyl)-acetamideyl] 1-benzyloxycarbonylamino-2-phenylethyl phosphonate* 4: To a solution of dimethyl 1-benzyloxycarbonylamino-2-phenylethyl phosphonate (3) (300 mg, 0.826 mmol) in CH_2Cl_2 (3.0 cm^3) was added bromotrimethylsilane (381 mg, 2.49 mmol) under an Ar atmosphere. The mixture was stirred at 35 °C for 5 h, and then evaporated to give a yellow oil. To a solution of the oil in CH_2Cl_2 (15 cm^3) was added thionyl chloride (0.602 cm^3, 8.25 mmol) under an Ar atmosphere at 25 °C. The mixture was stirred for 1 h, and then carefully evaporated to give an oil, which was co-evaporated with CH_2Cl_2 three times under an Ar atmosphere to afford a phosphonyl dichloride. To a solution of the phosphonyl dichloride in CH_2Cl_2 (3.1 cm^3) was dropped a solution of *N*-(2-hydroxyacetyl)-2-phenylethylamine (326 mg, 1.82 mmol) and triethylamine (0.465 cm^3, 3.34 mmol) in CH_2Cl_2 (12.5 cm^3) over 3 min. After stirring for 0.5 h at 25 °C, the mixture was diluted with $CHCl_3$. The solution was washed with 1 M HCl and brine. The organic layer was dried ($MgSO_4$) and evaporated. The product was purified by flash chromatography ($CHCl_3$-MeOH, 100:1) to give **4** (440 mg, 81%) as a colorless oil [Found: (FAB) $M^+ + 1$, 658.2729. $C_{36}H_{41}N_3O_7P$ requires $M + 1$, 658.2682; Found: C, 65.45; H, 6.07; N, 6.34. $C_{36}H_{40}N_3O_7P$ requires C, 65.74; H, 6.13; N, 6.39%]; ^1H NMR ($CDCl_3$) 2.78–2.84 (4 H, m), 2.86–2.98 (1 H, m), 3.15 (1 H, ddd, *J* 5.1, 9.3, 14.2), 3.43–3.53 (4 H, m), 4.31 (2 H, dd, *J* 7.5, 14.7), 4.36–4.45 (1 H, m), 4.50 (2 H, dd, *J* 8.5, 14.7), 4.95 (1 H, J_{AB} 12.4), 5.04 (1 H, J_{AB} 12.4), 5.89 (1 H, d, *J* 9.5, NH), 6.58 (1 H, t, *J* 5.6, NH), 6.81 (1 H, t, *J* 5.3, NH) and 7.16–7.32 (20 H, m, Ph); ^{31}P NMR ($CDCl_3$) 25.5.

O,O'-*Bis*[N-*(2-phenylethyl)-acetamideyl] 1-[(4-methoxycarbonyl)butanoyl]amino-2-phenylethyl phosphonate* 5: The suspension of **4** (178 mg, 0.271 mmol) and 10% palladium-on-charcoal (150 mg) in MeOH (8.0 cm^3) was stirred under 1 atm of hydrogen. After 0.5 h, the suspension was filtered to remove the insoluble material and the filtrate was evaporated. To the residue in CH_2Cl_2 (2.0 cm^3) were added *mono*-methyl glutarate (0.068 cm^3, 0.54 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (119 mg, 0.620 mmol). The mixture was stirred for 3.5 h and diluted with $CHCl_3$. The solution was washed with 1 M HCl and aq $NaHCO_3$. The organic layer was dried ($MgSO_4$) and evaporated. The product was purified by flash chromatography ($CHCl_3$-MeOH, 30:1) to give **5** (131 mg, 74%) as a colorless oil [Found: (FAB) $M^+ + 1$, 652.2814. $C_{34}H_{43}N_3O_8P$ requires $M + 1$, 652.2789; Found: C, 62.54; H, 6.45; N, 6.36. $C_{34}H_{42}N_3O_8P$ requires C, 62.66; H, 6.50; N, 6.45%]; ^1H NMR ($CDCl_3$) 1.74–1.82 (2 H, m), 2.04–2.19 (4 H, m), 2.85 (4 H, td, *J* 1.9, 7.3), 3.18 (1 H, ddd, *J* 5.1, 7.6, 14.4), 3.49–3.57 (4 H, m), 3.61 (3 H, s, OMe), 4.35 (1 H, dd, *J* 7.8, 14.8), 4.40 (1 H, dd, *J* 7.9, 14.8), 4.50 (1 H, dd, *J* 8.5, 14.8), 4.55 (1 H, dd, *J* 8.5, 14.8), 4.71–4.83 (1 H, m), 6.73 (1 H, t, *J* 5.6, NH), 6.93 (1 H, t, *J* 5.7, NH), 7.04 (1 H, d, *J* 9.3, NH) and 7.17–7.33 (15 H, m, Ph); ^{31}P NMR ($CDCl_3$) 25.5.

N-*[[2-[[[1-(4-carboxybutanoyl)amino]-2-phenylethyl]-hydroxyphosphinyl]oxy]-acetyl]-2-phenylethylamine* 1: A solution of LiOH (1 M, 0.85 cm^3) was added to a solution of **5** (111 mg, 0.170 mmol) in MeCN (3.0 cm^3) and the mixture was stirred for 1 h at 40 °C. After cooling to 25 °C, the MeCN was evaporated. The solution was diluted with MeOH (10 cm^3) and adjusted to pH 6.0 with 6 M HCl, and the resulting mixture was purified by preparative HPLC (MeCN-0.1% TFA, 4:6; 2.5 mL/min). The MeCN and TFA were evaporated, and the water was removed by lyophilization to give **1** (36 mg, 44%) as a white

powder [Found: (FAB) M^+ + Na, 499.1614. $C_{23}H_{29}N_2NaO_7P$ requires *M* + Na, 499.1610]; ^1H NMR (D_2O) 1.47–1.62 (2 H, m), 1.73–1.89 (2 H, m), 2.02–2.16 (2 H, m), 2.77 (1 H, ddd, *J* 7.2, 13.3, 14.1), 2.89 (2 H, t, *J* 7.0), 3.24 (1 H, td, *J* 3.5, 14.1), 3.47–3.62 (2 H, m), 4.30 (1 H, dd, *J* 6.1, 15.4), 4.36 (1 H, dd, *J* 6.2, 17.3) and 7.24–7.45 (10 H, m, Ph); ^{31}P NMR (D_2O) 20.0.

***N*-[[1-[(Benzyloxycarbonyl)amino]-2-phenylethyl]-methoxyphosphinyl]-glycyl-2-phenylethylamine 7:** To a solution of methyl hydrogen 1-benzyloxycarbonylamino-2-phenylethyl phosphonate (**6**)[9] (1.0 g, 2.9 mmol) in CH_2Cl_2 (20 cm^3) was added thionyl chloride (0.840 cm^3, 11.5 mmol) under an Ar atmosphere. The solution was stirred for 2 h at 25 °C and then carefully evaporated. The resulting oil was redissolved in CH_2Cl_2 and the solution was evaporated again to remove volatile compounds under an Ar atmosphere. To a solution of the resulting phosphonyl chloride in CH_2Cl_2 (5.0 cm^3) was added a solution of glycyl-2-phenylethylamine hydrochloride (830 mg, 3.88 mmol) and triethylamine (1.0 cm^3, 7.2 mmol) in CH_2Cl_2 (20 cm^3) at 0 °C. The mixture was warmed to 25 °C and stirred for 1 h and then diluted with CH_2Cl_2. The solution was washed with 1 M H_2SO_4, aq $NaHCO_3$ and brine, successively. The organic layer was dried ($MgSO_4$) and evaporated. The product was purified by flash chromatography ($CHCl_3$-MeOH, 20:1) to give diastereomers of **7** (1.07 g, 75%) as a white powder [Found: (FAB) M^+ + Na, 532.1973. $C_{27}H_{32}N_3NaO_5P$ requires *M* + Na, 532.1977; Found: C, 63.67; H, 6.31; N, 8.25. $C_{27}H_{32}N_3O_5P$ requires C, 63.64; H, 6.33; N, 8.25%]; One of the isomer; ^1H NMR ($CDCl_3$) 2.78 (2 H, t, *J* 7.0), 2.80–2.89 (1 H, m), 3.17 (1 H, td, *J* 4.6, 9.5), 3.40–3.74 (4 H, m), 3.59 (3 H, d, *J* 10.9, OMe), 4.20–4.32 (1 H, m), 4.89 (1 H, d, J_{AB} 12.4), 4.99 (1 H, d, J_{AB} 12.4), 6.02 (1 H, d, *J* 9.8, NH), 6.55 (1 H, brs, NH) and 7.12–7.30 (15 H, m, Ph); ^{31}P NMR ($CDCl_3$) 31.3. The other isomer; ^1H NMR ($CDCl_3$) 2.77–2.83 (2 H, m), 3.20–3.37 (2 H, m), 3.43–3.73 (4 H, m), 3.59 (3 H, d, *J* 10.8, OMe), 4.24–4.35 (1 H, m), 4.93 (1 H, d, J_{AB} 12.3), 5.00 (1 H, d, J_{AB} 12.3), 5.30 (1 H, brd, *J* 9.1), 6.60 (1 H, brs, NH) and 7.16–7.30 (15 H, m, Ph); ^{31}P NMR ($CDCl_3$) 29.8.

***N*-[[1-[(4-carboxybutanoyl)amino]-2-phenylethyl]-methoxyphosphinyl]-glycyl-2-phenylethylamine 2:** The diastereomers of **7** (375 mg, 0.736 mmol) and 10% palladium-on-charcoal (370 mg) in MeOH (16 cm^3) was stirred under 1 atm of hydrogen. After 15 min, the mixture was filtered and the filtrate was evaporated to give an amine. To a solution of the amine in CH_2Cl_2 (8 cm^3) were added triethylamine (0.19 cm^3, 1.33 mmol) and glutaric anhydride (152 mg, 1.33 mmol). The reaction mixture was stirred for 4 days and then diluted with $CHCl_3$. The solution was washed with 1 M HCl, brine and dried ($MgSO_4$). After evaporation, the products were purified by flash chromatography ($CHCl_3$-MeOH, gradient from 5:1 to 1:1) to afford the major product of hapten **2** (102 mg, 28%) and the minor product (59 mg, 16%) as a white powder; Major isomer [Found: (FAB) M^+ + 1, 490.2145. $C_{24}H_{33}N_3O_6P$ requires *M* + 1, 490.2107]; ^1H NMR (MeOH-d_4) 1.62–1.72 (2 H, m), 2.04 (2 H, t, *J* 7.4), 2.10–2.14 (2 H, m), 2.78 (1 H, ddd, *J* 7.8, 12.4, 14.2), 2.82 (2 H, t, *J* 7.3), 3.18 (1 H, ddd, *J* 3.7, 4.0, 14.2), 3.45–3.50 (2 H, m), 3.60 (2 H, d, *J* 10.7), 3.68 (3 H, d, *J* 11.0, OMe), 4.58 (1 H, ddd, *J* 3.6, 12.4, 14.4) and 7.14–7.30 (10 H, m, Ph); ^{31}P NMR (MeOH-d_4) 30.7; Minor isomer [Found: (FAB) M^+ + 1, 490.2156. $C_{24}H_{33}N_3O_6P$ requires *M* + 1, 490.2107; Found: C, 58.82; H, 6.45; N, 8.57. $C_{24}H_{32}N_3O_6P$ requires C, 58.89; H, 6.59; N, 8.58%]; ^1H NMR (MeOH-d_4) 1.63–1.72 (2 H, m), 2.04 (2 H, t, *J* 7.4), 2.10–2.16 (2 H, m), 2.77 (1 H, ddd, *J* 6.9, 12.4, 14.1), 2.84 (2 H, t, *J* 7.4), 3.22 (1 H, ddd, *J* 3.5, 4.3,

14.1), 3.44–3.50 (2 H, m), 3.56 (1 H, dd, *J* 11.1, 17.4), 3.70 (3 H, d, *J* 11.1, OMe), 3.75 (1 H, dd, *J* 11.1, 17.3), 4.48 (1H, ddd, *J* 3.5, 12.4, 14.2) and 7.13–7.32 (10 H, m, Ph); ^{31}P NMR (MeOH-d_4) 29.7.

***Preparation of KLH(BSA)-1*:** A solution of the hapten **1** (2.0 mg, 4.2 mmol) in 0.1 M MES, 0.9 M NaCl, pH 4.7 (0.50 cm^3) and a solution of 1% EDC (0.24 cm^3) were added to a solution of 2 mg of KLH (BSA) in water (0.20 cm^3) successively. The mixture was left for 2 h at 25 °C and the conjugate was purified by gel filtration (Sephadex G-25, PBS buffer) to give KLH(BSA)-**1**. The concentration of conjugates were determined by the bicinchoninic acid method (PIERCE).[20]

***Preparation of KLH(BSA)-2*:** The mixture of diastereomers of the hapten **2** (2.0 mg, 4.1 mmol) in DMF (0.15 cm^3) was diluted with 0.1 M MES, 0.9 M NaCl, pH 4.7 (0.33 cm^3). The resulting solution and a solution of 1% EDC (0.07 cm^3) were added to a solution of 2 mg of KLH (BSA) in water (0.20 cm^3) successively. The mixture was left for 2 h at 25 °C and the conjugate was purified by gel filtration (Sephadex G-25, PBS buffer) to give KLH(BSA)-**2**. The concentrations of the conjugates were determined by the same manner as described above.

***Hydroxyacetyl-2-phenylethylamine* 9:** Acetoxyacetyl chloride (3.45 g, 25.3 mmol) was added to a solution of 2-phenylethylamine (3.07 g, 25.3 mmol) and triethylamine (4.0 cm^3, 28.7 mmol) in CH_2Cl_2 (30 cm^3) at 0 °C under an Ar atmosphere. The mixture was stirred for 15 min, and then poured into water. The mixture was extracted with $CHCl_3$ (2 x 50 cm^3). The combined extracts were washed with brine, dried ($MgSO_4$) and then evaporated to give a yellow oil. To the solution of the oil in MeOH (80 cm^3) was added 28% NaOMe (1.52 cm^3). The mixture was stirred for 0.5 h, and the solution was evaporated to give a residue, which was dissolved in EtOAc (100 cm^3). The solution was washed with water and brine, dried ($MgSO_4$) and then evaporated. The product was crystallized from $CHCl_3$-hexane to give **9** (2.7 g, 60%) [Found: (FAB) M$^+$ + 1, 180.1040. $C_{10}H_{14}NO_2$ requires *M* + 1, 180.1025; Found: C, 66.86; H, 7.29; N, 7.85. $C_{10}H_{13}NO_2$ requires C, 67.02; H, 7.31; N, 7.82%]; mp 75 °C; ^{1}H NMR ($CDCl_3$) 2.83 (2 H, t, *J* 7.0), 3.55 (2 H, q, *J* 7.0), 4.03 (2 H, s), 6.17 (1 H, brs) and 7.19–7.34 (5 H, m, Ph); ^{13}C NMR ($CDCl_3$) 35.6, 40.0, 61.9, 126.5, 128.6, 138.4 and 172.3.

***[[(N-Benzyloxycarbonyl-D-phenylalanyl)oxy]-acetyl]-2-phenylethylamine* 10:** To a solution of *N*-benzyloxycarbonyl-D-phenylalanine (1.8 g, 6.0 mmol) and **9** (1.0 g, 5.6 mmol) in CH_2Cl_2 (55 cm^3) were added EDC (1.2 g, 6.3 mmol) and 4-dimethylaminopyridine (156 mg, 1.28 mmol). The mixture was stirred for 24 h at 25 °C, and quenched with 1 M HCl (15 cm^3). The mixture was extracted with $CHCl_3$ (3 x 50 cm^3), and the combined extracts were washed with aq $NaHCO_3$ and brine. The organic layer was dried ($MgSO_4$) and then evaporated. The product was purified by flash chromatography (hexane-EtOAc, 2:1) to give **10** (2.2 g, 85%) as a colorless oil [Found: (FAB) M$^+$ + 1, 461.2091. $C_{27}H_{29}N_2O_5$ requires *M* + 1, 461.2077; Found: C, 70.33; H, 6.18; N, 6.00. $C_{27}H_{28}N_2O_5$ requires C, 70.42; H, 6.13; N, 6.08%]; $[\alpha]_D^{24}$ −10 (*c* 1.7 in $CHCl_3$); ^{1}H NMR ($CDCl_3$) 2.79 (2 H, t, *J* 7.4), 3.07 (2 H, d, *J* 7.4), 3.40–3.46 (2 H, m), 4.40 (1 H, q, *J* 6.9), 4.51 (1 H, d, *J* 15.3), 4.61 (1 H, d, *J* 15.3), 5.06 (2 H, s), 5.12 (1 H, d, *J* 6.9), 6.42 (1 H, brs) and 7.11–7.36 (15 H, m); ^{13}C NMR ($CDCl_3$) 35.4, 37.6, 40.4, 55.4, 63.3, 67.3, 126.5, 127.4, 128.0, 128.4, 128.6, 128.7, 128.9, 135.2, 135.7, 138.7, 166.4 and 170.6.

***[[[N-(N-Benzyloxycarbonyl-4-aminobutanoyl)-D-phenylalanyl]oxy]-acetyl]-2-phenylethylamine* 11:** The suspension of **10** (1.00 g, 2.17 mmol) and 10% palladium-on-charcoal (0.90 g) in EtOAc (50 cm^3) was stirred under 3 atm of hydrogen. After 50 min, the mixture was filtered and the filtrate was evaporated to give a residue. To a solution of the residue in CH_2Cl_2 (21 cm^3) were added *N*-benzyloxycarbonyl-γ-amino-*n*-butyric acid (566 mg, 2.39 mmol) and EDC (497 mg, 2.59 mmol), successively. The mixture was stirred for 1.5 h, and diluted with $CHCl_3$. The solution was washed with 1 M HCl and aq $NaHCO_3$. The organic layer was dried ($MgSO_4$) and then evaporated. The product was crystallized from $CHCl_3$-hexane to give **11** (653 mg, 55%) [Found: (FAB) $M^+ + 1$, 546.2607. $C_{31}H_{36}N_3O_6$ requires $M + 1$, 546.2604; Found: C, 68.06; H, 6.47; N, 7.72. $C_{31}H_{35}N_3O_6$ requires C, 68.24; H, 6.47; N, 7.70%]; mp 134–135 °C; $[\alpha]_D^{24}$ –20 (*c* 1.0 in $CHCl_3$); ^1H NMR ($CDCl_3$) 1.69–1.78 (2 H, m), 2.16 (2 H, t, *J* 6.7), 2.79 (2 H, t, *J* 7.5), 3.02–3.22 (3 H, m), 3.35–3.55 (2 H, m), 4.39 (1 H, d, *J* 15.4), 4.57 (1 H, td, *J* 6.6), 4.60 (1 H, d, *J* 15.4), 5.07 (2 H, s), 5.29 (1 H, brt, *J* 5.9, NH), 6.91 (1 H, brt, *J* 5.3, NH) and 7.16–7.34 (10 H, m, Ph); ^{13}C NMR ($CDCl_3$) 26.4, 32.7, 35.3, 37.0, 39.6, 40.5, 54.6, 63.1, 66.8, 126.4, 127.3, 128.0, 128.2, 128.5, 128.7, 129.0, 135.7, 136.3, 138.8, 157.2, 166.7, 170.9 and 173.5.

***[[[[(5-Dimethylamino-1-naphthalenesulfonyl)-4-aminobutanoyl]-D-phenylalanyl]oxy]-acetyl]-2-phenylethylamine* 12:** The suspension of **11** (0.30 g, 0.55 mmol) and 10% palladium-on-charcoal (0.30 g) in EtOAc (50 cm^3) was stirred under 3 atm of hydrogen. After 40 min, the suspension was filtered to remove insoluble material and the filtrate was evaporated to give a residue. To a solution of the residue in CH_2Cl_2 (8.0 cm^3) were added 5-dimethylamino-1-naphthalenesulfonyl chloride (163 mg, 0.604 mmol) and triethylamine (0.085 cm^3, 0.61 mmol). The mixture was stirred for 40 min, and then evaporated. The product was purified by flash chromatography (hexane-EtOAc, 1:2) to give **12** (278 mg, 78%) as a green solid [Found: (FAB) $M^+ + 1$, 645.2754. $C_{35}H_{41}N_4O_6S$ requires $M + 1$, 645.2747; Found: C, 65.00; H, 6.04; N, 8.65. $C_{35}H_{40}N_4O_6S$ requires C, 65.20; H, 6.25; N, 8.69%]; $[\alpha]_D^{24}$ –9.2 (*c* 1.1 in $CHCl_3$); ^1H NMR ($CDCl_3$) 1.61–1.74 (2 H, m), 2.16–2.33 (2 H, m), 2.79 (2 H, t, *J* 7.4), 2.82–2.95 (2 H, m), 2.88 (6 H, s, Me), 2.92–3.13 (2 H, m), 3.37–3.48 (2 H, m), 4.42 (1 H, d, *J* 15.3), 4.59 (1 H, dq, *J* 5.6), 4.60 (1 H, d, *J* 15.3), 5.79 (1 H, t, *J* 6.2), 6.41 (1 H, d, *J* 6.4), 6.66 (1 H, brt, *J* 5.6), 7.12–7.32 (11 H, m), 7.48–7.57 (2 H, m), 8.18 (1 H, dd, *J* 1.2, 7.3), 8.25 (1 H, d, *J* 8.7) and 8.54 (1 H, d, *J* 8.5); ^{13}C NMR ($CDCl_3$) 25.3, 32.5, 35.3, 37.1, 40.5, 42.1, 45.3, 54.4, 63.1, 115.2, 118.4, 123.2, 126.3, 127.2, 128.4, 128.6, 128.7, 128.9, 129.5, 130.6, 131.8, 134.4, 135.7, 137.5, 152.0, 166.7, 170.9 and 173.4.

***Antibody production and purification*:** The types of mouse, adjuvant and injection used in antibody production are summarized in Table 1. Eight-week-old female Balb/c mice and MRL/MPJ-*lpr/lpr* mice were purchased from Immuno-Biological Laboratories and Charles River Laboratories, respectively. Four mice each received a subcutaneous (or foot-pad) injection of 50 μg of KLH-hapten mixed with adjuvant twice or three times at two-week intervals. Three days after the last injection, the spleens of the two mice that had the highest titers were excised and the cells were fused with myeloma cells. The hybridomas were incubated in medium containing HAT (hypoxanthine-aminopterin-thymidine) and 10% FBS in 96-well culture plates. After an appropriate period of growth, the plates were examined by ELISA for binding to the corresponding

BSA-hapten. The bound cells were selected and cloned. The monoclonal cells were incubated until cells reached confluence in TIL media, +10% FCS (Immuno-Biological Labo.). The supernatants were treated with ammonium sulfate at a final concentration of 60%, and the precipitates were dissolved and dialyzed against PBS at 4°C. Antibodies (IgG) were purified by a Hitrap protein G-Sepharose column (Pharmacia), followed by High Q anion-exchange chromatography (Bio-Rad) with a salt gradient from 0 to 1 M NaCl in 10 mM Tris-HCl, pH 7.5. The fractions containing antibody were concentrated by a Centriprep Concentrator (Amicon), and the solution was changed to the appropriate buffer for the kinetic assay. After purification, the samples were subjected to SDS-PAGE. No bands other than IgG were observed. The concentration of antibody was determined by absorbance at 280 nm with ε=1.4 and M.W.=150,000 for IgG.

***Catalytic assay and kinetic measurements*:** Initial screening for esterolytic activity was performed by antibody (20 μM, active site) in 25 mM Tris-HCl, 0.01% NaN_3, pH 7.0, with 5% DMSO, containing 0.20 mM ester ***R*-12** at 30 °C. Hydrolysis rates were measured by HPLC detection of the cleaved product by fluorescence (ex=340 nm, em=540 nm). The analysis was performed on a Shimadzu 10A VP system (YMC ODS column of 250 x 4.6 mm; MeCN-0.1% TFA, 6:4; flow rate of 1.0 mL/min). Retention times of ***R*-12** and the product were 3.6 and 6.5 min, respectively.

The kinetic parameters were determined by adding 5 μL of a stock solution of ***R*-12** in DMSO to 95 μL of antibody solution (1.05 μM) in 100 mM MES, 0.01% NaN_3, pH 7.0. The initial velocities were measured during the first 10% of hydrolysis. The k_{cat} and K_m were determined by a fitting of the Michaelis-Menten equation, using KaleidaGraph software (Synergy Software). All assays were performed at least in duplicate. The background hydrolysis (k_{un}) was determined by initial rates analysis and extrapolated to zero-buffer concentration.

***Measurement of dissociation constants*:** Dissociation constants (K_ds) were measured for the hapten **1** (**2**) and the modeled hapten **13** using a competitive ELISA for binding to the BSA-**1** (**2**). The final inhibitor concentrations were in the range 0.2 nM to 100 mM of the hapten **1** (**2**), and 0.1 mM to 500 mM of the modeled hapten **13**. K_ds were calculated by Scatchard plots.[14]

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References

1 (a) Pollack, S. J.; Jacobs, J. W.; Schultz, P. G. *Science* **1986**, 234, 1570. (b) Tramontano, A.; Janda, K. D.; Lerner, R. A. *Science* **1986**, 234, 1566.

2 (a) Thomas, N. R. *Appl. Biochem. Biotech.* **1994**, 47, 345. (b) Lerner, R. A.; Benkovic, S. J.; Schultz,

P. G. *Science* **1991**, 252, 659. (c) Iwabuchi, Y.; Miyashita, H.; Tanimura, R.; Kinoshita, K.; Kikuchi, M.; Fujii, I. *J. Am. Chem. Soc.* **1994**, 116, 771. (d) Fujii, I.; Tanaka, F.; Miyashita, H.; Tanimura, R.; Kinoshita, K. *J. Am. Chem. Soc.* **1995**, 117, 6199. (e) Tawfik, D. S.; Chap, R.; Green, B. S.; Sela, M.; Eshhar, Z. *Proc. Natl. Acad. Sci. USA* **1995**, 92, 2145. (f) Tanaka, F.; Kinoshita, K.; Tanimura, R.; Fujii, I. *J. Am. Chem. Soc.* **1996**, 118, 2332.

3 (a) Janda, K. D.; Weinhouse, M. I.; Danon, T.; Pacelli, K. A.; Schloeder, D. M. *J. Am. Chem. Soc.* **1991**, 113, 5427.(b) Suga, H.; Ersoy, O.; Tsumuraya, T.; Lee, J.; Sinskey, A. J.; Masamune, S. *J. Am. Chem. Soc.* **1994**, 116, 487.

4 (a) Shokat, K. M.; Ko, M. K.; Scanlan, T. S.; Kochersperger, L.; Yonkovich, S.; Thaisrivongs, S.; Schultz, P. G. *Angew. Chem. Int. Ed. Engl.* **1990**, 29, 1296. (b) Shen, R.; Priebe, C.; Patel, C.; Rubo, L.; Su, T.; Kahn, M. *Tetrahedron Lett.* **1992**, 33, 3417.

5 (a) Jacobsen, J. R.; Prudent, J. R.; Kochersperger, L.; Yonkovich, S.; Schultz, P. G. *Science* **1992**, 256, 365. (b) Wentworth, P.; Datta, A.; Blakey, D.; Boyle, T.; Partridge, L. J.; Blackburn, G. M. *Proc. Natl. Acad. Sci. USA* **1996**, 93, 799.

6 Gramatikova, S. I.; Christen, P. *J. Biol. Chem.* **1996**, 271, 30583.

7 (a) Jackson, D. Y.; Jacobs, J. W.; Sugasawara, R.; Reich, S. H.; Bartlett, P. A.; Schultz, P. G. *J. Am. Chem. Soc.* **1988**, 110, 4841. (b) Hilvert, D.; Carpenter, S. H.; Nared, K. D.; Auditor, M.-T. M. *Proc. Natl. Acad. Sci. USA* **1988**, 85, 4953. (c) Driggers, E. M.; Cho, H. S.; Liu, C. W.; Katzka, C. P.; Braisted, A. C.; Ulrich, H. D.; Wemmer, D. E.; Schultz, P. G. *J. Am. Chem. Soc.* **1998**, 120, 1945.

8 (a) Dive, V.; Yiotakis, A.; Nicolaou, A.; Toma, F. *Eur. J. Biochem.* **1990**, 191, 685. (b) Ger. Offen. DE 4036130/**1991** (*Chem. Abstr.* **1991**, 115, 232889t).

9 Bartlett, P. A.; Lamden, L. A. *Bioorg. Chem.* **1986**, 14, 356.

10 Bauminger, S.; Wilchek, M. in *Methods in Enzymology*, eds. Vunakis, H. V.; Langone, J. J. Academic Press, New York. **1980**, vol. 70, p. 151.

11 Anfinsen, C. B.; Sela, M.; Cooke, J. P. *J. Biol. Chem.* **1962**, 237, 1825.

12 Köhler, G.; Milstein, C. *Nature* **1975**, 256, 495.

13 Pollack, S. J.; Hsiun, P.; Schultz, P. G. *J. Am. Chem. Soc.* **1989**, 111, 5961.

14 Friguet, B.; Chaffotte, A. F.; Djavadi-Ohaniance, L.; Goldberg, M. E. *J. Immunol. Methods* **1985**, 77, 305.

15 Takahashi, N.; Kakinuma, H.; Hamada, K.; Shimazaki, K.; Takahashi, K.; Niihata, S.; Aoki, Y.; Matsushita, H.; Nishi, Y. *Eur. J. Biochem. in press.*

16 Frisch, M. j.; Trucks, G. W.; Schlegel, H. B.; Gill, P. M. W.; Johnson, B. G.; Robb, M. A.; Cheeseman, J. R.; Keith, T.; Petersson, G. A.; Montgomery, J. A.; Raghavachari, K.; Al-Laham, M. A.; Zakrzewski, V. G.; Ortiz, J. V.; Foresman, J. B.; Cioslowski, J.; Stefanov, B. B.; Nanayakkara, A.; Challacombe, M.; Peng, C. Y.; Ayala, P. Y.; Chen, W.; Wong, M. W.; Andres, J. L.; Replogle, E. S.; Gomperts, R.; Martin, R. L.; Fox, D. J.; Binkley, J. S.; Defrees, D. J.; Baker, J.; Stewart, J. P.; Head-Gordon, M.; Gonzalez, C.; Pople, J. A.; Gaussian Inc., Pittsburgh PA, **1995**.

17 Teraishi, K.; Saito, M.; Fujii, I.; Nakamura, H. *Tetrahedron Lett.* **1992**, 33, 7153.

18 Daggett, V.; Schröder, S; Kollman, P. *J. Am. Chem. Soc.* **1991**, 113, 8926.

19 (a) Silva, A. M.; Cachau, R. E.; Sham, H. L.; Erickson, J. W. *J. Mol. Biol.* **1996**, 255, 321. (b) Wladkowski, B. D.; Chenoweth, S. A.; Sanders, J. N.; Krauss, M.; Stevens, W. J. *J. Am. Chem. Soc.* **1997**, 119, 6423.

20 Brown, R. E.; Jarvis, K. L.; Hyland, K. J. *Anal. Biochem.* **1989**, 180, 136.