Pergamon

Bioorganic & Medicinal Chemistry, Vol. 4, No. 6, pp 901–916, 1996

0968-0896/96 $15.00 + 0.00

S0968-0896(96)00085-5

Synthesis of Five Enantiomerically Pure Haptens Designed for In Vitro Evolution of Antibodies with Peptidase Activity

Jürgen Wagner, Richard A. Lerner* and Carlos F. Barbas, III*

Department of Chemistry and Molecular Biology, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037, U.S.A.

Abstract—A series of five haptens have been synthesized for use in in vitro selection experiments from combinatorial antibody libraries. Haptens were designed for the recruitment of serine and cysteine protease reaction mechanisms for the cleavage of Phe–Ala and Phe–Phe (L,L) dipeptide analogues. For the selection of transition state stabilization, Phe^{P}(O)Ala (**7**) and PheP(O)Phe (**10**) derivatives were synthesized using the Mitsunobu approach where Phe^{P} represents the phosphonic acid analogue of phenylalanine and (O)Phe and (O)Ala represent (L)-β-phenyllactic and (L)-lactic acid, respectively. Optically pure peptidyl diazomethyl ketones **16** and **22** were synthesized for selection of the catalytic ensemble of cysteine proteases. An optically pure dipeptidyl boronic acid **26** was synthesized for the selection of the catalytic ensemble of serine proteases. A strategy for the evolution of catalytic antibodies using these haptens was developed which includes mechanism-based selections. Since mechanism based selections result in covalent trapping of species from libraries, diol and disulfide containing haptenic linkers were developed for the oxidative or reductive release of selected catalysts. Copyright © 1996 Elsevier Science Ltd

Introduction

The field of catalytic antibodies has expanded rapidly since the first reports of antibodies with catalytic activity in 1986. Since this time, a diverse set of chemical reactions ranging from ester hydrolysis to the aldol condensation have been catalyzed by antibodies.[1] Traditionally, antibodies have been prepared by immunization of mice with haptens which mimic the putative transition-state of the chosen reaction. For acyl-transfer reactions which proceed through an anionic tetrahedral transition-state, phosphonate analogues have provided the best approximation of the anionic and tetrahedral configuration of the transition-state. The utility of phosphonate haptens in this regard has been demonstrated in numerous studies.[2] Unfortunately, for many reactions, analogues that faithfully mimic the transition-state are not chemically feasible. Furthermore, transition state stabilization is only one of several mechanisms to affect catalysis. As a result the rate acceleration achieved by antibodies is in most cases several orders of magnitude lower than that displayed by natural enzymes. Indeed, the sequence-specific cleavage of peptides, an important target in this field, has remained an elusive goal, despite the utility of phosphonate haptens. Approaching this goal, efficient catalysis of the cleavage of an activated aryl amide has been reported ($k_{cat}/k_{uncat} = 250{,}000$).[3] More recently the cleavage of an unsubstituted amide has been induced with very modest acceleration ($k_{cat}/k_{uncat} = 132$).[4] Cofactor-based catalysis for peptide cleavage has been reported using a Co^{III} triethylene-tetramine–peptide hapten as the immunogen.[5]

Recent development of synthetic combinatorial antibody libraries has prompted us to propose new strategies for the induction and evolution of catalysis.[6] In this approach, vast libraries of antibodies are displayed on the surface of filamentous phage and sorted using selective procedures. As an alternative to the costly and lengthy preparation of antibodies through immunization, synthetic antibodies and directed molecular evolution should present fundamental advantages over the traditional hybridoma technique for producing catalytic antibodies. For example, it is possible to use haptens carrying functional groups that are sensitive and would be rapidly degradated in mice, for example by enzymes, or which are nonimmunogenic. In vitro selection schemes also allow for iterative selections to be developed. The successive selection with different haptens which embody partial features of the ideal analogue could greatly improve catalysis. This would circumvent the challenge of incorporating into a single hapten the features which might induce multiple mechanisms (transition-state stabilization, general acid–base catalysis, cofactors, proximity effects, etc.) used by natural enzymes to produce catalysis. This is hardly possible with the immunization of animals, although a few attempts have been made with some success.[7] In vitro selection provides the opportunity to examine the use of mechanism-based inhibitors or affinity labels to select for appropriately positioned functionalities within the combining site of an antibody. Appropriately placed functional groups, such as amino acid side chains with the suitable chemical characteristics and geometries, could then be recruited to promote the

desired chemical transformation. Such an approach would be either complementary to a transition-state analogue based strategy or it may stand alone. In preliminary investigations of the feasibility of the chemical event selection approach using phage display, a pyridyl disulfide affinity label was used to trap an appropriately positioned thiol (cysteine) in the active site of a synthetic antibody.[6g] A recent report from our group has also demonstrated the utility of this approach when combined with traditional immunization in providing covalent catalysis of the aldol condensation.[1c]

Hapten Design

Based on the strategies outlined above, we decided to investigate new approaches to evolve/select antibodies which mimic the cysteine and serine proteases in a mechanistic sense. The most studied member of these protease subgroups are papain[8] and chymotrypsin or subtilisin, respectively.[9] Papain, a cysteine protease, contains a catalytic triad of Asn, His, and Cys. Chymotrypsin and subtilisin, serine proteases, contain the analogous triad of Asp, His, and Ser. Both families function through a tetrahedral intermediate stabilized by hydrogen bonding in the oxyanion hole[10] (Fig. 1). The relative contribution of each member of the serine protease triad has been probed by site-directed mutagenesis studies of subtilisin.[11] Furthermore, it has been established that hydrogen bonds in the oxyanion hole account for an additional 2.0–3.5 kcal/mol of stabilization.[12]

The compounds shown in Figure 1 were designed for in vitro selection and evolution of the catalytic pocket of antibodies that would function in a manner analogous to these proteases. Phenylalanine, at position P_1, is used as a common recognition element in all haptens. This particular amino acid was selected, because the aromatic ring generally induces good hydrophobic interactions within antibodies and its UV absorbance will aid in assays of activity. Transition-state stabilization, in both families of antibodies, will be selected using phosphonate analogues. Additionally, they serve to program the side-chain specificities at P_1', which cannot be incorporated into the other haptens. In contrast, the pocket for P_2 recognition will be selected with either the diazomethyl ketones or the boronic acid. Two different residues, alanine and histidine, were incorporated at the P_2 position. Histidine was selected because of the potential to recruit 'substrate-assisted catalysis' as demonstrated by Carter and Wells with subtilisin.[13] Their results showed that the incorporation of a histidine residue into the substrate of a histidine deleted version of the enzyme allowed partial recovery of catalytic activity.

Among the wealth of protease inhibitors documented in the literature,[14] peptidyl diazomethyl ketones are among the most powerful inhibitors of proteases known (K_i values are in the nmol to pmol range).[15] The diazoketone functionality reacts specifically with thiol

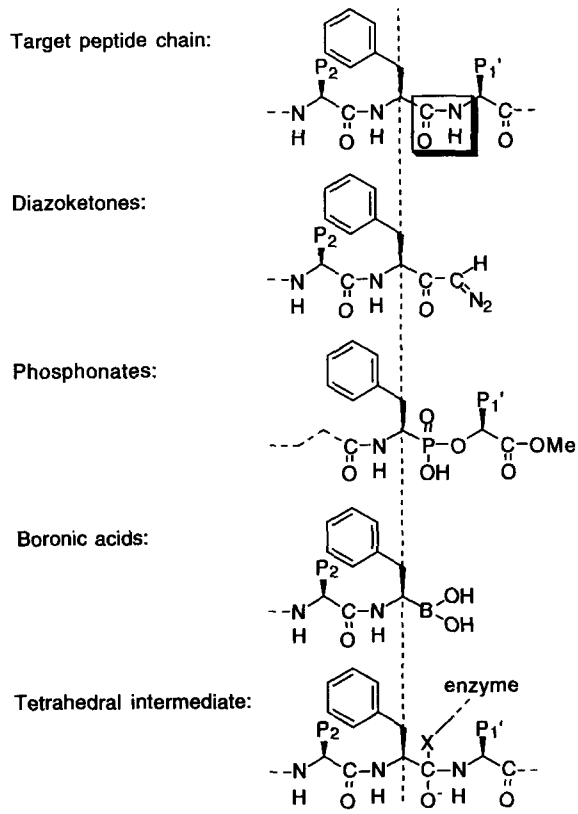


Figure 1. Comparison between substrate, haptens, and tetrahedral intermediate (serine: X = O; cysteine: X = S).

proteases in both model reactions and in tests on microorganisms. The diazoketone functionality is unreactive with other classes of proteases, as well as thiols such as mercaptoethanol and glutathione. Their ability to select specifically cysteine residues located in the binding pocket of thiol proteases is unique as even the closely related halomethyl ketones do not permit such a distinction. The shape and charge distribution of the peptide bond and diazomethylketone group are very similar.[16]

The mechanism of inactivation of cysteine proteases by diazomethyl ketones has been studied independently by Brocklehurst,[17] based on pH measurements, and more recently by Grzonka[16] using MNDO calculations. Although the details of their proposed mechanisms differ, it is clear that both His and Cys residues are indispensable and that the outcome of the reaction is alkylation of the thiol (Fig. 2). Selection with this mechanism-based inhibitor should recruit antibodies with a His/Cys catalytic diad. The formation of a covalent bond between the antibody and the inhibitor necessitates the introduction of a cleavable linker into the hapten structure. Cleavage allows for release of phage which carry and propagate the genetic information for the catalyst. Out of the various functionalities commonly used as cleavable units in protein linkage, we selected a disulfide or a diol which can be cleaved under reducing (DTT, $NaBH_4$) or oxidizing conditions ($NaIO_4$), respectively. Excellent water solubility and facile attachment to the peptide fragment through

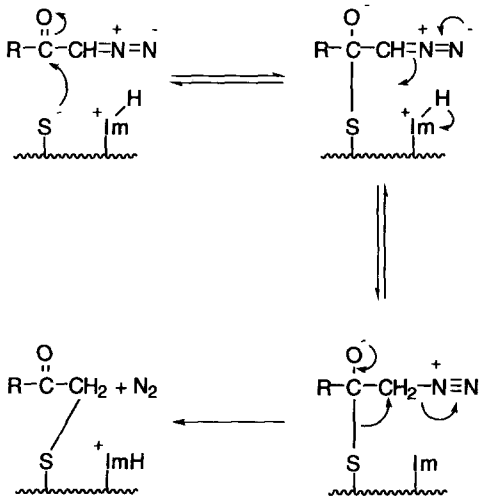


Figure 2. Postulated mechanism of inactivation of cysteine proteases by diazoketones (adapted from ref 17a).

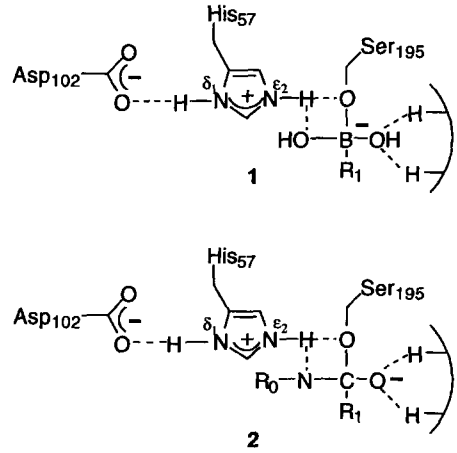


Figure 3. Comparison between boronic acid–serine tetrahedral adduct **1** and the transition state of amide bond hydrolysis **2** (adapted from ref 20b).

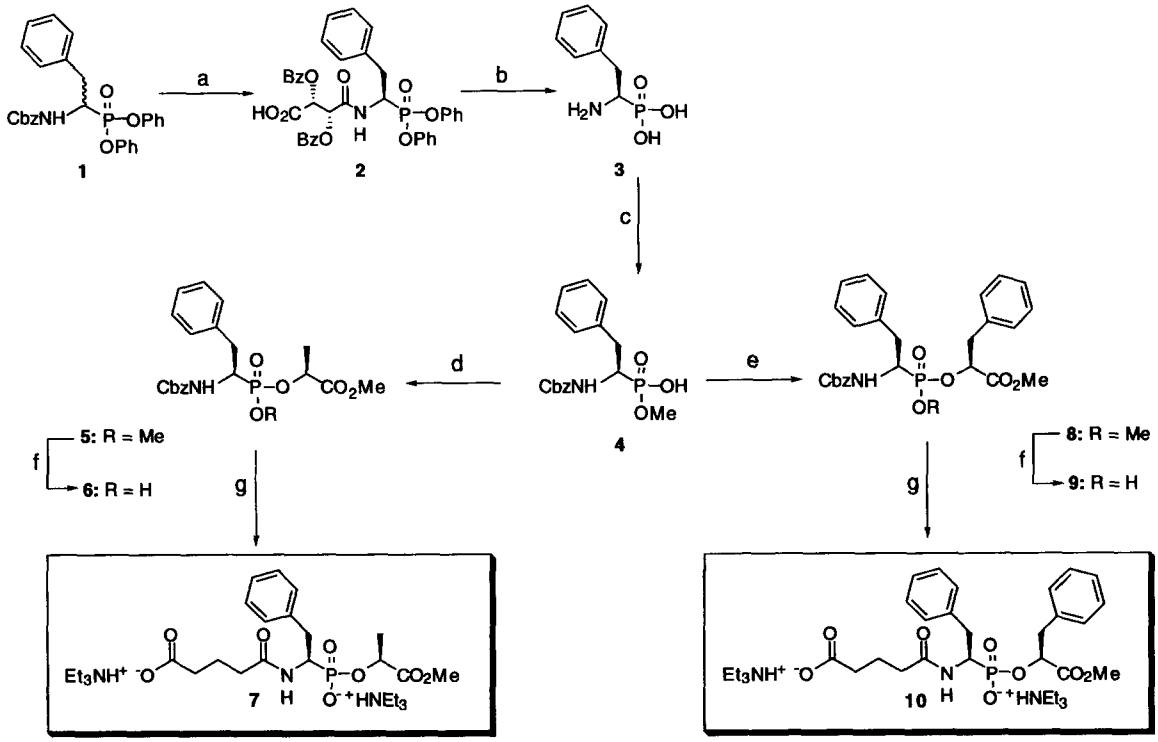
commercially available diacetyl-L-tartaric anhydride are important advantages of the diol. We have tested its cleavage using a fluoresceinamine derivative (see Experimental). Alternatively, the disulfide was particularly attractive due to its selective and smooth cleavage conditions.

In order to achieve complete complementarity of our system, we chose peptide boronic acid analogues to select the His/Ser diad of serine proteases. These analogues, which replace the C-terminal carboxylate by $-B(OH)_2$, are extremely good inhibitors of serine proteases[18] and became very popular inhibitors as soon as their synthetic challenge was solved by Matteson.[19] The interaction of peptidyl boronic acids with their target enzyme has been studied in detail by X-ray crystallography.[20] These studies as well as ^{11}B, ^{15}N, and ^1H NMR measurements[21] revealed that this class of inhibitors form transition-state-like tetrahedral complexes with the active site Ser. Structural similarity between the inhibitor complex and the tetrahedral intermediate formed during peptide hydrolysis is shown in Figure 3. The major difference between these two structures is the position of the partial negative charge which is located on oxygen in the cleavage reaction whereas boron is charged in the inhibitor complex. The negative charge on boron is partially stabilized by the catalytic His and may account for the higher affinities of this class of inhibitors than compounds such as peptidyl aldehydes or halomethyl ketones. The covalent radius of boron and the B—O length, 0.82 and 1.5 Å, respectively, compare favorably with those of carbon and C—O, 0.77 and 1.43 Å, respectively. Compounds of this type should allow for the direct selection of the His/Ser diad.

Hapten Synthesis

Phosphonates (Scheme 1)

The racemic phosphonate analogue **1** of phenylalanine was easily obtained in large amounts according to Oleksyszyn et al.[22] For our purposes, the chiral phosphonate possessing the '*S*' configuration was required in order to match the stereochemistry of the diazoketone and boronic acid haptens. A previous report by Kafarski et al.[23] used dibenzoyl-D-tartaric anhydride as a chiral auxiliary for resolution. Unfortunately, in our hands, high diastereoselective excess (de) could not be achieved directly according to ^1H NMR (500 MHz) and some modifications of the original procedure were necessary to obtain **2** with de > 97% (see Experimental). Optically pure **2** was completely deprotected and zwitterionic **3** was crystallized out by addition of propylene oxide in ethanol.[24] The L-phenylalanine phosphonic acid analogue **3** was partially protected to give **4**.[25] Rather than the more common two-step coupling strategy using an intermediate phosphonochloridate, we applied the Mitsunobu reaction recently extended to phosphonic acids by Campbell[26] to introduce the P_1 functionality in a single step under mild conditions. Phosphonates **5** and **8** were obtained using methyl (*R*)-(+)-lactate and methyl (*R*)-(+)-phenyllactate, respectively. In order to avoid a lengthy purification, **5** and **8** were deprotected in situ with bromotrimethylsilane to afford **6** and **9** in good overall yield (77% and 76% from **4**, respectively). Phosphonates **6** and **9** were deprotected by catalytic hydrogenolysis to cleanly afford the unstable free amines that were directly trapped by glutaric anhydride in the presence of triethylamine. The final phosphonate haptens **7** and **10** were purified by reverse-phase HPLC and immediately converted to their ditriethylamine salts. The free acids of **7** and **10** are highly unstable because the carboxylic acid functionality catalyzes the rapid elimination of the P_1' moiety. Phosphonate haptens **7** and **10** were obtained in 24% and 32% overall yield from **2**, respectively. The optical purities of haptens **7** and **10** were determined by comparison of the published optical rotation values of intermediates **3** and **4** and by HPLC analysis of **5** and **8**. Only two diastereoisomers were observed, corresponding to the racemic phosphonate center.



Scheme 1. (a) Resolution step using dibenzoyl-D-tartaric anhydride; (b) HBr 40%/H_2O, CH_3CO_2H, reflux, 80%; (c) i. benzyl chloroformate, aq NaOH, dioxane; ii. CH_2N_2, CH_2Cl_2; iii. NaOH, MeOH:H_2O, 1:1, 82%; (d) PPh_3, DIAD, methyl (*R*)-(+)-lactate, THF; (e) PPh_3, DIAD, methyl (*R*)-(+)-phenyllactate, THF; (f) TMSBrTHF [**6** (77%) and **9** (76%) from **4**]; (g) i. Pd/C, H_2, MeOH; ii. Et_3N, glutaric anhydride [**7** (50%) and **10** (67%)].

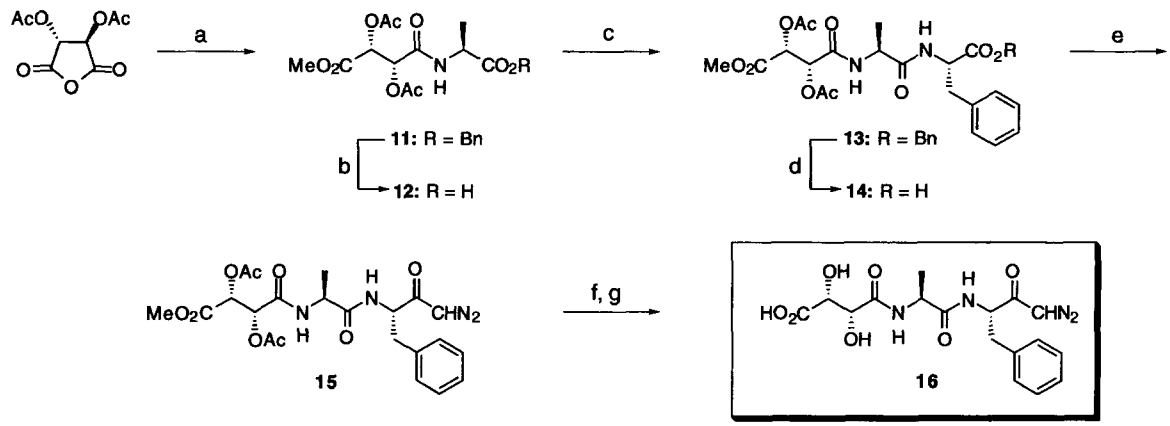
Diazoketones

The preparation of fully protected peptidyl diazoketones has been reported previously.[27] However, due to the sensitivity of the diazoketone functionality, the preparation of the unprotected compounds of type **16** and **22** in good yields represented a new synthetic challenge. For this reason, we attached the cleavable 'diol' linker to L-alanine benzyl ester using (+)-diacetyl-L-tartaric anhydride followed by diazomethane treatment during the initial step of the synthesis (Scheme 2). The benzyl ester **11** thus obtained in good yield (91%) can be subsequently completely deprotected under alkaline conditions, which are compatible with the diazoketone group. Catalytic hydrogenolysis of **11** and coupling of the resultant acid **12** with L-phenylalanine benzyl ester in the presence of DCC provided **13**. Catalytic reduction of benzyl ester **13** provided the acid **14**, which was converted to the peptidyl diazoketone **15** using the mixed anhydride activation method followed by reaction with diazomethane at low temperature. The moderate yield (40%) is probably related to the labile acetates on the linker, as much better results were obtained with structures lacking the 'diol' linker. Final deprotection was achieved using an excess of K_2CO_3. This base gave better results in our case than NaOH at low temperature previously reported.[28] Hapten **16**, purified by HPLC, was obtained in 14% overall yield.

Hapten **22**, incorporating a histidine instead of an alanine residue at P_2, was synthesized by a similar approach (Scheme 3). The choice of the protecting group on histidine proved to be crucial. Solubility problems required an inversion of the initial steps of the synthesis compared with **16**. *tert*-Butyl-*im*-tosylhistidine was coupled to L-phenylalanine benzyl ester in the presence of EDCI to provide dipeptide **17**. The *tert*-butyl group was deprotected with TFA and the crude amine salt was immediately reacted with (+)-diacetyl-L-tartaric anhydride followed by diazomethane treatment to afford fully protected **18** in 86% yield. Catalytic hydrogenolysis of **18** provided the acid **19**, which was converted to the peptidyl diazoketone **20** in moderate yield (31%) using the mixed anhydride method followed by diazomethane at low temperature. The imidazole ring of **20** was smoothly deprotected with 1-hydroxybenzotriazole to afford free histidine **21** (67%). Final deprotection of **21** with K_2CO_3 in H_2O and HPLC purification provided hapten **22** in 5% overall yield. As in previous cases,[28] no racemization was detected following the final deprotection of intermediates **15** and **21** under basic conditions.

Boronic acid

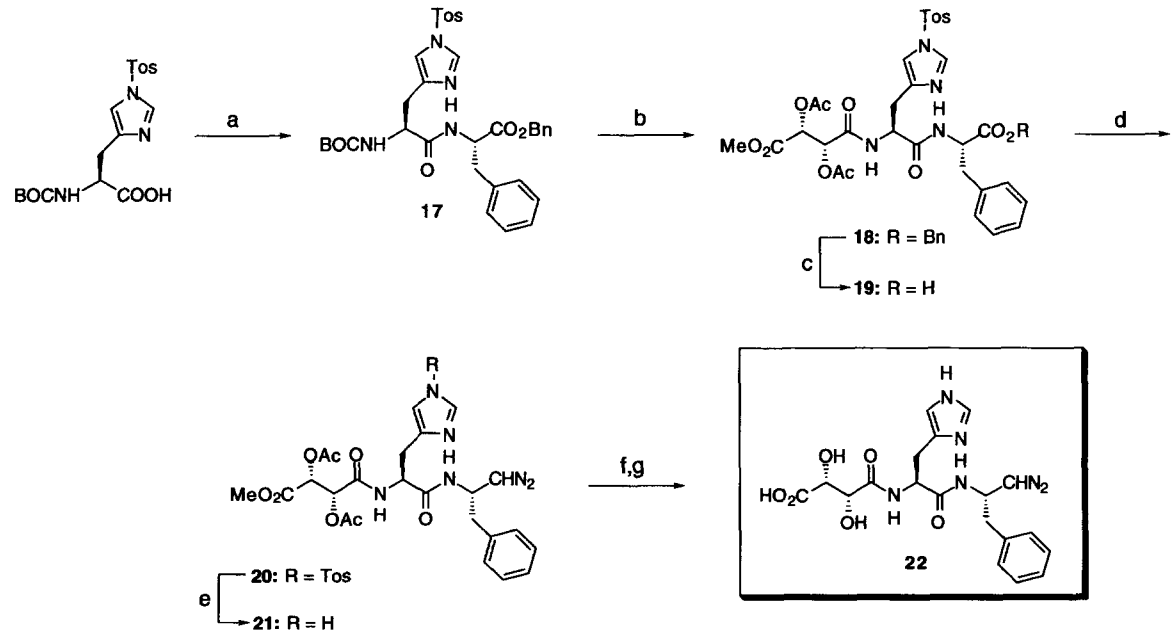
Though boronic acids are reversible inhibitors, we included into the design of hapten **26** a cleavable linker. The reducible disulfide bond should allow more flexibility during the conditions under which antibody selection is performed. The synthesis of the optically pure boronic acid analogue of phenylalanine (=boroPhe) was derived from Matteson's method[29] using (+)-pinanediol as a chiral auxiliary. A high de



Scheme 2. (a) i. L-Ala-OBn, CH_2Cl_2; ii. CH_2N_2, 91%; (b) Pd/C, H_2, THF/H_2O, 98%; (c) L-Phe-OBn, DCC, CH_2Cl_2, 67%; (d) Pd/C, H_2, THF/H_2O, 95%; (e) *N*-methylmorpholine, isobutyl chloroformate, CH_2N_2, THF, 40%; (f) K_2CO_3, MeOH, 95% (**15a**); (g) K_2CO_3, MeOH:H_2O, 1:3, 64%.

was obtained after the crucial homologation step affording (+)-pinanediol [1(*S*)-chloro-2-phenylethyl]-boronate by adding zinc chloride, as recommended in the original procedure.[19c] In the final step, the amine **27** was stabilized as its hydrochloride salt by treating directly the unstable hexamethyldisilazane intermediate with dry HCl in Et_2O (Scheme 4). Compound **27** could be stored at −20 °C over extended periods and was used directly in the coupling step.

The boronic acid hapten **26** was obtained from 3,3′-dithiopropionic acid in a five-step convergent synthesis. The bidirectional extension of 3,3′-dithiopropionic acid not only afforded two identical haptens simultaneously but had the advantage of blocking the thiol as a disulfide avoiding the need for additional protective group chemistry. L-Alanine benzyl ester was coupled to the diacid through the activated *N*-hydroxysuccinimide ester[30] to afford dibenzyl ester **23** in 58% yield. The preactivation of 3,3′-dithiopropionic acid proved necessary because direct coupling using various agents (DCC, EDCI, and BOP-Cl) gave lower yields and difficult purifications. Dibenzyl ester **23** was deprotected under basic conditions to afford the diacid **24**, which was directly coupled to boronate **27** using the mixed anhydride method. The boronate **25** was obtained in 95% yield from **23**. One attempt to prepare salt-free **24** resulted in a highly hygroscopic compound, which could not be used further. Reduction of the disulfide bond of **25** occurred smoothly in the presence of DTT to afford 2 equiv of hapten **26**. The 'boronic acid' hapten was obtained from 3,3′-dithiopropionic acid in 38% overall yield. Hapten **26** was linked to bovine serum albumin (BSA) by a two-step procedure.

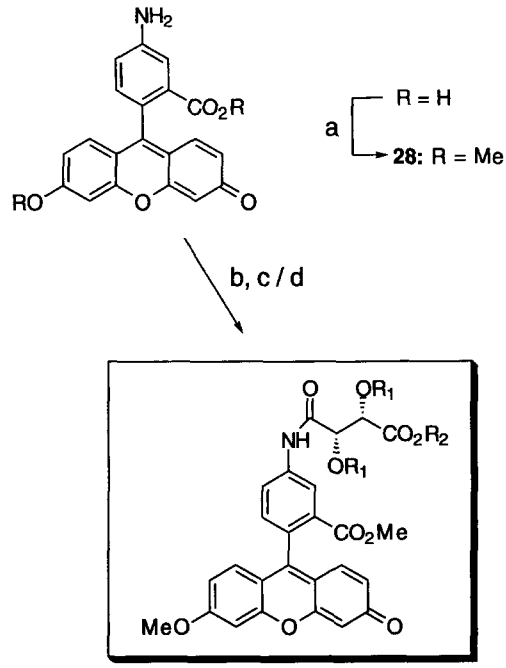


Scheme 3. (a) EDCI, DMF, 65%; (b) i. TFA; ii. wash with K_2CO_3; iii. (+)-diacetyl-L-tartaric anhydride, CH_2Cl_2; iv. CH_2N_2, 86%; (c) Pd/C, H_2, THF, 94%; (d) *N*-methylmorpholine, isobutyl chloroformate, CH_2N_2, THF, 31%; (e) HOBt, CH_2Cl_2, 67%; (f) K_2CO_3, MeOH, 90% (**21a**); (g) K_2CO_3, MeOH:H_2O, 1:3, 50%.

The heterobifunctional cross-linker SPDP (*N*-succinimidyl-3-[2-pyridyldithio]propionate) was attached to **26** restoring the cleavable disulfide linkage and providing the activated ester, which allowed hapten attachment to BSA in the second step. The (+)-pinanediol boronate was not deprotected due to several reports[18] stating the lability of this group under the phosphonate buffer (pH 7.5) conditions used during our selection procedure. The higher lability of peptidyl (+)-pinanediol boronates might be explained by the formation of cyclic species containing a B—N bond.[31]

Fluoresceinamine derivative

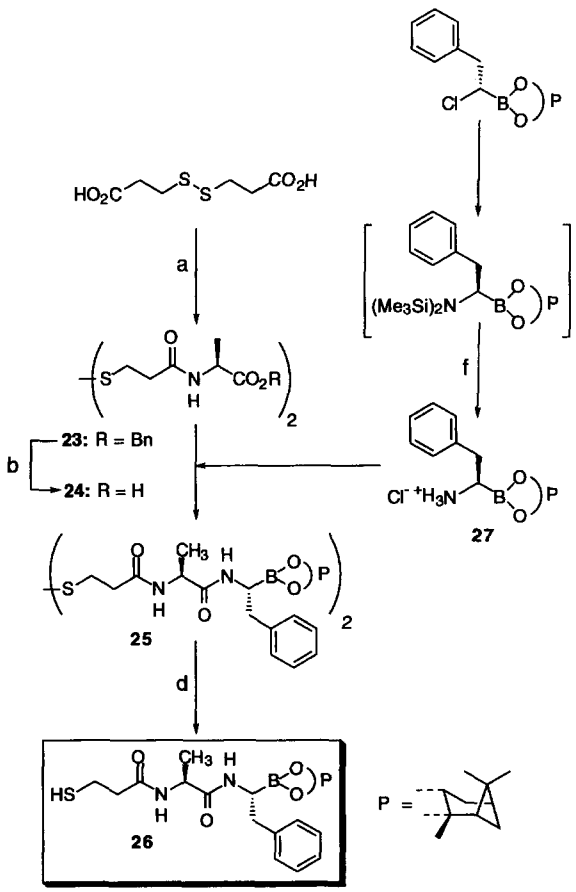
In order to optimize the cleavage of the diol group located on the linker, we needed a substrate possessing a strong UV absorption out of the range of BSA. Fluoresceinamine, absorbing at ~450 nm, fulfilled this requirement. Fluoresceinamine was treated with diazomethane to afford **28**, which was heated at reflux in the presence of (+)-diacetyl-L-tartaric anhydride (Scheme 5). The resulting acid, characterized as its methyl ester **29**, was deprotected under basic conditions (K_2CO_3) to afford test hapten **30** in 40% yield from **28**.



29: R_1 = Ac, R_2 = Me
30: R_1 = R_2 = H

Scheme 5. (a) CH_2N_2, acetone, 87%; (b) diacetyl-L-tartaric acid, acetone, reflux; (c) CH_2N_2, **29**; (d) K_2CO_3, MeOH:H_2O, 1:3, **30**: 40% (from **28**).

Compound **30** was conjugated to BSA under standard conditions via the *N*-hydroxysuccinimide ester. The protein solution was dialysed and the UV spectrum taken clearly indicated that the hapten **30** was attached to BSA. The solution was then treated with periodate for 5 h and dialysed. The UV spectrum of the resulting solution showed no absorbance at ~450 nm indicating cleavage of the diol. The general resistance of proteins to $NaIO_4$ had been previously described.[32]



Scheme 4. (a) i. DCC, *N*-hydroxysuccinimide, dioxane, 74%; ii. L-Ala-OBn, CH_2Cl_2, 78%; (b) NaOH, MeOH:H_2O, 1:3, 98%; (c) i. *N*-methylmorpholine, isobutyl chloroformate, THF; ii. boronate **27**, Et_3N, 95%; (d) DTT, CH_2Cl_2, 70%; (e) Li^+ $^-N(SiMe_3)_2$, THF, −78 °C to rt; (f) HCl/Et_2O, −78 °C, 72%.

Conclusions

In vitro protein evolution could become an important tool for the synthetic chemistry of the next century. This strategy will be successful if mechanistic and synthetic insight can be incorporated into advanced molecular selections. We have proposed an iterative evolution scheme based on two new sets of mechanism-based haptens, peptidyl diazoketones and boronic acid analogues of peptides, that will be used to induce sequence specific peptide cleavage in combination with phosphonate transition-state analogues. Five asymmetric haptens (**7**, **10**, **16**, **22**, and **26**) have been synthesized. The combinatorial antibody libraries developed in our laboratories should allow us to use any combination of these haptens during the course of antibody selection. This in vitro selection allows sensitive functionalities to be incorporated into the haptens used for selection and necessitates the incorporation of cleavable linkers into the hapten design. A cleavage site is required to recover phage displayed antibodies

which become covalently bound to the haptens. Diols and disulfides have been chosen as the cleavable sites and the cleavage of diols has been optimized with substrate **30**. This new strategy for the generation of catalytic antibodies is currently under investigation.

Experimental

General methods

Moisture-sensitive reactions were performed in flame-dried glassware under nitrogen. Anhydrous solvents were freshly distilled under argon as follows: THF and Et_2O from sodiumbenzophenone; CH_2Cl_2 from CaH_2. All reagents were purchased at highest commercial quality and used without further purification unless otherwise stated. Organic phases were dried over $MgSO_4$ before removal of volatile components at water aspirator pressure on a rotary evaporator.

All reactions were monitored by TLC carried out on aluminum sheets precoated (0.20 mm) with silica gel 60 F254. Materials were detected by visualization under a UV lamp (254 nm) and/or using potassium permanganate, vanilline, or ninhydrin solutions followed by heat as developing agents. Flash column chromatography (FCC) was performed according to Still et al.[33] with Merck silica gel 60 Å (230–400 mesh). Preparative TLC separations were carried out on glass plates (20 × 20 cm) precoated (0.50 mm) with silica gel 60 F254. All mixed solvent elements are reported as v/v solutions. Preparative reverse phase HPLC was performed using a Waters Delta Prep 4000 preparative system equipped with a 486 absorbance detector and an M1000 radial compression module containing a PrepPAK 500 column. Solvents and gradient conditions are indicated for each substrate.

Spectral data

Optical rotations were determined at ambient temperature on a Perkin–Elmer 241 polarimeter using a 1 mL, 10 dm cell; concentration (c) are reported in g/100 mL. Melting points (mp) were measured on a Thomas–Hoover capillary melting point apparatus and are uncorrected. FABMS were recorded on a VGZAB-VSE spectrometer; only partial data are reported. IR spectra were recorded on a Perkin–Elmer 1600 series FT-IR spectrometer. UV spectra were recorded on a Hewlett Packard 8542A spectrophotometer. Unless otherwise noted NMR spectra were measured at 500 MHz for ^1H and 125 MHz for ^{13}C. For ^1H NMR and ^{13}C NMR residual solvent peaks were employed as the internal standard ($CDCl_3$ 7.27 and 77.0 δ; CD_3OD 3.31 and 49.0 δ; DMSO 2.50 and 39.5 δ respectively). The ^1H NMR chemical shifts and coupling constants were determined assuming first-order behavior. Multiplicity is indicated by one or more of the following: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broad). The list of coupling constants (J) corresponds to the order of the multiplicity assignment and are reported to the nearest 0.5 Hz. In the case of ^{13}C NMR, only coupling constants between ^{31}P and ^{13}C are reported. Elemental analyses were performed by Raj K. Chadha at the Scripps Research Institute facility.

Diphenyl [1(*R*)-1-[*N*-(3-carboxy-2(*R*),3(*R*)-dibenzoyloxypropionyl)amino]-2-phenylethyl]phosphonate (2-*'R'*). Crude diphenyl [(4-amino)-2-phenylethyl]phosphonate hydrobromide[22] **1** (52.0 g, 0.12 mol) was suspended between $CHCl_3$ (450 mL) and 2 N NaOH (400 mL). The biphasic system was shaken until complete dissolution and the aqueous phase reextracted with $CHCl_3$ (50 mL). The combined organic phases were dried and the solvent was evaporated to afford diphenyl [(4-amino)-2-phenylethyl]phosphonate (40.0 g, 0.11 mol). This amine was redissolved in 1,4-dioxane (460 mL) and dibenzoyl-D-tartaric anhydride[34] (40.8 g, 0.12 mol) was added. After 48 h, the solvent was evaporated under vacuum (~40 °C, oil bath) and the residue was dissolved in benzene (200 mL; some heating may be necessary). Crystals of **2-*'R'*** fell out overnight at 4 °C. A second crop of crystals was obtained by redissolving the residue in benzene (100 mL). The two crops were combined and recrystallized in $CHCl_3$ (250 mL) until constant rotation and 'clean' ^1H NMR to afford optically pure **2-*'R'***[35] (27.0 g, 32%):[36] mp 185.5–186 °C; $[\alpha]_{589}+42.2°$, $[\alpha]_{578}+44.5°$, $[\alpha]_{546}+52.0°$, $[\alpha]_{436}+105.5°$, $[\alpha]_{365}+208.8°$ (*c* 0.992; acetone); ^1H NMR ($CDCl_3$): δ 7.95–7.99 (m, 4H), 7.51–7.55 (m, 2H), 7.37–7.41 (m, 4H), 7.21–7.25 (m, 3H), 7.00–7.15 (m, 11H), 6.89 (m, 2H), 5.95 (dd, J = 2.5, 1.0 Hz, 1H), 5.84 (d, J = 2.5 Hz, 1H), 5.15 (dddd, J = 22.0, 9.5, 7.0, 5.0 Hz, 1H), 3.24 (ddd, J = 20.5, 14.0, 5.0 Hz, 1H), 3.04 (ddd, J = 20.5, 11.0, 9.5 Hz, 1H).

Diphenyl [1(*S*)-1-[*N*-(3-carboxy-2(*R*),3(*R*)-dibenzoyloxypropionyl)amino]-2-phenylethyl]phosphonate (2-*'S'*). Partially optically pure **2-*'S'*** (30.0 g, 36%, de ~85% determined by ^1H NMR) could be precipitated out upon dissolution of the mother liquors in Et_2O (200 mL): mp 71–73 °C; $[\alpha]_{589}+56.5°$, $[\alpha]_{578}+59.3°$, $[\alpha]_{546}+69.1°$, $[\alpha]_{436}+136.6°$, $[\alpha]_{365}+261.7°$ (*c* 0.898; acetone); ^1H NMR ($CDCl_3$): δ 8.02, 7.90 (2m, 4H), 7.51–7.55 (m, 1H), 7.34–7.40 (m, 4H), 6.95–7.22 (m, 15H), 6.87 (m, 2H), 5.98, 5.79 (2d, J = 2.0 Hz, 2H), 5.11 (dddd, J = 22.0, 14.0, 9.5, 4.5 Hz, 1H), 3.24 (ddd, J = 19.5, 13.5, 4.5 Hz, 1H), 2.99 (ddd, J = 19.5, 14.0, 9.5 Hz, 1H); IR (neat): ν_{max} 3323, 3063, 1731, 1489, 1246, 1199, 1183, 1096, 948, 713 cm^{-1}; ^{13}C NMR ($CDCl_3$) δ 169.0, 165.5 (d, J = 5 Hz), 165.1, 164.5, 149.5, 149.4, 135.0 (d, J = 13 Hz), 133.8, 133.1, 130.0, 129.9, 129.7, 129.6, 129.0, 128.5, 128.4, 128.1, 127.0, 125.5, 125.3, 120.3 (d, J = 3 Hz), 120.1 (d, J = 4 Hz), 72.2, 71.6, 46.2 (d, J = 158 Hz), 35.3; FABMS (NBA): *m/z* (relative intensity) 1386 ($2M^+$, 5), 1149 (100), 693 (M^+, 100). Anal. ($C_{38}H_{32}NO_{10}P$) C, H, N.

[1(*R*)-1-Amino-2-phenylethyl]phosphonic acid (3). The optically pure phosphonate **2-*'R'*** (6.9 g, 9.95 mmol) was suspended in acetic acid (60 mL) and 40% HBr in H_2O (60 mL). The mixture was refluxed for 20 h. After cooling, the solvent was evaporated under high vacuum. The residue was taken up in H_2O (50 mL) and

the insoluble benzoic acid filtered out. The solution was decolorized with activated charcoal (Darco G-60) and the solvent was evaporated. The oil was dissolved in EtOH (25 mL) and propylene oxide was slowly added until crystalline **3** fell out. Crystallization was complete overnight at −20 °C and afforded **3** as a white solid (1.6 g, 80%): mp 264–266 °C; $[\alpha]_{589}$ −40.9° (*c* 2.03, 2 N NaOH);[37] ^{1}H NMR (D_2O, 300 MHz): δ 7.05–7.25 (m, 5H), 3.34 (ddd, *J* = 13.0, 12.0, 3.5 Hz, 1H), 3.18 (ddd, *J* = 15.0, 13.0, 3.5 Hz, 1H), 2.67 (ddd, *J* = 15.0, 12.0, 7.5 Hz, 1H); ^{13}C NMR (D_2O + K_2CO_3): δ 141.0 (d, *J* = 14 Hz), 129.7, 128.3, 125.9, 52.9 (d, *J* = 143 Hz), 38.5 (d, *J* = 4 Hz); IR (KBr) ν_{max} 3474, 3405, 3022 (br), 1739, 1170 cm^{-1}; FABMS (NBA): *m/z* (relative intensity) 403 ($2M^+$ + H, 51), 202 (M^+ + H, 63). $C_8H_{12}NO_3P$.

Dimethyl [(1(*R*)-1-[*N*-[(phenylmethoxy)carbonyl]-amino]-2-phenylethyl]phosphonate (3a). The phosphonic acid **3** (1.3 g, 6.46 mmol) was suspended in H_2O (60 mL) and the pH was adjusted between 9 and 9.5 with 2 N NaOH. The solution was cooled to 0 °C and benzyl chloroformate (975 μL, 6.85 mmol), dissolved in 1,4-dioxane (60 mL) was added slowly. The pH was maintained between 9 and 9.5 for 1.5 h at 0 °C by constant addition of 2 N NaOH. Then, the mixture was stirred 2 h at 0 °C, 2 N NaOH (5 mL) was added and the aqueous phase was washed twice with Et_2O (30 mL). The aqueous phase was acidified to pH ~ 1.0 with 6 N HCl and extracted three times with AcOEt (40 mL). The combined organic phases were dried and the solvent was evaporated to yield crude phosphonic acid as a white solid. The Cbz-protected amine was not purified further, but directly dissolved in acetone (55 mL). The solution was cooled to 0 °C and freshly prepared diazomethane in Et_2O was added until a yellow color persisted. The solvent was evaporated to yield **3a** as a yellowish oil (2.22 g, 95%): $[\alpha]_{589}$ −40.9°, $[\alpha]_{578}$ −42.8°, $[\alpha]_{546}$ −48.7°, $[\alpha]_{436}$ −85.9°, $[\alpha]_{365}$ −140.8°, (*c* 0.306, EtOH);[38] ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.12–7.33 (m, 10H), 5.18 (d, *J* = 10.0 Hz, 1H), 5.00 (s, 2H), 4.45 (dddd, *J* = 21.0, 10.0, 10.0, 4.5 Hz, 1H), 3.75 (d, *J* = 11.5 Hz, 3H), 3.69 (d, *J* = 10.5 Hz, 3H), 3.24 (ddd, *J* = 14.0, 8.5, 4.5 Hz, 1H), 2.89 (ddd, *J* = 20.0, 14.0, 10.0 Hz, 1H); ^{13}C NMR ($CDCl_3$): δ 155.7 (d, *J* = 6 Hz), 136.4 (d, *J* = 13 Hz), 136.2, 129.2, 128.5, 128.1, 127.9, 126.9, 67.0, 53.3 (d, *J* = 6 Hz), 53.1 (d, *J* = 6 Hz), 48.2 (d, *J* = 157 Hz), 35.9; IR (neat): ν_{max} 3245, 3032, 2954, 1719, 1539, 1261, 1229, 1038, 744, 698 cm^{-1}; FABMS (NBA): *m/z* (relative intensity) 386 (M^+ + Na, 36), 364 (M^+ + H, 55). $C_{18}H_{22}NO_5P$.

Methyl hydrogen [[1(*R*)-1-*N*-[(phenylmethoxy)carbonyl]amino]-2-phenylethyl]phosphonate (4). The phosphonate **3a** (2.22 g, 6.11 mmol) was dissolved in MeOH (22 mL) and 1 N NaOH (15 mL, 15.0 mmol) was added slowly. The mixture was stirred overnight, 1 N NaOH (10 mL) was added and the aqueous phase was washed twice with Et_2O (30 mL). The aqueous phase was acidified to pH ~ 1.0 with 6 N HCl and extracted three times with AcOEt (40 mL). The combined organic phases were dried and the solvent was evaporated to yield **4** as a white solid (1.75 g, 82%). In order to obtain a better yield in the subsequent step, the compound was purified by preparative reverse phase HPLC (35 min linear gradient; elution 20–80% H_2O:CH_3CN (1:1) in H_2O containing 0.1% TFA; flow rate 100 mL/min): mp 161–161.5 °C; $[\alpha]_{589}$ −47.9°; $[\alpha]_{578}$ −49.8°; $[\alpha]_{546}$ −57.0°; $[\alpha]_{436}$ −100.6°; $[\alpha]_{365}$ −165.4° (*c* 0.474, EtOH);[39] ^{1}H NMR ($CDCl_3$): δ major conformer 7.10–7.32 (m, 10H), 5.43 (d, *J* = 10.0 Hz, 1H), 5.01, 4.94 (2d, *J* = 12.5 Hz, 2H), 4.42 (m, 1H), 3.72 (d, *J* = 10.5 Hz, 3H), 3.23 (m, 1H), 2.82 (m, 1H); minor conformer 7.10–7.32 (m, 10H), 6.06 (d, *J* = 10.0 Hz, 1H), 4.95 (s, 2H), 4.22 (m, 1H), 3.74 (d, *J* = 10.5 Hz, 3H), 3.10 (m, 1H), 2.67 (m, 1H); ^{13}C NMR ($CDCl_3$): δ major conformer 156.0, 136.4 (d, *J* = 14 Hz), 136.2, 129.1, 128.4, 128.0, 127.7, 126.8, 67.0, 52.8 (d, *J* = 6 Hz), 45.7 (d, *J* = 159 Hz), 35.5; IR (neat): ν_{max} 3292, 3031, 2952, 1700, 1534, 1257, 1213, 1046, 741, 698 cm^{-1}; FABMS (NBA): *m/z* (relative intensity) 372 (M^+ + Na, 100), 350 (M^+ + H, 47), 210 (24). $C_{17}H_{20}NO_5P$.

2(*S*)-2-[[[1(*R*)-1-[*N*-[(Phenylmethoxy)carbonyl]amino]-2-phenylethyl]hydroxyphosphinyl]oxy]propanoate methyl ester (6). The phosphonate **4** (300 mg, 0.86 mmol) was dissolved in dry THF (18 mL). Methyl (*R*)-(+)-lactate (150 μL, 1.55 mmol) and triphenylphosphine (336 mg, 1.29 mmol) were added. Finally, diisopropylazodicarboxylate (DIAD; 255 μL, 1.29 mmol) was added and the mixture stirred for 30 min. When the formation of **5** was complete, bromotrimethylsilane (331 μL, 2.58 mmol) was added and the reaction stirred for 3.5 h. The solution was diluted with Et_2O (50 mL) and extracted with $NaHCO_3$ 5% (25 mL). The aqueous phase was washed with Et_2O (15 mL) and acidified to pH ~ 1.5 with 6 N HCl. The aqueous phase was extracted three times with AcOEt (25 mL). The combined organic phases were dried and the solvent was evaporated to yield **6** as a white foam (277 mg, 77%); ^{1}H NMR (DMSO): δ major conformer 7.61 (d, *J* = 10.0 Hz, 1H), 7.19–7.32 (m, 8H), 7.13–7.15 (m, 2H), 4.93, 4.86 (2d, *J* = 13.0 Hz, 2H), 4.81 (dq, *J* = 7.0, 7.0 Hz, 1H), 3.99 (m, 1H), 3.68 (s, 3H), 3.05 (m, 1H), 2.75 (m, 1H), 1.34 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (CD_3OD): δ 173.2, 158.2 (d, *J* = 3 Hz), 138.9 (d, *J* = 16 Hz), 138.2, 130.3, 129.4, 128.8, 128.5, 127.6, 72.0 (d, *J* = 7 Hz), 67.4, 53.0, 51.0 (d, *J* = 157 Hz), 36.4, 19.8; IR (neat): ν_{max} 3309, 3031, 2951, 1722, 1529, 1222, 1000, 743, 698 cm^{-1}; FABMS (NBA): *m/z* (relative intensity) 444 (M^+ + Na, 22), 422 (M^+ + H, 100), 210 (75). $C_{20}H_{24}NO_7P$.

2(*S*)-2-[[[1(*R*)-1-[*N*-[(Phenylmethoxy)carbonyl]amino]-2-phenylethyl]methoxyphosphinyl]oxy]propanoate methyl ester (5). A small amount of **6** was treated with a freshly prepared solution of diazomethane in Et_2O until the yellow color persisted. The solvent was evaporated to afford **5** quantitatively as a mixture of diastereoisomers. Compound **5** could be used to control the optical purity by ^{1}H NMR 500 MHz (4 methyl doublets between: δ 1.2 and 1.6). The de was found to be higher than 97%; ^{1}H NMR ($CDCl_3$): δ major diastereoisomer 7.15–7.35 (m, 10H), 5.36 (d, *J* = 10.0 Hz, 1H), 5.00 (s, 2H), 4.90 (dq, *J* = 7.5, 7.0 Hz,

1H), 4.45 (dddd, J = 21.0, 14.5, 10.0, 4.5 Hz, 1H), 3.82 (d, J = 11.0 Hz, 3H), 3.73 (s, 3H), 3.24 (ddd, J = 14.0, 9.5, 4.5 Hz, 1H), 2.89 (ddd, J = 20.0, 14.5, 9.5 Hz, 1H), 1.36 (d, J = 7.0 Hz, 3H); minor diastereoisomer 7.15–7.35 (m, 10H), 5.62 (d, J = 10.0 Hz, 1H), 5.08 (dq, J = 7.5, 7.0 Hz, 1H), 5.03, 4.95 (2d, J = 12.5 Hz, 2H), 4.52 (m, 1H), 3.75 (s, 3H), 3.69 (d, J = 11.0 Hz, 3H), 3.27 (m, 1H), 2.85 (m, 1H), 1.58 (d, J = 7.0 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ major diastereoisomer 171.4, 155.7 (d, J = 6 Hz), 136.4 (d, J = 8 Hz), 136.2, 129.2, 128.4, 128.0, 127.8, 126.8, 71.1 (d, J = 7 Hz), 66.8, 53.3 (d, J = 7 Hz), 52.5, 48.8 (d, J = 159 Hz), 35.7, 18.8 (d, J = 5 Hz); minor diastereoisomer 171.6, 155.9 (d, J = 7 Hz), 136.6 (d, J = 13 Hz), 136.3, 129.3, 128.3, 127.9, 127.7, 126.7, 70.6 (d, J = 7 Hz), 66.7, 53.2 (d, J = 7 Hz), 52.7, 49.0 (d, J = 157 Hz), 36.0, 19.2 (d, J = 4 Hz); IR (neat): ν_{max} 3251, 3032, 2954, 1754, 1723, 1536, 1259, 1230, 1042, 744, 698 cm^{-1}; FABMS (NBA): *m/z* (relative intensity) 436 (M^++H, 100), 408 (29), 210 (33). $C_{21}H_{26}NO_7P$.

2(*S*)-2-[[[1(*R*)-1-[*N*-(4-Carboxybutanoyl)amino]-2-phenylethyl]hydroxyphosphinyl]oxy]propanoate methyl ester ditriethylamine salt (7). The phosphonate **6** (130 mg, 0.31 mmol) was dissolved in MeOH (10 mL). Pd/C 10% (300 mg, 0.31 mmol) was added and the mixture was hydrogenated for 2.5 h under strong agitation. The slur was filtered through Celite®, washed with MeOH (~20 mL) and CH_2Cl_2 (~150 mL). Only 90% of the solvent was then evaporated to avoid polymerization. The residue containing the free amine was redissolved in CH_2Cl_2 (40 mL). Glutaric anhydride (53 mg, 0.47 mmol) and triethylamine (43 μL, 0.31 mmol) were added and the mixture was stirred overnight. The solvent was evaporated and the residue was purified by preparative reverse phase HPLC [35 min linear gradient; elution 0–50% H_2O:CH_3CN (1:1) in H_2O containing 0.1% TFA; flow rate 100 mL/min]. The fractions containing hapten **7** were immediately freeze-dried until ~5% H_2O was left. H_2O (10 mL) containing triethylamine (108 μL, 0.78 mmol) was added and freeze-dried again to afford **7** as a gummy oil (94 mg, 50%). The free diacid of **7** was found to be highly unstable; ^{1}H NMR (DMSO): δ 7.59 (d, J = 10.0 Hz, 1H), 7.08–7.20 (m, 5H), 4.63 (dq, J=7.0, 6.5 Hz, 1H), 4.04 (dddd, J = 22.0, 12.0, 10.0, 3.0 Hz, 1H), 3.58 (s, 3H), 3.01 (ddd, J = 14.5, 4.5, 3.0 Hz, 1H), 2.78 (q, J = 7.5 Hz, 12H), 2.61 (ddd, J = 14.5, 12.0, 6.0 Hz, 1H), 1.92–2.02 (m, 4H), 1.50–1.55 (m, 2H), 1.25 (d, J = 6.5 Hz, 3H), 1.08 (t, J = 7.5 Hz, 18H); ^{13}C NMR (MeOD): δ 179.8, 175.0, (d, J = 5 Hz), 174.4 (d, J = 5.2 Hz), 140.2 (d, J = 14.2 Hz), 130.3, 129.1, 127.2, 70.7 (d, J = 6 Hz), 52.6, 50.0 (d, J = 151 Hz), 47.4, 37.6, 36.9, 36.7, 23.2, 20.5, (d, J = 4 Hz), 9.2; IR (neat): ν_{max} 3296, 2986, 2947, 2487, 1738, 1662, 1199, 1059, cm^{-1}; FABMS (positive ion spray): *m/z* (relative intensity) 604 (M^++H, 15), 503 (M^++H−Et_3N, 100), 424 (12). $C_{29}H_{54}N_3O_8P$.

2(*S*)-2-[[[1(*R*)-1-[*N*-[(Phenylmethoxy)carbonyl]amino]-2-phenylethyl]hydroxyphosphinyl]oxy]-3-phenylpropanoate methyl ester (9). The phosphonate **4** (100 mg, 0.29 mmol) was dissolved in dry THF (6 mL). Methyl (*R*)-(+)-3-phenyllactate (77 mg, 0.44 mmol) and triphenylphosphine (112 mg, 0.44 mmol) were added. Finally, diisopropylazodicarboxylate (DIAD; 85 μL, 0.44 mmol) was added and the mixture was stirred for 30 min. When the formation of **8** was complete, bromotrimethylsilane (300 μL, 0.87 mmol) was added and stirred for 1.5 h. The solution was diluted with Et_2O (40 mL) and extracted with $NaHCO_3$ 5% (30 mL). The aqueous phase was washed with Et_2O (15 mL), acidified to pH ~1.0 with 6 N HCl and extracted twice with AcOEt (40 mL). The combined organic phases were dried and the solvent was evaporated to yield **9** as a colorless oil (110 mg, 76%); ^{1}H NMR ($CDCl_3$): δ major conformer 7.12–7.34 (m, 15H), 5.68 (d, J = 10.0 Hz, 1H), 5.16 (m, 1H), 5.01, 4.88 (2d, J = 12.5 Hz, 2H), 4.40 (m, 1H), 3.67 (s, 3H), 3.08–3.25 (m, 3H), 2.66 (m, 1H); minor conformer 9.95 (br s, 1H), 7.12–7.34 (m, 15H), 5.90 (br s, 1H), 5.16 (m, 1H), 4.87 (s, 2H), 4.10 (m, 1H), 3.70 (s, 3H), 3.08–3.25 (m, 3H), 2.58 (m, 1H); ^{13}C NMR ($CDCl_3$): δ major conformer 170.5, 156.1 (d, J = 6 Hz), 136.7 (d, J = 15 Hz), 136.3, 135.2, 129.5, 129.2, 128.4, 128.3, 128.2, 127.8, 127.6, 127.1, 126.5, 74.9 (d, J = 7 Hz), 66.7, 52.4, 49.6 (d, J = 159 Hz), 39.0 (d, J = 5 Hz), 35.4; IR (neat) ν_{max} 3322, 3031, 2952, 1727, 1526, 1224, 1079, 1000, 742, 699 cm^{-1}; FABMS (NBA/NaI): *m/z* (relative intensity) 542 (M^++2Na, 18), 520 (M^++Na, 100), 498 (M^++H, 33), 210 (25). $C_{26}H_{28}NO_7P$.

2(*S*)-2-[[[1(*R*)-1-[*N*-[(Phenylmethoxy)carbonyl]amino]-2-phenylethyl]methoxyphosphinyl]oxy]-3-phenylpropanoate methyl ester (8). A small amount of **9** was treated with a freshly prepared solution of diazomethane in Et_2O until the yellow color persisted. After evaporation of the solvent, **8** was obtained quantitatively as a colorless oil; ^{1}H NMR ($CDCl_3$): δ major diastereoisomer 7.03–7.35 (m, 15H), 5.05 (m, 1H), 4.98, 4.94 (2d, J = 12.5 Hz, 2H), 4.79 (d, J = 10.5 Hz, 1H), 4.29 (m, 1H), 3.79 (d, J = 11.0 Hz, 3H), 3.75 (s, 3H), 3.00–3.17 (m, 3H), 2.25 (m, 1H); ^{13}C NMR ($CDCl_3$): δ major diastereoisomer 170.3, 155.5 (d, J = 7 Hz), 136.3 (d, J = 10 Hz), 136.2, 135.5, 129.4, 129.0, 128.7, 128.4, 128.3, 127.9, 127.7, 127.4, 126.7, 75.4 (d, J = 8 Hz), 66.7, 53.4 (d, J = 7 Hz), 52.4, 48.9 (d, J = 158 Hz), 39.0 (d, J = 7 Hz), 35.4 (d, J = 3 Hz); IR (neat) ν_{max} 3252, 3031, 2954, 1756, 1723, 1230, 1040, 743, 698 cm^{-1}; FABMS (NBA/NaI): *m/z* (relative intensity) 534 (M^++Na, 79), 512 (M^++H, 100). $C_{27}H_{30}NO_7P$.

2(*S*)-2-[[[1(*R*)-1-[*N*-(4-Carboxybutanoyl)amino]-2-phenylethyl]hydroxyphosphinyl]oxy]-3-phenylpropanoate methyl ester ditriethylamine salt (10). The phosphonic acid **9** (144 mg, 0.29 mmol) was dissolved in MeOH (10 mL). Pd/C 10% (307 mg, 0.29 mmol) was added and the mixture was hydrogenated for 2 h under strong agitation. The slurry was filtered through Celite®, washed with MeOH (~15 mL) and CH_2Cl_2 (~100 mL). Only 90% of the solvent was evaporated to avoid polymerization. The residue containing the free amine was redissolved in CH_2Cl_2 (25 mL). Glutaric anhydride (50 mg, 0.44 mmol) and

triethylamine (40 μL, 0.29 mmol) were added and the mixture was stirred overnight. The solvent was evaporated and the residue was purified by preparative reverse phase HPLC [35 min linear gradient; elution 20–80% H_2O:CH_3CN (1:1) in H_2O containing 0.1% TFA; flow rate 100 mL/min]. The fractions containing hapten **10** were combined and immediately freeze-dried. Then, H_2O (10 mL) containing triethylamine (80 μL, 0.58 mmol) was added and freeze-dried again to afford **10** as a gummy oil (132 mg, 67%). The free diacid of **10** is highly unstable; ^{1}H NMR (DMSO): δ 7.51 (d, J = 9.5 Hz, 1H), 7.08–7.27 (m, 10H), 4.80 (ddd, J = 13.5, 13.0, 6.0 Hz, 1H), 4.05 (dddd, J = 22.5, 12.0, 9.5, 3.0 Hz, 1H), 3.53 (s, 3H), 2.91–2.98 (m, 3H), 2.84 (q, J = 7.5 Hz, 12H), 2.56 (ddd, J = 13.5, 12.0, 6.5 Hz, 1H), 1.94–2.02 (m, 4H), 1.48–1.56 (m, 2H), 1.09 (t, J = 7.5 Hz, 18H); ^{13}C NMR (MeOD): δ 179.6, 174.4 (d, J = 5 Hz), 173.6 (d, J = 4 Hz), 140.2 (d, J = 15 Hz), 137.6, 130.9, 130.3, 129.3, 129.1, 127.8, 127.2, 75.2 (d, J = 6 Hz), 52.4, 50.0 (d, J = 151 Hz), 47.4, 40.9 (d, J = 4 Hz), 37.7, 36.9, 23.2, 9.1; IR (neat): ν_{max} 3298, 2986, 2948, 1747, 1667, 1451, 1197, 1057, 701 cm^{-1}; FABMS (positive ion spray): *m/z* (relative intensity) 681 (M^++H, 16), 579 (M^++H−Et_3N, 100), 500 (M^++Na−2Et_3N, 8). $C_{35}H_{58}N_3O_8P$.

***N*-[2(*R*),3(*R*)-Diacetoxy-4-methoxybutanedioyl]-L-alanine benzyl ester (11).** L-Alanine benzyl ester hydrochloride (827 mg, 3.83 mmol) was suspended between Et_2O (20 mL) and H_2O (20 mL) containing K_2CO_3 (795 mg, 5.75 mmol). The biphasic system was shaken until complete dissolution and the aqueous layer was reextracted three times with Et_2O (20 mL). The combined organic phases were dried and the solvent evaporated. The residue was redissolved in CH_2Cl_2 (20 mL) and cooled to 0 °C. Finally, (+)-diacetyl-L-tartaric anhydride (885 mg, 4.10 mmol), dissolved in CH_2Cl_2 (20 mL), was added dropwise. After 30 min, the solvent was evaporated. The residue was redissolved in Et_2O (50 mL) and cooled to 0 °C. A freshly prepared solution of diazomethane in Et_2O was added dropwise until the yellow color persisted. Compound **11** fell out as a white solid (1.43 g, 91%): mp 142.5–143.5 °C; ^{1}H NMR ($CDCl_3$): δ 7.26–7.41 (m, 5H), 6.89 (d, J = 7.5 Hz, 1H), 5.75, 5.58 (2d, J = 2.5 Hz, 2H), 5.22, 5.17 (2d, J = 12.0 Hz, 2H), 4.65 (dq, J = 7.5, 7.0 Hz, 1H), 3.76, 2.18, 2.13 (3s, 9H), 1.45 (d, J = 7.0 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ 172.2, 169.4, 168.7, 167.1, 165.0, 134.9, 128.7, 128.6, 128.2, 71.6, 71.3, 67.5, 52.9, 48.2, 20.5, 20.3, 18.5; IR (neat): ν_{max} 3368, 2954, 1754, 1212 cm^{-1}; FABMS (NBA/NaI): *m/z* (relative intensity) 432 (M^++Na, 100), 410 (M^++H, 88). Anal. ($C_{19}H_{23}NO_9$) C, H, N.

***N*-[2(*R*),3(*R*)-Diacetoxy-4-methoxybutanedioyl]-L-alanine (12).** The benzyl ester **11** (1.38 g, 3.37 mmol) was dissolved in THF (60 mL) and H_2O (2 mL). Pd/C 10% (360 mg, 0.34 mmol) was added and the mixture was hydrogenated for 3 h under strong agitation. The slurry was filtered through Celite®, washed with AcOEt (~150 mL) and dried. After solvent evaporation, crude **12** was obtained as a white solid (1.07 g, quant.) and used directly for the next step without purification; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.00 (d, J = 7.5 Hz, 1H), 5.75, 5.57 (2d, J = 2.5 Hz, 2H), 4.58 (dq, J = 7.5, 7.0 Hz, 1H), 3.75, 2.20, 2.12 (3s, 9H), 1.46 (d, J = 7.0 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ 175.4, 169.5, 168.9, 167.1, 165.6, 71.5, 71.1, 52.9, 48.0, 20.4, 20.2, 17.9; IR (neat): ν_{max} 3350 (br), 2957, 1755, 1673, 1214 cm^{-1}; FABMS (NBA): *m/z* (relative intensity) 320 (M^++H, 14), 219 (28), 123 (69), 109 (100). $C_{12}H_{17}NO_9$.

***N*-[2(*R*),3(*R*)-Diacetoxy-4-methoxybutanedioyl]-L-alanyl-L-phenylalanine benzyl ester (13).** L-Phenyl-alanine benzyl ester *p*-toluenesulfonate salt (1.44 g, 3.37 mmol) was suspended between Et_2O (30 mL) and H_2O (40 mL) containing K_2CO_3 (700 mg, 5.06 mmol). The biphasic system was shaken until complete dissolution and the aqueous layer reextracted three times with Et_2O (30 mL). The combined organic phases were dried and the solvent evaporated. The residue was redissolved in CH_2Cl_2 (15 mL). The acid **12** (1.07 g, 3.37 mmol) was dissolved in CH_2Cl_2 (15 mL) and added to the previous solution. Finally, DCC (700 mg, 3.37 mmol) was added: the reaction was slightly exothermic and urea precipitated immediately. After 3 h, the mixture was filtered and the solvent evaporated. The residue was purified by FCC (CH_2Cl_2:Et_2O, 1:3) to afford **13** as a sticky foam (1.25 g, 67%); ^{1}H NMR ($CDCl_3$): δ 7.20–7.40 (m, 8H), 6.95–7.05 (m, 2H), 6.92 (d, J = 7.5 Hz, 1H), 6.53 (d, J = 8.0 Hz, 1H), 5.69, 5.58 (2d, J = 2.5 Hz, 2H), 5.19, 5.11 (2d, J = 12.5 Hz, 2H), 4.86 (dd, J = 8.0, 6.0, 6.0 Hz, 1H), 4.48 (dq, J = 7.5, 7.0 Hz, 1H), 3.75 (s, 3H), 3.14, 3.08 (2dd, J = 14.0, 6.0 Hz, 2H), 2.20, 2.12 (2s, 6H), 1.34 (d, J = 7.0 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ 171.0, 170.9, 169.4, 168.8, 167.1, 165.1, 135.2, 134.9, 129.2, 128.6, 127.2, 71.6, 71.3, 67.4, 53.3, 52.8, 48.6, 37.5, 20.6, 20.2, 18.7; IR (neat): ν_{max} 3309, 3064, 3091, 2955, 1755, 1659, 1530, 1213, 700 cm^{-1}; FABMS (NBA): *m/z* (relative intensity) 1113 ($2M^+$+H, 18), 557 (M^++H, 88), 256 (100). Anal. ($C_{28}H_{32}N_2O_{10}$) C, H, N.

***N*-[2(*R*),3(*R*)-Diacetoxy-4-methoxybutanedioyl]-L-alanyl-L-phenylalanine (14).** The benzyl ester **13** (980 mg, 1.76 mmol) was dissolved in THF (30 mL) and H_2O (1 mL). Pd/C 10% (187 mg, 0.18 mmol) was added and the mixture was hydrogenated for 3 h under strong agitation. The slurry was filtered through Celite®, washed with AcOEt (~100 mL) and dried. After evaporation of the solvent, **14** was obtained as a white solid (780 mg, 95%) and used without further purification: mp 29–31 °C; ^{1}H NMR ($CDCl_3$): δ 7.12–7.28 (m, 6H), 6.88 (d, J = 7.5 Hz, 1H), 5.68, 5.56 (2d, J = 2.5 Hz, 2H), 4.81 (ddd, J = 7.5, 6.5, 5.5 Hz, 1H), 4.54 (dq, J = 7.5, 7.0 Hz, 1H), 3.75 (s, 3H), 3.18 (dd, J = 14.0, 5.5 Hz, 1H), 3.03 (dd, J = 14.0, 6.5 Hz, 1H), 2.16, 2.11 (2s, 6H), 1.31 (d, J = 7.0 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ 173.7, 171.4, 169.4, 168.9, 167.1, 165.5, 135.4, 129.2, 128.5, 127.1, 71.4, 71.1, 53.3, 52.9, 48.6, 37.3, 20.5, 20.2, 18.4; IR (neat): ν_{max} 3326 (br), 2957, 1755, 1658, 1213, 733 cm^{-1}; FABMS (NBA): *m/z* (relative intensity) 467 (M^++H, 60), 302 (40), 231 (39), 166 (58), 120 (100). Anal. ($C_{21}H_{26}N_2O_{10}$) C, H, N.

3(*R*)-1-Diazo-3-[*N*-[2(*R*),3(*R*)-diacetoxy-4-methoxybutandioyl]-L-alanylamino]-4-phenyl-2-butanone (15). The acid **14** (1.0 g, 2.14 mmol) was dissolved in dry THF (15 mL). *N*-Methylmorpholine (0.25 mL, 2.25 mmol) was added and the mixture was stirred for 5 min at rt. After cooling to −15 °C, isobutyl chloroformate (0.29 mL, 2.25 mmol) was added; a white precipitate formed immediately. After 5 min at −15 °C, the solution was rapidly filtered and diluted with dry THF (20 mL). Finally, the mixed anhydride was added dropwise to a solution of freshly prepared diazomethane in Et_2O (20 mL, 6.5 mmol) cooled to −15 °C. After 30 min at −15 °C, argon was bubbled through the cold mixture for 10 min and the solvent was evaporated. The residue was purified by FCC (CH_2Cl_2:Et_2O:MeOH:petroleum ether, 3.5:3.5:0.25:0.5) to afford **15** as a yellowish solid (420 mg, 40%): mp 52–54 °C; ^{1}H NMR ($CDCl_3$): δ 7.21–7.30 (m, 3H), 7.15–7.17 (m, 2H), 6.93 (d, J = 7.0 Hz, 1H), 6.84 (d, J = 7.5 Hz, 1H), 5.66, 5.58 (2d, J = 2.5 Hz, 2H), 5.28 (br s, 1H), 4.68 (ddd, J = 7.0, 7.0, 7.0 Hz, 1H), 4.48 (dq, J = 7.5, 7.0 Hz, 1H), 3.76 (s, 3H), 3.09, 2.99 (2dd, J = 14.0, 7.0 Hz, 2H), 2.19, 2.12 (2s, 6H), 1.34 (d, J = 7.0 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ 192.0, 171.0, 169.4, 169.0, 167.1, 165.3, 135.8, 129.2, 128.6, 127.1, 71.7, 71.1, 57.3, 54.8, 52.9, 48.8, 38.1, 20.6, 20.2, 18.6; IR (neat): ν_{max} 3310, 3085, 2954, 2110, 1755, 1659, 1526, 1213, 1064; FABMS (NBA): *m/z* (relative intensity) 491 ($M^+ + H$, 4), 463 ($M^+ + H - N_2$, 100). Anal. ($C_{22}H_{26}N_4O_9$) C, H, N.

3(*R*)-1-Diazo-3-[*N*-[2(*R*),3(*R*)-dihydroxy-4-methoxybutandioyl]-L-alanylamino]-4-phenyl-2-butanone (15a). The diazoketone **15** (219 mg, 0.45 mmol) was dissolved in MeOH (20 mL). After cooling to 0 °C, K_2CO_3 (12.3 mg, 0.09 mmol) was added. After 30 min at 0 °C, two-thirds of the solvent was evaporated and the residue was filtered through a short bed of silica gel (5 cm). The solvent was evaporated to furnish **15a** as a yellowish solid (171 mg, 94%): mp >100 °C (dec); ^{1}H NMR (MeOD): δ 7.18–7.31 (m, 5H), 5.92[40] (br s, 1H), 4.59, 4.43 (2d, J = 2.0 Hz, 2H), 4.57 (dd, J = 9.0, 5.0 Hz, 1H), 4.33 (q, J = 7.0 Hz, 1H), 3.79 (s, 3H), 3.19 (dd, J = 13.5, 5.0 Hz, 1H), 2.91 (dd, J = 13.5, 9.0 Hz, 1H), 1.28 (d, J = 7.0 Hz, 3H); ^{13}C NMR (MeOD): δ 195.9, 174.6, 173.9, 173.7, 138.5, 130.3, 129.6, 127.8, 74.2, 73.7, 59.5, 55.7, 52.8, 50.4, 37.9, 18.3; IR (KBr): ν_{max} 3391 (br), 3082, 2930, 2110, 1740, 1659, 1528 cm^{-1}; FABMS (NBA/NaI): *m/z* (relative intensity) 429 ($M^+ + Na$, 21), 379 ($M^+ + H - N_2$, 100). $C_{18}H_{22}N_4O_7$.

3(*R*)-1-Diazo-3-[*N*-[3-carboxy-2(*R*),3(*R*)-dihydroxypropionyl]-L-alanylamino]-4-phenyl-2-butanone (16). The diol **15a** (171 mg, 0.42 mmol) was dissolved in MeOH (2.5 mL), H_2O (15 mL) was added and the solution was cooled to 0 °C. K_2CO_3 (400 mg, 2.90 mmol) was then added over a period of 1 h (every 15 min). The mixture was kept an additional 30 min at 0 °C before dilution with H_2O (25 mL). The solution was neutralized to pH 7.0 with 0.07 M HCl at 0 °C and was immediately purified by preparative reverse phase HPLC [30 min linear gradient; elution 10–40% H_2O:CH_3CN (1:1) in H_2O containing 0.1% ammonium acetate; flow rate 100 mL/min]. The fractions containing hapten **16** were combined and diluted with an equal amount of H_2O. The identical procedure was then repeated by replacing the buffered H_2O with pure H_2O in the eluant system in order to provide salt-free **16**. After freeze-drying, hapten **16** was obtained as a white solid (105 mg, 64%): mp 103–105 °C (dec); ^{1}H NMR (MeOD): δ 7.18–7.29 (m, 5H), 5.96[40] (br s, 1H), 4.57 (dd, J = 10.0, 5.0 Hz, 1H), 4.44, 4.41 (2d, J = 2.5 Hz, 2H), 4.27 (q, J = 7.0 Hz, 1H), 3.20 (dd, J = 14.0, 5.0 Hz, 1H), 2.90 (dd, J = 14.0, 10.0 Hz, 1H), 1.25 (d, J = 7.0 Hz, 3H); ^{13}C NMR (MeOD): δ 194.6, 176.5, 174.9, 174.7, 138.5, 130.3, 129.5, 127.8, 74.3, 74.2, 59.6, 55.6, 50.6, 37.8, 18.0; IR (KBr) ν_{max} 3297 (br), 2113, 1710, 1646, 1543, 1369, 699 cm^{-1}; FABMS (NBA): *m/z* (relative intensity) 393 ($M^+ + H$, 9), 365 ($M^+ + H - N_2$, 36), 238 (23). Anal. ($C_{17}H_{20}N_4O_7$) C, H, N.

***N*-[(*tert*-Butyloxy)carbonyl]-*im*-tosyl-L-histidyl-L-phenylalanine benzyl ester (17).** L-Phenylalanine benzyl ester *p*-toluenesulfonate salt (2.1 g, 4.93 mmol) was suspended between Et_2O (40 mL) and H_2O (15 mL) containing K_2CO_3 (1.0 g, 7.32 mmol). The biphasic system was shaken until complete dissolution and the aqueous layer was reextracted three times with Et_2O (30 mL). The combined organic phases were dried and the solvent was evaporated. The residue was redissolved in dry DMF (5 mL) and added to an ice-cold solution of *N*-*tert*-butyloxycarbonyl-*im*-tosyl-L-histidine (2.0 g, 4.88 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI; 1.4 g, 7.32 mmol) dissolved in dry DMF (10 mL). The solution was allowed to warm to rt and was stirred overnight. The mixture was diluted with AcOEt (100 mL) and washed twice with 1 M $NaHCO_3$ (30 mL). The organic phase was dried and the solvent evaporated. The residue was purified by FCC (CH_2Cl_2:MeOH, 9.0:0.5) to afford **17** as a white solid (2.05 g, 65%): mp 139–140 °C; ^{1}H NMR ($CDCl_3$): δ 7.89 (d, J = 1.5 Hz, 1H), 7.75–7.78 (m, 2H), 7.32–7.35 (m, 3H), 7.16–7.27 (m, 8H), 7.09 (s, 1H), 6.81 (m, 2H), 5.96 (d, J = 6.5 Hz, 1H), 5.07, 5.04 (2d, J = 12.5 Hz, 2H), 4.78 (m, 1H), 4.42 (m, 1H), 3.06 (dd, J = 15.0, 4.5 Hz, 1H), 2.94 (dd, J = 13.5, 5.5 Hz, 1H), 2.85 (dd, J = 15.0, 6.0 Hz, 1H), 2.82 (dd, J = 13.5, 6.0 Hz, 1H), 2.35 (s, 3H), 1.40 (s, 9H); ^{13}C NMR ($CDCl_3$): δ 170.7, 170.6, 155.4, 146.2, 140.5, 136.0, 135.4, 134.9, 134.5, 130.3, 129.1, 128.5, 128.4, 127.3, 126.9, 114.8, 80.4, 67.0, 53.7, 53.2, 37.8, 29.8, 28.1, 21.6; IR (neat): ν_{max} 3306, 3033, 2976, 1738, 1710, 1673, 1502, 1378, 1174, 1084, 675, 590 cm^{-1}; FABMS (NBA): *m/z* (relative intensity) 647 ($M^+ + H$, 100). Anal. ($C_{34}H_{38}N_4O_7S$) C, H, N.

***N*-[2(*R*),3(*R*)-Diacetoxy-4-methoxybutanedioyl]-*im*-tosyl-L-histidyl-L-phenylalanine benzyl ester (18).** The dipeptide **17** (3.79 g, 5.86 mmol) was dissolved in trifluoroacetic acid (35 mL) and stirred for 40 min at rt. All volatile components were evaporated. The residue was redissolved in AcOEt (200 mL), washed with H_2O (80 mL) containing K_2CO_3 (2.5 g, 18.2 mmol) and dried.

After solvent evaporation, the free amine was dissolved in CH_2Cl_2 (240 mL) and cooled to 0 °C. (+)-Diacetyl-L-tartaric anhydride (2.5 g, 11.7 mmol), dissolved in CH_2Cl_2 (20 mL), was added dropwise. The mixture was stirred at 0 °C for 30 min, then 3 h at rt. Finally, the mixture was cooled again to 0 °C and a freshly prepared solution of diazomethane in Et_2O was added until the yellow color persisted. After evaporation of the solvent, the residue was purified by FCC (CH_2Cl_2:Et_2O, 1:1) to afford **18** as a white solid (3.89 g, 86%): mp 46–48 °C; ^{1}H NMR ($CDCl_3$): δ 8.06 (d, J = 7.0 Hz, 1H), 7.77–7.80 (m, 2H), 7.58 (d, J = 7.5 Hz, 1H), 7.17–7.35 (m, 11H), 7.09 (s, 1H), 6.86–6.88 (m, 2H), 5.65, 5.57 (2d, J = 2.5 Hz, 2H), 5.10, 5.06 (2d, J = 12.0 Hz, 2H), 4.76 (ddd, J = 7.5, 6.5, 6.0 Hz, 1H), 4.63 (ddd, J = 7.0, 7.0, 4.0 Hz, 1H), 3.75 (s, 3H), 3.08 (dd, J = 15.0, 4.0 Hz, 1H), 2.95 (dd, J = 14.0, 6.5 Hz, 1H), 2.90 (dd, J = 14.0, 6.0 Hz, 1H), 2.75 (ddd, J = 15.0, 7.0, 1.0 Hz, 1H), 2.38, 2.19, 2.09 (3s, 9H); ^{13}C NMR ($CDCl_3$): δ 170.7, 169.7, 169.5, 169.2, 167.2, 165.6, 146.6, 140.2, 135.8, 135.6, 135.1, 134.5, 130.5, 129.2, 128.6, 128.5, 127.5, 127.1, 115.0, 72.0, 71.1, 67.2, 53.6, 52.9, 52.1, 37.5, 29.5, 21.7, 20.6, 20.4; IR (neat): ν_{max} 3322, 3032, 2954, 1755, 1675, 1213, 1076, 675, 589 cm^{-1}; FABMS (NBA): *m/z* (relative intensity) 777 (M^++H, 100). Anal. ($C_{38}H_{40}N_4O_{12}S$) C, H, N.

***N*-[2(*R*),3(*R*)-Diacetoxy-4-methoxybutanedioyl]-*im*-tosyl-L-histidyl-L-phenylalanine (19).** The benzyl ester **18** (200 mg, 0.26 mmol) was dissolved in MeOH (10 mL). Pd/C 10% (300 mg, 0.29 mmol) was added and the mixture was flushed with argon. 1,4-Cyclohexadiene (0.25 mL, 2.68 mmol) was added and the slurry was stirred during 2.5 h. The mixture was filtered through Celite® and washed with AcOEt (~100 mL). After evaporation of the solvent, **19** was obtained as a white foam (166 mg, 94%), which was not further purified; ^{1}H NMR (DMSO): δ 8.40 (d, J = 8.0 Hz, 1H), 8.28 (d, J = 1.5 Hz, 1H), 8.05 (d, J = 6.5 Hz, 1H), 7.92, 7.47 (2d, J = 8.0 Hz, 4H), 7.32 (s, 1H), 7.14–7.24 (m, 5H), 5.52, 5.45 (2d, J = 3.0 Hz, 2H), 4.52 (ddd, J = 8.5, 8.0, 5.0 Hz, 1H), 4.25 (ddd, J = 9.5, 6.5, 5.0 Hz, 1H), 3.66 (s, 3H), 3.00 (dd, J = 13.5, 5.0 Hz, 1H), 2.87 (dd, J = 15.5, 5.0 Hz, 1H), 2.84 (dd, J = 13.5, 8.5 Hz, 1H), 2.76 (dd, J = 15.5, 9.5 Hz, 1H), 2.38, 2.08, 1.91 (3s, 9H); ^{13}C NMR ($CDCl_3$) δ 174.6, 169.8, 169.6, 169.4, 167.2, 165.9, 146.7, 139.3, 136.3, 136.2, 134.3, 130.5, 129.3, 128.3, 127.4, 126.8, 115.4, 71.9, 70.9, 70.7, 52.9, 52.7, 37.4, 30.0, 21.7, 20.5, 20.3; IR (neat): ν_{max} 3321, 3032, 2955, 1757, 1673, 1214, 1078, 733, 675, 589 cm^{-1}; FABMS (NBA/NaI): *m/z* (relative intensity) 731 (M^+ + 2Na, 17), 709 (M^+ + Na, 100), 687 (M^+ + H, 24). $C_{31}H_{34}N_4O_{12}S$.

3(*R*)-1-Diazo-3-[*N*-[2(*R*),3(*R*)-diacetoxy-4-methoxybutanedioyl]-*im*-tosyl-L-histidylamino]-4-phenyl-2-butanone (20). The acid **19** (827 mg, 1.20 mmol) was dissolved in dry THF (25 mL). *N*-Methylmorpholine (0.14 mL, 1.27 mmol) was added and the mixture was stirred for 5 min. After cooling to −20 °C, isobutyl chloroformate (0.16 mL, 1.27 mmol) was added: a white precipitate appeared immediately. The reaction was kept at −20 °C over 5 min and was filtered rapidly into dry THF (30 mL). The diluted solution of the mixed anhydride was added dropwise to a freshly prepared solution of diazomethane in Et_2O (20 mL, 6.6 mmol) cooled to −20 °C. After 30 min at −20 °C, argon was bubbled through for 10 min and the solvent was evaporated. The residue was purified by FCC (CH_2Cl_2:Et_2O:MeOH:petroleum ether, 3.5:3.5:0.25:1.0) to afford **20** as a yellowish foam (267 mg, 31%); ^{1}H NMR ($CDCl_3$): δ 8.31 (d, J = 6.5 Hz, 1H), 7.78–7.80 (m, 3H), 7.31–7.33 (m, 2H), 7.23–7.25 (m, 3H), 7.08 (s, 1H), 7.00–7.04 (m, 3H), 5.59 (s, 2H), 5.39 (br s, 1H), 4.56–4.60 (m, 2H), 3.77 (s, 3H), 3.09 (dd, J = 15.0, 4.0 Hz, 1H), 2.88 (dd, J = 13.5, 6.0 Hz, 1H), 2.75 (dd, J = 13.5, 8.0 Hz, 1H), 2.73 (dd, J = 15.0, 5.5 Hz, 1H), 2.39, 2.22, 2.08 (3s, 9H); ^{13}C NMR ($CDCl_3$): δ 192.8, 169.8, 169.6, 169.3, 167.1, 166.1, 146.7, 139.9, 136.1, 135.9, 134.4, 130.5, 128.9, 128.5, 127.5, 127.0, 115.1, 72.4, 70.8, 57.5, 54.2, 53.0, 52.9, 37.2, 28.7, 21.7, 20.6, 20.3; IR (neat): ν_{max} 3311, 3107, 2954, 2928, 2110, 1756, 1674, 1213, 1077, 675, 589 cm^{-1}; FABMS (NBA): *m/z* (relative intensity) 711 (M^++H, 70), 701 (100), 683 (M^++H−N_2, 49). $C_{32}H_{34}N_6O_{11}S$.

3(*R*)-1-Diazo-3-[*N*-[2(*R*),3(*R*)-diacetoxy-4-methoxybutanedioyl]-L-histidylamino]-4-phenyl-2-butanone (21). The diazoketone **20** (200 mg, 0.28 mmol) was dissolved in THF (40 mL). 1-Hydroxybenzotriazole (HOBt; 76 mg, 0.56 mmol) was added and the mixture was stirred at rt overnight. The solvent was evaporated and the residue redissolved in AcOEt (50 mL), washed with 1 M $NaHCO_3$ (30 mL) and dried. After evaporation of the solvent, the residue was purified by FCC (CH_2Cl_2:MeOH, 7:1) to afford **21** as a yellowish foam (105 mg, 67%); ^{1}H NMR ($CDCl_3$): δ 7.37 (s, 1H), 7.20–7.27 (m, 4H), 7.06–7.10 (m, 3H), 6.69 (s, 1H), 5.64, 5.59 (2d, J = 2.5 Hz, 2H), 5.59 (br s, 1H), 4.58 (m, 2H), 3.79 (s, 3H), 3.14 (dd, J = 15.0, 4.0 Hz, 1H), 3.11 (dd, J = 12.5, 5.0 Hz, 1H), 2.85 (dd, J = 12.5, 7.5 Hz, 1H), 2.84 (dd, J = 15.0, 10.0 Hz, 1H), 2.24, 2.14 (2s, 6H); ^{13}C NMR ($CDCl_3$) δ 194.8, 170.6, 170.3, 169.5, 167.1, 166.3, 136.5, 134.6, 129.0, 128.5, 126.6, 72.9, 70.7, 57.9, 54.2, 53.8, 53.1, 37.2, 28.4, 20.7, 20.4; IR (neat): ν_{max} 3290, 2926, 2110, 1754, 1668, 1212, 1067, 733 cm^{-1}; FABMS (NBA): *m/z* (relative intensity) 557 (M^++H, 100), 529 (M^++H−N_2, 25). $C_{25}H_{28}N_6O_9$.

3(*R*)-1-Diazo-3-[*N*-[2(*R*),3(*R*)-dihydroxy-4-methoxybutanedioyl]-L-histidylamino]-4-phenyl-2-butanone (21a). The diacetate **21** (105 mg, 0.19 mmol) was dissolved in MeOH (7.5 mL). The solution was cooled to 0 °C and K_2CO_3 (5 mg, 0.04 mmol) was added. After 1.5 h at 0 °C, two-thirds of the solvent was evaporated and the residue was filtered through a bed of silica gel (CH_2Cl_2:MeOH, 7:3) to afford **21a** as a yellowish foam (80 mg, 90%); ^{1}H NMR (MeOD) δ 7.60 (d, J = 1.0 Hz, 1H), 7.18–7.28 (m, 5H), 6.84 (s, 1H), 5.79[40] (br s, 1H), 4.59 (m, 2H), 4.58, 4.42 (2d, J = 2.0 Hz, 2H), 3.78 (s, 3H), 3.15 (dd, J = 14.0, 5.0 Hz, 1H), 3.05 (dd, J = 15.0, 6.0 Hz, 1H), 2.98 (dd, J = 15.0, 7.0 Hz,

1H), 2.88 (dd, $J = 14.0$, 9.0 Hz, 1H); ^{13}C NMR (MeOD): δ 195.6, 173.9, 173.6, 172.7, 138.3, 136.4, 130.3, 129.6, 127.8, 119.0, 74.4, 73.7, 59.4, 55.6, 54.5, 52.8, 38.0, 30.3; IR (neat): ν_{max} 3273 (br), 2112, 1741, 1655, 1528 cm^{-1}; FABMS (NBA/NaI): *m/z* (relative intensity) 495 ($M^+ + Na$, 49), 473 ($M^+ + H$, 100), 445 ($M^+ + H - N_2$, 31). $C_{21}H_{24}N_6O_7$.

3(*R*)-1-Diazo-3-[*N*-[3-carboxy-2(*R*),3(*R*)-dihydroxypropionyl]-L-histidylamino]-4-phenyl-2-butanone (22). The diol **21a** (70 mg, 0.15 mmol) was dissolved in MeOH (0.5 mL) and H_2O (5 mL). After cooling to 0 °C, K_2CO_3 (400 mg, 2.93 mmol) was added portionwise over a period of 45 min. The mixture was stirred at 0 °C for an additional 45 min and diluted with H_2O (20 mL). The solution was neutralized to pH 7.0 with 0.07 N HCl at 0 °C and immediately purified by preparative reverse phase HPLC [30 min linear gradient; elution 0–35% H_2O:CH_3CN (1:1) in H_2O containing 0.1% ammonium formate; flow rate 100 mL/min]. The fractions containing hapten **22** were combined and diluted with an equal amount of H_2O. The identical procedure was then repeated by replacing the buffered H_2O with pure H_2O in the eluant system in order to provide salt-free **22**. After freeze-drying, hapten **22** was obtained as a white solid (35 mg, 50%): mp >130 °C (dec); ^{1}H NMR (MeOD): δ 8.24 (s, 1H), 7.18–7.29 (m, 5H), 7.08 (s, 1H), 5.90[40] (br s, 1H), 4.59–4.64 (m, 2H), 4.43, 4.32 (2d, $J = 2.0$ Hz, 2H), 3.21 (dd, $J = 15.0$, 6.0 Hz, 1H), 3.16 (dd, $J = 13.5$, 5.5 Hz, 1H), 3.06 (dd, $J = 15.0$, 7.0 Hz, 1H), 2.89 (dd, $J = 13.5$, 9.5 Hz, 1H); ^{13}C NMR (MeOD) δ 196.0, 177.2, 175.0, 171.9, 138.3, 135.6, 135.4, 130.3, 129.6, 127.9, 119.8, 74.9, 74.6, 59.4, 55.8, 53.6, 38.1, 28.8; IR (KBr) ν_{max} 3383 (br), 2113, 1633, 1528, 1374 cm^{-1}; FABMS (NBA): *m/z* (relative intensity) 459 ($M^+ + H$, 40), 431 ($M^+ + H - N_2$, 9). $C_{20}H_{22}N_6O_7$.

3,3′-Dithiodipropionyl-L-alanine benzyl ester (23). L-Alanine benzyl ester hydrochloride (3.14 g, 14.5 mmol) was suspended between Et_2O (50 mL) and H_2O (60 mL) containing K_2CO_3 (3.0 g, 21.8 mmol). The phases were shaken until complete dissolution and the aqueous phase was reextracted twice with Et_2O (60 mL). The combined organic phases were dried and the solvent was evaporated. The free amine was redissolved in CH_2Cl_2 (200 mL) and dithiobis(succinimidyl propionate)[29] (2.45 g, 6.06 mmol) was added. After 30 min, the solvent was evaporated and the residue redissolved in AcOEt (300 mL). The organic phase was washed twice with 2.5% $NaHCO_3$ (50 mL), dried and evaporated. The residue was recrystallized (CH_2Cl_2:Et_2O, 1:0.5) to afford **23** as a white crystalline solid (2.5 g, 78%): mp 121–122 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.30–7.45 (m, 10H), 6.58 (d, $J = 7.5$ Hz, 2H), 5.20, 5.15 (2d, $J = 14.5$ Hz, 4H), 4.67 (dq, $J = 7.5$, 7.0 Hz, 2H), 2.87–3.05 (m, 4H), 2.52–2.73 (m, 4H), 1.42 (d, $J = 7.0$ Hz, 6H); ^{13}C NMR ($CDCl_3$): δ 173.0, 170.5, 135.2, 128.5, 128.4, 128.0, 67.1, 48.0, 35.2, 33.6, 18.1; IR (neat): ν_{max} 3323, 3060, 1744, 1646, 1533, 1186, 1155, 729 cm^{-1}; FABMS (NBA/NaI): *m/z* (relative intensity) 555 ($M^+ + Na$, 86), 533 ($M^+ + H$, 100). Anal. ($C_{26}H_{32}N_2O_6S_2$) C, H, N.

3,3′-Dithiodipropionyl-L-alanine (24). The dibenzyl ester **23** (500 mg, 1.42 mmol) was dissolved in THF (15 mL) and H_2O (20 mL). 2 N NaOH (1.25 mL) was added over a period of 2.5 h (each 30 min) and the mixture was stirred an additional 2.5 h. Benzyl alcohol was then removed by washing twice with Et_2O (20 mL) and the aqueous phase acidified to pH ~1.0 with 6 N HCl before freeze-drying to afford crude diacid **24** (470 mg). Purification of **24** was not necessary for the next step, but could be achieved by reverse phase preparative HPLC [40 min linear gradient; elution 0–50% H_2O:CH_3CN (1:1) in H_2O containing 0.1% TFA; flow rate 100 mL/min]. Purified **24** was a highly hygroscopic white solid: mp 158.5–160 °C; ^{1}H NMR (DMSO, 300 MHz): δ 8.28 (d, $J = 7.2$ Hz, 2H), 4.19 (dq, $J = 7.2$, 7.0 Hz, 2H), 2.86, 2.50 (2t, $J = 7.0$ Hz, 8H), 1.24 (d, $J = 7.0$ Hz, 6H); ^{13}C NMR (MeOD) δ 176.1, 173.6, 49.3, 36.2, 34.9, 17.6; IR (KBr) ν_{max} 3342 (br), 1710, 1644, 1528, 1239, 1193, 646 cm^{-1}; FABMS (NBA): *m/z* (relative intensity) 353 ($M^+ + H$, 100), 282 (47). $C_{12}H_{20}N_2O_6S_2$.

3,3′-Dithiodi[(propionyl-L-alanyl)amino-2-phenylethyl]-boronate (+)-pinanediol (25). The crude diacid **24** (470 mg, ~1.4 mmol) was suspended in dry THF (40 mL). *N*-Methylmorpholine (328 µL, 3.0 mmol) was added and stirred for 5 min. The mixture was cooled to −15 °C and isobutyl chloroformate (387 µL, 3.0 mmol) was added; a white precipitate fell out immediately. After 5 min, dry THF (30 mL) and triethylamine (416 µL, 3.0 mmol) were added. Finally, the boronate **27** (950 mg, 2.84 mmol), suspended in dry THF (25 mL), was added slowly. The mixture was stirred 30 min at −10 °C and 1.5 h at rt. The salts were filtered out and the solvent was evaporated. Product **25** (820 mg, 95%) was induced to precipitate out of the residue by adding petroleum ether (30 mL) and scratching: mp 84–86 °C; ^{1}H NMR ($CDCl_3$) δ 7.16–7.27 (m, 10H), 7.11 (d, $J = 7.5$ Hz, 2H), 6.95 (d, $J = 3.5$ Hz, 2H), 4.49 (dq, $J = 7.5$, 7.0 Hz, 2H), 4.24 (dd, $J = 9.0$, 2.0 Hz, 2H), 3.15 (ddd, $J = 9.5$, 5.0, 3.5 Hz, 2H), 2.93 (dd, $J = 13.5$, 5.0 Hz, 2H), 2.88 (m, 4H), 2.79 (dd $J = 13.5$, 9.5 Hz, 2H), 2.46–2.57 (m, 4H), 2.29 (m, 2H), 2.09 (m, 2H), 1.95 (t, $J = 5.5$ Hz, 2H), 1.78–1.86 (m, 4H), 1.35 (d, $J = 7.0$ Hz, 6H), 1.32, 1.26 (2s, 12H), 1.18 (d, $J = 11.0$ Hz, 2H), 0.82 (s, 6H); ^{13}C NMR ($CDCl_3$): δ 173.9, 170.8, 139.5, 129.0, 128.4, 126.2, 85.2, 77.3, 51.4, 47.4, 40.6 (br), 39.5, 38.0, 36.8, 35.6, 35.5, 34.2, 28.6, 27.0, 26.1, 24.0, 18.0; IR (neat): ν_{max} 3286, 2924, 1625, 1538, 1374, 700 cm^{-1}; FABMS (NBA/NaI): *m/z* (relative intensity) 937 ($M^+ + Na$, 100), 915 ($M^+ + H$, 72). Anal. ($C_{48}H_{68}B_2N_4O_8S_2$) C, H, N.

(+)-Pinanediol [1*R*)-[*N*-(3-mercaptopropionyl)-L-alanyl]-amino-2-phenylethyl]boronate (26). The disulfide **25** (20 mg, 0.022 mmol) was dissolved in CH_2Cl_2 (1.5 mL). Triethylamine (1.2 µL, 0.009 mmol) and dithiothreitol (3.7 mg, 0.024 mmol) were added. The mixture was stirred for 2 days. The solvent was evaporated and the residue was purified by PLC (CH_2Cl_2:

Et_2O:MeOH:petroleum ether, 3.5:3.5:0.5:1.0) to afford **26** as a colorless oil (14.7 mg, 70%); ^1H NMR ($CDCl_3$, 300 MHz) δ 7.14–7.35 (m, 5H), 6.45 (d, J = 2.5 Hz, 1H), 6.38 (d, J = 7.5 Hz, 1H), 4.53 (dq, J = 7.5, 7.0 Hz, 1H), 4.32 (dd, J = 8.5, 2.0 Hz, 1H), 3.23 (ddd, J = 9.5, 7.0, 5.0 Hz, 1H), 2.98 (dd, J = 14.0, 5.0 Hz, 1H), 2.80 (dd, J = 14.0, 9.5 Hz, 1H), 2.68–2.76 (m, 2H), 2.36–2.54 (m, 2H), 2.32 (m, 1H), 2.15 (m, 1H), 2.00 (m, 1H), 1.80–1.94 (m, 2H), 1.38 (d, J = 7.0 Hz, 3H), 1.37, 1.29 (2s, 6H), 1.22 (d, J = 10.5 Hz, 1H), 0.85 (s, 3H); ^{13}C NMR ($CDCl_3$): δ 174.7, 170.8, 139.6, 129.0, 128.4, 126.3, 84.9, 77.0, 51.6, 47.0, 43.4 (br), 39.6, 39.5, 38.0, 36.9, 35.8, 28.8, 27.1, 26.2, 24.0, 20.5, 17.7; IR (neat): ν_{max} 3201 (br), 2917, 1651, 1538, 1373, 700 cm^{-1}; FABMS (NBA/NaI): *m/z* (relative intensity) 481 (M^+ + Na, 15), 459 (M^+ + H, 10). $C_{24}H_{35}BN_2O_4S$.

(+)-Pinanediol [1(*R*)-amino-2-phenylethyl]boronate hydrochloride (27). Hexamethyl-disilazane (2.8 mL, 13.2 mmol) was dissolved in dry THF (38 mL). The solution was cooled to −78 °C and 1.53 M butyllithium in hexane (8.6 mL, 13.2 mmol) was added slowly. The mixture was then allowed to warm to rt. After 10 min at rt, the LHMDS solution was cooled to −78 °C and (+)-pinanediol [1(*R*)-chloro-2-phenylethyl]boronate (**18**; 3.82 g, 12.0 mmol), dissolved in dry THF (20 mL), was added slowly. The mixture was allowed to warm to rt and stirred overnight. The solvent was evaporated and hexane (150 mL) was added to precipitate LiCl, which was filtered out under nitrogen. The filtrate was cooled to −78 °C and 1 M HCl in Et_2O (36 mL, 36.0 mmol) was added slowly. The solution was allowed to warm slowly to rt and was stirred for 2 h. Compound **27** was isolated by filtration as a white solid (2.40 g). The filtrate was evaporated and hexane (40 mL) was added to afford **27** (0.50 g) as a second crop. The total yield of **27** was 72% (2.90 g): mp 88–89 °C; ^1H NMR (DMSO) δ 8.12 (br s, 3H), 7.22–7.32 (m, 5H), 4.35 (dd, J = 9.0, 2.0 Hz, 1H), 3.03–3.07 (m, 2H), 2.91 (dd, J = 15.5, 10.5 Hz, 1H), 2.28, 2.04, 1.91, 1.82, 1.69, (5m, 5H), 1.29, 1.22 (2s, 6H), 0.94 (d, J = 11.0 Hz, 1H), 0.78 (s, 3H); ^{13}C NMR (DMSO) δ 137.0, 129.2, 128.5, 126.8, 86.8, 77.6, 50.6, 38.8, 37.8, 37.3 (br), 35.2, 34.6, 28.2, 26.8, 25.8, 23.6; IR (KBr) ν_{max} 3449, 3365, 2990 (br), 2073, 1501, 1417, 1394, 1265, 1025, 874, 732, 697 cm^{-1}; FABMS (NBA): *m/z* (relative intensity) 300 (M^+ − Cl, 100). $C_{18}H_{27}BClNO_2$.

Methyl 5-amino-2-[6-methoxy-3-oxo-3*H*-xanthen-9-yl]-benzoate (28). Fluoresceinamine (isomer I; 500 mg, 1.44 mmol) was dissolved in acetone (70 mL). The solution was cooled to 0 °C and a freshly prepared solution of diazomethane in Et_2O (30 mL, ~10.0 mmol) was added dropwise. The solvent was evaporated and the residue was purified by FCC (CH_2Cl_2:MeOH, 7.0:1.0) to afford **28** as a red solid (471 mg, 87%): mp >85 °C (dec); ^1H NMR ($CDCl_3$): δ 7.46 (d, J = 2.0 Hz, 1H), 6.95–7.03 (m, 3H), 6.94 (dd, J = 8.0, 5.5 Hz, 1H), 6.89 (d, J = 2.5 Hz, 1H), 6.72 (dd, J = 9.0, 2.5 Hz, 1H), 6.53 (dd, J = 10.0, 2.0 Hz, 1H), 6.42 (d, J = 2.0 Hz, 1H), 4.45 (br s, 2H), 3.86, 3.53 (2s, 6H); ^{13}C NMR ($CDCl_3$): δ 185.6, 165.9, 163.9, 159.1, 154.2, 152.1, 148.1, 131.4, 131.0, 130.7, 129.1, 122.5, 118.1, 117.6, 116.4, 115.3, 113.2, 105.2, 100.1, 55.8, 52.1; IR (KBr) ν_{max} 3332, 3213, 1718, 1598, 1485 cm^{-1}; FABMS (NBA/NaI): *m/z* (relative intensity) 398 (M^+ + Na, 14), 376 (M^+ + H, 100). $C_{22}H_{17}NO_5$.

Methyl-5-[*N*-[2(*R*),3(*R*)-diacetoxy-4-methoxybutanedioyl]amino]-2-[6-methoxy-3-oxo-3*H*-xanthen-9-yl]-benzoate (29). The amine **28** (471 mg, 1.26 mmol) was dissolved in acetone (50 mL). (+)-Diacetyl-L-tartaric anhydride (542 mg, 2.52 mmol) was added and the mixture was heated at reflux during 7 h. The solvent was evaporated to afford crude acid, which was not further purified but the product from a smaller scale reaction has been characterized as its methyl ester derivative **29** by adding diazomethane at 0 °C and purifying by FCC (CH_2Cl_2:MeOH, 7.0:1.0) to afford an orange solid: mp >135 °C (dec); ^1H NMR ($CDCl_3$, 300 MHz) δ 9.48 (d, J = 6.5 Hz, 1H), 8.30 (dd, J = 31.0, 2.0 Hz, 1H), 8.05 (ddd, J = 31.0, 8.5, 2.5 Hz, 1H), 7.25 (dd, J = 8.0, 6.0 Hz, 1H), 6.98 (s, 1H), 6.94 (dd, J = 7.5, 1.5 Hz, 1H), 6.80 (dd, J = 10.0, 7.0 Hz, 1H), 6.75 (dd, J = 9.0, 2.5 Hz, 1H), 6.49 (dd, J = 2.0, 1.0 Hz, 1H), 6.40 (ddd, J = 10.0, 3.0, 2.0 Hz, 1H), 5.96 (d, J = 2.5 Hz, 1H), 5.80 (dd, J = 2.5, 1.0 Hz, 1H), 3.95, 3.82 (2s, 6H), 3.60 (d, J = 9.0 Hz, 3H), 2.32 (s, 3H), 2.20 (d, J = 1.0 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ 185.5, 169.6, 169.5, 167.1, 165.0, 164.9, 164.4, 159.2, 154.3, 151.5, 138.9, 131.0, 130.6, 130.4, 130.0, 129.3, 129.1, 124.4, 122.8, 117.3, 114.8, 113.6, 105.4, 100.2, 72.0, 71.2, 55.9, 52.8, 52.3, 20.6, 20.4; IR (neat): ν_{max} 2954, 1757, 1598, 1511, 1213, 1110, 1073, 731 cm^{-1}; FABMS (NBA, CsI): *m/z* (relative intensity) 738 (M^+ + Cs, 15), 606 (M^+ + H, 100). $C_{31}H_{27}NO_{12}$.

Methyl 5-[*N*-[3-carboxy-2(*R*),3(*R*)-dihydroxypropionyl]-amino]-2-[6-methoxy-3-oxo-3*H*-xanthen-9-yl]benzoate (30). The above crude acid was redissolved in MeOH (20 mL) and K_2CO_3 (350 mg, 2.52 mmol) was added: the potassium salt slowly fell out. After 3 h at rt, the solvent was evaporated. The residue was retaken in H_2O (25 mL), washed with AcOEt (10 mL) and neutralized to pH 6.0 with 2 N HCl. The sample was freeze-dried. The residual powder was retaken in dry MeOH (15 mL) and the insoluble salts were centrifuged out at 3500 rounds/min for 10 min. After decanting, the solvent was evaporated and the residue redissolved in H_2O (10 mL). After freeze-drying, **30** was obtained as an orange solid (255 mg, 40%), which was attached to bovine serum albumin (BSA) without further purification; ^1H NMR (DMSO): δ 10.48 (s, 1H), 8.75 (s, 1H), 8.19 (d, J = 8.5 Hz, 1H), 7.39 (d, J = 8.5 Hz, 1H), 7.20 (d, J = 1.0 Hz, 1H), 6.86–6.93 (m, 3H), 6.37 (dd, J = 11.0, 1.0 Hz, 1H), 6.22 (d, J = 1.0 Hz, 1H), 4.48, 3.95 (2d, J = 1.5 Hz, 2H), 3.90, 3.57 (2s, 6H); ^{13}C NMR (MeOD): δ 187.0, 181.0, 178.3, 174.4, 167.2, 167.0, 161.3, 156.6, 156.3, 132.6, 132.2, 131.8, 130.7, 130.5, 129.0, 125.6, 123.7, 117.9, 116.0, 115.9, 105.3, 101.4, 75.0, 74.7, 57.2, 53.3; IR (KBr) ν_{max} 3429 (br), 1569, 1414 cm^{-1}; FABMS (negative ion spray): *m/z* (relative intensity) 506 ([M − H^+]-, 100). $C_{26}H_{21}NO_{10}$.

Diol cleavage conditions

The fluoresceinamine derivative **30** was coupled (via the *N*-hydroxysuccinimide ester) to the carrier protein bovine serum albumin (BSA; 5 mg/mL). The residual free hapten **30** was dialysed through a Spectra/Por membrane (cut off 12,000–14,000, size 10×6.4 mm) until no color change was noticed ($\sim$48 h). The yellowish color as well as the typical UV absorption of hapten **30** at 460 nm provided substantial evidence for the presence of linked hapten **30**. Sodium periodate (14 mg, 0.07 mmol) was added and the mixture was stored at rt for 5 h. The mixture was dialysed again under identical conditions: the solution decolorized completely, the UV absorption at 460 nm disappeared, indicating that the cleavage had occurred.

Acknowledgements

We thank the National Institute of Health (PO CA27489) for their support of this work. C.F.B. is the recipient of an Investigator Award from the Cancer Research Institute.

References and Notes

1. (a) Lerner, R. A.; Benkovic, S. I.; Schultz, P. G. *Science* **1991**, *252*, 659; (b) Lerner, R. A.; Schultz, P. G. *Acc. Chem. Res.* **1993**, *26*, 391; (c) Wagner, J.; Lerner, R. A.; Barbas, III C. F. *Science* **1995**, *270*, 1797.

2. (a) Tramontano, A.; Ammann, A A.; Lerner, R. A. *J. Am. Chem. Soc.* **1988**, *110*, 2282; (b) Shokat, K. M.; Ko, M. K.; Scanlan, T. S.; Kochersperger, L.; Yonkovich, S.; Thaisrivongs, S.; Schuyltz, P. G. *Angew. Chem. Int. Ed. Engl.* **1990**, *29*, 1296; (c) Landry, D. W.; Zhao, K.; Yang, G. X.-Q.; Glickman, M.; Georgiadis, T. M. *Science* **1993**, *259*, 1899; (d) Campbell, D. A.; Gong, B.; Kochersperger, L. M.; Yonkovich, S.; Gallop, M. A.; Schultz, P. G. *J. Am. Chem. Soc.* **1994**, *116*, 2165; (e) Guo, J.; Huang, W.; Scanlan, T. S. *J. Am. Chem. Soc.* **1994**, *116*, 6062.

3. Janda, K. D.; Schloeder, D.; Benkovic, S. J.; Lerner, R. A. *Science* **1988**, *241*, 1189.

4. Martin, M. T.; Angeles, T. S.; Sugasawara, R.; Aman, N. I.; Napper, A. D.; Darsley, M. J.; Sanchez, R. I.; Booth, P.; Titmas, R. C. *J. Am. Chem. Soc.* **1994**, *116*, 6508.

5. Iverson, B. L.; Lerner, R. A. *Science* **1989**, *243*, 1184.

6. (a) Barbas, III; C. F.; Bain, J. D.; Hoekstra, D. M.; Lerner, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*; 9339; (b) Lerner, R. A.; Kang, A. S.; Bain, J. D.; Burton, D. R.; Barbas, III; C. F. *Science* **1992**, *258*, 1313; (c) Barbas, III; C. F.; Rosenblum, J. S.; Lerner, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *14*, 6385; (d) Barbas, III; C. F.; Amberg, W.; Sominesitz, A.; Jones, T. M.; Lerner, R. A. *Gene* **1993**, *137*, 57, 62; (e) Barbas, III; C. F. *Curr. Opin. Biotech.* **1993**, *4*, 526; (f) Barbas, S. M.; Ghazal, P.; Barbas, III; C. F.; Burton, D. R. *J. Am. Chem. Soc.* **1994**, *116*, 2161; (g) Janda, K. D.; Lo, C.-H. L.; Li, T.; Barbas, III; C. F.; Wirsching, P.; Lerner, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 2532.

7. (a) Suga, H.; Ersoy, O.; Williams, S. F.; Tsumuraya, T.; Margolies, M. N.; Sinskey, A. J.; Masamune, S. *J. Am. Chem. Soc.* **1994**, *116*, 6025; (b) Suga, H.; Ersoy, O.; Tsumuraya, T.; Lee, J.; Sinskey, A. J.; Masamune, S. *J. Am. Chem. Soc.* **1994**, *116*, 487.

8. (a) Drenth, J.; Kalk, K. H.; Swen, H. M. *Biochemistry* **1976**, *15*, 3731; (b) Brocklehurst, K.; Malthouse, J. P. G.; Shipton, M. *Biochem. J.* **1979**, *183*, 223; (c) Kamphuis, I. G.; Kalk, K. H.; Swarte, M. B. A.; Drenth, J. *J. Mol. Biol.* **1984**, *179*, 233.

9. (a) Robertus, J. D.; Kraut, J.; Alden, R. A.; Birktoft, J. J. *Biochemistry* **1972**, *11*, 4293; (b) deHaen, C.; Neurath, H.; Teller, D. C. *J. Mol. Biol.* **1975**, *92*, 225.

10. Arad, D.; Langridge, R.; Kollman, P. A. *J. Am. Chem. Soc.* **1990**, *112*, 491.

11. Carter, P.; Wells, J. A. *Nature* **1988**, *332*, 564.

12. (a) Bryan, P.; Pantoliano, M. W.; Quill, S. G.; Hsiao, H.-Y.; Poulos, T. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 3743; (b) Menard, R.; Carriere, J.; Laflamme, P.; Plouffe, C.; Khouri, H. E.; Vernet, T.; Tessier, D. C.; Thomas, D. Y.; Storer, A. C. *Biochemistry* **1991**, *30*, 8934.

13. Carter, P.; Wells, J. A. *Science* **1987**, *237*, 394.

14. (a) *Proteinase Inhibitors*; Barrett, A. J.; Salvesen, G., Eds.; Elsevier: New York, 1986; pp 55–178; (b) Fischer, G. *Natl. Prod. Rep.* **1988**, 465; (c) Shaw, E. *Adv. Enzymol.* **1990**, *63*, 271; (d) Demuth, H.-U. J. *Enzyme Inhib.* **1990**, *3*, 249.

15. (a) Green, G. D. J.; Shaw, E. *J. Biol. Chem.* **1981**, *256*, 1923; (b) Shaw, E.; Wikstrom, P.; Ruscica, J. *Arch. Biochem. Biophys.* **1983**, *222*, 424; (c) Zumbrunn, A.; Stone, S.; Shaw, E. *Biochem. J.* **1988**, *250*, 621; (d) Crawford, C.; Mason, R. W.; Wikstrom, P.; Shaw, E. *Biochem. J.* **1988**, *253*, 751; (e) Hall, A.; Abrahamson, M.; Grubb, A.; Trojnar, J.; Kania, P.; Kasprsykowska, R.; Kasprsykowska, F. J. *Enzyme Inhibition* **1992**, *6*, 113.

16. Tarnowska, M.; Oldziej, S.; Liwo, A.; Kania, P.; Kasprzykowski, F.; Grzonka, Z. *Eur. Biophys. J.* **1992**, *21*, 217.

17. (a) Brocklehurst, K.; Malthouse, P. G. *Biochem. J.* **1978**, *175*, 761; (b) Sinnott, M. L. *CRC Crit. Rev. Biochem.* **1982**, 327.

18. (a) Kettner, C. A.; Shenvi, A. B. *J. Biol. Chem.* **1984**, *259*, 15106; (b) Kettner, C.; Mersinger, L.; Knabb, R. *J. Biol. Chem.* **1990**, *265*, 18289; (c) Elgendy, S.; Deadman, J.; Patel, G.; Green, D.; Chino, N.; Goodwin, C. A.; Scully, M. F.; Kakkar, V. V.; Claeson, G. *Tetrahedron Lett.* **1992**, *33*, 4209.

19. (a) Matteson, D. S.; Sadhu, K. M.; Lienhard, G. E. *J. Am. Chem. Soc.* **1981**, *103*, 5241; (b) Matteson, D. S.; Jesthi, P. K.; Sadhu, K. M. *Organometallics* **1984**, *3*, 1284; (c) Matteson, D. S. *Chem. Rev.* **1989**, *89*, 1535.

20. (a) Bone, R.; Shenvi, A. B.; Kettner, C. A.; Agard, D. A. *Biochemistry* **1987**, *26*, 7609; (b) Bone, R.; Frank, D.; Kettner, C. A.; Agard, D. A. *Biochemistry* **1989**, *28*, 7600.

21. (a) Bachovchin, W. W.; Wong, W. Y. L.; Farr-Jones, S.; Shenvi, A. B.; Kettner, C. A. *Biochemistry* **1988**, *27*, 7689; (b) Farr-Jones, S.; Smith, S. O.; Kettner, C. A.; Griffin, R. G.; Bachovchin, W. W. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 6922; (c) Zhong, S.; Jordan, F.; Kettner, C. A.; Polgar, L. *J. Am. Chem. Soc.* **1991**, *113*, 9429; (d) Tsilikounas, E.; Kettner, C. A.; Bachovchin, W. W. *Biochemistry* **1992**, *31*, 12839; (e) Tsilifounas, E.; Kettner, C. A.; Bachovchin, W. W. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *32*, 12651.

22. Oleksyszyn, J.; Subotkowska, L.; Mastalerz, P. *Synthesis* **1979**, 985.

23. Kafarski, P.; Lejczak, B.; Szewczyk, J. *Can. J. Chem.* **1983**, *61*, 2425.

24. Chambers, J. R.; Isbell, A. F. *J. Org. Chem.* **1964**, *29*, 832.

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

26. (a) Campbell, D. A. *J. Org. Chem.* **1992**, *57*, 6331; (b) Campbell, D. A.; Bermak, J. C. *J. Org. Chem.* **1994**, *59*, 658; (c) Malachowski, W. P.; Conrad, J. K. *J. Org. Chem.* **1994**, *59*, 7616.

27. Leary, R.; Larsen, D.; Watanabe, H.; Shaw, E. *Biochemistry* **1977**, *16*, 5857.

28. (a) DeWald, H. A.; Moore, A. M. *J. Org. Chem.* **1957**, *80*, 3941; (b) Handschumacher, R. E.; Bates, C. J.; Chang, P. K.; Andrews, A. T.; Fischer, G. A. *Science* **1968**, *161*, 62.

29. (a) Matteson, D. S.; Sadhu, K. M. *Organometallics* **1984**, *3*, 614; (b) Sylvia, L. A.; Gerig, J. T. *Biochim. Biophys. Acta.* **1993**, *1163*, 321. See also ref 19.

30. Lomant, A. J.; Fairbanks, G. *J. Mol. Biol.* **1976**, *104*, 243.

31. (a) Kelly, T. A.; Adams, J.; Bachovchin, W. W.; Barton, R. W.; Campbell, S. J.; Coutts, S. J.; Kennedy, C. A.; Snow, R. J. *J. Am. Chem. Soc.* **1993**, *115*, 12637; (b) Snow, R. J.; Bachovchin, W. W.; Barton, R. W.; Campbell, S. J.; Coutts, S. J.; Freeman, D. M.; Gutheil, W. G.; Kelly, T. A.; Kennedy, C. A.; Krolikowski, D. A.; Leonard, S. F.; Pargellis, C. A.; Tony, L.; Adams, J. *J. Am. Chem. Soc.* **1994**, *116*, 10860.

32. (a) Geoghegan, K. F.; Stroh, J. G. *Bioconj. Chem.* **1992**, *3*, 138; (b) Marburg, S.; Flanagan, M. E.; Tolman, R. L. *Analyt. Biochem.* **1989**, *181*, 242.

33. Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923.

34. Butler, C. L.; Cretcher, L. H. *J. Am. Chem. Soc.* **1933**, *55*, 2605.

35. '*R*' and '*S*' refer to the configuration of the carbon α to phosphorus. **2-'*R*'** and **2-'*S*'** can best be distinguished between: δ 5.7 and 6.0 by ^{1}H NMR (500 MHz).

36. This procedure was adapted from ref 23. They reported the following values using the L-tartaric acid derivative as a chiral auxiliary: **2-'*S*'** mp 160–161.5 °C, $[\alpha]_{578}^{20} -52 \pm 1°$; **2-'*R*'** mp 79–80 °C, $[\alpha]_{578}^{20} -47 \pm 1°$.

37. Previously observed optical rotations are: $[\alpha]_{578}^{21} -49 \pm 1°$ (*c* 1; 1 N NaOH), mp 267–268 °C, see ref 23; $[\alpha]_{589}^{21} -45.2°$ (*c* 2; 2 N NaOH), see ref 25; $[\alpha]_{589}^{20} -38.9°$ (*c* 2.0; 2 N NaOH); Kotynski, A.; Stec, W. J. *J. Chem. Res. (S)* **1978**, 41.

38. $[\alpha]_{589}^{21} -46°$ (*c* 2; EtOH) and all other characteristics of the Cbz-protected amine and **3a** were in agreement with ref 25.

39. $[\alpha]_{589}^{21} -46.9°$ (*c* 1, EtOH) was reported in ref 25.

40. This signal slowly disappeared through proton–deuterium exchange.

(*Received in U.S.A.* 27 *January* 1996; *accepted* 18 *March* 1996)