Macrocyclic Inhibitors of Penicillopepsin. 1. Design, Synthesis, and Evaluation of an Inhibitor Bridged between P1 and P3

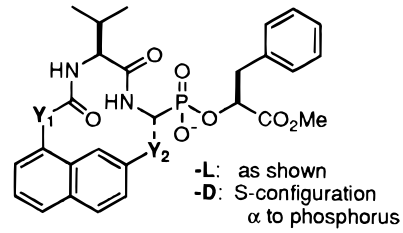
J. Hoyt Meyer and Paul A. Bartlett*

Contribution from the Department of Chemistry, University of California, Berkeley, California 94720-1460

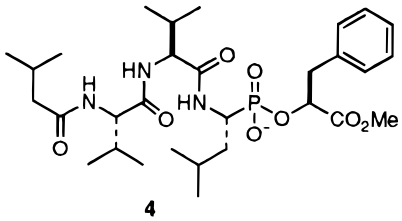
Received October 27, 1997

Abstract: The macrocyclic peptidyl phosphonate **1-L** was designed on the basis of the conformation of an acyclic analogue (**4**) bound to the aspartic protease penicillopepsin. This material and the two acyclic comparison compounds **2-L** and **3** were synthesized and evaluated as inhibitors; their binding affinity was found to be inversely related to the degree of conformational flexibility across the series: **3** (K_i = 110 μM), **2-L** (K_i = 7.6 μM), **1-L** (K_i = 0.80 μM). NMR methods in conjunction with molecular modeling were used to assign the stereochemical configurations of the precursor **16-L** and its diastereomer **16-D** and to determine the solution conformations of the macrocyclic ring systems. The conformation of the peptide backbone in **1-L** closely approximates that desired for a mimic of the lead inhibitor **4**, and it appears that the low-energy conformation of **1-L** can be accommodated in the pencillopepsin active site without significant distortion.

An important step in the rational design of enzyme inhibitors is the optimization of a known ligand using structural information from the enzyme−ligand complex. The aim of such "second-generation" designs may be to enhance the hydrophobic or electrostatic interactions between inhibitor and target, or to reduce the conformational flexibility of the ligand. If the predominant conformation of the free ligand is the same as it adopts in the binding site, the entropic disadvantage of complex formation is reduced and its affinity enhanced in comparison to a more conformationally mobile analogue. Conformational constraints have often been introduced in peptidase inhibitors by macrocyclization between the side chains, with recent examples in both zinc and aspartic proteases.[1,2] These constraints can lead to analogues of enhanced potency or selectivity; however, in only a few cases have the effects of the macrocyclic constraints on binding affinity or conformation been evaluated explicitly, and structurally related acyclic analogues are not often available to allow a quantitative comparison to be made. Both the structural and energetic effects of a macrocyclic constraint need to be determined to assess the success of the design. This report describes the design and synthesis of the macrocyclic phosphonate **1** and the acyclic analogues **2** and **3**, their evaluation as inhibitors of the aspartic protease penicillopepsin, and the determination of the solution conformations of the macrocyclic ring system in **1**. Accompanying papers report crystallographic analyses of the complexes of all three inhibitors with the enzyme[3] and the parallel investigation of a different macrocyclic inhibitor design for the same peptidase.[4]



1: $Y_1 = Y_2 = CH_2$
2: Y_1 = H,H, $Y_2 = CH_2$
3: $Y_1 = CH_2$, Y_2 = H,H



4

Design. Phosphorus-containing peptide analogues are known as potent inhibitors of the aspartic proteases.[5−7] Some phos-

(1) For leading references, see: Ksander, G. M.; de Jesus, R.; Yuan, A.; Ghai, R. D.; Trapani, A.; McMartin, C.; Bohacek, R. *J. Med. Chem.* **1997**, *40*, 495. Ksander, G. M.; de Jesus, R.; Yuan, A.; Ghai, R. D.; McMartin, C.; Bohacek, R. *J. Med. Chem.* **1997**, *40*, 506. March, D. R.; Abbenante, G.; Bergman, D. A.; Brinkworth, R. I.; Wickramasinghe, W.; Begun, J.; Martin, J. L.; Fairlie, D. P. *J. Am. Chem. Soc.* **1996**, *118*, 3375. Ettmayer, P.; Billich, A.; Hecht, P.; Rosenwirth, B.; Gstach, H. *J. Med. Chem.* **1996**, *39*, 3291. Podlogar, B. L.; Farr, R. A.; Friedrich, D.; Tarnus, C.; Huber, E. W.; Cregge, R. J.; Schirlin, D. *J. Med. Chem.* **1994**, *37*, 3684. Weber, A. E.; Steiner, M. G.; Krieter, P. A.; Colletti, A. E.; Tata, J. R.; Halgren, T. A.; Ball, R. G.; Doyle, J. J.; Schorn, T. W.; Stearns, R. A.; Miller, R. R.; Siegl, P. K. S.; Greenlee, W. J.; Patchett, A. A. *J. Med. Chem.* **1992**, *35*, 3755.

(2) Morgan, B. P.; Bartlett, P. A.; Holland, D. R.; Matthews, B. W. *J. Am. Chem. Soc.* **1994**, *116*, 3251.

(3) Ding, J.; Fraser, M.; Meyer, J. H.; Bartlett, P. A.; James, M. N. G. *J. Am. Chem. Soc.* **1998**, *120*, 4610.

(4) Smith, W. W.; Bartlett, P. A. **1998**, *120*, 4622.

(5) Bartlett, P. A.; Kezer, W. B. *J. Am. Chem. Soc.* **1984**, *106*, 4282. Williams, P. D.; Perlow, D. S.; Payne, L. S.; Holloway, M. K.; Siegl, P. K. S.; Schorn, T. W.; Lynch, R. J.; Doyle, J. J.; Strouse, J. F.; Vlasuk, G. P.; Hoogsteen, K.; Springer, J. P.; Bush, B. L.; Halgren, T. A.; Richards, A. D.; Kay, J.; Veber, D. F. *J. Med. Chem.* **1991**, *34*, 887. Thorsett, E. D.; Harris, E. E.; Aster, S. D.; Peterson, E. R.; Snyder, J. P. *J. Med. Chem.* **1986**, *29*, 251. Allen, M. C.; Fuhrer, W.; Tuck, B.; Wade, R.; Wood, J. M. *J. Med. Chem.* **1989**, *32*, 1652. Grobelny, D.; Wondrak, E. M.; Galardy, R. E.; Oroszlan, S. *Biochem. Biophys. Res. Commun.* **1990**, *169*, 1111. Peyman, A.; Budt, K.-H.; Spanig, J.; Stowasser, B.; Ruppert, D. *Tetrahedron Lett.* **1992**, *33*, 4549. Abdel-Meguid, S. S.; Zhao, B.; Murthy, K. H. M.; Winborne, E.; Choi, J.-K.; DesJarlais, R. L.; Minnich, M.; Culp, J. S.; Debouck, C.; Tomaszek, T. A., Jr.; Meek, T. D.; Dreyer, G. B. *Biochemistry* **1993**, *32*, 7972. Raddatz, P.; Minck, K.-O.; Rippmann, F.; Schmitges, C. J. *J. Med. Chem.* **1994**, *37*, 486.

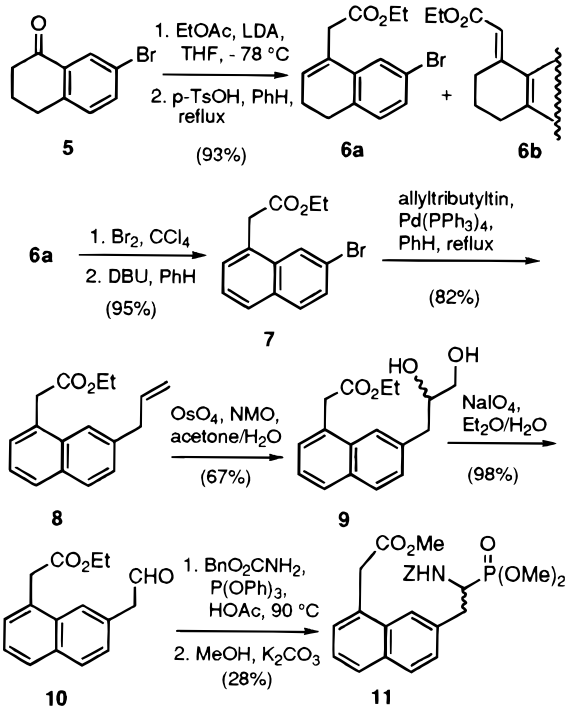
S0002-7863(97)03715-3 CCC: $15.00
Published on Web 04/30/1998

phonate penta- and hexapeptides inhibit penicillopepsin and pepsin with K_i values as low as 250 pM,[6] and a series of phosphonate tetrapeptides were recently shown to be transition-state analogues for pepsin.[7] The X-ray crystal structure of the complex between penicillopepsin and the phosphonate pentapeptide **4** ($K_i = 2.8$ nM) was determined by Fraser et al. and refined to an R value of 0.131 at 1.7 Å resolution.[8] The tetrahedral phosphonate of **4** is oriented between the catalytic aspartates of the enzyme, in imitation of the tetrahedral intermediate for substrate hydrolysis, and the backbone of the inhibitor is bound in the extended conformation that is typical for aspartic peptidase ligands. A salient feature of this conformation is the proximity of the side chains on alternating residues (e.g., P3 and P1, and P2 and P1′); these side chains share extended, hydrophobic binding pockets on opposite sides of the peptide, and there is room to connect them together.[9]

To design a bridge to link the P1 and P3 residues of **4**, CAVEAT was used to identify structural frameworks that match the Cα−Cβ bonds,[10] and standard modeling and minimization programs were used to elaborate and assess different possibilities. The naphthalene-bridged macrocycle of **1** was selected not only for its ability to adopt the desired conformation but also because of its rigidity and synthetic accessibility. The acyclic comparison compounds **2** and **3** were designed to distinguish the effect of macrocyclization from that of the hydrophobic bridging unit itself. These control compounds arise from replacement of methylene units with hydrogens at two positions in the macrocycle. The intent of this substitution was to enable the acyclic analogues to adopt conformations in the active site similar to the macrocycle, in contrast to the situation if one of the ring bonds were simply replaced with hydrogens. The difference in the C−C bond distance and the C−C separation of two C−H groups at van der Waals contact requires that the carbon atoms move apart by ca. 2.5 Å and could result in a different binding orientation in the active site, as is indeed observed for a macrocyclic thermolysin inhibitor and its seco analogue.[2] Even replacement of the methylene unit with two hydrogens leads to an increase in the distance between the remaining carbons of ca. 1.4 Å; however, the macrocyclic structure of **1** cannot lose two adjacent carbons and maintain the necessary connectivity between the remaining functionality.

Synthesis. The key unit for construction of the macrocyclic inhibitor **1** was the α-amino phosphonic acid derivative **11**, which was prepared by the Oleksyszyn procedure[11] from a suitably functionalized naphthaleneacetaldehyde precursor as shown in Scheme 1. Addition of ethyl lithioacetate to 7-bromo-2-tetralone **5** followed by dehydration of the tertiary alcohol afforded the endo- and exo-alkenes **6a** and **6b**. The mixture could be aromatized by dehydrogenation with palladium; however, this procedure resulted in considerable debromination. A two-step process involving bromine addition followed by double elimination with 1,8-diazabicyclo[5.4.0]undec-2-ene (DBU) is an effective alternative for producing the 7-bromonaphthalene-1-acetate ester **7**; both isomers of **6** can be

Scheme 1



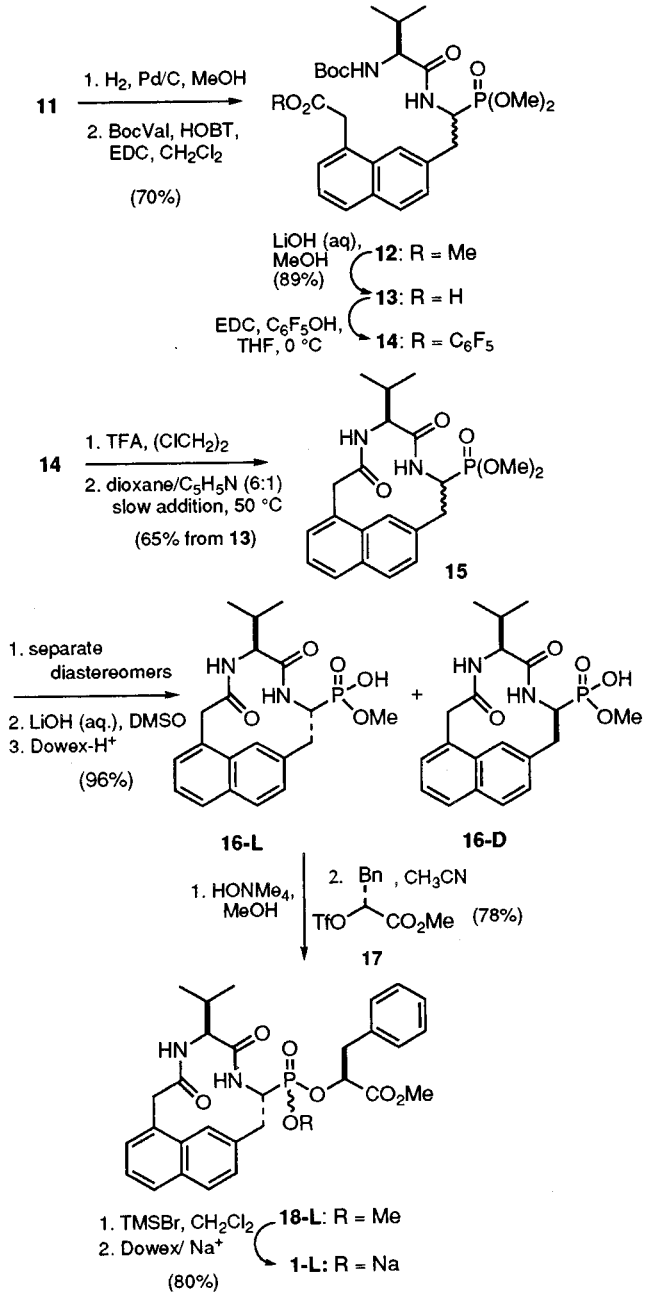
carried through the addition, elimination, isomerization sequence, although the product is cleaner starting with the endo isomer. Replacement of the bromine with an allyl group followed by two-step oxidative cleavage provided the naphthaleneacetaldehyde precursor **10** in good overall yield. Condensation with benzyl carbamate and triphenyl phosphite[11] followed by exchange of all esters for methyl was accomplished in modest yield to give the key intermediate **11**.

Phosphonate **11** was incorporated into the macrocycle as shown in Scheme 2. Standard procedures were employed to replace the Cbz group with Boc-valine and to convert the carboxylate methyl ester to the pentafluorophenyl ester **14**. After cleavage of the Boc group with trifluoroacetic acid (TFA), slow addition of the activated ester to dioxane and pyridine at 50 °C gave the macrocycle **15** as a mixture of diastereomers. After chromatographic separation, the two isomers were individually hydrolyzed to the monoesters **16-L** and **16-D** and coupled with methyl β-phenyllactate to give the corresponding diastereomers of **18**. Several procedures were investigated for this coupling step, including activation of **16** with diphenyl phosphoryl azide or benzotriazole-1-yloxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP), or condensation with methyl D-β-phenyllactate under Mitsunobu conditions.[12] Displacement of the triflate ester of methyl D-β-phenyllactate (**17**) with the tetramethylammonium salt of **16** proved to be the most reproducible and highest yielding. Synthesis of the macrocyclic inhibitors **1-L** and **1-D** was completed by cleavage of the phosphonate ester with trimethylsilyl bromide[13] and cation exchange. The absolute configurations of these analogues were assigned by NMR methods as described below.

Syntheses of the two acyclic comparison compounds are depicted in Scheme 3. The β-2-naphthylalanine phosphonate unit was built up from *tert*-butyl (dimethylphosphono)acetate and 2-(bromomethyl)naphthalene, with Schmidt degradation of the carboxyl group to provide the amino moiety. After introduction of the valine residue and formylation, the L- and

(6) Bartlett, P. A.; Hanson, J. E.; Giannousis, P. P. *J. Org. Chem.* **1990**, *55*, 6268.
(7) Bartlett, P. A.; Giangiordano, M. A. *J. Org. Chem.* **1996**, *61*, 3433.
(8) Fraser, M. E.; Strynadka, N. C. J.; Bartlett, P. A.; Hanson, J. E.; James, M. N. G. *Biochemistry* **1992**, *31*, 5201.
(9) Blundell, T. L.; Sibanda, B. L.; Hemmings, A.; Foundling, S. F.; Tickle, I. J.; Pearl, L. H.; Wood, S. P. In *Molecular Graphics and Drug Design*; Burgen, A. S. V., Roberts, G. C. K. , Tute, M. S., Eds.; Elsevier Science Publishers: Amsterdam, The Netherlands, 1986; p 321.
(10) Lauri, G.; Bartlett, P. A. *J. Comput.-Aided Mol. Design* **1994**, *8*, 51.
(11) Oleksyszyn, J.; Subotkowska, L.; Mastalerz, P. *Synth. Commun.* **1979**, 985.
(12) Campbell, D. A.; Bermak, J. C. *J. Org. Chem.* **1994**, *59*, 658.
(13) McKenna, C. E.; Higa, M. T.; Cheung, N. H.; McKenna, M.-E. *Tetrahedron Lett.* **1977**, 155.

Scheme 2

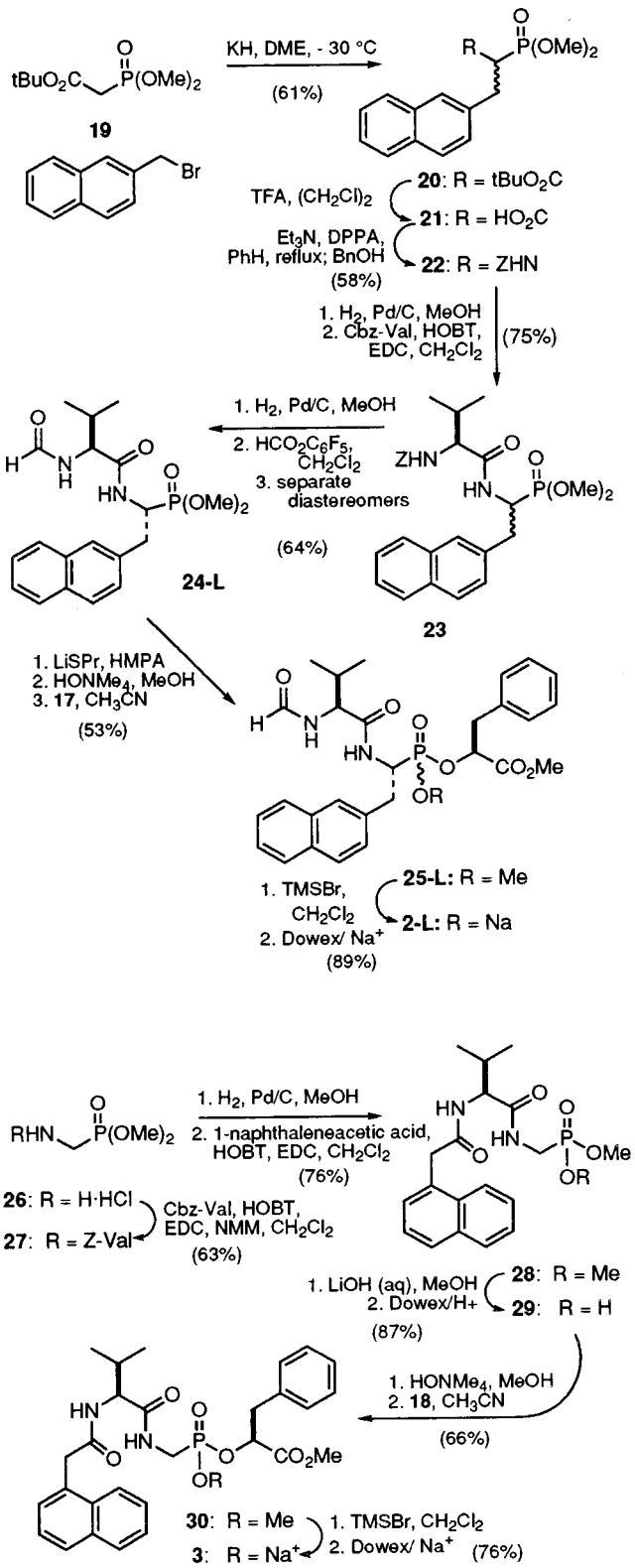


D-diastereomers of **24** were separated by chromatography. Deformylation competes with hydrolysis of the phosphonate ester under alkaline conditions; however, the ester is cleaved selectively under nucleophilic conditions with lithium propanethiolate in HMPA.[14] Formation of the β-phenyllactate ester, phosphonate ester cleavage, and isolation of the acyclic analogues **2-L** and **2-D** were accomplished as described above for the macrocyclic inhibitor. Deformylation was also a significant side reaction in the triflate alkylation step, but it could be minimized by using only a slight excess of triflate, limiting the reaction time, and purifying the product immediately after workup. The configurations of the diastereomers of **2** were inferred from their inhibition constants, as described below. The other comparison compound, **3**, was prepared from the phosphonate analogue of glycine, **26**, and 1-naphthaleneacetic acid using similar procedures (Scheme 3).

Inhibition of Penicillopepsin. The inhibition constants K_i were determined for the cyclic and acyclic phosphonates by

(14) Bartlett, P. A.; Johnson, W. S. *Tetrahedron Lett.* **1970**, 4459.

Scheme 3



established assay procedures using Ac-Ala-Ala-Lys-(*p*-nitro)-Phe-Ala-Ala-NH_2 as substrate (Table 1).[6] Although each inhibitor carries a significant amount of hydrophobic functionality, the phosphonate anion conveys adequate solubility at the assay pH of 3.5. Not surprisingly, there is a large difference in activity between the diastereomeric inhibitors **1-L** and **1-D** and between **2-L** and **2-D**. Our inference that the more inhibitory analogues have the natural L-configuration at the stereocenter adjacent to phosphorus was confirmed in the case of the

Table 1. Inhibition of Penicillopepsin by Acyclic and P1−P3-Linked Cyclic Phosphonates[a]

inhibitor	K_i (μM)
1-D	>1000
16-D	>1000
16-L	>1000
2-D	130
3	110
2-L	7.6
1-L	0.80

[a] Determined at 25 °C, pH 3.5, in 100 mM NaOAc.

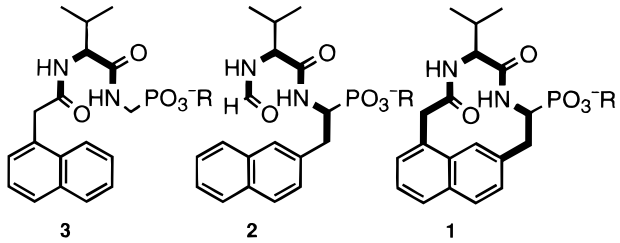


Figure 1. Bonds with significant conformational constraint.

macrocycles by NMR studies of the precursors **16-L** and **16-D**. Of the active inhibitors, the macrocycle **1-L** binds an order of magnitude more strongly than the acyclic comparison compound **2-L** and more than 2 orders of magnitude tighter than **3**. The importance of the P′ residues is underscored by the absence of inhibition observed for the macrocyclic analogues **16** which lack the β-phenyllactate residue.

Analysis. The inhibitory potency across the series **3** → **2-L** → **1-L** parallels the degree to which conformational freedom is reduced in the progression from linear to branched to cyclic topologies. Attachment of the 2-naphthylmethyl group to the middle of the chain, as in **2-L**, restricts its conformational mobility, as well as that of the neighboring bonds in the peptide backbone, to a much greater extent than occurs when this group is attached at the end of the chain, as in **3**. Cyclization of the molecule to **1-L** thus results in a larger loss of flexibility for **3** than for **2-L** (Figure 1).

Implicit in any quantitative assessment of the entropic factor in these binding differences is the assumption that the inhibitors bind in the same (congruent) orientation and share the same interactions with the enzyme and with solvent. In designing these analogues, we expected that the backbone atoms of the phosphonate peptides would adopt the same orientation in the active site as that found for the linear phosphonate **4** and related inhibitors.[8] Since the hydrophobic S1 and S3 binding pockets are well-defined, we considered it unlikely that the **1-L** or **2-L** side chains would bind in abnormal orientations. We were less confident of the bound conformation of the linear inhibitor **3**, since the penicillopepsin active site opens up near the S3 site and the naphthyl moiety could extend outward, rather than folding around toward the S1 pocket. As reported in the accompanying structural paper,[3] the conformations of the macrocycle **1-L** and acycle **2-L** in the enzyme active site proved to be comparable, but not identical, while that of **3** turned out to be quite different. Thus, the observed differences in affinity between the three inhibitors represent lower limits to the true differences were they to adopt congruent orientations.

The acyclic inhibitors **2** and **3** differ in composition from the cyclic analogue **1** by a carbon atom; that is, a methylene unit is replaced by two hydrogens. For comparison, the van der Waals surface of ethylbenzene is 183 Å^2, while benzene + methane have 194 Å^2 of surface area when they are in van der Waals contact and 209 Å^2 when they are separated (Figure 2). This difference is equivalent to that between the cyclic and acyclic inhibitors, showing that the latter have *higher* hydrophobic surface areas than the former. Thus, the enhanced binding of the macrocycle **1-L** over the controls **2-L** and **3** cannot be attributed to differences in hydrophobic surface area; indeed, this difference should favor the acyclic analogues by 0.5−1 kcal/mol.[15]

NMR Structure Determination. The degree to which the conformation of an unbound inhibitor corresponds to that in the binding site is also important, since the entropic advantage of a constrained inhibitor may be negated by the enthalpic penalty from a high-energy bound conformation. The solution structures of the macrocycles **16** and **1** were therefore determined using a combined 1D ^{1}H NMR and molecular modeling approach,[16] both to determine their conformations as well as to assign the configuration adjacent to phosphorus. 1D ^{1}H NMR spectra were obtained at several temperatures in pH 3.5 buffer to simulate the assay conditions. The nonaromatic resonances corresponding to the backbone hydrogens were assigned on the basis of coupling patterns and chemical shift (Table 2). A TOCSY experiment with DANTE water suppression was used to assign the amide hydrogens of **1-D** and **16-D**, since coupling of the NH of naphthylalanine phosphonate (Nala^P) to phosphorus was not observed. The diastereotopic hydrogens at the naphthalene benzylic positions could not be assigned stereospecifically.

From the 1D ^{1}H coupling constants, possible ϕ angles were calculated from the appropriate Karplus relationship (Figure 3). The αH−βH coupling patterns of the Nala^P moieties in both **16-L** and **16-D** indicate well-defined conformations for the macrocyclic rings with a χ_1 angle of +60° or −60°; the large coupling constants between valine αH and βH in turn suggest a predominant anti relationship between these hydrogens. The chemical shift dependencies ($\Delta\delta/\Delta$T) for the amide hydrogens indicate that they are not significantly shielded from solvent, as would be expected if they participate in intramolecular hydrogen bonds.

Molecular modeling was used to generate potential solution conformations for **16-L** and **16-D** that were energetically reasonable and in accord with the observed NMR spectra. Since the configuration adjacent to phosphorus was ambiguous, two separate Monte Carlo conformational searches were conducted for each diastereomer, applying each set of the experimentally determined ϕ angles to the (*R*)- and (*S*)-configurations. The trans geometry was deduced for both of the amide bonds in each macrocycle, since searches with the cis configurations in either or both of them yielded structures from 7 to 20 kcal/mol higher in energy than the minima for the all-trans conformers. The conformations generated from the four searches were clustered according to the backbone atoms, amide oxygens, and phosphorus atom. Three distinct families of structures within 5 kcal/mol of the global minimum and which satisfy the NMR data were obtained for the compound that proved to be **16-L** (Figure 4a); similarly, six families were found for **16-D** (Figure 4b). However, the configurations of the two isomers could not be determined unambiguously from these data alone: with each set of NMR data, low-energy conformations were found for both the (*R*)- and (*S*)-structures.

The stereochemical ambiguity was resolved by the NOESY spectra of the two isomers (Table 3). In the spectrum of **16-L**,

(15) Eriksson, A. E.; Baase, W. A.; Zhang, X.-J.; Heinz, D. W.; Blaber, M.; Baldwin, E. P.; Matthews, B. W. *Science* **1992**, *255*, 178. Sharp, K. A.; Nicholls, A.; Friedman, R.; Honig, B. *Biochemistry* **1991**, *30*, 9686.

(16) Sefler, A. M.; Lauri, G.; Bartlett, P. A. *Int. J. Pept. Protein Res.* **1996**, *48*, 129.

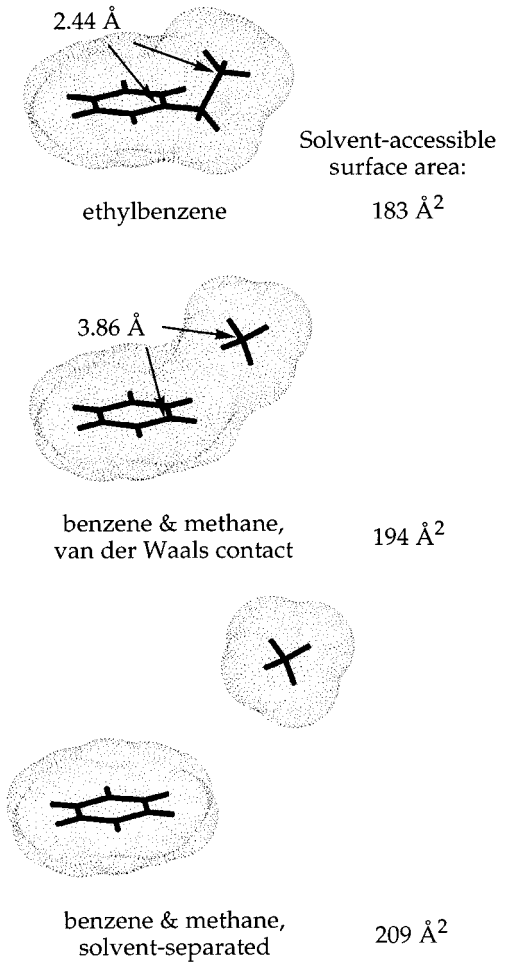


Figure 2. Effect on solvent-accessible surface area from removal of a methylene linkage.

Table 2. ^{1}H NMR Characterization of **16-L** and **16-D**[a]

hydrogen	δ (ppm) 16-L	δ (ppm) 16-D	coupled nucleus	J (Hz) 16-L	J (Hz) 16-D	Δδ/ΔT (ppb/K) 16-L	Δδ/ΔT (ppb/K) 16-D
Nala^P NH	8.16[b]	7.74	αH	10	10	8.0	9.0
Val NH	6.62	8.76	αH	10	8	4.4	5.4
Nala^P αH	3.92	3.96	NH	10	*c*		
			βH	2	2[c]		
			β′H	13	13[c]		
			P	17	13[c]		
Val αH	4.18	3.84	NH	10	8		
			βH	11	11		
Nac H	4.21	4.23	H′	18	15		
Nac H′	4.11	3.79	H	18	15		
Nala^P βH	3.32	3.22	αH	2	2		
			β′H	13	13		
			P	4	4		
Nala^P βH′	2.82	3.09	αH	13	13		
			βH	13	13		
			P	5	5		

[a] In 90% H_2O/10% D_2O, 100 mM CD_3CO_2D, 1 mN KH_2PO_4, pH 3.5 at 29 °C. Nala^P = 2-naphthyl-1-aminoethyl phosphonate moiety; Nac = naphthylacetamide CH_2 unit. [b] Chemical shift at 0 °C. [c] Measured in 100% D_2O since resonance was obscured in H_2O.

an NOE cross-peak is observed from Nala^P NH to Val αH, but none to Val βH; for **16-D**, the pattern is reversed, with a cross-peak for Nala^P NH to Val βH but not to Val αH. The low-energy conformers with the (*R*)-stereochemistry fit this pattern for **16-L**, and conversely, those with the (*S*)-configuration fit that for **16-D**.

The structures of the inhibitors **1-L** and **1-D** were determined

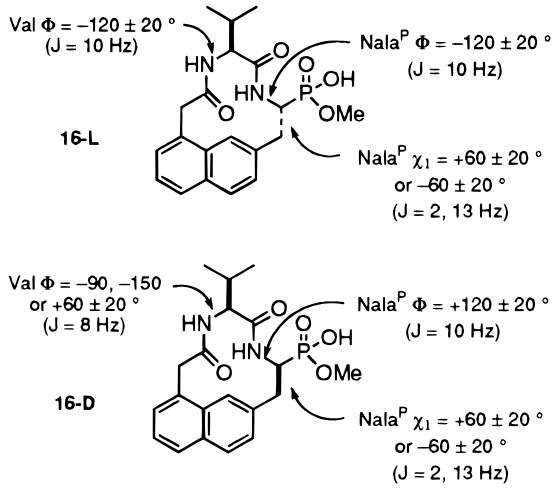


Figure 3. Dihedral constraints within the macrocyclic rings.

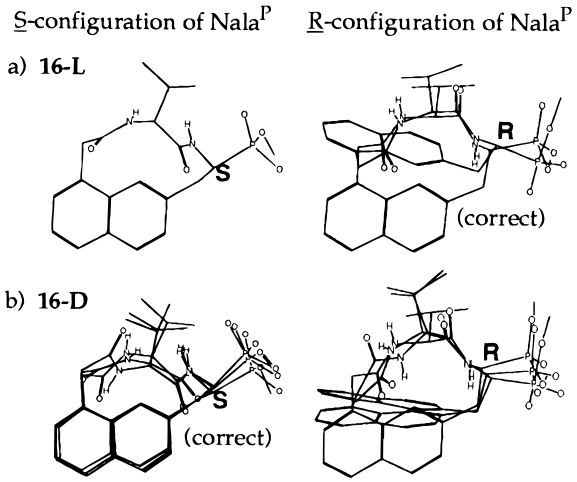


Figure 4. Low-energy conformer families for **16-L** that satisfy NMR constraints.

Table 3. Distances (Å) from Low-Energy Conformations[a] and NOE Cross-peaks Observed for **16-L** and **16-D**

structure[b]	Nala^P NH[c] to Val αH	Nala^P NH to Val βH
16-L, (*R*)-configuration	2.2	3.8−4.7
16-L, (*S*)-configuration	3.6	2.3−4.0
NOE observed for **16-L**	+	−
16-D, (*R*)-configuration	2.2−2.3	3.8−4.7
16-D, (*S*)-configuration	3.6	2.1−4.3
NOE observed for **16-D**	−	+

[a] Distance ranges encompass the separate families of minimum energy structures in Figures 2 & 3. [b] **16-L**, (*R*)-configuration indicates minimum energy conformations determined for (*R*)-configuration adjacent to phosphorus using 1D ^{1}H NMR data obtained with compound that proved to be **16-L**. [c] Nala^P = 2-naphthyl-1-aminoethyl phosphonate moiety.

using the same protocol, and the chemical shifts and coupling constants for the macrocyclic backbone hydrogens were very similar in the two series. A constrained Monte Carlo search of 10 000 steps was conducted for each isomer of **1** as described above, assuming the trans configuration of the amides and the (*R*)- and (*S*)-configurations adjacent to phosphorus for **1-L** and **1-D**, respectively. The structures generated were clustered and filtered against the experimentally determined χ_1 angles as described above. Six families of conformers within 5 kcal/mol of the global minimum were found for **1-L**, of which all but one were virtually superimposable with respect to the ring systems; furthermore, this ring conformation was identical to

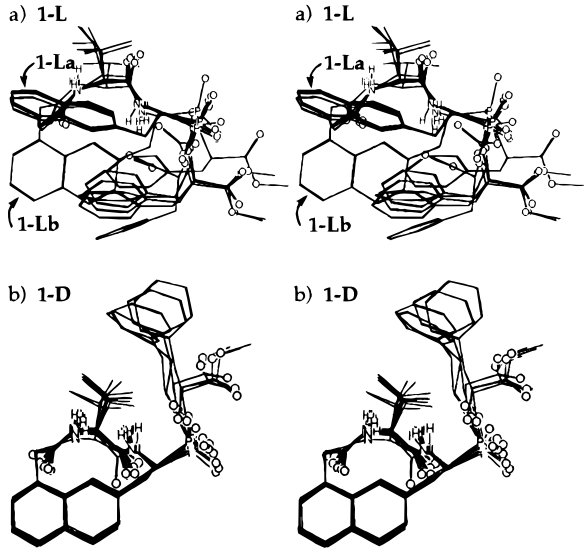


Figure 5. Low-energy conformer familes for **1-L** and **1-D** that satisfy NMR constraints.

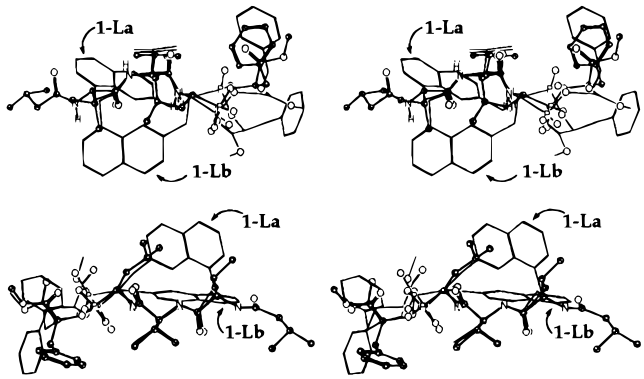


Figure 6. Comparison of bound form of acyclic peptide phosphonate **4** (bold) with solution conformations of **1-L**.

that in **16-L** (Figure 5a). In the case of diastereomer **1-D**, all eight families found were essentially the same, and the rings were superimposable with **16-D** (Figure 5b).

Success of the Design Strategy. Overlap of the solution conformation of the highest affinity inhibitor **1-L** and the bound conformations of the linear phosphonate **4** shows a high degree of similarity throughout the backbone atoms of these peptide analogues (Figure 6).[17] Thus, as a means of constraining the peptide backbone in these inhibitors to the conformation adopted in the binding site of penicillopepsin, the strategy appears to have been successful. On the other hand, the orientation adopted by the naphthalene moiety itself, relative to the peptide backbone, is displaced from that in our original model (Figure 7). To estimate the impact that this difference might have on the binding affinity, we docked each of the two low-energy solution conformations for **1-L** in the active site of penicillopepsin and minimized the structure while holding the protein fixed. The macrocyclic ring of the lower energy conformer, **1-La**, underwent very little movement and the energetic consequence was small (Figure 8a); the solvated energies calculated for the two ring conformers (with methyl ester in place of the phenyllactate ester) differed by only 0.1 kcal/mol

(17) The conformational differences depicted among the phosphonate ester and phenyllactate moieties are not significant; the conformers of **1-L** generated in the Monte Carlo search were clustered according to the macrocycle backbone and phosphorus atoms only. Hence, each conformer family contains many different conformers for the acyclic region of the structure.

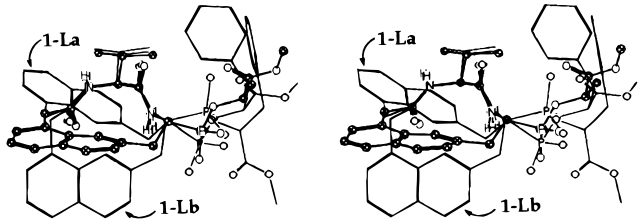


Figure 7. Comparison of original modeled structure (bold) with experimental conformations of **1-L**.

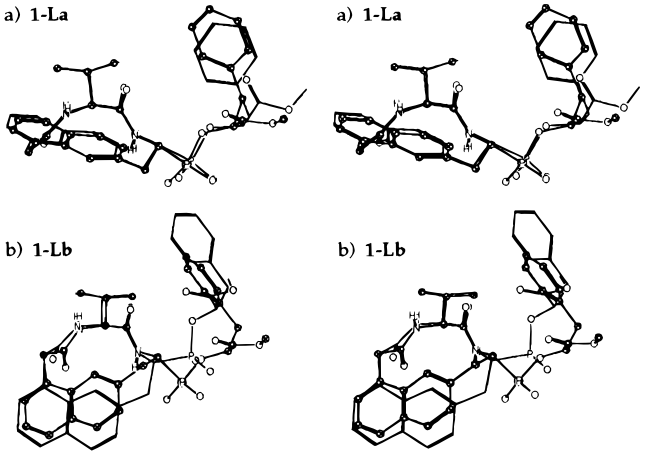


Figure 8. Conformational change on minimization in active site of penicillopepsin (minimized structures shown in bold with highlighted atoms).

(−106.4 → −106.5 kcal/mol). Thus, it does not appear that there is any significant enthalpic penalty for **1-L** to pay on binding to the enzyme. On the other hand, the higher energy solution conformer, **1-Lb**, is not as compatible with the active site and on minimization adopts a new conformation resulting from rotation about the Nala^{P} Cα−Cβ bond and movement of the aromatic linker away from one wall of the binding pocket (Figure 8b). The NMR data suggest that this conformation for **1-L** is not populated in solution; however, it is calculated to be 1.6 kcal/mol lower in energy than **1-Lb** itself (−103.4 → −105.0 kcal/mol).

In view of the close correspondence between the conformation determined for the macrocyclic inhibitor in solution (**1-La**) and that observed in the crystal structure of the complex with penicillopepsin, described in a following paper,[3] it appears that the design of **1-L** represents a successful application of the structure-based design strategy. On the other hand, since these structural studies show that the bound conformations for the cyclic and acyclic inhibitors differ in significant respects, we can only draw qualitative conclusions regarding the impact of conformational restriction from the observed differences in binding affinity.

Experimental Section[18]

Ethyl 7-Bromo-3,4-tetrahydro-1-naphthaleneacetate (6a) and Ethyl 7-Bromo-1,2,3,4-tetrahydronaphth-1-ylideneacetate (6b). A solution of diisopropylamine (5.8 mL, 29.6 mmol) in 15 mL of THF was cooled to −78 °C, and *n*-butyllithium (12.1 mL, 2.42 M in hexanes) was added. After 5 min, a solution of EtOAc (2.86 mL, 29.3 mmol) in 15 mL of THF was added to the preformed lithium diisopropylamide (LDA) solution by cannula over 10 min. The resulting mixture was stirred at −78 °C for 15 min before addition of tetralone **5** (6.28 g, 27.9 mmol) in 20 mL of THF over 20 min. After 2.5 h, the mixture, which had warmed to −25 °C, was recooled to −78 °C and 6 mL of 12 M HCl was added over 5 min with simultaneous precipitation of a

white solid. The mixture was allowed to warm to 25 °C and partitioned between 50 mL of H_2O and 75 mL of ether. The aqueous layer was extracted with 50 mL of ether, and the organic layers were combined, washed with 40 mL of 1 N HCl, 40 mL of H_2O, 40 mL of saturated $NaHCO_3$, and 40 mL of brine, dried, and concentrated to afford 8.49 g of the crude tertiary alcohol as a yellow oil. This oil (8.49 g) and 350 mg (1.8 mmol) of *p*-toluenesulfonic acid monohydrate were dissolved in 30 mL of benzene, and the resulting solution was heated at reflux for 1.5 h with azeotropic removal of H_2O using a Dean−Stark trap. The cooled solution was partitioned between 75 mL of ether and 25 mL of H_2O, and the organic layer was washed with 50 mL of H_2O, 50 mL of saturated $NaHCO_3$, and 50 mL of brine, dried, and concentrated. The resulting 7.70 g (96%) of amber oil proved to be a 3.5:1 mixture of the endo- and exocyclic alkenes **6a** (endo) and **6b** (exo), previously prepared by a different method and reported without complete characterization.[20] The two isomers were separable by column chromatography (10% EtOAc/hexanes). For **6a**: ^{1}H NMR (300 MHz) δ 1.26 (t, 3, J = 7), 2.31 (m, 2), 2.73 (t, 2, J = 8), 3.39 (s, 2), 4.16 (q, 2, J = 7), 6.04 (t, 1, J = 5), 7.00 (d, 1, J = 8), 7.25 (m, 1), 7.30 (d, 1, J = 2); ^{13}C NMR δ 14.1, 23.0, 27.4, 38.9, 60.8, 120.0, 125.7, 129.1, 129.4, 129.7, 130.4, 135.1, 136.2, 171.4; MS (FAB) *m*/*z* 294.0, 296.0 (M^+), 295.0, 297.0 (MH^+); a sample of this material was distilled bulb-to-bulb at 185 °C/2 Torr. Anal. Calcd for $C_{14}H_{15}O_2Br$: C, 56.97; H, 5.12. Found: C, 57.16; H, 5.43. For **6b**: ^{1}H NMR (300 MHz) δ 1.32 (t, 3, J = 7), 1.84 (tt, 2, J = 6, 6), 2.73 (t, 2, J = 6), 3.16 (dt, 2, J = 2, 6), 4.20 (q, 2, J = 7), 6.28 (t, 1, J = 2), 7.02 (d, 1, J = 8), 7.37 (dd, 1, J = 2, 8), 7.77 (d, 1, J = 2); ^{13}C NMR δ 14.3, 22.4, 27.6, 29.7, 59.8, 113.6, 119.9, 127.6, 130.7, 132.2, 136.2, 139.0, 153.0, 166.6; a sample of this material was distilled bulb-to-bulb at 110 °C/0.25 Torr.

Ethyl 7-Bromo-1-naphthaleneacetate (7). Bromine in CCl_4 (140 μL, 1.0 M, 0.14 mmol) was added to a stirred solution of the endo alkene **6a** (21 mg, 0.07 mmol) in 0.5 mL of CCl_4 at 25 °C until a red-orange color persisted. Stirring was continued for an additional 2 h at which time the solution was washed with 1 mL of 10% $NaHSO_3$, 1 mL of 5% $NaHCO_3$, and 1 mL of brine, dried, and concentrated. The resulting oil was redissolved in 0.5 mL of benzene, DBU (25 μL, 0.17 mmol) was added, and the resulting green, cloudy suspension was stirred at 25 °C for 12 h. The mixture was diluted with 2 mL of ether, washed with 2 mL of H_2O, 2 mL of 1 N HCl, 2 mL of 5% $NaHCO_3$, and 2 mL of brine, dried, and concentrated. The crude product was purified by column chromatography (50% $CHCl_3$/hexanes) to provide 20 mg (95%) of the aromatized product **7**. For **7**: ^{1}H NMR (300 MHz) δ 1.25 (t, 3, J = 7), 4.00 (s, 2), 4.16 (q, 2, J = 7), 7.42 (m, 2), 7.56 (m, 1), 7.71 (m, 2), 8.16 (m, 1); ^{13}C NMR δ 14.1, 39.0, 61.1, 120.6−133.3 (10 peaks), 171.1; MS (FAB) *m*/*z* 292.0, 294.0 (M^+), 293.0, 295.0 (MH^+); a sample of this compound was further purified by sublimation (mp 39−42 °C). Anal. Calcd for $C_{14}H_{13}O_2Br$: C, 57.36; H, 4.47. Found: C, 57.23; H, 4.62.

Ethyl 7-(2-Propenyl)-1-naphthaleneacetate (8). Allyltributyltin (2.3 mL, 7.4 mmol) was added by syringe to a solution of bromide **7** (2.16 g, 7.4 mmol) in 7 mL of benzene containing a catalytic amount of $Pd(PPh_3)_4$ (0.43 g, 0.37 mmol), and the resulting mixture was heated at reflux for 15 h. After cooling, the mixture was diluted with 50 mL of ether and added to 50 mL of 10% aqueous KF; the biphasic mixture was stirred vigorously for 30 min and filtered. The filter pad was washed with 50 mL of ether, the aqueous layer was separated, and the ethereal layer was dried and concentrated. The resulting cloudy yellow oil was purified by chromatography on a short silica plug (50 × 70 mm, 10% ether/hexanes) to afford 1.53 g (82%) of the product **8** as a pale yellow oil. For **8**: ^{1}H NMR δ 1.24 (t, 3, J = 7), 3.59 (d, 2, J = 7), 4.05 (s, 2), 4.16 (q, 2, J = 7), 5.13 (dd, 1, J = 2, 10), 5.15 (dd, 1, J = 2, 17), 6.06 (dddd, 1, J = 10, 17, 7, 7), 7.37 (m, 3), 7.79 (m, 3); ^{13}C NMR δ 14.1, 39.2, 40.6, 60.9, 116.1, 122.8−132.5 (9 peaks), 137.3, 138.0, 171.6; MS (FAB) *m*/*z* 254.0 (M^+), 255.0 (MH^+). Anal. Calcd for $C_{17}H_{18}O_2$: C, 80.28; H, 7.13. Found: C, 80.12; H, 7.03.

Ethyl 7-(2,3-Dihydroxypropyl)-1-naphthaleneacetate (9). Osmium tetroxide (23 mg, 0.09 mmol) was added to a solution of the alkene **8** (3.58 g, 14.1 mmol), *N*-methylmorpholine *N*-oxide (1.73 g, 14.8 mmol), and 3 mL of H_2O in 17 mL of acetone. The black mixture was stirred for 17 h and diluted with 150 mL of EtOAc, and the solution was washed with 10% $Na_2S_2O_4$ (100 mL and 25 mL), 100 mL of H_2O, 100 mL of 1 M HCl, and 100 mL of brine. The organic layer was dried and concentrated to leave a brown oil which was purified by chromatography through a silica plug (5% MeOH/CH_2Cl_2) and crystallization from ether to afford 2.25 g of the diol **9** as a light-brown solid (mp 70−73 °C). The mother liquor was purified by column chromatography to provide an additional 450 mg of product (combined yield 67%). For **9**: ^{1}H NMR ($CDCl_3$/0.1% H_2O) δ 1.23 (t, 3, J = 7), 2.96 (dd, 1, J = 8, 13), 3.00 (dd, 1, J = 5, 13), 3.58 (dd, 1, J = 7, 11), 3.73 (dd, 1, J = 3, 11), 4.04 (m, 1), 4.05 (s, 1), 4.15 (q, 2, J = 7), 7.38 (m, 3), 7.77 (m, 1), 7.84 (m, 2); ^{13}C NMR δ 14.1, 39.1, 40.1, 61.0, 65.8, 72.9, 123.9−135.9 (10 peaks), 171.8; MS (FAB) *m*/*z* 288.0 (M^+), 289.0 (MH^+). Anal. Calcd for $C_{17}H_{20}O_4$: C, 70.81; H, 6.99. Found: C, 70.45; H, 6.96.

Ethyl 7-(3-Oxopropyl)-1-naphthaleneacetate (10). A mixture of sodium periodate (200 mg, 0.93 mmol) and diol **9** (188 mg, 0.65 mmol) in 3 mL of ether and 300 μL of H_2O was stirred at 25 °C for 75 min and diluted with 5 mL of ether and 5 mL of H_2O. The aqueous layer was extracted with 3 mL of ether, and the combined organic layer was dried and concentrated to provide 163 mg (98%) of aldehyde **10** as an amber oil, which was carried on to the next reaction without purification. For **10**: ^{1}H NMR δ 1.22 (t, 3, J = 7), 3.86 (d, 2, J = 2), 4.04 (s, 2), 4.14 (q, 2, J = 7), 7.25−7.43 (m, 3), 7.75−7.86 (m, 3), 9.81 (t, 1, J = 2); ^{13}C NMR δ 14.1, 39.1, 51.0, 60.9, 124.6−132.9 (10 peaks), 171.3, 199.1; MS (FAB) *m*/*z* 257.1 (MH^+).

Methyl 7-[2-((*N*-Phenylmethoxycarbonyl)amino)-2-((dimethoxy)-phosphinyloxy)ethyl]-1-naphthaleneacetate (11). A solution of aldehyde **10** (163 mg, 0.64 mmol), benzyl carbamate (124 mg, 0.82 mmol), and triphenyl phosphite (262 mg, 0.85 mmol) in 0.7 mL of glacial HOAc was stirred at 25 °C for 45 min and at 90 °C for 45 min, cooled to 25 °C, and diluted with 8 mL of EtOAc. This solution was washed with 5 mL of H_2O, 3 mL of saturated $NaHCO_3$, and 3 mL of brine, dried, and concentrated, and the resulting yellow oil was purified by silica gel chromatography (25% to 50% EtOAc/hexanes), to give 157 mg of crude diphenyl phosphonate as a gummy semisolid. A portion of this material (52 mg, 0.085 mmol) was dissolved in 1.0 mL of MeOH with anhydrous K_2CO_3 (33 mg, 0.24 mmol), and the resulting suspension was stirred at 25 °C for 2.75 h. The mixture was diluted with 5 mL of ether and 5 mL of 0.5 N H_2SO_4, the aqueous fraction was extracted with 5 mL of ether, and the combined organic layer was washed with 5 mL of H_2O and 5 mL of brine, dried, and concentrated. The oily residue was purified by silica gel chromatography (10% to 30% ether/CH_2Cl_2), affording 29 mg (28% from **10**) of trimethyl ester **11** as a colorless, gummy semisolid. For **11**: ^{1}H NMR δ 3.06 (m, 1), 3.42 (m, 1), 3.62 (s, 3), 3.71 (d, 3, J = 11), 3.76 (d, 3, J = 11), 4.01 (s, 2), 4.56 (m, 1), 4.94 (s, 2), 5.18 (d, 1), 7.07−7.23 (m, 5), 7.36−7.43 (m, 3), 7.74−7.80 (m, 3); ^{13}C NMR δ 36.1, 38.7, 48.2 (d), 52.0, 53.0 (d), 53.2 (d), 66.8, 123.9−136.2 (16 peaks), 155.8, 171.8; ^{31}P NMR δ 27.1 (major rotamer), 26.5 (minor rotamer); MS (FAB) *m*/*z* 486.3

(18) **General**. Unless otherwise noted, reagents and solvents were obtained from commercial suppliers and used without further purification except for drying. All moisture or oxygen sensitive reactions were carried out under an atmosphere of N_2 in dry solvents. Molecular sieves were oven-dried before use. Unless otherwise noted, reaction workups culminated in drying the solution over $MgSO_4$ and concentrating under aspirator pressure using a rotary evaporator, followed by further evacuation under high vacuum. Flash chromatography was performed by the method of Still, Kahn, and Mitra,[19] using 60-mesh silica gel. Melting points were determined in Pyrex capillaries and are uncorrected. Unless otherwise indicated, ^{1}H and ^{13}C NMR spectra were obtained in $CDCl_3$ solution; all chemical shifts are reported in ppm relative to the solvent, except for ^{13}C NMR spectra acquired in D_2O, which were referenced to CH_3CN as internal standard (1.3 ppm). An external standard of trimethyl phosphate (δ 3.086 ppm) in $CDCl_3$ was used for ^{31}P spectra. ^{1}H spectra were acquired at 400 or 500 MHz and ^{13}C NMR spectra at 75 or 100 MHz, unless stated otherwise. NMR spectral data are reported as (MHz, solvent) chemical shift (multiplicity, number of hydrogens, coupling constants in Hz). Combustion analyses and mass spectra were obtained from the Mass Spectrometry Laboratory and the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley.

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

(20) Gruber, R.; Cagniant, D.; Cagniant, P. *Bull. Soc. Chim. Fr.* **1983**, 96.

(MH^+). Anal. Calcd for $C_{25}H_{28}NO_7P$: C, 61.85; H, 5.81; N, 2.89. Found: C, 61.53; H, 5.92; N, 2.87.

Methyl 7-[2-((*N*-*tert*-Butoxycarbonylvalyl)amino)-2-(dimethoxyphosphinyloxy)ethyl]-1-naphthaleneacetate (12). A suspension of carbamate **11** (250 mg, 0.51 mmol) and palladium on carbon (214 mg 5%, 0.10 mmol Pd) in 7 mL of MeOH was stirred under a hydrogen atmosphere at 25 °C for 3.5 h. The mixture was filtered through Celite and 0.45-μm nylon filter paper, and the pad was washed with 25 mL of MeOH. The combined filtrate was concentrated to give 154 mg of the free amine as a pale yellow oil which was dissolved along with Boc-valine (105 mg, 0.48 mmol) and 1-hydroxybenzotriazole (HOBt) (64 mg, 0.47 mmol) in 2.5 mL of CH_2Cl_2. This solution was cooled to 0 °C, EDC (102 mg, 0.53 mmol) was added, and the mixture was allowed to warm gradually to 25 °C and was stirred overnight. The solution was diluted with 40 mL of EtOAc, washed with 10 mL of 1 M HCl, 10 mL of H_2O, 10 mL of saturated $NaHCO_3$ and 10 mL of brine, dried, and concentrated. The crude product was purified by chromatography (70:28:2 EtOAc/hexanes/MeOH) and recrystallized from ether to provide 195 mg (70%) of the product **12** as a mixture of two diastereomers (mp 130−132 °C). For **12**: ^{1}H NMR (CD_3CN) δ 0.06 (d, 1.35, $J = 7$), 0.40 (d, 1.4, $J = 7$), 0.52 (d, 1.2, $J = 7$), 0.58 (d, 1.35, $J = 7$), 0.69 (d, 0.6, $J = 7$), 1.29 (s, 4.5), 1.33 (s, 4.5), 1.49 (m, 0.5), 1.78 (m, 0.5), 3.02 (m, 1), 3.36 (m, 1), 3.65 (s, 1.5), 3.67 (s, 1.5), 3.74 (d, 1.5, $J = 11$), 3.75 (d, 1.5, $J = 11$), 3.75 (d, 1.5, $J = 11$), 3.77 (d, 1.5, $J = 11$), 4.08 (m, 2), 4.73 (m, 1), 7.08 (d, 0.5, $J = 9$), 7.17 (d, 0.5, $J = 9$), 7.40 (m, 3), 7.77 (m, 3); ^{13}C NMR (CD_3CN) δ 16.4, 16.7, 18.5, 18.8, 28.1, 28.5, 30.4, 31.0, 35.7, 38.4, 38.6, 51.9, 52.0, 52.6, 52.7, 59.3, 59.7, 79.1, 124.1−134.4 (14 peaks), 155.6, 171.4, 171.8, 172.0; ^{31}P NMR (CD_3CN) δ 27.5, 27.3. Anal. Calcd for $C_{27}H_{39}N_2O_8P$: C, 58.90; H, 7.14; N, 5.09. Found: C, 58.95; H, 7.21; N, 5.14.

7-[2-((*N*-*tert*-Butoxycarbonylvalyl)amino)-2-(dimethoxyphosphinyloxy)ethyl]-1-naphthaleneacetic Acid (13). A solution of methyl ester **12** (183 mg, 0.33 mmol) and LiOH (450 μL, 0.87 M) in 1.1 mL of MeOH was stirred at 25 °C for 2.25 h, diluted with 15 mL of H_2O, and washed with 10 mL of ether. The ether layer was back-extracted with 10 mL of 10% $NaHCO_3$, and the aqueous layers were combined, acidified to pH 1 with 2 N H_2SO_4, and extracted with EtOAc (2 × 15 mL). The organic layers were combined, washed with 10 mL of brine, dried, and evaporated, and the residue was freeze-dried from benzene to provide 157 mg (89%) of the desired carboxylic acid **13** as a white solid. For **13**: ^{1}H NMR (($CD_3)_2SO$) δ 0.01 (d, 1.5, $J = 7$), 0.21 (d, 3, $J = 7$), 0.21 (d, 1.5, $J = 7$), 0.53 (d, 1.5, $J = 7$), 1.30 (s, 4.5), 1.31 (s, 4.5), 1.44 (m, 0.5), 1.52 (m, 0.5), 2.93 (ddd, 0.5, $J = 8$, 13, 13), 2.94 (m, 0.5), 3.22 (m, 0.5), 3.22 (m, 0.5), 3.60 (dd, 0.5, $J = 8$, 9), 3.65 (m, 0.5), 3.68 (d, 1.5, $J = 10$), 3.69 (d, 1.5, $J = 10$), 3.87 (d, 0.5, $J = 16$), 3.91 (d, 0.5, $J = 16$), 4.07 (d, 0.5, $J = 16$), 4.08 (d, 0.5, $J = 16$), 4.53 (m, 0.5), 4.54 (m, 0.5), 6.33 (d, 0.5, $J = 9$), 6.45 (d, 0.5, $J = 10$), 7.36 (m, 3), 7.71 (m, 0.5), 7.73 (m, 0.5), 7.76 (d, 0.5, $J = 8$) 7.77 (d, 0.5, $J = 8$), 7.85 (s, 0.5), 7.91 (s, 1), 8.16 (d, 0.5, $J = 9$), 8.50 (d, 0.5, $J = 9$); ^{13}C NMR (($CD_3)_2SO$) δ 16.6, 18.1, 18.5, 18.7, 28.1, 28.1, 30.1, 30.7, 34.9, 34.9, 45.7 (d, $J = 155$), 46.2 ($J = 154$), 52.6 (d, $J = 7$), 52.7 (d, $J = 7$), 53. 1 (d, $J = 7$), 53.3 (d, $J = 7$), 59.1, 60.2, 77.8, 78.1, 124.6, 124.9, 125.0, 125.1, 1266, 126.7, 126.8, 126.9, 127.6, 127.8, 128.2, 128.3, 131.6, 131.9,, 131.9, 132.2, 132.2, 134.5, 134.6, 134.8, 154.9, 155.3, 171.0, 171.2, 173.0; ^{31}P NMR (($CD_3)_2SO$) δ 27.4, 27.7; HRMS (FAB) *m*/*z* calcd $C_{26}H_{38}N_2O_8P$ (MH^+) 537.2366, found 537.2354.

Cyclo-7-[2-((*N*-valyl)amino)-2-(dimethoxyphosphinyloxy)ethyl]-1-naphthaleneacetamide (15-L and 15-D). A solution of pentafluorophenol (207 mg, 1.1 mmol), naphthaleneacetic acid **13** (157 mg, 0.29 mmol), and 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (90 mg, 0.47 mmol) in 7 mL of dry CH_2Cl_2 was stirred at 25 °C for 10 h and then evaporated. The residue was dissolved in 21 mL of an EtOAc/ether/MeOH mixture (15:5:1), and the solution was washed with 10 mL each of 1 N H_2SO_4, H_2O, 10% K_2CO_3, and brine, dried, and evaporated. The product was purified by chromatography (60% EtOAc/38% hexanes/2% MeOH) and lyophilization from benzene to give 152 mg of pentafluorophenyl ester **14** as a white solid. This activated ester was dissolved in 5 mL of 1,2-dichloroethane and 5 mL of TFA and stirred at 25 °C for 2 h. The solution was evaporated and the residue was lyophilized from benzene to afford 153 mg of the amine salt as a white solid. This material in turn was dissolved in 5 mL of dry 1,4-dioxane, and the solution was added by syringe pump over 9 h to a stirring mixture of 180 mL of 1,4-dioxane and 32 mL of pyridine at 50 °C. The mixture was stirred for an additional 2.5 h at 50 °C and then for 12 h at 25 °C. The solvent was evaporated and the glassy residue was chromatographed (2% MeOH/$CHCl_3$ to 1% HOAc/10% MeOH/$CHCl_3$) to yield 42 mg of cyclic diastereomer **15-L** and 37 mg of **15-D** (65% total yield from **13**). For **15-L**: ^{1}H NMR (($CD_3)_2SO$) δ 0.76 (d, 3, $J = 7$), 0.82 (d, 3, $J = 7$), 1.93 (m, 1), 2.96 (m, 1), 3.16 (m, 1), 3.57 (d, 3, $J = 11$), 3.68 (d, 3, $J = 11$), 3.89 (d, 1, $J = 17$), 4.08 (d, 1, $J = 17$), 4.14 (m, 1), 4.25 (m, 1), 7.38 (m, 3), 7.51 (d, 1, $J = 9$), 7.58 (s, 1), 7.62 (d, 1, $J = 9$), 7.79 (m, 2); ^{13}C NMR (($CD_3)_2SO$) δ 18.5, 19.3, 27.1, 34.9, 42.0, 44.7, 46.2, 52.7, 53.1, 53.12, 59.3, 125.1, 127.0, 127.6, 128.3, 128.7, 131.1, 132.0, 132.3, 133.7, 133.8, 169.9, 171.7; ^{31}P NMR (DMSO-d_6) δ 26.9. Anal. Calcd for $C_{21}H_{27}N_2O_5P$: C, 60.28; H, 6.51; N, 6.70. Found: C, 60.54; H, 6.56; N, 6.30.

For **15-D**: ^{1}H NMR (($CD_3)_2SO$) δ 0.89 (d, 3, $J = 7$), 0.99 (d, 3, $J = 7$), 1.97 (m, 1), 3.08 (m, 2), 3.63 (d, 1, $J = 14$), 3.71 (d, 3, $J = 11$), 3.73 (d, 3, $J = 11$), 3.76 (m, 1), 4.01 (m, 1), 4.05 (d, 1, $J = 14$), 7.35 (m, 3), 7.74 (dd, 1, $J = 2$, 9), 7.80 (d, 1, $J = 8$), 8.02 (d, 1, $J = 9$), 8.05 (s, 1), 8.26 (d, 1, $J = 10$); ^{13}C NMR (($CD_3)_2SO$) δ 18.7, 19.8, 29.2, 33.4, 41.8, 45.0, 46.6, 52.8, 52.9, 53.0, 53.1, 62.7, 125.2, 125.24, 126.5, 127.4, 128.1, 128.4, 131.2, 132.1, 133.6, 134.1, 170.5, 170.9; ^{31}P NMR (($CD_3)_2SO$) δ 27.8. Anal. Calcd for $C_{21}H_{27}N_2O_5P$: C, 60.28; H, 6.51; N, 6.70. Found: C, 60.48; H, 6.58; N, 6.49.

Cyclo-7-[(2*R*)-((*N*-valyl)amino)-2-(hydroxymethoxyphosphinyloxy)-ethyl]-1-naphthaleneacetamide (16-L). A solution of dimethyl phosphonate **15-L** (81 mg, 0.19 mmol) and LiOH (0.7 mL, 0.93 M) in 2 mL of DMSO was stirred at 25 °C for 20 h. The resulting white slurry was diluted with 10 mL of H_2O and lyophilized, and the residue was passed over a Dowex/H^+ column in 1:1 MeOH/H_2O. The eluate was partially concentrated and then lyophilized to provide 75 mg (96%) of the desired phosphonic acid **16-L** as a white powder. For characterization, a sample was converted to the sodium salt using Dowex/Na^+ and 1:1 MeOH/H_2O as the eluant. For **16-L**: ^{1}H NMR (D_2O) δ 0.62 (d, 3, $J = 7$), 0.67 (d, 3, $J = 7$), 1.48 (m, 1), 2.66 (ddd, 1, $J = 4$, 13, 13), 3.20 (ddd, 1, $J = 2$, 5, 13), 3.44 (d, 3, $J = 10$), 3.80 (ddd, 1, $J = 2$, 13, 17), 3.97 (d, 1, $J = 18$), 4.06 (d, 1, $J = 18$), 4.06 (d, 1, $J = 11$), 7.17 (s, 1), 7.34 (m, 3), 7.77 (m, 1), 7.79 (d, 1, $J = 9$); ^{13}C NMR (D_2O) δ 17.9, 18.7, 27.7, 37.5, 42.4, 48.1 (d, $J = 144$), 52.7 (d, $J = 6$), 61.5, 124.6, 125.8, 127.9, 128.8, 129.8, 130.0, 131.2, 132.9, 136.2, 136.3, 172.1 (d, $J = 7$), 176.4; ^{31}P NMR (D_2O) δ 20.4. HRMS (FAB) *m*/*z* calcd for $C_{20}H_{24}N_2O_5PNa$ (MH^+) 427.1399, found 427.1412.

Cyclo-7-[(2*S*)-((*N*-valyl)amino)-2-(hydroxymethoxyphosphinyloxy)-ethyl]-1-naphthaleneacetamide (16-D). In a similar fashion, diastereomer **15-D** (16 mg, 0.038 mmol) was converted to 15 mg (100%) of the sodium salt of acid **16-D**. For **16-D**: ^{1}H NMR (D_2O) δ 0.87 (d, 3, $J = 7$), 0.95 (d, 3, $J = 7$), 1.90 (m, 1), 2.96 (ddd, 1, $J = 5$, 13, 13), 3.09 (ddd, 1, $J = 2$, 4, 13), 3.45 (d, 3, $J = 10$), 3.62 (d, 1, $J = 15$), 3.72 (d, 1, $J = 11$), 3.83 (ddd, 1, $J = 2$, 13, 13), 4.14 (d, 1, $J = 15$), 7.29 (m, 3), 7.71 (m, 3); ^{13}C NMR (D_2O) δ 18.6, 19.6, 29.6, 35.1, 41.8, 48.1 (d, $J = 148$), 52.4 (d, $J = 5$), 64.4, 124.3, 125.9, 127.8, 128.1, 129.2, 129.4, 131.5, 132.6, 136.0, 136.1, 173.4 (d, $J = 4$), 175.9; ^{31}P NMR (D_2O) δ 21.2.

Methyl 3-Phenyl-(2*R*)-trifluorosulfonyloxypropanoate (17). A catalytic amount of concentrated sulfuric acid (approximately 100 μL) was added to a suspension of 3-phenyl-(2*R*)-hydroxypropanoic acid in 10 mL of MeOH. The mixture was heated at reflux overnight and concentrated. The residue was redissolved in 50 mL of ethyl ether, washed with 30 mL of saturated $NaHCO_3$ and 30 mL of brine, dried, and concentrated to leave 1.26 g of the methyl ester as a viscous oil, which solidified upon standing. Concurrently, trifluoromethanesulfonic anhydride was added to pyridine (360 μL, 4.47 mol) in 12 mL of CH_2Cl_2 at −20 °C, and the resulting white slurry was stirred for 5 min. A portion of the methyl ester (706 mg, 3.92 mmol) was added, and the mixture was stirred for 15 min and then warmed to 25 °C in a water bath. The suspension was filtered through glass wool and evaporated, and the residue was dissolved in 25 mL of ethyl ether and filtered through a plug of silica gel. The filtrate was evaporated to give 1.04 g (75%) of the triflate **17** as a pale yellow oily liquid. Fpr **17**: ^{1}H NMR ($CDCl_3$) δ 3.22 (dd, 1, $J = 9$, 15), 3.37 (dd, 1, $J = 4$, 15), 3.84

(s, 3), 5.27 (dd, 1, $J = 4, 9$), 7.23 (d, 2, $J = 8$), 7.31−7.35 (m, 3); ^{13}C NMR ($CDCl_3$) δ 38.1, 53.2, 83.7, 118.2 (q, $J = 320$), 127.8, 128.7, 129.3, 133.2, 167.0.

Methyl [Cyclo-7-[(2*R*)-((*N*-valyl)amino)-2-(methoxy-(1*S*)-1-methoxycarbonyl-2-phenylethoxy)phosphinyloxyethyl]-1-naphthaleneacetamide] (18-L). The macrocyclic acid **16-L** (53 mg, 0.13 mmol) was converted to the tetramethylammonium salt by dissolving it with an equivalent of tetramethylammonium hydroxide in 1 mL of MeOH, evaporating, and drying under vacuum for 2 h. A solution of triflate **17** (122 mg, 0.39 mmol) in 1 mL of CH_3CN was added to the residue by cannula, and the mixture, which had become a suspension within 1 h, was stirred at 25 °C overnight. The mixture was diluted with 15 mL of EtOAc, washed with 10 mL each of 1 M HCl, saturated $NaHCO_3$, and brine, dried, and concentrated to leave an oily residue which was purified by chromatography (50 to 100% EtOAc/ hexanes) to provide 57 mg (78%) of the desired phosphonate diester **18-L** as a mixture (1:1) of phosphonate diastereomers. For **18-L**: ^{1}H NMR δ 0.73 (d, 1.5, $J = 7$), 0.74 (d, 1.5, $J = 7$), 0.81 (d, 3, $J = 7$), 1.67 (m, 1), 1.95 (ddd, 0.5, $J = 6, 13, 13$), 2.86 (ddd, 0.5, $J = 6, 13, 13$), 3.05 (m, 0.5), 3.13 (dd, 0.5, $J = 9, 14$), 3.16 (d, 1.5, $J = 11$), 3.32 (m, 1), 3.81 (d, 1.5, $J = 11$), 3.82 (s, 1.5), 3.84 (s, 1.5), 3.94 (d, 0.5, $J = 18$), 3.97 (d, 0.5, $J = 18$), 4.19 (dd, $J = 5, 10$), 4.21 (dd, 10, $J = 5$), 4.25 (m, 0.5), 4.27 (d, 0.5, $J = 18$), 4.28 (d, 0.5, $J = 18$), 4.41 (m, 0.5), 5.17 (d, 0.5, $J = 10$), 5.12 (d, 0.5, $J = 10$), 5.15 (m, 0.5), 5.25 (ddd, 0.5, $J = 3, 9, 9$), 6.11 (d, 0.5, $J = 10$), 6.84 (dd, 0.5, $J = 2, 10$), 7.22−7.48 (m, 9), 7.76−7.83 (m, 2); ^{13}C NMR δ 18.2, 18.2, 19.0, 19.1, 27.7, 35.8, 37.0, 38.9 (d, $J = 6$), 39.1, 40.5, 43.1, 46.1 (d, $J = 159$), 46.3 (d, $J = 160$), 51.8 (d, $J = 8$), 52.5, 52.8, 53.6 (d, $J = 7$), 60.0, 60.4, 75.1 (d, $J = 7$), 75.7 (d, $J = 7$), 124.8−135.7 (28 peaks), 170.0, 170.0, 170.3, 170.7, 173.0, 173.3; ^{31}P NMR δ 24.9, 25.7.

Methyl [Cyclo-7-[(2*S*)-((*N*-valyl)amino)-2-(methoxy-(1*S*)-1-methoxycarbonyl-2-phenylethoxy)phosphinyloxyethyl]-1-naphthaleneacetamide] (18-D). In a similar fashion, the epimer **16-D** (9 mg, 0.022 mmol) was converted to 8 mg (67%) of ester **18-D**, isolated as a single diastereomer at phosphorus. For **18-D**: ^{1}H NMR (9:1 $CDCl_3$ /CD_3-OD) δ 0.79 (d, 3, $J = 7$), 0.84 (d, 3, $J = 7$), 1.86 (m, 1), 2.38 (ddd, 1, $J = 4, 4, 14$), 2.86 (ddd, 1, $J = 6, 13, 13$), 3.01 (dd, 1, $J = 8, 14$), 3.13 (m, 1), 3.50 (d, 1, $J = 14$), 3.61 (s, 3), 3.61 (d, 3, $J = 11$), 3.70 (ddd, $J = 2, 13, 15$), 3.79 (d, 1, $J = 11$), 3.97 (d, 1, $J = 14$), 5.01 (ddd, 1, $J = 4, 7, 8$), 6.80 (dd, 1, $J = 1, 8$), 7.10 (m, 5), 7.16 (m, 3), 7.52 (m, 2); ^{13}C NMR δ 18.8, 19.3, 28.9, 33.0, 39.1 (d, $J = 7$), 42.4, 47.3 (d, $J = 158$), 52.6, 54.2 (d, $J = 7$), 62.6, 75.5 (d, $J = 8$), 124.8, 125.4, 127.1, 127.14, 127.3, 128.5, 128.7, 129.3, 131.5, 132.6, 133.3, 133.5, 135.2, 171.3, 171.4, 172.9; ^{31}P NMR (9:1 $CDCl_3$/CD_3OD) δ 26.3.

Methyl [Cyclo-7-[(2*R*)-((*N*-valyl)amino)-2-(hydroxy-(1*S*)-1-methoxycarbonyl-2-phenylethoxy)phosphinyloxyethyl]-1-naphthaleneacetamide], Sodium Salt (1-L). Isobutylene was bubbled through 0.8 mL of CH_2Cl_2 for 20 min, and bromotrimethylsilane (30 μL, 0.23 mmol) was added. After 5 min, this solution was added to the methyl phosphonate **18-L** (25 mg, 0.043 mmol) by cannula, and the reaction solution was stirred overnight at 25 °C. The solvent had evaporated, leaving an off-white residue which was suspended in 3 mL of MeOH; this solution was concentrated, and the residue was dissolved in 1:1 MeOH/H_2O and passed over a Dowex/Na^+ column. The eluate was partially concentrated and lyophilized to provide 20 mg (80%) of the sodium salt **1-L** as a white powder. For **1-L**: ^{1}H NMR (D_2O) δ 0.59 (d, 3, $J = 7$), 0.66 (d, 3, $J = 7$), 1.46 (m, 1), 2.49 (ddd, 1, $J = 5, 13, 13$), 3.01 (d, 2, $J = 6$), 3.10 (ddd, 1, $J = 3, 6, 13$), 3.56 (s, 3), 3.74 (ddd, 1, $J = 3, 13, 17$), 3.98 (d, 1, $J = 18$), 4.02 (d, 1, $J = 10$), 4.06 (d, 1, $J = 18$), 4.85 (ddd, 1, $J = 6, 6, 8$), 7.12−7.34 (m, 9), 7.77 (m, 2); ^{13}C NMR (D_2O) δ 18.0, 18.8, 27.7, 37.5, 39.7 (d, $J = 6$), 42.3, 49.1 (d, $J = 150$), 53.2, 61.4, 74.3 (d, $J = 6$), 124.8, 125.8, 127.7, 128.0, 128.6, 128.7, 129.1, 129.5, 129.8, 130.2, 131.2, 132.9, 136.1, 136.3, 172.0 (d, $J = 8$), 174.1 (d, $J = 3$), 176.1; ^{31}P NMR (D_2O) δ 18.7; HRMS (FAB) *m*/*z* calcd for $C_{29}H_{33}N_2O_7PNa$ (MH^+) 575.1923, found 575.1922.

Methyl [Cyclo-7-[(2*S*)-((*N*-valyl)amino)-2-(hydroxy-(1*S*)-1-methoxycarbonyl-2-phenylethoxy)phosphinyloxyethyl]-1-naphthaleneacetamide], Sodium Salt (1-D). In a similar fashion, phosphonate **18-D** (5 mg, 0.009 mmol) was converted to 4 mg (77%) of the sodium salt **1-D**. For **1-D**: ^{1}H NMR (D_2O) δ 0.90 (d, 3, $J = 7$), 0.97 (d, 3, $J = 7$), 2.03 (m, 1), 2.92 (ddd, 1, $J = 4, 13, 13$), 2.97 (dd, 1, $J = 6, 13$), 3.10 (m, 2), 3.52 (s, 3), 3.67 (d, 1, $J = 15$), 3.76 (d, 1, $J = 11$), 3.84 (m, 1), 4.20 (d, 1, $J = 15$), 4.80 (ddd, 1, $J = 6, 6, 6$), 7.15−7.40 (m, 8), 7.73−7.77 (m, 3); ^{13}C NMR (D_2O) δ 20.9, 21.5, 31.6, 37.4, 41.9, 43.9, 51.1 (d, $J = 150$), 55.0, 66.3, 76.7, 126.3, 127.9, 129.7, 130.2, 131.1, 131.2, 131.3, 132.1, 133.5, 134.6, 134.7, 138.0, 138.2, 175.3, 176.3, 177.8; ^{31}P NMR (D_2O) δ 19.0; HRMS (FAB) *m*/*z* calcd for $C_{29}H_{33}N_2O_7PNa$ (MH^+) 575.1923, found 575.1933.

Enzyme Assays. General. All solutions were prepared with distilled and deionized water. Stock solutions were filtered through Rainin Nylon-66 0.45-μm filters. Assays were performed on a Kontron Uvikon 860 UV−vis spectrophotometer equipped with a data station. Temperature regulation (±0.2 °C) was provided by a Lauda Model RM 20 circulating constant-temperature bath connected to a water-jacketed sample cell holder. All assays were performed at 25 °C in 100 mM NaOAc at pH 3.5.

Enzyme Stock Solution. Penicillopepsin was obtained in the form of a lyophilized powder as a gift from Theo Hofmann (University of Toronto). A 0.8-mg sample of penicillopepsin was dissolved in 1.00 mL of 0.1 M KH_2PO_4 containing 10% (v/v) glycerol at 5 °C. The resulting solution was centrifuged for 5 min on an Eppendorf Model 5414 centrifuge. The upper 900 μL of this solution was removed and stored in a plastic tube at 5 °C. The concentration of penicillopepsin in this stock (14.1 μM) was determined by measurement of the absorbance at 280 nm. A working solution of the enzyme was prepared by dilution of 100 μL of the stock to 5.00 mL with 0.1 M KH_2PO_4 containing 10% (v/v) glycerol ([E] = 281 nM). The stock solutions were stored at 5 °C and were stable for at least 1 week.

Substrate Stock Solution. The substrate Ac-Ala-Ala-Lys-(*p*-NO_2)-Phe-Ala-Ala-NH_2 was prepared using an automated peptide synthesizer. The resin-bound substrate was cleaved and isolated and used without further purification. The substrate stock was prepared by dilution of the substrate in buffer solution, and the substrate concentration was determined from the absorbance at 277 nm ($\epsilon_{277} = 10\,970$ M^{-1} cm^{-1}).

Inhibitor Stock Solutions. Certified weights and ^{31}P combustion analyses were obtained for samples of inhibitors **1-L**, **2-L**, **3**, and **16-D**, each of which consisted of a single phosphorus-containing compound by ^{31}P NMR. Inhibitor concentrations in these stock solutions were calculated from the sample weight corrected for analytical purity according to phosphorus analysis. Extinction coefficients were measured for **1-L**, **2-L**, and **16-D** and employed in calculating the concentrations for stock solutions of the diastereomers **1-D**, **2-D**, and **16-L**, respectively.

Assay Procedure. Assays were initiated by addition of enzyme (typical [E] = 6−10 nM) to an appropriate mixture of substrate, inhibitor, and buffer. After a 3-min equilibration period, substrate hydrolysis was followed at $\lambda = 306$ nm ($\Delta\epsilon = -1710$ M^{-1} cm^{-1}); assays were followed for less than 15% of the total reaction.

Determination of Inhibition Constants. The initial velocity of the enzyme-catalyzed reaction was determined from a least-squares fit of the raw absorbance data using the program Enzfitter.[21] Velocity values, obtained in duplicate for the four inhibitors with measurable activity, were incorporated into Lineweaver−Burk plots. A replot of the slopes ($K_{m,app}/V_{max}$) versus [I] was linear for each of the four active inhibitors, and the K_i was taken from the absolute value of the [I] intercept.

Solution Structure Determination: NMR. All NMR samples were 5−20 mM in 90% H_2O/D_2O with 100 mM CD_3CO_2D and 1 mN KH_2PO_4. The buffer was adjusted to pH 3.5 with concentrated NaOD in D_2O. Chemical shifts were referenced to 1,4-dioxane at 3.73 ppm. All spectra were obtained using a Bruker AM 500 spectrometer equipped with an inverse probe and an Aspect 3000 computer. Variable temperature 1D spectra were acquired at 0, 29, and 50 °C using a jump and return selective excitation sequence, (π/2) − D2 − (π/2) − acquire.[22] The carrier frequency was set on the water signal and the delay time, D2, was set to 200 μs so that maxima occurred at frequency intervals 1.8 kHz from the water signal. All 2D experiments were recorded in the phase-sensitive mode using the time-proportional phase incrementation method[23] for quadrature detection in f_1. The spectral

(21) Leatherbarrow, R. J. *Enzfitter*, Elsevier Science Publishers BV: Amsterdam, 1987.

(22) Plateau, P.; Gueron, M. *J. Am. Chem. Soc.* **1982**, *104*, 7310.

widths used were the same as those in the 1D experiments. Typically between 8 and 32 transients of 2048 data points each were recorded in the f_2 dimension and 256−512 t_1 increments were recorded and zero-filled to 1024 data points. In cases where high digital resolution was required in f_1, up to 1024 t_1 increments were collected and zero-filled to 2048 data points. The NOESY 2D data were acquired at 30, 32, or 50 °C, and the TOCSY 2D data were acquired at 32 °C. All data were processed using the Bruker UXNMR software package. Prior to Fourier transformation, spectra were zero-filled once in the f_1 dimension and subjected to a phase-shifted sine bell weighting function in both dimensions.

Phase sensitive NOESY[24] spectra were recorded using a jump and return[22] read pulse so that the overall sequence was $(\pi/2) - t_1 - (\pi/2) - \tau_m - (\pi/2) -$ D2 $- (\pi/2) -$ acquire. The delay D2 was set to 200 μs. Appropriate phase cycling was used to select *z*-magnetization during the mixing time, τ_m. Mixing times (τ_m) of 700 ms were used with a 20 ms *z*-filter to remove zero quantum artifacts. The initial value of t_1 was set to 3 μs.

TOCSY[25] spectra were recorded with a 50 ms and a 2 kHz MLEV-17 spin-locking pulse.[26] Phase coherent solvent suppression was achieved using a DANTE[27] sequence (pulse width 25 μs and interpulse delay of 250 μs) applied for 1.7 s before the start of each transient.

Solution Structure Determination: Molecular Modeling. All modeling was performed with Macromodel/Batchmin[28] software version 4.5 including previously described modifications.[16] Monte Carlo searches (5000 iterations for **16-L** and **16-D**, 10 000 iterations for **1-L** and **1-D**) and minimizations were run with either double- or triple-well Φ angle torsional constraints, calculated from the NH−αH coupling constants.[29] Energy minimizations were performed with Macromodel's TNCG method, the Amber force field with modified phosphorus parameters,[30] and GB/SA water solvation,[31] until gradient convergence was achieved. The structures were clustered[10] into families of rmsd < 0.3 Å by comparing all of the backbone atoms, amide oxygens and NHs, and the phosphorus atom. Conformer families violating the Nap χ_1 angle torsional constraints were identified by visual inspection and discarded.

Acknowledgment. We thank Professor Theo Hoffman (University of Toronto) for providing the penicillopepsin, and Dr. David King (UC Berkeley, HHMI) for synthesis of the assay substrate. This work was supported by a grant from the National Institutes of Health (GM-30759); P.A.B. was on appointment as a Miller Research Professor in the Miller Institute for Basic Research in Science (1996). We also thank Jason Hataye for preparation of synthetic intermediates.

Supporting Information Available: Synthesis and characterization of acyclic analogues **2** and **3** and kinetic plots for K_i determinations (8 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

JA973715J

(23) Marion, D.; Wüthrich, K. *Biochem. Biophys. Res. Commun.* **1983**, *113*, 967.
(24) Bodenhausen, G.; Kogler, H.; Ernst, R. R. *J. Magn. Reson.* **1984**, *58*, 370.
(25) Braunschweiler, L.; Ernst, R. R. *J. Magn. Reson.* **1983**, *53*, 521.
(26) Davis, D. G.; Bax, A. D. *J. Magn. Reson.* **1985**, *65*, 355.
(27) Zuiderweg, E. R. P.; Hallenga, K.; Olejniczak, E. T. *J. Magn. Reson.* **1986**, *70*, 336. Morris, G. A.; Freeman, R. *J. Magn. Reson.* **1978**, *29*, 433.
(28) Mohamadi, F.; Richards, N. G. J.; Guida, W. C.; Liskamp, R.; Lipton, M.; Caufield, C.; Chang, G.; Hendrickson, T.; Still, W. C. *J. Comput. Chem.* **1990**, *11*, 440.
(29) Bystrov, V. F. *Prog. NMR Spectrosc.* **1976**, *10*, 41.
(30) Ferguson, D. M.; Kollman, P. A. *J. Comput. Chem.* **1991**, *12*, 620.
(31) Still, W. C.; Tempczyk, A.; Hawly, R. C.; Hendrickson, T. *J. Am. Chem. Soc.* **1990**, *112*, 6127.