Macrocyclic Inhibitors of Penicillopepsin. 3. Design, Synthesis, and Evaluation of an Inhibitor Bridged between P2 and P1′

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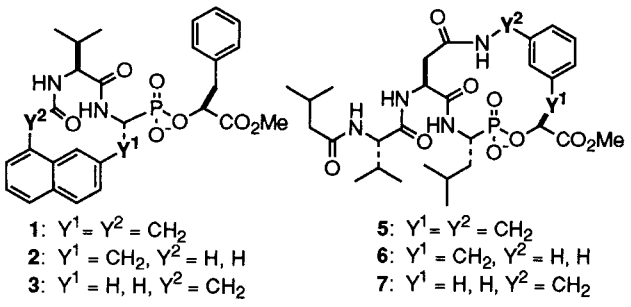
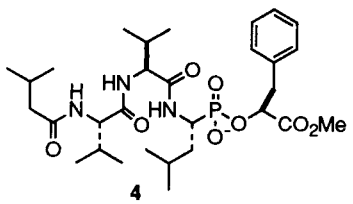
Abstract: Through application of a thorough design and modeling protocol, the macrocyclic peptidyl phosphonate **5** was derived from the bound structure of an acyclic inhibitor of penicillopepsin, **4**, by linking the P1′ and P2 side chains; the acyclic analogues **6** and **7** were also synthesized and evaluated for comparison. As observed for a related set of inhibitors, **1**−**3** [Meyer, J. H.; Bartlett, P. A. *J. Am. Chem. Soc.* **1998**, *120*, xxxx], the binding affinity at pH 4.5 was found to be inversely related to the degree of conformational flexibility across the series, **7** (K_i = 1300 nM), **6** (K_i = 42 nM), and **5** (K_i = 0.10 nM), although the differences are much greater. The structure of the macrocyclic ring of **5** in solution was determined by a combined NMR and molecular modeling method and found to correspond closely to the designed structure and to the backbone conformation of the original target, **4**. The binding enhancement conveyed by the macrocyclic constraint corresponds to 0.9−1.4 kcal/mol/deg of rotational freedom lost, indicating what can be gained from this structure-based design strategy.

An accompanying paper described the structure-based design of an inhibitor of the aspartic protease penicillopepsin in which conformational constraint was effected by linking the P1 and P3 side chains of a peptidyl phosphonate in a macrocyclic structure, **1**.[1] This strategy enhanced the affinity of the inhibitor by 1−2 orders of magnitude in comparison to the acyclic control compounds, **2** and **3**. The conformations adopted by the macrocycle in solution were shown to correspond to the original design with respect to the peptide backbone; moreover, the solution conformation proved to be the same as that found in the X-ray crystal structure of the enzyme/inhibitor complex.[2]





However, the bound conformations of the comparison compounds, **2** and **3**, are sufficiently different from that of **1** that this series cannot be used to quantitate the increase in binding affinity resulting from macrocyclic constraint. In this paper, we describe another series of macrocyclic and acyclic penicillopepsin inhibitors from which quantitative comparisons may be more valid.

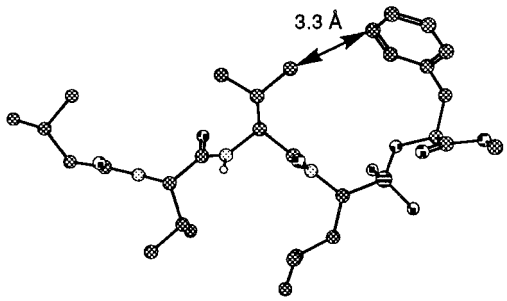


Figure 1. Conformation of isovaleryl-L-Val-L-Val-L-Leu-{PO_2^--O}-L-Phe-OMe (**4**) in its complex with penicillopepsin.[4]

The P1 and P3 side chains of a peptidic ligand are not the only ones brought into proximity as it binds in the extended conformation to an aspartic protease; for example, the side chains of the alternate residues P2 and P1′ share a binding pocket along the other wall of the active site.[3] In the case of penicillopepsin, the crystal structure of its complex with the phosphonate pentapeptide **4** shows a separation of only 3.3 Å between the phenyl group of the P1′ residue and a γ-methyl of the P2 valine (Figure 1).[4] In this paper, we describe the macrocyclic phosphonate **5**, in which the P2 and P1′ side chains have been bridged. The synthesis and evaluation of this inhibitor, and the acyclic controls **6** and **7**, were undertaken to explore further the generality of macrocyclization as a strategy for structure-based design and to provide additional insight into

(1) Meyer, J. H.; Bartlett, P. A. *J. Am. Chem. Soc.* **1998**, *120*, 4600.
(2) Ding, J.; Fraser, M.; Meyer, J. H.; Bartlett, P. A.; James, M. N. G. *J. Am. Chem. Soc.* **1998**, *120*, 4610.
(3) 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 BV: Amsterdam, 1986; pp 321−334.
(4) Fraser, M. E.; Strynadka, N. C. J.; Bartlett, P. A.; Hanson, J. E.; James, M. N. G. *Biochemistry* **1992**, *31*, 5201.

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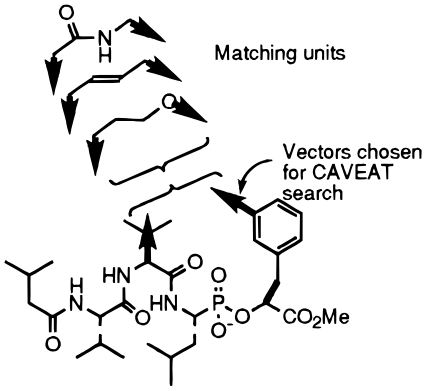


Figure 2. Use of CAVEAT to identify potential side chain-side chain connecting units for phosphonate **4**.

the quantitative enhancement in binding affinity that can be obtained by this approach.

Design. A CAVEAT[5] search for a unit that would link the Cα−Cβ bond of the P2-valine side chain with the meta C−H bond of the P1′ phenyl moiety, in the orientation they adopt in the bound complex of **4** with penicillopepsin, readily identified a 4-atom chain with a planar sp^2-sp^2 bond (Figure 2). We evaluated a number of possible linkers in the following fashion: models were constructed with acetyl in place of the isovaleryl-valine (Iva-Val) tail and with methyl in place of the leucine side chain and subjected to Monte Carlo conformational searches to estimate the likely low-energy conformations of the macrocyclic rings. Unique conformations within 3 kcal/mol of the lowest energy conformation were superimposed on the bound form of the acyclic inhibitor **4**, according to the backbone atoms of the leucine phosphonate moiety, and similarities in orientations of the rest of the backbone, the aromatic ring, and the side chain substituents were assessed. Ring systems were sought that adopted relatively few conformations overall, but with a high percentage matching the acyclic target.

The behavior of a number of linking groups is shown in Figure 3, which reveals some important and unanticipated trends. For example, the alkene **1** is clearly distinct from the corresponding amide **8**, despite their geometric resemblance. Most of the analogues were modeled with the β-methyl group corresponding to the Val *pro-S* methyl, on the expectation that this group would help control the conformation of the ring. Among those analogues, the amide-containing linkage **8** has the lowest number of conformers and the highest percentage that match the bound target. When the methyl is removed (**9**), the number of conformers remains the same, but many more of them position the backbone in the desired fashion.

Fully elaborated models of the most promising macrocycles were constructed, and their conformational behavior within the active site of the protein was explored by high-temperature (600 K) dynamics simulations. Representative structures along the trajectories were minimized, and selection of the final candidates was made by comparison to a model of **4** that had itself been minimized in the binding site. Macrocycle **5**, with a carboxamide unit for the sp^2-sp^2 bond, remained the most attractive analogue, not only because of its conformational behavior (Figure 4) but also because of its synthetic accessibility.

To allow us to gauge the significance of the macrocyclic constraint on the binding affinity of the peptidyl phosphonate, we prepared the acyclic analogues **6** and **7** for comparison. These control compounds differ from the macrocycle by loss of a carbon atom (replacement of a methylene group by two

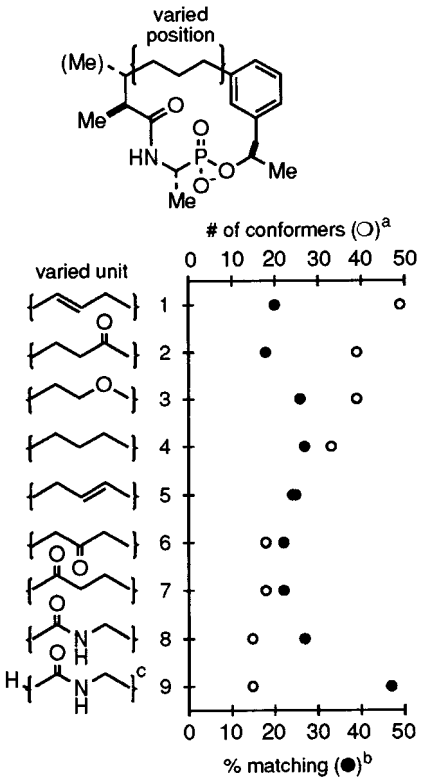


Figure 3. Relationship between structure and conformational properties of model macrocycles. (a) Number of discrete conformers within 3 kcal/mol of lowest energy. (b) Percentage of low-energy conformers that match backbone of inhibitor **4** in bound conformation. (c) Analogue without β-methyl at P2 position.

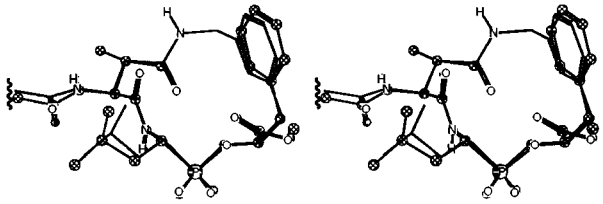


Figure 4. Lowest energy conformer predicted for macrocycle **5** (line and label) and bound conformation of phosphonate **4** (ball-and-stick) superimposed by backbone atoms of P2−P1′ residues.

hydrogens);[1] thus, in principle, they are able to adopt the same conformation in the active site as the macrocycle itself.

Synthesis. The key steps in the synthesis of the macrocyclic phosphonate **5** were preparation of methyl L-β-(*m*-(aminomethyl)phenyl)lactate, **12**, elaboration of the acyclic precursor, and cyclization. Several asymmetric approaches to the hydroxyacid **11** were explored, including alkylation of *N*-(diphenylmethylene)glycine *tert*-butyl ester[6] followed by diazotization of the amino acid[7] and alkylation of a chiral derivative of glycolic acid.[8] The most effective proved to be asymmetric hydroxylation of the chiral oxazolidinone **9**, as described by Evans et al.[9] *m*-Cyanohydrocinnamic acid, **8**, was prepared from *m*-cyanobenzaldehyde by Horner−Emmons reaction, hydrogenation, and hydrolysis, activated as the mixed anhydride with pivalic acid, and condensed with the lithium salt of benzylox-

(5) Lauri, G.; Bartlett, P. A. *J. Comput.-Aided Mol. Des.* **1994**, *8*, 51.

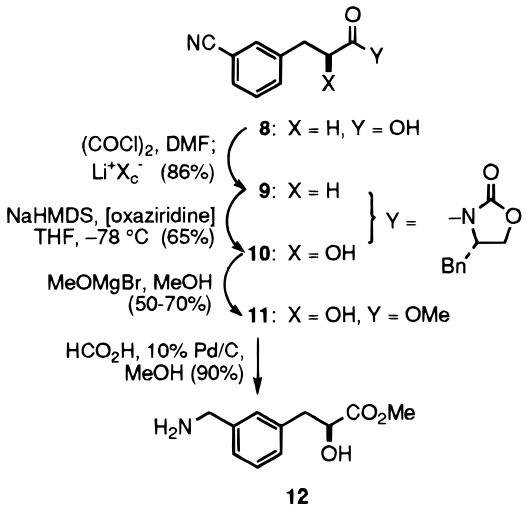
(6) O'Donnell, M. J.; Bennett, W. D.; Wu, S. *J. Am. Chem. Soc.* **1989**, *111*, 2353.

(7) Li, W.; Ewing, W.; Harris, B.; Joullie, M. *J. Am. Chem. Soc.* **1990**, *112*, 7659.

(8) Evans, D. A.; Bender, S. L.; Morris, J. *J. Am. Chem. Soc.* **1988**, *110*, 2506.

(9) Evans, D. A.; Morrissey, M. M.; Dorow, R. L. *J. Am. Chem. Soc.* **1985**, *107*, 4346.

Scheme 1

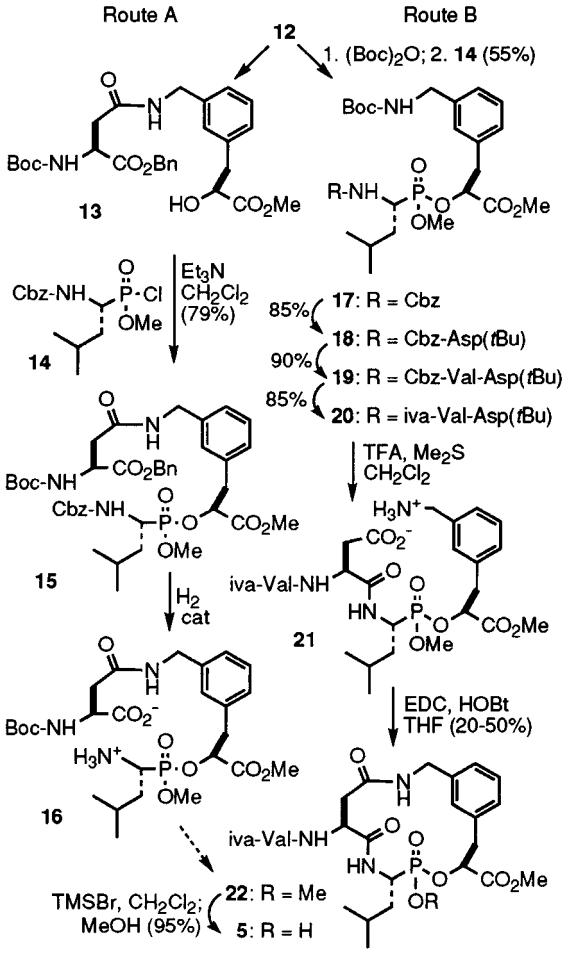


azolidinone to give imide **9** (Scheme 1). The enolate of this material was generated at −95 °C in the presence of 2-benzenesulfonyl-3-phenyloxaziridine by slow addition of sodium hexamethylsilazide,[10] and the resulting alkoxide was quickly quenched at low temperature by addition of a THF solution of camphorsulfonic acid prior to workup. The hydroxy ester **11** was liberated from the chiral auxiliary as described previously,[9] and the cyano moiety was reduced catalytically by transfer hydrogenation to afford the key intermediate **12**.

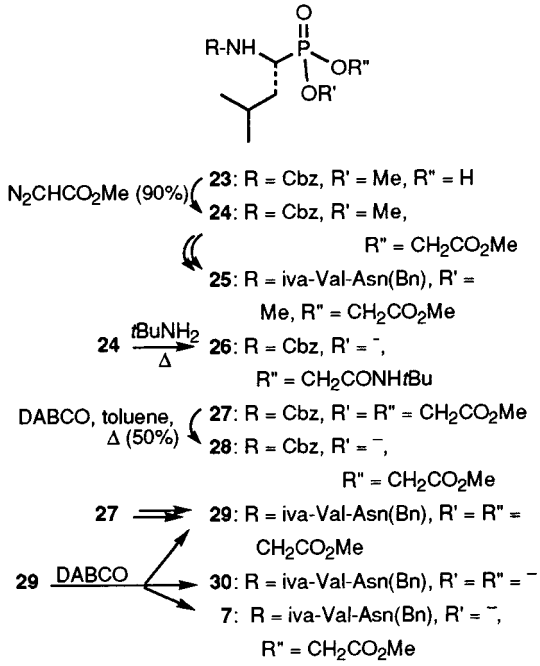
The sometimes capricious nature of phosphonate esterifications with hindered components suggested that the phosphonate linkage should be established early in the synthesis.[11] Sequences were therefore explored in which the Asn-Leu^P amide or the side chain-to-side chain linkages were formed in the macrocyclization step (Scheme 2). Route A was evaluated first. The protected precursor **15** was assembled by acylating the amine of intermediate **12** with an aspartic acid derivative and then acylating the hydroxyl group with the protected L-leucine-phosphonochloridate **14**.[12,13] Simultaneous deprotection of the benzyl ester and Cbz group proceeded in poor yield, and material of sufficient purity for subsequent cyclization proved difficult to isolate. We therefore pursued Route B, assembling the peptide backbone prior to ring closure.

The aminomethyl group of **12** was protected as the Boc derivative, and the hydroxyl moiety was phosphonylated to give **17** (Scheme 3). Elaboration of the peptide backbone proceeded straightforwardly to give the acyclic intermediate **20**.[14] Double deprotection of **20** was accomplished with trifluoroacetic acid (TFA) in the presence of dimethyl sulfide, and the TFA salt was converted to the zwitterion **21** by washing an ethyl acetate solution with small portions of pH 7.5 phosphate buffer. Several peptide coupling reagents were explored for cyclization of **21** to **22**; the reaction proceeded slowly but in reproducible yield

Scheme 2



Scheme 3



with ethyl dimethylaminoethyl carbodiimide (EDC), hydroxybenzotriazole (HOBt), and a tertiary amine in dilute THF solution (0.001 M) at room temperature. Clear differences were observed in the rates of reaction of the phosphonate diastereomers in both the cyclization (**21** → **22**) as well as earlier

(10) Davis, F. A.; Stringer, O. D. *J. Org. Chem.* **1982**, *47*, 1774.

(11) Hirschmann, R.; Yager, K. M.; Taylor, C. M.; Moore, W.; Sprengeler, P. A.; Witherington, J.; Phillips, B. W.; Smith, A. B., III *J. Am. Chem. Soc.* **1995**, *117*, 6370.

(12) Oleksyszyn, J.; Subotkowska, L.; Mastalerz, P. *Synth. Commun.* **1979**, 985. Kafarski, P.; Leijczak, B.; Szewczyk, J. *Can. J. Chem.* **1983**, *61*, 2425.

(13) Bartlett, P. A.; Hanson, J. E.; Giannousis, P. P. *J. Org. Chem.* **1990**, *55*, 6268.

(14) Deprotection of the side chain functionality and macrocyclization were evaluated at two points in this sequence, prior to and after introduction of the Iva-Val group. The cyclization itself proceeded in lower yield when carried out at the earlier stage, and it was difficult to acylate the macrocycle (H in place of Iva-Val in **22**) without racemization.

Table 1. Inhibition of Penicillopepsin by Constrained and Unconstrained Phosphonate Analogues

inhibitor	K_i (nM) at pH 4.5	K_i (nM) at pH 3.5
7	1300 ± 155	860 ± 3
6	42 ± 2.9	14 ± 5.6
4		2.8[a]
5	0.10 ± 0.002	

[a] From ref 13.

acylation (**18** → **19**) steps, although loss of the stereocenter at phosphorus at the end of the synthesis made these differences unimportant. As the last step, the phosphonate methyl ester was cleaved selectively with trimethylsilyl bromide and the macrocycle **5** was purified by reverse phase chromatography.

Synthesis of the acyclic comparison compound **6** was uneventful, in that similar coupling reactions were employed to link commercially available amino acids and hydroxy esters with the L-leucine phosphonate **14**. In contrast, synthesis of the other acyclic analogue **7** was unexpectedly difficult (Scheme 3). The glycolate ester **24** was conveniently prepared by esterification of monoacid **23** with methyl diazoacetate and readily elaborated to the full-length derivative **25**; however, we were not able to remove the phosphonate methyl ester selectively. Trimethylsilyl bromide[15] cleaved both P−O bonds at similar rates, and, in model experiments with dipeptide analogue **24**, refluxing *tert*-butylamine[16] generated the phosphonate monoester but transaminated the glycolate residue at the same time to give amide **26**. We were also frustrated in attempts to cleave only one of the phosphonate esters from symmetrical diester **27** with trimethylsilyl bromide, and no reaction was observed with *tert*-butylamine. However, 1,4-diazabicyclo-[2.2.2]octane (DABCO) gave the desired monoanion **28** as the major product.[17] After elaboration of **29**, treatment with DABCO in refluxing toluene for 1.5 h afforded 55% of the desired monoanion **7**, 19% of the dianion **30**, and 20% of recovered starting material after fractionation.

Inhibition of Penicillopepsin. The inhibition constants K_i for the acyclic comparison inhibitors were determined using the established assay procedure at the pH optimum of 3.5 as well as at pH 4.5 (Table 1). The K_i value for the macrocyclic phosphonate **5** was difficult to measure, since its high affinity required low enzyme and inhibitor concentrations for equilibrium experiments, with prolonged incubation times as a consequence.[18] By stabilizing the enzyme for overnight incubations with bovine serum albumin (BSA) and using a Henderson analysis to compensate for inhibitor depletion,[19] we were able to determine a K_i value at pH 4.5, where inhibitor affinity is reduced; tighter binding and enzyme instability prevented us from measuring an inhibition constant at pH 3.5.

Conformation of Macrocycle 5. The solution structure of the macrocyclic ring in phosphonate **5** was determined by a combined NMR−molecular modeling approach.[20] ^{1}H NMR spectra were obtained in aqueous buffer at 0, 27, and 40 °C, and there was no evidence of slow interconversion of conformational isomers. The temperature dependence of the N−H chemical shifts (8−9 ppb/K) indicated that the amide hydrogens are not shielded from solvent by intramolecular hydrogen bonds. Proton assignments were made from a TOCSY spectrum, and NH−αH coupling constants were used to define torsional constraints for a Monte Carlo conformational search (Table 2).[21] Several ranges for the N−Cα dihedral angles are consistent with the observed coupling constants for the valine and asparagine residues, but only one fits the large coupling constant of the leucine-phosphonate moiety.

Table 2. NH−αH Coupling Constants and Dihedral Constraints for Macrocycle **5**[a]

resonance	δ (ppm)	Δppb/K	J (Hz)	dihedral range for N−Cα bond
Asn αNH	8.55	8.3	8.1	+60° ± 20° −80° ± 20° −160° ± 20°
NH of Asn β-amide	8.5	8.3	5.4, 7.3[b]	(not assigned)
Val NH	8.08	9.4	7.85	+60° ± 20° −75° ± 20° −165° ± 20°
Leu^p NH	7.55	8.9	9.75	−120° ± 20°

[a] Determined at a concentration of 10 mM **5** in 0.1 M sodium acetate-d_3/1 mN KH_2PO_4 at pH 3.5. [b] Diastereotopic benzylic hydrogens not assigned.

These constraints were applied in a Monte Carlo conformational analysis of the complete inhibitor with a search protocol modified to include multiple angle constraints.[20] Some 20 000 different starting conformers were generated and minimized using an Amber force field with the GB/SA solvation treatment of MacroModel[22] and modified to include parameters for the phosphonate moiety developed by Merz and Kollman.[23,24] All minimizations reached convergence before 5000 steps, and multiple occurrences of the lowest energy structures suggested that the conformational ensemble was adequately sampled. The search resulted in 5469 distinct conformations that were clustered into only 240 groups when compared by the backbone atoms of the macrocycle (rmsd < 0.3 Å).[25] The lowest energy cluster, which contains 537 structures and 47 of the 48 lowest energy conformations, is 2.4 kcal/mol lower than the next ranked;[26] a representative set of these conformations is shown in Figure 5a. Of the other two clusters within 3 kcal/mol of cluster 1, only in cluster 2 (272 members) is the macrocyclic ring significantly different (Figure 5b); it may therefore represent a minor component of the ensemble.

The same force field and solvation treatment were used to predict the conformation of the macrocycle in advance of synthesis and to model it with constraints derived from the NMR data; the predicted conformation and that modeled with the NMR data are compared in Figure 5c. Finding the same structure in both approaches suggests that these protocols provide a realistic indication of the solution structure of **5**.

Analysis of Design Strategy: Quantitative Impact of Macrocyclization. In the absence of other torsional constraints, a linear chain of n-nodes has $n - 1$ independent rotations; connecting the termini to form a macrocycle adds another

(15) McKenna, C. E.; Higa, M. T.; Cheung, N. H.; McKenna, M. *Tetrahedron Lett.* **1977**, 155.
(16) Gray, M. D. M.; Smith, D. J. H. *Tetrahedron Lett.* **1980**, *21*, 859.
(17) Saady, M.; Lebeau, L.; Mioskowski, C. *J. Org. Chem.* **1995**, *60*, 2946.
(18) Morrison, J. F.; Walsh, C. T. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1987,** *61*, 201.
(19) Henderson, P. J. F. *Biochem. J.* **1972**, *127*, 321.
(20) Sefler, A. M.; Lauri, G.; Bartlett, P. A. *Int. J. Pept. Protein Res.* **1996,** *48*, 129.
(21) Bistrov, V. F. *Prog. NMR Spectrosc.* **1976**, *10*, 41.
(22) 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. Still, W. C.; Tempczyk, A.; Hawly, R. C.; Hendrickson, T. *J. Am. Chem. Soc.* **1990**, *112*, 6127.
(23) Merz, K. M., Jr.; Kollman, P. A. *J. Am. Chem. Soc.* **1989**, *111*, 5649.
(24) Ferguson, D. M.; Kollman, P. A. *J. Comput. Chem.* **1991**, *12*, 620.
(25) Lauri, G.; Bartlett, P. A. *J. Comput.-Aided Mol. Des.* **1994**, *8*, 51.
(26) The "energy" of a cluster is considered to be that of the lowest energy single conformation contained within it.

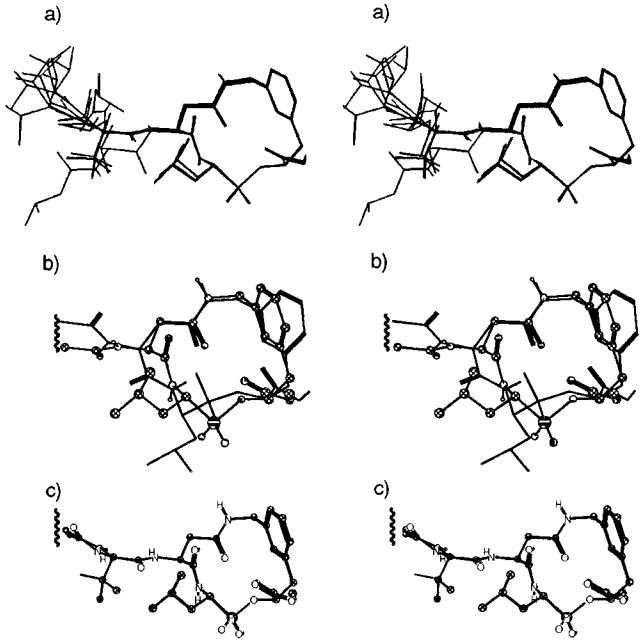


Figure 5. (a) A selection of conformers from the lowest energy cluster for macrocycle **5** consistent with the NMR data; (b) comparison of ring conformations from the low energy conformers from clusters 1 (ball-and-stick) and 2 (solid line); (c) comparison of conformation of **5** predicted in advance of synthesis (solid line) and that determined from NMR data (ball-and-stick).

Table 3. Effect of Constraining Bond Rotations

compd	no. of independent rotations in central structure[a]	effect of constraint on binding affinity per independent rotation (kcal/mol)
1	7 − 6 = 1	
2	5	0.34
3	5	0.74
5	11 − 6 = 5	
6	9	0.90[b]
7	9	1.41[b]

[a] Central structure corresponds to macrocycle and corresponding bonds in acyclic analogues, not including amides or bonds inside aromatic rings. [b] Calculated from inhibition constants determined at 25 °C and pH 4.5.

rotatable bond, but 6 degrees of freedom are lost as the bond rotations become dependent on each other.[27] Thus, the conformational constraint introduced by the macrocyclic rings in **1** and **5** can be gauged on a per-bond basis as shown in Table 3.

Although the same structural perturbation was applied to obtain each of the acyclic comparison compounds, the results are dramatically different. For both systems, the impact of cyclization is greater when comparing the cyclic inhibitor with the more linear acyclic analogue, since the comparison compounds are not equally *un*constrained. Bond rotation is clearly more restricted in branched analogues **2** and **6** than in linear analogues **3** and **7**. Moreover, there is a significant difference between the two macrocyclic systems: the effect is larger for **5** versus **6** and **7** than for **1** versus **2** and **3**. Since the latter compounds do not all adopt the same active site conformations,[2] the observed differences in their binding affinity only represent lower limits to what would be found for congruent binding modes.

(27) Go, N.; Scheraga, H. A. *Macromolecules* **1970**, *3*, 178. Guarnieri, F.; Cui, W.; Wilson, S. R. *J. Chem. Soc., Chem. Commun.* **1991**, 1542.

The presence of the macrocyclic ring has a dramatic effect on the binding of the inhibitors described in this report, enhancing the affinity of **5** by 3−4 orders of magnitude over the acyclic analogues **6** and **7** (Table 1). Moreover, **5** is a significantly better inhibitor than **4**, the original structure from which it was derived. The 3.6−5.6 kcal/mol in free energy represented by the K_i differences between macrocycle **5** and acycles **6** and **7** are among the highest values reported for a conformational constraint. Nevertheless, the per-bond effects are consistent with analyses from a variety of systems[28] and demonstrate the value of this structure-based design strategy.

Experimental Section[29]

(4*S*)-(3-(3-Cyanophenyl)propanoyl)-4-(phenylmethyl)-2-oxazolidinone (9). To a stirring solution of 3-(3-cyanophenyl)propanoic acid **8** (4.0 g, 23 mmol) in 75 mL of THF at −78 °C was slowly added triethylamine (TEA; 4.0 mL, 28.75 mmol) and pivaloyl chloride (3.1 mL, 25.3 mmol). After 10 min at −78 °C, the solution was brought to room temperature for 1.5 h, and cooled again to −78 °C. During this time, a solution of (*S*)-(−)-4-benzyl-2-oxazolidinone (4.4 g, 25.3 mmol) in 75 mL of THF was prepared and cooled to −78 °C, *n*-butyllithium (9.7 mL, 2.60 M in hexanes, 25.3 mmol) was added, and the mixture was stirred for 15 min. The lithium salt of the oxazolidinone was slowly added as a slurry to the mixed-anhydride solution via cannula. After 30 min, this mixture was allowed to warm to room temperature and stirred for 3.5 h. Half-saturated aqueous NH_4Cl (100 mL) was added, and most of the THF was removed by evaporation. The residue was extracted with CH_2Cl_2 (3 × 50 mL), and the combined organic layer was washed with saturated $NaHCO_3$ (50 mL) and brine (50 mL), dried, and evaporated to provide 8.0 g of a waxy yellow solid. Flash chromatography (20−50% EtOAc in hexanes) of this material provided 6.5 g (86% yield) of **9** as a thick, clear oil: ^{1}H NMR δ 7.17−7.58 (m, 9), 4.65−4.71 (m, 2), 4.17−4.24 (m, 1), 3.20−3.37 (m, 3), 3.06 (t, 2, J = 7.4), 2.78 (dd, 1, J = 9.5, 13.4); ^{13}C NMR δ 171.5, 153.3, 141.9, 135.0, 133.1, 132.1 130.0, 129.3, 129.2, 128.9, 127.3, 118.8, 112.4, 66.3, 55.0, 37.7, 36.6, 29.6. Anal. Calcd for $C_{20}H_{18}N_2O_3$: C, 71.84; H, 5.43; N, 8.38. Found: C, 71.44; H, 5.40; N, 8.23.

(2*S*,4*S*)-(3-(3-Cyanophenyl)-2-hydroxypropanoyl)-4-(phenylmethyl)-2-oxazolidinone (10). An equivalent of sodium bis(trimethylsilyl)-amide in THF was added via syringe pump over 1.5 h to a solution of amide **9** (3.63 g, 10.9 mmol) and 2-(phenylsulfonyl)-3-phenyloxaziridine (3.13 g, 12 mmol) in 55 mL of THF at −98 °C. During this time, a solution of camphorsulfonic acid (10.1 g, 43.6 mmol) in 90 mL of THF was prepared, cooled to −98 °C, and then transferred rapidly via cannula to the oxidation solution. The mixture was allowed to warm to room temperature and poured into 600 mL of 3:1 petroleum ether/CH_2Cl_2, and the cloudy suspension was washed with water until clear (3 × 200 mL), 1 M HCl (200 mL), and brine. The organic layer was dried and evaporated, and the residue was chromatographed (1:3:6 CH_2Cl_2/EtOAc/hexanes) to give 2.12 g (56% yield) of pure **10** as a clear oil, as well as 1.10 g of impure product. Repurification of the latter material (10% ether in CH_2Cl_2) gave an additional 0.40 g (10% yield) of hydroxyimide **10**: ^{1}H NMR δ 7.19−7.61 (m, 9), 5.16 (dd, 1, J = 3.6, 8.6), 4.66−4.71 (m, 1), 4.27−4.34 (m, 2), 3.29 (dd, 1, J = 3.3, 13.6), 3.20 (dd, 1, J = 3.5, 13.9), 2.83−2.89 (m, 2); ^{13}C NMR δ 73.4, 153.3, 138.7, 134.5, 134.2, 133.2, 130.5, 129.4, 129.1, 129.0, 127.6, 118.8, 112.4, 71.3, 67.1, 55.4, 39.7, 37.4; HRMS (FAB) calcd for $C_{20}H_{19}N_2O_4$ (MH^+) m/z 351.1345, found 351.1295.

Methyl (2*S*)-3-(3-Cyanophenyl)-2-hydroxypropanoate (11). A solution of bromomagnesium methoxide was prepared by slow addition of methylmagnesium bromide (2.7 mL, 8.0 mmol) to 60 mL of anhydrous methanol cooled to 0 °C. After 10 min, this solution was transferred via cannula into a flask containing a solution of hydroxy-

(28) Andrews, P. R.; Craik, D. J.; Martin, J. L. *J. Med. Chem.* **1984**, *27*, 1648. Gerhard, U.; Searle, M. S.; Williams, D. H. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 803. Williams, D. H.; Searle, M. S.; Mackay, J. P.; Gerhard, U.; Maplestone, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 1172.

(29) See footnote 18 in ref 1.

imide **10** (1.4 g, 4.0 mmol) in 60 mL of anhydrous methanol at 0 °C. The mixture was stirred for 20 min and acidified with 30 mL of 0.5 M HCl, and the methanol was removed under reduced pressure. The remaining aqueous solution was extracted with ether (3 × 30 mL), and the combined organic layer was washed with brine, dried, and evaporated. The residue was chromatographed (10% ether in CH_2Cl_2) to afford 0.513 g (63% yield) of hydroxyester **11** as a clear oil: $[\alpha]_D$ −23° (c = 0.012, $CHCl_3$); ^{1}H NMR δ 7.36−7.55 (m, 4), 4.44 (dd, 1, J = 4.2, 6.9), 3.79 (s, 3), 3.15 (dd, 1, J = 4.1, 14.1), 2.96 (dd, 1, J = 6.9, 14.1), 2.85 (br, 1); ^{13}C NMR δ 174.0, 138.1, 134.0, 133.0, 130.5, 129.0, 118.7, 112.3, 70.6, 52.6, 39.6. Anal. Calcd for $C_{11}H_{11}NO_3$: C, 67.38; H, 5.40; N, 6.83. Found: C, 64.01; H, 5.49; N, 6.64.

Methyl (2*S*)-3-(3-((*tert*-Butoxycarbonyl)aminomethyl)phenyl)-2-hydroxypropanoate. A suspension of palladium on carbon (10%, 80 mg, dried under vacuum and kept under dry nitrogen) and formic acid (0.20 mL) in ethanol (2 mL) was prepared, and a solution of hydroxyester **11** (80 mg, 0.39 mmol) in 1.6 mL of methanol, along with more formic acid (0.20 mL), was added. After 2 h of stirring, the suspension was passed through a 4.5-μm syringe filter, and the filtrate was evaporated to give 86 mg (87% yield) of the formate salt of **12** as a light yellow oil; this material was carried on without purification: ^{1}H NMR (CD_3OD) δ 8.27 (br s, 1), 7.27−7.36 (m, 4), 4.42 (dd, 1, J = 4.6, 7.9), 4.08 (s, 2), 3.70 (s, 3), 3.10 (dd, 1, J = 4.5, 13.9), 2.94 (dd, 1, J = 7.9, 13.9); ^{13}C NMR δ 175.5, 139.8, 134.3, 131.3, 131.2, 130.2, 128.2, 72.7, 52.5, 44.4, 41.2; LRMS (FAB) calcd for $C_{11}H_{15}NO_2$ (MH^+) m/z 210.2, found 210.1.

A 5.3-mmol sample of the formate salt of **12** was converted to the chloride salt by evaporation twice from a solution of ca. 5 equiv of HCl in methanol (prepared by from acetyl chloride (1.5 mL, 26.5 mmol) and 5 mL of methanol), and the material was dissolved in 50 mL of DMF. 2-(*tert*-Butoxycarbonyloxyimino)-2-phenylacetonitrile (2 g, 8.0 mmol) and di(isopropyl)ethylamine (2.7 mL, 10.6 mmol) were added, and the reaction mixture was stirred overnight. The solution was evaporated to ca. 5 mL, diluted with 50 mL of EtOAc, and washed with half-saturated aqueous $KHSO_4$ (10 mL), saturated $NaHCO_3$ (10 mL), and brine (10 mL), and dried. Evaporation provided 2.97 g of a yellow oil, which was purified by flash chromatography to yield 1.1 g (69% yield) of the Boc-protected derivative as a pale yellow oil: $[\alpha]_D$ = −7.1° (c = 0.0065, $CHCl_3$); ^{1}H NMR δ 7.08−7.25 (m, 4), 4.91 (br s, 1), 4.41 (dd, 2, J = 4.2, 6.9) 4.25 (br s, 2), 3.74 (s, 3), 3.08 (dd, 1, J = 4.2, 13.9), 2.91 (dd, 1, J = 7.0, 13.9), 2.75 (br s, 1), 1.43 (s, 9); ^{13}C NMR δ 174.5, 155.8, 139.0, 136.7, 128.6, 128.5, 128.4, 126.0, 79.4, 71.2, 52.4, 44.5, 40.4, 28.3. Anal. Calcd for $C_{16}H_{23}NO_5$: C, 62.12; H, 7.49; N, 4.53. Found: C, 61.86; H, 7.62; N, 4.49.

Methyl (2*S*)-2-[(1*R*)-1-(*N*-(Phenylmethoxycarbonyl)amino)-3-methylbutylmethoxyphosphinyloxy]-3-(3-(*tert*-butoxycarbonyl)aminomethyl)phenylpropanoate (17). A solution of Cbz-leucine phosphonate monomethyl ester[12] (208 mg, 0.66 mmol) in 2.2 mL of CH_2Cl_2 was cooled to 0 °C, and thionyl chloride (0.240 mL, 3.3 mmol) was added dropwise over 2 min. After 30 min, the solution was allowed to warm to room temperature, sparged with N_2 for 20 min, and placed under vacuum for 2 h to remove the remaining volatile material; the residue was then redissolved in 2.2 mL of CH_2Cl_2 and cooled to 0 °C under N_2. Meanwhile, a solution of the Boc-protected amine (265 mg, 0.86 mmol) and TEA (0.185 mL, 1.3 mmol) in 2.2 mL of CH_2Cl_2 was prepared in the presence of a few 4 Å molecular sieves and then added to the phosphonochloridate solution over 2 min. After 1 h, the mixture was allowed to warm to room temperature and stirred overnight. The reaction mixture was diluted with 10 mL of CH_2Cl_2, washed with half-saturated aqueous $KHSO_4$ (2 mL), saturated $NaHCO_3$ (2 mL), and brine (2 mL), dried, and evaporated; the oily residue was chromatographed (30% EtOAc in CH_2Cl_2) to give 287 mg (72% yield) of phosphonate **17** as a colorless oil as a ca. 70:30 ratio of diastereomers at phosphorus; each diastereomer appeared as a mixture of rotamers (ca. 10:1). For diastereomer 1: ^{1}H NMR δ 7.10−7.32 (m, 9), 5.45 (d, 1, J = 9.7), 4.98−5.15 (m, 4), 4.15−4.30 (m, 3), 3.73 (s, 3), 3.23 (dd, 1, J = <2, 14.5), 3.13 (d, 3, J = 10.9), 2.93−3.01 (m, 1), 1.44−1.70 (m, 3), 1.43 (s, 9), 0.90 (d, 6, J = 6.3); ^{31}P NMR δ 27.8. For diastereomer 2: ^{1}H NMR δ 7.10−7.32 (m, 9), 4.98−5.15 (m, 4), 4.71 (d, 1, J = 10.5), 4.15−4.30 (m, 3), 3.73 (d, 3, J = 10.9), 3.72 (s, 3), 3.07 (m, 1), 2.93−3.01 (m, 1), 1.44−1.70 (m, 3), 1.43 (s, 9), 0.80 (d, 3, J = 6.4), 0.79 (d, 3, J = 6.6); ^{31}P NMR δ 27.1. For both diastereomers: ^{13}C NMR δ 170.8, 170.6, 156.1, 156.0, 155.8, 155.7, 139.6, 139.3, 136.4, 136.0, 128.9, 128.7, 128.6, 128.4, 128.3, 128.1, 128.0, 127.9, 127.9, 126.2, 79.5, 79.4, 74.7, 74.4, 67.0, 66.8, 53.3, 52.7, 52.4, 51.8, 51.7, 46.5 (d, J = 150), 46.3 (d, J = 150), 44.4, 44.3, 38.9, 37.8, 28.3, 24.3, 24.2, 23.3, 23.1, 21.1, 20.9. Anal. Calcd for $C_{30}H_{43}N_2O_9P$: C, 59.40; H, 7.14; N, 4.62; P, 5.10. Found: C, 59.72; H, 7.21; N, 4.40; P, 5.09.

Methyl (2*S*)-2-[(1*R*)-1-(*N*-(L-*N*-Phenylmethoxycarbonyl-*β*-*tert*-butylaspartyl)amino)-3-methylbutylmethoxyphosphinyloxy]-3-(3-(*tert*-butoxycarbonyl)aminomethyl)phenylpropanoate (18). A suspension of dipeptide phosphonate **17** (270 mg, 0.45 mmol) and 10% Pd/C in 2.2 mL of EtOAc was stirred under a hydrogen atmosphere for 2 h and then filtered through a 4.5-μm nylon syringe filter directly into a solution of Cbz-aspartic acid, *β*-*tert*-butyl ester (173 mg, 0.54 mmol), EDC (120 mg, 0.63 mmol), and HOBt (120 mg, 0.90 mmol) in 2.2 mL of DMF at 0 °C. Another 2 mL of EtOAc was used to rinse the syringe filter then added to the reaction solution. The solution was allowed to warm, kept at room temperature for 16 h, diluted with 15 mL of EtOAc, washed with half-saturated aqueous $KHSO_4$ (2 mL), saturated $NaHCO_3$ (2 mL), and brine (2 mL), dried, and evaporated, and the crude product was chromatographed (0−2% methanol in $CHCl_3$) to give 303 mg (88% yield) of the tripeptide phosphonate **18** as a 2:1 mixture of diastereomers at phosphorus. Further purification allowed partial separation of the diastereomers for identification. For diastereomer 1: ^{1}H NMR δ 7.12−7.42 (m, 9), 6.93 (d, 1, J = 9.8), 5.99 (br s, 1), 5.08−5.15 (m, 4), 4.54−4.60 (m, 2), 4.28 (d, 2, J = 5.3), 3.74 (s, 3), 3.25 (dd, 1, J = 3.2, 14.4), 3.20 (d, 3, J = 10.9), 3.03 (dd, 1, J = 9.2, 14.2), 2.94 (dd, 1, J = 4.9, 17.1), 2.58 (dd, 1, J = 5.2, 17.1), 1.50−1.63 (m, 3), 1.45 (s, 9), 1.41 (s, 9), 0.90 (d, 3, J = 6.4), 0.85 (d, 3, J = 6.3); ^{13}C NMR δ 171.1, 170.7, 170.2, 158.1, 155.8, 139.4, 136.1, 136.0, 128.8, 128.6, 128.5, 128.4, 128.2, 128.0, 126.3, 81.6, 79.4, 74.8 (d, J = 7), 67.1, 52.6, 51.9, 51.5, 44.5, 44.5 (d, J = 125), 39.0, 38.7, 37.3, 28.4, 28.0, 24.2 (d, J = 11), 23.3, 21.1; ^{31}P NMR δ 26.5. For diastereomer 2: ^{1}H NMR δ 7.10−7.37 (m, 9), 6.46 (br s, 1), 6.46 (br s, 1), 5.49, (br s, 1), 5.13 (s, 2), 4.96−4.98 (m, 1), 4.20−4.40 (m, 4), 3.75 (s, 3), 3.74 (d, 3, $J \approx$ 10), 3.15 (dd, 1, J = <2, 15.2), 2.90 (m, 2), 2.55 (m, 1), 1.43 (s, 9), 1.41 (s, 9), 1.19−1.50 (m, 2), 0.82−0.90 (m, 1), 0.75 (d, 3, J = 8.6), 0.74 (d, 3, J = 6.6); ^{13}C NMR δ 171.1, 170.5, 170.1, 156.0, 155.9, 139.6, 136.0, 135.9, 128.8, 128.5, 128.2, 126.3, 81.7, 79.2, 75.6, 67.2, 53.4 (d, J = 6.4), 52.4, 51.2, 44.3, 44.2 (d, J = 157), 39.0, 37.5, 37.0, 28.3, 27.9, 24.0 (d, J = 14), 23.1, 20.8, 18.8; ^{31}P NMR δ 25.6; HRMS (FAB) calcd for $C_{38}H_{57}N_3O_{12}P$ (MH^+) m/z 778.3680, found 778.3682.

Methyl (2*S*)-2-[[(1*R*)-1-(*N*-(L-*N*-Phenylmethoxycarbonylvalyl-L-*β*-*tert*-butylaspartyl)amino)-3-methylbutyl]methoxyphosphinyloxy]-3-(3-(*tert*-butoxycarbonyl)aminomethyl)phenylpropanoate (19). A suspension of tripeptide phosphonate **18** (328 mg, 0.42 mmol) and 10% Pd/C in 4.2 mL of EtOAc was stirred under an atmosphere of hydrogen for 4 h, filtered through a 4.5-μm nylon syringe filter, and evaporated to give a quantitative yield (273 mg) of the free amine as a yellow oil. To a stirring solution of this material in 4.2 mL of DMF at 0 °C were added Cbz-valine (130 mg, 0.50 mmol), EDC (115 mg, 0.59 mmol), and HOBt (115 mg, 0.84 mmol). After 12 h, the mixture was diluted with 50 mL of EtOAc, washed with half-saturated aqueous $KHSO_4$ (5 mL), saturated $NaHCO_3$ (5 mL), and brine (5 mL), dried, and evaporated. The crude product was chromatographed (0−3% MeOH in $CHCl_3$) to give 270 mg (73% yield) of phosphonate **19** as a 2:1 mixture of diastereomers at phosphorus. For diastereomer 1: ^{1}H NMR δ 7.10−7.33 (m, 10), 6.88 (d, 1, J = 9.8), 5.26−5.49 (m, 2), 5.00−5.15 (m, 3), 4.70−4.74 (m, 1), 4.45−4.60 (m, 1), 4.20−4.35 (m, 2), 4.00−4.10 (m, 1), 3.76 (s, 3), 3.69−3.73 (m, 1), 3.20−3.24 (m, 1), 3.17 (d, 3, J = 10.9), 3.01 (dd, 1, J = 9.1, 14.2), 2.55 (dd, 1, J = 5.6, 16.9), 2.10−2.20 (m, 1), 1.42 (s, 9), 1.40 (s, 9), 1.23−1.57 (m, 3), 0.72−0.96 (m, 12); ^{13}C NMR δ 170.9, 170.3, 169.7, 169.5, 156.4, 155.8, 139.4, 136.0, 128.6, 128.4, 128.2, 128.1, 127.9, 126.2, 81.5, 79.4, 74.9, (d, $J \approx$ 7), 67.0, 60.4, 52.5, 51.8, 49.4, 44.5 (d, J = 157), 44.4, 38.9, 38.2, 36.6, 36.4, 31.3, 30.7, 28.3, 27.8, 24.2 (d, J = 14), 23.2, 20.9, 19.1, 17.4; ^{31}P NMR δ 26.2. For diastereomer 2: ^{1}H NMR δ 7.09−7.31 (m, 9), 6.67 (d, 1, J = 9.3), 5.56 (br s, 1), 5.43 (d, 1, J = 7.2), 5.07 (s, 2), 5.00 (br, s, 1), 4.50−4.60 (m, 1), 4.10−4.30 (m, 4), 3.95−4.05 (m, 1), 3.70 (s, 3), 3.70 (d, 3, J = 10.3), 3.16 (dd, 1, J = <3, 14),

3.01 (dd, 1, J = 8.1, 14.1), 2.75−2.85 (m, 1), 2.45−2.60 (m, 1), 2.05−2.20 (m, 1), 1.41 (s, 9), 1.40 (s, 9), 1.19−1.50 (m, 3), 0.92 (d, 3, J = 6.6), 0.86 (d, 3, J = 6.8), 0.75 (d, 3, J = 6.2), 0.71 (d, 3, J = 6.1); ^{13}C NMR δ 171.1, 171.0, 170.5, 169.7, 156.5, 156.0, 139.4, 135.8, 128.7, 128.4, 128.3, 128.1, 128.0, 126.3, 81.7, 79.1, 76.7, 75.4, 67.1, 60.4, 53.2, 52.3, 49.3, 44.4, 44.3 (d, J = 150), 39.0, 37.5, 36.5, 36.3, 31.3, 30.6, 28.3, 27.9, 24.1 (d, 15), 23.1, 20.8, 19.2, 17.4, 15.1; ^{31}P NMR δ 24.8; HRMS (FAB) calcd for $C_{43}H_{66}N_4O_{13}P$ (MH^+) m/z 877.4381, found 877.4377.

Methyl (2*S*)-2-[[(1*R*)-1-(*N*-(L-*N*-(3-methylbutanoyl)valyl-(*β*-*tert*-butyl ester)-L-aspartyl)amino)-3-methylbutyl]methoxyphosphinyloxy]-3-(3-(*tert*-butoxycarbonyl)aminomethyl)phenylpropanoate (20). This compound was prepared by a procedure similar to that above; thus, tetrapeptide phosphonate **19** (100 mg, 0.11 mmol) was deprotected by hydrogenolysis using 20 mg of 10% Pd/C in EtOAc (1.1 mL) and coupled to isovaleric acid (17 μL, 0.154 mmol) using EDC (35 mg, 0.176 mmol) in DMF (1.1 mL). The product was purified by chromatography (0−3% methanol in $CHCl_3$) to give 81 mg (86% yield) of phosphonate pentapeptide **20** as a 2:1 mixture of phosphorus diastereomers. For diastereomer 1: ^{1}H NMR δ 7.06−7.55 (m, 5), 6.97 (d, 1, J = 9.3), 5.25−5.33 (m, 1), 4.95−5.05 (m, 2), 4.68−4.73 (m, 1), 4.51−4.59 (m, 1), 4.21−4.25 (m, 2), 3.73 (s, 3), 3.66−3.71 (m, 1), 3.15−3.26 (m, 1), 3.16 (d, 3, J = 10.8), 2.95−3.08 (m, 1), 2.70−2.77 (m, 1), 2.54 (dd, 1, J = 5.7, 16.9), 2.05−2.20 (m, 4), 1.39 (s, 9), 1.35 (s, 9), 1.23−1.89 (m, 3), 0.75−0.95 (m, 18); ^{31}P NMR δ 25.4. For diastereomer 2: ^{1}H NMR δ 7.01−7.62 (m, 4), 6.68 (br s, 1), 6.17 (br s, 1), 5.53 (br s, 1), 4.95−5.02 (m, 1), 4.55−4.60 (m, 1), 4.11−4.28 (m, 4), 3.79 (s, 3), 3.73 (d, 3, J = 10.9), 3.20 (dd, 1, J = <2, 11.6), 3.04 (dd, 1, J = 8.1, 14.2), 2.68−2.72 (m, 1), 2.30−2.58 (m, 1), 2.05−2.28 (m, 4), 1.42 (s, 9), 1.41 (s, 9), 1.31−1.63 (m, 3), 0.74−0.95 (m, 18); ^{31}P NMR δ 24.9. For both diastereomers: ^{13}C NMR δ 173.0, 172.7, 171.0, 170.9, 170.3, 169.7, 169.6, 169.5, 169.3, 155.8, 155.5, 139.4, 136.4, 135.8, 128.6, 128.6, 128.3, 128.0, 126.5, 126.3, 126.0, 81.7, 81.5, 79.1, 79.0, 75.9 (d, $J \approx$ 7), 75.3 (d, $J \approx$ 7), 58.5, 58.4, 53.2, 52.6, 52.3, 52.0, 49.4, 49.3, 45.7, 44.5 (d, J = 160), 44.4, 44.2 (d, J = 158), 39.0, 38.9, 38.3, 38.0, 36.6, 36.3, 30.8, 30.5, 28.3, 27.9, 27.8, 26.0, 24.2 (d, J = 14), 24.1 (d, J = 14), 23.2, 23.1, 22.4, 22.3, 20.9, 20.8, 19.2, 19.1, 17.9, 14.1, 13.7; HRMS (FAB) calcd for $C_{40}H_{68}N_4O_{12}P$ (MH^+) m/z 827.4571, found 827.4592.

Methyl Cyclo[(2*S*)-2-[[(1*R*)-1-(*N*-(L-*N*-(3-methylbutanoyl)valyl-L-aspartyl)amino)-3-methylbutyl]methoxyphosphinyloxy]-3-(3-aminomethyl)phenylpropanoate (22). A solution of phosphonate pentapeptide **20** (40 mg, 0.048 mmol) in 540 mL of 4:4:1 TFA/CH_2Cl_2/DMS was stirred for 30 min, diluted with 1,2-dichloroethane, and evaporated. This residue was dissolved in 3 mL of pH 7.5 phosphate buffer and extracted with EtOAc (3 × 10 mL). The EtOAc solution was dried with $MgSO_4$ and evaporated to give 28 mg (88% yield) of the zwitterion **21**, which was further dried by lyophilization from benzene. A solution of this material in 48 mL of THF (0.001 M) was cooled to 0 °C, di(isopropyl)ethylamine (63 mL, 0.24 mmol), EDC (37 mg, 0.192 mmol), and HOBt (26 mg, 0.192 mmol) were added, and the mixture was allowed to warm to room temperature. After 5 d of stirring, the solution was evaporated, the residue was dissolved in 30 mL of EtOAc, and the mixture was washed with 1 M HCl (5 mL), saturated $NaHCO_3$ (5 mL), and brine (5 mL) and dried. After evaporation, the crude product was chromatographed (0−6% methanol in $CHCl_3$) to give 12 mg (38% yield) of macrocycle **22** as an opaque wax. For diastereomer 1: ^{1}H NMR δ 7.03−7.40 (m, 6), 6.76 (br s, 1), 6.21 (d, 1, J = 8.0), 5.08 (td, 1, J = 3.8, 7.5), 5.01 (dd, 1, J = 3.8, 7.5), 4.90−4.94 (m, 1), 4.69−4.74 (m, 1), 4.20 (dd, 1, J = 6.9, 7.6), 3.89 (dd, 1, J = 3.8, 15.7), 3.71 (s, 3), 3.28 (d, 3, J = 10.8), 3.18−3.22 (m, 1), 3.03−3.10 (m, 1), 2.94 (dd, 1, J = 10.8, 15.8), 2.78 (dd, 1, J = 2.1, 15.5), 2.03−2.15 (m, 4), 1.43−1.72 (m, 3), 0.89−1.00 (m, 18); ^{31}P NMR δ 26.5. For diastereomer 2: ^{1}H NMR δ 7.55 (d, 1, J = 9.2), 7.50 (s, 1), 7.28 (d, 1, J = 5.7), 7.16 (t, 1, J = 7.7), 7.03 (d, 1, J = 7.6), 6.94 (d, 1, J = 7.7), 6.68 (br s, 1), 6.16 (d, 1, J = 8.5), 5.15 (dt, 1, J = 4.4, 8.8), 4.87−4.95 (m, 2), 4.32−4.41 (m, 2), 3.85 (dd, 1, J = 5.0, 14.9), 3.70 (d, 3, J = 11.0), 3.67 (s, 3), 3.15−3.20 (m, 2), 2.84 (dd, 1, J = <2, 16.4), 2.61 (dd, 1, J = 9.8, 16.6), 2.06−2.14 (m, 4), 1.47−1.73 (m, 3), 0.87−0.95 (m, 18); ^{31}P NMR δ 26.4. For both diastereomers: ^{13}C NMR δ 172.9, 171.7, 171.5, 170.9, 170.8, 170.0, 169.9, 169.8, 169.9, 169.5, 138.7, 138.2, 135.8, 135.3, 130.2, 128.4, 128.3, 128.1, 127.9, 127.3, 126.4, 125.5, 74.6 (d, J = 7), 74.3 (d, J = 7), 58.3, 58.1, 53.6, 52.4, 52.1, 51.2, 49.5, 48.8, 45.6, 44.9 (d, J = 172), 43.5, 43.3 (d, J = 175), 39.0, 38.4, 38.0, 37.2, 31.1, 31.0, 30.9, 29.7, 28.0, 27.9, 26.1, 26.0, 24.6 (d, J = 13), 24.3 (d, J = 13), 23.0, 22.4, 22.4, 22.2, 21.0, 21.0, 19.2, 19.0, 18.1, 17.9, 14.3, 13.7; HRMS (FAB) calcd for $C_{31}H_{50}N_4O_9P$ (MH^+) m/z 653.3319, found 653.3307.

Methyl Cyclo[(2*S*)-2-[[(1*R*)-1-(*N*-(L-*N*-(3-methylbutanoyl)valyl-L-aspartyl)amino)-3-methylbutyl]hydroxyphosphinyloxy]-3-(3-aminomethyl)phenylpropanoate (5). Isobutylene was bubbled through 350 μL of CH_2Cl_2 for 5 min, bromotrimethylsilane (12 μL) was added, and, after 5 min, a solution of macrocycle **22** in 350 μL of CH_2Cl_2 was added. The solution was stirred for 4 h, evaporated from $CHCl_3$ (0.5 mL) and methanol (2 × 0.5 mL), and the residue was dissolved in 1 mL of 1:1 H_2O/methanol and eluted through a 1-mL syringe column of Li^+ Dowex with 5 mL of 1:1 H_2O/methanol. After evaporation, the residue was dissolved in 1 mL of H_2O, filtered through a 4.5-μm nylon syringe filter, and lyophilized to give 8.5 mg (75% yield) of the lithium salt of phosphonate **5** as a fluffy white powder: ^{1}H NMR (CD_3OD) δ 7.42 (s, 1), 7.14 (t, 1, J = 7.5), 7.05 (d, 1, J = 7.6), 6.94 (d, 1, J = 7.4), 4.96−4.99 (m, 1), 4.80−4.84 (m, 1), 4.60 (d, 1, J = 15.2), 4.23−4.30 (m, 1), 4.18 (d, 1, J = 7.1), 4.08 (d, 1, J = 15.2), 3.65−3.75 (m, 1), 3.62 (s, 3), 3.14 (dd, 1, J = 3.8, 14.1), 3.06 (dd, 1, J = 5.5, 14.2), 2.94 (dd, 1, J = 10.3, 15.2), 2.62 (dd, 1, J = <2, 12.7), 1.85−2.30 (m, 4), 1.35−1.62 (m, 3), 0.86−0.96 (m, 18); ^{13}C NMR (CD_3OD) δ 175.7, 173.7, 173.1, 172.1, 171.6, 139.6, 138.2, 130.4, 129.0, 128.9, 126.9, 74.4 (d, J = 6.4), 68.9, 60.2, 52.4, 51.5, 49.9, 47.2, 46.0, 43.9, 40.4, 39.7 (d, J = 187), 36.6, 31.7, 29.1, 27.4, 25.7 (d, J = 12.7), 24.1, 22.8, 22.7, 21.7, 19.9, 18.8; ^{31}P NMR (CD_3OD) δ 19.4; HRMS (FAB) calcd for $C_{30}H_{48}N_4O_9P$ (MH^+) m/z 639.315893, found 639.315130. Anal. Calcd for $C_{30}H_{46}N_4LiO_9P$: P, 4.81. Found: P, 2.45 (weight purity = 51%).

Determination of Inhibition Constants. Inhibition constants for acyclic inhibitors **6** and **7** were determined at 25 °C as described in the accompanying report.[1] Assay of the macrocyclic analogue **5** involved overnight incubation of inhibitor (1−5 nM) with enzyme (3−5 nM) to achieve equilibrium prior to addition of substrate.[30] The enzyme could be stabilized during these long incubations at pH 4.5 (but not at pH 3.5) by inclusion of BSA (0.1 mg/mL) in the buffer. Enzyme velocities were determined by duplicate or triplicate assays at 140 or 280 μM substrate, with the assumption that inhibitor dissociation was negligible during the course of the assay. The method of Henderson was employed to analyze the data.[19]

NMR Structure Determination. NMR spectra were obtained and analyzed according to the same protocols that are described in the preceding paper.[1]

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Supporting Information Available: Synthesis and characterization of acyclic comparison compounds **6** and **7**; preparation of **8**; kinetic plots for determination of inhibition constants (11 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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(30) Hanson, J. E.; Kaplan, A. P.; Bartlett, P. A. *Biochemistry* **1989**, *28*, 6294.