Transition State Analogy of Phosphonic Acid Peptide Inhibitors of Pepsin

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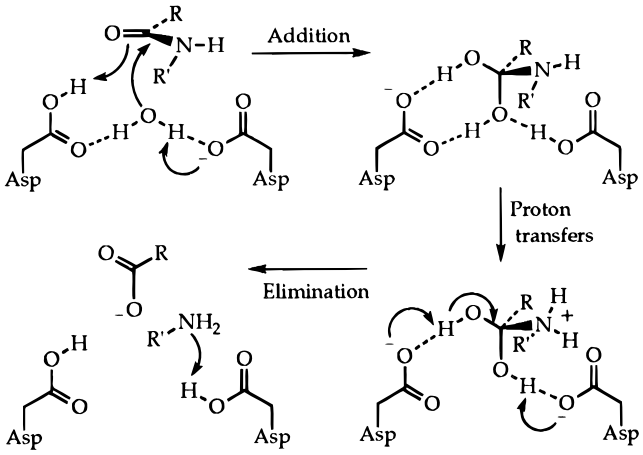
A series of 11 phosphonate peptide analogs, RO_2C-Xaa-Yaa-Phe-{PO_2^-−O}-Phe *O*-(3-(4-pyridyl)-propyl ester), were synthesized and evaluated as inhibitors of the aspartic peptidase pepsin. For the inhibitors with Gly or Ala at the P_2 position, the K_i values correlate with the K_m/k_{cat} values of the corresponding substrates, demonstrating that these analogs mimic the transition state in the way the P_2−P_4 residues bind. Deviations from the correlation for the other inhibitor/substrate pairs imply a different binding orientation as a result of N-substitution at P_2 (Pro), contamination with the more potent diastereomer (D-Ala), or a change in rate-limiting step for turnover (Ile).

The aspartic peptidases constitute one of the primary classes of proteolytic enzymes, utilizing two aspartic acid residues in the active site to catalyze the direct addition of water to the carbonyl of the scissile linkage.[1−3] Early investigations of pepsin and the fungal acid proteinases[4,5] gave rise to a variety of mechanistic proposals which were resolved in favor of the sequence shown in Scheme 1.[6,7] As a result of attempts to develop renin inhibitors as antihypertensive agents[8−10] and continuing efforts to exploit these and related inhibitor motifs for inactivation of the HIV protease,[11,12] there has been ongoing interest in understanding how these enzymes work and how they can be inhibited. Peptide analogs that incorporate tetrahedral phosphorus units in place of the scissile carbonyl group of a substrate are potent inhibitors of a variety of aspartic peptidases.[13−18] The activity of these derivatives has been understood to result from "transition state analogy": their mimicry of the high-energy, tetrahedral intermediate that lies along the reaction pathway (Scheme 1).[15] It was on this basis that such analogs were first studied, and the viewpoint has been supported by structural analysis of the complexes of these inhibitors with penicillopepsin[19] and with HIV protease.[20]

Scheme 1. Aspartic Peptidase Mechanism



However, potent inhibition does not of itself demonstrate that a molecule is a transition state analog.[21−25] Such evidence is obtained by showing that structural alterations produce similar effects on inhibitor binding (reflected in K_i) as they do on transition state stabilization, as inferred from the inverse second order rate constant K_m/k_{cat}.[22] This approach, first proposed by Wolfenden,[26] and by Thompson,[27] has been applied successfully in assessing the transition state analogy of phosphorus-containing inhibitors of the zinc peptidases thermolysin[28,29] and carboxypeptidase A,[21,30] among other enzymes.[31,32] We have recently described a complemen-

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(1) Fruton, J. S. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1976**, *44*, 1−36.
(2) Dunn, B. M. *Adv. Detailed React. Mech.* **1992**, *2*, 213−241.
(3) Davies, D. R. *Annu. Rev. Biophys. Biophys. Chem.* **1990**, *19*, 189−215.
(4) Wang, T.-T.; Hofmann, T. *Biochem. J.* **1976**, *153*, 691−699.
(5) James, M. N. G.; Sielecki, A. R. *Biochemistry* **1985**, *24*, 3701−3713.
(6) Suguna, K.; Padlan, E. A.; Smith, C. W.; Carlson, W. D.; Davies, D. R. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 7009−7013.
(7) James, M. N. G.; Sielecki, A. R.; Hayakawa, K.; Gelb, M. H. *Biochemistry* **1992**, *31*, 3872−3886.
(8) Lien, E. J.; Gao, H.; Lien, L. L. *Prog. Drug Res.* **1994**, *43*, 43−86.
(9) Raddatz, P. *Expert Opin. Ther. Pat.* **1994**, *4*, 1347−1359.
(10) Raddatz, P. *Expert Opin. Ther. Pat.* **1994**, *4*, 489−504.
(11) Thaisrivongs, S. *Annu. Rep. Med. Chem.* **1994**, *29*, 133−144.
(12) Huff, J. R. *J. Med. Chem.* **1991**, *34*, 2305−2314.
(13) Allen, M. C.; Fuhrer, W.; Tuck, B.; Wade, R.; Wood, J. M. *J. Med. Chem.* **1989**, *32*, 1652−1661.
(14) Dreyer, G. B.; Metcalf, B. W.; Tomaszek, T. A., Jr.; Carr, T. J.; Chandler, A. C., III; Hyland, L.; Fakhoury, S. A.; Magaard, V. W.; Moore, M. L.; Strickler, J. E.; Debouck, C.; Meek, T. D. *Proc. Nat. Acad. Sci. U.S.A.* **1989**, *86*, 9752−9756.
(15) Bartlett, P. A.; Hanson, J. E.; Giannousis, P. P. *J. Org. Chem.* **1990**, *55*, 6268−6274.
(16) Grobelny, D.; Wondrak, E. M.; Galardy, R. E.; Oroszlan, S. *Biochem. Biophys. Res. Commun.* **1990**, *169*, 1111−1116.
(17) Peyman, A.; Budt, K.-H.; Spanig, J.; Stowasser, B.; Ruppert, D. *Tetrahedron Lett.* **1992**, *33*, 4549−4552.
(18) Raddatz, P.; Minck, K.-O.; Rippmann, F.; Schmitges, C. J. *J. Med. Chem.* **1994**, *37*, 486−497.
(19) Fraser, M. E.; Strynadka, N. C. J.; Bartlett, P. A.; Hanson, J. E.; James, M. N. G. *Biochemistry* **1992**, *31*, 5201−5214.
(20) 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*, 7972−7980.
(21) Hanson, J. E.; Kaplan, A. P.; Bartlett, P. A. *Biochemistry* **1989**, *28*, 6294−6305.
(22) Wolfenden, R. *Annu. Rev. Biophys. Bioeng.* **1976**, *5*, 271−306.
(23) Wolfenden, R.; Radzicka, A. *Curr. Opin. Struct. Biol.* **1991**, *1*, 780−787.
(24) Morrison, J. F.; Walsh, C. T. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1987**, *61*, 201−301.
(25) Schloss, J. V. *Acc. Chem. Res.* **1988**, *21*, 348−353.
(26) Westerik, J. O.; Wofenden, R. *J. Biol. Chem.* **1971**, *247*, 8195−8197.
(27) Thompson, R. C. *Biochemistry* **1973**, *12*, 47−51.
(28) Bartlett, P. A.; Marlowe, C. K. *Biochemistry* **1983**, *22*, 4618−4624.
(29) Morgan, B. P.; Scholtz, J. M.; Ballinger, M.; Zipkin, I.; Bartlett, P. A. *J. Am. Chem. Soc.* **1991**, *113*, 297−307.
(30) Phillips, M. A.; Kaplan, A. P.; Rutter, W. J.; Bartlett, P. A. *Biochemistry* **1992**, *31*, 959−962.

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Table 1. Kinetic Parameters for Substrates, 1, and Inhibition Constants for Phosphonate Inhibitors, 2, of Porcine Pepsin[a]

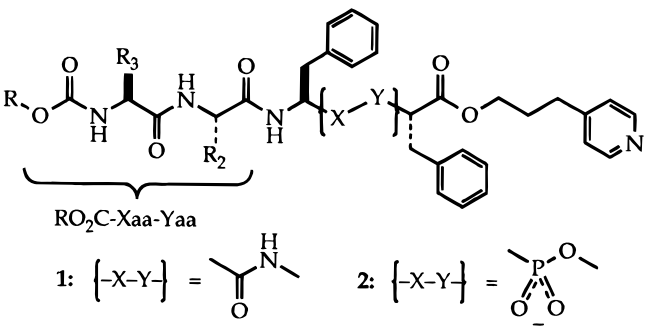
RO_2C-Xaa-Yaa[b]	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_i (nM)
ZGP	140	0.056	400	260
ZG-D-A	350	0.19	560	84
Z-D-AG	510	2.1	4 000	570
ZSarG	170	3.5	21 000	200
MocGG	750	19	25 000	67
ZGG	420	72	170 000	17
ZGI	70	12.6	180 000	0.31
ZFG	110	25	225 000	12
ZAG	250	145	580 000	4.2
ZGA	110	410	3 700 000	0.73
ZAA	40	280	7 000 000	0.34

[a] Kinetic data from Sachdev and Fruton at pH 3.5, 37 °C.[34] Inhibition constants were determined under the same conditions using Lys-Pro-Ala-Glu-Phe-(4-NO_2)Phe-Arg-Leu as substrate.[35] [b] One-letter amino acid codes used; Z = Cbz, Moc = methoxycarbonyl, Sar = sarcosyl.

tary correlation of inhibitor K_i versus substrate K_m values as evidence of "ground state analogy".[33] In this report, we provide evidence for the transition state analogy of phosphonate inhibitors of porcine pepsin.

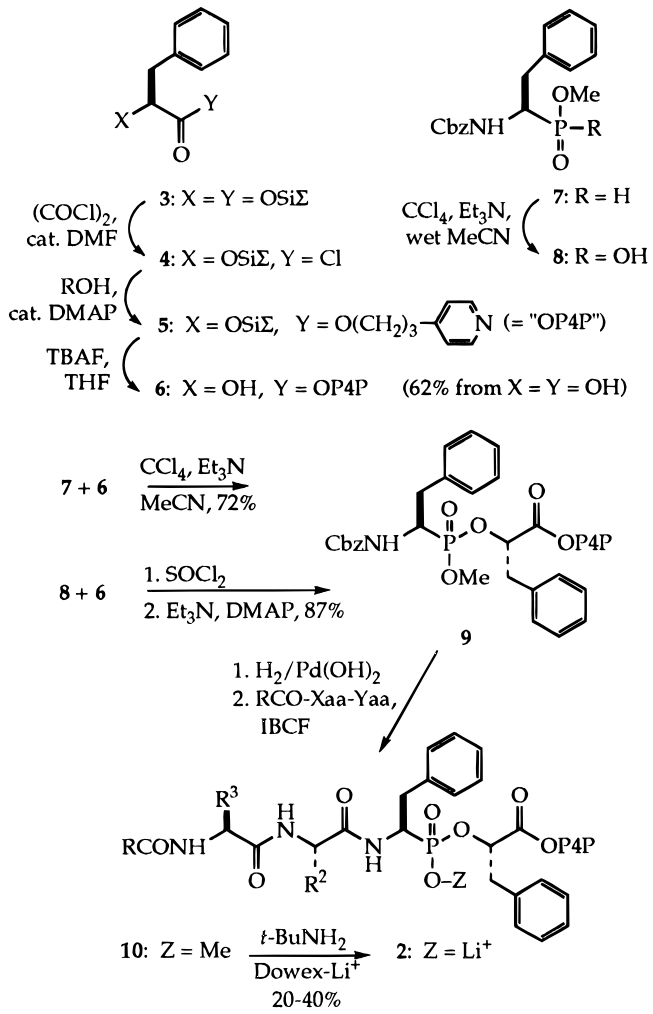
The validity of the K_i versus K_m/k_{cat} correlation depends on several key factors:[28] (1) the chemical step whose transition state is mimicked by the inhibitors is rate limiting for the enzymatic reaction, (2) the structural variation among the substrates does not alter their intrinsic reactivity (i.e., k_{noncat} is constant for the series), and (3) the invariant regions of the ligands bind to the enzyme in the same fashion across the series. The importance of the latter point was demonstrated in a recent study with phosphonate inhibitors of several carboxypeptidase A mutants, where parallel (rather than superimposed) correlation lines result from different binding modes for the transition states for acyl-tripeptides and acyl-Gly-Xaa dipeptides.

We selected 11 substrates of the form RO_2C-Xaa-Yaa-Phe-Phe 3-(4-pyridyl)propyl ester, **1**, which are cleaved between the phenylalanine residues with a broad range of K_m and k_{cat} values (Table 1).[34] These substrates differ in the S_2-S_4 positions, sufficiently remote from the Phe-Phe linkage to justify the assumption that k_{noncat} is invariant. In addition to a wide range in k_{cat}/K_m values, we selected this set of substrates to minimize any correlation between K_m and K_m/k_{cat} to allow a clear distinction between ground state and transition state analogy.



The analogous phosphonates, **2**, were synthesized as shown in Scheme 2. The 3-(4-pyridyl)propyl ester of L-β-phenyllactate, **6**, was prepared from the bis(*tert*-butyldimethylsilyl) derivative **3** by direct conversion to the

Scheme 2. Synthesis of Phosphonate Inhibitors



acid chloride with oxalyl chloride[36] and condensation with the alcohol. The phosphinic acid analog of Cbz-Phe was synthesized and resolved as described by Baylis et al.,[37] converted to the methyl ester **7**,[38] and oxidized with CCl_4 in the presence of water and triethylamine[39] to give the phosphonate monoester **8**. This procedure proved to be an operationally simpler route to this intermediate than an alternative sequence via the diphenyl phosphonate analog of Cbz-Phe.[40–42] Activation of **8** with thionyl chloride[43] and condensation with the hydroxy ester **6** in the presence of triethylamine gave phosphonate diester **9** in 87% yield. Alternatively, the phosphinate ester **7** could be oxidized with carbon tetrachloride in the pres-

(31) Mookthiar, K. A.; Grobelny, D.; Galardy, R. D.; Van Wart, H. E. *Biochemistry* **1988**, *27*, 4299–4304.
(32) Rahil, J.; Pratt, R. F. *Biochemistry* **1994**, *33*, 116–125.
(33) Bartlett, P. A.; Otake, A. *J. Org. Chem.* **1995**, *60*, 3107–3111.
(34) Sachdev, G. P.; Fruton, J. S. *Biochemistry* **1970**, *9*, 4465–4470.
(35) Dunn, B. M.; Valler, M. J.; Rolph, C. E.; Foundling, S. I.; Jimenez, M.; Kay, J. *Biochim. Biophys. Acta* **1987**, *913*, 122–130.
(36) Wissner, A.; Grudzinskas, C. V. *J. Org. Chem.* **1978**, *43*, 3972–3974.
(37) Baylis, E. K.; Campbell, C. D.; Dingwall, J. G. *J. Chem. Soc., Perkin Trans. 1* **1984**, 2845–2852.
(38) Karanewsky, D. S.; Badia, M. C. *Tetrahedron Lett.* **1986**, *27*, 1751–1754.
(39) Huber, J. W., III; Gilmore, W. F. *J. Med. Chem.* **1975**, *18*, 106–108.
(40) Oleksyszyn, J.; Subotkowska, L.; Mastarlerz, P. *Synth. Commun.* **1979**, 985–986.
(41) Kafarski, P.; Leijczak, B.; Szewczyk, J. *Can. J. Chem.* **1983**, *61*, 2425–2430.
(42) Bartlett, P. A.; Lamden, L. A. *Bioorg. Chem.* **1986**, *14*, 356–377.

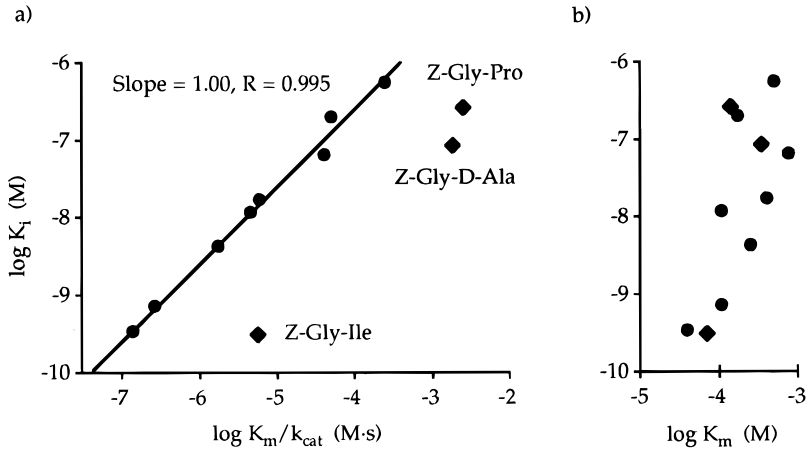


Figure 1. Correlation of substrate kinetic parameters and phosphonate inhibition constants.

ence of the hydroxy ester **6** and triethylamine to give diester **9** directly.[44] The Cbz protecting group was removed from **9** by hydrogenolysis, and the amine was acylated with the appropriate dipeptide activated with isobutyl chloroformate. Finally, the phosphonate methyl ester was cleaved nucleophilically in refluxing *tert*-butylamine,[45] and the inhibitor **2** was purified by chromatography on DEAE Sephadex and isolated as the lithium salt after ion exchange with Dowex-Li^+ and lyophilization.

Inhibition constants for the 11 analogs of **2** were measured with the chromogenic substrate Lys-Pro-Ala-Glu-Phe-(4-NO_2)Phe-Arg-Leu[35] under the same conditions utilized previously for determination of the substrate kinetic constants, pH 3.5 at 37 °C.[34] Simple Dixon analyses (eq 1)[46,47] were used to determine the inhibition constants for the weaker inhibitors (K_i > 10 nM), while the method of Henderson (eq 2)[48] was employed for the

$$\frac{v_0}{v_i} = 1 + \frac{[I]}{K_i}\left(1 + \frac{[S]}{K_m}\right) \quad (1)$$

$$\frac{I_t}{\left(1 - \frac{v_i}{v_0}\right)} = E_t + \frac{v_0}{v_i}K_i\left(1 + \frac{[S]}{K_m}\right) \quad (2)$$

more potent analogs where inhibitor concentrations on the order of $[E]_t$ had to be used. A full kinetic analysis of the Cbz-D-Ala-Gly analog demonstrated that it binds as a competitive inhibitor.[46] The more potent inhibitors were incubated with the enzyme for 5 min prior to addition of the substrate to overcome the slow binding step;[24,25] under these circumstances, the $[S]/K_m$ term was dropped from the analysis, since the E·I complex was not in equilibrium with the substrate during the timescale of the assay.

The inhibition constants are given in Table 1 and are compared graphically with the K_m and K_m/k_{cat} values of the corresponding substrates in Figure 1A and B, respectively. There is no meaningful correlation between the K_i values (which span more than 3 orders of magnitude) and the K_m values (which are within a factor of 20). In contrast, for eight of the 11 inhibitor/substrate pairs, there is a strong correlation between K_i and K_m/k_{cat}; linear regression gives a slope of 1.00 and a correlation coefficient R of 0.995. For these eight inhibitors, the P_2-P_4 residues bind to the enzyme active site the same way they do in the transition state complexes and distinctly differently than they do in the ground state, Michaelis complexes. This correlation is logically the result of the tetrahedral geometry of the phosphonate linkage, which conveys a different orientation to the remote side chains in the active site than the trigonal amide does. The potent binding of the Cbz-Ala-Ala inhibitor is therefore a manifestation of the remote substituent effects that are known to be important for activity of the aspartic peptidases.[49]

A surprising aspect to the correlation is the broad range over which it is observed: from substrates with turnover rates of 4×10^3 M^{-1} s^{-1} (Cbz-D-Ala-Gly-) to 7×10^6 M^{-1} s^{-1} (Cbz-Ala-Ala). As the second-order rate constant approaches 10^7 M^{-1} s^{-1}, the diffusion-limited association step becomes increasingly rate-limiting for a peptidase, and the "transition state" is no longer the tetrahedral species whose geometry is mimicked by the phosphonates. Tighter-than-expected binding of phosphonate analogs of very good substrates ($k_{cat}/K_m \approx 10^6$ M^{-1} s^{-1}) has indeed been observed for the zinc peptidases thermolysin[50] and carboxypeptidase A.[51] The absence of such a deviation for the Cbz-Ala-Ala and Cbz-Gly-Ala pairs in this study suggests that the chemical step remains rate-limiting for these substrates. This conclusion differs from that of Sachdev and Fruton, who found that nonchemical events may become kinetically significant for substrates with k_{cat} values greater than 20 s^{-1}.[52] Moreover, such behavior contrasts sharply with that

(43) 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*, 6370−6371.
(44) Sampson, N. S.; Bartlett, P. A. *J. Org. Chem.* **1988**, *53*, 4500−4503.
(45) Gray, M. D. M.; Smith, D. J. H. *Tetrahedron Lett.* **1980**, *21*, 859−862.
(46) Segel, I. H. *Enzyme Kinetics*; John Wiley and Sons: New York, 1975.
(47) Dixon, M. *Biochem. J.* **1972**, *129*, 197−202.
(48) Henderson, P. J. F. *Biochem. J.* **1972**, *127*, 321−333.
(49) Medzihradszky, K.; Voynick, I. M.; Medzihradszky-Schweiger, H.; Fruton, J. S. *Biochemistry* **1970**, *9*, 1154−1162.
(50) Bartlett, P. A.; Marlowe, C. K.; Giannousis, P. P.; Hanson, J. E. In *Cold Spring Harbor Symposia on Quantitative Biology*; Cold Spring Harbor Laboratory: Cold Spring Harbor 1987; Vol. LII; pp 83−90.
(51) Kaplan, A. P.; Bartlett, P. A. *Biochemistry* **1991**, *30*, 8165−8170.
(52) Sachdev, G. P.; Fruton, J. S. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 3424−3427.

observed with smaller peptides, whose anomalous kinetic properties and cleavage and recombination patterns have been explained by slow binding of substrates and release of products.[1,53,54] However, Pohl and Dunn have presented evidence that K_m represents the true dissociation constant even for substrates with $k_{cat} > 100\ s^{-1}$.[55]

Since eight of the derivatives appear to be transition state analogs, why aren't the other three? The points for the Cbz-Gly-Pro, Cbz-Gly-D-Ala, and Cbz-Gly-Ile analogs deviate significantly from the correlation line; these phosphonates are bound 20−50 times more tightly than predicted by the turnover rate of the corresponding substrates. Interestingly, these are the three analogs with amino acids other than glycine or L-alanine at the P_2 position. The enhanced binding of the Cbz-Gly-D-Ala analog (observed $K_i = 84$ nM) is probably due to contamination from the L-Ala diastereomer ($K_i = 0.73$ nM) formed during the coupling step (**10** → **11**); less than 1% of this material would account for the observed deviation.

Less obvious are the implications from the binding behavior of the P_2-proline and the Cbz-Gly-Ile derivatives. Peptide substrates and inhibitors with proline at the P_2 position are likely to bind in a different orientation than peptides with an unsubstituted NH. Structural studies of a variety of aspartic peptidases, including pepsin, reveal a favorable hydrogen-bonding interaction between this amide hydrogen and an active site carbonyl group.[3,19,56,57] Disruption of this interaction by introduction of an N-substituted amino acid at the P_2 position may alter the orientation of the Phe{X−Y}Phe-OR residues in the active site and thus explain why this point does not fit the correlation. This deviation does not imply that this phosphonate is not a mimic of the transition state, simply that the transition state for the proline-containing substrate is bound in a different orientation than the others; similar behavior has been seen with phosphonate inhibitors and substrates of carboxypeptidase A.[30] In contrast, an *N*-methyl group on the P_3 residue, in the Cbz-Sar-Gly analog, does not disturb the binding orientation because the active-site contact in pepsin is a more flexible threonine side chain at the protein surface.[57]

The deviation observed for the Cbz-Gly-Ile analog is more difficult to understand: the inhibition constant of 31 nM suggests that pepsin should be capable of hydrolyzing the corresponding peptide substrate at a rate of $8 \times 10^6\ M^{-1}\ s^{-1}$, rather than the observed rate of $1.8 \times 10^5\ M^{-1}\ s^{-1}$. Indeed, the corresponding Cbz-Gly-Leu analog is hydrolyzed at a rate of $4 \times 10^6\ M^{-1}\ s^{-1}$.[34] Thinking that the reported kinetic parameters for the Cbz-Gly-Ile analog may be incorrect, we resynthesized the peptide; however, our kinetic analysis did not reveal any meaningful errors in the reported K_m or k_{cat} values. Nor did examination of a model of the enzyme−substrate complex suggest any reason why the isoleucine side chain could not be accommodated sterically in the S_2 pocket.[57] The lack of transition state analogy in this case implies that the rate-limiting step for turnover of this substrate is not formation of the tetrahedral intermediate. Hydrophobic peptides and products are known to stick to pepsin, and the anomalously low turnover rate of the Cbz-Gly-Ile peptide may simply be the result of slow dissociation of product after the cleavage step.

Conclusion. The demonstration of transition state analogy for a closely related series of phosphonate pepsin inhibitors indicates that the binding orientation of these derivatives can serve as a model for that of the transition state. As such, they may help to elucidate the structural basis for remote substituent effects on catalysis. An understanding of these effects would be especially significant because of the structural and mechanistic similarities between pepsin, renin, and the fungal and viral aspartic peptidases.

Experimental Section[58]

3-(4-Pyridyl)propyl (2*S*)-2-[[[(1*R*)-1[*N*-[*N*-[[(Phenylmethoxy)carbonyl]glycyl]-L-alanyl]amino]-2-phenylethyl]hydroxyphosphinyl]oxy]-3-phenylpropanoate, Lithium Salt (2-GA, R_2 = Me, R_3 = H, R = Bn). A solution of 120 mg (0.16 mmol) of the methyl phosphonate **10-GA** (see below) in 10 mL of freshly-distilled *tert*-butylamine in the presence of 4 Å molecular sieves was heated at reflux for 14 h. The solvent was evaporated under a stream of N_2 and under high vacuum, and the residue was dissolved in 1:1 CH_2Cl_2/methanol (1:1) and filtered through a pad of Celite. The filtrate was concentrated to a minimum volume of solution and loaded onto a 20 × 1 cm column of DEAE-Sephadex (HCO_3^- form) and eluted with a linear gradient of 0−0.25 M triethylammonium bicarbonate buffer (pH 7.5; this eluting solvent contained up to 50% methanol depending on the analog). Fractions were monitored by UV absorbance, and those containing product were pooled and lyophilized to give an off-white, hygroscopic powder. This material was dissolved in water (with up to 50% methanol, again depending on the analog), passed through a 1 × 5 cm column (50 mequiv) of Dowex in the Li^+ form, and lyophilized to give 22 mg (18% yield) of **2-GA**, lithium salt, as a white powder: ^{1}H NMR (D_2O) δ 0.84 (d, 3, J = 7.1 Hz), 1.6 (m, 2), 2.32 (t, 2, J = 7.6 Hz), 2.45 (m, 1), 2.9 (m, 3), 3.56 (s, 2), 3.82 (m, 2), 4.1 (m, 2), 4.7 (q, 1), 4.9 (s, 2), 6.90−7.20 (m, 17), 8.15 (d, 2, J = 5.3 Hz); ^{31}P NMR (D_2O) δ 18.17; FAB MS 737 (MH^+); HRMS (FAB) calcd for $C_{38}H_{42}N_4LiO_9P$ + H *m/z* 737.2928, found 737.2938 (MH^+). Anal. Calcd for $C_{38}H_{42}N_4LiO_9P$: P, 4.21. Found: P, 3.91 (weight purity = 93%).

3-(4-Pyridyl)propyl *O*-(*tert*-Butyldimethylsilyl)-(2*S*)-3-phenyllactate (5). To a solution of 2.6 g (16 mmol) of L-β-phenyllactic acid and 10 g (67 mmol) of *tert*-butyldimethylsilyl chloride in 40 mL of CH_2Cl_2 at 0 °C was added 10 mL (67 mmol) of DBU *via* syringe. After 20 min, TLC analysis indicated that the diacid had been completely silylated; the reaction mixture was washed twice with 70-mL portions of saturated NH_4Cl, once with water, and once with brine. The organic layer was dried ($MgSO_4$), two drops of DMF from a disposable pipet were added, and the pale yellow solution was added to a flame-dried flask containing 4 Å molecular sieves and cooled in an ice bath. Freshly-distilled oxalyl chloride (5.0 mL, 57 mmol) was added carefully *via* syringe, with evolution

(53) Silver, M. S.; James, S. L. T. *Biochemistry* **1981**, *20*, 3183−3189.
(54) Silver, M. S.; James, S. L. T. *Biochemistry* **1981**, *20*, 3177−3182.
(55) Pohl, J.; Dunn, B. *Biochemistry* **1988**, *27*, 4827−4834.
(56) Lunney, E. A.; Hamilton, H. W.; Hodges, J. C.; Kaltenbronn, J. S.; Repine, J. T.; Badasso, M.; Cooper, J. B.; Dealwis, C.; Wallace, B. A.; Lowther, W. T.; Dunn, B. M.; Humblet, C. *J. Med. Chem.* **1993**, *36*, 3809−3820.
(57) Fujinaga, M.; Chernaia, M. M.; Tarasova, N.; Mosimann, S. C.; James, M. N. G. *Protein Sci.* **1995**, *4*, 960−972.

(58) Unless otherwise stated, all reactions were run in base-washed, oven-dried round-bottom flasks containing Teflon-coated stir bars, under house nitrogen atmosphere. Dichloromethane, THF, and triethylamine were distilled from CaH_2 prior to use. Unless otherwise indicated, NMR spectra were taken in $CDCl_3$; phosphorus chemical shifts are given with reference to an internal capillary containing trimethyl phosphite (δ 3.086) in $CDCl_3$. FAB mass spectra were performed using a matrix of thioglycerol/glycerol unless otherwise indicated. Silica gel column chromatography was performed according to Still[59] using the solvent system specified. Triethylammonium bicarbonate buffer ("TBK") was prepared by treating a solution of triethylamine in H_2O with CO_2 gas until a pH of 7.5 was achieved.

(59) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923−2925.

of gas. After 2 h, the clear yellow solution was evaporated *in vacuo*, 20 mL of benzene was added and the solution was lyophilized. The residue (**4**) was dissolved in 10 mL of CH_2Cl_2 and added *via* cannula to a flask containing 6.4 g (47 mmol) of 3-(4-pyridyl)propanol and 190 mg (1.6 mmol) of DMAP in 30 mL of CH_2Cl_2 cooled in a dry ice/acetone bath. After 14 h, the solvent was evaporated *in vacuo*, the residue was dissolved in ether, and the solution was filtered through a 0.5-in. pad of silica gel and evaporated *in vacuo* to give 6.0 g of **3** as a clear, colorless oil which can be purified by flash chromatography (70% ether/hexanes → ether). This material proved difficult to separate from the silyl ether of 3-(4-pyridyl)-1-propanol, so the crude product was carried on to the next step directly, when **6** can be separated easily from the alcohol by column chromatography: ^{1}H NMR δ −0.21 (s, 3), −0.11 (s, 3), 0.80 (s, 9), 1.90−1.98 (m, 2), 2.59−2.62 (m, 2), 2.97 (ddd, 2, J = 66.8, 13.4, 4.2 Hz), 4.10−4.13 (m, 2), 4.34 (dd, 1, J = 8.6, 4.3 Hz), 7.07 (d_{app}, 2), 7.20−7.30 (m, 5), 8.49 (d_{app}, 2); ^{13}C NMR δ −5.87, −5.55, 17.88, 25.34, 28.76, 31.12, 41.38, 63.42, 73.41, 123.41, 126.30, 127.85, 129.45, 136.91, 149.49, 149.55, 172.60; FAB MS 400 (MH^+). Anal. Calcd for $C_{23}H_{33}NO_3Si$: C 66.16; H 8.27, N 3.51. Found: C 66.21, H 7.77, N 3.31.

3-(4-Pyridyl)propyl (2*S*)-3-Phenyllactate (6). To a solution of 6.0 g (<15 mol) of the crude silyl ether **5** in 70 mL of THF in an ice bath was added 15 mL of a 1.0-M solution of tetra-*n*-butylammonium fluoride in THF. After 20 min, TLC (5% methanol/ether) indicated that the reaction had gone to completion, so 1.5 mL (25 mmol) of acetic acid was added, followed by pyridine (1.5 mL), and the reaction mixture was partitioned between ether and brine. The organic phase was dried ($MgSO_4$), and the solvent was evaporated *in vacuo* to give 5.0 g of crude alcohol which was purified by column chromatography (silica gel eluted with a gradient of ether → 5% methanol/ether) to give 2.4 g (54% overall from L-β-phenyllactic acid) of the hydroxy ester **6**: ^{1}H NMR δ 1.93−2.00 (m, 2), 2.60−2.64 (m, 2), 3.05 (ddd, 2, J = 50.2, 13.9, 4.8 Hz), 4.11−4.22 (m, 2), 4.45 (dd, 1, J = 6.7, 4.9 Hz), 7.00 (d, 2, J = 4.9 Hz), 7.13−7.35 (m, 5), 8.49 (d, 2, J = 4.9 Hz); ^{13}C NMR δ 28.37, 30.84, 40.40, 49.56, 63.62, 71.23, 123.48, 126.26, 127.90, 128.73, 129.05, 136.47, 148.85, 150.05, 173.73; FAB MS 286 (MH^+); $[\alpha]^{25}_D$ = −16.1° (c = 0.012 in $CHCl_3$); HRMS (FAB) calcd for $C_{17}H_{19}NO_3$ + H m/z = 286.1443, found 286.1442 (MH^+).

3-(4-Pyridyl)propyl 2-[[(1*R*)-1-[*N*-[(Phenylmethoxy)carbonyl]amino]-2-phenylethyl)methoxyphosphinyl]oxy]-3-phenylpropanoate (9). To a solution of 1.50 g (4.28 mmol) of the resolved phosphonic acid **8**[42] in 20 mL of dichloromethane was added 570 mL (6.42 mmol) of thionyl chloride *via* syringe, causing the cloudy solution to become clear. After 1 h, an aliquot was removed for ^{31}P NMR analysis (CH_2Cl_2, 82 MHz), which revealed that complete conversion to the acid chloride (δ 41.04, 42.07) had occurred; a trace of the dichloride (δ 50.98) was also present. The solvent was evaporated under a stream of nitrogen, 10 mL of benzene was added, and the solution was lyophilized to give a white solid. A solution containing 1.50 g (5.14 mmol) of hydroxy ester **6**, 1.0 mL (7.2 mmol) of triethylamine, and 50 mg (0.43 mmol) of DMAP in 20 mL of dichloromethane dried over 4 Å molecular sieves was added via cannula to the phosphonochloridate cooled in a dry ice/acetone bath. After being stirred for 20 h, the reaction mixture was loaded directly onto a chromatography column containing 150 mL of silica gel, slurry-packed in ether, and the column was eluted with an ether → 10% methanol/ether gradient. Fractions containing product were diluted with toluene and the solvent was removed in vacuo (in order to remove any water as an azeotrope) to afford 2.3 g (87%) of the diastereomeric phosphonates **9** as a clear, viscous oil: ^{1}H NMR δ 1.95 (quintet, 2), 2.6 (m, 2), 2.8−3.3 (m, 4), 3.15 and 3.75 (2d, Σ = 3, J = 11.4 Hz), 4.2 (m, 2), 4.3 and 4.5 (2m, Σ = 1), 4.7 and 5.5 (2d, Σ = 1), 4.9−5.0 (m, 2), 5.05 and 5.2 (2ddd, Σ = 1, J = 4, 4, 8 Hz), 7.0−7.4 (m, 17), 8.5 (2d, Σ = 2); ^{13}C NMR δ 28.69 and 31.08 (J = 8.5 Hz), 35.37 and 35.83, 38.95 (J = 7 Hz) and 39.07 (J = 7 Hz), 48.40 (J = 14 Hz) and 49.96 (J = 14 Hz), 52.09 (J = 7 Hz) and 53.22 (J = 7 Hz), 64.37 and 64.85, 66.43 and 66.57, 74.80 (J = 7 Hz) and 75.27 (J = 7.5 Hz), 123.71, 126.55−129.49 (17 peaks), 135.30 and 135.46, 136.38 and 136.56, 136.71 and 136.85, 136.94, 149.62 and 149.79, 155.80 and 155.91, 169.87, 170.34; ^{31}P NMR (from a prep with racemic **8**) δ 25.5, 26.2, 26.5, 27.0; FAB MS Calcd for $C_{34}H_{37}N_2O_7P$ + H m/z = 617, found m/z 617.1 (MH^+). This material proved to be unstable on storage and was therefore used directly in subsequent reactions.

Alternative Preparation of Phosphonate 9: Oxidative Coupling of 6 and 7. Methyl (1*R*)-1-[(Benzyloxycarbonyl)amino]-2-phenylethyl phosphinate (7). A solution of 3.2 g (10 mmol) of the phosphinic acid,[37] 4.0 mL (100 mmol) of methanol, and 2.1 g (11 mmol) of EDC in 50 mL of dichloromethane was stirred for 16 h, diluted with ethyl acetate, washed with saturated NaH_2PO_4 (pH 5) and saturated $NaHCO_3$, dried ($MgSO_4$), and evaporated to give 2.46 g (74%) of methyl phosphinate **7** as a waxy solid: ^{1}H NMR δ 2.95 (m, 1), 3.2 (m, 1), 3.73 and 3.76 (2d, Σ = 3, J = 5.0 Hz), 4.25 (m, 1), 5.0 (2br s, Σ = 2), 5.27 and 5.42 (2br d, Σ = 1, J = 9 Hz), 7.02 and 7.04 (2 dd, Σ = 1, J = 560 Hz), 7.1−7.4 (m, 10); ^{31}P NMR δ 36.32, 36.53.

A suspension of 53 mg (160 μmol) of methyl phosphinate **7**, 84 mg (300 μmol) of hydroxy ester **6**, and 4 Å molecular sieves in 1 mL of CCl_4 under nitrogen was cooled to −10 °C, 50 μL (0.59 mmol) of triethylamine was added, and the mixture was stirred at −10 °C for 18 h. The mixture was filtered and evaporated, and the residue was chromatographed to give 66 mg (72% yield) of phosphonate **9**.

3-(4-Pyridyl)propyl (2*S*)-2-[[(1*R*)-1-[[*N*-[*N*-[(Phenylmethoxycarbonyl)glycyl]-L-alanyl]amino]-2-phenyl-ethyl]methoxyphosphinyl]oxy]-3-phenylpropanoate (10-GA). A solution of 600 mg (ca. 1 mmol) of phosphonate **9** in 30 mL of ethanol was prepared, the reaction vessel was purged with N_2, and 70 mg of 20 wt % $Pd(OH)_2$ on carbon was added under a stream of N_2. A hydrogen balloon was then attached, and the progress of the hydrogenolysis was followed by TLC. After 1.5 h, the mixture was filtered through Celite, evaporated, and placed under high vacuum for 1 h to remove any residual ethanol. Half of this material was carried through the following coupling procedure.

A solution of 140 mg (0.5 mmol) of Cbz-Gly-Ala-OH was prepared in 3 mL of dry acetonitrile over 4 Å molecular sieves, and the suspension was cooled to −10 °C. Triethylamine (70 μL, 0.5 mmol) and 65 μL (0.5 mmol) of isobutyl chloroformate were added, followed after 3 min by a solution of the amine in acetonitrile, and the suspension was stirred for 15 h. Flash grade silica gel was added to the reaction mixture, and the solvent was removed in vacuo to give a silica gel composite which was loaded onto a column of 10 g of plate-grade silica packed into a 30-mL fritted filter funnel. Elution was performed using 40% THF/ethyl acetate to give 125 mg (34%) of the coupled product (**10-GA**) as a mixture of two diastereomers: ^{1}H NMR δ 1.20−1.30 (m, 3), 1.89−2.22 (m, 2), 2.57 and 2.64 (2 t, Σ = 2, J = 7.6 Hz), 2.88−2.95 (m, 1), 3.04−3.32 (m, 5), 3.20 (d, J = 11 Hz), 3.74−3.80 (m, 3), 3.78 (d, J = 11 Hz), 4.04 and 4.24 (2t, Σ = 2, J = 6.4 Hz), 4.40−4.46 (m, 1), 5.24 (dt, 1, J = 4, 9 Hz), 5.68−5.76 (m, 1), 6.90−7.46 (m, 19), 8.49−8.51 (br, 2); ^{31}P NMR δ 26.11 and 26.24; IR ($CDCl_3$) 3420 (wk), 3060, 2980, 1720, 1680, 1500, 1510 (wk), 1420 (wk), 1230 cm^{-1}; FAB MS 745 (MH^+).

Enzyme Assays. Porcine pepsin was obtained from Sigma Chemical Co. (P-6887, lot 10H8075) prepared as 100 mM stock solution, aliquots of which were diluted to 100 nM working solutions. The activity of the working solutions was determined prior to each assay, and fresh solution was prepared every 3−5 h. The substrate Lys-Pro-Ala-Glu-Phe-(4-NO_2)Phe-Arg-Leu[35] was prepared by standard solid phase methods and purified by HPLC (ϵ_{310} = 3770). Kinetic analysis[60] of this material gave values of K_m = 19 μM and k_{cat} = 66 s^{-1} (reported values: 50 μM and 100 s^{-1}, respectively[35]). Assays were initiated by addition of ca. 10 μL of working enzyme solution to a preequilibrated cuvette containing substrate (25−30 μM),

(60) Leatherbarrow, R. J. *ENZFITTER*; Elsevier Science Publishers BV: Amsterdam, 1987.

inhibitor, and buffer (0.1 M sodium acetate, pH 3.5); the final enzyme concentration was 0.5−2.0 nM. The decrease in absorbance at 310 nM ($\Delta\epsilon = -800\ M^{-1}\ cm^{-1}$) was followed over 6−30 min, as appropriate.

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Supporting Information Available: Characterization of other phosphonate analogs and kinetic plots for K_i determinations (7 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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