Phosphorus-containing Peptide Analogs as Peptidase Inhibitors

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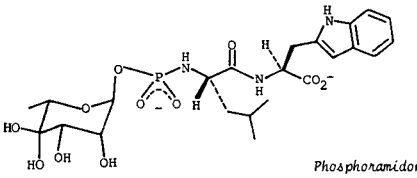
Peptidases play a variety of roles in living organisms, from the routine work of digestion to highly specific hormonal regulation. They are involved in such diverse processes as the blood-clotting and complement cascades, blood pressure regulation, egg fertilization, posttranslational processing in protein synthesis, tumor invasion, and degenerative diseases such as emphysema and arthritis. Synthetic and naturally occurring peptidase inhibitors have been useful for studying the function of these enzymes and as medicinal agents. Indeed, a successful treatment for hypertension involves the use of an inhibitor of the peptidase that produces the octapeptide pressor hormone angiotensin II from its decapeptide precursor.

The task of devising peptidase inhibitors is straightforward, since their mechanisms are relatively well understood. All peptidases whose mechanisms have been elucidated fall into one of four classes: the serine and cysteine peptidases, whose reactions proceed via a covalent acyl enzyme intermediate, and the zinc and aspartic peptidases, which catalyze the direct addition of water to the amide linkage. Moreover, inhibition strategies appear to be general within each class of peptidase. On the other hand, these generalities of mechanism and inhibition strategies make it more of a challenge to devise low-molecular-weight inhibitors of high specificity.

A general and highly effective approach for the design of an enzyme inhibitor is to devise a molecule that resembles the putative transition state or a high-energy intermediate along the reaction pathway (Wolfenden 1976; Stark and Bartlett 1984). Such a strategy is based on the recognition that the lower activation energy of the enzyme-catalyzed transformation reflects a corresponding increase in the binding energy between the enzyme and the substrate as their complex proceeds from the ground state to the transition state conformation. If a "transition state analog" can take advantage of even part of this additional binding energy, it will be a highly effective inhibitor (Frick et al. 1986; Jones and Wolfenden 1986).

The major change in the structures of the ground and transition states for hydrolysis of a peptidase substrate is the progression in geometry of the carbonyl carbon from trigonal to tetrahedral. Incorporation of a functional group that can adopt the tetrahedral geometry has therefore been crucial to the design of transition state analog inhibitors for these enzymes. For the zinc and aspartic peptidases, which do not involve a covalently bound intermediate, a particularly attractive design motif is to incorporate a phosphorus atom in place of the carbonyl carbon of the scissile linkage. The high valency of phosphorus allows a number of heteroatom substituents to be incorporated at this position while retaining a stable tetrahedral geometry. Several naturally occurring inhibitors have evolved on the basis of this strategy; a notable example is phosphoramidon, a phosphoryl dipeptide that is a potent inhibitor of the zinc endopeptidase thermolysin (Komiyama et al. 1975). In early work in this area, a number of simple *N*-phosphoryl amino acids and dipeptides were shown to be effective inhibitors of thermolysin (Kam et al. 1979; Nishino and Powers 1979) and the mechanistically related enzyme carboxypeptidase A (Holmquist 1977; Hofmann and Rottenberg 1980). More recently, in addition to our work reviewed below, phosphonic and phosphinic amino acid analogs have been incorporated into substrate sequences appropriate for a number of other zinc peptidases and have been shown to give rise to potent inhibitors (Galardy 1982; Thorsett et al. 1982; Galardy and Grobelny 1983; Galardy et al. 1983; Petrillo et al. 1983).



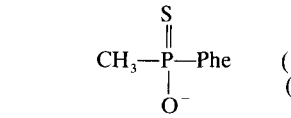
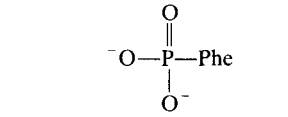
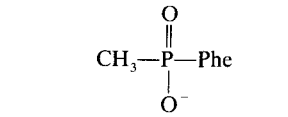
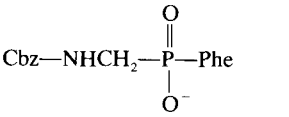
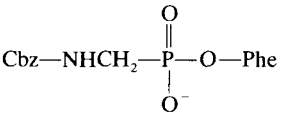
Phosphoramidon

PHOSPHORUS-CONTAINING INHIBITORS OF ZINC PEPTIDASES

Carboxypeptidase A

With the recognition that peptidyl phosphoramidates are effective inhibitors of thermolysin and carboxypeptidase A, a logical extension of the concept was the literal extension of the inhibitors in the direction of the P_1 and P_2 sites, i.e., with the incorporation of the tetrahedral phorphorus as an amino acid analog, rather than a phosphoric acid derivative. We therefore synthesized Cbz-Gly^P-Phe (Gly^P represents the phosphonic acid analog of glycine) and evaluated it as an inhibitor of carboxypeptidase A (see Jacobsen and Bartlett 1981a). This compound proved to be a good inhibitor of the peptidase, with a $K_i = 9 \times 10^{-8}$ M. A number of analogs and congeners were compared with this compound, as indicated in Table 1. It is clear that the

Table 1. Inhibition of Carboxypeptidase A

Inhibitor	K_i (nM)[a]	Reference
 (R_p isomer)	8500	Jacobsen and Bartlett (1981b)
(S_p isomer)	5300	Jacobsen and Bartlett (1981b)
	5000	Kam et al. (1979)
	1200	Jacobsen and Bartlett (1981b)
 (Cbz-Gly^P-Phe)	90	Jacobsen and Bartlett (1981a)
 (Cbz-Gly^P-(O)-Phe)	60	Jacobsen and Bartlett (1981b)
L-α-Benzylsuccinate	450	Byers and Wolfenden (1973)
3-Benzyl-2-mercaptopropanoate	11	Ondetti et al. (1979)

[a]pH 7.5, 25°C.

presence of the Cbz-amino-methyl moiety is a significant factor in the binding interaction. Other comparisons in Table 1 indicate that there is no significant difference in binding between the phosphonamidate derivative and the phosphonate ester, nor is any advantage to be gained by the incorporation of a sulfur ligand in the form of a thiophosphonamidate, regardless of the configuration at the phosphorus center (see Jacobsen and Bartlett 1981b).

Thermolysin

Phosphonamidate inhibitors. What is not revealed by the results of Table 1 is whether it is the tetrahedral nature of the phosphorus derivatives that accounts for their binding affinity or merely the anionic nature of the phosphonate moiety. To examine this broader question, we turned to thermolysin, a bacterial endopeptidase that is evolutionarily different from, but mechanistically analogous to, carboxypeptidase A. Although it is difficult to predict quantitatively what the binding affinity should be for a given transition state analog, theoretical considerations suggest that for a series of inhibitors and related substrates, there should be a proportionality between the inhibitor K_i and substrate K_m/k_{cat} values (Eq. 1) *if* the inhibitors take advantage of the binding interactions available in the transition state conformation (Westerik and Wolfenden 1972; Bartlett and Marlowe 1983; Thompson 1973).

$$K_i \propto K_m/k_{cat} \quad \text{(Assumptions: } K_S = K_m\text{, rate of nonenzymatic reaction constant over substrate series)} \qquad (1)$$

To explore the validity of this prediction and of the transition state analog appellation for the phosphonamidate derivatives, we synthesized a series of tripeptide inhibitors of thermolysin of the form Cbz-Gly^P-Leu-(amino acid) (Table 2) (Bartlett and Marlowe 1983). Within the corresponding series of substrates, there is relatively little variation in K_m values; however, there is a span in K_m/k_{cat} values of almost three orders of magnitude (Table 2, columns 3 and 4) (Morihara and Tsuzuki 1970). A clear distinction could therefore be made between correlation of inhibitor K_i with K_m/k_{cat} and K_m alone (Table 2, column 1; Fig. 1, open squares), indicating that these inhibitors are transition state analogs.

Phosphonate ester inhibitors. In addition to the phosphonamidates (Y = NH), we prepared the corresponding phosphonate esters (Y = O), primarily because they are more stable toward hydrolysis and because in our earlier work, we did not find a significant difference in the affinity of carboxypeptidase A for Cbz-Gly^P-Phe and Cbz-Gly^P-(O)-Phe (Table 1). Thermolysin, in contrast, discriminates against the phosphonate esters by a factor of 840, on average, reflecting a loss of 4.0 kcal/mole of binding energy as compared with the phosphonamidates (Bartlett and Marlowe

Table 2. Inhibition of Thermolysin by Cbz-Gly^P-Leu-X

Inhibitor	K_i (nM)[a] (Y = NH)	K_i (μM)[b] (Y = O)	K_m (mM)[c]	K_m/k_{cat} (μM sec)[c]
Cbz-Gly^P-(Y)-Leu-D-Ala[d]	>1700	—	16.6	3200
Cbz-Gly^P-(Y)-Leu-NH_2	760	660	20.6	196
Cbz-Gly^P-(Y)-Leu-Gly	270	230	10.8	165
Cbz-Gly^P-(Y)-Leu-Phe	78	53	2.4	20
Cbz-Gly^P-(Y)-Leu-Ala	16.5	13.0	10.6	13.6
Cbz-Gly^P-(Y)-Leu-Leu	9.1	9.0	2.6	7.0

pH 7.0, 25°C.
[a]Data from Bartlett and Marlowe (1983).
[b]Data from Bartlett and Marlowe (1987a).
[c]For corresponding substrate, see Morihara and Tsuzuki (1970).
[d]Contaminated with about 0.9% of L-Ala isomer.

1987a). The near constancy of this incremental binding loss across the series suggested to us that the inhibitors are all bound in a similar manner and that there is a specific binding interaction available to the amidates that is not available to the esters, most likely a hydrogen bond. Both of these inferences were substantiated by X-ray crystallographic investigations of the complexes of thermolysin with Cbz-Gly^P-Leu-Leu and Cbz-Gly^P-(O)-Leu-Leu (Tronrud et al. 1987). Between the two complexes, both the protein and inhibitor structures are virtually superimposable (largest difference <0.2 Å), demonstrating that the difference in binding affinity does not result from a different orientation of the inhibitors in the active site or a change in remote interactions. Moreover, the existence of a hydrogen bond between the phosphonamidate NH and the carbonyl oxygen of Ala-113 was supported by the observation of a 3-Å separation between the two heteroatoms. This hydrogen bond involves two formally uncharged groups, which is expected to result in a modest (0.5-1.0 kcal/mole) contribution to the binding energy (Fersht et al. 1985). That this hydrogen bond contributes 4.0 kcal/mole may reflect the fact that it is highly favored entropically, i.e., the interacting groups are positioned similarly whether or not the hydrogen bond is present.

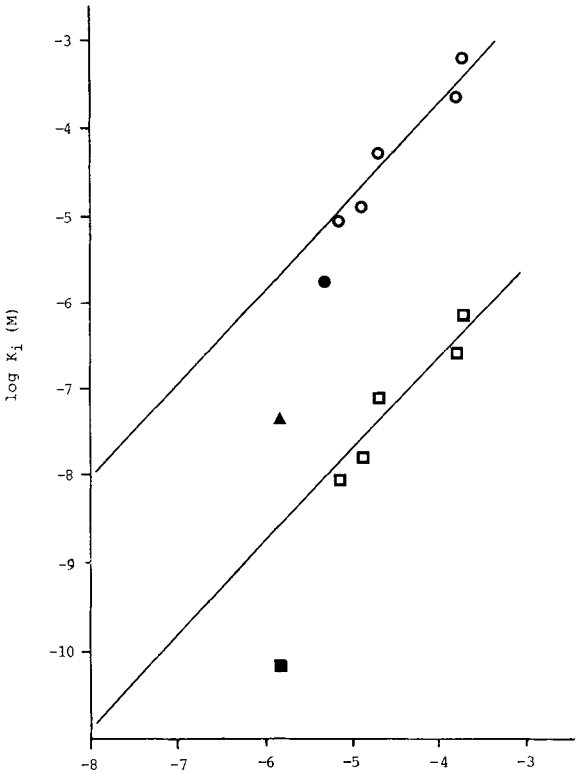


Figure 1. Comparison of peptide substrate K_m/k_{cat} values with the K_i values for Cbz-Gly^P-Leu-X (□), Cbz-Gly^P-(O)-Leu-X (○), and Cbz-X^P-(Y)-Leu-Ala (solid points). Point for Cbz-Gly^P-Leu-D-Ala is not depicted.

Slow-binding inhibitors. Equation 1 implies that to design a more potent inhibitor of thermolysin (lower K_i), one should look for a better substrate (higher k_{cat}/K_m = lower K_m/k_{cat}) and make the corresponding phosphonamidate or phosphonate analog. With this intention, we synthesized a number of phosphorus-containing tripeptides in which a phosphonic acid analog of a substituted amino acid is incorporated at the P_1 position (Table 3; Fig. 1, solid points) (Bartlett and Marlowe 1987b). The behavior of these inhibitors was striking in two respects. First, those inhibitors for which a comparison can be made (Cbz-Phe^P-Leu-Ala, Cbz-Ala^P-(O)-Leu-Ala, and Cbz-Phe^P-(O)-Leu-Ala) are seen to bind *more* tightly to thermolysin than the transition state correlation would predict. Second, *all* of the P_1-substituted inhibitors are slow binding.

The tighter than predicted binding of the P_1-substituted inhibitors reveals the importance of the assumptions made in deriving Equation 1 (Bartlett and Marlowe 1983), namely, that within the series (1) the substrates must be expected to have the same rate constants for the noncatalyzed transformation and (2) the same step must be rate-limiting for the enzymatic transformations. Neither of these may be true for the P_1-substituted inhibitors: The noncatalyzed rate constant for addition of water to the carbonyl group of a substituted amino acid is likely to be lower than that for addition of water to glycine. Moreover, an extrapolation of Figure 1 (solid square and triangle) reveals that thermolysin would have to turn over Cbz-Phe-Leu-Ala at a rate exceeding 10^7 $M^{-1}s^{-1}$ to match the correlation with the inhibitor K_i values. Since the on rate for

Table 3. Inhibition of Thermolysin by Cbz-X^P-(*Y*)-Leu-Ala

Inhibitor	Phosphonamidates (*Y* = NH)		Phosphonates (*Y* = O)	
	K_i (nM)[a]	k_{on} ($M^{-1}s^{-1}$)	K_i (nM)[a]	k_{on} ($M^{-1}s^{-1}$)
Cbz-Ala^P-(*Y*)-Leu-Ala	—		1800	1250
Cbz-Leu^P-(*Y*)-Leu-Ala	—		680	480
Cbz-Phe^P-(*Y*)-Leu-Ala	0.068[b]	1000	45	470
Cbz-D-Ala^P-(*Y*)-Leu-Ala	—		24,000	2.1
Cbz-D-Leu^P-(*Y*)-Leu-Ala	—		42,000	2.8
Cbz-D-Phe^P-(*Y*)-Leu-Ala	480	1300	30,000	400

[a]Data from Bartlett and Marlowe (1987b). Determined at pH 7.0, 25°C by steady-state kinetics, unless otherwise indicated; dash indicates compound not prepared.
[b]Calculated from $K_i = k_{off}/k_{on}$.

the related mansyl substrate is only 3×10^6 $M^{-1}s^{-1}$ (Morgan and Fruton 1978), it is possible that turnover of this substrate is limited by substrate association, not chemical catalysis.

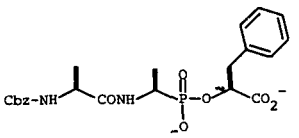
A greater puzzle is the origin of the slow-binding behavior observed for all of the tripeptide analogs depicted in Table 3. Whereas Cbz-Gly^P-Leu-Leu binds normally, with an on rate $\geq 10^5$ $M^{-1}s^{-1}$, the on rates for those with a substituent α to phosphorus are at least three orders of magnitude below that expected for a diffusion-limited process (Morgan and Fruton 1978; Brouwer and Kirsch 1982). This slow-binding behavior appears to be unrelated to the size of this substituent, its configuration, whether the inhibitor is a phosphonamidate or phosphonate ester, or whether it is a potent or weak inhibitor. Although slow binding is often associated with a two-step binding process and attributed to a protein conformational change required for attainment of the final, "tight" complex (Morrison and Walsh 1987), neither Cbz-Phe^P-Leu-Ala nor Cbz-Phe^P-(O)-Leu-Ala shows evidence for a "loose" intermediate complex with a K_D below 4 mM.

Determination of the structure of the complex of thermolysin with Cbz-Phe^P-Leu-Ala reveals that the protein has not undergone any significant conformational change in comparison with the native enzyme or the complex with the fast-binding inhibitor Cbz-Gly^P-Leu-Leu (Holden et al. 1987). We are therefore inclined to rule out a mechanism for slow binding in which such a conformational change is responsible. On the other hand, the P_1-substituted inhibitor occupies the S_1 and S_2 subsites in a different manner than the Gly^P derivatives do, with rotation around the C_α-N bond of the phosphorus amino acid residue placing the carbamate moiety deeper into the active-site cleft. Nevertheless, this inhibitor conformation is attained by a single bond rotation, and it does not represent an unfavorable conformation. It is therefore difficult to attribute the slow-binding phenomenon to this conformational feature. Binding of the inhibitor in this conformation does, however, require expulsion of an additional water molecule, in comparison to the fast-binding inhibitors. We have therefore explored the possibility that the slow-binding behavior of the P_1-substituted inhibitors may simply reflect a difficult exchange between bound water and bound inhibitor.

A binding sequence in which a specific water molecule must dissociate before the inhibitor can associate with the protein could lead to slow binding even if all of the individual steps are fast, simply because it is a rare form of the enzyme that the inhibitor must encounter. The results of a preliminary investigation of the effect of viscosity on the binding of Cbz-Phe^P-(O)-Leu-Ala are consistent with this interpretation: The rate of association of this inhibitor is almost completely diffusion-limited, despite the fact that $k_{on} = 470$ $M^{-1}s^{-1}$. Further studies of this effect may provide more understanding of the dynamic and static aspects of protein-ligand association processes.

Carboxypeptidase A Revisited

As indicated above, our work on zinc peptidase inhibitors began with the phosphonic acid analogs of Cbz-Gly^P-Phe, which showed inhibition constants toward carboxypeptidase A on the order of 60–90 nM (Table 1) (Jacobsen and Bartlett 1981a,b). Although these inhibitors are potent, they do not constitute the most tightly bound synthetic inhibitors reported for this enzyme (Ondetti et al. 1979). Tripeptides such as Cbz-Ala-Ala-Phe are more rapidly hydrolyzed by carboxypeptidase A than Cbz-Gly-Phe; therefore, a longer phosphonic acid peptide analog was an attractive target for an improved inhibitor. Since carboxypeptidase A does not appear to discriminate against phosphonate esters (in comparison to the phosphonamidates) the way thermolysin does, we prepared the more stable Cbz-Ala-Ala^P-(O)-Phe.



Cbz-Ala-Ala^P-(O)-Phe

Although this material is not particularly slow binding, compared with some of our thermolysin inhibitors, it is sufficiently tightly bound that its inhibition constant cannot be measured under steady-state conditions (Morrison and Walsh 1987). We were therefore required to determine separately the rates for association

and dissociation of the inhibitor from the enzyme. Since the dissociation rate proved to be very slow, it was necessary to find conditions under which the enzyme is stable for extended periods of time. Figure 2 depicts the results from two experiments, in which the equilibrium between enzymes and inhibitor was established in two directions, enabling us to determine the on and off rates and to verify the equilibrium constant. These incubations were carried out in the absence of substrate; the concentration of free enzyme was monitored by removing aliquots and measuring the residual (for k_{on}) or recovering activity (for k_{off}). That the K_i value for Cbz-Ala-Ala^P-(O)-Phe is indeed only 3 pM is underscored by the correspondence between the observed equilibrium constant and the value calculated from the on and off rates. This compound is, by several orders of magnitude, the most potent small-molecule inhibitor reported for carboxypeptidase A. As was the case for the P_1-substituted inhibitors of thermolysin, Cbz-Ala-Ala^P-(O)-Phe is more potent than expected based on extrapolation from Cbz-Gly^P-(O)-Phe, according to the K_i versus K_m/k_{cat} correlation of Equation 1. Again, however, this comparison involves substrates that differ substantially in structure.

Collagenase

Although collagenases as a group are poorly understood from the point of view of substrate specificity and mechanism, they belong to the zinc peptidases and appear to be inhibited by strategies that are effective against the other members of this class (Galardy and Grobelny 1983; Vencill et al. 1985). On the basis of emerging information on the substrate specificity of human neutrophil collagenase (Mookhtiar et al. 1986), the phosphonate-containing peptides listed in Table 4 were designed and synthesized; as indicated, they proved to be good inhibitors of the enzyme (Mookhtiar et al. 1987).

Table 4. Inhibition of Human Neutrophil Collagenase

Inhibitor	K_i (mM)
Cbz-Gly^P-Leu	2.1
Cbz-Gly^P-Leu-NH$_2$	1.3
Cbz-Gly^P-Leu-Ala	0.078
Cbz-Gly^P-Leu-D-Ala	2.6
Cbz-Gly^P-Leu-Gly	6.8
Cbz-Gly^P-Leu-Phe	0.98
Cbz-Gly^P-(O)-Leu-Ala	2.5
Cbz-Pro-Ala-Gly^P-Leu-NH$_2$	0.15
Cbz-Phe-Gly^P-Leu-Ala	0.071
Cbz-Gly^P-Leu-Ala-Gly	0.014

pH 7.5, 30°C. Data from Mookhtiar et al. (1987).

Leucine Aminopeptidase

In contrast to collagenase, the substrate specificity of leucine aminopeptidase is well understood, but the mechanistic details are not (Delange and Smith 1971). It is considered a member of the zinc peptidase class in view of its requirement for this metal and its inhibition by chelating agents and hydroxamic acid amino acid analogs. However, no structural comparison with thermolysin or carboxypeptidase A is available, nor is there a similar correspondence in effective inhibition strategies. The most potent inhibitors reported for leucine aminopeptidase are the naturally occurring dipeptide analog bestatin (Wilkes and Prescott 1985) and the aldehyde (Andersson et al. 1985) and boronic acid (Shenvi 1986) analogs of leucine. In view of the success of phosphonic acid peptide analogs as inhibitors of the more well-defined members of the zinc peptidase class, we synthesized and evaluated an analogous series of derivatives for leucine aminopeptidase (see Table 5) (Giannousis and Bartlett 1987).

Leu^P-(O)-Leu and Phe^P-(O)-Leu were patterned after the corresponding dipeptide substrates, which are hydrolyzed very effectively by the enzyme; the phos-

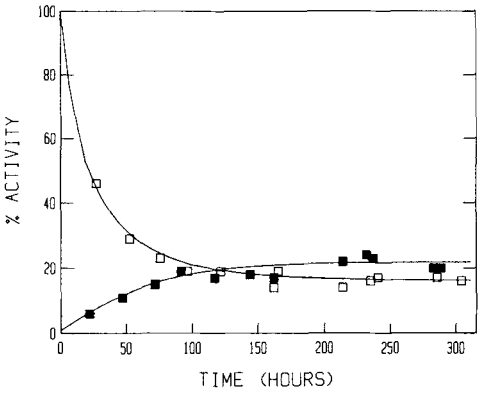


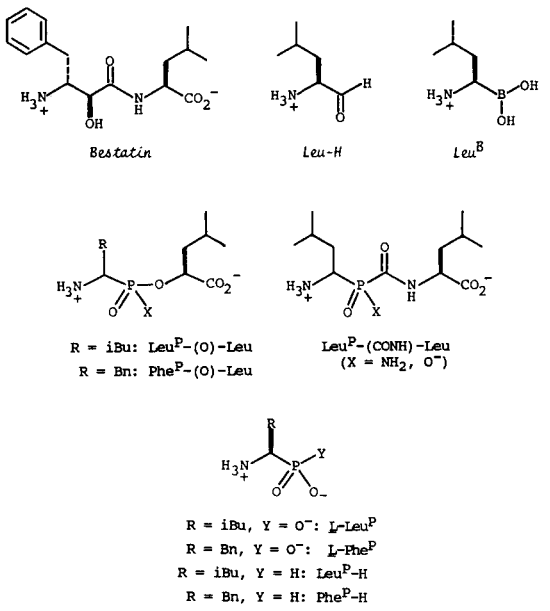
Figure 2. Establishment of equilibrium between Cbz-Ala-Ala^P-(O)-Phe (I) and carboxypeptidase A (E). Conditions: pH 7.5, 25°C, 50 mM Tris-HCl, 0.5 M NaCl, 0.1 mg/ml BSA, 1 μM $ZnCl_2$. Association reaction: 45 pM E + 50 pM I. Dissociation reaction: 45 pM EI complex. Theoretical curves represent $k_{on} = 2.5 \times 10^5$ M^{-1}s^{-1} and $k_{off} = 7.5 \times 10^{-7}$ s^{-1}.

Table 5. Inhibition of Leucine Aminopeptidase

Inhibitor	K_i (μM)
Leu^P-(O)-Leu	58[a]
Phe^P-(O)-Leu	340[a]
Leu^P-(CONH)-Leu, $X = O^-$	56[a]
Leu^P-(CONH)-Leu, $X = NH_2$	40[b]
L-Leu^P	0.23
D-Leu^P	220
L-Phe^P	0.42
D-Phe^P	15.4
Leu^P-H	87
Phe^P-H	59
Bestatin	0.0006[c]
Leu-H	0.06[d]
Leu^B	0.13[e]

pH 8.6, 25°C. Data from Giannousis and Bartlett (1987), unless otherwise indicated.
[a]Mixture of two diastereomers.
[b]Mixture of four diastereomers.
[c]Wilkes and Prescott (1985).
[d]Andersson et al. (1985).
[e]Shenvi (1986).

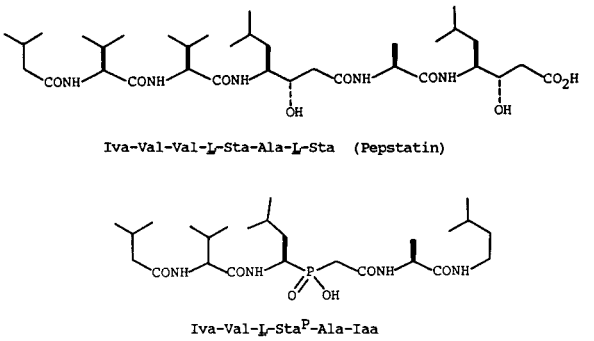
phonate esters were prepared instead of the phosphonamidates in view of the lability of the latter in the presence of a free α-amino group. In place of the secondary alcohol of bestatin, which may mimic the tetrahedral carbon of the transition state, the carbamoylphosphinates incorporate a tetrahedral phosphorus, as the phosphinate anion as well as neutral amide.



Neither the phosphonate dipeptide analogs nor the phosphorus derivatives similar to bestatin are particularly potent inhibitors of leucine aminopeptidase. In contrast, the simple phosphonic acid analogs of leucine and phenylalanine are good inhibitors of the enzyme ($K_i < 1$ μM). The affinity is strongest for the L-enantiomers, and they proved to be slow binding ($k_{on} \leq 500$ $M^{-1}s^{-1}$). The disparity between the potent inhibition by phosphonates of the classic zinc peptidases and the modest inhibition revealed in Table 5, coupled with the efficacy of leucinal and boroleucine against the serine peptidases, as well as leucine aminopeptidase, suggests that the mechanism of the latter may involve an enzyme-bound nucleophile, rather than direct addition of water to the scissile carbonyl group.

PHOSPHORUS-CONTAINING INHIBITORS OF ASPARTIC PROTEASES

The mechanism of the aspartic proteases, like that of the zinc peptidases addressed above, involves direct addition of water to the scissile carbonyl group. For these enzymes as well, evolutionary or intentional incorporation of a tetrahedral species into an oligopeptide, as a mimic either of the transition state or of the two substrates (peptide plus water), has led to effective inhibitors. Indeed, the most potent inhibitors for the aspartic peptidases have been analogs of the naturally occurring pepsin inhibitor, pepstatin, in which the unusual amino acid statine occupies the P_1 and P_1' positions and presents a secondary hydroxyl group to the catalytic residues in the enzyme active site (Rich 1985). In view of the intermediacy of a tetrahedral adduct in the mechanisms of both the zinc and aspartic peptidases, it appeared likely that tetrahedral phosphorus amino acid analogs should be effective as inhibitors of the latter class as well.



Pepsin

A series of analogs of pepstatin have been investigated by Rich et al. (1985) in order to define the requirements for tight-binding and slow-binding inhibition of pepsin. As indicated in Table 6, the truncated derivative Iva-Val-L-Sta-Ala-Iaa, although not showing the absolute affinity for the enzyme that pepstatin does, nevertheless shows the high affinity and two-step binding mechanism characteristic of the naturally occurring inhibitor.

The "phosphastatine" derivatives listed in Table 6 were synthesized and evaluated as pepsin inhibitors (Bartlett and Kezer 1984). As indicated, the L-derivative of Iva-Val-Sta^P-Ala-Iaa shows not only high affinity, but also unusual kinetic behavior. An initial, reversible complex is formed with a K_D of 7 nM. With time, this complex is converted to a more tightly bound form, whose affinity we have not yet determined accurately ($K_i < 0.07$ nM). Whether this represents a very tight but noncovalent complex or a covalent one has also not been determined, although the fact that the half-life for isomerization of the "loose" to the "tight" complex is on the order of 2 hours makes the former possibility seem remote.

Table 6. Inhibition of Pepsin

Inhibitor	K_D (nM)[a]	K_i (nM)
Iva-D-Sta^P-Ala-Iaa	—	25,000
Iva-L-Sta^P-Ala-Iaa	—	900
Iva-L-Sta-Ala-Iaa	—	350[b]
Iva-Val-D-Sta^P-Ala-Iaa	—	200
Iva-Val-L-Sta^P-Ala-Iaa	7	<0.07
Iva-Val-L-Sta-Ala-Iaa	60	3[b]
Iva-Val-Val-L-Sta-Ala-Sta	13	0.056[b]

pH 3.5, 37°C. Data from Bartlett and Kezer (1984), unless otherwise indicated.

[a]Dash indicates two-step binding not observed.

[b]Rich and Sun (1980); Rich (1985).

Table 7. Inhibition of Penicillopepsin

Inhibitor	K_i (nM)
Iva-Val-Val-Sta-OEt	47[a]
Iva-Val-Val-Sta^P-OEt (pH 4.5)	111[b]
Iva-Val-Val-Sta^P-OEt (pH 3.5)	24[b]
Iva-Val-Val-Sta-Ala-Sta	0.15[a]

[a]Data from Rich (1985).
[b]Data from J.E. Hanson and P.A. Bartlett (unpubl.).

Penicillopepsin

The bacterially derived but mechanistically and structurally related penicillopepsin shows a similar affinity for statine-containing oligopeptides; Iva-Val-Val-Sta-OEt, for example, inhibits this enzyme with a K_i of 47 nM (Rich 1985). Incorporation of phosphastatine into this sequence produces an effective inhibitor; however, the relative effect is considerably less than in the case of pepsin (Table 7). The reduced affinity of penicillopepsin for the phosphastatin derivative can be traced in part to the higher pH at which this enzyme is active in comparison to pepsin. The pK_a of the phosphinic acid is <3; hence, under the conditions of the assay, it is appreciably ionized. In contrast, the actual tetrahedral intermediate resulting from water addition to the enzyme-bound substrate is formally neutral. The fact that the K_i decreases with pH suggests that the enzyme binds only the neutral form of the inhibitor. In contrast to the behavior of the phosphastatine pepsin inhibitor, Iva-Val-Val-Sta^P-OEt shows no unusual binding behavior. Of significant encouragement are the preliminary results of a crystallographic analysis of the complex between the phosphinic acid analog Iva-Val-Val-Sta^P-OEt and penicillopepsin (James et al. 1982; M.N.G. James and A. Sielecki, unpubl.). This investigation has shown that the tetrahedral phosphorus is bound in the active site, as would be expected for a mimic of the tetrahedral intermediate, with the two active-site aspartic acid residues hydrogen-bonded (separately) to the two oxygens of the phosphinic acid moiety. As such, this complex provides a model for the actual transition state or reactive intermediate and support for the indicated mechanism.

CONCLUSIONS

The study of phosphorus-containing peptide analogs has led both to potent inhibitors for a variety of zinc and aspartic peptidases and to insight into some of the details of the enzymatic transformations and of the interactions between these proteins and their ligands. It is also our hope that they will provide an opportunity to elucidate some of the dynamic aspects of ligand-protein association as well, in particular the nature of slow-binding inhibition.

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