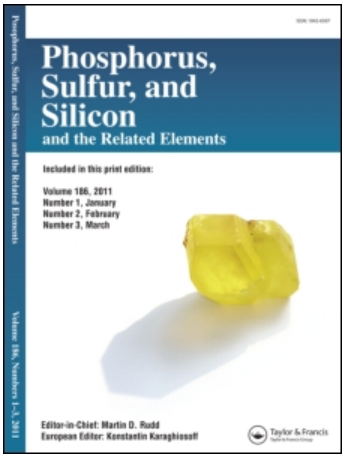

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AN ANALYSIS OF THE ENZYME-INHIBITOR BINDING INTERACTIONS FOR PHOSPHONIC ACID TRANSITION STATE ANALOGS OF THERMOLYSIN

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Abstract: Potent inhibitors of the zinc endopeptidase thermolysin are produced on replacement of the scissile peptide linkage with phosphonamidate or phosphonate ester moieties. These inhibitors have been shown to be transition state analogs, and a comparison between the esters and amidates reveals the intrinsic binding energy due to a specific hydrogen bonding interaction. Incorporation of an α-substituted phosphonic acid analog leads to slow-binding behavior on the part of the inhibitors, which is attributed to expulsion of a specific water molecule from the active site.

INTRODUCTION

The elucidation of enzyme mechanism and the development of mechanism-based[1] or transition state analog[2] enzyme inhibitors have had a synergistic influence on each other. On the one hand, an understanding of enzyme mechanism often points the way toward the design of potent inhibitors, and on the other, the evaluation of such inhibitors provides an indication of how substrate binding energy and specific active site interactions contribute to catalysis. The zinc endopeptidase thermolysin is a valuable target for application of the modern principles of inhibitor design because of the extensive structural and mechanistic work which has been carried out on this enzyme, as well as its relevance to medicinally

important systems.[3] In this report, an overview of our work on phosphorus-containing peptide analogs 1-3 as inhibitors of thermolysin is presented. We provide evidence that these inhibitors are transition state analogs,[4] an indication of the intrinsic binding energy due to an hydrogen bonding interaction,[5] and a possible structural explanation for the slow-binding inhibition which is observed for a specific class of these inhibitors.[6]

SYNTHESIS

The phosphonic acid analogs of amino acids are readily available in the form of their N-carbobenzoxy diphenyl esters according to Oleksyszyn.[7] Ester exchange in alkaline methanol followed by saponification provides the monomethyl esters. After activation of these compounds by formation of the acid chlorides, the fully protected phosphonamidates and phosphonates are prepared by coupling with the leucyl dipeptides or the corresponding L-2-hydroxy-4-methylpentanoyl derivatives. Deprotection is accomplished by nucleophilic dealkylation,[8] and the products are purified by ion exchange chromatography. The phosphonate esters can be converted to the diacids and fully characterized; the phosphonamidates, which are more labile hydrolytically, have been characterized as their dilithium salts. In the case of the α-substituted phosphonic acid derivatives, the tripeptide analogs are produced as a mixture of diastereomers, since the starting phosphonic acid moiety was used in racemic form. Separation of the isomers was achieved by HPLC at the diester stage, although this was complicated by the presence of an additional stereocenter at phosphorus.

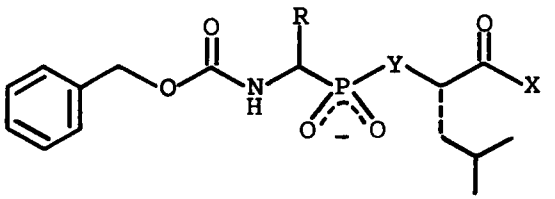
EVALUATION AS TRANSITION STATE ANALOGS

Phosphonic acid peptide analogs are potent inhibitors of thermolysin (Table 1). This inhibition was consistent with earlier reports in which it was shown that dipeptide phosphoramidates are also effective.[9] Of particular interest is the fact that there is a strong correlation between the inhibition constants K_i for the aminomethylphosphonamidate inhibitors **1a-e** (abbreviated Z-Gly^P-Leu-X) and the K_m/k_{cat} values for the corresponding peptide substrates (Figure 1).[4,10] Such a correlation is expected if the inhibitors mimic the transition state in the way they bind to the enzyme.[2]

INTRINSIC ENERGY OF A HYDROGEN-BONDING INTERACTION

The corresponding series of phosphonate ester analogs **2** was also prepared. A similar correlation of inhibitor K_i with substrate K_m/k_{cat} values is seen for this series as well (Table 1, **2a-e**; Figure 1); however, each ester inhibitor is bound about 840-fold less tightly than the corresponding amidate. The similarity in structure and correlation suggested that the esters and amidates are bound to the enzyme in the same way, a point which was confirmed on comparison of the thermolysin complexes of **1e** and **2e** by protein crystallography.[11] The crystallographic studies also reveal that the phosphonamidate N-H group of **1e** is hydrogen-bonded to a protein carbonyl group, an interaction which is missing in the complex with ester **2e**. Thus, the difference in affinities between the ester and amidate inhibitors, which corresponds to 4.0 ± 0.1 kcal/mole, can be attributed to the intrinsic binding energy due to this hydrogen bond.

TABLE 1. Inhibition of Thermolysin by Phosphonic Acid Tripeptide Analogs[a]



	R	Y	X	K_i (nM)	k_{on} (M^{-1} sec^{-1})
1a	H	NH	NH_2	760	
1b	H	NH	Gly	270	
1c	H	NH	L-Phe	78	
1d	H	NH	L-Ala	16.5	
1e	H	NH	L-Leu	9.1	> 10^6
2a	H	O	NH_2	660,000	
2b	H	O	Gly	230,000	
2c	H	O	L-Phe	53,000	
2d	H	O	L-Ala	13,000	
2e	H	O	L-Leu	9,000	> 10^6
3a	L-benzyl	NH	L-Ala	0.068[b]	1000
3b	L-Methyl	O	L-Ala	1,800	1250
3c	L-i-Butyl	O	L-Ala	680	480
3d	L-Benzyl	O	L-Ala	45	470
3e	D-Methyl	O	L-Ala	24,000	2.1
3f	D-i-Butyl	O	L-Ala	42,000	2.8
3g	D-Benzyl	O	L-Ala	30,000	400

[a] Unless otherwise indicated, determined at pH 7.0 as described in reference 4.
[b] Determined from k_{off}/k_{on}.

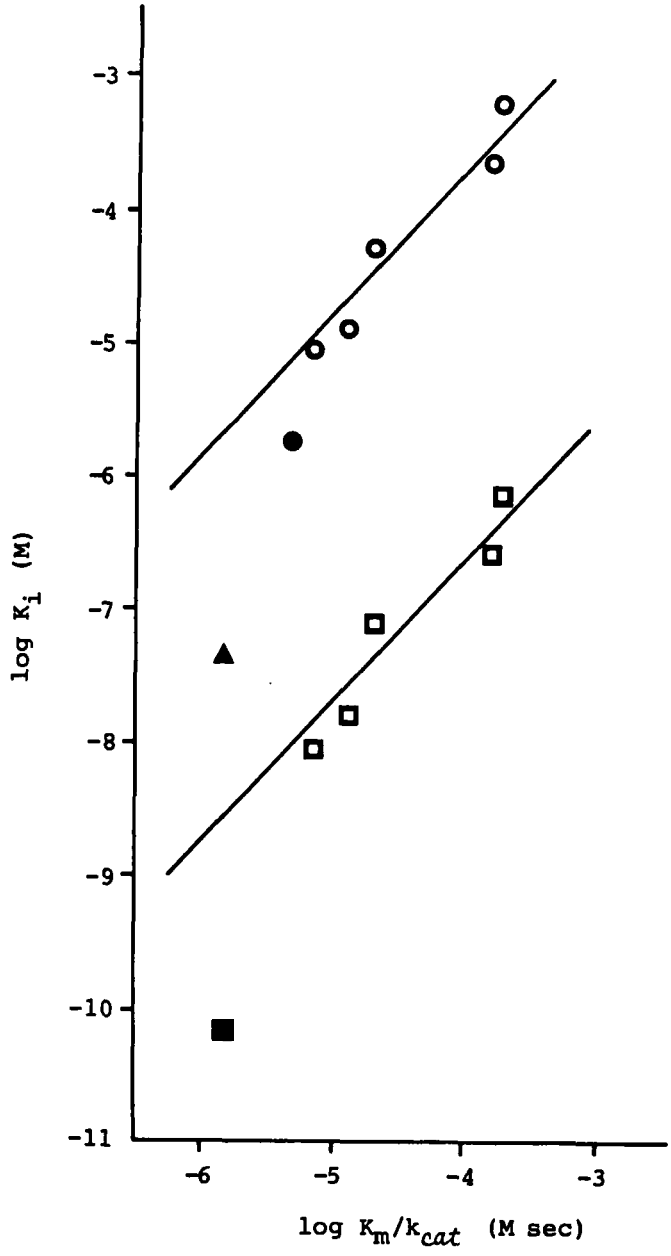


FIGURE 1. Comparison of inhibitor K_i values with K_m/k_{cat} values for the analogous peptide substrates: (○), Z-Gly^P-O-Leu-X; (□), Z-Gly^P-Leu-X; (●), Z-Ala^P-O-Leu-Ala; (▲), Z-Phe^P-O-Leu-Ala; (■), Z-Phe^P-Leu-Ala.

TIGHT- AND SLOW-BINDING INHIBITORS

The α-substituted inhibitors, **3**, are bound more tightly than predicted from the correlation of K_i vs. K_m/k_{cat} values (Figure 1). Indeed, Z-Phe^P-Leu-Ala, with an overall K_i value of 68 pM, is by far the most potent inhibitor yet reported for thermolysin. We attribute the deviations from the correlation to a breakdown in the assumptions made in deriving the relationship between these values,[4] namely that the rate of the non-enzymatic reaction is unaltered by the substitution.

The greatest contrast between the α-substituted inhibitors **3** and the Gly^P analogs **1** and **2** is the rate at which they bind to the enzyme. Whereas the latter compounds behave normally, with no discernible hindrance to binding, the α-substituted compounds are all slow-binding. Crystallographic studies of the thermolysin complex with Z-Phe^P-Leu-Ala rule out the conventional explanations for slow binding behavior, and suggest instead that expulsion of a specific water molecule from the active site is the barrier to association of these compounds.[6]

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