PEPTIDE-GAP INHIBITORS: I. COMPETITIVE INHIBITION OF AMINOPEPTIDASE M BY A HYDROLYTICALLY RESISTANT DIPEPTIDE ANALOGUE OF GLYCYLLEUCINE

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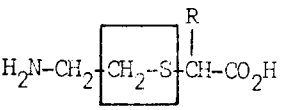
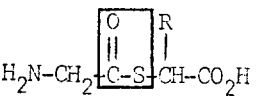
SUMMARY: A non-hydrolyzable analogue of the dipeptide glycylleucine has been prepared which contains a thiomethylene group substituted for the peptide linkage. The affinity of the analogue ((S)-2-(S-cysteaminyl)-4-methylpentoic acid) for aminopeptidase M is four-fold greater than that of Gly-Leu. Inhibition of the hydrolysis of glycine-p-nitroanilide is competitive. This analogue represents the first member of a new class of compounds which conserves both charge and conformation of the parent compound while presenting a non-hydrolyzable linkage at the normal point of cleavage in a peptide substrate. Analogues of this type may have potential for the design of inhibitors which utilize contiguous enzyme binding sites complementary to both sides of the point of cleavage in normal substrates of proteolytic enzymes.

A number of proteolytic enzymes have contiguous sites which bind up to six or more residues in peptide substrates (1). Design of proteolytic enzyme inhibitors which utilize substrate binding sites on both sides of the normal point of cleavage may prove advantageous. Such an approach is possible if a non-hydrolyzable linkage were to be substituted for the susceptible peptide bond in a normal substrate while maintaining compatibility of both stereochemistry and charge. Suggestions previously made for the substitution of such a group in Gly-X peptides either present synthetic difficulties or result in a change of charge character (2). Thioesters are now recognized to be very satisfactory substrates for some proteolytic enzymes (3). This observation, taken together with the high nucleophilicity of mercapto groups as well as the functional and stereochemical similarities of the methylene group to a divalent sulfur atom

(4,5), suggested the possibility of preparing dipeptide analogues of the type shown by structure I. Such an analogue might be viewed as arising



I



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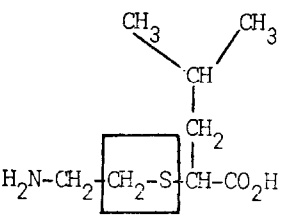

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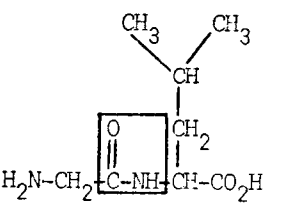

IV

from the thioester variant of a dipeptide (II) where the carbonyl group has been reduced to a methylene group. Inspection of molecular models (Plate 1) indicates a close stereochemical relationship between Gly-X and the dipeptide analogues generalized by structure I.

In this communication we report the interaction of (S)-2-(S-cysteaminyl)-4-methylpentoic acid (III) with aminopeptidase M (EC 3.4.1.2). Gly-Leu is shown by structure IV for reference.

Compound III was prepared by established chemical methods. D-Leucine was treated with nitrosyl bromide to produce (R)-2-bromo-4-methylpentoic acid (6,7). An earlier procedure was altered so that treatment of the bromoacid with cysteamine instead of cysteine (8) produced (S)-2-(S-cysteaminyl)-4-methylpentoic acid (III), m.p. 205-210°. (Anal. calcd for $C_8H_{17}NO_2S$: C, 50.23; H, 8.96; N, 7.32; S, 16.76. Found: C, 50.10; H, 9.08; N, 7.20; S, 16.60. The molecular weight (calcd: 191) found by mass spectrometry (chemical ionization) was 191; $[\alpha]_D^{22} = -24.5^\circ \pm 1.4^\circ$

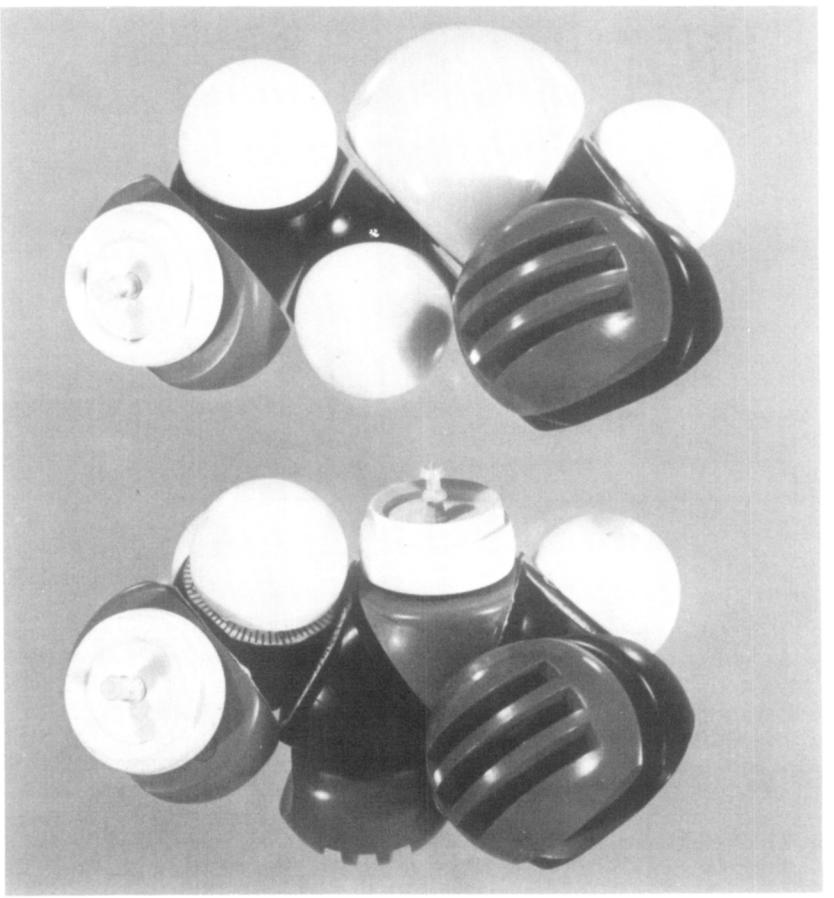

Plate 1. CPK model comparison of the peptide bond of a Gly-Gly dipeptide (lower) with the corresponding analogue (upper) where a thiomethylene linkage has replaced the peptide bond. The van der Waals radii of the N-H group and sulfur atom match closely, while a decrease in radius occurs in the substitution of a methylene group for a carbonyl group. Judging from the behavior of thioesters as substrates (3) loss of the N-H dipole may be of limited consequences for many proteolytic enzymes.

(c = 2, methanol). III is resistant to acid hydrolysis (91% recovery after 24 hrs, 6 N HCl, 110°) and appears near the position of arginine on the amino acid analyzer. Its ninhydrin constant is 0.82 times that of leucine.

Aminopeptidase M (Lot 16C-0108, 30.7 units per mg) and Gly-Leu (Lot 85C-0061) were products of Sigma Chemical Company. Glycine-p-nitroanilide was obtained from Vega-Fox Biochemicals. Rates of hydrolysis of the anilide were determined as described by Wachsmuth et al. (9) using a

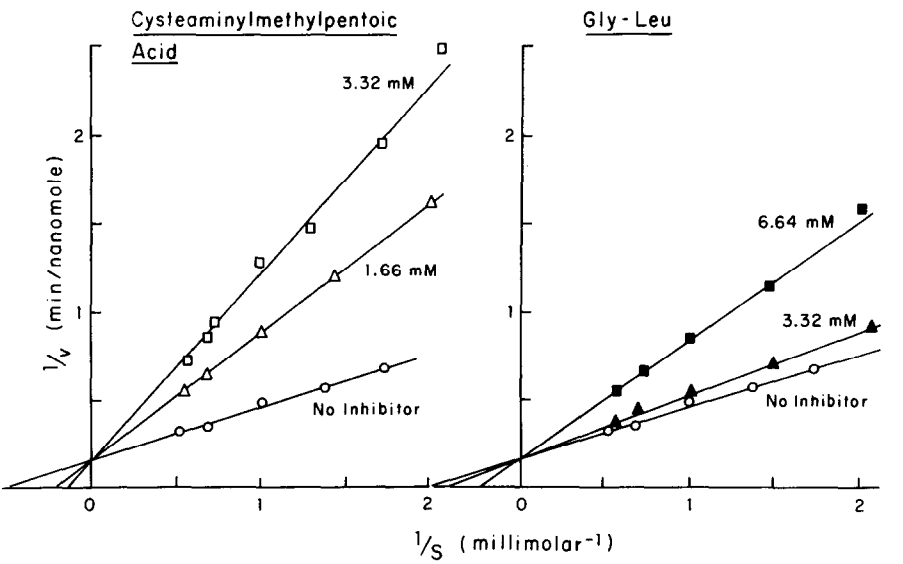

Figure 1. Lineweaver-Burk plots of the inhibition of glycine-p-nitroanilide hydrolysis by aminopeptidase M. Rates of hydrolysis were measured as described by Wachsmuth et al. (9). Aminopeptidase was maintained constant at approximately 10^{-8} M (10). Open circles indicate the hydrolysis of glycine-p-nitroanilide in the absence of inhibitor. Left frame: Open triangles and squares indicate same hydrolysis in the presence of 1.66 mM and 3.32 mM cysteaminylmethylpentoic acid, respectively. Right frame: Filled triangles and filled squares indicate hydrolysis of glycine-p-nitroanilide in the presence of 3.32 mM and 6.64 mM Gly-Leu, respectively.

Zeiss PMQ II spectrophotometer equipped with a cell holder thermostatted with 37° water.

Double reciprocal plots are shown in Figure 1 for the hydrolysis of glycine-p-nitroanilide by aminopeptidase M in the presence and absence of cysteaminylmethylpentoic acid. Gly-Leu was examined as a competitive inhibitor for purposes of comparison. The data permit a calculation of a K_m of 1.9 mM for glycine-p-nitroanilide in good agreement with 1.8 mM determined earlier (9). Inhibition by both Gly-Leu and its analogue (III) was competitive. K_I values of III and Gly-Leu were found to be 1.2 mM and 4.8 mM, respectively. Thus, cysteaminylmethylpentoic acid binds approximately 4 times more tightly to aminopeptidase M than the natural peptide it was designed to supplant.

The hydrolytic sensitivities of cysteaminylmethylpentoic acid and Gly-Leu to aminopeptidase M were compared by amino acid analysis (11). When 100 nanomoles of Gly-Leu were incubated with 32.6 µg of aminopeptidase M in a total volume of 120 µl of 0.1 M phosphate buffer (pH 7.0) at 37°, 95% of this peptide was hydrolyzed in 60 min. When cysteaminylmethylpentoic acid was incubated with aminopeptidase M under identical conditions no loss in this analogue was detectable by amino acid analysis.

We may conclude that this prototype of dipeptide analogues generalized by structure I is resistant to enzymatic cleavage while binding competitively to aminopeptidase M. We propose that members of this new class of inhibitors which contain thiomethylene linkages substituted for peptide bonds be termed "peptide-gap" inhibitors. Such analogues may have potential for a wide variety of applications which include mapping of active sites of enzymes (by kinetic measurements and by X-ray crystallography), affinity chromatography, chemotherapy, and studies on the mechanism of hormone action.

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