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A POTENT MERCAPTO BI-PRODUCT ANALOGUE INHIBITOR FOR HUMAN CARBOXYPEPTIDASE N

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SUMMARY: The bi-product analogue inhibitor, 2-mercaptomethyl-3-guanidinoethylthiopropanoic acid, has been synthesized in high yield and exhibits a K_i of 2.0 nM with human plasma carboxypeptidase N. The ease of synthesis and subsequent availability make it an ideal compound to study potentiation of bradykinin and other vasoactive peptides.

INTRODUCTION

Carboxypeptidase N is a potent bradykinin and anaphylatoxin inactivator in plasma (1-3). Other functions have also been proposed for the enzyme, such as inactivation of vasoactive peptides released by plasmin degradation of fibrin (4). Different forms of the enzyme appear to be present in tissues (5,6). In all cases it functions by cleavage of a COOH-terminal basic amino acid.

Byers and Wolfenden (7) first demonstrated that benzylsuccinic acid combines the binding modes of the two products of carboxypeptidase A hydrolysis of C-terminal phenylalanine. They termed this inhibitor a bi-product analogue. McKay and Plummer (8) prepared sulfur-containing bi-product analogues of lysine and arginine and demonstrated their efficacy as inhibitors for bovine and porcine carboxypeptidase B and also for human plasma carboxypeptidase N (9). Replacement of a carboxyl group with a sulfydryl group was later shown by Cushman et al. (10) and Ondetti et al. (11) to dramatically increase the efficiency of bi-product analogue inhibitors for angiotensin converting enzyme. Their subsequent synthesis of 2-mercaptomethyl-5-guanidinopentanoic acid yielded a com-

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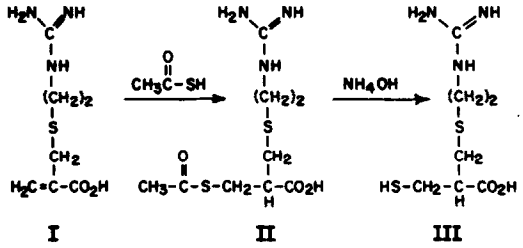

Figure 1. General method of synthesis of D,L-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid (Compound III).

pound with a K_i of 0.4 nM for porcine carboxypeptidase B (12). Synthesis of this inhibitor proceeded in five steps in a yield of less than 1%. By analogy (8,9), the mercapto-compound should be very effective for human plasma carboxypeptidase N. This communication reports a simple synthesis of 2-mercaptomethyl-3-guanidinoethylthiopropanoic acid and compares its activity with 2-mercaptomethyl-5-guanidinopentanoic acid.

MATERIALS AND METHODS

Thiolacetic acid (Sigma), α-(bromomethyl)acrylic acid, and S-2-aminoethylisothiouronium bromide hydrobromide (Aldrich) were used as supplied. Human plasma carboxypeptidase N was purified by the method of Plummer and Hurwitz (13).

Synthesis. D,L-2-Mercaptomethyl-3-guanidinoethylthiopropanoic acid was synthesized as outlined in Fig. 1. The overall yield was 43%, mp 208-209°C. Anal. Calcd for $C_7H_{15}H_3O_2S_2$: C, 35.43; H, 6.37; N, 17.70; S, 27.02. Found: C, 35.46; H, 6.32; N, 17.55; S, 27.02.

α-(Guanidinoethylthiomethyl)acrylic acid (I). α-(Bromomethyl)acrylic acid (20 mmol) was dissolved in 40 ml H_2O at 25°C under a nitrogen atmosphere. The pH was adjusted to 5.5, and S-2-aminoethylisothiouronium bromide hydrobromide (20 mmol) in 10 ml 2 N NaOH was added. The pH was adjusted to 7.25 and maintained at this pH. After 1 h the reaction was terminated by dropping the pH to 3.0 with 6 N HCl. The mixture was chromatographed in two portions on a 2.5 x 180-cm column of Sephadex G-10 equilibrated in 0.1 N acetic acid at a flow rate of 11.8 ml cm^{-2} h^{-1} at 4°C. Fractions of 10 ml were collected. Product was detected by optical density measurement at 250 nm and by Sakaguchi reaction of suitable aliquots (14). The major Sakaguchi-positive, sulfhydryl-negative (15) fractions were pooled and lyophilized to an oil.

2-Acetylthiomethyl-3-guanidinoethylthiopropanoic acid (II). Compound I was dissolved in 25 ml of thiolacetic acid (12) and stirred slowly for 72 h. Thiolacetic acid was removed in vacuo and three 40-ml portions of benzene were used to wash the residue, followed by removal in vacuo (12). The residue was washed from the flask with a mixture of 40 ml each of 0.1 N acetic acid and ethyl acetate. The aqueous phase was washed three times with 30 ml of ethyl acetate and then reduced in volume to 24 ml by concentration in vacuo.

The concentrate was chromatographed in three portions on a 2.5 x 180-cm column of Sephadex G-10 under the aforementioned conditions. The major Sakaguchi-positive fractions were pooled and lyophilized.

D,L-2-Mercaptomethyl-3-guanidinoethylthiopropanoic acid (III). Compound II was dissolved in 9.7 ml of H_2O and transferred to a conical vessel with a Teflon tube extended to the bottom to allow mixing by a stream of nitrogen. The solution was preflushed with nitrogen for 10 min in an ice bath at 0°C. Two aliquots of 4.85 ml each of cold, concentrated ammonium hydroxide were added, and the mixture was stirred by bubbling nitrogen for 1 h. The reaction was terminated by slow addition of 10 ml of cold 88% formic acid. The solution was diluted 17-fold with distilled H_2O and lyophilized. The resulting powder was relyophilized once in 1% formic acid. Excess ammonium salts were removed by chromatography in two portions on a 2.5 x 180-cm column of Sephadex G-10 as described previously. The Sakaguchi- and sulfhydryl-positive fractions were pooled, lyophilized, and chromatographed in two portions on a 2.0 x 108-cm column of CM-52 cellulose equilibrated in 0.2 M pyridine formate, pH 3.24, at a flow rate of 31.8 ml cm^{-2} h^{-1}. Fractions of 10 ml were collected, and product was detected on suitable aliquots by the Sakaguchi (14) and sulfhydryl (15) reactions. Fractions containing product were lyophilized. Yield was 8.6 mmol (1.92 g).

Determination of inhibition constants. Enzymatic activity was determined spectrophotometrically at 336 nm using furylacryloyl-alanyl-lysine (16). An appropriate dilution of inhibitor in nitrogen-flushed water was added to each reaction mixture as 1% of the final volume just prior to addition of enzyme. All molarities were adjusted accordingly. The final concentration of carboxypeptidase N was 1.6 nM. Inhibitor binding constants were determined graphically by Dixon plots (17) using substrate concentrations of 0.25 and 0.50 mM and four or five concentrations of inhibitor. The results were evaluated by linear regression analysis.

RESULTS AND DISCUSSION

The early studies by Byers and Wolfenden (7) established the principle of bi-product analogue inhibitors with the synthesis of benzylsuccinic acid for bovine carboxypeptidase A. This inhibitor contained the side-chain specificity of phenylalanine and its free carboxyl group. The β-carboxyl represented the carboxyl group newly released from amide linkage. Cushman, Ondetti, and co-workers (10,11) expanded the suggestion by Byers and Wolfenden (7) that the β-carboxy group might bind to the active site zinc of carboxypeptidase A. They reasoned that β-mercapto derivatives would be more efficient as zinc ligands and compared these derivatives with β-carboxyl inhibitors for angiotensin converting enzyme (10,11), bovine carboxypeptidase A (12), and porcine carboxypeptidase B (12). In all cases the β-mercapto derivative was approximately 1000-fold more efficient as a competitive inhibitor. Furthermore, each derivative was a specific inhibitor for the enzyme

TABLE I

BI-PRODUCT ANALOGUE INHIBITORS OF CARBOXYPEPTIDASE N

Inhibitor[a]	K_i μM	K_i nM
Carboxyl-type		
Guanidinopropylsuccinic acid	1.0[b]	
Guanidinoethylthiosuccinic acid	1.0[b]	
Guanidinopropylthiosuccinic acid	1.3[b]	
Mercapto-type		
2-mercaptomethyl-5-guanidinopentanoic acid		0.45[c]
2-mercaptomethyl-3-guanidinoethylthiopropanoic acid (III)		2.0[c]

[a]Racemic mixtures.

[b]Data of McKay et al. (9), Substrate: benzoyl-alanyl-lysine.

[c]Substrate: furylacryloyl-alanyl-lysine.

for which it was synthesized. The combination of the side chain preferred specificity and the mercapto group interacting with the zinc in the active site negated any possibility for nonspecific inhibition. Other workers have termed such derivatives "metal-coordinating substrate analogues" (18) and have provided direct evidence for interaction of the thiol with the active-site metal.

Our earlier work (8,9) demonstrated that a series of dicarboxylic acid bi-product analogues of arginine were good competitive inhibitors of human plasma carboxypeptidase N. The studies further revealed that efficient binding of these inhibitors to carboxypeptidase N is not affected by certain structural differences. First of all, a sulfur group substituted adequately for a methylene group in a position beta to the primary carboxyl group. Both guanidinopropylsuccinic acid and guanidinoethylthiosuccinic acid exhibited identical K_i's of 1 μM with carboxypeptidase N (Table I). Secondly,

guanidinopropylthiosuccinic acid, the homoarginine analogue, had a comparable K_i of 1.3 μM.

More recently Ondetti et al. (12) reported the synthesis of 2-mercaptomethyl-5-guanidinopentanoic acid. This compound had a K_i of 0.4 nM with porcine carboxypeptidase B (12), or 2500-fold more efficient than our observed K_i of 1.0 μM for guanidinoethylthiosuccinic acid with the same enzyme (8). By analogy with our previous comparisons of inhibitors with carboxypeptidases B and N (9), the Ondetti inhibitor should be very effective as an inhibitor for carboxypeptidase N. However, the reported synthesis (12) required five steps and provided only a low yield of product.

We decided to synthesize a mercapto derivative by a simpler procedure, replacing a methylene group with a sulfur atom. The availability of α-(bromomethyl)acrylic acid and the observation that S-2-aminoethylisothiouronium bromide hydrobromide undergoes an immediate intratransguanylation at neutral pH to give guanidinoethylmercaptan (19) allowed us to add a complete guanidinoethylthio side chain in one reaction by mild displacement of the bromine atom to yield Compound I. The next step was simple addition of the mercapto group to the methylene function by using thiolacetic acid (Compound II). Thus in two steps, followed by gentle release of the acetyl group in cold 50% ammonium hydroxide, D,L-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid (Compound III) was synthesized in satisfactory yield (43%).

Compound III was tested as a competitive inhibitor of carboxypeptidase N and was only 4.4-fold less efficient than a sample of D,L-2-mercaptomethyl-5-guanidinopentanoic acid kindly furnished by Dr. Ondetti (Table I). Dixon plots (17) were typical of competitive-type inhibition for both mercaptan compounds, as were the parallel lines given by Cornish-Bowden plots (20). Compound III is a mercaptan analogue of guanidinopropylthiosuccinic acid and is approximately 1000-fold more efficient.

The known biologic functions of plasma carboxypeptidase N depend on its cleavage of C-terminal basic amino acids from low-molecular-weight pep-

tides with very potent biologic activities such as bradykinin and anaphylatoxins. Recently Mathews et al. (21) reported on a patient with very low levels of carboxypeptidase N and an 11-year history of episodic angioedema. Concurrently Belew et al. (4) reported on structure-activity relationships of vasoactive peptides derived from fibrin by plasmin degradation that may contribute to pulmonary edema in some cases. These peptides are inactivated by release of C-terminal lysine and may be under physiologic control of carboxypeptidase N. Tissue enzymes with the specificity of carboxypeptidase N, but with unknown function have also been reported (5,6). It is hoped that the availability of potent, selective inhibitors such as 2-mercaptomethyl-3-guanidinoethylthiosuccinic acid will aid in assessing the physiologic contribution of carboxypeptidase N to normal and disease states.

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