# Communications to the Editor

## Application of a Conformationally Restricted Phe-Leu Dipeptide Mimetic to the Design of a Combined Inhibitor of Angiotensin I-Converting Enzyme and Neutral Endopeptidase 24.11

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*Received May 17, 1993*

We previously described the design and synthesis of rigid tricyclic phenylalanylleucine (PheLeu) mimetic 1 and its incorporation into 2, an inhibitor of angiotensin I-converting enzyme (ACE, EC 3.4.15.1).[1] Mimetic 1 was designed[2] to closely resemble the *anti* orientation ($X_1$ = 180°, $X_2$ = 0°) of the carboxy terminal histidylleucine (HisLeu) portion of angiotensin I (Chart I). The replacement of His by Phe in mimetic 1 was made with knowledge that His is not essential to ACE binding[3] and that neutral endopeptidase 24.11 (NEP, EC 3.4.24.11), a related zinc-containing proteinase, cleaves the PheLeu dipeptide from Leu-enkephalin.[4] The cleavage of bradykinin adjacent to Phe(8) by both ACE and NEP[5] further suggests that these two metalloproteinases could have shared active-site characteristics. We speculated early on that side chain constrained peptidomimetics would be useful tools to study the conformational preferences in peptide–protein interactions. The similarities and biological significance of these two enzymes made them ideal choices for evaluation by this approach. More recently, NEP has been shown to play a role in the degradation of the natriuretic peptides,[6] a family of hormones, some of which are secreted by the heart into the circulation in increased amounts in patients with congestive heart failure (CHF).[7] Because the renin–angiotensin–aldosterone system opposes[8] the beneficial natriuretic and diuretic actions of atrial natriuretic peptide (ANP), inhibition of ACE during NEP inhibition should be advantageous. The feasibility of simultaneous ACE and NEP inhibition was investigated utilizing our dipeptide mimic approach. Gros *et al.* have described close analogs of thiorphan which exhibit equipotent nanomolar ACE and NEP inhibition *in vitro* and demonstrate enzyme occupation upon oral dosing of a prodrug form.[9] We now report the design rationale and synthesis of a new class of subnanomolar dual ACE/NEP inhibitor, a member of which produces blood pressure lowering in animal models of both essential and salt dependent hypertension when orally administered in prodrug form.

Derivatives of 1 were used to probe the active site requirements of both ACE and NEP (Chart II). Whereas 2 was found to be an extremely selective inhibitor of ACE, epimeric tricyclic mercaptomethylene derivatives 3 and 4[10] gave the indication that simultaneous inhibition of ACE and NEP was feasible in spite of the intrinsic conformational constraints of mimetic 1. The mercaptoacetyl derivative of phenylalanylglycine had been reported to inhibit NEP ($K_i$ = 20 nM).[11] The mercaptoacetyl derivative 5 of mimetic 1 was found to be a potent inhibitor of ACE as well as NEP. This result, combined with the structural rigidity of mimetic 1, indicates that significant similarities in the $S_1'$ and $S_2'$ binding domains of these two enzymes exist and that an internal unsubstituted CO-NH function is not essential for binding to this region of NEP.[9,12]

**Chart I**

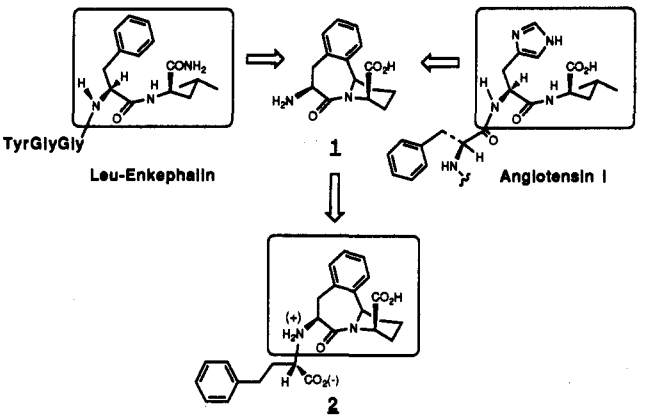
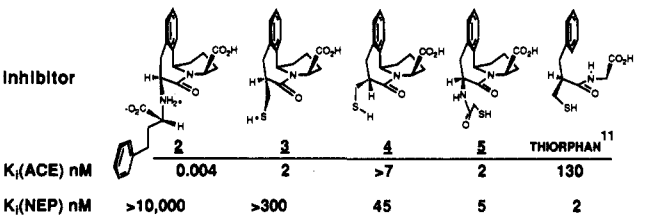



**Chart II**




Molecular modeling studies were used to derive heuristic substrate binding models for ACE and NEP in an attempt to account for the observed differences in inhibitor selectivity (Chart II). Differing orientations for the zinc coordinating ligands of inhibitors were correlated with low-energy conformations of the specific conformation depicted for the hypothetical enzyme substrate AcPhe-PheGlyOH (Chart III).

The derived correlation[13] is consistent with the stylized carbonyl interactions from the extremes of the low-energy (Φ) angle orientations determined for this substrate (Φ = −150° and Φ = −90°). Consequently, two substrate-bound enzyme active site models were envisioned which differ fundamentally in the positioning of their catalytic zinc ions (Chart IV). We believe that the limited conformational freedom of the mercaptoacetyl amide side chain of 5 plays a pivotal role in enabling this compound to inhibit both ACE and NEP. An implication of the NEP model is that the mercaptoacetyl amide side chain of 5 must adopt a higher energy *cis* amide conformation to achieve proper interaction of the thiol group with the active site zinc ion. Conversely, the preferred *trans* amide conformation of the side chain places the sulfur atom appropriately to interact with the zinc placement in the ACE model. Both models indicate that addition of an appropriate substituent, such as benzyl, onto the sulfur bearing

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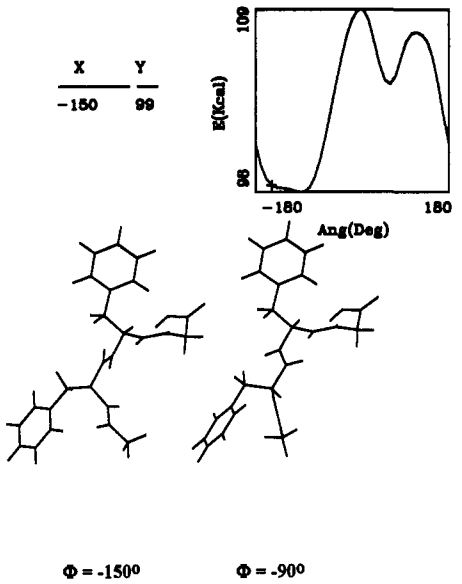
**Chart III**[a]



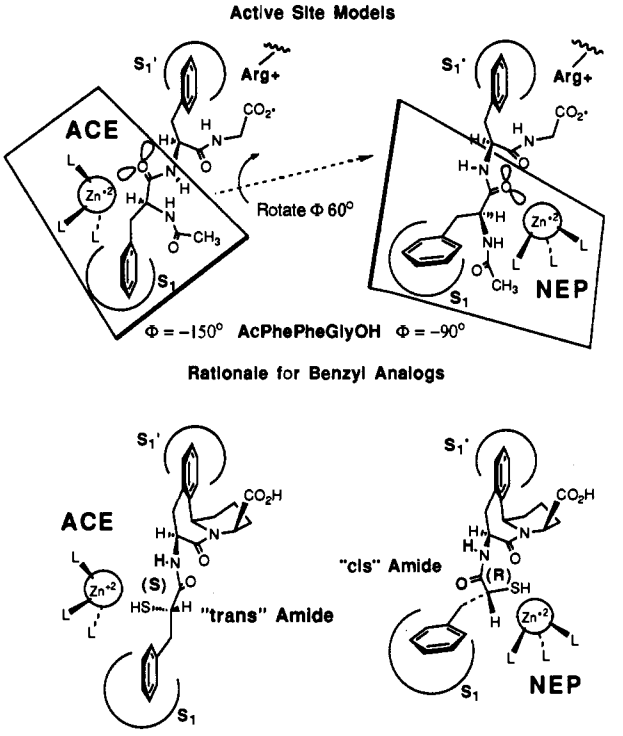

[a] Selection of the specific conformation depicted in Chart III for the hypothetical substrate (AcPhe-PheGlyOH) was founded on two key assumptions. First, based on the conformational constraint of inhibitor **5** and its activity toward both ACE and NEP, the PheGlyOH portion of the substrate was constrained ($X_1 = 180°$, $X_2 = 0°$, $\Psi = 120°$) into a low energy *anti* conformation similar to mimetic **1**. Secondly, due to the steric requirements of the catalytic machinery in metalloproteinases, as revealed in the crystal structure of thermolysin,[20] the side chain of the second Phe in this substrate was placed in a *gauche* (−) ($X_1 = -60°$) orientation and the C-$\alpha$ proton was forced to eclipse the scissile amide bond's carbonyl group ($\Psi = -120°$). These constraints provide the least congested environment for zinc coordination to the substrate's amide carbonyl oxygen. An energy vs $\Phi$ angle map was generated (Insight/Discover, Biosym Technologies Inc.) using 10° increments with steepest descent and conjugate gradient minimization at each step. Large forcing constants on the $X$ and $\Psi$ angles were used to maintain our two key assumptions through the course of the calculation. This experiment produced the narrow range (−90° to −150°) of low-energy $\Phi$ angle orientations indicated in Chart III. Other computational experiments with less rigid $X$ and $\Psi$ constraints indicate that a broader range of low energy $\Phi$ angles between −70° and −170° are accessible.

carbon of **5** could enhance binding affinity through interaction with the $S_1$ subsite of either enzyme.

The diastereomeric benzyl substituted analogs of **5** were prepared as the first two analogs in this series (Scheme I). (*R*)- and (*S*)-bromo acids **6a** and **6b**, prepared from D- and L-phenylalanine, respectively, by the method of Kellogg,[14] were coupled sequentially to tricyclic amino ester **7**[1] in high yield using standard coupling reagents such as EEDQ. Displacement of the resulting $\alpha$-bromo amides **8a** and **8b** with the cesium salt of thiolacetic acid in dimethyl formamide proceeded with clean inversion to give the corresponding (*S*)- and (*R*)-thioesters **9a** and **9b** in high yield. Cleavage of the benzhydryl esters with trifluoroacetic acid and anisole afforded thioester acids **10a** (MDL 100,240) and **10b** as potential prodrug forms. Alkaline hydrolysis under an inert atmosphere (LiOH, MeOH, 2 h) afforded the targeted (*S*)- and (*R*)-thiol inhibitors **11a** and **11b** in 48% and 53% overall yield, respectively. Representative experimental procedures for the synthesis of the (*S*)- diasteriomers **10a** and **11a** are available from the authors.

The properties of the substituted thiols and their acetate thioester prodrugs were assessed *in vitro* and *in vivo*. The *in vitro* evaluation is summarized in Table I. The (*S*)-benzyl-substituted thiol, **11a**, inhibited both enzymes substantially better than its unsubstituted progenitor **5**.

**Chart IV**[a]




[a] The energy calculation in Chart III coupled with the differences in enzyme selectivity observed with rigid tricyclic inhibitors having different ligand containing side chains (Chart II) led us to ask if these tendencies could be accounted for by dissimilar zinc positioning in ACE and NEP. Stylized carbonyl oxygen to zinc coordination trajectories for the two lowest energy $\Phi$ values calculated for our substrate were used to define the divergent substrate binding models illustrated above. We found the observed selectivity's of these tricyclic inhibitors to be qualitatively consistent with the accessibility of their ligands to the zinc locations proposed in these two models. Comparison of the ACE-bound substrate model with the *trans* mercaptoacetyl amide form of inhibitor **5** indicates that a side-chain substituent giving the (*S*)-configuration at the carbon bearing sulfur should interact best with $S_1$ subsite of ACE. Conversely, comparison of the NEP-bound substrate model with inhibitor **5** indicates that the *cis* (mercaptoacetyl)amide geometry is required for effective zinc coordination and that an (*R*)-substituted side chain might be preferred.

Conversely the (*R*)-diastereomer showed no improvement over **5** as an inhibitor of ACE, but rivaled the (*S*)-isomer as the most potent NEP inhibitor reported to date.[15] These results confirm the existence of a well defined $S_1$ subsite in ACE and suggest that the $S_1$ domain of NEP is more accommodating. However, the benefits of benzyl substitution on binding affinity were less pronounced for ACE than for NEP, regardless of side-chain stereochemistry. Our proposal that the *cis* amide geometry of mercaptoacetyl inhibitors is the NEP bound conformation could account for these significant increases in NEP affinity since the requisite *trans* to *cis* amide bond isomerization should be facilitated during the early stages of enzyme/inhibitor interaction by a side-chain substituent.

A 30 mg/kg oral dose of **10a**, the thioester prodrug of (*S*)-thiol **11a**, produced significant blood pressure lowering effects in spontaneously hypertensive rats (SHR)[16] and desoxycorticosterone acetate (DOCA)-salt hypertensive rats[17] that were sustained over a period of at least 5 h (Chart V). Although mixed inhibition has been described previously,[8b,9] thioester **10a** is the first orally effective form of a combined ACE/NEP inhibitor reported to display blood pressure lowering effects in these animal models of hypertension.[18] A detailed account of *ex vivo* and *in vivo*

**Scheme I**

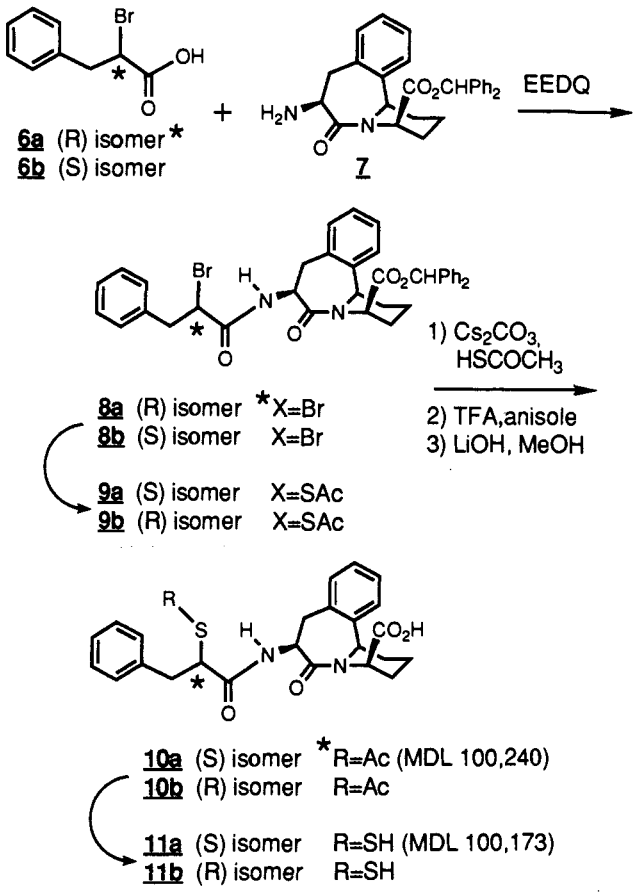



**Table I.** Binding Constants of (Mercaptoacetyl)amides[a]

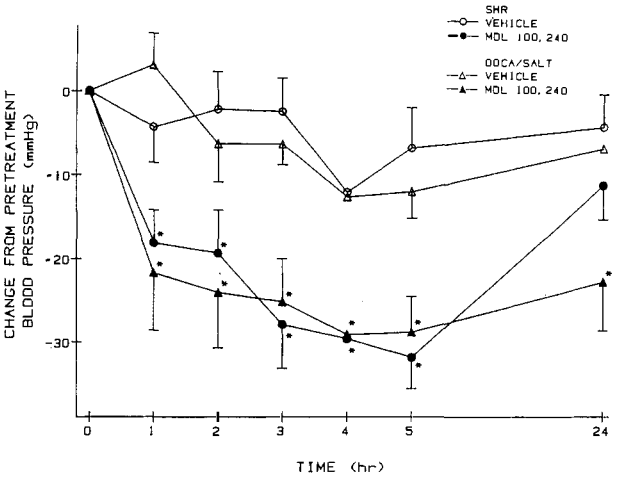
| MDL no. | side chain | $K_i$ (nM) ACE | $K_i$ (nM) NEP |
|---|---|---|---|
| 5 | | 2.0 | 5.0 |
| 11a | *S* | 0.11 (0.10–0.16) | 0.08 (0.05–0.18) |
| 11b | *R* | 4.5 (3.9–5.0) | 0.07 (0.05–0.15) |

[a] Inhibition potency was determined for rabbit lung ACE and rat kidney NEP. $K_i$ values are the median (with 68% nonsymmetric confidence intervals) of at least 13 determinations. Characterization of inhibition of ACE employed a spectrophotometric assay using the substrate (furylacryloyl)phenyllalanylglycylglycine (Sigma Chemical Co., St. Louis, MO) which was recrystallized in order to remove colored contaminants. Inhibition of NEP was assessed by using a fluorogenic assay with dansyl-D-Ala-Gly-(*p*-nitro)Phe-Gly-OH (Sigma Chemical Co.) as the substrate. Thiol content of inhibitor solutions was determined using Ellman's reagent just prior to testing: enzyme assays will be described in detail in another publication.[18d]

studies that further demonstrate the simultaneous inhibition of ACE and NEP by this agent is forthcoming.[18d]

In conclusion, a highly constrained *anti* phenylalanine-containing dipeptide mimetic was designed to mimic low-energy conformations of the HisLeu portion of angiotensin I and the PheLeu portion of leu-enkephalin conceivably adopted at the active sites of ACE and NEP, respectively. A mercaptoacetyl derivative of this mimetic was found to inhibit both metalloproteinases at low nanomolar concentrations. Molecular modeling studies provided divergent substrate binding models for ACE and NEP that contributed significantly to the design of a subnanomolar inhibitor of these two enzymes. This report suggests that the limited conformational freedom in the substituted mercaptoacetyl amide side chain conveys the mixed inhibition characteristic when coupled to an appropriate dipeptide recognition unit. This realization will likely lead

**Chart V.** Effect of **10a** (MDL 100 240) on Systemic Blood Pressure in Conscious SHR and DOCA/Salt Hypertensive Rats[a]




[a] **10a** (30 mg/kg, po, $n = 12$) or saline (5 mL/kg, po, $n = 12$) was administered at time zero and blood pressure recorded hourly for 5 h and at 24 h after treatment. DOCA/salt hypertensive rats were studied 9-weeks postimplant. Pretreatment blood pressures for **10a** and vehicle-treated rats were 232 ± 4 mmHg and 217 ± 4 mmHg, respectively. SHR were studied at 9–10 months of age. Pretreatment blood pressures for **10a** and vehicle-treated rats were 200 ± 4 mmHg and 195 ± 3 mmHg, respectively. Asterisks indicate a significant difference ($P < 0.05$) by a repeated measures analysis and Dunnett's test. Data is represented as mean ± SEM.

to a diversity of structures with similar properties. The acetate thioester prodrug of this dual action inhibitor produced blood pressure lowering in animal models of both essential and volume dependent hypertension. Future studies will focus on the benefits mercaptoacetyl amide dual inhibitors, such as **10a**, have for the treatment of CHF and hypertension. The design process described here demonstrates the utility of our side chain constrained dipeptide mimetic approach[19] as a method of inferring the bound conformations of peptides. The successful application of PheLeu mimetic 1 to the design of a dual inhibitor of ACE and NEP suggests that the specific *anti* conformation of phenylalanine rigidly defined in this mimetic may constitute a common structural motif in peptide recognition.

**Acknowledgment.** The authors wish to greatfully acknowledge the assistance of Dr. Herschel J. R. Weintraub and David A. Demeter in performing molecular modeling studies.

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