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MERCAPTOACYL DIPEPTIDES AS DUAL INHIBITORS OF ANGIOTENSIN-CONVERTING ENZYME AND NEUTRAL ENDOPEPTIDASE. PRELIMINARY STRUCTURE-ACTIVITY STUDIES

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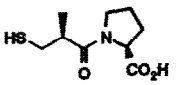
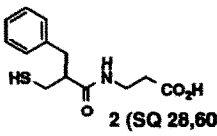
Abstract: Mercaptoacyl dipeptides were prepared as dual-acting ACE/NEP inhibitors. Inhibition of each enzyme may be explained by different binding models. Structure-activity studies determined that, in this series of compounds, the mercaptopropanoyl dipeptide framework leads to increased affinity for NEP but diminished ACE activity *in vivo*.

Atrial natriuretic peptide (ANP) is a 28-amino acid peptide hormone that induces diuresis, natriuresis, and vasodilatation.[1] ANP is rapidly cleaved and inactivated *in vivo*, predominantly by the zinc-containing metalloprotease neutral endopeptidase (NEP; E.C. 3.4.24.11).[2] Inhibitors of NEP potentiate the diuretic, natriuretic, and hypotensive effects of ANP and thus are of potential interest in the treatment of hypertension and congestive heart failure (CHF).[3] Angiotensin-converting enzyme (ACE) is the dipeptidyl carboxypeptidase responsible for cleavage of angiotensin I (AI) to the vasoconstrictor octapeptide angiotensin II (AII). ACE inhibitors are widely used for the treatment of hypertension and CHF.[4] Several studies have reported that inhibition of ACE augments the vascular and renal effects of both ANP and NEP inhibitors in heart failure suggesting that simultaneous inhibition of both enzymes might prove to be a useful therapeutic approach to CHF.[5]

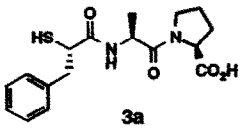
NEP and ACE have similar mechanisms of proteolytic action. The structural requirements of both enzymes allow the design of mercaptoacyl amino acid inhibitors which are selective for either enzyme or active against both.[6,7] Captopril (**1**) is a potent inhibitor of ACE with little activity against NEP whereas SQ 28,603[8] (**2**) has the opposite profile. Interestingly, the mercaptoacetyl dipeptide (**3a**)[9] has ACE activity comparable to captopril but is >1000-times more potent than **1** against NEP. Herein, we present a proposal to rationalize the ACE/NEP activities of **3a** and disclose preliminary SAR resulting in the discovery of potent inhibitors of both enzymes.



1 (Captopril)



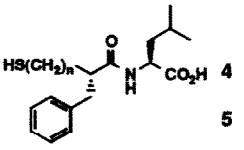
2 (SQ 28,603)



3a

NEP IC_{50} (nM)	830,000	9.4	400
ACE IC_{50} (nM)	23	32,000	30

Optimum binding requirements for both enzymes to mercaptopropanoyl amino acids related to **1** have previously been described.[6] NEP favors a hydrophobic substituent at S_1', most preferably, benzyl. ACE is less affected by variation at the P_1' position. Moreover, inhibitors which do not contain a P_1'-P_2' N-H display weakened against NEP whereas a P_2' proline residue is highly effective for inhibition of ACE. Considering the SAR, we formulated a "frame-shift" hypothesis to explain the binding of **3a** to the active sites of both enzymes: namely, that the benzyl, methyl, and prolyl groups of **3a** bind at the S_1, S_1' and S_2' subsites of ACE, and bind at the S_1', S_2', and S_3' subsites of NEP (Figure 1). Although the structural requirements for binding to zinc in ACE are stringent,[6] tight binding of **3a** could be explained by bidentate complexation *via* the thiol and carbonyl groups as shown. In contrast, the proposed binding of **3a** to NEP follows the classical active-site model for metalloproteases. Since we and others[6b] have found that NEP binds mercaptopropanoyl amino acids such as **5** more tightly than the corresponding mercaptoacetyl amino acids **4**, we reasoned that a similar structural modification might increase the NEP inhibitory activity of **3a**. We therefore undertook the synthesis of novel mercaptopropanoyl dipeptide analogs of **3a** in order to determine the optimum structural features required for potent inhibition of both enzymes.



		ACE (nM)	NEP (nM)
4	n = 0	1,600	87
5	n = 1	37	6.6

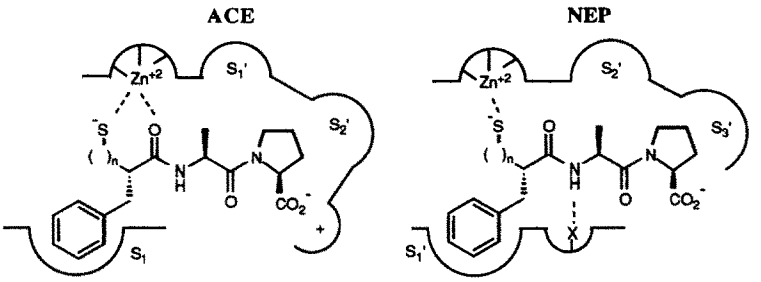
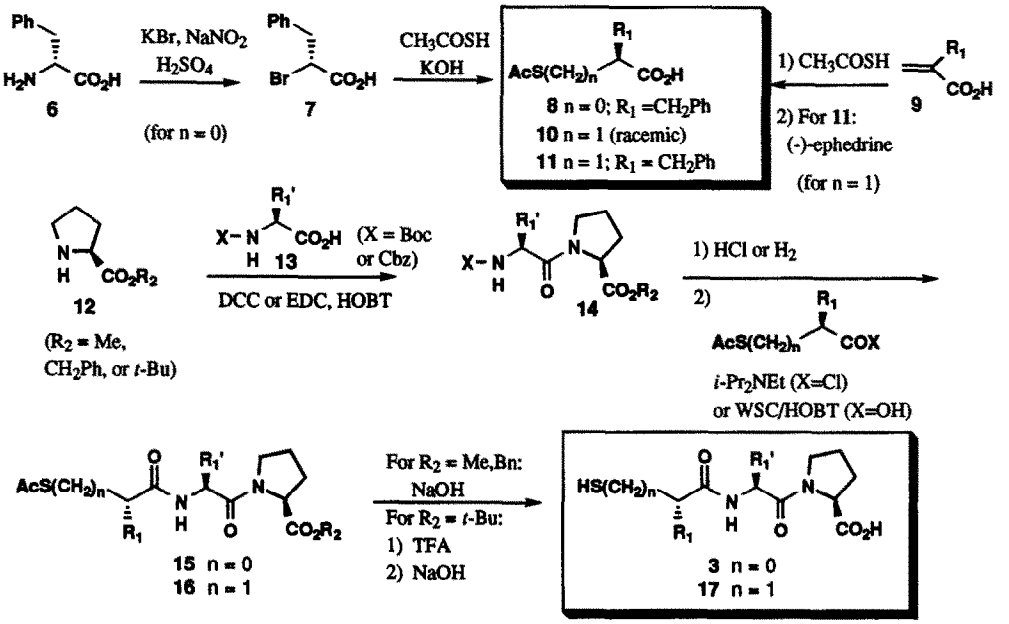


Figure 1. Proposed Binding of Mercaptoacyl Dipeptides to ACE and NEP

Mercaptoacyl dipeptides were prepared as shown in Scheme 1.[10] Synthesis of the S-protected mercaptoacetic acid **8**[11] proceeded from D-phenylalanine **6** *via* the α-bromoacid **7**, with overall inversion of configuration; the (R)-enantiomer was prepared from L-phenylalanine by an analogous procedure. Thiol-protected mercaptopropanoic acids **10**[12] were prepared by Michael addition of thiolacetic acid to the appropriate α-substituted acrylic acid. For the benzyl-substituted acid (**10**; $R_1 = CH_2Ph$), standard resolution with (-)-ephedrine gave the optically pure (S)-acid **11** (the (R) enantiomer was prepared by resolution with (+)-ephedrine). The required C-terminal amino acid (e.g. proline) was coupled as its ester with the N-protected amino acid **13** to afford **14**. N-Deprotection followed by coupling of the resulting dipeptide amino ester with the required S-acetyl mercaptocarboxylic acid and final deprotection gave **3** and **17**.

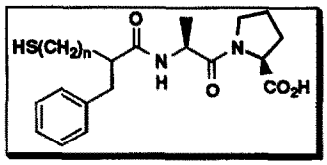
A direct comparison between mercaptoacetyl and mercaptopropanoyl dipeptides is shown in Table 1. The isomeric mercaptoacetyl dipeptides **3a** and **3b** are each more potent against ACE than NEP, with the activity displaying little dependence on the stereochemistry of the benzyl side chain. In contrast, the (S)-mercaptopropanoyl dipeptide **17a** has comparable activity against both enzymes and is substantially more

Scheme 1: Synthesis of Inhibitors



potent than its (R)-isomer, **17b**. Compound **17a** has ≥40-fold more affinity for NEP than **3a** or **3b**, but the mercaptoacetyl dipeptides show superior potency and duration (**17a**: $t_{1/2} \approx 10$ min @ 5 μmol/kg, iv; **3a**: $t_{1/2} \approx 45$ min @ 0.5 μmol/kg) against ACE in the AI-induced pressor response model in normotensive rats despite reduced activity in the ACE enzyme assay. Based on this data, our goal was to enhance the *in vivo* ACE activity of **17a** while retaining its good *in vitro* activity against NEP.

Table 1



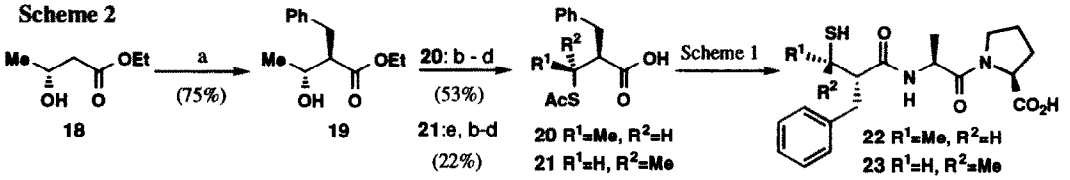
Compound	Stereochemistry	n	NEP IC_{50}[a] (nM)	ACE IC_{50}[b] (nM)	ACE i.v. ED_{50}[c] (μmol/kg)
3a	(S)	0	400	30	0.06
17a	(S)	1	6.6	4.0	0.5
3b	(R)	0	285	80	0.03
17b	(R)	1	45	210	8.1

a. 50% inhibition using a fluorometric assay with purified rat kidney NEP and Dansyl-Gly-Phe-Arg as substrate.[13] *b.* 50% inhibition using rabbit lung ACE and Hippuryl-His-Leu as substrate.[14] *c.* 50% inhibition of the angiotensin I - induced pressor response in normotensive rats; $t_{1/2}$ = time required to return to 50% inhibition of pressor response after AI-challenge.[15]

We speculated that the disparity in *in vivo* activity between **3a** and **17a** could partially be attributed to differences in S-methylation of the inhibitors. This process of metabolic deactivation is well-known with mercaptopropanoyl amino acid substrates.[16] A significant reduction in the S-methylation of **3a** might then be

explained by the steric environment around its secondary thiol group. As a means to circumvent this undesired metabolic pathway, we proposed the synthesis of secondary mercaptopropanoyl dipeptides related to **17a** (Scheme 2). Following literature precedent,[17] the dianion of (R)-(-)-ethyl 3-hydroxybutyrate **18** was benzylated to give **19** with high diastereoselectivity. Potassium hydroxide saponification (85%) followed by treatment of the resulting acid with *p*-toluenesulfonyl chloride (73%) and reaction of the intermediate β-lactone with cesium thioacetate in DMF (85%) gave the thiobutanoic acid **20**. The alternate (2R, 3R) stereoisomer **21** was prepared by initial Mitsunobu inversion on β-hydroxy ester **19** to give the inverted acetate in 65% yield along with byproducts formed from elimination of the activated intermediate. Saponification followed by treatment of the hydroxy acid as above gave **21**. S-Protected thiobutanoic acids **20** and **21** were subsequently coupled with Ala-Pro *t*-butyl ester, as described in Scheme 1, to give the substituted mercaptopropanoyl dipeptides **22** and **23**, respectively. Unfortunately, the intrinsic activities for **22** and **23** against both ACE and NEP were substantially reduced in comparison to **17a** (**22**: NEP = 32 nM, ACE = 452 nM; **23**: NEP = 182 nM, ACE = 78 nM). In addition, **23** displayed diminished potency against ACE *in vivo* (ED_{50} >5.0 μmol/kg).[18]

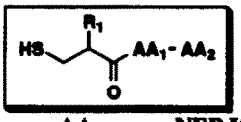
Scheme 2

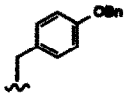
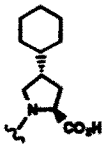
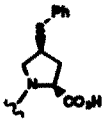
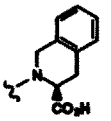
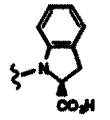


a. LDA, THF, -50°; BnBr, HMPA, -20°; b. KOH, H_2O, EtOH; c. *p*-TsCl, pyridine d. AcSCs, DMF; e. DIAD, PPh_3, HOAc, THF

In light of these results, we returned to **17a** as a template for optimizing the side-chain interactions with both enzymes, anticipating that a concurrent increase in *in vivo* ACE activity might ensue. Structure-activity studies centered on mercaptopropanoyl dipeptide **17a** are shown in Table 2. The phenethyl (**17c**) and methyl (**17d**) analogs of **17a** result in a substantial loss in activity against both enzymes while the isobutyl analog (**17e**) maintains potency against NEP. These results provide additional support for the "frame-shift" hypothesis; a β-branched hydrophobic group (**17e**) is preferred at $P_{1'}$ for NEP, and, in contrast to the expected SAR at $P_{1'}$ for ACE, a methyl group (**17d**) reduces affinity. Ortho substitution of the aromatic ring (**17f,g**) diminishes ACE activity both *in vitro* and *in vivo* with little effect on NEP activity; para-benzyloxy substitution (**17h**) reduces both activities but is worse for ACE than NEP. Replacement of the methyl side chain of **17a** with larger groups (**17i,j**) has only a small effect on *in vitro* activity but also results in reduced potency against ACE *in vivo*. Several replacements for the proline residue in mercaptopropanoyl amino acid and carboxyalkyl dipeptide ACE inhibitors have been reported to improve activity both *in vitro* and *in vivo*.[19] 4-Phenylthio substitution of the proline residue (**25**)[19a] is preferred over 4-cyclohexyl substitution (**24**)[19a] against both enzymes, resulting in activity essentially equivalent to **17a**. Replacement of the proline by a tetrahydroisoquinoline group (**26**)[19b] results in more than a 3-fold loss in activity against ACE *in vitro* compared to **17a** but equivalent potency *in vivo*. On the other hand, the indolinecarboxylic acid derivative, **27**[19c], displays comparable affinity compared to **17a** for both enzymes but appears more potent against ACE *in vivo*. However, the potency and duration of **27** ($t_{1/2}$ = 17 min @ 5 μmol/kg) in the AI-induced pressor response model remain less than that observed for **3a**. The critical nature of the C-terminal proline residue for ACE is underscored by the mercaptoacyl amino acid **28** which is equipotent to **17a** against NEP but 30-fold less active against ACE, a result also consistent with the "frame-shift" model.

Table 2



Compound	R_1	AA_1	AA_2	NEP IC_{50} (nM)	ACE IC_{50} (nM)	ACE i.v. ED_{50} (μmol/kg)
17a	(S)-CH_2Ph	Ala	Pro	6.6	4.0	0.5
17c	(R,S)-CH_2CH_2Ph	Ala	Pro	160	290	ND[a]
17d	(S)-CH_3	Ala	Pro	140	1,800	ND[a]
17e	(R,S)-CH_2CHMe_2	Ala	Pro	10	490	ND[a]
17f[b]		Ala	Pro	6.2	69	4.2
17g[b]		Ala	Pro	2.2	25	2.6
17h		Ala	Pro	40	880	ND[a]
17i	(S)-CH_2Ph	Trp	Pro	25	10	6.4
17j	(S)-CH_2Ph	NorVal	Pro	2.0	10	2.2
24	(S)-CH_2Ph	Ala		6.6	18	2.5
25	(S)-CH_2Ph	Ala		1.6	8.7	0.78
26	(S)-CH_2Ph	Ala		3.9	14	0.57
27	(S)-CH_2Ph	Ala		2.6	7.8	~0.15
28	(S)-CH_2Ph	Ala	-	5.7	120	15

[a] Not determined. [b] Prepared as a mixture of isomers; compound shown is more potent isomer against both ACE and NEP.

To summarize, inhibition of ACE and NEP by the mercaptoacyl dipeptides **3a** and **17a** may be explained by two different binding models: one in which the benzyl, methyl, and prolyl groups bind at the S_1, S_1' and S_2' subsites of ACE and the other in which the same groups bind at the S_1', S_2', and S_3' subsites of NEP.[20] Mercaptopropanoyl dipeptide **17a** has increased affinity for ACE and NEP compared to mercaptoacetyl dipeptide **3a** but diminished ACE activity *in vivo*. Additional structure-activity studies on **17a** led to the

indolinecarboxylic acid analog **27** which has excellent affinity for both enzymes and improved potency *in vivo* against ACE. Although we were unable to obtain a mercaptoacyl dipeptide with nanomolar affinity for NEP and *in vivo* potency and duration against ACE comparable to **3a**, this goal has subsequently been achieved *via* replacement of the alanyl-proline portion of **3a** by conformationally restricted dipeptide surrogates. This latter work is the focus of the following communications.[21]

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13. Incubation mixtures employed for the assay contained 0.5 mM substrate; 50 mM TRIS-HCl buffer (pH 7.5); enzyme preparation in 0.05% Triton X-100; and various concentrations of inhibitor, or vehicle, to a final volume of 0.25 mL. The inhibitor solutions were preincubated for 10 min with the enzyme preparation and buffer before the reaction was initiated by the addition of the substrate. After 30 min at 37°C, the reaction was stopped by the addition of 0.25 mL of 1N HCl and the Dansyl-Gly product was extracted into 1.5 mL of EtOAc. Relative fluorescent intensity (RFI) of the EtOAc extract was measured using a Perkin-Elmer LS-5B Luminescence Spectrometer at excitation and emission wavelengths of 342 and 508 nm, respectively, corrected for the RFI of a zero-time control, and compared with controls lacking inhibitors.
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16. Drummer, O.H.; Miach, P.; Jarrott, B. *Biochem. Pharmacol.* **1983**, *32*, 1557. The S-methyl metabolite of **19** and compounds derived from it form in significant amounts after dosing in monkeys. Although no direct comparison between **19** and **3** has been performed, we have determined that S-methylation is also a primary metabolic route in mercaptoacetyl dipeptides (Richard Morrison, unpublished results).
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20. The authors in Ref. 7b propose an analogous model for the binding of compounds related to **22** and **23** to NEP in which the C-terminal amino acid side chain is outside the active site. We believe that the increased affinity observed for analogs **25** and **27**, as well as for those containing dipeptide surrogates,[7c,21] provides evidence for the presence of an $S_{3'}$ binding pocket for NEP.
21. Robl, J.A., et al., following papers in this issue.

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