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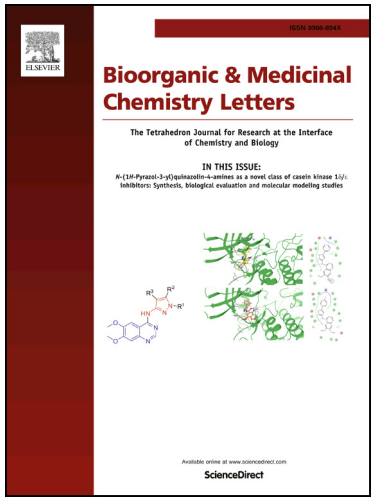

Substituted α-mercaptoketones, new types of specific neprilysin inhibitors

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Substituted α–mercaptoketones, new types of specific neprilysin inhibitors

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ABSTRACT

New neprilysin inhibitors containing an α–mercaptoketone HSC(R^1R^2)CO group, as zinc ligand were designed. Two parameters were explored for potency optimization: the size of the inhibitor which could interact with the S_1, S_1' or S_2' domain of the enzyme and the nature of the substituents R^1, R^2 of the mercaptoketone group. Introduction of a cyclohexyl chain in R^1, R^2 position and a (3-thiophen)benzyl group in position R^3 (compound **12n**) yielded to the most potent inhibitor of this series with a Ki value of 2 ± 0.3 nM. This result suggests that this new inhibitor interacts within the S_1, S_1' domain of NEP allowing a pentacoordination of the catalytic Zn^{2+} ion by the mercaptoketone moiety.

***Abbreviations*:** BTFA, Boron trifluoroacetate, DIEA, Diisopropylethylamine; EtOH, Ethanol; HPLC, High Performance Liquid Chromatography; LC/MS, Liquid Chromatography/Mass Spectroscopy; LDA, Lithium diisopropyl amidure; NEP, Neprilysin; NMM, N-Methylmorpholine; PMBSH, para-Methoxybenzenethiol; TFA, Trifluoroacetic acid.

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Neprilysin also called neutral endopeptidase (NEP, EC 3.4.24.11), enkephalinase or atriopeptidase, is a zinc dependent type II integral membrane protein belonging to the M13 family of metalloproteases. It is involved in the catabolism of biologically active peptides such as various vasoactive peptides[1,2] and essentially in the inactivation of enkephalins (Met-ENK: TyrGlyGly↓PheMet and Leu-ENK: TyrGlyGly↓PheLeu), the endogenous ligands of the mu and delta opioid receptors[1]. Neprilysin is an endopeptidase hydrolyzing peptide substrates at the N terminus of aromatic or large hydrophobic amino acids which constitute the P'_1 residue in the P_2-P_1-P'_1-P'_2 commonly accepted Schechter and Berger nomenclature[3]. It also possesses dipeptidyl carboxypeptidase activity thus releasing the C terminal hydrophobic dipeptides of small peptides such as enkephalins (Phe-Leu or Phe-Met). In all these cases, specificity is the result of specific interactions within the S'_1 and S'_2 subsites of the enzyme active site. The development of selective neprilysin inhibitors firstly required the characterization of these enzyme subsite specificities using various peptide substrates[4] and secondly the characterization of the best ligand among the different chemical entities able to chelate the catalytic zinc ion. Together these studies have led to the synthesis of very efficient inhibitors with inhibitory constant (Ki) values in the nanomolar range[1].

The co-crystallization of neprilysin[5] with phosphoramidon later allowed confirmation of the main hypotheses proposed for the active-site characteristics and also highlighted the stabilizing interactions (hydrogen bonds and hydrophobic interactions) between the inhibitor and the enzyme active site[6]. Two types of inhibitors have been co-crystallized with neprilysin: a modified dipeptide such as thiorphan[7] which was found to interact only with the S_1' and S_2' subsites of the enzyme and phosphoramidon[5], aminophosphinic[8] and mercaptoacyl dipeptides[8] which recognized the S_1, S_1' and S_2' subsites (Figure 1).

In these two-last series of molecules, the enzyme-inhibitor complex was characterized by a bidentation of the catalytic Zn^{2+} through two oxygens of the phosphinic moiety or the SH and CO groups of the α–mercapto acyl moiety[8].

These inhibitors possessed one or two amide groups which interacted respectively with the carbonyl of NEP Ala^{543} and with the CO and NH_2 of the terminal amide group of NEP Asn^{542} (Figure 1). These stabilizing interactions increased the enzyme recognition by these molecules but their polar groups could be unfavorable to their crossing the blood brain and/or gastrointestinal barriers[9]. Moreover, the presence of carboxamide bonds in these inhibitors could render these molecules sensitive proteolytic degradation thus decreasing their half-life. It could be also underlined that the amide bond planarity orients the spatial position of the inhibitor side chains and their adjustment within the enzyme active site.

Taking into account all these parameters, we decided to test the replacement of the amide bond by a ketone group $COCH_2$, "a classical replacement method"[10] in the synthesis of pseudopeptides. Starting from mercaptoacyl amino-acids or mercaptoacyl dipeptides NEP blockers[8], we have developed a series of α–mercaptoketones as new high affinity inhibitors of this enzyme.

The first α–Mercaptoketones have previously been described as matrix metalloproteinases inhibitors[11,12] and some of them were very efficient with nanomolar inhibitory potency against MMP1, MMP3 or MMP9. However, these molecules possessed an unsubstituted α–mercaptoketone group $HSCH_2CO$, to interact with the Zn^{2+} ion in the catalytic site.

For NEP inhibition, the modification of the mercaptoacyl- aminoacid or -dipeptide led to the synthesis of compounds bearing substituted α–mercaptoketones HS-CHR^{1}R^2-CO where the thiol and the carbonyl would be interacting with the catalytic Zn^{2+} and R would fit within a binding subsite of the enzyme. The sequence HS-CHR^{1}R^2-CO presents the disadvantage to be chiral and consequently to induce the formation of two supplementary stereoisomers this possible disadvantage could always be circumvented by stereoisomer separation.

The aim of this study was to characterize the impact of two parameters on inhibitory potency: i) the importance of the amide bond replacement, ii) the optimization of the hydrophobic interactions in each subsite.

Synthesis of pseudo-tripeptide inhibitors

Compounds **11** were obtained using a linear multi step scheme from an amino acid **1** of defined absolute configuration (R) (Scheme 1).

The amino acid **1** was converted to brominated derivative **2** by a reaction of deamination-halogenation, which was usually performed with retention of configuration[13].

The bromide derivative **2** was transformed into thioether **3** by nucleophilic substitution, with inversion of configuration, by reaction with 4-Methoxy-benzyl thiol.

The ketene **4**, obtained by addition of diazomethane to the mixed anhydride (prepared by the reaction of **3** with isobutyl chloroformate in presence of N-methylmorpholine), was then converted to halomethylketone **5** by reaction with bubbling gaseous HCl or HBr in 1,4-dioxane, diethyl ether or ethyl acetate.

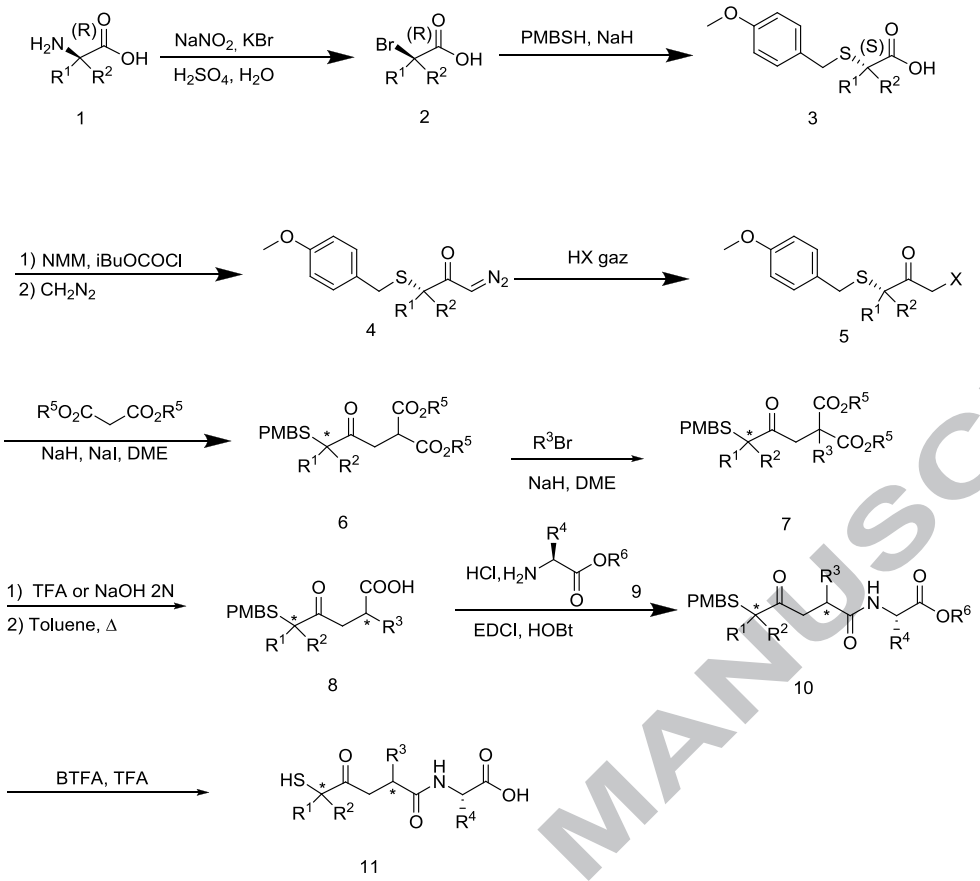
The halomethylketone **5**, treated with NaI, underwent a halogen exchange and then reacted with the dialkyl malonate sodium salt (R^5 may be a methyl, ethyl or tert-butyl group) leading to compound **6**. The basic conditions used in this reaction resulted in a racemization of compound **6**.

The R^3 substituent was introduced by reaction of the brominated derivative R^3Br with the malonate **6**, *in situ* deprotonated with NaH, to yield compound **7**.

After deprotection by action of TFA (when R^5 was a *tert*-butyl group) or hydrolysis of diester **7** (when R^5 was a methyl or ethyl group), a decarboxylation in refluxing toluene occurred leading to compound **8**.

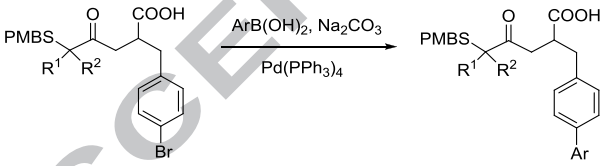
A usual amino-acid coupling reaction could be performed on compound **8**, as described in the literature, with the aminoester **9** leading to compound **10** (Scheme 1).

The ester **10** was then hydrolyzed and the thioether protection was removed by the action of BTFA (prepared from BBr_3 in trifluoroacetic acid)[14], to yield the expected compound **11**.



Scheme 1. Synthesis of pseudo-tripeptide inhibitor **11** starting from compound **1**.

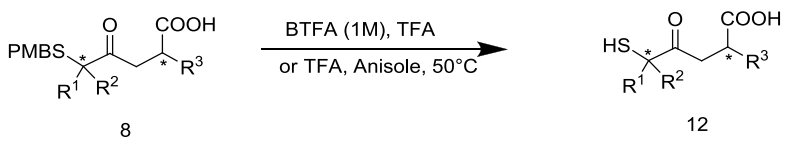
The biaryl group was obtained by Suzuki-Miyaura reaction on the 4-bromobenzyl **8** analog with boronic acid in presence of Pd catalyst as described in litterature[15] (Scheme 2). The following steps were as described in Scheme 1.



Scheme 2. Suzuki-Miyaura reaction

Synthesis of pseudo-dipeptide inhibitors

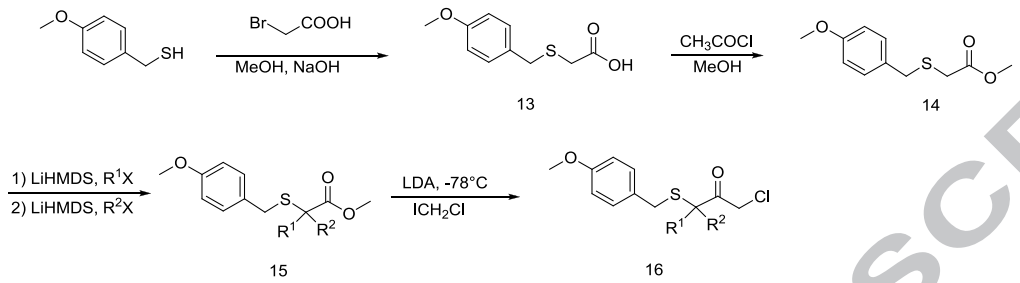
Starting from compound **8**, the thioether protection was removed by the action of BTFA (prepared from BBr_3 in trifluoroacetic acid)[14], to yield the expected compound **12** (Scheme 3).



Scheme 3. Synthesis of pseudo-dipeptide inhibitor **12** starting from compound **8**.

Synthesis of geminal inhibitors

Alternatively, in case of geminal compounds, the chloromethylketones **16** were prepared from a reaction between bromo-acetic acid and 4-methoxy benzyl mercaptan followed by the double alkylation leading to **15**. The synthesis of the chloromethylketones **16** was obtained by action of ICH_2Cl in presence of LDA as described in litterature[16] (Scheme 4).

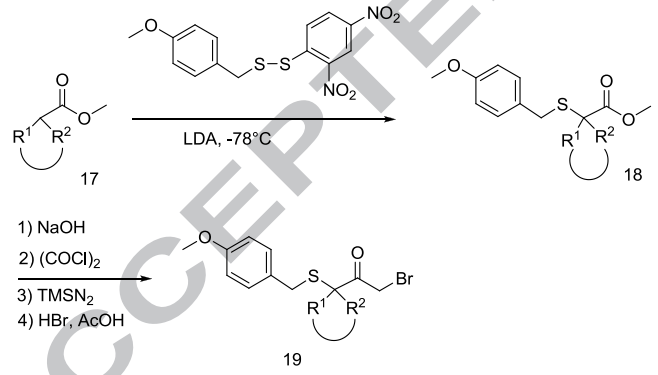


Scheme 4. Synthesis of geminal chloro α–mercaptoketones **16**

The following steps leading to geminal inhibitors were those described in the previous Scheme 1 and 3.

Synthesis of cyclic core inhibitors

The bromomethylketone **19** was synthesized from the cyclic methyl ester **17** by alkylation with PMB-S-S-DNP[17], leading to compound **18.** Saponification followed by reaction with oxalyl chloride gave the acid chloride[18] which could react with trimethylsilyldiazonium[19] and HBr in acetic acid to give the bromomethylketone **19** (Scheme 5).



Scheme 5. Synthesis of geminal bromo α-mercaptoketones **19**

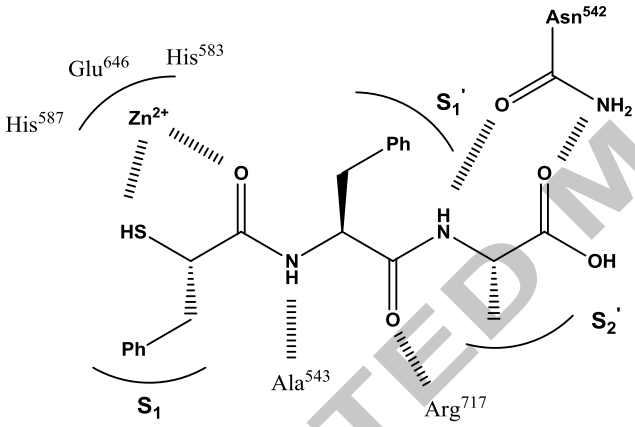
The following steps leading to geminal inhibitors were those described in the previous Scheme 1 and 3.

Inhibitory potency

Ki values of the different compounds towards neprilysin (NEP) were determined as described by Poras et al[20] (Tables 1-3). All these compounds were tested on NEP-2, ACE, ECE-1 and ECE-2 and were shown to be at least 20-fold more selective on NEP.

Biochemical and structural results

Compound **I** (Figure 1) has been crystallized in NEP[7] and was shown to fully occupy the enzyme active site. The three inhibitor side chains interacted respectively with the S_1, S_1' and S_2' binding subsites and the Zn^{2+} was bidentated by the mercapto acyl group leading to a pentacoordinated Zn^{2+} in this complex. The two amide NH groups are hydrogen bonded with the CO of Ala^{543} and Asn^{542} respectively and the C-terminal carboxylate interacted with the side chains of Arg^{102}, Asp^{107} and Arg^{110} at the surface of the enzyme. A new series of inhibitors based on this structure is reported in Table 1. These compounds (**11a-k**) contain three asymmetric carbons and only the C terminal aminoacid is optically pure. Consequently, they were synthesized as mixtures of four stereoisomers, which were initially not separated.



(Compound I) Ki = 2.3 nM

Figure 1: Positioning of compound I in Neprilysin[7]

The replacement of the mercapto amide in Compound **I** by an α–mercaptoketone in compound **11a** (Ki 95 ± 8 nM) significantly increased the Ki value by 40-fold. This reflects both the loss of the hydrogen bond involving Ala^{543} (Figure 1), but also the presence of four diastereoisomers in **11a** whose Ki values could be extremely different.

To improve NEP recognition, the side chains R^1, R^3 and R^4 were successively modified with the aim of optimizing their interaction with their respective subsites (Table 1). When the R^3 benzyl group was replaced by a bulkier aromatic side chain, 4-bromo benzyl in **11b** or 4-phenyl benzyl in **11c**, a notable increase in their potencies (78 ± 8 and 46 ± 4 nM respectively) was observed. This result is in accordance with the known preference of the NEP S_1' subsite for large aromatic residues as illustrated in the aminophosphinic series of NEP inhibitors[21].

The introduction of a tryptophan residue in R^4 position (compound **11d** (Ki 18 ± 1 nM) and **11e** (Ki 30 ± 4 nM) was also favorable (Table 1). This is in complete accordance with the

ability of the Trp indole NH to create a hydrogen bond with the carbonyl of Val^{541} in the S_2' subsite as shown in the crystallized NEP-phosphoramidon complex[5].

The R^1 side chain interacts with the S_1 subsite of NEP. As shown in the crystallographic structures[5,8], the R^1 chain is at the surface of the enzyme and is not in direct contact with the protein, reflecting the minor stabilizing role of the S_1 subsite in NEP. Consequently, as expected, a large increase in the size of hydrophobic R^1 (compound **11f**, Ki>10^{-5} M) was unfavorable, while decreasing side chain size *ie* aromatic (**11g**, Ki 22 ± 4 nM) or aliphatic (**11h**, Ki 12 ± 2 nM and **11i**, Ki 7 ± 0.5 nM) was well accepted. However, too small a side chain, like a CH_3 (compounds **11j** and **11k**), did not improve S_1 subsite recognition.

Taken together, these different results allowed structural parameters for NEP active site occupancy by these α-mercapto-ketones to be proposed: an aliphatic moiety such as an isobutyl chain in position S_1, a large aromatic residue as biphenyl methyl in S'_1 and a small aliphatic residue in S'_2.

In a second series of inhibitors, the C-terminal amino-acid has been deleted leading to a smaller molecule (compound **II**[5]**,** Table 2) with a Ki of 23nM, 10-fold higher than that of compound **I** (Figure 1). Based on compound **II** structure, a series of molecules in which the CO-NH group was replaced by a $CO\text{-}CH_2$ is reported in Table 2. These molecules contain two asymmetric carbons and were studied as a mixture of 4 diastereoisomers.

Compound **II** has only one amide bond and its reduction into $COCH_2$ leads to a derivative without peptide bond. This opens the question of the positioning of the analogues **12a-g** in NEP active site: i) interactions inside S_1' and S_2' subsites such as observed for thiorphan and its analogues[22] or ii) interactions inside S_1 and S_1' subsites, favored by bidentation of zinc, as previously described with compounds **11a-k.**

Compound **12a** (Ki 140 ± 10 nM) showed a 7-fold decrease in inhibitory potency as compared to **II** (Ki 23 nM)[5], suggesting a relatively weak participation of the amide NH group in the stabilization of the inhibitor-enzyme complex. Interestingly, an increase in size of the aromatic R^3 residue (**12b**, Ki 50 ± 8 nM) significantly favored NEP inhibition, while the same modification for R^1 (**12c**, Ki 1750 ± 80 nM) was detrimental. When both R^1 and R^3 were bulky aromatic groups (**12d** and **12e**), intermediate inhibitory potencies were observed (Ki 260 ± 40 and 252 ± 50 nM respectively). The replacement of the R^1 aromatic side chain in **12a** by an aliphatic one in **12f** led to an efficient, high affinity inhibitor with a Ki of 20 ± 5 nM, but the amidification of the C terminal carboxylic group by a morpholine in **12g** (Ki 1650 ± 40 nM) was not well accepted. All these results are in favor of an interaction of these inhibitors within the S_1, S_1' domain of the enzyme, enlightening the importance of Zn^{2+} pentacoordination for the complex formation.

To simplify the study of these α–mercaptoketones, the R^1 chain was modified in order to suppress one asymmetric center by introducing a symmetrical aliphatic geminal derivative (compounds **12h** and **12i**) (Table 3) or a cyclic residue based on symmetrical cyclopentyl or cyclohexyl structure (compounds **12j-n**) (Table 3). Such poorly flexible cycles have previously been introduced in NEP inhibitors in position S_1'[20,23] and were well recognized by this subsite. Moreover, it was interesting to test the ability of these cycles to fit within the largely solvent exposed S_1 subsite.

As shown in Table 3, the introduction of small aliphatic residues gem-dimethyl in **12h** (Ki 470 ± 40 nM) and gem-diethyl in **12i** (Ki 86 ± 9 nM) yielded inhibitory potencies in the 10^{-7}

M range. Conversely, the cyclic structures were more efficient. A comparison between **12j-m** revealed that the cyclohexyl moiety (**12l** and **12m)** in the S_1 position leads to a better inhibition than with the cyclopentyl one (**12j** and **12k)** and in all these cases, the biphenyl methyl S'_1 residue (**12k** and **12m**) is preferred to a bromobenzyl (**12j** and **12l**).

Due to this preference for large aromatic residues in S'_1, we tested a para-(3-thiophen)benzyl group in this position (**12n**). The inhibitory potency of this compound (Ki 4.6 ± 0.5 nM) was the best obtained for a racemic in this series of NEP inhibitors.

Compound **12n** was therefore resolved and the absolute configuration of the (3-thiophen)benzyl group determined by 1H-NMR spectroscopy after coupling with a (S)-Ala residue as described in the litterature[24]. Interestingly, the (R) stereoisomer (Ki 2 ± 0.3 nM) was found to be 30-fold more potent than the (S) stereoisomer (Ki 61 ± 2 nM).

In order to try and optimize the enzyme-inhibitor interactions within the S_1 subsite, modifications were introduced in position 4 of the cyclohexyl moiety of **12l** or **12m**. Inhibitory potencies obtained with these latter compounds, with an increased size of the R^1-R^2 cycle residue (**12p** to **12t** as compared to **12l** or **12o, 12u** as compared to **12m**), showed that the cyclohexyl group is significantly preferred even if the inhibitory potencies of **12o** to **12u** remained relatively low with Ki values in the 10^{-8} molar range (Table 3).

The 3D structure of NEP, shows that the essential parameters for a strong interaction of a small inhibitory molecule inside the active site are the Zn^{2+} ion and the S'_1 subsite. All the other possible interactions within the S_1 and S'_2 subsites, hydrogen bonds and ionic interactions are energetically less efficient but are essential for the correct positioning of peptide substrates or inhibitors inside the enzyme.

Potent inhibitors of NEP have systematically been obtained with compounds interacting with the Zn^{2+} ion and the hydrophobic S_1' subsite, associated with either occupancy of the S'_2[7,22] or the S_1 subsite[6,21,25].

Thus, with compounds interacting only with the S_1'- S_2' domains, the S_1' subsite is well recognized by a benzyl group as well as by a biphenyl group, due, for the latter, to the displacement of Met^{579} and Trp^{693}, increasing the size of S'_1 cavity[8]. With compounds recognizing also the S_1 domain, a biphenyl moiety is preferred to a benzyl one[8,21].

According to these results, we demonstrated in this paper that it is possible to obtain high affinity NEP inhibitors with compounds recognizing only the S_1 and S'_1 domains provided that Zn^{2+} bidentation was insured by suitable ligands such as α-mercapto ketones groups.

The pharmacological properties of these novel inhibitory molecules will be explored further using animal models.

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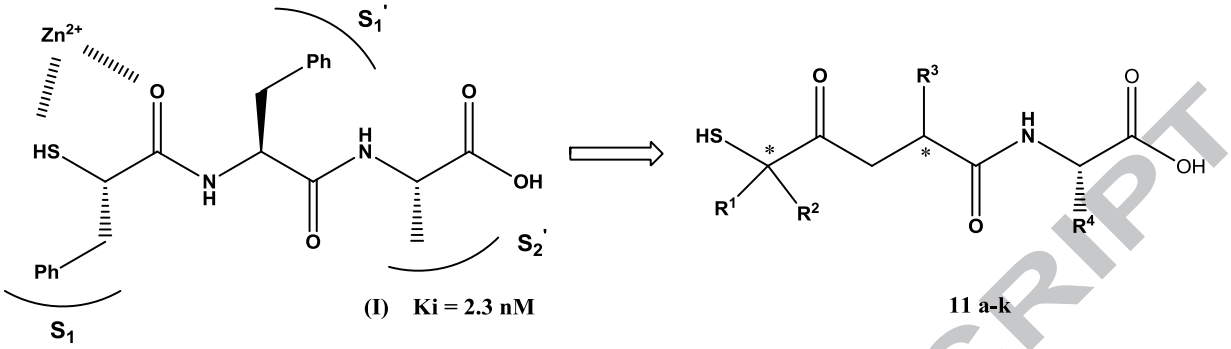
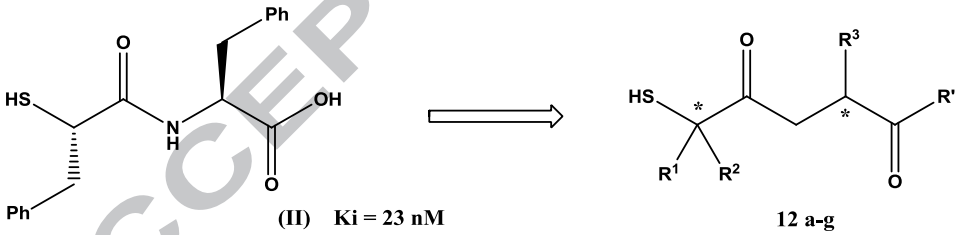
Table 1



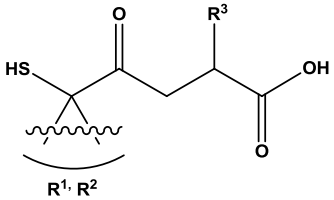
Compound	R^1	R^2	R^3	R^4	NEP Ki (nM)
11a	CH_2Ph	H	CH_2Ph	(S)-Ala	95 ± 8
11b	CH_2Ph	H	CH_2(4-Br-Ph)	(S)-Ala	78 ± 8
11c	CH_2Ph	H	CH_2(4-Ph-Ph)	(S)-Ala	46 ± 4
11d	CH_2Ph	H	CH_2Ph	(S)-Trp	18 ± 1
11e	CH_2Ph	H	CH_2(4-Br-Ph)	(S)-Trp	30 ± 4
11f	CH_2(4-Br-Ph)	H	CH_2Ph	(S)-Leu	> 10^{-5}
11g	Ph	H	CH_2(4-Br-Ph)	(S)-Ala	22 ± 4
11h	iBu	H	CH_2(4-Ph-Ph)	(S)-Ala	12 ± 2
11i	iBu	H	CH_2(4-Br-Ph)	(S)-Ala	7 ± 0.5
11j	CH_3	H	CH_2(4-Br-Ph)	(S)-Trp	162 ± 8
11k	CH_3	H	CH_2(4-Ph-Ph)	(S)-Ala	40 ± 5

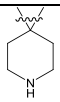
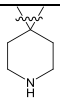
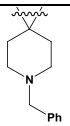
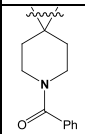
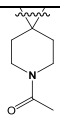
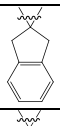
Table 2



Compound	R^1	R^2	R^3	R'	NEP Ki (nM)
12a	CH_2Ph	H	CH_2Ph	OH	140 ± 10
12b	CH_2Ph	H	CH_2(4-Br-Ph)	OH	50 ± 8
12c	CH_2(4-Br-Ph)	H	CH_2Ph	OH	1750 ± 80
12d	CH_2(4-Br-Ph)	H	CH_2(4-Br-Ph)	OH	260 ± 40
12e	CH_2(4-Ph-Ph)	H	CH_2(4-Br-Ph)	OH	252 ± 50
12f	iBu	H	CH_2(4-Br-Ph)	OH	20 ± 5
12g	iBu	H	CH_2(4-Br-Ph)	Morpholine	1650 ± 40

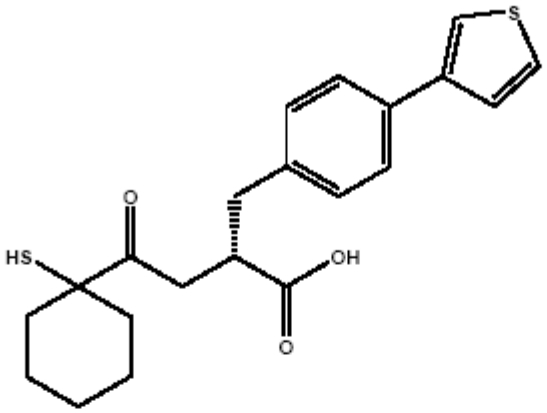
Table 3



Compound	Stereochemistry	R^1, R^2	R^3	NEP Ki (nM)
12h	Racemic	CH_3	CH_2(4-Br-Ph)	470 ± 40
12i	Racemic	C_2H_5	CH_2(4-Br-Ph)	86 ± 9
12j	Racemic		CH_2(4-Br-Ph)	52 ± 4
12k	Racemic		CH_2(4-Ph-Ph)	29 ± 5
12l	Racemic		CH_2(4-Br-Ph)	32 ± 7
12m	Racemic		CH_2(4-Ph-Ph)	9 ± 1
12m	*Stereo* 1		(R)-CH_2(4-Ph-Ph)	36 ± 1
12m	*Stereo* 2		(S)-CH_2(4-Ph-Ph)	4.2 ± 0.1
12n	Racemic		CH_2(3-Thiophen-Ph)	4.6 ± 0.5
12n	*Stereo* 1		(R)-CH_2(3-Thiophen-Ph)	61 ± 6
12n	*Stereo* 2		(S)-CH_2(3-Thiophen-Ph)	2 ± 0.3
12o	Racemic		CH_2(4-Ph-Ph)	61 ± 2
12p	Racemic		CH_2(4-Br-Ph)	54 ± 1
12q	Racemic		CH_2(4-Br-Ph)	54 ± 14
12r	Racemic		CH_2(4-Br-Ph)	49 ± 2
12s	Racemic		CH_2(4-Br-Ph)	95 ± 13
12t	Racemic		CH_2(4-Br-Ph)	36 ± 3
12u	Racemic		CH_2(4-Ph-Ph)	26 ± 1

Mercaptoketone vf2_tracked

Graphical abstract



12n Stereo 2, (*S*)-4-(1-mercaptocyclohexyl)-4-oxo-2-(4-(thiophen-3-yl)benzyl)butanoic acid

Neprilysin Ki = 2 +/- 0.3 nM