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CONCISE ARTICLE

Nitro-based inhibitors against thermolysin†

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A series of nitro-based compounds were synthesized and evaluated as inhibitors against thermolysin (TLN). The binding modes of the nitro group at the active site of TLN were characterized based on the structural analysis of TLN-(R)-2-benzyl-3-nitropropionic amide complex, which was suggested as a pseudo-transition state analogue. Further structure–activity relationship studies disclosed that both the stereochemistry of the P_1 chains and the bulkiness of the P_2' substituents were crucial to the potent inhibition of TLN.

Thermolysin (TLN, E.C. 3.4.24.27) is a heat-stable proteolytic enzyme isolated from *Bacillus thermoproteolyticus* and carries a catalytically essential zinc ion at the active site. It is an extra-cellular endopeptidase, hydrolyzing the peptide bond on the imino side of the amino acid residue having a large hydrophobic side chain, such as Leu, Ile, and Phe.[1] TLN is one of the proto-typical zinc proteases, and has played an important role as a model enzyme for the development of inhibitors for other zinc proteases of medicinal interest such as angiotensin I-converting enzyme (ACE, E.C. 3.4.15.1) and neprilysin (NEP, E.C. 3.4.24.11).[2]

The most popular approach to designing inhibitors of zinc proteases is the introduction of a zinc binding group (ZBG) in an optimized peptidic or non-peptidic scaffold.[3] However, it is still a challenge to obtain potent inhibition based on the known ZBG for a specific enzyme. For example, in the inhibition of a neutral proteinase isolated from *Saccharomonospora canescens* (NPS),[4] which shares many common features with TLN, a phosphor-amidon-based inhibitor is very weak with an inhibitory constant (K_i) value of 65.0 μM but potent inhibition was observed for TLN (K_i = 0.034 μM).[5] It is further complicated by the stereo-chemistry of inhibition. 2-Benzylsuccinic acids are inhibitors for TLN as well as carboxypeptidase A (CPA, E.C. 3.4.17.1), the other zinc-dependent enzyme whose active site structure, mechanism of action, and stereospecificity resemble those of TLN.[1a,6] The L-form of BSA binds CPA much more tightly than its enantiomer,[7] while the D-form is the preferred inhibitor for TLN

accompanied with the different coordinative modes to the zinc ion at the active site.[6c]

Being an isostere of the carboxylate, the nitro group has been investigated in various enzyme–inhibitor complexes.[8] A nitro-based inhibitor for CPA, L-2-benzyl-3-nitrosuccinic acid, exhibits slightly improved inhibitory activities (K_i = 0.15 μM) compared with L-BSA (K_i = 0.45 μM).[8b] However, nitro-based inhibitors have not been reported previously. As mentioned above, a prac-tical investigation is necessary to develop nitro-based TLN inhibitors. Our work was thus devoted to exploring the binding modes of the nitro group at the active site of TLN as well as to obtaining potent inhibition of TLN by the optimization of the side chains.

To examine the stereochemistry in the inhibition of TLN, 2-benzyl-3-nitropropionic amides (**1**) should be firstly screened as inhibitors for TLN due to their simple structure. In our opinion, their complexes with TLN could also provide enough structural information to disclose the binding modes of nitro group to the zinc ion at the active site of TLN. All optically active forms of **1** were then synthesized. For the dipeptide inhibitors against TLN, it is generally found that either benzyl or isobutyl was the opti-mized side chain for P_1' or P_2'.[9] We thus further synthesized four dipeptides **2**–**5** (Scheme 1). An alternative route to obtain opti-cally active **3** and **5** involved first formation of the peptide bond, followed by bromination through the mesylate.[10] The two dia-stereomers obtained could be separated by silica gel column
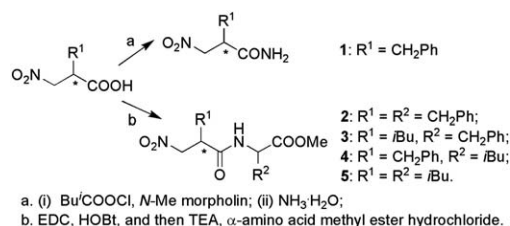
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a. (i) Bu^{i}COOCl, *N*-Me morpholin; (ii) NH$_3$·H$_2$O;
b. EDC, HOBt, and then TEA, α-amino acid methyl ester hydrochloride.

1: R^1 = CH$_2$Ph
2: R^1 = R^2 = CH$_2$Ph;
3: R^1 = *i*Bu, R^2 = CH$_2$Ph;
4: R^1 = CH$_2$Ph, R^2 = *i*Bu;
5: R^1 = R^2 = *i*Bu.

Scheme 1

chromatography with a mixture of *n*-hexane and ethyl acetate as eluent. Final introduction of the nitro group gave the optically active forms of **3** or **5** (Scheme 2). The stereochemical assignments for them were made on the basis of comparison of the [α] values with the compounds obtained using the first method. The corresponding configurations of the intermediates involved were thus correlated.

All compounds were assayed as competitive inhibitors for TLN using *N*-[3-(2-furyl)acryloyl]Gly-L-Leu-NH$_2$ as substrate.[9a] Their K_i values were obtained from the respective plot for v_0/v against the concentration of inhibitors and are collected in Table 1. Fig. 1 shows a Dixon plot for the inhibition of TLN.

Firstly, consistent to L-specificity of TLN, (*R*)-**1** showed more binding affinity to TLN than the (*S*)-form, which is different from the carboxylate-based inhibitor. TLN-(*R*)-**1** crystals for X-ray diffraction were then obtained by soaking the enzyme crystals into the solution containing the racemic **1** and determined at a resolution of 1.5 Å (PDB ID code: 3FOR). The final model including all residues of TLN and (*R*)-**1** was refined in the 38.48–1.93 Å (ESI†). Fig. 2 depicts the stereoview of the difference electron density in the region of the active site of TLN that is occupied by (*R*)-**1**. Distances of important interactions between TLN and (*R*)-**1** in the complex are listed in Table 2.

The electron density map for the TLN-(*R*)-**1** complex shows that one molecule of (*R*)-**1** is bound to the active site of TLN. Both oxygen atoms of the nitro group are involved in strong coordinative bonds to the zinc ion having the distances as 2.25 and 2.63 Å, respectively, which means that the nitro group also coordinates to the zinc ion in a bidentate mode (Table 2). One additional hydrogen bond between ON1 of (*R*)-**1** and the NE2 of His-231 could be inferred, which is believed to be the oxyanion hole to stabilize the transition-state intermediate occurring in the TLN-catalyzed hydrolysis of the substrate.[11] It is further found that the distance between ON2 of (*R*)-**1** and the OE1 of Glu-143 is measured as 3.68 Å. The hydrogen bond of the nitro group with Glu-143 could not be thus expected. Compared to the generally accepted transition-state-analogue inhibitors of TLN,[11a] which are structurally featured by the *gem*-diolate oxygens stabilized by chelation to the zinc ion and two respective hydrogen bonds with Glu-143 and His-203, we thus proposed that the nitro group acts as a pseudo-transition-state-analogue. In addition, the amide nitrogen atom of (*R*)-**1** formed a hydrogen bond with the amide oxygen atom of the side chain of Asn-112, which supported our design of the presented dipeptide compounds as TLN inhibitors.

Table 1 Inhibitory constants (K_i) of the presented compounds for TLN

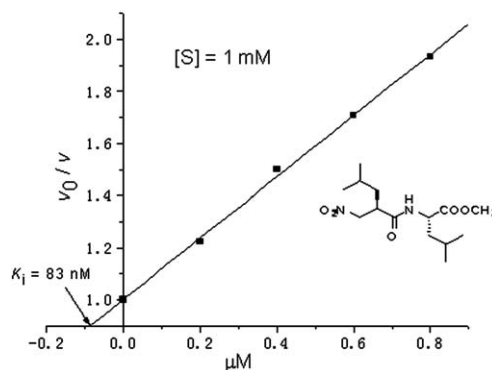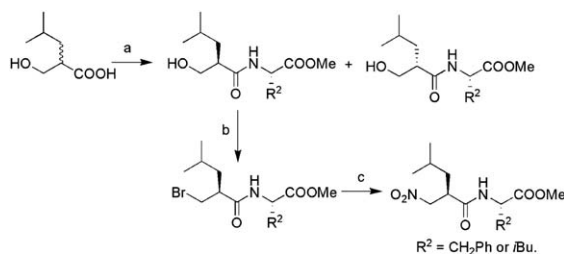| Compounds	K_i (μM)
(±)-**1**	36
(*R*)-**1**	7.6
(*S*)-**1**	663
(2′*R*, 2*S*)-**2**	0.95
(2′*S*, 2*S*)-**2**	79
(2′*R*, 2*R*)-**2**	1.40
(2′*S*, 2*R*)-**2**	86
(2′*R*, 2*S*)-**3**	0.84
(2′*S*, 2*S*)-**3**	7.2
(2′*R*, 2*S*)-**4**	0.37
(2′*S*, 2*S*)-**4**	2.4
(2′*R*, 2*S*)-**5**	0.083
(2′*S*, 2*S*)-**5**	0.77

Fig. 1

Of the four optically forms of **2**, (2′*R*, 2*S*)-**2** is the most potent inhibitors for TLN. Considering the benzyl as the same side chain for both S$_1$′ and S$_2$′ hydrophobic pockets of TLN, it could be reasonably believed that the enzyme showed preference to the nitro-based dipeptides compounds with L,L-configurations. The stereochemistry of P$_1$′ substituents seemed to have a great effect on the strong binding affinities to the enzyme, *i.e.*, significant differences could be observed in comparison with (2′*R*, 2*S*)- and (2′*S*, 2*S*)-**2**, (2′*R*, 2*R*)- and (2′*S*, 2*R*)-**2**, respectively. Similarly, it was found that (2′*R*, 2*S*)-**3**, (2′*R*, 2*S*)-**4**, and (2′*R*, 2*S*)-**5** are more than 7–9 fold more potent than (2′*S*, 2*S*)-**3**, (2′*S*, 2*S*)-**4**, and (2′*S*, 2*S*)-**5**, respectively. On the other hand, the right stereochemistry of the P$_2$′ chains slightly enhanced the inhibitory activities of (2′*R*, 2*S*)-**2** in comparison with (2′*R*, 2*R*)-**2**.
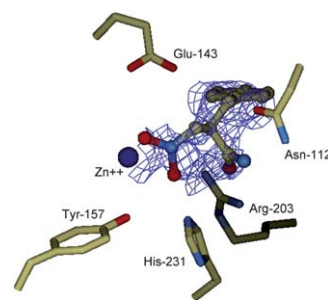

Fig. 2 Difference electron density map for TLN-(*R*)-**1** complex generated with Fourier coefficient $|F_o| - |F_c|$ phases calculated from the final model omitting the bound inhibitor.

a. EDC, HOBt, and then TEA, (*S*)-α-amino acid methyl ester hydrochloride;
b. (i) MsCl, TEA; (ii) LiBr; c. NaNO$_2$ / phloroglucinol.

Scheme 2

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Table 2 Polar interaction distances between TLN and inhibitors for the phosphonamide, silanediol and presented nitro compound[a]

	Contacting atom (inhibitors) and distance (Å)					
Enzyme residue	ZG^{p}LL (5TMN)		Silanediol (1Y3G)		(R)-2 (3FOR)	
Zn^{2+}	OP1	2.08	OSi1	1.93	ON1	2.25
Zn^{2+}	OP2	3.01	OSi2	3.28	ON2	2.63
His-231 NE2	OP1	2.9	OSi1	2.55	ON1	3.11
Glu-143 OE1	OP2	2.5	OSi2	2.76	ON2	3.68
Tyr-157 phenolic O	OP1	3.4	OSi1	3.44	ON1	3.96
Asn-112 amide O					N amide	3.09

[a] The contact distances between the enzyme residues and the inhibitor atoms were measured according to the coordination files obtained from the Protein Data Bank. The PDB IDs of 5TMN,[11a] 1Y3G[11c] and 3FOR correspond to the complexes of TLN with the inhibitor ZG^{p}LL, silanediol and (R)-1, respectively.

Replacement of the benzyl at the P$_1'$ position of (2$'$R, 2S)-2 by an isobutyl group gave (2$'$R, 2S)-3 accompanied with a small improvement of the binding affinities to TLN. A similar tendency could be found in the P$_2'$ position, where (2$'$R, 2S)-4 is about 3 fold more potent than (2$'$R, 2S)-2. It seemed that both the S$_1'$ and S$_2'$ pockets of TLN were preferred to the isobutyl group for the nitro-based dipeptide inhibitors. The most potent inhibition of TLN was achieved by (2$'$R, 2S)-5 with a K_i value of 83 nM, which was comparable to the silanediol-based inhibitor (K_i = 41 nM).[9c] The isobutyl as P$_2'$ chain led to (2$'$R,2S)-5 more than 10-fold more potent than (2$'$R,2S)-3. It is thus reasonable to assume that the bulkiness of the P$_2'$ chain more significantly affected the binding affinities of the dipeptides to TLN, that is, the isobutyl chain is better accommodated by the S$_2'$ pocket of TLN than the benzyl group.

In summary, the nitro group as ZBG was introduced in the inhibition of TLN. Its binding modes at the active site of TLN were investigated on the basis of structural analysis of the enzyme–inhibitor complex, which was featured by a pseudo-transition state analog. Subsequent studies on the relationships between the inhibitory activities and the structures of the side chains disclosed that both the stereochemistry of the P$_1'$ chain and the bulkiness of the P$_2'$ chains mainly contributed to the potent inhibition of TLN. Among the present nitro-based compounds, (2$'$R, 2S)-N-(2-isobutyl-3-nitropropionyl)leucine methyl ester is the most effective inhibitor of TLN with a K_i comparable to the reported transition state analog inhibitor using silanediol as zinc-binding group.

Acknowledgements

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