Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 323–326

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

INHIBITION OF THERMOLYSIN WITH NITRONE-BEARING SUBSTRATE ANALOGS: A NEW TYPE OF THERMOLYSIN INHIBITORS

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Received 31 October 1997; accepted 25 December 1997

Abstract: Nitrones are utilized as the active site zinc coordinating functionality in the design of inhibitors for thermolysin. This new type of thermolysin inhibitors are as potent as the existing inhibitors bearing a carboxylate or hydroxamate zinc ligating moiety.

Thermolysin (EC 3.4.24.4) is an extracellular thermostable zinc-contaning endopeptidase isolated from *Bacillus thermoproteolyticus*.[1] Along with the mammalian digestive enzyme carboxypeptidase A, thermolysin is one of prototypical zinc-containing proteases, and has played an important role as a model enzyme in the development of inhibitor design strategies that can be translated to zinc proteases of physiological importance.[2]

Most of thermolysin inhibitors are characterized as being analogs of substrate having a moiety which is capable of coordinating to the active site zinc ion. Carboxylate, aldehyde and ketone, sulfhydryl, hydroxamate, and phosphorus oxy acid of various oxidation states have served as the zinc ligand in the design of inhibitors for thermolysin.[3] In this communication we wish to report a new type of thermolysin inhibitors which are designed by incorporating a nitrone moiety into the substrate structural frame as the zinc coordinating functionality.

Nitrones (azomethine *N*-oxides) are highly valuable synthetic building blocks for the synthesis of various

0960-894X/98/$19.00
PII: S0960-894X(98)00023-7

types of nitrogen heterocycles,[4] and used as excellent 1,3-dipoles for cycloadditon reactions.[5] Reportedly, Lewis acid such as Mg^{2+} and Zn^{2+} brings about significant improvements of the rate as well as the regio- and stereo-specificity in the 1,3-dipolar cycloadditions, suggesting the negatively polarized oxygen atom of the nitrone to form a coordinative bond to the Lewis acid.[6] We have exploited this unique property of the nitrone to design a novel type of competitive reversible inhibitors for thermolysin. As illustrated in Figure 1, the nitrone moiety in the inhibitors is expected to ligate to the active site zinc ion.

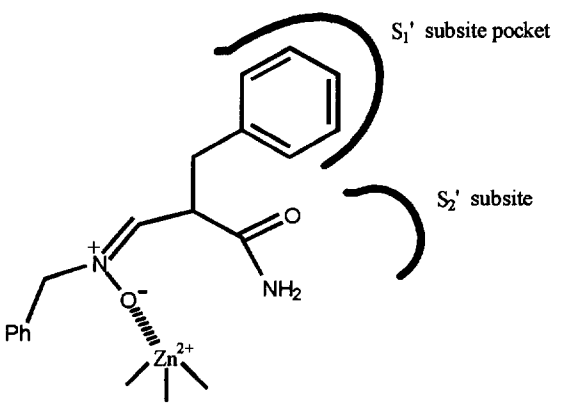


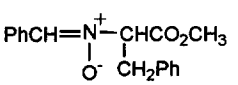
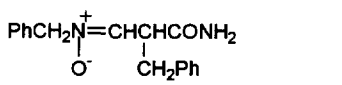
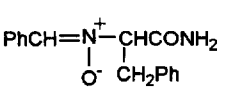
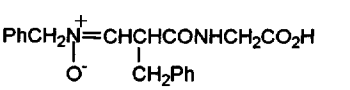
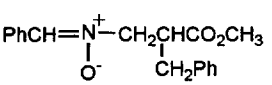
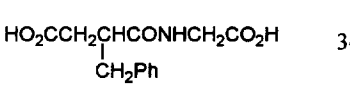
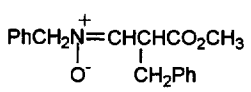
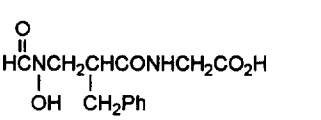
Figure 1. Postulated binding mode of a nitrone bearing inhibitor to the active site of thermolysin.

We have synthesized six representative potential inhibitors[7] by oxidation of the corresponding secondary amines with hydrogen peroxide in the presence of a catalytic amount of sodium tungstate.[8] In the case of *N*-benzyl-β-phenylalanine methyl ester, there was obtained a mixture of **3** and **4** (Table 1) in a nearly equal ratio, which was separated by column chromatography. Compound **6** was prepared by condensing *N*-β-phenyalanidenebenzylamine *N*-oxide with methyl glycinate by a standard method and subsequent treatment of the product with dilute NaOH solution. Aldonitrones such as those synthesized in this study are known to exist in the thermodynamically more stable *Z*-form.[9]

Inhibitory activities of the synthesized nitrone derivatives for thermolysin were estimated at pH 7.2 (Tris buffer) by the literature method[10] using 2-furylacryloyl-Gly-Leu-NH_2 as substrate, and are summerized in Table 1. No significant thermolysin inhibitory activity was observed with the amine precursor of **5** at concentration up to 3.3 mM, suggesting strongly that the enzyme inhibitory activity of these nitrone derivatives arises from the zinc coordinating propensity of the nitrone moiety. The most potent inhibitor in this study is shown to be **5** having the K_i value of 40 μM. In general, amides are more potent than esters,

suggesting that the hydrogen atom on the amide nitrogen is possibly involved in the formation of hydrogen bond with backbone peptide carbonyl oxygen atom. Unexpectedly, the incorporation of Gly into a most potent compound **5** increased the K_i value. The inhibitory potencies of our nitrone bearing substrate analogs are comparable to those of well known thermolysin inhibitors having a carboxylate or hydroxamate moiety such as **7** and **8** (Table 1).

Table 1. Inhibitory potencies of nitrone bearing inhibitors for thermolysin

Compd No.	Structure	K_i (μM)[a]	Compd No.	Structure	K_i (μM)[a]
1		1,910	**5**		40
2		774	**6**		54
3		244	**7**		340[b]
4		82	**8**		34[b]

[a] The inhibitory constants were determined according to the method reported by Feder *et al* using 2-furylacryloyl-Gly-Leu-NH_2 as the substrate at pH 7.2 (0.1 M Tris, 0.01 M $CaCl_2$, 25 ℃). [b] Yonghao Jin, Ph. D. Thesis, Department of Chemistry, Pohang University of Science and Technology, 1997.

In conclusion, we have demonstrated in this study that the readily obtainable nitrone is a valuable moiety that can be useful as an active site zinc coordination functionality in designing inhibitors for thermolysin. The novel design protocol may potentially be employed in designing inhibitors effective against zinc proteases of medicinal interest.

Acknowledgment: The Korea Science and Engineering Foundation is acknowledged for the financial support of this work.

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7. **1**: mp 174 - 175 ℃; IR (thin film) 1735, 1570 (C=N) cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.55 (dd, J = 14.2, 4.4 Hz, 1H), 3.66 (dd, J = 14.2, 10.3 Hz, 1H), 3.78 (s, 3H), 4.64 (dd, J = 10.3, 4.4 Hz), 7.06 (s, 1H, -C<u>H</u>=N), 7.17 - 7.38 (m, 8H), 8.11 (m, 2H, ArH ortho to C<u>H</u>=N^+-O^-); ^{13}C NMR ($CDCl_3$) δ 35.5, 53.6, 79.8, 127 - 137, 167; Anal. Calcd for $C_{17}H_{17}NO_3$: C, 72.07; H, 6.05; N, 4.94. Found: C, 71.72; H, 6.25; N, 5.14. **2**: mp 190 - 192 ℃; ^1H NMR (DMSO-d_6) δ 2.48 - 3.12 (m, 2H), 4.61 (m, 1H, α-H), 7.08 - 7.53 (m, 10H), 7.76 (d, J = 7.1 Hz, 2H), 8.45 (d, J = 8.5 Hz, 1H); ^{13}C NMR (DMSO-d_6) δ 38.1, 55.6, 127.0 - 138.4, 174.2. **3**: IR (neat) 1720, 1570, 1555 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.94 (m, 1H), 3.10 (m, 1H), 3.67 (s, 3H), 3.72 (m, 1H), 4.0 (dd, J = 12.0, 4.8 Hz, 1H), 4.25 (dd, J = 12.0, 8.7 Hz, 1H), 7.19 - 7.49 (m, 9H), 8.20 - 8.23 (m, 2H, ArH ortho to CH=N^+-O^-); ^{13}C NMR ($CDCl_3$) δ 36.2, 44.8, 52.5, 66.9, 127 - 137.0, 174.0. **4**: IR (neat) 1740, 1590, 1570 cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.11 (m, 1H), 3.21 (m, 1H), 3.68 (s, 3H), 4.21 (q, J = 6.9 Hz, 1H), 4.88 (s, 2H), 6.79 (d, J = 6.6 Hz, 1H, -C<u>H</u>=N), 7.04 - 7.07 (m, 2H), 7.19 - 7.41 (m, 8H); ^{13}C NMR ($CDCl_3$) δ 35.1, 44.9, 52.6, 69.9, 126.3 - 137.8, 171.7. **5**: mp 150 - 152 ℃; ^1H NMR (DMSO-d_6) δ 2.08 - 2.94 (m, 2H), 3.4 (m, 1H), 4.31 (m, 2H), 7.16 - 7.35 (m, 10H), 9.54 (t, J = 6.3 Hz, 1H); ^{13}C NMR (DMSO-d_6) δ 36.7, 47.7, 53.3, 123.0 - 141.5, 177.5. **6**: mp 186 - 188 ℃; ^1H NMR (DMSO-d_6) δ 2.96 - 3.15 (m, 2H), 3.49 (dd, J = 8.3, 6.2Hz, 1H), 3.76 (d, J = 5.7 Hz, 2H), 4.13 (dd, J = 15.3, 5.4 Hz, 1H), 4.30 (dd, J = 15.3, 5.4 Hz, 1H), 7.0 - 7.26 (m, 10H), 8.12 (t, J = 5.7 Hz, 1H), 8.32 (t, J = 6.1 Hz, 1H), 12.8 (br, 1H, CO_2H); ^{13}C NMR (DMSO-d_6) δ 36.1, 41.8, 43.0, 55.4, 124.5 - 128.7, 139.8, 170.0, 172.0; Anal. Calcd for $C_{19}H_{20}N_2O_4$: C, 67.05; H, 5.92; N, 8.23. Found: C, 67.62; H, 5.89; N, 8.49.

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