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Synthesis and inhibition properties of a series of pyranose derivatives towards a Zn-metalloproteinase from *Saccharomonospora canescens*

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ABSTRACT

The Zn-proteinase, isolated from *Saccharomonospora canescens* (NPS), shares many common features with thermolysin, but considerable differences are also evident, as far as the substrate recognition site is concerned. In substrates of general structure AcylAlaAlaPhe 4NA, this neutral proteinase cleaves only the arylamide bond (non-typical activity of Zn-proteinases), while thermolysin attacks the peptide bond Ala-Phe. Phosphoramidon is a powerful tight binding inhibitor for thermolysin and significantly less specific towards NPS. The K_i-values (65 μM for NPS vs 0.034 μM for thermolysin) differ nearly 2000-folds. This implies significant differences in the specificity of the corresponding subsites. The carbohydrate moiety is supposed to accommodate in the S_1-subsite and the series of arabinopyranosides and glucopyranosides (12 compounds), which are assayed as inhibitors in a model system: NPS with SucAlaAlaPhe4NA as a substrate could be considered as mapping the S_1-subsite of NPS. Members of the series with an additional ring (3,4-epithio, 3,4-anhydro-derivatives) turned out to be reasonably good competitive inhibitors ($K_i \approx 0.1$–0.2 mM are of the same order as the K_i value for phosphoramidon). The structure of these compounds (**8, 9, 11** and **12**) seems to fit the size of the S_1-subsite and due to an appropriately oriented OH-group in addition, to protect the active site Zn^{2+}.

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1. Introduction

Zinc proteinases (EC 3.4.24) have been recognized as a distinct class of proteolytic enzymes in which at least one ion of zinc is involved directly in catalysis.[1]

In recent years, the number of identified zinc proteinases has dramatically increased. A classification has been proposed on the basis of unique, repeated amino acid sequences, so called 'signatures' and also significant group similarities in their crystallographic structures.[2,3] Three groups of zinc metalloproteases were found to be encoded in *Trypanosoma brucei* genome, each of which contains ~30% amino acid identity with the major surface protease of *Leishmania*.[4]

A very big group of zinc proteases includes matrix metalloproteinases which degrade most of the components of the extracellular matrix. They also have a number of non-traditional roles in processing factors related to cell growth/proliferation and inflammation. There are 23 human and 23 mouse matrix metalloproteinases, most of which share orthology among most vertebrates; other examples have been found in invertebrates and plants.[5]

The interest in Zn-metalloproteinases from microorganisms is due to their diverse biological functions and promising ideas for practical application.[1] They are thought to play a role in many disease states, including arthritis, vascular disease, lung injury, wound repair, cancer and various neurodegenerative disorders. This family includes a growing number of biologically important enzymes which are attractive targets for rational drug design.

There is a lot of evidence for resemblance of the catalytic sites of most Zn-proteinases, accompanied with a considerable diversity in the substrate recognition sites. The special features of the zinc-binding environment of carboxypeptidase A were examined, focusing on the geometrical considerations. The results of these studies suggest that the zinc ion is important for both the binding and the catalytic activation of the substrate as well as for stabilization of the tetrahedral reaction intermediate.[6] The chemical mechanism of Zn-proteinase-catalyzed hydrolysis of peptide bonds has been elusive and is still under debate.

Abbreviations: NPS, neutral metalloproteinase from strain 5 of *Saccharomonospora canescens*; 4-NA, 4-nitroanilide; Suc, succinyl; Ant, anthraniloyl; FA, furylacryloyl.

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Therefore, mechanistic studies on new members of Zn^{2+}-proteinases are expected to be an additional source of information for this class. The recently isolated Zn-proteinase from *Saccharomonospora canescens* (NPS) was purified to homogeneity.[7] The intriguing properties of this enzyme are its high thermostability and the non-typical for this class arylamidase activity. This paper is a logic extension of the studies on this enzyme[7] in order to address it to some known Zn-proteinase family. We report in this paper on the comparative inhibition activities between NPS and thermolysin for the known inhibitor phosphoramidon.[8] New classes of carbohydrate inhibitors were synthesized and assayed towards NPS.

2. Results and discussion

The enzyme *Saccharomospora canescens* sp. *novus* is a thermostable neutral Zn-proteinase with a molecular mass of 33 kDa, consisting of a single polypeptide chain. The N-terminal sequence of NPS, including 26 amino acid residues, has no homology with other bacterial neutral proteinases such as themolysin and carboxypeptidase A. NPS is active towards casein. It is capable of hydrolyzing an anilide bond (a single cleavage) in peptide 4-nitroanilides of corresponding amino acid sequences, which is unusual for the great majority of the Zn-proteinases.[9,3] Thermolysin, a 34.6 kDa protease, isolated from *Bac. thermoproteolyticus*, being the most thoroughly studied Zn-metalloproteinase was used as an enzyme for comparison with the recently reported NPS. The data in Table 1 show that the two enzymes differ in the type of cleaved bond, recognized in the structure of the tripeptide nitroanilide substrates. Thermolysin attacks Ala-Phe peptide bond, while NPS causes only one cleavage at the nitroanilide bond, based on the HPLC analysis of the cleaved products of the substrates AntAlaAla-Phe4NA and SucAlaAlaPhe4NA. The arylamidase activity is typical for serine- and thiol-proteinases.[10] Common for a number of Zn-proteinases inhibitor phosphoramidon *N*-(α-L-rhamnopyranosyl-hydroxylphosphinyl)-L-leucyl-L-tryptophan is an inhibitor for both the compared proteinases. It is a powerful competitive (tight binding) inhibitor with a K_i-value of 0.034 μM for thermolysin.[11] Significantly weaker inhibitor is phosphoramidon for NPS, with a 2000-fold higher K_i-value (K_i = 65 μM). Nevertheless, we assume an analogy in the binding mode of phosphoramidon to both enzymes. The chelation of the active site zinc ion by phosphoryl group and a hydrophobic interaction between the side chain of the P_1^1-amino acid and the S_1^1 subsite of the enzyme (nomenclature of Schechter, Berger)[12] have been considered as the primary factors responsible for the effective inhibition. The crystallographic studies of the complex of thermolysin-phosphoramidon[11] have suggested the following mode of binding: (1) one of the phosphite oxygens chelates Zn^{2+} in the active site, displacing a structural water molecule; (2) the leucyl side chain of the inhibitor binds in the hydrophobic pocket S_1^1 (predilection to Leu, Ile, Phe as P_1^1 residue, (3) the Trp residue binds in what appears to be a secondary, less specific hydrophobic site P_2^1. Simultaneously a hydrogen bond is formed between the indole nitrogen and the protein backbone at Asn 111. The sugar moiety is supposed to accommodate at the S_1 subsite, it makes several contacts with the enzyme[11] and probably makes some contribution to the free energy of binding. It is supposed that a intermolecular hydrogen bond (2.4 Å) between the carboxyl terminal of phosphoramidon and the $C_{(2)}$ hydroxyl of the sugar is formed. This hydrogen bond may help 'lock' the inhibitor in a conformation optimal for binding to the enzyme.

In the light of the mode of phosphoramidon binding to Zn-proteinases, the series of compounds (Table 2) are assayed as NPS inhibitors and they could be considered as mapping the S_1 subsite. The values of K_i are an indication of the degree of structural fit of the corresponding structure to the S_1 subsite and also how much it hampers the alignment of the substrate to the active site Zn^{2+}. The series of carbohydrate compounds inhibited NPS in a competitive manner. Using Dixon plot of inhibition of NPS with inhibitor (**9**), the K_i value was calculated to be 1.5 mM. Based on the K_i values, the series could be divided into two groups: the first with K_i >1 mM and the second with K_i <1 mM. The common structural feature of the first group (compds **1**, **2**, **3**, **6** and **10**) is the presence of a number of OH groups (substituted or non-substituted), the preferred conformation being the 'boat conformation'. Maybe there is a hindrance for the appropriate accommodation of these compounds on the enzyme surface. The second group (compds **7**, **8**, **9**, **11** and **12**) is characterized with a presence of a second ring attached to the pyranose structure in positions 3 and 4 (3,4-epithio or 3,4-anhydro) and an OH group in position 2. They are of less flexible conformation. It sounds logic to ascribe the better inhibition constants to a better fit of the second group compounds to the S_1 enzyme cavity as well as to an ability to protect in some degree the active site Zn^{2+}. The K_i-value of compound **4**, lacking a second ring, implies a favourable role of the CH_2OH group serving as a 'handle' providing additional contacts with the active site.

Thermolysin, a 34.6 kDa protease, being the most thoroughly studied Zn-metalloproteinase, was included in our comparative kinetic studies.

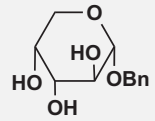
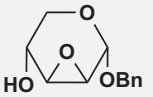
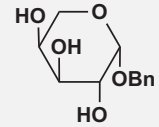
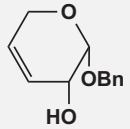
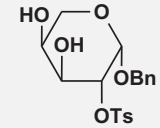
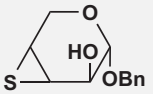
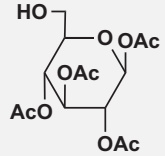
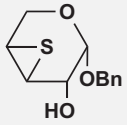
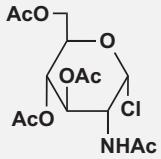
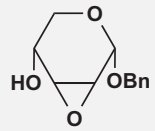
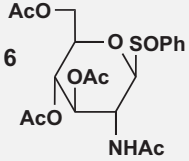
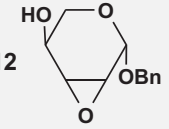
Using thermolysin isolated from *Bac. Thermoproteolyticus* in conditions analogous to the NPS-experiments, for comparison of enzyme kinetics with NPS, when assayed with compound (**9**) as an effector, the second ring was not affected at all. This implies a less importance of the S_1–P_1 interactions in the overall recognition process of the corresponding effectors in the case of thermolysin. It is known that in thermolysin, Zn^{2+} is tetra-coordinated, but there

Table 1
Kinetic data for NPS- and thermolysin-catalyzed hydrolysis of *N*-acyl-tripeptide-nitroanilides

Enzymatic Enzyme	Reaction Substrate	K_m[c] (μM)	k_{cat}[c] (s^{-1})	k_{cat}/K_m[c] ($M^{-1}\,s^{-1}$)	K_i (μM)	Ref.
NPS	AntAAF↓4NA	80	4.0	51,250		
NPS	SucAAF↓4NA	98	1.1	11,220		4
NPS	SucAAF↓4NA + phosph.				65.0[b]	
Thermolysin[d]	AntAA↓F4NA			4950		
Thermolysin[e]	SucAA↓F4NA			2700		
Thermolysin	FA-G↓L-NH_2			9460		17
Thermolysin	FA-G↓L-NH_2 + phosph.				0.034	18

K_i values for phosphoramidon inhibition of the corresponding enzymatic reactions.
[a] ↓Bond cleaved, proved by HPLC analysis of the reaction mixture.
[b] K_i value, determined using Dixon plot.
[c] The values of the kinetic parameters are evaluated with SD <10%.
[d] The hydrolysis of AntAA↓F4NA was followed fluorimetrically in the conditions of pseudo-first order kinetics $[S] \ll K_m$.
[e] The hydrolysis of SucAA↓F4NA was followed by HPLC kinetic experiment.

Table 2
Structure and inhibition constants (K_i) of a series pyranose derivative tested against NPS

Chemical structure	Name	K_i (mM)	Chemical structure	Name	K_i (mM)
1 	Benzyl α-D-arabino-pyranoside	2.80	7 	Benzyl 2,3-anhydro-α-D-lyxopyranoside	0.10
2 	Benzyl β-L-arabino-pyranoside	3.50	8 	Benzyl 3,4-dideoxy-α-D-glycero-pent-3-epopyranoside	1.15
3 	Benzyl 2-*O*-*p*-tosyl-β-L-arabinopyranoside	2.80	9 	Benzyl 3,4-dideoxy-3,4-epithio-α-D-arabinopyranoside	0.12
4 	1,2,3,4-Tetra-*O*-acetyl-β-D-glucopyranose	0.26	10 	Benzyl 3,4-didesoxy-3,4-epithio-β-L-arabinopyranoside	0.10
5 	2-Acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl chloride	0.90	11 	Benzyl 2,3-anhydro-α-D-ribopyranoside	1.50
6 	Phenyl 2-Acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-1-thiophenyl-β-D-glucopyranosyl-sulfoxide	1.90	12 	Benzyl 2,3-anhydro-β-L-lyxopyranoside	0.12

The inhibition constants (K_i) are determined by the method of Dixon (substrate: SucAlaAlaPhe pNA (100–700μM), NPS (0.1 μM), phosphate buffer: (0.1 M, pH 6.8, 2% DMSO), 25 °C. The hydrolysis of the substrate was followed at 405 nm (ε_{405} = 9600 M^{-1} cm^{-1}). Standard deviation was less than 10%.

are no data available about NPS. Therefore, we could not discussion probability for difference in the coordination number of the active site Zn^{2+} and the properties of the enzymes.

The experimental data reveal a significant difference between NPS and thermolysin as far as the substrate recognition site is concerned. It is hard to say which of the factors are (binding interactions, catalytic mechanism and differently coordinated Zn^{2+}) responsible for the different behaviour of the enzymes. On the basis of these experimental data, the design of a more potent NPS-inhibitor could be consider as a structure that combines the best 'sugar moiety' (compounds **9** and **12**) with an appropriate P_1'-residue.

3. Experimental

3.1. Purchased chemicals

SucAlaAlaPhe4NA, a substrate for subtilisins and chymotrypsin-like proteases and 3-(2-furylacryloyl)-glycyl-L-leucine amide (Fagla)—a substrate for thermolysin were from BACHEM Chemicals (Heidelberg, Germany). *N*-(α-rhamno-pyranosylphosphono)-L-leucyl-L-tryptophan Disodium salt (phosphoramidon disodium salt)—a highly specific, naturally occurring thermolysin inhibitor was from Fluka. Thermolysin with specific activity 40 units/mg lyophilizate (37 °C, casein as a substrate) was from Boehringer Mannheim. All other chemicals were of analytical grade.

3.2. The enzyme neutral proteinase from *S. canescens* sp. Novus

NPS was isolated from Bulgarian salt soils[7] using a Sephadex G-100 column (3.5 × 100 cm), equilibrated and eluted with a 10 mM phosphate buffer pH 7.0. The second step of the purification procedure was ion exchange chromatography on a DEAE 52 cellulose column (3.0 × 10 cm), equilibrated with 10 mM phosphate buffer, pH 7.0 and eluted under conditions of a linear gradient (0–0.06 M NaCl) for 10 h and further elution with 0.2 M NaCl. The final step of purification was ion exchange HPLC on a POROS HQ/M 4.6 × 100 mm column (Per Septive Biosystems, Germany) using a linear gradient (0–0.4 M NaCl). The samples, containing NPS, were pooled together, desalted on a Sephadex G-25 column, concentrated on an ultrafiltration membrane and lyophilized.

3.3. Substrate

AntAlaAlaPhe4Na, the best tripeptide 4-nitroanilide substrate for subtilisins with possibility for two independent methods of analysis of the enzyme-catalyzed hydrolysis of the arylamide bond was synthesized according to Ref. 13.

3.4. Synthesis of pyranose derivatives

The following pyranose derivatives were synthesized for studies of the inhibitory effect on NPS.

Benzyl α-D-arabinopyranoside (**1**) was prepared from 11.2 g (20 mmol) of benzyl 2,3,4-tri-*O*-benzoyl-α-D-arabinopyranoside, 25 ml of absolute MeOH and 2.5 g (10-mmol) of sodium as described in Ref. 14. Yield 47 g (96.1%), mp 138–139 °C (EtOH), $[\alpha]_D^{24}$ +12 (*c* 1, H_2O); Ref. 15: mp 140–141 °C (EtOH), $[\alpha]_D^{24}$ +12.3 (*c* 1, H_2O).

Benzyl β-L-arabinopyranoside (**2**) was prepared from 5 g (33 mmol) L-arabinose and 25 ml freshly distilled benzyl alcohol as described in Ref. 14. Yield 5.2 g (64%), mp 170–172 °C (EtOH/H_2O), $[\alpha]_D^{24}$ +215 (*c* 0.2, H_2O); Ref. 16: mp 168–171 °C (EtOH/H_2O), $[\alpha]_D^{24}$ + 206 (*c* 0.3, H_2O).

Benzyl 2-*O*-*p*-tosyl-β-L-arabinopyranoside (**3**) was prepared from 107 g (24 mmol) of benzyl 3,4-*O*-isopropylidene-2-*O*-*p*-tosyl-β-L-arabinopyranoside and 100 ml of 90% aqueous acetic acid, as described in Ref. 14. Yield 8.7 g (91%), mp 119–120 °C (MeOH/H_2O), $[\alpha]_D^{24}$ +130 (*c* 1, $CHCl_3$); Ref. 17: mp 118–120 °C (MeOH/H_2O), $[\alpha]_D^{24}$ +134 (*c* 1, $CHCl_3$).

1,2,3,4-Tetra-*O*-acetyl-β-D-glucopyranose (**4**) was purchased from Sigma Aldrich Chemie GmbH, Germany.

2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride (**5**) was prepared from 22.1 g (0.1 mol) of 2-acetamido-2-deoxy-D-glucose, 100 ml of acetic anhydride and the mixture was saturated with hydrogen chloride (0 °C) and kept (25 °C, three days) as described in Ref. 15. Yield 25.6 g (70%), mp = 120–123 °C; $[\alpha]_D^{24}$ +100.7 (*c* 0.9, CH_2Cl_2); Ref. 18: mp 126–127 °C.

2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-1-thio-β-D-glucopyranoside phenyl (*R*,*S*)-sulfoxide (**6**) was prepared from phenyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-1-thio-β-D-glucopyranoside and *meta*-chloroperbenzoic acid in dichloromethane. Yield 1.94 g (85%); mp = 156–158 °C, $[\alpha]_D^{24}$ +18.13 (*c* = 0.23, CH_2Cl_2).

Benzyl 2,3-anhydro-α-D-lyxopyranoside (**7**) was prepared from 3.75 g (10.5 mmol) of benzyl 2,3-anhydro-4-*O*-triflyl-β-L-ribopyranoside and 10 mmol of tetrabutyl-ammonium nitrate in DMF as described in Ref. 16. Yield 1.68 g (72%), mp 65 °C (EE /PE), $[\alpha]_D^{24}$ +60 (*c* 1, $CHCl_3$); Ref. 19: mp 65–66 °C (PE), $[\alpha]_D^{24}$ +60.1 (*c* 1, $CHCl_3$).

Benzyl 3,4-dideoxy-α-D-glycero-pent-3-enopyranoside (**8**) was synthesized from 2.48 g (10 mmol) of benzyl 3,4-dideoxy-2-*O*-acetyl-α-D-glyceropent-3-enopyranoside. Yield 1.82 g (90%), mp = 85 °C, $[\alpha]_D^{24}$ +141.5 (*c* 0.21, CH_2Cl_2).

Benzyl 3,4-dideoxy-3,4-epithio-α-D-arabinopyranoside (**9**) was synthesized from 0.177 g (0.5 mmol) of benzyl 4-*O*-triflyl-2,3-anhydro-β-L-ribopyranoside and 0.38 g (10 mmol) of lithium aluminium hydride in 25 ml EE as described in Ref. 20. Yield 0.113 g (95%), mp 101–103 °C (EE/PE), $[\alpha]_D^{24}$ + 49.9 (*c* 0.4, $CHCl_3$).

Benzyl 3,4-didesoxy-3,4-epithio-β-L-arabinopyranoside (**10**) was synthesized from 0.177 g (0.5 mmol) of benzyl 4-*O*-triflyl-2,3-anhydro-α-D-ribopyranoside as described in Ref. 20. Yield 0.109 g (92%), mp 92–95 °C (EE/PE), $[\alpha]_D^{24}$ +130.0 (*c* 0.1, $CHCl_3$).

Benzyl 2,3-anhydro-α-D-ribopyranoside (**11**) was prepared from 51.46 g (0.13 mol) of benzyl 2-*O*-*p*-tosyl-α-D-arabinopyranoside, 950 ml of absolute methanol and 3.6 g (0.15 mol) of sodium as described in Ref. 11. Yield 20.88 g (72%), mp 96–97 °C (EE/PE), $[\alpha]_D^{24}$ +190 (*c* 1, EE); Ref. 21: mp 94–96 °C (PE), $[\alpha]_D^{24}$ +202 (*c* 1, EtOAc).

Benzyl 2,3-anhydro-β-L-lyxopyranoside (**12**) was prepared from 3.75 g (10.5 mmol) of benzyl 4-*O*-triflyl-2,3-anhydro-α-D-ribopyranoside and 10 mmol of tetrabutyl-ammonium nitrate in DMF as described in Ref. 13. Yield 1.6 g (68%), mp 82–83 °C (EE/PE), $[\alpha]_D^{24}$ +68 (*c* 1, $CHCl_3$); Ref. 22: mp 83–85 °C (PE), $[\alpha]_D^{24}$ +68.6 (*c* 1, $CHCl_3$).

3.5. Kinetic studies

The enzyme-catalyzed (NPS and thermolysin) hydrolysis of the substrates AntAAF4NA and SucAAF4NA was studied in 50 mM phosphate buffer pH 7.0, 50 mM $CaCl_2$, 4% DMF at 25 °C. The bond cleaved was identified by HPLC analysis of aliquots, withdrawn from the reaction mixture at indicated time intervals, under the following conditions: WATERS equipment, RP-8/5 μm cartridge, elution with a gradient from 3.5% B to 60% B over 38 min at a flow rate of 1 ml/min. Solvent A was 0.1% trifluoroacetic acid in water and solvent B was 0.075% trifluoroacetic acid in acetonitrile. Elution was monitored at 220 nm and peak areas were determined by integration.

The arylamide bond cleavage was monitored at 410 nm ($\varepsilon = 9400\ M^{-1}\ cm^{-1}$) with a Shimadzu UV-3000 spectrophotometer. The ε value was determined by own experiment, considering the effect of the DMF present. The substrate concentration range was 50–600 μM. Thermolysin-catalyzed hydrolysis of peptide bond Ala-Phe in the substrate structure AntAAF4NA was followed

by a Perkin–Elmer spectrofluorimeter. The fluorescence pseudo-first order traces at 420 nm (excitation at 340 nm), due to the released AntAlaAlaOH were used to derive the kinetic parameter k_{cat}/K_m. The substrate concentration was kept low (1–10 μM) to avoid the problems caused by the 'inner filter effect'. The fluorescence changes were transformed into product concentration changes using a calibration curve plotting fluorescence versus concentration of the corresponding fluorophore solution. Thermolysin-catalyzed hydrolysis of peptide bond Ala-Phe in the substrate SucAAF4NA was followed by HPLC analysis of the withdrawn samples from the reaction mixture at indicated time intervals. The kinetic parameters were derived from initial rates (10% or less of substrate hydrolysis) using non-linear regression data analysis computer programme Enzfitter.[23] Thermolysin-catalyzed hydrolysis of Fagla was studied in Ref. 24. Traces at 345 nm under the conditions of pseudo-first order ($[S] \ll K_m$) have been recorded. The substrate concentration was kept low because of the high background of the substrate, so only the value of the specific constant k_{cat}/K_m could be estimated. The inhibition of thermolysin by phosphoramidon was studied in Ref. 25 using the formalism of Henderson[26] in a model system with Fagla as substrate. This approach is valuable for the case of tight binding inhibitors ($[I] \sim [E]$). The inhibition of NPS with phosphoramidon and also with the new synthesized for this purpose different pyranose derivatives, was studied in a model system with SucAAF4NA as substrate (concn range 50–700 μM), concentration range of the inhibitors (50–100 μM). The K_i values were derived using Dixon plot ($1/v_i$ vs [I]).[27] In all kinetic experiments SD evaluated was less than 10%.

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