Bioorganic & Medicinal Chemistry 17 (2009) 2920–2924

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Selectivity profiling of DegP substrates and inhibitors

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ARTICLE INFO

Article history:
Received 28 October 2008
Revised 21 January 2009
Accepted 25 January 2009
Available online 5 February 2009

Keywords:
HtrA protease
DegP
Allosteric regulation
Substrate
Inhibitor

ABSTRACT

Protein quality control factors are involved in many key physiological processes and severe human diseases that are based on misfolding or amyloid formation. Prokaryotic representatives are often virulence factors of pathogenic bacteria. Therefore, protein quality control factors represent a novel class of drug targets. The bacterial serine protease DegP, belonging to the widely conserved family of HtrA proteases, exhibits unusual structural and functional plasticity that could be exploited by small molecule modulators. However, only one weak synthetic peptide substrate and no inhibitors are available to date. We report the identification of a potent heptameric *p*NA-substrate and chloromethyl ketone based inhibitors of DegP. In addition, specificity profiling resulted in the identification of one strong inhibitor and a potent substrate for subtilisin as well as a number of specific elastase substrates and inhibitors.

1. Introduction

DegP is a key protein quality control factor of the bacterial unfolded protein response of the cell envelope and functions as an essential virulence factor of several pathogenic bacteria.[1,2] The unfolded protein response is believed to counteract the protein folding stress caused by the host's immune system attacking the bacterial invader. Consequently, inhibition of DegP might represent an alternative strategy for the development of novel antibiotics.[3] To date, the rational design of small molecule inhibitors failed due to the regulatory and mechanistic complexity as well as the unusual plasticity of the active site, illustrating that further systematic studies with small molecules probing DegP are required.

DegP belongs to the HtrA proteins,[4] an ubiquitously conserved family of serine proteases and is best characterized in *Escherichia coli*. The monomer consists of a protease domain resembling chymotrypsin and two C-terminal PDZ domains. PDZ domains are protein modules that mediate specific protein–protein interactions by binding preferentially the C-terminal 3 or 4 residues of target proteins. Recent studies revealed that DegP is a highly dynamic protein, assembling into distinct, multimeric particles that represent the resting[5] and active states.[6] Its proteolytic activity is regulated in at least two complementary modes. First, DegP functions in a temperature dependent manner.[7] At low temperatures, it acts as a molecular chaperone, while at elevated temperatures DegP switches into a protease, exhibiting a profound proteolytic activity at 37 °C. Second, DegP is allosterically regulated.[8,9] Upon binding the C-termini of hydrophobic peptides or proteins to the PDZ1 domain, the proteolytic activity of DegP is activated or amplified. This mechanism involves dramatic changes in the oligomeric states that is a change from the hexameric resting state into the proteolytically active 12 and 24-meric forms.[6] Importantly, the 'chemical' allosteric regulation displays the higher priority, overriding the temperature control. Recent structural studies revealed that DegP is also able to perform the antagonistic activities of protein repair and degradation in a single molecule. While in the large 12 and 24 mers, folded monomers of mislocalised outer membrane proteins are protected from degradation, unfolded substrates are at the same time efficiently degraded.[6]

In addition to its striking structural properties, DegP features an unusual substrate binding and processing mode. In contrast to classical serine proteases, DegP consists of an bipartite degradation system, in which the PDZ1 and protease domains collaborate during peptide cleavage.[10] Substrates are first recognized at their C-terminal end via binding to the PDZ1 domain, resulting in allosteric activation of DegP. After fixation at PDZ1, the N-terminal substrate segment then extends into an active site of a protease domain, where it is preferentially cleaved after valine, alanine, threonine

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doi:10.1016/j.bmc.2009.01.073

or isoleucine residues. This reaction produces a further binding site for a PDZ domain that holds the product of the first reaction for a second cycle of processing. This processive mechanism produces products of rather defined sizes.[10,11] These unusual and unique peculiarities in combination with its interesting biological relevance turn DegP into a highly interesting protease for probing its function with small molecules.

We have recently reported a first *para*-nitroaniline (*p*NA)-based substrate (**1**) for DegP,[8] which was also used in later mechanistic studies of substrate degradation.[10] The sequence of the substrate was derived from digestion experiments with native DegP substrates. However, this substrate displays only weak affinities and turnover rates, which can be explained by the substrate binding mode, in which a C-terminal binding to the PDZ1 domain is required for efficient substrate hydrolysis. However, the availability of a functional *p*NA-based substrate proves that besides the usual bipartite also other substrate binding modes are possible that could be exploited with small molecules. We therefore started a comprehensive investigation to discover further *p*NA-substrates 'violating' the DegP bipartite binding mode. Furthermore, we pursued the synthesis of a first series of irreversible DegP inhibitors based on the chloromethylketone (CMK) reactive group for co-crystallization and further biochemical experiments.

2. Results

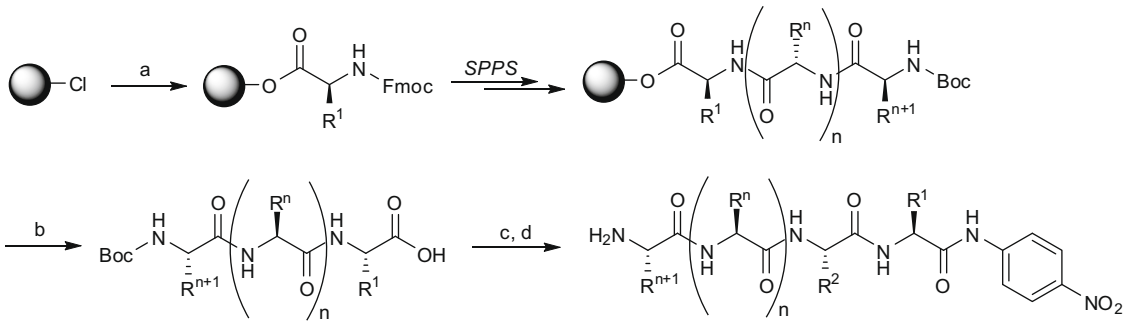
2.1. Identification of peptide sequences for the design of DegP substrates and inhibitors

To identify suitable peptide sequences for the design of substrates or inhibitors for *E. coli* DegP, complete digests of substrates of HtrA proteases were carried out. The following proteins were used for digestion experiments: alkaline phosphatase A (PhoA) and Sigma-E factor negative regulatory protein (RseA) from *E. coli*, porcine citrate synthase (cs) and human amyloid β-A4 protein (Aβ). Subsequent analysis of cleavage products by LC–MS usually resulted in numerous fragments, most of which featured alanine or valine as C-terminal residues corresponding to the P1 site. However, no further definite conserved sequence motifs could be detected, confirming the low cleavage specificity of DegP. In analogy to previous reports,[10] the majority of identified fragments had a length of 9–20 amino acids.

2.2. Chemical synthesis of potential DegP substrates

Due to their robust and broad applicability in biochemical assays, we decided to synthesize chromogenic substrates based on the *para*-nitroaniline motif performing straight-forward synthesis of *p*NA derivatives of the determined cleavage fragments. It consisted of a combination of Fmoc-based solid phase peptide synthesis and solution phase coupling, following essentially previously described methodologies (Scheme 1 and Table 1).[8,12]

In brief, 2-chlorotrityl chloride resin was loaded with a standard Fmoc-protected amino acid, using DIEA as a base in dry DCM. After capping of the resin with DCM/MeOH/DIEA (17:2:1), solid phase peptide synthesis with HBTU/HOBt as coupling reagents was performed. For all couplings but the N-terminal amino acid for which an N^{α}-Boc-protected amino acid was used, standard Fmoc-amino acid building blocks were employed. This led to fully protected precursor peptides that were cleaved from the solid support by addition of acetic acid/trifluoroethanol/DCM (2:2:6) under preservation of the side residue protecting groups. In a solution phase



Scheme 1. Combined solid phase and solution synthesis of chromogenic DegP substrates on 2-chlorotrityl chloride resin. Reagents and conditions: (a) (i) Fmoc-AA-OH (1.2 equiv), DIEA (4 equiv), DCM, rt, 2 h; (ii) DCM/MeOH/DIEA (17:2:1), rt, 10 min; (b) acetic acid/trifluoroethanol/DCM (2:2:6); (c) H-Ala-*p*NA × HCl or H-Val-*p*NA × HCl (2 equiv), EDC × HCl (1.5 equiv), HOBt (2 equiv), DIEA (3 equiv); rt, 16 h; (d) TFA/H_2O/ethandithiol/triisopropylsilane (94:2.5:2.5:1), rt, 2 h.

Table 1
Cleavage rate of *p*NA-based chromogenic DegP substrates and selectivity evaluation versus a panel of representative proteases

Substrate	Specific activity (nmol mg^{-1} min^{-1})[a]							
	DegP	Elastase	Subtilisin	Plasmin	Trypsin	Chymotrypsin	Thrombin	Kallikrein
SPMFKGV-*p*NA (**1**)	**18**	**7**	**3**	<1	<1	<1	<1	<1
SDAEFRHDSGYEV-*p*NA (**2**)	<1	**8**	**2**	<1	<1	<1	<1	<1
STDGGV-*p*NA (**3**)	<1	**5**	<1	**2**	<1	<1	<1	<1
SGRVVPGYGHA-*p*NA (**4**)	<1	**56**	**2971**	<1	<1	<1	<1	<1
SPLPEGV-*p*NA (**5**)	<1	**49**	**5**	<1	<1	<1	<1	<1
GLATGNVSTAELQDATPA-*p*NA (**6**)	<1	**24**	**1521**	<1	<1	**26**	<1	<1
KGKNSGSGATPV-*p*NA (**7**)	<1	**74**	**34**	<1	<1	**5**	<1	<1
KGASVPGAGLV-*p*NA (**8**)	<1	**6**	<1	<1	<1	<1	<1	<1
PMMGKASPV-*p*NA (**9**)	**1**	**96**	<1	**4**	<1	<1	<1	<1
VFNTLPMMGKASPV-*p*NA (**10**)	**32**	**89**	<1	<1	<1	**8**	<1	<1
PVFNTLPMMGKASPV-*p*NA (**11**)	**18**	**69**	<1	<1	**1**	**3**	<1	<1
SPAKGGEEPLPEGV-*p*NA (**12**)	<1	<1	**8**	<1	<1	<1	<1	<1
Bz-R-*p*NA (**13**)	<1	<1	<1	<1	**758**	<1	**3**	**24**
DPMFKLV-*p*NA (**14**)	**473**	**4**	**1**	<1	<1	<1	<1	<1

[a] Standard deviation was <15%.

coupling step, these peptides were coupled with commercially available H-Ala-*p*NA × HCl or H-Val-*p*NA × HCl to yield fully protected chromogenic substrates which upon addition of a TFA-based cleavage buffer were globally deprotected. Subsequent HPLC purification led to the desired chromogenic substrates **1**-**12** and **14**. Beside these synthesized chromogenic substrates, also the commercial available Bz-Arg-*p*NA (**13**) was employed for later biochemical testing.

2.3. Determination of cleavage rates of *p*NA-substrates and selectivity profiling

The synthesized *p*NA peptides were evaluated for their potential to act as DegP substrates. To obtain initial evidence for their selectivity, they were also tested in a panel of commercially available serine proteases, being elastase, subtilisin, plasmin, trypsin, chymotrypsin, thrombin, and kallikrein.

Following the optimization of assay conditions, all *p*NA-substrates **1**–**13** were tested for cleavage efficiency at a concentration of 0.5 mM (Table 1). As expected, most *p*NA-derivatives did not show any biochemical activity, as predicted from the DegP bipartite binding mode. However, three substrates, that is, **1**, **10** and **11**, with sufficient cleavage specificity were identified, of which substrate **1** with a cleavage rate of 18 nmol mg^{-1} min^{-1} displayed the best specificity versus the profiled proteases while substrate **10** showed the highest turnover with 32 nmol mg^{-1} min^{-1}, albeight with much lower specificity. As substrate **1** was the best short substrate but was cleaved only with low rates, a derivative **14** with improved features was synthesized by adapting its sequence to native citrate synthase. In comparison with **1**, DPMFKLV-*p*NA (**14**) had two modifications. P7 was changed from Ser to Asp and P2 from Gly to Leu. This modification increased DegP activity 26-fold at a substrate concentration of 0.5 mM, the k_m decreased from 2.6 to 0.6 mM, while V_{max} increased from 1.2 μmol min^{-1} to 8.7 μmol min^{-1} and a stronger cooperativity raising from 2.6 to 5.1 was observed. In addition, the specificity of the substrate was increased as the turnover rates by elastase and subtilisin were decreased (selectivity of >100:1) (Table 1).

Interestingly, the panel wide evaluation of all synthesized *p*NA-substrates allowed the identification of several elastase substrates that differed in specificity and activity. For example, the potent *p*NA-substrate **9** was rather specific (selectivity of >20:1). Moreover, substrate **10** also proved as a specific elastase substrate at least when compared to the other 5 mammalian serine proteases under investigation. *p*NA-substrate **5** was also specific when comparing elastase with the other 5 mammalian serine proteases tested but the turnover rate was lower. In addition, peptides **4** and **6** were excellent substrates for subtilisin displaying rates of about 3 and 1.5 μmol mg^{-1} min^{-1}.

2.4. Synthesis of chloromethyl ketone (CMK) based irreversible DegP inhibitors

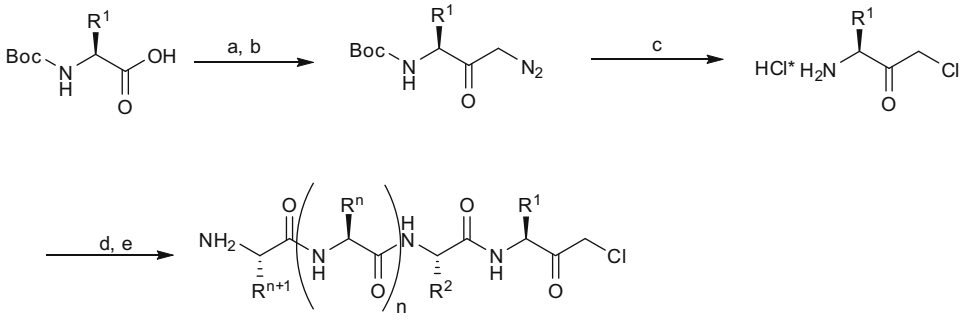
A series of CMK based inhibitors were synthesized some of which might prove useful for biochemical and structural studies. CMK derivatives **15**–**20** were prepared employing a subset of sequences used for the development of chromogenic substrates (Table 2). Compound **15** was derived from **4**; **16** is an extended version of **15**; **17** is a derivative of **1**; **18** of **11**; **19** of **10** and **20** of **9**. Synthesis involved coupling of the fully side chain protected peptides derived from solid phase synthesis with either H-Ala-CMK × HCl or H-Val-CMK × HCl to yield fully protected CMK precursors which upon protecting group cleavage and subsequent HPLC purification yielded the potential DegP chloromethylketone inhibitors **15**–**20**.

H-Ala-CMK and H-Val-CMK were derived from solution synthesis, starting from commercially available Boc-Ala-OH and Boc-Val-OH. Briefly, these amino acid derivatives were activated by the mixed anhydride method using isobutyl chloroformate in THF; followed by addition of freshly prepared diazomethane in diethyl ether. Subsequent addition of hydrogen chloride in dioxane resulted in the generation of the desired chloromethyl ketones as a hydrogen chloride salt (Scheme 2). These intermediates were then

Table 2
Remaining proteolytic activity after inhibition of DegP and selected proteases by 0.5 mM of chloromethyl ketone based inhibitors

Inhibitor	Specific activity (nmol mg^{-1} min^{-1})[a]							
	DegP (%)	Elastase (%)	Subtilisin (%)	Plasmin (%)	Trypsin (%)	Chymotrypsin (%)	Thrombin (%)	Kallikrein (%)
SGRVVPGYGHA-CMK (**15**)	102	83	15	62	285	69	131	135
IWNTLNSGRVVPGTGHA-CMK (**16**)	101	60	15	74	410	78	98	111
SPMFKGV-CMK (**17**)	3	<1	<1	81	354	92	96	101
PVFNTLPMMGKASPV-CMK (**18**)	63	<1	143	62	591	48	101	122
VFNTLPMMGKASPV-CMK (**19**)	57	<1	155	75	287	22	102	88
PMMGKASPV-CMK (**20**)	49	<1	5	71	348	53	122	108

[a] Specific activity was determined using SPMFKGV-*p*NA (DegP), KGKNSGSGATPV-*p*NA (elastase), SGRVVPGYGHA-*p*NA (subtilisin), Bz-R-*p*NA (plasmin, trypsin, thrombin and kallikrein), and GLATGNVSTAELQDATPA-*p*NA (chymotrypsin) as substrates (final concentration 0.5 mM). Standard deviation was <18%.



Scheme 2. Synthesis of chloromethyl ketone inhibitors ($R^1 = CH_3$ or $(CH_3)_2CH$). Reagents and conditions: (a) *i*-butylchloroformate (1.05 equiv), *N*-methylmorpholine (1.05 equiv), THF, −20 °C to 50 °C, 20 min; (b) (i) diazomethane (3 equiv), Et_2O, 0 °C, 2 h; (ii) HOAc (10 equiv), 0 °C, 0.5 h; (c) 4 M HCl in dioxane, dioxane, 0 °C, 2 h; (d) SPPS-derived peptides (0.5 equiv), EDC × HCl (0.75 equiv), HOBt (1 equiv), DIEA (1.5 equiv), DCM, rt, o/n; (e) TFA/H_2O/ethandithiol/triisopropylsilane (94:2.5:2.5:1), rt, 2 h.

coupled with the fully protected peptides derived from solid phase synthesis.

2.5. Evaluation of the CMK inhibitors

The synthesized CMK derivatives were evaluated in two steps. First, the residual proteolytic activity of several serine proteases after pre-incubation with a 0.5 mM solution of CMKs **15**–**20** was measured (Table 2). Of all serine proteases employed during this pre-screening, only three proteases, namely elastase, subtilisin and DegP were completely inhibited by some of the CMKs. Elastase was inhibited by **17**–**20**, subtilisin by **17** and DegP by **17**. Inhibitor **17** was chosen for further characterisation. A titration experiment was carried out using elastase, subtilisin and DegP revealing IC_{50} of 1 nM for subtilisin, 75 μM for DegP and 220 μM for elastase.

3. Discussion

3.1. Substrates

This study identified a short but surprisingly potent DegP substrate, DPMFKLV-*p*NA (**14**), increasing the specific activity of DegP versus the similar substrate **1** by a factor of 26. This unexpected finding suggests that a heptapeptide has the potential to convert DegP from its resting hexameric state into the active and higher oligomeric state. The availability of a short *p*NA-substrate is expected to facilitate the setup of a convenient enzyme assay for future high throughput screens that could be instrumental in identifying small molecule inhibitors of DegP. Such molecules could serve as leads for a novel class of antibiotics targeting a key protein quality control factor that was previously implicated in bacterial pathogenesis.[3]

Neutrophil elastase has an optimal pH of around 8 and the enzyme prefers small hydrophobic residues including Val, Ala and Leu at P1 position. At P2, Pro is preferred, while substrates with charged residues such as Lys, Arg and Asp are less well cleaved. Our data correlate well with these observations as elastase cleaves most substrates tested and the best ones have a Pro residue at P2. As expected, not all substrates carrying a small hydrophobic residue at P1 and a Pro at P2 are equally well processed as elastase has several binding sites for substrate.[13]

Subtilisin has a broad substrate specificity with a preference for aromatic or large nonpolar P1 and P4 substrate residues, even though substrate binding determinants extend over nine amino acids from P6 to P3′.[14] Interestingly, the two *p*NA-substrates with an Ala at P1 were cleaved several hundred fold better compared to the other *p*NA-substrates that had a Val residue at P1.

Chymotrypsin exhibits low specificity with a preference for Phe, Tyr, Trp, Met or Leu at P1. This protease cleaves 4 of the 13 *p*NA-substrates tested with GLATGNVSTAELQDATPA-*p*NA (**6**) being the best substrate. Since kallikrein and thrombin prefer an Arg and plasmin and trypsin a Lys or Arg at P1 position, respectively, the synthesized *p*NA-substrates were not cleaved by these proteases.

3.2. Inhibitors

The CMK derivative **17** was identified inhibiting DegP and should prove useful for future structural studies as to date there is no crystal structure of any HtrA1 protease with bound substrate or peptide inhibitor. Moreover, 4 of the 6 CMK derivatives inhibited elastase at high concentrations. In addition, the screen identified **17** as a potent inhibitor of subtilisin (IC_{50} of 1 nM). Therefore, this limited search for peptide based inhibitors yielded useful tools for further experimental studies. Unexpectedly, most CMK derivatives such as **18** activated trypsin by up to a factor of 6. Even though similar findings were reported previously, the underlying mechanism is not yet understood.[15]

Future directions of research will include to exploit substrate **14** as an inhibitor scaffold using either chlormethyl ketone or boronic acid derivatization. This inhibitor will allow us to address the open question of how substrates bind to HtrA proteases using X-ray crystallography. Such information is expected to be highly relevant for inhibitor optimization, which is required for practical application.

4. Experimental

4.1. General

All solvents and reagents were purchased from Fluka, Aldrich or Acros and were generally of peptide synthesis grade or p.a. quality and were used without further purification. Dry solvents were obtained from Aldrich while Fmoc-amino acids were purchased from IRIS biotech. H-Ala-*p*NA × HCl and H-Val-*p*NA × HCl were obtained from Bachem while 2-chlorotrityl chloride resin was purchased from Novabiochem.

LC-ESI-MS analysis was performed on a LCQ Advantage Max system from Thermo Finnigan, coupled with the Agilent 1200 series analytical HPLC. If not stated explicitly, a standard gradient of 10% acetonitrile in water (0.1% formic acid) to 100% acetonitrile (0.1% formic acid) on a RP-C_{18} column in 10 min was used for all analytical measurements. In certain cases, an alternative gradient (named gradient 2) of 5% acetonitrile in water (0.1% formic acid) to 100% acetonitrile (0.1% formic acid) was employed.

Preparative HPLC purifications were performed on a Varian ProStar HPLC, employing a 5 μ C_{18} reverse phase HPLC column from Macherey-Nagel and a gradient of 5% acetontrile in water (0.1% TFA) to 60% acetonitrile in water (0.1% TFA) in 60 min.

The serine protease DegP was prepared following previously published procedures.[7] Chymotrypsin, trypsin, elastase from porcine pancreas, kallikrein, plasmin, subtilisin, and thrombin were purchased from Sigma.

4.2. Synthesis of chromogenic substrates 1-12 and 14

Synthesis of chromogenic substrates **1**–**12** and **14** was performed on 2-chlorotrityl resin (0.4 g, 0.56 mmol, 100–200 mesh, 1% DVB, maximal loading 1.4 mmol g^{-1}) as a solid support. Loading of the first amino acid was performed with the corresponding amino acid (1.2 equiv) and DIEA (4 equiv) in dry DCM for 2 h, followed by capping with DCM/MeOH/DIEA (17:2:1, 5 mL). Initial loading efficiency as determined by UV absorption was between 0.50 mmol g^{-1} and 0.90 mmol g^{-1}. All reactions on solid support were done in syringe reactors in batches of 0.2 mmol and the amount of resin was adjusted according to the initial loading efficiency. Fmoc cleavage during peptide synthesis was achieved by addition of 20% piperidine in DMF for 20 min and was repeated to drive the reaction to completeness. Further amino acid couplings were performed with Fmoc-amino acids (4 equiv), HOBt (4 equiv), HBTU (3.5 equiv) and DIEA (4 equiv) in DMF and a coupling time of 2 h. Coupling efficiency of each step was verified by a Kaiser test or in case of Fmoc-Pro-OH with a chloranil test. For the last coupling step, N^{α}-Boc-protected amino acids were used. Resin cleavage was achieved with AcOH/TFE/DCM (2:2:6, 5 mL) to yield fully protected peptides which were used for solution phase couplings without any further purification.

Finally, the protected peptides were coupled with H-Val-*p*NA × HCl (2 equiv) or H-Ala-*p*NA × HCl (2 equiv), respectively, and EDC × HCl (1.5 equiv), HOBt (2 equiv) and DIEA (3 equiv) in DCM (15 mL). Some drops of DMF were added to facilitate dissolution of reagents if necessary. The resulting mixture was stirred over night at room temperature, evaporated to dryness and the residue was taken up with DCM (150 mL). The organic phase was extracted with brine (50 mL), dried over Na_2SO_4 and evaporated to dryness.

Cleavage of remaining protecting groups was achieved by addition of a mixture of TFA/H_2O/EDT/TIS (94:2.5:2.5:1, 6 mL) for 2 h, followed by precipitation of crude product by addition of cold diethylether. HPLC purification on a C_{18} reverse phase column with a gradient of 5–10% acetonitrile in water (0.1% TFA) to 60% acetonitrile in water (0.1% TFA) in 60 min yielded the final chromogenic substrates.

SPMFKGV-*p*NA (**1**): Yield = 130 mg (73%). LC–MS (ESI): t_R 5.93 min, *m/z* 885.93 $[M+H]^+$, 1770.33 $[2M+H]^+$, 885.43 calcd for $C_{41}H_{60}N_{10}O_{10}S^+$.

SDAEFRHDSGYEV-*p*NA (**2**): Yield = 138 mg (42%). LC–MS (ESI): t_R 5.43 min, *m/z* 1632.00 $[M+H]^+$, 817.00 $[M+2H]^{2+}$, 1631.67 calcd for $C_{70}H_{94}N_{20}O_{26}^+$.

STDGGV-*p*NA (**3**): Yield = 106 mg (82%). LC–MS (ESI): t_R 5.57 min, *m/z* 655.53 $[M+H]^+$, 1309.60 $[2M+H]^+$, 655.27 calcd for $C_{26}H_{38}N_8O_{12}^+$.

SGRVVPGYGHA-*p*NA (**4**): Yield = 146 mg (60%). LC–MS (ESI): t_R 4.73 min, *m/z* 1220.00 $[M+H]^+$, 611.00 $[M+2H]^{2+}$, 1219.60 calcd for $C_{54}H_{78}N_{18}O_{15}^+$.

SPLPEGV-*p*NA (**5**): Yield = 107 mg (66%). LC–MS (ESI): t_R 5.90 min, *m/z* 818.60 $[M+H]^+$, 1635.73 $[2M+H]^+$, 818.41 calcd for $C_{37}H_{55}N_9O_{12}^+$.

GLATGNVSTAELQDATPA-*p*NA (**6**): Yield = 167 mg (45%). LC–MS (ESI): t_R 5.79 min, *m/z* 1836.87 $[M+H]^+$, 919.00 $[M+2H]^{2+}$, 1835.88 calcd for $C_{77}H_{122}N_{22}O_{30}^+$.

KGKNSGSGATPV-*p*NA (**7**): Yield = 97 mg (40%). LC–MS (ESI): t_R 4.89 min, *m/z* 1222.87 $[M+H]^+$, 612.07 $[M+2H]^{2+}$, 1222.62 calcd for $C_{51}H_{83}N_{17}O_{18}^+$.

KGASVPGAGLV-*p*NA (**8**): Yield = 103 mg (48%). LC–MS (ESI): t_R 5.75 min, *m/z* 1075.87 $[M+H]^+$, 1075.59 calcd for $C_{48}H_{78}N_{14}O_{14}^+$.

PMMGKASPV-*p*NA (**9**): Yield = 76 mg (37%). LC–MS (ESI): t_R 5.59 min, *m/z* 1037.41 $[M+H]^+$, 519.42 $[M+2H]^{2+}$, 1037.49 calcd for $C_{45}H_{72}N_{12}O_{12}S_2^+$.

VFNTLPMMGKASPV-*p*NA (**10**): Yield = 87 mg (27%). LC–MS (ESI): t_R 6.06 min, *m/z* 1611.80 $[M+H]^+$, 1633.93 $[M+Na]^+$, 806.53 $[M+2H]^{2+}$, 1611.80 calcd for $C_{73}H_{114}N_{18}O_{19}S_2^+$.

PVFNTLPMMGKASPV-*p*NA (**11**): Yield = 104 mg (30%). LC–MS (ESI): t_R 6.19 min, *m/z* 1708.60 $[M+H]^+$, 855.33 $[M+2H]^{2+}$, 1708.86 calcd for $C_{78}H_{121}N_{19}O_{20}S_2^+$.

SPAKGGEEPLPEGV-*p*NA (**12**): Yield = 160 mg (54%). LC–MS (ESI): t_R 5.61 min, *m/z* 1487.67 $[M+H]^+$, 744.47 $[M+2H]^{2+}$, 1486.72 calcd for $C_{65}H_{99}N_{17}O_{23}^+$.

DPMFKLV-*p*NA (**14**): Yield = 158 mg (82%). LC–MS (ESI): t_R 6.76 min, *m/z* 969.80 $[M+H]^+$, 1938.73 $[2M+H]^+$, 969.49 calcd for $C_{46}H_{68}N_{10}O_{11}S^+$.

4.3. Synthesis of chloromethyl ketone inhibitors 15–20

The valine chloromethyl ketone building block was freshly prepared, adapting a previously published method.[16] Boc-Val-OH (15.2 g, 70 mmol) was activated with *iso*-butylchloroformate (9.53 mL, 73.5 mmol, 1.05 equiv) and *N*-methlymorpholine (8.08 mL, 73.5 mmol, 1.05 equiv) in dry THF (100 mL) at −20 °C under an argon atmosphere for 20 min and was then allowed to warm up to 5 °C. Addition of freshly prepared diazomethane (8.83 g, 200 mmol, 3 equiv) in diethylether (200 mL), followed by careful addition of acetic acid to destroy excess diazomethane after 5 h yielded after evaporation of the solvents Boc-Val-CH_2N_2 as a yellow oil. This intermediate was then redissolved in dry dioxane (50 mL) and cooled to 0 °C. 4 M HCl in dioxane (2 mL) was slowly added and the resulting mixture was stirred for 2 h. Addition of cold diethylether led to precipitation of H-Val-CMK × HCl (5.27 g, 28.3 mmol, 40% yield) which was used without further purification in the next coupling step.

The chloromethly ketone peptides (**15**–**20**) were then generated in an analogues manner to the synthesis of the peptide substrates (**1**–**12** and **14**) using instead of H-AA-*p*NA × HCl the freshly synthesized H-AA-CMK × HCl building block.

SGRVVPGYGHA-CMK (**15**): Yield = 84 mg (37%). LC–MS (ESI): t_R 8.09 min (gradient 2), *m/z* 1131.60 $[M+H]^+$, 566.75 $[M+2H]^{2+}$, 1131.55 calcd for $C_{49}H_{75}ClN_{16}O_{13}^+$.

IWNTLNSGRVVPGTGHA-CMK (**16**): Yield = 94 mg (25%). LC–MS (ESI): t_R 4.78 min, *m/z* 1875.07 $[M+H]^+$, 937.73 $[M+2H]^{2+}$, 1872.93 calcd for $C_{84}H_{126}ClN_{25}O_{22}^+$.

SPMFKGV-CMK (**17**): Yield = 46 mg (29%). LC–MS (ESI): t_R 5.38 min, *m/z* 797.40 $[M+H]^+$, 1594.33 $[2M+H]^+$, 797.38 calcd for $C_{36}H_{57}ClN_8O_8S^+$.

PVFNTLPMMGKASPV-CMK (**18**): Yield = 54 mg (17%). LC–MS (ESI): t_R 6.21 min, *m/z* 1620.63 $[M+H]^+$, 811.25 $[M+2H]^{2+}$, 1620.81 calcd for $C_{73}H_{118}ClN_{17}O_{18}S_2^+$.

VFNTLPMMGKASPV-CMK (**19**): Yield = 61 mg (20%). LC–MS (ESI): t_R 6.21 min, *m/z* 1523.57 $[M+H]^+$, 762.66 $[M+2H]^{2+}$, 1523.75 calcd for $C_{68}H_{111}ClN_{16}O_{17}S_2^+$.

PMMGKASPV-CMK (**20**): Yield = 63 mg (34%). LC–MS (ESI): t_R 5.47 min, *m/z* 949.36 $[M+H]^+$, 475.46 $[M+2H]^{2+}$, 949.44 calcd for $C_{40}H_{69}ClN_{10}O_{10}S_2^+$.

4.4. Enzyme assay conditions

After optimizing assay conditions, the following buffers were used for measuring the proteolytic activities: DegP: 50 mM NaH_2PO_4, pH 8.0; chymotrypsin and trypsin: 80 mM Tris, 20 mM $CaCl_2$, pH 8.0; elastase: 100 mM Tris, pH 8.0; kallikrein: 20 mM Tris, 100 mM NaCl, pH 7.8; plasmin: 50 mM NaH_2PO_4, 150 mM NaCl, pH 8.5; subtilisin: 50 mM K_2HPO_4, pH 7.5; thrombin: 50 mM NH_4HCO_3, pH 8.0.

4.5. Determination of cleavage efficiency of chromogenic substrates 1–14

For determination of the cleavage rate, 5 μg of the corresponding protease was dissolved in 100 μL buffer. Chromogenic substrates (**1**–**14**) were added to a final concentration of 0.5 mM and substrate cleavage was monitored at 405 nm for 60 min. The determined specific activities (sa) are derived from at least three independent duplicate measurements and calculated from $sa = \Delta OD_{405} \times V/(m \times \varepsilon \times t)$ (ΔOD_{405}: change of absorption at $\lambda = 405$ nm over 60 min; *V*: final volume of reaction, m: amount of protease (mg), ε: molar extinction coefficient of *para*-nitroaniline).

References and notes

1. Hasenbein, S.; Merdanovic, M.; Ehrmann, M. *Genes Dev.* **2007**, *21*, 6.
2. (a) Lipinska, B.; Fayet, O.; Baird, L.; Georgopoulos, C. *J. Bacteriol.* **1989**, *171*, 1574; (b) Strauch, K. L.; Johnson, K.; Beckwith, J. *J. Bacteriol.* **1989**, *171*, 2689.
3. Raivio, T. L. *Mol. Microbiol.* **2005**, *56*, 1119.
4. (a) Clausen, T.; Southan, C.; Ehrmann, M. *Mol. Cell* **2002**, *10*, 443; (b) Pallen, M. J.; Wren, B. W. *Mol. Microbiol.* **1997**, *26*, 209.
5. Krojer, T.; Garrido-Franco, M.; Huber, R.; Ehrmann, M.; Clausen, T. *Nature* **2002**, *416*, 455.
6. Krojer, T.; Sawa, J.; Schäfer, E.; Saibil, H. R.; Ehrmann, M.; Clausen, T. *Nature* **2008**, *453*, 885.
7. Spiess, C.; Beil, A.; Ehrmann, M. *Cell* **1999**, *97*, 339.
8. Meltzer, M.; Hasenbein, S.; Hauske, P.; Kucz, N.; Merdanovic, M.; Grau, S.; Beil, A.; Jones, D.; Krojer, T.; Clausen, T.; Ehrmann, M.; Kaiser, M. *Angew. Chem., Int. Ed.* **2008**, *47*, 1332.
9. Hauske, P.; Ottmann, C.; Meltzer, M.; Ehrmann, M.; Kaiser, M. *Chembiochem* **2008**, *9*, 2920.
10. Krojer, T.; Pangerl, K.; Kurt, J.; Sawa, J.; Stingl, C.; Mechtler, K.; Huber, R.; Ehrmann, M.; Clausen, T. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 7702.
11. Kolmar, H.; Waller, P. R. H.; Sauer, R. T. *J. Bacteriol.* **1996**, *178*, 5925.
12. Tonello, F.; Ascenzi, P.; Montecucco, C. *J. Biol. Chem.* **2003**, *278*, 40075.
13. Korkmaz, B.; Moreau, T.; Gauthier, F. *Biochimie* **2008**, *90*, 227.
14. Perona, J. J.; Craik, C. S. *Protein Sci.* **1995**, *4*, 337.
15. Radau, G.; Rauh, D. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 779.
16. Kato, D.; Boatright, K. M.; Berger, A. B.; Nazif, T.; Blum, G.; Ryan, C.; Chehade, K. A. H.; Salvesen, G. S.; Bogyo, M. *Nat. Chem. Biol.* **2005**, *1*, 33.