Bioorganic & Medicinal Chemistry Letters 23 (2013) 1412–1415

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Phosphonic analogues of glutamic acid as irreversible inhibitors of *Staphylococcus aureus* endoproteinase GluC: An efficient synthesis and inhibition of the human IgG degradation

Ewa Burchacka, Marcin Skoreński, Marcin Sieńczyk, Józef Oleksyszyn *

Division of Medicinal Chemistry and Microbiology, Faculty of Chemistry, Wrocław University of Technology, Wybrzeże Wyspiańskiego 27, 50-370 Wrocław, Poland

ARTICLE INFO

Article history:
Received 17 October 2012
Revised 17 December 2012
Accepted 21 December 2012
Available online 5 January 2013

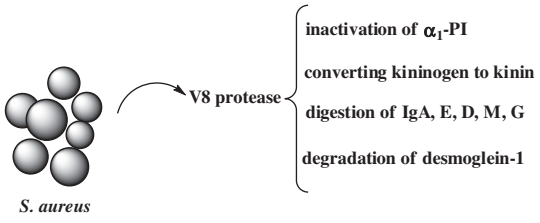
Keywords:
V8 protease
Staphylococcus aureus
α-Aminoalkylphosphonates
Serine protease inhibitors

ABSTRACT

Endoproteinase GluC (V8 protease) is one of many virulence factors released by the *Staphylococcus aureus* species in vivo. The V8 protease is able to hydrolyze some serpins and all classes of mammalian immunoglobulins. The application of specific and potent inhibitors of V8 protease may lead to the development of new antibacterial agents. Herein, we present the synthesis and the inhibitory properties of novel peptidyl derivatives of a phosphonic glutamic acid analogue. One of the compounds Boc-Phe-Leu-Glu-$^{P}(OC_6H_4)_2$ displayed an apparent second-order inhibition rate value of 8540 M^{-1} s^{-1}. The Boc-Phe-Leu-$Glu^{P}(OC_6H_4)_2$ compound with the highest inhibitory potency showed the ability to prevent V8-mediated human IgG proteolysis in vitro.

Extracellular bacterial proteases belong to the group of secreted virulence factors which are important for effective pathogenesis. Proteolysis, for which bacterial proteases are responsible, targets different elements of extracellular structures in infected organisms where fibronectin, collagen, elastin are just a few examples.[1–3] The presence of the active proteases allows circumvention of host defense mechanisms and modulation of the immune system during infection and inflammation by bacteria.[4,5] Moreover, most of the proteases released by bacteria are not only susceptible to inactivation by endogenous inhibitors present in plasma, but also can relatively quickly inactivate serpins, including α_1-PI, antithrombin III, and α_1-microglobulin.[2,6] Several bacterial species produce proteases (V8, 56K protease) capable of immunoglobulin degradation which in turn facilitates the progression of the infection and enhances the pathogenesis of infectious diseases.[2,7,8]

Endoproteinase GluC (also called V8 protease) from *Staphylococcus aureus* is one of the proteases that play an important role in evading host immune defense mechanisms (Scheme 1). V8 protease (EC 3.4.21.19) is a member of the glutamyl endopeptidase I family of the *Staphylococcus aureus* V8 strain (GluV8).[9,10] It specifically cleaves the peptide and ester bonds on the carboxylic side of Glu and Asp residues where the rate of Glu residue hydrolysis is approximately 100–1000 times more effective than hydrolysis of Asp residue.



Scheme 1. The role of V8 protease during infections.

Staphylococcus aureus contributes to globally important infections such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome (TSS), chest pain, bacteremia, and sepsis.[11] It has been shown that the activity of GluV8 protease increases the growth and survival of *S. aureus* in animal models.[12] Additionally, this enzyme inactivates α_1-Proteinase Inhibitor (α_1-PI) which protects tissues from inflammatory mediators and controls the activity of host proteases such as neutrophil elastase, trypsin and plasminogen activator.[6] Moreover, V8 protease converts kininogen to kinin which leads to blood vessel dilation and increases vascular permeability.[13] In vitro, *staphylococcal* glutamyl endopeptidase degrades human immunoglobulin A, IgG, IgM, IgE and IgD which results in immune system dysfunction.[7,8] GluV8 contributes to skin blistering disease via degradation of the desmoglein 1 protein.[14] Development of potent and specific V8 protease inhibitors may lead to potential therapeutics targeting *Staphylococcus aureus* infections.

* Corresponding author. Tel.: +48 71 320 4027; fax: +48 71 320 2427.
E-mail address: jozef.oleksyszyn@pwr.wroc.pl (J. Oleksyszyn).

0960-894X/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmcl.2012.12.074

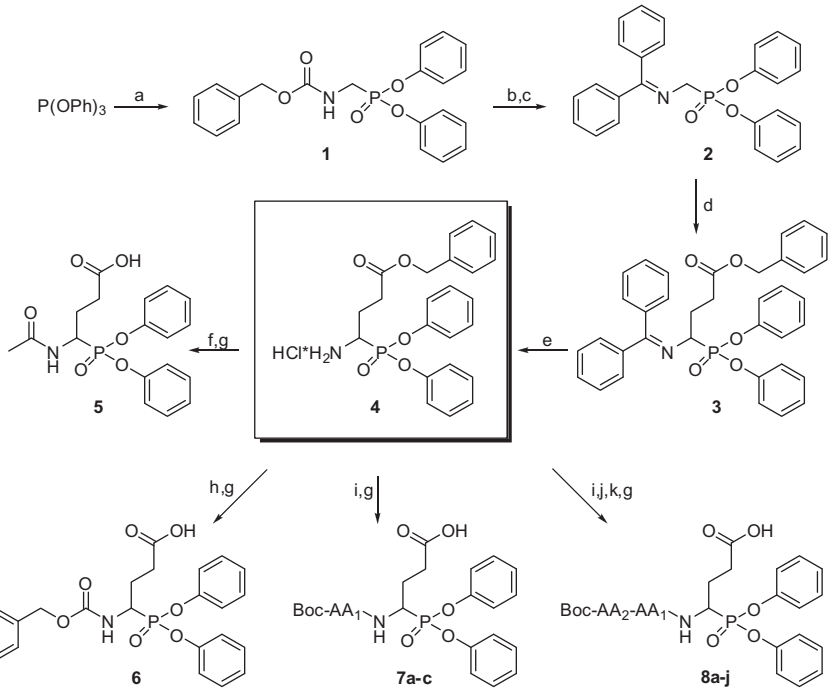
In this Letter we present the synthesis and biological evaluation of phosphonic di- and tripeptides towards V8 *Staphylococcus aureus* protease. The α-aminoalkylphosphonate diaryl esters and their peptidyl derivatives are known selective, potent and specific inhibitors which bind to the active site of serine proteases in an irreversible manner.[15–17]

The first Letter showing the application of aminophosphonates as V8 protease inhibitors was presented by Hamilton et al.[18] Simple acetylated phosphonic analogues of glutamic and aspartic acid displayed second-order inhibition rate values of 5.3×10^3 and $5.0 \times 10^2\ M^{-1}\ s^{-1}$ for Ac-Glu^P(OC$_6$H$_5$)$_2$ and Ac-Asp^P(OC$_6$H$_5$)$_2$, respectively. However, the inhibitory activity of peptidyl derivatives of phosphonic glutamic and aspartic acid against V8 protease has not yet been presented. The preferred substrate sequence of *Staphylococcus aureus* V8 protease is Phe/Leu/Ala-Leu-Asp/Glu (P3–P2–P1, Schechter nomenclature).[19,20] Based on the substrate recognition pattern we synthesized a series of di- and tripeptidyl α-aminophosphonate diaryl ester derivatives of phosphonic glutamic acid with various P2 and P3 residues in order to determine their influence on the inhibitory activity towards V8 and selectivity over different proteases produced by *S. aureus*, for example, the SplB protease. The SplB protease sequence reveals a high homology to the *S. aureus* V8 protease and epidermolytic toxins.[8–10] Introducing the particular amino acid residue into the structure of phosphonic peptide derivatives allowed a comparison of their inhibitory activity towards both serine proteases produced by *Staphylococcus aureus*. Studies have shown that V8 protease has a narrow substrate specificity (S1 binding pocket accommodated only/exclusively by Asp and Glu) whereas SplB protease mainly prefers Asp, Asn and Gln residues at P1.[10] Interestingly, investigations of SplB protease substrate mapping revealed that the glutamic acid side chain was not accepted at the P1 position. In this Letter we present the selectivity of novel peptidyl phosphonate inhibitors—analogues of glutamic acid—towards *staphylococcal* SplB and V8 protease.

For the synthesis of compound **4**, which was further used for the peptide derivative synthesis, we applied the procedure described by Hamilton et al. with modification in the synthesis of intermediate **2** (Scheme 1).[18] Briefly, an α-amidoalkylation reaction of triaryl phosphite with paraformaldehyde and benzyl carbamate led to Cbz-Gly^P(OC$_6$H$_5$)$_2$ (**1**) in which the protective group was removed using 33% hydrobromic acid solution in acetic acid. The resulting hydrobromide salt of H-Gly^P(OC$_6$H$_5$)$_2$ was condensed with benzophenone imine to obtain compound **2**.

Alkylation of compound **2** with benzyl-3-bromopropanoate and KHMDS gave compound **3** which, after treatment with 1 M HCl in diethyl ether solution, gave hydrochloride salt of H-Glu(OBzl)-^{P}(OC$_6$H$_5$)$_2$ **4**, a starting compound used for peptide derivative synthesis. The synthesis of Cbz-Glu^P(OPh)$_2$ (**6**), which was used as the reference inhibitor in our studies, started with the deprotection of a benzyl group by hydrogenolysis over 10% palladium on carbon leading to intermediate H-Glu^P(OC$_6$H$_5$)$_2$. To avoid phenyl ester group hydrolysis under basic conditions associated with the application of benzyl chloroformate (Cbz-Cl) as observed previously,[18] the introduction of a Cbz-protective group was achieved using *N*-(benzyloxycarbonyloxy)succinimide (CbzOSu) in the presence of *N*,*N*′-diisopropylethylamine (DIPEA) in acetonitrile which led to Cbz-Glu^P(OC$_6$H$_5$)$_2$ (**6**). In addition, we synthesized Ac-Glu^P(OC$_6$H$_5$)$_2$ (**5**) which was used as the control inhibitor (under the conditions of our assay the measured k_2/K_i value of Ac-Glu^P(OC$_6$H$_5$)$_2$ was identical to the value published by Hamilton group[18]).

The general synthetic procedure for generation of di- and tripeptidyl derivatives of α-aminoalkylphosphonate diphenyl esters, analogues of glutamic acid, is outlined in Scheme 1. Briefly, compound **4** was first coupled with *N*-Boc-protected amino acid using HBTU as the coupling agent in the presence of triethylamine, and the benzyl group in the resulting dipeptidyl derivatives was removed under standard conditions (H_2, 10% Pd/C) leading to the derivatives **7a–c**. Elongation of the peptidyl chain was achieved in a similar fashion—after removal of the *N*-Boc-protective group



Scheme 2. General synthesis of phosphonic glutamic acid derivatives. Reagents and conditions: (a) benzyl carbamate, acetic anhydride, paraformaldehyde, AcOH, 60–70 °C, 3 h; (b) 33% HBr/AcOH, rt, 2 h; (c) benzophenone imine, CH_2Cl_2, rt, 24 h; (d) KHMDS, benzyl-3-bromopropanoate, THF, −78 °C, 3.5 h; (e) 1 M HCl in Et_2O, rt, 24 h; (f) AcOH, HBTU, Et_3N, MeCN, rt, 12 h; (g) 10% Pd/C, H_2, MeOH, rt, 6 h; (h) CbzOSu, MeCN, DIPEA, rt, 48 h; (i) *N*-Boc-L-AA$_1$-OH, HBTU, Et_3N, MeCN, rt, 12 h; (j) 50% TFA/DCM (v/v), rt, 2 h; (k) *N*-Boc-L-AA$_2$-OH, HBTU, Et_3N, MeCN, rt, 12 h.

Table 1
The inhibition constant values of α-aminoalkylphosphonate diphenyl ester derivatives towards V8 and SplB proteases

No	Compound	V8 protease		SplB protease	
		K_i (μM)	k_2/K_i (M^{-1} s^{-1})	K_i (μM)	k_2/K_i (M^{-1} s^{-1})
5	Ac-Glu^P$(OC_6H_5)_2$	11.07 ± 1.2	5300	NI	–
6	Cbz-Glu^P$(OC_6H_5)_2$	8.1 ± 0.95	1384	NI	–
7a	Boc-Leu-Glu^P$(OC_6H_5)_2$	7.8 ± 1.2	792	NI	–
7b	Boc-Val-Glu^P$(OC_6H_5)_2$	23.3 ± 5.5	102	NI	–
7c	Boc-Ala-Glu^P$(OC_6H_5)_2$	39%[a]	nd	NI	–
8a	Boc-Phe-Leu-Glu^P$(OC_6H_5)_2$	**6.2 ± 0.98**	**8540**	NI	–
8b	Boc-Asp-Leu-Glu^P$(OC_6H_5)_2$	41%[a]	Nd	NI	–
8c	Boc-Glu-Leu-Glu^P$(OC_6H_5)_2$	9.5 ± 1.1	314	NI	–
8d	Boc-Phe-Ala-Glu^P$(OC_6H_5)_2$	9.4 ± 0.99	1708	NI	–
8e	Boc-Asp-Ala-Glu^P$(OC_6H_5)_2$	26%[a]	nd	NI	–
8f	Boc-Glu-Ala-Glu^P$(OC_6H_5)_2$	12.9 ± 0.19	238	NI	–
8g	Boc-Phe-Val-Glu^P$(OC_6H_5)_2$	8.15	5012	NI	–
8h	Boc-Asp-Val-Glu^P$(OC_6H_5)_2$	50%[a]	nd	NI	–
8i	Boc-Glu-Val-Glu^P$(OC_6H_5)_2$	9.95 ± 0.82	1002	NI	–
9	Boc-Glu-Leu-**Gln**^{P}$(OC_6H_5)_2$[a]	NI	–	4.2 ± 0.4[b]	500[b]

Bold values represent the most active compound in the presented studies.
[a] Percentage of inhibition observed after 30 min of measurement at a 200 μM concentration of the tested compound.
[b] Compound previously reported;[24] nd—not determined; NI—0% of inhibition observed after 30 min of measurement at a 200 μM concentration of the tested compound.

in dipeptidyl derivatives using 50% trifluoroacetic acid in dichloromethane, *N*-Boc-protected amino acid was coupled using the HBTU/Et_3N system. Finally, tripeptides **8a–j** were obtained by the hydrogenolysis of their benzyl-protected parent compounds over Pd/C in methanol (see Scheme 2).

All of the synthesized derivatives (**5**, **6**, **7a–c**, **8a–j**) were evaluated for their inhibitory activity against V8 and SplB proteases. The obtained inhibition kinetic data (K_i and k_2/K_i values) towards V8 and SplB proteases are summarized in Table 1. The observed rate of inhibition constants was determined by the progress curve method.[21] All measurements were performed using a Spectra Max Gemini XPS spectrofluorometer (Molecular Devices, USA) at 37 °C. The kinetics of V8 and SplB protease inhibition was determined by addition of the enzyme into the solution of substrate and tested inhibitor ($[E]_0 \ll [I]_0$) according to the following mechanism:

$$E + I \overset{K_i}{\rightleftarrows} EI \overset{k_2}{\rightarrow} Ei \quad (1)$$

where K_i is the reversible enzyme–inhibitor complex (EI) dissociation constant, and k_2 is the rate of an irreversible complex (Ei) formation.

The rates of V8 protease inhibition (75 nM, Sigma–Aldrich) and SplB protease (100 nM, expressed and purified as previously described [22]) were measured in 0.1 M Tris–HCl, 0.05% Tween 20 (pH 7.8) at 37 °C and 0.1 M Tris–HCl, 0.01% Triton X-100 buffer (pH 7.6) at 37 °C, respectively. Both proteases were assayed using fluorogenic substrates: Ac-Phe-Leu-Glu-ACC (15 μM, V8 protease, Ex. 355 nm, Em. 460 nm), and Ac-Trp-Glu-Leu-Gln-ACC (10 μM, SplB protease, Ex. 355 nm, Em. 460 nm). The calculated Michaelis constant (K_M) values for the substrate of V8 and SplB protease were 116 μM and 135 μM, respectively. All the compounds were screened for inhibitory activity against V8 and SplB protease at a concentration of 200 μM. For compounds for which less than 50% of inhibition was observed after 30 min of kinetic measurement, we expressed the percentage of inhibition (Table 1).

The kinetic investigation of the inhibitory potency towards V8 protease showed that the most active structure of phosphonic glutamic acid derivative was peptide **8a** (Boc-Phe-Leu-Glu^P$(OC_6H_5)_2$, $k_2/K_i = 8.5 \times 10^3$ M^{-1} s^{-1}) which was almost twice as active against V8 protease than control inhibitor Ac-Glu^P$(OC_6H_5)_2$ (**5**, $k_2/K_i = 5.3 \times 10^3$ M^{-1} s^{-1}). Among the obtained phosphonic dipeptides the highest activity was observed for compound **7a** which displayed a k_2/K_i value of 792 M^{-1} s^{-1}. Replacing Leu residue with Val at P2 position decreased the inhibitory activity by about 8-times (**7b**, $k_2/K_i = 102$ M^{-1} s^{-1}). For dipeptide Boc-Ala-Glu-^{P}$(OC_6H_5)_2$ (**7c**) only 39% of V8 inhibition was observed at 200 μM.

Among tripeptidyl derivatives of phosphonic glutamic acid the highest potency of action was observed for Boc-Phe-Leu-Glu-^{P}$(OC_6H_5)_2$ (**8a**) which displayed a k_2/K_i value of 8540 M^{-1} s^{-1}. Replacing Phe residue at P3 position with glutamic acid (**8c**) decreased the inhibitory activity by approximately 27-times ($k_2/K_i = 315$ M^{-1} s^{-1}). Interestingly, for derivative **8b**, which contains an aspartic acid residue at position P3, only 41% of V8 protease inhibition was observed. Additionally, replacing Leu residue at P2 position with Ala (**8d**) decreased the inhibitory activity by 5-times ($k_2/K_i = 1708$ M^{-1} s^{-1}). The obtained results indicate the importance of P3 and P1 residues in the inhibitor sequence. The highest inhibitory activity was observed for compounds containing Phe-X-Glu^P motif—hydrophobic residue occupying the S3 binding pocket and acidic side chain of glutamate interacting with the protease S1 pocket. Decreasing the length of the P2 residue aliphatic side chain decreases the inhibitor ability for V8 protease inactivation (Ala < Val < Leu). Importantly, all of the synthesized analogues of glutamic acids presented in this study were obtained as diastereoisomeric mixtures. Since only one diastereoisomer reacts with the protease active site, the true inhibitory potency should be at least twofold greater than the potency of the diastereoisomer mixture.[23]

The reason for introducing a particular amino acid residue into the structure of phosphonic peptide inhibitors was the generation of selective inhibitors which do not react with *Staphylococcus aureus* SplB protease, which specifically recognizes Gln at P1 position. Replacing phosphonic glutamine by phosphonic glutamic acid at P1 position made these compounds completely inactive against SplB protease leading to an absolute selectivity of action against V8 protease.

The influence of compound **8a** on human IgG degradation by V8 protease was evaluated using the following method. First, V8 protease (at final 1 μM) was pre-incubated for 1 h at 37 °C with **8a** (at final 100 μM) before addition of human IgG (at final 160 μg/ml, Sigma–Aldrich). The mixture was then incubated for 24 h at 37 °C. The control sample was prepared similarly with the exception that DMSO was added instead of the inhibitor solution. Control V8 protease and human IgG solutions were incubated under

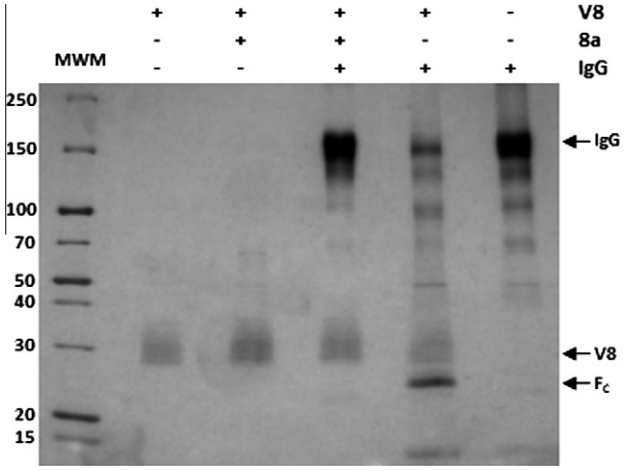


Figure 1. Inhibition of V8-mediated hydrolysis of human IgG: molecular weight marker (MWM, Lane 1); Control V8 and IgG samples (Lanes 2 and 6, respectively); degradation of IgG by V8 protease (Lane 5); the inhibitory effect of **8a** on IgG degradation (Lane 4).

the same conditions. All samples were quenched by the addition of non-reducing gel loading buffer and analyzed using SDS–PAGE (4–12%). Visualization of the bands was performed via the silver staining method (Fig. 1).

The results clearly demonstrated that compound **8a** prevents V8-mediated IgG proteolysis. IgG incubated with V8 protease in the absence of inhibitor undergoes proteolysis leading to formation of a 30 kDa band which corresponds to the F_C domain (monomer) of IgG (Fig. 1, Lane 5). These observations are in agreement with previously reported results.[8] Addition of **8a** efficiently prevents IgG degradation due to the V8 inhibition (Fig. 1, Lane 4).

In order to examine the antibacterial activity of **8a** the growth inhibition assay was performed. The results indicated that synthesized V8 inhibitor had no influence on *Staphylococcus aureus* growth under the conditions of our assay in which gentamicin displayed MIC value of 2 mg/L. The obtained data was not surprising since the activity of V8 protease is localized outside the cell. To establish the true potency of **8a** its activity needs to be examined in vivo and will be the subject of further investigations.

In summary, the most potent among all synthesized peptidyl derivatives of phosphonic glutamic acid was Boc-Phe-Leu-Glu-$^{P}(OC_6H_5)_2$ (**8a**, $k_2/K_i = 8540\ M^{-1}\ s^{-1}$) being the most active phosphonic V8 inhibitor published to date. Moreover, the results demonstrated that compound **8a** prevents V8-mediated IgG degradation in vitro. The presented preliminary data on new and selective inhibitors of V8 protease and it relationship to SplB protease activity could help to establish its role in vivo during *staphylococcal* infections, especially its influence on immune system dysfunction.

Acknowledgments

This work was supported by Grants N N401 596340 (to J.O.) from the Polish Ministry of Science and Higher Education. M.S. is thankful to Wroclaw University of Technology Statute Funds (S10156/0313).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.12.074.

References and notes

1. Bejrano, P. A.; Langeveld, J. P.; Hudson, B. G.; Noelken, M. E. *Infect. Immun.* **1989**, *57*, 3783.
2. Molla, A.; Matsumoto, K.; Oyamada, J.; Katsuki, T.; Maeda, H. *Infect. Immun.* **1986**, *53*, 522.
3. Sandholm, L.; Tolo, K. *J. Clin. Periodontol.* **1986**, *13*, 646.
4. Travis, J.; Potempa, J.; Maeda, H. *Ternds Microbiol.* **1995**, *3*, 1.
5. Potempa, J.; Travis, J. In *Handbook of Experimental Pharmacology: Proteases as Targets for Chemotherapy*; von der Helm, K., Korant, B. D., Eds.; Springer-Verlag: Berlin, 2000; pp 118–159.
6. Potempa, J.; Watorek, W.; Travis, J. *J. Biol. Chem.* **1986**, *261*, 14330.
7. Arvidson, S. *Microbiology* **2000**, *8*, 379.
8. Ryan, M. H.; Petrone, D.; Nemeth, J. F.; Barnathan, E.; Bjorck, L.; Jordan, R. E. *Mol. Immunol.* **1837**, *2008*, 45.
9. Drapeau, G. R.; Boily, Y.; Houmard, J. *J. Biol. Chem.* **1972**, *247*, 6720.
10. Stennicke, H. R.; Breddam, K. In *Handbook of Proteolytic Enzymes: Glutamyl Endopeptidase*; Barrett, A. J., Rawlings, N. D., Woessner, F. F., Eds.; Academic Press: San Diego, 1998; pp 243–246.
11. Michaell, R. D.; Roberts Baum *Clin. Microbiol. Rev.* **2010**, *3*, 616.
12. Karlsson, A.; Saravia-Otten, P.; Tegmark, K.; Morfeldt, E.; Arvidson, S. *Infect. Immun.* **2001**, *69*, 4742.
13. Maeda, H.; Yamamoto, T. *Biol. Chem.* **1996**, *377*, 217.
14. Hirasava, Y.; Taki, T.; Nakamura, T.; Mitsuishi, K.; Gunavan, H.; Suto, H. *J. Invest. Dermatol.* **2008**, *128*, 906.
15. Oleksyszyn, J.; Powers, J. C. *Biochem. Biophys. Res. Commun.* **1989**, *161*, 143.
16. Oleksyszyn, J.; Powers, J. C. *Biochemistry* **1991**, *30*, 485.
17. Oleksyszyn, J.; Powers, J. C. In *Methods in Enzymology*; Abelson, J. N., Simon, M. J., Barrett, A. J., Eds.; Academic Press: London, 1994; Vol. 244, pp 423–441.
18. Hamilton, R.; Walker, B.; Walker, J. B. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1655.
19. Kakudo, S.; Kikuchi, N.; Kitadokoro, K.; Fujiwara, T.; Nakamura, E.; Okamoto, H.; Shin, M.; Tamaki, M.; Teraoka, H.; Tsuzuki, H. *J. Biol. Chem.* **1992**, *267*, 23782.
20. Abramowitz, N.; Schechter, J.; Berger, A. *Biochem. Biophys. Res. Commun.* **1967**, *29*, 862.
21. Knight, C. G. In *Proteinase Inibitors*; Barett, A. J., Salvesen, G., Eds.; Elsevier: Amsterdam, 1986; pp 23–51.
22. Dubin, G.; Stec-Niemczyk, J.; Kisielewska, M.; Pustelny, K.; Popowicz, G.; Bista, M.; Kantyka, T.; Boulware, K.; Stennicke, H. R.; Czarny, A.; Phopaisarn, M.; Doughtry, P. S.; Thogerson, I. B.; Enghlid, J. J.; Thornberry, N.; Dubin, A.; Potempa, J. *J. Mol. Biol.* **2008**, *379*, 343.
23. Winiarski, L.; Oleksyszyn, J.; Sienczyk, M. *J. Med. Chem.* **2012**, *55*, 6541.
24. Burchacka, E.; Walczak, M.; Sieńczyk, M.; Oleksyszyn, J. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 5574.