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The development of first *Staphylococcus aureus* SplB protease inhibitors: Phosphonic analogues of glutamine

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ABSTRACT

Produced by *Staphylococcus aureus*, SplB belongs to the chymotrypsin-like serine protease family. Since the biological role of SplB protease is unknown, the design and application of its specific inhibitors may help to reveal the function of this enzyme. Until now no SplB inhibitors have been reported. Herein, we present the design and synthesis of novel α-aminophosphonic analogues of glutamine, as well as their peptidyl derivatives. The inhibitory effects of these compounds towards the newly discovered SplB serine protease from *S. aureus* are characterized. We have also investigated the influence of aromatic ester substituents on inhibitory potency towards SplB. One of the compounds—Cbz-Glu-Leu-Gln^{P}(OC_6H_4-4-*O*-CH_3)$_2$—displayed an apparent second-order inhibition rate value of 1400 M^{-1} s^{-1}.

Staphylococcus aureus is a Gram-positive spherical bacterium that forms microscopic clusters resembling grapes.[1] This pathogen is characterized by a worldwide spread and is responsible for many types of diseases including pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome (TSS), bacteremia, and sepsis.[2] At the same time, *S. aureus* asymptomatically colonizes the skin and mucous membranes of more than 30% of the human population, as well as skin and mucous membranes of animals.[3] Due to the increasing number of antibiotic-resistant *Staphylococcus aureus* strains, new strategies to treat staphylococcal infections need to be developed. One possible approach focuses on the application of synthetic inhibitors that block key bacterial proteases and may thus possibly overcome *S. aureus* infection.

Currently, genomes of several dozen different *Staphylococcus aureus* strains are available in publically accessible databases.[4] Analysis of this data demonstrates that some of the mobile genetic elements, including the pathogenicity islands (PAIs), encode several enterotoxins and other virulence factors. Among them a cluster of up to six genes organized into one operon encoding serine protease-like (Spl) proteins has been discovered.[5,6] The sequences of Spl proteins reveal their high homology to the *S. aureus* V8 protease and epidermolytic toxins.[5,7] This structural similarity to known staphylococcal virulence factors, clustering of the spl genes with genes encoding superantigens, and their location on PAI suggest that Spl proteases may contribute to *S. aureus* pathogenicity.[8] The first report on Spl proteins was provided by Reineck et al.[9] They discovered a high level of antibodies directed against the SplC protein produced by *S. aureus* in patients with endocarditis. Although the existence of Spl proteases was demonstrated more than a decade ago, their biological function is still unclear.

The SplB protease belongs to a chymotrypsin-like serine protease family.[7,8,10] The crystal structure of SplB protease reveals the presence of the Ser157, His39 and Asp77 catalytic triad in the active site of the protein.[8] Substrate mapping of SplB specificity using positional scanning synthetic combinatorial libraries revealed a narrow P1 residue selectivity towards Asp, Asn or Gln accommodated by the protease S1 binding pocket.[7] Interestingly, the side chain of the glutamic acid residue was not accepted at this position. Application of the CLiPS (cellular library of peptide substrates) method revealed Trp-Glu-Leu-Gln (P4-P1) as the optimal sequence recognized by the SplB protease.[10]

Since the role of the SplB protease for bacterial growth and the progression of infection is unknown, the application of specific and potent inhibitors may help to discover its function in vivo. Moreover, SplB inhibitors will allow the detailed characterization of the protease binding site (specificity determinants) by solving the crystal structure of the enzyme-inhibitor complex. Such information may be further used to determine potential natural substrates of SplB in the host organism.

An effective SplB inhibitor should be highly specific and stable under experimental conditions. One of the choices are the peptidyl

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derivatives of α-aminophosphonate diaryl esters. They are highly specific towards the target serine protease and do not react with acetylcholinesterase, cysteine proteases, or threonine proteases of proteasome, and do not inhibit metallo and aspartyl proteases.[11–13] The design of such inactivators of serine proteases is straightforward—the replacement of the C-terminal amino acid of the substrate of best fit by a structurally corresponding diaryl aminophosphonate analogue leads to generation of a potent inhibitor.[13,14] The best substrate is the one with the highest k_{cat}/K_M second order hydrolysis rate of the peptide bond, where the K_m value reflects only initial noncovalent binding of the substrate in the ground state. The initial step of inhibitor binding, the formation of a noncovalent reversible complex, is very likely similar to the binding of the substrate. In the next step, an irreversible reaction at the active site catalytic serine side chain nucleophile occurs. One of the aryl ester groups leaves the phosphorus phosphonate atom through nucleophilic substitution with a formation of the trigonal bipyramid transition state, in which the leaving group occupies the apical position in relation to the attacking serine hydroxylate. The resulting mixed diester undergoes hydrolysis (in a process called 'complex aging') and a final tetrahedral monoester (phosphonate-*O*-serine) complex is formed. Such a complex resembles the transition state tetrahedral intermediate observed during peptide bond hydrolysis by serine proteases. Therefore, a complex of phosphonate-based inhibitors with serine protease is extremely stable, with a half-life of hydrolysis in the range of a few hours to a few days.[12,13] The second order inhibition rate constant is the function of the initial noncovalent binding (K_i) and the rate of the irreversible step (k_2). Although its relation to K_i is evident and substrate mapping provides sufficient data for the inhibitors design, the influence of k_2 is more complex and depends on phosphorus atom electrophilicity. The K_i of phosphonate type inhibitors are not identical to the substrate K_M due to the fact that they are structurally different. The irreversible step (k_2) represents the energy of activation for the phosphonylation reaction of serine-O^- or stabilization of TS (transition state) for such a reaction—trigonal bipyramid. Such stabilization is provided by interaction with enzyme and by the electrophilicity of the phosphorus atom of the phosphonate molecule. The phosphorus atom of the inhibitor should possess some basic electrophilicity, since phosphonic dialkyl esters are devoid of reactivity with serine proteases.[11–13] The introduction of some electrowithdrawing substituents within the phenyl ester ring, such as NO_2, SO_2CH_3, COOR, significantly increases the electrophilic character of the phosphorus leading to extremely potent irreversible inhibitors of serine proteases.[15,16] However, increased electrophilicity results in decreased stability and higher susceptibility to water hydrolysis, which excludes them from practical application.

Although the influence of the electrowithdrawing character on the increased overall inhibition could be clearly noticed,[15,16] the electrodonating substituents could, in some cases, induce the perfect fitting of the phosphoryl oxygen to the oxyanion hole and increase the electrophilicity of the phosphorous atom by a 'steric' and not 'electronic' effect. In another words, the substituent on the phenyl ester ring could stabilize TS for the phosphonylation reaction and increase k_2/K_i despite its electrodonating character. Distinguishing the effect of each of these two factors is difficult and its understanding requires further studies.

Additionally, when the chemical stability of the 1-aminoalkylphosphonate diaryl esters and their outstanding selectivity of action, together with the stability of enzyme-inhibitor complex, are taken into consideration, it is clear that these compounds represent a perfect tool to study the role of the SplB protease in vivo.

In order to effectively inactivate a target protease, several types of short distance interactions are required that include not only the optimal structural fit of the P1 residue of the inhibitor into the S1 binding pocket, but also extended interactions within the S2 and S3 pockets.[11–13,17]

Herein, we present the first synthetic procedure for the generation of 1-aminophosphonate diaryl esters—phosphonic analogues of glutamine and their peptidyl derivatives; structures which were based on the optimal SplB substrate sequence (Glu-Leu-Gln/P3-P2-P1). In addition to our synthetic approach, we determined the inhibitory activity of these novel compounds against the SplB protease, including simple Cbz-protected phosphonates as well as their peptidyl derivatives.

The rate of SplB protease (100 nM, expressed and purified as previously described[8]) inhibition was measured in 0.1 M Tris–HCl, 0.01% Triton X-100 buffer (pH 7.6) at 37 °C. The inhibition of chymotrypsin (3 nM, Sigma–Aldrich, Poznan, Poland) and subtilisin (5 nM, Sigma–Aldrich, Poznan, Poland) was me assured in 0.1 M HEPES, 0.5 M NaCl, 0.03% Triton X-100 buffer (pH 7.5) at 37 °C. The enzymes were assayed using fluorogenic substrates: Ac-Trp-Glu-Leu-Gln-ACC (10 μM, SplB, Ex. 355 nm, Em. 460 nm) and Suc-Ala-Ala-Pro-Phe-AMC (5 μM, chymotrypsin and subtilisin, Ex. 350 nm, Em. 460, Calbiochem, Merck, Warszawa, Poland). The calculated Michaelis constant (K_M) values for SplB, chymotrypsin and subtilisin were 135 μM, 70 μM, and 60 μM, respectively.

The kinetics of SplB protease inhibition was determined by addition of the enzyme into the solution of the substrate and inhibitor tested ($[E]_0 << [I]_0$) according to the following mechanism:

$$E + I \underset{K_i}{\rightleftharpoons} \mathrm{EI} \xrightarrow{k_2} E_i \tag{1}$$

where K_i is the reversible enzyme-inhibitor complex (EI) dissociation constant, and k_2 is the rate of irreversible complex (E_i) formation. The observed rate of inhibition was determined by the progress curve method using the following (Eq. 2):

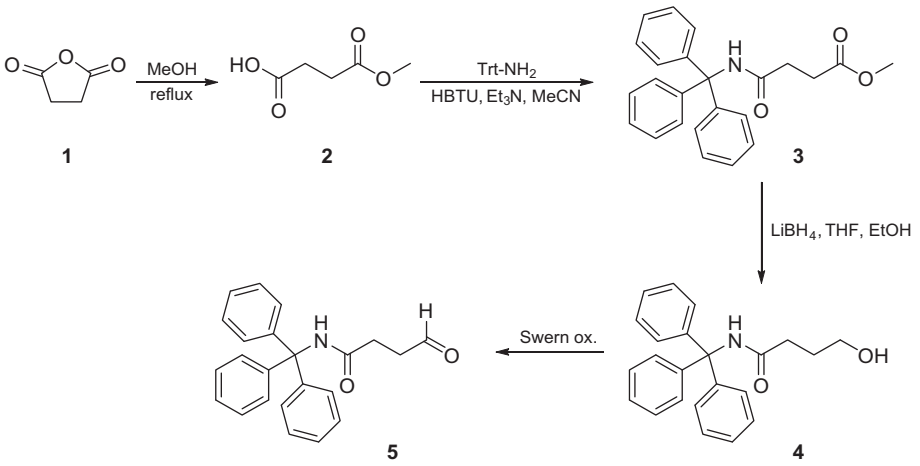
$$\ln([P]_\infty - [P]_t) = \ln[P]_\infty - k_{obs}t \tag{2}$$

where $[P]_\infty$ is the product concentration at the end of the reaction, $[P]_t$ is the product concentration at time t and k_{obs} is a pseudo-first-order rate constant.[18] Control curves in the absence of the inhibitor were linear at the evaluated conditions. For derivatives showing an inhibitory activity of less than 50% at 200 μM concentration we presumed a k_2/K_i value <10 M^{-1} s^{-1}. For inhibitors displaying a minimum 50% activity at 200 μM concentration kinetic parameters (k_{obs}, K_i and k_2/K_i) were examined according to the (Eq. 3)[18]:

$$\frac{k_{obs}}{[I]} = \frac{\frac{k_2}{K_i}}{1 + \frac{[S]}{K_m} + \frac{[I]}{K_i}} \tag{3}$$

All measurements were performed using a Spectra Max Gemini XPS spectrofluorometer (Molecular Devices, USA) at 37 °C.

The synthesis of phosphonic diaryl ester analogues of glutamine (Scheme 1) started with the preparation of a 4-oxo-*N*-tritylbutanamide. Briefly, the succinic anhydride (**1**) was suspended in methanol and was refluxed for 3 h until a single spot appeared on TLC.[19] After the reaction was completed the volatile products were evaporated. The resulting mono-methyl hydrogen succinate (**2**) was used directly in the next step without further purification. Coupling of triphenylmethylamine with mono-methyl hydrogen succinate was performed in acetonitrile using HBTU as the coupling agent in the presence of triethylamine leading to the methyl 4-oxo-4-(tritylamino)butanoate (**3**). The reduction of an ester group of **3** was achieved by the application of lithium borohydride leading to 4-hydroxy-*N*-tritylbutanamide (**4**). Further oxidation of 4-hydroxy-*N*-tritylbutyryloamid was performed under Swern conditions leading to 4-oxo-*N*-tritylbutanamide (**5**). Compound **5** was used as the starting material for the synthesis of all 1-aminoalkylphosphonate diaryl esters presented in this study.



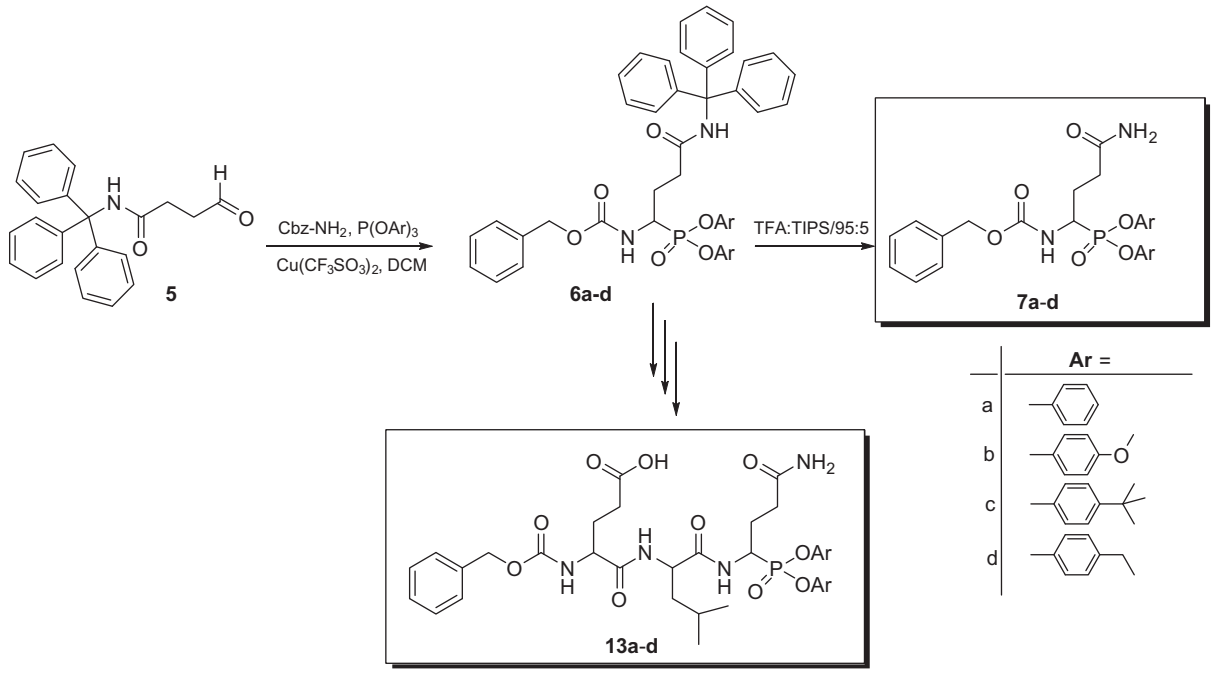
Scheme 1. General strategy of 4-oxo-*N*-tritylbutanamide synthesis.

N-benzyloxycarbonyl α-aminoalkylphosphonate diaryl esters were obtained as racemic mixtures of diastereoisomers in the α-amidoalkylation reaction of triaryl phosphite with benzyl carbamate and 4-oxo-*N*-tritylbutanamide in dry dichloromethane using cooper triflate as the catalyst (Scheme 2).[20] The Trt-protective group in resulting compounds was removed using a mixture of trifluoroacetic acid and triisopropylsilane (TIPS) (95:5, v/v).

The synthesis of target phosphonic glutamine peptidyl derivatives started with the removal of the benzyloxycarbonyl protective group of Cbz-(Trt)Gln^{P}(OAr)$_2$ by hydrogenolysis on Pd/C. The amino acid coupling steps were performed in acetonitrile using HBTU as the coupling agent in the presence of triethylamine. The obtained derivatives were purified on Silica Gel. In order to evaluate the inhibitory properties of the obtained dipeptides their Trt group was first removed by TFA:TIPS (95:5, v/v) solution and the resulting compounds were purified by HPLC prior to SplB inhibition studies.

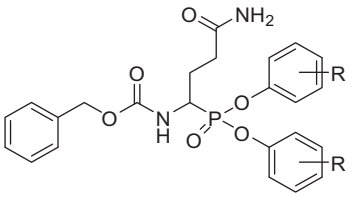
Several previous studies have demonstrated that the amino acid sequence of the most potent peptidyl inhibitor usually corresponds to the sequence of the best substrates.[12,15,21] Therefore, the structure of obtained SplB inhibitors was based on the protease substrate recognition pattern.[7]

We began our search for potential SplB inhibitors by screening the collection of different diaryl esters of phosphonic analogues of amino acids, including Ala^{P}, Leu^{P}, and Phe^{P}. None of the tested compounds influenced the activity of SplB at a concentration below 200 μM. Moreover, tripeptide Cbz-Glu-Leu-Glu^{P}(OPh)$_2$, which differs from the optimal SplB substrate only at the P1 residue, was also completely inactive against SplB [Skorenski, M.; unpublished results]. These observations are in agreement with the absolute substrate specificity observed for the SplB protease. Additionally, none of the synthesized simple Cbz-protected phosphonic analogues of glutamine (**7a**–**d**) displayed any inhibitory activity against SplB protease at concentrations below 200 μM. As expected, **7a–d** were also inactive against chymotrypsin. Weak subtilisin inhibition was observed for derivative **7b** ($k_2/K_i = 33\ M^{-1}\ s^{-1}$). The lack of inhibitory activity against SplB observed for simple Cbz-protected derivatives may be the consequence of weak hydrogen bond network formation between the protease and the inhibitor (Table 1).



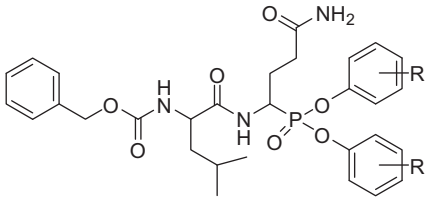
Scheme 2. Synthesis of analogues of phosphonic glutamine.

Table 1
Activity of phosphonic analogues of glutamine (Cbz-Gln^{P}(OAr)$_2$) against SplB, chymotrypsin and subtilisin



Compound	R	SplB		Chymotrypsin		Subtilisin	
		K_i (μM)	k_2/K_i (M^{-1} s^{-1})	K_i (μM)	k_2/K_i (M^{-1} s^{-1})	K_i (μM)	k_2/K_i (M^{-1} s^{-1})
7a	H	>200	<10	>200	<10	>200	<10
7b	4-*O*-Methyl	>200	<10	>200	<10	111 ± 3.2	33
7c	4-*t*-Butyl	>200	<10	>200	<10	>200	<10
7d	4-Ethyl	>200	<10	>200	<10	>200	<10

Table 2
Activity of dipeptidyl derivatives of phosphonic glutamine against SplB, chymotrypsin and subtilisin



Compound	R	SplB		Chymotrypsin		Subtilisin	
		K_i (μM)	k_2/K_i (M^{-1} s^{-1})	K_i (μM)	k_2/K_i (M^{-1} s^{-1})	K_i (μM)	k_2/K_i (M^{-1} s^{-1})
10a	H	>200	<10	>200	<10	>200	<10
10b	4-*O*-Methyl	>200	<10	>200	<10	35 ± 1.5	57
10c	4-*t*-Butyl	>200	<10	>200	<10	>200	<10
10d	4-Ethyl	>200	<10	>200	<10	>200	<10

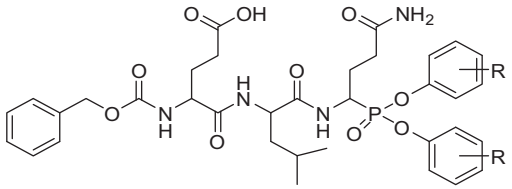
We have further tested the corresponding dipeptides (**10a–d**) but these also have not displayed inhibitory activity against SplB at concentrations lower than 200 μM. This observation is in close agreement with previously reported data showing that the Cbz-protected dipeptidyl α-aminoalkylphosphonate diaryl esters display poor inhibitory potency against serine proteases in comparison to simple Cbz-protected phosphonates.[12,13,22] For example, N-terminally Boc-protected dipeptidyl derivatives of α-aminoalkylphosphonate diaryl esters showed only moderate activity against subtilisin.[16] A similar observation is made in the current study, as **10b** displayed only weak inhibition of subtilisin with a k_2/K_i value of 57 M^{-1} s^{-1} (insignificant increase over **7b)** (Table 2).

Activity is significantly increased in phosphonic tripeptides (**13a–d**) compared to the amino acid analogue (**7a–d**) and dipeptide (**10a–d**) series. This is likely explained by the fact that the peptide chain of the inhibitor first directs the molecule in the proper binding conformation and then the active site nucleophile attacks the phosphorus atom of the inhibitor leading to formation of the enzyme-inhibitor complex. The peptidyl phosphonic glutamine derivative Cbz-Glu-Leu-Gln^{P}(OC$_6$H$_4$)$_2$ (**13a**) displayed a k_2/K_i value of 500 M^{-1} s^{-1} toward SplB protease. Introduction of electrodonating groups at the *para* position of the phenyl ester ring such as ethyl (**13d**) or *t*-butyl (**13c**) resulted in decreased inhibitory properties with k_2/K_i values of 180 M^{-1} s^{-1} and 15 M^{-1} s^{-1}, respectively. Despite the fact that isosteric ethyl and methoxy substituents at *para* position are electrodonating, the highest potency towards the SplB protease was observed for Cbz-Glu-Leu-Gln^{P}(OC$_6$H$_4$-4-*O*-CH$_3$)$_2$ (**13b**, k_2/K_i = 1400 M^{-1} s^{-1}) which was the most potent SplB inhibitor found in this study (Table 3). It is possible that some additional interactions may occur during **13b** binding to the enzyme such as hydrogen bond formation with the oxygen atom of the methoxy group leading to increased inhibitory potency.

In terms of inhibitor specificity, all phosphonic tripeptides obtained were completely inactive against chymotrypsin. However, compounds **13b** and **13d** inhibited subtilisin almost three times more potently than they inhibited SplB. An absolute selectivity of action against tested serine proteases was displayed only by compound **13a** which inhibited SplB and not chymotrypsin or subtilisin, however at a cost of potency of SplB inhibition. Compound **13a** showed the highest binding affinity to the SplB protease among all the obtained derivatives (K_i/K_M ratio of 0.031) however **13b**, the most potent inhibitor, displayed higher K_i value of 16 μM and it is highly likely the 4-*O*-methyl substituent provides additional interaction that stabilizes TS of the reaction.

In order to evaluate the role of the Cbz group in the inhibitory properties the activity of H-Glu-Leu-Gln^{P}(OPh)$_2$ and H-Glu-Leu-Gln^{P}(OC$_6$H$_4$-4-CH$_2$CH$_3$)$_2$ towards the SplB protease was measured. Both derivatives were found to be less potent as compared to the Cbz-protected parent compounds **13a** and **13d** displaying the apparent second-order inhibition rate values <10 M^{-1} s^{-1}. This could in part be explained by the formation of some additional bond networks inside the S4 binding pocket with the Cbz group of the inhibitor. These findings are consistent with previous reports showing that peptides devoid of the N-protective function (either Cbz or Boc group) are less potent inhibitors when compared to protected peptides.[23,24]

Table 3
Activity of tripeptidyl derivatives of phosphonic glutamine against SplB, chymotrypsin and subtilisin



Compound	R	SplB		Chymotrypsin		Subtilisin	
		K_i (μM)	k_2/K_i (M^{-1} s^{-1})	K_i (μM)	k_2/K_i (M^{-1} s^{-1})	K_i (μM)	k_2/K_i (M^{-1} s^{-1})
13a	H	4.2 ± 0.4	500	>200	<10	>200	<10
13b	4-*O*-Methyl	16 ± 0.9	1400	>200	<10	8.5 ± 0.7	4100
13c	4-*t*-Butyl	145 ± 3.1	15	>200	<10	49 ± 2.2	48
13d	4-Ethyl	12 ± 1.1	180	>200	<10	113 ± 2.3	440

In conclusion, we have provided the first ever example of the synthesis of phosphonic diaryl ester glutamine analogues and their peptidyl derivatives. Despite the lack of selectivity of the obtained compounds with highest potency between SplB and subtilisin (**13b** and **13d**), a highly specific SplB protease inhibitor was identified (**13a**). As such, our preliminary data opens the door to further optimization and the development of potent SplB protease inhibitors. Moreover, all of the synthesized analogues of glutamine presented in this study were obtained as diastereoisomer mixtures. Since only one diastereoisomer reacts with the protease active site, true inhibitory potency can be at least twofold greater than the potency of the diastereoisomer mixture.[21] It is worth noting that this is the first report on SplB protease inhibitor design ever described in the literature. Additionally, further investigation on obtaining the crystal structure of SplB protease complexed with inhibitor **13d** is currently underway and will be published separately in due course.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.07.011.

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