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Unnatural Amino Acid Side Chains as S1, S1′, and S2′ Probes Yield Cationic Antimicrobial Peptides with Stability toward Chymotryptic Degradation

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This work describes how the systematic incorporation of a range of unnatural amino acid derivatives in the P1, P1′, and P2′ positions allows for the generation of short lactoferricin based cationic antimicrobial peptides with a stability toward chymotryptic degradation. The necessary pharmacophore sets the peptides up for degradation by chymotrypsin, and a heavily truncated native tripeptide was rapidly digested despite its short sequence. Degradation studies indicated that increased half-lives could be obtained by altering the binding to each subsite surrounding the active site without sacrificing the antimicrobial activity. Important structural and mechanistic features were revealed in a fashion not feasible through the use of native peptide substrates. The results, which are generally applicable to a range of relevant peptides, further show that not only the S1 pocket, but also to the notably less studied S1′ site can be used to control the proteolytic stability by incorporating different analogues of tryptophan and arginine.

Introduction

Structural information on the interactions between the enzymes involved in the proteolysis of peptides and their target peptides is important from a fundamental biological viewpoint, but it is also essential in the search for general strategies to advance peptides and peptidomimetics as pharmaceuticals. Chymotrypsin and trypsin are key enzymes involved in protein and peptide degradation in the gastrointestinal (GI) tract, and as such they represent central targets to consider for any orally administered peptide pharmaceutical. They are serine protease endopeptidases with similar three-dimensional structure but with different substrate specificities.[1,2] The specificity of serine proteases is governed by the substrate recognition sites, which include the polypeptide binding site and the binding pockets for the peptide side chains according to the nomenclature shown in Figure 1.[3] The substrate specificity is mainly dictated by the interactions between the S1 binding pocket of the enzyme and selected amino acid side chains. Loop mediated interactions from loops L1 and L2 also play an important role for the substrate specificity.[4,5] Chymotrypsin cleaves C-terminally to large hydrophobic amino acids, while trypsin prefers cationic residues in the S1 subsite.[1,6] Stability toward both these enzymes is paramount for any orally administered drug.[a]

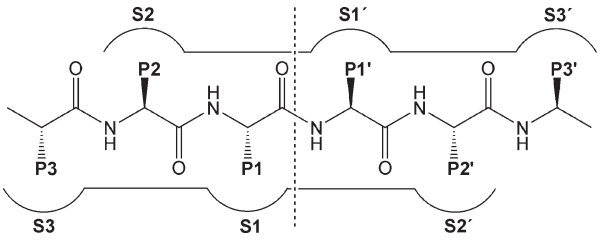

Figure 1. Schematic representation of the nomenclature used for serine proteases. The binding pockets are named S1−Sn and S1′−Sn′, where S1 is the binding site for the peptide residue (P1) on the acyl side of the scissile bond and S1′ is the binding site for the peptide residue (P1′) on the leaving group side of the scissile bond.

In response to the numerous challenges associated with peptide drugs, several strategies have been developed to improve their medicinal properties.[7−9] Methylation of the amide nitrogen atom or inversion of the stereochemistry of the amino acid responsible for substrate specificity in the enzyme are much used methods to inhibit degradation by endopeptidases.[10,11] Constrained cyclic analogues of linear peptides have also been shown to increase the stability toward degradation and the duration of action, as has incorporation of unnatural amino acids at selected positions.[12−14] Replacement of the scissile amide bond with peptidomimetic elements or coadministration of specific enzyme inhibitors are also strategies that have been successfully employed to generate peptides with both increased stability and activity.[15,16]

Antimicrobial peptides constitute a part of the innate immune system in most organisms, and several hundred have been described.[17] They represent attractive targets in the development of potential new drugs in the struggle against microorganisms resistant toward conventional antibiotics[18,19] due

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[a] Abbreviations: CAP, cationic antimicrobial peptide; MIC, minimal inhibitory concentration; ITC, isothermal titration calorimetry; RP-HPLC, reversed phase high performance liquid chromatography; Bip, L-biphenyl alanine; Dip, L-β-phenyl-phenylalanine; App, L-2-amino-3-(4-aminophenyl)propanoic acid; Gpp, L-2-amino-3-(4-guanidinophenyl)propanoic acid; Orn, L-ornithine; Har, L-2-amino-(6-guanidino)-hexanoic acid; Agp, L-2-amino-(3-guanidino)propanoic acid; ATCC, American Type Culture Collection; Bn, benzyl; hPEPT1, human intestinal di- and tripeptide transporter 1.

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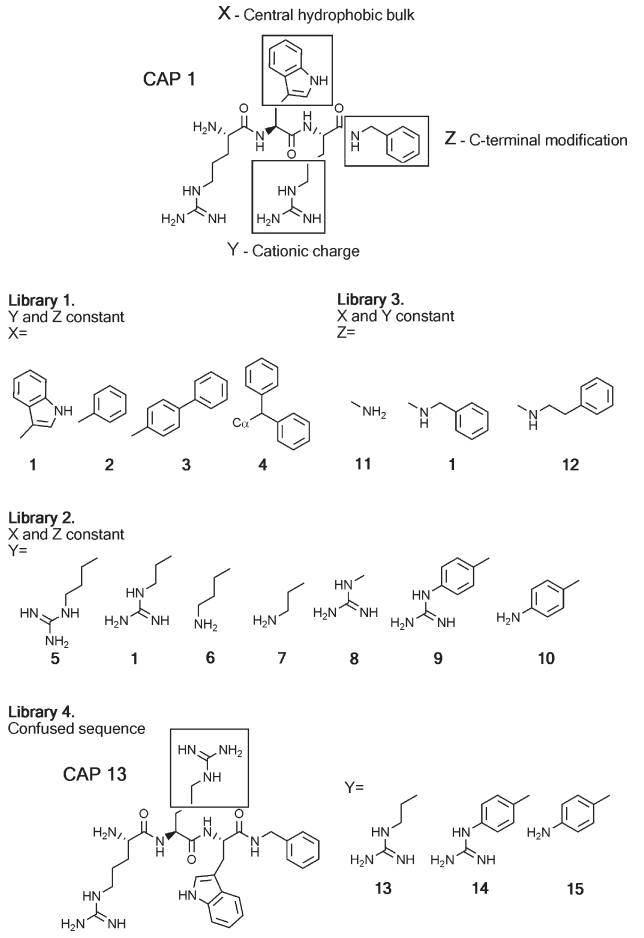

Figure 2. Structure of the four libraries analyzed. Libraries 1−3 investigate the role of hydrophobic bulk, cationic charge, and C-terminal capping (*X*, *Y*, *Z* kept constant according to CAP **1**). Library 4, here exemplified by CAP **13**, contains peptides with confused sequences (variation in *Y*).

to their general mechanism of action on the bacterial membrane, which is little likely to promote resistance. Short cationic antimicrobial peptides (CAPs), based on truncated analogues of bovine lactoferricin, have been developed by our group in an effort to generate promising potent antibiotic drugs with a low susceptibility for bacterial resistance development.[20] Currently, the definite mechanism of this particular class of peptides is unclear. It has been shown that they are bacterial membrane disruptors, but their small size excludes any likely membrane spanning pore formation.[21] A more likely mode of killing is through the carpet mechanism.[22] An intracellular target in combination with membrane disruption cannot be ruled out either. Several studies are presently aimed at trying to solve the definitive mechanism of these peptides, a mechanism that could also be strain specific.

The minimum motif for activity in this class of antimicrobial peptides has been defined as two cationic charges and two hydrophobic units. Several other groups have lately reported successful implementation of this pharmacophore.[23−25] The required structural motif theoretically also makes these peptides excellent substrates for both trypsin and chymotrypsin,[26] and a recent study of the metabolic fate in a range of organ extracts identified the GI-channel as the main metabolic obstacle for CAPs.[27] A more focused study described how introduction of bulky hydrophobic elements proved to be efficient strategies for creating CAPs with tryptic stability.[28] On the basis of the substrate specificity of chymotrypsin, it is important to establish the CAP stability toward chymotryptic degradation in order to further advance this class of promising compounds.[29]

For this purpose, four libraries of CAPs based on the lead Arg-Trp-Arg-NHBn displaying a medium antibacterial activity were prepared, each designed to explore a specific subsite in the active site of chymotrypsin (Figure 2). The peptide backbone was kept unaltered to increase the number of methodologies for making metabolically stable peptides within the CAP pharmacophore. In addition to analyzing the structural alterations earlier described as providing tryptic stability, the current libraries also contain several arginine analogues as a third tool for manipulating the structure and activity of the peptides. Collectively, the results from the degradation studies together with molecular modeling and calorimetric experiments on selected stable peptides describe the fate of these peptides within the active site of chymotrypsin. Several important structural features that can be used for the development of peptides with increased proteolytic stability in the future are identified and investigated.

Results and Discussion

After a decade of research, the performance (activity, selectivity, solubility, and production costs) have now reached satisfactory levels for short lactoferricin based CAPs. The size of the truncated active peptides is in the range that can allow for active uptake by hPEPT1 should they survive long enough in the smaller intestine.[30] The attention is thus now shifted toward strategies to maintain the activity while optimizing the ADME properties of the peptides. The first major obstacle for the peptides is represented by the GI-channel,[27] and the compounds in the present study were designed in an attempt to produce potent antimicrobial peptides with chymotryptic stability.

The fact that serine proteases generally prefer at least six residues for optimal substrate binding, and catalysis did not prevent a rapid degradation of tripeptide **1** to produce the inactive tripeptide carboxylic acid Arg-Trp-Arg-OH and benzylamine in a previous study aimed at stability toward tryptic degradation.[28] In that study, several design principles were suggested to solve the stability issues, and in the current paper we are applying analogous modifications to establish their effects on the chymotryptic stability while remaining within the structural borders defined by the pharmacophore. The effects of such modifications on the chymotryptic stability are to some extent expected to be adverse, considering the reversed structural requirements for substrate binding despite an otherwise very similar overall enzyme structure.[4,31]

The specificity of chymotrypsin is mainly defined by the hydrophobic P1−S1 interaction,[32] and thus the initial focus was on replacing the Trp in the lead compound. The first library was constructed by replacing the central (Trp) residue with either natural (Phe) or unnatural bulky amino acids (Bip and Dip). The second library consisted of peptides incorporating arginine analogues differing in length and basicity in the P1′ position. Previously, our group has shown that C-terminal amidation can provide both increased antibacterial activity, an altered albumin binding, and stability to tryptic degradation.[28,33] The third library in this study thus contains peptides with different C-terminal end-cappings ending up in S2′. It has been shown that the sequence not always has a big impact on the activity for these peptides, and the fourth library was

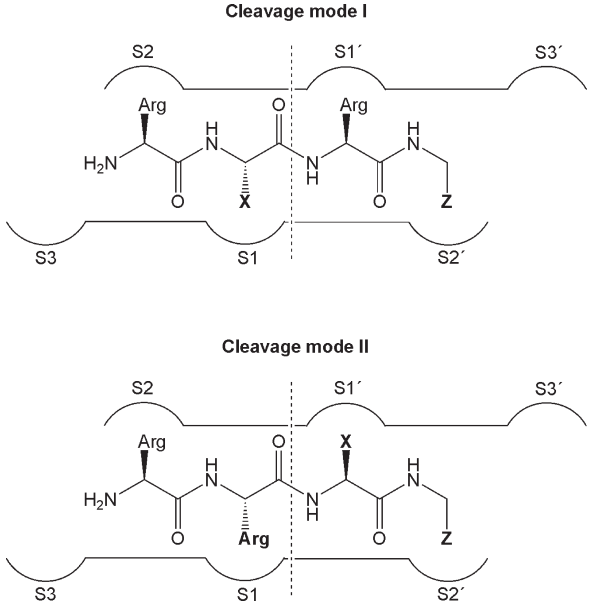

Figure 3. Schematic representation of the binding site in chymotrypsin and the mode of binding seen for the majority of the peptides with the bulky central unit in site S1 and C-terminal bulk in S2′ cleavage mode I. The lower cleavage mode II illustrates the binding mode of the confused peptides in library 4.

Table 1. MIC Values against Gram-Positive *S. aureus* and MRSA, Half-Lives against Chymotryptic Degradation and Hemolytic Activity of the Tested Peptides

		MIC (μM)		$\tau_{1/2}$	%
peptide	sequence	*S. aureus*[a]	MRSA[b]	(h)[c]	hemolysis[d]
1	R-W-R-NHBn	79	132	2	> 5
2	R-F-R-NHBn	165	83	3	nd[e]
3	R-Bip-R-NHBn	10	15	stable	> 5
4	R-Dip-R-NHBn	25	10	stable	> 10
5	R-W-Har-NHBn	78	52	1	nd
6	R-W-Lys-NHBn	163	54	4	nd
7	R-W-Orn-NHBn	110	55	20	> 1
8	R-W-Agp-NHBn	54	16	stable	> 2
9	R-W-Gpp-NHBn	50	10	4	nd
10	R-W-App-NHBn	157	79	stable	> 5
11	R-W-R-NH_2	> 200	> 200	5	> 1[34]
12	R-W-R-NHEtPh	52	26	0.5	nd
13	R-R-W-NHBn	79	26	7	nd
14	R-Gpp-W-NHBn	50	15	stable	> 2
15	R-App-W-NHBn	157	79	17	nd

[a] *S. aureus* strain ATCC 25923. [b] Methicillin resistant *S. aureus* strain ATCC 33591. [c] Calculated using Cornell Medical Calculator. [d] At a peptide concentration of 1 mM. [e] Not determined.

created to evaluate the possibility of increasing the stability by changing the amino acid sequence.[34] By using HPLC and MS analysis, we could also determine the products of the peptide cleavage to confirm the mode of cleavage suggested in Figure 3.

Peptides containing unnatural amino acids for stability testing were prepared on solid phase employing Fmoc based coupling chemistry using a range of coupling agents depending on the final peptide composition. The prepared peptides displayed an antibacterial activity toward Gram-positive *Staphylococcus aureus* and MRSA (Table 1.). No activity (> 200 μM) against the Gram-negative bacterium *Escherichia coli* was seen, which is in agreement with earlier studies.[26] Results from the antibacterial assay illustrate the beneficial effect of introduction of a bulkier central amino acid in peptides **3** and **4**, as it affords a near 10-fold decrease in MIC. Additional bulk can also be provided in direct conjunction with the charged side chain, and this favorable effect is seen for peptides **9** and **14**. Peptide **11**, without a C-terminal benzyl group, loses its antibacterial activity. The base strength also plays a role for these sequences as peptides containing guanidines are more active than those with the primary amines, Lys (**6**) and Orn (**7**). Removal of a positive charge by using App ($pK_a \sim 5$) instead of Arg lowers the potency, as is seen for peptides **10** and **15**. Peptides **5** and **8** contained analogues to Arg used for the first time for the preparation of antimicrobial peptides, and it was shown that both Har (**5**) and Agp (**8**) generate peptides with slightly improved antibacterial properties compared to Arg. The confused peptides **13**, **14**, and **15** behaved similarly to the native counterparts **1**, **9**, and **10**. Bringing the positive charge closer to the peptide backbone also seem to yield more efficient killers, as illustrated by peptides **7** and **8**. To ensure that the properties yielding a metabolic stabilization did not increase the potential cytotoxicity of the compounds, the hemolytic activity of a selection of the more stable peptides was also investigated as shown in Table 1 All the tested peptides displayed very favorable selectivities toward Gram positive bacterial cells and cannot be considered cytotoxic (less than 5% hemolysis at 30 × MIC for the most hemolytic peptides) within a relevant pharmaceutical concentration range. It has previously been shown that the cytotoxic activity is mainly attributed to an excess of hydrophobic bulk, and such a trend is clearly seen here as well[21,35] as peptides **3**, **4**, and **10** displayed the highest cytotoxic activities. The incorporation of arginine analogues did not produce peptides with an increased hemolytic activity; instead the opposite was seen at high concentrations (See Supporting Information).

Initial analysis of the half-life assay results indicated that the peptides became stable upon replacement of the central tryptophan with either Dip or Bip as P1 unit. Therefore, this study is mainly based on peptides with a central tryptophan in order to also evaluate the effect on stability using subunits S1′ and S2′. Such a strategy makes the findings much more general and applicable to a wider range of therapeutic peptides. The studied peptides are thus not optimized in terms of antibacterial effect although they can rapidly be transformed into highly potent compounds by exchanging the central tryptophan with a bulkier analogue. They still compare well with commonly used commercial antibiotics such as the aminoglycosides streptomycin and kanamycin.[36,37] Discussions of the events unfolding in each subsite follow below.

Library 1. R-**X**-R-NHBn (peptides **1**−**4**) S1-Pocket: The deep hydrophobic S1 pocket, composed of important structural contributions from residues Ser189, Gly216, and Gly226, dictates the specificity of chymotrypsin and it was natural to initially explore the central S1/P1 interaction.[38] Phe and Trp are the most preferred natural amino acids in the P1 position, and both peptides **1** and **2** are quickly degraded within 2−3 h. The Bip-containing peptide **3** exhibited at least a 10-fold increase in stability, and only trace amounts of products could be seen after 24 h. This positive outcome spurred us to also include the β-disubstituted Dip yielding peptide **4**, which was also resistant to degradation. Bip and Dip represent the structurally simplest hydrophobic amino acid analogues used to generate highly potent cationic antibacterial peptides, and it was concluded that further bulk is not needed in P1 to provide sufficient resistance. The fact that **3** actually produces trace

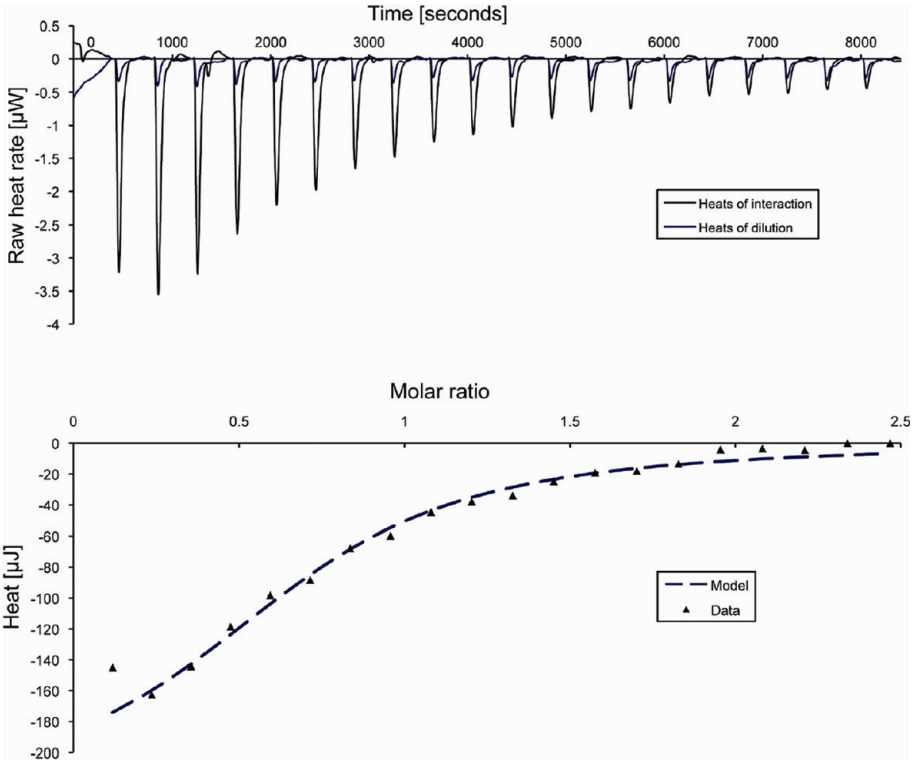

Figure 4. Representative raw data from the ITC analysis of R-Bip-R-NH_2 (top figure) into a buffered solution of chymotrypsin and into buffer only. The lower figure illustrates the binding isotherm derived from the same run yielding a stoichiometric interaction and perfect fit to the one-site binding model employed. The data from the first data point is not included due to slow syringe leakage during the equilibration step prior to the first injection.

Table 2. Binding Data from ITC Experiments

peptide	K_D (μM)[a]	n[a,b]	ΔH (kJ/mol)[a,c]	$-T\Delta S$ (kJ/mol)[a,c]	ΔG (kJ/mol)[a,c]
3	18.4	0.8	−7.5	−19.6	−27.1
8	61.1	0.7	−4.3	−19.7	−24.0
3′[d]	16.1	0.7	−22.4	−5.0	−27.4

[a] Data from the ITC experiments and the binding isotherms. Average values based on two parallel runs. [b] Stoichiometry of the interaction; experimental error is ±0.1. [c] Experimental error is ±15%. [d] Peptide R-Bip-R-NH_2, only included for comparative purposes in the ITC studies.

amounts of product during the analysis suggests that it binds in the correct conformation for proteolysis. Isothermal titration calorimetry (ITC) studies[39,40] were therefore performed on peptides **3**, **4**, **8**, and a derivative of **3** lacking the benzyl group (**3′**, not included in the main study) in an attempt to examine whether these compounds could act as inhibitors of chymotrypsin. Data from the ITC study indicates that chymotrypsin binds in a 1:1 complex with the injected peptides **3**, **3′**, and **8** (Figure 4). Table 2 shows that the dissociation constant is between 16.1 and 61.1 μM for the interacting peptides with similar free energies of binding but different enthalpic and entropic contributions. No binding was seen for peptide **4** (data not included in Table 2). The influence of the C-terminal capping for the binding mode is also illustrated by comparison with **3′**, which lacks the C-terminal benzyl group leading to a substantial decrease in $-T\Delta S$ from −19.6 kJ/mol to −5.0 kJ/mol. Peptides that were sensitive to degradation could not be analyzed by ITC due to the interfering heat contributions generated from the hydrolysis.

Computational docking experiments predict a binding mode for 3 with Bip inserted into the S1 pocket as illustrated in Figure 5. ITC experiments (K_D = 18.4 μM), together with the trace amounts of product observed in the degradation studies, provide additional support for the binding mode. However, it remains difficult to pinpoint the exact reason for the slower hydrolysis of 3, but it may result from suboptimal positioning of the scissile bond due to the length of the rigid Bip side chain. No docking results were obtained with Dip inserted into the S1 site for peptide **4**. ITC analysis of **4** did not yield a binding isotherm that differed from that of the heats of dilution, which is in support of the results from the docking experiments. The positive stabilizing effects seen for **3** and **4** were not as prominent in the recent trypsin study, where the central hydrophobic amino acid of the tested peptides is most likely to interact with S2. Of eight tested bulky units, only Dip generated stable peptides while the Bip containing peptide was rapidly degraded with a half-life of only 2 h.[28] This is a reflection of the flexibility in accommodation potential of the S2 site in trypsin.

Library 2. R-W-**Y**-NHBn (peptides **1**, **5**−**10**) S1′-pocket: In addition to the important S1/P1 interaction, chymotrypsin also prefers substrates with Arg or Lys at P1′.[41] Studies of this interaction have solely been based on naturally occurring structural determinants.[42,43] The unnatural synthetic amino acids, of importance for the development of peptidic drugs,[34] in this study have not been investigated before. Surface loops 40 (residues 34−41) and 60 (residues 58−69) act in synergy to generate the S1′ binding pocket that interacts, via electrostatic contributions from Asp35 and Asp64, with a positively charged P1′.[42] It represents a less studied but important binding epitope for the design of protease inhibitors, and library 2 was designed to further map this binding site by incorporation of a range of arginine

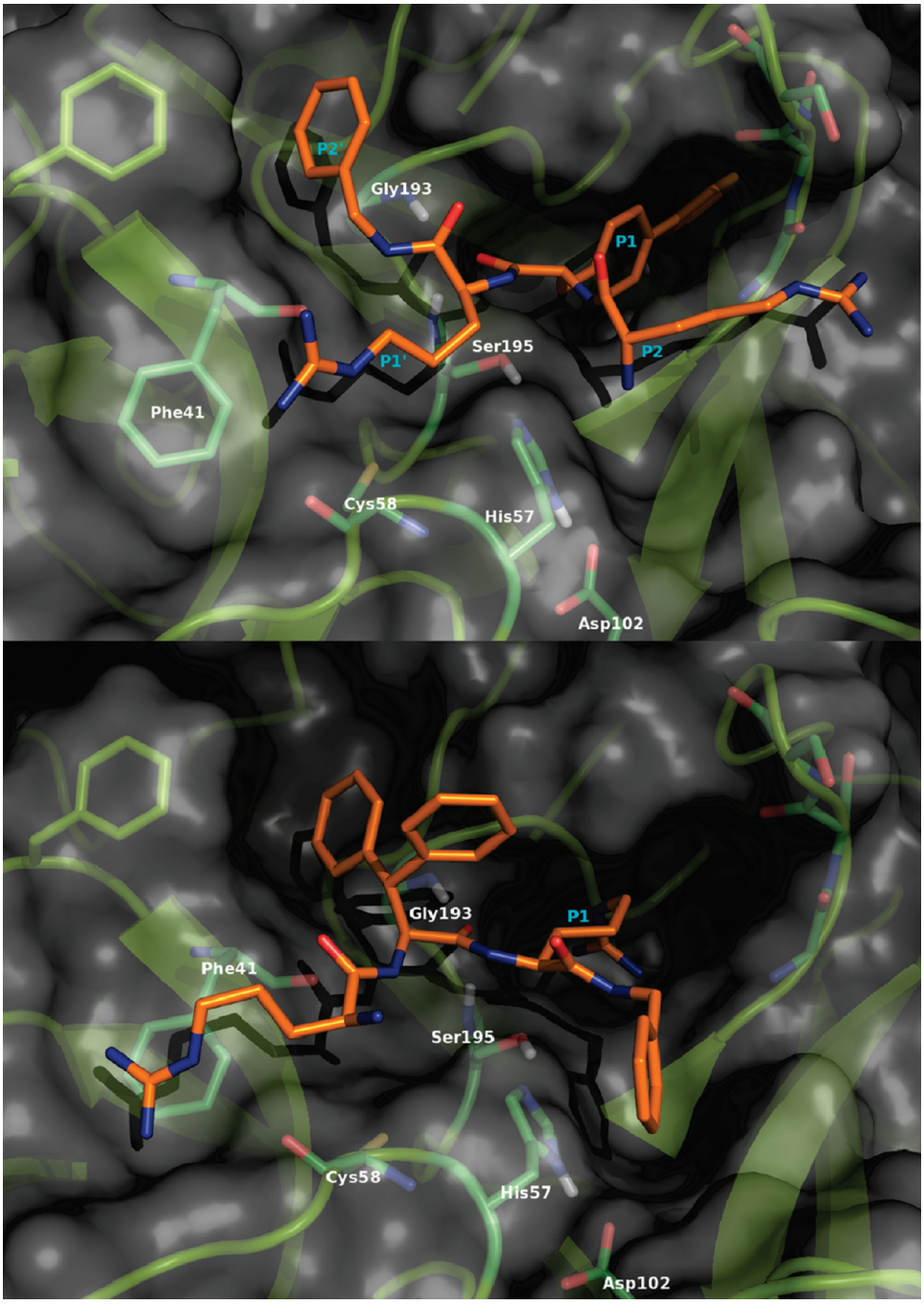

Figure 5. Docking of peptide **3** (upper) and peptide **4** (lower) into the active site of chymotrypsin reveals potential binding modes for Bip and Dip. Figure generated using PyMol.[62]

analogues. From the results of the stability assay in Table 1, it is obvious that the length of the side chain plays an important role. By replacing Arg (**1**) with Har (**5**), which has an additional methylene in its side chain, we can see a reduction in half-life. The opposite behavior is displayed when analogues containing shorter chains are introduced into the binding site. Peptide **8**, which contains Agp in P1′, is stable. Because of synthetic difficulties, we could unfortunately not include a guanidine containing analogue linked to the peptide backbone with two methylenes. The same trend is seen for side chains incorporating primary amines, which are generally more stable, as cationic elements (**6** and **7**). Peptide **10** is also stable due to the weaker basicity of the App moiety, which lowers the affinity for the S1′ binding pocket. For the tested peptides in this study, it seems that the deeper the basic residue can reach into the S1′ site the more the scissile amide bond is stabilized in the active site. Figure 6 shows a model of **1**, **5**, and **8** in complex with chymotrypsin with the tryptophan indole placed in S1 as supported by the degradation and ITC studies. Such a binding mode illustrates how most significant structural differences are observed for the interactions occurring at the S1′ site for these similar peptides. ITC control experiment on peptide **8** was performed in support of this hypothesis to establish that it actually bound to chymotrypsin in a stoichiometric manner similar to peptide **3** with a dissociation constant of 61.1 μM. These findings illustrate how the interactions in the S1′ pocket plays a decisive role for the degradation of short cationic peptides

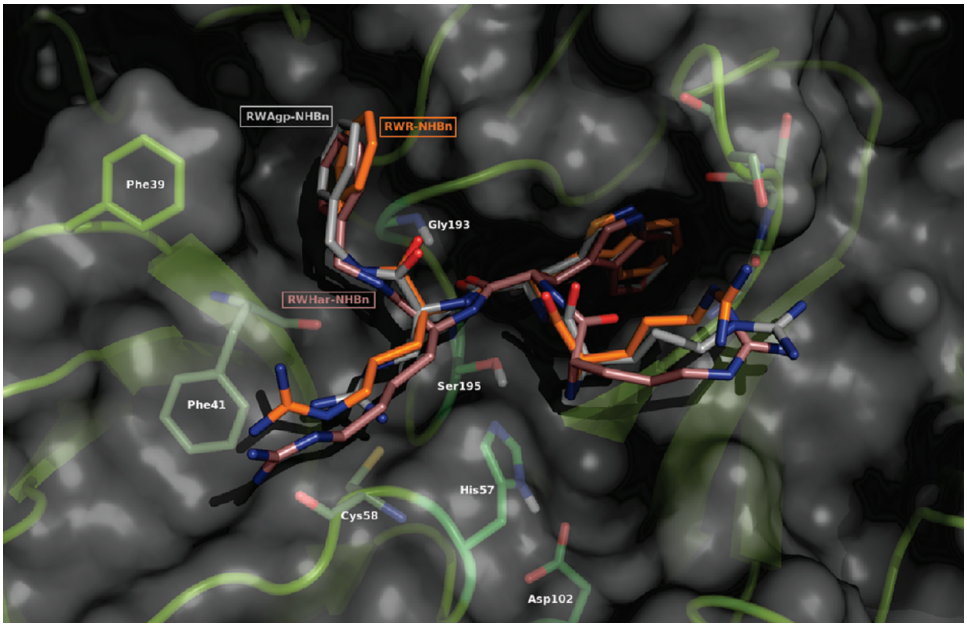

Figure 6. Overlay of R-W-Agp-NHBn (**8**), R-W-R-NHBn (**1**), and R-W-Har-NHBn (**5**) in the active site of chymotrypsin with the tryptophan indole placed in the S1 pocket. Figure generated using PyMol.[62]

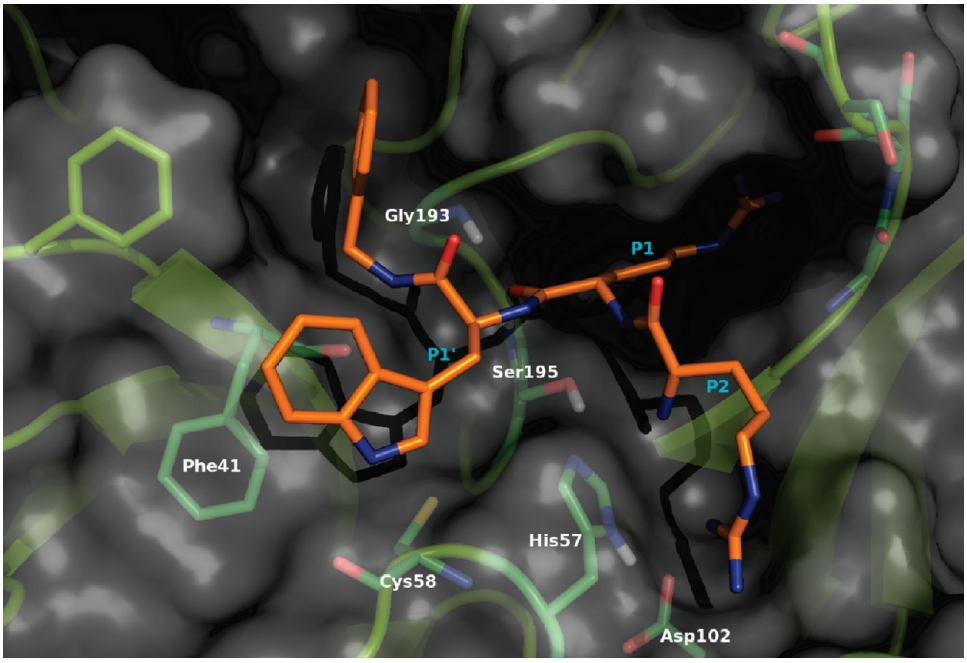

Figure 7. Model of confused peptide **13** in the active site of chymotrypsin. The degradation assay indicates that the S1 site interacts with the side chain of arginine in an unexpected manner yielding a trypsin-like activity with a bulky tryptophan indole in S1′. Figure generated using PyMol.[62]

with few other residues available to fixate the scissile bond in place over the active site. Longer peptides are held in place as antiparallel β-sheets by several additional substrate pockets as well as the polypeptide binding site.[44]

Library 3. R-W-R-**Z** (peptides **1**, **11**, and **12**) S2′-pocket: The S2′ site has not been studied in as much detail as the sites closer to the active site and library 3 was included to further explore the stabilizing effect a C-terminal capping group can have. In trypsin, the C-terminal group of similar CAPs either occupies S1′ or S3′ depending on which of the peptidic arginines reside in S1. The most common mode is with the capping group in S1′, and for those peptides a clear stabilizing effect was seen for the ethylphenyl group used in peptide **12** even if it can be considered as a fourth residue. In chymotrypsin, this part of the peptide is shifted to S2′ instead as a result of the central bulky unit in S1. Large hydrophobic groups have been suggested as favored P2′ moieties for chymotrypsin,[42] and in the present study we see a clear trend toward destabilization following incorporation of longer C-terminal additions. This behavior is in clear contrast to the results from the trypsin study and illustrates how the same structural modification can easily alter the stability toward similar enzymes in different ways. To remain within the pharmacophore of the peptides, other types of capping

groups displaying a wider spectrum of chemical functionalities were not considered.

Library 4. Confused peptides R-**Y**-W-NHBn (peptides **13**−**15**): Generally, antimicrobial peptides are regarded as amphiphilic compounds and there is a correlation between the distribution of charge and bulk within the sequence and the activity for most peptides and their peptidomimetics.[45,46] Concerning the very short unusual peptides included in this study, the sequence dependence is not as pronounced, and recent studies have shown that confused peptides can yield the same antibacterial activities.[34] From a stability perspective, this is an attractive drug design option, and library 4 was included to provide peptides with a different overall distribution of charge and bulk. As can be seen in Table 1, the confused peptides **13**, **14**, and **15** displayed similar antibacterial activities to the "native" **1**, **9**, and **10**. Their half-lives were, however, different. Both peptides **13** and **14** are superior to **1** and **9**, while peptide **15** is no longer considered as stable. The increased susceptibility of peptide **15** in the confused version can be explained by the fact that the uncharged, bulky App is likely a good ligand in S1. The mode of degradation remains the same though, illustrating that peptides **13** and **15** are degraded in the same way by chymotrypsin as trypsin would with a charged residue in S1 and a bulky unit in S1′ as illustrated in Figure 7. A binding mode previously described for chymotrypsin inhibitors containing lysine,[38,47] where the cationic side chain is bent in an attempt to minimize the interactions with the hydrophobic pocket in S1, may explain how the P1 arginine in **13** fits in S1. It further highlights that while sequence reversal may be employed to increase the stability, it is not always a general solution for these compounds. For other bioactive peptides with more specific structural requirements for target receptor binding, this option is less likely to be applicable.

Conclusion

Short potent antibacterial peptides containing several unnatural novel analogues to both tryptophan and arginine displaying a high selectivity against bacterial cells have been prepared. In addition, their stability against chymotryptic degradation has been investigated in detail. Through extensive studies it has become clear that the interactions between our short tested antibacterial peptides and subsites S1, S1′, and S2′ can all be used to tune the proteolytic stability while maintaining a high antibacterial activity and selectivity. For S1, the bulk provided by Dip and Bip are sufficient for stability, which opens up significant freedom in peptide design. The stability of peptides containing arginine analogues as S1′ binders suggest that short cationic side chains invoke a dramatic increase in stability. Bulky C-terminal capping groups that are essential for activity act as an additional residue and lower the stability due to substrate stabilizing interactions with S2′. Collectively, these represent important findings that allow for the design of short stable peptides, inhibitors, and peptidomimetics. In addition to these more readily applicable findings, it also provides fundamental insights into the structural determinants of several unnatural amino acid side chains and into the nature of the active site of chymotrypsin.

Experimental Section

Solid Phase Peptide Synthesis (SPPS). The peptides were prepared using SPPS, employing standard Fmoc coupling chemistry.[48] The peptide benzylamides and phenethylamide were synthesized on NovaSyn TG amino resin with BAL,[49] and the peptide amides were prepared on Rink amide resin. NovaSyn TG amino resin was washed with DMF. BAL (3 equiv), HOBt (4.5 equiv), and DIC (3.3 equiv) in enough DMF to dissolve and cover the resin were added and irradiated in a Biotage Initiator microwave oven at 60 °C for 20 min.[50] The solution was removed, and the resin was washed with DMF (×5) and DCM (×3). The resin was washed with MeOH (×2) before adding benzylamine (10 equiv) and $NaBH_3CN$ (10 equiv) in MeOH, covering the resin. The mixture was irradiated in a microwave oven at 60 °C for 20 min. Reductive amination was confirmed qualitatively by a positive chloranil test.[51] The peptide elongation for both the BAL-linker and Rink-linker was performed with 3 equiv of amino acid, PyBOP (3 equiv), and DIPEA (6 equiv). Some couplings, however, required stronger coupling reagents. Coupling Fmoc-Agp to the secondary amine on the BAL-linker was done with the same conditions but with PyClOP instead of PyBOP. The coupling completion was confirmed by a negative chloranil or Kaiser test on a small sample of the resin filtered and washed with DMF (×5) and DCM (×2). Recoupling was performed if the completion tests were positive. Removal of the Fmoc-protecting group between each coupling was achieved by adding 20% piperidine in DMF to the resin for 3 min. The resin was then washed with DMF (×5) before next coupling. The Rink amide resin required treatment with 20% piperidine in DMF before the first amino acid coupling to remove the Fmoc-protecting group. After the final coupling and deprotection, the resin was washed with DMF (×5), DCM (×2), and MeOH (×2) and dried. Cleavage of the peptides from the resin was performed employing a mixture of TFA:TIS:H_2O (95:2.5:2.5 v/v/v), which yielded a crude that was further purified.

CAP 3. ^{1}H NMR (600 MHz, D_2O) δ 7.48 (dd, J = 7.8, 19.3, 4H), 7.35 (t, J = 7.7, 2H), 7.27 (t, J = 7.4, 1H), 7.23−7.11 (m, 5H), 6.94 (d, J = 7.2, 2H), 4.54 (dd, J = 6.8, 9.5, 1H), 4.05−3.88 (m, 4H), 3.08 (dd, J = 6.7, 13.5, 1H), 3.04−2.96 (m, 2H), 2.94−2.87 (m, 3H), 1.80−1.68 (m, 2H), 1.62−1.39 (m, 4H), 1.33−1.16 (m, 2H). ESMS: calcd for $C_{34}H_{46}N_{10}O_3$ 642.8, found 643.4; Purity determined by HPLC: Retention time, 11.68 min; purity, 99.0%. See Supporting Information for data on remaining compounds.

Purification. The crude peptides were purified by reversed phase HPLC (RP-HPLC) on a Delta-Pak (Waters) C-18 column (100 Å, 15 μm, 25 mm × 100 mm) employing mixtures of water and acetonitrile (both containing 0.1% TFA) as eluent. The purified peptides were further analyzed by RP-HPLC using an analytical Luna (Phenomenex) C-18 column (100 Å, 5 μm, 4.6 mm × 150 mm). To ensure positive identification of the desired product, positive ion electrospray mass spectrometry on a Thermo Electron LTQ XL mass spectrometer (Thermo Fisher Scientific, Waltham MA) was performed.

Antibacterial Activity. The antibacterial activity of the peptides was tested against *Staphylococcus aureus* strain ATCC 25923, methicillin resistant *Staphylococcus aureus* (MRSA) strain ATCC 33591, and *Escherichia coli* strain ATCC 25922. The studies were performed by Toslab AS employing standard methods.[52]

Hemolysis. The hemolytic activity of selected peptides was tested against human red blood cells (hRBC) in a similar fashion as previously reported.[34] Fresh heparinized hRBC were rinsed three times with normal saline (NS) by centrifugation (10 min, 1500 rpm), followed by resuspension and dilution in NS (10% hematokrit). Various concentrations of the peptides (25−1000 μg/mL) were then dissolved in NS and added to the hRBC solution, yielding a final erythrocyte concentration of 1% v/v. The suspensions were incubated under agitation for 30 min at 37 °C, followed by centrifugation (5 min, 4000 rpm). Release of hemoglobin was monitored by measuring the optical density (OD) of the supernatant at 405 nm on a Thermo max microplate reader (Molecular Devices). Negative controls for zero hemolysis and positive controls (100% hemolysis) consisted of hRBC suspended in NS and Triton-X 1%, respectively. The degree of

hemolysis is defined as the ratio of the OD of the peptide sample relative to the OD of the difference between the positive and negative control for hemolysis.

Peptide Half-Life Assay. The half-lives of the peptides were tested using a modified version of a method established for both trypsin and chymotrypsin.[28,53] Each peptide was dissolved in a 0.1 M NH_4HCO_3 buffer (pH 8.2) to a final peptide concentration of 1 mg/mL. A chymotrypsin solution was prepared by dissolving 1 mg of chymotrypsin to 50 mL of 0.1 M NH_4HCO_3 buffer (pH 8.2). The peptide solution (150 μL), chymotrypsin solution (150 μL), and 0.1 M NH_4HCO_3 buffer (1200 μL) were combined and incubated at 37 °C. Then 150 μL aliquots were taken at given time intervals, diluted with 150 μL of water/acetonitrile (60/40 v/v) containing 0.5% TFA, and analyzed by RP-HPLC as described above. Samples without chymotrypsin were taken at 0 h and after 24 h at 37 °C and used as negative controls. Waters Millennium Chromatography Manager v. 4.0 was used for chromatogram handling. Integration of the peak area at 214 nm for samples taken during the first 5 h of the assay was used to calculate the peptide half-life ($\tau_{1/2}$) using Medical Calculator from Cornell University.[54] Peptides that displayed less than 10% degradation after 24 h were considered stable.

Molecular Modeling. Molecular models of peptides were built with Maestro version 9.0.[55] The geometries were then first relaxed with the UFF minimizer in Maestro to eliminate distortions. Further minimization of the peptide geometry was done with Macromodel version 9.6[56] to prepare the peptides for the docking experiment. The OPLS2005[57] force field was used for all optimiziations with the conjugate gradient minimization scheme PRCG[58] and continuum solvent (water). Long-range interactions were treated with the extended cutoff method with truncation of the potentials after 4, 8, and 20 Å for H-bonds, van der Waals, and electrostatic interactions, respectively. The minimization was set to converge on the gradient with the convergence threshold set to 0.05. The minimized peptides were then further prepared for the docking experiments with LigPrep[59] using the OPLS2005 force field.[57] LigPrep was set to generate possible states at target pH between 4.0 and 8.0, at most 32 per ligand. Coordinates for chymotrypsin were obtained from the crystal structure of the P1 Trp BPTI mutant-bovine-chymotrypsin complex with the entry code 1t8o.[60] The protein was prepared for the docking experiment in Maestro 9.0 by careful investigation of protonation state of ionizable residues and placement of hydrogen atoms. Water molecules beyond 5 Å from protein heavy atoms were removed. The hydrogen bonding network was optimized prior to docking.

The receptor grid was prepared with the Receptor grid generation in Glide.[61] The P1 Trp BPTI ligand was truncated to only include residues 14−16 where residue 15 is Trp. These three residues are expected to be similar to the target peptides in this study, and were therefore specified as a ligand molecule in the grid generation. This truncated tripeptide defined the center of a grid box, and glide was set to dock ligands similar in size to this peptide. The van der Waals radius scaling was set to a scaling factor of 1.00 and a partial charge cutoff of 0.25. The grid was also prepared to allow rotation of the receptor hydroxyl groups Ser195 and Ser189.

All calculations were performed with Extra Precision (XP). The calculations were set to dock flexibly and to sample ring conformations. Nonplanar amide bonds were penalized. Scaling of van der Waals radii on the ligands was done with a scaling factor of 0.80 and a partial charge cutoff of 0.15. For the Glide screens, 10000 poses per ligand were specified to be kept for the initial phase of the docking calculation. The scoring window for keeping the initial poses was set to 100 kcal/mol. The 1000 best poses per ligand were kept for energy minimization with a maximum number of conjugate gradient steps set to 5000. A maximum of one million ligand poses per docking run and 20 poses per ligand were collected. Glide was also set to perform postdocking minimization with 2000 poses per ligand included. The threshold for rejecting minimized poses was set to 0.50 kcal/mol.

Isothermal Titration Calorimetry (ITC). Heats of interaction were determined using a CSC 5300 nano-isothermal titration calorimeter III with a 1 mL cell volume (Calorimetry Sciences Corporation, UT). In a typical chymotrypsin titration experiment, the peptide (2.1 mM) was added in 20 aliquots (5 μL) to a stirred (150 rpm) solution of chymotrypsin (0.1 mM) in an aqueous buffer at 25 °C. The buffer used was a 0.1 M NH_4HCO_3 buffer (pH 8.2). An interval of 400 s between the injections was employed to reach equilibrium between the interacting species. The heats of dilution were determined in a similar fashion, where the peptides were added to a stirred buffer solution without protein. Subtraction of the dilution heat yielded the heat of interaction and a binding isotherm. A theoretical binding model (NanoAnalyze Software TA Instruments, LLC, New Castle, DE) was fitted to the binding isotherm, yielding the number of binding sites (stoichiometry), enthalpy, and as well as the binding constant.

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Supporting Information Available: List of chemical providers, hemolysis data, purity data, HPLC-traces, and ^{1}H NMR spectra for all the tested peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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