Targeting the S1 and S3 Subsite of Trypsin with Unnatural Cationic Amino Acids Generates Antimicrobial Peptides with Potential for Oral Administration

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Ⓢ Supporting Information

ABSTRACT: This study investigates how the S1 and S3 site of trypsin can be challenged with cationic amino acid analogues to yield active antimicrobial peptides with stability toward tryptic degradation. It is shown that unnatural analogues can be incorporated to generate stable peptides with maintained bioactivity to allow for a potential oral uptake. Selected peptides were studied using isothermal calorimetry and computational methods. Both stable and unstable peptides were found to bind stoichiometrically to trypsin with dissociation constants ranging 2−60 μM, suggesting several different binding modes. The stability of selected peptides was analyzed in whole organ extracts and the incorporation of homoarginine and 2-amino-(3-guanidino)propanoic acid re-

sulted in a 14- and 50-fold increase in duodenal stability. In addition, a 40- and 70-fold increase in stomach stability is also reported. Overall, these results illustrate how the incorporation of cationic side chains can be employed to generate bioactive peptides with significant systemic stability.
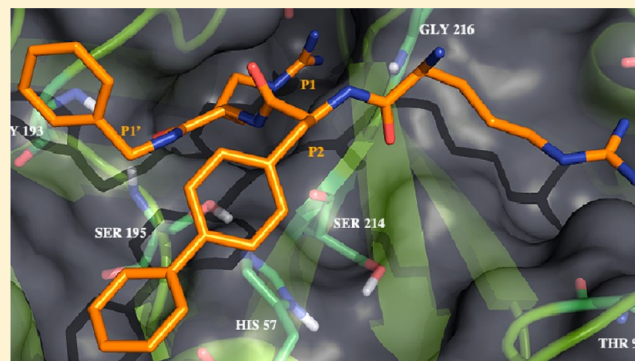
INTRODUCTION

Faced with more than 650 known and putative proteases encoded by the human genome with the sole purpose of digesting particular peptide substrates, it is not surprising that exogenous peptides are struggling to make their way to their site of action and subsequently onto the drug market.[1] The remarkable and diverse bioactivities of peptides are nevertheless enough driving force to pursue strategies with the aim of converting them into compounds with improved drug-like properties and a high bioavailability.[2,3] Despite the efforts made, the presystemic metabolism of peptides not administered via parenteral routes remain highly challenging to overcome.[4] Low stability in the gastrointestinal tract (GI) and a narrow therapeutic index are thus two major factors hampering the general applicability of peptides and proteins as orally available drugs.[5,6]

The serine proteases trypsin and chymotrypsin represent a particular obstacle in the GI tract for promising peptidic leads, effectively preventing oral bioavailability. The substrate specificity of the enzymes, as defined by the S1/P1 interaction in the active site, dictates the vulnerability of each individual amide bond within a peptide sequence and the k_{cat}/K_m can

differ over 10^5-fold upon optimization using natural amino acids.[7] Trypsin prefers a cationic side chain such as Arg or Lys in the S1 pocket in an electrostatic complex with anionic Asp189.[8] Conversely, chymotrypsin prefers a hydrophobic residue such as Phe or Tyr as optimized ligand for its deep hydrophobic S1 pocket formed by Ser189, Gly216, and Gly226.[9] The fact that the fringing S1′ site in both enzymes displays a pronounced selectivity for the opposite side chains makes the enzymes complementary and any peptide containing a sequential combination of a cationic and a hydrophobic amino acid are likely to be effectively hydrolyzed by either trypsin or chymotrypsin.[10]

Such susceptibility toward digestion was studied using a series of promising short cationic antibacterial tripeptides (CAP), incorporating both natural and unnatural cationic and hydrophobic amino acids shown to be rapidly digested by both trypsin[11] and chymotrypsin.[12] These peptides, derived from bovine lactoferricin,[13] have through structural optimizations been developed into promising pharmaceutical leads against
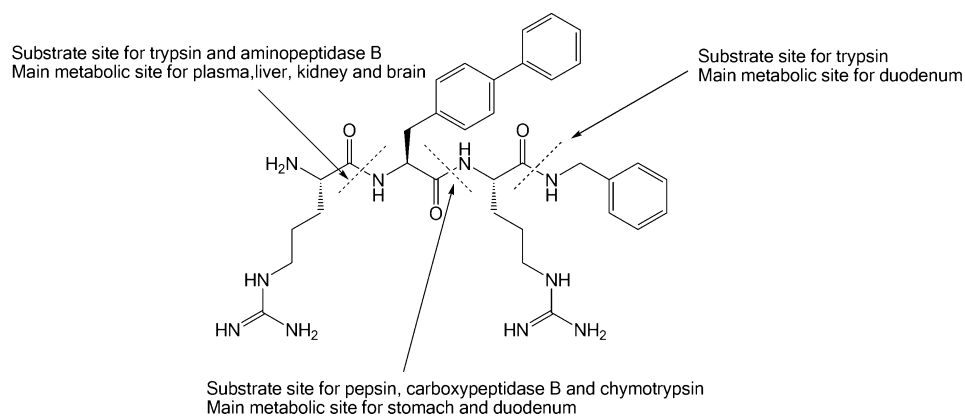
Figure 1. Illustration of short bioactive CAP that despite its small size, C-terminal benzyl amide capping, and unnatural bulky central amino acids is rapidly digested with a half-life of <10 min in both stomach and duodenum. Adapted from ref 20.

several pathogens including bacterial strains resistant to conventional antibiotics, and they are currently undergoing phase II trials (Lytix Biopharma).[14−18] A recent investigation illustrated their potential for oral absorption, as their small size allowed several to cross membranes via the passive route at rates suggesting an excellent oral in vivo absorption.[19] Their content of unnatural structural features did however prevent an active receptor (hPEPT1) mediated uptake. The initial trypsin study by Svenson et al. focused on evaluating different capping strategies and incorporation of a range of commercially available and synthetic unnatural hydrophobic amino acids to increase the tryptic stability.[11] All the studied peptides incorporated an S1 Arg, and the most studied sites were S2, S1′, and S3′.[11] A second study focused on chymotryptic stability, employed both unnatural cationic and hydrophobic amino acids, and described how the individual strategies could be employed to generate stability. Despite the fact that both reports illustrate how relatively simple synthetic alterations can yield bioactive peptides with increased stability against isolated enzymes,[11,12] the overall systemic effect is more complex. Studies of the systemic metabolism of the CAPs in the major metabolic compartments revealed that the structural requirements needed for activity also set the peptides up for rapid digestion,[20] preventing oral uptake. The stomach, duodenum, and liver were the main metabolic sites for the studied peptides. An illustration of the vulnerable regions of a model CAP with high antibacterial activity is shown in Figure 1.

Since it was shown that cationic analogues could be used to stabilize these peptides toward chymotryptic degradation via its S1′/P1′ interaction,[12] a beneficial dual protection against serine protease degradation for these peptides is potentially feasible by targeting the unexplored S1/P1 interaction of trypsin with similar analogues. Cationic analogues to Arg and Lys have successfully been incorporated in larger peptides by both Bruckdorfer and Hilpert to generate peptides with increased stability in dilute serum and in assays employing isolated proteases.[21,22] In addition, altered physicochemical properties upon replacements, leading to increased membrane permeation, insertion, and bioactivity, have also been reported.[18,23,24] In the current study, a library of 22 CAPs incorporating unnatural cationic S1 and S3 side chains is prepared and evaluated in an attempt to generate a complete picture of how their metabolic stability toward trypsin can be tuned. The study is focused on peptides displaying physicochemical properties, enabling them to transverse biological membranes.[19] The

antimicrobial effect of the CAPs is investigated and the specific interactions between the CAPs and trypsin are studied by in vitro degradation assays combined with isothermal calorimetry (ITC) and docking and molecular modeling of selected CAPs. The generality of the finds is further investigated in several whole organ extracts to establish the overall influence of the systemic metabolism in the major metabolic compartments for the most promising compounds.

■ RESULTS

Peptide Design and Synthesis. A total of 22 peptides were included in the present study to mainly probe the S1 and S3 site of trypsin as presented in Figure 2. The peptides were synthesized on solid support using Fmoc chemistry. Different synthetic strategies were used depending on the ease of the couplings. Selected unnatural amino acid analogues, in particular Agp and Har, presented lowered coupling yields. Peptides **1**, **14**, **15**, and **20−22** have been included in other studies.[11,12,19] Peptides **1**, **14**, **15**, **20**, and **21** have further been recently shown to display membrane permeation properties, suggesting excellent oral absorption for peptides **15** and **20**, moderate oral absorption for peptides **14** and **21**, and poor absorption for peptide **1** in a phospholipid vesicle-based passive permeation assay.[19]

Antibacterial Activity and Half-Life Assay. The antibacterial properties of the included peptides were determined by serial dilution and are presented in Table 1. All the peptides displayed powerful antibacterial properties against both Gram-positive *Staphylococcus* strains. To evaluate the stability of the included peptides, they were incubated with trypsin and sampled at regular intervals over a 24 h period. The majority of the peptides were rapidly digested by trypsin. Peptides **4−6**, **16**, **19**, and **22** showed no sign of degradation during the 24 h degradation assay and were classified as stable as shown in Table 1. The predominant site for degradation was the bond between the benzyl amide and the C-terminal arginine, yielding benzyl amine and H-Y1-Bip-Y2-OH for peptides **1−16** according to cleavage mode I shown in Figure 3 below. Peptides with scrambled sequences were instead cleaved between Y2 and either Bip or Trp to yield H-Arg-Y-OH and H-X-OH as products according to cleavage mode III in Figure 3. None of the peptides were degraded according to cleavage mode II, although docking studies suggest this binding mode for several peptides. Binding and cleavage according to cleavage
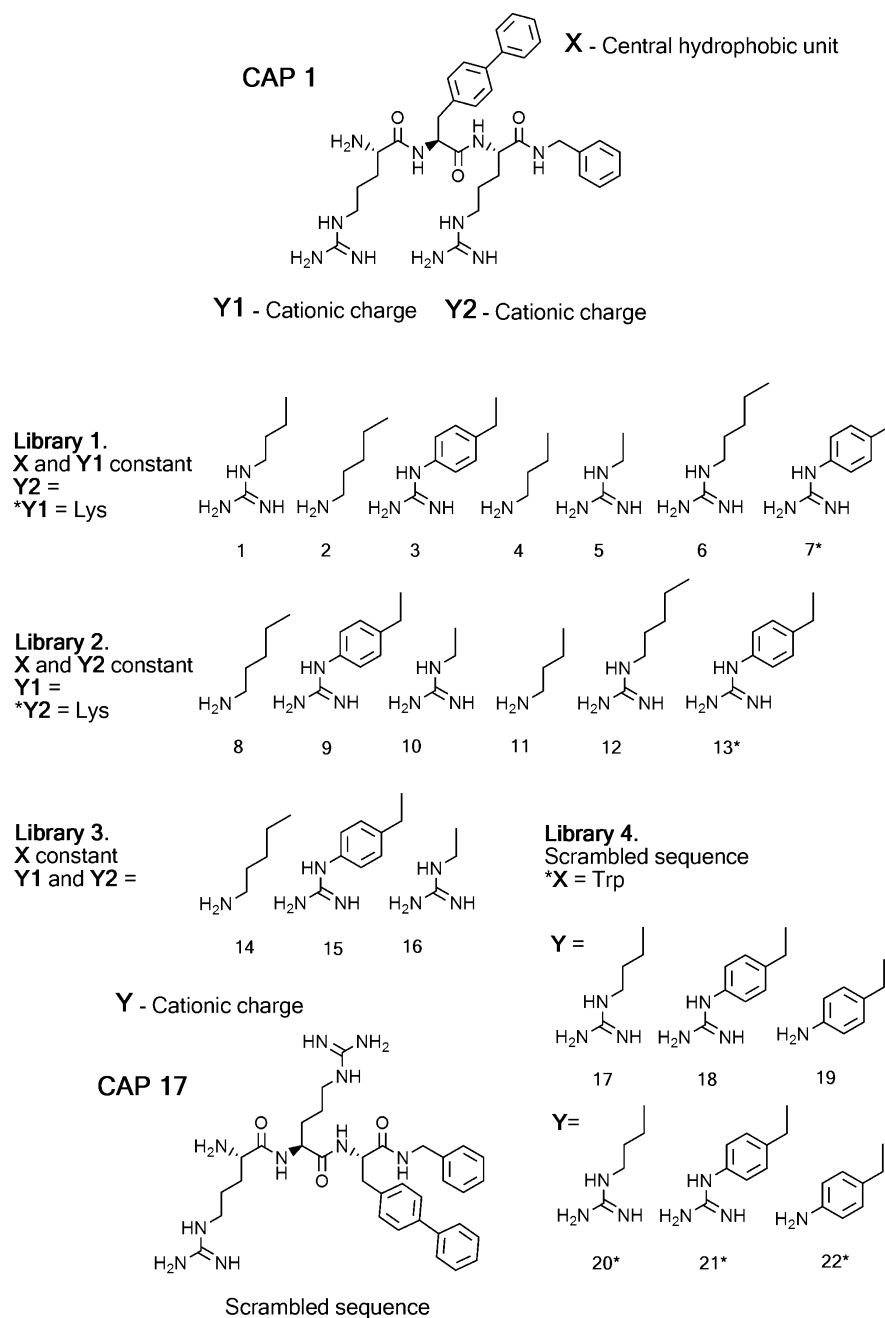
Figure 2. Structure of the antimicrobial peptides included in the current study. Library variation is mainly achieved by systematic replacing the Y1 and Y2 arginines with natural and unnatural cationic analogues employing peptide **1** as a reference peptide. The first carbon of each side chain represents the α-carbon of the peptide backbone. Library 4 also includes peptides with Bip replaced by Trp (peptides **20−22**) with a scrambled sequence, as exemplified by peptide **17**.

mode II by trypsin has been reported for other very similar peptides.[11]

Isothermal Titration Calorimetry. The interactions involved in the stabilization or destabilization of selected representative peptides toward tryptic degradation were studied by ITC.[25] The binding data from the ITC experiments is shown in Table 2. The unstable peptides (**1−3**, **8**, **11**, and **14**) all displayed similar interaction thermodynamics with excellent fit to a one-site binding model with a large binding contribution coming from $-T\Delta S$ but also with favorable binding enthalpies. Low micromolar dissociation constants ranging from 5.3 to 37.2 μM were seen for the unstable peptides which bound in a stoichiometric manner to trypsin. Several unstable peptides

could be analyzed without interfering background signals arising from the degradation. Nevertheless, peptides **17** and **20** could not be analyzed due to their rapid degradation and low amounts of peptides **5** and **16** prevented their inclusion. The stable peptides displayed two distinctly different binding modes to trypsin. Peptides **4** and **6** did not exhibit stoichiometric binding and a significantly smaller binding contribution from $-T\Delta S$. The overall binding affinity was further lower than for the other included peptides. The scrambled, stable peptides **19** and **22** interacted with trypsin in much the same way as the unstable peptides with good correlation with the one-site binding model. A comparison

Table 1. MIC Values against Gram-Positive *S. aureus* and MRSA together with Half-Lives against Tryptic Degradation of the Tested Peptides

| peptide | sequence | M_w | MIC (μg/mL) | | $\tau_{1/2}{}^c$ (h) |
			S. aureus[a]	MRSA[b]	
1	R-Bip-R-NHBn	642.8	10	10	1.0
2	R-Bip-K-NHBn	614.8	10	10	2.8
3	R-Bip-Gpp-NHBn	690.8	5	5	4.1
4	R-Bip-Orn-NHBn	600.8	5	10	stable
5	R-Bip-Agp-NHBn	614.7	5	5	stable
6	R-Bip-Har-NHBn	656.8	5	10	stable
7	K-Bip-Gpp-NHBn	662.8	5	5	5.0
8	K-Bip-R-NHBn	614.8	10	10	1.8
9	Gpp-Bip-R-NHBn	690.8	5	5	0.5
10	Agp-Bip-R-NHBn	614.7	10	10	1.4
11	Orn-Bip-R-NHBn	600.8	15	25	5.1
12	Har-Bip-R-NHBn	656.8	<2.5	10	1.9
13	Gpp-Bip-K-NHBn	662.8	5	10	2.2
14	K-Bip-K-NHBn	586.8	25	15	4.5
15	Gpp-Bip-Gpp-NHBn	738.9	<2.5	5	2.2
16	Agp-Bip-Agp-NHBn	586.7	10	15	stable
17	R-R-Bip-NHBn	642.8	5	5	≪0.5
18	R-Gpp-Bip-NHBn	690.8	2.5	5	3.5
19	R-App-Bip-NHBn	648.8	15	10	stable
20	R-R-W-NHBn	605.7	25	75	≪0.5
21	R-Gpp-W-NHBn	653.8	15	50	8.0
22	R-App-W-NHBn	611.7	75	>150	stable

[a]*S. aureus* strain ATCC 25923. [b]Methicillin resistant *S. aureus* strain ATCC 33591. [c]Calculated using Cornell Medical Calculator.[46]

between the binding isotherms for the different types of binding seen in the ITC studies is shown in Figure 4.

Molecular Modeling and Docking Experiments. A selection of the peptides was docked with trypsin and the binding was modeled and supported by the calorimetric and half-life data to generate an understanding of the underlying binding events of the different peptides. For a selection of the unstable peptides, the docking experiments suggested additional binding modes to trypsin not supported by degradation results (Supporting Information Table S2).

Whole Organ Extract Stability. The stability of the promising stable peptides **5** and **6** was further studied in whole organ extracts from mouse to establish the generality of the increased half-life seen in the initial isolated trypsin half-life assay. The main metabolic compartments, stomach (analyzed at both pH 7.4 and 2.0), duodenum, and liver, were included and the stability data is compiled in Table 3. The degradation of the peptides was continuously monitored and the formation of metabolites was followed to establish the major mode of degradation (Supporting Information Figure S1).

■ DISCUSSION

Peptide stability in the GI tract is essential for oral administration, and several strategies for dealing with the inherent metabolic instability of peptides have been developed over the years.[3,5,26] Stability toward endopeptidases can be achieved through cyclization, *N*-methylation, and incorporation of D-amino acids, β-amino acids, and amide bond isosteres.[26] Terminus selective exopeptidases can be targeted by several C- and N-terminal capping strategies.[27] Dimerization has also been showed to yield increased protease stability.[28] Each strategy comes with its own merits and drawbacks, and the major hurdle

Cleavage mode I
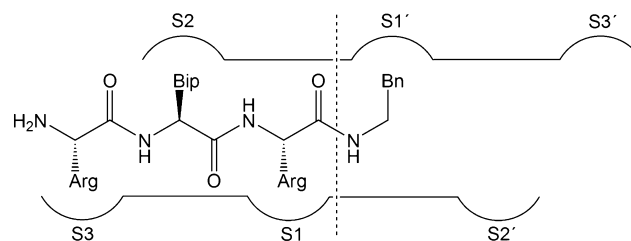
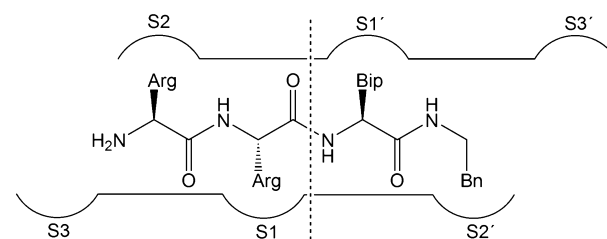**Cleavage mode II**
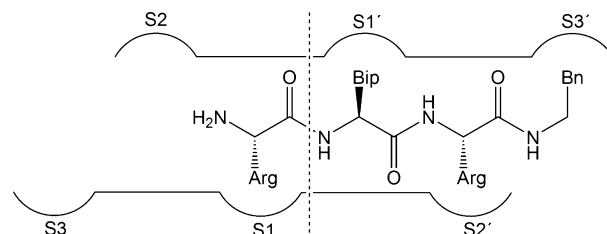
Cleavage mode III

Figure 3. Illustration of the potential modes of tryptic degradation of the tripeptides included in the present study based on metabolite analysis and docking. Cleavage mode I and II as shown with peptide **1** places either the C- or N-terminal Arg in the S1 pocket of trypsin. Cleavage mode III is exclusive for the scrambled peptides placing cationic side chains in both S1 and S2 as shown above with peptide **17**.

Table 2. Binding Data from ITC Experiments of Selected Peptides and Trypsin

peptide	$K_D{}^a$ (μM)	$n^{a,b}$	$\Delta G^{a,c}$ (kJ/mol)	ΔH^a (kJ/mol)	$-T\Delta S^{a,c}$ (kJ/mol)	$\tau_{1/2}$ (min)
			Unstable Peptides			
1	11.3	0.73	−28.3	−4.8	−23.4	1.0
2	20.0	1.06	−26.8	−5.2	−21.6	2.8
3	5.3	0.84	−30.1	−4.7	−25.4	4.1
8	11.6	0.99	−28.2	−3.4	−24.8	1.8
11	37.2	0.96	−25.3	−5.7	−19.6	5.1
14	21.0	0.97	−26.7	−2.5	−24.2	4.5
			Stable Peptides			
4	57.2	0.30	−24.2	−9.0	−15.2	stable
6	48.4	0.48	−24.6	−8.0	−16.6	stable
19	2.6	0.67	−31.9	−3.0	−28.9	stable
22	14.9	0.86	−27.6	−2.8	−24.8	stable

[a]Data from the ITC experiments and the binding isotherms. Average values based on two parallel runs. [b]Stoichiometry of the interaction; experimental error ±0.1. [c]Experimental error ±15%.

is to maintain the activity of the modified peptidomimetic.[3] Cyclization is not a general solution as the biological effect upon cyclization often is altered and challenging to predict.[29] It
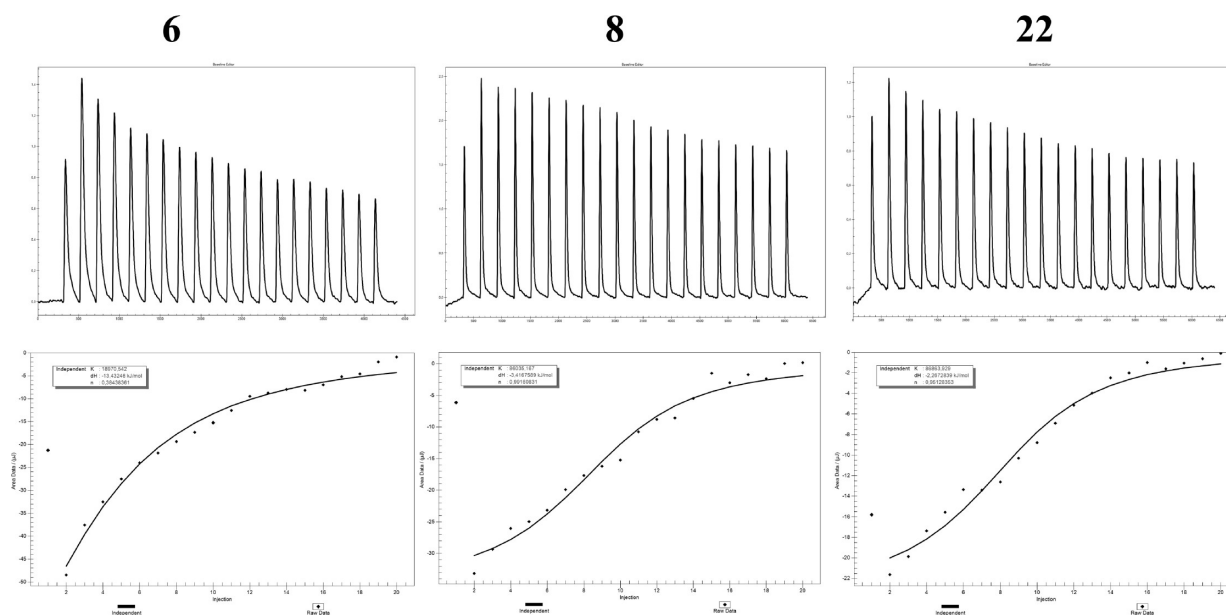
6 **8** **22**

Figure 4. Binding isotherms for the interaction of peptides **6**, **8**, and **22** and trypsin generated from the ITC experiments. The different binding mode of the stable peptides **6** and **22** is clear. The data from the first injection in each run is not included due to slow peptide diffusion from the syringe during the equilibration step prior to the first injection.

Table 3. Half-Lives of Selected Peptides in Whole Organ Extracts

	calculated peptide half-life (min)[a]		
tissue	peptide **1**[b]	peptide **5**	peptide **6**
stomach[c]	10.0	701.6	401.1
duodenum	6.1	323.6	82.1
liver	105.7	191.2	64.2

[a]See Supporting Information Tables S3−S5 for confidence interval and time profiles. [b]Data from ref 20. [c]See Table S4 in Supporting Information for stomach tissue data at pH 2.0.

is also synthetically challenging for certain peptide sequences. D-Amino acids are often an effective way to prevent hydrolysis although decreased half-lives have also been reported upon incorporation.[30,31] More chemically drastic approaches such as amide bond isosteres can be highly effective but are laborious, and also here it is difficult to maintain the bioactivity in a general fashion. Certain types of constrained peptidic scaffolds, for example, the cyclotides[32,33] and knottins,[34] are naturally resistant to proteolysis but they have only limited general use. At large, a strategy that can be implemented without extra synthetic steps or with a major cost elevation is particularly attractive.

Incorporation on unnatural amino acids is one such approach and it has previously been used to generate CAPs with improved stabilities toward trypsin and chymotrypsin with maintained bioactivities. The chymotrypsin study did however illustrate how several of the structural changes yielding tryptic stability generated CAPs with unaltered or even increased sensitivity toward chymotrypsin due to the complementary action of the enzymes. Most importantly, attachment of beneficial C-terminal amides, which was very beneficial for tryptic stability, generated peptides with short chymotryptic half-lives.[12] In contrast, other seemingly small changes such as the incorporation of biphenylalanine (Bip) instead of a central Trp rendered the CAPs completely stable toward chymotrypsin. Such incorporation had only a minor effect on the

tryptic stability. The CAPs investigated in the chymotrypsin study also contained analogues to arginine, and it was clear that those could be incorporated with very rewarding stability improvements via altered interactions with the S1′ site of chymotrypsin. The extent of this enzyme−substrate complex destabilization was unexpected, and several stable peptides were prepared by targeting this site alone. Those results encouraged us to explore if similar arginine analogues also can yield tryptic stability by targeting mainly S1 and S3. Because the S1−Arg association in trypsin is more pronounced than the S1′−Arg association in chymotrypsin, it is believed a broader general stability and sufficient half-lives to allow for oral bioavailability can be obtained. The P1 and P3 arginines were left unaltered in the initial trypsin study as the cationic charge is crucial to antibacterial activity to the same extent as hydrophobicity.[15] The present study is therefore aimed at in detail, investigating how natural and unnatural arginine replacements in the S1 and S3 position affect the tryptic stability. This study complements the previous trypsin study which was mainly focused on the S2, S1′, and S3′ subsites (see Figure S2 in Supporting Information for graphic library comparison).

Synthesis and Antibacterial Activity. The study included peptides specifically designed to probe the subsites surrounding the active site of trypsin. In addition, they were designed to remain within the pharmacophore requirement[15] for antibacterial activity and most of them represent highly bioactive compounds with MIC values against Gram-positive bacteria comparable to several commercial antibiotics.[35] Incorporation of synthetic unnatural amino acids were in many cases beneficial for the antibacterial activity (Table 1) in accordance with other studies.[12,18] The length of the side chains appeared to have little influence on the antibacterial properties. This has been reported earlier for a cationic antibacterial dodecameric peptide where six Arg were replaced by the shorter 2-amino-(3-guanidino)propanoic acid (Agp).[22] The analogues used have earlier been shown to have no effect on the unwanted hemolytic effect of the peptides or the therapeutic index.[12] Synthetic yields were generally high although some of the
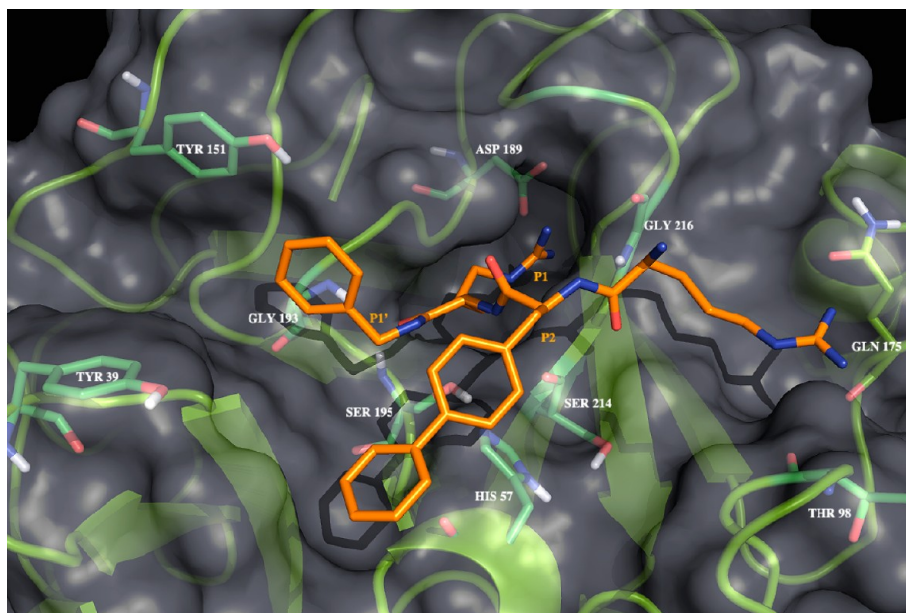
Figure 5. Peptide **1** docked in the active site of trypsin with its C-terminal arginine deeply buried in the S1 pocket and its C-terminal capping group in S1′.
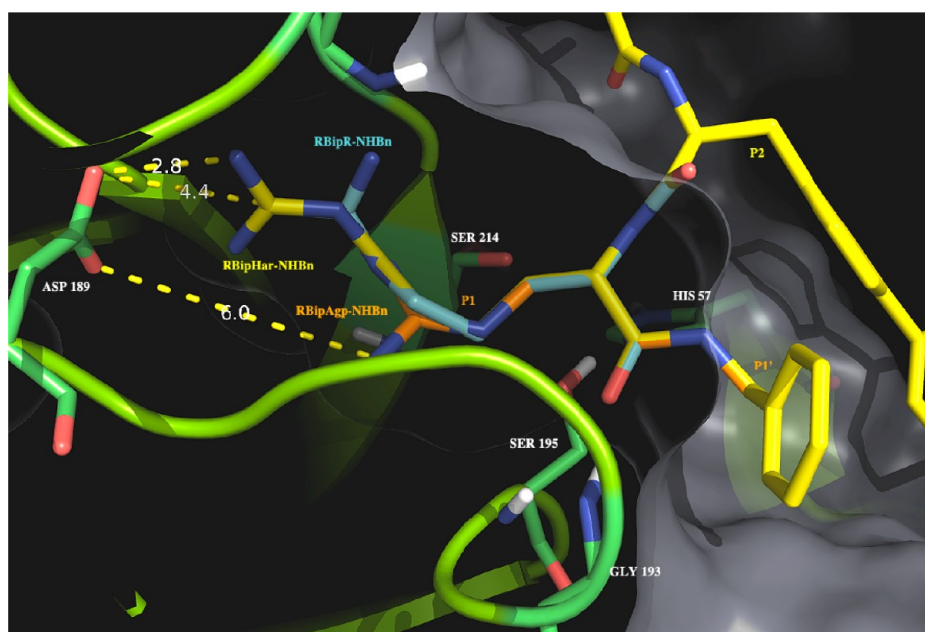

Figure 6. Close up of the bond distances for the cationic P1 unit for peptides **1** (blue), **5** (orange), and **6** (yellow) bound according to binding mode I illustrating the distances for bond formation with Asp189 at the bottom of S1.

arginine analogues gave lower coupling yields, in particular homoarginine (Har) and Agp. Agp often required long recouplings with DIC/HOBt in addition to PyBOP/DIPEA. Attempts to also include peptides incorporating norarginine with two methylenes in its side chain were unsuccessful as has been previously reported for similar peptides.[12] These shorter analogues represented a challenge and a possible cause is sterical interference between the short bulky basic side chain and the free N-terminus of the resin or bulky Bip during coupling. Other studies incorporating Agp into larger peptides do not mention low yields,[21,22] and it may be a phenomenon dependent on the unusual nature of these antibacterial tripeptides mainly incorporating unnatural amino acids. The peptides were divided into four libraries, each designed to focus on a particular structural feature in the active site of trypsin.

Library 1. R-Bip-Y-NHBn (peptides **1**−**7**) S1 pocket: The S1 specificity pocket of trypsin is negatively charged due to Asp189 at the bottom which is able to attract the cationic side chains of Arg and Lys in natural peptide substrates with high specificity.[8] The type of peptide studied carries multiple cationic residues to maintain a high bioactivity, and library 1 was designed with a range of different natural and unnatural cationic amino acids as C-terminal residues to study the S1 interaction. Peptide **1** has previously been shown to be rapidly degraded by trypsin with its C-terminal Arg as P1 residue, and it was therefore included as a reference peptide for the assays.[11]
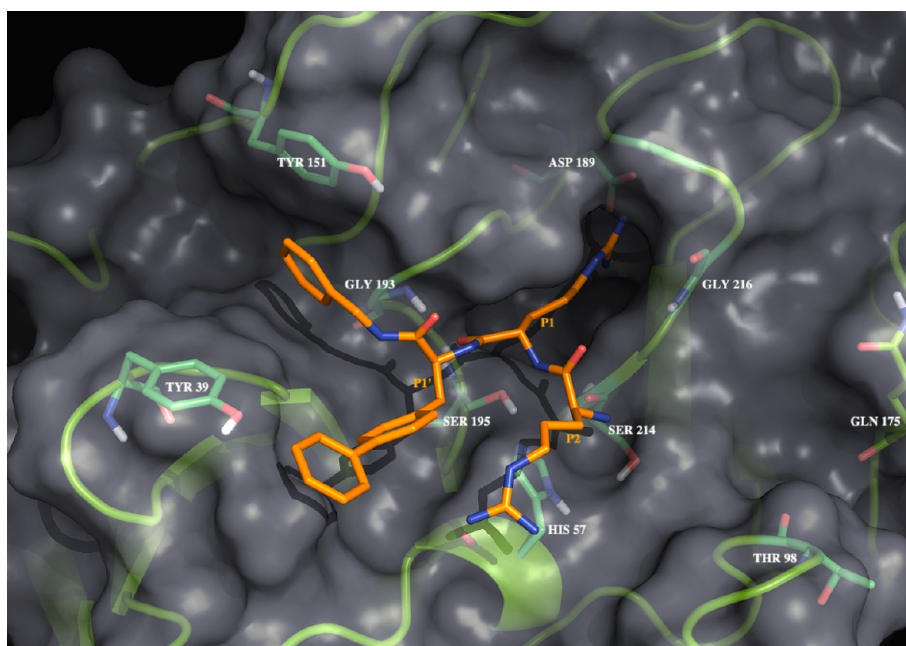
Figure 7. Illustration of the unusual binding mode of peptide **17** with Arg in both S1 and S2.

ITC studies indicate a stoichiometric interaction between trypsin and **1** with a dissociation constant of 11.3 μM. The docking suggested two plausible binding modes for **1** to trypsin with either the C- or the N-terminal Arg in the S1 pocket (see Table S2 Supporting Information for docking scores) with a 5.1 kJ/mol difference in ΔG in favor of binding mode I. The modeled interaction between **1** and trypsin, based on the experimental degradation products and the docking, is shown in Figure 5.

Peptide **2** incorporates Lys as P1 residue yielding an increased half-life from 1 to 2.8 h. This is likely due to the less favorable bonding between the primary amine of Lys in **2** and Asp189, which is further reflected in its 20 μM K_D. The difference in potential number of hydrogen bonds between Arg and Lys and carboxylates and phospholipid head groups does not only affect the stability of these peptides but also the antibacterial effect as has been shown recently.[18,23] Incorporation of the bulky unnatural 2-amino-3-(4-guanidinophenyl)-propanoic acid (Gpp) subunit of **3** further increases the half-life to nearly 5 h. Gpp bears a basic guanidine group, but the rigid and bulky phenyl group seems to lower the susceptibility to degradation. Peptide **3** nevertheless binds with a low 5.5 μM K_D and a ΔG of −30 kJ/mol, indicating an overall stronger affinity for trypsin in comparison with both **1** and **2**. Peptides **4**−**6** all incorporate unnatural side chains that, when placed in the C-terminal positions, yield peptides that are stable toward tryptic degradation. Peptides **4** and **5** both contain analogues (Orn and Agp, respectively) that are shorter than Lys and Arg, while the Har in **6** incorporates an additional methylene in comparison with Arg. The shorter P1 chain length of **4** and **5** effectively prevent optimal interactions with Asp189 in the S1 pocket. The side chain of Har is long enough to extend into the S1 pocket to generate a binding that theoretically would place the peptide bond within reach of the activated hydroxyl of Ser195. Docking of peptides **1**, **5**, and **6** into the active site of trypsin is shown in Figure 6 and illustrate how both **1** and **6** are within reach for electrostatic interactions while the guanidine of **5** is placed some 6 Å away from the Asp189 carboxylate, significantly

weakening the interaction and placing it out of reach for hydrogen bonding.[36]

Peptides **4** and **6** bind to trypsin with different ITC binding profiles compared to other analyzed peptides, both stable and unstable. Low amounts prevented inclusion of peptide **5** in the ITC study. The trypsin binding of **4** and **6** was not stoichiometric, and they displayed a larger binding contribution from ΔH. This behavior suggests a different binding mode than for the unstable peptides, which is further supported by the docking experiments which place the N-terminal Arg in S1 according to binding mode II for most stable peptides (Supporting Information Figure S3). For peptide **5**, the calculated gain in binding energy for binding mode II is 4.0 kJ/mol, illustrating how Agp is less preferred by trypsin in S1. Interestingly, this is also a suggested binding mode for peptide **1**, implying that it can bind in several ways. Peptide **6** can also bind according to both modes according to the docking. On the basis of the metabolite analysis, however, only binding mode I will induce tryptic degradation. These findings suggest that the stability induced by peptides **4**−**6** either arises from an S1 binding that is altered or the fact that these peptides instead bind to trypsin according to the stable binding mode II placing the N-terminal Arg in the S1 pocket. Other studies including Agp in larger peptides suggest that both situations are plausible and the binding modes are highly sequence dependent.[21,22] Peptide **7** was finally included to investigate the effect of overall hydrogen bonding. With Gpp in S1 and a Lys in S3, it is shown that the lower hydrogen bonding capacity of the primary amine yields higher stability in comparison with peptide **3**, indicating that the distant S3 binding site also may be used to tune the stability to some extent. Crystallization experiments to further investigate the binding modes have so far been unsuccessful for these peptides and serine proteases (unpublished data).

Library 2. Y-Bip-R-NHBn (peptides **1** and **8**−**13**) S3 pocket: The S3 site of trypsin is little studied and it is believed to have less effect on the role of trypsin as a digestive enzyme. In chymotrypsin substrates, the P3 side chain is pointing out of the active site and the S3 pocket itself, based around Gly216,

has been shown to display minimal substrate discrimination and have even been reported to harbor D-amino acids.[9] In other proteases, however, it acts as an important pocket as illustrated by elastase where the P3/S3 interaction is the dictating one.[37] All the included peptides of library 2 were effectively cleaved by trypsin according to binding mode I and no general stability trend was seen for the peptides. Peptide **9** was digested at twice the rate compared to **1** while the stability was increased for all remaining peptides in library 2. Peptides **8** and **11** were included in the ITC study and both bound stoichiometrically. Of interest is the significant 5-fold increase in half-life for peptide **11** incorporating Orn as P3 unit, which is also reflected in its elevated 37.2 μM dissociation constant compared to **1** and **8** with comparable half-lives. Peptides **10**, **12**, and **13** all displayed similar half-lives. This illustrates that the S3 site can be targeted to design improved peptides but in a much less obvious manner than the sites closer to Ser195 in the active site.

Library 3. Y-Bip-Y-NHBn (peptides **1** and **14−16**) S1 and S3 pocket: Library 3 was composed of peptides with identical cationic residues in both the S1 and S3 pockets to investigate the potential additive effects. Peptide **14**, with two Lys residues, was clearly more stable than reference peptide **1** and peptides **2** or **8** with single Lys residues in either the S1 or S3 sites. Peptide **15**, with two Gpp units, was less stable than peptide **3** due to the destabilizing effect seen for Gpp in S3 in **9** that counteracts the S1 stabilization of Gpp. Peptide **16**, incorporating two beneficial Agp units, was stable toward degradation.

Library 4. Scrambled peptides Y-R-Bip/Trp-NHBn (peptides **17−22**) S1 and S1′ pocket: While shown to be beneficial for activity, the hydrophobic bulk and cationic residues incorporated into the peptides do not have to be arranged in the manner represented by peptide **1**. The bioactivity is dependent on a certain degree of amphiphilicity, and this opens up for alternative assemblies with maintained biological activity and altered stabilities for this particular class of peptides.[12,19] Library 4 includes such "scrambled" peptides to evaluate if sequence scrambling could have an effect on the stability. Peptides including Trp were included to provide additional S1′ data from a naturally occurring hydrophobic amino acid side chain for comparison with Bip and the C-terminal benzylamide. Peptides **17** and **20** were very rapidly digested and generally degraded prior to the first data point. For this reason, they could not be studied using ITC. Analysis of the degradation products revealed degradation according to binding mode III illustrated in Figure 7 for peptide **17**.

This was unexpected as it places the N-terminal Arg in S2, a binding pocket which generally prefers hydrophobic residues as P2 substituents. Previous studies on chymotrypsin inhibitors have reported similar unforeseen binding of both Lys and Arg in S1 (hydrophobic in chymotrypsin). In those studies, the cationic side chains were bent in an attempt to minimize the interactions with the hydrophobic pocket in S1.[12,38] Studies of the tryptic stability of human growth hormone releasing hormone fragments further indicate that both Har and Orn can act as stabilizing P2 units when positioned next to a P1 Lys.[39] Placing the less favored Gpp in S1 significantly increased the half-life to 3.5 and 8.0 h for peptides **18** and **21**, respectively. The difference between **18** and **21** illustrates that S1′ prefers the large unnatural Bip side chain over the natural Trp indole. The preferential binding of such a side chain explains why trypsin accepts the unfavorable S2 Arg, and the contribution from a beneficial S1′ interaction in conjunction with a benzyl

group in S2′ is the believed to be the driving force for the trypsin binding of these scrambled peptides. Including peptides **19** and **22** with a central uncharged 2-amino-3-(4-aminophenyl)propanoic acid (App) (pK_a ∼5.0) yields stability although at the expense of a lowered biological activity. Peptide **22** has previously been shown to possess a high chymotryptic stability.[12] Both these peptides bound to trypsin with 2.6 and 14.9 μM dissociation constants without degradation, suggesting favorable interaction of the App with the S1′ pocket according to binding mode II.

Organ Extract Stability. The long-term goal is to generate peptide antibiotics with an overall metabolic stability, enabling oral administration, uptake, and sufficient half-life to reach the site of action. It has previously been shown that peptides of this class have high plasma stability,[40] and their binding to plasma proteins is currently under investigation.[41] In a recent study by Svenson et al., it was shown that the enzyme half-lives are not absolute but should only be viewed as relative stabilities for comparing the different peptides[20] as the in vivo situation in the GI tract is different from assays using isolated enzymes. To investigate the actual overall resilience toward the metabolic hurdles in stomach, duodenum, and the liver, whole organ extracts were used. This represents a situation closely mimicking the in vivo conditions. For this study, the stable and highly active peptides **5** and **6** were selected and compared with metabolic data for **1**.[20]

Despite being a tripeptide containing unnatural amino acids and a C-terminal benzyl amide capping, peptide **1** is targeted by several metabolic enzymes, yielding a half-life of a mere 10 min in the stomach extract and 6 min in the duodenum.[20] These are values preventing an uptake of this peptide in the intestine. Peptide **5**, incorporating a P1 Agp, displayed a substantial increase in organ extract half-life in comparison with **1**. In the stomach, the half-life was 702 min and in the duodenum 324 min, representing 70- and 54-fold increases. Peptide **6**, with a P1 Har, also displayed significant stabilities, with half-life values of 401 min in the stomach and 82 min in the duodenum. These are improved stabilities providing sufficient time to allow for uptake in the mammalian intestine. Analysis of the metabolite formation supports the data from the isolated enzyme assays. At tissue pH, the main degradation in the stomach is cleavage of the N-terminal Arg. Interestingly, no cleavage is seen in stomach at pH 2.0 (see Table S4 in Supporting Information), showing how these compounds also are resistant toward the main modes of pepsin cleavage in analogy to the serine proteases. In the duodenum, the N-terminal Arg is the most sensitive residue likely targeted by the aminopeptidase B exopeptidase. Tryptic cleavage of the benzyl amide, which would be the result of the unnatural cationic amino acids in the S1 site, is not seen, which demonstrates that these peptides do not bind according to binding mode I. The minute amount of R-Bip-OH in the duodenum extract (Supporting Information Figure S1) further illustrates the stability of these peptides toward chymotryptic degradation. In comparison with **1**, the liver stabilities were comparable, ranging from 64 min for peptide **6** to 191 min for peptide **5**. Again, aminopeptidase B is believed to be responsible for the majority of the N-terminal metabolism.[20]

■ CONCLUSION

In the current paper, the design, preparation, and evaluation of 22 antibacterial peptides is described. The stability of the peptides toward tryptic degradation has further been studied

using several analytical methods. The systematic incorporation of unnatural arginine analogues has been used as a strategy to evaluate mainly how the S1/P1 and S3/P3 interactions can be used to toggle the stability while maintaining bioactivity. The antibacterial activity of the peptides remained high upon incorporation of analogues, indicating that the length of the cationic side chains is of less significance for their mode of action. The interaction between peptides and trypsin was studied using half-life assays, ITC, and molecular modeling. It was shown that several of the unnatural cationic residues were able to generate stable peptides when incorporated in the P1 position. Both longer and shorter side chains could be used to yield stability. The P3 position could also be used to toggle the stability but in a less obvious manner. In combination with the previous trypsin study investigating the P2, P1′, and P3′ positions, a clear picture of how the individual binding pockets surrounding the active site of trypsin can be challenged to generate peptides with tryptic stability is now generated. A total of 46 different antibacterial peptide substrates incorporating both hydrophobic and cationic unnatural amino have been prepared and analyzed. Synthetically, most of the amino acid substitutions represent no major increased costs or additional synthetic steps, making such a strategy very appealing compared to other modes of generating metabolic stability. The most promising peptides were also studied in whole organ extracts, and their survival in the main metabolic compartments indicates that these subtle structural alterations also provide protection against other proteolytic enzymes. These bioactive peptides can now be prepared with sufficient activity and stability to allow for oral administration and potential uptake. The structural information gained about the preferential binding of unnatural amino acids in the binding pockets of the active site of trypsin allows the findings to be used in a general fashion to prepare stable peptides and peptidomimetics. The findings are believed to be particularly applicable to cationic antimicrobial peptides acting at the bacterial membrane level as they generally maintain their activity upon incorporation of unnatural cationic analogues.

■ MATERIALS AND METHODS

Materials. Essentially salt-free trypsin from bovine pancreas (T8802, 10000−15000 BAEE units/mg protein) was supplied by Sigma-Aldrich. Fmoc protected L-amino acids, Trp(Boc), Phe, and Lys(Boc), were purchased from Fluka. Biphenylalanine (Bip), Orn(Boc), Arg(Pbf), Homoraginine(Pmc) (Har), and 2-amino-3-(4-aminophenyl)propanoic acid(Boc) (App) were provided by Bachem. 2-Amino-3-(guanidino)propanoic acid(Bis-Boc) (Agp(Boc)₂) were supplied by Bapeks (Riga, Latvia). Aldrich and Fluka provided 2-amino-3-(4-guanidinophenyl)propanoic acid(Bis-Boc) (Gpp). 1-Hydroxybenzotriazole (HOBt), piperidine, N-ethyldiisopropylamine (DIPEA), benzylamine, and triisopropylsilane (TIS) were purchased from Fluka. (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) and 4-(4-formyl-3,5-dimethoxy-phenoxy)-butyric acid (Backbone amide linker, BAL) were purchased from Novabiochem. N,N′-Diisopropylcarbodiimide (DIC) was supplied by Aldrich, and Merck provided trifluoroacetic acid (TFA). The Rink amide resin and NovaSyn TG amino resin were purchased from NovaBiochem. Krebs−Henseleit buffer was purchased from Sigma. Solvents were purchased from Merck, Riedel-de Haën or Aldrich and used without further purification.

Solid Phase Peptide Synthesis (SPPS). The peptides were prepared using SPPS, employing standard Fmoc coupling chemistry on NovaSyn TG amino resin with BAL.[42,43] For C-terminal amide modification, NovaSyn TG amino resin was washed with DMF before BAL (3 equiv), HOBt (4.5 equiv), and DIC (3.3 equiv), dissolved in

enough DMF to cover the resin, were added. The mixture was subsequently irradiated in a Biotage Initiator microwave oven at 60 °C for 20 min.[44] The solution was removed, and the resin was washed with DMF (×5), DCM (×3), and MeOH (×2) before adding benzylamine (10 equiv) and NaBH₃CN (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. The peptide elongation for all the resins was performed with 3 equiv of amino acid, PyBOP (3 equiv), and DIPEA (6 equiv). 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 the next coupling. 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 with a mixture of TFA:TIS:H₂O (95:2.5:2.5 v/v/v), which yielded a crude that was further purified.

Peptide Purification and Analysis. The peptides were purified and isolated using reversed phase HPLC on a Delta-Pak (Waters) C₁₈ column (100 Å, 15 μm, 25 mm × 100 mm) with a mixture of water and acetonitrile (both containing 0.1% TFA) as eluent. The purity of the peptides was further analyzed by RP-HPLC using an analytical Delta-Pak (Waters) C₁₈ column (100 Å, 5 μm, 3.9 mm × 150 mm) and positive ion electrospray mass spectrometry on a VG Quattro quadrupole mass spectrometer (VG Instruments Inc., Altringham, UK).

Antibacterial Testing. MIC determinations on *Staphylococcus aureus*, strain ATCC 25923, and methicillin resistant *Staphylococcus aureus* (MRSA) strain ATCC 33591 were performed by TosLab AS using standard serial dilution methods.[45]

Measurements and Calculation of Peptide Half-Life. Each peptide was dissolved in a 0.1 M NH₄HCO₃ buffer (pH 8.2) to a final peptide concentration of 1 mg/mL. A trypsin solution was prepared by dissolving 1 mg of trypsin in 50 mL of 0.1 M NH₄HCO₃ buffer (pH 8.2). For the stability determination, 150 μL of freshly made trypsin solution and 150 μL of peptide solution were incubated in 1200 μL of 0.1 M NH₄HCO₃ buffer (pH 8.2) 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 2% TFA, and analyzed by RP-HPLC as described above. Samples without trypsin were taken at 0 h and after 24 h at 37 °C and used as negative controls. Peak areas at 214 nm for samples taken during the first 5 h of the assay were used to calculate the peptide half-life ($\tau_{1/2}$) using Medical calculator from Cornell University.[46] Peptides that displayed less than 10% degradation after 24 h were considered stable. MS analysis was employed to identify the formed metabolites.

In Vitro Metabolism in Mouse Stomach, Duodenum, and Liver. Organ homogenates were prepared as described previously by Vergote et al.[47] In brief, stomach, duodenum, and liver were collected from male CD-1 mice. Each organ was cleaned and washed in ice cold Krebs−Henseleit buffer pH 7.4 before about 1.5 g was transferred to 50 mL rotor−stator disperser tubes. Then 36 mL of ice cold Krebs-Henseleit buffer was added and the organ was homogenized with an IKA Ultra-Turrax (Staufen, Germany) for 1 min. After sedimentation for 30 min at 5 °C to remove the larger particles, 25 mL of the middle layer was taken as final homogenate, with aliquots stored at −35 °C until further use. Prior to use, the protein content of each homogenate was determined using the Pierce Modified Lowry Protein Assay method. A stock solution with a protein concentration of 0.6 mg/mL was prepared by dilution with Krebs−Henseleit buffer or 0.2 M HCl/KCl buffer pH 2 (for stomach analysis at pH 2.0 only).

In vitro metabolic stability testing was determined using established procedures.[20,47] Briefly, 100 μL of peptide dissolved in Krebs−Henseleit buffer pH 7.4 (1 mg peptide/mL) was added to 900 μL of organ extract (500 μL of homogenate + 400 μL of Krebs−Henseleit buffer pH 7.4 = 300 μg of protein in total) and kept at 37 °C while shaking at 750 rpm in an Eppendorf Thermomixer Comfort

(Hamburg, Germany). A 0.2 M HCl/KCl buffer pH 2 was used instead of Krebs−Henseleit buffer for the stomach analysis at pH 2.0. After 0, 15, 30, 60, 120, and 240 min of incubation, 100 μL samples were collected directly into tubes containing an equal volume of aqueous TFA (1% v/v). The enzymatic activity was terminated by additional heat inactivation at 95 °C for 5 min, after which the samples were cooled at 5 °C for 30 min. Centrifugation at 20000g for 5 min at 5 °C yielded a clear supernatant ready for HPLC analysis. Blank control solutions were prepared accordingly but without added peptide. Control reference solutions were prepared without tissue homogenate or with a prior heat inactivation to confirm chemical stability and mass balance, respectively. The degradation of the peptides was studied using an HPLC system consisting of a Waters Alliance 2695 separation module and a Waters 2996 photodiode array detector fitted with Empower 2 software for data handling (Waters, Milford, MA, USA). PDA detection was performed between 190 and 400 nm, with quantification at 254 nm. For a standard HPLC run, a 20 μL sample was injected and separated on an Alltima C_{18} (250 mm × 4.6 mm, 5 μm particle size) column (Alltech, Deerfield, IL, USA) at 1 mL/min in an oven set at 30 °C. Mixtures of water (0.1% TFA, (v/v)) and acetonitrile (0.1% TFA, (v/v)) were used to create appropriate gradients for separation of peptides and their metabolites.

Isothermal Titration Calorimetry Studies. Heats of interaction were determined using a CSC 5300 Nano-Isothermal Titration Calorimeter III with a 1 mL cell volume (Calorimetry Sciences Corporation, Utah, USA). In a typical titration experiment, the peptide (2.1 mM) was added in 20 aliquots (5 μL each) to a stirred (200 rpm) solution of trypsin (0.1 mM) in an aqueous buffer at 25 °C. The buffer used was 50 mM Tris-HCl, 10 mM $CaCl_2$ at pH 8.2. An interval of 300 s between the injections was allowed for the interacting species to reach equilibrium. The heats of dilution were determined in a similar fashion where the peptides were added to a stirred buffer solution without trypsin. Subtraction of the dilution heat yielded the heat of interaction, and a binding isotherm from which the association constant and complex stoichiometry was calculated using BindWorks analysis software.

Computational Studies. Molecular models of peptides were built with Maestro version 9.1.[48] The geometries were first relaxed with the UFF minimized in Maestro to eliminate distortions. Further minimization of the peptide geometries was done with Macromodel version 9.6[49] to prepare the peptides for the docking experiment. The OPLS2005[50] force field was used for all optimizations with the conjugant gradient minimization scheme PRCG[51] 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 0.005. The minimized peptides were then further prepared for the docking experiments with LigPrep[52] using the OPLS2005 force field. LigPrep was set to generate possible states at target pH between 6.5 and 8.5, at most 32 per ligand. ConfGen[53−55] was then used to generate a library of different peptide conformers (approximately 200 conformers for each CAP) using the fast search strategy set to minimize all output structures. Coordinates for trypsin were obtained from the crystal structure of the BPTI variant (Cys15 → Ser) in complex with trypsin with the entry code 2FI4.[56] The protein was prepared for the docking experiment in Maestro 9.1 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.[57] The BPTI ligand was truncated to only include residues 15 (P1) and 16 (P1′), where residue 15 is Lys. These two residues are expected to be oriented correctly in the active site for cleavage and were therefore specified as a ligand molecule in the grid generation. This truncated BPTI 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 Ser190,

Ser195, Ser214, and Ser228. A high throughput virtual screening (HTVS) was performed prior to calculations with Extra Precision (XP) to decrease the CAP library size. 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.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

Purity data, HPLC-traces, and ^{1}H NMR spectra for all the tested peptides together with docking scores, whole organ metabolism profiles, and additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

CAP, cationic antimicrobial peptide; ITC, isothermal titration calorimetry; DIPEA, diisopropylethylamine; 1-HOBt, 1-hydroxybenzotriazole; PyBOP, (benzotriazol-1-yloxy)-tripyrrolidinophosphonium hexafluorophosphate; MIC, minimal inhibitory concentration; DMF, dimethylformamide; App, L-2-amino-3-(4-aminophenyl)propanoic acid; Gpp, L-2-amino-3-(4-guanidinophenyl)propanoic acid; Orn, L-ornithine; Har, homoarginine; Agp, L-2-amino-(3-guanidino)propanoic acid; ATCC, American Type Culture Collection

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