Antimicrobial Peptides with Stability toward Tryptic Degradation[†]

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ABSTRACT: The inherent instability of peptides toward metabolic degradation is an obstacle on the way toward bringing potential peptide drugs onto the market. Truncation can be one way to increase the proteolytic stability of peptides, and in the present study the susceptibility against trypsin, which is one of the major proteolytic enzymes in the gastrointestinal tract, was investigated for several short and diverse libraries of promising cationic antimicrobial tripeptides. Quite surprisingly, trypsin was able to cleave very small cationic antimicrobial peptides at a substantial rate. Isothermal titration calorimetry studies revealed stoichiometric interactions between selected peptides and trypsin, with dissociation constants ranging from 1 to 20 μM. Introduction of hydrophobic C-terminal amide modifications and likewise bulky synthetic side chains on the central amino acid offered an effective way to increased half-life in our assays. Analysis of the degradation products revealed that the location of cleavage changed when different end-capping strategies were employed to increase the stability and the antimicrobial potency. This suggests that trypsin prefers a bulky hydrophobic element in S1′ in addition to a positively charged side chain in S1 and that this binding dictates the mode of cleavage for these substrates. Molecular modeling studies supported this hypothesis, and it is shown that small alterations of the tripeptide result in two very different modes of trypsin binding and degradation. The data presented allows for the design of stable cationic antibacterial peptides and/or peptidomimetics based on several novel design principles.

While it is possible to prepare a wide array of pharmacologically active peptides, their unfavorable properties, with regard to administration, narrow therapeutic index, toxicity, and stability, limit their applicability *in vivo*. Stability toward proteolytic degradation is important for therapeutic peptides and proteins as a means of increasing the plasma half-life (*1*). Furthermore, if the site of action is a metabolic active lesion (e.g., a wound), resistance against degradation in the lesion is of utmost importance for administration of the drug to be meaningful. In addition, for a drug to be orally active, stability toward degradation in the gastrointestinal (GI) tract is a required property. Peptides are usually metabolized in the body by enzymatic cleavage of the amide bonds joining the amino acids together. The proteolytic enzymes responsible for this degradation fall into two general classes, the exopeptidases, which cleave the terminal amino acids, and the endopeptidases, which cleave at more or less specific sites within the peptide sequence (*2*). The medicinal chemist has several tools to circumvent enzymatic degradation catalyzed by proteolytic enzymes (*3, 4*). The action of exopeptidases can usually be prevented by so-called end capping, i.e., chemical modifications of the N- and C-terminal amino or carboxylic acid functionalities (*5*). Typical end cappings include amidation of the C-terminus and acetylation or deamination of the N-terminus. The usual method of inhibiting degradation by endopeptidases is methylation of the amide nitrogen atom (*6, 7*) or inversion of the stereochemistry of the amino acid(s) in the vicinity of the scissile peptide bond (*8, 9*). The advantage with these methods is that the peptide retains most of its structure unaltered, which hopefully will lead to an unaffected bioactivity, although there are ample examples of changed and often diminished biological activity caused by these changes (*10*). Creating cyclic analogues of linear peptides can also increase the stability of the analogue (*11–13*).

A more radical approach to solve the problem of enzymatic degradation is to avoid hydrolyzable amide bonds altogether by introducing different peptidomimetic elements at strategic positions in the structure (*3, 14, 15*). A major drawback with the peptidomimetic approach is the need for a generally much more complex synthesis and an increased uncertainty in the prediction of the bioactivity. It should thus be evident that there is no panacea for obtaining stability against degrading enzymes in a peptide. However, the present methods at hand offer the chemist many possible ways, each with its own merits and drawbacks, of avoiding degradation on a more case-specific basis.

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For degradation of proteins and peptides, the most important endopeptidases in the human GI tract are pepsin, found in the stomach, and trypsin and chymotrypsin, found in the upper part of the intestine. Trypsin and chymotrypsin, mechanistically classified as serine proteases similar in their three-dimensional structure, although differing in substrate specificity, are responsible for the majority of this degradation. Chymotrypsin cleaves the peptide bond on the C-terminal side of large lipophilic amino acids (Phe, Tyr, or Trp) whereas trypsin cleaves on the C-terminal side of the cationic residues Lys and Arg (*16*).

Cationic antimicrobial peptides (CAP)[1] represent an important class of structurally diverse, potentially clinically useful peptides (*17–19*). Application areas that have been suggested include treatment of infections caused by both antibiotic-resistant bacteria as well as fungi. CAPs with antitumoral properties have also been identified and synthesized (*20*). The metabolic instability inherent in most CAPs is, however, a major cause of the problems associated with bringing these promising novel antibiotic drugs onto the market (*19*).

For more than a decade, our research group has focused on the transformation of naturally occurring antibacterial peptides into potential future antibiotic drugs (for a recent review see ref (*21*)). The results from our research into the medicinal chemistry of lactoferricin-based antibacterial peptides have enabled us not only to increase the potency but also to reduce the size of these antibacterial peptides (*21, 22*). Ultimately, we have identified a minimal set of structural motifs, i.e., a pharmacophore for antibiotic activity, and this has led to the development of extremely small CAPs. Against the common skin pathogen *Staphylococcus aureus* this motif can be defined as the presence of two cationic sites and two elements of lipophilic bulk each approximately the size of a phenyl group. The cationic sites in CAPs are usually furnished by the presence of cationic residues such as lysine or arginine in the sequence.

By being unavoidably cationic, CAPs are excellent substrates for trypsin. As the positively charged side chains are essential for the activity, other structural targets are needed to alter the stability of these peptides (*21–23*). It was thought that it would be possible to avoid tryptic degradation by reducing the size of the peptide since trypsin generally prefers its substrates to be of a certain length (*8, 24, 25*), but surprisingly it turned out that a large number of these short (<6 amino acids) chains were rapidly degraded by trypsin. One such peptide is Arg-Trp-Arg-NHBn (CAP 1), which comprises three positive charges and two hydrophobic elements and displays medium antibacterial activity. CAP 1 is, however, effectively hydrolyzed by trypsin to produce the tripeptide carboxylic acid derivative and benzylamine, with a half-life of just over 1 h in our assay system.

A larger investigation aimed at exploring potential molecular features in short cationic antimicrobial peptides that could confer an increased stability toward trypsin with the purpose of increasing the number of methodologies available to the biochemist for making metabolically stable peptides within the pharmacophore of CAPs was thus launched. By keeping the peptide backbone unaltered, the focus was on novel structural features such as hydrophobic side-chain modifications and different end-capping strategies to achieve increased stability toward trypsin. Results from the degradation and calorimetric studies in conjunction with molecular modeling experiments, which are presented in this paper, allowed for the generation of a series of guidelines useful for the future design of short cationic antimicrobial peptides with high resilience against tryptic degradation. It is also anticipated that these guidelines will be of general applicability for stabilization of other small cationic peptides against tryptic degradation.

MATERIALS AND METHODS

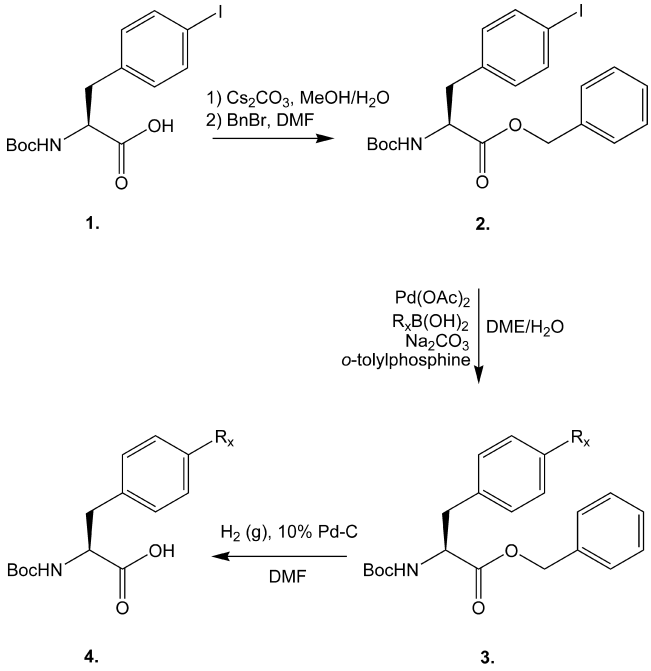
Chemicals. Essentially salt-free trypsin from bovine pancreas (T8802, 10000–15000 BAEE units/mg of protein) was supplied by Sigma-Aldrich. Protected amino acids Boc-Trp-OH, Boc-Arg-OH, Boc-4-phenyl-Phe-OH, and Ac-Arg-OH were purchased from Bachem AG while Boc-4-iodophenylalanine, Boc-3,3-diphenylalanine, and Boc-(9-anthryl)alanine were purchased from Aldrich. Benzylamine, 2-phenylethylamine, 3-phenylpropylamine, (*R*)-2-phenylpropylamine, (*S*)-2-phenylpropylamine, *N*,*N*-methylbenzylamine, *N*,*N*-ethylbenzylamine, and *N*,*N*-dibenzylamine, used in modifying the C-terminus of the peptides, were purchased from Fluka except for *N*,*N*-ethylbenzylamine, which was purchased from Acros. Diisopropylethylamine (DIPEA), 1-hydroxybenzotriazole (1-HOBt), chlorotripyrrolidinophosphonium hexafluorophosphate (PyCloP), and *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Fluka. 4-*n*-Butylphenylboronic acid, 4-*tert*-butylphenylboronic acid, 4-biphenylboronic acid, 2-naphthylboronic acid, tri-*o*-tolylphosphine, benzyl bromide, and palladium acetate were purchased from Aldrich. Solvents were purchased from Merck, Riedel-de Haën, or Aldrich and used without further purification with the exception of CH_2Cl_2, which was filtered through alumina before use.

Preparation of Amino Acids. Noncommercially available amino acid derivatives were prepared according to the general scheme outlined in Scheme 1.

*Benzylation of Boc-4-iodophenylalanine (**2**).* Boc-4-iodophenylalanine (**1**, 1 equiv) was dissolved in 90% methanol in water and neutralized by addition of cesium carbonate until it was weakly alkaline (determined with litmus paper). The solvent was removed by rotary evaporation, and any remaining water in the cesium salt of Boc-4-iodophenylalanine was further reduced by repeated azeotropic distillation with toluene. The resulting dry salt was dissolved in dimethylformamide (DMF). Benzyl bromide (1.2 equiv) was added, and the resulting mixture was stirred for 6–8 h. The solvent was removed under reduced pressure, producing an oil containing the title compound (**2**). The oil was separated between ethyl acetate and acetic acid (15% v/v), and the organic phase was washed with equal volumes of citric acid solution (5% v/v, two times), a saturated solution of $NaHCO_3$, and brine. The title compound was isolated as a pale yellow oil in 85% yield after purification by flash chromatography using CH_2Cl_2−ethyl acetate (95:5 v/v) as eluent. Crystalline benzyl Boc-4-iodophenylalanine could be

[1] Abbreviations: CAP, cationic antimicrobial peptide; ITC, isothermal titration calorimetry; DIPEA, diisopropylethylamine; 1-HOBt, 1-hydroxybenzotriazole; PyCloP, chlorotripyrrolidinophosphonium hexafluorophosphate; MIC, minimal inhibitory concentration; DMF, dimethylformamide.

Scheme 1: General Procedure for Preparation of Amino Acid Derivatives Using the Suzuki−Miyaura Reaction



obtained by recrystallization from *n*-heptane. Spectral data: ESMS 504.1 (calcd 504.1, M + Na^+); ^{1}H NMR ($CDCl_3$) δ 1.35 (s, 9H), 2.89–2.99 (m, 2H), 4.52 (d, J = 7.8 Hz, 1H), 4.90–4.92 (d, J = 7.8 Hz, 1H), 4.99–5.12 (AB system, J = 12.3 Hz, 2H), 6.68–7.46 (m, 9H); ^{13}C NMR ($CDCl_3$) δ 24.0, 28.0, 38.0, 54.0, 68.0, 128.6, 128.7, 131.4, 135.0, 135.6 137.6, 155.0, 172.0 ppm.

General Procedure for Suzuki−Miyaura Couplings. Benzyl Boc-4-iodophenylalanine (1 equiv), arylboronic acid (1.5 equiv), sodium carbonate (2 equiv), palladium acetate (0.05 equiv), and tri-*o*-tolylphosphine (0.1 equiv) were added to a degassed mixture of dimethoxyethane (6 mL/mmol of amino acid) and water (1 mL/mmol of amino acid). The reaction mixture was kept under argon and heated to 80 °C for 4–6 h. After being cooled to room temperature, the mixture was filtered through a short pad of silica gel and sodium carbonate. The filter cake was further washed with ethyl acetate and combined with the other fraction before the solvents were removed under reduced pressure. The products were purified using flash chromatography using mixtures of ethyl acetate and *n*-hexane as eluent.

*Preparation of Boc-Bip(t-Bu)-OBn (**3a**).* The title compound was prepared in 79% yield from 4-*tert*-butylphenylboronic acid using the general procedure for Suzuki−Miyaura couplings. **3a** was isolated after flash chromatography employing ethyl acetate−*n*-hexane (80:20 v/v) as eluent. Spectral data: ESMS 510.3 (calcd 510.3, M + Na^+); ^{1}H NMR ($CDCl_3$) δ 1.33 (s, 9H), 1.43 (s, 9H), 3.16 (m, 2H), 4.70 (m, 1H), 5.05 (d, J = 7.4 Hz), 5.14−5.24 (AB system, J = 12.3 Hz, 2H), 7.13–7.55 (m, 13H); ^{13}C NMR ($CDCl_3$) δ 28.0, 32.0, 35.0, 38.0, 54.0, 68.0, 125.7, 126.7, 127.1, 128.5, 128.6, 129.8, 134.6, 135.2, 137.9, 139.8, 150.3, 171.8 ppm.

*Preparation of Boc-Bip(4-Ph)-OBn (**3b**).* The title compound was prepared in 61% yield from 4-biphenylboronic acid using the general procedure for Suzuki−Miyaura couplings. **3b** was isolated by recrystallization of the crude product from *n*-heptane. Spectral data: ESMS, compound not soluble in suitable solvent; ^{1}H NMR ($CDCl_3$) δ 1.47 (s, 9H), 3.18 (m, 2H), 4.72 (m, 1H), 5.06 (d, J = 8.2 Hz, 1H), 5.15–5.25 (AB system, J = 12.3 Hz, 2H), 7.16–7.67 (m, 18H); ^{13}C NMR ($CDCl_3$) δ 28.0, 38.0, 54.0, 68.0, 127.0, 127.1, 127.4, 127.5, 128.5, 128.6, 128.8, 129.9, 135.1, 135.2, 138.2, 139.7, 140.1, 140.7, 171.8 ppm.

*Preparation of Boc-Bip(n-Bu)-OBn (**3c**).* The title compound was prepared in 53% yield from 4-*n*-butylphenylboronic acid using the general procedure for Suzuki−Miyaura couplings. **3c** was purified using 80:20 ethyl acetate−*n*-hexane as eluent. Spectral data: ESMS 510.3 (calcd 510.3, M + Na^+); ^{1}H NMR ($CDCl_3$) δ 0.88 (t, J = 7.8 Hz, 3H), 1.27–1.35 (m, 2H), 1.33 (s, 9H), 1.53–1.61 (m, 2H), 2.58 (t, J = 7.8 Hz, 2H), 3.04 (d, 2H), 4.59 (d, J = 7.8 Hz, 1H), 4.94 (d, J = 7.8 Hz, 1H), 5.02–5.13 (AB system, J = 12.3 Hz, 2H), 7.01–7.41 (m, 13H); ^{13}C NMR ($CDCl_3$) δ 14.0, 23.0, 28.0, 34.0, 35.0, 38.0, 55.0, 68.0, 126.8, 127.1, 128.5, 128.6, 128.8, 129.7, 134.6, 135.2, 138.1, 171.8 ppm.

*Preparation of Boc-Phe[4-(2-naphthyl)]-OBn (**3d**).* The title compound was prepared in 68% yield from 2-naphthylboronic acid using the general procedure for Suzuki−Miyaura couplings. **3d** was isolated by recrystallization of the crude product from *n*-heptane. Spectral data: ESMS 504.3 (calcd 504.2, M + Na^+); ^{1}H NMR ($CDCl_3$) δ 1.36 (s, 9H), 3.08 (m, 2H), 4.61 (m, 1H), 4.98 (d, J = 7.8 Hz, 1H), 5.04–5.15 (AB system, J = 12.3 Hz, 2H), 7.08–7.95 (m, 16H); ^{13}C NMR ($CDCl_3$) δ 28.0, 38.0, 55.0, 67.0, 125.5, 125.7, 126.0, 126.3, 127.5, 127.7, 128.2, 128.4, 128.5, 128.6, 129.9, 132.6, 133.7, 135.1, 135.2, 138.1, 165.0, 172.0 ppm.

General Procedure for Deesterification of Benzyl Esters. The benzyl ester was dissolved in DMF and hydrogenated for 2 days at ambient pressure and temperature using 10% Pd on carbon as catalyst. At the end of the reaction, the catalyst was removed by filtration and the solvent removed under reduced pressure. The free acid was isolated by recrystallization from diethyl ether.

*Preparation of Boc-Bip(4-t-Bu)-OH (**4a**).* The title compound was prepared in 65% yield from **3a** using the general procedure for deesterification. Spectral data: ESMS 420.2 (calcd 420.2, M + Na^+); ^{1}H NMR ($CDCl_3$) δ 1.40 (s, 9H), 1.46 (s, 9H), 3.14–3.30 (m, 2H), 4.68 (m, 1H), 5.00 (d, J = 7.4 Hz, 1H), 7.22–7.58 (m, 8H); ^{13}C NMR ($CDCl_3$) δ 28.0, 32.0, 38.0, 55.0, 128.8, 126.7, 127.2, 129.8, 134.5, 137.8, 139.9, 155.6, 175.6 ppm.

*Preparation of Boc-Bip(4-Ph)-OH (**4b**).* The title compound was prepared in 61% yield from **3b** using the general procedure for deesterification. Spectral data: ESMS 440.1 (calcd 440.2, M + Na^+); ^{1}H NMR ($CDCl_3$) δ 1.37 (s, 9H), 3.09 (m, 2H), 4.56 (m, 1H), 5.00 (d, J = 7.4 Hz, 1H), 7.20–7.59 (m, 13H); ^{13}C NMR ($CDCl_3$) δ 28.0, 38.0, 55.0, 127.1, 127.3, 127.4, 127.5, 128.8, 129.9, 139.6, 140.2, 140.7, 155.5, 175.6 ppm.

*Preparation of Boc-Bip(4-n-Bu)-OH (**4c**).* The title compound was prepared in 61% yield from **3c** using the general procedure for deesterification. Spectral data: ESMS 420.2 (calcd 420.2, M + Na^+); ^{1}H NMR ($CDCl_3$) δ 0.88 (t, J = 7.4 Hz, 3H), 1.27–1.35 (m, 2H), 1.33 (s, 9H), 1.52–1.60 (m,

2H), 2.58 (t, $J = 7.8$ Hz, 3H), 3.03–3.19 (m, 2H), 4.55 (d, $J = 7.8$ Hz, 1H), 4.90 (d, $J = 7.8$ Hz, 1H), 7.01–7.41 (m, 8H); ^{13}C NMR ($CDCl_3$) δ 14.0, 22.0, 28.0, 34.0, 36.0, 38.0, 54.0, 126.8, 127.1, 128.9, 129.8, 138.1, 139.9, 142.1, 155.0, 175.0 ppm.

*Preparation of Boc-Phe[4-(2-naphthyl)]-OH (**4d**).* The title compound was prepared in 68% yield from **3d** using the general procedure for deesterification. Spectral data: ESMS 414.2 (calcd 414.2, M + Na^+); ^{1}H NMR ($CDCl_3$) δ 1.36 (s, 9H), 3.08–3.21 (m, 2H), 4.60 (m, 1H), 4.95 (d, $J = 7.4$ Hz, 1H), 7.19–7.94 (m, 11H); ^{13}C NMR ($CDCl_3$) δ 28.0, 38.0, 54.0, 125.5, 125.7, 126.0, 126.3, 127.6, 127.7, 128.2, 128.5, 130.0, 132.6, 133.7, 135.1, 138.0, 139.9, 155.5, 175.6 ppm.

General Procedure for Solution Phase Peptide Synthesis Using HBTU as the Coupling Reagent. The peptides were prepared in solution by stepwise amino acid coupling using Boc-protecting strategy according to the following general procedure. The C-terminal peptide part with a free amino group (1 equiv), Boc-protected amino acid (1.05 equiv), and 1-HOBt (1.8 equiv) were dissolved in DMF (2–4 mL/mmol of amino component) before addition of DIPEA (4.8 equiv). The mixture was cooled on ice before HBTU (1.2 equiv) was added, and the reaction mixture was agitated at ambient temperature for 1–2 h. The reaction mixture was diluted with ethyl acetate and washed with a citric acid solution (5% v/v), a saturated $NaHCO_3$ solution, and brine. The solvent was removed under vacuum, and the Boc-protecting group of the resulting peptide was deprotected in the dark using 95% TFA or acetyl chloride in anhydrous methanol.

Solution Phase Amide Formation Using PyCloP (26): Synthesis of H-Arg-N(CH_2Ph)$_2$. A solution of Boc-Arg-OH (1 equiv), NH(CH_2Ph)$_2$ (1.1 equiv), and PyCloP (1 equiv) in dry CH_2Cl_2 (2 mL) and DMF (1 mL) was prepared. The solution was cooled on ice, and DIPEA (2 equiv) was added under stirring. The solution was stirred for 1 h at room temperature. The reaction mixture was evaporated, redissolved in ethyl acetate, and washed with citric acid solution (5% v/v), a saturated solution of $NaHCO_3$, and brine. The solvent was removed under reduced pressure, and the Boc-protecting group of the resulting amino acid derivative was removed in the dark using 95% TFA.

Peptide Purification and Analysis. The peptides were purified using reversed-phase HPLC on a Delta-Pak (Waters) C_{18} column (100 Å, 15 μm, 25 × 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) C_{18} column (100 Å, 5 μm, 3.9 × 150 mm) and positive ion electrospray mass spectrometry on a VG Quattro quadrupole mass spectrometer (VG Instruments Inc., Altringham, U.K.).

Measurements and Calculation of Peptide Half-Life (27). Each peptide was dissolved in a 0.1 M NH_4HCO_3 buffer (pH 6.5) to yield 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 NH_4HCO_3 buffer (pH 8.2). For the stability determination, 250 μL of freshly made trypsin solution and 250 μL of peptide solution were incubated in 2 mL of 0.1 M NH_4HCO_3 buffer (pH 8.6) at 37 °C on a rocking table. Aliquots of 0.5 mL were sampled at different time intervals, diluted with 0.5 mL of water−acetonitrile (60:40 v/v) containing 1% TFA, and analyzed by RP-HPLC as described above. Samples without trypsin addition taken at 0 h and after 20 h at 37 °C were used as negative controls. Integration of the peak area at 254 nm for samples taken during the first 5 h of the assay was used to generate the $\tau_{1/2}$ using Medical Calculator from Cornell University (*28*). Peptides that displayed no degradation during the first 24 h were classified as stable. The plotted kinetic profiles were made on GraphPad Prism 4c.

Determination of Metabolites. The identity of the metabolites from the tryptic degradation was determined either by preparative trypsin degradation of the peptide, purification, and structural determination by ESMS or by chemical synthesis of authentic reference samples.

Antibacterial Assay. MIC determinations on *S. aureus*, strain ATCC 25923, methicillin-resistant *S. aureus* (MRSA), strain ATCC 33591, and methicillin-resistant *Staphylococcus epidermidis* (MRSE), strain ATCC 27626, were performed by Toslab AS using standard methods (*29*).

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 Corp., UT). 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 and 10 mM $CaCl_2$ at pH 8.2. An interval of 400 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 were calculated using BindWorks analysis software.

Docking Studies and Molecular Modeling. Automated docking experiments were carried out using Autodock version 3.0 (*30*) in order to generate unbiased models of CAP 2 and CAP 19 when bound to trypsin. Bovine trypsin with PDB entry 3PTB was used as receptor in the docking experiments (*31*). The initial model of CAP 2 and CAP 19 was built from the previously used model of trypsin−BPTI with Arg as the P1 residue. The P1, P1′, and P2′ (Arg-Ala-Arg) residues were then used to construct CAP 19 from which CAP 2 was subsequently built (*32*). ArgusLab version 4.0.1 was used to finalize the three-dimensional structure of CAP 19 and CAP 2 (*33*). All crystallographic water molecules except residues 415, 416, 562, 704, and 705 in 3PTB were removed. These water molecules are often involved in bridging interactions between ligands and serine proteinases. Gasteiger atomic charges were assigned to the ligands while Kollman charges were used to describe the receptor. Grid maps were calculated with 60 × 60 × 60 points using a grid spacing of 0.375 Å centered at the Oγ atom of Ser195. Two runs were carried out for both CAP 2 and CAP 19 with 2.5 million energy evaluations (Lamarckian genetic algorithm), a population size of 500, and 250 runs. The resulting 500 conformations of CAP 2 and CAP 19 were clustered on the basis of the docking energy. The most favorable cluster contained four and six conformations for CAP 2 and CAP 19, respectively. When requiring one carbonyl oxygen of peptide to be located in the oxyanion hole, only one conformation of this cluster survived.

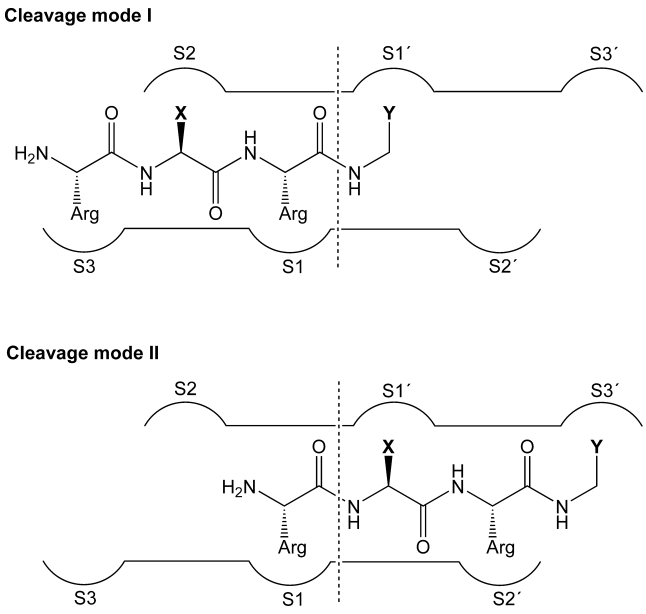


FIGURE 1: Potential modes of tryptic degradation of the tripeptides used throughout the study. Arg represents the positively charged side chain of arginine, Y denotes a C-terminal amide modification, and X represents a bulky hydrophobic side chain.

RESULTS

Degradation Studies. Trypsin cleaves peptides at the C-terminal side of positively charged residues, arginine, and lysine. All peptides in the libraries contain two arginine residues, one at the C-terminus and one at the N-terminus. The peptides thus contain two different conceivable scissile bonds, either between the C-terminal arginine and the Y-group corresponding to cleavage mode I in Figure 1 or, alternatively, between the N-terminal arginine and the X-residue corresponding to cleavage mode II. It was consequently decided to monitor the rate of peptide cleavage by the use of HPLC, as this method not only is capable of measuring the rate but, simultaneously, is capable of determining the mode of cleavage by characterizing the structure of the cleavage products.

The first library was constructed around alteration of the hydrophobic central amino acid. A series of homologous tripeptides with the general structure H-Arg-X-ArgNHBn, compounds CAP 2−CAP 8, where "X" represents a bulky nonnatural amino acid side chain as shown in Figure 2, was prepared as library 1.

The peptides were subjected to tryptic degradation, and their half-life and potential degradation products were determined. (See Materials and Methods for details about the assay.) The antibacterial activity of the peptides in library 1 was monitored against a series of staphylococci, and their minimal inhibitory concentration (MIC) is also shown in Table 1.

Replacing the indole side chain of Trp with bulkier, more hydrophobic groups has previously been shown to yield peptides with increased antibacterial activity, and the same effect was observed for this library as well (Table 1). The introduction of nonnatural amino acids did not, however, have a similarly large effect on the tryptic stability with the exception of CAP 4, which remained stable during the assay. All the remaining peptides were cleaved adjacent to the C-terminal Arg residue leading to metabolites with the general structure H-Arg-X-Arg-OH corresponding to cleavage mode I (Figure 1). The antibacterial efficacy of CAP 4 against *S. aureus* was, however, less than satisfactory, and therefore a second library of peptides was generated.

The second library of CAPs was designed to explore the influence of the C-terminal capping moiety on the stability toward trypsin while keeping the 4-phenylphenylalanine (biphenylalanine or Bip) residue as the central bulky and lipophilic residue. Both N-monosubstituted amides (CAPs 2, 9–12) and N,N-disubstituted amides (CAPs 13−15) were included in this library. The medium-sized noncoded amino acid Bip was used as the X moiety for all peptides (Figure 2). The compounds in library 2 fell into two groups; in analogy to N-methylation (*6*) of the scissile amide bond, the N,N-disubstituted amides were all stable against tryptic degradation, whereas the closely homologous series of N-monosubstituted amides were degraded by trypsin albeit at a rate that varied widely as shown in Table 2. All peptides of the second group were degraded according to cleavage mode I, where the amide adjacent to the C-terminal arginine was cleaved. Figure 3 shows plots of the relative amounts of peptide and metabolite (H-Arg-X-Arg-OH) versus time for a representative collection of peptides.

The length of the Y group influenced the stability, and the trypsin resistance could be increased by a factor of more than an order of magnitude when the phenyl group of the C-capping moiety was two carbon atoms away from the amide nitrogen (CAP 12), compared to the analogues with one (CAP 2) or three (CAP 11) carbon atoms between the amide nitrogen atom and the phenyl group. The stereochemistry of a branching group in the Y moiety also influenced the stability as CAP 9 was found to be some four times more stable than its diastereoisomer, CAP 10.

To further explore if the positive effect on peptide stability, seemingly induced by the ethylene unit, linking the phenyl group to the peptide in CAP 12, was general, a third library of CAPs was prepared. In library 3, the C-terminal end-capping "Y" was 2-phenylethylamine, whereas the internal lipophilic amino acid, "X", was varied. The results, which are compiled in Table 3, suggest that the positive effect of this end capping is general, at least within the present set of peptides.

Inspired by the increased stability arising from the proper choice of C-terminal modification and central lipophilic amino acid, a fourth library was prepared in order to probe whether a similar N-terminal end capping could further increase the peptide stability. The fourth library consisted of N-acetylated derivatives of CAPs 12 and 13 (CAPs 19 and 20, respectively). When these peptides were subjected to trypsin treatment, a rapid degradation was observed (Table 3). Interestingly, structural analysis of the metabolites revealed that trypsin now instead cleaved CAP 19 and CAP 20 between the N-terminal Arg and the X-amino acid corresponding to cleavage mode II. This is in sharp contrast to all the previously examined peptides in the present study where the tryptic cleavage occurred between the C-terminal Arg and the Y moiety. The rate of degradation for CAP 19 and CAP 20 was significantly faster than what was observed for any of the other CAPs.

Finally, a fifth group of peptides was included (Table 4). These substances have previously been synthesized using the Suzuki−Miyaura reaction on a common resin-bound trip-

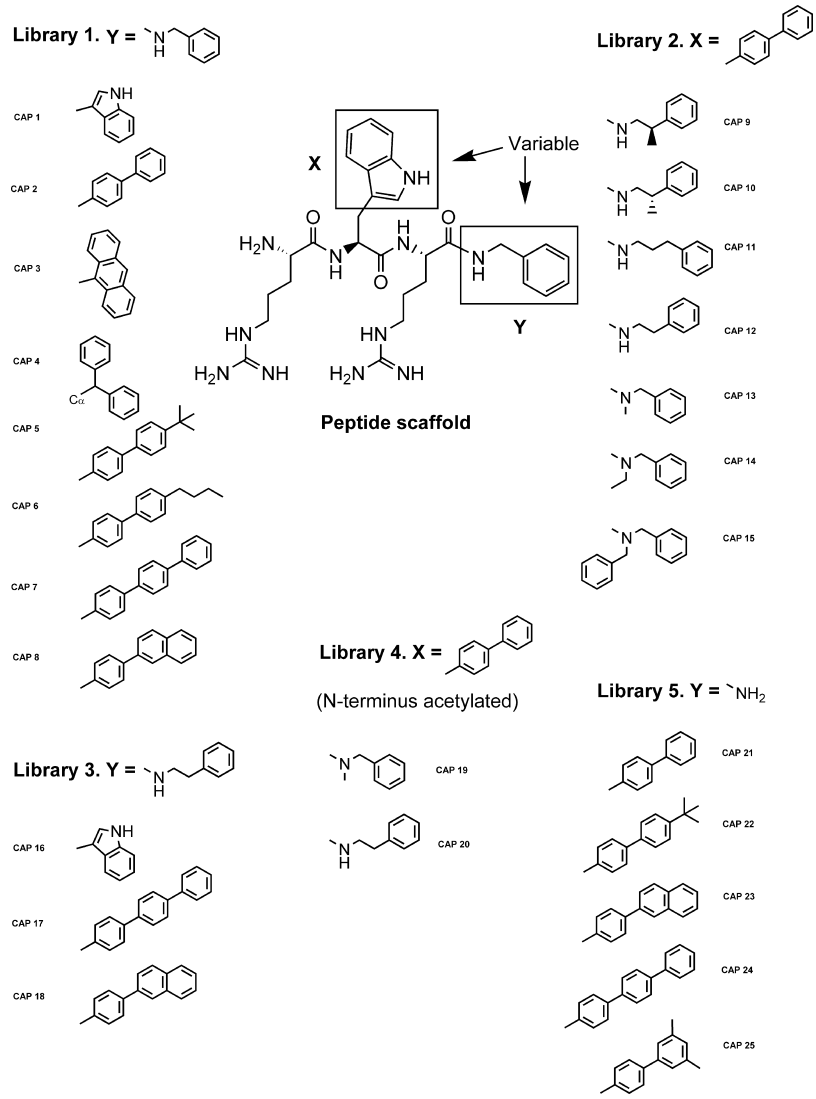


FIGURE 2: Structure of the five peptide libraries Arg-X-Arg-Y with variable side-chain groups (X) and C-terminal end-capping groups (Y). Note the N-terminal acetylation in library 4.

Table 1: Stability of H-Arg-X-Arg-NHBn Peptides toward Trypsin Measured as Half-Life ($\tau_{1/2}$) and Antibacterial Activities Displayed as MIC

	molar mass			MIC[b] (μM)		
peptide	calcd	obsd	$\tau_{1/2}$[a] (h)	*S. aureus*[c]	MRSA[d]	MRSE[e]
CAP 1	605.7	605.3	1	83	50	25
CAP 2	642.8	642.5	2	11	11	3
CAP 3	666.8	666.5	10	10	7	4
CAP 4	642.8	642.4	stable	47	6	11
CAP 5	698.9	699.4	1	11	9	6
CAP 6	698.9	699.6	1	14	9	6
CAP 7	718.9	719.4	7	11	7	7
CAP 8	692.9	692.5	3	6	4	3

[a] Medical Calculator from Cornell University was used to calculate the half-life. [b] Minimal inhibitory concentration. [c] *S. aureus* strain ATCC 25923. [d] Methicillin-resistant *S. aureus* ATCC 33591. [e] Methicillin-resistant *S. epidermis* ATCC 27626.

Table 2: Stability of H-Arg-Bip-Arg-Y Peptides toward Trypsin Measured as Half-Life ($\tau_{1/2}$) and Antibacterial Activities Displayed as MIC

	molar mass			MIC[b] (μM)		
peptide	calcd	obsd	$\tau_{1/2}$[a] (h)	*S. aureus*[c]	MRSA[d]	MRSE[e]
CAP 9	670.9	670.5	35	7	7	6
CAP 10	670.9	671.5	9	9	7	6
CAP 11	670.9	670.5	2	7	7	4
CAP 12	656.8	656.5	30	8	8	5
CAP 2	642.8	642.5	2	11	11	3
CAP 13	656.8	657.4	stable	12	12	8
CAP 14	670.9	670.8	stable	10	9	6
CAP 15	732.9	732.8	stable	8	5	4

[a] Medical Calculator from Cornell University was used to calculate the half-life. [b] Minimal inhibitory concentration. [c] *S. aureus* strain ATCC 25923. [d] Methicillin-resistant *S. aureus* ATCC 33591. [e] Methicillin-resistant *S. epidermis* ATCC 27626.

eptide scaffold to generate the X-group diversity. A comprehensive report on their antibacterial activity and preparation is described elsewhere (*34*). They were designed with a C-terminal primary amide (H-Arg-X-Arg-NH_2), lowering their antimicrobial activity somewhat compared to the compounds with N-substitution, but were included in the study to investigate the mode of degradation for this type of compound as well. Tryptic degradation of peptides in this library revealed that the cleavage mode of the peptides in library 5 was identical to that of the acetylated CAP 19 and CAP 20, i.e., between the N-terminal Arg and the X-amino acid (mode II).

Isothermal Titration Calorimetry. Isothermal titration calorimetry (ITC) studies (*35, 36*) were performed on the second set of peptides in an attempt to further unravel the

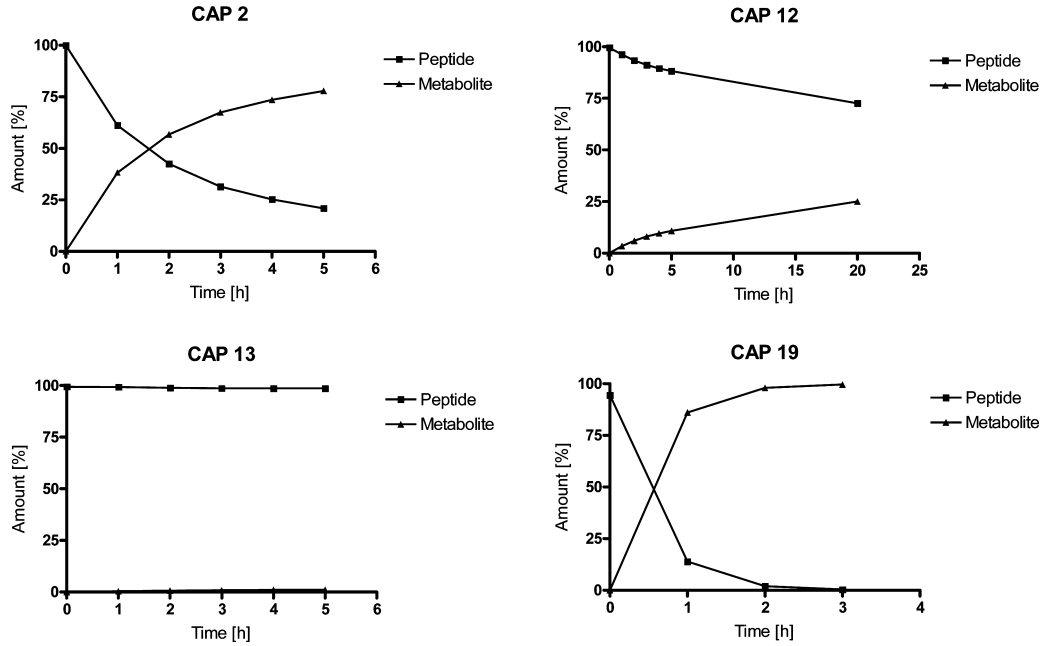


FIGURE 3: Variation of peptide and metabolite concentration versus time as a result of treatment with trypsin. Medical Calculator from Cornell University was used to calculate the half-life based on data gathered during the first 5 h of degradation.

Table 3: Stability of H-Arg-X-Arg-CH_2CH_2Ph and Ac-Arg-Bip-Arg-Y Peptides toward Trypsin Measured as Half-Life ($\tau_{1/2}$) and Antibacterial Activities Displayed as MIC

	molar mass			MIC[b] (μM)		
peptide	calcd	obsd	$\tau_{1/2}$[a] (h)	*S. aureus*[c]	MRSA[d]	MRSE[e]
CAP 16	619.8	619.8	7	145	97	81
CAP 17	732.9	732.8	stable	5	4	4
CAP 18	706.9	706.5	30	4	<3	<3
CAP 19	698.9	699.4	0.6	29	29	20
CAP 20	698.9	698.5	0.6	50	50	10

[a] Medical Calculator from Cornell University was used to calculate the half-life. [b] Minimal inhibitory concentration. [c] *S. aureus* strain ATCC 25923. [d] Methicillin-resistant *S. aureus* ATCC 33591. [e] Methicillin-resistant *S. epidermis* ATCC 27626.

Table 4: Stability of H-Arg-X-Arg-NH_2 Peptides toward Trypsin Measured as Half-Life ($\tau_{1/2}$) and Antibacterial Activities Displayed as MIC

	molar mass			MIC[b] (μM)		
peptide	calcd	obsd	$\tau_{1/2}$[a] (h)	*S. aureus*[c]	MRSA[d]	MRSE[e]
CAP 21	552.7	552.8	116	>100	>100	>100
CAP 22	608.8	608.6	17	11	10	4
CAP 23	602.8	602.7	67	25	25	5
CAP 24	628.8	628.5	17	9	8	5
CAP 25	580.7	580.6	88	>100	>100	9

[a] Medical Calculator from Cornell University was used to calculate the half-life. [b] Minimal inhibitory concentration. [c] *S. aureus* strain ATCC 25923. [d] Methicillin-resistant *S. aureus* ATCC 33591. [e] Methicillin-resistant *S. epidermis* ATCC 27626.

forces involved in binding of CAPs to trypsin. Data from the ITC studies (Figure 4 and Table 5) suggest a 1:1 complex between all the CAPs and trypsin. The data were, after subtraction of the heat of dilution, fitted to a one-site binding model developed using the BindWorks software. A correlation between the model and the data is shown in Figure 4. The dissociation constant ($1/K_a$) for the interactions was determined to be between 1 and 19 μM (Table 1) using the binding isotherm for each peptide (Figure 4). It seems as if the stable peptides are poorer substrates for trypsin as they in most cases display higher dissociation constants compared to those which are rapidly degraded, suggesting a weaker interaction with the active site. Table 5 also presents the thermodynamic parameters for binding of the peptides to trypsin and confirms a similar binding mechanism for the peptides. The free energy of binding (ΔG) varies from –28.8 to –34.1 kJ/mol with both favorable enthalpic and entropic contributions.

Docking Studies and Molecular Modeling. The different binding modes of CAP 2 and CAP 19 were investigated through automated docking experiments. The results are presented and discussed in conjunction with the degradation results in the Discussion section.

DISCUSSION

Trypsin is arguably the most thoroughly studied proteolytic enzyme (*37*). Trypsin is an endopeptidase with specificity for cleaving the amide bond between a cationic amino acid and the next amino acid in the C-terminal direction. Structurally the enzyme is composed of two β-barrels with the catalytic triad positioned at the interface of the two domains (*38*), and it hydrolyzes its substrates through a three-step mechanism involving substrate binding, acylation of a specific active site serine residue, and finally hydrolysis (deacylation) of the acyl-enzyme intermediate, with the acylation or deacylation step being the rate-determining step depending on the structure of the substrate (*25*). As is characteristic for an endopeptidase, trypsin prefers a certain length of the peptide in order to efficiently exert its catalytic action (*39*) and as many as six binding sites for the amino acid side chains in the peptide substrate have been characterized. These binding sites are denoted S1−S3 on the N-terminal side of the cleavage point and S1′−S3′ on the C-terminal side as shown in Figure 5.

The substrate specificity is primarily governed by a strong ion pairing interaction between the deeply buried Asp (189) carboxylate group in the trypsin S1 site and a cationic side chain (known as P1) adjacent to the cleavage point in the

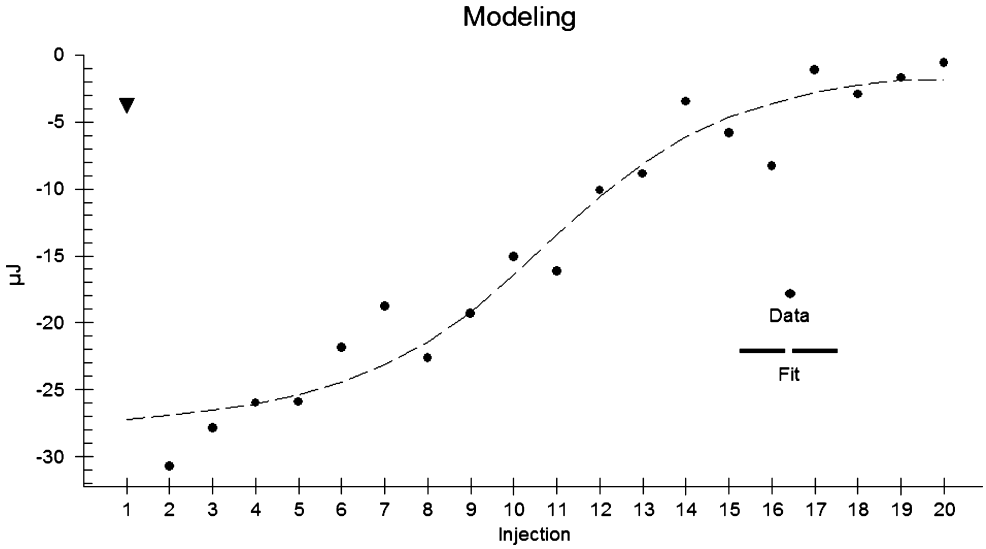


FIGURE 4: Binding isotherm for the interaction between CAP 10 and trypsin. The data from the first injection (▼) is not included to due slow peptide diffusion from the syringe during the equilibration step prior to the first injection.

Table 5: Binding Data from the ITC Runs

peptide	K_D (μM)[a]	n[a,b]	ΔH (kJ/mol)[a,c]	$-T\Delta S$ (kJ/mol)[a]	ΔG (kJ/mol)[a,c]
CAP 9	13.2	0.70	−4.4	−23.4	−27.8
CAP 10	7.4	0.97	−3.4	−25.9	−29.3
CAP 11	1.1	1.21	−7.8	−26.3	−34.1
CAP 12	10.6	0.68	−7.3	−21.1	−28.4
CAP 2	11.3	0.73	−4.8	−23.4	−28.2
CAP 13	19.0	0.68	−5.4	−21.5	−26.9
CAP 14	12.9	0.85	−4.1	−23.8	−27.9
CAP 15	15.1	1.03	−1.8	−25.7	−27.5

[a] Data from the ITC experiments and the binding isotherms. [b] Stoichiometry of the interaction; experimental error ± 0.1. [c] Experimental error ± 15%.

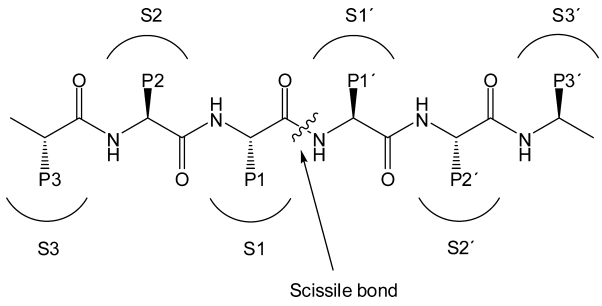


FIGURE 5: Schematic representation of the trypsin active site with nomenclature for both substrate and binding pockets. Adapted from ref *38*.

substrate, with additional loop-mediated interactions outside the active site (*40*). However, remote binding interactions on both sides of the active site lead to an increased acylation rate of the enzyme, both for substrates with a basic P1 moiety and for substrates that are nonspecific in P1 (*24*). Further interactions through hydrogen bonding between the substrate polypeptide backbone in an extended conformation forming an antiparallel β sheet and the backbone of the "polypeptide binding site" of trypsin are also expected (*41*).

Bearing in mind the size and shape of the active site, it was thus somewhat surprising to find that peptides composed of as little as three amino acids are cleaved at such a substantial rate by an endopeptidase as we see in this study and that even the mode of binding and cleavage differed between some of the libraries as is shown in Figure 6.

These findings motivated a more detailed study of the roles of the structural elements of the peptides and their interaction with the binding pockets of trypsin. As the overall aim of the present study was to investigate the possibility to increase the proteolytic stability of the CAPs while still maintaining the amide backbone intact and thus, hopefully, also the antibacterial activity, focus was shifted from peptide bond isosteres to side-chain modifications and different capping strategies.

The peptide libraries used in this investigation were based on a few design principles. Primarily, the peptides should all adhere to the pharmacophore for antimicrobial efficacy against *S. aureus*. The requirements for this pharmacophore are the presence of at least two units of lipophilic bulk, each approximately the size of a phenyl group, and the presence of at least two cationic charges (*21*). We chose to develop this pharmacophore in a tripeptide scaffold, where a lipophilic and bulky residue, X, was flanked by arginine residues. All peptides contained an amide-modified C-terminus to avoid the negatively charged carboxylate. Furthermore, the amide modification made it possible to introduce additional bulky and lipophilic modifications by the preparation of various monosubstituted and N,N-disubstituted amides.

An additional design feature was that the peptide should be viable substrates for trypsin; i.e., all peptides should have an amide bond on the C-terminal side of the arginine residues. Additionally, the stereochemistry of the backbone residues should be all L. Hence all peptides included in the various libraries in the present work are tripeptides with the general sequence Arg-X-Arg-Y, where the overall lipophilic bulk, as well as the three-dimensional shape of the X and Y moieties, is varied. The peptide Arg-Trp-ArgNHBn (CAP 1) was used as a basis for the library construction (Figure 2).

The advantage of these libraries is the relative ease of synthesis. The diversity in the Y moiety is created by performing a solution phase coupling between the C-terminal arginine and a variety of primary and secondary amines. The X diversity was created by utilizing a range of commercially available nongenetically coded amino acids such as 4-phenylphenylalanine (Bip), (9-anthryl)-

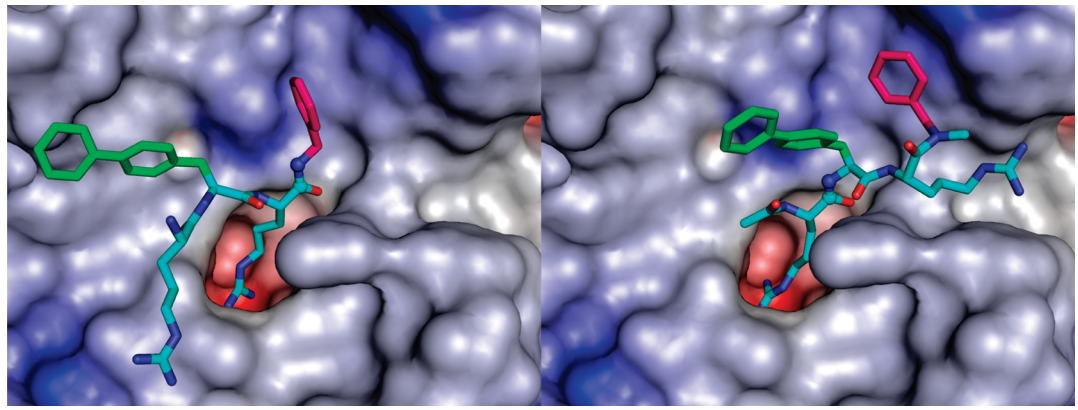


FIGURE 6: Generalized binding models for the CAPs to trypsin. Bulky X-amino acids are shown in green, and C-terminal end capping is shown in purple. The position of the scissile amide bond is indicated by a ball and stick bond. The left model (for libraries 1–3) yields H-Arg-X-Arg-OH and H_2N-Y products while the right one results in Ac-Arg-OH and H-X-Arg-Y for library 4 and H-Arg-OH and H-X-Arg-NH_2 for library 5.

alanine (Ath), or 3,3-diphenylalanine (Dip). These amino acids were supplemented by a series of novel biphenylalanine derivatives prepared through Suzuki−Miyaura coupling as outlined in Scheme 1.

The most important binding epitope of trypsin is the S1 site responsible for binding to Lys or Arg residues in the substrate, via a deep binding pocket with residue Asp189 at its bottom (*42*). However, since the overall aim was to develop bioactive stable CAPs and the antibacterial pharmacophore required the presence of two cationic sites, no changes were made in the position of the cationic residues. Thus, all of the peptides contained two Arg, and the S1 pocket of trypsin could therefore be occupied with any of these (which one depends on the rest of the substrate structure as is discussed below) in all of the compounds. We chose instead to focus on the other interactions surrounding the active site. The influence of the interaction with the S2 site was investigated with the first library of peptides (CAPs 1–8, Table 1). In trypsin, the shallow hydrophobic groove of the S2 site plays only a minor role for substrate specificity and catalytic action (*43*). It was nonetheless hypothesized that the extent of binding to this site could be altered by introduction of different bulky side chains. Peptides with a favorable interaction with the S2 site would be expected to be more readily cleaved than peptides with less favorable interactions. With a few exceptions, the stability of the peptides in library 1 was not much affected by substituting the tryptophan residue in CAP 1 by a variety of artificial bulky and lipophilic residues. The stability only increased significantly when (9-anthryl)alanine, diphenylalanine, and triphenylalanine were introduced as the second residue of the substrate. These results suggest that the S2 binding site indeed is quite flexible with respect to the structures it can accommodate as a great variety of side chains ranging from tryptophan (CAP 1) to 4-naphthy-phenylalanine (CAP 8) fit. Docking studies revealed a "snug" fit for these compounds, aligning the amide bond between P1 and P1′ with the activated hydroxyl on Ser195 as shown for CAP 2 in Figure 7. When the side chains were very rigid and elongated (CAP 7) or very wide (CAP 3 and CAP 4), docking studies suggest a hindered binding to S2, mainly sterical in nature. This hindered binding is beneficial for stability, but only CAP 4, which contains a Cβ disubstituted X moiety, was found to be stable.

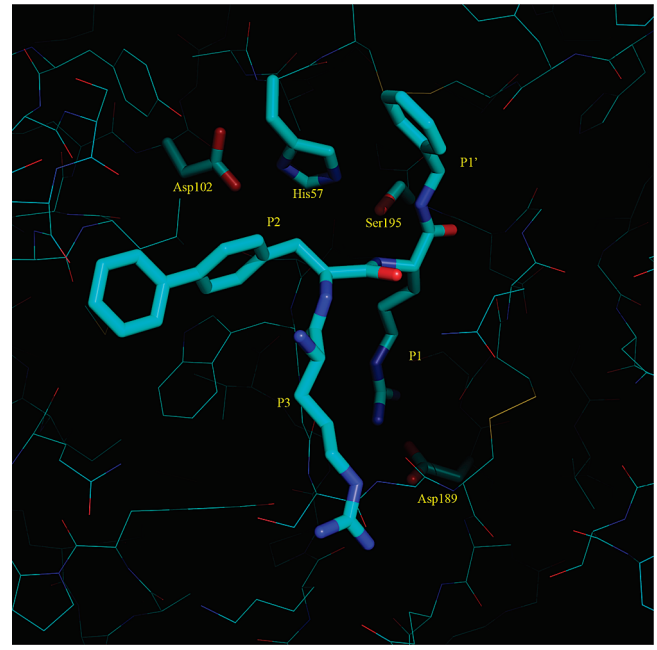


FIGURE 7: Docking of peptide CAP 2 in the active site of trypsin. The different side chains (P "X") bind to the corresponding pockets (S "X", not labeled in figure) in trypsin.

The next interaction site studied was the S1′ accommodating the C-terminal capping group Y. Little is known about the binding interactions between the S1′ site and the peptide substrate, mainly due to overusage of chromophoric substrates with limited variability in this region. The peptides of the second library were designed with the purpose of exploring this site in more detail. We had previously found that, for peptides of the type Arg-Bip-Arg-Y, no (or very slow) degradation takes place when the Y group is NH_2 (CAP 21) or NH-*i*-Pr (data not shown). The reason for this behavior may be connected to the size of the Y moiety. However, increasing the size to NHBn induces a hydrolysis pattern, which suggests that the larger Y moiety of CAP 2 may mimic a fourth amino acid which interacts with the S1′ in the active site of trypsin. A reasonable explanation for the high stability of the smaller analogues may thus be that NH_2 and NH-*i*-Pr are too small to produce any favorable binding interactions and are accordingly unable to mimic a fourth amino acid,

indicating that an S1′ interaction indeed plays an important role for this class of substrates.

Large stability differences were observed within this library. The most notable features are N,N-disubstituted amides (CAPs 13−15) are stable and peptides with an ethylene chain linking the phenyl group and the amide nitrogen display significantly higher stability than compounds that have either a methylene or propylene link.

The stability of the N,N-disubstituted amides is linked to the general reaction mechanism of serine proteases. This mechanism requires the presence of an amide proton, which the N,N-disubstituted amides lack. Although the N,N-disubstituted amides are stable to hydrolysis, results from the ITC analysis indicate that they still bind to the active site of trypsin (Table 5) in a fashion similar to those peptides that are digested. The trend among the monosubstituted amides (CAP 2 and CAPs 9−12) is more puzzling. The instability of CAP 11 is to be expected from the instability of CAP 2 whereas the unexpected high stability of CAP 12 can at present not be explained from our experimental data. It is reasonable to assume that the Y group of CAP 12 is either significantly less bound to the S1′ site than the Y group of CAP 2 and CAP 11 or that it, upon binding to the S1′ pocket, forces the scissile amide bond slightly out of plane, thereby slowing down the formation of the acyl-enzyme intermediate.

There are also minor variations within the library of compounds that have an ethylene chain between the amide nitrogen and the phenyl group. A methyl substituent on C_2 (counting from the nitrogen) seems to modulate the stability. If the absolute configuration on the C_2 carbon is (*S*) (i.e., CAP 10), the stability is slightly diminished compared to the unbranched analogue CAP 12, whereas the inverted configuration of CAP 9 enhances the stability. The observation of a stereochemical effect on peptide stability strongly suggests that this effect originates from differences in binding interaction with the S1′ binding site (which itself is chiral). It has been reported in the literature that binding interactions in the S1′ pocket of trypsin are nearly as important as the ionic interaction in the S1 pocket (*44*). On the other hand, it is also suggested that the S1′ pocket prefers small and/or hydrophilic side chains ($K_a = 1.3 \times 10^{13}$ and 7.8×10^9 M for Ala and Ser, respectively, in the P1′ position of peptidic inhibitor BPTI) when the substrate is a large peptide, a preferred group of epitopes which are very different from the bulky lipophilic Y groups of this study. The corresponding value for Trp is 1.1×10^6 M, indicating that S1′ also can bind hydrophobic residues albeit with less gain in association energy (*44*). Our results indicate that S1′ can bind even larger and more hydrophobic side chains than Trp, perhaps a somewhat surprising observation considering that some of the structures are not naturally occurring in peptides.

The third library of peptides, CAPs 16−18, was designed to confirm that the unexpected increase in stability induced by the Y group containing the ethylene link chain was a general effect and not necessarily limited to that specific X group (Bip) as is shown in Table 4. The results show that the substitution of a benzyl group to an ethylphenyl group in the Y moiety increased the stability toward tryptic degradation by an order of magnitude. Furthermore, the potentially protective action of the "X" and "Y" moieties seems to be additive as is evident when comparing the $\tau_{1/2}$ of CAPs 1, 7, and 8 in Table 1 with the $\tau_{1/2}$ for CAPs 16, 17, and 18. Even more advantageous, the increase in antimicrobial activity induced by these groups is also additive to some extent, yielding peptides displaying both improved stability and antibacterial effect compared to CAP 2, with CAP 18 being the most efficient bacterial killer in this study.

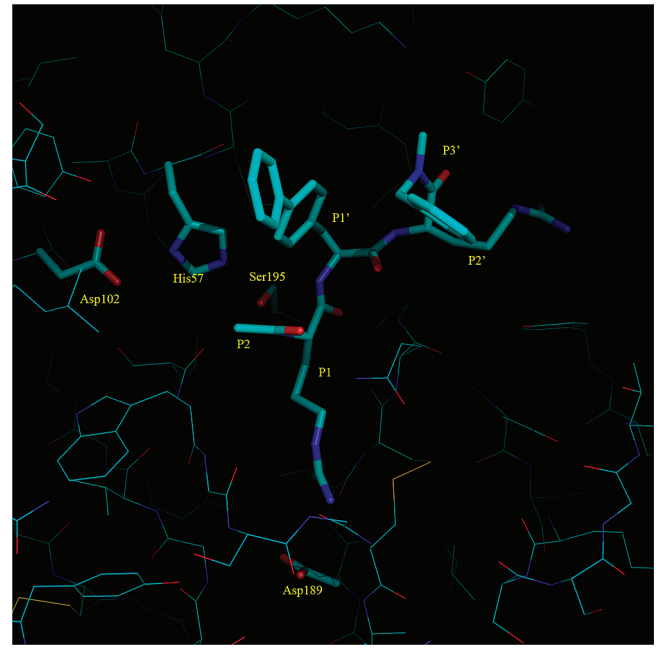


FIGURE 8: Docking of peptide CAP 19 in the active site of trypsin. The different side chains (P "X") bind to the corresponding pockets (S "X", not labeled in figure) in trypsin.

N-Terminal capping was the strategy behind the fourth small library since the C-terminal modification proved to be such a convenient and versatile way of altering the stability of the peptides. These CAPs were thus designed by acetylating the N-terminus of CAP 2 and CAP 13. However, this modification yielded peptides that were very rapidly degraded. Furthermore, by being hydrolyzed between the N-terminal Arg and the Bip residue, their degradation corresponded to binding mode II in the active site of trypsin as proposed in Figures 1 and 6. CAPs 19 and 20 were thus digested in a manner different from the previously investigated peptides. Docking studies of CAP 19 with trypsin verified the binding mode as is shown in Figure 8.

It seems, in parallel to the effect of a large Y group, that an N-terminal modification also can act as a mimic for an additional residue, effectively transforming the tripeptide into a pentapeptide mimic. It is interesting that such a small structural element as an acetyl group can change the binding mode to such an extent for these peptides. With the acetyl group acting as a P2 unit, the Bip moiety now occupies S1′ (yet again illustrating the potential of the S1′ binding pocket to accommodate large hydrophobic groups) while the N-terminal Arg binds to the S1 Asp189 setting up the new cleavage site for rapid hydrolysis. With the acetyl group as P2, the peptide can now access both S2′ and S3′, in a way (schematically shown in Figure 1) that was most likely not energetically favorable with the free N-terminal peptides (CAP 1−CAP 18) of the previous libraries. In the closely related serine protease plasmin, the S3′ pocket prefers hydrophobic targets, which perhaps can explain the binding mode of the CAPs of the fourth library (*15*). The study of

what effect further N-terminal modifications could have on stability was discontinued as the destabilizing effect of the acetyl group created the most unstable peptides within this study. ITC studies on the binding of these peptides to trypsin would be interesting, but impossible, due to the rapid degradation. An identical mechanism was seen for the stable peptides of library 5. These peptides lack a hydrophobic Y group, and their only possibility to access S1′ with a similar moiety is by binding with the N-terminal Arg in the S1 pocket, thereby placing the X group in S1′. It seems that a hydrophobic binding to S1′ for these compounds is more beneficial than the potential main-chain hydrogen bonding of the P1−P3 residues to the polypeptide site of trypsin, a set of interactions which are usually important for efficient binding and hydrolysis (*41, 45*). It should be noted, however, that some highly resilient peptides such as CAP 21, CAP 23, and CAP 25 (half-lives of 116, 67, and 88 h, respectively) were found within this last library.

The detailed binding mode of short peptides to trypsin is still partly uncharted waters even though much is known in minute detail (*25*). We have in the present study, through degradation and docking studies of a range of structurally diverse antimicrobial peptides, gained insight into how it is possible to alter the tryptic stability of this class of compounds. The CAPs display a slightly different binding mode than natural substrates, mainly due to the presence of hydrophobic, nonnatural side chains and capping groups. For hydrolysis, hydrophobic interactions with the S1′ site are central for this class of molecules. These proposed models are, however, not able to explain detailed variations within the experimental data such as why the S2 site cannot accommodate amino acid side chains such as those of CAP 3 and CAP 4 or why the ethylene chain of the Y group gives rise to unusually stable peptides. Irrespective of this, several guidelines for devising small peptides with stability toward degradation by trypsin can be extracted from the set of data generated through our work. (1) The peptides should be kept as small as possible in order to limit interactions with as many binding sites in trypsin as possible. End cappings at either the N-terminus or the C-terminus can act as additional residues increasing the binding to the active site. (2) The amino acid occupying the P2 site should have a side chain sterically preventing it from binding efficiently to the S2 unit of trypsin. Beneficial side chains in this respect include rigid elongated structures (longer than a para-alkylated biphenyl), wide structures (9-anthryl), and those that are "bulkily" Cβ-disubstituted. (3) The length and stereochemistry of the C-terminal end capping have a major influence on stability. Peptides with a phenyl group connected via an ethylene chain to the amide nitrogen are significantly more stable than those connected via a methylene or a propylene link. A small chiral substituent at C_2 of (*R*) configuration may further increase the stability. (4) Linking the Y group via a tertiary amide yields stable peptides in a manner analogous to peptide backbone N-methylation. The present work also shows that these modifications may be compatible with the pharmacophore of CAPs, as several of the stable peptides prepared in this study have good antimicrobial efficacy against *S. aureus*.

While tryptic stability is important for a potential peptidic drug, so is the stability toward degradation by α-chymotrypsin. Preliminary ITC experiments have revealed a similar degree of binding ($K_D \sim 10$ μM) of these CAPs to α-chymotrypsin, and on the basis of substrate specificity differences between the two degradative enzymes, different potential hydrolysis products are expected. We are currently undertaking a comprehensive study into which structural parameters will govern the proteolytic stability of CAPs toward α-chymotrypsin. These results will be reported in due course.

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