Bulky Nonproteinogenic Amino Acids Permit the Design of Very Small and Effective Cationic Antibacterial Peptides

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Received June 2, 2004

Abstract: The rate of multidrug-resistant infections is rapidly rising. Cationic antibacterial peptides are active against resistant pathogens and have low propensity for resistance development, but because of their unfavorable medicinal properties, cationic antibacterial peptides have been a limited clinical success. We have found that introduction of nongenetically coded amino acids and other lipophilic modifications opens the opportunity for development of extremely short and highly active antibacterial peptides with improved medicinal properties.

The near-explosive growth of multiresistant bacteria in hospitals have led to projections of an emerging crisis where an increasing number of antibiotics cease to be of clinical usefulness.[1−6] Despite this growing concern, only one new class of antibiotics, the oxazolidinones (Lineozolid), has entered the clinic during the past 2 decades.[7] Antibacterial peptides, with their ability to combat multiresistant bacteria combined with a unique low propensity for resistance development, have since their discovery in the late 1970s[8] been heralded as prospective new antibacterial drugs.[9−11] Numerous attempts to transfer cationic antibacterial peptides into clinical drugs have been undertaken over the years to little avail. The reasons for these failures are certainly diverse, but peptides within the size range typical for cationic antibacterial peptides are less than optimal drug targets. Most antibiotics in clinical use are small, stable, nontoxic, and inexpensive, while antibiotic peptides have unresolved issues regarding toxicity and stability, representing a potential lack of systemic applicability.[10] Furthermore, the size of cationic antibacterial peptides is so large that production costs represent additional concern.

Over a number of years, we have worked systematically to find a minimum antibacterial motif in cationic antibacterial peptides using lactoferricin derivatives as models.[12−19] Recently, we discovered the existence of a surprisingly small pharmacophore that could be expressed in units of lipophilic bulk (B) and cationic charge (Ch).[20] Although the exact nature of the pharmacophore may be dissimilar between different bacteria, it was discovered that the minimum motif for antibacterial activity against *Staphylococcus aureus*, even multiresistant strains (MRSA), was the presence of two cationic charges and two units of bulk. Activity against *Escherichia coli* required an additional unit of bulk.

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In the present work we have investigated further the influence of the bulk units on the antibacterial activities of cationic dipeptides and simple pseudodipeptides.

A series of dipeptides were prepared according to the general formula XRY, where X represents an amino acid with a lipophilic side chain of varying sizes, R is arginine, and the C-terminal Y moiety is methyl, benzyl or 4-phenylbenzyl esters, or isopropyl- or benzylamides. For these dipeptides the free N-terminus and the guanidinium side chain of arginine constitute the necessary two charged moieties of the pharmacophore, whereas the side chain of X or the ester or amide substituent Y conveys the required lipophilic bulk. The size of the side chain of amino acid X varied between that of a *tert*-butyl group and that of a 2,5,7-tri-*tert*-butylindol-3-ylmethylene group. The chemical structures of the side chains of X are given in Figure 1, and the antibacterial activity of the XRY dipeptides against *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) is compiled in Table 1. Some of the peptides were also tested against the methicillin-resistant *S. aureus* strain ATCC 33591, which generally was more sensitive to the peptides than the nonresistant strain (data not shown). This is a feature also noted previously for cationic antibacterial peptides.[10,21] The majority of the peptides in the series showed no activity[20] against human erythrocytes.

The antistaphylococcal motif of 2 B-units and 2 Ch-units previously reported[20] manifests itself in the benzyl ester series. The *tert*-butyl side chain of *tert*-butylglycine or the neopentyl side chain of *tert*-butylalanine and the benzyl group on the C-terminal ester are clearly insufficient for providing the necessary bulk for the antistaphylococcal motif because peptides **1** and **2** are inactive. Further, the cyclohexylmethyl side chain of cyclohexylalanine (or benzyl side chain of phenylalanine[20]) is in combination with the benzyl ester barely sufficient for satisfying the minimum motif because peptide **3** is moderately active, while increasing the size of the lipophilic side chain of X above this level considerably enhances antistaphylococcal activity, as can be seen for peptide **4** with a 4-*tert*-butylphenylalanine side chain. This effect is further substantiated for the 4-phenylphenylalanine (biphenylalanine) peptide **5**, which displayed a minimal inhibitory concentration (MIC) value of 5 μg/mL against *S. aureus*. Peptide **5** can in reality be considered to contain 3 B-units (2 in the biphenylalanine side chain and 1 in the benzyl ester moiety) and as such fulfills the minimum motif required against *E. coli*,[20] and peptide **5** was indeed found to have moderate activity against this Gram-negative bacterium.

When the size of the ester group is reduced to a methyl ester, and thus no longer constituting a unit of bulk, the side chain of 4-*tert*-butylphenylalanine becomes insufficient to provide antistaphylococcal activity. The larger side chain of biphenylalanine (Bip) with its two B-units does however fulfill the minimum condition of the pharmacophore, making BipROMe active against *S. aureus* albeit weakly so. When the size of the side chain is further increased, as in 2,5,7-tri-*tert*-butyltryptophan (Tbt), the antibacterial activity of the methyl

10.1021/jm049582b CCC: $27.50
Published on Web 07/16/2004

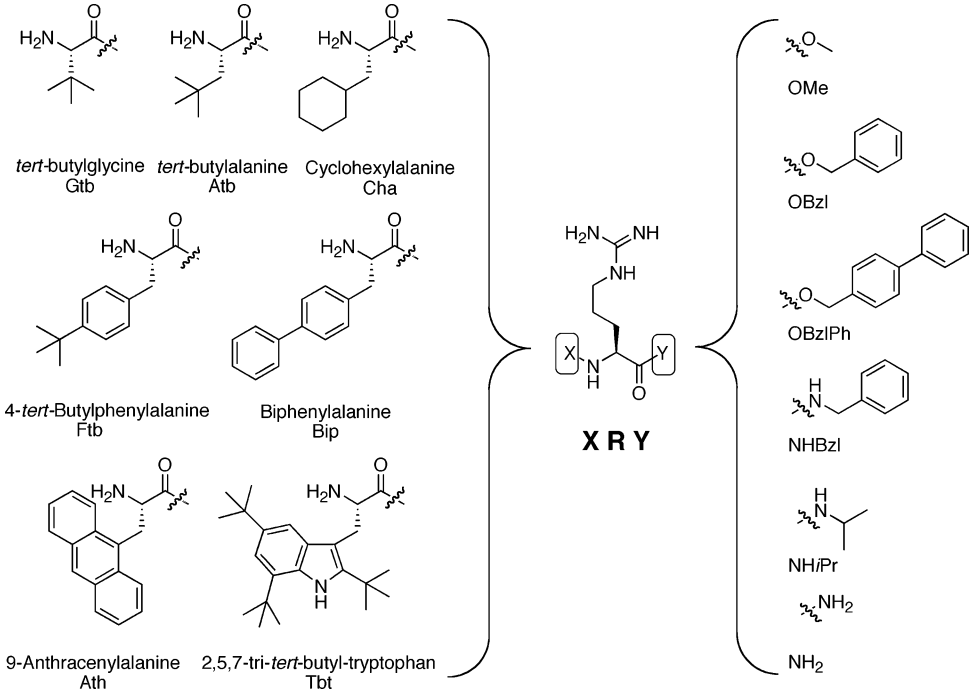

Figure 1. Structures of the nongenetically coded bulky and lipophilic amino acids (X, left) and C-terminal modifications (Y, right) used in the synthesis of antibacterial XRY peptides.

Table 1. Antibacterial Activity Measured as MIC of Dipeptide Derivatives

peptide sequence[a]		molecular mass calcd	molecular mass obsd	MIC[b] *E. coli*	MIC[b] *S. aureus*
GtbR−OBzl	**1**	377.5	378.4	>300	>300
AtbR−OBzl	**2**	391.5	392.1	>300	>300
ChaR−OBzl	**3**	417.5	418.3	>300	100
FtbR−OBzl	**4**	467.6	468.3	200	25
BipR−OBzl	**5**	487.6	488.4	150	5
FtbR−OMe	**6**	391.5	392.2	>300	>300
BipR−OMe	**7**	411.5	412.2	>300	150
TbtR−OMe	**8**	542.8	543.5	30	5
AR−OBzlPh[c]	**9**	411.5	412.3	300	100
FR−OBzlPh	**10**	487.6	488.4	100	12.5
BipR−OBzlPh	**11**	563.7	564.4	50	5
WR−NHBzl	**12**	449.5	449.0	>300	100
BipR−NHBzl	**13**	438.5	439.1	>50	15
AthR−NHBzl	**14**	510.6	511.4	150	20
TbtR−NHBzl	**15**	617.8	618.6	10	2.5
TbtR−NH*i*Pr	**16**	569.8	569.3	50	7.5
TbtR−NH_2	**17**	527.7	528.0	>150	15

[a] Amino acid abbreviations: Atb, *tert*-butylalanine; Ath, 9-anthracenylalanine; Bip, biphenylalanine; Ftb, *tert*-butylphenylalanine; Gtb, *tert*-butylglycine; Tbt, 2,5,7-tri-*tert*-butyltryptophan. [b] MIC in μg/mL. [c] OBzlPh = $-O-CH_2-C_6H_4-C_6H_5$.

ester peptide reaches 5 μg/mL against *S. aureus*, and considerable activity against *E. coli* was also observed.

In the 4-phenylbenzyl ester (OBzlPh) series, where the size of the ester moiety is nearly doubled with respect to the benzyl esters, the minimal requirement for lipophilic bulk is already fulfilled by the methyl group of the alanine side chain. In line with the observations presented above, a further increase of the side chain bulk of the lipophilic amino acid results in an increased antibacterial activity. It is, however, important to note that an upper limit of bulk may be reached because the BipROBzlPh peptide **11** did not display any higher antibacterial activity against *S. aureus* than did BipROBzl **5**. BipROBzl does not, however, represent an upper limit for antibacterial acitivity measured against *E. coli* because the larger BipROBzlPh peptide displayed a lower MIC value.

The C-terminal benzyl ester moieties could be exchanged with a benzylamide derivative, although with a slight loss in antibacterial activity. The overall most active peptide in the present study was TbtRNHBzl **15** with good activity against *E. coli* and an excellent effect against *S. aureus*. For peptides with this level of lipophilic bulk, loss of bacterial selectivity may become an issue and the cytolytic activity against human erythrocytes increases sharply and an EC_{50} value of 60 μg/mL is observed. Decreasing the lipophilic bulk of the amide part from benzyl to isopropyl derivatives and unsubstituted amide, as for derivatives **16** and **17**, reduced toxicity against erythrocytes to 85 and 305 μg/mL, respectively, followed by a slight loss of antibacterial activity.

The observation of the divergence in antibacterial activities between the ester and the amide series made us investigate how modifications of the peptide bond between X and R would influence the antibacterial activity. A medium-active peptide was chosen as a model because this would readily show an improvement in or a reduction of antibacterial activity. KWOBzl **18** was selected as the model dipeptide derivative, and the antibacterial activity of this peptide and the mimetics are compiled in Table 2. Initially, the peptide bond was replaced by an ester functionality in which pseudodipeptides were prepared from the benzyl ester of (±)-2-hydroxy-3-(1*H*-indol-3-yl)propionic acid and Boc-Lys(Boc)-OH, resulting in a 1:1 diastereomeric mixture of Kα(COO)WOBzl and Kα(COO)wOBzl (**20** and **21**). These were separable by RP-HPLC and displayed identical antibacterial activities. Furthermore, the MIC values did not differ from that of the model peptide (KWOBzl **18**). More surprising was the total loss of antibacterial activity of the reduced amide mimetic Kα-(CH_2NH)WOBzl **22**.

Table 2. Antibacterial Activity Measured as MIC of Pseudodipeptides

peptide sequence[a]		molecular mass calcd	molecular mass obsd	MIC[b] *E. coli*	MIC[b] *S. aureus*
KW−OBzl	**18**	422.5	423.3	>200	75
Kw−OBzl	**19**	422.5	423.3	>200	50
Kα(COO)W−OBzl	**20**	423.5	424.4	150	50
Kα(COO)w-OBzl	**21**	423.5	424.4	150	50
Kα(CH_2NH)W−OBzl	**22**	408.5	409.4	>300	150

[a] Lower case letters denote D-amino acids. [b] MIC in μg/mL.

Recently we reported the presence of a pharmacophore for short antibacterial peptides.[20] The minimum antibacterial motif against *S. aureus* was found to be 2 B-units each approximately the size of a phenyl group and 2 Ch-unit moieties. The motif was slightly larger for activity against *E. coli* where the minimum bulk was increased to 3 B-units. The results from the dipeptides containing nonproteinogenic bulky and lipophilic amino acids presented in this study corroborate the presence and nature of these pharmacophores. The results showed that the minimum size of a B-group is equivalent to a phenyl group and that larger B-groups increased antibacterial activity. Indeed, synthetic nonproteinogenic amino acids with "superbulky" side chains allows more than 1 B-unit to reside in one single amino acid side chain. Utilizing the knowledge that more bulk in very short cationic antibacterial peptides yields more active peptides, in combination with the repertoire of "superbulky" synthetic amino acids and C-terminal modifications available, permits sufficient bulk to produce unusual high antibacterial activity against *S. aureus* and also against the more resilient *E. coli* to be contained in peptides with as few as two amino acids. The present study also reveals that a peculiar, nearly arithmetic relationship between the B-units exists. The results presented show that the required 2 B-units can be evenly distributed with one in the side chain of the lipophilic amino acid and one in the C-terminal modification or can be concentrated in the C-terminal modification or in a superbulky lipophilic amino acid side chain, and as long as the combined size of these B-units is constant, the antibacterial activity of the peptides is hardly affected by how these B-groups are distributed. This effect can be seen in the following series of dipeptides containing 2 B-units where BipROMe **7**, AROBzlPh **9**, and FROBzl **10** have MIC values within one titer, or in the following pair of 3 B-unit peptides BipROBzl **5** and FROBzlPh **10** with similar MIC values. The increase in antibacterial activity by increasing the lipophilic bulk is followed by an increase in lytic activity against human erythrocytes. The hemolytic activity can be reduced by backtracking slightly the bulk units at a nominal cost in antibacterial activity. It is noted that the levels of hemolytic activity compared with antibacterial activity observed in this set of peptides compete favorably with that of other longer antibacterial peptides of this class.[22]

The apparently relaxed restraint on the distribution of the bulk units within the peptides was not conferred to the nature of the central peptide bond. Replacing the amide bond with an ester did not affect the antibacterial activity of the model peptide. On the other hand, replacement of the central amide by an amine to produce reduced amide pseudodipeptides almost extinguished the antibacterial activity. It is obviously important to have a carbonyl group in the center of the peptide, although whether this carbonyl group is of an ester or amide functionality is of minor importance. Whether the requirement of an ester or amide is due to a necessity of a hydrogen bond acceptor in the center of the molecule or is due to increased conformational rigidity of esters and amides compared with amines is still uncertain.

In conclusion, nonproteinogenic bulky and lipophilic amino acids allow the construction of highly active antibacterial dipeptides. The pharmacophore of short cationic antibacterial peptides seems to be robust enough to incorporate these non-natural amino acids and offer an unusual flexibility in the distribution of the bulky and lipophilic moieties. Taken together these findings indicate the existence of a vast drug optimizing space for this class of peptides, a property that may contribute very favorably for a putative drug. The present study does, however, point out the existence of some restrictions within the optimizing space.

Short cationic antibacterial peptides have now reached the realm of the classical drug molecules in terms of molecular size and complexity and should thus attract more research in pursuing the development of these molecules as future antibiotics against multidrug-resistant infections.

In terms of the experimental methods, the peptide derivatives were prepared in solution using Boc chemistry as reported previously.[20] The Kα(CH_2NH)WOBzl pseudodipeptide was purchased from Neosystem, Strasbourg, France. The peptides were purified using reversed-phase HPLC on a Delta-Pak (Waters) C_{18} column (100 Å, 15 μm, 25 mm × 100 mm) with a mixture of water and acetonitrile (both containing 0.1% TFA) as eluent. The peptides were analyzed by RP-HPLC using an analytical Delta-Pak (Waters) C_{18} 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, U.K.). The purity of all peptides was larger than 95%. The bacterial strains *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were grown in 2% Bacto Peptone water (DIFCO 1807-17-4) until exponential growth was achieved. A standard microdilution technique with an inoculum of 2×10^6 cfu/mL was used. The MIC of the peptides was determined in 1% Bacto Peptone water after incubation overnight at 37 °C. All peptides were tested in parallel using at least three independent dilutions. The susceptibility of the bacterial strains against gentamicin was used as an internal standard during MIC determinations. The results (MIC in μg/mL) were the following: *E. coli*, 0.12−0.25l *S. aureus*, 0.03−0.06. The activity of the peptides against human erythrocytes was measured as reported previously.[20]

Acknowledgment. Financial support from Alpharma AS, Lytix Biopharma AS, and the Research Council of Norway is gratefully acknowledged.

References

(1) Neu, H. C. The crisis in antibiotic resistance. *Science* **1992**, *257*, 1064−1073.

(2) Internal Task Force. *Antimicrobial Resistance: A Public Health Action Plan to Combat Antimicrobial Resistance*; CDC: Atlanta, GA, 2001; pp 1−46.
(3) Bren, L. Battle of the Bugs: Fighting Antibiotic Resistance. *FDA Consum.* **2002**, *36*, 28−34.
(4) Gold, H. S.; Moellering, R. C. Antimicrobial-drug resistance. *N. Engl. J. Med.* **1996**, *335*, 144−1453.
(5) Levy, S. B. The challenge of antibiotic resistance. *Sci. Am.* **1998**, *275*, 46−53.
(6) Levy, S. B. Multidrug resistance—a sign of the times. *N. Engl. J. Med.* **1998**, *338*, 1376−1378.
(7) Walsh, C. Molecular mechanisms that confer antibacterial drug resistance. *Nature* **2000**, *406*, 775−781.
(8) Steiner, H.; Hultmark, D.; Engström, Å.; Bennich, H.; Boman, H. G. Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature* **1981**, *292*, 246−248.
(9) Diamond, G. Nature's antibiotics—the potential of antimicrobial peptides as new drugs. *Biologist* **2001**, *48*, 209−212.
(10) Hancock, R. E. W. Host Defence (Cationic) Peptides. What Is Their Future Clinic Potential? *Drugs* **1999**, *57*, 469−473.
(11) Zasloff, M. Antimicrobial peptides of multicellular organisms. *Nature* **2002**, *415*, 389−395.
(12) Haug, B. E.; Svendsen, J. S. The role of tryptophan in the antibacterial activity of a 15-residue bovine lactoferricin peptide. *J. Pept. Sci.* **2001**, *7*, 190−196.
(13) Haug, B. E.; Skar, M. L.; Svendsen, J. S. Bulky aromatic amino acids increase the antibacterial activity of 15-residue bovine lactoferricin derivatives. *J. Pept. Sci.* **2001**, *7*, 425−432.
(14) Lejon, T.; Svendsen, J. S.; Haug, B. E. Simple Parameterisation of Non-proteinogenic Amino Acids for QSAR of Antibacterial Peptides. *J. Pept. Sci.* **2002**, *8*, 302−306.
(15) Rekdal, Ø.; Andersen, J.; Vorland, L. H.; Svendsen, J. S. Construction and synthesis of lactoferricin derivatives with enhanced antibacterial activity. *J. Pept. Sci.* **1999**, *5*, 32−45.
(16) Strøm, M. B.; Rekdal, Ø.; Svendsen, J. S. Antibacterial activity of 15-residue lactoferricin derivatives. *J. Pept. Res.* **2000**, *56*, 265−274.
(17) Strøm, M. B.; Haug, B. E.; Rekdal, Ø.; Skar, M. L.; Stensen, W.; et al. Important structural features of 15-residue lactoferricin derivatives and methods for improvement of antimicrobial activity. *Biochem. Cell Biol.* **2002**, *80*, 65−74.
(18) Strøm, M. B.; Rekdal, Ø.; Svendsen, J. S. The effects of charge and lipophilicity on the antibacterial activity of undecapeptides derived from bovine lactoferricin. *J. Pept. Sci.* **2002**, *8*, 36−43.
(19) Strøm, M. B.; Rekdal, Ø.; Svendsen, J. S. Antimicrobial activity of short arginine- and tryptophan-rich peptides. *J. Pept. Sci.* **2002**, *8*, 431−437.
(20) Strøm, M. B.; Haug, B. E.; Skar, M. L.; Stensen, W.; Stiberg, T.; et al. The pharmacophore of short cationic antibacterial peptides. *J. Med. Chem.* **2003**, *46*, 1567−1570.
(21) Hancock, R. E. W. Cationic peptides: effectors in innate immunity and novel antimicrobials. *Lancet Infect. Dis.* **2001**, *1*, 156−164.
(22) Fernandez-Lopez, S.; Kim, H.-S.; Choi, E. C.; Delgado, M.; Granja, J. R.; et al. Antibacterial agents based on the cyclic D,L-α-peptide architecture. *Nature* **2001**, *412*, 452−455.

JM049582B