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Improving the Selectivity of Antimicrobial Peptides from Anuran Skin

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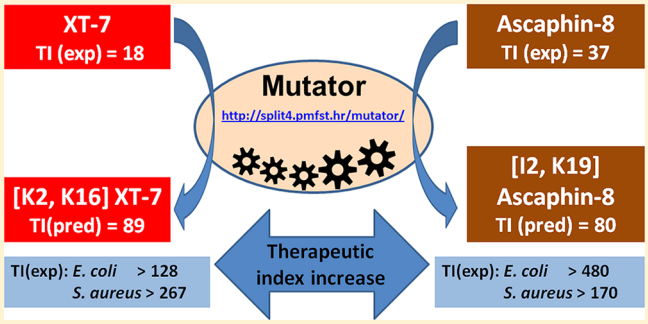
Supporting Information

ABSTRACT: Anuran skin is known to be a rich source of antimicrobial peptides although their therapeutic potential is often limited due to their toxicity against mammalian cells. The analysis of structure−activity relationships among anuran antimicrobial peptides provided the parameters to construct the "Mutator" tool for improving their selectivity for bacterial cells, by suggesting appropriate point substitutions. Double substitution analogues [K2, K16] of the *Xenopus tropicalis* peptide XT-7 and [I2, K19] of the *Ascaphus truei* peptide ascaphin-8 were predicted by this tool to have an increased 'therapeutic index' (TI = HC_{50}/MIC for erythrocytes with respect to bacteria) > 80. The mutated peptides were synthesized and respectively found to have experimental TI values > 130 for *S. aureus* or *E. coli*, a considerable improvement with respect to TI < 37 for the parent compounds. Circular dichroism studies of the mutated peptides suggested this may in part be due to variations in the α-helical structure. For *P. aeruginosa,* which is more resistant to XT-7, the TI increased in the mutated peptide from 5 to >270, also due to a significant improvement in minimal inhibitory concentration. We have shown that the Mutator tool is capable of suggesting limited variations in natural anuran peptides capable of increasing peptide selectivity, by decreasing toxicity against mammalian erythrocytes, in general without compromising antibacterial activity. The tool is freely available on the Mutator Web server at http://split4.pmfst.hr/mutator/.



1. INTRODUCTION

For over 20 years, peptide antibiotics have been put forward as new classes of antibiotics but with limited success for application in medical practice.[1] This expectation was based primarily on their ability to rapidly destroy the high membrane electric potential, which is essential for survival and reproduction of most bacterial strains.[2] The bacterial membrane potential is significantly higher than that of animal cells, but the cytoplasmic membrane is a much vaster and complex target than specific membrane-associated or cytoplasmic bacterial proteins targets. A highly specific molecular target of action can however be a disadvantage for long-term antibiotic use, as it can be modified or replaced, resulting in partly or fully resistant bacteria to that antibiotic. The capacity of antimicrobial peptides (AMPs) to permeabilize the cytoplasmic membrane to ions and metabolites, and dissipate its electric potential in the process,[3] is more difficult for bacteria to deal with. They cannot change their membrane in large proportion without compromising their survival. An indirect evidence for this is the persistence of antimicrobial peptide production for eons by all organisms examined so far, ranging from bacteria and unicellular eukaryotes to all types of multicellular organisms AMPs.[4]

Anurans (frogs and toads) often dwell in humid and microbe laden environments. Evolution endowed them with a rich armory of skin-secreted AMPs. These peptides are sometimes also toxic to host cells, but their activity is carefully orchestrated by the host, which releases the mature, active AMPs only at locations, such as epithelial surfaces, and times when damage will be minimal to host cells and maximal to microbial cells. To exploit the promise of peptide antibiotics derived from natural sources such as anuran skin, for human health, we therefore need to decrease their toxicity as much as possible without compromising their antibacterial activity. Limited, specific amino acid substitutions have been shown to result in both significantly reduced and increased eukaryotic cell toxicity.[5] The most promising

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amino acid substitutions in appropriate positions result in balanced changes in peptide overall properties such as cationicity, helicity, hydrophobicity, amphipathicity, and angle subtended by the polar face, but in general it is left to the investigator's instinct and expertise to choose what these substitutions should be. A classic example is pexiganan, conceived in the early 1990s,[6,7] in which the priority was always to enhance the antibiotic activity, without so much attention to the rational means of reducing the toxicity.[8] This limited use to a topical application, and it failed approval by the Food and Drug Administration.

A convenient measure of peptide selectivity is its 'therapeutic index' (TI) − the ratio of peptide concentration causing 50% lysis of red blood cells (HC_{50}) to minimal inhibitory concentration (MIC) against bacteria, so that TI = HC_{50}/MIC. The TI is thus a relative measure of the efficacy versus toxicity, and it can be increased either by decreasing peptide toxicity (i.e., an increase in HC_{50}) or by increasing its antibiotic activity (i.e., a decrease in MIC). Note that the higher the values of HC_{50} or MIC are, the less effective a peptide is in lysing blood cells or inhibiting bacterial growth, respectively. Unfortunately, the peptides' structural parameters underlying these two activities are tightly connected, so that an increase in HC_{50} is often accompanied by an increase in MIC and *vice versa*. For instance, an increase in the peptides' cationicity, while maintaining a good amphipathicity, can be easily implemented in the design of natural AMP analogues and normally results in a significant MIC reduction (increased efficacy), but the peptides' hemolytic activity is usually also increased (resulting in a lower HC_{50}), so that overall the TI is not improved. Only rarely do amino acid substitutions succeed in dissociating the antibacterial efficacy from the red blood cells toxicity.[5,9−14] We are not aware of the existence of dedicated algorithms that would assist in deciding which specific amino acid substitutions at particular sequence positions (either conservative or nonconservative) will produce significantly increased selectivity for bacterial cells.

We addressed this problem by developing software capable of objectively suggesting the amino acid substitutions expected to improve the selectivity of AMPs, i.e. with the potential to transform natural AMPs with good antimicrobial activity but unfavorable mammalian cell toxicity, into more promising lead compounds for the development of peptide antibiotics. This approach is made possible by previous results showing that a single descriptor is sufficient to distinguish potentially useful AMPs, with reasonable TI, from mediocre or quite toxic ones.[8] A functional asymmetry with respect to the primary structure of linear AMPs has been observed, namely that the N-terminal portion is often more important than the C-terminal one for activity and selectivity of AMPs,[15] and the lengthwise distribution of hydrophobic residues can also affect selectivity.[16] These observations suggest that the sequence profile for some physicochemical parameters of an AMP, such as hydrophobicity, may be more important for structure−activity correlations than mean values over the whole sequence. For this reason, we have defined a 'sequence moment'[8] that measures the lengthwise asymmetry in the hydrophobicity, to be distinguished from the more commonly used 'hydrophobic moment', that is a mean value derived from hydrophobicity distribution about the peptides' helical structure. Note that the sequence moment is not necessarily limited to the hydrophobicity of the residue side-chains but can in principle be derived from any amino acid attribute.

To derive the sequence moment, the sequence is bent into a 90° arc around a coordinate system (see Figure 1), and an index vector assigned to each residue position, with the same origin as the coordinate system (small vectors in Figure 1). The vector sum is then presented at the origin (large vectors in Figure 1) so that its direction and length results from summing the positional vectors for all amino acid residues (see Methods). The sequence moment hence contains information on the lengthwise asymmetry according to chosen attribute scale, such as the amino acid hydrophobicity. After extensive evaluation of 144 attribute scales, we found that in some cases using a pair of different hydrophobicity scales, the angle separating the obtained sequence moments correlated well with the measured TI values of the peptide being considered. The cosine of the angle between these vectors (which we term the D-descriptor)[8] could therefore be used as a parameter for predicting TI for AMPs similar to the anuran ones used to train and test the algorithm. In spite of being a simple one parameter linear fit, the D-descriptor structure-selectivity model is able to predict TI with respect to *E. coli* for nonhomologous AMPs, i.e. the homology to the specific anuran AMPs does not have to be high.[8]

The D-descriptor was implemented in a Designer module, which has previously been shown to successfully generate selective AMPs de novo.[8,17] We have now implemented it also in the Mutator algorithm, which is described in this paper together with its experimental verification. It contains the TI-predictor as its core, together with several methods designed to select only those amino acid substitutions (among the huge number of possible substitutions) that are likely to increase TI and unlikely to decrease peptide antibacterial activity. To this end, we reasoned that parent compounds used for testing the Mutator software should be broad-spectrum AMPs having a less than satisfactory therapeutic index. The existence of relevant published data would also be helpful in selecting lead compounds for which numerous analogues have already been reported together with extensive testing of their structure, activity, and selectivity, as such data would provide a useful benchmark. These selection criteria suggested ascaphin-8 and XT-7 from among the present in the recently published DADP database of anuran host defense peptides.[18]

After synthesis of the suggested analogues, tests were performed with Gram-positive and Gram-negative bacteria, including some resistant strains. Analogues with very high therapeutic index and good broad-spectrum antibacterial activity were found in this first attempt to use this dedicated software to achieve this objective.

2. MATERIALS AND METHODS

Software Description. The Algorithm and Its Input. The Mutator tool suggests limited amino acid substitutions for a given peptide in order to decrease toxicity for mammalian cells and to improve its therapeutic index. The Mutator algorithm can be considered as a simplified version of the Designer algorithm, which has been recently described.[8] Both training and testing sets used in the Designer algorithm and the corresponding peptide sequences (nonhomologous in the training set) are based on experiments performed in a uniform fashion, so that MIC and HC_{50} values are determined against standardized ATTC strains of *E. coli* and human red blood cells of healthy donors. The procedure used to train the algorithm's to predict therapeutic index was effected by using only anuran peptides that adopt mostly helical conformations in membrane-like environment. Because of this, the algorithm is most appropriate to work on linear peptides that likely adopt a helical conformation, while a resemblance to the anuran peptides is an advantage as some of

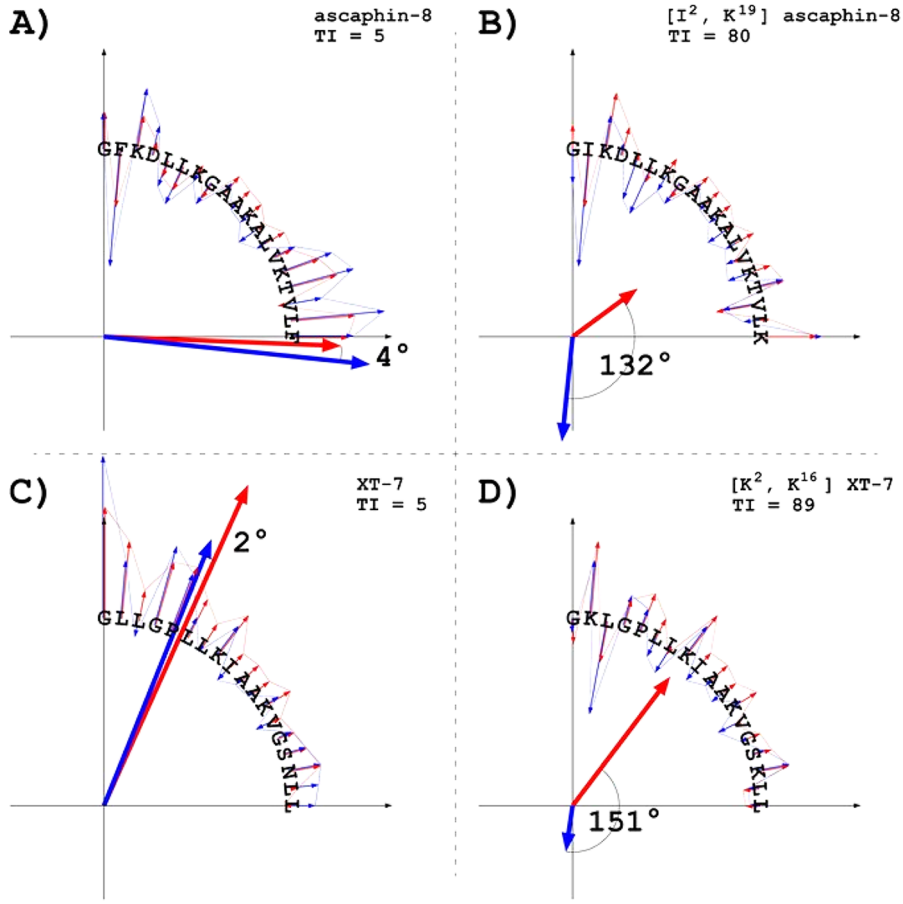

Figure 1. Relationship of sequence moments and predicted therapeutic index for pairs of parent peptide and its analogue: ascaphin-8, [I2, K19] ascaphin-8 (A, B) and XT-7, [K2, K16] XT-7 (C, D). Two vectors are calculated for each amino acid using Janin's[19] (red) and Guy's[21] (blue) amino acid scale. Large arrows represent sequence moments and are the vector sums of individual vectors for each residue. Predicted therapeutic index (TI) for each peptide is indicated. The increase of the angle between sequence moments when going from parent peptides to predicted analogues: (A→B), (C→D), is indicative of the increase in predicted therapeutic index.

the selection rules used by the algorithm are based on a statistical analysis of anuran AMPs. The input is the primary structure of an antimicrobial peptide, whose properties should be optimized. The algorithm then uses default input files with a set of 26 AMPs with a potent antimicrobial activity and measured TI > 20, defined as the SPB (Set of Best Peptides),[8] and three normalized hydrophobicity scales namely Janin's,[19] Eisenberg's,[20] and Guy's.[21] The Janin and Guy pair of hydrophobicity scales are used to predict TI by generating the D-descriptor, an essential parameter for TI prediction. The Eisenberg scale is used to determine hydrophobic moment, as one of the filtering parameters.

The Mutator algorithm is freely available for noncommercial usage at the Web site http://split4.pmfst.hr/mutator/ (accessed November 2, 2012). The server accepts peptide amino acid sequence expressed as single letter codes for standard amino acids as the only needed input. The output sequence can be reused in a copy-paste procedure should the first analysis not produce a high enough increase in the predicted TI. The tool contains five interconnected modules: Single Harsh Mutator, Single Mutator, Single Weak Mutator, Double Harsh Mutator, and Double Mutator, which introduce one or two amino acid substitutions into the parent sequence. Analogues of the query peptide sequence are presented as output only for those modules that are able to find a predicted TI increment of at least 10.

Methods Used by the Algorithm. - The D descriptor. This model was constructed by collecting the TI values derived from published HC_{50} and MIC (*E. coli*) data for anuran peptides in a dedicated database AMPad:http://pubs.acs.org/doi/suppl/10.1021/ci900327a/suppl_file/ci900327a_si_001.pdf (accessed Nov 2, 2012). Nonhomologous sequences (less than 70% pairwise identity among themselves) were used as a training set to correlate the amino acid attributes to this quantitative activity data, so as to develop an effective algorithm for predicting TI. It predicts TI values from the linear structure-selectivity fit: TI = 50.1−44.8D. Sequence moments (see the Introduction), used to calculate the D-descriptor, originate from application of Janin's[19] and Guy's[21] hydrophobicity scale and smoothing procedure, which were found to be the best way of extracting sequence hydrophobic environments during the training process. A sliding window smoothing procedure is used in which the central residue is omitted in the calculation of a mean hydrophobicity for that position, based on the scale in use. The window expands from three to 11 residues and then contracts in a similar manner while sliding from peptide N- to C-terminal. Each mean hydrophobicity value determines the length of the positional index vector for that position when the whole sequence is placed on 90° arc, with N- and C-terminal residues located respectively at y- and x-axis of a coordinate system (see Figure 1). A vector sum of all the positional vectors

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is the sequence moment for the corresponding amino acid scale. The chosen smoothing procedure results in a preference for suggesting amino acid substitution near peptide termini (see Table S1 provided as Supporting Information). The D-descriptor,[8] being the cosine of the angle between two sequence moments, ranges in value from −1 (TI = 95) to +1 (TI = 5). By only accepting mutations that result in an increase of TI > 10, together with other requirements (see below), Mutator drastically reduces the number of offered analogues. If for a given query peptide, none of software modules can suggest any amino acid substitution or substitutions that achieve this improvement, it is a good indication that the sequence may not be amenable to improvement, as such.

- Significant Succesor Index. Analysis of peptides in the AMPad database also allowed extracting mean and position-specific properties as well as motifs to guide the insertion of residues at each position. From these, an AASI (amino acid selectivity index) was generated. For the details please check Table 2 in ref 8 and the relevant text. Furthermore, this was used to generate a Significant Successor Index (SSIn), which is a score given to an amino acid in any given position, based on the amino acids that preceded it at positions *i-1* or *i-4* or succeed it at positions *i+1* and *i+4*. The significant successor index (*SSI*1) is the value analogous to that inscribed in Table 2 of ref 8 for immediately succeeding amino acid. Hence, the entry (x, y) of the table analogous to Table 2 from ref 8 will be denoted by $SSI1(x, y)$. The significant successor index (*SSI*4) is the value analogous to that inscribed in Table 3 of that reference. We shall call these "+4" successor. Hence, the entry (x, y) of the table analogous to Table 3 from ref 8 will be denoted by $SSI4(x, y)$. Most significant successor amino acids are generated by taking into account the residues at positions *i-4, i-1, i+1,* and *i+4,* as well as other relevant criteria.

- Peptide amphipathicity should not decrease as a result of mutated AA. For peptides with unknown 3D structure, amphipathicity, and hydrophobic moment calculations[20] usually involve making an unrealistic assumption that the whole peptide sequence is folded into a regular secondary structures. Examples of AMPs having more than 20 amino acids are quite frequent, and for these, mean hydrophobic moment differences after single substitution are quite small, and the values are strongly dependent on chosen hydrophobicity scale and the regular structure that the whole peptide is assumed to adopt. However, only the immediate sequence environment (to be defined below) of substituted AA is really relevant for an evaluation of the change in amphipathicity. By using this concept we can avoid having to compare mean hydrophobic moments of the whole sequence, before and after substitution.

The sequence environment calculation and its bearing on the hydrophobicity of a given (substituted) residue warrants a more detailed description. For the case of linear peptides with preference to form membrane associated helices, we can use a projection of the peptide amino acid sequence onto a helical-wheel projection, assuming that each subsequent amino acid is rotated 100° in the projection plane (the Edmundson projection for an ideal α helix). We now restrict our attention to 5 AA preceding the mutated position and to the five AA succeeding it. These residues form the sequence environment of the central residue considered for substitution. More formally, if we denote the sequence position of the mutated AA by i, then we observe amino acids from the set

$$S_i = \{j: \max\{1, i-5\} \le j \le \min\{l, i+5\} \text{ and } i \ne j\}$$

where l is the length of the observed peptide. We next assign to each of the AA a position on the helical wheel projection, so that the position to the ith AA (cos 0, sin 0) = (1, 0), then we rotate repeatedly clockwise for 100 degrees and assign successor positions and similarly rotate anticlockwise for −100 and assign predecessor positions. Then, a vector is assigned to each AA at one of the places in $S_i \cup \{i\}$, which is in the circle plane but orthogonal to the circles circumference. The length and orientation of this vector are defined according to the Eisenberg hydrophobicity scale.[20] Namely, if the value that corresponds to observed AA is positive, the vector is oriented outward, and if the value is negative, the vector is oriented inward. The length of the vector is the absolute value that corresponds to hydrophobicity index of observed AA. Let us denote by v_j the vector that corresponds to AA at the place j. Hence, the hydrophobicity of the "mutated" AA is v_i. The hydrophobicity of the remainder of the peptide near this amino acid is defined as $w_i = \sum_{j \in Si} v_j$. We assume that v_i and w_i are consistently oriented if the angle between them is an acute angle, i.e. if their scalar product is positive. Hence, by applying this test as the requirement for allowed substitutions, we ensure that we did not add hydrophobic AA in the hydrophilic, nor hydrophilic AA in the hydrophobic region of helical wheel projection near substituted residue. In this way we ensure that the amphipathicity of a parent (query) peptide is unlikely to decrease after the suggested substitution by the Mutator algorithm. The calculation of sequence environments is similar for finding sequence moments (to be used as peptide lengthwise asymmetry indicators) and for finding corresponding hydrophobic moment vectors (to be used as amphipathicity indicators).

Five Modules. *Single Harsh Mutator.* It substitutes each one of the AA in the sequence except the first one. Let us denote the original AA that will be "mutated" by *OAA* and mutated one by *MAA*. The substitution of the AA at the i^{th} place is successful if following requirements are satisfied:

1.1) *MAA* is one of the five most significant immediate successors of AA_{i-1};

1.2) If $i \ge 5$, then *MAA* is one of the five most significant "+4" successors of AA_{i-4};

1.3) If $i \ge 5$, then $SSI1\ (AA_{i-1}, MAA) + SSI4(AA_{i-4}, MAA) > SSI1\ (AA_{i-1}, OAA) + SSI4\ (AA_{i-4}, OAA)$, and if $i \le 4$, then $SSI1\ (AA_{i-1}, MAA) > SSI1\ (AA_{i-1}, OAA)$;

1.4) AA_{i+1} is one of the five most significant successors of *MAA*;

1.5) If $i \le l-4$, then AA_{i+4} is one of the five most significant "+4" successors of *MAA*;

1.6) If $i \le l-4$, then $SSI1(MAA, AA_{i+1}) + SSI4(MAA, AA_{i+4}) > SSI1\ (OAA, AA_{i+1}) + SSI4(OAA, AA_{i+4})$, and if $i \ge l-3$, then $SSI1(MAA, AA_{i+1}) > SSI1(OAA, AA_{i+4})$;

1.7) The amphipathicity of peptide segment is considered, with *MAA* being the central amino acid of the segment, such that it can increase but is unlikely to decrease an overall peptide amphipathicity;

1.8) Algorithm presents output only if this module passes all the criteria and able to find a predicted TI increment of at least 10 with respect to parent peptide.

Requirements 1−6 are there to ensure that small motives of type XY and X_ _ _Y are more regular in the "mutated" peptide than in the original one (namely, more in accordance with those that can be found in the default SBP set of good peptide antibiotics). Requirement 7 ensures that the peptide hydrophobic moment is unlikely to decrease. The algorithm tries all $19 \times (l-1)$ possible "mutations" for each position. If none satisfies all eight requirements the algorithm returns a

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"No significant mutation" message. Otherwise, it outputs the mutated sequence that results, in the greatest increase of the predicted TI, over the value of 10.

Single Mutator. This module works in the same way as the Single Harsh Mutator, but it does not use the requirements 1.4)−1.6). The reason for this is that it has been observed that peptide activity is often more determined by its N-terminal part than its C-terminal part,[15] hence we are more interested in the small motifs of the form XY and X_ _ _Y at the left side of the "mutated" AA.

Single Weak Mutator. This module works in the same way as the Single Mutator, but in condition 1.3) $i \geq 5$ is replaced by $i > 5$. The reason for this is that the first AA of peptide is fixed (to Gly for example), so the small motifs requirement X_ _ _ Y may not be applicable at the N-terminus.

Double Harsh Mutator. It works similarly as Single Harsh Mutator, just it mutates two AAs simultaneously.

Double Mutator. It works similarly as Single Mutator, but mutates two AAs simultaneously.

Selecting Mutator's Output. An obvious question is which output should be considered when more than one peptide analogue is suggested after a one-step application of a query peptide, if more than one output is presented. If the predicted improvement in TI is similar for output sequences offered by different modules, one should prefer the output sequence according to the strictness of the selection rules, so that the reliability of results can be probably be ordered as: Single Harsh Mutator > Single Mutator > Single Weak Mutator > Double Harsh Mutator > Double Mutator. However, the improvement of the predicted TI usually follows the opposite order, i.e. the Double Mutator generally produces analogues with much higher TI than other modules. Therefore, one should consider if the difference in TI is sufficiently larger to justify the use of the less reliable method. Furthermore, a human expert may take into account that some of suggested analogues have attributes, such as higher net positive charge, which may contribute to maintaining the antimicrobial activity, especially if the parent peptide had modest charge to begin with. The present version of the software has no direct requirements with respect to desirability of limited charge increase. It also has no requirement which would limit the peptide mean hydrophobicity (which tends to be higher in hemolytic peptides and toxins) or maintain it at a sufficient level to ensure membrane interaction. The source code allows for easy implementations of the above-mentioned additional restrictions. In the absence of such restrictions, the present version of the source code and corresponding web server however allows use even of peptide toxins as a query, although an iterative procedure is then required (repeatedly using the output sequence as a new input) to transform peptide toxin into a useable peptide antibiotic.

Convenient tools which can be used for further expert analysis of the output substitutions are the helical wheel tool http://cti.itc.virginia.edu/~cmg/Demo/wheel/wheelApp.html, membrane protein secondary structure prediction server: http://split.pmfst.hr/split/, and the peptide sequence analysis tool http://www.bbcm.univ.trieste.it/~tossi/HydroCalc/HydroMCalc.html (all accessed Nov 2, 2012).

Experimental Validation. Solid Phase Peptide Synthesis. Peptides were synthesized using solid phase FastMoc chemistry procedure on an Applied Biosystems 433A automated peptide synthesizer (Life technologies, Cergy-Pontoise, France). Resin and FMoc-protected amino acids were purchased from Iris Biotech GMBH (Marktredwitz, Germany), and solvents from SDS-Carlo Erba (Vitry, France). Carboxamidated peptides were prepared on 4-methylbenzhydrylamine polystyrene resin (Fmoc-Rink Amide PEG MBHA PS resin) substituted at 0.51 mmol/g. The syntheses were carried out as previously described.[22] Briefly, synthesis products were cleaved from the resin by a mixture of 95% trifluoracetic acid (TFA), 2.5% H_2O and 2.5% Triisopropylsilan, precipitated in ether, centrifuged, and then lyophilized. The lyophilized crude peptides were purified by reversed-phase HPLC (RP-HPLC) on a Luna C18(2) column (10 μm, 10 × 250 mm from Phenomenex, Le Pecq, France), eluted at 5 mL/min with a 35−70% linear gradient of acetonitrile (ACN) 0.07% TFA in 0.1% TFA/water over 35 min. The homogeneity and identity of the synthetic peptides were assessed by MALDI-TOF mass spectrometry (Voyager DE-Pro, Applied Biosystems, Life technologies, Cergy-Pontoise, France) and analytical RP-HPLC on a Luna C18(2) column (5 μm, 4.6 × 250 mm) eluted at a flow rate of 0.75 mL/min by a 0−60% linear gradient of ACN 0.07% TFA in 0.1% TFA/water (1%/min).

The purity of the peptides was greater than 90 to 95% (see chromatogram and spectra available as Supporting Information S2).

Antimicrobial Activity. Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *S. aureus multiresistant* ATCC BAA-44, *Enterococcus faecalis* ATCC 29212) and Gram-negative bacteria (*Escherichia coli* ATCC 25922, *E. coli ML35-p*,[23] *Pseudomonas aeruginosa* ATCC 27853) were cultured in LB medium. Logarithmic phase cultures of bacteria were centrifuged and diluted in Mueller-Hinton broth to an absorbance at 600 nm (*A*600) of 0.01 (10^6 cfu/ml). The minimal inhibitory concentration (MIC) was expressed as the lowest concentration of the peptide that completely inhibited bacterial growth and as the average value from three independent experiments, each performed in triplicate. MICs were determined by measuring *A*600 in 96-well microtitration plates by growing 50 μL of the microorganism suspension (10^6 cfu/ml) with 50 μL of 2-fold serial dilutions of synthetic peptides overnight at 37 °C (1−200 μM and if necessary 1−800 μM or 1−25 μM) . Positive (0.7% formaldehyde) and negative (H_2O) inhibition controls and sterility control (H_2O) were also performed.

Hemolytic Activity. The hemolytic activity was determined using fresh rat erythrocytes (Charles River Laboratories, L'Arbresle, France). Synthetic peptides (1−200 μM or 25−800 μM) were incubated with washed rat erythrocytes (2×10^7 cells) in Dulbecco's phosphate-buffered saline, pH 7.4 (100 μL) for 1h at 37 °C. After centrifugation (12000 g for 15 s), the absorbance at 450 nm of the supernatant was measured. Incubation with 0.1% v/v Triton was carried out to determine the absorbance associated with 100% hemolysis. The HC_{50} value corresponding to the mean concentration of peptide producing 50% hemolysis was determined from three independent experiments performed in triplicate.

CD Spectroscopy. Far-ultraviolet CD spectra were recorded at 20 °C with a Jobin Yvon CD6 spectropolarimeter (Longjumeau, France) using a quartz cell of 1-mm path length. Spectra were acquired between 185 and 260 nm with a spectral bandwidth of 2 nm and 0.5 nm steps. The instrument outputs were calibrated with *d*(+)-10-camphorsulfonic acid. Peptides were solubilized in H_2O Milli-Q or in 80 mM sodium dodecyl sulfate (SDS) at a concentration of 30 μM. Solvent spectra (H_2O, SDS) were subtracted, followed by baseline correction and smoothing. CD measurements are reported as $\Delta\epsilon$, which is the dichroic increment ($M^{-1}.cm^{-1}$) per amino acid residue.

Table 1. Predicted Substitutions and Corresponding Predicted Therapeutic Index Values Based on the Ascaphin-8 and XT-7 Query Sequences

peptide	sequence[a]	charge (pH 7)	software module[b]	predicted TI (*E. coli*)
ascaphin-8	GFKDLLKGAAKALVKTVLF	4	none	5
[W2] ascaphin-8	G**W**KDLLKGAAKALVKTVF	4	none *(Expert choice)*	10
[I2] ascaphin-8	G**I**KDLLKGAAKALVKTVLF	4	Single Harsh "Mutator"	27
[K19] ascaphin-8	GFKDLLKGAAKALVKTVL**K**	5	Single "Mutator" and Single Weak "Mutator"	30
[I2, K19] ascaphin-8	G**I**KDLLKGAAKALVKTVL**K**	5	Double "Mutator"	80
XT-7	GLLGPLLKIAAKVGSNLL	3	none	5
[K16] XT-7	GLLGPLLKIAAKVGS**K**LL	4	none *(Expert choice)*	6
[K2] XT-7	G**K**LGPLLKIAAKVGSNLL	4	Single "Mutator" and Single Weak "Mutator"	23
[K2, K16] XT-7	G**K**LGPLLKIAAKVGS**K**LL	5	Double "Mutator"	89

[a]All the peptides are amidated at the C-terminus. [b]Mutator's module used for predicting substitution.

Table 2. Experimental and Predicted Therapeutic Index Values of Ascaphin-8, XT-7, and Their Analogues for *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923)[b]

PEPTIDE	MIC(μM) *E. coli*	HC_{50} (μM)	TI-exp. Gram-	TI estimate[a] Gram-	MIC(μM) *S. aureus*	TI-exp. Gram+
Ascaphin-8	3	115	36.8	5	3	36.8
[W^2] ascaphin-8	3	40	12.8	10	-	-
[I^2] ascaphin-8	3	142	45.4	27	-	-
[K^{19}] ascaphin-8	5	>800	>160	30	18.8	>43
[I^2, K^{19}] ascaphin-8	1.7	>800	>480	80	4.7	>170
XT-7	6.3	110	17.6	5	6.3	17.6
[K^{16}] XT-7	6.3	142	22.7	6	-	-
[K^2] XT-7	12.5	>800	>64	23	-	-
[K^2, K^{16}] XT-7	6.3	>800	>128	89	3	>267

[a]TI-predictor: http://split4.pmfst.hr/split/dserv1/ (accessed November 2, 2012). [b]The [W2] ascaphin-8 and [K16] XT-7 analogues are expert human choice. Among measured selectivity values (TI-exp. = HC_{50}/MIC), those with correctly predicted TI increase are white on black background.

3. RESULTS

Analogues Found by the Mutator Software for Ascaphin-8 and Peptide XT-7. Using the ascaphin-8 sequence as the query, the Mutator software predicted three analogues of ascaphin-8 with estimated therapeutic indexes greater than 20 (Table 1). The first one corresponds to the substitution of phenylalanine in position 2 by an isoleucine.

The second analogue is mono substituted at the C terminus, with a phenylalanine replaced by a lysine. [K19] ascaphin-8 has a predicted TI that is 6-fold greater than that calculated for ascaphin-8. It is interesting that the Double Mutator's module, in this case, produced the same two substitutions predicted by "Single" mutation modules [I2, K19] ascaphin-8, with a predicted TI that is considerably increased (16-fold) with respect to ascaphin-8. Finally, to further probe the relevance of position 2, the analogue [W2] ascaphin-8 has also been considered, due to reports that inclusion of tryptophan tends to potentiate antibacterial activity and selectivity of AMPs.[24,25] This is our test case of an expert-choice for a substitution, as opposed to a "Mutator" generated one.

The Mutator software predicted two analogues of XT-7. [K2] XT-7 has a 4-fold greater predicted TI than the native peptide (Table 1). The second analogue [K2, K16] XT-7 (charge of +5), predicted by the Double Mutator's module, adds a substitution of an asparagine by a lysine at the carboxy end of the peptide. Its predicted TI is almost 18-fold greater than that of XT-7. [K16] XT-7, which corresponds to the expert-choice test case,[26] had a predicted TI = 6 calculated by the TI-predictor algorithm, which is close to that of the parent peptide.

Figure 1 compares sequence moments of parent peptides and corresponding disubstituted analogues. The greater separation of sequence moments is quite evident for the analogues. It allowed the D-descriptor structure-selectivity model to predict the high increase in therapeutic index for these peptides. Experimental confirmation of these and other TI predictions are the topic of the next section.

Experimental Determination of the Therapeutic Indexes. Ascaphin 8 and XT-7 peptides, as well as all their analogues, were synthetized by FastMoc solid phase synthesis (see Table 1 and Materials and Methods). The experimental therapeutic indexes (TI-exp. = HC_{50}/MIC) of the peptides were evaluated by determining their toxicity against red blood cells, their minimal inhibitory concentration against the Gram-negative strain *E. coli*, and in some cases their MIC against the Gram-positive strain *S. aureus* (Table 2).

The Ascaphin Set. All ascaphin-8 analogues proposed by the software have weak or undetectable hemolytic activity, with HC_{50} values being considerably higher than 115 μM value of the native peptide. Since MIC values against *E. coli* are similar for analogues and ascaphin-8, it follows that experimental TI values are appreciably better for all of the analogues proposed by Mutator and particularly for [K19] ascaphin-8 (TI greater than 160) and [I2, K19] ascaphin-8 (TI greater than 480). In contrast, the expert-choice analogue [W2] ascaphin-8 did not have better antimicrobial activity or selectivity in comparison

Table 3. Antibacterial Activities of [I2, K19] Ascaphin-8 and [K2, K16] XT-7, against Additional Gram-Positive and Gram-Negative Bacteria

PEPTIDE		Bacterial strains[a]							
		GRAM -				GRAM +			
		E. coli (ML35p)		*P. aeruginosa*		*S. aureus* (multiresistant)		*E. faecalis*	
	HC_{50} (μM)	MIC (μM)	TI-exp.	MIC (μM)	TI-exp.	MIC (μM)	TI-exp.	MIC (μM)	TI-exp.
Ascaphin-8	115	1.6	71.9	3	38.3	3	38.3	12.5	9.2
[I2, K19]ascaphin-8	>800	12.5	>64	25	32	6.3	>127	50	16
XT-7	110	3	36.7	25	4.4	1.6	68.8	6.3	17.5
[K2, K16] XT-7	>800	1.6	>500	3	>267	6.3	>127	50	16

[a]Gram-negative strains (*E. coli* ML-35p and the naturally resistant strain *P. aeruginosa* (ATCC 27853)) and Gram-positive strains (multiresistant *S. aureus* (ATCC BAA-44) and the resistant strain *E. faecalis* (ATCC 29212)). See Table 2 caption for the meaning of reverse color numbers. Values with gray background are cases when predicted amino acid substitutions for the TI increase do not achieve that goal (underpredictions).

with the parent peptide. The fact that measured TIs are higher than predicted TIs comes from the fact that predicted TIs are limited to the +5 to +95 range. However, the fold increase in TI after lysine K19 and [I2, K19] substitution is similar for predicted and measured selectivity values. The selectivity increase with respect to the parent compound was reasonable (about 5-fold) for the [I2, K19] analogue also in the case of the Gram-positive strain *S. aureus* (Table 2).

The XT-7 Set. The MIC values against *E. coli* of the XT-7 analogues were not better than that of the native peptide (Table 2), but this was not unexpected, as the Mutator software aims to predict or improve selectivity, not antibacterial activity. The HC_{50} values of the Mutator generated analogues were instead improved more than 7-fold, into >800 μM for both the [K2] XT-7 and [K2, K16] XT-7. The measured TI values are thus 4- to 7-fold better than the parent peptide. Experiments confirmed software prediction that the [K16] XT-7 analogue designed by Conlon et al.[26] would not result in a significant change in TI. The observed 7- and 15-fold TI increase of [K2, K16] XT-7 with respect to *E. coli* and *S. aureus*, respectively, suggests a generally increased selectivity for bacterial cells.

[I2, K19] Ascaphin-8 and [K2, K16] XT-7. Determination of experimental TI against other bacterial strains. The experimental therapeutic index of the two best analogues [I2, K19] ascaphin-8 and [K2, K16] XT-7 was determined against the Gram-negative *P. aeruginosa* (ATCC 27853) and two Gram-positive strains, *S. aureus* (ATCC BAA-44) and *E. faecalis* (ATCC 29212), as well as an additional *E. coli* strain ML35-p (Table 3).[23] The [I2, K19] ascaphin-8 analogue presents weaker activity (higher MIC values) against all of the additionally tested bacterial strains, but its TI is improved by more than 3-fold with regard to the native peptide for the *S. aureus* strain, which is multiresistant to antibiotics. The other disubstituted analogue, [K2, K16] XT-7, has much improved TI for the two Gram-negative bacteria: 14-fold for *E. coli* ML-35p and 60-fold for the *P. aeruginosa* strain (Table 3). The measured TI was instead not significantly improved for the [K2, K16] XT-7, tested against the multiresistant *S. aureus* and against *E. faecalis*, due to significantly higher MICs.

Secondary Structure of Disubstituted Analogues. The far-UV CD spectra of analogues in aqueous solution displayed a strong minimum at 200 nm that reflects a large content of unordered structure (not shown). Addition of 80 mM SDS to the aqueous solution strongly modifies the dichroic spectra (Figures 2A and 3A). Circular dichroism spectra showed that

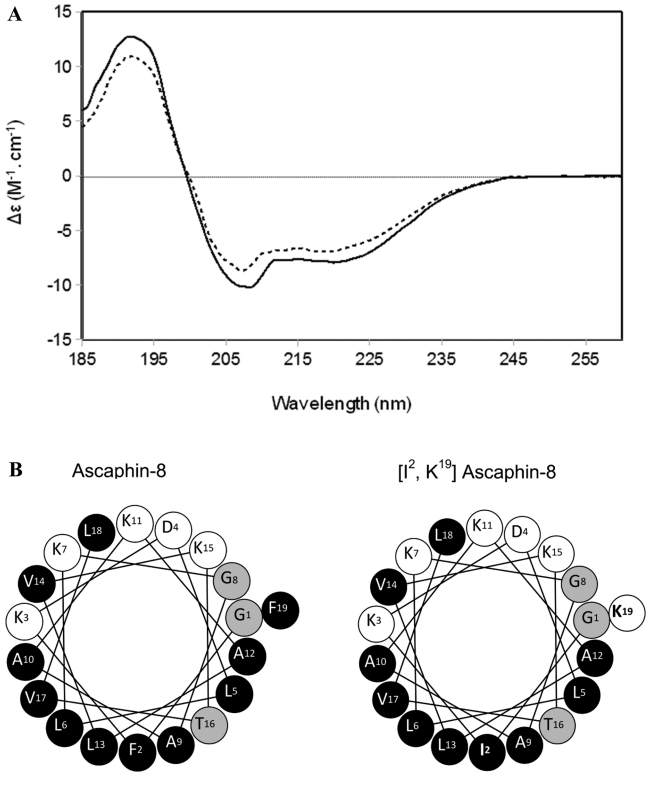

Figure 2. Ascaphin-8 and its analogue. A. Circular dichroism spectra. Ascaphin-8 (dotted line) and [I2, K19] ascaphin-8 (solid line) spectra were obtained in 80 mM SDS at a peptide concentration of 30 μM. No ordered structure was observed in H_20. B. Schiffer-Edmundson helical wheel projection. Apolar residues are represented in black, polar and neutral residues in gray, charged residues in white.

ascaphin-8 and its disubstituted analogue adopted an α-helix structure in 80 mM SDS (Figure 2, A). For both peptides, dichroic bands are typical of an α-helical structure with one absorption maximum at 190 nm and two minima at 208 and

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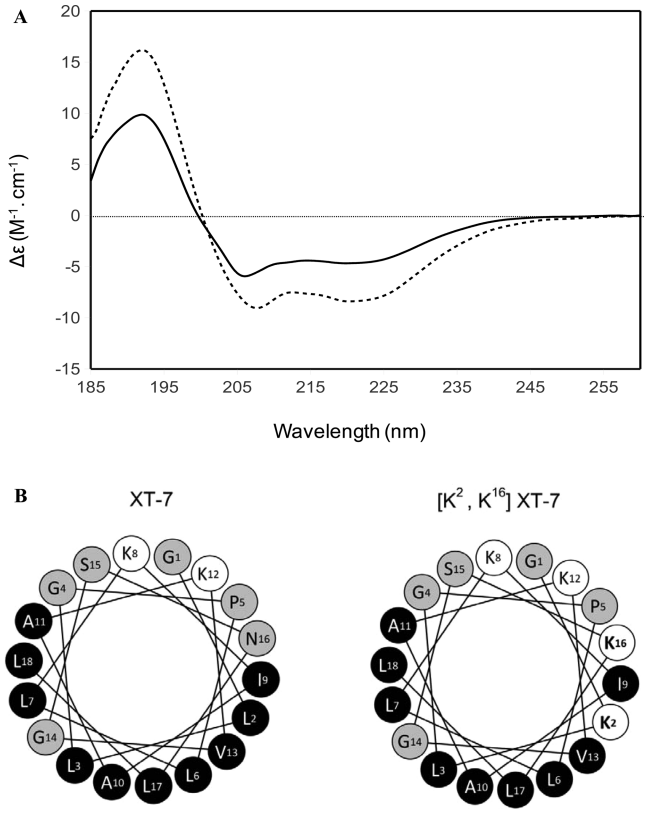

Figure 3. XT-7 and its analogue. A. Circular dichroism spectra. XT-7 (dotted line) and [K2, K16] XT-7 (solid line) spectra were obtained in 80 mM SDS at a peptide concentration of 30 μM. No ordered structure was observed in H_2O. B. Schiffer-Edmundson helical wheel projection. Apolar residues are represented in black, polar and neutral residues in gray, charged residues in white.

220 nm, respectively. When plotted on a Schiffer-Edmundson α-helical wheel (Figure 2, B), ascaphin-8 had a well-behaved amphipathic helical structure, i.e., almost all of the charged and polar residues were aligned on a portion of the helical cylinder and the lipophilic side chains occupy the remaining surface. The substitutions somewhat increase the asymmetry between the apolar and polar helix side (K3-G1 axis), thus increasing the amphipathic nature of the helix. The substitution of phenylalanine 19 by lysine strengthens the polar side of the helix, while putting isoleucine in the place of phenylalanine 2 maintains the hydrophobicity of the apolar helix side. Addition of 80 mM SDS to the aqueous solution strongly modifies the dichroic spectra of XT-7 and its analogue too (Figure 3, A), with ellipticity minima at 208 and 222 nm characteristic for helix structure in the case of XT-7. [K2, K16] XT-7 shows a lesser degree of helical structuring in a membrane-like environment,[27] but its amphipathicity is maintained (Figure 3, B).

Testing Algorithm Preferences for Suggesting Substitutions. We used the DADP database[18] of anuran defense peptides http://split4.pmfst.hr/dadp/ (accessed Nov 2, 2012) to test how often the algorithm is unable to offer any useful amino acid substitution and to test if the algorithm has a preference for suggesting substitutions near peptide termini (see Table S1 provided as Supporting Information). Out of 20 nonhomologous AMPs used as query, there were five for which none of the algorithm modules could suggest substitutions for improving selectivity. Only query peptides with predicted TI in the range from 5 to 20 were considered. Positional preferences of Mutator's predicted substitutions were analyzed by using 30 different anuran AMPs (with mean length of 25 residues) for which at least one of Mutator's modules suggested one or two amino acid replacements. Each peptide was divided into an N-terminal section (AA_{2-4}), C-terminal section (just four AA), and middle section (all of remaining residues). For the chosen 30 peptides there were 210 possible terminal positions for mutations (seven for each peptide) and 503 central positions for mutations (with mean length of 17 residues). Out of a total number of 98 nonredundant substitutions, 44 were located among terminal residues. It follows that the relative frequency for mutations near the termini (about 6.3% per residue) is twice that for internal residues (about 3.2% per residue). Of the 30 query peptides used, for five there were no substitutions in the middle section but only two with no suggested substitutions among terminal amino acids. Furthermore, N-terminal substitutions were more frequent than C-terminal ones. Counting only nonredundant substitutions of hydrophobic amino acids at positions two, three, and four with charged amino acid lysine, there are 14 such substitutions in 30 query peptides: four L2K, two F2K, two L3K, and one substitution for each of V2K, I2K, M2K, M3K, F3K, and F4K. The helical wheel projection of the N-terminal peptide section of these peptides indicates that the substituted lysines extend the polar sector into the hydrophobic sector, a pattern we observe also with ascaphin-8 and XT-7 substitutions (Figure 2 and Figure 3).

4. DISCUSSION

New concepts are clearly needed to dissociate antibacterial from hemolytic peptide activity and to increase peptide selectivity as expressed through the so-called "therapeutic index". Data mining and structure-selectivity relationships of anuran AMPs, which assume α-helical structure when associated with bacterial membranes, have been conducted recently to detect common properties shared by nonhomologous peptides.[8,17] Besides a net positive charge and amphipathic character, many AMPs displayed lengthwise asymmetry with their N-terminal part being more important with respect to biological activities[15] and having different physicochemical properties to the C-terminal part.[16] The introduction of the sequence moment concept (Figure 1), as a device for expressing lengthwise asymmetry, was an essential step for the construction of a linear structure-selectivity model fitting the D-descriptor to the therapeutic index. This TI predictor was first used as a key part of the Designer algorithm, which designed adepantins, a new class of peptide antibiotics with less than 50% identity to any known peptide and a very high therapeutic index. In laboratory tests, adepantins exhibited extraordinary specificity for Gram-negatives such as *E. coli* but were almost completely inactive against Gram-positives such as *S. aureus*.[8,17]

The same TI predictor was used as one of several devices for the construction of the Mutator algorithm, which we here show is able to predict amino acid substitutions that transform relatively unselective natural AMPs into potential peptide antibiotics with much higher selectivity but similar antimicrobial potency to their parent compounds. Analogues suggested by the Mutator tool maintain a similarly broad spectrum of antibacterial activity as the parent peptide, while their therapeutic index can be increased by 5 to 60 times, depending on the bacterial target (Tables 2 and 3). Biological assays on chemically synthesized ascaphin-8 and XT-7 analogues suggested by the Double Mutator module produced the best results for the disubstituted analogues [I2, K19] ascaphin-8 and [K2, K16] XT-7. HC_{50}

increased from about 100 μM for ascaphin-8 and XT-7 to over 800 μM for both analogues. As TI = HC_{50}/MIC, it follows that a roughly 10-fold TI increase occurs for *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923), and this is almost entirely due to a decreased toxicity for red blood cells. Due to the high number of possible substitutions for peptides containing 18 amino acids, we can safely state that without Mutator software, there would be very little chance of finding disubstituted analogues of ascaphin-8 and XT-7 with high antibacterial selectivity, even using expert considerations.

The disubstituted analogues had a rather different behavior with respect to *P. aeruginosa* (ATCC 27853). [I2, K19] ascaphin-8 was roughly 10 times less efficient than ascaphin-8 in inhibiting the growth of *P. aeruginosa* with similar TI of about 32. On the other hand, [K2, K16] XT-7 exhibits a marked increase in activity (8-fold) against *P. aeruginosa* and a very high TI (>267, a 60-fold selectivity increase) with respect to XT-7. Both disubstituted analogues inhibited the growth of the multiresistant strain *S. aureus* (ATCC BAA-44) with a MIC of about 6 μM and TI $\geq$ 127. The *S. aureus* ATCC 25923 strain and its multiresistant ATCC BAA-44 counterpart have similar cytoplasmic membrane organization and consequently a comparable sensitivity to the peptides, because the mechanism of AMP action is likely to be membranolytic; we indeed obtain comparable MIC (3 and 4.7 to 6.3 μM) and TI for multiresistant and commonly used *S. aureus* strains.

By comparing results in Table 2 and Table 3, we can see that predicted trends for increasing the antibacterial selectivity with respect to natural AMPs were experimentally confirmed in 13 out of 16 cases i.e. about 80% success rate. Since MIC data from Gram-positive and Gram-negative strains other than *E. coli* were not considered during the training procedure for the TI-predictor and Mutator software, the missed hits tended to be with these targets, while with *E. coli* it was quite accurate (five out of five predicted selectivity increases are confirmed in experiments as numbers in reverse colors, column 4, Table 2).

Results from Table 1 and Table 2 can be taken as evidence for algorithm robustness, because 8 out of 11 predicted selectivity increases with respect to other than standard *E. coli* strain are verified in experiments. One could suppose that the accuracy of the algorithm could be increased, if the input database of best anuran peptides is increased. We did increase it up to 53 peptides but found no difference in the suggested analogues for XT-7 and for ascaphin-8. For the final test of the algorithm's sensitivity, we used the normalized Wimley and White scale[28] as a replacement for the normalized Eisenberg consensus scale, in the subroutine calculating the change in a peptide's amphipathicity after each mutation. In the case of ascaphin-8, four out of five Mutator modules suggested identical substitutions as obtained with the Eisenberg scale. The Double Harsh Mutator module suggested one identical substitution, the replacement of F with I at the second position, and replacement of V with G at position 14 as the second substitution. The suggested ascaphin-8 analogue GIKDLLKGAAKALGKTVLF had a predicted therapeutic index of 40, a significant selectivity increase with respect to parent peptide (TI_{pred} = 5) and with respect to analogue suggested by the Double Harsh Mutator module using Eisenberg's scale: GIKDLLKGAGKALVKTVLF, with predicted TI = 30. Since none of analogues predicted by the Double Harsh Mutator module were tested in experiments for their selectivity (see Table 1) we cannot claim that the Wimley-White scale is any better than the Eisenberg scale in finding ascaphin-8 analogues with higher selectivity. Furthermore, the Wimley-White scale could not find any XT-7 analogue, while the Eisenberg scale finds two with an experimentally confirmed selectivity increase with respect to the parent peptide (Tables 1 and 2). For this reason, at the moment, we prefer to maintain the Eisenberg scale to produce the amphipathicity filter.

The results of Conlon et al.[26] can be used as a benchmark to further evaluate our Mutator tool, although in that publication only lysine and alanine were used for substitutions in ascaphin-8 and XT-7. Out of 15 XT-7 and ascaphin-8 analogues synthesized by Conlon et al.,[26] [K4] XT-7 and [K18] ascaphin-8 exhibited a significantly increased therapeutic index (7-fold and 9-fold, respectively) without loss of antimicrobial activity against *E. coli*, possibly due to position-specific net charge increase accompanied with helicity and hydrophobicity decreases.[9] XT-7 analogues with multiple lysine substitutions did not have improved selectivity. Compared with this example, Mutator's software application achieved a significant decrease in the cost and effort required for discovering new lead AMPs with low toxicity to mammalian erythrocytes and broad spectrum antibacterial activity.

Observations from this paper offer an additional insight into the nature and sequence location of toxicity decreasing substitutions. When we compare the MIC (*E. coli*) and HC_{50} of the two Mutator analogues [K2] XT-7 and [K2, K16] XT-7, it appears that a unique substitution of the N-terminal hydrophobic residue (leucine 2) by a lysine is enough to confer a nonhemolytic character to the analogue. Similarly, when we consider the Mutator's analogues [K19] ascaphin-8 and [I2, K19] ascaphin-8, a unique substitution of the C-terminal hydrophobic residue (phenylalanine 19) by lysine removes the hemolytic character of the peptide. These toxicity decreasing substitutions are located in terminal peptide regions that are likely unstructured, where the ends of a peptide helix are frayed. A partial explanation for the good performance of the Mutator algorithm is that it tends to place substitutions near peptide terminal ends with such a choice of amino acids that the net charge is either preserved or increased (see the last section from the Results chapter and Supporting Information Table S1). These results agree with the observations[29] that both N-terminal and C-terminal amino acid residues with hydrophobic side chains are good candidates for substitutions with positively charged amino acids if the goal is to decrease AMP toxicity without significant decrease in their antimicrobial activity.

Circular dichroism analysis of the disubstituted analogues shows that they are structured in α helix in 80 mM SDS, an environment which mimics bacterial membranes. For the [K2, K16] XT-7 analogue the percent helicity is calculated at about 40%,[27] about half that of the parent peptide, so that the helix may be terminally more frayed. NMR studies[30] showed that the peptide XT-7 is structured in α helix from residues 3 to 17. These studies also showed that the nonhemolytic analogue [K4] XT-7 has a shorter α helix encompassing residues 6 to 17, with an extended unstructured N-terminal region and diminished hydrophobicity with respect to its parent peptide XT-7. The flexibility and the cationicity of the AMP ends, associated with a decrease in helicity and hydrophobicity after appropriate substitutions, are probably acting together to produce a less toxic lead compounds.[30] The disruption of hydrophobic face of the parent peptide with lysine substitution in the nonpolar sector of helical wheel projection has been observed to increase peptide selectivity.[5]

The Mutator software serves to suggest designing more selective sequences and does not aim to produce more potent antibacterial peptides. It does not directly take into account the

possibility of different forms of peptide-membrane interactions when peptides are exposed to different types of membranes. These differences are presently taken into account in an indirect manner by using 26 nonhomologous peptides within a high range of known therapeutic indexes and a wide spectrum of AMP-relevant "amino acid motifs" for training the algorithm. The predicted amino acid substitutions would then be expected to produce peptide analogues with a good potential for the interaction with the *E. coli* membrane bilayer and poor potential for interactions with the mammalian erythrocyte membrane. When a significant selectivity increase is confirmed, this can be a first step in illuminating the mechanism of action. Some of the best peptides we found can now be used as tools to study the origin and mechanism of selectivity. Interestingly, point mutations found as a result of evolutionary change in natural anuran AMPs,[18] leading to single amino acid substitution, as a rule do not lead to noteworthy changes in peptide biological activities, nor do such mutations cause significant changes for the predicted or measured therapeutic index. Apparently, the point "mutations" suggested by the Mutator algorithm, which cause major selectivity increase, are difficult to find by other means, whether it is natural selection or human expert reasoning.

Many more candidate-peptides for the Mutator application can be found in the DADP database of anuran defense peptides.[18] For instance, the hemolytic peptide Brevinin-1-SPd might be converted into a good potential peptide antibiotic, according to the Double Mutator prediction of substitutions K2 and Q8. Similarly, this module predicts a 10-fold increase of the therapeutic index for Ranatuerin-2R-RA2 after K10 and G16 substitutions. The replacement of M with K at the second position leads to a predicted TI increase from 16 to 94 for Odorranain-G1. These are all short peptides amenable to peptide synthesis and suitable for additional testing of Mutator's predictions. Mutator's inability to predict MIC may be overcome by a combination of computer-assisted design strategies for increasing the potency of peptide antibiotics[31,32] with knowledge-based computational approaches for designing nonhemolytic AMPs, and this may be a promising future research avenue.

■ ASSOCIATED CONTENT

Supporting Information

The excel Table S1 contains 35 AMP sequences, all substitutions suggested by the Mutator software, and their analysis revealing algorithm preferences for sequence locations and residue types. Associated content S2 (the pdf file) supports the description of peptides purification and characterization with HPLC and MALDI results. 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

AMP, antimicrobial peptides; HC_{50}, concentration of peptide for 50% lysis of red blood cells; MIC, minimum inhibitory concentration; TI, therapeutic index

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