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Structure−Activity Relationship, Conformational and Biological Studies of Temporin L Analogues

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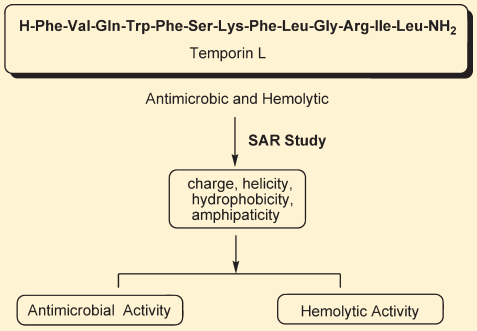
Supporting Information

ABSTRACT: Temporins are naturally occurring peptides with promising features, which could lead to the development of new drugs. Temporin-1Tl (TL) is the strongest antimicrobial peptide, but it is toxic on human erythrocytes and this fact makes the design of synthetic analogues with a higher therapeutic index vital.We studied the structure−activity relationships of a library of TL derivatives focusing on the correlation between the α-helix content of the peptides, the nature of their cationic residues, and their antibacterial/antiyeast/hemolytic activities. We found that the percentage of helicity of TL analogues is directly correlated to their hemolytic activity but not to their antimicrobial activity. In addition, we found that the nature of positively charged residues can affect the biological properties of TL without changing the peptide's helicity. It is noteworthy that a single amino acid substitution can prevent the antimicrobial activity of TL, making it a lytic peptide presumably due to its self-association. Last, we identified a novel analogue with properties that make it an attractive topic for future research.



INTRODUCTION

In recent decades, the widespread use and abuse of conventional antibiotics has led to the emergence of multidrug-resistant bacterial and fungal strains. The World Health Organization states that, worldwide, 95% of the *Staphylococcus aureus* isolates are now resistant to penicillin and up to 60% are resistant to methicillin.[1] As a consequence, infections due to resistant microbes have become the third most common cause of human death worldwide.[2]

In light of this, the search for new antibiotic agents with a new mode of action is extremely important. Gene-encoded cationic antimicrobial peptides (AMPs) that are produced in bacteria, insects, plants, and vertebrates including humans[3,4] are interesting compounds which could give rise to novel therapeutic approaches.

AMPs are evolutionarily conserved molecules of the innate immune system of a wide range of organisms and protect the host from the constant interactions with invading microbes before the adaptive immunity is activated.[5] In contrast with commonly used drugs which operate on specific intracellular targets,[6] many AMPs physically permeate and destroy the cell membrane, making it difficult for the bacteria to develop resistance. This is because, in order to become resistant to these AMPs, microbes have to change the composition of their membrane, which is a very expensive solution and not conducive to their survival.[7]

Although development of resistance to AMPs has been demonstrated experimentally in vitro, it occurs at rates that are many times lower than those observed for conventional antibiotics.[7−10] In addition, besides direct antimicrobial activity, AMPs display other biological effects including neutralization of endotoxins, chemokine-like activities, immunomodulating properties, and induction of both angiogenesis and wound repair.[11−15] Taken together, these properties make AMPs promising candidates for development as new anti-infective agents and food preservatives.

There are several sources for peptide antibiotics, but amphibian skin of the *Rana* genus is one of the richest.[16,17] Nowadays, temporins (first isolated from the skin secretions of specimens of the European red frog *Rana temporaria*)[18] are the largest family of AMPs, with more than 100 isoforms.[19] Temporins are among the smallest AMPs (10−14 residues long) found in nature to date.[20,21] They have an amidated C-terminus and a low net

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Table 1. Sequences of TL Analogues

compd	sequences[a]
TL	H-Phe^{1}-Val^{2}-Gln^{3}-Trp^{4}-Phe^{5}-Ser^{6}-Lys^{7}-Phe^{8}-Leu^{9}-Gly^{10}-Arg^{11}-Ile^{12}-Leu^{13}-NH$_2$
TL-1	H-**Leu^{1}**-**Leu^{2}**-Gln^{3}-Trp^{4}-**Leu^{5}**-Ser^{6}-Lys^{7}-**Leu^{8}**-Leu^{9}-Gly^{10}-Arg^{11}-**Leu^{12}**-Leu^{13}-NH$_2$
TL-2	H-**Leu^{1}**-**Leu^{2}**-Gln^{3}-Trp^{4}-**Leu^{5}**-Ser^{6}-Lys^{7}-**Leu^{8}**-Leu^{9}-Gly^{10}-Arg^{11}-**Trp^{12}**-Leu^{13}-NH$_2$
[Leu^{10}]TL	H-Phe^{1}-Val^{2}-Gln^{3}-Trp^{4}-Phe^{5}-Ser^{6}-Lys^{7}-Phe^{8}-Leu^{9}-**Leu^{10}**-Arg^{11}-Ile^{12}-Leu^{13}-NH$_2$
[Pro^{3}]TL[b]	H-Phe^{1}-Val^{2}-**Pro^{3}**-Trp^{4}-Phe^{5}-Ser^{6}-Lys^{7}-Phe^{8}-Leu^{9}-Gly^{10}-Arg^{11}-Ile^{12}-Leu^{13}-NH$_2$
[Leu^{2}, Pro^{3}]TL	H-Phe^{1}-**Leu^{2}**-**Pro^{3}**-Trp^{4}-Phe^{5}-Ser^{6}-Lys^{7}-Phe^{8}-Leu^{9}-Gly^{10}-Arg^{11}-Ile^{12}-Leu^{13}-NH$_2$
[Pro^{3}, Pro^{10}]TL	H-Phe^{1}-Val^{2}-**Pro^{3}**-Trp^{4}-Phe^{5}-Ser^{6}-Lys^{7}-Phe^{8}-Leu^{9}-**Pro^{10}**-Arg^{11}-Ile^{12}-Leu^{13}-NH$_2$
[Pro^{3}, DPro^{10}]TL	H-Phe^{1}-Val^{2}-**Pro^{3}**-Trp^{4}-Phe^{5}-Ser^{6}-Lys^{7}-Phe^{8}-Leu^{9}-**DPro^{10}**-Arg^{11}-Ile^{12}-Leu^{13}-NH$_2$
[Lys^{11}]TL	H-Phe^{1}-Val^{2}-Gln^{3}-Trp^{4}-Phe^{5}-Ser^{6}-Lys^{7}-Phe^{8}-Leu^{9}- Gly^{10}-**Lys^{11}**-Ile^{12}-Leu^{13}-NH$_2$
[Orn^{11}]TL	H-Phe^{1}-Val^{2}-Gln^{3}-Trp^{4}-Phe^{5}-Ser^{6}-Lys^{7}-Phe^{8}-Leu^{9}- Gly^{10}-**Orn^{11}**-Ile^{12}-Leu^{13}-NH$_2$
[Arg^{7}]TL	H-Phe^{1}-Val^{2}-Gln^{3}-Trp^{4}-Phe^{5}-Ser^{6}-**Arg^{7}**-Phe^{8}-Leu^{9}- Gly^{10}-Arg^{11}-Ile^{12}-Leu^{13}-NH$_2$
[Arg^{3}]TL	H-Phe^{1}-Val^{2}-**Arg^{3}**-Trp^{4}-Phe^{5}-Ser^{6}-Lys^{7}-Phe^{8}-Leu^{9}- Gly^{10}-Arg^{11}-Ile^{12}-Leu^{13}-NH$_2$
[Arg^{3}, Arg^{7}]TL	H-Phe^{1}-Val^{2}-**Arg^{3}**-Trp^{4}-Phe^{5}-Ser^{6}-**Arg^{7}**-Phe^{8}-Leu^{9}- Gly^{10}-Arg^{11}-Ile^{12}-Leu^{13}-NH$_2$
[Pro^{3}, Orn^{11}]TL	H-Phe^{1}-Val^{2}-**Pro^{3}**-Trp^{4}-Phe^{5}-Ser^{6}-Lys^{7}-Phe^{8}-Leu^{9}- Gly^{10}-**Orn^{11}**-Ile^{12}-Leu^{13}-NH$_2$
D-TL	H-phe^{1}-val^{2}-gln^{3}-trp^{4}-phe^{5}-ser^{6}-lys^{7}-phe^{8}-leu^{9}-Gly^{10}-arg^{11}-ile^{12}-leu^{13}-NH$_2$
RI-TL	H-leu^{1}-ile^{2}-arg^{3}-Gly^{4}-leu^{5}-phe^{6}-lys^{7}-ser^{8}-phe^{9}-trp^{10}-gln^{11}-val^{12}-phe^{13}-NH$_2$

[a] Residue variations compared to TL are highlighted in bold. [b] Already described in refs 29 and 30. Lower case indicates D-amino acids.

positive charge at neutral pH (2 to +3), due to the presence of only 1 or 2 basic residues in their sequence. Most of these peptides adopt an amphipathic α-helical-like preferential conformation in a hydrophobic environment. They act mainly on Gram-positive bacteria, including methicillin-resistant clinical isolates,[22−26] with minimal inhibitory concentrations ranging from 2.5 to 20 μM.[23] Interestingly, temporin-1Tl (TL, H-Phe^{1}-Val^{2}-Gln^{3}-Trp^{4}-Phe^{5}-Ser^{6}-Lys^{7}-Phe^{8}-Leu^{9}-Gly^{10}-Arg^{11}-Ile^{12}Leu^{13}-NH$_2$) has a higher and broader spectrum of activity than the other isoforms also being active against fungi and Gram-negative bacteria such as *Pseudomonas aeruginosa* and *Escherichia coli*.[27,28] In addition, TL is cytotoxic to three different human tumor cell lines (*Hut-78*, *K-562*, and *U-937*) but it also kills human erythrocytes at microbicidal concentrations. Otherwise, most of the temporins are practically nonhemolytic.[26]

By combining spectroscopic and computational analyses, we had previously obtained a molecular-level resolution of the interactions between TL and membrane mimicking micelles[29,30] and, on the basis of these results, we designed a novel analogue, [Pro^{3}]TL, which showed an increased antimicrobial activity toward Gram-positive bacteria and yeast cells and a decreased hemolytic activity compared to the native TL.[29]

However, to have a clearer understanding of the structural elements that are responsible for the cell selectivity of TL, we decided to perform an extensive SAR study of this peptide. We designed and synthesized a new library of TL derivatives and investigated the correlation between the α-helical content of the compounds (by CD analysis) and their antibacterial/antiyeast/hemolytic activities. Furthermore, we also analyzed the influence of positively charged residues of the TL template on its biological activity. The results of these experiments will assist in engineering analogues of TL with a better therapeutic index.

RESULTS

Design. Several TL analogues (Table 1) were synthesized in order to evaluate the impact of helical content and charge interactions on their biological activity. In compound TL-1, we replaced all the hydrophobic amino acids Phe^{1}, Val^{2}, Phe^{5}, Phe^{8}, and Ile^{12} of the TL template with leucine residues. Leucine was chosen because of its high helical propensity.[31] Starting from TL-1, we replaced Leu^{12} with Trp, thereby obtaining TL-2. Tryptophan membrane anchoring properties could significantly modulate the peptide antimicrobial activity and toxicity.[32] Then, to increase the helical character of the C-terminal region, we replaced the Gly^{10} residue of TL with a Leu, thus obtaining the compound [Leu^{10}]TL.

Replacement of Gln^{3} by Pro residue in TL led to [Pro^{3}]TL analogue, which was previously found to exhibit an increased antimicrobial activity and a decreased hemolytic activity.[29] This reduction was ascribed to the break of the N-terminal helical structure. Starting from [Pro^{3}]TL (Table 1), we changed Val^{2} with Leu ([Leu^{2}, Pro^{3}]TL) to get the Phe-Leu-Pro N-terminal sequence that is characteristic of many temporins including temporin-1Ta (H-Phe^{1}-Leu^{2}-Pro^{3}-Leu^{4}-Ile^{5}-Gly^{6}-Arg^{7}-Val^{8}-Leu^{9}-Ser^{10}-Gly^{11}-Ile^{12}-Leu^{13}-NH$_2$). Furthermore, to break the helical structure at the C-terminus of [Pro^{3}]TL too, we replaced Gly^{10} with a Pro ([Pro^{3}, Pro^{10}]TL) or with a DPro ([Pro^{3}, DPro^{10}]TL) residue. Subsequently, we focused our attention on charged residues of TL. Different modes of interaction were recently found for Arg^{11} and Lys^{7} with membrane mimicking micelles.[30] More specifically, Arg^{11} was found to interact mainly with DPC phosphate groups because of the very favorable electrostatic interaction between the protonated guanidinium group and the negatively charged phosphate groups. In contrast, the Lys^{7} side chain interacted mainly with water molecules. To obtain an insight into these different interaction propensities and their biological significance, we exchanged Arg^{11} for a Lys ([Lys^{11}]TL) or for an Orn ([Orn^{11}]TL), whereas Lys^{7} was replaced by an Arg ([Arg^{7}]TL). Because Gln^{3} also had previously shown itself to be highly capable of interacting with water molecules,[30] we substituted Gln^{3} for an Arg residue ([Arg^{3}]TL).

Moreover, both the water interacting Gln^{3} and Lys^{7} were simultaneously replaced by Arg ([Arg^{3}, Arg^{7}]TL). Finally, because of the interesting antimicrobial and toxicity profiles of [Orn^{11}]TL (see below), we combined Arg^{11}/Orn with Gln^{3}/Pro substitutions, obtaining the analogue [Pro^{3}, Orn^{11}]TL. All D-isomer (D-TL) and retro-inverse (RI-TL) classic analyses were also performed (Table 1) to verify the hypothesis that the

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Table 2. Antimicrobial Activity of the Synthesized Compounds Expressed as MIC (The Values Are Reported in μM)

strains	TL	TL-1	TL-2	[Leu^{10}] TL	[Pro^{3}] TL	[Leu^{2},Pro^{3}] TL	[Pro^{3},Pro^{10}] TL	[Pro^{3},DPro^{10}] TL	[Lys^{11}] TL	[Orn^{11}] TL	[Arg^{7}] TL	[Arg^{3}] TL	[Arg^{3},Arg^{7}] TL	[Pro^{3},Orn^{11}] TL	D-TL	RI-TL
							Gram-Negative Bacteria									
A. baumannii ATCC 19606	6	12	6	48	6	12	48	48	6	12	6	6	6	12	6	6
A. junii RT-4	3	24	6	>48	6	12	12	24	6	12	6	3	12	6	6	6
E. faecalis ATCC 29212	6	3	12	48	6	12	48	48	12	6	12	12	24	12	3	12
E. coli ATCC 25922	12	6	>48	>48	24	12	48	24	12	12	12	24	48	12	12	12
E. coli D21	12	12	>48	48	12	24	24	48	24	12	48	12	24	24	12	12
P. syringae pv tobaci	6	6	12	>48	6	12	24	>48	24	12	12	6	6	12	6	6
P. aeruginosa ATCC 15692	24	12	>48	>48	24	>48	>48	>48	24	24	24	12	24	48	48	>48
P. aeruginosa ATCC 27853	>48	24	>48	>48	>48	n.d.	>48	>48	48	24	48	24	24	48	>48	>48
Y. pseudotuberculosis YPIII	3	3	6	6	3	6	24	24	6	3	6	3	6	6	3	24
							Gram-Positive Bacteria									
B. megaterium Bm11	1.5	1.5	3	48	1.5	3	3	3	3	1.5	6	3	3	6	1.5	1.5
S. aureus ATCC 25923	3	3	6	48	3	3	24	48	6	3	6	6	6	12	3	12
S. aureus Cowan I	3	1.5	6	48	1.5	3	24	24	12	1.5	6	6	6	3	3	3
S. capitis 1	1.5	1.5	6	>48	1.5	3	12	24	6	3	6	3	6	6	3	6
S. epidermidis ATCC 12228	3	1.5	3	3	1.5	3	12	12	6	3	6	3	6	6	3	6
S. pyogenes ATCC 21547	6	6	6	6	6	6	12	24	6	24	6	3	3	6	6	6
							yeasts									
C. albicans ATCC 10231	12	6	12	>48	6	12	12	24	12	6	12	12	24	6	6	12
S. pombe	6	3	6	6	3	6	12	12	6	12	6	12	12	12	6	1.5
S. cerevisiae	6	3	12	nd[a]	6	12	12	12	6	12	12	24	24	6	6	>48

[a] nd, not determined.

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Table 3. Hemolysis (%) of TL Analogues

peptides (μM)	TL	TL-1	TL-2	[Leu^{10}] TL	[Pro^{3}] TL	[Leu^{2},Pro^{3}] TL	[Pro^{3},Pro^{10}] TL	[Pro^{3},DPro^{10}] TL	[Lys^{11}] TL	[Orn^{11}] TL	[Arg^{7}] TL	[Arg^{3}] TL	[Arg^{3},Arg^{7}] TL	[Pro^{3},Orn^{11}] TL	D-TL	RI-TL
24	94	100	100	83	92	93	5	2.5	100	89	98	100	100	72	90	81
12	92	73	100	80	42	64	5	2	59	62	90	100	92	12	88	49
6	48	25	78	80	10	19	4.5	0.6	14	22	29	52	43	2	46	15
3	13	9	13	78	6	9	2	1.5	5	7	9	20	14	1	13	6
1.5	3	4	8	69	4.5	4	2	0.4	2.6	3	4	7	6	0.7	5	1

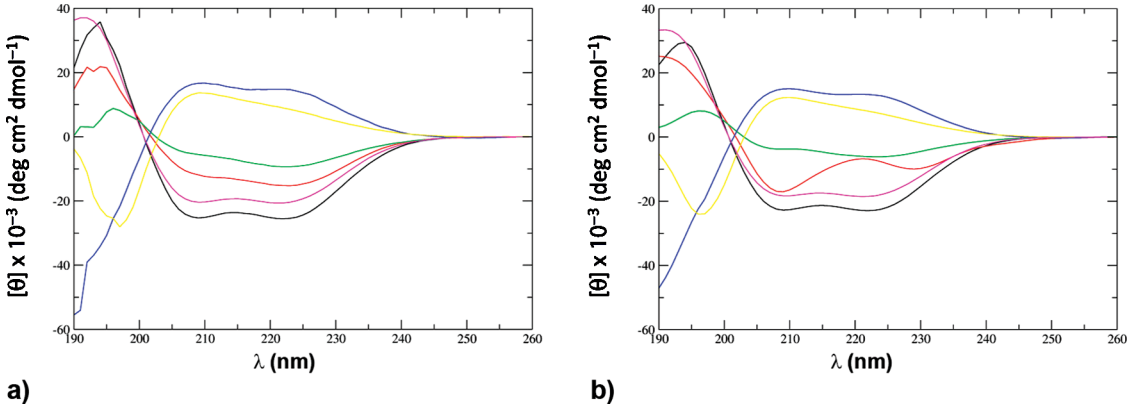

Figure 1. CD spectra of TL and some selected analogues in (a) DPC, (b) SDS (TL, pink line; [Leu^{10}]TL, black line; [Leu^{2}, Pro^{3}]TL, red line; [Pro^{3}, Pro^{10}]TL, green line; D-TL, blue line; RI-TL, yellow line). CD spectra of the other analyzed analogues are reported in Figure A of the Supporting Information.

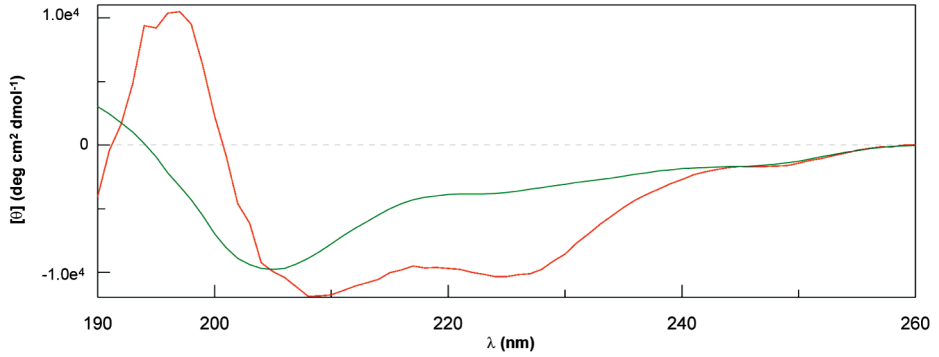

Figure 2. CD spectra of TL (red line) and [Leu^{10}]TL (green line) in LPS.

molecular mechanism underlying the antimicrobial activity of TL is not stereospecific or mediated by receptor recognition.

Biological Activity. All the synthesized compounds were evaluated for their biological activity against yeasts, Gram-positive and Gram-negative bacteria (Table 2), and human erythrocytes (Table 3).

Analogue TL-1 (Leu^{1}, Leu^{2}, Leu^{5}, Leu^{8}, Leu^{12} TL) had, in general, a higher antimicrobial activity and a lower hemolytic capacity (at a peptide concentration range from 3 to 12 μM) compared with the parent peptide TL. Differently, TL-2 analogue (Trp^{12} TL-1) showed a pronounced reduction in the antimicrobial activity against the majority of the microbial strains when compared to TL and turned out to be much more hemolytic (Table 3).

Surprisingly, the substitution of the Gly^{10} with Leu in [Leu^{10}]TL produced a compound devoid of activity against almost all the microorganisms tested but endowed with a potent hemolytic activity (69% hemolysis at 1.5 μM). The antimicrobial activity of [Leu^{2}, Pro^{3}]TL was found to be slightly lower than that of TL (about 2-fold higher MICs), whereas a marked reduction in the hemolytic activity was observed when compared with the parent peptide TL although it was more hemolytic than [Pro^{3}]TL. The analogue [Pro^{3}, Pro^{10}]TL showed a significantly lower antibacterial activity (2−8-fold higher MIC values with respect to the natural TL) and had a negligible lytic effect on red blood cells. Interestingly, the activity of [Pro^{3}, Pro^{10}]TL on *Candida albicans* was preserved. Likewise, the analogue [Pro^{3}, DPro^{10}]TL appeared to be nonhemolytic and with an antimicrobial activity even weaker than that of [Pro^{3}, Pro^{10}]TL.

Analogues [Lys^{11}]TL and [Orn^{11}]TL displayed an antimicrobial activity similar to that of TL being [Orn^{11}]TL generally more active than [Lys^{11}]TL. Moreover, both [Lys^{11}]TL and

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[Orn^{11}]TL showed a little (~2-fold) decrease in the hemolytic activity in comparison with TL, up to 12 μM.

Peptides [Arg^{7}]TL, [Arg^{3}]TL, and [Arg^{3}, Arg^{7}]TL had a reduced antimicrobial activity (approximately, 2-fold higher MICs than TL). The only exception was *Pseudomonas aeruginosa* ATCC 27853, toward which they all showed a higher activity than the native peptide. The hemolytic capacity of [Arg^{7}]TL was reduced by half with respect to TL at a peptide concentration of 6 μM, while [Arg^{3}]TL and [Arg^{3}, Arg^{7}]TL analogues had a toxic effect on human erythrocytes similar to that of TL at all concentrations used.

Finally, the antimicrobial activity of [Pro^{3}, Orn^{11}]TL was slightly lower than that of TL. However, of the most active antimicrobial compounds, [Pro^{3}, Orn^{11}]TL manifested the lowest hemolytic activity.

As expected, D-TL (all D-isomer) had very similar antimicrobial and hemolytic activities to those of the natural TL, whereas a lower biological activity was displayed by the retro-inverso analogue RI-TL.

Conformational Studies. To explore the conformational behavior of TL analogues, we performed a CD study of these peptides in water, SDS/water, and DPC/water solutions. CD spectra in water (pH 7.4) revealed the presence of disordered conformers for all compounds with a minimum close to 198 nm (data not shown). In contrast, in SDS and DPC micelles solution, the shape of CD spectra suggested the propensity to form defined secondary structures. In particular, CD spectra showed two minima around 209 and 222 nm, characteristic of α-helix structures (Figure 1 and Supporting Information).

Helical content was predicted from CD spectra using the SOMCD method[33] (Table 4). In the same Table 4, the hydrophobicity (H) and the mean hydrophobic moment (amphipathicity, M) are also reported. Two consensus scales were used: the Eisenberg consensus scale,[34] which is the most used in the scientific literature, and the combined consensus scale (CCS),[35] which is extended to nonproteinogenic amino acids and which allowed the calculation of H and M for ornitine-containing peptides.

Generally, helical contents observed in SDS and DPC micelle solutions were similar with a reduction in SDS of about 10% compared to DPC. The highest helical content was observed in TL-2 and in [Leu^{10}]TL (75% and 76%, respectively, in DPC). In the latter, Gly^{10} was replaced by a Leu residue, and this substitution probably enhanced the helical content of the C-terminal end. In contrast, peptides bearing one ([Pro^{3}]TL, [Leu^{2}, Pro^{3}]TL, and [Pro^{3}, Orn^{11}]TL) or two ([Pro^{3}, Pro^{10}]TL and [Pro^{3}, DPro^{10}]TL) proline residues showed the lowest helical content (about 30% and 5%, respectively, in DPC) in accordance with the design strategy. Substitutions carried out on peptides [Lys^{11}]TL/[Orn^{11}]TL did not change the helical content of the peptide (about 50−60% in DPC). D-TL showed a higher percentage of left-handed helix structure (60% in DPC) while only 30% α helix was observed in RI-TL analogue.

CD spectra of TL and [Leu^{10}]TL were also acquired in 100 μM LPS (lipopolysaccharide) solution (Figure 2). Note that LPS is the major component of the outer membrane of Gram-negative bacteria.[36] According to Rosenfeld et al.,[28] TL adopted an α-helix structure when added to LPS micelles in solution (two minima at 209 and 224 nm). In contrast, CD spectrum of [Leu^{10}]TL showed only one minimum at 205 nm. Using the SOMCD method, the secondary structure of [Leu^{10}]TL in LPS solution could be estimated as random coil (41%) and β-strand (49%) conformations.

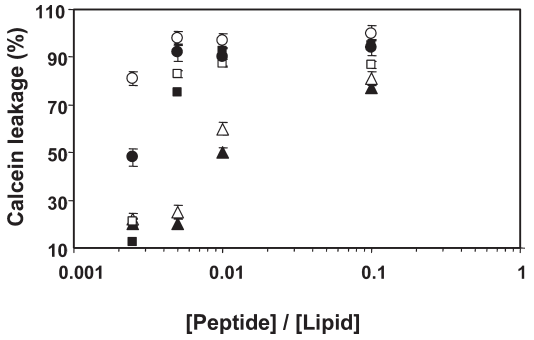

Figure 3. Calcein leakage from PE/PG (triangles), PG/CL (squares), and PC/Cho (circles) LUVs after addition of TL (filled symbols) or [Leu^{10}]TL (empty symbols) at different concentrations. Fluorescence recovery was measured for 10 min after the peptides were mixed with the vesicles, and its maxima was reported. Leakage was calculated as indicated in the Experimental Section. Values are means of three independent measurements with ±SD.

Permeabilization of Large Unilamellar Vesicles (LUV). Among the synthesized analogues, [Leu^{10}]TL revealed itself to be the peptide with the lowest antimicrobial activity especially against Gram-negative bacteria and with the highest toxic effect on human erythrocytes (Table 2), which is in agreement with its high content of α-helix (see Table 4). To check whether this drastic decrease in the antimicrobial activity was due to the peptide's inability to permeate bacterial membranes, we used calcein-loaded large unilamellar vesicles (LUV) of different lipid composition. More precisely, to mimic the lipid composition of the cytoplasmic membrane of a representative Gram-negative bacterium (e.g., *E. coli*), we used LUV made of phosphatidylethanolamine (PE)/phosphatidylglycerol (PG) (7:3, w-w). The membrane of a representative Gram-positive bacterium (e.g., *S. aureus*) was instead mimicked by PG/cardiolipin (CL) (6:4, w/w) LUV.[37] Liposomes made of phosphatidylcholine (PC)/cholesterol (Cho) (9:1, w/w), to mimic the composition of the outer leaflet of mammalian erythrocytes,[38] were also included for comparison. Different concentrations of peptide were added to the LUV suspension, and membrane permeability was measured by following fluorescence recovery due to calcein release from the vesicles.[39] As indicated in Figure 3, calcein leakage induced by [Leu^{10}]TL (empty symbols) from both PE/PG (triangles) and PG/CL (squares) liposomes was similar to that caused by the native peptide TL (filled symbols) and increased in a dose-dependent manner with a slightly higher leakage from PG/CL LUV. Overall, these results indicate that, despite lacking antimicrobial activity, [Leu^{10}]TL is able to bind and destabilize the two representative bacterial model membranes to a similar extent to that of TL. In contrast, when zwitterionic (PC/Cho) LUV were employed, the effect of [Leu^{10}]TL (empty circles) was double that of the native peptide (filled circles) at the lowest peptide/lipid molar ratio used (0.0025). This latter finding is in line with the stronger hemolytic activity of [Leu^{10}]TL compared to TL when tested at low concentrations (up to 6 μM) (Table 3).

■ DISCUSSION

The purpose of this study was to develop analogues of TL with a higher therapeutic index than the native peptide. Generally, activities of AMPs against bacteria, fungi, and mammalian cells

Table 4. Peptide Sequences, Percentage of α-Helix Character, Percentage of Hemolysis, Hydrophobicity (H), and Amphipathicity (M)

peptide	%helix[a]	%helix[b]	%hemolysis[c]	H Eisenberg[e]	M Eisenberg[e]	H CCS[f]	M CCS[f]
TL	59	52	48	0.06461	0.4188	3.0231	4.844
TL-1	42	37	25	0.03	0.3949	3.46	5.07
TL-2	75	68	78	0.01769	0.3851	3.46	5.07
[Leu^{10}]TL	76	69	80	0.09308	0.4029	3.95	4.13
[Pro^{3}]TL	25	22	10	0.1123	0.3804	3.46	4.42
[Leu^{2}, Pro^{3}]TL	35	32	19	0.1115	0.3798	3.9	4.63
[Pro^{3}, Pro^{10}]TL	10	6	4.5	0.09461	0.3899	3.64	4.29
[Pro^{3}, DPro^{10}]TL	3	1	0.6				
[Lys^{11}]TL	54	47	14	0.1185	0.3832	3.03	4.84
[Orn^{11}]TL	52	45	22			3.1	4.81
[Arg^{7}]TL	55	48	29	0.01077	0.4724	3.01	4.85
[Arg^{3}]TL	59	50	52	−0.02077	0.4917	2.71	5.14
[Arg^{3}, Arg^{7}]TL	52	45	43	−0.07462	0.5446	2.71	5.15
[Pro^{3}, Orn^{11}]TL	34	29	2			3.54	4.38
D-TL	60[d]	50[d]	46				
RI-TL	30[d]	24[d]	15				

[a] Measured in DPC micelle solution. [b] Measured in SDS micelle solution. [c] At peptide concentration of 6 μM. [d] Left-handed helices. [e] According to Eisenberg consensus scale.[34] [f] According to combined consensus scale (CCS).[35]

are determined by a complex interaction between cationicity, hydrophobicity, α-helicity, and amphipathicity.[6,40,41] Hence, we designed a first group of analogues with the aim of increasing (TL-1, TL-2, and [Leu^{10}]TL) or decreasing ([Leu^{2}, Pro^{3}]TL, [Pro^{3}, Pro^{10}]TL, [Pro^{3}, DPro^{10}]TL) the percentage of helicity. Actually, TL-1 turned out to have less helical content than TL. In a second group of analogues ([Lys^{11}]TL, [Orn^{11}]TL, [Arg^{7}]TL, [Arg^{3}]TL, [Arg^{3}, Arg^{7}]TL), we investigated the importance of the nature of the positively charged side chains by exchanging guanidinium groups with amine groups and vice versa ([Lys^{11}]TL, [Orn^{11}]TL, [Arg^{7}]TL) or by inserting a further positive charge in the peptide sequence ([Arg^{3}]TL, [Arg^{3}, Arg^{7}]TL).

Helical contents of the peptides were evaluated by CD spectroscopy in water, SDS, and DPC micelle solutions (Figure 1 and Table 4). SDS and DPC micelles are considered to be good models of negatively charged (microbial) and zwitterionic (eukaryotic) membranes respectively.[29,30]

All peptides exhibited a helical content ranging from 3% to 76% in DPC (Table 4). Considering the first group of peptides (TL-1, TL-2, [Leu^{10}]TL, [Leu^{2}, Pro^{3}]TL, [Pro^{3}, Pro^{10}]TL, [Pro^{3}, DPro^{10}]TL) and the lead compounds TL and [Pro^{3}]TL, a clear and direct correlation between the helical content and the hemolytic activity could be detected at 6 μM (Table 4). This is consistent with the "barrel stave" mechanism proposed for the hemolytic activity of TL and its derivatives.[29,30]

In contrast, the antimicrobial activity was not dependent on the helicity of the peptide; for instance, TL-1 (37% helix in SDS) exhibited a higher antimicrobial activity than TL-2 (68% helix in SDS) against the tested microbial strains except for *Acinetobacter* spp. (Table 2). However, for the analogues [Pro^{3}, Pro^{10}]TL or [Pro^{3}, DPro^{10}]TL bearing a Pro or a DPro residue at position 10 (in addition to Pro^{3}), to also break the helical structure at the C-terminus, the antibacterial activity was significantly reduced compared to that of [Pro^{3}]TL (Table 2). These results indicate that a helical structure at the C-terminus of the peptide sequence, rather than at its N-terminal end, is crucial in order to confer antibacterial activity on TL. Nevertheless, disruption of the C-terminal helix does not affect the anti-*Candida* activity of the peptide (Table 2). Taken together, these findings suggest a "carpet-like" or a "dynamic peptide−lipid supramolecular pore" mechanism underlying the antimicrobial activities of temporin L analogues.[29] Indeed, unlike the "barrel-stave" model, a helical structure encompassing the entire peptide sequence is not required for these two types of mechanisms.

Interestingly, [Leu^{10}]TL (76% helical content in DPC) was the analogue with the highest hemolytic capacity and the lowest antimicrobial activity. This could be due to the peptide's oligomeric state, probably induced by contact with LPS in Gram-negative bacteria or with teichoic acids in Gram-positive bacteria. The large size of the oligomers would make it more difficult for the peptide to diffuse through the cell wall into the target cytoplasmic membrane.[42] This would explain the very low antimicrobial activity of [Leu^{10}]TL despite its ability to permeate bacterial membranes to a similar extent to that found with TL (Figure 3). It is worthwhile noting that our CD spectra in LPS solution (Figure 2) revealed a contribution of ∼50% of β-sheet structures for [Leu^{10}]TL, whereas the native peptide TL adopted an α-helix structure in LPS solution. Such β-sheet structures might be associated with amyloid-like aggregates. In fact, small β-sheets are unstable in aqueous solution without aggregation unless they are stabilized by, for example, disulfide bridges.[43]

In the second group of peptides ([Lys^{11}]TL, [Orn^{11}]TL, [Arg^{7}]TL, [Arg^{3}]TL, [Arg^{3}, Arg^{7}]TL), the helical content, in both DPC or SDS, was almost constant and similar to that of the parent TL although the hemolytic activity was not preserved. More precisely, replacement of Arg^{11} by Lys ([Lys^{11}]TL) or by Orn ([Orn^{11}]TL) gave rise to an approximately 2-fold reduction in hemolytic activity. This means that structural features of arginine (e.g., the bifurcated structure or high hydrogen-bonding potential of the guanidinium group), rather than cationicity per se, do favor the interaction between TL and the eukaryotic cell membrane (as already demonstrated for cathepsin G derivatives).[44]

Replacement of Lys^7 by Arg ([Arg^7]TL) also decreased hemolytic activity by half (Table 3). This indicates different functions of Lys vs Arg charged residues in the peptide interaction with zwitterionic membranes depending on their position along the peptide sequence, which is in agreement with our previous MD simulations results.[30]

Analogously, the exchange of guanidinium with amine groups at position 11 ([Lys^{11}]TL and [Orn^{11}]TL) or the substitution of Lys with Arg in position 7 ([Arg^7]TL) were found to be responsible for a slightly reduced antimicrobial activity. Bearing in mind what was stated above, these results underline the fact that side chains of basic residues also have a different role in the interaction of TL with anionic membranes depending on their position along the peptide sequence.

Note also that [Orn^{11}]TL resulted more active than TL against the Gram positive *S. aureus* Cowan I, the Gram-negative *P. aeruginosa* ATCC 27853, and the yeast *C. albicans* ATCC 10231 (Table 3).

Replacement of Gln^3 by Arg ([Arg^3]TL) increased the net positive charge of the peptide to +4. Helical percentage and hemolytic and antimicrobial activity of this analogue results (Table 2 and 4) results were very similar to those of TL, suggesting that a positive charge at this position does not affect the helicity or the biological activity of the peptide. Probably, residue in position 3 is not involved in the interaction with a negative counterpart but rather it is confined in a hydrophilic environment.

This is in line with our previous data showing that Gln^3 of TL mainly interacts with water molecules when the peptide is bound to a DPC micelle.[30] The results obtained with [Arg^3]TL have also indicated that, in contrast to what is observed in magainins and other host defense peptides,[45,46] a direct relationship between cationicity and antimicrobial activity is not a general rule. This is also supported by the results found with [Arg^3, Arg^7]TL (net charge +4), a peptide with a similar hemolytic but a slightly reduced antimicrobial activity compared to that of TL. Moreover, [Arg^3, Arg^7]TL showed the highest hydrophobic moment (*M*) and the lowest hydrophobicity (*H*) among the peptides reported in this study (Table 4). Hydrophobic moment, which measures the amphiphilicity of the peptide in an α-helix, is considered to be one of the most important factors governing antimicrobial activity.[46,47] However, the results found with [Arg^3, Arg^7]TL and other analogues have clearly shown that the hydrophobic moment does not control the antimicrobial activity of temporins. In addition, data reported in Table 4 do not show any correlation between hydrophobicity and hemolytic (or antimicrobial) activity in temporin L analogues. Note that [Pro^3, Orn^{11}]TL, which combines [Pro^3]TL and [Orn^{11}]TL substitutions, showed the lowest hemolytic effect among the most active antimicrobial analogues (Table 3). These results confirm that both the breakage of the N-terminal helical structure of TL and the absence of a guanidinium group at position 11 can almost abolish the hemolytic activity of the peptide without causing drastic changes in its antimicrobial activity (Table 2). Finally, we found that the antimicrobial and the hemolytic activities of the D-isomer were similar to those of TL, demonstrating that antimicrobial activity of temporins is not mediated by receptor recognition and that peptide−membrane interactions are the major step in the mechanism of action of these peptides.

CONCLUSION

Overall, our studies have indicated that the α-helical content and the nature of positively charged residues in the sequence of TL induce different effects on the hemolytic and the antimicrobial activity of the peptide. Regarding the hemolytic activity, the helical content of the peptide does play an important role as proved by the existence of a direct correlation between the percentage of helicity and the lytic effect of the peptide on human erythrocytes. On the contrary, no direct correlation has been detected between the helicity of the peptide and its antimicrobial activity despite the finding that disruption of the C-terminal α-helix drastically reduces the toxic effect of the peptide on different species of bacteria but not on *Candida*. This finding can address the design of further selective antifungal agents starting from the TL template by breaking its helical structure both at the N- and at the C-terminal regions.

We have also demonstrated that the nature of positively charged residues can differently affect the antimicrobial/hemolytic activity of TL (depending on their position along the peptide's sequence) without modifying its helical content. More particularly, the presence of an amine instead of a guanidinium group at position 11 reduces the lytic effect of the peptide on red blood cells but does not significantly alter its antimicrobial activity. Note, too, that insertion of a further positively charged residue (i.e., Arg) in position 3 does not improve the antimicrobial potency of the peptide despite its higher cationicity and amphiphilicity nor does it induce changes in the helical content/hemolytic activity of TL.

Remarkably, our studies have also indicated that replacement of Gly^{10} with a leucine almost abolishes the antimicrobial activity of TL and causes a dramatic increase in hemolytic activity, which might be due to an oligomerization of the peptide.

Finally, it is important to underline that when the breakage of the N-terminal helical structure of TL (due to Pro^3) is combined with the replacement Arg^{11}/Orn ([Pro^3, Orn^{11}]TL), the hemolytic activity of the peptide is suppressed by up to 12 μM without significant changes in the antimicrobial activity of the native peptide. These findings, together with the unique properties of temporins (short size and low positive charge), have contributed to making [Pro^3, Orn^{11}]TL an interesting template for the future development of temporin-based anti-infective agents with a better therapeutic index.

EXPERIMENTAL SECTION

Synthesis. Nα-Fmoc-protected amino acids, HBTU, HOBt, and Rink amide resin, were purchased from GL Biochem Ltd. (Shanghai, China). Peptide synthesis solvents, reagents, as well as CH_3CN for HPLC, were reagent grade and were acquired from commercial sources and used without further purification unless otherwise noted. Peptides were synthesized according to the solid phase approach using standard Fmoc methodology in a manual reaction vessel and in a stepwise fashion.[48] For example, for the synthesis of TL, Nα-Fmoc-Leu-OH was coupled to Rink amide resin (0.5 g, 0.7 mmol NH_2/g). The following protected amino acids were then added stepwise Nα-Fmoc-Ile-OH, Nα-Fmoc-Arg(Pbf)-OH, Nα-Fmoc-Gly-OH, Nα-Fmoc-Leu-OH, Nα-Fmoc-Phe-OH, Nα-Fmoc-Lys-(Boc)-OH, Nα-Fmoc-Ser(tBu)-OH, Nα-Fmoc-Phe-OH, Nα-Fmoc-Trp-(Boc)-OH, Nα-Fmoc-Gln(Trt)-OH, Nα-Fmoc-Val-OH, and Nα-Fmoc-Phe-OH. Each coupling reaction was accomplished using a 3-fold excess of amino acid with HBTU and HOBt in the presence of DIEA.

The N^{α}-Fmoc protecting groups were removed by treating the protected peptide resin with a 25% solution of piperidine in DMF

(1 × 5 min and 1 × 20 min). The peptide resin was washed three times with DMF, and the next coupling step was initiated in a stepwise manner. All reactions were performed under an Ar atmosphere. The peptide resin was washed with DCM (3×), DMF (3×), and DCM (4×), and the deprotection protocol was repeated after each coupling step. The N-terminal Fmoc group was removed as described above, and the peptide was released from the resin with TFA/Et_3SiH/H_2O (90:5:5) for 3 h. The resin was removed by filtration, and the crude peptide was recovered by precipitation with cold anhydrous ethyl ether to give a white powder which was purified by RP-HPLC on a semipreparative C18-bonded silica column (Vydac 218TP1010, 1.0 cm × 25 cm) using a gradient of CH_3CN in 0.1% aqueous TFA (from 10% to 90% in 30 min) at a flow rate of 1.0 mL/min. The product was obtained by lyophilization of the appropriate fractions after removal of the CH_3CN by rotary evaporation. Analytical RP-HPLC indicated a purity >98% and the correct molecular weights were confirmed by LC/ESI-MS (6110 Quadrupole, Agilent Technologies). The peptide concentration was determined by quantitative amino acid analysis using an Applied Biosystems model 420A. (Table A and B, Supporting Information).

Microorganisms. The strains used for the antimicrobial assays were the following: the Gram-negative bacteria *Acinetobacter baumannii* ATCC 19606, *Acinetobacter junii* RT-4, *Enterobacter faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *E. coli* D21, *Pseudomonas aeruginosa* ATCC 15692, *P. aeruginosa* ATCC 27853, *Pseudomonas syringae* pv tobaci 1918NCPPB, *Yersinia pseudotuberculosis* YPIII, the Gram-positive bacteria *Bacillus megaterium* Bm11, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* Cowan I, *Staphylococcus capitis* 1, *Staphylococcus epidermidis* ATCC 12228, *Streptococcus pyogens* ATCC 21547, and the yeasts *Candida albicans* ATCC 10231, *Saccharomyces cerevisiae*, and *Saccharomyces pombe*.

Antimicrobial Assay. Susceptibility testing was performed by adapting the microbroth dilution method outlined by the Clinical and Laboratory Standards Institute using sterile 96-well plates (Falcon NJ, USA). Aliquots (50 μL) of bacteria in midlog phase at a concentration of 2×10^6 colony-forming units (CFU)/mL in culture medium (Mueller-Hinton, MH) were added to 50 μL of MH broth containing the peptide in serial 2-fold dilutions in 20% ethanol. The range of peptide dilutions used was 0.75−48 μM. The same procedure was followed with yeast strains but using Winge medium.[49] Inhibition of microbial growth was determined by measuring the absorbance at 600 nm after an incubation of 18−20 h at 37 °C (30 °C for yeasts), with a 450-Bio-Rad Microplate Reader. Antimicrobial activities were expressed as the minimal inhibitory concentration (MIC), the concentration of peptide at which 100% inhibition of microbial growth is observed after 18−20 h of incubation.

Hemolytic Assay. The hemolytic activity was measured on human red blood cells as reported previously.[50] Briefly, aliquots of a human erythrocyte suspension in 0.9% (w/v) NaCl were incubated with serial 2-fold dilutions of peptide (dissolved in 20% ethanol prior to use) for 40 min at 37 °C with gentle mixing. The samples were then centrifuged for 5 min at 900*g*, and the absorbance of the supernatant was measured at 415 nm. Complete lysis was measured by suspending erythrocytes in distilled water.[51]

Calcein-Loaded LUV and Leakage Assay. Lipid films of PE/PG (7:3, w/w), PG/CL (6:4, w/w), and PC/Cho (9:1) were prepared by dissolving dry lipids (2 mg of each lipid mixture) in chloroform/methanol (2:1, v/v) and evaporating the solvents under a nitrogen stream. The lipid film was then hydrated with 10 mM Tris and 150 mM NaCl (pH 7.4) containing 60 mM calcein solution. The liposome suspension was extruded 10 times through a polycarbonate filter (pore size, 0.1 μm), and free calcein was removed by gel filtration by using a Sephadex G-25 column (1.5 cm × 10 cm; Pharmacia Biotech AB) at room temperature. Calcein entrapped in the vesicles was highly concentrated, and the fluorescence was self-quenched. Calcein release from LUVs (final lipid concentration, 30 μM) due to membrane permeation induced by the peptides was monitored at 37 °C for 10 min after peptide addition, by the fluorescence increase ($\lambda_{excitation}$ = 485 nm; $\lambda_{emission}$ = 535 nm). Complete dye release was obtained by using 0.1% Triton X-100, which caused total destruction of lipid vesicles.[52] The percentage of calcein leakage was calculated as $100(F_1 - F_0)/(F_t - F_0)$, where F_0 represents the fluorescence of intact vesicles, and F_1 and F_t denote the intensities of the fluorescence achieved after peptide and Triton-X-100 treatment, respectively.

Circolar Dicroism. All CD spectra were recorded using a JASCO J710 spectropolarimeter at 25 °C with a cell of 1 mm or 10 mm path length. The CD spectra were acquired by the range from 260 to 190 nm 1 nm bandwidth, 4 accumulations, and 100 nm/min scanning speed. The CD spectra of the TL and TL derivates, at a concentration of 100 μM, were performed in water (pH = 7.4), in SDS (20 mM) and in DPC (20 mM) micellar solutions. We acquired these CD spectra also at lower (10 and 1 μM) peptide concentrations (data not shown), and we did not observe any significant variation of the spectra, indicating that no self-assembly phenomena occur within this concentration range. CD spectra of TL and [Leu^{10}]TL (100 μM) were also acquired in a 100 μM LPS solution.

■ ASSOCIATED CONTENT

Supporting Information. Analytical data of the synthesized peptides. CD spectra of TL analogues. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

SDS, sodium dodecylsulphate; DPC, dodecylphosphocholine; SAR, structure−activity relationship; CD, circular dichroism; DCM, dichloromethane; DIPEA, *N*,*N*-diisopropylethyl-amine; DMF, *N*,*N*-dimethylformamide; Et_3SiH, triethylsilane; Fmoc, 9-fluorenyl-methoxycarbonyl; HOBt, *N*-hydroxy-benzotriazole; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; RP-HPLC, reversed-phase high performance liquid chromatography; ESI, electrospray ionization; LC-MS, liquid chromatography−mass spectrometry; TL, temporin-1Tl; TA, temporin-1Ta; PE, phosphatidyl-ethanolamine; PG, phosphatidylglycerol

■ ADDITIONAL NOTE

Abbreviations used for amino acids and designation of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* **1972**, *247*, 977−983. Amino acid symbols denote L-configuration unless indicated otherwise.

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