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De Novo Cyclic Pseudopeptides Containing Aza-β^3-amino Acids Exhibiting Antimicrobial Activities

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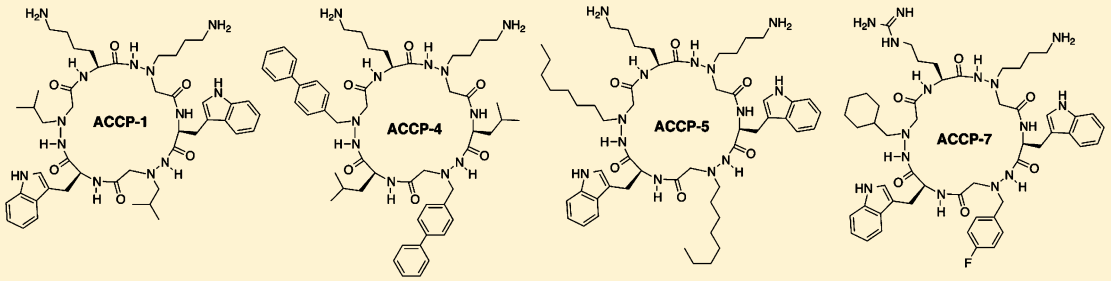

ABSTRACT: *De novo* cyclic pseudopeptides composed of α-amino and aza-β^3-amino acids were designed with the aim to obtain potential new antimicrobial agents. Antimicrobial cyclic pseudopeptides (ACPPs) are based on the properties of antimicrobial peptides (AMPs), so they are cationic and amphiphilic. Aza-β^3-amino acids enhance the *in vivo* half-life of these compounds and offer the possibility to incorporate a large variety of side chains. Most of the 13 ACPPs exert antimicrobial activities in rich media with broad spectrum of antibacterial activities. Selectivity for bacterial over mammalian cells was determined by testing the hemolytic activities of ACPPs against sheep red blood cells (sRBC). We examined the ratio of cationic to hydrophobic residues as well as the type of hydrophobic side chains essential for biological activity of this class of ACPPs. These results will be useful for designing potential candidates for a therapeutic application.

INTRODUCTION

Increasing resistance of bacteria to conventional antibiotics has posed a serious threat to human health and generated a growing need for new drugs to combat microbial infection. AMPs from either microbial or eukaryotic sources have been proposed as an alternative, their lethal mechanism (based on disruption of the pathogen's cytoplasmic membrane) differs from those of most conventional antibiotics in clinical use and makes induction of resistance rather unlikely.[1−5] These short cationic peptides are usually unstructured in solution and adopt a highly amphipathic structure in cell membranes. Most AMPs act by nonspecifically binding and disrupting the bacterial cell membrane through formation of small pores or complete membrane solubilization; however, recent studies have suggested the existence of specific cellular targets.[4−6]

Although several AMPs (pexiganan, omiganan) have advanced into phase III clinical trials none has been approved for medical use.[7−11] This is largely due to relative toxicity and limited bioavailability and stability issues. To address these questions, numerous approaches have been proposed including the development of peptidomimetic polymer molecules,[12,13] introduction of non-natural amino acids (such as D-isomers),[14−17] *de novo* and rational peptide design,[18] and truncation of AMP in order to improve peptide selectivity and membrane binding and to reduce their length.[19,20]

With the aim of studying the minimal number of residues to obtain an antibacterial peptidic sequence and to reduce the production costs, small synthetic peptides (three to six residues) consisting mainly of tryptophan and arginine residues were constructed. These studies revealed that a relatively short peptide chain length is sufficient to maintain the antibacterial activity.[21] Indeed, it was demonstrated that to be active on Gram-positive bacteria, a pentapeptide carrying an amide function at the C-terminus (like H-WRWRW-NH_2) is sufficient, whereas a hexapeptide (like H-WRWRWR-NH_2) is needed to be active on Gram-negative bacteria.[22] These small peptides have to contain at least two positively charged residues and two aromatic hydrophobic residues. It was also shown that the size of this antimicrobial pharmacophore could be lowered to three or two residues by incorporating hydrophobic substituants at the N- and C-termini.[22]

The effect of the cyclization on a series of hexapeptides based on the Ac-RRWWRF-NH_2 sequence was studied afterward. The cyclization of these hexapeptides significantly increased the antibacterial activity, especially for sequences presenting at least three consecutive aromatic residues.[23,24] The hemolytic activity was also increased by the cyclization, although by a lower order of magnitude.[25] The later incorporation of D-residues within

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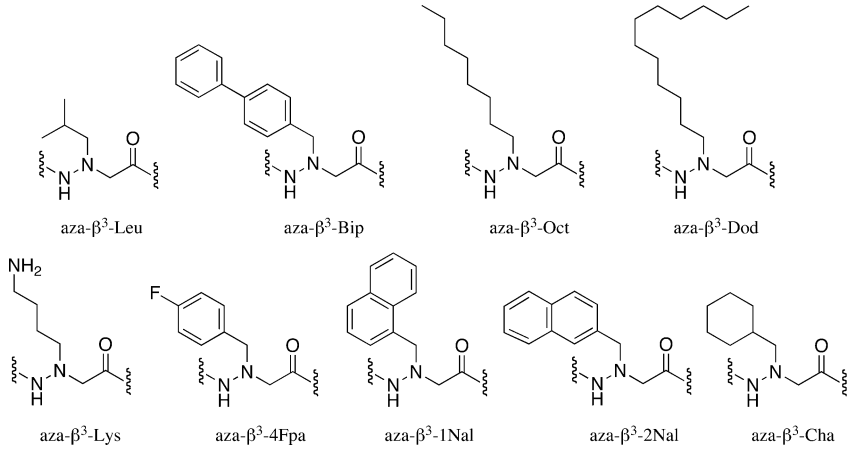

Figure 1. Aza-β^3-amino acid structures and their designators used in this study: aza-β^3-Leu, aza-β^3-leucine; aza-β^3-Bip, aza-β^3-4,4′-biphenylalanine; aza-β^3-Oct, aza-β^3-octylalanine; aza-β^3-Dod, aza-β^3-dodecylalanine; aza-β^3-4Fpa, aza-β^3-4-fluoro-phenylalanine; aza-β^3-1Nal, aza-β^3-1-naphthylalanine; aza-β^3-2Nal, aza-β^3-2-naphthylalanine; aza-β^3-Cha, aza-β^3-cyclohexylalanine.

Table 1. Analytical Data on Cyclopseudopeptidic Sequences ACPP1-13

			RP-HPLC[b]		mass spectrometry		
ACPP	cyclopseudopeptidic sequences	ratio[a]	R_t (min)	purity (%)	calcd[c]	obsd[d]	yields (%)
1	c[Lys-aza-β^3Lys-Trp-aza-β^3Leu-Trp-aza-β^3Leu]	2/4	20.0	99.9	899.32	900.55	36
2	c[Lys-aza-β^3Lys-Lys-aza-β^3Leu-Trp-aza-β^3Leu]	3/3	14.0	99.8	841.42	842.57	44
3	c[aza-β^3Lys-Lys-aza-β^3Lys-Trp-aza-β^3Leu-Trp]	3/3	16.6	99.9	914.41	915.56	46
4	c[aza-β^3Lys-Lys-aza-β^3Bip-Leu-aza-β^3Bip-Leu]	2/4	24.7	99.7	974.02	974.59	39
5	c[Lys-aza-β^3Lys-Trp-aza-β^3Oct-Trp-aza-β^3Oct]	2/4	24.0	99.9	1011.31	1012.67	31
6	c[Lys-aza-β^3Lys-Cha-aza-β^3Dod-Trp-aza-$\beta^3$1-Nal]	2/4	28.3	99.7	1062.39	1063.71	38
7	c[Arg-aza-β^3Lys-Trp-aza-$\beta^3$4Fpa-Trp-aza-β^3Cha]	2/4	24.0	99.6	1019.23	1020.56	29
8	c[aza-$\beta^3$1Nal-Phe-aza-β^3Lys-Thr-aza-β^3Lys-Ser-aza-β^3Lys-Ser]	6/2	14.8	99.5	1063.40	1064.60	19
9	c[Leu-aza-β^3Arg-Arg-aza-$\beta^3$1-Nal-Leu]	2/3	18.8	99.9	765.34	766.48	8
10	c[aza-$\beta^3$1Nal-Lys-aza-β^3Lys-Leu-aza-β^3Leu]	2/3	18.2	99.7	724.00	725.47	7
11	c[aza-β^3Lys-Lys-aza-β^3Bip-Leu-aza-β^3Bip]	2/3	22.3	99.8	860.21	861.51	8
12	c[aza-β^3Lys-Arg-aza-$\beta^3$2-Nal-Phe-aza-$\beta^3$2-Nal]	2/3	21.7	99.7	869.97	871.47	10
13	c[aza-β^3Lys-Lys-aza-$\beta^3$2-Nal-Leu-aza-$\beta^3$2-Nal]	2/3	20.6	99.9	808.28	809.47	8

[a]The "ratio" illustrated sequence composition based on the formula P/H, where P is the number of hydrophilic polar residues and H is the number of hydrophobic residues. [b]Analytical HPLC analysis(see Experimental Section). [c]Theoretical monoisotopic molecular weight. [d]The m/z value assessed by HR-MS and ESI.

these cyclopeptides does not have a strong influence on biological activities,[25] whereas the introduction of residues such as naphthylalanine increases the biological activities for linear as well as cyclic peptides.[25−27]

Aza-β^3-amino acids bear their side chains on a nitrogen atom with nonfixed configuration.[28−30] However with the possible inversion of this nitrogen atom, these aza-β^3-monomers are able to mimic orientations of the L- or D-α-amino acid side chains and adopt preferentially one of these configurations according to the environment, the interactions, and the sterical constraints. By cyclization of hybrid pseudopeptides composed of aza-β^3-amino acids and L-α-amino acids, we envisage that it is possible to decelerate these configuration inversions or to fix the orientation of the analogue side chains in a membrane environment. Besides, aza-β^3-amino acid residues are able to form additional hydrogen bonds that may participate in potential intermolecular associations, and they are also known to improve serum stability.[31] Moreover, aza-β^3-amino acids are very suitable for rapid solid phase peptide synthesis. In addition, the numerous Fmoc-α-amino acids that are commercially available means we have build more than 30 Fmoc-aza-β^3-amino acids with proteinogenic and nonproteinogenic side chains, which allowed us to significantly improve the number of accomplishable sequences.[32−35]

Hybrid cyclopseudopeptides composed of L-α-amino acids and of aza-β^3-amino acids appear as serious candidates to favor a possible structuration or self-association that would be responsible for antimicrobial activity. The purpose of this study is to explore the biological effects of 13 ACPPs containing aza-β^3-amino acids and to compare their antimicrobial activities. The long-term aim of this research is to find a key for the rational design of novel ACPPs using aza-β^3-amino acids. We examined the most favorable cationic/hydrophobic ratio and the positioning of aza-β^3-amino acid residues. More concretely, we investigated the influence of different hydrophobic side chains such as aromatic residues or acyl chains (Figure 1).[35] Our results show that it is possible to distinguish the shape of an ideal candidate for a therapeutic application.[3]

RESULTS

Design. We subfractioned the pseudopeptide library in three parts as a function of length, residue composition, and hydrophilic/hydrophobic ratio (Table 1, Figure 2).

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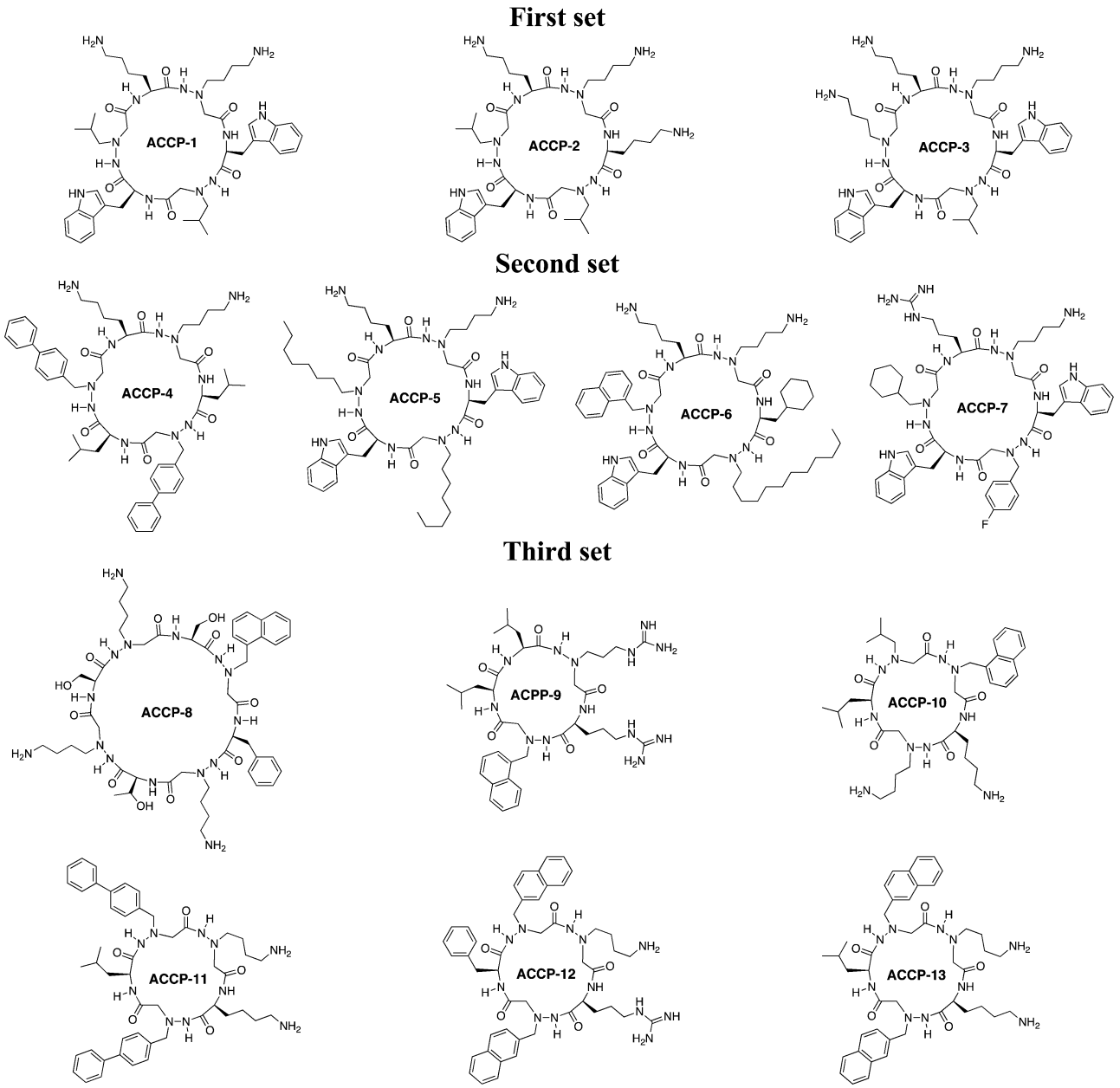

Figure 2. Structures of cyclic pseudopeptides (ACPPs).

The first set of pseudopeptides consisted of three hexapseudopeptides with alternating α-amino acids and aza-β^3-amino acids (ACPP1, ACPP2, ACPP3), in which we modified the ratio between hydrophilic cationic residues and hydrophobic residues. In this context, we built the hexacyclopseudopeptide ACPP1 on the basis of an existing antimicrobial cyclic peptidic sequence with alternating D- and L-α-amino acids, described by Ghadiri and co-workers and used *in vivo* in mice to protect them from bacterial infection (cyclopeptide 22, *c*[k-K-w-L-w-L]).[36] We examined in this first series the optimal ratio between aliphatic and aromatic residues among the hydrophobic domain of the cycle (2/2, 2/1, or 1/2) and the number of cationic charges (two or three lysyl side chains).

The second group was composed of four cyclohexapseudopeptides displaying more original sequences and containing some aza-β^3-amino acids bearing hydrophobic nonproteinogenic side chains (Figure 1). Based on our preliminary results obtained with ACPP1, we designed these unnatural side chains with the aim of improving their antibacterial activities.[35] It has been previously described that incorporating hydrophobic fluorinated α-amino acids,[37,38] bulky aromatic amino acids such as naphthylalanine,[23,25,39] or long lipophilic residues[40−42] in AMPs dramatically improved their activities. Concerning the aromatic residues, due to the aza-β^3-tryptophan side chain instability in standard deprotection conditions (TFA/TIS/H_2O) highlighted during this study, we investigated the influence of the substitution of the natural tryptophanyl side chains (ACPP1) by an aza-β^3-amino acid bearing a bulky nonproteinogenic hydrophobic side chain, the aza-β^3-4-biphenylalanine (ACPP4), leading to aza-β^3-leucine/L-α-leucine substitution in this pseudopeptide. Then, for the acyl part, two ACPPs possessed aza-β^3-amino acids bearing long alkyl side chains. ACPP5 differed from ACPP1 by the simple substitution of the aza-β^3-leucine by aza-β^3-octylalanine. ACPP6 with its aza-β^3-dodecylalanine bore a long aliphatic side chain and three cyclic side chains: α-cyclohexylalanine, aza-β^3-(1)-naphthylala-

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Table 2. MIC (μM) against Gram-Negative and Gram-Positive Bacteria, HC_{50} (μM) against sRBC, and Selectivity (SR and MHC/MIC) for Three Sets of ACPPs[a]

		first set			second set				third set					
ACPPs	control, melittin	1	2	3	4	5	6	7	8	9	10	11	12	13
						MIC								
						Gram-Positive								
B. megaterium	6.25	25	50	25	0.78	6.25	12.5	1.56	>100	25	50	3.12	3.12	12.5
Enc. faecalis	12.5	>100	>100	>100	6.25	12.5	12.5	50	>100	>100	>100	50	>100	>100
Str. equinus	3.12	100	>100	>100	1.56	6.25	12.5	6.25	>100	100	100	6.25	12.5	25
M. luteus	1.56	50	100	100	0.78	3.12	12.5	3.12	>100	25	50	3.12	6.25	6.25
Lac. garvieae	6.25	>100	>100	>100	3.12	6.25	50	25	>100	>100	>100	50	100	>100
L. monocytogenes	3.12	>100	>100	>100	1.56	3.12	12.5	6.25	>100	>100	>100	12.5	25	100
S. aureus	25	>100	>100	>100	1.56	6.25	25	25	>100	>100	>100	25	50	>100
						Gram-negative								
E. coli	6.25	>100	>100	>100	12.5	>100	>100	25	100	>100	>100	50	100	>100
Sal. enterica	25	>100	>100	>100	100	>100	>100	25	>100	>100	>100	50	100	100
P. aeruginosa	50	>100	>100	>100	12.5	25	>100	50	100	>100	>100	50	50	100
K. oxytoca	25	>100	>100	>100	25	>100	>100	25	>100	>100	>100	50	>100	>100
Enb. aerogenes	12.5	>100	>100	>100	25	>100	>100	12.5	>100	>100	>100	25	100	>100
A. caviae	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
HC_{50} sRBC		>100	>100	>100	15	90	50	>100	>100	>100	>100	>100	>100	>100
% hemolysis at 100 μM		0	0	0	90	55	62	2.5	0	0	0	30	1	0
						SR:[b] MIC/HC_{50}								
S. aureus		*c*	*c*	*c*	9.6	14.4	2	$\gg 4^d$	*c*	*c*	*c*	$\gg 4^d$	$\gg 2^d$	*c*
E. coli		*c*	*c*	*c*	1.2	*c*	*c*	$\gg 4^d$	*c*	*c*	*c*	$\gg 2^d$	$\gg 1^d$	*c*
						MHC/MIC								
S. aureus		*c*	*c*	*c*	1	1	0.12	4	*c*	*c*	*c*	1	2	*c*
E. coli		*c*	*c*	*c*	0.12	*c*	*c*	4	*c*	*c*	*c*	0.5	1	*c*

[a]All ACPPs were isolated as their trifluoroacetate salts, and the molar concentrations were calculated as such. Bacterial strains: *Bacillus megaterium* (ATCC 10778); *Enterococcus faecalis* (CIP 186); *Listeria monocytogenes* (SOR 100); *Steptococcus equinus* (ATCC 5623); *Micrococcus luteus* (ATCC 10240); *Lactococcus garvieae* (ATCC 43921); *Staphylococcus aureus* (ATCC 25923); *Escherichia coli* (ATCC 25922); *Salmonella enterica* (ATCC 13076); *Pseudomonas aeruginosa* (ATCC 27853); *Klebsiella oxytoca* (CIP 7932); *Enterobacter aerogenes* (ATCC 13048); *Aeromonas caviae* (ATCC15648). Abbreviations: sRBC, sheep red blood cells; MHC, minimal hemolytic concentration corresponding to the first concentration where more than 1% of significant hemoglobin release was observed. [b]SR was calculated by dividing HC_{50} sRBC values by MIC values against bacterial strain. [c]No MIC values to calculate the selectivity ratio within the concentration range tested. [d]The "$\gg$" denotes that there were no HC_{50} values to calculate the SR within the concentration range tested, so the reported values were minimal SR calculated with 100 μM.

nine, and α-tryptophan. ACPP6 appeared to be the most hydrophobic ACPP attributed to its longest retention time (R_t) in our analytical RP-HPLC conditions. Halogenated aza-β^3 residues can also be incorporated as in the original sequence of ACPP7 with an aza-β^3-(4)-fluorophenylalanine. This atypical sequence derived from ACPP1 included three modifications: an aza-β^3-(4)-fluoro-phenylalanine (three consecutive aromatic residues), an aza-β^3-cyclohexylalanine, and an α-arginine instead of the α-lysine. ACPP7 was designed to demonstrate the infinitely possible α/aza-β^3-amino acid substitutions for further larger screening (Table 1 and Figures 1 and 2).

The last group of tested ACPPs allowed us to consider the influence of cycle size (residue number), as well as the importance of alterning α- and aza-β^3-amino acids. This group contained one octacyclopseudopeptide, ACPP8, designed to be relatively more hydrophilic than the others to ensure noncytotoxicity. This was built on the basis of an existing antimicrobial cyclic peptidic sequence alternating D- and L-α-amino acids described to have systemic prolonged antibacterial activity *in vivo* against methicillin-resistant *Staphylococcus aureus* (cyclopeptide 6752, *c*[S-w-F-k-T-k-S-k]).[43] In this group, we also tested five cyclic pentacyclopseudopeptides. We chose to evaluate the biological activities of these pseudopeptides to investigate the effect of the size reduction of the pharmacophore. Penta-ACPPs also allowed us to investigate the consequence of breaking α/aza-β^3-amino acid alternation (ACPP9 had two adjacent α-amino acids whereas the others had two adjacent aza-β^3-amino acids). All of them contained two cationic residues. ACPP9 and ACPP10 contained only one aromatic residue in their hydrophobic domains, whereas ACPP11, ACPP12, and ACPP13 had two aromatic residues. In most of the ACPPs described here, cationic charges were provided by lysyl side chains. However, it was reviewed that primary amines could not form hydrogen bonds while engaged in cation$-\pi$ interactions with aromatic side chains such as tryptophan.[44] Indeed, it was instead suggested that arginine residues could keep their ability to form hydrogen bonds when shielded by tryptophan residues and this difference could facilitate peptide–membrane interactions and therefore could enhance antimicrobial activities. To investigate a possible effect of such cationic substitutions, we selected the pentapeptide ACPP9 with two arginyl side chains and incorporated one α-arginine in ACPP7 and ACPP12, two ACPPS with aromatic hydrophobic clusters.

Synthesis. All ACPPs were synthesized using solid phase peptide synthesis with fluorenylmethyloxycarbonyl (Fmoc)/*t*-butyl (*t*-Bu) protection strategy and 2(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate (TBTU)/1-hy-

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droxy-benzotriazole (HOBt)/diisopropylethylamine (DIEA) procedure and cyclized at high dilution in a "head-to-tail" fashion. The Fmoc-protected aza-β^3-amino acids were synthesized by the same overall method that we have already reported, based on the reductive amination of glyoxylic acid with a protected Fmoc hydrazin.[32−35] To avoid possible problems of epimerization, we chose to load Fmoc-protected aza-β^3-amino acids on an acid-sensitive solid support, 2-chlorotrityl chloride resin. This solid support allowed us to cleave pseudopeptides without side chain deprotection and then to easily perform the cyclization at high dilution with a 1-ethyl-3-(3′-dimethylaminopropyl)carbodimide (EDC)/HOBt/DIEA procedure before the final side chain deprotection in standard TFA/TIS/H_2O conditions. Due to the tryptophanyl side chain instability in these standard deprotection conditions for aza-β^3-tryptophan residues, we selected powerful aromatic hydrophobic aza-β^3 residues such as aza-β^3-4-biphenylalanine and aza-β^3-naphthylalanine, already known to enhance antimicrobial activity.[39] This strategy is suitable for automated, parallel, and high-throughput synthesis. All ACPPs were obtained as the major product of synthesis with good yields (29−46% for cyclohexapseudopeptides, Table 1). The major secondary byproducts that were isolated (7−10% yields) were cyclopentapseudopeptides that lacked a hydrophobic residue due to steric hindrance. These compounds were easily separated and tested for their antimicrobial properties.

Antibacterial Activity in Rich Media. All minimal inhibitory concentrations (MIC_{48h}'s) in rich media were reported in Table 2. All ACPPs have been tested on 13 bacterial strains (7 Gram-positive strains and 6 Gram-negative strains) and compared with the well-known AMP melittin. Some ACPPs were inactive whereas other ACPPs exhibited low-micromolar MICs against the quasi-totality of the tested bacterial strains. First of all, the initial set of three ACPPs (ACPP1, ACPP2, and ACPP3) demonstrated that such cyclic pseudopeptides could exhibit low but significant antimicrobial activities against Gram-positive bacteria. Gram-negative bacterial growth was not inhibited by these ACPPs. These three ACPPs appeared less potent against target bacteria than mellitin ($MIC_{ACPP1-3} > MIC_{melittin}$). Greater antibacterial activity was observed for ACPP1 revealing that two positively charged residues for four hydrophobic residues (ratio 1/2) appeared to be the optimal ratio to obtain antimicrobial molecules. Indeed, the most hydrophobic cyclohexapseudopeptides were the most potent. So ACPP1 (R_t 20 min) was active on three bacterial strains, whereas ACPP2 (R_t 14 min) and ACPP3 (R_t 16.5 min) only inhibited the growth of the most sensitive Gram-positive strains. For this structural framework, antimicrobial potency appeared as a function of hydrophobicity. Furthermore, among the hydrophobic domain of the cycle, aromatic residues that exalt the hydrophobicity were more effective than aliphatic ones as demonstrated by the higher activity of ACPP3 on *Bacillus megaterium* compared with ACPP2.

The second set focused on incorporation of nonproteinogenic side chains. Overall, the presence of hydrophobic nonproteinogenic side chains had an impressive effect on antibacterial activity, MICs being in the same range or lower than those of melittin. L-α-Tryptophan substitution by aza-β^3-4-biphenylalanine drastically increases the antimicrobial activity. ACPP4 (R_t 24.7 min) is particularly effective on Gram-positive bacteria with MICs in the range of <10 μM. Moreover MICs on *B. megaterium* and *M. luteus* are submicromolar (780 nM). ACPP4 is also able to inhibit the growth of all Gram-negative bacteria except *A. caviae*. In particular, ACPP4 was active on *P. aeruginosa* (MICs $\leq$ 12.5 μM) and more active than melittin. Results revealed that the most potent ACPP was ACPP4.

ACPP5 (R_t 24 min), which differs from ACPP13 by only an aza-β^3-leucine substitution by aza-β^3-octylalanine, displayed a specific antimicrobial profile. ACPP5 exhibited substantial MICs on all Gram-positive bacteria (MICs $\leq$ 12.5 μM) and was moderately potent against one Gram-negative bacterium, *P. aeruginosa* (MIC $\leq$ 25 μM). Interestingly, introducing one long alkyl side chain (aza-β^3-dodecylalanine) to get ACPP6 resulted in a specificity shift. ACPP6 was active on all tested Gram-positive bacteria (MICs ranging from 12.5 to 50 μM), whereas it appeared inert toward all tested Gram-negative bacteria up to 100 μM. It is noteworthy that the two ACPPs (5 and 6) including at least one aza-β^3-residue bearing a long aliphatic side chain appeared more specific because they showed high antimicrobial potencies against all Gram-positive bacteria but poor anti-Gram-negative activity.

Although ACPP7 had the same retention time as ACPP5 (R_t 24 min), this cyclopseudopeptide bearing two L-α-tryptophan residues and two original hydrophobic aza-β^3 residues behaved in a different way. ACPP7 showed the second highest antimicrobial potency against all Gram-positive and Gram-negative bacteria. Like ACPP4, ACCP7 exhibited a broad-spectrum antimicrobial activity; within the concentration range tested, only the Gram-negative bacteria *A. caviae* was not affected.

The last set focused on ACCPs with different numbers of residues. The only octacyclopseudopeptide tested in this study, ACPP8, showed poor activity against Gram-negative bacteria and no antimicrobial activity was detected against Gram-positive bacteria. ACPP8 only displayed measurable activity against *E. coli* and *P. aeruginosa* at the highest tested concentration (MIC = 100 μM).

Concerning the five tested penta-ACPPs, the results reported in Table 2 confirmed a better potency against Gram-positive bacteria. It was possible to divide penta-ACPPs into two groups according to the number of aromatic residues within their sequences.

Penta-ACPPs with only one aromatic residue (aza-β^3-(1)-naphthylalanine) within their hydrophobic domains, ACPP9 and ACPP10, were only potent against the three most susceptible Gram-positive bacterial strains of this study (*B. megaterium, S. equinus, M. luteus*) with MICs $\geq$ 25 μM. Neither compound ACPP9 nor ACPP10 showed antimicrobial activity against Gram-negative bacteria.

Conversely, the final three penta-ACPPs with two or three aromatic residues (ACPP11, ACPP12, and ACPP13) displayed an increased potency on Gram-positive bacteria. With two or three aromatic residues, antibacterial spectra against Gram-positive bacteria were broadened, and some exhibited MICs $\leq$ 6.25 μM. ACCP12 was active against all assayed Gram-positive bacteria. Moreover, these three ACPPs were moderately potent against some tested Gram-negative bacteria (MICs $\geq$ 25 μM). These results demonstrated that five residues were sufficient to obtain ACPPs with antimicrobial potency.

To summarize, some ACPPs assayed in this study were antibacterial against all targeted bacteria except a Gram-negative one, *A. caviae*. Overall, ACPP antibacterial activity was higher against Gram-positive bacteria than Gram-negative ones. Five ACPPs possessed a higher antibacterial activity than melittin ($MIC_{ACPPs} \leq MIC_{melittin}$), underlining the fact that cyclic

peptides containing aza-β^3-amino acids can be as active as a naturally occurring AMP.

Hemolytic Activity and Selectivity. As a preliminary evaluation of the toxicity in mammalian cells, hemolytic activities of ACPPs were tested on commercially available sheep erythrocytes (sRBC). ACPP concentrations causing 50% hemolysis (HC_{50}) were derived from the dose−response curves. HC_{50} and hemolysis (%) at 100 μM were reported in Table 2.

Among 13 ACPPs, 9 of them displayed less than 5% of hemolytic activity on sRBC at 100 μM; only ACPP7 and ACPP12 showed no noteworthy hemolysis at the same concentration (2.5% and 1%, respectively).

Among the four significantly hemolytic ACPPs, ACPP4 and ACPP11 (both bearing two hydrophobic aza-β^3-4-biphenylalanine) displayed 90% and 30% hemolysis, respectively, at 100 μM. The highest hemolytic activity was detected with the hexacyclopseudopeptide ACPP4 (HC_{50} = 15 μM). ACPP5 and ACPP6, two hexacyclopseudopeptides containing long acyl side chains on the aza-β^3-residues, also exhibited hemolytic activities with HC_{50} of 90 and 50 μM, respectively. It is important to note that all of the more hydrophobic ACPPs of this study (R_t > 22 min) displayed a minimal hemolytic activity at 100 μM.

To rationalize these values and to envisage a therapeutic application, selectivity ratio, the ratio between HC_{50} on sRBC and MIC on relevant pathogenic bacterial strains (SR = HC_{50}/MIC), had to be considered and are reported in Table 2.[45] We focused our attention on two strains, *S. aureus* ATCC 25923 and *E. coli* ATCC 25922, Gram-positive and Gram-negative references, respectively.

SR can be calculated for ACPPs with detectable HC_{50} (ACPPs 4, 5, 6). In other cases (HC_{50} > 100 μM), a minimal value can be defined. Investigating accurately the four more hemolytic ACPPs, it was clear that their SR was better on Gram-positive bacteria over Gram-negative bacteria. Especially in the case of ACPP5 and ACPP6 (containing long acyl side chain aza-β^3-residues), no antimicrobial activities were detected on *E. coli* whereas they displayed HC_{50} values for sRBC. On the other hand, ACPP5 and ACPP6 exhibited positive SR values on Gram-positive *S. aureus* of 14.4 and at least 2, respectively.

Considering ACPP4, the more potent antimicrobial agent of this study, the SR was greater than 1 but was lowered by its high hemolytic activity. Against the selected Gram-positive strain, ACPP4 SR was remarkable (9.6), whereas it was limited on Gram-negative *E. coli* (1.2). SR of the related corresponding cyclopentapseudopeptide ACPP11 (two hydrophobic aza-β^3-4-biphenylalanine, with a lower absolute lipophilicity, R_t 22.3 min) was at least 4 on *S. aureus*.

For compounds displaying a possible good SR despite having high hemolytic concentrations, a more stringent parameter could be used as the ratio between the MHC (minimum hemolytic concentration defined as the minimal peptide concentration that produced hemolysis) and the MIC. The MHC/MIC ratio was described to give more rigorous information on the selectivity of hypothetical antimicrobial agents.[45] For example, the MHC/MIC ratio of ACPP11 was only 1 against *S. aureus* because the MHC was observed at 25 μM in our experimental conditions. It appeared in this preliminary screening of ACPPs that ACPP7, with a MHC/MIC ratio of 4 on both *S. aureus* and *E. coli* (MHC = 100 μM), displayed a significant selective profile between prokaryotic cells and mammalian sRBCs, and such cyclopseudopeptides appeared as a good basis for further improvements.

Susceptibility of Cyclic Pseudopeptides to Enzymatic Degradation. When incorporated into a linear peptide, aza-β^3-amino acids are known to enhance the resistance of the pseudopeptide to enzymatic degradation.[46] To compare the metabolic stability of ACPPs to that of melittin, the peptides were incubated *in vitro* with bovine serum and analyzed by RP-HPLC. As with most natural peptides, melittin had a short half-life, 3.6 min. The half-life of ACPPs in bovine serum (≫120 min for the three tested ACPPs) was >80 times higher than that of melittin (Figure 3).

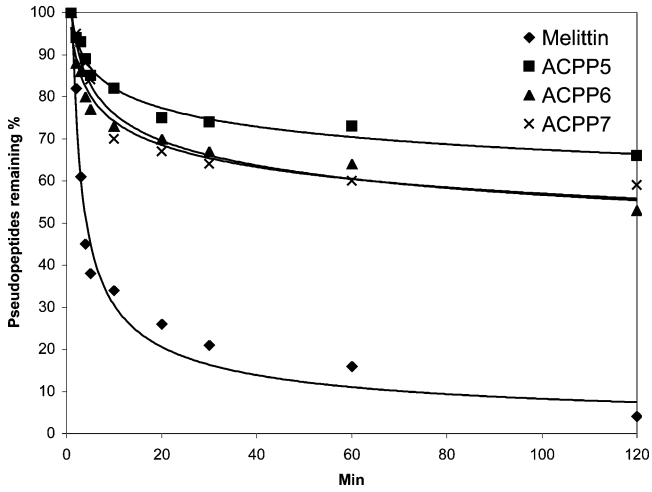

Figure 3. Degradation kinetics of melittin, ACPP5−7 evaluated by HPLC analysis after incubation of the peptide and pseudopeptides in bovine serum.

Discussion: Selection of the Ideal Candidate. Based on already described antimicrobial cyclic L-α-peptides[25] or cyclopeptides alternating L- and D-α-amino acids,[36,43,47,48] we have chosen to evaluate the feasibility and the potential efficiency of putative antimicrobial pseudopeptides alternating aza-β^3-amino acids and L-α-amino acids. In a recent study, we showed that incorporation of a single aza-β^3-amino acid in an antimicrobial peptidic sequence could lead to an inactivation or an improvement of the biological activity.[49] The incorporation of an aza-β^3-amino acid modified parameters such as the flexibility of the backbone or the overall absolute lipophilicity. This hypothesis is confirmed by our first significant observed *in vitro* antibacterial activity obtained with ACPP1. Indeed, ACPP1 (c[K-aza-β^3K-W-aza-β^3L-W-aza-β^3L]) revealed to be potent against three Gram-positive strains and inert against Gram-negative strains, whereas cyclopeptide (c[k-K-w-L-w-L]) with a very similar peptidic sequence was reported as efficient on both Gram-negative and Gram-positive bacteria.[36]

In order to extend our ACPP library, we initially decided to focus on hexacyclopseudopeptides, because their size is sufficient for cyclopeptides to exert antimicrobial activities associated with good selectivity profiles.[25,36] We designed sequences segregated in two parts, a hydrophilic cationic and a hydrophobic cluster alternating aromatic and aliphatic side chains.

The antimicrobial series of ACPP1−3 had three similar sequences, which included various numbers of the same type of residues, allowing us to obtain new insights into structural elements essential for activity. These concern the hydrophilic and hydrophobic cluster. For the former, it appeared that two cationic residues were sufficient to target Gram-positive

bacteria since ACPP2 and ACPP3 appeared less effective than ACPP1. Additionally, concerning the hydrophobic residues, increasing the number of tryptophan aromatic residues tended to enhance activity; hence, two tryptophan residues were better than one (ACPP3 displayed a better MIC on *B. megaterium* than ACPP2).

Previous studies have demonstrated that incorporation of nonproteinogenic hydrophobic bulky aromatic residues (such as naphthylalanine, biphenylalanine) could enhance antimicrobial properties of antimicrobial peptides.[39] However, it could also decrease cell selectivity if the overall peptide hydrophobicity was too high.[25]

By substituting L-α-tryptophan with aza-β^3-4-biphenylalanine, we confirmed that the nature of the aromatic side chains could lead to a strong modification of the biological activity. ACPP4 was very active against Gram-positive bacteria (all tested MIC < 10 μM), and displayed a very broad spectrum inhibiting the growth of five Gram-negative bacterial strains. The elongated aromatic side chain of aza-β^3-4-biphenylalanine appeared to be very effective for increasing antibacterial activity and overall lipophilicity. The cell selectivity was also affected by this substitution because we can measure hemolytic activity for ACPP4, with HC_{50} of 15 μM. Aza-β^3-4-biphenylalanine conferred enhanced lytic properties to ACPPs, probably due to stronger interactions with cell membranes.

Modulation of the antibacterial activity profile by side chain substitutions of the ACPPs was confirmed with ACPP5, an analogue of ACPP1 containing two aza-β^3-octylalanine residues instead of two aza-β^3-leucine ones. This simple substitution of long alkyl lipophilic side chains instead of isopropyl side chains enhanced activity against all Gram-positive bacteria. Surprisingly, ACPP6, which also contained a long alkyl "fatty acid-like" aza-β^3-dodecylalanine among its hydrophobic cluster, was active only against Gram-positive bacteria and was inert against tested Gram-negative bacteria. With this observation, it appeared that the two tested ACPPs bearing long alkyl side chains seemed to display a reinforced selectivity for Gram-positive bacteria. Lockwood and co-workers saw the same observation by acylation of the SC4 dodecapeptide (H-KLFKRHLKWKII-NH_2) with C-12 and C-18 fatty acid tails; they only increased bactericidal potency against Gram-positive bacteria.[50] They demonstrated that the presence of a fatty acid tail increased the ability of the peptide to neutralize LPS endotoxin and favored the capacity to penetrate the thick peptidoglycan layer in Gram-positive bacteria. Such specificity between Gram-positive and Gram-negative bacteria was also observed with short lipopeptides such as C16−K-L-l-K.[40] However, it was shown that amino acid substitution or length diminution of the aliphatic acid for this type of lipopeptide could drastically change the selectivity profile.

In this work, we fixed a maximum of three aromatic residues in the hydrophobic cluster. An interesting bacterial/mammalian cell selectivity profile was observed for ACPP7 possessing three adjacent aromatic residues (Trp-aza-β^3-4-Fpa-Trp). This enhanced selectivity profile could be attributed to the clustering of three aromatic residues in a cyclic sequence, as was reported with the three adjacent aromatic residues of *c*[R-R-W-W-F-R] and *c*[R-R-W-F-W-R] peptides.[25] However, the sequence of ACPP7 was too original to make a conclusion regarding its selectivity profile because of the presence of an α-arginine,[44] as well as a fluorine atom,[38] which was also known to increase the activity on bacteria. On the other hand, ACPP7 demonstrated that interesting results could be obtained by combining several parameters described here in order to increase cell selectivity.

The size of the cyclopseudopeptide rings should have an important impact on the antimicrobial activity. The ACPP8 did not exhibit any significant *in vitro* antimicrobial activity against Gram-positive bacteria. Only slight antibacterial activity (MIC = 100 μM) was observed on two Gram-negative strains (*E. coli* and *P. aeruginosa*). Once again, it clearly demonstrates that sequence transposition from a peptidic model is not sufficient to obtain high antimicrobial activities. Nevertheless, it is interesting to note that this larger ACPP8 was the more effective against Gram-negative bacteria.

To extract supporting information of this work, we checked activity profiles of penta-ACPPs. First, we had to conclude that five residues were sufficient to obtain antimicrobial cyclopseudopeptides. These results were not surprising considering there are other studies where antimicrobial pharmacophores can be reduced to two or three residues[22] or even to very short organic compounds.[51] The second observation was that alternating the aza-β^3-amino acids and L-α-amino acids was not necessary because antibacterial activity was obtained for ACPPs possessing two adjacent L-α-amino acids (ACPP9) or two subsequent aza-β^3-amino acids (ACPP10, 11, 12, and 13). ACPP11 was the more potent penta-ACPP, as well as being the only one with significant measurable hemolytic activity below 100 μM. As observed with hexa-ACPP4, incorporation of two aza-β^3-4-biphenylalanine drastically enhanced antibacterial activity associated with an increase of hemolysis. In the same way as hexa-ACPPs, two bulky aromatic residues among hydrophobic residues were necessary to observe MIC on Gram-negative bacteria. Finally, from comparison of the activities of ACPP12 and ACPP13, two penta-ACPPs including two aza-β^3-2-nahphtylalanine, a hydrophobic aromatic cluster appeared slightly more efficient than an alternation of aliphatic/aromatic side chains.[25] The same observation could be made by comparing ACPP11 and ACPP12; ACPP11 has two aromatic residues and one aliphatic residue in its hydrophobic face, while ACPP12 has three aromatic residues, and ACPP12 showed a 2-fold increased selectivity considering MHC/MIC.

From comparison of the activities of ACPP9 and ACPP10, it was difficult to conclude the impact of arginyl/lysyl side chain substitutions concerning the cationic residues because of their low antibacterial activities; no drastic enhancement was observed. Moreover, it was already suggested that the impact of arginyl/lysyl substitutions could be linked with several parameters, such as the positioning of the cationic charges, the peptide structuration at membrane interfaces, and the AMP modes of action. Thus, further investigations are needed in the case of our ACPPs.[52]

While Gram-negative bacteria have shown to be less sensitive than the Gram-positive ones, the growth of all the bacterial strains that were assayed was found to be inhibited by at least one cyclic pseudopeptide with MIC $\leq$ 25 μM, except for a single example, *A. caviae* ATCC 15468. This apparent selectivity of ACPPs between Gram-positive and Gram-negative strains points out the important differences in membrane characteristics and metabolism (lipid composition, secreted proteins, etc.) between the two types of pathogens. Gram-negative cell walls are significantly more complex than Gram-positive ones, which also possess a cytoplasmic membrane, a periplasmic space rich in hydrolytic enzymes, a thin peptidoglycan, and an outer membrane composed of phospholipids (inside layer) and lipopolysaccharides (LPS)

(outside layer). LPS with their particular oligosaccharide moiety had been described to be responsible for the low permeability of hydrophobic compounds.[53] This Gram-negative specific barrier against hydrophobic compounds would explain why highly hydrophobic ACPPs have, on average, poor activity against Gram-negative strains. However, the hydrophobic parameter alone was not sufficient because two ACPPs possessing the same HPLC retention time (ACPP5 and ACPP7) displayed significantly different antimicrobial activity on Gram-negative strains, which was already demonstrated for cyclohexapeptides.[25] These results underlined the fact that, in this context, each side chain exhibited its own properties on antimicrobial activity, demonstrating the benefit of screening nonproteinogenic side chains along with their positioning.[25]

A further peculiarity of LPS was the shielding effect against cationic AMPs via electrostatic interaction that was recently reviewed as an important aspect of Gram-negative bacterial resistance to AMPs.[54] The particular case of *A. caviae* ATCC 15468 may be related to the presence of specific LPS. Indeed, this bacteria belonging to the *Aeromonadaceae* family completely evades the pseudopeptide antimicrobial potency. It may result from its particular composition of the *O*-chain from the LPS, which was shown to be of a high-molecular-mass, acidic, and branched polymer.[55] Such a bacterial surface would generate a dreadful electrostatic trap for cationic antimicrobial peptides. Explored in *K. pneumoniae*,[56,57] it has been shown that capsule polysaccharides increase the MIC of polymyxin B, an antibiotic lipocyclopeptide, by electrostatic binding. Moreover, authors have shown that a polycation treatment of bacteria resulted in MIC restoration of cationic antimicrobial peptides, by passing this protection strategy.

The selectivity ratio (SR) observed for this preliminary library of short ACPPs symbolizes the potential in terms of selective antimicrobial agents. Because the HC_{50} values of many ACPPs were above 100 μM, many SR values were not determined and could possibly be even higher than the indicated values. Although hemolytic activity on mammalian cells was measured for the most potent ACPP of this study, ACPP4 displayed remarkable SR on *S. aureus* (9.6). Such values indicated the tendency of ACPPs to kill Gram-positive bacterial cells without exerting significant lytic activity toward sheep erythrocytes. However, it is important to note that the *in vitro* antibacterial assays were carried out in a rich medium with many possible interactive molecules, whereas *in vitro* hemolysis had been assayed in a simple PBS solution. Furthermore, it was already demonstrated for cyclopeptides that antibacterial activities remained unchanged in the presence of abundant natural blood components such serum albumin whereas hemolytic activities were reduced.[36]

CONCLUSION

The easy synthesis on solid support developed during this work and the large variety of Fmoc-protected aza-β^3-amino acids constructed in our laboratory could lead to original cyclo-pseudopetidic sequences. Moreover, these hexa-ACPPs (ACPP1 to ACPP7) displayed remarkable antimicrobial activities and specific selectivities. Incorporation of original aza-β^3-amino acids could lead to Gram-positive specific antimicrobial pseudopeptides (ACPP6). In particular, ACPP7, with three consecutive aromatic side chains among its hydrophobic part, appeared as the more interesting potential therapeutic agent of this work. ACPP7 had a good antimicrobial potency with a broad-spectrum activity against both Gram-positive and Gram-negative bacteria. Additionally, ACPP7 presented a good selectivity profile between prokaryotic and eukaryotic cells with a MHC/MIC ratio of 16 on the potentially pathogen strain (*L. monocytogenes*).

Our results show that a number of factors, such as oligomer length, type of cation, polar−hydrophobic ratio, folding propensity, and overall lipophilicity, are important for antimicrobial activity as well as selectivity. Furthermore, as we had already shown, incorporation of aza-β^3-amino acids modifies the overall hydrophobicity and constraints of the backbone, two crucial determinants of biological activity.[49] It is, however, apparent that several challenges must be met in the future in order to extend the scope of these peptide mimics.

Another very interesting property was the great enhancement of the ACPP's resistance against proteases and enzymatic degradation compared with natural occurring AMPs. The three tested ACPPs exhibited a half-life more than 80 times higher than that of melittin in bovine serum.

Further studies are being conducted to improve the potency and selectivity of these ACPPs, as well as studies to determine their mechanism of action and mode of interaction with model bilayers using, for example, NMR measurements, in analogy to studies of other types of peptidomimetics.

EXPERIMENTAL SECTION

Automated Solid-Phase Synthesis of Mixed Aza-β^3-peptides. Linear pseudopeptides were synthesized on a Pioneer peptide synthesis system with standard Fmoc solid phase peptide synthesis protocol. Synthesis was accomplished using commercially available N^{α}-Fmoc-amino acids, N^{β}-Fmoc-aza-β^3-amino acids,[29−32] and 2-chlorotrityl chloride resin. Typically, 2-chlorotrityl chloride resin (100−200 mesh; 1.2 mmol/g, 1 g) was swelled in dry dichloromethane (DCM) (10 mL) for 10 min. The first monomer was attached onto the resin by adding a solution of N^{α}-Fmoc-amino acid or N^{β}-Fmoc-aza-β^3-amino acid (1.2 equiv) in dry DCM (10 mL) and DIPEA (4 equiv) on a shaker platform for 4 h at room temperature under nitrogen. The loading resin was washed with dimethylformamide (DMF) (5 × 10 mL), dry DCM (3 × 10 mL), and then with a mixture of DCM/MeOH/DIPEA (17/2/1) (2 × 10 mL), and finally with DMF (3 × 10 mL). The yield of the loading step was determined on the absorption of dibenzofulvene−piperidine adduct (λ_{max} = 301 nm). Peptides were synthesized via Fmoc solid phase synthesis methods using a 4-fold excess of amino acid, TBTU, and HOBt in the presence of a 8-fold excess of DIPEA for 1 h for standard residues and 2 h for aza-β^3 residues. The Fmoc group was removed with 20% piperidine in DMF for 10 min. At the end of the synthesis, the resin was washed with DCM, dried, and then treated with a solution of 3% TFA in DCM (30 mL) for 20 min at room temperature. The resin slurries were then filtered. The cleavage solution was neutralized with a solution of *N*-methyl morpholine and concentrated *in vacuo*, and the resulting oil was dissolved in DCM (170 mL). The linear peptides were slowly added to a solution of EDC, HOBt (4 equiv), and DIEA (4 equiv) in DCM (to a final concentration of 10^{-4} M). The resulting mixture was stirred for 2 days. The crude mixture was concentrated *in vacuo*. The resulting oily residue was dissolved in DCM and washed with 0.5 M HCl, water, and saturated sodium chloride. The organic phases were dried under sodium sulfate then concentrated. Side chain deprotection and cleavage of peptides from the resin were performed simultaneously by treatment with trifluoroacetic acid (TFA)/H_2O/triisopropylsilane (TIS) (95/2.5/2.5, v/v/v) for 3 h. After filtration of the resin, the TFA solutions were concentrated *in vacuo*, and peptides were precipitated by addition of cold diethyl ether. Peptides were purified by RP-HPLC on a C18 XTerra semipreparative column (10 μm, 19 mm × 300 mm, Waters) with a linear gradient of water, 0.08% TFA (A)/acetonitrile, and 1% TFA (B) (5−60% B in 40 min and 60−100% B in 20 min, 8 mL/min, 215 nm) to a final purity of ≥95% and lyophilized. Characterization of purified peptides by RP-

HPLC analyses were performed on a C18 XTerra (4.6 × 250 mm, 5 μm) column using water 0.08% TFA (A)/acetonitrile, 1% TFA (B) linear gradient (5−60% B in 20 min, 1 mL/min, 30 °C, 215 nm). Peptide concentrations for all experiments were calculated as the TFA salt (assuming association of one molecule of TFA per cationic residue, determined by ^{13}C NMR). The analytical laboratory from the Centre Régional de Mesures Physiques de l'Ouest performed electrospray mass spectrometry (HR-MS, ESI) studies using MS/MS mass spectrometer ZAB Spec TOF.

Antibacterial Assays in Rich Medium. The microtiter broth dilution method used to assess antimicrobial potency of ACCPs in this study is based on the NCSLA guidelines.[58] This method was only slightly modified. Indeed, assay volumes were increased to 200 μL to improve reproducibility. Incubation time from 16 to 20 h was extended to 48 h since growth delay was observed leading to MIC underestimation. MIC values were evaluated by optical density (600 nm) to confirm unaided-eye observations (determined by a lack of turbidity). Briefly, MICs of pseudopeptides were measured by liquid growth inhibition assay, performed in sterile 96-well microtiter plates (Nunc). Microbial growth was assessed after 48 h incubation in TSB (tryptone soy broth, agar 16 g/L, pH 7.4, Biokar Diagnostics, Beauvais, France) at the optimal growth temperature depending on the strain. The bacterial strains tested were Gram-positive *Bacillus megaterium* (American Type Culture Collection ATCC 10778), *Enterococcus faecalis* (Collection of "Institut Pasteur" CIP A186), *Listeria monocytogenes* (SOR 100, a strain isolated from food meat product), *Streptococcus equinus* (ATCC 5623), *Micrococcus luteus* (ATCC 10 240), *Lactococcus garvieae* (ATCC 43921), and *Staphylococcus aureus* (ATCC 25923) and Gram-negative *Escherichia coli* (ATCC 25922), *Salmonella enterica* (ATCC 13076), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella oxytoca* (CIP 7932), *Enterobacter aerogenes* (ATCC 13048), *Aeromonas caviae* (ATCC 15468). All bacteria were grown at 37 °C except *Bacillus megaterium*, *Lactococcus garvieae*, and *Aeromonas caviae*, which were incubated at 25 °C. Bacteria from a single colony were grown overnight in TSB at the optimal growth temperature under shaking. An aliquot was diluted in fresh broth and cultured up to an exponential growing phase. Bacteria were collected and inoculated at 2×10^5 CFU/mL in TSB 2×. A 2-fold serial dilution of pseudopeptide in sterile distilled water was then added. After 48 h incubation at the optimal growth temperature of the target cells, bacterial growth was measured at 600 nm for optical density. Evaluation was carried out in triplicate. MIC was defined as the lowest pseudopeptide concentration displaying 100% growth inhibition after 48 h of incubation. The well-known antimicrobial peptide melittin was also evaluated as control of the integrity of the antibacterial assay.

Hemolytic activity. The hemolytic activity of analogs was determined with commercially available sheep erythrocytes (sRBCs) (Sigma). Erythrocytes were washed by centrifugation with phosphate-buffered saline (PBS 1×, pH 7.4) at 900*g* for 5 min until the supernatant was clear. The sRBCs were resuspended and diluted with PBS to a final concentration corresponding to $OD_{540nm} = 0.8$ for the 100% hemolysis control well (1% Triton X-100). Two-fold serial dilution of pseudopeptides in PBS in a 96-well plate resulted in a final volume of 100 μL per well, in which 100 μL of sRBCs were added. Plates were incubated for 1 h at 37 °C, followed by centrifugation at 500*g* for 5 min using a tabletop centrifuge. Supernatants were collected and hemolysis was determined by measuring the optical density of the supernatant at 540 nm. Zero hemolysis (blank) and 100% hemolysis were determined with PBS buffer alone and PBS containing 1% Triton X-100, respectively. Percentage of hemolysis was calculated using the following equation:

$$\%\ \text{of hemolysis} = 100 \times [(A_{540\text{pseudopeptide}} - A_{540\text{blank}}) / (A_{540\text{tritonX-100}} - A_{540\text{blank}})]$$

For each concentration and control, the experiments were set in triplicate.

Susceptibility of Cyclo-aza-β^3-peptides to Enzymatic Degradation. The stability of melittin and ACCPs were tested *in vitro*.[59] Typically, 1.35 mL of 25% bovine serum in a 1.5 mL Eppendorf tube was preincubated at 37 ± 1 °C for 15 min prior to addition of 150 μL of pseudopeptide stock solution (10^{-2} M in water) to make the final peptide concentration 10^{-4} mmol/mL. The initial time was recorded, and at known time intervals, 100 μL of reaction solution was removed and quenched by adding 200 μL of 95% EtOH. The cloudy reaction sample was cooled (4 °C) for 15 min and then spun at 14 000 rpm (Eppendorf centrifuge) for 4 min to pellet the precipitated serum proteins. After centrifugation, the supernatants were collected, evaporated, and diluted in 100 μL of H_2O and then analyzed by RP-HPLC on a C18 XTerra (4.6 mm × 250 mm, 5 μm) column using water−0.08% TFA (A)/acetonitrile−1% TFA (B) linear gradient (5−95% B in 45 min, 1 mL/min, 25 °C, 215 nm and 280 nm). For melittin and ACPPs, HPLC peak areas were used to calculate the percentage of intact compound remaining at the various time points during the incubation.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS[a]

AMPs, antimicrobial peptides; ACPPs, antimicrobial cyclic pseudopeptides; ATCC, American Type Culture Collection; CIP, Collection of "Institut Pasteur"; DCM, dichloromethane; DMF, dimethylformamide; DIEA, diisopropylethylamine; HC_{50}, hemolytic activity; EDC, 1-ethyl-3-(3′-dimethylaminopropyl) carbodimide; Fmoc, fluorenylmethyloxycarbonyl; HOBt, 1-hydroxy-benzotriazole; MIC, minimal inhibitory concentration; MHC, minimum hemolytic concentration; R_t: HPLC retention time; SR, selectivity ratio; sRBCs, sheep red blood cells; *t*-Bu, tertiobutyl; TBS, tris-buffered saline; TBTU, 2(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate; TFA, trifluoroacetic acid; TIS, triisopropylsilane; TSB, tryptone soy broth

ADDITIONAL NOTE

[a]Abbreviations for amino acids are based on the following sources: Nomenclature and Symbolism for Amino Acids and Peptides. *Biochem. J.* **1984**, *219*, 345−373 and A Short Guide to Abbreviations and Their Use in Peptide Science. *J. Pept. Sci.* **2003**, *9*, 1−8.

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