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Biological and structural effects of the conjugation of an antimicrobial decapeptide with saturated, unsaturated, methoxylated and branched fatty acids

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The increasing bacterial resistance against conventional antibiotics has led to the search for new antimicrobial drugs with different modes of action. Cationic antimicrobial peptides (AMPs) and lipopeptides are promising candidates to treat infections because they act on bacterial membranes causing rapid destruction of sensitive bacteria. In this study, a decapeptide named A2 (IKQVKKLFKK) was conjugated at the N-terminus with saturated, unsaturated, methoxylated and methyl -branched fatty acids of different chain lengths (C8 – C20), the antimicrobial and structural properties of the lipopeptides being then investigated. The attachment of the fatty acid chain significantly improved the antimicrobial activity of A2 against bacteria, and so, endowed it with moderated antifungal activity against yeast strains belonging to genus *Candida*. Lipopeptides containing hydrocarbon chain lengths between C8 and C14 were the best antibacterial compounds (MIC = 0.7 to 5.8 μM), while the most active compounds against yeast were A2 conjugated with methoxylated and enoic fatty acids (11.1 to 83.3 μM). The improvement in antimicrobial activity was mainly related to the amphipathic secondary structure adopted by A2 lipopeptides in the presence of vesicles that mimic bacterial membranes. Peptide conjugation with long hydrocarbon chains (C12 or more), regardless of their structure, significantly increased toxicity towards eukaryotic cells, resulting in a loss of selectivity. These findings suggest that A2-derived lipopeptides are potential good candidates for the treatment of infectious diseases caused by bacteria and opportunistic pathogenic yeast belonging to genus *Candida*.

Additional supporting information may be found in the online version of this article at the publisher's web site.

Keywords: lipopeptides; linear fatty acids; branched fatty acids; unsaturated fatty acids; antimicrobial activity; hemolytic activity; critical micelle concentration; secondary structure

Introduction

Antimicrobial peptides (AMPs) are produced by all living organisms, including bacteria, fungi, plants, invertebrated and vertebrated animals. They are considered important effector molecules of the innate immune system, which is the main defense mechanism for most living organisms during the initial stages of infection, and complement the highly specific adaptive immune system [1,2].

In the last decades, AMPs have received special attention as these molecules are candidates for the treatment of infections produced by different pathogenic microorganisms, together with their low probability of generating microbial resistance.

Most AMPs contain between 12 and 50 amino acid residues, possess an overall positive net charge and, in the proximity of the bacterial membranes, are capable of adopting amphipathic α-helix or β-sheet structures. These structures are characterized by a clear segregation of hydrophobic and hydrophilic surfaces, allowing the cationic peptides to target negatively charged bacterial membranes via electrostatic and hydrophobic interactions [3,4].

AMPs can destroy bacteria, fungi, parasites and cancer cells [1]. Initially, cationic AMPs were believed to act only by disrupting the integrity of the bacterial membrane, causing rapid killing of the microorganisms [5]. Additional studies have demonstrated that some AMPs may translocate to the cytoplasm without disrupting the membrane and inhibit multiple internal targets including

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DNA/RNA synthesis, cell wall synthesis, cell division, translocation and protein folding [5,6]. Although there is no universal agreement regarding the precise mechanism of action of AMPs, it is accepted that cytolytic activity is not mediated by interaction with specific receptors [5].

There are many short peptides with broad applications reported, and some of them are in different phases of clinical trials that hold promise for drug development. In most cases, these short peptides are the derivatives of natural ones [7].

However, only a few AMPs have been approved by the Food and Drug Administration (USA). Therefore, there is a need to develop strategies to identify new AMPs that may have therapeutic applications.

AMPs generated from lactic acid bacteria known as bacteriocins have gained great importance due to their potential use in food biopreservation, replacing chemical preservatives, because they are safe for incorporation into different food matrices. These peptides are ribosomally synthesized, although some of them are extensively posttranslationally modified [8]. Bacteriocin family includes a diversity of peptides in terms of size, primary and secondary structure, and microbial targets [9].

Listeria monocytogenes is one of the most important food-borne pathogens. Consumption of food contaminated with this bacterium can lead to Listeriosis, an infection whose severe form has a case-fatality rate of 15% to 30%. *L. monocytogenes* is an emerging pathogen with a strong impact on pregnant women, children and elderly people and its virulence having increased in AIDS patients [10,11]. Because one promising method to control *L. monocytogenes* is the use of bacteriocins and similar metabolites as antimicrobial agents, these AMPs can be considered as alternatives to the use of chemical preservatives [12].

According to the pharmacophore model of short AMPs, positive charges as well as bulky and lipophilic moieties are necessary for antimicrobial activity. An optimal balance of these factors governs the antimicrobial potency and spectrum of activity [13]. In this sense, conjugation of tetrapeptides with *p*-hydroxy cinnamic acid, cinnamic acid and 3-(4-hydroxyphenyl) propionic acid has been shown to improve the antimicrobial activity, increasing the hydrophobicity of the peptide sequence [14].

Previous studies have demonstrated that the conjugation of a fatty acid to a linear AMP leads to an increase in antimicrobial activity [15,16]. Moreover, fatty acid conjugation to non-active cationic peptides endowed them with antimicrobial capacity [17,18]. This effect is primarily due to a change in the peptide hydrophobicity. Lockwood *et al.* have shown that N-terminal acylation of SC4, a potent bactericidal helix-forming peptide, with C12 and C18 saturated fatty acids, generates more potent AMPs and extends their spectrum of action to drug-resistant Gram (+) bacteria and anthrax strains [19]. In addition, Nasompag *et al.* found that C12-KYR-NH_2 and C14-KYR-NH_2 presented the highest antimicrobial activity of a series of ultrashort lipopeptides acylated with C10 to C16 fatty acids, indicating that hydrophobicity is the key parameter dictating biological activity of these compounds [20].

In recent years, Zhang *et al.* reported in the World Intellectual Property Organization a series of short lipopeptides corresponding to the formula C12–18 lipid—KXZ-NH2 (wherein K = lysine; X = proline, d-proline, histidine or arginine; Z = valine, threonine, alanine or leucine) for its used as antimicrobial compound to treat inflammations or bacterial infections affecting the skin and other related mucosal body surfaces [21].

On the other hand, polymyxin's family are an interesting group of natural lipopeptides that contain branched fatty acids. They comprise cyclic cationic decapeptides conjugated with 6-methylheptanoic or (S)-6-methyloctadecanoic acids in the N-terminal, which have high antimicrobial activity against Gram (−) bacteria [22]. A series of synthetic analogs based on polymyxin structure showed an extended spectrum of action toward Gram (+) and Gram (−) bacteria [23,24].

For many years, the antimicrobial activity of saturated and unsaturated fatty acids has been well documented. Several investigations demonstrated the antifungal activity of short, medium and long chain unsaturated fatty acids against a broad variety of yeast and fungi [25–27]. In addition, some long chain unsaturated fatty acids were found to inhibit the growth of *Staphylococcus aureus* strains [28]. On the other hand, it has been demonstrated that saturated fatty acids possess antifungal and antibacterial activity [25,29]. In a study done with a series of linear chain saturated fatty acids containing between 2 and 20 carbon atoms, lauric and decanoic acids were shown to be the most active against mycobacteria strains [30].

Methoxylated fatty acids are a series of saturated and unsaturated fatty acids that contain a methoxy group bound to the alkyl chain. When isolated from bacteria and marine organisms and also synthesized, they presented interesting biological activities, such as antibacterial, antifungal, antiviral and anticancer activity [31–33].

Another attractive group of fatty acids, because they display antiprotozoal activity [34], are those constituted by iso-methyl-branched fatty acids isolated from marine sponges and then synthesized [35].

Acetylenic fatty acids, a group of natural and synthetic compounds with triple carbon–carbon bonds in their structure, are widely distributed in nature and have been isolated from higher plants, fungi, microorganisms and marine organisms, especially sponges [36]. These compounds have an extensive variety of biological activity that includes antibacterial activity, mainly against mycobacteria strains, antifungal activity, antiprotozoal activity, antimalarial and antileishmanial activity in particular, as well as potential anticancer properties [37–42].

The majority of published studies related to synthetic lipopeptides focused on conjugation with saturated fatty acids, as far as we know, there are no reports of linear cationic peptides conjugated to unsaturated or methyl-branched fatty acids.

In turn, Plantaricin 149 (YSLQMGATAIKQVKKLFKKKGG), a bacteriocin produced by *Lactobacillus plantarum* NRCI 149 isolated from pineapple, has a narrow spectrum of activity, inhibiting the growth of lactic acid bacteria (LAB), such as *L. plantarum*, *Lactobacillus delbrueckii* subsp. *delbrueckii*, *L. delbrueckii* subsp. *bulgaricus*, *Lactobacillus helveticus*, *Lactobacillus casei*, *Leuconostoc mesenteroides*, *Pediococcus acidilactici*, *Pediococcus cerevisiae*, *Enterococcus hirae* and *Lactococcus*. However, this natural bacteriocin does not inhibit other Gram (+) and Gram (−) bacteria [43]. In a previous work, we demonstrated that a synthetic C-terminal amidated analog of Plantaricin 149 (Pln 149a) inhibited the growth of *S. aureus* and *L. monocytogenes* strains, and also exhibited antifungal activity against a strain of *Saccharomyces cerevisiae* [44,45]. Pln 149a contains 41% of hydrophobic amino acids and six Lys residues, giving it a net positive charge of +6 at neutral pH. It presents an amphipathic α-helix structure, extended from Ala7 to Lys20 residues [44].

Peptides covering region 6–22 of Pln 149a (named CT) were also synthesized and studied. We also found that the CT analog retains antimicrobial activity against the Gram (+) bacteria *S. aureus* and *L. monocytogenes* and the conjugation with octanoic and dodecanoic acids improved the CT inhibitory activity [46].

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In this work, our studies focused on the antimicrobial properties of a peptide which covers region Ile10 to Lys19 of Pln149a (IKQVKKLFKK, named A2), as this corresponds to the most amphipathic region of this bacteriocin (hydrophobic moment, μH = 0.837), because it is estimated that the antimicrobial activity may be centered in this region. The fatty acid conjugation effects on the biological activity and therapeutic properties were investigated.

Materials and Methods

Peptide Synthesis

Peptide A2 and its derived lipopeptides were synthesized as C-terminal amides by Fmoc solid-phase peptide synthesis (Table 1) on Rink Amide MBHA Resin (0.4 mmol g^{-1}) at 0.016-mmol scale. Amino acid couplings were performed by *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate and diisopropylethylamine. Fmoc-deblockings were carried out with 20% piperidine in *N*,*N*-dimethylformamide (v/v). Fatty acids were attached to the N-terminus of a resin-bound peptide by standard protocols using as coupling reagent (benzotriazol-1-yloxy) tris (pyrrolidino) phosphonium hexafluorophosphate in the presence of diisopropylethylamine. Final cleavage from the resin was achieved by a mixture of trifluoracetic acid/H_2O/triisopropylsilane (95 : 2.5 : 2.5) (v/v). The crude peptides were precipitated in dry cold diethyl ether, centrifuged and washed several times with cold diethyl ether until scavengers were removed. The products were then dissolved in water and lyophilized twice.

The following saturated fatty acids were conjugated to the N-terminal amino group of A2: n-octanoic acid (C8:0), n-decanoic acid (C10:0), dodecanoic acid (lauric acid, C12:0), tetradecanoic acid (myristic acid, C14:0) and hexadecanoic acid (palmitic acid, C16:0). The unsaturated and substituted fatty acids used for conjugation to A2 were (±) 4-methoxydecanoic acid (4-OMe-C10:0), 10-undecenoic acid (C11:1), 17-methyl-13-(Z)-octadecenoic acid (17-Me-C18:1), 6-heptadecynoic acid (C17:1) and 6-icosynoic acid (C20:1). Saturated fatty acids (C8 to C16) and undecenoic acid were obtained from Sigma, while acetylenic, methoxylated and isomethyl branched fatty acids were synthesized by Dr. Carballeira's research group [34,41,47].

The structures of unsaturated and substituted fatty acids are shown in Figure 1. Additionally, the structure of A2 and some of derived lipopeptides are shown in Figure S1, in Supporting Information section.

Peptides and lipopeptides were purified by High Performance Liquid Chromatography (HPLC) (Waters) using a semi-preparative reverse-phase (RP) C18 column (Jupiter-Proteo Phenomenex, 10 μm, 90 Å, 250 × 10 mm) and analyzed by RP-HPLC using a Jupiter (Phenomenex) C4 (5 μm, 300 Å, 250 × 4.60 mm) analytical column. Both peptides and lipopeptides were eluted with a linear gradient of 15 to 60% of acetonitrile in H_2O containing 0.1% trifluoracetic acid, at a flow rate of 0.8 ml/min. Absorbance was measured at 220 nm. Mass spectrometric data were obtained using a MALDI-TOF-TOF spectrometer, Ultraflex II (Bruker), in the Mass Spectrometry Facility CEQUIBIEM, Argentina.

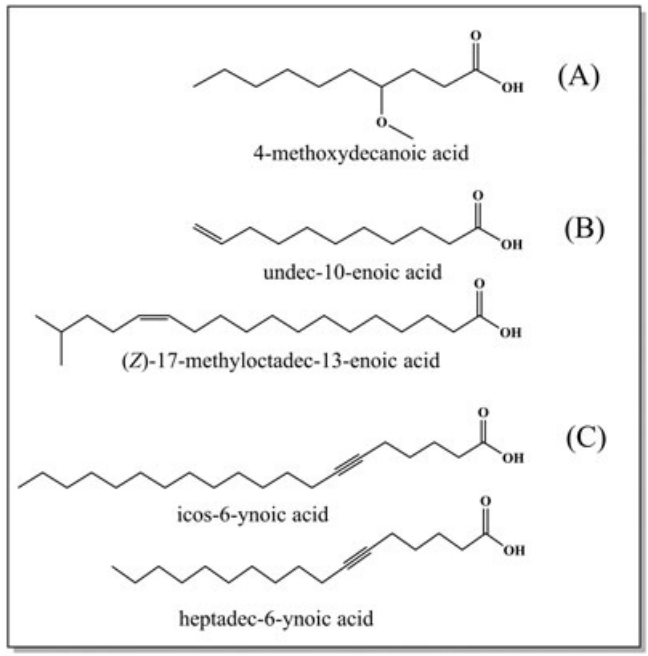

Figure 1. Structures of unusual fatty acids used in this work. A) Methoxylated fatty acid. B) Monoenoic fatty acids. C) Acetylenic fatty acids.

Table 1. Synthetic peptides and properties

Peptide identification	Peptide A2 and lipopeptides	Theoretical MW	Experimental MW[a]	rt (min)	CMC (mM)[b]
A2	IKQVKKLFKK-NH2	1258.7	1258.83	5.9	ND
C8:0-A2	Octanoic acid-A2	1384.9	1384.99	16.3	5.0
C10:0-A2	n-Decanoic acid-A2	1413.0	1413.08	17.8	5.7
4-OMe-C10:0-A2	4-Methoxydecanoic acid-A2	1442.9	1443.06	17.5	>12.5
C11:1-A2	Undec-10-enoic acid-A2	1424.9	1424.09	18.7	4.5
C12:0-A2	Dodecanoic acid-A2	1441.0	1441.08	19.6	5.6
C14:0-A2	Tetradecanoic acid-A2	1469.0	1469.28	21.9	3.8
C16:0-A2	Hexadecanoic acid-A2	1497.1	1497.11	24.7	2.4
17-Me-C18:1- A2	17-Methyl-octadec-13-(Z)-Enoic acid-A2	1537.2	1537.22	22.5	1.5
C17:1- A2	Heptadec-6-ynoic acid-A2	1506.7	1506.25	23.3	ND
C20:1- A2	Icos-6-ynoic acid-A2	1548.7	1548.36	26.3	1.2

rt: retention time determined by RP-HPLC in C4 analytical column (minutes); all peptides were synthesized as C-terminal amides.
ND: not determined
[a]The experimental MW was determined by MALDI-TOF mass spectrometry.
[b]Critical micellar concentration determined in Water Milli Q at 25 °C.

Antimicrobial Activity

Minimal Inhibitory Concentration (MIC) determination against bacteria

MIC determinations against bacterial strains were performed by the modified broth microtiter dilution assay, according to the procedures proposed by R.E.W. Hancock Laboratory for testing AMPs [48]. The target strains *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella* sp. DBFIQ S 6, *Enterococcus faecalis* ATCC 29212, *S. aureus* ATCC 25929, *Bacillus cereus* DBFIQ B 28 and *L. monocytogenes* DBFIQ LM 3, belong to the American Type Culture Collection (ATCC) and to the Culture Collection of Microbiology and Biotechnology Sections-FIQ-UNL. The Methicillin-Resistant *S. aureus* BSF FBCB1313 strain (MRSA) belongs to the Culture Collection of Clinical Bacteriology Section of FBCB-UNL. All strains were activated by culture for 24 h at 37 °C in Mueller-Hinton Broth (MHB) (Biokar Diagnostics). Each inoculum was taken and adjusted to cellular concentrations of 5×10^7 colony-forming units/ml in diluted MHB. All the peptides were dissolved in bovine serum albumin buffer with the addition of 0.01% acetic acid in order to favor their solubilization; 100 μl of each inoculum was added to 11 μl of peptide solution in serial twofold dilutions and was incubated for 18–24 h at 37 °C. The MIC was the lowest peptide concentration that completely inhibited the growth of each bacterial strain, compared with the growth in the control well. Assays were done in triplicate.

Minimal Inhibitory Concentration (MIC) determination against yeast

MIC against yeast strains was measured by the broth microtiter dilution assay according to the conditions of NCCLS document M27-A. The target strains *Candida albicans* DBFIQ CA 1, C. *albicans* PEEC 2 and *Candida tropicalis* DBFIQ 3, all of them belonging to the Culture Collection of Microbiology and Biotechnology Sections-FIQ-UNL, were activated by culture for 24 h at 30 °C in Sabouraud Dextrose Agar (SDA) (Biokar Diagnostics). Each inoculum was taken, and the cellular concentration was adjusted to 2×10^3 colony-forming units in Sabouraud Dextrose Broth (Biokar Diagnostics); 50 μl of these inocula was added to 50 μl of peptide solution in serial twofold dilutions. The plates were incubated for 48 h at 30 °C. The considered MIC was the lowest peptide concentration that completely inhibited the growth of each yeast strain, compared with the growth in the control well. Assays were done in triplicate.

Hemolysis Assay

The assay was performed using human red blood cells and following previously optimized protocols [46,49]. Human erythrocytes were isolated from EDTA anticoagulated blood by centrifugation (3000 rpm for 10 min), after washing three times with saline solution. Erythrocyte solutions were prepared at a concentration of 0.4% (v/v) in isotonic–saline solution. Test tubes containing 200 μl of erythrocyte solution were incubated at 37 °C for 60 min with 200 μl of peptide solution at concentrations ranging from 6 to 400 μM. After centrifugation at 3000 rpm for 5 min, the supernatant absorbance was measured at 405 nm. Lysis induced by 1% Triton X-100 was taken as 100% reference value.

In vitro Therapeutic Index

The *in vitro* Therapeutic Index (TI) or specificity is defined as the relationship between the Lowest Hemolytic Concentration (LHC is the lowest peptide concentration that produces 100% hemolysis) and the MIC. Thus, larger values of therapeutic index indicate greater antimicrobial specificity. When 100% of hemolysis was not detected at 400 μM, a value of 800 μM was used to calculate the LHC/MIC ratio. The index was calculated for each peptide and strain tested.

Critical Micelle Concentration (CMC) Determination

A lipopeptide solution in Milli Q water was prepared, and the specific conductivity of each lipopeptide solution at different concentrations (range of concentration: 0.06 to 18 mg/ml) was measured using a drop conductivity-meter (HORIBA), at 25 °C. Conductivity values were plotted against lipopeptide concentration ($mS \times cm^{-1}$ vs mg/ml), and CMC was determined graphically [50].

Circular Dichroism (CD) Analyses

Far-UV CD measurements were performed on a Jasco J-810 CD spectrometer (Tokyo, Japan) in a 0.1-cm path quartz cuvette (Hellma) and recorded after accumulation of five runs. CD analyses were recorded in the presence of dipalmitoylphosphatidylglycerol (DPPG) and dipalmitoylphosphatidylcholine (DPPC) vesicles. For the preparation of small unilamellar vesicles, the lipid dispersion in Milli-Q H_2O was sonicated, with a tip sonicator, until the solution became transparent. A final lipid concentration of 3 mM and a peptide concentration of 0.2 mg/ml were achieved in all samples. Spectra were corrected for background scattering caused by the vesicles by subtracting the spectrum of a single vesicle solution from that of the peptides in the presence of vesicles [51].

Additional spectra were obtained both in H_2O and in the presence of trifluorethanol [50% TFE/H_2O (v/v)], with a final peptide concentration of 0.2 mg/ml in all cases. Deconvolution of CD spectra was performed by means of the CDPro software package (Colorado State University) using the SELCOM 3 and CONTILL methods [52].

Results and Discussion

The model peptide (A2) contains 10 amino acid residues (IKQVKKLFKK-NH_2), a net positive charge of +6, 40% of hydrophobic amino acid residues and was synthesized as C-terminal amidated peptide, a common feature of many AMPs isolated from different organisms.

The analysis of A2 sequence by SPARK- X software predicted a predominant α-helical structure (Figure 2A). The Schiffer–Edmundson wheel projection of A2, drawn using the online tool of the Web site: http//heliquest.ipmc.cnrs.fr/ ComputParamsV2.py (Figure 2B), showed that the five positively charged Lys residues (in positions 2, 5, 6, 9 and 10) and the polar uncharged Gln3 appear on the hydrophilic face of the helix, while Ile1, Val4, Leu7 and Phe8 form the hydrophobic face. As previously reported in literature, the ability to assume an amphipathic structure favored insertion into microbial membranes [3,53].

Synthesis of A2 was achieved with a crude yield of 60%. For the most of the lipopeptides, yields were lower, mainly due to an incomplete acylation (see HPLC Figures S2 to S12, in the Supporting Information). Particularly, C11:1-A2 yield was low owing to hydroxylation of the double bond C-C of 10-undecenoic acid, which occurred during the cleavage step, favored by the strong acidic conditions (Figure S9). This fact was only observed with C11:1 acid, as other analogs that contained double and triple C—C bonds were not susceptible to hydroxylation.

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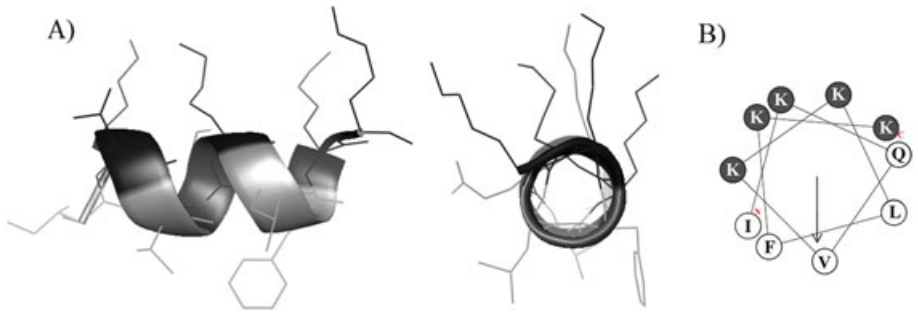

Figure 2. A) Molecular model of A2 (IKQVKKLFKK) was obtained with SPARK-X software. PYMOL 1.7.2 was used for structure visualization. The charged and polar residues on the helix are in black and the hydrophobic ones in gray. B) Schiffer–Edmundson wheel projection of A2. Light gray circles indicate hydrophobic amino acids, and the black circles represent hydrophilic amino acids (drawn by using the online tool of the Web site: http:// heliquest.ipmc.cnrs.fr/cgi-bin/ComputParamsV2.py).

In general, N-terminal acylation with saturated fatty acids increased the rt determined by RP-HPLC of lipopeptides in a chain length-dependent manner (Table 1). Lipopeptides C10:0-A2 and 4-OMe-C10:0-A2 showed similar rt, suggesting the substitution by a methoxy group in position 4 of the C10 chain did not affect the interaction with the hydrophobic stationary phase of the C4 column. The most hydrophobic peptide was the one conjugated with 6-icosynoic acid (C20:1-A2) as it contains the longest acyl chain. For lipopeptide 17-Me-C18:1-A2 (enoic), the presence of the doble carbon–carbon bond and the methyl branching affected the interaction with the stationary phase of the RP-HPLC column, as it showed lower rt than C16:0-A2 (saturated) and C17:1-A2 (acetylenic).

The CMC of the lipopeptides was determined by conductimetry in Milli-Q water, and the results are shown in Table 1. Most lipopeptides were able to adopt a micellar supramolecular assembly, with CMC ranging from 1.2 to 5.7 mM; these values were lower than those found for the SDS surfactant (CMC = 8.9 mM).

It is known that lipopeptide CMC decreases as the fatty acid chain length increases. Nevertheless, for lipopeptides containing saturated and unsaturated hydrocarbon chains between C8 and C12, similar CMC were found. The analysis of CMC values obtained for lipopeptides containing 14 or more carbon atoms (Table 1) suggested that, besides the particular structural characteristics of each fatty acid, the physicochemical parameter that determined the differences in CMC was the size of the hydrocarbon chain.

On the other hand, lipopeptide 4-OMe-C10:0-A2 displayed the highest CMC (>12.5 mM) of all studied compounds, suggesting that the presence of the methoxy group at carbon 4 of the hydrocarbon chain affects considerably and reduces the ability to adopt a micellar organization.

Because the fatty acid hydrocarbon chain of 17-Me-C18:1-A2 is branched and unsaturated, it was expected that these structural factors might affect the CMC as it is well established that unsaturated fatty acids have much lower molecular packing properties than saturated hydrocarbon chains, mostly because of the kink in the hydrocarbon chain at the *cis* double- bond sites [54,55]. Nevertheless, 17-Me-C18:1-A2 (CMC = 1.5 mM) and the saturated lipopeptide C16:0-A2 (CMC = 2.4 mM) showed similar CMC values.

Antibacterial Activity

A2 conjugated with saturated fatty acids

MIC values of A2 derived lipopeptides against Gram (+) and Gram (−) bacterial strains are shown in Table 2. A2 showed good inhibitory activity against the majority of the tested strains, with MIC values ranging from 6.4 to 25.4 μM, although it was

Table 2. Antibacterial activity of A2 and its conjugated analogs

Peptide	MIC (μM)								
	E. coli ATCC 25922	*E. coli* ATCC 35218	*P. aeruginosa* ATCC 27853	*S.* sp. DBFIQ S 6[a]	*S. aureus* ATCC 25929	*S. aureus* (MRSA)[b]	*E. faecalis* ATCC 29212	*B. cereus* DBFIQ B 28	*L. monocytogenes* DBFIQ LM 3
A2	12.7	25.4	25.4	6.4	12.7	12.7	50.8	12.7	12.7
C8:0-A2	0.7	2.2	2.2	0.7	1.4	2.2	2.2	2.8	5.8
C10:0-A2	1.4	1.4	1.4	1.4	1.4	2.1	1.4	2.8	5.7
C12:0-A2	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4
C14:0-A2	0.7	1.4	2.7	1.4	1.4	1.4	1.4	1.4	1.4
C16:0-A2	4.0	5.3	5.3	5.3	5.3	2.7	2.7	5.3	2.7
4-OMe-C10:0-A2	2.8	2.8	0.7	2.8	5.5	2.8	5.5	5.5	0.7
C11:1- A2	1.4	2.8	1.4	2.8	2.8	2.8	2.8	2.8	5.6
17-Me-C18:1-A2	1.3	10.4	1.3	2.6	2.6	2.6	10.4	20.8	2.6
C17:1-A2	1.3	21.2	2.7	2.7	10.6	2.7	1.3	5.3	10.6
C20:1-A2	5.2	20.7	10.3	10.3	10.3	5.2	10.3	10.3	10.3

[a]*S.* sp.: *Salmonella* sp.
[b]MR MRSA: Methicillin resistant *Staphylococcus aureus.*

less active against *E. faecalis* ATCC 29212 (MIC = 50.8 μM). These results suggest that despite its small size, the physicochemical properties of the sequence allow good interaction with bacterial membranes.

Conjugation of A2 with saturated fatty acids enhanced the antibacterial activity of the peptide against all bacterial strains tested, and especially against *E. faecalis* ATCC 29212 (MIC = 2.7 – 1.4 μM), for which acylation increased significantly the inhibitory activity of this strain (between 23 and 35-fold). The best MIC results against all bacterial strains tested were obtained with lipopeptides C8:0-A2, C10:0-A2, C12:0-A2 and C14:0-A2 (MIC values ranging from 0.7 to 5.8 μM) (Table 2).

Conjugation with palmitic acid also increased the antibacterial activity, particularly against Methicillin resistant *S. aureus*, *E. faecalis* and *L. monocytogenes* strains (MIC = 2.7 μM), although it was less effective than conjugation with lauric or myristic acids (Table 2). Also, it is interesting to note that for *L. monocytogenes* strain, C16:0-A2 was more active than C8:0-A2 and C10:0-A2 (MIC = 5.7 and 5.8 μM, respectively). This suggests an optimal fatty acid chain length for *Listeria* between C12 and C16. These results are consistent with those previously found for CT analogs of Pln149a (region 6–22) against *L. monocytogenes*, which state that conjugation with dodecanoic acid was much more effective than with octanoic acid (4 and 16 μM, respectively) [46].

In a recent determination of anti-Listeria activity of surfactin, results evidenced that this microbial compound could be considered another natural tool in the development of new strategies to prevent or control *L. monocytogenes* in the food industry [56]. Surfactin produced by strains of *Bacillus subtilis* is one of the cyclic lipopeptides lactonized by a heptapeptide and a β-hydroxy fatty acid. The β-hydroxy fatty acids are n-, iso- or anteiso-3-hydroxy fatty with 12–16 carbons [57,58].

Our findings partially agree with that reported by Chu-Kung *et al.*, in a study about the effect of fatty acid conjugation of a peptide containing 18 amino acid residues and with a predominance of Lys and Ala. The authors found that conjugation with lauric (C12:0) and myristic (C14:0) acids improved the antimicrobial activity against Gram (+) and Gram (−) bacteria, while conjugation with palmitic (C16:0) and stearic (C18:0) acids enhanced activity only against Gram (−) bacteria [59].

A2 conjugated with unsaturated, substituted and branched fatty acids

The conjugation of A2 with 4-OMe-C10:0 increased the inhibitory activity against Gram (+) and Gram (−) bacteria, and particularly against *L. monocytogenes* DBFIQ LM 3 and *Pseudomonas aeruginosa* ATCC 27853 (MIC = 0.7 μM), being more active than C10:0-A2 against these strains (Table 2). It appeared that the methoxy group favored the interaction with these specific bacterial membranes.

Most of the data reported in the literature about methoxylated fatty acids and their antimicrobial activity refer to those substituted at position 2. Carballeira *et al.* (2004) found that 2-methoxydecanoic acid was the most active against *Mycobacterium tuberculosis* H37Rv strain in a series of four saturated 2-methoxylated fatty acids (C8 to C14), but had similar activity to decanoic acid, indicating that α-methoxylation does not contribute to enhance the antimycobacterial activity [60,61]. In another work, Carballeira *et al.* (1998) stated that two unsaturated 2-methoxylated fatty acids, (Z)-2-methoxy–6-hexadecenoic and (Z)-2-methoxy–5-hexadecenoic acids, were active against Gram (+) bacteria (MIC = 0.35 mM), but had no activity against Gram (−) strains. However, a saturated 2-methoxylated fatty acid, 2-methoxyhexadecanoic acid (C16), was not active against any bacteria [32].

We found that, in most cases, the conjugation of A2 with the monoenoic fatty acids, 10-undecenoic acid (C11:1) and 17-methyl-13-(Z)-octadecenoic acid (17-Me-C18:1-A2), improved the antimicrobial activity of A2. The unsaturated lipopeptide C11:1-A2 and the saturated C10:0-A2 showed similar MIC values against *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* (MRSA1), *B. cereus* DBFIQ B28 and *L. monocytogenes* DBFIQ LM3, and for the other ones, C11:1-A2 was only onefold dilution less active (Table 2). According to these results, the presence of a double carbon–carbon bond in position 10 had no additional effect on the antimicrobial activity.

Comparing the results obtained with 17-Me-C18:1-A2 and C16:0-A2, it was found that the conjugation with 17-Me-C18:1 was more favorable for some bacterial strains than for others; in this sense, for *B. cereus*, it was totally ineffective, because 17-Me-C18:1-A2 was less active than A2 (Table 2). For *L. monocytogenes*, similar inhibitory activity was found by conjugation with these both fatty acids.

It is well known that unsaturated long chain fatty acids, oleic, linoleic and linolenic acids, in particular, have antibacterial activity against important pathogenic microorganisms, such as methicillin-resistant *S. aureus*, *Helicobacter pylori* and mycobacteria [62,63]. Zheng *et al*. have shown that oleic, linoleic, linolenic and arachidonic acids inhibited Gram (+) bacterial strains with MIC values ranging from 0.05 to 0.4 mM though they did not inhibit Gram (−) bacteria. The authors demonstrated that this activity correlated with the inhibition of enoyl-acil carrier protein reductase (FabI), an essential component of bacterial fatty acid biosynthesis [64].

Conjugation with the acetylenic fatty acids 6-heptadecynoic (C17:1) and 6-icosynoic (C20:1) acids also enhanced A2 antimicrobial activity against most strains tested, except for *E. coli* ATCC 35218 and *S. aureus* ATCC 25929. C17:1-A2 was particularly active against *E. coli* ATCC 25922 and *E. faecalis* ATCC 29212 strains, showing MIC values similar to those found for the saturated lipopeptides C10:0-A2 and C12:0-A2 (MIC = 1.4 μM).

On the other hand, C20:1-A2 showed lower activity compared to other lipopeptides also containing long fatty acid chains. The loss in activity may occur due to the self-assembly of the lipopeptide into micelles in solution. This may be explained considering that the CMC of the peptide in the growth media (CMC_{MHB} = 0.2 mM, data not shown) was lower than the CMC measured in water (CMC_{H20} = 1.2 mM). These aggregates would have lower affinity for the bacterial membrane than the monomeric peptide. These results agree with those reported about conjugation effect of a peptide containing 18 amino acid residues with arachidic (C20:0) acid. The lipopeptide had no measured activity against any strain of bacteria. The authors suggested that the loss in activity occurred due to the self-assembly of the fatty acid conjugated peptide into micelles in solution [59].

In addition, antimicrobial activity of the free fatty acids C10:0, 4-OMe-C10:0, C11:1, 17-Me-C18:1, C17:1 and C20:1 was determined against *S. aureus* (MRSA1) and *Salmonella* sp. MIC assay was carried out on saline solution with 3% of dimethyl sulfoxide. We found that all fatty acids were inactive against these bacterial strains (data not shown) in the concentration range tested (250 to 1 μg/ml).

Antifungal Activity

Results of MIC against yeast strains are shown in Table 3. A2 was not active against any yeast strain in the range of concentrations tested,

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Table 3. Antifungal activity of A2 and its conjugated analogs

Peptide	MIC (μM)		
	C. albicans PEEC 2	*C. albicans* DBFIQ 1	*C. tropicalis* DBFIQ 3
A2	>203.4	>203.4	>203.4
C8:0-A2	46.2	92.4	46.2
C10:0-A2	90.6	90.6	90.6
C12:0-A2	177.0	177.0	177.0
C14:0-A2	174.3	174.3	174.3
C16:0-A2	171.0	171.0	171.0
4-OMe-C10:0-A2	11.1	44.3	22.1
C11:1- A2	44.9	44.9	44.9
17-Me-C18:1-A2	41.6	83.3	41.6
C17:1-A2	>170.0	>170.0	>170.0
C20:1-A2	82.7	>165.3	165.3

but conjugation with fatty acids endowed A2 with moderate antifungal activity. In this respect, best results were obtained by conjugation with 4-methoxydecanoic, 10-undecenoic and 17-methyl-13-(Z)-octadecenoic acids, with MIC values ranging from 11.1 to 83.3 μM. 4-OMe-C10:0-A2 was two to eightfold more active than C10:0-A2 against all yeast strains tested (Table 3).

In agreement with our results, it was reported that 4-OMe-decanoic acid inhibited the growth of *C. albicans* ATCC 60193 (MIC = 1457 μM) and *Cryptococcus neoformans* ATCC 66031 (MIC= 1457 μM), being more active than the unsubstituted decanoic acid (MIC = 25.5 mM for both strains). The authors suggested that the addition of the methoxy group in position 4 could increase its solubility, facilitating the interaction with their targets [47]. It was also demonstrated that the α-methoxylation of myristic acid increases its antifungal activity [65].

When comparing the results obtained by conjugation with the monoenoic fatty acids, 10-undecenoic and 17-methyl-13-(Z)-octadecenoic acid, both derived lipopeptides proved effective to inhibit *Candida* strains; MIC values were similar against *C. albicans* PEEC 2 and *C. tropicalis* DBFIQ 3, but C11:1-A2 was twofold more active than 17-Me-C18:1-A2 against *C. albicans* DBFIQ 1. Moreover, inhibitory activity of C11:1-A2 was twofold higher than C10:0-A2 against all yeast strains tested.

According to these results, the presence of a double bond in fatty acids would have a significantly positive effect in the antifungal activity. Many studies demonstrated that free unsaturated fatty acids display antifungal activity, and, in agreement with our results, the unsaturated fatty acids are more potent than saturated ones against fungal pathogens [25–27,29].

Undecylenic acid, an economical antifungal agent, is the active ingredient in many topical antifungal formulations for the treatment of dermatomycosis caused by *Trichophyton rubrum*, *Epidermophyton inguinale* and *Microsporum audouini*, onychomycosis caused by *T. rubrum,* as well as tinea pedis caused by *Trichophyton mentagrophytes* and *T. rubrum* [66,67]. In addition, it was found to inhibit the growth of *C. albicans* and *C. glabrata* strains (MIC = 256 μg/ml) [68], and hyphal formation of *C. albicans* [69].

Moreover, Avis *et al.* (2000 and 2002) found that an iso-methyl branched monounsaturated acid, 6-methyl-9-(Z)-heptadecenoic acid, produced by the yeast *Pseudozyma flocculosa*, inhibits the phytopathogen powdery mildew *Sphaerotheca fuliginea*. The authors suggested that this fatty acid induces disorder in the fungal membrane due to its bulkiness caused by the *cis* double bond and the methyl branching which occupies a large cross section in the membrane [70,71]. These data could explain the better inhibitory activity of 17-Me-C18:1-A2 compared to C16:0-A2, which contains a large and saturated alkyl chain.

Conjugation of A2 with the acetylenic fatty acid 6-icosynoic slightly increased the antifungal activity of A2, approximately 1.2 times for most strains tested (Table 3), while C17:1-A2 was inactive. This finding demonstrated that acylation of A2 with acetylenic acids was not effective in improving the interaction with the antifungal membranes.

Nevertheless, acetylenic fatty acids have been known to be fungitoxic [25,38,39]. It has been reported that 2-alkynoic fatty acids, particularly 2-hexadecynoic acid, has antifungal activity, which depends on the fatty acid chain length and pH of the medium.

Furthermore, Li *et al.* (2008) investigated the antifungal activity of a series of 6-acetylenic fatty acids, with chain lengths between C16 and C20, isolated from roots of *Sommera sabiceoides*. They found that 6-nonadecynoic acid was the most active, in particular against the dermatophytes *T. mentagrophytes* and *T. rubrum* and the opportunistic pathogens *C. albicans* and *Aspergillus fumigatus*, with MIC around 0.7 to 11 μM, whereas 6-hexadecynoic acid, 6-heptadecynoic acid and 6-icosynoic acid were inactive or marginally active [39]. The authors suggested that 6-nonadecynoic acid interferes with sphingolipid biosynthesis [72]. However, Carballeira *et al.* (2005) found that a synthetic 6-nonadecynoic acid was active against *C. neoformans* ATCC 66031 (MIC < 4.3 μM) but was inactive against *C. albicans* ATCC 14053 and ATCC 60193 strains [73].

Hemolytic Activity

The hemolytic activity of A2 and lipopeptides against human erythrocytes was determined as a measure of peptide toxicity towards eukaryotic cells. The effect of peptide concentration on erythrocyte lysis is shown in Figure 3 (see also Table S1, in the Supplementary Information section.)

The lowest hemolytic activity (less than 20% at 400 μM) was found for A2 in the whole range of concentrations tested (6.0 to 400 μM).

Conjugation of A2 with fatty acids containing 12 and more carbon atoms produced a high increase in their lytic activity against eukaryotic membranes. Analogs 17-Me-C18:1-A2, C17:1-A2, C20:1-A2, C16:0-A2, C14:0-A2 and C12:0-A2 displayed 80–90% of hemolysis at very low concentrations. This finding may be explained by the increase in the mean hydrophobicity of the lipopeptide (Table 1). Indeed, it is well known that an increase in the hydrophobicity of an AMP decreases its selectivity for bacterial membranes [74]. Our findings are consistent with these trends.

On the other hand, conjugation of A2 with shorter fatty acid chains did not produce a significant lytic activity at low concentration, as was found for analogs C8:0-A2, C10:0-A2, 4-OMe-C10:0-A2 and C11:1-A2 (10% and 20% at 25 μM).

Graphs of hemolysis percentage *versus* peptide concentration (Figure 3) show the different behavior of lipopeptides containing 8 to 11 carbon atoms and those having 12 to 18 carbon atoms, either saturated or unsaturated.

Mean Therapeutic Index against Bacteria and Yeast

The major limitation to the use of AMPs as antibiotics is their ability to lyse eukaryotic cells. Many studies including α-helical and β-sheet peptides have evidenced different features responsible

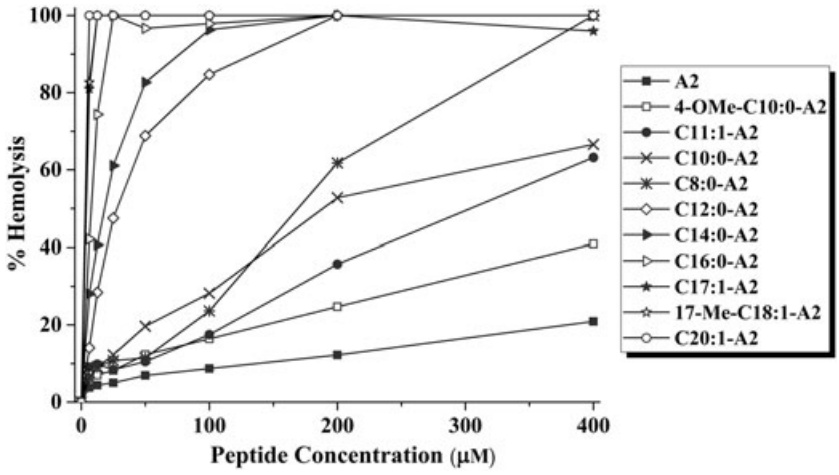

Figure 3. Hemolytic activity of A2 and its conjugated analogs against human red blood cells.

for high toxicity to normal cells that include high amphipathicity, high hydrophobicity and high helicity or beta-sheet structure [75–77].

In vitro therapeutic index or specificity, which is defined as the ratio of LHC and MIC, could be increased in one of the following three ways: increasing antimicrobial activity, decreasing hemolytic activity, or by a combination of both [78].

The average therapeutic index against Gram (+) and Gram (−) bacteria are shown in Table 4. In relation to A2, average TI values were 39.4 and 45.8 against Gram (+) and Gram (−) bacteria, respectively. The TI of A2 against yeast was very low (TI < 4) in accordance with its low antifungal activity.

Furthermore, A2 conjugated with the medium size fatty acid chains C10 and C11 showed the best TI against Gram (+) and Gram (−) bacteria (Table 4); these results agree with their high antibacterial activity and moderate hemolytic activity.

Conjugation with C12 to C20 fatty acids, although in most cases significantly increased the antimicrobial activity, also increased the hemolytic activity. Thus, these analogs showed the lowest therapeutic indices against all microorganisms tested.

Circular Dichroism Analysis

CD spectra corresponding to A2 and derived lipopeptides are shown in Figures 4 and 5.

CD analyses were recorded in the presence of DPPG and DPPC vesicles. These vesicles were used as a simplest membrane model to study the behavior of peptides near to biological membrane environments. DPPG vesicles mimic the bacterial membranes that are composed predominantly of anionic phospholipids, while zwitterionic DPPC vesicles mimic eukaryotic membranes [23,46].

Additional spectra were obtained both in H_2O and in the presence of trifluorethanol [50% TFE/H_2O (v/v)], a solvent that induces α-helical conformation.

In water, A2 did not show any preferential conformation, as expected for a short linear peptide. This is consistent with the presence of a minimum at 198 nm. Similarly, none of the synthetic lipopetides show any preferential conformation in water, except C20:1-A2 that was partially folded in a helical structure with minor contributions of turn (Figures 4A and 5A). This finding may be related to the increased ability of C20:1-A2 to assemble in water, as demonstrated by its low CMC value (CMC=1.2 mM).

In the presence of DPPC vesicles (Figures 4B and 5B), spectra deconvolution indicated that A2 and its lipopeptides were predominantly unstructured, with more than 55% unordered structure, except for C8:0-A2, C17:1-A2 and C20:1-A2 which were partially structured, showing contributions of α-helix (between 40 and 50%) and β-turn (around 20%).

In the presence of DPPG vesicles, all the lipopeptides adopted an α-helical conformation, consistent with the presence of two minima

Table 4. Therapeutic Index of synthetic analogs

Peptide	Average MIC (μM) bacteria Gram (+)	Average MIC (μM) bacteria Gram (−)	Average MIC (μM) yeast	LHC (μM)	TI against bacteria Gram (+)	TI against bacteria Gram (−)	TI against Yeast
A2	20.3	17.5	>203.4	800	39.4	45.8	<4.0
C8:0-A2	2.88	1.45	61.6	400	138.9	275.9	6.5
C10:0-A2	2.7	1.4	90.6	800	298,1	571.4	8.8
C12:0-A2	1.4	1.4	177.7	200	142.8	142.8	0.1
C14:0-A2	1.36	1.5	174.3	100	73.5	66.7	0.6
C16:0-A2	3.75	5.0	171.0	25	6.7	5.0	0.2
4-OMe-C10:0-A2	4.0	2.28	25.8	800	200	351	30.9
C11:1-A2	3.36	2.1	44.9	800	238.1	381.0	17.8
17-Me-C18:1-A2	7.8	3.9	55.5	12.5	1.6	3.2	0.2
C17:1-A2	6.1	7.0	>170.0	12.5	2.0	1.8	< 0.07
C20:1-A2	11.4	11.6	137.7	6.25	0.6	0.5	0.04

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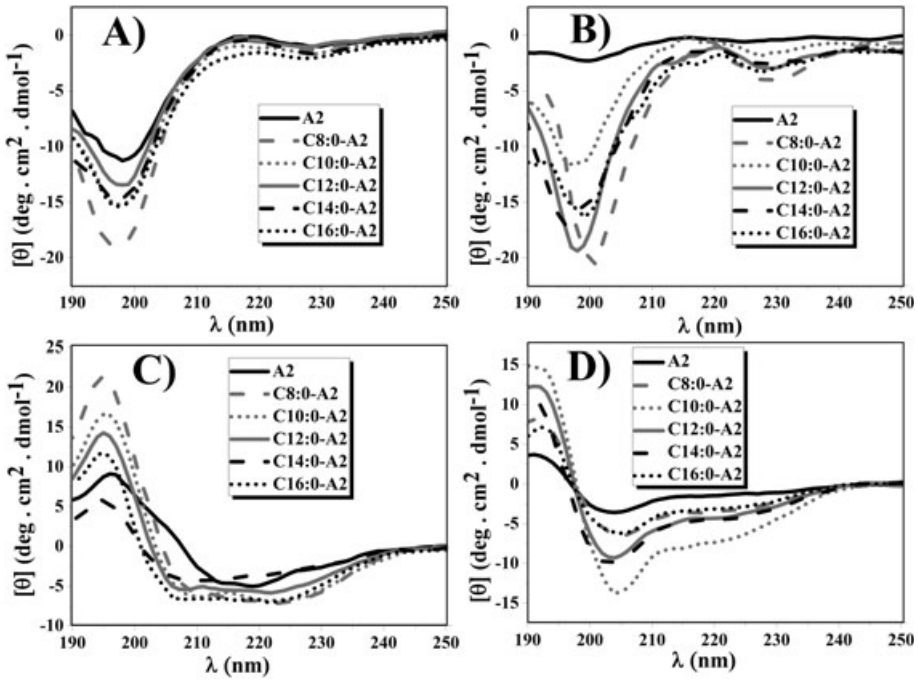

Figure 4. Circular dichroism spectra of saturated lipopeptides and peptide A2. A) Water. B) DPPC vesicles. C) DPPG vesicles. D) TFE/water (50%, v/v). Peptide concentration: 0.2 mg/ml.

at 205−207 nm and 215−220 nm and a maximum near 195 nm (Figures 4C and 5C). A2 secondary structure was significantly stabilized by conjugation with fatty acids of different hydrocarbon size, and with acetylenic and monoenoic acids. Spectra deconvolution indicated α-helix contributions of 70–90% for the majority of the lipopeptides, while 4-OMe-C10:0-A2 and C14:0-A2 showed lower contributions of α-helix (60% and 55%, respectively). These results agree with previous studies, indicating that Pln149a and lipopeptides of CT analog adopt α-helical structure in the presence of DPPG vesicles [46].

In the presence of TFE (Figures 4D and 5D), A2 was partially structured. The spectra deconvolution indicated 32% β sheet and turn, 21% α-helix and 34% of unordered structure. Spectra deconvolution of lipopeptides suggested contributions of more than 70% α-helix for most analogs, except for 17-Me-C18:1-A2, which showed lower contributions of α-helix (45%).

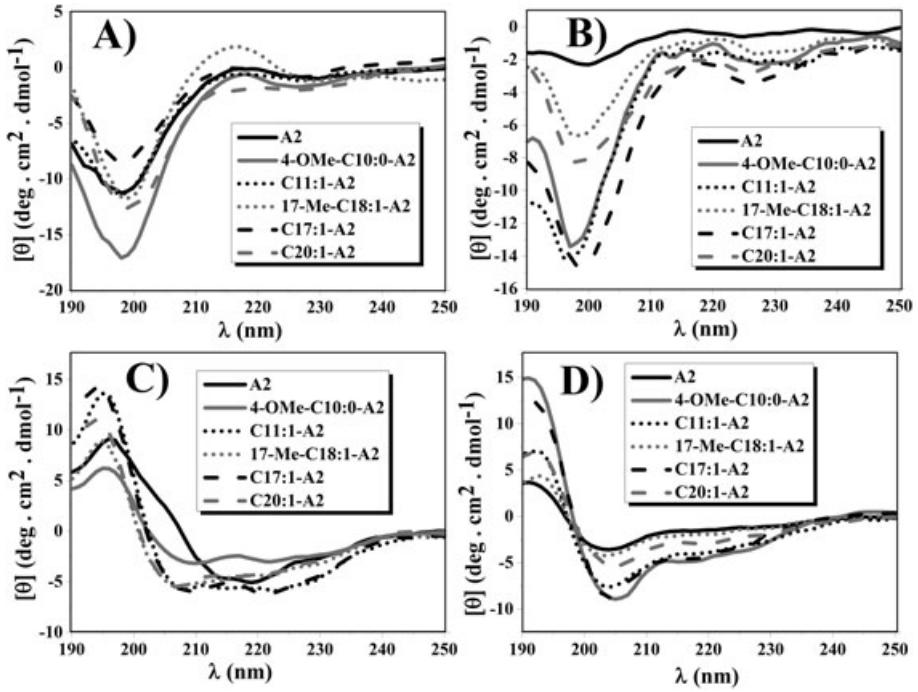

Figure 5. Circular dichroism spectra of lipopeptides containing unusual fatty acids (methoxylated, unsaturated and methyl branched) and peptide A2. A) Water. B) DPPC vesicles. C) DPPG vesicles. D) TFE/water (50%, v/v). Peptide concentration: 0.2 mg/ml.

The finding that most lipopeptides adopt an α-helical structure in the presence of DPPG vesicles, while in DPPC vesicles most were unstructured, suggests that the adoption of a stable secondary structure is a key factor for antibacterial activity, while the hemolytic activity is governed by the lipopeptide hydrophobicity.

Conclusions

Conjugation of A2 with saturated and unsaturated fatty acids significantly increased the inhibitory activity against all tested bacterial and yeast strains. In this sense, acylation with medium size fatty acid chains was the most effective one.

Results clearly show that C10:0-A2, 4-OMe-C10:0-A2, C11:1-A2, C12-A2, C14:0-A2 and C17:1-A2 lipopeptides are potential good candidates for treatment of infectious diseases caused by Gram (+) and (−) bacteria, and 4-OMe-C10:0-A2 should be considered as a potential antifungal compound for the treatment of infections caused by opportunistic pathogens of genus *Candida*.

Finally, many compounds shown here were able to inhibit the growth of *L. monocytogenes* DBFIQ LM3 and are promising candidates for use as food preservatives. In this sense, 4-OMe-C10:0-A2, C12:0-A2 and C14:0-A2 showed the best antimicrobial properties against this foodborne pathogenic bacteria.

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