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Abstract	Antimicrobial peptides (AMPs) play a key role in the defence mechanism of living organisms against microbial pathogens, displaying both bactericidal and immunomodulatory properties. They are considered as a promising alternative to the conventional antibiotics towards which bacteria are becoming highly resistant. Recently, a derivative of the frog skin AMP Esculentin-1a, Esculentin-1a(1–21)NH_2 [Esc(1–21)], showed a strong and fast membranolytic activity against Gram-negative bacteria but with a lower efficacy against Gram-positive ones. Here, with the aim to increase the α-helicity of Esc(1–21) and the expected potency against Gram-positive bacteria, we designed an analog bearing three α-aminoisobutyric acid (Aib) residues at positions 1, 10, and 18 of its primary structure. We demonstrated that the incorporation of Aib residues: (1) promoted the α-helix conformation of Esc(1–21), as confirmed by circular dichroism and two-dimensional nuclear magnetic resonance spectroscopies; (2) was sufficient to make this analog more active than the parent peptide against several Gram-positive bacterial strains without affecting its activity against Gram-negative bacteria; and (3) resulted to be devoid of toxic effect toward epithelial cells at the active antimicrobial concentrations. These results suggest that replacement of L-amino acids with Aib residues has beneficial effects on the structure and properties of the membrane-active peptide Esc(1–21), making it a better candidate for the design and development of selective drugs against Gram-positive bacteria.

Keywords (separated by '-')	Antimicrobial peptides - α-Aminoisobutyric acid - Gram-positive bacteria - NMR
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ORIGINAL ARTICLE

Effects of Aib residues insertion on the structural–functional properties of the frog skin-derived peptide Esculentin-1a(1–21)-NH_2

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Abstract Antimicrobial peptides (AMPs) play a key role in the defence mechanism of living organisms against microbial pathogens, displaying both bactericidal and immunomodulatory properties. They are considered as a promising alternative to the conventional antibiotics towards which bacteria are becoming highly resistant. Recently, a derivative of the frog skin AMP Esculentin-1a, Esculentin-1a(1–21)NH_2 [Esc(1–21)], showed a strong and fast membranolytic activity against Gram-negative bacteria but with a lower efficacy against Gram-positive ones. Here, with the aim to increase the α-helicity of Esc(1–21) and the expected potency against Gram-positive bacteria, we designed an analog bearing three α-aminoisobutyric acid (Aib) residues at positions 1, 10, and 18 of its primary structure. We demonstrated that the incorporation of Aib residues: (1) promoted the α-helix conformation of Esc(1–21), as confirmed by circular dichroism and two-dimensional nuclear magnetic resonance spectroscopies; (2) was sufficient to make this analog more active than the parent peptide against several Gram-positive bacterial strains without affecting its activity against Gram-negative bacteria; and (3) resulted to be devoid of toxic effect toward epithelial cells at the active antimicrobial concentrations. These results suggest that replacement of L-amino acids with Aib residues has beneficial effects on the structure and properties of the membrane-active peptide Esc(1–21), making it a better candidate for the design and development of selective drugs against Gram-positive bacteria.

Keywords Antimicrobial peptides · α-Aminoisobutyric acid · Gram-positive bacteria · NMR

Abbreviations

CD	Circular dichroism
DIEA	*N*,*N*-Diisopropylethylamine
DMEM	Dulbecco's modified Eagle's medium
FBS	Heat-inactivated fetal bovine serum
HATU	1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate
HBTU	*N*,*N*,*N*′,*N*′-Tetramethyl-*O*-(1H-benzotriazol-1-yl) uronium hexafluorophosphate
HOBt	1-Hydroxybenzotriazole
MBHA	4-Methylbenzhydrylamine
MH	Mueller–Hinton
MTT	3(4,5-Dimethylthiazol-2yl)2,5-diphenyltetrazolium bromide
NMR	Nuclear magnetic resonance
SDS	Sodium dodecylsulfate
TFA	Trifluoroacetic acid
TFE	Trifluoroethanol

Introduction

Ribosomally made antimicrobial peptides (AMPs) are produced by all species of life throughout the evolutionary scale as principal components of their innate defence system against invading microorganisms (Gonzalez-Navajas et al. 2014; Mangoni and Shai 2011; Mookherjee and Hancock 2007; Nicolas and Mor 1995). Furthermore, they are endowed with immunomodulatory properties (Choi et al.

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Journal : Large 726 | Dispatch : 30-9-2016 | Pages : 12
Article No : 2341 | LE | TYPESET
MS Code : AMAC-D-16-00328 | CP | DISK

2012; Hemshekhar et al. 2016) which have led to the more appropriate designation of "host-defence peptides" (Hancock et al. 2016; Mansour et al. 2014). More specifically, amphibian skin dermal glands, controlled by sympathetic nerves, are among the richest sources of biologically active peptides with pharmacological and antimicrobial activities (Chen et al. 2003; Conlon 2011; Erspamer 1971; Haslam et al. 2014; Konig et al. 2014; Mangoni et al. 2015). They are stored within granules and released on the skin surface by a holocrine mechanism, upon alarm or physical injury (Mangoni et al. 2001, 2007). Each frog species produces its own unique set of AMPs encompassing families of 2–100 closely related members (Mangoni 2006). Esculentins-1 are a class of frog skin AMPs, characterized by a 46 amino acids primary structure and a broad range of antimicrobial activity (Gamberi et al. 2007; Mangoni et al. 2003; Ponti et al. 2003; Simmaco et al. 1994). Studies on their mode of action pointed out the bacterial membrane as the major target. Esculentins-1 possess features common to most linear AMPs, i.e., an overall positive charge at neutral pH and a considerable proportion of hydrophobic residues (Simmaco et al. 1994). These properties are instrumental in allowing an electrostatic interaction between the cationic AMP and the negatively charged components of the microbial cell surface followed by the peptide's folding into an amphiphilic structure, with a resulting perturbation of the cell membrane permeability and hence cell death (Ganz and Lehrer 1998; Haney et al. 2010; Lohner and Blondelle 2005; Shai 2002). Importantly, in contrast with the conventional antibiotics that interfere with biological events by processes involving specific recognition of chiral targets (Bai et al. 2011; Levy 2002; Savjani et al. 2009), the mechanism of action underlying the killing activity of AMPs is generally based on the physical disruption of the target cell membrane, thus limiting the induction of microbial resistance (Hancock and Rozek 2002; Lohner 2016). Indeed, to become resistant to AMPs, microbes should drastically change the composition of their membrane, an event that could not be achieved without causing a significant harm to the microorganism itself (Mangoni 2006; Mangoni and Shai 2011). It is worth recalling that the membrane of mammalian cells is much richer in zwitterionic phospholipids as compared with that of microbial cells and this difference is one of the major reasons accounting for the preferential activity of AMPs towards bacterial and fungal cells (Epand and Vogel 1999).

The previous studies reported that the N-terminal derivative of esculentin-1a, Esc-1a(1–21)NH_2, [Esc(1–21)] corresponding to its first 20 amino acids followed by an amidated Gly residue (H-Gly-Ile-Phe-Ser-Lys-Leu-Ala-Gly-Lys-Lys-Ile-Lys-Asn-Leu-Leu-Ile-Ser-Gly-Leu-Lys-Gly-NH_2) adopts an alpha-helical conformation in a membrane-mimicking environment and retains the antimicrobial activity of the full-length peptide esculentin-1a (Gamberi et al. 2007; Ghosh et al. 2016). More recently, Esc(1–21) was shown to display a fast membranolytic activity against both planktonic and biofilm forms of the multi-drug resistant (MDR) opportunistic Gram-negative bacterium *Pseudomonas aeruginosa* (Breidenstein et al. 2011; Drenkard and Ausubel 2002; Kolar et al. 2015; Luca et al. 2013; Uccelletti et al. 2010). This bacterium has the ability to colonize both inert surfaces (such as those of medical devices, e.g., contact lenses) and biological tissues, forming biofilm communities (Hoiby et al. 2011; Parsek and Tolker-Nielsen 2008; Rybtke et al. 2015) which can easily lead to acute and chronic infections, including otitis, pneumonia, and keratitis (Abbouda et al. 2014; Bodey et al. 1983). However, a lower efficacy has been shown by Esc(1–21) against Gram-positive bacteria (Kolar et al. 2015).

In this connection, with the aim of enlarging the spectrum of activity of Esc(1–21) especially against Gram-positive bacteria as well as its biostability to proteases, we explored the effects of the incorporation of α-aminoisobutyric acid (Aib) residues into the peptide sequence. When inserted into the primary structure of peptides, this strongly helicogenic, non-coded, C^{α}-tetrasubstituted α-amino acid is expected to increase the α-helical content of the molecule (Karle and Balaram 1990; Toniolo et al. 2001) and potentially confers it a higher resistance against enzymatic degradation (De Zotti et al. 2009, 2012; Yamaguchi et al. 2003). Furthermore, it was previously demonstrated that a stabilized α-helical structure is an essential requirement to enhance the microbicidal activity of a peptide against Gram-positive bacteria and fungi (Giangaspero et al. 2001). In this work, we report on the synthesis of an analog of Esc(1–21), bearing three Aib residues at sequence position 1, 10, and 18 [($Aib^{1,10,18}$)-Esc(1–21)]; its structural characterization in different environments, by circular dichroism (CD) and two-dimensional nuclear magnetic resonance spectroscopies (2D-NMR) techniques, as well as its biological activity, and compared these results with those of the parent peptide Esc(1–21).

Materials and methods

Materials

Fmoc-amino acids were supplied from Novabiochem (Merck Biosciences, La Jolla, CA, USA), and all other amino-acid derivatives and reagents for peptide synthesis were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate (HATU) was purchased from GLS (Shanghai, China). Trypsin–EDTA was purchased from Invitrogen (Life-Technologies Europe,

Journal : Large 726 | Dispatch : 30-9-2016 | Pages : 12
Article No : 2341 | LE | TYPESET
MS Code : AMAC-D-16-00328 | CP | DISK

Monza, Italy); 3(4,5-dimethylthiazol-2yl)2,5-diphenyltetrazolium bromide (MTT) was from Sigma-Aldrich (St. Luis, MO). Dulbecco's modified Eagle's medium (DMEM), heat-inactivated fetal bovine serum (FBS), glutamine, gentamycin, and penicillin/streptomycin were from Euroclone (Milan, Italy). Esc(1–21) was purchased from Selleck Chemicals (Houston, TX, USA) and purified according to (Di Grazia et al. 2015a, b).

Synthesis of [Aib^{1,10,18}]-Esc (1–21)

Assembly of the peptide on the Advanced ChemTech (Louisville, KY, USA) 348 Ω peptide synthesizer was performed on a 0.06-mmol scale by the FastMoc methodology [HBTU, HOBt, DIEA, single acylation protocol, 45 min coupling time, *N*,*N*-dimethylformamide (DMF) as the solvent], starting with Rink Amide MBHA resin (Iris Biotech, Marktredwitz, Germany) (95 mg, loading 0.65 mmol g^{-1}). The deprotection of the Fmoc group was performed with a 20 % piperidine solution in DMF in two steps of 5 and 15 min, respectively. The coupling steps involving Aib residues, carried out in presence of HATU, were repeated twice. Boc and *t*Bu side-chain protections were used for Lys and Ser residues, respectively. Cleavage of the peptide from the resin, concomitantly with side-chain deprotections, was achieved by treatment with trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/water (95:2.5:2.5 v/v). The crude peptide was purified by reverse-phase flash chromatography using a Biotage Isolera Prime (Uppsala, Sweden) purification system. The chromatrographically homogeneous, final peptide was characterized by electrospray ionization mass spectrometry (ESI–MS) and NMR.

Microorganisms

The microorganisms used for the antimicrobial assays were the reference Gram-negative bacteria *Acinetobacter baumannii* ATCC 19606, *Escherichia coli* D21, *E. coli* ATCC 25922; *Pseudomonas aeruginosa* ATCC 27853, *Yersinia pseudotuberculosis* YPIII, and the Gram-positive bacteria *Bacillus megaterium* Bm11, *Staphylococcus epidermidis* ATCC 12228, as well as the clinical isolates *Staphylococcus aureus* 6938; *Staphylococcus capitis* 1; *Staphylococcus epidermidis* 21; and *Staphylococcus hominis* 1. In addition, two *Candida* strains were employed: the reference *Candida albicans* ATCC 10231 and *C. guillier mondii* from the frog natural flora (Mangoni et al. 2001).

Antimicrobial assay

Susceptibility testing was performed by adapting the microbroth dilution method outlined by the Clinical and Laboratory Standards Institute, using sterile 96-well plates (Falcon NJ, USA). The bacterial growth was aseptically measured by absorbance at 590 nm with a spectrophotometer (UV-1700 Pharma Spec Shimadzu, Tokyo, Japan). Aliquots (50 µl) of bacteria in mid-log phase at a concentration of 2×10^6 colony-forming units (CFU)/mL in culture medium (Mueller–Hinton, MH) were added to 50 µl of MH broth containing the peptide in serial twofold dilutions ranging from 64 to 0.25 µM. Inhibition of microbial growth was visually observed, after 18-h incubation at 37 °C. Antibacterial activity was expressed as the minimal inhibitory concentration (MIC), the concentration of peptide causing 100 % inhibition of microbial growth. The same procedure was followed with yeasts in Winge medium (Valenti et al. 1985) using a final cell concentration of 3.5×10^4 CFU/ml and an incubation time of 18 h at 30 °C.

Cell cultures

The human type II alveolar epithelial cell line A549 cells (from the American Type Culture Collection) and the human immortalized keratinocytes (HaCaT) cell line were employed. Cells were cultured in DMEM containing 10 % heat-inactivated fetal bovine serum (FBS) and supplemented with L-glutamine (2 mM or 4 mM for A549 or HaCaT cells, respectively) and antibiotics (0.1 mg/ml of penicillin and streptomycin for A549 cells; 0.05 mg/ml of gentamicin for HaCaT cells) at 37 °C and 5 % CO_2 in 25-cm^2 flasks.

Peptides' effect on cell viability

The effect of both peptides on the viability of mammalian cells was determined by the inhibition of MTT reduction to insoluble formazan, by mitochondrial reductases. Cells suspended in the corresponding culture medium supplemented with glutamine and 2 % FBS without antibiotics were plated in triplicate wells of a microtiter plate, at 4×10^4 cells/well. After overnight incubation at 37 °C in a 5 % CO_2 atmosphere, the medium was replaced with 100-µl fresh serum-free medium containing the peptides at different concentrations. The plate was incubated for 2 h or 24 h at 37 °C in a 5 % CO_2 atmosphere (Paiva et al. 2012). Then, the culture medium was removed and replaced with Hank's buffer (136-mM NaCl; 4.2-mM Na_2HPO_4; 4.4-mM KH_2PO_4; 5.4-mM KCl; 4.1-mM $NaHCO_3$, pH 7.2, supplemented with 20-mM D-glucose) containing 0.5 mg/ml MTT. After 4 h incubation, the formazan crystals were dissolved by adding 100 µl of acidified isopropanol and viability was determined by absorbance measurements at 570 nm using a microplate reader (Infinite M200; Tecan, Salzburg, Austria). Cell viability was calculated with respect to the control (cells not treated with peptide). The percentage of viable cells was calculated according to the formula:

Journal : Large 726 | Dispatch : 30-9-2016 | Pages : 12
Article No : 2341 | ☐ LE | ☐ TYPESET
MS Code : AMAC-D-16-00328 | ☑ CP | ☑ DISK

$$\frac{(\text{Absorbance sample} - \text{Absorbance blank})}{(\text{Absorbance control} - \text{Absorbance blank})} \times 100,$$

where the blank is given by samples without cells and not treated with the peptide.

Circular dichroism spectroscopy

The CD spectra were measured on a Jasco (Hachioji City, Japan) model J-715 spectropolarimeter equipped with a Haake thermostat (Thermo Fisher Scientific, Waltham, MA, USA). Milli-Q grade water, spectrograde methanol, and TFE (Acros Organic, Geel, Belgium) were used as solvents. Peptide concentrations were determined by UV absorption at 254 nm. For each spectrum, a total of eight scans were averaged. Baselines were corrected by subtracting the solvent contribution. Fused quartz cell of 0.1-mm path length (Hellma, Mühlheim, Germany) was used. For the experiments carried out in mixed solvents, the proper ratio of the individual solutions at the same concentration was mixed. The values are expressed in terms of $[\theta]_T$, the total molar ellipticity (deg × cm^2 × $dmol^1$).

Nuclear magnetic resonance spectrometry

Samples for NMR spectrometry were dissolved in TFE-d_2 solution (peptide concentrations: about 1 and 1.5 mM, respectively, for Esc(1–21) and its $Aib^{1,10,18}$ analog. The spectra were recorded at 298 K. All NMR experiments were performed on a Bruker Avance DMX-600 spectrometer using the TOPSPIN 1.3 software package. Presaturation of the H_2O signal was obtained using a WATERGATE gradient program. All homonuclear spectra were acquired by collecting 512 experiments, each one consisting of 64–80 scans and 2 K data points. The spin systems of the protein amino-acid residues were identified using the standard DQF-COSY (Rance et al. 1983) and CLEAN-TOCSY (Bax and Davis 1985; Griesinger et al. 1988) spectra. In the latter case, the spin-lock pulse sequence was 70-ms long. NOESY experiments were used for sequence-specific assignment (Wüthrich 1986), the mixing time used was 150 ms to avoid spin-diffusion problems.

Statistical analysis

Data were collected from three independent experiments. Quantitative data are expressed as the mean ± SEM. Statistical analysis was performed using the two-way analysis of variance (ANOVA), with the PRISM software (GraphPad, San Diego, CA, USA). Differences were considered to be statistically significant for $p < 0.05$. The levels of statistical significance are indicated in the legend to figures.

Results and discussion

Synthesis

We synthesized an analog of Esc(1–21) in which we inserted three Aib residues at positions 1, 10, and 18. The choice of the sequence positions of Esc(1–21) to be replaced by Aib was based on the following considerations: (1) the placement of a non-coded Aib residue at position 1 might prevent proteolytic degradation by aminopeptidases, whereas an amidated peptide C-terminus is known to confer protection against carboxypeptidases (Rink et al. 2010; Veber and Freidinger 1985), thus suggesting that the presence of a C-terminal Aib would not be necessary. Concerning the protection by endopeptidases, the introduction of a few additional Aib replacements, possibly quite evenly distributed along the primary structure, is expected to be (at least to some extent) beneficial. (2) The helix-promoting capabilities of Aib are more effective when this residue is placed *internal* to the peptide sequence, where it can display its influence on both the preceding and the following residues in the primary sequence. (3) A helical wheel plot of the primary structure of Esc(1–21), where residues are arranged according to an ideal α-helical folding, is shown in Fig. 1a. In this putative fully α-helical conformation, two faces can be identified, one possessing a more pronounced hydrophobic character and the other a more hydrophilic one. However, strongly hydrophobic residues, such as Ile2, Leu6, and Ile16, are located on the same face occupied by the five charged Lys residues. (4) It is worth recalling that in naturally occurring Aib-rich amphipathic helical peptides of fungal origin known as peptaibiotics (Toniolo and Brückner 2009), the Aib residues are, in general, located within the hydrophobic face but also at its boundary with the hydrophilic one. The most striking example of this latter disposition is provided by the lipopeptide trichogin (Toniolo et al. 1994). All together, these observations suggested us to place three Aib residues in the Esc(1–21) sequence; two of them at positions 1 and 18 (both as a replacement for Gly), and one in substitution for Lys^{10}, i.e., at the boundary between the hydrophilic and hydrophobic faces (Fig. 1b). As a result of this latter replacement, the overall net charge decreases by one unit if compared with that of the parent peptide, and the overall hydrophilic/hydrophobic profile becomes slightly modified. We principally preferred to give priority to the increase of the helical propensity and proteolytic stability of the central part of the sequence expected as a result of the $Lys^{10} \rightarrow Aib^{10}$ substitution, even if accompanied by the potentially unfavorable effects outlined above. For the solid-phase peptide synthesis of $[Aib^{1,10,18}]$-Esc (1–21), we exploited a well-established protocol concerning the use of a strong activating

Journal : Large 726 | Dispatch : 30-9-2016 | Pages : 12
Article No : 2341 | LE | TYPESET
MS Code : AMAC-D-16-00328 | CP | DISK

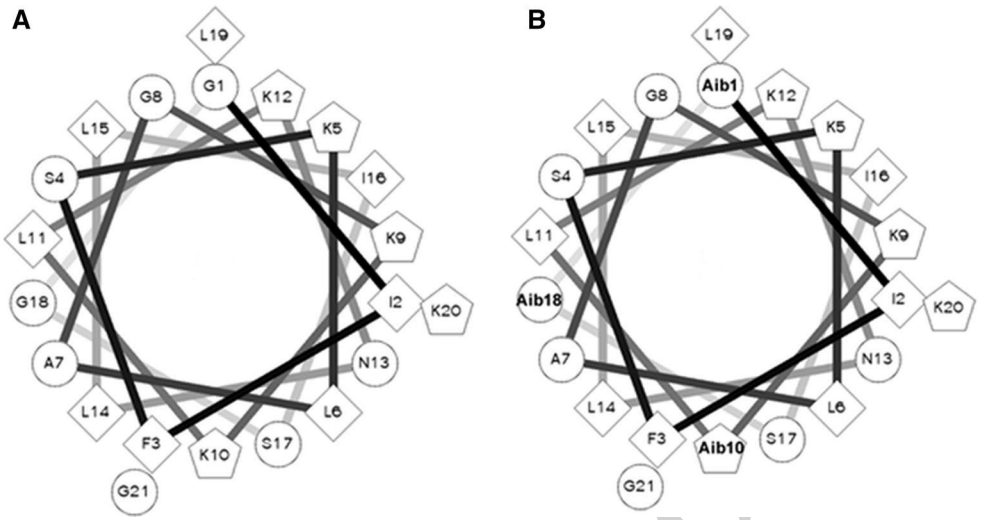

Fig. 1 Helical wheel plots of the primary structure of Esc(1–21) (**a**) and its ($Aib^{1,10,18}$) analog (**b**)

agent, HATU, in the coupling reactions involving Aib residues (De Zotti et al. 2012). The synthesis was performed on a Rink amide MBHA resin using an Fmoc N^{α} protection protocol as described in "Materials and methods". The cleavage of the peptide from the resin was achieved using a mixture of TFA/TIS/H_2O. The crude peptide was obtained in 80 % yield with a purity of 85 %, as evidenced by RP-HPLC. Reverse-phase flash chromatography allowed the isolation of the peptide with 97 % purity.

H-Aib^{1}-Ile-Phe-Ser-Lys-Leu-Ala-Gly-Lys-Aib^{10}-Ile-Lys-Asn-Leu-Leu-Ile-Ser-Aib^{18}-Leu-Lys-Gly-NH_2

	MW (calcd for $C_{104}H_{185}N_{27}O_{24}$)	MW (experimental)	t_r (min)	Purity
[$Aib^{1,10,18}$]-Esc(1–21)	2196.41	2196.40	9.38[a]	97 %

[a] Elution conditions: Jupiter C18 column, 300 Å, 5 μm; 30–60 % B in 20 min (A: 9:1 water/acetonitrile, 0.05 % TFA; B: 9:1 acetonitrile/water, 0.05 % TFA)

Antimicrobial activity

The activity of [$Aib^{1,10,18}$]-Esc(1–21) against different microorganisms, including Gram-negative, Gram-positive bacteria, and yeasts, was tested by the microdilution broth assay to determine the MIC. In comparison with the parent peptide, the incorporation of Aib residues sharply increases the activity of the peptide against Gram-positive bacteria, as shown by its lower MICs in Table 1. More specifically, the MIC of the analog carrying Aib residues is eight-fold lower against *S. epidermidis* strains or 16-fold lower against *S. capitis* 1. Furthermore, the antibacterial activity of [$Aib^{1,10,18}$]-Esc(1–21) becomes even stronger against *S. aureus*, with a 32-fold lower MIC than that of Esc(1–21). Interestingly, in line with what previously found for the de novo designed P19 peptide (Giangaspero et al. 2001),

Table 1 Antimicrobial activity of Esc(1–21) and [$Aib^{1,10,18}$]-Esc(1–21)

Microorganisms	MIC (μM)	
	Esc(1–21)	[$Aib^{1,10,18}$]-Esc(1–21)
Gram-negative bacteria		
Acinetobacter baumannii ATCC 19606	2	2
Escherichia coli ATCC 25922	4	2
Escherichia coli D21	2	2
Pseudomonas aeruginosa ATCC 27853	4	4
Yersinia pseudotuberculosis YPIII	1	1
Gram-positive bacteria		
Bacillus megaterium Bm11	2	0.5
Staphylococcus aureus 6938	64	2
Staphylococcus capitis 1	64	4
Staphylococcus epidermidis ATCC 12228	16	2
Staphylococcus epidermidis 21	16	2
Staphylococcus hominis 1	1	1
Yeasts		
Candida albicans ATCC 10231	4	2
Candida guillier mondii	1	1

[a] Values are those obtained from at least three of four independent experiments

the presence of Aib residues within the peptide sequence does not significantly affect the activity of the peptide against Gram-negative bacteria, as indicated by the same MIC values to those of the parent peptide (Table 1), with the exception of *E. coli* ATCC 25922 towards which the analog results to be only twice as powerful as Esc(1–21). In addition, the anti-yeast activity is not significantly influenced by the presence of Aib residues, and the MIC against

Journal : Large 726 | Dispatch : 30-9-2016 | Pages : 12
Article No : 2341 | LE | TYPESET
MS Code : AMAC-D-16-00328 | CP | DISK

Candida strains is equal or twofold lower than that of Esc(1–21). It is very well known that the cell selectivity of AMPs is governed by several biophysical and biochemical factors, including not only the peptide's cationicity, amphipathicity, hydrophobicity, chain length, helicity, and oligomeric state, but also the properties of the target cell surface (Glukhov et al. 2005; Matsuzaki 2009). In the last 15 years, several studies reported that an amphipathic structure is a primary requirement for AMPs to be able to kill Gram-positive bacteria and fungi, while Gram-negative bacteria remain susceptible to both non-helical and scrambled peptides (Dathe et al. 1996, 1997; Giangaspero et al. 2001). However, it is not easy to provide an unequivocal explanation for the difference in the activity profile of Esc(1–21) and its Aib-containing analog against the three classes of microorganisms. The different lipid composition existing between the membrane of Gram-positive and Gram-negative bacteria or fungi certainly plays a crucial role in determining the feasibility of the peptide's insertion into the hydrophobic core of the phospholipid bilayer, which results in membrane destabilization/perturbation and microbial death (Epand et al. 2007; Epand and Vogel 1999). In addition, differences in the cell wall architecture of the target microorganism would contribute to variations in the peptides' antimicrobial efficacy. Indeed, before reaching the target, cytoplasmic membrane AMPs need to interact with lipopolysaccharides of the outer membrane that surrounds the cell wall in Gram-negative bacteria (Bhunia et al. 2009, 2010; Domadia et al. 2010). Differently, a thicker peptidoglycan or a glucan–rich layer is present in the cell wall of Gram-positive bacteria or fungi, respectively (Free 2013; Schaffer and Messner 2005). Presumably, according to the previous studies (Giangaspero et al. 2001), more stringent structural requirements of a peptide may favor its translocation through the peptidoglycan barrier of Gram-positive bacteria into the cytoplasmic membrane. This observation may at least in part justify the lower MIC values found for the more helical [Aib^{1,10,18}]-Esc(1–21) compared with the parent Esc(1–21) against these bacterial strains.

Peptides' effect on viability of mammalian cell lines

The effect of Aib introduction within the primary structure of Esc(1–21) on the viability of eukaryotic cells was studied by the MTT assay on different types of mammalian epithelial cell lines: the human alveolar lung epithelial A549 cells and the human keratinocyte HaCaT cells. As reported in Fig. 2a, viability of A549 cells after exposure to [Aib^{1,10,18}]-Esc(1–21) at concentrations 2–4 μM is not significantly reduced and there is no significant difference between the two peptides.

Similarly, when [Aib^{1,10,18}]-Esc(1–21) is analyzed against HaCaT cells (Fig. 2b), any marked reduction in the number of metabolically active cells is obtained within the peptide concentrations of 2–4 μM compared with the harmless parent peptide. These findings suggest that [Aib^{1,10,18}]-Esc(1–21) is not toxic to mammalian cells when used at its growth inhibitory concentrations against Gram-positive bacteria (Table 1). Note, however, that when the Aib-containing analog is tested at higher concentrations, i.e., 16, 32, and 64 μM, against A549 cells, cell viability decreases to ~60, 20, and 5 %, respectively (Fig. 2a) or even further when the peptide is assayed against keratinocytes (Fig. 2b). As reported in Fig. 2c and d, the cytotoxicity of [Aib^{1,10,18}]-Esc(1–21) is only slightly increased after a long-term peptide treatment (24 h), indicating that an irreversible damage has been caused to the cells.

Note that the higher cytotoxicity of the more helical Aib-containing Esc(1–21) is in agreement with the previous findings showing that the ease of α-helix formation and stability are important factors for the mammalian membrane perturbation and cell lysis (Gazit et al. 1994; Pouny et al. 1992; Shai and Oren 1996; Strahilevitz et al. 1994).

Circular dichroism

Far-UV CD spectra of Esc(1–21) and its (Aib^{1,10,18}) analog were acquired in three different solvents: water, TFE, and 100-mM sodium dodecyl sulfate (SDS) aqueous solution (Fig. 3a, b). In water, both peptides exhibit a random coil AQ4 structure, while in TFE and micellar SDS aqueous solution, they adopt an overall helical conformation. In both membrane-mimicking environments, each spectrum shows two negative maxima near 205 and 222 nm and one positive maximum at 195 nm, indicative of a right-handed helical conformation for both peptides (Beychock 1967). The ellipticity ratio $R = [\theta]_{222}/[\theta]_{205}$ calculated in TFE and SDS solution evidenced a predominant α-helical conformation for both analogs (Mangoni and Shai 2011).

	R_{SDS}	R_{TFE}
Esc(1–21)	0.76	0.76
[Aib^{1,10,18}]-Esc(1–21)	0.92	0.75

The CD results outlined above would seem to suggest that the two compounds are conformationally similar. However, a significant difference emerges from the analysis of the CD spectra collected in water/TFE mixtures of varying composition (Fig. 3c, d). Indeed, for each peptide, the set of spectra is characterized by an isodichroic point at 203 nm, consistent with a two-state transition from the unordered conformation in water to the helical structure in 100 % TFE. The helical content of Esc(1–21) increases sharply with increasing TFE percentage from 20 to 50 % and to a lower extent from 50 to 100 % TFE (Fig. 3c), whereas the Aib-containing analog appears to be much more helical

Journal : Large 726 | Dispatch : 30-9-2016 | Pages : 12
Article No : 2341 | LE | TYPESET
MS Code : AMAC-D-16-00328 | CP | DISK

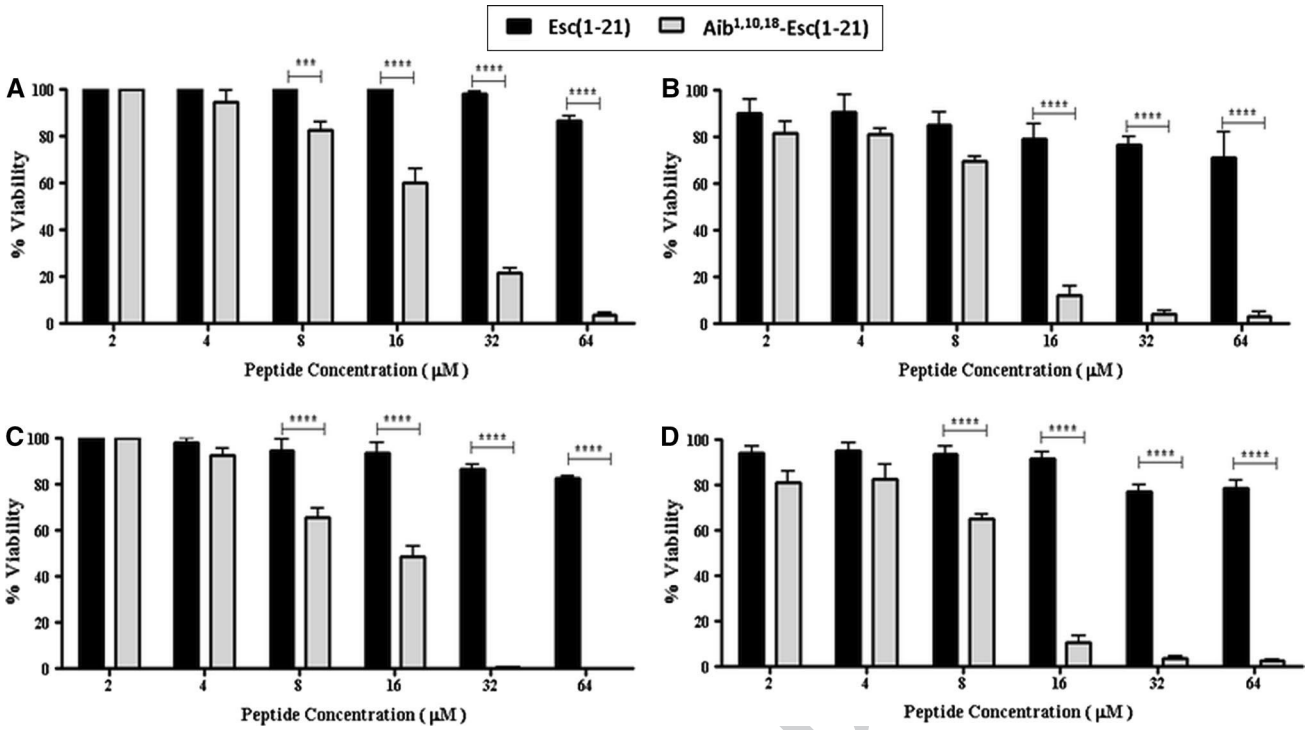

Fig. 2 Peptides' effect on the viability of A549 cells (**a**, **c**) or HaCaT cells (**b**, **d**). Cells were plated in wells of a microtiter plate, at 4×10^4 cells/well in culture medium, as described in the Experimental section. After overnight incubation at 37 °C in a 5 % CO_2 atmosphere, the medium was replaced with 100-μl fresh medium supplemented with the peptides at different concentrations. After 2 h (**a**, **b**) or 24 h (**c**, **d**) of peptide treatment, cell viability was determined by the MTT reduction to insoluble formazan. Cell viability is expressed as percentage with respect to the control (cells not treated with the peptide). Data points represent the mean of triplicate samples ± SEM. The data of the wild-type peptide Esc(1–21) on HaCaT cells were taken from our previous work (Di Grazia et al. 2015a). The levels of statistical significance between the two peptides are: ***$p < 0.001$; ****$p < 0.0001$

than the parent peptide already at 20 % TFE, and its CD spectra vary little at higher TFE percentages (Fig. 3d).

Nuclear magnetic resonance analysis

The 2D-NMR spectra of Esc(1–21) and its ($Aib^{1,10,18}$) analog were recorded in TFE solution. The proton resonances were fully assigned following the Wüthrich procedure (Wüthrich 1986).

The NOESY spectra in TFE solution of both peptides evidenced the presence of most of NH_i–NH_{i+1} sequential cross peaks, indicative of the occurrence of helical structure (Fig. 4), thus confirming the information obtained from CD spectra.

The NOESY fingerprint region of Esc(1–21) shows the $C^{\alpha}H_i \rightarrow NH_{i+2}$ and $C^{\alpha}H_i \rightarrow NH_{i+3}$ cross peaks, diagnostic of helical conformation (Fig. 4a), even if a $C^{\alpha}H_i \rightarrow NH_{i+4}$ cross peak, characteristic of α-helical structure could be detected only in the Phe^3–Ala^7 segment (although the extensive overlapping of the signals might hamper their detection in other portions of the sequence).

The fingerprint region of the NOESY spectrum of the ($Aib^{1,10,18}$) analog results better resolved (Fig. 4b). A number of connectivities are necessarily missing, because the quaternary Aib residues (at sequence positions 1, 10, and 18) lack the α hydrogen atom. Nevertheless, in addition to the $C^{\alpha}H_i \rightarrow NH_{i+2}$ and $C^{\alpha}H_i \rightarrow NH_{i+3}$ cross peaks (the latter in a larger number if compared to the parent peptide), two $C^{\alpha}H_i \rightarrow NH_{i+4}$ are also evident at the level of the Phe^3–Ala^7 and Leu^{15}–Leu^{19} segments.

A comparison of the conformationally relevant signatures extracted from the NOESY spectra of Esc(1–21) and its ($Aib^{1,10,18}$) analog is reported in Fig. 5. Overall, in the C-terminal portion of the ($Aib^{1,10,18}$) analog, the number of connectivities consistent with a helical conformation is more abundant than in the corresponding region of Esc(1–21). Therefore, both peptides are largely helical, but the introduction of Aib residues appears to increase the population and stability of the helix in the otherwise more flexible C-terminal domain of Esc(1–21).

Conclusions and perspectives

In this work, we demonstrated that the increased alpha-helical content of Esc(1–21), obtained by incorporation of

Journal : Large 726 | Dispatch : 30-9-2016 | Pages : 12
Article No : 2341 | ☐ LE | ☐ TYPESET
MS Code : AMAC-D-16-00328 | ☑ CP | ☑ DISK

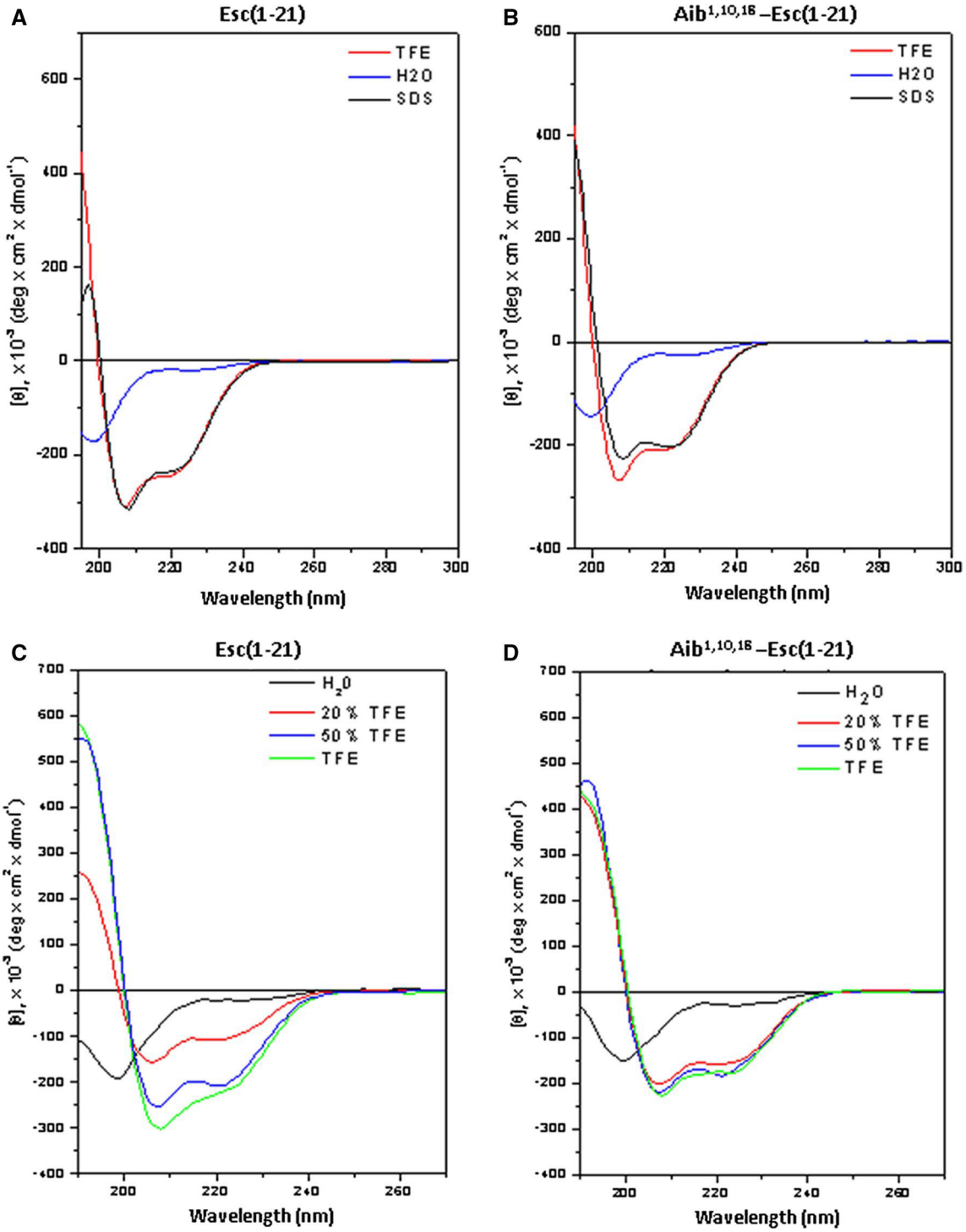

Fig. 3 Far-UV CD spectra of: **a** Esc(1–21) and **b** [Aib[1,10,18]]-Esc(1–21) in three different environments: water, TFE, and 100 mM SDS solution; **c** Esc(1–21) and **d** [Aib[1,10,18]]-Esc(1–21) in water, 20 % TFE, 50 % TFE, and 100 % TFE (peptide concentration 1 mM)

three non-proteinogenic Aib residues at positions 1, 10, and 18 (as shown by CD and NMR studies) is sufficient to provoke a dramatic increase in the peptide's activity against Gram-positive bacteria without significantly increasing its toxicity towards epithelial cells at antimicrobial concentrations. This suggests that the Aib designed analog is a better candidate than the wild-type peptide for the development of a new drug against Gram-positive bacterial infections, such

Journal : Large 726 | Dispatch : 30-9-2016 | Pages : 12
Article No : 2341 | ☐ LE | ☐ TYPESET
MS Code : AMAC-D-16-00328 | ☑ CP | ☑ DISK

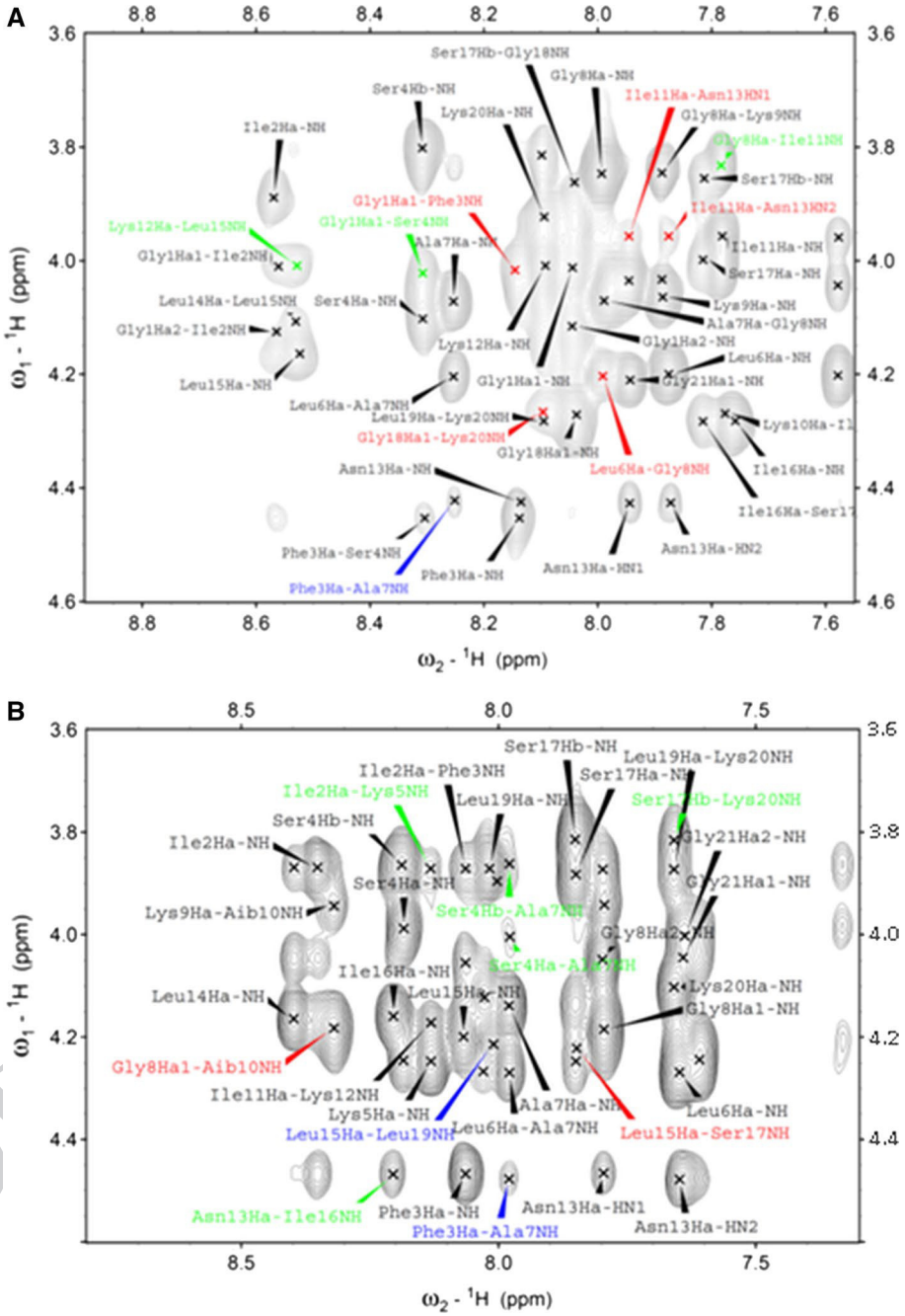

Fig. 4 Fingerprint region of the H/H-NOESY spectrum of Esc(1–21) (**a**) and [Aib[1,10,18]]-Esc(1–21) (**b**) (600 MHz, 1.1 and 1.4 mM, respectively, in TFE-d_2 solution, 298 K). The $C^{\alpha}H_i \rightarrow NH_{i+2}$ (*red*), $C^{\alpha}H_i \rightarrow NH_{i+3}$ (*green*), and $C^{\alpha}H_i \rightarrow NH_{i+4}$ (*blue*) cross peaks, diagnostic of helical conformation, are *highlighted*

as those associated with the human skin or the lung (Lee et al. 2015; Soufi and Soufi 2016). Nevertheless, considering the toxicity of this analog at concentrations higher than the MICs, it would also be useful to develop this peptide for further applications, such as those related to the usage of friendly biocides against Gram-positive bacterial communities on metal surfaces in marine engineering systems, e.g., pipelines of the offshore oil and gas industry, to

Journal : Large 726 | Dispatch : 30-9-2016 | Pages : 12
Article No : 2341 | ☐ LE | ☐ TYPESET
MS Code : AMAC-D-16-00328 | ☑ CP | ☑ DISK

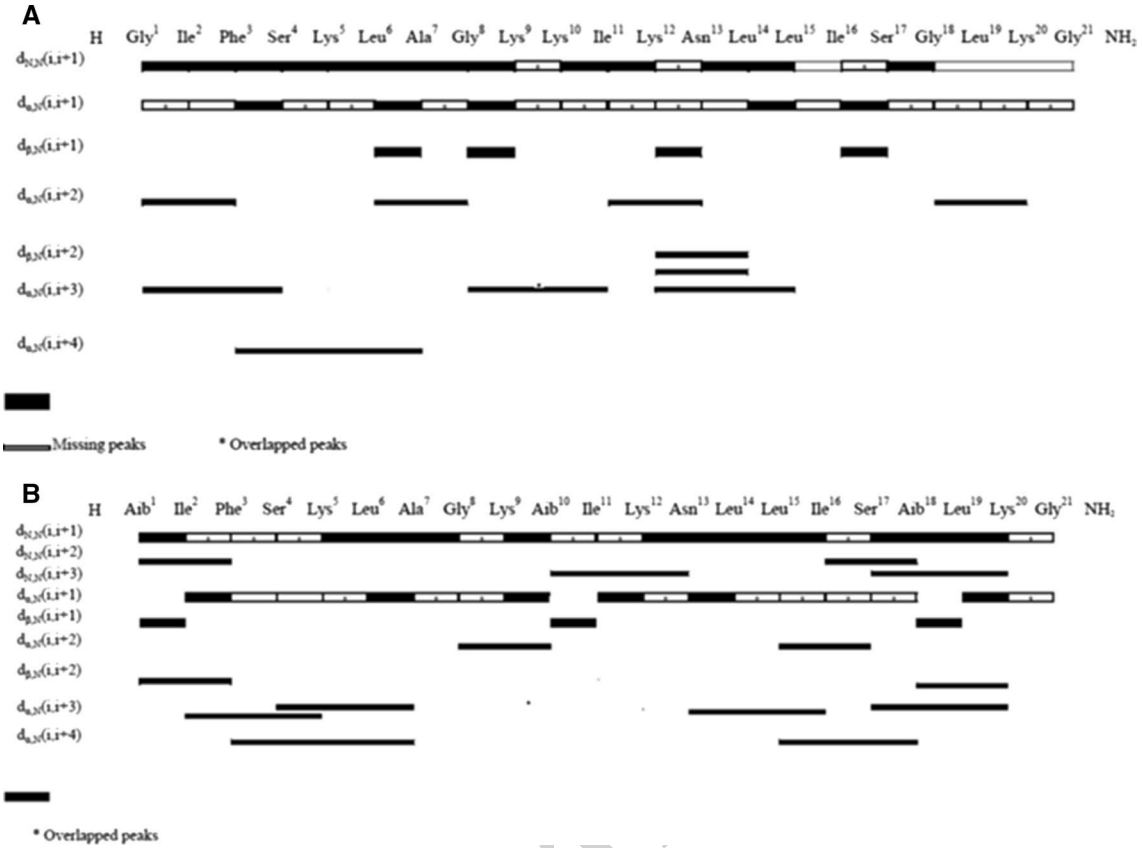

Fig. 5 Summary of the significant interresidue NOESY cross peaks for Esc(1–21) (**a**) and [Aib[1,10,18]]-Esc(1–21) (**b**) in TFE-d_2 solution. Peptide concentration 1.1 and 1.4 mM, respectively

prevent substantial corrosion problems and contamination of agricultural lands (Godwin and Akpan 2014; Schwermer et al. 2008).

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Article No : 2341 | LE | TYPESET
MS Code : AMAC-D-16-00328 | CP | DISK

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Article No : 2341 | ☐ LE | ☐ TYPESET
MS Code : AMAC-D-16-00328 | ☑ CP | ☑ DISK

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