Isolation and structural characterization of epilancin 15X, a novel lantibiotic from a clinical strain of *Staphylococcus epidermidis*

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Received 10 November 2004; revised 14 January 2005; accepted 22 January 2005

Available online 24 February 2005

Edited by Christian Griesinger

Abstract The potential application of lantibiotics as food-preserving agents and more recently as antibiotics has strongly increased the interest in these antibacterial peptides. Here, we report the elucidation of the primary and three-dimensional structures of the novel lantibiotic epilancin 15X from *Staphylococcus epidermidis* using high-resolution nuclear magnetic resonance spectroscopy and tandem mass spectrometry. The molecule contains ten post-translationally modified amino acids, three lanthionine ring structures and a hydroxy-propionyl N-terminal moiety. The primary and tertiary structure and the distribution of positive charges are closely similar to the previously identified lantibiotic epilancin K7, most likely indicative of a common mode of action.

Keywords: Lantibiotics; Nuclear magnetic resonance; Mass spectrometry; Antibacterial peptides; Primary sequence determination; *Staphylococcus epidermidis*

1. Introduction

Lantibiotics is the name for a group of bacterial peptides with antibacterial action, which are ribosomally synthesized as prepeptides and post-translationally modified to contain the dehydrated amino acids α,β-didehydroalanine (Dha) and α,β-didehydrobutyric acid (Dhb) as well as the thioether bridges formed by lanthionine (Lan) and β-methyllanthionine (MeLan) [1–3] (see also Fig. 1). Nisin, a member of the lantibiotic family, has been commonly used as a food preservative (known as E234) for its strong activity against food pathogens [4]. Recently, the application of lantibiotics, as antibiotic reagents, has moved a big step forwards as the structure of nisin in complex with its natural target lipid II revealed a possible common motif that may pave the way to the design of novel antibiotics [5]. The potential application of other lantibiotics as food preservatives or as antibiotics has led so far to the discovery of over 40 different lantibiotics, produced by bacteria isolated from different sources.

Lantibiotics are sub-divided into type A and type B peptides. Type A lantibiotics are flexible, cationic and elongated peptides which exert their function by pore formation in the bacterial membrane, whereas type B are generally more rigid globular molecules that act by disruption of enzyme functions such as peptidoglycan biosynthesis and phospholipase activity [2].

The lantibiotic described in this paper is produced by *Staphylococcus epidermidis* 15X154, a clinical strain isolated from a wound infection. *S. epidermidis* is a Gram positive, katalase positive and coagulase negative coccus and is a colonizer of the human skin. However, it may cause serious infections in immuno-compromised patients and in patients with indwelling devices [6]. Thus far, four lantibiotic peptides produced by *S. epidermidis* strains have been identified, namely pep5 [7], epidermin [8], epicidin 280 [9] and epilancin K7 [10,11], all of which are type A lantibiotics.

Structural characterization of novel lantibiotics remains a challenge, primarily due to the problematic designation of the thioether bridges [3]. Nuclear magnetic resonance (NMR) spectroscopy is often employed to complement DNA sequencing in order to obtain a definitive primary sequence. In this paper, we combined NMR spectroscopy and nano-scale liquid chromatography/tandem mass spectrometry (MS) to determine the primary sequence and molecular structure of the newly isolated active peptide. The peptide was found to highly resemble the previously identified epilancin K7 [10,11] with 68% sequence identity, three nearly identical lanthionine rings and a modified amino acid at the N-terminus. This prompted us to name the new lantibiotic *epilancin 15X*.

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Abbreviations: HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single-quantum correlation; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser enhancement spectroscopy; TOCSY, total correlation spectroscopy; Dha, dehydroalanine; Dhb, dehydrobutyrine; MeLan, 3-methyllanthionine; Lan, lanthionine; Hop, hydroxy-propionyl

0014-5793/$30.00
doi:10.1016/j.febslet.2005.01.083

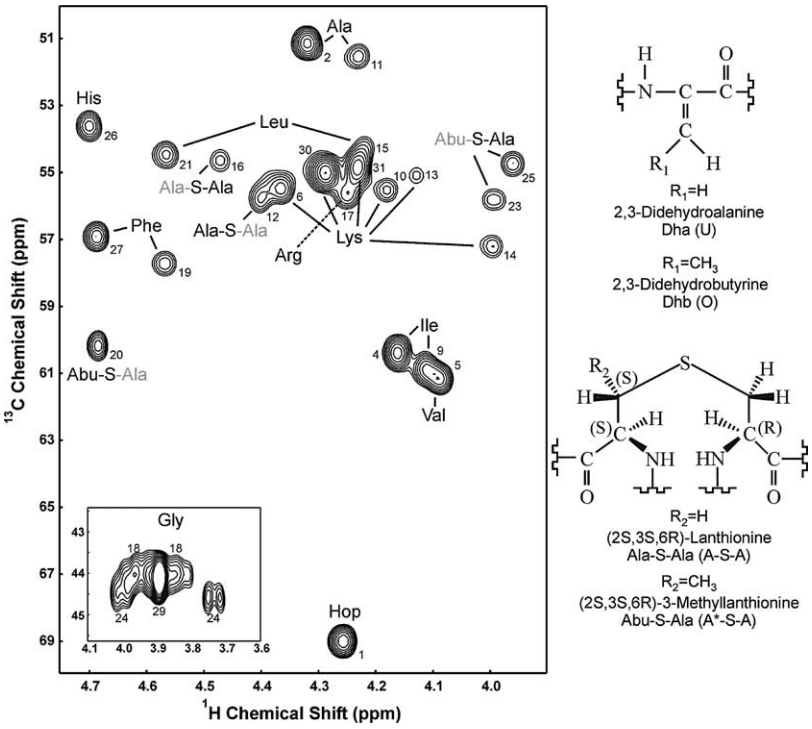

Fig. 1. The ^{1}Hα–^{13}Cα region of a natural abundance ^{1}H–^{13}C-HSQC spectrum of epilancin 15X. Peaks are labeled with the corresponding residue number and identity. The inset shows the glycine region. The structure of the post-translational modified residues is shown in the right. The N-terminus is a Hop group indicated as *Hop*.

2. Materials and methods

2.1. Isolation and purification of the active peptide

1.5 liters of Mueller Hinton broth were inoculated with 100 μl of overnight *S. epidermidis* 15X154 culture and incubated for 16 h at 37 °C on a shaker. After centrifugation, the supernatant was filter sterilized and purified on an Äkta FPLC system (Amersham, Uppsala, Sweden) by cation exchange chromatography (HiTrap SP XL), hydrophobic interaction chromatography (Source 15 PHE) and reverse phase chromatography (Source 15 RPC, Amersham), essentially as described previously [12]. Activity in the FPLC fractions was determined as described previously [13], using *Staphylococcus aureus* strain 29213 as the indicator strain. The purified compound was stored at 4 °C.

2.2. NMR spectroscopy

Two-dimensional homonuclear ^{1}H total correlation spectroscopy (TOCSY) and nuclear Overhauser enhancement spectroscopy (NOESY) spectra were recorded on a Bruker DRX 750 MHz spectrometer at 305 and 283 K with 1.25 mg of epilancin 15X dissolved in 500 μl of 90% H_2O/10% D_2O, 10 mM d3-sodium acetate at pH 4. Mixing times were 100, 200 and 300 ms in the NOESY experiments and 80 ms in the TOCSY experiments. Natural abundance ^{1}H–^{13}C heteronuclear single-quantum correlation (HSQC) and ^{1}H–^{13}C heteronuclear multiple bond correlation (HMBC) NMR spectra were acquired on a Bruker Avance 600 MHz spectrometer equipped with a cryoprobe system at 283 K with 3 mg epilancin 15X in 300 μl D_2O using a Shigemi tube. The ^{1}H and ^{13}C spectral widths were 10 ppm and 150 or 200 ppm, respectively. All spectra were processed using NMRPipe [14] and analyzed using NMRView [15].

The assigned chemical shifts were deposited in BioMagResonBank under Accession No. 6352.

2.3. Structure calculation and analysis

Structure calculations were performed with CNS [16] using the ARIA setup and protocols [17]. Most peaks in the NOESY spectra were unambiguously assigned, except for those that show spectral overlap, which were assigned as ambiguous with a lower weighing factor. The semi-automated NOE assignment with ARIA and additional parameters and topologies introduced to define Dha, Dhb, MeLan and Lan were done as described previously [18]. The topology for hydroxy-propionyl (Hop) was constructed based on alanine with comparison of available databases. Three thioether bridges between residues 12 and 16 (ring A), 20 and 23 (ring B) and 22 and 25 (ring C) were introduced. A simulated annealing protocol was performed using torsion angle dynamics as described before [18]. All 100 structures were subjected to explicit solvent refinement [19] and the 20 lowest-energy structures were kept for structural analysis.

The structural coordinates were deposited in the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB) under Accession Code 1W9N.

2.4. Mass spectrometry

Nano-scale liquid chromatography MS and tandem MS (MS/MS) were performed on a 1100 series liquid chromatography system (Agilent Technologies, Palo Alto, CA, USA) and a Q-Tof Ultima API hybrid quadrupole/time of flight mass spectrometer (Waters corporation, Milford, MA, USA) as described previously [20]. Samples were diluted to 1 fmol/μl in 5% DMSO/5% formic acid and 10 μl were injected onto a C18 trapping column. Analyte separation was performed on a C18 column using a linear gradient from 0% to 60% acetonitril + 0.1 M acetic acid (both columns manufactured by Nano-separations, Nieuwkoop, The Netherlands). Data were analyzed using the MassLynx 3.4 software.

2.5. Peptide digestion

The peptide was hydrolyzed with modified trypsin according to the procedure provided by the supplier (Boehringer Mannheim, Germany). Approximately 100 pmol of peptide was incubated with 20 pmol enzyme in a buffer solution (100 μl) for 4 h at 37 °C. Digestion was terminated by acidification with 1 μl of acetic acid.

3. Results and discussion

3.1. Isolation and purification

Pure epilancin 15X was obtained in a three-step liquid chromatography setup as often described for lantibiotics [9,21]. The yield was typically 0.5 mg peptide/l culture. Antibiotic activity remained stable during the purification process and partly purified peptide solutions could be stored for several weeks at 4 °C without significant loss of activity. Epilancin 15X was found to be active against a range of gram positive micro-organisms, such as *staphylococci*, *enterococci* and *streptococci* (data not shown).

3.2. Spin system analysis & sequence determination by NMR

The number and nature of residues of the peptide was determined using the 2D TOCSY and NOESY spectra recorded at

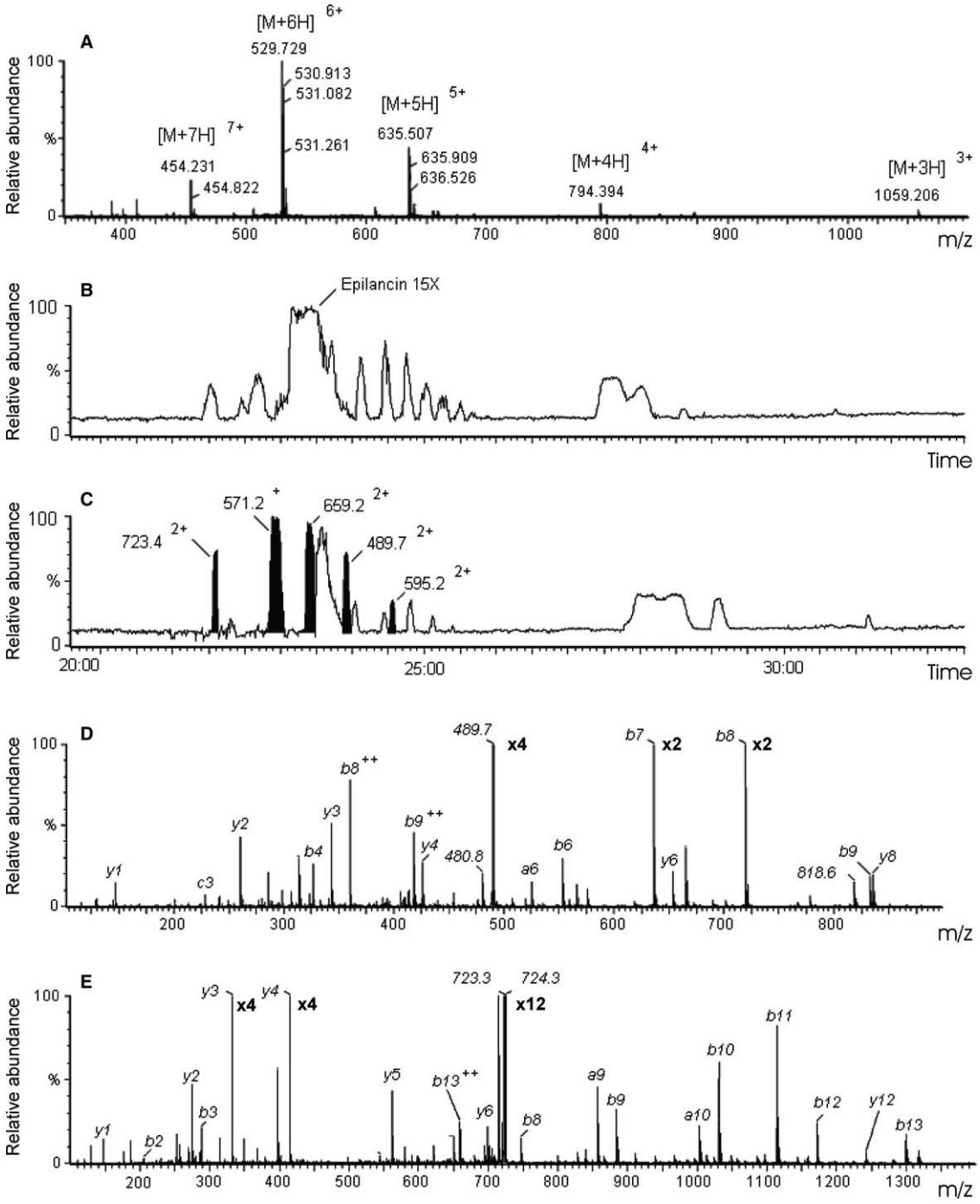

Fig. 2. Analysis of epilancin 15X by MS. (A) Mass spectrum of epilancin 15X. The $[M + 6H]^{6+}$ ion (*m*/*z* ratio 529.729) is most abundant. (B) Chromatogram of epilancin 15X and (C) of the peptide digest fragments. After digestion, five new peaks can be retrieved. (D) MS/MS spectra of fragments 1–10 (489.7^{2+}) and (E) of 18–31 (723.4^{2+}). Fragment ions are marked.

283 K. No signs of spin diffusion were observed in the 300 ms NOESY spectrum so it was used for the structure calculation. For initial identification of each spin system, chemical shifts were compared with random coil values [22]. A similar approach was applied for the modified amino acids by comparison with other lantibiotics [5,10,23,24]. Sequential assignment was achieved using standard NOE connectivity-based protocol [25]. Spectral overlaps were resolved by inspection of either homonuclear NMR spectra at 305 K or by the ^{13}C heteronuclear spectra. All residues could be unambiguously identified and sequentially assigned in this way. The results were then confirmed by the $^{1}H–^{13}C$ HMBC experiment. In addition, the combined use of the $^{1}H–^{13}C$ HSQC and $^{1}H–^{13}C$ HMBC spectra enabled complete ^{13}C chemical shift assignment of the backbone Cα and side chain carbon atoms, and 87% of the carbonyl atoms. Fig. 1 shows the assigned $^{1}H\alpha–^{13}C\alpha$ region of the $^{1}H–^{13}C$ HSQC.

The observation of the Ala2 amide proton (which was first believed to be the N-terminal residue) led to the idea of an additional residue located prior to it. Comparison with Pep5, epicidin 280 and epilancin K7 [26] and close inspection of all NMR spectra in conjunction with the molecular weight obtained from MS (see the following section) allowed us to unambiguously assign the N-terminus to *Hop*. Analysis of the NOE connectivities between the residue pairs of the lanthionine rings allowed for the localization of the thioether bridges.

The combined analysis of all NMR data established the primary structure of the active peptide with a calculated mass of 3173 Da: a total number of 31 residues, including 10 modified amino acids, three lanthionine rings (denoted as A, B and C in Fig. 3) and *Hop* at the N-terminus.

3.3. Analysis by MS

In order to verify the sequence obtained by NMR, mass spectra of digested and undigested peptide samples were analyzed by MS. The mass spectrum of the undigested peptide yielded an envelope of multiple charges ranging from 3 to 7 charges, the $[M + 6H]^{6+}$ ions with a *m/z* of 529.7 being the most abundant (Fig. 2A). A molecular weight of 3172.75 Da was calculated, which is in agreement with the mass determined from the NMR-derived sequence.

In the analysis of the tryptic digest, five major fragments were detected (Fig. 2B and C). These ions were selected for MS/MS analysis (Fig. 2D and E) and were found to correspond to residues 18–31, 1–5, 18–30, 1–10 and 18–29, respectively. No digestion fragment corresponding with residues 11–17 could be identified. In the MS/MS spectrum of the 1–5 digest fragment, a- and b-ions were accompanied by ions at 14 Da lower *m/z* value, which can be explained by the loss of a CH_2 group by the *Hop* moiety. As observed for other lantibiotics [27,28], little cleavage occurred in the part of the molecule containing two MeLan rings (between Abu20 and Ala25). Ion masses of 288.1 and 401.2 were found in the spectra of all three digest fragments 18–29, 18–30 and 18–31 corresponding to the b3 and b4-ions formed by cleavage between Abu20 and Leu21, and between Leu21 and Abu22, respectively. The mass difference of 83 Da between the b2 (205.1) and b3-ion suggests loss of a hydrogen atom and formation of Dhb from Abu after cleavage of the thioether bridge.

The cleavage sites retrieved by MS/MS are indicated in Fig. 3. A total of 21 out of 30 expected cleavage sites (70%) could be confirmed for epilancin 15X.

3.4. The 3D solution structure of epilancin 15X and comparison to epilancin K7

The solution structure of epilancin 15X was determined based on the NOE-derived distance restraints (Fig. 4 and Table 1). Like many type A lantibiotics, it exhibits no conventional secondary structure but well-defined local ring structures.

The temperature dependency of amide proton chemical shifts (temperature coefficient) provides information on the involvement in hydrogen bond formation or sequestering from the solvent and has been used in studies of other lantibiotics [11,29]. The temperature coefficients of individual amide protons were measured (see Fig. 5) and compared with those measured for epilancin K7 [11]. Generally, in the regions of the ring structures, the lower temperature coefficients correlate with the lower surface accessibility of the amide protons that was calculated from the ensemble of 20 structures (data not shown). The sequential profiles of the temperature coefficients of the two homologous lantibiotics are very similar, indicating that epilancin 15X resembles epilancin K7 also in 3D structure. Both molecules are also highly positively charged (about 20% of the total number of amino acids) and the distribution of these charges are almost identical except for the missing charge at the C-terminus in epilancin K7 (see Figs. 3 and 4B). The homology

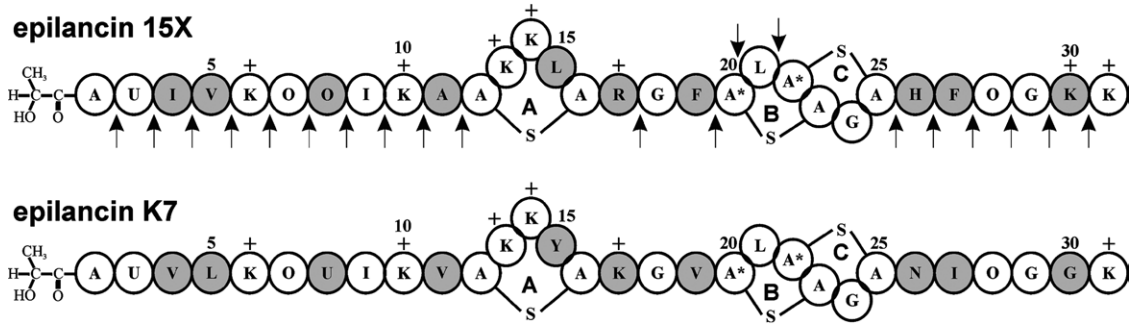

Fig. 3. Primary structure of epilancin 15X compared to that of epilancin K7. Lanthionine rings A, B and C are indicated. The residues that are different between the two lantibiotics are shaded grey. The positively charged residues are indicated by a plus sign. The cleavage sites that were detected by MS are indicated by arrows.

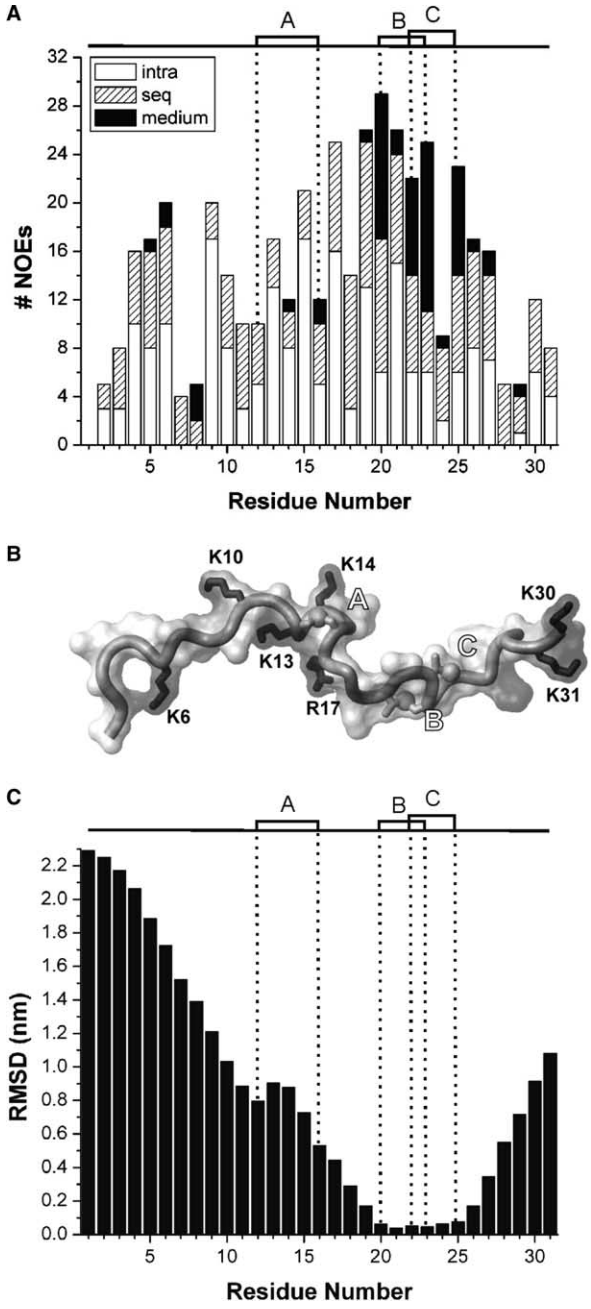

Fig. 4. The three-dimensional solution structure of epilancin 15X. (A) Distribution of NOEs along the sequence divided into intra-residual (white bars), sequential (hashed bars) and medium range NOEs (black bars). Note that most medium-range NOEs were identified for residues in rings B and C. (B) A combined ribbon/surface presentation of the lowest-energy structure. The side chains of the positively charged residues are shown in stick representation with the corresponding residue numbers. The sulfur atoms in the three rings A, B and C are shown in spheres. The figure was generated using MolMol [33]. (C) The main-chain RMS displacements from the mean structure of the ensemble of 20 lowest-energy structures showing that the structure is mostly well-defined for rings B and C, in agreement with the larger number of medium-range NOEs observed in this region (panel A). The three ring-systems A, B and C are indicated at the top of panels (A) and (C).

between epilancin K7 and epilancin 15X suggests that they may, as is the case for nisin A and nisin Z [30,31], be natural variants.

Table 1
Structural statistics for the ensemble of twenty lowest-energy water-refined structures of epilancin 15X

Distance restraints[a]	
Intraresidue ($i - j = 0$)	209
Sequential ($\|i - j\| = 1$)	92
Medium range ($2 \leqslant \|i - j\| \leqslant 4$)	30
All	331
RMSD from idealized covalent geometry	
Bonds (Å)	0.0034 ± 0.00019
Angles (°)	0.58 ± 0.03
Impropers (°)	1.27 ± 0.15
CNS energies (kcal/mol)	
E_{total}	−806 ± 37
E_{vdw}	−170 ± 5
E_{elec}	−886 ± 36
Backbone (N,Cα and C′) pair wise RMSD (Å)[b]	
All residues	7.63 ± 2.14
Residues 20–25 (rings B + C)	0.61 ± 0.34

[a]Distance restraint statistics reported for unique, unambiguous assigned NOEs. No NOE distance restraint was violated by more than 0.3 Å in any of the structures.
[b]Coordinate precision is given as the average pairwise Cartesian coordinate root mean square deviations over the ensemble.

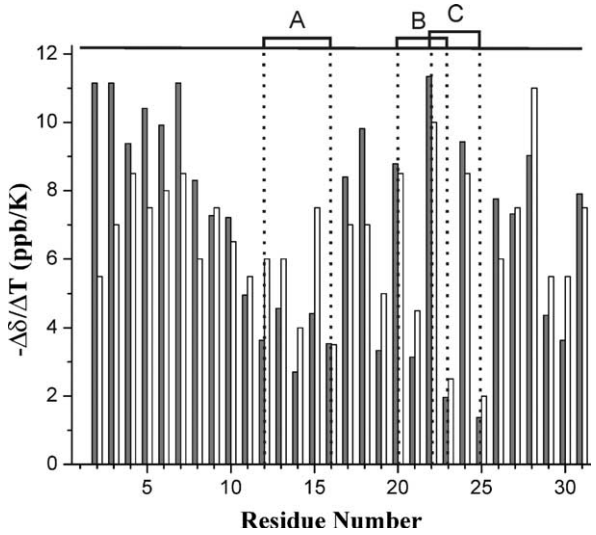

Fig. 5. NMR-derived structural data for epilancin 15X and epilancin K7. The temperature-coefficients ($\Delta\delta/\Delta T$) of epilancin 15X were derived from TOCSY experiments at 283 and 305 K, and are shown by dark-grey bars. The temperature-coefficients of epilancin K7 [11] are shown by white bars. Note the typical up-down pattern for the residues in rings B and C in both peptides. The three ring-systems A, B and C are indicated at the top of the figure.

3.5. Concluding remarks

Because of the presence of post-translationally modified amino acids, a reliable determination of lantibiotic amino acid sequence and secondary structure depends on a combination of techniques, usually DNA sequencing combined with NMR spectroscopy. Initial difficulties with DNA sequencing, which are not uncommon with lantibiotics, prompted us to use MS, which was especially helpful in the identification of the N-terminal *Hop*. The molecular mass as measured by

MS matched the mass derived by NMR. In addition, MS/MS was capable of confirming several (stretches of) amino acid residues. NMR was also successfully applied previously to determine the primary and tertiary structure of the nonadecapeptide cinnamycin, a type B lantibiotic [32].

The high similarity in primary and tertiary structure as well as in the distribution of positive charges, most likely reflects a similar way in which epilancin K7 and the novel lantibiotic epilancin 15X recognize their targets. These general properties might be of great importance as they can provide the basis for future design of antibiotic reagents.

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