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ORIGINAL ARTICLE

Melectin MAPs: the influence of dendrimerization on antimicrobial and hemolytic activity

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Abstract The recently described antimicrobial peptide melectin (MEP, GFLSILKKVLPKVMAHMK-NH_2) exhibits high antimicrobial activity against Gram-positive and Gram-negative bacteria. Here we describe the synthesis and biological activities of 23 new analogues of MEP. We studied the influence of dimerization and tetramerization (MAP-constructs of MEP) on the antimicrobial and hemolytic activities, as well as the role of Met in positions 14 and 17 of the peptide chain. Oxidation of the Met to Met(O) and Met(O_2) decreases antimicrobial activity of all tested bacteria if the peptide is in the monomeric form, however, only to *Staphylococcus aureus* if in the form of dimer or tetramer. Dimerization and tetramerization increase the undesirable hemolytic activity of the peptides. Interestingly, substitution of Leu for Val in position 6 leads to the decrease of hemolytic activity. Introduction of the isosteric amino acid Nle into positions 14 or 17 or both leads to slight increase of hemolytic activity under preservation of high antimicrobial activities. Unfortunately, dimerization again leads to an increase of hemolytic activity.

Keywords Melectin · Antibacterial peptides · Dendrimers · Hemolytic activity · Methionine sulfone · Methionine sulfoxide · Multiple antigen peptides (MAPs)

Standard abbreviations have been used throughout this paper (*J. Pept. Sci.*, 2006; **12**:1–12). Amino acids are of L-configuration unless stated otherwise.

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Introduction

Melectin (MEP), a novel antimicrobial peptide, was isolated as a major component from the venom of the cleptoparasitic bee *Melecta albifrons*. Its primary structure was established as H-Gly-Phe-Leu-Ser-Ile-Leu-Lys-Lys-Val-Leu-Pro-Lys-Val-Met-Ala-His-Met-Lys-NH_2 by Edman degradation and ESI-QTOF mass spectrometry (Čeřovský et al. 2008). The primary structure of MEP is quite unique because it contains Pro in position 11 unlike other AMPs found in hymenoptera venom (Čeřovský et al. 2008). Synthetic MEP exhibits antimicrobial activity against both Gram-positive and Gram-negative bacteria and it degranulates rat peritoneal mast cells. Its hemolytic activity is low. The CD spectra of MEP measured in the presence of trifluoroethanol and sodium dodecyl sulfate showed its ability to adopt an amphipathic α-helical secondary structure.

In comparison with linear peptides, in dendritic constructs (multiple antigenic peptides, MAPs) the multiple peptide copies are in close proximity. This may not only allow multivalent binding but also may generate a higher local concentration of the antimicrobial peptide leading to higher antimicrobial activity and better aqueous solubility (Pini et al. 2005; Tam et al. 2002). Higher stability (including the enzymatic one), increased biocompatibility and lower toxicity represent also advantages of the dendrimeric antimicrobial peptides (Pini et al. 2005; Tam et al. 2002; Bracci et al. 2003). Regarding cytotoxicity (expressed as hemolytic potency), the situation is more complicated. In some cases, the MAPs are more cytotoxic than linear peptides and in other cases not. With some simplification, the toxicity of dendritic structure depends on the polarity and charge of the surface. Positively charged dendrimers are mostly toxic both in vitro and in vivo (Boas et al. 2006; Tomalia et al. 2007). On the other hand, anionic dendrimers and non-charged polar

dendrimers are in most cases not toxic [for more details see (Niederhafner et al. 2008)]. In most cases, the optimal branching related to antimicrobial activity and to molecular size for further development of therapeutics appears to be tetravalency (Pini et al. 2005; Tam et al. 2002; Niederhafner et al. 2005). These data are valid also for other biological activities. There is a very important phenomenon with dendrimers namely the cluster effect (Lunquist and Toone 2002). Increasing of the valency from 1 to 2, 4 or 8 (dendrimerization of linear compounds) may bring about 10- to 1,000-fold increase of the given activity. For example, multivalent glycosides (glycoside dendrimers) bind to their polyvalent protein receptors with affinities much higher than those that can be rationalized solely on the basis of valency (Corbell et al. 2000; Damm and Brewer 2004). In general, we can say with some exaggeration, that in the case of cluster effect, one octavalent dendrimer is a few orders of magnitude more active than eight copies of the linear peptide. The results depend on many factors, dendrimer structure, character of dendrimer surface groups, testing model, etc. In some cases, for the given activity, valency 4 is better than 16; in other cases it can be the opposite (steric reasons) (Lindhorst 2002; Roy and Baek 2002). The optimal valency has, however, to be determined for any dendrimer and desired activity. Another advantage of MAPs over linear peptides with comparable size is that they require much less steps for their chemical synthesis.

With the aim to increase biological activity and to prolong biological half-life of MEP peptides, we decided to synthesize MEP analogues in the MAP format and compare their biological activities with that of original linear ones. We prepared dendrimeric peptides with valency 2 and 4. We also studied the influence of Met^{14} and/or Met^{17} replacement by Met(O), Met(O_2) or norleucine (Nle) in both the linear and dendrimeric forms of MEP. The idea behind the replacement of Met for Met(O) or Met(O_2) was that partial local decrease of cationicity due to polarity of the sulphoxide or sulphone groups (negative character of the oxygen) could lower hemolytic activity. This difference may be multiplied in the case of dimeric and tetrameric peptides by factor 2 or 4, respectively. In addition to determination of biological activity, some of the prepared peptides were also studied using CD spectroscopy and NMR.

Materials and methods

General procedures

Materials

The solvents acetonitrile (ACN), *N*,*N*′-dimethylformamide and TFA were purchased from Fluka (Buchs, Switzerland). Fmoc-protected amino acids (Fmoc-Lys(Boc)-OH, Fmoc-Lys(Fmoc)-OH, Fmoc-His(Trt)-OH, Fmoc-Ser(OBu^t), Fmoc-Cys(Trt)-OH, Fmoc-Met(O)-OH and H-Met(O_2)-OH) were obtained from IRIS Biotech GmBH (Marktredwitz, Germany). Protected amino acid Fmoc-Met(O_2)-OH was prepared from H-Met(O_2)-OH by reaction with Fmoc-ONSu. The support (Fmoc-Rink amide MBHA, 0.45 mmol NH_2/g) was purchased from Merk-Novabiochem (Darmstad, Germany). Tetracycline, *p*-nitrophenyl-*N*-acetyl-*β*-D-glucosaminidine, LB broth and LB agar were from Sigma-Aldrich. All other reagents and peptide synthesis solvents were of the highest purity from commercial sources.

Peptide synthesis

MEP and its analogues were synthesized manually using the solid-phase method in 5 mL polypropylene syringes with a bottom Teflon filter. Synthesis was done using the N^{α}-Fmoc chemistry protocol (Fields and Noble 1990) on a Rink Amide MBHA resin (120 mg) with 0.45 mmol/g substitution. Protected amino acids were coupled in fourfold excess in DMF as solvent and DIPC (7 equiv)/HOBt (5 equiv) as coupling reagents. The peptides which contain Met or Cys were deprotected and cleaved from the resin with a mixture of TFA/H_2O/1,2-ethanedithiol/triisopropylsilane (TIS) (94:2.5:2.5:1) for 3.5 h. Peptides which do not contain Met or Cys were deprotected and cleaved from the resin with a mixture of TFA/H_2O/TIS (95:2.5:2.5) for 3.5 h. The peptides were precipitated with tert-butyl methyl ether. The MEP dimers were synthesized using Fmoc-Lys(Fmoc)OH as a branching unit which was coupled first to the resin. Tetrameric peptides were prepared by oxidation of *C*-terminal extension (-Pro-Cys-Pro-NH_2) of dimeric peptides via S–S bridge. The oxidation was done in aqueous ammonium acetate buffer (pH 7.2) under oxygen atmosphere for 48 h. The oxidation process was monitored by HPLC. The purity of the peptides was followed using analytical HPLC and capillary electrophoresis (CE). This methodology afforded peptides with high purity as illustrated in the case of tetravalent peptides in Fig. 1.

High-performance liquid chromatography

Analytical RP HPLC was performed with a Spectra-Physics 8800 apparatus (Darmstadt, Germany) equipped with a Discovery R, C-18, Supelco (Bellefonte, USA) 150 × 4.6 mm column, particle size 5 μm, flow rate 1 ml/min, detection at 222 nm, using a 5–100% gradient of ACN in 0.05% aqueous TFA for 40 min (gradient A) or 5–50% gradient of ACN in 0.05% aqueous TFA for 60 min (gradient B). The purification of crude peptides was carried out by means of preparative RP HPLC using the

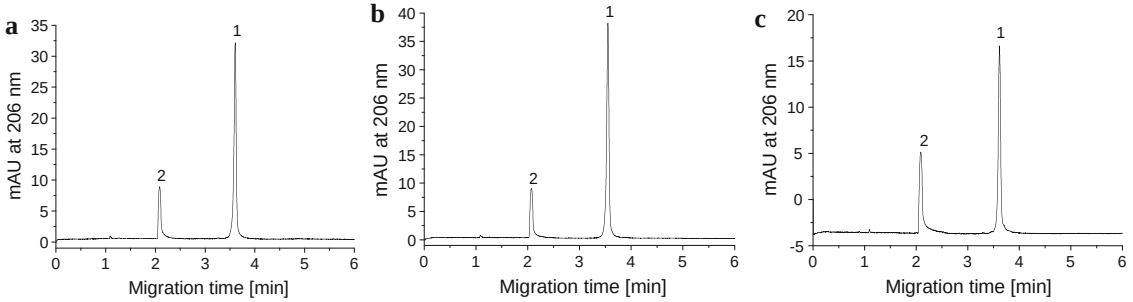

Fig. 1 Capillary electrophoretic analysis of compound **III** (**a**), compound **VI** (**b**) and compound **IX** (**c**), i.e. tetramer of MEP **I**, tetramer of MEP **IV** and tetramer of MEP **VII**, respectively. Peak assignment: *1* **III** (**a**), **VI** (**b**) and **IX** (**c**); *2* DMSO (electroosmotic flow marker)

above-mentioned apparatus and Vydac C-18 (The Separations Group, Hesperia, CA, USA) 250 × 10 mm column, particle size 5 μm, flow rate 3 ml/min, detection at 222 nm, in a 5–50% gradient of ACN in 0.05% aqueous TFA for 60 min.

Capillary electrophoresis

CE analyses of all analogues were performed in a home-made CE analyser (Kašička et al. 1999) equipped with bare fused-silica capillary with outer polyimide coating, 320 mm total length, 210 mm effective length (from injection end to the detector), 50/375 μm inner/outer diameter (Polymicro Technologies, Phoenix, AR, USA) and UV-absorption detector operating at 206 nm. Peptides were dissolved in water in the concentration range 0.5–1.0 mg/ml and were analysed as cations in acidic background electrolyte 30 mM H_3PO_4, 25 mM Tris, 0.1 mM didodecyldimethylamonium bromide, pH 2.8. Nanolitre sample volumes were introduced into the capillary hydrodynamically with pneumatically induced overpressure (500–700 Pa) for 5–10 s. The applied voltage was 8.0 kV (cathode at injection end of the capillary). Analyses were performed at ambient temperature of 22–25°C. The purity degree was quantified by relative peak area, i.e. peak area of the main component was related to the sum of all peaks present on electrophoreogram (Šolínová et al. 2004).

Mass spectrometry

Positive-ion FAB spectra were recorded on a ZAB-EQ mass spectrometer (VG Analytical, Manchester, UK) with an IonTech gun and glycerol or glycerol + thioglycerol matrix. MALDI-TOF spectra were recorded on mass spectrometer Lasermat (Finnigan, USA) with Lasermat 2000 programme. Matrix used were 2,5-dihydroxy benzoic acid or 2-cyano-4-hydroxycinnamic acid. All measurements were done in positive mode.

Antimicrobial activity determination

As test organisms we used *Bacillus subtilis* 168 (*B.s.*), kindly provided by Prof. Yoshikava (Princeton University, Princeton, NJ), *Escherichia coli B* (*E.c.*), from the Czech Collection of Microorganisms (Brno, Czech Republic), *Staphylococcus aureus* (*S.a.*) and *Pseudomonas aeruginosa* (*P.a.*) as a multi resistant clinical isolates. Fresh bacterial cultures were always prepared in the LB broth. The minimal inhibitory concentration (MIC) was established by observing bacteria growth in multi-well plates as a concentration at which there is no growth of bacteria for 20 h (Mendes et al. 2004; Oren and Shai 1997; Lequin et al. 2006). Bacteria in mid-exponential phase were added to individual wells containing solutions of different concentrations of tested peptides (final volume 0.2 mL, final peptide concentration ranged from 0.5 to 100 μM) in the LB broth. These were incubated at 37°C for 20 h while being shaken continuously in a Bioscreen C instrument (Helsinki, Finland). The absorbance was measured at 540 nm every 15 min, and each peptide was tested at least 3 times in duplicates. Routinely, 1.2×10^3–7.5×10^3 colony forming units (CFU) of bacteria per well were used for the activity determination. Tetracycline (0.5–50 μM) was tested as a standard.

Determination of the hemolytic activity

Peptides were incubated with rat red blood cells for 1 h at 37°C (final volume 0.2 mL) in a physiological solution (final erythrocyte concentration 5%, v/v, and final peptide concentration 1–200 μM) (Souza et al. 2005). The samples were then centrifuged for 5 min at 250*g*, and absorbance of the supernatant was determined at 540 nm. Controls for zero hemolysis (blank) and 100% hemolysis consisted of supernatants of red blood cells suspended in physiological solution and 0.2% Triton X-100 in physiological solution, respectively. Each peptide was tested at least in two independent experiments in duplicates.

CD spectra measurement

Far-UV CD spectra were recorded on a Jasco 815 spectropolarimeter (Tokyo, Japan). All peptides were measured in water, in the TFE/water mixture (10, 20, 30, 40 and 50%, v/v), and in the presence of SDS at a concentration interval 0.16 mM to 16 mM, i.e. below and above the critical micelle concentration (CMC). For all of the studied peptides, the concentration was 0.25 mg/ml. Spectra were collected from 190 to 350 nm at room temperature as average of four scans using 0.1 cm path length. A 0.5-nm step resolution, a 50-nm/min speed and 2-s response time and 1-nm bandwith were generally used. The final spectra were expressed as molar elipticity per residue. Assuming the two-state model, the observed mean residue elipticity at 222 nm was converted into a α-helix fraction (f_H) using the method proposed in literature (Rohl and Baldvin 1998; Backlund et al. 1994).

NMR spectroscopy

NMR experiments were carried out using Bruker AVANCE 600 spectrometer with cryo-probe. Protected amino acids and peptides **IV**, **V** and **VII** (5–10 mg of each) were dissolved in the mixture of H_2O/D_2O (9:1); trace amount of dioxane was added as internal reference and pH in the range 3–4 was adjusted using HCl. For each sample, under given conditions, the 1D-spectra and set of homonuclear 2D-NMR spectra (COSY, TOCSY and NOESY) were collected at 27 and 7°C. The spin-lock time of 90 ms was used for TOCSY and a mixing time of 300 ms was used for NOESY spectra. A typical 2D NMR data set consists of 2,048 complex points in t_2 dimension with 512 complex t_1 increments and spectral width 6,600 in both dimensions. After complete set of NMR experiments, the CF_3CD_2OH (TFE) was added to produce 30% TFE solution and the same set of spectra was collected.

Results and discussion

Antimicrobial activity and hemolysis

We have studied the effect of dendrimerization on antimicrobial activities and hemolytic activity of native MEP and its analogues modified in position 14 and 17 (replacement of one or both methionines by methionine sulfoxides or sulfones or by norleucins) and position 6 (replacement of Leu for Val). The formulas of the synthesized peptides are given in Table 1 and the biological activities in Table 2.

In the case of monomers, replacement of Met by Met(O) or Met(O_2) (methionine sulfoxide or sulfone, respectively) led to the decrease of antimicrobial activity against all four tested microorganisms. Also the toxicity to rat red blood cells was decreased. Replacement of methionine by norleucin in one of the two positions (peptides **XX**, **XVI)** or in both positions (**XI)** resulted in analogues having slightly improved antimicrobial activities (up to 3 times) but also led to slight increase of toxicity to rat red blood cells. Dimerization of MEP worsened the antimicrobial activity towards *S. aureus*; potency towards the other three bacteria was more or less the same as that of the monomer. In the case of the dimers having oxidized methionine **(V** and **VIII)**, the activities improved almost to the level of MEP monomer with the exception of the activity towards *S. aureus*, which remained low. However, the toxicity towards rat red blood cells increased with the exception of dimerization of **IV**. Surprisingly, the dimer **V** (both Met replaced by Met(O)) demonstrated high antimicrobial activity and low hemolytic activity (Table 2). All tetravalent MAPs (**III**, **VI** and **IX**) showed very good MIC values with the exception of the antimicrobial activity against *S. aureus* (see Table 2), but unfortunately very high hemolytic activity ($LC_{50} < 6.3$, 14.3, and 9.1 μM, respectively). The improvement of MIC values for all kinds of tested microorganisms was achieved by dimerization of **IV**. Antimicrobial activities of dimer **V** are better towards all four kinds of tested microorganisms: towards *B. subtilis* 5.5 times, towards *P. aeruginosa* 2.6 times and towards *E. coli* even 75 times in comparison with its monomeric form (cluster effect). Similar behaviour was found in the case of dimerization of **VII**. Both the dimer **V** and the tetramer **VI** have better antimicrobial activity than the monomeric form. As the sulfoxide moiety is chiral, the methione sulfoxide (Fmoc-Met(O)-OH) used for the synthesis is a mixture of isomers in the ratio almost 1:1, as showed by NMR. The peptides having one Met(O), two Met(O) or four Met(O) can be resolved into two, four or eight sulfoxide diastereoisomers, respectively (see ^{1}H NMR spectra of **IV**, **V** and **VII**, Fig. 2). Analogue of MEP with methionine sulfone instead of both Met in position 14 and 17 **(VII)** was also dimerized and tetramerized. The resulting peptides **VIII** and **IX** have better MIC values than the linear one, but their hemolytic activity is high (see Table 2). The dimer **VIII** where both Met are replaced by methionine sulfone have slightly higher antimicrobial activity than dimeric **V** where both Met are replaced by methionine sulfoxide, but comparison of hemolytic activities of **VIII** and **V** favours the second one ($LC_{50} = 35$ and >200 μM, respectively).

Our study shows that exchange of Met in native sequence for methionine sulfoxide or methionine sulfone led to the decrease of antimicrobial and hemolytic activities in monomer peptides. The exchange of both methionines in native sequence for norleucines leads to potent

Table 1 Synthetized derivatives of melectin

I	**MEP (melectin)**	G-F-L-S-I-L-K-K-V-L-P-K-V-Met-A-H-Met-K-NH_2
II	**MEP-D**	G-F-L-S-I-L-K-K-V-L-P-K-V-Met-A-H-Met-K-K-NH_2 G-F-L-S-I-L-K-K-V-L-P-K-V-Met-A-H-Met-K/
III	**MEP-T**	G-F-L-S-I-L-K-K-V-L-P-K-V-Met-A-H-Met-K- K-P-C-P-NH_2 G-F-L-S-I-L-K-K-V-L-P-K-V-Met-A-H-Met-K/ | G-F-L-S-I-L-K-K-V-L-P-K-V-Met-A-H-Met-K- K-P-C-P-NH_2 G-F-L-S-I-L-K-K-V-L-P-K-V-Met-A-H-Met-K/
IV	[$Met(O)^{14, 17}$] **MEP**	G-F-L-S-I-L-K-K-V-L-P-K-V-**Met(O)**-A-H-**Met(O)**-K-NH_2
V	[$Met(O)^{14, 17}$] **MEP-D**	G-F-L-S-I-L-K-K-V-L-P-K-V-**Met(O)**-A-H-**Met(O)**-K-K-NH_2 G-F-L-S-I-L-K-K-V-L-P-K-V-**Met(O)**-A-H-**Met(O)**-K/
VI	[$Met(O)^{14, 17}$] **MEP-T**	G-F-L-S-I-L-K-K-V-L-P-K-V-**Met(O)**-A-H-**Met(O)**-K- K-P-C-P-NH_2 G-F-L-S-I-L-K-K-V-L-P-K-V-**Met(O)**-A-H-**Met(O)**-K/ | G-F-L-S-I-L-K-K-V-L-P-K-V-**Met(O)**-A-H-**Met(O)**-K- K-P-C-P-NH_2 G-F-L-S-I-L-K-K-V-L-P-K-V-**Met(O)**-A-H-**Met(O)**-K/
VII	[$Met(O_2)^{14, 17}$] **MEP**	G-F-L-S-I-L-K-K-V-L-P-K-V-**Met(O_2)**-A-H-**Met(O_2)**-K-NH_2
VIII	[$Met(O_2)^{14, 17}$] **MEP-D**	G-F-L-S-I-L-K-K-V-L-P-K-V-**Met(O_2)**-A-H-**Met(O_2)**-K-K-NH_2 G-F-L-S-I-L-K-K-V-L-P-K-V-**Met(O_2)**-A-H-**Met(O_2)**-K/
IX	[$Met(O_2)^{14, 17}$] **MEP-T**	G-F-L-S-I-L-K-K-V-L-P-K-V-**Met(O_2)**-A-H-**Met(O_2)**-K- K-P-C-P-NH_2 G-F-L-S-I-L-K-K-V-L-P-K-V-**Met(O_2)**-A-H-**Met(O_2)**-K/ | G-F-L-S-I-L-K-K-V-L-P-K-V-**Met(O_2)**-A-H-**Met(O_2)**-K- K-P-C-P-NH_2 G-F-L-S-I-L-K-K-V-L-P-K-V-**Met(O_2)**-A-H-**Met(O_2)**-K
X	[Val^6, $Met(O_2)^{14, 17}$] **MEP-D**	G-F-L-S-I-**V**-K-K-V-L-P-K-V-**Met(O_2)**-A-H-**Met(O_2)**-K- K-NH_2 G-F-L-S-I-**V**-K-K-V-L-P-K-V-**Met(O_2)**-A-H-**Met(O_2)**-K/
XI	[$Nle^{14, 17}$] **MEP**	G-F-L-S-I-L-K-K-V-L-P-K-V-**Nle**-A-H-**Nle**-K-NH_2
XII	[$Nle^{14, 17}$] **MEP-D**	G-F-L-S-I-L-K-K-V-L-P-K-V-**Nle**-A-H-**Nle**-K-K-NH_2 G-F-L-S-I-L-K-K-V-L-P-K-V-**Nle**-A-H-**Nle**-K/
XIII	[Val^6, $Nle^{14, 17}$] **MEP**	G-F-L-S-I-**V**-K-K-V-L-P-K-V-**Nle-**A-H-**Nle**-K-NH_2
XIV	[$Met(O)^{14,}$ Nle^{17}] **MEP**	G-F-L-S-I-L-K-K-V-L-P-K-V-**Met(O)**-A-H-**Nle**-K-NH_2
XV	[$Met(O)^{14,}$ Nle^{17}] **MEP-D**	G-F-L-S-I-L-K-K-V-L-P-K-V-**Met(O)**-A-H-**Nle**-K-K-NH_2 G-F-L-S-I-L-K-K-V-L-P-K-V-**Met(O)**-A-H-**Nle**-K/
XVI	[Nle^{17}] **MEP**	G-F-L-S-I-L-K-K-V-L-P-K-V-Met-A-H-**Nle**-K-NH_2
XVII	[Nle^{17}] **MEP-D**	G-F-L-S-I-L-K-K-V-L-P-K-V-Met-A-H-**Nle**-K-K-NH_2 G-F-L-S-I-L-K-K-V-L-P-K-V-Met-A-H-**Nle**-K/
XVIII	[Nle^{14}, $Met(O)^{17}$] **MEP**	G-F-L-S-I-L-K-K-V-L-P-K-V-**Nle**-A-H-**Met(O)** -K-NH_2
XIX	[Nle^{14}, $Met(O)^{17}$] **MEP-D**	G-F-L-S-I-L-K-K-V-L-P-K-V-**Nle**-A-H-**Met(O)**-K-K-NH_2 G-F-L-S-I-L-K-K-V-L-P-K-V-**Nle**-A-H-**Met(O)**-K/
XX	[Nle^{14}] **MEP**	G-F-L-S-I-L-K-K-V-L-P-K-V-**Nle**-A-H-Met -K-NH_2
XXI	[Val^6, Nle^{14}, $Met(O)^{17}$] **MEP**	G-F-L-S-I-**V**-K-K-V-L-P-K-V-**Nle**-A-H-**Met(O)**-K-NH_2
XXII	[Val^6, Nle^{14}] **MEP**	G-F-L-S-I-**V**-K-K-V-L-P-K-V-**Nle**-A-H-Met-K-NH_2
XXIII	[Lys^7(εMEP(1-6)] **MEP**	G-F-L-S-I-L-K-K-V-L-P-K-V-Met-A-H-Met-K-NH_2 G-F-L-S-I-L/
XXIV	[Lys^7(εMEP(1-11)] **MEP**	G-F-L-S-I-L-K-K-V-L-P-K-V-Met-A-H-Met-K-NH_2 G-F-L-S-I-L-K-K-V-L-P/

Table 2 Antimicrobial and hemolytic activity of MEP and its analogues

Peptide	MIC (μM)				LC_{50} (μM)[a]	M calc.	$[M + H]^+$ found	RT (min)	
	B.s.	S.a.	E.c.	P.a.				A[c]	B[d,e]
I	0.83	6.9	1.97	23.7	>100	2,038.23	2,039.2	18.2	44.9
II	0.64	100	1	18.8	16.4	4,187.65	4,188.4	19.4	52.0
III	0.50	>100	0.88	18.8	<6.3	8,967.3 8,973.6[f]	8,973.2[f]	21.0	54.3
IV	4.92	>100	>100	100	>200	2,070.22	1,036.4	14.6	39.9
V	0.89	100	1.33	37.5	>200	4,251.51	4,252.5	15.6	42.8
VI	0.5	>100	1.3	20	14.3	9,095.26 9,101.62[f]	9,102.2[f]	16.6	43.0
VII	1.75	>100	70	>100	>200	2,102.21	2,103.2	14.9	38.1
VIII	0.92	>100	0.92	25	35.0	4,315.51 4,318.43[f]	4,317.9	16.1	42.5
IX	0.83	≫100	0.75	50	9.1	9,223.26 9,229.62[f]	9,230.7[f]	17.8	44.8
X	0.46	≫100	1.17	13.8	>200	4,287.46 4,290.38[f]	4,290.5	14.9	39.0
XI	0.51	2.5	0.88	17.3	50	2,002.32	2,003.2	17.9	45.5
XII	1.27	>100	3.83	80.0	12.7	4,115.71	4,116.3	19.6	52.3
XIII	0.67	8.0	1.5	50	>200	1,988.3	1,989.2	16.8	42.3
XIV	0.69	100	6.3	85	>200	2,036.27	2,037.3	15.4	40.0
XV	0.51	73.3	0.45	43.3	18	4,183.6	4,184.0	16.9	44.6
XVI	1.0	4.0	1.6	30.0	80	2,020.27	2,021.4	17.7	45.0
XVII	0.51	100	0.45	27.5	11	4151.62	4,156.2	19.2	51.2
XVIII	0.75	100	6	>100	>200	2,036.27	2,037.4	17.3	44.2
IXX	0.63	40.0	0.91	33.3	11.8	4,183.6	4,184.1	17.4	49.3
XX	0.37	6	1.5	50.0	56.3	2,020.27	2,021.3	17.3	43.6
XXI	0.80	>100	12.8	100	>200	2,022.25	2,023.0	15.2	37.4
XXII	0.58	7.5	1.17	22.5	>200	2,006.25	2,007.0	16.9	42.2
XXIII	1.42	100	100	100	18.5	2,668.6	2,669.6 2,691.8[b]	19.2	50.5
XXIV	0.62	40	1.0	22	6.8	3,234.0	3,234.8 3,257.0[b]	19.0	49.0
Tetracyclin	8.8	0.4	1.2	75.7	>200	444.4			

MIC minimal inhibitory concentration, *B.s. Bacillus subtilis*, *S.a. Staphylococcus aureus*, *E.c. Escherichia coli*, *P.a. Pseudomonas aeruginosa*

[a] Hemolytic activity, concentration leading to 50% hemolysis

[b] Monoisotopic molecular mass with sodium

[c] *Gradient A*, an elution gradient of solvents from 5 to 100% acetonitrile in water/0.05% TFA was applied for 40 min at a 1 ml/min flow rate, 222 nm; Discovery R, C-18, 150 × 4.6 mm column, particle size 5 μm

[d] *Gradient B*, an elution gradient of solvents from 5 to 5 to 50% acetonitrile in water/0.05% TFA was applied for 0–5–60 min at a 1 ml/min flow rate, 222 nm; Discovery R, C-18, 150 × 4.6 mm column, particle size 5 μm

[e] All analogues synthesized and RP HPLC purified showed purity higher than 95% (analytical HPLC integration) and according to capillary electrophoresis were also more than 95% pure (manuscript in preparation)

[f] Average molecular mass

antimicrobial peptide **XI** having all tested antimicrobial activities better than the native MEP, but its hemolytic activity is increased. The attempt to increase antimicrobial potential of **XI** via synthesis of its dimer was not successful. The dimeric **XII** shows much lower values of antimicrobial activities and higher hemolysis (see Table 2). It is interesting that the exchange of Leu for Val in position 6 in our peptides (**X**, **XIII**, and **XXII**) resulted in minimizing their hemolytic activity without affecting antimicrobial properties (in Table 2 compare antimicrobial

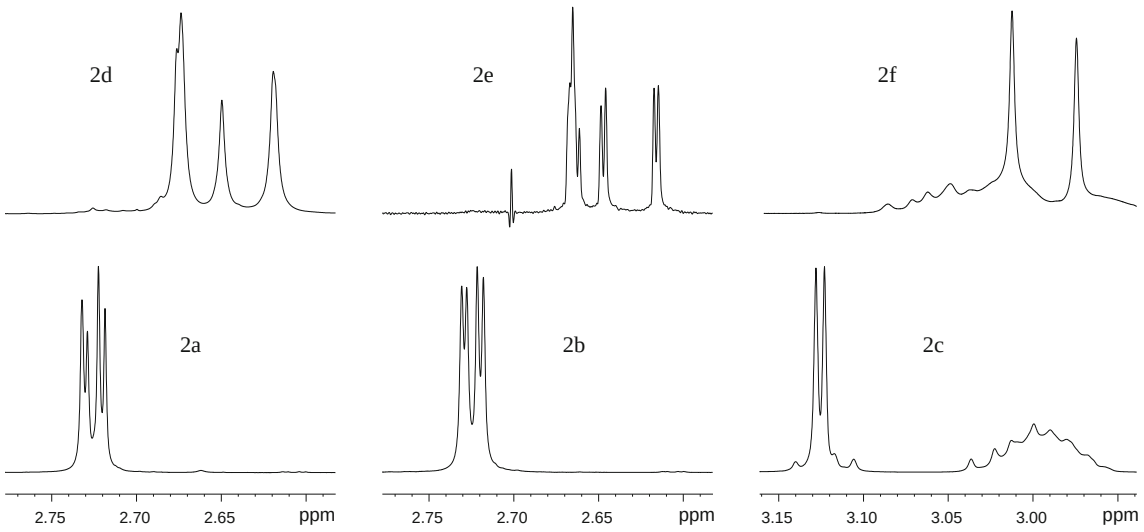

Fig. 2 Singlets of S-CH_3 protons in ^{1}H NMR spectra of studied peptides in water: **a IV**, **b V** (dimer of **IV**), **c VII**, and in 30% TFE: **d IV**, **e V** (dimer of **IV**), **f VII**

properties and hemolytic activities of peptides **X** and **VIII**, **XIII** and **XI** and **XXII** and **XX**). The influence of different branching position of native MEP was also studied. Peptides **XXIII** and **XXIV** with branching in positions 7 or 12, respectively, related to native MEP sequence **I** (see Table 1) show high toxicity to the rat red blood cells (see Table 2).

CD spectroscopy

Biological activities of studied peptides were carefully correlated with their α-helix forming ability expressed by α-helical fraction f_H (Tables 3, 4). First we studied the influence of oxidation of methionine in primary structure of linear peptide. It was shown that these changes have only feeble influence on the secondary structure measured in membrane-mimicking environment (see Table 3). The percentage of helical structure for all measured peptides without addition of TFE or SDS is practically the same. As can be seen, all peptides formed α-helix in the presence of TFE (maximum of the α-helical content for 40%, v/v TFE). The propensity to form α-helix was for most peptides slightly lower than for MEP. The exceptionally high tendency to form α-helix in this medium showed **VII** and **VIII**, two analogues having quite different profile of biological activities. These two peptides also show the highest propensity to form α-helix in SDS at concentrations when micelles are formed. In the presence of SDS below critical micelle concentration (2–4 mM) the formation of the β-structure was observed for the peptides with low hemolytic activity. Surprisingly, the CD did not reveal any substantial changes in structural behaviour of peptides having replaced Leu in position 6 by Val, even if this change is leading to substantial reduction of hemolytic activity which is a measure for toxicity to eukaryotic cells. The folding of studied peptides in various model membranes should be studied and correlation with their biological activities should be performed in more detail to explore the potential to use peptide folding properties as a marker of their biological activity.

Table 3 Content of α-helices as calculated from CD spectra in the presence of SDS or TFE for selected linear peptides and their dimeric and tetrameric forms

	α-helical fraction (f_H)						
	I	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**
SDS conc. (mM)							
0.0	11.8	10.4	9.1	11.5	11.6	12.1	12.14
0.016	18.6	9.0	9.8	11.6	9.4	12.1	22.96
0.16	41.1	10.8	32.5	17.7	12.8	20.4	32.79
2.0	43.0	35.4	34.6	31.7	46.1	38.5	33.70
8.0	41.7	36.5	35.0	32.2	45.2	45.9	35.79
16.0	44.8	37.3	34.4	32.1	47.2	40.1	35.42
TFE conc. (%)							
0	11.8	10.4	9.1	11.5	11.6	12.1	12.1
10	15.8	9.5	9.8	11.5	12.8	12.1	16.8
20	38.4	33.1	35.5	32.3	35.6	20.4	35.6
30	38.7	42.7	42.6	36.0	52.8	38.5	42.5
40	40.7	40.7	43.8	37.1	50.6	51.8	38.6
50	40.1	38.0	39.5	35.2	48.6	45.9	32.3

NMR studies

Using ^{1}H NMR, peptides **IV** and **VII** (sulfoxide and sulfone of MEP, respectively) and the dimer of the sulfoxide

Table 4 Content of α-helices as calculated from CD spectra in the presence of SDS or TFE for native MEP, its linear analogues and the analogues branched in positions 7 and 12

	α-helical fraction (f_H)							
	I	**XIII**	**XVI**	**XIV**	**XXII**	**XXI**	**XXIII**	**XXIV**
SDS conc. (mM)								
0.0	11.8	10.6	11.1	11.1	11.9	11.8	11.8	11.8
0.016	18.6	23.0	11.5	10.8	11.5	11.2	13.6	13.5
0.16	41.1	32.8	12.3	11.3	12.7	11.5	15.1	17.4
2.0	43.0	33.7	39.3	35.0	37.7	34.0	40.7	42.8
4.0	43.6	38.8	36.4	35.6	35.9	33.7	46.0	41.9
8.0	41.7	35.8	37.0	33.2	38.2	33.8	43.9	40.1
16.0	44.8	35.4	37.5	33.6	39.2	33.7	43.9	37.1
TFE conc. (%)								
0.0	11.8	10.6	11.1	11.1	11.9	11.8	11.8	11.8
10.0	15.8	14.6	14.3	12.8	13.3	12.0	17.8	23.0
20.0	38.4	35.1	34.5	32.3	34.5	33.5	37.4	39.5
30.0	38.7	44.4	35.3	36.4	35.8	36.3	37.5	34.4
40.0	40.7	44.0	36.2	33.1	35.5	34.4	39.2	40.5
50.0	40.1	39.3	36.1	34.5	38.5	34.5	37.7	42.3

IV (**V**) were studied in water and in 30% TFE solution. To estimate the effect of oxidation of L-Met to L-Met-sulfoxide and L-Met-sulfone, we checked the isomer composition of the commercial Fmoc-L-Met(O)-OH (used for preparation of methionine sulfoxide analogues). Spectra of commercial Fmoc-L-Met(O)-OH showed fine splitting of most signals in NMR spectra corresponding to almost equivalent diastereomeric mixture (ratio about 54:46). Due to this composition of L-Met(O) residues used for the synthesis, the peptides **IV** and **V** should be in solution a mixture of approximately equally populated diastereoisomers [Met(O) in two positions 14 and 17]. In water, the situation is clearly confirmed by observation of four sharp –SO–CH_3 signals in ^{1}H NMR spectrum (Fig. 2a). In analogy, dimeric dendrimer **V** should contain eight diastereoisomers due to L-Met(O) residues in positions 14 and 17 in two present chains. However, only four sharp –SO–CH_3 peaks are observed (Fig. 2b) at nearly identical positions as in **IV**, obviously due to symmetry equivalence. The presence of two L-Met(O_2) residues in **VII** is manifested by two –SO_2–CH_3 peaks shifted downfield to 3.128 and 3.123 ppm (Fig. 2c). In 30% TFE solution, the two L-Met(O) residues in **IV** give also four –SO–CH_3 signals (Fig. 2d) and two L-Met(O_2) residues in **VII** give two –SO_2–CH_3 signals (Fig. 2f) similarly as in water; the pattern of –SO–CH_3 signals in **V** is more complex (Fig. 2e). In dimer **V**, at least six peaks are resolved from eight theoretically expected. It means that symmetry equivalence observed in water is lost, obviously due to formation of helical conformation of both chains (see below).

Similarly to water, the presence of diastereomer mixtures in **IV** and **V** leads to observation of very small differences in chemical shift of NH and αH protons that appear as broadening and/or fine splitting of these signals for residues 12–18 [in the neighborhood of L-Met(O)] while those more distant (residues 1–11) are not influenced.

The negative CSI values larger than −0.1 ppm indicate significant population of helical conformation of a fragment from Phe-2 until Lys-18 with some flexibility of the *C*-end (less negative $\Delta\delta\alpha$H values for residues 17 and 18). The only slightly positive $\Delta\delta\alpha$H value for Lys-8 in the middle of helical peptide chain is probably caused by proline in position 11 leading to the absence of H-bonding to C=O of Lys-7. Very small differences of NMR parameters between compared peptides are caused by the presence of slightly structurally different residues in positions 14 and 17 and not by the different conformation.

Conclusion

The results clearly confirmed that the effect of dendrimerization depends on the structure of the linear peptide unit. While in the case of methionine or oxidized methionine-containing dimers and tetramers the antimicrobial potency was the same or higher than that of the monomer (with the exception to the potency towards *S.a.*), their undesired hemolytic activity increased. The activity of Nle-containing peptides was dependent on the fact whether the Nle was substituted in position 14, 17, or both. Dimerization of the analogue **XVI** having Nle in position 17 led to decrease of antimicrobial activity only to *S.a.*, but very high hemolytic activity. Dimerization of the analogue **XI** having Nle in both positions led to decrease of antimicrobial activity to all four bacteria tested as well as to increase of the hemolytic activity. The Nle ^{14,17} analogue **XI** has the best antibacterial qualities; however, it also has high hemolysis. Substantial reduction of the hemolytic activity was reached if in the Nle-containing monomers **XI** and **XX** and dimer **VIII** containing four Met(O_2) the Leu in position 6 was replaced by Val (see Table 2 analogues **XIII**, **XXII**, and **X**, respectively).

The relationship between the biological data and structural analyses using CD and NMR is not clear. We were not able to find any unambiguous dependence between the physicochemical data and biological activities. Moreover, the study of the oxidized methionine-containing analogues is complicated by the diastereoisomerism of the starting amino acid which leads to complex mixture of diastereoisomers in the final products as confirmed using NMR. More detailed study should be carried out before we could make any correlation.

Finally, we may state that the three analogues **XIII**, **XXII**, and **X** are highly active and may be considered a lead for the synthesis of stable and highly active antimicrobial peptides.

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