

Effect of hydrocarbon stapling on the properties of α -helical antimicrobial peptides isolated from the venom of hymenoptera

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Abstract The impact of inserting hydrocarbon staples into short α -helical antimicrobial peptides lasioglossin III and melectin (antimicrobial peptides of wild bee venom) on their biological and biophysical properties has been examined. The stapling was achieved by ring-closing olefin metathesis, either between two *S*-2-(4'-pentenyl) alanine residues (*S*₅) incorporated at *i* and *i* + 4 positions or between *R*-2-(7'-octenyl) alanine (*R*₈) and *S*₅ incorporated at the *i* and *i* + 7 positions, respectively. We prepared several lasioglossin III and melectin analogs with a single staple inserted into different positions within the peptide chains as well as analogs with double staples. The stapled peptides exhibited a remarkable increase in hemolytic activity, while their antimicrobial activities decreased. Some single stapled peptides showed a higher resistance against proteolytic degradation than native ones, while the double stapled analogs were substantially more resistant. The CD spectra of the singly stapled peptides measured in water showed only a slightly better propensity to form α -helical structure when compared to native peptides, whereas the doubly stapled analogs exhibited dramatically enhanced α -helicity.

Keywords Antimicrobial peptides · Stapled peptides · Analogs · Amphipathic helix · CD spectroscopy

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Introduction

Antimicrobial peptides (AMPs) that have been identified in almost all forms of life represent the first line of defense against infection. They are active against both Gram-positive and -negative bacteria, mycobacteria, protozoa, fungi, and even against some cancer cells. As they kill microbes by a fundamentally different mechanism of action that does not induce bacterial resistance, they are considered as a promising supplement to, or substitute for, conventional antibiotics (Baltzer and Brown 2011; Giuliani et al. 2007; Oyston et al. 2009; Yeung et al. 2011; Zaiou 2007). Many of them kill bacteria by permeabilization of the cytoplasmic membrane that leads to the leakage of cytoplasmic components and cell death, whereas others target putative key intracellular compartments. The large and well-studied group of AMPs is represented by cationic linear peptides whose biological activity is linked to their propensity to adopt an amphipathic α -helical structure within the environment of a target bacterial cell membrane, or in a membrane-mimicking environment. In their secondary structure, amino acid residues are segregated into hydrophobic and cationic faces, the feature prerequisite for their biological activity. It is generally considered that the cationic face of the α -helical peptide interacts with the anionic surface of a bacterial cell membrane. The peptide then infiltrates the lipid bilayer of the membrane where the hydrophobic face is partitioned in the hydrophobic environment of the membrane lipid, and leads to the disruption of membrane structure via different mechanisms (Amiche and Galanth 2011; Epand and Epand 2011; Huang et al. 2010; Toke 2005; Tossi et al. 2000; Wimley and Hristova 2011; Yeaman and Yount 2003).

Despite the pharmaceutical potential of AMPs and efforts to develop them commercially, none of these

peptides are yet available on the drug market. The reasons for this include high production costs, possible immunogenicity or toxicity, and the susceptibility to rapid *in vivo* degradation that dramatically reduces their bioavailability. The most common strategies to circumvent the limited bioavailability include the design of various non-natural mimics of AMPs (Godballe et al. 2011), the substitution of natural L-amino acids with D-amino acid residues (Hong et al. 1999; Lee and Lee 2008; Pag et al. 2004) or, head to tail cyclization (Monroc et al. 2006).

Another promising possibility for improving the pharmacological properties of AMPs may utilize the concept of reinforcement of their α -helical conformation using the methodology of hydrocarbon stapling (Blackwell and Grubbs 1998; Blackwell et al. 2001; Schafmeister et al. 2000). Several recent reports have shown that incorporation of a hydrocarbon staple into pharmaceutically interesting peptides increases their α -helicity. This leads to an improvement of their pharmacological properties such as cell permeability, receptor affinity and proteolytic stability (Bird et al. 2010; Estieu-Gionnet and Guichard 2011). Usually the staple is introduced into a peptide by the incorporation of two non-natural amino acid residues with olefinic side chain at positions separated by one (*i* and *i* + 4) or two (*i* and *i* + 7) helical turns (Kim and Verdine 2009; Schafmeister et al. 2000). This is followed by ruthenium-catalyzed ring-closing metathesis (RCM) as shown schematically in Fig. 1. Up to date, such an approach has been used often for peptides that can access targets engaged in intra-cellular protein–protein interactions (Bernal et al. 2007; Blattacharia et al. 2008; Henchey et al. 2008; Kutchukian et al. 2009; Moellering et al. 2009; Stewart et al. 2010; Walensky et al. 2004; Wilder et al. 2007). In recent paper, stapling of an amphipathic peptide derived from apolipoprotein, playing the role in cholesterol efflux process, improved its biological properties (Sviridov et al. 2011).

To the best of our knowledge, the peptide stapling technique has been applied in the field of AMPs only once when intramolecular disulfide bridge of brevinin-1BYa was replaced by dicarba bond (Hossain et al. 2011). In this study, we investigated the effect of hydrocarbon stapling on the antimicrobial and hemolytic activities, proteolytic stability and the propensity to form helical structure of two short AMPs, lasioglossin III (LL-III) and melectin (MEP) formerly isolated in our laboratory from the venom of wild bees (Čeřovský et al. 2008, 2009). This study was done in an effort to elucidate whether or not the method of hydrocarbon stapling may improve the pharmacological properties of these AMPs.

Materials and methods

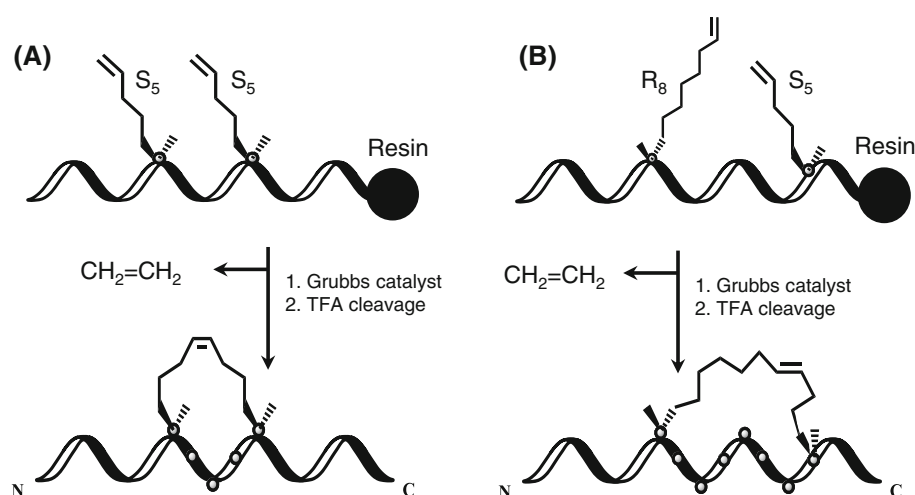
Materials

Fmoc-(*S*)-2-(4'-pentenyl)alanine and Fmoc-(*R*)-2-(7'-octenyl)alanine were purchased from AAPPTec LLC, Louisville, KY, USA. Grubbs 1st generation catalyst bis(tricyclohexylphosphine)benzylidene ruthenium(IV) dichloride, 1,2-dichloroethane, trypsin (DPCC treated, Type XI: from bovine pancreas) and LB-broth were supplied by Sigma-Aldrich. Other Fmoc-protected L-amino acids and Rink Amide MBHA resin were purchased from IRIS Biotech GmbH, Marktredwitz, Germany. All other reagents, peptide synthesis solvents, and HPLC-grade acetonitrile were of the highest purity available from commercial sources.

Peptide synthesis

The LL-III and MEP analogs shown in Tables 1 and 2 were prepared by the method of Fmoc solid-phase peptide synthesis protocol, i.e., in 5 mL polypropylene syringes with a Teflon filter at the bottom on a Rink Amide MBHA resin

Fig. 1 Schematic illustration of ring-closing olefin metathesis reactions between two *S*-2-(4'-pentenyl)alanine residues (S_5) incorporated at *i* and *i* + 4 positions (a), and between *R*-2-(7'-octenyl) alanine (R_8) and S_5 incorporated at *i* and *i* + 7 positions, respectively, (b). In the latter case stapled peptides are produced as *cis* and *trans* double bond isomers (not shown). The reactions were carried out on protected peptides bound to the Rink Amide resin



(110 mg, 0.7 mmol/g substitution). Standard Fmoc amino acids (4 eq, 0.28 mmol) were coupled using *N,N'*-diisopropylcarbodiimide (DIPC, 7 eq, 0.49 mmol) and 1-hydroxybenzotriazole (HOBt, 5 eq, 0.35 mmol) as coupling reagents in *N,N'*-dimethylformamide (DMF, 0.6 mL) as a solvent used in the non-destructive monitoring of free amino groups conversion with 1 % bromophenol blue indicator in DMF (1 μ L). The Fmoc olefinic amino acids (3 eq, 0.21 mmol) were coupled similarly using the same coupling reagents (DIPC 0.37 mmol/HOBt 0.26 mmol) but at an extended reaction time. When the olefinic amino acid was coupled to the proline as a foregoing residue, it was re-coupled with HATU reagent. After the coupling of the Boc-protected N-terminal amino acid, the resin-bound peptide was washed properly, dried and removed from the syringe. An aliquot amount of the unstapled peptide was de-protected and cleaved from the resin (to get the sample of unstapled peptide), while most of the material was used for the synthesis of stapled peptides by RCM.

Ring-closing metathesis

Typically, 100 mg of resin-bound peptide was added to the catalyst suspension (20 mg, 0.024 mmol) in 1,2-dichloroethane (2 mL), and then stirred at room temperature for 2 h

under argon. The resin bound peptide was washed on a glass filter and the reaction repeated once again. The stapled peptides were de-protected and cleaved from the resin (in the syringe) with a mixture of trifluoroacetic acid (TFA)/triisopropylsilane/H₂O (95:2.5:2.5) for 3.5 h and then precipitated with diethyl ether. The crude peptides were further purified by preparative RP-HPLC. The dominant fraction containing the required stapled peptide was lyophilized, and its purity and identity were checked by analytical HPLC and electrospray ionization mass spectrometry (Tables 1, 2); see the Supplementary Material for the mass spectra.

Mass spectrometry

Mass spectra of the peptides were acquired on a Micromass Q-ToF micro mass spectrometer (Waters) equipped with an electrospray ion source. A mixture of acetonitrile/water 1:1 with 0.1 % formic acid as a mobile phase was continuously delivered to the ion source at a 20 μ L/min flow rate. Samples dissolved in the 20 μ L of the mobile phase were introduced using a 2 μ L loop. The measurement was performed in the positive mode and the capillary voltage, cone voltage, desolvation temperature and ion source temperature were 3.5 kV, 20 V, 150 °C and 90 °C, respectively.

Table 1 Primary structures, RP-HPLC retention times (t_R) and MS data for lasioglossin (LL-III) and its stapled analogs

Peptide	Sequence	t_R (min)	Molecular mass [Da]	
			calcd	found
LL-III	VNWKKILGKIIKVVK-NH ₂	31.93	1764.19	1763.9
LL-IIIs-1	VNWKK ^{S₅} LGK ^{S₅} IKVVK-NH ₂	33.63	1788.19	1788.3
LL-IIIs-2	VNWKKILGK ^{S₅} IKV ^{S₅} K-NH ₂	35.89	1802.20	1802.0
LL-IIIs-3	V ^{S₅} WKKS ^{S₅} LGKIIKVVK-NH ₂	36.44	1787.23	1787.5
LL-IIIs-4	VNS ^{S₅} KKIS ^{S₅} GK ^{S₅} IKV ^{S₅} K-NH ₂	36.55	1753.21	1753.0
LL-IIIs-5 <i>cis</i>	VNR ^{S₅} KKILGK ^{S₅} IKVVK-NH ₂	33.98	1757.24	1756.8
LL-IIIs-5 <i>trans</i>	VNR ^{S₅} KKILGK ^{S₅} IKVVK-NH ₂	35.01	1757.24	1756.8
LL-IIIs-6a ^{a)}	VNS ^{S₅} KKIS ^{S₅} PK ^{S₅} IKV ^{S₅} K-NH ₂	33.13	1793.24	1793.2
LL-IIIs-6b ^{a)}	VNS ^{S₅} KKIS ^{S₅} PK ^{S₅} IKV ^{S₅} K-NH ₂	34.01	1793.24	1793.2

^{a)} stapling resulted in two products, a and b, probably due to the *cis/trans* isomerisation around the S₅-Pro8 peptide bond

Table 2 Primary structures, RP-HPLC retention times (t_R) and MS data for melectin (MEP), [Nle^{14,17}]-melectin (MEP-N) and its stapled analogs

Peptide	Sequence	t_R (min)	Molecular mass [Da]	
			calcd	found
MEP	GFLSILKKVLPKVMAHMK-NH ₂	34.33	2038.23	2039.2
MEP-N ^{a)}	GFLSILKKVLPKVXAHXK-NH ₂	37.12	2002.32	2002.2
MEP-Ns-1	GFLSILKKVLPKS ₅ XAH ₅ S ₅ K-NH ₂	37.20	2040.33	2040.4
MEP-Ns-2	GFLSS ₅ LKK ₅ LPKVXAHXK-NH ₂	38.35	2040.33	2040.3
MEP-Ns-3	GFLSS ₅ LKK ₅ LPKS ₅ XAH ₅ S ₅ K-NH ₂	39.11	2078.35	2078.2
MEP-Ns-4 <i>cis</i>	GFR ₈ SILKKV ₅ PKVXAHXK-NH ₂	39.98	2068.36	2068.4
MEP-Ns-4 <i>trans</i>	GFR ₈ SILKKV ₅ PKVXAHXK-NH ₂	40.77	2068.36	2068.4
MEP-Ns-5	GFLSS ₅ LKK ₅ LGKS ₅ XAH ₅ S ₅ K-NH ₂	37.83	2038.32	2038.8
MEP-Ns-5x	GFLSS ₅ LKK ₅ LGKS ₅ XAH ₅ S ₅ K-NH ₂	39.14	2066.35	2067.1
MEP-Ns-6	GFLSS ₅ LKK ₅ LAKS ₅ XAH ₅ S ₅ K-NH ₂	37.88	2052.33	2053.1
MEP-Ns-6x	GFLSS ₅ LKK ₅ LAKS ₅ XAH ₅ S ₅ K-NH ₂	39.18	2080.36	2081.3

^{a)} [Nle^{14,17}]-melectin (MEP-N), analog of MEP where Met residues were replaced by Nle (X)

¹H NMR spectroscopy

Proton NMR spectra were measured using a Bruker AVANCE II 600 NMR instrument (at 600.13 MHz frequency) equipped with a triple-resonance cryo-probe (5 mm CPTCI ¹H-¹³C/¹⁵N/D Z-GRD) in D₂O at a temperature of 300 K. Chemical shifts are referenced to dioxane (added in a trace amount as an internal standard), and recalculated using the relation $\delta(\text{dioxane}) = 3.75$ ppm. The strong residual water peak was suppressed by pre-saturation (by a 2 s irradiation before data acquisition).

Determination of antimicrobial and hemolytic activity

Minimum inhibitory concentrations (MICs) were established by observing bacterial growth in multi-well plates (Čeřovský et al. 2008, 2009; Monincová et al. 2010). Midexponential phase bacteria were added to individual wells containing solutions of the peptides at different concentrations in LB broth (0.5 % NaCl) in final volume of 0.2 mL and final peptide concentration in the range of 0.5 to 100 μ M. The plates were incubated at 37 °C for 20 h

while being continuously shaken in a Bioscreen C instrument (Oy Growth Curves AB Ltd., Helsinki, Finland). The absorbance was measured at 540 nm every 15 min and each peptide was tested at least 3 times in duplicate. Routinely, 1×10^4 to 5×10^4 CFU of bacteria per well were used to determine activity. Tetracycline in the 0.5–50 μ M concentration range was tested as a standard. As test organisms we used *Bacillus subtilis* (B.s.) 168, kindly provided by Prof. Yoshikawa (Princeton University, Princeton, NJ, USA). *Escherichia coli* (E.c.) B and *Micrococcus luteus* (M.l.) No. CCM 144 were from the Czech Collection of Microorganisms, Brno, Czech Republic; *Staphylococcus aureus* (S.a.) and *Pseudomonas aeruginosa* (P.a.) were obtained as multi-resistant clinical isolates; *Candida albicans* (C.a.) (F7-39/IDE99) came from the mycological collection of the Faculty of Medicine, Palacky University, Olomouc, Czech Republic.

Hemolytic activity was expressed as a concentration of a peptide able to lyse 50 % of human erythrocytes in the assay (LC₅₀ values) as described (Monincová et al. 2010). Briefly, the peptides were incubated with red blood cells of healthy volunteers for 1 h at 37 °C in a physiological

solution at a final volume of 0.2 mL (final erythrocyte concentration 5 % (v/v) and final peptide concentration 1–200 μ M). The samples were then centrifuged for 5 min at 250g, and the supernatant absorbance determined at 540 nm. Supernatants of red blood cells suspended in physiological solution and 0.2 % Triton \times 100 in physiological solution served as controls for zero and 100 % hemolysis, respectively. Each peptide was tested in duplicate in at least two independent experiments.

Circular dichroism

Circular dichroism (CD) experiments were carried out using a Jasco 815 spectrometer (Tokyo, Japan). The final peptide concentration was kept constant (0.25 mg/mL) for all peptides studied. The spectra were collected from 190 to 300 nm using a 0.1 cm quartz cell at room temperature. We used 0.5 nm step resolution, 20 nm/min scanning speed, 8 s response time and 1 nm spectral band width. All peptide samples were measured in water and in water/trifluoroethanol (TFE) mixtures (from 10 to 50 % v/v of TFE), and in the presence of SDS at concentrations of 0.016–8 mM (below and above the critical micelles concentration). After baseline correction, the final spectra were expressed as a molar ellipticity θ (deg cm² dmol^{−1}) per residue. The α -helix fraction (f_H) was calculated by assuming a two state model (Backlund et al. 1994; Rohl and Baldwin 1998; Whitmore and Wallace 2008).

Digestion of peptides with trypsin

After adding 5 μ L of trypsin stock solution (1 mg/mL) in water to the peptide solution (0.2 mg/0.1 mL) in 50 mM ammonium bicarbonate, the mixture was incubated at 37 °C. At given times, aliquots (5 μ L) were taken and quenched by a stop solution of 50 % aqueous acetonitrile containing 1 % TFA (5 μ L). These were then analyzed by HPLC using detection at 220 nm. The peptide degradation at each time was calculated in terms of percent of peptide peak area at time zero (100 %).

Results and discussion

Design of analogs

As already reported, native lasioglossin III (LL-III) as well as melectin (MEP) exhibit potent antimicrobial activity against both Gram-positive and -negative bacteria and yeasts, low hemolytic activity and potency to kill various cancer cells in vitro (Čerovský et al. 2008, 2009; Slaninová et al. 2011, 2012). They belong to the category of linear amphipathic α -helical peptides that are highly cationic, due to the presence

of several lysine residues and the absence of acidic residues. Their cationicity in combination with a high number of hydrophobic amino acid residues contribute to their antimicrobial properties. To keep the net positive charge unaffected, we designed such stapled analogs of LL-III and [Nle^{14,17}]-MEP (MEP-N) that have olefinic amino acid residues incorporated into the hydrophobic face of the amphipathic α -helix (Fig. 2), so that the presence of any staple in the sequence does not affect the number of Lys residues or hinder the presumed electrostatic interaction of the cationic face of the α -helix with the negatively charged surface of the bacterial cell membrane. Figure 2a and b shows the sequences of LL-III and MEP-N in a α -helical wheel projection, with different positions of the staples related to all the analogs used in this study. Due to the hydrophobic hydrocarbon staples that cross-linked the hydrophobic region (Fig. 2) of amphipathic α -helices, the hydrophobicities and hydrophobic moments of all stapled analogs increased. That increase is reflected in the increment of their RP-HPLC retention times with respect to the native LL-III and MEP (MEP-N) (Tables 1, 2).

Synthesis of stapled peptides

The results of earlier studies (Schafmeister et al. 2000; Kim and Verdine 2009) show that the conversion of the RCM

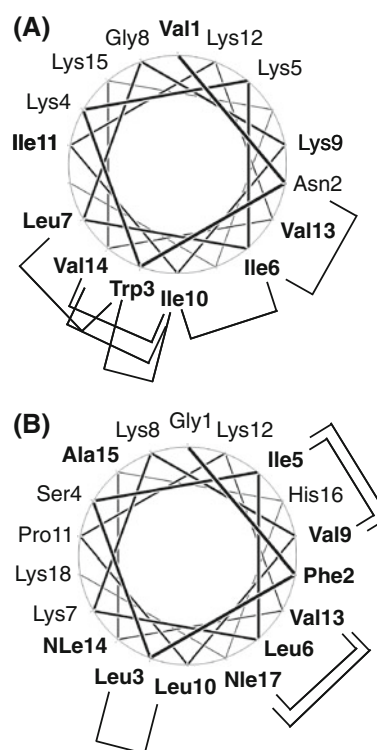


Fig. 2 Wheel diagram of LL-III (a) and MEP-N (b) and their stapled analogs. The hydrophobic amino acid sector is shown in **bold letters**. Different positions of the staples correspond to the analogs shown in Tables 1 and 2

reaction strongly depends on the ring size in the macrocyclic crosslink and on the configuration of olefinic amino acid residues and their position in the peptide chain. The highest conversions were generally achieved in those cases forming $S_{i,i+4}S(8)$ and $R_{i,i+7}S(11)$ staples. That is, peptides with two olefinic amino acid residues in the *S* configuration and 8 carbons in the metathesized crosslink, and peptides with olefinic amino acid residues in the *R* and *S* configuration and 11 carbons in the crosslink, respectively (Schafmeister et al. 2000). We therefore designed several analogs of LL-III and [Nle^{14,17}]MEP (MEP-N) based on these ring patterns utilizing 2-(4'-pentenyl) alanine in the *S* configuration (S_5), and 2-(7'-octenyl) alanine in the *R* configuration (R_8) as olefinic amino acid residues incorporated in the appropriate positions within the peptide sequences. The [Nle^{14,17}]MEP (MEP-N) analog of melectin, in which sulfur-containing methionines at positions 14 and 17 are replaced by norleucine, was chosen for the study of MEP peptides in order to avoid possible poisoning of the ruthenium containing catalyst during the RCM reaction.

The RCM reaction of peptides with two pentenyl side chains ($i, i + 4$ staple), or four pentenyl side chains (double $i, i + 4$ staple) led to a complete conversion of unstapled peptides to stapled ones as analyzed by HPLC. In each case, the stapled peptides eluted earlier than their unstapled precursors as shown in Fig. 3 for some LL-III analogs. The reaction involving double stapling of LL-III-4 (four pentenyl side chains) resulted in the desired doubly stapled product LL-III-4 (Table 1; Fig. 3b) and excluded possible $i, i + 3$ stapling between two internal pentenyl side chains. Usually, this arrangement represents very unfavorable conditions for $i, i + 3$ stapling as already published (Kim et al. 2010).

The double stapling of MEP-N-3, MEP-N-5 and MEP-N-6 may theoretically give rise to two peptide products, since all pentenyl side chains are at $i, i + 4$ positions relative to each other as shown schematically in Fig. 1 of Supplementary Material. We observed that the presence of proline residue in MEP-N-3 prevents the stapling between two internal pentenyl side chains, as the RCM reaction resulted only in one product characterized as the doubly stapled peptide MEP-Ns-3 (Table 2). However, when the proline residue was substituted by glycine (MEP-N-5) or alanine (MEP-N-6) the RCM reaction gave rise to a mixture of two compounds—the desired doubly stapled peptides (MEP-Ns-5, MEP-Ns-6) and singly stapled peptides with two external unstapled pentenyl side chain residues—MEP-Ns-5 \times and MEP-Ns-6 \times (Table 2; see the Fig. 1 of Supplementary Material).

The $i, i + 7$ stapled peptides (LL-III-5 and MEP-Ns-4) were produced as *cis* and *trans* double bond isomers as separable products by HPLC in the ration 1:1 and had identical molecular masses. Figure 3c (lower panel) shows

the HPLC profile of the crude LL-III-5. The earlier eluted peak was identified by NMR (see the paragraph below) as a *cis* isomer, and the later one as a *trans* isomer (Fig. 2 of Supplementary Material). Also, NMR analysis of the two products of the RCM reaction in the MEP-Ns-4 case confirmed *cis* and *trans* isomerisation (Fig. 3 of Supplementary Material).

The RCM reaction of the LL-III-6 analog (Gly8 to Pro substitution) resulted surprisingly in the formation of two products, LL-III-6a and LL-III-6b, as indicated by the HPLC profile (Fig. 3d). The short ($i, i + 4$) stapling approach in principle avoids the incidence of *cis/trans* isomerisation within the rings. The presence of two compounds of the same molecular mass could thus be explained by the fact that the presence of proline residue adjacent to the staple induces the formation of *cis/trans* isomerisation around the S_5 -Pro8 peptide bond (Table 1).

NMR identification of *cis* and *trans* double bond isomers of the $i, i + 7$ stapled analogs

Cross-linking of the LL-III-5 and MEP-N-4 produced two RP-HPLC separable products of identical molecular masses, considered as *cis* and *trans* double bond isomers as known from the literature. The structure of both isomers was determined from ¹H NMR spectra. The olefinic protons in the structure fragment $-\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2-$ of each isomer gave two doublet of triplets due to one larger coupling between olefinic protons $^3J(\text{CH}=\text{CH}-)$ and two smaller couplings with CH_2 protons in vicinal position $^3J(=\text{CH}-\text{CH}_2-)$. In the case of LL-III-5 stapled analogs, the earlier eluted compound showed olefinic protons at 5.41 and 5.30 ppm with $^3J(\text{CH}=\text{CH}-) = 10.5$ Hz (Fig. 2a of Supplementary Material), that can be assigned to the *cis*-configuration of the olefinic protons (LL-III-5 *cis*), while the later eluted compound showed olefinic protons at 5.42 and 5.33 ppm with $^3J(\text{CH}=\text{CH}-) = 15.4$ Hz (Fig. 2b of Supplementary Material). This indicates a *trans*-configuration of the olefinic protons (LL-III-5 *trans*). Similarly, in the MEP-Ns-4 analogs case (Fig. 3a, b of Supplementary Material), the earlier eluted compound showed $^3J(\text{CH}=\text{CH}-) = 10.4$ Hz and could be assigned to a *cis*-isomer (MEP-Ns-4 *cis*) while the later eluted compound with $^3J(\text{CH}=\text{CH}-) = 15.4$ Hz was assigned to *trans*-isomer (MEP-Ns-4 *trans*).

Biological activity

The values of biological activities of the prepared peptides are summarized in Tables 3 and 4. Antimicrobial activity of all stapled peptides against sensitive Gram-positive bacteria *M. luteus* and *B. subtilis* was high, and practically

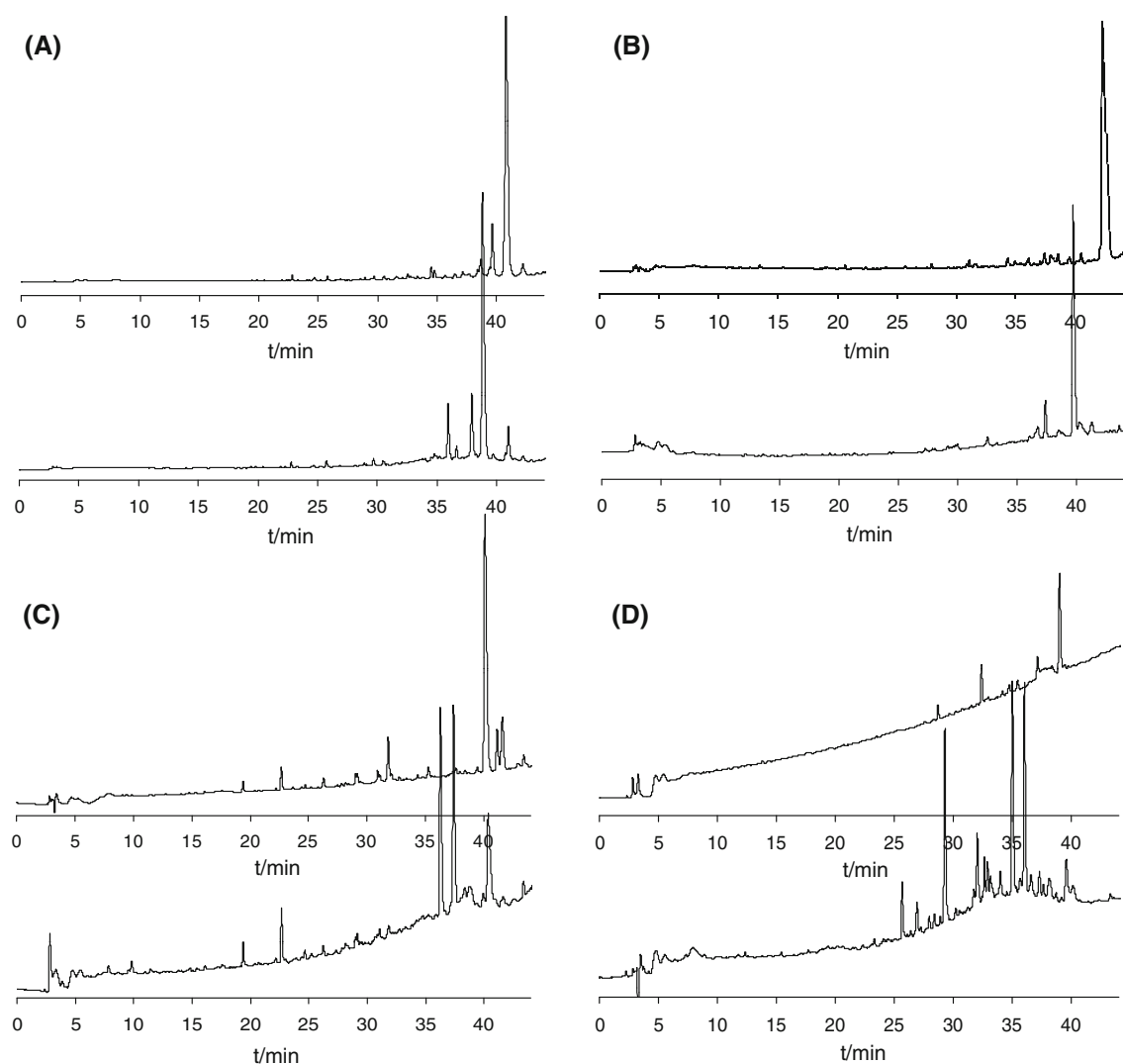


Fig. 3 Examples of RP-HPLC profiles of the crude unstapled LL-III derived peptides with olefinic amino acids incorporated, compared to their crude stapled peptide versions. In each case the stapled peptides (lower profiles) eluted earlier than their unstapled precursors (upper profiles). **a** LL-III-3/LL-IIIs-3, **b** LL-III-4/LL-IIIs-4, **c** LL-III-5/LL-IIIs-5 *cis* and *trans*; **d** LL-III-6/LL-IIIs-6a and LL-IIIs-6b; in this case stapling rendered two products, probably due to the *cis/trans*

the same as those of native peptides, regardless of their structure (Tables 3, 4). Introduction of a single staple into the LL-III as well as into the MEP-N peptides decreased their antimicrobial potency most significantly against pathogenic *P. aeruginosa* followed by *S. aureus*. Apparently, the stapling of both peptides did not affect the antimicrobial activity against *E. coli* as strongly. LL-III and MEP-N stapled across two helix turns at the *i*, *i* + 7 positions exhibited two to four times weaker activity against *S. aureus* compared to analogs stapled across one helix turn (*i*, *i* + 4 stapling). Their activities against *P. aeruginosa* were comparable. Similarly, double stapling of natural peptides rendered significantly less potent

isomerisation around the S_5 -Pro8 peptide bond. Chromatography was carried out on an Agilent Technologies 1200 Series module with a Vydac C-18, 250 \times 4.6 mm; 5 μ m, column (Grace Vydac, Hesperia, California) at a 1 mL/min flow rate using a solvent gradient ranging from 5 to 70 % acetonitrile/water/0.1 % TFA over 60 min. The elution was monitored by absorption at 220 nm

analogs against *S. aureus* and *P. aeruginosa*. All the stapled peptides are more hemolytic than natural peptides, and this can be attributed to the increase of their hydrophobicity and α -helicity, a phenomenon known from the literature (Chen et al. 2005). Slightly different results were obtained in the case of dicarba-brevinin-1BYa in which the stapling was achieved by ring-closing metathesis between two allylglycines incorporated at the *i* and *i* + 6 positions thus replacing the metabolically labile disulfide bridge at the C-terminal part of the peptide (Hossain et al. 2011). The dicarba analog showed a two-fold increase in antimicrobial activity, was more toxic, and its helical content was lower than that of native brevinin-1BYa.

On one hand, stapling enhances the α -helicity of peptides in water, but on the other, it increases their hydrophobicity and decreases their conformational freedom—the flexibility of the molecules. In agreement with the literature, antimicrobial activity of α -helical peptides depends, above all, on their net cationic charge, moderate hydrophobicity, and flexibility. Hemolytic activity is related to hydrophobicity, and helical structure that is important as a means of adopting an amphipathic structure. Our results confirmed that even if the cationicity in stapled peptides is preserved, the negative influence of hydrophobicity and reduction of flexibility prevails, resulting in lower antimicrobial potency and high hemolysis.

CD analyses and structural features

To assess the impact of stapling on the helicity of peptides, we studied the peptide analogs by CD spectroscopy in both unstapled (with olefinic amino acids incorporated) and stapled forms. The CD-UV spectra were acquired in both the absence and presence of the helix promoting solvent TFE, and in the anisotropic environment of SDS micelles.

In water, the LL-III, MEP and MEP-N exhibit spectra (Figs. 4, 5) that are characteristic of an unstructured peptide with a broad minimum below 200 nm, and containing α -helix in the range 12 % (MEP, MEP-N)–15 % (LL-III) (Tables 1 and 2 of Supplementary Material). The incorporation of two or four helix-promoting α,α -disubstituted olefinic amino acids as α -helix supporters into the peptides (unstapled variants) is expected to increase their helical content (f_H) with respect to unmodified LL-III and MEP-N. This should be indicated by the appearance of double minimum bands at 208 and 222 nm in the CD spectra. In

Table 3 Antimicrobial and hemolytic activity of lasioglossin (LL-III) and its stapled analogs

Peptide	Antimicrobial activity MIC (μ M)						Hemolytic activity LC ₅₀ (μ M)
	<i>M.l.</i>	<i>B.c.</i>	<i>S.a.</i>	<i>E.c.</i>	<i>P.a.</i>	<i>C.a.</i>	
LL-III	1	1	8	2	10	10	>200
LL-III _s -1	0.7	0.8	12.5	4.4	78.7	100	31.3
LL-III _s -2	0.9	1.2	20.3	8.8	86.7	100	69
LL-III _s -3	1.4	1.1	21.7	1.2	76.3	93.3	30
LL-III _s -4	2	1.1	80	20	>100	>100	>100
LL-III _s -5 <i>cis</i>	0.9	0.9	45	3.5	80	>100	100
LL-III _s -5 <i>trans</i>	1.1	1.3	65	5.5	80	>100	>100
LL-III _s -6 a	1	1.1	93	7.8	93	>100	>100
LL-III _s -6 b	0.9	0.8	50	7.8	63	>100	82

M.l. *Micrococcus luteus*, *B.s.* *Bacillus subtilis*, *S.a.* *Staphylococcus aureus*, *E.c.* *Escherichia coli*, *P.a.* *Pseudomonas aeruginosa*, *C.a.* *Candida albicans*

Table 4 Antimicrobial and hemolytic activity of melectin (MEP), [Nle^{14,17}]-melectin (MEP-N) and its stapled analogs

Peptide	Antimicrobial activity MIC (μ M)						Hemolytic activity LC ₅₀ (μ M)
	<i>M.l.</i>	<i>B.s.</i>	<i>S.a.</i>	<i>E.c.</i>	<i>P.a.</i>	<i>C.a.</i>	
MEP	1	0.8	6.8	2	18.5	8.8	>100
MEP-N	1	0.5	2.5	0.9	17.3	15	50
MEP-N _s -1	0.8	1.1	10.8	2.5	77	30	18.1
MEP-N _s -2	1.5	1.2	37	7.8	≥ 100	≥ 100	14.7
MEP-N _s -3	1.3	1.2	≥ 100	46.7	>100	>100	13.9
MEP-N _s -4 <i>cis</i>	1.5	1.3	37	8.1	≥ 100	≥ 100	11
MEP-N _s -4 <i>trans</i>	2	1.7	63	16.3	≥ 100	≥ 100	20
MEP-N _s -5	1.5	1.2	57	5.6	>100	>100	29
MEP-N _s -6	1.5	1.2	43	5.6	>100	>100	39.5

M.l. *Micrococcus luteus*, *B.s.* *Bacillus subtilis*, *S.a.* *Staphylococcus aureus*, *E.c.* *Escherichia coli*, *P.a.* *Pseudomonas aeruginosa*, *C.a.* *Candida albicans*

the *i*, *i* + 4 unstapled peptide series of LL-III (LL-III-1, LL-III-2, and LL-III-3), the insertion of two olefinic amino acids resulted in a 6–9 % increase of helical content, while

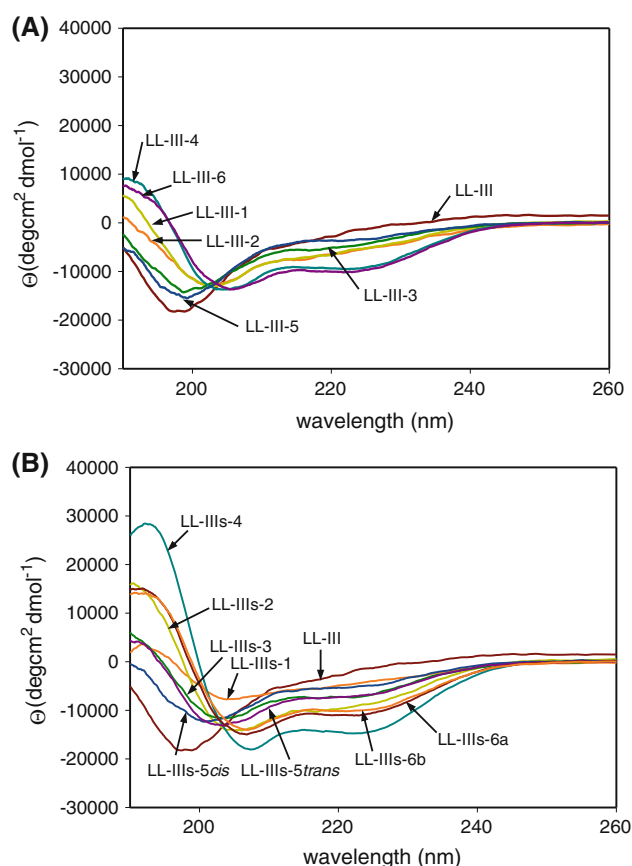


Fig. 4 UV-CD spectra of LL-III analogs measured in water. **a** Unstapled analogs with incorporated olefinic amino acids; **b** stapled analogs

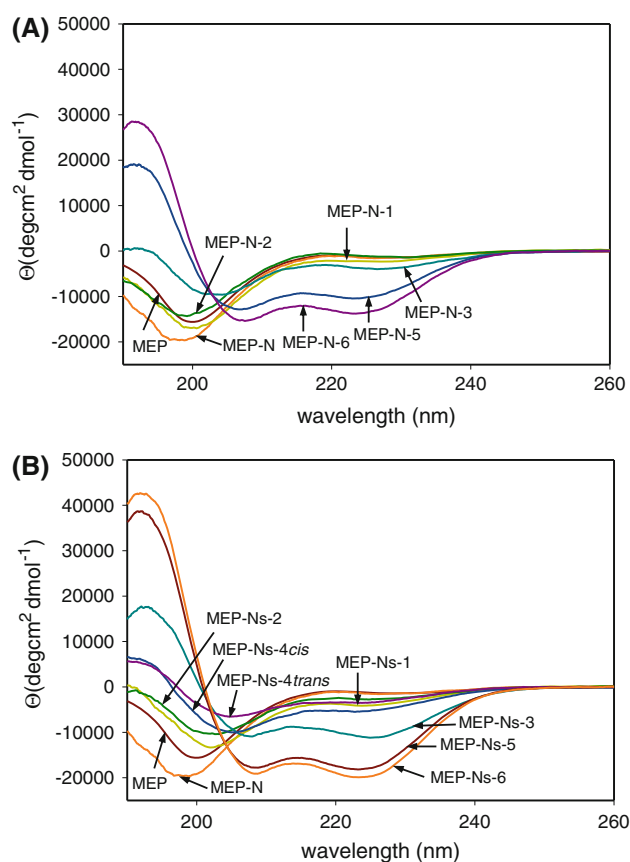


Fig. 5 UV-CD spectra of MEP and MEP-N analogs measured in water. **a** Unstapled analogs with incorporated olefinic amino acids; **b** stapled analogs

in the case of MEP-N-1 and MEP-N-2 insertion resulted only in a negligible increase of helical content compared to MEP-N (Tables 1 and 2 of Supplementary Material). On the other hand, the insertion of four olefinic amino acids into the peptides (LL-III-4, MEP-N-3, MEP-N-5, and MEP-N-6) had a noticeable effect on the helical content increase (by 18–29 %) (Tables 1 and 2 of Supplementary Material). The only exception was MEP-N-3, where the low increase of helical content might be caused by the kink imposed by the Pro residue at the central position of that peptide. In the case of unstapled LL-III-5 with olefinic amino acids inserted at $i, i + 7$ positions, the increase of helical content was insignificant.

The CD spectra of the singly stapled peptides of the $i, i + 4$ type acquired in water show a slight increase (by 5 %) of helical content in the case of MEP-Ns-1, MEP-Ns-2 and LL-III-3 compared to their unstapled precursors. For the LL-III-2 analog, the helical content increment was slightly higher (8 %). It seems that the staple in the central part of the LL-III-1 analog that crosslink the bend of the α -helix on its concave site around the Gly8 residue (Čerňovský et al. 2009) resulted in slight helix destabilization. On the other hand, the difference in α -helical content

between doubly stapled analogs (LL-III-4, MEP-Ns-3, MEP-Ns-5, and MEP-Ns-6) and their unstapled precursors was apparently higher, on average by 15 % (Tables 1 and 2 of Supplementary Material). CD spectra analyses of the structurally related MEP-Ns-3, MEP-Ns-5 and MEP-Ns-6 (Fig. 5b) show that the substitution of Pro11 by Gly (MEP-Ns-5) or Ala (MEP-Ns-6) increased significantly their propensity for α -helix formation. The stapling of the LL-III-5 in the $i, i + 7$ fashions that produced the *cis* and *trans* double bond isomers resulted in an insignificant increase (by 4 %) in the f_H of the *cis* isomer. However, in the case of the *trans* isomer, an increase in f_H of 8 % was more apparent.

The CD spectra of all the peptides studied underwent a considerable change with increasing concentration of TFE or SDS, thereby demonstrating a predominantly α -helical structure, as indicated by the appearance of distinct double minimum bands at 208 and 222 nm (See the CD spectra, and Tables 1 and 2 of Supplementary Material). The singly stapled analogs and their unstapled precursors adopted α -helical conformation with a α -helical content above 50% at TFE 30 %, or in the presence of SDS around critical micelles concentrations (2–4 mM). The CD spectra of doubly stapled peptides and their unstapled precursors show that they become structured at lower TFE or SDS concentrations (f_H around 30 %). Analysis of CD spectra in the presence of TFE or SDS micelles shows that stabilization of the α -helix, resulting from the incorporation of olefinic amino acids, is very similar, and independent of whether or not the peptides were stapled. In the presence of high concentrations of those promoters, the helical content of stapled peptides was the same as for native LL-III and MEP. Overall, the introduction of a single staple into the LL-III and MEP-N did not noticeably stabilize their α -helical conformation in water. However, introduction of a double staple had a greater effect on the stabilization of their α -helical structure. In the presence of high concentrations of the α -helix promoting solvent TFE, and in the anisotropic environment of SDS micelles, the native peptides LL-III and MEP adopt a α -helical conformation, independent of stapling their structure.

Digestion of peptides with trypsin

The stability against proteolytic degradation is an important feature of biologically active peptides also valid for AMPs. Since proteases bind peptides in an extended form rather than in helical conformation, the reinforcement of helical conformation by stapling is expected to enable the peptides proteolytic stability. LL-III, MEP and their stapled analogs have several trypsin cleavage locations due to the numerous Lys residues in their sequences. So we chose trypsin as a model protease for this study. We assumed that

the Lys-X scissile peptide bonds inside the stapled regions should be protected against digestion (Kim et al. 2010). Because the cross-linked region of single stapled peptides of the $i, i + 4$ type does not cover each cleavage site of these peptides, LL-III_s-1, LL-III_s-2, LL-III_s-3, MEP-Ns-1, and MEP-Ns-2 were readily digested by trypsin, similarly to native peptides (Fig. 6). The notably higher resistance of centrally stapled LL-III_s-1 compared to other singly stapled peptides may be due to the immediate vicinity of two protease cleavage sites (Lys5, Lys9) to the staple. The fact that the -Lys-Lys- peptide bond is theoretically less susceptible to cleavage by trypsin than the -Lys(Ile)Val- peptide bonds, (Kurth et al. 1997) may also account for the slightly higher stability of stapled analogs in which the -Lys(Ile)Val- cleavage site was included in the stapled region (LL-III_s-2 was slightly more resistant than LL-III_s-3). The peptides cross-linked by a longer $i, i + 7$ staple exhibited somewhat better resistance to proteolysis. In doubly stapled LL-III_s-4, all trypsin cleavage sites are protected against trypsin digestion by their location inside the staple (Lys4, Lys5, Lys12) and proximity to a cleavage site (Lys9). This accounts for its total resistance even at extended times (Fig. 6a). In doubly stapled MEP-Ns-3,

MEP-Ns-5, and MEP-Ns-6, the cleavage sites at Lys7 and Lys8 residues are situated within the staple thus being protected against the enzyme, and the site at the Lys12 residue is protected by the proximity of the staple. However, of these three analogs, only the MEP-Ns-6 analog was completely resistant, while the other two MEP-Ns-3 and MEP-Ns-5 were slightly cleaved (Fig. 6b). This is probably due to their conformational flexibility caused by a kink (Čeřovský et al. 2008) at Pro11 or Gly11 residues, respectively, situated between two staples. This may cause better insertion into the protease active site.

Conclusion

Introducing all-hydrocarbon staple/s in the $i, i + 4$ fashion to short α -helical amphipathic antimicrobial peptides lasioglossin III and melectin increases their hydrophobicity, hydrophobic moment and rigidity (stabilization of a turn/s of the α -helix). As expected, the stapled peptides generally show higher α -helical structure content in water, and in low concentrations of helix-promoting compounds TFE and SDS. However, the content of α -helical structure at high concentrations of TFE and at critical micelles concentrations (2–4 mM) is the same, whether or not these peptides have in their structure incorporated olefinic amino acid residues, and whether or not they are stapled. Stapling generally makes the peptides more resistant to proteolytic degradation, particularly those that are doubly stapled. As far as biological activities are concerned, the introduction of olefinic amino acid residues and conformational restriction by stapling make the peptides significantly more hemolytic, and lead to a decrease of their antimicrobial potency against pathogenic bacteria and the yeast *C. albicans*. Unfortunately, the stapling of our peptides is not rendering compounds that would have better pharmacological qualities than the native unmodified potent peptides.

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Conflict of interest The authors declare that they have no conflict of interest.

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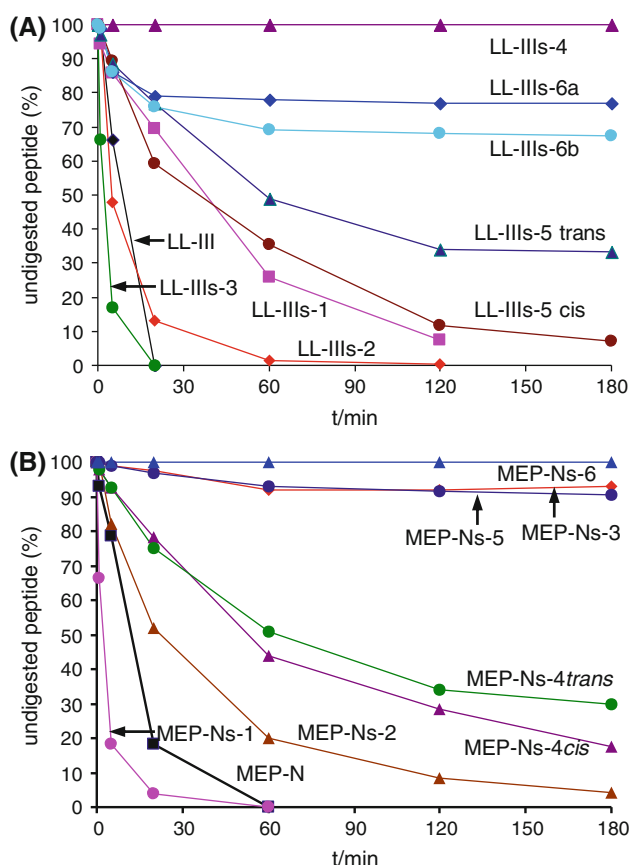


Fig. 6 Digestion of stapled LL-III peptides (a) and MEP-N peptides (b) by trypsin

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