Journal of Peptide Science
J. Pept. Sci. 2006; **12**: 491–496
Published online 20 April 2006 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.755

JOURNAL OF PEPTIDE SCIENCE

Design of a minimized cyclic tetrapeptide that neutralizes bacterial endotoxins

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Received 28 December 2005; Accepted 27 January 2006

Abstract: Septic shock is a leading cause of mortality in intensive care patients, and no specific drugs are as yet available for its treatment. Therefore, new leads are required in order to increase the number of active molecules that may develop into efficacious and safe LPS-neutralizing molecules during pre-clinical stages. We used peptides, derived from the binding regions of known LPS-binding proteins, as scaffolds to introduce modifications at the amino acid level. Structure–activity relationship studies have shown that these modifications generate highly active peptides. Thus, from a bioactive peptide with an initial 16 amino acid residues, a tetrapeptide sequence was determined. After inserting this sequence in a Cys cyclic peptide, it showed the same biological activity as the parent peptide. This sequence could provide the basis for the design of small molecules with LPS-binding properties. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: endotoxin; septic shock; sepsis; LALF; peptide mapping; lipid A; disulfide bridge formation; molecular recognition; solid phase; peptides

INTRODUCTION

Sepsis, a systemic inflammatory response to infection, can lead to multiple organ failure known as *septic shock*, the first cause of mortality in intensive care units [1]. Recognition of the bacterial LPS by immune system cells is detected on the basis of the pathology [2]. LPS is a pathogen-associated molecular pattern (PAMP) present in the outer leaflet of Gram-negative bacteria [3]. Continuous exposure to LPS in mammalian bloodstream induces the deregulation of inflammatory cytokine release, thereby leading to the pathological condition. The cascade of events is initiated by the recognition and binding of LPS to circulating LPS-binding proteins. Among other proteins, the LPS-binding protein (LBP) binds to LPS and transfers it to the CD14 receptor [4,5]. Although LBP and CD14 are at the top of the cell responsive pathway to LPS, a cell membrane receptor must interact with the complex in order to transduce the signal into the cell. TLR2 and TLR4 receptors, members of the toll-like receptors family (TLR), participate in the transduction of the LPS signal to the cell nucleus; an event that initiates the transcription of cytokine genes [6].

Research efforts have been directed towards the characterization of all the members involved in cascade recognition events and the full elucidation of the LPS-signalling pathway in order to define pharmacological targets. However, although inhibitors of TNF-α factor and other inflammatory mediators have been targeted for inhibition, to date this approach has not increased the survival of patients with septic shock [7,8]. Therefore, increased interest has been devoted to the inhibition of early events of the process. Compounds that could neutralize LPS or its toxic part, the lipid A moiety, may provide a potential source of useful lead compounds of pharmacological relevance [8].

Recent developments in identifying novel strategies to overcome endotoxic shock involve LPS-neutralizing peptides. Of these, special interest has been focused on Limulus anti-LPS factor (LALF), a small (101 amino acids) basic protein that binds and neutralizes LPS with high affinity [9]. From the analysis of the crystal structure of recombinant LALF (rLALF) [9], it was proposed that an amphipathic loop that spans residues 31 to 52 is the true LPS-binding domain [9]. In particular, the minimal LPS-binding domain is a 14-amino acid cyclic peptide (residues 36–47 – named LALF-14c), which binds LPS with an activity comparable to the high-affinity endotoxin-binding peptide polymyxin B (PMB), whereas its linear counterpart has a lower activity [10].

Herein, we report on the minimization of the LPS-binding domain of LALF-14c. We focused on

Abbreviations: BPI, bactericidal/permeability-increasing protein; DPLA, 1-4′-diphosphoryl lipid A; EDT, 1,2-ethanedithiol; LALF, *Limulus* anti-LPS factor, LAL, *Limulus* amebocyte lysate; LPS, lipopolysaccharide; LBP, lipopolysaccharide-binding protein; TBME; *tert*-butyl methyl ether; TIPS, triisopropylsilane

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hexapeptide mapping and alanine scanning of LALF-14c, which together provided data on the most relevant amino acids for biological activity.

MATERIAL AND METHODS

Materials and HPLC

Rink amide MBHA resin (0.66 mmol/g) and protected Fmoc-L-amino acids were purchased from Iris Biotech GmbH (Marktredwitz, Germany), Luxembourg Industries (Tel-Aviv, Israel), Neosystem (Strasbourg, France), Calbiochem-Novabiochem AG (Laüfelfingen, Switzerland) and Bachem AG (Bubendorf, Switzerland). Diisopropylcarbodiimide (DIC) was obtained from Fluka Chemika (Buchs, Switzerland), HOAt from GL Biochem (Shanghai, China), PyBOP from Calbiochem-Novabiochem AG and *N*,*N*-diisopropylethylamine (DIEA) from Albatros Chem. Inc. (Montreal, Canada). Solvents for peptide synthesis and RP-HPLC equipment were obtained from Scharlau (Barcelona, Spain). Trifluoroacetic acid (TFA) was supplied by KaliChemie (Bad Wimpfen, Germany). Other chemicals used were obtained from Aldrich (Milwaukee, WI, USA) and were of the highest purity commercially available. Buffers and solutions used in the *in vitro* LPS-neutralizing assays were endotoxin-free. Endotoxin-free water and LPSs from *Escherichia coli* 0111:B4 were from BioWhittaker (Rockland Maine, Walkersville, USA). HPLC was performed using a Waters Alliance 2695 (Waters, MA, USA) chromatography system with a photodiode array (PDA 995) detector, a reverse-phase Symmetry C_{18} (4.6 × 150 mm) 5-μm column and linear gradient MeCN with 0.036% TFA into H_2O with 0.045% TFA. The system was run at a flow rate of 1.0 ml/min. HPLC-MS was performed using a Waters Alliance 2796 with a UV–Vis detector 2487 and ESI-MS Micromass ZQ (Waters) chromatography system, a reversed-phase Symmetry 300 C_{18} (3.9 × 150 mm) 5-μm column, and H_2O with 0.1% formic acid and MeCN with 0.07% formic acid as mobile phases. Mass spectra were recorded on a MALDI Voyager DE RP time-of-flight (TOF) spectrometer (PE Biosystems, Foster City, CA, USA).

Solid-phase Synthesis

Linear hexapeptides and their cyclic derivatives were synthesized using the Fmoc solid-phase strategy performed manually in polypropylene syringes fitted with polyethylene porous disks. Side chains of Fmoc amino acids were protected as follows: Tyr and Thr were protected with the *tert*-butyl group (tBu), Lys and Trp with the *tert*-butyloxycarbonyl group (Boc), Cys with the trityl group (Trt) and Arg with the 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl group (Pbf). Solvents and soluble reagents were removed by suction. Washings between deprotection, couplings and subsequent deprotection steps were carried out with DMF and DCM using 10 ml of solvent per gram of resin each time. The Fmoc group was removed by treatment with piperidine–DMF (1 : 4) for 20 min. All syntheses were performed on Rink Amide MBHA resin (300 mg) by a Fmoc solid-phase strategy. Couplings of all Fmoc-aa-OH (4 equiv.) were performed with DIC (4 equiv.) and HOAt (4 equiv.) in DMF for 2 h at room temperature. Recouplings were done either with HATU (4 equiv.) and DIEA (8 equiv.) in DMF for 30 min at 25 °C or with PyBOP (4 equiv.), HOAt (4 equiv.) and DIEA (12 equiv.) for 2 h at room temperature. The resin was washed with DMF and DCM after each coupling. Couplings were monitored using the Kaiser [11] or de Clercq [12] method. After each coupling, the capping steps were performed with HOAc–DIEA–DMF (4 : 2 : 94). For the deprotection of side-chain groups and concomitant cleavage of the peptide from the support, the resin was washed with DCM (3 × 1 min), dried and treated with a TFA–H_2O–TIS (95 : 2.5 : 2.5) mixture for a range of times depending on the peptide sequence (1 to 2 h). When necessary, a thiol-containing cleavage mixture, TFA–H_2O–TIS–EDT (95 : 2 : 2 : 1), was used instead. TFA was then removed by evaporation with nitrogen, and peptides were precipitated with cold anhydrous TBME, dissolved in H_2O–MeCN (distinct mixtures used) and then lyophilized. The crude peptides were purified either by semi-preparative or preparative HPLC. The alanine scanning–derived peptides were synthesized using an automatic peptide synthesizer ABI 433A (Applied Biosystem) and a *fast*Fmoc solid-phase strategy on polystyrene aminomethyl Rink Amide AM resin (RAM) resin (0.76 meq/g, Rapp polymer).

LALF-14c and the alanine scanning derived–peptides were cyclized upon dissolution in a HOAc–DMSO–H_2O (1 : 3 : 16) solution at a concentration of 0.5 mg/ml. A neutral pH was achieved after treatment with ammonium carbonate. The solution was then stirred at room temperature for 24 h [13,14]. Alternatively, LALF07c, LALF08c and LALF09c peptides were dissolved with H_2O–MeCN (1 : 1) in a round-bottom flask at a concentration of 0.5 mg/ml. The pH was then adjusted to 9 with a 20% solution of NH_3. The solution was stirred at room temperature for one or two days to allow air oxidation. Cyclization was easily monitored either by Ellman's test [15] and/or by RP-HPLC.

LPS-neutralizing Activity

The chromogenic LAL test assay [16] was used following the manufacturer's instructions (BioWhittaker). LAL contains a clottable enzyme that is activated in the presence of non-neutralized LPS [17] and is an extremely sensitive indicator of the presence of endotoxin. LPS-activated enzyme catalyses the release of *p*-nitroaniline (pNA) from the colourless chromogenic substrate Ac-Ile-Glu-Ala-Arg-pNA. The pNA released was measured photometrically at 405 nm in a Rosys Anthos 2010 microtiter plate reader (Tecnomara AG, Zurich, Switzerland). LPS-neutralization assays were performed at a fixed concentration of LPS (100 pg/ml) and using a range of peptide concentrations in a PBS saline buffer (137 mM NaCl, 2,7 mM KCl, 4,3 mM Na_2HPO_4, 1,4 mM KH_2PO_4 pH 7.0) in a 96-well microtiter plate. Peptides were pre-incubated with LPS for 45 min at 37 °C. The colorimetric reaction was started by adding LAL (50 μl) for an incubation period of 6 min, followed by the addition of the chromogenic substrate (100 μl) and incubation for 10 min. Finally, the reaction was stopped by the addition of acetic acid up to 25% of the total reaction volume.

RESULTS AND DISCUSSION

Hexapeptide Mapping Analysis of LALF-14c

LALF-14c has characteristics similar to PMB in terms of structure and charge. PMB is an amphipathic,

positively charged cyclic oligopeptide, which belongs to a family of antibiotics that binds lipid A with high affinity [18]. LALF-14c adopts a positively charged amphipathic hairpin loop with a β-turn stabilized by a disulfide bridge (Figure 1). In fact, Cys^{35} and Cys^{48}, which are not present in the natural protein, were added at the *N*- and *C* terminus to obtain the cyclic peptide that stabilizes the secondary structure [10].

Previous studies to reduce the length of the active peptides derived from the LPS-binding domain of LALF were performed with peptides 10-amino acids long and no improvement in LPS-binding activity was achieved [10]. In an attempt to identify the minimal LALF peptide sequence required for LPS inhibition, we mapped the LALF14c region with a series of overlapping linear hexapeptides (Table 1). The resulting seven hexapeptides comprised the whole LPS-binding site of LALF-14c. Peptide characterization is shown in Table 2. The linear peptides were dissolved in PBS saline buffer and their concentration was measured spectrophotometrically. The LPS-binding activity of each peptide was measured at 200 μM in the presence of 100 pg/ml of LPS (Figure 2). Although all the hexapeptides showed a reduced LPS-neutralizing activity compared to LALF-14c, all except LALF-09 displayed biological activity. These results indicate that particular peptides containing sequence residues Arg^{41},

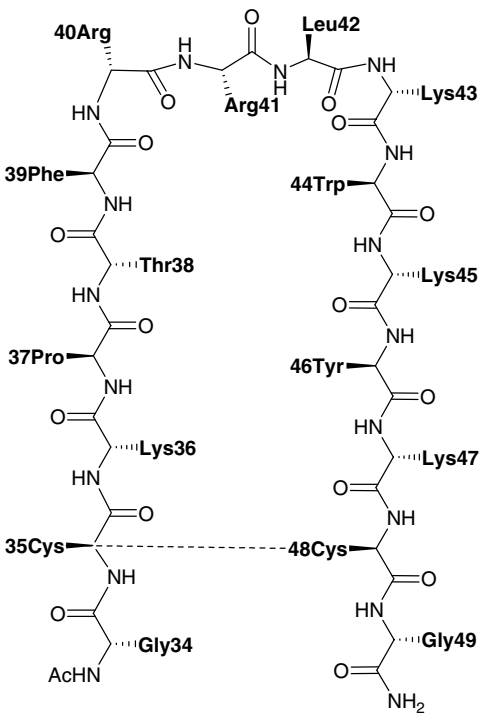

Figure 1 Schematic representation of the amphipathic β-hairpin of LALF14c. Amino acids are represented with the three-letter code. Wedged bonds and hashed wedged bonds indicate the relative position of side chains. A dashed bond represents the disulfide bridge between the two Cys introduced.

Table 1 Design of synthetic hexapeptides mapping the LALF14 domain

Name	Sequence[b]
LALF[a]	ECHYRIKPTFRRLKWKYKGKFWCP
LALF-14c	G-(CKPTFRRLKWKYKC)-G
LALF-03	KPTFRR
LALF-04	PTFRRL
LALF-05	TFRRLK
LALF-06	FRRLKW
LALF-07	RRLKWK
LALF-08	RLKWKY
LALF-09	LKWKYK

[a] LALF stands for the proposed LPS-binding domain of the LALF protein [10].
[b] All the peptides are acetylated and amydated at the *N*- and *C*-terminus, respectively. Sequence in parenthesis indicates cyclized.

Table 2 Characterization of Linear Hexapeptides

Name	t_R/min (HPLC)	Calculated mass	Experimental mass (MALDI)		
			M + H	M + Na	M + K
LALF-03	4.2	844.50	845.54	867.43	—
LALF-04	5.5	829.49	830.58	852.55	868.33
LALF-05	4.9	860.53	861.55	883.54	899.50
LALF-06	5.9	945.57	946.60	—	—
LALF-07	4.5	926.59	928.16	950.14	966.12
LALF-08	5.0	933.55	934.70	—	—
LALF-09	5.0	905.55	906.71	928.70	944.68

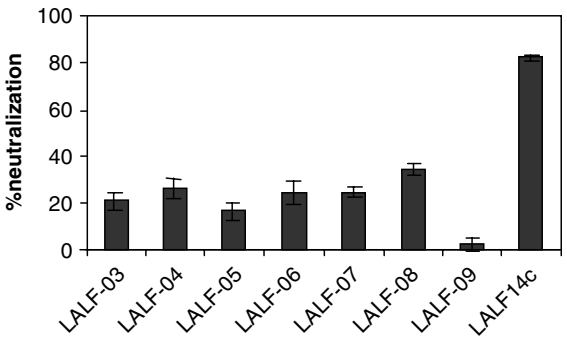

Figure 2 Inhibition of LPS-inducing gelling of chromogenic *Limulus* amebocyte lysate by LALF14c synthetic derived linear peptides or by control peptide (LALF14c) (200 μM) using 100 pg/ml of LPS. The assay was performed as described under 'Material and Methods'. The LPS-binding activity is performed in three independent assays, and the data is represented with ±SD.

Leu^{42}, Lys^{43} and Trp^{44} were slightly more active, and the absence of Arg^{41} in peptide LALF-09, together with the decrease observed in activity, shows that this residue may have a relevant role in the biological activity.

Table 3 Alanine Scanning in LALF14 Domain

Name	Sequence
LALFK47Ac	AC-GCKPTFRRLKWKY**A**CG-NH2
LALFY46Ac	AC-GCKPTFRRLKWK**A**KCG-NH2
LALFK45Ac	AC-GCKPTFRRLKW**A**YKCG-NH2
LALFW44Ac	AC-GCKPTFRRLK**A**KYKCG-NH2
LALFK43Ac	AC-GCKPTFRRL**A**WKYKCG-NH2
LALFR41Ac	AC-GCKPTFR**A**LKWKYKCG-NH2
LALFR40Ac	AC-GCKPTF**A**RLKWKYKCG-NH2
LALFF39Ac	AC-GCKPT**A**RRLKWKYKCG-NH2

Alanine Scanning of LALF-14c Sequence

As mentioned earlier, anti-LPS peptides are amphipathic and positively charged [19,20]. The sequence of LALF-14 is rich in basic amino acids (lysine and arginine) and in hydrophobic residues (phenylalanine, tryptophan and tyrosine). Results from our laboratories indicate that the amino acids located at the *C*-terminus of the β-hairpin loop of LALF-14c (Figure 1) are of relevance for biological activity (Mora *et al.*, to be published). To analyse the contribution of each amino acid from this particular sequence, we performed an alanine scan (Table 3). The peptides were synthesized with *N*- and *C*-terminal cysteines in order to obtain cyclic peptides. Peptide characterization is shown in Table 4. The LPS-binding activity of each peptide was measured at a range of peptide concentrations using 100 pg/ml of LPS, and the IC_{50} of each peptide was then evaluated (Figure 3). The substitution of the aromatic residues Trp^{44} and Tyr^{46} induced a dramatic decrease in biological activity. Surprisingly, the alanine substitution of basic residues was not deleterious to the activity. Given the proposed structure for LALF-14c [10], residues Trp^{44} and Tyr^{46} were found to contribute to the partial hydrophobic inner face of the β-hairpin strand and to the stabilization of the structure by both H-bonding and aromatic interactions. These contributions may therefore occur early in the folding process of the active structure and may contribute to the orientation of the positively charged amino acids at the loop and *C*-terminal regions, thereby favouring productive peptide–LPS binding. These results are in agreement with previous studies on the LPS-binding domain of related proteins [21]. In a very recent study, it has been proposed that the presence of the pair Arg^{40}-Arg^{41} plays an important role for the structure stabilization of LALF-14c bound to LPS complex [22]. These observations are consistent with our findings in the hexapeptide mapping (see above), where the peptide LALF-09, which holds no arginine residues, showed a reduced LPS-neutralizing activity. However, the output of the alanine-scanning substitution indicates that a single point mutation was easily overcome and did not affect LPS-neutralizing activity.

Table 4 Alanine-scanning Characterization

Name	t_R/min (HPLC)	Calculated mass	Experimental mass (MALDI)		
			M + H	M + Na	M + H
LALFK47Ac	12.0	1953.58	1955.50	—	—
LALFY46Ac	11.5	1919.86	1920.50	—	—
LALFK45Ac	12.2	1954.86	1954.38	—	—
LALFW44Ac	10.9	1895.79	1897.35	—	—
LALFK43Ac	12.2	1953.00	1955.38	—	—
LALFR41Ac	12.2	1924.87	1926.37	—	—
LALFR40Ac	12.2	1927.93	1927.37	—	—
LALFF39Ac	11.0	1934.71	1935.37	—	—

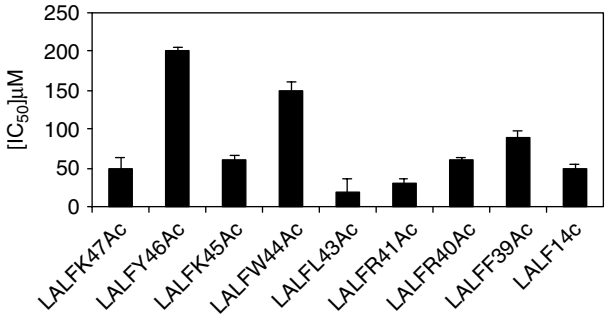

Figure 3 The inhibition of the different peptides determined using a chromogenic *Limulus* amebocyte lysate assay. The inhibition activity of LPS by peptides derived from the alanine scanning is represented as the IC_{50}. IC_{50} is the concentration necessary to neutralize *in vitro* 50% of LPS as determined by a serial dilution assay (12, 5, 25, 50, and 100 μM) using 100 pg/ml of LPS. The determined IC_{50} is the average of three independent assays, and the data is represented with ±SD.

Design of Minimized LALF14-based LPS-neutralizing Cyclic Peptides

Peptides LALF-07 and LALF-08, which cover the *C*-terminus of LALF-14-containing aromatic residues and arginines, were chosen for cyclization. In addition, peptide LALF-09 was also included in the following procedures as a putative control peptide to analyse the role of arginine residues in the biological activity of the minimized cyclic peptides. Cysteines were added at the terminal of the linear peptides LALF-07, LALF-08 and LALF-09 in order to oxidize the peptides via a disulfide bridge formation (Table 5). To facilitate the formation of the cyclic hexapeptides, we performed a distinct procedure to that used for the synthesis of LALF-14c and its alanine scanning–derived peptides (Air oxidation assisted by DMSO at neutral pH). Cys

Table 5 Design of Cyclic Hexapeptides

Name	Sequence
LALF-14c	G-(CKPTFRRLKWKYKC)-G
LALF-07	RRLKWK
LALF-08	RLKWKY
LALF-09	LKWKYK
LALF-07c	(C-RRLKWK-C)
LALF-08c	(C-RLKWKY-C)
LALF-09c	(C-LKWKYK-C)

Table 6 Characterization of Cyclic Hexapeptides

Name	t_R/min (HPLC)	Calculated mass	Experimental mass (MALDI)		
			M + H	M + Na	M + H
LALF-07c	7.6	1130.60	1131.71	1153.70	1169.68
LALF-08c	8.7	1137.56	1138.81	1160.78	1176.77
LALF-09c	8.1	1109.55	1110.71	1132.68	1148.66

was protected with the acid-labile trityl (Trt) protecting group, which is easily removed by the acidic conditions used in the final cleavage step. However, tritylation of several functional groups was observed in some crude samples, indicating that an optimized selection of scavengers should be critical at this point. Crude peptides were purified before cyclization. After all these manipulations, a small amount of cyclic peptide was observed in all cases. Peptides were dissolved in aqueous NH_3 solution at pH 9 and monitored by the Ellman's test and RP-HPLC. Reactions were followed for 3 days; a conversion higher than 90% was consistently obtained after 24–30 h of reaction. Finally, the reaction mixture was purified to afford the desired cyclic peptides. Although both methods work well, the second one (absence of DMSO) allows easier control of the reaction by HPLC and facilitates the work-up. On the other hand, a careful neutralization is required in this second method because liofilization at high pH leads to the scrambling (intermolecular reactions between thiol groups) as shown by the complexity of the HPLC. The characterization of cyclic peptides is shown in Table 6.

The LPS-binding activity of each peptide was measured at 100 μM using 100 pg/ml of LPS (Figure 4). Compared to the biological activity of the original peptides (see Figure 1), the cyclic peptides showed increased activity, indicating that both the amino acid sequence and a restricted conformation are of special relevance for LPS-binding. These results also highlight the importance of the amino acids located at the *C*-terminus of the loop region of the LALF-14c sequence for LPS-neutralizing activity (Figure 5). LALF-08c and

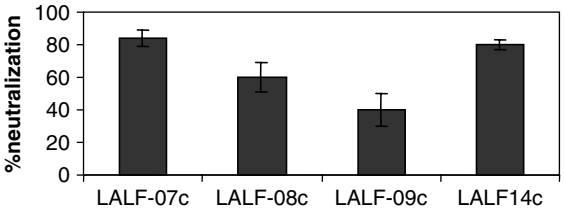

Figure 4 Anti-LPS activity of cyclic peptides determined using chromogenic *Limulus* amebocyte lysate assay described previously. Cyclic peptides were assayed at a fixed concentration (100 μM) and LPS (100 pg/ml). The neutralization activity of each peptide is compared with that of LALF14c (100 μM), the control peptide. The LPS-binding activity represented for each cyclic peptide is the average value obtained in three independent assays, and the data is represented with ±SD.

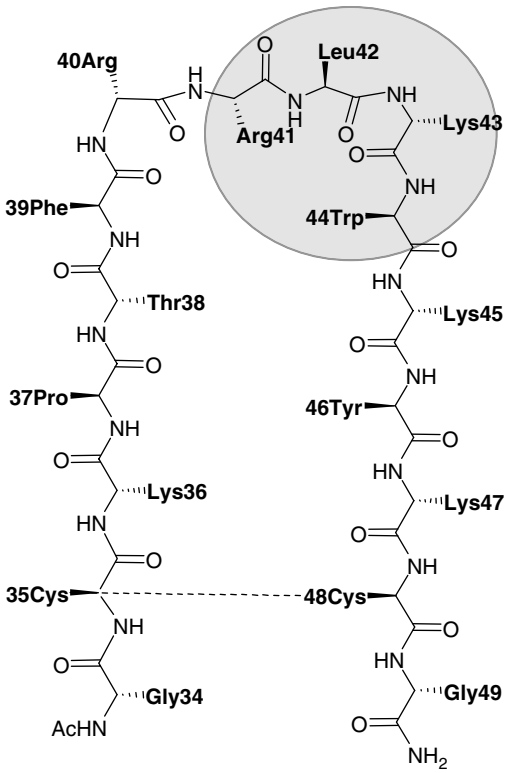

Figure 5 Schematic drawing of the loop structure of rLALF. The directions of the amino acid (three-letter code) side chains are indicated. Solid bonds indicate side chains pointing out of the plane of the diagram and dashed bonds into the plane. Cysteines were introduced pairwise instead of the authentic amino acid residues at positions indicated with a dotted line representing the resulting disulfide bridge. Amino acids inside the circle are postulated as the most relevant for the LPS-binding capability. They are contained in the sequence of the most active hexapeptides (LALF-07c, LALF-08c and RLKWc).

LALF-07c, which contain the four amino acids of the Arg-Leu-Lys-Trp sequence that were proposed as critical residues for the activity of the linear peptides, showed improved LPS-binding activity over LALF-09c,

which contains only three of the four amino acids of the same sequence. Moreover, LALF-07c showed higher activity than LALF-08c. The extra arginine residue in the former might enhance the LPS-binding capacity, as reported in previous studies [22].

The peptide LALF-07c, which showed LPS-neutralizing activity similar to that of LALF-14c, was selected for further analysis and an IC_{50} value of 60 μM was obtained, indicating that the peptide proposed earlier is a minimal LPS-binding domain of LALF, i.e. the peptide LALF-14c was susceptible to further minimization. To confirm the importance of the Arg-Leu-Lys-Trp sequence, we designed a new cyclic peptide with these four active residues (peptide named RLKWc). We added two alanines at the *N*-terminal and *C*-terminus of the RLKW sequence to keep the hexapeptide length. Two cysteines were also added to the *N*-terminus and *C*-terminus to obtain the cyclic peptide. The peptide RLKWc showed the same LPS-binding affinity at 100 μM peptide concentration as LALF14c, and IC_{50} values of 30 and 40 μM were observed for peptides RLKWc and LALF-14c, respectively.

In conclusion, our results indicate that a detailed analysis of bioactive peptides could render a minimization of the biologically active sequences, which could be of interest for further optimization studies, such as those addressing the design of small organic molecules. In fact, from an initial bioactive peptide (LALF-14c) with 16 amino acid residues and after a detailed analysis of sequence–activity relationships, we obtained a tetrapeptide sequence, which when inserted in a Cys-aided cyclic peptide, displayed the same biological activity.

Acknowledgements

This work was partially supported by the *Marató de TV-3*, CICYT (BQU2003-00089, BIO2004-998), the *Centro de Investigación Príncipe Felipe*, the *Generalitat de Catalunya* and the Barcelona Science Park.

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