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All-D-magainin: chirality, antimicrobial activity and proteolytic resistance

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All-D-magainin-2 was synthesized to corroborate experimentally the notion that the biological function of a surface-active peptide stems primarily from its unique amphiphilic α-helical structure. Indeed, the peptide exhibited antibacterial potency nearly identical to that of the all-L-enantiomer. Being highly resistant to proteolysis and non-hemolytic all-D-magainin might have considerable therapeutic importance.

All-D-magainin; Surface-activity; Proteolytic stability; Clinical potential

1. INTRODUCTION

Surface-active peptides, i.e. peptides that bind to and affect amphiphilic surfaces, such as membranes, receptors, etc., have been extensively studied in recent years [1,4]. A common feature of many of these compounds is their characteristic helical amphiphilic secondary structure, which is often induced by the respective target. Affinity of such peptides to the surface is enhanced in parallel to the increase complementarity between the two counterparts [5]. Some of these peptides, like hormones, which exert their biological activity through specific cellular receptors require, for manifestation of optimal function, meticulous conformation. The action of others, however, which do not seem to be mediated via such loci, is far less dependent upon strict structural requirements. Representatives of the latter family of compounds are cationic wide-range cytolytic peptides isolated from mammalian phagocytes, e.g. defensins [6], insects, e.g. melilittins [7], cecropins [8], sarcotoxins [9], and amphibians, e.g. magainins [10]. The target of these surface-active peptides appears to be the cellular lipid bilayer membrane. They have been reported to act almost exclusively by virtue of their unique structural features, which allow them to associate with respective cells and thereby to modulate membranal potential, permeability and function [6,11]. Mediation of these events through generation of voltage-dependent ion channels was also proposed [12-15].

If the overall structure of the peptide is the most important factor in the manifestation of the activity, then the all-D-enantiomer might possess biological properties similar to those of the respective natural L-enantiomer. This consideration does not hold true for receptor-oriented peptides [e.g. 16-18]. Enantiomeric conformation, however, in a non-chiral environment may, in principle, generate entirely different diastereoisomeric interactions in a chiral environment, such as the 'living' milieu of biological surfaces. Absence of such differences implies that, indeed, peptide-cell interactions should be interpreted primarily in amphiphilic terms. To examine the above considerations, we chose, for reasons of relatively simple synthesis and significant clinical potential, to study all-D-magainin.

The magainins, also known as PGS peptides [19], are a family of cationic 23-amino acid peptides recently isolated from the skin of the clawed African toad *Xenopus laevis* [10]. They are stored within the granular glands of the toad's skin and released in response to challenges with foreign invaders as part of the immune-defense mechanism. The peptides exhibit a wide range of potent anti-bacterial activity against a variety of Gram-negative and Gram-positive bacteria [10,20,21]. They can also inhibit the growth of fungi and viruses [21] and induce osmotic lysis in protozoa [10,20,21].

2. EXPERIMENTAL

2.1. *Synthesis of all-D-magainin*

This was carried out manually on a chloromethylated polystyrene/2% divinylbenzene resin (Chemalog, South Plainfield, NJ) [22]. Protected amino acid derivatives were purchased from Bachem (Bubendorf, Switzerland). α-Amino groups of amino acids were protected by *t*-butyloxycarbonyl. Side chain protecting groups were as follows: serine, *O*-benzyl; glutamic acid, γ-benzyl; lysine,

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N^{ε}-2-chloro-benzyloxycarbonyl; histidine, N^{im}-benzyloxycarbonyl. All coupling stages were performed with a 3-fold excess of protected amino acid derivatives with an equimolar mixture of *N,N'*-dicyclohexylcarbodiimide and 1-hydroxybenzotriazole as reagents. Deprotection and cleavage from resin was achieved by treatment with anhydrous HF [23]. The product was purified to homogeneity by initial chromatography on Sephadex G-15, using 0.1 N acetic acid as an eluent, followed by preparative HPLC (Lichrosorb RP-8; 7 μm; 240-10 mm) employing a linear gradient of acetonitrile (10-60%) in 0.1% aqueous trifluoroacetic acid. Amino acid analysis was performed on a Dionex Automatic Amino Acid Analyzer. Sequence determination was accomplished using an Applied Biosystems 470A Gas-Phase Microsequencer, coupled to Applied Biosystems 120A PTH Analyzer.

2.2. *Circular dichroism studies*

All-D- and all-L-magainin-2 ($2.5\text{-}5 \times 10^{-5}$ M) were dissolved each in either 50 mM potassium phosphate buffer, pH 7.0, or 50% (v/v) trifluoroethanol (Merck, Darmstadt, FRG) in the same buffer. A quartz cell of 1.0 cm path length was employed. Scans were performed on a Jasco model J-500C spectrophotometer with Jasco NP-500 data processor at 24°C, over a wavelength range of 240-200 nm. Calculation of α-helix and β-sheet contents was performed according to S.W. Provencher [24].

2.3. *Proteolytic studies*

The two enantiomeric peptides (20 μg each) were dissolved in PBS (pH 7.4; 90 μl) and the solutions incubated at 37°C. To each enantiomer a solution (10 μl) containing either a mixture of trypsin (EC 3.4.2.4., Merck; 2 μg) and chymotrypsin (EC 3.4.21.1., Merck; 2 μg) or pronase (EC 3.4.24.4., Merck; 2 μg) in 0.001 N HCl was added. Aliquots (25 μl) were taken from each mixture after incubation at 5 min, 30 min and 60 min, and monitored by HPLC at 220 nm.

HPLC analyses were performed on a Merck, Lichrospher 100 RP-8 (5 μm) column (125 × 4 mm). Elution gradient started ($T=0$) with 90% solvent A (water - 0.1% TFA)/10% solvent B (75% acetonitrile in water/0.1% TFA) and continued: $T=10$ min, 80% A/20%B; $T=25$ min, 55%A/45%B; $T=40$ min, 40%A/60%B.

2.4. *Antibacterial activity*

The antimicrobial activity of all-D- and all-L-magainin-2 (designated D and L, respectively) was determined by in vitro tests performed according to standard techniques. The in vitro MIC and MBC determinations were performed by the microbroth dilution method, at pH 7.0, in Tryptose Broth (Difco Laboratories, U.S.A.) as a growth medium.

2.5. *Hemolysis*

Three ml of packed human erythrocytes were washed 3 times with isotonic phosphate buffered saline (PBS, pH 7.4) and diluted to a final volume of 20 ml in the same buffer. Aliquots (190 μl) of cell suspension were placed in Eppendorf tubes and solutions (10 μl) of different concentrations of the tested peptides, in PBS, were added. Following gentle mixing, while incubated for 30 min at 37°C, the tubes were centrifuged at 4000 × *g* for 5 min. Aliquots (100 μl) of supernatants were taken, diluted to 1 ml with PBS and absorptions at 576 nm were measured. Hemolysis effected by 0.1% Triton X-100 was considered as 100%. All experiments were performed in duplicates.

3. RESULTS AND DISCUSSION

All-D-magainin (Fig. 1) was synthesized, employing the solid-phase strategy [22]. The peptide was purified to homogeneity using high performance liquid chromatography (HPLC) and its correct composition and primary structure were ascertained by amino acid and sequence analyses, respectively. The anticipation

H-Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Lys-Lys-Phe-

-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Met-Asn-Ser-OH

Fig. 1. Amino acid sequence of all-D-magainin-2.

that the all-D-peptide can exhibit a conformation that is the mirror image of that of the all-L-enantiomeric peptide was confirmed by CD measurements (Fig. 2). Thus, it is apparent that both the D- and L-enantiomers displayed random conformation in aqueous buffer, but adopted, in the range of 200-240 nm, a positive and negative ellipticity, respectively, in the presence of 50% trifluoroethanol. Identical α-helices (32%) were calculated from the CD spectra [24].

The antimicrobial activity of all-D-magainin was compared to that of all-L-magainin, against a wide

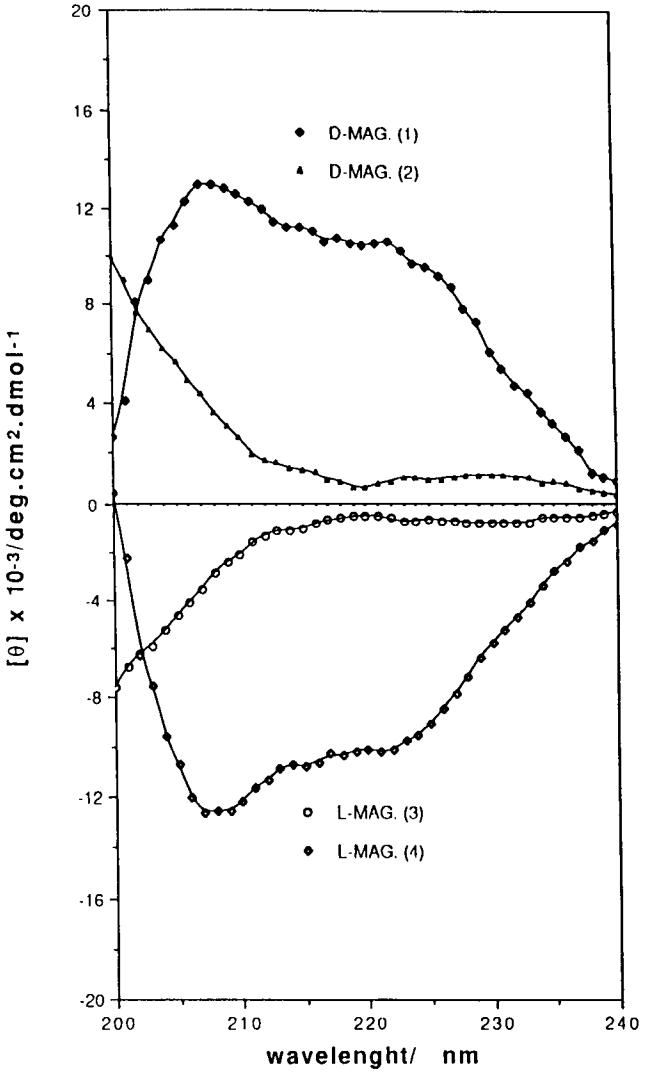

Fig. 2. Far-ultraviolet circular dichroism of all-D- and all-L-magainin-2. (1) and (3) refer to D- and L-magainin-2 in 50% TFE, respectively. (2) and (4) refer to D- and L-magainin-2 in phosphate buffer, respectively.

Table I

Antimicrobal activity of all-D- and all-L-magainin-2

Microorganism*		Minimal inhibitory concentration (μg/ml)	Minimal bactericidal concentration (μg/ml)
Escherichia coli	L	12.5	12.5
	D	12.5	12.5
Enterobacter cloacae	L	25	50
	D	50	100
Proteus mirabilis	L	>400	>400
	D	>400	>400
Shigella flexineri	L	3.12	3.12
	D	3.12	3.12
Salmonella anatum	L	50	>50
	D	50	>50
Pseudomonas aeruginosa	L	100	200
	D	100	400
Klebsiella oxytoca	L	50	>50
	D	50	50
Streptococcus fecalis	L	400	400
(ATCC 29212)	D	>400	>400
Staphylococcus aureus	L	25	50
(ATCC 29213)	D	50	50
Escherichia coli	L	12.5	25
(ATCC 25922)	D	12.5	25
Pseudomonas aeruginosa	L	6.25	6.25
(ATCC 27953)	D	3.12	12.5

*Non-ATCC strains are recent clinical isolates from a tertiary-care hospital.

spectrum of Gram-negative and Gram-positive bacteria (Table I). As is evident from the table, minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values are rather similar for both peptides with all tested microorganisms. As shown in Table II, all-D-magainin was as non-hemolytic as all-L-magainin towards human erythrocytes.

Considering the antimicrobial potency of magainin, coupled with its antifungal, antiprotozal and antivirus activities, as well as low hemolyticy, the peptide has a rather significant therapeutic potential. It would be beneficial to have an analog with enhanced stability towards proteolyis and thus, presumably, long active duration. As a model to examine the susceptibility of all-D-magainin towards proteolysis, its resistance to the digestive action of pronase, trypsin and chymotrypsin was studied in parallel to that of all-L-magainin (Fig. 3). As shown, the all-D-enantiomer remained intact even after 1 h of incubation with the enzymes, while the all-L-enantiomer was extensively degraded rather rapidly (5 min). These results suggest that all-D-magainin or a relevant derivative may be of significant therapeutic potential. Indeed in vitro experiments with magainin-2 showed that the biological activity decreased due to the presence of proteases in the medium [11,25,26].

Table II

% Hemolysis of Human Red Blood Cells

Peptide	Concentration (μg/ml)			
	25	80	100	200
all-L-magainin-2	0	1	2	2
all-D-magainin-2	0	0	1	2

Mellitin (Sigma Chemical Co., St. Louis, MO, USA) at concentrations of 2.5, 5, 10 and 20 μg/ml effected 1, 5, 20 and 70% hemolysis, respectively.

Based on theoretical considerations, i.e. Chou-Fasman principles and the 'helical-wheel' projection of Schiffer-Edmundson, it was proposed that the magainins might have an amphiphilic α-helical structure [27]. This assumption was corroborated by two-dimensional NMR spectroscopy, which demonstrated that, in fact, magainin assumes such a characteristic while dissolved in a mixture of trifluoroethanol and water [28]. It was further postulated that this structural feature, which can be enhanced by sequence alterations with a consequent augmentation of bioactivity, is important for the expression of antimicrobial activity [27].

It may be assumed that the initial interaction between the positively charged magainin and the bacterial surfaces is of an electrostatic nature through the multitude of negatively charged groups on the surfaces of cells, notably among them those contained in lipopolysaccharides (LPS) and teichoic acids in Gram-negative and Gram-positive bacteria, respectively [29]. Following this encounter the microbial membrane is perturbed, in a manner which is not yet clearly understood, with eventual cell death [30–31].

Our present data show that, as postulated, the unique architectural feature of magainin, i.e. amphiphilic α-

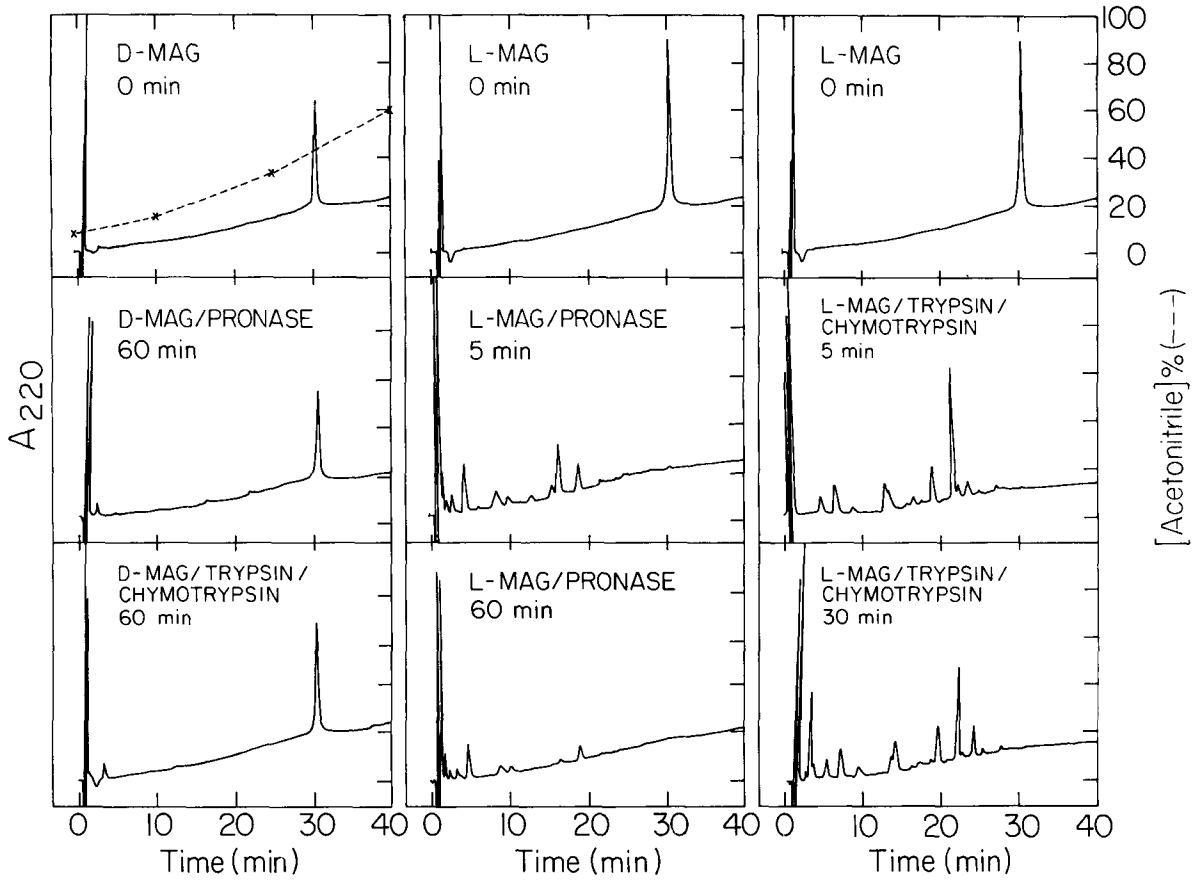

Fig. 3. HPLC analysis of the susceptibility of all-D-magainin-2 towards enzymatic degradation.

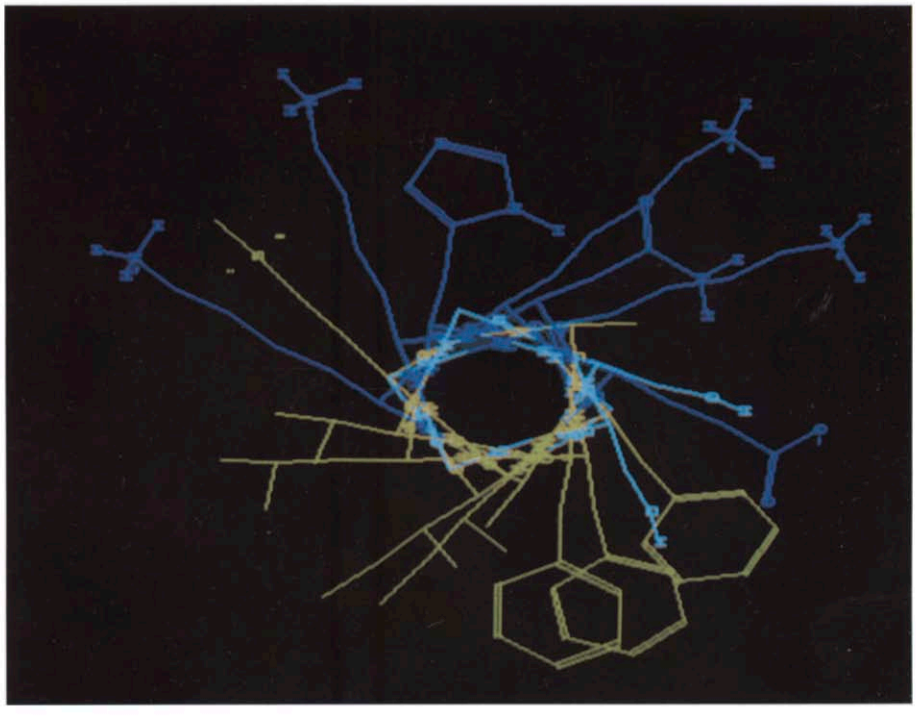

Fig. 4. Projection in two-dimension of the α-helical wheel conformation of magainin-2 according to Macromodel computer modelling program (Clark Still, Columbia University, USA).

helical structure (Fig. 4) is crucial in the manifestations of antimicrobial activity. It is possible that initial peptide-bacterial cell surface association is somewhat distinct for the L- and D-enantiomers due to different chiralic-related interactions. However, further adaptation of the peptide within the amphipathic bacterial membrane has eventually led to similar cellular activation. Finally, we suggest that the consequences of our studies with all-D-magainin may hold true for other surface-active peptides.

While the present study was being summarized for publication, the synthesis of the amide of all-D-magainin-2 was described by Wade et al. [32]. This analog was essentially identical in its antibacterial specificity to that of the carboxy-free all-L-magainin-2 which occurs naturally and was resistant towards proteolytic digest.

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