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Chemical Synthesis, Characterization and Activity of RK-1, a Novel α-Defensin-related Peptide

NICOLA F. DAWSON[a], DAVID J. CRAIK[b], AILSA M. MCMANUS[b], STUART G. DASHPER[c], ERIC C. REYNOLDS[c], GEOFFREY W. TREGEAR[a], LASZLO OTVOS JR[d] and JOHN D. WADE[a,*]

[a] Howard Florey Institute of Experimental Physiology and Medicine, University of Melbourne, Parkville, Victoria 3052, Australia
[b] Centre for Drug Design and Development, University of Queensland, Brisbane, Queensland 4072, Australia
[c] The School of Dental Science, University of Melbourne, Melbourne, Victoria 3000, Australia
[d] The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104, USA

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Abstract: The 32-residue peptide, RK-1, a novel kidney-derived three disulfide-bonded member of the antimicrobial α-defensin family, was synthesized by the continuous flow Fmoc-solid phase method. The crude, cleaved and *S*-reduced linear peptide was both efficiently folded and oxidized in an acidic solution of aqueous dimethyl sulfoxide. Following purification of the resulting product, it was shown by a variety of analytical techniques, including matrix assisted laser desorption time of flight mass spectrometry, to possess a very high degree of purity. The disulfide bond pairing of the synthetic peptide was determined by ^{1}H-NMR spectroscopy and confirmed to be a Cys^1-Cys^6, Cys^2-Cys^4, Cys^3-Cys^5 arrangement similar to other mammalian α-defensin peptides. The synthetic RK-1 was also shown to inhibit the growth of *Escherichia coli* type strain NCTC 10418.

Keywords: antibacterial assay; α-defensin; disulfide bond assignment; Fmoc-solid phase peptide synthesis; ^{1}H-NMR spectroscopy; mass spectroscopy; RK-1

INTRODUCTION

Defensins are small (*ca* 30 amino acids) cystine-rich, polycationic antimicrobial peptides that are involved in eukaryotic host defences against gram-positive bacteria, fungi and enveloped viruses [1,2]. They are also believed to contribute to the regulation of the inflammatory response. There are two classes of mammalian defensins known as the α- and β-defensins, each of which possesses a distinct cystine motif [2]. A recently isolated rabbit kidney-derived peptide, termed RK-1, was shown to have an identical cysteine residue number and spatial distribution to human defensin suggesting it may be a *bona fide* member of the corticostatin/α-defensin family of peptides [3] (Figure 1). The peptide was demonstrated to have significant growth inhibitory activity against *Escherichia coli*, the pathogen most commonly associated with kidney and urinary tract infections, and is postulated to play an important role in renal pathophysiology. A second α-defensin-like peptide, RK-2, has also been recently identified in the rabbit kidney establishing the existence of a new subfamily of α-defensins in this organ [4].

Abbreviations: CD spectroscopy, circular dichroism spectroscopy; CSI, chemical shift indice; DMF, dimethylformamide; DMSO, dimethylsulfoxide; DQF-COSY, double-quantum filtered correlation spectroscopy; MALDITOF-MS, matrix-assisted laser desorption time of flight mass spectrometry; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; RP-HPLC, reversed-phase high performance liquid chromatography; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; TFA, trifluoroacetic acid; TFE, trifluoroethanol; TOCSY, total correlation spectroscopy.

* Correspondence to: Howard Florey Institute of Experimental Physiology and Medicine, University of Melbourne, Parkville, Victoria 3052, Australia.
E-mail: j.wade@hfi.unimelb.edu.au

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Figure 1 Primary structure of rabbit RK-1 showing the predicted disulfide bond pairings. For comparison, the primary structures of the related defensin, rabbit myeloid α-defensin, NP-1, and defensin cystine motif-like peptides, decorsin and ShK toxin, are also shown.

The three disulfide bond pairs of peptide members of the α-defensin family have a distinctive pairing arrangement of Cys^1-Cys^6, Cys^2-Cys^4 and Cys^3-Cys^5 [5]. Furthermore, the solution structure of α-defensin has been determined and shown to consist almost exclusively of β-sheets and reverse turns [6,7]. As yet, there is no information regarding the disulfide bond concatenation of RK-1 although its primary structure strongly suggests that it, too, adopts a pattern identical to that of human α-defensin. In order to confirm this as well as to more fully study its mode of action, we undertook the solid phase chemical synthesis of the peptide. Following its folding and oxidation in solution and subsequent chemical and biological characterization, the disposition of the disulfide bonds was then determined by ^{1}H-NMR spectroscopy.

MATERIALS AND METHODS

Solid Phase Peptide Synthesis

This was carried out on a 0.1 mmol scale using the continuous flow Fmoc-polyamide method and a MilliGen 9050 automated synthesizer (Bedford, USA) as previously described [8,9]. Side chain protection was afforded by *tert*-butyl esters and ethers for Asp, Glu, Ser and Tyr, trityl for His, Asn and Cys, 2,2,5,7,8-pentamethylchromane-6-sulfonyl for Arg and *tert*-butoxycarbonyl for Lys and Trp. Glycine-17 was introduced as its bis-Fmoc(2-hydroxy-4-methoxybenzyl[Hmb]) pentafluorophenyl (OPfp) ester derivative [10]. The following residue, Asp^{16}, was double-coupled as its pentafluorophenyl ester for 0.5 h each time. All amino acids were purchased from Auspep (Melbourne, Australia). The exception, bis-Fmoc(Hmb)-Gly-OPfp, was obtained from Novabiochem (Läufelfingen, Switzerland). Following synthesis, the peptide was cleaved and deprotected by 2.5 h treatment with 82% trifluoroacetic acid (TFA)/5% thioanisole/2.5% ethanedithiol/5% phenol/5% water and four drops of triethylsilane at room temperature. The crude cleaved peptide was allowed to stand in 0.1 N aqueous acetic acid overnight prior to freeze drying to complete deprotection of the N^{in}-function.

Folding and Oxidation

The crude *S*-reduced peptide was subjected to folding and oxidation in 20% aqueous dimethyl sulfoxide (DMSO) in 5% aqueous acetic acid [11]. The peptide concentration was 1.0 mg/ml. The progress of oxidation was monitored by analytical reversed phase high performance liquid chromatography (RP-HPLC) on a Vydac C18 column (Hesperia, USA) using a gradient of CH_3CN in 0.1% aqueous TFA, and, after 21 h, the reaction was stopped by further acidification of the solution with neat TFA. The peptide was isolated by preparative RP-HPLC purification on a Vydac C18 column.

Characterization

The purity of the synthetic oxidized peptide was assessed by capillary zone electrophoresis (capillary length 50 cm, 10 kV) in 20 mM sodium citrate buffer (pH 4.0), analytical RP-HPLC, and matrix-assisted laser desorption time of flight (MALDITOF) mass spectrometry using a Bruker Biflex instrument (Bremen, Germany) in the linear mode at 19.5 kV. Peptide quantitation was by amino acid analysis of a 24 h acid hydrolysate. Circular dichroism (CD)

spectroscopy was carried out using a Jasco J-720 spectropolarimeter using a 0.2 mm path length cell in the 178–260 nm range. The peptide concentration was 0.3 mg/ml. Double-distilled water and spectroscopy grade trifluoroethanol (TFE) were used as solvents. Mean residue ellipticity is expressed in deg cm^2/dmol by using a mean residue mass of 110.

^{1}H-Nuclear Magnetic Resonance Experiments

Samples of RK-1 prepared for NMR spectroscopy contained approximately 1.5 mM of the peptide dissolved in 90% H_2O:10% D_2O and 100% D_2O at pH 3.5. ^{1}H-NMR spectra were recorded on a Bruker DMX 750 spectrometer at temperatures of 298 and 308 K. Two-dimensional NMR spectra were recorded in the phase-sensitive mode using time proportional phase incrementation for quadrature detection in the f_1-dimension [12]. Two-dimensional homonuclear experiments included TOCSY [13] using a MLEV-17 spin-lock sequence [14] with a mixing time of 80 ms, NOESY [15] with mixing times of 100, 250 and 300 ms and DQF-COSY [16]. For the DQF-COSY experiment, the water protein signal was suppressed by low power irradiation during the relaxation delay (1.8 s). Solvent suppression in the TOCSY and NOESY experiments was achieved using a modified WATERGATE sequence [17] in which two gradient pulses of 1 ms duration and 6 G/cm strength were applied on either side of the binomial pulse. Two-dimensional spectra were collected over 4096 data points in the f_2-dimension and 512 increments in the f_1-dimension over a spectral width corresponding to 13 ppm. TOCSY spectra were acquired with 16 scans per increment, DQF-COSY spectra with 80 scans per increment and NOESY spectra with 64 or 80 scans per increment.

The data were processed and analysed on a Silicon Graphics SGI 4D/30 computer using the UXNMR software package in conjunction with X-EASY [18]. The f_1-dimension was zero-filled to 2048 real data points with the f_1- and f_2-dimensions being multiplied by a sine-squared function shifted by 90° prior to Fourier transformation. Polynomial baseline correction was used in selected regions to improve the appearance of the spectra.

Antimicrobial Assay

The effect of synthetic RK-1 on the growth of *E. coli* NCTC 10418 was determined in sterile 96-well microtitre plates with nutrient broth (pH 7.0) as the growth medium. To each well was added 250 μl of media containing the peptide to give final concentrations of between 5 and 50 μg/ml. To this was added 50 μl of inoculum which was prepared by diluting exponentially growing *E. coli* cells in growth medium to give $\sim 2.7 \times 10^4$ colony forming units/ml. Microplates were incubated aerobically at 37°C and growth was monitored by measuring optical density (OD) at a wavelength of 650 nm.

RESULTS AND DISCUSSION

The solid phase synthesis of RK-1 presented no particular difficulties. The use of *S*-Trt protection was chosen to allow acquisition of the peptide in the fully *S*-reduced form following cleavage and deprotection and prior to folding and oxidation. Possible base-mediated aspartimide formation of the single Asp-Gly within the peptide sequence was prevented by the use of the *N*-(2-hydroxy-4-methoxybenzyl) (Hmb) function [10,19]. Following cleavage and deprotection, the crude *S*-reduced peptide was observed by RP-HPLC to be of good purity (data not shown). The peptide was readily and successfully folded and oxidized by use of DMSO in aqueous acetic acid solution. The reaction was monitored by analytical RP-HPLC and was well advanced after 6 h and was complete overnight. This rate of folding and oxidation compares favorably to about 8 h in the case of synthetic human defensin [11]. This ease of production of RK-1 and defensin in aqueous DMSO contrasts with that for other tricylic peptides such as conotoxins and α-amylase inhibitor [20,21] for which elaborate oxidation methods were required for generation of target peptide. The folded and oxidized RK-1 was purified by RP-HPLC in an overall yield of 5.1%. Comprehensive chemical characterization, which included analytical RP-HPLC (Figure 2B; for comparison, purified *S*-reduced peptide is shown in Figure 2A) and capillary electrophoresis (data not shown), confirmed the high purity of the product. MALDITOF-MS gave a MH^+ value of 3702.4 (calculated MH^+ 3702.4). Nevertheless, none of these techniques provide definite proof of absence of isomeric impurities. While the RP-HPLC data strongly suggested that such impurities were not present, it was necessary to confirm this by unequivocal elucidation of the disulfide bond pairings of the peptide. For most multi-cystine peptides, this can be achieved by controlled proteolytic digestion of the peptide followed by mass spectrometric and/or sequencing analysis of the resulting fragments [22–24]. In the case of the synthetic RK-1, however, its primary structure did not lend itself

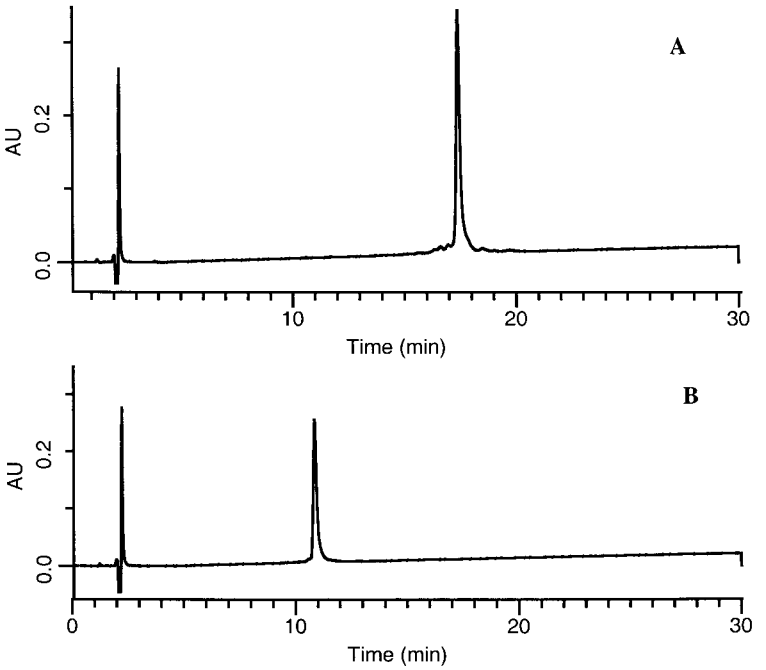

Figure 2 RP-HPLC profile of synthetic purified *S*-reduced RK-1 (A) prior to and (B) after oxidation in aqueous DMSO.

to ready digestion and liberation of clearly defined fragments that would allow determination of the cystine pairings. Attempts were made to overcome this problem by use of controlled slow and sequential *S*-reduction of the cystines by tris(2-carboxyethyl)phosphine hydrochloride (TCEP) followed by *S*-alkylation of the resulting free thiol groups, enzymic digestion and fragment analysis [25]. However, these were not successful due to *S*-reduction of the cystines failing to occur sequentially. For this reason, it was necessary to resort to tertiary structure analysis using ^{1}H-NMR spectroscopy, an approach that was instrumental for the determination of the cystine pairings of Ac-AMP, a three cystine antibiotic peptide derived from the seeds of *Amaranthus caudatus* [26].

The first step in determining the disulfide connectivities of RK-1 by NMR was to make a full spectral assignment and this was achieved from a combination of DQF-COSY, TOCSY and NOESY spectra using standard methods [27]. The D_2O NOESY and H_2O NOESY spectra were then examined for NOEs between all possible pairs of Cys residues. βH^i-βH^j and αH^i-βH^j NOEs (hereafter referred to as $\beta\beta$ and $\alpha\beta$ NOEs) have been shown to be indicative of a disulfide bond between cysteines i and j, whereas the presence of an $\alpha H^i NH^j$ NOE between cysteines is considered to be 'anti-diagnostic' of disulfide bonding [28], that is, the observation of an NOE between αH of one Cys and NH of another normally indicates that they are not disulfide bonded. In the case of RK-1, $\alpha\beta$ NOEs were found between Cys^3 and Cys^{29}, Cys^5 and Cys^{19} and Cys^9 and Cys^{28} (Figure 3). $\beta\beta$ NOEs were also present between Cys^5 and Cys^{19}. No other $\beta\beta$ NOEs were observed between any of the other cysteines although their presence could not be excluded as, if present, they would be obscured by peak overlap as is apparent from Figure 3. In addition to these diagnostic NOEs, no 'anti-diagnostic' αN NOEs between any cysteine pairs were observed. The observed NOEs are summarized in Figure 4. These results strongly indicate that the correct disulfide connectivity is indeed Cys^3-Cys^{29}, Cys^5-Cys^{19} and Cys^9-Cys^{28}, a similar arrangement determined for other members of the corticostatin/α-defensin family [5,29].

To provide additional confirmation of the likely disulfide connectivity, the probable secondary structure of RK-1 was determined from an analysis of chemical shift indices (CSIs) [30]. The data showed that there are three regions (residues 3–6, 12–19 and 26–30) containing three or more sequential CSI values of $+1$ (results not shown). Such stretches of positive CSI values are highly characteristic of β-strands [30]. The positions of these β-strands are identical to those in other members of corticostatin/α-defensin family [29] and strongly suggest that RK-1 shares the common fold

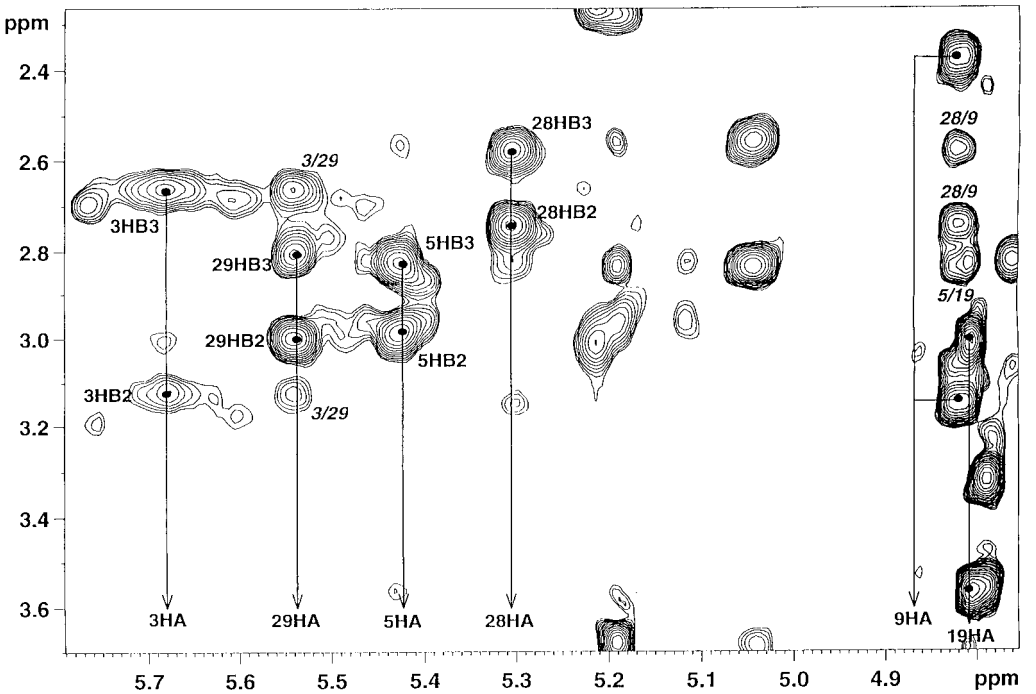

Figure 3 The aliphatic region of the NOESY spectrum of RK1 (100% D_2O, 308 K, pH 3.5, 750 MHz) showing some of the NOEs used to confirm the disulfide connectivity.

of this family of a triple stranded β-sheet with the strands connected by turns.

Further support for this conclusion was provided by the circular dichroism (CD) spectra data obtained for the synthetic peptide. The fully aqueous and the 50% TFE spectra are virtually identical (Figure 5) and the data clearly show a mixture of unordered and β-sheet structures. That the aqueous conformation of the peptide was not at all altered by addition of TFE to 50% indicates that (i) the structure in water is well stabilized by the disulfide bridges, and (ii) the tentatively assigned type U spectrum, characteristic for unordered structures, is in reality a type D spectrum, indicative of the presence of distorted turns [31] that are likely generated by the multiple disulfide bridges within the peptide. Types U and D spectra are very similar particularly if other secondary structural elements are also present in the conformational equilibrium [32]. The shoulder at 216 nm representing the β-sheet structure is clear in each solvent. Taken together, CD spectroscopy data fully supports the NMR findings. Finally, the similar spacing of cysteine residues (Figure 1) and the similar secondary structure to other members of the family further indicate that the disulfide connections are identical.

Preliminary bioassays showed the synthetic RK-1 inhibited *E. coli* growth by 28% at a concentration of 50 μg/ml (Table 1). This was comparable to that of natural RK-1 that was shown to decrease *E. coli* viability by 74% at a concentration of 150 μg/ml [4]. The unusually relatively noncationic structure of RK-1 suggests that it will likely act via a process different to the arginine-rich α-defensins. The availability of the synthetic peptide is presently enabling further studies to determine its precise mechanism of action.

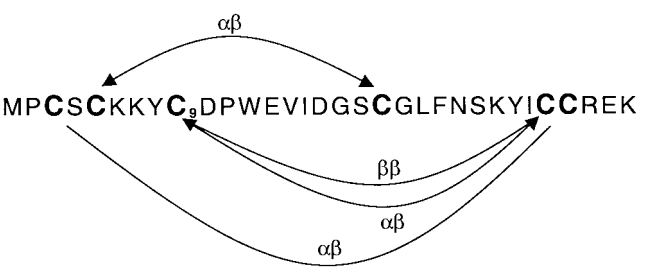

Figure 4 A schematic diagram of the NOEs used to confirm the disulfide connectivity as Cys^3-Cys^{29}, Cys^5-Cys^{19} and Cys^9-Cys^{28}.

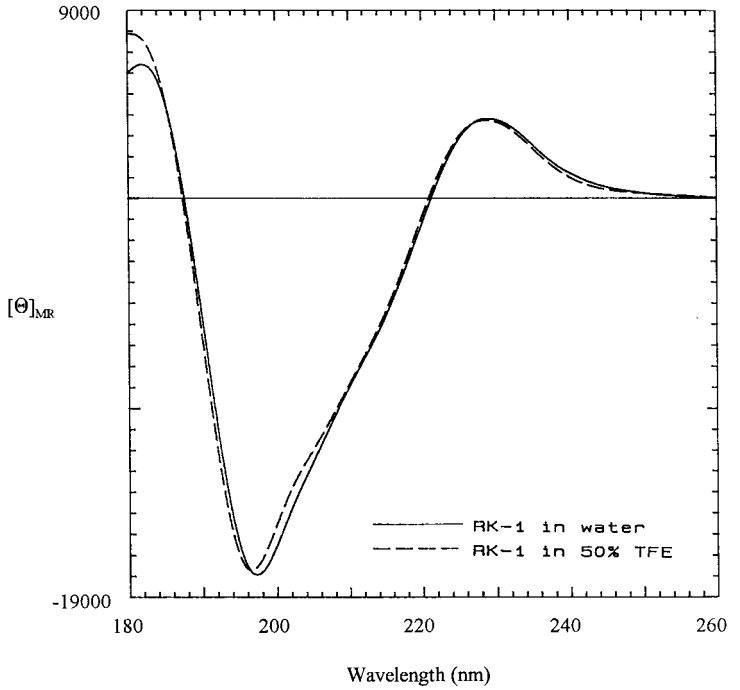

Figure 5 Circular dichroism spectra of synthetic RK-1 in water (solid line) and in 50% aqueous TFE solution (dashes).

Table 1 The Effect of Synthetic RK-1 on the Growth of *Escherichia coli* Determined in a Microtitre Plate Assay

Peptide concentration (μg/ml)	Growth of *E. coli* (%)
0	100 ± 8
5	105 ± 3
10	95 ± 3
50	72 ± 9

CONCLUSION

The synthetic defensin-like peptide, RK-1, was obtained in good overall yield and shown to be active in inhibiting the growth of the microbial pathogen, *E. coli*. Use of ^{1}H-NMR also demonstrated that the peptide possesses a three cystine pairing arrangement identical to other known antimicrobial α-defensin peptides.

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