Structure of microcin C51, a new antibiotic with a broad spectrum of activity

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Abstract The structure of microcin C51, a new antibiotic produced by *E. coli*, has been determined. This antibiotic was shown to be a 1.18 kDa nucleotide peptide. It consists of a heptapeptide with formylmethionine as the N-terminus and a C-terminal asparagine linked with nebularin-5′-monophosphate through the three-methylene bridge. The OH-group of threonine is substituted. The peptide chain of microcin C51 synthesized on ribosomes is the longest among the known biologically active nucleotide peptides.

Key words: Microcin; Peptide antibiotic; Nucleotide peptide

1. Introduction

The microcins are a growing family of low-molecular mass antibiotics produced by enterobacteria. There are seven microcin types which differ in their cross-immunity patterns and other properties [1,2]. Microcin synthesis and immunity have been the subject of detailed genetic investigations [3–6], but there are few data on the chemical structure of microcins [7–9].

In the present report we describe the structure of microcin C51 (MccC51) which is produced by a natural isolate of *E. coli* and inhibits the growth of an unusually wide range of bacterial genera: the antibiotic is active against Gram-negative bacteria of various taxonomic groups as well as some Gram-positive bacteria [2]. The genetic determinants for MccC51 synthesis and preliminary data on its structure have been described previously [6].

2. Materials and methods

The isolation of crude extracts of microcin was as described previously [6]. Purification was performed using an ion-exchange analog of DOWEX 50X2 (SDW 3 BioLar; Latvia) followed by chromatography on an hydrophobic sorbent MPS (Russia) and HPLC, column Zorbax ODS C-18 (Du Pont). Microcin activity was assayed by the growth inhibiting effect on a plate seeded with *E. coli* B. Paper electrophoresis and amino acid analysis were carried out after acid hydrolysis of microcin (5.7 N HCl at 105°C for 18 h). Paper electrophoresis was performed in 2 N ACOH at 550 V for 1.5 h using a set of diaminoalkyls and hydroxyaminoalkyls as standards. The amino acid composition and sequence were determined in an Hitachi 835 analyzer and in a Knauer Model 816 sequencer, respectively. Mass spectra were recorded using a ^{252}Cf time-of-life plasma desorption mass-spectrometer (Experimental model, Ukraine). UV absorption spectra were recorded on a Specord M40 spectrometer (Karl Zeiss, Germany). Carbohydrate composition was determined in a Model LC200 analyzer (Biotronic, Germany) after hydrolysis in 3 M TFA at 105°C for 3 h. ^{1}H NMR spectra were taken using a Bruker spectrometer AMX-400 in D_2O with acetone as an internal reference at room temperature. HMQC and ^{31}P/^{1}H 2D spectra were collected on a Bruker AM-300 spectrometer using standard Bruker software [10]. Trypsin, subtilisin, and carboxypeptidases B and Y were obtained from Sigma; proteinase K was obtained from Boehringer-Mannheim.

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Abbreviations: ACOH, acetic acid; 1D, one dimensional; 2D, two dimensional; COSY, correlation spectroscopy; HQMC, heteronuclear multiple quantum coherence; Mcc, microcin; NMR, nuclear magnetic resonance; ORF, open reading frame; ppm, parts per million; TFA, trifluoroacetic acid.

3. Results and discussion

The final purification step of microcin preparations by HPLC usually showed two peaks eluting at 20.8 and 24.3 min (Fig. 1). The two fractions demonstrated the same antibacterial activity. Molecular masses were determined by plasma desorption mass spectrometry as 1195.4 and 1177.5, respectively. Most of the subsequent experiments were carried out with the second peak. Amino acid analysis showed the following amino acid composition of both microcin peaks: Ala 1.0; Arg 1.1; Asx 2.2; Gly 1.0; Met 0.95; Thr 1.1. Carbohydrate analysis demonstrated the presence of one ribose residue in the microcin molecule. A fragment with mobility corresponding to that of 3-amino-1-propanol was revealed following the acid hydrolysis of microcin.

The UV absorption spectrum, with a maximum at 258 nm, and the pH dependence characteristic for purine derivatives indicated that MccC51 contains an aromatic moiety, perhaps a nucleoside or a nucleotide. The extinction coefficient was estimated to be about $7–9 \times 10^{-3}$.

In order to elucidate the antibiotic structure, the microcin was treated by several proteases, and the analysis of proteolytic fragments fractionated by HPLC chromatography was performed.

The trypsin digestion resulted in two major fragments designated as T1 and T2. The sum of their molecular masses (334 + 862 − 18) was equal to that of native microcin (1178).

Fragment T1 (molecular mass 334) contained equal amounts of Arg and Met. Trypsin cleaves preferentially at the Arg–X peptide bond, and T1, like the native microcin, did not react with ninhydrin; these facts allowed us to consider T1 to be the N-terminal dipeptide with the Met–Arg sequence. The high hydrophobicity and molecular mass of T1 indicate that the NH_2-blocking group may be formyl. Actually, in the ^{1}H NMR spectrum of microcin a signal at 12.2 ppm, typical for a formyl

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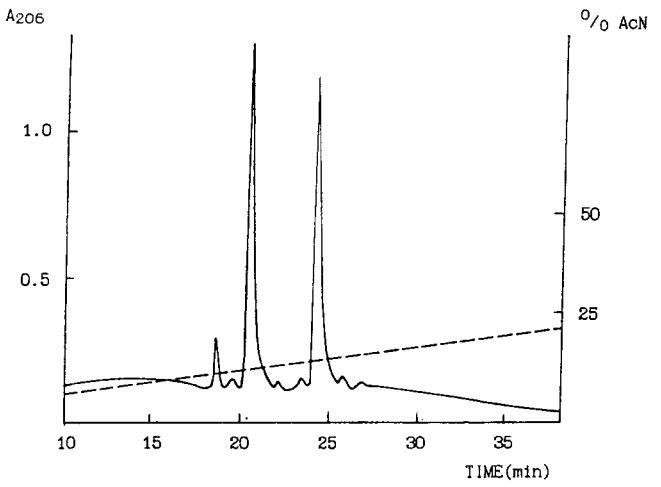

Fig. 1. HPLC elution profile of microcin C51. MccC51 was eluted from a C-18 column with a gradient of acetonitrile in 0.1% TFA. The flow rate was 1 ml/min.

group, was seen. Thus the structure of T1 could be formyl-Met-Arg.

Amino acid sequencing of the C-terminal T2 fragment showed the sequence Thr-Gly-Asn-Ala-Asx. The product of the first cycle of Edman degradation revealed some deviation from normal threonine mobility (not shown). The mass spectrometry of threonine-containing proteolytic fragments revealed the molecular mass increasing by 15, indicating, along with NMR data (not shown), that the OH-group of threonine is substituted by an hydroxylamine residue.

The UV absorption spectrum of T2 fragment corresponded to that of native microcin. The MccC51 resistance to digestion by carboxypeptidases B and Y indicated that the peptide lacked a free carboxyl-terminus. These data allowed us to localize a nucleotide-containing moiety at the C-terminus of the molecule. The characterization of fragments obtained after subtilisin and proteinase K digestion confirmed these conclusions.

The nature of the nucleotide was established using NMR spectroscopy. The ^{1}H NMR spectrum (Fig. 2) contained three one-proton singlets in the aromatic proton region (8.18, 8.40 and 8.44 ppm) and a set of ribofuranose residue signals with chemical shifts H-1 6.20 ppm ($J_{1,2}$ = 8.0 Hz); H-2 4.80 ppm ($J_{2,3}$ = 8.6 Hz); H-3 4.51 ppm ($J_{3,4}$ = 4.0 Hz); H-4 4.43 ppm; H-5 4.49 ppm and H-5′ 4.43 ppm. Analysis of the 2D $^{1}H/^{13}C$ HMQC spectrum [10] showed that signals at 89.2, 75.2, 71.0, 83.8 and 68.1 belong to C-1, C-2, C-3, C-4 and C-5 of the ribofuranosidic residue, respectively. The last two signals were split into doublets with coupling constants 8 (C-4) and 4 (C-5) Hz. The splitting of the ^{13}C signals was a sign of localization of the residue of phosphoric acid at C-5 of the ribofuranose residue. The presence of the last in the microcin molecule was confirmed by direct measurement of the ^{31}P NMR spectrum, where the only signal with chemical shift was seen at −0.2 ppm. The 2D heteronuclear $^{31}P/^{1}H$ COSY spectrum (Fig. 3) showed the correlation peak in coordinates of the chemical shifts of the ^{31}P (−0.2 ppm) and H-5 and H-5′ of the ribose residue (4.49 and

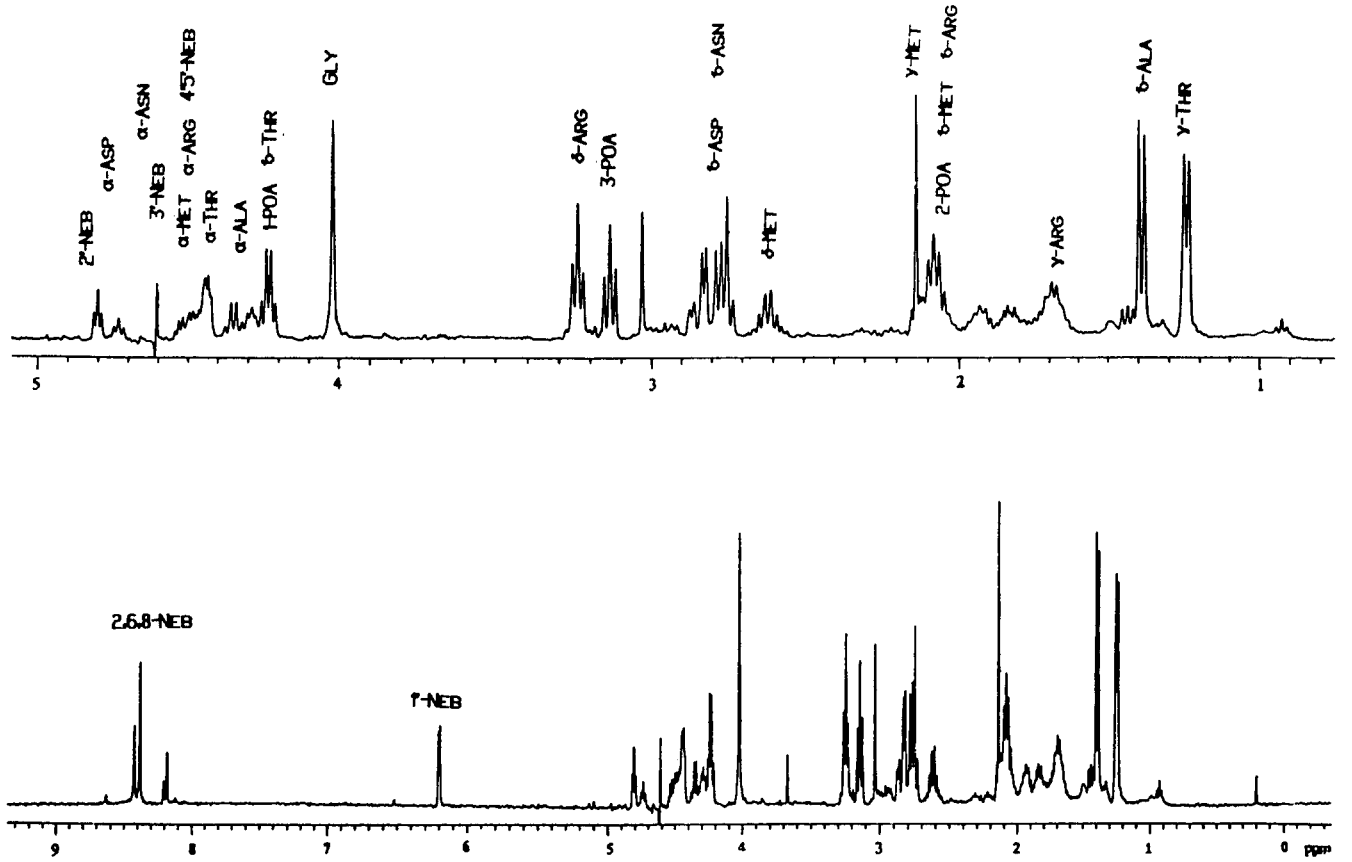

Fig. 2. 400 MHz ^{1}H NMR spectrum of MccC51 in D_2O at 40°C. The signal at 12.2 ppm of C(O)H is not shown. POA, the residue of 3-amino-1-propanol; Neb, the residue of nebularin.

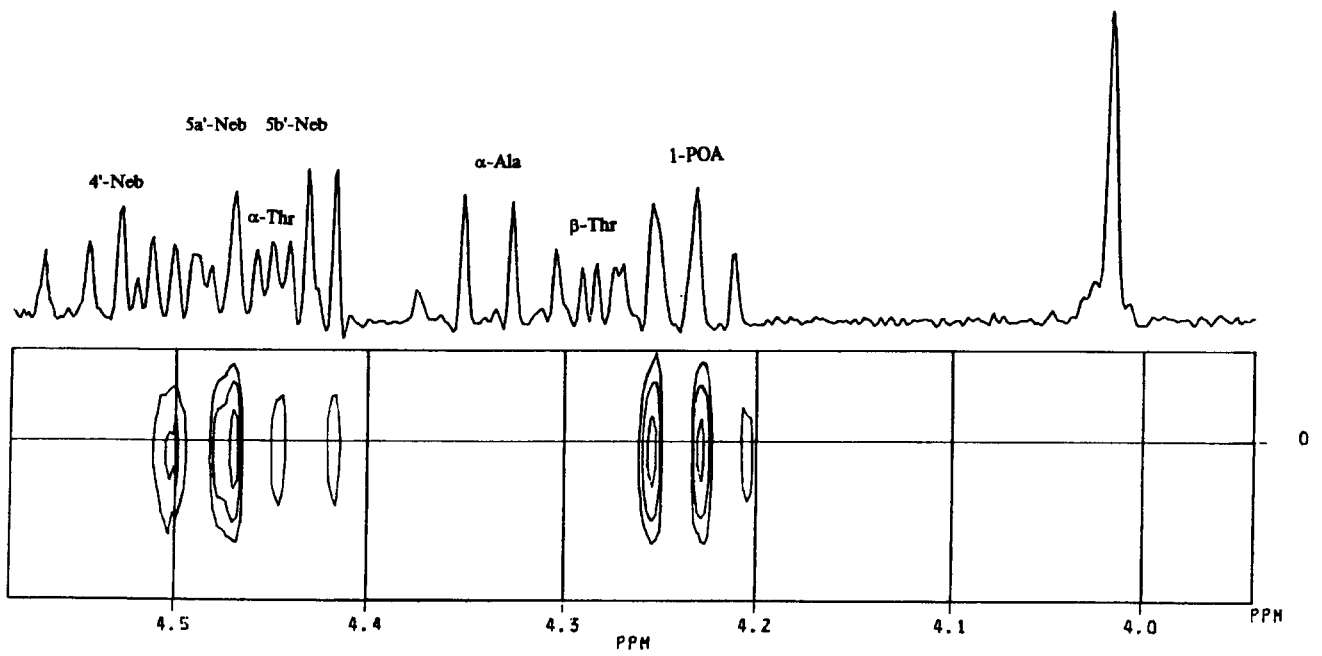

Fig. 3. 300 MHz $^{1}H/^{31}P$ HMQC [10] spectrum of MccC51.

4.43 ppm). The spectrum also contained one additional correlation peak with a chemical shift for protons of 4.23 ppm. In the ^{1}H 1D spectrum a broadened triplet (J = 5 Hz) presented at the same chemical shift. The homonuclear $^{1}H/^{1}H$ COSY spectrum revealed the correlation of the last triplet with the multiplet at 2.09 ppm, which correlated in turn with a triplet at 3.13 ppm. All three of the above mentioned signals were of two-proton integral intensity, i.e. belong to $-CH_2-$ groups. According to the analysis of the HMQC spectrum, the 1D ^{13}C NMR spectrum contained the signals of the carbons bearing these three groups of protons at 67.1 ppm (doublet $^{2}J_{31p\text{-}13c} = 4$ Hz), 28.5 ppm (doublet, $^{3}J_{31p\text{-}13c} = 8$ Hz) and 54.5 ppm (singlet).

Thus, the nucleotide was represented by a nebularin-5′-monophosphate residue which bears the 3-amino-1-propanol residue at C-5′ of the ribose. The chemical shift of the carbon linked with nitrogen (54.5 ppm) was typical for that of alkylated or acylated amino groups.

Therefore, the chemical composition of the non-peptide moiety of MccC51 determined by analytic methods was completely confirmed by NMR spectroscopy. It allowed us to propose the following structure for MccC51 (Fig. 4).

The existence of two active forms of MccC51 (Fig. 1) with molecular masses differing by 18 is believed to be due to formation of *N*-acylhydroxamate.

The unusually broad antibacterial spectrum of MccC51 could be due to the presence of some chemically heterogenic groups. Indeed, as can be seen from Fig. 5, MccC51 includes 9-β-ribosofuranosylpurine, known as the antibiotic nebularin [11] but its activity and antibacterial spectrum are unlike those of native microcin. It was shown that the only other representative of this microcin group C7, the complete structure of which is as yet unknown, contains the same heptapeptide [7], but there are some functional differences from MccC51 [6]. These factors make MccC51 a useful model for researching the role of diverse chemical groups in antibacterial activity.

The presence of formylmethionine at the N-terminus of the molecule suggests that MccC51 is the unprocessed product of ribosomal translation. The plasmid genes determining MccC51 synthesis and immunity were cloned in our previous work [6]. Hybridization of oligonucleotides set corresponding to the amino acid sequence of the heptapeptide with plasmid restriction fragments revealed a plasmid region containing a MccC51

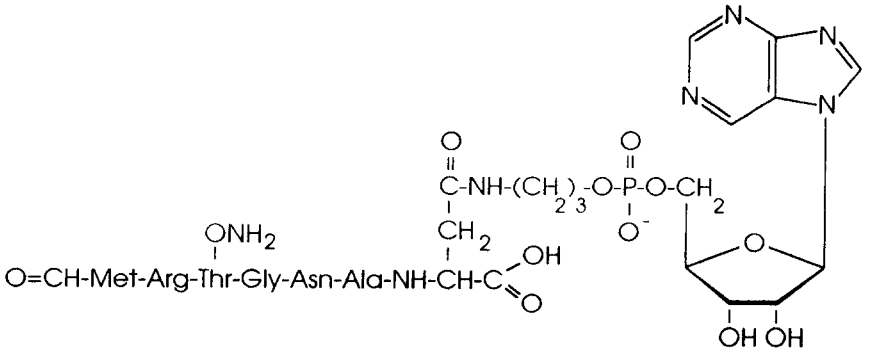

Fig. 4. Structure of microcin C51.

structural gene (Basyuk et al., unpublished). The ORF corresponding to the structural gene for heptapeptide of MccC7 was recently discovered by Moreno et al. by sequencing of corresponding DNA region [12]. These findings confirm the ribosomal origin of the peptide moiety of C-microcins. Two groups have recently demonstrated post-translation modifications of MccB17 [13,14]. The linear peptide, including 69 amino acid residues, undergoes modifications involving the peptide backbone to form oxazoles and thiazoles. The post-translational modifications of MccC51 heptapeptide and genetic determinants controlling these processes are the subject of our further research.

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