Chemical Structure and Translation Inhibition Studies of the Antibiotic Microcin C7*

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Escherichia coli microcin C7 (MccC7) is an antibiotic that inhibits protein synthesis in vivo. It is a heptapeptide containing unknown modifications at the N and C termini (García-Bustos, J. F., Pezzi, N., and Méndez, E. (1985) Antimicrob. Agents Chemother. 27, 791-797). The chemical structure of MccC7 has been characterized by use of 1H homonuclear and heteronuclear (13C,15N,31P) nuclear magnetic resonance spectroscopy as well as mass spectrometry (1177 ± 1 Da). The heptapeptide Met-Arg-Thr-Gly-Asn-Ala-Asp is substituted at the N terminus by a N-formyl group. The C-terminal substituent consists of the phosphodiester of 5'-adenylic acid and n-aminopropanol (AMPap), which is linked via the phosphorus atom to an amide group, thus forming a phosphoramidate. The main chain carbonyl of the C-terminal aspartic acid residue is connected via this amide bond to the modified nucleotide unit. MccC7 and the peptide unit inhibit protein translation in vitro while a synthetic analog of the AMPap substituent is not active. Neither the peptide nor the AMPap molecule has an effect on the growth of MccC7-sensitive cells. Our results strongly suggest that the peptide is responsible for MccC7 antibiotic activity while the C-terminal substituent is needed for MccC7 transport. Implications of the structure determined in this work for MccC7 synthesis and mode of action are discussed.

Microcin C7 (MccC7)* is a small peptide antibiotic produced by Escherichia coli during the stationary phase of growth (1). The spectrum of activity of this microcin includes several members of the Enterobacteriaceae family (2). MccC7 exerts its bacteriostatic action by blocking protein synthesis. Like for other microcins, the bacterial strains that produce MccC7 are immune (resistant) to this microcin (3). The heptapeptide of MccC7 is synthesized in ribosomes (4) and undergoes post-translational modifications to yield the mature molecule.

The genetic determinants for MccC7 synthesis, export, and immunity have been cloned from the 43 kilobases E. coli pMccC7 plasmid into multicopy plasmids that overproduce MccC7 and express MccC7 immunity (5). These determinants lie on a 6.2-kilobase region of pMccC7, which has been entirely sequenced. Different complementary approaches, such as physical and phenotypical characterization of insertion mutations and complementation studies, have shown that this region contains six genes (mccABCDEF) (5). Genes A, B, C, D, and E are involved in the production of mature extracellular microcin. Genes C, E, and F code for self-immunity bestowing products. Genes mccA-D are directly involved in the synthesis and export of MccC7 and constitute an operon transcribed from a promoter (mccp), located upstream of mccA (4, 6). Expression of these genes is regulated by the cAMP-CAP regulatory protein complex and by the stationary phase factor RpoS (also called AppR) (6, 7).

mccA codes for the unmodified peptide of MccC7, MRTGNAN (MccA) (4). The predicted gene polypeptide product of mccB (350 residues) is strikingly homologous to a 81-residue fragment of the ubiquitin-activating enzyme from different eucaryotic species (UBA1) (8), ThfF (9) and Chn1 (10) from E. coli which participate, respectively, in the biosynthesis of thiamine pyrophosphate and of molybdopterin, and HesA, an enzyme required by Anaabaena for nitrogen fixation (11). The predicted mccC product (404 residues) contains 11 potential transmembrane domains and displays significant similarity with stretches of transport proteins; suggesting that MccC is responsible for MccC7 export and explaining why it also confers resistance to exogenous MccC7. The carboxy end of the expected MccC peptide which is highly similar to RimL, an enzyme that acetylates the ribosomal protein L12 from E. coli (12). Principally on the basis of this homology with an acetylating protein, and because target alteration is a common mechanism of antibiotic resistance (13), it has been proposed that MccE might confer MccC7 immunity to producing cells by acetylating the target of microcin C7. No similarity was found for the predicted MccD (267 residues) and MccF (334 residues) polypeptides.

The knowledge of the chemical structure of MccC7 is neces-
Necessary to understand its mode of action, biosynthesis, export out of the producing cell and intake into the target cell, and the self-immunity mechanisms. For these reasons, we undertook the elucidation of the microcin C7 chemical structure, one of the best genetically and functionally characterized microcins.

Amino acid analysis and sequencing of peptides derived from MccC7 tryptic digestions showed that the sequence of the MccC7 peptide is MRTGNAD (2). The N and C termini are both modified, and it has been suggested that the N-terminal might be acetylated, while the C-terminal modification remains unknown (2).

Here, we report on the chemical structure of microcin C7 (Fig. 1) based on complete NMR assignment of 1H, 13C, 15N, and 31P resonances at natural abundance, as well as on the determination of the molecular mass of the molecule (1177 ± 1 Da). We also show that MccC7 inhibits protein synthesis in a cell-free coupled transcription-translation system. Furthermore, we demonstrate that the peptide of MccC7 is responsible for protein synthesis blocking function. Thus, the N- and C-terminal substituents do not have any effect on this function. Finally, some implications of the chemical structure of MccC7 relative to its biosynthesis and mode of action are discussed.

### MATERIALS AND METHODS

**Microcin Production and Purification**—Microcin was prepared from E. coli MC4100 strain containing the multicopy PMM550 plasmid coding for MccC7 (5). After 28 h of incubation at 37°C in M63 minimal medium, the growth culture was centrifuged. Supernatant was passed through a Sep-Pak C18 (Waters) column previously washed with 3 ml of ethanol and 30 ml of deionized water. Once the supernatant had passed through the silica bed, the column was washed with 3 ml of H2O and MccC7 activity was eluted with 2 ml of methanol. A 4:1 (v/v) mixture of chloroform and 50 mM KCl was added to the methanolic solution (2.5 milliliters of mixture per milliliter of methanol). The aqueous phase was then passed through a Sephadex G-25 column equilibrated with a 50 mM KCl aqueous solution. Antibiotic activity and the absorbance at 259 nm were used throughout the purification to identify the microcin containing fractions. Active fractions were pooled, lyophilized, dissolved in H2O, and further purified by RP-HPLC on a Delta-Pak C18 column (Waters). The column was eluted with a 10–40% isocratic gradient of CH3CN in 0.1% trifluoroacetic acid. Fractions containing MccC7 were pooled and then freeze-dried. Typically, 1 mg of microcin was purified from 2 liters of culture.

**Synthetic Molecules—Peptides f-MRTGNAD, f-MRTGNAN, and MRTGNAD were synthesized by the solid phase method of Merrifield (14) using an Applied Biosystems 430A synthesizer. P. methylbenzydrylamine resins were used to obtain C terminus-amidated peptides. After HF cleavage, the crude peptides were purified by C18 reverse-phase medium pressure liquid chromatography followed by semi-preparative C18 RP-HPLC using linear gradients of CH3CN in 0.1% trifluoroacetic acid.

The phosphodiester of 5'-adenylic acid and N-aminopropanol (AMPap) was obtained by reacting overnight the tetraethyl ammonium salt of 5'-adenylic acid with 1-bromo-3-aminopropane in dry CH3CN at 80°C. After evaporation, the reaction mixture was dissolved in H2O and desalted on a G-25 column. The first eluted fractions that were ninhydrin-positive and contained the AMPap molecule, were pooled and lyophylized. AMPap was then purified by C18 RP-HPLC using a linear gradient of CH3CN in 0.1% trifluoroacetic acid.

Final purity (>95%) of synthetic molecules was verified by analytical RP-HPLC. The molecular mass of the formylated peptides was determined by electrospray mass spectrometry and differed from expected mass in accordance with the expected mass. NMR spectra analysis of the peptides and the AMPap molecule was also in agreement with the expected formulas.

**NMR Spectroscopy**—Dried powder of purified microcin was dissolved either in 700 μl of H2O (10% D2O), D2O (100%) (SDS, France), or MeSO-d6 (100%) (Commissariat à l'Énergie Atomique, France). Sample concentration ranged from 0.9 to 9 mM. When needed, the pH was adjusted by adding DCl/HCl or NaOD/NaOH solutions (0.33 N) to D2O/H2O microcin samples. The acidity/basicity of MccC7 samples in MeSO was also changed with these solutions. pH and pD values are reported without correction for isotopic effects. All experiments were run at 27°C.

NMR experiments were performed on a Varian Unity 500 MHz spectrometer equipped with a Sun Sparc 2 station. An indirect detection probe or a multinuclear broad band direct probe were used. Spectra were processed on a Sparc 2 station with Varian's Vnmr software (Varian, Inc.). Sweep widths were for 1H, 5200 Hz in H2O or D2O, and 7200 and 5200 Hz in MeSO; for 13C, 32,000 Hz; for 15N, 4000 Hz; and for 31P, 7500 Hz (indirect detection) and 6000 Hz (direct detection). Chemical shifts were referenced to: 3-(trimethylsilyl)-propane sulfonic acid, sodium salt, for 1H and 13C, 15NH4Cl for 15N, and 85% H3PO4 for 31P, used as external references. All heteronuclear experiments were carried out at isotope natural abundance. When needed, the water peak was eliminated by presaturation during the appropriate delays.

Phase-sensitive COSY (15, 16) and TOCSY (17) using the MLEV17 sequence for spin-locking (18) and mixing times of 80 or 100 ms were performed to identify short and long range scalar couplings. NOESY experiments (19) were run with a 500-ms mixing time. A π pulse at variable times was used during the mixing time to reduce zero quantum contributions.6 ROESY experiments were carried out with a mixing time of 300 ms and a spin lock field of 2.5 kHz to reduce Hartmann Hahn transfers (20). Two-dimensional spectra were obtained with quadrature detection in both dimensions using the hypercomplex method in the rf dimension (19). Usually, 2048 points were acquired in the rf dimension, and 400 or 512 complex points in the size dimension. For each complex data point in the first dimension 8, 16, or 24 free induction decays were accumulated with a relaxation delay of at least 2.4 s. All spectra were apodized with a shifted sine-bell function in both dimension or with a sine-bell function for the COSY experiments.

One bond, 1H-13C, 1H-15N, and one to three bonds, 1H-31P, correlation maps were obtained from 1H detected HSQC experiments (21, 22). For 1H-13C HSQC experiments, a BIRD pulse sequence followed by a 0.9-s delay was used to increase the canalization of signals of protons attached to 13C (23). 1H-15N spectra in dimethyl sulfoxide were acquired without water suppression to observe the maximum of 15N nuclei bearing a labile hydrogen. 1H-31P HSQC spectra were acquired in one and two dimensions for various conditions of solvent and pH. Multiple bond 1H-13C interactions were obtained from HMBC experiments (22, 24).

The delay preceding the 13C pulse for the creation of multiple quanta coherences through several bonds was set to 60 ms. Heteronuclear coupling constant values used in the HSQC and HMBC experiments to establish the delays needed to select the protons coupled to the heteronuclei were the following: 150 Hz (1H-13C), 90 Hz (1H-15N), and 15 Hz (1H-31P). All reverse detection spectra were obtained with the WALTZ-16 pulse scheme for heteronuclear decoupling (25). Heteronuclear two-dimensional spectra were obtained with quadrature detection in both dimensions using the hypercomplex method (19). An exponential function in the t1 dimension and a shifted sine bell in the t2 dimension were used to Fourier transform the heteronuclear spectra.

One-dimensional 13C direct detection spectra were obtained in D2O at different pD values (pD 3.4, 4.3, and 7.3) with microcin sample concentrations higher than 10−3M. A long delay (15 s, as well as a π/3 pulse, were used to allow an efficient relaxation of the quaternary carbons, thus permitting a reliable integration of the signals. Spectra were recorded with 5,800 (pD 3.4), 8,944 (pD 4.3), and 20,032 (pD 7.3) scans over 64 K data points using broad band proton decoupling. One-dimensional 31P spectra were acquired in different conditions of pH and solvent, always with a 3.5-s relaxation delay. 8

4. P. Sodano, unpublished data.
Mass Spectrometry—Electrospray mass spectrometry was performed at the Laboratoire de Spectrométrie de Masse Bio-Organique (Strasbourg) and at the Université de Paris VII (Paris). In order to avoid fragmentation of labile bonds, MccC7 mass spectra were obtained in conditions similar to those used during purification, that is a mixture of 1:1 H2O/CH3CN (v/v), 0.1% trifluoroacetic acid. The observed mass for microcin was 1177 ± 1 Da. The experiment, repeated under different conditions (50% H2O, 25% CH3OH, and 25% CH3COOH) gave the same result.

In Vitro Coupled Transcription-Translation Assays—S30 extracts prepared from E. coli (strain MRE600, RNase I−), and the reaction mixture for coupled transcription-translation were purchased from Amersham (Procaryotic DNA-directed translation kit N.380Z). Circular plasmid DNA template was purified by gradient centrifugation in CsCl, and was added at 4 μg/50 μl of reaction mixture. MccC7 and peptides were added to the concentrations indicated in Fig. 11. Reactions were allowed to proceed for 30 min at 37°C in the presence of L-[35S]methionine (15 mCi, 1000 Ci/mmol). Proteins were then precipitated and the incorporation of radioactivity was determined by liquid scintillation counting.

MccC7 concentration was determined by measuring the absorbance at 259 nm, using an extinction coefficient of 16,500 M−1 cm−1, determined through the quantitative amino acid analysis of a MccC7 solution of known absorbance. The concentration of peptides was determined with the BCA protein assay reagent (Pierce), using a solution of MccC7 of known concentration as standard.

RESULTS

1H homonuclear shift correlation spectra COSY and TOCSY allowed the identification of three distinct units in the molecule: the peptide chain modified in its N-terminal (X-peptide), a sugar ring, and an aliphatic chain. In addition, there are two singlets in the low field region of the spectrum resonating at 8.41 and 8.35 ppm, and integrating for one proton each (Fig. 2). We will describe these different units in the following sections.

X-peptide Structure—All proton resonances in the peptide chain were assigned by analysis of COSY, TOCSY, and ROESY spectra following the method proposed by Wüthrich (26). Proton chemical shifts in H2O are reported in Table I. The sequence found agree with the previously published sequence MRTGNA (2). The C-terminal residue is an AMX spin system in agreement with amino acid composition analysis (N or D) (1), peptide microsequencing (D) (2), and the gene sequence (N) (4). As there are only two amide signals (7.55 and 6.89 ppm) corresponding to an asparagine residue (Fig. 2a) there must be only one asparagine in MccC7. The distinction between an asparagine and an aspartic acid residue was further done from the study of 1H chemical shift variation with pH, as asparagine is neutral while aspartic acid is not. Proton chemical shift variation of the C-terminal residue as a function of pD in D2O or as a function of NaOD added in Me2SO-d6 showed that this residue has an ionizable group. The observed pKa of ~4.2 in D2O is in agreement with an aspartic acid at this position.

One-bond 1H-13C correlation maps (HSQC) and two- or three-bond 1H-13C heteronuclear couplings (HMBC) obtained in D2O allowed the assignment of 13C signals including those of the quaternary carbons. Chemical shifts are reported in Table II.

In previous studies (1), it was not possible to perform the
N-terminal sequence of MccC7 by the Edman degradation method, suggesting the presence of an NH₂ blocking group at the N terminus of the peptide. The singlet observed at 8.12 ppm in the one-dimensional proton spectrum is in fact scalar coupled (\(J = 1.5\) Hz) to the methionine amide proton resonating at 8.45 ppm as observed in COSY spectra. In addition, these two protons are in dipolar contact (rOe). The 8.12 ppm proton is borne by a carbon resonating at 166.8 ppm as shown in a \(1H-13CHCHSQC\) experiment run in D₂O (pD 4.3). This position is compatible with a carbonyl group such as that of formamide (27). From the HMBC experiment, it was possible to (i) determine a \(1H, 13C\) coupling constant of 198 Hz, in agreement with aldehydes such as formamide whose coupling constant is 188 Hz (28) and (ii) to link the proton resonating at 8.12 ppm with the methionine \(\alpha\)-carbon at 54.1 ppm. Taken together, these data are in favor of a N-formyl-Met concatenation. This is further confirmed by analysis of the NMR spectra of the synthetic peptide f-MRTGNAD. Indeed, the one-dimensional proton spectrum of this synthetic peptide also contains a singlet at 8.12 ppm which shows the same coupling pattern (COSY, TOCSY, and ROESY) as the analogous signal in the MccC7 spectra.

In addition to the N- and C-terminal substitutions, the peptide of MccC7 might be modified at other positions. It is thus necessary to identify all the peptide signals, especially those of exchangeable protons. It must be mentioned though, that the \(1H\) and \(13C\) chemical shifts are in agreement with values expected for nonsubstituted amino acids in unstructured peptides (26, 29). As rapidly exchanging proton signals might not be observed in aqueous solvents, proton as well as nitrogen 15 \(1H-15N, HSQC\) assignments were completed in dimethylsulfoxide (Me₂SO-\(d_{6}\)). For this purpose, MccC7 in aqueous solution at pH 3.2 was freeze-dried and dissolved in Me₂SO-\(d_{6}\). Assignments are reported in Table III. All the expected exchangeable protons could be identified. Indeed, the threonine hydroxyl proton (4.95 ppm), the arginine NHₐ (7.56 ppm), and guanidinium group (7.15 ppm) integrating, respectively, for one and four protons, and the two asparagine side chain amide protons (7.52 and 7.03 ppm) were assigned via chemical shift analysis, through-bond (COSY, TOCSY) and through-space (NOESY, ROESY).

### Table I

| Residue | NH | NH-NH₂ | \(\alpha\) | \(\beta\) | \(\gamma\) | \(\delta\alpha\) | Others |
|---------|----|--------|----------|----------|----------|-------------|--------|
| f-Met   | 8.45 | 4.52 | 2.07-2.01 | 2.59-2.54 | 2.08 | CHO: 8.12 |
| Arg     | 8.57 | 4.45 | 1.88-1.77 | 1.63 | 3.18 |
| Thr     | 8.17 | 4.38 | 4.24 | 1.19 |
| Gly     | 8.42 | 3.97 | |
| Asn     | 8.32 | 4.68 | 2.80-2.71 |
| Ala     | 8.36 | 4.28 | 1.34 |
| Asp     | 8.39 | 4.56 | 2.82-2.73 |
| Aliphatic | 8.41 | 4.19 | 2.03 | 3.09 | \(13P: -1.63\) |

### Table II

| Residue | CO | \(\alpha\) | \(\beta\) | \(\gamma\) | \(\delta\alpha\) | Others |
|---------|----|----------|----------|----------|-------------|--------|
| f-Met   | \(\delta 1H\) | 4.53 | 2.08-2.01 | 2.59-2.55 | 2.09 | 8.13 |
|         | \(\delta 13C\) | 176.9 | 54.1 | 33.1 | 31.7 | 16.8 | 166.8 |
| Arg     | \(\delta 1H\) | 4.46 | 1.88-1.79 | 1.64 | 3.19 |
|         | \(\delta 13C\) | 176.2 | 55.9 | 30.6 | 27.0 | 43.1 | 159.3 |
| Thr     | \(\delta 1H\) | 4.39 | 4.25 | 1.21 |
|         | \(\delta 13C\) | 174.9 | 61.5 | 69.7 | 21.3 |
| Gly     | \(\delta 1H\) | 3.98 |
|         | \(\delta 13C\) | 173.7 | 45.1 |
| Asn     | \(\delta 1H\) | 4.69 | 2.80-2.72 |
|         | \(\delta 13C\) | 174.9\(a\) | 53.0 | 38.9 |
| Ala     | \(\delta 1H\) | 4.30 | 1.35 |
|         | \(\delta 13C\) | 177.3 | 52.5 | 18.8 |
| Asp     | \(\delta 1H\) | 4.56 | 2.82-2.73 |
|         | \(\delta 13C\) | 177.7\(a\) | 54.8 | 40.1 |
| Aliphatic | \(\delta 1H\) | 4.18 | 2.03 | 3.09 | 179.0\(a\) |
|         | \(\delta 13C\) | 68.5 | 29.9 | 39.1 |
| Ribose  | \(\delta 1H\) | 6.13 | 4.76 | 4.49 | 4.41 | 4.41-4.43 |
|         | \(\delta 13C\) | 90.2 | 76.5 | 72.5 | 85.1 | 69.3 |
| Adenine | \(\delta 1H\) | 8.29 | 8.37 |
|         | \(\delta 13C\) | 154.4 | 151.6 | 121.3 | 157.4 | 142.7 |

\(a\) Main and side chain carbonyl carbons of Asn and Asp residues were, respectively, assigned from the H-\(\alpha\)-C at H-\(\beta\)-C correlations peaks in the HMBC spectrum. In agreement with this, the main chain carbonyl carbon signal of Asp is coupled to the phosphorus nucleus of the Z substituent while the side chain carbonyl signal is not.
ROESY) interactions (Fig. 3a). The broad signal at 4.95 ppm corresponding to the threonine hydroxyl proton, sharpened upon addition of a small amount of NaOH and became a doublet with a coupling constant of 4.2 Hz, as a result of through-bond coupling with the threonine Hβ proton.

All these signals are also present in the spectra of the synthetic peptide f-MRTGNAD (data not shown). Hence, the peptide is not substituted and the remaining spin systems identified in the proton homonuclear experiments must belong to the C-terminal modification.

C-terminal Modification, Z—In addition to the peptide chain, a sugar ring, an aliphatic chain, and two low field singlets were identified. All these spin systems must belong to the C-terminal modification and have to be linked together to form the Z substituent. Hence, in this section we describe (i) the sugar ring (and the associated base, see below), (ii) the aliphatic chain, and (iii) the structure of the Z substituent. (i) The six non-exchangeable sugar ring protons were assigned mainly from COSY and TOCSY spectra analysis and their chemical shifts are reported in Tables I (H2O, pH 3.4), II (D2O, pH 4.3), and III (Me2SO-d6). The sugar corresponds to a ribose ring. The chemical shift values obtained for the H5 and H4 sugar protons, downfield shifted by −0.7 ppm relative to ribose, suggest that the corresponding carbon is substituted (26). For the same reason, the C2 and C3 carbons do not seem to be substituted. This is validated by the presence of two broad signals of exchangeable protons at 5.58 and 5.38 ppm (Fig. 3b), chemical shifts that are in agreement with that of hydroxyl protons. These two broad signals sharpened upon addition of NaOH. Using a short mixing time of 10 ms in TOCSY spectra to limit the transfer to two or three bonds, it was possible to observe cross-peaks from the signal at 5.58 ppm to the H2′ sugar proton at 4.57 ppm, and from the signal at 5.38 ppm to the H3′ sugar proton at 4.19 ppm.

Having established the proton network in the 1H spectra, the sugar ring carbon signals were identified from a 1H-detected 1H-13C HSQC experiment carried out in D2O (Table II). The chemical shifts values obtained are in accordance with a C5′ substituted ribose bearing a N-glycosyl bond in C1′. The H1′ ribose proton (6.13 ppm) gives an interaction with a quaternary carbon at 151.6 ppm and with a signal at 142.7 ppm (HMBC, Fig. 4a) to which the low field proton resonating at 8.37 ppm is attached (HSQC). In addition, in ROESY experiments, a dipolar interaction between the ribose H1′ sugar proton and the singlet at 8.37 ppm is observed, suggesting that the latter could correspond to a purine proton. Furthermore, this low field proton resonating as a singlet at 8.37 ppm showed an interaction in the HMBC spectrum with two quaternary carbons resonating at 151.6 and 151.6 ppm (Fig. 4b). Finally, the remaining low field proton resonating as a singlet at 8.29 ppm and attached to the carbon resonating at 154.4 ppm (HSQC) is linked via scalar interactions to two quaternary carbons resonating at 157.4 and 151.6 ppm (Fig. 4b). Thus, the proton singlets at 8.37 and 8.29 ppm are both coupled to a carbon resonating at 151.6 ppm and belong to the same coupling network. The coupling pattern observed for the base and the sugar carbons and protons, the proton and carbon chemical shifts (27, 30), and the number of protons and carbons for this unit are characteristic of an adenine or a hypoxanthine linked to a ribose via a N-glycosyl bond. The fact that the MccC7 UV absorbance spectrum presents a maximum at 259 nm (H2O, pH 7) is in favor of an adenine rather than hypoxanthine. Indeed, adenosine presents a UV absorbance maximum at 260 nm at neutral pH, while hypoxanthosine, under the same conditions, absorbs maximally at 249.5 nm (31). These results are strengthened by the identification of the two adenine amino protons (Fig. 3a) as well as of the nitrogen bearing those protons in an 1H-15N HSQC experiment in dimethyl sulfide (Fig. 5). Under more basic conditions, the signal corresponding to the two amino protons at 7.82 ppm (Fig. 3a) sharpened up to become a singlet integrating for two protons while moving upfield at the same time that the H2 and H8 singlets titrated. Under these conditions, a through-bond interaction (TOCSY) with the H2 base proton is observed. Titration of the base amino, H2 and H8 protons is surely caused by the imino nitrogen deprotonation.

![Table III](image-url)
(ii) The aliphatic chain contains three methylene groups coupled one to another, as shown by integration curves and COSY spectra. In Me2SO-<sup>d6</sup>, these methylene proton signals were assigned at 4.07, 1.88, and 2.89 ppm (Fig. 3b). The methylene group at 2.89 ppm is scalar coupled to three exchangeable protons resonating at 7.78 ppm (COSY), and which are attached to a nitrogen resonating at 88.4 ppm (1H-15N HSQC, see Fig. 5). Thus, this group is a n-aminopropanechain (CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-<sup>3</sup>NH<sub>3</sub>).</p>

(iii) For the Z substituent, taking into account the chemical shift values observed for the ribose H<sub>5</sub> and the C<sub>5</sub> signals, as well as the presence of heteronuclear couplings in the one-dimensional proton-decoupled carbon spectrum for the C<sub>5</sub> and C<sub>4</sub> signals (Fig. 6), it was important to check if the molecule contained any phosphorus atom. One signal is indeed observed, at —2.36 ppm in the one-dimensional phosphorus spectrum obtained in Me<sub>2</sub>SO. From a <sup>1</sup>H-<sup>31</sup>P HSQC experiment, it was possible to link the phosphorus nucleus to the ribose H<sub>5</sub> proton (4.12 and 4.26 ppm), to the aliphatic chain (CH<sub>2</sub>)<sub>a</sub> protons (4.07 ppm) as well as to a signal at 9.46 ppm (Fig. 7). The intensity of the signal at 9.46 ppm corresponds to that of one proton. Its coupling constant of 10.5 Hz (Fig. 3a) disappears in proton one-dimensional spectra acquired with phosphorus broadband decoupling, indicating that this signal is coupled to the phosphorus nucleus but not to any other proton. A <sup>1</sup>H-<sup>31</sup>N HSQC shows that this proton is attached to a nitrogen resonating at 92.8 ppm (Fig. 5). Thus, MccC7 contains a P-NH group. In addition, the C<sub>5</sub> ribose and the C<sub>4</sub> aliphatic chain chemical shifts (69.3 and 68.5 ppm, respectively) in D<sub>2</sub>O (pD 4.3), together with the phosphorus chemical shift (—1.63 ppm) are in favor of P-O-C bonds rather than of phosphine bonds (P-C) (27). The <sup>13</sup>C-<sup>31</sup>P coupling constants C<sub>5</sub>-P (4.7 Hz) and C<sub>a</sub>-P (5.5 Hz) are also in agreement with two-bond C-O-P coupling constants (27). This holds true for the three-bond coupling constants C<sub>4</sub>-P (7.9 Hz) and C<sub>b</sub>-P (7.6 Hz). Hence, the MccC7 phosphorus group is a phosphoramide. Together, these results lead to the formula shown in Fig. 1 for the Z substituent.

The comparative analysis of the chemical shifts (and coupling patterns) of the proton signals of the MccC7 Z substituent and of the analogous molecule AMPap (phosphodiester of 5'-adenylic acid and n-aminopropanol) is in agreement with this chemical structure. Indeed, in D<sub>2</sub>O and Me<sub>2</sub>SO, chemical shifts are similar for the corresponding proton signals in both molecules, with the exception of those protons that are closer to the differently substituted phosphorus atom: the ribose H<sub>5</sub> proton and the (CH<sub>2</sub>)<sub>a</sub> aliphatic protons.

We will now describe how the Z substituent is linked to the peptide.

The Microcin C7 Structure—The phosphoramide NH signal...
which could only be seen in Me$_2$SO, is not scalar coupled to other protons in the molecule. Since all the exchangeable proton signals of the Z substituent were identified, the only possibility to append the X-peptide is via this NH group. On the other hand, microcin C7 is not degraded by carboxypeptidase A, B, and Y, suggesting that the C-terminal residue is cyclized, modified, or bears an end blocking group (2). There are two possible positions in the C-terminal residue for branching of the Z substituent: the main chain or the side chain carboxylic groups. The existence of a 10.6 Hz coupling constant between the 31P and the 13C$^\alpha$ of the aspartic residue is in favor of a main chain branching of the Z substituent (Fig. 6).

The proton one-dimensional spectrum in Me$_2$SO displays a broad signal at 12.5 ppm, a typical position for a carboxylic proton (not shown). However, this signal is too broad to produce scalar or dipolar interactions. The chemical shift variation as a function of pH in D$_2$O, or of NaOD added in Me$_2$SO, indicates that the aspartic residue contains an ionizable group. In addition, in the one-dimensional 13C spectrum (D$_2$O), nine carbonyl signals are observed (Fig. 8): six belong to the peptide amide groups f-M, R, T, G, N, and A, and one to the asparagine side chain. Thus, the two remaining carbons must belong to the aspartic residue. In agreement with this hypothesis, the 1H-13C HMBC experiment shows an interaction between the Asp $\alpha$ proton and a carbon signal at 177.5 ppm as well as an interaction between the Asp $\beta$ protons and a carbon signal at 177.8 ppm.$^5$ It should be mentioned though, that the digital resolution in the carbon dimension was quite low (2.65 ppm/point) and it is not possible to know if this correlation peaks correspond to one or two carbons. Finally, one of these two carbons is scalar coupled to the phosphorus ($^1$J$_{13C-31P}$ = 3.2 Hz, see Fig. 8). Hence, we can conclude that the aspartic residue contains a carboxylic and a carbonyl group.

Taking into account the MccC7 molecular weight, the number of carbons determined from the one-dimensional 13C spectrum and the assignments already established, there is only one solution for the Z substituent branching: an amide peptide bond to the phosphoramide NH as shown in Fig. 1.

Several experimental evidences support the proposed branching and lead to the conclusion that the side chain carboxylic group is free. Indeed, (a) the Asp C$\alpha$ is scalar coupled to the phosphorus nucleus (10.6 Hz) while the C$\beta$ is not. This coupling constant value is of the same order of magnitude as the three-bond 13C-31P coupling constant observed between the phosphorus and the sugar ring C4$^\prime$ or the aliphatic chain C$\beta$. In agreement with this observation, the sugar ring C3$^\prime$ and the aliphatic chain C$\gamma$, separated by four bonds from the phosphorus atom, are not scalar coupled to it. Furthermore, in D$_2$O, the Asp C$\beta$ signal broadened at pH 4.3, that is, at a pH value close to the carboxylic group pK$_a$ value, whereas the C$\alpha$ signal remained sharp. In addition, the Asp C$\alpha$ signal broadened (23 Hz at half-height) at pH 7.3 in the phosphoramide pK$_a$ value range.

(b) In Me$_2$SO, a larger chemical shift variation is observed upon the carboxylic group ionization for the Asp H$\beta$ protons ($-0.26$ ppm and $-0.13$ ppm) than for the $\alpha$ proton ($-0.10$ ppm). It is well known that for protons, the inductive effect decreases as the number of bond increases. This observation is also valid

$^5$ The chemical shifts reported in this paragraph (177.5 and 177.8 ppm) were determined from the HMBC spectrum and differ slightly from those presented in Table I, which were obtained from the one-dimensional 13C spectrum. This difference is caused by the low resolution in the HMBC spectrum.
in aqueous solution but the interpretation is not straightforward, as in this environment the aspartic residue and the adenine base titrated with identical $pK_a$ values.

(c) In D$_2$O, the Asp $\beta$ proton chemical shifts remained constant when the pH was changed in the phosphoramidate group $pK_a$ range (Fig. 9), while the Asp $H\alpha$ chemical shift varied (−0.09 ppm) between pH 7.2 and 9. Hence, the Asp $\beta$ protons are not in the direct environment of the phosphoramidate group. Moreover, the Asp $\alpha$ proton displayed the same chemical shift variation pattern as a function of pH than the H5'5' protons and the aliphatic chain $\alpha$ protons which are at a two-bond distance from the phosphorus (Fig. 9).

(d) There is a dipolar coupling between the Asp $\alpha$ proton and the phosphoramidate NH$_{\alpha}$ proton in Me$_2$SO (Fig. 10). This interaction is analogous to the interaction observed in the peptide of MccC7 between the $H\alpha$ proton of residue i and the amide proton of residue i+1.

It was previously reported that MccC7 does not react with ninhydrin (2), a compound that is commonly used to reveal aliphatic amines. However, in the MccC7 structure determined here, there is a primary amine group that should react with ninhydrin. We thus performed the ninhydrin test on thin layers and found that MccC7 is ninhydrin-positive.

Chemical Shift Variations with pH—There are three distinct pH regions for which chemical shifts vary: 3 to 5, 7 to 9, and 10 to 12 (Fig. 9). The transition observed for the aliphatic chain $\beta$ and $\gamma$ protons between −10 and −12, centered at 10.7 corresponds to a typical $pK_a$ value for primary amines or for a lysine side chain, for instance. The same $pK_a$ value was observed for the amine group in the analog molecule AMPa. The transition observed between −3 and −5 involves all the protons close to the adenine imino group or to the $\gamma$ carbonyl of the aspartic residue: the purine H2 and H8 protons, the aliphatic chain methylene protons (CH$_2$)$_{\alpha}$ and (CH$_2$)$_{\beta}$, the ribose ring H5'5' protons, and the aspartic $H\alpha$ and H$\beta$ protons. The transition between −7 and −9 and centered at pH 7.8 involves protons close to the phosphorus atom. This transition must be the result of the phosphoramidate nitrogen deprotonation. Indeed, the magnitude of the induced shift upon titration (13.3 ppm) as well as the direction of it (downfield shift upon ionization) are in accordance with nitrogen deprotonation (33).

It is interesting to note that the phosphorus signal splits into two broad signals when the pH value is near 6. Close to pH 7.8, the phosphorus signal spreads over several units of ppm. At pH values higher than 9, it becomes a single sharp peak once again. These line width changes and splitting are reproducible.
Microcin C7 Structure

**DISCUSSION**

Results described here allow us to conclude on the structure of microcin C7 as presented in Fig. 1. The interpretation of the MccC7 NMR spectra is reinforced through comparative analysis with NMR spectra of the synthetic molecules f-MRTGNAD and AMPap, analogs of the MccC7 formylated peptide and of the C-terminal substituent Z, respectively.

Implication of Our Results for Microcin C7 Biosynthesis —

The polypeptide predicted from the gene mccB, which is known to be directly involved in MccC7 synthesis, is highly similar to a central segment of 158 residues of the enzyme UBA1 (8). The identity is as high as 40% for a sequence of 76 residues that includes the motif Gly-X-Gly-X-Gly, a hallmark of nucleotide binding domains. If conservative substitutions are allowed, the similarity increases to 60%. The ubiquitin-activating enzyme UBA1 (also called E1) catalyzes two reactions: first, the adenylation of the ubiquitin C-terminal glycine, and then the formation of a thioester bond between the adenylylated glycine and a cysteine residue of the enzyme, thus forming a ubiquitin-UBA1 covalent complex (34). In the adenylylated ubiquitin that is formed after the first reaction, the C-terminal carboxyl of ubiquitin is linked via an acyl-phosphate anhydride to the 5’-adenyl-lic acid (34, 35). The homology between this peptidoacyl adenylate and the structure of MccC7, together with the homology found between MccB and UBA1, and the fact that the gene mccB is essential for MccC7 synthesis (but does not participate in immunity), supports very strongly that MccB is responsible for the adenylation of the peptide of MccC7. It is attractive to think that, as in the case of ubiquitin, ATP may act as an AMP donor. We do not know, however, if the N-amino-propane group is incorporated before, after, or simultaneously with the AMP group.

That the C-terminal residue of mature MccC7 is an aspartic acid while the gene mccA codes for an asparagine at this position is not surprising to us: chemical deamidation of Asn has been recognized in proteins and peptides for many years (36–38). In principle, the side chain carboxyl carbon of the C-terminal asparagine should be susceptible to nucleophilic attack by the phosphoramidate nitrogen. Hence, chemical deamidation, which is not usually observed on peptide C-terminal asparagines lacking a neighboring main chain amide nitrogen, is conceivable for MccC7. However, we do not know whether this deamidation occurs enzymatically or chemically, in vivo or during MccC7 purification, whether it is important for peptide modification or not, or whether it is concomitant with peptide modification (adenylation) or not.

Microcin C7 Mode of Action —

MccC7 blocks protein synthesis in vivo without affecting the incorporation of precursors into DNA, RNA, and the cell wall, as shown in previous work (2). Although this result does not permit us to exclude that MccC7 might alter other cellular processes, it does indicate that the antibiotic activity of MccC7 is due, at least in part, to its translation inhibition activity. Hence, that the synthetic peptide f-MRTGNAD inhibits protein translation in vitro with the same efficiency than MccC7, implies that the antibacterial activity of MccC7 is due to its peptide unit. That AMPap, an analog of the modified nucleotide unit of MccC7, does not inhibit protein synthesis in vitro (and in vivo) is in agreement with this proposal. This is further corroborated by the fact that cells carrying the gene mccA coding for the peptide, but lacking the other genes necessary for the synthesis of mature extracellular MccC7 and for microcin immunity, show a decreased growth rate and enter the stationary phase before nutrient depletion.2 These two observations strongly suggest that the unsubsti-
The peptide coded by the gene *mccA*, f-MRTGNAN, inhibits protein synthesis in vitro like the peptide found in mature microcin: f-MRTGNAD. Hence, the deamidation of the asparagine residue is not important for translation inhibition. However, as already noted, this deamidation might be necessary for (or parallel to) the modification of the peptide.

The peptide of MccC7 (f-MRTGNAD) is similar to the leader peptide (MSTSKNAD) involved in the regulation by translation attenuation of the gene cmlA from *E. coli*, which codes for non-enzymatic resistance to chloramphenicol (39). This peptide inhibits the peptidyl transferase activity of the ribosomal 50 S subunit, and binds the peptidyl transferase center of the 23 S rRNA. Given that the peptide of MccC7 inhibits protein synthesis, that both peptides have five residues in common, that the side chain of residue 2 in f-MRTGNAD is not important for protein synthesis inhibition, and that the unformylated peptide (MRTGNAD) has the same in vitro activity that the formylated peptide, it is tempting to think that the target of MccC7 might be the same as that of the cmlA leader peptide. Further

6 J. E. González-Pastor, unpublished data.
experimental work is needed to test this hypothesis.

Comparison of Microcins C7 and C51—It seems important to us to discuss here about microcin C7 and microcin C51 (MccC51) structures, because these independently isolated antibiotics are very similar, and might in fact be the same. The resemblance of the physical and genetic maps of MccC7 (5) and MccC51 (40) is striking. In addition, E. coli strains producing MccC7 and MccC51 exhibit cross-immunity (40). The antibacterial spectrum of activity of both molecules also appears to be similar. However, as different bacterial strains and testing conditions were used in the studies of MccC7 (2) and MccC51 (40), it is not possible to assess if the spectra of action are actually the same or not. Some functional differences might exist; while microcin C7 has been described as a bacteriostatic agent that inhibits protein synthesis (Ref. 2, and this article), microcin C51 has been reported to be a bactericidal agent that inhibits incorporation of precursors into DNA, RNA, and proteins (40). To our knowledge, as of this writing, the announced results of MccC51 inhibition of DNA, RNA, and protein synthesis have not been published. Although, these functional differences might result in being significant, at present it is difficult to ascertain if this is the case; classification of an antibiotic as a bactericidal or bacteriostatic agent depends on experimental conditions such as drug concentration, susceptible strains used, time of incubation in the presence of the drug, and culture conditions. These experimental conditions also have an important influence on the outcome of incorporation of radiolabeled precursors into macromolecules experiments.

Principally based on the molecular mass of MccC51 (1177.5 Da), analysis of MccC51 NMR spectra obtained in D2O at 40 °C, and amino acid sequencing (and mass determination) of MccC51 proteolytic peptides (MRTGNAX, where X represents N or D), Metlitskaya and co-workers (41) have proposed a chemical structure for MccC51. MccC51 would consist of the peptide f-MRTGNAX, a three-methylene chain, and the nucleotide nebularine 5'-monophosphate. The side chain of the N-terminal asparagine would be linked via an amide bond to the three-methylene chain. The aliphatic chain would be linked to the nucleotide phosphate. Finally, the hydroxyl oxygen of threonine would be involved in an hydroxylamine group (-O-NH2). However, we have several arguments to conclude that this proposed structure is not correct. Indeed, the one-dimensional proton spectra of MccC7 (40 °C, H2O, pH 3.2, not shown) and of MccC51 (40 °C, D2O, pH not specified) are very similar. The differences can be accounted for by impurities, labile proton signals which are not present in D2O spectra, line shape of proton signals coupled to exchangeable protons, and a systematic ~0.05 ppm shift for all proton signals (which is probably linked to the different references used in both studies).

In the region between 8.0 and 8.5 ppm, the proton spectrum of MccC51 contains three singlets that integrate for one proton each (8.44, 8.40, and 8.18 ppm). These signals were assigned to the H2, H6, and H8 protons of the nucleoside nebularine, without any experimental proof that these belong to the same system of connectivities. It is astonishing to note that the chemical shift of these resonances corresponds, respectively, to the adenine H8, H2 protons, and to the formyl proton of f-Met in the MccC7 spectrum. A resonance at 12.20 ppm was attributed to the formyl proton of f-Met, arguing that 12.20 ppm is a chemical shift compatible with a formyl signal. No information of scalar coupling with other protons or carbons of the methionine residue is mentioned. However, for MccC7, we were able to demonstrate unambiguously that the signal at 8.12 ppm corresponds to the formyl proton. This assignment is grounded on the scalar (and dipolar) connectivities observed for this proton signal and the methionine protons (and even the α carbon), its intensity and the chemical shift of the carbon attached to it. Furthermore, this signal is also present in spectra of the synthetic peptide f-MRTGNAX and shows the same pattern of connectivities than that seen in MccC7 spectra. That the chemical shift of the formyl proton (and of the rest of the methionine residue protons) is the same in the synthetic peptide and in MccC7, indicates that the C-terminal substituent has no influence on it. Given the similarity of MccC7 and MccC51 spectra (and molecules) it is very unlikely that the chemical shift of the formyl proton could shift as much as ~4

![Figure 10](image1.png)

**Figure 10.** Footprint region of the MccC7 ROESY spectrum (Me2SO, 27 °C). Eight scans were acquired for each of the 400 complex points that constitute the t2 dimension. Only negative contours are shown. The NH-Da cross-peak shows two components separated by the proton-phosphorus coupling constant of NHp (10.5 Hz). This signal belongs to a minor form of the formyl group.

![Figure 11](image2.png)

**Figure 11.** Protein synthesis inhibition by MccC7 (1) and the peptides f-MRTGNAN (2), f-MRTGNAD (3), and MRTGNAD (4) at three different concentrations, using a coupled transcription-translation system. An unrelated synthetic octapeptide, which is a modulator of a neuronal-membrane opioid receptor, was used as negative control and, as expected, did not have any influence on protein synthesis.
 Nevertheless, it should be pointed out that small variations in elution time are often observed for amino acid phenylthiohydantoin derivatives relative to reference compounds during the sequence of peptides. In addition, there is a priori no reason to expect that the mobility of a threonine-phenylthiohydantoin derivative might be similar to that of a phenylthiohydantoin derivative of a hydroxylamine-substituted threonine.

In summary, although at present we cannot know if MccC7 and MccC51 are the same molecule or not, they are very similar. The comparative analysis of the results presented in this work (obtained in different conditions of solvent and pH, with many NMR experiences that produced complementary data, and with synthetic analogs of the peptide and the C-terminal substituent of MccC7), with those reported for MccC51 (41) (only in D₂O at a single pD value and with relatively few NMR data), permits us to conclude that the chemical structure proposed for MccC51 is not correct. It is noteworthy that it has been suggested that the antibiotic activity of MccC51 might be due, at least in part, to its nebulinarine unit (41), nebulinarine being a well known antibiotic (42). However, as stated by Metlitskaya et al. (41), MccC51 and nebulinarine have different antibiotic activity spectra. This apparent contradiction might be easily explained, if as argued above, MccC51 does not contain a nebulinarine unit.

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