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Sequence determination and resonance assignments of an *Azomonas* siderophore using ^{13}C natural abundance ^{13}C–^{1}H HNCA experiment

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Abstract – The determination of the amino acid sequence of small peptides as siderophores is commonly performed using long-range HMBC correlation experiments. However, this type of experiment can lead to ambiguities in the assignment resulting in erroneous primary sequence determination. In this paper, we propose to use an HNCA type experiment on a ^{15}N-labelled sample in order to avoid these ambiguities. This experiment was successfully used to determine the primary sequence of azoverdin, a siderophore produced by *Azomonas*. © 2001 Académie des sciences / Éditions scientifiques et médicales Elsevier SAS

azoverdin / NMR / assignment / iron metabolism

Résumé – La détermination de séquences primaires de petits peptides comme les sidérophores bactériens s'appuie en général sur des spectres RMN de corrélation carbone–proton à longue distance de type HMBC. Cependant, ce type d'expérience ne permet pas de résoudre toutes les ambiguïtés d'attribution et peut conduire à des erreurs de séquence primaire. Dans cet article, nous proposons d'utiliser une expérience de type HNCA sur un échantillon enrichi à l'azote 15 afin de lever ces ambiguïtés. Cette expérience a été appliquée avec succès pour déterminer la séquence primaire de l'azoverdine, un sidérophore d'*Azomonas*. © 2001 Académie des sciences / Éditions scientifiques et médicales Elsevier SAS

azoverdine / RMN / attribution / métabolisme du fer

The abbreviations used are: common amino acids 3-letter code; AcOHOrn, Nδ-acetyl-Nδ-hydroxy-ornithine; Dab, 2,4-diaminobutyric acid; Hse, homoserine; HMBC, Heteronuclear Multiple Bond Correlation spectroscopy; HSQC, Heteronuclear Single Quantum Coherence spectroscopy.

1. Introduction

Siderophores are small organic molecules that are used by bacteria to obtain iron under limiting conditions [1]. A group of these molecules named pyoverdins is composed of a short peptide bound to a fluorescent chromophore derived from 2,3-diamino-6,7-dihydroxyquinoline. The investigation of the chemical structure of these molecules for different micro-organisms has led to the discovery of a wide variety of peptide sequences that differ in their amino acid composition (unusual amino acids are used) and length. The determination of the amino acid sequence is performed using mass spectrometry of limited proteolysis fragments and

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NMR. For the latter, the sequential assignments of the backbone carbon nuclei are commonly obtained using long-range HMBC correlation experiments. However, the use of this type of experiment can lead to ambiguities in the assignments resulting in erroneous primary sequence determination. In this paper, we propose to use the HNCA experiment on ^{15}N-labelled compounds in order to determine the primary sequence of pyoverdins. This type of experiments was designed for assignment of backbone atoms of ^{15}N,^{13}C-labelled proteins [2, 3] and is now widely used in protein structure determination. We show here that HNCA spectra can be recorded on chromopeptides that are enriched only in ^{15}N. We have successfully used this approach to determine the primary sequence of azoverdin, a siderophore produced by *Azomonas macrocytogenes* ATCC 12334, which is composed of a six amino acid peptide chain linked to a chromophore. Two chemical structures were published that differed in the sequence of the peptide chain and in the site of covalent linkage on chromophore [4, 5]. The use of a HNCA experiment at natural ^{13}C abundance allowed us to identify the correct chemical structure and to confirm that azoverdin defines a new class of pyoverdins: the isopyoverdins. We report here the complete assignment of azoverdin bound to gallium, a diamagnetic substitute for iron.

2. Materials and methods

2.1. NMR sample

Azomonas macrocytogenes strain ATCC 12334 was grown in a rotary shaker in aerobic and iron-limited conditions, at 301 K, during 48 h. The siderophore was labelled uniformly with ^{15}N. NMR experiments were conducted on a sample containing 10.4 mM azoverdin in H_2O:D_2O 11:1, pH 3.6, at 298 K on a Bruker DRX-600 spectrometer.

2.2. The ^{13}C–^{1}H HNCA/CO experiment

The pulse sequence used to assign the backbone atoms of azoverdin is shown in *figure 1*. This sequence is basically a slightly modified 2D version of the HNCA sequence proposed by Kay et al. [2], which is adapted to take advantage of the low isotopic abundance of the ^{13}C nuclei within the molecule. The HNCA sequence was designed to observe correlations between the amide proton of one residue and alpha carbons of the same and the preceding residues, allowing the sequential assignment of these resonances throughout a polypeptide chain. In ^{13}C natural abundance samples, the absence of carbon homonuclear couplings allows the use of high-power square pulses in the carbon channel in order to cover both carbonyl and aliphatic carbon frequencies. In addition, decoupling

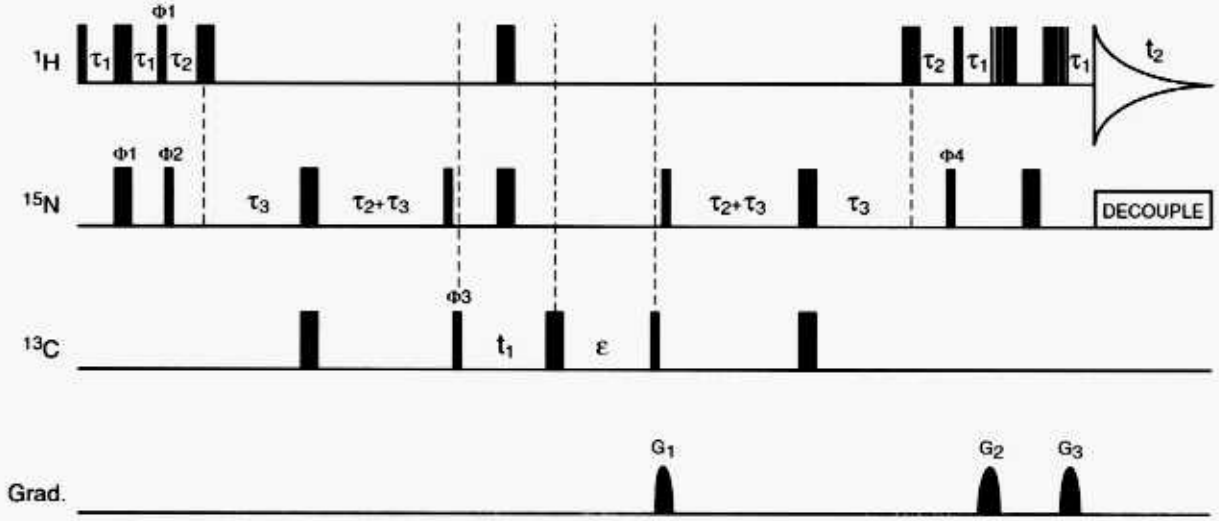

Figure 1. Pulse sequence of the HNCA/CO experiment. Narrow pulses correspond to a 90° flip angle and wide pulses to 180°. Pulses for which the RF phase is not marked are applied along the *x* axis. Delay durations are τ_1 = 2.6 ms, τ_2 = 2.4 ms and τ_3 = 12.6 ms. ε is set to the first t_1 increment. Gradient lengths are G1 = 400 µs, G2 and G3 = 800 µs. All gradients are sine-bell shaped, with a gradient strength of 2 G·cm^{-1} at the G1 midpoint and 12.5 G·cm^{-1} at the G2 and G3 midpoints. The phase cycling used is as follows: Φ_1 = *y*, *y*, –*y*, –*y*; Φ_2 = *x*,–*x*; Φ_3 = *x*, *x*, *x*, *x*, –*x*, –*x*, –*x*, –*x*; Φ_4 = *x*, *x*, *x*, *x*, *x*, *x*, *x*, *x*, –*x*, –*x*, –*x*, –*x*, –*x*, –*x*, –*x*, –*x*; receiver = *x*, –*x*, –*x*, *x*, –*x*, *x*, *x*, –*x*, –*x*, *x*, *x*, –*x*, *x*, –*x*, –*x*, *x*.

of the carbonyl frequencies is not required during the carbon chemical shift evolution period. These modifications together with the similar $^1J_{NC'}$ and $^1J_{NC\alpha}$ (15 and 11 Hz respectively) allows both the HN–CO and HN–CA correlations to be observed in the same experiment, referred to below as ^{13}C–^1H HNCA/CO.

2.3. Experimental parameters

For the 2D HNCA/CO experiment, a total of 1 024 fids with 32 scans of 2 048 complex data points in t2 were collected during 25.5 hours. Quadrature detection in F1 was achieved using the time-proportional phase incrementation scheme (TPPI) [6]. The spectral widths were 29 411 Hz (194.88 ppm) in F1 and 8 012 Hz (13.35 ppm) in F2. The carbon-offset frequency was set to 96.426 ppm. The 90° pulse lengths were 13.1, 30.0 and 12.5 µs for the proton, nitrogen and carbon nuclei respectively. The delays τ_1, τ_2 and τ_3 were set to 2.6, 2.4 and 12.6 ms respectively. The water signal was eliminated using a Watergate sequence [7] during

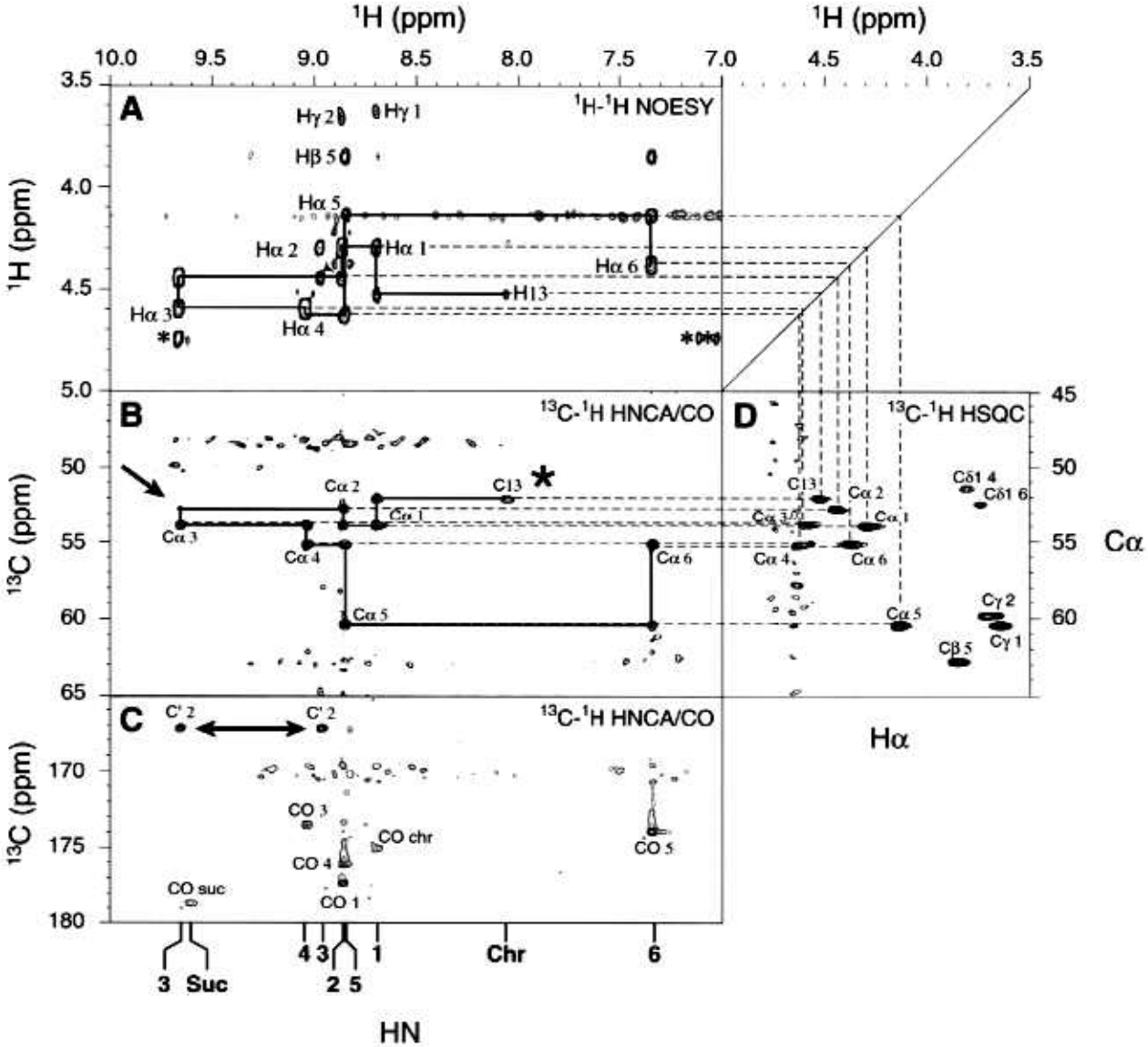

Figure 2. Spectra obtained for a sample of azoverdin (10.4 mM in H_2O/D_2O 11:1), ^{15}N labelled. **A.** Hα-HN correlations region of NOESY spectrum. **B.** Cα-HN correlations region of HNCA/CO spectrum. **C.** CO–HN correlations region of HNCA/CO spectrum. **D.** Cα–Hα correlation region of ^{13}C–^1H HSQC spectrum. Sequential assignment pathway is shown in both the NOESY and the ^{13}C HNCA/CO spectra. The amide proton assignment is indicated at the bottom of *figure 2C*. In *figure 2A*, stars indicate correlation with water signal. In *figure 2B*, the connectivity seen between the chromophore C13 carbon and its amide H14 proton is pointed out by a star and the sequential connectivity between the amidine proton HNα of 3-Dab and the Cα of 2-Hse is shown by an arrow. In *figure 2C*, common correlations between the two amidine proton of 3-Dab with the carbon C' of 2-Hse is indicated by an arrow.

the last proton refocalisation spin echo sequence. Nitrogen decoupling was performed during acquisition using a GARP sequence [8]. Processing was performed on an SGI INDY R5000 computer using XWIN–NMR software (Bruker). A 90°-shifted sine-bell apodization function was applied in both dimensions before Fourier transform. Spectra were analysed with the XEASY program [9]. The proton and carbon frequencies were referenced using 2,2-dimethylsilapentane-5-sulfonate (DSS) as an external reference.

For the HMBC experiment, a total of 800 fids with 64 scans of 2 048 complex data points in t2 were collected during 48 h. The spectral widths were 32 000 Hz (212.02 ppm) in F1 and 5 482 Hz (9.14 ppm) in F2, and the long range coupling evolution delay was set to 80 ms.

3. Results

The 2D ^{13}C–^{1}H HNCA/CO spectrum recorded on azoverdin complexed with gallium is shown in *figures 2B* and *2C*, together with the fingerprint region of a NOESY spectrum and the Cα–Hα correlation region of a ^{13}C–^{1}H HSQC (*figures 2A* and *2D* respectively). The sequential assignment pathway is shown in both the fingerprint region of the NOESY and the ^{13}C–^{1}H HNCA/CO spectra. The high resolution used in the carbon dimension allowed an unambiguous assignment of the alpha carbons avoiding the use of a third nitrogen dimension. The side chain resonances were subsequently assigned using a TOCSY spectrum combined with the ^{13}C–^{1}H HSQC. The chemical structure of azoverdin deduced from the heteronuclear NMR experiments is shown in *figure 3*. The sequential Cα(*i*)-HN(*i*+1) connectivities observed in the ^{13}C–^{1}H HNCA/CO spectrum indicates a primary sequence in that is in agreement with Michalke et al. [5]:

Hse–Hse–Dab–AcOHOrn–Ser–AcOHOrn

In this sequence, the Dab residue, which results from a condensation of the two amino groups of the diaminobutyric acid with the carboxyl group of the homoserine is located in the third position of the sequence invalidating its first C-terminal localisation (4). The erroneous assignment was due to the lack of sequential ROE cross peaks as well as the lack of long-range HMBC correlations between the homoserine and the carbon belonging to the Dab. The connectivity observed between the amidine proton HNα of the Dab and the Cα of homoserine 2, although weak (see arrow in *figure 2B*), provides unambiguous proof of the sequential link between the second homoserine and the Dab. The unique intra-residual HN–Cα connectivity allows the backbone HN resonances of

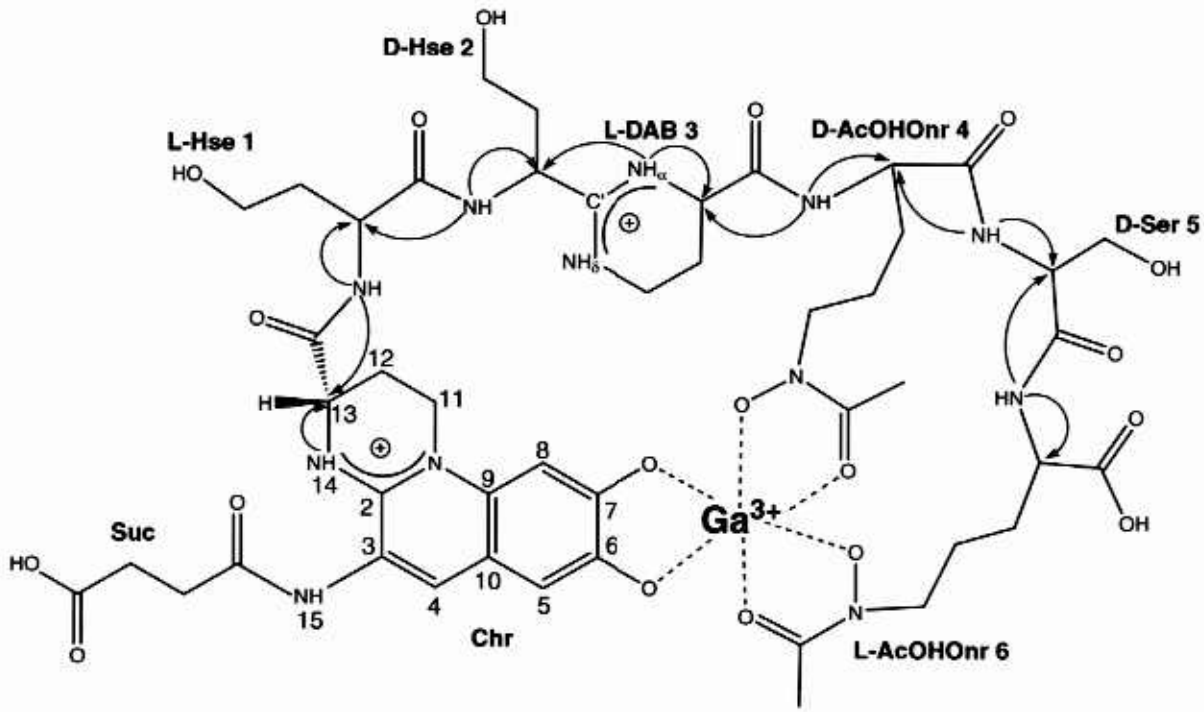

Figure 3. Chemical structure of azoverdin checked by HNCA/CO experiment results. Observed connectivities between HN and Cα in the ^{13}C HNCA/CO spectrum are shown by arrows.

Table. Assignments of ^{1}H,^{15}N and ^{13}C resonances for ^{15}N-labelled azoverdine-Ga(III) at 298 K, pH 3.6, in H_2O/D_2O 11:1, using 2,2-dimethylsilapentane-5-sulfonate (DSS) as an external standard.

	^{1}H chemical shifts (ppm)							^{15}N chemical shifts (ppm)	
	HN	Hα	Hβ	Hγ	Hδ	Others		NH	Others
1-Hse	8.69	4.29	1.86	3.63				135.07	
2-Hse	8.85	4.43	1.97	3.69–3.64				133.09	
3-Dab	9.65	4.58	2.37–2.16	2.81–2.66		HNδ: 8.96		126.55	NHδ: 124.06
4-AcOHOrn	9.03	4.61	1.89–1.76	1.60	3.80–3.35	H acetyl: 2.05		136.08	Nε: 204.17
5-Ser	8.84	4.13	3.84					132.02	
6-AcOHOrn	7.34	4.38	1.84–1.62	1.10	3.73–3.34	H acetyl: 213		133.57	Nε: 199.65
	^{1}H chemical shift (ppm)								
	H4	H5	H6	H11–H11'	H12	H13	H14		
Chr	7.80	6.77	6.81	2.41–2.58	3.81	4.53	8.05	102.09	N1: 157.19
	H15	Hα	Hβ						
Suc	9.60	2.73	2.69						N15: 135.81

	^{13}C chemical shifts (ppm)					
	CO	Cα	Cβ	Cγ	Cδ	Others
1-Hse	177.41	53.93	35.34	60.34		
2-Hse		52.75	36.45	59.60		C': 167.05
3-Dab	175.08	53.83	24.66	38.89		
4-AcOHOrn	176.06	55.22	33.56	25.33	51.42	CO acetyl: 165.56 C acetyl: 18.80
5-Ser	173.97	60.29	62.70			
6-AcOHOrn	177.64	55.10	29.97	24.19	52.44	CO acetyl: 164.66 C acetyl: 18.80

	^{13}C chemical shifts (ppm)						
	CO	C2	C3	C4	C5	C6	C7
Chr	173.34	150.82	116.12	141.46	110.91	154.53	164.23
		C8	C9	C10	C11	C12	C13
Chr		99.51	136.46	136.46	25.42	45.57	51.98
	CO	Cα	Cβ	Cγ			
Suc	179.95	33.01	45.57	31.82			

two amidine protons of the Dab (HNα and HNδ) to be assigned. There are clearly identified by their common correlation to the C' carbon atom of homoserine 2 in the HNCO region (see arrow in *figure 2C*). The C-terminal linkage of the Dab Cα carbon with the following acetylhydroxyornithine (AcOHOrn) is also clearly shown by a cross peak between this carbon and the amide proton of the AcOHOrn.

A further difference between the two reported structures of azoverdin was the location of the peptide anchoring point to the chromophore. The connectivity seen between the C13 of the chromophore and an amide proton at 8.05 ppm (indicated by a star in *figure 2B*) clearly defines the C13 position as the binding point and distinguishes azoverdin from other known pyoverdins where the peptide moiety is linked to the chromophore at position C11. This identifies the azoverdin as an isopyoverdin, as suggested by Michalke et al. [5].

Figure 2C shows the correlations between amide protons and the carbonyl atoms of preceding residues. This region of the ^{13}C–^{1}H HNCA/CO spectrum allowed the assignment of all carbonyl atoms (*table*), with the exception of the C-terminal AcOHOrn.

In order to assess the advantage of the ^{13}C–^{1}H HNCA/CO over the ^{13}C–^{1}H HMBC that is usually used to identify sequential connectivities in peptides, we compared the two spectra recorded on the same azoverdin sample. In the HMBC spectrum, the carbonyl resonance assignment is obtained using the intra-residual correlation between the Hα and the carbonyl atom. For azoverdin, these correlations display a large range of intensities. If high signal-to-noise ratios were obtained for residues 1-Hse, 2-Hse and 5-Ser, no peak could be detected for 3-Dab and the correlation between the H13 and the carbonyl atom of the chromophore is very weak. In contrast, all inter-

residual correlations are present in the spectrum shown in *figure 2C*.

4. Conclusion

We have shown that the use of HNCA-type experiment could be used on ^{15}N-labelled peptides to provide unambiguous sequential assignments. This removes the need of expensive ^{13}C-labelling, provided that the peptide can withstand high concentrations. Under these conditions, HNCA provides a powerful alternative to the widely used HMBC experiment. Using this technique, we established the azoverdin chemical structure and determined the full assignment of its gallium complex.

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