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Isolation and characterization of the siderophore *N*-deoxyschizokinen from *Bacillus megaterium* ATCC 19213

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N-Deoxyschizokinen, a novel siderophore, was isolated from stationary phase cultures of *Bacillus megaterium* ATCC 19213 and identified as 4-[(3(acetylhydroxyamino)propyl)amino]-2-[2-[(3-(acetylamino)propyl)amino]-2-oxoethyl]-2-hydroxy-4-oxo-butanoic acid. The siderophore was purified by HPLC and its structure determined using ^{1}H and ^{13}C NMR, ^{1}H–^{1}H COSY and electrospray mass spectroscopy. The monohydroxamate siderophore has the same carbon skeleton as schizokinen but the hydroxyl group on one hydroxamate is replaced by a hydrogen. A detailed ^{1}H NMR study of schizokinen, *N*-deoxyschizokinen and their imides, schizokinen A and *N*-deoxyschizokinen A is presented.

Keywords: *Bacillus megaterium*, *N*-deoxyschizokinen, schizokinen, schizokinen A, siderophore

Introduction

Many organisms produce highly specific chelators called siderophores in response to iron limitation. These siderophores chelate and transport iron via specific transport systems to promote cell growth. A specific siderophore is often synthesized by more than one species of microbes. For example, the siderophore ferrichrome has been widely detected in both basidiomycete and ascomycete fungi, including *Ustilago* spp., *Penicillium* spp., *Aspergillus niger* and *Tilletiaria anomala* (Neilands 1981, Winkelmann & Huschka 1987). Organisms may also produce multiple siderophores under iron limitation. Examples include ferrichrome and ferrichrome A isolated from *Ustilago maydis* (Wang *et al.* 1989), and parabactin and parabactin A identified from *Paracoccus denitrificans* (Tait 1975, Peterson & Neilands 1979). In these examples, the chemical structures of the siderophores are closely related. *Erwinia herbicola* FM13 produces enterobactin, ferrichrome and coprogen, three siderophores with quite different chemical structures (Berner *et al.* 1988, Berner & Winkelmann 1990). Production of the uptake receptors necessary to use the individual siderophore is often separate from production of the siderophore itself. *Escherichia coli* synthesizes the uptake receptors for six different siderophores, even though it apparently only produces enterobactin (Winkelmann 1991). *Ustilago sphaerogena* produces both ferrichrome and ferrichrome A, but only has the uptake receptor for ferrichrome (Emery 1971).

Bacillus megaterium ATCC 19213 produces the dihydroxamate siderophore schizokinen. This compound was originally isolated as a factor active in the initiation of cell division (Lankford *et al.* 1966, Byers *et al.* 1967) and later shown to be important in iron uptake (Mullis *et al.* 1971). Schizokinen has been isolated from the cyanobacteria *Anabaena* sp. ATCC 27898 (Simpson & Neilands 1976, Lammers & Sanders-Loehr 1982) and *Anabaena* sp. strain PCC 7120 (Goldman *et al.* 1983), and is involved in iron uptake by these species. It has also been found in the soil of rice fields after annual flooding (Akers 1983). Schizokinen is composed of a single residue of citric acid symmetrically substituted with 1-amino-3-(*N*-hydroxy-*N*-acetyl)-aminopropane (Figure 1a). The two hydroxamate groups and the hydroxyl and carboxyl groups on the citrate backbone form the six coordination sites for ferric iron (Plowman & Loehr 1982, Plowman *et al.* 1984). A second neutrally charged compound called schizokinen A can be separated from schizokinen by ion exchange chromatography. The imide linkage of schizokinen A (Figure 1b) readily forms under acidic conditions and schizokinen A may represent an artifact formed from schizokinen.

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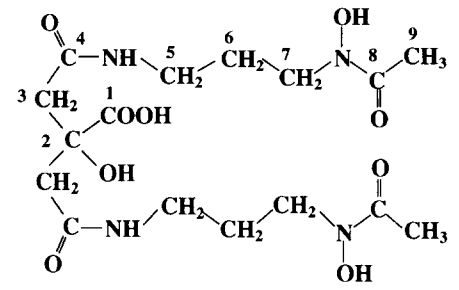
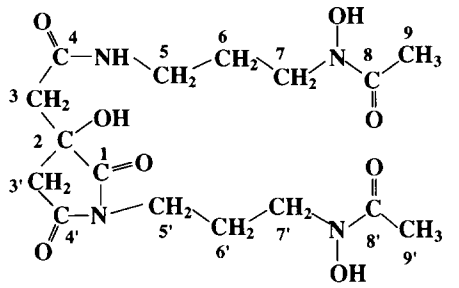
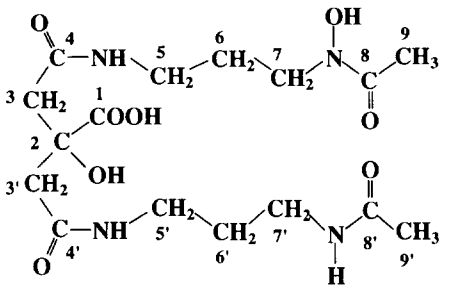


a. Schizokinen



b. Schizokinen A



c. Deoxyschizokinen

Figure 1. The structures of schizokinen, schizokinen A and *N*-deoxyschizokinen.

In this paper, we describe the isolation and structural characterization of a third siderophore from iron-limited cultures of *B. megaterium* ATCC 19213. The structure of this siderophore was determined to be *N*-deoxyschizokinen (Figure 1c) on the basis of high resolution NMR and electrospray mass spectroscopy. Spectral data for schizokinen and schizokinen A are presented for comparative purposes.

Materials and methods

Isolation of N-*deoxyschizokinen and schizokinen*

B. megaterium ATCC 19213 was obtained from the American Type Culture Collection (Rockville, MD) and grown in 2 l acid-washed glass Erlenmeyer flasks containing 1400 ml media (pH 7.0) consisting of 1.0 g K_2SO_4, 3.0 g Na_2HPO_4, 3.0 g NH_4OAc, 20 g sucrose, 800 mg $MgSO_4 \cdot 7H_2O$, 8.6 mg $ZnSO_4 \cdot 7H_2O$, 0.113 mg $MnSO_4 \cdot H_2O$ and 1.5 g arginine hydrochloride per liter as described in Mullis *et al.* (1971). The cells were inoculated with 1.0 ml of an iron-replete (64 mg $FeSO_4 \cdot 7H_2O$ per liter) suspension of *B. megaterium* and grown at 37°C with shaking (130 r.p.m). Cultures generally showed a 1–2 day lag phase followed by exponential growth, reaching stationary phase in 4–7 days. After 10 days, the cells were removed via centrifugation at 12 000 r.p.m. and the supernatant concentrated by lyophilization. The lyophyllate was resuspended in distilled water, applied to a Bio-Gel P-2 column (2.5 × 80 cm; Bio-Rad, Hercules, CA) and eluted with 10% (v/v) methanol/water. Each fraction was monitored for siderophore activity using the ferric perchlorate assay and positive fractions confirmed using the ^{55}Fe-binding assay. The fractions with siderophore activity were lyophilized, suspended in distilled water and extracted with an equal volume of 1:2 (w/w) chloroform:phenol. Four volumes of diethyl ether were added to the organic layer and this phase was extracted twice with 1 volume distilled water. The resulting aqueous phase was lyophilized, dissolved in distilled water and applied to a 1.3 × 12 cm Dowex AG 2-X10 column in the acetate form. The column was eluted with 40 ml of distilled water and then a 40 ml each step gradient of 0.1–1.0 M ammonium chloride in 0.1 M increments. Individual fractions were tested for siderophore activity using the ferric perchlorate assay, and positive fractions were lyophilized, suspended in a minimum volume of distilled water and purified by isocratic HPLC using a Hamilton PRP-1 column (10 μm, 7.0 × 250 mm; Hamilton, Reno, NV) and 15% methanol in water as eluant. The eluant was monitored at 220 nm. Individual peaks were collected, tested using both the ferric perchlorate and the HPLC ^{55}Fe-binding assay, and those fractions with siderophore activity lyophilized.

Measurement of siderophore activity

For the ferric perchlorate asay (Neilands & Nakamura 1991), 0.5 ml of a 5 mM ferric perchlorate solution was mixed with 0.1 ml of sample and the absorbance at 453 nm measured. For the ^{55}Fe-binding assay (Speirs & Boyer 1991), samples were labeled using an ^{55}Fe-NTA solution containing 7.27 nM $^{55}FeCl_3$ (1.96 GBq per mg Fe, Amersham International, Amersham, UK) in 5 mM nitrilotriacetic acid. Samples were mixed with an equal volume of ^{55}Fe-NTA solution and 0.2 N sodium bicarbonate. The mixture was allowed to stand for 30 min before injecting 20 μl on a Shim-pack CLC-ODS (M) column (5 μm, 4.6 × 150 mm; Shimadzu Scientific Instruments, Inc., Columbia, MD) and eluting with a 20 min linear gradient of distilled water to 50% acetonitrile containing 1% acetic acid.

NMR and mass spectral analysis

^{1}H, ^{13}C NMR and ^{1}H–^{1}H COSY spectra for schizokinen and *N*-deoxyschizokinen were recorded on a Bruker AMX

300 spectrometer in deuterium oxide. The ^{1}H NMR spectrum of schizokinen A was obtained by subtracting the schizokinen peaks from the spectrum of a mixture of schizokinen and schizokinen A until the two methylene doublets on the citrate residue of schizokinen disappeared. Electrospray mass spectrometry was performed using a Finnigan TSQ-70 spectrometer. The samples were dissolved in 1:1 methanol:distilled water (v/v) and were introduced by a syringe pump at a flow rate of 4 μl min^{-1}. The stainless needle was held at 2 kV and the source temperature was 120°C. All siderophores were purified by HPLC immediately before mass spectrometry. The trace amount of *N*-deoxyschizokinen A needed for electrospray mass spectrometry was obtained as a side fraction during the purification of *N*-deoxyschizokinen.

Results

During the isolation of siderophores from culture medium of *B. megaterium* ATCC 19213, three fractions with siderophore activity were found in the eluant from ion exchange chromatography. The first fraction eluted with distilled water and was identified after HPLC purification as schizokinen A. The second eluted with 0.2 M ammonium chloride and was later identified as *N*-deoxyschizokinen. Schizokinen eluted in the third fraction between 0.3 and 0.4 M ammonium chloride. After HPLC purification, approximately 18 mg schizokinen, 6 mg *N*-deoxyschizokinen and a small amount of schizokinen A were obtained from 6 l of culture medium.

The electrospray mass spectra for schizokinen and its three related siderophores are shown in Figure 2. The ion pattern for schizokinen revealed peaks at *m/z* (% base peak) = 421 (100) and 443 (81) for $[M+H]^+$ and $[M+Na]^+$ respectively, agreeing with a molecular weight for schizokinen of 420 a.m.u. The molecular weight of schizokinen A is 18 a.m.u. less than schizokinen and gave molecular ions of *m/z* = 425 (100) for $[M+Na]^+$ and *m/z* = 403 (61) for $[M+H]^+$. Schizokinen and schizokinen A are easily transformed to each other so that the molecular ions corresponding to both compounds often appeared in the individual mass spectrum of schizokinen and schizokinen A. The mass spectrum of the third siderophore, *N*-deoxyschizokinen, gave strong peaks at *m/z* = 427 (100) for $[M+Na]^+$ and *m/z* = 405 (67) for $[M+H]^+$, corresponding to a molecular weight of 404 a.m.u. The parent peaks for *N*-deoxyschizokinen A at *m/z* = 387 (100) and 409 (86) indicated a loss of water from *N*-deoxyschizokinen, similar to that observed for schizokinen A.

The ^{1}H NMR spectra for schizokinen, *N*-deoxyschizokinen and schizokinen A are shown in Figures 3(a), 3(b) and 5(b), and summarized in Table 1. Schizokinen is a symmetrical siderophore, showing five sets of proton peaks in its ^{1}H NMR spectrum (Figure 3a). The protons on carbons 5, 6 and 7 were assigned on the basis of their COSY NMR spectrum (not shown). The signal at 1.57 p.p.m. assigned to the protons on C-6 appeared as a quintet due to the coupling of four magnetically equivalent vicinal protons on C-5 and C-7. Two doublets (J = 14.7 Hz) at 2.42 and 2.57 p.p.m. resulted from the two non-equivalent protons on the methylenes of the citrate backbone.

The ^{1}H NMR spectrum of *N*-deoxyschizokinen is shown in Figure 3(b). It contains 22 non-exchangable protons, the same number as in schizokinen. The two doublets assigned to the citrate protons are similar in both *N*-deoxyschizokinen and schizokinen. However, the triplet at 3.4 p.p.m. integrated for only two protons while the multiplet at 2.97 p.p.m. now

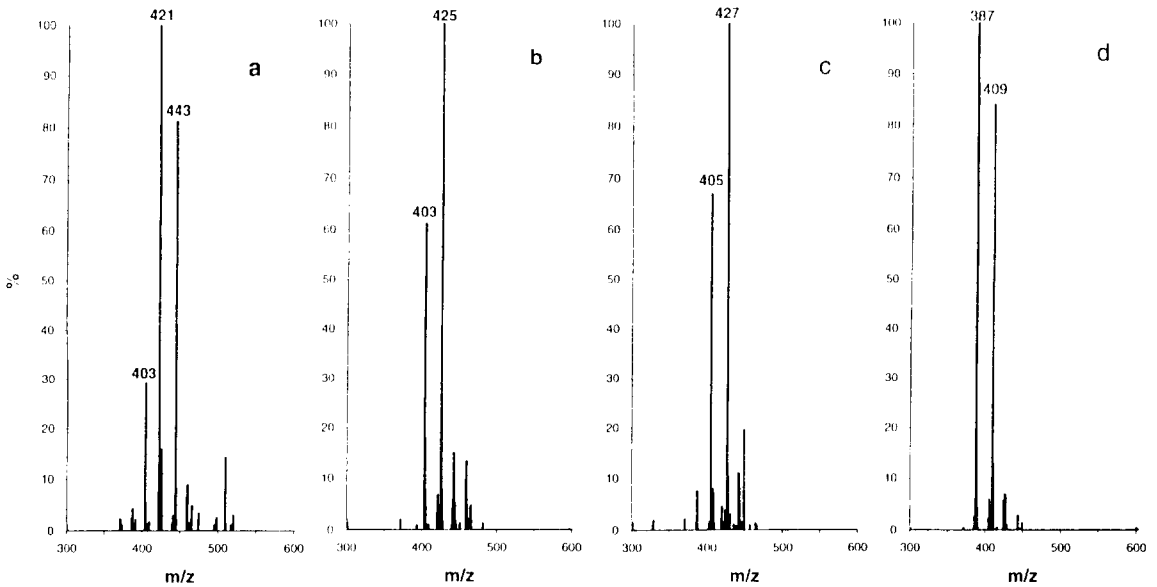


Figure 2. The electrospray mass spectra of (**a**) schizokinen, (**b**) schizokinen A, (**c**) *N*-deoxyschizokinen and (**d**) *N*-deoxyschizokinen A.

Table 1. ^{1}H NMR spectral data for schizokinen, schizokinen A, *N*-deoxyschizokinen and *N*-deoxyschizokinen A

Carbon no.	Schizokinen	Schizokinen A	*N*-Deoxyschizokinen	*N*-Deoxyschizokinen A
		Acetyl residue		
9	1.89(s, 6H)[a]	1.88(s, 6H)	1.89(s, 3H)	1.87(s, 3H)
9′	—		1.73(s, 3H)[d]	1.73(s, 3H)
		Amino propane residue		
7	3.40(t, 4H, $J=6.6$)	≈3.4(2H)[b]	3.40(t, 2H, $J=6.8$)	≈3.3(2H)
7′	—	≈3.4(2H)[b]	≈2.9(2H)[c]	≈2.9(2H)
6	1.57(m, 4H, $J=6.6$)	1.56(m, 2H, $J=6.5$)	1.58(m, 2H, $J=6.8$)	1.54(m, 2H, $J=6.9$)[e]
6′	—	1.68(m, 2H, $J=7.1$)	1.44(m, 2H, $J=6.8$)	1.42(m, 2H, $J=6.8$)[e]
5	2.96(t, 4H, $J=6.5$)	2.94(t, 2H, $J=7.4$)	≈2.9(2H)[c]	≈3.3(2H)[f]
5′	—	≈3.4(2H)[b]	≈2.9(2H)[c]	≈2.9(2H)[f]
		Citrate residue		
3	2.42(d, 2H, $J=14.7$)	2.63(d, 1H, $J=15.3$)	2.34(d, 2H, $J=14.5$)	2.62(d, 1H, $J=15.1$)
	2.57(d, 2H, $J=14.7$)	2.71(d, 1H, $J=15.3$)	2.45(d, 2H, $J=14.5$)	2.72(d, 1H, $J=15.3$)
3′	—	2.59(d, 1H, $J=18.6$)	—	2.58(d, 1H, $J=18.7$)
		2.86(d, 1H, $J=18.6$)		2.85(d, 1H, $J=20.9$)

[a] Chemical shifts are in p.p.m. downfield from TMS and coupling constants are in Hz. Those signals marked with a (—) are the same on both sidearms due to symmetry.

[b,c] These signals are overlapping with a total integration of 6H.

[d] The subtraction spectrum shows two closely spaced peaks with a total integration of 3H. This may represent the presence of two diasteromeric forms.

[e,f] This assignments may be interchanged depending on the structures of *N*-deoxyschizokinen A (Figure 6).

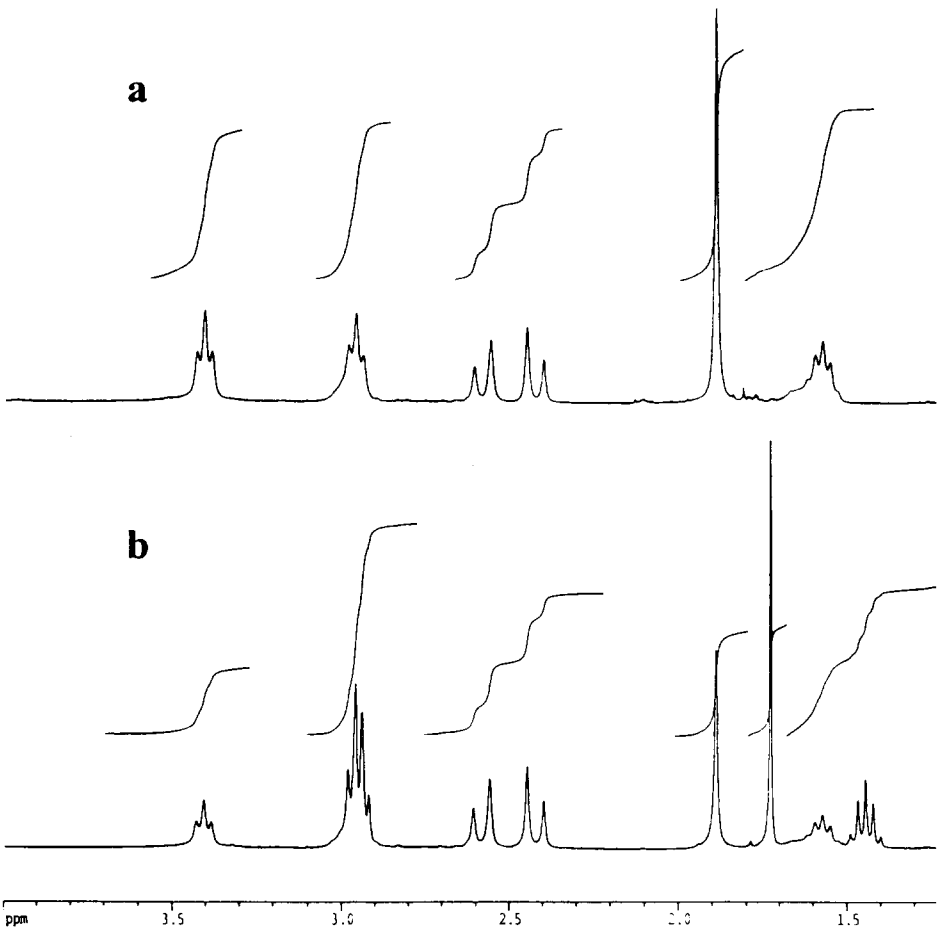


Figure 3. The 300 MHz ^{1}H NMR spectra of (**a**) schizokinen and (**b**) *N*-deoxyschizokinen.

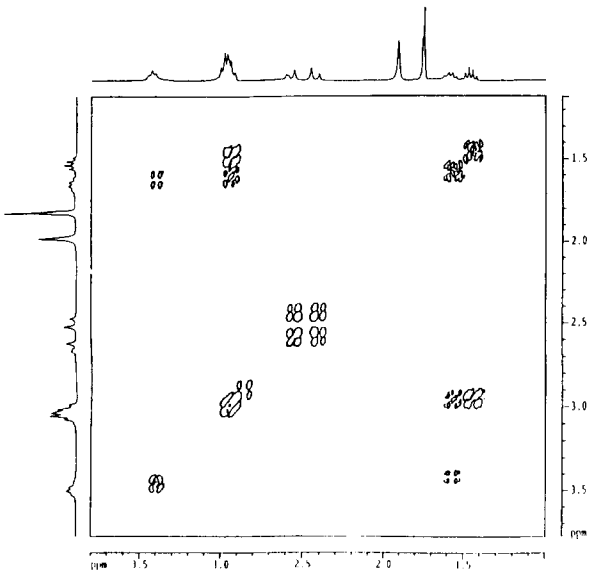


Figure 4. The ^{1}H–^{1}H COSY NMR spectrum of *N*-deoxyschizokinen.

integrated to six protons. The acetyl methyl signals on the two arms are now resolved, showing up as two singlets at 1.89 and 1.73 p.p.m. This indicated that half the structure of *N*-deoxyschizokinen was similar to schizokinen, but the molecule was no longer symmetrical in structure. These changes in the NMR spectra, coupled with a molecular ion 16 mass units less than schizokinen observed in the mass spectrum, suggested that a proton was substituted for a hydroxyl group on one hydroxamate of schizokinen. The protons on C-6′, C-7′ and C-9′ are now more shielded than those on the other side. This assignment were clearly shown in ^{1}H–^{1}H COSY spectrum of *N*-deoxyschizokinen (Figure 4) and similar changes were observed in the ^{13}C spectra (Table 2). Therefore, the structure of *N*-deoxyschizokinen is 4-[(3-(acetylhydroxamino)propyl)amino]-2-[2-[(3-(acetylamino)propyl)amino]-2-oxoethyl]-2-hydroxy-4-oxo-butanoic acid (Figure 1c).

Schizokinen A could easily be separated from schizokinen by HPLC, but it was partially transformed to schizokinen during the work-up for ^{1}H NMR. The NMR spectrum

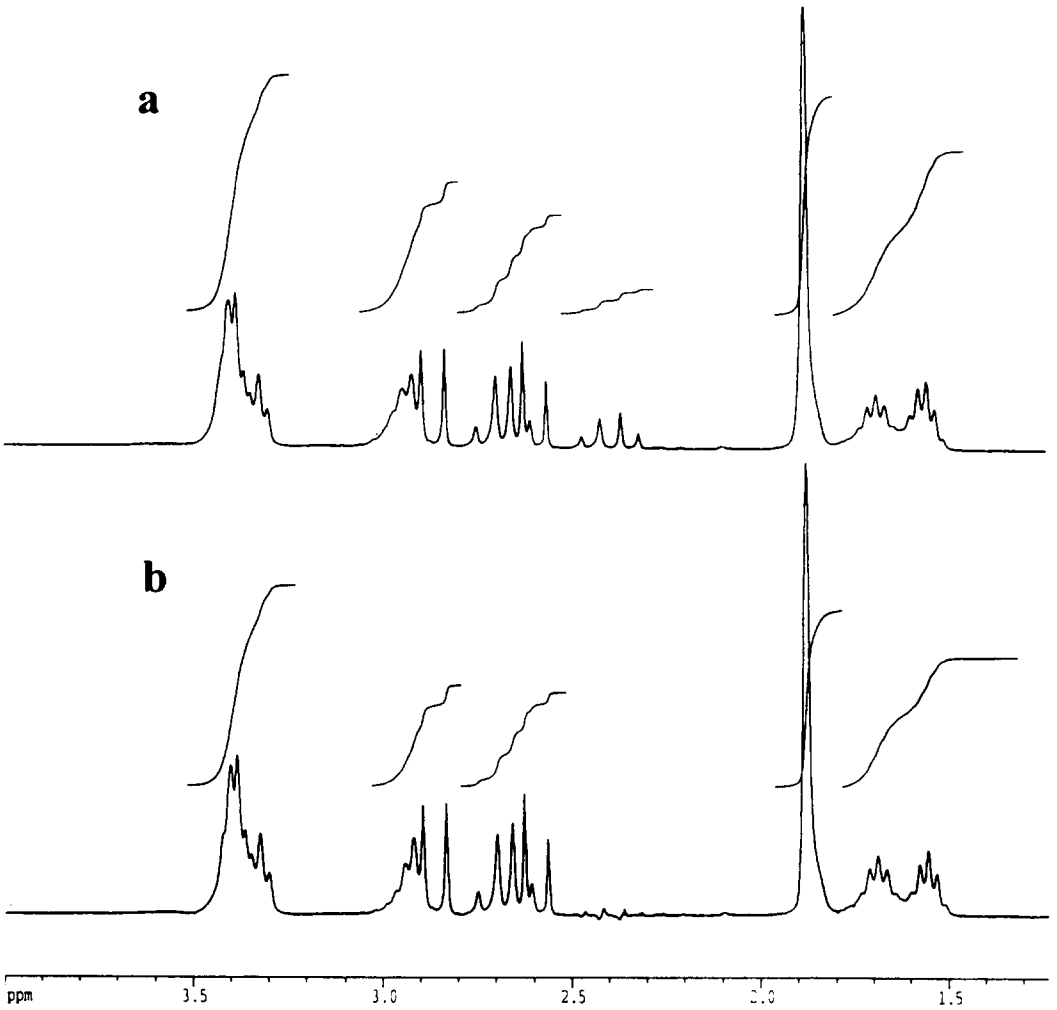


Figure 5. The 300 MHz ^{1}H NMR spectra of (**a**) the mixture of schizokinen A and a small amount of schizokinen and (**b**) schizokinen A obtained by subtraction of the spectrum of schizokinen from the spectrum of the mixture.

of schizokinen A consistently showed small amounts of schizokinen (Figure 5a). These signals assigned to schizokinen could be subtracted from the spectrum of the mixture to give the spectrum of schizokinen A (Figure 5b). The total number of non-exchangable protons in schizokinen A were the same as schizokinen and *N*-deoxyschizokinen. The proton peaks of C-5′ appeared at 3.32 p.p.m., partially overlapping the triplet from the C-7 and C-7′ protons. Methylene protons in the citric residue in schizokinen A give a more complicated spectrum than observed with schizokinen. The protons at C-3 appeared as two doublets with a coupling constant similar to that observed in schizokinen (15.3 versus 14.7 Hz) but shifted about 0.2 p.p.m. downfield. The protons at C-3′ showed a much greater change. One doublet moved down to 2.86, partially overlapping the signal from the C-5 protons. The large difference in the two C-3′ protons from that observed in schizokinen suggests that these protons are in a ring structure. The five-membered cyclic imide formed by condensation of citrate carboxyl with an amide of schizokinen (Figure 1b) is in agreement with the NMR data and supported by the loss of water observed in the mass spectrum of schizokinen A. The ring structure of the imide increased both the coupling effect and non-equivalence of the two protons on C-3′ and resulted in two widely separated doublets. The protons on C-3′, C-5′, C-6′ and C-3 were more shielded than those in schizokinen and their chemical shifts moved downfield in the ^{1}H NMR spectrum.

Table 2. ^{13}C NMR spectral data for schizokinen and *N*-deoxyschizokinen in p.p.m. downfield from TMS

Carbon number	Schizokinen	*N*-Deoxyschizokinen
	Acetyl residue	
9	20.1[a]	20.1
9′		22.7
8	174.7	174.9
8′		174.7
	Amino propane residue	
7	46.4	46.3
7′		37.4
6	26.4	26.4
6′		28.6
5	37.4	37.6
5′		37.5
	Citrate residue	
4	172.6	172.3
4′		172.3
3	45.3	45.4
3′		45.4
2	73.5	73.5
1	172.6	172.3

[a] The signals are equivalent on both sidearms of schizokinen due to symmetry.

The analogous compound, *N*-deoxyschizokinen A, was also observed in isolated samples of *N*-deoxyschizokinen. Too little material was obtained to get clean NMR data, but the observed spectrum (not shown) was generally a hybrid of schizokinen A and *N*-deoxyschizokinen. The chemical shifts for this compound are summarized in Table 1. Formation of an imide ring was confirmed by parent ions 18 mass units less than *N*-deoxyschizokinen in the electrospray mass spectrum. Two possible imide ring structures (Figure 6) exist for *N*-deoxyschizokinen A. One-dimensional NMR failed to resolve these two possibilities. The isolated *N*-deoxyschizokinen A sample may be an equilibrium mixture of both structures.

Discussion

B. megaterium ATCC 19213 produces two or three siderophores. These compounds are closely related in structure. There are strong similarities in their ^{1}H NMR spectra and they are easy to confuse with each other. However, a direct comparison of the three spectra shows distinct differences. The two multiplets around 1.5 p.p.m. distinguish *N*-deoxyschizokinen and schizokinen A from schizokinen. The two singlets at 1.73 and 1.89 p.p.m. are characteristic of *N*-deoxyschizokinen.

Besides schizokinen, other citrate-based dihydroxamate siderophores include aerobactin (Gibson & Magrath 1969), arthrobactin (Linke *et al.* 1972), nannochelin A and C (Kunze *et al.* 1992), and acinetoferrin (Okujo *et al.* 1994). They are

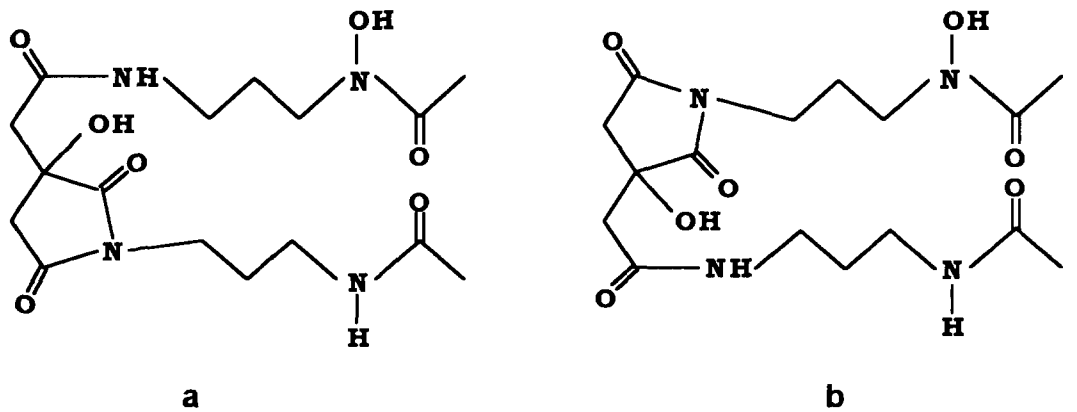


Figure 6. Two diasteriomeric structures for *N*-deoxyschizokinen A.

all symmetrically substituted around the citric acid residue. Annochelin B (Kunze *et al.* 1992) and rhizobactin 1021 (Persmark *et al.* 1993) are examples of dihydroxamate citrate-based siderophores which are not symmetrical. *N*-Deoxyschizokinen is unusual in that it is a monohydroxamate siderophore. Other monohydroxamate siderophores such as anguibactin (Jalal *et al.* 1989) and maduraferrin (Keller-Schierlein *et al.* 1988) are known, but they do not contain citrate. This change from a dihydroxamate to monohydroxamate should change its affinity for iron. The ferric perchlorate and ^{55}Fe-binding HPLC assays both indicate that *N*-deoxyschizokinen does bind iron; however, we do not know at this time if the complex can be used for iron uptake by *B. megaterium*.

Schizokinen A represents another interesting compound. Mullis *et al.* (1971) initially reported that this compound contained a six membered ring formed between the carboxylate (C-1) and the C-5 methylene. This is in disagreement with the total number of non-exchangable protons observed in the ^{1}H NMR, suggesting rather an imide ring structure. This cyclic imide structure has been confirmed by synthesis (Lee & Miller 1983). Of more interest is the origin of schizokinen A in natural samples. Schizokinen and schizokinen A are very easily transformed to each other under acidic conditions. Therefore, the schizokinen A found in culture medium from *B. megaterium* could be formed from schizokinen during isolation or extraction.

In contrast, *N*-deoxyschizokinen is not formed from schizokinen during isolation. Purified samples of *N*-deoxyschizokinen stored for up to 1 year showed traces of the imide assigned to *N*-deoxyschizokinen A but never traces of schizokinen. *N*-Deoxyschizokinen may represent an intermediate in the pathway for either the biosynthesis or the degradation of schizokinen. It was isolated from stationary phase cultures and may be due to an enzymatic cleavage of schizokinen brought about by cell lysis. A study of the production of *N*-deoxyschizokinen with time in culture by *B. megaterium* would help to determine its role. Unfortunately, while schizokinen and *N*-deoxyschizokinen can be separated by HPLC in their iron-free forms, the ferric complexes are not resolved. This causes difficulty in using the extremely sensitive HPLC iron-binding assay to study *N*-deoxyschizokinen production. Work on this problem is currently in progress.

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