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The stereoisomers of pyochelin, a siderophore of *Pseudomonas aeruginosa*

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Abstract

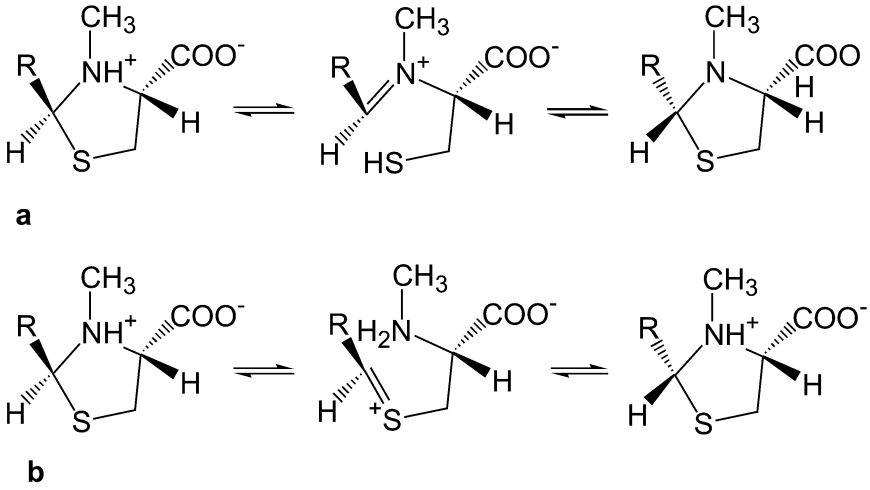
The *Pseudomonas aeruginosa* siderophore pyochelin is obtained from the bacterial culture medium as a mixture of two epimers. Chromatically isolated pure stereoisomers equilibrate readily in most solvents. Experiments will be reported which allow to isolate one of the isomers in pure form and which shed some additional light on the epimerization reaction.

Introduction

Pyochelin is one of the siderophores of *Pseudomonas aeruginosa* and of some related bacterial species (Budzikiewicz, 2003). Biogenetically it is a condensation product of salicylic acid and two molecules of cysteine (Serino *et al.* 1997). The structure of pyochelin was established (Cox *et al.* 1981) as 2-(2-*o*-hydroxyphenyl-2-thiazolin-4-yl)-3-methylthiazolidine-4-carboxylic acid, forming a mixture of two easily interconvertible stereoisomers (pyochelin I and II) (Ankenbauer *et al.* 1988; Rinehart *et al.* 1995). The ratio of pyochelin I : II differs somewhat with the solvent, but I prevails always. The methyl esters do not isomerize readily.

The nature of the two stereoisomers and the mechanism of their interconversion has been a matter of discussion over the years. One of the suggestions was that the two forms are actually rotamers with and without a hydrogen bridge between the phenolic hydroxyl group and the nitrogen atom of the adjacent thiazoline ring (Namiranian *et al.* 1997). The more generally accepted idea assumes an inversion at C-2″, based on the following chain of arguments (Ankenbauer *et al.* 1988; Rinehart *et al.* 1995):
– The methyl ester of the 4-methyl analog of pyochelin I (obtained by feeding 4-methylsalicylic acid to a bacterial strain not able to produce salicylic acid (Ankenbauer & Cox 1988)) was correlated with pyochelin I by NMR and optical rotational data. An X-ray analysis demonstrated either 4′*R*,2″*R*,4″*R*- or 4′*S*,2″*S*,4″*S*-configuration.
– Synthesis starting from salicylic acid nitrile, L-cysteine and L-N-methylcysteine gave four stereoisomers which were transformed into their methyl esters and separated. Two of them were identical with the methyl esters of pyochelin I and II.
– It is known from literature that 2-substituted thiazolidin-4-carboxylic acids epimerize at C-2 and not at C-4 (Luhowy & Meneghini 1979; Ponticelli *et al.* 1982; Szilágyi & Györgydeák 1979; Nagasawa *et al.* 1981).

Hence, pyochelin I should be 4′*R*,2″*R*,4″*R*- (**1**) and pyochelin II 4′*R*,2″*S*,4″*R*-configured. The two additional synthetic isomers (neopyochelin I and II) must then be the 4′-*S*-epimers (Rinehart *et al.* 1995). Indirect support for the isomerization at C-2″ came from the observation that the *Streptomyces* metabolites watasamycin **2** (Sasaki *et al.* 2002) and thiazostatin **3** (Shindo *et al.* 1989) which can not epimerize at C-4″ due to the additional methyl group there, also form equilibria by inversion of C-2″. For the isomerization at C-2″ intermediate zwitterionic structures formed by opening either of the C,S- (Luhowy & Meneghini 1979; Ponticelli *et al.* 1982; Szilágyi and Györgydeák 1979) or the C,N-bond (Nagasawa *et al.* 1981) are assumed (Scheme 1).



Scheme 1. Isomeriztion of 2-substituted thiazolidine-4-carboxylic acid compounds (**a**) by opening of the C-2″/S-bond; (**b**) by opening of the C-2″/N-bond.

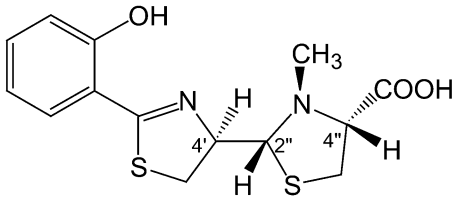
We wish now to report some additional observations related to this problem.

Materials and methods

Instruments. NMR: Bruker (Karlsruhe, Germany) DRX 500, solvents as indicated in the text. Mass spectrometry Finnigan MAT (Bremen, Germany) 900ST with an electrospray ion source. HPLC: Knauer (Berlin) 64 with a variable wave length monitor (detection 254 nm), column nucleosil 100-C_{18}, solvents (unless indicated otherwise) 0.02 M acetic acid/methanol, gradient 40% to 99% methanol in 20 min. Column chromatography: Biogel P-2 (Bio-Rad, Richmond, USA), Polyamide < 0.07 mm (Macherey-Nagel, Düren, Germany), Sep-Pak RP18 cartridges (Waters, Milford, USA).

Isolation of pyochelin. Pseudomonas aeruginosa PAO1 was grown in an artificial medium (Briskot *et al.* 1986). After removal of the cells from the iron-free medium the solution was brought to pH 1-2. Portions of 1 l supernatant were extracted three times with 250 ml acetic acid ethyl ester. After removal of the solvent *i. v.* the residue was dissolved in 250 ml water and extracted three times with 200 ml acetic acid ethyl ester. The organic phase was washed with 100 ml water and the solvent was removed *i. v.* It remained an orange oil. To a solution of the oily residue from 1 l culture medium in methanol/glacial acetic acid 5:1 (v/v) 5 ml of a 5% solution of Fe(III) citrate in water was added and the mixture was concentrated *i. v.* to 5 ml. After addition of 5 ml glacial acetic acid the solution was applied to a Biogel P-2 column equilibrated with a 0.2 M pyridinium acetate buffer (pH 5.0). Salts etc. were removed with the same buffer solution and the red Fe(III) complex was finally desorbed with a 2 M buffer and re-chromatographed under the same conditions. The solution was concentrated *i. v.*, dissolved in water/methanol and concentrated again several times to remove the buffer completely. 50 mg of the complex were dissolved in 4 ml methanol and applied to a polyamide column. Contaminants were removed with 300 ml methanol and 300 ml acetone/methanol/water 5:2:1 (v/v). Desorption was achieved with acetone/methanol/0.2 M acetic acid 5:2:1 (v/v) and the solvent was removed *i. v*. For decomplexation 30 mg of the complex in 2 ml methanol were added drop by drop to a 10% potassium oxalate buffer (pH 4.3). After 30 min the solution was applied to a Sep-Pak RP18 cartridge. The adsorbed material was washed with 15 ml water and pyochelin was desorbed with methanol. The solvent was removed *i. v.* yielding a yellow oil. In this way a mixture of pyochelin I + II is obtained.

To obtain pure pyochelin I 2 mg of the Fe(III) complex in 0.1 ml methanol were applied to a Sep-Pak RP18 cartridge equilibrated with water. With a 10% potassium oxalate buffer (pH 4.3) solution partial decomplexation was achieved and the remaining complex was eluted while the free pyochelin was retained. After washing with 15 ml water it was desorbed with 2 ml methanol and immediately brought to dryness



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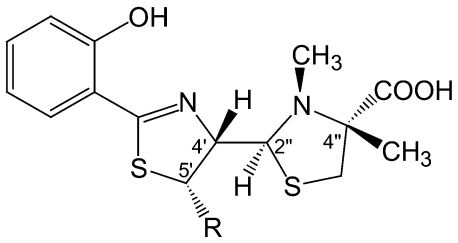


2, R = CH_3
3, R = H

Fig 1. Pyochelin I (**1**), absolute configuration; watasemycin (**2**), relative configuration; thiazostatin (**3**), relative configuration

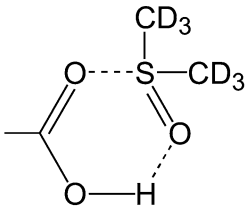


Fig 2. Proposed pyochelin-DMSO-adduct

i. v. Control by HPLC (0,2 M acetic acid/ methanol) demonstrated the presence of pure pyochelin I.

Ga-pyochelin. To a pyochelin I/II mixture was dissolved in methanol a double molar excess of $Ga_2(SO_4)_2$ in water was added at 20 °C. After 2 h the yellow solution was concentrated *i. v.*, brought onto a polyamide column equilibrated with methanol and desorbed with methanol. Purity control was achieved by HPLC.

Results

Isolation of the pyochelin isomers

In the original publications it was reported that a separation of pyochelin I and II was barely possible on chromatographic plates, and that after elution the original equilibrium was restored quickly (Cox *et al.* 1981). Separation can be achieved by analytical HPLC (see Experimental), but partial re-epimerization in the eluates can not be suppressed. An isolation of pure pyochelin I is possible by adsorption of its Fe^{3+} complex (see below) on a Sep-Pak RP18 column and decomplexation with a pH 4 oxalate buffer. The methanolic eluate has to be brought to dryness immediately. In methanol the typical equilibrium is restored quickly, but in DMSO-d_6 the pure isomer I can be kept for an extended period of time. The isolation of the pure enantiomer allows *i. a.* a NMR analysis which confirms earlier assignments obtained with the stable methyl ester (see above).

Temperature studies

To exclude the possibility that pyochelin I and II are actually rotamers (Namiranian *et al.* 1997) the mixture was dissolved in dimetylsulfoxide-d_6 (DMSO-d_6) and changes of the ^{1}H-NMR spectrum upon heating were recorded. Up to 130 °C only shifts of various signals, but no coalescence could be observed. The shift values lie mainly between ca. 0.0 and 0.1 ppm downfield for both isomers; outstanding exceptions are the signals for H-4′ (I: 0.10 ppm upfield, II: 0.05 ppm downfield) and H-4″ (I: 0.10 ppm downfield, II: 0.10 ppm upfield). Most striking is the observation that the signals of H-2″ change place (see Table 1). The reason for the shifts is the increased flexibility of the heterocyclic rings which changes the positions of the various protons relative to the shielding and deshielding spheres of the benzene ring and the carbonyl group. The rotamer theory can thus be excluded.

^{1}*H-NMR studies*

For NMR studies the mixture of pyochelin I and II was used. The various signals are well separated and identical conditions for both isomers are thus maintained. ROESY experiments confirm the assignments reported in the literature (Rinehart *et al.* 1995; Zamri & Abdallah, 2000) obtained in $CDCl_3$ solution. Of importance are two observations. For pyochelin II a ROESY cross peak is observed between H-4″ and H-4′, but not for pyochelin I. In addition, NOE difference spectroscopy showed that for pyochelin I the H-2″ and H-4″ are situated on the same side of the thiazolidine ring, in contrast to pyochelin II.

Table 1. ^{1}H chemical shifts (δ ppm) of the aliphatic part of pyochelin (blanks at high temperature experiments indicate that the center of the signal could not be determined exactly).

Solvent	DMSO-d_6				CD_3COOD				CF_3COOD/ $CDCl_3$ 1:2	
°C	25°	130°	25°	130°	25°	85°	25°	85°	25°	
isomer	I		II		I		II		I	II
H-4′	5.18	5.09	4.93	4.98	5.32	5.14	5.32		5.66	5.84
H-5′	3.41		3.60		3.37		3.35		3.86	4.20
	3.39		3,34		3.63		3.68		3.46	3.66
CH_3	2.47	2.57	2.34	2.46	3.10		2.91		3.34	3.46
H-2″	4.51	4.51	4.44	4.53	4.82	4.64	4.97	4.75	5.29	5.42
H-4″	3.50		4.14	4.09	4.40		4.42		4.83	5.02
H-5″	3.12		3.11		3.57		3.38		3.79	3.69
	3.02							3.77	3.61	

Isomerisation in acidic media

a. CD_3COOD at different temperatures

When the pyochelin I/II mixture is slowly heated up in a NMR tube to 65 °C a sharpening of the signals and upfield shifts are observed for most signals in contrast to the observations reported above for the heating experiments in DMSO-d_6. Starting from 85 °C the H-4″ signals lose in intensity and at 100 °C they have disappeared completely. At the same time additional singulets start to appear in the vicinity of the N-methyl signals, which partially remain after cooling to 25 °C. Otherwise the original habitus of the spectrum is restored with the following exceptions: the H-4″ signals are still missing and the H-5″ signal shows no ^3J splitting any more. Changes in the H-4′ and I-H-2″ signals suggest that in addition to the complete exchange of H-4″ there was a partial exchange of H-4′.

Obviously the H-4″ hydrogen atoms in both isomers have been replaced by deuterium. An LC-MS analysis of the sample recovered after the NMR experiment showed the presence of four fractions which according to their UV/Vis and mass spectra were pyochelin isomers. The observation of four LC peaks demonstrates that at least at one additional chiral center isomerisation had taken place: if C4′ had remained unaffected, four diastereomers with differing chiralities at C-2″ and C-4″ had been formed; if C-4′ had also been racemized, eight different steric isomers were formed, but pairwise two of them were enantiomers which could not be separated on a non-chiral column.

The mass spectra of the four fractions showed incorporation (after back-exchange of OD and COOD to OH and COOH) of one and partially of two deuterium atoms, in either case one of them is located in the third (viz. the thiazolidine) ring of pyochelin.

b. $CF_3COOD/CDCl_3$ (1:2)

As compared with the shift data obtained for pyochelin in CD_3COOD appreciable down-field shifts are observed. This could be explained by protonation of the nitrogen functions. Remarkably, low intensity signals of a third pyoverdin species are evident. Partial isomerisation at C-4′ could be invoked as an explanation for the third set of signals, an alternative reason could be the degree of protonation of the two nitrogen functions (a mixture of mono- and di-protonated species). For pyochelin I, but not for II and III ROESY cross peaks are observed between H-4′ and H-2″. The most important observation is that heating to 55 °C did not lead to H/D exchanges in agreement with the results above.

Metal complexes

Fe^{3+} complex

A X-ray analysis of the Fe^{3+} complex (Schlegel *et al.* 2004) of pyochelin demonstrated a structure comprising two 4′*R*,2″*R*,4″*R*-(pyochelin I) units binding Fe^{3+} by the carboxylate and the phenolate anion and the two nitrogen atoms. The two moieties are held together by an acetate and a hydroxyl ion satisfying the remaining two sites of the octahedral iron center. Stereochemically pure pyochelin I could be obtained by oxalic acid decomplexation (see above). Titration experiments described in literature (Visca *et al.* 1992) reporting a

Table 2. ^{1}H chemical shifts (δ ppm) of the aliphatic part of the Ga^{3+} complex of pyochelin

Solvent	DMSO-d_6	
°C	25°	
isomer	I	II
H-4′	4.26	4.30
H-5′	3.62	3.68
	3.23	3.30
CH_3	2.76	2.69
H-2″	4.52	4.61
H-4″	4.08	4.32
H-5″	3.69	3.66
	3.51	3.58

complex stoichiometry ligand-to-iron of 2:1 at pH 2.5 are probably due to a partial protonation of the ligand sites.

Ga^{3+} complex

Ga^{3+} is used frequently in structural studies as a substitute for the Fe^{3+}. It also forms octahedral complexes, the ionic radii are very similar (62 *vs.* 65 pm), and it is amenable to NMR analysis. Ga^{3+} complexes of pyochelin can be obtained by addition of $Ga_2(SO_4)_2$ to a methanolic solution of pyochelin I + II. The solution shows a dark blue fluorescence at 366 nm. The UV absorption maxima in methanol lie at 220, 270 and 365 nm.

In DMSO-d_6 Ga^{3+}-complexes of both pyochelin isomers are present (Table 2). The ^3J-coupling constant between H-4′ and H-2″ is 10,8 Hz for both isomers suggesting identical dihedral angles. Comparison of the ^{13}C data of the free ligands with those of the complexes show that the phenolate anion and N″ are definitely involved in the complexation (I: C-2: 158.4, Ga-I 165.6 ppm, II: C-2 158.4, Ga-II 165.1 ppm; I: N-CH_3 I 40.1, Ga-I 45.1, II: N-CH_3 37.3, Ga-II 45.2 ppm). The shift differences of C-2′, C-4′, C2″, C-4″ and of the carboxyl-C are less significant. It may well be that in contrast to the crystalline Fe^{3+}-complex occupation of free ligand sites by solvent molecules plays a more pronounced role. Interestingly ROESY cross peaks are observed between the CH_3 signal of pyochelin I and H-2″/H4″ of pyochelin II and *vice versa*. This indicates that mixed complexes are formed. In contrast in D_2O only one set of signals can be detected. As in the case of the Fe^{3+} complex in aqueous solution only one isomer is involved.

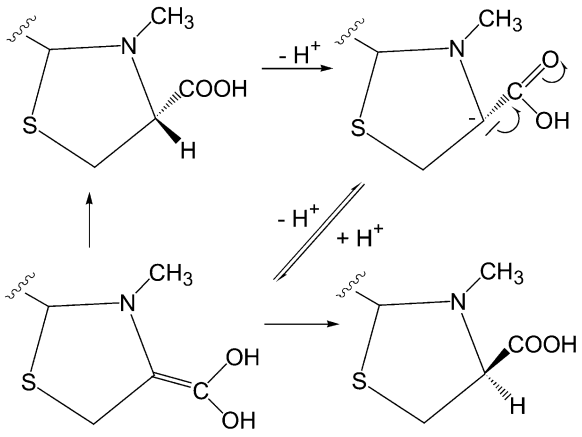
Discussion

In essentially neutral solvents as methanol the proton of the C-4″ carboxyl group seems to play an important role for the isomerization since the methyl esters are stable. It allows the formation of the zwitterionic forms invoked in the isomerization schemes. The stability of pyochelin I in DMSO must then be explained by the formation of strong hydrogen bonds to the solvent molecules. Experiments with CF_3COOD and CD_3COOD show that even under warming to 50 °C H/D-exchange at ring positions does not occur. NOE correlations show that in pyochelin I H-2″ and H-4″ are on the same side, in pyochelin II on opposite sides of the thiazolidine ring. The absolute configuration of pyochelin I being known from X-ray analyses of the methyl analog and of the Fe^{3+} complex (see above) as 4′*R*,2″*R*,4″*R* pyochelin II must be epimeric either on C-2″ or on C-4″.

Complete H/D exchange of H-4″ in both isomers was observed after heating the pyochelin I/II mixture in CD_3COOD to 100 °C, while additional stereoisomers of pyochelin were formed. After cooling to 25 °C the H-4′ and H-2″ signals were found with the same shift values as before the experiment though some changes in the splitting pattern were observed. This should exclude a complete isomerization at C-4′ provided the shift values of pyochelin I and II in DMSO-d_6 differ from those of the corresponding neopyochelins (C-4′ epimers) as they do in $CDCl_3$ solution. To a minor part H/D exchange did occur at C-4′ in agreement with the mass spectrometric data which indicated a complete exchange of one and a partial exchange of two protons.

It had been pointed out above that an opening of the N/C-4 bond during the isomerization of 2-substituted thiazolidin-4-carboxylic acids had never been observed. For the epimerization at C-4″ with H/D exchange at high temperatures in the case of pyochelin I and II the intermediacy of an en-diol structure could be invoked (Scheme 2).

Summing up, in solvents where zwitterionic tautomers can be formed isomerization at C-2″ without H/D exchange occurs readily at room temperature. The high temperature experiments show that heating alone does not lead to a deprotonation at C-4″ in DMSO-d_6 (130 °C). In this solvent the abstraction of



Scheme 2. Isomerization of pyochelin at C-4″

H^+ from C-4″ could be impeded by an adduct formation between the carboxyl group and DMSO-d_6 (**4**). This would also explain the stability of pyochelin I in DMSO-d_6 at room temperature. In CD_3 COOD hydrogen bridge formation between the solvent and the carbonyl group of the C-4″ carboxyl group on the other hand could facilitate the H-abstraction from C-4″.

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