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Bacterial Siderophores: Synthesis and Biological Activities of Novel Pyochelin Analogues

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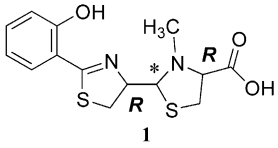
Abstract—The synthesis and biological activities of four pyochelin analogues substituted in different parts of the molecule are reported: 5-*N*HBoc-pyochelin, 3″*N*-Boc-pyochelin, 3″-nor-*N*H-pyochelin and neopyochelin II, the enantiomer of natural pyochelin. All these compounds complex iron(III) and transport it at different rates into the cells of *Pseudomonas aeruginosa*.

Introduction

Pyochelin **1** is a siderophore isolated for the first time from the iron-deficient cultures of *Pseudomonas aeruginosa* ATCC 15692 by Liu and Shokrani.[1] Its structure was established later by Cox et al. (1981):[2] it is a hydroxyphenylthiazolinylthiazolidine type of siderophore which chelates iron with a 2:1: stoichiometry. Although its association constant with iron(III) is very weak (estimated to be 5.10^5 M L in methanol),[3] the pyochelin-mediated iron transport is very efficient.[4]



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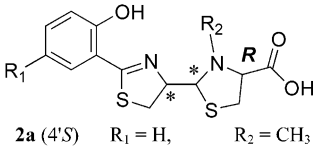

2a (4′*S*) R_1 = H, R_2 = CH_3
2b (4′*S*) R_1 = H, R_2 = H
2c (4′*S*) R_1 = H, R_2 = Boc
2d (4′*R*,*S*) R_1 = NHBoc, R_2 = CH_3

This siderophore was shown later to be produced by a great number of clinically isolated strains of *P. aeruginosa* and *Burkholderia* (ex *Pseudomonas*) *cepacia*,[5,6] both species being involved in severe lung infections occurring in cystic fibrosis patients. From the almost omnipresence of pyochelin in these two bacterial species we could assume that most of these strains shared a common or at least a very closely related pyochelin outer-membrane receptor, called FptA. In order to perform a topological study of this receptor by photoaffinity labelling, we decided to prepare pyochelin analogues bearing different functionalities in various positions, using the efficient, simple and versatile synthesis of pyochelin **1** we have recently reported.[7,8]

We present here the synthesis and biological activities of four pyochelin analogues substituted in different parts of the molecule: 5-*N*-Boc-pyochelin **2d** (*t*-butyloxycarbonyl-amino group at position 5 of the aromatic ring), 3″-*N*-Boc-pyochelin **2c** (*t*-butyloxycarbonyl-amino group on the nitrogen atom of the thiazolidine ring), 3″-nor-*N*H-pyochelin **2b** (where the *N*-methyl group of the thiazolidine ring is replaced by an NH group) and neopyochelin II **2a** (the enantiomer of natural pyochelin). We show that all these compounds chelate iron(III) and transport it with different rates into the bacteria.

Results and Discussion

Synthesis of the substituted pyochelins

In order to compare the influence of the configuration of the asymmetric center 4′ on the transport activities of pyochelin analogues, neopyochelin II **2a**, and 3″-nor-*N*H-pyochelin **2b** were prepared as described previously

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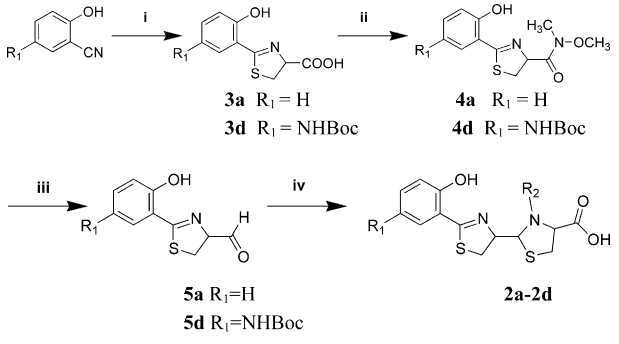
for pyochelin **1**, in four steps starting from 2-hydroxybenzonitrile,[7,8] which was treated with (*R*)-cysteine (instead of (*S*)-cysteine) in phosphate buffer pH 6.4/methanol. The carboxythiazoline **3a** obtained was coupled with *N,O*-dimethyl hydroxylamine in the presence of DECP, to yield the corresponding hydroxamate **4a**, which was reduced by lithium aluminum hydride into the key aldehyde **5a** (Scheme 1).

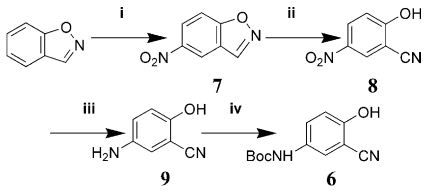
This aldehyde was further cyclized either with *R*-methylcysteine to yield neopyochelin II **2a** (61% overall yield from hydroxamate **4a**), or with (*R*)-cysteine to yield 3″-nor-*N*H-pyochelin **2b** (59% overall yield from hydroxamate **4a**). The treatment of this latter with *t*-butyloxycarbonyl anhydride yielded 3″-*N*-Boc-pyochelin **2c**. Pyochelins **2a**, **2b** and **2c** thus obtained were found to be each constituted with four diastereisomers (4′*S*,2″*R*,4″*R*), (4′*S*,2″*S*,4″*R*) and (4′*R*,2″*R*,4″*R*) and (4′*R*,2″*S*,4″*R*) in a ratio 5:2:2:1 (measured by ^{1}H NMR).

Analogue **2d** was prepared similarly but starting from 5-*t*-butyloxycarbonylamino-2-hydroxybenzonitrile **6** which was prepared in four steps starting from 1,2-benzisoxazole. This latter was first converted into 5-nitro-benzo[*d*]isoxazole **7** using fuming nitric acid (69% yield),[9] which was further hydrolyzed into 2-hydroxy-5-nitrobenzonitrile **8** (94% yield).[10] This nitro compound was reduced into the corresponding amine **9** (88% yield),[11] then treated with *t*-butyloxycarbonyl anhydride, to yield 5-*t*-butyloxycarbonylamino-2-hydroxybenzo-nitrile **6** (50% yield) (Scheme 2).[12]

Since the configuration of the asymmetric center 4′ did not seem to be determining in the transport process (see below), treatment of this latter was performed with



Scheme 1. Synthesis of pyochelin analogues: (i) cysteine, phosphate buffer pH 6.4/methanol 1:1, 50 °C; (ii) DECP/DMF, MeNH–OMe hydrochloride/TEA; (iii) LAH/THF; (iv) cysteine or *N*-methylcysteine, potassium acetate, 5:1 ethanol/water, 25 °C.



Scheme 2. Synthesis of 5-substituted 2-hydroxy-benzonitriles: (i) H_2SO_4, HNO_3 fuming, 0 °C, 1 h; (ii) ethanol/water 3:2, 2 N aqueous NaOH, 25 °C; (iii) ethanol, $SnCl_2$, 70 °C; (iv) dioxane/water (5:1, v/v), Na_2CO_3, Boc_2O, 25 °C.

(*R*,*S*) cysteine and gave the corresponding thiazoline **3d** (67% yield) which was converted to hydroxamate **4d** (95% yield), then reduced into aldehyde **5d**. This was finally treated with (*R*)-*N*-methylcysteine to yield the analogue **2d** (41% overall yield from hydroxamate **4d**).

Chelation of iron(III) by pyochelin and its analogues

Treatment of pyochelin **1**, or its analogues **2a**, **2b** and **2c** with iron(III) chloride in methanol/water (1:1 v/v) showed a 10 nm shift of the maximum of the corresponding free ligand from 310 to 320 nm and the presence of two maxima at 420 and 520 nm. 5-*N*-Boc-pyochelin **2d** showed slightly different spectral properties with an absorption maximum at 340 nm. The corresponding iron(III) complex had a maximum at 340 nm (no shift), two maxima at 420 and 585 nm (Fig. 1).

Iron uptake with pyochelin and its analogues

Iron uptake experiments performed with pyochelin **1** and its analogues **2a**–**2d** on both strains of *P. aeruginosa* ATCC 15692 and its mutant CDC5(pPVR2) gave very similar results from analogue to analogue and showed:

1. that the uptake rates of ^{55}Fe(III) with pyochelin **1** and neopyochelin II **2a** were very similar, in agreement with former results reported;[13]
2. the absence of the methyl group at position 3″ in 3″-nor-*N*-pyochelin **2b** increased the amount of transported ^{55}Fe(III) by nearly 30% after 30 min, making analogue **2b** a more efficient transporter than pyochelin;
3. surprisingly the presence of bulky substituents such as Boc at positions 3″*N* (analogue **2c**) and *N*HBoc at position 5 (analogue **2d**) increases the rate of uptake.

These results show clearly that the configuration of the asymmetric center 4′ is not determining in the recognition with the receptor FptA and in the transport process. The very comparable rates of transport observed for pyochelin **1** and neopyochelin II **2a** are thus easy to understand. Moreover the results observed in the course of the complexation with zinc show that a mixture of 4′*R*,2″*R*,4″*R* and 4′*R*2″*S*4″*R* pyochelins gives exclusively rise to the 2″*R* diastereoiomer, very likely via an asymmetric induction due to a template effect directed by both the

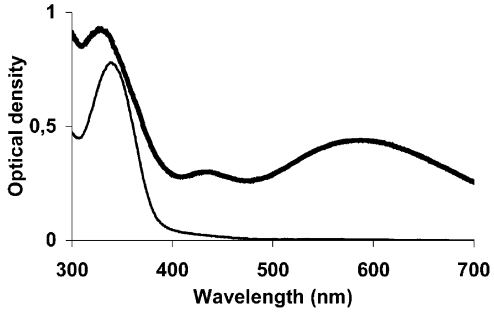


Figure 1. UV–visible spectra of 5-*N*-Boc-pyochelin **2d** (thin line) and its iron(III) complex (thick line) in aqueous methanol (1:1, v/v).

metal and the C4″ atom.[14] This explains why the pyochelin diastereoisomers show a very similar rate of transport.[13]

Moreover the shape of the uptake curves of both *N*-Boc pyochelins **2c** and **2d** suggest that the transport takes place faster at the beginning of the process. However if for analogue **2c** the amount of $^{55}Fe(III)$ incorporated after 30 min. is in the same range as for pyochelin, for analogue **2d** this amount is about 30% smaller.

Conclusions and Prospects

In conclusion, we prepared four analogues of pyochelin which chelate iron(III) and transport it into the bacterial cells of *P. aeruginosa*. We showed that the substitution of the aromatic ring at position 3″ or at position 5 does not decrease dramatically the biological properties of the corresponding analogues compared to pyochelin. The substitution at position 5 will be used to bind photoactivatable groups allowing the preparation of structurally pyochelin based photoactivatable probes which will be useful tools in the topological mapping of FptA, the pyochelin-specific iron transport receptor in *P. aeruginosa* and *B. cepacia*.

In addition we are starting synthesizing and investigating a new set of pyochelins in our laboratory in order to localize and confirm the atoms involved in the iron (III) chelation.

Experimental

2-(5-*t*-Butyloxycarbonylamino-2-hydroxyphenyl)-4′, 5′-dihydrothiazole-4′-carboxylic acid 3d. FAB-MS (negative); *m/z* (%): 337.1 $[M–H]^-$ (65), 293.1 (100). HR-FAB-MS (positive) (*m/z*): 339.1004 $[M+H]^+$ (calcd 339.1015 for $C_{15}H_{19}N_2O_5S$). ^1H NMR (500 MHz, acetone-d_6): 1.48 (9H, s), 3.11 (1H, s, br), 3.71.78 (2H, m), 5.54 (1H, q, $J=7.9$ Hz), 6.90 (1H, d, $J=8.9$ Hz), 7.53 (1H, d, $J=8.3$ Hz), 7.83 (1H, s). ^{13}C NMR (125.7 MHz, acetone-d_6): 28.59, 34.21, 77.83, 117.81, 117.92, 120.59, 125.27, 132.44, 153.03, 153.96, 155.33, 171.46, 174.40.

{4-Hydroxy-3-[4′-(*N*-methoxy,*N*-methyl carboxamido-4′,5′-dihydrothiazol-2′-yl]-phenyl}-*t*-butyl carbamate 4d. HR-FAB-MS (positive) (*m/z*): 382.1426 $[M+H]^+$ (calcd 382.1436 for $C_{17}H_{24}N_3O_5S$). ^1H NMR (500 MHz, $CDCl_3$): 1.48 (9H, s), 3.28 (3H, s), 3.47 (1H, dd, $J=9.2$, 10.8 Hz), 3.77 (1H, t,$J=8.8$ Hz), 3.82 (3H, s), 5.68 (1H, t, $J=9.0$ Hz), 6.37 (1H, s, br), 6.92 (1H, d, $J=13.8$ Hz), 7.35 (1H, s, br), 7.44 (1H, d, $J=2.6$ Hz), 12.04 (1H, s, br). ^{13}C NMR (125.7 MHz, $CDCl_3$): 28.39, 33.00, 61.87, 74.79, 115.81, 117.46, 120.95, 125.27, 129.87, 153.08, 155.14, 169.72, 173.91.

2′-(5-*t*-Butyloxycarbonylamino-2-hydroxyphenyl)-3″-methyl-2″,3″,4″,4′,5′-hexahydro-[2,4′]-bisthiazolyl-4″-carboxylic acid 2d. FAB-MS (negative); *m/z* (%): 438.0 $[M-H]^-$ (100), 392.0 (3), 337.0 (6). HR-FAB-MS (positive) (*m/z*): 440.1307 $[M+H]^+$ (calcd) 440.1314 for $C_{19}H_{26}N_3O_5S_2$).

Major isomers

4′*R*, 2″*R*, 4″*R*. ^1H NMR (500 MHz, $CDCl_3$): 1.51 (9H, s), 2.7 (3H, s), 3.30.38 (2H, m), 3.27.48 (2H, m), 3.83 (1H, t, $J=6.9$ Hz), 4.35 (1H, d, $J=7.8$ Hz), 4.85 (1H, m), 6.89 (1H, m), 6.93 (1H, m), 7.30 (1H, m), 7.45 (1H, m). ^{13}C NMR (125.7 MHz, $CDCl_3$): 28.37, 33.25, 33.35, 33.90, 43.94, 73.67, 77.95, 80.17, 115.81, 117.42, 120.82, 125.32, 129.95, 152.92, 170.22, 171.74, 173.76.

4′*S*, 2″*R*, 4″*R*. ^1H NMR (500 MHz, $CDCl_3$): 1.51 (9H, s), 2.63 (3H, s), 3.28.37 (2H, m), 3.39.53 (2H, m), 3.82 (1H, t, $J=6.6$ Hz), 4.35 (1H, d, $J=7.8$ Hz), 4.95 (1H, m), 6.89 (1H, m), 7.38 (1H, m), 7.41 (1H, m). ^{13}C NMR (125.7 MHz, $CDCl_3$): 28.37, 33.28, 33.57, 44.37, 74.00, 77.24, 80.89, 115.81, 117.42, 120.82, 125.32, 129.95, 152.92, 170.22, 171.22, 173.95.

2′-(2-Hydroxyphenyl)-3″-methyl-2″,3″,4″,5″,4′,5′-hexahydro-[2,4′]-bisthiazolyl-4″-carboxylic acid 2a (Neopyochelin II). FAB-MS (positive); *m/z* (%): 325.1 $[M+H]^+$ (100), 192.1 (5). HR-FAB-MS (positive) (*m/z*): 325.0683 $[M+H]^+$ (calcd 325.0681 for $C_{14}H_{17}N_2O_3S_2$).

4′*S*, 2″*R*, 4″*R* (Neopyochelin II, major isomer). ^1H NMR (500 MHz, $CDCl_3$): 2.65 (3H, s), 3.30.38 (2H, m), 3.39.54 (2H, m), 3.82 (1H, t, $J=6.7$ Hz), 4.37 (1H, d, $J=5.2$ Hz), 4.97 (1H, m), 6.89 (1H, m), 7.01 (1H, m), 7.38 (1H, m), 7.41 (1H, m). ^{13}C NMR (125.7 MHz, $CDCl_3$): 33.28, 33.57, 44.35, 74.09, 77.24, 80.69, 116.00, 117.33, 119.08, 130.72, 133.58, 159.07, 171.2, 173.95.

4′*R*, 2″*R*, 4″*R* (Pyochelin). ^1H NMR (500 MHz, $CDCl_3$): 2.71 (3H, s), 3.34.40 (2H, m), 3.27.48 (2H, m), 3.85 (1H, t, $J=7.1$ Hz), 4.36 (1H, d, $J=7.8$ Hz), 4.87 (1H, q, $J=8.3$ Hz), 6.89 (1H, m), 7.01 (1H, m), 7.38 (1H, m), 7.41 (1H, m). ^{13}C NMR (125.7 MHz, $CDCl_3$): 33.25, 33.55, 33.85, 44.07, 73.60, 77.95, 80.06, 116.00, 117.33, 119.08, 130.72, 133.58, 159.07, 171.74, 173.95.

2′-(2-Hydroxy-phenyl)-2″,3″,4″,5″,4′,5′-hexahydro-[2,4′]-]thiazolyl-4″-carboxylic acid 2b. FAB-MS (negative); *m/z* (%): 309.0 [M-H]$^-$ (100). HR-FAB-MS (positive) (*m/z*): 311.0529 $[M+H]^+$ (calcd 311.0524 for $C_{13}H_{15}N_2O_3S_2$).

4′*S*, 2″*R*, 4″*R* (major isomer). ^1H NMR (500 MHz, $CDCl_3$): 3.30.38 (2H, m), 3.39.54 (2H, m), 4.00 (1H, t, $J=6.7$ Hz), 5.01 (1H, d, $J=5.2$ Hz), 5.17 (1H, m), 6.89 (1H, m), 7.01 (1H, m), 7.38(1H, m), 7.41 (1H, m).

4′*R*, 2″*R*, 4″*R*. ^1H NMR (500 MHz, $CDCl_3$): 3.34.40 (2H, m), 3.49.74 (2H, m), 4.05 (1H, t, $J=7.1$ Hz), 4.73 (1H, d, $J=7.8$ Hz), 5.08 (1H, q,$J=8.3$ Hz), 6.89 (1H, m), 7.01 (1H, m), 7.38 (1H, m), 7.41 (1H, m).

2′-(2-Hydroxyphenyl)-4″,5″,4′,5′-hexahydro-[2,4′]-bisthiazolyl-3″,4″-dicarboxylic acid 3″-*t*-butyl ester 2c. HR-FAB-MS (positive) (*m/z*): 411.1049 $[M+H]^+$ (calcd 411.1048 for $C_{18}H_{23}N_2O_5S_2$).

^1H NMR (500 MHz, $CDCl_3$): 1.47 (9H, s), 3.20.40 (2H, m), 3.45 (2H, m), 4.70 (1H, m), 4.90 (1H, m), 5.30 (1H,

m), 6.86 (1H, t, $J = 7.7$ Hz), 6.98 (1H, d, $J = 7.16$ Hz), 7.34 (1H, t, $J = 6.8$ Hz), 7.40 (1H, d, $J = 7.5$ Hz).

Strains, cultures and iron uptake experiments

The strains used were *P. aeruginosa* ATCC 15692 and one of its pyoverdin defective mutant CDC5(pPVR2).[15] They were cultivated during 48 h aerobically in conical flasks in iron-deficient succinate medium as described previously.[16] The cells were harvested, centrifugated during 10 min at 8000 rpm at 4 °C, washed three times with 50 mM MOPS buffer pH 8.0, then suspended in the same buffer, and the optical density at 600 nm of the suspension was adjusted to 2.0 by addition of buffer.

Pyochelin or its analogues were dissolved in methanol (analytical grade) to a concentration of 10 mM (measured by spectrophotometry on a double beam Uvikon 930, Kontron spectrophotometer). The ^{55}Fe solution was prepared by dilution of 5.0 μL commercial $^{55}FeCl_3$ (2 mCi) in 95 μL 0.5 N aqueous hydrogen chloride. The radioactive pyochelin (or analogues)–^{55}Fe(III) complex was prepared after mixing 12.5 μL of the siderophore solution and 2.0 μL of the $^{55}FeCl_3$ solution. After 15 min incubation, the volume was adjusted to 1 mL by addition of 50 mM MOPS buffer pH 8.0. 500 μL of this solution and 500 μL of the cell suspension were mixed. 100 μL of this suspension were withdrawn at varying times (1, 5, 10, 15, 20, 30, 40 and 50 min), filtered on Whatman cellulose nitrate membrane filters 0.45 μm and washed with 0.5 N aqueous hydrogen chloride.[17] The filters were then counted. The same experiment was performed without cells and the values obtained were substracted from those obtained in the presence of cells.

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