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From a total synthesis of cepabactin and its 3:1 ferric complex to the isolation of a 1:1:1 mixed complex between iron (III), cepabactin and pyochelin

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Abstract—A novel and straightforward total synthesis of cepabactin and its iron (III) complex is described. The latter compound was compared and identified to that obtained from the cultures of *Burkholderia cepacia*. On treatment of the growth medium of two different strains of *B. cepacia* with ferric chloride, we have isolated and characterized an unexpected mixed complex of iron (III), cepabactin and pyochelin.

Iron is one of the most essential elements for aerobic life. Although extremely abundant in earth crust, in aerobic conditions and at physiological pH, iron (III) yields insoluble polymeric oxides in aqueous media. When grown in iron deficient conditions, microorganisms synthesize and excrete into extracellular medium, small molecules called siderophores in order to strongly chelate iron (III).[1] In Gram-negative bacteria, the ferrisiderophore is translocated through the membranes into the cytoplasm by a multiproteic system.[2] *Burkholderia* (ex-*Pseudomonas*) *cepacia*[3] is an emerging opportunistic pathogen responsible of the '*cepacia* syndrome', characterized by severe lung infections, often lethal especially for cystic fibrosis patients.[4] In the last decade, this bacterium increased its resistance against most of the antibiotics currently used. In a programme to develop new therapeutic strategies against *B. cepacia*, we have focused our attention on iron uptake systems in this bacterium. *B. cepacia* excretes mainly three siderophores: cepabactin **1**,[5] pyochelin **2**[6] and ornibactins **3**,[7] in strain depending proportions (Fig. 1).[8]

In due course to our studies of iron uptake in *Pseudomonas aeruginosa*,[9] we have described a few years ago a stereocontrolled synthesis of pyochelin **2**.[10] This protocol gave us an access to a panel of analogues currently under investigation for their biological activities.[11] Similarly we wish to develop a comparable strategy based on cepabactin **1**, which would be applied to *B. cepacia*. In this respect we describe in this paper, a new and straightforward synthesis of cepabactin and its iron (III) complex. As a matter of comparison we have also isolated the natural ferricepabactin from *B. cepacia*

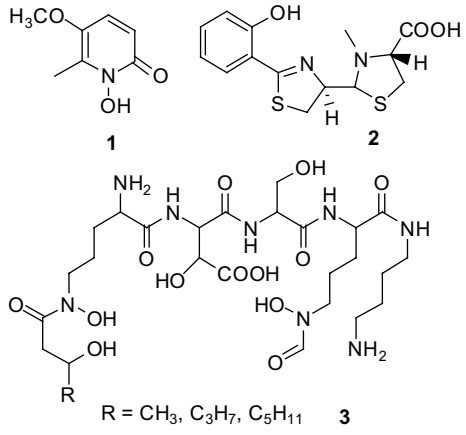

Figure 1. The main siderophores of *B. cepacia*.

Keywords: *Burkholderia cepacia*; Siderophore; Cepabactin; Pyochelin; Microwave-assisted reaction.
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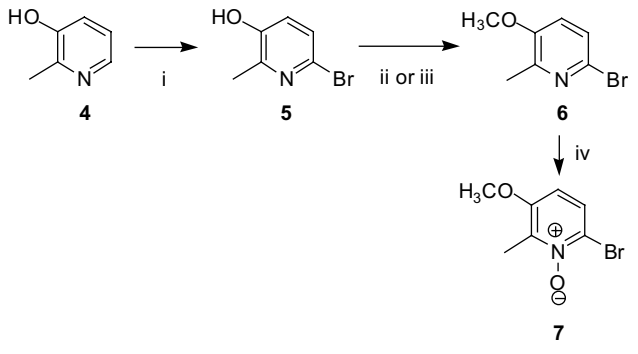
cultures. After treatment of the culture medium with ferric chloride, during the purification process, an unprecedented type of mixed complex between iron (III), cepabactin and pyochelin was isolated and is described as well in this article.

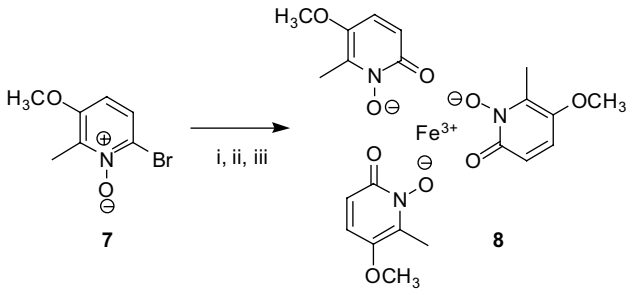
Ohta et al. described the first total synthesis of cepabactin **1**.[12] Unfortunately this protocol, which gave **1** only in poor yield, is based on an expensive commercially available starting material.[13] Therefore our synthesis starts with the bromination of commercial 3-hydroxy-2-methylpyridine **4** in alkaline medium according to a procedure reported by Hassberg and Gerlach.[14] Although under these conditions the yield of the brominated product **5** was low (22%), this latter could be easily isolated from a complex mixture by selective precipitation. The hydroxyl function of the substituted pyridine **5** was then methylated using either trimethylsilyldiazomethane or iodomethane. Both protocols led to the expected compound **6** with approximately the same yield, 91% and 95%, respectively.[15] The oxidation of the pyridine nitrogen atom was performed using *m*CPBA in refluxing chloroform leading to the expected *N*-oxide **7** isolated in 84% yield.[16] Other oxidizing systems like potassium perborate in acetic acid or aqueous hydrogen peroxide in trifluoroacetic acid appeared to be poorly efficient (Scheme 1).

In the last step of our protocol, the halogen/hydroxide exchange generated the expected cepabactin **1**. Among the different conditions tested, the best results were obtained when the reaction was performed in a 10% aqueous sodium hydroxide solution using microwave assisted reaction. The crude mixture was then acidified and continuously extracted with chloroform yielding crude cepabactin **1**. If ferric chloride was added at this stage, after extraction and purification, the expected ferricepabactin **8** was isolated in 58% yield.[17] Without microwaves, the same reaction gave **8** only in 27% yield after one week with a classical reflux (Scheme 2).

To confirm the structure of **8** we decided to compare its properties with the natural molecule. For this purpose two different *B. cepacia* strains (ATCC 17754 and ATCC 25416) were grown in succinate containing med-



Scheme 1. Reagents and conditions: (i) Br_2, $NaOH_{aq}$, 20 °C (yield: 22%); (ii) $TMSCHN_2$, MeOH, CH_2Cl_2, 20 °C (yield: 91%); (iii) CH_3I, K_2CO_3, acetone, reflux (yield: 95%); (iv) *m*CPBA, $CHCl_3$, reflux (yield: 84%).



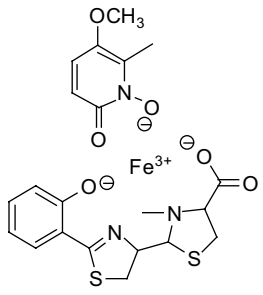
Scheme 2. Reagents and conditions: (i) 10% $NaOH_{aq}$, μ-waves; (ii) HCl; (iii) $FeCl_3$.

ium under iron depleted conditions.[18] When the supernatant was complemented with ferric chloride, the corresponding metal (III)–siderophore complexes were extracted with CH_2Cl_2. Under these conditions, two different complexes, an orange and a purple one, were isolated and purified by chromatography.

The orange complex showed two maxima in UV at 445 nm ($\varepsilon = 4880\ M^{-1}\ cm^{-1}$) and at 314 nm ($\varepsilon = 14800\ M^{-1}\ cm^{-1}$) in methanol suggesting that this compound is the ferricepabactin according to previously reported spectrophotometric properties.[5] This was confirmed by the following mass spectrometry experiments. Thus, high resolution mass measurements performed on this complex gave a mass of 518.0860 as expected for iron (III)–cepabactin complex (calcd mass: 518.0862). The electron impact spectra gave a main signal at *m*/*z* 364 corresponding to a fragment with two molecules of ligand bound to one metallic ion. In a last experiment, submitted to electrospray, the orange complex gave two major signals at *m*/*z* 541 and at *m*/*z* 1059 corresponding, respectively, to $M+Na^+$ and $2M+Na^+$. Submitted to the same UV and mass spectrometry measurements, the synthetic complex **8** proved to be identical to the natural ferricepabactin.

In UV, the purple complex dissolved in methanol showed three maxima, respectively, at 469 nm ($\varepsilon = 2400\ M^{-1}\ cm^{-1}$), 398 nm ($\varepsilon = 2700\ M^{-1}\ cm^{-1}$) and 319 nm ($\varepsilon = 9900\ M^{-1}\ cm^{-1}$). Using FAB mass spectrometry measurements this complex gave a signal at *m*/*z* 534 and some of the corresponding clusters. By adding dilute acid to the sample, this signal disappeared giving rise to a signal at *m*/*z* 519 corresponding to the ferricepabactin and a signal at *m*/*z* 364 corresponding to the fragment already observed above (iron chelated by two molecules of cepabactin). These signals were accompanied by a signal at *m*/*z* 325 corresponding to free pyochelin ($M+H^+$). These results proved that this purple complex contains iron (III), cepabactin and pyochelin. After calibration, MS electrospray gave two signals at *m*/*z* 533.037 and *m*/*z* 555.019 and mainly some of the corresponding adducts. These calculations agreed with a 1:1:1 stoichiometry between iron (III), cepabactin and pyochelin in the complex **9** (calcd mass for $M+H^+$: 533.038 and for $M+Na^+$: 555.019).

These mass spectrometry experiments are quite consistent with the low stability of the pyochelin metal com-



9

Figure 2. The 1:1:1 complex of iron (III), cepabactin and pyochelin.

plexes in solution and show that the most stable complexes involve the presence of cepabactin either as a 3:1 iron complex **8** or as a mixed complex **9**. Nevertheless this latter seems somehow less stable than the former since it tends to equilibrate to it. Finally in such a mixed complex **9**, pyochelin apparently contributes to the complexation giving four of the six dentates necessary to achieve an octahedral ferric complex. Some very recent results based on the tridimensional structure of the specific outer membrane receptor FptA loaded with ferripyochelin seem to confirm this observation.[20] Taken together these results support the structure shown above for the complex **9** (Fig. 2).

In conclusion, we have described in this paper a novel and straightforward microwave assisted synthesis of cepabactin **1**, a siderophore of *B. cepacia* and of its iron (III) complex **8**. A strong point is that this protocol will give an easy access to cepabactin analogues with a diversity point located on position 5 using alkylation reactions on the key compound **5**. These types of analogues are, to the best of our knowledge, only poorly documented in the literature and are currently under investigation in our group. In addition we have isolated and characterized a new type of mixed complex **9** formed by two siderophores of *B. cepacia*, pyochelin and cepabactin, and iron (III). The occurrence and stability of such a complex raises the question of the species that effectively transport iron into bacterial cells.

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15. *6-Bromo-3-methoxy-2-methylpyridine* **6**: a solution of **5** (332 mg, 1.77 mmol), CH_3I (132 μL, 301 mg, 2.13 mmol), K_2CO_3 (489 mg, 3.54 mmol) in acetone (15 mL) was refluxed 16 h. The mixture was cooled to room temperature, filtered through a Celite pad and adsorbed on silica before being purified on a silica gel column (20 g SiO_2, hexane/Et_2O: 2:1) leading to the expected methylether **6** (338 mg, 1.68 mmol, yield: 95%) isolated as a white solid. Mp 63–65 °C; R_f 0.55 (hexane/Et_2O: 2:1); ^1H NMR (300 MHz, $CDCl_3$): δ 2.49 (s, 3H), 3.86 (s, 3H), 6.71 (d, $J = 9.1$ Hz, 1H), 7.43 (d, $J = 9.1$ Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 11.8, 56.4, 108.5, 124.6, 125.8, 142.3, 154.8. MS (ES+) *m/z* 202 ($M+H^+$, 100), 203 (7), 204 (100),

205 (7); IR (neat, cm^{-1}): 3078, 3011, 2926, 2845, 2541, 2019, 1870, 1633, 1576, 1447, 1427, 1387, 1364, 1305, 1280, 1252, 1198, 1176, 1124, 1011, 970, 940, 931, 873, 820, 809, 749, 727. Anal. Calcd for C_7H_8BrNO: C, 41.61; H, 3.99; N, 6.93. Found: C, 41.93; H, 4.21; N, 6.75.

16. *6-Bromo-3-methoxy-2-methylpyridine-1-oxide* **7**: a solution of **6** (44 mg, 0.22 mmol), *m*CPBA 70% (108 mg, 0.44 mmol) in $CHCl_3$ (1 mL) was refluxed 18 h. The mixture was cooled to room temperature then a 0.5 N aqueous NaOH solution (10 mL), was added and the mixture was vigorously stirred for 1 h. After dilution with water (10 mL), the mixture was extracted with $CHCl_3$ (3×25 mL) and the organic layers were collected, dried over Na_2SO_4, and filtered. After evaporation under reduced pressure, the pure *N*-oxide **7** was isolated (40 mg, 0.18 mmol, yield: 84%) as a white solid. Mp 142–144 °C; R_f 0.46 (CH_2Cl_2/MeOH: 95:5); ^1H NMR (300 MHz, $CDCl_3$): δ 2.41 (s, 3H), 3.80 (s, 3H), 6.94 (d, $J = 8.5$ Hz, 1H), 7.21 (d, $J = 8.5$ Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 19.0, 55.6, 119.4, 125.4, 130.0, 150.0, 153.5. MS (ES+) *m*/*z* 240 ($M+Na^+$, 38), 241 (4), 242 (38), 243 (7), 277 (24), 278 (4), 279 (24), 280 (4), 457 ($2M+Na^+$, 48), 458 (7), 459 ($2M+2H^+ + Na^+$, 100), 460 (17), 461 (48), 462 (7). IR (neat, cm^{-1}): 3073, 3006, 2939, 2840, 2015, 1863, 1593, 1554, 1470, 1454, 1351, 1291, 1236, 1201, 1175, 1107, 1009, 930, 855, 801, 717. Anal. Calcd for $C_7H_8BrNO_2$: C, 38.56; H, 3.70; N, 6.42. Found: C, 39.02; H, 3.89; N, 6.26.
17. *Cepabactin* **1** *and ferricepabactin* **8**: in an adapted reaction tube, the *N*-oxide **7** (28 mg, 0.13 mmol) was dissolved in 10% aqueous solution of NaOH (4 mL). The mixture was irradiated 20 min (200 W, 150 °C, 12 bar) in a microwave reactor. The mixture was cooled to room temperature and the pH was adjusted to 2.0 with concentrated HCl. At this stage, the ligand **1** could be isolated by an overnight liquid–liquid extraction with $CHCl_3$ whereas the ferricepabactin **8** was prepared by adding a 2 M $FeCl_3$ solution (200 μL). The complex was then isolated by an overnight liquid–liquid extraction with $CHCl_3$. The resulting deep red solution was dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The crude (54 mg) was dissolved in a minimum amount of CH_2Cl_2 and hexane was dropwise added until a red precipitate appears. This precipitate was filtered off and dilution in MeOH induced the formation of a white precipitate. The latter was eliminated by filtration and the resulting deep orange filtrate was evaporated under reduced pressure. The final purification could be performed on Sephadex LH 20.[19] The expected ferricepabactin **8** (13 mg, yield: 58%) was isolated as orange-red crystals. Analytical and spectrophotometric data were consistent with those described previously for the natural ferrisiderophore.[5,19]
18. *Culture conditions for B. cepacia*: the standard succinate culture medium: Meyer, J.-M.; Abdallah, M. A. *J. Gen. Microbiol.* **1978**, *22*, 878–890, contained no added source of iron. *B. cepacia* ATCC 17754 or ATCC 25416 were grown for 24 h at 25 °C in this medium for preculture. This culture (2 mL) were added to 500 mL of culture medium as an inoculum, and bacteria were grown for 48 h at 25 °C.
19. *Isolation and purification of the iron-complexes*: 5 L of bacterial culture grown as above were treated after centrifugation with a 2 M $FeCl_3$ solution (10 mL) and the supernatant was extracted with methylene chloride (3×1.0 L). The organic solution was washed with water (3×0.4 L), dried, and evaporated to yield 500 mg of a crude mixture. This was dissolved in acetonitrile/water (70:30) and chromatographed on a Sephadex LH 20 column (900 mL) made up in the same eluent, which separated the orange ferricepabactin complex **8** (210 mg) from the purple mixed complex **9** (165 mg). Both compounds could be easily distinguished by TLC on silica gel eluted with MeOH/$CHCl_3$ 1:9 with respective R_f of 0.50 and 0.25.
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