Pergamon

Tetrahedron Letters, Vol. 38, No. 1, pp. 97-100, 1997

0040-4039/97 $17.00 + 0.00

PII: S0040-4039(96)02224-1

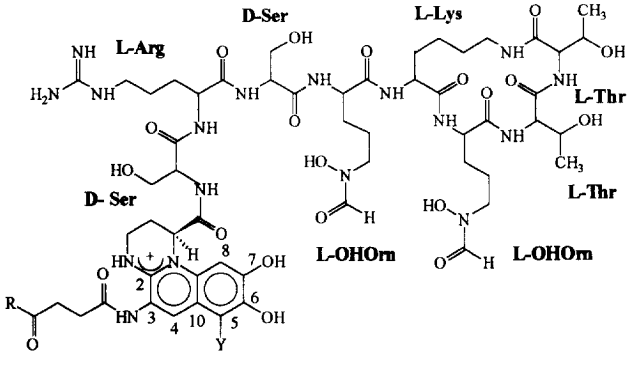
Pseudomonas Siderophores: A Mild and Selective Halogenation Procedure of the Chromophoric Moiety of Pyoverdins

Christophe Hennard[a], Bachir Machi[a], Pavel Kyslík[b] and Mohamed A. Abdallah[a*1]

[a]Laboratoire de Chimie Microbienne, Associé au C.N.R.S., Faculté de Chimie, Université Louis Pasteur, 1 rue Blaise Pascal, F-67008-Strasbourg, France; [b] Institute of Microbiology, Academy of Sciences of the Czech Republic, Videňská 1083, Prague, Czech Republic.

Abstract: *In the course of the purification procedure of pyoverdins produced by Pseudomonas aeruginosa ATCC 15692 in large batch cultures, a minor peptidic chlorinated siderophore was isolated. This compound possesses the same peptidic moiety as the three major pyoverdins isolated from this strain, but it is bound to a chlorinated chromophore derived from 5-chloro-2,3-diamino-6,7-dihydroxyquinoline. A mechanism for the formation of these compounds is suggested and a procedure for the preparation of halopyoverdins is described.*

When grown in iron-deficient conditions, *Pseudomonas aeruginosa* ATCC 15692 produces 3 pyoverdins: pyoverdin Pa, pyoverdin Pa A and pyoverdin Pa B **1 a-c**.[1,2] The purification procedure of these siderophores was earlier reported in details[3] and has been recently improved[4]. It involves successively centrifugation, ultrafiltration, hydrophobic chromatography on octadecylsilane[5], ion exchange chromatography on CM-Sephadex of the crude free pyoverdins. This is followed by complexation of each pyoverdin with ferric chloride, hydrophobic chromatography on octadecylsilane of the corresponding ferric complexes, ion exchange chromatography on CM-Sephadex and HPLC on octadecylsilane. The final steps are successively decomplexation by EDTA, hydrophobic chromatography on octadecylsilane and CM-Sephadex chromatography of the pure ligand. During the HPLC purification step of large amounts of ferripyoverdin Pa A (iron(III) complex of **1b**), a very small proportion of a closely related ferripyoverdin was isolated, the structure of which is shown here in **1d**. This siderophore which we call chloropyoverdin Pa A differs from pyoverdin Pa A by the presence of a chlorine atom. We show here the precise location of this chlorine atom,



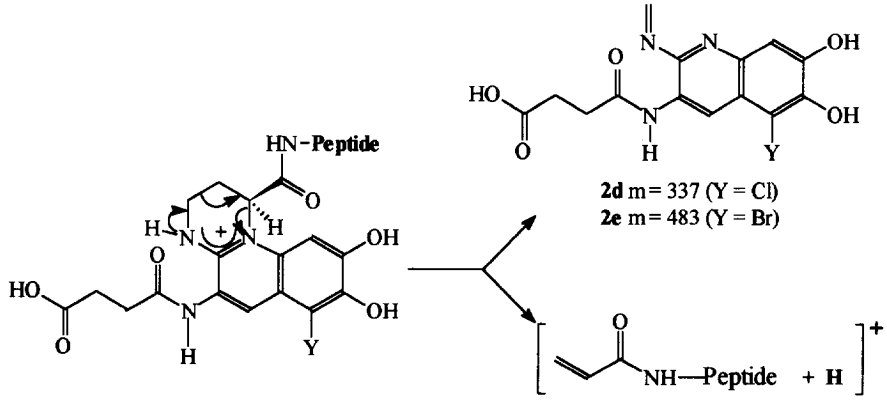
1 a	R = NH_2,	Y = H (pyoverdin Pa)
1 b	R = OH,	Y = H (pyoverdin Pa A)
1 c	R = COOH,	Y = H (pyoverdin Pa B)
1 d	R = OH,	Y = Cl (5-chloropyoverdin Pa A)
1 e	R = OH,	Y = Br (5-bromopyoverdin Pa A)

[1]Fax: (0033) 3 88 60 44 07; E-mail: abdallah@chimie.u-strasbg.fr

suggesting a mechanism for this halogenation. We also describe a procedure for the synthesis of 5-chloro- and -5-bromopyoverdin (**1d** & **1e**).

The UV-visible absorption spectrum of the ferric complex of chloropyoverdin Pa A (**1d**) shows one maximum at 400 nm (ε = 19500 M^{-1}x L) as well as two broad bands with shoulders at 460 nm (ε = 6000 M^{-1} x L) and 540 nm (ε = 3000 M^{-1}x L) and is identical to the spectrum of ferripyoverdin Pa A (**1b**)[1]. The spectrum of chloropyoverdin Pa A as free ligand is similar in shape to the spectrum of pyoverdin Pa A and pH-dependent as well. However it is shifted to higher wavelengths, with two maxima at respectively 382 nm (ε = 16000 M^{-1}x L) and 397 nm (ε = 16500 M^{-1}x L), at pH 5.0.[6]

FAB-MS performed on the free ligand shows a molecular peak at m/z 1368 (M^+), 34 mass units above the molecular peak of pyoverdin Pa A. The ratios of the signals at m/z 1368, 1369 & 1370 forming a molecular cluster are identical to those calculated for $C_{55}H_{83}N_{17}O_{22}Cl$, confirming that this increment of 34 mass units is precisely due to the substitution of a hydrogen atom by a chlorine atom on the molecule. The signal at m/z 1368 loses 337 mass units (**2d**) to yield a major fragment **3** at m/z 1031 which is a well known characteristic fragment common to all the pyoverdins produced by this bacterial strain and differing in their chromophore[1,2] (Scheme 1). It is characteristic of the release of the peptidic moiety possessing an additional acrylamide group, occurring from the cleavage across the chromophore[1,2].



Scheme 1

After the loss of the chromophoric part, the C-terminal fragmentation pattern shown by chloropyoverdin Pa A (**1d**) was identical to that determined for pyoverdin Pa A[1], whereas most of the signals forming the N-terminal fragmentation pattern were present but 34 m.u. above the corresponding ones in pyoverdin PaA. These FAB-MS results show clearly that the chlorine atom is located on the chromophore.

The 1D ^1H NMR spectrum of chloropyoverdin Pa A (**1d**) shows in addition that either proton H-8 or proton H-5 is replaced by another atom since in chloropyoverdin Pa A only one proton is present between 7.5 and 6.5 ppm, at precisely 6.85 ppm . The ^1D ^{13}C NMR spectrum confirms these results, and the DEPT 135 ^{13}C NMR spectrum shows in addition that it is indeed the position C-5 which is substituted by the chlorine atom (Table 1).

The 2D NMR ^1H-^{13}C long distance correlation spectrum confirms this result showing predominently the ^3J correlations given by H-4 with C-2, C-9, C-3 and C-5, and by H-8 with C-6 and C-10. The chemical shifts of the signals for carbon atoms C-4, C-5, C-8 and C-10, shifting respectively from 141.5, 115.6, 102.9 and 115.8 ppm in pyoverdin Pa A to 138.6, 117.2, 101.2 and 112.4 ppm in 5-chloropyoverdin, are also consistent with the replacement of H-5 in pyoverdin Pa A by a chlorine atom (Table 1)[7].

The hypothesis of the substitution by a chlorine atom of a biogenic precursor of pyoverdins during its biosynthesis in the culture was ruled out since

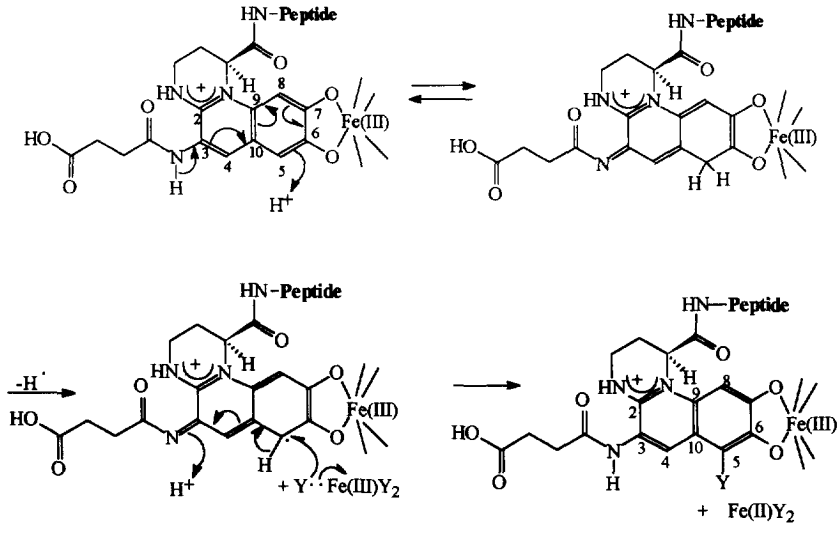
1- there were only traces of chloride ions present as a contaminant in the culture media,

2- addition of increasing amounts of sodium chloride (concentrations up to 17 mM) to the culture medium did not modify the proportion of this chlorinated compound.

Table 1 : Chemical shifts of the chromophoric carbon atoms of 5-chloropyoverdin Pa A compared to those of pyoverdin Pa A[1]

Carbon atom	Pyoverdin Pa A	Chloropyoverdin Pa A
C2	151.8	151.7
C3	118.4	118.3
C4	**141.5**	**138.6**
C5	**115.6**	**117.2**
C6	148.0	147.5
C7	158.8	158.7
C8	**102.9**	**101.2**
C9	135.4	136.6
C10	**115.8**	**112.4**

The values were determined in H_2O in the presence of 1% deuterated *t*-butanol as an internal standard. The values in bold correspond to the largest shift differences, consistent with the substitution of H-5 by Cl.



$$Fe(III)Y_3 + H^{\cdot} \longrightarrow Fe(II)Y_2 + H^+ + Y^- \quad (Y = Cl, Br)$$

Scheme 2

Then, the other hypothesis for obtaining a chlorinated pyoverdin is during the treatment with aqueous ferric chloride in the complexation step of the purification procedure.

Therefore, pure pyoverdin Pa A was treated with increasing amounts of ferric chloride, and the solution was analyzed by HPLC, where ferripyoverdin Pa A **1b** and 5-chloroferripyoverdin Pa A **1d** were present and easy to separate. The reaction of chlorination of ferripyoverdin Pa A increased and was quantitative when a 30 fold excess of ferric chloride was used[8]. The same treatment performed on pyoverdin Pa A using ferric bromide instead of ferric chloride gave also very high yields of the corresponding 5-bromoferripyoverdin Pa A **1e**. The corresponding free ligand showed a similar electronic spectrum as the chloro analogue, and its FAB-MS spectrum gave a molecular ion at m/z 1414 (M^+). The ratios of the signals at 1414, 1415 & 1416 forming a molecular cluster are identical to those calculated for $C_{55}H_{83}N_{17}O_{22}Br$.

The privileged electrophilic position for this halogenation would be position C-5 which is ortho to a phenolic hydroxyle and meta to the positively charged nitrogen of the chromophore. A plausible mechanism is a two step electron transfer oxidation[9] with a homolytic cleavage of a hydrogen at the bisallylic position of the tautomeric form of the chromophore, namely C-5, resulting in the formation of both an $H^{\cdot}$ radical and a bisallylic aromatic radical. Both radicals would reduce ferric halide to ferrous halide and give respectively a halide anion and the halogenated chromophore (Scheme 2)[10]. This mechanism illustrates the general character of this reaction: when applied to other pyoverdins, it yielded readily 5-halopyoverdins, independently of the peptidic moieties and independently of the side chains attached to the chromophore. It also explains the occurrence of 5-chloropyoverdins during the usual purification steps of pyoverdins which involve a step of complexation with iron(III) chloride[3], illustrating how easily artefactual 5-substituted pyoverdins can sometimes be isolated from bacterial cultures.

Acknowledgements
We acknowledge the expert technical assistance of Roland Graff in the determination of the NMR spectra.

References

1. Wendenbaum, S., Demange, P., Dell, A., Meyer, J. M., Abdallah, M. A. *Tetrahedron Lett.*, **1983**, *24*, 4877-4880.
2. Demange, P.; Wendenbaum, S.; Linget, C.; Mertz, C.; Cung, M. T.; Dell, A.; Abdallah, M. A. *Biol. Metals*, **1990**, *3*, 155-170.
3. Abdallah, M. A. In *Handbook of Microbial Iron Chelates*; Winkelmann, G. Ed., CRC Press Inc., Boca Raton, Florida, U.S.A., **1991**; pp. 139-153.
4. Albrecht-Gary, A. M.; Blanc, S.; Rochel N.; Ocaktan, A. Abdallah, M. A., *Inorg. Chem.*, **1994**, *33*, 6391-6402.
5. Demange, P.; Bateman, A.; Dell, A.; Abdallah, M. A. *Biochemistry*, **1988**, *27*, 2745-2752.
6. The molecular extinction coefficients of the iron complexes were deduced from the iron(III) concentration present as determined by atomic absorption. Those of the free ligand were calculated after titration of the free ligand with iron(III) (see reference 5).
7. Kalinowski, H. O.; Berger, S.; Braun, S. In "^{13}C NMR Spektroskopie", Kalinowski, H. O.; Berger, S.; Braun, S. Editors, Thieme G. Verlag Publishers, Stuttgart, Germany, **1984**, pp. 88-199.
8. In a typical experiment 1.0 mg of pyoverdin Pa A (**1b**) as a free ligand was dissolved in 1.0 ml water and treated with 30 equivalents of a $FeCl_3$ (or $FeBr_3$) solution in 5 mM HCl. The solution was kept 18 h at room temperature, then chromatographed on an octodecylsilane column before HPLC on octadecylsilane. The pure free ligand was obtained by decomplexation of the purified complexes with EDTA as described previously.
9. Kochi, J. K. In "Free radicals", Kochi, J. K., Editor, Wiley-Interscience, New York, **1973**, Vol. 1., pp.591-683
10. The ferric halide would exist in solution as an octahedral coordination complex. However the ligands shown (Y = halogen) will only be those pertinent to the discussion of the mechanism.

(*Received in France* 22 *October* 1996; *accepted* 13 *November* 1996)