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Structural characterization of vanchrobactin, a new catechol siderophore produced by the fish pathogen *Vibrio anguillarum* serotype O2

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Abstract—Vanchrobactin, a new catecol-type siderophore produced by cells of the fish pathogen *Vibrio anguillarum* serotype O2, has been isolated from the supernatants of iron-deficient cultures. Its structure was characterized from spectral data and established as *N*-[*N*′-(2,3-dihydroxybenzoyl)-arginyl]-serine.

Iron acquisition is an indispensable process for most living organisms because it is required as a cofactor for enzymes involved in general metabolism, DNA replication, as well as in the electron transport chain.[1] In spite of its abundance (5% of the earth crust), the availability of iron is dramatically limited by the very high insolubility of Fe^{+3} at physiological pH. Since there is virtually no free iron available for bacterial growth,[2] bacterial pathogens have evolved a number of mechanisms to overcome this iron restriction. The most common 'biomachinery' involves the synthesis and secretion of low molecular weight (300–2000 Da) high-affinity chelators to sequester Fe^{+3}, termed siderophores.[3] These molecules, generally excreted into the culture medium, are able to strongly chelate in a specific manner to solubilize and deliver Fe^{+3} into the cells. This process occurs via specific cell surface receptors using an ATP-dependent high-affinity transport system.[4]

The bacterial fish pathogen *Vibrio anguillarum* is the causative agent of vibriosis, an extremely fatal hemorrhagic septicaemia. This disease affects marine and freshwater fish species throughout the world that results in considerable economic losses in aquaculture farming worldwide.[5] Although there are more than 20 recognized serotypes,[6] those named O1 and O2 are the main ones implicated in the infections.[7] It is known that the ability to scavenge iron through the utilization of siderophores is a key factor in the virulence of this fish pathogen.[8]

At least two different siderophore-mediated systems have been described so far in *V. anguillarum*. Most pathogenic strains belonging to serotype O1 possess a system that is encoded by the 65-kb plasmid pJM1, which harbors genes for the synthesis and utilization of the catecholate-type siderophore anguibactin (**2**).[9,10] However, serotype O2 strains and some plasmidless serotype O1 strains produce a siderophore,[11a] given the trivial name of vanchrobactin (**1**), encoded by chromosomal genes and that is biologically unrelated to the anguibactin-mediated system.[11] The structure of vanchrobactin is still not determined, since attempts to reveal its complete structure have been hindered by problems associated with its purification in sufficient quantities and decomposition during isolation.

In this letter, we wish to present the isolation, and the planar structural characterization of the mentioned siderophore vanchrobactin (**1**).

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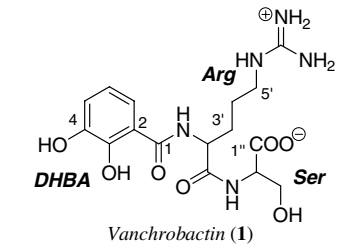
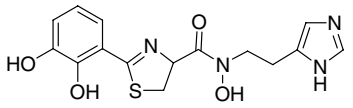


Vanchrobactin (**1**)



Anguibactin (**2**)

This compound was isolated from iron-deficient cultures in CM9 medium of *V. anguillarum* serotype O2 strain RV22 by a bioguided fractionation using several assays. Siderophore activity of fractions was tested using plate assays (CM9 medium supplemented with the iron chelator 2,2′-dipyridyl 100 μM) with strain RV22 as indicator. In addition, siderophore activity was measured by the CAS assay,[12] along with the Arnow test[13] that was used for catechol determination.

The cell-free culture supernatants (18 L) were loaded onto a XAD-7 resin, and then, after washing with distilled water, the siderophore was eluted with a methanol/water (1:1) mixture followed by methanol. The active fractions were first dissolved in water and then extracted with CH_2Cl_2 and *n*-BuOH. The bioactive aqueous fraction was evaporated under reduced pressure and then chromatographed on a Sephadex LH-20 column in 10% MeOH/H_2O. Finally, repeated RP-HPLC purification of the active fraction on Atlantis C18 ($100 \times 4{,}6$ mm, 5 μm) column using a 10 min 0–50% gradient of acetonitrile in water with 0.05% TFA gave 0.8 mg (retention time 5 min, flow 1 mL/min) of compound **1**.

Compound **1** was obtained as an amorphous solid with an $[\alpha]_D$ of −13.6 (c 25×10^{-3}, MeOH). The strong IR band at 1679 cm^{-1} was characteristic of an amide C=O stretch, while the broadband near 3349 cm^{-1} was indicative of the presence of O–H stretching and an hydrogen bonded amide NH.[14] The prominent pseudomolecular ion $[M+H]^+$ at m/z 398 along with $[M+Na]^+$ at m/z 420 in the (+)-ESIMS of **1** and the corresponding $[M-H]^-$ at m/z 396 in the (−)-ESIMS were consistent with a molecular weight of 397 for vanchrobactin. The HRESIMS of the pseudomolecular $[M+H]^+$ ion at m/z 398.1658 was in good agreement with that expected for $C_{16}H_{24}N_5O_7$ (calculated 398.1670). The ^{13}C NMR and DEPT spectra of **1** in D_2O displayed 16 distinct resonances including three carbonyl groups, one sp^2 quaternary carbon at 157.24 characteristic of a guanidine moiety, six aromatic carbons (three CH and three quaternary), two methines, and four methylenes. The presence of a catechol moiety in **1** was easily deduced from the proton and carbon chemical shifts, and the coupling of the three proton aromatic signals. The combined use of ^1H–^1H DQF-COSY and edited *g*HSQC experiments measured in a cryoprobe, allowed the assignment of three spin systems, which clearly suggested the presence of a dipeptide composed by serine and arginine aminoacid residues linked to the 2,3-dihydroxybenzoyl moiety (DHBA). The downfield shifted of the arginine α-proton at 4.67 ppm suggested that DHBA was attached to the free amino group of the arginine. This was confirmed by the long range C–H correlation observed in the *g*HMBC spectrum between the carbonyl carbon at 170.55 ppm of the DHBA moiety and the H2′ proton of the arginine residue which showed also HMBC correlations to C1′ and C3′. Further long range C–H correlations observed in the *g*HMBC allowed us to assemble the three fragments (Fig. 1). The absolute configurations of the aminoacid residues have not been determined.

On the basis of these data, the structure of vanchrobactin (**1**) was thus established as *N*-[*N*′-(2,3-dihydroxybenzoyl)-arginyl]-serine.

Very few containing-arginine siderophores were reported so far.[15] Since it is very unlikely that arginine serves as metal-binding ligand at pH 7–8 because its pK_a is too high, it was postulated that it plays a role in molecular recognition with the outer membrane receptors.[16]

Interestingly, our recent work,[17] on the identification and characterization of the genes and enzymes involved in the biosynthesis of vanchrobactin in strain RV22, confirms the structure reported here. The analysis of the adenylation domains of enzymes VacE and VacF, two non ribosomal peptide synthetases, as well as the mutation of the respective genes, confirm that arginine and serine are in fact the aminoacids incorporated in vanchrobactin. The assembly order of the three components (DHBA, arginine, serine) was also confirmed by the module arrangement predicted by the enzymes aminoacid sequences. On the other hand, the identification of other genes that codify for enzymes involved in DHBA biosynthesis clearly demonstrates that both, DHBA and vanchrobactin, are products of the iron metabolism of *V. anguillarum* serotype O2 strains.

According to the structure reported here, vanchrobactin (**1**) shares several structural similarities with anguibactin (**2**), the siderophore typically produced by strains of *V. anguillarum* serotype O1. Both are linear molecules

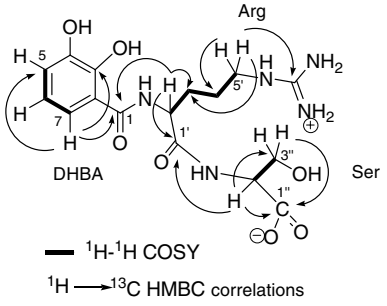


Figure 1. Selected COSY and HMBC correlations observed for **1**.

Table 1. ^{13}C NMR (125 MHz), ^{1}H NMR (500 MHz), and HMBC spectral data of vanchrobactin (**1**) in D_2O

Position	δ_C	DEPT	δ_H mult (*J* in Hz)	HMBC (H → C)
DHBA				
C1	170.55	C		
C2	117.83	C		
C3	146.87	C		
C4	145.08	C		
C5	120.45	CH	6.83 d (6.9)	C: 3,4,7
C6	120.23	CH	6.65 t (6.9)	C: 2,4
C7	120.18	CH	7.01 d (6.9)	C: 1,3,5
Arginine				
C1′	174.23	C		
C2′	54.08	CH	4.67 dd (7.4, 4.9)	C: 1(DHBA), 1′, 3′(Arg)
C3	28.86	CH_2	2.33 m/2.22 m	
C4′	24.82	CH_2	2.08 m	
C5′	40.98	CH_2	3.41 t (5.9)	C: 3′,4′,6′(Arg)
C6′	157.24	C		
Serine				
C1″	174.57	C		
C2″	56.14	CH	4.52 br t (4.4)	C: 1″,3″(Ser), 1′(Arg)
C3″	61.79	CH_2	4.02 dd (4.4 and 10.5)/3.97 dd (3.3 and 10.5)	

derived from dipeptide derivatives bound to 2,3-dihydroxybenzoic acid. However, the structure of vanchrobactin differs from that of anguibactin by the lack of the hydroxamate functionality and by the aminoacid residue precursors: arginine and serine for vanchrobactin instead of cysteine and decarboxylatehistidine residues for anguibactin. This suggests that both siderophores may have different evolutive origins.

It has been reported,[11a] that functional and genetic similarities exist between vanchrobactin and enterobactin (a triscatechol derivative of a cyclic triserine lactone)[18] produced by *Escherichia coli* and other Enterobacteria, some of them being human pathogens. The results reported here corroborate these previous observations since enterobactin, like vanchrobactin, is made from DHBA and serine (see Table 1).[19]

The observation that a fish pathogen synthesizes a siderophore chemically related to that produced by human pathogens, opens new interesting perspectives to study the evolution of bacterial pathogens and their metabolic functions. From a practical point of view, and since siderophores are critical for growth and virulence of the producer pathogens, the knowledge of its structure and biosynthesis pathways can be useful for the development of a novel class of antimicrobials.[20] In this regard, the determination of vanchrobactin structure, together with the already known anguibactin, may lead to new strategies for either, interfering with the iron-acquisition mechanisms of *V. anguillarum*, or be used as 'trojan horses' for development of new rationally designed antibacterial agents against vibriosis.

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