Synthesis and biological activity of analogues of vanchrobactin, a siderophore from *Vibrio anguillarum* serotype O2

Raquel G. Soengas,[a] Marta Larrosa,[a] Miguel Balado,[b] Jaime Rodríguez,*[a] Manuel L. Lemos[b] and Carlos Jiménez*[a]

Received 21st December 2007, Accepted 11th February 2008
First published as an Advance Article on the web 29th February 2008
DOI: 10.1039/b719713f

Several analogues of vanchrobactin, a catechol siderophore isolated from the bacterial fish pathogen *Vibrio anguillarum* serotype O2 strain RV22, have been synthesized. The biological evaluation of these novel compounds showed that most of them are active as siderophores, as determined by growth promotion assays using the producer strain, as well as *V. anguillarum* serotype O1, *Salmonella enterica*, and *Erwinia chrysanthemi.* These compounds also gave a positive chrome azurol-S (CAS) test. On the basis of these results, we were able to deduce some structure–activity relationships. Furthermore, we found an analogue with siderophore activity that has appropriate functionality (an amino group) for use as an antibiotic vector to be employed in a "Trojan horse strategy".

Introduction

All living creatures require iron in their cellular reactions. Since iron is unavailable for uptake due to the low solubility of Fe^{3+} at the neutral to alkaline pH conditions in soil or in aqueous media, organisms have developed different mechanisms to acquire it from the environment. During infection, most bacteria encounter within the host an iron-limited environment: higher eukaryotes contain substantial amounts of this metal but it is tightly associated with transport and storage proteins and not freely available for pathogens. Consequently, the level of free iron in biological fluids is usually estimated to be only 10^{-18} M. In an attempt to cope with this insolubility, many microorganisms have evolved specific iron uptake mechanisms. One of these mechanisms involves the production of iron chelators named siderophores, which are low molecular weight (300 to 2000 Da) ferric-specific ligands. These molecules, which are generally excreted into the culture medium, are able to strongly chelate Fe^{3+} in a specific manner to solubilize it and deliver it to the cells. In bacteria, the siderophore–iron(III) complex is recognized and transported by specific outer membrane receptors. The uptake process is thermodynamically driven from the cytoplasm through the TonB system that transduces the necessary energy from the cytosol to the outer membrane receptor. Natural siderophores are usually not very selective and many bacteria and fungi can utilize exogenous siderophores made by different microorganisms.[1]

Vibrio anguillarum is the causative agent of vibriosis in fish, a fatal hemorrhagic septicaemia. This disease affects marine and freshwater fish species and results in considerable economic losses in aquaculture farming worldwide.[2] There are more than twenty recognized serotypes, but those denoted O1 and O2 are the main ones implicated in vibriosis outbreaks.[3] Although the virulence mechanisms of *V. anguillarum* are not fully understood, it is known that the ability to scavenge iron through the utilization of siderophores contributes significantly to the virulence of this bacterium.[4] The best characterized siderophore produced by *V. anguillarum* is anguibactin, which was isolated from a strain belonging to serotype O1. In the course of our studies into siderophores from marine microorganisms, a novel siderophore, vanchrobactin (**1c**), was isolated (and characterized) from iron-deficient cultures of *V. anguillarum* serotype O2 strain RV22.[5] The stereochemistry of **1c** was elucidated by chiral capillary electrophoresis analysis and total synthesis, showing the compound to be *N*-[*N*′-(2,3-dihydroxybenzoyl)-D-arginyl]-L-serine.[6] The increasing antibiotic-mediated selective pressure has led to the emergence of multiresistant strains in many bacterial pathogens, and fish pathogens are no exception. Thus, more novel and efficient antimicrobial drugs are necessary to treat these emerging infections that are resistant to common antibiotics. One way as increase the efficiency of antimicrobials against bacterial infections could be the "Trojan horse strategy", where antimicrobial drugs are coupled to siderophores and transported across the bacterial membranes through the iron uptake pathways.[7] Such an approach has already been developed and has given promising results with the pyoverdine-mediated iron uptake system[8] and conjugated siderophore/β-lactamase inhibitors.[9] Our working hypothesis was that vanchrobactin from *V. anguillarum* could be a very interesting candidate for such a prodrug strategy aimed at the development of new antimicrobials against vibriosis and probably against other related infectious diseases.

Previous examples of natural and synthetic catecholate-type compounds based on amino acids or peptides, such as chrysobactin,[10] were shown to be active as siderophores. Although those studies allowed some structure–activity correlations to be deduced,[11] very few systematic investigations into this type of compound have been carried out.[12] We report here the synthesis of several analogues of vanchrobactin (**1c**) using the efficient, simple and versatile synthesis that we reported recently.[5] The aim was

[a]*Departamento de Química Fundamental, Facultade de Ciencias, Universidade da Coruña, Campus da Zapateira, 15071, A Coruña, Spain. E-mail: carlosjg@udc.es, jaimer@ udc.es; Fax: +34 9811 67065; Tel: +34 9811 67065*
[b]*Departamento de Microbiologia y Parasitologia, Instituto de Acuicultura, Universidade de Santiago de Compostela, Santiago de Compostela, E-15782, Spain. E-mail: mlemos@usc.es; Fax: +34 9815 47165; Tel: +34 9815 63100*

to evaluate their siderophore activity and to study the structure-siderophore activity relationships. Moreover, in view of its possible use as an antibiotic vector, another goal was to obtain an analogue with siderophore activity for *V. anguillarum* that incorporated appropriate functionality (such as an amino group) for use as an anchor to be attached directly (or *via* spacers) to other biologically active agents, like antibiotics, for the design of iron transport-mediated drug delivery.

Results and discussion

In order to compare the influence of the configuration of the asymmetric centres, the amino acid sequence and the presence of the guanidine moiety on the siderophore activity of vanchrobactin analogues, compounds **1–11** were prepared using a strategy similar to that employed for the total synthesis of vanchrobactin.[6]

Synthesis of *N*-(2,3-dihydroxybenzoyl)-arginyl/ornithinyl derivatives

Firstly, we studied the preparation of the analogues with the DHBA directly linked to ornithine or arginine residues (Scheme 1), *i.e.*, analogues **1**, **3**, **4** and **6**.

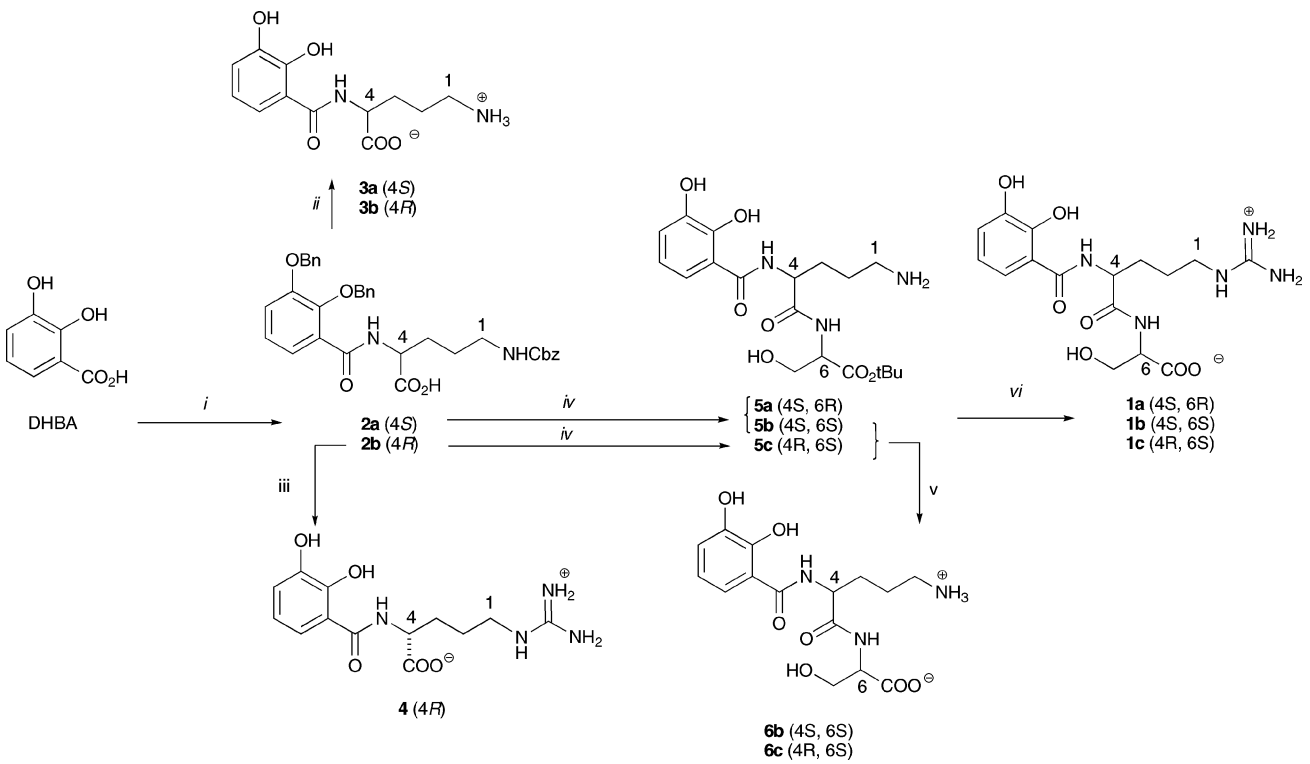
N^{δ}-Cbz-D-ornithine-OMe, prepared from D-ornithine,[13] was coupled with 2,3-dibenzyloxybenzoic acid (DHBA)[14] using TBTU as the coupling agent to give the corresponding ester in very good yield. The resulting ester was hydrolyzed with barium hydroxide, giving the acid **2b** in a 78% yield.[15] The same sequence was carried out using N^{δ}-Cbz-L-ornithine-OMe, which was prepared by esterification of the commercially available N^{δ}-Cbz-L-ornithine,[16] to afford the enantiomeric acid **2a** (79% yield). Both acids were submitted to catalytic hydrogenation, giving rise to analogues **3a** (DHBA-L-Orn) and **3b** (DHBA-D-Orn) in quantitative yield. Alternatively, protection of the acid **2b** as the *tert*-butyl ester, catalytic hydrogenation followed by the introduction of the guanidine function by reaction with 1,3-di-Boc-2-(trifluoromethanesulfonyl) guanidine,[17] and acidic hydrolysis afforded analogue **4** (DHBA-D-Arg) in 35% yield.

Both D- and L-serine *tert*-butyl esters were coupled with compound **2a** using TBTU, after removal of the benzyl and Cbz protecting groups, to afford compounds **5a** (28% yield) and **5b** (29% yield), respectively. Following the same procedure, L-serine *tert*-butyl ester was coupled with **2b** to give compound **5c** in 82% yield. Derivatives **5b** and **5c** were submitted to acidic hydrolysis of the *tert*-butyl ester to afford analogues **6b** (DHBA-L-Orn-L-Ser) and **6c** (DHBA-D-Orn-L-Ser), respectively, in quantitative yields.

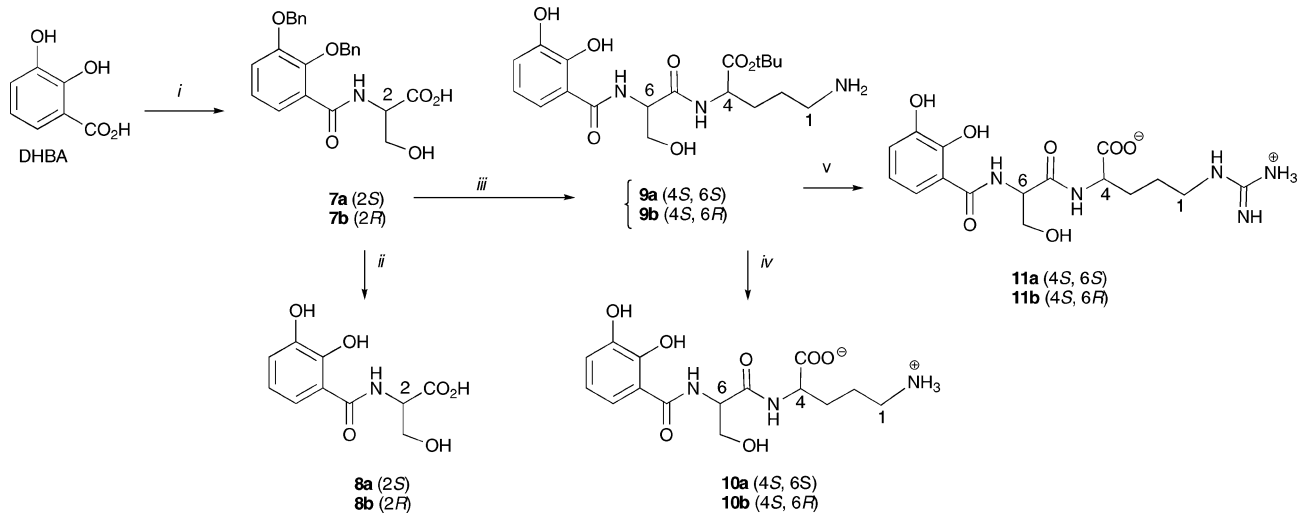
Finally, guanidinylation of **5a**, **5b** and **5c** as before followed by acidic hydrolysis gave **1a** (DHBA-L-Arg-D-Ser), **1b** (DHBA-L-Arg-L-Ser) and **1c** (DHBA-D-Arg-L-Ser, vanchrobactin) in 73%, 62% and 61% yield, respectively.

Synthesis of 2,3-dihydroxybenzoyl-serinyl derivatives

In an effort to evaluate the influence of the amino acid sequence on the siderophore activity, we designed the synthesis of



Scheme 1 *Reagents and conditions*: i: 1. $SOCl_2$/MeOH (89%); 2. BnBr/K_2CO_3 (99%); 3. $Ba(OH)_2$, THF, H_2O, 50 °C, 12 h, quant.; 4. N^{δ}-Cbz-L-ornithine-OMe or N^{δ}-Cbz-D-ornithine-OMe, TBTU, Et_3N, DMF, rt, 16 h (78% and 79%, respectively); 5. $Ba(OH)_2$, THF, H_2O, 50 °C, 16 h, quant.; ii: H_2, Pd/C, MeOH, rt, 3 h, quant.; iii: 1. *t*-BuOAc, $HClO_4$; 2. H_2, Pd/C, MeOH, rt, 3 h, (48%); 3. $(NHBoc)_2NTf$, Et_3N, $CHCl_3$, rt, 1 h (73%); 4. TFA/DCM 3:7, rt, 16 h, quant.; iv: 1. L-Ser-O^{t}Bu or D-Ser-O^{t}Bu, TBTU, Et_3N, DMF, rt, 16 h; 2. H_2, Pd/C, MeOH, rt, 3 h (28%, 29% and 82%); v: TFA/DCM 3:7, rt, 16 h, quant.; vi: 1. $(NHBoc)_2NTf$, Et_3N, $CHCl_3$, rt, 1 h (73%, 62% and 61%); 2. TFA/DCM 3:7, rt, 16 h, quant.



Scheme 2 *Reagents and conditions*: i: 1. $SOCl_2$/MeOH (89%); 2. $BnBr/K_2CO_3$ (99%); 3. $Ba(OH)_2$, THF, H_2O, 50 °C, 12 h, quant.; 4. L-Ser-OMe or D-Ser-OMe, TBTU, Et_3N, DMF, rt, 16 h (84% and 82% respectively); 5. $Ba(OH)_2$, THF, H_2O, 50 °C, 16 h, quant.; ii: H_2, Pd-C, MeOH, rt, 3 h, quant.; iii: N^{δ}-Cbz-L-ornithine-O^{t}Bu, TBTU, Et_3N, DMF, rt, 16 h, (81% and 93% respectively); iv: 1. H_2, Pd/C, MeOH, rt, 3 h, quant.; 2. TFA/DCM 3:7, rt, 16 h, quant.; v: 1. $(NHBoc)_2NTf$, Et_3N, $CHCl_3$, rt, 1 h (71% and 79% respectively); 2. TFA/DCM 3:7, rt, 16 h, quant.

2,3-dihydroxybenzoyl derivatives linked directly to a serine (instead of arginine residues) using a similar strategy to that described above (analogues **8**, **10** and **11**)

Coupling of the L- and D-serine methyl esters with 2,3-dibenzyloxybenzoic acid using TBTU, followed by hydrolysis of the corresponding esters with barium hydroxide, afforded compounds **7a** (84% yield) and **7b** (82% yield), respectively. Catalytic hydrogenation of acids **7a** and **7b** gave analogues **8a** (DHBA-L-Ser)[18] and **8b** (DHBA-D-Ser) in quantitative yields.

The N^{δ}-benzyloxycarbonyl-L- and D-ornithine *tert*-butyl esters[19] were coupled with compound **7a** using TBTU, and the resulting products were submitted to catalytic hydrogenation to give compounds **9a** (81% yield) and **9b** (83% yield), respectively. Acidic hydrolysis of the *tert*-butyl ester afforded analogues **10a** (DHBA-L-Ser-L-Orn) and **10b** (DHBA-L-Ser-D-Orn), both in quantitative yield..

Finally, guanidinylation of **9a** and **9b** as before followed by acidic hydrolysis gave **11a** (DHBA-L-Ser-L-Arg) and **11b** (DHBA-L-Ser-D-Arg) in 71% and 69% yield, respectively.

Biological evaluation

Compounds **1**, **3**, **4**, **6**, **8**, **10** and **11** were evaluated for their siderophore activities by growth promotion assays with several bacteria that are well defined in their ability to transport and utilize natural siderophores (siderophore indicator strains). The following indicator strains were used: *Vibrio anguillarum* serotype O2 RV22 (strain producing vanchrobactin), *V. anguillarum* serotype O1 775 (strain producing siderophore anguibactin, but that can also use vanchrobactin as siderophore), *Vibrio alginolyticus* TA15[20] (strain producing a hydroxamate siderophore that cannot use vanchrobactin as an exogenous siderophore), *Erwinia chrysanthemi* PPV20 (strain that produces chrysobactin, a siderophore structurally related to vanchrobactin)[21] and *Salmonella enterica* enb-1 (a mutant strain that does not produce the natural siderophore enterobactin but that can use vanchrobactin as an exogenous siderophore).[22] The results of these growth promotion assays are given in Table 1.

Growth promotion of *Vibrio anguillarum* strains

All analogues were tested for growth promotion under iron-deficient conditions for two strains of *V. anguillarum*: (a) one strain (RV22 from serotype O2) from which vanchrobactin was isolated, and (b) one strain (775 from serotype O1) that produces the siderophore anguibactin. All analogues stimulated growth of both strains with the exception of **3a** (DHBA-L-Orn), which was inactive. These results are consistent with the catecholate and amino acidic structural nature of the natural siderophores biosynthesized by both strains. Furthermore, they indicate that these microorganisms are not very specific in the use of siderophores and they can use several different ones to supply iron to the cell, even siderophores with very different structures.

The natural siderophore **1c**, along with its stereoisomers **1a** and **1b**, having the sequence DHBA-Arg-Ser, was found to be the most active compound in the growth promotion of *V. anguillarum* RV22 (serotype O2). The similar values obtained for the three analogous stereoisomers **1a–c** indicate that the absolute configuration of the amino acid moieties has little influence on the siderophore activity. Interestingly, compounds **6b** and **6c**, which have an ornithine instead of an arginine residue, also promoted the growth of both strains under iron-deficient conditions. This result could be very useful because these vanchrobactin derivatives possess an amino group that can be attached to other bioactive agents in the Trojan horse strategy. Compounds in which DHBA is linked directly to serine and to D-ornithine were found to be the most active analogues in the growth promotion of *V. anguillarum* 775 (serotype O1). These differences between the two strains could be due to the different outer membrane proteins involved in ferric-siderophore transport in strains RV22 and 775.[3]

Table 1 Growth promotion is represented as a growth halo diameter (average values after three independent experiments) of the indicator strains as follows: –, no growth; +, halo <12 mm; ++, halo diameter 12–14 mm; +++, halo diameter 15–17 mm; ++++, halo diameter >17 mm. The CAS test gives negative values: higher numbers indicate higher iron chelating activity. The natural compound (**1c**, vanchrobactin) is shown in italics

Analogues	*Vibrio anguillarum* RV22 (serotype O2)	*Vibrio anguillarum* 775 (serotype O1)	*Erwinia chrysanthemi* PPV20	*Vibrio alginolyticus* TA15	*Salmonella enterica* enb-1	CAS Test
Series Orn(Arg)-Ser						
$Fe_2(SO_4)_3$ (growth control)	+++	++++	++++	++++	++	—
DHBA-L-Arg-D-Ser (**1a**)	+++	++	+	–	–	−0.655
DHBA-L-Arg-L-Ser (**1b**)	+++	++	++	–	–	−0.736
DHBA-D-Arg-L-Ser (**1c**)	++++	++	+++	–	+	−0.714
DHBA-L-Orn (**3a**)	–	–	–	–	–	−0.353
DHBA-D-Orn (**3b**)	++	+++	+	–	++	−0.672
DHBA-D-Arg (**4**)	++++	++	++	–	+	−0.476
DHBA-L-Orn-L-Ser (**6b**)	++	+	+	–	++	−0.621
DHBA- D -Orn- L–Ser (**6c**)	++	++	++	–	+	−0.679
Series Ser-Orn(Arg)						
DHBA-L-Ser (**8a**)	++	+++	–	–	–	−0.589
DHBA-D-Ser (**8b**)	++	+++	–	–	–	−0.788
DHBA-L-Ser-L-Orn (**10a**)	++	+	+	–	+++	—
DHBA-L-Ser-D-Orn (**10b**)	++	+	+	–	+++	—
DHBA-L-Ser-L-Arg (**11a**)	++	+	–	–	–	−0.476
DHBA-L-Ser-D-Arg (**11b**)	++	+	+	–	+	−0.547

Growth promotion of *Vibrio alginolyticus* TA15

In order to test the specificity of each analogue, all compounds were tested for growth promotion of a strain belonging to the Vibrionaceae family, but producing a different type of siderophore. The lack of biological activity in *V. alginolyticus* TA15 of all analogues probably confirms the presence of a completely different structure for the still unknown natural siderophore synthesized by this pathogen. In fact, a recent study suggested a structure that is closely related to vibrioferrin,[23] which is a non-catecholate siderophore belonging to a member of the carboxylate class of siderophores and contains two α-hydroxy acid groups.[24]

Growth promotion of *Erwinia chrysanthemi* PPV20

This strain produces chrysobactin, a catechol siderophore bearing lysine and serine residues linked to DHBA.[21] Thus, the only difference in the structure with respect to vanchrobactin is the substitution of the arginine by a lysine residue. Due to these highly related structures, we tested whether *E. chrysanthemi* could use vanchrobactin and its analogues as siderophores. The synthetic compounds in which the DHBA residue is directly linked to ornithine or arginine were the most active analogues for the growth promotion of *E. chrysanthemi.* However, those analogues in which the DHBA is linked directly to serine showed weaker growth promotion ability or were inactive. This result suggests that the ferric-siderophore receptors present in *E. chrysanthemi* are probably more structure-specific than those present in *V. anguillarum.*

Growth promotion of *Salmonella enterica* serovar *Typhimurium* enb-1

All salmonellas produce the well-known siderophore enterobactin, which is a cyclic trimer of 2,3-dihydroxy-N-benzoyl-L-serine. Our previous studies[3] showed that the enterobactin-deficient mutant enb-1 can use vanchrobactin to overcome iron limitation. We wished to determine whether this utilization is exclusive for the natural siderophore. The results showed that besides vanchrobactin, other derivatives containing ornithine instead of arginine were also active as growth promoters under iron-limiting conditions.

Siderophore activity of vanchrobactin analogues in CAS assays

In parallel to the growth promotion assays, the relative iron complexing capacities of the siderophore derivatives were evaluated by reactivity with chrome azurol-S (CAS),[25] and the results are shown in Table 1. Most analogues gave a positive reaction in this test with a range of values from −0.353 for **3a** to −0.788 for **8b**; the exceptions are compounds **10a** and **10b**, which lack reactivity for unknown reasons. It is worth noting that a high CAS reactivity does not necessarily reflect high growth promotion ability. This is explained by the fact that the CAS test is based on the iron chelating activity of each compound, while the growth promotion assays reflect the ability of each strain to actually transport the different compounds into the cell, a process that is related to the recognition of each ferric-compound by an appropriate outer membrane receptor. Thus, there is generally a qualitative match between the two methods but not a quantitative correlation, and high biological activity can be seen along with relatively low CAS

reaction and *vice versa*. This same observation was previously reported for analogues of exochelin from *Mycobacterium*.[26]

Conclusions

Very few systematic investigations into structure–activity correlations of siderophores have been performed to date on amino acids and dipeptides. We have prepared a new series of analogues of vanchrobactin (**1c**) in order to determine the influence on the iron transport activities of the configuration of the asymmetric centres, the presence of the amino acid residues linked to the dihydroxybenzoic acid moiety, and the presence of the guanidine or amine moiety on the arginine or ornithine residues. The results reveal the lack of stereoisomeric influence of the amino acid scaffold on the siderophore activity in both serotypes of *V. anguillarum*, although some differences can be observed in *Erwinia chrysanthemi* PPV20 and *Salmonella enterica* serovar *Typhimurium* enb-1.

V. anguillarum and other Gram-negative bacteria tested were able to use several different analogues as siderophores, with the exception of *V. alginolyticus* TA15, which did not use any of them. The siderophore activity found for most of the synthetic analogues in the growth promotion of *Erwinia chrysanthemi* PPV20 is consistent with the catecholate amino acid structure of chrysobactin, its natural siderophore. This finding is in agreement with previous observations concerning the importance of the aromatic ring in catecholate siderophores binding to the outer membrane receptors. In this regard, the *Escherichia coli* FepA receptor transports certain catecholate ferric siderophores but not others, nor any noncatecholate compounds.[27].

Our observations confirm previous reports in the sense that microorganisms are not always selective in the use of siderophores and they can use several different ones to supply iron to the cell, even siderophores with very different structures. This lack of specificity could be an evolutionary adaptation of bacterial pathogens to increase the number of possibilities to obtain iron from the environment.

A vanchrobactin analogue has been prepared that has siderophore activity and an amino functionality that could be used as an anchor group to attach it to other bioactive agents. This opens new and interesting possibilities for the use of the Trojan horse strategy to develop new drugs that are effective against vibriosis and other related infections. The relatively low specificity of siderophore utilization could enable the design of drugs that are effective against different Gram-negative bacteria. Experiments aimed at this objective are currently under way.

Experimental

N^{α}-2,3-Dibenzyloxybenzoyl-N^{δ}-benzyloxycarbonyl-L-ornithine (2a)

N^{δ}-Benzyloxycarbonyl-L-ornithine methyl ester hydrochloride (0.19 g, 0.66 mmol) and TBTU (0.32 g, 0.99 mmol) were added to a stirred solution of 2,3-dibenzyloxybenzoic acid (0.22 g, 0.66 mmol) in anhydrous DMF (6 mL). After 10 min, triethylamine (0.23 mL, 1.65 mmol) was added and the reaction mixture was stirred at room temperature for 20 h under an atmosphere of argon. T.l.c. (ethyl acetate–hexane 1:1) indicated complete conversion of the acid starting material (R_f 0.0) to a major product (R_f 0.32). The solvent was removed *in vacuo* and the residue was preabsorbed onto silica and purified by flash column chromatography (ethyl acetate–hexane 1:2→1:1) to yield N^{α}-2,3-dibenzyloxybenzoyl-N^{δ}-benzyloxycarbonyl-L-ornithine methyl ester (0.31 g) as a clear oil, which was immediately dissolved in THF (10 mL) and water (10 mL). To the resulting solution, barium hydroxide (0.27 g, 1.58 mmol) was added and the mixture was stirred for 16 h at 50 °C, when t.l.c. (ethyl acetate–hexane 1:1) indicated complete conversion of starting material (R_f 0.32) to a major product (R_f 0.0). DOWEX 50 W (H^+) was added to the solution to give a pH = 6 and the resin was then removed by filtration. The filtrate was evaporated *in vacuo* to give N^{α}-2,3-dibenzyloxybenzoyl-N^{δ}-benzyloxycarbonyl-L-ornithine (**2a**) (0.29 g, 0.51 mmol, 79%), which was used without further purification.

N^{α}-2,3-Dibenzyloxybenzoyl-L-ornithine (3a)

N^{α}-2,3-Dibenzyloxybenzoyl-N^{δ}-benzyloxycarbonyl-L-ornithine (**2a**) (82 mg, 0.12 mmol) was dissolved in methanol (4 mL) and the flask was flushed with argon. 5% Pd/C (0.01 g) was added and the flask was again flushed with argon. The reaction mixture was flushed with hydrogen and stirred under a hydrogen atmosphere for 3 h, after which t.l.c. (dichloromethane–methanol 95:5) indicated complete consumption of the starting material. The reaction mixture was filtered through Celite and the solvent was removed *in vacuo* to afford N^{α}-2,3-dibenzyloxybenzoyl-L-ornithine (**3a**) (65 mg, 0.15 mmol, quantitative), which was used without further purification; $[\alpha]_D^{23}$: +2.3 (*c*, 0.8 in H_2O); ^{1}H NMR (200 MHz, D_2O) δ_H: 1.73–1.98 (m, 4H, OrnH-3, OrnH-3′, OrnH-4, OrnH-4′), 2.91–2.99 (m, 2H, OrnH-2, OrnH-2′), 4.43–4.45 (m, 1H, OrnH-1), 6.86–7.42 (m, 3H, DHBH-4, DHBH-5, DHBH-6); ^{13}C NMR (50 MHz, D_2O): 25.33, 30.88, 40.44 (OrnC-3, OrnC-4, OrnC-5), 49.88 (OrnC-2), 118.91, 128.79, 128.75 (DHBC-4, DHBC-5, DHBC-6), 129.52, 135.12, 135.14 (DHBC-1, DHBC-2, DHBC-3), 170.32 (CO_2H); (+)-LRESIMS (*m*/*z*, %): 269.11 ($[M + H]^+$, 100%); (+)-HRESIMS: calculated for $C_{12}H_{17}N_2O_7$ $[M + H]^+$ 269.1131, found 269.1129.

N^{α}-2,3-Dihydroxybenzoyl-L-ornithinyl-L-serine *tert*-butyl ester (5b)

L-Serine *tert*-butyl ester (0.09 g, 0.44 mmol) was coupled to 2,3-dibenzyloxybenzoyl-N^{δ}-Cbz-L-ornithinic acid (**2a**) (0.06 g, 0.12 mmol) in anhydrous DMF (2 mL) and triethylamine (0.15 mL, 1.11 mmol) using TBTU as coupling agent (0.21 g, 0.67 mmol). The solvent was removed *in vacuo* and the residue was preabsorbed onto silica and purified by flash column chromatography (ethyl acetate–hexane 1:2→2:3) to yield N^{α}-2,3-dibenzyloxybenzoyl-N^{δ}-benzyloxycarbonyl-L-ornithinyl-L-serine *tert*-butyl ester, which was hydrogenated in methanol (5 mL) using 5% Pd–C (0.01 g) as catalyst. The reaction mixture was filtered through Celite and the solvent was removed *in vacuo* to afford N^{α}-2,3-dihydroxybenzoyl-L-ornithinyl-L-serine *tert*-butyl ester (**5b**) (54 mg, 0.14 mmol, 28%), which was used without further purification; $[\alpha]_D^{25}$: +15.8 (*c*, 0.3 in $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$) δ_H: 1.42 (s, 9H, -C(CH_3)$_3$), 1.67–1.91 (m, 4H, OrnH-3, OrnH-3″, OrnH-4, OrnH-4′), 2.94–2.96 (m, 2H, OrnH-5, OrnH-5′), 3.52 (m, 1H, SerH-3), 3.76 (m, 1H, SerH-3′), 4.55–4.67 (m, 2H, OrnH-2, SerH-2), 6.61 (bs, 1H,

-NH-), 7.66–7.91 (m, 3H, DHBH-4, DHBH-5, DHBH-6), 8.22 (bs, 1H, -NH-); ^{13}C NMR (50 MHz, $CDCl_3$) δ_C: 23.21 (OrnC-4), 27.30, 27.97 (-C(CH_3)$_3$), 27.97 (OrnC-5), 52.13 (OrnC-3), 53.15, 62.24 (OrnC-2, SerC-2), 73.31 (SerC-3), 82.11 (-C(CH$_3$)$_3$), 118.41, 119.00, 119.19 (DHBC-5, DHBC-6, DHBC-7), 145.25, 148.37 (DHBC-2, DHBC-3, DHBC-4), 169.41, 169.80, 171.63 (3 × C=O); (+)-LRFABMS (*m*/*z*, %): 434.2 [M + Na]$^+$, 412.2 [M + H]$^+$.

N^{α}-2,3-Dihydroxybenzoyl-L-ornithinyl-L-serine (6b)

N^{α}-2,3-Dihydroxybenzoyl-L-ornithinyl-L-serine *tert*-butyl ester (**5b**) (31 mg, 0.075 mmol) was stirred at room temperature in a solution of TFA and DCM (3:7, 1 mL). After 16 h, evaporation of the solvent under reduced pressure and coevaporation with toluene afforded N^{α}-2,3-dihydroxybenzoyl-L-ornithinyl-L-serine (**6b**) (25 mg, 0.07 mmol, quantitative) as a dark yellow oil; $[\alpha]_D^{26}$: −8.0 (*c*, 0.11 in H_2O); ^{1}H NMR (200 MHz, D_2O) δ_H: 1.78–2.09 (m, 4H, OrnH-3, OrnH-3′, OrnH-4, OrnH-4′), 3.04–3.09 (m, 2H, OrnH-5, OrnH-5′), 3.90 (dd, 1H, $J_{H3,H2} = 4.0$ Hz, $J_{H3,H3'} = 11.7$ Hz, SerH-3), 3.99 (dd, 1H, $J_{H3',H2} = 5.1$ Hz, $J_{H3,H3''} = 11.7$ Hz, SerH-3′), 4.57–4.60, 4.68–4.70 (2 × m, 2H, SerH-2, OrnH-2), 6.89 (t, 1H, $J_{H4,H5} = J_{H5,H6} = 8.1$ Hz, DHBH-5), 7.10, 7.30 (2 × d, 2H, $J_{H4,H5} = J_{H5,H6} = 8.1$ Hz, DHBH-4, DHBH-6); ^{13}C NMR (50 MHz, D_2O) δ_C: 23.93 (OrnC-4), 28.85 (OrnC-3), 39.56 (OrnC-5), 53.94, 55.49 (OrnC-2, SerC-2), 61.59 (SerC-3), 120.12, 120.33, 120.44 (DHBC-4, DHBC-5, DHBC-6), 117.44, 145.16, 147.20 (DHBC-1, DHBC-2, DHBC-3), 170.61, 173.66 (2 × *C*ONH), 174.18 (*C*O$_2$H); (+)-LRESIMS (*m*/*z*, %): 356.14 ([H]$^+$, 100%); (+)-HRESIMS calculated for $C_{15}H_{22}N_3O_7$ [M + H]$^+$ 356.1452, found 356.1457.

2,3-Dihydroxybenzoyl-L-argininyl-L-serine (1b)

To a stirred solution of N^{α}-2,3-dihydroxybenzoyl-L-ornithinyl-L-serine *tert*-butyl ester (**5b**) (29 mg, 0.07 mmol) in chloroform (1 mL) was added 1,3-di-*tert*-butoxycarbonyl-2-(trifluoromethanesulfonyl)guanidine (26 mg, 0.065 mmol) and triethylamine (0.01 mL, 0.07 mmol) and the resulting mixture was stirred at room temperature. After 1 h, methanol (0.5 mL) was added and the mixture was stirred for a further 30 min. The mixture was then preabsorbed onto silica and purified by flash column chromatography (methanol–DCM 5%) to yield N^{α}-2,3-dihydroxybenzoyl-N^{ω}-*tert*-butoxycarbonyl-$N^{\omega'}$-*tert*-butoxycarbonyl-L-argininyl-L-serine *tert*-butyl ester, which was then stirred at room temperature in a solution of TFA and DCM (3:7, 1 mL). After 16 h, evaporation of the solvent under reduced pressure and coevaporation with toluene afforded 2,3-dihydroxybenzoyl-L-argininyl-L-serine (**1b**) (25 mg, 0.06 mmol, 73%) as a dark yellow oil; $[\alpha]_D^{24}$: +12.0 (*c*, 0.7 in MeOH); ^{1}H NMR (200 MHz, D_2O) δ_H: 1.61–1.95 (m, 4H, ArgH-3, ArgH-3′, ArgH-4, ArgH-4′), 3.13–3.17 (m, 2H, ArgH-5, ArgH-5′), 3.78–3.97 (m, 2H, SerH-3, SerH-3′), 4.43–4.59 (m, 2H, SerH-2, ArgH-2), 6.77 (t, 1H, $J_{H4,H5} = J_{H5,H6} = 8.1$ Hz, DHBH-5), 6.99, 7.21 (2 × d, 2H, $J_{H4,H5} = J_{H5,H6} = 8.1$ Hz, DHBH-4, DHBH-6); ^{13}C NMR (50 MHz, D_2O) δ_C: 24.92 (ArgC-4), 29.10 (ArgC-3), 41.16 (ArgC-5), 54.04, 55.44 (ArgC-2, SerC-2), 61.63 (SerC-3), 117.35, 120.16, 120.41 (DHBC-4, DHBC-5, DHBC-6), 145.18, 147.25 (DHBC-1, DHBC-2, DHBC-3), 157.30 (*C*=N), 170.39, 173.70 (2 × *C*ONH), 174.39 (*C*O$_2$H); (+)-LRESIMS (*m*/*z*, %): 398.10 ([M + H]$^+$, 84%); (+)-HRESIMS: calculated for $C_{16}H_{24}N_5O_{97}$ [M + H]$^+$ 398.1670, found 398.1674.

N^{α}-2,3-Dihydroxybenzoyl-L-ornithinyl-D-serine *tert*-butyl ester (5a)

D-Serine *tert*-butyl ester (0.10 g, 0.53 mmol) was coupled with N^{α}-2,3-dibenzyloxybenzoyl-N^{δ}-benzyloxycarbonyl-L-ornithine (**2a**) in a similar way as D-serine *tert*-butyl ester to yield N^{α}-2,3-dibenzyloxybenzoyl-N^{δ}-benzyloxycarbonyl-L-ornithinyl-D-serine *tert*-butyl ester (0.11 g), which was hydrogenated in a similar way as the corresponding L-ornithinyl-L-serine derivative to afford N^{α}-2,3-dihydroxybenzoyl-L-ornithinyl-D-serine *tert*-butyl ester (**5a**) (57 mg, 0.14 mmol, 29%), which was used without further purification; $[\alpha]_D^{24}$: −12.0 (*c*, 1.5 in H_2O); ^{1}H NMR (200 MHz, $CDCl_3$) δ_H: 1.40 (s, 9H, -C(CH$_3$)$_3$), 1.49–2.47 (m, 6H, OrnH-3, OrnH-3′, OrnH-4, OrnH-4′, OrnH-5, OrnH-5′), 3.40–3.71 (m, 2H, SerH-3, SerH-3′), 4.45–4.53 (m, 2H, SerH-2, ArgH-2), 6.28–6.34 (m, 1H, DHBH-5), 6.71–7.27 (m, 2H, DHBH-4, DHBH-6); (+)-LRFABMS (*m*/*z*, %): 434.2 [M + Na]$^+$, 412.2 [M + H]$^+$.

2,3-Dihydroxybenzoyl-L-argininyl-D-serine (1a)

N^{α}-2,3-Dihydroxybenzoyl-L-ornithinyl-D-serine *tert*-butyl ester (**5a**) (57 mg, 0.15 mmol) was reacted with 1,3-di-*tert*-butoxycarbonyl-2-trifluoromethanesulfonylguanidine (52 mg, 0.13 mmol) in a similar way as **5b** to yield N^{α}-2,3-dihydroxybenzoyl-N^{ω}-*tert*-butoxycarbonyl-$N^{\omega'}$-*tert*-butoxycarbonyl-L-argininyl-D-serine *tert*-butyl ester (60 mg) which was hydrolysed in a similar way as the L-argininyl-L-serine derivative to afford 2,3-dihydroxybenzoyl-L-argininyl-D-serine (**1a**) (27 mg, 0.06 mmol, 62%) as a dark yellow oil; $[\alpha]_D^{24}$: −16.4 (*c*, 0.7 in MeOH); ^{1}H NMR (200 MHz, D_2O) δ_H: 1.56–1.94 (m, 4H, ArgH-3, ArgH-3′, ArgH-4, ArgH-4′), 3.10–3.14 (m, 2H, ArgH-5, ArgH-5′), 3.80 (dd, 1H, $J_{H3,H2} = 3.9$ Hz, $J_{H3,H3'} = 10.1$ Hz, SerH-3), 3.80 (dd, 1H, $J_{H3',H2} = 5.1$ Hz, $J_{H3,H3'} = 10.1$ Hz, SerH-3′), 4.47–4.57 (m, 2H, SerH-2, ArgH-2), 6.74 (t, 1H, $J_{H4,H5} = J_{H5,H6} = 8.1$ Hz, DHBH-5), 6.96, 7.17 (2 × d, 2H, $J_{H4,H5} = J_{H5,H6} = 8.1$ Hz, DHBH-4, DHBH-6); ^{13}C NMR (50 MHz, D_2O) δ_C: 24.98 (ArgC-4), 29.02 (ArgC-3), 41.13 (ArgC-5), 54.13, 55.48 (ArgC-2, SerC-2), 61.60 (SerC-3), 120.17, 120.32, 120.46 (DHBC-4, DHBC-5, DHBC-6), 117.41, 145.19, 147.17 (DHBC-1, DHBC-2, DHBC-3), 157.32 (*C*=N), 170.51, 173.72 (2 × *C*ONH), 174.42 (*C*O$_2$H); (+)-LRESIMS (*m*/*z*, %): 398.10 ([M + H]$^+$, 84%); (+)-HRESIMS: calculated for $C_{16}H_{24}N_5O_{97}$ [M + H]$^+$ 398.1670, found 398.1674.

N^{α}-2,3-Dibenzyloxybenzoyl-N^{δ}-benzyloxycarbonyl-D-ornithine (2b)

N^{δ}-Benzyloxycarbonyl-D-ornithine methyl ester (0.40 g, 1.39 mmol) was coupled with 2,3-dibenzyloxybenzoic acid in a similar way as the corresponding L-ornithine derivative to yield N^{α}-2,3-dibenzyloxybenzoyl-N^{δ}-benzyloxycarbonyl-D-ornithine methyl ester (0.65 g), which was hydrolysed in a similar way as the corresponding L-ornithine derivative to give N^{α}-2,3-dibenzyloxybenzoyl-N^{δ}-benzyloxycarbonyl-D-ornithine (**2b**) (0.60 g, 1.06 mmol, 79%), which was used without further purification.

N^{α}-2,3-Dihydroxybenzoyl-D-ornithine (3b)

N^{α}-2,3-Dibenzyloxybenzoyl-N^{δ}-benzyloxycarbonyl-D-ornithine (**2b**) (80 mg, 0.11 mmol) was hydrogenated in a similar way as **2a** to afford N^{α}-2,3-dihydroxybenzoyl-D-ornithine (**3b**) (65 mg, 0.11 mmol, quantitative), which was used without further purification; $[\alpha]_D^{23}$: −4.6 (*c*, 0.55 in D_2O); ^{1}H NMR (200 MHz, D_2O) δ_H: 1.73–1.98 (m, 4H, OrnH-3, OrnH-3′, OrnH-4, OrnH-4′), 2.91–2.99 (m, 2H, OrnH-2, OrnH-2′), 4.43–4.45 (m, 1H, OrnH-1), 6.86–7.42 (m, 3H, DHBH-4, DHBH-5,DHBH-6); ^{13}C NMR (50 MHz, D_2O) δ_C: 25.33, 30.88, 40.44 (OrnC-3, OrnC-4, OrnC-5), 49.88 (OrnC-2), 118.91, 128.79, 128.75 (DHBC-4, DHBC-5, DHBC-6), 129.52, 135.12, 135.14 (DHBC-1, DHBC-2, DHBC-3), 170.32 (*C*O_2H); (+)-LRESIMS (*m*/*z*, %): 269.11 ([M + H]$^+$, 100%); (+)-HRESIMS: calculated for $C_{12}H_{17}N_2O_7$ [M + H]$^+$ 269.1131, found 269.1129.

2,3-Dihydroxybenzoyl-D-arginine (4)

N^{α}-2,3-Dibenzyloxybenzoyl-N^{δ}-benzyloxycarbonyl-D-ornithine (**2b**) (0.39 g, 0.69 mmol) was suspended in *tert*-butyl acetate (2.30 mL, 16.70 mmol) and perchloric acid (70%, 0.07 mL, 0.77 mmol) was added. The mixture was stirred at room temperature for 16 h and added to saturated aqueous sodium bicarbonate (10 mL). After stirring for a further 30 min, the mixture was extracted with diethyl ether (3 × 20 mL) and the combined organic layers were dried, filtered and evaporated *in vacuo* to obtain N^{α}-2,3-dibenzyloxybenzoyl-N^{δ}-benzyloxycarbonyl-D-ornithine *tert*-butyl ester (0.44 g, 48%) as a clear oil, which was hydrogenated using 5% Pd-C (0.07 g) in a similar way as **2a** to afford N^{α}-2,3-dibenzyloxybenzoyl-D-ornithine *tert*-butyl ester, which was used immediately in the next reaction. This D-ornithine derivative ester was treated with 1,3-di-*tert*-butoxycarbonyl-2-trifluoromethanesulfonyl guanidine (0.12 g, 0.30 mmol) in a similar way as **5b**. The product mixture was then preabsorbed onto silica and purified by flash column chromatography (methanol/DCM 5%), to yield N^{α}-2,3-dibenzyloxybenzoyl-N^{ω}-*tert*-butoxycarbonyl-$N^{\omega'}$-*tert*-butoxycarbonyl-D-arginine *tert*-butyl ester (0.133 g), which was then stirred at room temperature in a solution in TFA and DCM (3:7, 1 mL). After 16 h the solvent was evaporated under reduced pressure and coevaporation with toluene afforded 2,3-dihydroxybenzoyl-D-arginine (**4**) (74 mg, 0.25 mmol, 73%) as a dark yellow oil; $[\alpha]_D^{24}$: +3.5 (*c*, 2.3 in H_2O); ^{1}H NMR (200 MHz, D_2O) δ_H: 1.50–1.60, 1.68–1.94 (2 × m, 4H, ArgH-3, ArgH-3′, ArgH-4, ArgH-4′), 3.03–3.07 (m, 2H, ArgH-5, ArgH-5′), 4.43–4.59 (dd, 1H, $J_{H2,H3}$ = 5.4 Hz, $J_{H2,H3'}$ = 8.7 Hz, ArgH-2), 6.67 (t, 1H, $J_{H4,H5} = J_{H5,H6}$ = 8.1 Hz, DHBH-5), 6.90, 7.12 (2 × d, 2H, $J_{H4,H5} = J_{H5,H6}$ 8.1 Hz, DHBH-4, DHBH-6); ^{13}C NMR (50 MHz, D_2O) δ_c: 24.54 (ArgC-4), 27.83 (ArgC-3), 40.58 (ArgC-5), 52.60 (ArgC-2), 116.15, 119.30, 119.71 (DHBC-4, DHBC-5, DHBC-6), 144.67, 147.15 (DHBC-1, DHBC-2, DHBC-3), 156.74 (*C*=N), 170.00, 1756.29 (2 × *C*ONH), 174.39 (*C*O_2H); (+)-LRESIMS (*m*/*z*, %): 311.13 ([M + H]$^+$, 81%); (+)-HRESIMS: calculated for $C_{13}H_{18}N_4O_5$ [M + H]$^+$ 311.1354, found 311.1349.

N^{α}-2,3-Dihydroxybenzoyl-D-ornithinyl-L-serine *tert*-butyl ester (5c)

N^{α}-2,3-Dibenzyloxybenzoyl-N^{δ}-benzyloxycarbonyl-D-ornithine methyl ester (**2b**) (0.51 g, 0.90 mmol) was coupled with L-serine in a similar way as **2a** to yield N^{α}-2,3-dibenzyloxybenzoyl-N^{δ}-benzyloxycarbonyl-D-ornithinyl-L-serine *tert*-butyl ester, which was hydrogenated as previously reported for the L-ornithinyl-L-serine derivative to afford N^{α}-2,3-dihydroxybenzoyl-D-ornithinyl-L-serine *tert*-butyl ester (**5c**) (0.33 g, 0.83 mmol, 82%), which was used without further purification; $[\alpha]_D^{23}$: +14.6 (*c*, 0.14 in Cl_3CH); ^{1}H NMR (200 MHz, $CDCl_3$) δ_H: 1.40 (s, 9H, -C(CH_3)$_3$), 1.49–2.47 (m, 6H, OrnH-3, OrnH-3′, OrnH-4, OrnH-4′, OrnH-5, OrnH-5′), 3.40–3.71 (m, 2H, SerH-3, SerH-3′), 4.45–4.53 (m, 2H, SerH-2, ArgH-2), 6.28–6.34 (m, 1H, DHBH-5), 6.71–7.27 (m, 2H, DHBH-4, DHBH-6); (+)-LRFABMS (*m*/*z*, %): 434.2 [M + Na]$^+$, 412.2 [M + H]$^+$.

N^{α}-2,3-Dihydroxybenzoyl-D-ornithinyl-L-serine (6c)

N^{α}-2,3-Dihydroxybenzoyl-D-ornithinyl-L-serine *tert*-butyl ester (**5c**) (45 mg, 0.11 mmol) was hydrolysed in a similar way as **5a** to afford N^{α}-2,3-dihydroxybenzoyl-D-ornithinyl-L-serine (**6c**) (36 mg, 0.11 mmol, quantitative) as a dark yellow oil; $[\alpha]_D^{22}$: +8.7 (*c*, 0.16 in H_2O); ^{1}H NMR (200 MHz, D_2O) δ_H: 1.71–2.04 (m, 4H, OrnH-3, OrnH-3′, OrnH-4, OrnH-4′), 3.00–3.05 (m, 2H, OrnH-5, OrH-5′), 3.86 (dd, 1H, $J_{H3,H2}$ = 4.1 Hz, $J_{H3,H3'}$ = 12.0 Hz, SerH-3), 3.95 (dd, 1H, $J_{H3',H2}$ = 5.0 Hz, $J_{H3,H3'}$ = 12.0 Hz, SerH-3′), 4.53–4.56, 4.62–4.65 (2 × m, 2H, SH-2, OrnH-2), 6.81 (t, 1H, $J_{H4,H5} = J_{H5,H6}$ = 8.0 Hz, DHBH-5), 7.03, 7.23 (2 × d, 2H, $J_{H4,H5} = J_{H5,H6}$ = 8.0 Hz, DHBH-4, DHBH-6); ^{13}C NMR (50 MHz, D_2O) δ_C: 23.92 (OrnC-4), 28.85 (OrnC-3), 39.53 (OrnC-5), 53.94, 55.97 (OrC-2, SerC-2), 61.78 (SerC-3), 120.22, 120.35, 120.52 (DHBC-4, DHBC-5, DHBC-6), 117.45, 145.20, 147.07 (DHBC-1, DHBC-2, DHBC-3), 170.63, 174.06 (2 × *C*ONH), 174.26 (*C*O_2H); (+)-LRESIMS (*m*/*z*, %): 356.14 ([M + H]$^+$, 100%); (+)-HRESIMS calculated for $C_{15}H_{22}N_3O_7$ [M + H]$^+$ 356.1452, found 356.1458.

2,3-Dihydroxybenzoyl-D-argininyl-L-serine (1c)

N^{α}-2,3-Dihydroxybenzoyl-D-ornithinyl-L-serine *tert*-butyl ester (**5c**) (95 mg, 0.25 mmol) reacted with 1,3-di-*tert*-butoxycarbonyl-2-trifluoromethanesulfonylguanidine (85 mg, 0.21 mmol) in a similar way as **5a** to yield N^{α}-2,3-dihydroxybenzoyl-N^{ω}-*tert*-butoxycarbonyl-$N^{\omega'}$-*tert*-butoxycarbonyl-D-argininyl-L-serine *tert*-butyl ester, which was hydrolysed in a similar way as the corresponding L-argininyl-L-serine derivative to afford 2,3-dihydroxybenzoyl-D-argininyl-L-serine (**1c**) (69 mg, 0.16 mmol, 61%) as a dark yellow oil; $[\alpha]_D^{24}$: −20.8 (*c*, 0.1 in H_2O) [natural sample: $[\alpha]_D^{24}$: −15.9 (*c*, 0.08 in H_2O)]; ^{1}H NMR (500 MHz, D_2O) δ_H: 1.56–1.94 (m, 4H, ArgH-3, ArgH-3′, ArgH-4, ArgH-4′), 3.10–3.14 (m, 2H, ArgH-5, ArgH-5′), 3.80 (dd, 1H, $J_{H3,H2}$ = 3.9 Hz, $J_{H3,H3'}$ = 10.1 Hz, SerH-3), 3.90 (dd, 1H, $J_{H3',H2}$ = 5.1 Hz, $J_{H3,H3'}$ = 10.1 Hz, SerH-3′),4.47–4.57 (m, 2H, SerH-2, ArgH-2), 6.74 (t, 1H, $J_{H4,H5} = J_{H5,H6}$ = 8.1 Hz, DHBH-5), 6.96, 7.17 (2 × dd, 2H, *J* = 1.5 Hz, *J* = 8.1 Hz, DHBH-4, DHBH-6); ^{13}C NMR (120 MHz, D_2O) δ_C 24.98 (ArgC-4), 29.02 (ArgC-3), 41.13 (ArgC-5), 54.13, 55.48 (ArgC-2, SerC-2), 61.60 (SerC-3), 120.17, 120.32, 120.46 (DHBC-4, DHBC-5, DHBC-6), 117.41, 145.19, 147.17 (DHBC-1, DHBC-2, DHBC-3), 157.32 (*C*=N), 170.51, 173.72 (2 × *C*ONH), 174.42 (*C*O_2H); (+)-LRESIMS (*m*/*z*, %): 398.10 ([M + H]$^+$, 84%); (+)-HRESIMS: calculated for $C_{16}H_{24}N_5O_{97}$ [M + H]$^+$ 398.1670, found 398.1674.

2,3-Dibenzyloxybenzoyl-L-serine (7a)

L-Serine hydrochloride methyl ester (0.34 g, 2.16 mmol) was coupled to 2,3-dibenzyloxybenzoic acid (0.72 g, 2.16 mmol) in anhydrous DMF (20 mL) and triethylamine (0.75 mL, 5.39 mmol) using TBTU as the coupling agent (1.04 g, 3.23 mmol). The solvent was removed *in vacuo* and the residue was preabsorbed onto silica and purified by flash column chromatography (ethyl acetate–hexane 1:1) to yield 2,3-dibenzyloxybenzoyl-L-serine methyl ester, which was then dissolved in THF (40 mL) and water (80 mL). To the resulting solution, barium hydroxide (0.79 g, 4.58 mmol) was added and the mixture was stirred for 3 h at 50 °C, after which t.l.c. (ethyl acetate–cyclohexane 2:3) indicated complete conversion of starting material (R_f 0.28) to a major product (R_f 0.0). DOWEX 50 W (H^+) was added to the solution to give a pH = 6, the resin was removed by filtration and the filtrate was evaporated *in vacuo* to give 2,3-dibenzyloxybenzoyl-L-serine (**7a**) (0.84 g, 2.00 mmol, 84%), which was used for the next step without further purification.

2,3-Dihydroxybenzoyl-L-serine (8a)

2,3-Dibenzyloxybenzoyl-L-serine (**7a**) (0.04 g, 0.10 mmol) was hydrogenated using 5% Pd/C (1 mg) in a similar was as **2b** to afford 2,3-dihydroxybenzoyl-L-serine (**8a**) (23 mg, 0.1 mmol, quantitative); $[\alpha]_D^{21}$: +12.2 (*c*, 0.6 in H_2O); ^{1}H NMR (200 MHz, D_2O) δ_H: 4.39 (m, 2H, SerH-3, SerH-3′), 5.04 (m, 1H, SerH-2), 7.14 (t, 1H, J = 7.0 Hz, DHBH-5), 7.35, 7.73 (2 × d, 2H, J = 7.0 Hz, J = 7.2 Hz, DHBH-4, DHBH-6); ^{13}C NMR (200 MHz, D_2O) δ_C: 49.53 (SerC-2), 62.18 (SerC-3), 116.96, 120.02, 120.24 (DHBC-4, DHBC-5, DHBC-6), 145.12, 147.54 (DHBC-1, DHBC-2, DHBC-3), 170.21 (*C*ONH), 177.64 (*C*O_2OH); (+)-LRESIMS (*m*/*z*, %): 264.05 $[M + Na]^+$, 242.06 $[M + H]^+$; (+)-HRESIMS: calculated for $C_{10}H_{12}NO_6$ $[M + H]^+$ 242.0659; found 242.0659.

2,3-Dihydroxybenzoyl-L-serinyl-L-ornithine *tert*-butyl ester (9a)

N^δ-Benzyloxycarbonyl-L-ornithine *tert*-butyl ester (0.07 g, 0.21 mmol) was coupled to 2,3-dibenzyloxybenzoyl-L-serine (**7a**) (0.06 g, 0.12 mmol) in anhydrous DMF (2 mL) and triethylamine (0.07 mL, 0.52 mmol) using TBTU as coupling agent (0.10 g, 0.30 mmol). The solvent was removed *in vacuo* and the residue was preabsorbed onto silica and purified by flash column chromatography (ethyl acetate–hexane 2:1) to yield 2,3-dibenzyloxybenzoyl-L-serinyl-N^δ-benzyloxycarbonyl-L-ornithine *tert*-butyl ester, which was hydrogenated in methanol (6 mL) using 5% Pd/C (0.02 g) as catalyst. The reaction mixture was filtered through Celite and the solvent was removed *in vacuo* to afford 2,3-dihydroxybenzoyl-L-serinyl-L-ornithine *tert*-butyl ester (**9a**) (64 mg, 0.16 mmol, 81%), which was used without further purification; $[\alpha]_D^{21}$: +15.5 (*c*, 0.55 in MeOH); ^{1}H NMR (200 MHz, CD_3OD) δ_H: 1.40 (s, 9H, -$C(CH_3)_3$), 1.51–1.86 (m, 4H, OrnH-3, OrnH-3′, OrnH-4, OrnH-4′), 2.78 (m, 2H, OrnH-5, OrnH-5′), 3.89–3.90 (m, 2H, SerH-3, SerH-3′), 4.35 (m, 1H, SerH-2), 4.66 (bs, 1H, ArgH-2), 6.30–6.38 (m, 1H, DHBH-5), 6.76, 7.28 (2 × d, 2H, J = 7.0 Hz, J = 7.3 Hz, DHBH-4, DHBH-6), (+)-LRFABMS (*m*/*z*, %): 434.2 $[M + Na]^+$, 412.2 $[M + H]^+$.

2,3-Dihydroxybenzoyl-L-serinyl-L-ornithine (10a)

2,3-Dihydroxybenzoyl-L-serinyl-L-ornithine *tert*-butyl ester (**9a**) (51 mg, 0.012 mmol) was stirred at room temperature in a solution of TFA and DCM (3:7, 1 mL). After 16 h, evaporation of the solvent under reduced presure and coevaporation with toluene afforded 2,3-dihydroxybenzoyl-L-serinyl-L-ornithine (**10a**) (44 mg, 0.12 mmol, quantitative) as a clear oil; $[\alpha]_D^{23}$: +3.9 (*c*, 1.1 in MeOH); ^{1}H NMR (200 MHz, D_2O) δ_H: 1.32–1.87 (m, 4H, OrnH-3, OrnH-3′, OrnH-4, OrnH-4′), 2.85–2.99 (m, 2H, OrnH-5, OrnH-5′), 3.86–3.94 (m, 2H, SerH-3, SerH-3′), 4.51–4.61 (m, 2H, SerH-2, OrnH-2), 6.64 (t, 1H, $J_{H4,H5} = J_{H5,H6}$ = 7.8 Hz, DHBH-5), 7.04, 7.21 (2 × d, 2H, $J_{H4,H5} = J_{H5,H6}$ = 7.8 Hz, DHBH-4, DHBH-6); ^{13}C NMR (50 MHz, D_2O) δ_C: 23.87 (OrnC-4), 29.17 (OrnC-3), 39.67 (OrnC-5), 49.51, 56.28 (OrnC-2, SerC-2), 62.04 (SerC-3), 117.73, 118.59, 120.52 (DHBC-4, DHBC-5, DHBC-6), 145.66, 147.01 (DHBC-1, DHBC-2, DHBC-3), 169.89, 171.44 (2 × *C*ONH), 173.57 (*C*O_2H).

2,3-Dihydroxybenzoyl-L-serinyl-L-arginine (11a)

2,3-Dihydroxybenzoyl-L-serinyl-L-ornithine *tert*-butyl ester (**9a**) (64 mg, 0.15 mmol) was treated with 1,3-di-*tert*-butoxycarbonyl-2-trifluoromethanesulfonylguanidine (52 mg, 0.13 mmol) in a similar was as **5b**. The product mixture was then preadsorbed onto silica and purified by flash column chromatography (methanol/DCM 5%), to yield N^α-2,3-dihydroxybenzoyl-L-serinyl-N^ω-*tert*-butoxycarbonyl-$N^{\omega'}$-*tert*-butoxycarbonyl-L-arginine *tert*-butyl ester, which was hydrolysed with TFA as before to afford 2,3-dihydroxybenzoyl-L-serinyl-L-arginine (**11a**) (40 mg, 0.10 mmol, 71%) as a dark yellow oil; $[\alpha]_D^{20}$: +3.2 (*c*, 0.8 in MeOH); ^{1}H NMR (200 MHz, D_2O) δ_H: 1.60–1.98 (m, 4H, ArgH-3, OrnH-3′, OrnH-4, OrnH-4′), 3.12–3.18 (m, 2H, ArgH-5, ArgH-5′), 3.78–3.84 (m, 2H, SerH-3, SerH-3′), 4.35 (dd, 1H, $J_{H2,H3}$ = 4.8 Hz, $J_{H2,H3'}$ = 9.0 Hz, SerH-2), 4.69–4.75 (m, 1H, ArgH-2), 6.74 (t, 1H, $J_{H4,H5} = J_{H5,H6}$ = 7.8 Hz, DHBH-5), 6.96, 7.18 (2 × d, 2H, $J_{H4,H5} = J_{H5,H6}$ = 7.8 Hz, DHBH-4, DHBH-6); ^{13}C NMR (50 MHz, $CDCl_3$) δ_C: 24.75 (ArgC-4), 27.83 (ArgC-3), 40.25 (ArgC-5), 52.12, 55.43 (ArgC-2, SerC-2), 61.14 (SerC-3), 117.94, 119.06 (DHBC-4, DHBC-5, DHBC-6), 143.88, 146.75 (DHBC-1, DHBC-2, DHBC-3), 156.33 (*C*=N), 170.12, 171.35 (2 × *C*ONH), 174.03 (*C*O_2H); (+)-LRESIMS (*m*/*z*, %): 398.10 ($[M + H]^+$, 84%); (+)-HRESIMS: calculated for $C_{16}H_{24}N_5O_{97}$ $[M + H]^+$ 398.1670, found 398.1678.

2,3-Dibenzyloxybenzoyl-D-serine (7b)

D-Serine methyl ester hydrochloride (0.34 g, 2.16 mmol) was coupled with 2,3-dibenzyloxybenzoic acid (0.72 g, 2.16 mmol) in a similar way as L-serine methyl ester to yield 2,3-dibenzyloxybenzoyl-D-serine methyl ester, which was hydrolysed in a similar way as the corresponding L-serine derivative to give 2,3-dibenzyloxybenzoyl-D-serine (**7b**) (0.83 g, 1.97 mmol, 84%), which was used in the next step without further purification.

2,3-Dihydroxybenzoyl-D-serine (8b)

2,3-Dibenzyloxybenzoyl-D-serine (**7b**) (31 mg, 0.07 mmol) was hydrogenated in a similar way as **7a** to afford 2,3-dihydroxybenzoyl-D-serine (**8b**) (17 mg, 0.07 mmol, quantitative); $[\alpha]_D^{23}$: −10.8 (*c*,

0.8 in H_2O); ^{1}H NMR (200 MHz, D_2O) δ_H: 4.39 (m, 2H, SerH-3, SerH-3′), 5.04 (m, 1H, SerH-2), 7.14 (t, 1H, J 7.0 Hz, DHBH-5), 7.35, 7.73 (2 × d, 2H, J = 7.0 Hz, J = 7.2 Hz, DHBH-4, DHBH-6); ^{13}C NMR (50 MHz, D_2O) δ_C 49.53 (SerC-2), 62.18 (SerC-3), 116.96, 120.02, 120.24 (DHBC-4, DHBC-5, DHBC-6), 145.12, 147.54 (DHBC-1, DHBC-2, DHBC-3), 170.21 (*C*ONH), 177.64 (*C*O_2OH); (+)-LRESIMS (*m*/*z*, %): 264.05 [M + Na]$^+$, 242.06 [M + H]$^+$; (+)-HRESIMS: calculated for $C_{10}H_{12}NO_6$ [M + H]$^+$ 242.0659; found 242.0659.

2,3-Dihydroxybenzoyl-D-serinyl-L-ornithine *tert*-butyl ester (9b)

2,3-Dibenzyloxybenzoyl-D-serinic acid (**7b**) was coupled with N^δ-benzyloxycarbonyl-L-ornithine *tert*-butyl ester (0.07 g, 0.21 mmol) in a similar way as **7a** to yield 2,3-dibenzyloxybenzoyl-D-serinyl-N^δ-benzyloxycarbonyl-L-ornithine *tert*-butyl ester, which was hydrogenated in a similar way as the corresponding D-serinyl-L-ornithine derivative to afford 2,3-dihydroxybenzoyl-D-serinyl-L-ornithine *tert*-butyl ester (**9b**) (64 mg, 0.15 mmol, 81%), which was used without further purification; $[\alpha]_D^{21}$: +15.5 (*c*, 0.55 in MeOH); ^{1}H NMR (200 MHz, CD_3OD) δ_H: 1.40 (s, 9H, -C(CH_3)$_3$), 1.51–1.86 (m, 4H, OrnH-3, OrnH-3′, OrnH-4, OrnH-4′), 2.78 (m, 2H, OrnH-5, OrnH-5′), 3.89–3.90 (m, 2H, SerH-3, SerH-3′), 4.35 (m, 1H, SerH-2), 4.66 (bs, 1H, ArgH-2), 6.30–6.38 (m, 1H, DHBH-5), 6.76, 7.28 (2 × d, 2H, J = 7.0 Hz, J = 7.3 Hz, DHBH-4, DHBH-6); (+)-LRESIMS: 434.2 [M + Na]$^+$, 412.2 [M + H]$^+$.

2,3-Dihydroxybenzoyl-D-serinyl-L-ornithine (10b)

2,3-Dihydroxybenzoyl-D-serinyl-L-ornithine *tert*-butyl ester (**9b**) (45 mg, 0.01 mmol) was hydrolysed in a similar way as **9a** to give 2,3-dihydroxybenzoyl-D-serinyl-L-ornithine (**10b**) (39 mg, 0.01 mmol, quantitative) as a clear oil; $[\alpha]_D^{23}$: −3.1 (*c*, 1.8 in MeOH); ^{1}H NMR (200 MHz, D_2O) δ_H: 1.32–1.87 (m, 4H, ArgH-3, OrnH-3′, OrnH-4, OrnH-4′), 2.85–2.99 (m, 2H, ArgH-5, ArgH-5′), 3.86–3.94 (m, 2H, SerH-3, SerH-3′), 4.51–4.61 (m, 2H, SerH-2, ArgH-2), 6.64 (t, 1H, $J_{D4,D5}$ = $J_{D5,D6}$ = 7.8 Hz, DHBH-5), 7.04, 7.21 (2 × d, 2H, $J_{D4,D5}$ = $J_{D5,D6}$ = 7.8 Hz, DHBH-4, DHBH-6); ^{13}C NMR (50 MHz, D_2O) δ_C: 23.87 (OrnC-4), 29.17 (OrnC-3), 39.67 (OrnC-5), 49.51, 56.28 (OrnC-2, SerC-2), 62.04 (SerC-3), 117.73, 118.59, 120.52 (DHBC-4, DHBC-5, DHBC-6), 145.66, 147.01 (DHBC-1, DHBC-2, DHBC-3), 169.89, 171.44 (2 × *C*ONH), 173.57 (*C*O_2H).

2,3-Dihydroxybenzoyl-D-serinyl-L-arginine (11b)

2,3-Dihydroxybenzoyl-D-serinyl-L-ornithine *tert*-butyl ester (**9b**) (64 mg, 0.15 mmol) was reacted with 1,3-di-*tert*-butoxycarbonyl-2-(trifluoromethanesulfonyl)guanidine in a similar way as **9a** to yield N^α-2,3-dihydroxybenzoyl-D-serinyl-N^ω-*tert*-butoxycarbonyl-$N^{\omega'}$-*tert*-butoxycarbonyl-L-arginine *tert*-butyl ester, which was hydrolysed in a similar way as the corresponding L-serinyl-L-arginine derivative to give 2,3-dihydroxybenzoyl-D-serinyl-L-arginine (**11b**) (42 mg, 0.10 mmol, 71%) as a dark yellow oil; $[\alpha]_D^{23}$: −6.2 (*c*, 0.4 in MeOH); ^{1}H NMR (200 MHz, D_2O) δ_H: 1.50–1.99 (m, 4H, ArgH-3, ArgH-3′, ArgH-4, ArgH-4′), 3.04–3.08 (m, 2H, ArgH-5, ArgH-5′), 3.89–3.91 (m, 2H, SerH-3, SerH-3′), 4.37 (dd, 1H, $J_{H2,H3}$ = 4.8 Hz, $J_{H2,H3'}$ = 9.0 Hz, SerH-2), 4.69–4.71 (t, 1H, $J_{H2,H3}$ = $J_{H2,H3'}$ = 5.2 Hz, ArgH-2), 6.74 (t, 1H, $J_{H4,H5}$ = $J_{H5,H6}$ = 7.8 Hz, DHBH-5), 6.96, 7.19 (2 × d, 2H, $J_{H4,H5}$ = $J_{H5,H6}$ = 7.8 Hz, DHBH-4, DHBH-6); ^{13}C NMR (50 MHz, D_2O) δ_C: 24.26 (ArgC-4), 27.69 (ArgC-3), 40.36 (ArgC-5), 52.20, 55.62 (ArgC-2, SerC-2), 61.02 (SerC-3), 118.02, 119.66 (DHBC-4, DHBC-5, DHBC-6), 144.46, 146.75 (DHBC-1, DHBC-2, DHBC-3), 156.55 (*C*=N), 169.85, 171.84 (2 × *C*ONH), 174.78 (*C*O_2H); (+)-LRESIMS (*m*/*z*, %): 398.10 ([M + H]$^+$, 84%); (+)-HRESIMS: calculated for $C_{16}H_{24}N_5O_{97}$ [M + H]$^+$ 398.1670, found 398.1676.

Biological assays

The growth promotion assays were performed using the disc diffusion method in Petri dishes. All tests were carried out using CM9 minimal medium containing the non-assimilable iron chelator 2,2′-dipyridyl at the appropriate concentration for each test strain to make the medium iron depleted and to hinder normal bacterial growth. Strains to be tested were suspended in this medium before plate pouring. After agar solidification, 10 μL of siderophore solutions (all adjusted to a concentration of 1 mg mL^{-1}) were applied to sterile paper discs of 6 mm diameter onto the surface of the agar plates. The presence of growth zones around discs after 24 h or 48 h incubation at 25 °C or 37 °C (for *Salmonella enterica*) indicates utilization of the compound as an iron source. Measurement of the growth halo diameter was used as a quantitative estimation of utilization of each derivative. All assays were repeated at least three times and the average values are given in the results. A disc with 10 μL of a 10 μM solution of $Fe_2(SO_4)_3$ was used as the growth control.

In addition, the iron complexation capacity of each compound was tested by the chrome azurol S (CAS) liquid assay developed by Schwyn and Neilands. An aqueous solution of each compound at a concentration of 1 mg mL^{-1} was diluted to 1:10 in purified water and mixed with 1 vol of the CAS mixture. After 20 min the colour change was measured in a UV-VIS spectrophotometer at 630 nm. A positive siderophore activity produces a colour change from blue to orange with the corresponding reduction in absorbance values with respect to the blank.

Acknowledgements

This work was financially supported by Xunta de Galicia (PGIDIT05RMA10302PR, PGIDIT06PXIC103118PN and PGIDIT04RMA261014PR) and Ministry of Education (MEC) of Spain (cofunded by FEDER) (CQT2005-00793 and AGL2006-00697). R.G.S. thanks the Parga Pondal Programme. M.B. is the recipient of a FPI predoctoral fellowship from the MEC.

References

1 (*a*) C. Ratledge and L. Dover G., *Annu. Rev. Microbiol.*, 2000, **54**, 881–941; (*b*) C. Wandersman and P. Delepelaire, *Annu. Rev. Microbiol.*, 2004, **58**, 611–647.
2 M. Balado, C. R. Osorio and M. L. Lemos, *Microbiology*, 2006, **152**, 3517–3528.
3 R. F. Conchas, M. L. Lemos, J. L. Barja and A. E. Toranzo, *Appl. Environ. Microbiol.*, 1991, **57**, 2956–2962.
4 M. K. Wolf and J. L. Crosa, *J. Gen. Microbiol.*, 1986, **132**, 2949–2952.
5 R. G. Soengas, C. Anta, A. Espada, V. Paz, I. R. Ares, M. Balado, J. Rodríguez, M. L. Lemos and C. Jiménez, *Tetrahedron Lett.*, 2006, **47**, 7113–7116.

6 R. G. Soengas, C. Anta, A. Espada, R. M. Nieto, M. Larrosa, J. Rodríguez and C. Jiménez, *Tetrahedron Lett.*, 2007, **48**, 3021–3024.
7 (*a*) M. J. Miller and F. Malouin, *Accts. Chem. Res.*, 1993, **26**, 241–249; (*b*) A. Ghosh, M. Ghosh, C. Niu, F. Malouin, U. Moellmann and M. J. Miller, *Chem. Biol.*, 1996, **31**, 1011–1019; (*c*) H. Budzikiewicz, *Curr. Top. Med. Chem.*, 2001, **1**, 73–82; (*d*) F. Rivault, C. Liebert, A. Burger, F. Hoegy, M. A. Abdallah, I. J. Schalk and G. L. Mislin, *Bioorg. Med. Chem. Lett.*, 2007, **17**, 640–644.
8 C. Hennard, Q. C. Truong, J. F. Desnottes, J. M. Paris, N. J. Moreau and M. A. Abdallah, *J. Med. Chem.*, 2001, **44**, 2139–2151.
9 J. D. Buynak, *Curr. Med. Chem.*, 2004, **11**, 1951–1964.
10 C. Lu, J. S. Buyer, J. F. Okonya and M. J. Miller, *BioMetals*, 1996, **9**, 377–283.
11 (*a*) M. Schnabelrauch, S. Wittmann, K. Rahn, U. Möllmann, R. Reissbrodt and L. Heinisch, *BioMetals*, 2000, **13**, 333–348; (*b*) S. Wittmann, L. Heinisch, I. Scherlitz-Hofmann, T. Stoiber, D. Ankel-Fuchs and U. Möllmann, *BioMetals*, 2004, **17**, 53–64.
12 (*a*) A. J. Walz, U. Möllmann and M. J. Miller, *Org. Biomol. Chem.*, 2007, **5**, 1621–1628; (*b*) R. Schobert Rainer, A. Stangl and K. Hannemann, *Tetrahedron*, 2008, **64**, 1711–1720.
13 E. Masiukiewicz, B. Rzeszotarska and J. Szczerbaniewicz, *Org. Prep. Proc. Int.*, 1992, **24**, 191–194.
14 S. K. Sharma, M. J. Miller and S. M. Payne, *J. Med. Chem.*, 1989, **32**, 357–367.
15 W. H. Rastetter, T. J. Erickson and M. C. Venuti, *J. Org. Chem.*, 1981, **46**, 3579–3590.
16 T. T. Charvat, D. J. Lee, W. E. Robinson and A. R. Chamberlin, *Bioorg. Med. Chem.*, 2006, **14**, 4552–4567.
17 K. Feichtinger, H. L. Sings, T. J. Baker, K. Matthews and M. Goodman, *J. Org. Chem.*, 1998, **63**, 8432–8439.
18 I. G. O′Brien, G. B. Cox and F. Gibson, *Biochim. Biophys. Acta*, 1969, **177**, 321–328.
19 M. J. Milewska and A. Chimiak, *Synthesis*, 1990, **3**, 233–234.
20 J. Gómez-León, L. Villamil, M. L. Lemos, B. Novoa and A. Figueras, *Appl. Environ. Microbiol.*, 2005, **71**, 98–104.
21 M. Persmark, D. Expert and J. B. Neilands, *J. Biol. Chem.*, 1989, **264**, 3187–3193.
22 J. R. Pollack, B. N. Ames and J. B. Neilands, *J. Bacteriol.*, 1970, **104**, 635–639.
23 Q. Wang, Q. Liu, Y. Ma, L. Zhou and Y. Zhang, *Arch. Microbiol.*, 2007, **188**, 433–439.
24 S. A. Amin, F. C. Küpper, D. H. Green, W. R. Harris and C. J. Carrano, *J. Am. Chem. Soc.*, 2007, **129**, 478–479.
25 B. Schwyn and J. B. Neilands, *Anal. Biochem.*, 1987, **160**, 47–56.
26 L. Dong, M. J. Miller and U. Möllmann, *BioMetals*, 2004, **17**, 99–104.
27 R. Annamalai, B. Jin, Z. Cao, S. M. Newton and P. E. Klebba, *J. Bacteriol.*, 2004, **186**, 3578–3589.