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Complexes formed by the siderophore-monosulfactam conjugate BAL30072 and their interaction with the outer membrane receptor PiuA of *P. aeruginosa* --Manuscript Draft--

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Abstract:	<p>Nuclear magnetic resonance and infrared spectroscopy have been used to investigate the formation of complexes of BAL30072 with Fe³⁺ and Ga³⁺ in solution and to collect geometrical parameters supporting reliable 3D structure models. Structural models for the ligand-metal complexes with different stoichiometries have been characterized using density functional theory calculations. Blind ensemble docking to the PiuA receptor from <i>P. aeruginosa</i> was performed for the different complexes to compare binding affinities and statistics of the residues most frequently contacted. When compared to analogues, BAL30072 was found to have an intrinsic propensity to form complexes with low ligand-to-metal stoichiometry. By using one of the sulfate oxygen atoms as a third donor in addition to the bidentate pyridinone moiety, BAL30072 can form a L2M complex, which was predicted to be the one with the best binding affinity to PiuA. The example of BAL30072 strongly suggests that a lower stoichiometry might be the one recognized by the receptor, so that to focus only on the highest stoichiometry might be misleading for siderophores with less than six donors.</p>	

Complexes formed by the siderophore-monosulfactam conjugate BAL30072 and their interaction with the outer membrane receptor PiuA of *P. aeruginosa*

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ABSTRACT

Nuclear magnetic resonance and infrared spectroscopy have been used to investigate the formation of complexes of BAL30072 with Fe^{3+} and Ga^{3+} in solution and to collect geometrical parameters supporting reliable 3D structure models. Structural models for the ligand-metal complexes with different stoichiometries have been characterized using density functional theory calculations. Blind ensemble docking to the PiuA receptor from *P. aeruginosa* was performed for the different complexes to compare binding affinities and statistics of the residues most frequently contacted. When compared to analogues, BAL30072 was found to have an intrinsic propensity to form complexes with low ligand-to-metal stoichiometry. By using one of the sulfate oxygen atoms as a third donor in addition to the bidentate pyridinone moiety, BAL30072 can form a L_2M complex, which was predicted to be the one with the best binding affinity to PiuA. The example of BAL30072 strongly suggests that a lower stoichiometry might be the one recognized by the receptor, so that to focus only on the highest stoichiometry might be misleading for siderophores with less than six donors.

KEYWORDS

Nuclear Magnetic Resonance, Molecular Docking, Infrared Spectroscopy, Antibiotic Resistance.

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INTRODUCTION

The problem of multi-drug resistant pathogens is increasing worldwide at a rapid pace.(WHO 2014) The ‘ESKAPE’ organisms (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species) are of particular concern, being responsible for many serious infections in hospitals from which numerous deaths have already been registered (Tommasi et al. 2015; Brown and Wright 2016). The design of novel antibiotics and/or the discovery of new scaffolds is widely recognized to be particularly difficult for Gram-negative bacteria (The PEW Charitable Trusts 2018). The presence of an outer membrane provides these microorganisms with a very efficient barrier to the permeation of even small hydrophilic antibiotics. By slowing down the translocation into the periplasmic space, Gram-negative bacteria greatly impair the efficacy of a drug by ensuring that its periplasm concentration is orders of magnitude lower than in the extracellular space. Thus, although efflux pumps(Nikaido 1994; Vaccaro et al. 2006; Blair et al. 2015) and degradative enzymes(Drawz and Bonomo 2010) are certainly important and are object of numerous studies, the problem of antibiotic permeation is widely recognized as being of primary importance (Stavenger and Winterhalter 2014; The PEW Charitable Trusts 2018).

The polar character of most of the commonly used antibiotics requires that their main route for penetration is mediated by the outer membrane porins. These may be either general pore-forming proteins or more specific protein channels allowing the passage of nutrients, ions and water molecules (O’Shea and Moser 2008; Mach et al. 2008; Mahendran et al. 2010; Cama et al. 2015). Resistant strains that have mutations which affect either the expression of the porins or their transport properties have been reported (Pagès et al. 2008; Fernandez and Hancock 2012). Several attempts have been made to hijack active iron-transport systems as an alternative to exploiting the passive diffusion through porins to accelerate uptake of the drug into the bacterial cell (Page et al. 2010; Page 2013; Katsube et al. 2017; Ito et al. 2018). Iron is essential to life, being fundamental for a variety of redox systems. In humans, iron is transported tightly bound to proteins, such as those belonging to the transferrin family and to heme proteins. Bacteria compete with the host for the available Fe^{3+} , either by expressing receptors for host’s proteins or by secreting low-molecular weight scavengers, called siderophores (Braun and Hantke 2013; Wilson et al. 2016). Some bacteria use exogenous compounds secreted by other species, referred to as xenosiderophores in addition to their intrinsic siderophores (Matzanke 2006). Siderophores form highly stable complexes with Fe^{3+} that are recognized by specific receptors in the outer membrane of Gram-negative

bacteria and are taken up through an energy-coupled transport system typically driven by the TonB protein, which transduces energy from the electrochemical potential of the cytoplasmic membrane to the receptor (Braun and Hantke 2013). The TonB-dependent receptors typically have high-affinity binding sites for their cognate iron-siderophore complex, which can be thus recognized and transported even at very low concentrations.

The monosulfactam BAL30072 (Fig. 1A), is endowed with one 1,3-dihydroxy-4-pyridinone chelating moiety. It showed potent activity against many species across the Enterobacteriaceae family as well as *S. maltophilia*, *P. aeruginosa* and *Acinetobacter* spp., among others (Page et al. 2010). The activity of BAL30072 also includes different multidrug-resistant *Acinetobacter* species that are not inhibited by other β -lactams (Page et al. 2010). Resistance was found to evolve slowly: mutations in both siderophore receptors and TonB were infrequent, while overexpression of β -lactamases was more commonly encountered (Page et al. 2010; Page 2013). The receptors, PiuA and PirA, in both *P. aeruginosa* and *A. baumannii* have been recently identified and their crystal structures determined (PDB IDs: 5FOK, 5FP1, 5FP2, 5FR8 (Moynié et al. 2017)). However, BAL30072-receptor co-crystals have not been reported so far, meaning that we still have no molecular level information on the complex binding and the ligand-induced structural changes in the receptor.

In the present work, we have combined experiments and computer modelling to gain a deeper characterization of BAL30072-Fe³⁺ complexes and their potential interactions with the putative receptor PiuA of *P. aeruginosa*.

MATERIALS AND METHODS

Materials

BAL30072 and the two derivatives, compound 1 (PTX-2616) and compound 2, were synthesized and purified at Basilea Pharmaceutica International Ltd. All the other chemicals and solvents were purchased from Sigma-Aldrich at purity $\geq 98\%$ and used without further purification. Phosphate buffer saline (PBS) was prepared by dissolving the appropriate amounts of KH₂PO₄ and K₂HPO₄ in D₂O to give a final total phosphate concentration of 50 mM, which was supplemented with 100 mM NaCl. The pD was finally adjusted to 7.4 (7.0 pH meter reading (Covington et al. 1968)) using small volumes of 0.1 M HCl or 0.1 M NaOH. Acetate buffer saline (ABS) was prepared by dissolving the appropriate amount of CH₃COONa in D₂O (or H₂O) for NMR (or FTIR) analysis, (respectively), together with CH₃COOH to give a final total acetate concentration of 50 mM and the buffer was supplemented with 100 mM NaCl. When using D₂O, the pD was finally adjusted to 5.4 (5.0

pH meter reading (Covington et al. 1968)) and for H₂O, pH was adjusted to 5.4 (meter reading) using small volumes of 0.1 M HCl or 0.1 M NaOH. All the salts were dried to constant weight before solubilization. MarvinSketch (ChemAxon 2015a) was used for drawing and characterizing chemical structures, and the Calculator Plugins (ChemAxon 2015b) were used for structure properties prediction and calculation, Marvin 15.7.13, 2015, ChemAxon (<http://www.chemaxon.com>).

Nuclear Magnetic Resonance

Spectra were acquired at 300K with an Agilent UNITY INOVA spectrometer operating at a ¹H frequency of 500 MHz. The one-dimensional ¹H spectra were typically acquired with a pulse of 6.3 μs (90°), 5 s recycle delay, 48 transients over a spectral window of 6500 Hz. Homonuclear ¹H gCOSY was acquired with the same acquisition parameters and sampling each of the 256 increments with 2048 complex points. The same parameters were applied also for homonuclear ¹H ROESY experiments, with 200 ms mixing time of mlev17 spin-lock scheme. The ¹H chemical shift scale was referenced to the solvent residual resonance at 2.50 and 4.81 ppm for DMSO and D₂O, respectively. Titrations were performed at constant ligand concentration in the 2-3 mM range, by varying metal concentration up to 2 equivalents. Relative intensities of selected resonances from the same proton group in the different species formed along the titration were measured and normalized for each spectrum. Normalized NMR intensities were analyzed by fitting model equations through a Monte-Carlo minimization scheme (see below). Acquisition of heteronuclear ¹H-¹³C gHSQC and gHMBC was performed with the same parameters as for homonuclear sequences, carbon spectral window of 28750 Hz and a ¹³C(90°) pulse of 11.6 μs.

¹H resonances were initially assigned on the basis of their relative chemical shift, intensity and their multiplicity, as well as by comparison with the NMR predictor tool within the software Marvin Sketch (ChemAxon 2015b). Assignments were then confirmed with 2D homonuclear ¹H-¹H gCOSY and ROESY. The ¹³C resonances of nuclei directly attached to H atoms were assigned by 2D heteronuclear ¹H-¹³C gHSQC. The chemical shift of the other ¹³C nuclei were identified through 2D heteronuclear ¹H-¹³C gHMBC.

Structure calculations were performed using the simulated annealing molecular dynamics algorithm implemented in DYNAMO (Delaglio 2012). The temperature was increased to 4000 K in 1000 initialization steps, then kept constant for 4000 steps, and finally slowly decreased to 0 K during the 20000 steps cooling stage. Gromos-53a6 force field parameters

(Oostenbrink et al. 2004) were obtained through the PRODRG server (Schüttelkopf and van Aalten 2004).

Infrared Spectroscopy

Fourier-transform infrared spectroscopy (FTIR) was applied in the attenuated total reflectance mode (ATR). Spectra were acquired with a Bruker Vector-22 equipped with a diamond single-reflection ATR accessory (Platinum ATR module) and a liquid nitrogen cooled MCT detector. Ligands were studied in ABS solution prepared with H₂O at a concentration ranging from 2 to 3 mM, in the presence of different equivalents of Fe³⁺. Spectra were acquired from 10 µL of each sample deposited on the ATR crystal and dried under a gentle N₂ stream. Acquisition of the background (clean ATR crystal) was always performed before each sample for automatic removal. All spectra were acquired in the 3500-600 cm⁻¹ spectral range, with 4 cm⁻¹ resolution, automatic atmosphere compensation and 32 scans.

Solution equilibria and fitting procedure

The following stepwise equilibria were taken into consideration at constant pH:

where, L is the ligand, M the metal, K_n is the apparent equilibrium constant, n is an integer from 1 to 3, and molar concentrations are indicated with brackets.

Equilibria can be expressed also in terms of formation constants:

where $\beta_n = \prod_{i=1}^n K_i$. In addition to these mass-action laws, also the two following mass-balance relationships must be applied:

$$(3) \quad [L_0] = [L] + \sum_{n=1}^3 n \cdot [L_nM]$$

$$(4) \quad [M_0] = [M] + \sum_{n=1}^3 [L_nM]$$

where, $[L_0]$ and $[M_0]$ are the total ligand and metal molar concentration, respectively. Finally, the following quartic equation is obtained for $[L]$, i.e. the free ligand at equilibrium:

$$(5) \quad K_1 K_2 K_3 [L]^4 + (K_1 K_2 - K_1 K_2 K_3 [L_0] + 3 K_1 K_2 K_3 [M_0]) [L]^3 + (K_1 - K_1 K_2 [L_0] + 2 K_1 K_2 [M_0]) [L]^2 + (1 - K_1 [L_0] + K_1 [M_0]) [L] - [L]_0 = 0$$

which was solved numerically with the Newton-Raphson method.

A Monte-Carlo scheme was implemented to minimize the sum of square difference between the experimental and calculated data points, by using the K_n as fitting parameters. One parameter was randomly selected at each iteration and randomly varied between -10% and +10%. Only the steps resulting in a decrease of the sum of square difference were accepted, rejecting the last move otherwise. All the datasets pertaining to the different species along the same titration were fitted simultaneously.

Quantum mechanical calculations

The Gaussian09 computer code was used (Frisch et al. 2009). We adopted the Density Functional Theory (DFT) (Jones and Gunnarsson 1989), which offers a superior ratio of accuracy to computational effort as compared to other methodologies. The molecular systems have been optimized in vacuum at the B3LYP level of theory (Becke 1993; Stephens et al. 1994). A preliminary optimization was performed for both the doublet and quartet multiplicity states with the computationally inexpensive 3-21G basis set. In all the investigated cases, the ground-state was found to be the quartet. For this electronic state only, optimization was refined by using the larger 6-31G* basis set with polarization functions on heavy atoms. Geometry optimizations were followed by full vibrational analyses. To assess the impact of solvation effects we started from the optimized ground-state geometries to minimize the structures by using the Polarizable Continuum Model (Tomasi et al. 2005) as implemented in Gaussian09.

Molecular docking

A systematic blind docking campaign was performed by using AutoDock Vina (Trott and Olson 2010) on the receptor PiuA from *P. aeruginosa*. Starting from the recently solved X-ray structure (Moynié et al. 2017), missing loops were modelled by homology with Modeller (Webb and Sali 2014), using other TonB dependent transporters as templates (PDB id: 1XKH, 2IAH, 2O5P, 2W16, 2O5P). A total of 20 different protein models were considered to

cover an ample range of loop flexibility. As far as the ligand is concerned, different models were considered, ranging from a single BAL30072 molecule without Fe^{3+} , to complexes of BAL30072 and Fe^{3+} with stoichiometry 1:1, 2:1 and 3:1. Each receptor model was kept fixed during docking, while all possible torsions of the ligand detected by the program were considered. A rectangular docking box of 60x60x60 Å was used. The exhaustiveness of the search was set to 4096 (default 8). Only the top 5 best poses from each one of 20 independent runs were selected for the analysis. Statistics of the most contacted residues was collected by adopting a distance cut-off of 3.5 Å.

RESULTS AND DISCUSSION

Nuclear Magnetic Resonance

Fig. 1 shows the ^1H spectrum of BAL30072 in both DMSO and D_2O at two different pH values, namely 7.4 and 5.4, together with resonance assignments. Position and multiplicity are summarized in table S1. Titration of BAL30072 with Fe^{3+} in DMSO was limited by the paramagnetic character of ferric ions, which enhances spin relaxation (Jacobsen 2007), such that all resonances showed a marked broadening and intensity loss with titration and it was only possible to titrate up to 0.9 equivalents of Fe^{3+} (Fig. S1). The multiplicity of resonances did not change and no new resonances appeared in the spectrum. On the other hand, the chemical shifts showed significant changes, indicating the presence of an equilibrium between the free BAL30072 and some complexes formed with the metal ion. Progressive shifts were observed with increasing metal equivalents, with a different extent depending on the ^1H nuclei position in the molecular structure. In particular, the most affected protons were those belonging to, or close to, the pyridinone moiety (*h*, *e* and *d* resonances, Fig. 1), while the effect was much less evident for the others. The trend of chemical shift as a function of metal equivalents (Fig. S1) was clearly not compatible with the formation of a simple LM complex (where L is the ligand and M the metal). Higher stoichiometries had to be considered, such as L_2M and L_3M . We tried to fit the experimental NMR data through a Monte-Carlo minimization scheme, but the limited range of metal equivalents sampled, together with uncertainty on the relative chemical shift for the different species formed, made the fitting procedure questionable.

Therefore, we moved to Ga^{3+} , which is diamagnetic and widely reported to show coordination properties very similar to Fe^{3+} (Matzanke 2006; Weaver et al. 2008; Eldik and Harvey 2010). In contrast to the titration with Fe^{3+} , new resonances appeared in the spectrum during titration with Ga^{3+} (Fig. S2), reflecting the formation of different complexes and

indicating their remarkably slower exchange rate. An additional resonance for each ^1H signal appeared at the beginning of titration, with relative intensity increasing with increasing Ga^{3+} . Then, with further increasing metal ion concentration, a second species emerged in the spectrum, while the former one progressively disappeared. The intensity of resonances due to the free L decreased with increasing Ga^{3+} . Finally, it was possible to obtain a speciation plot by integrating the homologue resonance for the different species in equilibrium and then calculating their relative area. Fig. S3A shows the results obtained, for instance, in the case of both resonance *h* and *a''*, showing very good agreement. The fact that the first formed species reached maximum concentration at 0.3 metal equivalents, suggested that this highest stoichiometry species was the L_3M , while the second one has to correspond to a lower stoichiometry. The most affected ^1H resonances in the presence of Ga^{3+} were those belonging to, or close to, the pyridinone moiety, namely, protons *h*, *e* and *d* as was the case with Fe^{3+} . Fig. S3B shows the heteronuclear ^1H - ^{13}C gHMBC spectrum acquired at the beginning and at the end of the titration. The most affected carbons were *n* and *j*, while, interestingly, the other oxygen bonded ^{13}C nuclei, namely *l* and *o*, were almost unaffected by the presence of the metal ions.

In the same conditions of metal excess, where L was almost completely converted into the lowest stoichiometry complex (Fig. S3A), a ^1H - ^1H ROESY was acquired. The presence of certain cross-peaks with sign opposite to the diagonal ones could be attributed to the corresponding inter-proton through-space dipolar interactions (Bax and Davis 1985) and converted into distances ($< 5\text{\AA}$). Results are reported in Fig. S4 and Table S2. It was very interesting to find such interactions between protons belonging to the β -lactam moiety and the other two ring systems, namely, the pyridinone and the thiazole. These findings suggested that BAL30072 assumed a rather compact conformation when involved in metal coordination, different to that found in the absence of metal for which the same ROESY cross-peaks were not observed. In particular, only the ^1H nuclei on one side of the β -lactam ring, the *HN* and *a''*, were found to interact with ^1H nuclei located in the other rings, namely *e* and *f*, whereas the absence of cross-peaks for *a'* and *b* suggested that these were at an average distance $>5\text{\AA}$ from the latter.

By including these interatomic distances as constraints in a molecular model, the 3D conformation of BAL30072 in the presence of Ga^{3+} was calculated. A hundred structures were generated via simulated annealing (Delaglio 2012) and only those compatible with the experimental NMR observations were extracted (Fig. 2). It is important to stress here that the metal center was not included in the model at this stage. Nevertheless, a dominant

conformation was obtained, which was characterized by both the pyridinone oxygens and the sulfate group on the same side of the molecular structure. The same structure was found by clustering the BAL30072 all-atom conformers obtained along a 1 μ s MD simulation in explicit water with no restraints applied (Mallocci et al. 2015). This particular conformation suggested that, while the pyridinone moiety was shown to be the main metal coordination group of BAL30072, also sulfate could be involved in the chelation of the metal ion.

In order to shed light on this point, since NMR sensitivity of both ^{17}O and ^{33}S nuclei is very low, we decided to compare two BAL30072 derivatives, namely, compound 1 and compound 2. In the former, the sulfate was substituted by the shorter sulfonate group, whereas in the latter, the N-hydroxyl group of pyridinone was replaced by a simple N-H (Fig. 1). Titration in DMSO with Ga^{3+} was performed for all the three siderophore-antibiotic conjugates. Results were analyzed in terms of relative intensity of the NMR signals attributed to the different species formed with increasing metal equivalents. Model curves for complex formation equilibria were fitted to the experimental results through the Monte-Carlo minimization algorithm illustrated in the methods section, in order to determine stoichiometry and estimate equilibrium constants. Fig. 3 shows the results.

In all of the cases, the highest stoichiometry complex showed maximum intensity close to 0.3 metal equivalents, indicating the formation of L_3M . A total of three species was observed along the titrations, with the only acceptable fitting model being the one where the low stoichiometry species was described as the sum of L_2M and LM . This means that the chemical exchange of the latter was relatively rapid with respect to the difference in their resonance frequency (Jacobsen 2007).

The results showed that the three molecules had a rather different relative stability for the three possible stoichiometries, despite the apparently slight chemical difference. In particular, a remarkable difference was observed as far as the relative stability of the L_2M complex is concerned. BAL30072 showed the highest propensity, which was substantially reduced in compound 1, although the chemical modification was far from the pyridinone. Quite surprisingly, the preference for L_2M complex formation was greatly suppressed in compound 2, despite its similarity to BAL30072.

A more effective comparison was obtained by using the estimated equilibrium constants (Table 1) to calculate the molar fraction of the metal in the different species as a function of its equivalents along the three titrations (Fig. 3D-F). When ligand was in large excess, in the case of BAL30072, up to 10% of M was involved in L_2M , while in the case of compound 1 it was halved and it was even lower in the case of compound 2.

Titration experiments were also performed in D₂O at pD 7.4 by using a PBS buffer to test a more physiologic condition (Fig. 4). At neutral pH and high [L]/[M], hydroxypyridinones usually form L₃M complexes, and the three ligands under investigation are not an exception (Harrington et al. 2012; Lachowicz et al. 2016). However it is interesting to note that, despite L₃M is largely preferred, its stability relative to L₂M follows the same trend observed in DMSO, which was BAL30072 < compound 1 < compound 2. Finally, it is also important to note that in these conditions, free M rapidly increased with decreasing [L]/[M], since L is consumed by formation of the highest stoichiometry complex.

Generally speaking, stability of the L₃M usually decreases with decreasing pH and, in turn, lower stoichiometry complexes are formed. Since acidic pH can be found at infection sites (Bennison et al. 2017), we completed the study by performing the titrations also in ABS at pD 5.4 (Fig. 5). The L₃M was clearly predominant at the lowest [L]/[M] but, in contrast to neutral pH, L₂M also appeared as soon as [L]/[M] decreased. While in the case of BAL30072, we had still to consider rapid exchange between L₂M and LM, slower exchange between all the species was evident for the two derivatives and up to four different species were observed along the titration. For the sake of completeness, indeed, it has to be mentioned that actually, in the case of compound 2, the fourth species was observed only in the last two spectra (metal equivalents ~1.50 and ~1.75), thus, these were omitted from the fitting procedure and from the figure. When Fig. 5 is compared to the results obtained at neutral pH (Fig. 4), we can clearly see that L₃M was destabilized at lower pH, and more L₂M was correspondingly formed. The stability order observed at neutral pH with respect to L₃M formation applied to the L₂M complex at mild acidic pH, which was BAL30072 < compound 1 < compound 2. However, BAL30072 showed an interesting high stability of the LM complex when compared to the two derivatives (Fig. 5D-F). In the case of BAL30072, LM started to form already at 0.3 equivalents with such a high formation constant that free metal was absolutely negligible up to 1 metal equivalent, differently from the other two conjugates. In other words, BAL30072 was able to effectively retrieve Ga³⁺ from the solution even when it was not in large excess compared to the metal ions. Finally, Table 1 reports all the K_n (and the correspondingly calculated β_n) determined from the present work.

Table 1. Both stepwise equilibrium and formation constants obtained for the three ligands under investigation in the three different environments considered.						
	<i>BAL30072</i>		<i>compound 1</i>		<i>compound 2</i>	
<i>DMSO</i>	K ₁ 5.48E+05	β ₁ 5.48E+05	K ₁ 1.24E+06	β ₁ 1.24E+06	K ₁ 9.79E+04	β ₁ 9.79E+04
	K ₂ 5.57E+03	β ₂ 3.05E+09	K ₂ 4.53E+03	β ₂ 5.62E+09	K ₂ 1.25E+02	β ₂ 1.23E+07
	K ₃ 2.46E+03	β ₃ 7.51E+12	K ₃ 4.18E+03	β ₃ 2.35E+13	K ₃ 6.88E+03	β ₃ 8.45E+10

	pM ^a = 5.58		pM ^a = 6.05		pM ^a = 3.65	
D₂O-PBS (pD 7.4)	K ₁ 2.74E+01	β ₁ 2.74E+01	K ₁ 4.91E+02	β ₁ 4.91E+02	K ₁ ---	β ₁ ---
	K ₂ 1.49E+05	β ₂ 4.08E+06	K ₂ 2.54E+07	β ₂ 1.25E+10	K ₂ ---	β ₂ ---
	K ₃ 3.09E+04	β ₃ 1.26E+11	K ₃ 1.50E+06	β ₃ 1.87E+16	K ₃ ---	β ₃ 5.10E+13
	pM ^a = 3.74		pM ^a = 8.91		pM ^a = 6.34	
D₂O-ABS (pD 5.4)	K ₁ 2.24E+18	β ₁ 2.24E+18	K ₁ 5.92E+06	β ₁ 5.92E+06	K ₁ ---	β ₁ ---
	K ₂ 5.22E+04	β ₂ 1.17E+23	K ₂ 2.13E+05	β ₂ 1.26E+12	K ₂ ---	β ₂ 3.52E+14
	K ₃ 1.68E+04	β ₃ 1.96E+27	K ₃ 1.05E+04	β ₃ 1.32E+16	K ₃ 2.68E+06	β ₃ 9.42E+20
	pM ^a = 19.94		pM ^a = 8.77		pM ^a = 13.61	
^a pM = -log[M _{free}] was calculated from the obtained equilibrium constants with [L _{tot}] = 5.0E-03 M and [L _{tot}]/[M _{tot}] = 10.						

Complex formation by BAL30072 was strongly influenced by the environment. In DMSO, the most interesting observation was that BAL30072 had the highest formation constant (K₂) for the L₂M. BAL30072 was the least effective ligand in water at neutral pH, but it became the best one at mildly acidic pH. Despite compound 1 having the same 1,3-dihydroxy-4-pyridinone chelating moiety, it was far less affected by solvent and pH, bolstering the hypothesis of a possible role of the sulfate group in the case of BAL30072. On the other hand, compound 2 was more comparable to BAL30072, with the differences observed in water being most likely attributable to difference in protonation equilibria of the pyridinone moiety (Santos 2002; Matzanke 2006). However, in DMSO, BAL30072 showed a marked preference for L₂M stoichiometry compared to compound 2 and the same trend was found in water, especially in the acidic environment, where BAL30072 showed a higher stability for the lowest stoichiometry complex.

BAL30072 showed dipolar interactions between protons belonging to the β-lactam moiety and both the pyridinone and thiazole rings both in DMSO and water (Fig. S4 and S5), indicating a comparable compact conformation when involved in metal coordination (Fig. 2). A similar cross-peak pattern was found in the presence of both 0.6 and 1.0 equivalents of Ga³⁺, suggesting the same conformation either in the case of L₂M and LM complex. Conversely, no correlation was found for compound 1 in the presence of 0.6 metal equivalents, while in the case of compound 2 the β-lactam ring showed dipolar interaction only with thiazole (Fig. S5).

Infrared Spectroscopy

In order to collect more direct information on possible involvement of the sulfate group in metal coordination, infrared spectra of BAL30072 were acquired in water, both in the absence and presence of Fe³⁺ at different [L]/[M] ratios. Peak assignments were obtained for the most intense absorption bands on the basis of DFT harmonic vibrational analysis and data

from the literature (Yamaji et al.; Ahuja et al. 1979; Smith 1999; Bruker Corporation 2012), as reported in the SI (Fig. S6 and S7, Table S3 and S4). In particular, the stretching modes of the β -lactam and amide carbonyl groups were found at 1777 and 1655 cm^{-1} , respectively, while contribution from pyridinone carbonyl was hidden in the intense band centered at 1564 cm^{-1} . Sulfate vibrational modes were identified at 1175 and 1121 cm^{-1} , respectively. In Fig. 6, the FTIR spectrum of BAL30072 is compared to that obtained after addition of 0.3, 0.6 and 1.2 metal equivalents.

Differences were clearly observed between the spectra of BAL30072 measured in the absence and in the presence of the metal ions. However, while at 0.3 equivalents differences were relatively small and almost limited to the pyridinone moiety, spectra at 0.6 and 1.2 equivalents were remarkably different but comparable with each other. The band assigned to amide C=O stretching and N-H in-plane bending shifted from 1655 for L alone to 1661, 1670 and 1672 cm^{-1} for L_3M , L_2M and LM, respectively, which might be attributable to a significant change in molecular plasticity. Interestingly, profile of the multi-component bands at ~ 1560 and ~ 1400 cm^{-1} was not significantly affected in the L_3M , while marked differences were observed for both the L_2M and LM. These were likely due to modification of pyridinone moiety vibration modes, especially the OH and C=O groups. Differences were also observed in the spectral range 1250-1300 as well as 900-1100 cm^{-1} , where different vibration modes of the pyridinone moiety were identified, thus, bolstering the evidence of differences in metal coordination between L_3M and the other two stoichiometries. However, the most interesting result was the shift found for the sulfate bands (Fig. 6B). In particular, the band at 1121 cm^{-1} was almost unaffected in the case of L_3M when compared to the spectrum recorded in the absence of metal, while it moved to 1127 and 1130 cm^{-1} for L_2M and LM, respectively. Overall, these results confirmed that sulfate group was involved in metal coordination for both L_2M and LM.

Quantum mechanical calculations

Summarizing the results obtained with both FTIR and NMR, a compact conformation is proposed for BAL30072, which exploits both the oxygen atoms of the pyridinone group and one oxygen atom from the sulfate group to coordinate the metal ion. Thus, three out of six positions of the optimal octahedral configuration can be occupied by a single BAL30072 molecule, meaning that just two of them are sufficient to complete the coordination sphere. By including the NMR derived geometrical parameters reported above in the simulated

annealing scheme (Delaglio 2012), we obtained the “scorpion-like” conformation shown in Fig. 7, assumed by BAL30072 both in the LM and L₂M.

The ground-state optimized geometries corresponding to the L₂M complexes displayed in Fig. 7 are shown in Fig. S6. At the B3LYP/6-31G* level of theory, the trans isomer was found to be about 10 kcal mol⁻¹ lower in energy than the cis one. The same energy difference was found by including a polarized continuum model of water to take also solvation effects into account (Tomasi et al. 2005). In the molecular docking calculations reported hereinafter we therefore considered only the trans isomer.

Size and shape of the complex are crucial to receptor-siderophore recognition. By considering the average value from different 3D model structures of the L₃M complex of BAL30072 (Fig. S7) we found that, interestingly, this complex would be rather large, with both Van der Waals volume and minimal projection area considerably larger than other well characterized natural siderophores, namely, pyochelin, enterobactin and pyoverdine. Conversely, the trans isomer of the L₂M complex (Fig. 7A,B and S8 bottom) appeared to be more suitable to access the binding site of the identified receptors, with a Van der Waals volume of 784 Å³ and minimal projection area of 95 Å², thus, slightly larger than enterobactin.

The most studied natural siderophores are polydentate ligands able to complete the metal coordination sphere and form, thus, very stable and compact LM complexes, such as ferrichrome, enterobactin and pyoverdin (see also Fig. S9) (Matzanke 2006; Page 2013). When the siderophore does not have a sufficient number of donors, like BAL30072 as well as a number of natural compounds (Page 2013), missing positions in the coordination sphere can be either occupied by solvent molecules (e.g. ethylene glycol and pyochelin bound to FptA from *P. aeruginosa* in PDB ID 1XKW) (Cobessi et al. 2005) or higher stoichiometry L₂M and L₃M complexes have to be envisaged. However, when the complex-protein interaction is taken into account, donors can be also provided by the amino acid residues of the binding site. In the case of hexadentate siderophores, where all the six coordination positions of Fe³⁺ are occupied by the ligand, the interactions with the protein binding site are given by other chemical groups of the ligand itself, as seen with enterobactin (PDB ID 2XUZ) (Peuckert et al. 2011) or ferrichrome (PDB ID 1FCP) (Ferguson et al. 1998). On the other hand, although co-crystals with outer membrane receptors are not yet available for siderophores with a lower number of donors, a series of tetradentate siderophore mimics have been recently shown to interact with the periplasmic binding protein CeuE (PDB IDs: 5A1J, 5A5D, 5A5V, 5AD1). This is part of the Fe³⁺ transport system in *Campylobacter jejuni*, and makes use of one Tyr

and one His residues to complete the metal's octahedral coordination sphere (Wilde et al. 2017).

Molecular docking

In the absence of a BAL30072-PiuA co-complex crystal structure, we undertook an extended molecular docking campaign to compare the predicted binding affinity and binding modes of the possible complexes formed by this siderophore-antibiotic conjugate and its identified receptor PiuA in *P. aeruginosa* (Moynié et al. 2017). In order to improve the statistics and significance of the results, we looked at 100 predicted ligand-receptor configurations (the 5 best docking poses out of 20 totally independent runs) for each case, rather than focusing on one single pose.

Fig. 8 compares the spatial distribution of all the docking poses for the different cases examined, ranging from BAL30072 alone to the complexes formed with Fe^{3+} with different stoichiometry. The corresponding statistics of protein residues more frequently contacted in all the 100 predicted poses are displayed in Fig. S10. The three smallest examples (Fig. 8A-C) populated different regions of the extracellular vestibule of the receptor, with small difference in binding energy regardless of the presence of the metal ion and direct coordination by the sulfate group. However, it is worth noting the progressive stabilization of the interaction with the receptor by moving towards the scorpion-like configuration. In particular, an increase of about 10% is observed for the average binding free-energy when moving from L free to LM with the scorpion-like configuration.

Conversely, the three high stoichiometry cases (Fig. 8D-F) showed a higher degree of selectivity for the putative binding site (Moynié et al. 2017), together with a remarkable decrease of the binding free energy. It is very interesting that L_2M and L_3M showed a comparable energy, with a further decrease observed for the proposed scorpion-like configuration adopted by L_2M . If we consider the net charge of these complexes, i.e. +1, -1 and -3 for LM, L_2M and L_3M , respectively, correlation with binding energy appeared only poor. Likely, the major contribution has to be looked for in the different size and shape and in the different exposure of specific chemical groups, especially the negatively charged sulfates.

CONCLUSIONS

Investigation of complex formation equilibria are typically performed at a relatively high $[\text{L}]/[\text{M}]$ ratio (between 10 and 5) in this field, as well as in other pathologic contexts related to chelation therapy (Santos 2002; Matzanke 2006; Harrington et al. 2012; Lachowicz et al.

2016). This choice is motivated by the paucity of Fe^{3+} in most physiologic environments. In such conditions, at neutral pH, BAL30072 was shown to form a rather large L_3M complex utilizing only one bidentate chelating group. However, when the interaction with the receptor is considered, other factors (such as local pH, electrostatics effects, dehydration upon binding to the receptors) have to be taken into consideration. The equilibria observed in the bulk solution might be significantly shifted towards lower stoichiometries.

In the present work we showed how the behavior of BAL30072 is sensitive to the environment by changing the pH or moving to a less polar solvent like DMSO. When compared to other derivatives, its peculiar propensity to form low stoichiometry complexes, owing to its ability to coordinate the metal ion by using also the sulfate group in addition to the hydroxypyridinone group, is particularly interesting. When only the L_3M complex was taken into consideration, it was quite hard to realize how it can approach and fit into the receptor's binding site. Our calculations showed that L_2M in the proposed scorpion-like configuration is significantly more compact and it is comparable in size to the complexes formed by well-known natural hexadentate siderophores. Further, the L_2M adduct was found to be the one with the most favorable binding affinity among all the ones tested.

A siderophore is able to form high stability complexes with Fe^{3+} but the complex has to be finally recognized and captured by the specific receptor in the outer membrane (Matzanke 2006; Braun and Hantke 2013). This has two main direct implications, i) shape and size of the complex are of crucial importance and ii) the cognate siderophore complex can be thus recognized and transported even at low concentrations. The actual species transported may not necessarily be the one formed at the highest concentration in the bulk solution.

SUPPORTING INFORMATION

Table S1-S4 and Fig. S1-S10. This material is available free of charge via the Internet.

CONFLICT OF INTEREST

Eric Desarbres is employee of Basilea Pharmaceutica International Ltd. Malcolm Page is employee and owns stock of Basilea Pharmaceutica International Ltd.

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FIGURE CAPTION

Fig. 1 (A) Molecular structure of BAL30072 is shown (ChemAxon 2015a) with C atoms labeled by lower case letters. (B) ¹H NMR spectra of BAL30072 recorded in DMSO-d₆ (blue), D₂O pD 7.4 (green) and D₂O pD 5.4 (red) are shown together with resonance assignments. For the sake of clarity, left portion of the spectra was magnified (x2). It has to be noted that relative position of resonances e and f is inverted when moving from DMSO to D₂O. Molecular structure of the BAL30072 derivatives studied in this work, namely, (C) compound 1 and (D) compound 2

Fig. 2 Structure model (30 conformers) obtained for BAL30072 through simulated annealing by including NMR geometrical restraints corresponding to the end of titration in Fig. S3A. Metal ion was not present in the model

Fig. 3 Results of curve fitting for titration of (A) BAL30072, (B) compound 1 and (C) compound 2 in DMSO with Ga^{3+} . For each case, results are shown for the complexation model with the lowest total square deviation between the calculated (solid lines) and the experimental data. The corresponding speciation plots are shown in (D), (E) and (F), respectively, as the molar fraction of the metal ion as a function of metal equivalents. Key: L, ligand; M, metal

Fig. 4 Results of curve fitting for titration of (A) BAL30072, (B) compound 1 and (C) compound 2 in PBS prepared in D_2O at pD 7.4 with Ga^{3+} . For each case, results are shown for the complexation model with the lowest total square deviation between the calculated (solid lines) and the experimental data. The corresponding speciation plots are shown in (D), (E) and (F), respectively, as the molar fraction of the metal ion as a function of metal equivalents. Key: L, ligand; M, metal

Fig. 5 Results of curve fitting for titration of (A) BAL30072, (B) compound 1 and (C) compound 2 in ABS prepared in D_2O at pD 5.4 with Ga^{3+} . For each case, results are shown for the complexation model with the lowest total square deviation between the calculated (solid lines) and the experimental data. The corresponding speciation plots are shown in (D), (E) and (F), respectively, as the molar fraction of the metal ion as a function of metal equivalents. Key: L, ligand; M, metal

Fig. 6 (A) FTIR spectrum of BAL30072 acquired in acetate buffer saline (pH 5.4) either in the absence and presence of Fe^{3+} at different relative concentrations. The region of sulfate stretching modes is indicated by the dashed rectangle and zoomed in (B), where a dashed line is used as visual guide to appreciate better the shift of the S-O stretching band

Fig. 7 Proposed structure of the L_2M complex formed by BAL30072 in two different configurations. Two possible isomers are shown with the sulfate groups in (A, B) trans and (C, D) cis position, respectively. Atoms are color coded as follows: C, black; N, blue; O, red; S, yellow. Hydrogens are not shown for the sake of clarity. Iron is shown as a grey sphere embedded in a shaded octahedron defined by the six coordinating O atoms. Each of the two isomers is shown either from one (A, C) edge or (B, D) face of the octahedron

Fig. 8 The 100 best poses of molecular docking are shown (ligand in blue; PiuA receptor's backbone trace in gray), together with a representative molecular structure and average binding free energy in descending order: **(A)** free BAL30072, **(B)** bidentate LM complex, **(C)** scorpion-like LM complex, **(D)** bidentate L₂M complex, **(E)** L₃M complex and **(F)** scorpion-like L₂M complex

Figure 1

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Figure 3

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Figure 4

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Figure 5

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Figure 6

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Complexes formed by the siderophore-monosulfactam conjugate BAL30072 and their interaction with the outer membrane receptor PiuA of *P. aeruginosa*

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SUPPLEMENTARY MATERIAL

Table S1. Resonance assignment for BAL30072 in different solvents. Labels refer to Figure 1 in the main paper. Multiplicity of ^1H resonances is reported in parenthesis (d: doublet; dd: doublet of doublets; s: singlet).

label	DMSO-d ₆		D ₂ O pD 7.4		D ₂ O pD 5.4
	^1H [ppm]	^{13}C [ppm]	^1H [ppm]	^{13}C [ppm]	^1H [ppm]
a'	1.449 (s)	23.303	1.635 (s)	24.901	1.631 (s)
a''	1.247 (s)	20.188	1.290 (s)	22.132	1.321 (s)
b	4.646 (d)	60.570	5.007 (s)	63.455	4.998 (s)
c		67.766		73.812	
d	5.274 (dd)	68.625	5.325 (dd)	72.991	5.374 (dd)
e	7.071 (s)	110.403	6.756 (s)	115.135	7.027 (s)
f	6.838 (s)	110.403	7.088 (s)	116.365	7.085 (s)
g		141.870		142.717	
h	8.145 (s)	127.264	7.731 (s)	129.080	7.954 (s)
i		168.505		173.582	
j		144.340		150.921	
k		141.441		140.359	
l		161.847		166.814	
m		n.o.		n.o.	
n		151.106		151.536	
o		155.510		161.892	
HN	9.573 (d)		n.o.		n.o.

Figure S1. Chemical shift variation is shown for ^1H resonances of BAL30072 along the titration performed with Fe^{3+} in DMSO. Lower case letters are used to identified different protons according to Figure 1 in the main paper. Lines are not due to any fitting procedure but were included just as a guide to better visualize the trend.

Figure S2. Stacking plot for the titration of BAL30072 with Ga^{3+} in DMSO. Selected resonances are labeled (according to Figure 1) to show the progressive disappearance of the signals due to the free molecule, the appearance of a first species ('1' as subscript), which then disappeared and, finally, the progressive rising of a second species ('2' as subscript).

Figure S3. (A) Normalized intensity of the NMR resonances assigned proton h and protons a'' for the different species formed along the titration of BAL30072 with Ga^{3+} in DMSO. (B) A selected region of the heteronuclear ^1H - ^{13}C gHMBC spectrum is shown to compare resonances position at the beginning and at the end of the titration. Labels refer to Figure 1 in the main paper.

Figure S4. (A) Selected Regions of Interest (RoI) from an homonuclear ^1H - ^1H ROESY with 200 ms mixing time, performed in DMSO on BAL30072 at the end of the titration with Ga^{3+} . Cross-peaks are labeled according to Figure 1a of the main paper, which is also reproduced in (B) together with dotted lines indicating ^1H nuclei couples corresponding to cross-peaks in (A).

Table S2. Proton-proton distances [Å] obtained from relative ROESY intensities.^a

Reference distance ^b $d(a'-b) = 2.9$						
$d(a''-b)$	$d(a''-e)$	$d(a''-f)$	$d(a''-\text{HN})$	$d(b-\text{HN})$	$d(e-\text{HN})$	$d(f-\text{HN})$
3.9	3.3	3.6	3.0	3.4	3.7	3.7
Reference distance ^b $d(a''-b) = 3.9$						
$d(a'-b)$	$d(a''-e)$	$d(a''-f)$	$d(a''-\text{HN})$	$d(b-\text{HN})$	$d(e-\text{HN})$	$d(f-\text{HN})$
2.9	3.3	3.6	2.9	3.3	3.7	3.7

^aAccording to the formula $d_{ij} = d_{\text{ref}} \sqrt{\frac{I_{\text{ref}}}{I_{ij}}}$; where, d_{ij} and d_{ref} are the distance between the two nuclei i and j , and a reference intramolecular distance, respectively, I is the corresponding cross-peak intensity.

^bReferences distances were obtained from molecular mechanics minimization with the software Avogadro (<https://sourceforge.net/projects/avogadro/>) and the MMFF94 force field (Halgren 1996).

Comments to table S2:

In this work, we calculated proton-proton distances by using two different reference distances, namely, $d(a'-b)$ and $d(a''-b)$. The shaded cells of table S2 show the results of the cross-check, by using the former as a reference we obtained an accurate value for the latter and vice-versa. The values obtained for the other distances by using either of the two reference distances were in very good agreement with each other.

Figure S5. Selected Regions of Interest (RoI) from different homonuclear ^1H - ^1H ROESY with 200 ms mixing time, performed in D_2O at pD 5.4 on **(A)** BAL30072 with 0.6 eq. of Ga^{3+} , **(B)** BAL30072 with 1.0 eq. of Ga^{3+} , **(C, D)** compound 1 with 0.6 eq. of Ga^{3+} and **(E)** compound 2 with 0.75 eq. of Ga^{3+} . Equivalents of metal were selected in order to maximize the relative concentration of complex **(A, C, D, E)** L_2M , **(B)** LM , respectively (see corresponding titrations in the main paper). Cross-peaks are labeled with arabic numbers: **(1,5,11)** $\text{a}''\text{-f}$; **(2,6)** $\text{a}''\text{-e}$; **(3,7,12)** $\text{a}''\text{-b}$; **(4,8,13)** $\text{a}'\text{-b}$; **(9)** $\text{a}'\text{-a}''$; **(10)** $\text{a}''\text{-a}'$. Proton nuclei are labeled according to Figure S4.

Figure S6. FTIR spectrum (ATR mode) of the acetate buffer saline ($\text{CH}_3\text{COOH} / \text{CH}_3\text{COO}^-\text{Na}^+$ 50 mM + NaCl 100 mM; pH 5.4). Main absorption bands are labeled with arabic numbers. Assignments are reported in Table S3.

Table S3. Assignments^a of main absorption bands from the FTIR spectrum of acetate buffer saline (Figure S6).

Label ^b	Wavenumber [cm^{-1}]	Assignment
1	1558	-COO^- asymmetric stretching
2	1407	-COO^- symmetric stretching
3	1344	CH_3 - umbrella bending
4	1049	C-C-O asymmetric and symmetric stretching
5	1018	
6	926	
7	647	-COO^- bending
8	621	
*		residual H_2O bending

^aAssignments were obtained by comparison with data from the literature (Yamaji et al.; Ahuja et al. 1979; Smith 1999; Bruker Corporation 2012).

^bLabels refer to Figure S6.

Figure S7. FTIR spectrum (ATR mode) of BAL30072 in acetate buffer saline ($\text{CH}_3\text{COOH} / \text{CH}_3\text{COO}^-\text{Na}^+$ 50 mM + NaCl 100 mM; pH 5.4). Main absorption bands are labeled with arabic numbers. Assignments are reported in Table S4.

Table S4. Assignments^a of main absorption bands from the FTIR spectrum of BAL30072 in acetate buffer saline (Figure S7).

Label ^b	Wavenumber ^c [cm^{-1}]	Assignment
1	1564	acetate: $-\text{COO}^-$ asymmetric stretching; BAL30072: pyridinone $\text{C}=\text{O}$ stretching; ^d NH in-plane bend, NH_2 scissoring, pyridinone OH bending; BAL30072-complex: pyridine ring including stretching $\text{C}-\text{O}(-\text{M})$. ^e
2	1406	acetate: $-\text{COO}^-$ symmetric stretching; BAL30072: pyridinone OH bending; pyridinone CH in-plane bending.
3	1344	CH_3 - umbrella bending.
4	1100-900	acetate: $\text{C}-\text{C}-\text{O}$ asymmetric and symmetric stretching; BAL30072: unsaturated CH out-of-plane bending, $\text{C}-\text{O}$ bending.
5		
6		
7	660-600	acetate: $-\text{COO}^-$ bending.
8		
9	1777	BAL30072: β -lactam $\text{C}=\text{O}$ stretching.
10	1655	BAL30072: amide $\text{C}=\text{O}$ stretching; NH in-plane bend.
11	1292	BAL30072: phenol $\text{C}-\text{O}$ stretching; pyridinone $\text{C}-\text{N}$ stretching and other modes of pyridinone ring.
12	1264	BAL30072: pyridinone ring.
13	1175	BAL30072: sulfate modes.
14	1121	BAL30072: sulfate modes.
15	720	Found in many functional groups, unambiguous assignment not possible.

^aAssignments were obtained by comparison with data from the literature (Yamaji et al.; Ahuja et al. 1979; Smith 1999; Bruker Corporation 2012).

^bLabels refer to Figure S7.

^cIn the case of groups of bands the most intense one is reported.

^dEstimated at $\sim 1624\text{--}1626 \text{ cm}^{-1}$ (Yamaji et al.).

^e $\text{C}-\text{O}$ bonds with the O atom bound to Fe^{3+} .

Figure S8. Stick representations of the B3LYP/6-31G* optimized geometries of cis (**top**) and trans (**bottom**) isomers of the L_2M complex formed by BAL30072 with Fe^{3+} . The latter was found to be lower in energy by about 10 kcal mol⁻¹.

Figure S9. Stick representations of the B3LYP/6-31G* optimized geometries of the LM complexes formed by (A) pyochelin (with ethylene glycol), (B) enterobactin and (C) pyoverdine with Fe^{3+} . The L_3M complex formed by BAL30072 is shown in (D). The calculated minimal projection area and Van der Waals volume are reported in each panel. Donors are highlighted with red shaded rectangles.

Figure S10. Statistics of residues contacts for the 100 best poses of molecular docking shown in Figure 8 (main paper), together the calculated binding free energy in descending order: **(A)** free BAL30072, **(B)** bidentate LM complex, **(C)** scorpion-like LM complex, **(D)** bidentate L₂M complex, **(E)** L₃M complex and **(F)** scorpion-like L₂M complex.

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