Synthesis of novel Iron(III) chelators based on triaza macrocycle backbone and 1-hydroxy-2(H)-pyridin-2-one coordinating groups and their evaluation as antimicrobial agents

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Several novel chelators based on 1-hydroxy-2(1H)-pyridinone coordinating groups decorating a triaza macrocyclic backbone scaffold were synthesised as potential powerful Fe³⁺ chelators capable of competing with bacterial siderophores. In particular, a novel chloromethyl derivative of 1-hydroxy-2(1H)-pyridinone exploiting a novel protective group for this family of coordinating groups was developed. These are the first examples of hexadentate chelators based on 1-hydroxy-2(1H)-pyridinone to be shown to have a biostatic activity against a range of pathogenic bacteria. Their efficacy as biostatic agents was assessed revealing that minor variations in the structure of the chelator can affect efficacy profoundly. The minimal inhibitory concentrations of our best tested novel chelators approach or are comparable to those for 1,4,7-tris(3-hydroxy-6-methyl-2-pyridylmethyl)-1,4,7-triazacyclononane, the best Fe³⁺ chelator known to date. The retarding effect these chelators have on microbial growth suggests that they could have a potential application as a co-active alongside antibiotics in the fight against infections.

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1. Introduction

Bacterial resistance to once effective antibiotics has emerged as a major health threat of the 21st century [1,2]. There is therefore an urgent need to develop new strategies to combat the spread of multi-drug resistant infections. One of the potential options currently being studied is the use of biostatic agents (i.e. inhibitors of bacterial growth) that could work synergistically with existing antibiotics and boost their efficacy [3–7]. Metal chelators can be used to that effect as their biostatic activity upon microorganisms has long been known. Their mode of action is thought to be the imposition of metal starvation on the microorganisms [8–10].

The Fe³⁺/Fe²⁺ redox couple is able to catalyse a broad range of biological reactions [11] thus iron is an essential element ubiquitous to virtually all organisms, making it a desirable target for the prevention of microbial growth by chelation [12–15]. In the case of infections, the pathogen’s source of iron is known to be the hosts themselves [16–18]. Although iron is plentiful, from the microbial perspective there is limited bioavailability: pathogenic bacteria use efficient iron acquisition mechanisms, often based on small molecules called siderophores [19]. Siderophores are predominantly hexadentate ferric chelators; their Fe³⁺ affinities/binding strengths can be very high and thus they are able to acquire otherwise unavailable iron from sequestered host sources [20–24]. Achieving a biostatic effect by iron starvation therefore appears to depend upon a deceptively simple thermodynamic and kinetic competition in the binding of Fe³⁺ cations by the bacterial siderophores and the added chelator. Alongside the difficult design of chelators that can compete effectively with siderophores, great care must also be taken to avoid toxic demetallation of host metalloenzymes, making development of this technology a non-trivial matter.

To compete with strong siderophores, the right choice of coordinating groups is crucial. When considering only the thermodynamic competition between bacterial siderophores and an added chelator, one must primarily consider the respective pFe³⁺ values (defined as −log[Fe³⁺]free, usually calculated at pH 7.4, with [Chelator]total = 10 μM and [Fe³⁺]total = 1 μM and cited herein in these conditions) of the two chelators to estimate which is most likely to be potent in complexing the metal [25]. It would appear that three isomers of the hydroxypyridinone (HOPO) family (Fig. 1) possess the right combination of pKa and log(⟨Fe³⁺⟩) to give high pFe³⁺ values and therefore thermodynamically compete with siderophores (that are commonly based on coordinating groups such as α-hydroxycarboxylic acids, hydroxamic acids and catechols) [24,26].

These hydroxypyridinones also offer attractive additional prospects. Many bacteria can use siderophores elaborated by those of another species [18,27]; to be effective biostatic agents, synthetic chelators must not suffer from this “siderophore piracy” and actually promote bacterial
growth. Because very few HOPO are found in nature and only one has been described as a siderophore ligand (1-hydroxy-5-methoxy-6-methyl-2(1H)-pyridinone, also called cepabactin), the likelihood of the metallated chelators being recognised by bacterial receptors and used as a source of Fe\(^{3+}\) is expected to be small. Additionally, infected human hosts are known to use various Fe\(^{3+}\) withholding strategies to limit bacterial growth. One of these is based on the activity of siderocalin, a protein that in essence acts as a trap for some siderophores [28–31]. A therapeutic chelator must not interact with siderocalin and inhibits its protective action. It is known that some 1-hydroxy-2(1H)-pyridinone (1.2-HOPO) based chelators do not bind strongly to siderocalin, suggesting that this class of coordinating groups would complement rather than overwhelm this defence strategy [32].

Members of the 3-hydroxy-4(1H)-pyridinone (3.4-HOPO) sub-family have been extensively studied in chelation therapies [33]. A significant number of reports have also described growth inhibition of a range of pathogenic bacteria by bidentate or hexadentate chelators based upon 3.4-HOPO [34–43]. To achieve high pFe\(^{3+}\), hexadentate chelators are preferred to bidentate ones, especially since their mode of action as biostatic agents is expected to be extracellular and therefore do not suffer from size restriction to penetrate the microbes. A very small number of compounds belonging to the 1.2-HOPO sub-family were described as antimicrobial agents [44,45] but these are all bidentate chelators. No hexadentate member of that 1.2-HOPO sub-family has been described as antimicrobial agent. This absence of reports is surprising considering that a few hexadentate chelators based on 1.2-HOPO were described (for complexation of non-biologically relevant metals) whose structural features and chelation properties make them attractive candidates for this purpose (for example compounds 1 and 2, Fig. 2) [46–49]. Although slightly weaker coordinating groups than their 3-hydroxy-2(1H)-pyridinone (3.2-HOPO) and 3.4-HOPO isomers, 1.2-HOPO coordinating groups possess another advantage that makes them more attractive than their isomers. Their lower pKa values (typically 6 versus 8.5–10 depending on substitution [26]) make them charged molecules at physiological pH and therefore less likely to penetrate cells of the host. This could have beneficial safety benefits in treating systemic infections.

Although not based on HOPO, compound 3 has the highest known value of pFe\(^{3+}\) (see below) and is therefore expected to be able to compete favourably with siderophores for ferric cations at low concentration and therefore show high biostatic efficacy [50]. Despite its high pFe\(^{3+}\) value, to the best of our knowledge this compound has never been described as a biostatic agent. It was therefore considered necessary to synthesise, screen and use compound 3 as a benchmark.

Our interest in developing powerful Fe\(^{3+}\)-optimised chelators as biostatic agents prompted us to investigate a range of hexadentate chelators based on 1.2-HOPO and a triaza macrocyclic backbone scaffold to try to identify novel microbiostatic chelators. Very few polyaza macrocycles bearing HOPO coordinating groups have been described [48,51–54]. Macrocycles offer the potential to fine-tune the metal chelating properties by varying the cycle’s size and flexibility. Our aim was to combine key structural elements of siderophores, HOPO and 1,4,7-tris(3-hydroxy-6-methyl-2-pyridylmethyl)-1,4,7-triazacyclononane (TACN-MeHP, 3) into a novel series of chelators. In particular by analogy with enterobactin (Fig. 3, compound 4), it is speculated that the effect of the macrocyclic backbone scaffold could be beneficial to the efficacy of metal binding and therefore to the minimum inhibitory concentration (MIC) of the chelator. Moreover, no previous synthesis of hexadentate chelators where 1.2-HOPO moieties were linked to the molecular scaffold via a methylene bridge were ever reported, the focus having been on a carbonyl linker. The impact of that linker on metal chelation efficacy can be dramatic and therefore deserves in-depth study. Reported herein is the synthesis of novel triaza macrocyclic chelators bearing 1.2-HOPO moieties, linked via methylene or carbonyl groups and the study of their biostatic effect on a range of microorganisms, including a comparison with that of known compounds 1–3.

2. Results and discussion

2.1. Organic synthesis

Our chelator design has focused on thermodynamic (pFe\(^{3+}\)) rather than kinetic considerations. One of the most remarkable bacterial siderophores is enterobactin (4), whose scaffold is composed of a triserine macrocycle (Fig. 3), and has been extensively studied owing to its very powerful Fe\(^{3+}\) chelation (log\(\beta_{15}\) = 49 and pFe\(^{3+}\) = 34.3, where \(\beta_{15}\) is defined as the equilibrium constant for the reaction \(L + mM + nH^+ \rightleftharpoons L_mM_nH_n\) [25]) [55]. The origin of its efficient binding has been elucidated and shown to be influenced by the pre-organisation
of the coordinating groups, itself largely influenced by the cyclic structure of its scaffold and intramolecular hydrogen bonding [55–59]. Interestingly, bacillibactin (5), a siderophore analogue of enterobactin with glycine spacers linked to a tris-threonine macrocyclic scaffold also shows large logβ₁₁₀ and pFe³⁺ values (47.6, 33.1 respectively) [55]. However, synthetic analogue 6, also composed of a glycine spacer but of a tris-serine scaffold has poorer logβ₁₁₀ and pFe³⁺ values (44.1 and 29.6 respectively). It would appear that the nature of the spacer and the nature of the scaffold have a profound impact on pFe³⁺ that we hypothesise as having a key influence on growth inhibition.

Compound 3 has a Fe³⁺ coordination environment in complexes that is very different to that of enterobactin or HOPO-based hexadentate chelators. Contrary to the O₆ donor set of 1, 2, 4, the coordination of 3 on Fe³⁺ occurs through an N₃O₃ donor set involving the three nitrogen atoms of the 1,4,7-triazacyclononane (TACN) ring. As mentioned earlier, 3 has the highest known logβ₁₁₀ and the highest pFe³⁺ values of any known ligand (logβ₁₁₀ = 49.98, pFe³⁺ = 39.4) and therefore is also worthy of consideration as a potential template to improve metal chelating ability of the molecules [50]. Although Fe³⁺ coordination studies with hexadentate analogues with a larger triaza macrocyclic cores were not fully described, it would appear that the three nitrogen atoms are sensibly at the right distance to efficiently bind the metal atom.

Inspired by these observations, and wanting to study a range of chelators based on 1,2-HOPO and bearing structural similarities to compounds 1–3 we first synthesised compound 7 from TACN as depicted in Scheme 1.

The first step in the preparation of 7 was performed based upon an existing procedure by reacting known acyl chloride 8 with TACN in THF [60]. Protected chelator 9 was obtained in low yield (25%). Removal of the benzyl groups from 9 using a mixture of concentrated hydrochloric and glacial acetic acid yielded 7 in excellent yield (97%). An analogue of that compound with the larger triaza macrocycle (i.e. 1,5,9-triazacyclodecane, TACD) was also synthesised to allow for comparison of the effect of the ring size upon bacterial growth inhibition (Scheme 1). Reaction of acyl chloride 8 with TACD in DMF allowed for the isolation of the larger core protected molecule 11 in 49% yield. A similar acid deprotection gave 10 in good yield (71%).

It was anticipated that the amide linkage used in 7 and 10 would have a large impact on the conformational flexibility of the chelator [61] and also possibly an electronic effect on the 1,2-HOPO coordinating groups. That in turn could have an important impact (either beneficial or detrimental) on Fe³⁺ binding but the exact effect of these linkers is still unreported. Therefore, we also investigated the synthesis of an analogue of 7, replacing the carbonyl groups by methylene units. A novel HOPO protection strategy away from the benzyl protection traditionally used for 1,2-HOPO would be required. Indeed, it was predicted that benzyl protection would not be suitable as the most common methods used for its removal could also cleave the linkage between our HOPO group and the molecular backbone. Of the potential protective methods identified, protection of the N-hydroxyl group as the allyloxy group was undertaken. It was considered that deprotection can be afforded under a range of relatively mild conditions by double bond isomerisation and subsequent hydrolysis without compromising other bonds in the chelator [62]. The key allyl protected 6-hydroxymethyl intermediate 17 was synthesised in five steps from commercially available 12 (Scheme 2) [63]. This hydroxymethyl derivative could then be converted to the novel chloromethyl derivative 17 by thionyl chloride for incorporation onto ligand cores.
Oxidation to the expensive 1.2-HOPO-6-carboxylate 12 was performed using commercially available peroxycetic acid, a modification to the existing procedure of Xu et al. [60], resulting in a slightly increased yield (77%) without the use of also expensive trifluoroacetic acid. Treatment of the free acid 13 with thiomyl chloride in methanol, following the method of Burgada et al. [64], gave the methyl ester 14 in excellent yield (96%). Subsequent protection of the N-hydroxyl group, using allyl bromide and potassium carbonate, yielded the novel methyl ester 15 (94%). Reduction of the ester 15 to the methyl alcohol 16 was accomplished by slow addition of methanol to sodium borohydride in THF, remarkably without reduction of the allyl group [65,66]. Notably, most of these steps gave acceptable to excellent yields. Workup of the reaction mixtures were easy to perform and the products either needed no purification at all or were easy to purify.

Reaction of a stoichiometric quantity of 17 with TACN in the presence of potassium carbonate produced the allyl protected macrocyclic product 18 in a yield of 93% (Scheme 3). The removal of the allyl protective group was performed using boron trichloride, without cleavage of the newly formed C—N bond, to give compound 19 as the said analogue of 7. To the best of our knowledge, compound 19 is the first example of a 1,2-HOPO metal chelator anchored to its scaffold via a methylene link in position 6.

Finally, in order to discern any correlation of scaffold rigidity and biostatic effect, the acyl chloride intermediate 8 was also reacted with diethylenetriamine, a linear analogue of the cyclic TACN, to give chelator 20 after acid deprotection (Scheme 4).

Although chelators 1–3 were previously described in the literature they have not been evaluated as biostatic agents therefore their synthesis was also undertaken in order to assess their antimicrobial properties [48,49,67].

Chelator 19 was considered to be an interesting compound in several respects. One of them concerns the way in which it can chelate Fe³⁺. The typical mode of coordination of 1,2-HOPO-based hexadentate chelators on trivalent metals such as Fe³⁺ and Ga³⁺ is via the two oxygen donors of the carboxylate group, using allyl bromide and potassium carbonate produced the allyl protected macrocyclic product 18 (Scheme 3). The removal of the allyl protective group was performed using boron trichloride, without cleavage of the newly formed C—N bond, to give compound 19 as the said analogue of 7. To the best of our knowledge, compound 19 is the first example of a 1.2-HOPO metal chelator anchored to its scaffold via a methylene link in position 6.

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To further study the exact coordination mode of ligand 19, in particular by NMR, Ga³⁺ was used as a surrogate for Fe³⁺ [71]. No in-depth study by MS or NMR of 1,2-HOPO based complexes of Ga(III) exist in the literature. For example, only one derivative of 1,2-HOPO complex (based on the 1-oxo-2-hydroxy-isoquinoline-3-carboxylic acid) of Ga(III) has been characterised by NMR in CDCl₃ [72]. Critically, no study of the chelation by NMR or MS was performed in water, a solvent more relevant to our growth media and where protonation reactions can have a dramatic impact on the species formed [73]. To try and establish spectroscopic features that would allow us to distinguish an N₃O₃ from an O₆ coordination mode, we were forced to first investigate by MS and NMR the complexation of Ga(III) on a model system. Compound 22 that we described for another type of application has been selected as such a model of ligand 19 [63].

It was judged that compound 22 in the presence of Ga(III) would form a 3:1 complex via the six oxygen atoms of three 1,2-HOPO bidentate ligands (Fig. 5). Therefore, compound 23 would be a good model of the coordination environment around Ga(III) if ligand 19 coordinated Ga(III) in an O₆ mode (Fig. 4A) and by extension, would give us some information on the way Fe³⁺ is chelated by 19.

Proton and carbon NMR were first performed on solutions of 22 and 19 in D₂O. To these solutions, a stoichiometric amount of Ga(acac)₃ (acac = acetylacetonate) was then added and the solutions incubated at room temperature for 24 h. The 1H and 13C NMR of the resulting complexes were then recorded. Also, these solutions, after dilution with methanol (non-deuterated), were analysed by MS.

Starting with MS, 22 + Ga(acac)₃ (3:1 molar ratio) gave major peaks that match (m/z values and isotopic distribution) the formula [22 – 3H]/[Ga + Na⁺] at m/z 511.93 (expected 512.02), 512.93 (expected 513.02), 513.93 (expected 514.02), 515.00 (expected 515.02) and 516.00 (expected 516.02) (Fig. S1), confirming the displacement of the three acac ligands and strongly suggesting the formation of the 3:1 complex 23 as expected.
Ga(III). Ascribed to rapid exchange between the different from that of the parent ligand and that of the analogous to the three C

\[ \Delta \text{ppm} = 3.3 \] 

Interestingly, the most impacted signals correspond assigned to the carbon atom of the carbonyl is also shifted, up

\[ \Delta \text{ppm} = 0.6 \] 

These differences could sug-

gest a different coordination mode. The presence of only one set of signals for the TACN scaffold are much more impacted by the presence of the metal. Instead of well-defined singlet in the ligand, various broad signals are seen between 3.1 and 4.1 ppm. Appearance of broad signals for the TACN's methylene has been observed at elevated temperature [contrary to the Ga(III)/NOKA complex where the signals remained as well defined multiplets even at 85 °C] [70]. This has been ascribed to rapid exchange between the \( \Delta \) and \( \Lambda \) isomers. The broad signals observed in our complex suggest that this rapid exchange exist even at room temperature.

In the \( ^{13} \text{C} \) spectrum of 22 with and without Ga(acac)\(_3\), the signal assigned to the carbon atom of the carbonyl is also shifted, upfield this time by 3.3 ppm. Interestingly, the most impacted signals correspond to the three C—H groups. The 13C NMR of 19 + Ga(acac)\(_3\) is drastically different from that of the parent ligand and that of the analogous 23. Again, focusing on the signals for the TACN's methylene group, several signals are now seen, confirming the distorted nature of the scaffold. The signals associated with the carbon atoms in the 1,2-HOPO groups are also drastically shifted upon complexation. For example, one signal was found at 195.4 ppm corresponding to a quaternary carbon atom that did not appear in the spectrum of the free ligand. The differences between the 1H and 13C NMR of 19 and 22 with and without Ga(III) suggest that the two ligands provide a different coordination mode. Because 22 is expected to provide an \( \text{O}_6 \) coordination environment, we tentatively suggest that an \( \text{N}_3\text{O}_3 \) coordination mode exists for ligand 19 + Ga(III) and therefore by extension to 19 + Fe(III) but further work will be necessary to draw a firmer conclusion.

2.2. Biostatic activity

We selected a small panel of diverse microbes to assess the efficacy of these chelators; these included bacteria from both Gram negative and Gram positive lineages as well as the pathogenic dimorphic fungus *Candida albicans*. Some species were selected for their implication in healthcare-associated infections, where possible strains previously used in the assessment of iron chelators or pharmaceuticals were used. *Bacillus subtilis* strain DSM-23,778 lacks a key phosphopantetheinyl transferase activity [74] required for siderophore biosynthesis and generates the bidentate itoic acid rather than the hexadentate bacillibactin [75]. Both microdilution tests were undertaken based upon a standard procedure [76]. The results obtained are shown in Table 1. The commercially available \( \text{N,N,N',N'',N'''}-\text{diethylenetriaminepentaaacetic acid (DTPA)} \) was also assayed for comparison purposes.

Across the range of microorganisms tested, it would appear that compounds 1 and 3 are systematically the best inhibitors of microbial growth (supplementary material, Table S). Compound 19 also appears to be one of the better chelators but it failed to equal the former

**Fig. 4.** Possible coordination modes for compound 19 with trivalent metal M (e.g. Fe\(^{3+}\), Ga\(^{3+}\)).

**Fig. 5.** 6-hydroxymethyl-1-hydroxy-2(1H)-pyridinone (22) and its expected 3:1 complex with Ga(III) (23).
two. On the contrary, compounds 2, 7 and 20 appear to be the worst performing chelators of this series. Interestingly, compound 10 performs poorly against E. coli but very well against C. albicans. If the efficacy of the chelators was simply linked to the thermodynamics of their Fe$^{3+}$ chelation (e.g. pFe$^{3+}$), one could expect to observe the same rank ordering across the microorganism panel, the interspecies variations in MIC being indicative of the capacity of each microbe to deal with the finite competitive challenge defined by the chelator dose. The fact that 1, 3, 19 appear to be the most effective inhibitors and 2, 7 and 20 the poorest but that the efficacy of 10 appears variable suggests that strength of metal chelation, if dominant, is not the only factor that may have a biostatic effect on that microorganism or that our chelator also interferes with the thermodynamics and kinetics of their metal binding.

As the siderophores produced by each of these bacterial strains have not all been formally identified and/or their Fe$^{3+}$-binding efficacy measured, it is not yet possible to determine with a high level of confidence whether the bacteria most resistant to external chelators are systematically the ones that produce the strongest siderophores (highest pFe$^{3+}$). Also, for each siderophore, its pFe$^{3+}$ is influenced by its total concentration and these can vary over time and as a function of the ability of the microorganism to excrete them in large quantity.

The poor range of hexadentate chelators based on 1,2-HOPO described in the literature needs to be addressed if these are to be seriously considered in therapeutic applications. Consideration of the high efficacy of chelator 19, where the HOPO group is linked to the TACN core via a methylene group, and the poor efficacy of 7, where the linker is a carboxyl, suggests that optimisation of the linker will be critical. Comparison of chelators 1, 7 and 19 suggest that the use of a more rigid macrocyclic scaffold compared to the tris(2-aminoethyl)amine core (to promote chelation via entropic effects) was not successful in improving biological activity.

All the HOPO-based chelators tested herein were designed with the goal of optimising Fe$^{3+}$ chelation. This does not mean that trapping of other essential metals is not concomitantly achieved that leads to the biostatic effect. The amount of key metals in the growth medium was measured and found to be 65.7 μM for iron, 31.5 μM for zinc, 13.7 μM for manganese, 8.3 μM for cobalt and 4.5 μM for copper. For all the microorganisms tested except C. albicans, the MIC is above the concentration of iron, as would be expected if our hypothesised mechanism of action is valid. However, the MIC observed on C. albicans of 39 μg/mL for 3, 10 and 19 suggests either that partial chelation of iron is enough to have a biostatic effect on that microorganism or that our chelator also targets other metals that have a much more dramatic effect on its growth or that it affects the organism in a manner not related to metal limitation.

Finally, the effect of added chelators on the availability of other biologically important metals must also be taken into consideration as discussed above. It is therefore unwise to try and rationalise the data presented herein based on relative pFe$^{3+}$ values. Progress towards an understanding of their mode of action however can be made by considering the siderophores that are known to be produced by the tested organisms. Further work is in progress to investigate in depth the mechanism of action of these chelators and to correlate their activity to the thermodynamics and kinetics of their metal binding.
The impact of sub-MIC concentrations on the growth of the microorganisms was followed by optical density (Fig. 6).

As expected each microorganism in the absence of the added chelator, shows a lag phase before entering exponential growth. In the presence of the added chelator, the lag phase appears longer in some cases (e.g. *K. pneumoniae* + 3 at MIC/4) but is not substantially increased in the other cases tested, although a decrease in growth rate is evident. In all the tested cases and by definition, the lag phase was over 24 h when the chelator was used at the MIC value derived from the previous assays. What is apparent for all tested combination is that the rate of growth is significantly reduced with increasing concentrations of chelators. These growth profiles are consistent with the expected mode of action of the chelators. The microorganisms that suffer from the biostatic effect of a metal chelator find themselves in iron deprivation conditions due to the presence of the said chelator. They therefore have to produce a large amount of siderophore to compete and only when the chelator’s pFe$^{3+}$ is overcome by the siderophore’s pFe$^{3+}$ value will any growth be initiated. The higher the amount of chelator, the longer it takes the microorganisms to overcome the effect of the biostatic agent. This is consistent with the hypothesis the compounds operate as an extracellular chelator.

3. Conclusion

We report the synthesis of a range of hexadentate chelators based on triaza macrocycles, including the first report of the use of a methylene as a linker between a 1.2-HOPO coordinating group and the molecular scaffold. The ligands have a demonstrable biostatic effect upon the growth of a range of microbes. It is suggested that metal chelation is the main mode of action of these chelators but that a simple thermodynamic competition between the chelator and bacterial siderophore for Fe$^{3+}$ is too simple a picture. Further work is in progress to understand the mode of action of these chelators in detail and to synthesise a wider range of hexadentate chelators based on 1.2-HOPO to assess their efficacy as biostatic agents. These compounds will also form the basis for the study of the effect of the linker between the 1.2-HOPO moiety and the molecular backbone on the thermodynamic efficacy of Fe$^{3+}$ chelation via measurements of pK$_{a}$ and $|eta|_{110}$ values.

4. Experimental

4.1. Preparation of stock solutions

Glassware was rinsed with a deionised aqueous solution of EDTA (0.1 M) then rinsed thoroughly with deionised water (18 mΩ) before ligands were dissolved in deionised water to the desired concentration (5 mM; 5 mL). The stock solution was then passed through a membrane filter (0.22 μM) into a sterile bijoux tube (7 mL) and stored at 4 °C until required.

4.2. Bacterial strains

All strains were purchased from DSMZ. *E. coli* DSM-18,039, *K. pneumoniae* DSM-30,104, *P. aeruginosa* DSM-19,880 and *S. aureus* DSM-1104 were cultured onto brain heart infusion (BHI) agar and incubated at 37 °C for 24 h. Similar procedures were conducted for strains of *A. baumannii* DSM-30,007 and *C. albicans* DSM-1386 incubated at 30 °C and *B. subtilis* incubated at 25 °C, all for 48 h. The cultured plates were then stored at 4 °C until needed.

4.3. Antimicrobial assay

The assay conducted was based upon a similar literature procedure [76]. Stock solutions of ligands (5000 μM) were added to the first wells of a 96 well-microtitre plate (200 μL) and sterile BHI broth (100 μL) was added to the remaining wells in the row. Ligand solution from the first well (100 μL) was added to the next well in the row and mixed. The procedure was then repeated along the row from the dilute solutions and discarded after the penultimate well. Inoculum (10$^{5}$ CFU/mL; 100 μL) was then added to all wells and the plate incubated without agitation at 37 °C. Readings were taken after 24 and 48 h, depending upon the microorganism, and the MIC determined on the basis of visual turbidity of the well. Assays were performed in triplicate.
4.4. Organic synthesis

All solvents and reagents were purchased from Sigma-Aldrich, Acros Organics or Alfa-Aesar and used without further purification unless otherwise specified. Reactions were followed by TLC using silica gel with UV254 fluorescent indicator and column chromatography was conducted using 0.060–0.20 mm silica gel (70–230 mesh), where automated flash column chromatography was conducted using a Biotage Isola One ISOLV. Hydrogenations performed using an H-cube® continuous flow hydrogen generator was operated as specified.

4.5. Physical measurements

Melting points were taken on a SRS DigiMelt MPA161 digital melting point apparatus with samples prepared in SAMCO soda glass capillary tubes 100 mm. NMR spectra were recorded using a JEOL JNM Ecs270 instrument at 270 MHz and 68 MHz or a JEOL JN-NCS400 instrument at 400 MHz and 100 MHz, as specified, for 1H and 13C NMR respectively, and are reported in ppm (δ). Infrared spectra were obtained using Durasecond diamond ATR system on a Perkin Elmer RX1 FTIR spectrometer. Positive and negative electrospray ionisation mass spectrometry (ESI-MS) was conducted using a Thermo LCQ Advantage mass spectrometer by direct injection. High resolution mass spectrometry were conducted using a Varian Cary 50 UV–vis spectrophotometer (range 200–1100 nm) using silica gel (70–230 mesh), where automated flash chromatography was conducted using a Biotage Isolera-One 250. Hydrogenations performed using an H-cube® continuous flow hydrogen generator was operated as specified.

4.5.4 4-Hydroxymethyl)-1-(allyloxy)pyridin-2(1H)-one (16)

To a suspension of compound 15 (19.12 g, 92 mmol) in THF (200 mL) was added solid sodium borohydride (25.07 g, 663 mmol) in small portions. The solution was heated under reflux for 15 min. Methanol (14 mL) was then added dropwise at reflux over 2 h. The solution was then cooled to 0 °C, quenched by careful addition of saturated aqueous ammonium chloride (25 mL) and stirring was continued for 15 min. The solvents were removed in vacuo and the residue was extracted with dichloromethane (3 × 25 mL). The combined organic extracts were dried and evaporated to afford the title compound 16 as an off-white solid (10.83 g, 65%). Mp 101–104 °C (from DCM). Found: C, 57.06; H, 5.38; N, 6.67%; C10H11NO4 requires 57.41; H, 5.30; N, 6.70%.

54.5.3 Methyl 6-oxo-1-(allyloxy)-1,6-dihydropyridine-2-carboxylate (15)

To a solution of compound 14 (16.47 g, 97 mmol) in acetonitrile (200 mL) was added potassium carbonate (32.11 g, 232 mmol), followed by allyl bromide (28.10 g, 232 mmol). The flask was heated under reflux for 4 h before the reaction mixture was filtered and the solvent removed under high vacuum. The residue was dissolved in toluene (100 mL) and the solvent was evaporated in vacuo to afford the title compound 15 as a white crystalline solid (19.12 g, 94%). Mp 65–67 °C (from toluene). Found: C, 75.06; H, 5.38; N, 6.67%; C10H11NO4 requires C, 75.41; H, 5.30; N, 6.70%. v\textsuperscript{max}(neat)/cm\textsuperscript{-1} 3462, 3078, 2953, 1735 (CO), 1586 (CC), 1445, 1249, 1136. δ\textsubscript{H}(399.8 MHz, DMSO-d\textsubscript{6}) 3.94 (3H, s, CO\textsubscript{2}H), 4.90 (2H, d, J 6.9, CH\textsubscript{2}CH\textsubscript{2}), 5.42 (2H, m, CH\textsubscript{2}CH\textsubscript{2}), 6.08 (1H, m, CH\textsubscript{2}CH\textsubscript{2}), 6.53 (1H, dd, J 6.9, 1.8, 3-H), 6.80 (1H, dd, J 9.2, 1.8, 5-H), 7.31 (1H, dd, J 9.6, 6.9, 4-H). δ\textsubscript{C}(100.5 MHz, DMSO-d\textsubscript{6}) 53.3 (CO\textsubscript{2}CH\textsubscript{3}), 105.8 (ArC), 122.6 (ArC), 137.8 (ArC), 138.8 (quat), 158.1 (quat), 161.4 (quat).

54.5.5 N,N'-N'-tris-(1-benzyloxy-6-(1H)-pyridinone-2-carbonyl)-1,4,7-triazacyclononane (9)

Following a reported procedure [60] a solution of TACN (0.21 g; 1.6 mmol) and methyl 6-oxo-1-(allyloxy)-1,6-dihydropyridine-2-carboxylate (14) [64] to a solution of acid 13 (15.73 g, 101 mmol) in methanol (200 mL) at 0 °C was added triethylamine (39.89 mmol; 50 mL) and the solution was heated under reflux for 4 h. The solution was then allowed to cool to room temperature and the solvent was removed in vacuo to afford the title compound 14 as a cream solid (16.47 g, 96%). Mp 164–170 °C. δ\textsubscript{H}(400 MHz; CDCl\textsubscript{3}, Me\textsubscript{Si}) 2.07–4.26 (12H, mm) 8.40 (1H, d, J 8.2 Hz) 4.81 (1H, d, J 8.7 Hz) 5.04 (1H, m) 5.49–6.06 (5H, mm) 6.68–6.89 (7H, mm) 7.28–7.60 (16H, m), δ\textsubscript{C}(100 MHz) 47.9, 48.8, 49.2, 49.8, 77.1, 79.2, 79.7, 80.0, 102.7, 103.3, 123.4, 123.5, 123.6, 128.6, 128.7, 128.9, 129.1, 129.4, 130.1, 130.6, 131.0, 131.3, 132.4, 133.1, 133.8, 137.9, 138.3, 138.4, 140.8, 144.5, 145.1, 159.7, 158.9, 162.9, 163.0, 163.2: (+) ESIMS: m/z 833.01 (M + Na\textsuperscript{+}); NESI: requires m/z 811.3092, found 811.3086 (M + H\textsuperscript{+}).
9.2 Hz); 180 °C (decomp.).

\[ \text{m} \text{s} \] 3.72 (6H, s) 4.79 (6H, d,

\[ \text{H} \] (400 MHz; \[\text{D}_6\]-DMSO; Me4Si) 3.03–3.82 (12H, m) 6.12–6.35 (2H, m) 6.37–6.63 (4H, m) 7.11 & 7.23 (1H, m) 7.29–7.48 (4H, m); \( \eta \) (100 MHz) 48.4–49.6 (m), 103.1 (m), 121.0, 138.4 (m), 141.7, 157.8–158.0, 163.3; (+)-ESI-MS: m/z 541.13 (M + H⁺) 563.10 (M + Na⁺); NESI: requires; m/z 563.1502, 541.1683, found; 563.1490 (M + Na⁺) 541.1672 (M⁺).

4.5.8. \( \text{N,N,N}^\prime,\text{N}^\prime\prime\)-tris(1-allyloxy-6(1H)-pyridinone-2-carbonyl)-1,4,7-triazacyclonane (18)

A mixture of 17 (0.448 g, 2.24 mmol), potassium carbonate (0.41 g, 3.0 mmol) and TACN (0.95 g, 0.74 mmol) in acetonitrile (20 mL) was refluxed overnight. The mixture was then added to water (50 mL) before extraction with dichloromethane (3 × 50 mL). The organic extracts were washed with brine (50 mL) before being dried with magnesium sulfate and the solvent removed in vacuo to yield a dark orange highly viscous liquid (0.431 g, 93%). \( \eta \) (400 MHz; CDCl3; MeSi) 2.87 (12H, s) 3.72 (6H, s) 4.79 (6H, d, J 6.4 Hz) 5.38 (6H, m) 6.05 (3H, m) 6.15 (3H, dd, J 1.4, 6.9 Hz) 6.56 (3H, dd, J 1.4, 9.2 Hz) 7.24 (3H, dd, J 6.9, 9.2 Hz); \( \eta \) (100 MHz) 55.9, 57.1, 76.7, 104.8, 120.4, 122.1, 130.5, 137.9, 147.7, 159.7; (+)-ESI-MS: m/z 619.05 (M + H⁺); NESI: requires; m/z 619.3244, found; 619.3243 (M + H⁺).

4.5.9. \( \text{N,N,N}^\prime,\text{N}^\prime\prime\)-tris(1-hydroxy-6(1H)-pyridinone-2-carbonyl)-1,4,7-triazacyclonane. 3 HCl (19)

Under an atmosphere of nitrogen, a solution of 18 (0.759 g, 1.2 mmol) in dry dichloromethane (8 mL) at 0 °C was added boron trichloride in hexane (1.0 M; 8.6 mL, 8.6 mmol) and vigorously stirred overnight before the mixture was added methanol (8 mL) and stirred for 30 min further. The solution was then evaporated in vacuo and re-evaporated with methanol (10 mL) 5 times, yielding the crude as brown flakes. Dissolution in a minimum volume of methanol and precipitation using diethyl ether yielded a cream solid following collection by Büchner filtration and washing with diethyl ether (0.534 g, 87%). mp 180 °C (decomp.). \( \eta \) (400 MHz; CDCl3; MeSi) 3.01 (12H, s) 4.18 (6H, s) 6.34 (3H, dd, J 1.4, 6.9 Hz) 6.54 (3H, dd, J 1.4, 9.2 Hz) 7.39 (3H, dd, J 6.9, 9.2 Hz); \( \eta \) (100 MHz) 49.3, 53.1, 106.8, 118.5, 137.5, 141.9, 158.5.

4.5.10. \( \text{N,N,N}^\prime,\text{N}^\prime\prime\)-tris(1-benzoyloxy-6(1H)-pyridinone-2-carbonyl)-1,5,9-triazacyclodecane (21)

Product was prepared based upon a similar literature procedure [60]. A mixture of 8 (1.29 g, 4.9 mmol) and triethylamine (1.35 g, 13.3 mmol) in dry tetrahydrofuran (20 mL) was added diethylamine (0.15 g, 1.5 mmol) and stirred in a stopped flask overnight at 60 °C. The mixture was then concentrated to dryness in vacuo and the residue dissolved in a mixture of water (100 mL) and dichloromethane (100 mL). Separation of the phases and washing of the organic layer with aqueous sodium hydroxide (1 M; 100 mL), aqueous hydrochloric acid (1 M; 100 mL) then brine (100 mL) before drying over magnesium sulfate, filtration and solvent removal in vacuo yielded the crude residue which was purified by automated flash chromatography (dichloromethane: methanol, 1–11%) yielding the product as a hygroscopic solid (1.04 g, 91%); mp 84–89 °C; \( \eta \) (400 MHz; CDCl3; MeSi) 3.04–3.28 (6H, m) 3.48 (1H, d, J 4.1 Hz) 3.84 (1H, m) 4.94 (1H, d, J 7.8 Hz) 5.21 (1H, d, J 8.7 Hz) 5.29–5.40 (4H, m) 5.63 (1H, d, J 7.8 Hz) 6.03 (1H, dd, J 1.8, 6.9 Hz) 6.14 (1H, dd, J 1.4, 6.9 Hz) 6.19 (1H, dd, J 1.4, 9.2 Hz) 6.60 (1H, dd, J 1.4, 9.2 Hz) 6.65 (1H, t, J 5.5 Hz) 6.71 (1H, dd, J 1.4, 9.2 Hz) 7.17 (1H, dd, J 6.4, 9.2 Hz) 7.22 (1H, dd, J 6.4, 9.2 Hz) 7.25 (1H, dd, J 6.4, 9.2 Hz) 7.30–7.33 (10H, m) 7.41–7.44 (4H, m) 7.49–7.52 (2H, m); \( \eta \) (100 MHz) 38.2, 45.8, 48.5, 79.2, 79.3, 79.4, 103.1, 105.6, 105.9, 123.3, 124.3, 124.7, 128.7, 128.7, 129.5, 129.6, 129.7, 130.1, 130.4, 130.5, 133.2, 133.3, 137.9, 137.9, 138.4, 141.9, 142.0, 145.6, 158.0, 160.7, 161.0, 163.0; (+)-ESI-MS: m/z 807.02 (M + Na⁺); NESI: requires; m/z 785.3093, found; 785.3272 (M + H⁺).

4.5.13. \( \text{N,N,N}^\prime,\text{N}^\prime\prime\)-tris(1-hydroxy-6(1H)-pyridinone-2-carbonyl)-bis(2-aminoethyl)amine (20)

A solution of 21 (0.35 g, 0.46 mmol) in a mixture of concentrated hydrochloric acid and glacial acetic acid (1: 1; 12 mL) was stirred at room temperature for 4 days before solvent removal in vacuo. The crude solid was then dissolved in a minimum volume of methanol and precipitated.
by addition of diethyl ether, yielding an off-white powder collected by Büchner filtration ([0.16 g, 70%], δs (400 MHz) [D$_2$]-DMSO: Me$_2$Si 3.37 (4H, s, br) 3.48 (4H, s, br) 6.28 (3H, m) 6.56 (2H, m) 7.33–7.39 (3H, m) 8.85 (1H, t, br) 8.93 (1H, t, br) δc (100 MHz) 37.0, 37.8, 44.5, 47.8, 102.9, 104.3, 104.6, 119.8, 120.1, 137.7, 137.9, 138.3, 142.0, 142.3, 142.6, 157.9, 158.0, 160.9, 161.1, 162.4, 162.9, 172.6; (+)-ESI-MS: m/z 537.0 (M + Na$^+$); HRMS NSE N: requires; m/z 513.1370, found; m/z 513.1376 (M + H$^+$).

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Appendix A. Supplementary data

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References