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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis, physico-chemical properties, and antimicrobial evaluation of a new series of iron(III) hexadentate chelators

Yuan-Yuan Xie · Mu-Song Liu · Pan-Pan Hu · Xiao-Le Kong · Di-Hong Qiu · Ji-Lin Xu · Robert C. Hider · Tao Zhou

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Abstract A series of related 3-hydroxypyridin-4-one hexadentate ligands have been synthesized. These chelators were found to possess a high affinity for iron(III), with a pFe value of about 30. As iron is a critical element to the survival of bacteria, these chelators were predicted to inhibit the growth of bacteria by disrupting bacterial iron absorption. Indeed, they were demonstrated to possess appreciable inhibitory activity against both Gram-positive and Gram-negative bacteria, and therefore, they have potential as antimicrobial agents. **1c** and **1g** were found to be particularly effective against Gram-negative species.

Keywords Iron chelator · 3-Hydroxypyridin-4-one · Hexadentate · Antimicrobial activity

Y.-Y. Xie · M.-S. Liu
College of Pharmaceutical Sciences,
Zhejiang University of Technology,
Hangzhou 310014, People's Republic of China

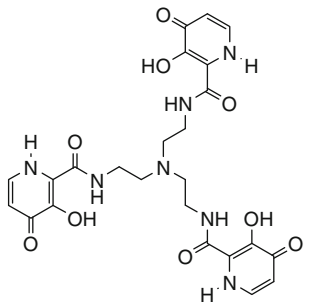
P.-P. Hu · T. Zhou (✉)
School of Food Science and Biotechnology,
Zhejiang Gongshang University, Hangzhou,
Zhejiang 310035, People's Republic of China
e-mail: taozhou@zjgsu.edu.cn

X.-L. Kong · R. C. Hider
Division of Pharmaceutical Science,
King's College London, Franklin–Wilkins Building,
150 Stamford Street, London SE1 9NH, UK

D.-H. Qiu · J.-L. Xu
Faculty of Life Science and Biotechnology,
Ningbo University, Ningbo, Zhejiang 315211,
People's Republic of China

Introduction

Bacterial and fungal resistance to antimicrobial agents is a growing problem worldwide (Nikaido and Zgurskaya, 1999; Mitscher *et al.*, 1999; Hogan and Kolter, 2002). Consequently, there is an urgent need for the development of novel types of antimicrobial agents targeting unique mechanisms and pathways. Iron is an essential element for the growth of virtually all bacteria and fungi. Thus, limiting the iron absorption of microorganism should in principle be an effective strategy to inhibit microbial growth. Most microorganisms have developed efficient methods of absorbing iron from the environment and many microorganisms secrete siderophores to scavenge iron (Lewin, 1984; Hider and Kong, 2010). In principle, such uptake can be interrupted by the introduction of high affinity iron selective chelating agents (Lowe and Phillips, 1962; Bergan *et al.*, 2001). The iron affinity of these agents must be extraordinally high, so that they can efficiently outcompete siderophores. Furthermore, the structure of chelators should differ appreciably from those of siderophores, otherwise the iron-chelator complex will be able to supply iron to the microorganism via the iron-siderophore transporter. Thus, iron(III)-selective hexadentate chelators with a high affinity for iron are predicted to inhibit the growth of a wide range of bacteria. We have previously demonstrated that compound **1a** (Fig. 1) and other two compounds, which have the structure of a hexadentate 3-hydroxypyridin-4-one, inhibit the growth of both Gram-positive and Gram-negative bacteria (Qiu *et al.*, 2011; Xu *et al.*, 2011). Further more, it has also been demonstrated that the iron complex of **1a** cannot be utilized by a range of bacteria, including *Staphylococcus aureus* and *Escherichia coli* (Zhou *et al.*, 2011). However, some chelators with high iron-binding constants, for instance, ethylenediamine-*N*,



1a

Fig. 1 Structure of hexadentate hydroxypyridinone **1a**

N′-bis(2-hydroxyphenyl) acetic acid (EDHPA, logK 34), *N,N′-bis*(2-hydroxybenzyl) ethylenediamine-*N,N′*-diacetic acid (HEBD, logK 40), and desferrioxamine (logK 30.4) (Anderson, 1980) have been reported to only possess a weak ability to inhibit Gram-positive and Gram-negative bacterial growth (Chew *et al.*, 1985), indicating that iron affinity of the chelator is not the only factor which affects the antimicrobial potential of an iron chelator. In an attempt to investigate the influence of hydrophobicity on the antimicrobial efficacy of hexadentate hydroxypyridinones, we synthesized a range of **1a** analog possessing different oil–water partition coefficients.

Experimental section

General procedures

All chemicals were of AR grade and used without any further purification. Melting points were determined using an SGW X-4A Digital Melting Point Apparatus and were uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance 400 or a Bruker Avance 500 spectrometer with TMS as an internal standard. Electrospray ionization (ESI) mass spectra were obtained by infusing samples into an LCQ Deca XP ion trap instrument. High resolution mass spectra (HRMS) were determined on Waters QTOF micro.

3-(Benzyloxy)-2-methyl-4H-pyran-4-one (**3**)

To a solution of **2** (10 g, 79.4 mmol) in methanol (150 mL) was added 35 % sodium hydroxide (10 mL) dropwise with stirring vigorous at 80 °C. Benzyl chloride was then added dropwise to the above mixture with stirring over 30 min and refluxed for 8 h. After cooled and filtered, the filtrate was concentrated on a rotatory evaporator to remove most of the organic solvent, and extracted with dichloromethane (3 × 50 mL). The combined organic extracts were washed with 5 % sodium hydroxide (2 × 50 mL) and brine, dried over anhydrous sodium sulfate. After removal of the solvent, the residue was purified by recrystallization from diethyl ether to obtain the product as a white solid (14.75 g, 68.12 mmol). Yield: 85.8 %; ^{1}H NMR ($CDCl_3$) δ 2.08 (s, 3H, CH_3), 5.15 (s, 2H, $ArCH_2$), 6.35 (d, $J = 5.6$ Hz, 1H, Pyridinone C5-H), 7.31–7.39 (m, 5H, Ar), 7.58 (d, $J = 5.6$ Hz, 1H, Pyridinone C6-H). ESI–MS *m/z* 217 ($[M + H]^+$).

3-(Benzyloxy)-4-oxo-4H-pyran-2-carbadehyde (**4**)

A mixture of **3** (5 g, 23.15 mmol) and SeO_2 (7.1 g, 69.4 mmol) in bromobenzene (80 mL) was stirred vigorously at 159 °C for 14 h. The reaction mixture was cooled to room temperature and filtered, the filtrate was concentrated under vacuum. The residue was purified by chromatography (SiO_2; EtOAc/hexane 1:1) to give the product as a brown oil (4.15 g, 18.06 mmol). Yield: 78 %; ^{1}H NMR ($CDCl_3$) δ 5.46 (s, 2H, $ArCH_2$), 6.47 (d, $J = 5.6$ Hz, 1H, Pyridinone C5-H), 7.32 (m, 5H, Ar), 7.72 (d, $J = 5.6$ Hz, 1H, Pyridinone C6-H), 9.83 (s, 1H, CHO), and ESI–MS *m/z* 231 ($[M + H]^+$).

3-(Benzyloxy)-4-oxo-4H-pyran-2-carboxylic acid (**5**)

To a mixture of **4** (4.60 g, 20 mmol) in acetone and water (1:1) was added sodium hypochlorite (2.18 g, 24 mmol) and sulfanic acid (2.92 g, 30 mmol) with vigorous stirring. The stirring was continued at room temperature for 2 h. The precipitate was collected by filtration, washed with acetone and dried in vacuo to give product **5** as a white solid (4.77 g, 19.4 mmol). Yield: 97 %; m.p. 150.1–151.8 °C. ^{1}H NMR (DMSO-d_6) δ 5.10 (s, 2H, CH_2), 6.53 (d, $J = 5.6$ Hz, 1H, Pyridinone C5-H), 7.44–7.41 (m, 5H, Ar), 8.19 (d, $J = 5.6$ Hz, 1H, Pyridinone C6-H). ESI–MS *m/z* 247 ($[M + H]^+$).

N,N′,N″-(2,2′,2″-nitrilotris(ethane-2,1-diyl) tris(3-methyl-4-oxo-4H-pyran-2-carboxamide) (**7**)

The mixture of compound **5** (4 g, 16.26 mmol), *N*-hydroxysuccinmide (NHS, 1.87 g, 16.26 mmol) in THF (70 mL) was stirred at room temperature for 0.5 h, *N,N′*-dicyclohexylcarbodiimide (DCC, 2.35 g, 16.26 mmol) was then added. The stirring was continued for 3 h, N^1,N^1-*bis*(2-aminoethyl)ethane-1,2-diamine (0.66 g, 4.52 mmol) in THF (10 mL) was added dropwise. The reaction mixture was stirred at room temperature overnight, and the formed precipitate was filtered off and washed with THF (20 mL). The filtrate was concentrated under vacuum to give a yellow oil which was then dissolved in CH_2Cl_2 (100 mL), washed with 0.5 % sodium hydroxide (2 × 40 mL) and brine successively, dried over anhydrous sodium sulfate, purified by chromatography (SiO_2, CH_2Cl_2/CH_3OH, 10:1), giving the product **7** as a pale yellow powder (2.17 g, 2.62 mmol). Yield: 58 %. ^{1}H NMR ($CDCl_3$) δ 2.29 (t, $J = 6.0$ Hz, 6H, CH_2), 3.11 (t, $J = 6.0$ Hz, 6H,

CH_2), 5.34 (s, CH_2, 6H), 6.47 (d, $J = 5.6$ Hz, 3H, Pyridinone C5-H), 7.35–7.33 (m, 15H, Ph), 7.69 (s, 3H, NH), and 7.82 (d, $J = 5.6$ Hz, 3H, Pyridinone C6-H). ESI–MS *m/z* 831 ($[M + H]^+$).

General procedure for the synthesis of N,N′, N″-(2,2′,2″-nitrilotris(ethane-2,1,diyl)tris(3-(benzyloxy)-4-oxo-4H-pyran-2-carboxamide) derivatives (**8a**–**i**)

A mixture of compound **7** (0.50 g, 0.602 mmol) and various amines (2.166 mmol) in ethanol was refluxed for 3 h. After removal of the solvent, the residual brown oil was purified by chromatography (SiO_2) using CH_2Cl_2/CH_3OH as an eluent to provide product **8**.

*N,N′,N″-(2,2′,2″-nitrilotris(ethane-2,1-diyl)tris (3-(benzyloxy)-4-oxo-1,4-dihydropyridine-2-carboxamide) (**8a**)*

Yield: 57 %. ^{1}H NMR ($CDCl_3$) δ 2.27 (t, $J = 6.0$ Hz, 6H, CH_2), 3.06 (q, $J = 6.0$ Hz, 6H, CH_2), 5.32 (s, 6H, CH_2), 6.46 (d, $J = 7.2$ Hz, 3H, Pyridinone C5-H), 7.27–7.32 (br, 15H, Ph), 7.54 (d, $J = 7.2$ Hz, 3H, Pyridinone C6-H),7.95 (t, $J = 6.4$ Hz, 3H, NH). ESI–MS *m/z* 828 ($[M + H]^+$).

*N,N′,N″-(2,2′,2″-nitrilotris(ethane-2,1-diyl)tris (3-(benzyloxy)-1-methyl-4-oxo-1,4-dihydropyridine-2-carboxamide) (**8b**)*

Yield: 45 %. ^{1}H NMR ($CDCl_3$) δ 2.14 (t, $J = 6.0$ Hz, 6H, CH_2), 2.82 (t, $J = 6.0$ Hz, 6H, CH_2), 3.57 (s, 9H, CH_3), 5.05 (s, 6H, CH_2), 6.24 (d, $J = 7.2$ Hz, 3H, Pyridinone C5-H), 7.14 (d, $J = 7.2$ Hz, 3H, Pyridinone C6-H), 7.32 (m, 9H, Ph), 7.44 (m, 6H, Ph). ESI–MS *m/z* 870 ($[M + H]^+$).

*N,N′,N″-(2,2′,2″-nitrilotris(ethane-2,1-diyl)tris (3-(benzyloxy)-1-ethyl-4-oxo-1,4-dihydropyridine-2-carboxamide) (**8c**)*

Yield: 42 %. ^{1}H NMR ($CDCl_3$) δ 1.38 (t, $J = 6.0$ Hz, 9H, CH_3), 2.39 (t, $J = 6.0$ Hz, 6H, CH_2), 3.05 (t, $J = 6.0$ Hz, 6H, CH_2), 4.05 (q, $J = 6.0$ Hz, 6H, CH_2), 5.16 (s, 6H, CH_2), 7.14 (d, $J = 6.8$ Hz, 3H, Pyridinone C5-H), 7.21–7.32 (m, 15H, Ph), 8.15 (d, $J = 6.8$ Hz, 3H, Pyridinone C6-H). ESI–MS *m/z* 912 ($[M + H]^+$).

*N,N′,N″-(2,2′,2″-nitrilotris(ethane-2,1-diyl)tris (3-(benzyloxy)-1-butyl-4-oxo-1,4-dihydropyridine-2-carboxamide) (**8d**)*

Yield: 38 %. ^{1}H NMR ($CDCl_3$) δ 0.92 (t, $J = 7.2$ Hz, 9H, CH_3), 1.28 (m, 6H, CH_2), 1.85 (m, 6H, CH_2), 2.17 (t, $J = 6.0$ Hz, 6H, CH_2,) 2.88 (t, 6H, $J = 6.0$ Hz, CH_2), 3.73 (t, $J = 7.2$ Hz, 6H, CH_2), 5.01 (s, 6H, CH_2), 6.30 (d, $J = 7.2$ Hz, 3H, Pyridinone C5-H), 7.15 (d, $J = 7.2$ Hz, 3H, Pyridinone C6-H), 7.30 (m, 9H, Ph), 7.44 (m, 6H, Ph). ESI–MS *m/z* 996 ($[M + H]^+$).

*N,N′,N″-(2,2′,2″-nitrilotris(ethane-2,1-diyl)tris (3-(benzyloxy)-1-hexyl-4-oxo-1,4-dihydropyridine-2-carboxamide) (**8e**)*

Yield: 32 %. ^{1}H NMR($CDCl_3$) δ 0.89 (t, $J = 6.8$ Hz, 9H, CH_3), 1.28 (m, 12H, CH_2), 1.76 (m, 6H, CH_2), 1.85 (m, 6H, CH_2), 2.14 (t, $J = 6.4$ Hz, 6H, CH_2), 2.84 (t, $J = 6.4$ Hz, 6H, CH_2), 3.74 (t, $J = 6.4$ Hz, 6H, CH_2), 5.02 (s, 6H, CH_2), 6.27 (d, $J = 7.6$ Hz, 3H, Pyridinone C5-H), 7.15 (d, $J = 7.6$ Hz, 3H, Pyridinone C6-H), 7.29 (m, 9H, Ph), 7.43 (m, 6H, Ph). ESI–MS *m/z* 1080 ($[M + H]^+$).

*N,N′,N″-(2,2′,2″-nitrilotris(ethane-2,1-diyl)tris (3-(benzyloxy)-1-(2-hydroxyethyl)-4-oxo-1, 4-dihydropyridine-2-carboxamide) (**8f**)*

Yield: 25 %. ^{1}H NMR (DMSO-d_6) δ 2.51 (t, $J = 6.0$ Hz, 6H, CH_2), 3.15 (t, $J = 6.0$ Hz, 6H, CH_2), 3.74 (t, $J = 5.6$ Hz, 6H, CH_2), 3.86 (t, $J = 5.6$ Hz, 6H, CH_2), 5.06 (s, 6H, CH_2), 6.19 (d, $J = 7.2$ Hz, 3H, Pyridinone C5-H), 7.31 (d, $J = 7.2$ Hz, 3H, Pyridinone C6-H), 7.45–7.49 (m, 15H, Ph). ESI–MS *m/z* 960 ($[M + H]^+$).

*N,N′,N″-(2,2′,2″-nitrilotris(ethane-2,1-diyl)tris(3-(benzyloxy)-1-(2-methoxyethyl)-4-oxo-1,4-dihydropyridine-2-carboxamide) (**8g**)*

Yield: 40 %. ^{1}H NMR ($CDCl_3$) δ 2.11 (t, $J = 6.0$ Hz, 6H, CH_2), 2.82 (t, $J = 6.0$ Hz, 6H, CH_2), 3.31 (s, 9H, CH_3), 3.61 (t, $J = 4.8$ Hz, 6H), 3.90 (t, $J = 4.8$ Hz, 6H, CH_2), 5.03 (s, 6H, CH_2), 6.24 (d, $J = 7.2$ Hz, 3H, Pyridinone C5-H), 7.29 (m, 12H, Ph and buried Pyridinone C6-H), 7.40 (m, 6H, Ph). ESI–MS *m/z* 1002 ($[M + H]^+$).

*N,N′,N″-(2,2′,2″-nitrilotris(ethane-2,1-diyl)tris (3-(benzyloxy)-1-(2-hydroxyethoxyl)-4-oxo-1, 4-dihydropyridine-2-carboxamide) (**8h**)*

Yield: 25 %. ^{1}H NMR (DMSO-d_6) δ 2.67 (t, $J = 6.0$ Hz, 6H, CH_2), 3.22 (t, $J = 6.0$ Hz, 6H, CH_2), 3.32 (m, 6H, CH_2), 3.47 (m, 6H, CH_2), 3.65 (t, $J = 5.2$ Hz, 6H, CH_2), 3.96 (t, $J = 5.2$ Hz, 6H, CH_2), 5.05 (s, 6H, CH_2), 6.21 (d, $J = 7.6$ Hz, 3H, Pyridinone C5-H), 7.29–7.38 (m, 15H, Ph), 7.62 (d, $J = 7.6$ Hz, 3H, Pyridinone C6-H), 8.82 (t, $J = 6.0$ Hz, 3H, NH). ESI–MS *m/z* 1092 ($[M + H]^+$).

*N,N′,N″-(2,2′,2″-nitrilotris(ethane-2,1-diyl)tris (3-(benzyloxy)-1-(2-(2-hydroxyethylamino)ethyl)-4-oxo-1,4-dihydropyridine-2-carboxamide) (**8i**)*

Yield: 21 %. ^{1}H NMR (DMSO-d_6) δ 2.65 (t, $J = 5.6$ Hz, 6H, CH_2), 2.82 (d, $J = 5.6$ Hz, 6H, CH_2), 3.18 (t, $J = 5.6$ Hz, 6H, CH_2), 3.56 (t, $J = 7.0$ Hz, 6H, CH_2), 3.82 (m, 12H, CH_2), 5.03 (s, 6H, CH_2), 6.21 (d, $J = 7.2$ Hz, 3H, Pyridinone C5-H), 7.16–7.34 (m, 15H, Ph), 7.65 (d, $J = 7.2$ Hz, 3H, Pyridinone C6-H), 8.59 (t, $J = 6.0$ Hz, 3H, NH). ESI–MS *m/z* 1089 ($M + H^+$), 1111 ($M + Na^+$).

General procedures for the preparation of hexadentate (**1**)

To a suspension of **8** (**8a–i**) (1 mmol) and concentrated hydrochloric acid (1 mL) in MeOH (30 mL) was added 5 % Pd/C (0.15 g). Hydrogenation was carried out at 30 psi H_2 for 5–6 h. After filtration to remove the catalyst, the filtrate was concentrated to dryness. The residue was purified by crystallization from methanol/acetone. Hydrochlorides of hexadentate **1** were obtained as white solids.

*N,N′,N″-(2,2′,2″-nitrilotris(ethane-2,1-diyl)tris(3-hydroxy-4-oxo-1,4-dihydropyridine-2-carboxamide) (**1a**)*

Yield: 80 %; ^{1}H NMR (DMSO-d_6) δ 3.41 (t, $J = 7.5$ Hz, 6H, CH_2), 3.79 (t, $J = 7.5$ Hz, 6H, CH_2), 7.02 (d, $J = 7.5$ Hz, 3H, Pyridinone C5-H), 7.82 (d, $J = 7.5$ Hz, 3H, Pyridinone C6-H), 9.38 (br, 3H, NH); ^{13}C NMR (DMSO-d_6) δ 34.40 (CONHCH_2), 51.69 (NHCH_2), 113.05 (C-5H in pyridinone), 127.56 (C-2 in pyridinone), 136.80 (C-6H in pyridinone), 147.72 (C-3 in pyridinone), 161.50 (C-4 in pyridinone), 163.35 (CO). ESI–MS *m/z* 558 ($[M + H]^+$).

*N,N′,N″-(2,2′,2″-nitrilotris(ethane-2,1-diyl)tris(3-hydroxy-1-methyl-4-oxo-1,4-dihydropyridine-2-carboxamide) (**1b**)*

Yield: 89 %; IR (KBr) ν_{max} 3387, 3025, 2930, 1671, and 1623 cm^{-1}. ^{1}H NMR (DMSO-d_6) δ 3.44 (s, 9H, CH_3), 3.81 (t, $J = 9.0$ Hz, 6H, CH_2), 3.93 (t, $J = 9.0$ Hz, 6H, CH_2), 7.38 (d, $J = 9.0$ Hz, 3H, Pyridinone C5-H), 8.22 (d, $J = 9.0$ Hz, 3H, Pyridinone C6-H), 9.68 (s, 3H, NH); ^{13}C NMR (DMSO-d_6) δ 34.54 (CH_3), 44.39 (CH_2), 51.51 (CH_2), 112.33 (C-5H in pyridinone), 136.60 (C-2 in pyridinone), 139.87 (C-6H in pyridinone), 143.87 (C-3 in pyridinone), 159.89 (C-4 in pyridinone), 161.91 (CO). HRMS *m/z* Calcd. for $C_{27}H_{34}N_7O_9$ $[M + H]^+$ 600.2418, found: 600.2418.

*N,N′,N″-(2,2′,2″-nitrilotris(ethane-2,1-diyl)tris(1-ethyl-3-hydroxy-4-oxo-1,4-dihydro-pyridine-2-carboxamide) (**1c**)*

Yield: 91 %; IR (KBr) ν_{max} 3404, 3234, 3048, 2978, 2930, 1652, and 1620 cm^{-1}. ^{1}H NMR (DMSO-d_6) δ 1.40 (t, $J = 7.5$ Hz, 9H, CH_3), 3.43 (t, $J = 7.5$ Hz, 6H, CH_2), 3.81 (t, $J = 7.5$ Hz, 6H, CH_2), 4.23 (q, $J = 7.5$ Hz, 6H, CH_2), 7.37 (d, $J = 7.5$ Hz, 3H, Pyridinone C5-H), 8.30 (d, $J = 7.5$ Hz, 3H, Pyridinone C6-H), 9.65 (t, $J = 7.5$ Hz, 3H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 16.48 (CH_3), 34.35 (CH_2), 51.38 (CH_2), 51.99 (CH_2), 112.35 (C-5H in pyridinone), 135.39 (C-2 in pyridinone), 138.09 (C-6H in pyridinone), 143.14 (C-3 in pyridinone), 159.50 (C-4 in pyridinone), 161.99 (CO). ESI–MS *m/z* 642 ($[M + H]^+$), HRMS *m/z* Calcd. for $C_{30}H_{40}N_7O_9$ $[M + H]^+$ 642.2886, found: 642.2890.

*N,N′,N″-(2,2′,2″-nitrilotris(ethane-2,1-diyl)tris(1-butyl-3-hydroxy-4-oxo-1,4-dihydro-pyridine-2-carboxamide) (**1d**)*

Yield: 95 %; IR (KBr) ν_{max} 3189, 3029, 2955, 2929, 2872, 1635, and 1619 cm^{-1}. ^{1}H NMR (DMSO-d_6) δ 0.81 (t, $J = 7.5$ Hz, 9H, CH_3), 1.26 (m, 6H, CH_2), 1.75 (m, 6H, CH_2), 3.44 (t, $J = 8.5$ Hz, 6H, CH_2), 3.81 (t, $J = 8.5$ Hz, 6H, CH_2), 4.18 (t, $J = 8.0$ Hz, 6H, CH_2), 7.21 (d, $J = 9.0$ Hz, 3H, Pyridinone C5-H), 8.21 (d, $J = 9.0$ Hz, 3H, Pyridinone C6-H), 9.53 (s, 3H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 13.84 (CH_3), 19.25 (CH_2), 32.97 (CH_2), 34.93 (CH_2), 51.77 (CH_2), 56.34 (CH_2), 112.45 (C-5H in pyridinone), 134.59 (C-2 in pyridinone), 139.11 (C-6H in pyridinone), 144.24 (C-3 in pyridinone), 160.34 (C-4 in pyridinone), 163.81 (CO). ESI–MS *m/z* 726 ($[M + H]^+$); HRMS *m/z* Calcd. for $C_{36}H_{52}N_7O_9$ $[M + H]^+$ 726.3826, found: 726.3812.

*N,N′,N″-(2,2′,2″-nitrilotris(ethane-2,1-diyl)tris(1-hexyl-3-hydroxy-4-oxo-1,4-dihydro-pyridine-2-carboxamide) (**1e**)*

Yield: 86 %; IR (KBr) ν_{max} 3194, 3025, 2967, 2850, 1675, and 1619 cm^{-1}. ^{1}H NMR (DMSO-d_6) δ 0.88 (t, $J = 7.5$ Hz, 9H, CH_3), 1.25 (br, 18H, CH_2), 1.74 (br, 6H, CH_2), 3.42 (t, $J = 7.0$ Hz, 6H, CH_2), 3.79 (q, $J = 7.0$ Hz, 6H, CH_2), 4.14 (t, $J = 7.5$ Hz, 6H, CH_2), 7.20 (d, $J = 7.5$ Hz, 3H, Pyridinone C5-H), 8.20 (d, $J = 7.5$ Hz, 3H, Pyridinone C6-H), 9.58 (t, $J = 7.0$ Hz, 3H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 13.82 (CH_3), 14.22 (CH_2), 19.36 (CH_2), 22.27 (CH_2), 25.69 (CH_2), 31.10 (CH_2), 51.88 (CH_2), 56.16 (CH_2), 111.91 (C-5H in pyridinone), 133.48 (C-2 in pyridinone), 139.08 (C-6H in pyridinone), 144.41 (C-3 in pyridinone), 160.64 (C-4 in pyridinone), 164.86 (CO). ESI–MS *m/z* 810 ($[M + H]^+$). HRMS *m/z* Calcd. for $C_{42}H_{64}N_7O_9$ $[M + H]^+$ 810.4764, found: 810.4756.

*N,N′,N″-(2,2′,2″-nitrilotris(ethane-2,1-diyl)tris (3-hydroxy-1-(2-hydroxylethyl)-4-oxo-1,4-dihydro-pyridine-2-carboxamide) (**1f**)*

Yield: 83 %; ^{1}H NMR (DMSO-d_6) δ 3.25 (t, $J = 6.5$ Hz, 6H, CH_2), 3.43 (t, $J = 6.5$ Hz, 6H, CH_2), 3.71 (t, $J = 6.5$ Hz, 6H, CH_2), 3.91 (t, $J = 6.5$ Hz, 6H, CH_2), 6.31 (d, $J = 9.5$ Hz, 3H, Pyridinone C5-H), 7.84 (d, $J = 9.5$ Hz, 3H, Pyridinone C6-H); ^{13}C NMR (DMSO-d_6) δ 30.76 (CH_2), 51.22 (CH_2), 56.38 (CH_2), 71.61 (CH_2), 120.02 (C-5H in pyridinone), 127.67 (C-2 in pyridinone), 139.32 (C-6H in pyridinone), 144.88 (C-3 in pyridinone), 170.19 (C-4 in pyridinone), 173.23 (CO). ESI–MS *m/z* 690 ($[M + H]^+$); HRMS *m/z* Calcd. for $C_{30}H_{40}N_7O_{12}$ $[M + H]^+$ 690.2734, found: 690.2741.

*N,N′,N″-(2,2′,2″-nitrilotris(ethane-2,1-diyl)tris (3-hydroxy-1-(2-methoxyethyl)-4-oxo-1,4-dihydro-pyridine-2-carboxamide) (**1g**)*

Yield: 83 %; IR (KBr) ν_{max} 3385, 3203, 3042, 2950, 2800, 1685, 1671, and 1620 cm^{-1}. ^{1}H NMR (DMSO-d_6) δ 3.20 (t, $J = 6.4$ Hz, 6H, CH_2), 3.43 (t, $J = 6.4$ Hz, 6H, CH_2), 3.66 (s, 9H, CH_3), 3.78 (t, $J = 7.2$ Hz, 6H, CH_2), 4.28 (t, $J = 7.2$ Hz, 6H, CH_2), 7.38 (d, $J = 7.2$, 6H, Pyridinone C5-H), 8.15 (d, $J = 7.2$, 6H, Pyridinone C6-H), 9.71 (s, 3H, NH); ^{13}C NMR (DMSO-d_6) δ 34.66 (CH_2), 51.60 (CH_2), 56.40 (CH_2), 58.66 (CH_2), 70.61 (CH_3), 111.94 (C-5H in pyridinone), 136.11 (C-2 in pyridinone), 139.97 (C-6H in pyridinone), 140.01 (C-3 in pyridinone), 159.80 (C-4 in pyridinone), 162.96 (CO). ESI–MS *m/z* 732 ($[M + H]^+$). HRMS *m/z* Calcd. for $C_{33}H_{46}N_7O_{12}$ $[M + H]^+$ 732.3204, found 732.3228.

*N,N′,N″-(2,2′,2″-nitrilotris(ethane-2,1-diyl)tris(3-hydroxy-1-(2-(2-hydroxyethoxy)ethyl)-4-oxo-1,4-dihydro-pyridine-2-carboxamide) (**1h**)*

Yield: 79 %; IR (KBr) ν_{max} 3400, 3035, 2970, 2926, 1670, and 1619 cm^{-1}. ^{1}H NMR (DMSO-d_6) δ 3.40 (m, 18H, CH_2), 3.71 (m, 12H, CH_2), 4.19 (m, 6H, CH_2), 7.16 (d, $J = 7.5$ Hz, 3H, Pyridinone C5-H), 8.05 (d, $J = 7.5$ Hz, 3H, Pyridinone C6-H), 9.46 (br, 3H, NH); ^{13}C NMR (DMSO-d_6) δ 34.24 (CH_2), 48.52 (CH_2), 60.01 (CH_2), 66.32 (CH_2), 68.64 (CH_2), 72.15 (CH_2), 111.47 (C-5H in pyridinone), 135.21 (C-2 in pyridinone), 139.71 (C-6H in pyridinone), 143.38 (C-3 in pyridinone), 159.71 (C-4 in pyridinone), 163.53 (CO). ESI–MS *m/z* 822 ($[M + H]^+$). HRMS *m/z* Calcd. for $C_{36}H_{52}N_7O_{15}$ $[M + H]^+$ 822.3521, found 822.3543.

*N,N′,N″-(2,2′,2″-nitrilotris(ethane-2,1-diyl))tris (3-hydroxy-1-(2-(2-hydroxyethylamino)ethyl)-4-oxo-1,4-dihydropyridine-2-carboxamide) (**1i**)*

Yield 86 %; IR (KBr) ν_{max} 3369, 3021, 2961, 2788, 1672, and 1618 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 3.25 (t, $J = 4.8$ Hz, 6H, CH_2), 3.42 (t, $J = 6.0$ Hz, 6H, CH_2), 3.60 (t, $J = 4.8$ Hz, 6H, CH_2), 3.72 (t, $J = 5.6$ Hz, 6H, CH_2), 3.83 (t, $J = 5.6$ Hz, 6H, CH_2), 4.09 (t, $J = 5.6$ Hz, 6H, CH_2), 7.29 (d, J = 8.0 Hz, 3H, Pyridinone C5-H), 8.10 (d, $J = 8.0$ Hz, 3H, Pyridinone C6-H), 10.03 (s, 3H, NH); ^{13}C NMR (DMSO-d_6) δ 39.76 (CH_2), 40.09 (CH_2), 40.26 (CH_2), 49.08 (CH_2), 49.90 (CH_2), 56.73 (CH_2), 112.90 (C-5H in pyridinone), 136.35 (C-2 in pyridinone), 139.78 (C-6H in pyridinone), 148.79 (C-3 in pyridinone), 159.76 (C-4 in pyridinone), 162.36 (CO). ESI–MS *m/z* 819 ($[M + H]^+$). HRMS *m/z* Calcd. for $C_{36}H_{53}N_{10}O_{12}$ $[M-H]^-$ 817.3843, found 817.3855.

Physico-chemical properties of hexadentate HPOs

p*K*a determination

The titration system used in this determination comprised of an autoburette (Metrohm Dosimat 765 l mL syringe) and a HP 8453 UV–Visible spectrophotometer. 0.1 M KCl electrolyte solution was used to maintain the ionic strength. The temperature of the test solutions was maintained in a thermostatic cuvette holder at 25 ± 0.1 °C using a Cary 1 controller, an argon atmosphere was applied to the entire titration equipment. The initial sample concentration was approximately 7×10^{-5}M. p*K*a values were analyzed from these data by pHab.

Determination of iron(III) affinity

The automatic titration system used in this study comprised of an autoburette (Metrohm Dosimat 765 l mL syringe) and Mettler Toledo MP230 pH meter with Metrohm pH electrode (6.0133.100) and a reference electrode (6.0733.100). 0.1 M KCl electrolyte solution was used to maintain the ionic strength. The temperature of the test solutions was maintained in a thermostatic jacketed titration vessel at 25 ± 0.1 °C using a Techne TE-8 J temperature controller. The solution under investigation was stirred vigorously during the experiment. A Gilson Miniplus#3 pump with speed capability (20 mL/min) was used to circulate the test solution through a Hellem quartz flow cuvette. For stability constant determinations, a 50 mm path length cuvette was used, and for p*K*a determinations, a cuvette path length of 10 mm was used. The flow cuvette

was mounted on an HP 8453 UV–Visible spectrophotometer. All instruments were interfaced to a computer and controlled by a Visual Basic program. Automatic titration and spectral scans adopted the following strategy: the pH of a solution was increased by 0.1 pH unit by the addition of KOH from the autoburette; when pH readings varied by <0.001 pH unit over a 3 s period, an incubation period was activated. For p*K*a determinations, a period of 1 min was adopted; for stability constant determinations, a period of 5 min was adopted. At the end of the equilibrium period, the spectrum of the solution was then recorded. The cycle was repeated automatically until the defined end point pH value was achieved. All the titration data were analyzed with the pHab program (Gans *et al.*, 1999). The species plot was calculated with the HYSS program (Alderighi *et al.*, 1999). Analytical grade reagent materials were used in the preparation of all solutions

Antimicrobial assay

Bacterial strains

Pseudomonas aeruginosa, Staphylococcus aureus, and *Escherichia coli* were purchased from CGMCC. *Bacillus subtilis* and *Bacillus cereus* were separated from mussels (see below). All bacteria were inoculated in a tube containing an inclined plane of Brain–Heart Infusion (BHI) agar and cultured at 37 °C for 24 h. This gel was then used to inoculate into 5 mL of BHI broth and incubated at 37 °C for 24 h before transfer 50 μL into another tube of fresh BHI broth. This transfer was incubated at 37 °C to an optical density of approximately 10^7 colony-forming units (cfu/mL).

Isolation and identification of *B. subtilis* and *B. cereus*: *Mytilus edulis linne* was obtained from a local fishing company and was transported to the laboratory on ice. Samples of 25 g muscle were homogenized in 250 mL of 0.1 % physiological peptone salt [PFZ 0.85 %NaCl (w/v) and 0.1 % peptone (w/v)] for 60 s in a stomacher bag. Suitable decimal dilutions were pour-plated on modified plate count agar (PCA) for bacteria species. PCA agar plates were incubated for 48 h at 30 °C. Representative colonies were picked up randomly and purified by repeatedly streaking on appropriate agar medium. The isolates were identified following the criteria outlined in Bergey's Manual of Systematic Bacteriology (Holt and Krieg, 1994). Further characterization and confirmation was carried out using a 6850 automated identification method (MIDI) and PCR identification method.

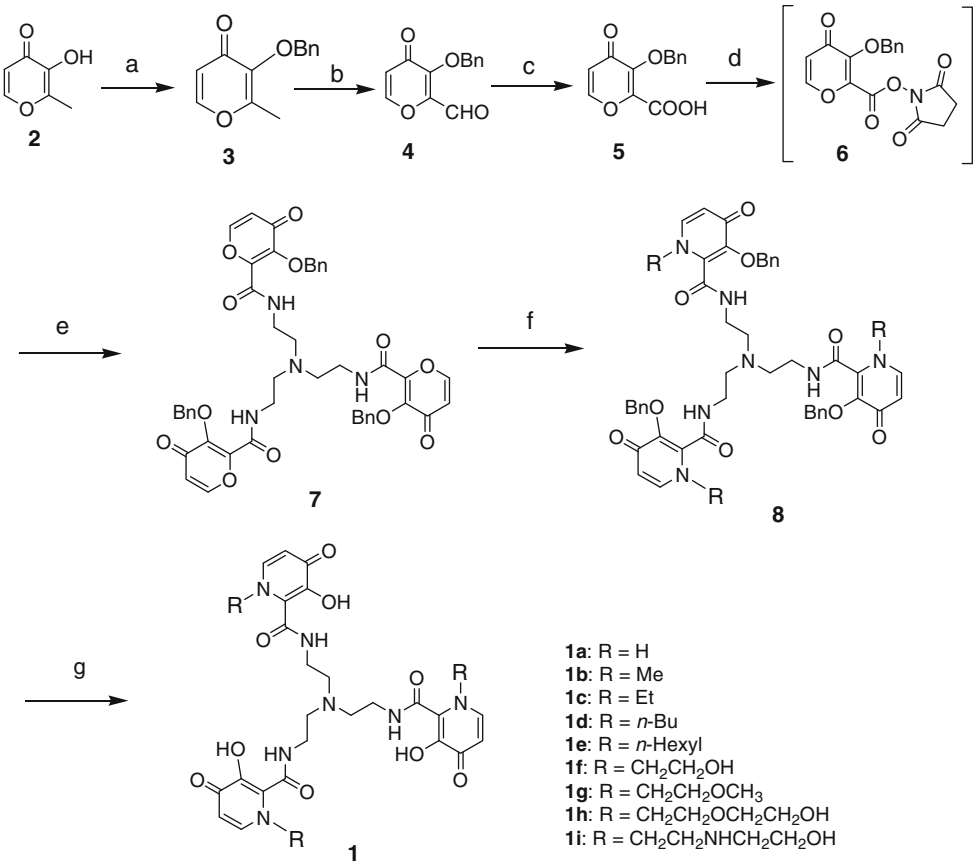
MIC determination

All assays were cultured at 37 °C for 24 h in 15 × 75-mm tubes. The incubation medium was BHI broth. All tubes contained 80 μL of antimicrobial agent with a different concentration ranging from 0 to 3,000 μg/mL, 20 μL of bacterial inoculum, with a total volume of 100 μL. After incubation at 37 °C for 2 h, 900 μL of sterilized water was added to each tube to reach a final volume of 1 mL, and cultured at 37 °C for 24 h. Minimum inhibitory concentrations (MIC) were determined by visual inspection of the turbidity of broth in tubes (Barry, 1976). All assays were carried out in triplicate.

Result and discussion

Chemistry

Considering that both iron(III) affinity and hydrophobicity of iron chelators may affect their antimicrobial activity, we have designed and synthesized a series of hexadentate 3-hydroxypyridin-4-one derivatives (**1a–1i)** which have different structures to those of naturally occurring siderophores, so as to investigate the structure-antimicrobial activity relationship. The synthetic route of compound **1** is outlined in Scheme 1. The benzylation of the starting material, maltol (**2**) was achieved by the treatment with benzyl chloride to obtain **3**, which underwent the selective oxidation of the methyl group at position-2 with selenium dioxide to generate the aldehyde **4**. Further oxidation of aldehyde **4** with sodium hypochlorite and sulfanic acid provided carboxylic acid **5** in good yield (Pace *et al.*, 2004; Puerta *et al.*, 2006). Activation of carboxyl group of **5** was achieved in the presence of *N*-hydroxysuccinmide (NHS) and *N,N'*-dicyclohexylcarbodiimide (DCC) to give the active ester **6**, which was used in the next reaction without isolation. Coupling of the active ester **6** with *N,N*-bis(2-aminoethyl)ethane 1,2-diamine (TREN) gave **7**, which was reacted with different amines to provide a series of benzyl protected hexadentate ligands **8**. Finally, hydrogenation of **7** was carried out in the presence of Pd/C and hydrochloric acid, providing the hydrochlorides of hexadentate 3-hydroxypyridin-4-ones **1** (**a–i**). All the hexadentate hydroxypyridinones have been characterized by ^{1}H NMR, ^{13}C NMR, IR, ESI–MS, and HRMS. It is worthy to note that in comparison with our reported synthesis of compound **1a**, which also starts from maltol (Piyamongkol *et al.*, 2005), the present synthetic method has the advantages of a shorter synthetic procedure and applicability for the preparation of a range of analog.



Scheme 1 Reagents and conditions: (*a*) CH_3OH, NaOH, BnCl. reflux 8 h; (*b*) PhBr, SeO_2, 159 °C, 12 h; (*c*) CH_3COCH_3/H_2O, NH_2SO_3H, $NaClO_2$, rt, 2 h; (*d*) THF, DCC, NHS, rt, 3.5 h; (*e*) THF, $N(CH_2CH_2NH_2)_3$, rt, overnight; (*f*) CH_3CH_2OH, reflux, RNH_2, 3 h; (*g*) CH_3OH, Pd/C, H_2. 5–6 h, rt

Physico-chemical characterization

In order to further demonstrate the high affinity for iron(III), we selected some of the hexadentate chelators, and evaluated their p*K*a values and stability constants for the corresponding iron(III) complexes using an automated titration system (Liu *et al.*, 1999; Dobbin *et al.*, 1993; Rai *et al.*, 1998), the titration data were analyzed with the pHab (Gans *et al.*, 1999).

p*K*a values

The pH dependence UV spectra of **1b** (Fig. 2) was recorded between 250 and 390 nm over the pH range 2–12 for the free ligand. The speciation spectra demonstrate a clear shift in λ_{max} from 285 to 320 nm, which reflects the pH dependence of the ligand ionization equilibrium. The ligand group **1b** can be considered as trimers of the corresponding bidentate ligands, and they, therefore, possess two sets of intrinsic p*K*a values. Using the spectrophotometric titration method, the p*K*a values of **1b** obtained from nonlinear least-squares regression analysis were found to be 1.91, 2.56, 4.13, 6.92, 8.06, and 9.06. Of the six p*K*a values, the three lower values correspond to the 4-oxo function and the higher three correspond to the 3-hydroxyl function. The p*K*a values of chelators **1c**, **1g** and **1h** were determined using the same method as that for **1b**, and in similar fashion, all were found to possess six p*K*a values (Table 1). For these four compounds, the p*K*a for the tertiary amine function could not be detected using spectrophotometric titration. However, we previously determined the p*K*a values of **1a** by potentiometric titration, and the tertiary amine p*K*a value was found to be 4.25. We assume that this value will be close to the p*K*a values of the corresponding amine functions in compounds (**1b**–**1i**).

Iron(III) affinity

The stability constant of an iron-ligand complex is one of the key parameters related to the chelation efficacy of a ligand. The log stability constants of the series of chelators **1**-iron(III) complexes were determined by spectrophotometric titration against the hydroxyl anion. The $\log K_1$ values were found to be similar and all close to 32 (Table 1). As an example, a series of UV spectra of **1b** in the presence of iron at different pH values is shown in Fig. 3. The pFe^{3+} value, defined as the negative logarithm of concentration of the free iron(III) in solution (when $[Fe^{3+}]_{total} = 10^{-6}$M; $[Ligand]_{total} = 10^{-5}$M; pH = 7.4), is a more suitable factor for comparison than the stability constant, since this parameter takes into account the effect of ligand basicity, denticity, and the degree of protonation.

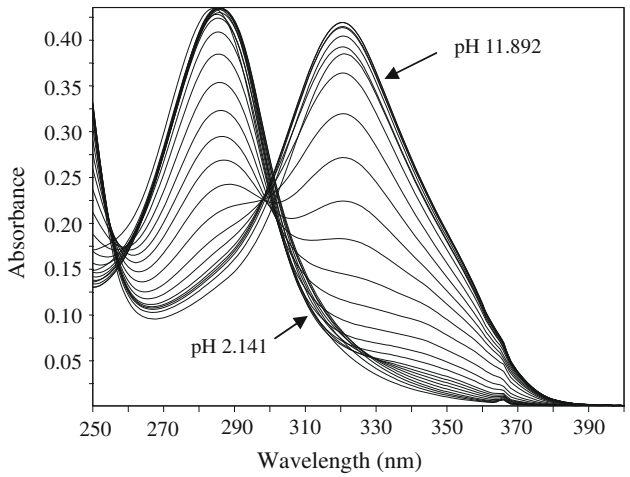


Fig. 2 UV spectra of **1b**. [**1b**] = 222.6 μM (in 15.067 mL of 0.1 M KCl), pH was changed from 2.141 to 11.892 by the addition of KOH at 25 °C

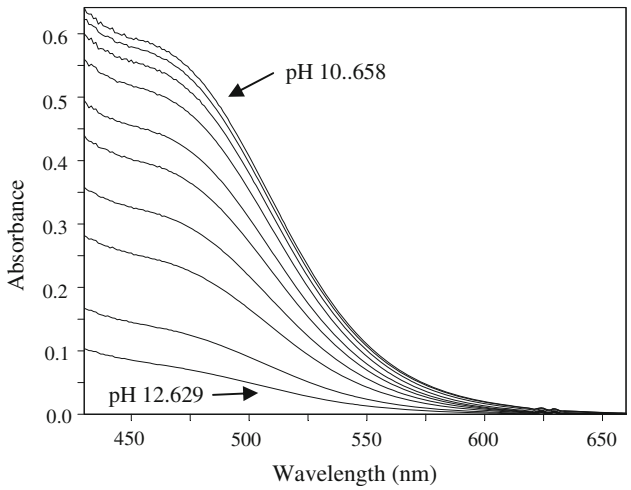


Fig. 3 UV spectra of **1b** with iron. [**1b**] = 32.7 μM, [Fe^{3+}] = 29.7 μM (in 18.059 mL of 0.1 M KCl), pH was changed from 10.658 to pH 12.629 by the addition of KOH at 25 °C

Table 1 p*K*a values and iron affinities of hexadentate ligands

Ligands	p*K*a	$logK_1$	pFe
1a	0.66, 1.88, 3.6, 6.58, 7.65, 8.10	30.7	30.5
1b	1.91, 2.56, 4.13, 6.92, 8.06, 9.06	32.1	30.5
1c	1.91, 2.58, 4.23, 7.45, 8.26, 9.01	32.6	30.7
1g	2.40, 3.76, 4.54, 7.30, 8.61, 9.27	32.3	29.9
1h	1.89, 2.93, 4.31, 7.10, 7.75, 8.83	32.0	30.8

The pFe^{3+} values of **1** (**1b**, **1c**, **1g** and **1h**), calculated using the measured stability constant and p*K*a values, are extremely high, ranging between 29.9 and 30.8. These values are close to that of **1a** (pFe^{3+} 30.5), suggesting that substituent in the 1-position of the pyridinone ring does not appreciably influence the iron(III) affinity. This is probably because of the group at 1-position has less effect on the p*K*a value of 3-hydroxyl than the group at 2-position, and the electron-withdrawing ability does not vary much among the introduced substituents.

Antimicrobial evaluation

The antimicrobial activity of these chelators was evaluated using minimum inhibition concentration (MIC) assay. It was found that all the iron chelators exhibited strong inhibition against three Gram-positive bacteria (*Staphylococcus aureus, Bacillus subtilis* and *Bacillus cereus*) and two Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) (Table 2). In the case of *S. aureus*, the effect of **1a**, **1b**, **1c**, **1e**, and **1h** are similar to that of commercially available chelator diethylene-triamine pentaacetic acid (DTPA), with a MIC of 40 μg/mL, whereas **1d**, **1f**, and **1g** have a MIC of 80 μg/mL. Against *B. cereus and B. subtilis,* most of the chelators showed stronger inhibitory activity than DTPA, with a MIC of 40 μg/mL. In the case of *E. coli* and *P. aeruginosa*, almost all the chelators exhibited stronger inhibition than DTPA which has a MIC of 80 μg/mL. **1g** showed the strongest activity with a MIC of 16 μg/mL. **1c** exhibited the strongest overall

Table 2 Antimicrobial activity of chelators (MIC, μg/mL)

Compound	*S. aureus*	*B. cereus*	*B. subtilis*	*E. coli*	*P. aeruginosa*	Molecular weight	ClogP
DTPA	40	80	80	80	80	393	−5.596
1a	40	40	40	80	24	557	−4.505
1b	40	40	40	24	24	599	−4.569
1c	40	40	40	24	16	641	−3.638
1d	80	80	80	40	40	725	−0.452
1e	40	40	40	80	40	809	2.579
1f	80	40	40	40	40	689	−5.298
1g	80	80	40	16	16	731	−4.595
1h	40	40	40	40	40	821	−5.384

inhibitory activity when considering the entire group of bacteria.

Most of the chelators investigated in this study were found to possess stronger antimicrobial activity than DTPA. This is presumably due to these chelators possessing a higher affinity for iron(III) than DTPA (logK = 28.6) (Sohnle *et al.*, 2001). The calculated partition coefficients (clogP) of the chelators (http://www.molinspiration.com/cgi-bin/properties), are also presented in Table 2. These chelators are highly hydrophilic with the exception of **1e** (clogP 2.579). This hydrophilicity, together with the relatively high molecular weights (Table 2) and would suggest that these chelators and their iron complexes will not be able to cross bilayer membranes by nonfacilitated diffusion. Thus, it is proposed that they inhibit the growth of Gram-positive bacteria by scavenging iron in the immediate environment around the bacteria and for Gram-negative bacteria by entering and scavenging iron in the periplasmic space. It is surprising that there is not a marked difference between the antimicrobial properties of the hydrophobic **1e** (clogP 2.579) and the hydrophilic **1f** (clogP −5.298), and this further supports the concept of extracellular iron chelation being responsible for the antimicrobial effect. Most of the chelators are more effective against Gram-negative bacteria than Gram-positive bacteria. This is probably due to the ready access to the periplasmic space in Gram-negative bacteria.

In conclusion, the inhibition of bacterial iron uptake represents a promising alternative area of research for the design of new antimicrobials, the hexadentate 3-hydroxypyridin-4-one were found to possess strong inhibitory activity against the growth of both Gram-positive and Gram-negative bacteria. Overall, **1c** showed optimal bacterial growth inhibitory effect. These hexadentate chelators have potential as antimicrobial agents, particularly in the treatment of external infections.

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