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ARTICLE *in* BIOORGANIC & MEDICINAL CHEMISTRY LETTERS · DECEMBER 2005

Impact Factor: 2.42 · DOI: 10.1016/j.bmcl.2005.08.008 · Source: PubMed

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Bioorganic & Medicinal Chemistry Letters 15 (2005) 5007–5011
Bioorganic & Medicinal Chemistry Letters

High affinity iron(III) scavenging by a novel hexadentate 3-hydroxypyridin-4-one-based dendrimer: Synthesis and characterization

Tao Zhou,[a] Zu Dong Liu,[a] Hendrik Neubert,[b] Xiao Le Kong,[a] Yong Min Ma[a] and Robert C. Hider[a,*]

[a]*Department of Pharmacy, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London SE1 9NH, UK*
[b]*Drug Control Cent Centre/Mass Spectrometry Facility, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London SE1 9NH, UK*

Received 10 June 2005; revised 1 August 2005; accepted 2 August 2005
Available online 8 September 2005

Abstract—The synthesis of a novel iron(III)-selective hydroxypyridinone hexadentate-terminated dendritic chelator based on a benzene tricarbonyl core polyamine dendrimer is described. The iron-chelating ability of the dendritic chelator was demonstrated by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and UV–vis spectroscopy. The physicochemical properties of the isolated hexadentate unit were also investigated. The dendrimer was found to possess an extremely high affinity for iron(III), namely log$K$ = 34.8, pFe^{3+} = 30.6.

The most frequent treatment of haemoglobinopathic disorders, such as β-thalassaemia major, is to maintain high levels of haemoglobin by regular blood transfusion. Because man lacks the physiological means of eliminating excess iron, iron associated with transfused red cells progressively accumulates, in the liver and other highly perfused organs, leading to tissue damage, organ failure, and eventual death.[1] Complications associated with elevated iron levels can be largely avoided by the use of iron-specific chelators. As a result of our interest in the design and synthesis of orally active iron(III)-selective chelators centred on hydroxypyridinones,[2] we previously reported the synthesis of a range of dendritic chelators by conjugating amino functionalized hydroxypyridinones to carboxyl group-terminated dendrimers.[3] Dendrimers, by virtue of their relatively high molecular weight, are not absorbed by the mammalian gastrointestinal tract, thus by chelating iron from the lumen of the intestine their presence will decrease iron availability for absorption into the blood supply. The particular attraction of dendrimers is that, unlike polymers, they have a unique molecular structure and are therefore amenable to precise quality control. Alternative approaches to the synthesis of dendrimers of this type offer structural flexibility and the potential to incorporate modified properties. To this end, we report the synthesis, characterization and iron-chelating properties of a novel hexadentate 3-hydroxypyridinone-terminated dendrimer by coupling hydroxypyridinone containing activated carboxyl functions to the amino group-terminated dendrimer.

The 3-hydroxypyridinone hexadentate ligand **4**[4] was synthesized by attaching three bidentate ligands **2** prepared from maltol in eight steps[5] to a tripodal molecule triamine **1**, followed by the removal of benzyl groups (Scheme 1). The 2-amido group adjacent to the 3-hydroxyl group in the hydroxypyridinone moieties can form a coplanar intramolecular hydrogen bond, as there is no N-alkyl substitution in the ring. This results in an increase in the stability of the iron complex at pH 7.4.[6] Such an intramolecular hydrogen bond is not so well favoured in the presence of N-alkyl substitution, as appreciable steric repulsion exists between the 1-alkyl group and the amide oxygen atom.[5] To obtain a hydroxypyridinone chelator with an extremely high affinity for iron(III), a dendritic chelator containing

Keywords: Iron chelator; Dendrimers; Hexadentate; Hydroxypyridinone; Fluorescence.
* Corresponding author. Tel.: +44 20 78 48 48 82; fax: +44 20 78 48 48 00; e-mail: robert.hider@kcl.ac.uk

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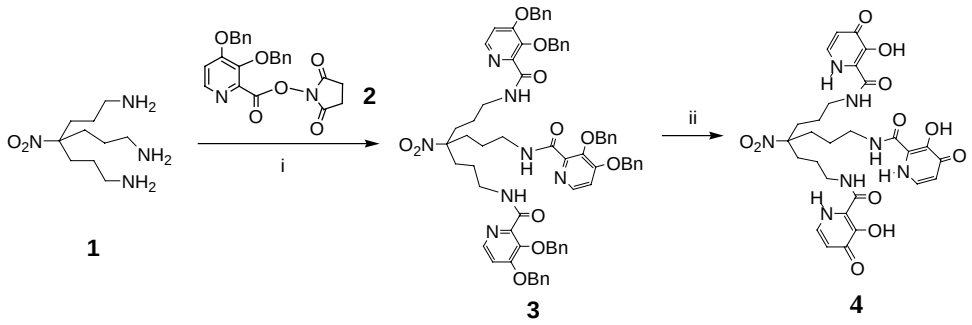
doi:10.1016/j.bmcl.2005.08.008

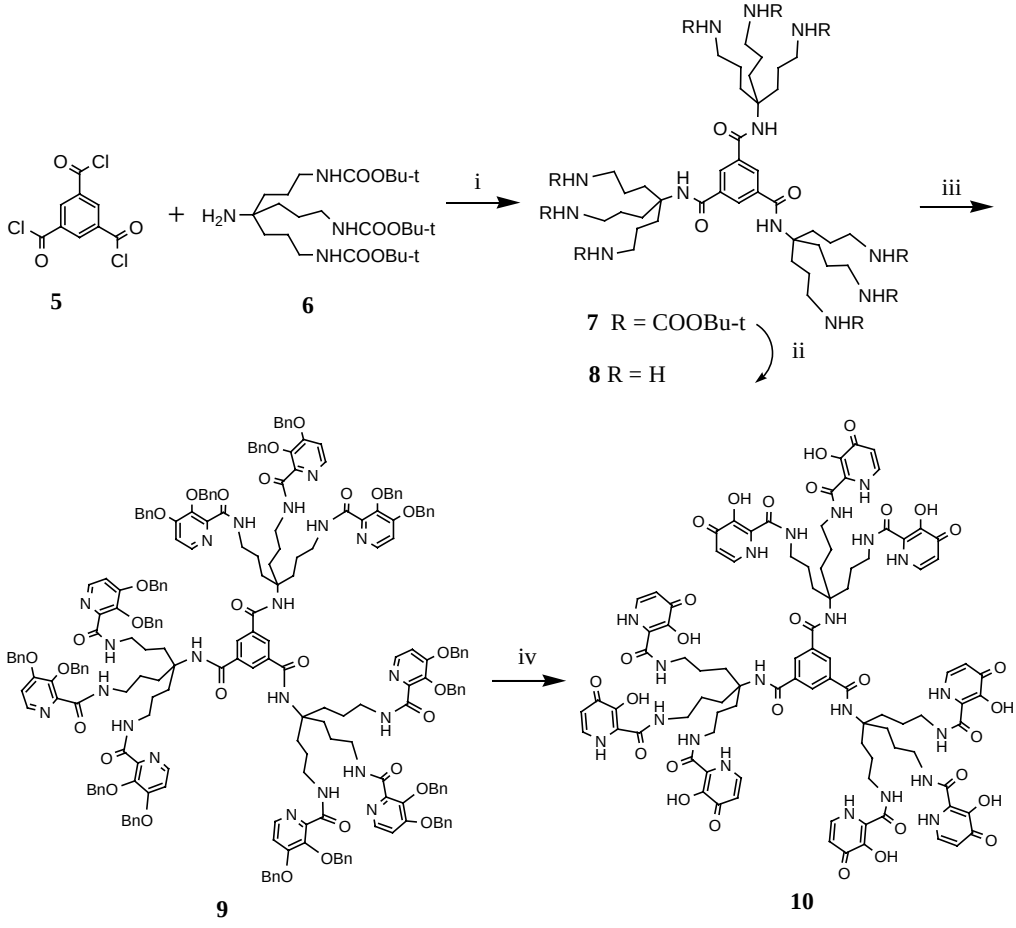


Scheme 1. Synthesis of hexadentate chelator **4**. Reagents and conditions: (i) DMF, rt, 2 d, 77% yield; (ii) BCl_3, 0 °C–rt, 3 h, 82% yield.

three of the above hexadentate ligand analogues was synthesized by a divergent strategy (Scheme 2).[7] In this approach, we selected benzenetricarbonyl trichloride **5** as a core, and the amine **6**, which was synthesized from 4-(2-cyanoethyl)-4-nitroheptanedinitrile in three steps,[7b] as a building block. The reaction of **5** and **6** afforded the BOC-protected nonamine **7**, which was followed by the hydrolysis in formic acid and neutralization with dilute sodium hydroxide to generate the nonamine dendrimer **8**. The coupling of **8** and activated pyridinone **2** produced the benzyl group protected dendritic chelator **9**,[8] which was then subjected to deprotection in boron trichloride to give the hydrochloric acid salt of dendritic chelator **10**.[9]

To estimate the ligand affinity of the dendritic chelator **10**, both pK_a values of **4** and stability constant of the corresponding iron(III) complex were evaluated using an automated titration system.[10]

The pH dependence UV spectra of **4** (Fig. 1) demonstrate a clear shift in λ_{max} over the pH range 1.75–12.1, which displays the pH dependence of the ligand ionization equilibrium. The pK_a values obtained from



Scheme 2. Synthesis of dendritic chelator. Reagents and conditions: (i) CH_2Cl_2, 0 °C–rt, 6 h, 68% yield; (ii) 96% HCOOH, rt, 24 h, 95% yield; (iii) DMF, rt, 3 d, 83% yield; (iv) BCl_3, CH_2Cl_2, 0 °C–rt, 2 d, 88% yield.

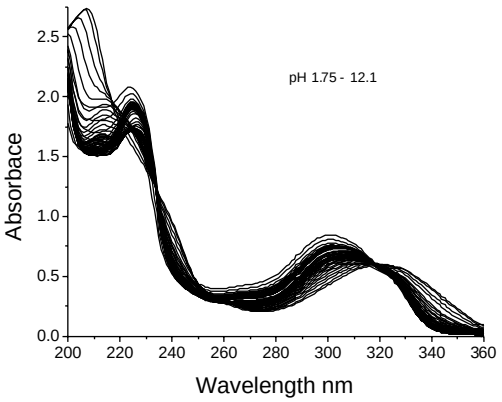


Figure 1. The pH dependence UV spectra of **4** over the pH range 1.75–12.1.

non-linear least-squares regression analysis are 9.40, 9.07, 8.91, 4.01, 3.86, and 1.39. The stability constant of the **4**–iron(III) complex was determined spectrophotometrically by competition with the well-characterized aminocarboxylate hexadentate ligand, HBED. The absolute stability constant (log*K*) for the iron(III) complex of **4** was determined to be 35.0 ± 0.1, as compared with HBED (logK = 39.7).[11] All the above investigations were undertaken in aqueous DMSO solutions (50%, v/v).

The speciation plot of compound **4**–iron(III) complexes (Fig. 2) demonstrates that the 1:1 ligand–iron(III) complex is the dominant species over the pH range 2–12. The pFe^{3+} value, defined as the negative logarithm of concentration of the free iron(III) in solution, is a more suitable comparator than the stability constant since it takes into account the effect of ligand basicity, denticity, degree of protonation and difference in metal–ligand stoichiometries.[12] Under biological conditions, pFe^{3+} values are typically calculated for total [ligand] = 10^{-5} M and total [iron] = 10^{-6} M at pH 7.45. Chelators with high pFe^{3+} values are predicted to scavenge iron more efficiently at low ligand concentrations. The pFe^{3+} value of **4** is 30.9 at pH 7.45, which is over 8 log units higher than that of the bidentate ligand analogue 3-hydroxy-*N*-(2-hydroxyethyl)-4-oxo-1,4-dihydropyridine-2-carboxamide (pFe^{3+} value at pH 7.45 = 22.0).[5]

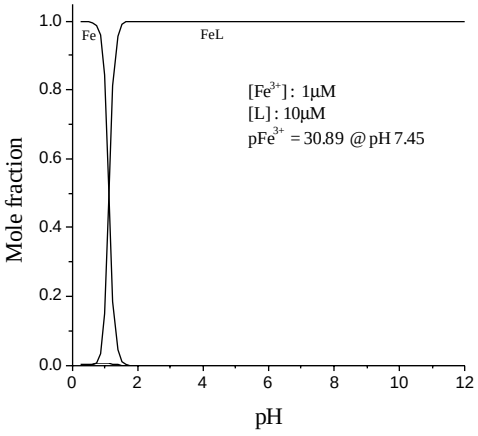


Figure 2. Speciation plot of **4** in the presence of iron(III). The pFe^{3+} value was determined by calculating the equilibrium concentration of free hexa-aquoiron(III) in a solution of pH 7.45 containing 10^{-6} M iron(III) and 10^{-5} M ligand.

The iron(III) affinity of dendrimer **10** was directly investigated by both dendrimer **10** and hexadentate **4** competing with a fluorescent probe CP691,[13] which is capable of binding iron(III) with high affinity. The fluorescence of CP691 is strongly quenched in the presence of iron(III). To ensure that chelators **10** and **4** possess the same amounts of iron-binding site in the iron competition studies, the final concentrations of chelators **10** and **4** were 2 and 6 μM, respectively. The iron-free CP691 fluorescence was set at 100% probe fluorescence intensity, and the fluorescence intensity of CP691 in the presence of equimolar amount of iron(III) was set at 0%. When CP691 was mixed with iron, the fluorescence intensity was quenched to a low level. However with an addition of a competing ligand, the fluorescence gradually increased. After the competition reached equilibrium (typically three days), the relative fluorescence intensity of the two solutions was found to be similar (Table 1), which indicated that the hexadentate moieties on dendrimer **10** possess a similar affinity for iron(III) as the hexadentate ligand **4**. As the hexadentate moieties on dendrimer **10** are composed of the same pyridinone bidentate ligands as hexadentate **4**, it is assumed that these hexadentate moieties possess the same pK_a values as those of **4**, the iron(III) affinity of the hexadentate pyridinone moieties on **10** can be calculated, namely log*K* = 34.8, pFe^{3+} = 30.6.

As described above, the three 3-hydroxypyridin-4-one ligands with the 2-position groups attached to the same building block could effectively constitute one hexadentate ligand. The dendritic chelator **10** effectively contains three such hexadentate centres, indeed the existence of the one-to-three dendrimer–iron complex was confirmed by using MALDI-TOF mass spectrometry. The MALDI-TOF mass spectrum obtained from a one-to-three mixture of dendritic chelator **10** and iron contains one signal at *m/z* 2155.6, which corresponds to the proton adduct of one-to-three dendrimer–iron complex also annotated as $[M+(Fe^{III}-3H)_3+H]^+$ (Fig. 3). Evidently,

Table 1. Comparison of the affinities of **4** and **10** for iron(III) by the fluorescence method

Time	Relative fluorescence intensity (%)	
	CP691 + Fe(III) + **4**	CP691 + Fe(III) + **10**
3 h	49.8	48.3
1 d	64.5	60.4
2 d	66.8	63.4
3 d	69.2	65.7
4 d	71.7	67.2
5 d	71.9	67.6

Experimental conditions: DMSO solutions of either **4** (600 μM) or **10** (200 μM) were added to a solution of CP691 and $FeCl_3$ in MOPS buffer (50% aqueous DMSO, v/v, pH 7.4). The final concentrations in 50% aqueous DMSO (v/v): [CP691] 6 μM, [Fe(III)] 6 μM, [**4**] 6 μM, [**10**] 2 μM.

three protons are released upon complexation of each iron(III). No evidence for the 1:2 and 1:1 dendrimer–iron complexes was obtained, indicating ideal stereochemistry of the nine hydroxypyridinones.

To further evaluate the iron(III) binding properties of dendrimer **10**, UV–vis spectra of a range of solutions containing different dendrimer and iron(III) ratios were determined scanning between 300 and 600 nm (Fig. 4). The intensity of absorbance at 400 nm increased until a 3:1 ratio of iron(III): **10** was obtained. With the iron(III): **10** ratios less than 3, the line is linear, confirming that chelator **10** possesses a high affinity for iron(III).

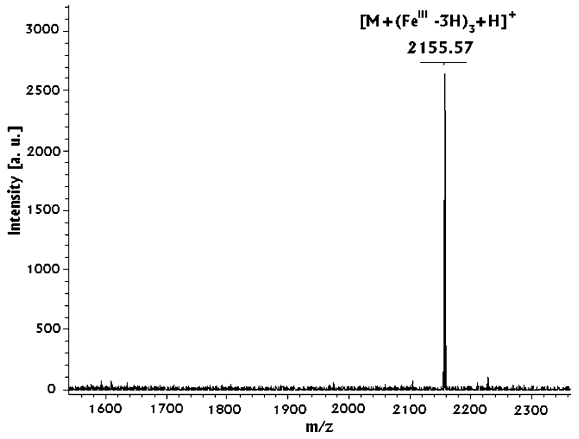


Figure 3. MALDI-TOF mass spectrum of the proton adduct of the 1:3 dendrimer–iron(III) complex $[M+(Fe^{III}-3H)_3+H]^+$ recorded from a MBT matrix.

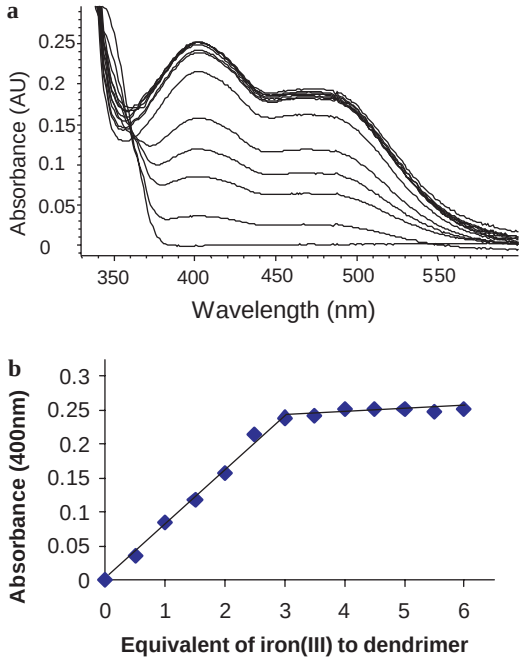


Figure 4. (a) UV–vis spectrophotometric titrations scanned between 300 and 600 nm; (b) plots of absorbance versus equivalent ratios of iron(III) to dendrimer **10** at 400 nm. Experimental conditions: a range of different ratio mixtures of dendrimer **10** and iron(III) were prepared by adding an iron solution (100 μM, 500 μM NTA in DMSO) to dendrimer (0.5 mL,100 μM in DMSO), followed by the addition of DMSO to a total volume of 3.5 mL, pyridine (0.3 mL) was added as a base. All samples were equilibrated at room temperature for 5 h.

In summary, a novel iron(III)-selective hydroxypyridinone hexadentate-terminated dendritic chelator has been demonstrated to possess a high affinity for iron(III). The potential of this molecule in iron-overload therapy is currently under investigation.

Acknowledgment

We are grateful for the financial support from EC Framework 5 project QLK1-2001-00444.

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4. Data for hydrochloric acid salt of hexadentate ligand **4**: ^{1}H NMR (DMDO-d_6, 360 MHz) δ 1.46 (m, CH_2, 6H), 1.93 (m, CH_2, 6H), 3.36 (m, CH_2, 6H), 5.24 (br, OH), 7.21 (d, J = 6.0Hz, pyridinone C-5H, 3H), 7.97 (d, J = 6.0 Hz, pyridinone C-6H, 3H), 9.04 (m, NH, 3H); ^{13}C NMR (DMDO-d_6, 90 MHz) δ 23.6 ($CCH_2CH_2CH_2NH$), 32.6 ($CCH_2CH_2CH_2NH$), 39.5 ($CCH_2CH_2CH_2NH$), 94.5 (O_2NC), 112.8 (*C*-5H in pyridine ring), 127.5 (*C*-2 in pyridine ring), 136.5 (*C*-6H in pyridine ring), 147.5 (*C*-3 in pyridine ring), 161.4 (*C*-4 in pyridine ring), 162.1 (*C*O). MS *m/z* calcd for $C_{28}H_{34}N_7O_{11}$ (M+H), 644.2311; found: MALDI-TOF MS (2-mercaptobenzothiazole matrix): 644.1; HRMS: 644.2320.
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8. Synthetic procedure: A solution of **8** (0.5 mmol) in DMF (10 mL) was added slowly to a solution of 2-[(succinimidyloxy)-carbonyl]-3,4-dibenzyloxypyridine (**2**) (5.4 mmol) in DMF (30 mL). After the mixture was left to stir for 3 days at room temperature, the solvent was removed under reduced pressure. The residue was chromatographed on silica gel eluting with $CHCl_3$/MeOH/40%NH_4OH (9:1:0.1) to give the compound **9** (83%) ^{1}H NMR (DMDO-d_6, 360 MHz) δ 1.46 (br, CH_2, 18H), 1.77 (br, CH_2, 18H), 3.17 (br, CH_2, 18H), 4.97 (s, CH_2, 18H), 5.21 (s, CH_2, 18H), 7.22 (m, total 36H; ArH, 27H, pyridinoneC5-H, 9H), 7.36 (m, ArH, 45H), 7.44 (m, ArH, 18H), 7.71 (br, ArH, 3H),

8.13 (d, J = 5.4Hz, pyridinoneC6-H, 9H), 8.32 (m, NH, 9H), 8.37 (br, NH, 3H). ^{13}C NMR (DMDO-d_6, 90 MHz) δ 23.5 ($CCH_2CH_2CH_2NH$), 32.5 ($CCH_2CH_2CH_2NH$), 39.6 ($CCH_2CH_2CH_2NH$), 70.4 ($PhCH_2O$), 75.4 ($PhCH_2O$), 110.9 (*C*-5H in pyridine ring), 128.2 (*C*H in Ar), 128.4 (*C*H in Ar), 128.5 (*C*H in Ar), 128.9 (*C*H in Ar), 136.2 (*C* in Ar), 137.5 (*C* in Ar), 142.9 (*C*-2 in pyridine ring), 145.6 (*C*-6H in pyridine ring), 148.3 (*C*-3 in pyridine ring), 158.8 (*C*-4 in pyridine ring), 165.2 (*C*O). ESIMS *m*/*z* calcd for $C_{219}H_{213}N_{21}O_{30}$ (M), 3619.2; found: *m*/*z* 1810.7 $[M+2H]^{2+}$, 1207.9 $[M+3H]^{3+}$.

9. The hydrochloric acid salt of the dendritic chelator **10**: In the atmosphere of nitrogen, 1 M boron trichloride in dichloromethane (27 mL, 27 mmol) was added slowly dropwise into an ice-bath cooled solution of **9** (0.5 mmol) in dichloromethane (10 mL). The mixture was stirred at room temperature for 2 days. Methanol (20 mL) was added to quench the reaction. After removal of the solvent, the residue was precipitated with methanol/acetone three times to afford hydrochloric acid salt of the dendritic chelator **10** as a white powder (88% yield).^{1}H NMR (DMDO-d_6, 360 MHz) δ 1.54 (br, CH_2, 18H), 1.84 (br, CH_2, 18H), 3.34 (br, CH_2, 18H), 6.82 (br, OH), 7.22 (d, J = 5.9Hz, pyridinoneC5-H, 9H), 7.94 (d, J = 5.9Hz, total 12H; pyridinoneC6-H, 9H, 3ArH are buried), 8.37 (s, CONH, 3H), 8.97 (s, CONH, 9H). ^{13}C NMR (DMDO-d_6, 100 MHz) δ 23.0 ($CCH_2CH_2CH_2NH$), 31.8 ($CCH_2CH_2CH_2NH$), 39.5 ($CCH_2CH_2CH_2NH$), 58.6 (NH*C*), 112.4 (*C*-5H in pyridine ring), 127.0 (*C*-2 in pyridine ring), 129.2 (*C*H in Ar), 135.1 (*C* in Ar), 135.9 (*C*-6H in pyridine ring), 147.2 (*C*-3 in pyridine ring), 161.6 (*C*-4 in pyridine ring), 162.0 (*C*O), 165.5 (Ar*C*O). ESIMS *m*/*z* calcd for $C_{93}H_{105}N_{21}O_{30}$ (M), 1997.0; found: *m*/*z* 1997.7 $[M+H]^+$, 999.7 $[M+2H]^{2+}$.

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13. CP691 was supplied by Yong Min Ma. The compound (>95% purity) is a hexadentate pyridinone and has been fully characterized by ^{1}H NMR, mass spectroscopy, and elemental analysis.