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Synthesis, iron(III)-binding affinity and in vitro evaluation of 3-hydroxypyridin-4-one hexadentate ligands as potential antimicrobial agents

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ABSTRACT

Iron is a critical element for the survival of bacteria. We have designed and synthesized two novel 3-hydroxypyridin-4-one hexadentate ligands with high affinity for iron(III), which disrupt bacterial iron absorption. Biological studies demonstrate that these two chelators have significant inhibitory effect against both Gram-positive and Gram-negative bacteria, and therefore have potential as antimicrobial agents.

As metal ions are essential for all living processes, using selective metal chelators as therapeutical agents, namely chelation therapy, has received increasing attention. Iron chelators, for instance desferrioxamine B (DFO),[1] deferiprone[2] and exjade[3] have been used for the treatment of iron overload disorders associated with β-thalassaemia and sickle cell anaemia. Iron is an essential element for the growth of virtually all bacteria and fungi, consequently microorganisms have developed efficient methods of absorbing iron from the environment. Many microorganisms secrete siderophores in order to scavenge iron.[4] Such methods of uptake can be circumvented by the introduction of high affinity iron selective chelating agents. The iron affinity of these agents must be extraordinally high, so that they can efficiently compete with siderophores. Furthermore, the structure of chelators should differ appreciably from those of siderophores, otherwise the iron-chelator complex will be recognized by the iron-siderophore receptors and utilized by the microorganism. Antimicrobial activity of chelators has been previous reported,[5–9] however, most chelators investigated for this purpose have been bidentate ligands, which possess a relatively low affinity for iron(III).[10] In contrast hydroxypyridinone hexadentate ligands, possess a high affinity for iron(III)[11,12] and have different structures and net charges to those of typical siderophores. Thus, in principle, they should inhibit the growth of bacteria. Indeed, our recent work has demonstrated that the hexadentate hydroxypyridinone (**1**) exhibits a stronger inhibitory effect on the growth of bacteria than the commercially available chelator DTPA.[13] Herein we report the synthesis, iron-chelating properties and antimicrobial activity of two structurally related 3-hydroxypyridin-4-one hexadentate ligands.

The synthesis of the two 3-hydroxypyridin-4-one hexadentate ligands, begins with commercially available kojic acid (**2**) and is presented in Scheme 1. The benzyl-protected bidentate hydroxypyridinone, which contains a free amino group, 2-(aminomethyl)-3-(benzyloxy)-1,6-dimethylpyridin-4(1*H*)-one (**6**) was synthesized from kojic acid by following an established procedure with slight modifications.[14] Kojic acid was treated with thionyl chloride, followed by reduction with zinc in hydrochloric acid to give allomaltol (**3**). The α-position with respect to the ring hydroxyl was then functionalized in an analogous fashion to that of the aldol condensation, followed by benzyl protection of the 3-hydroxyl group, giving compound **4**. The 2-hydroxyl function of **4** was protected using 3,4-dihydro-2*H*-pyran before reaction with methylamine. The pyran protecting group was subsequently removed under mild acid conditions, yielding product **5**. Amine **6** was obtained by the treatment of **5** with thionyl chloride, followed by the reaction with ammonium solution. Amine **6** was conjugated to the two triacids, nitrilotriacetic acid and 3,3′,3″-nitrilotripropionic acid, via amide bonds in the presence of 1-hydroxybenzotriazole (HOBt) and 1,3-dicyclohexylcarbodiimide (DCC) in *N*,*N*-dimethylformamide (DMF) at room temperature, providing the protected hexadentate ligands **7**.[15] Deprotection of the benzyl groups on **7** was

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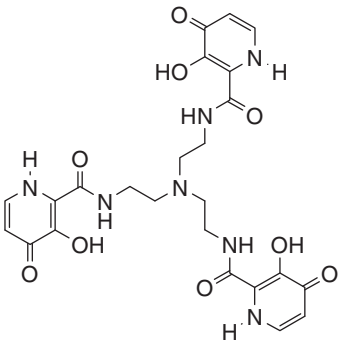
achieved by hydrogenation in the presence of palladium/charcoal, generating the hexadentate chelators **8**.[16] All the compounds have been fully characterized by ^{1}H NMR, ^{13}C NMR, MS and HRMS.



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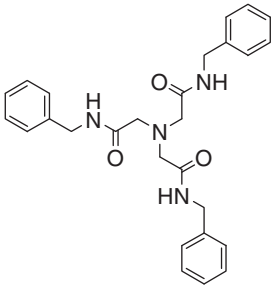

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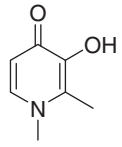

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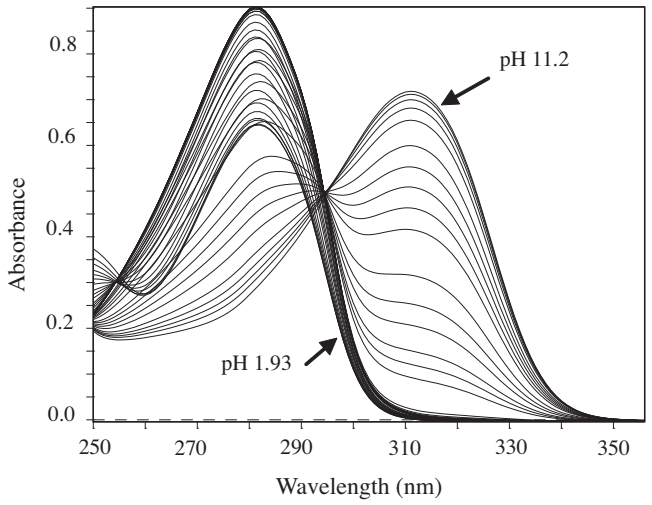
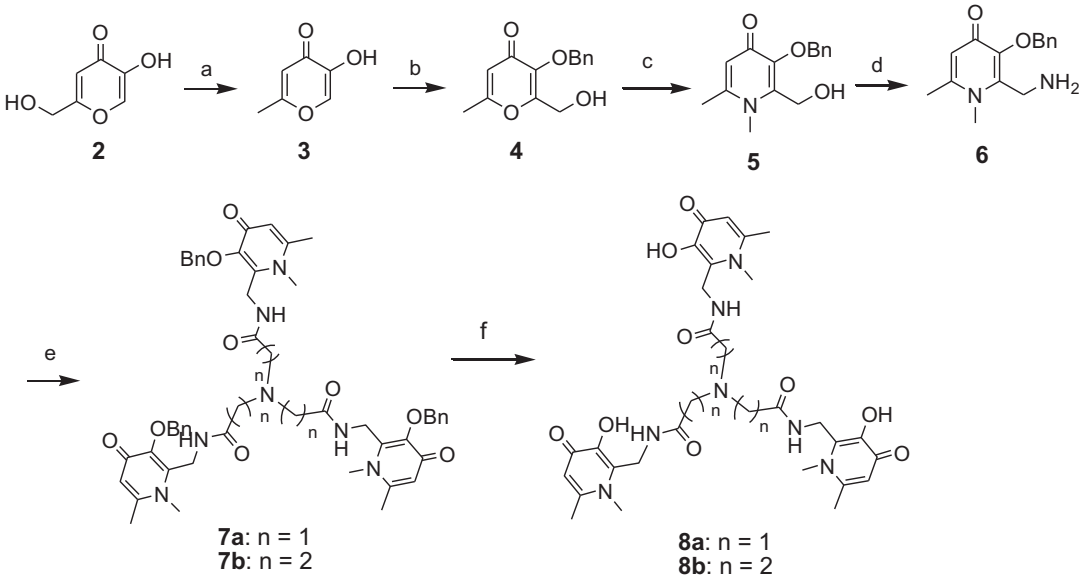


Figure 1. UV spectra of **8a**. [**8a**] = 24.5 μM, pH was changed from 1.93 to 11.20 by the addition of KOH.

The carbon chain of the tripodal scaffold in the hexadentate chelator **8a** is shorter than that of **8b**. Thus, although the hexadentate chelators **8a** and **8b** possess the same chelating group, the stability of their iron complexes is predicted to be slightly different.

In order to further demonstrate the ligand affinity, both pK_a values of the hexadentate chelators **8a** and **8b** and their stability constants for the corresponding iron(III) complexes were evaluated using an automated titration system.[17–19] All the titration data were analyzed using the pHab software.[20] The pH dependence UV spectra of **8a** (Fig. 1) was recorded between 250 and 350 nm over the pH range 1.93–11.20 for the free ligand. The speciation spectra demonstrate a clear shift in λ_{max} from 280 to 310 nm, which reflects the pH dependence of the ligand ionization equilibrium. The pK_a values of **8a** obtained from nonlinear least-squares regression analysis are 2.58, 3.30, 3.97, 8.41, 9.30 and 10.12. Of the six pK_a values, the lower three values correspond to the 4-oxo functions and the higher three correspond to the 3-hydroxyl functions. Surprisingly, the pK_a for the tertiary amine function of



Scheme 1. Reagents and conditions: (a) (i) $SOCl_2$, (ii) Zn/HCl, 70%; (b) (i) HCHO, (ii) BnCl, 65%; (c) (i) 2,3-dihydro-4*H*-pyran, TsOH, (ii) $MeNH_2$, (iii) 6 M HCl, 85%; (d) (i) $SOCl_2$, (ii) 37% NH_3, MeOH, 72% in two steps; (e) triacid, HOBt, DCC, DMF, rt, 88% for **7a** and 83% for **7b**; (f) H_2 (30 psi), 5% Pd/C (20% W/W), MeOH, HCl, 93% for **8a** and 90% for **8b**.

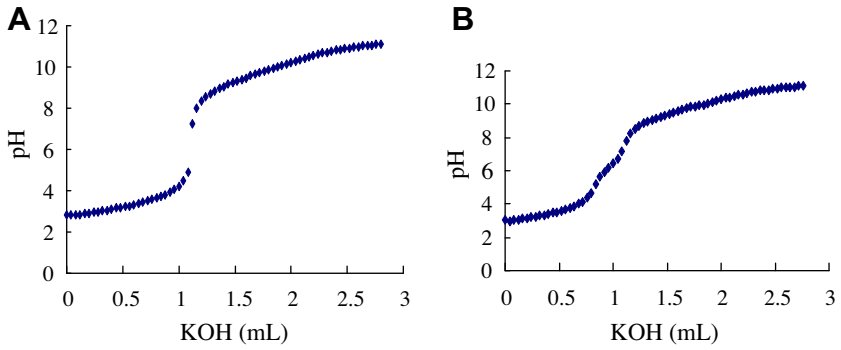


Figure 2. A: Potentiometric titration of **8a** (18.5 mg in 20 mL KCl) at 25 °C. B: Potentiometric titration of **8b** (18.9 mg in 20 mL KCl) at 25 °C.

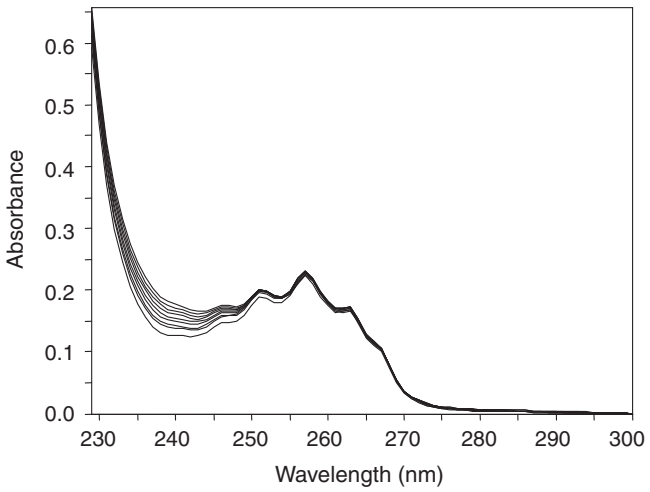


Figure 3. Spectrophotometric titration of **9** (140.6 μM in 20.006 mL 0.1 M KCl) at 25 °C, pH was changed from7.17 to 0.192.

8a was not detected, even with potentiometric titration (Fig. 2A). The corresponding pK_a values of **8b** were determined as: 2.98, 3.59, 3.89, 8.03, 8.94 and 9.81. Again, the tertiary amine pK_a value was not detected during the spectrophotometric titration although it was clearly identified by potentiometric titration (Fig. 2B) where a buffer range with a corresponding pK_a value (6.18+/−0.072) was observed. In order to investigate further the pK_a value associated with the tertiary amine function of compound **8a**, an analogue based on **8a** which has the 3-hydroxypyridin-4-one groups replaced by benzene (**9**) was also synthesized using a reported method.[21] Only one pK_a value exists for this compound and by spectrophotometric titration a surprisingly low pK_a value (1.41+/−0.05) was identified (Fig. 3). This indicates that the three amide functions adjacent to the tertiary amine function, exert a strong electron withdrawing effect and considerably decrease the charge density on the central nitrogen atom. In contrast with **8b**, due to the longer carbon chains there is a much weaker electron withdrawing effect. Significantly the pK_a value of **1** is intermediate between the two values obtained for **8a** and **8b**, namely 4.52.[11]

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 **8a** and **8b** for iron(III) were determined to be 35.07 and 33.76, respectively, using a spectrophotometric titration against the hydroxyl anion (Fig. 4A). Under exactly the same experimental conditions, the **8a-**iron(III) complex was found to be stable at higher pH values than that of the **8b-**iron(III) complex. As depicted in Figure 4B (455 nm) **8b** starts to dissociate at pH 12 while **8a** starts at pH 12.5. In order to comprehend the superior iron(III) affinity of **8a**, we applied molecular dynamics simulation studies on these two iron complexes.[22] After 48,000 simulations, an optimized structure was obtained for each iron(III) complex model (Fig. 5). The corresponding bidentate iron(III) complex with 1,2-dimethyl-3-hydroxypyridin-4-one (deferiprone, **10**) was also investigated using the same process. We found that **8a** has less octahedron distortion than **8b**, when compared with the bidentate counterpart (Table 1). For bidentate ligands there is no

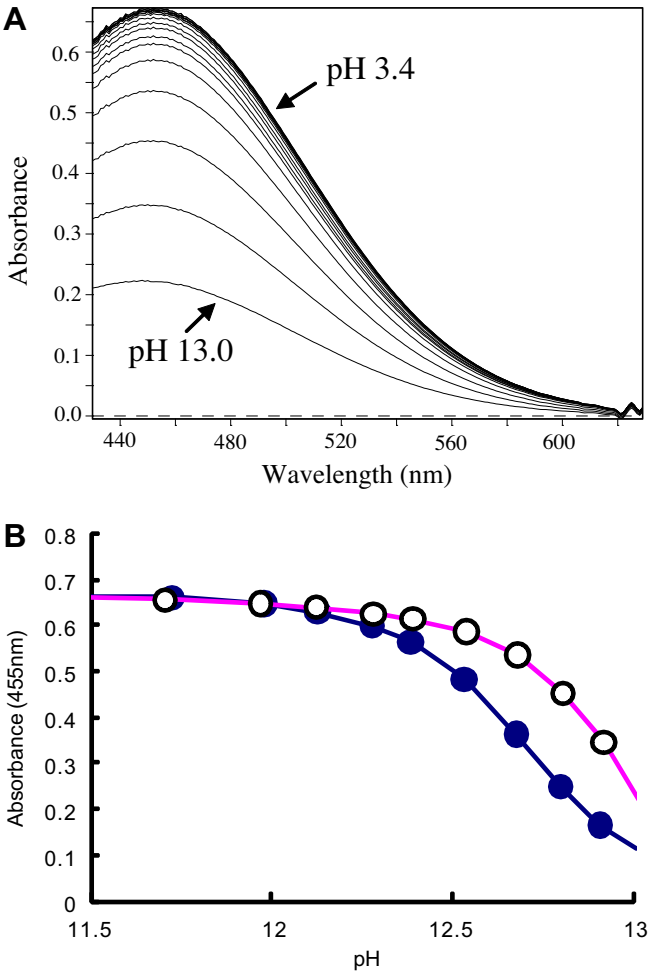


Figure 4. A: UV spectra of **8a** in the presence of iron(III). [**8a**] = 32.7 μM, [Fe^{3+}] = 29.8 μM. The pH was changed from 3.436 to 13.016 by the addition of KOH. B: The stability of the iron complexes of **8a** (○) and **8b** (●) as a function of pH, as monitored by the absorption at 455 nm.

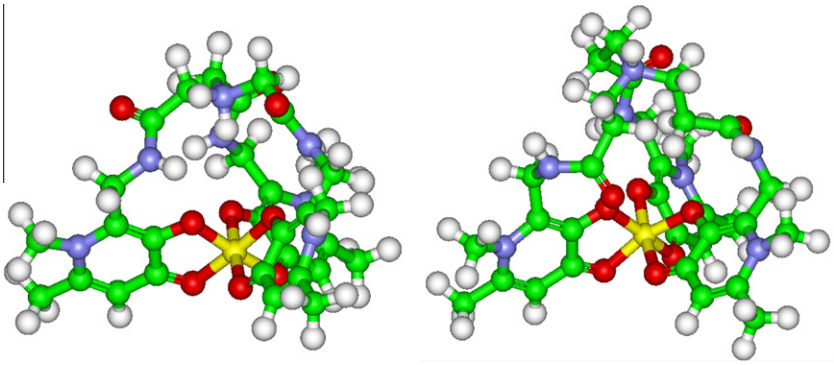


Figure 5. A: Optimized structures of **8a**-iron complex (left) and **8b**-iron complex (right) obtained by molecular dynamic simulation studies.[21] C(green); O(red); iron(yellow) N(blue) H(white).

Table 1
Calculated octahedron angle for iron(III) complexes

	Angle 1	Angle 2	Angle 3	Average	Difference with deferiprone
Deferiprone	174.7605	173.6917	172.0701	173.5074	0
8a	175.2007	175.1695	175.2263	175.1988	1.6914
8b	176.3598	176.4442	176.3909	176.3983	2.8909

Table 2
Antimicrobial activity of chelators (MIC)[a]

Chelators	*S. aureus*		*B. cereus*		*B. subtilis*		*E. coli*		*P. aeruginosa*	
	μg/mL	μM	μg/mL	μM	μg/mL	μM	μg/mL	μM	μg/mL	μM
1	40	56.9	40	56.9	40	56.9	80	113.7	24	34.1
8a	40	50.8	40	50.8	40	50.8	40	50.8	40	50.8
8b	40	48.2	80	96.4	80	96.4	40	48.2	40	48.2
DTPA	40	101.7	80	203.4	80	203.4	80	203.4	80	203.4

[a] MICs for bacteria were determined on BHI agar after 24 h of incubation at 37 °C, according to the standard agar dilution technique and with visual inspection of bacterial growth.[23] The tubes were seeded at a density of approximately 1.0×10^7 colony-forming units per milliliter (CFU/mL).

limitation resulting from intramolecular links, as the ligands will position themselves with respect to the central metal ion in order to achieve maximum bonding overlap for coordination bond formation. In contrast, when joined by a covalent structure, the chelating moieties will experience a restrictive inflexibility and will probably be unable to achieve optimal distribution around the coordinated metal. Significantly the arm length of **8a** provides an angle (175.2) which is closer to that of deferiprone (173.5) than that of **8b** (176.4).

By using the determined pK_a values and log stability constants, the pFe^{3+} value, which is defined as the negative logarithm of concentration of free iron(III) in solution under conditions total [ligand] = 10^{-5} M and total [iron] = 10^{-6} M at pH 7.45 was calculated. The corresponding pFe^{3+} values of **8a** and **8b**, under these conditions, are 30.36 and 30.04, respectively, indicating **8a** possesses a moderately higher affinity for iron(III) than **8b**, which is in agreement with the result of the molecular dynamics simulation studies of the iron complexes.

The antimicrobial activity of chelators **8a** and **8b** was evaluated in direct comparison with that of diethylene-triamine pentaacetic acid (DTPA). It was found that both chelators demonstrated a strong inhibitory effect on the growth of the three tested Gram positive bacteria (*Staphyloccocus aureus*, *Bacillus subtilis* and *Bacillus cereus*) and the two Gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) (Table 2). In the case of *S. aureus*, the effect of both **8a** and **8b** was similar to that of DTPA, with a MIC of 40 μg/mL. Against *B. cereus and B. subtilis*, **8a** showed the strongest inhibitory activity with a MIC of 40 μg/mL. In the case of *E. coli* and *P. aeruginosa*, both **8a** and **8b** exhibited relatively stronger inhibition than DTPA with a MIC of 40 μg/mL. In comparison with **1**, **8a** exhibited similar inhibitory effect against the three tested Gram positive bacteria. **8a** and **8b** showed stronger inhibition than **1** against *E. coli*, but a relatively weaker inhibitory activity against *P. aeruginosa*.

Overall, of the chelators investigated, **8a** and **8b** showed very similar antimicrobial activity and they were found to possess stronger antimicrobial activity than DTPA in most cases. This is primarily due to the fact that **8a** and **8b** possess a higher affinity for iron(III) than DTPA (pFe = 24.6).[24] The calculated partition coefficients ($c \log P$) of **8a** and **8b**, obtained by MarvinView 5.4.0.1 software,[25] were found to be −2.27 and −1.56, respectively, this hydrophilicity together with the relatively high molecular weights (**8a**: 641, **8b**: 683) would suggest that these complexes will not be able to cross bilayer membranes by non-facilitated diffusion. Thus with Gram negative bacteria they may gain access to the periplasmic space and there restrict the availability of iron to the bacterium. These chelators may also inhibit the growth of bacteria by scavenging iron in the environment around the bacteria. **8a** and **8b** may find application in the treatment of external infections such as those associated with wounds.

Acknowledgments

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15. Data for **7a**: ^{1}H NMR (DMSO-d_6) 2.22 (s, CH_3, 9H), 3.30 (s, CH_2, 6H), 3.34 (s, CH_3, 9H), 4.35 (d, J = 5.0 Hz, CH_2, 6H), 5.07 (s, CH_2, 6H), 6.18 (s, Pyridinone 5C-H, 3H), 7.29–7.35 (m, Ph, 9H), 7.42 (m, Ph, 6H), 8.39 (t, J = 5.0 Hz, NH, 3H); ^{13}C NMR (DMSO-d_6) δ 20.0 (CH_3), 34.1 (NHCH$_2$-pyridinone), 35.6 (NCH$_3$), 57.4 (NCH$_2$CO), 72.1 (CH$_2$Ph), 117.4 (*C*-5H in pyridinone), 127.7 (*C*H in Ph), 128.1 (*C*H in Ph), 128.3 (*C*H in Ph), 137.6 (*C* in Ph), 140.0 (*C*-2 in pyridinone), 145.6 (*C*-3 in pyridinone), 147.7 (*C*-6 in pyridinone), 170.5 (*C*-4 in pyridinone), 171.9 (CONH). ESI-MS: 912 ([M+H]$^+$); HRMS: Calcd for $C_{51}H_{58}N_7O_9$ ([M+H]$^+$) 912.4295, found 912.4270. Data for **7b**: ^{1}H NMR (DMSO-d_6) 2.18 (m, CH_2, 6H), 2.25 (s, CH_3, 9H), 2.60 (m, CH_2, 6H), 3.37 (s, CH_3, 9H), 4.33 (d, J = 4.9 Hz, CH_2, 6H), 5.07 (s, CH_2, 6H), 6.17 (s, Pyridinone 5C-H, 3H), 7.28–7.36 (m, Ph, 9H), 7.42 (m, Ph, 6H), 8.13 (t, J = 4.9 Hz, NH, 3H); ^{13}C NMR (DMSO-d_6) δ 20.0 (CH_3), 32.8 (NCH$_2$CH$_2$), 34.3 (NHCH$_2$-pyridinone), 35.6 (NCH$_3$), 48.9 (NCH$_2$CH$_2$), 72.2 (CH$_2$Ph), 117.4 (*C*-5H in pyridinone), 127.8 (*C*H in Ph), 128.2 (*C*H in Ph), 128.3 (*C*H in Ph), 137.6 (*C* in Ph), 140.3 (*C*-2 in pyridinone), 145.6 (*C*-3 in pyridinone), 147.8 (*C*-6 in pyridinone), 171.2 (*C*-4 in pyridinone), 172.0 (CONH). ESI-MS: 954 ([M+H]$^+$); HRMS: Calcd for $C_{54}H_{64}N_7O_9$ ([M+H]$^+$) 954.4764, found 954.4751.
16. Data for **8a**: ^{1}H NMR (DMSO-d_6) 2.55 (s, CH_3, 9H), 3.55 (br, CH_2, 6H), 3.85 (s, CH_3, 9H), 4.62 (d, J = 4.5 Hz, CH_2, 6H), 7.33 (s, Pyridinone 5C-H, 3H), 9.29 (br, NH, 3H); 10.70 (br, 1H, HN$^+$); ^{13}C NMR (DMSO-d_6) δ 20.6 (CH_3), 34.6 (NHCH$_2$-pyridinone), 39.3 (NCH$_3$), 56.8 (NCH$_2$CO), 112.6 (*C*-5H in pyridinone), 139.4 (*C*-2 in pyridinone), 142.6 (*C*-3 in pyridinone), 148.3(*C*-6 in pyridinone), 159.3 (*C*-4 in pyridinone), 211.0 (CONH). ESI-MS: 642 ([M+H]$^+$), 664 ([M+Na]$^+$); HRMS: Calcd for $C_{30}H_{40}N_7O_9$ ([M+H]$^+$) 642.2886, found 642.2883. Data for **8b**: ^{1}H NMR (DMSO-d_6) 2.58 (s, CH_3, 9H), 2.75 (t, J = 7.0 Hz, CH_2, 6H), 3.28 (m, CH_2, 6H), 3.90 (s, CH_3, 9H), 4.62 (d, J = 4.8 Hz, CH_2, 6H), 7.35 (s, Pyridinone 5C-H, 3H), 9.01 (t, J = 4.7 Hz, NH, 3H), 11.00 (br, HN$^+$, 1H); ^{13}C NMR (DMSO-d_6) δ 20.5 (*C*H$_3$), 28.8 (NCH$_2$CH$_2$), 34.5 (NHCH$_2$-pyridinone), 39.2 (NCH$_3$), 48.2 (NCH$_2$CH$_2$), 112.5 (*C*-5H in pyridinone), 139.4 (*C*-2 in pyridinone), 142.6 (*C*-3 in pyridinone), 148.3(*C*-6 in pyridinone), 159.3 (*C*-4 in pyridinone), 169.3 (CONH). ESI-MS: 684.3 ([M+H]$^+$), 342.7 ([M+2H]$^{2+}$); HRMS: Calcd for $C_{33}H_{46}N_7O_9$ ([M+H]$^+$) 684.3356, found 684.3320.
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