Electronic supplementary information

Novel 3-Hydroxypyridin-4-one Hexadentate Ligand-Based Polymeric Iron Chelator: Synthesis, characterization and Antimicrobial Evaluation

Ying-Jun Zhou [a], Xiao-Le Kong [b], Jun-Pei Li [b], Yong-Min Ma [c], Robert C Hider [b], Tao Zhou[a]*

[a] *School of Food Science and Biotechnology, Zhejiang Gongshang University, Xiasha, Hangzhou, Zhejiang, 310018, P. R. China.*

[b] *Division of Pharmaceutical Science, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London, SE1 9NH, UK.*

[c] *College of Pharmaceutical Science, Zhejiang Chinese Medical University, Hangzhou, Zhejiang, 310053, P. R. China.*

Experimental

Instruments. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance 500 spectrometer (Bruker Corp.,Germany) with TMS as an internal standard. Electrospray ionization (ESI) mass spectra were obtained by infusing samples into an LCQ Deca XP ion-trap instrument (ThermoFinnigan, San Jose, CA). High resolution mass spectra (HRMS) were determined on a QTOFMicro (Waters, U.S.) by direct infusing samples into the ESI source.

General synthesis. All chemicals were of AR grade and used without any further

purification.

*Synthesis of 3-(benzyloxy)-2-methyl-4H-pyran-4-one (**2**)*. To a solution of maltol (**1**) (100 g, 0.79 mol) in methanol (500 mL) was added NaOH solution (35 g in 100 mL of H_2O) dropwise with stirring vigorously. The mixture was heated to reflux, and BnCl (120g, 0.94 mol) was added dropwise over 0.5 h. The reaction was monitored by TLC and the reflux was continued for about 8 h. The reaction mixture was cooled to room temperature, filtered to remove the inorganic salt, and the filtrate was concentrated under reduced pressure to remove most of the organic solvent. After extraction with CH_2Cl_2 (3×100 mL), the combined organic extracts were washed with 5% NaOH (2×100 mL) and brine, and 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. Yield: 82%. ^{1}H NMR ($CDCl_3$, 500MHz) δ 2.08 (s, 3H, CH_3), 5.16 (s, 2H, CH_2), 6.35 (d, *J*=5.5 Hz, 1H, C5-H in Pyridinone), 7.30-7.40 (m, 5H, Ph), 7.58 (d, *J*=5.5 Hz, 1H, C6-H in Pyridinone). ESI-MS: *m/z* 217 ($[M+H]^+$), calcd. for $C_{13}H_{13}O_3$ 217.

*Synthesis of 3-(benzyloxy)-1-(2-hydroxyethyl)-2-methylpyridin-4(1H)-one (**3**)*. A mixture of **2** (5g, 23.15mmol), ethanolamine (1.7g, 27.87mmol), and NaOH (2g, 50mmol) in methanol/water (20mL/20mL) was refluxed. The reaction was monitored by TLC. After completion of the reaction (about 2h), the reactant was concentrated under reduced pressure to about half volume. Extracted with dichloromethane (3×40mL), the combined organic layers were washed with brine twice and dried over anhydrous sodium sulfate. After removal of the solvent, the crude product **3** was

obtained as a brown solid. Yield: 85%. ^{1}H NMR ($CDCl_3$, 500MHz) δ 2.11 (s, 3H, CH_3), 3.84 (m, 4H, NC***H₂***C***H₂***), 4.95 (s, 2H, PhC***H₂***), 6.15 (d, *J* = 7.5 Hz, 1H, C5-H in Pyridinone), 7.29-7.35 (m, 5H, Ph), 7.38 (d, *J* = 7.5Hz, 1H, C6-H in Pyridinone). ESI-MS: *m/z* 260 ($[M+H]^+$), calcd. for $C_{15}H_{18}NO_3$ 260.

*Synthesis of 3-(benzyloxy)-1-(2-(benzyloxy)ethyl)-2-methylpyridin-4(1H)-one (**4**)***.* To a solution of **3** (10g, 38.6mmol） in dry THF (80mL) was added sodium hydride (2.3g, 96.5mmol) and BnCl (5.9g, 46.32mmol). The mixture was refluxed for about 5h. Water was added dropwise cautiously to quench the reaction. The reactant was concentrated and then was dissolved in dichloromethane, washed with brine twice and dried over anhydrous sodium sulfate. After removal of the solvent, the crude product **4** was obtained as a brown oil. Yield: 90%. ^{1}H NMR ($CDCl_3$, 500MHz) δ 2.07 (s, 3H, CH_3), 3.58 (t, *J* = 5.0 Hz, 2H, NC***H₂***), 3.92 (t, *J* = 5.0 Hz, 2H, C***H₂***OBn), 4.42 (s, 2H, CH_2OC***H₂***Ph), 5.19 (s, 2H, OC***H₂***Ph), 6.38 (d, *J* = 7.5Hz, 1H, C5-H in Pyridinone), 7.15-7.40 (m, 11H, C6-H in pyridinone and 2Ph). ESI-MS: *m/z* 350 ($[M+H]^+$), calcd. for $C_{22}H_{24}NO_3$ 350.

*Synthesis of 3-(benzyloxy)-1-(2-(benzyloxy)ethyl)-2-(methylaldehyde)pyridine -4(1H)-one (**5**)***.* A mixture of **4** (10g, 28.65mmol), SeO_2 (9.54g, 85.95mmol) in acetic acid/acetic anhydride (60mL/60mL) was heated at 90-100^{o}C for 3-4h. The reactant was filtered, and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography using EtOAc/MeOH (50:1-20:1) as an eluent to provide aldehyde **5** as a brown oil. Yield: 63%. ^{1}H NMR ($CDCl_3$, 500MHz) δ 3.46 (t, *J* = 5.0 Hz, 2H, NC***H₂***), 4.36 (t, *J* = 5.0 Hz, 2H, C***H₂***OBn), 4.52 (s,

2H, $CH_2OC\boldsymbol{H_2}Ph$), 5.49 (s, 2H, $OC\boldsymbol{H_2}Ph$), 6.60 (d, *J* = 7.5Hz, 1H, C5-H in pyridinone), 7.15-7.37 (m, 11H, 2Ph and C6-H in Pyridinone), 9.98 (s, 1H, CHO). ESI-MS: *m/z* 364 ($[M+H]^+$), calcd. for $C_{22}H_{22}NO_4$ 364.

Synthesis of 3-(benzyloxy)-1-(2-(benzyloxy)ethyl)-2-(hydroxymethyl)pyridine -4(1H)-one (**6**). To a solution of **5** (5g, 13.8mmol) in ethanol (28mL) was added $NaBH_4$ (0.26g, 6.9mmol). The mixture was stirred at room temperature for 3h. The reactant was concentrated and then was dissolved in CH_2Cl_2, washed with 5% $NaHCO_3$ twice and brine, dried over anhydrous sodium sulfate. After removal of the solvent, the residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH, 50:1-10:1) to give product **6** as a white powder. Yield: 85%. ^1H NMR ($CDCl_3$, 500MHz) δ 3.62 (t, *J* = 5.0Hz, 2H, $NC\boldsymbol{H_2}$), 4.14 (t, *J* = 5.0Hz, 2H, $C\boldsymbol{H_2}OBn$), 4.39 (s, 2H, $C\boldsymbol{H_2}OH$), 4.49 (s, 2H, $CH_2OC\boldsymbol{H_2}Ph$), 5.01 (s, 2H, $OC\boldsymbol{H_2}Ph$), 6.34 (d, *J* = 7.5Hz, 1H, C5-H in Pyridinone), 7.14 (m, 2H, Ph), 7.20 (d, *J* = 7.5Hz, 1H, C6-H in Pyridinone), 7.24-7.30 (m, 8H, Ph). ESI-MS: *m/z* 366 ($[M+H]^+$), calcd. for $C_{22}H_{24}NO_4$ 366.

Synthesis of 2-(aminomethyl)-3-(benzyloxy)-1-(2-(benzyloxy)ethyl)pyridine-4(1H) -one (**7**). Thionyl chloride (40mL) was added slowly to a flask containing **6** (5g, 13.7mmol). The resulting solution was stirred at room temperature for 2h. The reaction mixture was concentrated to dryness under reduced pressure. The residue was dissolved in methanol (40mL), followed by the addition of ammonia solution (25%, 30mL). The resulting solution was stirred at 50°C for 4h. After removal of the solvent, the residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH, 20:1

to 5:1) to provide amine **7** as a brown oil. Yield: 59%. ^{1}H NMR ($CDCl_3$, 500MHz) δ 3.66 (t, *J* = 5.0Hz, 2H, NC***H*****$_2$**), 3.70 (s, 2H, C***H*****$_2$**NH_2), 4.18 (t, *J* = 5.0Hz, 2H, C***H*****$_2$**OBn), 4.45 (s, 2H, CH_2OC***H*****$_2$**Ph), 5.31 (s, 2H, OC***H*****$_2$**Ph), 6.45 (d, *J* = 7.5 Hz, 1H, C5-H in Pyridinone), 7.18 (m, 2H, Ph), 7.29-7.41 (m, 9H, 1H from C6-H in pyridinone, 8H from Ph). ESI-MS: *m/z* 365 ($[M+H]^+$), calcd. for $C_{22}H_{25}N_2O_3$ 365.

Synthesis of 3-acryloylamino-propionic acid (**9**). Acryloyl chloride (2.17g, 24mmol) was added to a solution of β-alanine ethyl ester hydrochloride (3.07g, 20mmol) in dry CH_2Cl_2 (40mL) cooled with an ice bath, and then triethylamine (4.44g, 44mmol) was added dropwise. The mixture was stirred at 0^{o}C for 2h, and then at room temperature overnight. The reaction mixture was washed with 5% $NaHCO_3$, brine, and dried in anhydrous Na_2SO_4. After removal of the solvent, the residue was chromatographed on silica gel using EtOAc/Hexane (1:1) as an eluent to provide a colorless liquid product. The product was then dissolved in methanol (20mL) cooled with an ice bath, followed by the addition of sodium hydroxide (20mL, 2M). The mixture was stirred at 0^{o}C for 2h. The reaction mixture was neutralized with Amberlite® IR120 (H form) ion exchange resin to pH below 4. After filtration, the filtrate was concentrated to provide the product **9** as a colorless oil. Yield: 67% (two steps). ^{1}H NMR (DMSO, 500 MHz) δ 2.42 (t, *J* = 6.8 Hz, 2H, CH_2), 3.32 (m, 2H, CH_2), 5.56 (m, 1H, vinyl), 6.07 (m, 1H, vinyl), 6.21 (m, 1H, vinyl), 8.15 (s, 1H, NH), 12.20 (s, 1H, COOH). ESI-MS: *m/z* 144 ($[M+H]^+$), calcd. for $C_6H_{10}NO_3$ 144.

*Procedure for preparation of **11***. To a solution of **9** (1.32g, 9.25mmol) in DMF (45mL) was added amine **10** (3.84g, 9.25mmol) and triethylamine (1.87g, 18.5mmol)

cooled with an ice bath, and then HCTU (3.83g, 9.25mmol) was added in three portions. The mixture was stirred at room temperature overnight. The reactant was concentrated under reduced pressure and then was dissolved in CH_2Cl_2, washed with 5% $NaHCO_3$ twice and brine, dried over anhydrous sodium sulfate. After removal of the solvent, the residue was purified by silica gel column chromatography (EtOAc/Hexane, 1:1 to EtOAc) to give product **11** as a white solid. Yield: 64%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.45 (s, 27H), 1.98 (m, 6H, CH_2), 2.21 (m, 6H, CH_2), 2.40 (t, J = 5.5Hz, 2H, CH_2), 3.59 (m, 2H, CH_2), 5.62 (m, 1H, vinyl), 6.13 (m, 1H, vinyl), 6.28 (m, 1H, vinyl), 6.70 (s, 1H). ESI-MS: *m/z* 541 ($[M+H]^+$), calcd. for $C_{28}H_{49}N_2O_8$ 541.

*Procedure for preparation of **12***. A solution of **11** (3g, 5.55mmol) in formic acid (40mL, 88%) was stirred at room temperature overnight. After concentration and removal of residual formic acid by the addition of toluene (3×15 mL), the product **12** was obtained as a white powder. Yield: 100%. ^{1}H NMR (DMSO, 500 MHz) δ 1.83 (m, 6H, CH_2), 2.11 (m, 6H, CH_2), 2.29 (t, J = 7.1 Hz, 2H), 2.51 (m, *2*H, CH_2), 5.55 (m, 1H, vinyl), 6.06 (m, 1H, vinyl), 6.21 (m, 1H, vinyl), 7.21 (s, 1H, NH), 8.06 (t, J = 5.4 Hz, 1H, NH). ESI-MS: *m/z* 373 ($[M+H]^+$), calcd. for $C_{16}H_{25}N_2O_8$ 373.

*Synthesis of protected hexadentate ligand **13***. A mixture of **7** (1.78g, 4.89mmol), **12** (0.5g, 1.36mmol) in dry DMF (10mL) cooled with an ice bath was added DIPEA (1.9g, 14.7mmol), and then HCTU (2.03g, 4.9mmol) was added in three portions. The mixture was stirred at room temperature overnight. The reactant was concentrated under reduced pressure and then was dissolved in CH_2Cl_2, washed with 5% $NaHCO_3$

twice and brine, dried over anhydrous sodium sulfate. After removal of the solvent, the residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH, 20:1 to 5:1) to give product **13** as a light yellow powder (1.45g). Yield: 75%. ^{1}H NMR (DMSO, 500 MHz) δ 1.77 (m, 6H, CH_2), 1.99 (m, 6H, CH_2), 2.24 (t, J = 7.0 Hz, 2H, CH_2), 3.28 (m, 2H, CH_2), 3.60 (t, J = 4.9 Hz, 6H), 4.10 (t, J = 4.8 Hz, 6H), 4.30 (d, J = 5.0 Hz, 6H), 4.43 (s, 6H, CH_2), 5.10 (s, 6H, CH_2), 5.47 (m, 1H, vinyl), 6.01 (m, 1H, vinyl), 6.16 (m, 1H, vinyl), 6.23 (d, J = 7.5 Hz, 3H, C5-H in Pyridinone), 7.19-7.42 (m, 30H, Ph), 7.61 (d, J = 7.5 Hz, 3H, C6-H in Pyridinone), 8.04 (t, J = 4.8 Hz, 4H, NH). ESI-MS: *m/z* 1412 ([M+H]$^+$), calcd. for $C_{82}H_{91}N_8O_{14}$ 1411.7.

Hexadentate ligand hydrochloride salt (**14**). Under an atmosphere of nitrogen, 1 M boron trichloride in CH_2Cl_2 (8mL, 8mmol) was dropped slowly onto an ice-bath cooled solution of **13** (0.63g, 0.45mmol) in CH_2Cl_2 (10mL). The mixture was stirred at room temperature for about 6h. Methanol (20 mL) was added to quench the reaction. After filtration, the filtrate was concentrated to remove the solvent. The residue was recrystallized with methanol/acetone to afford the hydrochloric acid salt of **14** as a white powder. Yield: 92%. ^{1}H NMR (DMSO, 500 MHz) δ 1.78 (m, 6H, CH_2), 2.05 (m, 6H, CH_2), 2.27 (t, J = 7.0 Hz, 2H), 3.28 (q, J = 6.5 Hz, 2H, CH_2), 3.75 (t, J = 4.7 Hz, 6H, CH_2), 4.56 (m, 12H, CH_2), 5.52 (m, 1H, vinyl), 6.03 (m, 1H, vinyl), 6.23 (m, 1H, vinyl), 7.28 (s, 1H, NH), 7.41 (d, J = 6.9 Hz, 3H, C5-H in pyridinone), 8.21 (t, J = 5.6 Hz, 1H, NH), 8.25 (d, J = 7.0 Hz, 3H, C6-H in Pyridinone), 8.83 (t, J = 5.0 Hz, 3H, NH). ^{13}C NMR δ 29.71 (CH_2), 31.14 (CH_2), 34.71 (NH*C*H_2-pyridinone), 36.03 (NHCH_2), 36.32 (CH_2), 57.29 (NHC), 58.32 (NCH_2), 60.77 (OCH_2), 111.90 (*C*-

5H in pyridinone), 125.26 (CH_2), 132.27 (CH), 139.97 (*C*-2H in pyridinone), 140.28 (*C*-3H in pyridinone), 144.74 (*C*-6H in pyridinone),160.68 (*C*-4H in pyridinone), 165.15 (CO), 170.55 (CO), 173.86 (CO). ESI-MS: *m/z* 871 ($[M+H]^+$). ESI-HRMS: calcd. for $C_{40}H_{55}N_8O_{14}$ 871.3838, found 871.3848 ($[M+H]^+$).

Preparation of polymeric chelators **(16).** The iron chelating copolymers were prepared by the polymerization of **14** hydrochloride salt and 2-hydroxyethylacrylate (HEA) (**15**) with different mole ratios (0%, 1%, 3%, 5%, 7% and 9%). For instance, for the 1% mole ratio, the mixture of **14** hydrochloride salt (0.01mmol) and **15** (0.99mmol) was purged with oxygen-free dry nitrogen. The initiator, AIBN (10mg), dissolved in absolute ethyl alcohol, was added and the mixture was heated at 60-70^oC for 12h. The reaction mixture is then subjected to dialysis (MW cutoff 3500 Daltons) for 24h with frequent changes of water. The solution in the dialysis bag was then freeze-dried to give the copolymers **16** as pale red solids.

Physico-chemical properties of chelating monomer 14.

Determination of pK_a. The hexadentate **14** is readily water soluble. The titration system comprised of an autoburette (Metrohm Dosimat 765 l mL syringe) and a HP 8453 UV-visible spectrophotometer. 0.1M 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. A cuvette path length of 10 mm was used. An argon atmosphere was applied to the entire titration

equipment. The initial sample concentration was approximately 7×10^{-5}M. pKa values were analyzed from these data by pHab.[1] *Determination of iron(III) affinity*. The automatic titration system used in this study comprised of an autoburette (Metrohm Dosimat 765 liter 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 ^{o}C ± 0.1 ^{o}C by using a Techne TE-8J temperature controller. The solution under investigation was stirred vigorously during the experiment. A Gilson Mini-plus#3 pump with speed capability (20 mL/min) was used to circulate the test solution through a Hellem quartz flow cuvette. A 50 mm path length cuvette 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 pKa 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.[1] The species plot was calculated with the HYSS program.[2] Analytical grade reagent materials were used in the preparation of all solutions.

Measurement of iron(III) chelating capacity of the copolymers.

Determination of iron(III) chelating capacity and utilization rate of monomeric chelator was carried out with iron standard solution-nitrilotriacetic acid (NTA; 1:3 mol ratio) (200μM) in 3-(*N*-morpholine)-propanesulfonic acid (MOPS) buffer solution (25mM, pH=7.5) by spectrophotometry. The maximum absorption wavelength of iron(III)-polymer complex was 458nm by measurement on a UV spectrophotometer scanning from 280 to 700 nm.

A range of solutions with different ratios of copolymer and iron were prepared by adding iron standard solution to 0.2mL of copolymers (certain concentrations in MOPS) followed by the addition of MOPS to a certain volume. All samples were equilibrated at room temperature for at least 0.5h before spectral acquisition. When with increasing the amount of iron standard solution added, the absorbance at 458nm was no longer increasing, it suggested that all hexadentate ligands incorporated in the polymers bound with iron. The amount of iron(III) chelated by the copolymer was calculated from the amount of iron standard solution added.

Determination of pFe for monomer 14 and copolymer 16-4 using the fluorescence method: The fluorescent probe CP691[3] was selected for this purpose. Fe(III) (final concentration: 6 μM) prepared from atomic absorption iron standard solution in the presence of 2.5-fold NTA solution was added to MOPS buffer (50mM) containing CP691 (final concentration: 6 μM). After 10 minutes, the competing chelator at a concentration yielding an equal amount number of iron binding units [**14**

(final concentration: 6 μM), **16-4** (7.64 mg/L, namely 6 μmol iron binding units/L for final concentration)] in DMSO was mixed with the iron-probe complex at 1:1 ratio volume (50% DMSO in MOPS). The mixture was incubated in a sealed 4-clear side cuvette for one month in dark before measuring the fluorescence intensity. 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%. The percentage of fluorescence intensity of the iron-probe complex in the presence of the competing ligands could be thus calculated. Based on the standard curve of the relationship between pFe and relative fluorescence intensity, the pFe value of **14** and **16-4** could be determined.

Determination of molecular weight of the polymer.

The average molecular weight of the iron chelating polymer was measured with gel permeation chromatography (GPCmax, Viscotek Corp., Houston, TX, USA) equipped with two Agilent® columns (PL 1149-6801, PL 1149-1840), RI detector (VE 3580, Viscotek Corp.), and data module at 36 °C. The molecular weights were determined from the refractive index data, which were analyzed with the Viscotek OmniSEC Omni-01 software. The columns were operated at 30 °C and polyethylene glycol standard samples were used for narrow peak calibration. The eluent was water with a flow rate of 1.0 mL/min during analysis.

Antimicrobial assay

Reagents. The media used in this study were nutrient agar medium (NA) and tryptone

soybean broth (TSB), which were purchased from Beijing Land Bridge Technology Co. Ltd. The semisolid medium incorporates NA (pH 7.3±0.1) and TSB (pH 7.4±0.2) each half. Antimicrobial agents were tested in triplicate at several appropriate concentrations for their antimicrobial effects. The solutions of these antimicrobial agents were prepared by dissolving in deionized water and stored at 4°C.

Bacterial strains. *Escherichia coli* (CGMCC1.0907), *Staphyloccocus aureus* (CGMCC1.0089), *Bacillus subtilis* (CGMCC1.0108), *Salmonella spp.* (CGMCC1.1552) *and Pseudomonas aeruginosa* (CGMCC1.2342) were purchased from China General Microbiological Culture Collection (CGMCC). These five bacteria were inoculated in a tube containing an inclined plane of NA and cultured at 37°C for 24 h. This gel was then used to inoculate into 5 mL of TSB and incubated at 37°C for 24 h before transfer of 50 μL into another tube of fresh TSB. This transfer was incubated at 37°C to an OD (600nm) corresponding to approximately 5.0×10^5 colony-forming units (CFU/mL).

Measurement of inhibition zone. The inhibitory zone assay was performed by Oxford Cup method and used EDTA as a positive control. To a sterile petri dish was added 15mL of NA, after it was congealing, an aliquot of 1mL bacterial inoculum (the viable cells were about 5.0×10^5 CFU/mL) and 10mL of semisolid medium were added on NA plate and then made them thoroughly blended. Steriled oxford cup (6mm diameter) was placed on the agar surface followed by the addition of 0.2mL of antimicrobial agent solution (6.53 mM in deionized water; the control was used sterile water instead) into oxford cup. The average diameter of inhibition zone was measured

after incubated at 37°C for 24h. Each treatment and the corresponding controls were carried out in triplicate.

Measurement of MIC. All assays were cultured at 37°C for 24h in 10×100mm tubes. The incubation medium was TSB (pH 7.4±0.2). All tubes contained 80μL of antimicrobial agent with different concentrations (0-3.3mM), and 20μL of bacterial inoculum (5.0×10^5 cells/mL). After incubation at 37°C for 2h, 900μL of sterilized TSB was added to each tube to reach a final volume of 1mL and cultured at 37°C for 24h. Minimum inhibitory concentrations (MIC) were determined by visual inspection of the turbidity of broth in tubes.[4] All assays were carried out in triplicate.

Statistical analysis. All the experimental data concerning the various antimicrobial treatments were statistically analyzed using analysis of variance test (SPSS version 16.0). Statistical analyses were performed using one-way ANOVA. Significant differences between the treatments were examined by Duncan's multiple range test and $p < 0.05$ was considered statistically significant.

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