Journal of Reports in Pharmaceutical Sciences 2013, 2(1), 5-15

Hydroxypyridinone Derivatives: Synthesis And Cytotoxic Evaluation

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ARTICLE INFO

Article Type:
Research Article

Article History:
Received: 2013-01-08
Revised: 2013-01-18
Accepted: 2013-01-29
ePublished: 2013-02-08

***Keywords*:**
3-Hydroxypyridin-4-ones
Iron chelating agents
Lipophilicity
MTT assay
MCF-7 and MDA-MB-231 Cells

A B S T R A C T

A series of 3-hydroxypyridin-4-one derivatives (HPOs) as bidentate iron (III) chelating agents were synthesized from 3-hydroxypyran-4-ones (maltol and ethyl maltol) in three steps through protection of hydroxyl group. The protected compounds were then reacted with suitable primary amines to give benzylated pyridinones. Finally, the benzyl group was removed by catalytic hydrogenation to produce the desired products. The partition coefficient of the free ligands and their iron (III) complexes were determined in an aqueous/octanol system using shake-flask method. The cytotoxic effects of these iron chelators against MCF-7 and MDA-MB-231 cancer cells were also evaluated using MTT assay. The results revealed that cytotoxicity of synthesized compounds were closely related to the lipophilycity of them so that the most lipophilic compound (**4f**) showed the highest activity; whereas compound **4a** as a more hydrophilic agent showed the lowest cytotoxic effect; Although these compounds were cytotoxic at high concentration (≥ 0.1 mM).

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Introduction

Cancer known medically as a malignant neoplasm, is a broad group of various diseases, all involving unregulated cell growth. In cancer, cells divide and grow uncontrollably, forming malignant tumors, and invade nearby parts of the body [1]. Like other cells, the tumor cells need iron for cell division and DNA synthesize. Cancer cells divide rapidly and therefore they have a higher requirement for iron than normal cells. For this reason they are more sensitive to iron depletion and hence, iron ligands could be used in cancer chemotherapy [2]. Chelating the required iron for tumor cells may interfere with tumor growth and its proliferation [3]. A wide variety of studies in vitro, in vivo, and in clinical trials have demonstrated that desferrioxamine (DFO), a chelator currently used to treat iron overload disease, has anti-proliferative effects against both leukemia and neuroblastoma. However, the efficacy of DFO is severely limited due to its poor ability to permeate cell membranes and chelate intracellular iron pools. [4]. Other limiting factor of DFO is that it is not orally active and has to be administered parentally [5]. For this reasons, many patients find it difficult to comply with the treatment, so there is an urgent need for an orally active iron chelating agent [6].

Hydroxypyridinone (HPOs) derivatives are good candidates for development as orally active iron chelators for replacement of DFO. These derivatives possess many advantages including high selectivity for iron *in vivo* [7]. One of the key enzymes that plays an important role in the synthesis of DNA is ribonucleotide reductase. This enzyme needs iron for its proper function [8]. HPOs inhibit enzyme activity by their iron chelation properties. This, consequently leads to a cessation of cell division in tumor cells [9].

There are three classes of HPOs: 1-hydroxypyridin-2-ones (1,2-HPOs) [10], 3-hydroxypyridin-2-ones (3,2- HPOs) [11] and 3 hydroxypyridin-4-ones (3,4- HPOs) [12] .Of the three classes of HPO ligands, the 3 hydroxypyridin-4-one class (Fig. 1) possesses the highest affinity for Fe(III) that is 10^{37} . This is the direct consequence of the elevated pK_a value associated with the 4- oxo group compared with the 2-oxo congeners (Table 1) [13].

Table 1. The pK_a Values and Fe (III) affinity constants for bidentate ligands

Ligands	pKa1	pKa2	Log β3 (Fe3+)
1-Hydroxypyridin-2-ones (1,2-HPOs)	**-**	**5.8**	**27**
3-Hydroxypyridin-2-ones (3,2-HPOs)	**0.2**	**8.6**	**32**
3-Hydroxypyridin-4-ones (3,4-HPOs)	**3.6**	**9.9**	**37**

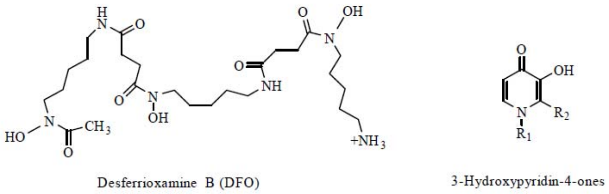

Fig.1. Structures of two iron(III) chelators

In this study, we synthesized some derivatives of 3-hydroxypyridine-4-ones as iron chelating agents and evaluated *in vitro* cytotoxic activity of these compounds against MCF-7 and MDA-MB-231 Cell lines.

Materials and Methods

Chemistry

All chemicals were obtained from Sigma-Aldrich without any further purification. Melting points were determined on a Mettler capillary melting point apparatus *(Electro thermal9200*) (England) and were uncorrected. The IR spectra were recorded with a WQF-510 Ratio Recording FT-IR spectrometer (China) as KBr disc (γ, cm^{-1}). The ^{1}H-NMR spectra were determined with a Bruker 400 MHz spectrometer (Germany). Chemical shifts (δ) are reported in ppm downfield from the internal standard tetramethylsilane (TMS). The purity of the compounds was checked by thin layer chromatography (TLC) on silica gel plate using chloroform and methanol. The procedure for the synthesis of the desired compounds is described in Fig 2.

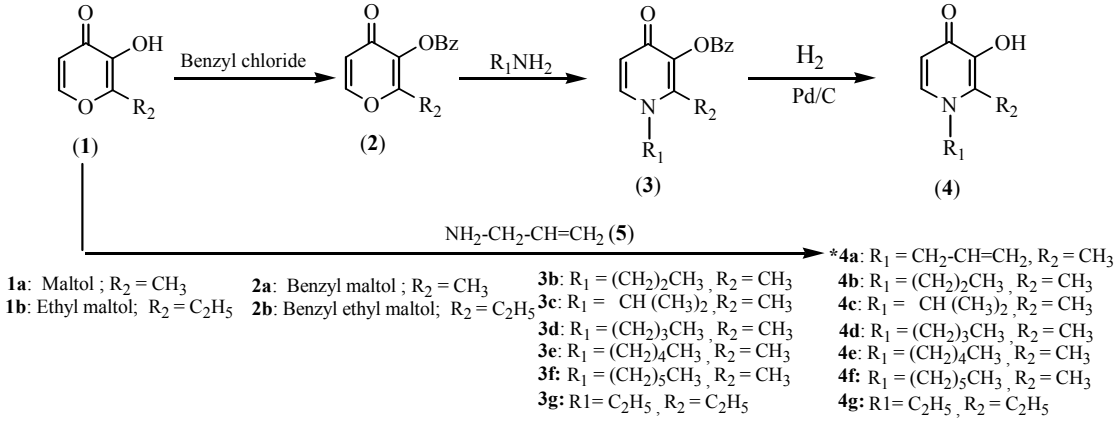

Fig.2. Synthesis scheme of 1-substituted-3-hydroxypyridin-4-ones derivatives**.**
***** Ligand **4a** was synthesized via a single-step synthetic pathway.

Experimental procedures

Synthesis of 2- methyl -3- benzyloxypyran -4- one (Benzyl maltol) (2a):

To a solution of 2-methyl-3-hydroxypyran-4-one (**1a**) (24 g, 0.2 mol) in methanol (200 ml) was added 20 ml of sodium hydroxide solution (8.8 g, 0.22 mol) followed by addition of benzyl chloride (27.8 g, 0.22 mol). The mixture was

then refluxed for 12 h. After removal of solvent by rotary evaporation, the residue was mixed with water (50 ml) and extracted into dichloromethane (3 × 100 ml). The combined extracts were washed with 5% sodium hydroxide (3 × 150 ml) and then with water (2 × 150 ml). The organic fraction was dried over anhydrous sodium sulphate, filtered and rotary evaporated to yield an oily orange residue which became solid on cooling. Recrystallization from diethyl ether gave the pure product as colorless needles, 25.4 g (82%). mp 52-53 ℃. ^{1}H-NMR (DMSO-d_6): δ 2.1 (s, 3H, 2-CH_3), 5.1 (s, 2H, O-CH_2-Ph), 6.4 (d, 1H, 5-H), 7.2-7.5 (m, 5H, Ph), 8.0 (d, 1H, 6-H): IR (KBr): 1640 (C=O) cm^{-1}.

***Synthesis of 1-allyl-2-methyl-3-hydroxypyridin-4-one (4a) via* a single-step synthetic pathway:**

Maltol 1a (6.31g, 0.05 mol) was added to a solution of allylamine (5.7 g, 0.1 mol) in 150 ml water. The mixture was refluxed for 24 h. Decolorizing charcoal was added and the mixture left for 0.5 h. This mixture was filtered and the filtrate evaporated to give a brown solid. Recrystallization from ethanol/ether gave a white crystalline solid, (2.7g, 35%), mp: 175 -176 ºC (lit. value 177-178 °C [14]). ^{1}H-NMR (DMSO-d_6): δ 2.4 (s, 3H, 2-CH_3), 4.5 (d, j = 6.8Hz, 2H, N-CH_2-), 4.8 (d, j = 6.8 Hz, 1H, N-CH_2-CH=C**H**H), 5.2 (d, j = 6.8Hz, 1H, N-CH_2-CH=CH**H**),6.1-6.3 (m, 1H, N-CH_2-C**H**=), 6.10 (d, j = 7.3 Hz, 1H, 5-H), 7.20 (d, j =7.3 Hz, 1H, 6-H); IR (KBr) (cm^{-1}): 3170 (OH), 1628 (C=O),1050 (C-N). ESI-MS (positive): m/z 166 $[M+H]^+$ (Calcd for $C_9H_{11}NO_2$, 165).

Synthesis of 1-propyl-2-methyl-3-benzyloxypyridin-4-0ne hydrochloride (3b):

To a solution of compound **2a** (2.16 g,0.01 mol) in ethanol (100 ml)/water (100 ml) was added propylamine (1.3g, 0.03 mol) followed by 2 N sodium hydroxide solution to adjust to pH 13 and the mixture refluxed for 12h. After removal of solvent by rotary evaporation, the residue was mixed with water (50 ml) and extracted into dichloromethane (3 × 50 ml). Obtained orange oil was dissolved in ethanol (20 ml) and adjusted to pH 1 with HCl and rotary evaporated. Recrystallization from ethanol/ diethyl ether gave the pure product as colorless needles, 2.4g (82%) mp:167-168 °C(lit. value 169-170 °C [14]). ^{1}H-NMR (DMSO-d_6): δ 0. 8 (t, 3H, , j =7.3 Hz, N-CH_2-CH_2-**CH_3**), 1.6 (m, 2H, N-CH_2-**CH_2**-CH_3), 2.3 (s, 3H, 2-CH_3), 4.3 (t, 2H, j =7.3 Hz, N-**CH_2**-CH_2-CH_3), 5.2 (s, 2H, O-CH_2-Ph), 7.3-7.5 (m, 5H, Ph), 7.7 (d, j =7.2 Hz, 1H, 5-H), 8.5 (d, j =7.2 Hz, 1H, 6-H); IR (KBr) (cm^{-1}): 1624 (C=O),1051(C-N).

Synthesis of 1-propyl-2-methyl-3-hydroxypyridin-4-0ne hydrochloride (4b):

Compound **3b** (2g , 0.007mol) dissolved in 80 ml ethanol (99%) and subjected to hydrogenation for 4 h in the presence of 5% Pd/C catalyst . Filtration which was followed by rotary evaporation gave a white solid. Recrystallization of resulting compound from ethanol/diethyl ether yielded a white powder, (1.1 g, 78%); mp: 203-204 °C (lit. value 206-207 °C [14]). ^{1}H-NMR (DMSO-d_6): δ 0. 8 (t, 3H , N-CH_2-CH_2-**CH_3**), 1.5 (m, 2H, N-CH_2-**CH_2**-CH_3), 2.45 (s, 3H, 2-CH3), 4.3 (t, 2H, N-**CH_2**-CH_2-CH_3), 7.4 (d, j =7.2 1H, 5-H), 8.2 (d, j =7.2 1H, 6-H) 9-10.5 (br, 1H,3-OH). IR (KBr): 1630 (C=O), 1026(C-N), 2400-3242 (O-H) cm^{-1}. ESI-MS (positive): m/z 168 $[M+H]^+$ (Calcd for $C_9H_{13}NO_2$, 167).

Synthesis of 1-isopropyl-2-methyl-3-benzyloxypyridin-4-one hydrochloride (3c):

The procedure used was as described for synthesis of **3b** except isopropylamine was used in place of ethylamine in the reaction mixture. Recrystallization from ethanol/diethyl ether gave the desired product as a white powder in a 71% yield (25.0 g), mp: 171-172ºC (lit. value170-171 °C [14]). ^{1}H-NMR (DMSO-d_6): δ 1.4 (d, j= 6.7 Hz, 6H, N-CH(C**H**$_3$)$_2$), 2.5 (s, 3H, 2-CH_3), 4.6-

4.8 (m, 1H, N-C**H**$(CH_3)_2$), 5.3 (s, 2H, O-CH_2-Ph), 7.3-7.5 (m, 5H, Ph), 7.6 (d ,j = 7.3 Hz, 1H, 5-H), 8.6 (d,j = 7.3 Hz, 1H, 6-H). IR (KBr) (cm^{-1}): 1634 (C=O), 1045(C-N).

Synthesis of 1-isopropyl-2-methyl-3-hydroxypyridin-4-one hydrochloride (4c):

The procedure used was as described for synthesis of **4b** except **3c** was used in place of **3b** in the reaction mixture. Recrystallization from ethanol/diethyl ether gave the desired product as a white powder in a 70% yield (10.7 g), mp 223-224 ºC (lit. value 225-226 ºC [14]). ^{1}H NMR (DMSO-d_6): δ 1.4 (d, j = 6.8 Hz, 6H, N-CH ($C\mathbf{H}_3)_2$), 2.5 (s, 3H, 2-CH_3), 4.6-4.8 (m, 1H, N-C**H** $(CH_3)_2$), 7.3 (d, j = 7.2 Hz, 1H, 5-H), 8.2 (d, j = 7.2 Hz, 1H, 6-H). IR (KBr) (cm^{-1}): 1630 (C=O), 1026 (C-N), 2400-3242(O-H). ESI-MS (positive): m/z 168 $[M+H]^+$ (Calcd for $C_9H_{13}NO_2$, 167).

Synthesis of 1-butyl-2-methyl-3-benzyloxypyridin-4-one hydrochloride (3d):

The procedure used was as described for synthesis of **3b** except butylamine was used in place of propylamine in the reaction mixture. Recrystallization from ethanol/diethyl ether gave the desired product as a white powder in a 77% yield (2.4g). mp: 162-163 °C**.** ^{1}H-NMR (DMSO-d_6): δ 0.9 (t, j = 7.2 Hz, 3H, N-CH_2-CH_2-CH_2-**CH_3**), 1.3 (m, 2H, N-CH_2-CH_2-**CH_2**-CH_3), 1.7 (m, 2H, N-CH_2-**CH_2**-CH_2-CH_3), 2.3 (s, 3H, 2-CH_3), 4.4 (t, j = 7.2 Hz, 2H, N-**CH_2**-CH_2-CH_2-CH_3), 5.2 (s , 2H, O-CH_2-Ph), 7.4 (m , 5H , Ph), 7.6 (d, j = 7.1 Hz, 1H, 5-H), 8.5 (d, j = 7.1 Hz, 1H ,6-H). IR (KBr) (cm^{-1}): 1632 (C=O), 1040 (C-N)

Synthesis of 1-butyl-2-methyl-3-hydroxypyridin-4-one hydrochloride (4d):

The procedure used was as described for synthesis of **4b** except **3d** was used in place of **3b** in the reaction mixture. Recrystallization from ethanol/diethyl ether gave the desired product as a white powder in a 73% yield (1.1g). mp: 202-203°C (lit. value199-200 °C[14]). ^{1}H-NMR (DMSO-d_6): δ 0.9 (t, j = 7.2 Hz, 3H , N-CH_2-CH_2-CH_2-CH_3), 1.32 (m, 2H, N-CH_2-CH_2-**CH_2**-CH_3) , 1.74 (m, 2H, N-CH_2-**CH_2**-CH_2-CH_3) , 2.4 (s, 3H, 2-CH_3), 4.3 (t, j = 7.2 Hz , 2H, N-**CH_2**-CH_2-CH_2-CH_3), 7.4 (d, j = 7.0 Hz, 1H, 5-H), 8.3 (d, j = 7.0 Hz, 1H ,6-H) 10-11 (br, 1H, 3-OH). IR (KBr): 1630 (C=O), 1030 (C-N), 3072 (O-H) cm^{-1}. ESI-MS (positive): m/z 182 $[M+H]^+$ (Calcd for $C_{10}H_{12}NO_2$, 181).

Synthesis of 1-pentyl-2-methyl-3-benzyloxypyridin-4-one hydrochloride (3e):

The procedure used was as described for synthesis of **3b** except pentylamine was used in place of propylamine in the reaction mixture. Recrystallization from ethanol/diethyl ether gave the desired product as a white powder in a 65% yield (2.1g). mp: 158-159 °C. ^{1}H-NMR (DMSO-d_6): δ 0.85 (t, j = 7.2 Hz, 3H, N-CH_2-CH_2-CH_2-CH_2-**CH_3**), 1.2-1.4 (m, 4H, N -CH_2-**CH_2-CH_2**-CH_2-CH_3), 1.7 (m, 2H, N-CH_2-CH_2-CH_2-**CH_2**-CH_3), 2.5 (s, 3H, 2-CH_3) , 4.3 (t, j = 7.2 Hz, 2H, N-**CH_2**-CH_2-CH_2-CH_2-CH_3) , 5.1 (s, 2H, O-CH_2-Ph), 7.3-7.5 (m, 5H, Ph) , 7.5 (d, j = 7.1, 1H, 5-H) , 8. 5 (d, j = 7.1 1H, 6-H). IR (KBr): 1625 (C=O), 1040 (C-N).

Synthesis of 1-pentyl-2-methyl-3-hydroxypyridin-4-one hydrochloride (4e):

The procedure used was as described for synthesis of **4b** except **3e** was used in place of **3b** in the reaction mixture. Recrystallization from ethanol/diethyl ether gave the desired product as a white powder in a 76% yield (1.2g). mp: 162-163°C (lit. value 165-166 °C[14]).. ^{1}H-NMR (DMSO-d_6): δ 0.85 (t, j = 7.3 Hz, 3H, N-CH_2-CH_2-CH_2-CH_2-CH_3), 1.3 (m, 4H, N -CH_2-**CH_2-CH_2**-CH_2-CH_3), 1.7 (m, 2H, N-CH_2-CH_2-CH_2-**CH_2**-CH_3), 2.5 (s, 3H,2-CH_3), 4.3 (t, j = 7.3 Hz, 2H, N-**CH_2**-CH_2-CH_2-CH_2-CH_3), 7.4 (d, j = 6.9 1H, 5-H), 8.27 (d, j = 6.9 1H, 6-H), 9.5-11.5 (br,

1H, 3-OH). IR (KBr): 1624 (C=O), 1032 (C-N), 3064 (O-H) cm^{-1}. ESI-MS (positive): m/z 196 $[M+H]^+$ (Calcd for $C_{11}H_{14}NO_2$, 195).

Synthesis of 1-hexyl-2-methyl-3-benzyloxypyridin-4-one hydrochloride (3f):

The procedure used was as described for synthesis of **3b** except hexylamine was used in place of propylamine in the reaction mixture. Recrystallization from ethanol/diethyl ether gave the desired product as a white powder in a 59% yield (2g). mp: 149-150. ^{1}H-NMR (DMSO-d_6): δ 0.9 (t, j = 7.3 Hz, 3H, N-CH_2-CH_2-CH_2-CH_2-CH_2-**CH_3**), 1.3 (m, 6H, N-CH_2-**CH_2-CH_2-CH_2**-CH_2-CH_3), 1.7 (m, 2H, N-CH_2-CH_2-CH_2-CH_2-**CH_2**-CH_3), 2.6 (s, 3H, 2-CH_3), 4.3 (t, j = 7.3 Hz, 2H, N-**CH_2**-CH_2-CH_2-CH_2-CH_2-CH_3), 5.1 (s, 2H, O-CH_2-Ph), 7.3-75 (m, 5H, Ph), 7.4 (d, j =7.1, 1H, 5-H), 8.5 (d, j =7.1 1H, 6-H). 1631 (C=O), 1039 (C-N).

Synthesis of 1-hexyl-2-methyl-3-hydroxypyridin-4-one hydrochloride (4f):

The procedure used was as described for synthesis of **4b** except **3f** was used in place of **3b** in the reaction mixture. Recrystallization from ethanol/diethyl ether gave the desired product as a white powder in a76% yield (1.3g). mp: 164-165°C (lit. value 166-167 °C [14]). ^{1}H-NMR (DMSO-d_6): δ 0.87 (t, j = 7.2 Hz, 3H, N-CH_2-CH_2-CH_2-CH_2-CH_2-CH_3), 1.3 (m, 6H, N-CH_2-**CH_2-CH_2-CH_2**-CH_2-CH_3), 1.74 (m, 2H, N-CH_2-CH_2-CH_2-CH_2-**CH_2**-CH_3), 2.5 (s, 3H, 2-CH_3), 4.33 (t , j = 7.3 Hz, 2H, N-**CH_2**-CH_2-CH_2-CH_2-CH_2-CH_3), 7.3 (d, j =7.1 Hz, 1H, 5-H), 8.25 (d, j = 7.1 Hz, 1H ,6-H) , 9-12 (br , 1H, 3-OH) IR (KBr): 1630 (C=O), 1040(C-N), 3074 (O-H) cm^{-1}. ESI-MS (positive): m/z 210 $[M+H]^+$ (Calcd for $C_{12}H_{16}NO_2$, 209).

Synthesis of 1, 2-diethyl-3-benzyloxypyridin-4-one hydrochloride (3g):

Sodium hydroxide (6.0 g, 0.15 mol) dissolved in water (20 ml) followed by benzyl chloride (19.0 g, 0.15 mol) were added to a solution of ethyl maltol **1b** (21.0 g, 0.15 mol) in methanol (180 ml) and the mixture was refluxed for 12 h. After removal of solvent by rotary evaporation, the residue was mixed with water (75 ml) and extracted into dichloromethane (2 × 75 ml). The combined extracts were washed with 5% sodium hydroxide (3 × 150 ml) and then with water (2 × 150 ml). The organic fraction was rotary evaporated to yield orange oil. The oily product was dissolved in a mixture of ethanol /water (400 ml; 1:1) and was added to it 90% aqueous ethylamine (29.5 ml, 0.45 mol) followed by 2N sodium hydroxide solution (12 ml), and the mixture was refluxed for 18 h. After adjustment to pH=1 with HCl, volume was reduced to 200 ml by rotary evaporation prior to addition of water (200 ml) and washing with diethyl ether (400 ml). Subsequent adjustment of the aqueous fraction to pH 7 with 10 N NaOH solution was followed by extraction into dichloromethane (3×400 ml). The organic layers added to each other and dried over anhydrous sodium sulphate, filtered and rotary evaporated to give a brownish oily product. The product was then dissolved in ethanol/hydrochloric acid and the solvent was evaporated using rotary. The resulting white solid was recrystallized from ethanol/diethyl ether to give a white powder (24.5 g, 55%); mp: 173-174 °C. ^{1}H-NMR (DMSO-d_6): δ 1.1 (t, j = 7.4 Hz, 3H, N-CH_2-**CH_3**), 1.4 (t, j = 7.2 Hz, 3, 2-CH_2-**CH_3**), 2.9 (q, 2H, 2-**CH_2**-CH_3), 4.4 (q, 2H, N-**CH_2**-CH_3), 5.8 (s, 2H, O-CH_2-Ph), 7.3-7.5 (m, 5H, Ph), 7.5 (d, j = 7.1 Hz, 1H, 5-H), 8.5 (d, j = 7.1 Hz, 1H, 6-H). IR (KBr): 1640 (C=O), 1578 (C=C) cm-1.

1, 2-diethyl-3hydroxypyridin-4-one hydrochloride (4g):

The procedure used was as described for synthesis of **4b** except **3g** was used in place of **3b** in the reaction mixture. Recrystallization from ethanol/diethyl ether gave the desired product as a white powder in a 84% yield (1.2 g), mp 185-186 ºC (lit. value 188- 189 ºC [14]). ^{1}H-NMR (DMSO-d_6): δ 1.2 (t, j = 7.3 Hz 3H, N-CH_2-CH_3) , 1.4 (t, j = 7.2 Hz 3H, 2-CH_2-**CH_3**), 3.0 (q, 2H, 2-**CH_2**-CH_3), 4.4 (q, 2H, N-**CH_2**-CH_3), 7.4 (d, 1H, 5-H), 8.3 (d, 1H, 6-H), 9-12 (br, 1H, 3-OH). IR (KBr):

1630 (C=0), 1032 (C-N), 3064 (O-H) cm^{-1}. ESI-MS (positive): m/z 168 $[M+H]^+$ (Calcd for $C_9H_{13}NO_2$, 167).

Cell lines

MCF-7 and MDA-MB-231(both related to breast cancer ER positive & negative, respectively) cell lines were purchased from Pasture Institute (Tehran, Iran). Cells were grown in RPMI-1640 [each 500 ml of RPMI-1640 was supplemented with 10% of fetal calf serum, 5 ml of penicillin/ streptomycin (50 IU/ml and 50 µg/ml, respectively), 5 ml of sodium pyruvate (1 mM), $NaHCO_3$ (1 g) and 5 ml of L-glutamine (2 mM)]. Completed media were sterilized by 0.22 µm microbiological filter after preparation and kept at 4°C before using.

Preparation of stock solutions

Stock solution (10 mM) of each compound was prepared in 1 ml of DMSO and 9 ml of PBS and was sterilized with filtration. The final solutions (1, 10 and 100 µM) were then obtained by diluting these solutions with PBS or culture media.

MTT-based cytotoxicity assay

The cytotoxic effects of synthesized compounds against human tumor cell line were determined by a rapid colorimetric assay, using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) which compared with untreated controls. This assay is based on the metabolic reduction of soluble MTT by mitochondrial enzyme activity of viable tumor cells into an insoluble colored formazan product, which can be measured spectrophotometrically after dissolution in DMSO. Briefly, 200 µl of cells (5×10^4 cells/ml) were seeded in 96 well micro- plates and incubated for 24 h (37°C, 5% CO2 air humidified). Then 20 µl of final concentration of each compound (1, 10 and 100 µM) was added and incubated for another 48h in the same condition. Doxorubicin (2.3 µM) was used as a positive control. Cell suspension (5×10^4 cells/ml) was considered as negative control with 100% viability and RPMI-1640 used as blank [15]. Cell survival was determined as previously described [16].

Determination of partition coefficients (K_{part})

The K_{part} values of the synthesized compounds were determined using the shake-flask method [17]. The two phases used in determination were tris buffer (50 mM, pH 7.4) and n-octanol, each of which was pre-equilibrated with the other phase before use (to avoid the slight solubility of water in n-octanol which is 2.3 M) [18].

a) Determination of K_{part} values of ligands

A solution of ligands with concentration of 10^{-4} M was prepared in tris buffer (pH 7.4) and the absorbance of solution was measured in the ultraviolet region at maximum wavelength (λ_{max}) 180 nm using the buffer as a blank. A 10 ml of sample solution was stirred vigorously with 10 ml of n-octanol in a glass vessel for 1 h. The two layers were separated by centrifugation for 5 minutes. An aliquot of the aqueous layers was then carefully removed using a Pasteur pipette ensuring that the sample was not contaminated with n-octanol. The absorbance of the sample was measured as above and the K_{part} was then calculated using the following equation:

$$K_{part} = \frac{A_1 - A_2}{A_2} \times \frac{V_w}{V_0} \qquad (1)$$

Where

A_1 = Absorbance reading in the aqueous layer before partitioning.

A_2 = Absorbance reading in the aqueous phase after partitioning.

V_0 = Volume of n-octanol layer used in partitioning

V_v = Volume of aqueous layer used in partitioning

For each sample, the experiment was repeated at least four times which led to calculation of a mean K_{part} value and standard deviation (Table 2).

Table 2. The ligands K_{part} values and their corresponding iron (III) complexes obtained by shake-flask method (mean ± SD).

Ligands	K_{part} of the ligand (n=4)	K_{part} of the Fe-complex (n=4)
4a	0.81 ± 0.08	0.30 ± 0.05
4b	1.52 ± 0.40	0.53± 0.04
4c	1.41 ± 0.12	0.50 ± 0.06
4d	4.2 ± 0. 20	16.3±1.40
4e	15.50 ± 1.0	480± 60
4f	60 ± 8.0	1900 ± 120
4g	1.3 ± 0.15	0.39±0.05

b) Determination of K_{part} values of iron (III) complexes

The shake-flask technique was also used to determine the K_{part} values of the iron (III) complexes of the bidentate ligands. Partition co-efficients were measured using a 10:1 molar ratio of ligand to iron to ensure that the 3:1 neutral complexes were completely formed. A solution of iron complex was prepared using a ligand/iron concentration ratio of 10^{-3} M/10^{-4} M and the absorbance of the iron complexes in the region between 300-900 nm was measured using the buffer as blank. The K_{part} values were determined at the λ_{max} of the iron (III) complex which typically was close to 460 nm. The partitioning was carried out as described for ligands. Obtained results are shown in Table (2).

Results and Discussion

General procedure for preparation of 3-hydroxypyridin-4-ones:

The general methodology [19] which has been adopted for the synthesis of 1-substituted-3-hydroxypyridin-4-ones is summarized in Fig. 2 .The corresponding 3-hydroxypyran-4-ones (maltol **1a** or ethyl maltol **1b**) were benzylated to give compounds **2a-b**. Reaction of **2a-b** with alkylamines prod- uced the benzylated pyridinones **3b-g**, which was subsequently subjected to catalytic hydro- genation under acidic conditions to remove the protecting group, yielding the corresponding bidentate 1-substituted -3- hydroxypyridin-4-ones **4b-g.** Although the 3-hydroxy substituent of 3-hydroxypyran- 4-ones can also be protected by methyl ether formation, the corresponding 2-alkyl-3-methoxy-4-pyrones are oils which are less convenient to work with than the crystalline 2-alkyl-3-benzyloxy-4-pyrones. Furthermore, the benzyl protecting group can be removed by hydrogenation under acidic, neutral or basic conditions. For these reasons the benzyl group was selected for the work described in this study. The conversion of pyran-4-one to pyridin-4-one involves an initial Michael reaction followed by ring opening and ring closure. Mesomerisation of α, β-unsaturated carbonyl compound causes the β-carbon to be electron deficient and therefore susceptible to nucleophilic attack. When the nucleophile is a primary amine, a double attack at both α, β-unsaturated functions of the pyran-4-one leads to the formation of pyridin-4-one with the loss of a molecule of water. Conversion of 3-hydroxypyran-4-one derivatives to the corresp-onding 3-hydroxypyridin- 4-one analogues can be achieved without protection of the 3-hydroxyl group [19]. However, the synthetic utility of this

reaction is limited to small primary amines since larger amines result in yields less than 10% [20].
It is interesting to note that, for the synthesis of compound **4a** the same general method was employed, but some difficulties were encountered during hydrogenation in the third step. Under catalytic hydrogenation step, not only the benzyl group was cleaved but also, the N-substituent group (allyl group) was saturated and converted to propyl chain (compound **4b**). In other words, compound **4b** (1-propyl-2-methyl-3-hydroxypyridin-4-one) was synthesized instead of desired product of 1-allyl-2-methyl-3-hydroxypyridin-4-one (**4a**). This problem, prompted us to attempt a direct one-step preparation. In this method, maltol (instead of benzyl maltol) was reacted with an excess of the primary amine under reflux conditions in water. Although, the yield for synthesis of **4a** *via* single-step method is lower than the "three-step synthetic pathway"; this method is much easier and less expensive overall. This low yield is due to the fact that, under basic conditions employed in the amination reaction, the unprotected 3-hydroxyl group will predominantly exist as an anion, and hence will act as a competing nucleophile in the amination step. For this reason, the consumption of maltol is high and therefore leads to the low yield.

The K_{part} values of ligands

The partition coefficient of the free ligands and their iron (III) complexes between an aqueous phase buffered at pH 7.4 and 1-octanol are presented in Table 2. The K_{part} values of both bidentate ligands and their Fe-complexes were determined by using the shake-flask method [17]. In general, as expected, the introduction of a more hydrophobic substituted group on the heterocyclic nitrogen results in an increase in the K_{part} values of both the ligands and the iron complexes. In compounds **4a-c** and **4g**, the free ligands are more hydrophobic than their corresponding iron (III) complexes. However, this trend did not hold for those compounds which have K_{part} values greater than 3 (i.e. **4d**, **4e** and **4f**). Among the ligands, compounds **4f** and **4a** possess the highest and the lowest K_{part} values respectively, and not surprisingly they form the most hydrophobic and hydrophilic iron (III) complexes, respectively.
It should be noted that, for iron (III) complexes which possess K_{part} values less than 100, an aqueous layer/n-octanol volume ratio of 1:1 was used. Difficulties arose however, on attempting to determine K_{part} values of iron (III) complexes with a K_{part} value greater than 100. For this type of compounds, the absorbance of the aqueous layer after partitioning was found to be low owing to the high solubility of the molecule in the 1-octanol layer. As a consequence the results obtained were not reliable. For this reason, the partitioning of the iron (III) complexes with K_{part} values greater than 100 (iron complexes of ligands **4e-f**) was monitored using an aqueous layer/n-octanol volume ratio of 100:1. Solubility problems also occurred on the addition of some ligand solutions to iron (III) nitrate solution resulting in cloudiness. This problem was avoided by dilution of the sample. The iron (III) complex of **4f** was, for instance, prepared with the ligand and iron concentrations of 10^{-4} M and 10^{-5} M respectively.

Cytotoxic activity

The cytotoxicity of all compounds was determined by MTT assay. Data are given in Fig.3 and Fig.4. All compounds were tested for cytotoxic properties against MDA-MB-231 and MCF-7 cells. At concentrations of 0.001 and 0.01 mM no cytotoxic effect were observed against tested cells (cells survival were more than 90%); but at concentration of 0.1 mM ,the compounds were effective as showed in Fig.3 and Fig.4. Compounds **4b, 4d, 4e** and **4f** showed highest cytotoxic activity against MCF-7 and MDA-MB-231 cells as seen in Fig.3 and Fig.4, respectively.

As previously reported for the most cytotoxic activity in 3,4-HPOs, R was substituted with an alkyl group. Bigger alkyl side chains resulted in greater cytotoxic activity [15].

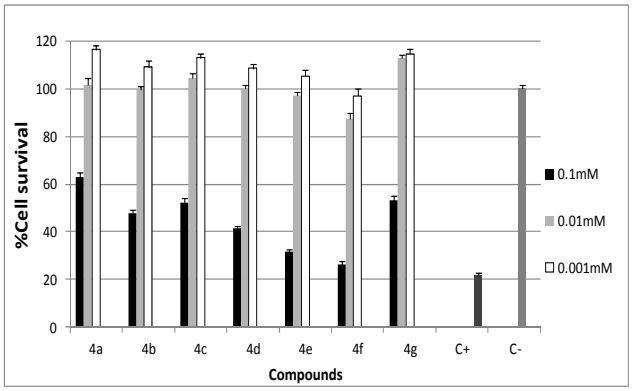

Fig. 3. Viability of MCF-7 breast cancer cell line treated with a panel of HPO compounds for 48h at 3 different concentrations. Cell viability was assessed using MTT assay. Data are presented as mean ± SD of cell survival compared to negative control (Cell survival of 100%) P<0.05 (significantly different from control). Doxorubicin in a concentration of 2.3 µM was used as positive control(C+) to ensure cells are sensitive to a well known cytotoxic agent (n=3).

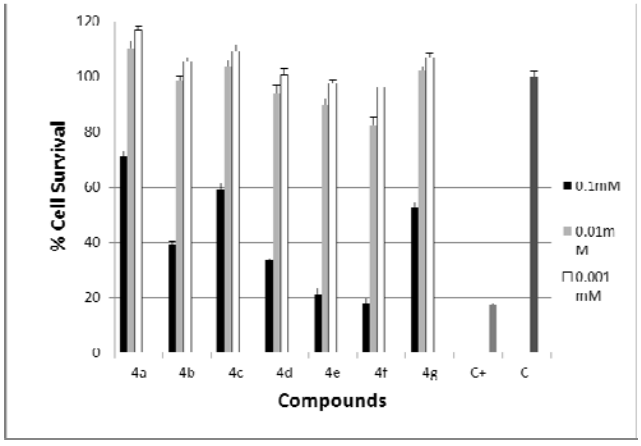

Fig. 4. Viability of MDA-MB-231 breast cancer cell line treated with a panel of HPO compounds for 48h at 3 different concentrations. Cell viability was assessed using MTT assay. Data are presented as mean ± SD of cell survival compared to negative control (Cell survival of 100%) P<0.05 (significantly different from control). Doxorubicin in a concentration of 2.3 µM was used as positive control(C+) to ensure cells are sensitive to a well known cytotoxic agent (n=3).

Where **R** is an alkyl group, membrane permeability (as an important factor for cytotoxic activity) is more than non-alkyl groups like amides and carboxyphenyls. On the other side HPOs skeleton which have alkyl side chain are neutral at physiologic pH and posses low molecular weight and high lipophilicity [21] and therefore they may have acceptable biological activity inside the cells. There are two important factors that influence the cytotoxic effect of 3,4-HPOs including membrane permeability and partition coefficient [22].

As seen in compounds **4b**, **4d**, **4e** and **4f** the bigger alkyl groups the more cytotoxic compound. Compound **4f** has the greatest activity against tested cell lines among other compounds. The biggest side chain in **4f** leads to higher lipophilicity and higher partition coefficient. It has been shown that cell penetration and cytotoxicity in compounds with higher K_{part} are more than compounds with lower K_{part} value [23]. These facts are consistent with our results in this study. The orders of cytotoxic effects of compounds in this study were as follows:

4f > 4e > 4d > 4b

In this regard compound **4a** that was the most hydrophilic one while the lowest K_{part} showed the weakest cytotoxic effect.

Conflict of interest

Authors certify that no actual or potential conflict of interest in relation to this article exists.

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