Research in Pharmaceutical Sciences, August 2013; 8(3): 185-195
Received: Aug 2012
Accepted: Sep 2012

School of Pharmacy & Pharmaceutical Sciences
Isfahan University of Medical Sciences

Original Article

Synthesis, analysis and cytotoxic evaluation of some hydroxypyridinone derivatives on HeLa and K562 cell lines

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Abstract

A range of iron bidentae ligands containing the chelating moiety 3-hydroxypyridin-4-ones (HPOs) have been synthesized via a single or a three-step synthetic pathway. In the single-step reaction, maltol was directly reacted by suitable primary amine and in the second synthetic method; benzylated maltol was reacted with related amines to give 1-substuted-2-methyl-3-benzyloxypyridin-4-one derivatives. Finally, removal of the benzyl group under acidic conditions was performed by catalytic hydrogenation to yield the favored bidentate chelators as HCl salt. The partition coefficient of the free ligands and their iron (III) complexes between an aqueous phase buffered at pH 7.4 and 1-octanol were also determined. The cytotoxic effects of these iron chelators against HeLa and K562 cell lines were evaluated using MTT assay and the results showed that cytotoxicity was closely related to the lipophilicity of compounds so that the most lipophilic compound (4g) revealed the highest activity and compound 4e as a more hydrophilic agent (K_{part}; 0.05) showed the lowest cytotoxic effect.

Keywords: 3-Hydroxypyridin-4-ones; Iron chelating agents; Lipophilicity; Cytotoxicity; HeLa and K562 cells

INTRODUCTION

Iron (Fe) is an essential nutritional element for a variety of important biological processes including cell growth and differentiation, electron transfer reactions, oxygen transport, activation, and detoxification (1). In addition, iron is required for neoplastic cell growth because of its effect on promotion of cell proliferation (2,3). The importance of iron in cell division is related to the Fe-dependent activity of ribonucleotide reductase (4). In the absence of iron, cells cannot proceed from the G1- to the S-phase of the cell cycle. Iron accumulation in many organs has been shown to correlate with the process of carcinogenesis (5). As a result, restricting cellular iron supply via the use of iron chelators has been shown to play a role in cancer therapy (6).

In this regards, several studies have indicated that iron deprivation by chelation could potentially correlated to the prevention and management of cancer (2,3). Deferoxamine (DFO, Fig. 1), a bacterial siderophore, is one such chelator that shows anti-proliferative activity against cancer cells including HL-60 leukemic cells (6). Iron chelation by other types of chelators is also known to induce apoptotic cell death in human breast cancer cells (T47D-YB and MCF-7) and human neuroblastoma cells (7,8). Diethylenetriamine pentaacetic acid (DTPA, Fig. 1), a membrane impermeable iron chelator, has also been shown to inhibit growth and proliferation of several cancerous cell types such as lung, colon, and liver (9,10). However, DTPA has mainly been used for the purpose of tumor imaging using radionucleotides (10). Traditionally, iron chelators have been investigated as potential therapeutic agents for diseases of iron overload, e.g., thalassemia. And DFO is the only chelator in regular clinical use for the treatment of iron overload conditions (8). However studies to understand the efficacy of iron chelators in gynecologic malignancies are limited.

It has been previously shown that the key non-heme iron containing enzymes 5-lipoxygenase (5-LO) and ribonucleotide

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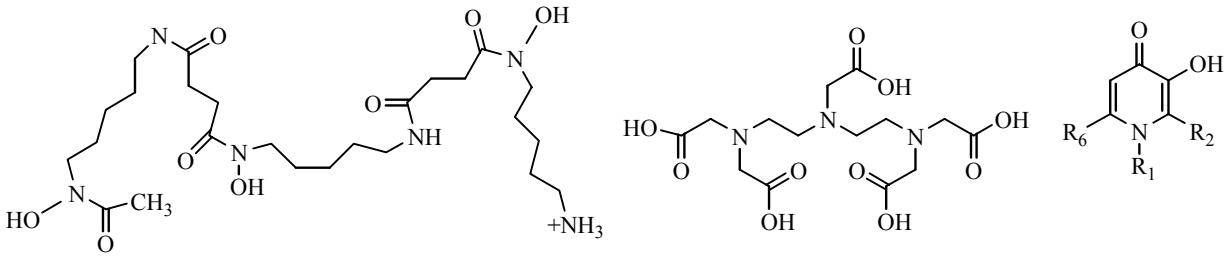

Fig. 1. Structures of desferrioxamine (DFO), diethylenetriamine pentaacetic acid (DTPA) and 3-hydroxypyridin-4-ones (HPOs)

reductase (RR), in which the iron center is dominated by oxygen and imidazole ligands, are inhibited rapidly by bidentate 3-hydroxypyridin-4-one chelators (HPOs, Fig. 1) such as deferiprone (CP20), but significantly more slowly by the larger hexadentate iron chelator DFO (11,12). Earlier studies suggested that the hydrophobicity of bidentate hydroxypyridinones was important in determining the inhibition rate of these enzymes, but the larger size of DFO might also limit inhibition activity of this chelator. In these studies, however, substitution effect was only examined in the position 1 of the pyridinone ring (R1), and then it was not possible to differentiate the effects of size and shape from those of hydrophobicity.

The HPO derivatives known as excellent bidentate iron chelators were designed by Hider's research group as a new candidate for curing iron overload (13-16).

Iron chelation ability of these compounds was proved *in vitro* (in heart and hepatocyte cell cultures) and *in vivo* in some animal species (17-20). Moreover these compounds have been investigated for their positive effects for the treatment of diseases such as malaria (21), aluminium toxic overload (22) and antimicrobial effects (23).

The aim of this study is the synthesis of some derivatives of HPO with the different substitutions at the position 1 of dihydro-pyridinone ring to increase their diffusion into the cell membranes and thus improve their iron chelating ability inside the cells. The partition coefficients (K_{part}) of these compounds were measured to show their hydrophobicity as a quantitative variable (24). To correlate the structure activity with the biological effects, the cytotoxicity of the synthesized iron chelating compounds were evaluated on two cancer cell lines including K562 and HeLa (25).

MATERIALS AND METHODS

Chemistry

All chemicals were obtained from Sigma-Aldrich and used without any further purification. Melting points were determined on a Mettler capillary melting point apparatus *(Electro thermal 9200*) (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}HNMR 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 silicagel plate using chloroform and methanol. The procedure for the synthesis of the desired compounds is described in Fig. 2.

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

The general methodology (26) which has been adopted for the synthesis of 1-substituted-3-hydroxypyridin-4-ones is summarized in Fig. 2. The commercially available maltol **1** was benzylated to give compound **2**. Reaction of **2** with alkylamines produced the benzylated pyridinones **3a-e**, which were subsequently subjected to catalytic hydrogenation under acidic condition to remove the protecting group, yielding the corresponding bidentate 1-substituted -3-hydroxypyridin-4-ones **4a-e**. The purity of

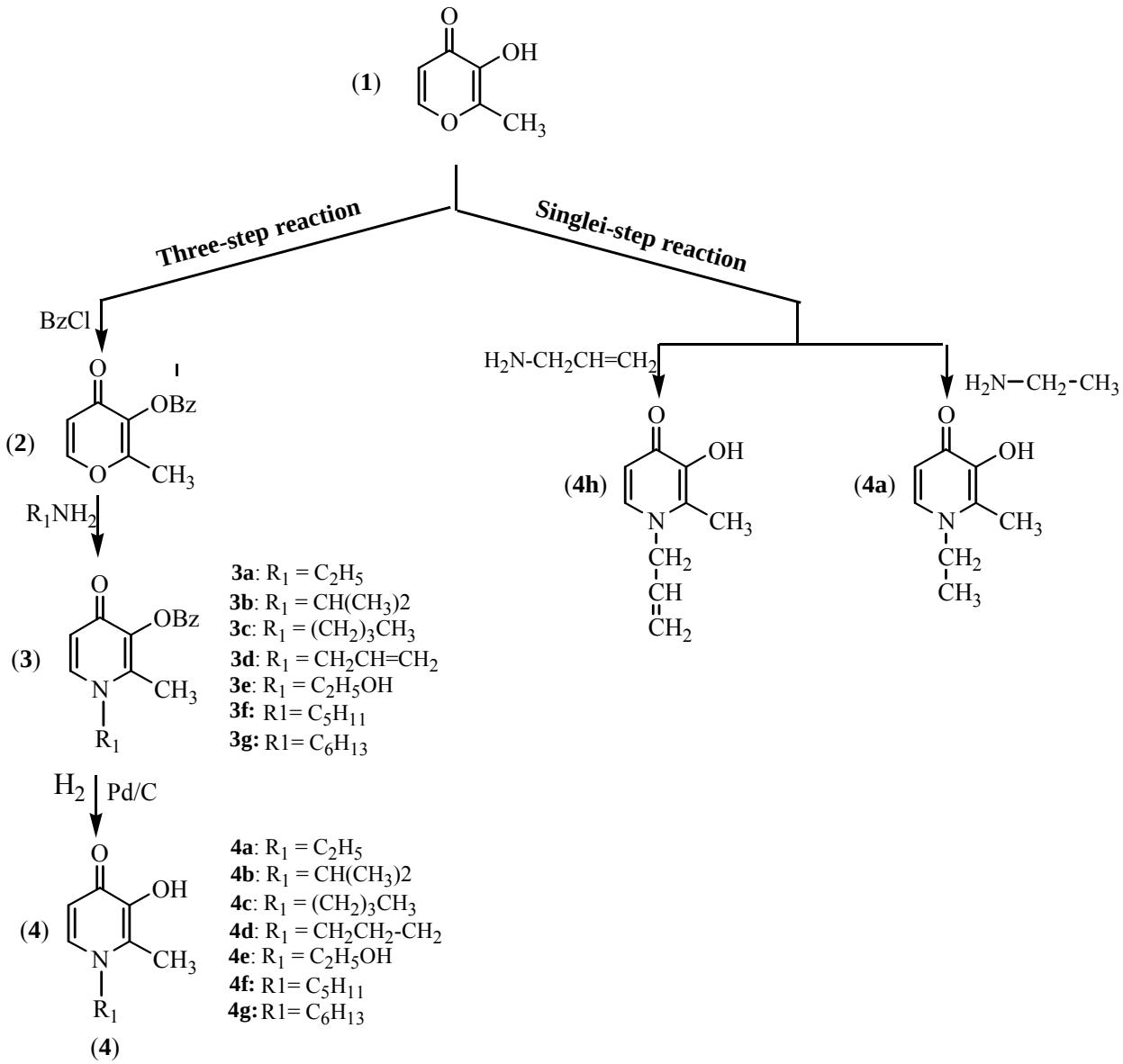

Fig. 2. Synthesis of substituted 1-alkyl-3-hydroxypyridin-4-ones *via* a single or three steps synthetic pathway.

ligands was confirmed by spectroscopic methods. In this study ligand **4h** was synthesized via a single-step synthetic pathway and **4a** synthesized based on single and three-step reaction method. Other resulted products (**4f-g**) were previously reported.

Cell lines

HeLa (Human cervix carcinoma) and K562 (Human myelogenous leukemia) cells 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, 1% of penicillin/ streptomycin (50 IU/ml and 50 µg/ml, respectively), $NaHCO_3$ (1 g) and 1% 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 (1 mM) of each compound was prepared in 1 ml of DMSO and 9 ml of PBS. The final solutions (1, 10 and 100 µM) were then obtained by serial dilution of 1 mM solution, using PBS or culture media and stored at -20°C before using.

MTT based cytotoxicity assay

The cytotoxic effects of synthesized compounds against cells 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 cells into an insoluble colored formazan product, which can be measured spectrophotometrically at 540 nm after dissolution in DMSO. Briefly, 180 µl of cells (5×10^4 cells/ml) were seeded in 96 well microplates and incubated for 24 h (37°C, 5% CO_2 air humidified). Then 20 µl of final concentration of each compound (1, 10 and 100 µM) was added and incubated for another 72 h in the same condition. Doxorubicin (2.3 µM) was used as a positive control. HeLa cells (5×10^4 cells/ml) and K562 cells (2×10^4 cells/ml) were considered as negative control with 100% viability. Cell survival was determined as previously described (25) as following equation:

$$\text{Cell survival (\%)} = [(AT-AB)/ (AC-AB)] \times 100$$

where, AC is the absorbance of control, AT is the absorbance of the treated samples, and AB is the absorbance of the blank.

Determination of partition coefficients (K_{part})

The K_{part} values of the synthesizd compounds were determined using the shake-flask method (24). The two phases used in determination were tris buffer (50 mM, pH 7.4) and 1-octanol, each of which was pre-equilibrated with the other phase before use (because the solubility of water in 1-octanol is 2.3 M) (27).

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 a wavelength of approximately 280 nm using the buffer as a blank. 50 ml of sample solution was stirred vigorously with 50 ml of 1-octanol in a glass vessel for 1 h. The two layers were separated by centrifugation for 5 min. An aliquot of the aqueous layers was then carefully removed using a Pasteur pipette ensuring that the sample was not contaminated with 1-octanol. The absorbance of the sample was measured as above and the K_{part} was then calculated using the following equation:

$$K_{part} = \frac{A_1 - A_2}{A_2} \times \frac{V_w}{V_0}$$

where,

A_1, Absorbance of the aqueous layer before partitioning.
A_2, Absorbance of the aqueous phase after partitioning.
V_0, Volume of 1-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 1).

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 coefficients 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 except for those complexes which present a solubility problem where a ligand/iron concentration ratio of 10^{-4} M/ 10^{-5} M was used. 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 1.

Table 1. Ligand K_{part} values and their corresponding iron (III) complexes obtained by shake-flask method (mean ± SD).

Ligand	K_{part} of the ligand (n=4)	K_{part} of the Fe-complexes (n=4)
4a	0.60 ± 0.07	0.04 ± 0.006
4b	1.41 ± 0.12	0.50 ± 0.06
4c	4.10 ± 0.50	25.20 ± 2
4e	0.05 ± 0.007	0.003 ± 0.0005
4f	15.50 ± 1.0	480± 60
4g	60 ± 8.0	1900 ± 120
4h	0.81 ± 0.08	0.30 ± 0.05

RESULTS

Chemistry results

I) Synthesis of ligands 4a-e via the three-steps synthetic pathway

*Synthesis of 2- methyl -3 -benzyloxypran- 4-one (Benzyl maltol)(**2**).*

To a solution of 2-methyl-3-hydroxypyran-4-one **1** (24 g, 0.2 mol) in methanol (200 ml) was added sodium hydroxide aqueous solution (8.8 g, 0.22 mol in 20 ml water), followed by benzyl chloride (27.8 g, 0.22 mol) and was refluxed for 12h. After removal of solvent by rotary evaporation, the resultant orange oil was taken up in dichloromethane (300 ml) and washed with 5% sodium hydroxide (3×300 ml) followed by water (2×300 ml). The organic fraction was dried over anhydrous sodium sulphate, filtered and rotary evaporated to yield an orange oil which became solid on cooling. Recrystallization form diethyl ether afforded **2** as colorless needles. 25.4 g (82%);. mp 54-55 ℃. ^{1}H NMR (DMSOd6): δ 2.10 (s, 3H, 2-CH_3), 5.10 (s, 2H, O-CH_2-Ph), 6.39 (d, j=5.60 Hz, 1H, 5-H), 7.23-7.48 (m, 5H, Ph), 7.94 (d, j=5.60 Hz, 1H, 6-H). IR (KBr) (cm^{-1}): 1640 (C=0), 1574 (C=C), 1188 (C-O).

*Synthesis of 1-ethyl-2-methyl-3-benzyloxypyridin - 4 -one hydrochloride (**3a**).*

To a solution of compound **2** (25 g, 0.12 mol) in ethanol (200 ml)/water (200 ml) was added 90% aqueous ethylamine (11.8 ml, 0.18 mol) followed by 2N sodium hydroxide solution (10 ml), and the mixture was refluxed for 12 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 after being dried over anhydrous sodium sulphate, was 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/diethylether to give a white powder (24.5 g, 73%); mp 176-177 ^{o}C ((lit. value (28) 178-179^{o}C)). ^{1}H NMR (DMSO-d_6): δ 1.2 (t,j=7.30 Hz, 3H, N-CH_2CH_3), 2.20 (s, 3H, 2-CH_3), 4.30 (q,j=7.30 Hz, 2H, N-CH_2CH_3), 5.10 (s, 2H, O-CH_2-Ph), 7.35-7.50 (m, 5H, Ph), 7.60 (d, j=7.20 Hz, 1H, 5-H), 8.50 (d, j=7.20 Hz, 1H, 6-H). IR (KBr) (cm^{-1}): 1631 (C=0), 1039 (C-N), 1522 (C=C).

*Synthesis of 1-ethyl-2-methyl-3-hydroxypyridin-4-one hydrochloride (**4a**).*

Compound **3a** (18.30 g, 0.075 mol) was dissolved in ethanol / water (270 ml /30 ml) and subjected to hydrogenation in the presence of Pd/C catalyst. Filtration followed by rotary evaporation gave a white solid. Recrystallization from ethanol/diethyl ether yielding a white powder (10.71 g, 75%); mp 206-207ºC ((lit. value (28) 205-206^{o}C)). ^{1}HNMR (DMSO-d_6): δ 1.40 (t, j=7.20 Hz, 3H, N-CH_2CH_3), 2.60 (s, 3H, 2-CH_3), 4.40 (q, j=7.20 Hz , 2H, N-CH_2CH_3), 7.40 (d, j=7.00 Hz , 1H, 5-H), 8.30 (d, j=7.00 Hz , 1H, 6-H). IR (KBr) (cm^{-1}): 1630 (C=0), 1053(C-N), 3093(O-H).

*Synthesis of 1- isopropyl- 2- methyl- 3-benzyl oxypyridin -4-one hydrochloride (**3b**).*

The procedure used was as described for synthesis of **3a** 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 (26.4 g, 75%); mp 170-171 ºC .^{1}H NMR (DMSO-d_6): δ 1.40 (d,j=6.40 Hz, 6H, -CH(C**H**$_3$)$_2$), 2.60 (s, 3H, 2-CH_3), 4.72- 4.95 (m, 1H, N-C**H**$(CH_3)_2$),5.20 (s, 2H, O-CH_2-Ph), 7.33-7.41 (m, 5H, Ph), 7.50 (d ,j=7.40 Hz, 1H, 5-H), 8.6 (d,j=7.40 Hz, 1H, 6-H). IR (KBr) (cm^{-1}): 1616 (C=0), 1039(C-N), 1533 (C=C).

*Synthesis of 1- isopropyl- 2-methyl- 3-hydroxy pyridin -4-one hydrochloride (**4b**).*

The procedure used was as described for synthesis of **4a** except **3b** was used in place of **3a** in the reaction mixture. Recrystallization from ethanol/diethyl ether gave the desired product as a white powder (11.8 g, 77%): mp 223-224ºC ((lit. value (28) 225-226 ^{o}C)). ^{1}H NMR (DMSO-d_6): δ 1.42 (d, j=6.80 Hz, 6H, -CH (C**H**$_3$)$_2$), 2.63 (s, 3H, 2-CH_3), 4.70-4.91 (m, 1H, N-C**H** $(CH_3)_2$), 7.40 (d, j=7.20 Hz, 1H, 5-H), 8.33 (d, j=7.20 Hz, 1H, 6-H). IR

(KBr) (cm^{-1}): 1630 (C=0), 1026 (C-N), 3116 (O-H).

*Synthesis of 1- isobutyl -2-methyl- 3-benzyl oxypyridin -4-one hydrochloride (**3c**).*

The procedure used was as described for synthesis of **3a** except isobutylamine was used in place of ethylamine in the reaction mixture. Recrystallization from ethanol/diethyl ether gave the desired product as a white powder (26.1 g, 71%): mp 193-194°C. ^{1}HNMR (DMSO-d_6): δ 0.94 (d, j=7.00Hz, 6H,-CH(C**H**)$_3$), 1.91-2.10 (m, 1H, -C**H**$(CH_3)_2$), 2.45 (s, 3H, 2-CH_3), 4.21 (d, j=7.00 Hz, 2H, N-CH_2-), 5.25 (s, 2H,O-CH_2- Ph), 7.29-7.39 (m, 5H, Ph), 8.05 (d, j=7.20 Hz, 1H, 5-H), 8.25 (d, j=7.20 Hz, 1H, 6-H). IR (KBr) (cm^{-1}): 1624(C=O),1039 (C-N), 1535 (C=C).

*Synthesis of 1- isobutyl- 2- methyl- 3-hydroxy pyridin- 4-one hydrochloride (**4c**).*

The procedure used was as described for synthesis of **4a** except **3c** was used in the reduction reaction instead of **3a**. Recrystallization from ethanol/diethyl ether gave the desired product as a white powder (11.8 g, 77%); mp 240-241ºC. ^{1}HNMR (DMSO-d_6): δ 0.92 (d, j=70 Hz, 6H,-CH(C**H**$_3)_2$), 1.93-2.15 (m, 1H, -C**H**$(CH_3)_2$), 2.50 (s, 3H, 2-CH_3), 4.21 (d , j=7.00 Hz, 2H, N-C**H**$_2$), 7.50 (d, j=7.00 Hz, 1H, 5H), 8.22 (d, j=70 Hz, 1H, 6-H). IR (KBr) (cm^{-1}): 1630 (C=0), 1059(C-N), 3200(O-H).

*Synthesis of 1- allyl -2 -methyl- 3-benzyl oxypyridin -4-one hydrochloride (**3d**).*

The procedure used was as described for synthesis of **3a** except allylamine was used in place of ethylamine in the reaction mixture. Recrystallization from ethanol/diethyl ether gave the desired product as a white powder (23.8 g, 68%); mp 167-169°C. ^{1}HNMR (DMSO-d_6): δ 2.32 (s, 3H, 2-CH_3), 4.60 (d, j=6.40 Hz, 2H, N-CH_2-), 4.84 (d, j=14.00 Hz, 1H, N-CH_2-CH=C**H**H), 5.30 (d, j=14.00 Hz, 1H, N-CH_2-CH=CH**H**), 5.93-6.14 (m, 1H, N-CH_2-C**H**=), 6.15 (d, j=7.20 Hz, 1H, 5-H), 7.15 (d, j=7.20 Hz, 1H, 6-H). IR (KBr) (cm^{-1}):1618(C=O), 1045 (C-N), 1539 (C=C).

*Synthesis of 1- propyl -2-methyl-3-hydroxy pyridin-4-one hydrochloride (**4d**).*

The procedure used was as described for synthesis of **4a** except **3d** was used in place of **3a** in the reaction mixture. Recrystallization from ethanol/diethyl ether gave the desired product as a white powder (9.8 g, 65%); mp 203-204ºC ((lit. value (28) 206-207°C)). ^{1}HNMR (DMSO-d_6): δ 0.82 (t,j=7.20 Hz, 3H,N-CH_2CH_2C**H**$_3$),1.52- 1.83 (m, 2H, N-CH_2C**H**$_2CH_3$), 2.51(s, 3H, 2-CH_3), 4.22 (t, j=7.20 Hz, 2H, N-C**H**$_2CH_2CH_3$), 7.33 (d, j=6.80 Hz, 1H, 5H), 8.22 (d, j=6.80 Hz, 1H, 6-H); IR (KBr) (cm^{-1}): 1632 (C=O),1050 (C-N), 3100(O-H).

*Synthesis of 1-(2´-hydroxyethyl)-2-methyl-3-benzyloxypyridin-4-one hydrochloride (**3e**).*

The procedure used was as described for synthesis of **3a** except ethanolamine was used in place of ethylamine in the reaction mixture. Recrystallization from ethanol/diethyl ether gave the desired product as a white powder (29.8 g , 88%) ; mp 207-208ºC ((lit. value (28) 205-206°C)). ^{1}HNMR (DMSO-d_6): δ 2.42 (s, 3H, 2-CH_3), 3.83 (t, j=5.20Hz, 2H, N-CH2-), 4.42 (t, j=5.20 Hz, 2H, N-CH_2C**H**$_2$OH), 5.10 (s, 2H, O-CH_2-Ph), 7.35-7.55 (m, 6H, Ph & 5-H), 8.32 (d, j=7.20 Hz, 1H, 6-H). IR (KBr) (cm^{-1}): 1637(C=0), 1078(C-N), 1522(C=C).

*Synthesis of 1-(2´-hydroxyethyl)-2-methyl-3-hydroxypyridin-4-one hydrochloride (**4e**).*

The procedure used was as described for synthesis of **4a** except **3e** was used in the process of reduction. Recrystallization from ethanol/diethyl ether gave the desired product as a white powder (11.4 g , 79 %); mp 151-152ºC ((lit. value (28) 156-157°C)). ^{1}HNMR (DMSO-d_6): δ 2.13 (s, 3H, 2-CH_3), 3.82 (t, j=5.20 Hz, 2H, N-CH2), 4.42 (t, j=5.20 Hz, 2H, N-CH_2C**H**$_2$OH), 7.32 (d, j=6.80 Hz,1H, 5-H), 8.23 (d, j=6.80 Hz, 1H, 6-H) .IR (KBr) (cm^{-1}): 1643(C=0), 1084(C-N), 3232(O-H).

***II) Synthesis of ligands 4h and 4a via* the single- step synthetic pathway**

*Synthesis of 1-allyl-2-methyl-3-hydroxypyridin -4-one (**4h**)*

Maltol (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. Decolourizing charcoal was added and the mixture stirred for 0.5 h prior to filtration. The filtrate was evaporated to give a brown solid.

Recrystallization from ethanol/ether gave a white crystalline solid. (2.4g, 31%); mp 176.6-178ºC. ^{1}HNMR (DMSO-d_6): δ 2.32 (s, 3H, 2-CH_3), 4.60 (d, j=6.80 Hz, 2H, N-CH_2-), 4.82 (d, j=6.80 Hz, 1H, N-CH_2-CH=C**H**H), 5.33 (d, j=6.80 Hz, 1H, N-CH_2-CH=CH**H**),5.92-6.13 (m, 1H, N-CH_2-C**H**=), 6.15 (d, j=7.20 Hz, 1H, 5-H), 7.15 (d, j=7.20 Hz, 1H, 6-H); IR(KBr) (cm^{-1}): 3167 (OH), 1626 (C=O),1047(C-N).

*Synthesis of 1-ethyl-2-methyl-3-hydroxypyridin -4-one hydrochloride (**4a**).*

Maltol (24 g, 0.2 mol) was added to a solution of ethylamine (24.2 ml, 0.4 mol) in 300 ml water. The mixture was refluxed for 24 h. Decolorizing charcoal was added and left for 0.5 h. The mixture was filtered and the filtrate evaporated to give a brown solid. This solid was then dissolved in ethanol/hydrochloric acid and rotary evaporated, the resulting yellow solid was recrystallized from ethanol/ether to give a white powder(11.4 g, 33%); mp 207-208ºC ((lit. value (28) 205-206^{o}C)). ^{1}HNMR (DMSO-d_6): δ 1.42 (t, j=7.20 Hz, 3H, N-CH_2CH_3), 2.63 (s, 3H, 2-CH_3), 4.40 (q , j=7.20 Hz , 2H, N-CH_2CH_3), 7.42 (d, j=7.00Hz , 1H, 5-H), 8.33 (d, j=7.00 Hz , 1H, 6-H). IR (KBr) (cm^{-1}): 1630 (C=0), 1053(C-N), 3093(O-H).

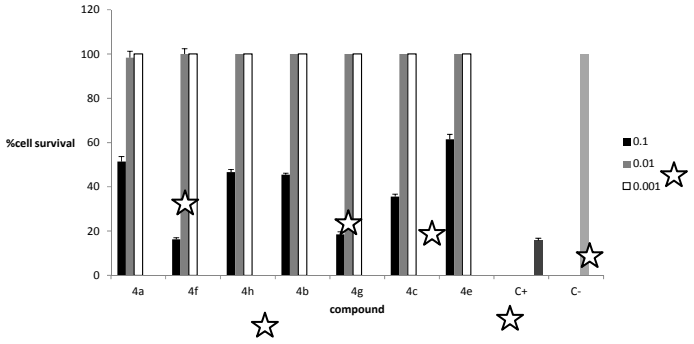

Fig. 3. Cytotoxic effects of HPO compounds against Hela cell line following exposure of 3 different concentrations of each compound. Cell viability was assessed using MTT assay. Data are presented as mean ± SD of cell survival compared to negative control (Cell survival of 100%) P<0.05(significantly different from control)

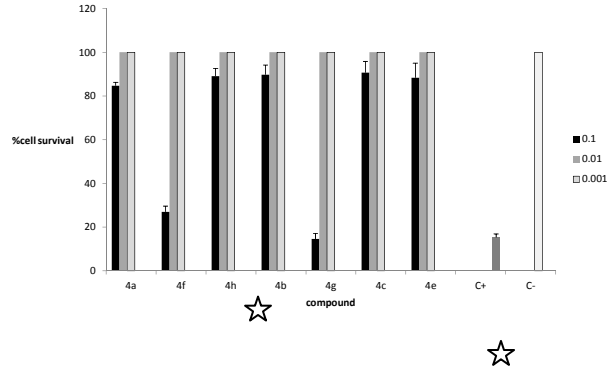

Fig. 4. Cytotoxic effects of HPO compounds against K562 cell line following exposure of 3 different concentrations of each compound. Cell viability was assessed using MTT assay. Data are presented as mean ± SD of cell survival compared to negative control (Cell survival of 100%) P<0.05 (significantly different from control).

Cytotoxic activity

The cytotoxicity of all compounds was determined by MTT assay as already described (25). Data are given in Fig. 3 and Fig. 4. All compounds were tested for cytotoxic properties against K562 and HeLa cells. At concentrations of 0.001 and 0.01 mM no cytotoxic effect was 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. As seen in Fig. 3 and Fig. 4, IC50 values for all compounds were measured to be less than 0.1 mM with the exception of 4e. Agents **4g** and **4f** showed highest cytotoxic activity against HeLa and K562 cells (P<0.05). It appears that the cytotoxic activity of compounds against HeLa cells is in the following order: 4f> 4g> 4c> 4b> 4h> 4a> 4e; whereas the K562 cells were more resistant to these compounds.

DISCUSSION

The hydroxypyridinone ligands were synthesized from 2-methyl-3-hydroxypyran-4-one (maltol, **1**) in three steps via protection of the hydroxyl group. Then the protected compound was reacted with an alkyl amine RNH_2, in which R represents the group on the nitrogen atom of the pyridinone ring Fig. 2 (26).

The protection of the 3-hydroxyl group proved to be essential since under the basic conditions employed in the amination reaction, it is likely that the unprotected hydroxyl group undergoes a Michael-type reaction Fig. 5. Further condensation products lead to significant consumption of starting material and therefore influence the overall yield.

A protective group must fulfill a number of requirements: (I) It must react selectively in good yield to give a protected substrate that is stable to the projected reaction conditions, (II) the protected group must be selectively removed in good yield by readily available, preferably non toxic reagents, which do not attack the regenerated functional group, and (III) the protective group should have a minimum of additional functionality to avoid further sites of reaction.

For this work, benzyl and methyl protective groups fulfill these requirements, but benzyl protective group was preferred. Although the 3-hydroxy substituents of 3-hydroxypyran-4-ones can also be protected by methyl ether formation, the corresponding 3-methoxy-2-ethyl-4-pyrane (methoxy maltol) is oil which is less convenient to work with than the crystalline 3-benzyloxy-2-methyl-4-pyrone (benzyl maltol). Furthermore, the benzyl protecting group can be removed by hydrogenation under acidic, neutral or basic conditions. For these reasons the benzyl group was selected as protecting group in this study.

The conversion of pyran-4-one to pyridin-4-one involves Michael addition of amine 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, double attack at both α,β-unsaturated functions of the pyran-4-one leads to formation of pyridin-4-one with the loss of a water molecule as seen in Fig. 6.

Conversion of the pyranone derivatives to the corresponding pyridinone analogues was achieved without protection of the 3-hydroxyl group in different solvents (such as water, methanol and ethanol) via the one step reaction. However, this reaction in water was limited to primary alkyl amines with short chain length (i.e. ethylamine and allylamine), since bulky alkyl amines with longer or branched chain (i.e. isopropylamine and isobutylamine) gave low yield (less than 10%). 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. As mentioned above, further condensation of products resulted in high consumption of maltol and therefore lead to the low yield (Fig. 6). Although the reported yield for the preparation of 1-ethyl-2-methyl-3-hydroxypyridin-4-one (**4a**) via single step synthetic pathway is lower (30% yield) than that the three-step method reaction (45%) but single step reaction is much easier and less expensive overall.

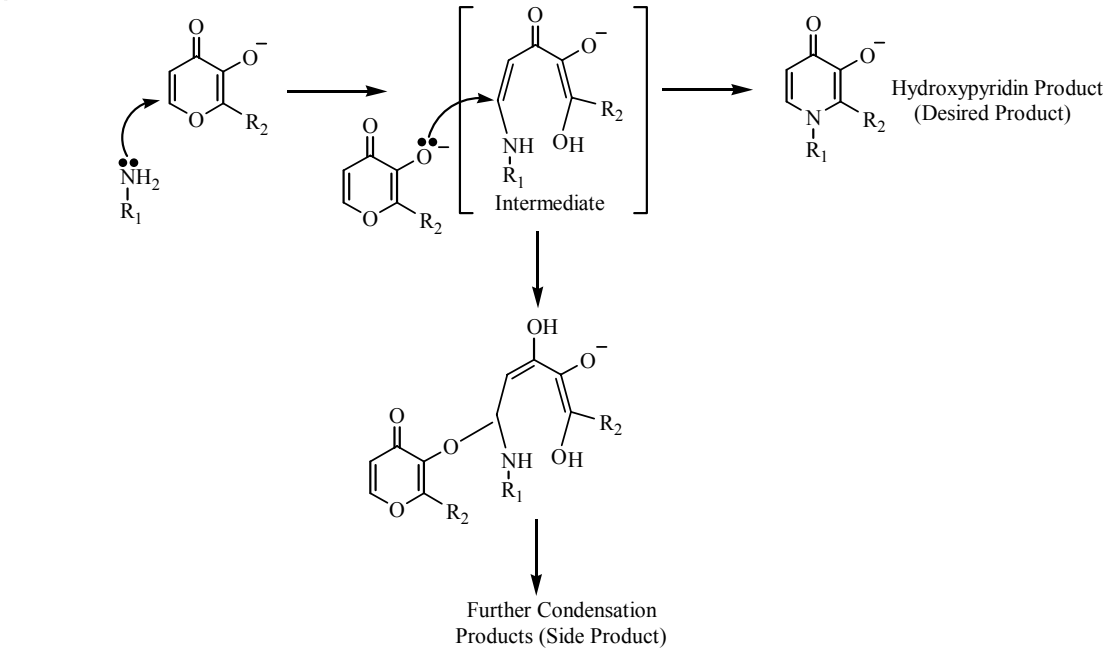

Fig. 5. A possible condensation product in the synthesis of bidentate pyridin-4-ones from reaction of unprotected maltol with primary amines under basic conditions.

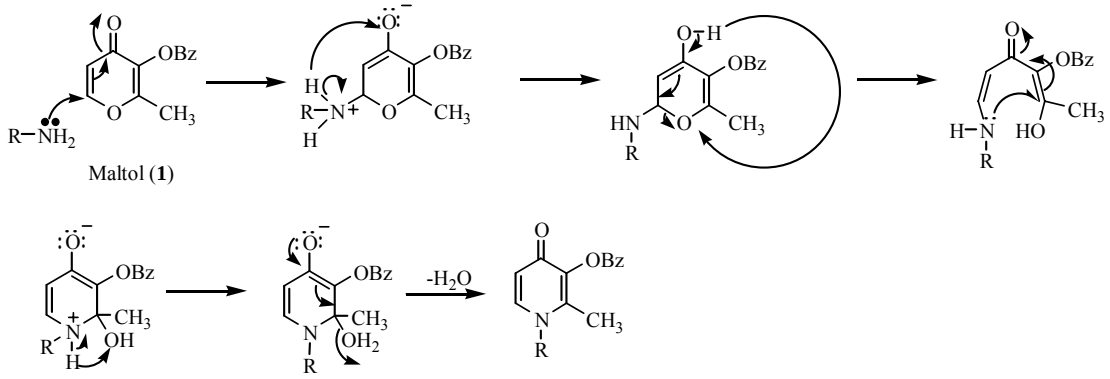

Fig. 6. Formation of 1-alkyl-3- hydroxypyridin-4-ones from reaction of protected maltol with alkyl amines under basic conditions.

It should be noted that for the synthesis of 1-allyl-2-methyl-3-hydroxypyridin-4-one **4h** ligand via three-step reaction, under catalytic hydrogenation step, allyl group was saturated and converted to propyl chain (compound **4d**). In other words, compound **4d** (1-propyl-2-methyl-3-hydroxypyridin-4-one) was synthesized instead of desired product of 1-allyl-2-methyl-3-hydroxypyridin-4-one (**4h**). For this reason, the ligand 4h was prepared based on a single step synthetic pathway.

Conversion of maltol **1** with aryl amines can also be achieved without protection of the 3-hydroxyl group in acidic conditions (Fig. 7). Under the acidic conditions employed in the amination reaction, it is unlikely that the unprotected hydroxyl function could undergo a Michael-type reaction with intermediates formed during the amination step. This investigation, prompted us to attempt a direct one-step reaction of maltol **1** (instead of benzylmaltol, **2** with bulky or branched chain alkyl amines (such as isopropyl and isobutyl amines) in dilute hydrochloric acid solutions with different pH (pH 1-6), which consequently resulted in no reaction. Unreactivity of the alkyl amines may be attributed to this fact that, in acidic conditions, alkyl amines, unlike aryl amines are fully protonated and therefore the nitrogen atom of this species would not act as an nucleophile to attack at $C_{(6)}$ [or $C_{(2)}$] of the maltol (Fig. 7).

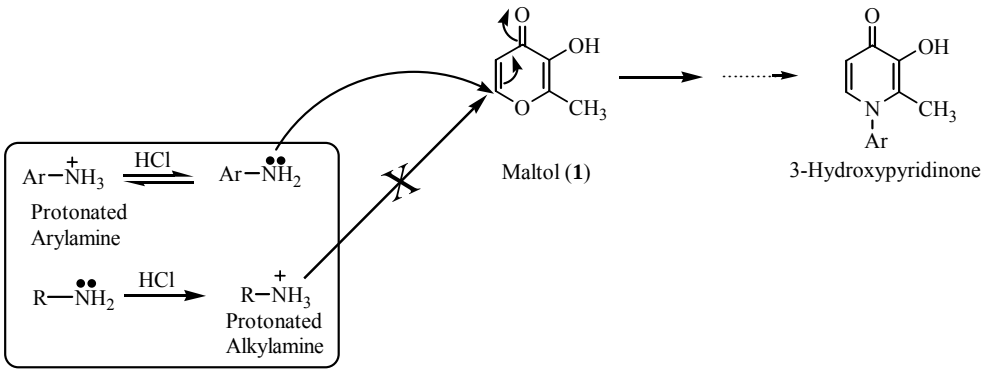

Fig. 7. Formation of 1-aryl-3- hydroxypyridin-4-ones from reaction of unprotected maltol with aryl amines under acidic conditions. In these conditions, alkyl amines are completely protonated and therefore the nitrogen atom of this moiety would not act as a nucleophile to attack the maltol.

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 1. The K_{part} values of both bidentate ligands and their Fe-complexes were determined by using the shake-flask method (24). 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 previous studies, we have shown that in most cases iron (III) complexes are more hydrophilic than their corresponding free ligands. However, this trend did not hold for those compounds which have K_{part} values greater than 3 (i.e. **4c**, **4g** and **4f**). Among the ligands, **4g** and **4e** possess the highest and the lowest Kpart values respectively, and not surprisingly they form the most hydrophobic and hydrophilic iron (III) complexes, respectively.

In vitro cytotoxicity

In vitro cytotoxic evaluation results showed that there is a strong straight correlation between the effects and lipophilicity of the N-1 moiety of tested compounds. The k_{part} of the substances also proved our theory regarding their effects. In fact the diffusion of the ligands to the cells through their membrane need more hydrophobic or small molecules, that in the present investigation both of these conditions come together. The weakest cytotoxic effect was observed with the most hydrophilic one (**4e**), while the highest cytotoxicity was seen with compound 4g that is the most lipophilic compound.

The results also showed that the critical part of these derivatives was their hydroxyl and carbonyl group; but the group on N-1 was also important. The correlations between effects and structures were disappeared when N-1 groups were changed.This conclusion supports our initial idea of the existence of a relationship between cytotoxic activity and iron chelation ability.

As shown in Fig. 3 and Fig. 4 compound 4f and 4e showed maximum and minimum effects, respectively.

There are two important factors that influence the cytotoxic effect of 3-hydroxypyridin-4-ones including membrane permeability and partition coefficient (29,30); although the former itself depends on the size of the compounds. These results were consistent with Saghaie *et al* findings (31).

CONCLUSION

So it would be concluded that highly lipophilic small molecules of 3-hydroxypyridin-4-one with higher iron chelating ability may be potentially effective in cancer therapy.

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