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3-Hydroxy-(4*H*)-benzopyran-4-ones as Potential Iron Chelating Agents In Vivo

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Abstract—Increasing evidence suggests that iron plays an important role in tissue damage both during chronic iron overload diseases (i.e., hemochromatosis) and when, in the absence of actual tissue iron overload, iron is delocalised from specific carriers or intracellular sites (inflammation, neurodegenerative diseases, post-ischaemic reperfusion, etc.). In order to be used for therapeutical purposes in vivo, a reliable iron chelator should be capable of preventing the undesired effects that follow the electrochemical activation of iron (see below). Bearing in mind the molecular structure of some flavonols that are able to chelate iron, we synthesised a new oral iron-chelator, 2-methyl-3-hydroxy-4*H*-benzopyran-4-one (MCOH). We demonstrate that MCOH chelates iron in a 2:1 ratio showing a stability constant of $\sim 10^{10}$. MCOH is able to cross cell membranes (erythrocytes, ascite tumour cells) in both directions. Following intraperitoneal administration to rats, it is quickly taken up by the liver and excreted in the urine within 24 h. A similar behaviour has been documented after oral administration. We propose that MCOH may represent the prototype of a new class of iron chelating agents to be developed for iron-removal therapy in vivo with the goal of preventing tissue damage caused by the iron redox cycle.

Introduction

It is well know that iron plays an important role in mammalian life and in all living cells. In mammals, iron is trapped by specific proteins and maintained in a form that is unsuitable for reaction with active oxygen species. When, for different reasons, iron is mobilised from these proteins, it becomes extremely dangerous for cells and tissues entering a redox cycle that generates toxic free radicals.[1,2] The generation of such species induces an oxidative stress in the cell leading to lipid peroxidation,[3] protein oxidation[4] and DNA damage.[5] Since delocalised iron has the capacity of generating cellular damage, the direct or indirect involvement of the redox active metal has been hypothesised in several disease states. In fact, there is evidence of iron involvement in the pathogenesis of neoplasia,[6] neurodegenerative diseases,[7] rheumatoid arthritis,[8] xenobiotic intoxication,[9] post-ischaemic reperfusion,[10] malaria,[11] haemochromatosis[12] and thalassemias.[13] The redox activity of free iron is inhibited, both in vitro and in vivo, by the presence of iron chelators, represented by two different groups. One is constituted by iron chelators isolated from bacteria or plants, the other by artificially generated products. Desferrioxamine and hydroxyamates belong to the first group; pyridoxalhydrazones and hydroxypyridones belong to the second group. All these chelators are currently used in both in vitro and in vivo studies related to iron metabolism and pathophysiology. In particular desferrioxamine and hydroxypyridones are used in the treatment of human iron overload.[14,15] Desferrioxamine presents some disadvantages because of the need for continuous subcutaneous infusion and the high cost involved. The hydroxypyridones, despite the advantage of oral administration, show a number of serious complications after prolonged therapy.[16] At present, pyridoxalhydrazones appear to be the most promising iron chelators to be used in the treatment of iron overload in humans.[17] The advantages and disadvantages of the iron chelators have been elegantly

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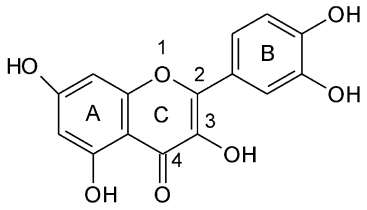
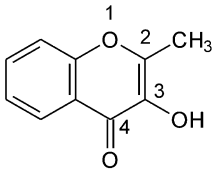


Quercetin



2-Methyl-3-hydroxy-(4H)-benzopyran-4-one (MCOH)

Scheme 1.

summarised by Tilbrook and Hider.[18] In view of all the above considerations, the development of novel orally active and safer iron chelators appears to be very important.[19]

To design an iron chelator for clinical use it is very important to pay particular attention to at least five points: (a) its specificity for iron; (b) it should not be toxic for living cells; (c) its affinity for iron should be not so high as to mobilise the metal from specific protein carriers; (d) it should be suitable for oral administration; (e) it should be rapidly eliminated after chelation of iron before it becomes redox active. Our interest in developing novel iron chelators originates from early observations made on quercetin.[20] The flavonol quercetin belongs to the class of flavonoids and it is taken up by humans from vegetable foods like tea, onions, apples, and from red wine, together with smaller amounts of others similar molecules such as kaempferol, or the glycone rutin. The antioxidant activity of flavonoids is well known,[21] although the mechanism(s) by which they act in vivo is not clarified. In a clinical study, the risk of coronary heart disease was shown to inversely correlate with the extent of flavonoid intake.[22]

We have recently shown that the flavonoid quercetin chelates iron by the 3-hydroxyl and 4-keto groups of the C ring (Scheme 1). Quercetin easily crosses the erythrocyte membranes and prevents the iron redox cycle inside the cell. Because kaempferol and rutin contain the hydroxyl and keto groups in the same position, they chelate iron in the same way as quercetin.[20] Thus, the neighbouring positions of a hydroxyl and a keto group in a pyranyl ring appear to account for the iron chelation capability. Maltol, too, is a good iron chelator even though it is not able to cross cell membrane in vitro (personal observation). Quercetin is not absorbed following subcutaneous or intraperitoneal injection and it is minimally absorbed after gastric intubation in rats

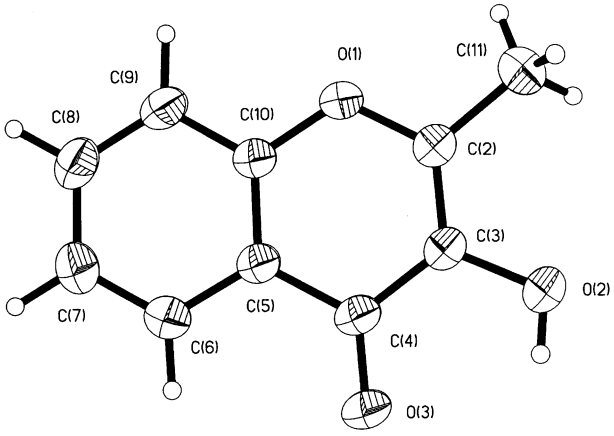


Figure 1. X-ray crystal structure of MCOH.

(personal observations). Therefore it is unlikely that flavonoids constitute a suitable class of compounds for development in the clinical setting, as previously suggested.[23]

The aim of this study was to synthesise and test the iron chelating capability of a quercetin-like molecule, which contains the same iron chelating groups but with a lower molecular weight. To this end we synthesised and characterised the iron chelating activity of the compound 2-methyl-3-hydroxy-(4*H*)-benzopyran-4-one and evaluated its capacity for cell penetration.

Results and Discussion

The molecular structure of MCOH determined by X-ray crystallography is reported in Figure 1. The molecule is planar with the largest deviation (0.14 Å) shown by O(1) from the least-square plane defined by the heterocyclic system. The distance O(2)···O(3), the two most probable sites for iron chelation, is equal to 2.734(3) Å. In the crystal lattice, O(2)–H participates in intermolecular hydrogen bonding with O(3) and stacking interactions occur between the heterocyclic systems. Crystal data details will be given elsewhere.

We performed different experiments to ascertain the ability of MCOH to chelate iron. The original idea was that MCOH chelates iron by the oxygen atoms at positions 3 and 4 of the pyranyl ring, as quercetin appears to do. In this perspective, two molecules of MCOH should chelate one atom of iron. The UV–vis spectrophotometric analysis of the MCOH in water solution confirmed this hypothesis (Fig. 2). As can be seen, the addition of $FeCl_3$ to the 100 μM solution of MCOH produces a shift in the absorption peak from 316 to 360 nm. When the concentration of the iron reaches half that of MCOH, the maximum absorbance is reached at 360 nm. This strongly indicates that a MCOH–Fe complex is formed with 2:1 ligand to metal stoichiometry. The addition of 50 μM desferrioxamine to the 50 μM [$(MCOH)_2$–Fe] complex restores the original absorbance spectrum of 100 μM MCOH (not shown). This not only indicates that desferrioxamine shows a higher

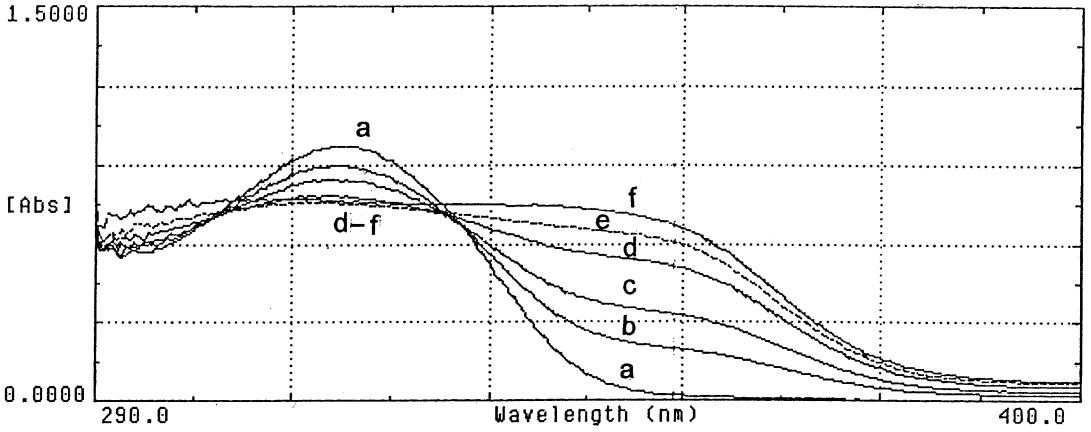


Figure 2. Spectrophotometric detection of Fe complexation with MCOH: (a) spectrum of 100 μM MCOH in water containing 5% DMSO; (b–f) spectra of MCOH after the addition of 6, 12, 25, 50 and 100 μM $FeCL_3$, respectively. See Experimental for details.

affinity for iron than MCOH, but also that desferrioxamine extracts iron from a former complex. It is noteworthy that, unlike quercetin, MCOH seems to chelate Fe^{3+} despite the fact that the molecular sites engaged in the bond are the same.

Further and stronger evidence of the iron chelating properties of MCOH was obtained by electrochemical and mass spectrometric measurements. As can be seen in Figure 3, when the cyclic voltammogram of a solution of 1 mM Fe^{3+} (as anhydrous ferric chloride) in 0.15 M NaCl plus 5% DMSO is recorded, the reduction potential of Fe^{3+} is 0.4 V (Fig. 3, A, peak R_1). Based on the fact that the reduction of the ferrous iron to the metal occurs at about −1.3 V versus SCE, the second reduction in Figure 3A (Ep = −0.04 V) is attributed to electrode absorption phenomena. The addition of half molar equivalents of MCOH to the 1 mM solution of Fe^{3+} determinates the appearance of a new reduction potential peak at 0.2 V suggesting that part of Fe^{3+} is in a complexed form (Fig. 3, B, peak R_2). When the molar ratio between iron and MCOH is 1 (Fig. 3, C), R_2 peak increases and R_1 is still present. This means that not all Fe^{3+} is sequestered. R_1 disappears completely (Fig. 3, D) when the MCOH concentration is double that of iron. It is also noteworthy that the oxidation peak (O) of Fe^{2+} to Fe^{3+} is not proportional to the free iron in solution, as it is also present when no iron is present in free form and R_1 has disappeared (Fig. 3, D). This indicates that the iron that can be still oxidised comes from the complex after its electrochemical reduction, suggesting that MCOH does not chelate Fe^{2+}. Moreover, based on the shift in the reduction process of the MCOH-sequestered Fe^{3+} with respect to free Fe^{3+} ion at different molar ratios, the β_2 ($\beta_2 = \frac{[Fe(MCOH)_2]}{[Fe][MCOH]^2}$) of the reaction $Fe^{3+} + 2MCOH \rightarrow Fe(MCOH)_2$ has been calculated, by electrochemical data, according to literature procedure.[24] β_2 was found to be $10^{10}\,dm^6\,mol^{-2}$, suggesting that MCOH has a significant affinity for iron(III), even if this is lower than that of desferrioxamine or pyridones.

The electron ionisation mass spectrum obtained by mixing $FeCl_3$ and MCOH in acetone shows that MCOH has strong chelating properties towards the metal ion. The most abundant complexes have 2:1 ligand/iron stoichiometry (Fig. 4, top). They are constituted by a species at *m/z* 406 due to $[Fe(MCOH–H)_2]^+$ and a further complex at *m/z* 441 containing a chlorine atom. A complex with ligand/metal ratio equal to 1 is also detected at *m/z* 301 with a quite low relative intensity in comparison to the species containing two ligand molecules. To complete the coordination sphere of the iron, two chlorine atoms are also present in the complex at *m/z* 301 (Fig. 4, top). The species at *m/z* 266 and 231 are attributable to ions produced by fragmentation reactions. These occur both in the ion source and in metastable decompositions, as shown by MS/MS experiments. The MS/MS spectrum of the complex $[Fe(MCOH–H)_2]^+$ shows that the only metastable decomposition consists of the loss of a ligand molecule producing the species at *m/z* 231 (Fig. 4, bottom), also detected in the mass spectrum.

In view of a possible use of MCOH in biological environments, preliminary studies have been carried out on the lipophilicity and cellular availability of the new iron chelating agent. The distribution coefficient of MCOH between water/octanol and saline phosphate buffer (pH 7.4)/octanol has been calculated as log[MCOH] octanol/[MCOH] water. This ratio, usually expressed as P, is 1.59 for water and 0.9 for the buffer (pH 7.4) suggesting that MCOH is suitable for cell membrane penetration, as suggested by Hider et al.[25] After these encouraging

Table 1. Percent distribution of MCOH between medium and intracellular environment in two different cell types[a]

Incubation time	Erythrocytes				EATC			
	MCOH		$[MCOH]_2$–Fe		MCOH		$[MCOH]_2$–Fe	
	Plasma	Cell	Plasma	Cell	Buffer	Cell	Buffer	Cell
30 min	49	51	44	56	41	59	34	66
(30 + 30) min	48	52	50	50	45	55	46	54

[a]Erythrocytes and Ehrlich ascite tumor cells (EATC) were incubated first with 100 μM MCOH or $[MCOH]_2$–Fe (30 min). Loaded cells were sedimented and reincubated in fresh medium for an additional 30 min (30 + 30) min. For experimental design see Experimental.

observations, we tested MCOH for its ability to cross cell membrane. Thus we incubated MCOH (100 μM) and $[MCOH]_2$–Fe^{3+} complex with rat erythrocytes and Ehrlich ascite tumour cells, separately, at 37 °C. Table 1 shows that both MCOH and $[MCOH]_2$–Fe^{3+} are able to reach the intracellular environment in a short time. As can be seen the repartition of the tested compounds between water and the intracellular environment is almost the same.

However, when MCOH or the MCOH-iron complex are incubated with EATC, both clearly prefer the intracellular environment and their return in the medium is slower than in the case of the erythrocytes. It is possible that this behaviour is due to the different architecture of the membranes used or to the known greed of tumour cells for iron. In fact, when loaded cells were re-incubated in fresh media, both MCOH and its iron complex re-crossed the erythrocyte and EATC membrane with different kinetics. All these data strongly indicate that MCOH crosses the cell membrane and, possibly, after intracellular iron chelation, the MCOH–iron complex turns back easily. This aspect could be of great interest if the chelator is considered suitable for experimental and/or clinical use in iron overload diseases or in the prevention of the secondary effects due to iron delocalisation.

In order to verify whether MCOH is absorbed in rodents after parenteral administration, a preliminary trial was carried out to gain information on its biodistribution. As can be seen in Table 2, after intraperitoneal administration MCOH appears in the liver within 30 min, remains there for several hours and is no longer detected after 24 h. MCOH is already present in the urine at 2 h from the start of treatment and excretion is completed within 24 h. MCOH was not found in the faeces at any of the examined time points. These data indicate that MCOH is rapidly absorbed in rodents, extracted by the liver and excreted by the urine within 24 h.

MCOH has been also administered to rats by gastric intubation (100 mg/kg body weight) after dissolving in DMSO at the same concentration as that used intraperitoneally. MCOH appears in urine already after 4 h and disappears after 24 h. About 30–40% of the orally administered MCOH was recovered by this way thus demonstrating that the chelator is absorbed by the alimentary tract. Studies on the capacity of MCOH to chelate excess iron in tissues and to vehicle it into the urine are ongoing. Studies on the specificity of MCOH for chelation of iron rather than that of other biological ions are also in progress.

In conclusion, the original idea that MCOH chelates iron in a 2:1 molar ratio through its protruding oxygens turned out to be realistic. The affinity between MCOH and Fe^{3+} is high but not dramatic. As a consequence, it is presumed that in vivo MCOH will not mobilise iron from storage proteins (i.e., ferritin) or from those which contain it as a catalytic site (i.e., cytochromes, hydroxylases). This may make MCOH particularly useful in the pathologic states where the detrimental effects of the catalytical properties of iron are due to its 'delocalisation' from

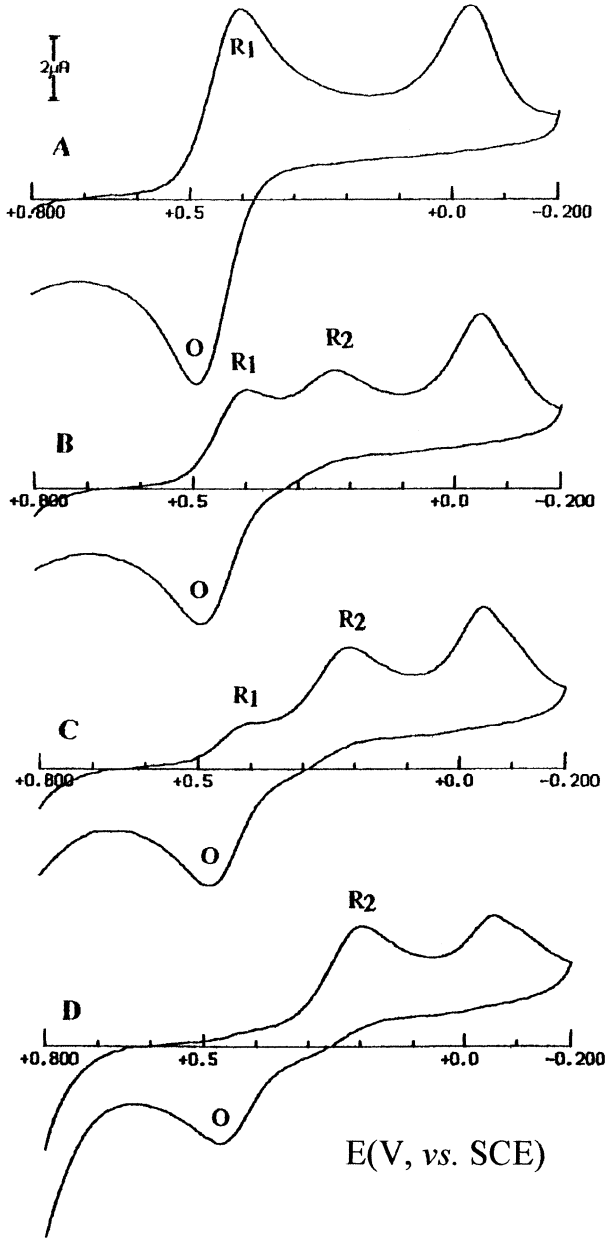


Figure 3. Cyclic voltammetry of the MCOH–Fe^{3+} complex during its formation: (A) 10^{-3} M $FeCl_3$ in 0.15 M NaCl containing 5% DMSO; (B–D) 10^{-3} M $FeCl_3$ after addition of 5×10^{-4} M, 10^{-3} M, 2×10^{-3} M MCOH, respectively. R_1, R_2, reduction peaks of Fe^{3+} to Fe^{2+}; O, oxidation peak of Fe^{2+} to Fe^{3+}. See Experimental for details.

storage proteins rather than to an actual iron overload.

Since two molecules of MCOH chelate iron using four oxygen atoms, two co-ordination sites of Fe^{3+} might be available for other ligands. This might foster metal ion reduction and detachment. For this reason studies are in progress to modify the basal molecule (chromone) to obtain a molecule able to chelate Fe^{3+} by blocking all six co-ordination sites. The chromone ring of MCOH, which is also common to tocopherols, should ensure liposolubility and low toxicity. This reinforces our idea on the usefulness of developing this new class of iron chelators and their promising use in the treatment of human diseases due to iron toxicity.

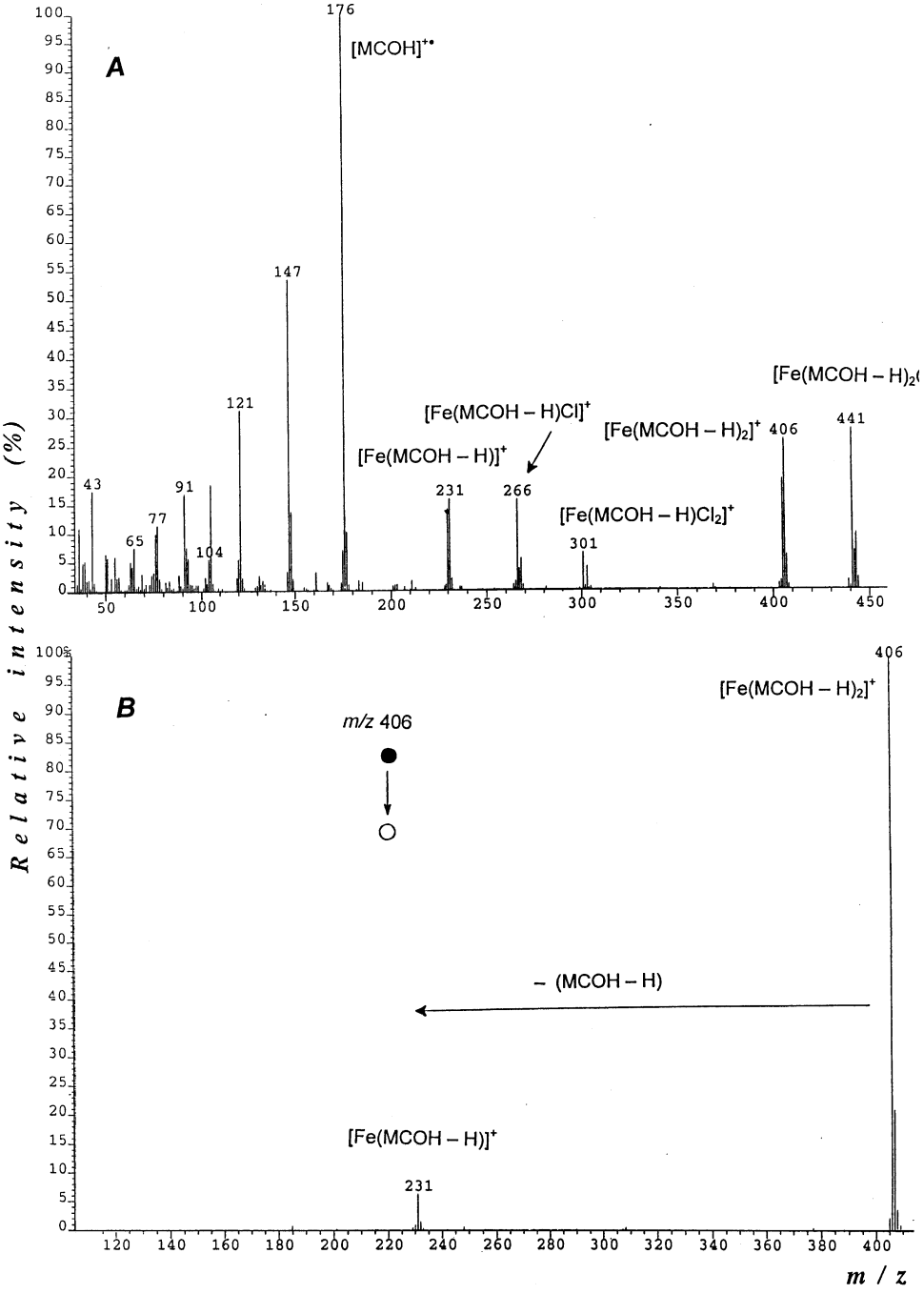


Figure 4. Electron ionization mass spectrometry of MCOH–Fe^{3+} complex: (A) MS of MCOH + $FeCl_3$ in acetone; (B) MS/MS of the species at m/z 406.

Table 2. Detection of MCOH in liver and excretions[a]

Time after MCOH administration	Liver	Urine	Faeces
30 min	p	—	—
2 h	p	p	a
12 h	p	p	a
24 h	a	p	a
48 h	a	a	a

[a]Rats were killed at indicated times after an intraperitoneal injection of 50 mg/kg of MCOH in DMSO. Samples of various tissues and excretions were extracted with ethyl acetate, the solvent concentrated, and MCOH detected by TLC and U.V. Further details on the experimental design are given in Experimental. a, absent; p, present.

Experimental

2′-Hydroxyacetophenon, palladium (5%) on calcium carbonate, and isoamyl nitrite were supplied by Aldrich (Sigma Aldrich, Milan, Italy). All the other reactants and solvents used were of analytical grade. The NMR analysis were performed using a Bruker AC 200 MHz instrument; melting point was detected using a Mettler TA instrument DSC 12 E; mass spectrometry (MS) and MS/MS analyses were performed with a VG 70-250S instrument using electron ionisation (EI) and liquid secondary ion mass spectrometry (LSI-MS) techniques; crystallographic analysis was carried out

using an X-ray singlecrystal diffractometer (Siemens P4).

Synthesis of 2-methyl-3-hydroxy-(4H)-benzopyran-4-one

The following steps 1 and 2 are performed according to ref 26 and reported with some details not found in the literature.

Step 1. Synthesis of 2-acetyl-2′-hydroxyacetophenon. 20 g of 2′-hydroxyacetophenon were dissolved in ethylacetate (200 mL) in a round bottom flask equipped with vigorous mechanical stirrer and a reflux column. 16 g of granular metallic sodium were gradually added to maintain a moderate reflux. At the end of addition (within 15–20 min) the flask was heated to reflux for about 90–120 min. After this time, the mixture was cooled and 200 g of ground ice were added to the flask and left to stand at 4 °C until the formation of a white solid precipitate. This product was recovered after vacuum filtration and petroleum ether washing, dissolved and re-precipitated (at 4 °C) in 200 mL of 40% acetic acid which desalts it. The product has been characterised by melting point (95 °C)[26] and about 15 g of acetyl-2′-hydroxyacetophenon were obtained.

Step 2. Cyclisation of 2-acetyl-2′-hydroxyacetophenon to 2-methylchromone. To 15 g of 2-acetyl-2′-hydroxyacetophenon, 30 g concentrated H_2SO_4 were added. After spontaneous cooling, cold water was added until a white precipitate was formed. The mixture was maintained at 4 °C for about 1 h and then filtered by vacuum suction. The residue was washed by cold water, dried, and the product characterised by melting point (71 °C).[26] The yield was about 70%.

The following steps 3 and 4 were performed according to ref 27 with some modifications.

Step 3. Conversion of 2-methylchromone to 2-methyl-2,3-dihydrochromone. 3.2 g of Pd (5%) on $CaCO_3$ were suspended in 15 mL of benzene in a flask reliable for gaseous hydrogenation. 3.2 g of 2-methylchromone dissolved in about 15 mL benzene were added and left to absorb 400–420 mL of H_2 at room temperature and pressure (this takes about 16–18 h). After this, the mixture was filtered and the benzene evaporated obtaining a pale yellow oleous residue. The major component of the residue was purified on a silica gel column (60–200 μm) eluting with a mixture of petroleum ether/ethyl ether (1:1) and characterised as 2-methyl-2,3-dihydrochromone [colourless oil; ^{1}H NMR ($CDCl_3$): 1.51(d, 6H, Me); 2.68(d, 2H, CH_2); 4.55(m, 1H, C*H*(CH_3); 7.30/8.30 (m, 4H, Ar)]; yield about 60%.

Step 4. Hydroxylation of 2-methyl-2,3-dihydrochromone to MCOH. 1.6 g of 2-methyl-2,3-dihydro-chromone were dissolved in 20 mL of 95% ethanol and heated to reflux in a three-neck round bottom flask, under stirring. 8 mL of isoamyl nitrite and 40 mL of concentrated HCl were separately and slowly poured in the flask (15–20 m). When this was completed, heating and stirring were stopped and the mixture was allowed to stand for about 2 h. Water (about 200 mL) was added until a colourless precipitate was formed. The solvent was removed by filtration and the residue purified on a silica gel column (60–200 μm) eluting with petroleum ether/ethyl ether (60:40). The major fluorescent product (TLC detected using a 360 nm lamp) was MCOH. This was further purified by crystallisation in acetone. The purified, colourless crystals were characterised by melting point (182.3/183.3 °C) (179/181 reported by ref 27). ^{1}H NMR ($CDCl_3$): 2.48 (3H, Me); 6.58 (1H, OH, exch); 7.3/8.3 (4H, Ph). The purity grade was determined by GC–MS and its X-ray structure by crystallographic analysis. Fluorescence measurements in $CHCl_3$ revealed an Ex_{max} at 340 nm and an Em_{max} at 490 nm.

X-ray crystallography

Single crystals of MCOH were obtained by dissolving some mg of powder in acetone and allowing the solution to concentrate at room temperature. A colourless single crystal ($C_{10}H_8O_3$ mol wt 176.2) of approximate dimensions $0.10\times0.25\times0.15$ mm was submitted to X-ray analysis using a Siemens P4 four-circle diffractometer with graphite monochromated Mo-K_α radiation ($\lambda = 0.71069$ Å). Lattice parameters were determined by least-squares refinement on 44 randomly selected and automatically centred reflections. The $\omega/2\theta$ scan technique was used in the data collection in the $4 \leq 2\Theta \leq 50°$ scan range. Crystal system: triclinic; space group: $P\bar{1}$ (n. 2); $a = 5.566(1)$, $b = 7.830(2)$, $c = 9.740(1)$ Å, $V = 403.2(1)$Å^3, $Z = 2$, $Dc = 1.451$ g/cm^3. 1877 reflections were collected at 22 °C of which 1410 are unique ($R_{int} = 0.01$). No absorption correction was applied.

The structure was solved by direct methods implemented in the SHELXS-97 program.[28] The refinement was carried out by full-matrix anisotropic least-squares on F^2 for all reflections for non-H atoms by using the SHELXL-97 program.[29] The final refinement converged to $R_1 = 0.037$, $wR_2 = 0.102$ for $I > 2\sigma I$, goodness-of-fit = 1.08. Min/max height in last $\Delta\rho$ map of −0.22 and 0.19 e Å^{-3}. Full crystallographic details will be given elsewhere.

Investigations on the iron-chelating capacity of 2-methyl-3-hydroxy-4*H*-benzopyran-4-one (MCOH)

Spectrophotometric analyses. A solution of 100 μm MCOH in water containing 5% of dimethylsulfoxide (DMSO) was made using a 10 mM stock solution in DMSO. The absorbance spectrum was recorded between 400 and 290 nm. Then 12.5, 25, 50 and 100 μm anhydrous $FeCl_3$ was added, using a 20 mM stock solution in 5% DMSO, and absorbance recorded after each addition.

Cyclic voltammetry. 20 μmol of anhydrous $FeCl_3$ were dissolved in 20 mL of 150 mM NaCl containing 5% DMSO in a reliable cell and the voltametric cycle recorded using a Pt electrode versus a calomelan-saturated electrode (SCE) as reference. Then 5 μmol of MCOH withdrawn by a 20 mM solution in DMSO, were added into the cell and the cycle recorded. The

measurements were repeated after further additions of 5, 10 and 20 μmol of MCOH, respectively.

Mass spectrometry of the MCOH–Fe^{3+} complex. One drop of a solution obtained by dissolving few micrograms of MCOH and ferric chloride or sulphate in acetone was put on the stainless steel tip of the LSI-MS probe, mixed with glycerol as a matrix, and exposed to the Cs^+ beam for the desorption. In other experiments, electron ionisation was also used. Metastable decompositions occurring in the first field-free region were studied by computer-controlled B/E linked scans.

Lipophylicity of MCOH

Octanol–water repartition. 3 mL of octanol were added to a 100 μm solution of MCOH in water (3 mL, triplicate) and vigorously shaken. The two phases allowed to separate by gentle centrifugation. The concentration of MCOH was determined spectrophotometrically using appropriate standards in octanol and water. The measures were repeated with saline-phosphate buffer (pH 7.4) instead of water. The respective repartition coefficients were calculated as log [MCOH oct]/[MCOH wat].

Erythrocyte and Erlich ascite tumor cell (EATC) membrane permeability. 24.3 mg of anhydrous $FeCl_3$ were dissolved in 30 mL of acetone. To 3 mL of this solution, 5.3 mg of MCOH were added to obtain a concentrated stock solution of MCOH–Fe^{3+} complex; a further 5 mM solution of MCOH in acetone was prepared. 300 nmol of MCOH and 150 nmol of $MCOH^{3+}$ complex withdrawn from these solutions, were pipetted into the flasks, the solvent evaporated and 3 mL of 50% (v/v) rat erythrocyte suspension in Tris-buffered saline solution added. Alternatively, MCOH and its iron complex, at the same concentrations, were dissolved in 3 mL of 50% (v/v) rat erythrocytes (washed in saline solution) re-suspended in their own plasma. All the flasks were shaken for 1 h at 37 °C. Then the samples were centrifuged (2500 RPM for 10 min) and the supernatants separated from erythrocytes. Aliquots of both, supernatant and red cells were extracted by two volumes of ethyl acetate and MCOH and its complex measured spectrophotometrically. One mL of MCOH–Fe^{3+} complex loaded cells were re-incubated for 1 h at 37 °C after their re-suspension (50%; v/v) in new saline and fresh plasma respectively. Again, cells and media were separated, extracted by ethyl acetate and the MCOH or MCOH–Fe^{3+} complex measured spectrophotometrically. The above described experiments were accurately repeated using EATC, re-suspended in Tris-buffered saline solution, instead erythrocytes.

In vivo test

Male Sprague–Dawley rats, between 220 and 250 g body weight and maintained by a common chow, were fasted for 16 h before the treatment. MCOH was dissolved in dimethylsulfoxide at the concentration of 50 mg/mL. Rats were treated intraperitoneally at the dose of 50 mg MCOH/kg body weight.

After the treatment, rats were placed in metabolic cages and re-fed after the second hour. At the times reported in Table 2 rats were anaesthetised and their livers withdrawn and homogenised (1 g) in two volumes of ethyl acetate. Urine samples were collected and (1 mL) similarly extracted with ethyl acetate. After centrifugation the organic solvent was collected, evaporated and residue dissolved in about 50 μL of ethyl acetate. Using glass capillary tubes, a few μL were applied on silica gel plates (250 μm) with fluorescein. After running with ethyl acetate: petroleum ether (30:70) as element, a pale blue fluorescent spot ($R_f \simeq 0.6$), was detected under irradiation at 360 nm in urine and liver of treated animals.

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