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PAPER

New conjugates of β-cyclodextrin with manganese(III) salophen and porphyrin complexes as antioxidant systems†

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Oxidative stress is the hallmark of several pathologies like arthritis, hypertension and many neurodegenerative disorders such as Parkinson's and Alzheimer's diseases. In this scenario, antioxidant compounds can play a pivotal role in treating these severe pathologies. The synthesis of molecules able to mimic physiologically-relevant proteins is nowadays of particular interest. Several transition metal complexes, especially manganese(III) complexes with porphyrin and salen-type ligands, have been reported to be superoxide scavengers. Here we report the synthesis and spectroscopic characterization of manganese(III) complexes of 5[4-(6-*O*-β-cyclodextrin)-phenyl],10,15,20-tri(4-hydroxyphenyl)-porphyrin and of 6^A-deoxy-6^A[(*S*-cysteamidobenzoyl(3,4-diamino)-*N*,*N*′-bis(salicylidene))]-β-cyclodextrin. The superoxide dismutase activity of the metal complexes was investigated by indirect methods. The catalase and peroxidase activities were tested using ABTS assays.

Introduction

Oxidative stress has become widely viewed as an underlying condition of a number of diseases, such as ischemia-reperfusion injury,[1] chronic inflammation,[2] diabetes,[3] neurodegenerative disease,[4,5] cancer[6] and aging.[7] Therefore, natural and synthetic antioxidants have been actively sought.

Superoxide dismutase is the first line of defence against oxidative stress under physiological and pathological conditions. Therefore, mimetics of antioxidant enzymes such as superoxide dismutase (SOD) and catalase are reported as potential new drugs able to reduce oxidative stress damage.[2,8,9] Among these systems, metal complexes of manganese(II) or (III) are the most widely investigated molecules.[10] One reason for favouring manganese is that catalyst decomposition can release free metal; in the case of metal ions such as copper[10] and iron[11] this can give rise to severe toxicity *in vivo*.

Particularly, the manganese(III) complexes of porphyrins[12,13] or salen type ligands[9,14–16] have been reported as both SOD and catalase mimetics. Generally the SOD-like activity investigated *in vitro* is considered to be the operative mechanism *in vivo*.[9,17,18] It has been shown that salen complexes exert beneficial effects in several *in vitro* and *in vivo* animal models of human diseases which involve oxidative stress ranging from Alzheimer's,[19,20] Parkinson's,[21] prion disease[22] and motor neuron disease to multiple sclerosis and excitotoxic neuronal injury.[23] The salen-type systems investigated from Eukarion have also been shown to protect from ischemia-reperfusion injury of the brain,[17] heart[24] and kidney[25,26] in animal models. On this basis some complexes are used in cosmetics as anti-wrinkle agents.

Manganese(III) porphyrin complexes have been widely investigated and their activities with regard to the nature of the side chain have been recently discussed in a comprehensive review.[27] Manganese porphyrins have been shown to be SOD mimics, $ONOO^-$ scavengers and redox modulators of cellular transcriptional activity. These compounds could also be mimics of the cyt P450 family of enzymes. Metalloporphyrin SOD/catalase mimetics have also been shown to protect against radiation injury.[28–30] In addition, metalloporphyrins should be effective in treating peripheral diseases such as diabetes[31] and heart disease.[32] Manganese-containing mesoporphyrins have demonstrated efficacy in cellular and animal models of neurodegenerative diseases.[33] Clinical trials are now under way for manganese(III) *tetrakis*(*N*,*N*′-diethylimidazolium-2-yl)porphyrin (AEOL 10150), an injectable Mn porphyrin, for treatment of ALS (amyotrophic lateral sclerosis).[34] Metalloporphyrins may be advantageous over other types of compounds because of their stability.

We have already reported some SOD mimetics based on metal complexes of cyclodextrin (CD) conjugates and have found that the CD cavity, in some cases greatly, improves the SOD activity of the complexes.[35–39]

CDs can be used either as drug solubilizers by forming inclusion complexes or to form covalent conjugates. The chemical conjugation could confer on the linked moiety new properties: improved stability, solubility and site specificity. CD–drug bioconjugates are reported in the literature as very promising site specific anti-inflammatory pro-drugs for colon diseases.[40] The synthesis of a SOD *synzyme* containing CDs could be a novel approach to deliver

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$O_2^{\bullet-}$ scavenger to the colon.[41] Reactive Oxygen Species (ROS) such as $O_2^{\bullet-}$ and $^{\bullet}OH$ have been reported to have a role in mediating intestinal damage in inflammatory bowel diseases. The CD ability to quench the $^{\bullet}OH$ radicals could render the conjugates able to act against a cocktail of radicals.[42]

In this paper we report the synthesis of two new manganese(III) complexes obtained conjugating two members of the most investigated SOD mimics such as porphyrin and salophen with β-cyclodextrin in order to further investigate the effects of the oligosaccharidic moiety. In particular, we report the synthesis and spectroscopic characterization of the manganese(III) complexes of 5[4-(6-*O*-β-cyclodextrin)-phenyl],10,15,20-tri(4-hydroxyphenyl)-porphyrin (CDL_1) and of 6^A-deoxy-6^A[(*S*-cysteamidobenzoyl(3,4-diamino)-*N*,*N*′-bis(salicylidene))]-β-cyclodextrin (CDL_2) (Chart 1). The SOD-like activity of the metal complexes was investigated by indirect methods.[43] The catalase and peroxidase activities were tested using the ABTS assays.[44]

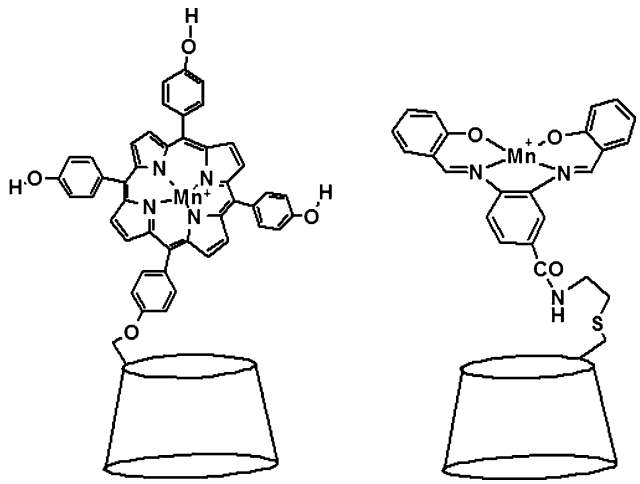

Chart 1

Experimental

General

β-Cyclodextrin (Fluka), anhydrous *N*,*N*-dimethylformamide (DMF) (Aldrich) and 5,10,15,20-tetra(4-hydroxyphenyl)porphyrin (*p*-THPP, Porphyrin Systems) were used without further purification.

3,4-diaminobenzoic acid (Fluka) was purified by chromatography on silica gel with acetone as the eluent.

Merck Lichroprep RP-8 (40–60 μm) was used for reversed phase column chromatography. Analytical and preparative HPLC was carried out on a Varian Prepstar instrument equipped with a Prostar 330 photodiode array detector. Preparative (5 μm, 22 × 250 mm) and analytical (5 μm, 2.0 × 250 mm) Econosphere ODS (Alltech) columns were used. CD derivatives were detected on TLC by UV and by an anisaldehyde test. TLC was carried out on silica gel plates (Merck 60-F254). 5[4-(6-*O*-β-cyclodextrin)-phenyl],10,15,20-tri(4-hydroxyphenyl)-porphyrin was synthesised as reported elsewhere.[45]

NMR spectroscopy. ^{1}H NMR spectra were recorded at 25 °C in D_2O or CD_3OD with a Varian UNITY PLUS spectrometer at 499.883 MHz. The ^{1}H NMR spectra were obtained by using standard pulse programs from a Varian library. In all cases the length of the 90° pulse was 7 ms. The 2D experiments were acquired using 1 K data points, 256 increments and a relaxation delay of 1.2 s. T-ROESY spectra were obtained using a 300 ms spin-lock time. DSS was used as external standard.

UV/Visible and circular dichroism spectroscopy. UV/Visible spectra were recorded with an Agilent 8452A diode array spectrophotometer. The spectra were recorded at 25 °C, on freshly prepared aqueous solutions. Circular dichroism measurements were performed at 25 °C under a constant flow of nitrogen on a JASCO model J-810 spectropolarimeter. The spectra represent the average of 5 or 10 scans.

Mass spectrometry. The ESI-MS measurements were performed on a Finnigan LCQ-Duo ion trap electrospray mass spectrometer.

Superoxide dismutase assay

SOD-like activity was determined by the indirect method of cytochrome c (cyt *c*) or Nitro Blue Tetrazolium (NBT) reduction.[43] Superoxide radical anion was enzymatically generated by the xanthine–xanthine oxidase (XO/XOD) system and spectrophotometrically detected by monitoring the formation of reduced cyt *c* or NBT at 550 nm. The reaction mixture was composed of cyt *c* 50 μM or NBT 250 μM, xanthine 50 μM, catalase 30 μg ml^{-1} in phosphate buffer 10 mM, at pH 7.4. An appropriate amount of xanthine oxidase was added to 2.0 ml reaction mixture to produce a $\Delta A_{550\ nm}$ min^{-1} of 0.024. This corresponded to a $O_2^{\bullet-}$ production rate of 1.1 μmol min^{-1}. The cytochrome or NBT reduction rate was measured in the presence and in the absence of the investigated complex for 500 s. Stock solutions of complexes were prepared in water or in methanol, depending on solubility. Where present, at a final concentration that did not exceed 7%, methanol did not affect assay activity. All measurements were carried out at 25 ± 0.2 °C using 1 × 1 cm thermostated cuvettes in which solutions were magnetically stirred. In separate experiments urate production by xanthine oxidase was spectrophotometrically monitored at 295 nm, ruling out any inhibition of xanthine oxidase activity. The I_{50} (the concentration which causes the 50% inhibition of cyt *c* or NBT reduction) of manganese(III) complexes at pH 7.4 was determined. Three determinations were carried out.

Catalase activity assay

Catalase activity was measured by the method described elsewhere.[37] A calibration curve for absorbance against hydrogen peroxide concentration was obtained. Complexes were dissolved in phosphate buffer (10 mM, pH 7.4). Hydrogen peroxide solution (30 μM) was incubated at various concentrations of the complexes (ranging from 10^{-5} to 10^{-6} M) in 0.010 M phosphate buffer at pH 7.4 at 25 °C.

After 50 min, ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (50 μM) and horseradish peroxidase (1.3 μg ml^{-1}) were added. After 5 min, the reaction went to completion and the absorbance at 735 nm was read. The data analysis gives the amount of hydrogen peroxide removed per minute per mmole of compound. Three determinations were carried out.

Peroxidase activity assay

Peroxidase activity was assessed by monitoring the hydrogen peroxide-dependent oxidation of ABTS spectrophotometrically.[46] The assay mixture consisted of ABTS (0.2 mM), hydrogen peroxide (0.5 mM) and manganese(III) complex in phosphate buffer (50 mM) at pH 7.4. Assays were conducted at 25 °C. The ABTS oxidation was monitored at 735 nm to eliminate interference with the manganese complex absorption. The amount of oxidized ABTS was estimated using the value ε (at 735 nm) of 1.5×10^4 M^{-1} cm^{-1}. No ABTS oxidation due to the complexes or the hydrogen peroxide was observed in the conditions of the assay.

Ligand synthesis

***N*,*N*′-Di-Boc-3,4-diaminobenzoic acid.** 3,4-diaminobenzoic acid (2.0 g, 13.1 mmol) was dissolved in methanol (30 ml). Di-*tert*-butyl pyrocarbonate (11.21 g, 51.4 mmol) was added and the mixture was stirred at room temperature for 2 h. The solvent was evaporated to dryness *in vacuo*, the solid was purified by column chromatography on silica gel (ethyl acetate–n-hexane 6 : 4). The appropriate fractions were concentrated to give the product. Yield: 1.48 g (32%).

TLC: R_f 0.78 (AcOEt/n-hexane 3 : 2).

ESI-MS: m/z = 374.9 $[M+Na]^+$, 726.9 $[2M+Na]^+$.

^{1}H NMR: (CD_3OD, 500 MHz) δ (ppm): 7.85 (1H, s, H-2 of aromatic moiety), 7.62 (1H, dd, $J_{5\text{-}6}$ = 9.0 Hz, H-6 of aromatic ring), 6.75 (1H, dd, $J_{5\text{-}6}$ = 9.0 Hz, H-5 of aromatic ring), 1.29 (18 H, s, H of Boc).

6^A-deoxy-6^A[(*S*-cysteamidobenzoyl(3,4-diamino))]-β-cyclodextrin (CDL_0). Di-Boc-3,4-diaminobenzoic acid (104 mg, 0.29 mmol) was dissolved in dry DMF. 1-hydroxybenzotriazole (HOBt) (40 mg, 0.30 mmol) and *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-bis(tetramethylene)uronium hexafluorophosphate (HBPyU) (130 mg, 0.30 mmol) were added. This mixture was stirred at room temperature for 30 min. 6^A-deoxy-6^A[(*S*-cysteamine)]-β-cyclodextrin[38] (350 mg, 0.29 mmol) was added to the solution and the reaction mixture was stirred at 35 °C under nitrogen for 22 h and was followed by TLC ($PrOH/AcOEt/H_2O/NH_3$ 4 : 3 : 2 : 1 R_f 0.43). The solvent was evaporated *in vacuo* and the solid was purified by chromatography on RP-8 column (gradient H_2O–CH_3CN 0→50%). The isolated product was deprotected by stirring with CF_3COOH (10 ml) for 1.5 h. The reaction was followed by TLC analysis ($PrOH/AcOEt/H_2O/NH_3$ 4 : 3 : 2 : 1 R_f 0.20). The CF_3COOH was evaporated and the obtained solid was purified by CM-Sephadex C-25 (NH_4^+ form), eluting with a linear gradient of a NH_4HCO_3 solution 0→0.2 M.

Appropriate fractions were collected, (R_f 0.23, $PrOH/H_2O/AcOEt/NH_3$, 4 : 3 : 2 : 1) and the solvent was evaporated. Yield: 77 mg (20%).

ESI-MS: m/z = 1350.5 $(M+Na)^+$.

^{1}H NMR (500 MHz, D_2O) δ (ppm): 7.15 (1H, s, H *ortho* to the amide and amine groups), 7.07 (1H, dd, H *ortho* to the amide group), 6.80 (1H, dd, H *meta* to the amide group), 5.01–4.89 (7H, m, H-1 of CD), 3.95–3.63 (26H, m, H-3, H-5 and H-6 of CD), 3.59–3.36 (14H, m, H-2 and H-4 of CD), 3.29 (1H, t, H-4A of CD), 2.87 (3H, m, H-6A of CD, and 2H α to the amide group in cysteamine moiety), 2.70 (2H, m, H β to the amide group in cysteamine moiety).

6^A-deoxy-6^A[(*S*-cysteamidobenzoyl(3,4-diamino)-*N*,*N*′-bis(salicylidene))]-β-cyclodextrin (CDL_2). Salicylaldehyde (3.2 μl, 0.030 mmol) was added to CDL_0 (20 mg, 0.015 mmol) in anhydrous methanol (about 25 ml) under stirring at room temperature. The mixture rapidly became yellow. After 4.5 h, the solvent was evaporated *in vacuo*. The residue was suspended in a small volume of acetonitrile and centrifuged off. The supernatant was taken away with a pipette and this washing procedure was repeated twice more. Yield: 19 mg (82%).

ESI-MS: m/z = 1536.3 $(CDL_2+H)^+$, 1558.4 $(CDL_2+Na)^+$.

^{1}H NMR (500 MHz, CD_3OD) (ppm) 8.90 (1H, s, iminic proton *meta* to the amidic bond), 8.86 (1H, s, other iminic proton), 7.89–7.87 (2H, m, H *ortho* to the iminic group and H *ortho* to the carbonylic group and iminic group), 7.59 (1H, d, $J_{1,2}$ = 8.0 Hz, H *ortho* to the iminic group and *meta* to the OH), 7.57 (1H, d, $J_{1,2}$ = 8.0 Hz, other proton *ortho* to the iminic group and *meta* to the OH), 7.48 (1H, d, $J_{1,2}$ = 9.0 Hz, H *meta* to the carbonylic group), 7.42 (1H, d, $J_{1,2}$ = 7.0 Hz, H *ortho* to the OH), 7.40 (1H, d, $J_{1,2}$ = 7.0 Hz, other proton *ortho* to the OH), 7–6.96 (4H, m, Hs *meta* and *para* to the OH), 4.96 (7H, d, other H-1 of CD), 3.97 (H, m, H-5A), 3.86–3.75 (25 H, m, H-3, H-5, H-6 of CD), 3.61 (2H, m, CH_2 α to the amide group), 3.49 (7H, m, H-2 of CD), 3.44 (7H, m, H-4 of CD), 3.18 (1H, m, H-6′-A of CD), 2.97(1H, t, H-6A of CD), 2.93 (2H, t, CH_2 β to the amide group).

^{13}C NMR (125 MHz, CD_3OD) 171.0 (C═O), 166.5 (C═N), 166.3 (other C═N), 161.6 (C–OH), 161.8 (other C–OH), 143.2 (C–C═N), 144.0 (C–C═N), 135.7 (C *ortho* to OH), 135.4 (other C *ortho* to OH), 134.2 (C *ortho* to C═N), 133.8 (other C *ortho* to C═N), 134.0 (C *meta* to C═O), 126.3 (C *ortho* to N═C and *meta* to C═O), 120.5–116.6 (other Cs of aldehyde moieties), 104.0 (C-1 of cyclodextrin), 103.7 (C-1A 85.4 (C-4A), 83.0 (C-4A), 74.8 (C-3), 74.4 (C-2), 73.8 (C-5), 61.6 (C-6), 40.8 (CH_2 alpha to NHCO), 34.4 (CH_2 beta to NHCO), 34.24 (C-6A).

Elemental analysis for $C_{65}H_{89}N_3O_{37}S{\cdot}4H_2O$: calc. C 48.54; H 6.08; N 2.61; found C, 48.41; H, 6.12; N, 2.55.

Preparation of manganese(III) complexes

Manganese(III) complex of L_1 (MnL_1). L_1 (70 mg, 0.10 mmol) was dissolved in anhydrous methanol (10 ml). $Mn(CH_3COO)_3{\cdot}2H_2O$ (27 mg, 0.10 mmol) was added and the mixture was stirred for 4 h under reflux. The solvent was evaporated to dryness *in vacuo*, the solid was purified by chromatography on silica gel with $AcOEt/CH_3OH$ (4 : 1) as the eluent. Yield: 54 mg (68%).

TLC: R_f 0.39 ($AcOEt/CH_3OH$ 4 : 1).

ESI-MS: m/z = 731.4 $[MnL_1]^+$.

UV-Vis: (MeOH) λ/nm (ε/M^{-1} cm^{-1}) 225 (35616), 320 (13377), 347 (16743), 384 (27313), 402 (26859), 427 (26996), 471 (46732), 573 (5669), 610 (6345).

Manganese(III) complex of CDL_1 ($MnCDL_1$). CDL_1 (100 mg, 0.056 mmol) was dissolved in dry DMF (5 ml). $Mn(CH_3COO)_3{\cdot}2H_2O$ (15 mg, 0.056 mmol) was added and the mixture was stirred for 1 h under reflux. The solvent was evaporated to dryness *in vacuo*, the solid was purified by

preparative HPLC (linear gradient $H_2O \rightarrow CH_3CN$). Yield: 96 mg (90%).

TLC: R_f 0.62 ($PrOH/H_2O/AcOEt/NH_3$ 5 : 3 : 1 : 2).

ESI-MS: $m/z = 1848$ $[Mn(CDL_1)]^+$.

UV-Vis: (MeOH) λ/nm (ε/M^{-1} cm^{-1}) 224 (38791), 319 (15993), 348 (18195), 384 (25949), 404 (26220), 425 (30784), 471 (41630), 572 (5964), 609 (6391).

Elemental analysis for $MnC_{88}H_{100}N_4O_{40}\cdot 4H_2O$: calc. C, 53.36; H, 5.50; N, 2.83; found C, 53.01; H 5.55, N, 2.79.

Manganese(III) complex of CDL_2 ($MnCDL_2$). CDL_0 (17.8 mg, 0.0134 mmol) was dissolved in anhydrous methanol (5 ml). $Mn(CH_3COO)_3\cdot 2H_2O$ (3.6 mg, 0.0134 mmol) and salicylaldehyde (2.9 µl, 0.0268 mmol) were added. The colourless solution turned to brown immediately and was refluxed for 3 h. The mixture was left at room temperature, the precipitated solid was filtered off and washed with acetonitrile. Yield: 17.7 mg (80%).

ESI-MS: $m/z = 1588.3$ $[Mn(CDL_2)]^+$, 805.8 $[Mn(CDL_2)+Na]^{2+}$.

UV-Vis: (H_2O) λ/nm (ε/M^{-1} cm^{-1}): 194 (34828), 246 (29333), 306 (18469), 335 (20509), 434 (7577), 547 (1351).

Elemental analysis for $MnC_{67}H_{91}N_3O_{39}S\cdot 4H_2O$: calc. C, 46.75; H, 5.80; N, 2.44; found C, 46.51; H, 5.71; N, 2.39.

Results and discussion

Ligands

In order to prepare Mn(III) complexes, porphyrin and salen-type ligands were synthesised. 10,15,20-tri(4-hydroxyphenyl)-porphyrin (CDL_1) was synthesised for comparison as reported elsewhere.[45]

CDL_0 was synthesised starting from *N*,*N*′-di-Boc-3,4-diaminobenzoic acid and 6^A-deoxy-6^A[(*S*-cysteamidobenzoyl(3,4-diamino))]-β-cyclodextrin (CDMEA), in the presence of activating and condensating agents typically used for these kinds of reactions.[47] After activation of the carboxylic group of the benzoic acid, the CDMEA forms an amide bond by a nucleophilic attack *via* its amino group. In order to prevent uncontrolled amide bond formation, the amino groups of 3,4-diaminobenzoic acid were protected with Boc groups.

The Boc groups were removed by CF_3COOH. The final product CDL_0 was fully characterised. NMR spectra confirm the identity of the ligand. The H-1 protons of the CD moiety are divided into six groups as often observed in the case of other derivatives.[38] In addition to the signals due to the CD protons, the signals of the chain are evident: the protons of the ethylenic chain of the cysteamine moiety appear at 2.87 ppm and at 2.70 ppm, while the protons of the aromatic ring resonate at 7.15, 7.07 and 6.80 ppm.

The new CDL_2 ligand was characterised in methanol by ESI-MS and NMR.

On the ^{1}H NMR spectrum (Fig. 1), the signals due to the chain, the aromatic rings and CD moiety are evident. The signals due to the cysteamine moiety are shifted to downfield compared to those of CDL_0. The iminic protons appear as different signals at 8.90 and 8.86 ppm for the asymmetry of the salophen moiety.

Complexes

The manganese complex of L_1 and CDL_1 were synthesised starting from the ligands and manganese acetate under reflux. MnL_1 and

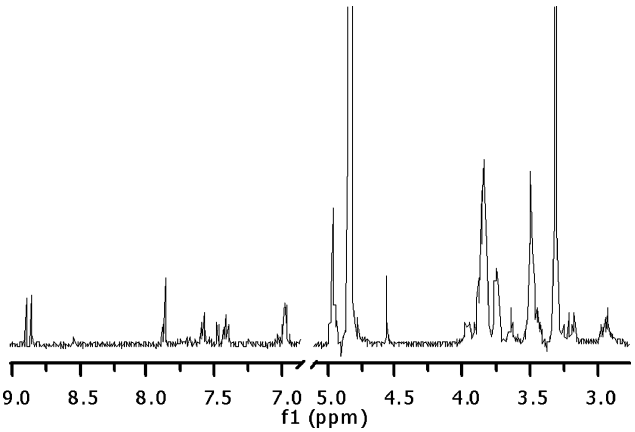

Fig. 1 ^{1}H NMR spectrum of CDL_2 (CD_3OD, 500 MHz).

$MnCDL_1$ were characterised by ESI-MS, UV-Vis and circular dichroism. The ESI-MS spectrum of MnL_1 shows one peak at 731.4 m/z due to $[MnL_1]^+$.

The ESI-MS spectrum of $MnCDL_1$ shows two peaks at 1848.3 and 935.9 m/z due to $[Mn(CDL_1)]^+$ and the double charged species $[Mn(CDL_1)+Na]^{2+}$. No peaks were observed at m/z lower than 400. The peaks in this region have been assigned to manganese clusters due to manganese impurities.[48]

The UV-Vis spectrum (Fig. 2) shows typical absorptions of manganese(III) porphyrin complexes.[49]

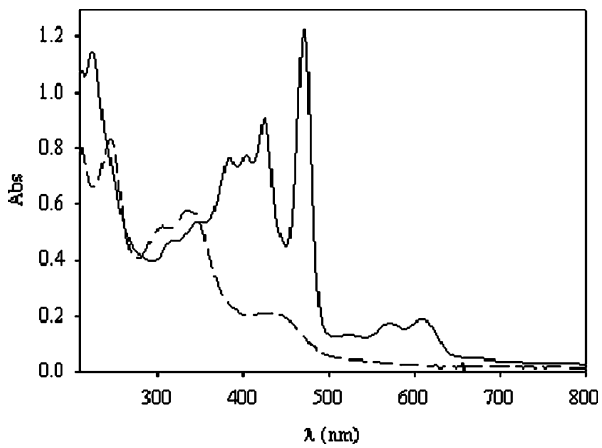

Fig. 2 UV-Vis spectra of $MnCDL_2$ (---) and $MnCDL_1$ (–) (2.8×10^{-5} M).

The circular dichroism spectrum shows two weak negative bands in the Soret region, due to the dipole–dipole coupling of the porphyrin moiety and the CD cavity. This behaviour has been generally reported for functionalised CDs and is due either to the inclusion of the functionalizing moiety in the cavity or its proximity.

In the case of the $[Mn(CDL_1)]^+$, the circular dichroism spectrum is not modified by the addition of various amounts of 1-adamantanol, suggesting that the porphyrin is out of the cavity. The negative sign of the induced circular dichroism band suggests that the porphyrin moiety is near to the cavity, oriented with the plane of the porphyrin parallel to the symmetry axis of CD.[50] In comparison with the ligand CDL_1, the complex shows a different behaviour. In line with previous results,[45] the complexation of the manganese(III) ion destroys the supramolecular dimeric

structure of the ligand. The manganese(III) complex of CDL_2 was synthesised at room temperature as reported elsewhere for similar compounds.[51] It was characterised by UV-Vis and ESI-MS.

The ESI-MS spectrum of $MnCDL_2$ shows three peaks at 1588.3, 805.8 and 542.8 m/z due to $[Mn(CDL_2)]^+$, $[Mn(CDL_2)+Na]^{2+}$ and $[Mn(CDL_2)+H+K]^{3+}$ species, respectively.

The UV-Vis spectrum of $MnCDL_2$ (Fig. 2) is similar to the MnL_2 one.[52] This suggests that the coordination of manganese(III) in the CD–salophen conjugate is similar to that of manganese(III) salophen. The circular dichroism spectrum of $MnCDL_2$ does not show any dichroic band, ruling out any interaction of the aromatic moiety with the CD cavity.

SOD-like activity

SOD-like activity of the manganese(III) complexes was determined by competition kinetics using NBT or cytochrome *c* target as reported elsewhere.[53]

Possible interferences of the tested compounds with the xanthine/xanthine oxidase reaction were assessed by following the rate of urate accumulation at 295 nm in the absence of the target. The complexes, at the concentration used in the assay, had no effect on the reaction of the XO/XOD system. Inhibition of target reduction by the complex, when plotted as $V_0/V_{complex}$-1 against the [complex] yielded a straight line (Fig. 3). V_0 is the uninhibited reduction rate of cyt *c* or NBT by $O_2^{\bullet-}$ and V_{cat} is the rate of reduction of cyt *c* or NBT inhibited by the complex. When [complex] is equal to I_{50}, k_{cat}[complex] = $k_{detector}$[detector] and k_{cat} can be determined. $k_{detector}$ is the constant for the reaction between the $O_2^{\bullet-}$ and the detector molecule and it is 2.6×10^5 and 5.88×10^4 M^{-1} s^{-1} when the detector is cyt *c* and NBT, respectively.[54] In the case of $MnCDL_2$ we reported data obtained using both cyt *c* and NBT as targets to rule out interferences with the target as reported by us for similar systems.[38] The results obtained with NBT are higher than those obtained with cyt *c* as typically observed in the case of simple Mn-salophen complex.[55] Reduced NBT is reoxidated by O_2 and this determines higher antioxidant activity[56]

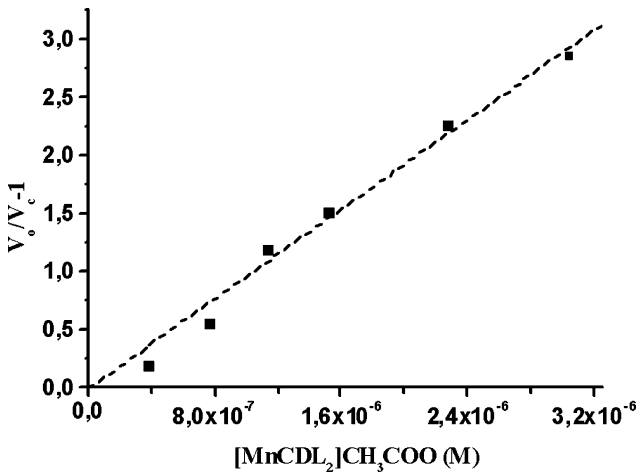

Fig. 3 The SOD-like activity of $MnCDL_2$. V is the reduction rate of NBT (V_o) alone and (V_c) in the presence of the complex.

The I_{50} values are reported in Table 1. $MnCDL_2$ shows a good I_{50}, similar to the values reported for this class of compounds. I_{50} of $MnCDL_2$ is only slightly higher than that of the simple analogue without CD (MnL_2).[52] Unlike the other CD-conjugates in which the proximity of the cavity and the complex significantly improves the catalytic activity,[39] in this system the catalytic moiety is rigid and too far away from the cavity to show any improvement in activity.

Table 1 I_{50} and k_{cat} values of manganese complexes

Complex	I_{50} (μM)	k_{cat} ($M^{-1}s^{-1}$)	Reference
$MnCDL_2$	1.11(±0.05)[a]	$1.32(\pm0.06) \times 10^7$[a]	This paper
	1.4(±0.2)[b]	$1.0(\pm0.1) \times 10^7$[b]	This paper
MnL_2	1.3[a]	1.2×10^7[a]	52
	1.7(±0.2)[b]	$8(\pm1) \times 10^6$[b]	This paper
$MnCDL_1$	7.5(±0.9)[b]	$1.7(\pm0.2) \times 10^6$[b]	This paper
MnL_1		$< 1.0 \times 10^5$[b]	This paper
$MnTPP^+$		6.8×10^4[b]	27
$MnTPFP^+$		1.0×10^5[b]	27

[a] Determined by NBT, [b] Determined by cyt c.

$MnCDL_1$ also shows a good IC_{50} (Table 1) even if it is less effective than the $MnCDL_2$ complex. This behaviour is in line with the general trend observed for neutral porphyrin complexes which are less active than salen complexes. In order to assess the effect of the cavity, we synthesised the MnL_1 complex and tested its SOD-like activity. The MnL_1 showed a $k_{cat} < 1 \times 10^5$ $M^{-1}s^{-1}$. A more accurate determination of SOD-like activity was not possible due to its low solubility under assay conditions.

The k_{cat} value for this system is in the range of values reported for other neutral porphyrins.[27] It is interesting to note that the k_{cat} value for $MnCDL_1$ is significantly higher than those reported for neutral porphyrin complexes[27] (Table 1). The improvement of the SOD activity as a consequence of the conjugation of the CD has been already observed in other metal complexes of functionalised CDs.[39] In the case of $MnCDL_1$, the improvement of SOD activity is higher than that observed for salen CD-conjugates.[38]

On the basis of the data in the literature[36,37,57] we can hypothesize that the CD cavity improves the catalytic activity for the presence, in proximity to the catalytic moiety, of a microenvironment which can trap radical species[58–61] so favouring their reaction with the catalytic centre. The hydroxylic groups of the CD might play a synergic role in the catalytic mechanism. The increased SOD activity for $MnCDL_1$ compared to MnL_1 might therefore be explained by the spatial proximity of the porphyrin moiety to the CD cavity.

Peroxidase activity

Peroxidase-like activity of synthesised manganese complexes was evaluated. The peroxidase activity of MnL_2 was also determined for comparison.

The following mechanism is reported in the literature[15] for the peroxidase activity, where A is a typical substrate:

$$Mn(III) + H_2O_2 \rightleftarrows Mn(V){=}O + H_2O$$

$$Mn(V){=}O + AH_2 \rightleftarrows Mn(IV){-}OH + AH^+$$

$$Mn(IV){-}OH + AH_2 \rightleftarrows Mn(III) + H_2O + AH^+$$

Table 2 Catalase and peroxidase activities of manganese complexes

Complex	μM ABTS·min^{-1}	μmol of H_2O_2/min/mmole
$MnCDL_1$	—	8.9(±0.4)
MnL_2	18(±1)	40.2(±1.6)
$MnCDL_2$	15(±3)	36.0(±0.9)

In order to assess the peroxidase activity of manganese complexes, ABTS was used as substrate. The reaction of ABTS with H_2O_2 in the presence of complex generates radical cation:

$$2\ ABTS + H_2O_2 + 2H^+ \rightleftarrows 2ABTS^{\bullet+} + 2H_2O$$

The reduced form of ABTS is colourless, while the oxidized ABTS, the $ABTS^{\bullet+}$ radical cation, is green and its visible spectrum shows several characteristic absorption bands at 415, 650, 735 and 815 nm. In the absence of the complex, a solution of ABTS and H_2O_2 is stable for several hours at 25 °C, in the dark, without showing any formation of radical cation.

In order to compare our results with the data in the literature, the amount of $ABTS^{\bullet+}$ formed per minute was determined at the complex concentration of 1.0×10^{-5} M. MnL_2 and $MnCDL_2$ exhibit similar peroxidase activity and their activities (Table 2) are very similar to those of the Eukarion compounds.[15] $MnCDL_1$ did not show peroxidase activity at the investigated concentrations (1.0×10^{-5} M and 1.0×10^{-4} M).

Catalase activity

The catalase activity was determined by an indirect assay (Table 2).

The mechanism by which the Mn complex acts as a catalase mimetic involves the oxidation of Mn(III) to oxomanganese by H_2O_2, releasing water. The oxomanganese is then reduced to Mn(III) by another hydrogen peroxide molecule to form oxygen and water:

$$Mn(III) + H_2O_2 \rightleftarrows Mn(V){=}O + H_2O$$

$$Mn(V)O + H_2O_2 \rightleftarrows Mn(III) + H_2O + O_2$$

The catalase activity of MnL_2 was also determined for comparison. Both salophen derivatives showed higher activity than $MnCDL_1$. This trend is reported in the literature[62] and shows that neutral porphyrins are not particularly efficient H_2O_2 scavengers.

For all these new systems the values are significantly higher than those obtained with the method reported for some Eukarion compounds based on salens and porphyrins.[62]

Conclusions

In this paper new manganese(III) porphyrin and salophen conjugates with β-cyclodextrin are reported.

Apart from significantly improving the aqueous solubility of both systems compared to the simple manganese-complexes, the cyclodextrin might play a synergic role in the catalytic process.

In the case of the salophen derivative the functionalisation gives rise to a slight increased antioxidant activity of the system *in vitro* compared to the system not linked to the cyclodextrin. The new SOD mimic based on porphyrin–CD conjugate shows SOD activity about 15 times higher than the reference compounds. The greater proximity of the catalytic centre to the CD cavity for the porphyrinic system leads us to assume that the saccharidic residue of the CD is somehow involved and improves the reaction with superoxide radicals.

The functionalisation with the CD is also an efficient strategy to target the antioxidant bioconjugates to the colon for orally usable drugs such as the metalloporphyrins.[62]

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