Selective Inhibition of Fe- *versus* Cu/Zn-Superoxide Dismutases by 2,3-Dihydroxybenzoic Acid Derivatives

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Received September 21, 2001; accepted January 15, 2002

A series of catechol derivatives were synthesised and tested for their ability to inactivate the iron-containing superoxide dismutase (Fe-SOD) from *Escherichia coli* and the bovine erythrocytes Cu/Zn-SOD. Incubation of catechols with Fe- or Cu/Zn SODs resulted in a time-dependent loss of enzyme activity with highly selective inhibition for the iron-dependent enzyme. Catechol-induced inactivation of SODs was correlated with the auto-oxidation of the catechol compounds to their corresponding *ortho*-quinone derivatives, which was found to be non-dependent on the presence of enzymes. Mass electrospray experiments on catechol-incubated Fe-SOD provided evidence for the irreversible nature of the inhibition process, yielding to a complex mixture of modified proteins.

Key words catechol; quinone; Fe-SOD; Cu/Zn-SOD; inhibitor

Metalloenzymes superoxide dismutases (SODs) are key enzymes in the metabolism of oxygen free radicals whose primary activity is the dismutation of superoxide radical anion into hydrogen peroxide and molecular oxygen.[1)] The iron-dependent superoxide dismutases (Fe-SODs), structurally distinct from the Cu/Zn family of SODs and, to a lesser extent, from the manganese family of SODs (Mn-SODs),[2)] constitute in a number of parasitic protozoan organismsthe primary enzymatic defence against damage caused by the superoxide anion radical $O_2^{\cdot-}$, and its derivatives such as peroxynitrite. As a consequence, it has been proposed that the development of specific inhibitors of parasitic iron superoxide dismutases, which are different from their host's Cu/Zn or Mn-dependent enzymes, may lead to the perturbation of the antioxidant defence mechanism, and thus could contribute to the development of new drugs with anti-parasitic properties.[3)] The bis-catechol derivative N^1,N^6-bis(dihydroxybenzoyl)-1,6-diaminohexane **8** (Chart 1) has been shown to be a selective and irreversible inhibitor of the iron-SOD from *Crithidia fascicula*, a non-parasitic trypanosomatid related to trypanosomes.[4)] The authors showed that inhibition was caused by a mechanism other than chelation, and hypothesized that this bis-catechol was oxidized to a quinone before forming a covalent bond with the protein. In addition to any inhibitory effects, there is an interest in studying the reactivity of catechol derivatives towards biological molecules such as proteins, whose structural modifications are often associated in the development of many diseases and in aging. Indeed, a number of drugs can undergo oxido-reductive transformations in cells, such as *O*-demethylation reactions catalyzed by cytochrome P450-dependent mono-oxygenases, or one- or two-electron oxidation mediated by peroxidases and tyrosinases, leading to the formation of catechol and/or *ortho*-quinone moieties as major metabolites.[5—7)]

In the present work, as part of a program to develop novel iron-superoxide dismutase inhibitors, we synthesised and evaluated a series of catechol derivatives. The influence of added SOD on the rate of auto-oxidation reaction of these compounds to their corresponding *ortho*-quinones was also investigated.

Results

Synthesis of Catechol Derivatives (Chart 1) Commercially available 2,3-dihydroxybenzoic acid **1** was used as starting material for the synthesis of all catechol derivatives **2**—**8**. Catechol **2** was prepared from **1** and [(*tert*-butyloxycarbonyl)amino]ethylamine[8)] with 1,3-dicyclohexylcarbodiimide (DCC) as coupling reagent. Removal of the butyloxy carbonyl protecting group (Boc) from **2** under acidic conditions using trifluoroacetic acid gave hydrochloride compound **3**. Compounds **4** and **6** were obtained from 2,3-dihydroxybenzoic acid using the DCC method in the presence of glycine ethyl ester or *N*-hydroxysuccinimide, respectively. Treatment of **4** in aqueous sodium hydroxide and subsequent acidification gave compound **5**, and reaction of **6** with 1,2-diaminoethane and 1,6-diaminohexane yielded the bis-catechol derivatives **7** and **8**, respectively. All compounds were then fully characterised by mass spectrometry, ^{1}H-, ^{13}C-NMR and IR spectroscopies.

Oxidation Kinetic Measurements The oxidation of catechols to their corresponding *ortho*-quinone derivatives was determined by UV-visible spectroscopy by monitoring the increase in absorbance at $\lambda = 280$ nm. As shown in Fig. 1A, autoxidation of compound **5** at 400 μM occurred through a very slow process in a 50 mM Tris buffer (pH 8.2) at 25 °C, with a first order constant value of 0.079 h^{-1} ($t_{1/2} = 9$ h). The addition of either *Escherichia coli* Fe-SOD or bovine erythrocytes Cu/Zn-SOD had no significant effect on the oxidation rate with half-life time values of about 11 and 10 h, respectively (Fig. 1A). A similar effect was observed on all other compounds (data not shown), thus providing proof that this class of enzyme neither catalyses nor inhibits the conversion of catechol compounds to their corresponding quinones. In these experiments, it should be noted that catechols (400 μM) were in large excess compared to enzyme amount (0.3 μM), explaining why an inhibition of auto-oxidation rate, which could be due to substrate consumption by enzyme through covalent bonding, was not observed. In comparison, when tyrosinase, known to catalyse the production of *ortho*-quinones from phenol and catechol compounds, was added, oxidation of compound **5** occurred much more rapidly (Fig. 1B).

Inhibition Studies The activities of catechol-incubated

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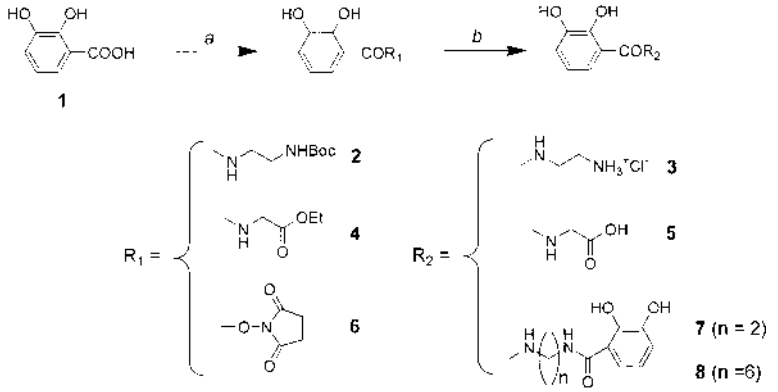

(a) **2**: [(*tert*-Butyloxycarbonyl)amino]ethylamine, DCC, THF, 36%; **4**: glycine ethyl ester hydrochloride, Et_3N, DCC, THF, 40%; **6**: *N*-hydroxysuccinimide, DCC, THF, 36%. (b) **3**: TFA, CH_2Cl_2, 72%; **5**: 1 M aq. NaOH, 84%; **7**: ethylene diamine, THF, 0 °C, 41%; **8**: 1,6-hexane diamine, THF, 0 °C, 35%.

Chart 1

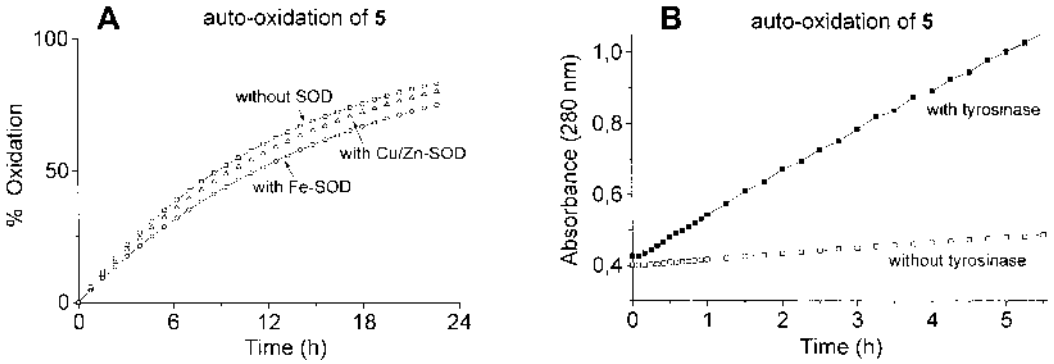

Fig. 1. (A) Effect of *E. coli* Fe-SOD (0.3 μM) and Bovine Erythrocyte Cu/Zn-SOD (0.3 μM) on the Auto-oxidation of Compound **5** (400 μM) and (B) Effect of Tyrosinase (5 U/ml) on the Auto-oxidation Initial Rate of Compound **5** (400 μM)

All reactions were performed in a 50 mM Tris buffer pH 8.2, and spectrophometrically monitored at 280 nm.

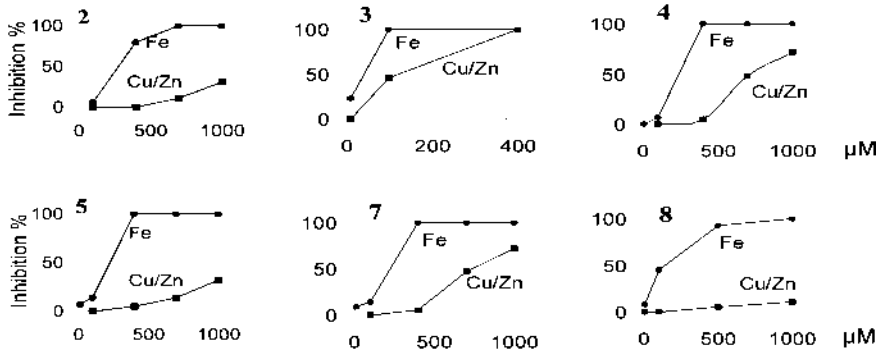

Fig. 2. Dose-Dependence of the Inhibition of *E. coli* Fe-SOD and Bovine Erythrocyte Cu/Zn-SOD by Catechol Derivatives **2**—**5**, **7** and **8** after 24 h at 25 °C in a 50 mM Tris Buffer pH 8.2 with 1 mM EDTA

Superoxide dismutase activities were determined using a standard SOD assay based on the auto-oxidation of pyrogallol.

SODs were determined using the SOD assay based on pyrogallol auto-oxidation in a 50 mM Tris buffer (pH 8.2) containing 1 mM EDTA. As shown in Fig. 2, all compounds of this study demonstrated a selective inhibitory effect on the iron-dependent enzyme in a dose-dependent manner. Except for compound **2**, all catechol derivatives at concentrations of 400—500 μM fully inhibited the activity of the iron-dependent enzyme, whereas bovine erythrocyte Cu/Zn-SOD was slightly inactivated at these concentrations (with the only exception being for compound **3**). Inhibition by all compounds (400 μM) was found to be time-dependent, suggesting that catechol-induced inactivation is an irreversible process. For compounds **3** and **5**, the $t_{1/2}$ values (time corresponding to 50% inhibition) were found to be 25 min and 11 h, respectively. However, the corresponding kinetic data (K_i and k_i or k_{inact}) could not be determined due to the uncertainty of *ortho*-quinone concentrations, which are generated *in situ* by spontaneous catechol oxidation, and which are known to decompose rapidly in aqueous solution. In the attempt to identify the modifications induced by catechol derivatives, *E. coli* Fe-SOD (30 μM) was incubated with compound **5** (40 mM) for 24 h, extensively dialysed against water, and analysed by

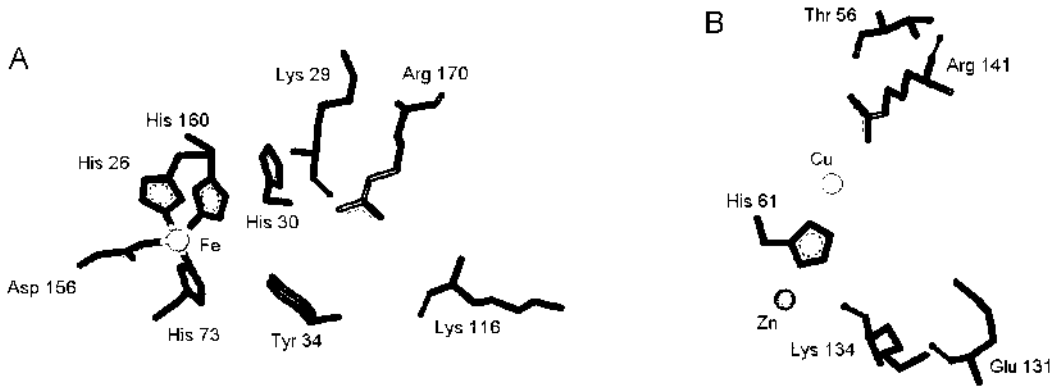

Chart 2. Comparative Schematic Views of the Shape of Active Site Channels of *E. coli* Fe-SOD (A) and Bovine Erythrocyte Cu/Zn-SOD (B)

The data files were obtained from the Protein Data Bank at Brookhaven National Laboratory (PDB access codes for the coordinate sets are *E. coli* Fe-SOD, 1ISA and bovine erythrocyte Cu/Zn-SOD, 1CBJ).

electrospray mass spectrometry. After this treatment, native SOD (apo subunit 21136 Da) was not recovered on the mass spectrum which displayed a complex molecular mass distribution, corresponding to calculated deconvoluted masses up to around 23500 Da, suggesting extensive product heterogeneity.

Discussion

The primary activity of superoxide dismutases is the dismutation of superoxide radical anion into hydrogen peroxide and molecular oxygen. However, other interesting catalytic properties comprise the ability of SODs to either increase or decrease the rate of some oxidation reactions. For instance, the auto-oxidation of pyrogallol,[9] epinephrine[10] and 6-hydroxydopamine[11] is inhibited by SODs because superoxide anion acts as a chain propagation species. In contrast, when superoxide is the product of a reversible process, autoxidation rates can be enhanced in the presence of SODs. This behaviour, for example, is observed in the auto-oxidation of 3-hydroxyanthranilic acid to cinnabarinate.[12] Conversely, the auto-oxidation of 1,4-naphthohydroquinone has been shown to occur rapidly in a phosphate buffer (pH 7.4), and the addition of Mn- or Cu/Zn-SOD has no effect on the course of its auto-oxidation, except a slight increase in the lag phase.[13] In recent studies, it has been shown that the rate of auto-oxidation of some substituted naphthohydroquinone derivatives, including 2,3-dimethyl-1,4-naphthohydroquinone, decreases with increasing concentration of bovine erythrocytes Cu/Zn SOD,[14,15] but the same enzyme has no effect on the rate of auto-oxidation of some halogenated 1,4-naphthohydroquinones, while that of the corresponding 5-hydroxy derivative is increased.[15] In our context, we observed that *E. coli* Fe-SOD and Cu/Zn-SOD had little or no effect on the rate of the auto-oxidation of catechol derivatives to their corresponding quinones. The kinetic data indicated a time-dependent inactivation, suggesting an irreversible inhibition process of superoxide dismutases. To confirm the covalent modification on Fe-SOD by catechol derivatives, both native and modified enzymes were analysed by electrospray mass spectrometry. In good agreement with previously published work[4] showing that inhibition of Fe-SOD by catechol **8** was associated with the formation of a complex which could not be dissociated by gel-filtration, this spectrometry experiment clearly demonstrated the irreversible modification of Fe-SOD. However, this method did not provide information regarding the nature of the residues associated with the irreversible process. It can be assumed that cysteines are more susceptible to *ortho*-quinone-induced modifications,[16] but the large number of adducts formed relative to the single available cysteine residue per subunit requires that other nucleophilic amino acid residues, such as lysines or histidines, also be modified. In all known Fe-SODs, the iron is coordinated to three histidines, one aspartate and a solvent molecule as fifth ligand, forming a trigonal bipyramid around the metal ion.[17] These four inaccessible ligand residues are highly conserved, as are His30 and Tyr34 located at the vertex of the funnel, which block access to the active site of the enzyme. Consequently, it seems unlikely that one residue of the active site could be responsible for the nucleophilic attacks leading to irreversible modifications of the protein. Several studies, based on chemical modification and site-directed mutagenesis on charged residues of SODs, showed the existence of an electrostatic control of substrate diffusion involving positively charged residues.[18—22] It can be assumed that the catechol-induced loss of activity of *E. coli* Fe-SOD is attributable to covalent modifications occurring at these residues located at the entrance of the channel conducting towards the active metal ion (*i.e.* Lys 29, Lys 116 or Arg 170; Chart 2A). In particular, Arg 170, which is conserved in the large majority of Fe-SOD sequences, and which has been shown to be critical for catalytic activity,[23,24] could be one of the major target residues. The difference that we observed in the inhibition of the two classes of enzymes by the oxidation products of catechols should be related to binding at this specific arginine residue, which is likely to be more accessible in the Fe-SOD (Arg 170) than in the Cu/Zn-SOD (Arg 141) (Chart 2).

Experimental

General Nuclear magnetic resonance spectra were recorded on a Bruker AC 250 at 250 MHz (^{1}H) or 60 MHz (^{13}C), ultraviolet spectra with a UV mc^2 spectrophotometer (SAFAS), and IR spectra on a Perkin-Elmer 1600 FTIR spectrophotometer. Merck silica gel 60 (70—230 mesh) was used for column chromatography. All chemicals were obtained from Aldrich, and used without further purification. Superoxide dismutases from bovine erythrocytes and from *E. coli*, and tyrosinase from mushroom were purchased from Sigma.

Catechol Synthesis. *tert*-Butyl *N*-{2-[2,3-Dihydroxybenzoyl)amino]-ethyl}carbamate 2 To a solution of 2,3-dihydroxybenzoic acid (0.96 g, 6.2 mmol) and [(*tert*-butyloxycarbonyl)amino]ethylamine (1 g, 6.2 mmol) in

dry tetrahydrofuran (THF, 8 ml) was added dropwise at 0 °C a solution of 1,3-dicyclohexylcarbodiimide (1.42 g, 6.9 mmol) in THF (3 ml). The solution was stirred at room temperature for 18 h. The reaction mixture was filtered, and the filtrate evaporated to dryness. The residue was treated with ethyl acetate and then washed with 5% aq. sodium hydrogencarbonate. The organic layer was dried over magnesium sulfate, and the solvent evaporated under reduced pressure. The residue was column chromatographed using a mixture of dichloromethane and ethyl acetate (9 : 1) to yield 0.66 g of **2** (yield: 66 %). ^{1}H-NMR ($CDCl_3$) δ: 1.43 (9H, s), 3.49 (4H, m), 5.09 (1H, m), 5.95 (1H, s), 6.72 (1H, t, J=8.0 Hz), 7.02 (2H, d, J=8.0 Hz). ^{13}C-NMR ($CDCl_3$) δ: 28.3, 39.5, 42.4, 80.5, 114.0, 116.7, 118.0, 118.6, 145.7, 149.1, 158.0, 170.6. IR (KBr) cm^{-1}: 3356, 1695, 1637. Desorption chemical ionization (DCI)-MS (NH_3) m/z: 297 [M+H$^+$]; 314 [M+NH_4^+].

***N*^{1}-(2-Ammonioethyl)-2,3-dihydroxybenzamide Chloride 3** A solution of **2** (0.3 g, 1 mmol) in dichloromethane (5 ml) and trifluoroacetic acid (5 ml) was stirred for 16 h at room temperature. The reaction mixture was then evaporated to dryness under reduced pressure, and several co-evaporations with water were carried out. The residue was dissolved in ethanol (5 ml), and 12 M hydrochloric acid (84 μl) was added. The solution was filtered to give 0.17 g of **3** (yield: 72%). ^{1}H-NMR (DMSO-d_6) δ: 3.01—3.02 and 3.56—3.58 (4H, m), 6.68 (1H, t, J=8.0 Hz), 6.96 and 7.43 (2H, d, J=8.0 Hz), 8.23 (3H, s), 9.13 (1H, s). ^{13}C-NMR (DMSO-d_6) δ: 36.7, 38.2, 114.8, 117.6, 117.8, 118.9, 146.1, 149.5, 170.2. IR (KBr) cm^{-1}: 1640. DCI-MS (NH_3) m/z: 197 [M+H$^+$].

Ethyl 2-[(2,3-Dihydroxybenzoyl)amino]acetate 4 To a solution of glycine ethyl ester hydrochloride (0.905 g, 6.48 mmol), triethylamine (0.657 g, 6.49 mmol) and 2,3-dihydroxybenzoic acid (1 g, 6.49 mmol) in dry THF (8 ml) was added dropwise at 0 °C 1,3-dicyclohexylcarbodiimide (1.47 g, 7.14 mmol) in dry THF (3 ml). The solution was stirred at room temperature for 16 h. The reaction mixture was filtered and the filtrate evaporated to dryness. The residue was diluted with ethyl acetate, washed with 0.01 M aq. hydrochloric acid and water. The organic layer was dried over magnesium sulfate and evaporated to dryness. The product was chromatographed using dichloromethane as eluent to give 0.62 g of **4** as a white powder (yield: 40%). ^{1}H-NMR (DMSO-d_6) δ: 1.21 (3H, t, J=7.0 Hz), 4.04 (2H, d, J=6.0 Hz), 4.17 (2H, q, J=7.0 Hz), 6.72 (1H, t, J=8.0 Hz), 6.94 (1H, dd, J=8.0, 1.0 Hz), 7.30 (1H, dd, J=8.0, 1.0 Hz), 9.17 (1H, t, J=7.0 Hz). ^{13}C-NMR (DMSO-d_6) δ: 14.0, 40.0, 80.5, 114.8, 117.5, 118.2, 119.0, 146.1, 149.2, 169.4, 169.8. IR (KBr) cm^{-1}: 1723, 1655. DCI-MS: (NH_3) m/z: 240 [M+H$^+$], 257 [M+NH_4^+].

2-[(2,3-Dihydroxybenzoyl)amino]acetic Acid 5 Ester **4** (0.5 g, 2.1 mmol) was dissolved in 1 M aq. sodium hydroxide (25 ml) and the solution was stirred at room temperature for 4 h. After filtration, the filtrate was acidified with diluted sulfuric acid, and the residue was extracted with ethyl acetate. The organic layer was dried over magnesium sulfate and evaporated to dryness to yield 0.37 g of **5** as a brown powder (yield: 84%). ^{1}H-NMR (DMSO-d_6) δ: 3.97 (2H, d, J=6.0 Hz), 6.71 (1H, t, J=8.0 Hz), 6.93 (1H, dd, J=8.0, 1.0 Hz), 7.31 (1H, dd, J=8.0, 1.0 Hz), 9.12 (1H, t, J=6.0 Hz), 12.31 (1H, s). ^{13}C-NMR (DMSO-d_6) δ: 40.9, 114.8, 117.4, 118.1, 118.9, 146.1, 149.2, 169.7, 170.8. IR (KBr) cm^{-1}: 1731, 1650. DCI-MS (NH_3) m/z: 212 [M+H$^+$], 224 [M+NH_4^+].

2,5-Dioxotetrahydro-1*H*-1-pyrrolyl 2,3-Dihydroxybenzoate 6 To a solution of *N*-hydroxysuccinimide (1.495 g, 13 mmol) and 2,3-dihydroxybenzoic acid (2 g, 13 mmol) in dry THF (16 ml) was added dropwise at 0 °C a solution of 1-3-dicyclohexylcarbodiimide (3.21 g, 15.6 mmol) in dry THF (6 ml). The solution was stirred at room temperature for 16 h. The reaction mixture was filtered, and the filtrate evaporated to dryness. The residue was taken up with ethanol, and the insoluble material was filtered to give 1.26 g of pure **6** (yield: 36%). ^{1}H-NMR (DMSO-d_6) δ: 2.87 (4H, s), 6.81 (1H, t, J=8 Hz), 7.13 (1H, dd, J=8.0, 1.0 Hz), 7.30 (1H, dd, J=8.0, 1.0 Hz). ^{13}C-NMR (DMSO-d_6) δ: 25.5, 110.9, 119.1, 120.6, 120.8, 146.4, 148.8, 161.5, 170.4. IR (KBr) cm^{-1}: 1729. DCI-MS (NH_3) m/z: 269 [M+NH_4^+], 286 [M+$N_2H_7^+$].

***N*^{1}-{2-[2,3-Dihydroxybenzoyl)amino]ethyl}-2,3-dihydroxybenzamide 7** To a solution of ethylenediamine (0.06 g, 0.99 mmol) in THF (5 ml) was added dropwise at 0 °C a solution of **6** (0.5 g, 1.98 mmol) in THF (5 ml). The solution was stirred overnight at room temperature. The solvent was evaporated to dryness and the residue was taken up with ethyl acetate and washed with 0.01 M hydrochloric acid. The organic layer was dried over magnesium sulfate and evaporated to dryness. Recrystallisation from dichloromethane gave 0.135 g of **7** as a white powder (yield: 41%). ^{1}H-NMR (DMSO-d_6) δ: 3.49 (4H, s), 6.68 (2H, t, J=8.0 Hz), 6.92 (2H, dd, J=8.0, 1.0 Hz), 7.27 (2H, dd, J=8.0, 1.0 Hz), 8.93 (1H, s). ^{13}C-NMR (DMSO-d_6) δ: 38.5, 114.9, 117.1, 117.8, 118.8, 146.1, 149.6, 170.1. IR (KBr) cm^{-1}: 1644. DCI-MS (NH_3) m/z: 333 [M+H$^+$], 350 [M+NH_4^+].

***N*^{1}-{6-[2,3-Dihydroxybenzoyl)amino]hexyl}-2,3-dihydroxybenzamide 8** To a solution of 1,6-hexanediamine (0.069 g, 0.6 mmol) in THF (5 ml), was added dropwise at 0 °C a solution of **6** (0.3 g, 1.12 mmol) in THF (5 ml). The reaction mixture was stirred at room temperature for 18 h, and the solvent evaporated. The residue was chromatographed on silica gel using a mixture of dichloromethane/methanol (9 : 1) to give 0.08 g of **8** (yield: 35%). ^{1}H-NMR (DMSO-d_6) δ: 1.20—1.34 and 3.24—3.31 (12H, m), 6.67 (2H, t, J=8.0 Hz), 6.90 and 7.28 (4H, d, J=8.0 Hz), 8.77 (1H, s). ^{13}C-NMR (DMSO-d_6) δ: 26.1, 26.9, 28.7, 114.8, 117.0, 117.7, 118.6, 146.1, 149.6, 169.6. IR (KBr) cm^{-1}: 1638. DCI-MS (NH_3) m/z: 389 [M+H$^+$], 406 [M+NH_4^+].

Inhibition Studies Superoxide dismutase activities were determined by UV–visible spectroscopy at pH 8.2 by measuring the rate of the auto-oxidation of pyrogallol (4—5 mM) at 420 nm. Enzymatic inhibition studies were carried out as follows: a solution of inhibitor (10 μl) in dimethylsulfoxide (1 to 100 mM) was added to a solution (990 μl) of Fe-SOD (0.30μM) or Cu/Zn-SOD (0.34 μM) in 50 mM Tris buffer (1 mM EDTA, pH adjusted to 8.2 with cacodylic acid). The percentage of inhibition was calculated as follows: I %=[$V_{I\text{-}SOD}-V_{SOD}$]/[V_I-V_{SOD}]×100, where V_I is the rate of auto-oxidation of pyrogallol in the presence of inhibitor, $V_{I\text{-}SOD}$ in the presence of SOD and inhibitor, and V_{SOD} in the presence of SOD.

To determine the dose-dependence inhibition by catechol compounds, various concentrations were incubated with Fe-SOD or Cu/Zn-SOD (or 0.34 μM) for 24 h at 25 °C. The time-dependence was determined by periodical assays of incubation with or without catechol compounds at various concentrations with Fe-SOD.

Catechols Oxidation Oxidation reactions of compound **5** (400 μM) in the absence of the enzyme, or in the presence of Cu/Zn-SOD (0.34 μM), Fe-SOD (0.30 μM) or tyrosinase (5 U/ml) were monitored by UV–visible spectrophotometry at 280 nm in a 50 mM Tris buffer pH 8.2.

Electrospray Mass Spectrometry Compound **5** (40 mM) was dissolved in a 100 mM ammonium hydrogenocarbonate buffer pH 8.0 containing Fe-SOD (30 μM) at 25 °C. After 24 h, the solution (25 μl) was then diluted in 25 μl of a mixture of acetonitrile and water (1/1) containing 0.1% formic acid. The solution was directly injected into the electrospray interface of a LC MS/MS mass spectrometer (API 365, Perkin Elmer SCIEX). Spectra were scanned over the range of 500—1600 m/z.

Acknowledgments We thank Catherine Claparols for excellent technical assistance in the mass spectrometry field. Laurent Soulère is the recipient of a grant from the Ministère de la Recherche (France).

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