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EDTA Bis-(ethyl phenylalaninate): A Novel Transition Metal-Ion Chelating Hydroxyl Radical Scavenger with a Potential Anti-inflammatory Role

Declan P. Naughton[a,]* and Martin Grootveld[b]

[a]*School of Pharmacy and Biomolecular Sciences, University of Brighton, Cockcroft Building, Moulsecoomb, Brighton BN2 4GJ, UK*
[b]*The Inflammation Research Group, Saint Bartholomew's and the Royal London School of Medicine and Dentistry, London E1 2AD, UK*

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Abstract—Conjugation of ethylenediaminetetraacetic acid (EDTA) to ethyl phenylalaninate generates a novel radical scavenging metal-ion chelator EDTA bis-(ethyl phenylalaninate) (EBEP). The oxidation products *o*-, *m*- and *p*-tyrosine were isolated from hydrolysed, aqueous and aerated solutions containing EBEP, Fe(II) and H_2O_2. Data obtained demonstrate the potential of EBEP to act as a radical scavenging, iron-ion chelating antioxidant under physiologically relevant conditions. © 2001 Elsevier Science Ltd. All rights reserved.

The transition metal ion-dependent formation of hydroxyl radical ($^{\bullet}$OH) from hydrogen peroxide (H_2O_2) in the presence of a reducing agent such as superoxide ion ($O_2^{\bullet -}$) or ascorbate (at low concentrations) is an important mechanism of 'oxidative stress' leading to irreversible cell and tissue damage.[1,2] In particular, metal-ion mediated oxidative stress has been attributed a role in inflammation, atherosclerosis and Alzheimer's disease. Indeed, recent reports illustrate that amyloid protein in Alzheimer's plaques bind copper and iron and produce H_2O_2 from oxygen via Udenfriend's system.[3] Subsequent reaction between the generated H_2O_2 and adjacent reduced metal ions generates $^{\bullet}$OH radicals in vitro. Iron chelators function as antioxidants to diminish plaque and aggregate formation in neurodegenerative diseases and atherosclerosis.[4,5] In addition to disease states, much interest has been shown in the contribution of oxidative damage to ageing. A recent report outlined the extension of lifespan by a mean of 44% in one species by treating with superoxide dismutase/catalase mimetics.[6] Clearly, new approaches to the suppression of oxidative damage are a priority in both health and disease.

Aromatic compounds such as phenols, phenolic acids and the amino acid phenylalanine react extremely rapidly with $^{\bullet}$OH radical to form a mixture of hydroxylated products (for example, phenylalanine is attacked by $^{\bullet}$OH radical to produce three products: *o*-, *m*- and *p*-tyrosines). Indeed, aromatic hydroxylation has been successfully employed as a method for measuring $^{\bullet}$OH radical production both in vivo and in vitro.[7,8] Hence, the design of an effective 'site-specific' [directed] $^{\bullet}$OH radical scavenger can in principle be achieved by covalently attaching an aromatic moiety to a chelator that is able to form a thermodynamically-stable complex with iron ions. In this study, the $^{\bullet}$OH radical-scavenging potential of the powerful metal-ion chelator EDTA bis-(ethyl phenylalaninate) (EBEP) has been investigated.[9,10] The $^{\bullet}$OH radical-mediated hydroxylation of aromatic rings with the subsequent formation of relatively stable phenolate–iron(III) charge-transfer bonds would favour a particular ligand for the role of an $^{\bullet}$OH radical-scavenging chelator. These properties may be useful in the development of novel dual-function antioxidants to treat inflammation, atherosclerosis and Alzheimer's disease.

The ligand is accessible in good yield using standard amide preparation following protection of the amino acid carboxylic acid moiety. Using the method developed by Grootveld and Halliwell,[11] the products *o*-, *m*- and *p*-tyrosine were isolated from an aqueous, aerated solution containing EBEP, Fe(II) and H_2O_2. No detectable levels of the above hydroxylation products were detected in the absence of added iron(II) ions. The

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*Corresponding author. Tel.: +44-1273-642036; fax: +44-1273-679333; e-mail: d.p.naughton@bton.ac.uk

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selectivity of the hydroxylation process afforded by the proximity of the intramolecular aromatic rings is apparent on comparison of the hydroxylation pattern detected for the intact phenylalanine containing ligand EBEP relative to phenylalanine itself (Fig. 1). In the case where the aromatic rings were not constrained in a close locality to the iron (i.e., where the metal ion was complexed by EDTA), hydroxylation sites were in the order *o*-$\gg$*m*-$\sim$*p*-. However, for EBEP, hydroxylation occurred in the order *p*- > *o*- > *m*-. Furthermore, exposure of phenylalanine to Fenton-derived $^{\bullet}$OH radical generated a similar pattern of isomeric tyrosines irrespective of whether EDTA was present in solution. Thus, site-specific hydroxylation occurs for EBEP.

^{1}H NMR provided further evidence for hydroxylation of the phenylalanine aromatic rings by Udenfriend's system. The influence of added iron(III) chloride (1.00 molar equivalent in aqueous solution) and ascorbate (5.00 mol equiv) on the ^{1}H NMR spectrum of a solution of EBEP in H_2O was investigated. For this purpose, the reaction mixture was incubated at room temperature for a 30 min period. The electronic absorption spectrum of this solution exhibits a broad shoulder at ca. 550 nm indicative of the presence of phenolate–Fe(III) binding (data not shown). Removal of iron ions as hydrated iron(III) oxide was achieved by raising the pH, incubating at 60 °C for 2.5 h and centrifugation. The resulting solution was lyophilised and the ^{1}H NMR spectrum exhibits characteristic splitting patterns of the aromatic proton resonances corresponding to *p*- and *o*-tyrosine isomers, demonstrating that $^{\bullet}$OH radical attack on the aromatic ring has occurred in two or more positions (data not shown). Control studies show that hydroxylation does not occur in the absence of ascorbate.

The ability of EBEP to trap $^{\bullet}$OH radicals was assessed by the deoxyribose assay.[12] As expected, addition of EBEP resulted in a marked decrease in $^{\bullet}$OH radical attack on ribose. A reduction of 20% in hydroxyl radical attack on deoxyribose in the presence of EBEP (0.104 mM) contrasts to a reduction of only 2% on addition of EDTA and phenylalanine. The inhibition of greater than 50% of $^{\bullet}$OH-mediated damage at 5.2 mM reflects the ability of this metal ion chelating scavenger to limit Fe^{3+} mediated ROS damage in vitro (Table 1).

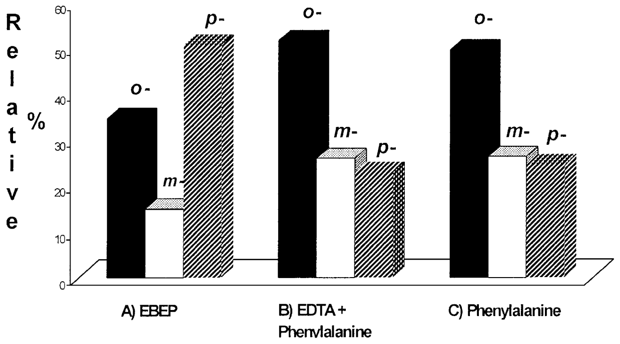
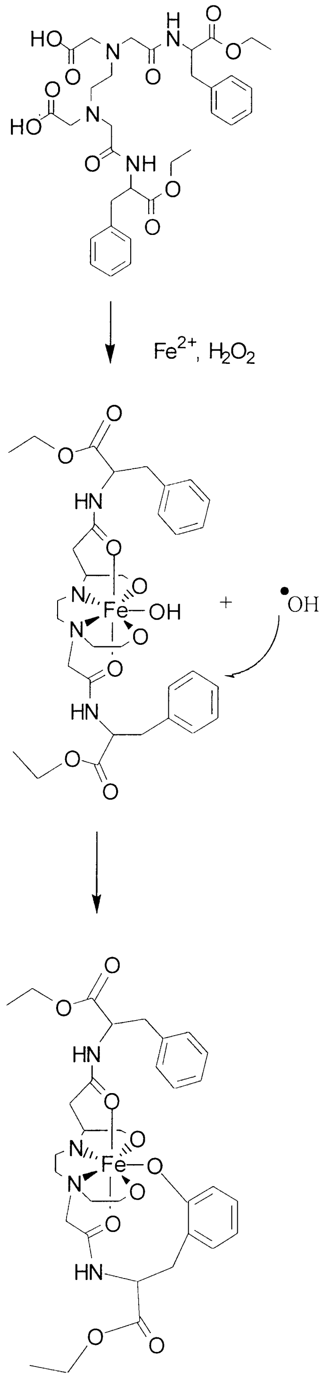


Figure 1. Products derived from the hydroxylation of EDTA bis(ethyl phenylalaninate): (A) contains molar equivalents of EBEP and Fe(II) and three molar equivalents of H_2O_2; (B) contains molar equivalents of PA, Fe(II) and EDTA, and three equivalents of H_2O_2; and (C) contains molar equivalents of PA, Fe(II) and three equivalents of H_2O_2.

Table 1. Antioxidant efficacy of EBEP as assessed by the deoxyribose assay

Compound	Concentration (mM)	Absorbance	% Inhibition
—	0.000	2.13	0.0
EBEP	0.104	1.71	20
EBEP	1.040	1.23	42
EBEP	5.200	1.04	51
EDTA + Phenylanine	0.104 0.208	2.09	0.2



Scheme 1.

The 'site-specific' hydroxylation of the *o*-position of tyrosine is evident from examination of space filling models. However, hydroxylation of the *p*-position is accounted for by the relative position of the aromatic rings. In the case where the aromatic rings interact with the metal orbitals the proximity of the *p*-position to the metal orbital would be expected to cause an enhanced degree of hydroxylation at this site.[13] The results from visible absorption spectroscopy studies indicate that hydroxylation of the aromatic ring followed by phenolate binding to the ferric ion occurs. From examination of space-filling models, *o*-tyrosine is the only phenolate species, resulting from $^{\bullet}OH$ attack on the phenylalanine moiety of EBET, which can participate in intramolecular phenolate–iron(III) bonding (Scheme 1).

These results show the potential of EBEP as a radical scavenging chelator in biological systems. The stabilising effect and steric crowding caused by phenolate binding to the ferric ion may prevent the occurrence of potentially deleterious redox chemistry at the active metal ion centre.

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9. **Experimental**: L-Phenylalanine (50 g, 0.3026 mol) was refluxed in dry ethanol (350 mL, 80 °C, 3 h) with a slow stream of dry HCl gas bubbling continuously. The hydrochloride salt was recovered by evaporating the excess ethanol. The free ester was then obtained by neutralisation with aqueous NaOH solution (1.0×10^{-1} mol dm^{-3}) and then extracted with ethyl acetate. EDTA bis-anhydride (3.306 g, 0.0129 mol), phenylalanine ethyl ester (4.26 mL, 0.0258 mol) and pyridine (2.0 mL) were refluxed in tetrahydrofuran (THF) for 3.5 h. The white product was filtered and recrystallised from hot ethanol (mp 170 °C). Elemental analysis: C, 59.99% (59.96), H, 6.27% (6.62), N, 8.74% (8.58). ^{1}H NMR analysis (D_2O: 200 MHz, 298 K): δ 1.23 [t, 3H, $^3J = 7$ Hz, CH_3], 2.5 [s, 4H, CH_2CH_2], 3.15 [m, 4H, $^3J = 11$ Hz, CH_2], 3.05 [s, 4H, CH_2CONH], 3.18 [s, 4H, CH_2COO], 4.19 [q, $^3J = 7$ Hz, OCH_2CH_3], 4.65 [dt, 1H, $^3J = 11$ Hz, CH], 7.30 [m, 8H, Ar].
10. **HPLC studies**: To an aqueous solution of the ligand (EBEP, 2.20×10^{-2} mol dm^{-3}), one molar equivalent of iron(II) sulphate (in an aqueous, N_2-purged solution) was added followed by 3.00 mol equiv of H_2O_2 solution. Subsequent to incubation at 40 °C for a 40 min period, the samples were treated with 1.0 mol dm^{-3} NaOH solution at 60 °C for a period of 2.5 h in order to hydrolyse the amide bond. This procedure was followed by centrifugation to remove the hydrated iron(III) oxide precipitate. The resulting supernatants were then subjected to HPLC analysis for separation and measurement of aromatic hydroxylation products. Detection of these products was by electrochemical detection with the detector potential set in the +0.70 to +0.85 V range. Authentic *o*-, *m*- and *p*-tyrosine standards were subjected to HPLC analysis to pre-calibrate the system.

   In order to ensure that the proximity of the ligand to the metal ion's coordination sphere demonstrated an enhanced potential with respect to the scavenging of $^{\bullet}OH$ radicals generated from chelated iron ions relative to that of a 2:1 molar ratio of the 'free' ligand (phenylalanine)/EDTA, suitable control experiments were conducted. A solution of iron(II) sulphate was added to an aqueous solution containing 2.0 mol equiv of phenylalanine, followed by an EDTA solution (1.00 mol equiv). Finally, a solution of 3.30 mol equiv of H_2O_2 was added and the reaction mixture was then equilibrated at a temperature of 40 °C for a 2.5 h period. Following removal of hydrated iron(III) oxide as described above, the sample was subjected to HPLC analysis and the products were detected via electrochemical oxidation. The above experiments were also performed in the absence of added iron as controls.
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