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EDTA Bis-(Methyl Tyrosinate): A Chelating Peptoid Peroxynitrite Scavenger

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Abstract—Conjugation of ethylenediaminetetra-acetic acid (EDTA) to methyl tyrosinate generates a chelating peptoid EDTA bis-(methyl tyrosinate), (EBMT). Peroxynitrite-mediated nitration was studied for the free peptoid and its ferric and cupric complexes. The nitration products were monitored by electronic absorption spectroscopy at λ_{max} of 420 nm (mono-nitrated) and 440 nm (di-nitrated). Peak deconvolution was effected by pH manipulation as the mono-nitrated analogue of tyrosine exhibited a bathochromic shift from 365 nm (below its pK_a of 6.8) to 420 nm. Rates of nitration were: free peptoid $<$ Cu(II) complex $<<$ Fe(III) complex. These results demonstrate the potential of EBMT to act as a radical scavenging chelating peptoid antioxidant.

Peroxynitrite ($ONOO^-$) has been implicated with a major role in oxidative stress in a number of diseases including arthritis, inflammatory bowel disease, atherosclerosis and pulmonary diseases.[1] It is generated from the reaction of superoxide ($O_2^{\bullet-}$) with nitric oxide ($NO^\bullet$) at near diffusion controlled rates. In inflamed tissues both macrophages and enzymes can generate peroxynitrite by the co-production of $O_2^{\bullet-}$ and $NO^\bullet$.[2,3] Peroxynitrite-mediated oxidative damage involves (i) copper release from caeruloplasmin, (ii) enzyme dysfunction via nitrotyrosine formation, (iii) DNA adduct formation through deamination and subsequent nitration of bases, (iv) lipid peroxidation and (v) activation of haem oxygenase.[1,4–7]

Aromatic compounds such as phenols, phenolic acids and the amino acid tyrosine react rapidly with $ONOO^-$ to form mono- and possibly di-nitrated products. Indeed, tyrosine nitration has been successfully employed as an assay for $ONOO^-$ determination in biological systems. To date, two methods have been used extensively: detection of free nitrotyrosine by HPLC[8] and immuno-histochemical localization of nitrated tyrosines in proteins.[9] Redox-active transition metal ions catalyse the nitration of tyrosine by $ONOO^-$[10] and have been ascribed with a role in $ONOO^-$ mediated oxidative stress.[1]

In this report, we describe an effective $ONOO^-$ scavenging peptoid comprising a chelator conjugated to two tyrosine moieties. The $ONOO^-$ radical-scavenging potential of the powerful metal-ion chelator EDTA bis-(methyl tyrosinate) (EBMT) has been investigated. To optimise $ONOO^-$ scavenging EBMT is designed to (i) bind redox active metal ions that catalyse the decomposition of $ONOO^-$ and (ii) to direct $ONOO^-$ scavenging to the peptoid. These properties are required for the development of dual-function antioxidants to treat conditions such as inflammation, atherosclerosis and pulmonary diseases.

The peptoid is accessible in good yields using standard amide preparation and is readily purified by recrystallisation from ethanol as described previously.[11] UV–visible absorption spectroscopy is a convenient accessible technique which allows rapid analysis of the mono- and di-nitration products of tyrosine. We have devised a system by manipulation of pH which allows simultaneous detection of both the mono- and di-nitrated tyrosine. At high pH the peaks merge impeding analysis. Above pH 8.0 the λ_{max} and molar absorbtivity (ε) values are 420 nm and 4189 M cm^{-1} for

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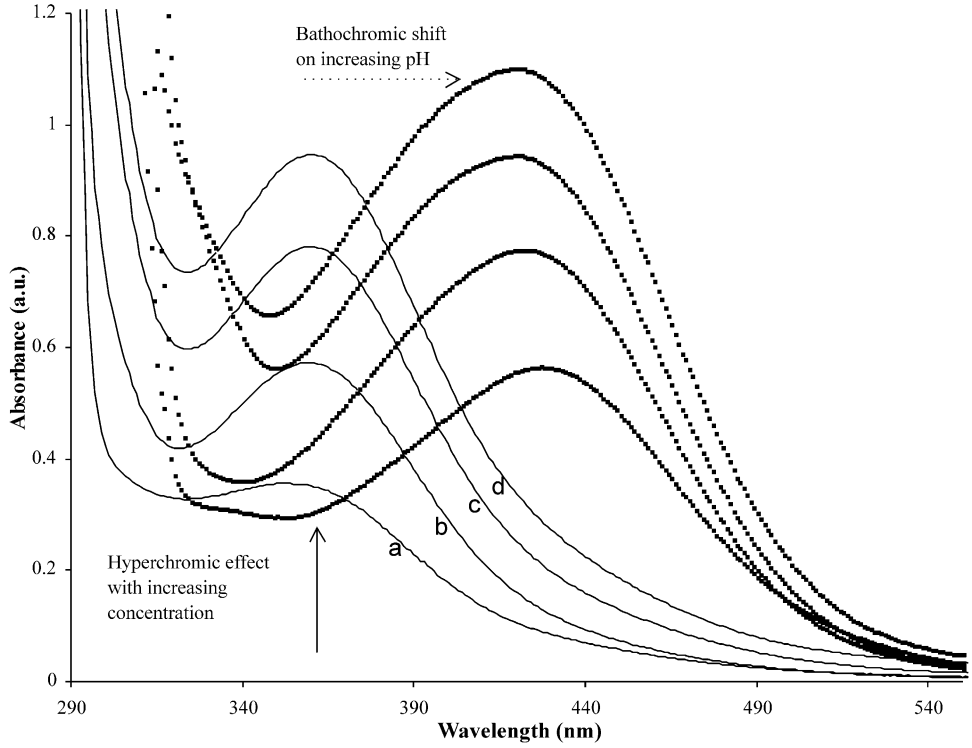


Figure 1. Spectral changes reflecting nitration of EDTA bis(methyl tyrosinate) upon exposure to $ONOO^-$ and effects of pH adjustments from 6.0 — to 8.0 - - -. $ONOO^-$ additions were: (a) 20 μL, (b) 40 μL, (c) 60 μL, (d) 80 μL of 0.16 M $ONOO^-$ added to 3 mL 10 mM EBMT.

3-nitrotyrosine and 440 nm and 7,322 M cm^{-1} for 3,5-dinitrotyrosine. By adjusting the pH to below the pK_a of nitrotyrosine (pH 6.8) a bathochromic shift results in two distinct peaks. Below pH 6.8 the λ_{max} for 3-nitrotyrosine appears at 365 nm ($\varepsilon = 2761$ M cm^{-1}) whilst the peak for 3,5-dinitrotyrosine does not shift until its pK_a value at ca. pH 3 ($\varepsilon = 4900$ M cm^{-1}).[12] Using this system, interfering peaks from residual amounts of $ONOO^-$ (λ_{max} 302 nm) and its decomposition products nitrite (λ_{max} 355 nm) and nitrate (λ_{max} 302 nm) are avoided.

The interactions between $ONOO^-$ and neat peptoid and peptoid–metal complexes in aqueous solutions were studied by UV–visible absorption spectroscopy.[13–15] As for the mono- and di-nitrotyrosines, adjustments to pH were required to determine the relative amounts of mono- and di-nitrated EBMT formed (Fig. 1). For the nitration products of EBMT, the λ_{max} values and spectral changes as a function of pH are consistent with 3-nitrotyrosine. Under the conditions employed, di-nitration of the free peptoid or its metal complexes was not observed. By exploiting pH induced spectral changes, all readings are made at 420 and 440 nm to avoid background absorbance originating from the peptoid, peptoid–metal ion complexes or residual $ONOO^-$.

Ferric complexes of EDTA have been shown to enhance the nitration rates of phenolic compounds by $ONOO^-$.[16] For EBMT, the effects of redox active metal binding on the nitration process was studied using UV–visible absorption spectroscopy. The percentage nitration increased with raised concentrations of $ONOO^-$ and

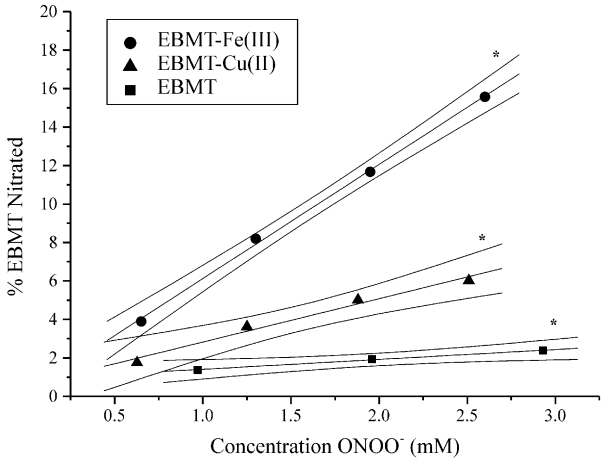
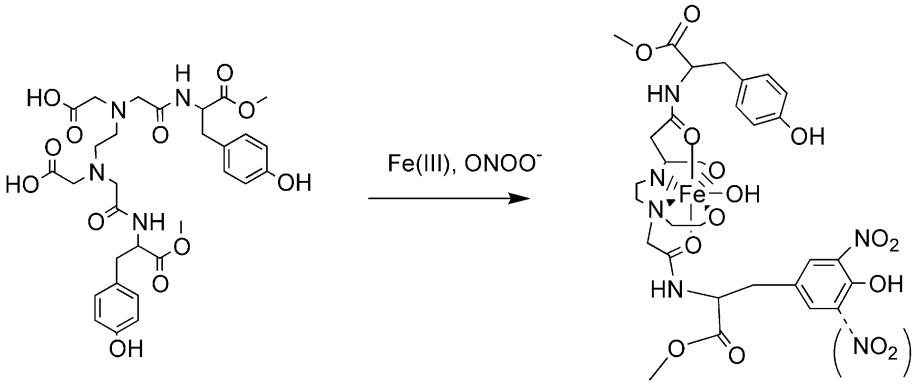


Figure 2. Enhancement of ligand nitration in the presence of metal ions. Owing to the excess of EBMT used relative to $ONOO^-$, maximum nitration rates expected in the event of 100% $ONOO^-$ capture would be 42% [Fe(III)–EBMT], 33% [Cu(II)–EBMT] and 32% (EBMT) at points indicated (*). Confidence limits at 95% are given.

complexation with both Cu(II) and Fe(III) ions. Optimal nitration of EBMT was observed for the ferric complex (Fig. 2). With an excess of EBMT, mono-nitration was favoured over di-nitration. Maximal nitration was exhibited with the Fe(III) complex giving 16% peptoid nitration which corresponds to 37% $ONOO^-$ scavenging. The Cu(II) complex afforded 6% peptoid nitration relating to 18% $ONOO^-$ capture. In the absence of co-ordinated redox-active metal ions only minimal nitration (2%) was observed giving only 7% $ONOO^-$ capture.



Scheme 1. Proposed route for formation of nitrated products of EBMT.

Nitration of the EBMT tyrosine moieties is consistent with previous results[10,16] and examination of space filling models led to a proposed mechanism of nitration (Scheme 1). The presence of a 'free' co-ordination site on the seven co-ordinate ferric ion allows $ONOO^-$ binding to occur.

Work is currently in progress to investigate the fate of the ca. 60% $ONOO^-$ not captured as nitrated tyrosine. Peroxynitrite is known to hydroxylate aromatic moieties through a proposed hydroxyl radical intermediate.[17] The formation of hydroxyl radical-mediated hydroxylation forming isomeric catechols is currently under investigation.[11] In addition, conditions favouring the formation of di-nitrotyrosine analogues are under investigation.

This study clearly shows the deleterious effects that low-molecular-mass redox-active metal ions can play in inflammatory conditions where $ONOO^-$ is generated. This system is also being developed as an assay to differentiate between the various components of oxidative stress.

Acknowledgements

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References and Notes

1. Free Radicals in Biology and Medicine, Halliwell, B. Gutteridge, J. M. C., Eds. 3rd ed.; Oxford University Press, Oxford, UK, 1999.
2. Babior, B. M. *Am. J. Med.* **2000**, *109*, 33.
3. Zhang, Z.; Naughton, D. P.; Winyard, P. G.; Benjamin, N.; Blake, D.; Symons, M. C. R. *Biochem. Biophys. Res. Comm.* **1998**, *249*, 767.
4. Swain, J. A.; Darley-Usmar, V.; Gutteridge, J. M. C. *FEBS Lett.* **1994**, *342*, 49.
5. Gergel, D.; Misik, V.; Riesz, P.; Cederbaum, A. I. *Arch. Biochem. Biophys.* **1997**, *337*, 239.
6. Spencer, J. P.; Wong, J.; Jenner, A.; Aruoma, O. I.; Cross, C. E.; Halliwell, B. *Chem. Res. Toxicol.* **1996**, *7*, 1152.
7. Hashimo, M.; Hsu, L. J.; Xia, Y.; Takeda, A.; Sisk, A.; Sundsmo, M.; Masliah, E. *Neuroreport* **1999**, *10*, 717.
8. Van der Vliet, A.; O'Neill, C. A.; Halliwell, B.; Cross, C. E.; Kaur, H. *FEBS Lett.* **1994**, *339*, 89.
9. Mapp, P. I.; Klocke, R.; Walsh, D. A.; Chana, J. K.; Stevens, C. R.; Gallagher, P. J.; Blake, D. R. *Arthritis Rheum.* **2001**, *44*, 1534.
10. Ramezanian, M. S.; Padmaja, S.; Koppenol, W. H. *Chem. Res. Toxicol.* **1996**, *9*, 232.
11. Naughton, D. P.; Grootveld, M. *Bioorg. Med. Chem. Lett.* **2001**, 2573.
12. De Caro, J. D.; Behnke, W. D.; Bonicel, J. J.; Desnuelle, P. A.; Rovery, M. *Biochim. Biophys. Acta* **1983**, *747*, 253. Other pK_a values were measured spectrophotometrically where they were unavailable in the literature.
13. Peroxynitrite was prepared by treating isoamyl nitrite with a basic solution of H_2O_2 and assayed at 302 nm. Uppu, R. M.; Pryor, W. A. *Anal. Biochem.* **1996**, *236*, 242.
14. % nitration of EBMT was calculated from new peak formation at 420 and 440 nm.
15. 1:1 ferric and cupric complexes of EBMT were prepared in aqueous solutions and the stoichiometry was verified by Job's method (data not shown).
16. Beckman, J. S.; Ischiropoulos, H.; Zhu, L.; van der Woerd, M.; Smith, C.; Chen, J.; Harrison, J.; Martin, J. C.; Tsai, M. A. *Arch. Biochem. Biophys.* **1992**, *298*, 438.
17. Crow, J. P.; Spruell, C.; Chen, J.; Gunn, C.; Ischiropoulos, H.; Tsai, M.; Smith, C. D.; Radi, R.; Koppenol, W. H.; Beckman, J. S. *Free Radic. Biol. Med.* **1994**, *16*, 331.