Bioorganic & Medicinal Chemistry Letters 19 (2009) 1173–1176

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Design, solid phase synthesis and evaluation of cationic ferrocenoyl peptide bioconjugates as potential antioxidant enzyme mimics

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ARTICLE INFO

Article history:
Received 29 October 2008
Revised 16 December 2008
Accepted 19 December 2008
Available online 25 December 2008

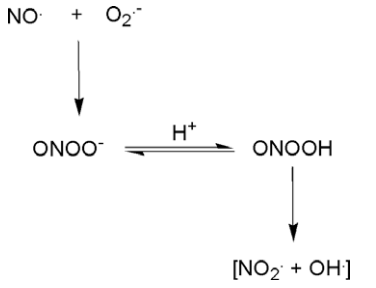
Keywords:
Ferrocene
Peptide bioconjugate
Peroxynitrite
Tyrosine nitration
Superoxide dismutase
Antioxidant

ABSTRACT

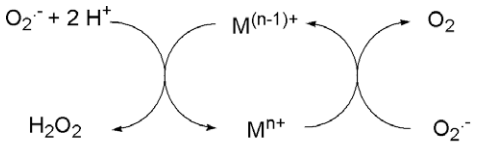
Synthetic C-terminal amidated cationic ferrocenoyl peptide bioconjugates Fc-Orn-Orn-Orn (**1**) and Fc-Tyr-Orn-Orn-Orn (**2**) were rationally designed as superoxide dismutase (SOD) mimics based on the structure of the iron SOD from *Escherichia coli*. Ferrocenoyl peptide bioconjugates **1**, **2** and ferrrocenecarboxylic acid (**4**) were subsequently evaluated as SOD mimics and as inhibitors of peroxynitrite-mediated tyrosine nitration. Due to their cationic character, ferrocenoyl peptide bioconjugates **1** and **2** exerted an acceptable SOD activity (EC_{50} = 575 μM and 310 μM, respectively) in comparison with **4** (EC_{50} = 1.4 mM). The C-terminal amidated cationic peptide Ac-Tyr-Orn-Orn-Orn (**3**), designed as marker of peroxynitrite, was used to evaluate the inhibitory activity of **1** and **4** towards peroxynitrite-mediated tyrosine nitration. Both compounds proved to inhibit the nitration especially the cationic ferrocenoyl peptide bioconjugates **1**. The ferrocene moiety of conjugate **2** displayed a strong inhibitory activity of peroxynitrite-mediated nitration of the neighboring tyrosine.

The combination of superoxide anion ($O_2^{-\cdot}$) and nitric oxide ($NO^{\cdot}$) at diffusion-controlled rates results in the formation of peroxynitrite ($ONOO^-$), a toxic and powerful oxidant that provokes cellular damages.[1,2] Indeed, the peroxynitrous acid, the conjugate acid of $ONOO^-$ (pK_a = 6.8) leads to the release of $NO_2^{\cdot}$ and $OH^{\cdot}$ radicals through homolytic cleavage (Scheme 1).[3–5] These radicals are particularly involved in the oxidation and the nitration of DNA, proteins and lipids.[6] The main effects of peroxynitrite on proteins are the nitration and the hydroxylation of tyrosine and tryptophane residues, the formation of dityrosine and the modification of sulfur containing residues.[7,8]

During the last decade, antioxidant enzymes such as peroxidase, reductase and selenoproteins[9–11] have emerged as relevant species preventing the oxidative action of peroxynitrite. Among these species, metallo-enzymes superoxide dismutases (SODs) that inhibit the peroxynitrite formation by detoxifying cells from $O_2^{\cdot-}$ and thus subtracting the radical to its reaction with nitric oxide[12] have been extensively studied. These enzymes are dimeric or tetrameric and



Scheme 1. Mechanism of formation of $NO_2^{\cdot}$ and $OH^{\cdot}$ from the combination of nitric oxide and superoxide.



M = Cu, Fe or Mn and n = 2 (Cu) ou 3 (Fe, Mn)

Scheme 2. Cyclic mechanism of metallo superoxide dismutases.

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doi:10.1016/j.bmcl.2008.12.084

exhibit in their active site a coordinated metal atom involved in the dismutation catalysis of $O_2^{\cdot-}$ into water and molecular oxygen according to a cyclic mechanism (Scheme 2).[13,14] In addition, SODs are also known as peroxynitrite scavengers because they catalyze the decomposition of peroxynitrite by nitrating their own tyrosine residues, or tyrosine residues of other proteins.[15–17]

The defensive role of SODs is now well established in several pathologies such as degenerative diseases or ischemia–reperfusion injury.[10,11] Therefore, the design, the synthesis and the evaluation of antioxidant enzyme mimics for potential therapeutic applications has recently received a significant interest.[18–21] Among the potential SOD mimics, metalloporphyrin derived compounds and nitrogenated metalloheterocycle complexes have been particularly studied.[22–24] Some of these metallocomplexes have also been shown to inhibit the peroxynitrite-mediated tyrosine nitration.[9,25] Polyphenols such as gallic acid and catechin have been reported to exhibit this activity.[26–28]

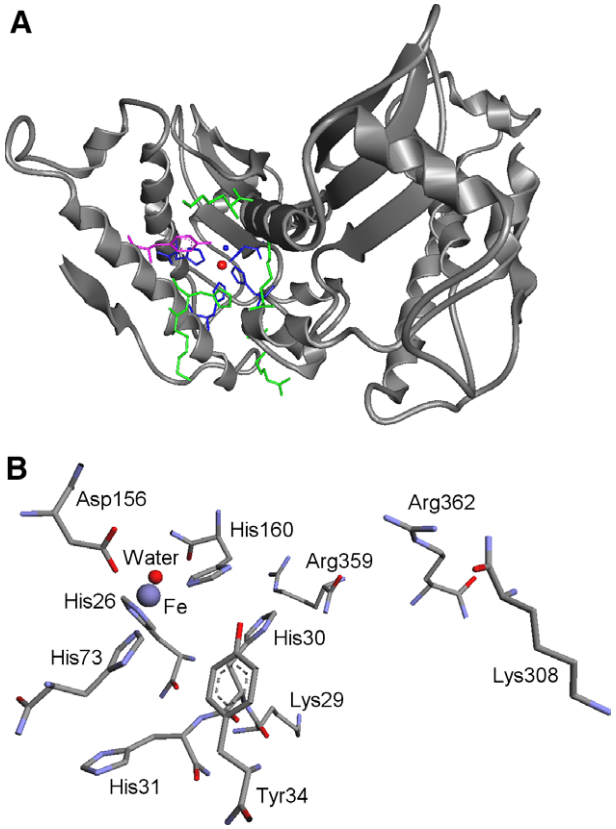
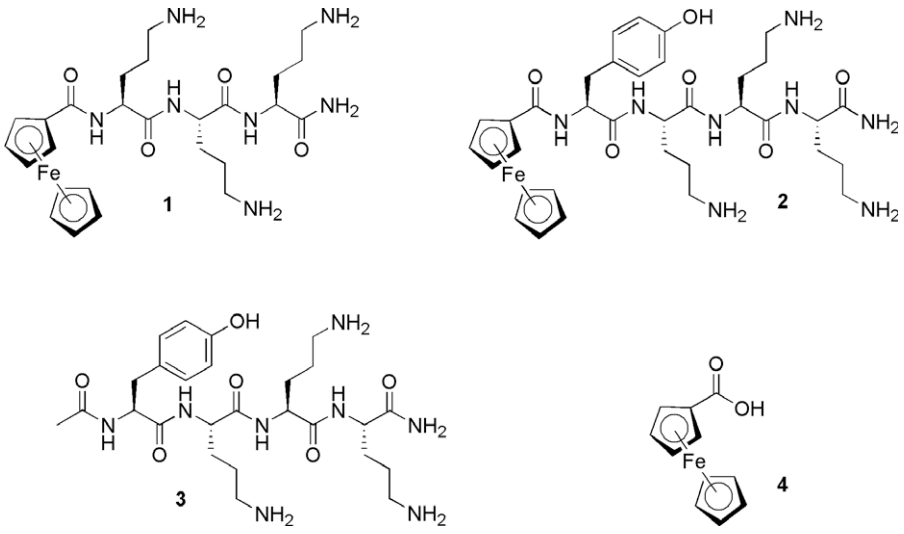


Figure 1. (A) Tri-dimensional structure of Fe-SOD with α-helixes and β-sheets forming a funnel as access to the metal of the active site with positively charged residues (in green) and residues coordinated with the metal (in blue). (B) Overview of the electrostatic funnel as access to the metal.

Ferrocene-derived compounds display a broad range of biological activities.[29,30] Particularly, ferrocene containing peptide conjugates have been synthesised and evaluated for their antiproliferative activity against human leukemia cells.[31] The design and the synthesis of cationic ferrocenoyl peptide bioconjugates have been reported as antibacterial agents.[32] However, although metallocenes have been suggested as SOD mimics,[33] to the best of our knowledge, no study has been reported so far. Aiming at the development of new antioxidant enzyme mimics based on the structure of the iron SOD (Fe-SOD) from *Escherichia* coli (pdb code 1ISA) (Fig. 1), the synthesis of ferrocenoyl peptide bioconjugates and their evaluation as SOD mimics and inhibitors of peroxynitrite-mediated tyrosine nitration were investigated.

Fe-SOD is a homodimeric protein with predominant α-helices. The interface between the two subunits forms a funnel like access to the active site constituted by the metal center, an atom of iron coordinated by three histidine residues and a molecule of water (colored in blue in Figure 1). This funnel embodies positively charged residues (histidine, arginine and lysine residues colored in green in Figure 1) responsible for the electrostatic guidance of the substrate. The tyrosine residue Tyr34 (colored in pink in Figure 1) has also been identified as an important residue due to its strong reactivity with the peroxynitrite anion leading to the corresponding nitrated tyrosine.

Based on these observations, cationic ferrocene-peptide bioconjugates Fc-Orn-Orn-Orn (**1**) and Fc-Tyr-Orn-Orn-Orn (**2**) were designed (Scheme 3). In order to exert an attractive effect towards the two anions $O_2^{\cdot-}$ and $ONOO^-$, a cationic tripeptide sequence Orn-Orn-Orn was introduced on bioconjugates **1** and **2**. Ornithine (pK_a δ-$NH_2 \approx 10.5$) was preferred over other natural cationic amino acids to prevent biodegradation during potential in vivo applications. A tyrosine residue was introduced on peptide **2** as potential nitration site and a ferrocene moiety was placed next to the tyrosine residue to evaluate the effect of the metallocene moiety on the peroxynitrite-mediated tyrosine nitration. In both cases, the ferrocene moiety was anchored to the N-terminal via an amide



Scheme 3. Structures of ferrocenoyl peptide conjugates **1** and **2**, peptide **3** and ferrocenecarboxylic acid **4**.

bond by using ferrocenecarboxylic acid. In order to evaluate the activity towards peroxynitrite-mediated tyrosine nitration, the peptide Ac-Tyr-Orn-Orn-Orn **3** (Scheme 3) was designed to be used as peroxynitrite marker with the same cationic character of bioconjugates **1** and **2** and with a tyrosine residue as potential site of nitration.

The synthesis of peptides **1**–**3** was achieved using Fmoc strategy by solid phase peptide synthesis (SPPS) on Rink-amide resin in order to obtain an amidated C-terminal and thus mask the negative charge of the carboxylate group. Amino acids (4 equivalents) were coupled using the HBTU/DIEA method for 1 h. As previously described,[31] the ferrocenecarboxylic acid was reluctant to couple to N-terminal peptide anchored on resin. Consequently, a double coupling procedure was performed with a mixture HBTU/HOBt/DIEA and the reaction time was extended to 3 h. After standard cleavage procedures, peptides **1**–**2** were isolated as orange solids.

Peptides **1**, **2** and ferrocenecarboxylic acid (**4**) were first tested as potential SOD mimics using the SOD assay based on pyrogallol autooxidation.[34] They were thus evaluated for their ability to inhibit the $O_2^{\cdot-}$-mediated reaction of autooxidation. The SOD assay was slightly modified compared with the initial assay by measuring the autooxidation rate at 325 nm[35] (instead of 420 nm) with a concentration of 0.4 mM of pyrogallol instead of 0.2 mM to increase the autooxidation rate.[36] All tested ferrocene-derived compounds exerted a moderate SOD activity with IC_{50} values of 1.4 mM, 575 μM and 310 μM for compounds **4**, **1** and **2**, respectively. In the same conditions the enzyme Fe-SOD from *E. coli* exhibited a high activity with an IC_{50} of 0.1 nM (Fig. 2). In comparison, Durot et al.[37] reported literature data for porphyrin or other nitrogenated ligand metal complexes with SOD activity ranging from 6.5 nM for $[Mn^{III}Cl_4$ TE-2-PyP$]^{5+}$ to 270 μM for $[Fe^{III}(IPG)Cl_2]$ (non-exhaustive data). However the comparison of SOD activities between compounds **1**, **2** and **4** clearly indicates an enhancement which can be attributed to the attractive effect of the cationic peptide moiety.

In order to evaluate how the presence of ferrocenoyl moieties or cationic (Orn-Orn-Orn) motifs impacts the peroxynitrite-mediated nitration of tyrosine groups, we compared the nitration of tyrosine groups of **2**, **3** and L-tyrosine. For this purpose, the nitration of peptide **3** and L-tyrosine at 100 μM was first explored (Fig. 3). While peptide **3** appeared to be highly sensitive to the addition of peroxynitrite, lower yields of nitrotyrosine were obtained from L-tyrosine (74% vs 33%, respectively, with 375 μM of cumulative doses of peroxynitrite). These results are probably the consequence of the attractive effect of the positively charged moiety of peptide **3** towards peroxynitrite and underline the potential of peptide **3** to serve as peroxynitrite marker.

The nitration of the ferrocenoyl peptide bioconjugate 2 and of (ferrocenoyl-free) peptide 3 were subsequently compared (Fig. 3). The presence of the ferrocenoyl moiety on 2 strongly reduced the nitration of the neighboring tyrosine. Indeed, only 20% of tyrosine were nitrated with 375 μM of cumulative doses of peroxynitrite whereas in the case of 3, 74% of this residue were nitrated in the same conditions.

The ability of ferrocene-derived compounds to modulate the peroxynitrite-mediated tyrosine nitration was further investigated in the presence of peptide **3** used as a marker. Exposure of **3** (100 μM) to 75 μM and 300 μM of peroxynitrite led, respectively, to 32% and 67% of the corresponding nitro-tyrosinyl peptide (Fig. 4). The same experiments were then performed in the presence of ferrocenecarboxylic acid **4** or ferrocenoyl peptide bioconjugate **1** (100 μM). In contrast or in comparison with either, other metal complexes[9,16,39,40] or superoxide dismutases,[41,42] both compounds inhibited the peroxynitrite-mediated tyrosine nitration of **3**. Indeed, in this range of peroxynitrite concentrations, a drastic decrease of the nitration yield was observed in the presence of the ferrocenoyl peptide bioconjugate **1** or compound **4**. Exposure to 725 μM of peroxynitrite led to almost quantitative tyrosine nitration of peptide **3** whereas only 42% and 23% of the corresponding nitro-tyrosinyl peptide were obtained, respectively, in the presence of compounds **4** and **1**. Considering the high reactivity of peptide **3** towards peroxynitrite, these results confirm the ability of ferrocenoyl derivatives to inhibit peroxynitrite-mediated nitration especially in the case of the ferrocenoyl peptide bioconjugate **1**.

To further examine the influence of ferrocenoyl derivatives on the inhibition of peroxynitrite-mediated tyrosine nitration, the

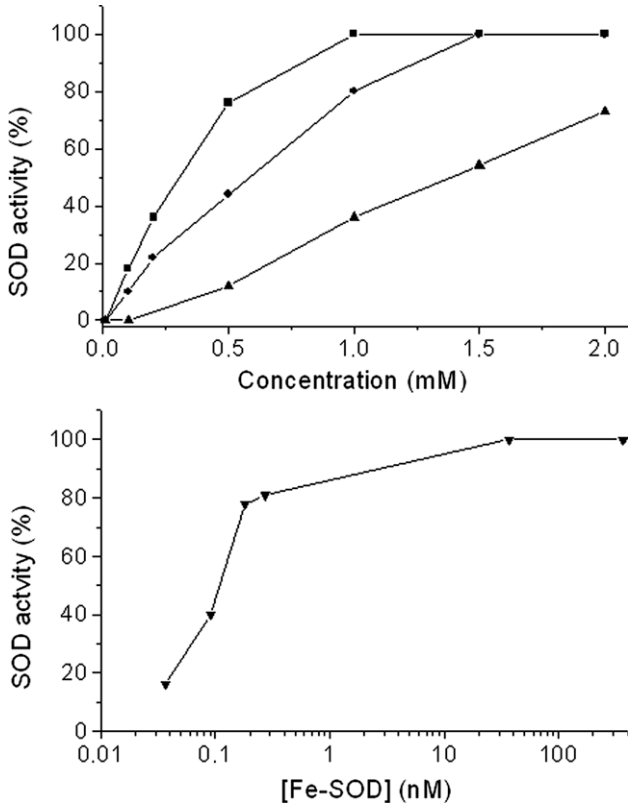


Figure 2. SOD activity (%) based on the inhibition of the $O_2^{\cdot-}$-mediated autooxidation of pyrogallol with increasing concentrations of ferrocenoyl peptide bioconjugate **1** (♦), **2** (■), ferrocenecarboxylic acid **4** (▲) and Fe-SOD from *E. coli* (▼).[38]

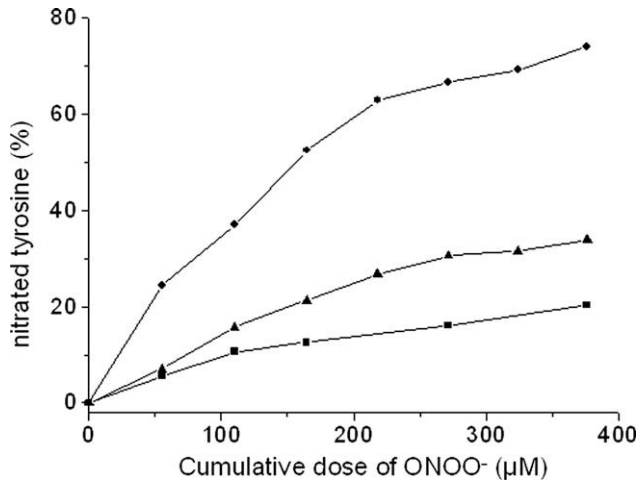


Figure 3. Nitration of peptide **3** (100 μM, ●), of L-tyrosine (100 μM, ▲) and of ferrocenoyl peptide bioconjugate **2** (100 μM, ■) induced by cumulative doses of peroxynitrite.[38]

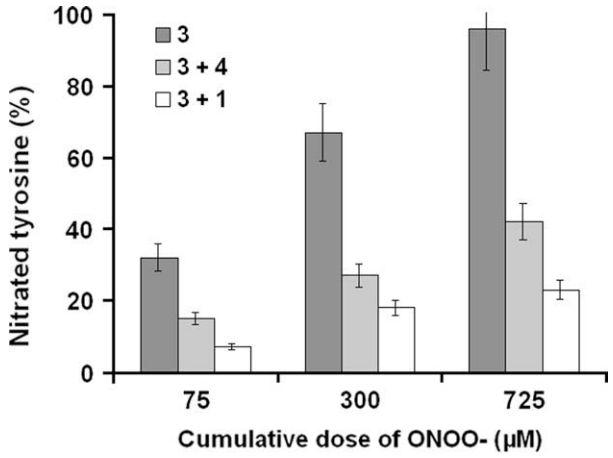


Figure 4. Yield of peroxynitrite-mediated nitration of peptide **3** (100 μM) obtained in the presence and in the absence of ferrocenecarboxylic acid **4** or ferrocenoyl peptide bioconjugate **1** (100 μM).

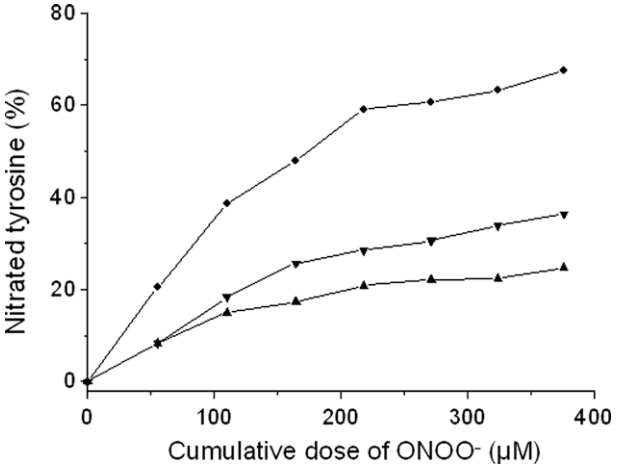


Figure 5. (A) Yield of tyrosine nitration of peptide **3** (100 μM) induced by cumulative doses of peroxynitrite without (●) or in the presence ferrocenoyl peptide bioconjugate **1** [50 μM (▼) or 100 μM (▲)].[38]

nitration of peptide **3** (100 μM) was subsequently carried out with increasing doses of peroxynitrite in the presence of ferrocenoyl peptide bioconjugate **1** (100 or 50 μM see Fig. 5). As expected, the increase of the concentration of **1** strongly affected the reaction of nitration of peptide **3**. Indeed, after the cumulative addition of 375 μM of peroxynitrite, the yield of nitration of peptide **3** decreased from 68% to 36% and 25% in the presence of, respectively, 50 and 100 μM of **1**.

In conclusion, the rational design and the solid phase synthesis of ferrocenoyl peptide bioconjugates were achieved. In vitro evaluation of ferrocenoyl peptide bioconjugates **1** and **2** as SOD mimics revealed that these compounds exerted an acceptable SOD activity. Moreover, examination of the ability of the ferrocenoyl moiety to modulate peroxynitrite-mediated nitration showed the potent activity of the metallocene to inhibit nitration of peptide **3**, a highly reactive peptide or of a tyrosine located at its vicinity. Ferrocenoyl substituents should be thus considered as potent inhibitor of peroxynitrite-mediated nitration with potential SOD activity and should be useful for the development of antioxidant enzyme mimics.

Acknowledgments

This research was supported by the MENESR and CNRS. The authors thank Pr. A. Doutheau and Dr. Y. Queneau for reading the manuscript.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.12.084.

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