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Bio-Inspired Casein-Derived Antioxidant Peptides Exhibiting a Dual Direct/Indirect Mode of Action

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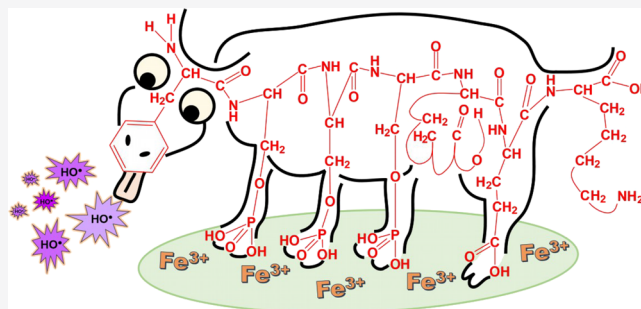


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Supporting Information

ABSTRACT: Antioxidant compounds are chemicals of primary importance, especially for their applications in nutrition and healthcare, thanks to their abilities to prevent oxidation processes and to limit and/or rebalance the oxidative stress, well-known for its impact on a wide variety of diseases. While several biomolecules are well-known for their antioxidant properties (e.g., ascorbic acid, carotenoids, phenolic derivatives), bio-sourced antioxidants have drawn considerable attention in the last decades, especially bioactive peptides, mainly obtained by the hydrolysis process. Antioxidant peptide sequences are mainly identified a posteriori, thanks to fastidious and time-consuming approaches and techniques, limiting the discovery of new efficient peptides. In this context and taking inspiration from nature, we report herein on a new series of three bio-inspired antioxidant peptides derived from the milk protein casein. These phosphopeptides, designed to chelate the redox-active iron(III) and forming highly soluble complexes up to pH 9, act both as *indirect* (i.e., inhibition of the metal redox activity) and *direct* (i.e., radical scavenging) antioxidants.



effectiveness, safety, and tolerability,^{15,16} and biologically relevant peptides can be synthesized using proven, fast, and efficient methods.¹⁷ Up until now, the majority of antioxidant peptides have been identified through tandem LC/MS/MS analyses from protein hydrolysates of natural resources^{12–14,18,19} known for their antioxidant properties. However, this serendipitous approach is hazardous, fastidious, and time-consuming, severely limiting the discovery of new effective antioxidant peptides. Thus, we believe that a more rational approach based on a finely tailored bio-inspired molecular design could achieve the same goal in a more judicious and efficient way. While a large body of work concerning copper-chelating peptides has been reported in the recent years,^{20,21} the development and studies of iron-chelating peptides are still in their infancy and remain challenging. However, iron is present in the body in iron labile pools in which iron is not or slightly chelated and therefore accessible to exogenous antioxidants,^{22,23} including in the lysosomes where iron is in its free Fe(III) form.^{22,24} For these reasons and because Fe(III) is in a biologically unusual +III oxidation state, we focus our attention on the development of antioxidant Fe(III)-

INTRODUCTION

Reactive oxygen species (or ROS) are essential highly reactive components for the proper functioning of the body, acting as signaling molecules to regulate biological and physiological processes.^{1,2} In healthy organisms, the control of the ROS concentration is ensured by a complex endogenous antioxidant defense system involving enzymes, proteins, or other chemicals.³ However, the presence of endogenous or exogenous stresses can trigger a dysregulation of the ROS homeostasis, inducing the so-called oxidative stress reported to cause a wide variety of diseases.^{4,5} In order to prevent oxidative stress and its detrimental effects, the intake of exogenous antioxidants is an efficient strategy to rebalance the concentration of ROS. Depending on their inherent structure, antioxidants act according to two main modes of action: (i) a *direct mode of action* whereby the antioxidants directly react with the ROS;^{3,6,7} (ii) an *indirect mode of action* whereby the antioxidants hinder the biometal-induced redox aerobic process *via* the chelation of specific transition metals. Indeed, *in vivo*, copper and iron are involved in the production of ROS through the catalytic reduction of molecular oxygen (O₂) to the superoxide anion radical (O₂^{•-}), and subsequently to hydrogen peroxide (H₂O₂) and a hydroxyl radical (HO[•]) (Figure 1a). Thus, their chelation induces a decrease of the ROS production and positive effects on ROS-mediated pathologies.^{3,8–10} Numerous metal-chelating antioxidants have been reported^{3,8–11} and, recently, peptides have emerged as new bio-sourced antioxidants.^{12–14} Peptides have been already considered as therapeutics offering high selectivity and

effectiveness, safety, and tolerability,^{15,16} and biologically relevant peptides can be synthesized using proven, fast, and efficient methods.¹⁷ Up until now, the majority of antioxidant peptides have been identified through tandem LC/MS/MS analyses from protein hydrolysates of natural resources^{12–14,18,19} known for their antioxidant properties. However, this serendipitous approach is hazardous, fastidious, and time-consuming, severely limiting the discovery of new effective antioxidant peptides. Thus, we believe that a more rational approach based on a finely tailored bio-inspired molecular design could achieve the same goal in a more judicious and efficient way. While a large body of work concerning copper-chelating peptides has been reported in the recent years,^{20,21} the development and studies of iron-chelating peptides are still in their infancy and remain challenging. However, iron is present in the body in iron labile pools in which iron is not or slightly chelated and therefore accessible to exogenous antioxidants,^{22,23} including in the lysosomes where iron is in its free Fe(III) form.^{22,24} For these reasons and because Fe(III) is in a biologically unusual +III oxidation state, we focus our attention on the development of antioxidant Fe(III)-

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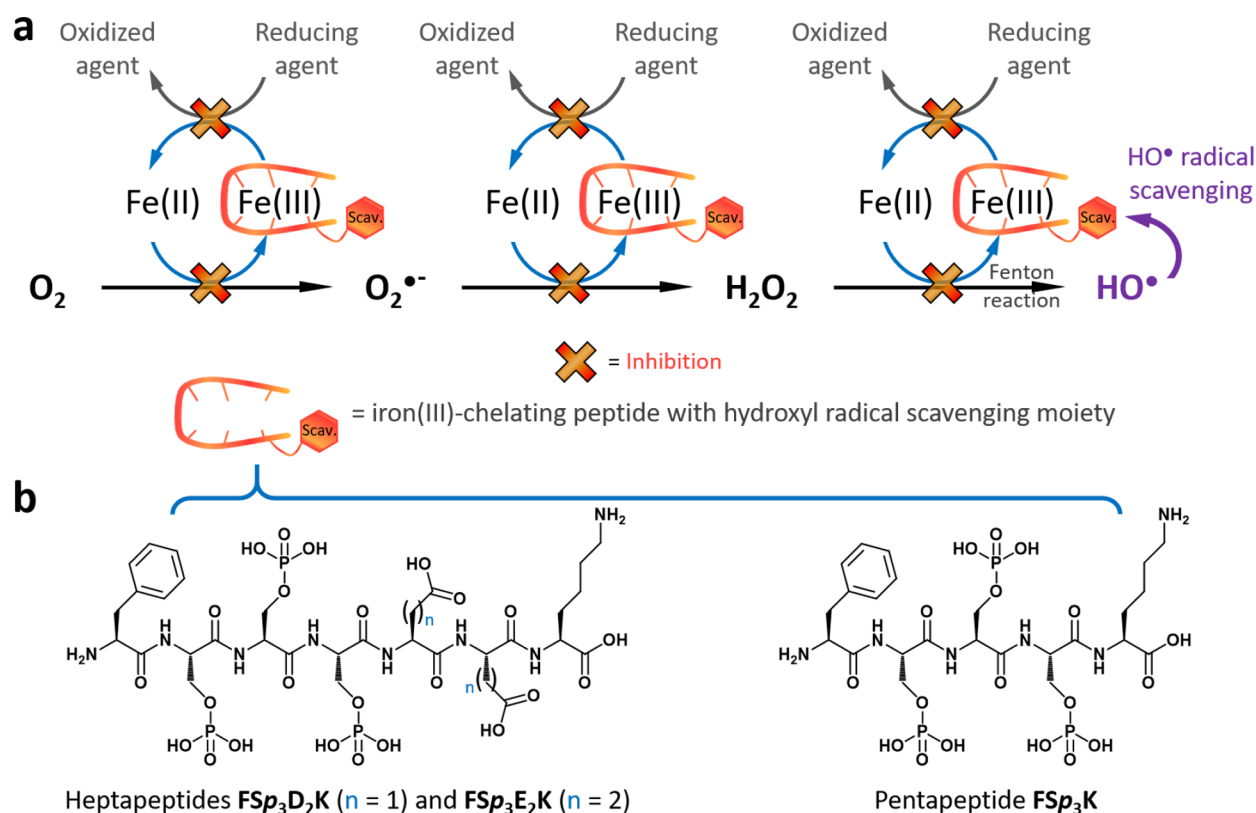


Figure 1. (a) Schematic representation of the *indirect* (i.e., inhibition of the metal redox activity) and *direct* (i.e., radical scavenging) antioxidant mode of actions and (b) chemical structures of the three phosphopeptides studied.

69 chelating peptides with both indirect and direct modes of
70 action (Figure 1a). While a first series of efficient antioxidant
71 pentapeptide has been previously reported by our group,²⁵ we
72 would like to report herein on new bioinspired phosphopep-
73 tides offering both high antioxidant properties and high
74 Fe(III)-chelating capabilities.

75 ■ RESULTS AND DISCUSSION

76 **Bioinspired Design and Synthesis of Antioxidant**
77 **Phosphopeptides.** To design such compounds, we took
78 inspiration from dietary phosphoproteins well-known for their
79 iron-chelating abilities, such as the egg yolk phosvitin²⁶ or the
80 milk caseins.²⁷ In particular, caseins are a family of four
81 proteins termed $\alpha_{\text{S1-}}$, $\alpha_{\text{S2-}}$, β -, and κ -caseins in which
82 phosphorylation occurs at the serine residues and reported
83 for their antioxidant properties.^{28,29} Interestingly, a specific
84 recurring sequence of five amino acids, termed cluster,
85 comprising three phosphoserines (noted Sp) followed by two
86 glutamic acids (E), i.e., an SpSpSpEE cluster, appears once in
87 the β -casein and $\alpha_{\text{S1-}}$ -casein, and twice in the $\alpha_{\text{S2-}}$ -casein.^{30–32}
88 Based on these observations and on our previous work,²⁵ we
89 design a first heptapeptide $\text{FSp}_3\text{E}_2\text{K}$ (Figure 1b) comprised of
90 the above-mentioned casein-derived cluster on which we
91 grafted (i) a lysine (K) at the C-term bringing an additional
92 charge to improve the peptide solubility at neutral pH, and (ii)
93 a phenylalanine (F) at the N-term to provide a further
94 antioxidant property to the peptide, i.e., a direct mode of action
95 based on the ability of phenylalanine to entrap hydroxyl
96 radicals (*vide infra*). To evaluate the impact of the two acidic
97 side chains on the chelation and antioxidant properties, two
98 other compounds have been considered: the heptapeptide
99 $\text{FSp}_3\text{D}_2\text{K}$ in which the glutamic acids have been replaced by

aspartic acids, and the acidic side chain-lacking pentapeptide
100 FSp_3K in which the two E have been removed. The three
101 phosphopeptides were synthesized, purified, and fully charac-
102 terized (Supporting Information (SI), part 2).
103

104 Evaluation of the Direct and Indirect Antioxidant

Properties. To evaluate the antioxidant performance of the
105 three Fe(III)-chelating peptides, we carried out a series of
106 three complementary assays, namely, ascorbate,³³ Amplex
107 Red,³⁴ and 3-CCA (for coumarin-3-carboxylic acid)³⁵ tests to
108 measure the iron redox activity (i.e., the reducibility of Fe(III)
109 to Fe(II)) and the formation of H_2O_2 and HO^\bullet , respectively.
110 To assess the antioxidant power of the three Fe(III)-chelating
111 phosphopeptides, two compounds were selected as references:
112 the positive control is the deferoxamine (DFO), an approved
113 siderophore drug used to treat iron overload and exhibiting
114 antioxidant properties both *in vitro*^{36–38} and *in vivo*,³⁹ whereas
115 the negative control is the ethylenediaminetetraacetic acid
116 (EDTA), well-known for its good chelation abilities but weak
117 antioxidant properties.^{36,37,40}
118

119 Thus, employing the ascorbate test, a first series of
120 experiments were carried out with 1 equiv Fe(III) for 1
121 equiv ligand at physiological pH 7.4. As depicted in Figure 2a,
122 the maximum AsCH^- consumption is observed for EDTA
123 (negative control) with $64.2 \mu\text{M}$ consumed after 10 min.
124 Interestingly, when phosphopeptides were used, this value
125 drastically drops to $15.0 \mu\text{M}$ for the casein cluster-derived
126 heptapeptide $\text{FSp}_3\text{E}_2\text{K}$, to $12.7 \mu\text{M}$ for the other heptapeptide
127 $\text{FSp}_3\text{D}_2\text{K}$, and to $11.0 \mu\text{M}$ for the shortest FSp_3K . These data
128 highlight the efficiency of the three peptides to reduce the
129 redox process of the metal ion from 77% ($\text{FSp}_3\text{E}_2\text{K}$) to 83%
130 (FSp_3K) compared to EDTA, even if the positive control DFO
131 appears slightly more efficient (-96% , $2.5 \mu\text{M}$ AsCH^-

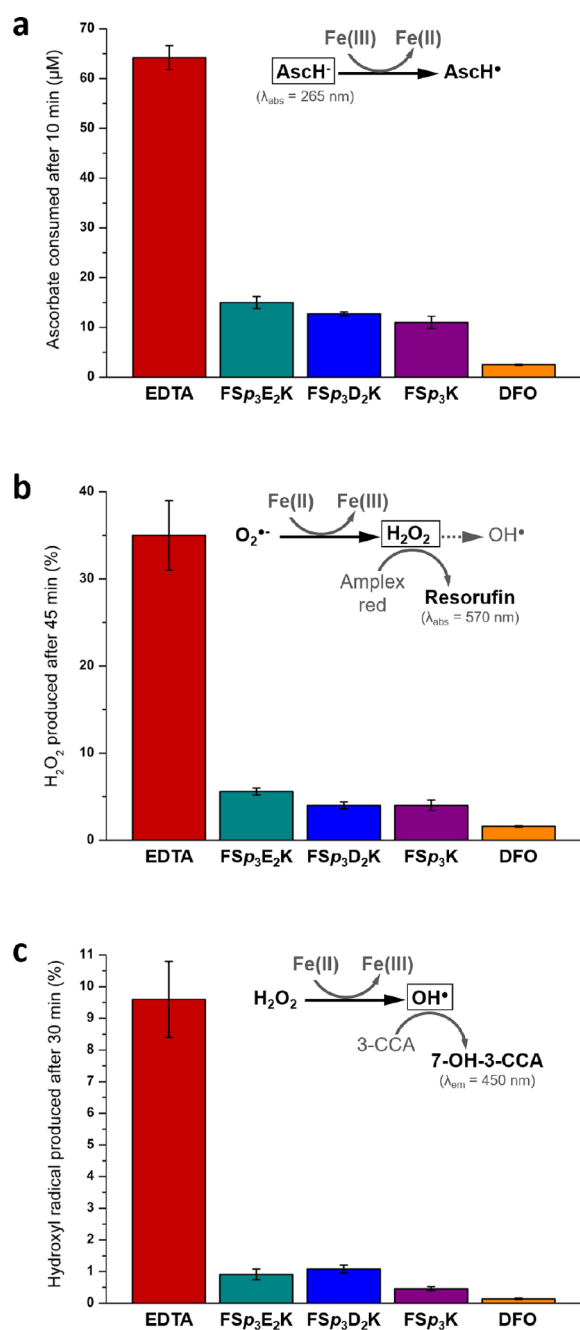


Figure 2. Evaluation of the antioxidant properties of all the phosphopeptides and of the negative (EDTA) and the positive (DFO) reference compounds. The corresponding reactions involved for each assay are inserted in each graph, with their corresponding absorbance or emission wavelengths. All experiments were carried out at pH 7.4 with (a) $[\text{AscH}^-] = [\text{Ligand}] = [\text{Fe(III)}] = 100 \mu\text{M}$, in 50 mM HEPES, (b) $[\text{AscH}^-] = 200 \mu\text{M}$, $[\text{Ligand}] = [\text{Fe(III)}] = 50 \mu\text{M}$ (except $[\text{EDTA}] = [\text{Fe(III)}] = 10 \mu\text{M}$), in 50 mM HEPES, and (c) $[\text{AscH}^-] = 125 \mu\text{M}$, $[\text{Ligand}] = [\text{Fe(III)}] = 50 \mu\text{M}$ (except $[\text{EDTA}] = [\text{Fe(III)}] = 5.0 \mu\text{M}$ and $[\text{AscH}^-] = 12.5 \mu\text{M}$) in water.

132 consumed, Table S1). To gain further insights into the
133 antioxidant properties of the three phosphopeptides, we then
134 measured their abilities to limit the formation of two distinct
135 ROS, namely, H_2O_2 and HO^\bullet . Considering the negative
136 control EDTA in the presence of 1 equiv Fe(III), the amount
137 of H_2O_2 formed after 45 min is equal to 35.0% relative to the
138 concentration of the complex (Figure 2b, Table S2). By

contrast, this value drops to 1.6% when the positive control 139
DFO is used. Phosphopeptides also exhibit significant 140
antioxidant properties, with an amount of H_2O_2 produced 141
from 5.6% for $\text{FSp}_3\text{E}_2\text{K}$ to 4.0% for both $\text{FSp}_3\text{D}_2\text{K}$ and FSp_3K . 142
Interestingly, the glutamic acid-incorporating peptide $\text{FSp}_3\text{E}_2\text{K}$ 143
with the closest casein cluster-like sequence has the weaker 144
antioxidant efficiency compared to the two others, as 145
previously observed in the AscH^- test (Figure 2a). 146
Subsequently, 3-CCA experiments were carried out, and 147
results (Figure 2c) highlight once more that the highest 148
quantity of HO^\bullet (9.6%, corresponding to the formed HO^\bullet / 149
Fe(III)-complex ratio) is formed in the presence of EDTA. 150
Oppositely, DFO and phosphopeptides show better antioxi- 151
dant activities, with a ratio of HO^\bullet produced of 0.14% for 152
DFO, while this value spans from 1.08 (for $\text{FSp}_3\text{D}_2\text{K}$) to 0.46% 153
(FSp_3K) for our compounds (Table S3). These experiments 154
confirm that all the three phosphopeptides act as effective 155
antioxidants, slightly less efficient than DFO but highly better 156
than EDTA. 157

However, these peptides have also been designed to behave 158
as direct antioxidants through the entrapment of the most 159
reactive ROS, *i.e.*, the hydroxyl radical HO^\bullet . Indeed, the 160
oxidation of phenylalanine to tyrosine by HO^\bullet has already been 161
reported as a marker of oxidative stress.^{41,42} To follow the 162
oxidation of the Phe moiety, a first series of four experiments 163
per peptide was performed by HRMS/MS.²⁵ Thus, the MS 164
spectrum (direct injection) of a stoichiometric peptide/ 165
Fe(III)/ $\text{AscH}^-/\text{H}_2\text{O}_2$ solution for the heptapeptide $\text{FSp}_3\text{D}_2\text{K}$ 166
(Figure S8, SI for $\text{FSp}_3\text{E}_2\text{K}$ (Figure S14), and FSp_3K (Figure 167
S2)) shows the presence of the native heptapeptide ($m/z =$ 168
513.124 $[\text{M} + 2\text{H}]^{2+}$), the $\text{FSp}_3\text{D}_2\text{K}$ -iron(III) complex ($m/z =$ 169
539.579 $[\text{M-H} + \text{Fe}]^{2+}$), which confirms the Fe(III)-chelating 170
properties of the peptide (discussed hereinafter), and of 171
another peak at $m/z = 521.123$ corresponding to the mono- 172
oxidized peptide $[\text{M} + 2\text{H} + \text{O}]^{2+}$. To separate and precisely 173
analyze each of these species, all the samples were injected in 174
RP-HPLC/HRMS/MS, and the chromatogram for $\text{FSp}_3\text{D}_2\text{K}$ 175
(Figure 3a, SI part 4 for $\text{FSp}_3\text{E}_2\text{K}$ and FSp_3K) reveals the 176
presence of the native peptide (retention time $t_R = 13.4$ min), 177
preceded by a set of three peaks with shorter retention times 178
($t_R = 10, 11,$ and 12.2 min). The latter ones correspond to the 179
mono-oxidized $[\text{M} + 2\text{H} + \text{O}]^{2+}$ species attributable to the 180
oxidation of the phenyl ring of the N-term phenylalanine to its 181
monohydroxy-equivalent as determined by HRMS/MS 182
fragmentation (Figure 3b). The use of HPLC allows us to 183
separate the three phenolic isomers,^{43,44} *i.e.*, the peptides 184
containing the *ortho*-, *meta*-, and *para*-hydroxyphenylalanine, 185
indicative of the reaction of the HO^\bullet with all positions of the 186
phenyl ring. Interestingly, working with harsher conditions (*i.e.*, 187
 $\text{FSp}_3\text{D}_2\text{K}/\text{Fe(III)}/\text{AscH}^-/\text{H}_2\text{O}_2$ 1/1/10/10) in order to shift 188
the reaction equilibrium, we observed the formation of 189
additional broad peaks (in green, Figure 3c) with shorter t_R 190
 $= 7.5$ to 8.9 min characterized by $m/z = 529.116$ 191
corresponding to the di-hydroxylated peptide $[\text{M} + 2\text{H} +$ 192
 $2\text{O}]^{2+}$, *i.e.*, the peptide in which the phenylalanine has been 193
oxidized twice (Figure 3d). These experiments confirm the 194
ability of our phosphopeptides to selectively react with HO^\bullet 195
(control experiments were carried out to discard the possibility 196
of a H_2O_2 -induced oxidation, SI part 4) *via* the N-term 197
phenylalanine, validating the relevance of our approach and the 198
direct antioxidant mode of action of our designed peptides. 199
Lastly, the HPLC/HRMS chromatograms also highlight the 200
presence of the mononuclear peptide-Fe(III) complex ($t_R = 201$

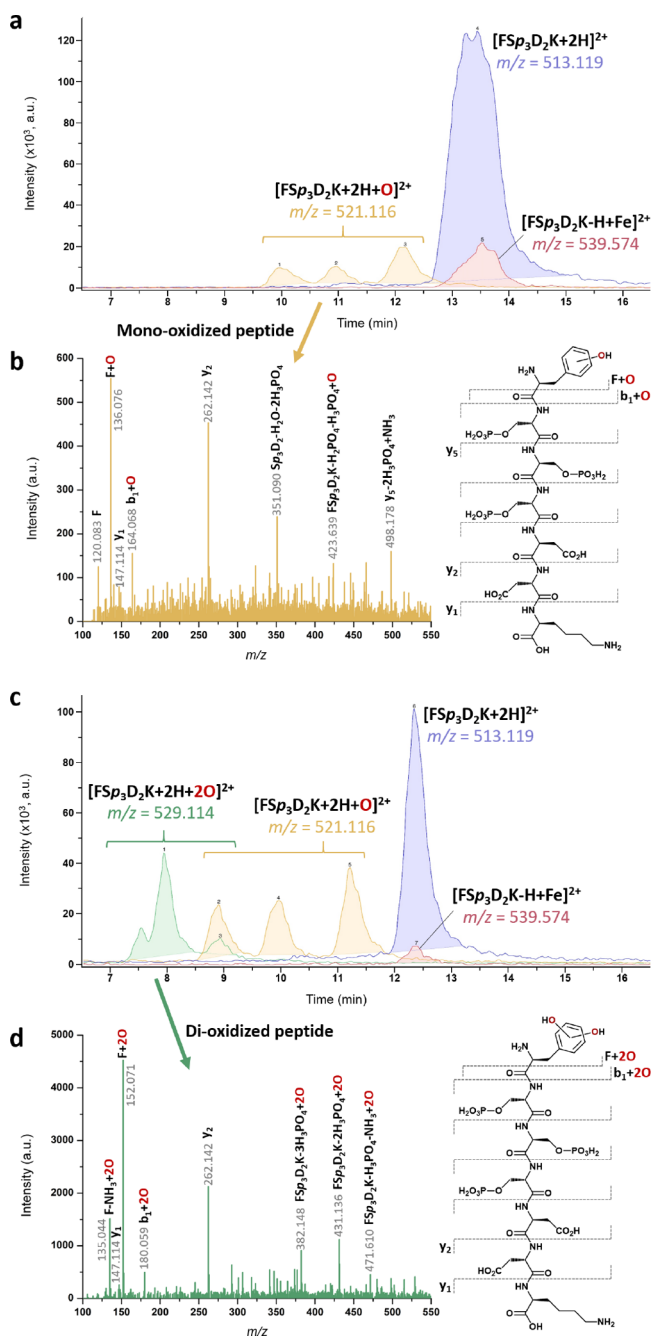


Figure 3. HPLC-HRMS chromatograms highlighting (a) the mono-oxidation of $\text{FSp}_3\text{D}_2\text{K}$ ($[\text{Fe(III)}] = [\text{FSp}_3\text{D}_2\text{K}] = [\text{AscH}^-] = [\text{H}_2\text{O}_2] = 1 \text{ mM}$), and (c) the di-oxidation of $\text{FSp}_3\text{D}_2\text{K}$ ($[\text{Fe(III)}] = [\text{FSp}_3\text{D}_2\text{K}] = 1 \text{ mM}$, $[\text{AscH}^-] = [\text{H}_2\text{O}_2] = 10 \text{ mM}$). Corresponding HRMS/MS spectra of the (b) mono-oxidized peptide at $m/z = 521.116$ and (d) di-oxidized peptide at $m/z = 529.114$. Experiments were carried out at pH 7.4 in water.

202 13.5 min, $m/z = 539.574$ $[\text{M-H} + \text{Fe}]^{2+}$ for $\text{FSp}_3\text{D}_2\text{K}$ (in red,
 203 Figure 3a); see SI part 4 for $\text{FSp}_3\text{E}_2\text{K}$ and FSp_3K , which have
 204 been further characterized using potentiometric measurements,
 205 circular dichroism (CD), and ^{31}P NMR spectroscopies (SI part
 206 5A,B).

207 **Studies on the Phosphopeptides Chelation Proper-**
 208 **ties.** First, potentiometric studies were carried out on the
 209 synthesized compounds, in the presence and absence of
 210 Fe(III) , to both access the species distributions as a function of

pH and to obtain characteristic thermodynamic constants 211
 (protonation, complexation) of the peptides and iron(III) 212
 complexes. The deprotonation constants of each ligand alone 213
 (i.e., in the absence of Fe(III)) are reported in Table 1. 214 11

Table 1. Overall Protonation Constants ($\log\beta$, H_L) and Deprotonation Constants (pK) of the Studied Peptides; $T = 298 \text{ K}$, $I = 0.1 \text{ M NaNO}_3$ (Standard Deviations Are in Parentheses)

$\log\beta$	FSp_3K	$\text{FSp}_3\text{E}_2\text{K}$	$\text{FSp}_3\text{D}_2\text{K}$
HL	10.90 (4)	11.36 (1)	11.43 (1)
H ₂ L	19.16 (6)	19.90 (2)	20.01 (2)
H ₃ L	26.31 (6)	27.28 (2)	27.43 (2)
H ₄ L	32.49 (7)	33.86 (2)	34.02 (2)
H ₅ L	38.18 (8)	39.66 (3)	39.82 (3)
H ₆ L	42.27 (12)	44.74 (3)	44.72 (3)
H ₇ L		49.12 (3)	48.85 (3)
H ₈ L		52.78 (5)	52.37 (5)
pK			
$\text{COOH}_{\text{term}}$	4.09 (12)	3.66 (5)	3.52 (5)
COOH (1)		4.38 (3)	4.13 (3)
COOH (2)		5.08 (3)	4.90 (3)
$\text{PO}_3(\text{OH})^-$ (1)	5.69 (8)	5.80 (3)	5.80 (3)
$\text{PO}_3(\text{OH})^-$ (2)	6.18 (7)	6.58 (2)	6.59 (2)
$\text{PO}_3(\text{OH})^-$ (3)	7.15 (6)	7.38 (2)	7.42 (2)
NH_3^+ (N_{term})	8.26 (6)	8.54 (2)	8.58 (2)
ϵNH_3^+ (Lys)	10.90 (4)	11.36 (1)	11.43 (1)

Depending on the peptide sequence, these ligands have 9 215
 (FSp_3K) or 11 ($\text{FSp}_3\text{D}_2\text{K}$, $\text{FSp}_3\text{E}_2\text{K}$) protonable groups, 216
 notably one or three carboxylate (COOH/COO^-), three 217
 phosphates ($\text{PO}_2(\text{OH})_2/\text{PO}_4^{2-}$), and two amino ($-\text{NH}_3^+/-$ 218
 NH_2) groups. The first pK of phosphate groups ($\text{PO}_2(\text{OH})_2/$ 219
 $\text{PO}_3(\text{OH})^-$) is too low to be easily determined in titration 220
 experiments. Thus, the first measured dissociation step 221
 corresponds to the deprotonation of terminal COOH whose 222
 value is considerably higher ($\text{pK}_{\text{COOHterm}} = 3.52-4.09$) than 223
 the pK value of free amino acids (2.0–2.5). This increase in 224
 the pK values is probably due to the large network of hydrogen 225
 bonds formed between the negatively charged phosphate 226
 groups (as H acceptors) and the protonated amine and 227
 COOH groups (as H donors). This H-bond network has also 228
 an impact on the deprotonation process of terminal NH_2 229
 ($\text{pK}_{\text{Nterm}} = 8.26-8.58$) and lysine NH_2 ($\text{pK}_{\text{Lys}} = 10.90-11.43$) 230
 groups, whose pK values are therefore higher than those 231
 observed for the first series of pentapeptide FD_3K ($\text{pK}_{\text{Nterm}} =$ 232
 7.64, $\text{pK}_{\text{Lys}} = 10.98$) and FE_3K ($\text{pK}_{\text{Nterm}} = 7.50$, $\text{pK}_{\text{Lys}} =$ 233
 10.82).²⁵ In the case of $\text{FSp}_3\text{D}_2\text{K}$, $\text{FSp}_3\text{E}_2\text{K}$ peptides, two 234
 additional deprotonation steps were measured corresponding 235
 to the dissociation of aspartic and glutamic acid side chains 236
 ($\text{pK}_{\text{COOH}(1)} = 4.13$ and 4.38, $\text{pK}_{\text{COOH}(2)} = 4.90$ and 5.08 for 237
 $\text{FSp}_3\text{D}_2\text{K}$ and $\text{FSp}_3\text{E}_2\text{K}$, respectively). These pK values are 238
 similar to those measured for the previously studied peptides,²⁵ 239
 showing that these groups are not involved in inter- or 240
 intramolecular interactions. Regarding the $\text{PO}_3(\text{OH})^-/\text{PO}_4^{2-}$ 241
 dissociation processes of three phosphate groups in the peptide 242
 sequence, the consecutive pK values are well separated (ΔpK 243
 $\approx 0.6-0.9$) and the first $\text{pK}_{\text{PO}_3(\text{OH})-(1)}$ value (5.69–5.80) 244
 corresponds well to those of phosphoserine alone 245
 ($\text{pK}_{\text{PO}_3(\text{OH})-\text{serine}} = 5.61$). 246

Then, stability constants ($\log\beta$) and stepwise deprotonation 247
 constants (pK) of iron(III) complexes were assessed and are 248 14

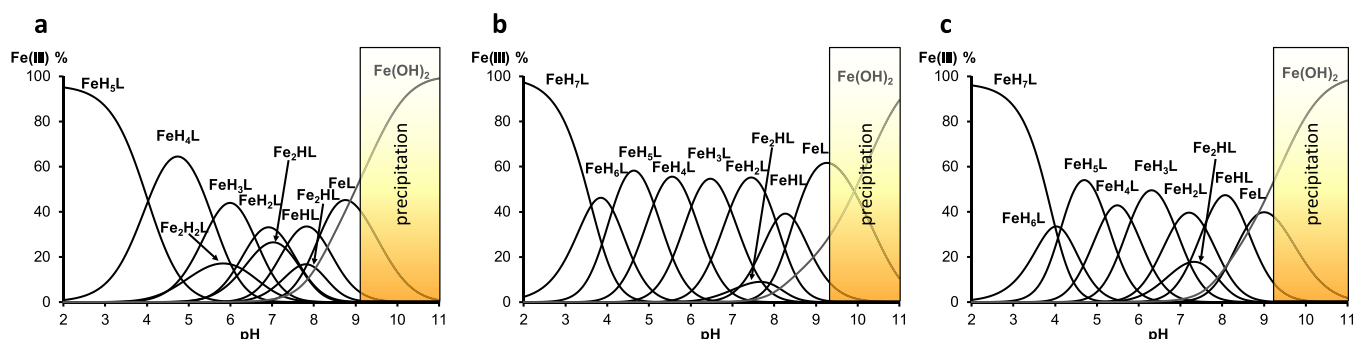


Figure 4. Speciation diagrams of Fe(III)-phosphopeptides 1:1 systems: (a) Fe(III)-FSp₃K, (b) Fe(III)-FSp₃D₂K and (c) Fe(III)-FSp₃E₂K. For all experiments, [peptide]_{tot} = 2.0 mM, T = 298 K, and I = 0.1 M NaNO₃.

summarized in Table 2 and Figure 4. The results indicate that mono- and binuclear complexes were formed, while bis(ligand) complexes were not observed. For all the three phosphopeptides and at pH 2, the second deprotonation of one phosphate group occurs, meaning that the complexation of iron(III) is already performed by mono- and di-deprotonated phosphate groups. Accordingly, potentiometric titrations start with the species FeH₃L for FSp₃K and FeH₇L for both FSp₃D₂K and FSp₃E₂K. The following steps up to the formation of FeH₄L species correspond to the deprotonation of COOH_{term} for the three phosphopeptides and also to the deprotonations of COOH(1) and COOH(2) in the case of FSp₃D₂K and FSp₃E₂K. The next two dissociation steps belong to the formation of the FeH₃L and FeH₂L complexes where the other two phosphate groups are deprotonated. All these pK values from pK(FeH₇L/FeH₆L) to pK(FeH₃L/FeH₂L) are close to the corresponding pK values in the ligands alone (Table 1), demonstrating that the metal ion-promoted dissociation process does not occur. The H-bond network, operating in

Table 2. Stability Constants (logβ) and Derived Data (pK) for Fe(III) Complexes of the Studied Peptides; T = 298 K, I = 0.1 M NaNO₃ (Standard Deviations Are in Parentheses)

logβ	FSp ₃ K	FSp ₃ E ₂ K	FSp ₃ D ₂ K
FeH ₇ L		59.24 (5)	60.32 (4)
FeH ₆ L		55.24 (7)	56.70 (4)
FeH ₅ L	48.19 (6)	51.10 (5)	52.57 (4)
FeH ₄ L	44.16 (5)	45.90 (6)	47.45 (3)
FeH ₃ L	38.57 (6)	40.08 (5)	41.43 (3)
FeH ₂ L	31.99 (7)	33.22 (6)	34.48 (3)
FeHL	24.62 (6)	25.65 (3)	26.44 (2)
FeL	16.48(5)	17.07 (5)	17.96 (3)
Fe ₂ H ₆ L		62.05 (7)	62.83 (6)
Fe ₂ H ₅ L		58.78 (7)	59.99 (4)
Fe ₂ H ₄ L	50.71 (8)	54.81 (6)	56.20 (4)
Fe ₂ H ₃ L	47.70 (6)	49.87 (6)	51.54 (4)
Fe ₂ H ₂ L	43.07 (6)	44.33 (5)	45.86 (4)
Fe ₂ HL	36.78 (6)	37.73 (5)	39.09 (4)
Fe ₂ L	29.13 (10)		
pK (FeH ₇ L/FeH ₆ L)		4.00	3.62
pK (FeH ₆ L/FeH ₅ L)		4.14	4.13
pK (FeH ₃ L/FeH ₄ L)	4.03	5.20	5.12
pK (FeH ₄ L/FeH ₃ L)	5.59	5.82	6.02
pK (FeH ₃ L/FeH ₂ L)	6.58	6.86	6.95
pK (FeH ₂ L/FeHL)	7.37	7.57	8.04
pK (FeHL/FeL)	8.14	8.58	8.48

the solution of phosphopeptide alone, cannot be formed with terminal NH₃⁺ because the phosphate groups are coordinated to the metal ion; thus, the pK_{Nterm} (FeHL formation) value is decreased to 7.37–8.04, a value close to that observed with the previous FD₃K series.²⁵ The last dissociation step corresponds to water deprotonation coordinated to the metal ion with a pK value of 8.14, 8.48, and 8.58 for FSp₃K, FSp₃D₂K, and FSp₃E₂K, respectively. At a concentration of 100 μM and at pH 7.4, while small precipitation occurred in the presence of FD₃K, homogeneous solutions were detected with the phosphorylated FSp₃K, FSp₃D₂K, and FSp₃E₂K peptides. At this pH, the pFe value is, for instance, 1.5 logarithmic units higher for FSp₃D₂K (pFe = 14.3) than for FD₃K (pFe = 12.8),²⁵ highlighting that the presence of phosphoserines in the sequences significantly increases the stability of the formed iron(III) complexes and are able to prevent metal ion hydrolysis up to pH 9.2. Based on all these data (potentiometric and spectroscopic, SI part 5), it can be reasonably assumed that the mononuclear 1:1 Fe(III):peptide complexes are formed through the chelation of the metal ion mainly by the phosphate groups, even if other functions (e.g. carboxylate) and/or water can be involved in the chelation as previously reported.²⁵ However, to decipher the precise complex structures of these phosphopeptides, further investigations are required. Interestingly, such data both confirm and bring new insights into the role of the casein cluster Sp₃E₂ on the chelation and antioxidant properties of milk casein proteins, as already reported.^{27–32}

Finally, since our phosphopeptides are derived from a specific sequence of casein, a milk protein well-known for its ability to bind calcium mainly through its phosphoserines,^{45,46} we performed competition experiments in the presence of calcium ions. Using the AsCH⁻ test, no loss of antioxidant activity is observed upon addition of 1 and 10 equiv of the non-redox active Ca(II) to a 1:1 mixture of Fe:peptide (Table S1); conversely, a sensitive decrease of the ascorbate consumption, i.e., a better antioxidant activity, appears. Additional CD, ³¹P NMR, and ESI-MS studies of the Fe(III):Ca(II):phosphopeptide ternary systems (Figures S23–S27) show that iron remains chelated even with excess calcium and suggest a possible reorganization of the peptides around Fe(III) in the presence of Ca(II) (SI part 5B), confirming the efficiency and selectivity of our bioinspired antioxidant phosphopeptides.

CONCLUSIONS

Thus, we reported herein on the rational design of an original series of bioinspired casein-derived phosphopeptides exhibiting

315 significant antioxidant properties thanks to a dual mode of
316 action. Indeed, these compounds show high Fe(III)-chelating
317 abilities with stability of the complexes formed up to pH 9.2,
318 leading to indirect antioxidant activities. Concomitantly, the
319 direct antioxidant behavior is provided by the phenylalanine
320 moiety and its inherent abilities to act as radical scavenger,
321 especially for the highly reactive hydroxyl radical, as observed
322 by HRMS experiments. Altogether, these two combined
323 antioxidant effects brought by the phosphopeptides reduce,
324 *inter alia*, the metal redox process to 83% and the formation of
325 HO[•] to -95%.

326 Also, our study highlights that to design effective antioxidant
327 peptides, the incorporation of aromatic amino acid(s) (herein,
328 phenylalanine) is an easy way to induce a direct antioxidant
329 mode of action (*i.e.*, radical scavenging), while the presence of
330 highly chelating amino acids (herein, phosphoserines) are
331 required to assess both an efficient Fe(III) chelation and
332 effective indirect antioxidant properties.

333 ■ MATERIAL AND METHODS

334 **Peptide Synthesis (General Procedure).** The three peptides
335 were synthesized at a 200 μmol scale using an automated ResPep XL
336 synthesizer (Intavis AG) using an Fmoc/tBu strategy and double
337 couplings for each amino acid. Preloaded Wang-resin (0.70 $\text{mmol}\cdot\text{g}^{-1}$,
338 200–400 mesh) was used. The standard conditions for each coupling
339 were Fmoc-amino acid (5 equivalents), 2-(1*H*-benzotriazol-1-yl)-
340 1,1,3,3-tetramethyl-uronium tetrafluoroborate (HBTU, 5 equiva-
341 lents), and 4-methylmorpholine (NMM, 10 equivalents) in
342 dimethylformamide (DMF) and *N*-methyl-2-pyrrolidone (NMP),
343 with a coupling time of 60 min. Fmoc-Ser(PO(OBzl)OH)-OH was
344 introduced at 4.0 equiv using HBTU (4.0 equiv), NMM (8.0 equiv)
345 in DMF, and NMP. Fmoc-deprotection steps were carried out using a
346 20% piperidine solution in DMF (3 \times 15 min), and final cleavages
347 were achieved using a trifluoroacetic acid/triisopropylsilane/water
348 (92.5/5/2.5) mixture (3 h, 500 rpm). The crude peptides were
349 precipitated from cold diethylether (-20 $^{\circ}\text{C}$), centrifuged (3 \times 5000
350 rpm, 5 min) washed with cold diethylether, dried under reduced
351 pressure, resolubilized in water, and finally, lyophilized. Then,
352 peptides were dissolved in a solvent A, sonicated, and purified by
353 semi-preparative HPLC equipped with a Nucleosil (Macherey–
354 Nagel) 100-5 C₁₈ 250 \times 21 mm column using solvent A (95% water,
355 5% acetonitrile, 0.1% trifluoroacetic acid) and solvent B (100%
356 acetonitrile, 0.1% trifluoroacetic acid) with a UV detection at 214 nm.
357 For FSp₃K, the same procedure was applied except solvent A, which is
358 97% water, 3% acetonitrile, and 0.1% trifluoroacetic acid. The resulting
359 solutions were evaporated under reduced pressure and were double-
360 lyophilized. The purity of each peptide was evaluated by analytical
361 reversed-phase HPLC equipped with a Nucleosil (Macherey–Nagel)
362 100-5 C₁₈ 250 \times 4.6 mm column using solvent A' (97% water, 3%
363 acetonitrile, 0.1% formic acid) and solvent B' (100% acetonitrile, 0.1%
364 formic acid) with a UV detection at 214 nm. Peptides were obtained
365 with 31% (FSp₃E₂K), 37% (FSp₃D₂K), and 38% (FSp₃K) yield. All
366 chemical characterizations (NMR, HRMS, and HPLC) are provided
367 in the SI.

368 **Antioxidant Activity Assays (General Procedure).** HEPES
369 (50 mM, pH 7.4) buffer was treated on a Chelex100 to remove trace
370 metal contaminants from the solution and prevent unwanted reactions
371 before the different tests. The stock solution at 2 mM of iron(III)-
372 nitrate was prepared from an analytical grade reagent in an acidic
373 media (0.1 M HNO₃) to keep iron(III) ion in solution; its exact
374 concentration was checked spectrophotometrically using complexo-
375 metric titration. The stock solution at 2 mM of calcium(II)-chloride
376 was prepared in H₂O, and the concentration was checked as the same
377 method as in the case of iron(III). Peptides, EDTA, and DFO ligand
378 stock solutions at 2 mM were prepared using HEPES buffer. To
379 perform the tests, a semi-micro quartz cell with a 1 cm optical
380 pathway was used; the sample holder in the spectrophotometer was

thermostated at 25 $^{\circ}\text{C}$ by a Peltier temperature controller. For all the
381 experiments, the iron(III)-complexes were prepared 20 min before
382 each antioxidant analysis to assure a complete chelation of the metal
383 ion. 384

Ascorbate Tests. For a standard assay, a 5 mM ascorbate stock
385 solution was freshly prepared using HEPES buffer at pH 7.4. The
386 absorbance of ascorbate was followed over 10 min at 265 nm ($\epsilon =$
387 14,500 $\text{M}^{-1}\text{cm}^{-1}$), and at least three independent measurements were
388 carried out. In a total volume of 1 mL, the final concentration of the
389 components was 100 μM with 1:1:1 AscH⁻:ligand:Fe(III) ratio. In
390 the ascorbate test and in the competition study with Ca(II), the final
391 concentration of the components was also 100 μM with a 1:1:1 (or 2
392 or 10) AscH⁻:ligand:Ca(II) ratio or with a 1:1:1:1 (or 10)
393 AscH⁻:ligand:Fe(III):Ca(II) ratio. The pH was checked at the end
394 of each assay. The reaction was triggered by the addition of ascorbate.
395 As a reference reaction, AscH⁻ oxidation by ligands alone was also
396 studied. The initial ascorbate oxidation rate was calculated from the
397 slope of $[\text{AscH}^-] = f(t)$ (Figure S1). 398

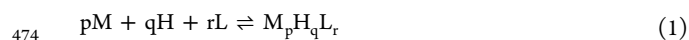
Amplex Red Assays. The H₂O₂ production was detected by an
399 Amplex Red reagent in the presence of horseradish peroxidase (HRP)
400 forming the compound Resorufin with $\lambda_{\text{abs}} = 570$ nm. For this assay,
401 all components except the iron(III) ion and Amplex Red reagent were
402 dissolved in 50 mM HEPES buffers. The Amplex Red reagent was
403 dissolved in a DMSO:buffer mixture at a 1:4 ratio. A working solution
404 was first prepared containing 0.8 U/mL HRP and 200 μM Amplex
405 Red reagent in a light-protected tube. A total of 50 μL of this solution
406 was placed in the total volume of 1 mL of reaction mixture. The final
407 concentration of the AscH⁻ was 200 μM , and the ligand (peptides or
408 DFO):Fe(III):AscH⁻ ratio was 1:1:4. In the case of EDTA, a ratio of
409 1:1:20 of EDTA:Fe(III):AscH⁻ was applied. The AscH⁻ was the last
410 reagent added, thus initiating the reaction between O₂ and Fe(III);
411 the pH was checked at the end of each reaction. A negative control
412 containing only the working solution in buffer was necessary in order
413 to monitor the background absorbance of the Amplex Red itself. The
414 Resorufin formation was monitored by visible absorption spectroscopy
415 at 570 nm at RT over 45 min. To determine the H₂O₂
416 concentration, a standard calibration curve was used. To obtain this
417 calibration curve, a 20 mM hydrogen peroxide solution was previously
418 titrated by permanganometry and then diluted successively. The
419 results are summarized in Table S2. 420

Coumarin-3-carboxylic Acid (3-CCA) Assay. The HEPES
421 buffers disturbed the fluorescence spectra and also had efficient
422 HO[•] radical scavenging activity, thus pure water treated on Chelex100
423 was used as solvent and the pH was adjusted by adding 0.1 M NaOH.
424 Thus, all the reagents were dissolved in Milli-Q water except the 3-
425 CCA, since it was only soluble in phosphate buffer (50 mM, pH 7.4).
426 The concentration of the 3-CCA stock solution was 5 mM. In the
427 total volume of 2 mL, the final concentrations of the ascorbate and 3-
428 CCA were both 125 μM , and the ratio of the reaction mixture was
429 ligand (peptides, EDTA or DFO):Fe(III):AscH⁻ = 1:1:2.5. The pH
430 was checked at the end of the measurement. In the case of EDTA, the
431 amount of all components was 10 times less than that of the other
432 ligand systems (in order to avoid the saturation of the signal due to
433 the high activity of EDTA). For the greater reproducibility of the
434 measured data, each measurement was started by monitoring a blank
435 sample (without any ascorbate) for 5 min, and then the ascorbate was
436 added to the sample. The reaction between 3-CCA and hydroxyl
437 radical generating the fluorescent compound 7-OH-3-CCA was
438 followed over 30 min at 25 $^{\circ}\text{C}$ at $\lambda_{\text{em}} = 450$ nm ($\lambda_{\text{ex}} = 395$ nm) by
439 spectrofluorometry. The HO[•] concentration was determined by a
440 standard calibration curve using commercially available 7-OH-3-CCA.
441

High Resolution Mass Spectroscopy Studies. For direct
442 injections (HRMS, HRMS/MS, and LC-HRMS experiments), the
443 reaction mixtures were prepared at room temperature in a total
444 volume of 1 mL at pH 7.4 in water (pH was adjusted by 0.1 M
445 NaOH) and analyzed after 24 h. Five different mixtures were studied,
446 including peptide alone, peptide + oxidant (H₂O₂), peptide + Fe(III)
447 + oxidant (H₂O₂), peptide + Fe(III) + oxidant (H₂O₂) + ascorbate,
448 and also, as another control without oxidant, peptide + Fe(III) +
449 ascorbate. Similarly to antioxidant assays, the iron(III)-complexes
450

451 were prepared 20 min before the addition of the redox agent(s)
452 (H₂O₂ and/or ascorbate), to assure a complete chelation of the metal.
453 LC-HRMS experiments were carried out using solvent A (95% water,
454 5% acetonitrile, 0.1% trifluoroacetic acid) and solvent B (100%
455 acetonitrile, 0.1% trifluoroacetic acid) with a 15 min linear gradient
456 (0.5% to 8% solvent B) followed by another 10 min linear gradient
457 (up to 50% B).

458 **Potentiometric Measurements.** The pH-potentiometric titra-
459 tions were investigated in the pH range of 2.0–11.0 at *I* = 0.1 M
460 NaNO₃ ionic force and at *T* = 298.0 ± 0.1 K. A Dosimat 715
461 (Metrohm) automatic burette and pH-meter equipped with a semi-
462 micro combined electrode (Metrohm) was used for the titration. The
463 initial concentration of peptides was 1.5 × 10⁻³ M, using 1:1, 1:2, and
464 2:1 metal-to-ligand ratios. The titrations were performed with a
465 carbonate free stock solution of sodium hydroxide at known
466 concentration. During the titration, argon was bubbled through the
467 samples to ensure the absence of oxygen and carbon dioxide. The
468 recorded pH readings were converted to hydrogen ion concentration
469 as described by Irving *et al.*⁴⁷ Protonation constants of the ligands and
470 the overall stability constants (logβ_{pqr}) of the complexes were
471 calculated from at least three independent titrations (*ca.* 70 data
472 points per titration) by means of the general computational programs,
473 PSEQUAD⁴⁸ and SUPERQUAD using eqs 1 and 2.



$$475 \quad \beta_{pqr} = \frac{[M_pL_qH_r]}{[M]^p[L]^q[H]^r} \quad (2)$$

476 ■ ASSOCIATED CONTENT

477 ■ Supporting Information

478 The Supporting Information is available free of charge at
479 <https://pubs.acs.org/doi/10.1021/acs.inorgchem.1c03085>.

480 Chemical analysis of synthesized peptides, results of
481 antioxidant activity assays, high-resolution mass spec-
482 troscopy studies, and complexation studies (PDF)

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