Carlos Bravo-Diaz
Editor

Lipid Oxidation in Food and Biological Systems

A Physical Chemistry Perspective

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Amino Acids, Amino Acid Derivatives and Peptides as Antioxidants

Luís S. Monteiro and Fátima Paiva-Martins

1 Introduction

The biological concept of antioxidant refers to any compound that is able to delay or prevent the oxidation of easily oxidizable substrates, such as DNA, proteins and lipids. Antioxidants reduce oxidative stress and mutations in DNA as well as other parameters associated with cell damage. Epidemiological studies have proven the ability of antioxidants to contain the effects of reactive oxygen species (ROS) and of reactive nitrogen species (RNS) activities and decrease the incidence of cancer, as well as other degenerative diseases (Morales-Gonzalez 2013).

The demand for natural antioxidants has gained great importance in recent years, since some synthetic antioxidants have health risks, mainly liver damage (Ndhlala et al. 2010; Bast and Haenen 2002). Phenolic compounds are the most abundant class of natural antioxidants (Shahidi and Ambigaipalan 2015). The potential of these compounds as antioxidants has long been recognized due to their great ability to break chains and eliminate radicals, protecting cells from the harmful effects of ROS. The presence and disposition of multiple hydroxyl groups in the chemical structure of polyphenols is important for their antioxidant capacity (Bast and Haenen 2002).

Phenolic amino acids or amino acids coupled with phenolic or catecholic groups are bioactive substances involved in suppressing the harmful effects caused by oxidative stress (Wei et al. 2012a; Kwak et al. 2009, 2012; Seo et al. 2010; Son

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and Lewis 2002), having biological activities such as anti-cancer (De Baltas and Bedos-Belval 2011), antimicrobial (Wei et al. 2012b; Narasimhan et al. 2004; Fu et al. 2010; Chochkova et al. 2012; Georgiev et al. 2012; Heijnen et al. 2001), anti-atherogenic (Wei et al. 2012b), among others. Studies confirm that the conjugation of amino acids with phenolic acids is useful as a strategy to improve antioxidant efficiency and bioactivity (Monteiro et al. 2019).

The mitochondria play a vital role in regulating cellular energy metabolism. Their ability to regulate the redox/oxidative balance is critical in controlling cellular life and death. Thus, mitochondrial dysfunction caused by oxidative damage has been implicated in several human pathologies such as neurodegenerative diseases and metabolic syndromes (Reddy et al. 2017; Teixeira et al. 2018). The development of new therapeutic strategies involving the minimization of mitochondrial dysfunction is of major importance. In fact, great progress has been made in the development and functional testing of mitochondria-targeted molecules. Special attention has been given to small peptides capable of regulating mitochondrial reactive oxygen species production and facilitating mitochondrial respiration and ATP synthesis (Sorriento et al. 2014; Apostolova and Victor 2015). Thus, in the last two decades, many structurally modified peptides with antioxidant properties and improved ability to cross the cell membrane while maintaining low toxicity and immunogenicity have been synthesized (Cerrato et al. 2015).

2 Antioxidant Amino Acids

2.1 Phenolic Amino Acids

2.1.1 L-Tyrosine and Its Derivatives

Natural phenols constitute a diverse class of compounds with biological interest. Phenolic compounds have been studied as antioxidants, due to their stable structure after the elimination of free radicals (Shahidi and Ambigaipalan 2015).

L-tyrosine, a phenolic proteinogenic amino acid, has an effective antioxidant activity which has been assessed by several in vitro antioxidant activity assays, such as the oxygen radical absorbance capacity assay (ORAC) and the trolox equivalent antioxidant capacity assay (TEAC) (Torkova et al. 2015).

Tyrosine residues have been shown to accumulate in the transmembrane domains of integral membrane proteins, particularly in the high lipid density region. This region is formed by the inner portion of the polar head groups and the beginning of the hydrocarbon tails. It is believed that these tyrosine residues play a vital role as antioxidants inside lipid bilayers, protecting cells against oxidative destruction (Ndhlala et al. 2010). Tyrosines acylated with long-chains are capable of inhibiting lipid peroxidation and thus reduce oxidative cell death. Low-protein neuronal membranes are observed in neurodegenerative disorders. These membranes have a high vulnerability to oxidative stress, which may be due to lack of tyrosine residues

(Moosmann and Behl 2000). This is further corroborated by the high vulnerability to oxidants of low-protein membranes and artificial lipid-only membranes. The higher resistance to oxidative stress of the inner membranes of mitochondria, which have a high protein content, when compared to membranes with low protein content such as myelin sheaths, further supports the importance of tyrosine residues as antioxidants (Moosmann and Behl 2000).

The capacity of the phenolic group to act as hydrogen radical donor inside lipidic phases and thus interfere with peroxidising free radical chain reactions, may explain the cytoprotective antioxidant effects of membrane-anchored tyrosine. Tyrosine residues, in the same way as many nonpeptide low molecular mass antioxidants, such as oestrogen, serotonin, and tocopherol are converted to nonreactive and relatively stable phenoxyl radicals. These more stable radicals have longer lifetimes than simple peroxyl radicals and can reverse reactions or inhibited propagation of the radical-mediated peroxidising chain reaction. When tyrosine is radicalized, it becomes more polar, facilitating its diffusion into zone one of the lipid bilayer where it is exposed to hydrophilic reducing molecules, such as, ascorbate or glutathione (Moosmann and Behl 2000). Thus, tyrosinyl lipids may become a new class of cytoprotective antioxidants, since the accumulation of tyrosine in transmembrane proteins protects the surrounding lipid bilayer from peroxidation.

L-3,4-diHydroxyphenylalanine (L-DOPA) is obtained from tyrosine and is the immediate precursor of the natural neurotransmitter dopamine. With the aim of alleviating the symptoms in Parkinson's disease caused by decreased dopamine levels in the brain, L-DOPA has been widely used as medication. In addition, the reduction of chronic diseases, mutagenesis and carcinogenesis have been shown to be due to the antioxidant activity of L-DOPA, which prevents hydrogen peroxide induced oxidative damage to DNA (Shi et al. 2002).

Several in vitro assays have shown that L-tyrosine and L-DOPA are effective antioxidants with activities that compare with the reference antioxidants butylated hydroxyanisol (BHA), butylated hydroxytoluene (BHT) and α-tocopherol (Gülçin 2007). Of these assays, the antilipid peroxidation test, the determination of reducing capacity, the elimination of radicals such as 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,2-diphenyl-1-picryl hydrazyl ($DPPH^{\bullet}$), superperoxide and hydrogen peroxide can be highlighted. Of these two compounds L-Dopa has shown to be much more effective than L-tyrosine. This indicates that the number of hydroxyl groups present in the aromatic moiety is fundamental in controlling radical elimination and antioxidant activity.

2,6-Dimethyl-L-tyrosine (Dmt) is a non-natural amino acid widely used in the synthesis of opioid peptides and other small molecules. Typically, opioid ligands containing Dmt instead of tyrosine at the amine-terminal exhibit a greater affinity for μ-opioid receptors (Schiller 2010). In addition, small peptides containing a Dmt residue can be absorbed by cells and help to mitigate oxidative stress (Sinha and Eudes 2015). Other studies with peptides containing this amino acid also highlight the antioxidant properties of Dmt (Cerrato et al. 2015) and will be discussed further on.

2.1.2 Hydroxyphenylglycines

Non-proteinogenic amino acids can have a variety of applications such as antiviral, antitumor, anti-inflammatory, immunosuppressor and antioxidant (Gilon et al. 2003; Kotha 2003). Non-natural α-amino acids have been used to modify the conformation and thus the activity of peptides and proteins. Non-proteinogenic phenolic amino acids play an important role in several natural peptide products such as antibiotics (Hubbard et al. 2000) and cell-penetrating peptides (CPPs) (Apostolova and Victor 2015).

The phenolic non-proteinogenic amino acid 4-hydroxyphenylglycine (HPG) is found in several natural glycopeptides, namely in some antibiotics, as is the case of vancomycin and its derivatives, in antimicrobial compounds, such as ramoplanin, and in calcium-dependent antibiotics (CDA) (Hubbard et al. 2000). The rigid structure characteristic of vancomycin, results from oxidative cross-linking of HPG with the aromatic rings of L-hydroxytyrosines and this rigidity plays an important role in the structure and function of the final molecule (Hubbard et al. 2000).

Catechols are a class of compounds endowed with a wide range of properties and important biochemical functions, which are conferred by the *ortho*-dihydroxyaryl moiety (Sedó et al. 2013), namely:

- At moderate redox potentials and pHs they are able to establish reversible equilibria;
- They cross-link irreversibly through complex oxidation mechanisms;
- They possess excellent chelating capacities;
- They interact with surfaces of different natures due to their vicinal hydroxyl groups.

Regardless of these properties, amino acids with the *ortho*-dihydroxyaryl function, such as 3,4-dihydroxyphenylglycine, have attracted reduced attention. This non-natural amino acid has been studied as copper ligand (Gordon and Jameson 1972) and as a substrate for tyrosinase, being converted to 3,4-dihydroxybenzaldehyde via spontaneous decarboxylation of the enzymatically generated *ortho*-quinone (Sugumaran et al. 1996).

The multicomponent Ugi reaction, which involves a carboxylic acid, an amine, an aldehyde and an isocyanide gives amino acids in the form of their bis-amide derivatives. 4-Hydroxyphenylglycine and 4-hydroxy-3-methoxyphenylglycine have been prepared through this reaction by using a hydroxyl substituted benzaldehyde as reactant and their effect on radical quenching and DNA oxidation determined (Wang and Liu 2013).

Recently, in addition to the above mentioned hydroxyphenylglycines, this methodology allowed the synthesis of derivatives of 3,4-dihydroxyphenylglycine (Fig. 1) (Monteiro et al. 2019). Their radical-scavenging activity was determined by the $DPPH^{\bullet}$ assay and the oxidation peak potentials by cyclic voltammetry. The results show that the 4-hydroxyphenylglycine derivative has radical scavenging activity

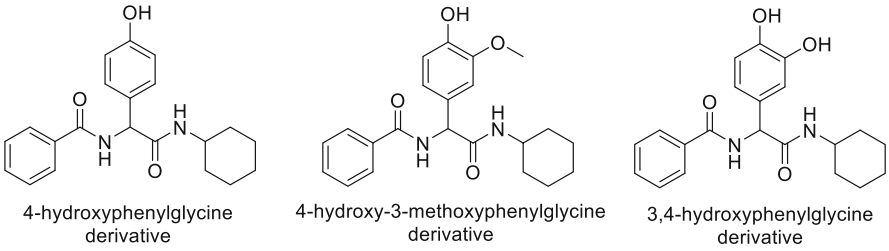


Fig. 1 Structure of hydroxyphenylglycines derivatives

(determined after 5 min) and first anodic peak potential comparable to that of tyrosine. In the case of 4-hydroxy-3-methoxyphenylglycine derivative, a significant rise in radical scavenging activity and decrease in first anodic peak potential was found. For the 3,4-dihydroxyphenylglycine derivative an approximately 100-fold rise in radical scavenging activity when compared to tyrosine was registered with a further decrease in first anodic peak potential.

2.2 Other Natural Amino Acids with Antioxidant Properties

In addition to tyrosine, other natural amino acids have antioxidant capacity (Davalos et al. 2004; Sarmadi and Ismail 2010). Amino acid antioxidant capacity has been investigated and compared with the chain-breaking antioxidant activity of known compounds such as, ascorbic acid and trolox (Meucci and Mele 1997). No radical scavenging activity has been observed for basic, acidic and most neutral amino acids. On the contrary, tryptophan, tyrosine, cysteine and homocysteine showed antiradical scavenging ability at concentrations which are within the usually reported physiological ranges. Davalos et al. established the following decreasing order in amino acid antioxidant activity: tryptophan (Trp), tyrosine (Tyr), methionine (Met), cysteine (Cys), histidine (His), phenylalanine (Phe) (Davalos et al. 2004).

The radical-scavenging properties of aromatic amino acids such as His, Trp and Phe is attributed to their proton donating capacity to electron deficient radicals (Rajapakse et al. 2005). Additionally, the imidazole ring of histidine has shown to have hydrogen donating, lipid peroxyl radical trapping and metal ion-chelating abilities (Wade and Tucker 1998; Chan et al. 1994). Cysteine contributes to antioxidant power since, due to its reducing power, the sulfydryl group can act as a radical scavenger (Patterson and Rhoades 1988).

The acidic amino acids, aspartic acid (Asp) and glutamic acid (Glu) and the basic amino acids, arginine (Arg) and lysine (Lys) can use their side chain carbonyl and amino groups as chelators of metal ions (Suetsuna et al. 2000). Although, having intrinsic antioxidant activities, these amino acids have not shown to be effective antioxidants in food and biological systems (Davalos et al. 2004). However, despite having little or no antioxidant effect as free amino acids, they can exhibit high

antioxidant activity in peptides and this will be addressed later (Nagasawa et al. 2001; Kawashima et al. 1979).

2.3 α,β-Dehydroamino Acids

Non-proteinogenic amino acids containing a double bond between the α and the β carbons are designated as α,β-dehydroamino acids (Δaa) and have several important biological activities (Siodlak 2015). They are also intermediates in the synthesis of new amino acids and peptides (Siodlak 2015; Bierbaum et al. 1996; Dawid 2015; Gupta and Chauhan 2011). They appear in some bacteria, or yeasts and are constituents of many natural antibiotics (Jiang et al. 2015). They may also play a vital role in the active centre of some enzymes (Jain and Chauhan 1996). The most common α,β-dehydroamino acids are dehydroalanine (ΔAla), dehydroaminobutyric acid (ΔAbu), dehydrophenylalanine (ΔPhe), dehydrovaline (ΔVal) and dehydroleucine (ΔLeu) (Fig. 2) (Jiang et al. 2015).

Among the activities of dehydroamino acid are antioxidant properties. When reacting with oxygen or hydroxyl radicals, these compounds form stabilized free radical adducts and thus function as radical scavengers. In order to establish structure-antioxidant activity relationships that can lead to new analogues with increased activities, Suzen et al. prepared the amides of several *N*-acetyl dehydroalanine derivatives (Fig. 3) (Suzen et al. 2006). Their free radical scavenging activity against the DPPH radical and their antioxidant properties against rat liver lipid peroxidation were evaluated (Suzen et al. 2006). Little or no effect on DPPH• was detected, however a strong inhibitory effect on rat liver lipid peroxidation was

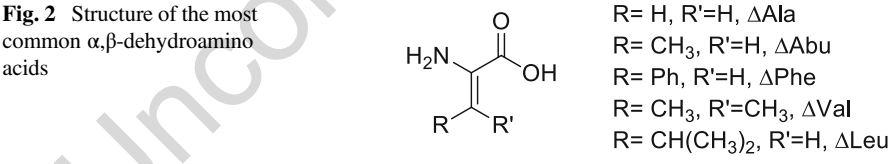


Fig. 2 Structure of the most common α,β-dehydroamino acids

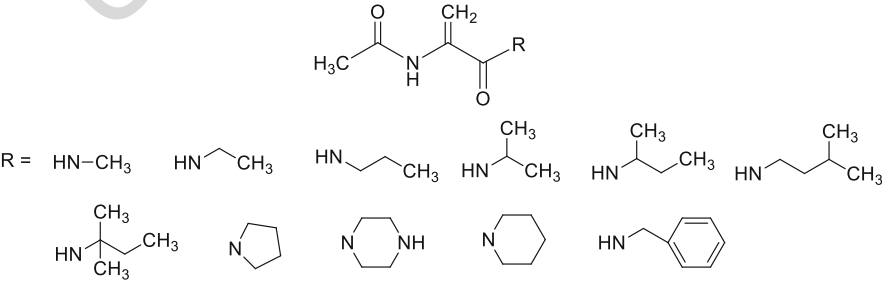


Fig. 3 Structure of *N*-acetyl dehydroalanine amides

observed. The highest effect was found for the amides of *N*-acetyl dehydroalanine with aliphatic chains of 3 or 4 carbons and cyclic 5 member rings.

Several methyl esters of dehydroalanine with various *N*-substituting groups were subject to a similar study using both DPPH$^{\bullet}$ and superoxide ($O_2^{\bullet-}$) radical scavenging activity assays (Ferreira et al. 2009). Again, no significant activity was observed. However, all the compounds were effective in lipid peroxidation experiments. These results led to the conclusion that these dehydroalanine derivatives are not able to scavenge the DPPH or superoxide radical but can scavenge the hydroxyl radical. In fact, for many compounds no radical scavenging activity for several radicals used to evaluate radical scavenging activity, such as DPPH$^{\bullet}$ or ABTS is observed. However, during oxidative injury, highly reactive radicals in vivo can be formed, and can be neutralized by these compounds and therefore protect cells (Senoner and Dichtl 2019). This is the case of tyrosol (Fernandes et al. 2020). Most ROS exert their pathological effects by giving rise to the hydroxyl radical or closely related species, the final mediators of most free radical induced tissue damage. This is due to the hydroxyl radical being capable of reacting, with extremely high rate constants, with almost every type of molecule found in living cells, such as lipids and nucleotides. Hydroxyl radical formation can occur in several ways, however, the transition metal catalysed decomposition of superoxide anion and hydrogen peroxide is likely to be most important mechanism in vivo (El Haouari 2019).

3 Amino Acids Coupled with Phenols

Several studies indicate that, due to the synergic effects between different types of molecules, a mixture of antioxidants, with different molecular structures and mechanisms of action, result more effective than a single antioxidant (Milde et al. 2004, 2007; Shi and Kakuda 2006; Gonzalez-Perez and Gonzalez-Castaneda 2006; Yogeeta et al. 2006; Trombino et al. 2004; Cirico and Omaye 2006). In order to better understand mechanistic aspects and possible synergic actions, the design of modified and/or dualistic molecules is an important approach. Coupling of compounds with different functionalities, such as, amino acids and phenolic acids is a strategy that can lead to improved antioxidant efficiency and bioactivity and also allow the establishment of structure-activity relationships (Silvia et al. 2012).

Fruits, vegetables and beverages are natural sources of phenolic acids coupled with amines or amino acids but they can also be obtained synthetically. The deleterious effects of oxidative stress (Kwak et al. 2009, 2012; Seo et al. 2010; Son and Lewis 2002) can be suppressed by these bioactive substances which also have a broad scope of other biological activities, such as, anticancer (De Baltas and Bedos-Belval 2011) and antimicrobial (Wei et al. 2012b; Narasimhan et al. 2004; Fu et al. 2010; Chochkova et al. 2012; Georgiev et al. 2012; Heijnen et al. 2001). For example, accumulation of hydroxycinnamic acid amides in plants occurs in a response to deleterious environmental stimuli such as wounding, fungal infection

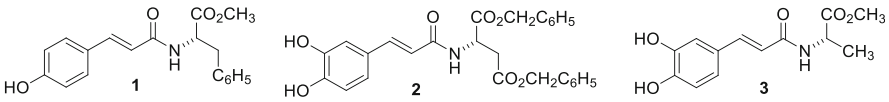


Fig. 4 Structure of *N*-(hydroxycinnamoyl) amino acid derivatives

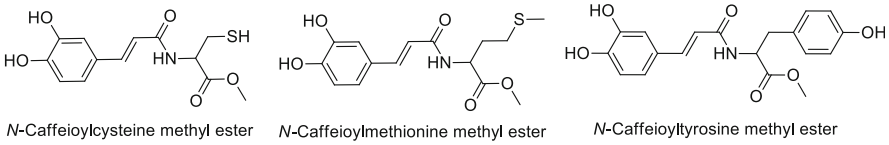


Fig. 5 Chemical structure of phenolic acid-amino acid conjugates with highest antioxidant activity

or heavy metal ions (Negrel et al. 1993; Peipp et al. 1997; Fink et al. 1990; Negrel et al. 1995).

Synthesis of a series of hydroxycinnamic amino acid derivatives and evaluation of their biological activities in lipoprotein metabolism was carried out by Lee et al. (2004) (Fig. 4).

These authors found that compounds **1** and **2** inhibited human acyl-CoA: cholesterol acyltransferase (ACAT) activities. These compounds also acted as antioxidants against copper induced low-density lipoprotein (LDL) oxidation. Compound **3** presented a potent in vivo activity. In hypercholesterolemic rabbits, this compound showed an important reduction in the formation of atherosclerotic type lesions, with an improvement in the serum lipid profile.

Wei et al. (2012a) also synthesized a series of *N*-hydroxycinnamoyl amino acid esters with the aim of finding more active antioxidants with these moieties. DPPH radical scavenging and human red blood cells haemolysis methods evaluated their antioxidative activities. It was found that:

- *N*-hydroxycinnamoyl amino acid derivatives exhibited stronger antioxidative activity than the free acids or their esters.
- Of the three hydroxycinnamamides studied (caffeoylamides, feruloylamides, and *p*-coumaroylamides), *N*-caffeoyl amino acid derivatives exhibited the highest DPPH radical scavenging activities, whereas *N*-feruloylamides had the highest antihaemolysis activities.

Silvia et al. proposed the synthesis of a series of natural amino acid derivatives with possibly enhanced antioxidant activities (Silvia et al. 2012). These authors prepared several combinations of amino acids with phenolic acids and some of these conjugates were additionally coupled with dopamine. With the conjugates it was possible to investigate the effect on the antioxidant activity of the different phenolic moieties (in particular caffeic acid and 3,4-dihydroxyphenylacetic acid). The results obtained indicate that, in order to observe significant antioxidant activity, the phenolic acid must have at least two hydroxyl groups and a conjugated spacer between the aromatic ring and the amide (Fig. 5).

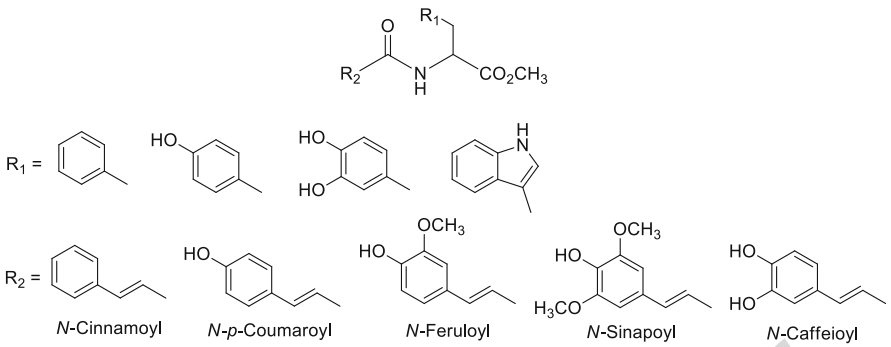


Fig. 6 Methyl esters of *N*-cinnamoylamino acids

Coupling of dopamine with the phenolic acid-amino acid conjugates did not improve significantly the antioxidant activity. This result suggests that, not only is the shape and complexity of the molecule important for its antioxidant activity, but that, also, this approach is limited by a "saturation effect" that limits the maximum potency achievable. However, the results obtained indicate that, combinations of molecules with different moieties increase the antioxidant efficiency of natural antioxidants, having the authors referred to this methodology as the "Centaurus tactic" (Silvia et al. 2012).

Georgiev et al. prepared a library of *N*-cinnamoylamino acids by combining cinnamic, *p*-coumaric, ferulic, sinapic and caffeic acids with the esters of phenylalanine, tyrosine, DOPA and tryptophan (Fig. 6) (Georgiev et al. 2013).

In vitro studies of the antioxidant activity of these compounds was carried out by two antioxidant assay systems, the DPPH$^{\bullet}$ assay and inhibition of lipid peroxidation (LPO) (Georgiev et al. 2013). From the results obtained, the authors could conclude that:

- The most active compounds contained the catechol moiety, whereas the presence of a methoxy groups decreased the activity;
- The conjugation of the catechol type amino acid DOPA to the less active cinnamic and *p*-coumaric acids leads to an increase in their radical scavenging activity;
- The radical scavenging activities of all compounds was dose-dependent and correlated positively with the concentrations, except for the *N*-caffeoyl-DOPA-OMe. This correlates with the saturation effect described above (Silvia et al. 2012).
- The radical scavenging activity of the aromatic amino acids (phenylalanine and tryptophan) showed that even for compounds with the weaker sinapoyl moiety, a positive influence of the phenyl and the indole moiety.

Using an innovative strategy, Monteiro et al. prepared *N*-phenolic and *N*-catecholic dehydroalanine and dehydrophenylalanine derivatives in order to study

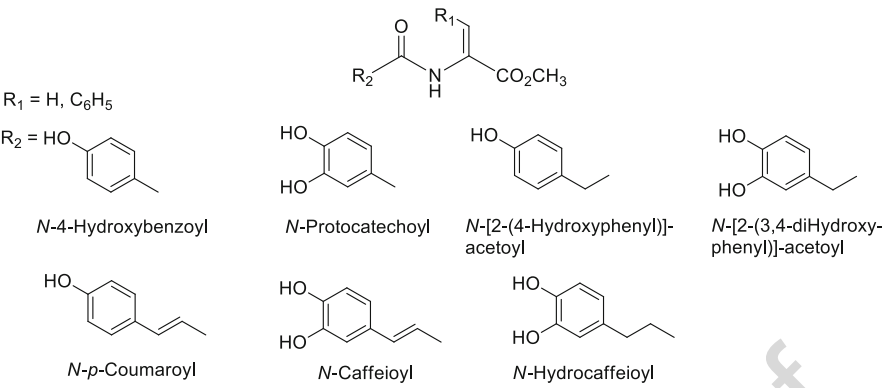


Fig. 7 Methyl esters of *N*-phenoyl and *N*-catechoyl dehydroalanines and dehydrophenylalanines

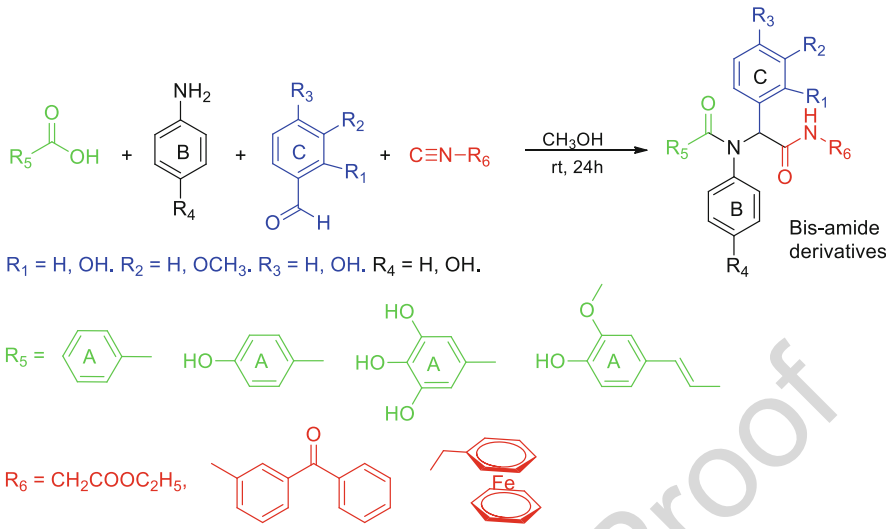
the effect of conjugation of dehydroamino acids with phenolic or catecholic acids (Fig. 7) (Monteiro et al. 2017).

The toxicity of the dehydroalanine and dehydrophenylalanine derivatives was evaluated using cancerous and non-cancerous cell cultures. These tests showed higher toxicity in relation to cancer cells than to non-cancer cells, being the most potent molecules the dehydrophenylalanine derivatives. This can be attributed to their greater liposolubility (Monteiro et al. 2017).

Phenolic conjugates of hydroxyphenylglycines could be obtained using the Ugi reaction in a similar fashion as described before to obtain hydroxyphenylglycines, but with the additional use of hydroxyl substituted benzoic acids. The effect of these hydroxylated bis-amides on the scavenging of radicals and in the protection of DNA oxidation was determined (Scheme 1) (Wang and Liu 2013). This allowed the study of the effects on antioxidant activity of hydroxyl groups attached to the different aromatic rings, as well as the influence of the isocyanide component, on the radical scavenging activity and protective effect of bis-amides.

The results showed a marked influenced of the structural feature derived from the isocyanide used in the Ugi reaction on the antioxidant effectiveness of the hydroxylated phenylglycines (Wang and Liu 2013). It was actually found that the isocyanide moiety at one end of the molecule strongly influences the antioxidant properties of the phenolic hydroxyl groups at the other end of the molecule. The most effective was the ferrocenylmethyl group, which enhances both radical scavenging activity and DNA oxidation inhibition of the bis-amides. Additionally, comparison of a series of bis-amide derivatives with this ferrocenylmethyl group indicate that the hydroxyl groups at phenyl ring C play the major role in inhibiting DNA oxidation, followed by the hydroxyl groups attached to aromatic rings B and A.

New therapeutic strategies based on multitarget-directed ligands have been proposed as a possible approach for the treatment of Alzheimer's disease. The goal is to bind simultaneously at diverse enzymatic systems or receptors involved in the progress and development of the disease. Lambruschini et al. (2017) using the Ugi



Scheme 1 Synthesis of hydroxylated bis-amides obtained by Ugi condensation

reaction, were able to prepare a series of complex polyphenols containing two to four hydroxy-substituted aryl groups linked to the main structure through linkers of different lengths. Some compounds showed highly promising capacity to inhibit aggregation of two β-amyloid peptides and potentially can be used as prevention or therapy for Alzheimer's disease.

Novel multifunctional tacrine derivatives (tacrine is the first FDA-approved drug for the treatment of Alzheimer's disease) have been obtained by the Ugi-reaction and proposed as a therapeutic strategy to modulate oxidative stress in Alzheimer's (Benchekroun et al. 2016). To inforce the antioxidant additive approach, reaction between ferulic or lipoic acid, a melatonin-like isocyanide, formaldehyde, and tacrine derivatives, gave glycine bis-amides. Thus, these glycine bis-amides contained two antioxidant motifs (the ferroloyl or lipoyl, and the melatonin fragments). Biological evaluation of these ferulic (or lipoic) acid-tacrine-melatonin glycine derivatives as antioxidants, and neuroprotective agents for the potential treatment of Alzheimer's disease were carried out. From these studies, a ferulic acid-tacrine-melatonin glycine derivative was identified as a potent cholinesterase inhibitor and a strong antioxidant, being likely to penetrate the blood brain barrier. Furthermore, this compound showed the best neuroprotective profile (Fig. 8) (Benchekroun et al. 2016).

Using the Ugi reaction to combine hydroxybenzaldehydes and phenolic or cathecolic acids with 4-methoxybenzylamine and cyclohexyl isocyanide, Monteiro et al. prepared a series of *N*-phenoyl and *N*-catechoyl hydroxyphenylglycine amides (Scheme 2) (Monteiro et al. 2019).

Radical-scavenging activities and anodic oxidation peak potentials of the compounds prepared were determined. The radical-scavenging activity studies showed a

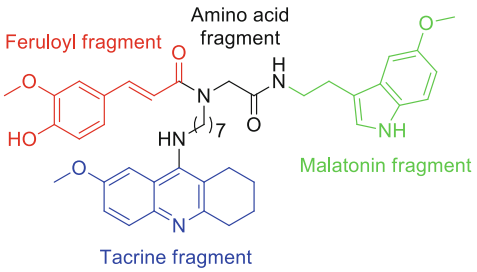
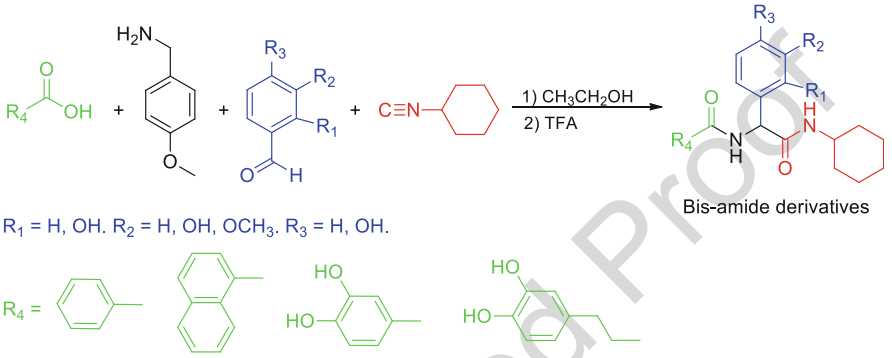


Fig. 8 Structure of the most active ferulic acid-tacrine-melatonin glycine derivative



Scheme 2 Synthesis of hydroxylated bis-amides obtained by Ugi condensation

sharp increase in activity with the increase in number of hydroxyl or catechol groups present. On the other hand, a high correlation between the oxidation peak potentials determined cyclic voltammetry and radical-scavenging activity could be established (Monteiro et al. 2019).

4 Antioxidant Peptides

Antioxidant peptides are gradually being accepted as food ingredients that positively regulate oxidative stress in the human body against lipid and protein oxidation (Liu et al. 2016). The commercial use of antioxidant peptides covers several areas, such as, functional foods, nutraceuticals and cosmeceuticals (Liu et al. 2016).

In recent years, characterization of antioxidant peptides from several food sources, such as, meat (Liu et al. 2016), fish (Sila and Bougatef 2016), cereals (Esfandi et al. 2019), seeds (Ye et al. 2018; Yang et al. 2018), among others, has been conducted in a growing number of studies. Both in vitro and in vivo assay methods have been used for assessing these antioxidant properties of peptides (Liu et al. 2016). There is solid evidence that high antioxidant activities of purified peptides can be determined in in vitro assays, however, degradation and modification by the intestine, vascular system and liver, challenge whether these function in

the human body. Thus, to confirm the bioavailability, in vivo assays, such as, animal studies and clinical trials must be carried out.

The determination of DPPH radical scavenging activity, hydroxyl radical scavenging, ferric-reducing antioxidant power, superoxide ion scavenging activity and linoleic acid peroxidation inhibition activity are among the in vitro methods used (Liu et al. 2016; Sila and Bougatef 2016). In vivo tests have included ethanol-induced cardiotoxicity in rats (Kamoun et al. 2012) and the study of the protective effects of peptides on mice skin subject to photoageing induced by UV irradiation (Sun et al. 2013).

Protein hydrolysates (peptides) are more potent antioxidants than free amino acids, which results from their chemical composition and physical properties (Liu et al. 2016; Elias et al. 2008). Even some free amino acids which have little or no effect individually can exhibit high antioxidant capacity in peptides (Nagasawa et al. 2001; Kawashima et al. 1979). Thus, the amino acid composition and sequence play important roles in determining the antioxidant activity of peptides. However, the relationship between structural characteristics of peptides, such as molecular size, amino acid composition and sequence, hydrophobicity and their activities as antioxidants is still unclear (Liu et al. 2016).

Several studies have shown that crude peptides of smaller molecular weights tend to have higher antioxidant activity. Peptide chains of 4–16 amino acids, corresponding to molecular weights of 0.5–2 kDa correspond to maximum activity (Meisel and FitzGerald 2003; Tang et al. 2009; Bougatef et al. 2012). Lower molecular weight peptides in the 1–3 kDa range were reported by Samaranayaka and Li-Chan (2011) to interact more effectively with radicals leading to termination of lipid peroxidation propagation cycles. The molecular weight ideal for antioxidant activities was further lowered by Chi et al. (2015) which studied smaller molecular size peptides with hydrophobic and/or aromatic amino acids in their sequences. The highest scavenging activities on $DPPH^{\bullet}$, $HO^{\bullet}$ and $O_2^{-\bullet}$. was found for a peptide with molecular weight 432.52 Da. Molecular weight also affects the antioxidant capacity in vivo since it influences the routes by which bioactive peptides are transferred into target sites (Rubas and Grass 1991). Peptides with 2–6 amino acids, when compared with proteins or single amino acids, can be more easily absorbed through the gastrointestinal barrier and enter peripheral blood, with an increase in bioavailability at tissue level (Roberts et al. 1999; Grimble 1972).

Histidine-containing peptides have also been reported to have antioxidative activity (Murase et al. 1993). This activity can be attributed to the, previously stated, hydrogen donating ability of the side chain imidazole ring, and also to metal ion-chelating and lipid peroxyl radical trapping properties (Chan et al. 1994). Peptides containing His in the N-terminus show higher scavenging ability on $DPPH^{\bullet}$, $OH^{\bullet}$ and superoxide suggesting that this feature contributes to their high antioxidant capacity (Liu et al. 2010; Lee et al. 2012). On the other hand, Yamaguchi et al. (1975) reported stronger antioxidant activity for dipeptides consisting of Tyr and Trp at the amino terminus, and His and Met at the carboxyl terminus.

Several studies of structure and activity of antioxidant peptides have reported that peptides with hydrophobic amino acids, Val, Leu or Ile and having Ala, Tyr, His, Pro

or Met at the N-terminal have potent inhibitory activity (Liu et al. 2016; Chen et al. 1998).

In conclusion, the structure-antioxidant activity relationship of peptides has not been completely established and structural information of antioxidant peptides from various protein sources is still lacking. On the other hand, progress in testing the bioavailability and thus, the application in the consumer markets of antioxidant peptides has been limited due to limited animal model experiments and human clinical trials. Further research has to be conducted in vivo to establish the potential antioxidant effects of peptides.

5 Antioxidants Targeting the Mitochondria

The mitochondria is a cytoplasmic organelle that regulates cell metabolism (Nelson and Cox 2017). This organelle is involved in oxidative energy metabolism through the catabolism of nutrients, ATP synthesis by oxidative phosphorylation and heat production. Structurally, the mitochondria consists of two different components: the inner mitochondrial matrix and the intermembrane space, with an internal mitochondrial membrane that separates them. The intermembrane space is limited by the outer mitochondrial membrane, which plays the role of signal transport and transduction between the organelle and the cytosol.

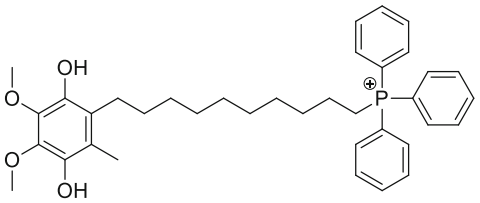
Mitochondria are an important source of reactive oxygen species and reactive nitrogen species (Murphy 2009). Initial studies showed that the respiratory chain produces ROS. Subsequently it was determined that isolated mitochondria produce hydrogen peroxide and that this hydrogen peroxide comes from the dismutation of superperoxide generated in the mitochondria (Galley 2011).

Mitochondria have natural processes to eliminate ROS in order to keep the cell active. This network of antioxidant defence systems, consisting of a combination of enzyme and non-enzyme pathways is tightly regulated. Under normal conditions, the effect of ROS is cancelled out by antioxidants. When this balance is broken and the effect of ROS is more potent than antioxidant power, damage occurs in the mitochondria. This unbalance has been linked to several degenerative diseases, such as Alzheimer's, Parkinson's, neural death, cancer and cardiovascular diseases (Sorriento et al. 2014; Szeto 2006; Hoye et al. 2008; Anders et al. 2006; Victor and Rocha 2007).

5.1 Non-peptidic Mitochondrial Antioxidants

Several strategies to direct antioxidants to the mitochondria have been reported (Anders et al. 2006; Victor and Rocha 2007). One such strategy is covalently linking antioxidant molecules to lipid cations that accumulate in the mitochondria as a result of the potential of the mitochondrial membrane. MitoQ (Fig. 9) consists of binding

Fig. 9 Structure of MitoQ



the ubiquinone antioxidant, also known as coenzyme 10, to the lipophilic triphenylphosphonium cation (TPP) (Galley 2011; Murphy 2008).

The negative charge present within the mitochondrial membrane results in the accumulation of MitoQ within the mitochondria in about 500 times the levels present in the cytoplasm (Galley 2011; Murphy 2008). The side chain of MitoQ allows it to penetrate deeply into the membrane. Thus, as soon as MitoQ enters the mitochondria, it is absorbed by the inner membrane of the mitochondria and is recycled as active uniquinol through the respiratory chain.

Tests in isolated mitochondria have verified the effectiveness of MitoQ against lipid peroxidation. Animal experiments indicate that MitoQ is efficient against sepsis-induced organ dysfunction, opening perspectives for the study of this substance in human diseases (Galley 2011).

Other antioxidants have been conjugated with TPP and tested against mitocondrial dysfunctions, such as, tocopherol (MitoVitE) (Minter et al. 2020) and lipoic acid (MitoLipoic acid) (Smith et al. 2008).

5.2 Cell Penetrating Peptides

The plasma membrane is a barrier that protects the cell from the unregulated influx of bioactive molecules and ions, thus controlling the stability of its internal environment (Nelson and Cox 2017). Most drugs need to cross one or more cell membranes to reach their targets and thus have a therapeutic effect. Small molecules are able to penetrate the membrane, however larger molecules, due to their physical-chemical properties, are unable to do so (Cerrato et al. 2015). The ability to transport macromolecules into cells is important both in drug administration and in biotechnological applications (therapy genetics) (Rodriguez-Plaza et al. 2014).

In the last decades, new biotherapeutic agents, such as peptides and proteins with cell-penetrating capability have contributed to the treatment of several diseases and have been designated as cell penetrating peptides (CPPs). These peptides have less than 10 amino acids and contain chemical features that permit these molecules to freely penetrate by passive diffusion into cells membranes (Zhao et al. 2004). CPPs have opened a new pathway for delivering a wide variety of bioactive compounds across the cell membrane—from proteins to therapeutic molecules—due to their high efficiency in the internalization of CPPs and low cytotoxicity (Derakhshankhah and Jafari 2018; Khafagy and Morishita 2012).

Although several studies on CPPs have been carried out, the mechanism by which they penetrate the cell is still not completely understood (Nasrollahi et al. 2012). It is suggested that their entry into the cell can be influenced by several factors, such as, length of the molecule, delocalization of charge, hydrophobicity and concentration (Jones et al. 2005). When linked to bioactive cargos the nature of this cargo (size and charge) also influences their internalization (Fonseca et al. 2009).

Two mechanisms have been proposed for the entry of CPPs into the cell: direct translocation across the cell membrane and through the endocytic route (Ram et al. 2008).

There are two types of CPPs that have been used to cross the cell membrane: (1) cationic CPPs, consisting of short chains with various amino acid residues such as arginine, lysine and histidine that provide positive charge to the peptides and allow their interaction with anionic structures, present in the plasma membrane; (2) amphipathic peptides, which contain lipophilic and hydrophilic tails responsible for a direct translocation mechanism of peptides across the cell membrane (Aroui and Kenani 2020).

CPPs have been used to deliver numerous classes of molecules, including DNA, proteins, drugs and nanoparticles, many of which are sometimes larger than the CPP itself (Langel 2019; Wagstaff and Jans 2006). However, it has not yet been determined whether the coupling of a CPP to a molecule of considerable size interferes with the translocation mechanism (Magzoub and Gräslund 2004).

The conjugation of bioactive charges with a CPP can occur in two ways: (1) covalent conjugation, where the bioactive molecules bind to the CPP through a covalent bond; and (2) through an electrostatic interaction, in which aggregates or nanoparticles are formed (Derakhshankhah and Jafari 2018; Foged and Nielsen 2008). These different conjugation processes can impact the route of administration, the mechanism of entry into the cell, the distribution within the cell and other different effects at the cellular level. Furthermore, based on the therapeutic use and the nature of the target in which the drug acts, the choice of the form of conjugation plays a very important role (Feni and Neundorf 2017).

5.3 Mitochondrial Penetrating Peptides

As stated before, mitochondria are an important target in the treatment of various diseases, due to their role in energy production and cell death (Sorriento et al. 2014; Hoye et al. 2008; Anders et al. 2006; Victor and Rocha 2007). Mitochondrial penetrating peptides (MPPs) represent a new direction for the development of vectors targeting the mitochondria. They are short peptides, with high uptake by mitochondria. MPPs are generally small synthetic, positively charged, basic, hydrophobic peptides of less than 10 amino acids that freely penetrate by passive diffusion into cells and are taken up into the mitochondria and accumulate in the matrix (Galley 2011). Additionally, MPPs exhibit low cytotoxicity (Zhao et al. 2019).

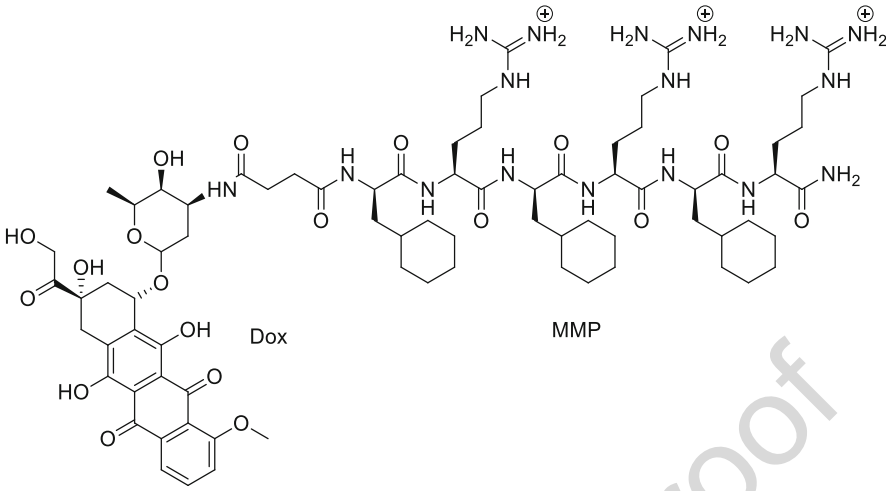


Fig. 10 Structure of the complex doxorubicin (Dox)-MPP

MPPs have been used as vehicles for the transport of bioactive molecules into the mitochondria.

With the aim of studying if the interference of nucleic acid synthesis in mitochondria would have significant cellular effects, Chamberlain et al. (2013) complexed doxorubicin (Dox) with a mitochondrial penetrating peptide (Fig. 10).

Doxorubicin is an anti-cancer drug, which inhibits the DNA topoisomerase II enzyme present in both the cell nucleus and the mitochondria. Although the potency of the Dox-MPP complex in the mitochondria decreased somewhat in sensitive cells, when compared to doxorubicin, the complex demonstrated an ability to overcome the mechanisms of resistance to multiple drugs (Chamberlain et al. 2013).

The existence of excess iron is a concern in a number of clinical conditions, as excess iron is involved in the production of pro-oxidant species by reaction with oxygen and nitrogen substrates giving rise to oxidative stress (Halliwell and Gutteridge 2015). One such disease is Friedreich's ataxia where excess iron is found in the mitochondria (Alta et al. 2017). Excess iron must be removed through the use of natural or synthetic iron chelators, with deferoxamine (DFO) being the most clinically used chelator (Kwiatkowski 2011).

Alta et al. (2017) synthesized four MPP-DFO conjugates using succinic acid as a binder, in order to have a strong iron chelator and be permeable to mitochondria. The four MPP-DFO conjugates were studied for their chelating and antioxidant properties. Results showed that the iron binding and affinity of the four conjugates are identical to free DFO and that they effectively suppress iron-catalysed oxidation (Alta et al. 2017). MPP-DFO conjugates were labelled to demonstrate mitochondrial localization in cells and all conjugates entered the mitochondria. In addition, MPP-DFO exhibited low levels of toxicity, cell cycle disruption, mitochondrial DNA damage and apoptosis (Alta et al. 2017).

The advance of research in the area of mitochondrial targeting and some positive results in clinical trials, make it necessary to continue studying the targeted delivery of different molecules to the mitochondria, in order to develop useful tools for therapy and research (Galley 2011).

5.4 Antioxidant Mitochondrial Penetrating Peptides

An alternative approach to targeting antioxidants to the mitochondria is the use of small positively charged peptides with antioxidant properties capable of protecting the mitochondria from oxidative stress. Peptides of this type were developed by Szeto and Schiller having acquired the designation of SS peptides (Szeto 2006, 2008; Rocha et al. 2010; Smith and Murphy 2011).

SS peptides are synthetic tetrapeptides originally developed as opioid analgesics with alternating aromatic residues and basic amino acids (Cerrato et al. 2015; Anders et al. 2006). Of these compounds the peptide SS-02 (Dmt-D-Arg-Phe-Lys-NH_2) and SS-31 (D-Arg-Dmt-Lys-Phe-NH_2) have been prepared and studied in cell cultures and isolated mitochondria (Szeto 2008).

Despite these peptides being water soluble, with a net charge of +3, they are readily taken up by all cells via passive diffusion (Szeto 2008). Both SS-02 and SS-31 have a 2,6-dimethyl-L-tyrosine residue (Dmt) but in a different position in the peptide chain. The presence of a D-amino acid renders them resistant to aminopeptidase activity.

These SS peptides may be viewed as "cloaked" or "stealth" as they can evade cellular membranes, even penetrating cell barriers with tight junctions including the blood-brain barrier (Sedó et al. 2013). Absorption studies with SS-02 show rapid absorption (<30 min) producing a beneficial effect, with a greater concentration in the mitochondria than in the cytosol. These results and the membrane's permeability to these peptides, suggest that they can freely pass the membrane in both directions (Cerrato et al. 2015; Rocha et al. 2010). The mechanism behind their cell permeability is unclear, but the aromatic rings may serve as electron cages to shield the cationic charges via cation-π interaction.

Studies with cell cultures and isolated mitochondria have proven that these peptides are capable of eliminating mitochondrial ROS production, reduce their production and inhibiting the mitochondrial permeability transition, and are therefore potent in preventing apoptosis induced by oxidative stress. These peptides have shown excellent efficacy in animal models of neurodegeneration and renal fibrosis, with no toxicity (Szeto 2006, 2008).

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