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[d] Abbreviations: AGEs, advanced glycation end products; HNE, 4-hydroxy-*trans*-2,3-nonenal; RCS, reactive carbonyl species; ROS, reactive oxygen species; RNS, reactive nitrogen species; LDL, low density lipoprotein; HBTU, O-Benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; DIPEA, di-isopropylethylamina; DMF, dimethylformamide; DCC, dicyclohexylcarbodiimide; TBARS, thiobarbituric acid reactive substances. hPepT1, human PepT1 transporter.

Abstract. The synthesis and the physico-chemical and biological characterisation of a series of carnosine amides bearing on the amido group alkyl substituents endowed with different lipophilicity are described. All synthesised products display carnosine-like properties differentiating from the lead for their high serum stability. They are able to complex Cu^{2+} ions at physiological pH with the same stoichiometry as carnosine. The newly synthesised compounds display highly significant copper ion sequestering ability and are capable of protecting LDL from oxidation catalysed by Cu^{2+} ions, the most active compounds being the most hydrophilic ones. All the synthesised amides show quite potent carnosine-like HNE quenching activity; in particular, **7d**, the member of the series selected for this kind of study, is able to cross the blood-brain barrier (BBB) and to protect primary mouse hippocampal neurons against HNE-induced death. These products can be considered metabolically stable analogues of carnosine and are worthy of additional investigation as potential neuroprotective agents.

KEYWORDS: carnosine, antioxidants, copper complexes, HNE-scavenging.

Introduction

Carnosine, β-alanyl-L-histidine (**1**) (chart 1) is a naturally occurring dipeptide that in humans is preferentially localized in skeletal muscles and brain.[1] Carnosine can display a variety of physiological roles including that of a cytosolic buffering agent, that of a regulator of content of transition metal ions in biological fluids and tissues, owing to its ability to form complexes with these ions, and that of a regulator of macrophage function.[2-4] It is a potent scavenger of both reactive oxygen species (ROS) and reactive nitrogen species (RNS) which induce peroxidation of unsaturated lipids present in membranes as well as of toxic reactive α,β-unsaturated aldehydes deriving from this oxidation.[1,5-8] Acrolein, 4-hydroxy-*trans*-2,3-nonenal (HNE), and malondialdehyde are examples of such products. They are potent bifunctional electrophiles able to bind nucleophilic centers present in both DNA and proteins. In addition, the second electrophilic center may undergo additional attack affording protein-protein and DNA-protein cross-linking. The final result is an amplification of the cellular damage induced by oxidative attack on membranes. Another source of cellular damage is the glycation of proteins, namely, the process whereby reducing sugars react with protein amino groups generating Schiff's bases. These latter products, in turn, can afford advanced glycation end products (AGEs) able to incorporate additional proteins through stable crosslinks.[9] AGEs are toxic for those cells that are able to recognize them. Carnosine is capable of protecting proteins against glycation and of reacting with protein carbonyl groups to form carnosylated proteins.[10-12] Since ROS, RNS, lipidic oxidation and glycation products may contribute to the development of many neurodegenerative and cardiovascular diseases, today there is a great interest in carnosine and related structures as potential therapeutic tools.

It is known that carnosine is rapidly cleaved to its constituents, histidine and β-alanine, by the carnosinases, which are a group of ubiquitous dipeptidases, belonging to the family of metalloproteases. So far, two principal isoforms of carnosinase are known, one is a cytosolic form (EC 3.4.13.3), the other (EC 3.4.13.20) is known as serum carnosinase since it is mostly present in the plasma and brain.[13,14] The facility of enzymatic hydrolysis of carnosine and of many related

compounds limits their therapeutic potential. The nature of the metal ion in serum carnosinase is not known with precision, but it is likely that two Zn^{2+} ions are present in the catalytic site. A recent computational study shows that one of these two ions is important in recognizing the carboxylic group of carnosine.[15] If this interaction is lost, it is reasonable to think that the affinity for the enzyme is strongly reduced. By contrast, the carboxylic group of carnosine should be less involved in recognition of the binding site of hPepT1, which is the main intestinal transporter involved in the absorption of both dietary peptides and peptidomimetics.[16]

On these bases we designed a series of carnosine carbonamide derivatives bearing on the amide nitrogen alkyl or aryl groups characterized by different lipophilicity (compounds **7a-n**). This paper reports synthesis, dissociation constants and lipophilicity determination, the ability to complex Cu^{2+} ions and the stability in human serum of these products. Their capacity to inhibit human LDL autoxidation induced by Cu^{2+} and to sequester HNE, chosen as a model of reactive carbonyl species (RCS), is also considered. Finally, the ability to cross the BBB in an in vivo rat model and the cytoprotective efficacy on primary mouse hippocampal neurons is shown in the case of **7d**, one of the most promising members of the series.

Results and Discussion

Chemistry. The synthetic pathway used to prepare compounds **7a-n** is reported in Scheme 1. The commercially available N^{α}-Fluorenylmethoxycarbonyl-N-trityl-L-histidine (Fmoc-His(Trt)-OH) (**2**) was coupled with the appropriate amines **3b-n** using the O-Benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU)/1-hydroxybenzotriazole (HOBt)/di-isopropylethylamina (DIPEA) protocol to obtain the corresponding intermediate amides **4b-n**. Fmoc deprotection with piperidine in DMF was achieved at room temperature (r.t.) to afford the expected amines **5b-n**. The simple amido derivative **5a** was obtained directly by action of ammonia on **2**. Free amines so obtained were coupled with *tert*-butoxycarbonyl protected β-alanine (Boc-β-Ala(OH)) using dicyclohexylcarbodiimide (DCC) as the coupling agent and then deprotected using 10% (v/v) CF_3COOH in CH_2Cl_2 at RT to afford the expected di-trifluoroacetates **7a-n**. In Scheme 2

the synthetic pathway followed for the preparation of the ether **12** that was used for a comparison in the Cu^{2+} complexing studies is reported. Di-trityl protected alcohol **8**[17] underwent Williamson etherification with ethylbromide in the presence of an excess of NaH in dry DMF giving intermediate **9**. Selective detritylation of this product carried out using 1% (v/v) CF_3COOH in CH_2Cl_2 at 0 °C afforded the expected free amine **10**. This intermediate was coupled with Boc-β-Ala(OH), by the usual DDC-mediated procedure, obtaining **11**. Removal of the protecting groups was achieved with 10% (v/v) CF_3COOH in CH_2Cl_2 to afford the target compound **12**.

Dissociation constants (p*K*$_a$s) and lipophilicity. Dissociation constants (p*K*$_a$s) of the new carnosine carbonamides and of ether derivative **12** were determined by potentiometric titration in aqueous solution using a GLpKa apparatus. Their values are collected in Table 1, with those of carnosine measured in the same conditions. For solubility reasons, in the case of compounds **7h** and **7i**, the measurements were carried out using methanol as a co-solvent and the results were extrapolated to zero co-solvent. As expected, p*K*$_a$ values of imidazole rings lie in a very narrow range, just a little lower than the value of the carnosine imidazole ring. The values of the dissociation constants of the basic center in the lateral chain are the same, within the experimental errors. At physiological pH (7.4) all the products exist prevalently as a mixture of mono-charged and di-charged cations, unlike carnosine which is a mixture of a zwitterion and a tri-charged ion (++−).

The lipophilicity studies were carried out by shake-flask technique using as partition pair buffered water (pH 7.4)/*n*-octanol. The obtained distribution coefficients (log $D^{7.4}$) are reported in Table 1. The very high hydrophilicity of the compounds **7a-c** prevented the direct measurement of their log $D^{7.4}$. Consequently these molecular descriptors were calculated from the related p*K*$_a$ and CLOGP values (CLOGP for windows, v.1.0 Biobyte Corp., Claremont, CA, USA). As shown in Table 1, the lipophilicity of the series is modulated over a very large range (about 5.5 log units). As expected it increases in the aliphatic series with the length of the chain: the simple amide **7a** is the most hydrophilic member and its dodecyl derivative **7i** the most lipophilic.

Stability in human serum. As aforementioned, carnosine is rapidly hydrolyzed by carnosinases. Therefore, one of the aspects that must be addressed when working with potential drugs structurally related to carnosine is their metabolic stability. A number of structural modifications of carnosine were carried out in order to obtain products with higher stability in biological media. The most common approach to obtain carnosinase-stable derivatives consisted in the removal of the carboxylic group from carnosine structure thus obtaining β-alanylhistamine (carcinine). Carcinine and some of its N-substituted derivatives proved stable to enzymatic hydrolysis and were endowed with free radical scavenging ability.[18,19] Other modifications have regarded the peptide bond present in the parent molecule which was transformed into a sulfonamide affording derivatives stable to carnosinase activity.[20] Acetylation on primary amine afforded the prodrug derivative N-acetylcarnosine which proved more stable to carnosinase activity than parent L-carnosine,[13,19] moreover substitution of the β-alanyl moiety with 2,3-diamino propionic acid, with different degrees of N-acetylation, afforded carnosinase stable compounds which were able to exert protective action against hydroxyl radical and peroxynitrite anion.[21] The inversion of configuration of the COOH group switching from L to D-carnosine proved a successful approach. D-carnosine and some synthesised derivatives were stable to hydrolysis in human serum and shared some common features with natural L-carnosine; in particular D-carnosine was able to inhibit α-crystallin fibrillation and of disassembling α-crystallin amyloid fibrils.[22] Some D-carnosine analogues showed efficient sequestering of RCS.[23] Surprisingly enough only few approaches involving carnosine COOH group manipulation have been reported so far. In particular amide formation with β-cyclodextrin affording carnosinase stable macromolecules endowed with antioxidant properties in different biological media was reported.[24,25] Other fluorinated amphiphilic alkylamides were synthesized and proposed as metal coordinating agents, however their stability against carnosinase activity was not tested.[26] Carnosine and the carnosine amides **7a-n** were studied by RP-HPLC for their stability in human serum at 37 °C. Unlike the lead ($t_{1/2}$ = 5 min), all the products were completely stable over 3 h. This result is in keeping with the previously mentioned hypothesis that the carnosine carboxylic group binds a Zn^{2+} ion present in the active cleft of the enzyme, so playing a paramount role in the hydrolysis of the substrate.

Copper complexes. Carnosine is able to form complexes with a number of metals. This property has been widely studied and the complexes with Cu^{2+} have received particular attention in

view of their physiological roles.[27,28] At neutral pH values at 35 °C, when the molar concentrations of carnosine and Cu^{2+} are equal, the predominant complex is represented by the neutral dimeric species $[Cu_2L_2H_2]^0$. A structure in solution related to the structure found in the solid state has been proposed for this complex, namely two Cu^{2+} ions, each bound to an amino group, dissociated amido function, N^3 imidazole nitrogen and carboxylate oxygen.[29] Compounds **7a**, **7d**, **7g**, **7l**, **7m**, were chosen as representative members of the structural variety of carnosine amides here described to investigate the capacity of complexing Cu^{2+} of the series. Compound **12** and carnosine were also studied for comparison. The complex formation between copper(II) and the different carnosine derivatives has been investigated by means of the classical pH-metric technique. The stability constants of complexes have been expressed by $\beta_{pqr} = [Cu_pL_qH_r]/[Cu]^p[L]^q[H]^r$ (for reaction pCu + qL + rH = $Cu_pL_qH_r$, where L is the carnosine derivative, p, q, and r are the stoichiometric coefficients and charges are omitted for sake of simplicity). It must be pointed out that for ligands here studied the dissociation of both peptide and amide hydrogens takes place at pH values too high to allow a reliable evaluation of the relative pK_a in aqueous solution. As a consequence, the concentration [L] in the expression of β_{pqr} takes into account only the hydrogen ion dissociation from protonated imidazole and amino nitrogens. If the presence of a metal ion promotes further dissociation of hydrogen ion from peptide and amide group, in the above reaction the species H^+ is subtracted and the index *r* becomes negative. Thus, for all the complexes for which dissociation takes place from peptides and/or amide groups the *r* index is negative. The measurements were carried out at t = 25 °C and ionic strength I = 0.15 M. The elaboration of experimental pH-metric data, in order to calculate the values of formation constants, has been performed by BSTAC program.[30] The results are listed in Table 2. From an inspection of these data it can be observed that for all derivatives in which the carboxylate group has been transformed into amido or an N-substituted amido group, the stoichiometry of complexes is the same as for carnosine, but the stability is always significantly lower. This indicates that the contribution of carboxylate to the formation of the different species is more relevant, if compared to that of amido group; moreover

the different N-substituents on amido group does not affect to a great extent the stability of copper (II) complexes. The stability of 11-1 complex ($\log\beta_{11-1}$ = 0.16) for the ligand **12**, which does not contain an amido group, is further lowered with respect to all the other ligands studied and, in addition, **12** does not form dimeric species, suggesting a participation, however less significant with respect to carboxylate, of an amido group in the complex formation for all the above ligands (a weak coordination by carbonyl in 22-2 species and a more significant coordination by deprotonated amido group in 22-3 or 22-4 species). It is very likely that the structure of 22-2, 22-3, 22-4 is similar to that reported for carnosine.[28]

The stability constants of Table 2 allow us to calculate the species distribution for the different complexes and the ratio between the free and total copper(II) concentration. If assuming $[Cu]_{total}$ = 2.5 x 10^{-6} M and $[carnosine]_{total}$ = 1 x 10^{-4} M, the values of $\log([Cu]_{total}/[Cu]_{free})$ at pH 7.4, range from 2.89 to 3.19 for all carnosine derivatives (Table 2). These values, if compared with the value calculated for carnosine (3.80), clearly indicate that, although it is weaker than that in lead, the sequestering ability of all the above ligands is highly significant. The value of $\log([Cu]_{total}/[Cu]_{free})$ is significantly lower for **12** (1.95) in which N-substituted amido group is not present.

Antioxidant activity. Oxidative stress occurs in cells, tissues and organs, when the balance between the concentration of the prooxidant reactive species and the antioxidant capacity is broken in favour of the prooxidant forces. Oxidative stress is involved in many pathological processes including aging, cancer, chronic inflammation, diabetic complications, cardiovascular diseases, including atherosclerosis and stroke, as well as a number of neurodegenerative disorders. [31,32] In particular there is strong evidence that LDL-oxidation is increased in neurodegenerative and cognitive impairing diseases such Alzheimer's disease (AD) and vascular dementia.[33-36] As aforementioned, carnosine displays antioxidant properties; in particular it is reported that carnosine was able to increase lag time for TBARS appearance and reduce the maximal rate of LDL oxidation catalysed by Cu^{2+}. In the same work conflicting results were obtained when hydroperoxides formation was monitored.[37] In our work we tested carnosine and all the newly synthesised amides

7a-n (100 μM) for their ability to suppress conjugated dienes formation during copper–mediated LDL (50 μg protein mL^{-1}) oxidation. The time course of autooxidation, initiated by the addition of 2.5 μM $CuSO_4$, was followed spectrophotometrically by detecting the formation of conjugated dienes at 234 nm. Typical examples of such experiments are reported in Figure 1 for carnosine, the simple amide derivative **7a** and its *n*-decyl analogue **7h**. According to literature,[38] three parameters were used to characterise the kinetics of LDL oxidation: the maximal accumulation of oxidation products (OD_{max}), the Δlag-time (Δt_{lag}), i.e. the duration of the period prior to onset of rapid lipidic peroxidation (propagation phase) compared to the control, and the propagation rate of the oxidation (*R*). OD_{max} of all the amides was the same as the control. *R* and Δt_{lag} derived for each compound from the corresponding curves are collected in Table 3, together with those measured for carnosine and the control. Analysis of the data indicates that the more hydrophilic products **7a**-**f**, **l**-**n**, bearing shorter alkyl substituent groups or the cyclohexylmethyl and benzyl substituents, are able to reduce *R* with respect to the control in a manner similar to carnosine. By contrast, all the remaining more lipophilic members of the series behave similarly to the control, with the only partial exception of **7h** which induced a slight increase of the propagation rate of the oxidation. Analysis of Δt_{lag} values again indicates an influence of lipophilicity. Hydrophilic compounds are the most active members of the series, showing a Δt_{lag} near that of carnosine, while the less hydrophilic ones display lower activity. When Δt_{lag} is plotted against log $D^{7.4}$ the diagram of Figure 2 is obtained. It shows that the ability of carnosine amides to increase the lag-time of the copper-catalysed LDL peroxidation linearly increases with hydrophilicity and then reaches a plateau. It is known that Cu^{2+} ions can form LDL-copper complexes with binding sites of apolipoprotein and the amount of copper bound to LDL increases with increasing concentrations of copper ions until the copper binding sites become saturated.[39] These complexes are able to produce free radicals at the LDL surface by interacting with pre-formed LDL-associated hydroperoxides (LOOH), thus inducing peroxidation.[40] As aforementioned, the ability of the amide analogues of carnosine to sequester Cu^{2+} ions at physiological pH is roughly similar. The lipophilicity of compounds, expressed by log $D^{7.4}$, should

reasonably mimic their capacity of distribution between the LDL and the aqueous phase. Consequently, the linear tract of the plot in Figure 2 could be justified by the fact that, under the adopted experimental conditions, the higher the hydrophilicity is, the higher the amount of ligand in aqueous phase free to complex Cu^{2+} ions and thus able to decrease the onset of rapid lipidic peroxidation. Under the threshold log $D^{7.4}$ value of about -1.5, the amount of ligand sequestered by the LDL phase is so small that any further reduction of lipophilicity does not produce any appreciable variation in ligand aqueous concentrations, and consequently, in the amount of chelated Cu^{2+} ions; this could justify the presence of the plateau. The different ability to scavenge Cu^{2+} of the products as consequence of their different lipophilicity could also partly justify their different ability in influencing the propagation rate of the oxidation. Compound **12,** which does not contain amido group, was also tested for its ability to reduce LDL oxidation. It proved unable to modify the three parameters which characterise the kinetics of LDL oxidation. This result concurs with the copper complexes formation test result in which **12** showed a very low Cu^{2+} sequestering ability.

HNE-quenching activity. The products formed by lipid peroxidation are degraded to reactive aldheydes such as HNE, malondialdehyde and alkenals. HNE has been demonstrated to cause neuronal death.[41-44] Moreover HNE-protein adducts have been detected in the brain of patients with AD, therefore HNE is considered to play a crucial role in oxidative injury of biomolecules related to AD.[45-47] In the introduction we have already briefly discussed the biological role of carnosine as a quencher of α,β-unsaturated aldehydes. This reaction has been object of detailed studies.[6,7,48] Two adducts have been isolated as principal reaction products working in phosphate buffer, pH 7.4, using HNE as a model of reactive aldehyde. The former is an imidazole-based Michael adduct, stabilized as a 5-membered cyclic hemi-acetal and the latter a 13-membered ring Schiff base Michael adduct.[6,7,23,49] In order to evaluate whether carnosine amides display carnosine-like properties as quenchers of HNE, all the products were incubated in phosphate buffer, pH 7.4, with HNE at 37 °C. Samples relative to different incubation times were directly analyzed by RP-HPLC to measure HNE consumption. The % HNE quenched increased with the time and after

24 h reached a plateau. The results after 1, 6, 24 h are summarized in Table 3. Carnosine, taken as reference, after 24 h was able to block about 89% of HNE. The most active product among the carnosine amides was the n-octyl substituted compound **7g,** which was as active as carnosine within the experimental error. For all the other compounds the quenching effect on HNE fell in the range 62-81% These results indicate that all the canosine amides here described display quite potent carnosine-like HNE quenching activity.

Protection from HNE-induced cell death. On the bases of the results we obtained from the experiments of HNE-quenching activity, we decided to test the ability of compound **7d**, chosen as an example of this new series of carnosine derivatives, to protect primary mouse hippocampal neurons against HNE-induced death. Carnosine was taken as reference. At first, the adverse effects of **7d** (100 μM), HNE (10 and 20 μM), and carnosine (100 μM) on hippocampal neurons were evaluated. (Figure 3A). Then, mouse hippocampal neurons were pretreated either with the selected compound or with carnosine and then exposed to HNE 10 μM concentration. After 24 h exposure, the percentage of cell death was evaluated (Figure 3B) by tripan blue exclusion, in order to stain just dead cells due to loss of membrane integrity. Inspection of the Figure 3A indicates that, when compared to the control, HNE was able to kill 61-84% of treated cells in a concentration dependent manner while no adverse effect was seen either with carnosine or **7d**. Analysis of Figure 3B shows that carnosine has, if any, a very low cytoprotective efficacy (6% ± 4), this is probably due to its instability to carnosinase which is known to be present in mouse brain,[50] while **7d** triggers highly significant protective effect (40% ± 17). Figure 3C shows the damage induced by HNE on the neural network (middle image) compared to the control (left image). The destruction is visible at the axonal level and also in the matrix surrounding the processes; moreover, there is a reduction in the total number of neurons and visible signs of necrosis. The presence of **7d** clearly reduces these effects (right image).

HPLC-detection of 7d in rat serum and brain.

In order to further explore the neuroprotective potential of this class of compounds we determined whether **7d** enters the brain by administering **7d** (20 mg/kg) intravenously to a group of rats. Blood and brain tissues samples were collected from rats assigned to 1.5 or 3 h post-treatment time points. Samples were analyzed by RP-HPLC (see experimental) and concentration of **7d** was determined. At 3 h level of **7d** in brain was 1.1 ± 0.4 μg/g tissue, at 1.5 h level of **7d** was about 0.6 μg/g tissue, approaching the detection limit of this technique. In blood **7d** was only detectable at 1.5 h at a concentration of 0.26 μg/mL. The obtained data indicates how levels of **7d** in brain increased between 1.5 and 3 h of administration, whereas levels of **7d** in the serum decreased during this time interval.

Conclusion. This paper describes synthesis, physico-chemical and biological characterisation of a series of carnosine amides bearing on the amido group alkyl substituents endowed with different lipophilicity. All the products were able to display carnosine-like properties, morover they were stable over 3 h of incubation in human serum at 37 °C, unlike lead that was rapidly cleaved into its constituents. All the synthesised compounds were capable of affording copper complexes at physiological pH with the same stoichiometry as carnosine, and of displaying highly significant copper ion sequestering ability. The products were capable of protecting LDL from oxidation catalysed by Cu^{2+} ions, the most active compounds being the most hydrophilic ones. All the amides triggered quite potent carnosine-like HNE quenching activity. **7d**, the compound chosen as representative example of the series, was able to protect primary mouse hippocampal neurons against HNE-induced death. **7d** was also capable of penetrating rat brain after in vivo administration. These products can be considered metabolically stable analogues of carnosine, worthy of additional investigation as potential neuroprotective agents.

Experimental Section

Chemistry. Melting points were measured with a capillary apparatus (Büchi 540). Compounds **7a-h**, **12** were highly hygroscopic amorphous semisolids or foams. The determination of their melting point was

affected by the complex thermal behaviour of these compounds; consequently, the melting point was not reported. All the compounds were routinely checked by ^{1}H and ^{13}C-NMR (Bruker Avance 300) at 300 and 75 MHz respectively, and mass spectrometry (Finnigan-Mat TSQ-700). The following abbreviations are used to indicate the peak multiplicity: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Flash column chromatography was performed on silica gel (Merck Kieselgel 60, 230-400 mesh ASTM) using the reported eluents. Thin layer chromatography (TLC) was carried out on 5 x 20 cm plates (Fluka) with a 0.2 mm layer thickness. Unless otherwise stated anhydrous magnesium sulfate was used as the drying agent for the organic phases. Analysis (C, H, N) of the target compounds was performed by Service de Microanalyse, Université de Genève, Genève (CH) and REDOX (Monza) and the results were within ± 0.4% of the theoretical. HNE (4-hydroxy-*trans*-2,3-nonenal) was prepared by acid treatment (1mM HCl) of HNE-DMA (4-hydroxy-*trans*-2,3-nonenal-dimethylacetal; Sigma). Compound **8**[17] was synthesized according to the literature. Preparative HPLC was performed on a Lichrospher C_{18} column (250 × 25 mm, 10 μm) (Merck Darmstadt, Germany) with a Varian ProStar mod-210 with Varian UV detector mod-325 with a flow rate of 39 mL/min.; the detection was performed at 224 nm.

General procedure for preparation of 4b-n. To a stirred solution of Fmoc-His-(Trt)-OH (**2**) (2.5 g; 4 mmol) in dry DMF (40 mL) DIPEA (1.03 mL; 6 mmol), HBTU (2.29 g; 6 mmol) and HOBt (0.08 g; 0.6 mmol) were added. After 10 min the appropriate amine **3a-n** (6 mmol) was added; the reaction mixture was allowed to stir at r.t. until TLC showed complete consumption of starting material (1-18 h). The solvent was removed under reduced pressure (oil pump) and the residue taken up with CH_2Cl_2 (40 mL) and washed with water (3 x 30 mL), brine (30 mL), then dried and evaporated under reduced pressure. The residual oil was purified by flash chromatography eluting with CH_2Cl_2/MeOH 9.9/0.1 to 9.5/0.5 to afford the desired intermediate **4b-n** (56-100%). To a stirred solution of the obtained intermediate (2.59 mmol) in dry DMF (23 mL), piperidine (1.15 mL; 11.6 mmol) was added and the reaction mixture was stirred at r.t. for 1 h. The solvent was evaporated under reduced pressure and the solid residue was taken up with CH_2Cl_2 (30 mL) and washed with water (3 x 30 mL) and brine (30 mL). The organic phase was dried (Na_2SO_4) and the crude product was purified by flash chromatography eluting with CH_2Cl_2/MeOH 9.8/0.2 to 8/2 to yield the desired intermediates **5b-n** (50-100%). When ammonia was used as the amine nucleophile in the coupling reaction with activated **2**, amidation and fmoc-deprotection were achieved in one step leading to desired **5a** which

was isolated as a white solid after filtration from cold (0 °C) CH_2Cl_2 in 86% yield. To a stirred solution of the free amines **5a-n** (1.75 mmol) and Boc-β-Ala(OH) (0.35 g; 1.83 mmol) in dry CH_2Cl_2 (30 mL) kept at 0 °C DCC (0.36 g; 1.75 mmol) was added, the ice bath was removed and the reaction was stirred at r.t. for 2-18 h. The reaction mixture was cooled to 0 °C. The precipitate was filtered and washed with cold (0 °C) CH_2Cl_2. The liquid phase was washed with water (3 x 30 mL), brine (30 mL), then dried and evaporated under reduced pressure to leave a white solid. The crude material was purified by flash chromatography eluting with CH_2Cl_2/MeOH 9.8/0.2 to 9.5/0.5 to give the desired derivatives **6a-n** (41-98%) as white foams. The obtained products were dissolved in CH_2Cl_2 (21 mL), treated with CF_3COOH (2.1 mL) and stirred at r.t. for 20 h. The solvent was evaporated under reduced pressure and the semisolid residue was treated with water (30 mL). The formed precipitate was filtered-off through a sintered glass funnel and the water phase was washed with CH_2Cl_2 (2 x 20 mL), then with EtOAc (2 x 20 mL) and evaporated to afford the final product.

β-Alanyl-L-histidinamide ditrifluoroacetate (7a). The product was recrystallised from dry MeOH/Et_2O and freeze-dried to afford **7a** as an amorphous semisolid material; overall yield: 28%; ^{1}H-NMR (DMSO): δ, 8.81 (s, 1H, ImH_2); 8.46 (d, 1H, *J*=8.4 Hz, *NH*CH); 7.86 (s, br, 3H, NH_3^+); 7.52 (s, br, 1H, CON*H*H); 7.28 (s, 1H, ImH_5); 7.26 (s, br, 1H, CONH*H*); 4.55-4.48 (m, 1H, C*H*); 3.17-2.85 (m, 4H, 2 CH_2); 2.61-2.43 (m, 2H, COCH_2). ^{13}C-NMR (DMSO):δ, 172.1; 169.7; 134; 130.3; 116.9; 51.8; 35.3; 32.2; 27.3.

β-Alanyl-*N*-metil-L-histidinamide ditrifluoroacetate (7b). The product was recrystallised from dry MeOH/Et_2O and freeze-dried to afford **7b** as a white amorphous foam; overall yield 29%; ^{1}H-NMR (DMSO): δ, 8.89 (s, 1H, ImH_2); 8.52 (d, 1H, *J*=8.1 Hz, N*H*CH); 8.03-8.01 (m, 1H, N*H*CH_3); 7.89 (s, br, 3H, NH_3^+); 7.30 (s, 1H, ImH_5); 4.56-4.49 (m, 1H, C*H*); 3.17-2.83 (m, 4H, 2 CH_2); 2.57 (d, 3H, *J*=4.5 Hz, CH_3); 2.51-2.41 (m, 2H, COCH_2). ^{13}C-NMR (DMSO): δ, 170.1; 169.5; 134; 133.7; 116.6; 51.7; 35; 32; 27; 25.6.

β-Alanyl-*N*-ethyl-L-histidinamide ditrifluoroacetate (7c). The product was recrystallised from dry MeOH/Et_2O and freeze-dried to afford **7c** as a white amorphous foam; overall yield 78%; ^{1}H-NMR (CD_3OD): δ, 8.68 (s, 1H, ImH_2); 7.29 (s, 1H, ImH_5); 4.70-4.60 (m, 1H, C*H*); 3.21-3.04 (m,

6H, 3 CH_2); 2.66-2-64 (m, 2H, COCH_2); 1.10-1.06 (t, 3H, *J*=7.2 Hz, CH_3). ^{13}C-NMR (CD_3OD): δ, 172.3; 171.9; 135.1; 131.5; 118.3; 53.8; 36.8; 35.4; 32.7; 28.5; 14.7.

β-Alanyl-*N*-propyl-L-histidinamide ditrifluoroacetate (7d). The product was recrystallised from dry MeOH/Et_2O and freeze-dried to afford **7d** as a white amorphous foam; overall yield: 41%; ^{1}H-NMR (DMSO): δ, 8.84 (s, 1H, ImH_2); 8.50 (d, 1H, *J*=8.4 Hz, N*H*CH); 8.06 (t, 1H, *J*=10.8 Hz, CON*H*CH_2); 7.90 (s, br, 3H, NH_3^+); 7.28 (s, 1H, ImH_5); 4.57-4.50 (m, 1H, C*H*); 3.16-2.85 (m, 6H, 3 CH_2); 2.58-2.42 (m, 2H, COCH_2); 1.39 (q, 2H, *J*=7.2 Hz, CH_2CH_3); 0.91 (t, 3H, *J*=7.2 Hz, CH_3). ^{13}C-NMR (DMSO): δ, 170.5; 170.4; 134.6; 130.7; 117.6; 52.8; 41.3; 36; 32.9; 28.2; 23; 12.1.

β-Alanyl-*N*-butyl-L-histidinamide ditrifluoroacetate (7e). The product was recrystallised from dry MeOH/Et_2O and freeze-dried to afford **7e** as a white amorphous foam; overall yield: 31%; ^{1}H-NMR (CD_3OD): δ, 8.74 (d, 1H, *J*=1.2 Hz, ImH_2); 7.30 (d, 1H, *J*=1.2 Hz, ImH_5); 4.68-4.64 (m, 1H, C*H*); 3.30-3.02 (m, 6H, ImCH_2, NHCH_2, C$H_2NH_3^+$); 2.71-2.58 (m, 2H, COCH_2); 1.49-1.35 (m, 4H, CH_2CH_2); 0.93(t, 3H, *J*=7.2 Hz, CH_3). ^{13}C-NMR CD_3OD): δ, 172.3; 172; 135.1; 131.4; 118.4; 53.8; 40.3; 36.8; 32.7; 32.4; 28.41; 21; 14.1.

β-Alanyl-*N*-hexyl-L-histidinamide ditrifluoroacetate (7f). The product was purified by RP-HPLC eluting with MeOH/H_2O 60/40 + 0.1% TFA to give pure **7f** as a white amorphous foam; overall yield: 49%; ^{1}H-NMR (CD_3OD): δ, 8.81 (s, 1H, ImH_2); 7.33 (s, 1H, ImH_5); 4.70-4.65 (m, 1H, C*H*); 3.31-3.03 (m, 6H, ImCH_2, NHCH_2, C$H_2NH_3^+$); 2.73-2.56 (m, 2H, COCH_2); 1.49-1.44 (m, 2H, NHCH_2CH_2); 1.33-1.28 (m, 6H, 3 CH_2); 0.90 (t, 3H, *J*=6.9 Hz, CH_3). ^{13}C-NMR (CD_3OD):δ, 172.3; 171.9; 135; 131.1; 118.4; 53.7; 40.6; 36.8; 32.69; 32.66; 30.3; 28.3; 27.7; 23.6; 14.4.

β-Alanyl-*N*-octyl-L-histidinamide ditrifluoroacetate (7g). The product was recrystallised from dry MeOH/Et_2O and freeze-dried to afford **7g** as white amorphous foam; overall yield: 41%; ^{1}H-NMR (CD_3OD): δ, 8.78 (s, 1H, ImH_2); 7.33 (s, 1H, ImH_5); 4.70-4.65 (m, 1H, C*H*); 3.30-3.08 (m, 6H, NHCH_2, ImCH_2, C$H_2NH_3^+$); 2.68-2.62 (m, 2H, COCH_2); 1.49-1.44 (m, 2H, NHCH_2CH_2); 1.30-1.28 (m, 10H, 5 CH_2); 0.90 (t, 3H, *J*= 6.9 Hz, CH_3). ^{13}C-NMR (CD_3OD): δ, 172.3; 171.9; 135; 131.2; 118.4; 53.7; 40.7; 36.8; 33.0; 32.7;

30.42; 30.41; 30.36; 28.3; 28; 23.7; 14.5. ^{1}H-NMR in agreement with those reported for the hydrochloride derivative.[26]

β-Alanyl-*N*-decyl-L-histidinamide ditrifluoroacetate (7h). The product was purified by RP-HPLC eluting with MeOH/H_2O 70/30 + 0.1% TFA to give **7h** as a white amorphous solid; overall yield 29%; ^{1}H-NMR (CD_3OD): δ, 8.81 (s, 1H, ImH_2); 7.33 (s, 1H, ImH_5); 4.70-4.65 (m, 1H, C*H*); 3.34-3.03 (m, 6H, NHCH_2, ImCH_2, C$H_2NH_3^+$); 2.71-2.56 (m, 2H, COCH_2); 1.48-1.44 (m, 2H, NHCH$_2$CH_2); 1.31-1.29 (m, 14H, 7 CH_2); 0.90 (t, 3H, *J*=6.9 Hz, CH_3). ^{13}C-NMR (CD_3OD): δ, 172.3; 171.9; 135; 131.2; 118.4; 53.7; 40.8; 36.8; 33.1; 32.7; 30.8; 30.7; 30.49; 30.46; 30.37; 28.3; 28; 23.8; 14.5. ^{1}H-NMR in agreement with those reported for the hydrochloride derivative.[26]

β-Alanyl-*N*-dodecyl-L-histidinamide ditrifluoroacetate (7i). The product was purified by RP-HPLC eluting with MeOH/H_2O 80/20 + 0.1% TFA to give the desired **7i** as a white amorphous solid; overall yield 14%; mp: 72.2 - 80.7 °C; ^{1}H-NMR (CD_3OD): δ, 8.79 (s, 1H, ImH_2); 7.36 (s, 1H, ImH_5); 4.75-4.70 (m, 1H, C*H*); 3.26-3.02 (m, 6H, ImCH_2, NHCH_2, C$H_2NH_3^+$); 2.71-2.58 (m, 2H, COCH_2); 1.48-1.44 (m, 2H, NHCH$_2$CH_2); 1.30-1.29 (m, 18H, 9 CH_2); 0.90 (t, 3H, *J*=6.9 Hz, CH_3). ^{13}C-NMR (CD_3OD): δ, 172.5; 172.1; 134.9; 131.1; 118.4; 53.9; 40.8; 36.8; 33.0; 32.7; 30.77; 30.75; 30.71; 30.6; 30.5; 30.4; 30.3; 28.7; 28; 23.7; 18.4.

β-Alanyl-*N*-cyclohexyl-L-histidinamide ditrifluoroacetate (7l). The product was purified by RP-HPLC eluting with MeOH/H_2O 70/30 + 0.1% TFA and freeze-dried to give **7l** as a white amorphous solid; overall yield 37%; mp: 74.5-84.2 °C. ^{1}H-NMR (CD_3OD): δ, 8.81 (s, 1H, ImH_2); 7.34 (s, 1H, ImH_5); 4.71-4.66 (m, 1H, C*H*); 3.30-2.94 (m, 6H, ImCH_2, NHCH_2cHex, C$H_2NH_3^+$); 2.66-2.59 (m, 2H, COCH_2); 1.73-0.90 (m, 11H, cHex*H*). ^{13}C-NMR (CD_3OD): δ, 172.5; 172.2; 135.2; 131.3; 118.6; 54; 47.1; 39.3; 37.1; 32.9; 32.1; 28.5; 27.7; 27.2.

β-Alanyl-*N*-benzyl-L-histidinamide ditrifluoroacetate (7m). The product was recrystallised from MeOH/Et_2O and freeze-dried to give **7m** as a white amorphous solid; overall yield 20%; mp: 158.7-161.9 °C; ^{1}H-NMR (CD_3OD): δ, 8.74 (s, 1H, ImH_2); 7.32-7.21 (m, 6H, ImH_5, 5 Ar*H*); 4.76-4.71 (m, 1H, C*H*); 4.45-4.30 (m, 2H, NHCH_2); 3.20-2.95 (m, 4H, ImCH_2, C$H_2NH_3^+$); 2.76-2.58 (m, 2H,

COCH_2). ^{13}C-NMR (CD_3OD): δ, 172.4; 172; 139.7; 134.9; 129.6; 128.6; 128.4; 118.42; 112.2; 53.8; 44.2; 36.8; 32.7; 28.1.

β-Alanyl-*N*-(4-butoxbenzyl)-L-histidinamide ditrifluoroacetate (7n). The product was purified by preparative RP-HPLC eluting with MeOH/H2O 60/40 + 0.1% TFA and freeze-dried to give the desired product as a white solid; overall yield 17%; mp: 70.8 – 73.2 °C; ^{1}H-NMR (CD_3OD): δ, 8.73 (s, 1H, ImH_2); 7.23 (s, 1H, ImH_5); 7.10 (d, 2H, *J*=8.7 Hz, Ar$H_{2',6'}$); 6.82-6.79 (d, 2H, *J*=8.7 Hz, Ar$H_{3',5'}$); 4.67 (m, 1H, C*H*); 4.26-4.23 (m, 2H, NHCH_2); 3.91 (t, 2H, *J*=6.3 Hz, OCH_2); 3.15-3.10 (m, 4H, ImCH_2, C$H_2NH_3^+$); 2.62-2.61 (m, 2H, COCH_2); 1.72-1.68 (m, 2H, $CH_2CH_2CH_2CH_3$); 1.47-1.45 (m, 2H, $CH_2CH_2CH_2CH_3$); 0.94 (t, 3H, *J*=7.2 Hz, CH_3). ^{13}C-NMR (CD_3OD): δ, 172.3; 171.8; 160; 134.9; 131.5; 131; 130; 118.4; 115.5; 68.7; 53.8; 43.7; 36.8; 32.7; 32.5; 28.2; 20.3; 14.2.

3-amino-*N*-[(1S)-2-ethoxy-1-(1*H*-imidazol-4-ylmethyl)ethyl]-propanamide ditrifluoroacetate (12). In a flame dried flask equipped with a $CaCl_2$ guard tube NaH 60% in mineral oil (0.83g; 20.8 mmol) suspended in dry DMF (12.5 mL) was stirred for 1 h. A solution of **8** (2.50 g; 4.0 mmol) in dry DMF (15 mL) was added and the reaction mixture was stirred at r.t. for 1 h, then bromoethane (1.09 g; 10 mmol) was added and the reaction mixture stirred overnight. The mixture was cooled to 0 °C and excess NaH was destroyed by a slow addition of water. The obtained suspension was extracted with EtOAc (3 x 40 mL), the organic phase was dried (Na_2SO_4) and evaporated under reduced pressure. The residue was purified by flash chromatography eluting with CH_2Cl_2/EtOAc 9.5/0.5 to 9/1 to yield **9** as a white foam (2.21 g; 85%). To an ice-cooled solution of **9** (2.16 g, 3.3 mmol) in dry CH_2Cl_2 (73 mL), CF_3COOH (0.73 mL; 9.82 mmol) was added and the reaction mixture was stirred for 2 min. The mixture was diluted with 10% (w/v) Na_2CO_3 aqueous solution and extracted with EtOAc (3 x 40 mL). The combined organic layers were washed with water (50 mL) and dried (Na_2SO_4) to afford a crude product which was purified by flash chromatography eluting with CH_2Cl_2/MeOH 9.5/0.5 to 8.5/1.5 to yield 1.17 g (87%) of **10** as a colourless oil. To a stirred solution of **10** (1.17 g; 2.84 mmol) and Boc-β-Ala(OH) (0.67 g; 3.54 mmol) in dry CH_2Cl_2 (20 mL) kept at 0 °C DCC (0.73 g; 3.54 mmol) was added, the ice bath was removed and the reaction was stirred at r.t. for 15 h. The reaction mixture was cooled to 0 °C and the obtained precipitate was filtered and washed with cold (0 °C) CH_2Cl_2. The liquid phase was washed with water (3 x 30 mL), brine (30 mL), then dried and evaporated under reduced pressure to leave a white solid. The crude material was purified by flash chromatography

eluting with CH_2Cl_2 + 2% MeOH, to give 1.60 g (97%) of **11** as a white foam. The obtained product was dissolved in CH_2Cl_2 (41 mL), treated with CF_3COOH (4.1 mL; 55 mmol) and stirred at r.t. for 20 h. The solvent was evaporated under reduced pressure and the dark oily residue was treated with water (20 mL). The formed precipitate was filtered-off through a sintered glass funnel and the liquid phase was extracted with EtOAc (3 x 20 mL). The aqueous layer was evaporated under reduced pressure to leave 1.12 g (87%) of **12** as a colourless semisolid material. The product was recrystallised twice from dry MeOH/Et_2O and freeze-dried. ^{1}H-NMR (CD_3OD): δ, 8.74 (s, 1H, ImH_2); 7.3 (s, 1H, ImH_5); 4.33-4.29 (m, 1H, CH); 3.56-3.45 (m, 4H, 2 CH_2O); 3.15-3.11 (t, 2H, J = 6.6 Hz, C$H_2NH_3^+$); 3.07-2.87 (m, 2H, ImCH_2); 2.59 (t, 2H, J= 6.6 Hz, COCH_2); 1.19 (t, 3H, J= 6.9 Hz, CH_3). ^{13}C-NMR (CD_3OD): δ 172.1; 134.9; 132.3; 118; 72.2; 67.8; 49.9; 37; 32.8; 27.9; 15.4.

Ionisation constants and lipophilicity descriptors. The ionisation constants of compounds were determined by potentiometric titration with the GLpKa apparatus (Sirius Analytical Instruments Ltd, Forrest Row, East Sussex, UK). Ionisation constants of carnosine **1** and compounds **7a-g, 7l-n** and **12,** were obtained by aqueous titrations by at least four separate titrations for each compound: different aqueous solutions (ionic strength adjusted to 0.15 M with KCl) of the compounds (20 mL, about 1 mM) were initially acidified to pH 1.8 with 0.5 N HCl; the solutions were then titrated with standardized 0.5 N KOH to pH 12.2. Because of the low aqueous solubility, ionisation constants measurement of compounds **7h-i** required titrations in the presence of methanol as a cosolvent: at least five different hydro-organic solutions (ionic strength adjusted to 0.15 M with KCl) of the compounds (20 mL, about 1 mM in 20–34 wt% methanol) were initially acidified to pH 1.8 with 0.5 N HCl; the solutions were then titrated with standardized 0.5 N KOH to pH 12.2. The initial estimates of the p_sK_a values (the apparent ionization constants in the water–methanol mixtures) were obtained and aqueous pK_a values were determined by extrapolation to zero content of the cosolvent according to the Yasuda-Shedlovsky procedure.[51] All the titrations were performed under nitrogen at 25.0 ± 0.1 °C. The apparent partition coefficients log $D^{7.4}$ were obtained by shake-flask procedure at pH 7.4 (phosphate buffer solutions with ionic strength adjusted to 0.15 M with KCl) n-octanol was added to the buffers and the two phases were mutually saturated by shaking for 4 h.

The compounds were solubilized in the buffered aqueous phase at a concentration of about 0.1 mM and an appropriate amount of n-octanol was added. The two phases were shaken for about 20 min, by which time the partitioning equilibrium of solutes was reached, and then centrifuged (10,000 rpm, 10 min). The concentration of the solutes in the aqueous phase was measured by UV spectrophotometer (UV-2501PC, Shimadzu) at 230 nm. For each compound at least seven log *D* values were measured.

Copper complexes. The complex formation between copper(II) and carnosine and derivatives **7a, 7d, 7g, 7l, 7m, 12** has been investigated by means of the classical pH-metric technique with the GLpKa apparatus. For each compound at least three separate titrations were performed: different aqueous solutions of the compounds and of $CuCl_2$ equimolar (1 mM) were initially acidified to pH 1.8 with 0.5 N HCl; the solutions were then titrated with standardized 0.5 N KOH to pH 12.2. The measurements were carried out under nitrogen at 25.0 ± 0.1 °C and ionic strength adjusted to 0.15 M (KCl). The elaboration of experimental pH-metric data, in order to calculate the values of formation constants, has been performed by BSTAC program.[30]

Stability in human serum. A solution of each compound (10 mM) in water was added to human serum (Sigma) preheated at 37 °C, the final concentration of the compound was 0.5 mM. Resulting solutions were incubated at 37 ± 0.5 °C and at appropriate time intervals 500 μL of reaction mixture was withdrawn and added to 500 μL of acetonitrile containing 0.1% trifluoroacetic acid in order to deproteinize the serum. The sample was sonicated, vortexed and then centrifuged for 10 min at 2150 g, The clear supernatant was filtered by 0.45 μm PTFE filters (Alltech) and analysed by RP-HPLC. HPLC analyses were performed with a HP 1100 chromatograph system (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump (model G1311A), a membrane degasser (G1379A), a diode-array detector (DAD) (model G1315B) integrated in the HP1100 system. Data analysis was done using a HP ChemStation system (Agilent Technologies). The analytical column was a Purospher C18-endcapped (250 × 4.6 mm, 5 μm particle size) (Merck Darmstadt, Germany). The mobile phase consisting of methanol/20 mM CH_3COONa pH 4.5 - 5 mM SDS (80/20 to 60/40 in accordance with the polarity of compounds) and the flow-rate was 0.7 mL/min. The injection volume was 20 μL (Rheodyne, Cotati, CA). The column effluent was monitored at 210 and 223 nm

referenced against a 360 nm wavelength. Quantitation was done by comparison of peak areas with standards chromatographed under the same conditions.

Antioxidant Activity.

LDL isolation and oxidation. Human plasma from healthy donors was provided by Blood Bank (A.O. San Giovanni Battista Turin) and added with 0.1% EDTA. The LDL fraction was isolated by ultracentrifugation through NaCl discontinuous gradients and collected as the fraction floating at a density of 1.019-1.063 g/mL. The determination of the lag phase (t_{lag}) and of the propagation rate (R) was carried out as previously described.[52] EDTA was removed by rapid filtration through disposable desalting columns Econo-Pac 10 DG (Bio-Rad). Filtered LDL were diluted with PBS (10 mM phosphate buffer, pH 7.4) to give a final concentration of 50 μg LDL protein/mL and transferred to a 1-cm cuvette with 50 μL of water alone or 50 μL of tested compound solution in water at a final concentration of 100 μM. The formation of conjugated dienes was measured spectrophotometrically in a Varian Cary 50 Bio spectrophotometer, equipped with a thermostatic control (37 °C) and an automatically exchangeable multi-positions cuvettes holder, operating at 234 nm. Oxidation was initiated by the addition to the LDL suspension of $CuSO_4$ at a final concentration of 2.5 μM.

In vitro HNE scavenging studies

HNE Incubation and LC Analysis. HNE (final concentration 50 μM in 1 mM phosphate buffer, pH 7.4) was incubated with solution of **7a-n**, **12** or with carnosine (final concentration 1 mM in 1 mM phosphate buffer, pH 7.4) for different periods (up to 24 h) at 37 °C. Samples for each different incubation time were directly analyzed by HPLC to measure HNE consumption, as previously described.[6,49] HNE was determined by reverse-phase HPLC using a HP 1100 chromatograph system (Agilent Technologies, Palo Alto, CA, USA). Reaction mixture (20 μL) were eluted on a Agilent Zorbax Eclipse XDB-C18 column (150 x 4.6 mm; particle size 5 μm). The mobile phase was 60% A (water/acetonitrile/formic acid; 9:1:0.01, v/v/v) and 40% B (water/acetonitrile; 1:9, v/v) delivered at a flow rate of 1 mL/min. The column effluent was monitored at 223 nm.

Protection from HNE-induced cell death

Cell culture. All experiments were performed in accordance with the guidelines established by the National Council on Animal Care and approved by the local Animal Care Committee of Turin University. Hippocampal cells were obtained from black-six mouse 18-day embryos. The hippocampus was rapidly dissected under sterile conditions, kept in cold HBSS (4ºC) with high glucose, and then digested with papain (0.5 mg/ml) dissolved in HBSS plus DNAsi (0,1 mg/ml). Isolated cells were plated onto Petri dishes, coated with poly-DL-lysine and laminine, at the final density of 260 cells/mm^2. The cells were incubated with 1% penicillin/streptomycin, 1% glutamax, 2% B-27 supplemented neurobasal medium in a humidified 5% CO_2 atmosphere at 37 ºC for 6 days before performing the experiments.

Cytotoxicity assays. Carnosine and **7d** were dissolved in the culture medium, containing: Neurobasal (Invitrogen), 2% B-27, 1% pen-strep (Lonza), 1% ultraglutamine (Lonza) and used at the final concentration of 100 μM. HNE (Cayman) was stored at –80 °C in ethanol and then tested at the final concentration of 10 μM and 20 μM. After incubating hippocampal cells with the substances for 24 h, cell viability was determined by trypan blue exclusion, in order to stain just dead cells, due to loss of membrane integrity. Cells were counted with knowledge of the treatment history of the culture, by comparing the number of living cells before incubation and after 24 h of treatment. Substances were tested alone and combined to HNE to assess not only the cytoprotective activity of carnosine and the carnosine analogue **7d**, but also to exclude a possible toxicity of the compound. The percentage of cytoprotection was then evaluated by comparing the percentage of cells death after exposure to HNE and to HNE + **7d**. Data are given as the mean ± SEM for 4-7 experiments. Statistical significance was calculated by using Student's paired t test. Values of $p<0.05$ were considered significant.

HPLC detection of 7d in rat blood and brain. Male Wistar rats weighing 200-250 g were used for this experiment. Each rat received 20 mg/kg of **7d** by i.v. injection of 200 μL of compound dissolved in 0.9% normal saline. After 1.5 or 3 h animals were sacrificed for decapitation, 3 rats were used for any time point. Blood was collected in polystyrene tubes with 500 U.I. eparine and brain was rapidly removed, rinsed with cold distilled water, weighed and homogenized at 4 °C in an equal volume of cold distilled water using a Potter-Elvehjem homogenizer. An equal volume of acetonitrile containing 0.1% trifluoroacetic acid was

added to blood samples or brain tissue homogenates to precipitate proteins. The samples were sonicated, vortexed and then centrifuged for 10 min at 2150 g. The clear supernatant was filtered by 0.45 μm PTFE filters and analysed by RP-HPLC. HPLC analyses were performed with the same chromatograph system and stationary phase used for stability in human serum analyses the supernatant (100 μL) was eluted with methanol/20 mM CH_3COONa pH 4.5 - 5 mM SDS (70/30) at a flow-rate of 0.7 mL/min. The column effluent was monitored at 210 and 223 nm (ref. 360 nm). Samples from untreated rats were used either as control or, after adding a known amount of **7d**, as standard.

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Supporting information available. Elemental analyses, HPLC and MS detection of **7d** in rat brain homogenate. This material is available free of charge via the Internet at http://pubs.acs.org.

Figure 1. Effect of carnosine and compounds **7a** and **7h** on kinetics of conjugated diens formation during copper-induced LDL oxidation. The figure shows typical experimental kinetic profiles obtained by incubating the compounds (100 μM) at 37 °C with 50 μg mL^{-1} of LDL in PBS in the presence of 2.5 μM $CuSO_4$. Conjugated diene formation was assessed monitoring over 6 h the changes in absorbance at 234 nm.

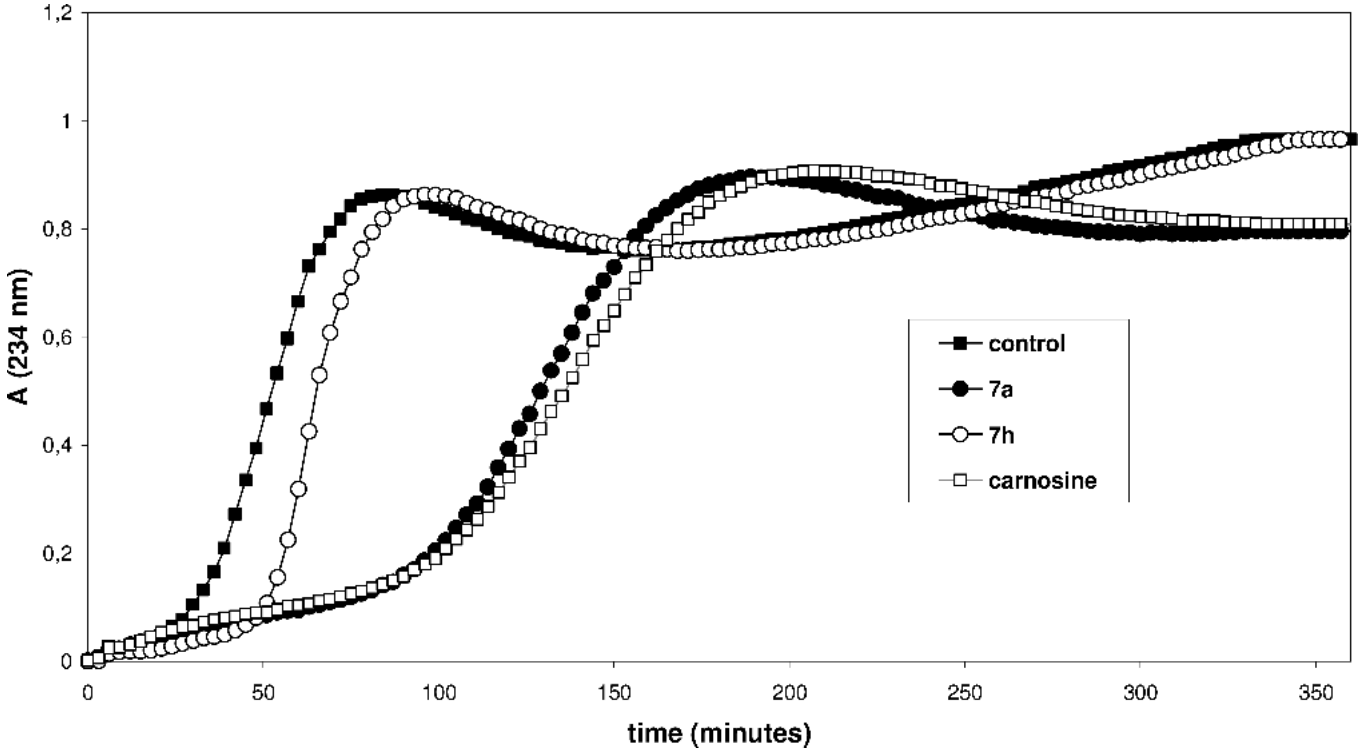


Figure 2. Relationship between antioxidant activity (expressed as Δt_{lag}) and log $D^{7.4}$ for carnosine amide derivatives **7a**-**n**; compound **12** is also showed for comparison.

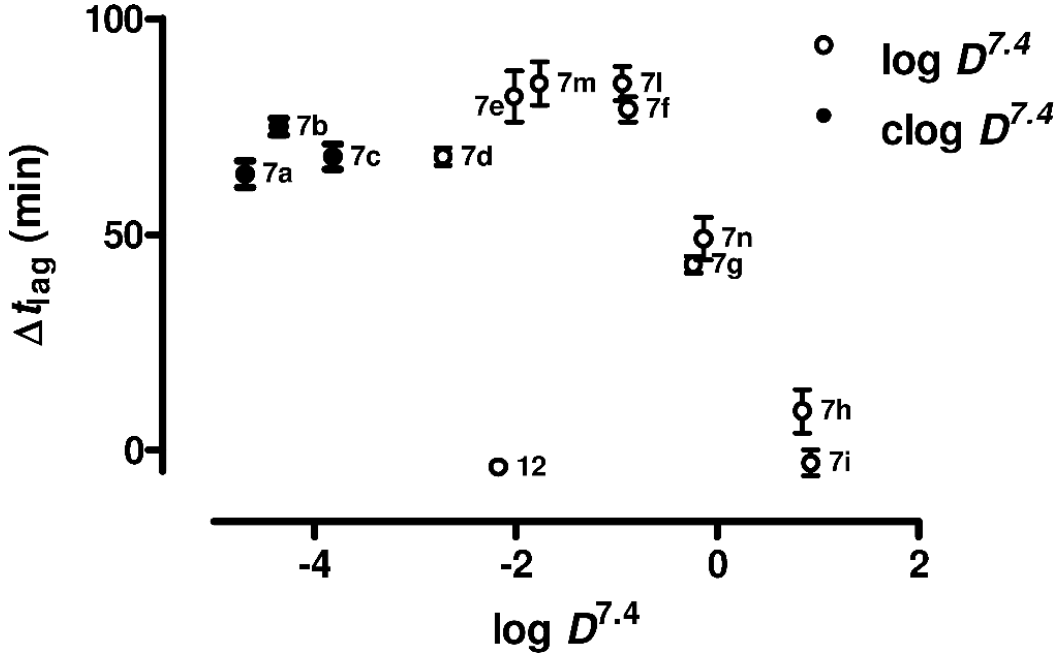
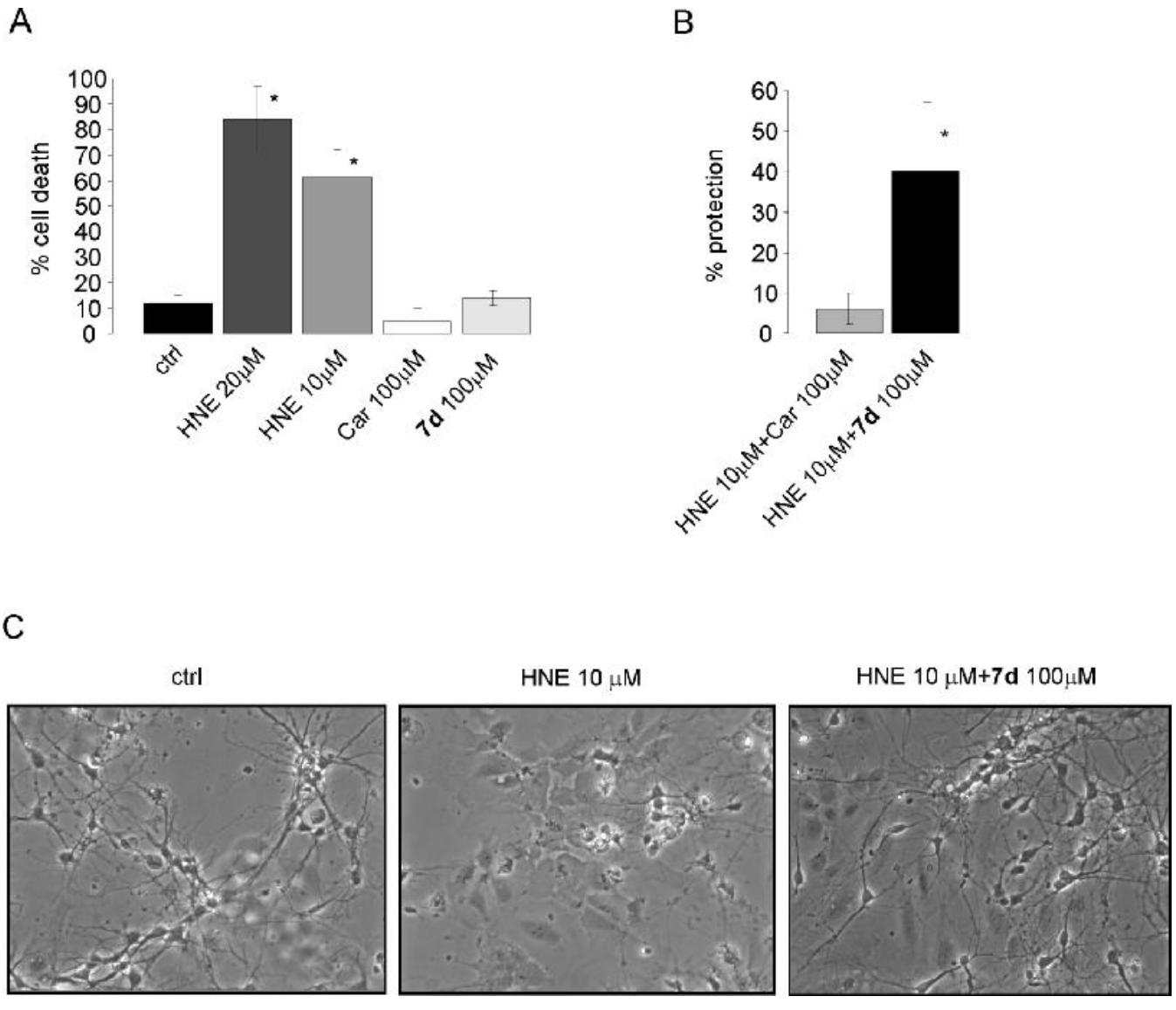
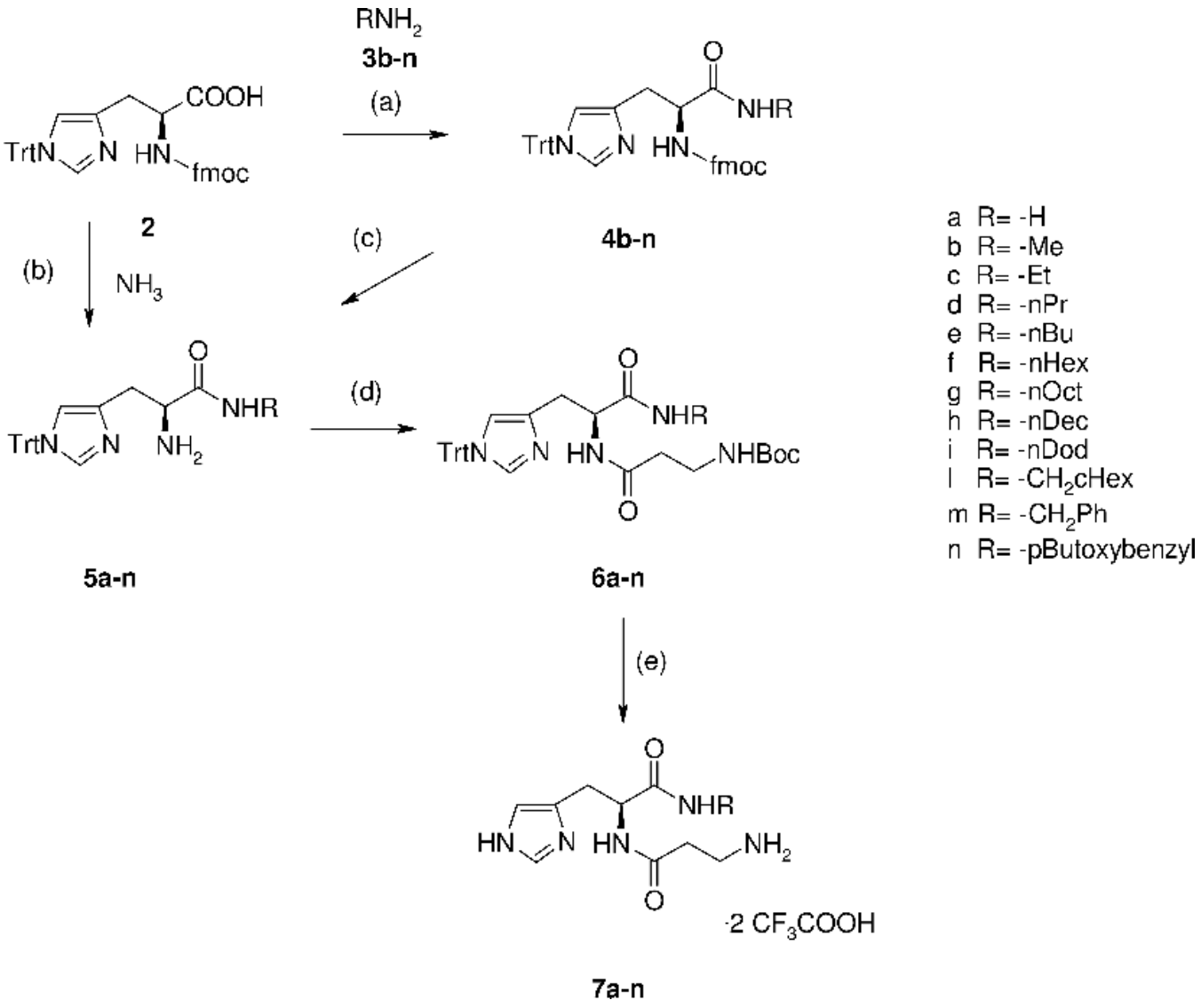


Figure 3. In vitro cytotoxicity on hippocampal neurons. Panel A. Percentage of cell death induced by different compounds on hippocampal neurons after 24 h exposure; **Panel B**. Percentage of cytoprotection after 24 h exposure of hippocampal cells to HNE (10 μM) and carnosine 100 μM (grey bar) or **7d** 100 μM (black bar). **Panel C**. Images at the optical microscope at 20X magnification. On the left a group of control cells after 24 h. In the middle a group of cell exposed for 24 h to HNE 10 μM. Cell death is evident. In the last panel, on the right, a group of cells after 24 h incubation with HNE 10 μM plus **7d** 100μM. Reported results are given as mean ± SEM for n (n= 4-7) experiments. Statistical significance was calculated by using Student's paired t test. Values of $p<0.05$ were considered significant.

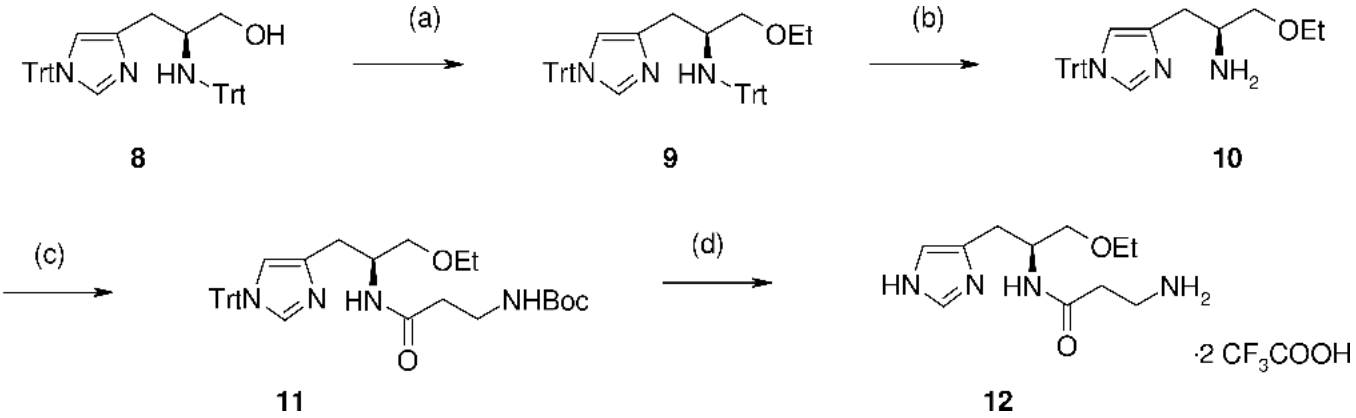


Scheme 1. Preparation of carnosine carbonamides **7a-n**[a]



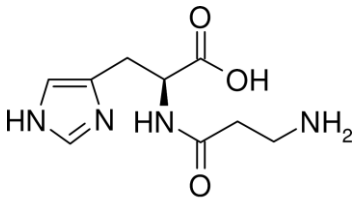
[a] Reagents and conditions: a) DIPEA, HBTU, $HOBt_{cat}$, 10 min r.t., then **3b-n** 1-18 h, r.t. b) DIPEA, HBTU, $HOBt_{cat}$, 10 min r.t. then NH_3 1 h, r.t. c) Piperidine , dry DMF, 1 h, r.t. d) Boc-β-Ala(OH), DCC, dry CH_2Cl_2, 0 °C to r.t. 2-18 h. e) CF_3COOH 10 % in CH_2Cl_2, 20 h , r.t.

Scheme 2. Preparation of carnosine ethylether **12**[a]



[a] Reagents and conditions: a) NaH, dry DMF, 1h, r.t., then EtBr 18 h, r.t. b) CF_3COOH 1 % in CH_2Cl_2, 0 °C, 2 min. c) Boc-β-Ala(OH), DCC, dry CH_2Cl_2, 0 °C to r.t., 18 h. d) CF_3COOH 10 % in CH_2Cl_2, 20 h , r.t.

Chart 1.



1

β-alanyl-L-histidine (Carnosine)

Table 1. Dissociation constants and lipophilicity parameters for carnosine, **7a-n** and **12**

Compd.	pK_{a1} [a]	pK_{a2} [a]	pK_{a3} [a]	Clog $D^{7.4}$ [b]	Log $D^{7.4}$ [c]
Carnosine[d]	2.60	6.79	9.42	ND	ND
7a	-	6.17	9.16	- 4.68	ND
7b	-	6.18	9.19	- 4.35	ND
7c	-	6.17	9.16	- 3.81	ND
7d	-	6.18	9.19	- 3.30	-2.72
7e	-	6.18	9.19	- 2.78	- 2.01
7f	-	6.21	9.18	- 1.72	- 0.88
7g	-	6.21	9.18	- 0.66	- 0.23
7h	-	6.17[e]	9.21[e]	0.39	0.85
7i	-	6.16[e]	9.20[e]	1.46	0.93
7l	-	6.19	9.20	- 1.73	- 0.94
7m	-	6.22	9.19	- 2.49	- 1.76
7n	-	6.19	9.20	- 0.99	- 0.13
12	-	6.60	9.18	- 2.88	- 2.17

[a] Determined by potentiometric titration with GLpKa apparatus; $n \geq 4$, SD < 0.03. [b] Calculated according to the equation Clog $D^{7.4}$ = CLOGP - log $[1+10^{(pKa_3-pH)}+10^{(pKa_3-pKa_2-2pH)}]$; CLOGP for windows, v.1.0 Biobyte Corp., Claremont, CA, USA; [c] Determined by shake-flask technique; $n \geq 6$, SD < 0.2. [d] Reported values are in agreement with those reported in literature[28] (pK_{a1} = 2.59, pK_{a2} = 6.77, pK_{a3} = 9.37). [e] Data obtained with 20-34 % methanol as cosolvent; aqueous pK_a values were obtained by extrapolation at 0% methanol using the Yasuda-Shedlovsky procedure.[51] ND = not determined.

Table 2. Formation constants ($\log \beta_{pqr}$)[a] of copper(II) complexes for carnosine and derivatives **7a**, **7d**, **7g**, **7l**, **7m**, **12** and logarithm of the ratio between $[Cu^{2+}]_{total}$ and $[Cu^{2+}]_{free}$

Compd.	**logβ_{111}**	**logβ_{110}**	**logβ_{11-1}**	**logβ_{22-2}**	**logβ_{22-3}**	**logβ_{22-4}**	**logβ_{120}**	**log ($[Cu^{2+}]_{tot}$ / $[Cu^{2+}]_{free}$) [b]**
7a	12.62(5)	6.82(3)	1.15(8)	5.09(3)	-2.82(4)	-12.23(5)	13.86(4)	2.89
7d	12.22(4)	6.72(2)	1.25(7)	5.40(2)	-3.09(5)	-12.67(6)	13.47(4)	2.93
7g	12.47(5)	6.59(3)	1.55(6)	5.52(2)	-2.46(4)	-11.68(5)	13.81(4)	3.19
7l	12.71(4)	7.05(3)	1.53(7)	6.35(2)	-2.41(4)	-12.32(5)	13.27(4)	3.00
7m	12.69(5)	7.01(3)	1.50(7)	6.20(2)	-2.84(4)	-12.74(6)	13.26(4)	3.09
12	13.58(4)	7.30(2)	0.16(5)	-	-	-	13.30(4)	1.95
carnosine	13.30[c]	8.47[c]	2.44[c]	8.35[c]			14.05[d]	3.80

[a] In parentheses errors (± 3σ) in the last significant figures. [b] Calculated for $[Cu^{2+}]_{total} = 2.5 \times 10^{-6}$ M and $[ligand]_{total} = 1 \times 10^{-4}$ M at pH 7.4.[c] According to lit.[28] [d] According to lit.[53]

Table 3. Antioxidant activity and HNE scavenging ability of derivatives **7a-n**, **12** and carnosine

	Antioxidant activity		**HNE scavenging ability**		
Compd	**Δt_{lag} (min) ± SEM** [a]	**R (nmol min^{-1} mg^{-1} LDL prot) ± SEM** [a]	**HNE scavenged (%) ± SEM** [b] **1 h**	**HNE scavenged (%) ± SEM** [b] **6 h**	**HNE scavenged (%) ± SEM** [b] **24 h**
Carnosine	67 ± 2	5.8 ± 0.3	20.4 ± 1.7	59.6 ± 1.6	88.8 ± 1.6
7a	64 ± 3	7.7 ± 0.4	10.2 ± 1.7	31.6 ± 1.4	77.7 ± 1.7
7b	75 ± 2	6.8 ± 0.6	8.0 ± 1.6	35.2 ± 0.8	75.4 ± 2.4
7c	68 ± 3	7.0 ± 0.3	8.4 ± 1.0	32.9 ± 1.1	70.9 ± 2.2
7d	68 ± 2	7.7 ± 0.3	12.5 ± 1.9	42.7 ± 2.0	81.0 ± 1.6
7e	82 ± 6	5.7 ± 0.3	12.5 ± 1.7	36.2 ± 1.2	75.2 ± 1.3
7f	79 ± 3	6.7 ± 0.4	12.0 ± 1.0	35.4 ± 1.9	75.9 ± 2.3
7g	43 ± 2	11.8 ± 0.4	12.8 ± 2.3	49.7 ± 1.9	86.7 ± 1.8
7h	9 ± 5	21.0 ± 1.0	ND	ND	ND
7i	-3 ± 3	14.5 ± 0.2	ND	ND	ND
7l	85 ± 5	5.6 ± 0.1	12.8 ± 3.2	28.2 ± 2.8	62.5 ± 2.1
7m	85 ± 4	5.2 ± 0.2	7.2 ± 1.4	29.3 ± 1.6	72.8 ± 0.6
7n	49 ± 5	10.0 ± 0.3	8.8 ± 1.6	32.6 ± 1.6	80.7 ± 0.8
12	- 4 ± 1	12.2 ± 0.4	17.9 ± 1.6	60.7 ± 1.2	86.2 ± 0.3

[a] Obtained by $CuSO_4$-induced human LDL oxidation assay in the presence of compounds at 100 μM. *R* values were calculated from ΔA_{234} as a function of time, using ε_{234} = 29500 M^{-1} cm^{-1} for conjugated lipid peroxides. For control LDL samples *R*= 12.1 ± 0.5 nmol min^{-1} mg^{-1} LDL prot. [b] Determined in phosphate buffer (pH 7.4, 1 mM) at 37 °C; HNE (50 μM), test compound (1 mM). Scavenging % was calculated according to the following formula: scavenging (%) = 100 –{[(amount of HNE left after *t* h in the presence of the scavenger) / (amount of HNE left after *t* h in the control)] x 100}. ND = not determined.

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Table of Contents graphic

SYHNTHESIS, PHYSICO-CHEMICAL CHARACTERIZATION AND BIOLOGICAL PROPERTIES OF NEW CARNOSINE DERIVATIVES STABLE IN HUMAN SERUM AS POTENTIAL NEUROPROTECTIVE AGENTS

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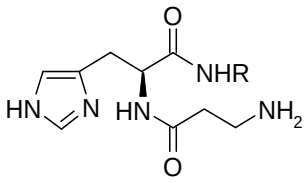

R = alkyl chain or aryl-substituted alkyl chain.