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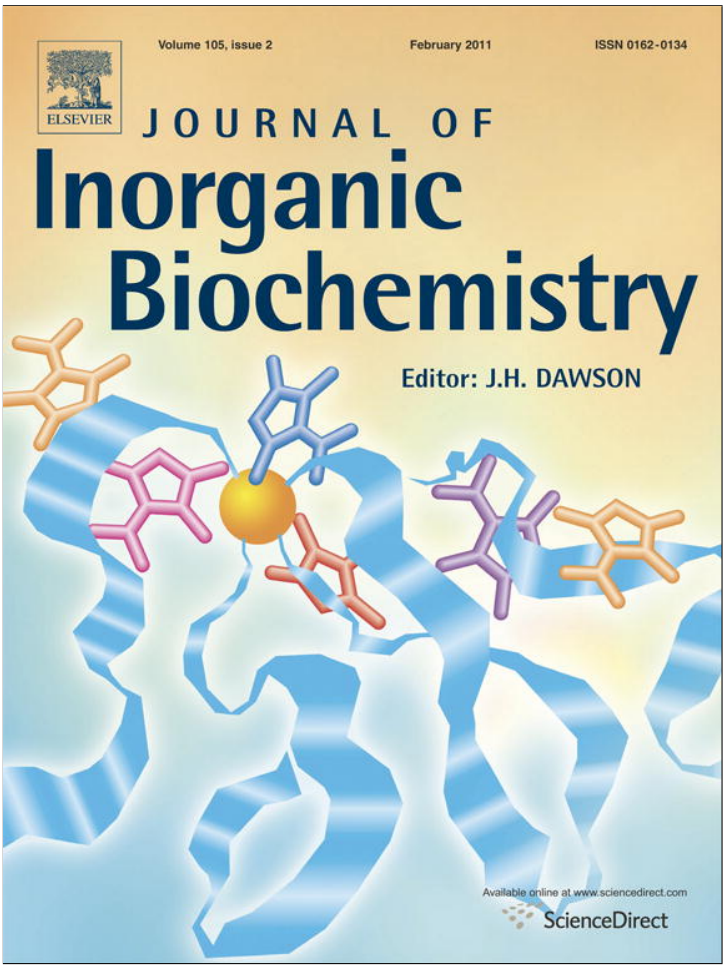

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New glycoside derivatives of carnosine and analogs resistant to carnosinase hydrolysis: Synthesis and characterization of their copper(II) complexes

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ABSTRACT

Carnosine (β-alanyl-L-histidine) is an endogenous dipeptide widely and abundantly distributed in muscle and nervous tissues of several animal species. Many functions have been proposed for this compound, such as antioxidant and metal ion-chelator properties. However, the main limitation on therapeutic use of carnosine on pathologies related to increased oxidative stress and/or metal ion dishomeostasis is associated with the hydrolysis by the specific dipeptidase carnosinase. Several attempts have been made to overcome this limitation. On this basis, we functionalized carnosine and its amide derivative with small sugars such as glucose and lactose. The resistance of these derivatives to the carnosinase hydrolysis was tested and compared with that of carnosine. We found that the glycoconjugation protects the dipeptide moiety from carnosinase hydrolysis, thus potentially improving the availability of carnosine. The copper(II) binding properties of all the new synthesized compounds were investigated by spectroscopic (UV–Visible and circular dichroism) and ESI-MS studies. Particularly, the new family of amide derivatives that are not significantly hydrolyzed by carnosinase is a very promising class of carnosine derivatives. The sugar moiety can act as a recognition element. These new derivatives are potentially able to act as chelating agents in the development of clinical approaches for the regulation of metal homeostasis in the field of medicinal inorganic chemistry.

1. Introduction

Carnosine (β-alanyl-L-histidine, AH) is an endogenous dipeptide widely and abundantly distributed in muscle and nervous tissues of several animal species [1]. This peptide is extensively used for nutraceutical applications [2–5].

Many functions have been proposed for this compound, including physiological buffer, wound healing promoter, ion-chelating agent, especially for Cu^{II} and Zn^{II}, antioxidant and free-radical scavenger [6,7]. In fact, this dipeptide prevents cellular toxicity *in vitro*, showing anti-peroxidative activity on proteins [8], lipids [9] and DNA bases [10]. The carnosine efficacy as an antioxidant and anti-inflammatory agent has been observed on lung injury caused by bleomycin administration [11] and ischemia/reperfusion liver injury in rats [12].

The metal binding ability of AH especially for copper(II) and zinc (II) ions has extensively been studied [13]. The copper- and zinc-mediated neurotoxicity involved in several pathologies, such as ALS, Alzheimer's and Parkinson's diseases [14], might be reduced or prevented by endogenous metal-chelating agents, such as AH and its derivatives [15,16]. Other diseases with neuropathological components in which copper has been implicated include Alzheimer's, Menkes's, Wilson's, and Pick's disease [17]. Therefore, understanding the role of endogenous compounds that are able to modulate copper availability and that have putative neuromodulatory and/or neuroprotective actions, such as carnosine, may help in the development of clinical approaches for the treatment of neuropathologies that involve metals and free radicals. Recently, it has been also proved that the Zn^{II}-carnosine complex (polaprezinc) is effective for the repair of ulcers and other lesions in the alimentary tract [18,19].

The peptidic nature of carnosine compromises its therapeutical uses and its chelating action, mainly for the breakdown by specific dipeptidases. The carnosine concentration in the animal species is regulated by the activity of the metalloprotease carnosinases. In mammals, two dipeptidases have been characterized: the serum-circulating form ('serum carnosinase', CN1), secreted by brain cells into the cerebrospinal fluid [20–22] and the cytosolic isoform ('tissue carnosinase', CN2), a non-specific dipeptidase distributed in several human tissues and in the rodent brain [20,23,24].

Recently, several carnosine derivatives with saccharides, such as β-cyclodextrin [25–28], and trehalose [29], have been synthesized. All these compounds are able to scavenge hydroxyl radicals and their copper(II) complexes exhibit SOD (superoxide dismutase) activity [30,31]. Furthermore, they are resistant to the hydrolysis of the carnosinase [29,32] and have an antioxidant efficacy at concentrations 10–20 times lower than that reported for other synthetic derivatives [33].

An important physiological role of the conjugating moiety in the carnosine derivatization is enhancing the bioavailability of the

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dipeptide by facilitating the site-specific transport to different tissues. In recent years, it has been shown that the animal lectins, galectins, are important mediators in inflammatory diseases [34–36]. The important role that lectins played in recognition processes has prompted efforts to synthesize glycoconjugates of small molecules [37–39] or proteins, such as albumin or SOD [40], to be specifically bound to a selected lectin. In this way the chelation therapy approach based on carnosine action could be successful in specific tissues.

On this basis, we functionalized carnosine and its amide derivative ($AHNH_2$) with small sugars such as glucose and lactose and their structural characterization by NMR was carried out (Chart 1). The resistance of these derivatives to the carnosinase hydrolysis was tested and compared with that of the AH. The Cu^{II} binding properties of all the synthesized compounds were investigated by spectroscopic (UV–Visible and circular dichroism).

ESI-MS studies. Coherently with our previously reported results, the glycoconjugation protects the dipeptide moiety from the carnosinase hydrolysis, thus potentially improving its availability. Moreover, the characterization of the Cu^{II} complexes has brought to light similarities and differences among all the glycoside conjugates and the unconjugated dipeptides.

2. Experimental

2.1. Chemicals

Commercially available reagents were used directly unless otherwise noted.

α-D-lactose (Fluka), anhydrous N,N-dimethylformamide (DMF) and dichloromethane (Aldrich), L-carnosine (Sigma) were used without further purification. Pentacetyl- α- D- glucose (Fluka) was dried with P_2O_5, for 24 h at 90 °C.

Thin layer chromatography (TLC) was carried out on silica gel plates (Merck 60-F254). Glycoside derivatives were detected on TLC by anisaldehyde test. The histidine derivatives were detected on TLC by Pauli test.

Merck Silica Gel (40–60 μm) and Merck Lichroprep Rp-8 (40–63 μm) were used for column chromatography. CM Sephadex C-25 (Sigma) NH_4^+ form and Sephadex-DEAE A-25 (Sigma) HCO_3^- form were used for ion exchange chromatography.

Carnosine ethyl ester was synthesized from AH in ethanol at 0 °C with acetylchloride. 2-bromoethyl-(2,3,4,6-tetraacetyl)-β-D-glucopyranoside and 2-bromoethyl-(2,3,4,6-tetraacetyl-β-D-galactopyranosyl)-(1→4)-2,3,6-triacetyl-β-D-glucopyranoside were synthesized as reported elsewhere [41,42].

2.2. Synthesis of carnosine amide ($AHNH_2$)

Boc-β-Ala (0.100 g, 0.53 mmol) (boc = tert-butyloxycarbonyl) was dissolved in 4 mL of anhydrous DMF, along with the coupling agents HOBT (0.0846 g, 0.53 mmol) and HBPyU (0.229 g, 0.53 mmol). $HisNH_2 \cdot 2HCl$ (0.122 g, 0.53 mmol) was separately dissolved in DMF after having added triethylamine (150 μL). After 30 min at room temperature under stirring, the solutions were combined and the coupling reaction was carried out for 3 h. The solvent was then evaporated at 40 °C under vacuum and the crude product was purified by reversed-phase chromatography on Lichroprep Rp-8 column (20×300 mm). Boc-$AHNH_2$ was eluted by a linear gradient of H_2O–CH_3OH (0 to 80%). The fractions containing the product were pooled, concentrated under vacuum and the residue was dissolved in trifluoroacetic acid (2 mL) in order to remove the t-Boc protecting group. After 2 h under stirring at room temperature, the final product was purified by cation-exchange chromatography on CM Sephadex C-25 (25×160 mm, NH_4^+ form). The amidated product was eluted by a linear gradient of NH_4HCO_3 (0–0.5 M). The fractions were collected and analysed by TLC and those containing the product were pooled and concentrated under vacuum at 40 °C.

Yield: 62%. $R_f = 0.72$ (PrOH-H_2O-NH_3-AcOEt, 5:3:2:1).

ESI-MS *m/z* [Found (Theoretical)] = 226.1 (226.1) $(M + H)^+$; 248.1 (248.1) $(M + Na)^+$. Elemental Analysis calcd. for $C_9H_{15}N_5O_2$ C 48.0, H 6.7, N 31.1; found C 47.5, H 6.5, N 30.7.

Abbreviations used for the reported ^1H NMR spectra are as follows: s = singlet, dd = double of doublets, t = triplet, dt = double of triplets, m = multiplet.

^1H NMR (500 MHz, D_2O) δ (ppm): 7.48 (s; 1H, H-2 of imidazole ring); 6.68 (s; 1H, H-5 of imidazole ring); 4.38 (dd; 1H, X of His, $J_{XA} = 8.3$ Hz, $J_{XB} = 6.0$ Hz); 2.96 (t; 2H , CH_2 in α to the NH_2); 2.90 (dd; 1H; B of His, $J_{AB} = 14.9$ Hz, $J_{BX} = 5.8$ Hz, 1H); 2.80 (dd; 1H; A of His, $J_{BA} = 14.9$, J_{AX} 8.4 Hz); 2.45 (t; 2H, CH_2 in β to NH_2).

2.3. Synthesis of 1-[2-(β-alanyl-L-histidine) ethoxy)]-β-D-glucopyranoside (GluAH)

Carnosine ethyl ester (1.0 g, 3.93 mmol) and 2-bromoethyl-(2,3,4,6-tetraacetyl)-β-D-glucopyranoside (2.0 g, 4.40 mmol) were added in anhydrous DMF (10 mL). The reaction was carried out at 70 °C, under nitrogen and under stirring. After 20 h, DMF was evaporated under vacuum at 40 °C. The residue was purified by a CM Sephadex C-25 column (40×900 mm, NH_4^+ form) using water as the eluent and then a linear gradient of NH_4HCO_3 (0–0.2 M). The fractions containing the product were concentrated under vacuum at 40 °C. The acetyl and the ethyl groups were hydrolysed by adding NaOH solution (1%), under stirring and under nitrogen for 2 h. The reaction mixture was purified by a CM Sephadex C-25 column (30×600 mm, NH_4^+ form) using water as the eluent.

Yield: 25%. Rf = 0.45 (PrOH-H_2O-NH_3-AcOEt, 5:3:2:1).

ESI-MS *m/z* [Found (Theoretical)] = 433.1 (433.2) $(M + H)^+$. Elemental Analysis calcd. for $C_{17}H_{28}N_4O_9 \cdot 2H_2O$ C 43.6, H 6.9, N 12.0; found C 43.2, H 6.8, N 12.2.

^1H NMR (500 MHz, D_2O) δ (ppm): 7.71 (s; 1H, H-2 of imidazole ring); 6.87 (s; 1H, H-5 of imidazole ring); 4.40 (d; 1H; H-1 Glu $J_{1,2} = 7.90$ Hz); 4.35 (dd; 1H, X of His $J_{XA} = 8.70$ Hz, $J_{XB} = 4.80$ Hz); 4.03 (dt; 1H, one H del CH_2 in α to O in 1 of Glu); 3.85 (dt; 1 H, other H of CH_2 in α to O in 1 of Glu); 3.81 (dd;1H, H-6 of Glu); 3.61 (dd; 1H, H-6' of Glu); 3.41 (t, 1H, H-3 of Glu); 3.36 (m, 1H; H-5 of Glu); 3.29 (t, 1H, H-4 of Glu); 3.20 (m, 5H, H-2, CH_2 in α to NH_2 of Ala, CH_2 in β to O of Glu); 3.03 (dd; 1H; A of His, $J_{BX} = 4.70$ Hz, $J_{AB} = 15.0$ Hz); 2.88 (dd; 1H; B of His, $J_{AX} = 8.70$ Hz; $J_{AB} = 15.0$ Hz); 2.60 (m, 2H; CH_2 in β to NH_2 of alanine).

2.4. Synthesis of 1-[2-(β-alanyl-L-histidine)ethoxy]-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranoside (LacAH)

The reaction was carried out as for GluAH, starting from 2-bromoethyl-(2,3,4,6-tetraacetyl-β-D-galactopyranosyl)-(1→4)-2,3,6-triacetyl-β-D-glucopyranoside.

Yield: 20%. Rf = 0.35 (PrOH-H_2O-NH_3-AcOEt, 5:3:3:1).

ESI-MS *m/z* [Found (Theoretical)] = 595.3 (595.2) $(M + H^+)$. Elemental Analysis calcd. for $C_{23}H_{38}N_4O_{14} \cdot 3H_2O$ C 42.6, H 6.8, N 8.6; found C 42.3, H 6.6, N 8.5.

^1H NMR (500 MHz, D_2O) δ (ppm): 7.70 (s, 1H; H-2 of imidazole ring); 6.88 (s, 1H; H-5 of imidazole ring); 4.43 (d, 1H; H-1 of Glu); 4.35 (m, 2H, X of His., H-1 of Gal); 4.03 (dt, 1H; H of CH_2 in α to O of Glu); 3.86 (m, 2H, H del CH_2 in α to O of Glu, H-6 of Glu); 3.83 (d, 1H, H-4 of Gal,); 3.70–3.58 (m, 4H, H-6' of Glu; H-5, H-6, H-6' of Gal), 3.56 (m, 3H, H-3 and H-4 of Glu and H-3 of Gal); 3.51 (m, 1H, H-5 of Glu); 3.45 (dd, 1H; H-2 of Gal); 3.21 (t, 1H; H-2 of Glu); 3.18 (m, 4H; CH_2 in α to NH_2 of β- Ala, CH_2 in β to O of Glu); 3.10 (dd; 1H; B of His); 2.90 (dd; 1H; A of His); 2.65 (m. 2H; CH_2 in β to NH_2 of β- Ala).

2.5. Synthesis of 1-[2-(β-alanyl-L-histidylamino)ethoxy]-β-D-glucopyranoside (GluAHNH$_2$)

Carnosine ethyl ester conjugate of the acetylated glucose was dissolved in NH_3 solution under stirring. After 4 h the solvent was evaporated under vacuum and the reaction mixture was purified by a CM Sephadex C-25 column (30×600 mm, NH_4^+ form) using water as the eluent and then a linear gradient of NH_4HCO_3 (0→0.1 M).

Yield: 25%. Rf = 0.58 (PrOH-H_2O-NH_3-AcOEt, 5:3:2:1).

ESI-MS *m/z* [Found (Theoretical)] = 432.2 (432.2) (M + H^+). Elemental Analysis calcd. for $C_{17}H_{29}N_5O_8 \cdot 2H_2O$ C 43.7, H 7.1, N 15.0; found C 43.3, H 7.0, N 14.7.

^{1}H NMR (500 MHz, D_2O) δ (ppm): 7.55 (s, 1H, H-2 of imidazole ring); 6.85 (s, 1H, H-5 of imidazole ring); 4.45 (dd, 1H, X of His, J_{BX} = 5.3 Hz, J_{AX} = 8.9 Hz); 4.31 (d, 1H, H-1 of glu); 3.85 (m, 1H, one H of CH2 in α to O in 1 of Glu), 3.77 (dd, 1H, H-6 of Glu); 3.63 (m, 1H, other H of CH_2 in α to O in 1 of Glu), 3.59 (dd, 1H, H-6 of Glu), 3.36 (t, 1H, H-3 of Glu), 3.32 (m, H-5 of Glu); 3.24 (t, 1H, H-4 of Glu), 3.15 (dd, 1H, H-2 Glu); 2.99 (dd, 1H, B of His, J_{BA} = 14.8, J_{BX} = 5.2 Hz); 2.85 (dd, 1H, A of His, J_{AB} = 14.9, J_{AX} 8.9Hz); 2.69 (m, 4H, CH_2 in β to O in 1 of Glu and CH_2 in α to NH_2 of Ala); 2.34 (m, 2H, CH_2 in β to the NH_2 of β-Ala).

2.6. Synthesis of 1-[2-(β-alanyl-L-histidylamino)ethoxy)]-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranoside (LacAHNH$_2$)

The synthesis was carried out as reported for the glucose conjugate, starting from carnosine ethyl ester conjugate of the acetylated lactose.

Yield: 20%. Rf = 0.42 (PrOH-H_2O-NH_3-AcOEt, 5:3:3:1).

ESI-MS *m/z* [Found (Theoretical)] = 594.5 (594.3) (M + H^+). Elemental Analysis calcd. for $C_{23}H_{39}N_5O_{13} \cdot 4H_2O$ C 41.5, H 7.1, N 10.5; found C 41.1, H 7.0, N 10.3.

^{1}H NMR (500 MHz, D_2O) δ (ppm): 7.56 (s, 1H, H-2 of imidazole ring); 6.83 (s, 1H, H-5 of imidazole ring); 4.44 (dd, X of His, J_{XB} = 8.6, J_{XA} = 5.7 Hz); 4.33 (d, 1H, H-1 of Glu, $J_{1,2}$ = 7.9 Hz, 1H); 4.30 (d, 1H, H-1, Gal, $J_{1,2}$ = 7.8 Hz,); 3.81 (m, 2H, one H of CH_2 in α to O in 1 and H-6 of Glu) 3.77 (d, 1H, H-4 of Gal); 3.70–3.55 (m, 5H, other H of CH_2 in α to O in 1, H-6' of Glu, H-6, H-6', H-5 of Gal); 3.51 (m, 3H, H-3 and H-4 of Glu and H-3 of Gal); 3.45 (m, 1H, H-5 of Glu); 3.40 (t, 1H, H-2 of Gal), 3.17 (t, 1H, H-2 of Gal); 2.96 (dd, 1H, H of CH_2 of His, J_{BA} = 14.9 Hz, J_{BX} = 5.8 Hz,); 2.83 (dd, 1H, H of CH_2 of His, dd, J_{AB} = 14.8 Hz, J_{AX} 8.5 Hz, 1H); 2.61 (m, 4H, 2H del CH_2 in β to O in 1 of Glu, 2H del CH_2 in α to NH_2 of β- Ala); 2.21 (m, 2H, 2H of CH_2 in β to NH_2 of β-Ala).

2.7. Copper(II) complexes of carnosine derivatives

The copper(II) complexes were prepared by adding a solution of copper nitrate to a ligand water solution in 1:1 ratio.

2.8. Spectroscopic measurements

^{1}H NMR spectra were recorded at 25 °C in D_2O with a Varian Inova 500 spectrometer at 499.883 MHz. The ^{1}H NMR spectra were obtained by using standard pulse programs from Varian library. In all cases the 90° pulse length was ca. 7 ms. The 2D experiments were acquired using 1K data points, 256 increments and a relaxation delay of 1.2 s. DSS was used as external standard.

UV-Visible spectra were recorded with Agilent 8452A diode array spectrophotometer. The spectra were recorded at 25 °C, on freshly prepared aqueous solutions. The spectral range (200–700 nm) was covered using quartz cells of various path lengths.

Fluorescence spectra were recorded by combining Cary Eclipse fluorescence spectrophotometer (Varian Inc., Palo Alto, CA) and microplate recorder accessory (Varian Inc., Palo Alto, CA) at an excitation wavelength of 340 nm and emission wavelength of 450 nm.

All the ESI-MS measurements were carried out by using a Finnigan LCQ DECA XP PLUS ion trap spectrometer operating in the positive ion mode and equipped with an orthogonal ESI source (Thermo Electron Corporation, USA). Sample water solutions were injected into the ion source without the addition of any other solvent at a flow rate of 5 μL/min, using nitrogen as the drying gas. All the other experimental parameters were the same as described elsewhere [43,44]. Xcalibur software was used for the elaboration of mass spectra. Each species is indicated in the following with the *m/z* value of the first peak of its isotopic cluster. For a more accurate structural assignment, the relative intensity of the peaks in each cluster was compared with that of the peaks in the corresponding simulated modelling. To simplify the isotopic pattern, $^{63}Cu^{II}$ was used.

2.9. Carnosinase activity

The human carnosinase was purified from the culture medium of stably transfected Hela cells, as previously reported [45]. The carnosinase solution (3 μl) was incubated with any substrate (AH, GluAH, LacAH, $AHNH_2$, $GluAHNH_2$, $LacAHNH_2$) (1 mM) in 50 μL of 25 mM Tris/HCl (pH 8.0) for 1 h at 37 °C. The reaction was stopped by adding 10 μL of 3 M trichloroacetic acid (TCA). After centrifugation (15,000×*g* for 5 min), the final mixture was used to assay the histidine or histidine-amide content by a spectrofluorimetric method,[21] in 96-well plates. 90 μL of 2 M NaOH and 90 μL of 0.05% *o*-phthaldialdehyde were added to the deproteinized sample, incubated at 37 °C for 15 min until the addition of 90 μL of 4 M H_3PO_4. After 15 more min at 37 °C, the solution was left at room temperature for 30 min before fluorescence measurement (λ_{exc}: 340 nm and λ_{em}: 450 nm). The fluorescence readings were converted into concentration of histidine or histidine-amide by reference to a calibration curve prepared from various mixture of AH (or $AHNH_2$), β-alanine and His (or $HisNH_2$). The hydrolysis extent of any substrate was reported as percentage of the AH hydrolysis and calculated as the mean of five different experiments.

The time-dependent stability of all the substrates (AH, GluAH, LacAH, $AHNH_2$, $GluAHNH_2$, $LacAHNH_2$) was obtained by their incubation (100 μM) at 37 °C in the human serum, kindly gifted by the Central Analysis Laboratory (University Hospital of Catania). Aliquots of 100 μL were collected at intervals until 120 min, deproteinized with TCA (10 μL, 3 M), diluted with 40 μL of 25 mM Tris/HCl (pH 8.0) and then treated as above reported.

3. Results and discussion

3.1. The ligands

The new conjugates of AH were synthesized with the aim of protecting the biological peptide from the carnosinase action, thus improving the potential pharmacological role of carnosine. This approach has been reported in the literature for biologically active peptides [46–49]. The synthesis of $AHNH_2$ was carried out with the same purpose; the glycoside moiety conjugated to both AH and $AHNH_2$ may potentially improve the bioavailability of the dipeptides by means of the site-specific transport to different tissues.

The new carnosine conjugates were synthesized by alkylation of carnosine ethyl ester instead of the underivatized carnosine in order to improve the reaction yield. The AH and $AHNH_2$ conjugates were characterized by NMR and ESI-MS (see supplementary data section).

3.1.1. Carnosinase activity

The new glycoside derivatives of carnosine and carnosine amide were tested as substrates of human serum carnosinase in order to compare their hydrolysis extent to that of AH. The reported results (Table 1) clearly show very different efficiency of hydrolysis when using the histidine-containing dipeptides, indicating an almost 50

Table 1
Hydrolysis percentage of the dipeptide carnosine (AH) and carnosine amide ($AHNH_2$) and their glycoside derivatives with glucose (Glu) and lactose (Lac). The hydrolysis extent of any substrate was reported as percentage of the AH hydrolysis.

	AH	GluAH	LacAH	$AHNH_2$	$GluAHNH_2$	$LacAHNH_2$
% hydrolysis	100 ± 17	11 ± 2	10 ± 1	2.1 ± 0.2	1.0 ± 0.1	1.1 ± 0.1

times higher enzymatic activity with AH as substrate than that with $AHNH_2$. Furthermore, the results demonstrate that the glycoside derivatives of AH and $AHNH_2$ (GluAH, LacAH, $GluAHNH_2$, $LacAHNH_2$) were hydrolyzed in negligible amounts with respect to AH. The hydrolysis extent of carnosine derivatives (GluAH and LacAH) was nearly 10 times lower than that of AH, whereas the glycoside conjugates with carnosine amide ($GluAHNH_2$ and $LacAHNH_2$) were even less hydrolysed than $AHNH_2$.

Similar results were obtained when the time-dependent stability of all the substrates was assayed in the human serum. Fig. 1 clearly shows that AH (100 μM) is completely hydrolyzed within 30 min, but its derivatives are more resistant within the explored interval (2 h). LacAH and GluAH are at most about 50 and 40% hydrolyzed, respectively. $AHNH_2$ and its glycoside derivatives ($GluAHNH_2$ and $LacAHNH_2$) are more resistant to the serum hydrolysis with respect to the above-mentioned substrates and they keep about 80% of the initial concentration at the end of the incubation time.

All these experiments confirm that the chemical modification of both the histidine-containing dipeptides significantly reduces the carnosinase hydrolysis.

3.2. Copper complexes

The Cu^{II}-AH system has largely been investigated by different techniques [25,50–53]. A detailed thermodynamic characterization of the complex species has been also reported [25,54–56]. The main species formed at physiological pH and mM concentration of both the ligand and the Cu^{II} ion is the monomeric species $[Cu(AH)H_{-1}]$, together with a secondary dimeric species $[Cu_2(AH)_2H_{-2}]$.

The new Cu^{II}-glycoconjugate systems were characterized by ESI-MS, UV–Vis and CD spectroscopy. The experiments were carried out at different pH, from 5.0 to 9.0.

3.2.1. ESI-MS characterization

ESI-MS has been recognized as a powerful method for determining the stoichiometry and binding strength of metal–peptide [57] or metal–protein complexes [58,59]. A proper picture of the liquid-phase complexation processes can be obtained as long as the mass spectrometric data adequately reflects the metal–ligand complexes formed in solution. This is usually the case as the gentle nature of the ESI process permits some non-covalent intermolecular interactions to be studied, while the higher charge density of multiple-charge ions detected in ESI increases the effectiveness of collision-induced dissociation (CID), so that MS/MS spectra of appropriate ions might reveal sequence and structural information for peptides [60,61].

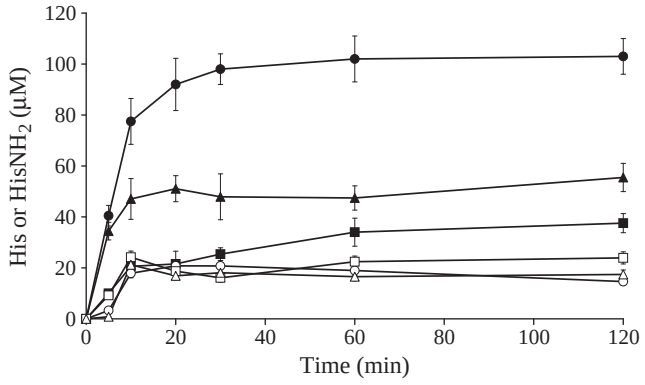


Fig. 1. Time-dependent hydrolysis of AH (●), GluAH (■), LacAH (▲), $AHNH_2$ (○), $GluAHNH_2$ (□) and $LacAHNH_2$ (△) in the human serum.

In Table 2 the ESI-MS characterizations of all the metal complexes with the glycoside derivatives of AH and $AHNH_2$ are reported, while in Fig. 2 representative ESI-MS spectra of Cu^{II} complexes with GluAH (a) and $GluAHNH_2$ (b) are shown.

The ESI-MS spectra of Cu^{II}-systems with AH derivatives (GluAH and LacAH) show the existence of the same complex species. $[CuLH_{-1}]$ is the main Cu^{II} species over the explored pH range. Its formation percentage increases at higher pH because of the amide deprotonation, as reported for analogous Cu^{II} systems with AH and its derivatives [25]. Noteworthy, its expected *m/z* value is equal to that of $[CuL]^+$ species. However, the latter can be reasonably excluded at pH ≥7 and considered a minor component of the observed peak at acid pH, as previously obtained by similar studies [52,62]. The species distribution diagram of the Cu^{II} complexes with analogous AH derivatives shows $[CuLH_{-1}]$ as the main species over pH 5.0 and $[CuL]^+$ as a minor species [25,54,55], further confirming our species assignment. At pH 7.0 the dimeric species $[Cu_2L_2H_{-2}]$ was observed for both Cu^{II} systems with GluAH and LacAH and it is more abundant at pH 9.0 (Fig. 2a). This is in agreement with the copper speciation of AH: the main complex species at pH >6 are $[CuLH_{-1}]$ and $[Cu_2L_2H_{-2}]$ [25].

The complex systems with $GluAHNH_2$ and $LacAHNH_2$ show the same pattern of species. Also in this case, $[CuLH_{-1}]$ is the main complex species from pH 7.0 to 9.0. However, two main features differ the spectra of both Cu^{II} systems with $GluAHNH_2$ and $LacAHNH_2$ from those with the AH derivatives: the absence of the dimeric species (Fig. 2b) and the presence of the $[CuLH_{-2}]$ species, detected for both Cu^{II}-$GluAHNH_2$ (Fig. 2b) and Cu^{II}-$LacAHNH_2$ systems. This finding suggests that in carnosine derivatives the formation of dimeric species is assisted by the carboxylate coordination with the copper(II) ion. In the case of the amide derivatives, the monomeric and the dimeric species ($CuLH_{-1}$ and $Cu_2L_2H_{-2}$) would have the same *m/z* value, but different charges (+1 and +2, respectively). Therefore, the *m/z* values of the relative isotopic cluster would differ by 1.0 or 0.5, respectively. Fig. 2b (inset) clearly shows only single-charged peaks. The deprotonation of both the backbone and terminal amide groups of the peptide moiety occurs in the copper complexes with the $AHNH_2$ derivatives and the formation of $[CuLH_{-2}]$ is in keeping with that analogous complex species found for similar systems [63,64].

The ESI-MS spectra of the Cu^{II}-$AHNH_2$ system (data not shown) are very similar to those found for the metal complexes with $GluAHNH_2$

Table 2
ESI-MS characterization of all the ^{63}copper(II) complexes with the glycoside derivatives of AH and $AHNH_2$. $[Cu^{2+}] = [ligand] = 1 \cdot 10^{-4}$M.

Ligand (L)	Assignment	Theoretical (m/z)	Observed (m/z)	Relative intensity (%)		
				pH 5.0	pH 7.0	pH 9.0
GluAH	LH_2^+	433.2	433.1	100	75	47
	$[CuLH_{-1}] + H^+$	494.1	494.2	13	100	100
	$[Cu_2L_2H_{-2}] + Na^+$	1009.2	1009.1	–	21	65
	$[Cu_2L_2H_{-2}] + K^+$	1025.2	1025.1	–	28	37
LacAH	LH_2^+	595.2	595.3	100	50	22
	$[CuLH_{-1}] + H^+$	656.2	656.2	34	100	100
	$[Cu_2L_2H_{-2}] + 2Na^+$	678.1	678.2	–	9	10
	$[Cu_2L_2H_{-2}] + 2K^+$	694.1	694.1	–	8	18
$GluAHNH_2$	LH^+	432.2	432.2	100	45	42
	$[CuLH_{-1}]^+$	493.1	493.2	–	100	100
	$[CuLH_{-2}] + Na^+$	515.1	515.2	–	11	36
	$[CuLH_{-2}] + K^+$	531.1	531.2	–	24	28
$LacAHNH_2$	LH^+	594.3	594.5	100	100	39
	$[CuLH_{-1}]^+$	655.2	655.1	9	83	100
	$[CuLH_{-2}] + Na^+$	677.2	677.3	–	–	24
	$[CuLH_{-2}] + K^+$	693.1	693.3	–	–	29

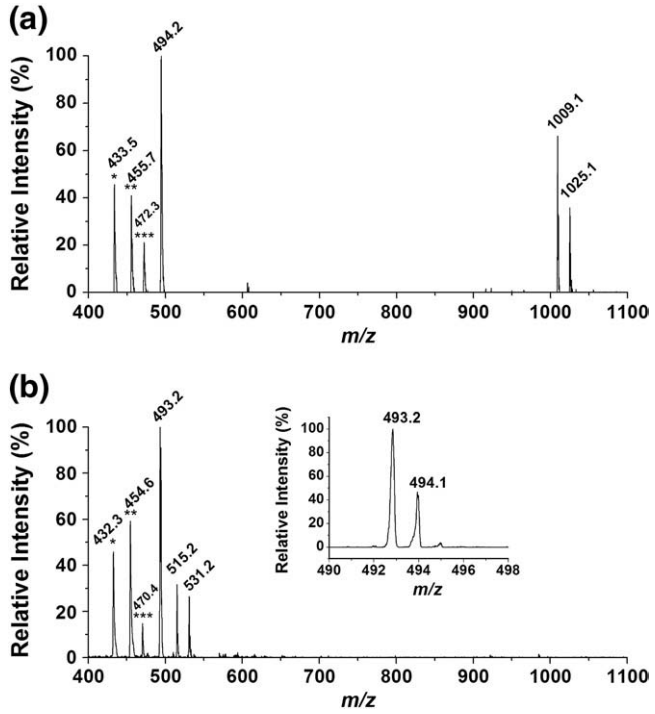


Fig. 2. ESI-MS spectra of the Cu^{II} complexes with GluAH (a) and $GluAHNH_2$ (b) at pH 9. In both cases, the free ligand appears as adduct with H^+ (*), Na^+ (**) and K^+ (***). The MS Zoom Scan of the main peak detected for the Cu^{II}-$GluAHNH_2$ system at pH 9 is also reported (b, inset).

and $LacAHNH_2$: [$CuLH_{-1}$] is the main complex species over pH 7, [$CuLH_{-2}$] appears at pH 7.0 and [$Cu_2L_2H_{-2}$] was not found.

In the course of our ESI-MS studies of complex systems, the formation of Cu^I species was also observed. In Fig. 2b (inset) the MS Zoom Scan of the main peak detected for the Cu^{II}-$GluAHNH_2$ system at pH 9 is reported. Beside the peak relative to the [$CuLH_{-1}$] species (*m/z* 493.2), the spectrum shows a peak at *m/z* 494.1, having a higher intensity than that expected for the relative isotopic peak. It corresponds to the CuL species, where copper is in the +1 oxidation state. The reduction of copper(II) during ESI-MS experiments has been observed earlier by others [65] and could be partially attributed to charge transfer, either because of ligand to metal electron transfer reactions or through a mechanism that happens in the electrospray source, when a high electric field is applied between the capillary and the counter electrode. In any case, the copper reduction was only observed for the Cu^{II}-$GluAHNH_2$ and Cu^{II}-$LacAHNH_2$ systems and not for the Cu^{II} complexes with GluAH and LacAH. We explain these interesting results by hypothesizing that the glycoside conjugates of carnosine amide species form copper complexes where the reaction $Cu^{2+} + e- \rightleftarrows Cu^+$ is more favourite than for non-amidated ones, as reported in the literature [66,67]. However, the copper reduction only occurs during the ESI-MS analysis as no reduction of copper(II) complexes was observed in solution.

3.2.2. Spectroscopic characterization

In Table 3 the spectroscopic parameters of all the new synthesized carnosine derivatives are reported, while the representative CD spectra of Cu^{II}-$LacAHNH_2$ and Cu^{II}-LacAH systems are reported in the Fig. 3.

The UV–Vis and CD spectra of the Cu^{II} complexes with carnosine derivatives (GluAH and LacAH) are not significantly different from those of the Cu^{II}-AH complex system. They do not show significant variations when pH changes from 7.0 to 9.0. Therefore, the same coordination behaviour of carnosine moiety for the new glycoconjugates can be suggested. The longer wavelength of the d–d band in the Vis spectra of copper(II) complexes with GluAH (622 nm) and LacAH (624 nm) in comparison with AH (604 nm) [68] has been typically found in other similar carnosine conjugates with β-cyclodextrin [25,68]. Similarly, we can assume that the alkylation of the AH produces a slight distortion of the metal coordination plane, producing a red shift and an increase of the ε values. The CD spectra show the positive charge transfer (CT) broad band at about 278 nm due to N-3d transition from the amino to the Cu^{II}. A shoulder at 326 nm due to the N^-–Cu^{II} and to the π_1 of the imidazole is also evident. As in the case of free AH, the monomeric species [$CuLH_{-1}$] together with a secondary dimeric species [$Cu_2L_2H_{-2}$] are the main species [51], in keeping with the ESI-MS results. The same coordination found for carnosine could be hypothesized (Fig. 4) in keeping with our previous investigation on similar conjugates [25].

Stability constants similar to that of carnosine may be reasonably hypothesized for the copper(II) complexes of new glycosylated carnosine derivatives, in keeping with the stability constant values reported for cyclodextrin [69,70] or other sugar [71] derivatives. On the basis of our spectroscopic investigation a similar speciation could be proposed. We have carried out some mass spectrometric experiments in order to confirm this hypothesis (see supplementary data section).

The UV–Vis and CD spectra of the amide derivatives are slightly different from those of carnosine derivatives. At pH 7, in the Vis spectra of Cu-$AHNH_2$ system, the λ (624 nm) and ε (93) values are consistent with the coordination of three nitrogen atoms with the copper(II) ion [72]. The slightly blue shifted λ_{max} value in comparison to that of the AH derivatives is due to the absence of the carboxylate group [73]. The intensities of all the dichroic bands in the CD spectra are lower than that of carnosine derivatives. The two bands due to the d–d transition have the same magnitude and opposite sign at 615 nm (negative) and 727 nm (positive), as generally found for the L-amino

Table 3
Spectroscopic data for the copper(II) complexes with GluAH, LacAH, $AHNH_2$, $GluAHNH_2$, $LacAHNH_2$.

System	pH	UV–vis λ/nm ($\varepsilon/M^{-1}cm^{-1}$)	CD λ/nm ($\Delta\varepsilon/M^{-1}cm^{-1}$)
Cu-GluAH	7.0	622 (113)	698 (0.35); 576 (−2.17); 326 (0.78); 278 (1.90)
	9.0	622 (113)	698 (0.35); 576 (−2.04); 325 (0.79); 278 (2.35)
Cu-LacAH	7.0	624 (112)	697 (0.42); 577 (−1.82); 327 (0.56); 275 (1.91)
	9.0	623 (109)	697 (0.42); 577 (−1.94); 327 (0.56); 275 (1.91)
Cu-$AHNH_2$	7.0	624 (93)	709 (0.25); 584 (−0.55); 322 (0.60); 252 (1.8)
	9.0	610 (92)	711 (0.48); 552 (−0.40); 321 (0.74); 310 (sh); 248 (2.56)
Cu-$GluAHNH_2$	7.0	631 (98)	723 (0.48); 612 (−0.49); 367 (0.07); 304 (−0.14); 256 (1.16); 228 (−2.06)
	9.0	619 (110)	711 (0.48); 599 (−0.35); 334 (0.21); 293 (−0.42), 257 (1.77); 228 (−1.07)
Cu-$LacAHNH_2$	7.0	629 (99)	726 (0.63); 616 (−0.64); 367 (0.07); 304 (−0.19); 258 (1.71); 229 (−2.40)
	9.0	616 (111)	711 (0.54); 596 (−0.46); 340 (0.26); 293 (−0.42), 258 (2.91); 228 (−3.52)

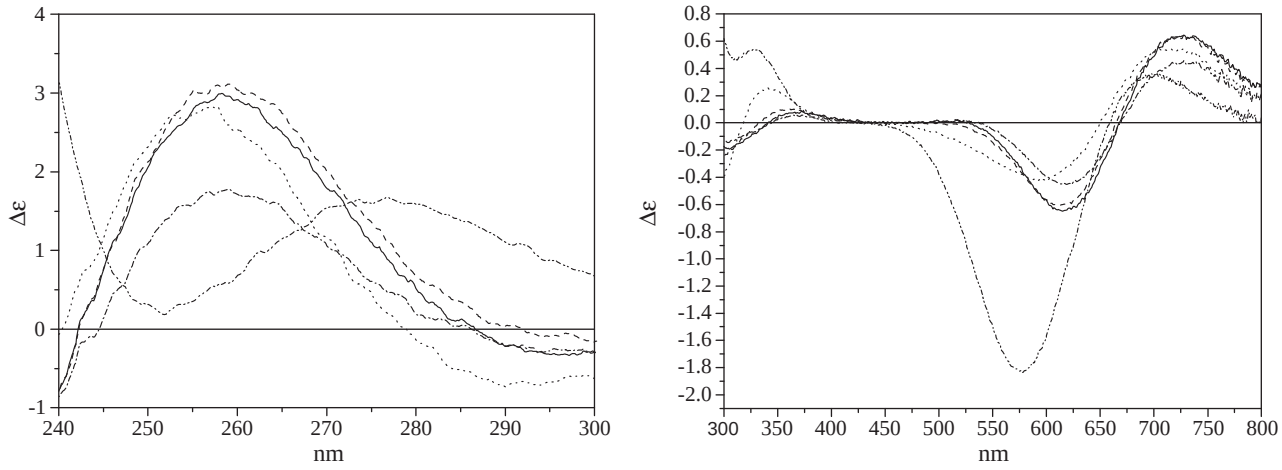


Fig. 3. The CD spectra for the Cu-LacAHNH$_2$ system at different pH 6.0 (-·-·), 7.0 (—), 8.0 (——), 9.0 () and for the Cu-LacAH system at pH 7.0 (-··-··-··).

acid and their amides [64]. In the UV-CD spectra the broad band at 248 nm originates from NH_2–Cu^{II} and π_2 imidazole to Cu^{II} CT transition, while the N^-–Cu^{II} and the π_1 of the imidazole CT transition occur at 321 nm [74]. The coordination of the amide derivatives by the amino group, amide deprotonated group and imidazole is suggested, in a similar way to the copper(II) coordination of carnosine (Fig. 5). The observation that $[Cu(AHNH_2)H_{-1}]^+$ is the main complex species in the ESI-MS spectra confirms the hypothesized coordination environment.

For these amide carnosine derivatives, the formation constant of the monomeric species $CuLH_{-1}$, that is the main species formed at physiological pH, should not be significantly different from that of carnosine in keeping with data reported for comparable systems [63,72].

Increasing the pH, a blue shift of the d–d band occurred in the Vis spectra. In the CD-UV spectra, the bands changes in the CT region: a new negative band appears at 286 nm and the band at 321 nm appears with increased intensities. The distinct intensity increase of the latter due to the $N^- \rightarrow 3d$ CT [75] suggests the involvement of primary amide function in the formation of the complex species $[Cu(AHNH_2)H_{-2}]$, as found in the case of other dipeptide amides [63,64,72]. Molecular models demonstrate that the four potential coordinating nitrogens cannot coordinate together in the equatorial plane. On this basis, the formation of a monomeric species involving the deprotonated primary amide group together to the amino and amido secondary groups could be proposed (Fig. 5). This new species is formed at basic pH in addition to the main species $[CuLH_{-1}]^+$, in keeping with the ESI-MS results. The imidazole might act as an apical ligand [76].

The spectra of the amide glycoconjugate copper(II) complexes are similar to those of carnosine amide. For the copper(II) complexes with $LacAHNH_2$ and $GluAHNH_2$, the band at c.a. 259 nm in the UV-CD spectra can be assigned to the CT transition from NH_2 and π_2 imidazole to Cu^{II}, the transition at c.a. 297 nm to the CT N^-–Cu^{II}. The low intensity band at 340 nm can be ascribed to the π_1 components of the imidazole [74]. These amide derivatives act as a tridentate ligand with three nitrogen donor atoms (amino group, deprotonated peptide amide, and imidazole), such as carnosine derivatives. When the pH increases, the d–d bands in the Vis spectra show a shift at lower λ with an increase in the molar absorptivity. These findings indicate a coordination of the primary amide group with the copper(II), as reported for similar systems [63] and consistently with the ESI-MS.

4. Conclusions

Carnosine is a widely studied dipeptide with potential pharmacological activity. Its potential applications are limited by carnosinase

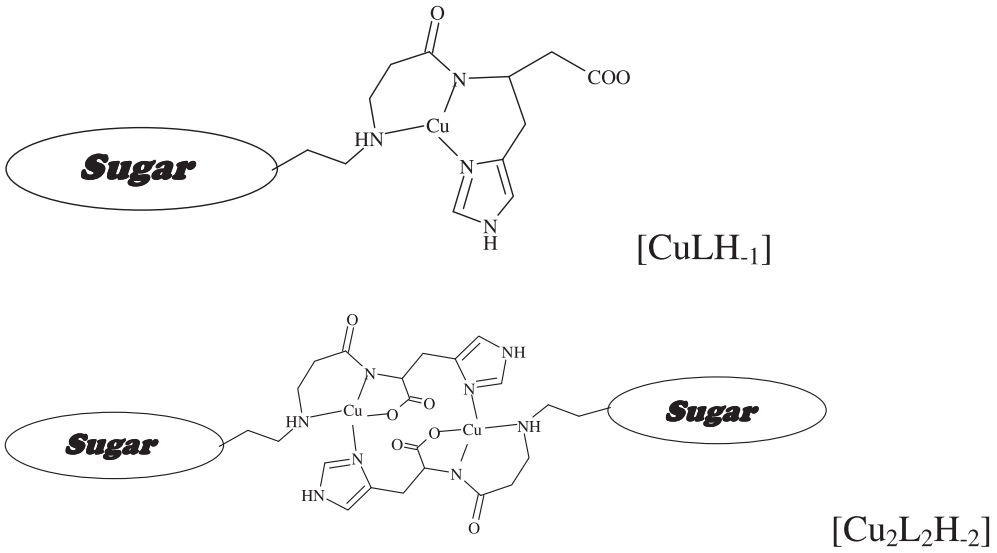


Fig. 4. Copper(II) complexes of AH derivatives.

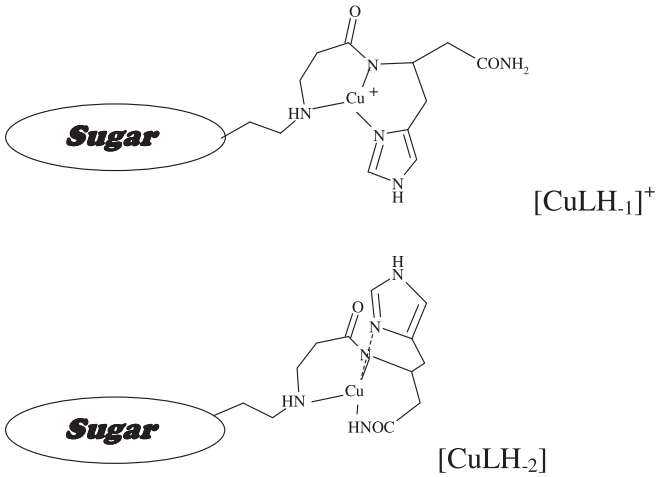
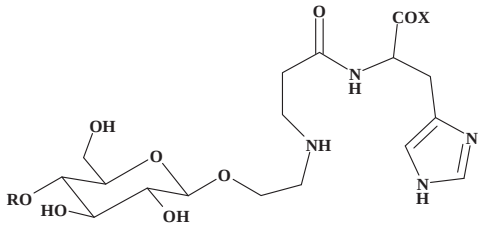


Fig. 5. Copper(II) complexes of $AHNH_2$ derivatives.

enzymes that strongly control carnosine homeostasis. The newly synthesized carnosine derivatives are very promising systems that are able to increase carnosine bioavailability and to maintain the coordination ability of metal ions such as copper(II).

Particularly, the new family of amide derivatives that are poorly hydrolyzed by carnosinase in the human serum, is a very promising class of carnosine derivatives. They are potentially able to act as chelating agents in the development of clinical approaches for the regulation of copper(II) homeostasis in the field of medicinal inorganic chemistry [77,78].

The presence of the sugar renders these derivatives capable of recognizing important biological systems such as the lectins. This feature should localize copper(II) chelating activity to the target tissue or even to the target cell compartment of interest.



	R	X
GluAH	H	OH
LacAH	D-galactopyranosyl	OH
$GluAHNH_2$	H	NH_2
$LacAHNH_2$	D-galactopyranosyl	NH_2

Abbreviations

AH	β-alanyl-L-histidine
Boc	tert-butyloxycarbonyl
CN	Carnosinase
COSY	Correlation spectroscopy
CT	Charge Transfer
DMF	N,N-dimethylformamide
ESI-MS	ElectroSpray Ionization Mass Spectrometry
HBPyU	*O*-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-bis(tetramethylene)uronium hexafluorophosphate
$HisNH_2$	L-Histidine amide
HOBT	1-Hydroxybenzotriazole
HSQC	Heteronuclear Single Quantum Coherence
PrOH	Propanol
SOD	Superoxide dismutase
TLC	Thin layer chromatography
TOCSY	Total correlation spectroscopy
Tris	Tris(hydroxymethyl)aminomethane

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jinorgbio.2010.10.014.

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