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Original article

New glycosidic derivatives of histidine-containing dipeptides with antioxidant properties and resistant to carnosinase activity

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Abstract

Synthesis, antioxidant properties and resistance to carnosinase hydrolysis of histidine-containing dipeptides are reported in this study. Carnosine (β-alanyl-L-histidine), homocarnosine (γ-aminobutyryl-L-histidine) and anserine (β-alanyl-3-methyl-L-histidine) were covalently derivatized with β-cyclodextrin to form different OH- or NH-bound conjugates. Mass spectroscopic and ^{1}H NMR data were used to determine the structure and the purity of the various β-cyclodextrin derivatives. The inhibitory effect towards oxidation of human LDL induced by Cu^{2+} ions, was estimated by measuring malondialdehyde formation as a function of increasing concentrations of these newly synthesized compounds (the β-cyclodextrin-anserine conjugated in 3 had the highest antioxidant effect). All derivatives had higher antioxidant effects than those of the corresponding free histidine-containing dipeptides. Resistance to rat brain carnosinase hydrolysis of the most active derivatives indicated that these compounds are good candidates for further studies in more complex cellular and animal models. Their possible applications for remedies in neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases, are discussed.

Keywords: Carnosine; Anserine; Homocarnosine; β-Cyclodextrin; Antioxidant activity; Neurodegeneration

1. Introduction

Carnosine (β-alanyl-L-histidine), homocarnosine (γ-aminobutyryl-L-histidine) and anserine (β-alanyl-3-methyl-L-histidine) (Chart 1) are the three most representative compounds of the family of the so called histidine-containing dipeptides, widely distributed in mammals in different amounts depending on the species and the tissue considered [1]. In human beings, carnosine is the most abundant of the aforementioned substances and it is localized in skeletal muscles [2] and in brain tissue, where it can reach in some specific areas amounts as high as those of the neurotransmitter glutamate [3,4]. As it occurs for other low molecular weight molecules of amino acidic nature present in relevant quantities in the cerebral tissue, such as *N*-acetylaspartate [5,6], *N*-acetylaspartylglutamate [7], *N*-acetylhistidine [8], there is not an unanimous opinion as far as the biological role of carnosine is concerned. Several evidences have been produced suggesting that carnosine might play an important role in the general brain antioxidant defenses [9–12], due to its ability to scavenge reactive oxygen species (ROS). On the other hand, different data supported a significant role for carnosine in contributing to intracellular

Abbreviations: ANS, anserine; AH, carnosine; HC, homocarnosine; CD, β-cyclodextrin; CDANS3, β-cyclodextrin-anserine conjugated in 3; CDANS6, β-cyclodextrin-anserine conjugated in 6; CDAH3, β-cyclodextrin-carnosine conjugated in 3; CDAH6, β-cyclodextrin-carnosine conjugated in 6; CDHC3, β-cyclodextrin-homocarnosine conjugated in 3; CDHC6, β-cyclodextrin-homocarnosine conjugated in 6; CDNHAH6, 6-amino-β-cyclodextrin conjugate with carnosine through the carboxyl group; LDL, low density lipoproteins; MDA, malondialdehyde.

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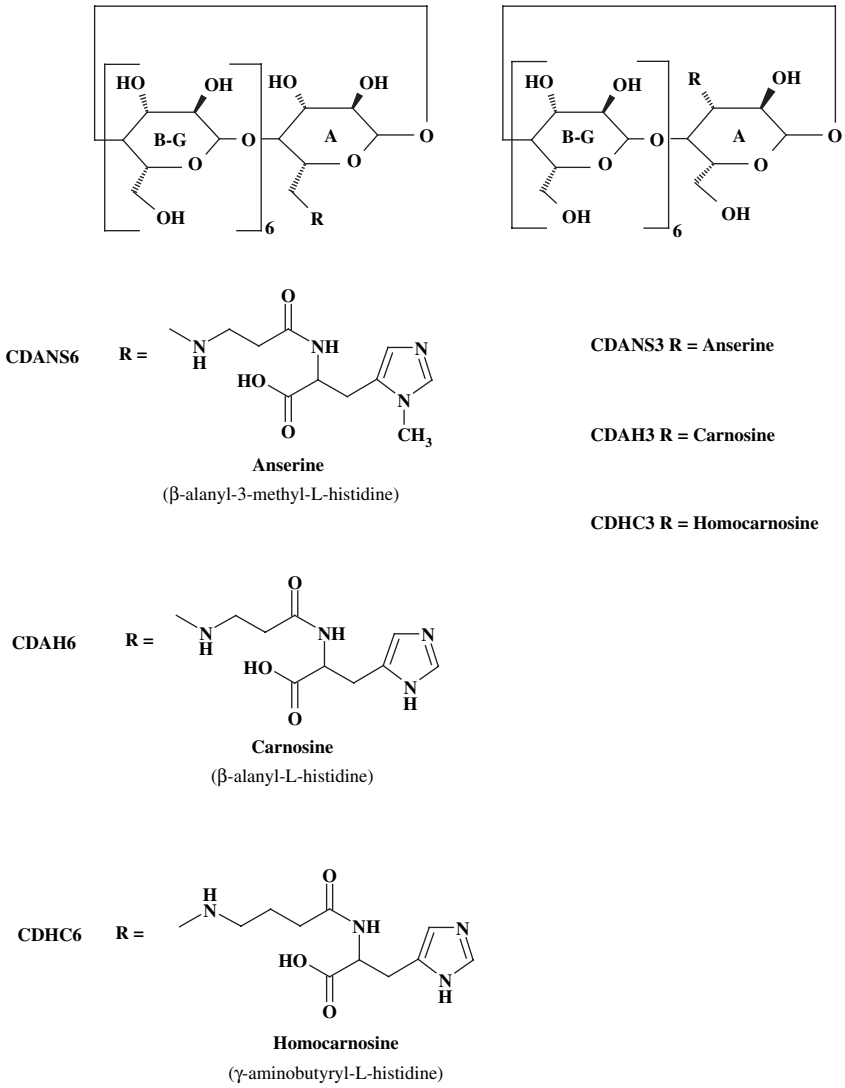

Chart 1. Molecular structures of carnosine, homocarnosine, anserine and their cyclodextrin conjugates.

pH maintenance, due to its pH buffering capacity [13], or to play a major function as an antiglycating agent with significant involvement in the aging process [14,15]. Recently, it has been evidenced that several aging-related molecular processes occurring to the cerebral tissue during neurodegenerative disorders (Alzheimer, Parkinson, etc.) are positively influenced by the members of the histidine-containing dipeptide family, with carnosine often showing the highest effectiveness [16–19].

The intracellular concentration of carnosine is regulated by the activity of the metalloprotease carnosinases. In mammals, two isoforms of these dipeptidases have been characterized. The serum-circulating form ('serum carnosinase', CN1, EC 3.4.13.20), secreted by brain cells into CSF, is rather selective for carnosine, homocarnosine and anserine [20–22]. The cytosolic isoform ('tissue carnosinase', CN2, EC 3.4.13.18) is a non-specific dipeptidase distributed in several human tissues and in rodent brain [22–24]. For this reason, the enzymatic hydrolysis of carnosine, homocarnosine and anserine is the main limitation for their possible effective pharmacological applications.

According to our previously synthesized β-cyclodextrin derivatives of carnosine [25,26] and homocarnosine [27] with significant ROS scavenging activity, we here describe the synthesis, chemical characterization and antioxidant activity of anserine cyclodextrin derivatives (see Chart 1) towards copper-induced human LDL oxidation. Having in mind the aim of generating carnosine and carnosine-like derivatives resistant to the enzymatic hydrolysis, we also determined the capacity of anserine cyclodextrin derivatives to survive the carnosinase hydrolysis induced by rat brain homogenate, extending this study also to the previously synthesized derivatives of carnosine [26] and homocarnosine [27].

2. Materials and methods

2.1. Chemicals

Purified human LDL and $CuSO_4 \cdot 5H_2O$, used to catalyze their oxidation, were obtained from Sigma (St. Louis, MO,

USA). Tetrabutylammonium hydroxide, used as the ion-pairing reagent for the HPLC detection of MDA, was obtained as a 55% water solution from Nova Chimica (Cinisello Balsamo, Milan, Italy).

β-Cyclodextrin (Fluka), anhydrous *N*,*N*-dimethylformamide (Aldrich), carnosine, homocarnosine and anserine (Sigma) were used without further purification. Thin layer chromatography (TLC) was carried out on silica gel plates (Merck 60-F254). CD derivatives were detected on TLC by UV and by anisaldehyde test or by Pauli test for the histidine derivatives.

Anserine methylester (ANSOMe) was synthesized from anserine (Sigma) with HCl in MeOH at 0 °C, using acetylchloride as HCl source. All other reagents were of the highest purity available from commercial sources.

2.2. NMR apparatus and conditions for spectral analysis

^{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 measured by using standard pulse programs from Varian library. In all cases the length of 90° pulse was ca. 7 μs. The 2D experiments were acquired using 1K data points, 256 increments and a relaxation delay of 1.2 s. T-ROESY spectra were obtained using a 300 ms spin-lock time. DSS was used as external standard.

2.3. Synthesis of 6^A-[(3-{[(1S)-1-carboxy-2-(1-methyl-1H-imidazol-5-yl)ethyl]amino}-3-oxopropyl)amino]-6^A-deoxy-β-cyclodextrin [CDANS6]

ANSOMe (1 g) was added to a solution of 6-deoxy-6-iodo-β-cyclodextrin (1 g) [28] in anhydrous DMF (10 ml). The reaction was carried out at 70 °C, under nitrogen and constant stirring. After 14 h, DMF was evaporated under vacuum at 40 °C. The crude product was purified using a CM Sephadex C-25 column (20 × 600 mm, NH_4^+ form). Water and then a linear gradient 0–0.1 M of NH_4HCO_3 solution (400 ml) were used as the eluent. The appropriate fractions containing the methylester of CDANS6 were combined. The product was hydrolyzed in NaOH solution (1%) in water/methanol for 1 h. The solvent was evaporated and the product was isolated by column chromatography on a CM-Sephadex C-25 (20 × 600 mm, NH_4^+ form) using water as the eluent. Yield: 20%, $R_f = 0.40$, eluent PrOH/H_2O/AcOEt/NH_3 5:3:2:3. FAB MS 1358 (M + 1).

^{1}H NMR (D_2O, 500 MHz) δ (ppm) 7.50 (s, 1H, H-2 of Im), 6.70 (s, 1H, H-5 of Im), 5.02–4.90 (m, 7H, H-1 of CD), 4.33 (dd, 1H, CH of His, $J = 4.4$ Hz, $J = 8.4$ Hz), 3.92–3.67 (m, 28H, H-3,-5,-6), 3.60–3.44 (m, 13H, H-2,-4), 3.34 (t, 1H, H-4A), 3.08 (dd, 1H, H of CH_2 of His, $J = 4.4$ Hz, $J = 15.6$ Hz), 3.03 (d, 2H, H-6A), 2.86 (dd, 1H, CH_2 of His, $J = 8.4$ Hz, $J = 15.6$ Hz), 2.80 (m, 3H, CH_2 of β-Ala in α to the NH and H-6A); 2.38 (m, 2H, CH_2 of β-Ala α to the CO).

2.4. Synthesis of (2^AS,3^AR)-3^A-[(3-{[(1S)-1-carboxy-2-(1-methyl-1H-imidazol-5-yl)ethyl]amino}-3-oxopropyl)amino]-3^A-deoxy-β-cyclodextrin [CDANS3]

The CDANS3 derivative was synthesized from the 2,3-mannoepoxide of β-CD formed in situ from 2-deoxy-2-[(*p*-tosyl)oxy]-β-cyclodextrin in aqueous solution of $NaHCO_3$ (15 ml) [29]. ANS (0.9 g) was added to the solution containing the mannoepoxide. The reaction was carried out at 60 °C, under nitrogen for 40 h. The solvent was evaporated and the solid was resuspended in water, loaded on a Rp8 column and eluted with water followed by a linear gradient water/MeOH (0 → 10%). Yield: 37%. $R_f = 0.55$, eluent PrOH/H_2O/AcOEt/NH_3 5:3:3:1. FAB MS 1358 (M + 1).

^{1}H NMR (D_2O, 500 MHz) δ (ppm) 7.47 (s, 1H, H-2 of Im), 6.70 (s, 1H, H-5 of Im), 5.04 (m, 2H, other H-1), 4.95–4.93 (m, 2H, H-1), 4.88 (d, 1H, H-1G, $J_{1G,2G} = 3.8$ Hz), 4.75 (d, 1H, H-1A, $J_{1A,2A} = 6.6$ Hz), 4.39 (dd, 1H, C*H* of His, $J = 4.6$ Hz, $J = 9.5$ Hz), 4.18 (m, 1H, H-5A), 3.90 (m, 1H, H-4A), 3.90–3.68 (m, 25H, H-3,-5,-6), 3.65 (dd, 1H, 6A); 3.58–3.46 (m, 17H, H-2,-4, CH_3), 3.10 (dd, 1H, CH_2 of His, $J = 4.6$ Hz, $J = 15.4$ Hz), 2.85 (dd, 1H, CH_2 of His $J = 15.4$ Hz, $J = 9.5$ Hz), 2.78 (m, 1H, CH_2 in α to the amino group of β-Ala); 2.74 (m, 1H, H-3A, $J_{2A,3A} = 10.1$ Hz, $J_{3A,4A} = 3.8$ Hz); 2.58 (m, 1H, CH_2 in α to the amino group of β-Ala); 2.32 (t, 2H, CH_2 in α to the CO of β-Ala).

2.5. Oxidation of human LDL

Lyophilized LDL were resuspended in water (2 mg protein/ml) and extensively dialyzed against 20 mM CH_3COONH_4 buffer, pH 7.4, to remove any phosphate and EDTA traces. LDL suspension was used at a final concentration of 1 mg/ml. Oxidation was started by the addition of $CuSO_4$ (40 μM final concentration) to the protein suspension and carried out by incubation at 37 °C for 4 h. The inhibitory effects on lipid peroxidation of ANS only, CD only, ANS + CD, CDANS3, CDANS6 were evaluated by incubating 40 μM $CuSO_4$-challenged LDL suspensions with increasing concentrations of each of the aforementioned compounds (0, 2, 5, 10, 20, 50, 100 and 200 μM) and for a fixed time (4 h). In addition, experiments for evaluating the influence of pH on the antioxidant efficacy of the various compounds of interest, were also performed. To this purpose, LDL were suspended in 20 mM CH_3COONH_4, pH 5.0 and incubated at 37 °C for 24 h with increasing concentrations of the different compounds (2, 5, 10, 20, 50, 100 and 200 μM). For any pH values, LDL incubated in buffer solution only were used as controls.

In all experiments, incubations were stopped by adding a double volume of HPLC-grade acetonitrile and samples were processed for the HPLC determination of MDA to evaluate the extent of LDL lipid peroxidation.

2.6. HPLC analysis of MDA

After acetonitrile addition, samples of LDL suspensions were extracted twice with chloroform (2:1; v/v) and, after each extraction, they were centrifuged at 20,190 × g for 5 min at 4 °C. The upper aqueous phase was collected, filtered through a 0.45 μm Millipore filter and then loaded (200 μl) onto a C-18, 250 × 4.6 mm, 5 μm particle size column (Kromasil, Bohus, Sweden) for the HPLC detection of MDA. The HPLC apparatus consisted of a Spectra System P2000 pump (ThermoElectron, Rodano, Milan, Italy) connected to a highly sensitive UV6000 LP diode array spectrophotometric detector (ThermoElectron, Rodano, Milan, Italy), equipped with a 5 cm light path flow cell and set up between 200 and 300 nm for data acquisition. The direct MDA determination was carried out on the organic solvent-extracted samples according to a previously described ion-pairing method [30], which does not require sample derivatization prior to HPLC analysis. MDA quantification was performed at 267 nm wavelength (MDA maximum of absorbance).

2.7. Tissue preparation and spectrofluorimetric analysis of carnosinase

Male Wistar rats of 350 g b.w. fed with standard laboratory diet and water ad libitum in a controlled environment, were used in this study. They were anesthetized with an intraperitoneal injection of 23 mg/kg b.w. propofol. An in vivo craniectomy was performed under anesthesia consistent with the method previously described elsewhere [31] and the whole brain was directly dropped into liquid nitrogen using a surgical spatula. After the wet weight determination, the brain was homogenized for 60 s in 20 mM ice-cold tris–HCl, pH 8.0, using an Ultra-Turrax (Janke & Kunkel, Staufen, Germany) at the maximal speed, so as to obtain a final 20% homogenate (w/v). After centrifugation at 21,000 × g for 15 min at 4 °C, the supernatant (100 μl) was added to 300 μl of 50 mM tris–HCl, pH 7.80. The reaction was started by adding 100 μl of one of the different substrates (ANS, AH, HC, CDANS3, CDANS6, CDAH3, CDAH6, CDNHAH6, CDHC3, CDHC6), each added to have a 1 mM final concentration. The incubation lasted for 120 min at 37 °C under constant shaking. The reaction was stopped by adding 1.5 ml of HPLC-grade acetonitrile (Carlo Erba, Milan, Italy), followed by centrifugation at 20,690 × g for 15 min at 4 °C. The supernatant was then extracted with large volumes of HPLC-grade chloroform, centrifuged again and the upper aqueous phase removed and subjected to two additional chloroform extraction. The deproteinized samples were used to assay the histidine content deriving from to the carnosinase activity by using the spectrofluorimetric method of Lenney et al. [21].

3. Results

3.1. Synthesis of the ligands and NMR characterization

The bioconjugates of ANS were synthesized with the aim of stabilizing the biological peptide towards the protease action, as reported for other bioconjugates with biologically active peptides [32,33]. In the CD-derivatives grafting involves the amino group of the peptides which becomes a secondary amino group. The conjugates cyclodextrin–peptides were synthesized following the methods reported in the literature [34]. The products were characterized by NMR and ESI-MS spectroscopies. The 1D spectra were assigned by COSY, TOCSY, HSQC, T-ROESY. The notation system in which the glucose rings are named A, B, C, D, E, F, and G, counter clockwise and viewed from the primary hydroxyl side, is adopted.

3.1.1. NMR spectra of CDANS6

^{1}H NMR spectrum confirms (see Supplementary data, Fig. S1) the identity of the product. The spectrum is characterized by the signals of the CD moiety protons in the 4.1–3.4 ppm region; the signals of the ANS moiety are also evident. H-2 and H-5 protons of imidazole moiety are also seen to resonate in the aromatic region. The protons of the ABX system of *N*-methyl His resonate at 4.34 ppm (X), at 3.07 (B) and 2.86 ppm (A), and the ethylenic chain protons at 2.78 ppm and at 2.38 ppm. The 6A protons resonate at 2.99 ppm and at around 2.77 ppm and are diastereotopic, as typically observed for this kind of derivatives [35,36]. The substitution of the 6-OH group with an amino group produces an upfield shift of the 6A proton signals [37–39]. The signal due to 5A is evident at 3.81 ppm. ROESY spectra suggest that the dipeptide is not included in the cavity, as in the case of the carnosine and homocarnosine 6-derivatives [27].

3.1.2. NMR spectra of CDANS3

The ^{1}H NMR spectrum shows (see Supplementary data, Fig. S2) the signals due to the functionalized A ring, together with those of the other CD protons at 4.0–3.4 ppm. As a consequence of the synthetic route followed to functionalize CDs on the C-3, the configuration inversions of C-2 and C-3 occur and an altrose unit replaces a glucose unit in the CD molecule [40]. Thus the cavity became asymmetric and this is evident especially in the H-1 region: other four groups of signals are observed in addition to the H-1A signal. The signals due to the protons of the altrose unit are easily identified on the COSY spectra: 2A ($\delta = 3.56$ ppm), 3A ($\delta = 2.73$ ppm) and 4A ($\delta = 3.93$ ppm), 5A ($\delta = 4.17$ ppm) and the 6A protons which are diastereotopic at 3.72 and 3.64 ppm. The coupling constant values $J_{1A,2A} = 6.59$ Hz, $J_{2A,3A} = 10.06$ Hz and $J_{3A,4A} = 3.84$ Hz indicate that 1A and 2A are both axial in the altrose unit, this being in keeping with its predominately $^1C_4$ conformation [41,42] as observed in other altrocyclodextrin derivatives [43–46].

The CH_2 protons of the ethylenic chain in α to the β-alanine amino group are diastereotopic and appear as two multiplets as typically found in the 3-derivatives [26].

ROESY spectra of CDANS3 show correlation peaks between the imidazole protons and H-3, H-5 and H-6 region. Though it is not straightforward which glucose unit interacts with the imidazole moiety because of the overlapping of respective signals, the disposition of the imidazole inside the cavity could be hypothesized. This behaviour was found in the case

of other carnosine 3-derivatives, where the self-inclusion of the chain was proposed on the basis of the NMR spectra [26].

3.2. LDL oxidation

In Table 1, data referring to the protective effects of ANS, as well as the equimolar combination of CD with ANS, are summarized. ANS, as well as CD, have only modest protective effects towards copper-induced human LDL oxidation. The inhibition of MDA formation by ANS was higher than that of CD. At the highest concentration tested (200 μM), the dipeptide reached 18.7% of protection, whereas CD at the same concentration had a protective effect only slightly higher than 10%. Moreover, the concomitant presence of equimolar amounts of CD and ANS produced antioxidant effect that was merely the sum of the effects of the two compounds considered.

In Fig. 1, the effects of the anserine conjugates are illustrated. In both cases, a much higher antioxidant activity than that observed in the case of ANS, CD, or equimolar mixture of ANS and CD (see Table 1) was recorded at any concentration tested. The comparison of the antioxidant potency of the two cyclodextrin derivatives, in the concentration range 20–200 μM, clearly showed that the 3-derivative of anserine had significantly higher effect than that of the corresponding 6-derivative ($p < 0.01$). Consequently, the apparent IC_{50} varied from 41.6 μM for CDANS3 to 88.7 μM for CDANS6.

The pH dependency of the capacity of the different β-cyclodextrin derivatives to inhibit MDA formation was tested by challenging human LDL with 40 μM Cu^{2+} for 4 h at pH 5.00. For the sake of simplicity, only results referring to the most effective β-cyclodextrin derivative (CDANS3) are summarized in Table 2. It is evident from the data reported that mild acidification of the incubation medium had no effects either on the extent of copper-induced LDL oxidation (same amount of MDA generated either at pH 7.40 or 5.00) or on the inhibitory capacity of the β-cyclodextrin conjugates of ANS.

3.3. Resistance to the carnosinase enzymatic hydrolysis

The carnosinase hydrolytic activity was determined by means of rat brain homogenate containing different potential substrates (1 mM of ANS, AH, HC, CDANS3, CDANS6, CDAH3, CDAH6, CDNHAH6, CDHC3 or CDHC6). The data (Fig. 2) clearly show very different efficiency of hydrolysis when using the natural histidine-containing dipeptides, indicating an almost 10 times higher enzymatic activity with AH as substrate than that with either ANS or HC. Furthermore, the results also demonstrate that the cyclodextrin derivatives of the histidine-containing dipeptides (CDANS3, CDANS6, CDAH3, CDAH6, CDNHAH6, CDHC3 or CDHC6) are hydrolyzed in very minimal amounts with respect to the corresponding parental molecules.

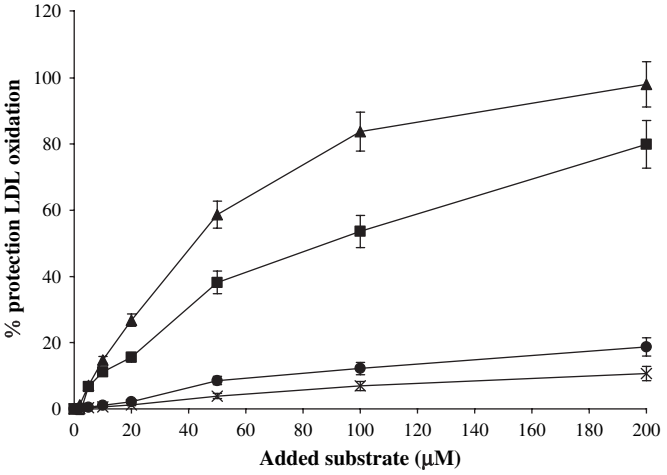

Fig. 1. Dose response protective effect of β-CD (×), ANS (●) and ANS derivatives, conjugated in C-3 (▲) or in C-6 (■) with β-cyclodextrin, on 40 μM copper-induced lipid peroxidation of human LDL. Peroxidation was evaluated by the HPLC determination of MDA. Control LDL were incubated in presence of 40 μM Cu^{2+} and buffer only, with no added antioxidant (0% protection). Each point represents the mean of four different experiments. Standard deviations are represented by vertical bars.

4. Discussion

The histidine-containing dipeptide family is a class of compounds of still unclear biological role. In spite of this, several experimental studies showed, very recently, significant pharmacological activities for the main representative substance of the histidine-containing dipeptide family, AH, which might find possible practical application in pathological conditions of relevant clinical interest, such as diabetes [47,48], neurodegeneration [49,50], cataract [51,52]. In most of these studies, effectiveness of AH was mainly related to its antioxidant or

Table 1
Dose response effect of histidine-containing dipeptides and β-cyclodextrin on lipid peroxidation induced in human LDL by 40 μM Cu^{2+}, as evaluated by HPLC in terms of MDA produced in the incubation medium

Concentration (μM)	ANS	CD	CD + ANS
2	0.23 (0.05)	0.11 (0.01)	0.46 (0.08)[a]
5	0.51 (0.07)	0.38 (0.06)	1.27 (0.20)[a]
10	1.05 (0.31)	0.65 (0.13)	2.02 (0.26)[a,b]
20	2.14 (0.30)	1.26 (0.18)	3.87 (0.54)[a]
50	8.55 (1.07)	3.87 (0.64)	14.91 (1.66)[a,b]
100	12.21 (1.74)	6.94 (0.83)	25.62 (3.52)[a,b]
200	18.72 (0.92)	10.72 (1.55)	29.77 (3.44)[a,b]

Values are the mean (s.d.) of four different experiments and are expressed as percent protection (0% protection = control LDL incubated with buffer only).
[a] Significantly different from samples incubated with CD only.
[b] Significantly different from samples incubated with the corresponding dipeptide only.

Table 2
Dose response effect of CDANS3 on lipid peroxidation induced in human LDL by 40 μM Cu^{2+} at pH 5.00 and 7.40

CDANS3 (μM)	MDA (μM), pH 5.00	MDA (μM), pH 7.40
10	7.03 (1.32)[a]	7.54 (1.06)[a]
20	5.85 (1.55)[a]	6.75 (0.91)[a]
50	4.52 (0.70)[a]	4.61 (0.31)[a]
100	1.94 (0.26)[a]	2.80 (0.16)[a]
200	0.89 (0.13)[a]	0.71 (0.08)[a]

[a] Significantly different from respective 0 time ($p < 0.05$).

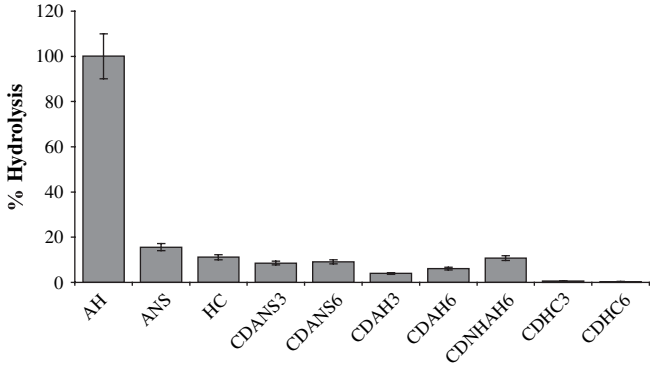

Fig. 2. Hydrolytic activity determined in a rat brain homogenate as a function of different types of potential substrates (10 mM). Values are the mean (s.d.) of four different experiments and are expressed as percentage of carnosine hydrolysis value (100%).

antiglycating activity capable to counteract oxidative and/or glycating processes operative in the aforementioned pathological conditions [53].

The possible involvement of AH in neurodegeneration was hypothesized by the finding that cerebral carnosinase activity varied as a function of the age [21] and such a variation was inversely correlated with the different AH concentration [54]. However, it is worth underlining that the multifunctional and uncertain biological role of AH renders unclear how and where this dipeptide might be involved in the physiopathological processes of neurodegeneration. In this scenario, all these information, including the capacity of AH to reduce nitrosative stress in astroglial cell cultures [55], prompted to test carnosine-based compounds as possible effective pharmacological remedies in chronic neurodegenerative disorders, such as Parkinson's and Alzheimer's diseases. To this purpose, a consistent variety of AH derivatives has been synthesized in the past, with the specific aim of obtaining potential new drugs with significantly higher pharmacological activity than the parental molecules [56].

In the present study, we demonstrated that the chemical modification of anserine, one of the most representative members of the histidine-containing dipeptide family, obtained by covalently linking a β-cyclodextrin residue, conferred to the different derivatives a significantly higher antioxidant activity than that of the parental molecule. Furthermore, the cyclodextrin conjugation of carnosine, homocarnosine and anserine greatly reduced the enzymatic hydrolysis induced by the rat brain dipeptidases, including carnosinase.

As far as the antioxidant activity of the various derivatives is concerned, the substantial difference in the inhibition of MDA production seems to be related to the functionalizing position of the β-cyclodextrin involved in the link with the dipeptides, as found for other similar conjugates [27]. As illustrated in Fig. 1, CDANS3 had higher antioxidant potency than that of the corresponding 6-conjugate, as reported in the case of analogous bioconjugates with carnosine [27]. The functionalization on the secondary rim could favor the interaction of the peptide with some intermediate radicals by easier inclusion in the cavity. This effect may also be related to the distorted cavity of the 3-derivative, where a glucose is modified in altrose unit.

Our previous data on the activity of CDAH3 and CDAH6 indicated that the imidazole ring and the β-cyclodextrin moiety serve as competitive scavenging sites for hydroxyl radicals [26]. Results of the experiments carried out at pH 5.00 (see Table 2) clearly indicate that, in the overall antioxidant mechanism of the β-cyclodextrin derivatives, copper sequestration plays a minor role (if any), at least in the concentration ranges of both oxidants and antioxidants tested and in the experimental model of human LDL oxidation. The data showed in this work strengthen our previously reported hypotheses on the mechanism by which the cyclodextrin conjugates of AH and HC inhibit the copper(II)-mediated LDL oxidation [27]. Moreover, the similar antioxidant effect showed by both CD-anserine conjugates and the analogous carnosine derivatives could be due to the comparable conformations of the dipeptide moieties grafted to the cyclodextrin, especially for the 3-conjugates, as we found on the basis of NMR spectra. This is in agreement with the higher antioxidant potency of CDAH3 and CDANS3 with respect to the other tested cyclodextrin conjugates.

The enzymatic hydrolysis assay of the natural dipeptides (AH, HC and ANS) and their cyclodextrin conjugates pointed out two important results: (i) the enzymatic activity is due to the cerebral carnosinase, in agreement with previous data that attribute the carnosine hydrolysis activity of rat brain mainly to CN1 [22,24]; (ii) the resistance of all glycosidic derivatives of AH, HC and ANS to the enzymatic hydrolysis with respect to the natural dipeptides confirms the effectiveness of our synthetic strategy, aimed to obtain potential new drugs with significantly higher pharmacological activities than AH, HC and ANS, as well as with a concomitant resistance to carnosinase hydrolysis. In the last decade, a consistent variety of AH derivatives has been synthesized with the same purpose [56]. For many of them, the biological activity tested was the measurement of the antioxidant activity in various in vitro experimental models. For all of those tested, antioxidant effectiveness was significant in the millimolar concentration range only, thereby rendering difficult their eventual clinical application because of the impossibility to reach such a high concentration in the cell or tissue targets.

Results reported in the present study clearly demonstrate that some of the β-cyclodextrin derivatives of histidine-containing dipeptides that we synthesized (CDAH3, CDANS3) have a remarkable antioxidant activity already in the micromolar range, as indicated by the values of the IC_{50} (23.4 and 41.6 μM for CDAH3 and CDANS3, respectively), i.e., they are effective at concentrations 10–20 times lower than those reported for other synthetic derivatives [57]. This finding, coupled with the resistance towards the hydrolytic activity of the rat cerebral carnosinase, strongly indicates that the most active compounds synthesized (CDAH3 and CDANS3) are good candidates to carry out further studies devoted to characterize their pharmacological profiles in more complex cellular and animal models. The final goal would be to evaluate their activities in neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases. Studies on cell and/or animal models to

characterize the pharmacological profile of the most effective AH derivatives here reported are in progress.

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2007.03.038.

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