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Original article

Synthesis and antioxidant activity of new homocarnosine β-cyclodextrin conjugates

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Abstract

Several *in vitro* and *in vivo* studies have suggested that carnosine (β-alanil-L-histidine) and homocarnosine (β-aminobutyril-L-histidine) can act as scavengers of reactive oxygen species. β-Cyclodextrin was functionalized with homocarnosine, obtaining the following new bioconjugate isomers: 6^A-[(4-{[(1*S*)-1-carboxy-2-(1*H*-imidazol-4-yl)ethyl]amino}-4-oxobutyl)amino]-6^A-deoxy-β-cyclodextrin and (2^AS,3^AR)-3A-[(4-{[(1*S*)-1-carboxy-2-(1*H*-imidazol-4-yl)ethyl]amino}-4-oxobutyl)amino]-3^A-deoxy-β-cyclodextrin. Pulse radiolysis investigations show that the β-cyclodextrin homocarnosine bioconjugates are scavengers of $^{\bullet}$OH radicals because of the formation of stable imidazole-centered radicals and the scavenger ability of glucose molecules of the macrocycle. The ability of these new β-cyclodextrin derivatives to inhibit the copper(II) driven LDL oxidation was determined in comparison with that displayed by the analogous carnosine derivatives. Both the β-cyclodextrin carnosine isomers show a higher protective effect than that of free dipeptide and homocarnosine derivatives, bringing into light the role of the β-CD cavity.

The ability of these new β-cyclodextrin derivatives to inhibit the copper(II) driven LDL oxidation was determined in comparison with that displayed by the analogous carnosine derivatives. Both the β-cyclodextrin carnosine isomers show a higher protective effect than that of free dipeptide and homocarnosine derivatives, bringing into light the role of the β-CD cavity.

Keywords: β-Cylodextrin; Homocarnosine; Carnosine; Low density lipoprotein; Pulse radiolysis

1. Introduction

Cyclodextrins (CDs) are cyclic non-reducing, water soluble oligosaccharides, which present a hydrophobic cavity with the appropriate size to accommodate another molecule forming inclusion compounds through host–guest interactions [1]. CDs derive their nomenclature from the number of glucose residues in their structure, such that the glucose hexamer is referred to as α-CD, the heptamer to β-CD and the octamer to γ-CD. Molecular encapsulation of drugs into CDs has been studied with the aim of improving characteristics of pharmaceutical interest such as stability and bioavailability [2]. Chemically modified CDs generate more efficient systems to delivery drugs by: (i) increasing oral bioavailability; (ii) decreasing toxicity; and (iii) preventing an immune response in mammals [3]. The covalent linkage of bioactive peptides to cyclodextrins has recently been proposed as a means of achieving good results in terms of solubility and reduced catabolism [4–11].

Homocarnosine (γ-aminobutyrilhistidine, HC) is a good antioxidant molecule as carnosine [12,13], and possesses similar neuroprotective abilities as carnosine (AH) [14]. It acts as

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a GABA reservoir and may mediate the antiseizure effects of GABAergic therapies [15,16]. Increasing concentration of the histamine containing dipeptides in the body could be a promising strategy for the treatment of disorders which are related to the oxidative stress process [17]. Specific dipeptidases can modulate the concentration of these dipeptides [18]; these dipeptidases are present in plasma, liver and kidneys and they do not permit to accumulate exogenous homocarnosine and carnosine in tissues [19]. In order to overcome this limitation, conjugates of carnosine with cyclodextrins have been synthesized [10,20] and their ability to scavenge •OH radicals has been determined [21]. This capability is maintained in the cyclodextrin conjugates and it is cooperative with the ability of cyclodextrin to react with the •OH. In addition these functionalized compounds are able to survive to carnosinase activity [22].

This paper reports the synthesis, the NMR characterization and the antioxidant ability of the new bioconjugates: 6^{A}-[(4-{[(1*S*)-1-carboxy-2-(1*H*-imidazol-4-yl)ethyl]amino}-4-oxobutyl)amino]-6^{A}-deoxy-β-cyclodextrin (CDHC6) and ($2^{A}S$, $3^{A}R$)-3^{A}-[(4-{[(1*S*)-1-carboxy-2-(1*H*-imidazol-4-yl)ethyl]amino}-4-oxobutyl)amino]-3^{A}-deoxy-β-cyclodextrin (CDHC3). In addition to the ability to act as •OH scavenger measured by means of pulse radiolysis also the inhibitory effect on copper-induced LDL oxidation has been determined. The antioxidant activity towards LDL oxidation has been determined also for the carnosine–cyclodextrin conjugates previously synthesized [10,21]: CDAH6, CDAH3 and CDNHAH6 (see Chart 1). It has been also proved by means of pulse radiolysis technique that these β-cyclodextrin carnosine bioconjugates are scavengers of •OH radicals [21].

2. Experimental procedures

2.1. Chemicals

Purified human LDL 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 was purchased from Fluka; anhydrous *N*, *N*-dimethylformamide was purchased from Aldrich. 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 the Pauli test for the homocarnosine derivatives. Homocarnosine ethylester (HCEt) was synthesized from homocarnosine (HC) (Sigma) with HCl in EtOH at 0 °C, using acetylchloride as HCl source. All other reagents were of the highest purity available from commercial sources.

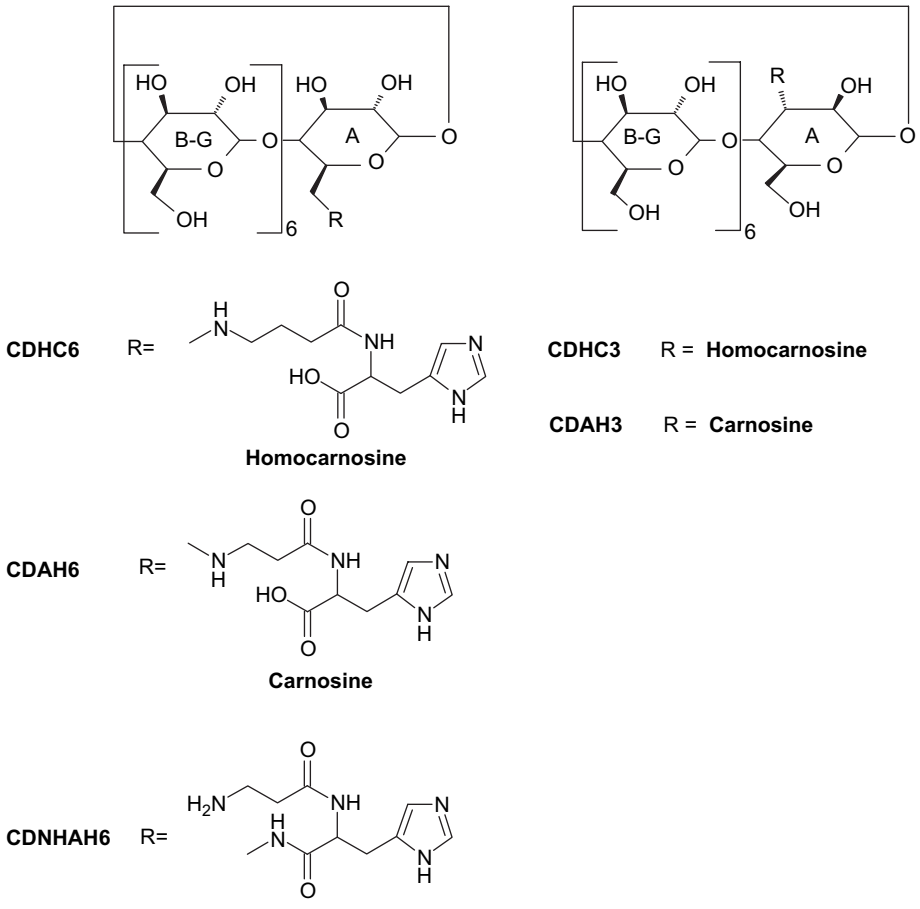


Chart 1.

^{1}H NMR spectra were recorded at 25 °C in D_2O with a Varian Inova 500 instrument at 499.883 MHz. The ^{1}H NMR spectra were obtained by using standard pulse programs from Varian library. In all cases the length of 90° pulse was c.a. 7 μs. Two-dimensional 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.

2.1.1. Synthesis of 6^A-[(4-{[(1S)-1-carboxy-2-(1H-imidazol-4-yl)ethyl]amino}-4-oxobutyl)amino]-6^A-deoxy-β-cyclodextrin (CDHC6)

Synthesis was carried out starting from the 6-deoxy-6-iodo-β-cyclodextrin (CDI) [23]. HCEt (1 g) was added to a solution of CDI (1 g) in anhydrous DMF (1 ml). The reaction was carried out at 70 °C, under nitrogen and under stirring. After 12 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 0–0.2 M of NH_4HCO_3 solution (400 mL) were used as the eluent. The appropriate fractions containing the ethylester of CDHC6 were combined. The product was hydrolyzed by treatment with 1% NaOH in water/methanol (1:1) for 1 h. The solvent was evaporated and the product was isolated by column chromatography of CM-Sephadex C-25 (20 × 600 mm, NH_4^+ form) using water as the eluent. Yield: 20%, $R_f = 0.32$, (PrOH/H_2O/AcOEt/NH_3 5:3:2:3). ESI-MS *m/z* 1358.2 (M + 1). Elemental analysis for $C_{52}H_{84}O_{37}N_4 \cdot 6H_2O$: calcd (%): C 42.98, H 5.83, N 3.86; found: C 42.7, H 5.7, N 3.7.

^{1}H NMR (D_2O, 500 MHz) δ (ppm) 7.69 (s, 1H, H-2 of Im), 6.85 (s, 1H, H-5 of Im), 5.0–4.96 (m, 7H, H-1 of CD), 4.33 (dd, 1H, CH of His, $J_{XB} = 4.58$ Hz, $J_{XA} = 8.97$ Hz), 3.97 (m, 1H, H-5A), 3.88–3.67 (m, 26H, H-3, -5, -6), 3.58–3.35 (m, 15H, H-2, -4, -6′A), 3.15 (dd, 1H, H-6A, $J_{6A,5A} = 8.97$ Hz, $J_{6A,6'A} = 13.91$ Hz), 3.04 (dd, 1H, H of CH_2 of His, $J_{BX} = 4.49$ Hz, $J_{AB} = 14.83$ Hz), 2.85 (m, 3H, other H of His, CH_2 in α to NH); 2.26 (m, 2H, CH_2 in α carbonyl group), 1.80 (m, 2H, CH_2 in β to NH).

^{13}C NMR (D_2O, 125 MHz) 104.2 (C-1), 85.6 (C-4A), 83.7 (C-4), 75.8 (C-5), 74.6 (C-2), 74.2 (C-3), 70.2 (C-5A), 63.2 (C-6), 57.5 (CH of His), 51.5 (C-6A), 50.3 (CH_2 in α to NH); 35.6 (CH_2 in α carbonyl group), 32.2 (CH_2 of His), 25.9 (CH_2 in β to NH).

2.1.2. Synthesis of (2^{A}S,3^{A}R)-3^A-[(4-{[(1S)-1-carboxy-2-(1H-imidazol-4-yl)ethyl]amino}-4-oxobutyl)amino]-3^A-deoxy-β-cyclodextrin CDHC3

The 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) [24]. HC (0.9 g) was added to the solution containing the mannoepoxide. The reaction was carried out at 60 °C, under nitrogen for 12 h. The solvent was evaporated and the solid was purified by column chromatography using a column of Rp8 eluted with water and then with a linear gradient water/MeOH (0 → 20%). The isolated product was further purified by a CM-Sephadex C-25 column using water as eluent. Yield: 41%, $R_f = 0.44$ (PrOH/H_2O/AcOEt/NH_3 5:3:1:2). ESI-MS *m/z* 1358.4 (M + 1). Elemental analysis for $C_{52}H_{84}O_{37}N_4 \cdot 8H_2O$: calcd (%): C 41.60, H 6.71, N 3.73; found: C 41.2, H 6.6, N 3.5.

^{1}H NMR (D_2O, 500 MHz,) δ (ppm) 7.61 (s, 1H, H-2 of Im), 6.83 (s, 1H, H-5 of Im), 5.05 (m, 2H, H-1), 4.96 (d, 2H, H-1); 4.93 (d, 1H, H-1, $J_{12} = 3.41$ Hz), 4.92 (d, 1H, H-1G, $J_{1G,2G} = 4.01$ Hz); 4.80 (d, 1H, H-1A $J_{1A,2A} = 6.90$), 4.36 (dd, 1H, CH of His, $J_{XB} = 4.62$ Hz, $J_{XA} = 9.03$ Hz), 4.21 (m, 1H, H-5A), 4.00 (m, 1H, H-4A), 3.95–3.72 (m, 27H, H-3,-5,-6, H-2A), 3.76 (m, 1H, 6A), 3.68 (m, 1H, 6′A); 3.62-3.44 (m, 12H, H-2, -4), 3.04 (dd, 1H, one proton of CH_2 of His, $J_{BX} = 4.62$ Hz, $J_{BA} = 15.00$ Hz), 2.99 (dd, 1H, H-3A, $J_{3A,2A} = 10.50$, $J_{3A,4A} = 3.00$), 2.85 (dd, 1H, proton of CH_2 of His, $J_{AX} = 9.13$ Hz, $J_{AB} = 15.00$ Hz); 2.72 (m, 1H, proton of CH_2 in α to amino group); 2.54 (m, 1H, other of CH_2 in α to amino group); 2.21 (m, 2H, CH_2 in α to carbonyl group); 1.67 (m, 2H, CH_2 in β to amino group).

^{13}C NMR (D_2O, 125 MHz) 106.5 (C-1A), 105.3, 104.7, 104.5 (C-1), 84.0–83.5 (C-4), 79.0 (C-5A), 78.60 (C-4A), 76.2 (C-5), 74.6 (C-2), 74.2 (C-3), 72.0 (C-2A), 63.8 (C-6), 61.4 (C-3A), 58.2 (CH of His), 49.19 (C-6A), 59.2 (CH_2 in α to NH); 36.4 (CH_2 in α carbonyl group), 32.5 (CH_2 of His), 27.5 (CH_2 in β to NH).

2.2. Pulse radiolysis

Pulse radiolysis was performed by using electron pulses (≈20 ns duration) of the 12 MeV electron linear accelerator at the ISOF-CNR Institute in Bologna. The irradiations were carried out at room temperature, 22 ± 2 °C, on samples contained in Spectrosil cells of 2 cm optical path length. Solutions were protected from the analyzing light by means of a shutter and appropriate cutoff filters. The monitoring light source was a 450 W Xe arc lamp. The radiation dose per pulse was monitored by means of a charge collector placed behind the irradiation cell and calibrated with a N_2O-saturated solution containing 0.1 M HCO_2^- and 0.5 mM methyl viologen (1,1′-dimethyl-4,4′-bipyridinium dication; MV^{2+}) using $G\varepsilon = 9.66 \times 10^{-4}$ m^2 J^{-1} at 602 nm [25]. *G*(X) represents the number of moles of species X formed or consumed per joule of energy absorbed by the system.

2.3. 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. The inhibitory effects on lipid peroxidation of AH, HC, CDAH3, CDAH6, CDNHAH6, CDHC3, CDHC6, were evaluated by incubating 40 μM Cu^{2+}-challenged LDL suspensions with increasing concentrations of each of the aforementioned compounds (2, 5, 10, 20, 50, 100 and 200 μM) at 37 °C for 4 h. In addition, experiments for evaluating the influence of pH on the antioxidant efficacy of the various compounds of interest, were also performed. For this purpose, LDL were suspended in 20 mM CH_3COONH_4, pH 5.0 and incubated at 37 °C

for 4 h with increasing concentrations of the different compounds (2, 5, 10, 20, 50, 100 and 200 μM). For any pH values, LDL incubated in presence of buffer 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.

2.4. 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 an ion-pairing method described in detail elsewhere [26], which does not require sample derivatization prior to HPLC analysis. MDA quantification was performed at 267 nm wavelength (MDA maximum of absorbance).

3. Results and discussion

The major objective of this research was to evaluate the antioxidant activity of conjugates of cyclodextrin with biological dipeptides histidine containing, carnosine and homocarnosine.

We synthesized two new conjugates of β-cyclodextrin with homocarnosine (Chart 1) to better investigate the role of the bioconjugation on the activity of the peptide, encouraged from some results obtained in the case of carnosine conjugates. In addition to the data on the scavenger ability of the homocarnosine conjugates against the $^{\bullet}$OH, we also investigated the antioxidant activity of the carnosine and the homocarnosine conjugates in the copper(II) dependent LDL oxidation assay, where other free radical species are involved.

The bioconjugates of carnosine and homocarnosine were investigated with the aim of stabilizing the biological peptides towards the protease action, as reported for other bioconjugates with biologically active peptides [4–9,11,22].

3.1. Synthesis and NMR characterization

In these derivatives, the link involves the amino group of HC which becomes a secondary amino group. The new compounds were synthesized following the methods reported in the literature [10,21]. The products were characterized by NMR spectroscopy and ESI-MS.

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 rings counter clockwise and viewed from the primary hydroxyl side is adopted.

3.1.1. NMR spectra of CDHC6

^{1}H NMR (Fig. 1a) spectra confirm the identity of the product. In addition to the signals due to the protons of CD moiety in the 4.1–3.4 ppm region, the spectrum shows the signals of the HC moiety. H-2 and H-5 protons of the imidazole are also seen to resonate in the aromatic region at 7.69 ppm and 6.85 ppm. The protons of the ABX system of His resonate at 4.33 ppm (X), at 3.04 ppm (B) and at 2.85 ppm (A), and the propylenic chain protons at 2.86 ppm, at 2.26 ppm and at 1.80 ppm. The 6A protons resonate at 3.34 ppm and at around 3.15 ppm and are diastereotopic as typically observed for these kinds of derivatives [23,27,28]. The substitution of the 6-OH group with an amino group produces an upfield shift of the 6A proton signals [23,27–30]. The signal due to 5A is evident at 3.97 ppm. The chemical shift values of 5A and 6A suggest the zwitterionic form for this compound [20,23,29,30]. ROESY spectra suggest that the homocarnosine chain is not included in the cavity, as in the case of the carnosine 6-derivative. The chain ethylenic or propylenic together with

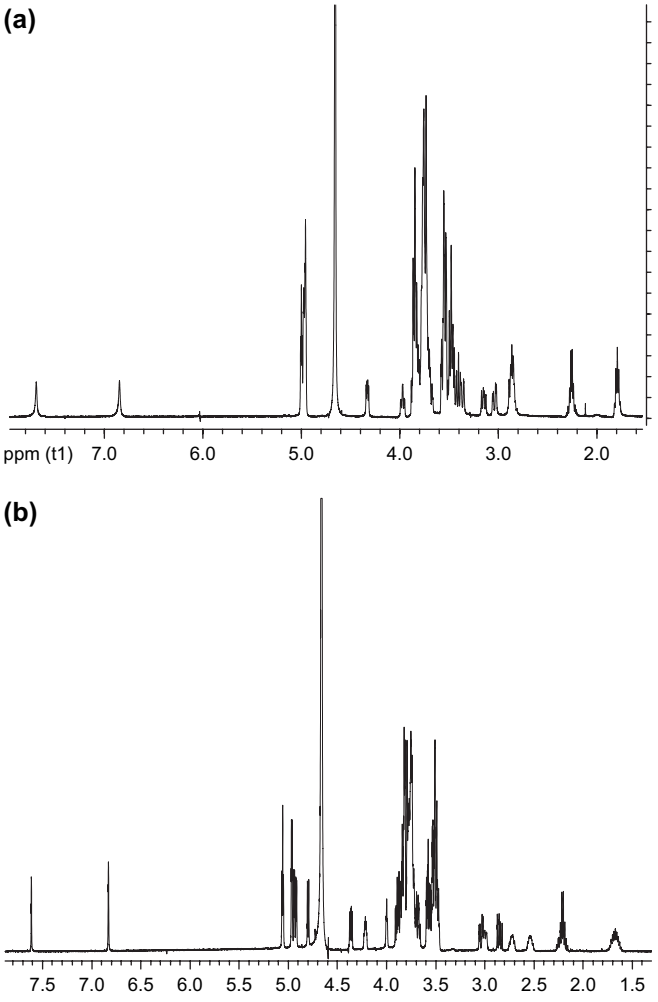


Fig. 1. ^{1}H NMR spectra (500 MHz, D_2O) of HC derivatives: (a) CDHC6, (b) CDHC3.

the presence of the imidazole which is a small aromatic ring does not determine the intra or the inter-molecular inclusion in these kinds of systems.

3.1.2. NMR spectra of CDHC3

The ^{1}H NMR spectrum (Fig. 1b) shows the signals due to the functionalized A ring together with those for the CD protons at 4.0–3.4 ppm. The signals due to the 2A ($\delta = 3.66$ ppm), 3A ($\delta = 2.99$ ppm) and 4A ($\delta = 4.00$ ppm), and of the protons of the CH_2 in γ (2.72 ppm and 2.54 ppm), β (1.67 ppm) and α (2.21 ppm), are evident. The γ-CH_2 protons are diastereotopic and show two different signals.

The chemical shift values of the 4A and 3A protons suggest that CDHC3 is in zwitterionic form [20,31–35]. 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 [36]. 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 coupling constant values $J_{1A,2A} = 6.8$ Hz, $J_{2A,3A} = 10.5$ and $J_{3A,4A} = 3.0$ Hz indicate that 1A and 2A are both axial in the altrose unit, this being in keeping with its predominately $^1C_4$ conformation [37,38], as observed in other altrocyclodextrin derivatives [21,31–35].

The ROESY spectra do not show correlation peaks between the imidazole protons and H-3, H-5, H-6 regions, suggesting the disposition of the moiety outside the cavity. This behaviour is different to that observed for the carnosine 3-derivative, where the self-inclusion of the chain has been hypothesized on the basis of NMR spectra [21]. We can assume that, in the case of the analogous homocarnosine derivative, the presence of the propylenic chain disfavours the self-inclusion rendering the moiety more flexible.

3.2. Pulse radiolysis

The pulse radiolysis technique is a direct method to gain insight into the interaction of substances with the active radicals produced since it allows in general measurement of both kinetic formation and decay as well as spectra of transient intermediates.

The radiolysis of water predominantly produces the species shown in Eq. (1) where the values in parentheses represent the yields expressed in terms of G-values (μmol J^{-1}) [39].

$$H_2O \rightarrow e^-_{aq}(0.27), {}^{\bullet}OH(0.28) \text{ and } H^{\bullet}(0.062), \quad H_2(0.047), H_2O_2(0.073), H^+(0.27) \qquad (1)$$

3.2.1. Reaction of ˙OH radicals with CDHC6 and CDHC3

In order to investigate the scavenging properties of the synthesized compounds towards ˙OH radicals, samples containing either CDHC6 or CDHC3 were irradiated after saturating the aqueous solutions with N_2O. Under these experimental conditions the e^-_{aq} are converted into ˙OH according to reaction (2)

$$e^-_{aq} + N_2O + H_2O \rightarrow {}^{\bullet}OH + OH^- + N_2 \qquad (2)$$

and the yield of this species increases to ca. 0.55. Since the dose used in our experiments was ≈16 Gy, the concentration of ˙OH generated is $\approx 10^{-5}$ M.

Fig. 2 shows the transient absorption spectrum recorded 6 μs after the pulse and obtained after the reaction of ˙OH radicals with a 10^{-4} M aqueous solution of CDHC6. The spectrum is characterized by a relevant absorption below 280 nm and by two better-defined bands around 300 and 350–450 nm, respectively. The time profile of the formation of this transient (inset Fig. 2) is described fairly well by a first order fit with a pseudo-first order rate constant, $k_{obs} = 8 \times 10^5$ s^{-1}. A bimolecular quenching constant $k_1 = 8 \times 10^9$ M^{-1} s^{-1} related to the reaction of ˙OH with CDHC6 is obtained by using Eq. (3).

$$k_1 = k_{obs}/[1] \qquad (3)$$

Such a value did not change significantly with the concentration of CDHC6, confirming that under these experimental conditions ˙OH radicals react almost exclusively with the quencher.

The band at 300 nm and the broad absorption in the 350–450 nm region are very indicative for an active role of the homocarnosine unit of CDHC6 as a scavenger of ˙OH radicals. This is in very good agreement with the literature data showing that these radicals readily react with carnosine [40] leading to a transient spectrum quite similar to that reported in Fig. 2. In particular, it has been demonstrated that the reaction is consistent with an attack of the radical species at level of the imidazole ring, preferentially at C(2) position, giving an adduct (a resonance-stabilized radical) characterized by a well-defined band at 310 nm and a weaker absorption in the region 380–400 nm [40,41]. The bimolecular rate constant related to this process is $k_{carnosine} = 5 \times 10^9$ M^{-1} s^{-1} and it is only slightly dependent on pH [41]. Such a value is smaller than that obtained in our case (*vide supra*). This finding along with the remarkable absorption extending below 280 nm, not

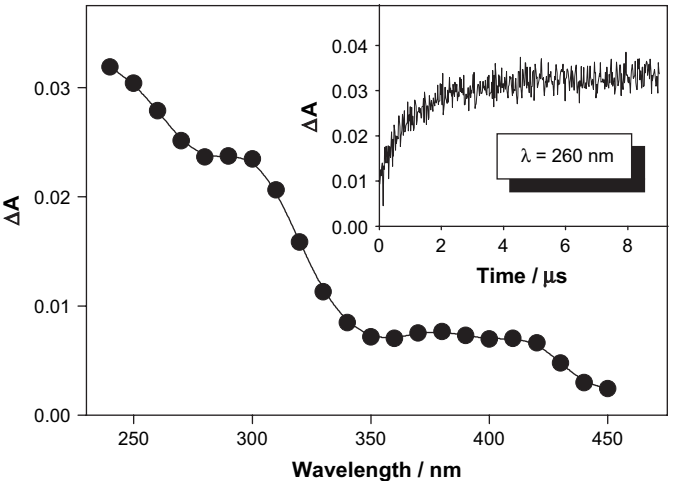


Fig. 2. Absorption spectrum obtained from the pulse radiolysis of N_2O-saturated 10^{-4} M solution of CDHC6, taken 6 μs after the pulse. The inset shows the build-up monitored at 260 nm.

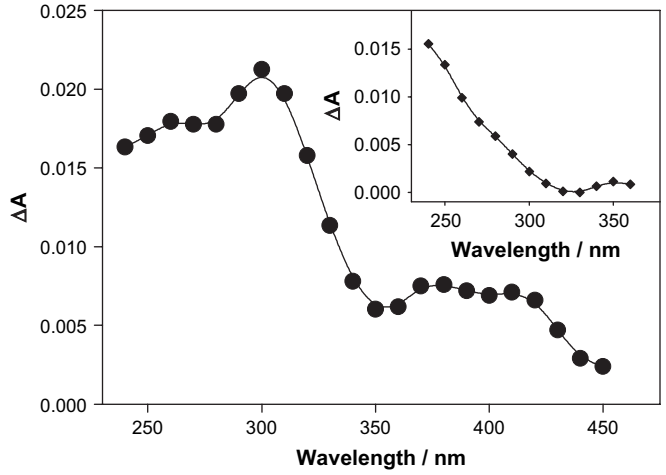


Fig. 3. Difference transient absorption spectrum between CDHC6 (see Fig. 2) and β-CD (see inset). Inset: absorption spectrum obtained from the pulse radiolysis of N_2O-saturated solution of 10^{-4} M β-CD, taken 6 μs after the pulse.

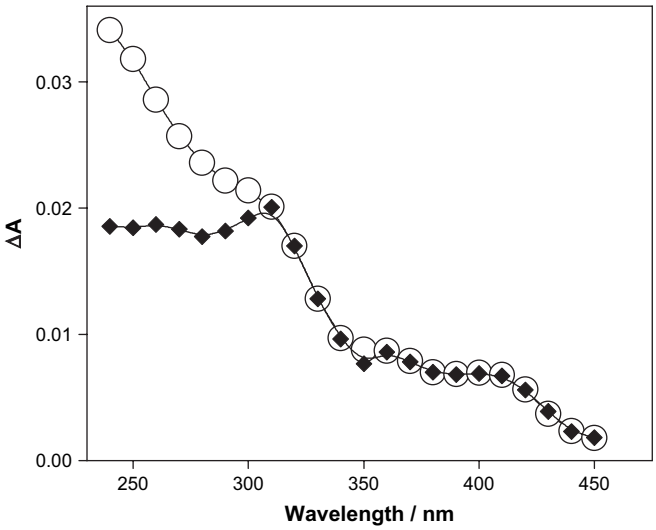
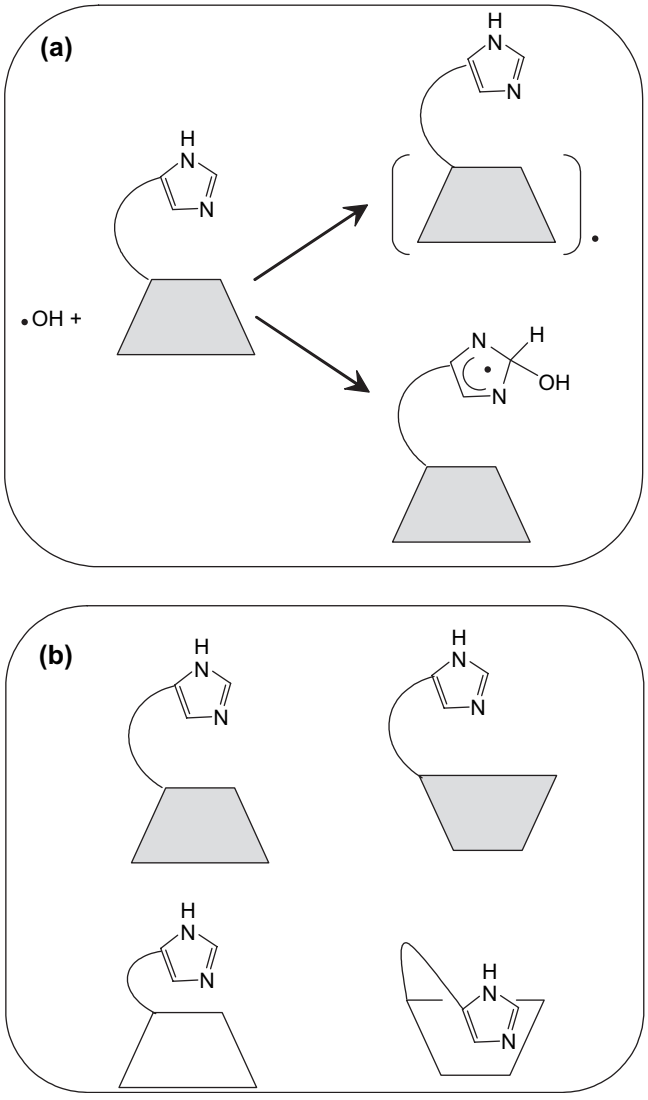


Fig. 4. Absorption spectrum obtained from the pulse radiolysis of N_2O-saturated 10^{-4} M solution of CDHC3, taken 6 μs after the pulse (○) and difference transient absorption spectrum between CDHC3 and β-CD (see inset Fig. 3) (◆).

present in the case of either homocarnosine, carnosine or imidazole alone [40,41], suggests that the imidazole ring of CDHC6 is not the unique scavenging site of the molecule. In this regard, abstraction hydrogen process involving $^{\bullet}$OH radical and glucose, the monomer species of cyclodextrin, are reported [41] to take place efficiently with a rate constant of $1.5 \times 10^9\ M^{-1}\ s^{-1}$. Thus, a direct participation of the cyclodextrin unit of CDHC6 in scavenging $^{\bullet}$OH radical can be reasonably conceived. This proposal is in agreement with our recent findings concerning carnosine derivatives of β-CD [21]. Indeed, we demonstrated that the reaction between $^{\bullet}$OH and β-CD itself produces a transient absorption in the region 240–360 nm, assigned to carbon-centered radicals generated after hydrogen abstraction from the macrocycle (see inset of Fig. 3 for sake of clarity). Despite the low extinction coefficient of the carbon-centered radicals, we could estimate a rate constant of $k_{\beta\text{-CD}} \approx 9 \times 10^9\ M^{-1}\ s^{-1}$ for the reaction between $^{\bullet}$OH and β-CD by the pseudo-first order kinetic formation monitored at 240 nm. Note that the estimated value is ca. 7-fold higher than that reported for glucose [40] in good agreement with the fact that each molecule of β-CD contains seven glucose units. Fig. 3 shows the difference spectrum between CDHC6 and β-CD after reaction with $^{\bullet}$OH. It can be noticed that the spectral features are virtually identical to those exhibited by the $^{\bullet}$OH–imidazole adduct [40,41]. On the basis of these results, we can safely propose that the transient absorption of CDHC6 (Fig. 2) reflects independent contributes of the intermediates produced upon reaction of both the imidazole center and the β-CD unit of CDHC6 with $^{\bullet}$OH radicals. Furthermore, by taking into account the ΔA value at 310 nm and the extinction coefficient for the $^{\bullet}$OH–imidazole adduct at this wavelength [40], we can estimate that the amount of this species formed is $\approx 3 \times 10^{-6}$ M. Given that the concentration of the $^{\bullet}$OH radicals generated in our experiments is $\approx 10^{-5}$ M (*vide supra*) the concentration of the carbon-centered radicals formed after hydrogen abstraction from β-CD would be $\approx 7 \times 10^{-6}$ M. This picture rules out, of course, any attack of the $^{\bullet}$OH radicals to the side chain of the imidazole. According to literature data, such a process is only a minor pathway [42].

The transient absorption spectrum obtained after the reaction of $^{\bullet}$OH radicals with a 10^{-4} M aqueous solution of CDHC3 is shown in Fig. 4 (circles). The shape of the transient spectrum observed is very similar to that of compound CDHC6 (see Fig. 2 for comparison). Thus, in light of the above picture we can infer that also in this case the $^{\bullet}$OH radicals that react with both β-CD and imidazole ring. In particular, by the ΔA of the difference transient spectrum between CDHC3 and β-CD alone related to the $^{\bullet}$OH–imidazole adduct (diamonds in Fig. 4), it is possible to note that the amount of this latter species formed is essentially the same as in the case of compound CDHC6 ($\approx 3 \times 10^{-6}$ M). As far as the kinetic of the reaction between $^{\bullet}$OH and CDHC3 is concerned, the bimolecular rate constant obtained from the build-up monitored at 260 nm (data not shown), was $k_2 = 7 \times 10^9\ M^{-1}\ s^{-1}$, a value very close to that observed for CDHC6.

The overall picture emerging from both spectroscopic and kinetic behaviours suggests that, likewise to carnosine derivatives of β-CD [21], the imidazole and β-CD sub-units of both homocarnosine derivatives behave as competitive sites for the scavenging of the $^{\bullet}$OH radicals. The former being a radical trap leading to the formation of a resonance-stabilized radical adduct, the latter providing a source of 14 hydrogen atoms (Scheme 1a). However, in contrast to the analogous carnosine derivatives of β-CD [21], in the present case the different position of the imidazole sub-unit (upper or lower position of the β-CD rim) does not affect significantly the relative amounts of the $^{\bullet}$OH radicals reacting with the two active scavenging sites. This finding is strictly related to the structure of CDHC6 and CDHC3. In fact, NMR analysis (*vide supra*) pointed out that in both the homocarnosine derivatives the



Scheme 1. Pictorial view of the different products formed by reaction of •OH radicals with the two sub-units of CDHC6 (a) and idealized structures of the homocarnosine (top) and carnosine (bottom) derivatives of β-CD (b).

imidazole ring is located outside the β-CD cavity, probably due to the presence of the additional methylene group, and thus not hindered against the scavenging of the •OH radicals (Scheme 1b).

3.3. LDL oxidation

The effect of carnosine, homocarnosine and their cyclodextrin conjugates on copper-induced LDL oxidation has been determined.

The oxidative modification of LDL is an important event in the pathogenesis of atherosclerosis [43] and is evidenced by alteration in the protein and lipid components of the particle. Compared to pulse radiolysis process, LDL oxidation is featured by a radical reaction series that yields more than a free radical (•OH) [44]. Thus, all the tested compounds may perform their antioxidant activity through several ways, depending on the reaction that they inhibit.

Table 1 shows the data referring to the protective effects of AH, HC, CD, as well as the equimolar combination of CD with any of the considered dipeptides. The considered dipeptides, as well as CD, have only low protective effects towards copper-induced human LDL oxidation. The highest efficacy was observed for AH (22.5% inhibition of MDA production), whereas HC and CD reached 17.6% and 10.7% of inhibition, respectively, at the lowest metal-to-ligand ratio. The concomitant presence of equimolar amounts of CD and any of the histidine-containing dipeptides produced antioxidant effects that were merely the sum of the activities of the two considered compounds.

Fig. 5 reports the dose–response effects of increasing concentrations of CDAH3, CDAH6, CDNHAH6 towards human LDL oxidation induced by 40 μM Cu^{2+}. CDAH3 reduced the control MDA amount by 3–98% when the ligand concentration ranged from 2 to 200 μM. In the same concentration range, the inhibition activity of CDAH6 ranged from 4% to 75%. The antioxidant activity of CDNHAH6 also increased as the metal-to-ligand ratio decreased and reached 50% at highest ligand concentration (200 μM). All the AH derivatives, therefore, inhibited the MDA production more than CD, AH and the equimolar combination of both. Moreover, the C-3 cyclodextrin conjugate with AH gave a more effective antioxidant than the corresponding C-6 derivative. This was particularly evident for concentrations higher than 20 μM. The apparent IC_{50} of CDAH3 was 23.4 μM and that of CDAH6 was 85.2 μM.

As shown in Fig. 6, the chemical modification of HC with CD linked at 3 or at 6 position produced resulting compounds

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)	AH	HC	CD	CD + AH	CD + HC
2	0.28 (0.07)	0.31 (0.08)	0.11 (0.01)	0.41 (0.11)	0.54[a] (0.13)
5	0.93 (0.10)	1.02 (0.21)	0.38 (0.06)	1.42[a] (0.18)	1.59[a] (0.23)
10	1.38 (0.27)	1.86 (0.29)	0.65 (0.13)	2.27[a,b](0.35)	2.55[a] (0.31)
20	2.72 (0.41)	2.50 (0.22)	1.26 (0.18)	4.31[a,b] (0.46)	4.71[a,b] (0.39)
50	9.13 (1.21)	8.28 (1.36)	3.87 (0.64)	14.66[a,b] (1.94)	16.32[a,b] (2.03)
100	15.06 (2.63)	12.10 (1.19)	6.94 (0.83)	23.18[a,b] (2.78)	28.09[a,b] (3.14)
200	22.47 (2.05)	17.56 (2.12)	10.72 (1.55)	35.47[a,b] (4.21)	38.12[a,b] (2.97)

Values are the mean (s.d.) of four different experiments and are expressed as protection percentage (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.

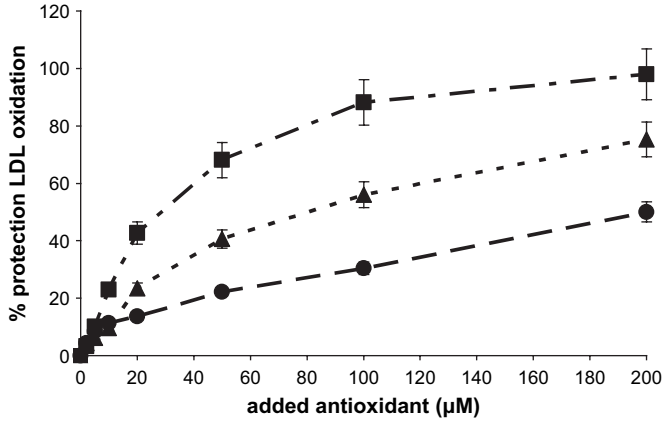


Fig. 5. Dose–response protective effect of CDNHAH6 (●), CDAH3 (■) and CDAH6 (▲) on 40 μM copper-induced lipid peroxidation of human LDL. Peroxidation was evaluated by the HPLC determination of MDA. Control LDL were incubated in presence of 40 μM Cu^{2+} and buffer only, with no added antioxidant (0% protection). Each point represents the mean of four different experiments. Standard deviations are represented by vertical bars.

with lower antioxidant potency than that of corresponding AH derivatives. CDHC3 and CDHC6 inhibited the MDA production at most of 39% and 27%, respectively. Thus, the antioxidant activity towards the copper-induced LDL oxidation was not higher than that due to the equimolar combination of CD and HC.

The pH dependency of the capacity of the different cyclodextrin derivatives to inhibit MDA formation was tested by challenging human LDL with 40 μM Cu^{2+} for 4 h at pH 5.0, instead of pH 7.4. For the sake of simplicity, only results referring to the most effective cyclodextrin derivative (CDAH3) are summarized in Table 2. The difference of MDA amount generated at the two tested pHs for any metal-to-ligand ratio was not greater than 3%. It is therefore evident that the mild acidification of the incubation medium had no significative effects either on the extent of copper-induced LDL oxidation or on the inhibitory capacity of the β-cyclodextrin conjugate.

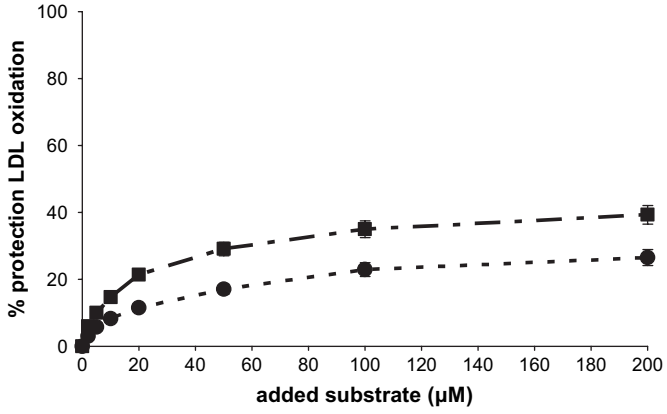


Fig. 6. Dose–response protective effect of CDHC3 (■) and CDHC6 (●), on 40 μM copper-induced lipid peroxidation of human LDL. Peroxidation was evaluated by the HPLC determination of MDA. Control LDL were incubated in presence of 40 μM Cu^{2+} and buffer only, with no added antioxidant (0% protection). Each point represents the mean of four different experiments. Standard deviations are represented by vertical bars.

Table 2
Dose–response effect of CDAH3 on lipid peroxidation induced in human LDL by 40 μM Cu^{2+} at pH 5.0 and 7.4

CDAH3 (μM)	MDA (μM) pH 5.0	MDA (μM) pH 7.4
0	0.00 (0.31)	0.00 (0.20)
2	6.36 (0.61)	5.59 (0.55)
5	10.96 (0.96)	10.40 (0.98)
10	22.92[a] (1.90)	18.46[a] (1.51)
20	40.24[a] (3.55)	40.38[a] (3.42)
50	64.69[a] (5.27)	61.19[a] (5.02)
100	83.11[a] (7.01)	79.98[a] (6.61)
200	90.24[a] (7.94)	90.72[a] (7.46)

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

It is known that strong metal chelating molecules such as EDTA are able to prevent the copper-catalyzed peroxidation [45]. In order to compare the protective effect towards the Cu^{2+}-induced LDL oxidation with the chelating property of free and CD-conjugated dipeptides, the amounts of free and complex species of metal and some tested compounds were calculated (Table 3). All these values, expressed as percent of total copper concentration (40 μM), were obtained by using the previous determined copper-binding constants [20]. As shown in Table 3, the copper chelating effect of AH and their derivatives increases with their concentration and the amount of free copper ions come to zero when the ligand concentration is higher than 50 μM. Moreover, AH, CDAH3 and CDAH6 produce the same amount of free and complex species for any metal-to-ligand ratio at pH 7.4, but their inhibition efficacy towards MDA formation show a different pattern. On the contrary, the percentage of MDA inhibition of CDAH3 at pH 5.0 and 7.4 are not significantly different, while the copper-binding ability is distinctly pH-dependent. HC protects the copper-mediated LDL oxidation as much as AH, but its ability to chelate free copper ions is lower.

On the basis of these results, we could hypothesize which are the molecular events that involve the inhibition of copper-induced LDL oxidation from AH, HC and their cyclodextrin derivatives.

The copper complex species formed with the tested histidine-containing dipeptides contribute to their antioxidant activity by two distinct ways: by decreasing the amount of free metal ions available for the oxidizing LDL induction [46] and by acting as scavengers towards $O_2^{\bullet -}$ radicals [47]. Because the superoxide ion is not the major radical species of the copper dependent LDL oxidation [44], the SOD-like activity of the complexes should not significantly contribute to the observed antioxidant effect. Moreover, as we observe in Table 3, there is not a straightforward correlation between the LDL protection and the concentration decrease of free copper ions, caused by the different ligands. For example, the protection percentages of AH and HC are not significantly different, even if AH is a copper(II) ligand stronger than HC [20,48]. Results of the experiments carried out at pH 5.0 (see Table 2) clearly confirm that, in the overall antioxidant mechanism of the β-cyclodextrin derivatives, copper sequestration plays a minor role, at least in the concentration ranges of both

Table 3
Total free and complex species percentages of copper and ligand in comparison with the inhibition MDA formation of any tested compound

Ligand	Cu^{2+}:ligand molar ratio	LDL protection[a]	Total ligand[a]	Free Cu^{2+a}	Free ligand[a]	Complexed Cu^{2+a}
AH	20:1	0	5	95	0	5
	8:1	1	13	87	0	13
	4:1	1	25	75	0	25
	2:1	3	50	50	0	50
	1:1.25	9	125	0	25	100
	1:2.5	15	250	0	150	100
	1:5	22	500	0	400	100
HC	20:1	0	5	99	4	1
	8:1	1	13	97	10	3
	4:1	2	25	94	19	6
	2:1	3	50	89	39	11
	1:1.25	8	125	75	100	25
	1:2.5	12	250	58	208	42
	1:5	18	500	39	434	61
CDAH6	20:1	4	5	95	0	5
	8:1	6	13	87	0	13
	4:1	10	25	75	0	25
	2:1	24	50	50	0	50
	1:1.25	41	125	0	25	100
	1:2.5	56	250	0	150	100
	1:5	75	500	0	400	100
CDAH3 (pH 7.4)	20:1	13	5	95	0	5
	8:1	26	13	87	0	13
	4:1	48	25	75	0	25
	2:1	58	50	50	0	50
	1:1.25	71	125	0	25	100
	1:2.5	82	250	0	150	100
	1:5	97	500	0	400	100
CDAH3 (pH 5.0)	20:1	15	5	97	2	3
	8:1	28	13	93	5	7
	4:1	45	25	86	11	14
	2:1	60	50	74	24	26
	1:1.25	75	125	48	73	52
	1:2.5	83	250	28	178	72
	1:5	91	500	14	414	86

[a] Values expressed as percentage of total copper concentration (40 μM).

oxidants and antioxidants tested and in the experimental model of human LDL oxidation.

In the activity of CDAH3 and CDAH6, previous observation from our laboratories indicated that the imidazole ring and the β-cyclodextrin moiety serve as competitive scavenging sites for hydroxyl radicals [21]. As reported in Table 3, the inhibition percentage increases as the copper-to-ligand ratio decreases. Thus, the scavenging activity of the tested compounds should affect the protection of LDL oxidation more than their copper-binding affinity.

Further molecular events that may play an important role in preventing LDL oxidation could reasonably involve the inclusion ability of the cyclodextrin cavity. The CD moiety could reduce the LDL oxidation by inclusion of cholesterol intermediate oxidation products [49]. It is also known that the initiation of Cu^{2+}-mediated LDL oxidation involves tryptophan radicals formed at Cu^{2+} binding sites on apolipoprotein B-100 [50]. Because cyclodextrins are able to interact with protein aromatic amino acids [51], it is likely that the inclusion events involving cyclodextrin moieties and Cu^{2+} accessible tryptophan residues prevent the oxidation of such amino acids and, then, the lipid peroxidation.

As illustrated in Figs. 5 and 6, all 3-derivatives had higher antioxidant potency than that of the corresponding 6-conjugates. According to the different structures of the two types of derivatives, this result allows to hypothesize that CD-grafted dipeptides, where the antioxidant activity is most presumably elicited, are somehow less available for the reaction with ROS when the link with β-cyclodextrin is on the upper rim rather than on the lower rim. We can speculate that this could be due to the different size of the two rims of the β-cyclodextrin. It has been reported that β-CD is able to include free [52] and protein-included aromatic amino acids [51,53]. Because the cyclodextrin cavity includes the indole group of tryptophan from the upper rim [52], the steric hindrance of 6-conjugated dipeptides could likely reduce the inclusion complex formation and, then, the inhibitory effect towards the LDL oxidation.

CDHC3 and CDHC6 showed an efficacy to inhibit MDA formation lower than that of corresponding AH derivatives. It remains unclear how an additional methylne group can change significantly the inhibition activity. The unknowing of the copper-binding constants of CDHC3 and CDHC6 does not allow to understand if the different inhibition of copper-induced LDL oxidation could depend on the amount of free metal ions.

However, it has been reported that the complex species of AH and HC cyclodextrin derivatives have a different structure [20,47]. At physiological pH, CDAH3 forms two fused six-membered chelate rings instead more stable than two fused seven- and six-membered rings of the Cu^{2+}–CDHC3 complex. Thus, CDAH3 could be more effective of the corresponding HC derivative for decreasing the oxidizing effects of free metal ions.

Stability of copper complex species and inhibitory effects towards the Cu^{2+}-induced LDL oxidation seem also correlated to all six-derivatives. At pH 7.4 the main species of copper(II)–CDAH6 system is a dimeric complex, while CDHC6 is not able to form such stable species [20].

4. Conclusions

The syntheses of the new bioconjugates of β-CD with homocarnosine are reported together with an investigation of their scavenger ability towards $^{\bullet}OH$ radicals. Furthermore the antioxidant activity of the new bioconjugates in comparison with the carnosine–cyclodextrin derivatives was also investigated in the LDL oxidation assay. The new compounds are effective $^{\bullet}OH$ radical scavengers, as confirmed by the diffusion-controlled value of the bimolecular quenching constant related to the reaction with these radical species. The different functionalization way does not appear to influence the spectroscopic and kinetic behaviours of the investigated compounds which exhibit similar scavenging capability and quenching mechanisms, in agreement with the disposition of the homocarnosine sub-unit outside the cavity. In particular, as already reported in the case of carnosine derivatives [21], the imidazole and β-CD can be viewed as independent sub-units of the same molecule, both playing an active role in the scavenging of the $^{\bullet}OH$ radicals.

In light of the relatively stable nature of the radical species formed following the reaction of these compounds with $^{\bullet}OH$ radicals if compared with the $^{\bullet}OH$ radicals themselves, a protective action of the synthesized compounds towards the harmful effects of this species can be reasonably expected.

Results reported in the present study clearly demonstrate that the homocarnosine and carnosine–cyclodextrin conjugates show also an antioxidant activity already in the micromolar range in the LDL assay. Particularly, CDAH3 has a remarkable activity, as indicated by the values of the IC_{50}, (23.4 μM), i.e. it is effective at concentrations 10–20 times lower than those reported for other synthetic derivatives previously tested [54].

This finding strongly indicates that these compounds 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 its activities in neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases. Studies in cell and/or animal models to characterize the pharmacological profile of the most effective AH derivatives here reported are in progress.

Acknowledgements

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