Synthesis of New Carnosine Derivatives of β-Cyclodextrin and Their Hydroxyl Radical Scavenger Ability

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Several *in vitro* and *in vivo* studies have suggested that carnosine can act as a scavenger of reactive oxygen species and intracellular proton buffer. On the other hand, carnosinase is a specific peptidase able to destroy the biological active dipeptide. To overcome this constraint, β-cyclodextrin (β-CD) was functionalized with carnosine to give the following new compounds: 6^A-[(3-{[(1*S*)-1-carboxy-2-(1*H*-imidazol-4-yl)ethyl]amino}-3-oxopropyl)amino]-6^A-deoxy-β-cyclodextrin (**1**), 6^A-[(β-alanyl-L-histidyl)amino]-β-cyclodextrin (**2**), and (2^A*S*,3^A*R*)-3^A-[(3-{[(1*S*)-1-carboxy-2-(1*H*-imidazol-4-yl)ethyl]amino}-3-oxopropyl)amino]-3^A-deoxy-β-cyclodextrin (**3**). Pulse-radiolysis investigation showed that the β-CD derivatives **1**–**3** are excellent scavengers of OH· radicals. Their activity is not only due to the formation of the stable imidazole-centered radical, but also to the scavenger ability of the glucose moieties of the macrocycle (*Scheme*). This effect is independent of the disposition of the imidazole ring. In fact, the quenching constant values are similar for the three compounds.

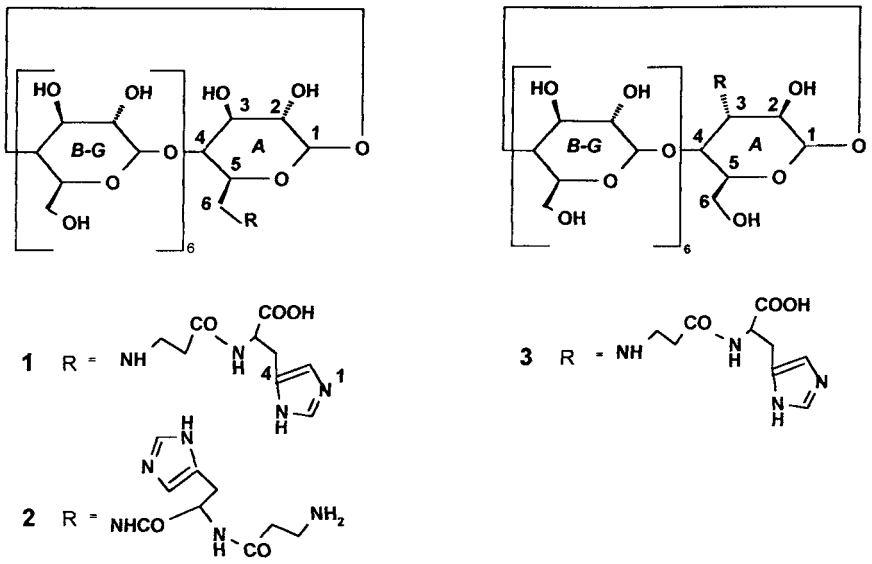
Introduction. – Cyclodextrins (CDs), cyclic oligomers of α-D-glucopyranose, have attracted interest in a variety of contexts [1–7], which include studies of their role in the realization of delivery systems [6–12]. This is also reflected in the increasing number of patents in this field. Drug targeting commonly involves the use of cyclomaltosaccharides as CD inclusion complexes [7–13]. It has been pointed out that the use of CD as a drug carrier may sometimes provide a simple, cheap, and effective strategy to increase drug solubility [6][7][12][13], stability [6][7][12][13], and photostability [14–16] to modulate drug photoreactivity as well as to minimize the photoinduced toxic effects of a drug on biosubstrates [17][18]. The bio-availability and effectiveness of the drug itself [6][7][12] may also be improved, especially in the case of lipophilic drugs.

Cyclodextrin complexation has proved promising in stabilizing and increasing the absorption of growth hormones [19], interleukin-2 [19], aspartame [20], albumine [21], γ-globuline [21], cyclosporine A [22], calcitonin [23], and insulin [6][24][25]. Rapid plasma clearance and problems regarding immunogenicity [6–8] may limit the practical use of peptides or proteins of therapeutic importance. 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 [26–35]. The data reported support an interest in developing the design and investigation of such molecules. Biological peptides such as enkephalin [26][31], enkephalin analogue DPDPE [33], neuropeptide substance P [30], and gastrin peptides [27] have been grafted onto CDs.

The spacer used to graft bioactive molecules onto the CD cavity may be expected to play an important role in improving the accessibility of the bioactive component. There is, however, little recent data available for the biological properties of these systems. Some of the biological properties of tetra- and heptapeptides bonded to β-CD, *i.e.*, of artificial receptor agonists of the CCK-B/gastrin receptor, have been reported [27]. The enzymatic digestion rate of these bioconjugates with proteases has been tested and, in some cases, increased stability has been reported [27]. Slow enzymatic-degradation rates in plasma have also been reported for substance P derivatives [30]. Carnosine (= *N*-β-alanyl-L-hystidine-βAH) was discovered one hundred years ago, and in the last few years, a number of its biological roles have been discovered [36]. These take into consideration the regulation of the intracellular concentration of protons, metal ions, and ROS (reactive oxygen species), as well as that of active sugars. A number of papers and patents describe βAH as a potential drug able to protect against oxidative stress and related diseases [37–42], including ocular disorders [42]. Exogenous carnosine does not accumulate in tissues, and it is destroyed by carnosinase [43], a very specific dipeptidase present in plasma, in the liver, and in the kidneys. To overcome this limitation, modified carnosine, such as *N*-acetylcarnosine [42], has been tested successfully as a pro-drug for the treatment of ocular disorders related to the oxidative-stress process (*i.e.*, cataracts, glaucoma, retinal degeneration, corneal disorders, ocular inflammation). *N*-Acetylcarnosine is digested slowly in comparison to the carnosine present in biological fluids. The functionalization of carnosine with cyclodextrins may make it possible to stabilize the carnosine while maintaining its biological and pharmaceutical properties. These systems might have the same therapeutic applications, expecially in the protection against ROS, which are responsible for oxidative stress.

This paper reports the synthesis and the NMR characterization of the following new compounds: 6^A-[(3-{[(1*S*)-1-carboxy-2-(1*H*-imidazol-4-yl)ethyl]amino}-3-oxopropyl)-amino]-6^A-deoxy-β-cyclodextrin (**1**), 6^A-[(β-alanyl-L-histidyl)amino]-6^A-deoxy-β-cyclodextrin (**2**), and (2^A*S*,3^A*R*)-3^A-[(3-{[(1*S*)-1-carboxy-2-(1*H*-imidazol-4-yl)ethyl]-amino}-3-oxopropyl)amino]-3^A-deoxy-β-cyclodextrin (**3**). Before testing their ability to survive carnosinase, an enzyme that is not yet fully characterized, the OH$^\bullet$ scavenger features of these compounds were determined by means of pulse radiolysis. To the best of our knowledge, no investigation of the scavenger ability of functionalized CDs against OH$^\bullet$ radicals has been reported up to now.

Results and Discussion. – 1. *Syntheses.* The three derivatives of carnosine were synthesized with the aim of conjugating stabilization due to functionalization as reported for other bioconjugates with biologically active carnosine. In derivatives **1** and **3**, grafting involved the amino group of βAH, which thus became a secondary amino group. The grafting of βAH to form derivative **2** instead involved the carboxylic group; thus a peptide bond was formed between the amino group inserted onto β-CD and βAH. Derivative **2** has a primary amino group, and the antiglycating activity reported for βAH may be maintained [41]. Derivatives **1** and **3** were synthesized by methods reported in the literature [44][45]. Compound **2** was synthesized as described for the Boc derivative [46], removing the Boc group. The products were characterized by NMR and mass spectroscopy.



2. *NMR Characterization*. The 1D spectra were assigned by COSY, TOCSY, HSQC, and T-ROESY data.

NMR Spectra of **1**. ^{1}H- and ^{13}C-NMR Spectra confirm the identity of product **1**. In addition to the signals due to the protons of the CD moiety in the 4.1–3.4 ppm region, the signals of the βAH moiety are also evident. The protons H–C(2) and H–C(5) of Im resonate in the aromatic region at 7.84 and 6.95 ppm. H–C(α) of His appears at 4.40 ppm, while $CH_2(\beta)$ of His give rise to signals at 3.10 and 2.95 ppm, and the CH_2CH_2 moiety appears at 2.95 and 2.60 ppm. The CH_2 (6^A) protons resonate at 3.47 and at *ca*. 3.10 ppm and are diastereotopic, as typically observed for this kind of derivative [46][48]. The signal due to H–C(5^A) is evident at 4.02 ppm. The chemical-shift values of H–C(5^A) and $CH_2(6^A)$ suggest the zwitterionic form for compound **1**. ROESY Experiments suggest that the carnosine chain is not included in the cavity. No NOE peaks are evident in the ROESY plot.

NMR Spectra of **2**. The signals of the βAH moiety are evident. The protons H–C(2) and H–C(5) of Im resonate in the aromatic region at 7.73 and 6.97 ppm. H–C(α) of His resonates at 4.64 ppm, $CH_2(\beta)$ of His at *ca*. 3 ppm, and the CH_2CH_2 moiety at 3 and 2.60 ppm. Some protons of the A ring can be assigned as follows: H–C(4^A) at 3.22 ppm, 1 H–C(6^A) at 3.48 ppm, and the other H–C(6^A) at *ca*. 3.6 ppm. ROESY Plots show a cross peak between the Im protons and some protons of the β-CD int he H–C(3)/CH_2(6)/H–C(5) region. The signals overlap and the resonances have thus been assigned, but the correlation between H–C(5) of Im and H–C(5^A) is also evident. Furthermore, H–C(2) of Im shows a correlation with the H–C(3)/H–C(5)/CH_2(6) region at *ca*. 3.82 ppm. This may suggest the preferred axial disposition of the imidazole moiety with respect to the cavity near the upper rim of β-CD, *i.e.*, H–C(5) of Im might preferentially be oriented towards the H–C(5^A) protons. An H-bond between the NH of imidazole and the OH of the upper rim could stabilize this conformation.

NMR Spectra of **3**. The ^{1}H-NMR spectrum of compound **3** shows the signals due to the functionalized ring A together with those for the CD protons at 4.0–3.4 ppm. As a consequence of 3-functionalization, signals of H–C(5^A), H–C(4^A) and H–C(3^A) appear at 4.17, 4.12, and 2.26 ppm, respectively. All the other protons at ring A are easily identified by COSY experiments. The H–C(4^A) and H–C(3^A) chemical-shift values suggest that **3** is in zwitterionic form. The synthetic route followed to functionalize CDs at C(3) involves configuration inversions at C(2) and C(3), thus replacing a glucose by an altrose unit. The coupling constant $J(1^A, 2^A) = 6.5$ Hz indicates that H–C(1^A) and H–C(2^A) are both axial in the altrose unit, this being in accord with its predominate $^1C_4$ conformation [48][49]. The signals due to the aliphatic protons of the βAH moiety appear at 3.0–2.65 ppm, and the CH_2 protons of the CH_2CH_2 moiety of the β-alanine residue are diastereotopic, unlike those of free βAH. This suggests a rigid disposition of the chain. An H-bond between NH of the functionalized ring or the peptidic bond and the OH of the adjacent ring can be formed. This can determine the chain rigidity together with the interaction of the imidazole with the cavity. The ROESY plot (*Fig. 1*), in fact, shows

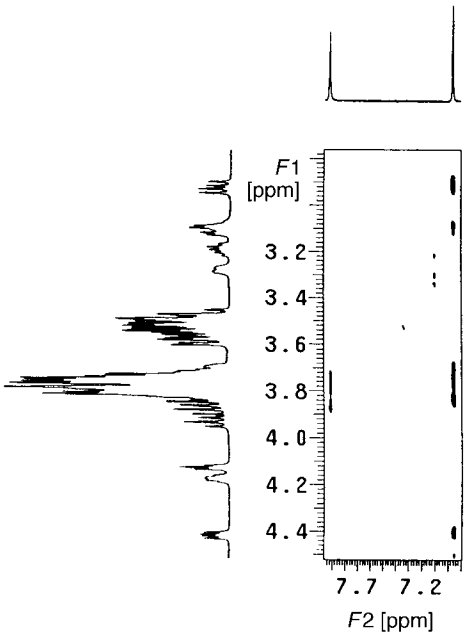


Fig. 1. *Partial contour plot of the T-ROESY experiment* (500 MHz, D_2O) *with* **3**

correlation peaks between the imidazole protons and H–C(3) and H–C(5) of the cyclodextrin moiety at 3.7–3.9 ppm. Due to the overlap of the CD signals in this region, the protons which correlate with imidazole protons were not identified. However, the interaction of the aromatic ring with the β-CD cavity is evident. The ^{13}C-NMR spectra show a large upfield shift of C(3^A) in comparison to β-CD, thus confirming substitution at the C-(3^A) position.

3. *Pulse Radiolysis. Preamble.* The pulse-radiolysis technique is a direct method to gain insight on 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 *Eqn. 1* where the values (in parentheses are the yields expressed in terms of *G* values in μmol J^{-1}) [50]:

$$\begin{aligned} H_2O \rightarrow e^-_{aq}\ (0.27),\ OH^{\cdot}\ (0.28),\ \text{and}\ H^{\cdot}\ (0.062),\\ H_2\ (0.047),\ H_2O_2\ (0.073),\ H^+\ (0.27) \end{aligned} \tag{1}$$

Reaction of OH· Radicals with **1**–**3**. To investigate the scavenging properties of the synthesized compounds towards OH· radicals, samples containing **1**, **2**, or **3** were irradiated after saturating the aqueous solutions with N_2O. Under these experimental conditions, the e^-_{aq} are converted into OH· according to *Eqn. 2*, and the yield of this species increases to *ca.* 0.55. Since the dose used in our experiments was *ca.* 16 Gy, the concentration of OH· generated was *ca.* 10^{-5} M.

$$e^-_{aq} + N_2O + H_2O \rightarrow OH^{\cdot} + OH^- + N_2 \tag{2}$$

Fig. 2 shows the transient absorption spectrum taken 6 μs after the pulse and obtained after the reaction of OH· radicals with 10^{-4} M aqueous **1**. The spectrum is characterized by a relevant absorption below 280 nm and by two better-defined bands around 300 and 350–400 nm. 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, $k_{obs} = 1 \cdot 10^6\ s^{-1}$. A bimolecular quenching constant $k_{\mathbf{1}} = 1 \cdot 10^{10}\ M^{-1}\ s^{-1}$ related to the reaction of OH· with **1** is obtained from *Eqn. 3*. This value did not change significantly with the concentration of **1**, confirming that under these experimental conditions, OH· radicals react almost exclusively with the quencher.

$$k_{\mathbf{1}} = k_{obs}/[\mathbf{1}] \tag{3}$$

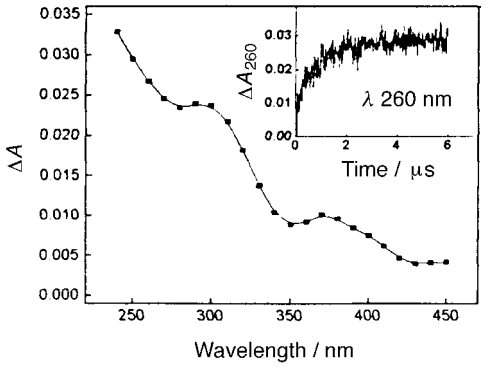


Fig. 2. *Absorption spectrum obtained from the pulse radiolysis of N_2O-saturated 10^{-4} M* **1**, *taken 6 μs after the pulse.* The inset shows the build-up monitored at 260 nm.

The bands at 300 and 350–400 nm are very indicative for an active role of the carnosine unit of **1** as a scavenger of OH· radicals. This is in excellent agreement with literature data showing that these radicals readily react with carnosine 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 the imidazole ring, preferentially at position C(2), 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 [51–53]. The bimolecular rate constant related to this process is $k_{carnosine} = 5 \cdot 10^9\ M^{-1}\ s^{-1}$, and it is only slightly dependent on pH [51]. This value is smaller than that obtained in our case (*vide supra*). This finding along with the remarkable absorption extending below 280 nm, not present in the case of either carnosine or imidazole alone [51–53], suggests that the imidazole ring of **1** may not be the unique scavenging site of the molecule. In this connection, a H-abstraction process involving the OH· radical and glucose, the monomer species of cyclodextrin, is reported to take place efficiently with a rate constant of $1.5 \cdot 10^9\ M^{-1}\ s^{-1}$ [51]. Thus, a direct participation of the β-CD unit of **1** in scavenging the OH· radical can be reasonably conceived. This suggestion was confirmed by an experiment carried out in the presence of 10^{-4} M β-CD itself. *Fig. 3* shows that the reaction between OH· and β-CD produces a transient absorption in the region 240–360 nm, probably attributable to C-centered radicals generated after H-abstraction from the macrocycle.

Due to the low extinction coefficient of the C-centered radicals, the build-up of this transient was significantly affected by the initial absorption of the OH· radicals [54].

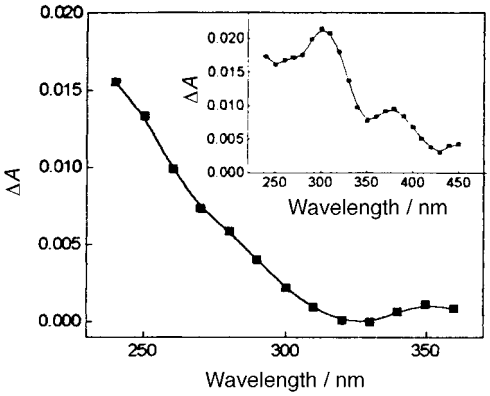


Fig. 3. *Absorption spectrum obtained from the pulse radiolysis of N_2O-saturated 10^{-4} M β-CD, taken 6 μs after the pulse.* The inset shows the difference transient absorption spectrum between **1** and β-CD, taken 6 μs after the pulse.

Nevertheless, we could estimate a rate constant of $k_{\beta\text{-CD}} \approx 9 \cdot 10^9$ M^{-1} s^{-1} for the reaction between OH$^\bullet$ and β-CD by the pseudo-first-order kinetic formation monitored at 240 nm. Although the aforementioned inconvenience precluded better accuracy, and, to the best of our knowledge, no values related to the reaction between OH$^\bullet$ radicals and β-CD have been previously published, a comparison of the obtained rate constant with that reported for glucose supports our data. In fact, the value found for $k_{\beta\text{-CD}}$ is *ca.* 6-fold higher than that reported for glucose [50], in good agreement with the fact that each molecule of β-CD contains 7 glucose units. Furthermore, it is noteworthy that $k_{\beta\text{-CD}}$ is very close to that found for the reaction of OH$^\bullet$ with **1**.

On the basis of these results, the transient absorption reported in *Fig. 2* may reflect independent contributions of the transient intermediates produced after reaction of the OH$^\bullet$ radicals with both imidazole and β-CD. This proposal is confirmed by the difference spectrum between **1** and β-CD shown in the inset of *Fig. 3*. Indeed, it can be noticed that this spectrum is virtually identical to that reported for the (OH$^\bullet$)·imidazole adduct [51–53]. Based on the ΔA at 310 nm and on the extinction coefficient for the (OH$^\bullet$)·imidazole adduct at this wavelength [53], we can estimate that the amount of this species formed is *ca.* $3 \cdot 10^{-6}$ M. Given that the concentration of the OH$^\bullet$ radicals generated in our experiments is *ca.* 10^{-5} M (*vide supra*), the concentration of the C-centered radicals formed after H-abstraction from β-CD would be *ca.* $7 \cdot 10^{-5}$ M. The proposed scenario rules out, of course, any attack of the OH$^\bullet$ radicals at the side chain of the imidazole in accordance with the literature. Indeed, it has been pointed out that such a process is only a minor pathway [55].

Fig. 4 shows the transient absorption spectrum taken 6 μs after the pulse and obtained after the reaction of OH$^\bullet$ radicals with 10^{-4} M aqueous **2**. As it can be seen, the shape of the transient obtained is very similar to that observed in the case of compound **1** (see *Fig. 2* for comparison). Thus, in the light of what was proposed above, we can safely infer that also in this case, the OH$^\bullet$ radicals react with both the β-CD and the imidazole ring. In particular, from the ΔA of the difference transient spectrum between **2** and β-CD related to the (OH$^\bullet$)·imidazole adduct and shown in the inset of *Fig. 4*, one can deduce that the amount of this latter species formed is essentially the same as in the case of compound **1** (*ca.* $3 \cdot 10^{-6}$ M). Finally, it is also worth to be noted that the bimolecular rate constant of the reaction between OH$^\bullet$ and **2**, obtained from the build-

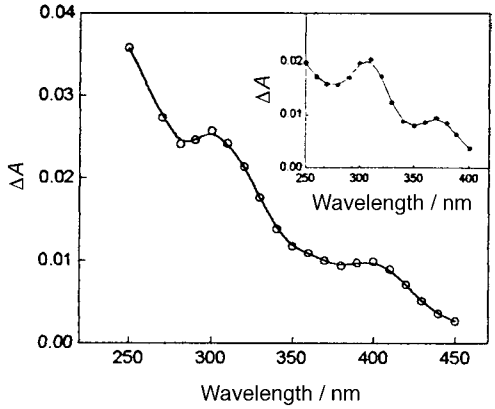


Fig. 4. *Absorption spectrum obtained from the pulse radiolysis of N_2O-saturated 10^{-4} M* **2**, *taken 6 μs after the pulse*. The inset shows the difference transient absorption spectrum between **2** and β-CD, taken 6 μs after the pulse.

up monitored at 260 nm, is $k_2 = 9 \cdot 10^9$ M^{-1} s^{-1}. This value is very similar to that obtained for **1**.

A scavenging process for OH$^\bullet$ radicals involving both the β-CD and the imidazole ring was found also in the case of compound **3**. In fact, the difference transient spectrum between **3** and β-CD after reaction with OH$^\bullet$ radicals and recorded 6 μs after the pulse (*Fig. 5*) is characterized by the typical absorptions around 300 and 370–400 nm, which may be safely attributable to the (OH$^\bullet$) · imidazole adduct. The bimolecular quenching constant, obtained from the growth monitored at 240 nm (see inset *Fig. 5*), is not different if compared to those of **1** and **2** ($k_3 = 9 \cdot 10^9$ M^{-1} s^{-1}). Nevertheless, the observed spectral behavior suggests clearly that an important quantitative aspect differentiates **3** from the previous compounds. Actually, from the values of the ΔA obtained, we can estimate that the amount of the (OH$^\bullet$) · imidazole adduct generated in this case is about two times lower than that formed in the cases of both **1** and **2**. We believe that this finding can be due to the particular structural conformation of **3** when compared with either **1** or **2**. Concerning this, the NMR experiments showed above provide clear evidences that in compound **3**, the imidazole moiety is included within the CD cavity, and it could be hindered against OH$^\bullet$ reaction.

The overall picture emerging from both the spectroscopic and kinetic behavior suggests that the imidazole and β-CD subunits of the compounds studied behave as

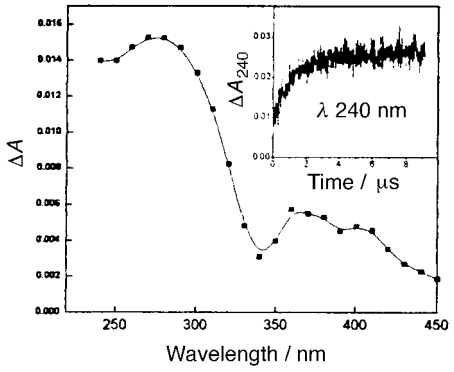
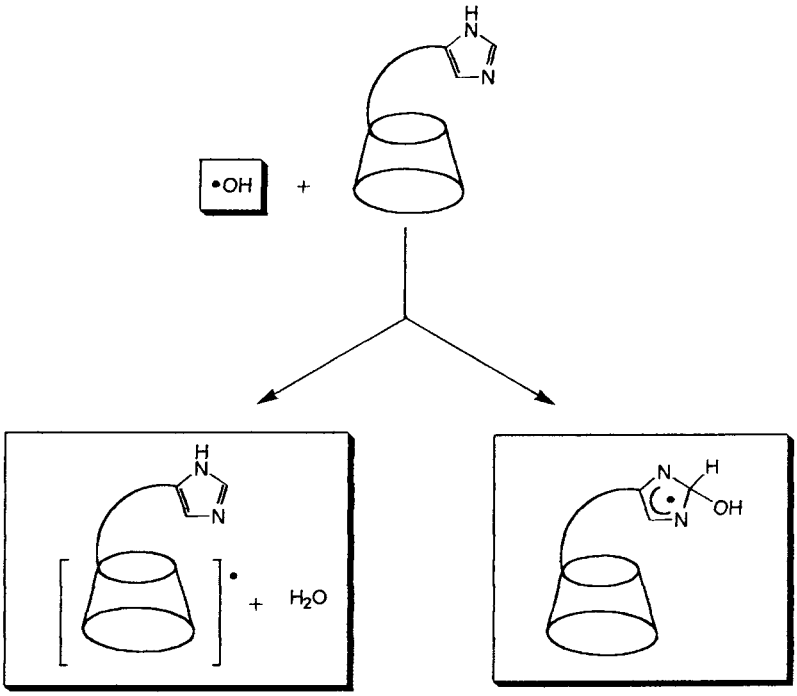


Fig. 5. *Difference transient absorption spectrum between 10^{-4} M aqueous* **3** *and β-CD obtained from the pulse radiolysis in N_2O, taken 6 μs after the pulse.* The inset shows the build-up monitored at 240 nm.

competing sites for scavenging the 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 H-atoms promptly available for a highly efficient H-abstraction process, as schematically depicted in the *Scheme*.

Scheme



The similarity in OH• scavenging capability displayed by **1**–**3** provides clear evidence that the different position of the imidazole subunit does not affect significantly the overall kinetic of scavenging. On the other hand, the structural features of the three molecules play a role in the relative amounts of OH• radicals reacting with the two active scavenging sites.

Conclusions. – The syntheses of the bioconjugates **1**–**3** of β-CD with carnosine are reported together with an investigation of their ability to scavenge OH• radicals. These compounds are efficient OH• radical scavengers, as confirmed by the high value of the quenching constant related to the reaction with this radical species. Derivatives **1**–**3** have different structural features, *i.e.*, **1** and **3** bear a carboxylic group, whereas **2** is characterized by the presence of a primary amino group. However, the different functionalization in **1** and **2** apparently does not influence their spectroscopic and kinetic behavior, and both **1** and **2** have similar scavenging capabilities as well as comparable quenching mechanisms. In particular, the imidazole and β-CD moieties can be considered as independent subunits of the same molecule, both playing an active role in scavenging the OH• radicals. The presence of the oligosaccharide moiety thus

enhances the ability of these systems to tune OH· radicals in comparison with free carnosine.

In the light of the relatively stable nature of the radical species formed in the reaction of these **1**–**3** with OH· radicals, compared to the OH· radicals themselves, a protective action of the synthesized compounds towards the harmful effects of this species can be reasonably expected. On the basis of the stabilization towards enzymatic hydrolysis reported for peptides bound to CDs [27][30] or only included in CDs [11], enzymatic stabilization may also be expected for these new derivatives. Functionalization with cyclodextrins could thus stabilize the carnosine moiety against the carnosinases (which are very specific dipeptidases), thus preserving the pharmacological activity reported for carnosine.

Experimental Part

General. β-Cyclodextrin was purchased from *Fluka* and anh. dimethylformamide from *Aldrich*; they were used without further purification. L-Carnosine methyl ester (βAHMe) was synthesized from L-carnosine (=βAH; (*Sigma*) with HCl in MeOH at 0° and AcCl as HCl source. The 6^A-amino-6^A-deoxy-β-cyclodextrin was synthesized as reported for the 6^A,6^B-diamino-6^A,6^B-dideoxy-β-cyclodextrin [56] from 6-tosyl-β-cyclodextrin [44]). TLC: silica gel plates (*Merck 60-F254*); detection of CD derivatives by UV and by the anisaldehyde test, or by the *Pauli* test for the derivatives of carnosine. CC = column chromatography. NMR Spectra: at 25° in D_2O; ^{1}H: *Varian-Inova-500* spectrometer at 499.883 MHz, standard pulse programs from the *Varian library*, length of 90° pulse *ca*. 7 µs; ^{13}C: *Bruker AC-200* spectrometer at 50.9 MHz; 2D experiments with 1K data points, 256 increments, and a relaxation delay of 1.2 s; TROESY with a 300-ms spin-lock time; DSS (sodium 3-(trimethylsilyl)propane-1-sulfonate) as external standard, δ in ppm, J in Hz.

*6^A-[(3-{[(1S)-1-Carboxy-2-(1*H*-imidazol-4-yl)ethyl]amino}-3-oxopropyl)amino]-6^A-deoxy-β-cyclodextrin* (**1**). βAHMe (1 g) was added to a soln. of 6^A-deoxy-6^A-iodo-β-cyclodextrin [44] (1 g) in anh. DMF (1 ml). The reaction was carried out at 70° under N_2 and under stirring. After 12 h, DMF was evaporated at 40°. The crude product was purified by CC (*CM Sephadex C-25* (20 × 600 mm), NH_4^+ form; H_2O, then linear gradient of 0–0.2M $(NH_4)HCO_3$ (400 ml)). The eluate containing the methyl ester of **1** was hydrolyzed in 1% NaOH soln. in H_2O/MeOH for 1 h. The solvent was evaporated an the product isolated by CC (*CM-Sephadex C-25* (20 × 600 mm), NH_4^+ form, H_2O): **1** (20%). R_f 0.56 (PrOH/H_2O/NH_3 5:3:1).

^{1}H-NMR (D_2O): 7.84 (*s*, H–C(2) of Im); 6.95 (*s*, H–C(5) of Im); 5.1–5.0 (*m*, 7 H, H–C(1) of CD); 4.40 (*m*, CH(α) of His); 4.02 (*m*, H–C(5^A), of CD); 4.1–3.7 (*m*, 26 H, H–C(3), H–C(5), CH_2(6) of CD); 3.7–3.4 (*m*, 14 H, H–C(2), H–C(4) of CD); 3.47 (*m*, 1 H–C(6^A) of CD); 3.22–3.05 (*m*, 4 H, 1 H–C(6^A) of CD, 1 H–C(β) of His, 1 CH_2 of Ala); 2.95 (*dd*, 1 H–C(β) of His); 2.60 (*m*, 1 CH_2 of Ala). ^{13}C-NMR (D_2O): 179.9 (COO); 174.5 (CONH); 137.7 (C(2) of Im); 135.1 (C(4) of Im); 119.9 (C(5) of Im); 104.5–103.8 (C(1) of CD); 85.8 (C(4^A) of CD); 83.4–83 (other C(4) of CD); 75.7–74.5 (C(2), C(3), C(5) of CD); 70.6 (C(5^A) of CD); 63.2–62.8 (C(6) of CD); 57.5 (CH(α) of His); 50.8 (C(6^A) of CD); 46.9 (CH_2 of Ala); 34.3 (CH_2 of Ala); 31.2 (CH_2 of His). FAB-MS: 1343 ($[M+1]^+$). Anal. calc. for $C_{51}H_{82}N_4O_{37}\cdot 8\,H_2O$: C 41.2, H 6.6, N 3.8; found: C 40.9, H 6.4, N 3.7.

6^A-[(β-Alanyl-L-histidyl)amino]-6^A-deoxy-β-cyclodextrin (**2**). The 6^A-({*N*-[(*tert*-butoxy)carbonyl]-β-alanyl-L-histidyl}amino)-6^A-deoxy-β-cyclodextrin [46] was deprotected in CF_3COOH under stirring. After 2 h, the solvent was evaporated and the final product purified by CC (*CM-Sephadex C-25* (20 × 600 mm), NH_4^+ form, H_2O): **2** (25%). R_f 0.28 (PrOH/H_2O/AcOEt/NH_3 5:3:3:1). ^{1}H-NMR (D_2O): 7.73 (*s*, H–C(2) of Im); 6.97 (*s*, H–C(5) of Im); 5.15–4.96 (*m*, 7 H, H–C(1) of CD); 4.64 (*m*, CH(α) of His); 4.00–3.76 (*m*, 26 H, H–C(3), H–C(5), CH_2(6) of CD); 3.76–3.57 (*m*, 14 H, H–C(2), H–C(4) of CD); 3.48 (*m*, 1 H–C(6^A) of CD); 3.22 (*m*, 1 H–C(6^A) of CD); 3.10–2.97 (*m*, 4 H, CH_2(β) of His, 1 CH_2 of Ala); 2.60 (*m*, 1 CH_2 of Ala). FAB-MS: 1342 ($[M+1]^+$). Anal. calc. for $C_{51}H_{83}N_5O_{36}\cdot 6\,H_2O$: C 42.2, H 5.7, N 4.8; found: C 41.5, H 5.5, N 4.6.

*(2^AS,3^AR)-3^A-[(3-{[(1S)-1-Carboxy-2-(1*H*-imidazol-4-yl)ethyl]amino}-3-oxopropyl)amino]-3^A-deoxy-β-cyclodextrin* (**3**). The 2^A,3^A-mannoepoxide of β-CD was formed *in situ* from 2^A-deoxy-2^A-tosyloxy)-β-cyclodextrin in aq. $NaHCO_3$ soln. (15 ml) [45]. β-AH (0.9 g) was then added and the mixture stirred at 60° under N_2 for 12 h. The solvent was evaporated and the solid purified by CC (*RP 8*, H_2O, then linear gradient of H_2O/MeOH 0 → 20%). The product isolated was further purified by a second CC (*CM-Sephadex C-25*, H_2O): **3**

(30%). R_f 0.56 ($PrOH/H_2O/NH_3$ 5:3:1). ^{1}H-NMR (D_2O): 7.91 (*s*, H–C(2) of Im); 6.95 (*s*, H–C(5) of Im); 5.07–4.94 (*m*, 6 H, H–C(1) of CD); 4.85 (*d*, $J(1^A,2^A) = 6.5$, H–C(1^A) of CD); 4.51 (*dd*, CH(α) of His); 4.17 (*m*, H–C(5^A) of CD); 4.13 (*t*, H–C(4^A) of CD); 3.95–3.09 (*m*, 27 H, H–C(3), H–C(5), CH_2(6), H–C(2^A) of CD); 3.95–3.45 (*m*, 13 H, H–C(2), H–C(4) of CD); 3.26 (*dd*, H–C(3^A)); 3.17 (*m*, 1 H–C(β) of β-Ala); 3.19 (*m*, 2 H, 1 H–C(β) of β-Ala, 1 H–C(β) of His); 2.92 (*dd*, 1 H–C(β) of His); 2.56 (*m*, 1 H–C(α) of β-Ala); 2.51 (*m*, 1 H–C(α) of β-Ala). ^{13}C-NMR (D_2O): 179.9 (COO); 175.2 (CONH); 137.3 (C(2) of Im); 134.4 (C(5) of Im); 120.3 (C(4) of Im); 105.8 (C(1^A) of CD); 105–103 (other C(1) of CD); 83–81 (C(4) of CD); 76.7 (C(4^A) of CD); 76–73.6 C(2), C(3), C(5) of CD); 62.9 (C(6) of CD); 62.4 (C(6^A) of CD); 61.0 (C(3^A) of CD); 57.4 (CH(α) of His); 45.1 (CH_2 of Ala); 43.6 (CH_2 of Ala); 31.0 (CH_2 of His). FAB-MS 1343 ($[M+1]^+$). Anal. calc. for $C_{51}H_{82}N_4O_{37} \cdot 4\,H_2O$: C 43.3, H 6.4, N 3.9; found: C 41.2, H 6.2, N 3.7).

Pulse Radiolysis. Pulse radiolysis was performed with electron pulses (*ca.* 20 ns duration) generated by the 12-MeV electron linear accelerator at the FRAE-CNR Institute in Bologna. The irradiations were carried out at r.t. ($22 \pm 2°$) on samples contained in *Spectrosil* cells of 2-cm optical-path length. Solns. were protected from the analyzing light by means of a shutter and appropriate cut-off 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-sat. soln. containing 0.1M HCO_2^- and 0.5 mM methylviologen (=1,1′-dimethyl-4,4′-bipyridinium dication; MV^{2+}) and on the basis of $G\varepsilon = 9.66 \cdot 10^{-4}$ m^2 J^{-1} at 602 nm [57]. G(X) represents the number of mol of species X formed or consumed per joule of energy absorbed by he system.

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REFERENCES

[1] 'Cyclodextrins', Eds. V. T. O'Souza and K. B. Lipkowitz, *Chem. Rev.* **1998**, *98*, 1741.
[2] 'Comprehensive Supramolecular Chemistry', Eds. J. L. Atwood, J. E. Davies, D. D. MacNicol, F. Vögtle, J. Szejtli, and J. Osa, Pergamon, Oxford, 1996, Vol. 3.
[3] M. L. Bender, M. Komiyama, 'Cyclodextrin Chemistry', Springer Verlag, Berlin, 1978.
[4] E. Rizzarelli, G. Vecchio, *Coord. Chem. Rev.* **1999**, *188*, 343.
[5] V. T. Souza, M. L. Bender, *Acc. Chem. Res.* **1987**, *20*, 146.
[6] V. J. Stella, V. M. Rao, E. A. Zannou, V. Zia, *Adv. Drug Deliv. Rev.* **1999**, *36*, 3.
[7] F. Hirayama, K. Uekama, *Adv. Drug Deliv. Rev.* **1999**, *36*, 125.
[8] L. R. Moses, K. J. Dileep, C. P. Sharma, *J. Appl. Polym. Sci.* **2000**, *75*, 1089.
[9] G. Trapani, C. Altomare, E. Sanna, G. Biggio, G. Liso, *Curr. Med. Chem.* **2000**, *7*, 249.
[10] D. Q. Ma, R. A. Rajewski, V. J. Stella, *Int. J. Pharm.* **1999**, *189*, 227.
[11] I. Tetsumi, K. Uekama, *Adv. Drug. Deliv. Rev.* **1999**, *36*, 101.
[12] C. LeBourlais, L. Acar, H. Zia, P. A. Sado, T. Needham, R. Leverge, *Recent Adv. Prog. Retinal Eye Res.* **1998**, *17*, 33.
[13] T. Irie, K. Uekama, *J. Pharm. Sci.* **1997**, *86*, 147.
[14] S. Sortino, G. De Guidi, G. Marconi, S. Monti, *Photochem. Photobiol.* **1998**, *67*, 603.
[15] S. Sortino, J. C. Scaiano, G. De Guidi, S. Monti, *Photochem. Photobiol.* **1999**, *70*, 549.
[16] S. Monti, S. Sortino, S. Encinas, G. Marconi, G. De Guidi, M. A. Miranda, 'Photoprocesses in Photosensitizing Drugs Containing a Benzophenone-Like Chromophore', in 'Drugs Photochemistry and Photostability', Eds. A. Albini and E. Fasani, The Royal Society of Chemistry, 1998, p. 150.
[17] S. Sortino, S. Giuffrida, S. Fazio, S. Monti, *New J. Chem.* **2001**, *25*, 707.
[18] S. Monti, S. Sortino, G. De Guidi, G. Marconi, *New J. Chem.* **1998**, *22*, 599.
[19] M. E. Brewster, M. S. Hora, J. W. Simpkin, N. Bodor, *Pharm. Res.* **1991**, *8*, 792.
[20] R. J. Prankerd, H. W. Stone, K. B. Sloan, J. H. Perrin, *Int. J. Pharm.* **1992**, *88*, 189.
[21] M. Katakam, A. K. Banga, *J. Pharm. Pharmacol.* **1995**, *47*, 103.
[22] K. Miyake, T. Irie, F. Hirayama, K. Uekama, *Drug Deliv. Syst.* **1998**, *13*, 369.
[23] N. G. M. Schipper, J. C. Verhoef, S. G. Romeijn, F. W. H. M. Merkus, *Calcif. Tissue Int.* **1995**, *56*, 280.
[24] F. W. H. M. Merkus, N. G. M. Schipper, J. C. Verhoef, *J. Controlled Release* **1996**, *41*, 69.
[25] K. Tokihiro, A. Hidetoshi, T. Shinichirou, I. Tetsumi, F. Hirayama, K. Uekama, *J. Pharm. Pharmacol.* **2000**, *52*, 911.

[26] F. Djedaini, B. Perly, A. W. Coleman, H. Parrot-Lopez, H. Galons, M. Miocque, *Tetrahedron Lett*. **1990**, *31*, 1999.
[27] N. Schaschke, S. Fiori, E. Weyher, C. Escrieut, D. Fourmy, G. Muller, L. Moroder, *J. Am. Chem. Soc*. **1998**, *120*, 7030.
[28] V. Cucinotta, F. D'Alessandro, G. Impellizzeri, G. Pappalardo, E. Rizzarelli, G. Vecchio, *J. Chem. Soc., Chem. Commun*. **1991**, 293.
[29] B. Di Blasio, V. Pavone, F. Nastri, C. Isernia, M. Saviano, C. Pedone, V. Cucinotta, G. Impellizzeri, E. Rizzarelli, G. Vecchio, *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 7218.
[30] C. Péan, C. Créminon, A. Wijkhuisen, J. Grassi, P. Guenot, P. Jéhan, J.-P. Dalbiez, B. Perly, F. Djedaïni-Pilard, *J. Chem. Soc., Perkin Trans. 2*, **2000**, 853.
[31] F. Djedaïni-Pilard, J. Desalos, B. Perly, *Tetrahedron Lett*. **1993**, *34*, 2457.
[32] F. Rossi, L. Zaccaro, B. Di Blasio, V. Pavone, O. Maglio, M. Saviano, C. Pedone, V. Cucinotta, G. Impellizzeri, E. Rizzarelli, in 'Peptides 1992', Eds. C. H. Schneider and A. N. Eberle, ESCOM Scienze Publisher, B. V. p. 577.
[33] M. K. Hristova-Kazermieski, P. Horan, P. Davies, H. I. Yamamura, T. Kramer, R. Horvath, W. M. Kazermieski, F. Porreca, V. J. Hruby, *Bioorg. Med. Chem. Lett*., **1993**, *3*, 831.
[34] R. P. Bonomo, G. Impellizzeri, G. Pappalardo, E. Rizzarelli, G. Vecchio, *Gazz. Chim. Ital*. **1993**, *123*, 593.
[35] D. La Mendola, E. Rizzarelli, G. Vecchio, Università di Catania, Pat. Appl. No. MI2000A01696, 25 July, 2000; Eur. Appl. No. 01117259.0, 17 July, 2001.
[36] 'Biological Role of Carnosine in the Functionalizing of Excitable Tissues', Ed. A. A. Boldrev, *Biochemistry (Moscow)* **2000**, *65*, 749.
[37] P. L. Quin, A. A. Boldyrev, V. H. Formaziuk, *Mol. Aspects. Mod*. **1992**, *13*, 379.
[38] J. Preston, A. R. Hipkiss, D. T. Himsworth, *Neurosci. Lett*. **1998**, *242*, 105.
[39] A. Hipkiss, *Neurosci. Lett*. **1997**, *135*, 138.
[40] A. Hipkiss, *Int. J. Biochem. Cell Biol*. **1998**, *30*, 863.
[41] C. Brownson, A. S. Hipkiss, *Free Radical Biol. Med*. **2000**, *28*, 1564.
[42] M. A. Babizhayev, V. N. Yermakova, N. L. Sakina, R. P. Evstigneeva, E. A. Rozhkova, G. A. Zheltukhina, *Clin. Chim. Acta* **1996**, *254*, 1.
[43] M. C. Jackson, C. M. Kucera, J. F. Lenney, *Clin. Chim. Acta* **1991**, *196*, 193.
[44] R. P. Bonomo, V. Cucinotta, F. D'Alessandro, G. Impellizzeri, G. Maccarrone, G. Vecchio, E. Rizzarelli, *Inorg. Chem*. **1991**, *30*, 2708.
[45] R. Breslow, A. W. Czarnik, M. Lauer, R. Leppkes, J. Winkler, S. Zimmerman, *J. Am. Chem. Soc*. **1986**, *108*, 1969.
[46] G. Vecchio, D. La Mendola, E. Rizzarelli, *J. Supramol. Chem*. **2001**, *1*, 87.
[47] V. Cucinotta, F. D'Alessandro, G. Impellizzeri, G. Maccarrone, E. Rizzarelli, G. Vecchio, *J. Chem. Soc., Perkin Trans. 2* **1992**, 1785.
[48] C. Donzè, E. Rizzarelli, G. Vecchio, *Supramol. Chem.* **1998**, *10*, 33.
[49] K. Fujta, O. Kazuko, Y. Ykegami, H. Shimada, T. Tahara, Y. Nogami, T. Koga, K. Saito, T. Nakajima, *Tetrahedron Lett*. **1994**, *35*, 9577.
[50] G. V. C. Buxton, L. Greenstock, W. P. Helman, A. B. Ross, *J. Phys. Chem. Ref. Data* **1988**, *17*, 513.
[51] A. Tamba, A. Torregiani, *Int. J. Radiat. Biol.* **1998**, *74*, 333.
[52] K. M. Nansal, R. M. Sellers, *J. Phys. Chem*. **1975**, *79*, 1275.
[53] P. S. Rao, M. Simic, E. Hayon, *J. Phys. Chem*. **1975**, *79*, 1260.
[54] S. O. Nielsen, B. D. Michael, E. J. Hart, *J. Phys. Chem*. **1976**, *80*, 2482.
[55] C. von Sonntag, 'Amino Acids, Oligopeptides and Protein', in 'The Chemical Basis of Radiation Biology', Taylor and Francis, London, 1987, p. 394.
[56] B. Di Blasio, S. Gualdiero, M. Saviano, C. Pedone, E. Benedetti, E. Rizzarelli, S. Pedotti, G. Vecchio, W. Gibbons, *Carbohydr. Res.* **1991**, *282*, 41.
[57] Q. G. Mulazzani, M. D'Angelantonio, M. Venturi, M. Z. Hoffman, M. A. Rodgers, *J. Phys. Chem*. **1986**, *90*, 5347.

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