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Biochemical properties of new synthetic carnosine analogues containing the residue of 2,3-diaminopropionic acid: the effect of N-acetylation

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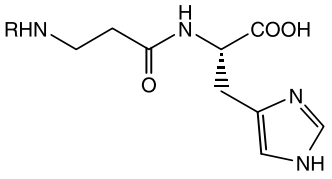
Summary. Three novel carnosine analogues **7–9** containing the residue of L(+)2,3-diaminopropionic acid with different degree of N-acetylation instead of β-alanine have been synthesized and characterized. Comparative analysis of hydrolysis by carnosinase revealed that the mono- and bis-acetylated compounds **8** and **9** are resistant to enzymatic hydrolysis and act as competitive inhibitors of this enzyme. The hydroxyl radical scavenging potential of the three analogues was evaluated by their ability to inhibit iron/H_2O_2-induced degradation of deoxyribose. The second-order rate constants of the reaction of compounds **7–9** with hydroxyl radical were almost identical to that of carnosine. These compounds were also found to act as protective agents against peroxynitrite-dependent damage as assessed by their ability to prevent nitration of free tyrosine induced by this species.

Keywords: Carnosine – Scavengers – Peroxynitrite – Free radicals – Antioxidants – Peptidomimetics

Abbreviations: The nomenclature and symbols of amino acids follow Recommendations of the IUPAC/IUB Joint Commission on Biochemical Nomenclature. Eur. J. Biochem (1984); 138: 9–37. Abbreviations listed in the guide recently published in J. Peptide Sci. (1999); 5: 465–471 are used without explanations. Additional abbreviations: Dap, L(+)2,3-diaminopropionic acid; EtOAc, ethyl acetate

Introduction

Carnosine (β-alanyl-L-histidine, Fig. 1) and structurally related compounds such as anserine (β-alanyl-3-methyl-L-histidine) and homocarnosine (γ-aminobutyryl-L-histidine) are natural histidine-containing dipeptides widely distributed in vertebrate organisms and present in relatively high concentrations (2–20 mM) in mammalian excitable tissues such as muscle and nervous system (Crush, 1970; Jackson et al., 1996; Bonfanti et al., 1999). Numerous biological functions have been attributed to carnosine including a role as a cytosolic buffer especially in the muscle (Okuma et al., 1992), a neurotransmitter (Rochel et al., 1982), and an antiglicating agent (Hipkiss et al., 2000; 1998; 1995). In addition, several studies have been reported indicating that carnosine and related compounds possess antioxidant and free radical-trapping properties (Boldyrev et al., 2002; Decker et al., 2000). These dipeptides have been shown to be good scavengers of peroxyl (Kohen et al., 1988) and hydroxyl radicals (Auroma et al., 1989; Babizhayev et al., 1994), efficient chelating agents for copper and other transition metal (Brown, 1981), and quenchers of singlet oxygen (Hartman et al., 1990; Egorov et al., 1997). Furthermore, in a recent paper (Fontana et al., 2002) we have reported that carnosine and analogues are able to prevent damage induced by peroxynitrite, a potent cytotoxic oxidant produced *in vivo* by the reaction of nitric oxide ($^{\bullet}NO$) and superoxide anion ($O_2^{\bullet -}$) (Huie et al., 1993). Carnosine and related compounds, therefore, behaving as multifunctional antioxidants, have been suggested to play an important role in the defense against damage induced by oxidative stress *in vivo* especially in skeletal muscle and brain, two tissues where these dipeptides are present in large amount (Boldyrev et al., 1988; 1997). The efficient ability of carnosine to protect biomolecules and cells from deleterious action of products accumulated during oxidative stress (Seidler et al., 2004;



Carnosine R = H-

N-acetylcarnosine R = CH_3CO-

Fig. 1. Structure of carnosine and N-acetylcarnosine

Hobart et al., 2004; Boldyrev et al., 2004; Stvolinsky et al., 1999) has also suggested its pharmacological use in those diseases where oxidative reactions have been implicated (Quinn et al., 1992). However, *in vivo*, the physiological and antioxidant activities of carnosine are limited by its susceptibility to hydrolysis by human cytosolic and serum carnosinases (Pegova et al., 2000). Consequently, there has been a considerable interest in the development of carnosine-related structures with increased metabolic stability in view of a possible therapeutic application (Babizhayev et al., 1998). In previous studies, we have described the synthesis and the bioactivity of carnosine-related pseudodipeptides characterized by the presence of a sulphonamide junction which confers stability towards enzymatic hydrolysis and preserve the antioxidant properties of the natural dipeptide (Fontana et al., 2002; Calcagni et al., 1999).

In tissues, carnosine is also subjected to enzymatic modification by acetylation to N-acetylcarnosine (NAC, Fig. 1) (O'Dowd et al., 1988). This natural carnosine derivative has been used as ophthalmic prodrug of carnosine in the long-term therapy of human cataracts (Babizhayev et al., 2002). The efficacy of NAC as therapeutic agent has been related to its resistance to carnosinase hydrolysis (Quinn et al., 1992; Jackson et al., 1991), with consequent prolongation of its lifetime. Moreover, because of the relative hydrophobicity compared with carnosine, NAC can pass more efficiently across the cellular membrane.

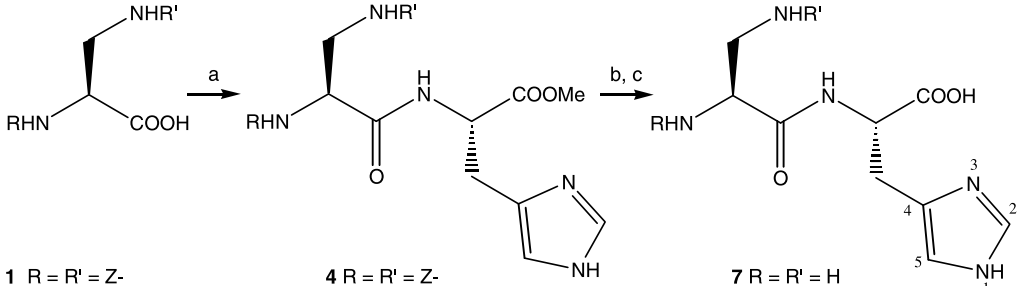
The present study has been carried out in order to investigate in more detail the effect of N-acetylation on the biochemical properties of carnosine. For this purpose, it seemed interesting to synthesize and study carnosine analogues obtained by replacing the residue of β-alanine with L(+)2,3-diaminopropionic acid. This chemical modification leads in fact to derivatives which combine a close structural similarity to the native model with the possibility to bear two N-acetylamino groups or to maintain a free extra amino group. Hence, we have synthesized three novel carnosine analogues **7–9** containing 2,3-diaminopropionic acid with a different degree of N-acetylation instead of β alanine (Fig. 2). The compounds have been tested either as substrates or inhibitors of human serum carnosinase. The antioxidant and free radical scavenger properties of these compounds have been also investigated by their ability to inhibit reactions induced by hydroxyl radicals and by peroxynitrite.

Materials and methods

A General methodology

Z-Dap(Z)-OH (**1**), H-Dap-OH, Z-Dap-OH and H-His-OMe · 2HCl were obtained from Novabiochem. Carnosine, L-histidine and *o*-phthaldialdehyde were from Fluka. The commercial preparation of synthetic carnosine was free of hydrazine as possible contaminant (Zhou et al., 1998). Human serum was obtained from the Centre of Transfusion of Rome. Hydroxylapatite (Bio-Gel HTP) resin and the protein assay kit were from Bio-Rad. DE-52 resin (pre-swollen) was from Whatman. Diaflo YM30 membranes were from Amicon. Peroxynitrite was synthesized from potassium nitrite and hydrogen peroxide under acidic conditions, as previously described (Beckman et al., 1994). All other reagents were of the highest purity commercially available.

TLC was performed on Merck 60 F_{254} silica gel plates developed with the following solvent system: (a) $CHCl_3$:MeOH (7:3), (b) *n*-BuOH:



1 R = R' = Z-
2 R = Z-, R' = CH_3CO-
3 R = R' = CH_3CO-

4 R = R' = Z-
5 R = Z-, R' = CH_3CO-
6 R = R' = CH_3CO-

7 R = R' = H
8 R = H-, R' = CH_3CO-
9 R = R' = CH_3CO-

Fig. 2. **a** H-His-OMe · 2HCl, NMM, DCC, DMF, 0°C, 3 h then 5°C, 16 h; **b** H_2, 10% Pd-C, (2:1) MeOH–H_2O, rt, 3 h (for analogues **7** and **8**); **c** NaOH 1N, MeOH, rt, 3 h. Numbering of the imidazole ring follows the IUPAC-IUB recommendations

AcOH:H_2O (4:5:1), (c) $CHCl_3$:MeOH (9:1). Column chromathography was carried out using Merck 60 silica gel (230–400 mesh). Optical rotations were taken at 25°C with Perkin-Elmer 241 polarimeter. IR spectra were recorded employing a Perkin-Elmer 1600 FTIR spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were determined on a Varian VXR 300 MHz instrument (δ expressed in ppm).

B Synthesis of carnosine analogues

Preparation of N-acetyl-aminoacids **2**–**3**

By following the procedure Chenault et al. (1989), Z-Dap-OH and H-Dap-OH were acetylated by using acetyl chloride under Schotten-Baumann conditions to give the mono- and bis-acetyl derivatives **2** and **3**, respectively, as amorphous solids.

Z-Dap(Ac)-OH (**2**)

1.5 g (yield 75%). Rf (a) = 0.59; $[\alpha]_D = -25°$; ^{1}H-NMR (D_2O) $\delta = 1.74$ (3H, s, CH_3), 3.32 (1H, m, Dap β-CH_A), 3.48 (1H, m, Dap β-CH_B), 4.19 (1H, m, Dap α-CH), 4.93 (2H, s, $PhCH_2$), 7.25 (5H, aromatics). For $C_{13}H_{16}N_2O_5$ (280.11) calculated: 55.71%C, 5.75%H, 9.99%N; found 55.32%C, 5.86%H, 9.97%N.

Ac-Dap(Ac)-OH (**3**)

1.1 g (yield 83%). Rf (b) = 0.68; $[\alpha]_D = -18.6°$ ($c = 1$, H_2O); ^{1}H-NMR (D_2O) $\delta = 1.77$ (3H, s, CH_3), 1.83 (3H, s, CH_3), 3.30 (1H, m, Dap β-CH_A), 3.48 (1H, m, Dap β-CH_B), 4.34 (1H, m, Dap α-CH). For $C_7H_{12}N_2O_4$ (188.08) calculated: 44.68%C, 6.43%H, 14.89%N; found 44.92%C, 6.63%H, 14.07%N.

Synthesis of dipeptide methyl esters **4**–**6**

To a stirred solution of H-His-OMe · 2HCl (1.3 g, 5.3 mmol) and NMM (1.2 ml, 10.6 mmol) in dry DMF (7 ml), the corresponding protected aminoacid (**1** for compound **4**, **2** for compound **5** and **3** for compound **6**, see Fig. 2) (5.3 mmol) were added at 0°C followed by portionwise addition of DCC (1.1 g, 5.3 mmol) in dry DMF (3 ml). After 3 h at 0°C and 16 h at 5°C, the reaction mixture was filtered and the resulting solution was evaporated under *vacuum*. The residue was taken up in $CHCl_3$ and the organic layer washed with saturated aqueous $NaHCO_3$ and H_2O. The residue obtained after drying and evaporation was cromatographed on silica gel using $CHCl_3$:MeOH (95:5) as eluant for compound **4**, $CHCl_3$/MeOH (9:1) for compound **5** and $CHCl_3$/MeOH (7:3) for compound **6** to give the corresponding dipeptide methylesters **4**–**6** as white foams.

Z-Dap(Z)-His-OMe (**4**)

1.6 g (yield 57%). Rf (c) = 0.46; $[\alpha]_D = -19.3°$ ($c = 1$, MeOH); ^{1}H-NMR (DMSO-d_6) $\delta = 2.86$–2.90 (2H, m, His β-CH_2), 3.17 (1H, m, Dap β-CH_A), 3.32 (1H, m, Dap β-CH_B), 3.54 (3H, s, OCH_3), 4.13 (1H, m, Dap α-CH), 4.43 (1H, m, His α-CH), 4.99 (4H, 2 × s, $PhCH_2$), 6.85 (1H, s, His H-5), 7.27–7.48 (10H, m, aromatics and 2 × Dap NH), 7.48 (1H, s, His H-2), 8.38 (1H, d, His NH, J = 7.50 Hz), 11.8 (1H, s, His H-1). For $C_{26}H_{29}N_5O_7$ (523.21) calculated: 59.65%C, 5.58%H, 13.38%N; found 59.72%C, 5.66%H, 13.29%N.

Z-Dap(Ac)-His-OMe (**5**)

1.1 g (yield 48%). Rf (c) = 0.35; $[\alpha]_D = -28.2°$ ($c = 1$, MeOH); ^{1}H-NMR (D_2O) $\delta = 1.76$ (3H, s, Ac CH_3), 2.86–2.90 (2H, m, His β-CH_2), 3.15 (1H, m, Dap β-CH_A), 3.28 (1H, m, Dap β-CH_B), 3.56 (3H, s, OCH_3), 4.10 (1H, m, Dap α-CH), 4.45 (1H, m, His α-CH), 5.01 (2H, s, $PhCH_2$), 6.81 (1H, m, His H-5), 7.27-7.37 (6H, aromatics and Dap OCONH), 7.49 (1H, s, His H-2), 7.88 (1H, br t, Dap NH), 8.37 (1H, d, His NH, J = 7.47 Hz), 11.75 (1H, s, His H-1). For $C_{20}H_{25}N_5O_6$ (431.18) calculated: 55.68%C, 5.84%H, 16.23%N; found 55.72%C, 5.86%H, 16.03%N.

Ac-Dap(Ac)-His-OMe (**6**)

0.9 g (yield 52%). Rf (c) = 0.44; $[\alpha]_D = -20.6°$ ($c = 1$, H_2O); ^{1}H-NMR (DMSO-d_6) $\delta = 1.76$ (3H, s, CH_3), 1.83 (3H, s, CH_3), 2.84–2.89 (2H, m, His β-CH_2), 3.14 (1H, m, Dap β-CH_A), 3.28 (1H, m, Dap β-CH_B), 3.57 (3H, s, OCH_3), 4.29 (1H, m, Dap α-CH), 4.42 (1H, m, His α-CH), 6.81 (1H, s, His H-5), 7.54 (1H, s, His H-2), 7.77 (1H, br t, Dap β-CH_2 NH), 7.99 (1H, d, Dap α-CH NH, J = 5.5 Hz), 8.39 (1H, d, His NH, J = 7.47), 11.82 (1H, s, His H-1). For $C_{14}H_{21}N_5O_5$ (399.15) calculated: 49.55%C, 6.24%H, 20.64%N; found 49.72%C, 6.56%H, 20.89%N.

Synthesis of the carnosine analogs **7**–**9**

According to the procedure previously reported by us (Calcagni et al., 1999) the above described N-protected dipeptide esters **4** and **5** were hydrogenated to give the corresponding dipeptide esters in 90% yield which were used as such.

To a solution of the corresponding amino ester or **6** (1.5 mmol) in MeOH (3 ml) 1N NaOH (1.8 mmol) was added under stirring at room temperature. After 3 h, the pH was adjusted to 6 by 2N HCl and the aqueous solution was concentrated and subjected to column chromatography on Sephadex LH-20 using H_2O:MeOH (2:1) as eluant to afford the corresponding dipeptides **7**–**9** as foams.

H-Dap-His-OH (**7**)

0.2 g (yield 60%). Rf (b) = 0.36; $[\alpha]_D = +11.8°$ ($c = 1$, H_2O); ^{1}H-NMR (D_2O) $\delta = 2.95$–3.02 (2H, m, Dap β-CH_A and His β-CH_A), 3.06–3.13 (2H, m, Dap β-CH_B and His β-CH_B), 3.64 (1H, m, Dap α-CH), 4.35 (1H, m, His α-CH), 7.04 (1H, s, His H-5), 8.12 (1H, s, His H-2). For $C_9H_{15}N_5O_3$ (241.12) calculated: 48.81%C, 6.27%H, 29.03%N; found 48.92%C, 6.46%H, 29.27%N.

H-Dap(Ac)-His-OH (**8**)

0.25 g (yield 58%). Rf (b) = 0.34; $[\alpha]_D = +25°$ ($c = 1$, H_2O); ^{1}H-NMR (D_2O) $\delta = 1.78$ (3H, s, CH_3), 2.90–3.05 (2H, m, Dap β-CH_2), 3.20–3.43 (2H, m, His β-CH_2), 3.75 (1H, m, Dap α-CH), 4.25 (1H, m, His α-CH), 6.91 (1H, m, His H-5), 8.10 (1H, s, His H-2). For $C_{11}H_{17}N_5O_4$ (283.13) calculated: 46.64%C, 6.05%H, 24.72%N; found 46.72%C, 6.12%H, 24.83%N.

Ac-Dap(Ac)-His-OH (**9**)

0.3 g (yield 57%). Rf (b) = 0.58; $[\alpha]_D = +20.6°$ ($c = 1$, H_2O); ^{1}H-NMR (D_2O) $\delta = 1.74$ (3H, s, CH_3), 1.82 (3H, s, CH_3), 2.80–3.34 (4H, m, Dap β-CH_2 and His β-CH_2), 4.20–4.26 (2H, m, His α-CH and Dap α-CH), 6.95 (1H, d, His H-5), 8.19 (1H, s, His H-2). For $C_{13}H_{19}N_5O_5$ (325.14) calculated: 48.0%C, 5.89%H, 21.53%N; found 48.09%C, 5.79%H, 21.39%N.

C Biological studies

Serum carnosinase preparation

Human serum was obtained from freshly clotted blood without haemolysis and immediately used or stored at −80°C. The partial purification of the carnosinase was carried out according to Jackson et al. (1991) using the first two steps of the reported procedure (DEAE-cellulose chromatography and hydroxylapatite chromatography). The final enzymatic solution, obtained from various pooled preparations, was split up into small portions

and stored at −20°C until use. The enzyme retained its activity for at least two months. The specific activity, measured as described below, was 80 ± 5 μmoles of histidine formed per h per mg of protein.

Carnosinase assay

Carnosinase activity was measured essentially as previously reported (Bando et al., 1984; Lenney et al., 1982). Briefly, the enzymatic assay was carried out in 50 mM Tris-HCl buffer, pH 8.5, 0.75 mM $CdCl_2$ in a final volume of 0.5 ml using 8 μg of carnosinase prepared as described above. The enzyme was activated by incubation with the buffer and $CdCl_2$ for 10 min at 37°C before starting the reaction by addition of carnosine (10 mM final concentration). After 60 min at 37°C the reaction was stopped by adding 0.5 ml of 0.6 N trichloroacetic acid; the protein was removed by centrifugation and the amount of histidine released was determined by reacting 100 μl of the supernatant with *o*-phthaldialdehyde. The resulting fluorescence ($\lambda_{ex} = 344$ nm, $\lambda_{em} = 420$ nm) was measured and the concentration of histidine calculated from a standard curve. Reaction blank values were determined by adding the trichloroacetic acid stop solution prior to substrate addition.

The hydrolytic activity of carnosinase on compound **7–9** was determined under the same assay conditions but with 10 mM of **7–9** instead of carnosine.

The inhibitory effect of compounds **8** and **9** on the carnosinase activity was determined at carnosine concentrations ranging from 1 to 6 mM and at inhibitor concentrations of 10, 15 and 20 mM. Reciprocals of velocities were plotted against the reciprocal concentrations of carnosine in absence and in the presence of inhibitor. Replot of the apparent Km values against inhibitor concentrations was used to determine the inhibition constant (Ki) according to equation: slope = Km/Ki.

Assay for Fe(II)-H_2O_2-induced degradation of deoxyribose

This assay was carried out in the presence of EDTA essentially as previously reported (Halliwell et al., 1987). Briefly, the reaction mixture, in a final volume of 0.65 ml, contained the following reagents added in the order indicated: 0.1 ml of 200 mM K-phosphate buffer, pH 7.4, 0.2 ml of 5 mM deoxyribose, 0.1 ml of varying concentrations (1.0–10 mM) of scavenger (carnosine or compound **7–9**), 0.1 ml of 5 mM H_2O_2, 0.05 ml of 4 mM EDTA and 0.1 ml of a solution of 2 mM $(NH_4)_2Fe(SO_4)_2$ made up, immediately before use, in distilled water purged with N_2. The reaction mixture was incubated for 1 h at 37°C and the extent of deoxyribose degradation was monitored by the formation of malondialdehyde (MDA) determined by the addition of 0.5 ml of 1% (w/v) thiobarbituric acid (TBA) in 0.05 M NaOH and of 0.5 ml of 2.8% (w/v) trichloroacetic acid. The solution was heated for 10 min at 100°C, cooled, and the resulting absorbance was read at 532 nm against appropriate blanks. A control, without scavengers, was carried out in parallel and taken as 100% deoxyribose degradation. None of the compounds tested produced TBA-reactive material as shown by controls in the absence of deoxyribose. Concentrations of carnosine and of compound **7–9** up to 10 mM (the maximum used in these studies) produced no interference with the assay as tested by adding the scavengers at the end of the incubation, just before addition of TBA reagent.

Assay for peroxynitrite-induced nitration of tyrosine

Peroxynitrite (final concentration 100 μM) was added to a solution containing L-tyrosine (100 μM) in the presence of varying concentrations (0.5–10 mM) of compounds **7–9** in 0.5 M K-phosphate buffer, pH 7.4, in a final volume of 1 ml. After incubation for 10 min at room temperature, the reaction mixtures were analysed for 3-nitrotyrosine formation by high-performance liquid chromatography (HPLC). Analyses were carried out with a Waters Chromatograph equipped with a Waters 996 photodiode array detector. The column was a Nova-pack C18 (3.9 × 150 mm), 4 μm. The mobile phase was: A, 50 mM K-phosphate/H_3PO_4, pH 3; B, acetonitrile:water (50:50, v/v). A linear gradient from A to 33% B for 10 min was used at flow rate of 1 ml/min. Tyrosine and 3-nitrotyrosine were monitored at 274 and 360 nm, respectively, and concentrations were calculated from standard curves. A control, without **7–9** was carried out in parallel to estimate the extent of tyrosine nitration and taken as 100% nitration.

Results and discussion

Enzymatic studies

Hydrolysis of **7–9** by carnosinase

The three compounds under study have been tested as substrates of human serum carnosinase. As shown in Table 1, the rate of enzymatic hydrolysis of non acetylated compound (**7**) is only 30% of that of carnosine while hydrolysis of mono- (**8**) and bis-acetylated (**9**) compound is almost negligible being 3 and 6%, respectively. In agreement with previous studies on N-acetylcarnosine (Pegova et al., 2000; Jackson et al., 1991), these results indicate that the presence of one or two N-acetyl groups on the backbone of carnosine increases the resistance of the molecule toward enzymatic hydrolysis.

Inhibition of carnosinase activity by **8** and **9**

The relatively high resistance of **8** and **9** to degradation by carnosinase prompted us to investigate whether these compounds could act as inhibitors of the enzymatic hydrolysis of carnosine. In Fig. 3 is reported a representative Lineaweaver-Burk plot indicating that compound **8** behaves as competitive inhibitor of human serum carnosinase. The insert of Fig. 3 shows the secondary plot used to determine the inhibition constant (Ki). A very similar pattern of inhibition was obtained with compound **9** used in the same range of concentration of compound **8** (not shown). The Ki values determined for compounds **8** and **9** were 4 mM and 3.45 mM, respectively. These values are of the same order of the magnitude of the Km of the enzyme for the carnosine (2.7 mM under our experimental conditions) indicating that carnosinase affinity for the inhibitors is similar to that for carnosine.

Table 1. Hydrolysis of compounds **7–9** by serum carnosinase. Substrate hydrolysis was measured using 8 μg of partially purified serum carnosinase as described under Materials and methods

Substrate (10 mM)	Rate [a] (μmoles of histidine/h)	%
Carnosine	0.64 ± 0.07	100
7	0.19 ± 0.03	30
8	0.02 ± 0.01	3
9	0.04 ± 0.01	6

[a] Results are the means ± SD of three or more determinations

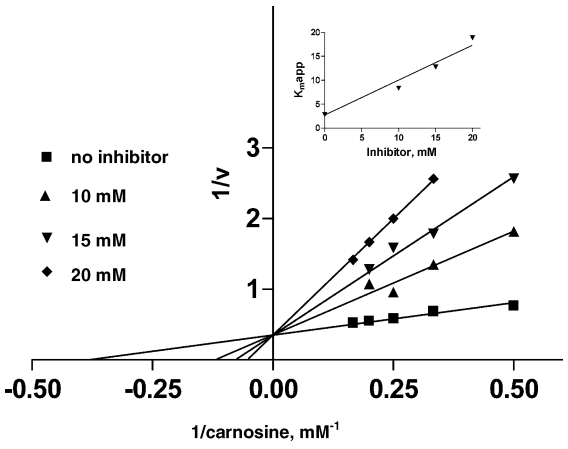


Fig. 3. Inhibition of serum carnosinase by compound **8**. The reaction mixture contained 50 mM Tris-HCl buffer (pH 8.5), 0.75 mM $CdCl_2$, 8 μg of enzyme in a final volume of 0.5 ml. Carnosine concentrations varied from 1 to 6 mM and the compound **8** as inhibitor was 10, 15 and 20 mM. The data are presented as a double reciprocal plot; in the insert is shown the replot of the apparent Km values against inhibitor concentrations to determine the inhibition constant (Ki)

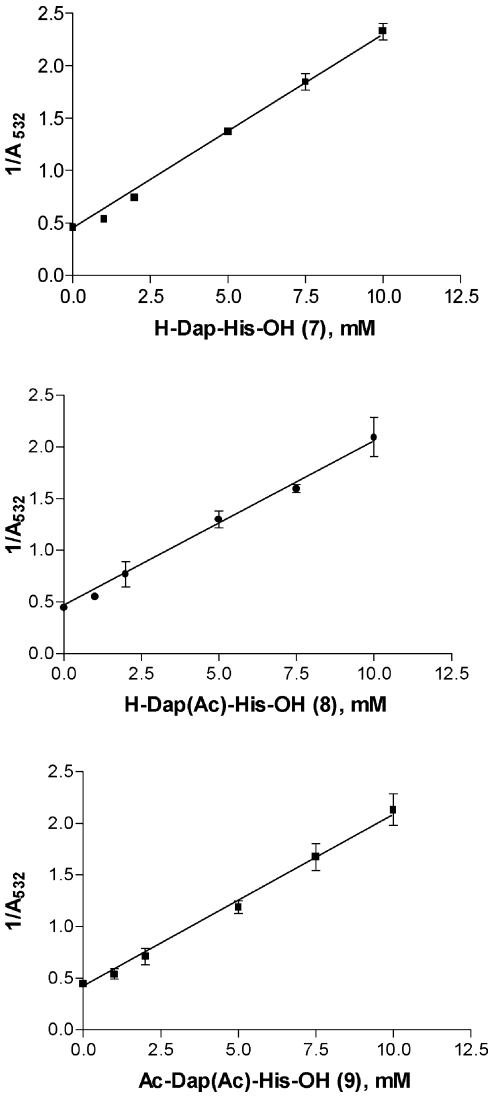


Fig. 4. Inhibition of •OH-induced degradation of deoxyribose by compounds **7–9**. Deoxyribose (DR) degradation in the presence of the indicated concentrations of **7–9** was followed as described under Materials and methods. The rate constants were determined from the slopes of the lines ($k = \text{slope} \times k_{DR} \times [DR] \times A_o$), where, $k_{DR} = 3.1 \times 10^9\ M^{-1}\ s^{-1}$, [DR] = 1.54 mM and A_o is the absorbance measured in the absence of scavenger

Antioxidant activity

The •OH radical scavenging activity of 7–9

Compounds **7–9** were tested on their ability to scavenge hydroxyl radicals by means of deoxyribose (DR) degradation test. Upon exposure to hydroxyl radical, generated by the Fenton reaction, deoxyribose is degraded into malondialdehyde (MDA) which is determined spectrophotometrically at 532 nm by reaction with thiobarbituric acid (Halliwell et al., 1987). Hydroxyl radical scavengers will compete with deoxyribose resulting in a reduced amount of MDA. Figure 4 shows the dose-dependent inhibitory effect of compounds **7–9** on the deoxyribose degradation in this system. From the slopes of the competition plots and literature data, second-order rate constants have been calculated for the reaction of **7–9** with •OH. These values and the percentage of inhibition of deoxyribose degradation at 5 mM concentration of **7–9** are reported in Table 2. It may be seen that the efficiency of the three compounds as •OH scavengers is similar (if not identical) to that of carnosine also tested for comparison.

Inhibition by 7–9 of peroxynitrite-dependent tyrosine nitration

The toxicity of peroxynitrite has been related to its ability to react with a variety of biological molecules including thiols, lipids, amino acids, proteins, antioxidants and nucleic acids. In particular peroxynitrite is able to nitrate free and protein residues of tyrosine with production of 3-nitrotyrosine (Beckman, 1996; Alvarez et al., 2003). In a previous paper we have reported that carnosine may also act as protective agent able to prevent peroxynitrite-dependent damage (Fontana et al., 2002). The peroxynitrite-scavenging activities of compounds **7–9** were evaluated by their ability to prevent nitration of free tyrosine induced by peroxynitrite. Figure 5 shows that all three compounds decrease nitration of tyrosine dose-dependently in the range 0.5–10 mM. The concentration of compound **7**, **8** and **9** required for 50% inhibition of 3-nitrotyrosine formation are 3.3 ± 0.3 mM, 2.3 ± 0.2 mM and 2.5 ± 0.2 mM, respectively. These results show that compounds **7–9** are

Table 2. The hydroxyl radical scavenging ability of compound **7–9** as determined by the deoxyribose assay. Results are the means of three or more determinations that differed by no more than 10%

Scavenger	Second-order rate constant ($M^{-1}\,s^{-1}$)	Inhibition of deoxyribose degradation [scavenger] = 5 mM (%)[a]
7	1.94×10^9	66.2
8	1.78×10^9	61.2
9	1.87×10^9	62.2
Carnosine[b]	2.07×10^9	66.4

[a] Percentage of inhibition was obtained by comparison with a control experiment with no scavenger
[b] The value of $4.0 \times 10^9\,M^{-1}\,s^{-1}$ has been previously reported for carnosine (Kohen et al., 1988; Auroma et al., 1989)

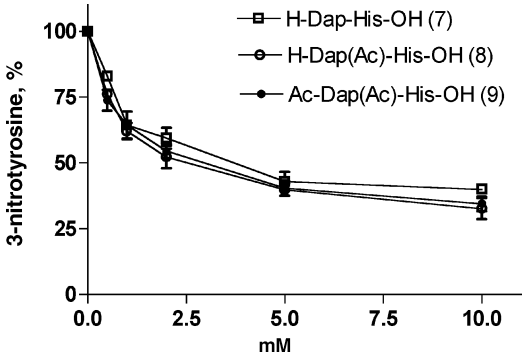


Fig. 5. Prevention of peroxynitrite-dependent nitration of tyrosine. Compounds **7–9**, at the indicated concentrations, were mixed with tyrosine (100 μM) in 0.5 M K-phosphate buffer, pH 7.4, before peroxynitrite addition (100 μM). After 10 min at room temperature, 3-nitrotyrosine formation was measured by HPLC as described in Materials and Methods. 100% nitration corresponds to 9.2 ± 0.5 μM 3-nitrotyrosine. Results are the mean ± SEM of three experiments performed in duplicate

able to counteract the nitrating properties of peroxynitrite with similar efficiencies, comparable to that previously reported for carnosine (Fontana et al., 2002). These findings indicate that the presence of N-acetyl groups does not affect the peroxynitrite-scavenging potential of the molecule.

As reported, under physiological conditions, peroxynitrite predominantly reacts with carbon dioxide to give the short-lived adduct nitrosoperoxycarbonate ($ONOOCO_2^-$) capable of generating secondary reactive species which can participate in the nitration process (Gow et al., 1996). Therefore, we examined the ability of compounds **7–9** to inhibit the peroxynitrite-mediated nitration of tyrosine in the presence of added bicarbonate. The results reported in Fig. 6 demonstrate that the compounds under study have similar protective effect in counteracting the nitrating properties of peroxynitrite in the absence and in the presence of 25 mM bicarbonate.

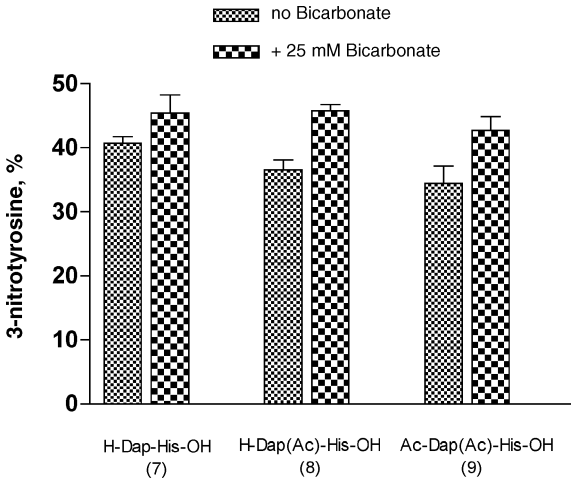


Fig. 6. Inhibition of peroxynitrite-dependent tyrosine nitration by compounds **7–9**: effect of added bicarbonate. The assay conditions, in the absence and in the presence of 25 mM bicarbonate, were as in the legend for Fig. 5 with compounds **7–9** at a final concentration of 5 mM. Results are the mean ± SEM of three separate experiments performed in duplicate

Conclusions

In conclusion, we have shown here that the new synthetic carnosine analogues bearing one (compound **8**) or two *N*-acetyl groups (compound **9**) in the molecule not only can resist to hydrolysis by human serum carnosinase but they can also inhibit the enzymatic degradation of carnosine. Furthermore, they inhibit reactions induced by hydroxyl radicals and by peroxynitrite in a way similar to that exhibited by the corresponding natural dipeptide. These properties make these compounds interesting mimics of the natural compound, with possible useful therapeutic applications.

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References

Alvarez B, Radi R (2003) Peroxynitrite reactivity with amino acids and proteins. Amino Acids 25: 295–311

Aruoma OI, Laughton MJ, Halliwell B (1989) Carnosine, homocarnosine and anserine: could they act as antioxidants *in vivo*? Biochem J 264: 863–870

Babizhayev MA, Seguin MC, Gueyne J, Evstigneeva RP, Ageyeva EA, Zheltukhina GA (1994) L-carnosine (beta-alanyl-L-histidine) and

carcinine (beta-alanylhistamine) act as natural antioxidants with hydroxyl-radical-scavenging and lipid-peroxidase activities. Biochem J 304: 509–516

Babizhayev MA, Courbebaisse Y, Nicolay JF, Semiletov YA (1998) Design and biological activity of imidazole-containing peptidomimetics with a broad spectrum antioxidant activity. Lett Pept Sci 5: 163–169

Babizhayev MA, Deyev AI, Yermakova VN, Semiletov YA, Davydova NG, Doroshenko VS, Zhukotskii AV, Goldman IM (2002) Efficacy of N-acetylcarnosine in the treatment of cataracts. Drug Develop Res 3: 87–103

Bando K, Shimotsuji T, Toyoshima H, Hayashi C, Miyai K (1984) Fluorometric assay of human serum carnosinase activity in normal children, adults and patients with myopathy. Ann Clin Biochem 21: 510–514

Beckman JS (1996) Oxidative damage and tyrosine nitration from peroxynitrite. Chem Res Toxicol 9: 836–844

Beckman JS, Chen J, Ischiropoulos H, Crow JP (1994) Oxidative chemistry of peroxynitrite. Methods Enzymol 233: 229–240

Boldyrev A, Johnson P (2002) Carnosine and related compounds: antioxidant dipeptides. In: Johnson P, Boldyrev A (eds) Oxidative stress at molecular, cellular and organ levels. Res Signpost 101–114

Boldyrev A, Dupin AM, Pindel EV, Severin SE (1988) Antioxidative properties of histidine-containing dipeptides from skeletal muscles of vertebrates. Comp Biochem Phys B 89: 245–250

Boldyrev A, Stvolinsky SL, Tyulina OV, Koshelev VB, Hori N, Carpenter DO (1997) Biochemical and physiological evidence that carnosine is an endogenous neuroprotector against free radicals. Cell Mol Neurobiol 17: 259–270

Boldyrev A, Bulygina E, Leinsoo T, Petrushanko I, Tsubone S, Abe H (2004) Protection of neuronal cells against oxygen species by carnosine and related compounds. Comp Biochem Phys B 137: 81–88

Bonfanti L, Peretto P, De Marchis S, Fasolo A (1999) Carnosine-related dipeptides in the mammalian brain. Prog Neurobiol 59: 333–353

Brown CE (1981) Interactions among carnosine, anserine, ophidine and copper in biochemical adaptation. J Theor Biol 88: 245–256

Calcagni A, Piattini PG, Di Stefano A, Duprè S, Luisi G, Pinnen F, Rossi D, Spirito A (1999) $\psi(SO_2NH)$ transition state isosteres of peptides. Synthesis and bioactivity of sulfonamido pseudopeptides related to carnosine. Il Farmaco 54: 673–677

Chenault HK, Dahmer J, Whitesides GM (1989) Kinetic resolution of unnatural and rarely occurring amino acids: enantioselective hydrolysis of N-acyl amino acids catalyzed by acilase I. J Am Chem Soc 111: 6354–6364

Crush KG (1970) Carnosine and related substances in animal tissues. Comp Biochem Physiol 34: 3–30

Decker EA, Livisay SA, Zhou S (2000) A re-evaluation of the antioxidant activity of purified carnosine. Biochemistry-Moscow 65: 766–770

Egorov SY, Kurella EG, Boldyrev A, Krasnovsky AA (1997) Quenching of singlet molecular oxygen by carnosine and related antioxidants. Monitoring 1270-nm phosphorescence in aqueous media. Biochem Mol Biol Int 41: 687–694

Fontana M, Pinnen F, Lucente G, Pecci L (2002) Prevention of peroxynitrite-dependent damage by carnosine and related sulphonamido pseudopeptides. Cell Mol Life Sci 59: 863–870

Gow A, Duran D, Thom SR, Ischiropoulos H (1996) Carbon dioxide enhancement of peroxynitrite-mediated protein tyrosine nitration. Arch Biochem Biophys 333: 42–48

Halliwell B, Gutteridge JMC, Aruoma OI (1987) The Deoxyribose method: a simple ''Test-Tube'' assay for determination of rate constants for reactions of hydroxyl radicals. Anal Biochem 165: 215–219

Hartman PH, Hartam Z, Ault KT (1990) Scavenging of singlet molecular oxygen by imidazole compounds: high and sustained activity of imidazole-4-acetic acid. Photochem Photobiol 51: 59–66

Hipkiss AR, Brownson C (2000) A possible new role for the anti-ageing peptide carnosine. Cell Mol Life Sci 57: 747–753

Hipkiss AR, Michaelis J, Syrris P (1995) Non-enzymatic glycosylation of the dipeptide L-carnosine, a potential anti-protein cross-linking agent. FEBS Lett 371: 81–85

Hipkiss AR, Worthington VC, Himsworth DTJ, Herwig W (1998) Protective effect of carnosine against protein modification mediated by malondialdehyde and hypochlorite. Biochim Biophys Acta 1380: 46–54

Hobart LJ, Seibel I, Yeargans GS, Seidler NW (2004) Anti-crosslinking properties of carnosine: significance of histidine. Life Sci 75: 1379–1389

Huie RE, Padmaja S (1993) The reaction of NO with superoxide. Free Radic Res Commun 18: 195–199

Jackson MC, Kucera CM, Lenney JF (1991) Purification and properties of human serum carnosinase. Clin Chim Acta 196: 193–206

Jackson MC, Lenney JF (1996) The distribution of carnosine and related dipeptides in rat and human tissues. Inflamm Res 45: 132–135

Kohen R, Yamamoto Y, Cundy KC, Ames BN (1988) Antioxidant activity of carnosine, homocarnosine, and anserine present in muscle and brain. Proc Natl Acad Sci 85: 3175–3179

Lenney JF, George RP, Weiss AM, Kucera CM, Chan, PWH, Rinzler GS (1982) Human serum carnosinase: characterization, distinction from cellular carnosinase, and activation by cadmium. Clin Chim Acta 123: 221–231

O'Dowd JJ, Robins DJ, Miller DJ (1988) Detection, characterization, and quantification of carnosine and other histidyl derivatives in cardiac and skeletal muscle. Biochim Biophys Acta 967: 241–249

Okuma E, Abe H (1992) Major buffering constituents in animal muscle. Comp Biochem Phys A 102: 37–41

Pegova A, Abe H, Boldyrev AA (2000) Hydrolysis of carnosine and related compounds by mammalian carnosinases. Comp Biochem Phys B 127: 443–446

Quinn PJ, Boldyrev AA, Formazuyk KV (1992) Carnosine its properties, functions and potential therapeutic applications. Mol Aspect Med 13: 379–444

Rochel S, Margolis FL (1982) Carnosine release from olfactory bulb synaptosomes is calcium-dependent and depolarization stimulated. J Neurochem 38: 1505–1514

Seidler NW, Yeargans GS, Morgan TG (2004) Carnosine disaggregates glycated alpha-crystalline: an *in vitro* study. Arch Biochem Biophys 427: 110–115

Stvolinsky SL, Kukley ML, Dobrota D, Matejovica M, Tkac I, Boldyrev A (1999) Carnosine: an endogenous neuroprotector in the ischemic brain. Cell Mol Neurobiol 19: 45–56

Zhou S, Dickinson LC, Yang L, Decker EA (1998) Identification of hydrazine in commercial preparations of carnosine and its influence on carnosine's antioxidative properties. Anal Biochem 261: 79–86

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