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# Malondialdehyde scavenging and aldose-derived Schiff bases' transglycation properties of synthetic histidyl-hydrazide carnosine analogs

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**Abstract**—Second-generation carnosine analogs bearing the histidyl-hydrazide moiety have been synthesized and tested for their efficiency in scavenging malondialdehyde (MDA) derived from lipid peroxidation and for their ability to reverse the glycation process in the glucose–ethylamine Schiff base model. The data obtained indicate that this class of compounds maintains the activity profile of carnosine and is a suitable candidate for the treatment of disorders caused by oxidative stress.


## 1. Introduction

Carnosine (β-alanyl-L-histidine) is a dipeptide commonly present in mammalian tissues, and in particular in skeletal muscle and central nervous system cells; it is responsible for a variety of activities related to the detoxification of free radical species and the by-products of membrane lipid peroxidation,[1] but recent studies have shown that this small molecule also has membrane-protecting activity, proton buffering capacity, forms complexes with transition metals, and regulates macrophage function.[2] It has been proposed that carnosine could act as a natural scavenger of dangerous reactive aldehydes from the degradative oxidative pathway of endogenous molecules such as sugars, polyunsaturated fatty acids (PUFAs), and proteins.[3,4] In particular, Hipkiss et al. recently demonstrated that carnosine is a potent and selective scavenger of α,β-unsaturated aldehydes (hydroxynonenal (HNE), malondialdehyde (MDA), acrolein), typical by-products of membrane lipid peroxidation (also known as ALEs, Advanced Lipoxidation Endproducts) that mediate adverse effects of oxidative stress; it also inhibits aldehyde-induced protein–protein and DNA–protein cross-linking.[3,5] These data further suggest that carnosine reduces α,β-unsaturated aldehydes' effects on cellular macromolecules in neurodegenerative disorders such as Alzheimer's disease, in cardiovascular ischemic damage, and in inflammatory diseases.[6]

Many reports recognize that carnosine has strong anti-glycating properties[7–9] and, most interestingly, is able to reverse pre-existing glycation[10] and formation of Advanced Glycation Endproducts (AGEs). Recently Szwergold demonstrated that both carnosine and anserine act as efficient transglycating agents[11]: the inhibition of non-enzymatic protein glycation (Maillard reaction) and the consequent improvement of cell function might depend not only on their ability to prevent the reaction of glucose and other carbonyl-containing compounds with protein amino groups, but also on the promotion of decomposition of such intermediates by nucleophilic attack.

We recently synthesized a series of carnosine analogs based on the 1,3-diol and hydrazide moieties, and demonstrated their ability to bind to reactive unsaturated aldehydes in vitro.[12] In particular, the histidyl-hydrazides were more efficient than carnosine in *trans*-2-nonenal binding assay.

These results prompted us to further investigate the carnosine-like properties of this new class of antioxidants, both in scavenging active and toxic aldehydes and in

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reversing the protein glycation process. We prepared a series of histidyl-hydrazide analogs **2**–**9** (Fig. 1) with different hydrophilic/lipophilic properties using neutral and aromatic aminoacids (leucine, phenylalanine), benzyloxycarbonyl (Z) group, cholesterol and the natural lipophilic antioxidant α-tocopherol, and tested them both for malondialdehyde (MDA) scavenging in iron/ascorbate induced oxidation of phosphatidylcholine liposomes,[13–15] and for transglycation efficiency in a glucose–ethylamine Schiff base model of protein glycation.[11]

## 2. Results and discussion

### 2.1. Synthesis of hydrazide compounds

Compounds **2**–**9** were synthesized in our laboratories following standard peptide chemistry procedures and were purified by LC or HPLC to obtain pure specimens as confirmed by NMR and mass spectroscopy. log *P*

**Table 1.** Physicochemical data of carnosine and synthetic analogs

| Compound | Formula | Mass (Calculated for $M+1$) | log *P*[a] |
|---|---|---|---|
| Carnosine (**1**) | $C_9H_{14}N_4O_3$ | — (227.1066)[b] | −1.54 |
| **2** | $C_6H_{11}N_5O$ | 170.2714 (170.1924) | −2.28 |
| **3** | $C_{14}H_{17}N_5O_3$ | 304.1320 (304.1331) | −0.10 |
| **4** | $C_{15}H_{20}N_6O_2$ | 317.1753 (317.3657) | −0.63 |
| **5** | $C_{12}H_{22}N_6O_2$ | 283.1807 (283.3481) | −0.88 |
| **6** | $C_{10}H_{13}N_3O_3$ | 224.1039 (224.2365) | −0.03 |
| **7** | $C_2H_7N_3O$ | 89.4821 (90.0589) | −2.88 |
| **8** | $C_{34}H_{55}N_5O_3$ | 582.4272 (582.8401) | 6.98 |
| **9** | $C_{39}H_{63}N_5O_5$ | 682.4761 (682.9560) | 6.32 |

[a] log *P* calculated with algorithm provided by Interactive Analysis (Bedford, MA; www.logP.com).
[b] Commercial specimen (Aldrich, Inc.).

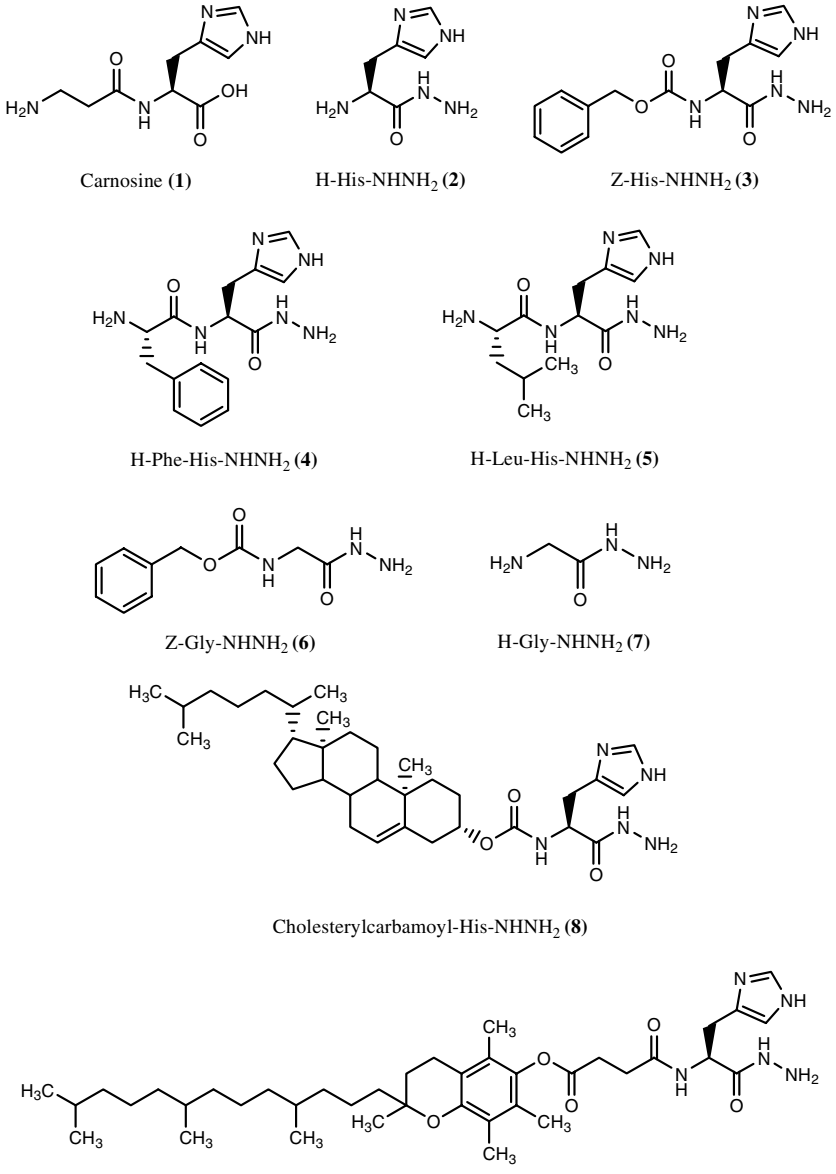


**Figure 1.** Synthesized hydrazide compounds.

values were calculated using a free software facility provided by Interactive Analysis (Bedford, MA; www.logP.com) (Table 1).

### 2.2. Iron–ascorbate peroxidation of liposomes and TBARs test

Original procedure from Murase and et al.[13] was adapted in our laboratory as described in the Experimental section. Briefly, phosphatidylcholine (PC) unilamellar vesicles suspension (1 mg/mL) in 0.1 M Tris–HCl buffer, pH 7.4, was prepared by sonication, and lipoperoxidation was initiated adding iron sulfate and ascorbic acid. Carnosine and compounds **2**–**9** were then added in separate experiments to the mixture, that was incubated at 37 °C. Aliquots were sampled and MDA concentration was measured with the TBARs (thiobarbituric acid reactive substance) test.

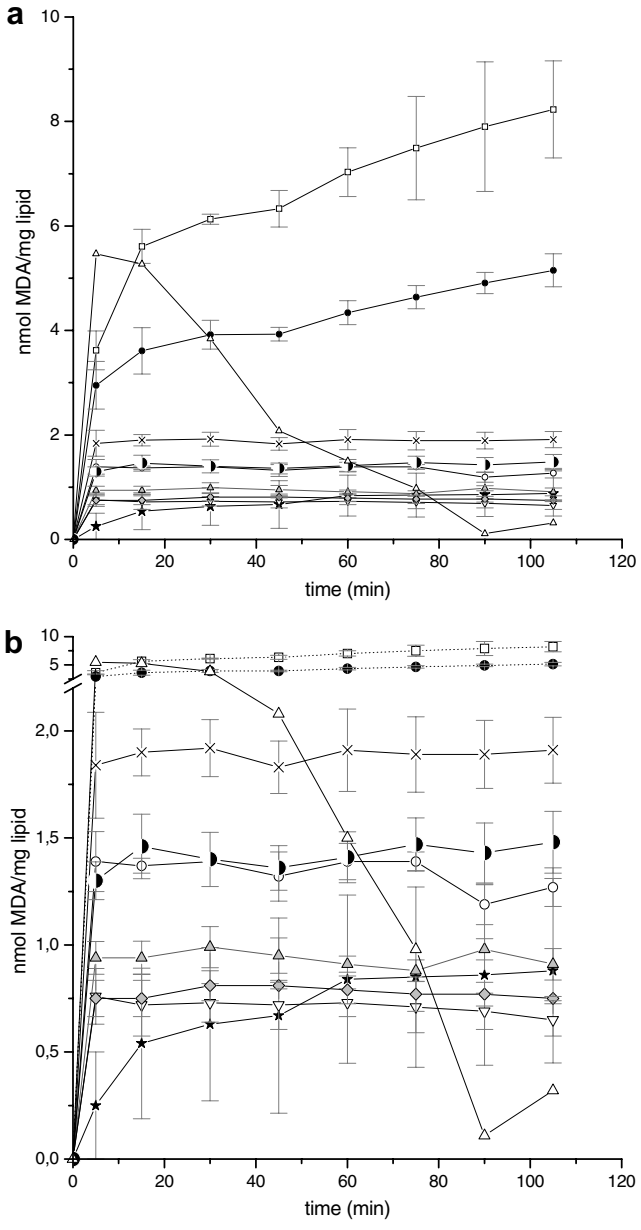


**Figure 2.** (a) Accumulation of MDA from lipoperoxidation of PC liposomes induced by $Fe^{3+}$/ascorbate and scavenging activity of carnosine and hydrazides: liposomes control (□); carnosine **1** (•); His-$NHNH_2$ **2** (★); *Z*-His-$NHNH_2$ **3** (◆); Phe-His-$NHNH_2$ **4** (▲); Leu-His-$NHNH_2$ **5** (○); *Z*-Gly-$NHNH_2$ **6** (▽); Gly-$NHNH_2$ **7** (×); cholesterylcarbamoyl-His-$NHNH_2$ **8** (◗); tocopheryl-succinyl-His-$NHNH_2$ **9** (△). All curves are means of three independent experiments, except **9** that was run as single experiment. (b) Enlargement of Graph (a) magnifying 0–2.5 vertical scale portion.

The data shown in Figure 2 clearly indicate that all hydrazides are excellent scavengers of MDA produced by iron/ascorbate peroxidation of PC liposomes in the experimental conditions reproducing the MDA levels in oxidative stress conditions (up to 20 nmol/mL in serum of patients suffering for various chronic liver diseases,[16] 1–10 mmol/mL in diseases associated with oxidative stress like cancer, heart failure, Alzheimer, acute abdominal pain, etc.[17]) and the average concentration of carnosine in tissues (from 0.3–5 mM to 2–20 mM in human olfactory bulb and skeletal muscle, respectively[18]).

The peculiar behavior of tocoferyl-histidylhydrazide (**9**), with a delayed reactivity toward MDA, could be explained taking into account its poor solubility in the experimental conditions: insoluble in water, in the liposomes suspension it dissolves very slowly and only after sonication, concentrating in the lipid hydrophobic bilayer; this behavior is in contrast with that of the other derivatives, readily dissolved in the liposomal aqueous suspension. MDA generated inside the liposomal membrane immediately after iron/ascorbate addition diffuses in the buffer solution, and while for the water-soluble species **2**–**7** its scavenging involves a homogeneous reaction, **9** does not react while suspended in the buffer solution, but only after dissolution in the lipid bilayer. Cholesterol-bound histidyl-hydrazide **8**, with a similar log *P* value, was immediately picked up by liposomes allowing an immediate scavenging effect.

Tentative correlation of the hydrophilic/lipophilic nature (expressed as log *P*) with MDA scavenging efficiency (Fig. 3) resulted in poor curve fit ($R^2 = 0.08$).

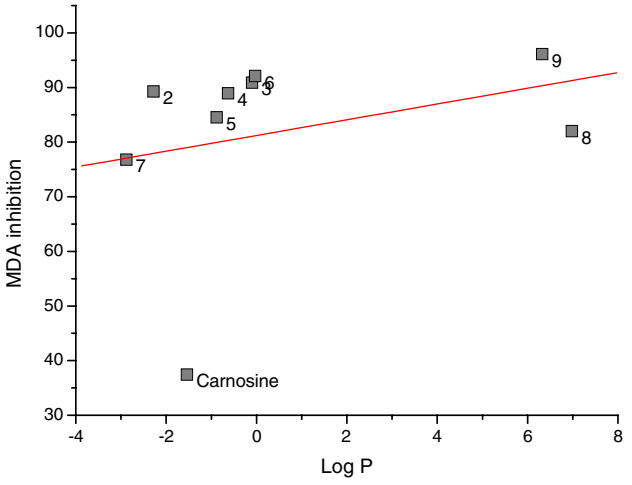


**Figure 3.** log *P*/MDA inhibition efficiency correlation curve of tested compounds. $R^2 = 0.08$.

Although glycine derivatives **6** and **7**, lacking the imidazole ring, were unable to scavenge unsaturated aldehydes like *trans*-2-nonenal in previous experiments,[12] the simple presence of the hydrazide moiety is sufficient to produce high reactivity toward MDA as compared with carnosine.

### 2.3. $^{13}$C NMR experiments

Transglycation efficiency of hydrazide derivatives **2**–**7** was assessed following Szwergold protocol,[11] using the Schiff base glucosyl–ethylamine (G–E) as a model of the first intermediate in the glycation process of side chain primary amines of proteins. $^{15}$N labeled ethylamine was used to minimize electric quadrupole moment and obtain a C-1 peak of glucose as a sharp doublet centered at 90.00 ppm. The kinetics of the transglycation reaction for the control reaction, for carnosine, and for compounds **2**–**7** are illustrated in Figure 4.

For a better evaluation of the transglycation kinetics of the compounds, for each $^{13}$C spectrum the integral of the buffer Hepes signals (50–55 ppm range) was set as = 1, then the integral of the C-1 glucose peak at 90.00 ppm was measured and integration values, normalized and corrected for the natural decay of the G–E Schiff base (control curve), were plotted against time in Figure 5. The data show that the transglycating efficiency of the hydrazide derivatives is generally lower than that of carnosine, with the exception of leucyl-histidylhydrazide (**5**).

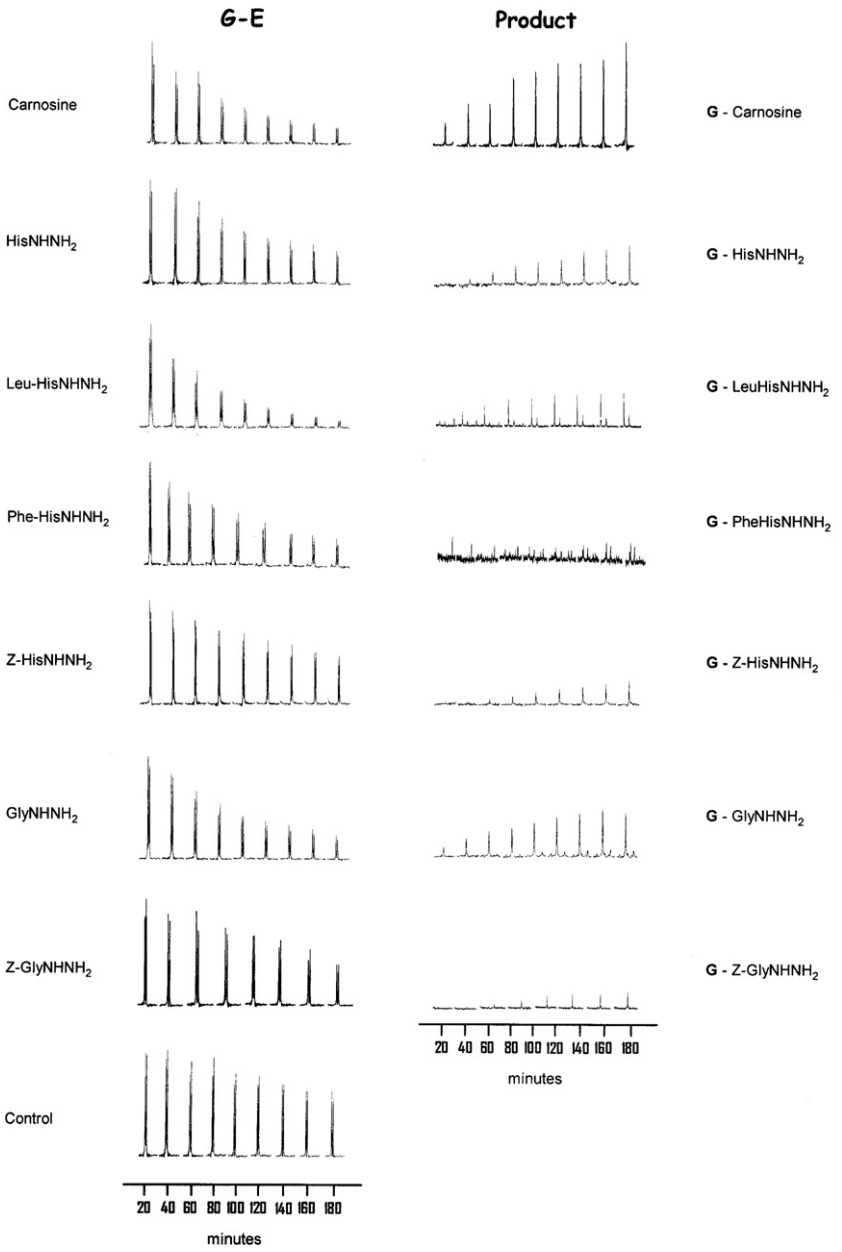


**Figure 4.** Kinetics of transglycation of G–E by carnosine and related compounds (left column: serial spectra of G–E as a function of time; right column: serial spectra of the respective transglycation products).

As in the MDA scavenging test, the presence of the imidazole moiety does not seem to influence the reactivity, while the free $N^{\alpha}$-amino group is mandatory (both *Z*-histidylhydrazide **3** and *Z*-glycylhydrazide **6** are less efficient). log *P* value and transglycating efficiency of the derivatives show a good correlation ($R^2 = 0.38$) (Fig. 6) but highly lipophilic compounds **8** and **9** were insoluble in the experimental conditions and were not tested.

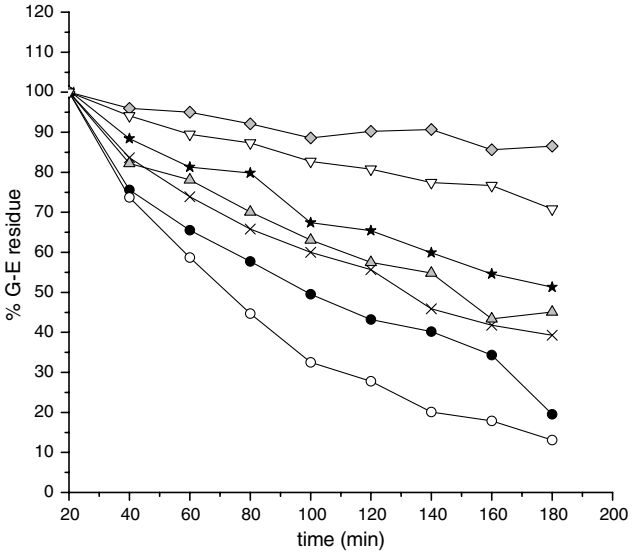


**Figure 5.** Transglycation efficiency of tested compounds: carnosine **1** (•); His-$NHNH_2$ **2** (★); *Z*-His-$NHNH_2$ **3** (◆); Phe-His-$NHNH_2$ **4** (▲); Leu-His-$NHNH_2$ **5** (○); *Z*-Gly-$NHNH_2$ **6** (▽); Gly-$NHNH_2$ **7** (×). The area of the G–E doublet at 90 ppm was plotted against time and corrected for the G–E Schiff base decay measured in the control experiment.

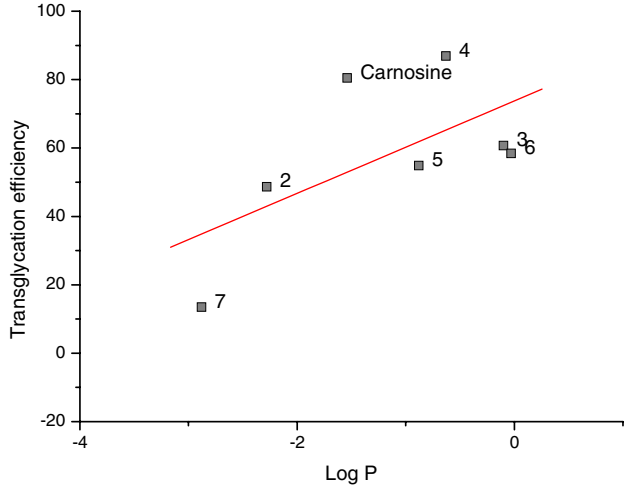


**Figure 6.** log *P*/Transglycation efficiency (% of G–E removal after 180 min incubation) correlation for carnosine and compounds **2**–**7**. $R^2 = 0.38$.

## 3. Conclusions

The discovery of new molecular entities able to scavenge the by-products of oxidative stress causing damage to cell components (lipids, proteins, etc.) is a fertile and rather young area that addresses several human disorders (CNS degenerative impairments, diabetes, reperfusion damage after stroke, ocular disorders such as cataract to name but a few) in which excess of ALEs and AGEs, at the same time bio-markers of disease and toxic species concurring in its outcome, seems to be a common etiological factor.

In a recent paper, we discussed the neuroprotective actions of histidylhydrazide **2** in models of ischemic stroke, demonstrating that the molecule crosses the blood–brain barrier after iv injection and reduces brain damage improving functional outcome at a dose of 20 mg/kg either 30 min before or 60 min and 3 h after the onset of ischemic injury.[19]

In this paper, we demonstrate that histidylhydrazides **2**–**9** are very potent scavengers for toxic aldehydes produced by lipoperoxidation and maintain the ability of carnosine to revert the glycation process at its early stages. In particular, one compound (leucyl-histidylhydrazide, **5**) is markedly more active than carnosine in both experiments. While the role of the imidazole ring in both MDA scavenging and transglycation seems marginal (as opposed to its pivotal role in unsaturated aldehyde scavenging, cf. Ref. 12), the hydrazide moiety boosts the MDA scavenging efficiency and, in combination with a free $N^{\alpha}$-amino group, concurs in the disruption of the Schiff base adduct G–E as a model of protein glycation. Further structure/activity relationship details will need in vivo evaluation to determine the real influence of the hydro/lipophylicity on the pharmacokinetic and pharmacodynamic parameters of this class of compounds.

## 4. Experimental

### 4.1. Materials and methods

ESI-MS spectra were acquired with a Mariner (PerSpective Biosystems) mass spectrometer instrument using a mixture of neurotensin, angiotensin, and bradykinin at concentration of 1 pmol/μL as external standard. Samples were prepared by dissolving the compound ($10^{-5}$ M) in acetonitrile/water 1:1 mixture with 1% acetic acid. $^1H$ and $^{13}C$ NMR spectra were recorded with a Bruker Advance DRX 400 spectrometer. Chemical shifts ($\delta$) are given in parts per million (ppm) using solvent ($CDCl_3$ or DMSO-$d_6$) as internal standard. Reaction courses and product mixtures were routinely monitored by TLC on silica gel (precoated Polygram Sil G/UV 254 from Macherey-Nagel) and visualized with UV lamp (254 nm) or iodine vapors. Reagents and solvents were of high-purity grade and were purchased from Sigma–Aldrich, J.T. Baker, and Carlo Erba. Carnosine was purchased from Fluka.

### 4.2. Liposome preparation and TBARS test

Phosphatidylcholine (PC) unilamellar vesicles were prepared dissolving PC in chloroform, evaporating solvent and adding 0.1 M Tris–HCl buffer, pH 7.4. The mixture was sonicated for 30 min obtaining the desired solution of liposomes at a final concentration of 1 mg/mL.

Lipoperoxidation was achieved by addition of $FeSO_4$ (2.5 μM) and ascorbic acid (200 μM) and incubation at 37 °C. Aliquots of the reaction mixture were sampled (the first after 5 min, then at 15 min intervals), diluted with citrate/HCl buffer, pH 3, and added with thiobarbituric acid (0.125%); after incubation (95 °C, 30 min) MDA concentration was measured by UV–vis absorption at 535 nm.

The experiment was repeated adding carnosine and compounds **2**–**9** to the liposomes immediately after iron/ascorbate addition at a final concentration of 10 mM and the resulting data were plotted against time (Fig. 2).

### 4.3. $^{13}C$ NMR experiments

Glucose–ethylamine (G–E) was synthesized by incubating 500 mM $^{13}C$-glucose and $^{15}N$-ethylamine at pH 12 and 37 °C for 3 h. At the end of the incubation period, about 75% of the starting material was converted to glucose–ethylamine in equilibrium with the starting materials. NMR experiments were conducted under conditions which stabilized Schiff base enough to be able to observe them by NMR over several hours. The reaction mixture (0.5 mL in a 5 mm NMR tube) included 250 mM Hepes, pH 8.5, 10% $D_2O$, and 20 mM concentration of carnosine or one histidylhydrazide derivative. The reaction was performed at room temperature and it was initiated by adding an aliquot of G–E to produce a final concentration of 20 mM. At that time consecutive NMR spectra of 20 min duration were acquired using 580 scans, 60° pulses, and an interpulse delay of 2.05 s. Spectra were analyzed using information from model compounds and chemical shifts from literature. The area of the G–E doublet at 90.00 ppm was calculated and plotted against time after subtraction of the natural G–E Schiff base decay measured in a blank experiment.

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