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New derivative of carnosine for nanoparticle assemblies

Francesco Bellia [a,*], Valentina Oliveri [a,b,1], Enrico Rizzarelli [b,1], Graziella Vecchio [b,1]

[a] Institute of Biostructure and Bioimaging, CNR, viale A. Doria 6, 95125 Catania, Italy
[b] Department of Chemical Sciences, University of Catania, viale A. Doria 6, 95125 Catania, Italy

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ABSTRACT

Carnosine (β-alanyl-L-histidine) is an endogenous dipeptide, extensively studied owing to its multifunctional activity exhibited in tissues of several animal species. This natural compound may act as a physiological buffer, ion-chelating agent (especially for copper(II) and zinc(II)), antioxidant and anti-glycating agent. The main limit for the therapeutical uses of carnosine is the rapid hydrolysis mostly in human plasma by carnosinase. The chemical derivatization of carnosine is a promising strategy to improve the bioavailability of the dipeptide and facilitating the site-specific transport to different tissues. On this basis, a new carnosine derivative with biotin was synthesized and structurally characterized by NMR and MS measurements, with aim of exploiting the avidin–biotin technology that offers a universal system for selective delivery of any biotinylated agent. The stability of the new carnosine derivative towards the hydrolytic action of serum carnosinase as well as the copper(II) binding ability of the carnosine–biotin conjugate were also assessed. The binding affinity of the new molecular entity to avidin and streptavidin, investigated by a spectrophotometric assay, was exploited to functionalize avidin– and streptavidin–gold nanoparticles with the carnosine–biotin conjugate.

1. Introduction

Among all the dipeptides nowadays studied in scientific research, carnosine (β-alanyl-L-histidine, Car) occupies an important position because its increasingly ascertained biological properties are an intriguing stimulus for trying to understand its still unknown physiological functions.

Carnosine is the first peptide ever isolated from natural material [1] and reaches very high concentrations (up to 20 mM) in muscle and nervous tissues of several animal species [2]. The massive presence of carnosine could be ascribed to numerous functions, such as physiological buffer, wound healing promoter, ion-chelating agent, especially for Cu^{II} and Zn^{II}, antioxidant and anti-glycating agent [3,4], all being important in physiological and pathological conditions [5].

It has been shown that carnosine retards cancer growth in animal models [6] and protect against alcohol-induced oxidative stress [7] as well as ethanol-induced chronic liver damage [8]. The neuroprotective action of carnosine in oxidative driven diseases has been recently reviewed [9]. All these properties make this natural dipeptide an interesting compound for several application in biomedical field [10].

The peptidic nature of carnosine compromises its therapeutical uses mainly for the breakdown by specific dipeptidases. The carnosine concentration in the animal species is regulated by the activity of the metalloprotease carnosinases. In mammals, two dipeptidases have been characterized: the serum-circulating form ('serum carnosinase', CN1), secreted by brain cells into the cerebrospinal fluid [11,12] and the cytosolic isoform ('tissue carnosinase', CN2), a non-specific dipeptidase distributed in several human tissues and in the rodent brain [13,14].

Recently, several carnosine derivatives with saccharides [15], such as β-cyclodextrin [16,17] and trehalose [18], have been synthesized. All these compounds are able to scavenge hydroxyl radicals and their copper(II) complexes exhibit SOD (superoxide dismutase) activity [19,20]. Furthermore, they are resistant to the hydrolysis of the carnosinase [21,18] and have an antioxidant efficacy at concentrations 10–20 times lower than that reported for other synthetic derivatives [22].

An important physiological role of the conjugating moiety in the carnosine derivatization is enhancing the bioavailability of the dipeptide by facilitating the site-specific transport to different tissues.

One of the largely used molecules for selective delivery is vitamin H, better known as biotin (Bio). The extensive biomedical investigation of biotin both *in vitro* and *in vivo* is mainly due to its specific

* Corresponding author. Tel.: +39 095 738 5047; fax: +39 095 337678.
E-mail address: fbellia@unict.it (F. Bellia).
[1] Tel.: +39 095 738 5064; fax: +39 095 337678.

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interaction with avidin (Av) and streptavidin (SAv). These tetrameric proteins have a high-affinity biotin-binding site ($K_d \approx 10^{-15}$ M^{-1}) for each subunit [23] and they selectively binds to specific cells, tissues or organs. Thus avidin–biotin technology offers a universal system for selective delivery of any biotinylated agent to the target using the same affinity carrier. For this reason, a large panel of biomolecules have been biotinylated, usually without significant loss of their biological properties, and they have been investigated for biomedical applications *in vitro* (cross-linking, staining or targeting biomolecules, etc) [24] and *in vivo* (drug targeting [25–27], radioimmunoimaging [28], stimulation of immune response [29], etc).

Another approach employed in modern scientific era for the selective delivery consists in the use of nanoparticles (NPs). They are attracting considerable and growing interest because of their unique physical and chemical properties. The integration of nanotechnology with biology and medicine has led to further developments of a new emerging research area, nanobiotechnology, which offers opportunities for discovering new materials, processes, and phenomena.

Recently, carnosine-coated iron oxide [30] and gold NPs have been obtained [31,32]. These NPs have been investigated as activators of carbonic anhydrase and nickel sensors. As for carnosine-NPs as activators of carbonic anhydrase, carnosine has been functionalized with L-lipoic acid in order to synthesize gold NPs through gold–sulfur bond formation. The role of carnosine could be related to the presence of histidine, being that the activity of carnosine nanoparticles is very similar to that of histidine nanoparticles.

On this basis, a new carnosine derivative with biotin (BioCar) was synthesized (Fig. 1) and structurally characterized by NMR and MS measurements. The stability of BioCar towards the hydrolytic action of serum carnosinase as well as the copper(II) binding ability of the new carnosine derivative were also assessed. The binding affinity of BioCar to avidin and streptavidin, investigated by a spectrophotometric assay, was exploited to functionalize gold nanoparticles with BioCar via Av- and SAv-coated NPs.

2. Materials and methods

2.1. Chemicals

In order to synthesize the new carnosine derivative, the following reagents have been used: carnosine (Sigma–Aldrich), Biotin succinimidyl ester (Bio-NHS, Carbosynth), *N*,*N*-Dimethylformamide (DMF, Sigma–Aldrich) and Triethylamine (TEA, Fluka). Phosphate Buffered Saline (PBS) was prepared with the following composition: phosphate buffer (12 mM, pH 7.4), NaCl (137 mM) and KCl (2.7 mM).

Thin layer chromatography (TLC) was performed on silica gel (60F-254, 0.20 mm, Macherey–Nagel). The compounds not detectable under UV light were revealed with a 1% solution of ninhydrine in acetone and the Pauly's reagent (10% of Fast Red B salt (Fluka) in deionized water) for detecting primary amino and imidazole groups, respectively. Acetonitrile and heptafluorobutyric acid (HFBA) were from Sigma (HPLC grade).

Hydrogen tetrachloroaurate ($HAuCl_4 \cdot 3H_2O$), and sodium citrate were purchased from Alfa Aesar. Streptavidin from *Streptomyces avidinii* and Avidin from egg white were from Sigma–Aldrich. Millipore-Q Ultrapure water was used to prepare nanoparticle solutions. Dedicated glassware was cleaned before each reaction with aqua regia and then rinsed with ultra-pure deionized water. All solutions used for nanoparticle preparation were filtered through a 0.45 μm membrane filter (Cellulose Nitrate Membrane Filter).

Copper(II) nitrate was prepared from copper(II) basic carbonate by adding a slight excess of HNO_3; the concentration of stock solutions was determined by ethylenediaminetetraacetic acid titrations using murexide as the indicator [33]. The HNO_3 excess in metal stock solutions was determined by Gran's method [34,35]. High-purity water (Millipore, Milli-Q Element A 10 ultrapure water) and grade A glassware were employed.

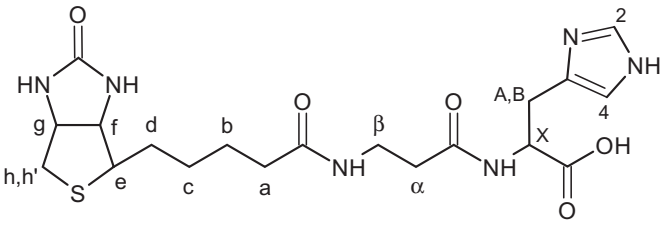

Fig. 1. Structure of the new carnosine–biotin conjugate. Symbols, letters and numbers are used for the NMR peak assignment.

2.2. Synthesis of biotin–carnosine (BioCar)

Car (35 mg, 0.15 mmol) was dissolved in water (300 μl) and a solution of Bio-NHS (51 mg, 0.15 mmol) in DMF (1.5 ml) was added drop by drop. 20 μl of TEA (0.15 mmol) were finally added to the mixture and the reaction was carried out at room temperature. After 20 h the solvent was evaporated under vacuum at 40 °C. The solid obtained was purified with a Sephadex-DEAE-A25 anion exchange column (HCO_3^- form) using water as the eluent and a linear gradient of NH_4HCO_3 (0–0.3 M). The fractions collected were analyzed by thin-layer-chromatography (TLC PrOH-H_2O-AcOEt-NH_3 5:3:1:2), and those containing the product were concentrated under vacuum at 40 °C. Yield 68%. ESI–MS [BioCar] $m/z = 453.1911$ (M + 1). Calculated m/z: 453.1915.

^{1}H NMR (D_2O, 500 MHz) δ (ppm): 8.51 (s, 1H, H-2 Im), 7.18 (s, 1H, H-5 Im), 4.52 (dd, 1H, $J = 7.5$, 5.4 Hz, g Bio), 4.45 (m, 1H, X His), 4.33 (dd, 1H, f Bio, $J = 7.9$, 4.4 Hz), 3.34 (m, 2H, CH_2 Ala), 3.23 (m, 1H, e Bio), 3.16 (dd, 1H, A His, $J = 15.3$, 5.0 Hz), 3.01 (dd, 1H, B His $J = 15.4$, 8.1 Hz), 2. 90 (ddd, 1H, h' Bio, $J = 13.0$, 4.9, 1.2 Hz), 2.69 (d, h Bio, $J = 13.0$ Hz), 2.39 (t, 1H, CH2 Ala, $J = 6.6$ Hz), 2.10 (m, 1H, a Bio), 1.62 (m, 2H, d Bio), 1.50 (m, 2H, b Bio), 1.29 (2H, c Bio).

^{13}C NMR (D_2O, 125 MHz) δ (ppm): 62.3 (f Bio), 60.3 (g Bio), 55.7 (e Bio), 53.9 (X His), 39.7 (α Ala), 35.7 (β Ala), 35.4 (a Bio), 35.1 (CH_2 His), 27.9 (c Bio), 27.5 (h Bio), 27.4 (d Bio), 25.4 (b Bio).

2.3. Spectroscopic and spectrometric measurements

^{1}H NMR spectra were recorded at 25 °C in D_2O with a Varian Unity Plus 500 spectrometer at 499.883 MHz. The ^{1}H NMR spectra were recorded by using the standard pulse programs from the Varian library. In all cases, the length of 90° pulse was ca. 7 μs. The two-dimensional (2D) experiments were acquired using 1 K data points, 256 increments, and a relaxation delay of 1.2 s. 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) was used as the external standard.

Circular dichroism spectra of the ligand and its copper(II) complexes were recorded on a Jasco 810 spectropolarimeter at a scan rate of 50 nm min^{-1} and a resolution of 0.1 nm. The path lengths were 1 or 0.1 cm, in the 190–800 nm range. The spectra were recorded as an average of 10 or 20 scans. Calibration of the instrument was performed with a 0.06% solution of ammonium camphorsulfonate in water ($\Delta\varepsilon = 2.40$ M^{-1} cm^{-1} at 290.5 nm). The 200–800 nm spectral range was covered by using quartz cells of various path lengths. The results are reported as $\Delta\varepsilon$ (molar dichroic coefficient) in M^{-1} cm^{-1}.