Journal of Inorganic Biochemistry 131 (2014) 56–63

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

Copper(II)-chelating homocarnosine glycoconjugate as a new multifunctional compound

Giuseppa I. Grasso [a], Giuseppe Arena [b], Francesco Bellia [a,*], Enrico Rizzarelli [a,b], Graziella Vecchio [b]

[a] Institute of Biostructure and Bioimaging, National Research Council (CNR), Viale A. Doria 6, Catania, Italy
[b] Department of Chemical Sciences, University of Catania, Viale A. Doria 6, Catania, Italy

ARTICLE INFO

Article history:
Received 29 July 2013
Received in revised form 23 October 2013
Accepted 23 October 2013
Available online 31 October 2013

Keywords:
Dipeptide
Copper
Carnosinase
Amyloid
Trehalose
Superoxide dismutase mimics

ABSTRACT

Homocarnosine is an endogenous dipeptide distributed in cerebral regions and cerebrospinal fluid. Homocarnosine may serve as an antioxidant, free radical scavenger, neurotransmitter, buffering system and metal chelating agent, especially for copper(II) and zinc(II). The homeostasis of homocarnosine is regulated by carnosinases; the serum-circulating isoform of these metallodipeptidases partially hydrolyses homocarnosine in the blood. The enzyme activity is also inhibited by homocarnosine itself in a dose-dependent manner. We synthesized a new multifunctional homocarnosine derivative with trehalose, a disaccharide that possesses several beneficial properties, among which the inhibition of protein aggregation (i.e. Aβ amyloid and polyglutamine proteins) involved in widespread neurodegenerative disorders. We studied the copper(II) binding features of the new conjugate by means of potentiometric and spectroscopic techniques (UV–visible and circular dichroism) and the superoxide dismutase-like activity of the copper(II) complexes with homocarnosine and its trehalose conjugate was evaluated. The inhibitory effect of the new homocarnosine derivative on the carnosinase activity and its effects on Aβ aggregation were also investigated.

1. Introduction

Homocarnosine (γ-aminobutyryl-L-histidine, HCar) is a dipeptide related to a group of natural histidine containing molecules, the archetype of which is carnosine (β-alanyl-L-histidine, Car) [1]. These dipeptides are present in considerable amounts in several vertebrate tissues including the skeletal muscles, eyes, olfactory system and brain [2,3]. Carnosine is found in low concentrations (below 100 nM) in the blood and the cerebrospinal fluid (CSF). Homocarnosine concentration is also low in the blood, while its concentration in the brain is 100-fold larger than that of carnosine [4]. Moreover, as for carnosine, the cerebral content of homocarnosine is age-related, being three to six times larger in adults than in infants [5]. Thus, it has been suggested to be a precursor for the neurotransmitter GABA (γ-aminobutyric acid), hence serving as an important inhibitory neuromodulator in human neocortex [6].

However the physiological role of these histidine-containing dipeptides has not been fully understood yet [7], although different hypotheses have been postulated on the basis of their in vitro and in vivo properties. Indeed, they may serve as antioxidants, free radical scavengers, neurotransmitters and also possess a buffering effect [8]. These natural compounds are efficient metal chelating agents (mainly for copper(II) and zinc(II)) and their copper complexes exhibit superoxide dismutase (SOD)-like activity [9]. For these reasons, they may act as neuroprotective agents in copper-dependent toxic conditions and as antioxidants in physiological and pathological conditions [10,11]. Indeed, the copper- and zinc- mediated neurotoxicity involved in several pathologies, such as Amyotrophic lateral sclerosis, Alzheimer's, Menkes's, Parkinson's, Pick's and Wilson's diseases [12,13], might be reduced or prevented by endogenous metal-chelating agents, such as homocarnosine and its homologues [14,15]. Therefore, understanding the role of endogenous compounds that are able to modulate copper availability and that have putative neuromodulatory and/or neuroprotective actions, such as homocarnosine, may help in the development of clinical approaches for the treatment of neuropathologies that involve metals and free radicals.

The homeostasis of homocarnosine and other histidine-containing dipeptides is regulated by some M20 metalloprotease family members, known as carnosinases. The serum-circulating isoform (CN1) [16,17] shows high specificity for carnosine and also hydrolyses homocarnosine though to a lesser extent. Only recently, it has been reported that CN1 activity towards carnosine is inhibited by homocarnosine in a dose-dependent manner [18]; the large homocarnosine concentration in CSF may explain the reduced carnosine degradation compared to that in serum [18].

The high activity of carnosinase in serum drastically reduces the potential applications of homocarnosine and much more of carnosine for pharmaceutical and nutraceutical applications. The chemical derivatization of carnosine has been largely employed *i.* to prevent the

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

0162-0134/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jinorgbio.2013.10.020

carnosinase action on the peptide moiety; *ii.* to improve the multifunctional activity resulting from the beneficial effect of the conjugated moiety and *iii.* to allow for the recognition of the conjugated peptide by a specific target [19,20]. Among all the organic molecules covalently linked to the carnosine and homocarnosine moieties, several glycosides have been used [21,22], owing to both their biocompatibility and their being recognized by a specific receptor. Moreover, glycosides have important beneficial properties on several physiological and pathological processes [23].

Trehalose (Tr), a non-reducing disaccharide present in a wide variety of animal and plant organisms, is a source of energy and carbon and also protects proteins and cellular membranes from inactivation or denaturation caused by a variety of stress conditions, including desiccation, dehydration, heat, cold, and oxidation [24]. Trehalose has also been shown to be very effective in inhibiting the aggregation of the β-amyloid peptide (Aβ) and in reducing its cytotoxicity [25]. Other studies have revealed that it can accelerate the clearance of α-synuclein [26] and inhibit the formation of fibrillar aggregates of insulin [27]. The trehalose treatment of prion-infected cells has been shown to decrease the size of de novo produced PrP^{Sc} aggregates, to modify their subcellular localization and to protect prion-infected cells against induced oxidative stress [28]. Finally, the efficacy of trehalose to inhibit polyglutamine aggregation makes it a good candidate for the treatment of Huntington's disease as well as other polyglutamine-dependent diseases [29].

For all these reasons, in this paper we report the synthesis of a new homocarnosine derivative with trehalose (TrHCar) (Fig. 1). The characterization of its copper(II) complex species was carried out by means of potentiometric and spectroscopic techniques (UV–visible and circular dichroism). The SOD-like activity of the copper(II) complex systems with HCar and TrHCar was also assessed. Finally, the inhibitory effect of TrHCar on the CN1 activity and its effects on Aβ aggregation were evaluated.

2. Experimental

2.1. Chemicals

Commercially available reagents were used directly unless otherwise specified. Trehalose (Fluka) was dried under vacuum (10^{-2} mm Hg) for 24 h at 80 °C using a P_2O_5 trap. Homocarnosine was purchased from Sigma. Triphenylphosphine (PPh_3) and N-bromosuccinimide (NBS) were purchased from Sigma and Fluka, respectively. Thin layer chromatography (TLC) was performed on silica gel (60 F–254, 0.20 mm, Macherey-Nagel); products that were not detectable under UV light were revealed with a 5% solution of anisaldehyde in ethanol (containing 5% H_2SO_4) and Pauli's reagent for peptide derivatives.

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 [30]. The HNO_3 excess in metal stock solutions was determined by the Gran's method [31,32]. High-purity water (Millipore, Milli-Q Element A 10 ultrapure water) and grade A glassware were employed. Homocarnosine ethyl ester (HCarOEt) was synthesized from homocarnosine (1.00 g, 3.0 mmol) in ethanol (30 ml) at 0 °C using acetyl chloride as HCl source. After 2 h, the solvent was evaporated under vacuum, and the crude product was purified using a Sephadex-DEAE A25 anion-exchange column (20 × 600 mm, HCO_3^- form) and water as the eluent.

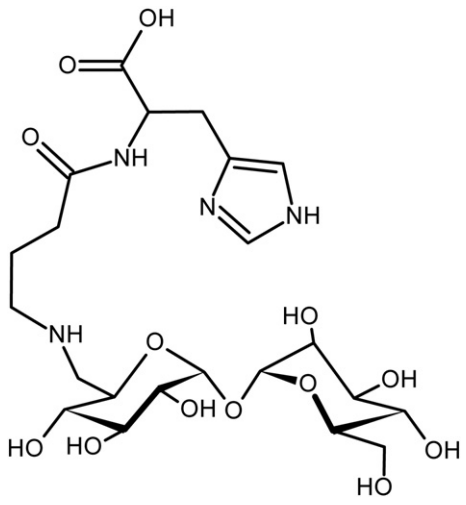

Fig. 1. Structure of TrHCar.

2.2. Synthesis procedures

2.2.1. Synthesis of 6^A-bromide-6^A deoxy-α,α′-trehalose (TrBr)

PPh_3 (16.6 g) was added to a solution of anhydrous trehalose (10.8 g) in N,N-dimethylformamide (DMF) in a 2:1 molar ratio. The reaction was cooled under stirring. An equimolar ratio of N-bromosuccinimide (11.4 g) was slowly added to the PPh_3 under stirring. The reaction mixture was kept at room temperature (usually ca for 48 h) until TLC indicated the formation of the maximum amount of TrBr. 100 ml of methanol was added to the solution to decompose excess reagent. Methanol and DMF were evaporated under vacuum at 35 °C. The oily product was washed with ethanol; the white crystals formed were filtered off using a Millipore. The filtered solution was evaporated under vacuum at 35 °C. The solid was purified by chromatography on an RP_8 column, using a linear gradient H_2O–EtOH (0–25%) as the eluent. TrBr yield: 40%. TLC: Rf = 0.54 (PrOH/H_2O/AcEt/NH_3 5:3:1:1).

^{1}H NMR = (D_2O, 500 MHz): δ (ppm) 5.12 (d, 1H, H-1 bromide ring), 5.10 (d, 1H, H-1′), 3.90 (m, 1H, H-5 bromide ring), 3.66–3.78 (m, 6H, H-3, H-6a, H-6b, bromide ring H-3′, H-5′, H-6b′), 3.54–3.62 (m, 3H, H-2, bromide ring H-2′, H-6a′), 3.40 (t, 1H, H-4 bromide ring), 3.35 (t, 1H, H-4′).

2.2.2. Synthesis of 6^A-[(4-{[(1S)-1-carboxy-2-(1H-imidazol-4-yl)ethyl]amino}-4-oxobutyl)amino]-6^A-deoxy-α,α′-trehalose (TrHCar)

HCarOEt (HCarOEt:TrBr 4:1) was added to a solution of TrBr (0.25 g) in anhydrous DMF (5 ml) under N_2 flow. The reaction was carried out at 70 °C under continuous stirring and under nitrogen; after 24 h, DMF was evaporated under vacuum at 40 °C. The solid obtained was purified with a CM-Sephadex C-25 cation exchange column (NH_4^+ form) using water as the eluent and a linear gradient of NH_4HCO_3 (0–0.3 M). The fractions collected were analyzed by TLC (PrOH–H_2O–AcOEt–NH_3 5:3:1:1), and those containing the product were concentrated under vacuum at 40 °C. TrHCarOEt thus obtained was hydrolysed with 1% NaOH in water for approx. 1 h at room temperature. The solid obtained after evaporation of the solvent was further purified with a CM-Sephadex C-25 column using water as the eluent. Yield 30%. ESI-MS [TrHCar] m/z = 565.3 (M + 1). ^{1}H-NMR (500 MHz, D_2O): δ (ppm) 7.66 (1H, s, H-2 Im), 6.85 (1H, s, H-5 Im), 5.11 (1H, d, $J_{1',2}$ = 3.8 Hz, H-1′), 5.07 (1H, d, J = 3.7 Hz, H-1); 4.35 (1H, dd, J_{AX} = 8.9, J_{BX} = 4.7 Hz, X of His), 3.92 (1H, td, $J_{5',6'b}$ = 2.6 Hz, $J_{5',4}$ = $J_{5',6'a}$ = 9.4 Hz, H-5 functionalized ring), 3.80–3.70 (4H, m, H-3, H-3′, H-5, H-6a), 3.65 (1H, dd, $J_{6a,6b}$ = 12.9, $J_{5,6b}$ = 6.0 Hz, H-6_a), 3.58 (1H, dd, $J_{2,3}$ = 9.9 Hz, $J_{1,2}$ = 3.9 Hz, H-2′), 3.55 (1H, dd, $J_{2',3}$ = 10.0, $J_{1',2}$ = 3.9 Hz, H-2), 3.34 (1H, t, $J_{3,4}$ = 9.4 Hz, H-4), 3.29 (1H, dd, $J_{6a,6b}$ = 13.1, $J_{6,5}$ 2.4 Hz, H-6′$_b$), 3.25 (1H, t, $J_{3,4}$ = 9.5 Hz, H′-4), 3.04 (2H, m, H-6′$_a$, CH_2 histidine), 3.87 (3H, m, CH_2 histidine, γ CH_2), 2.26 (2H, m, α CH_2), and 1.80 (2H, m, β CH_2).