Amino Acids (2013) 45:821–833
DOI 10.1007/s00726-013-1525-x

ORIGINAL ARTICLE

A new method to synthesize creatine derivatives

Patrizia Garbati · Annalisa Salis · Enrico Adriano · Andrea Galatini · Gianluca Damonte · Maurizio Balestrino · Enrico Millo

Received: 4 April 2013 / Accepted: 23 May 2013 / Published online: 7 June 2013

Abstract Creatine is an amino acid that has a pivotal role in energy metabolism of cells. Creatine acts as an "ATP shuttle", carrying ATP to the sites where it is utilized, through its reversible phosphorylation by creatine kinase. Moreover, the creatine-phosphocreatine system delays ATP depletion during anoxia or ischemia, thus exerting a neuroprotective role during those pathological conditions. Thus, its administration has been advocated as a treatment or prevention of several conditions involving the central nervous system. However, creatine crosses poorly the blood–brain barrier and the cell plasma membrane, thus its administration has but a limited effect. The use of more lipophilic creatine derivatives has thus been suggested. However, such a synthesis is complicated by the intrinsic characteristics of the creatine molecule that hardly reacts with other molecules and easily cyclizes to creatinine. We obtained amide derivatives from creatine starting from a new protected creatine molecule synthesized by us, the so-called $(Boc)_2$-creatine. We used a temporary protection only on the creatine guanidine group while allowing a good reactivity on the carboxylic group. This temporary protection ensured efficient creatine dissolution in organic solvents and offered simultaneous protection of creatine toward intramolecular cyclization to creatinine. In this manner, it was possible to selectively conjugate molecules on the carboxylic group. The creatine guanidine group was easily released from the protection at the end of the reaction, thus obtaining the desired creatine derivative.

Keywords Creatine · $(Boc)_2$-creatine · Creatine derivatives synthesis · Guanidine group · Creatine reactivity

P. Garbati · E. Adriano · M. Balestrino
Department of Neuroscience, Ophthalmology, Genetics, Maternal–Infantile Sciences, University of Genova, Largo Paolo Daneo 3, 16132 Genoa, Italy

A. Salis
Department of Hearth Environmental and Life Science (DISTAV), University of Genova, Corso Europa 26, 16132 Genoa, Italy

A. Salis · G. Damonte · E. Millo
Center of Excellence for Biomedical Research, University of Genova, Viale Benedetto XV 5, 16132 Genoa, Italy

A. Galatini
Department of Chemistry and Industrial Chemistry, University of Genova, Via Dodecaneso 31, 16146 Genoa, Italy

G. Damonte · E. Millo (✉)
Department of Experimental Medicine, Section of Biochemistry, University of Genova, Viale Benedetto XV 1, 16132 Genoa, Italy
e-mail: enrico.millo@unige.it

Introduction

Creatine is an amino acid that has a pivotal role in energy metabolism of cells. It is reversibly phosphorylated to phosphocreatine (Andres et al. 2008), creating a creatine/phosphocreatine system that acts as an "adenosine triphosphate (ATP) shuttle", carrying ATP to the sites where it is utilized (Greenhaff 2001), an action so important that hereditary conditions where brain creatine is absent or reduced cause severe brain malfunction (Nasrallah et al. 2010; Schulze 2003). Moreover, the creatine phosphocreatine system delays ATP depletion during anoxia or ischemia, thus exerting a neuroprotective role during those pathological conditions (Balestrino et al. 2002). Thus, its administration has been advocated as a treatment or prevention of several conditions involving the central nervous

system, the main ones being creatine deficiency syndromes (Nasrallah et al. 2010; Schulze 2003), anoxia or ischemia (Perasso et al. 2013), neurodegenerative diseases (Adhihetty and Beal 2008; Beal 2011). However, creatine crosses poorly the blood–brain barrier (Perasso et al. 2003) and the cell plasma membrane (Lunardi et al. 2006) despite the presence of a specific transporter (Ohtsuki et al. 2002; Snow and Murphy 2001), thus its administration has but a limited effect. The use of more lipophilic creatine derivatives has thus been suggested (Lunardi et al. 2006; Perasso et al. 2008; Perasso et al. 2013).

To this aim, we decided to modify the creatine molecule to allow it to cross the phospholipid bilayer of biological membranes without the use of its specific transporter.

However, modifying the creatine molecule is difficult because it hardly reacts with other molecules because of its intrinsic characteristics. Creatine is a guanidine zwitterionic molecule that, forming a sort of internal salt, binds with difficulty with other compounds.

Creatine solubility is a key aspect relative to its ability to react with other compounds. Under normal conditions, creatine monohydrate exhibits low aqueous solubility (Singh and Dash 2009; Windholz et al. 1976). The aqueous solubility increases with the increase of temperature; even at 60 °C a great abundance of sub-products is present (Pischel and Gastner 2008).

At a pH between 3 and 12, creatine is principally in its zwitterionic form (Edgar and Shiver 1925) and this fact adversely affects the bonds with other molecules. An acidic environment is the best condition to promote the creatine solubility, such that at pH < 3 creatine is mainly present as a cation. Furthermore, the creatine shows low solubility in a large part of organic solvents (Windholz et al. 1976).

Preparations in which the creatine is linked to other compounds by forming a salt are well documented (Negrisoli and Del Corona 1999; Pischel et al. 1999). It is necessary that these compounds keep the undissociated form during the passage through biological membranes so that such compound can use the carrier of the molecule linked to creatine or the characteristics that the lipophilic molecule partner offers. In addition, there are other data on the creatine prodrug where it is covalently linked in the form of esters or anhydrides (Burov et al. 2011b; Heuer et al. 2009; Peters et al. 2007) with different substances.

Although creatine esters are more lipophilic than creatine molecule, they are not stable enough and their enzymatic or non-enzymatic cleavage results in the formation of creatinine instead of creatine release (Adriano et al. 2011). As an alternative the possibility of a covalent link between creatine and other substances was investigated; this was done by exploiting direct acylation of the compound with particular creatine salts (Burov et al. 2011a). Using this strategy, the problem of solubility of creatine can be overcome by its conversion to an appropriate salt form. Infact the addition of particular lipophilic counterion represents a simple and convenient procedure for dissolution of creatine and other different guanidino or amidino compounds in organic solvents. In our experience, this method gives good results only with substances at high nucleophilicity (example with some aliphatic or cyclic amine), moreover this strategy involves low yields and long times of purification.

Moreover, the synthesis of creatine derivatives can be performed starting from a secondary amine called sarcosine. These conjugates can be synthesized by the guanidinylation of sarcosyl derivatives. This strategy comprises different stages, but the yields of the products in literature are quite low (Burov et al. 2011a).

To overcome these problems, and to ameliorate the creatine reactivity, our strategy was to design a creatine molecule with a temporary protection only on the guanidine group (Powell et al. 2003; Robinson and Roskamp 1997) while allowing a good reactivity on the carboxylic group. Indeed, this temporary protection ensured efficient creatine dissolution in organic solvents offering simultaneous protection of creatine toward intramolecular cyclization to creatinine. In this manner, it was possible to conjugate selectively several molecules with different characteristics, on the carboxylic group while the guanidine group had to be easily released from the protecting group to allow the final molecule to be phosphorylated upon reaching the site of action.

We therefore decided to build a creatine with the guanidine group temporarily protected; tert-butoxycarbonyl group (t-Boc) was the most suitable protective group found for the purpose. The protection with t-Boc also provided a high solubility of the molecule in organic solvents, moreover, allowing a high reactivity of the molecule on the carboxyl group.

Starting from this particular kind of creatine, called $(Boc)_2$-creatine, some creatine derivatives with different aminogroups in high yield and purity were obtained.

Materials and methods

All chemicals were purchased from Sigma-Aldrich and used as received without further purification. All untreated solvents used were of HPLC grade.

The thin layer chromatography (TLC) was performed on UV plates (Fluka Analytical, Silica on TLC Alu foils, with fluorescent indicator 254 nm). Four different eluent phases were used for the TLC: *n*-butanol:acetic acid:water (6:2:2), MeOH:$CHCl_3$:CH_3COOH (8:1.75:0.25), CH_2Cl_2:MeOH:CH_3COOH (9.25:0.5:0.25) and hexane:ethyl acetate (5:5).

Revelation: solution of ninhydrin (0.2 g) in ethanol (50 ml) spraying before putting on hot plate over 100 °C.

Electrospray ionization mass spectrometry and high performance liquid chromatography coupled to electrospray mass spectrometry methods

The analysis of the crude products was performed by electrospray ionization mass spectrometry (ESI-MS) full scan and MS^2 analysis (ESI-MS^2) were carried out in positive ion mode on an Agilent 1100 series LC/MSD ion trap XCT instrument (Agilent Technologies, Palo Alto, CA, USA) in direct infusion (DIA) using the infusion pump NE1000 (KF Technology, Roma, Italy). The flow rate was set at 5 µl/min. Each product was diluted in acetonitrile: water 50:50 containing 0.1 % formic acid to get a final concentration of 0.01 mg/ml.

The qualitative analysis of creatine derivatives and the $(Boc)_2$-creatine stability were carried out on an Agilent 1100 HPLC system coupled to a MSD Ion Trap XCT mass spectrometer, equipped with an electrospray ion source (HPLC–ESI-MS). Separations were performed on a HILIC silica 2.1 × 150 mm^2 Atlantis column with 3-µm particle size (Waters Corporation, Milford, MA, USA). Eluents were acetonitrile (A) and 50 mM ammonium acetate (B) whose pH was adjusted to 5 with acetic acid. The flow rate was set to 0.20 ml/min. The column temperature was set at 30 °C. The best chromatographic conditions were outlined as follows: injection at 80 % A, hold for 3 min, then decrease by step to 50 % A at 3.01 min and hold for 1 min, then reconditioning to initial conditions at 4.01 min and hold for 12 min. Injection volume was 5 µl. Ions were detected in ion-charged control with a target ions value of 50,000 and an accumulation time of 300 ms, using the following operation parameters: capillary voltage: 3,000 V; nebulizer pressure: 40 psi; drying gas: 10 L/min; dry temperature: 325 °C; rolling averages 3, averages 8. Mass spectra were acquired in positive ion mode in the 100–400 m/z mass range. An amplitude voltage of 1.0 V was typically used for fragmentation in the ion trap MS^2 analysis.

Preparative HPLC method

The products were purified by preparative reverse phase high performance liquid chromatography (RP-HPLC) on a Shimadzu LC-9A preparative HPLC equipped with a Phenomenex C18 Luna column 21.20 id × 250 mm (Phenomenex Italia, Castel Maggiore, Bologna, Italy).The separation was performed in gradient starting with 10 % solvent B for 5 min, linearly increasing to 70 % solvent B in 30 min and up to 100 % B in 10 min. The solvent used was 0.1 % formic acid (HCOOH) in water A and 0.1 % HCOOH in acetonitrile (B).

Nuclear magnetic resonance (NMR) analysis method

^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Varian MercuryPlus spectrometer at 300 and 75 MHz, respectively. Chemical shifts are reported as δ values (ppm).

Synthesis of N^1,N^2-bis(tertbutoxycarbonyl)-N^3-methylguanidino-N^3-acetic acid ($(Boc)_2$-creatine).

Method A using N^1,N^2-Bis(tert-butoxycarbonyl)-S-methylisothiourea as guanylating agent

To a solution of sarcosine ethyl ester (1 g; 6.5 mmol; 1.2 equivalents), N^1,N^2-Bis(tert-butoxycarbonyl)-S-methylisothiourea (1.575 g; 5.4 mmol; 1 equivalent), and triethylamine (16.3 mmol; 2.27 µl; 3 equivalents) in 10 ml anhydrous dimethylformamide (DMF) was added $HgCl_2$ (1,620 g; 6 mmol; 1.1 equivalents). The suspension was stirred at room temperature for about 18 h. Then the crude reaction mixture was taken up in diethyl ether, a white precipitate was formed and then filtered through a filter vacuum. The filtrate was washed two times with deionized water and after other three times with a solution of NaCl 0.1 M. The organic phase was evaporated to dryness and finally lyophilized. Analysis of the crude product was performed by ESI-MS. The result showed the expected molecular weight of N^1,N^2-bis(tertbutoxycarbonyl)-N^3-methylguanidino-N^3-acetic acid ethyl ester.

A solution of acetonitrile and NaOH 1 N 1:1 was added and the mixture was stirred at room temperature. The reaction was carried out until the complete hydrolysis of the ethyl group, monitored by TLC, to obtain the desired compound. At the end the pH was adjusted to 6 using HCl 1 N. The compound obtained was dried in vacuum to obtain a white powder and then purified by preparative RP-HPLC. Fractions containing the molecule were collected, vacuum dried and finally lyophilized to afford colorless powder of N^1,N^2-bis(tertbutoxycarbonyl)-N^3-methylguanidino-N^3-acetic acid. Yield 86 %. The compound purity was finally verified by HPLC–MS analysis as described above. The qualitative analysis of the product was finally confirmed by ESI-MS and ESI-MS^2 analysis.

^{1}H NMR (300 MHz, $CDCl_3$) δ 9.54 (s, broad 1H), 3.89 (s, 2H), 3.01 (s, 3H), 1.44 (s, 18H).

^{13}C NMR (75 MHz, $CDCl_3$) δ 175.43, 161.53, 154.63, 151.53, 81.72, 79.66, 55.05, 38.41, 28.34.

ESI-MS: m/z 332.00 $[M + H]^+$; ESI-MS^2: m/z (rel. int.): 275 $[M-C_4H_9]^+$ (100); 220 $[M-2 \times C_4H_9]^+$ (40); 176 $[M-(2 \times C4H9)-CO_2] +$ (10).

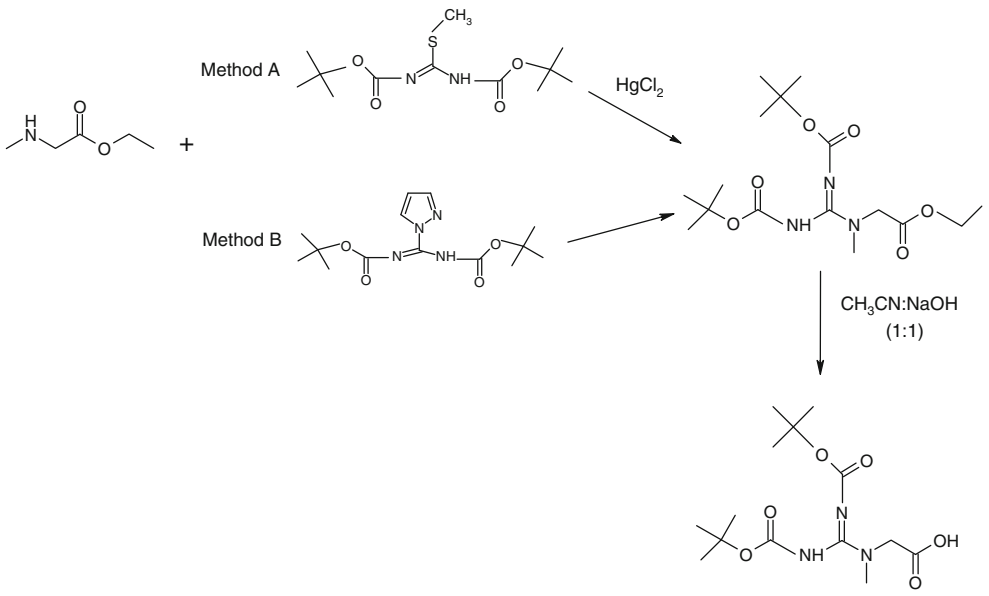


Fig. 1 $(Boc)_2$-Creatine synthesis. Sarcosine ethyl ester reacts with N^1,N^2-Bis(tert-butoxycarbonyl)-S-methylisothiourea (method A) or with N^1,N^2-Bis(tert-butoxycarbonyl)-1H-pyrazole-1-carboxamidine (method B) as guanylating agent. This reaction gives $(Boc)_2$-creatine ethyl ester that is subjected to basic hydrolysis leading to the formation of $(Boc)_2$-creatine, able to react with other molecules through its carboxyl group

Method B using N^1,N^2-Bis(tert-butoxycarbonyl)-1H-pyrazole-1-carboxamidine as guanylating agent

To a solution of sarcosine ethyl ester (1 g; 6.5 mmol; 1.2 equivalents), in 10 ml of anhydrous DMF, N^1,N^2-Bis(tert-butoxycarbonyl)-1H-pyrazole-1-carboxamidine (1.68 g; 5.4 mmol; 1 equivalent), and triethylamine (2.26 ml; 16.25 mmol; 3 equivalents) were added. The suspension was stirred at room temperature for about 48 h. Then the crude reaction mixture was centrifuged at 4,800 rpm for 10 min. The supernatant obtained was taken up in diethyl ether and washed three times with deionized water. The organic solution was evaporated to minimum volume and finally lyophilized. Analysis by ESI-MS was performed. The result showed the expected molecular weight of N^1,N^2-bis(tertbutoxycarbonyl)-N^3-methylguanidino-N^3acetic acid ethyl ester.

A solution of acetonitrile and NaOH 1 N 1:1 was added and the mixture was stirred at room temperature. The reaction was carried out until the complete hydrolysis of the ethyl group, monitored by TLC, to obtain the desired compound. At the end the pH was adjusted to 6 using HCl 1 N. The compound was evaporated to dryness under vacuum and then purified by preparative RP-HPLC. Fractions containing the molecule were collected, vacuum dried and finally lyophilized to afford colorless powder of N^1,N^2-bis(tertbutoxycarbonyl)-N^3-methylguanidino-N^3-acetic acid. Yield 83 %. The compound purity was finally verified by HPLC–MS as described above.

The qualitative analysis of the product was finally confirmed by ESI-MS and ESI-MS^2 analysis.

^{1}H NMR (300 MHz, $CDCl_3$) δ 9.54 (s, broad 1H), 3.89 (s, 2H), 3.01 (s, 3H), 1.44 (s, 18H).

^{13}C NMR (75 MHz, $CDCl_3$) δ 175.43, 161.53, 154.63, 151.53, 81.72, 79.66, 55.05, 38.41, 28.34.

ESI-MS: m/z 332.00 $[M + H]^+$; ESI-MS^2: m/z (rel. int.): 275 $[M-C_4H_9]^+$ (100); 220 $[M-C_8H_{18}]^+$ (40).

ESI-MS: m/z 332.00 $[M + H]^+$; ESI-MS^2: m/z (rel. int.): 275 $[M-C_4H_9]^+$ (100); 220 $[M-2 \times C_4H_9]^+$ (40); 176 $[M-(2 \times C4H9)-CO_2] + (10)$.

Synthesis of amino acids esters

Some of amino acids esters were purchased; others have been esterified by us following the methods in literature with some modifications (Chen et al. 1999; Li and Sha 2008).

Briefly, the amino acid (1 equivalent) was suspended in chlorotrimethylsilane (2 equivalents) and a suitable volume of methanol was added. The reaction mixture was stirred at room temperature monitoring the reaction with TLC analysis ($MeOH:CHCl_3:CH_3COOH$ 8:1.75:0.25). The reaction was carried out for 12–48 h, depending on the amino acid, and mainly monitored until the reaction was complete. The organic solution was then evaporated under vacuum and finally repeatedly lyophilized to obtain the final product. The qualitative analysis of the products was

confirmed by ESI-MS analysis. The aminoacid esters were used without further purification.

General procedure for synthesis of creatine–amino acids esters

$(Boc)_2$-creatine (1 equivalent), was dissolved in anhydrous DMF. Isobutyl chloroformate (1 equivalent) and *N*-methylmorfoline (1 equivalent) were added to this compound while holding the reaction at 0 °C. After 10 min the reaction was held at room temperature protected from light. At this point, amino acid ester (1.5 equivalents) was prepared by stirring commercially available or synthesized ester hydrochloride and triethylamine (1.5 equivalents) in dry DMF for 30 min. The mixture was centrifuged to remove the resulting salt, before adding it to the activated $(Boc)_2$-creatine. The solution was stirred for 24–48 h depending on the aminoacid used monitoring the reaction by TLC. When the reaction was completed the mixture was centrifuged and the supernatant was lyophilized. After that, the compound was dissolved in diethyl ether or ethyl acetate depending on the aminoacid used, and the organic solution was washed three times with deionized water. Indeed the organic phase was evaporated under vacuum until dryness. The crude product was then purified by preparative RP-HPLC.

A solution of dichloromethane (DCM) and trifluoroacetic acid (TFA) 1:1 was indeed added to the purified product at 0 °C. When the reaction was completed the compound was treated with ice cold diethyl ether. If a precipitate was formed, it was separated by centrifugation (4,800 rpm per 10 min), washed with diethyl ether and finally lyophilized to obtain a solid or oil. In absence of any precipitate the organic mixture was washed twice with deionized water and then evaporated under vacuum. The final derivative was then lyophilized to obtain a powder or oil. The compounds purity was finally verified by HPLC–MS analysis as described above. The qualitative analysis of the products was finally confirmed by ESI-MS and ESI-MS^2 analysis.

Creatine–alanine methyl ester

The product was obtained as a white powder in 84 % yield. Rf = 0.41 (MeOH:CHCl3:CH3COOH 8:1.75:0.25). ESI-MS: m/z 217.08 $[M + H]^+$; ESI-MS^2: m/z (rel. int.): 200 $[M-H_2O]^+$ (5); 114 $[M-C_4H_9NO_2]^+$ (100).

^{1}H NMR (300 MHz, H_2O/D_2O 9/1) δ 8.49 (d, broad $J = 5.1$ Hz, 1H), 6.76 (s, broad 3H), 4.39–4.22 (m, 1H), 4.03 (s, 2H), 3.59 (s, 3H), 2.87 (s, 3H), 1.26 (d, $J = 7.3$ Hz, 3H).

^{13}C NMR (75 MHz, H_2O/D_2O 9/1) δ 174.82, 169.52, 157.79, 52.82, 52.21, 48.63, 36.87, 15.79.

Creatine–valine ethyl ester

The product was obtained as a white powder in 76 % yield. Rf = 0.34 (MeOH:CHCl3:CH3COOH 8:1.75:0.25). ESI-MS: m/z 259.08 $[M + H]^+$; ESI-MS^2: m/z (rel. int.): 241 $[M-H_2O]^+$ (5); 114 $[M-C_7H_{15}NO_2]^+$ (100).

^{1}H NMR (300 MHz, H_2O/D_2O 9/1) δ 8.40 (d, broad $J = 6.9$ Hz, 1H), 6.75 (s, broad 3H), 4.20–3.98 (m, 5H), 2.86 (s, 3H), 2.14–1.94 (m, 1H), 1.10 (t, $J = 7.1$ Hz, 3H), 0.78 (d, $J = 6.6$ Hz, 3H), 0.77 (d, $J = 6.6$ Hz, 3H).

^{13}C NMR (75 MHz, H_2O/D_2O 9/1) δ 173.39, 169.54, 157.87, 62.51, 58.86, 52.30, 36.97, 29.79, 18.12, 17.15, 13.24.

Creatine–threonine methyl ester

The product was obtained as a white powder in 85 % yield. Rf = 0.39 (*n*-butanol:acetic acid:water 6:2:2). ESI-MS: m/z 247.08 [M + H] + ; ESI-MS^2: m/z (rel. int.): 229 [M−H2O] + (5); 114 [M−C5H11NO3] + (100).

^{1}H NMR (300 MHz, H_2O/D_2O 9/1) δ 8.39 (d, broad $J = 8.3$ Hz, 1H), 6.77 (s, broad 3H), 4.42 (dd, $J = 8.3$, 2.8 Hz, 1H), 4.25 (dd, $J = 6.3$, 2.8 Hz, 1H), 4.12 (s, 2H), 3.62 (s, 3H), 2.89 (s, 3H), 1.03 (d, $J = 6.3$ Hz, 3H).

^{13}C NMR (75 MHz, H_2O/D_2O 9/1) δ 172.21, 169.83, 157.87, 66.94, 58.31, 53.00, 52.43, 37.01, 18.68.

General procedure for synthesis of creatine–amides

$(Boc)_2$-creatine (1 equivalent) was dissolved in anhydrous DMF. Isobutyl chloroformate (1 equivalent) and *N*-methylmorfoline (1 equivalent) were added to this compound holding the reaction at 0 °C. After 10 min the reaction was held at room temperature protected from light. At this point, a solution of amine (1.5 equivalents) in anhydrous DMF was added to the activated $(Boc)_2$-creatine. The mixture was stirred for 24–48 h, depending on the amine, monitoring the reaction by TLC. When the reaction was completed, the mixture was centrifuged and the supernatant was lyophilized. The compound was dissolved in diethyl ether and then washed three times with deionized water. Indeed the organic phase was evaporated under vacuum until dryness. The crude product was then purified by preparative RP-HPLC.

A solution of DCM and TFA 1:1 was indeed added to the purified product at 0 °C. When the reaction was completed the compound was treated with ice cold diethyl ether. If a precipitate was formed, it was separated by centrifugation (4,800 rpm per 10 min), washed with diethyl ether and finally lyophilized to obtain a solid or an oil. In absence of any precipitate, the organic mixture was washed twice with deionized water and then evaporated under vacuum. The final derivative was then lyophilized to

obtain a powder or an oil. The qualitative analysis of the product was finally confirmed by ESI-MS analysis.

Stability of $(Boc)_2$-creatine in aqueous solution

For the measurement of the $(Boc)_2$-creatine stability the molecule was dissolved, at a final concentration of 2 mM, in artificial cerebrospinal fluid (ACSF) at 36 °C and the solution was diluted 400 times in eluent A:B (80:20 %). The ACSF had the following composition: NaCl 130 mM, KCl 3.5 mM, NaH_2PO_4 1.25 mM, $NaHCO_3$ 24 mM, $CaCl_2$ 2.4 mM, $MgSO_4$ 1.2 mM, glucose 10 mM. Then, 5 µl of solution was injected in HPLC–MS system, and the different peaks were quantified. We were interested to know the amount of $(Boc)_2$-creatine that was still intact at time 0 and after 5, 60, 120,150 and 180 min of treatment. Thus, at these times, the area under each peak was measured, and normalized as a percentage of the area of $(Boc)_2$-creatine peak at time 0 min.

Results and discussion

The chemical synthesis of creatine derivatives is quite difficult because of its intrinsic characteristics. Normally, creatine hardly reacts with other molecules because of zwitterionic form which creates a sort of internal salt, decreasing its reactivity.

Our goal was to synthesize creatine derivatives able to cross the phospholipid bilayer of biological membranes without using the specific transporter of creatine. Creatine has two possible groups able to form bonds with other molecules: carboxylic and guanidine groups.

Creatine derivatives with drastic alteration of guanidine nitrogen present a high degree of lipophilicity but their function in the brain may have negative aspects such as the impediment of the creatine phosphorylation. At this regard, it is necessary that the molecule that we are going to combine selectively is covalently bound with the creatine carboxylic group while the guanidine group must be free to interact with the creatine kinase enzyme.

Our strategy was to design a creatine molecule with a temporary protection only on the guanidine group that allows us to obtain a new precursor which had both a high solubility in organic solvents and good reactivity of the carboxylic part.

Moreover, the carboxyl group could not be activated with standard activating agents like carbodiimide such as dicyclohexylcarbodiimide (DCC) or ethyl-(*N*',*N*'-dimethylamino)propylcarbodiimide hydrochloride (EDC) or aminium-based reagents such as *O*-(benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HBTU) if the guanidine group was free.

The reason for this poor reactivity is probably due to the hydrogen bonds that are formed between the carboxyl group and the two amino groups of the guanidine moiety. In a molecule in which the guanidine group is protected, you can activate the carboxyl group with the same activating reagents.

Initially, we tried using some temporary protective groups for the guanidine group described in the literature: 2,2,4,6,7,pentamethyldihydrobenzofuran-5-sulfonyl group (Pbf), 4-toluensulfonyl group (Tosyl) tryphenylmethyl group (Trt) and others (Carpino et al. 1993; Isidro-Llobet et al. 2009; Ruskin 1946), but these were not convenient because attempts to clamp the guanidine group lead to constructs with too low yields. Indeed, even using the Pbf blocking group the product was completely degraded when put in an acid environment intended to remove the protective group. The best protecting group found was t-Boc which binds to both the nitrogen atoms of the guanidine group. Heuer et al. (2009) describe a method to synthesize $(Boc)_2$-creatine starting from creatine itself using agents able to insert the t-Boc group such as tert-butoxycarbonyl anhydride. Following this protocol, we did not find the desired intermediate because of the instability and low solubility of creatine at the pH of the experimental procedure.

Therefore, we synthesized the $(Boc)_2$-creatine, which is a form of creatine where the two nitrogen atoms of its guanidine group are protected by two t-Boc groups. This form of creatine has several advantages: it is soluble in organic solvents, reacts easily and it is stable and does not degrade in creatinine. This particular derivative cannot be synthesized starting from creatine itself, but using sarcosine a similar compound lacking the guanidine moiety. The first step of the process involved the use of sarcosine ethyl ester as a precursor which was converted to $(Boc)_2$-creatine by the use of a guanylating agent which was protected on both its nitrogen atoms with t-Boc. This allowed its direct synthesis. Two methods were used to synthesize the $(Boc)_2$-creatine using two different guanylating agents; the two reactions were more or less similar. The sarcosine ethyl ester was easily converted to a protected guanidine compound through a simple procedure. Introducing a guanidine group to sarcosine ethyl ester gives a higher yield if the guanylating agent used already has the t-Boc protection. The same reaction without t-Boc on the guanylating agent gives a very low yield. The guanylating agents used were N^1,N^2-Bis(tert-butoxycarbonyl)-S-methylisothiourea (Powell et al. 2003) and N^1,N^2-Bis(tert-butoxycarbonyl)-1H-pyrazole-1-carboxamidine (Robinson and Roskamp 1997). The yields obtained with these two guanylating agents were essentially the same. The final ethyl ester of $(Boc)_2$-creatine was easily isolated in high yield.

In the next step of the procedure, the ester of $(Boc)_2$-creatine was subjected to alkaline hydrolysis to form the $(Boc)_2$-creatine (Fig. 1). The method advantageously allowed to obtain a good yield (>80 %) and excellent purity of the final product as shown in the ESI-MS and ESI-MS^2 spectra of Fig. 2.

In addition, we tested the $(Boc)_2$-creatine stability. We found that this molecule has a great stability, indeed after 6 months at room temperature about 100 % of the product was present. Its stability is also evaluated in an aqueous medium, ACSF: after 3 h 96.6 % of the initial value was still present. The stability was verified using ACSF because this is the incubation medium that we will use in future biological experiments. Furthermore the brain is the target for these kinds of molecules where the aqueous medium present is the cerebrospinal fluid. The $(Boc)_2$-creatine was subsequently used to synthesize various derivatives of creatine, by conjugating a molecule having a functional group capable of reacting with the free carboxyl group of creatine. Due to their greater stability, we preferred to synthesize amide derivatives of creatine. In the Table 1 was summarized all the derivatives of creatine synthesized.

We obtained various compounds where the creatine is linked to amino acids or amines with yields ranging from 35 to 85 % depending on the type of aminoacid or amine used. The compound containing histidine has a low yield probably due to the poor reactivity of amino group of histidine (see Table 1).

The reaction between the carboxylic group and the amino group of amino acids or amines obviously requires the activation of the carboxyl group. We tried different standard activating agents as DCC, EDC and HBTU and others. Even if these agents allow us to obtain the coupling of amino groups the final yield of the entire process is low

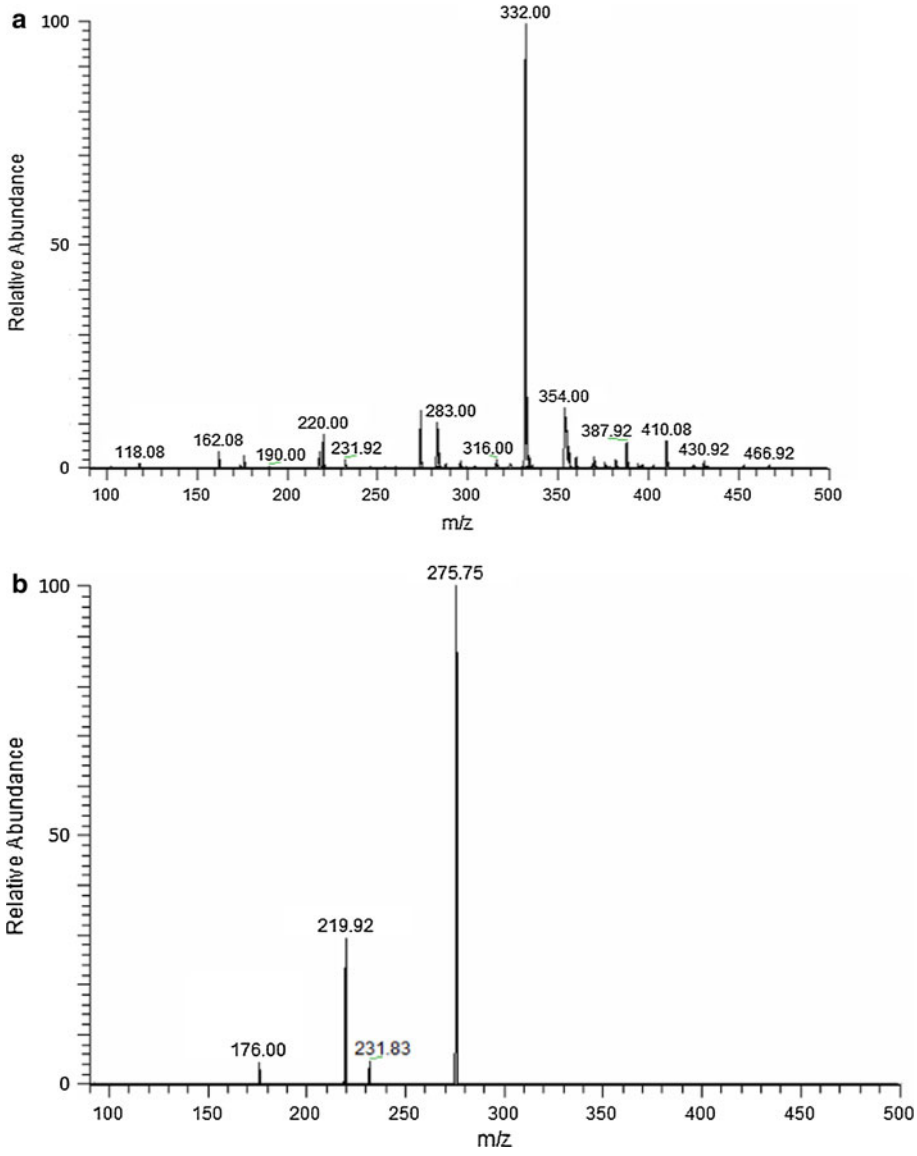
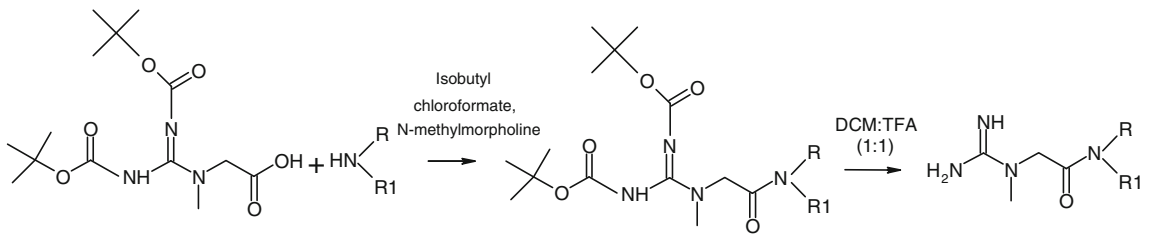
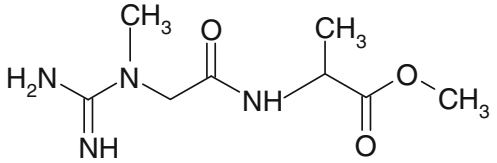
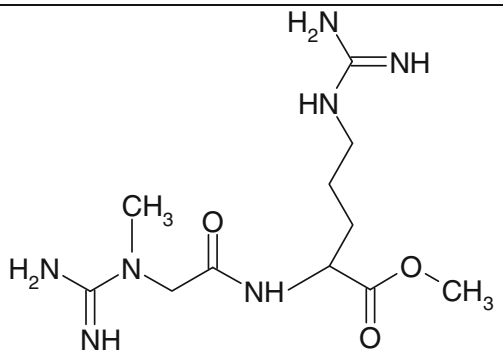
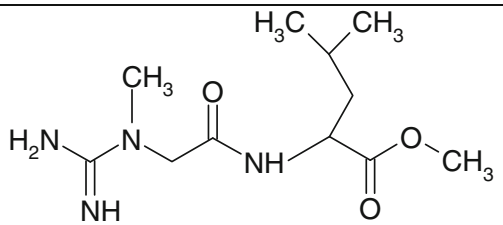
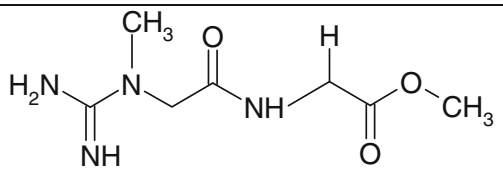
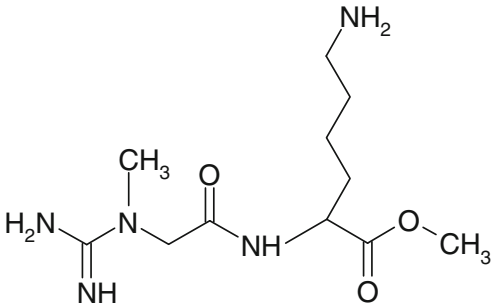


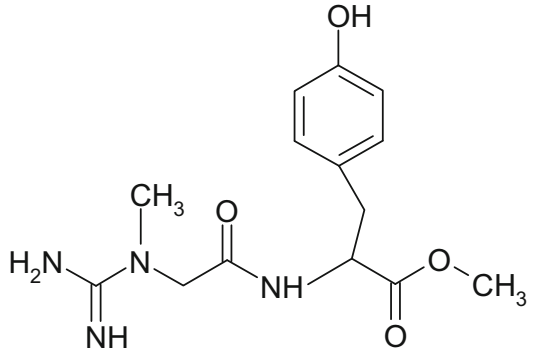
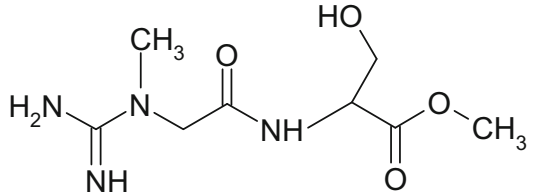
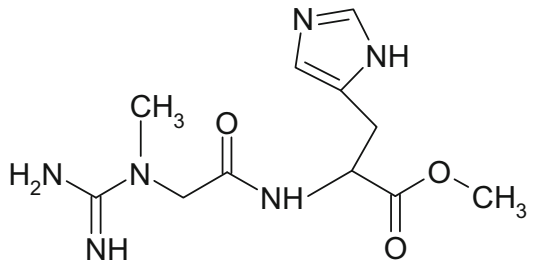
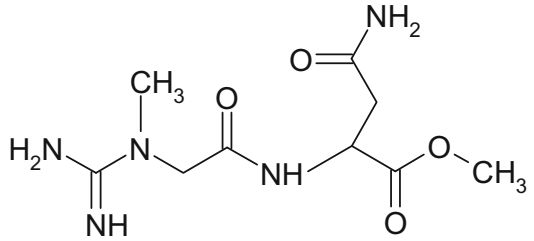
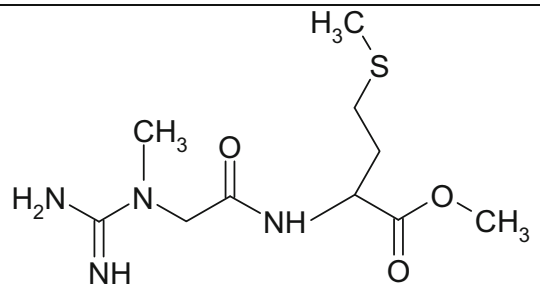
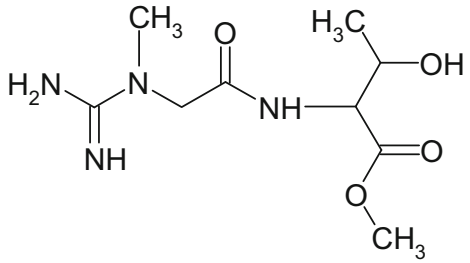
Fig. 2 ESI-MS and ESI-MS^2 spectra of $(Boc)_2$-creatine. **a** Protonated $(Boc)_2$-creatine molecular ion (m/z 332.00). **b** $(Boc)_2$-Creatine ESI-MS^2 spectra (see details in "Materials and methods" section)

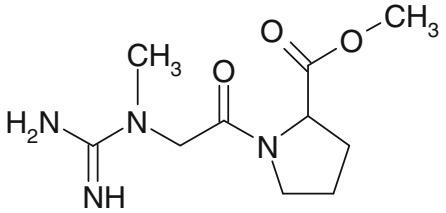
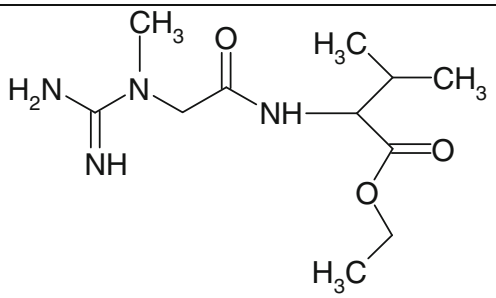
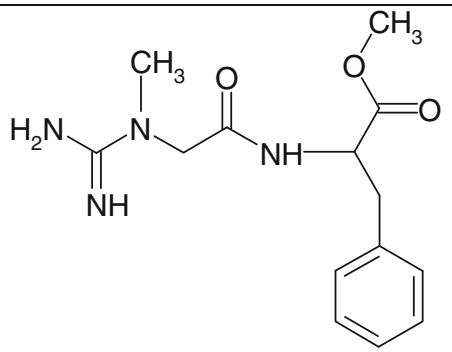
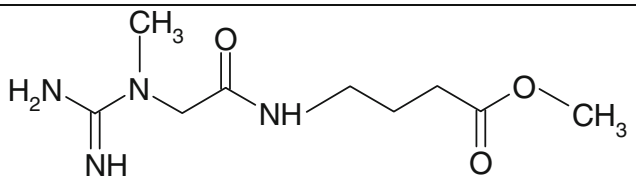
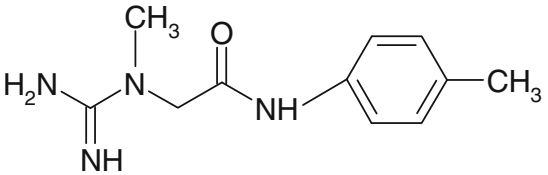
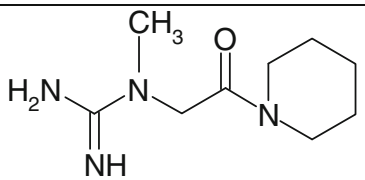
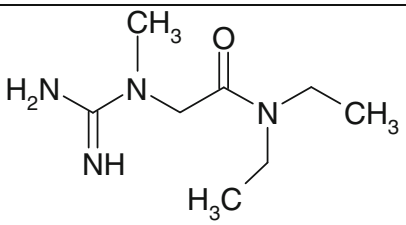
Table 1 Synthesis of creatine derivatives starting from $(Boc)_2$-creatine

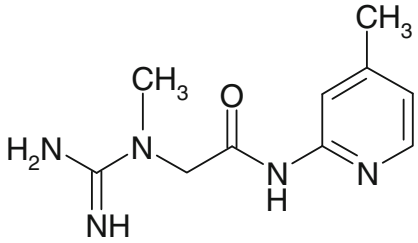
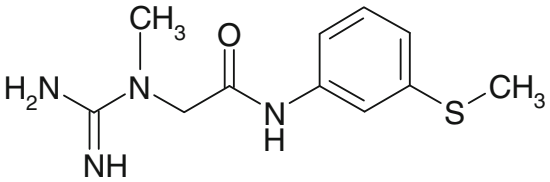


		MW	Creatine derivatives structure	Yield
1	Creatine-Alanine methyl ester	216.23		84%
2	Creatine-Arginine methyl ester	301.35		65%
3	Creatine-Leucine methyl ester	258.31		68%
4	Creatine-Glycine methyl ester	202.21		82%
5	Creatne-Lysine methyl ester	273.33		78%

On the top is described the synthetic pathway for the preparation of the various derivatives. The creatine derivatives synthesized are summarized in the table with their molecular weight and yield

6	Creatine-Tyrosine methyl ester	308.33		66%
7	Creatine-Serine methyl ester	232.23		74%
8	Creatine-Histidine methyl ester	282.29		28%
9	Creatine-Asparagine methyl ester	259.26		46%
10	Creatine-Methionine methyl ester	276.35		66%
11	Creatine-Threonine methyl ester	246.26		85%

12	Creatine-Proline methyl ester	242.27		62%
13	Creatine-Valine ethyl ester	258.32		76%
14	Creatine-Phenylalanine methyl ester	292.34		82%
15	Creatine-γ-Aminobutyric acid methyl ester	230.26		68%
16	Creatine-p-Toluidine	220.27		52%
17	Creatine-Piperidine	198.26		59%
18	Creatine-Diethylamine	186.25		38%

19	Creatine-2-amino-4-picoline	221.26		35%
20	Creatine-3-(methylthio)aniline	252.33		42%

and the contemporary presence of sub-products discourages their use.

The mixed anhydride method with isobutyl chloroformate was most advantageous in terms of reaction completion and simplicity of the subsequent purification. Better results were obtained by using 1 equivalent of isobutyl chloroformate and dropwise addition of 1 equivalent of *N*-methylmorfoline to the reaction mixture of $(Boc)_2$-creatine followed by the addition of the amino component. The entry sequence of the various reagents was fundamental. It prevented the formation of the side reaction that gives a urethane by-product and liberates the amino acid or the amine group. These products were formed if the amino group was added before using the alkyl chloroformate (Chaudhary et al. 2003).

In our experience, we also observed that the addition of the amino acid in its hydrochloride form gives a low yield or in same cases even absence of the reaction. To overcome this problem, we transformed the hydrochloride form into an amino acid free form (Todoroki et al. 2011), thus increasing the amount of the final product.

A further advantage offered by the $(Boc)_2$-creatine amino intermediates was the fact that the product was easily purified by preparative RP-HPLC before removing the t-Boc group.

The presence of the t-Boc group greatly increases the lipophilicity of the compound favoring the purification by RP-HPLC with water and acetonitrile. This advantage is particularly important when considering the difficulty in analyzing, and hence purifying creatine and its derivatives with chromatographic methods. The purification of creatine and its derivatives without the t-Boc group involves using salt buffers which, however, cause the final problem of having to remove the salts formed (Dash and Sawhney 2002; Smith-Palmer 2002).

However, to be active the creatine derivatives must have their guanidine groups free to interact with the creatine kinase enzyme. The final construct had to be released from the t-Boc protection to become active. But often, the t-Boc removal in acidic environment could cause the degradation of the molecule with the formation of sub-products, so this step is crucial in the entire protocol.

We tried different methods to detach the t-Boc groups from the guanidine group of creatine to select the optimal condition. We used TFA/DCM (1:1) at 0 °C, H_2O/TFA (1:1) at room temperature, HCl 1 N at room temperature and CH_3COCl:MeOH (1:1) at 0 °C (Cheguillaume et al. 2001; Nudelman et al. 1998; Zhu et al. 2000). Only the first method detached the two t-Boc groups on creatine providing the final product without further problems.

Deprotection by TFA/DCM at 0 °C seemed to be the method of choice to obtain the desired product without forming creatinine or other sub-products in all the derivatives tested.

As an example, we characterized three specific creatine derivatives (creatine–alanine methyl ester, creatine–valine ethyl ester, creatine–threonine methyl ester). In addition to HPLC–MS analysis, performed on the derivatives described in table 1, for these three derivatives we also reported NMR and ESI-MS^2 analysis. We chose to deepen the analysis on such compounds because we will use them in future biological experiments.

Conclusions

Creatine derivatives may have an important role for the treatment of neurological diseases. Unfortunately, it is a zwitterionic molecule which hardly reacts with other compounds and it is also practically insoluble in organic solvents. We solved these two problems by synthesizing a particular kind of creatine with the guanidine group protected by two t-Boc groups. The $(Boc)_2$-creatine was then

used to synthesize derivatives of creatine. In this paper, we show the synthesis method of the $(Boc)_2$-creatine and a new simple method to synthesize creatine derivatives with molecules having a reactive amino group. This method allows obtaining the products synthesized in high yield and with a purity greater than 95 %, due to the ease with which they can be purified by RP-HPLC before removal of the t-Boc groups. This method is especially suitable for synthesis of amide derivatives, but does not exclude the ability of $(Boc)_2$-creatine to react with other nucleophilic groups.

Acknowledgments The authors would like to thank Dr. Marta Bellotti for her valuable help in verifying $(Boc)_2$-creatine stability.

Conflict of interest The authors declare that they have no conflict of interest.

Bibliography

Adhihetty P, Beal M (2008) Creatine and its potential therapeutic value for targeting cellular energy impairment in neurodegenerative diseases. Neuromolecular Med 10(4):275–290. doi:10.1007/s12017-008-8053-y

Adriano E, Garbati P, Damonte G, Salis A, Armirotti A, Balestrino M (2011) Searching for a therapy of creatine transporter deficiency: some effects of creatine ethyl ester in brain slices in vitro. Neuroscience 199:386–393. doi:10.1016/j.neuroscience.2011.09.018

Andres RH, Ducray AD, Schlattner U, Wallimann T, Widmer HR (2008) Functions and effects of creatine in the central nervous system. Brain Res Bull 76:329–343. doi:10.1016/j.brainresbull.2008.02.035

Balestrino M, Lensman M, Parodi M, Perasso L, Rebaudo R, Melani R, Polenov S, Cupello A (2002) Role of creatine and phosphocreatine in neuronal protection from anoxic and ischemic damage. Amino Acids 23(1–3):221–229. doi:10.1007/s00726-001-0133-3

Beal M (2011) Neuroprotective effects of creatine. Amino Acids 1–9. doi:10.1007/s00726-011-0851-0

Burov S, Leko M, Dorosh M, Dobrodumov A, Veselkina O (2011a) Creatinyl amino acids—new hybrid compounds with neuroprotective activity. J Pept Sci 17(9):620–626. doi:10.1002/psc.1379

Burov S, Khromova NV, Khromova AA, Khromov PA (2011b) Patent US 2011/0269986 A1 Amides of creatine, method of their preparation, and remedy possessing a neuroprotective activity, Pub. Date Nov. 3, 2011

Carpino LA, Shroff H, Triolo SA, Mansour ESME, Wenschuh H, Albericio F (1993) The 2,2,4,6,7, pentamethyldihydrobenzofuran-5-sulfonyl group (Pbf) as arginine side chain protectant. Tetrahedron Lett 34(49):7829–7832. doi:10.1016/S0040-4039(00)61487-9

Chaudhary A, Girgis M, Prashad M, Hu B, Har D, Repič O, Blacklock TJ (2003) Using mixed anhydrides from amino acids and isobutyl chloroformate in *N*-acylations: a case study on the elucidation of mechanism of urethane formation and starting amino acid liberation using carbon dioxide as the probe. Tetrahedron Lett 44(29):5543–5546. doi:10.1016/S0040-4039(03)01329-7

Cheguillaume A, Salaün A, Sinbandhit S, Potel M, Gall P, Baudy-Floćh M, Le Grel P (2001) Solution synthesis and characterization of aza-beta(3)-peptides (*N*(alpha)-substituted hydrazino acetic acid oligomers). J Org Chem 66(14):4923–4929

Chen BC, Skoumbourdis AP, Guo P, Bednarz MS, Kocy OR, Sundeen JE, Vite GD (1999) A facile method for the transformation of *N*-(tert-butoxycarbonyl)α-amino acids to *N*-unprotected α-amino acids methyl esters. J Org Chem 64:9294–9296. doi:10.1021/jo990311w

Dash AK, Sawhney A (2002) A simple LC method with UV detection for the analysis of creatine and creatinine and its application to several creatine formulations. J Pharm Biomed Anal 29(5):939–945. doi:10.1016/S0731-7085(02)00167-X

Edgar G, Shiver HE (1925) The equilibrium between creatine and creatinine in aqueous solution. The effect of the hydrogen ion. J Am Chem Soc 47(4):1179–1188

Greenhaff PL (2001) The creatine-phosphocreatine system: there's more than one song in its repertoire. J Physiol (Lond) 537(3):657. doi:10.1113/jphysiol.2001.013478

Heuer MA, Maceougall J, Molins M (2009) Patent US 2009/0297685A1 Preparations containing creatine and imino sugars, Pub. Date Dec. 3, 2009

Isidro-Llobet A, Alvarez M, Albericio F (2009) Amino acid-protecting groups. Chem Rev 109:2455–2504. doi:10.1021/cr800323s

Li J, Sha Y (2008) A convenient synthesis of amino acid methyl esters. Molecules 13(5):1111–1119. doi:10.3390/molecules13051111

Lunardi G, Parodi A, Perasso L, Pohvozcheva AV, Scarrone S, Adriano E, Florio T, Gandolfo C, Cupello A, Burov SV, Balestrino M (2006) The creatine transporter mediates the uptake of creatine by brain tissue, but not the uptake of two creatine-derived compounds. Neuroscience 142(4):991–997. doi:10.1016/j.neuroscience.2006.06.058

Nasrallah F, Feki M, Kaabachi N (2010) Creatine and Creatine Deficiency Syndromes: biochemical and clinical aspects. Pediatr Neurol 42(3):163–171. doi:10.1016/j.pediatrneurol.2009.07.015

Negrisoli G, Del Corona L (1999) Hydrosoluble organic salts of creatine, Patent 5,973,199, Date of patent: Oct. 26, 1999

Nudelman A, Bechor Y, Falb E, Fischer B, Wexler BA, Nudelman A (1998) Acetyl chloride-methanol as a convenient reagent for: A) quantitative formation of amine hydrochlorides; B) carboxylate ester formation; C) mild removal of *N*-t-Boc-protective group. Synth Commun 28(3):471–474. doi:10.1080/00397919808005101

Ohtsuki S, Tachikawa M, Takanaga T, Shimizu H, Watanabe TM, Hosoya K, Terasaki T (2002) The blood-brain barrier creatine transporter is a major pathway for supplying creatine to the brain. J Cereb Blood Flow Metab 22(11):1327–1335

Perasso L, Cupello A, Lunardi GL, Principato C, Gandolfo C, Balestrino M (2003) Kinetics of creatine in blood and brain after intraperitoneal injection in the rat. Brain Res 974(1–2):37–42. doi:10.1016/S0006-8993(03)02547-2

Perasso L, Lunardi G, Risso F, Pohvozcheva A, Leko M, Gandolfo C, Florio T, Cupello A, Burov S, Balestrino M (2008) Protective effects of some creatine derivatives in brain tissue anoxia. Neurochem Res 33(5):765–775. doi:10.1007/s11064-007-9492-9

Perasso L, Spallarossa P, Gandolfo C, Ruggeri P, Balestrino M (2013) Therapeutic use of creatine in brain or heart ischemia: available data and future perspectives. Med Res Rev 33(2):336–363. doi:10.1002/med.20255

Peters J, MacDougall J, Chaudhuri S (2007) Creatine-fatty acid, Patent CA 2 577 439 A1, Pub. Date 07.05.2007

Pischel I, Gastner T (2008) Creatine—its chemical synthesis, chemistry, and legal status. In: Salomons GS, Wyss M (eds) Creatine and creatine kinase in health and disease subcellular biochemistry. Springer, Berlin, pp 291–307

Pischel I, Weiss S, Gloxhuber C, Mertschenk B (1999) Creatine ascorbates and method of producing them, Patent 5,863,939, Date of patent Jan. 26, 1999

Powell DA, Ramsden PD, Batey RA (2003) Phase-transfer-catalyzed alkylation of guanidines by alkyl halides under biphasic conditions: a convenient protocol for the synthesis of highly functionalized guanidines. J Org Chem 68(6):2300–2309. doi:10.1021/jo0265535

Robinson S, Roskamp EJ (1997) Solid phase of guanidines. Tetrahedron 53(19):6697–6705. doi:10.1016/S0040-4020(97)00225-1

Ruskin LS (1946) Sulphanilamide derivatives and method of preparing same, Patent US 2,407,686. Sept. 17, 1946

Schulze A (2003) Creatine deficiency syndromes. Mol Cell Biochem 244(1–2):143–150. doi:10.1023/A:1022443503883

Singh S, Dash AK (2009) Creatine Monohydrate. In: Brittain HG (ed) Profiles of drug substances, excipients, and related methodology, vol 34, 1st edn. Elsevier, Oxford, pp 18–19

Smith-Palmer T (2002) Separation methods applicable to urinary creatine and creatinine. J Chromatogr B Analyt Technol Biomed Life Sci 781(1–2):93–106. doi:10.1016/S1570-0232(02)00617-7

Snow RJ, Murphy RM (2001) Creatine and the creatine transporter: a review. Mol Cell Biochem 224(1–2):169–181. doi:10.1023/A:1011908606819

Todoroki Y, Narita K, Muramatsu T, Shimomura H, Ohnishi T, Mizutani M, Ueno K, Hirai N (2011) Synthesis and biological activity of amino acid conjugates of abscisic acid. Bioorg Med Chem 19:1743–1750. doi:10.1016/j.bmc.2011.01.019

Windholz M, Budavari S, Stroumtsos Y, Fertig MN (1976) The Merck index: an encyclopedia of chemicals, drugs, and biologicals, 9th edn. Merck, NJ

Zhu XF, Williams HJ, Scott AI (2000) Aqueous trifluoroacetic acid—an efficient reagent for exclusively cleaving the 5′-end of 3′,5′-TIPDS protected ribonucleosides. Tetrahedron Lett 41(49):9541–9545