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Creatinyl amino acids – new hybrid compounds with neuroprotective activity

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Prolonged oral creatine administration resulted in remarkable neuroprotection in experimental models of brain stroke. However, because of its polar nature creatine has poor ability to penetrate the blood–brain barrier (BBB) without specific creatine transporter (CRT). Thus, synthesis of hydrophobic derivatives capable of crossing the BBB by alternative pathway is of great importance for the treatment of acute and chronic neurological diseases including stroke, traumatic brain injury and hereditary CRT deficiency. Here we describe synthesis of new hybrid compounds – creatinyl amino acids, their neuroprotective activity *in vivo* and stability to degradation in different media. The title compounds were synthesized by guanidinylation of corresponding sarcosyl peptides or direct creatine attachment using isobutyl chloroformate method. Addition of lipophilic counterion (p-toluenesulfonate) ensures efficient creatine dissolution in DMF with simultaneous protection of guanidino group towards intramolecular cyclization. It excludes the application of expensive guanidinylating reagents, permits to simplify synthetic procedure and adapt it to large-scale production. The biological activity of creatinyl amino acids was tested *in vivo* on ischemic stroke and $NaNO_2$-induced hypoxia models. One of the most effective compounds – creatinyl-glycine ethyl ester increases life span of experimental animals more than two times in hypoxia model and has neuroprotective action in brain stroke model when applied both before and after ischemia. These data evidenced that creatinyl amino acids can represent promising candidates for the development of new drugs useful in stroke treatment. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: creatinyl amino acids; sarcosine guanidinylation; creatine p-toluenesulfonate; brain stroke; neuroprotective activity

Introduction

Creatine (α-methylguanidino acetic acid; Cr) plays an essential role in the cellular energy metabolism. It is phosphorylated by creatine kinase, producing phosphocreatine, a high-energy compound that takes part in synthesis of ATP, replenishing the energy stores even in the absence of oxygen and glucose (Figure 1). Cr is synthesized presumably in the kidney, liver and pancreas with subsequent delivery to the energy consuming tissues like muscles and brain. Under normal circumstances its penetration through the blood–brain barrier (BBB) is mediated by specific protein, creatine transporter (CRT), containing 635 amino acids [1].

It was shown that oral Cr administration resulted in remarkable neuroprotection in experimental models of brain stroke, amyotrophic lateral sclerosis, Huntington and Parkinson diseases [2–5]. However because of the limited capacity of CRT and poor ability of polar Cr molecule to penetrate the BBB, neuroprotective effect can be achieved only after prolonged treatment lasting at least several weeks. Thus, synthesis of hydrophobic derivatives capable of crossing the BBB by alternative pathway is of great importance for the therapy of acute and chronic neurological diseases including stroke, traumatic brain injury and hereditary CRT deficiency.

Numerous Cr analogs synthesized so far comprise different homologs, cyclic structures and compounds with modified functional groups (Figure 2). One of the most active and well-known derivatives – cyclocreatine (2-amino-4,5-dihydro-1H-imidazole-1-acetic acid) can serve as a substrate for creatine kinase, suppress cancer cell growth and exhibit neuroprotective activity in case of prolonged administration [3,6]. Vennerstrom and Miller [7] suggested Cr esterification as a convenient method for the prevention of intramolecular cyclization and formation of creatinine (2-amino-3-methyl-4H-imidazol-5-one). Authors postulated better stability of Cr ethyl ester and related derivatives in acidic media and their cleavage by esterases resulted in Cr release; however they did not mention any experimental data confirming these statements. Although Cr esters are less polar as compared with natural molecule and can penetrate the BBB irrespective to CRT, recently it was shown that their enzymatic or non-enzymatic cleavage is accompanied by creatinine formation instead of expected free Cr [8–10].

In this article we describe synthesis of new hybrid compounds – creatinyl amino acids, their neuroprotective activity *in vivo* and stability to degradation in different media.

Materials and Methods

General

All the amino acids and reagents were obtained from Acros Organics (Morris Plains, NJ, USA), Aldrich (Steinheim, Germany), Merk Chemicals (Darmstadt, Germany) and IRIS Biotech (Marktredwitz, Germany). Amberlite IRA-67 was purchased from Sigma (St. Louis, MO, USA). SP Sephadex C-25 was purchased from GE Healthcare Bio-Science AB (Uppsala, Sweden). TLC was performed on

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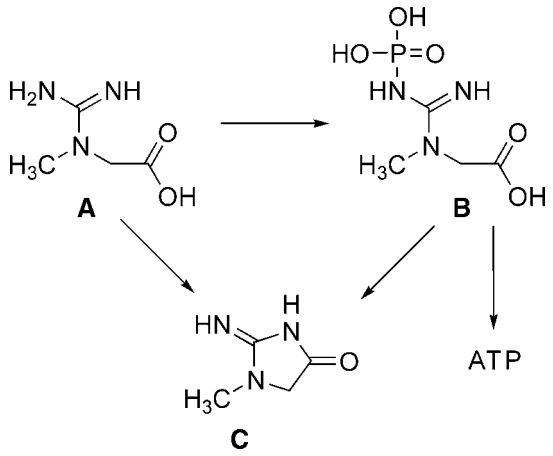

Figure 1. Structures of creatine (A), phosphocreatine (B) and creatinine (C).

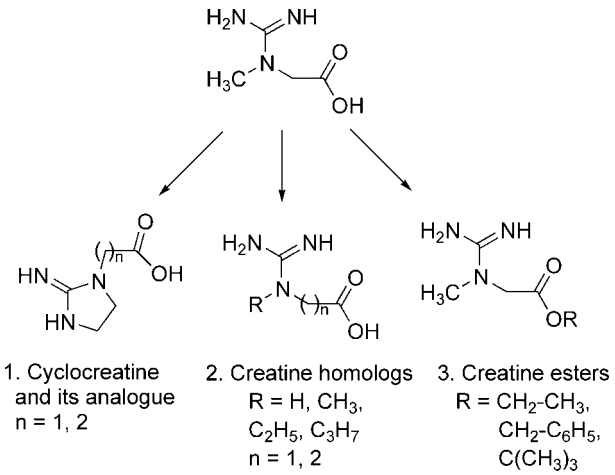

Figure 2. The main classes of creatine analogs, described in the literature.

Merck F 254 and Silufol plates using specified solvent system. For visualization of creatine, creatinyl amino acids and creatinine sodium nitroprusside [11] spray was utilized. Analytical RP-HPLC was carried out using Beckman System Gold with a Phenomenex Luna C_{18} column (4.6 mm × 150 mm, 5 µm) using UV detection at 210 nm, solvent system: A: 0.1% H_3PO_4 in H_2O, B: 0.1% H_3PO_4 in 50% MeCN/H_2O at gradient rate of 1% per min and flow rate of 1 ml/min. Mass spectra were recorded on Finnigan LCQ ion-trap mass spectrometer with an ESI source in the positive mode. Analytical RP-HPLC and electrospray MS confirmed the purity and molecular mass of the synthesized compounds. ^{1}H NMR spectra of final products were recorded at 400 MHz on a Bruker Avance 400 spectrometer. Calibration was carried out with solvent residual signals as internal standards.

Preparation of Creatinyl Amino Acids by Guanidinylation of Sarcosyl Peptides. General Procedure

Protected peptide esters

Boc-Sar-OH (9.5 g, 50 mM) and HOBt (6.75 g, 50 mM) were dissolved in DMF (40 ml) at 0 °C. DCC (10.3 g, 50 mM) solution in DMF (20 ml) was added and the reaction mixture was stirred for 10 min in an ice bath. Then hydrochloride of amino acid ester (45 mM) and triethylamine (6.3 ml, 45 mM) were added. The reaction mixture was stirred overnight at room temperature then filtered and filtrate was diluted with EtOAc (300 ml) and washed successively with 1N H_2SO_4, 5% $NaHCO_3$ and brine. The organic layer was dried over anhydrous $MgSO_4$ and solvent was evaporated in vacuum.

Protected peptide amides

N^{α}-BOC protected peptide ester (20 mM) was dissolved in 6M methanolic ammonia (200 ml), closed tightly and allowed to stay at room temperature till the disappearance of the starting material controlled by TLC (about 48h). Then the solvent was evaporated in vacuum.

Trifluoroacetates of sarcosyl peptides

N^{α}-BOC protected peptide (20 mM) was dissolved in TFA (40 ml) at room temperature and stirred for 15 min. Then TFA was evaporated in vacuum at 25 °C and residue was crystallized from diethyl ether (200 ml).

Creatinyl amino acids and peptides

DIEA (3.48 ml, 20 mM) was added to stirred suspension of sarcosyl peptide trifluoroacetate (10 mM) and benzotriazole-1-carboxamidinium tosylate (10 mM) in DMF (5 ml) at room temperature. The reaction mixture was stirred for 48 or 72 h and then DMF was evaporated in vacuum. The residue was dissolved in 0.001 M pyridine–acetate buffer (50 ml) and loaded on SP Sephadex C-25 column (25 mm × 150 mm) equilibrated with 0.001 M pyridine–acetate buffer. The product was eluted with a linear gradient of 0.001–0.5 M pyridine–acetate buffer containing 20% of isopropyl alcohol. The solvent was evaporated in vacuum and residue was crystallized from MeCN.

Preparation of Creatinyl Amino Acids using Creatine p-Toluenesulfonate. General Procedure

p-Toluenesulfonic acid (10 mM, 1.9 g) was added to a suspension of Cr monohydrate (10 mM, 1.49 g) in DMF (10 ml). The suspension was stirred for 10 min till complete Cr dissolution and then hydrochloride of C-terminally protected amino acid derivative (10 mM) was added. The reaction mixture was cooled to −10 °C and isobutyl chloroformate (1.38 ml, 10 mM) was added followed by NMM (2.2 ml, 20 mM) dropwise addition for 10 min. After stirring for 1 h at −10 °C and 3 h at room temperature NMM hydrochloride was filtered off and filtrate was evaporated in vacuum. The residue was dissolved in water and treated by Amberlite IRA-67 (acetate form) to remove p-toluenesulfonate ions. The water was evaporated in vacuum and residue was crystallized from MeCN (5 ml). The obtained white solid was dissolved in absolute ethanol (5 ml) and allowed to stay for 24 h at −20 °C. The precipitate containing creatine and creatinine was filtered off and filtrate was evaporated in vacuum. The residue was crystallized from diethyl ether.

RP-HPLC Evaluation of Creatinyl Amino Acids Stability in Water Solution and Blood Plasma

Stock solutions of creatinyl amino acids were prepared by dissolving test compounds in bidistilled water at a final concentration 2 mg/ml. All solutions were used immediately upon preparation. An aliquot of fresh stock solution was diluted tenfold, analyzed

by RP-HPLC and then incubated for 3 h at room temperature. The amount of intact creatinyl amino acid was assayed by RP-HPLC.

The stability study in human and rat blood plasma were carried out in analogous way. The assay was initiated upon the addition of test compound stock solution (200 µl) to 1 ml of plasma and the mixture was incubated at 37 °C. 200 µl aliquots of the incubations were taken for the following time points: 0, 60 and 180 min. The aliquots were mixed with 18 µl of 100% TCA and incubated at −24 °C for at least 15 min to precipitate serum proteins. The supernatant was collected for each sample after centrifugation at 6000 *g* for 5 min and analyzed by RP-HPLC. These assays were performed in triplicate.

Biological Activity Studies

The antihypoxic activity of creatinyl amino acids was determined on white outbred male mice (19–25 g body weight; Pavlov Institute of physiology RAS) using hemic hypoxia model [12]. The animals were randomized into treatment groups consisting of 10 mice each. Each group received intraperitoneal (i.p.) injection contained tested compounds (100, 200 and 500 mg/kg) or vehicle (control, 10 ml/kg of physiological solution), 30 min before the experiment. The acute hemic hypoxia was produced by the subcutaneous administration of $NaNO_2$ in a dose of 300 mg/kg. Then the survival time was recorded.

The neuroprotective activity of synthesized creatine derivatives was tested on male Wistar rats (220–240 g body weight; Pavlov Institute of physiology RAS) using ischemic stroke model [13]. Reversible middle cerebral artery occlusion was performed as described by Longa *et al*. [14] and Belayev *et al*. [15]. Tested compounds were dissolved in 1 ml of distilled water and injected i.p. to deliver a dose of 55, 137 or 275 mg/kg 1 h before the initiation of ischemia. Vehicle-treated controls received an equal volume of physiological solution. After 48 h, the animals were killed and the brains examined. Infarct volume was assessed using 2,3,5-triphenyltetrazolium chloride (TTC) (MP Biomedicals, Solon, OH, USA) staining [16]. The brain was removed and cut into 2-mm sections. The slices were placed in a petri dish containing 0.1% TTC for 15 min at 37 °C. Lesion areas were calculated from summed, measured areas (ImageJ software, National Institute of Mental Health, Bethesda, MD, USA) of unstained tissue in mm^2. Data are expressed as mean ± SD.

Statistical analysis of the data was performed using Statistica software (StatSoft Inc., Tucsa, USA). All comparisons between treatment groups and the corresponding vehicle groups were performed using the nonparametric Mann–Whitney *U* test. Differences were considered statistically significant at $P < 0.05$.

Results and Discussion

Conjugation of Cr with amino acid derivatives permits to modify physico-chemical and biological properties of synthesized compounds within wide range and can improve their hydrolytic stability as compared with Cr esters. For the synthesis of new drugs potentially useful in the treatment of acute and chronic neurological diseases such as stroke and traumatic brain injury it is necessary to attach Cr to hydrophobic moiety ensuring its delivery through the BBB irrespective to the presence of CRT. Ideally, preferable amino acid derivatives should be not only safe for the brain cells but also provide additional advantage of synergistic neuroprotective action.

Guanidinylation of Sarcosyl Peptides

Preparation of Cr derivatives with neuroprotective properties encounters several common problems including low Cr solubility in water and organic solvents, its propensity for cyclization resulted in creatinine formation and strong influence of any structural modifications on biological activity. To avoid solubility limitations and exclude the possibility of intramolecular cyclization, in our initial experiments synthesis of creatinyl amino acids was performed by the guanidinylation of sarcosyl peptides [17]. The typical procedure of Cr preparation described in the

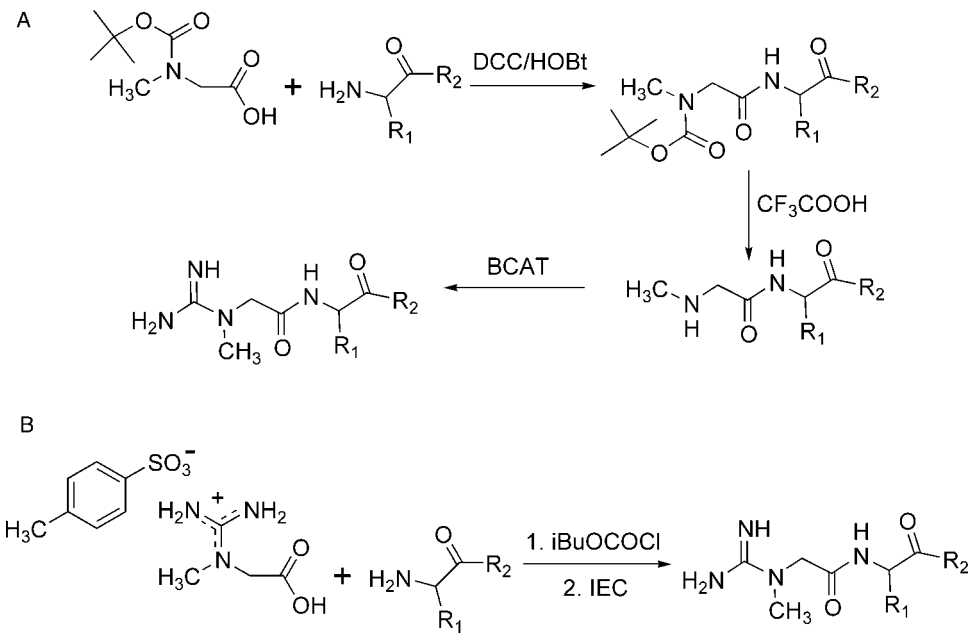

Figure 3. Synthesis of creatinyl amino acids by guanidinylation of sarcosyl peptides (A) or acylation by creatine p-toluenesulfonate (B).

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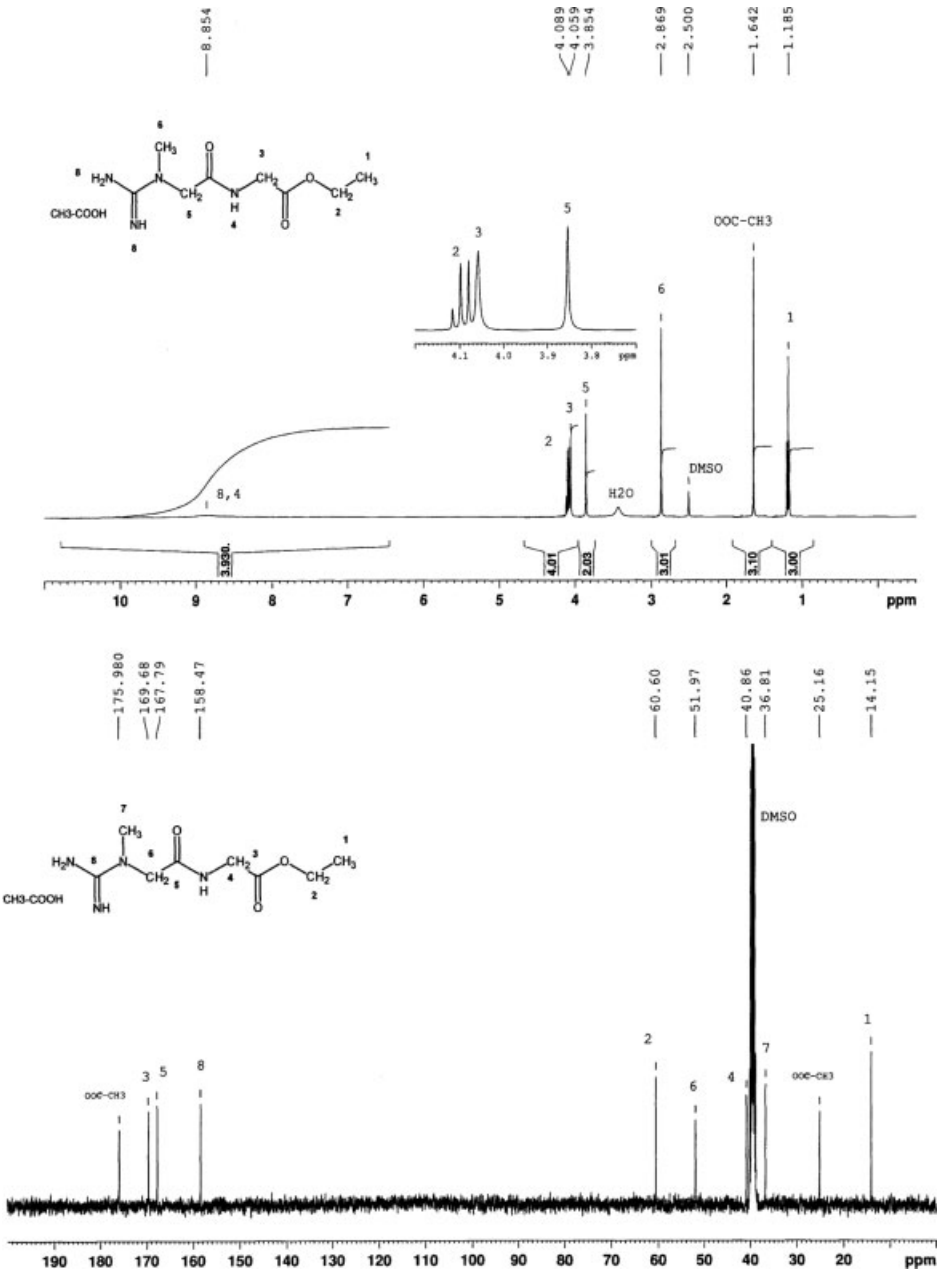

Figure 4. ^{1}H and ^{13}C NMR spectra of Cr-Gly-OEt.

literature comprises sarcosine treatment by cyanamide or S-methylisothiourea in water solution at pH 8.5–9.5 [18,19]. However, our attempts of H-Sar-Gly-OBzl guanidinylation at the same conditions failed because of the instability of final product in water–alkaline solution.

Application of protected guanidinylating reagents such as N,N′-di-tert-butoxycarbonyl-1H-benzotriazole-1-carboxamidine [20] and N,N′-di-benzyloxycarbonyl-1H-benzotriazole-1-carboxamidine in DMF with 1 equiv. of TEA permits to obtain Cr(BOC)$_2$-Gly-OBzl, Cr(BOC)$_2$-Phe-OBzl and Cr(Z)$_2$-Phe-NH$_2$ in a yield of 60–70%. However, BOC group removal using TFA, HCl/dioxane, HCOOH or HCl/Et$_2$O is accompanied with partial decomposition of product. Deprotection by HCl/Et$_2$O seems to be the method of choice, but the yield of desired compounds was only 10–25%. In the case of benzyloxycarbonyl protection, hydrogenolysis over Pd black resulted in Cr-Phe-NH$_2$ formation in a yield of 50%. Nonetheless, this approach to the synthesis of creatinyl amino acids is apparently impractical because of relatively high price of protected guanidinylating reagents and possibility of side reactions at the stage of final deprotection.

Creatinyl amino acids and related derivatives were obtained by guanidinylation of peptide esters, amides or substituted amides using benzotriazole-1-carboxamidinium tosylate [21] (Figure 3A). Synthesized compounds were converted to acetate salts by cation exchange chromatography on SP Sephadex C-25 and crystallized from acetonitrile. To elucidate the influence of attached amino acid on physico-chemical properties and stability of Cr derivatives, we prepared a set of analogs, shown in Table 1. The purity of synthesized compounds was determined by ^{1}H NMR spectroscopy, analytical RP-HPLC and ESI MS (Figure 4; Table 1).

Table 1. List of creatinyl amino acids and peptides

Cr-containing compounds	Yield[a] (%)	ESI MS MW	ESI MS $[M+H]^+$
1a Cr-Gly-OEt	22(1), 25(2)	216.12	217.15
1b Cr-Gly-OH	12(1)	188.09	189.11
1c Cr-Gly-NHEt	29(1), 18(2)[b]	215.14	216.16
1d Cr-Gly-OBzl	21(1)	278.14	279.14
1e Cr-Gly-OiPr	20(2)	230.14	231.15
2a Cr-Phe-OEt	22(1)	306.17	307.18
2b Cr-Phe-NH_2	46(1), 10(2)[b]	277.15	278.17
2c Cr-Phe-OH	20(1)	278.14	279.15
3a Cr-Tyr-NH_2	38(1)	293.15	294.16
3b Cr-Tyr-OH	16(1)	294.13	295.15
4 Cr-GABA-OEt	29(1)	244.15	245.18
5 H-Lys(Cr)-OEt	26(1), 6(2)[b]	287.20	288.16
6 Cr-Gly-Gly-OEt	15(2)	273.14	274.16
7 Cr-Glu-Arg-OEt	24(1)[c]	444.24	445.26
8 Cr-Phe-Arg-Gly-OEt	37(1)[c]	519.29	520.33

[a] Total yield of products, obtained using different synthetic approaches: (1) guanidinylation of sarcosyl peptides; (2) acylation by Cr p-toluensulfonate.
[b] Significant loss of product at the stage of crystallization.
[c] Yields were calculated starting from sarcosyl peptides.

According to HPLC analysis extent of sarcosyl peptide conversion was about 90–100%, however the yield of purified product was only 40–60% because of its partial loss in the course of IEC and subsequent crystallization. It should be mentioned that some creatinyl amino acids can be precipitated in pure form as free bases by the addition of n-butyl alcohol to the reaction mixture but, in contrast to acetate salts, their solubility in water is very low.

Acylation of Amino Acids by Creatine p-Toluenesulfonate

Considering that preparation of creatinyl amino acids by guanidinylation of sarcosyl peptides comprises three stages the opportunity to minimize the number of synthetic steps and reagents requirements seems to be very attractive. To simplify the procedure we investigated the possibility of direct amino group acylation by anhydrous Cr or Cr hydrate (Figure 3B). The problem of low Cr solubility in organic solvents can be overcome by its conversion to an appropriate salt form. It is known that solubility of peptides and proteins, in organic solvents can be improved using p-toluenesulfonate as hydrophobic counterion [22]. In our experience, addition of p-TSA represents simple and convenient procedure for dissolution of Cr and other different guanidino or amidino compounds in DMF and related solvents.

The second obstacle to be overcome is high propensity of Cr to cyclization in acidic media or in the course of carboxylic group activation. To minimize side products formation and simplify the purification of creatinyl amino acids, we investigated different coupling methods including DCC, active esters and isobutyl chloroformate. In all experiments application of p-toluenesulfonate counterion ensures Cr solubility and significant suppression of intramolecular cyclization. As a model compound to optimize the reaction conditions we have chosen Cr-Gly-OEt. While our attempts to prepare Cr active esters failed, DCC condensation resulted in the formation of desired product. The completeness of the coupling reaction was determined by RP-HPLC. The influence of reagents ratio on the yield of Cr-Gly-OEt is summarized in Table 2. Although an excess amount of Cr p-toluenesulfonate increases the yield of final product, the purification procedure becomes difficult because of side products formation and the presence of starting material in the reaction mixture. The mixed anhydride method was most advantageous in terms of reaction completeness and simplicity of subsequent purification. Better results were obtained by using 1 equiv. of Cr hydrate instead of anhydrous Cr and dropwise addition of NMM to the reaction mixture.

Table 2. Influence of reagents ratio on the yield of Cr-Gly-OEt

Cr	p-TSA	DCC	DIEA	HCl*H-Gly-OEt	Yield (%)[a]
1	1	1	1	1	11
1	1	1	2	2	13
2	2	2	1	1	45
2	2	1	1	1	44
2	2	1	2	2	26

[a] Quantified by RP-HPLC at 220 nm.

Purification of synthesized creatinyl amino acids primarily comprises removal of p-toluenesulfonic acid, Cr and creatinine. Our preliminary experiments revealed that application of cation exchange chromatography on Rexyn 101(H), SP Sephadex C-25 or Toyopearl SP-650M in pyridine–acetate buffer permitted to obtain Cr-Gly-OEt in acetate form, but the purity was low. Moreover, the procedure requires the use of large buffer volumes. To simplify the treatment of crude product we investigated utility of anion-exchange resin (IRA-67) at the stage of p-toluenesulfonic acid removal. Application of IRA-67 in free-base form resulted in significant decomposition of Cr-Gly-OEt accompanied by creatinine formation, while resin in acetate form does not influence the product stability.

In attempts to simplify the procedure and exclude the stage of ion-exchange chromatography we investigated utility of different organic and inorganic acids for Cr solubilization and its protection from intramolecular cyclization. It was found that addition of 1 Equiv. of TFA or HCl, but not CH_3COOH and H_3PO_4 ensures complete Cr dissolution in DMF. While trifluoroacetate is not pharmaceutically acceptable salt, it can be easily converted to hydrochloride using well-known techniques [23]. However our attempts of Cr-Gly-OEt purification by the crystallization in the form of TFA or HCl salt failed. In addition, application of Cr hydrochloride decreased the yield of final product by 30–40% as compared with Cr p-toluenesulfonate. Thus, in our hands application of p-toluenesulfonic acid for Cr solubilization was the method of choice both in terms of yield and purity of crude product.

Efficient Cr and creatinine removal is the most problematic stage of creatinyl amino acids purification. In the case of Cr-Gly-OEt simple recrystallization from water seems to be impossible because of its high solubility. Commonly, the crude product obtained after ion-exchange chromatography contains about 5–10% of creatinine. Its content can be reduced up to 3–5% by crystallization from MeCN, followed by the dissolution of Cr-Gly-OEt in ethyl alcohol and removal of precipitated creatinine. This protocol afforded the desired product in a yield of 20–25%. However, in the cases of hydrophobic creatinyl amino acid derivatives purification procedure should be modified in order to ensure the maximum recovery.

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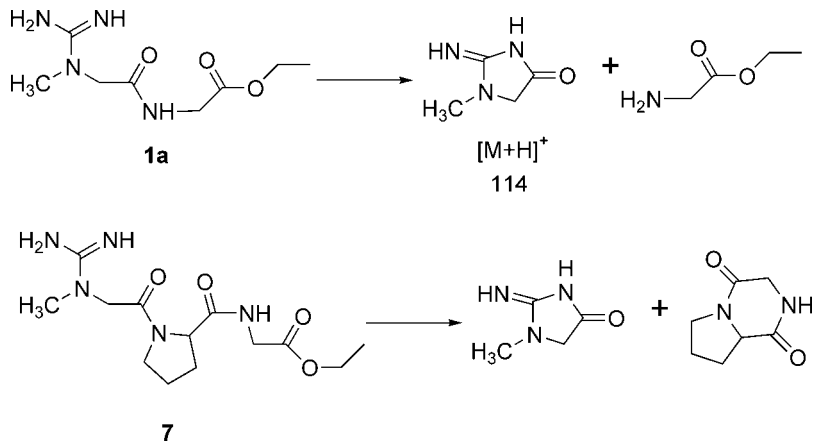

Figure 5. Suggested mechanism of creatinyl amino acids degradation.

Stability of Creatinyl Amino Acids in Water Solutions and Blood Plasma

Chemical stability of synthesized compounds in aqueous solutions and their susceptibility to enzymatic degradation in blood plasma was investigated by means of RP-HPLC and ESI MS. It was shown that stability of Cr derivatives is strongly dependent on the structure of adjacent amino acid residue, the kind of counterion and pH of solution. Creatinyl-glycine esters seem to be less stable as compared with other analogs and can undergo partial decomposition in the course of RP-HPLC purification. These data can be explained by the assumption that the degradation mechanism comprises intramolecular cyclization resulted in cleavage of amide bond and creatinine formation (Figure 5). Creatinine can be easily identified in water solution by means of HPLC and by the intensive signal in the mass spectrum (*m/z* 113). In contrast to creatinyl-glycine esters, phenylalanine derivatives containing bulky benzyl side chain are relatively stable irrespectively to the presence of counterion. The less stable of synthesized Cr derivative is Cr-Pro-Gly-OEt which easily undergoes decomposition both in water and organic media resulted in creatinine and Pro-Gly diketopiperazine formation. However, it should be mentioned that surprisingly high stability of Cr-GABA-OEt remains unclear.

On the basis of these preliminary results we studied resistance of glycine and phenylalanine derivatives to hydrolysis in water solution and enzymatic cleavage in blood plasma. It was found that in distilled water both Cr-Gly-OBzl and Cr-Phe-NH_2 are completely stable during 3 h at room temperature, while Cr-OBzl at the same conditions undergoes degradation (up to 60%). In agreement with the literature data, the main decomposition product was identified as creatinine using RP-HPLC and ESI MS technique. In human and rat blood plasma Cr-Gly-OBzl was stable during at least 3 h at 37 °C, whereas Cr-Phe-NH_2 concentration decreased up to 85%. These data suggest that creatinyl amino acids can reach the target cells without significant decomposition and function as intact molecules or undergo enzymatic cleavage near the sites of their action.

Neuroprotective Activity of Creatinyl Amino Acids

Conjugation of Cr with different amino acid derivatives improves its solubility in water and potentially can facilitate its penetration via cell membrane and BBB. Considering neuroprotective activity of Cr molecule one can expect similar biological properties of creatinyl amino acids in the case of their action as substrates of creatine kinase or ability to release free creatine as a result of enzymatic degradation. It should be mentioned that in spite of numerous biological experiments with free Cr, the exact mechanism of its protective action is still unclear. Recent studies have shown that in brain stroke model Cr-mediated protection can occur independent of changes in the bioenergetic status of neurons and involve improved cerebrovascular function [24].

The neuroprotective activity of creatinyl amino acids was tested *in vivo* using two different models: $NaNO_2$-induced hypoxia (mice) and ischemic stroke (rats). In the first case, sodium nitrite injection reduces oxygen-carrying capacity of hemoglobin because of its conversion to methemoglobin and induces severe vasodilatation resulted in cerebral hypoxia. All tested compounds were administered intraperitoneally at doses of 100–500 mg/kg. It was shown that efficacy of antihypoxic action depends on the structure of amino acid moiety. Thus, Cr-Phe-NH_2 and Cr-GABA-OEt possessed moderate activity, while Cr-Tyr-NH_2 and Cr-Gly-OEt increase life span of experimental animals about two times (Figure 6).

Similar results were obtained using ischemic brain injury model. The efficiency of neuroprotective action was estimated as the ratio of damaged tissue area to the total area of brain slice (Figure 7). I.p. administration of Cr-GABA-OEt (1 mmol/kg) and Cr-Phe-NH_2 reduces the size of necrotic zone by 25% in contrast to more than 50% in the case of intravenous Cr-Gly-OEt injection 1 h before or after ischemia. These data evidenced that creatinyl amino acids possess pronounced neuroprotective properties. The mechanisms of their biological action and potential synergistic effect of attached amino acid residue are under investigation.

Conclusions

Conjugation of Cr with amino acid derivatives permits to alter physico-chemical properties of the molecule in wide range. These conjugates can be synthesized in reasonable yield by the guanidinylation of sarcosyl peptides or direct acylation of amino acids using Cr p-toluenesulfonate. The structure of attached amino acid derivative determines stability of synthesized compound in water solution and their susceptibility to enzymatic degradation in blood plasma. Synthesized creatinyl amino acids possess neuroprotective action *in vivo* and can represent promising

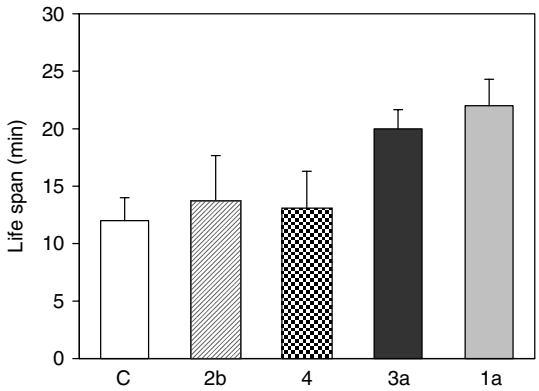

Figure 6. The influence of creatinyl amino acids on the life span of experimental animals in hypoxia model. C, control; **1a**, Cr-Gly-OEt; **2b**, Cr-Phe-NH_2; **3a**, Cr-Tyr-NH_2; 4, Cr-GABA-OEt.

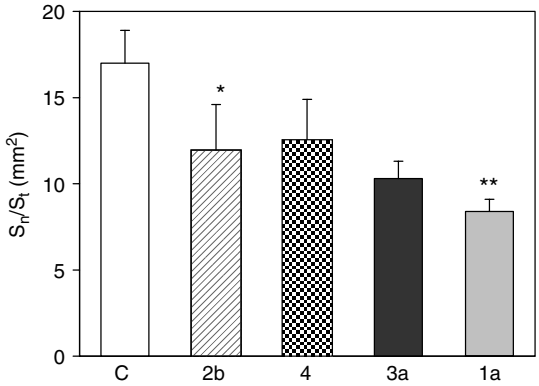

Figure 7. The ratio of necrosis area to total brain area after 48 h of creatinyl amino acid injection. C, control; **1a**, Cr-Gly-OEt; **2b**, Cr-Phe-NH_2; **3a**, Cr-Tyr-NH_2; 4, Cr-GABA-OEt. *Compound **2b** was administered at well-tolerated dose (0.5 mmol/kg). **I.v. injection (i.p. administration of compound **1a** produce similar effect).

candidates for the development of new drugs useful in stroke treatment.

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