Evaluation of the Impact of Proline on the Folding of α-Conotoxins

KUI 15/2004
Received January 13, 2004
Accepted April 22, 2004

A. K. Croskey and B. Hargittai

Department of Chemistry, Mathematics and Physical Sciences, Saint Francis University, Loretto, Pennsylvania 15940, USA

Evaluation of the role of disulfide bridges is essential in our understanding of protein folding. Our research group is interested in exploring how slight changes in the sequences of small peptides affect their folding properties. The compounds under consideration were multiple disulfide bridge-containing peptides belonging to the class of α-conotoxins, α-conotoxins GI, GII (found in *Conus Geographus*), SI, SIA (found in *Conus Striatus*), and MI (found in *Conus Magus*), and their analogs. These are all thirteen or fourteen amino acid-containing peptide amides having four cysteine residues. The four cysteine residues can form two disulfide bridges, leading to three possible regioisomers. The amino acid in position 9 of these peptides plays an important role in the biological characteristics of these compounds. In our studies we have investigated the effects of different amino acids, primarily proline, in this position on the folding of the peptides. Our results indicate that proline's cyclic nature may impose steric constraints for the folding of α-conotoxins.

Keywords: *α-Conotoxins, folding, disulfide-paired regioisomers*

Introduction

Part of the challenge of understanding the folding of peptides and proteins is to evaluate the effects of intramolecular disulfide bridges between two cysteine amino acids in small disulfide rich peptides.[1] A number of naturally occurring neurotoxic peptides utilize two or more disulfide bridges to achieve a compact, highly stable conformation. This structure is filled out with amino acid residues at particular positions to generate a given biological function for the peptide.[2] Folding is a self-assembly process, which occurs spontaneously and most likely proceeds by the formation of secondary structures first. The structures are held together by hydrogen bonds, along with more random structural elements that result from hydrophobic interactions. This final folding arrangement can be stabilized by disulfide bridges between cysteine residues at different points in the peptide/protein chain.[1] Conotoxins, isolated from cone snail venom, represent a large class of peptides having a unique ability to differentiate between various types of ion channels. They are small, highly stable peptides with multiple disulfide bridges, and have the potential of being ideal leads for peptide therapeutics due to their high specificity.[3]

All conotoxins reported in the literature contain multiple disulfide bridges. It has been shown that most reduced forms of native conotoxins are capable of folding predominantly to the natural, bioactive, isomers.[3] Under certain conditions, however, folding yields this natural isomer in a mixture of the other possible, "mispaired", isomers.[4–6] These non-native isomers are different from the natural one and from each other only in the orientation of the disulfide bridges (Fig. 1). Prior studies have indicated that the folding of these peptides is defined mainly by the placement of the cysteine residues.[5] The sequences also contain a variety of residues that influence the biological activity or selectivity of the peptides rather than the folding characteristics.[5] To fully understand the effects on folding of specific amino acid residues in different positions in the peptide chain, we set out to study a number of α-conotoxins, peptides that contain two disulfide bridges, exhibiting small, yet significant differences in their sequences.[7,8]

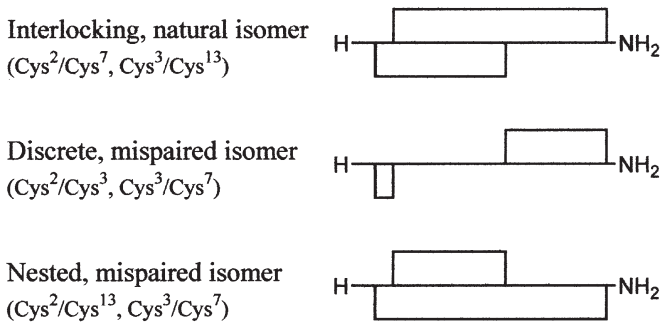

Fig. 1 – *α-Conotoxin SI disulfide regioisomer nomenclature*

Materials and methods

Most of the materials, solvents, instrumentation, and general methods relating to the subject have been described and summarized in our previous publications.[6,9–11] Most protected Fmoc-amino acids were purchased from Advanced ChemTech (Louisville, Kentucky), Bachem Bioscience (Philadelphia, Pennsylvania) or Chem-Impex International (Wood Dale, Illinois). Fmoc-PAL-PEG-PS resin was from PerSeptive Biosystems GmbH (Hamburg, Germany). *N*,*N*-dimethylformamide (DMF) was purchased from Aldrich

* Correspondence: Dr. Balazs Hargittai, Department of Chemistry, Mathematics and Physical Sciences, Saint Francis University, 117 Evergreen Drive, Loretto, PA 15940, U.S.A.; Phone: (814) 472-2814; Fax: (814) 472-2773; E-mail: bhargittai@francis.edu

Chemicals (Milwaukee, Wisconsin), and dried over 0.4 nm (4 Å) molecular sieves for 24 h prior use. Other chemicals, including CH_2Cl_2, piperidine, trifluoroacetic acid (TFA), *N*,*N*′-diisopropylcarbodiimide (DIPCDI) and 1-hydroxybenzotriazole (HOBt), were also purchased from Aldrich Chemicals and used without purification.

Manual solid-phase peptide synthesis was carried out in sterile plastic syringes (6 or 12 mL volume), containing porous polypropylene frits.[6] Peptides were synthesized on PEG-PS supports (0.3 g each synthesis), using base-labile 9-fluorenylmethyloxycarbonyl (Fmoc) N^{α}-amino protection, acid-labile trityl (Trt) or metal ion-labile acetamidomethyl (Acm) protecting groups for cysteine, and acidolyzable *tert*-butyl (*t*-Bu) and trityl type side-chain protecting groups [*t*-Bu ether for Ser and Tyr, O*t*-Bu ester for Glu, *tert*-butyloxycarbonyl (Boc) urethane for Lys, Trt for Asn and His, and the 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) group for Arg], and the acidolyzable tris(alkoxy)benzylamide (PAL) anchoring linkage (0.16 – 0.20 mmol g^{-1} loading). N^{α}-Fmoc groups were removed by treatment with piperidine–DMF (1 : 4, 8 + 3 min), followed by washes with DMF (5 x 1 min). Fmoc-amino acids (4 equiv) were incorporated by 1 h couplings mediated by DIPCDI–HOBt (4 equiv each) in DMF. Peptide chains were removed from the solid support (90–100 mg per experiment for orthogonal syntheses of disulfide regioisomer standards; 45–50 mg per experiment for folding oxidation studies) with concomitant removal of all acid-labile side-chain protecting groups, prior to any orthogonal disulfide bridge formation, by treatment with Reagent K, TFA–phenol–H_2O–thioanisole–1,2-ethanedithiol (82.5 : 5 : 5 : 5 : 2.5), at 25 °C for 90 min.[12] The resultant bis(thiol) or tetra(thiol) intermediates were precipitated with ether, and used directly for the first oxidation step in solution.

The various peptide mixtures were characterized by HPLC injections and the relevant regioisomers were verified by HPLC co-injections with standards of each correct isomer, synthesized in parallel experiments. Analytical HPLC was performed using a Waters Symmetry analytical C-18 reverse phased column (5 μ particle diameter; 0.46 × 15.0 cm) on a Waters system configured with Waters 1525 binary pumps and a Waters 2487 dual wavelength absorbance detector controlled from a Pandya computer with Waters Breeze Software. Peptide samples were chromatographed at 1.2 mL min^{-1} using 0.1 % aqueous TFA – 0.1 % TFA in CH_3CN (1:0 to 11:9 over 20 min), detection at 220 nm.

Formation of first disulfide bridge from free bis(thiol) precursor (orthogonal syntheses)

The crude bis(thiol), bis(Acm) peptides were dissolved in 0.01 mol L^{-1} Na_2HPO_4 buffer, pH 7.5 (3.0 mL), at a final concentration of 2.5 μmol L^{-1}, and DMSO [30 μL, φ = 1 %] was added.[6,13,14] After continuous and vigorous magnetic stirring at 25 °C for 18 h, the oxidation mixtures were lyophilized to provide the monocyclic peptides.

Formation of second disulfide bridge from protected thiol precursors (orthogonal syntheses)

The monocyclic, bis(Acm)-intermediates were dissolved in TFA (3 mL) followed by addition of DMSO (3 μL, 10 equiv), Me_3SiCl (60 μL, 150 equiv), and anisole (40 μL, 100 equiv).[6,15–17] Reactions proceeded for 30 min, and were stopped by addition of Bu_4NF (1.0 mol L^{-1} solution in THF, 1.2 mL, 300 equiv). Crude products were precipitated with a large excess of dry diethyl ether (45 mL), dissolved in φ = 1 % aq. HOAc (10 mL), and lyophilized to give the crude bicyclic peptides.

Formation of disulfide bridge under folding conditions (oxidation studies)

The crude tetra(thiol) peptides were dissolved in 0.01 mol l^{-1} Na_2HPO_4 buffer, pH 7.5 (1.5 mL), at a final concentration of 2.5 μmol l^{-1}, and DMSO [15 μL, φ = 1 %] was added.[6] After continuous and vigorous magnetic stirring at 25 °C for 15 h, the oxidation mixtures were lyophilized to provide the bicyclic peptides.

Formation of disulfide bridge under denaturing conditions (oxidation studies)

The crude tetra(thiol) peptides were dissolved in 0.01 mol L^{-1} Na_2HPO_4 buffer, pH 7.5 (1.5 mL), at a final concentration of 2.5 μmol L^{-1}, and DMSO [15 μL, φ = 1 %] and guanidine hydrochloride (0.860 g, 6 moL l^{-1}) was added.[5,6] After continuous and vigorous magnetic stirring at 25 °C for 15 h, the oxidation mixtures were lyophilized to provide the bicyclic peptides.

Results and discussion

Disulfide bridges represent important evolutionarily conserved structural motifs in many biologically important peptides and proteins.[1] Intramolecular disulfide bridges covalently cross-link portions of the peptide chain, that might be far apart in the linear sequence. Formation of disulfide bridges does not appear to be the stimulus behind protein folding, but has an important role in stabilizing bioactive conformations.[18]

The overall plan for this work followed earlier precedents.[5,6,9,19] Standards of the three regioisomers of the peptide were synthesized by forming the large disulfide loop first (Cys in position 13 protected by Trt), and the small loop second. Reagent K cleavage gave the bis(thiol), bis(Acm)-intermediate, which was oxidized further to the monocyclic bis(Acm)-peptide using solution DMSO oxidation at pH 7.5. The monocyclic intermediates were formed using Me_3SiCl (150 equiv), DMSO (10 equiv), and anisole (100 equiv) in TFA at 25 °C for 30 min. Tetra(thiol) precursor peptides in the folding studies were oxidized either in the absence (folding conditions) or in the presence (denaturing conditions) of guanidine hydrochloride using DMSO oxidation at pH 7.5.

Earlier work on the folding of synthetic α-conotoxin SI indicated that the presence of a proline residue in a strategic position gives the expected natural isomer with good selectivity, regardless of the reaction conditions.[6] Studies with α-conotoxins GI, containing a basic amino acid (arginine) in the same position as the proline in SI, gave a mixture of all three possible disulfide-paired isomers under conditions that did not promote folding of the peptide

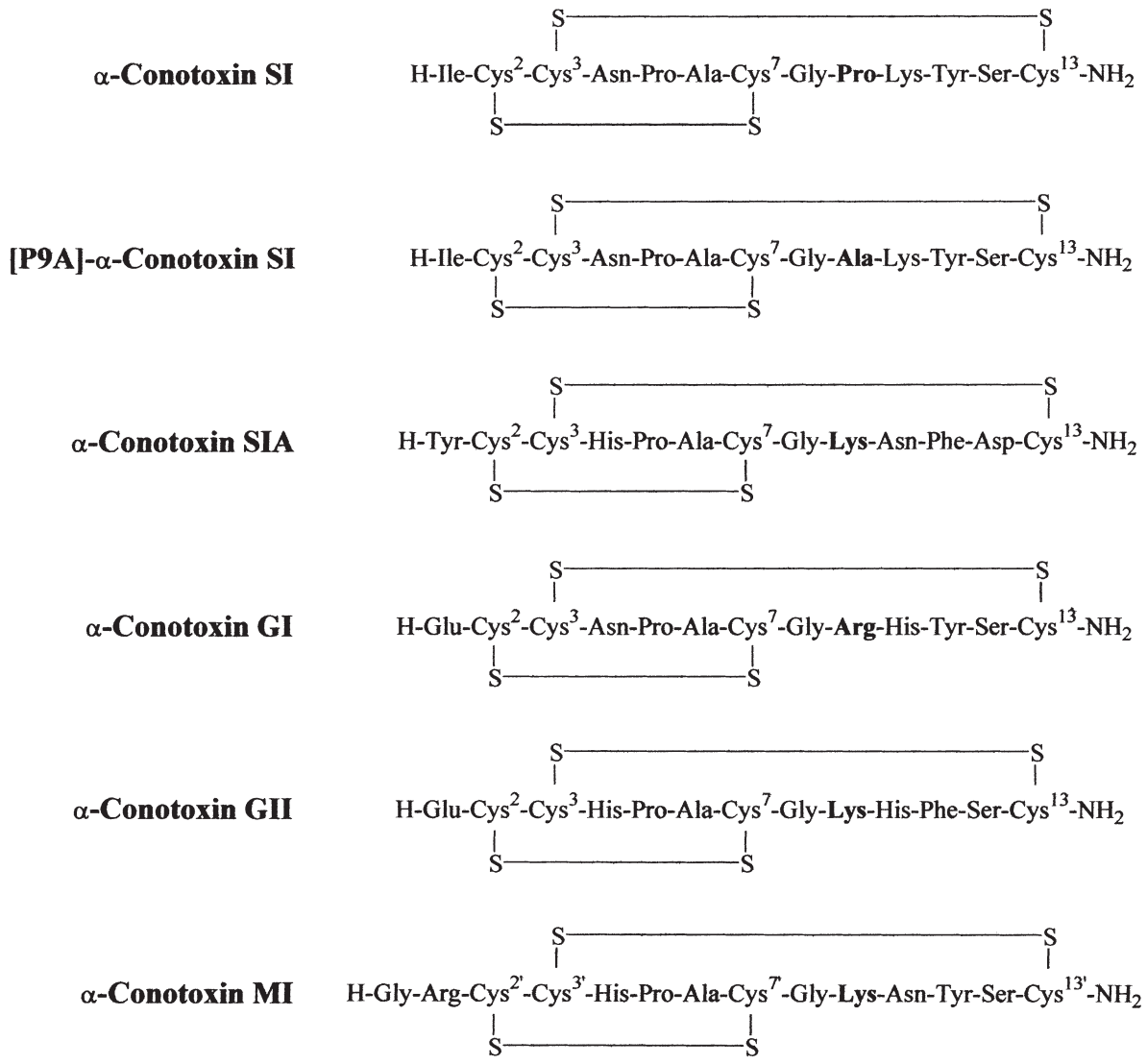

Fig. 2 – *Sequences of peptides discussed*

chain.[5] In this study, we compared these results to those of α-conotoxins SIA, GII and MI (all containing lysine in position 9), along with an analogue of α-conotoxin SI, [P9A]-α-conotoxin SI, where an alanine residue replaces the proline in position 9 of α-conotoxin SI (Fig. 2).

Under highly optimized conditions random oxidation can be successful for the formation of the major natural isomer, since the reduced polypeptide chain might fold into the native-like conformation to favor the proper alignment of the disulfide bridges.[11] Studies using folding conditions (see Materials and Methods for details) demonstrated that all α-conotoxins studied had a preference for one specific orientation, the natural "interlocking" isomer (Table 1). It is interesting to note that in all cases the "nested" mispaired isomer was the major by-product – in the case of α-conotoxin GI a quite significant one.[5]

Application of denaturing conditions (see Materials and Methods for details) yields completely different results (Table 1). While α-Conotoxin SI gave similar results to the case when folding conditions are applied, the rest of the α-conotoxins, including [P9A]-α-conotoxin SI, showed greatly reduced selectivity for the formation of the natural isomer (Fig. 3). In the case of [P9A]-α-conotoxin SI, the only change in the amino acid sequence from α-conotoxin SI was the absence of the proline residue from position 9, indicating that this amino acid indeed plays an important role in the folding of these short compact peptides. The natural "interlocking" isomer was the major product, but not the predominant one. In these experiments the "nested" mispaired isomer was not as dominant a by-product as in the folding cases.

It is also interesting to compare the results from non-orthogonal and orthogonal syntheses studies done on α-conotoxin SI and α-conotoxin SIA.[6,19] During non-orthogonal syntheses of α-conotoxin SIA, the natural isomer was the major product, with the nested isomer being the major by-product. On the other hand, during orthogonal syntheses the best results were obtained when the protection scheme was oriented towards the synthesis of the discrete isomer. Somewhat similar results were seen with SI, but the differences were less striking.

It is noteworthy that in the amino acid sequence of these analogues there are conspicuous differences that correspond to their selectivity during oxidation. As mentioned

Table 1 – *Relative yields observed for the syntheses of α-conotoxins*

	Cys^2/Cys^7, Cys^3/Cys^{13} natural isomer	Cys^2/Cys^3, Cys^7/Cys^{13} mispaired isomer	Cys^2/Cys^{13}, Cys^3/Cys^7 mispaired isomer
		Folding solvent[a]	
α-Conotoxin SI[8]	88	3	9
[P9A]-α-Conotoxin SI	79	8	13
α-Conotoxin SIA	93	2	5
α-Conotoxin GI[7]	71	8	21
α-Conotoxin GII	77	1	22
α-Conotoxin MI	85	9	6
		Denaturing solvent[a]	
α-Conotoxin SI[8]	82	6	12
[P9A]-α-Conotoxin SI	57	24	19
α-Conotoxin SIA	51	23	26
α-Conotoxin GI[7]	47	31	22
α-Conotoxin GII	49	22	29
α-Conotoxin MI	52	28	20

[a]: For experimental conditions see Materials and Methods section.

before, α-conotoxin SI has a hydrophobic cyclic residue, Pro, in position 9, while the rest of the peptides in the study have basic amino acids (Lys in α-conotoxin SIA, GII, MI and Arg in α-conotoxin GI) in this position (Fig. 2). According to the biological activities reported about these compounds, and studies done on sequence effects in these peptides, this position is significant in determining the biological activity of these compounds.[20] Proline, due to its cyclic nature is limited by steric constraints and therefore has a strong structural effect, orienting the *C*-terminal of the peptide chain in one specific direction and limiting the possible structures. The absence of proline's structural limitations in the sequences of, e.g., α-conotoxin SIA and α-conotoxin GI, can give rise to a higher degree of scram-

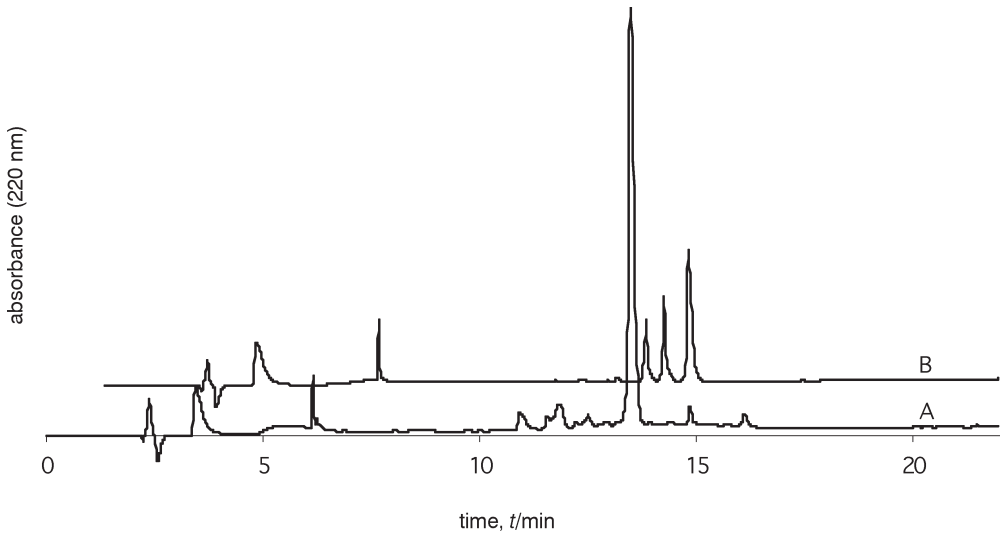

Fig. 3 – *HPLC analyses of α-conotoxin SIA.* [*A*]*: Folding oxidizing conditions;* [*B*]*: Denaturing oxidizing conditions.* t_R *(Cys^2/Cys^{13}, Cys^3/Cys^7 "nested" mispaired isomer): 12.3 min;* t_R *(Cys^2/Cys^3, Cys^7/Cys^{13} "discreet" mispaired isomer): 12.8 min;* t_R *(Cys^2/Cys^7, Cys^3/Cys^{13} "interlocking" natural isomer): 13.5 min.*

bling of disulfide bridges, especially in non-folding/denaturing conditions.

Studies done on the solution structure of α-conotoxin SI[21] have shown that the lowest energy structure of this peptide is stabilized by six hydrogen bonds. The most significant of these are the ones connecting the side-chain nitrogen of lysine in position 10 to its main chain oxygen, and to the main chain oxygen of cysteine in position 2. This arrangement has the effect of burying this side-chain in the core of the molecule. Peptides like α-conotoxin GI and SIA do not have a residue in this position that is able to make such hydrogen bonds.[22,23] Burying the lysine side-chain in the core of the molecule separates the termini of α-conotoxin SI more than the termini of the other two conotoxins.

The topology of α-conotoxin SI is different from those of α-conotoxins SIA and GI due to the hydrogen bonds of the side-chain of lysine in position 10, and to the presence of a proline in a position where α-conotoxins SIA and GI have basic amino acid residues. This has an important effect on the biological activity, selectivity, and binding to the nicotinic acetylcholine receptors. The separation of the two chain termini causes an overall expansion of the α-conotoxin SI molecule, and leads to a slightly altered alignment of the side-chains.[21] This difference causes α-conotoxin SI to have a considerably different surface topology and electrostatics for binding to nAChRs compared to the other α-conotoxins discussed in this study.

Earlier studies[24] showed that the side-chain of the amino acid in position 9 has a significant interaction with the receptor binding site and that the addition of a positive charge in this position has a highly important role in determining binding selectivity. It appears that size, charge, and conformational restrictions imposed by side-chain geometries, and hydrogen bonds formed by the residues in positions 9 and 10, are all significant for the affinities and relative selectivities of the different α-conotoxins for various types of nAChRs binding sites.[21]

ACKNOWLEDGEMENTS

The authors would like to thank Drs. Stewart N. Abramson, Ioana Annis, George Barany, Robert W. Janes and Ms. Sarah A. Rebar for helpful discussions and Dr. Jonathan Southard (Indiana University of Pennsylvania) for assistance in the purification of the peptides.

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SAŽETAK

Procjena utjecaja disulfidnih veza na sekundarnu strukturu peptida

A. K. Croskey i B. Hargittai

U našem smo laboratoriju istraživali ulogu disulfidnih mostova u svijanju proteina. Osim toga, istraživali smo kako male promjene u slijedu aminokiselina u peptidima utječu na njihova svojstva svijanja. Peptidi našeg interesa su α-conotoxini s mnogobrojnim disulfidnim mostovima, α-conotoxini GI, GII (*Conus Geographus*), SI, SIA (*Conus Striatus*), i MI (*Conus Magus*) i analozi spomenutih peptida. Ti su peptidi trinaest ili četrnaest aminokiselina dugi peptidni amidi koji sadrže četiri cisteinske aminokiseline. Te četiri cisteinske kiseline u peptidima sposobne su formirati dva ili tri disulfidna mosta koja su odgovorna za formiranje tri moguća regioizomera. Aminokiselina na mjestu 9 u tim peptidima igra vrlo važnu ulogu u biološkim karakteristikama peptida. U našem radu je istraživan utjecaj različitih aminokiselina, posebno prolina, na mjestu 9 u slijedu aminokiselina na svijanje peptida. Rezultati pokazuju da struktura prolina u obliku prstena vjerojatno uzrokuje steričku smetnju za svijanje peptida.

Odjel za kemiju, matematičke i fizikalne znanosti,
Sveučilište Saint Francis, Loretto, Penssylvania 15940, SAD

Prispjelo 13. siječnja 2004.
Prihvaćeno 22. travnja 2004.