Conotoxins: Disulfide-rich Small Peptides

KUI 17/2004
Received January 13, 2004
Accepted April 22, 2004

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Various naturally occurring peptide toxins and antibiotics contain one or more rings, often through disulfide bridges. α-Conotoxins are a family of small disulfide-rich peptides that contain thirteen or fourteen amino acids and have two disulfide bridges, leading to three possible regioisomers. Most reduced forms of native conotoxins are able to fold forming the isomer found in nature. However, under certain conditions the oxidation of these peptides will yield a mixture of all three isomers. This paper gives an overview of the family of conotoxin peptides, including their synthesis and the contributions that I have made to the field. We have successfully synthesized all three regioisomers of α-conotoxins SI (a thirteen amino acid-containing peptide amide having four cysteine residues, found in *Conus Striatus*), along with the four lactam analogues of the natural isomer. We have also looked at how slight changes in the sequence of these small peptides could influence their folding properties.

Keywords: *α-Conotoxin SI, disulfide bridges, orthogonal synthesis, structure-activity relationships*

Introduction

The synthesis of peptides containing one or more disulfide bridges have been attractive targets of research ever since the pioneering work of *Vincent du Vigneaud*.[1] It has been a long standing goal in peptide science to develop methods for the chemical syntheses of these peptides, and throughout the years, a number of them have been prepared by either solution or solid-phase methods. Considerable effort has been devoted towards the controlled synthesis of disulfide bridges, which requires optimal protection strategies for chain assembly and for selective cleavage of the thiol protecting groups.[2,3] Introduction of disulfide bridges into peptide and protein sequences has been carried out with the goal to improve biological activities/specificities,[2,4–7] and stabilities.[2,8–12] The possibility of linking two (or more) separate chains by intermolecular disulfide bridges has numerous implications for biological research, including conjugation of peptides to carriers for immunological studies,[13] generation of active site models and preparation of standards corresponding to proteolytic fragments isolated during structural elucidation work on large proteins.[14–16]

Conotoxins, isolated from cone snail venom, represent a large class of peptides having a unique ability to differentiate between various types of ion channels. These are small, multiple disulfide bridge-containing, and highly stable peptides, with the potential of being ideal leads for peptide therapeutics.[17] Their specificity is what makes them important diagnostic tools in the characterization of neural pathways, as well as in drug development. Every *Conus* species has its own very distinct pharmacological profile; a large variety of peptides are present in every cone snail venom. These peptides are believed to serve major roles in three areas: (i) capturing prey; (ii) defense and escape from predators; and (iii) interaction with potential competitors.[18,19] It should be mentioned that there are peptides in the *Conus* venom, called conantokins that are not disulfide rich, but also have significant (and in many cases very similar) biological characteristics.[20]

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Discussion

Classes of conotoxins

The venoms of Conus snails are very complex, containing 50–200 distinct, biologically active components.[17–26] These components are short peptides, 12–30 amino acids in length, highly constrained, and generally contain multiple disulfide bridges. Although conotoxins exhibit very diverse biological profiles, a relatively constant arrangement of cysteine residues can be found in their sequences.[24] Based on their disulfide frameworks conotoxins can be grouped into three major classes.[20,22,23] It is important to note that these three classes are also different in their receptor targets (Fig. 1).

α-Conotoxins (Fig. 1, Fig. 2) have two disulfide bridges in a "2-loop" framework.[27–36] Most of these peptides target neuromuscular nicotinic acetylcholine receptors, that are made up of four homologous subunits in a pentameric arrangement ($\alpha_2\beta\gamma\delta$).[30–32,35,37–42] There are two acetylcholine binding sites in these receptors, located near the α/γ and α/δ subunit interfaces. Some α-conotoxins are of interest due to their ability to differentiate between the two binding sites on the nicotinic acetylcholine receptor, selectively inhibiting only one site. It has been shown that in mammalian muscle cells α-conotoxin GI, MI, and SI specifically target the α/δ binding site.[7,37,38] This is in contrast to

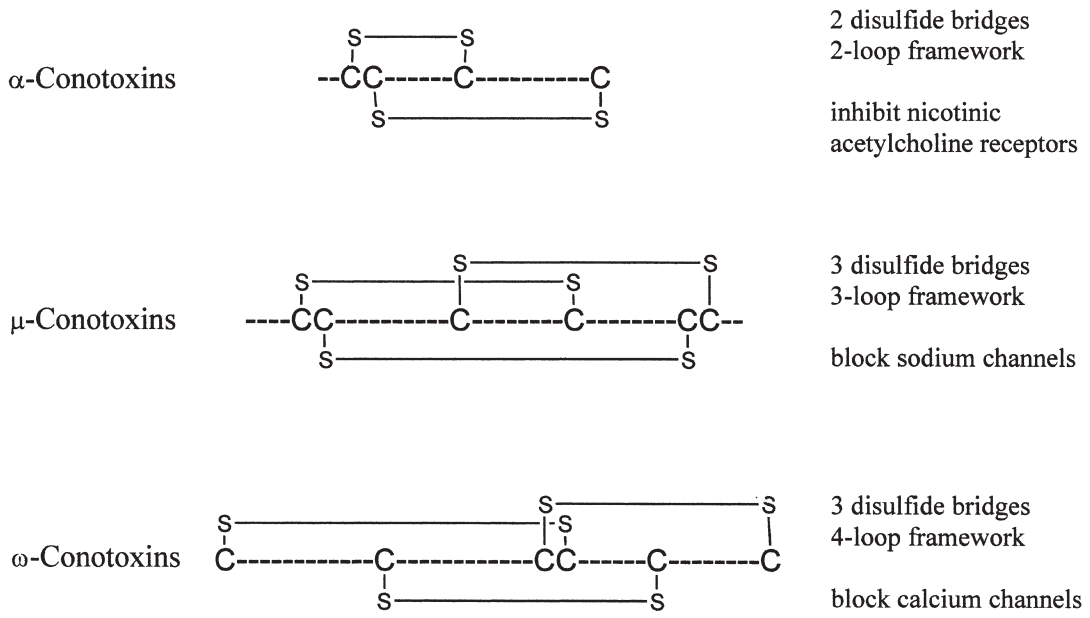

Fig. 1 – *Three major classes of conotoxins based on disulfide loop positions and on biological target specificity. The frameworks also try to indicate an average distance in the sequence between cysteine residues.*

GI	H-Glu-**Cys**-**Cys**-Asn-Pro-Ala-**Cys**-Gly-Arg-His-Tyr-Ser-**Cys**-NH_2
GIA	H-Glu-**Cys**-**Cys**-Asn-Pro-Ala-**Cys**-Gly-Arg-His-Tyr-Ser-**Cys**-Gly-Lys-NH_2
GII	H-Glu-**Cys**-**Cys**-His-Pro-Ala-**Cys**-Gly-Lys-His-Phe-Ser-**Cys**-NH_2
MI	H-Gly-Arg-**Cys**-**Cys**-His-Pro-Ala-**Cys**-Gly-Lys-Asn-Tyr-Ser-**Cys**-NH_2
SI	H-Ile-**Cys**-**Cys**-Asn-Pro-Ala-**Cys**-Gly-Pro-Lys-Tyr-Ser-**Cys**-NH_2
SIA	H-Tyr-**Cys**-**Cys**-His-Pro-Ala-**Cys**-Gly-Lys-Asn-Phe-Asp-**Cys**-NH_2

Fig. 2 – *Amino acid sequences of selected α-conotoxins, considered in our research*[27,28,30,31]

μ-conotoxins

PIIIA	H-Arg-Leu-**Cys**-**Cys**-Gly-Phe-Hyp-Lys-Ser-**Cys**-Arg-Ser-Arg-Gln-**Cys**-Lys-Hyp-His-Arg-**Cys**-**Cys**-NH_2
GIIIA	H-Arg-Asp-**Cys**-**Cys**-Thr-Hyp-Hyp-Lys-Lys-**Cys**-Lys-Asp-Arg-Gln-**Cys**-Lys-Hyp-Gln-Arg-**Cys**-**Cys**-Ala-NH_2
GIIIB	H-Arg-Asp-**Cys**-**Cys**-Thr-Hyp-Hyp-Arg-Lys-**Cys**-Lys-Asp-Arg-Arg-**Cys**-Lys-Hyp-Met-Lys-**Cys**-**Cys**-Ala-NH_2

ω-conotoxins

MVIIA	H-**Cys**-Lys-Gly-Lys-Gly-Ala-Lys-**Cys**-Ser-Arg-Leu-Met-Tyr-Asp-**Cys**-**Cys**-Thr-Gly-Ser-**Cys**-Arg-Ser-Gly-Lys-**Cys**-NH_2
GVIA	H-**Cys**-Lys-Ser-Hyp-Gly-Ser-Ser-**Cys**-Ser-Hyp-Thr-Ser-Tyr-Asn-**Cys**-**Cys**-Arg-Ser-**Cys**-Asn-Hyp-Tyr-Thr-Lys-Arg-**Cys**-Tyr-NH_2

Fig. 3 – *Amino acid sequences of selected μ-conotoxins and ω-conotoxins, mentioned in paper*[44-46,49-51,54,56]

the observation that in cells from the *Torpedo* electric organ α-conotoxin GI and MI have a higher affinity for the α/γ binding site,[38,43] while α-conotoxin SI does not differentiate between the two sites. Some α-conotoxins, *e.g.*, α-conotoxin ImI, are high affinity ligands for neuronal nicotinic acetylcholine receptors, which are made up of two types of subunits (α and β).[33,34,36] The diversity in these two types of subunits allows for targeting a great variety of receptors by multiple combination of subunits.

μ-Conotoxins have three disulfide bridges in a "3-loop" framework (Fig. 1, Fig. 3).[44–49] These peptides reversibly block sodium channels through binding to receptor site 1 skeletal muscle cells. They inhibit these muscle sodium channels without effecting neuronal sodium channels.[45,46] Recently, a novel type of μ-conotoxin, μ-conotoxin PIIIA, has been isolated, having the same structure as previously reported members of the family, however, the binding to sodium channel receptor site 1 is not reversible. μ-Conotoxin PIIIA is also different in that it reversibly binds to neuronal sodium channels.[49]

The third large class, which is the most common, contains ω-conotoxins, targeting presynaptic calcium channels, and having three disulfide bridges in a "4-loop" framework (Fig. 1, Fig. 3).[21,32,50–55] ω-Conotoxins have been important tools for the characterization of different types of calcium channels in nerve cells and chemical synopses, due to their high specificity. The most thoroughly investigated peptide of this class is the first one that was isolated, ω-conotoxin GVIA, which irreversibly blocks calcium channels in various mammalian neuronal cells.[56,57] It inhibits neuro-

transmitter releases at amphibian neuromuscular junctions, but has no effect on mammalian neuromuscular junctions.[58–60] Another widely investigated toxin, ω-conotoxin MVIIA, also blocks calcium channels, but the effects are irreversible in all cases.[50]

Each of these classes of conotoxins is represented in every venom with a number of peptides. Generally, only the cysteines and one or two other amino acids can be found in every member of a class present in a venom. Recently a number of smaller classes of conotoxins with fewer members have also been reported; these classes might not be present in every *Conus* venom. These peptides have structures similar to one of the three large classes while their biological characteristics resemble another one of the major classes.[20] There are also some other small classes of conotoxins that either have a distinct framework or unique biological characteristics.[20]

Application of conotoxins in medicine

Conotoxins combine a high affinity for the macromolecular receptor with a narrow target receptor specificity.[24] This could prove to be very useful in medicine, where side effects of drugs is a major problem. These side effects may occur if a drug candidate binds to receptor types that closely resemble its own target receptor, but are therapeutically irrelevant, and this may cause undesirable physiological effects. In contrast to most drugs, conotoxins are able to discriminate between receptor types that are similar and closely related.[25] By having such refined target specificity, the *Conus* snails that produce conotoxins, are able to perform a number of different physiological phases of prey immobilization at the same time, but on different sets of the same type of receptors.[61] Different classes of conotoxins are also able to work in synergy for faster and very specific effects. To use strategies like this in medicine would involve the use of combinatorial peptide libraries to develop drugs with such highly refined target selectivity. Having highly refined target selectivity would also ensure that the drug administered binds to the desired receptor and not one that is similar, but has no medicinal use.

The different classes of conotoxins are significant tools in neuroscience, due to their special characteristics. For example, α-conotoxins that target nicotinic acetylcholine receptors with very high specificity, have great importance both in neuroscience and in drug development, since these receptors have been shown to play significant roles in the physiology of a number of neuropsychiatric disorders, such as Alzheimer's disease, Parkinson's disease, Tourette's syndrome, or schizophrenia.[62] α-Conotoxins have also been reported as useful tools for phylogenetic discrimination between nicotinic acetylcholine receptors of different species, and as effective compounds for probing the surfaces of these receptors.[30] The characterization of already known α-conotoxins and the discovery of new highly specific ligands should help the understanding of the pharmacology and physiology of nicotinic acetylcholine receptors. Most μ-conotoxins, *e.g.*, μ-conotoxin GIIIA, are useful tools for immobilizing skeletal muscle without affecting axonal or synaptic events, due to their ability to preferentially block muscle sodium channels, but not axonal sodium channels.[46,63] These peptides can also be used to study synaptic transmission mechanisms at neuromuscular junctions.

While α-conotoxins block synaptic transmissions and μ-conotoxins block muscle action potentials in all vertebrates tested, ω-conotoxins have different actions depending on the animal. ω-Conotoxins are used in the investigation of voltage-sensitive calcium channels and to block presynaptic termini and neurotransmitter releases.[64] These have been the most successful conotoxins to date in medical studies, since calcium channels are important in heart muscle function, and can also be found in most neurons in the brain. The understanding of the physiological function of calcium channels has been made possible by studies on ω-conotoxins from Conus snails. It has been shown that the wide variety of amino acids, found between the cysteine residues in the sequences of ω-conotoxins, are responsible for the specificity of these neurotoxins on different types of calcium channels, therefore, these peptides should be considered as invaluable tools for identifying new calcium channel subtypes and for pharmacologically distinguishing known subtypes.

Laboratory synthesis of conotoxins

For the formation of conotoxins, solid-phase peptide synthesis of the fully reduced precursor, followed by folding under oxidative conditions, has been the most successful approach.[17] All conotoxins reported in the literature contain multiple disulfide bridges. Most reduced forms of native conotoxins are capable of folding in the "correct" way, leading predominantly to the desired natural isomers,[17] but on occasions, under certain conditions, oxidation yields the desired isomer in a mixture of the other, "mispaired," isomers.[65–67] These non-native isomers are different only in the orientation of the disulfide bridges, and sometimes it can be difficult to separate them from the native isomer.

As discussed above, α-conotoxins have two disulfide bridges, while μ- and ω-conotoxins have three disulfide bridges. There are three different orientations for two disulfide bridges, while three disulfide bridges can be arranged in 15 different combinations. To circumvent the problem of mispaired isomer formation, orthogonal syntheses of the natural isomers of a variety of α-conotoxins have been reported,[65,67–70] using orthogonally removable cysteine protecting groups.[3] Orthogonal syntheses have also been performed in order to form the mispaired isomers that are not found in nature.[65,67,70] For conotoxins having three disulfide bridges, no fully orthogonal synthesis has been reported to date.

Our work on the synthesis of α-conotoxin SI described the orthogonal synthesis of all three regioisomers of this peptide.[67,70] These investigations indicated that the overall yields for the desired peptides and the selectivities for the desired peptides were both the highest when both disulfide bridges were formed in solution. When either one or both bridges were formed while the peptide was still attached to the solid support the yields greatly decreased and considerable amount of disulfide bridge scrambling was observed.[2,67,70] We compared numerous methods for the formation of both disulfide bridges – forming the first bridge from the free thiol precursor, and the second one from

protected thiol precursors. These experiments showed that for the formation of the first disulfide bridge from free thiol precursors the solid-phase bound Ellman reagent[71] gave high yields (the yields were comparable to the traditional DMSO mediated oxidation[72] method) and the highest selectivities.[70] Using iodine[73] for the formation of the second disulfide bridge from protected thiol precursors proved to be the most successful reagent with respect to both yield and selectivity.[67,70] These studies also showed that in orthogonal syntheses of the three disulfide-paired isomers, the best results are achieved for the "interlocking" natural isomer and the least favorable results for the formation of the "nested" mispaired isomer (for designation of regioisomers see *A. K. Croskey, B. Hargittai* in this issue). This refers to, both, final yield and selectivity for the desired regioisomer.

Some studies[66] have also speculated on what residues are important in α-conotoxins for the formation of the different isomers, or more specifically, the natural isomer, by exchanging amino acids at various positions, and looking at the ratio of the three isomers following oxidation of the tetrathiol precursors. Folding of α-conotoxins is defined mainly by the placement of the four cysteine residues, forming the two disulfide bridges. The presence of these bridges, along with proline in position 5 and glycine in position 8 already favor the natural isomer, but only slightly.[66] The sequences also contain a variety of residues in positions 4, 9, and 10 that effect the biological activity or selectivity of the peptide.[66] Our studies on the folding of different α-conotoxins indicate that the presence of a proline residue in position 9 of α-conotoxin SI will greatly influence the folding properties of this peptide.[74] Most α-conotoxins will form their natural "interlocking" isomer with only small amounts of the other two regioisomers when their tetrathiol precursor is oxidized under folding conditions (φ = 1 % aqueous DMSO), but will form a complete mixture of all three regioisomers under denaturing conditions (φ = 1 % DMSO in the presence of 6 mol L^{-1} guanidine hydrochloride), as all three isomers will be present in great amounts.[66,74] α-Conotoxin SI, which has a proline in position 9 of its sequence, forms the natural isomer as the dominant product regardless of the oxidizing conditions.[74]

Sakakibara and coworkers[75] reported that during oxidation of the hexathiol precursor of μ-conotoxin GIIIB (22 amino acids, three disulfide bridges), three major products are formed in a ratio of 1:4:3. According to their results, the minor component is the natural isomer (disulfide bridges: Cys^3/Cys^{15}, Cys^4/Cys^{20}, Cys^{10}/Cys^{21}), while the other two are isomers that contain the Cys^{10}/Cys^{15} disulfide bridge, which is formed most rapidly during the oxidation reaction. Once this disulfide bridge is formed in the center of the molecule, the other cysteine residues, closer to the two termini, are able to form the remaining two bridges only with each other. They also report that increasing the peptide concentration in the oxidation mixture favors the formation of the natural isomer, although this process can lead to undesired oligomer formation.

Structure-activity relationships in conotoxins

It has been shown by several groups of investigators[29,30,76] that variations at some positions in the amino acid sequence can have significant effects on the receptor binding ability of the conotoxin, while changes at other positions have no noticeable effect. In the case of α-conotoxin GI, changing Glu^1, Asn^4, and His^{10} has very small effects on the biological activity of the peptide, while permutations at Pro^5 and Gly^8 lower the activity significantly, and the presence of a positively charged amino acid (lysine or arginine) in the vicinity of positions 9 and 10 are important for the activity.[29,30,76] Since for a few α-conotoxins, all three disulfide-paired isomers have been synthesized successfully, biological activity studies were performed on all of them. These studies show that the natural isomers are the only ones that have significant biological activity, while the effects of the mispaired isomers range from no activity to activity reduced to one-forth of the natural isomers.[7,66,76]

Gray et al.[28] compared the activities of α-conotoxins GI and MI, two peptides with similar biological targets (nicotinic acetylcholine receptors), but isolated from venoms of different *Conus* snails. They found that although the two peptides have a strong sequence homology, especially due to the disulfide bridges present in the peptides, α-conotoxin MI has a higher biological activity. The difference is probably due to the different amino acids in their sequence. One major difference suggested to be significant is that α-conotoxin GI has an acidic glutamic acid in position 1, while α-conotoxin MI has a basic dipeptide, glycine-arginine, in the same position (Fig. 1). This seems to be important, since potent antagonists of nicotinic acetylcholine receptors are often highly basic molecules. An even more astonishing finding was that α-conotoxin MI, unlike other α-conotoxins, equilibrates between two conformational forms.[28] It is yet to be seen if the sequence differences, which may account for biological activity differences, are also responsible for the conformational transitions.

α-Conotoxin SI contains a proline in position 9, while other investigated α-conotoxins have a basic amino acid (lysine or arginine) in this position (Fig. 1). The results of various studies show that substitution of proline for the basic amino acid accounts for the special phylogenetic discrimination exhibited by α-conotoxin SI.[30] 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 α-conotoxins.[38] Positions 9 and 11 are crucial for the activities of α-conotoxins, since even the changing of the basic amino acid for another basic one in position 9, or changing one aromatic amino acid for another aromatic one in position 11, can have a significant effect on the activity.[38] Therefore, it is not surprising that in comparison to most other α-conotoxins, the affinity of α-conotoxin SI for nicotinic acetylcholine receptor sites is significantly lower. α-Conotoxin SIA, on the other hand, has an activity in mice that is intermediate between α-conotoxins SI and GI.[31] This peptide does have a basic amino acid in position 9, but it also contains a negatively charged aspartic acid residue in position 12.

In order to explore the effects of the presence of multiple bridges in the structure of the peptides and to investigate the significance of disulfide bridges, we have designed and synthesized four lactam analogues of the natural isomer of α-conotoxin SI. These analogues exchanged one of the two paired cysteines of the original peptide for a lactam bridge, formed by a glutamic acid and lysine side-chain.[7]

Our studies have shown that replacement of the Cys^2-Cys^7 (small) disulfide bridge resulted in complete loss of activity, indicating that changing this loop results in some slight differences in the structure that affect the activity of the peptide. Exchanging the larger (Cys^3-Cys^{13}) bridge, on the other hand, led to analogues that exhibited considerable affinities for the receptor sites. The Glu^3-Lys^{13} analogue was about 70-fold more potent than the natural isomer of α-conotoxin SI, although the Lys^3-Glu^{13} analogue showed about 60-fold lower affinity. These results led us to assume that the larger disulfide loop in α-conotoxin SI plays a global structural role.

In ω-conotoxins, the most important residue is the tyrosine in position 13, due to the phenolic hydroxyl side chain.[54] It has also been shown,[77] that replacing any one of the three lysines with alanine in the *N*-terminal region of ω-conotoxin MVIIA (positions 2, 4, and 7) results in decreased activity, suggesting that the basic residues are important for the biological characteristics of ω-conotoxins similarly to α-conotoxins. Most ω-conotoxins contain up to four basic amino acids in the *N*-terminal region of their sequence. *Norton* and coworkers[54] have preformed extensive studies looking at the contributions of key amino acids in ω-conotoxin GVIA. By substituting unnatural amino acids and arginine for lysine in position 2, they found that the length and charge of the amino acid is indeed an important factor in the biological activity of the peptide. The roles of the modified amino acid, hydroxyproline, in positions 10 and 21 were studied by proline substitution, showing that the effect of these amino acids seem to be structural rather than functional. Finally, the importance of the orientation of the tyrosine side chain in position 13 has been shown by substituting D-tyrosine and a highly constrained analogue for L-tyrosine.[54]

ACKNOWLEDGEMENTS

The author would like to thank the organizers of the 2nd Central European Chemistry Meeting for the opportunity to present the talk that formed the bases for this paper, Dr. George Barany for helpful guidance, and Drs. Stewart N. Abramson, Ioana Annis, Duncan R. Groebe, Núria Solé and Ms. Amy K. Croskey who were involved in various aspects of these studies.

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SAŽETAK

Konotoksini: mali peptidi bogati disulfidima

B. Hargittai

Mnogi peptidni otrovi i antibiotici koji se mogu naći u prirodi izgrađeni su od jednog ili više prstenova i disulfidnih mostova. α-Konotoksini pripadaju skupini malih peptida bogatih disulfidnim mostovima. Većina dotičnih peptida sadrži trinaest do četrnaest aminokiselina i dva disulfidna mosta. Takve sheme kemijskog vezivanja vode do stvaranja tri moguća peptidna regioizomera. Većina reduciranih izvornih konotoksina sposobni su svijati se na način koji rezultira formiranjem prirodnog konotoksin izomera. Međutim, u sklopu posebnih eksperimentalnih uvjeta, proces oksidacije tih peptida rezultira sintetiziranjem sva tri peptidna izomera. U članku se raspravlja o generalnim detaljima o konotoksin peptidima uključujući i detalje o sintezi takvih konotoksina i našim doprinosima ovom polju znanstvenog istraživanja. Uspješno su sintezirana tri α-konotoksin SI regioizomera i četiri cirkularna amidna analoga prirodnih izomera. Također je istraživano i kako male promjene u slijedu aminokiselina u peptidima utječu na njihova svojstva svijanja.

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