ABSTRACT:

Rearrangement of disulfide bonds during the synthesis of α-conotoxin GI using PhS(O)Ph/CH₃SiCl₃ oxidation procedure was observed. We have demonstrated that the protecting scheme (order of acetamidomethyl (Acm) and t-Bu protecting groups) of the Cys residues as well as the reaction time influenced the ratio of the native and the mispaired compounds, while the temperature of the reaction mixture had no significant effect. However, in all cases the nonnative derivative was produced in high amount. The structure of the isomers was identified by the combination of enzymatic digestion and mass spectrometry measurements. We conclude that the air oxidation followed by the application of Tl(TFA)₃ for the regioselective formation of disulfide bonds leads up to the appropriate compound in the case of the synthesis of α-conotoxin GI, while the oxidation procedure using PhS(O)Ph/CH₃SiCl₃ system resulted in the nonnative disulfide isomer. © 2006 Wiley Periodicals, Inc.


Keywords: α-conotoxin GI; oxidation procedures; disulfide isomers; rearrangement of disulfide bonds; mass spectrometry; enzymatic degradation

INTRODUCTION

The α-conotoxin GI from Conus geographus is a potent reversible blocker for the nicotinic acetylcholine receptor at the postsynaptic neuromuscular junction, thus inhibiting neuron stimulated muscular contractions.¹,² It consists of 13 amino acid residues (ECCN-PACGRHYSC) with two ‘interlocking’ disulfide bonds in positions 2–7 and 3–13, respectively. In most publications, the synthesis of α-conotoxin derivatives with two disulfide bridges are described as a two-step regioselective oxidation procedure using orthogonal cysteine protecting groups. Acetamidomethyl (Acm) group, stable under acidic and alkaline conditions, is usually applied with a combination of Cys protecting groups that are removable under the final cleavage procedure of SPPS. First, the free thiol groups are oxidized by either air, DTNB, or DMSO.³–⁵ The second disulfide bond is formed in one step simultaneously by the removal of Acm protecting groups using either Tl(TFA)₃ or I₂.⁶,⁷ Akaji et al. investigated the silyl chloride-sulfoxide system for disulfide bond formation of cysteine peptides.⁸ They obtained the best result in case of the application of PhS(O)Ph/CH₃SiCl₃ mixture. Disulfide bond formation from t-Bu protected cysteines was completed in 10 min. The conversion of two Cys(Acm)
residues to cystine needed a somewhat longer period of time (30 min). This procedure was used for the synthesis of α-conotoxin MI (GRCCHPACGKNYSC with 3–8, 4–14 disulfide bond pattern). The peptide was built up by Fmoc chemistry on Rink-Amide resin and Bu protection was applied for Cys3 and Cys8, while Acm for Cys4 and Cys14. The peptide was cleaved from the resin with HF either in the presence of m-cresol (4°C, 20 min) or in the presence of anisole (4°C, 60 min). In the first case, all Cys protecting groups remain intact ([Cys(Bu)3, Cys(Acm)4,14]-conotoxin MI). Using the second procedure, the Bu protection was removed from Cys residues ([Cys3,8, Cys(Acm)4,11]-conotoxin MI). The peptide containing both Bu and Acm protection was oxidized first by iodine, resulting in 4–14 cystine followed by the formation of the 3–8 disulfide bridge from the Bu protected Cys derivatives using the PhS(O)/Ph/CH3SiCl3 mixture. The second linear precursor peptide was air oxidized, resulting in 3–8 cystine from free Cys residues, after which the Cys(Acm) residues were connected by PhS(O)/Ph/CH3SiCl3 oxidation.

The conformation of α-conotoxin GI is stabilized by the two ‘interlocking’ disulfide bridges resulting in a rigid 3D structure. Solution conformation of α-conotoxin GI studied by nuclear magnetic resonance (NMR) spectroscopy and crystal structure determined at 1.2-Å resolution by X-ray crystallography suggested turn-like structure in the 8–13 part. This rigid and well-accessible region of the peptide provides an opportunity for replacement by the “guest” epitope sequence having turn structure and could result in chimeric peptides as synthetic antigens.

In our laboratory, we have synthesized chimera peptides of α-conotoxin GI. In these compounds, RHYS part of the molecule was replaced by an epitope sequence of 281DPVG284 derived from glycoprotein D of herpes simplex virus type 1 or by PDTR from mucin 1 glycoprotein. Considering that the replacement of RHYS resulted in a loss of aromatic (Tyr) residue, useful for the determination of peptide concentration, the glutamic acid at the N-terminus was replaced by a tyrosine. We found that the desired alignments could be achieved either by the formation of the smaller loop first or by the preparation of the larger loop before the small one. We have also demonstrated that the disulfide bond between Cys3 and Cys13 was formed much faster than that between Cys2 and Cys7 during the first regioselective oxidation step. Several oxidation methods were compared. The best result was obtained with the application of DTNB (Ellman’s reagent) for the oxidation of Cys3 and Cys13 and Tl(tfa)3 for connection of Acm protected Cys derivatives in positions 2 and 7. Similar results were obtained by Hargittai and Barany, who prepared all of the disulfide isomers of α-conotoxin SI, which differs from GI version in position 1 (Ile instead of Glu) and in positions 9–10 (Pro-Lys instead of Arg-His). The authors showed that the formation of the large loop prior to the small one produced higher yield than in the “vice versa” case resulting in the same isomers. However, the NMR studies of α-conotoxin GI derivatives, in which Cys3 and Cys13 or Cys7 and Cys2 were replaced by alanines, showed that in the presence of single disulfide bond between cysteines in position of 2 and 7 resulted in some elements of secondary structure, which might facilitate formation of the second disulfide bond.

For NMR studies, α-conotoxin GI as reference compound was prepared. Two synthetic routs were compared. In the first case, the combination of air and Tl(tfa)3 oxidation, respectively, was used for regioselective disulfide bond formation. In the second approach, Akaji’s method was applied. To our surprise, the products that resulted were different. Here we report on our findings indicating that the compounds obtained were disulfide isomers. In this article, we present data on the identification of the disulfide pairing of the respected isomers. The influence of protecting scheme and reaction conditions on the appearance of disulfide isomers under PhS(O)/Ph/CH3SiCl3 oxidation is also discussed.

**EXPERIMENTAL**

**Chemicals**

All amino acid derivatives and Rink Amide MBHA resin were purchased from NovaBiochem (Lülfingen, Switzerland) or Reanal (Budapest, Hungary). Scavengers (ethanedithiol (EDT), thioanisole), coupling agents (1-hydroxybenzotriazole (HOBt), N,N-diisopropylcarbodiimide (DIC)) and cleavage reagents (piperidine, 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU)) were Fluka (Buchs, Switzerland) products. Solvents for synthesis and trifluoroacetic acid (TFA) were obtained from Reanal, while acetonitrile for HPLC and phenol were from Sigma (Sigma–Aldrich, Budapest, Hungary).

**Solid Phase Peptide Synthesis**

The linear precursor peptide of α-conotoxin GI was built up on Rink Amide MBHA resin (0.69 mmol/g capacity) using standard Fmoc-chemistry. The synthetic protocol was as follows: (i) DMF washing of the resin (3 times for 1 min each); (ii) removal of Fmoc-group with 2% DBU and 2% piperidine in DMF (4 times for 2 + 2 + 5 + 10 min); (iii) DMF washing (8 times for 1 min each); (iv) coupling of Fmoc-amino acid derivative—HOBt–DIC (3 equiv each for 60 min); (v) DMF washing (3 times for 1 min each); (vi) DCM washing (2 times for 0.5 min each); (vii) ninhydrine assay. Two of the cysteine residues were protected with tert-butyl (Bu) or trityl (Trt) groups (Cys 3 and 13 or Cys 2 and 7), while the other two with Acm (Cys 2 and 7 or Cys 3 and 13), respectively, depending on...
the synthetic strategy used. The further protecting groups were trityl for Asn and His, tert-butyl for Glu, Tyr and Ser, and 2,2,4,6,7-pentamethyl-dihydrobenzofuran-6-sulfonyl (Pbf) for Arg. After the removal of the last Fmoc-group from the N-terminus, the peptide was cleaved from the resin with TFA/phenol/thioanisole/EDT/ water (10 ml:0.75 g:0.5 ml:25 ml:0.5 ml) mixture at RT for 1.5 h. All protecting groups, except Acm and Bu from cysteine residues, were removed under this condition. The resin was filtered off and the crude product was precipitated with ice-cold dry diethyl ether. The precipitate was separated by centrifugation and washed three times with diethyl ether. The solid material was dissolved in 10% acetic acid followed by freeze-drying. The crude products were purified by RP-HPLC, and the purified compounds were characterized by analytical HPLC and ESI-MS.

**Formation of Disulfide Bridges**

**Method A.** Air oxidation, used for the first disulfide bridge formation in case of peptide H-EC(Acm)CNPAC(Acm)GRHYSC-NH$_2$, was carried out overnight under vigorous stirring in 0.1 M C(Acm)GRHYSC-(Bu)NH$_2$ or H-EC(Bu)C(Acm)NPAC(Bu)GRHYSC(Acm)-NH$_2$ dissolved in TFA at a peptide concentration of 1 μmol/ml. The mixture was acidified prior to purification by RP-HPLC. The purified compound was obtained in 45% yield calculated to the purified linear precursor peptide.

**Method B.** The mono–disulfide peptide was dissolved in TFA containing 2% anisole in a peptide concentration of 100 μmol/ml. The solution was cooled down to 4°C prior to the addition of 0.6 equiv TI(tfa)$_3$ to each Acm protected cysteine residue. The reaction was continued for 1 h at 4°C and then the peptide was precipitated with dry ether. The product was collected by centrifugation and it was washed with ether three times. The crude peptide was purified by RP-HPLC. The yield of the purified product was 77% to the mono–disulfide peptide.

**Method C.** The Cys protected peptides (H-EC(Acm)C(Bu)NPAC(Acm)GRHYSC-(Bu)-NH$_2$ or H-EC(Bu)C(Acm)NPAC(Bu)GRHYSC(Acm)-NH$_2$) were 28 and 33%, respectively.

**Method D.** The mono–disulfide peptides with Bu protecting groups at Cys$^{1,10}$ or Cys$^{2,2}$ as well as fully Cys protected linear peptide (H-EC(Acm)C(Bu)NPAC(Acm)GRHYSC(Bu)-NH$_2$) were dissolved in TFA at a peptide concentration of 1 μmol/ml, then treated with CH$_3$SiCl$_3$ (150 equiv) and PhSH(O)Ph (10 equiv) mixture at RT for 10 or 20 min for development of disulfide bond(s). The reaction was carried out also at 4°C. The products were precipitated by adding dry ether and the solid materials were separated by centrifugation. The crude products were purified by RP-HPLC and characterized by analytical HPLC and ESI-MS. The yields of the purified bis-disulfide peptide isomers were 25–30% of the starting compounds. The ratio of the isomers corresponding to the different experimental steps are indicated later.

**Purification and Analysis by RP-HPLC**

Analytical RP-HPLC was performed on a Knauer (H. Knauer, Bad Homburg, Germany) system using a Phenomenex Jupiter C$_{18}$ column (250 × 4.6 mm) with a 5-μm silica (300 Å pore size) (Torrence, CA, USA) as a stationary phase. Linear gradient elution (0 min 0% B; 5 min 0% B; 50 min 90% B) with eluent A (0.1% TFA in water) and eluent B (0.1% TFA in acetonitrile–water (80:20, v/v)) was used at a flow rate of 1 ml/min at ambient temperature. Peaks were detected at λ = 214 nm. The samples were dissolved in eluent A. The crude products were purified on a semipreparative Phenomenex Jupiter C$_{18}$ column (250 × 10 mm) with 10-μm silica (300 Å pore size) (Torrence, CA). The flow rate was 4 ml/min. The same eluents with a linear gradient of 10–50% B in 40 min were applied.

**Enzymatic Digestion**

Enzymatic digestion was performed by subtilisin, using a peptide/enzyme ratio of 15:1 (mol/mol). Peptides were dissolved in 50 mM ammonium bicarbonate in a concentration of 35 pmol/μl, and the reaction mixture was incubated at 37°C for 2 h. After incubation, samples were diluted to the appropriate concentration and analyzed by mass spectrometry without further purification.

**Mass Spectrometry**

MALDI-TOF mass spectrometry was carried out on a Voyager DE-Pro mass spectrometer (Applied Biosystems, Framingham, MA), equipped with a 337-nm nitrogen laser, in positive, reflectron acceleration mode. Spectra were acquired by 20 kV acceleration voltage, 75% grid voltage, 0.005% guide wire, and 120 ns delay time in the range of m/z 200–3000. Mass to charge (m/z) calibration was performed externally using a set of peptides as a standard (Perceptive Biosystems). α-Cyano-4-hydroxycinnamic acid (CCA) matrix were prepared at 10 mg/ml concentration in acetonitrile/water = 1:1 (v/v) containing 0.1% TFA. Samples were diluted to a peptide concentration of 10 pmol/μl using 0.1% TFA (v/v) solution, mixed with the matrix in 1:1 ratio, spotted onto the target, and allowed to dry on air. Approximately 150 laser shots were averaged to improve signal-to-noise.

Electrospray ionization mass spectrometric experiments and tandem mass spectrometry were carried out either on a hybrid, quadrupole orthogonal acceleration time-of-flight mass spectrometer (Micromass Q-ToF Micro, Manchester, UK), or on a Bruker Daltonics Esquire 3000plus (Bremen, Germany) ion trap mass spectrometer. Spectra were acquired in the range of m/z 50–2000. Multiple tandem mass spectrometry was carried out on a Bruker Daltonics Esquire 3000plus ion trap mass spectrometer. Samples were dissolved in acetonitrile/water = 1:1 (v/v) solvent mixture, containing 0.1% acetic acid.

**Reversed-Phase Capillary HPLC-ESI-MS**

Peptides were separated using a Waters CapLC HPLC system, equipped with low flow capillary HPLC pumps and autosampler. Eluent A was 0.1% TFA, 2% acetonitrile, 97.9% H$_2$O, while eluent B was 0.1% TFA, 2% acetonitrile, 97.9% H$_2$O, while eluent B.
was 0.1% TFA, 5% H₂O, 94.9% acetonitrile. A 5-µl sample (5 pmol) was injected in partial loop mode onto a C₁₈ precolumn (5 x 0.3 mm). Separation was carried out on a C₁₈ (PepMap, 150 x 0.3 mm, 3 µm) column (LCPackings, Amsterdam, The Netherlands) with a flow rate of 5 µl/min, at room temperature. Linear gradient of eluents (0 min 5% B; 2 min 5% B; 30 min 50% B) was used for separation. The HPLC was directly coupled to a hybrid, quadrupole orthogonal acceleration time-of-flight mass spectrometer equipped with an orthogonal Z-spray type electrospray ionization source. Mass spectra were acquired in positive ion mode, in the m/z region 100–2200. Optimized operating conditions were as follows: capillary voltage 3000 V, sample cone voltage 30 V, extraction voltage 1 V, collision cell voltage 8 V.

RESULTS AND DISCUSSION

Regioselective Disulfide Bond Formation of α-Conotoxin GI

The synthesis of linear precursor peptides of α-conotoxin GI was performed on Rink-Amide MBHA resin by Fmoc chemistry. Two different types of orthogonal protecting scheme were used for cysteine derivatives. In the first experiment, Acm (Cys₂,₇) and Trt (Cys₃,₁₃) groups were applied, while in the second case the Trt protecting group was replaced by tBu group of Cys residues in position 3 and 13 (see Figure 1). Under the final TFA cleavage, both Acm (Cys²,⁷) and tBu (Cys³,₁₃) protection remained intact. The purified linear peptide was dissolved in 80% acetic acid at 1/2 mol/ml concentration and it was treated with 20% I₂ in methanol (5 equiv/Acm group) at RT for 1 h. After elimination of the excess of iodine, the peptide was purified by HPLC and was characterized by mass spectrometry. The isolated product was dissolved in TFA (at c = 1 µmol/ml) and was treated with PhS(O)Ph (10 equiv) in the presence of CH₃SiCl₃ (150 equiv) at RT for 20 min.

The products were characterized by MALDI-TOF mass spectrometry, ESI-MS, and HPLC-MS. Both samples gave the same mass value ([M+H]+ = 1437.6) using either MALDI-TOF or ESI mass spectrometry; however, their retention times detected by HPLC-MS were different (18.9 and 17.2 min, respectively, Figure 2). These data indicate that the disulfide alignment of the peptides is different.
Identification of the Disulfide Alignments in Isomers of \( \alpha \)-Conotoxin GI

To identify the disulfide alignments of the isomers, the peptides were digested by an unspecific protease, subtilisin, and the mixture was analyzed by mass spectrometry. After enzymatic digestion, in the case of \( \alpha \)-conotoxin GI isomer 1 prepared by oxidation procedure A and B, only a single degradation product was detected by MALDI-TOF mass spectrometry \((\text{[M+H]}^+) = 1155.4\) (Figure 3A). The product corresponds to a peptide fragment with the loss of His\(^{10}\)-Tyr\(^{11}\) dipeptide. Using ESI-MS, the peak corresponding to this dipeptide was also detected \((\text{[M+H]}^+) = 319.1\); Figure 3B). This shows that subtilisin catalyzed hydrolysis at two main sites in this peptide; the enzyme cleaves between Arg\(^2\)-His\(^{10}\) and also Tyr\(^{11}\)-Ser\(^{12}\). However, the cleaved peptide fragments are still connected via the disulfide bond. In contrast, the isomer 2 of \( \alpha \)-conotoxin GI prepared by oxidation procedure C and D provide two intensive peaks in the spectrum after incubation with subtilisin (see Figure 4). One of them corresponds to a peptide fragment \(^1\text{ECCNPAC}^\circ\) containing disulfide bridge between neighboring cysteines \((\text{[M+H]}^+) = 633.5\), while the other peak corresponds to the complementary peptide CGRHYSC with disulfide bond between the N- and C-terminal cysteine residues \((\text{[M+H]}^+) = 822.6\). These data suggest that in isomer 2, the enzyme cleaves the amide bond between \(^6\text{Ala}^-\text{Cys}\). An additional peak \((\text{[M+H]}^+) = 822.6 + 18 \text{ Da}\) was also detected in the mass spectrum. The presence of this fragment is indicative of an additional enzymatic

![Figure 2](image-url)

**FIGURE 2** HPLC-MS chromatogram and the corresponding MALDI-TOF spectrum of the two isomers of \( \alpha \)-conotoxin GI prepared by method A and B (A) or by C and D (B) as regioselective disulfide bond formation.

![Figure 3](image-url)

**FIGURE 3** MALDI-TOF (A) and ESI-MS (B) spectra of \( \alpha \)-conotoxin GI (isomer 1) digest after 2 h incubation with subtilisin at 37°C with a peptide:enzyme ratio = 15:1 (mol/mol).
cleavage site in the \(^7\text{CGRHYSC}^{13}\) peptide. These results suggest that the disulfide alignment of isomer 2 is unexpected and unusual, 2–3 and 7–13.

Results of the analysis of the subtilisin digest were confirmed by tandem mass spectrometry of the intact peptides. Doubly and triply charged molecular ions of both isomers were subjected to collision induced dissociation (CID) analysis. Tandem mass spectra of the isomer 1 show limited level of fragmentation, showing no characteristic fragment ions. However, the analysis of isomer 2 clearly shows the appearance of intensive fragment ions corresponding to a characteristic \(y\) series: \(y_7, y_8, y_9, y_{10}, y_{12}\) (see Figure 5). The presence of these fragment ions is in good correlation with the 2–3 and 7–13 pattern of the disulfide bridges. In agreement with the data in the literature, HPLC analysis of \(\alpha\)-conotoxin GI disulfide isomers showed the following order of retention times: ‘nested’ (2–13,3–7) < ‘discrete’ (2–3,7–13) < ‘interlocking’ (2–7,3–13).\(^{20,21}\) This confirms that the isomer 1 having the retention time of 18.9 min is the natural \(\alpha\)-conotoxin GI (see Figure 2).

Identification of Disulfide Pairing in Monocyclic Derivative of \(\alpha\)-Conotoxin GI

To verify the structure of the mono–disulfide peptide prepared by iodine oxidation (H-ECC(\(^t\)Bu)NPACGRHYSC(\(^t\)Bu)-NH\(_2\), with disulfide bridge between Cys\(^2\) and Cys\(^7\)), it was also subjected to enzymatic digestion by subtilisin. After incubation at 37°C for 2 h, the digested peptide mixture was analyzed by tandem mass spectrometry (see Figure 6). The main peaks in the mass spectra showed the presence of singly and doubly protonated molecular ions of fragment...
ECC('Bu)NPACGR with a disulfide bridge between cysteines in position 2 and 7 ([M+H] + = 1006.3 and [M+2H] 2+ = 503.7). Two small peptide fragments of HY ([M+H] + = 319.1) and SC('Bu) ([M+H] + = 264.1) were also detected in the spectrum. The lack of involvement of cysteine in a disulfide bridge at the C-terminal was also confirmed by tandem mass spectrometry experiments. MS/MS cleavage of the triply charged molecular ion of the 'Bu protected cyclic peptide (518.1 m/z) resulted in the rapid loss of the 'Bu groups and a water molecule.

One-Step Oxidation Using PhS(O)Ph/CH3SiCl3 Mixture

The PhS(O)Ph/CH3SiCl3 oxidation procedure could result in cystine not only from 'Bu protected cysteines, but also from Acm protected Cys residues.8 To analyze whether the silyl chloride–sulfoxide oxidation provides different isomer composition from linear precursor peptide, one-step oxidation was performed next. The oxidation of peptide H-EC(Acm) C('Bu)NPAC(Acm)GRHYSC('Bu)-NH2 was carried out according to method D at RT for 20 min. Similar to the regio-selective disulfide bond formation, one main peak was observed in the HPLC chromatogram and this proved to be related to the mispaired (2–3,7–13) disulfide bond containing peptide.

Influence of the Reaction Time on the Ratio of the Resulted α-Conotoxin GI Isomers

To investigate the influence of the reaction time on rearrangement of disulfide bonds, 10-min oxidation using the PhS(O)Ph/CH3SiCl3 system was performed. It should be noted that Akaji and coworkers suggested a 10-min reaction time for disulfide bond formation from 'Bu protected cysteines. The oxidation of mono–disulfide peptide (2–7 disulfide bridge) resulted in a reaction mixture containing two main peaks with a ratio of 2/3 and 1/3 corresponding to the isomers with 2–3, 7–13 and 2–7, 3–13 disulfide pattern, respectively. This result suggests that besides the expected 2–7, 3–13 disulfide isomer nonnative mispaired disulfide bonds could also be created in higher amount under these conditions. Reshuffling of the native disulfide bridges resulting in the mispaired isomer can be proposed because the isomer with the 2–3 and 7–13 disulfide bonds was the only detectable compound after 20 min.

Influence of the Protecting Group Order of Cysteine Residues

Our previous results in agreement with the observation of Hargittay and Barany18 showed that the formation of larger

![FIGURE 7](image_url) MS/MS spectrum of the triply protonated molecular ion (518.1 m/z) of one-loop α-conotoxin GI (2–7 disulfide bridge, H-ECC('Bu)NPACGRHYSC('Bu)-NH2). The spectrum shows the characteristic loss of the 'Bu groups and a water molecule.

![FIGURE 8](image_url) MS/MS/MS spectrum of the doubly charged fragment ion ([M-2 'Bu-H2O+2H] 2+, 711.7 m/z) derived from one-loop α-conotoxin peptide (2–7 disulfide bridge, H-ECC('Bu)NPACGRHYSC('Bu)-NH2). The most characteristic fragment ions are labeled. Please note that simplified a/bx labeling is used for a/bx-2 'Bu-H2O fragment ions.

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loop prior to the smaller one gave the desired peptide with a better yield. It is worth mentioning that linear precursor peptide of H-GRC(’Bu)C(Acm)HPAC(’Bu)GKNYSC(Acm)-NH₂ was used for regioselective disulfide bond arrangement by Akaji et al. during the synthesis of α-conotoxin MI. The iodine oxidation (4–14 disulfide bridge formation) followed by silyl chloride–sulfoxide procedure (3–8 disulfide bond formation) resulted in the native compound as the main product with a moderate yield. To follow this synthetic route, the Cys protecting groups were changed in our next experiment. For this, Cys 2 and 7 were protected with ’Bu group, while Cys 3 and 13 were blocked with Acm group. In the first step, the disulfide bridge between Cys 3 and 13 was prepared by using the iodine oxidation procedure. In the second step the purified mono-disulfide peptide was oxidized by the application of the PhS(O)Ph/CH₃SiCl₃ system. In this experiment, 2 mg of mono–disulfide peptide was dissolved in TFA (c = 1 μmol/ml) and was treated with PhS(O)Ph (10 equiv) in the presence of CH₃SiCl₃ (150 equiv) at RT for 10 min. In the crude product, two main peaks were detected by RP-HPLC, one peak corresponded to the peptide with the native 2–7, 3–13 disulfide pattern, and another one proved to be the mispaired product containing the 2–3, 7–13 disulfide bridges. The native α-conotoxin GI was present at 70% and peptide with 2–3, 7–13 disulfide isomer was in the mixture at 30% according to the area under the peak values. Surprisingly, the ratio between these two components was dependent on the amount of precursor peptide used for oxidation. The oxidation step with 20 mg peptide using the same 1 μmol/ml peptide concentration resulted in higher amount of the bis-disulfide peptide with the nonnative 2–3, 7–13 disulfide alignment (60%) compared with the native α-conotoxin GI (35%). In this case, 5% of isomer containing 2–13 and 3–7 disulfide bridges was also detected by retention time and ESI-MS.

**Influence of the Temperature on Formation of Disulfide Isomers**

The temperature used for the reaction might also have an influence on the disulfide bond rearrangement. To study the effect of temperature for disulfide reshuffling, the oxidation using silyl chloride–sulfoxide mixture was carried out also at 4°C. The results in comparison with those obtained at RT suggested that the lowering of the temperature has no significant influence on the isomer composition.

**Stability of Mispaired Derivative of α-Conotoxin GI**

Stability of the nonnative peptide containing the 2–3, 7–13 disulfide bridges as a function of time was also analyzed under two conditions in deionized water as well as in 0.1M Tris buffer (pH 8). No changes were detected by RP-HPLC, neither after 7 days in deionized water nor after 3 days in the buffer. However, in both cases by adding a catalytical amount of cysteine to the solutions, almost quantitative (95%) rearrangement of the disulfide bridges was observed in less then 1 h (see Figure 9). The isolated compound was identical to the native 2–7, 3–13 isomer. It is interesting to note, that the disulfide alignments of nonnative α-conotoxin GI isomer were rearranged even in freeze-dried state at 4°C after several months.

**CONCLUSION**

In this article, we have described our observation concerning the formation of a mispaired α-conotoxin GI during the synthesis with PhS(O)Ph/CH₃SiCl₃ mixture for oxidation. The main product was the α-conotoxin GI derivative with the disulfide bridges between cysteines in positions 2 and 3 and in positions 7 and 13. We have also demonstrated that the protecting group scheme, the order of the formation of disulfide bridges, and the reaction time have a marked influence on the amount as well as on the isomer ratio of the disulfide isomers produced. The unusual disulfide pattern was demonstrated by tandem mass spectrometry as well as by MS analysis of fragments derived from the subtilisin digests. From the data presented, we conclude that the unusual disulfide alignment was formed by the rearrangement of disulfide bonds under PhS(O)Ph/CH₃SiCl₃ condition used for the regioselective establishment of disulfide bond. The analysis of the mechanism of the reshuffling of disulfide bonds needs more detailed studies. However, it is worth mentioning that the 8-membered-ring cycle formed between...
adjacent cysteine residues might have some sequential criteria. In a number of natural and nonnatural peptides/proteins one can identify Xxx-Cys-Cys-Yyy sequences containing disulfide bridge between the neighboring cysteine residues. The analysis of the frequency of amino acids in Xxx and Yyy position show that Ser, Thr, Leu, Gly, Glu and Pro, Asp, Arg are the most frequent amino acid residues in position Xxx and Yyy, respectively. It is interesting to note that in the case of α-conotoxin GI, Glu and Asn are present in the position of 1 and 4 (Glu-Cys-Cys-Asn).

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