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Formation of multiple intramolecular disulfide bonds in peptides using the reagent *trans*-[Pt(ethylenediamine)$_2$Cl$_2$]$^{2+}$

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Abstract—The disulfide-bond forming reagent *trans*-[Pt(en)$_2$Cl$_2$]$^{2+}$ cleanly, rapidly and quantitatively converts fully reduced α-conotoxin GI and α-conotoxin SI to their three disulfide bond-containing regioisomers in one-step in media of pH 3–7, enabling the convenient synthesis of the three isomers. © 2001 Elsevier Science Ltd. All rights reserved.

Many biologically active peptides are characterized by their high density of intramolecular disulfide bonds. Examples include peptide toxins and peptides with anti-microbial and anti-fungal activities. The sites of action of these peptides are frequently proteins that control movement of ions through cell membranes, which makes them useful as neurological tools to probe receptor sites. For example, peptide toxins in the venoms of fish-hunting cone snails (genus *Conus*) block synaptic transmission by inhibition of the nicotinic acetylcholine receptor (α-conotoxins), presynaptic voltage-sensitive calcium channels (ω-conotoxins), and muscle sodium channel potentials (μ-conotoxins).[1]

Because only minute quantities of these peptides can be isolated from venoms and other natural sources, it is important to develop efficient methods for their synthesis. There is also interest in analog peptides with non-native disulfide bond pairings for their therapeutic potential and as a way of increasing the diversity of peptide libraries.[2,3] The usual approach is to synthesize the precursor linear peptide using standard solid phase peptide synthesis methods, and then form the intramolecular disulfide bonds.[4] In spite of extensive research on methods for forming peptide disulfide bonds, formation of multiple intramolecular disulfide bonds is still a challenge. Several strategies have been used, including simultaneous formation of all the disulfide bonds by oxidation of the fully deprotected linear peptide in the sulfhydryl form and sequential formation of disulfide bonds by use of selectively removable sulfhydryl protecting groups.[4] These strategies, together with various oxidation methods developed during the last 40 years,[4b] have been used with varying degrees of success to synthesize multiple disulfide bond-containing peptides.

Recently, we discovered a new class of reagents for the formation of intramolecular peptide disulfide bonds.[5] The reagents, which are *trans*-dichloro-Pt(IV) complexes, oxidize dithiol peptides selectively and efficiently to their corresponding intramolecular disulfide forms under mild reaction conditions. Application of the *trans*-dichloro-Pt(IV) reagents was demonstrated with the formation of single disulfide bonds in a variety of peptides, where the ring size formed by the disulfide bond varied from 4 to 17 amino acids (14–53 atoms). No side reactions were observed with the reagent *trans*-[Pt(en)$_2$Cl$_2$]$^{2+}$, where en is ethylenediamine.[5b] In all cases, essentially quantitative formation of the intramolecular disulfide bond was observed, with no detectable formation of dimers or higher oligomers.

In this Letter, we describe application of the reagent *trans*-[Pt(en)$_2$Cl$_2$]$^{2+}$ to the formation of multiple disulfide bonds in peptides, using as examples α-conotoxin GI and α-conotoxin SI. Both are peptide toxins that inhibit the nicotinic acetylcholine receptor.[1b] Both also have been used to test other protocols for the synthesis of peptides with multiple disulfide bonds.[6]

The side-chain protected α-conotoxin GI peptide H-Glu(O*t*Bu)-Cys(Trt)-Cys(Trt)-Asn(Trt)-Pro-Ala-Cys(Trt)-Gly-Arg(Pbf)-His(Trt)-Tyr(*t*Bu)-Ser(*t*Bu)-Cys(Trt)-NH$_2$ was assembled on a Millipore model 9050 Plus peptide synthesizer using solid phase Fmoc peptide synthesis methodology. Fmoc-PAL-PEG-PS (0.50 g)

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resin with a loading capacity of 0.22 mmol/g and a five times excess of Fmoc-protected amino acid were used for the synthesis. The Fmoc group was removed from the N-terminal amino group of the resin-bound peptide before each coupling step by reaction with 20% piperidine in *N*,*N*-dimethylformamide for 8 min. Entering amino acids were preactivated with 1-hydroxy-7-azabenzotriazole (HOAt) and *N*,*N*′-diisopropylcarbodiimide (DIPCDI), and couplings were run for 90 min. Freshly prepared reagent (20 mL) with a composition of 88% TFA, 2% triisopropylsilane, 5% phenol and 5% water were reacted with the peptide resin for 3 h to cleave and deprotect the peptide. The resin was removed by filtration and washed with 5 mL of TFA, which was combined with the filtrate; the filtrate was then diluted with 200 mL of water. After two extractions of the filtrate with ether, it was lyophilized to give 147 mg of crude product. The major peak in the chromatogram of the crude product (Fig. 1, top) was identified as fully reduced α-conotoxin GI (corresponding to peptide **1** in Scheme 1, with an HPLC purity >80%) by mass spectrometry.[7] Synthesis by the same procedure yielded 165 mg of crude fully reduced α-conotoxin SI. The major peak was assigned to the fully reduced peptide (peptide **5** in Scheme 2, with an HPLC purity >85%) by mass spectrometry.[7]

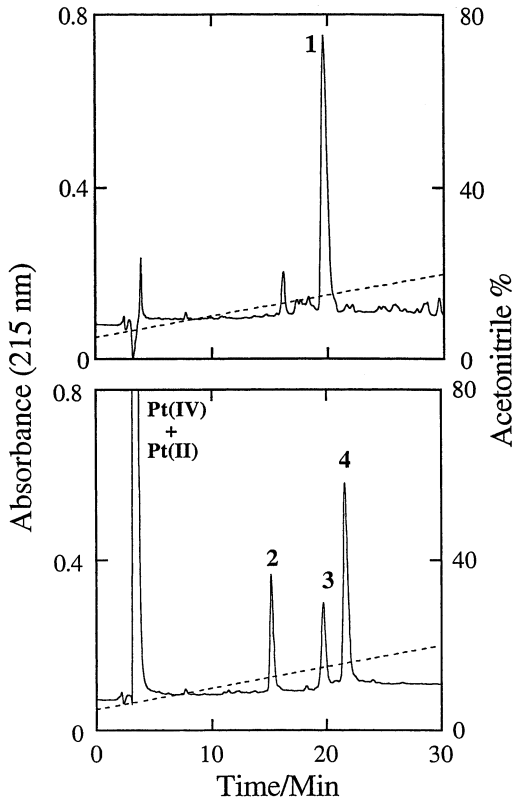
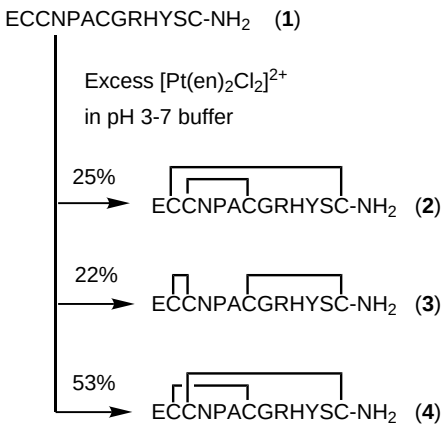
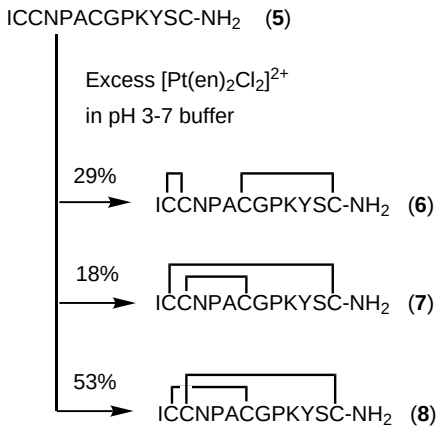


Figure 1. Chromatograms of ~1 mM crude reduced α-conotoxin GI (top) and a reaction mixture containing ~1 mM crude peptide and 3.0 mM $[Pt(en)_2Cl_2]^{2+}$ in a pH 4.0 phosphate buffer after reaction for 60 min (bottom). A 10× 250 mm Vydac C18 column was used with an acetonitrile–water gradient mobile phase (dashed lines indicate the acetonitrile gradient). Peak assignments are given in Scheme 1.

Oxidation of a ~1 mM solution of crude peptide **1** in pH 4.0 phosphate buffer with 3.0 mM *trans*-$[Pt(en)_2Cl_2]^{2+}$ for 60 min resulted in three major products, as indicated by the chromatogram in Fig. 1, bottom. The three products have identical mass, corresponding to monomeric peptides with two disulfide bonds (monoisotopic mass found: 1436.6; theoretical: 1436.5). The peaks were assigned to the disulfide bond regioisomers **2**–**4** in Scheme 1 using the retention times for an authentic sample of **4** and retention data reported by Nishiuchi and Sakakibara.[6a] It is noteworthy that not only is the fully reduced peptide **1** completely converted to the three regioisomers by the Pt(IV) oxidation, but the peptides giving the much smaller peaks in its chromatogram, which apparently are partially folded intermediates, are also converted, as demonstrated by the cleaner chromatogram for the oxidation product (Fig. 1). The clean nature of the oxidation was further confirmed by oxidation of a 1 mM solution of pure fully reduced α-conotoxin GI with 4 mM *trans*-$[Pt(en)_2Cl_2]^{2+}$ (reaction time 30 min, pH 4–7); separation of the reaction mixture on an analytical column with a mobile phase gradient up to 40% acetonitrile and an elution time of 60 min gave no



Scheme 1. Formation of the three disulfide bond regioisomers of α-conotoxin GI by one-step oxidation with $[Pt(en)_2Cl_2]^{2+}$.



Scheme 2. Formation of the three disulfide bond regioisomers of α-conotoxin SI by one-step oxidation with $[Pt(en)_2Cl_2]^{2+}$.

late-eluting peaks due to side products or oligomers. Conversion of fully reduced α-conotoxin GI to its three disulfide bond regioisomers was determined to be 100% by quantitation of chromatographic peak areas.[8]

Oxidation of a ~1 mM solution of crude peptide **5** in pH 5.0 phosphate buffer with 3.0 mM *trans*-$[Pt(en)_2Cl_2]^{2+}$ also resulted in three major products that have identical molecular weights. They were assigned to disulfide bond isomers **6**–**8** in Scheme 2 (monoisotopic mass found: 1352.5; theoretical: 1352.6) based on the work of Hargittai and Barany.[6g] Conversion of the reduced peptide to the three disulfide bond isomers was found to be quantitative.[8]

Several features of the formation of multiple disulfide bonds in α-conotoxin GI and α-conotoxin SI with *trans*-$[Pt(en)_2Cl_2]^{2+}$ should be noted. (i) Oxidation of the crude peptides to form the three regioisomers is clean, with no dimer or higher oligomers formed. (ii) More than 50% of the product had the native disulfide pairing for both α-conotoxin GI and α-conotoxin SI (peptides **4** and **8** in Schemes 1 and 2), which suggests that the reduced peptides have some secondary structure that favors formation of the native disulfide pairings. For reduced α-conotoxin GI, this is consistent with the results of NMR studies of its structure in solution.[9] (iii) The results in Fig. 1 and Schemes 1 and 2 indicate that oxidation with *trans*-$[Pt(en)_2Cl_2]^{2+}$ is an efficient way to obtain the regioisomers of α-conotoxin GI and α-conotoxin SI with non-native disulfide pairings, including those with the rare structural element of a disulfide bond between two sequential cysteines. (iv) Variation of the solution pH from 3 to 7 does not alter the distribution of the products among the three regioisomers. (v) Formation of the disulfide bonds is rapid, even in acidic media; for instance, oxidation of 1 mM α-conotoxin GI with 4.0 mM *trans*-$[Pt(en)_2Cl_2]^{2+}$ was found to be complete in 30 min and 3 min, respectively, in pH 4.0 and 7.0 phosphate buffer.

For comparison, fully reduced α-conotoxin GI was also oxidized with other reagents (dimethyl sulfoxide (DMSO), air and oxidized glutathione (GSSG)) used for disulfide bond formation. Oxidation of 0.8 mM α-conotoxin GI by DMSO in pH 4.0 and 7.0 phosphate buffers (within the range of pH 3–8 suggested by Tam et al.[10]) was found to proceed via formation of at least four partially folded species containing one disulfide bond and a four disulfide bond-containing dimer (confirmed by MALDI-MS[7]). After 48 h, disulfide bond formation was still incomplete at pH 4, while at pH 7 the products are the three disulfide bond-containing regioisomers and the dimer (Fig. 2). It is interesting to note that only a single dimer is formed. Air oxidation in pH 7.4 buffer was also slow, and resulted in a product distribution similar to that obtained with DMSO oxidation after 48 h. Oxidation by GSSG results in the formation of an equilibrium mixture that contains glutathione-conotoxin GI mixed disulfide intermediates and the three disulfide bond-containing regioisomers.[9,11]

A limitation compared to methods based on sequential disulfide bond formation using selectively removable protecting groups is that either standards are required or additional analyses must be performed to determine the disulfide bonding patterns of the regioisomers formed. However, once the disulfide bonding patterns are known, oxidation with *trans*-$[Pt(en)_2Cl_2]^{2+}$ to simultaneously form the three regioisomers is considerably faster and more convenient than sequential deprotection/bond formation methods.

In summary, one-step oxidation with *trans*-$[Pt(en)_2Cl_2]^{2+}$ cleanly and rapidly converts the crude reduced forms of α-conotoxin GI and α-conotoxin SI to their three disulfide bond-containing regioisomers, enabling the convenient synthesis of all three isomers. Moreover, use of the methodology described here gives a higher yield than procedures described previously.[2,6] Synthesis of regioisomers of other multiple disulfide bond-containing peptides are in progress.

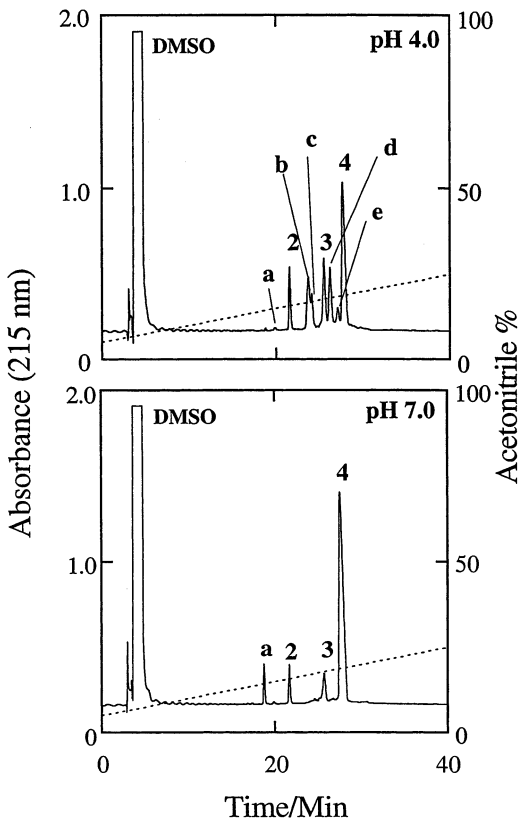


Figure 2. Chromatograms of reaction mixtures containing 0.8 mM pure fully reduced α-conotoxin GI and 4% DMSO in pH 4.0 phosphate buffer (top) and pH 7.0 phosphate buffer (bottom) after reaction for 48 h. A 4.6×250 mm Zorbax C18 column was used with an acetonitrile–water gradient mobile phase (dashed lines indicate the gradients). Peak assignments: peaks **1**–**4** are assigned according to Scheme 1; peak **a** is a peptide dimer containing four disulfide bonds; and peaks **b**–**e** are partially folded intermediates (monomers with one disulfide bond).

Acknowledgements

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