Chemical synthesis of venom peptides using directed-disulfide bond formation

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Abstract

We herein describe a method to direct disulfide bond formation of peptides containing up to three disulfide bridges. This method is based on the use of several protecting groups of cysteine. The selective and sequential formation of two disulfide bridges was first developed and optimized on the conotoxin analogue NMB-1, allowing formation of disulfide bonds in a one-pot fashion. We finally successfully applied this strategy to the synthesis of the spider toxin SGTx1 that contains 3 disulfide bridges.

Introduction

Venoms are the result of thousands of years of evolution that allowed venomous species to develop complex mixtures of peptides and proteins known as toxins. Such molecules have received a growing interest due to their potential as drugs in the medical field. These toxins are particularly well folded due to the high abundance of cysteines. Usually, toxins are first isolated from venoms, but the low quantity of material that can be extracted remains a major hurdle to overcome. To address this issue, chemical peptide synthesis is needed, and the key step is then to correctly reproduce the fold of the native biomolecule. Random folding using oxidative buffers is the easiest way to fold a cysteine-containing peptide, but generally lead to a complex mixture containing undesired products.[1]

We herein describe a chemical scheme to direct disulfide bond formation that involves the introduction of cysteines containing orthogonal protecting groups during solid phase peptide synthesis (SPPS). Among the wide range of commercially available protected cysteines, we selected three protecting groups: trityl (Trt), acetamidomethyl (Acm) and methoxybenzyl (Mob). Trityl is generally used as protecting group of cysteine for Fmoc based SPPS. This acid labile group is removed using TFA and gives a pair of unprotected cysteines that can be oxidized. This group determines the first disulfide bond to be formed. The Acm group is removed using oxidative agent like iodine,[2] which forms the second disulfide bridge by a concerted mechanism. The Mob group is deprotected using trifluoromethanesulfonic acid (TFMSA) before oxidation.[3] The strategy was first developed for the synthesis of NMB-1,[4] an analogue of conotoxin containing two disulfide bonds, using Trt and Acm groups, and adapted by adding the Mob group to the synthesis of SGTx1, toxin isolated from the spider *Scodra Griseipes* , that contains three disulfide bonds.[5]

Materials and Methods

Peptide syntheses. SPPS was carried out on an automated Symphony synthesizer from Gyros Protein Technologies using standard Fmoc/t-Bu chemistry with HCTU as coupling reagent (0.1 mmol scale).

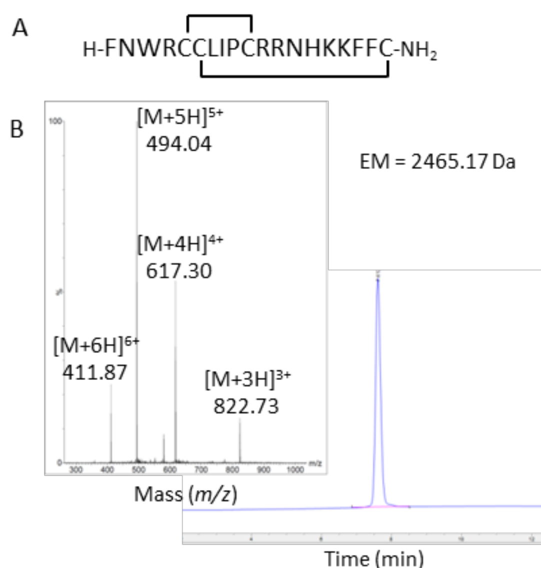
General protocol for directed-disulfide bond formation of NMB-1 in a one-pot fashion. After SPPS, crude NMB-1 was dissolved in H_2O/MeCN (1:1) at 10 mg/mL and added dropwise to a solution containing 0.1M citric acid, 2M Gn.HCl and 20% DMSO, at pH 7, to a final concentration of 0.1mg/mL in peptide. After one night under gentle stirring, pH was adjusted to 1-2, and 1 eq. of iodine 50mM in MeCN was added every five minutes, for a total of four additions. Five minutes after the last addition, the excess of iodine was quenched with sodium ascorbate and the solution was filtered and purified by preparative HPLC. Isolated yield: 57% over two steps. MS calculated 2465.17; found 2465.18

General protocol for directed-disulfide bond formation of SGTx1. After SPPS, crude SGTx1 was dissolved in H_2O/MeCN (1:1) at 10 mg/mL and added dropwise to a solution containing 0.1M citric acid and 10% DMSO, at pH 7, to a final concentration of 0.1mg/mL in peptide. After one night under gentle stirring, pH was adjusted to 1-2, and 1 eq. of iodine 50mM in MeCN was added every five minutes, for a total of four additions. Five

minutes after the last addition, the excess of iodine was quenched with sodium ascorbate and the solution was filtered and purified by preparative HPLC. The freeze dried peptide was dissolved in TFA/phenol at 0°C and TFMSA was added to reach a concentration of 5mg/mL of peptide in TFMSA/phenol/TFA (1:1:8). The mixture was stirred for 10 min at 0°C and then the peptide was precipitated with ice-cold diethyl ether, recovered by centrifugation and washed twice with diethyl ether. The peptide was dissolved in H2O/MeCN (1:1) at 10 mg/mL and added dropwise to a solution containing 0.1M citric acid and 10% DMSO, at pH 2, to a final concentration of 0.1mg/mL in peptide. After 48h, the solution was filtered and purified by preparative HPLC. Isolated yield: 3% based on resin loading. MS calculated 3773.58; found 3773.59

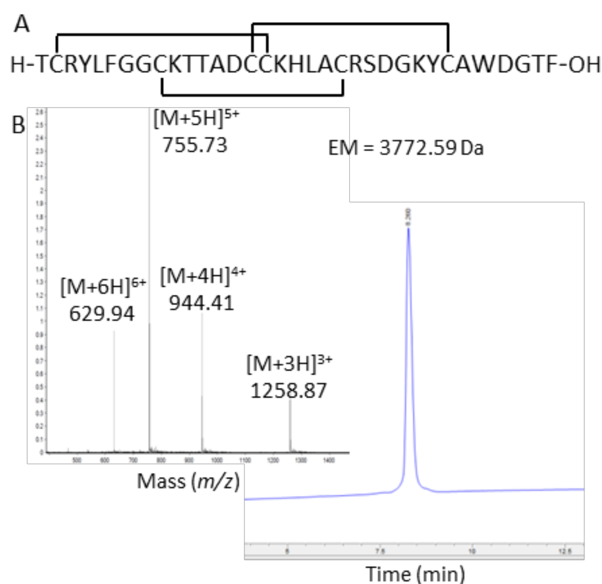
Results and Discussion

To develop our strategy, we first decided to work on NMB-1. We synthesized this toxin with a pair of cysteine (C5 and C19) protected by Trt groups and the other one (C_6 and C_{10}) by Acm groups, according to the native pattern.[4] The position of Acm groups was judiciously chosen as several side reactions[6] can occur during SPPS such as the migration of Acm on an adjacent tyrosine, or the formation of aspartimide promoted by the sequence Asp(OtBu)-Cys(Acm). The formation of each disulfide bond was achieved sequentially with an intermediate purification. The first one was formed in a Tris buffer (pH 8) containing DMSO as oxidative agent and guanidinium as denaturantand in diluted conditions avoiding intermolecular disulfide bonds. The second one was then formed in an acidic buffer to avoid scrambling of disulfide bonds which can lead to peptide misfolding.[7] After several attempts, the combination of citrate buffer and iodine in presence of guanidinium proved to be better than other acidic solutions (acetic acid, TFA, formic acid. . .). To increase the overall yield, we decided to use citric acid as buffer for both steps. First disulfide bond was formed in citrate buffer (pH 7) containing DMSO as oxidative agent and guanidinium as denaturant. The complete formation of disulfide bridge was monitored by HRMS. Interestingly, the presence of DMSO did not interfere with Acm deprotection. NMB-1 was obtained after a single RP-HPLC purification improving the isolated overall yield from 31% to 57% (Scheme 1).



Scheme 1: (A) Sequence and disulfide pattern of NMB-1 ; (B) Analytical RP-HPLC (λ = 214 nm) and ES-MS of purified NMB-1. Observed mass 2465.18 Da vs calculated mass 2465.17 Da

We then used this strategy to prepare SGTx-1 toxin that contains three disulfide bonds. As problematic sequences (Tyr-Cys, Asp-Cys) for Acm group are present, we chose to protect cysteines, according to the native pattern describe in the literature,[5] as followed: C_2 and C_{16} with Trt, C_9 and C_{21} with Acm, C_{15} and C_{28} with Mob. The first two disulfide bridges were formed as described previously, then, Mob groups were cleaved with TFMSA to give the last cysteine pair that was finally oxidized in cystine in citrate buffer at pH 2 containing DMSO. The final product was isolated by RP-HPLC (Scheme 2).



Scheme 2: (A) Sequence and disulfide pattern of SGTx1 ; (B) Analytical RP-HPLC (λ = 214 nm) and ES-MS of purified SGTx1. Observed mass 3772.59 Da vs calculated mass 3772.58 Da

Conclusion

Herein, we report a convenient strategy to direct disulfide formation of three cysteine pairs by using Trt, Acm and Mob groups. This approach has been applied with success to relevant toxins. Interestingly, regioselective formation of two disulfide bonds was carried out in one-pot, avoiding fastidious and time-consuming purification of intermediates.

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