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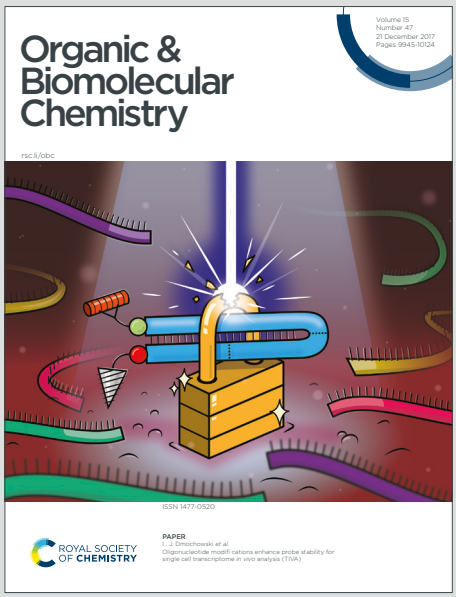




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ARTICLE

# Facile synthesis of the sulfotyrosine-containing α-Conotoxins†

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α-Conotoxins (Ctx) can selectively target distinct subtypes of the nicotinic acetylcholine receptors (nAChRs), which are closely related to a number of neurological diseases, and they have been considered as ideal probes and model peptide drugs. Sulfotyrosine (sY) is an important post-translational modification and believed to modulate certain key protein-protein interactions. Although sY modification has been indicated in several α-Ctx, its biological consequence has largely remained unexplored, mostly because of the difficulties in both the extraction from biological samples and chemical synthesis. Herein, we report a facile synthesis and folding strategy for obtaining the sY modified α-Ctx. This strategy is based on the development of a simple and controlled deprotection of the neopentyl protecting group of the sulfate ester as well as its compatibility with a step-wise oxidative folding of the two disulfide bonds. Eight sY modified α-Ctx peptides were successfully synthesized in high purity and yield, and their serum stabilities were almost comparable with the non-modified peptides.

## Introduction

Conotoxins (Ctx) belong to a large family of peptide toxins derived from the venoms of cone snail, which act on a variety of ion channels and receptors.[1] α-conotoxin (α-Ctx) is a subtype of Ctx, which typically has 12-19 amino acid residues and two disulfide bonds (Cys1-Cys3 and Cys2-Cys4, respectively), forming a rigid two-loop helical framework.[2,3] α-Ctx can selectively target ion channels and distinct subtypes of the nicotinic acetylcholine receptors (nAChRs). Since nAChRs are closely related with neurological diseases such as nicotine addiction, severe pain, epilepsy, and Alzheimer's disease, α-Ctx have been regarded as ideal molecular probes for target validation and peptide drug development.[4-6] The diverse selectivity of α-Ctx has been largely attributed to the comprising amino acid residues, where mutating key residues can fine-tune their nAChR subtype specificity.[7,8] Moreover, the extracted native α-Ctx have been shown to have gone through a number of post-translational modifications (PTM),[7] especially the tyrosine sulfation that leads to sulfotyrosine (sY)-containing α-Ctx.[9-11] While sY modification has been considered a common and important mode of protein PTM,[12] its biological impact on α-Ctx, be it the subtype specificity or affinity, remains largely unexplored.

It has been a general problem to produce and study the sulfated peptides/proteins from biological systems due to the lability of the sulfate ester group, fortunately, a number of key synthetic advancements have made it possible to construct the sY modified peptides/proteins in a highly controllable fashion.[13] Several acid-stable orthogonal protection groups of the sY have been developed, which enables the direct synthesis of sulfopeptides via standard Fmoc-SPPS.[13-19] Among them, the most widely used is the so-called cassette strategy developed by Widlanski and co-workers, where the neopentyl (nP) moiety is employed as a protection group in the monomer Fmoc-Tyr($OSO_3$-nP)-OH.[16] As shown in **Scheme 1**, the monomer can be conveniently installed in a peptide sequence, after which the nP protection group can be removed by either sodium azide or ammonium acetate treatment, at elevated temperatures, e.g. 37–70 °C. The resulting sulfopeptides can then be purified by HPLC, typically using ammonium acetate and acetonitrile (MeCN) as eluents. A number of sY containing peptides/proteins have been successfully obtained with this strategy, however, with the presence of free Cys in the peptide sequence only in rare cases.[20-24]

The ammonium acetate solution used for both the nP deprotection and HPLC purification is, in principle, not a buffer, and may subject to pH changes when different solutes are used.[25] It can be imagined (also proved from this work, see below) that free thiols, like in the case of α-Ctx, may be susceptible to oxidation during the nP removal or the HPLC purification, which complicates the disulfide pairing process. As such, the current cassette strategy may not be compatible with the synthesis of sY-modified peptides having multiple Cys residues, like α-Ctx. As such, an alternative strategy will be required, which is the aim of the present work.

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†Electronic Supplementary Information (ESI) available: monomer and peptide analytical data; table of conotoxin sequence and total synthetic yields. See DOI: 10.1039/x0xx00000x

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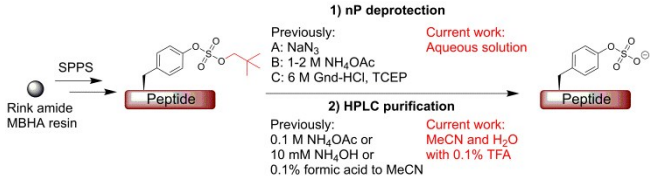
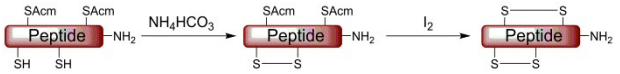



**Scheme 1.** Overview of the cassette strategy for the synthesis of sulfopeptides using nP protecting group, highlighting the major discoveries of the current work.

## Results and discussion

### Synthesis of non-sY modified conotoxin

We selected eight α-Ctx peptides, namely EpI, PnIA, PnIB, PnIC, AnIA, AnIB, AuIA and PnMGMR-02, that have either been reported or predicted (according to Sulfinator[26]) to be sY modified, and their amino acid sequences are summarized in **Table S1**. Initially, a direct synthetic strategy was employed, where four Cys residues were left unprotected after SPPS. Under a variety of folding conditions tested, multiple disulfide isomers could always be observed (data not shown, which may affect the overall yield) — a known major challenge in the folding of these peptides.[27] We therefore switched to a step-wise disulfide pairing strategy as highlighted in **Scheme 2**.[28] Here, the Cys residues 2 and 4 were side-chain protected with Acm and the first disulfide bond was selectively formed between Cys 1 and 3, after which the Acm group can be easily removed using $I_2$ and the second disulfide bond formed between Cys 2 and 4. Note that the protection of the other disulfide pair (i.e. Cys 1 and 3) initially with Acm works just as well. More importantly, all these steps can be carried out in a one-pot fashion, which significantly improved the overall yield. With this strategy, we obtained all eight non-sY modified α-Ctx in good yield and high purity. (**Fig. S1, Table S2**)




**Scheme 2**. Regio-selective folding strategy using Acm protecting group.

### Removal of nP from Cys-rich peptides by ammonium acetate

With the synthetic route developed for the non-modified α-Ctx, we proceeded to the synthesis of the sY modified peptides. Using AnIC (GGCCSHPACFASNPDYC) as an example, we placed the Cys 4,17-Acm protected sY-peptide **1** under the literature conditions, i.e. 1-2 M $NH_4OAc$ solution, for nP deprotection. Although the desired species **3** was seen as the major product in the solution **(Fig. 1D)**, significant precipitation, however, occurred soon after dissolving the peptide **(Fig. 1E)**. HPLC analysis indicates the presence of at least 4 major species, **1**-**4** (**Fig. 1**). As mentioned earlier, we reasoned that $NH_4OAc$ solution itself is not a probable buffer and precipitation may occur depending on the property of the solute peptides, which could also complicate the HPLC purification were it to be used as the eluent, as in the literature.[13] As such an alternative nP deprotection strategy is needed in order to synthesize the sY-containing α-Ctx. During the processing of peptide **1**, we found that the nP group is not stable if stored longer in aqueous solutions (data not shown). It was also reported that the nP group can be removed after native chemical ligation if left longer in the ligation buffer,[21,22] we were interested to see whether the deprotection can just happen in any aqueous (buffer) system. If this is the case, we may achieve one-pot nP deprotection while folding of the α-Ctx.

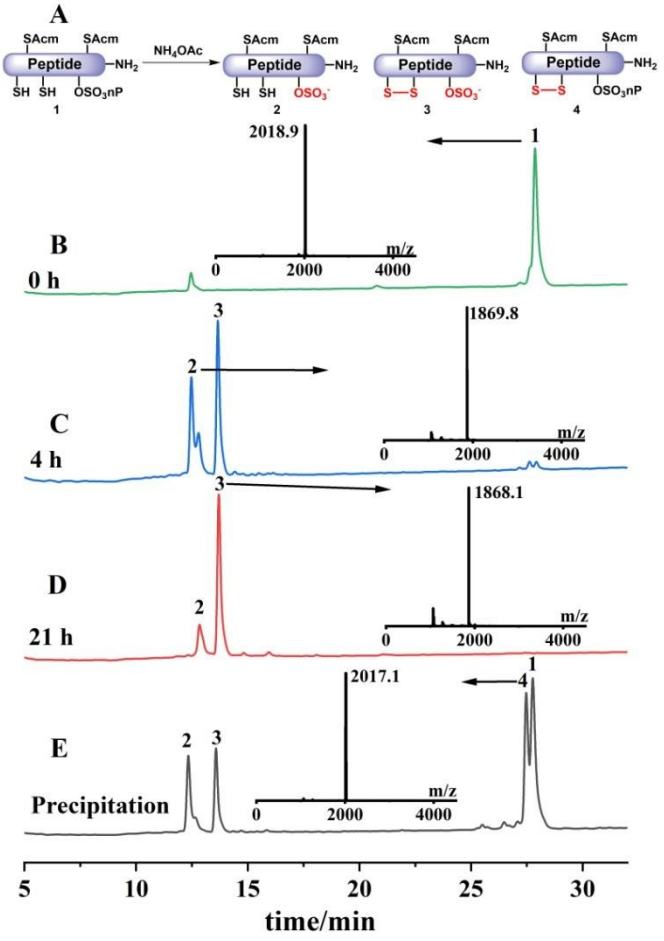



**Figure 1**. nP removal in peptide **1** using $NH_4OAc$ solution. **(A)** Schematic presentation of the major species formed. **(B)-(D):** Analytical HPLC trace (214 nm) of the reaction after 0-21 h, respectively. Insert: MALDI-TOF-MS characterizations, **1** ($[M+H]^+_{obs}$: 2018.9 Da, $[M+H]^+_{calc}$: 2021.6 Da); **2** ($[M+H]^+_{obs}$: 1869.8 Da, $[M-SO_3+H]^+_{calc}$: 1871.6 Da); **3** ($[M+H]^+_{obs}$: 1868.1 Da, $[M-SO_3+H]^+_{calc}$: 1869.6 Da) Note that the sulfate group will be cleaved off under the laser used in MALDI. **(E)** Analytical HPLC trace (214 nm) of the precipitation re-dissolved in 6 M Gnd-HCl and MALDI-TOF-MS characterization of **4** ($[M+H]^+_{obs}$: 2017.1 Da, $[M+H]^+_{calcd}$: 2019.6 Da). HPLC condition: 15%-40% MeCN (with 0.1% TFA) in 25 min.

### Removal of nP in aqueous solutions

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We firstly tested the stability of the Fmoc-Tyr($OSO_3$-nP)-OH monomer in MeCN/$H_2O$ system as well as a number of buffer solutions (**Fig. S7**). It shows clearly that as the water content increases, the extent of nP deprotection increases, with almost complete removal when only $H_2O$ is used (Note that the monomer is completely stable in MeCN, **Fig. S7C**). Further, a number of buffer solutions (inclusive 20% MeCN for the solubilization of the monomer), ranging from the routinely used phosphate buffer, 6 M Gnd-HCl buffer and acetate buffer to refolding buffer like $NH_4HCO_3$ buffer with oxidizing agents were tested, and 55-70% of removal could be achieved within 24 hrs of the incubation. These results encouraged us to proceed further with the deprotection of nP in a model peptide **5** containing an N-terminal sY modification (**sY**DC(Acm)STNISPKQGLDK) using selected buffers. Gratifyingly, it was shown that under the conditions tested, ranging from acidic to basic pHs, nP can be successfully removed within 24 hrs, with very high conversion yields in $H_2O$ and $NH_4HCO_3$ buffer (both over 90%, **Fig. 2 C&F**). It suggests that nP removal might be achieved essentially in any aqueous buffer, provided that the pH is probably controlled such that the peptide itself is stable throughout the process.

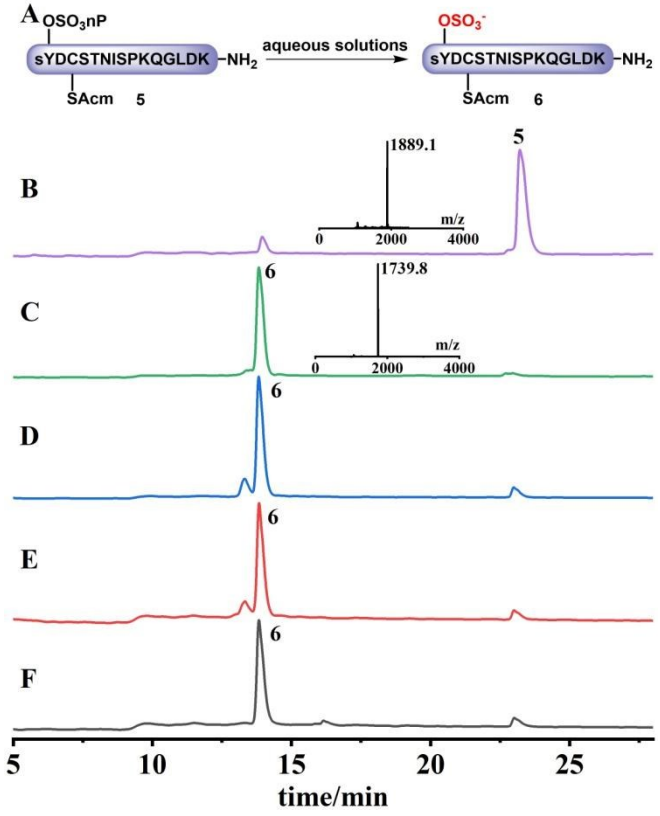


**Figure 2** Analytical HPLC (214nm) and MALDI-TOF-MS of nP removal from model peptide **5** in different solutions for 24h. **(A)**. Schematic presentation of the nP removal. **(B)**. Purifeid model peptide **5**. **(C)**. nP removal in $H_2O$, pH 7.1. **(D)**. nP removal in 0.1 M NaOAc and 0.1 M NaCl, pH 5.5. **(E)**. nP removal in 2 M $NH_4OAc$, pH 7.0. **(F)**. nP removal in 0.1 M $NH_4HCO_3$, pH 8.4. MS of **5**: $[M+H]^+_{obs}$: 1889.1 Da, $[M+H]^+_{calc}$: 1889.8 Da. MS of **6**: $[M+H]^+_{obs}$: 1739.8 Da, $[M-SO_3+H]^+_{calc}$: 1739.8 Da. HPLC condition: 10%-40% MeCN (with 0.1% TFA) in 20 min.

**One-pot synthesis of sY modified α-Ctx**

Having established that $NH_4HCO_3$ buffer is compatible with sY deprotection, we employed a similar two-step oxidative folding strategy as for the synthesis of the non-modified α-Ctx. Taken the synthesis of sY-AnIC as an example, the nP protected peptide **1** was incubated in 0.1 M $NH_4HCO_3$, pH 8.4 for nP removal as well as the formation of the disulfide bond between Cys 1 and 3 under air oxidation. As shown in **Fig. 3C**, the nP group was completely removed after 4 hrs and the disulfide bond was partially formed. When left longer, e.g. 24 hrs, the complete formation of disulfide bond could be established (**Fig. 3D**). It is noted that for several α-Ctx peptides, i.e. AnIA, AnIB and AuIA, the formation of the disulfide Cys1-Cys3 was too slow and incomplete even after 48 hrs. In these cases, the addition of oxidizing agent $K_3Fe(CN)_6$ (10 mM) was found to be very effective in promoting the disulfide formation (**Fig. S8-S10**). Care should be taken when adding $K_3Fe(CN)_6$, cause excess of it might lead to peptide dimerization.

Next, the solution was acidified and the Acm deprotection as well as the formation of the second disulfide bond was promoted via the addition of $I_2$. This process was usually very efficient, e.g. in 5-10 min, and ascorbic acid was added to quench the reaction. As shown in **Fig. 3E**, the $I_2$ promoted Acm deprotection and disulfide formation was very clean and the transformation was almost quantitative. It is worth mentioning that for peptides containing oxidation sensitive residues, i.e. Met, the $I_2$ treatment time should be kept minimum, which is the case for sY-EpI (**Fig. S11**). Through this one-pot stepwise disulfide pairing strategy, all eight sY α-Ctx containing peptides were obtained with high purity and good overall yield (30-70%, **Fig. S8-S14, Table S3**).

To our surprise, we did not find instability of the sulfate ester on the modified α-Ctx peptides when the solution was acidified (to pH 3) for the removal of Acm group, which encouraged us to further purify the folded peptides using normal MeCN/$H_2O$ (0.1% TFA) via semi-preparative HPLC (the presence of sY modification was clearly indicated in the MS traces under negative mode, **Fig. 3C-E** insert). While most literature claim that sulfate ester is acid-labile,[13,14,16,29,30] we reason that it may be because of the tight tertiary structure of α-Ctx, and with the help of H-bonding, that protects sY from hydrolysis. Nevertheless, the present work suggests that the sulfate ester group of the modified Tyr might not be acid-labile in all the cases, which makes the purification process much more convenient.

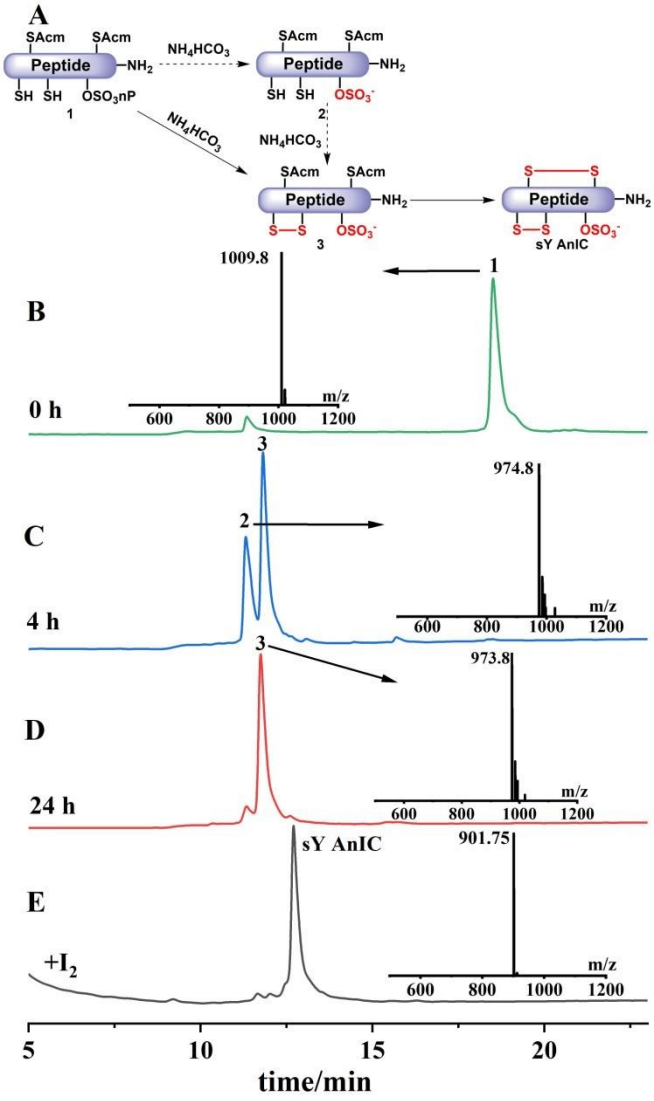


**Figure 3** Synthesis of sY-AnIC. **(A)** Schematic presentation of nP removal and regio-selective folding strategy. **(B)-(D)** Analytical HPLC trace (214 nm) of the folding in $NH_4HCO_3$ after 0-24 hrs incubation, respectively. Insert: ESI-MS characterization, **1** ($[M-2H]^{2-}_{obs}$: 1009.8 Da, $[M-2H]^{2-}_{calc}$: 1009.8 Da); **2** ($[M-2H]^{2-}_{obs}$: 974.8 Da, $[M-2H]^{2-}_{calc}$: 974.8 Da); **3** ($[M-2H]^{2-}_{obs}$: 973.8 Da, $[M-2H]^{2-}_{calc}$: 973.8 Da). **(E)** Analytical HPLC trace (214 nm) following $I_2$ treatment and ESI-MS characterization of sY AnIC peptide ($[M-2H]^{2-}_{Obs}$: 901.75 Da, $[M-2H]^{2-}_{calc}$: 901.8 Da). HPLC condition: 15%-50% MeCN (with 0.1% TFA) in 15 min.

#### Serum stability test of conotoxin

With all sY-containing α-Ctx in hand, we further tested and compared the stability of the modified peptides with those unmodified. The peptides were incubated together with 50% serum solution at 37 °C for 24 hrs, the resulting solution was analyzed by HPLC for peptide integrity (**Figs. S15-S22**). As seen from **Fig. 4**, similar to the non-modified peptides, at least 60% of the sY-containing α-Ctx was intact after 24 hrs of the digest by serum. It indicates that in all the cases sY modification does not affect the serum stability, which may be crucial for the execution of its biological function under complex physiological conditions.

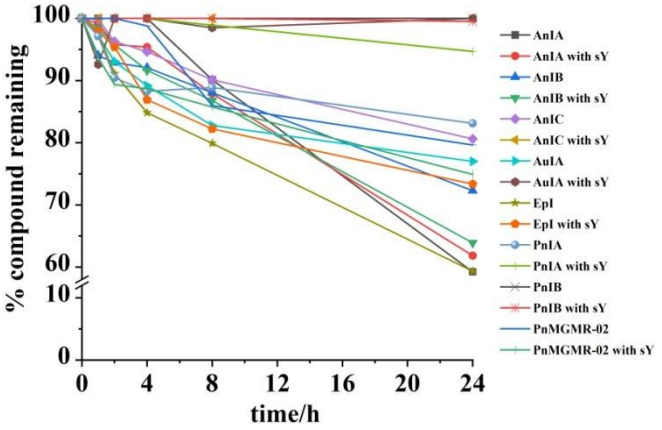


**Figure 4** Comparison of the serum stability between non-modified α-Ctx and sY-containing α-Ctx.

## Experimental

#### General methods

Amino acid, coupling reagent and resins for peptide synthesis were obtained from GL Biochem. Fmoc-Tyr($OSO_3$nP)-OH was synthesized by our own lab according to the literature (Fig. S2-S6)[16]. General reagents were obtained from Sigma or Sangon, HPLC grade MeCN was from Fisher. Horse serum was obtained from Sangon. Analytical HPLC (Agilent 1260) was performed on a Phenomenex Jupiter 4.6 µm C18 4.6×250 mm column running at a flow rate of 1 mL/min with UV detection at 214 nm. The semi-preparative HPLC (Shimadzu AR-20) was performed using a Phenomenex Jupiter 4.6 µm C18 10×250 mm column running at a flow rate of 5 mL/min with UV detection at 214 nm. Both instruments were run using a mobile phase composed of 0.1% TFA in $H_2O$ (Solvent A) and 0.1% TFA in MeCN (Solvent B) in a linear gradient as indicated. The LC-MS was performed on Agilent LC/MSD (ESI) operating in negative mode to detect the sulfate group. Low Resolution MALDI-TOF mass spectra (Shimadzu 8020) were operated in linear mode using a matrix of 10 mg/mL HCCA in water/MeCN (1:1 v/v) with no TFA.

#### Peptide Synthesis and folding

Peptides were synthesized by manual solid-phase Fmoc chemistry on Rink Amide MBHA resins. Couplings were performed using 4 eq of activated amino acid (HOBT: DIC: amino acid, 1:2:1) relative to resin for about 2 hrs, While Fmoc-Tyr($OSO_3$nP)-OH were performed using 2 eq of the resins. In order to form two disulfides (Cys1-Cys3, Cys2-Cys4) selectively, we used Trt group for the protection of Cys1 and Cys3, and Acm group for Cys2 and Cys4. Fmoc deprotection was carried out using 20% piperidine 2×5 min. Kaiser reagent were used to test the extent of coupling, and typically a 0.5

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mmol/g loading was used. Following each coupling, capping or deprotection step, the resin was washed with DMF×2, DCM×2, DMF×2.

Upon complete assembly of the peptide chain, the peptides were cleaved from the resin with TFA, DODT and $H_2O$ (95:2.5:2.5, v/v/v) and incubated for 2h. At this point, the Acm groups of Cys and the nP group of Tyr were intact. Next, the resin was removed by filtration and volatile substances were removed by vacuum. The released peptides were precipitated and washed by cold ether, then centrifuged at 8,000 rpm for 10 min. The precipitate was collected and lyophilized. The obtained crude peptides were purified using RP-HPLC (This step must be fast to prevent nP group from being removed in $H_2O$/MeCN solution).

Afterwards, the linear peptides can be folded with correct disulfide pairs by using a two-step oxidation strategy. To remove nP group and form the first disulfide, we used a 0.1 M $NH_4HCO_3$, pH 8.4 buffer and air-oxidation was applied here. Peptides were dissolved to 1 mg/mL. While this condition was enough for most α-Ctx, some α-Ctx peptides (AnIA, AnIB and AuIA) couldn't fold completely. In these cases, 10 mM $K_3Fe(CN)_6$ (excessive amounts will cause peptide dimerization) was added to promote the disulfide formation, and peptides were dissolved to 0.3 mg/mL. In both of the above conditions, the reaction was left for several hours and almost complete conversion could be observed. Later, HCl was added the solution to remove $NH_4HCO_3$ and pH adjusted to about 3. Then, 0.05 eq of 0.1 M $I_2$/MeOH was added to remove the Acm group as well as to promote the second disulfide formation and the solution immediately became cloudy. This reaction takes just several minutes (The reaction time of EpI should be kept as short as possible, because the Met contained in it can be easily oxidized). Next, 1 M ascorbic acid was added until the solution became clear. Finally, the folded peptides were purified by semi-preparative HPLC.

#### Serum stability test

Horse serum was centrifuged at 16,000 rpm and 4°C for 15 min to separate lipid layer and serum. Each peptide was dissolved in serum at a concentration of 500 μg/mL and incubated at 37°C. A 100 μL aliquot extracted at a range of incubation times (0, 1, 2, 4, 8, 24 hrs) was quenched with 15 μL trichloroacetic acid. Then, the samples were centrifuged at 10,000 rpm for 10 min, and 50 μL of the supernatant was analyzed on analytical HPLC, and the percentage of intact peptide was calculated based on the area under its HPLC peak. Peptide samples in saline solution were run for each time point as a control.

## Conclusions

In conclusion, we have established that the nP protection group can essentially be removed in any aqueous solution tested. This discovery leads to the development of a one-pot nP deprotection and peptide folding strategy, which was harnessed for the facile synthesis of eight sY-containing α-Ctx peptides. Further, in contrast to literature that claims the sY ester as instable under acidic conditions, the modified α-Ctx is pretty robust when purified with eluents containing 0.1% TFA in our hands, and this will be crucial in obtaining the modified peptides in large quantities. While previous reports have indicated the presence of sY modification in α-Ctx either via LC-MS analysis or a possible sulfate electron density in the X-ray crystallography structure,[31] the investigation of its biological consequence has rarely been possible. With the strategy developed herein, these key questions can now be approached. While these studies are ongoing in our group, the results will be reported should they become available. Moreover, the current strategy may find general applicability in the synthesis of other sY-containing peptides that also have multiple Cys residues.

## Conflicts of interest

A Chinese patent is filed covering the synthetic method for sY modified α-Ctx described in this manuscript (Application No. CN201910944921.6).

## Acknowledgements

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## Facile synthesis of the sulfotyrosine-containing α-Conotoxins

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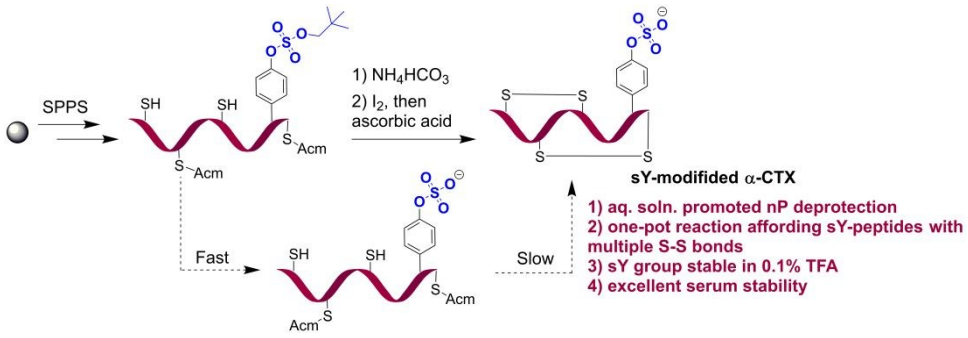


A one-pot neopentyl deprotection and oxidative disulfide pairing strategy was developed for the facile synthesis of sulfotyrosine (sY)-containing α-Conotoxins (Ctx).