Synthesis of Scyllatoxin-Based BH3 Domain Mimetics with Diverse Patterns of Native Disulfide Bonds

Matthew J. K. Vince[1,2,3] and Justin M. Holub[1,4,5,6]

[1]Department of Chemistry and Biochemistry, Ohio University, Athens, Ohio
[2]Institut für Bioanalytische Chemie, Fakultät für Chemie und Mineralogie, Universität Leipzig, Leipzig, Germany
[3]Biotechnologisch-Biomedizinisches Zentrum, Universität Leipzig, Leipzig, Germany
[4]Edison Biotechnology Institute, Ohio University, Athens, Ohio
[5]Molecular and Cellular Biology Program, Ohio University, Athens, Ohio
[6]Corresponding author: *holub@ohio.edu*

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This article outlines the design and development of scyllatoxin (ScTx)–based BH3 domain mimetics with diverse patterns of native disulfide bonds. More specifically, this method summarizes the total chemical synthesis of ScTx-based peptides that contain zero, one, two, or three disulfide linkages, including techniques to generate variants with any combination of native disulfides. Each peptide reported herein is generated on solid-phase support using microwave-assisted coupling procedures, and all reaction parameters related to the peptide synthesis are described in detail. The various disulfide patterns of the ScTx-based constructs are established during peptide synthesis and are ultimately verified by mass analysis of trypsin-digested fragments. The BH3 domain mimetics developed herein were generated by transposing residues from the helical BH3 domain of the pro-apoptotic BCL2 protein Bax to the α-helix of wild-type ScTx. Interestingly, we found that the relative binding affinities of ScTx-Bax peptides for the anti-apoptotic BCL2 protein Bcl-2 (proper) were heavily influenced by the number and position of disulfide linkages within the ScTx-Bax sequence. As a consequence, we were able to utilize ScTx-Bax BH3 domain mimetics with varied patterns of disulfide bonds to survey how structural rigidity within the helical Bax BH3 domain affects binding to promiscuous anti-apoptotic BCL2 proteins. More broadly, the ability to generate ScTx-based molecules that contain any combination of native disulfide bonds expands the utility of such constructs as tools to study the molecular nature of protein-protein interactions. © 2022 Wiley Periodicals LLC.

Basic Protocol 1: Synthesis and characterization of ScTx-based Bax BH3 domain mimetics
Basic Protocol 2: Oxidation of ScTx-Bax BH3 domain mimetics containing one, two, or three disulfide linkages
Support Protocol: Mapping of disulfide linkages in oxidized ScTx-Bax BH3 domain mimetics

Keywords: BCL2 proteins • BH3 domain mimetic • disulfide bond • protein-protein interaction • scyllatoxin

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INTRODUCTION

The procedure outlined in this article uses solid-phase peptide synthesis (SPPS) techniques in conjunction with several distinct oxidation reactions to generate scyllatoxin (ScTx)–based molecules that include diverse patterns of native disulfide linkages (Arachchige & Holub, 2018; Arachchige, Margaret Harris, Coon, Carlsen, & Holub, 2017; Harris, Coon, Alqaeisoom, Swords, & Holub, 2016). Introducing native disulfides at different positions within the ScTx sequence enables the flexibility of the molecule to be finely tuned, and allows such constructs to be used as tools to study the molecular nature of protein-protein interactions (PPIs) (Arachchige et al., 2017; Zhu et al., 2002). Furthermore, the inclusion of natural cysteine residues allows ScTx-based peptides to be oxidized (folded) without the need for non-natural amino acids (Henchey, Jochim, & Arora, 2008). Specifically, we were able to use ScTx-based molecules to study how flexibility within the helical BH3 domains of pro-apoptotic BCL2 proteins affects targeting of anti-apoptotic BCL2 proteins *in vitro*. While this strategy was used specifically to develop ScTx-based BH3 domain mimetics, this modular yet straightforward procedure could, in principle, be used to develop structural variants of ScTx that are designed to interact with other biomolecular targets (Li et al., 2008; Vita et al., 1999; Vita, Roumestand, Toma, & Menez, 1995).

Miniature proteins are a class of short-chain oligopeptides (less than 50 amino acids) that fold into stable three-dimensional structures. Despite their small size, the globular folds of miniature proteins are often stabilized by the same forces (e.g., electrostatic interactions, salt bridges, metal-ion complexation, hydrophobic effects, and disulfide linkages) found in much larger proteins. Owing to their synthetic tractability and well-defined architectures, miniature proteins have emerged as attractive alternatives to small-molecule- and protein-based drugs for targeting therapeutically-relevant PPIs (Holub, 2017; Li et al., 2008; Vita et al., 1999). Indeed, miniature proteins occupy a unique 'middle-space', combining compact size with elements of well-folded secondary/tertiary structures that can be used to target protein interaction surfaces. The overall utility of miniature proteins as chemical genetics agents can be further expanded through a technique known as protein grafting (Harris et al., 2016; Rutledge, Volkman, & Schepartz, 2003; Sia & Kim, 2003). In this strategy, select residues within the native miniature protein are replaced by residues from another protein that are important for molecular recognition. Provided that specific sequence and structural elements are retained, the re-engineered miniature protein can be applied to modulate various types of PPIs. Over the past 20 years, protein grafting has been applied to diverse structural elements within miniature proteins (e.g., α-helices, turns, β-sheets, and polyproline helices), and has facilitated the development of miniature protein–based mimetics that have been used to inhibit such interactions as CD4:gp120, p53:MDM2/MDMX, and Mena:ActA (Golemi-Kotra et al., 2004; Li et al., 2008; Vita et al., 1999).

ScTx is a small, 31-amino-acid protein that folds into an α/β structural motif stabilized by three native disulfide linkages between residues C3-C21, C8-C26, and C12-C28 (Figure 1; Martins, Van de Ven, & Borremans, 1995). While wild-type ScTx contains three disulfide linkages, it has been shown previously that just two are necessary to fold ScTx into its α/β structure (Zhu et al., 2002). ScTx-based molecules with two or three disulfide linkages are well-folded and have limited flexibility within their respective tertiary structures (Arachchige & Holub, 2018; Harris et al., 2016; Martins et al., 1995; Zhu et al., 2002). On the other hand, ScTx-based molecules with zero or one disulfide linkage are inherently more flexible and capable of adopting a greater number of structural configurations than ScTx-based variants with two or three disulfide bonds (Arachchige et al., 2017; Harris et al., 2016). From a structural perspective, the native disulfides within the ScTx sequence are positioned along the α-helix at three distinct locations. For example, one is placed near the N-terminus of the helix (C3-C21), one near the middle of the helix

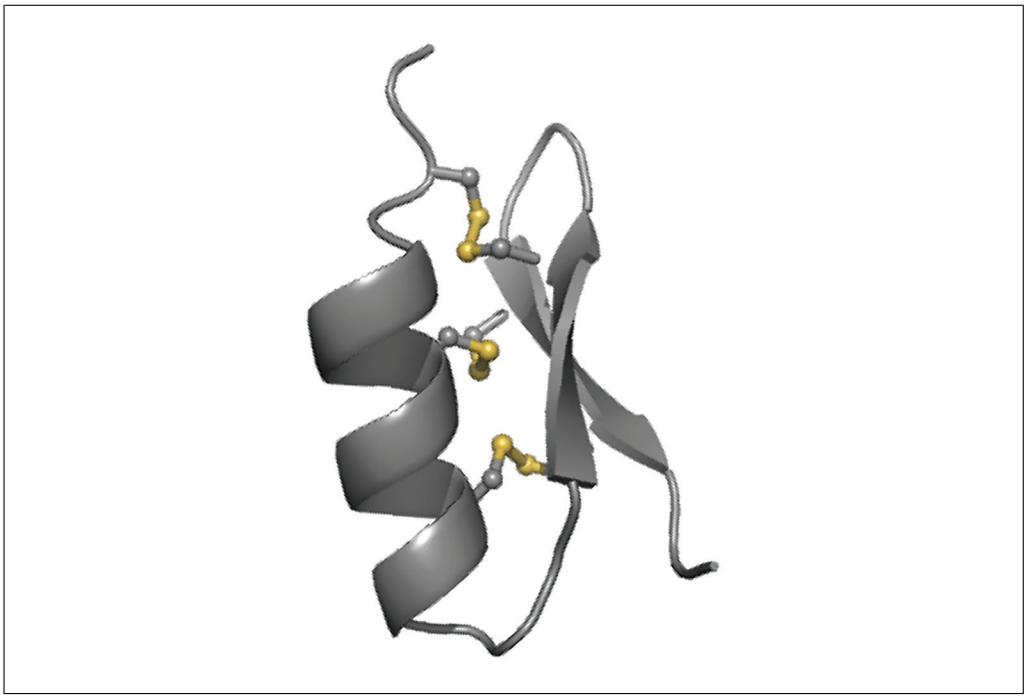

Figure 1 NMR structure of ScTx showing well-defined α/β structural motif. N-terminus is at top left; disulfide bonds are rendered as ball and stick between cysteines C3-C21, C8-C26, and C12-C28. Adapted from PDB ID: 1SCY. The primary sequence of wild-type ScTx is: AFCNLRMCQLSCRSLGLLGKCIGDKCECVKH.

(C8-C26), and one near the C-terminus of the helix (C12-C28). This led us to speculate that the α-helix of ScTx proteins with just one native disulfide will have comparatively different flexibilities depending on where the disulfide is positioned. We further hypothesized that the protein-grafting approach could be combined with a so-called 'disulfide patterning' strategy to develop unique molecular tools that can be applied to study the structural requirements of discrete PPIs that involve helical interaction domains. This concept has recently been used by our group to characterize the molecular requirements for favorable BH3:BCL2 interactions between anti- and pro-apoptotic BCL2 proteins (Arachchige & Holub, 2018; Arachchige et al., 2017; Harris et al., 2016). Here, we developed a library of ScTx-based peptides with different patterns of native disulfide linkages, and grafted residues of the pro-apoptotic BCL2 protein Bax to the α-helix of each ScTx variant. We then applied our constructs to study how flexibility within the helical BH3 domain affected binding to the anti-apoptotic BCL2 protein Bcl-2 (proper) *in vitro.* Importantly, these studies allowed us to determine discrete structural properties that are important for favorable BH3:BCL2 interactions.

Herein, we outline a basic protocol to synthesize ScTx-based BH3 domain mimetics containing zero, one, two, or three disulfide linkages, including all combinations thereof. Specifically, Basic Protocol 1 describes the microwave-assisted synthesis of ScTx-Bax peptides on solid-phase support. It should be noted that this synthesis scheme can be applied to any ScTx peptide that is generated on solid support, and will ultimately determine the disulfide linkage pattern of the final construct. Despite containing similar covalent linkages (the disulfide bond), the synthetic strategies to oxidize one, two, or three disulfides within the ScTx-based variants require moderately different approaches. Basic Protocol 2 describes three distinct oxidation (folding) reactions for ScTx-based BH3 domain mimetics containing one, two, or three disulfide linkages. Here, native disulfides are installed between different combinations of complementary cysteine pairs (C3-C21, C8-C26, or C12-C28). Finally, the Support Protocol outlines techniques to map the disulfide linkages within the oxidized ScTx-based BH3 domain mimetics using trypsin digestion

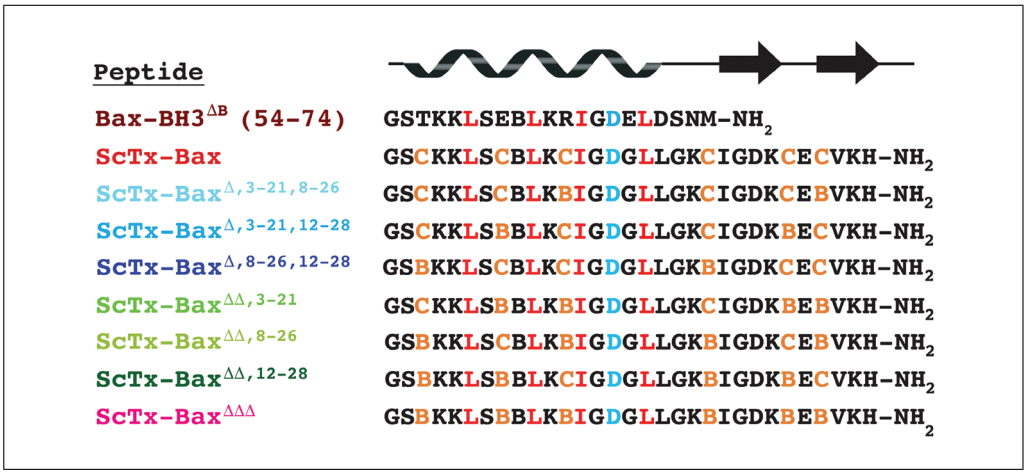

Figure 2 Sequence alignment of the Bax BH3 domain peptide (residues 54-74) and ScTx-Bax structural variants developed in this work. BH3 residues required for targeting anti-apoptotic BCL2 proteins are shown in red; structural cysteines and aminobutyric acids are orange; conserved BH3 aspartate residue is colored cyan. Peptide nomenclature indicates number and position of disulfide bonds (e.g., ScTx-Bax$^{\Delta\Delta,3\text{-}21}$ includes one cysteine pair between C3 and C21). B, aminobutyric acid. Cysteine thiol protecting groups for each isoform are outlined in Table 1.

Table 1 Thiol Protecting Groups of ScTx-Bax Isoforms[a]

Peptide	Native Cys residues with protecting groups
ScTx-Bax	C3(Trt); C8(Trt); C12(Trt); C21(Trt); C26(Trt); C28(Trt)
ScTx-Bax$^{\Delta,3\text{-}21,8\text{-}26}$	C3(Acm); C8(Trt); C21(Acm); C26(Trt)
ScTx-Bax$^{\Delta,3\text{-}21,12\text{-}28}$	C3(Acm); C12(Trt); C21(Acm); C28(Trt)
ScTx-Bax$^{\Delta,8\text{-}26,12\text{-}28}$	C8(Acm); C12(Trt); C26(Acm); C28(Trt)
ScTx-Bax$^{\Delta\Delta,3\text{-}21}$	C3(Trt); C21(Trt)
ScTx-Bax$^{\Delta\Delta,8\text{-}26}$	C8(Trt); C26(Trt)
ScTx-Bax$^{\Delta\Delta,12\text{-}28}$	C12(Trt); C28(Trt)
ScTx-Bax$^{\Delta\Delta\Delta}$	None

[a] Trt, trityl; Acm, acetamidomethyl.

and mass spectrometry (Arachchige & Holub, 2018; Zhu et al., 2002). Collectively, these protocols will allow users to synthesize a library of eight ScTx-based structural variants that include all possible combinations of native disulfide linkages. We anticipate that this method will be broadly applicable to any ScTx-based molecule that includes native disulfide bonds, and will allow researchers a wide range of options to optimize disulfide patterning when developing ScTx-based mimetics to investigate the molecular nature of discrete PPIs.

CAUTION: All chemical reactions described herein must be run in a suitable fume hood with efficient ventilation. Many of the reactions in this article are exothermic and use reagents that are caustic or harmful. Safety glasses, reagent-impermeable protective gloves, and appropriate laboratory outerwear should be worn at all times when handling these reagents.

STRATEGIC PLANNING

Rational Design of ScTx-Bax BH3 Domain Mimetics

In this article, we describe the synthesis and characterization of a library of ScTx-based BH3 domain mimetics that contain diverse patterns of native disulfide linkages.

Specifically, these ScTx-based BH3 domain mimetics are designed to mimic the N-terminal region (residues 55-70) of the helical BH3 domain of the pro-apoptotic BCL2 protein Bax (ScTx-Bax). In order to generate ScTx-Bax constructs that could target the anti-apoptotic BCL2 protein Bcl-2 (proper), we first considered several possible sequence alignments between the Bax BH3 domain and the α-helical portion of ScTx (Figure 2). Arrangements were scored based on their ability to align BH3 residues important for Bcl-2 recognition with solvent-exposed residues within the ScTx α-helix. ScTx-Bax sequences were also designed so that native cysteine residues required for folding aligned with BH3 residues that point away from the Bax:Bcl-2 binding interface. Here, we used the x-ray crystal structure of the Bax BH3 domain in complex with Bcl-2 to identify such residues (Czabotar, Lessene, Strasser, & Adams, 2014; Ku, Liang, Jung, & Oh, 2011). Structural alignments between the ScTx-Bax α-helix (residues 5-15) and the N-terminus of the Bax BH3 domain (residues 58-68) showed that these regions overlay with an RMSD of 0.496 across 52 atoms (Arachchige & Holub, 2018). This design strategy resulted in ScTx-based BH3 domain constructs that are near-direct sequence mimics of the helical Bax BH3 domain.

Importantly, such protein-grafting approaches can, in principle, be applied to any helical interaction domain that is of comparable size to the ScTx α-helix (18 residues, 5 turns). To develop similar ScTx-based mimetics adapted from other helical interaction domains, the user should align residues important for molecular recognition with the solvent-exposed surface of the ScTx α-helix. Furthermore, residues within the protein ligand that point away from the binding interface can be substituted for structural cysteines in the designed ScTx mimetic.

SYNTHESIS AND CHARACTERIZATION OF ScTx-BASED Bax BH3 DOMAIN MIMETICS

BASIC PROTOCOL 1

This protocol describes the solid-phase peptide synthesis (SPPS), purification, and characterization of ScTx-based peptides that are designed to mimic the helical BH3 domain of the pro-apoptotic BCL2 protein Bax. In these experiments, ScTx-Bax proteins that include Bax BH3 domain residues are synthesized using standard Fmoc-based SPPS protocols (Fields & Noble, 1990). During synthesis, Bax BH3 domain residues important for Bcl-2 recognition are grafted onto the solvent-exposed portion of the ScTx α-helix, while Bax BH3 domain residues that point away from the Bcl-2 binding interface are replaced with cysteines or aminobutyric acid (Abu, B). Abu is a structural isostere of Cys and has been used previously as a placeholder for cysteine in ScTx-Bax mimetics containing zero, one, or two disulfide linkages (Arachchige & Holub, 2018; Arachchige et al., 2017; Zhu et al., 2002). In addition to its overall structural similarity to Cys, Abu is an aliphatic amino acid that is likely to contribute to a hydrophobic core in folded ScTx-Bax proteins (Farber & Mittermaier, 2008; Zhu et al., 2002). To ensure the proper location of the native disulfide linkages, Cys (or Abu) residues are incorporated at complementary positions 3-21, 8-26, or 12-28 of the ScTx-Bax sequence. Once, synthesized, the reduced (linear) ScTx-Bax proteins are cleaved from the resin and purified by reversed-phase high-performance liquid chromatography (HPLC). All peptide products are identified using MALDI-TOF or ESI mass spectrometry. Following synthesis and purification, the reduced peptides will be oxidized (folded) using techniques outlined in Basic Protocol 2.

All peptides outlined in this protocol are synthesized using standard Fmoc-based SPPS procedures as described previously (Harris et al., 2016). Typically, peptides are synthesized on Fmoc-PAL-AM resin at a 25-μmol scale based on the resin loading level. Synthesis on Fmoc-PAL-AM resin affords final peptide products that are functionalized with

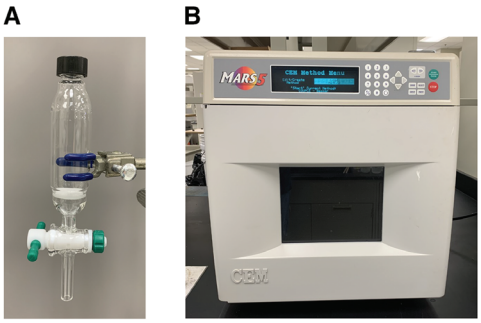

Figure 3 **(A)** Fritted glass reaction vessel with assembled stopcock and cap; no stir bar is present. All deprotections, amino acid couplings, N-terminal modifications, and cleavage reactions are performed in the fritted reaction vessel. **(B)** CEM microwave-accelerated reaction system (MARS) 5. All deprotections, amino acid couplings, and cleavage reactions are performed in the CEM MARS 5, using software programs written in-house (Table 2).

Table 2 Microwave Program Settings and Parameters

Program name	Power (W)	Power (%)	Ramp time	PSI	Temperature (°C)	Hold time
Couple	300	66	2:00	800	60	6:00
Couple 50°C[a]	300	66	2:00	800	50	6:00
Deprotect	300	66	2:00	800	70	4:00
DBU deprotect[b]	300	66	2:00	800	40	2:00
Cleave	300	66	2:00	800	38	30:00
Cleave rinse	300	66	2:00	800	38	5:00

[a] Lower reaction temperatures should be used when coupling Cys and His residues.
[b] Stronger bases such as DBU may be used for more efficient Fmoc deprotections.

a C-terminal amide, which enhances the overall stability and shelf life of the peptides (Kuzmin, Emelianova, Kalashnikova, Panteleev, & Ovchinnikova, 2017). All amino acid coupling and deprotection reactions are performed in fritted glass reaction vessels (Figure 3A) within a microwave-accelerated reaction system (MARS, Figure 3B) using software programs written in-house (Table 2).

Materials

Standard Fmoc-protected amino acids (EMD Millipore): Fmoc-Abu-OH (aminobutyric acid), Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(O*t*-Bu)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(O*t*-Bu)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(*t*-Bu)-OH, Fmoc-Thr(*t*-Bu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(*t*-Bu)-OH, Fmoc-Val-OH

Orthogonally protected amino acids (Bachem Americas): Fmoc-Cys(Acm)-OH (acetamidomethyl cysteine)

6-Chloro-benzotriazole-1-yloxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyClock; Millipore-Sigma, cat. no. 8510870025)

Fmoc-PAL-AM resin (Millipore-Sigma, cat. no. 8551330005 for 0.61 mmol/g)

Piperidine (Chem-Impex, cat. no. 02351)

N-Methyl-2-pyrrolidinone (NMP; Lab Depot, cat. no. BDH11-4LP)

1-Hydroxybenzotriazole hydrate (HOBt; anhydrous; ApexBio, cat. no. A7025)

Sigmacote® (Millipore-Sigma, cat. no. SL2)

N,N-Diisopropylethylamine (DIPEA; Millipore-Sigma, cat. no. D125806)

Acetic anhydride (Millipore-Sigma, cat. no. 320102)
4-Methylmorpholine (Millipore-Sigma, cat. no. M56557)
5(6)-carboxyfluorescein (Chem-Impex, cat. no. 00472)
2-(6-Chloro-1H-benzotrizaole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU; Peptides International, cat. no. KHC-1018-PI)
Dichloromethane (DCM; Fisher Scientific, cat. no. D37SK4)
Methanol (Fisher Scientific, cat. no. A4124)
Diethyl ether (Fisher, cat. no. 309966)
Trifluoroacetic acid (TFA; Millipore-Sigma, cat. no. T6508)
Deionized water
Phenol (Amresco, cat. no. 0735)
Triisopropylsilane (TIPS; Millipore-Sigma, cat. no. 233781)
Nitrogen source
Acetonitrile (ACN; HPLC-grade, Lab Depot, cat. no. LSS1110)

Glass reaction vials (2 dram)
Set of adjustable pipettes (0.5-10, 2-20, 20-200, 200-1000) and tips.
Polypropylene conical tubes (15-ml and 50-ml)
Vortex mixer
Fritted glass reaction vessels with stopcocks and caps (Yale Glass)
Miniature stir bars (Fisher)
Ring stand and clamps
Pasteur pipettes
Stir plate
Microwave Accelerated Reaction System (CEM)
Filter flask
Vacuum pump (e.g., Welch)
Centrifuges (e.g., Beckman Coulter; tabletop and floor)
Bath sonicator
Lyophilizer, canisters, and adaptors (Labconco)
0.45-μm filters (Fisher)
Polypropylene microcentrifuge tubes (2 ml with caps)
HPLC system
Semi-preparative-scale reversed-phase HPLC column (C18)
Analytical-scale reversed-phase HPLC column (C18)
MALDI-TOF mass spectrometer
Norm-Ject syringes

1. Following the rational design of the ScTx-based mimetics, plan the synthesis by first choosing proper reaction conditions for each amino acid. Here, you should choose equivalencies (based on resin loading level), reaction time, reaction temperature, coupling (activating) agent, and total number of coupling steps required for the synthesis of the peptide.

   Typically, single couplings using 5 eq. of amino acid are sufficient for most syntheses at the 25-μmol scale (e.g., resin to amino acid mole ratio = 1:5). However, it is advised to double-couple any amino acids that follow sterically hindered residues (e.g., proline) and amino acids with bulky protecting groups (e.g., Fmoc-Arg(Pbf)-OH). It is also advised to double-couple all residues after the tenth amino acid. Expensive or exotic (non-natural) amino acids can be coupled at 3 eq., to conserve resources.

2. Calculate the weight for the amount of amino acid (based on equivalencies and resin loading level) and add each amino acid separately to individual 2-dram glass reaction vials.
   A typical equation to determine the amount of amino acid required is:

5 equivalents × synthesis scale (mmol) × total number of couplings for the amino acid × molecular weight of the amino acid (mg $mmol^{-1}$) = mass (mg) of amino acid.

3. Calculate the weight for the amount of coupling agent (based on equivalencies and resin loading level) and add the coupling agent to each 2-dram glass reaction vial containing the amino acids (step 2).
   A typical equation to determine the amount of coupling agent required is:

   5 equivalents × synthesis scale (mmol) × total number of couplings for the coupling agent × molecular weight of coupling agent (mg $mmol^{-1}$) = mass (mg) of coupling agent.

4. Calculate the volume for an appropriate amount of 25% (v/v) piperidine in NMP and prepare the solution in a 50-ml polypropylene conical tube.

   It is advised to perform double-deprotections after each amino acid coupling.

The equation to determine the amount of deprotection solution required is:

total number of Fmoc-deprotections (number of amino acids × 2) × 3 (ml per treatment) = total volume (ml) of 25% piperidine solution in NMP.

If there are aspartate residues in the peptide sequence, it is strongly recommended to add HOBt to the deprotection solution at a final concentration of 0.15 M to mitigate unwanted aspartimide formation (Palasek, Cox, & Collins, 2007).

5. Calculate the weight for an appropriate amount of resin for the desired synthesis: synthesis scale (mmol) ÷ resin loading level (mmol g^{-1}) = mass (g) resin.

   For example, the amount of Fmoc-PAL-AM resin (loading level: 0.61 mmol g^{-1}) needed for a 25-μmol-scale synthesis = 0.025 mmol ÷ 0.61 mmol g^{-1} = 0.041 g.

6. Coat the entire inside surface of a glass fritted reaction vessel with Sigmacote®. Discard any excess Sigmacote® through the top of the reaction vessel and dry the interior of the vessel using a light stream of nitrogen or air. Assemble the reaction vessel by installing the stopcock, and add one miniature stir bar to the vessel.

   Reaction vessels coated with Sigmacote® can be stored in a drying oven (without the stopcock or cap) for future use.

7. Transfer the resin to the coated, dry glass reaction vessel and use a clamp to mount the vessel on a ring stand or cross-bars in a fume hood. Close the vessel stopcock.

8. Using a Pasteur pipette, add 2-3 ml of NMP to the reaction vessel and use the NMP to rinse any resin from the sides of the vessel. Cap the reaction vessel and gently agitate (stir) the resin with the stir bar by placing the reaction vessel over a magnetic stir plate.

   Ensure the stir plate is set on a low spin setting to minimize mechanical damage to the resin.

9. Allow the resin to incubate (swell) in the NMP for 30-60 min with gentle mixing at room temperature. Once the resin is sufficiently swollen, loosen the vessel cap and drain the NMP into an appropriate waste beaker through the fritted end of the reaction vessel. This can be facilitated by connecting the reaction vessel spigot to a filter flask using chemically-resistant tubing and pulling a light vacuum through the filter flask. Once drained, close the vessel stopcock.

10. If using an Fmoc-protected resin, add 2-3 ml of 25% (v/v) piperidine in NMP to the reaction vessel, cap the vessel, and place it into the microwave reactor. Set the

reaction program to 'Deprotect' (see Table 2 for reaction conditions). Close the door and run the program.

You can place the reaction vessel into an appropriately sized Griffin beaker to ensure stability during the microwave reaction. It should be noted that commercial microwave reactors may have different power settings fixed by the manufacturer; users are encouraged to read the instruction manual supplied with their specific microwave reactor to ensure proper temperature of the reaction.

11. Once the program is complete, remove the vessel from the microwave reactor and loosen the vessel cap. Drain the deprotection solution from the reaction vessel by connecting the reaction vessel spigot to a filter flask using chemically resistant tubing and pulling a light vacuum through the filter flask. Once drained, close the vessel stopcock.

    Note that a small amount of positive pressure can build inside the reaction vessel during microwave-assisted deprotection; use caution when opening the stopcock or loosening the cap from the vessel. The efficiency of Fmoc deprotection can be quantified at this step by monitoring the absorbance of the used deprotection solution at 300 nm (Luna et al., 2016).

12. Repeat steps 10 and 11 to double-deprotect the resin or peptide.

13. Wash the resin by adding 2-3 ml of NMP through the top of the reaction vessel, and allow the resin to stir at room temperature for 1 min. Following washing, loosen the vessel cap and open the stopcock. Use a light vacuum to drain the NMP into an appropriate waste beaker through the fritted end of the reaction vessel using the same setup as described in step 9. Repeat this washing step three times.

14. Dissolve the amino acid and coupling agent (steps 2 and 3) in an appropriate volume of NMP to yield a 1.0 M concentration of amino acid.
    A typical equation to determine the amount of NMP required is:

    mass (g) of amino acid (calculated from step 2) ÷ molecular weight of amino acid (g mol^{-1}) ÷ 1.0 M (mol l^{-1}) = final volume (l) of NMP for a 1.0 M stock solution.

15. Add 10 eq. DIPEA to the amino acid solution and thoroughly mix the reaction; all equivalencies are based on resin loading level.

16. Add the amino acid coupling solution to the reaction vessel containing the deprotected resin and cap the vessel. Place the vessel into the microwave reactor and set the reaction program to 'Couple' (see Table 2 for reaction conditions). Close the door and run the program.

    It is advised to perform double-couplings for amino acids added immediately after bulky amino acids, amino acids with large protecting groups, and each amino acid after the tenth residue.

For double couplings, the equation to determine the amount of coupling solution required is:

the total number of couplings (number of times the amino acid appears in the sequence × 2) × 3 (ml per treatment) = total volume (ml) of coupling solution.

17. Once the program is complete, remove the vessel from the microwave reactor, loosen the vessel cap, and use a light vacuum to drain the coupling solution into an appropriate waste beaker through the fritted end of the reaction vessel using the same setup as described in step 9. Repeat steps 14, 15, and 16 to double-couple the amino acid (if desired).

Please note that a minimal amount of positive pressure can build up inside the reaction vessel during microwave-assisted coupling; use caution when opening the stopcock or loosening the cap from the vessel.

18. Wash the resin by adding 2-3 ml of NMP through the top of the reaction vessel and allow the resin to stir at room temperature for 1 min. Following washing, loosen the vessel cap and use a light vacuum to drain the wash NMP into an appropriate waste beaker through the fritted end of the reaction vessel. Repeat this washing step three times.

    If necessary, the peptide synthesis can be paused after a coupling step and before the next Fmoc-deprotection. To pause the synthesis here for short term (12-48 hr) storage, it is recommended to follow the above washing steps and store the resin in NMP at 4°C. For long-term storage (protected peptide-resins can be stored for up to 1 year), we recommend performing the above NMP washing procedure, followed by washing three times with DCM and three times with methanol. Following the final wash, the resin can be dried by pulling a light vacuum through the vessel at room temperature for at least 2 hr. Once dried, the resin can be stored in a sealed container at 4°C for later use.

19. Repeat steps 10-18 (iterative deprotections and amino acid couplings) until a peptide of desired sequence/length has been obtained.

20. For the final Fmoc deprotection, remove the Fmoc from the N-terminal amino acid using methods described in steps 10 and 11. Wash the resin as described in step 13.

    The N-terminus of the resin-bound peptides can be modified before cleavage. Below, we describe two options for N-terminal modification: acetylation (step 21) and fluorescent labeling with 5(6)-carboxyfluorescein (step 22).

21. For acetylation of the N-terminus:
    i. Dissolve 6% (v/v) acetic anhydride and 6% (v/v) 4-methylmorpholine in 3 ml of NMP.
    ii. Add the mixture to the reaction vessel and gently stir the resin for 15 min at room temperature.
    iii. Drain the capping solution into an appropriate waste beaker as described above.
    iv. Following incubation, repeat steps 21(i), 21(ii), and 21(iii), above.
    v. Wash the resin as described in step 13.

22. For fluorescent labeling of the N-terminus:
    i. Dissolve 3 eq. 5(6)-carboxyfluorescein, 3 eq. HCTU, and 7.5 eq. DIPEA in 3 ml of NMP; all equivalencies are based on resin loading level.
    ii. Add the mixture to the reaction vessel and gently stir the resin for 24 hr in the dark at room temperature.
    iii. Drain the labeling solution into an appropriate waste beaker as described above.
    iv. Wash the resin as described in step 13.

23. To prepare the resin-peptide for cleavage or storage, wash the resin three times with DCM and then three times with methanol. Following washing, loosen the vessel cap and use a light vacuum to drain the wash solutions into an appropriate waste beaker through the fritted end of the reaction vessel using the same setup as described in step 9. Following the final wash, dry the resin under a light vacuum for at least 4 hr.

24. Prior to running the cleavage reaction, add 37.5 ml of diethyl ether to a 50-ml conical polypropylene tube and cap the tube tightly. Place the tube on dry ice for at least 30 min.

    CAUTION: *Dry ice is extremely cold and can burn exposed skin; wear appropriate personal protective equipment when handling dry ice. Dry ice will also release substantial volumes of CO_2 which can quicky displace oxygen and cause an asphyxiation hazard; work in an efficient fume hood when handling dry ice.*

25. For cleavage and global deprotection of the peptide, prepare 5 ml of cleavage cocktail (88% TFA, 5% water, 5% phenol, and 2% TIPS, v/v/v/v) in an appropriately sized, acid-resistant glass vial and tightly seal the cap.

    CAUTION: *TFA is highly corrosive; wear appropriate personal protective equipment and work in an efficient fume hood when handling TFA.*

26. Add 2.5 ml of the cleavage cocktail to the reaction vessel containing the dried resin-peptide; cap the vessel. Place the vessel into the microwave reactor and set the reaction program to 'Cleave' (see Table 2 for reaction conditions). Close the door and run the program.

27. Once the cleavage program is complete, remove the vessel from the microwave reactor, and secure it on a ring stand or cross bars over the (open) tube containing the cold diethyl ether. Loosen the vessel cap and open the stopcock. Drain the full volume of the cleavage solution directly into the cold diethyl ether by applying a light nitrogen stream through the top of the reaction vessel to obtain positive pressure.

    CAUTION: *Please note that a minimal amount of positive pressure can build inside the reaction vessel during microwave-assisted cleavage; use caution when opening the stopcock or loosening the cap from the vessel.*

28. Add the remaining 2.5 ml of the cleavage cocktail to the reaction vessel containing the resin-peptide; cap the vessel. Place the vessel into the microwave reactor and set the reaction program to 'CleaveRinse' (see Table 2 for reaction conditions). Close the door and run the program.

    While not explicitly necessary, we have found that performing a double cleavage of the resin (CleaveRinse) significantly improves final yields of the peptide.

29. Once the second cleavage program is complete, remove the vessel from the microwave reactor and secure it on a ring stand or cross bars over the (open) tube containing the cold diethyl ether. Loosen the vessel cap and open the stopcock. Drain the full volume of the cleavage solution directly into the cold diethyl ether by applying a light nitrogen stream through the top of the reaction vessel to obtain positive pressure.

30. Cap the tube and allow the ether-peptide solution to incubate on dry ice for an additional 10 min.

31. Centrifuge the sample 10 min at 3200 $\times$ *g*, 4°C, to pellet the precipitated peptide.

32. Carefully decant the supernatant and discard in an appropriate waste container, place the tube on its side, and air dry the open container at room temperature for 10 min.

    CAUTION: *Please note that there may be residual TFA fumes remaining in the tube following decanting of the ether supernatant.*

33. Resuspend the crude peptide mixture in an appropriate amount of aqueous ACN (20% v/v) and gently sonicate to dissolve the products.

34. Once the peptide is dissolved, evaporate all residual TFA and organic solvents from the solution by passing a light nitrogen stream over the sample for 30-60 min in an efficient fume hood.

    To facilitate subsequent freezing and lyophilization, it is advised to replace any evaporated solvent with deionized water to lower the concentration of dissolved peptide and ACN.

35. Cap the tube tightly and freeze the sample at −80°C for at least 4 hr.

To facilitate efficient freezing and lyophilization, it is advised to resuspend the peptides in a volume of aqueous ACN that is less than or equal to 50% of the maximal conical tube volume (e.g., 25 ml aqueous ACN solution in a 50-ml tube). It is also advised to freeze the tube on its side to maximize the surface area of the frozen sample during lyophilization.

36. Once the sample is frozen, lyophilize the sample to dryness.

    Crude peptide powders can be stored at −20°C in sealed tubes until further use.

37. To prepare the samples for purification, resuspend the crude peptide powder in an appropriate amount of aqueous ACN (10% v/v). Ensure that the peptide is fully dissolved by gently sonicating the mixture. Filter the sample through a 0.45-μm filter to remove any undissolved material.

38. Purify the crude samples by reversed-phase HPLC using parameters appropriate for your system. Collect all major peaks that elute off the column.

    Optimized conditions for HPLC purification (e.g., sample volume, gradient slope, resolution time) should be determined by first performing a "scout run" on a small amount of crude peptide sample. For peptides not containing chromophores, the absorbance should be monitored at 214 nm; all other peptides can be monitored at 280 nm. Fluorescein-labeled peptides can be monitored at 450 nm.

39. Determine identities of all collected peaks using an appropriate mass spectrometry method (e.g., MALDI-TOF, ESI-LC/MS). Combine all fractions that contain the products and aliquot if desired.

    CAUTION: *TFA is highly corrosive and can damage direct-injection ESI mass spectrometry equipment. If TFA is being used as a counter-ion in your HPLC solvents, we advise using MALDI-TOF for mass analysis or processing your samples to remove all residual TFA from the solution prior to ESI mass analysis.*

40. Following purification, repeat steps 34-36 to dry the purified peptides. Store all dry, purified peptides in tightly capped, sealed tubes in a desiccated vessel at −20°C protected from light, until further use.

    Lyophilization may be repeated to remove residual TFA salts by resuspending the peptide powder in an appropriate amount of water and repeating steps 35 and 36. Final peptide purities can be determined through analytical-scale HPLC analysis of the purified samples.

    Note that ScTx-based peptides containing no disulfides (e.g., ScTx-Bax$^{\Delta\Delta\Delta}$) will not require the subsequent oxidation reactions described in Basic Protocol 2. However, such peptides may require a second round of purification (steps 37-40) if they are not sufficiently pure (>95%) following the first round of HPLC. Typically, peptides undergoing subsequent oxidation will not require a second round of purification at this step. However, it is recommended that such peptides be purified to >85% before performing the oxidation reactions outlined in Basic Protocol 2.

BASIC PROTOCOL 2

OXIDATION OF ScTx-Bax BH3 DOMAIN MIMETICS CONTAINING ONE, TWO, OR THREE DISULFIDE LINKAGES

This protocol outlines general procedures to oxidize (fold) ScTx-Bax peptides containing two, four, or six native cysteine residues. As mentioned above, ScTx-Bax peptides containing no disulfides (e.g., ScTx-Bax$^{\Delta\Delta\Delta}$) do not require subsequent oxidation and can be purified and stored directly following synthesis (Harris et al., 2016). Conversely, reduced (linear) ScTx-Bax proteins that include complementary Cys residues at positions C3-C21, C8-C26, and/or C12-C28 must be oxidized to facilitate the formation of native disulfide linkages found in wild-type ScTx. Here, we describe separate protocols to oxidize each of the three classes (one, two, or three disulfides) of ScTx-Bax variant. For example, ScTx-Bax peptides containing one disulfide (designated

ScTx-Bax$^{\Delta\Delta}$) can be oxidized with linkages at positions C3-C21, C8-C26, or C12-C28, depending on where the two complementary Cys are located within the primary sequence. It has been demonstrated by our group that single disulfide linkages within ScTx-Bax peptides can be efficiently formed using platinum catalysts such as $[Pt(en)_2Cl_2]^{2+}$ (Figure 4; Arachchige et al., 2017). Constructs with two disulfides (designated ScTx-Bax$^{\Delta}$) can have linkages positioned at either C3-C21 and C8-C26; C3-C21 and C12-C28; or C8-C26 and C12-C28. Successful oxidation of these constructs requires that two complementary Cys residues within the primary sequence be functionalized with orthogonal protecting groups (e.g., acetamidomethyl, Acm) during synthesis (Table 1; Arachchige & Holub, 2018). Acm protecting groups are stable under standard acidic conditions that cleave trityl (Trt) groups, and also provide protection for cysteine thiols within peptides that are subjected to oxidative conditions (Lamthanh, Virelizier, & Frayssinhes, 1995). Separate oxidations of the complementary cysteine pairs ensures that no disulfide shuffling (incorrect disulfide formation) occurs during the reaction (Figure 5). Finally, ScTx-Bax peptides containing three native disulfides at positions C3-C21, C8-C26, and C12-C28 (designated ScTx-Bax) can be fully oxidized using glutathione (Figure 6; Arachchige et al., 2017). Once oxidized, the peptides are purified by reversed-phase HPLC and characterized by mass spectrometry. Following synthesis and characterization, the disulfide patterning of each construct should be confirmed by analyzing the mass signatures of trypsin digested fragments, as outlined in the Support Protocol.

Materials

Reduced ScTx-based peptides including two, four, or six native cysteine residues (see Basic Protocol 1)
NaH_2PO_4
NaCl
Bis(ethylenediamine)platinum (II) chloride ($[Pt(en)_2Cl_2]^{2+}$; Strem Chemicals, cat. no. 78-0100)EDTA
Acetonitrile (ACN; HPLC-grade, Lab Depot, cat. no. LSS1110)
Nitrogen source
Dimethyl sulfoxide (DMSO; Santa Cruz Biotechnology, cat. no. sc-202581)
Iodine (I_2; Millipore-Sigma, cat. no. 207772)

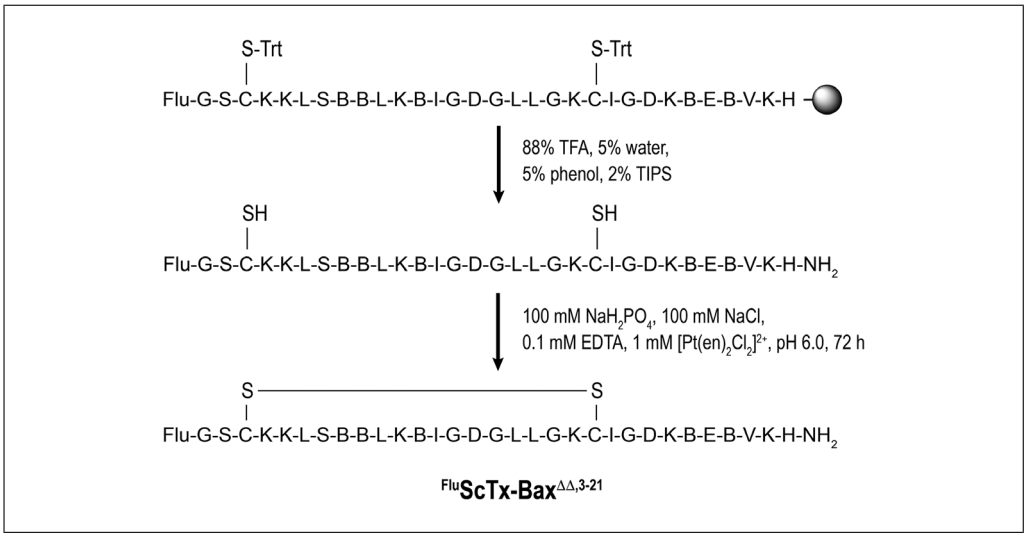

Figure 4 Representative reaction scheme for the total chemical synthesis of a ScTx-Bax BH3 domain mimetic containing one disulfide linkage. Top sequence shows resin-bound peptide with S-Trt protected cysteine thiols. Reaction with aqueous TFA cleaves the peptide from the resin and removes all acid-labile protecting groups. Subsequent oxidation using $[Pt(en)_2Cl_2]^{2+}$ in aqueous buffer generates the oxidized ScTx-Bax$^{\Delta\Delta}$ product.

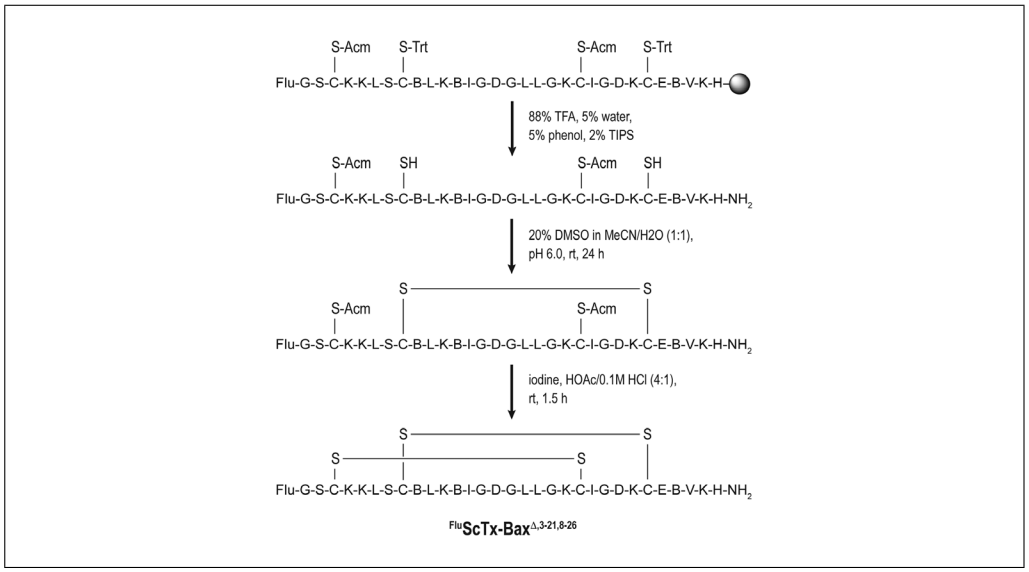

Figure 5 Representative reaction scheme for the total chemical synthesis of a ScTx-Bax BH3 domain mimetic containing two disulfide linkages. Top sequence shows resin-bound peptide with S-Trt- and S-Acm-protected cysteine thiols. Reaction with aqueous TFA cleaves the peptide from the resin and removes all acid-labile protecting groups. Selective oxidation using DMSO in aqueous acetonitrile generates the semi-oxidized species. Concomitant deprotection of the Acm groups and oxidation of the remaining cysteine thiols with iodine in acetic acid yields the fully oxidized ScTx-Bax$^{\Delta}$ product. Figure adapted from Arachchige & Holub (2018).

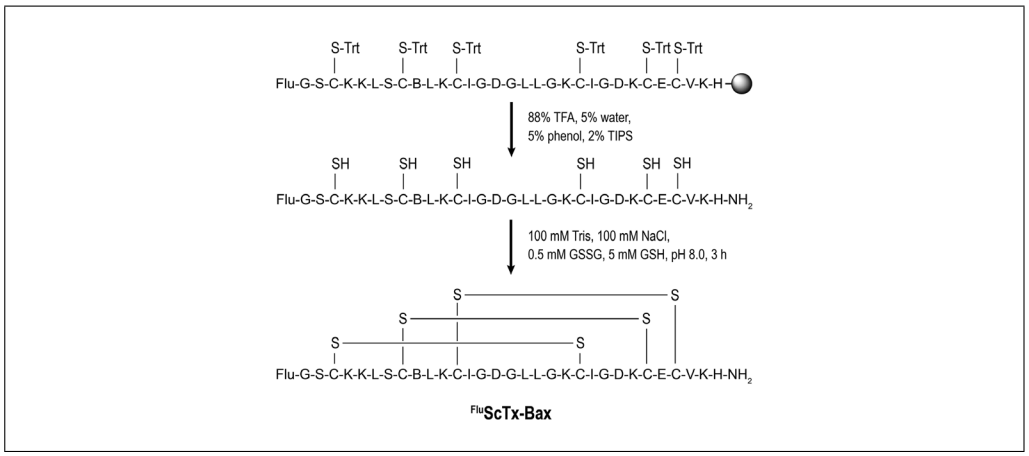

Figure 6 Representative reaction scheme for the total chemical synthesis of a ScTx-Bax BH3 domain mimetic containing three disulfide linkages. Top sequence shows resin-bound peptide with S-Trt protected cysteine thiols. Reaction with aqueous TFA cleaves the peptide from the resin and removes all acid-labile protecting groups. Subsequent oxidation using oxidized (GSSG) and reduced (GSH) glutathione in aqueous buffer generates the fully oxidized ScTx-Bax product.

Acetic acid, glacial (Fisher Scientific, cat. no. A38S-500)
Hydrochloric acid (1 N; Fisher Scientific, cat. no. AC124210010)
Oxidized glutathione (GSSG; Gold Biotechnology, cat. no. G-060)
Reduced glutathione (GSH; Gold Biotechnology, cat. no. G-155)
Tris base (Fisher Scientific, cat no. BP1521)
Trifluoroacetic acid (TFA; Millipore-Sigma, cat. no. T6508)

Set of adjustable pipettes (0.5-10, 2-20, 20-200, 200-1000) and tips.
Polypropylene conical tubes (15-ml and 50-ml)
Bath sonicator
Lyophilizer, canisters and adaptors (Labconco)
Polypropylene microcentrifuge tubes (2 ml with caps)

Desiccator
HPLC system
Semi-preparative scale reversed-phase HPLC column (C18)
Analytical-scale reversed-phase HPLC column (C18)
MALDI-TOF mass spectrometer
Norm-Ject syringes
Orbital shaker

a. Oxidation of ScTx-Bax peptides with one disulfide linkage (ScTx-Bax$^{\Delta\Delta}$)

1a. Prepare 25 ml of folding buffer (100 mM NaH_2PO_4, 100 mM NaCl, 0.1 mM EDTA, 1 mM $[Pt(en)_2Cl_2]^{2+}$, pH 6.0) using deionized water.

2a. Weigh out an appropriate amount of ScTx-Bax$^{\Delta\Delta}$ peptide containing two reduced cysteines (peptide may be acetylated or fluorescently labeled; see Basic Protocol 1, steps 21 or 22) and dissolve in 25 ml folding buffer at a final concentration of 100 μM. Ensure that the peptide is fully dissolved by gently sonicating the mixture.

All cysteine thiols (C3 and C21; C8 and C26; or C12 and C28) in the ScTx-Bax$^{\Delta\Delta}$ variants were protected with an acid-labile Trt group and are fully deprotected following cleavage from the resin with TFA.

3a. Allow the reaction to gently mix for up to 72 hr at room temperature in the dark on an orbital shaker.

Optional: The reaction progress can be monitored by removing a small amount of sample and analyzing the mixture by HPLC. Product formation can be confirmed by collecting peaks and performing mass analysis; a loss in mass corresponding to two hydrogen atoms should be observed. Typically, oxidized ScTx peptides have shorter retention times than their reduced counterparts on reversed-phase HPLC chromatograms.

4a. Once the reaction is complete, purify the oxidized peptide by reversed-phase HPLC and collect all major product peaks.

Optimized conditions for HPLC purification (e.g., sample volume, gradient slope, resolution time) should be determined by first performing a "scout run" on a small amount of crude peptide sample. For peptides not containing chromophores, the absorbance should be monitored at 214 nm; all other peptides can be monitored at 280 nm. Fluorescein-labeled peptides can be monitored at 450 nm.

5a. Determine collected peak identities using an appropriate mass spectrometry method such as ESI or MALDI-TOF. Combine all fractions that contain the desired product and aliquot if desired.

All major peaks should be collected following the oxidation reaction, and their identities should be confirmed by mass spectrometry. As mentioned above, fully oxidized ScTx peptides will typically elute faster (that is, have shorter retention times) than their reduced counterparts on reversed-phase HPLC chromatograms. Mass differences between fully oxidized and fully reduced ScTx-Bax$^{\Delta\Delta}$ peptides will correspond to a loss of two hydrogen atoms.

It should be noted that only a single major product peak was present following oxidation of our ScTx-Bax$^{\Delta\Delta}$ peptides under these conditions (see Figure 7A). However, this oxidation reaction has the potential to generate multimeric species, and the formation of higher-order products cannot be explicitly ruled out for other peptide sequences (see Table 4 In the Troubleshooting section of the Commentary for troubleshooting scenarios).

6a. Once the samples are purified, evaporate all residual TFA and organic solvents (Basic Protocol 1, step 34) from the solution by passing a light nitrogen stream over the sample for 30-60 min in an efficient fume hood.

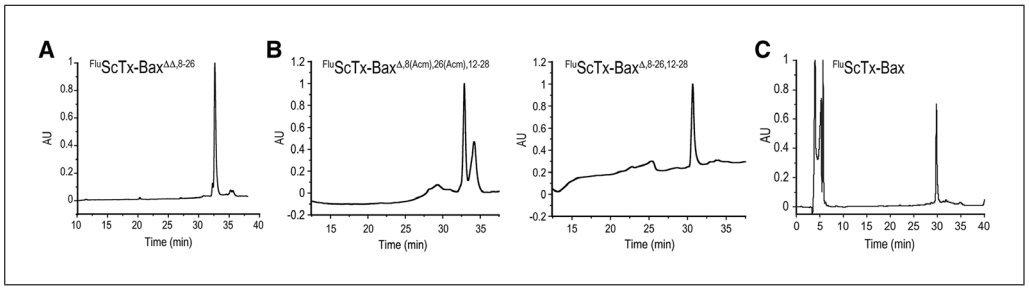

Figure 7 Representative preparatory reversed-phase HPLC chromatograms of oxidized ^{Flu}ScTx-Bax BH3 domain mimetics. All peptides were purified using a reversed-phase C18 column (Grace, 10 μM, 250 × 10 mm) on an Agilent ProStar HPLC system. Peptides were eluted over 45 min with a linear gradient of 15%-60% solvent B (0.1% TFA in ACN) over solvent A (0.1% TFA in water). All chromatograms were monitored at 214 nm and show crude peptide product following each respective oxidation reaction as outlined in Basic Protocol 2. **(A)** ^{Flu}ScTx-Bax$^{\Delta\Delta,8\text{-}26}$ peptide following 72-hr oxidation in the presence of $[Pt(en)_2Cl_2]^{+2}$. Dominant peak at 32.5 min represents the fully oxidized product. **(B)** ^{Flu}ScTx-Bax$^{\Delta,8\text{-}26,12\text{-}28}$ peptide following each separate round of oxidation. Left figure in panel B shows crude chromatogram of first round of oxidation (DMSO/ACN); dominant peak at 32.5 min represents semi-oxidized product; peak at 34 min represents fully reduced starting material. Right figure in panel B shows crude chromatogram of second round of oxidation (iodine/acetic acid); dominant peak at 31 min represents fully oxidized product. **(C)** ^{Flu}ScTx-Bax peptide following 2 hr oxidation in the presence of glutathione. Dominant peak at 30 min represents fully oxidized product. Note the absence of any multimeric species in all crude HPLC chromatograms.

To facilitate subsequent freezing and lyophilization, it is advised to replace any evaporated solvent with deionized water to lower the concentration of dissolved peptide and ACN.

7a. Cap the tube tightly and freeze the sample at −80°C for at least 4 hr.

To facilitate efficient lyophilization, it is advised to resuspend the peptides in a volume of aqueous ACN that is less than or equivalent to 50% of the maximal conical tube volume (e.g., 25 ml aqueous ACN solution in a 50-ml tube). It is also advised to freeze the tube on its side to maximize the surface area of the frozen sample during lyophilization.

8a. Following freezing, lyophilize the sample to dryness.

Lyophilization may be repeated to remove residual TFA salts by resuspending the dried peptide powder in an appropriate amount of deionized water and repeating steps 7a and 8a.

9a. Once dry, store purified peptide powders in tightly capped, sealed tubes in a desiccated vessel at −20°C protected from light until further use. Determine final peptide purities using analytical-scale reversed-phase HPLC.

b. Oxidation of ScTx-Bax peptides with two disulfide linkages (ScTx-Bax$^{\Delta}$)

1b. Prepare 25 ml of folding buffer 1 (50% v/v aqueous ACN containing 20% v/v DMSO, pH 6.0) using deionized water.

2b. Weigh out an appropriate amount of ScTx-Bax$^{\Delta}$ peptide containing four reduced cysteines (peptide may be acetylated or fluorescently labeled; see Basic Protocol 1, steps 21 or 22) and dissolve in 25 ml folding buffer 1 at a final concentration of 50 μM. Ensure the peptide is fully dissolved by gently sonicating the mixture.

Peptides with four disulfides require two separate rounds of oxidation to mitigate disulfide shuffling. To facilitate oxidation of the native disulfides, we synthesized ScTx-Bax$^{\Delta}$ peptides that included two complementary cysteines protected by acetamidomethyl (Acm) protecting groups and two complementary cysteines that contained trityl (Trt) protecting groups (Table 1). The Trt protecting group is acid-labile and is removed during the initial cleavage reaction. The Acm functionality is stable under standard acidic conditions and provides protection of the remaining cysteine thiols during the initial

oxidation reaction (Arachchige & Holub, 2018; Veber, Milkowski, Varga, Denkewalter, & Hirschmann, 1972). Acm groups can subsequently be removed from the remaining cysteine thiols by treating the peptide with iodine under mild oxidative conditions (see below).

It has also been reported that Acm groups can be removed in the presence of hindered hydrosilane compounds including triisopropylsilane (TIPS; Ste. Marie & Hondal, 2018). Because TIPS is utilized as a cation scavenger in our aqueous TFA cleavage protocol, the user should be aware that orthogonal Acm groups may potentially be removed during this step. We did not observe any premature Acm deprotection following cleavage of ScTx-Bax$^{\Delta}$ peptides from the resin; however, only major product peaks were collected and analyzed during our HPLC purifications. While it is possible that some Acm groups were removed during the initial cleavage reaction, the fact that the semi-deprotected derivatives did not show up as major products during the initial purification indicates that TIPS-mediated removal of Acm protecting groups was not a significant issue under these conditions. Nevertheless, premature Acm deprotection should be carefully monitored by the user when using TIPS, and alternative approaches (e.g., removing TIPS from the cleavage cocktail or using another scavenger) may be needed when synthesizing other ScTx-based molecules containing two disulfide linkages.

3b. Allow the reaction to stir in the dark for 24 hr at room temperature.

This reaction affords semi-oxidized ScTx-based peptide products that contain a native disulfide linkage between the complementary cysteine thiols that are deprotected (Trt) during the TFA cleavage reaction.

Optional: The progress of the initial oxidation reaction can be monitored by removing a small amount of sample and analyzing the mixture by HPLC. Product formation can be confirmed by collecting peaks and performing mass analysis (a loss in mass corresponding to two hydrogen atoms should be observed). Typically, oxidized ScTx peptides have shorter retention times than their reduced counterparts on reversed-phase HPLC chromatograms.

4b. Once the reaction is complete, purify the semi-oxidized peptide by reversed-phase HPLC and collect all major product peaks.

Optimized conditions for HPLC purification (e.g., sample volume, gradient slope, resolution time) should be determined by first performing a "scout run" on a small amount of crude peptide sample. For peptides not containing chromophores, the absorbance should be monitored at 214 nm; all other peptides can be monitored at 280 nm. Fluorescein-labeled peptides can be monitored at 450 nm.

5b. Determine collected peak identities using an appropriate mass spectrometry method such as ESI or MALDI-TOF. Combine all fractions that contain pure product, and aliquot if desired.

All major peaks should be collected following the oxidation reaction, and their identities should be confirmed by mass spectrometry. As mentioned above, oxidized ScTx peptides will typically elute faster (that is, have shorter retention times) than their reduced counterparts on reversed-phase HPLC chromatograms. Mass differences between semi-oxidized and fully reduced ScTx-Bax$^{\Delta}$ peptides should correspond to a loss of two hydrogen atoms.

It should be noted that only a single major product peak was present following oxidation of our ScTx-Bax$^{\Delta}$ peptides under these conditions (see Figure 7B). However, this oxidation reaction has the potential to generate multimeric species, and the formation of higher-order products cannot be explicitly ruled out for other peptide sequences (see Table 4 in the Troubleshooting section of the Commentary for troubleshooting scenarios).

6b. Evaporate any residual TFA and organic solvents from the solution by passing a light nitrogen stream over the sample for 30-60 min in an efficient fume hood.

To facilitate subsequent freezing and lyophilization, it is advised to replace any evaporated solvent with deionized water to lower the concentration of dissolved peptide and ACN.

7b. Cap the tube tightly and freeze the sample at −80°C for at least 4 hr.

To facilitate efficient lyophilization, it is advised to resuspend the peptides in a volume of aqueous ACN that is less than or equivalent to 50% of the maximal conical tube volume (e.g., 25 ml aqueous ACN solution in a 50-ml tube). It is also advised to freeze the tube on its side to maximize the surface area of the frozen sample during lyophilization.

8b. Following freezing, lyophilize the sample to dryness.

9b. Prepare 25 ml of folding buffer 2 [0.05 M iodine in acetic acid/0.1 M HCl (4:1)] using deionized water.

10b. Weigh out an appropriate amount of semi-oxidized ScTx-Bax$^{\Delta}$ peptide containing two Acm-protected cysteines (peptide may be acetylated or fluorescently labeled; see Basic Protocol 1, steps 21 or 22) and dissolve in 25 ml folding buffer 2 at a final concentration of 25 μM. Ensure the peptide is fully dissolved by gently sonicating the mixture.

11b. Allow the mixture to incubate in the dark for 1.5 hr at room temperature with gentle shaking.

Optional: The progress of the second oxidation reaction can be monitored by removing a small amount of sample and analyzing the mixture by HPLC. Product formation can be confirmed by collecting peaks and performing mass analysis (a loss in mass corresponding to two hydrogen atoms should be observed). Typically, oxidized ScTx peptides have shorter retention times than their reduced counterparts on reversed-phase HPLC chromatograms.

12b. Once the reaction is complete, purify the fully oxidized peptide by reversed-phase HPLC.

Optimized conditions for HPLC purification (e.g., sample volume, gradient slope, resolution time) should be determined by first performing a "scout run" on a small amount of crude peptide sample. For peptides not containing chromophores, the absorbance should be monitored at 214 nm; all other peptides can be monitored at 280 nm. Fluorescein-labeled peptides can be monitored at 450 nm.

13b. Determine collected peak identities using an appropriate mass spectrometry method. Combine all fractions that contain pure product and aliquot if desired.

All major peaks should be collected following the oxidation reaction, and their identities should be confirmed by mass spectrometry. As mentioned above, oxidized ScTx peptides will typically elute faster (that is, have shorter retention times) than their reduced counterparts on reversed-phase HPLC chromatograms. Mass differences between fully oxidized and semi-oxidized ScTx-Bax$^{\Delta}$ peptides should correspond to a loss of two hydrogen atoms; mass differences between fully oxidized and fully reduced ScTx-Bax$^{\Delta}$ peptides should correspond to a loss of four hydrogen atoms.

It should be noted that only a single major product peak was present following oxidation of our ScTx-Bax$^{\Delta}$ peptides under these conditions (see Figure 7B). However, this oxidation reaction has the potential to generate multimeric species, and the formation of higher-order products cannot be explicitly ruled out for other peptide sequences (see Table 4 in the Troubleshooting section of the Commentary for troubleshooting scenarios).

14b. Evaporate any residual TFA and organic solvents from the solution by passing a light nitrogen stream over the sample for 30-60 min in an efficient fume hood.

To facilitate subsequent freezing and lyophilization, it is advised to replace any evaporated solvent with deionized water to lower the concentration of dissolved peptide and ACN.

15b. Cap the tube tightly and freeze the sample at −80°C for at least 4 hr.

To facilitate efficient lyophilization, it is advised to resuspend the peptides in a volume of aqueous ACN that is less than or equivalent to 50% of the maximal conical tube volume (e.g., 25 ml aqueous ACN solution in a 50-ml tube). It is also advised to freeze the tube on its side to maximize the surface area of the frozen sample during lyophilization.

16b. Following freezing, lyophilize the sample to dryness.

Lyophilization may be repeated to remove residual TFA salts by resuspending the peptide in an appropriate amount of deionized water and repeating steps 15b and 16b.

17b. Once dry, store purified peptide powders in tightly capped, sealed tubes in a desiccated vessel at −20°C protected from light until further use. Determine final peptide purities using analytical-scale reversed-phase HPLC.

c. Oxidation of ScTx-Bax peptides with three disulfide linkages (ScTx-Bax)

1c. Prepare 25 ml of folding buffer (100 mM Tris, 100 mM NaCl, 0.5 mM oxidized glutathione, 5 mM reduced glutathione, pH 8.0) using deionized water.

2c. Weigh out an appropriate amount of ScTx-Bax peptide containing six reduced cysteines (peptide may be acetylated or fluorescently labeled; see Basic Protocol 1, steps 21 or 22) and dissolve in 25 ml folding buffer at a final concentration of 280 μM. Ensure the peptide is fully dissolved by gently sonicating the mixture.

All cysteine thiols (C3, C8, C12, C21, C26, and C28) in the ScTx-Bax variant were protected with an acid-labile Trt group and are fully deprotected following cleavage from the resin with TFA. Oxidized glutathione (GSSG) functions as an oxidant in the formation of disulfide bonds in peptides and proteins. Reduced glutathione (GSH) serves to reduce any mis-formed disulfide linkages during the reaction. This process results in the formation of the thermodynamically stable conformation of the peptide or protein in vitro and in vivo (Hwang, Sinskey, & Lodish, 1992; Zhu et al., 2002).

3c. Allow the reaction to stir for 2 hr at room temperature in the dark on an orbital shaker.

Optional: The progress of the oxidation reaction can be monitored by removing a small amount of sample and analyzing the mixture by HPLC. Product formation can be confirmed by collecting peaks and performing mass analysis (a loss in mass corresponding to six hydrogen atoms should be observed). Typically, oxidized ScTx peptides have shorter retention times than their reduced counterparts on reversed-phase HPLC chromatograms.

4c. Once the reaction is complete, acidify the reaction mixture by adding 2.5 ml of a 50% (v/v) aqueous TFA, and gently mix the sample.

CAUTION: *TFA is highly corrosive; wear appropriate personal protective equipment and work in an efficient fume hood when handling TFA. Adding acid to the aqueous solution is exothermic and may cause pressure buildup in the reaction tube. Be sure to vent the cap of the reaction vessel in an efficient fume hood periodically while gently mixing the solution.*

5c. Purify the crude oxidized peptide by injecting the reaction mixture onto a reversed-phase HPLC system and collect all major product peaks.

Optimized conditions for HPLC purification (e.g., sample volume, gradient slope, resolution time) should be determined by first performing a "scout run" on a small amount of crude peptide sample. For peptides not containing chromophores, the ab-

sorbance should be monitored at 214 nm; all other peptides can be monitored at 280 nm. Fluorescein-labeled peptides can be monitored at 450 nm.

6c. Determine collected peak identities using an appropriate mass spectrometry method such as ESI or MALDI-TOF. Combine all fractions that contain pure product and aliquot if desired.

All major peaks should be collected following the oxidation reaction, and their identities should be confirmed by mass spectrometry. As mentioned above, oxidized ScTx peptides will typically elute faster (that is, have shorter retention times) than their reduced counterparts on reversed-phase HPLC chromatograms. Mass differences between fully oxidized and fully reduced ScTx-Bax peptides should correspond to a loss of six hydrogen atoms.

It should be noted that only a single major product peak was present following the full oxidation of our ScTx-Bax peptides (Figure 7C). However, this reaction has the potential to generate multimeric species and disulfide shuffling; therefore, the formation of non-native disulfides, along with higher-order products, cannot be explicitly ruled out for other peptide sequences (see Table 4 in the Troubleshooting section of the Commentary for troubleshooting scenarios).

7c. Evaporate any residual TFA and organic solvents from the solution by passing a light nitrogen stream over the sample for 30-60 min in an efficient fume hood.

To facilitate subsequent freezing and lyophilization, it is advised to replace any evaporated solvent with deionized water to lower the concentration of dissolved peptide and ACN.

8c. Cap the tube tightly and freeze the sample at −80°C for at least 4 hr.

To facilitate efficient lyophilization, it is advised to resuspend the peptides in a volume of aqueous ACN that is less than or equivalent to 50% of the maximal conical tube volume (e.g., 25 ml aqueous ACN solution in a 50-ml tube). It is also advised to freeze the tube on its side to maximize the surface area of the frozen sample during lyophilization.

9c. Following freezing, lyophilize the sample to dryness.

Lyophilization may be repeated to remove residual TFA salts by resuspending the peptide in an appropriate amount of deionized water and repeating steps 8c and 9c.

10c. Once dry, store purified peptide powders in tightly capped, sealed tubes in a desiccated vessel at −20°C protected from light until further use. Determine final peptide purities using analytical-scale reversed-phase HPLC.

SUPPORT PROTOCOL

MAPPING OF DISULFIDE LINKAGES IN OXIDIZED ScTx-Bax BH3 DOMAIN MIMETICS

This support protocol outlines a general procedure to map the disulfide linkages of fully oxidized ScTx-Bax peptides that contain one, two, or three disulfide linkages (Arachchige & Holub, 2018, Zhu et al., 2002; Arachchige et al., 2017; Harris et al., 2016). It is worth mentioning that ScTx-based peptides that contain no native cysteines (e.g., ScTx-Bax$^{\Delta\Delta\Delta}$) do not contain any disulfide linkages and therefore do not require mapping of their disulfide bonds. However, oxidized (folded) ScTx-Bax proteins containing native cysteines require confirmation that the disulfides were formed between the correct complementary cysteine residues during the oxidation reactions outlined in Basic Protocol 2. Here, we employ a straightforward protocol that utilizes enzymatic digestion of the oxidized products and determines the corresponding disulfide patterning by mass spectrometry. Notably, digestion of our ScTx-Bax BH3 domain mimetics with trypsin results in peptide fragments that are unique to each sequence (Figure 8). This allowed us to determine the discrete disulfide linkage patterns between each ScTx-Bax sequence

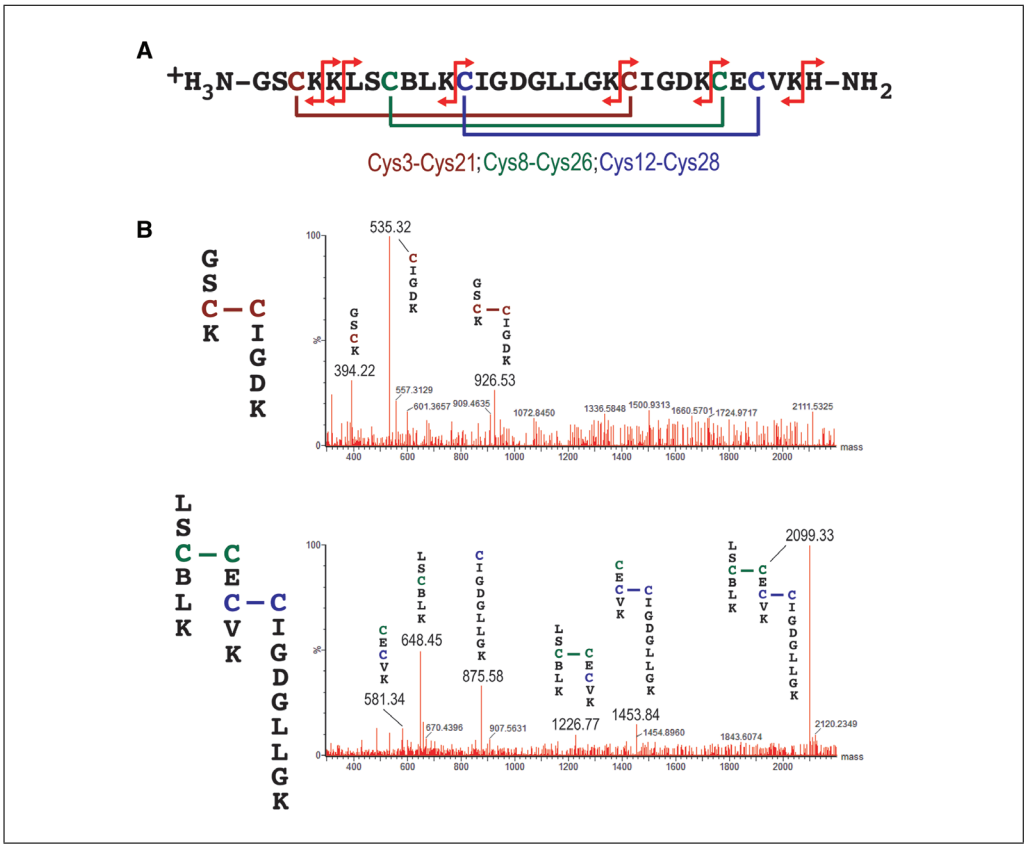

Figure 8 **(A)** Sequence of ScTx-Bax peptide containing three disulfide bonds; linked Cys residues are shown below primary sequence. Trypsin cut sites are indicated with red arrows. **(B)** Mass spectra of peptide fragments isolated from trypsin digest. All peptide fragments are shown above corresponding masses; dicysteine-linked peptide sequences are shown to the right of the mass spectra. Figure adapted from Harris et al. (2016).

variant. Importantly, these experiments confirmed that the oxidation reactions outlined in Basic Protocol 2 were sufficient to generate ScTx-Bax peptides with the desired number and position of disulfide linkages.

Materials

Oxidized (folded) ScTx-Bax peptide containing one, two, or three disulfide linkages (peptide may be acetylated or fluorescently labeled; see Basic Protocol 1, seps 21 or 22)
Tris base (Fisher Scientific, cat no. BP1521)
Calcium chloride ($CaCl_2$; Fisher Scientific, cat. no. AC349610250)
Trypsin, lyophilized powder (Millipore-Sigma, cat. no. T8658-1VL)
Trifluoroacetic acid (TFA; Millipore-Sigma, cat. no. T6508)
Acetonitrile (ACN; HPLC-grade, Lab Depot, cat. no. LSS1110)

Set of adjustable pipettes (0.5-10, 2-20, 20-200, 200-1000) and tips
Polypropylene microcentrifuge tubes (2 ml with caps)
Incubator or water bath
HPLC system
Semi-preparative scale reversed-phase column (C18)
Analytical scale reversed-phase column (C18)
MALDI-TOF mass spectrometer
Norm-Ject syringes

1. Prepare 1 ml of digestion buffer (100 mM Tris, 1 mM $CaCl_2$, pH 7.8) using deionized water. Warm the mixture to 37°C for at least 30 min prior to use.

   The digestion buffer used at this step should be optimized for the specific proteolytic enzyme being used; the buffer outlined above has been optimized for trypsin.

2. Weigh out an appropriate amount of oxidized (folded) ScTx-Bax peptide containing one, two, or three disulfide linkages (peptide may be acetylated or fluorescently labeled; see Basic Protocol 1, seps 21 or 22) and dissolve in 100 μl digestion buffer at a final concentration of 30 μM.

3. Add an appropriate amount of trypsin to bring the final concentration to 5% (w/w) compared to the oxidized peptide.

   Trypsin cleaves peptide bonds on the C-terminal side of cationic amino acids. For our ScTx-Bax peptides, this affords fragmentation patterns that will allow for the determination of the position of dicysteines (Figure 8). While the general concept is similar, the specific enzymatic digestion outlined in this Support Protocol should be optimized for each unique ScTx-based peptide sequence by using a proteolytic enzyme that will cleave at appropriate sites to observe proper disulfide linkage patterning among the produced fragments.

4. Incubate the digestion reaction at 37°C for 2 hr.

   Be sure to include a 'trypsin only' sample with no peptide for comparative analysis with the peptide samples, and to rule out any autolytic fragments forming during the digestion reaction.

5. Once the reaction is complete, add 100 μl 50% (v/v) TFA in digestion buffer to stop the proteolysis reaction.

   CAUTION: *TFA is highly corrosive; wear appropriate personal protective equipment and work in an efficient fume hood when handling TFA.*

6. Analyze the reaction by injecting the full volume onto a reversed-phase HPLC system and collect all major product peaks.

   Optimized conditions for HPLC analysis (e.g., sample volume, gradient slope, resolution time) should be determined by first performing a "scout run" on a small amount of digested peptide sample. For peptides not containing chromophores, the absorbance should be monitored at 214 nm; all other peptides can be monitored at 280 nm. Fluorescein-labeled peptides can be monitored at 450 nm.

7. Determine collected peak identities using an appropriate mass spectrometry method.

   To rule out any autolytic trypsin fragments, a sample containing trypsin alone should be analyzed in parallel under similar conditions and compared to the peptide samples as mentioned above.

COMMENTARY

Background Information

The ability to target and inhibit discrete protein-protein interactions (PPIs) in complex environments, such as the cytoplasm of live cells, remains one of the most pressing challenges in modern chemical biology research (Mabonga & Kappo, 2019). This issue becomes further compounded when multiple proteins involved in similar or redundant pathways bind promiscuously to the same ligand (Schreiber & Keating, 2011). Over the past 30 years, attempts to target and inhibit therapeutically relevant PPIs has been met with varying degrees of success. On the one hand, the ability to modulate large-scale PPIs with small molecules has been especially challenging, as small molecules often cannot inhibit biomolecular interactions that occur over large surface areas (Wilson, 2009). To circumvent this problem, researchers have gravitated towards using protein-based molecules, such as miniature proteins or antibody fragments, to target and inhibit larger-scale PPIs (Chrunyk et al., 2000; Zabrady et al., 2014). Such approaches have been especially successful for inhibiting PPIs involving extracellular targets

(Laraia, McKenzie, Spring, Venkitaraman, & Huggins, 2015). At the same time, many labs have expanded the utility of miniature proteins through "protein grafting", a re-engineering technique that replaces sequences of structured peptides or small proteins with recognition elements that are complementary to the targeted protein surface (Holub, 2017; Sia & Kim, 2003; Tsomaia, 2015). This strategy has gained considerable traction among chemical biologists over the last 20 years and has been used to successfully target PPIs previously considered to be "undruggable." Since being introduced in the early 2000s, the protein grafting strategy has become a well-established approach to develop molecules that can be used for therapeutic intervention or as tools to help researchers better understand the molecular nature of PPIs.

The B cell lymphoma 2 (BCL2) proteins are a class of outer mitochondrial membrane proteins that regulate the intrinsic apoptosis pathway (Chao & Korsmeyer, 1998; Cory, Huang, & Adams, 2003). There are currently 25 known genes in the BCL2 family, each of which can be classified into three subcategories based on their inherent function. The "anti-apoptotic" (pro-survival) BCL2 proteins are responsible for binding and sequestering "pro-apoptotic" (pro-death) BCL2 members on the mitochondrial surface (Czabotar et al., 2014). This interaction is typically manifested between the helical BH3 domain of pro-apoptotic BCL2 members and a shallow, hydrophobic binding groove on anti-apoptotic BCL2 proteins. A third class of BCL2 proteins, designated the "BH3 only" or "activator" proteins, are upregulated during times of cellular stress and act to inhibit the interaction between pro- and anti-apoptotic BCL2 members (Chen et al., 2005). Upon disruption of the BH3:BCL2 interaction, pro-apoptotic BCL2 proteins, such as Bax and Bak, oligomerize on the mitochondrial surface, where they form pores that facilitate the release of cytochrome c. Owing to their profound influence on regulating the intrinsic apoptosis pathway, it is perhaps not surprising that dysregulated or aberrant activity of BCL2 proteins can lead to the onset of disease. For instance, overexpression of anti-apoptotic BCL2 proteins, such as Bcl-2 (proper) and Bcl-X_L, has been linked to cancerous phenotypes (Farsinejad, Gheisary, Ebrahimi Samani, & Alizadeh, 2015; Kang & Reynolds, 2009). As a result, significant effort has been dedicated to developing selective inhibitors of anti-apoptotic BCL2 function. Unfortunately, high incidences of crosstalk among the various BCL2 family members have made determining specific functions of discrete BCL2 proteins a considerable challenge (Su et al., 2014; Zhou, Yang, & Xing, 2011). Consequently, there is a pressing need for molecules that can act as selective modulators of BCL2 function.

In an effort to develop highly selective modulators of anti-apoptotic BCL2 proteins, our lab has recently applied a protein-grafting strategy to generate BH3 domain mimetics that are based on the small protein ScTx (Arachchige & Holub, 2018; Arachchige et al., 2017; Harris et al., 2016). ScTx is a 31-amino-acid protein that folds into a stable α/β structural motif stabilized by three disulfide linkages between residues C3-C21, C8-C26, and C12-C28 (Figure 1; Martins et al., 1995). We hypothesized that we could develop molecules that selectively target discrete anti-apoptotic BCL2 family members by replacing residues on the α-helix of wild-type ScTx with residues from the helical BH3 domain of the pro-apoptotic BCL2 protein Bax. Furthermore, because the ScTx scaffold has three native disulfides that can be added or removed during synthesis, we speculated that ScTx-Bax constructs could be used as unique molecular tools to better understand how flexibility within the Bax BH3 domain affects the BH3:BCL2 interaction. To that end, we have developed a series of ScTx-based Bax BH3 domain mimetics that vary in the number and location of native disulfide linkages. For these experiments, we used fluorescence polarization (FP) direct binding assays with fluorescently labeled ScTx-Bax constructs (^{Flu}ScTx-Bax) targeting Bcl-2-ΔTM. Bcl-2-ΔTM is a truncated form of Bcl-2 that has no transmembrane domain, which facilitates expression and purification of the mature protein (Stewart, Fire, Keating, & Walensky, 2010). Notably, we found that ScTx-Bax peptides having no disulfide bonds (ScTx-Bax$^{\Delta\Delta\Delta}$) displayed nanomolar binding affinity when targeting recombinant Bcl-2-ΔTM *in vitro* (Figure 9A, Table 3; Harris et al., 2016). Contrarily, we found that binding to Bcl-2-ΔTM was effectively eliminated under similar conditions when using an ScTx-Bax construct containing three native disulfides (Figure 9A, Table 3). It should be noted that the fully oxidized ScTx-Bax protein was found to adopt a similar fold in solution compared to wild-type ScTx (Harris et al., 2016), which strongly suggested

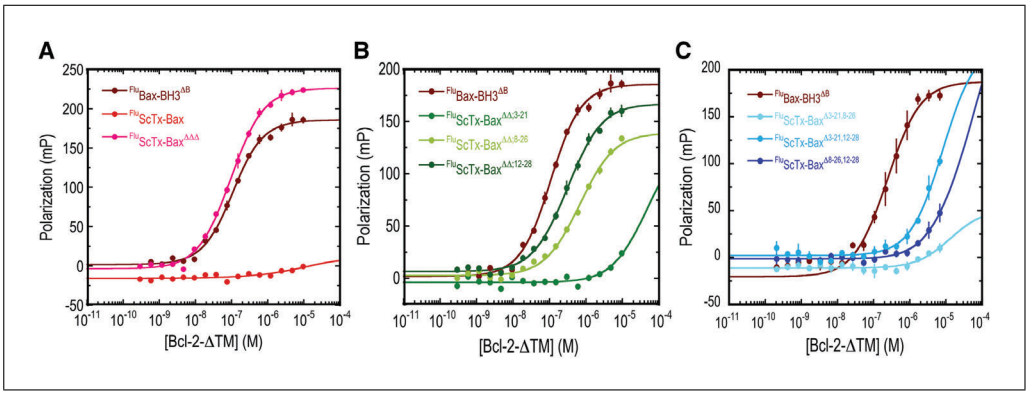

Figure 9 Results from *in vitro* fluorescence polarization (FP) direct binding assays of fluorescently labeled ScTx-Bax BH3 domain mimetics targeting the anti-apoptotic BCL2 protein Bcl-2-ΔTM in binding buffer (50 mM Tris, 100 mM NaCl, pH 7.4) at 25°C. Data points represent an average of three independent experiments; error bars are standard deviation. **(A)** FP direct binding data for ScTx-Bax peptides containing zero or three disulfide linkages; **(B)** FP direct binding data for ScTx-Bax peptides containing one disulfide linkage; and **(C)** FP direct binding data for ScTx-Bax peptides containing two disulfide linkages. All plots include the Bax BH3 domain peptide ^{Flu}Bax-BH3$^{\Delta B}$ as a positive control. Figures adapted from Arachchige et al. (2017) and Harris et al. (2016).

Table 3 Summary of Direct FP Binding Experiments and Structural Analysis of ScTx-Bax Peptides Developed Herein

Peptide	Disulfide bonds	K_d (μM)[a]	Structure[b]
^{Flu}Bax-BH3$^{\Delta B}$	0	0.21	-
^{Flu}ScTx-Bax	3	ND	ScTx-like
^{Flu}ScTx-Bax$^{\Delta 3\text{-}21,8\text{-}26}$	2	ND	ScTx-like
^{Flu}ScTx-Bax$^{\Delta 3\text{-}21,12\text{-}28}$	2	9.51	ScTx-like
^{Flu}ScTx-Bax$^{\Delta 8\text{-}26,12\text{-}28}$	2	63.28	ScTx-like
^{Flu}ScTx-Bax$^{\Delta\Delta 3\text{-}21}$	1	ND	Unstructured
^{Flu}ScTx-Bax$^{\Delta\Delta 8\text{-}26}$	1	0.38	ScTx-like
^{Flu}ScTx-Bax$^{\Delta\Delta 12\text{-}28}$	1	0.28	Unstructured
^{Flu}ScTx-Bax$^{\Delta\Delta\Delta}$	0	0.27	Unstructured

[a] Values determined from direct *in vitro* FP binding assays against Bcl-2 (Arachchige & Holub, 2018; Arachchige et al., 2017; Harris et al., 2016). ND, not determined.
[b] Determined from circular dichroism spectropolarimetry (Arachchige & Holub, 2018; Arachchige et al., 2017; Harris et al., 2016).

that binding to Bcl-2-ΔTM was abolished due to the structural rigidity of the molecule and not because the helical BH3 epitope was disrupted. To the best of our knowledge, these studies were among the first to demonstrate that an induced-fit binding mechanism is required for targeting Bcl-2 by Bax BH3 domains.

Subsequent studies by our laboratory showed that the number and position of the disulfide linkages significantly influenced the ability of ScTx-Bax proteins to target Bcl-2-ΔTM *in vitro*. For instance, ScTx-Bax proteins containing just one disulfide (ScTx-Bax$^{\Delta\Delta,3\text{-}21}$, ScTx-Bax$^{\Delta\Delta,8\text{-}26}$, and ScTx-Bax$^{\Delta\Delta,12\text{-}28}$) each bound Bcl-2-ΔTM effectively, albeit with varying affinities (Figure 9B, Table 3) (Arachchige et al., 2017). Here, it was observed that binding to Bcl-2-ΔTM was diminished when the disulfide linkage was placed near the N-terminus of the ScTx-Bax α-helix (as with the C3-C21 variant). On the other hand, tighter binding was observed when the disulfide linkage was positioned near the middle (C8-C26) or at the C-terminus (C12-C28) of the ScTx-Bax α-helix. This indicated that Bcl-2 recognition is better tolerated when the N-terminus of the Bax BH3 α-helix is more flexible. It is also worth mentioning that ScTx-Bax$^{\Delta\Delta,8\text{-}26}$ was the only ScTx-Bax$^{\Delta\Delta}$ variant observed to adopt a fold similar to wild-type ScTx

(Arachchige et al., 2017; Harris et al., 2016). This indicated that the C8-C26 disulfide linkage was important for establishing an α/β structural fold in our ScTx-Bax$^{\Delta\Delta}$ variants. Alternatively, none of the variants containing two disulfides (ScTx-Bax$^{\Delta,3\text{-}21,8\text{-}26}$, ScTx-Bax$^{\Delta,3\text{-}21,12\text{-}28}$, or ScTx-Bax$^{\Delta,8\text{-}26,12\text{-}28}$) bound Bcl-2 with high affinity (Figure 9C, Table 3), despite all ScTx-Bax$^{\Delta}$ peptides having similar structural configurations to wild-type ScTx (Arachchige & Holub, 2018). As was observed with our fully oxidized ScTx-Bax variant, these results indicated that the ScTx-Bax$^{\Delta}$ constructs were too rigid to bind Bcl-2 under these conditions.

From a molecular design standpoint, the ability to generate ScTx-based mimetics with varied disulfide linkage patterns is highly advantageous. Indeed, this strategy allows for the rigidity of secondary and tertiary structural elements within the ScTx-based peptide (e.g., helices, turns or sheets) to be finely tuned depending on the molecular interaction being investigated. In this regard, such ScTx-based molecules could be used in an exploratory context when studying PPIs in which the mechanism of binding is unknown or poorly understood. In addition, if the molecular mechanism is well known, this approach could be used to develop highly specific inhibitors of such molecular interactions by controlling the flexibility within the targeting domains. Finally, because ScTx-based mimetics can be constructed using entirely proteogenic amino acids, such constructs could be generated recombinantly in bacteria or expressed in cultured cells, thus facilitating their use as chemical genetics agents to study biochemical pathways in living systems.

Critical Parameters

The number and position of disulfide linkages within each ScTx-based peptide will determine what oxidation methods (Basic Protocol 2a, 2b, or 2c) are used during the course of the synthesis. For example, ScTx-Bax proteins containing three disulfides can be generated by reacting the reduced peptide with glutathione (Harris et al., 2016; Liang, Shu, Wang, & Zong, 1999; Vita et al., 1999). On the other hand, a two-step oxidation with orthogonal protecting groups (Acm) on complementary native cysteine thiols is required when generating ScTx-Bax peptides with two disulfide linkages (Arachchige & Holub, 2018; Veber et al., 1972). Finally, a platinum catalyst ($[Pt(en)_2Cl_2]^{2+}$) is employed when synthesizing ScTx-Bax constructs containing one disulfide (Arachchige et al., 2017; Shi & Rabenstein, 2000). Surprisingly, we found that disulfide linkages in our ScTx-Bax$^{\Delta\Delta}$ peptides did not form using standard oxidizing agents such as glutathione. We therefore utilized a moderately stronger oxidizing agent (e.g., $[Pt(en)_2Cl_2]^{2+}$) to affect disulfide formation in these ScTx-Bax variants. While the explicit mechanistic basis for the more efficient oxidation remains to be fully elucidated, we attribute this to several possible factors: (1) oxidation of thiols by *trans*-Pt(IV) reagents is highly selective and occurs without any significant side reactions (Shi & Rabenstein, 2000; Shi, Berglund, & Elding, 1996), while glutathione-mediated oxidation can result in numerous disulfide-linked side products (Okumura, Saiki, Yamaguchi, & Hidaka, 2011); (2) the reaction conditions using *trans*-Pt(IV) reagents are mild compared to those of glutathione reactions, which may result in more efficient intramolecular disulfide formation; and (3) the Pt(IV) complex and its reduced Pt(II) product are highly stable and easily separated from oxidized peptides, while the tripeptide glutathione may associate with the parent peptide in ways that are not conducive to efficient oxidation of the cysteine thiols.

It should also be noted that the positions of the disulfide(s) in ScTx-Bax$^{\Delta}$ and ScTx-Bax$^{\Delta\Delta}$ peptides can influence the efficiency of the oxidation reactions (Arachchige & Holub, 2018; Arachchige et al., 2017). For example, experiments conducted with ScTx-Bax$^{\Delta}$ peptides showed that the double oxidation was most efficient when the disulfides were positioned near the middle and C-terminus of the α-helix (e.g., ScTx-Bax$^{\Delta,8\text{-}26,12\text{-}28}$). It was also observed that the reaction rate was significantly affected by the number of disulfide linkages present in the final sequence. For instance, all reactions involving ScTx-Bax$^{\Delta\Delta}$ (one-disulfide) variants required up to 72 hr for efficient oxidation, despite being reacted with a relatively strong oxidizer and requiring only one linkage to be formed. The extent of oxidation and percent of product formed can be determined from reversed-phase HPLC and mass spectrometry analysis of the oxidized peptides. Finally, it is critical to note that while the synthesis conditions outlined in these protocols are likely to be applicable to any ScTx-based peptide, they have been optimized for ScTx-Bax sequences. It is likely that the efficiency of disulfide oxidation will depend on the primary sequence of the ScTx-based peptide itself, and the user should be prepared to optimize the reaction conditions when

working with ScTx-based mimetics with different primary sequences.

Following synthesis and purification, the number and position of the disulfide linkages within each oxidized construct is determined from mass spectrometry analysis of trypsin-digested peptides (Arachchige & Holub, 2018; Zhu et al., 2002). More specifically, peptide fragments containing disulfide linkages are analyzed by mass spectrometry for unique mass signatures that correspond to the crosslinked fragments. When analyzing such mass fragments, it is important to ensure that the ionization energy used by the mass spectrometer is not strong enough to further fragment the peptides (Brodbelt, 2016). Optimization of the mass spectrometry method may be needed to enhance the resolution of the product masses. Furthermore, use of an in-line LC/MS system may facilitate resolving the individual peaks and allow determination of their masses without the need for a separate reversed-phase HPLC step prior to mass analysis (see Support Protocol). Appropriate proteolytic digestion conditions must also be determined for each individual ScTx-based mimetic. Careful analysis of the peptide sequences should be performed to ensure that a suitable protease is chosen that will produce fragments to properly map the disulfide linkages within the oxidized construct (Luo et al., 2011).

Finally, if disulfide shuffling (e.g., formation of non-native disulfides) is observed following the oxidation reactions, it likely indicates that the oxidation reaction is not optimized for that specific peptide sequence. To be sure, this is likely only going to be an issue with ScTx-based mimetics containing three disulfides, as the protocols outlined herein employ separate oxidation reactions to generate ScTx-Bax peptides containing two disulfide linkages (see Basic Protocol 2b), and intramolecular disulfide shuffling is not possible with the single disulfide constructs. Nevertheless, it is critical that the oxidation conditions be optimized when disulfide shuffling is observed with any ScTx-based peptide. When disulfide shuffling is suspected, the first step should be to determine what non-native disulfide bonds are being formed. This knowledge can then be used to optimize reaction conditions for proper disulfide formation, including positioning of orthogonally protected cysteines (He, Pan, Mayer, & Liu, 2020). If disulfide shuffling becomes apparent in ScTx-based constructs containing three disulfide linkages, we suggest first installing orthogonal protecting groups (Acm) on two or four of the native cysteines during synthesis and running a two-step oxidation as described in Basic Protocol 2b. This process can be performed systematically, testing different combinations of orthogonal protection until optimized reaction conditions are found. Alternatively, researchers may take advantage of the multitude of orthogonal Cys protecting groups available, including acid-labile, reducing agent–labile, enzyme-labile, hydrazine-labile, or photolabile protecting groups (He et al., 2020; Postma & Albericio, 2014). Here, one could envision placing orthogonal protecting groups on each of the three complementary native cysteine pairs and running three separate oxidation reactions to form each disulfide separately. It should be noted that this approach may require up to four rounds of purification by HPLC to obtain the final oxidized product, so care should be taken to generate enough material during the initial peptide synthesis to carry through the final purification step.

Troubleshooting

See Table 4 for common problems associated with peptide synthesis/purification, oxidation reactions, and disulfide mapping. Solutions for such common problems are outlined in Table 4.

Understanding Results

The successful completion of Basic Protocols 1 and 2 will result in the synthesis of a library of eight ScTx-Bax BH3 domain mimetics that include all combinations of native disulfide linkages. From an analysis perspective, the key characterization experiments that are used to confirm differences in disulfide linkage patterns are: (1) reversed-phase HPLC, which shows changes in retention times between reduced and oxidized constructs; (2) mass spectrometry, which verifies successful synthesis of the initial reduced peptide and loss in masses corresponding to two, four, or six hydrogen atoms upon oxidation; and (3) disulfide mapping, which is used to confirm the position of disulfides within the oxidized products. Importantly, the disulfide mapping step is critical to differentiate between oxidized ScTx-based peptides that have the same mass (e.g., ScTx-Bax$^{\Delta,3\text{-}21,\ 8\text{-}26}$ and ScTx-Bax$^{\Delta,8\text{-}26,12\text{-}28}$) and ScTx-based molecules that contain non-native disulfide linkages. Importantly, the mass signatures obtained from these experiments will be unique for each variant and will ideally allow the number and positioning of the

Table 4 Troubleshooting of Commonly Encountered Problems

Problem	Potential cause	Solution
Deleted/truncated peptide sequence	Incomplete coupling of amino acids	Apply double coupling of amino acids
		Try different activating or coupling reagent
		Allow the amino acid to react with the activating agent longer before adding it to the resin
		Try longer coupling times in the microwave reactor
	Incomplete deprotection	Apply double or triple deprotection of Fmoc group
		Try different deprotection reagents such as DBU (Tickler, Barrow, & Wade, 2001)
		Use a higher percentage of piperidine in the deprotection solution
		Use longer time for deprotection reaction in microwave reactor
Aspartimide formation	HOBt is compromised (wet)	Use fresh HOBt
	Peptide sequence is conducive to aspartimide formation	Check that Asp residue is not adjacent to residues that facilitate aspartimide formation (Lauer, Fields, & Fields, 1995)
Low conversion rate during oxidation	Oxidizing agent is compromised	Use fresh oxidizing agent
	Oxidizing agent is not strong enough	Use stronger oxidizing agent
		Change reaction buffer and or pH
		Use longer reaction time during oxidation reaction
Disulfide shuffling observed	Deprotected thiols cross-react with non-complementary cysteines	Install orthogonal thiol protecting groups that will allow for separate oxidation reactions
Formation of multimeric species	Peptides aggregate and facilitate interstrand crosslinking upon oxidation	Run oxidation reaction with a lower concentration of peptide
		Add detergent to the reaction mixture to mitigate aggregation
		Lower the reaction temperature

disulfide bonds in the various members of the ScTx-based library to be determined. Finally, analytical HPLC and mass spectrometry of the final purified products can be used to verify that the ScTx-based constructs were effectively synthesized.

While we did not observe multimerization or higher-order species in any of the experiments outlined in this article, the presence of high-molecular-weight products may indicate interstrand crosslinking of the ScTx-based peptides. This result suggests that the peptides are associating in solution and establishing an environment with high local concentration of proximal interstrand thiols that result in non-native disulfides upon oxidation. The presence of such higher-order oligomers would likely be observed as a larger-molecular-weight species during HPLC purification (Mant et al., 2007). Formation of such unwanted side products may be minimized by: (1) lowering the concentration of peptide or oxidizing agent in the initial reaction; (2) increasing the temperature of the reaction; or (3) introducing detergents to alleviate unwanted aggregation of the peptide starting materials. Furthermore, the primary sequence of the peptide may facilitate interstrand association through salt bridges, electrostatic interactions, or hydrophobic effects

(Zapadka, Becher, Gomes Dos Santos, & Jackson, 2017). Therefore, additional experimental analysis may be required to determine if the sequence of the ScTx-based molecule itself facilitates aggregation of the reduced starting material or misfolding of the oxidized product (Houshdar Tehrani, Bamoniri, Mirjalili, & Gholibeikian, 2018).

The ScTx-based proteins developed in this procedure are derived from the helical Bax BH3 domain, and are capable of targeting anti-apoptotic BCL2 proteins *in vitro* (Figure 9). We envision that such ScTx-Bax proteins can be used as tools to study the molecular nature of the BH3:BCL2 interaction. For instance, direct and competitive binding assays could be used to evaluate the affinity (and specificity) that such ScTx-Bax constructs have for anti-apoptotic, pro-apoptotic, or activator members of the BCL2 family. To the best of our knowledge, this specific approach for ScTx-based disulfide patterning has only been applied to study BH3:BCL2 interactions. Nevertheless, this technique could, in principle, be applied to virtually any ScTx-based molecule that has been re-engineered to include recognition elements from other proteins of interest (POIs). In this context, a POI and its target ligand will be determined by the user's interest. Once a suitable protein-ligand pair has been established, the user could apply ScTx-based ligands with varied patterns of disulfide linkages to explore the molecular nature of the PPI. It is also worth mentioning that the protein grafting/disulfide patterning strategy is not limited to ScTx α-helices. We envision that this technique could be applied to study the molecular nature of interactions that involve other elements of secondary structure, including β-sheets and turns. For example, this could be accomplished by grafting residues important for molecular recognition onto the β-sheet of ScTx-based mimetics and evaluating how structural rigidity affects recognition by varying the number and position of disulfide linkages within these constructs (Vita et al., 1995, 1999). Users may also find it advantageous to test different experimental conditions, such as stronger oxidizing agents or the use of different orthogonal protecting groups, to optimize the protocols outlined herein for their own specific applications.

Time Considerations

Depending on the synthesis and oxidation schemes, Basic Protocols 1 and 2 can be completed in approximately 1-3 weeks. The peptide synthesis protocol for a 31-amino acid protein will take up to 4 days to complete using a manual method (assuming an 8-hr workday and 1 hr per amino acid coupling) and less than 2 days to complete using an automated peptide synthesizer or flow chemistry setup (Hartrampf et al., 2020; Mäde, Els-Heindl, & Beck-Sickinger, 2014). Additional time (3-5 days) will be needed for cleavage, lyophilization, and purification of the reduced peptide. Generally speaking, peptides will take 2-3 days to lyophilize to dryness, and HPLC purification of a suitable amount of sample can take 1-2 days.

The remaining time for the synthesis will be determined by the type of oxidations being performed. As mentioned in Basic Protocol 2a, the oxidation step for a single disulfide-containing ScTx-Bax mimetic can take up to 72 hr (3 days). This is followed by a final HPLC purification (1-2 days) and lyophilization (2-3 days). Basic Protocol 2b, outlines the synthesis of ScTx-Bax peptides containing two disulfides. Here, the first oxidation step takes 24 hr, followed by 3-5 days for purification and lyophilization. Removal of the orthogonal Acm protecting groups and subsequent oxidation take 2 hr. The second round of oxidation is then followed by an additional round of purification and lyophilization, which can take up to 3-5 days. Finally, the oxidation step for a peptide containing three disulfide linkages is outlined in Basic Protocol 2c and takes 2 hr to complete. This one-step oxidation reaction is then followed by one round of lyophilization and purification (3-5 days). Once the purified peptides have been lyophilized for the final time and characterized for purity, they can be used immediately in subsequent experiments or stored in sealed tubes in a desiccated container at −20°C.

The Support Protocol can be completed in 1 day. Here, peptides including one, two, or three disulfide linkages are incubated in proteolysis buffer for 2 hr. Following the reaction, the peptide fragments are separated by analytical HPLC (1 hr). Finally, the peptide fragments are analyzed by an appropriate mass spectrometry method (2 hr). Sequencing and mass profiling may take up to 2 hr depending on the user's analysis procedure.

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Author Contributions

Matthew J. K. Vince: formal analysis, funding acquisition, investigation, writing original draft, figure/table generation, review of writing and editing. **Justin M. Holub**: conceptualization, funding acquisition, project administration, supervision, writing review, figure/table generation, editing, and writing final draft.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

No new data was produced during this project.

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