SUPPORTING INFORMATION

Biocatalytic detoxification of paralytic shellfish toxins

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I. Cloning

**General considerations.** *E. coli* cloning strain DH5α (Invitrogen) was used for DNA propagation. Phusion HF polymerase and SspI restriction enzyme were purchased from New England BioLabs. *Taq* DNA polymerase, 10x PCR buffer, MgCl₂, and dNTPs were purchased from Invitrogen. Ligation independent cloning (LIC) qualified T4 DNA polymerase was purchased from EMD Millipore. QiAquick PCR Purification, Gel Extraction and Spin Miniprep kits were purchased from Qiagen. LIC vectors pMCSG7 (N-terminal 6xHis tag) and pMCSG9 (N-terminal 6xHis tag and maltose binding protein, MBP) were obtained from the Center for Structural Biology (CSB) at the University of Michigan.¹ The primers listed in Table S1 were used to clone the genes listed in Table S2 into LIC vectors following previously described procedures.² Ambient temperature, where noted, indicates 22 °C. A TC9610 Multigene Optimax thermocycler from Labnet International was used for all PCR reactions and cloning procedures. All primers were purchased from Integrated DNA Technologies (IDT). Codon-optimized genes were purchased as gBlocks from IDT. ddH₂O was sourced from a MilliQ Biocel water purification unit from Millipore.

**DNA and protein sequences**

*sxtSUL* from *Microseira wollei*

**DNA**

ATGAAAAAGCCCAACCTTTTCGTTGTGGTGTCACCGACAAAGTCATGAGTAATGGCACAATTATATCTGGGCCAGACAGCGACCAATCCCAGAAGACTACATATCGCATGTTGGGCTTGTCCACGTTCCCGTAGTACCGTGATCACTCGTGCTTTGAAAATCTGGATGGCTGCATCGTGTACGACGAACCGCTTTTTGCTCCGAGCGTATTCATCACAACCTA

**Protein**

MKKPNLFVVGAPKCTTSMHNYLQHPHEIFMSSPHELHYFSPIDYFPQKITGINSYLQFFDSAGDAKYGESSPELYSEVAAQQIKEFCSEAIIMLRNPEDMLYLHGDLLWKLDEPDESEIALAQERKQGRKIPESSKSHHEIKFLLYFDWKVYSTQVYRFKMKFENGHIILFNEFVKDTANIRYQTLFELGAVPDFPEIKVKNPSKFPNIAVRSFRFKYPHPHLVDKLESSLYYYADLVKFLFYDLGKFGQPRPKIDPELRLKLMKHFQPEIELAILLNWDEQRLQLWLQ

*sxtN* from *Aphanizomenon* sp. NH-5

**DNA**

ATGAAAAAGCCCAACCTTTTCGTTGTGGTGTCACCGACAAAGTCATGAGTAATGGCACAATTATATCTGGGCCAGACAGCGACCAATCCCAGAAGACTACATATCGCATGTTGGGCTTGTCCACGTTCCCGTAGTACCGTGATCACTCGTGCTTTGAAAATCTGGATGGCTGCATCGTGTACGACGAACCGCTTTTTGCTCCGAGCGTATTCATCACAACCTA
Protein

**gxtA from *Microseira wollei***

**DNA**

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ATGACCACCGCAGATCTGATTCTGATTAATAACTGGTATGTTGTGGCCAAGGTTGAAGATTGTCGTCCGG
GTAGCATTACACACAGCACATCTGCTGGGTGTTAAACTGGTTCTGTGGCGTAGCCATGAACAGAATAGCCCG
GTTCAGGTTTGGCAGGATTATTGTCCGCATCGTGGTGTTCCGCTGAGCATGGGT
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Protein

**MTTADLILINNWYVVAKVEDCRPGSITTAHLLGVKLVLWRSHEQNSPIQVWQDYCPHRGVPLSMGEVANN
TLVCPYHGWRYNQAGKCVQIPAHPDMVPPASAQAKTYHCQERYGLVWVCLGNPVNDIPSFEWDPNYHK
TYTKSYLIQASPRFVMDSINSVDHPFHEIGLDRNHEVEDELVHKDGLMKGYQHTSFKNSSTK
DSDMMNVFLSHPLCQYCTESAERMRTVDLMVTPIDEANDSVLRYLMWNGSKTLLESKILADYDQVIEED
IRILHSQQPTRLPLLSPKQINTQGLPQEIHVPDRCSTVAYRRLKELGVTYGVC**

**vanB**

**DNA**

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TACCTTCCAAATCCTAGATCGAATGGGTTTTTGTGAGCGCCAGCCACCAGCCAGGCGGCTGAGTTTTCTCG
GCTACGAACGTGCTGTTGACGTCAGCTCAGCTGCGCCGCAATTACGCAGGCTGACACATTTTTCGAG
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TACTAACATTAAATAGCAGCAGCTTTGGAAGGAAAATCTTAAGCATTGTCGCAAAAAACAAATTCCGAG
CGGATTTAAAGTATCGCAAGAATCTGCTGTTGATGTTCTGAGATCGCTGGAAGATTGTCGTCCGG
GTAGCATTACACACAGCACATCTGCTGGGTGTTAAACTGGTTCTGTGGCGTAGCCATGAACAGAATAGCCCG
GTTCAGGTTTGGCAGGATTATTGTCCGCATCGTGGTGTTCCGCTGAGCATGGGT
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Protein

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MKTKHIAMWACPRSRSTVITRAFENLDGCIVYDEPLFAPSVFITYTNINSRTLEEENLQLAQNFE
PDIKKVIEKIGNLPGKFLSFQMRMTGDARSEFGIDWVKKLNFIHDPQDIILSDFIAEKGTITEP
FTQQIMGKTYQFQIQIELLTQPFPLVHISDIKINPSALEWLCNKLEALFDKMLTWKANLDSWNL
LYQLFANFEFSSEAWFETLRSLQTKTFLPYEKIEIKLPDHILPILDESIIPYGLQHCHSEFVSEHRV
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CCTTCCTACTGGGATGATTCGCCAATATTCTTTATGCAATCACCCTGAGGAAACGCCATCGCTACCTTATTGTTGGTTCTGCGTGACCCCGCATCCCGTGGTGGGTCGCGCGCGATGCACGAATTGATCGAGCCTGGTACAC
GTTTGCAAATCTCAGAGCCGCGCAATCTTTTTCTTTGTCGCTGAGCCGCTCTGACTTCACTTTACCGGAGCCGTGCTTCAATGGCCGAGTTTCTGGCCCAAGACGGTGCCGCTTTCGAGCTGCACTACTGTGCGCGTTCCCGTGAGCGCGCAGCTTTCGTAGAACGTTTGCGTTTGTCACCTTACGGGACCGTGTCTTCCTTCATTCTCGATGAAAGACCCGCGACACCCTTGCTGAGCGGCCAGTCGTCTGTCCACATGCAATATATTTTAGATACGGCGCGC
CATCATGGGTGGGAAGAGACCCGCCTTCACCGTGAGTATTTTTCCGCAGCGCCAGTTGATACGCGTGCGGATGGTTCTTTCAGTGTAAAGCTGGCGCGCTCGGGGCAGGTTTTCGACATCCCGGCAGATCGCTCTGTA
GCAAGTGTTGGAATCACACGGGATCGAAATTCCAATTAGTTGTGAGCAGGGCATCTGTGGAACCTGCTTGACCCGCGTCTTGGAAGGGGTCCCAGAACATCGCGATATGTTTTTAACTGAAGCAGAGCAGGCGTGTAATG
ATCAATTCACGCGGCTTTGCTACGCCTAAGACGCCTCTCTCTGTACCCTATGGAACTG
Protein
MIEVIVGAI RLEARQDIHF SFE LDLHPNLVQRQYSLC GPAERP RYRI AVLRCR

Table S1. PCR primers used in this study.

| Primer name  | Primer DNA sequence (LIC extension regions are underlined) |
|--------------|-----------------------------------------------------------|
| SxtSUL_F     | 5’ – TACTTCCAATCCAAATGCAATGAAAGCCCAACCTTTTC – 3’         |
| SxtSUL_R     | 5’ – TTATCCACTTTCAATGCTTAATGAAAGCCCTATTGAGACG – 3’      |
| SxtN_F       | 5’ – TACTTCCAATCCAAATGCAATGAAACTAAATCATATCGCAATG – 3’  |
| SxtN_R       | 5’ – TTATCCACTTTCAATGCTTAATGAAAGCCCTATTGAGACG – 3’      |
| GxtA_F       | 5’ – TACTTCCAATCCAAATGCAATGAGACCGCAGATC – 3’            |
| GxtA_R       | 5’ – TTATCCACTTTCAATGCTTAATGAAAGCCCTATTGAGACG – 3’      |
| VanB_F       | 5’ – TACTTCCAATCCAAATGCAATGAGACCGCAGATC – 3’            |
| VanB_R       | 5’ – TTATCCACTTTCAATGCTTAATGAAAGCCCTATTGAGACG – 3’      |

Table S2. Protein sequence accession numbers (GenBank), DNA sources, and vectors used in this study.

| Protein      | Organism of origin | Accession   | DNA Source                     | Vector   |
|--------------|--------------------|-------------|--------------------------------|----------|
| SxtSUL       | Microseira wollei  | ACG63834.1  | IDT, fragment, codon-optimized | pMCSG7   |
| SxtN         | Aphanizomenon sp. NH-5 | ACG63812.1  | IDT, fragment, codon-optimized | pMCSG7   |
| GxtA         | Microseira wollei  | ACG63835.1  | IDT, fragment, codon-optimized | pMCSG9   |
| VanB         | Pseudomonas aeruginosa | NP_253592.1 | IDT, fragment, codon-optimized | pMCSG7   |
II. Protein expression and purification

**General considerations.** Proteins were expressed in *E. coli* strains BL21(DE3), BL21(DE3)-pGro7, and C41(DE3). Cells were grown in LB supplemented with the corresponding antibiotics ampicillin (100 μg/mL) and/or chloramphenicol (35 μg/mL in 95% ethanol) (Gold Biotechnology). HisPur nickel-nitriotriacetic acid (Ni-NTA resin) was purchased from Thermo Scientific. Fast protein liquid chromatography (FPLC) was conducted on a GE Healthcare ÄKTA Pure FPLC with 5 mL HisTrap, 5 mL MBPTrap, or HiPrep 16/60 Sephacryl S-200 High Resolution columns (GE Healthcare). Proteins were concentrated using Amicon centrifugal filters purchased from EMD Millipore at 4,500 x g at 4 °C. PD-10 desalting columns were purchased from GE Healthcare. Protein samples were analyzed on 10% Tris SDS-PAGE gels and visualized with Protein Ark Quick Coomassie Stain from Anatrace. All purification steps were performed at 4 °C. Protein and iron quantifications were performed according to previously described methods.

**Expression protocol for SxtSUL.** pMCSG7 plasmid containing sxtSUL was transformed by heat-shock into BL21(DE3) *E. coli* cells containing the pGro7 plasmid and plated on an ampicillin/chloramphenicol plate. A single colony was used to inoculate 10 mL LB containing 100 μg/mL ampicillin and 35 μg/mL chloramphenicol and incubated at 37 °C, 200 rpm overnight. The overnight culture was used to inoculate a 1 L LB culture in a 2.8 L flask containing 100 μg/mL ampicillin and 35 μg/mL chloramphenicol. Cultures were incubated at 37 °C and 250 rpm until an optical density at 600 nm (OD$_{600}$) of 0.6 was achieved. Expression of GroEL and GroES was induced by the addition of 0.5 mg/mL arabinose, freshly prepared. The culture was incubated in a shaker cooling to 20 °C for 1 h, 200 rpm. Expression of SxtSUL was induced by the addition of 0.1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and the culture was incubated at 20 °C, 200 rpm overnight (~18 h) before harvesting. The typical wet mass of a pellet from a 1 L culture was 4 g.

**Expression protocol for SxtN and VanB.** pMCSG7 plasmid containing sxtN or vanB was transformed by heat-shock into BL21(DE3) *E. coli* cells. A single colony was used to inoculate 10 mL LB containing 100 μg/mL ampicillin and incubated at 37 °C, 200 rpm overnight. The overnight culture was used to inoculate a 1 L LB culture in a 2.8 L flask containing 100 μg/mL ampicillin. Cultures were incubated at 37 °C and 250 rpm until an OD$_{600}$ of 0.6–0.8 was achieved. Flasks were chilled at 20 °C for 1 h before induction by addition of 0.1 mM IPTG (100 μL of 1 M stock). Cultures were incubated overnight at 20 °C and 200 rpm overnight (~18 h) before harvesting. The typical wet mass of a pellet from a 1 L culture was 6 g.

**Expression protocol for GxtA.** pMCSG9 plasmid containing gxtA was transformed by standard heat-shock protocols into chemically competent C41(DE3) *E. coli* cells. A single colony was used to inoculate 10 mL of LB containing 100 μg/mL ampicillin and incubated overnight at 37 °C, 200 rpm. The overnight culture was used to inoculate a 1 L LB culture containing 100 μg/mL ampicillin in 2.8 L flasks. Cultures were grown at 37 °C and 250 rpm until the OD$_{600}$ reached 0.6. Flasks were then briefly cooled to 20 °C before addition of 0.1 mM IPTG, 0.2 mg/mL ferric ammonium citrate, and 0.4 mg/mL ferrous sulfate heptahydrate. Cultures were incubated at 20 °C and 200 rpm overnight (~18 h) before harvesting. The average pellet for a 1 L culture was 5 g wet cell mass.
Purification protocol for SxtSUL and SxtN. Pellet from 1 L of culture (~4 g) was resuspended in 50 mL lysis buffer (50 mM TrisHCl pH 7.4, 300 mM NaCl, 10% glycerol, 10 mM imidazole). Cells were lysed by sonication, 5 min total “on” time, 10 s on, 20 s off. Lyed cells were centrifuged at 40,000 x g for 30 min and the supernatant was filtered using 0.45 µm syringe filters (CellTreat). The sample was loaded onto an ÄKTA Pure FPLC system fitted with a 5 mL HisTrap column, where Buffer A was the lysis buffer and Buffer B was the lysis buffer with the addition of 400 mM imidazole. Lysate was loaded onto the column at 2.5 mL/min, washed with 5 CV of 35 mM imidazole buffer at 2.5 mL/min, and eluted in a 15 CV gradient to 100% Buffer B at 1 mL/min. 2.5 mL fractions were collected during elution. The fractions were analyzed by gel and those containing SxtSUL were pooled and concentrated to 2 mL using a 10 kDa cut-off centrifugal filter.

SxtSUL was desalted using a PD-10 column equilibrated with 20 mM TrisHCl pH 7.4, 200 mM NaCl buffer. The eluted protein was concentrated to 4.5 mg/mL (130 µM) for SxtSUL and 10.3 mg/mL (294 µM) for SxtN, aliquoted, and flash-frozen in liquid nitrogen for long-term storage at –80 °C.

Purification protocol for GxtA.2 Approximately 20 g cell pellet was resuspended in 80 mL lysis buffer (20 mM TrisHCl pH 7.4, 1 M NaCl, 1 mM DTT). Cells were lysed by sonication, 5 min total “on” time, 10 s on, 20 s off. Lyed cells were centrifuged at 40,000 x g for 30 min and the supernatant was filtered using 0.45 µm syringe filters (CellTreat). The sample was loaded onto an ÄKTA Pure FPLC system fitted with a 5 mL MBPTrap column, where Buffer A was the lysis buffer and Buffer B was the lysis buffer with the addition of 10 mM maltose. Lysate was loaded onto the column at 2.5 mL/min, washed with 5 CV Buffer A at 2.5 mL/min, and eluted in a gradient to 100% Buffer B over 5 CV at 1 mL/min. 2.5 mL fractions were collected during elution. The fractions containing the desired protein were pooled and diluted to 30 mL. 2 mg of TEV protease was added to the diluted protein, and the mixture was dialyzed overnight in 20 mM TrisHCl pH 7.4, 50 mM NaCl, 1 mM DTT, 10% glycerol buffer. The contents of the dialysis bag were combined with 1 mL Ni-NTA resin that had been washed with water and incubated for 1 h. The mixture was loaded onto a 12 mL column and allowed to drain completely. The flowthrough containing proteins cleaved from MBP were collected and concentrated to 2 mL using a 30 kDa cutoff centrifugal filter. 2 mL protein was loaded onto a Sephacryl S-200 HR gel filtration column equilibrated with 20 mM TrisHCl pH 7.4, 200 mM NaCl and separated at 0.5 mL/min. 2 mL fractions were collected. The final purified protein was visibly reddish-brown in color upon concentration to 5.9 mg/mL (154 µM) and flash-frozen in liquid nitrogen for long-term storage at –80 °C.

Purification protocol for VanB.2 Cell pellet was resuspended in 4 mL of lysis buffer (50 mM TrisHCl pH 7.4, 300 mM NaCl, 10 mM imidazole, 10% glycerol) per gram of pellet. The mixture was sonicated for 5 min total “on” time, 10 s on, 20 s off. Lyed cells were centrifuged at 40,000 x g for 30 min and the clarified supernatant was combined with 4 mL Ni-NTA resin. The mixture was incubated for 2 h and poured over a 35 mL column. The packed resin was washed with 10 mL lysis buffer followed by 10 mL each of buffer containing increasing amounts of imidazole: 25 mM, 30 mM, and 35 mM imidazole. Proteins were eluted with 20 mL elution buffer (50 mM TrisHCl pH 7.4, 300 mM NaCl, 250 mM imidazole, 10% glycerol). Fractions containing protein were run on an SDS-PAGE gel and those containing VanB were pooled and exchanged into storage buffer (50 mM TrisHCl pH 7.4, 10% glycerol) using a PD-10 desalting column. Proteins were concentrated to 206 µM and flash frozen in liquid nitrogen for long-term storage at –80 °C.
**Figure S1.** Gel of SxtN, SxtSUL, VanB, and GxtA. Expected sizes as predicted by ProtParam: SxtN (35594.74 Da, including His-tag), SxtSUL (38138.77 Da, including His-tag), VanB (37341.06 Da, including His tag), GxtA (38199.45 Da without His-tag).
III. Enzymatic reactions

**General considerations.** Stock solutions of all substrates were prepared to final concentrations of 20 mM in dimethyl sulfoxide (DMSO, analytical grade). Enzyme aliquots were stored in the buffers outlined in Part II and discarded after one freeze-thaw cycle. 3'‐phosphoadenosine‐5'-phosphosulfate (PAPS) stocks were prepared to 50 mM in 1 M TrisHCl pH 8.0 buffer and stored in 2 µL aliquots at –80 °C. Stocks of 1 mM Fe(NH₄)₂(SO₄)₂•6H₂O were prepared fresh in MilliQ water before each use. 10 mM stock solutions of nicotinamide adenine dinucleotide hydride (NADH) were prepared in water and stored at –20 °C, undergoing no more than 10 freeze-thaw cycles. All reactions were conducted in 1.5 mL plastic tubes.

**SxtSUL reactions.** 50 µL reactions consisting of 5 µM SxtSUL, 200 µM of a mixture of 11-α-hydroxySTX (16) and 11-β-hydroxySTX (17), 100 µM PAPS, and 50 mM HEPES pH 7.0 buffer were combined and incubated at room temperature for 2 h and quenched by the addition of 150 µL acetonitrile. Reactions were centrifuged at 12,000 x g for 20 min and 100 µL of the supernatant was diluted with a mixture of 50% v/v acetonitrile and 50% v/v sterile-filtered ddH₂O containing 1% formic acid.

**Cascade reactions with SxtSUL and GxtA.** 50 µL reactions consisting of 20 µM SxtSUL, 20 µM GxtA, 20 µM VanB, 200 µM STX (1), 100 µM PAPS, 500 µM NADH, 100 µM Fe(NH₄)₂(SO₄)₂, and 50 mM TrisHCl pH 8.0 buffer were combined and incubated at room temperature for 2 h and quenched by the addition of 150 µL acetonitrile. Reactions were centrifuged at 12,000 x g for 20 min and 100 µL of the supernatant was diluted with a mixture of 50% v/v acetonitrile and 50% v/v sterile-filtered ddH₂O containing 1% formic acid.

Reactions monitoring epimerization over time were conducted using the same reaction concentrations as described above but were scaled to 500 µL and incubated at 30 °C for 8 days. 50 µL aliquots were taken every 24 h to evaluate epimerization of GTX3 (8) to GTX2 (7) by LC-MS. Control reactions replacing SxtSUL with protein storage buffer (20 mM TrisHCl pH 7.4, 200 mM NaCl) were conducted to assess epimerization of the GxtA product 11-β-hydroxySTX (17) to 11-α-hydroxySTX (16) under the same reaction conditions. Reactions were prepared for analysis by LC-MS as described above.

**SxtN reactions.** 50 µL reactions consisting of 50 µM SxtN, 200 µM substrate (STX (1), α-STOH (20), β-STOH (19), or ddSTX (18)), 100 µM PAPS, and 50 mM HEPES pH 7.0 buffer were combined and incubated at room temperature for 2 h and quenched by the addition of 150 µL acetonitrile. Reactions were centrifuged at 12,000 x g for 20 min and 100 µL of the supernatant was diluted with a mixture of 50% v/v acetonitrile and 50% v/v sterile-filtered ddH₂O containing 1% formic acid.

**Cascade reactions with SxtSUL, GxtA, and SxtN.** 50 µL reactions consisting of 20 µM SxtSUL, 20 µM SxtN, 5 µM GxtA, 5 µM VanB, 200 µM STX (1), 200 µM PAPS, 500 µM NADH, 100 µM Fe(NH₄)₂(SO₄)₂, and 50 mM TrisHCl pH 7.0 buffer were combined and incubated at room temperature overnight (~10 h) and quenched by the addition of 150 µL acetonitrile. Reactions were centrifuged at 12,000 x g for 20 min and 100 µL of the supernatant was diluted with a mixture of 50% v/v acetonitrile and 50% v/v sterile-filtered ddH₂O containing 1% formic acid.
IV. LC-MS and MS/MS Analysis

**General considerations.** Liquid chromatography-mass spectrometry analysis was performed on an Agilent G6545A quadrupole-time of flight (Q-TOF) or Agilent 6230 time of flight (TOF) mass spectrometer equipped with a dual AJS ESI source and an Agilent 1290 Infinity series diode array detector, autosampler and binary pump. Solvent A = water with 0.1% formic acid. Solvent B = 95% acetonitrile, 5% water and 0.1% formic acid. An Acquity UPLC BEH Amide 1.7 µm, 2.1 x 100 mm hydrophobic interaction liquid chromatography (HILIC) column from Waters was used for all separations. The chromatographic method was as follows: 18% solvent A 0–5 min and gradient 18% to 40% A 5–7 min followed by an 8 min re-equilibration at 18% A, with 0.4 mL/min flow rate. 0.5–2 µL injections were made for each sample. Targeted MS/MS was performed using the Q-TOF, and the same column and method described above to obtain fragmentation patterns. Methods were augmented to target each mass. Collision energies were set to 10, 20, and 30 eV and the resulting chromatograms were the averages of the three trials.

**SxtN conversions with substrates.** To determine which substrate was preferred by SxtN, endpoint reactions were conducted on 50 µL scale in triplicate. 50 µM SxtN, 200 µM substrate (ddSTX (18), α-STOH (20), β-STOH (19), STX (1)), 300 µM PAPS, and 50 mM HEPES pH 7 buffer were combined and incubated at room temperature for 2 h. Reactions were quenched by the addition of 450 µL acetonitrile. Standards (Figure S2) were prepared in duplicate under the same conditions with the omission of PAPS. All standards and samples were centrifuged in a 96-well plate at 2000 x g for 2 min. 10 µL of each centrifuged sample was diluted with 190 µL of a mixture of of 50% v/v acetonitrile and 50% v/v sterile-filtered ddH₂O containing 1% formic acid and 6 µg/mL ¹⁵N-arginine. Samples were filtered using a 96-well 0.22 µm filter plate. The plate was centrifuged over a new 96-well plate at 2000 x g for 2 min. 1 µL each sample was injected on the TOF LC-MS instrument for analysis. Total turnover number (TTN) was determined by dividing the quantity substrate consumed by the amount of total enzyme added to the reaction.
**Figure S2.** Standard curves of A) ddSTX (18), B) $\alpha$-STOH (20), C) $\beta$-STOH (19), and D) STX (1) for quantification of substrate consumption in endpoint assay. Peak area ratio = (peak area substrate)/(peak area $^{15}$N-arginine internal standard).

**Figure S3.** Relative conversions and total turnover numbers (TTNs) of ddSTX (18), $\alpha$-STOH (20), $\beta$-STOH (19), STX (1), neoSTX (2), 11-$\alpha/\beta$-hydroxySTX (16/17), and GTX2/3 (7/8) in reaction with SxtN.
**Steady-state kinetics of SxtN.** To determine the steady-state kinetic parameters of the SxtN reaction, reactions were conducted on 50 µL scale with PAPS ranging 1 µM – 40 µM in duplicate with 10 µM SxtN, 200 µM STX (1), and 50 mM HEPES pH 7 buffer in a 96-well plate. For determining kinetic parameters of STX (1), the substrate range was 25 µM – 2 mM with 50 µM PAPS held constant. Reactions were initiated by the addition of 10 µM SxtN (10 µL distributed by multichannel pipette). Reactions were quenched after 5 min by the addition of 150 µL of a mixture of 50% v/v acetonitrile and 50% v/v sterile-filtered ddH2O containing 1% formic acid and 6 µg/mL 15N-arginine. 96-well plates were centrifuged at 2000 x g for 2 min, then 100 µL of the centrifuged mixture was added to a clean 96-well 0.22 µm filter plate. The plate was centrifuged over a clean 96-well plate at 2000 x g for 2 min. Product standards were prepared in the same manner, in duplicate, with SxtN storage buffer (20 mM Tris HCl pH 7.4, 200 mM NaCl) replacing enzyme. 3 µL each sample and standard was injected on the TOF LC-MS. GTX5 (10) standard curve used to quantify product is shown in Fig. S2.

![Figure S4](image-url)

**Figure S4.** Standard curve of GTX5 (10). Peak area ratio = (peak area GTX5)/(peak area 15N-arginine internal standard).

For reactions with α-STOH (20) and β-STOH (19), reaction conditions were identical to those described above for determination of STX (1) kinetic parameters. For these two substrates, the reactions failed to reach a V_max up to 2.5 mM substrate and parameters could not be reliably determined. The K_M for each substrate is anticipated to be >1 mM based on the data shown in Figure S4.

For reactions with ddSTX (18) no product could be detected using the reaction conditions specified for STX (1), α-STOH (20), and β-STOH (19) and cannot be compared. Product formation is only observed in reactions with high enzyme concentrations (e.g. 50 µM). Based on percent conversion and TTN, it is anticipated that the K_M of ddSTX (18) would be similar to or higher than those of α-STOH (20) and β-STOH (19).
Figure S5. Michaelis-Menten plots of A) α-STOH (20) and B) β-STOH (19) substrates in reaction with SxtN. Peak area ratio = (peak area product)/(peak area $^{15}$N-arginine internal standard).
SxtSUL reactions

Figure S6. SxtSUL reaction with a mixture of 11-α-hydroxySTX (16) and 11-β-hydroxySTX (17). A) LC-MS trace, B) MS/MS spectra comparing the major SxtSUL product to the GTX3 (8) standard.
Figure S7. Assessment of epimerization. A) Epimerization of GTX3 (8), product of the SxtSUL + GxtA reaction, over time to generate GTX2 (7). B) GxtA-only reactions comparing 11-β-hydroxySTX (17) product over time. Epimerization to 11-α-hydroxySTX (16) was not observed.

SxtN reactions

Figure S8. Reaction of SxtN with STX (1) to yield GTX5 (10). A) LC-MS traces, B) MS/MS spectra comparing the SxtN reaction product to an authentic standard of GTX5 (10).
Figure S9. Reactions of SxtN with A) ddSTX (18) and B) a mixture of α-STOH (20) and β-STOH (19).
Figure S10. Reaction of SxtN + GxtA. A) Reaction scheme and LC-MS traces of products and standards. B) MS/MS spectrum of product 4, identified as M1β, compared to the MS/MS spectrum of GTX3 (8). Both compounds have the same exact mass, m/z = 396.0932. For comparison, M1 compounds were previously observed to have the same fragmentation pattern observed in this study.4
V. Mouse whole-brain binding assays

[^3]H-saxitoxin competition binding. To measure the competitive binding of PSTs vs. saxitoxin (STX, 1) to sodium channels, whole brain membranes were prepared from adult (postnatal day (P) 30–150) wildtype C57Bl/6J mice as described previously. Equilibrium[^3]H-STX binding in the presence or absence of PST was measured at 4 °C for at least one hour using a vacuum filtration assay with a saturating concentration (5 nM) C11 labeled[^3]H-STX (20 Ci/mmol, American Radiolabeled Chemicals Inc.). In a subset of samples, 10 µM unlabeled tetrodotoxin (TTX, Alomone Labs) was added to assess nonspecific binding. To quantify[^3]H-STX binding, counts per minute (CPM) values obtained from liquid scintillation counting (Packard Tri-carb 1900TR) were corrected for specific binding by subtraction of nonspecific (in the presence of 10 µM TTX) values and then converted to decays per minute (DPM) before normalization to total protein concentration using the bicinchoninic acid (BCA) protein assay (Thermo-Fisher). A stock of each unlabeled toxin was diluted in binding buffer to give a final concentration of 10000, 1000, 100, 10, 1, 0.1 and 0.01 nM. A 7-point concentration-response curve was generated for each unlabeled PST in competition with 5 nM[^3]H-STX. Two independent experiments were conducted for each unlabeled PST and, in each experiment, each sample was tested in duplicate. The average DPM reading of duplicate values was used for analysis.

Data analysis. Competitive binding data were analyzed using Microsoft Excel and Graph Pad Prism v7.0. The specific binding curve in[^3]H-STX (fmol/ mg protein) was initially generated using the following one-site IC_{50} equation:

\[ Y = \frac{\text{Bottom} + (\text{Top} - \text{Bottom})}{1 + 10^{x\cdot \log(\text{IC}_{50})}} \]

where Top and Bottom are plateaus in the units of the Y-axis. IC_{50} represents the concentration in nanomolar of unlabeled competitor that results in binding half-way between Top and Bottom values. Data were then normalized for each concentration point to the Top value obtained in the assay for each unlabeled toxin to obtain fraction of total binding. Each Y value thus represents the fraction of maximal[^3]H-STX displacement at the corresponding concentration (X value). To obtain K_i values from the IC_{50}, the following equation was used:

\[ K_i = \frac{\text{IC}_{50}}{1 + \frac{[\text{radioligand}]}{K_d}} \]

where[^3]H-STX] = 5 nM in each assay at equilibrium. K_d of[^3]H-STX = 2.5 nM. K_i = equilibrium dissociation constant of the unlabeled competitor in nM.
Table S3. Specific binding of PSTs to mouse whole-brain membrane samples in competition with 5 nM [3H]-STX. Values are expressed in femtomoles of [3H]-STX per milligram of total protein present in the mouse whole-brain membrane preparation.

| [toxin], nM | STX   | GTX5 | 11-α/β-hydroxySTX | GTX2/3 | C1/C2   |
|-------------|-------|------|-------------------|--------|---------|
| 1000        | -3.540142 | -0.769596 | 29.95667685 | 10.280332 | 9.58921429 | 6.65476396 | 1.86901914 | 6.4265629 | 69.4398763 | 57.3058915 |
| 1000        | 1.0114692 | 2.308783 | 131.1755689 | 62.7874072 | 68.2832792 | 56.2279332 | 15.8460318 | 36.0805737 | 218.0223 | 191.883291 |
| 100         | 12.665353 | 17.898607 | 314.5265888 | 224.288115 | 237.479708 | 249.524715 | 86.0019876 | 192.631704 | 299.467175 | 276.579309 |
| 10          | 88.503553 | 99.145968 | 417.9010435 | 390.707906 | 405.722403 | 471.35018 | 189.691899 | 345.712489 | 310.547132 | 297.459381 |
| 1           | 354.27807 | 399.75021 | 421.9726447 | 448.742041 | 454.078734 | 524.048196 | 214.232933 | 426.929513 | 305.551901 | 314.638085 |
| 0.1         | 410.20023 | 507.86747 | 395.7928016 | 435.200743 | 478.033247 | 466.527888 | 215.727231 | 375.182882 | 310.917149 | 283.611909 |
| 0.01        | 484.47175 | 563.74015 | 442.2569567 | 413.516242 | 489.853896 | 515.638117 | 220.490084 | 375.293051 | 325.635607 | 281.129814 |

Figure S11. Concentration response curves showing specific binding of PSTs to mouse whole-brain membrane samples in competition with 5 nM [3H]-STX. Unlabeled STX (1), GTX5 (10) and mixtures of 11-α (16) & 11-β-hydroxySTX (17), GTX2 (7) & GTX3 (8), and C1 (12) & C2 (13) were tested at 10 µM, 1 µM, 100 nM, 10 nM, 1 nM, 100 pM and 10 pM. Each data point represents the mean of displaced [3H]-STX in femtomoles per milligram of total protein. Error bars represent the range of all values. n=4.

Table S4. Specific binding of PSTs to mouse whole-brain membrane samples in competition with 5 nM [3H]-STX. fmol [3H]-STX/mg protein values were normalized to the total specific binding (top) value for each experiment and expressed as a fraction of total.

| [toxin], nM | STX   | GTX5 | 11-α/β-hydroxySTX | GTX2/3 | C1/C2   |
|-------------|-------|------|-------------------|--------|---------|
| 1000        | 0.007680933 | 0.001397233 | 0.071122193 | 0.023775052 | 0.020387031 | 0.013146511 | 0.008556524 | 0.016415237 | 0.221498808 | 0.194785491 |
| 1000        | 0.002194552 | 0.0041917 | 0.311432975 | 0.145206767 | 0.145036702 | 0.110784893 | 0.072621594 | 0.092159831 | 0.695445932 | 0.652220568 |
| 100         | 0.027479612 | 0.032495655 | 0.74673929 | 0.518705618 | 0.504417391 | 0.492937011 | 0.39414264 | 0.492035004 | 0.955238199 | 0.94016419 |
| 10          | 0.192023331 | 0.180003573 | 0.99216772 | 0.903578901 | 0.861772308 | 0.93115405 | 0.869348798 | 0.88304596 | 0.990580963 | 1.011078792 |
| 1           | 0.768665817 | 0.72576291 | 1.001834389 | 1.03779304 | 0.964483292 | 1.035259177 | 0.981819124 | 1.090487645 | 0.974647212 | 1.069470036 |
| 0.1         | 0.889998323 | 0.922054232 | 0.939679016 | 1.004772704 | 1.015300014 | 0.9921625783 | 0.988646795 | 0.958321537 | 0.991761241 | 0.964010566 |
| 0.01        | 1.051142871 | 1.023493372 | 1.049927775 | 0.956328035 | 1.04071317 | 1.018645035 | 1.010495343 | 0.958602941 | 1.038710071 | 0.955573808 |
VI. Synthesis and standard preparation

Product standards of ddSTX (18), α-STOH (20), β-STOH (19), STX (1), neoSTX (2), 11-α-hydroxySTX (16), 11-β-hydroxySTX (17), GTX2 (7), and GTX3 (8) were prepared as previously described.\(^2\) GTX5 (10), C1 (12), and C2 (13) were authentic standards purchased from National Research Council Canada.
VII. References

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