SUPPORTING INFORMATION

C–H hydroxylation in paralytic shellfish toxin biosynthesis

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I. Cloning

General Considerations. *E. coli* cloning strains DH10B or DH5α (Invitrogen) were used for DNA propagation. Phusion HF polymerase and *Ssp*I restriction enzyme were purchased from New England BioLabs. *Taq* DNA polymerase, 10x PCR buffer, $MgCl_2$, 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 2 into LIC vectors. 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). ddH_2O was sourced from a MilliQ Biocel water purification unit from Millipore.

Codon-Optimized DNA and Protein Sequences

<u>*sxtT* from *Microseira wollei*</u>

DNA

```
ATGACCACCGCAGATCTGATTCTGATTAATAACTGGCATGTTGTGGCCAATGTGGAAGATTGTAAACCGG
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Protein

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IRILHSQQPARLPLLAPKQINTQGLPQEIHVPSDRGTVAYRRWLKELGVTYGVC
```

<u>*sxtT* from *Cylindrospermopsis raciborskii*</u>

DNA

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AGGTGTTACCTATGGTGTTTGCTAA

Protein

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

DNA

ATGACCACCGCAGATCCGATTCTGATTAATAACTGGTATGTTGTGGCCAAGGTGGAAGATTGTAAACCGG
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Protein

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sxtT from *Dolichospermum circinale*

DNA

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Protein

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gxtA (formerly *sxtDiox*) from *Microseira wollei*

DNA

ATGACCACCGCAGATCTGATTCTGATTAATAACTGGTATGTTGTGGCCAAGGTTGAAGATTGTCGTCCGG
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Protein

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sxtH from *Microseira wollei*

DNA

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Protein

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DESWTLWQRISHPLCQYYITKSSEIRTADLMLVTPIDEDNSLVRMLITWNCPEMLDSKTLETLESKLLAE
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sxtV from *Microseira wollei*

DNA

ATGAAACTGACCGCCATTAAAGAAGAACGTCAGCTGTTTACCGCATATAGCGATACCAAACTGCAGCTGA
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Protein

MKLTAIKEERQLFTAYSDTKLQLTADVLVIGGGPAAAWAAYAAAAQGVKVIIVDKGFLGTSGAAAASGNS
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sxtW from *Microseira wollei*

DNA

ATGATGATTGAACTGGTGAGCCATAAACTGTGCATTAATTGCAATGTTTGCGTTCAGGTTTGTCCGACCA
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Protein

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ddmA1

DNA

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A

Protein

MSKADVVIVGAGHGGAQCAIALRQNGFEGTITVIGREPEYPYERPPLSKEYFAREKTFDRLYIRPPTFWA
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ddmB

DNA

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Protein

MPQITVVNQSGEESSVEASEGRTLMEVIRDSGFDELLALCGGCCSCATCHVHIDPAFMDKLPEMSEDEND
LLDSSDHRNEYSRLSCQIPVTGALEGIKVTIAQED

vanB

DNA

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GATAA

Protein

MIEVIVGAIRLEAQDIHSFELFRADGAALPSFEPGAHIDLHLPNGLVRQYSLCGPAERPRHYRIAVLRCR
DSRGGSATLHAELRVGQRLHIGEPRNLFPLSPEPGPHLLFAGGIGITPLLAMAERLARDGADFQLHYCAH
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ASRLHREHFAAAPRDASADGTFEVQLASNGALIRVAAGQTVLAALREAGVDLPASCEQGICGTCLTRVLD
GEPEHRDLYLSEEEQAANDCFTPCCSRSRSPRLVLDL

ndmD

DNA

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GGACTTATGAAAGCGGGACTGGTGGCTGCACTTTTGTCCCAGCCCATCGCGATGCACCACCCCCAAATGC
CGCGCGGGTTAATACTTTTCCTGTCCGCGAAAAGCACGGCTTTATCTGGACGACATTAGGTCAGCCGCCA
GGAGAGCCCATTTCAATCCTCGATGACGCTCAGCTTGTAAACGCTGTAAAAACAAATCTGCATAGCGTAG
TTATAGATGCTGATATTGACGGAGTTGTCAGCGTCCTACGTCAGAATCTTTCAGCGTTCATCGATGTGTT
TGGTGCGGCCAGCGCTGAAGATCTGCATTTGAAATCCATGCTGCAAGATCGAGGGATTCTGGTAACAAGA
TCAGGCTCTATTGCTATTCATTTTTATATGCAGCGCTCAACCATTAGTAAATGCGTTGTACATGCGCAAG
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GGCAGCAGAAGCTGTAGCTACCGACTTGATTAGCATTACAGATATCAGCGATCAGACTATCGAAAAGCTT
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TCTGTGA

Protein

MNKLDVNQWFPIATTEDLPKRHVFHATLLGQEMAIWRDDSGSVNAWENRCPHRGLRLTLGANTGNELRCQ
YHGWTYESGTGGCTFVPAHRDAPPPNAARVNTFPVREKHGFIWTTLGQPPGEPISILDDAQLVNAVKTNL
HSVVIDADIDGVVSVLRQNLSAFIDVFGAASAEDLHLKSMLQDRGILVTRSGSIAIHFYMQRSTISKCVV
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TRTQETGDINSYWLKPIGYPLPAFSPGMHISITTPEGSIRQYSLVNGPDERESFIIGVKKEIQSRGGSRS
MHEDVKVGTQLKVTLPRNGFPLVQTRKHPILVAGGIGITPILCMAQALDQQGSSYEIHYFARAFEHVPFQ
DRLTALGDRLNVHLGLGPDETRAKLPDIMEIHNAQDVDVYTCGPQPMIETVSAVALAHGIAEESIRFEFF
SKKNDVPVSDEEYEVELKKTGQIFTVSPGSTLLQACLDNDVRIEASCEQGVCGTCITPVVSGDLEHHDTY
LSKKERESGKWIMPCVSRCKSKKIVLDL

PDR

Protein

TTPQEDGFLRLKIASKEKIARNIWSFELTNPQGAPLPPFEAGANLTVAVPNGSRRTYSLCNESSERDRYT
IAVKRDSNGRGGSISFIDDTSEGDAVEVSLPRNEFPLDKRAKSFILVAGGIGITPMLSMARQLRAEGLRS
FRLYYLTRDPEGTAFFDELTSDEWQSDVKIHHDHGDPTKAFDFWSVFEKSKPAQHVYCCGPQALMDTVRD
MTGHWPSGTVHFESFGGAAAAAAANTADTVRDARSGTSFEIPANRSINQVLRDANVRVPSSCESGTCGSC
KTGLCSGAADHRDDVLAAAAKGTQIMVCVSRAKSAELVLDL

vioC

DNA

ATGACTGAGAGCCCCACGACGCACCACGGCGCCGCGCCGCCGGATTCGGTCGCCACGCCGGTCCGGCCGT
GGAGTGAGTTTCGGCTGACGCCTGCGGAAGCCGCAGCCGCCGCCGCGCTCGCGGCCCGGTGCGCACAGCG
GTACGACGAGACCGACGGCCCCGAGTTCCTTCTCGACGCCCCCGTCATCGCCCACGAACTGCCCAGGCGG
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GCCGCGGCGGCTCTCGGGACACTGCGCAAGCTGATCGACGCGTCGCTGTACGAGCTCGTACTCGACCAGG
GCGACGTGGCCTTCATCGACAACCGCAGGGCCGTACACGGCAGGCGCGCCTTCCAGCCCCGCTACGACGG
CCGGGACCGCTGGCTCAAGCGCATCAACATCACCCGTGATCTGCACCGGTCGCGCAAGGCGTGGGCCGGC
GACTCGCGGGTCCTGGGGCAGCGATGA

Protein

MTESPTTHHGAAPPDSVATPVRPWSEFRLTPAEAAAAAALAARCAQRYDETDGPEFLLDAPVIAHELPRR
LRTFMARARLDAWPHALVVRGNPVDDAALGSTPVHWRTARTPGSRPLSFLLMLYAGLLGDVFGWATQQDG
RVVTDVLPIKGGEHTLVSSSSRQELGWHTEDAFSPYRADYVGLLSLRNPDGVATTLAGVPLDDLDERTLD
VLFQERFLIRPDDSHLQVNNSTAQQGRVEFEGIAQAADRPEPVAILTGHRAAPHLRVDGDFSAPAEGDEE
AAAALGTLRKLIDASLYELVLDQGDVAFIDNRRAVHGRRAFQPRYDGRDRWLKRINITRDLHRSRKAWAG
DSRVLGQR

Table S1. PCR primers used in this study.

Primer Name	Primer DNA Sequence (LIC extension regions are underlined)
Rieske Primers	
SxtT_wollei_F	5'-TACTTCCAATCCAATGCAATGACCACCGCAGATCTGAT-3'
SxtT_wollei_R	5'-TTATCCACTTCCAATGTTAACAAACACCATAGGTCACACCC-3'
SxtT_raciborskii_F	5'-TACTTCCAATCCAATGCAATGACCACCACCGATCC-3'
SxtT_raciborskii_R	5'-TTATCCACTTCCAATGTTAGCAAACACCATAGGTAACAC-3'
SxtT_aphan_F	5'-TACTTCCAATCCAATGCAATGACCACCGCAGATC-3'
SxtT_aphan_R	5'-TTATCCACTTCCAATGTTAACAAACACCATAGGTCAC-3'
SxtT_circinale_F	5'-TACTTCCAATCCAATGCAATGACCAATGCAGATCAG-3'
SxtT_circinale_R	5'-TTATCCACTTCCAATGTTAACAAACACCATAGGTCAC-3'
GxtA_wollei_F	5'-TACTTCCAATCCAATGCAATGACCACCGCAGATC-3'
GxtA_wollei_R	5'-TTATCCACTTCCAATGTTAACAAACACCATAGGTCAC-3'
SxtH_wollei_F	5'-TACTTCCAATCCAATGCAATGACCACCGCAGATCTGAT-3'
SxtH_wollei_R	5'-TTATCCACTTCCAATGTTATTAACAAACACCATAGGTCACACC-3'
Redox Partner Primers	
SxtV_F	5'-TACTTCCAATCCAATGCAATGAAACTGACCGCCATTAAAGAAGAACG-3'
SxtV_R	5'-TTATCCACTTCCAATGTTATTACAGTTTCAGAACGCTTGCTGTGG-3'
SxtW_F	5'-TACTTCCAATCCAATGCAATGATGATTGAACTGGTGAGCCATAAACTG-3'
SxtW_R	5'-TTATCCACTTCCAATGTTATTATTCAAACAGGCGCAGTTTATGATC-3'
DdmA1_F	5'-TACTTCCAATCCAATGCAATGTCGAAGGCAGATGTTG-3'
DdmA1_R	5'-TTATCCACTTCCAATGCTACAGCAACTCTTTCAGGGG-3'
DdmB_F	5'-TACTTCCAATCCAATGCAATGCCTCAAATCACTGTGGTCAATCAATC-3'
DdmB_R	5'-TTATCCACTTCCAATGTCAATCTTCTTGCGCAATAGTCACCTTGATTCC-3'
VanB_F	5'-TACTTCCAATCCAATGCAATGATCGAGGTTTTGGTGGCA-3'
VanB_R	5'-TTATCCACTTCCAATGCTACAGGTCAAGGACCAGAAGAGG-3'

Ligation Independent Cloning (LIC) procedures. pMCSG7 and pMCSG9 DNA was prepared by extracting the DNA from 5 mL overnight cultures grown in Luria-Bertani (LB) broth supplemented with 100 μg/mL ampicillin using the QIAprep Spin Miniprep Kit according to the manufacturer's instructions. 7 μg DNA was divided into two *Ssp*I restriction digestion reactions. To each aliquot, 20 U *Ssp*I was added. The reactions were incubated at 37 °C for 1 h before an additional 20 U *Ssp*I was added. The digestion proceeded for 18 h before denaturing at 65 °C for 5 min. Reactions were pooled and purified using the QIAquick PCR Spin Kit, eluting with 30 μL ddH_2O. The purified *Ssp*I-digested DNA was treated with LIC-qualified T4 DNA polymerase in 60 μL reactions containing 10% v/v T4 polymerase reaction buffer, 5 mM dithiothreitol (DTT), 4 mM dGTP, 3.75 U T4 DNA polymerase, and 20% v/v digested DNA. Reactions were incubated at 22 °C for 30 min followed by 75 °C for 20 min and held at 4 °C.

Gene insert fragments were prepared following a similar protocol to that outlined above. DNA sequences were amplified using the corresponding primers listed in Table S1, which include complementary LIC overhangs. In general, sequences were amplified in 50 μL PCR reactions containing 20% v/v Phusion HF buffer, 0.2 mM dNTPs, 500 nM forward primer, 500 nM reverse primer, 1 U Phusion DNA polymerase and 100 ng DNA template. Reactions were run using the following program: 98 °C 30 s, (98 °C 10 s, 58 °C – 63 °C 30 s (as determined for each primer pairing by the NEB T_m Calculator v 1.9.6), and 72 °C 1 min) for 25 cycles, and 72 °C 10 min. The entire volume for each reaction was loaded onto a 0.8% agarose gel containing 0.2 μg/mL ethidium bromide and bands at the corresponding sizes were excised and purified using the QIAquick Gel Extraction Kit. Purified fragments were treated with LIC-qualified T4 polymerase in 20 μL reactions containing 10% v/v T4 polymerase reaction buffer, 5 mM DTT, 4 mM dCTP, 1.25

U T4 polymerase, and the remaining volume of purified amplification product. Reactions were incubated at 22 °C for 30 min followed by 75 °C for 20 min and held at 4 °C.

To anneal the gene inserts into prepared LIC vectors, 2 μL insert and 2 μL vector were incubated at ambient temperature in PCR tubes overnight (~18 h). The reaction was quenched by the addition of 1 μL sterile 25 mM EDTA and incubation at ambient temperature for 5 min. The entire 5 μL volume of the annealing mixture was transformed immediately into chemically competent DH5α *Escherichia coli* (*E. coli*) cells. Cloning procedures typically resulted in 15-25 colonies with approximately 50% cloning efficiency as determined by colony PCR. Colony PCR methods were generally similar to those described above for the amplification of gene insert fragments with the exception of using *Taq* DNA polymerase instead of Phusion DNA polymerase and replacing the volume of DNA template added with water. All constructs were verified by sequencing through the University of Michigan DNA Sequencing Core.

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

Protein	Organism of Origin	Accession	DNA Source	Vectors
SxtT	*Microseira wollei*	ACG63840.1	GeneArt, subcloned	pET151, pMCSG9
SxtT	*Cylindrospermopsis raciborskii*	ABI75109.1	GeneArt, fragment	pMCSG7, pMCSG9
SxtT	*Aphanizomenon sp. NH-5*	ACG63810.1	GeneArt, fragment	pMCSG7, pMCSG9
SxtT	*Dolichospermum circinale*	ABI75133.1	GeneArt, fragment	pMCSG7, pMCSG9
GxtA	*Microseira wollei*	ACG63835.1	IDT, fragment	pMCSG7, pMCSG9
SxtH	*Microseira wollei*	ACG63831.1	GeneArt, subcloned	pET151, pMCSG9
SxtV	*Microseira wollei*	ACG63837.1	GeneArt, subcloned	pET151, pMCSG9
SxtW	*Microseira wollei*	ACZ26230.1	GeneArt, subcloned	pET151, pMCSG9
DdmA1	*Stenotrophomonas maltophilia*	AAV53700.1	IDT, fragment	pMCSG7
DdmB	*Stenotrophomonas maltophilia*	AAV53698.1	IDT, fragment	pMCSG7
VanB	*Pseudomonas aeruginosa*	NP_253592.1	IDT, fragment	pMCSG7
NdmD	*Pseudomonas putida*	H9N291.1	Subramanian lab, agar stab	pET28-His-NdmD
PDR	*Pseudomonas cepacia*	AAB24396.1	Ballou lab, protein*	N/A
VioC	*Streptomyces vinaceus*	Q6WZB0.1	Thomas lab, pure plasmid	pET28b-His-VioC

*Purified protein sample was obtained from Professor Emeritus David P. Ballou, University of Michigan, Department of Biological Chemistry.

Table S3. Rieske oxygenase systems most closely related to SxtT and their associated redox partners. Query cover and sequence identity percentages are based on the SxtT from *M. wollei* protein sequence. *SxtW and SxtV are putative redox partners for SxtT based on Neilan and coworkers.[2-4]

Rieske Oxygenase	Query Cover	Sequence Identity	Redox Partner	Cofactors
SxtT from *M. wollei*	100%	100%	SxtW/SxtV*	FAD/2[4Fe-4S]
vanillate *O*-demethylase (VanA)	98%	36%	VanB	FMN, [2Fe-2S]
methylxanthine *N*-1 demethylase (NdmA/NdmB)	97%	33%	NdmD	FAD, [2Fe-2S]
dicamba *O*-demethylase (DdmC)	49%	32%	DdmA1/DdmB	FAD/[2Fe-2S]
phthalate dioxygenase (PDO)	54%	29%	PDR	FMN, [2Fe-2S]

II. Protein Expression and Purification

General Considerations. Proteins were expressed in *E. coli* strains BL21(DE3) and C41(DE3). Cells were grown in either LB or Terrific broth (TB) supplemented with the corresponding antibiotics kanamycin (50 µg/mL) or ampicillin (100 µg/mL) (Gold Biotechnology). HisPur nickel-nitrilotriacetic acid (Ni-NTA resin) was purchased from Thermo Scientific. Fast protein liquid chromatography (FPLC) was conducted on a GE Healthcare ÄKTA Pure FPLC with 1 mL and 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 Mini-PROTEAN TGX Gels (4-15%) from BioRad and visualized with Protein Ark Quick Coomassie Stain from Anatrace. All purification steps were performed at 4 °C.

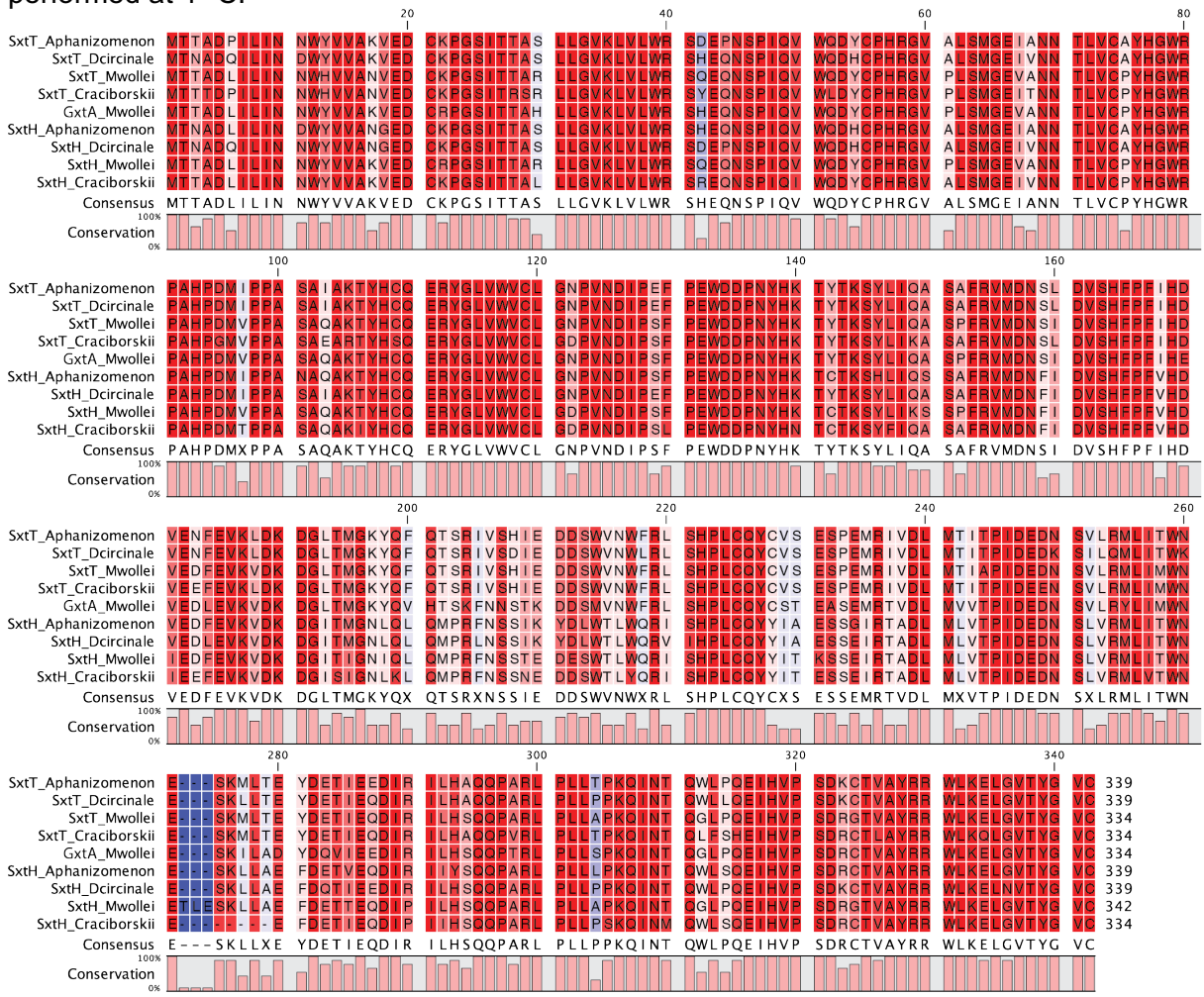


Figure S1. Multiple sequence alignment of SxtT, SxtH, and GxtA proteins by CLC Sequence Viewer (v7.6.1). Results are on a spectrum of red (most conserved) to blue (least conserved).

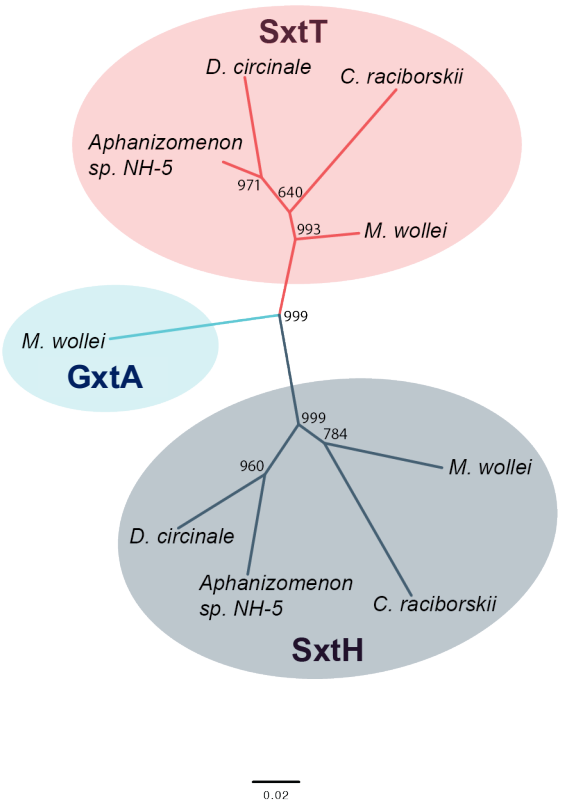


Figure S2. Phylogenetic tree of Rieske oxygenases in cyanobacterial saxitoxin biosynthetic gene clusters. The ClustalX algorithm with a Gonnet 250 weight matrix was used to obtain the pairwise distance matrix. The tree was generated in ClustalX using the neighborjoining (NJ) clustering algorithm and visualized in FigTree v1.4.3. Bootstrap values are represented out of 1000 replicates.[5]

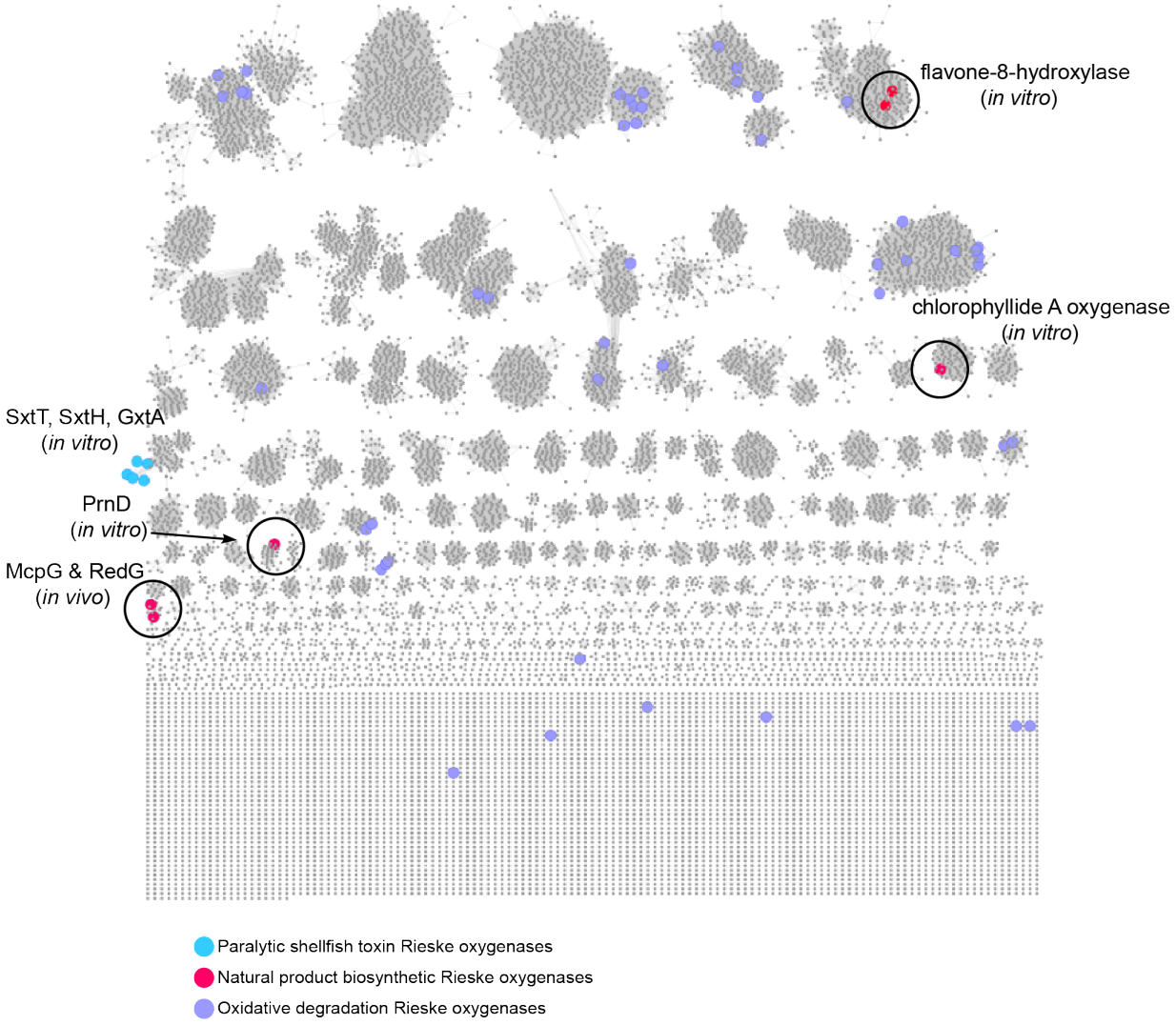


Figure S3. Network analysis of Rieske oxygenase protein family (Pfam: PF00355). Dataset was generated using the EFI-EST (Enzyme Function Initiative Tools – Enzyme Similarity Tool) using an alignment score of 70. The data was visualized in Cytoscape v 3.6.1 as a representative node network where each node contains proteins with 40% sequence identity. Blue circles indicate the locations of SxtT, SxtH, and GxtA homologs, red circles are representative of the characterized Rieskes from secondary metabolic pathways (flavone-8-hydroxylase[6], chlorophyllide A oxygenase[7], RedG and McpG[8], and PrnD[9]) and purple circles highlight all other characterized Rieske oxygenases involved in oxidative degradation pathways, either *in vivo* or *in vitro*.

Expression protocol for SxtT, MBP-SxtT, GxtA, MBP-GxtA, SxtH, MBP-SxtH, MBP-SxtW, and MBP-SxtV. pMCSG9 plasmids containing *sxtW* and *sxtV* were transformed by standard heat-shock protocols into chemically competent BL21(DE3) *E. coli* cells. pMCSG7 and pMCSG9 plasmids containing *sxtH, sxtT* and *gxtA* were transformed by heat-shock into C41(DE3) chemically competent cells to afford a higher percentage of soluble protein than observed from overexpression in BL21(DE3) *E. coli* cells. A single colony was used to inoculate 5 mL of LB containing 100 µg/mL ampicillin and incubated overnight at 37 °C, 200 rpm. Overnight cultures were used to inoculate 500 mL TB cultures containing 4% glycerol (v/v) and 100 µg/mL ampicillin

in 2.8 L flasks. Cultures were grown at 37 °C and 250 rpm until the optical density at 600 nm (OD_{600}) reached 0.6. Flasks were then incubated at 20 °C for 1 h followed by addition of 0.1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) with the additives 0.2 mg/mL ferric ammonium citrate and 0.4 mg/mL ferrous sulfate heptahydrate for all proteins except SxtV. Cultures were incubated at 20 °C and 200 rpm overnight (~18 h) before harvesting. The average pellet for a 500 mL culture was 25 g wet cell mass.

Expression protocol for VanB. pMCSG7 plasmids containing *vanB* were 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, 250 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. 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 5 g.

Expression protocol for NdmD.[10] A single colony from an agar stab of His_6-NdmD in BL21(DE3) prepared by Ryan Summers (University of North Dakota) was used to inoculate 5 mL LB media containing 30 µg/mL kanamycin. The culture was grown at 37 °C, 250 rpm for ~18 h. The overnight culture was used to inoculate 500 mL TB containing 4% glycerol (v/v) and 30 µg/mL kanamycin in a 2.8 L flask. Cultures were grown at 37 °C and 250 rpm until the OD_{600} reached 0.5. At this stage, sterile ferric chloride was added to a final concentration of 10 µM and ethanol was added to 0.1% (v/v). The cultures were incubated at 18 °C until an OD_{600} of 0.8 was reached followed by the addition of 0.2 mM IPTG. Cultures were incubated at 18 °C for 18 h, 200 rpm. The average wet mass of pellet for 500 mL culture was 14 g.

Expression protocol for DdmA1 and DdmB. pMCSG7 plasmids containing *ddmA1* and *ddmB* were transformed into chemically competent C41(DE3) cells using standard heat-shock protocols. Single colonies were used to inoculate 5 mL LB containing 100 µg/mL ampicillin, and cultures were grown overnight at 37 °C, 200 rpm. Using the starter cultures, 500 mL LB cultures containing 100 µg/mL ampicillin in a 2.8 L flask were inoculated and incubated at 37 °C and 250 rpm until an OD_{600} of 0.6 was reached. The cultures were induced with 1 mM IPTG and incubated at 18 °C overnight at 200 rpm for DdmA1 and for 72 h at 200 rpm for DdmB. The wet pellet mass of DdmB was 2.5 g, and 3 g for DdmA1.

Expression protocol for VioC.[11] pET28b plasmid containing *vioC* was transformed into chemically competent BL21(DE3) cells using standard heat-shock protocols. Single colonies were used to inoculate 40 mL LB containing 50 µg/mL kanamycin, and cultures were grown overnight at 37 °C, 200 rpm. 4 x 1 L LB cultures were prepared in 2.8 L flasks and inoculated with 10 mL overnight culture. Cultures were grown at 37 °C, 250 rpm until OD_{600} = 0.9. Flasks were cooled to 20 °C and induced by the addition of 0.1 mM IPTG before being incubated overnight at 20°C, 200 rpm.

Purification protocols for SxtT, GxtA, and SxtH. Approximately 50 g cell pellet was resuspended in 200 mL lysis buffer (50 mM TrisHCl pH 7.4, 300 mM NaCl, 10 mM imidazole, 10% glycerol) with the addition of 1 mg/mL lysozyme, 1 mM phenylmethane sulfonyl fluoride (PMSF), and 0.1 mg/mL benzamidine HCl. Resuspended cells were incubated for 20 min with rocking before being sonicated in two 100 mL batches for 4 min total, 10 s on, 20 s off. Lysed cells were centrifuged at 40,000 x *g* for 30 min. The clarified lysate was light brown in color. Supernatant was combined with 4 mL Ni-NTA resin that had been equilibrated with lysis buffer

and was incubated with rocking for 30 min to 2 h. The mixture was loaded onto a 35 mL column and allowed to drain completely before passing over the resin an additional time. The resin was washed with 20 mL lysis buffer followed by 10 mL each of buffer containing increasing amounts of imidazole: 25 mM imidazole, 30 mM imidazole, 35 mM imidazole, 40 mM imidazole, and 45 mM imidazole. Proteins were eluted with 20 mL elution buffer (50 mM TrisHCl pH 7.4, 300 mM NaCl, 400 mM imidazole, 10% glycerol). Fractions containing protein of interest were pooled, diluted to 20 mL using lysis buffer, and added to a dialysis bag containing 2 mg TEV protease. The cleavage reaction was dialyzed for at least 18 h in 50 mM TrisHCl, 300 mM NaCl, 2 mM dithiothreitol (DTT), and 10% glycerol.

The contents of the dialysis bag were combined with 1 mL Ni-NTA resin that had been washed with lysis buffer and incubated for 20 min to 1 h. The mixture was loaded onto a 12 mL column and allowed to drain completely. The flowthrough contained light brown protein, which was concentrated to 2 mL using a 30 kDa cutoff centrifugal filter and loaded onto an ÄKTA Pure FPLC system fitted with a Sephacryl S-200 HR gel filtration column equilibrated with 20 mM TrisHCl pH 7.4 and 200 mM NaCl buffer at a flow rate of 0.5 mL/min. SxtT, SxtH, and GxtA typically eluted between 47 and 48 mL, corresponding to the ~111 kDa trimer state. Fractions surrounding the putative trimer were run on an SDS-PAGE gel and the cleanest fractions were pooled, concentrated to at least 50 μM protein, and flash frozen in liquid nitrogen for long-term storage at -80 °C.

Purification protocols for MBP-SxtT, MBP-GxtA, MBP-SxtH, and MBP-SxtW. Approximately 20 g cell pellet was resuspended in 80 mL lysis buffer (20 mM TrisHCl pH 7.4, 200 mM NaCl, 1 mM DTT). 5 μg/mL leupeptin, 5 μg/mL pepstatin, 1 mM PMSF, 0.1 mg/mL benzamidine HCl, 20 U DNaseI, 10 mM tris(2-carboxyethyl)phosphine (TCEP, GoldBio), and 1 mg/mL lysozyme were added, and the resuspended cells were incubated with rocking for 30 min. Cells were lysed using an Avestin Emulsiflex pressure homogenizer for four passes at 15,000 psi. Lysed 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 lysis buffer.

The contents of the dialysis bag were combined with 1 mL Ni-NTA resin that had been washed with lysis buffer and incubated for 20 min to 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 following the protocols outlined in the section above. The final purified proteins were visibly reddish-brown in color upon concentration and were flash frozen in liquid nitrogen for long-term storage at -80 °C.

Purification protocols for MBP-SxtV. 25 g wet cell mass was resuspended in 100 mL lysis buffer (50 mM TrisHCl pH 7.4, 300 mM NaCl, 10% glycerol) with the addition of 1 mg/mL lysozyme, 1 mM PMSF, and 40 μM flavin adenine dinucleotide (FAD). The mixture was incubated with rocking for 30 min before lysing using an Avestin Emulsiflex pressure homogenizer for four passes at 15,000 psi. The lysed cells were centrifuged at 40,000 x *g* for 30 min. The clarified supernatant was sterile filtered through 0.45 μm syringe filters and loaded onto an ÄKTA Pure FPLC system fitted with a 5 mL HisTrap column. Lysis buffer was used as Buffer A, and lysis buffer with the addition of 400 mM imidazole was used as Buffer B. The sample was loaded at a rate of 2.5 mL/min, washed with 5 CV of 6.3% Buffer B at 2.5 mL/min, and eluted over 10 CV in

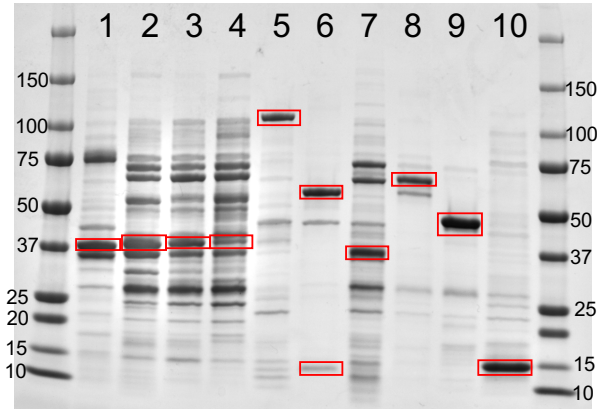
a gradient to 100% Buffer B at 1 mL/min, collecting 2.5 mL fractions. Fractions containing SxtV, as determined by SDS-PAGE analysis, were pooled and concentrated to 2.5 mL using a 30 kDa cutoff centrifugal filter. The protein was desalted using a PD-10 desalting column equilibrated with 50 mM TrisHCl pH 7.4 and 10% glycerol following the manufacturer's directions. Protein was aliquoted, flash frozen in liquid nitrogen, and stored at -80 °C.

Purification protocols for VanB, DdmA1, and DdmB. Cell pellets were 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 with the addition of 1 mM PMSF, 0.1 mg/mL benzamidine HCl, and 1 mg/mL lysozyme. The mixture was incubated with rocking for 1 h before sonicating for 4 min total at 10 s on, 20 s off. Lysed 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 30 min 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, DdmB, or DdmA1 were pooled and dialyzed into storage buffer (50 mM TrisHCl pH 7.4, 10% glycerol). Proteins were concentrated and stored at -80 °C.

Purification protocol for NdmD.[10] 15 g cell pellet was resuspended in 60 mL lysis buffer (25 mM potassium phosphate pH 7.0, 300 mM NaCl, 10 mM imidazole) with the addition of 1 mg/mL lysozyme and incubated for 30 min with rocking. The cells were lysed by four passes at 15,000 psi through an Avestin Emulsiflex pressure homogenizer, and the total lysate was centrifuged at 40,000 x *g* for 30 min. The clarified lysate was combined with 2 mL Ni-NTA resin and incubated for 1.5 h. The mixture was poured over a 35 mL column followed by washing with 50 mL lysis buffer and 20 mL of buffer containing 50 mM imidazole. The protein was eluted with 10 mL of elution buffer (25 mM potassium phosphate pH 7.4, 300 mM NaCl, 250 mM imidazole) and concentrated to 2.5 mL for desalting on a PD-10 column according to the manufacturer's instructions. The desalting buffer was 50 mM potassium phosphate pH 7.5, 5% glycerol, 1 mM DTT. Protein was concentrated, aliquoted, and stored at -80 °C.

Purification protocol for VioC.[11] Cell pellets were resuspended in 4 mL lysis buffer (20 mM TrisHCl pH 8.0, 300 mM NaCl, 10% glycerol) per gram wet mass. The mixture was sonicated for 5 min total in a 5 sec on, 10 sec off cycle before being centrifuged at 40,000 x *g* for 20 min. The supernatant was collected and combined with 1 mL Ni-NTA resin per L culture and incubated at 4 °C for 2 h with gentle rocking. The mixture was poured over a column and the resin was washed with lysis buffer. VioC was eluted with elution buffer (lysis buffer + 400 mM imidazole), desalted using a PD-10 column equilibrated with 50 mM TrisHCl pH 8.0, 100 mM NaCl, 10% glycerol buffer, and stored at -80 °C.

Protein quantification and analysis. The Pierce660 assay kit (Thermo Scientific) was used to determine the concentrations of all purified proteins by comparison to a standard curve using bovine serum albumin (BSA) generated following the manufacturer's instructions. All absorbance assays were conducted using a Spectramax M5 spectrophotometer from Molecular Devices.



Lane #	Protein	Predicted Size (Da)
1	SxtT *M. wollei*	38391.73
2	SxtT *Aphanizomenon* sp. NH-5	38737.07
3	SxtT *D. circinale*	39150.85
4	SxtT *C. raciborskii*	38794.25
5	MBP-SxtV	104315.11
6	MBP-SxtW & SxtW	56001.41 & 12250.94
7	VanB	35985.89
8	NdmD	66061.15
9	DdmA1	44637.88
10	DdmB	12392.74

Figure S4. SDS-PAGE gel of proteins purified in this study outlined in red boxes corresponding to the table on the right. Predicted sizes were determined by the ProtParam tool on the Expasy server. Activity was abolished upon further purification of SxtT proteins.

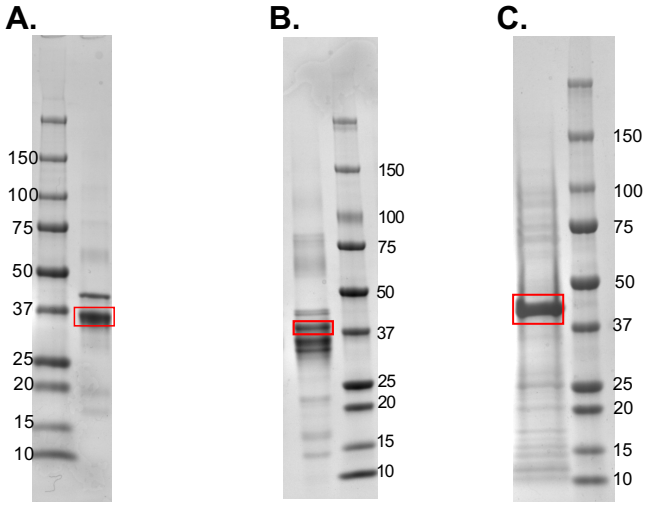


Figure S5. (A) SDS-PAGE gel of GxtA. The size, as predicted by the ProtParam tool on the Expasy server, is 38199.45 Da. (B) SDS-PAGE gel of SxtH from *M. wollei*. The size, as predicted by the ProtParam tool on the Expasy server, is 38953.32 Da. (C) SDS-PAGE gel of VioC. The predicted size is 41.6 kDa with His-tag attached.

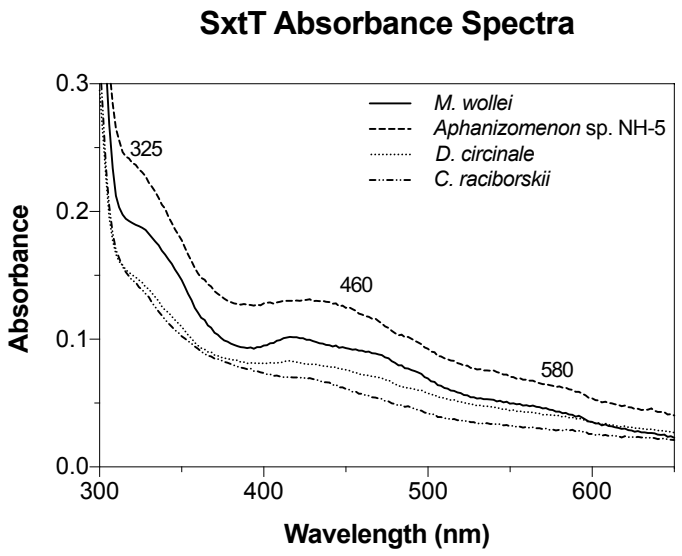


Figure S6. Absorbance spectra of 100 μM total protein from SxtT purifications. Relevant absorbance maxima indicative of an oxidized Rieske cluster are 325 nm, 460 nm, and a low shoulder at 560-580 nm.[12] Weaker signals (i.e. *C. raciborskii*) are due to lower relative abundance of protein in the 100 μM sample.

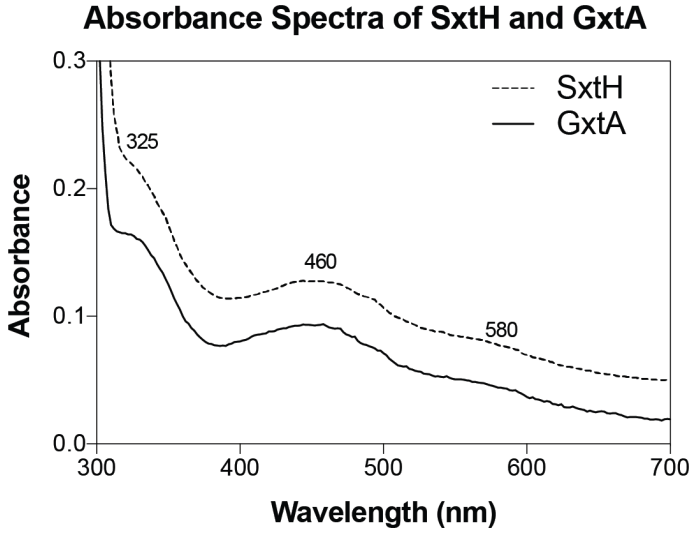
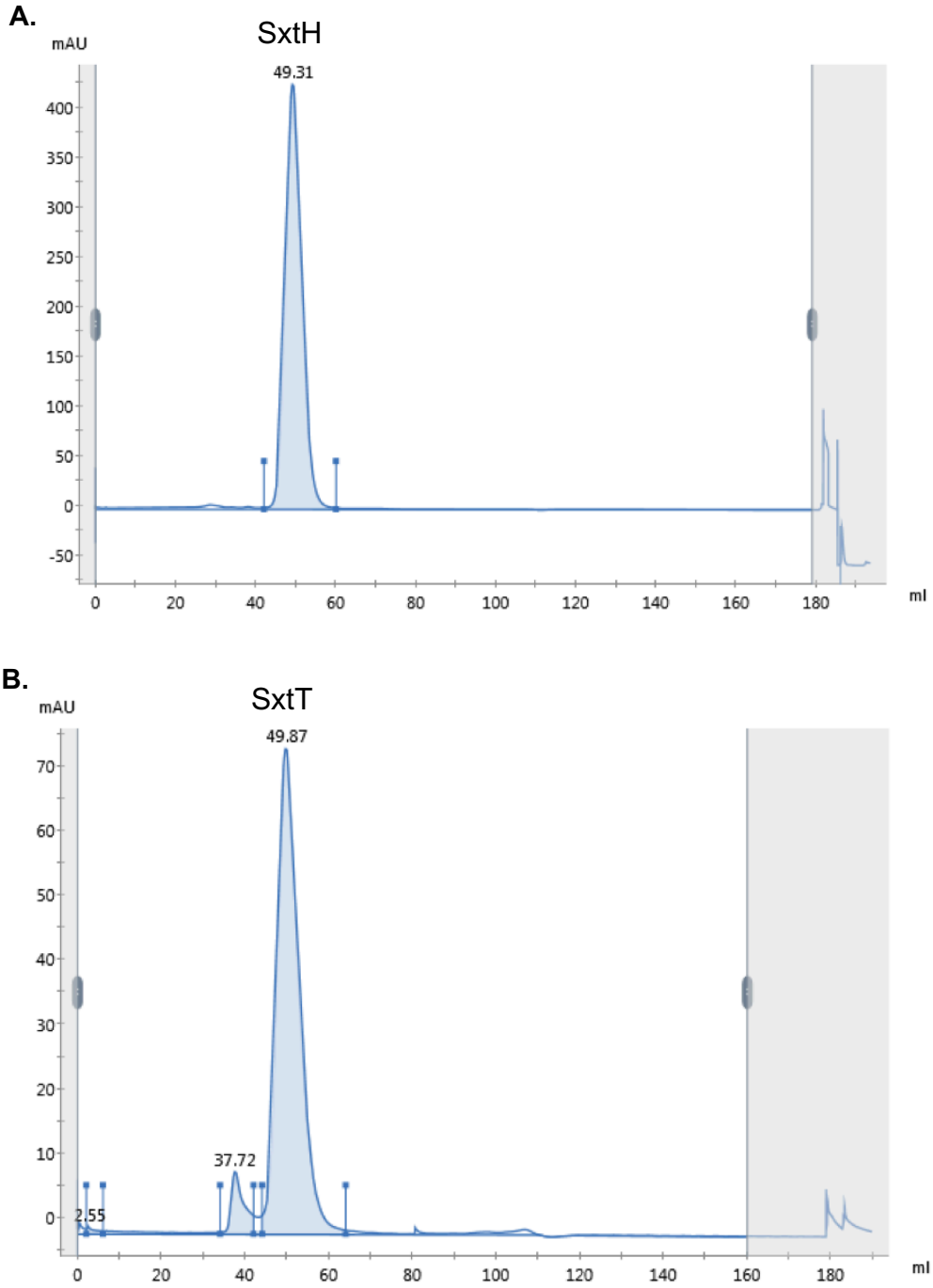


Figure S7. Absorbance spectra of 100 μM total protein from purifications of GxtA and SxtH from *M. wollei*. Relevant absorbance maxima indicative of an oxidized Rieske cluster are 325 nm, 460 nm, and a low shoulder at 560-580 nm.[12]

Oligomeric state determination. Separations were conducted as described in respective purification procedures using a Sephacryl S-200 HR gel filtration column. Approximate sizes were determined relative to a calibration curve generated using the Gel Filtration Standards Kit from Sigma. According to the calibration curve where $y = 10^{(-0.7496(x/39.88)+3.068)}$, SxtH was determined to be 138 kDa, SxtT was determined to be 135 kDa, and GxtA was determined to be 145 kDa. The estimated molecular weight of the trimer form of each protein is 114 kDa. Based on this analysis and error in measurement, it can be concluded that the proteins are forming trimers in solution after cleavage of MBP or His tags.



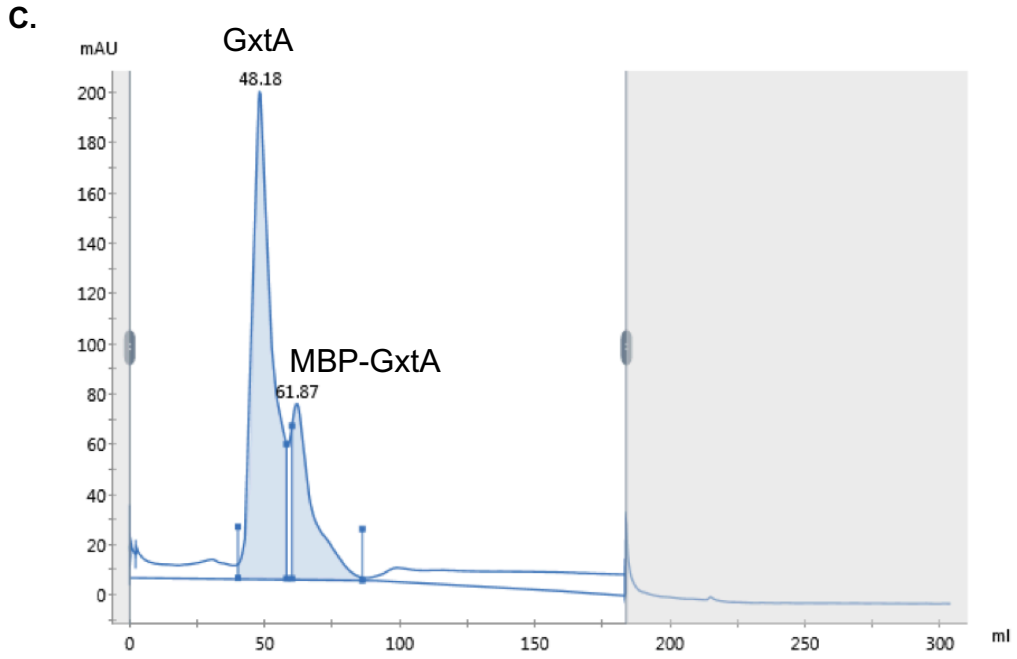


Figure S8. Gel filtration elution profiles of (A) SxtH, (B) SxtT, and (C) GxtA.

Iron quantification in SxtT, SxtH, GxtA, and VanB.[13-14] Iron content was determined according to published procedures using ferene (ε_{593} = 34,500 M^{-1} cm^{-1}) as a spectrophotometric reagent for iron analysis. The iron incorporation for SxtT, SxtH, GxtA, and VanB were found to be 3.2:1, 2.4:1, 2.3:1, and 2:1, respectively. These results are consistent with SxtT, SxtH, and GxtA having three irons on average as anticipated for Rieske oxygenases. VanB is proposed to have a [2Fe-2S] site, which is confirmed by these results.

Determination of VanB cofactor identity. The flavin content of VanB was assessed by diluting protein to 10 μM in 1 mL for UV-vis analysis using a PMMA cuvette.[15] The absorbance spectrum was recorded from 300 nm to 700 nm before being boiled for 10 min and reading again. The absorbance at 473 nm for the denatured enzyme and the extinction coefficient of free FAD/FMN at 473 nm (9,200 M^{-1} cm^{-1}) was used to calculate the concentration of flavin in the protein sample. The flavin incorporation was 60.8% relative to the purified protein mixture in Figure S4 Lane 7.

The identity of the flavin cofactor was found to be FMN upon treatment with phosphodiesterase (crude, Type IV, *Crotalus atrox*, Western diamondback rattlesnake, Sigma).[15] Phosphodiesterase (PDE) cleaves the phosphodiester bond in FAD to generate FMN and adenosine monophosphate, resulting in a spectral shift of maximum absorbance from 450 nm to 446 nm with an isosbestic point around 474 nm (Figure S9A-C). 6 mU of PDE was added to 10 μM of heat-denatured VanB and the reaction was monitored by UV-vis over 10 min. No spectral shift was observed (Figure S9D), confirming the identity of the flavin to be FMN.

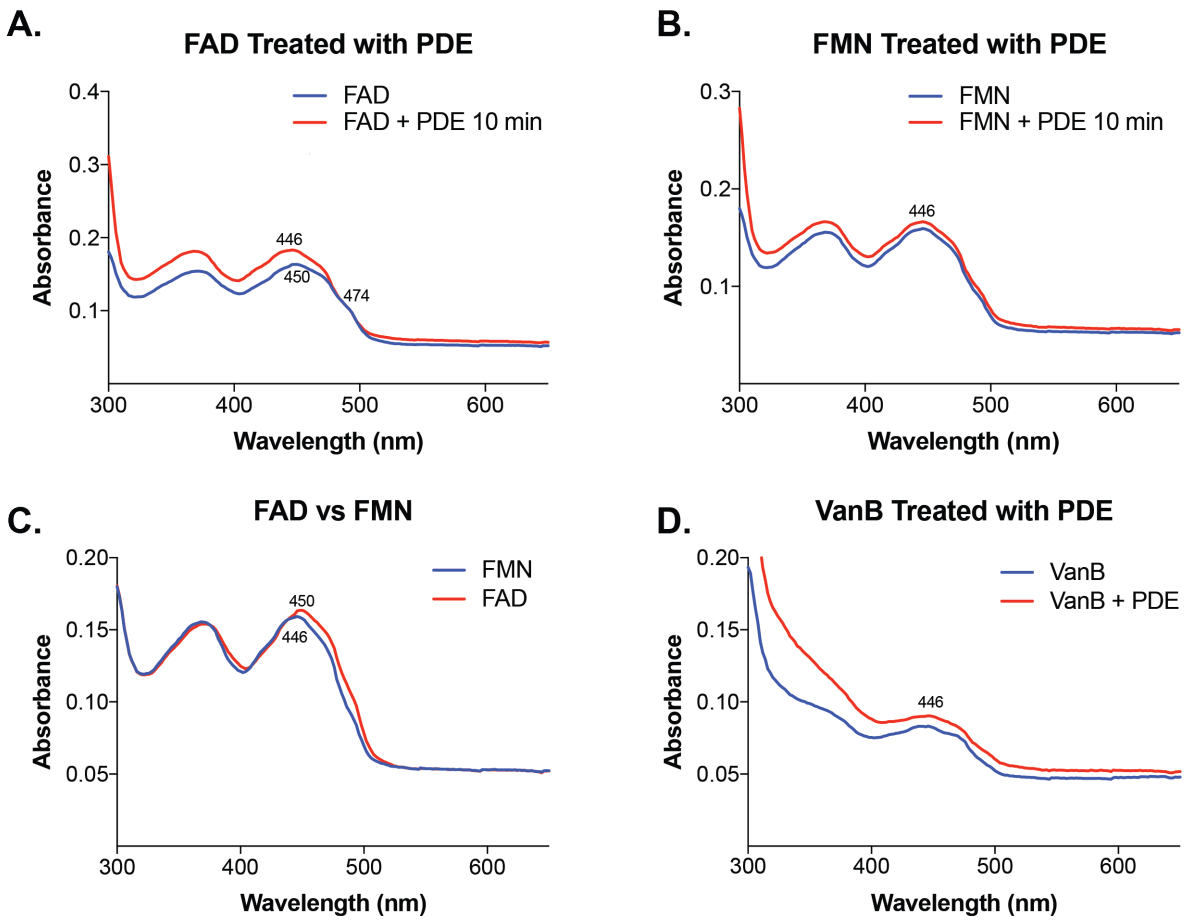
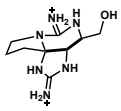
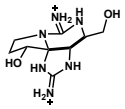
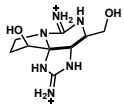
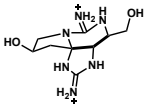
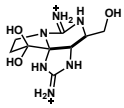
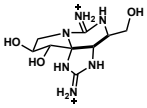
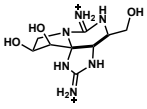
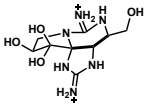
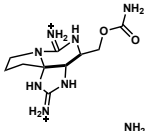
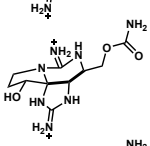
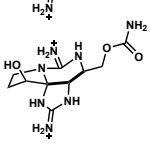


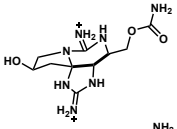
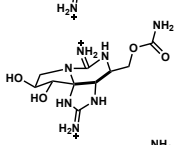
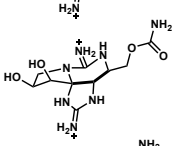
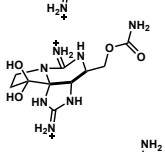
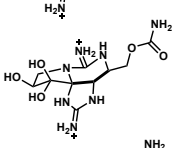
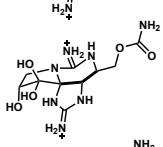
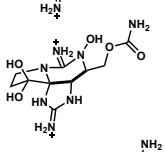
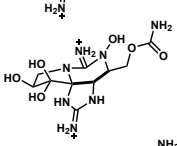
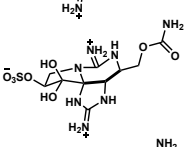
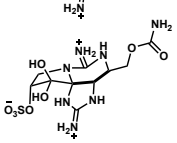
Figure S9. (A) Spectra of FAD before and after treatment with phosphodiesterase (PDE). (B) Spectra of FMN before and after treatment with PDE. (C) Comparison of FAD and FMN absorbance spectra. (D) Spectra of heat-denatured VanB before and after treatment with PDE.

III. Enzymatic Reactions

General Considerations. Substrates used in this study are referred to under the abbreviations listed in Table S4. Stock solutions of all substrates were prepared to final concentrations of 20 mM in dimethyl sulfoxide (DMSO, analytical grade), with the exception of α-saxitoxinol (**14**) and β-saxitoxinol (**13**), which were prepared to 20 mM in 0.1% acetic acid water. Enzyme aliquots were stored in the buffers outlined in Part II and discarded after one freeze-thaw cycle. Hydrogen peroxide was used from a 30% stock solution (~9.8 M). Stocks of 1 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ 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 for no more than 10 freeze-thaw cycles. 10 mM nicotinamide adenine dinucleotide phosphate ($NADP^+$) stocks were also prepared under the same conditions as NADH. Glucose-6-phosphate (G6P, 500 mM) and glucose-6-phosphate dehydrogenase (G6PDH, 100 U/mL) were prepared in water and stored at -20 °C.

Table S4. Saxitoxin and related analogs included in this study. Hydrolysis conditions are noted as applicable.[16-17]

	Compound #	Name	Abbreviation	Source
	**11**	decarbamoyl dideoxysaxitoxin	dc-ddSTX	formed by hydrolysis of **12**
	**15**	decarbamoyl α-saxitoxinol	dc-α-STOH	formed by hydrolysis of **14**
	**16**	decarbamoyl β-saxitoxinol	dc-β-STOH	formed by hydrolysis of **13**
	**17**	decarbamoyl 11-β-saxitoxinol	dc-11-β-STOH	biocatalytic reaction, this work
	**1**	decarbamoyl saxitoxin	dc-STX	formed by hydrolysis of **9**
	**18**	decarbamoyl 11-β-hydroxy-α-saxitoxinol	dc-11-β-hydroxy-α-STOH	biocatalytic reaction, this work
	**19**	decarbamoyl 11-β-hydroxy-β-saxitoxinol	dc-11-β-hydroxy-β-STOH	biocatalytic reaction, this work
	**20**	decarbamoyl 11-β-hydroxy-saxitoxin	dc-11-β-hydroxy-STX	biocatalytic reaction, this work
	**12**	dideoxysaxitoxin	ddSTX	synthesized by Du Bois lab
	**14**	α-saxitoxinol	α-STOH	standard supplied by the FDA
	**13**	β-saxitoxinol	β-STOH	standard supplied by the FDA

	Compound #	Name	Abbreviation	Source
	**21**	11-β-saxitoxinol	11-β-STOH	biocatalytic reaction, this work
	**22**	11-β-hydroxy-α-saxitoxinol	11-β-hydroxy-α-STOH	biocatalytic reaction, this work
	**23**	11-β-hydroxy-β-saxitoxinol	11-β-hydroxy-β-STOH	biocatalytic reaction, this work
	**9**	saxitoxin	STX	standard supplied by the FDA
	**7**	11-β-hydroxy-saxitoxin	11-β-hydroxy-STX	formed by hydrolysis of **3**
	**S1**	11-α-hydroxy-saxitoxin	11-α-hydroxy-STX	formed by hydrolysis of **2**
	**4**	neosaxitoxin	neoSTX	standard supplied by the FDA
	**24**	11-β-hydroxy-neosaxitoxin	11-β-hydroxy-neoSTX	biocatalytic reaction, this work
	**3**	gonyautoxin 3	GTX3	standard supplied by the FDA
	**2**	gonyautoxin 2	GTX2	standard supplied by the FDA

SxtT reactions with hydrogen peroxide. SxtT was prepared from MBP-SxtT at a stock concentration of 60 μM. 20 μM SxtT, 250 μM dideoxysaxitoxin (ddSTX), 50 mM H_2O_2, 100 μM $Fe(NH_4)_2(SO_4)_2$, and 50 mM TrisHCl pH 7.0 were combined, adding SxtT last. Reactions were incubated at 30 °C for 7 h without agitation and quenched by the addition of 150 μL acetonitrile (UPLC-grade). Quenched reactions were centrifuged at 12,000 x *g* for 20 min and 100 μL of the supernatant was diluted with 80 μL acetonitrile and 20 μL sterile-filtered ddH_2O containing 0.1% formic acid.

Redox partner assessment. SxtT was prepared from MBP-SxtT at a stock concentration of 60 μM. Stock concentrations of redox partners are as follows: SxtW (MBP-SxtW prep,142 μM), SxtV (65 μM), phalate dioxygenase reductase (PDR, 125 μM), VanB (103 μM), NdmD (57 μM), DdmA1 (55 μM), and DdmB (250 μM), all prepared as described above. All redox partners were NADH-dependent, aside from SxtW/SxtV, which was reliant on NADPH generated via the G6P/G6PDH recycling system (500 μM $NADP^+$, 2 mM G6P, 1 U/mL G6PDH). 10 μM SxtT, 20 μM each redox partner, 200 μM ddSTX, 500 μM NADH or noted quantities of recycling system, 100 μM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, and 50 mM TrisHCl pH 7.0 were combined, adding SxtT last. Reactions were incubated at 30 °C for 7 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 80 μL acetonitrile and 20 μL sterile-filtered ddH_2O containing 0.1% formic acid.

SxtT homolog activity with substrate panel and VanB. SxtT from each homolog was prepared from His-SxtT at the following stock concentrations: *M. wollei* (60.0 μM), *Aphanizomenon sp. NH-5* (223 μM), *D. circinale* (97 μM), and *C. raciborskii* (116 μM). Reactions consisting of 20 μM SxtT, 5 μM VanB, 200 μM each substrate (dc-ddSTX, dc-α-STOH, dc-β-STOH, dc-STX, ddSTX, α-STOH, β-STOH, STX), 500 μM NADH, 100 μM $Fe(NH_4)_2(SO_4)_2$, and 50 mM TrisHCl pH 7.0 buffer were mixed and incubated at 30 °C for 18 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 80 μL acetonitrile and 20 μL sterile-filtered ddH_2O containing 0.1% formic acid.

GxtA reactions with VanB. GxtA was prepared from MBP-GxtA to a final concentration of 100 μM. Reactions consisting of 20 μM GxtA, 5 μM VanB, 200 μM each substrate (dc-ddSTX, dc-α-STOH, dc-β-STOH, dc-STX, neoSTX, ddSTX, α-STOH, β-STOH, STX, neoSTX), 500 μM NADH, 100 μM $Fe(NH_4)_2(SO_4)_2$, and 50 mM TrisHCl pH 7.0 buffer were mixed and incubated at 30 °C 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 80 μL acetonitrile and 20 μL sterile-filtered ddH_2O containing 0.1% formic acid.

SxtH reactions with arginine methyl ester. SxtH was prepared from MBP-SxtH at a stock concentration of 685 μM. 20 μM SxtH, 5 μM VanB, 200 μM arginine methyl ester, 500 μM NADH, 100 μM $Fe(NH_4)_2(SO_4)_2$, and 50 mM TrisHCl pH 7.0 buffer were mixed and incubated at 30 °C 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 100 μL sterile-filtered ddH_2O containing 1% formic acid.

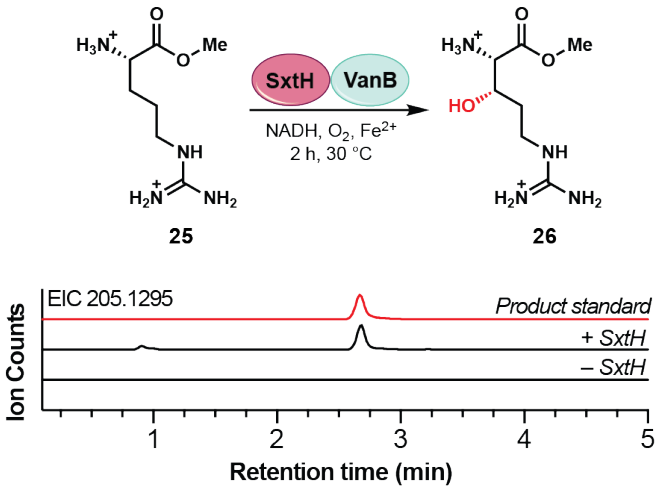
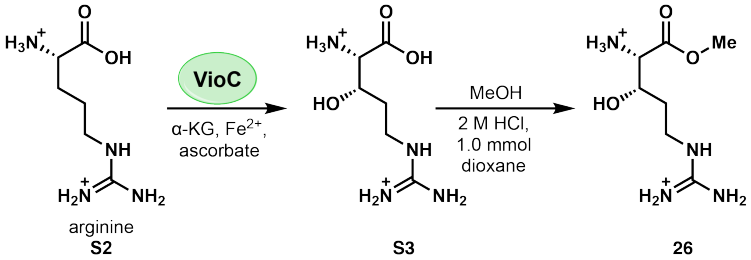


Figure S10. Reaction of SxtH with arginine methyl ester (**25**) to generate hydroxylated arginine methyl ester (**26**).

Analytical-scale VioC reactions. VioC was used from a 378 μM stock solution. Reactions contained: 5 μM VioC, 2 mM arginine, 10 mM L-ascorbic acid sodium salt, 10 mM α-ketoglutaric acid, 1 mM DTT, 50 μM $Fe(NH_4)_2(SO_4)_2$, 0.1 M NaCl, 10% glycerol, and 0.1 M phosphate pH 7.5 buffer. The reaction components were combined, mixed and incubated at 30° C for 16 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 100 μL sterile-filtered ddH_2O containing 1% formic acid.

Preparative-scale VioC reaction on arginine and elaboration to methyl ester (26).



VioC was used from a stock concentration of 378 μM. Reactions contained: 8 μM VioC, 2 mM arginine (**S2**), 10 mM L-ascorbic acid sodium salt, 10 mM α-ketoglutaric acid, 1 mM DTT, 50 μM $Fe(NH_4)_2(SO_4)_2$, 0.1 M NaCl, 10% glycerol, and 0.1 M phosphate pH 7.5 buffer. Reaction components were mixed and incubated at 30° C for 16 h and quenched by acidifying to pH 2.5 with HCl. Precipitated protein was removed by centrifugation at 12,000 x g for 20 min. The crude reaction mixture was loaded onto Dowex 50WX8-100 (H^+ form) resin that had been equilibrated with ddH_2O then washed with ddH_2O. Elution was carried out with a step gradient of 1, 2, 3, 4, and 5 M NH_4OH, fractions were neutralized with HCl and concentrated under reduced pressure. 3-hydroxyarginine (**S3**) was dissolved in 2 mL of MeOH and treated with 2 M HCl in dioxane (0.5

mL, 1.0 mmol). The mixture was refluxed for 4 h. Removal of the solvent under reduced pressure afforded **26** as a white solid.

Preparative-scale GxtA reaction. 1 mg of saxitoxin (STX, **9**) was used in a preparative-scale GxtA reaction to generate 11-β-hydroxySTX (**7**). The reaction was conducted on 12.8 mL scale with 20 μM GxtA, 5 μM VanB, 200 μM STX (**9**), 500 μM NADH, 100 μM $Fe_2(NH_4)_2(SO_4)_2$, and 50 mM Tris HCl pH 7 buffer in a small evaporation dish and was incubated at room temperature overnight (~12 h).

Product isolation from GxtA reactions. Products were effectively separated from protein in reactions without quenching with organic solvent using Centrifree ultrafiltration devices (EMD Millipore). For analytical-scale reactions, 50 μL enzymatic reactions were diluted to 200 μL with MilliQ water. The total volume was loaded into the reservoir and centrifuged at 2,000 x *g*. The presence and quantification of product in the flowthrough was verified by LC/MS. Compounds subjected to this workup protocol were sufficiently clean for direct use in sodium channel binding assays.

SxtT and GxtA reactions with $H_2{}^{18}O_2$. SxtT was prepared from MBP-SxtT at a stock concentration of 60 µM and GxtA was prepared from MBP-GxtA to a final concentration of 100 µM. 20 µM SxtT or GxtA, 200 µM substrate, 50 mM $H_2{}^{18}O_2$ (Sigma Aldrich), 100 µM $Fe(NH_4)_2(SO_4)_2$, and 50 mM TrisHCl pH 7.0 were combined, adding enzyme last. Reactions were incubated at 30 °C for 2 h without agitation and quenched by the addition of 150 µL acetonitrile (UPLC-grade). Quenched reactions were centrifuged at 12,000 x *g* for 20 min and 100 µL of the supernatant was diluted with 80 µL acetonitrile and 20 µL sterile-filtered ddH_2O containing 0.1% formic acid.

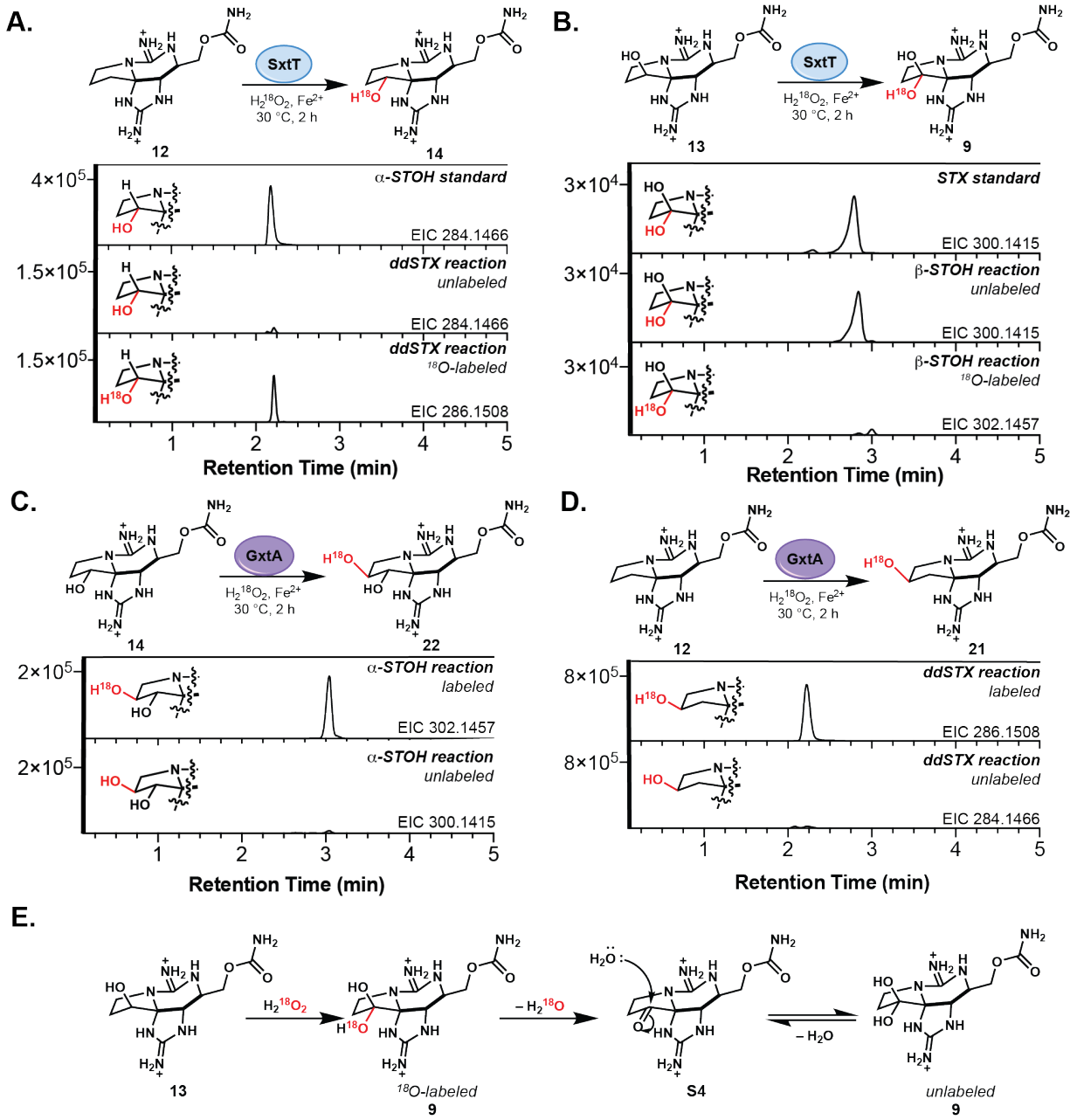


Figure S11. Reactions with SxtT and GxtA employing ^{18}O labeled hydrogen peroxide. (A) Reaction of SxtT with ddSTX (**12**). (B) Reaction of SxtT with β-STOH (**13**). (C) Reaction of GxtA with α-STOH (**14**). (D) Reaction of GxtA with ddSTX (**12**). (E) Proposed mechanism of ^{18}O-exchange of the vicinal diol of STX (**9**) via intermediate **S4** in solution.

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 for all substrates except dc-β-STOH 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. The method for separation of dc-β-STOH and products was 27% solvent A 0 – 1 min (to waste), 27% solvent A 1 – 4 min (to MS), and gradient 27% - 40% A (to waste) followed by an 8 min re-equilibration at 27% A, all at a constant flow of 0.4 mL/min. 0.1-1 µL injections were made for each sample. Targeted MS/MS was performed using the same mass spectrometer, 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.

Total turnover number (TTN) determination for SxtT and GxtA. SxtT from *D. circinale* was prepared from MBP-SxtT to a final concentration of 130 µM and GxtA was prepared from MBP-GxtA to a final concentration of 100 µM. 50 µL reactions consisting of 1 µM SxtT or 2.5 µM GxtA (total protein), 5 µM VanB, 200 µM each respective substrate (dc-ddSTX, dc-α-STOH, dc-β-STOH, dc-STX, ddSTX, α-STOH, β-STOH, STX, neoSTX), 500 µM NADH, 100 µM $Fe(NH_4)_2(SO_4)_2$, and 50 mM Tris HCl pH 7.0 buffer were mixed and incubated at 30 °C for 18 h. Reactions were quenched by the addition of 150 µL acetonitrile. Reactions were centrifuged at 12,000 x *g* for 20 min and 10 µL of the supernatant was diluted with 190 µL dilution mix containing 10 µL of 10 µg/mL ^{15}N-arginine in 1% formic acid water and 180 µL acetonitrile, prepared as a master mix for all reactions and standards. TTNs were determined by comparison to standard curves of the respective reaction products (SxtT reactions only) or the respective reaction starting materials (GxtA reactions only). Standard curves were constructed using the following relationship:

$$\left(\frac{\text{peak area product (or starting material)}}{\text{peak area internal standard}}\right) = \text{ratio}$$

The ratio obtained from reactions was used to solve for the concentration of product generated using the equations from product or starting material standard curves. To obtain product generated in GxtA reactions, where starting material standard curves were used, the value obtained from the standard curve was subtracted from the initial quantity of starting material (200 µM). TTNs were calculated using the following:

$$\frac{\text{µM product generated}}{\text{1 µM SxtT or 2.5 µM GxtA}} = \text{TTN}$$

Reactions and standards were prepared and analyzed in duplicate. Standard curves of products and starting materials are shown.

SxtT Reactions and Standard Curves

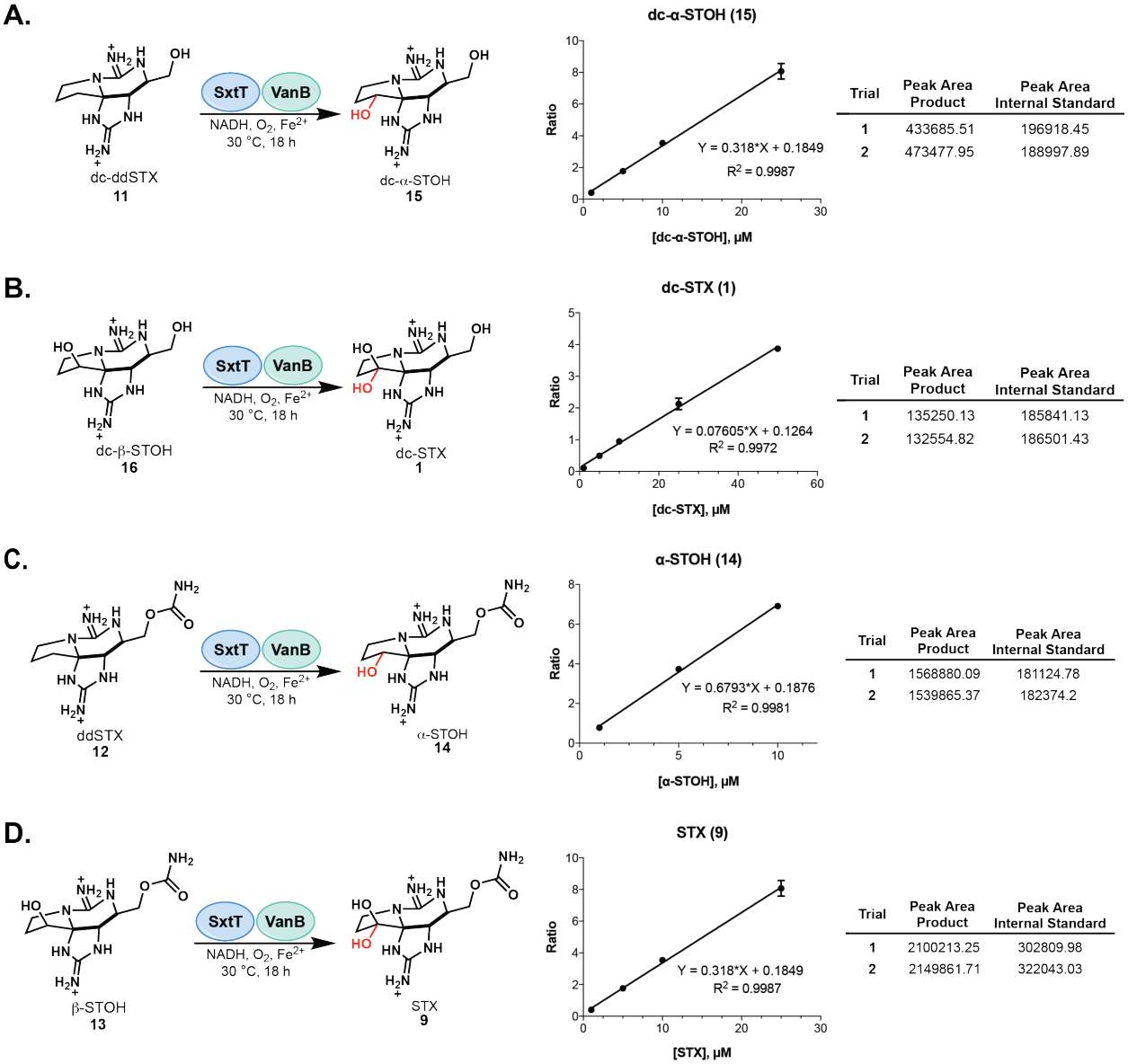


Figure S12. (A) Reaction of SxtT with dc-ddSTX with dc-α-STOH standard curve. (B) Reaction of SxtT with dc-β-STOH with dc-STX standard curve. (C) Reaction of SxtT with ddSTX with α-STOH standard curve. (D) Reaction of SxtT with β-STOH with STX standard curve.

GxtA Reactions and Standard Curves: Decarbamoylated Substrates

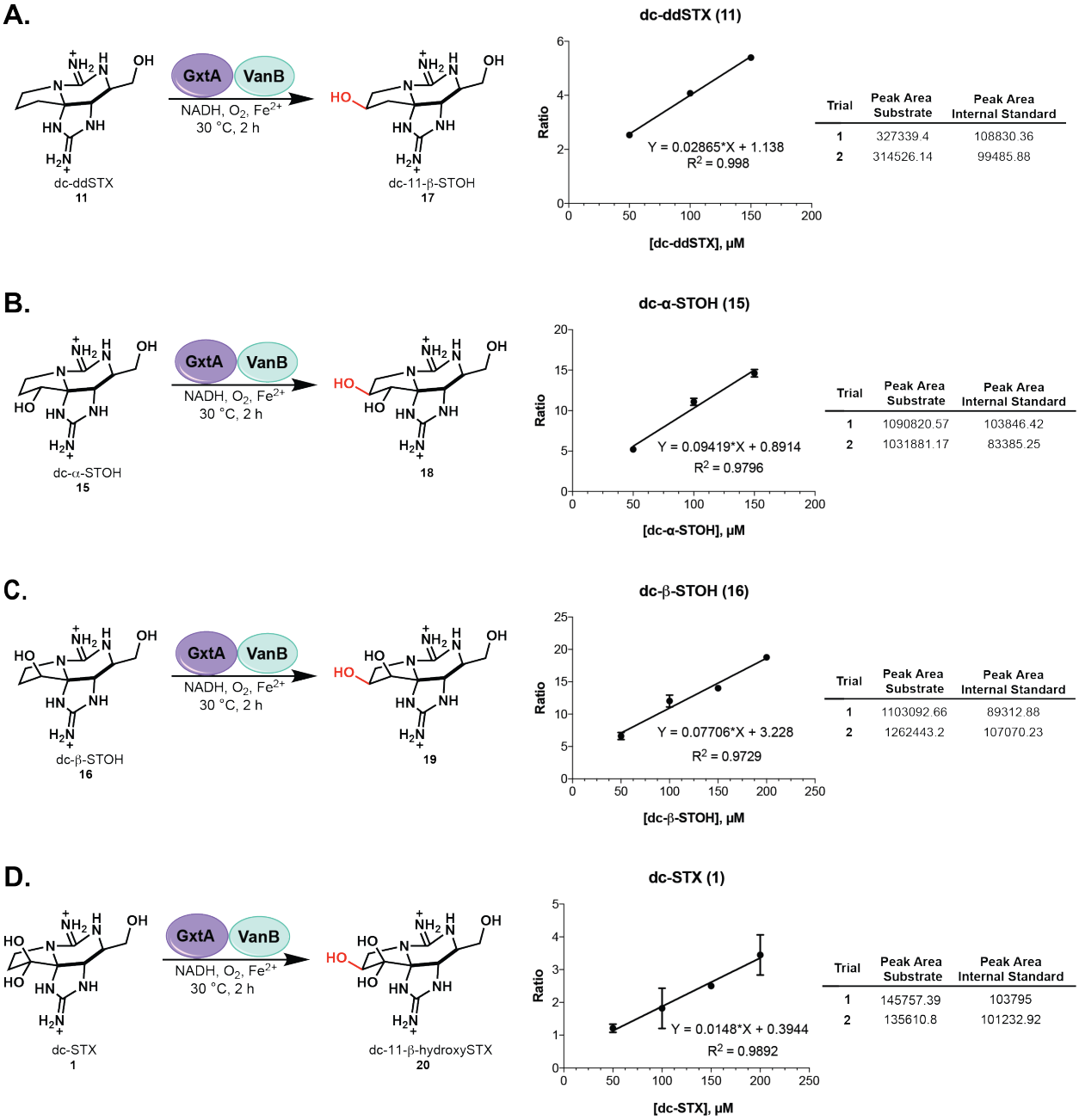


Figure S13. (A) Reaction of GxtA with dc-ddSTX with dc-ddSTX standard curve. (B) Reaction of GxtA with dc-α-STOH with dc-α-STOH standard curve. (C) Reaction of GxtA with dc-β-STOH with dc-β-STOH standard curve. (D) Reaction of GxtA with dc-STX with dc-STX standard curve.

GxtA Reactions and Standard Curves: Carbamoylated Substrates

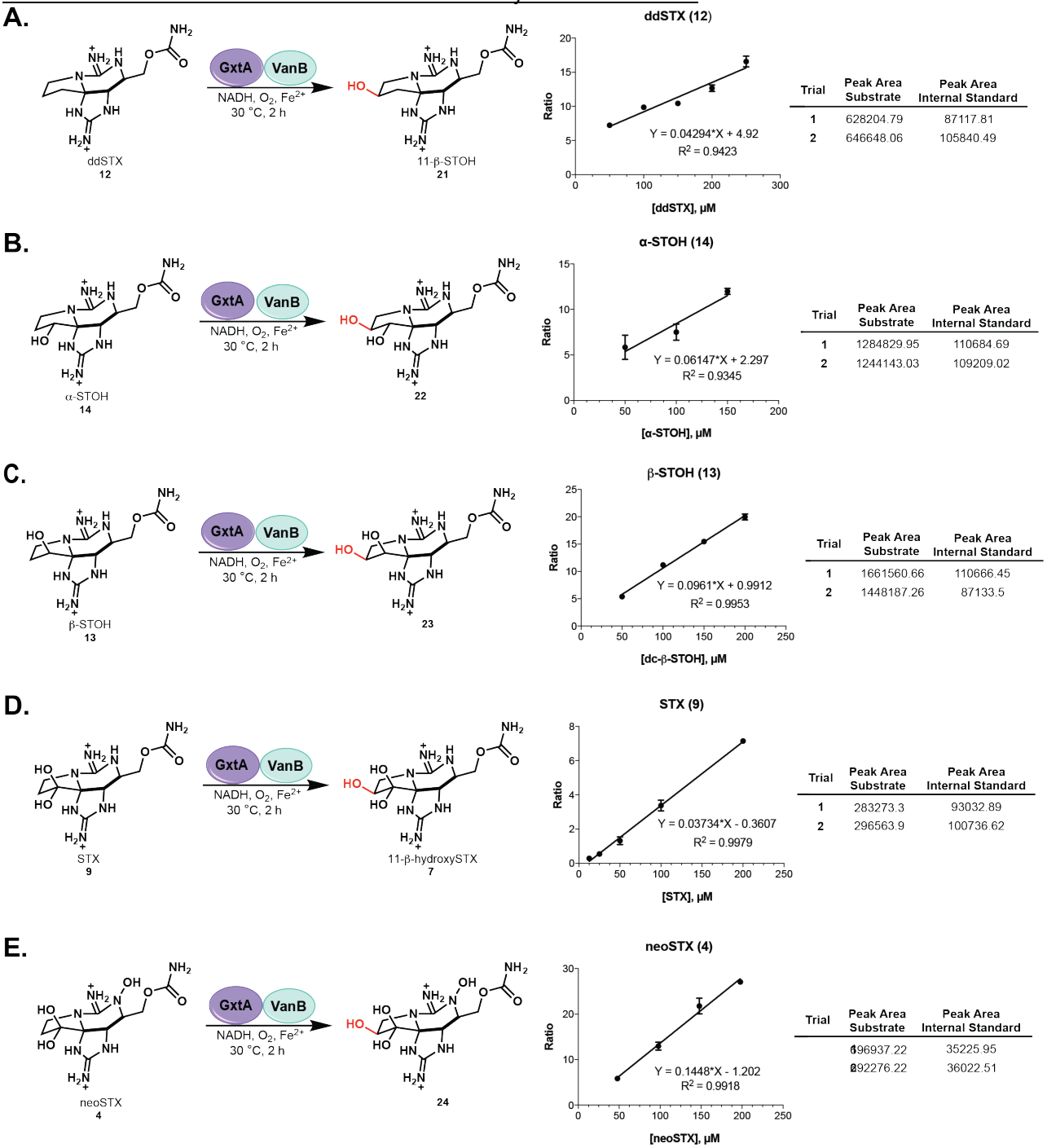


Figure S14. (A) Reaction of GxtA with ddSTX with ddSTX standard curve. (B) Reaction of GxtA with α-STOH with α-STOH standard curve. (C) Reaction of GxtA with β-STOH with β-STOH standard curve. (D) Reaction of GxtA with STX with STX standard curve. (E) Reaction of GxtA with neoSTX with neoSTX standard curve.

Steady-state kinetics of SxtT with ddSTX (12) and β-STOH (13). SxtT from *D. circinale* was prepared from MBP-SxtT to a final concentration of 130 μM. To determine the time initial rates could be recorded, duplicate 50 μL reactions with 5 μM β-STOH (**13**) and 50 μM ddSTX (**12**) were performed and analyzed compared to an internal standard (10 μg/mL ^{15}N-arginine) by LC/MS. The resulting timecourse is showed in Fig S13, demonstrating that 1 min is the optimal time to capture the initial reaction rates for a variety of concentrations of ddSTX (**12**) and β-STOH (**13**).

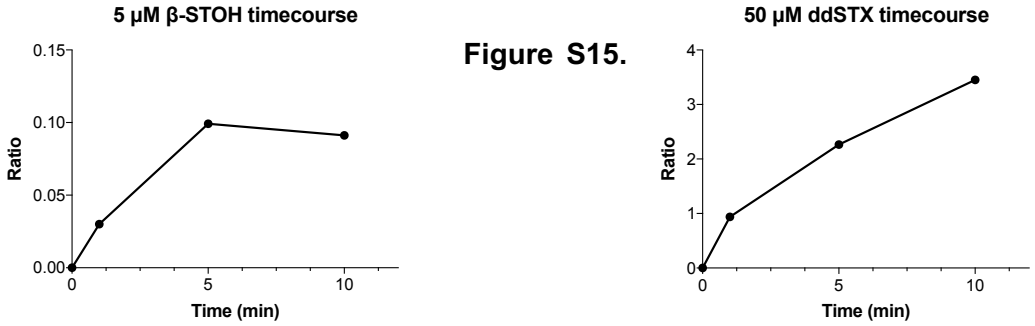


Figure S15.

Timecourses of SxtT with β-STOH (**13**) and ddSTX (**12**).

To determine the steady-state kinetic parameters of the SxtT with ddSTX (**12**), reactions were conducted on 50 μL scale with substrate ranging 1 μM – 200 μM in duplicate with 5 μM SxtT, 5 μM VanB, 100 μM $Fe_2(NH_4)_2(SO_4)_2$, and 50 mM Tris HCl pH 7 buffer in a 96-well plate. For b-STOH (**13**), the substrate range was 500 nM – 100 μM with all other reaction components the same as the ddSTX (**12**) reactions. Reactions were initiated by the addition of 500 μM NADH (10 μL distributed by multichannel pipette). Reactions were quenched after 1 min by the addition of 150 μL acetonitrile. 96-well plates were centrifuged at 2000 x *g* for 2 min, then 100 μL of the centrifuged mixture was added to 100 μL of a dilution mix containing 10 μL 10 μg/mL ^{15}N-arginine in 1% formic acid water and 90 μL acetonitrile in 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 with SxtT and VanB storage buffer (20 mM Tris HCl pH 7.4, 200 mM NaCl) replacing SxtT and VanB enzymes. 2 μL each sample was injected on the TOF LC/MS. The resulting Michaelis-Menten curves for each substrate are shown in Fig S14. Standard curves used to quantify product are shown in Fig S15.

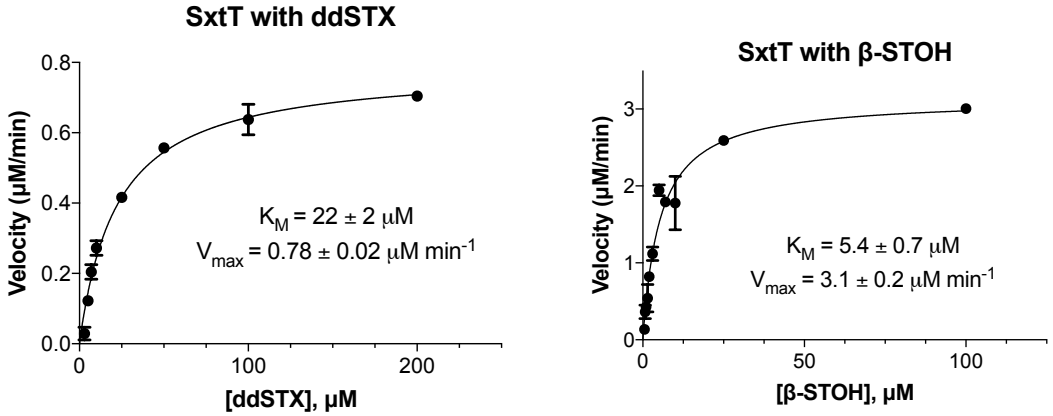


Figure S16. Michaelis-Menten plots of SxtT reactions with ddSTX (**12**) and β-STOH (**13**).

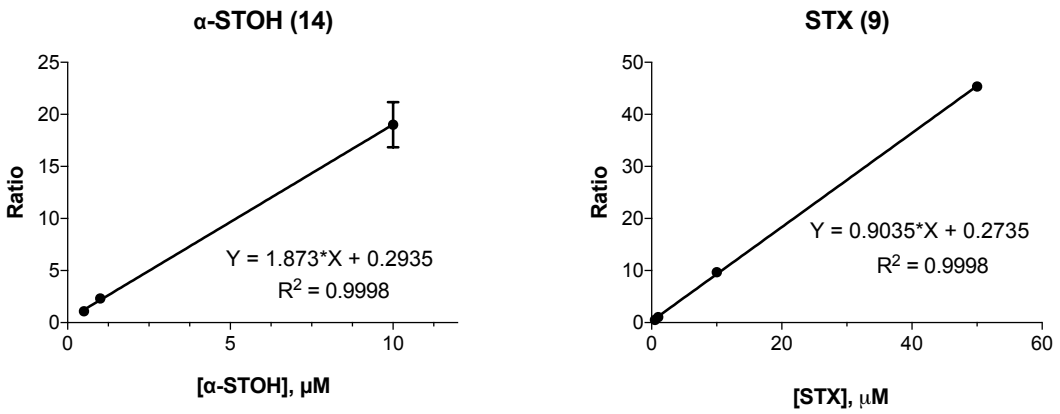


Figure S17. Standard curves of α-STOH (**14**), the product of the SxtT reaction with ddSTX (**12**), and STX (**9**), the product of the SxtT reaction with β-STOH (**13**).

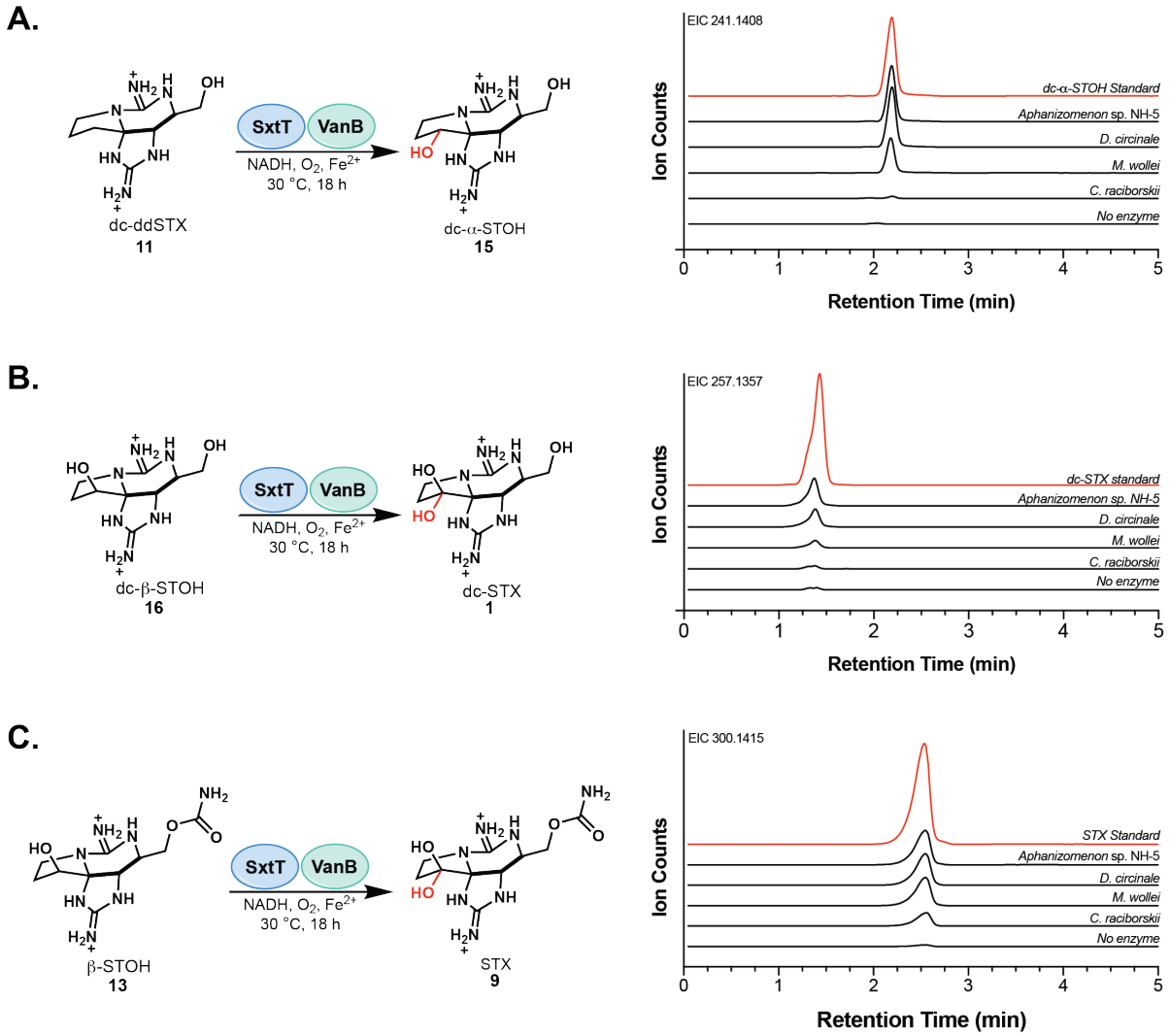


Figure S18. (A) Reaction of SxtT homologs with dc-ddSTX, (B) Reaction of SxtT homologs with dc-β-STOH, (C) Reaction of SxtT homologs with β-STOH.

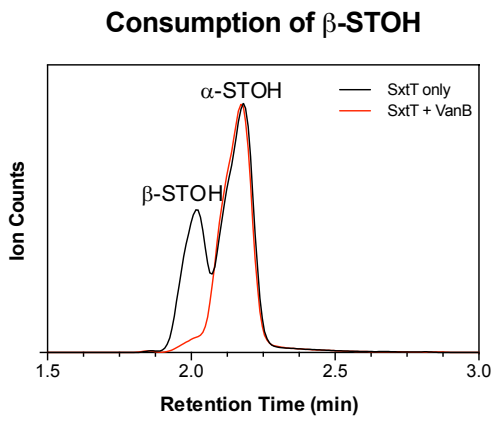


Figure S19. SxtT reaction with a mixture of α-STOH (**14**) and β-STOH (**13**) compared to a reaction containing both SxtT and VanB. SxtT selectively acts on β-STOH if both isomers are present.

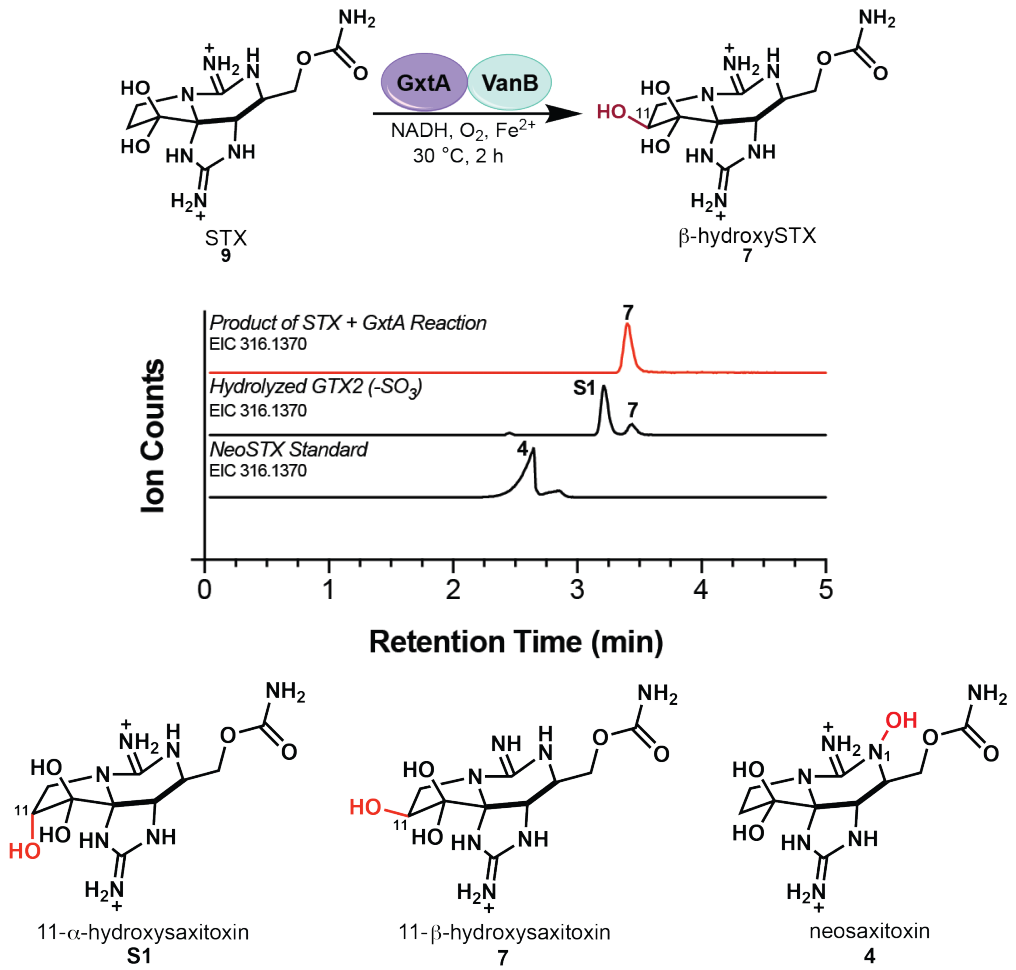


Figure S20. Comparison of the product of the GxtA reaction with STX (**9**) to a mixture of 11-α-hydroxysaxitoxin and 11-β-hydroxysaxitoxin (**S1**, **7**) and neoSTX (**4**) standards by LC/MS.

MS/MS of SxtT + VanB Reaction Products. Fragmentation parameters are as described above. The average fragmentation patterns from recordings at collision energies of 10 eV, 20 eV, and 30 eV are shown.

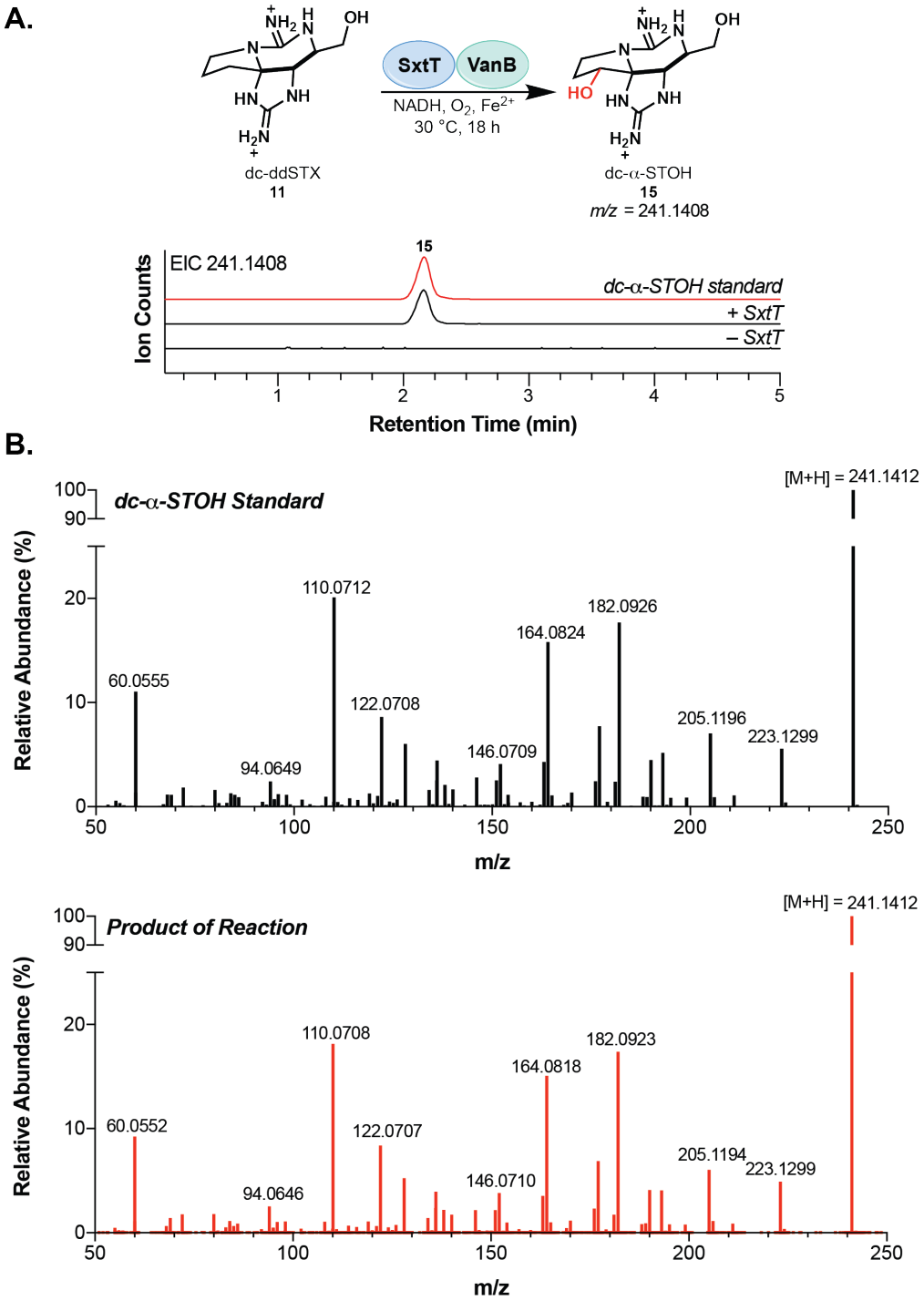


Figure S21. A) Extracted ion chromatogram (EIC) and B) MS/MS spectrum of the product of the reaction of SxtT with decarbamoyl dideoxysaxitoxin (dc-ddSTX, **11**) compared to the decarbamoyl α-saxitoxinol (dc-α-STOH, **15**) standard. The targeted mass of **15** is *m/z* = 241.1408.

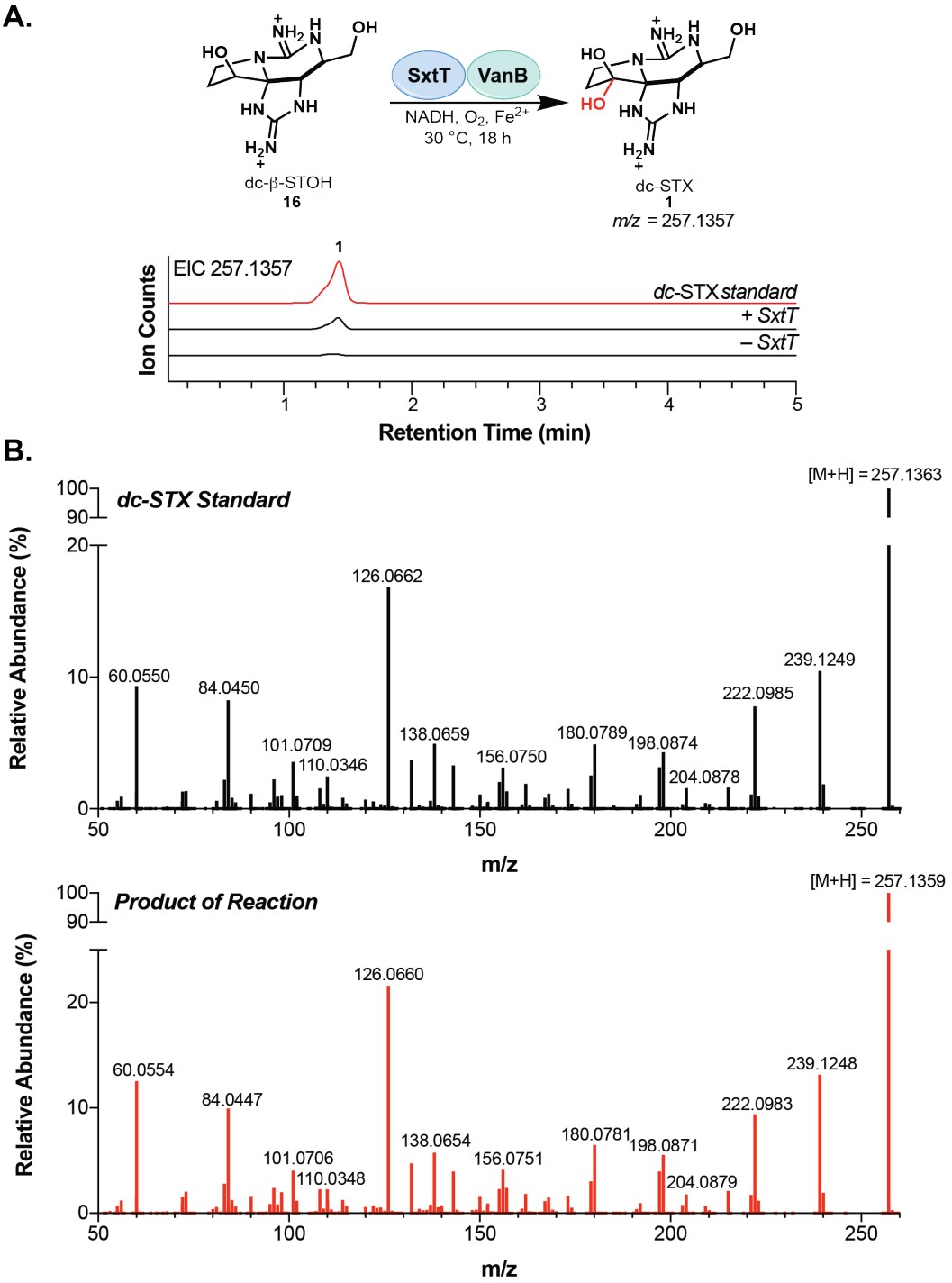


Figure S22. A) Extracted ion chromatogram (EIC) and B) MS/MS spectrum of the product of the reaction of SxtT with decarbamoyl β-saxitoxinol (dc-β-STOH, **16**) compared to the decarbamoyl saxitoxin (dc-STX, **1**) standard. The targeted mass of **1** is *m/z* = 257.1357.

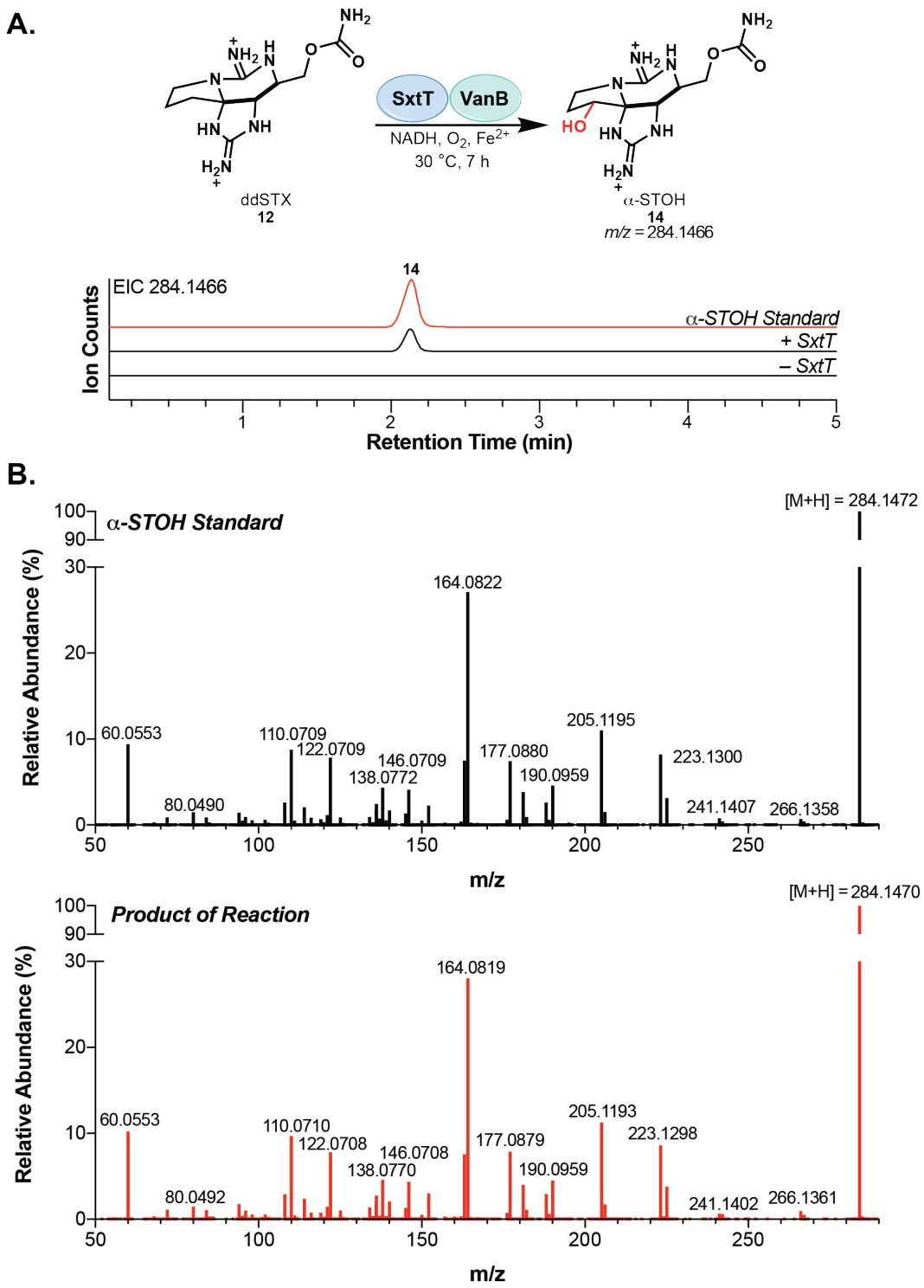


Figure S23. A) Extracted ion chromatogram (EIC) and B) MS/MS spectrum of the product of the reaction of SxtT with dideoxysaxitoxin (ddSTX, **12**) compared to the α-saxitoxinol (α-STOH, **14**) standard. The targeted mass of **14** is *m/z* = 284.1466.

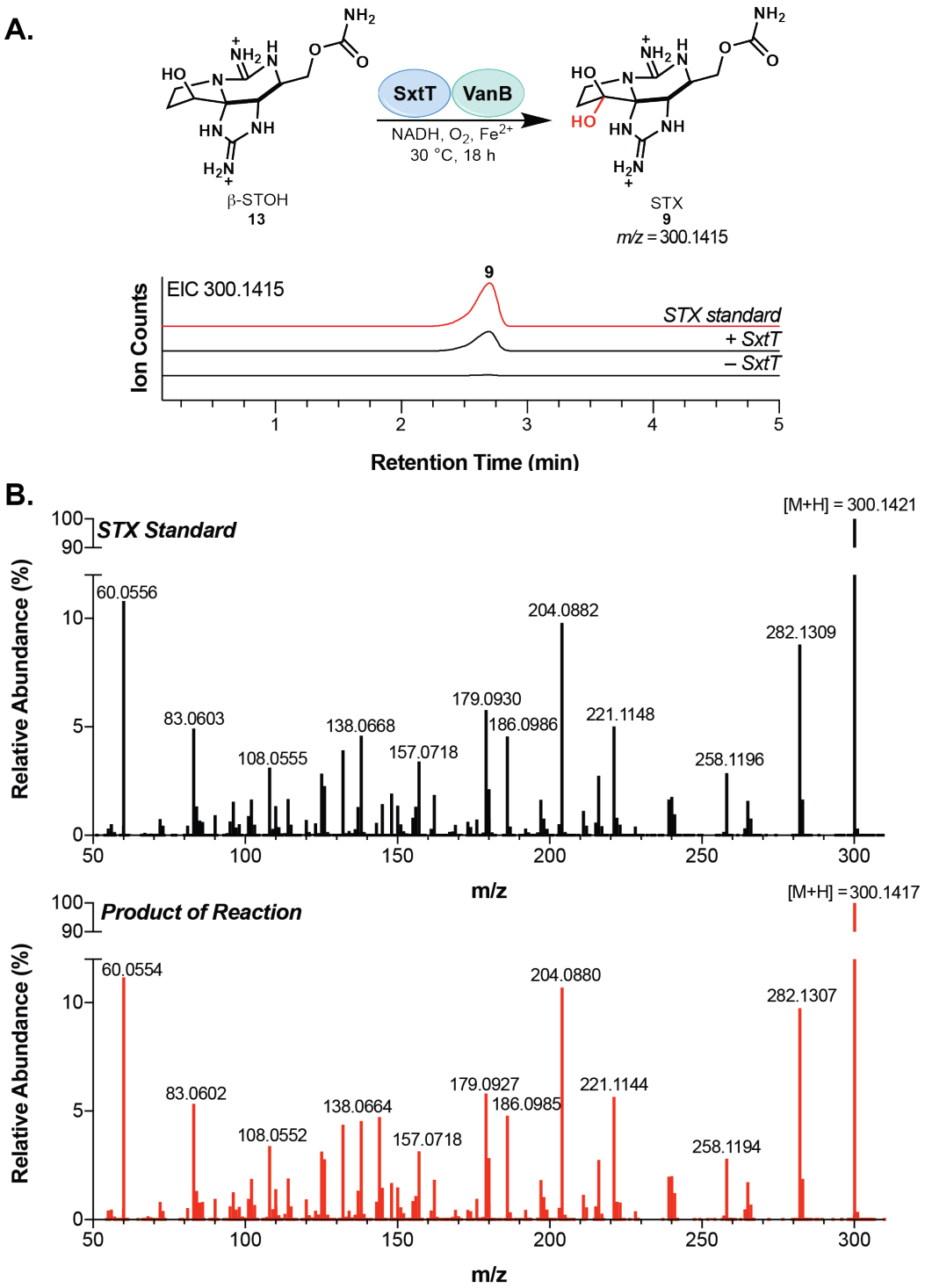


Figure S24. A) Extracted ion chromatogram (EIC) and B) MS/MS spectrum of the product of the reaction of SxtT with β-saxitoxinol (β-STOH, **13**) compared to the saxitoxin (STX, **9**) standard. The targeted mass of **9** is *m/z* = 300.1415.

MS/MS of GxtA + VanB Reaction Products. Fragmentation parameters are as described above. The average fragmentation patterns from recordings at collision energies of 10 eV, 20 eV, and 30 eV are shown.

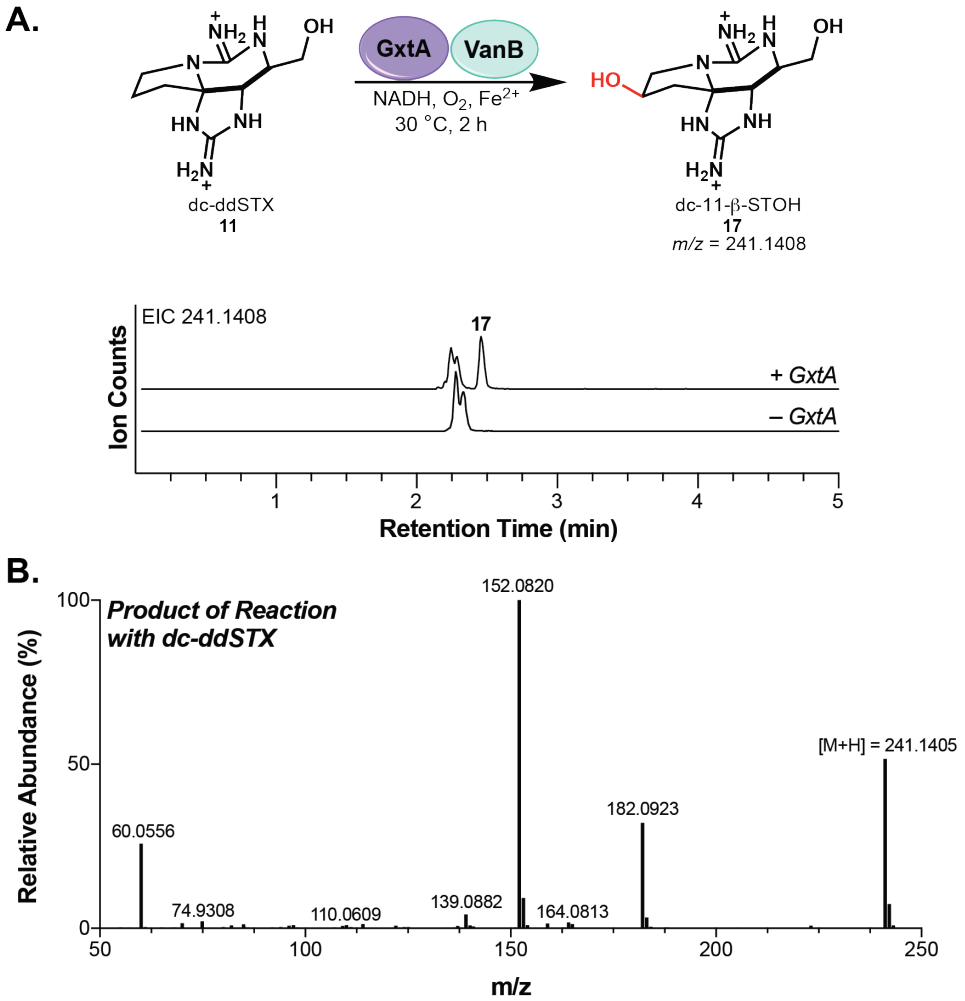


Figure S25. A) Extracted ion chromatogram (EIC) and B) MS/MS spectrum of the product of the reaction of GxtA with decarbamoyl dideoxysaxitoxin (dc-ddSTX, **11**). The product is decarbamoyl 11-β-saxitoxinol (dc-11-β-STOH, **17**) and the targeted mass is *m/z* = 241.1408. The contaminant at the same exact mass as **17** has not been identified.

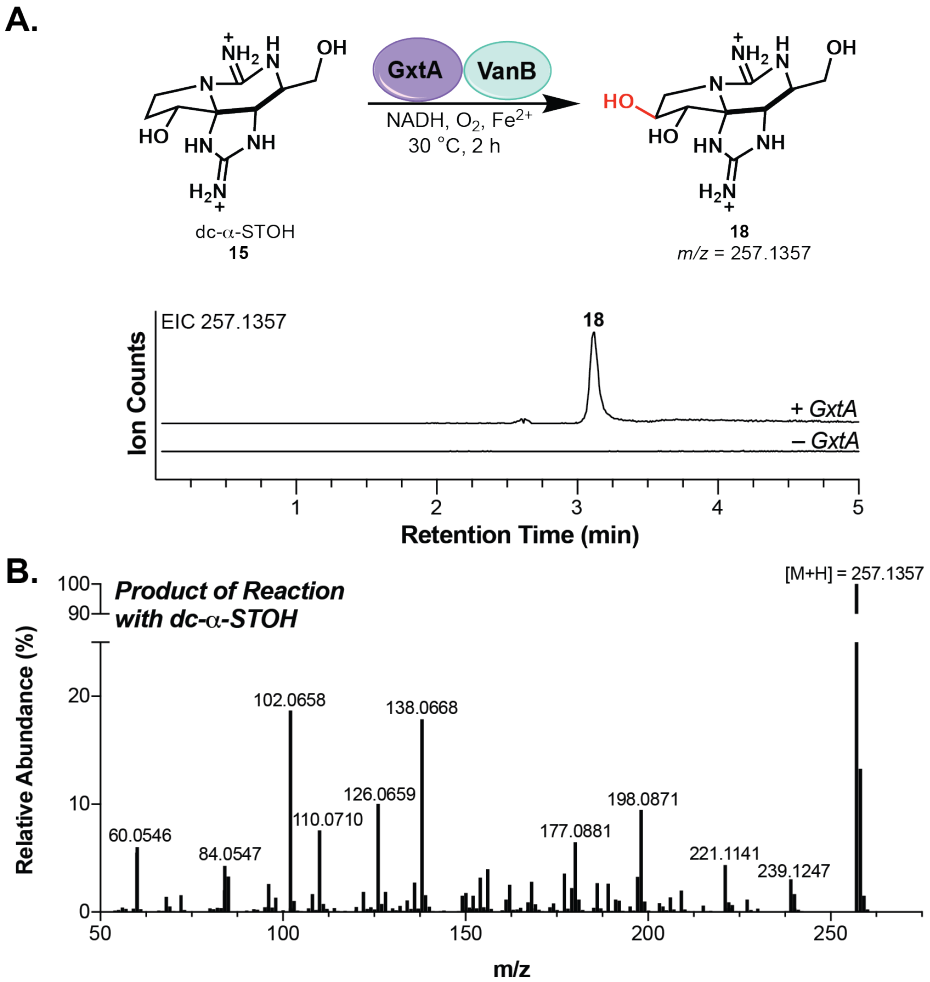


Figure S26. A) Extracted ion chromatogram (EIC) and B) MS/MS spectrum of the product of the reaction of GxtA with decarbamoyl α-saxitoxinol (dc-α-STOH, **15**). The product is decarbamoyl 11-β-hydroxy-α-saxitoxinol (**18**) and the targeted mass is *m/z* = 257.1357.

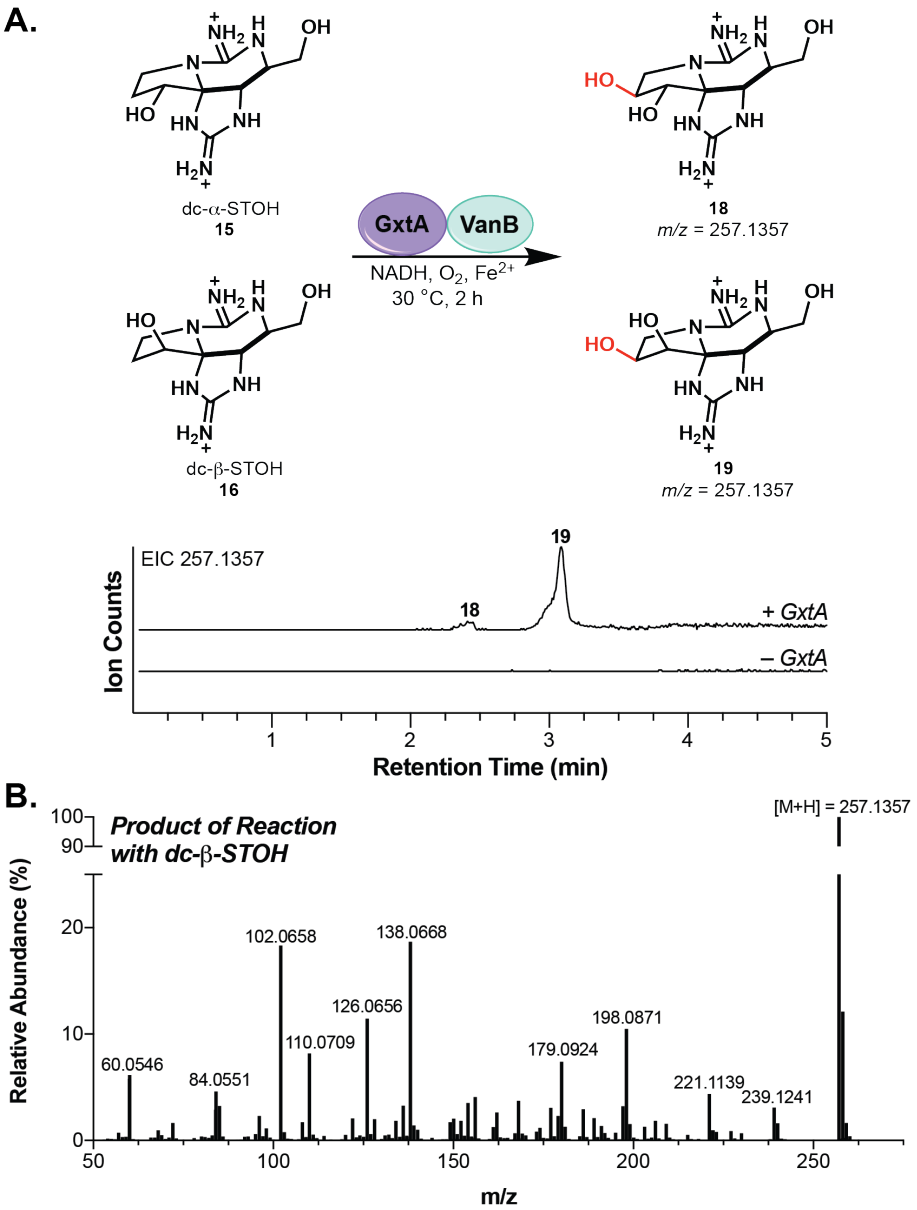


Figure S27. A) Extracted ion chromatogram (EIC) and B) MS/MS spectrum of the product of the reaction of GxtA with decarbamoyl β-saxitoxinol (dc-β-STOH, **16**). The product is decarbamoyl 11-β-hydroxy-β-saxitoxinol (**19**) and the targeted mass is *m/z* = 257.1357. The stock of dc-β-STOH was a mixture of **16** and dc-α-STOH (**15**), where **15** was the predominant species. Two products are observed, and the MS/MS spectrum shown is taken from the peak corresponding to **19**. The peak corresponding to **18** is decarbamoyl 11-β-hydroxy-α-saxitoxinol.

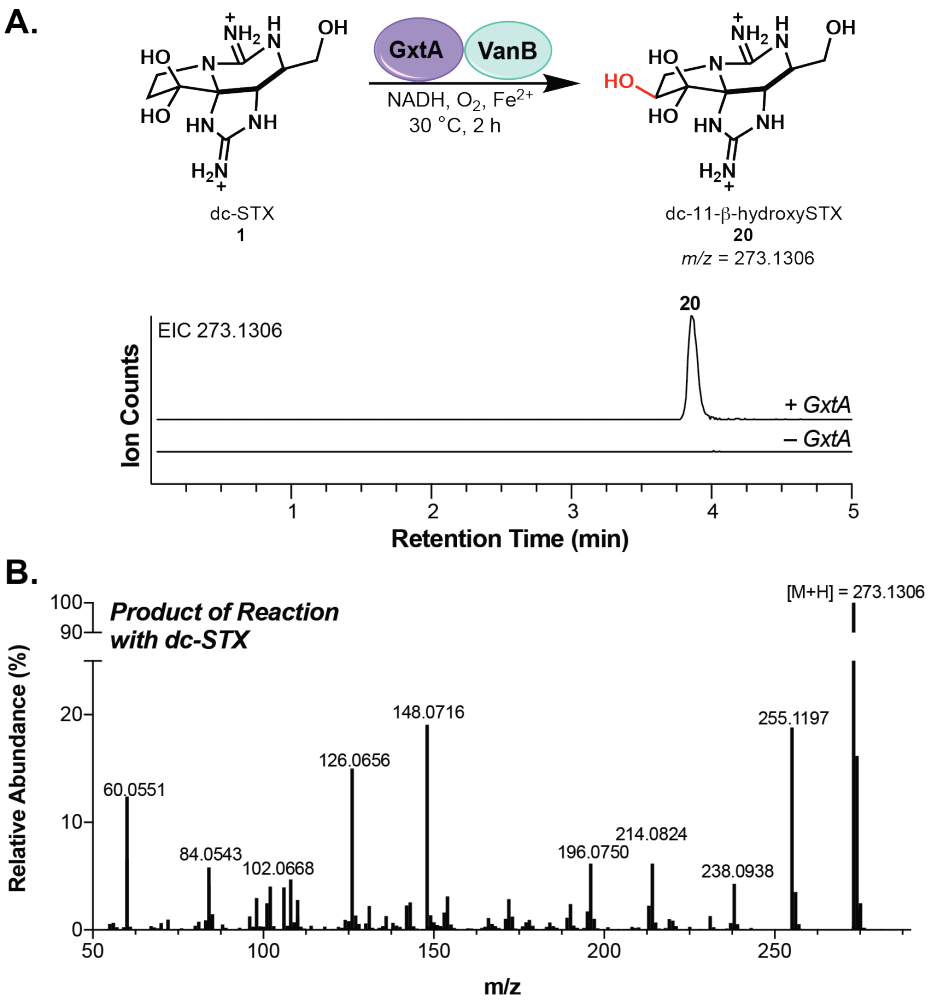


Figure S28. Extracted ion chromatogram (EIC) and B) MS/MS spectrum of the product of the reaction of GxtA with decarbamoyl saxitoxin (dc-STX, **1**). The product is decarbamoyl 11-β-hydroxysaxitoxin (**20**) and the targeted mass is *m/z* = 273.1306.

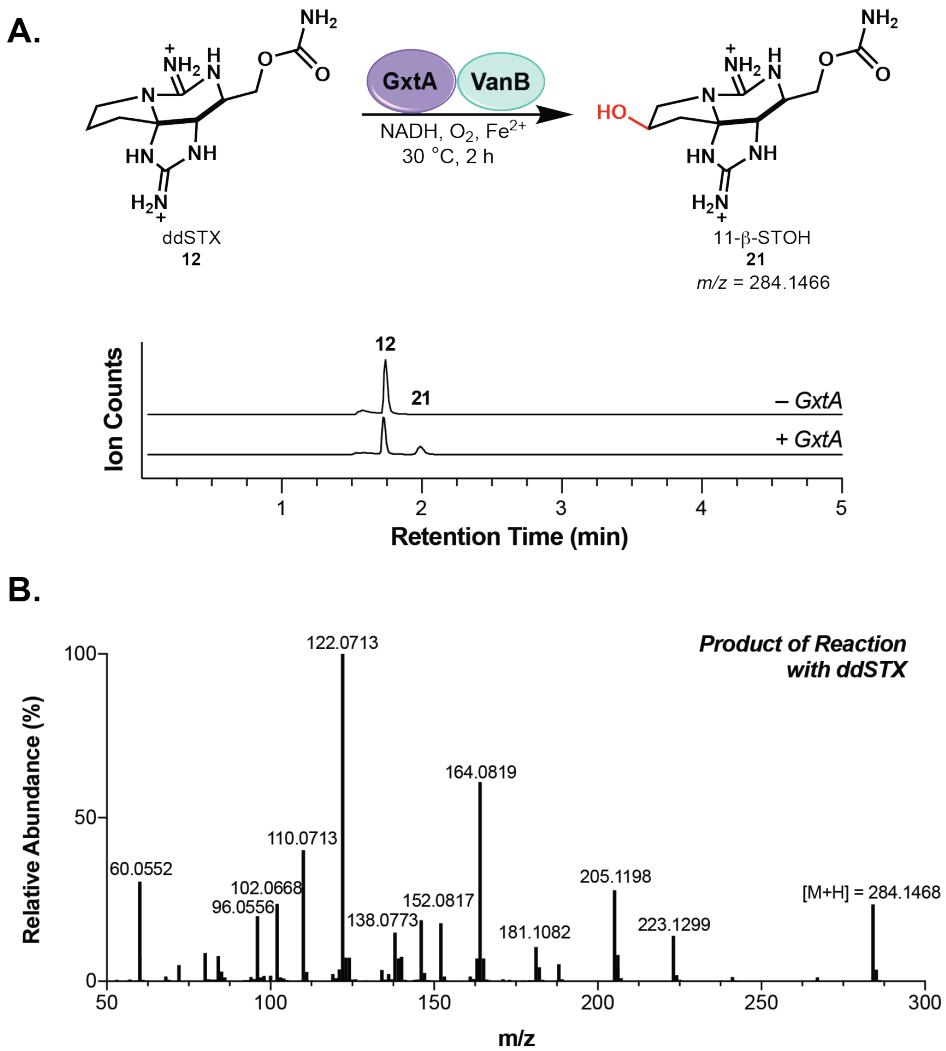


Figure S29. A) Extracted ion chromatogram (EIC) and B) MS/MS spectrum of the product of the reaction of GxtA with dideoxysaxitoxin (ddSTX, **12**). The product is 11-β-saxitoxinol (**21**) and the targeted mass is *m/z* = 284.1466.

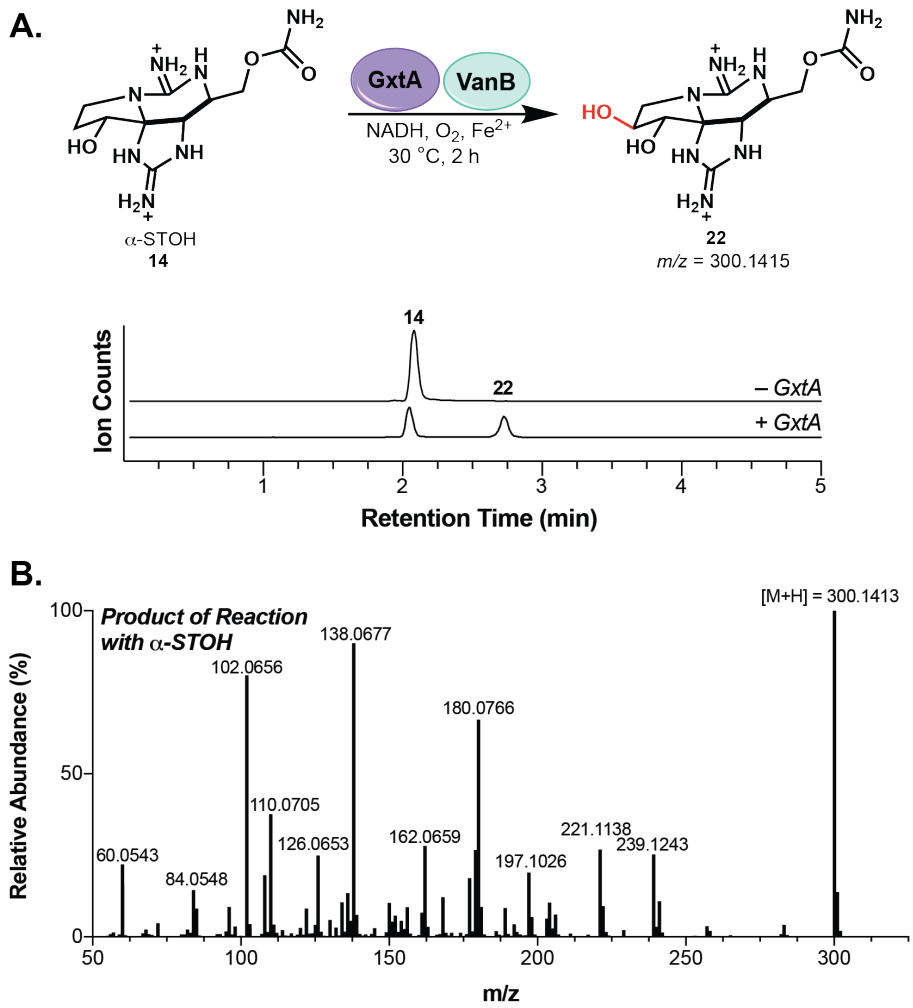


Figure S30. A) Extracted ion chromatogram and B) MS/MS spectrum of the product of the reaction GxtA with α-saxitoxinol (α-STOH, **14**). The product is 11-β-hydroxy-α-saxitoxinol (**22**) and the targeted mass is *m/z* = 300.1415.

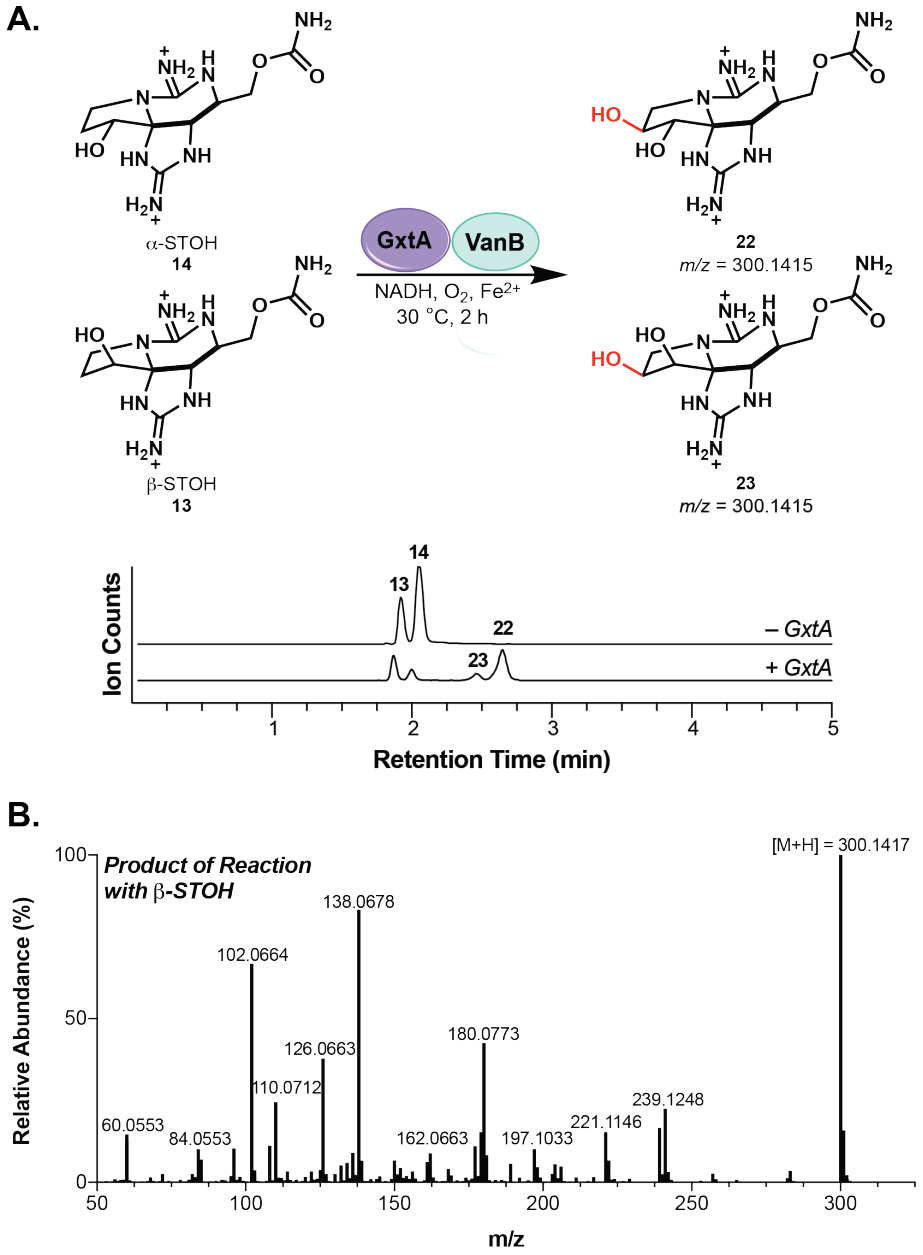


Figure S31. A) Extracted ion chromatogram and B) MS/MS spectrum of the product of the reaction of GxtA with β-saxitoxinol (β-STOH, **13**). The product is 11-β-hydroxy-β-saxitoxinol (**23**) and the targeted mass is *m/z* = 300.1415. The stock of β-STOH was a mixture of **13** and α-STOH (**14**), where **14** was the predominant species. Two products are observed, and the MS/MS spectrum shown is taken from the peak corresponding to **23**.

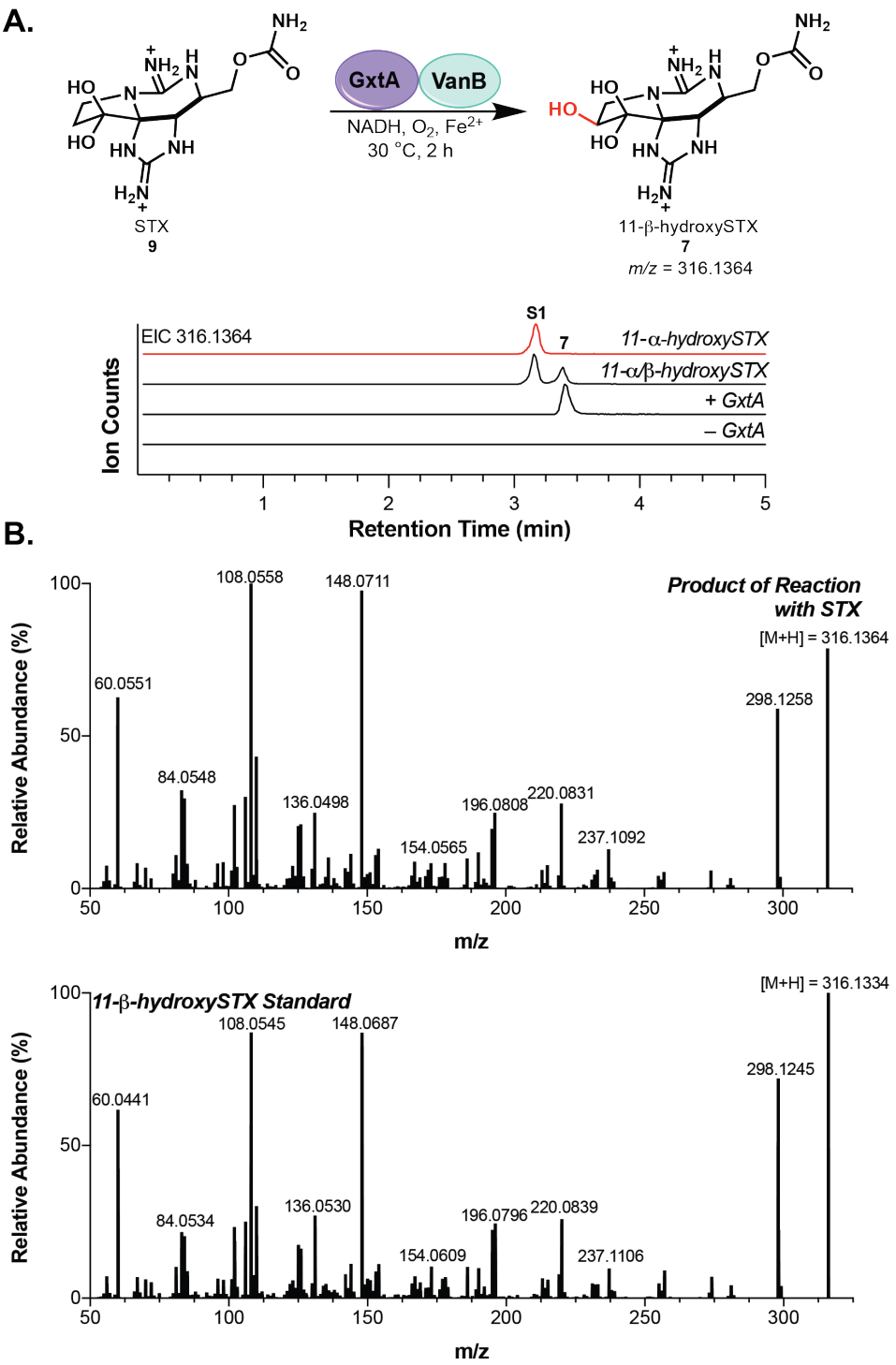


Figure S32. A) Extracted ion chromatogram (EIC) and B) MS/MS spectrum of the product of the reaction of GxtA with saxitoxin (STX, **9**) compared to the 11-β-hydroxysaxitoxin (11-β-hydroxySTX, **7**) standard in a mixture of 11-α-hydroxysaxitoxin (**S1**) and **7**, where only the MS/MS spectrum of the peak representative of **7** was obtained for comparison. The targeted mass of **7** is *m/z* = 316.1364.

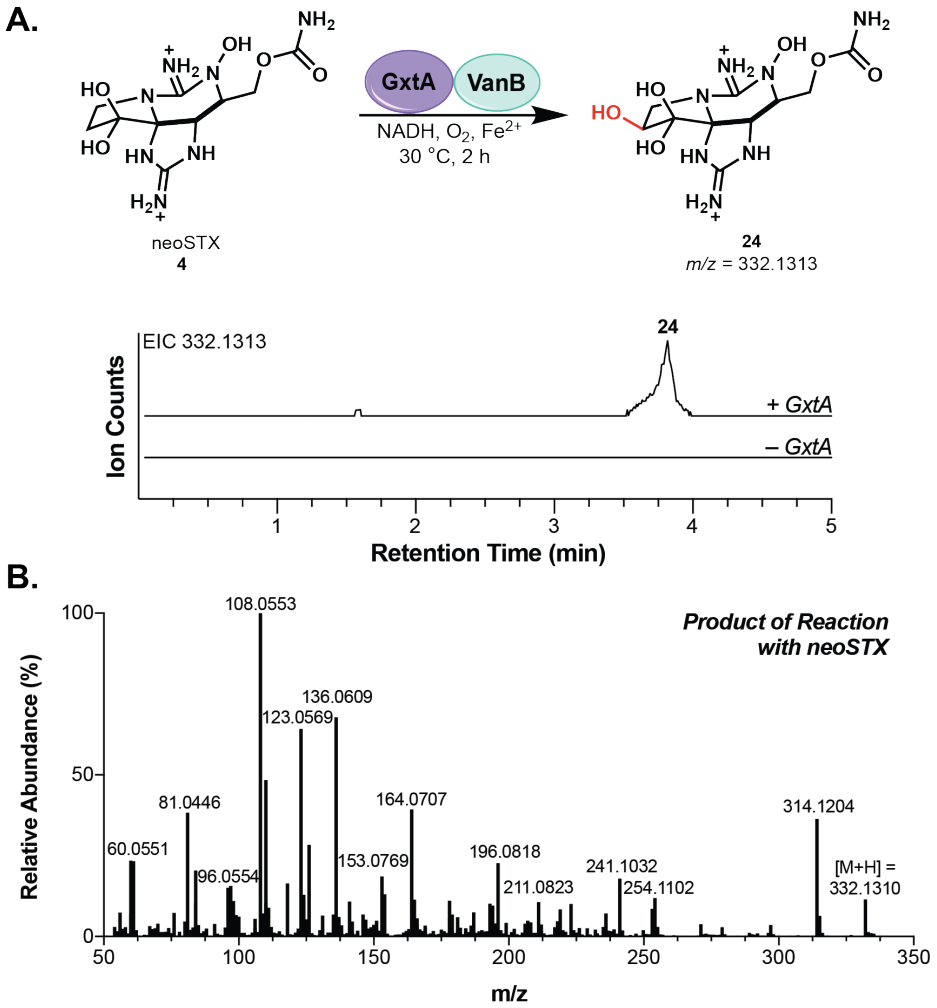


Figure S33. A) Extracted ion chromatogram (EIC) and B) MS/MS spectrum of the product of the reaction of GxtA with neosaxitoxin (neoSTX, **4**). The product is 11-β-hydroxyneosaxitoxin (**24**) and the targeted mass is *m/z* = 332.1313.

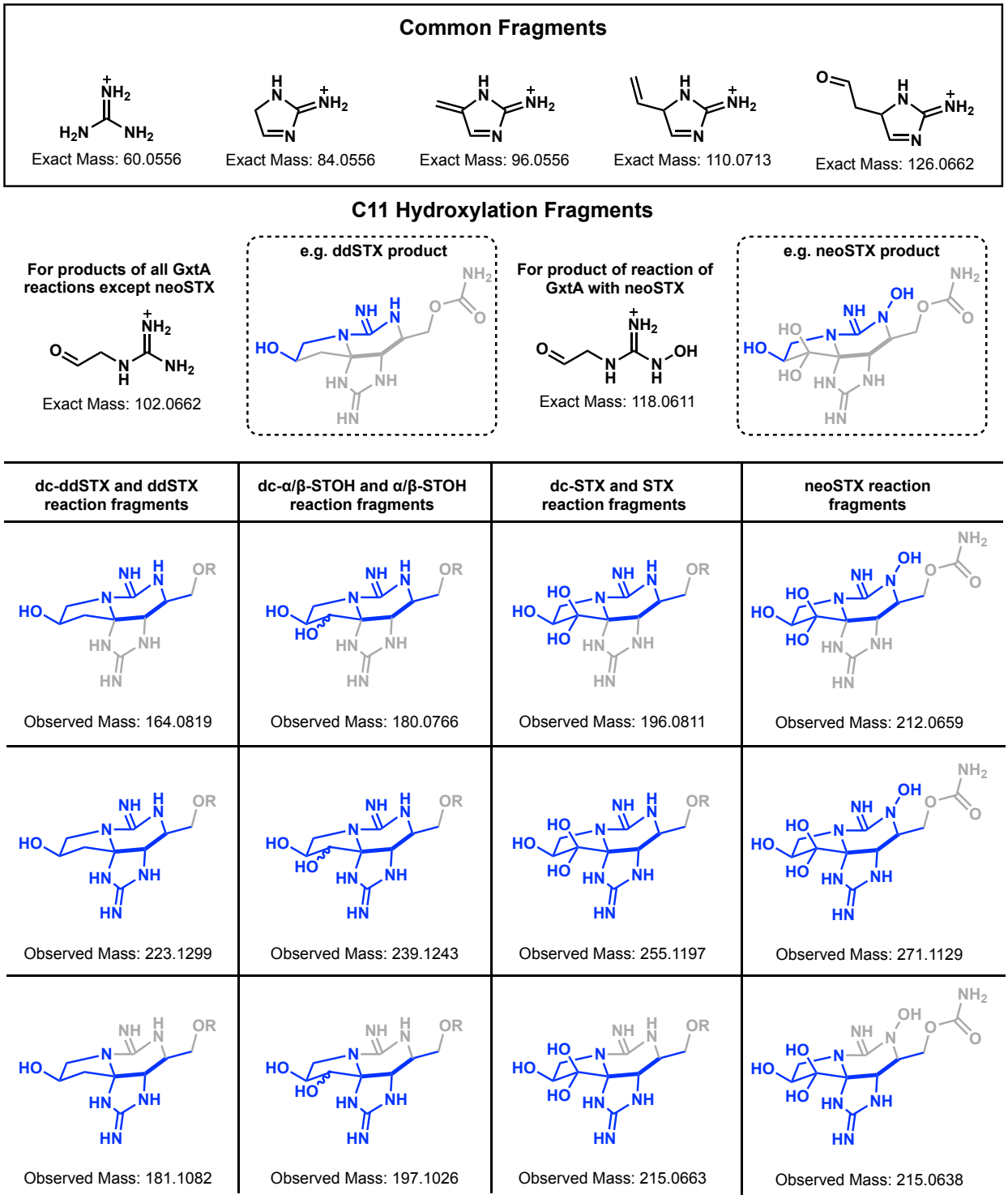
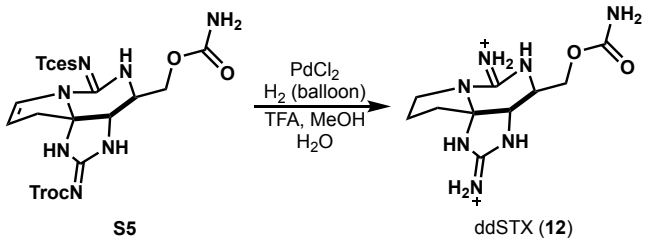


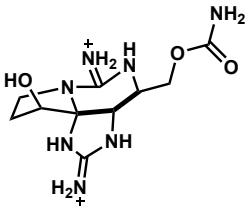
Figure S34. Proposed identities of key MS/MS fragmentation peaks and trends indicating C11 hydroxylation by GxtA. R = H for decarbamoylated compounds and R = $CONH_2$ for carbamoylated compounds. Fragment structures are based on Agilent MassHunter Molecular Structure Correlator (MSC) analysis.[18]

V. Synthesis and Standard Preparation

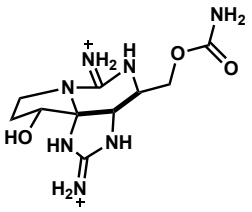
General Considerations. All reagents were used as received unless otherwise noted. Reactions were carried out under a nitrogen atmosphere unless otherwise noted. ^{1}H NMR spectra were obtained in D_2O at rt (25 °C), unless otherwise noted on a Varian 600 MHz spectrometer. Chemical shifts of ^{1}H NMR spectra were recorded in parts per million (ppm) on the δ scale. General considerations for mass spectra were in accordance to those listed on S22. Saxitoxin (**9**), β-saxitoxinol (**13**), α-saxitoxinol (**14**), neosaxitoxin (**4**), and gonyautoxin 2 (**2**) and gonyautoxin 3 (**3**) mixtures were generously provided by Sherwood Hall.



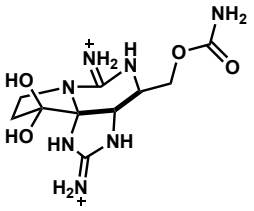
dideoxysaxitoxin (ddSTX, 12):
S4 was synthesized according to the procedures described by Mulcahy et al.[19]
To a solution of **S5** (10 mg, 15.3 μmol) in 6.2 mL of a 3:1 MeOH/H_2O mixture was added trifluoroacetic acid (176 μL, 2.3 mmol, 150 equiv). The mixture was sparged with N_2 for 10 min before $PdCl_2$ (1.4 mg, 7.7 μmol, 0.5 equiv) was added. The solution was then sparged with N_2 for 2 min, and then with H_2 for 5 min. The flask was fitted with a balloon of H_2 and the contents stirred for 3 h. Following this time, the mixture was filtered through a 0.45 μm PTFE filter. The reaction flask and filter were rinsed with 5 mL of 1.0 N aqueous HCl, and the combined filtrates were stirred for 30 min. The aqueous solution was frozen and lyophilized to remove all volatiles. Purification of the isolated material was performed by reversed-phase HPLC (Silicycle SiliaChrom Aq C18, 5 μm, 10 x 250 mm column, eluting with a 5 min prerun of 0:100 MeCN/10 mM aqueous $C_3F_7CO_2H$ then a gradient flow over 40 min of 0:100→40:60 MeCN/10 mM aqueous $C_3F_7CO_2H$, 2-fraction/min time-slice collection, 214 nm UV detection). At a flow rate of 4 mL/min, dideoxysaxitoxin (ddSTX, **12**) had a retention time of 22 min and was isolated as a white hygroscopic solid (5.4 μmol, 3.7 mg, 35%). ^{1}H NMR (500 MHz, D_2O): δ 4.66 (s, 1H), 4.14 (d, *J* = 5.9 Hz, 2H), 3.76 (t, *J* = 5.9 Hz, 1H), 3.63 (td, *J* = 10.2, 2.6 Hz, 1H), 3.52 (dt, *J* = 9.9, 8.1 Hz, 1H), 2.34 (dd, *J* = 11.4, 7.0 Hz, 1H), 2.23 – 2.13 (m, 2H), 2.12 – 2.02 (m, 1H) ppm. ^{13}C NMR (150 MHz, D_2O): δ 158.1, 156.9, 154.5, 79.6, 63.0, 60.4, 52.1, 45.1, 37.4, 19.5. HRMS (ES$^+$) *m/z* calcd for $C_{10}H_{18}N_7O_2$ $(M+H)^+$: 268.1516, found : 268.1524.



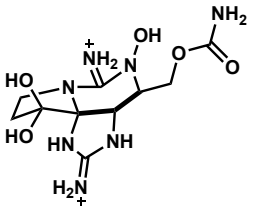
β-saxitoxinol (β-STOH, 13):
Saxitoxin diacetate (36 mg) extracted from cultures of *Alexandrium* sp., clone PW06, was divided into 6 portions of approximately 6 mg for reduction with sodium borohydride (30 mg) under various conditions, each in 0.5 mL solvent. After one hour each was quenched with acetone (0.2 mL) and acidified with glacial acetic acid (0.3 mL). Following evaluation, the six batches were combined and applied to a column (6.5 cm x 0.45 cm^2 = 2.9 cm^3 bed volume) of carboxylate cation exchange resin (IRP64) in the H^+ form with a preload of 1 mEq ammonium hydroxide. Following application, the column was eluted with aqueous acetic acid, 0.1 M followed by 1.0 M. Progress of the elution was followed by applying spots (5 µL) to a strip of E. Merck silica 60 F-254 aluminum-backed TLC plate, drying, and spraying with Weber reagent.[17, 20] Fractions that were Weber spot positive were combined and dried to 33 mg clear glaze. This was taken up in about 2 mL of 0.1 M aqueous acetic acid and applied to a BioGel P2 column, bed 2.4 cm^2 x 110.7 cm long, isocratic elution with 0.1 M aqueous acetic acid.[21-22] Fractions (3.68 mL, 1.37 % bed volume) were collected, spotted, and sprayed with Weber reagent. Effluent conductivity was monitored. Weber spot positive fractions were dried and residue weights determined. The residue mass peak coincided with the conductivity peak at about 120 % bed volume. The peak fractions were combined and dried to 9.6 mg. ^{1}H-NMR (600 MHz) in D_2O revealed that the β-STOH content of this material was approximately 16% with α-STOH making up the balance of the material. All spectra obtained were consistent with literature values.[23]



α-saxitoxinol (α-STOH, 14):
Saxitoxin diacetate, extracted from cultures of *Alexandrium* sp., clone PW06, was reduced in various trials with sodium borohydride or sodium cyanoborohydride in aqueous solutions and the resulting mixtures of α and β-STOH purified on columns of IRP64, BioGel P2, and Sephadex G-10-100.[22] Fractions were spotted on strips of E. Merck Silica 60 F-254 plate and visualized with Weber reagent. Fractions of interest were run on E. Merck Silica 60 F-254 plates using a Buckley E solvent system[21], grouped, and dried accordingly. A group of fractions identified by TLC as probable α-STOH was combined and a portion removed for ^{1}H NMR, which revealed that the preparation was relatively clean α-STOH. All spectra obtained were consistent with literature values.[23]

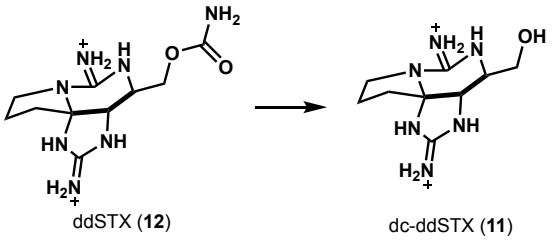


saxitoxin (STX, 9):
Saxitoxin diacetate was extracted from cultures of *Alexandrium* sp., clone PW06. The extract was purified using a succession of carboxylate ion exchange columns (IRC-50, IRP64), BioGel P2, and then Chelex 100 to remove copper and other heavy metals. The concentrated extract was treated with ammonium oxalate to precipitate Ca^{2+} and Mg^{2+} and with ethanol to precipitate protein and other large biomolecules. Following removal of the ethanol the residue was taken up in water and applied to a final IRP64 column. Heart fractions from this were combined, dried, and applied to a final BioGel P2 column. Heart fractions from this were evaluated by ^{1}H NMR and the heart fractions combined and dried. All spectra obtained were consistent with literature values.[22-23]



neosaxitoxin (neoSTX, 4):
Neosaxitoxin diacetate was extracted from cultures of *Alexandrium* sp., clone PI07 and purified on columns of IRP64 and BioGel P2 (Hall, 1982). Several portions of purified neosaxitoxin diacetate, used in various NMR experiments, were combined and cleaned up, again on columns of carboxylate ion exchange resin (IRP64) and BioGel P2, eluting with aqueous acetic acid. ^{1}H NMR of the resulting material confirmed that it was relatively clean neosaxitoxin diacetate. All spectra obtained were consistent with literature values.[24]

General procedure for carbamate cleavage. Conditions for the decarbamoylation of saxitoxin scaffolds were adapted from Koehn and Ghazarossian et al.[16-17] Starting materials were obtained from an aqueous stock solution unless otherwise noted. To a 20 mM aqueous stock solution of starting material was added MeCN (1 mL) and concentrated under reduced pressure to afford a film. This residue was dissolved in a solution of 7.5 M HCl and heated at 100 °C for the designated amount of time. An aliquot was taken for MS analysis to determine extent of carbamate hydrolysis. After addition of MeCN (1 mL), the solution was concentrated under reduced pressure to afford a film. The product was used without further purification unless otherwise noted.



decarbamoyl dideoxysaxitoxin (dc-ddSTX, 11):
The title compound was prepared according to the procedure above using 10 µL of a 20 mM stock solution of dideoxysaxitoxin (ddSTX, **12**) in DMSO and 100 µL of 7.5 M HCl and was heated for 6 h.

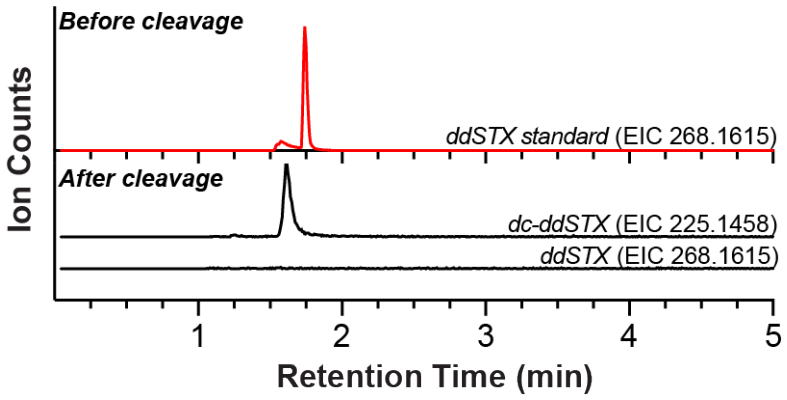
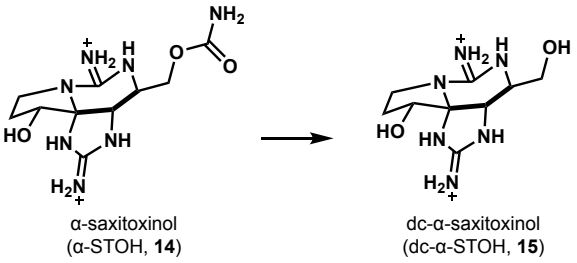


Figure S35. Extracted ion chromatograms (EICs) of dideoxysaxitoxin (ddSTX, **12**) and decarbamoyl dideoxysaxitoxin (dc-ddSTX, **11**) before and after carbamate cleavage.



decarbamoyl α-saxitoxinol (dc-α-STOH, 15):
The title compound was prepared according to the procedure above using 20 µL of a 20 mM stock solution of α-saxitoxinol (α-STOH (**14**) in water and 200 µL of 7.5 M HCl and was heated for 6 h.

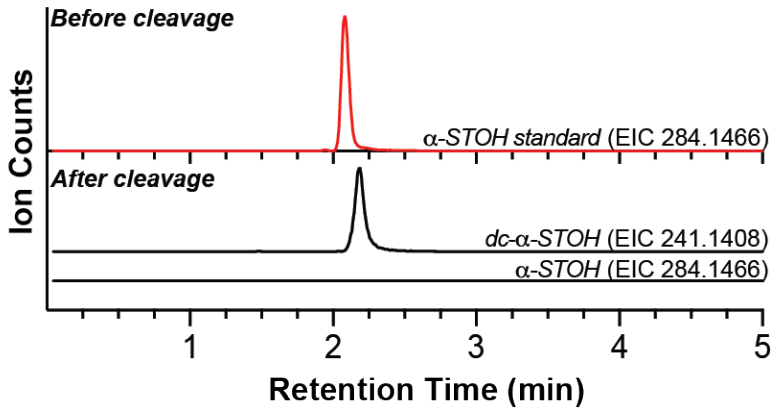
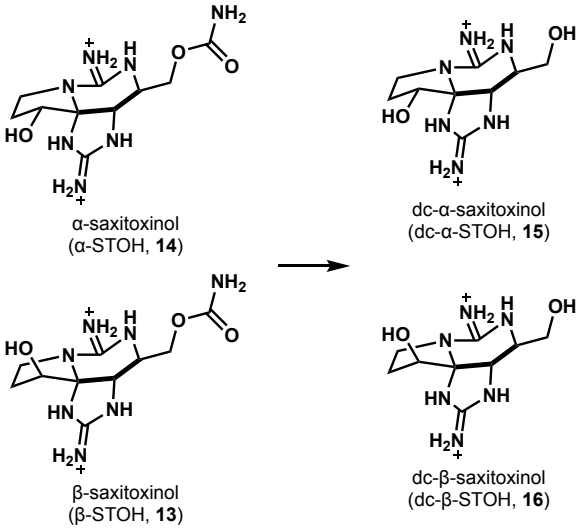


Figure S36. Extracted ion chromatograms (EICs) of α-saxitoxinol (α-STOH, **14**) and decarbamoyl α-saxitoxinol (dc-α-STOH, **15**) before and after carbamate cleavage.



decarbamoyl β-saxitoxinol (dc-β-STOH, 16):
The title compound was prepared according to the procedure above using 20 µL of a 20 mM stock solution of a mixture of β-saxitoxinol (β-STOH, **13**) and α-saxitoxinol (α-STOH, **14**) in water and 200 µL of 7.5 M HCl and was heated for 6 h.

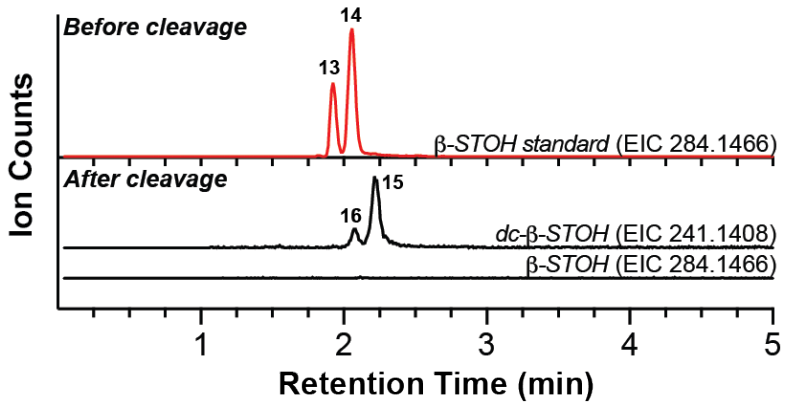
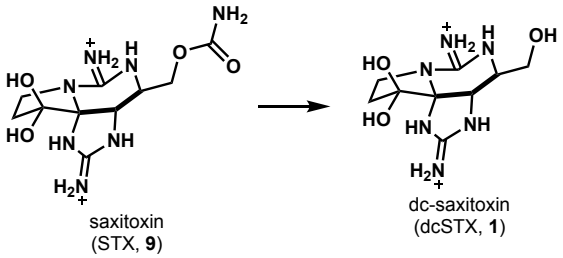


Figure S37. Extracted ion chromatograms (EICs) of β-saxitoxinol (β-STOH, **13**) and decarbamoyl β-saxitoxinol (dc-β-STOH, **16**) before and after carbamate cleavage. The β-STOH stock was a mixture of α-saxitoxinol (α-STOH, **14**) and β-STOH where the more abundant species was α-STOH.



decarbamoyl saxitoxin (dc-STX, 1):
To saxitoxin (STX, **9**, 4.4 mg) was added 1.0 mL of 7.5 M HCl, and the solution subjected to the conditions of the procedure above and heated for 8.6 h. The product was purified by reversed-phase HPLC (Phenomenex Kinetex 5 µm C18, 150 x 10 mm column) under the following conditions: mobile phase A = deionized water + 0.1% TFA and B = acetonitrile + 0.1% TFA; method = 99% A for 2 min, 99% A to 90% A over 6 min, 90% A to 5% A over 20 min, 5% A to 95% A over 2 min, 95% A for 5 min, 214 nm UV detection; a flow rate of 5 mL/min with a retention time of 1.2-1.7 min afforded 3.4 mg of dcSTX (**1**).

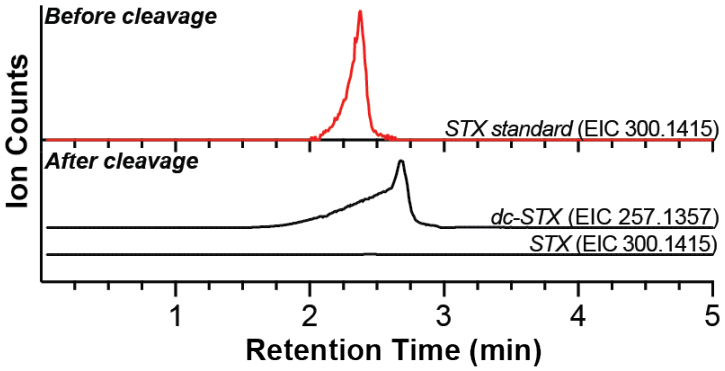
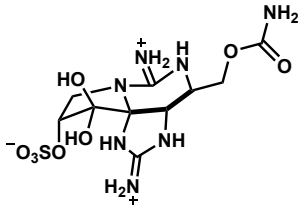
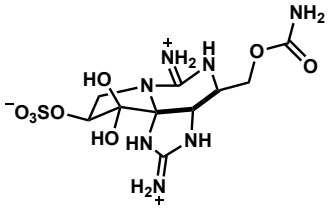


Figure S38. Extracted ion chromatograms (EICs) of saxitoxin (STX, **9**) and decarbamoyl dideoxysaxitoxin (dc-STX, **1**) before and after carbamate cleavage.



gonyautoxin 2
(GTX2, **2**)



gonyautoxin 3
(GTX3, **3**)

gonyautoxin 2 (GTX2, 2) and gonyautoxin 3 (GTX3, 3):
Gonyautoxin 2 (11-α-hydroxysaxitoxin sulfate) was extracted from cultures of *Alexandrium* sp., clone PI07 and purified on columns of IRP64 and BioGel P2.[22] A large batch (520 mg) of mixed gonyautoxin 2 and 3 was applied to a final BioGel P2 column to provide fractions enriched in each epimer. Fractions were monitored with a spot plate and TLC according to Buckley.[21] Fractions 28 and 29, near the front of the toxin peak, were highly enriched in gonyautoxin 2. They were combined and dried to 26.94 mg residue. All spectra obtained were consistent with literature values.[24-25]

gonyautoxin 2 (2) and **gonyautoxin 3 (3),** 4:1 mixture**: ^{1}H NMR** (600 MHz, D_2O) δ 4.98 (dd, *J* = 8.2, 6.9, 1H), 4.84 (d, *J* = 5.0, 5H), 4.82 (d, *J* = 1.0, 4H), 4.32 (dd, *J* = 11.7, 9.2, 1H), 4.28 (dd, *J* = 11.7, 9.3, 4H), 4.18 (overlapped, dd, *J* = 10.7, 8.3 and d, *J* = 12.0, 5H), 4.08 (dd, *J* = 11.7, 5.3, 1H), 4.02 (overlapped, dd, *J* = 11.7, 5.5 and dd, *J* = 12.1, 5.0, 8H), 3.86 (dd, *J* = 9.2, 5.2, 5H), 3.61 (dd, *J* = 10.8, 6.9, 1H).

Gonyautoxin 3 (11-α-hydroxysaxitoxin sulfate) was extracted from cultures of *Alexandrium* sp., clone PI07 and purified on columns of IRP64 and BioGel P2.[22] A large batch (520 mg) of mixed gonyautoxin 2 and 3 was applied to a final BioGel P2 column to provide fractions enriched in each epimer. Fractions were monitored with a spot plate and TLC according to Buckley.[21] Fraction 36, near the back of the toxin peak, was highly enriched in gonyautoxin 3. It was dried to 4.69 mg residue. All spectra obtained were consistent with literature values.[24-25]

gonyautoxin 2 (2) and **gonyautoxin 3 (3),** 1:1 mixture**: ^{1}H NMR** (600 MHz, D_2O) δ 4.98 (dd, *J* = 8.1, 6.9, 1H), 4.84 (d, *J* = 4.9, 1H), 4.82 (d, *J* = 1.3, 2H), 4.32 (dd, 11.7, 9.3, 1H), 4.28 (dd, *J* = 11.7, 9.2, 1H), 4.18 (overlapped, dd, *J* = 10.7, 8.3 and d, *J* = 12.5, 2H), 4.08 (dd, *J* = 11.7, 5.3, 1H), 4.02 (overlapped, dd, *J* = 11.7, 5.5 and dd, *J* = 12.0, 5.0, 2H), 3.85 (td, *J* = 8.8, 5.2, 2H), 3.60 (dd, *J* = 10.8, 6.9, 1H).

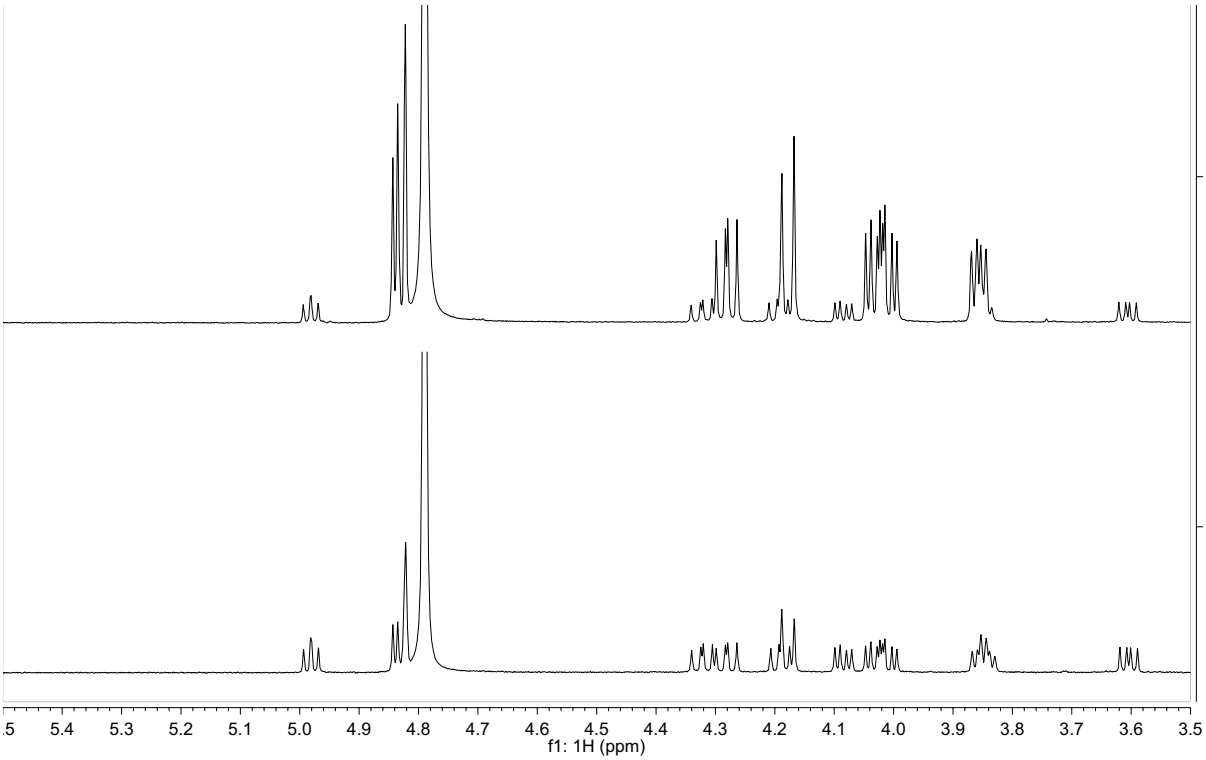
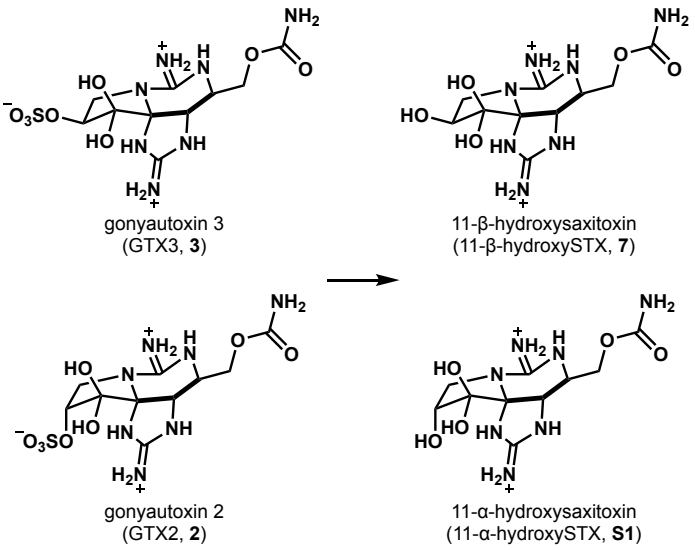


Figure S39. ^{1}H NMR spectra of gonyautoxin 2/gonyautoxin 3, 4:1 mixture (upper, 600 MHz, D_2O) and gonyautoxin 2/gonyautoxin3, 1:1 mixture (lower, 600 MHz, D_2O).



11-β-hydroxysaxitoxin (11-β-hydroxySTX, 7) and 11-α-hydroxysaxitoxin (11-α-hydroxySTX, S1):

Conditions for sulfate hydrolysis were adapted from Koehn.[17]

To a mixture of GTX3 and GTX2 (**3, 2**, 5.0 mg) was added 1 mL of 2.5 M HCl and the mixture was heated at 80 °C for 50 min. An aliquot was taken for MS analysis to determine extent of

sulfate hydrolysis and C11 epimerization. After addition of MeCN (1 mL), the solution was concentrated under reduced pressure to afford a film. The products were purified by reversed-phase HPLC (Phenomenex Kinetex 5 µm C18, 150 x 21.2 mm column) under the following conditions: mobile phase A = deionized water + 0.1% formic acid and B = acetonitrile + 0.1% formic acid; method = 99% A for 10 min, 99% A to 70% A over 30 min, 70% A for 5 min, 214 nm UV detection. A mixture of 11-β-hydroxySTX (**7**) and 11-α-hydroxySTX (**S1**) co-eluted (in approximately a 2:1 ratio, ^{1}H NMR) with a retention time of 4.5-8.2 min to afford 1.4 mg of the mixture as a white film with minor impurities. 11-α-hydroxySTX (**S1**) also eluted with a retention time of 12.7-15.8 min to afford 1.5 mg of 11-α-hydroxySTX (**S1**) as a white film with minor impurities. All spectra obtained were consistent with literature values.[19, 24-25]

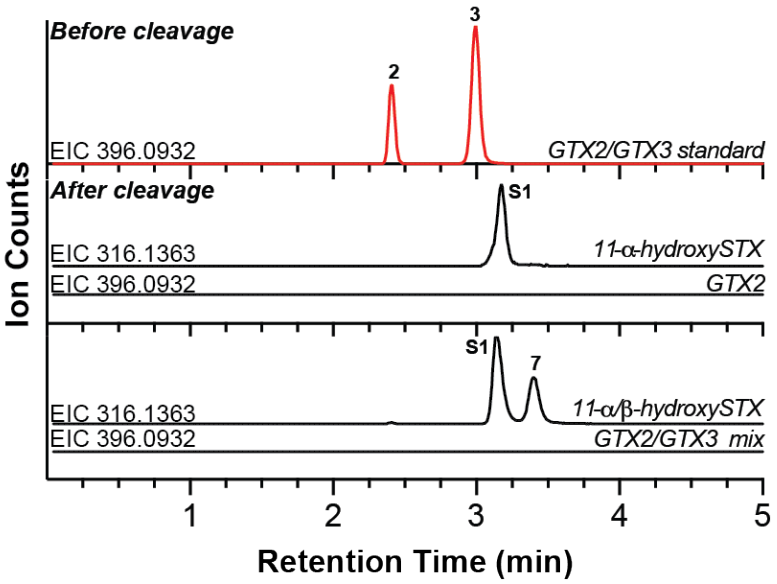


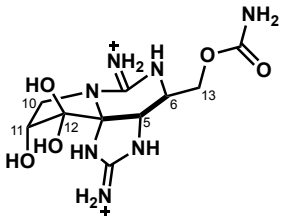
Figure S40. Extracted ion chromatograms (EICs) of GTX2 (**2**), GTX3 (**3**), 11-α-hydroxySTX (**S1**), and 11-β-hydroxySTX (**7**) before and after sulfate hydrolysis.

11-β-hydroxySTX (7) and **11-α-hydroxySTX (S1)**, 2:1 mixture**: ^{1}H NMR** (600 MHz, D_2O) δ 4.84 (s, 3H), 4.48 (t, *J* = 7.7, 1H), 4.35 (dd, *J* = 11.4, 9.6, 1H), 4.32-4.26 (m, 5H), 4.07 (dd, *J* = 11.5, 5.3, 1H), 4.03 (dd, *J* = 11.8, 5.7, 3H), 3.89 (dd, *J* = 11.5, 5.1, 3H), 3.86 (dd, *J* = 9.2, 5.6, 4H), 3.79-3.74 (m, 5H), 3.30 (dd, *J* = 10.4, 7.2, 1H).

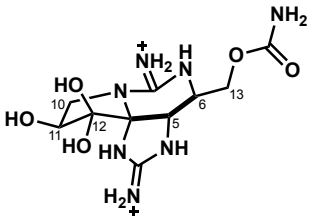
11-α-hydroxySTX (S1): ^{1}H NMR (600 MHz, D_2O) δ 4.82 (s, 1H), 4.32 (d, *J* = 5.0, 1H), 4.29 (d, *J* = 9.6, 1H), 4.04 (dd, *J* = 11.7, 5.1, 1H), 3.91 (dd, *J* = 11.4, 4.9, 1H), 3.90-3.84 (m, 1H), 3.77 (d, *J* = 11.1, 1H).

Table S5. Comparison of spectral data for 11-β-hydroxySTX **(7)** and 11-α-hydroxySTX **(S1)**.[19, 24-25]

Note: Chemical shifts of Ref. 24 were adjusted in Ref. 25 by adding 0.37 ppm to the values based on an internal reference. Values have been corroborated from Ref. 24.



11-α-hydroxysaxitoxin
(11-α-hydroxySTX, **S1**)



11-β-hydroxysaxitoxin
(11-β-hydroxySTX, **7**)

position	^{1}H (Schnoes)[24-25]	^{1}H (Narayan)	position	^{1}H (Schnoes)[24-25]	^{1}H (Du Bois)[19]
H5	4.76 (s, 1H)	4.82 (s, 1H)	H5	4.80 (d, *J* = 1.1, 1H)	4.80 (s, 1H)
H11	4.27 (d, *J* = 5.1, 1H)	4.32 (d, *J* = 5.0, 1H)	H11	4.44 (dd, *J* = 8.1, 7.0, 1H)	4.45 (dd, *J* = 8.0, 7.2, 1H)
H13	4.22 (dd, *J* = 11.4, 9.2, 1H)	4.29 (d, *J* = 9.6, 1H)	H13	4.25 (dd, *J* = 11.4, 9.2, 1H)	4.33 (dd, *J* = 11.6, 9.6, 1H)
H13	4.00 (dd, *J* = 11.4, 5.1, 1H)	4.04 (dd, *J* = 11.7, 5.1, 1H)	H13	4.02 (dd, *J* = 12.0, 5.5, 1H)	4.07-3.98 (m, 2H)
H10	3.85 (dd, *J* = 11.8, 5.1, 1H)	3.91 (dd, *J* = 11.4, 4.9, 1H)	H10	4.01 (dd, *J* = 10.3, 8.4, 1H)	
H6	3.83 (m, 1H)	3.90-3.84 (m, 1H)	H6	3.81 (ddd, *J* = 9.6, 5.5, 1.1, 1H)	3.82 (dd, *J* = 9.6, 4.4, 1H)
H10	3.72 (d, *J* = 11.4, 1H)	3.77 (d, *J* = 11.1, 1H)	H10	3.26 (dd, *J* = 10.6, 7.3, 1H)	3.27 (dd, *J* = 10.4, 7.2, 1H)

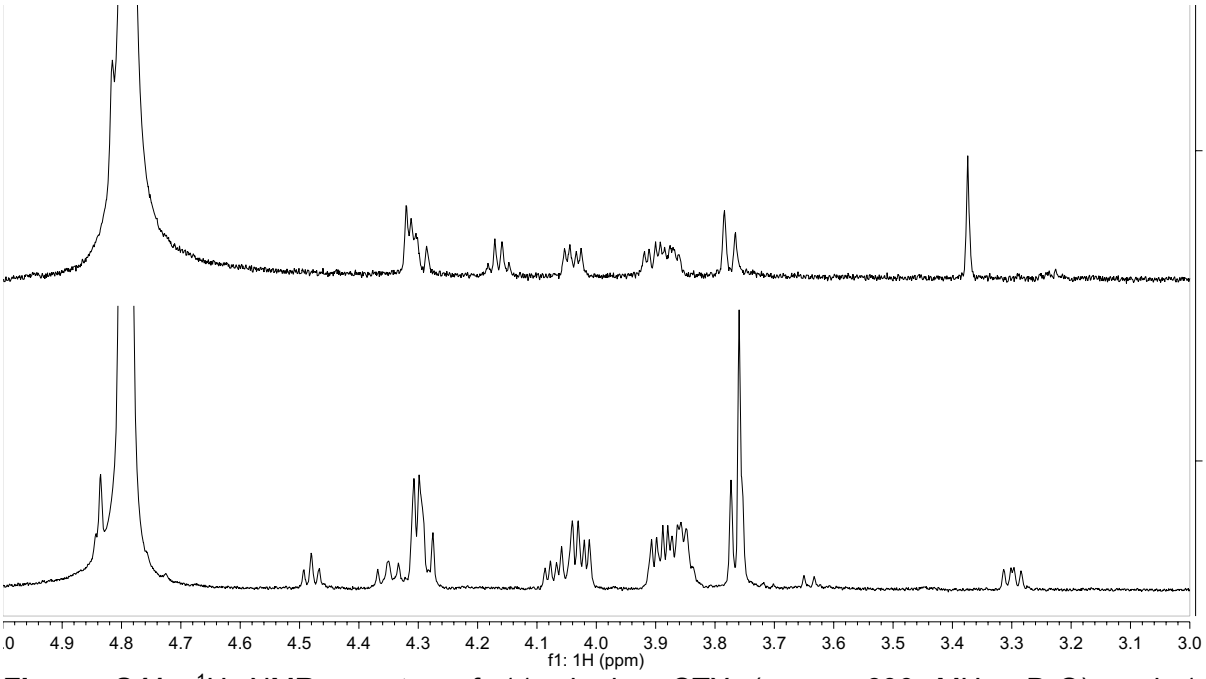
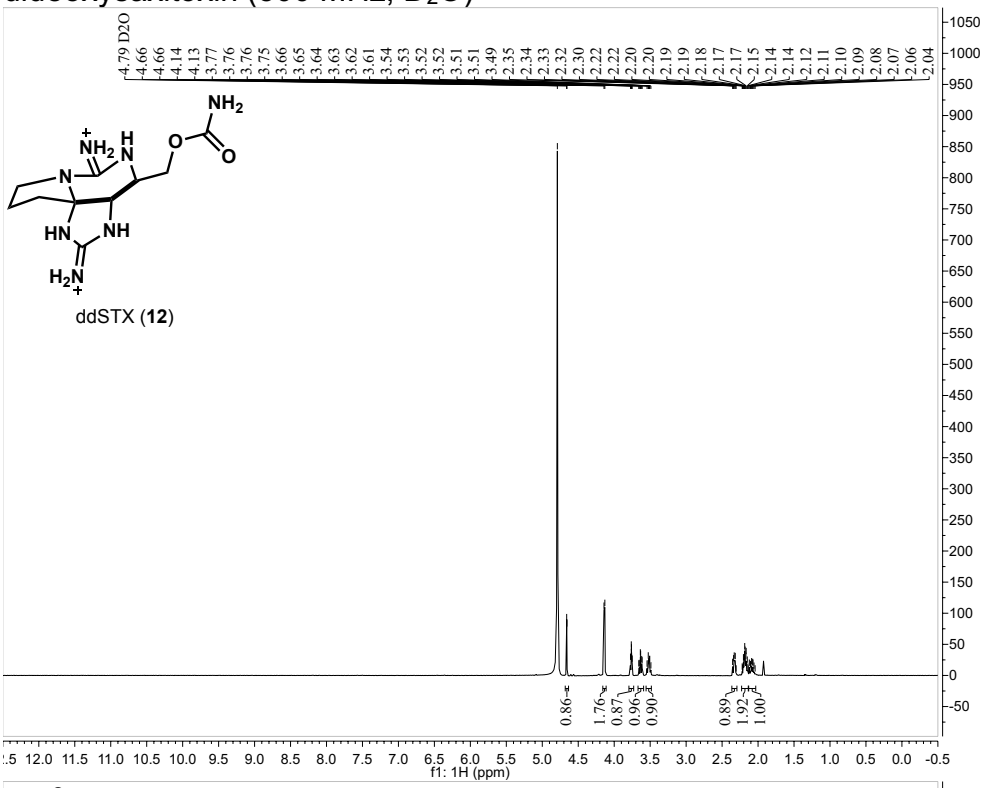
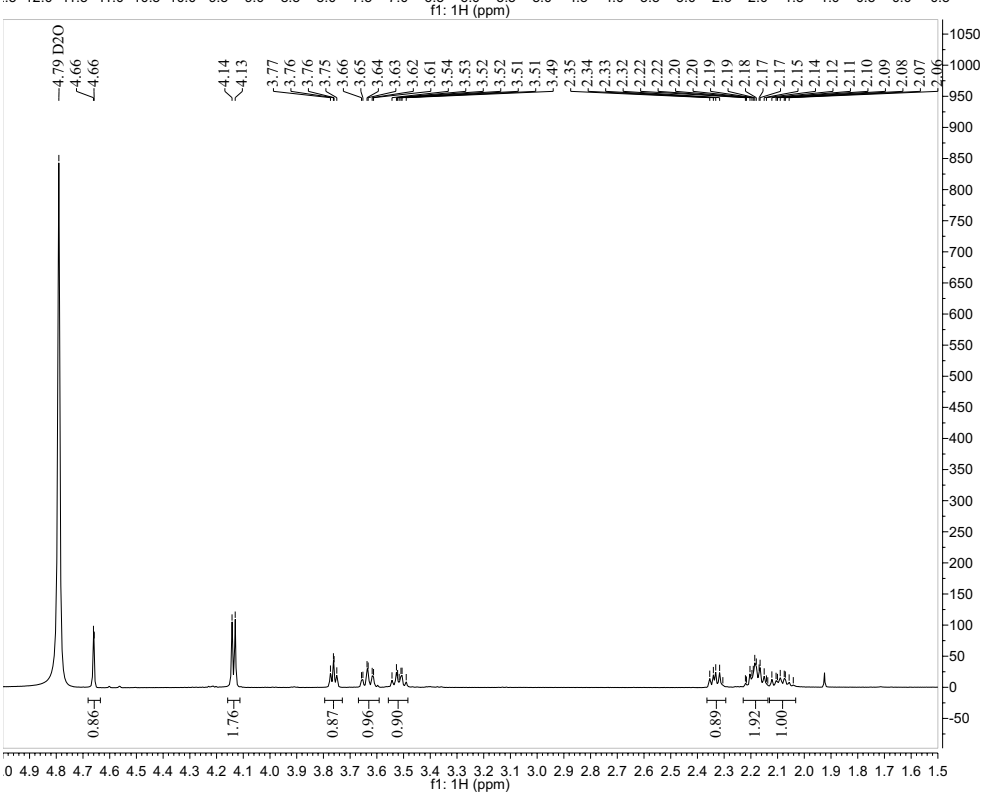


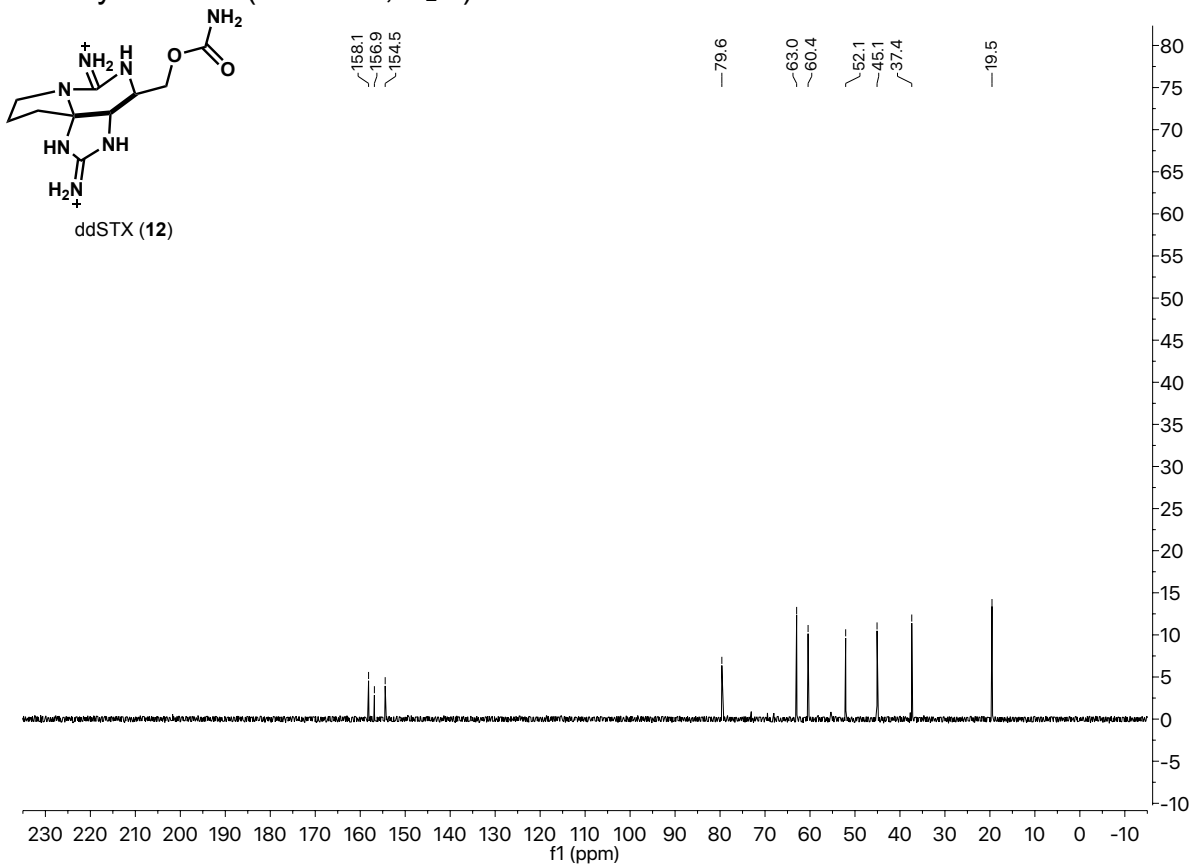
Figure S41. ^{1}H NMR spectra of 11-α-hydroxySTX (upper, 600 MHz, D_2O) and 11-α-hydroxySTX/11-β-hydroxySTX mixture (lower, 600 MHz, D_2O).

dideoxysaxitoxin (500 MHz, D_2O)

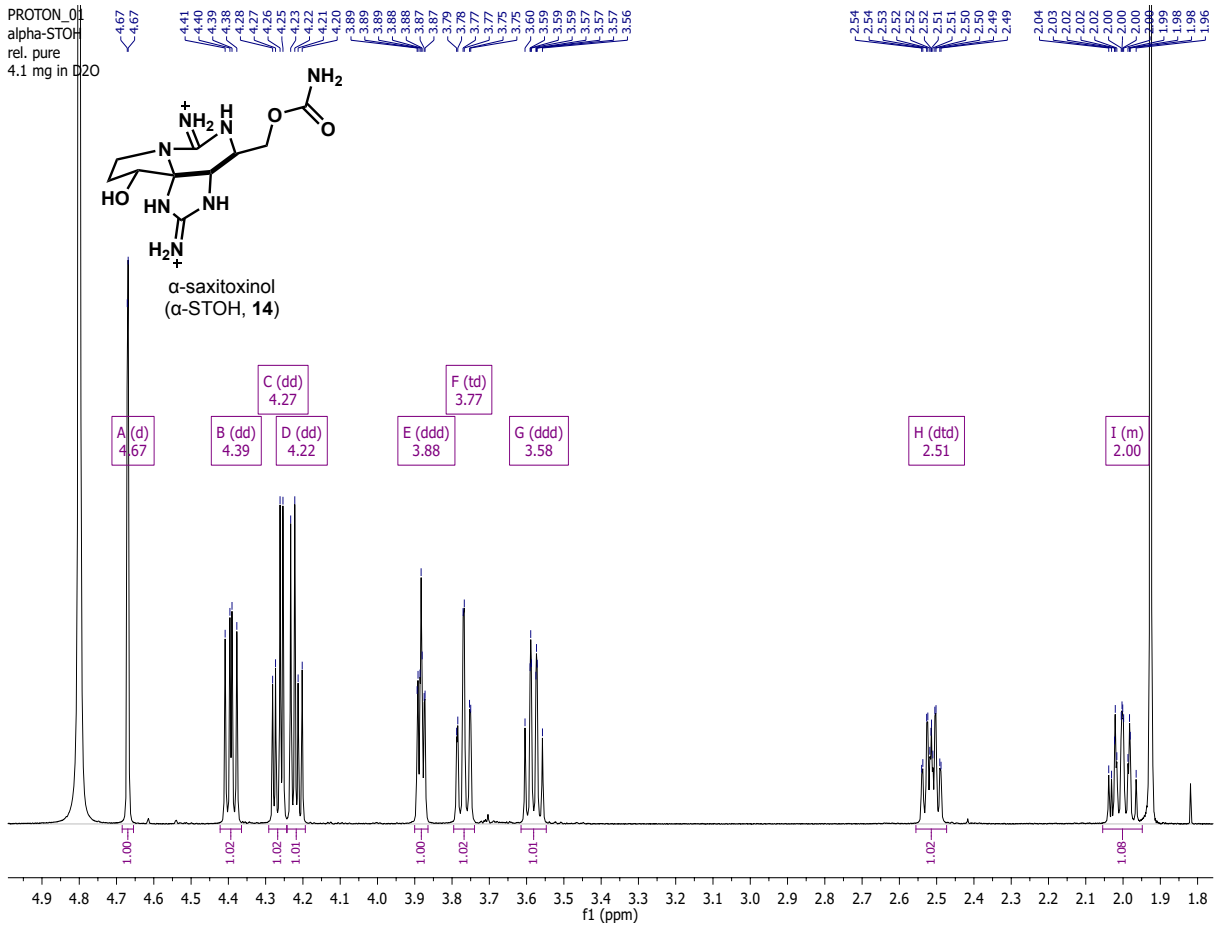




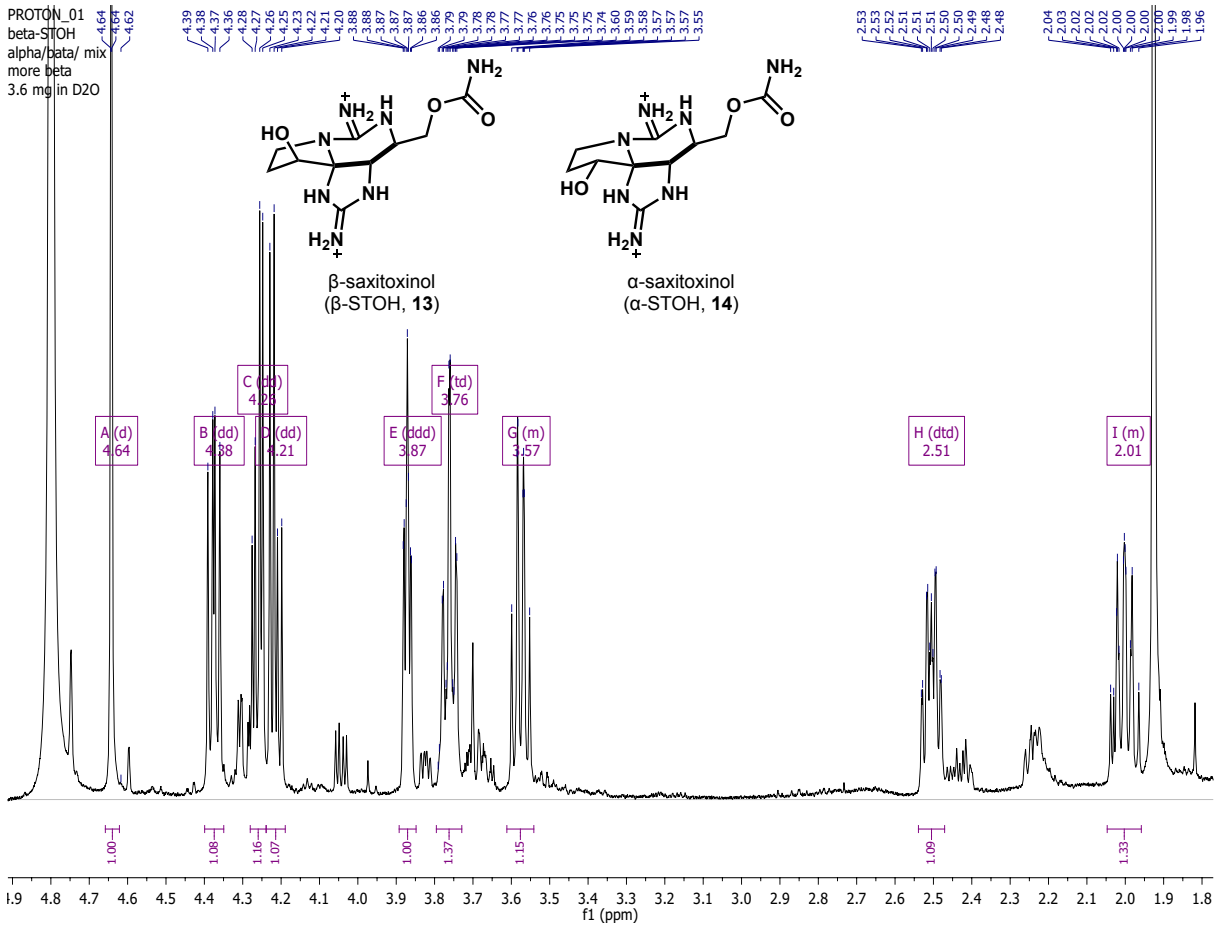
dideoxysaxitoxin (150 MHz, D_2O)



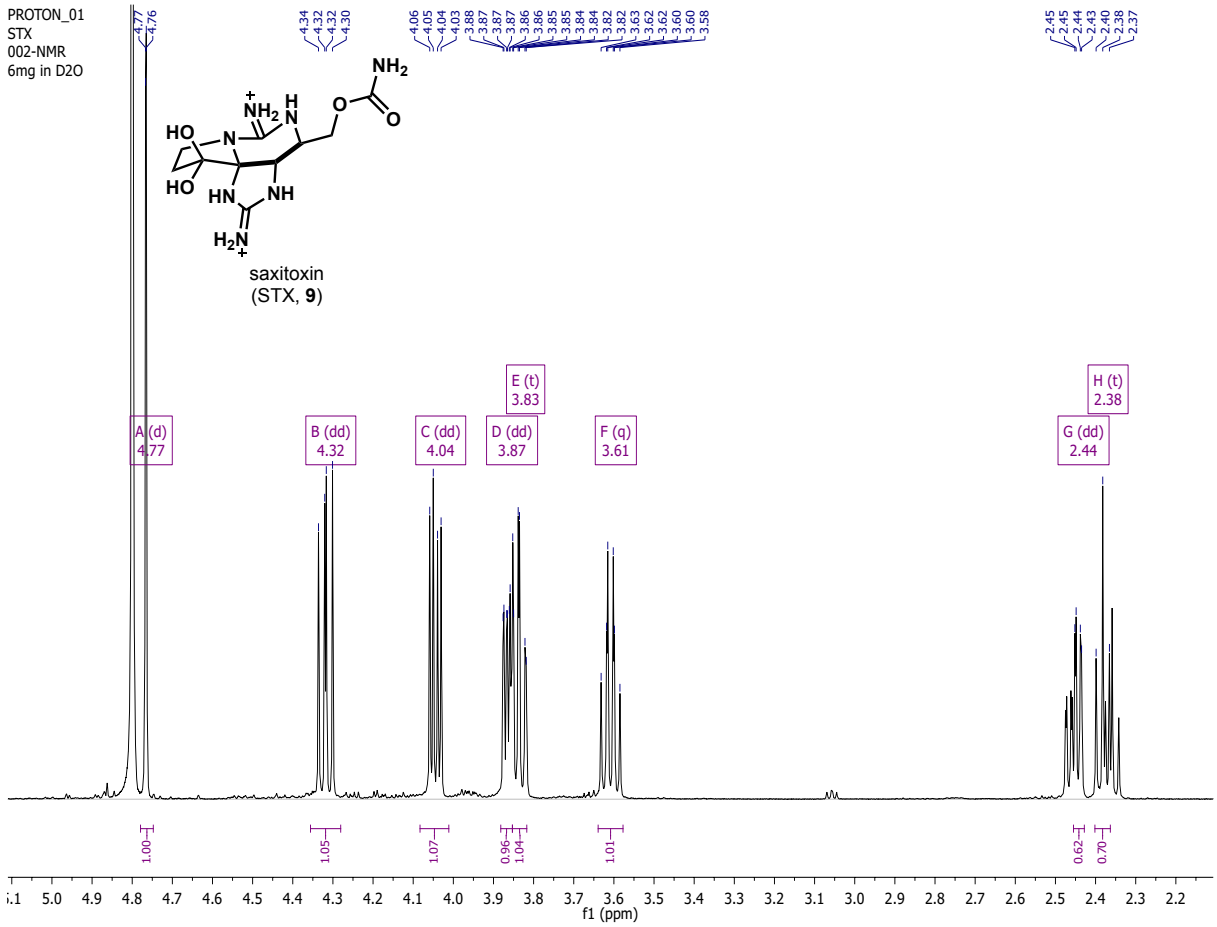
α-saxitoxinol (600 MHz, D_2O)



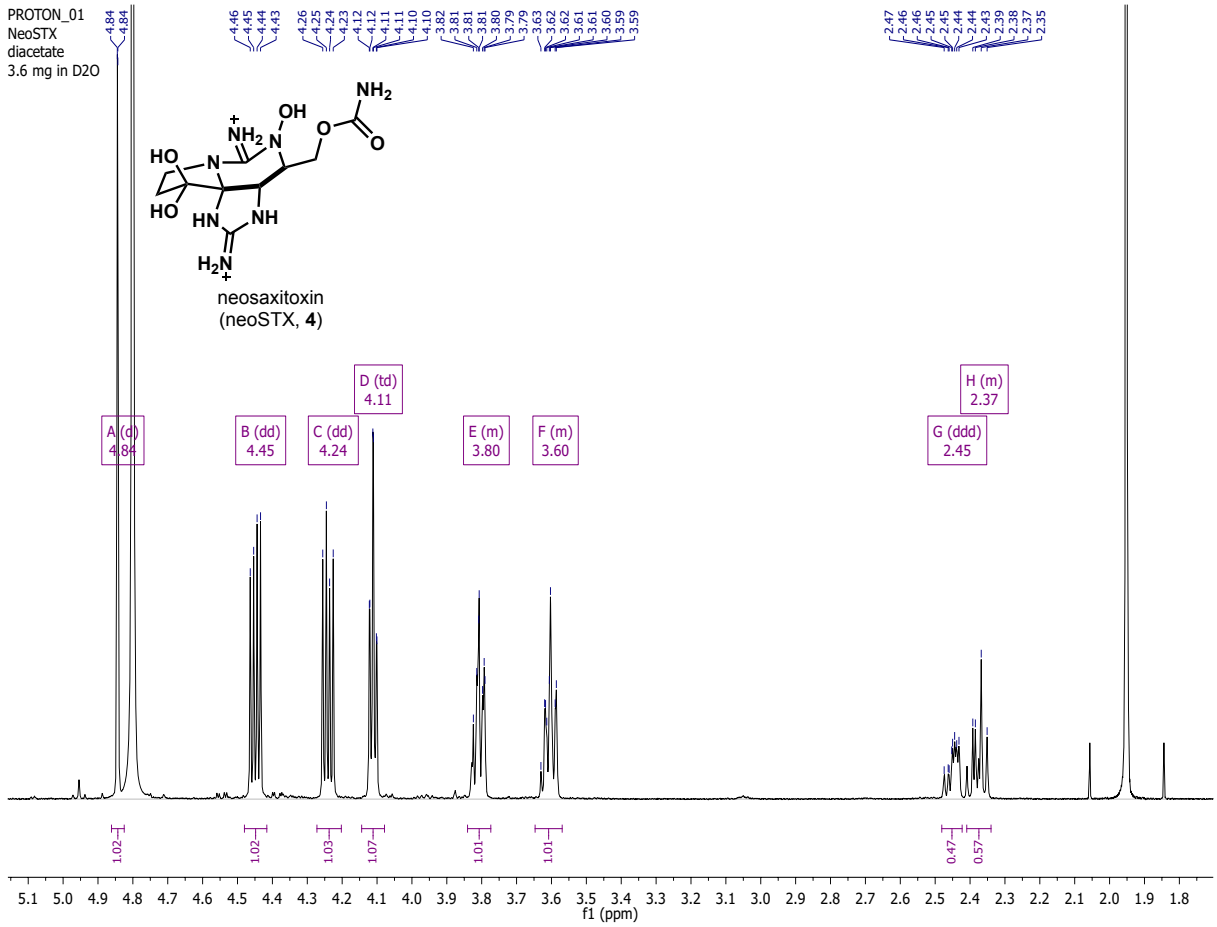
α-saxitoxinol and β-saxitoxinol mixture (600 MHz, D_2O)



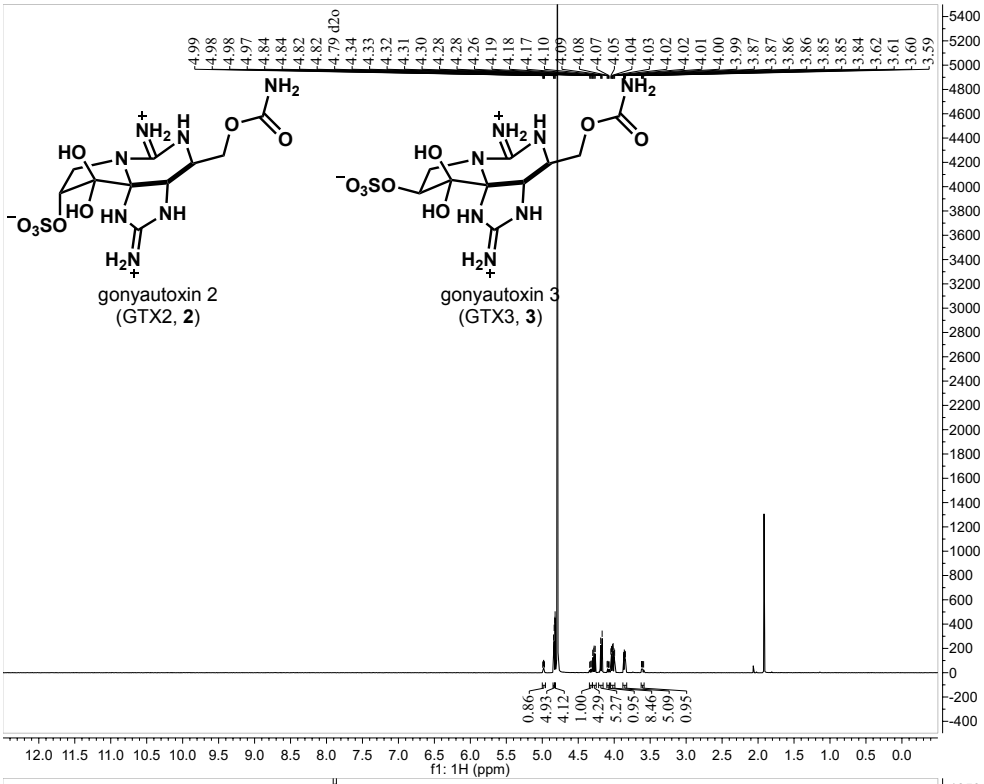
saxitoxin (600 MHz, D_2O)

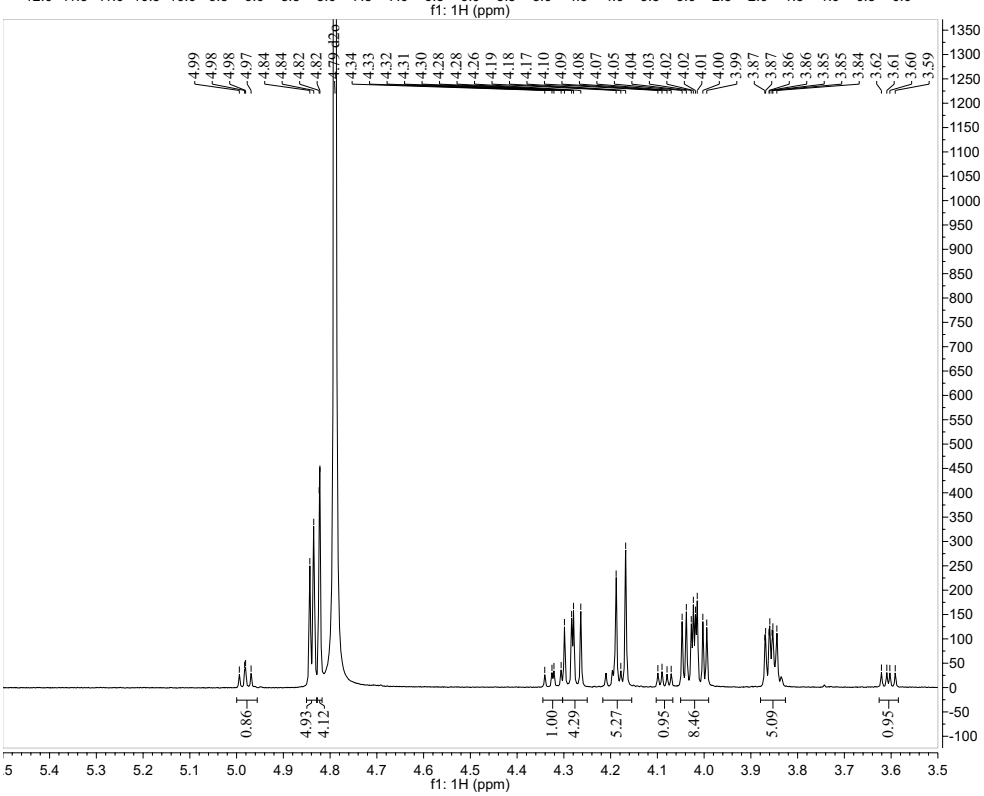


neosaxitoxin (600 MHz, D_2O)

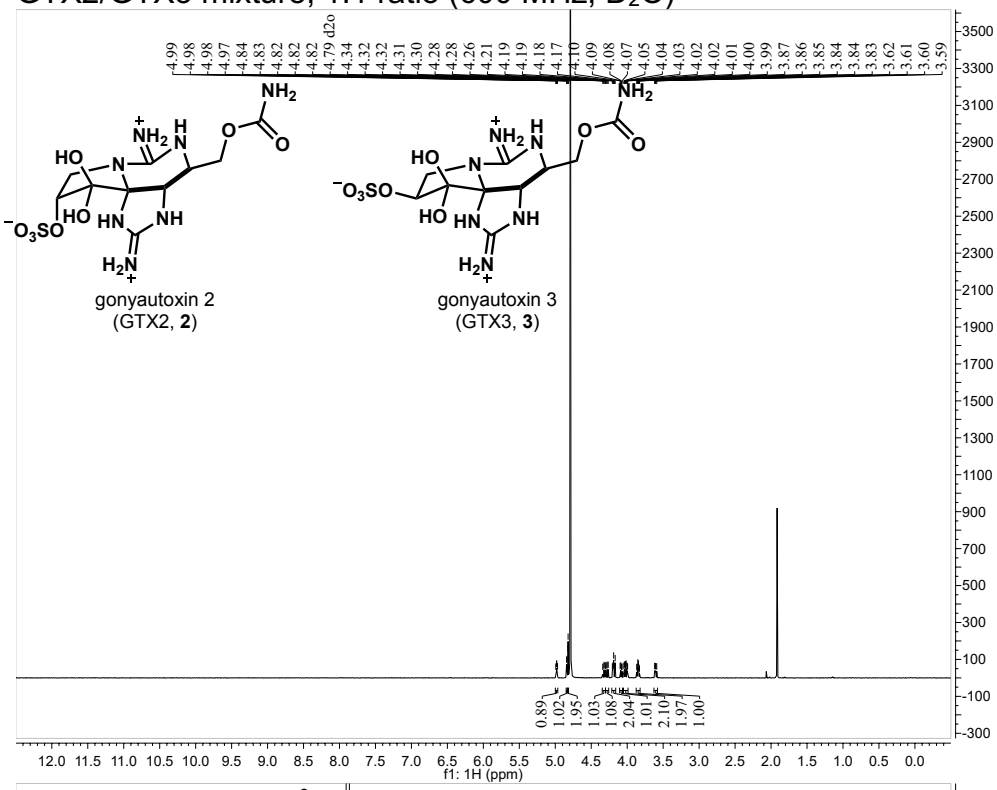


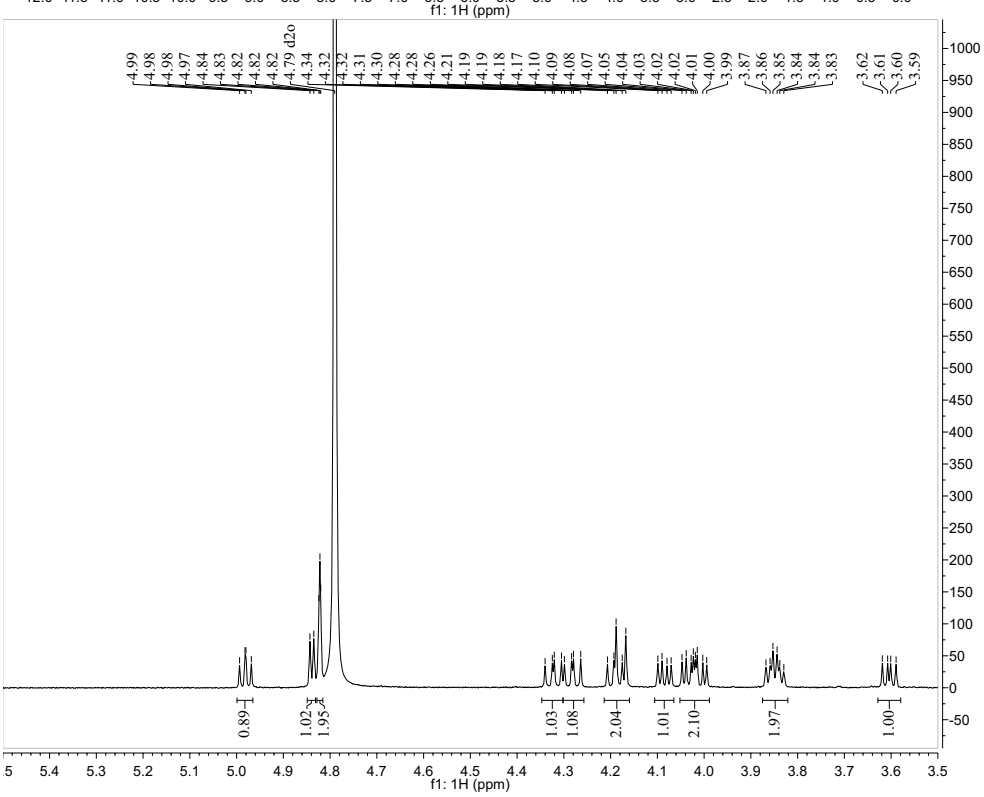
GTX2/GTX3 mixture, 4:1 ratio (600 MHz, D_2O)



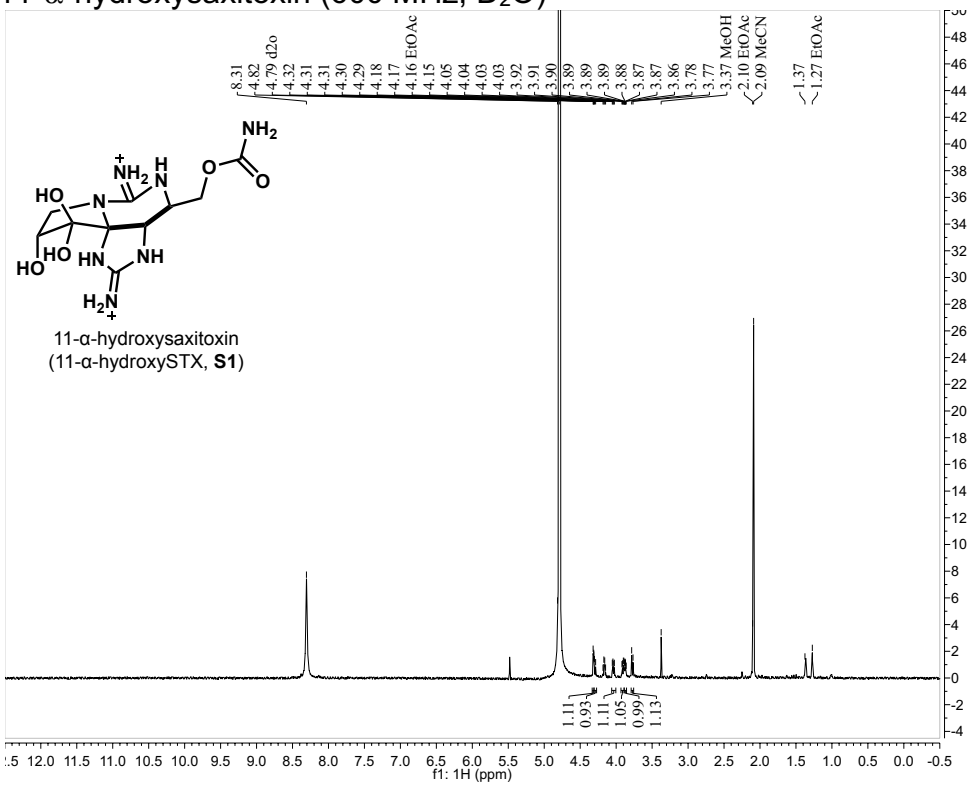


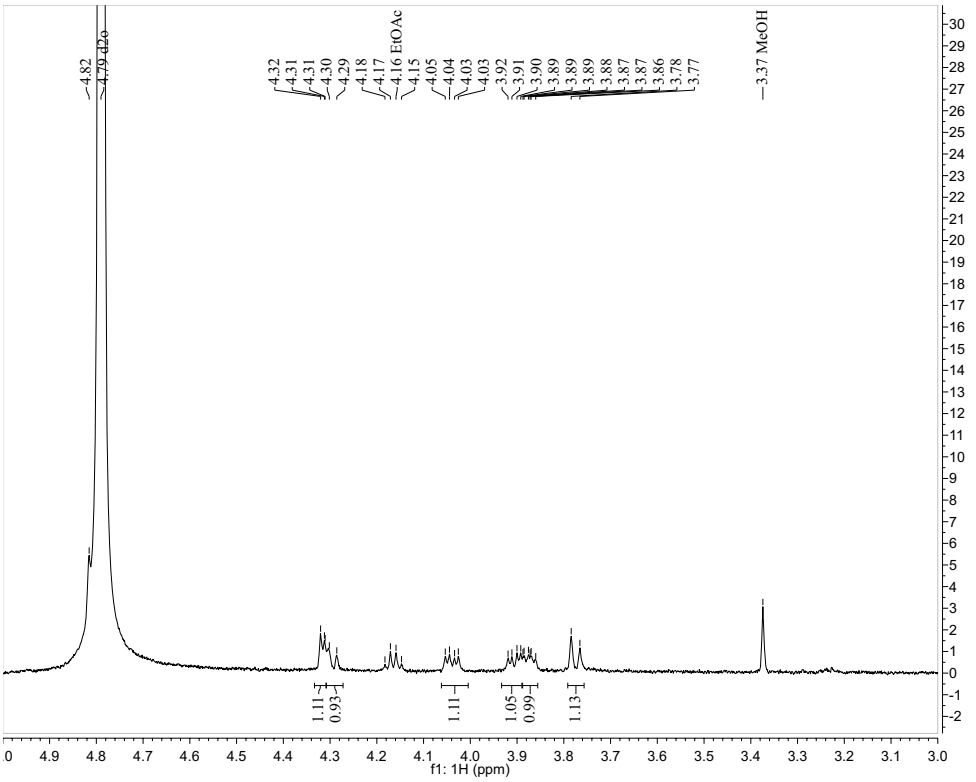
GTX2/GTX3 mixture, 1:1 ratio (600 MHz, D_2O)



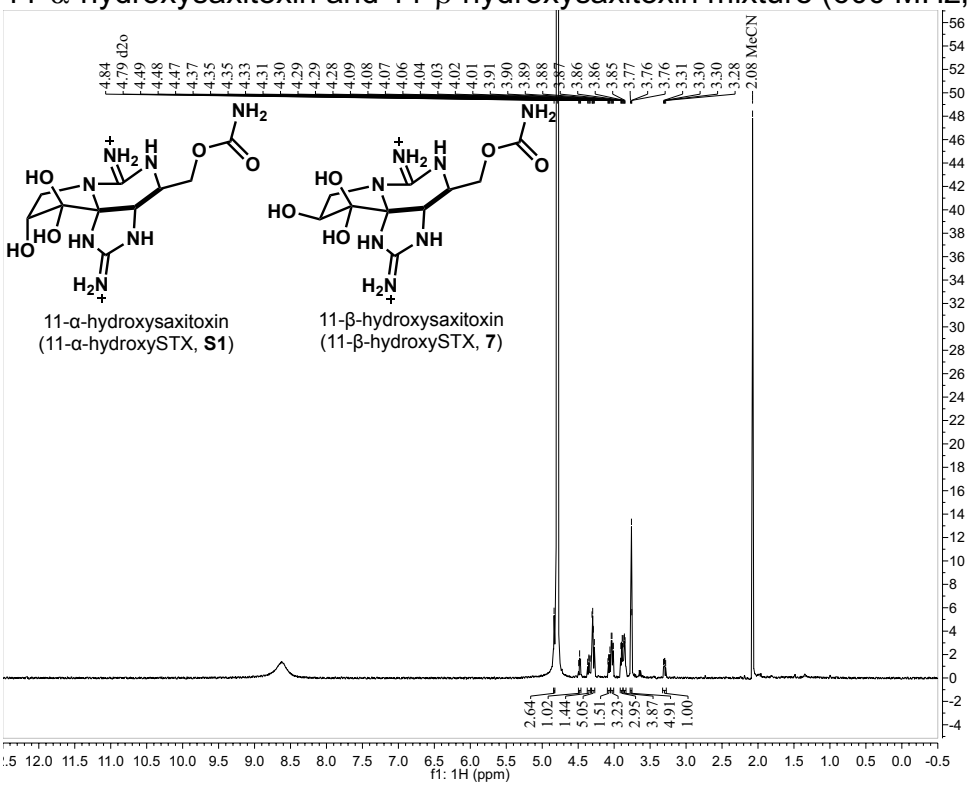


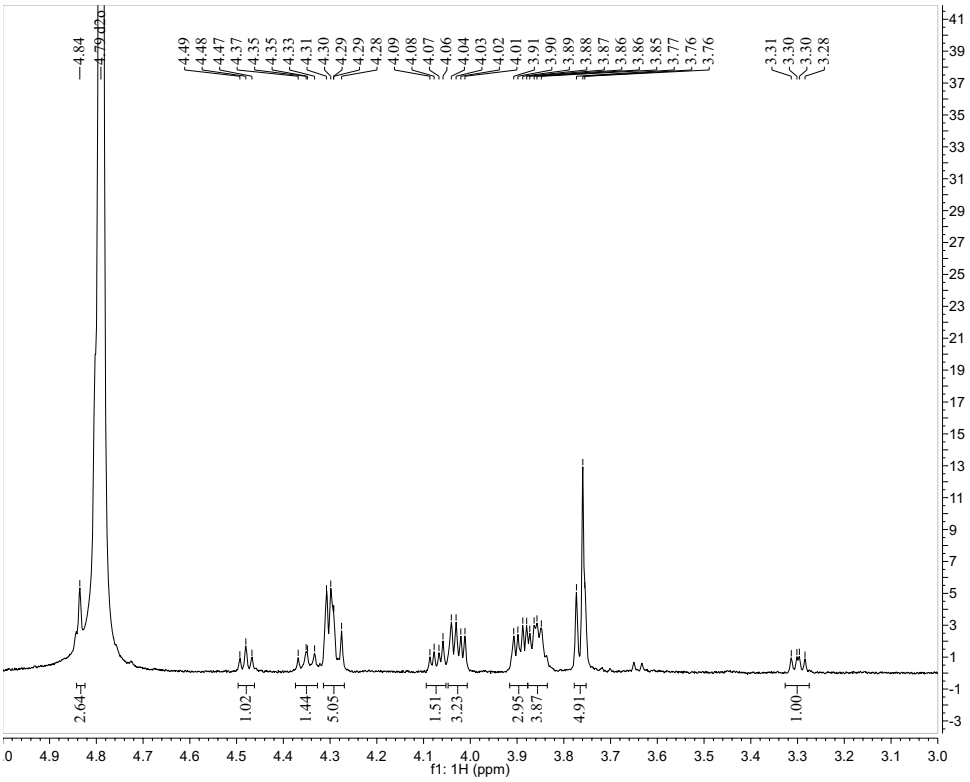
11-α-hydroxysaxitoxin (600 MHz, D_2O)





11-α-hydroxysaxitoxin and 11-β-hydroxysaxitoxin mixture (600 MHz, D_2O)





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