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De Novo Synthesis of Modified Saxitoxins for Sodium Ion Channel Study

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Voltage-gated Na^+ ion channels (Na_V) serve an obligatory role in the generation of bioelectricity and are essential for all of life's processes.[1] Genes that encode for 10 unique channel isoforms ($Na_V1.1-1.9$, Na_X) have been identified in mammalian cells.[2] Differences in the biophysical properties of these protein subtypes, their membrane concentrations and spatial distribution define the signaling characteristics of a neuron.[3] Aberrant Na_V function and/or expression is thought to be associated with numerous disease states, including arrhythmia, epilepsy, neuropathic pain, and congenital analgesia.[4] Accordingly, chemical tools for exploring protein structure, modulating the activity of specific Na_V isoforms, and tracking dynamic events associated with Na_V regulation and expression are sought to further understand the pathophysiologies associated with channel function.[5] Herein, we describe our initial efforts to develop such agents, for which the shellfish poison (+)-saxitoxin (STX) (**1**), a single-digit nanomolar inhibitor of certain Na_V subtypes, provides the molecular blueprint (Figure 1).[6] Our findings delineate a path for preparing novel carbamate-modified forms of the toxin and establish that such structural changes do not significantly influence substrate−receptor binding affinity.[7]

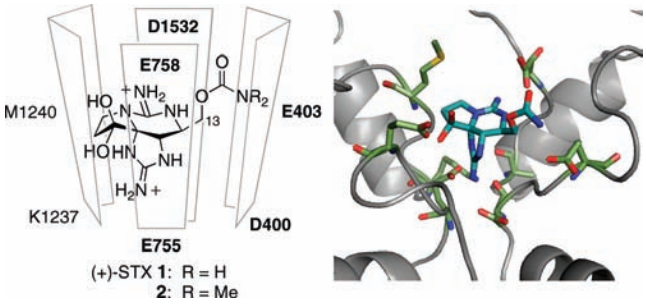

Figure 1. Model of (+)-STX bound in the Na_V channel pore.[9b]

The fully functional voltage-gated Na^+ channel consists of a large heteromeric α-subunit (~260 kDa) and one or two auxiliary β-subunits (33−36 kDa).[2] In the absence of protein crystallographic data, small molecule pharmacological probes together with protein mutagenesis and electrophysiology have provided much of the structural insights that currently exist for this family of macromolecules.[8] These data together with X-ray structures of associated K^+ ion channels, Kcsa and MthK, have made possible the construction of homology models of the Na_V α-subunit.[9] The outer mouth of the channel includes the ion selectivity filter and is considered to be the receptor site for STX and related guanidinium poisons (Figure 1).[2] Five carboxylate residues line this pore region [D400, E755, E403, E758, and D1532 ($Na_V1.4$ numbering)]; their presence is critical for high-affinity STX binding, as shown by site-directed mutagenesis studies.[8,9b,10] Computational models by Lipkind and Fozzard, Dudley, and Zhorov all posit that the 7,8,9-guanidine of STX points toward the ring of four amino acids that comprise the selectivity filter (also known as the DEKA loop).[8,9] Specific contacts between the C13-carbamate unit, the C12-hydrated ketone, the 1,2,3-guanidinium moiety, and the carboxylate residues of the outer vestibule loop (E403, E758, D1532) are also highlighted.

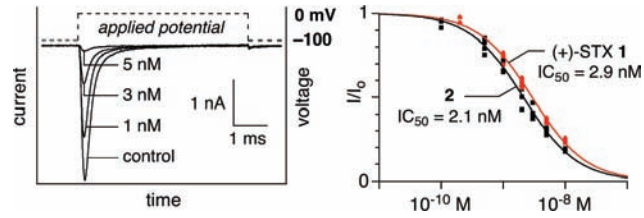

Figure 2. (left) Current recordings at varying concentrations of **2** on $rNa_V1.4$ expressed in CHO cells. (right) Dose−response curves for (+)-STX **1** (red) and **2** (black).

As a starting point for our investigations, we chose to examine the contribution of the C13-carbamate as a hydrogen-bond donor to the overall binding affinity of the toxin.[8,9b] Naturally occurring decarbamoyl STX (dc-STX) displays a modest reduction (<10-fold) in potency relative to STX.[6] Previous efforts to alter this functional group through semisynthetic modification of dc-STX have been limited to a single succinate derivative.[7a,c] The availability of a de novo synthesis of STX (see below) remedies this problem. Accordingly, *N*,*N*-dimethyl-STX (**2**) was prepared and evaluated for its ability to block Na^+ current. Electrophysiology measurements were performed in a whole-cell voltage-clamp format against the α-subunit of the rat skeletal channel $Na_V1.4$ ($rNa_V1.4$) heterologously expressed in CHO cells.[11] Figure 2 shows current recordings following a 10 ms depolarizing pulse to 0 mV from a holding potential of −100 mV. Increasing concentrations of **2** were perfused into the external solution, resulting in decreased peak current. These data were fit to a Langmuir isotherm to give an IC_{50} of 2.1 ± 0.1 nM for **2**, a value nearly equal to the IC_{50} recorded for our synthetic (+)-STX (2.9 ± 0.1 nM). This result seems to indicate that the role of the C13-carbamate in the natural product is not as a hydrogen-bond donor.[9b] Such a finding also provides the motivation for exploring further the steric environment of the protein pore in the vicinity of the carbamoyl residue.

A strategic modification to one of our previously published routes to (+)-STX was devised in order to prepare alternate C13-carbamate forms (Scheme 1).[12] Tricyclic oxazolidinone **5** represents the cornerstone of this new synthetic plan, our assumption being that nucleophilic amines would open selectively this strained heterocycle. This structure can be fashioned in just three steps from the nine-membered-ring guanidine **3**, a material that we now routinely synthesize on >5 g scales. To access **5**, sequential formation of the C13-Troc carbonate and ring closure proved necessary; use of other carbonylating agents (i.e., phosgene, carbonyldiimidazole) gave almost exclusively the C4−C13 alkene. Catalytic ketohydroxylation of **4** proceeded efficiently, albeit with modest selectivity, to generate hemiaminal **5**.[13] Oxazolidinone ring opening with a 1° amine at

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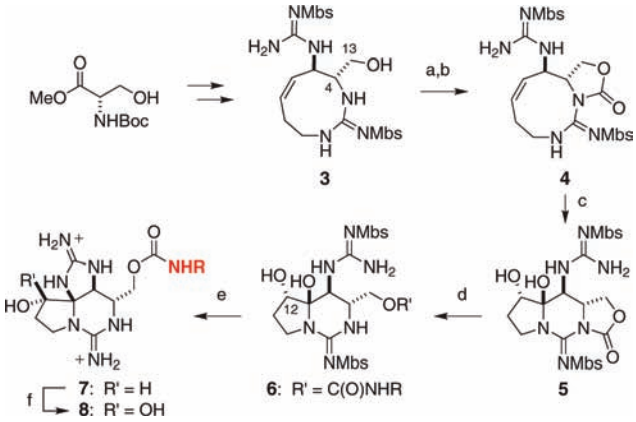
ambient temperature then furnished the corresponding carbamate **6**. Two subsequent transformations, Lewis acid-mediated guanidine ring closure and deprotection and C12 oxidation, completed the assembly of the tailored saxitoxins.

Scheme 1. Synthetic Route to C13-Modified Saxitoxins[a]

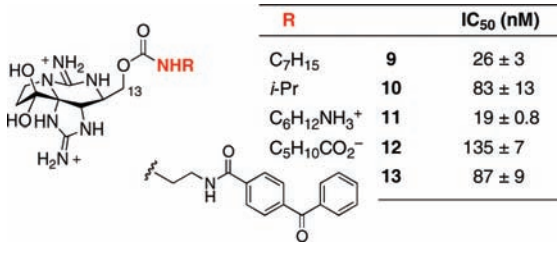


[a] (a) $Cl_3CCH_2C(O)Cl$, C_5H_5N, 0 °C, 93%; (b) *i*-Pr_2NEt, CH_3CN, 86%; (c) 10 mol % $OsCl_3$, Oxone, 44%; (d) RNH_2, THF, 77−99%; (e) $B(O_2CCF_3)_3$, 75–92%; (f) DCC, DMSO, $C_5H_5NH^+CF_3CO_2^-$, 25–63%. Mbs = *p*-methoxybenzenesulfonyl.

IC_{50} values were determined for STX C13-derivatives **9**−**13** against $rNa_V1.4$ (Figure 3). In spite of the steric, electronic, and polar modifications to the C13-carbamate, all of these compounds exhibited channel-blocking potency within 1−1.5 orders of magnitude of the parent toxin. The most severe loss in affinity was noted with acid **12**, possibly the result of a charge interaction between the carboxylate residue and one of the guanidinium moieties.[14] Other data, namely results from voltage-clamp recordings with **9** and **10**, suggest that the carbamate appendage sits in a narrow gorge between protein domains. Future experiments will continue to test this hypothesis. Finally, we note that, to the best of our knowledge, the benzophenone-labeled toxin **13** represents the first such STX photoaffinity probe.[15]

The power of de novo synthesis to provide novel forms of STX is further underscored with compounds such as **11** (Figure 4). The presence of two guanidinium groups notwithstanding, **11** undergoes selective coupling with an *N*-hydroxysuccinimide (NHS) benzoate ester to give the desired amide **14**. This final-step, "post-synthetic" coupling reaction makes possible the attachment of structurally complex payloads (e.g., fluorogenic groups, cofactors) to the STX core, side-chain elements that might otherwise be incompatible with the chemistries employed for guanidine deprotection and/or C12 oxidation (see Scheme 1).

Access to (+)-STX through asymmetric total synthesis has empowered the development of unnatural analogues of this unique



R		IC_{50} (nM)
C_7H_{15}	**9**	26 ± 3
i-Pr	**10**	83 ± 13
$C_6H_{12}NH_3^+$	**11**	19 ± 0.8
$C_5H_{10}CO_2^-$	**12**	135 ± 7
	13	87 ± 9

Figure 3. Recorded IC_{50} values for novel STXs against $rNa_V1.4$.

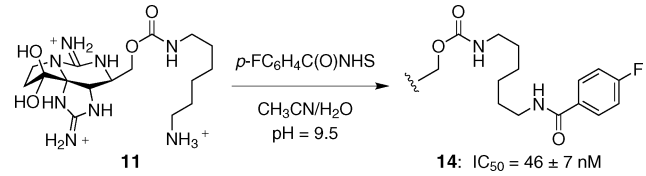


Figure 4. Final-step ligation strategy for STX modification.

natural product. New saxitoxins have been evaluated for their efficacy in blocking Na_V function using whole-cell voltage-clamp techniques. These findings have revealed opportunities for re-engineering the C13-carbamoyl unit of STX with any one of a number of different structural groups. Studies of this type together with the tools of molecular biology should allow us to map in greater detail the three-dimensional arrangement of the channel pore. We view these studies as a necessary step in a larger plan to utilize designed chemical tools to interrogate dynamic processes associated with Na_V function.

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Supporting Information Available: General experimental protocols and characterization data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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