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Asymmetric synthesis of batrachotoxin: Enantiomeric toxins show functional divergence against NaV

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The steroidal neurotoxin (−)-batrachotoxin functions as a potent agonist of voltage-gated sodium ion channels (NaVs). Here we report concise asymmetric syntheses of the natural (−) and non-natural (+) antipodes of batrachotoxin, as well both enantiomers of a C-20 benzene–modified derivative. Electrophysiological characterization of these molecules against NaV subtypes establishes the non-natural toxin enantiomer as a reversible antagonist of channel function, markedly different in activity from (−)-batrachotoxin. Protein mutagenesis experiments implicate a shared binding side for the enantiomers in the inner pore cavity of NaV. These findings motivate and enable subsequent studies aimed at revealing how small molecules that target the channel inner pore modulate NaV dynamics.

The phenotypic effects of acute poisons found among the rich pharmacopeia of terrestrial and marine life have been documented from antiquity. Isolation and characterization of toxic compounds have made available important chemical reagents for studying complex biochemical circuits (I). Studies of this type have revealed a large number of peptide and small-molecule agents that target voltage-gated sodium ion channels (NaVs), an obligate class of membrane proteins for bioelectrical signaling (1–4). Among the collection of known NaV modulators are three structurally related agents, (−)-batrachotoxin (−-BTX), veratridine, and aconitine (Fig. 1A)—sterically large, lipophilic amine derivatives believed to share a common binding locus in the inner pore region of NaV (5) (site 2, Fig. 1B). The influence of these toxins on ion gating, however, differs distinctly. On one extreme, (−)-BTX, the primary toxic constituent of Colombian poison dart frogs (genus Phyllobates), is a full NaV-agonist, causing the channel to open more readily at hyperpolarized membrane potentials and blocking fast inactivation (among other characteristic effects) (3–5). Conversely, the activities of veratridine and aconitine are best described as partial agonism and inhibition of channel function, respectively (5). Despite recent insights from structural biology into the three-dimensional architecture of prokaryotic NaV (6–9), a molecular understanding of the influence of the site 2 toxins on ion conduction and ion gating kinetics is lacking. Toxin structure-activity studies, in combination with protein mutagenesis experiments, can address questions related to the dynamical nature of channel function and may guide the rational design of small-molecule modulators of NaV activity (I).

The potency of (−)-BTX (10), its storied history as the archetypical small-molecule site 2 probe (4), and its unparalleled effects on channel gating render it an optimal “lead” compound for such investigations. (−)-BTX binding to NaV alters every aspect of channel function, resulting in a hyperpolarized shift in the voltage dependence of activation, inhibition of both fast and slow inactivation, a decrease in single-channel conductance, and reduction of ion selectivity (3, 4). The utility of this natural product as a NaV activator has led to a substantial depletion in the world supply, which once exceeded 1 g but was less than 170 mg as of 2009 (11, 12). Since the toxin was first isolated in 1963 by Márki and Witkop from poisonous frogs collected in the northern rain forest of Colombia (13), Phyllobates has been placed on the endangered species list, and thus collection of natural (−)-BTX from this source is restricted. (−)-BTX has also been identified in select species of birds (genus Pitohui and Icteria) (14) and beetles (genus Chlosyne) (15), but only in small quantities (e.g., ~1.8 µg of (−)-BTX per beetle). Although semi-(6) and racemic syntheses (17) of BTX-A (Fig. 1C), a compound lacking the C-20 pyrrole ring, have been described, the closed channel at each of these works (~45 linear steps) precludes the facile production of (−)-BTX or select analogs. Accordingly, our desire to use BTX and modified forms thereof for examining channel dynamics and ion gating mechanisms has motivated our efforts to obtain the natural product through de novo synthesis.

Retrosynthetic analysis of (−)-BTX led us to outline a plan that would enable late-stage assembly of the homomorpholine E ring and elaboration of the C-20 allylic ester (Fig. 1C), thereby facilitating access to modified forms of the toxin. Previous structure-activity relationship studies using a small number of semisynthetic BTX derivatives (10, 18) and C/D/E-ring BTX analogs (19) revealed the importance of the C-20 ester, tertiary amine, and tetracyclic skeleton for NaV.
agonist activity. Unraveling BTX-A exposes a steroid-like frame 1, the assembly of which is con-
found by two angular groups at C/D-ring junction, the C-11 exo-methylene and the C-8/C-9 alkene. To maximize convergence in our synthetic plan, we conceived a disconnection strategy for 1 across the C ring. This idea would reduce the problem of constructing 1 into two fragments, expressing A/B-ring system (3, 20) and a second comprising the D-ring cyclopentane (4, 21).

Our synthesis of (-)-BTX commenced with the coupling of methyleneacyclopenatonane 4 (21) (fig. S1A) and vinyl bromide 3, obtained from (S)-(-)-Hajos-Parrish ketone through a modified sequence of steps originally outlined by Parsons and co-workers (20) (fig. S1B). Conjoining fragments 3 and 4 to generate the linked A/B/D-tricycle 5 presented the first in a series of process develop-
ment challenges. An initial attempt to effect this transformation involved LiBr exchange of 3 with n-BuLi (Bu, butyl) and sequential addition of enone 4. Although 5 was delivered under these conditions, product yields never exceeded 30%. Deuterium quenching experiments with D$_2$O validated our hypothesis that α-deprotonation of 4 was competitive with the desired ketone addition pathway. Transmetalation reactions of the vinyl-lithium species with ZnCl$_2$, MgBr$_2$, and CeCl$_3$ (Et, ethyl), CeCl$_3$, Yb(OTf)$_3$ (Tf, trifluoromethanesulfonate), CeCl$_3$-2LiCl, and LaCl$_3$ were examined, but none of these measures proved effective (22, 23). The addition of one equivalent of anhydrous LiBr to the reaction media of 3 improved the coupling efficiency by >20% (24). Following this lead, an optimized protocol using 2 equivalents of n-BuLi, which presumably generates one equivalent of LiBr in situ, reproducibly afforded 5 as a single diastereomer in 65% yield on a multi-
gram scale. The ease of synthesis of this material and its desilylated form 6 enabled subsequent efforts to identify conditions for tandem annula-
tion of the C ring and installation of the quater-
nary C-13 center.

An evaluation of available methods for ring closure of 1,6-enynes led us to consider radical-initiated processes (25). Under such conditions, an incipient C-13 3° radical could be intercepted to forge the angular aminomethylene unit (or a suitable surrogate). Efforts to first examine C-ring formation on 6, however, revealed the potential fallacy of this plan. Using n-Bu$_3$SnH and triethylborane (Et$_3$B) to promote the cyclization event resulted in the generation of two isomers, 7 and 8, in a 1.5 ratio favoring the undesired product (Fig. 2A). Studies by Stork and Beckwith and co-workers have demonstrated that substrate concentration and reaction temperature can influence the mode of cyclization (i.e., 5-exo-trig versus 6-endo-trig) in radical-mediated enyne reactions (26, 27). At elevated temperature (130°C) and with fivefold dilution of 6, a reversal in selectivity was observed, affording a slight excess of the desired tetracycle (1:3.1 ratio of 7 to 8; Fig. 2A). The combined yield of this transformation exceeded 90%, thus encouraging further exploration of this chemistry, despite the modest selectivity results.

Repeated attempts to capture the intermediate C-13 radical with oxime and hydrazine derivatives generated from formaldehyde failed to deliver the expected aminomethylation product (28). Forced to consider alternative solutions, we recognized that a modified silyl ether group appended from the neighboring C-H alcohol would be aptly positioned to intercept the 3° radical (29). Based on available precedent, an alkynylsil(1) chloride, Me$_2$SiCH=CSiEt$_2$Cl (Me, methyl), was selected for modification of the C-14 alcohol in 6 (30, Fig. 2A). Treatment of the resulting silyl ether 9 with n-Bu$_3$SnH and Et$_3$B at 150°C resulted in a cyclization cascade to give pentaerythritol 10 as the exclusive product (31). Within the limits of proton nuclear magnetic resonance (1H NMR) detection, none of the corresponding five-membered C-ring isomer was generated in this process. Our preliminary efforts to understand the role of C-14 substituent groups on reaction selectivity suggest that silyl protection of the alcohol (along with the elevated reaction temperatures) favors 6-endo-trig ring closure. Although additional studies are warranted to appreciate these structure-selectivity data, our enyne cyclization cascade offers a convergent approach for synthesizing substituted steroid scaffolds and should facilitate access to a wide range of such compounds.

Close inspection of the radical cyclization products derived from either 6 or 9 revealed an unexpected outcome pertaining to the structure of the resulting organostannane moiety (Fig. 2, A and B). Carbostannylation of the alkene group would afford a vinyl-tin product, as noted in the reaction of 6. Unexpectedly, when 9 was sub-
jected to the reaction conditions, allylstannane 10 was the sole product, a result confirmed by both NMR and x-ray crystallography. Formation of allylstannane 10 can be rationalized through a mechanism involving 1,4-H-atom transfer of an intermediate vinyl radical (32) (Fig. 2B), a proposal supported by a deuterium labeling experiment (fig. S5). Although this result was unplanned, the efficiency and selectivity of the cyclization reaction compelled our decision to advance this material. Looking forward, the versatility of the allylstannane group should serve future efforts to prepare C ring–modified BTXs.

The availability of 10 in nine steps from the Hajos-Parrish ketone enabled the production of

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**Fig. 1. Background and synthetic plan.** (A) The structures of lipophilic site 2 toxins (−)-batrachotoxin (BTX), aconitine, and veratrione. (B) A pore model of Na$_V$ with (−)-BTX (depicted as spheres) docked at site 2. The structure is based on Magnetococcus marinus Na$_V$ crystallographic data (Protein Data Bank accession code 4F4L) (9, 19). Domain I, orange; domain II, red; domain III, gray; domain IV, teal. (C) Retrosynthetic analysis of BTX and BTX C-20 ester analogs. LD$_{50}$, half-
maximal lethal dose; Me, methyl; Bu, tert-butyl; Et, ethyl; TBS, tert-butyl-dimethyl silyl.
substantial quantities of material to complete the target synthesis. Exclusion of the bridging silyl ether in 10 was accomplished with excess n-Bu4NF, revealing a diol intermediate that was subsequently advanced to 11 through 2-iodoxybenzoic acid-mediated alcohol oxidation and chemoselective vinylsilane cleavage (57%; Fig. 2B). Conversion of aldehyde 11 to chloroacetamide 12 was performed by following a three-step, single-flask sequence mediated alcohol oxidation and chemoselective advanced to revealing a diol intermediate that was subsequently converted to C-11 ketone 15 by following a series of functional group interconversion steps highlighted by a Curtius rearrangement (37). The absence of a viable chromophore on batrachotoxinin A (BTX-A) makes purification of this material difficult; accordingly, in the sequence leading to 15, the C-3 methoxy acetal was exchanged with p-methoxyphenethyl alcohol.

Completion of the carbon skeleton of (-)-BTX was accomplished through a palladium-catalyzed cross-coupling of tributyl(1-ethoxyvinyl)tin to vinyl triflate 15 (Fig. 3A) (38). In situ hydrolysis of the incipient enol ether with 1 M oxalic acid supplied enone 16 (77%). Following an extensive screen of reducing agents, successful stereoselective global reduction of enone 16 was accomplished in 33% yield by treatment with freshly prepared AlH3 (39). We hypothesize that the Lewis-basic lactam (or a reduced form) acts as a pivotal stereocontrolling element, as treatment of enone 15 with alternative hydride reducing agents [e.g., AlH3•NMMe3•Et, NaBH4, NH2•BH3, (S)-Me-CBS-oxazaborolidine/BH3, or t-selectride] delivered the undesired C-11β alcohol exclusively. The use of AlH3 also favored generation of the correct C-20 allylic alcohol epimer, a stereochemical outcome that can be rationalized through a model invoking chelation control (38). Deprotection of the product from AlH3 reduction under acidic conditions afforded (-)-BTX-A in 83% yield (17). Finally, by employing a modification of Tokuyama, Daly, and Witkop’s (-)-BTX-A acylation protocol with the mixed anhydride prepared from ethyl chloroformate and 2,4-dimethyl-pyrole-3-carboxylic acid (10), the synthesis of 2 mg of (-)-BTX was completed (79%, 0.25% overall yield, 24 steps from 14-ilepoxybenzyl ketone). The product was identical in all respects [as assessed by high-resolution mass spectrometry, thin-layer chromatography, and high-performance liquid chromatography (HPLC) cojection] with a sample of the natural material and with previously recorded spectroscopic data (40, 41). Our synthetic plan also enabled milligram-scale preparation of the unnatural toxin antipode, (+)-BTX, the known benzoate ester of (-)-BTX-A (BTX-B; Fig. 3B) (42, 43), and the enantiomer of this compound (ent-BTX-B).

Electrophysiological characterization of synthetic (-)-BTX and BTX-B against rat NaV1.4 (rNaV1.4) confirmed that the latter also functions as an agonist and is similar in potency to the natural product (Fig. S6 and Table S12). Previous reports and our own studies indicate that the ester group of BTX-B

**Fig. 2.** Enyne radical cyclization to furnish the steroidal core of BTX. Reagents, conditions, and product yields for steps a to p are as follows: (A) a, t-BuLi, THF, −90°C, then 4 (see Fig. 1) (65%); b, K2CO3, MeOH (94%); c, Et3B, air, n-Bu3SnH. (B) d, Me3SiC=CSiEt3Cl, imidazole, CH2Cl2 (93%); e, O2, n-Bu3SnH, Et3B, Ph2O, 150°C (75%); f, n-Bu3NF, THF, 60°C (94%); g, 2-iodoxybenzoic acid, t-BuOH, 65°C, then OsO4 (7 mol %), NaIO4, pyridine, H2O (57%); h, MeNH2, CH2Cl2, Na(B(0)COCF3)2•H, CH2Cl2, −78°C, then Cl(0)COCl, 2,6-lutidine, −78 to 0°C (52%); i, NaOEt, EtOH, 11 THF/C6H6 (92%); j, KN(SiMe3)2, PhNTf2, THF, −78 to 0°C (94%); k, CuCl2, O2, 1,4-dioxane, 73°C (85%); l, NaClO2, NaH2PO4, dimethyl sulfoxide/H2O; m, SOCl2, pyridine, CH2Cl2, n, NaOAc, acetone/H2O; o, aqueous AcOH, 1,4-dioxane, 90°C (57% over four steps; p, t-SClOH, 4-Å molecular sieves, p-methoxyphenethyl alcohol (PMBCO2H), C6H6 (89%), THF, tetrahydrofuran; Ph, phenyl; Tf, trifluoromethanesulfonate; Ts, p-toluenesulfonate; Ac, acetate.
is more stable than the oxidatively sensitive acyl-pyrrole of BTX; thus, additional experiments were performed with the former compound (42, 43).

Synthetic BTX-B was tested against a subset of representative NaV isoforms including rNaV1.4, human NaV1.5, and human NaV1.7. Application of 10 μM BTX-B to Chinese hamster ovary cells expressing a single NaV subtype resulted in sustained sodium current in all cases (Fig. 4A and figs. S7 and S8). Use-dependent agonism of NaV isoforms by BTX-B prevented steady-state inactivation of >80% of the sodium channel population (Fig. 4A and fig. S8). BTX-B also induced a characteristic hyperpolarizing shift (−44.9 mV to −51.5 mV) in the half-maximal voltage (V0.5) of activation of wild-type NaV isoforms (Fig. 4B and table S13). The similarity of these data is consistent with the high protein sequence conservation between NaV subtypes in the inner pore–lining S6 helices that form the putative toxin binding site (fig. S9).

Following earlier work from our laboratory (19) and others (44, 45), we questioned whether the enantiomer form of BTX would bind with high affinity to NaV with analogous functional effects. Such a question can only be answered with the availability of a de novo synthesis of the toxin. Accordingly, electrophysiological recordings with ent-BTX-B were performed against rNaV1.4. These data revealed ent-BTX-B to be a use- and state-dependent channel antagonist, with a measured half-maximal inhibitory concentration of 5.3 ± 0.6 μM [Fig. 4C and fig. S10; (+)-BTX also displays antagonistic activity (fig. S11)]. The concentration for half-maximal inhibition of NaV by ent-BTX-B is similar in magnitude to the half-maximal effective concentration for BTX-B agonism (1.0 ± 0.1 μM; fig. S10) measured under identical conditions. Notably, unlike the natural antipode, ent-BTX-B binding caused only a minimal shift in the V0.5 of activation and the V0.5 of steady-state inactivation (table S14).
addition, channel block was fully reversible by this inhibitor.


REFERENCES AND NOTES


31. K. Nozaki, K. Oshima, K. Utimoto, J. Am. Chem. Soc. 109, 2547–2549 (1987). ACKNOWLEDGMENTS We are grateful to M. Modaque (Stanford University) for generous use of her laboratory space and equipment. We thank S. Lynch (Stanford University) for assistance with NMR experiments and analysis, Y. Kishi (Harvard University) for graciously providing NMR spectra of synthetic BTX-A. J. K. Maclaren (Stanford Nano Shared Facilities) for solving the crystal structure of 1 (supported by the NSF under award ECCS-1542512). G. Dick (Stanford University) for assistance with HPLC conjuction of natural and synthetic BTX, and the Vincent Coates Foundation Mass Spectrometry Laboratory. Stanford University Mass Spectrometry (https://mass-spec.stanford.edu). Metrical parameters for the structure of compound 1 are available free of charge from the Cambridge Crystallographic Data Centre under reference number CSDC-1509206. This work was supported in part by the NIH (RO1NS045684) and by gifts from Pfizer and Amgen. T.T. was sponsored as a Japan Society for the Promotion of Science Fellow for research abroad. R.T.-T. is a NSF predoctoral fellow. M.M.L. and T.T. contributed to the synthesis of BTX, and R.T.-T. was responsible for electrophysiology experiments. The manuscript was prepared by M.M.L., R.T.-T., and J.D.B. J.D.B. is a co-founder of and owns equity shares in SiteOne Therapeutics, a pharmaceutical startup company aimed at developing sodium channel subtype-selective inhibitors as antiincoptive agents.

SUPPLEMENTARY MATERIALS www.sciencemag.org/content/354/6314/865/suppl/DC1 Materials and Methods Figs. S1 to S12 Tables S1 to S14 References (SI–58) 13 June 2016; accepted 14 October 2016 10.1126/science.aag2981

GEOPHYSICS Coseismic rupturing stopped by Aso volcano during the 2016 M_w 7.1 Kumamoto earthquake, Japan A. Lin, T. Satsukawa, M. Wang, Z. Mohammadi Asl, R. Fueta, F. Nakajima

Field investigations and seismic data show that the 16 April 2016 moment magnitude (M_w) 7.1 Kumamoto earthquake produced a ~40-kilometer-long surface rupture zone along the northeast-southwest-striking Hinagui-Futagawa strike-slip fault zone and newly identified faults on the western side of Aso caldera, Kyushu Island, Japan. The coseismic surface ruptures cut Aso caldera, including two volcanic cones inside it, but terminate therein. The data show that northeastward propagation of coseismic rupturing terminated in Aso caldera because of the presence of magma beneath the Aso volcanic cluster. The coseismic faults of the 2016 Kumamoto earthquake may require reassessment of the volcanic hazard in the vicinity of Aso volcano.

Large earthquakes and active volcanoes are closely related natural phenomena resulting from plate tectonic processes (1–3). Large earthquakes often accompany or precede volcanic eruptions (4–5). Seismic analyses and geological observations reveal that the distribution and segmentation of active faults are mainly controlled by the presence of magma bodies in volcanic regions (6) and that fault segment boundaries play important roles in a number of aspects of earthquake behavior, including rupture initiation and termination (7). Fault segment boundaries are associated with a buildup of heterogeneous fault stress (8) and large changes in earthquake-induced surface offset (9). In volcanic regions, a magma chamber may affect seismicity and the rupture process of an earthquake through the presence of a high-temperature area (10), a heterogeneous fault plane on a crater wall (11), and/or rectified diffusion (12). However, because of a lack of geological data, it is unknown whether a volcano can affect coseismic fault rupturing processes and mechanisms. The 16 April 2016 Kumamoto earthquake of magnitude M_w 7.1

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**Pluses and minuses of BTX behavior**

Batrachotoxin is a potent neurotoxin produced by the endangered Colombian poison dart frog and is an agonist of voltage-gated sodium ion channels (NaVs). Logan *et al.* developed a chemical synthesis of this molecule, denoted (−)-BTX, by taking advantage of a tin hydride–mediated radical cyclization to stitch together the polycyclic framework. Using an analogous route, they also prepared the non-natural mirror image, (+)-BTX. Conversely to the natural product, (+)-BTX antagonized NaVs.

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