A novel potassium channel antagonist has been purified from the defensive mucus secreted by Calliostoma canaliculatum, a marine snail found in the temperate coastal waters of the western Pacific. The toxin is expelled from the hypobranchial gland as part of a defensive response and is contained within a viscous matrix that minimizes dilution and degradation. The active compound was isolated by multistage microbore HPLC separations followed by bioactivity assays. Nuclear magnetic resonance, combined with electrospray ionization Fourier-transform ion cyclotron resonance and electrospray ionization ion trap mass spectrometry indicate that the active component is a heretofore unknown indole-derivative, a disulfide-linked dimer of 6-bromo-2-mercaptotryptamine (BrMT). Exudates from the hypobranchial glands of various marine mollusks have been sources for dye compounds such as 6–6 dibromoindigo, the ancient dye Tyrian purple. BrMT represents the first correlation of a hypobranchial gland exudate with a molecular response. Voltage clamp experiments with a number of K channel subtypes indicate that BrMT inhibits certain voltage-gated K channels of the Kv1 subfamily.

Natural toxins are valuable tools for investigating ion channels. They expedite identification, purification and cloning of native channel types, permit in vivo manipulation of specific channels in complex tissues, such as the brain or heart, and can provide the basis for useful pharmaceuticals. In addition, they can serve as biophysical probes of channel function and permit structural deductions.

In the case of voltage-gated potassium (Kv) channels, many highly selective peptide toxins from a variety of venomous organisms have been described (1), and different toxins display distinct mechanisms of action. For example, scorpion Kc-toxins, which have been extensively used to map the extracellular region around the mouth of the conducting pore for Kv1 (Shaker-type) channels, block channels by occluding the conducting pore (2–5). In contrast, hanatoxin, a peptide isolated from a spider (6), alters the gating process that opens and closes Kc,2.1 and Kc,4.2 channels (7) and does not bind directly over the pore (8). Thus, peptide toxins inhibit K channels by either blocking conduction through the pore or by stabilizing the gating machinery in one or more closed states. The vast majority of peptides display the former mechanism.

We describe here a new natural product that produces inhibition of certain Kv channels. This toxin was isolated from Calliostoma canaliculatum, a common snail that inhabits kelp forests of the Pacific coast of the United States and Canada (9). When provoked by predatory starfish (Pycnopodia heliothoides or Pisaster giganteus) this snail repell the attacker by secreting a brilliant yellow mucus from its hypobranchial gland out of the shell aperture (10). We find that this mucus inhibits activation of K currents (Ik) through Shaker and other Kv,1 channels. The bioactive compound was purified by following bioactivity through successive rounds of reverse-phase liquid chromatography and the final structure characterized via NMR and mass spectrometry. The active compound is not a peptide but a disulfide-linked dimer of 6-bromo-2-mercaptotryptamine (BrMT). BrMT thus represents a new class of K channel inhibitor from a gastropod mollusc and appears to exert its effects on channel gating rather than by blocking the pore.

EXPERIMENTAL PROCEDURES

Purification of the Active Compound

Calliostoma canaliculatum was collected from Monterey Bay, CA. Snails were anesthetized in a 1:1 mixture of 7.5% MgCl₂ and sea water. The shell of an individual snail was cracked with a vise-grip and removed. The hypobranchial gland was surgically removed, immediately frozen in liquid nitrogen and stored at −80 °C. Hypobranchial glands from ~100 animals were pooled and homogenized in an acidified acetone solution. This homogenate was centrifuged at 12,000 rpm for 5 min, and the yellow-brown supernatant stored at −20 °C in glass test tubes. The pellet was repeatedly extracted with acidified acetone in this fashion until the supernatant appeared clear. These extracts were pooled, dried under a stream of nitrogen gas, and resuspended in an aqueous solution of 5% acetonitrile (ACN) and 0.1% (v/v) trifluoroacetic acid. This material was loaded onto 10 g C₁₈ separatory cartridges in an effort to remove salts and other non-bioactive components. The car-
tridides were then rinsed with water followed with a 40% ACN solution. The bioactive fraction was eluted using a 90% ACN + 0.1% trifluoroacetic acid solution and stored at −20 °C.

**HPLC Purification**

An aliquot (600 µl, equivalent to about 2 glands worth of material) of the 90% ACN fraction obtained from the C18 cartridges was dried under a stream of nitrogen gas and resuspended in a 25 µl volume of 5% ACN and 0.1% (v/v) trifluoroacetic acid. Separation was then performed using 20 µl with a reversed-phase, microbore LC (Magic 2002, Michrom BioResources, Auburn, CA) and a Reliastat C8 column (150 × 2.0 mm, 5 µm particle diameter and 300 Å pore size). For the first-stage separation, the mobile phase consisted of solvent A (98% H2O and 2% ACN + 0.1% trifluoroacetic acid (v/v)) and solvent B (95% ACN and 5% H2O + 0.1% trifluoroacetic acid (v/v)). A gradient was developed by increasing the fraction of solvent B from 5 to 98% over 34 min at a uniform flow rate of 60 µl/min per minute. Separation was monitored by absorption at both 240 and 280 nm. Fractions from the separation were obtained by using a fraction collector (Gilion FC 203B, Midleton, WI) and assayed for activity against Shaker channels heterologously expressed in mammalian HEK-293 cells (see below). The active fraction was dried under a stream of nitrogen gas and resuspended to a volume of 50 µl in 5% ACN and 0.1% (v/v) hepta-fluorobuturylic acid (HFBA).

For the second-stage separation, the HPLC system was equilibrated using a mobile phase consisting of solvent C (98% H2O and 2% ACN + 0.1% HFBA (v/v)), and solvent D (95% 2:2:1 ACN-isopropyl alcohol-methanol and 5% H2O + 0.1% HFBA (v/v)). Again, 20 µl of sample was injected for analysis. At a uniform flow rate of 60 µl per minute, a gradient was developed by increasing solvent D from 5 to 45% over 5 min, holding steady for 5 min, then changing from 45 to 65% over 20 min, holding steady for 5 min, and finally increasing from 65 to 98% over 5 min. Absorbance at both 240 and 280 nm was monitored, and fractions were assayed as described above.

After initially developing the above procedures, a larger scale, single-stage separation was performed with freshly prepared extract using a Waters Analytical HPLC system and a 4.6 × 250 mm Sigma-Aldrich peptide column (5-µm particle diameter and 300 Å pore size). Performing the extraction and separation within hours eliminated the necessity of the second-stage separation to isolate the compound from degradation products. Gradient and solvent conditions were similar to the first-stage microbore procedure described above. Absorbance was monitored with a photodiode array detector scanning wavelengths from 280 to 600 nm. Absorbance at 433 nm was found to be optimal for selecting the fraction of interest.

**NMR Experiments**

All NMR experiments were conducted using a Varian Unity-Inova 500 MHz narrow-bore NMR spectrometer with a Z-gradient 1H-13C-15N inverse-detection probe. Bioactive HPLC fractions were combined after a stream of nitrogen gas and resuspended in a volume of 50 µl in 5% ACN and 0.1% (v/v) hepta-fluorobuturylic acid (HFBA). For the second-stage separation, the HPLC system was equilibrated using a mobile phase consisting of solvent C (98% H2O and 2% ACN + 0.1% HFBA (v/v)), and solvent D (95% 2:2:1 ACN-isopropyl alcohol-methanol and 5% H2O + 0.1% HFBA (v/v)). Again, 20 µl of sample was injected for analysis. At a uniform flow rate of 60 µl per minute, a gradient was developed by increasing solvent D from 5 to 45% over 5 min, holding steady for 5 min, then changing from 45 to 65% over 20 min, holding steady for 5 min, and finally increasing from 65 to 98% over 5 min. Absorbance at both 240 and 280 nm was monitored, and fractions were assayed as described above.

**ESI-MS of Fractions**

Electrospray ionization mass spectrometry (ESI-MS) was performed for the initial examination of the various peaks from the two stages of HPLC separation utilizing a Micromass Quattro (quadrupole-hexapole-quadrupole (QHQ) mass spectrometer). Data acquisition and processing were controlled by the Micromass MassLynx NT data system running on a PC platform. Fractones were dried under a stream of nitrogen gas and reconstituted in 10–500 µl of ACN with 1% formic acid for injection into the ESI-MS system.

Identification of BrMT and assessment of purity within HPLC fractions were facilitated by the use of an electrospray ion-trap mass spectrometer fitted with a static nanospray source and using the Xcalibur data system (LCQ Deca, Thermo-Finnigan). An aliquot of ~10 µl was taken from a fraction, dried under a stream of nitrogen gas and reconstituted in 10 µl of 50/50 (v/v) CH3OH and H2O with 0.1% formic acid. The sample was loaded into a tapered, gold coated, borosilicate nanospray emitter tip (PicoTIP™, New Objective, Woburn MA) with capillary temperature of 220 °C and potential of 4 V. An optimized spray voltage of 1.3 kV was employed, and the tube-lens offset was adjusted to minimize source-induced fragmentation of the parent peak. This method was also used for ion trap tandem MS experiments.

All high-resolution mass spectrometry experiments were conducted on a home-built 2.7 Tesla Fourier-transform mass spectrometer described elsewhere (11). Ions are generated using nano-electrospray ionization (nano-ESI) from 50/50 water/acetonitrile solution. A 0.78-mm inner diameter borosilicate capillary pulled to a fine (~4 µm) aperture is filled with several µl of the analyte-containing solution. A platinum wire running down the center of the solution-filled capillary is used as an electrode with an applied potential of ~0.8–1.2 kV.

Exact mass measurements are made using four internal standards, arginine, tryptophan, dilysine, and the pentapeptide leucine-enkephalin, which produce (M + H+) ions with m/z values ranging from 175 to 556. The possible elemental compositions for the ions in the samples are determined from exact mass measurement using the Spectral Interpreter module provided with the Odyssey software.

For the tandem mass spectrometry experiments, an ion of interest was isolated using a series of stored waveform inverse Fourier-transform, chirp, and single frequency excitation waveforms. These mass-selected ions are fragmented either by IRMPD using a continuous wave CO2 laser at 10.6 µm (12) or by SORI-CAD (13). Both of these techniques deposit energy into the ion slowly and produce relatively gentle dissociation, resulting in a limited number of fragment ions. A broadband radio frequency chirp is used to excite all ions for detection. Data are acquired using a Finnigan Odyssey system (Finnigan MAT; Bremen, Germany).

**BrMT Solutions Used for Physiological Experiments**

Two BrMT stock solutions were used. One contained 3.8 mM BrMT in deuterated methanol and had previously been utilized for NMR analysis. The other contained 3.7 mM BrMT in a mixture of H2O/ACN/TFA resulting from HPLC purification. These solvents had no significant effect on channel properties at the dilutions used to study BrMT.

**Channel Expression**

**Oocytes, Xenopus laevis** oocytes were surgically removed, defolliculated with collagenase and stored at 17 °C in ND96 solution (in mM): 96 NaCl, 2 KCl, 1.8 CaCl2, 2 MgCl2, and 5 mM HEPES (pH 7.8 with NaOH). K channel cRNAs were transcribed using a kit following the manufacturer’s protocols (mMessage Machine, Ambion; Austin, TX), and oocytes were injected 2–7 days prior to recording. The following clones (gifts as indicated) were utilized: Drosophila ShakerB3 (6–46) (ShB3 14) from R. Aldrich, Stanford University), squid sqK1.1 (15; Kv.1.1 (16) from J. Adelman, Oregon Health Sciences University), K.2.1 (drk1 17) from Kenton Swartz, NIH), K.3.1 (mK.3.1b 18) from S. Grissmer, University of Ulm), K.4.1 (mShal 19) from M. Corvarrubias, Thomas Jefferson University), K.4.2 (SrK 20) from L. Jan, University of California, San Francisco), and Drosophila ether-a-go-go (EAG 21) from D. Papazian, University of California, Los Angeles.

**Mammalian Cells—ShB3 channels were transiently expressed in CHO-K1 or HEK 293 cells. Cells were plated onto untreated glass coverslips and transfected using standard CaPO4 methods (22). Recordings were carried out 1–4 days post transfection. The ShB3 channel expressed in mammalian cells contained the additional mutations C301S, C308S and T449V (23) and was a gift of G. Yellen, Harvard University.**

**Electrophysiology**

**Whole Oocytes—**A two-electrode voltage clamp (Dagan TEY-200; Minneapolis, MN) was used, and data were acquired with a Macintosh computer and ITC-16 interface (Instrutech Corp; Elmont, NY) running either the Pulse Control extension package (Herrington and Bookman, 1995) with Igor Pro (WaveMetrics Inc.; Lake Oswego, OR) or the Pulse software package (HEKA Electronic). Records were filtered at 1–5 kHz and digitized at 10–20 kHz. P/4 leak subtraction protocols were used. Microelectrodes were filled with 3 m KCl and had tip resistances of <1.5 MΩ.

Recordings from EAG, K.4.1, K.4.2, K.3.1 and hK.1,1 used a bath...
with a pipette after stimulating a snail with the tube foot from a predatory starfish. Effects of this mucus were tested on two extensively studied K channel preparations after diluting the material an additional 50-fold in the appropriate external recording solution. Calliostoma mucus slows the apparent time-course of activation and reduces peak amplitude of $I_{\text{K}}$ through both native delayed-rectifier K channels in the squid giant axon system (Fig. 1A) and heterologously expressed Shaker (ShBΔ) channels (Fig. 1B). Although the effects are qualitatively similar, they clearly differ in degree. In neither case are deactivation kinetics affected.

**Isolation of a Non-peptidic K Channel Inhibitor**—Details of the purification of the active compound in the yellow defensive mucus are given under “Experimental Procedures.” After extraction of pooled glands, the homogenized material was subjected to several clean-up steps in order to reduce the high concentrations of salts and lipids from the matrix. Slowling of $I_{\text{K}}$ activation in voltage-clamp experiments as described above was used as the criterion for screening fractions. The active compound was found to retain an intense yellow color, absorbing at wavelengths in the violet region of the visible spectrum, and can thus be visually followed throughout the purification process.

Representative chromatograms of microbore HPLC elution of the active compound are presented in Fig. 2. The first-stage separation (Fig. 2A) yields a peak of sufficient purity for structural identification. Once separated from other components of the extract, the active compound degrades. Should partial degradation occur, the remaining active compound can be isolated from degradation products by a second-stage separation (Fig. 2B). Bioactivity tests using ShBΔ channels expressed in mammalian cells clearly identified the peak eluting at ~23 min as that containing the compound of interest.

Structural information on the isolate was gained via nuclear magnetic resonance (NMR) and mass spectrometry (MS). MS techniques were also used to corroborate the structural identity and purity of bioactive isolates used in subsequent physiological experiments. Results obtained with each technique are described below.

$^1$H-NMR——Table I gives the NMR data used in the structural determination of the bioactive agent. The deduced structure is presented in Fig. 2C with the corresponding NMR assignments. Fig. 2D displays one-dimensional $^1$H NMR spectra of the compound dissolved in CD$_3$CN. Addition of D$_2$O to eliminate resonance arising from exchangeable protons (Fig. 2E) prompted the disappearance of resonances at 6.62 (peak H$_C$) and 9.94 ppm (peak H$_D$) as well as the collapse of the sextet at 3.07 ppm (peak H$_B$) to a triplet. The exchangeable proton resonance near 10 ppm integrates to a relative value of one, suggesting the compound is an indole derivative. This putative indole accounts for the 6 double-bond equivalents obtained from Fourier-transform ion cyclotron resonance mass spectrometry (FTICR-MS) (see below).

The molecular formula, C$_{20}$H$_{20}$N$_4$S$_2$Br$_2$, obtained from exact mass measurements of the fragments of the molecule using FTICR-MS, is consistent with the peaks at 2.86, 3.07, and 6.62 ppm corresponding to an ethylamine moiety and with the peaks at 7.27, 7.51, and 7.59 ppm corresponding to aromatic resonances. From the $^1$H-$^1$H coupling constants, all three aromatic peaks are assigned to the larger ring subunit such that either the 5 or 6 indole position is occupied by another substituent. Integration and chemical shift data indicate that the primary amine is protonated when the sample is dissolved in CD$_3$CN. In addition, the spectrum in Fig. 2D does not display any peak assignable as a thiol resonance, consistent with a disulfide bond between two identical indole derivatives as illustrated in the assigned structure (Fig. 2C).

**RESULTS**

**Effects of Yellow Defensive Mucus on Native and Cloned K Channels**—Naturally secreted defensive mucus was collected from isolated squid giant fiber lobe cells were carried out as described previously (22).

![Figure 1](callio.png)

**Fig. 1.** Calliostoma defensive mucus at a 1:50 dilution slows $I_{\text{K}}$ activation and reduces peak amplitude. A, $I_{\text{K}}$ at +60 mV through native delayed-rectifier channels in a squid giant fiber lobe neuron studied with whole-cell patch-clamp. Holding potential = -80 mV. B, $I_{\text{K}}$ at +20 mV through ShBΔ channels expressed in a CHO cell studied with whole-cell patch-clamp. Holding potential = -100 mV.

Solutions were applied to excised patches using a delivery manifold (DAD-12, ALA Scientific Instruments; Westbury, NY) with a 100-μm diameter port and a pressure of 200 mm Hg. The effect of 5 mM BrMT and 200 mM HEPES (pH 7.2 with HCl). Internal solutions contained 90 mM NaCl, 20 mM CaCl$_2$, 20 HEPES (pH 7.2 with methanesulfonic acid). In addition, 1 mM diamide was added to these solutions to buffer oxidative conditions in experiments with ShBΔ, sqKv1A, Kv2.1 and hKv1.1. Addition of diamide did not alter the effects of BrMT with ShBΔ, but the other channels were not tested.

A low-volume flow-through chamber was used for recording. Solution exchange was calibrated with TEA-containing solutions to achieve >95% solution exchange with 0.5 ml. Typically, this volume was flushed through to add BrMT, and several minutes were required for the effects of BrMT on K currents to stabilize. The effects of BrMT were not readily reversed by washout with whole oocytes.

**Oocyte Patches**—Excised patch recordings were made in the outside-out configuration using an Axopatch 1-B amplifier (Axon Instruments; Foster City, CA). Data were collected as described above for whole oocytes, except filtering was at 2 kHz, and sampling was at 20 kHz. External solutions contained (in mM) 115 NaCl, 10 KCl, 2 CaCl$_2$, 20 HEPES (pH 7.2 with HCl). Internal solutions contained 90 KF, 20 KCl, 30 KOH, 10 EGTA, 20 HEPES (pH 7.2 with HCl). Pipette resistances with these solutions was less than 3 MΩ.

Solutions were applied to excised patches using a delivery manifold (DAD-12, ALA Scientific Instruments; Westbury, NY) with a 50-μm diameter port and a pressure of 200 mm Hg. The effect of 5 μM BrMT in this configuration required several seconds to reach completion and several minutes to washout.

Mammalian Cells—Ionic currents from ShBΔ channels were recorded from transfected mammalian cells in the whole-cell patch-clamp mode using an Axopatch 200A amplifier (Axon Instruments; Foster City, CA). Data were collected as described above for whole oocytes, except filtering was at 2 kHz, and sampling was at 50 kHz. Internal and external solutions were the same as used for excised patches.

**Squid Giant Fiber Lobe Cells**—Whole-cell patch-clamp recordings from isolated squid giant fiber lobe cells were carried out as described previously (22).
Multidimensional and Heteronuclear NMR—To complete the structural assignment, heteronuclear correlation experiments were conducted in CD<sub>3</sub>OD, and results are listed in Table I. The combined ps-gHMQC and gHMBC data indicate the presence of ten carbon resonances, in accordance with the MS studies, and provide strong evidence for the ethylamine moiety being at the 3-position of the indole ring and for the 6-position of the indole ring being substituted. Of particular importance for the gHMBC data analysis considered here, aromatic <sup>1</sup>H-<sup>13</sup>C coupling constants across two bonds (geminal) (24) are smaller than those across three bonds (vicinal). Although the empirically based <sup>13</sup>C chemical shift calculations were inconclusive, the <sup>1</sup>H chemical-shift calculations clearly suggest that the 6-position of the indole is occupied by the bromine substituent over the alternate choice of sulfur (24). Sulfur is consequently assigned to the 2 position.

**Table I**

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<th>Mult. (J in Hz)</th>
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<th>Int. (CD&lt;sub&gt;3&lt;/sub&gt;CN)</th>
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**Fig. 2.** Microbore HPLC purification of the bioactive compound. A, reverse-phase HPLC chromatogram illustrating the first-stage purification of BrMT (see “Experimental Procedures” for details). The illustrated trace represents absorbance at 240 nm. The peak of interest is observed at ~23 min. B, reverse-phase HPLC chromatogram illustrating the second-stage purification. Absorbance was monitored at 240 nm. The BrMT peak is observed at 23 min. C, final deduced structure of the active component that inhibits K channel activation. The compound is a disulfide-bonded dimer of 6-bromo-2-mercaptotryptamine. Capital letters on protons in left half correspond to <sup>1</sup>H NMR peaks in panel D and Table I. Roman numerals correspond to <sup>13</sup>C NMR peaks in Table I. Numbered atoms on the right half of the dimer indicate the scheme followed. D, <sup>1</sup>H NMR spectra of the purified, bioactive compound dissolved in CD<sub>3</sub>CN. E, <sup>1</sup>H NMR spectra of the purified, bioactive compound after addition of D<sub>2</sub>O. See text for additional details.

Fourier-Transform Ion Cyclotron Resonance Mass Spectrometry (FTICR-MS)—FTICR-MS analysis identified the active
component by comparing electrospray ionization spectra from HPLC fractions of differing bioactivity. Three ions of interest, corresponding to a singly brominated ion at \( m/z \) 237/239 and 270/272 and a doubly brominated ion at \( m/z \) 539/541/543, were identified. The nature of the isotope patterns of the \( m/z \) 270/272 and 539/541/543 clusters indicates the presence of one and two sulfurs, respectively. Assignment of the \( m/z \) 270/272 peaks (exact mass 269.9832 ± 0.0011) as the compound of interest yielded a putative molecular formula of \( \text{C}_{10}\text{H}_{11}\text{N}_2\text{SBr} \), a double bond equivalent (DBE) of 6 with a +4.1 ppm error from the calculated exact mass. Assignment of the \( m/z \) 237/239 peaks (exact mass 237.0019 ± 0.0015) yielded a likely molecular formula of \( \text{C}_{10}\text{H}_{10}\text{N}_{2}\text{Br} \), a double bond equivalent (DBE) of 6.5 with a −1.2 ppm error from the calculated exact mass. Using the NMR and FTICR-MS data, the bioactive agent has been assigned the structure of a disulfide bonded dimer of 6-Br-2-m商aptopotryptamine (BrMT) (Fig. 2C).

Reduction and Oxidation Reveal BrMT Dimerization—MS analysis of the sample used for the NMR work strongly suggests that the bioactive compound has mass of 538 Da (protonated mass 539). The odd electron ion, \( \text{C}_{10}\text{H}_{11}\text{N}_{2}\text{SBr}^+ \) (\( m/z \) 539/541/543; Fig. 3A), is not always present in the initial electrospray spectra, but grows in with time, as observed by FTICR-MS. This indicates that it is formed by electrochemical reduction in the electrospray solution. The even electron ion, \( \text{C}_{10}\text{H}_{10}\text{N}_{2}\text{Br}^+ \) (\( m/z \) 539/541/543; Fig. 3A), is formed by gas-phase dissociation of the parent ion (\( m/z \) 539/541/543). The measured mass spectra of BrMT plus reducing agent TCEP (tris (2-carboxyethyl) phosphine; Fig. 3B) or DTT (dithiothreitol; not illustrated) is consistent with chemical reduction of a single disulfide bond in BrMT, thus forming ions at \( m/z \) 271/273. This reduction is reversed by addition of \( \text{H}_2\text{O}_2 \) to the BrMT + TCEP solution, resulting in the reappearance of the protonated BrMT (\( m/z \) 539/541/543; Fig. 3C).
ally Activated Dissociation (SORI-CAD) was employed to dissociate all three principle ions in the mass spectra of the bioactive isolate in Fig. 3A as well as the m/z 271 molecular ion peak from the sample reduced by TCEP in Fig. 3B. These dissociation experiments result in fragmentation products consistent with the proposed structure for BrMT, in particular the assignments of bromine on the six-member ring and sulfur on the 5-member ring.

**BrMT Is Active Only as a Dimer**—Reversible reduction/oxidation was also utilized in conjunction with an absorbance assay and the standard bioassay to determine the active form of the molecule. The yellow color of BrMT is produced by absorption in the violet region of the visible spectrum. Reduction of the BrMT disulfide bond by TCEP shifts this violet absorbance into the ultraviolet, resulting in loss of color (Fig. 3D). This colorless monomer has little effect on IK through ShB/H9004 channels (thin trace in Fig. 3E). Re-oxidation of the BrMT dimer with H2O2 reconstitutes the yellow color, absorption spectrum, and activity against K channels (thick traces in Fig. 3, D and E). This pattern mirrors the disappearance and reappearance of the m/z 539/541/543 cluster under similar oxidizing and reducing conditions (Fig. 3, A–C).

These observations are thus consistent with the BrMT dimer predominating in aqueous solutions and being primarily, if not entirely, responsible for activity against ShBΔ channels. Further references to BrMT in this paper assume a disulfide-bonded dimer, and all concentrations cited are appropriate for the dimer.

**Tandem Mass Spectrometry Experiments**—Ion-trap MS was used to confirm the assignment of the bioactive compound (see “Experimental Procedures” for details). Fig. 4A illustrates a full-scan spectrum of a single-stage HPLC fraction corresponding to the bioactive substance. The signal-to-noise ratio of the parent cluster (539/541/543 m/z) could be optimized over that of the FTICR-MS conditions by fine adjustment of the ion trap parameters. Initial FTICR-MS experiments suggested that the single brominated cluster at 270/272 m/z was formed by electrochemical reduction in the electrospray solution. The higher signal-to-noise ratio of the parent dimer allowed a comparison of the observed isotopic cluster to a simulated spectrum based...
on the proposed formula of C_{20}H_{20}N_{4}S_{2}Br_{2} (Fig. 4, B and C). The results clearly indicate a closely matching mass and isotopic distribution pattern.

Tandem experiments, MS^2 and MS^3, were performed in order to give a complete explanation for the 237/239, 255/257 m/z peaks seen in the spectrum. Fig. 5A depicts the results of an MS^2 experiment with the parent ion at 541 m/z isolated in the trap. The resulting MS^2 spectrum can be seen to yield the 237/239 m/z ion cluster. The proposed rearrangement product is depicted in Fig. 5B. The peaks at 158 and 208/210 m/z seen in the collisional spectrum are the result of further rearrangement of the 237/239 fragment as evidenced by MS^3 results of the isolated 541 m/z parent ion and its 237 m/z fragment (not shown). The peak at 158 m/z corresponds to the loss of bromine from the 237/239 m/z ion cluster. Isolation of the electrospray-reduced monomer within the ion trap (270/272 m/z) and subsequent MS^2 unambiguously demonstrated that the peak cluster at 253/255 m/z, seen in the full scan MS, is due exclusively to fragmentation of the monomer (not shown).

The tandem MS experiments demonstrate that the remaining peaks seen in Fig. 4A are products of instrumentally induced and/or dissociation fragments from the identified parent cluster at 541 m/z.

**Action of BrMT against ShBD Channels**—The effects of bath-applied 10 μM BrMT on I_K at +40 mV through ShBD channels expressed in a Xenopus oocyte are illustrated in Fig. 6A. The time course of I_K rise is greatly slowed and peak current is depressed. Deactivation kinetics as channels close are not altered (data not illustrated; see Fig. 1). Fig. 6B illustrates the relationship between K conductance (g_K) and voltage from the same oocyte, and the effect of 10 μM BrMT can be described as decrease in maximum g_K of about 50% and a modest shift in voltage dependence of -10 to -15 mV. Similar results were obtained in every oocyte studied.

Exponential fits to the final 50% of the time course of I_K rise provide a means of quantifying the degree of slowing imparted by exposure to BrMT in a model-independent manner. In the case of the traces in Fig. 6A, the ratio of time constants is close to 100. Measurements at other voltages between -10 mV and +50 mV indicated that this ratio shows no significant voltage dependence (data not illustrated). Extending this comparison to more negative voltages is complicated by the steep dependence of g_K on voltage in this region and the apparent shifting of this voltage dependence produced by BrMT.

An important feature of the action of BrMT is that the slowing of I_K becomes more profound as the concentration of
BrMT increases. Fig. 6C illustrates examples of $I_K$ at 0 mV for a series of BrMT concentrations. Slowing of the $I_K$ waveform clearly becomes more pronounced as concentration increases. The reduction in peak $I_K$ produced by BrMT also increases over this concentration range, and this reflects an increasing reduction in the maximum $g_K$ attainable with large voltage steps.

Specificity of BrMT for $K$ Channels—Several voltage-gated $K$ channel types were tested for sensitivity to 10 $\mu$M BrMT (Fig. 7). The effects of BrMT on kinetics and amplitude of $I_K$ are summarized in Fig. 7B. Of the channels tested, only K,2.1 and K,3.1 were essentially insensitive to 10 $\mu$M BrMT. All of the other channels tested showed a reduction in $I_K$ amplitude at least as large as that to that described for ShB$, but the degree of $I_K$ slowing differed greatly. Although they are closely related to ShB$, the K,1 isoforms from human (hK,1,1) and squid (sQK,1A) sources were affected much less. Other channel types were even less sensitive. Thus, $I_K$ kinetics appear to be most strongly affected in certain K,1 channel types. In the case of K,4 channels, the rapid inactivation process could obscure effects on activation, but this possibility was not examined further.

**DISCUSSION**

In this paper, we identify BrMT as a novel, natural product that inhibits certain voltage-gated $K$ channels, most notably ShB$ and other K,1 members. Fundamental differences exist in the structure of BrMT that obviously distinguish it from peptidic K channel toxins. Similarly, its mode of action appears to be unusual in that it may primarily inhibit channel opening. This hypothesis is discussed further below. Although BrMT shows some selectivity among K channel subtypes, we have not investigated its effects on other classes of voltage- or ligand-gated channels.

**Does BrMT Inhibit Kc1 channels by a Pore-blocking Mechanism?**—Naturally occurring toxins that specifically inhibit K,1 channels are peptides isolated from scorpions (25), snakes (1) Conus snails (26, 27) and sea anemones (28–30). BrMT, a novel indole derivative, obviously differs in not being a peptide. In every case where the mechanism of inhibition has been investigated, these peptide toxins have been found to block K conduction by directly occluding the entrance to the pore (31). However, some peptides (perhaps most) lead to an appearance of slowed $I_K$ activation due to voltage-dependent unbinding at positive voltages (27, 32,33 and unpublished data on TTX-Ko, a scorpion toxin (34)). This important mechanism (35) predicts that, at a given voltage, the rate of $I_K$ rise will be faster at higher toxin concentrations because of faster equilibrium binding kinetics (33). This pattern is clearly opposite to that observed in the present study for BrMT (Fig. 6C), and the action of BrMT on $I_K$ kinetics is thus not compatible with voltage-dependent unbinding of a pore blocker.

Instead, we propose that BrMT effective slows activation of ShB$ channels through an interaction with the voltage-dependent gating process. Slowing of $I_K$ as observed might result from a stabilization of closed channels by binding of BrMT to resting or partially activated subunits, or it could involve an allosteric mechanism that directly impairs gating. Based on the similar range of BrMT concentrations over which $I_K$ slowing and reduction become apparent for ShB$ channels (Fig. 6C), we believe that both effects are likely to stem from the same BrMT binding event. A rigorous test of this idea will require a deeper understanding of the mechanism by which BrMT acts. Whether a qualitatively different mechanism of $I_K$ reduction exists for those channels that are not kinetically sensitive, e.g. EAG, can only be speculated at present.

**Comparison of BrMT with Natural Kc Gating Modifiers**—Although none of the peptide toxins discussed above directly interacts with voltage-dependent gating, hana toxin clearly does so (7). This spider toxin slowly slows activation kinetics of K,2.1 and K,4,2 channels, markedly accelerates deactivation, and strongly shifts the $g_K$-voltage relationship to more positive voltages. These effects contrast to those produced by BrMT on K,1 channels, and it seems unlikely that this peptide and BrMT would share a similar mechanism of action. Hana toxin binding to K,2.1 is thought to occur well outside of the pore region (8), as do peptide gating modifiers of voltage-gated Na and Ca channels (36). Whether BrMT exerts its effects on ShB$ from an analogous area remains to be determined.

BrMT contains two indole moieties, and the actions of indole itself on Shaker channels merit comparison. Indole also slows activation and reduces peak $I_K$ in a concentration-dependent manner (37), but important differences exist in the effects of indole versus BrMT. First, indole at mM concentrations clearly accelerates deactivation kinetics. Second, peak $I_K$ reduction is quite voltage-dependent. Third, indole strongly decreases the steepness of the $g_K$-voltage relationship. BrMT shows none of these effects. Despite structural similarity between the two compounds, these functional differences strongly suggest different mechanisms of channel inhibition.

**Do Other Toxins of the BrMT-type Exist?**—Hypobranchial gland (HG) extracts from several marine gastropods have been known and utilized since antiquity. The ancient dye Tyrian purple (also known as Royal purple) is obtained from the HG of Murex (Trunculariopsis) trunculus sea snails, and recipes for preparation of this dye date back to 1600 B.C. (38), including a manuscript from Pliny in the first century A.D. (39). The major component of the dye itself is 6,6'-dibromoindigo and is obtained by boiling the viscous extract containing the precursors to fix a stable color (39, 40). A major component of the natural exudate from the snail is a precursor of Tyrian purple, tyri-ndoxyl sulfate (38, 39), which is structurally similar to BrMT. To our knowledge, actions of tyriodxyl sulfate or other components of HG exudates on ion channels have not been explored, and BrMT represents the first such case.

Interestingly, 6-bromination of tryptophan-related compounds is not unique to the BrMT class of toxins in gastropod mollusces and also exists as a post-translational modification in more than one peptide toxin (41–43). In one case, the selectivity of an inhibitor of a serotonin-gated channel may be due to the 6-bromo modification (42). In addition, 6-bromo indole-like compounds are found in other marine phyla (43). For example, sponges contain many brominated metabolites (44). None of these, however, closely resembles the structure of the marine HG exudates, nor have they been implicated in any similar biological roles. Bryzoa also contain 6-bromo indole-based compounds of unknown specific biological activity (45).

Structural similarities between BrMT and 6-bromo trypto-phan in gastropod mollusces and other 6-bromo metabolites in distant taxa strongly suggest a widespread biological role for brominated indolamines. Future work directed at further testing other HG extracts such as tyriodxyl sulfate and other 6-bromo indole-related natural compounds for biological activity against both voltage- and ligand-gated ion channels should prove productive. Such compounds may represent a heretofore unrecognized and rich group of natural toxins.

**Acknowledgements**—We thank Natalie Lu for carrying out preliminary physiological studies with crude Calliostoma mucus, Philip Floyd for his initial work on this project, and Rick Aldrich and members of his laboratory for helpful discussions and general support.

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A Novel Gastropod Toxin that Inhibits K⁺ Channels

271, 31013–31016