A Maurotoxin with Constrained Standard Disulfide Bridging

Maurotoxin (MTX) is a 34-residue toxin that has been isolated initially from the venom of the scorpion Scorpio maurus palmatus. It presents a large number of pharmacological targets, including small conductance Ca2+-activated and voltage-gated K+ channels. Contrary to other toxins of the α-KTx6 family (P11, P14, P17, and HsTx1), MTX exhibits a unique disulfide bridge organization of the type C1-C5, C2-C6, C3-C4, and C7-C8 (instead of the conventional C1-C5, C2-C6, C3-C7, and C4-C8, herein referred to as P11-like) that does not prevent its folding along the classic αβ scaffold of scorpion toxins. Here, we developed an innovative strategy of chemical peptide synthesis to produce an MTX variant (MTXₚ₁₁) with a conventional pattern of disulfide bridging without any alteration of the toxin chemical structure. This strategy was used solely to address the impact of half-cystine pairings on MTX structural properties and pharmacology. The data indicate that MTXₚ₁₁ displays some marked changes in affinities toward the target K⁺ channels. Computed docking analyses using molecular models of both MTXₚ₁₁ and the various voltage-gated K⁺ channel subtypes (Shaker B, K₁,2, and K₁,3) were found to correlate with MTXₚ₁₁ pharmacology. A functional map detailing the interaction between MTXₚ₁₁ and Shaker B channel was generated in line with docking experiments.

Maurotoxin (MTX) is a basic 34-residue toxin that has been initially isolated from the venom of the Tunisian chactidae

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The abbreviations used are: MTX, synthetic maurotoxin (toxin from the scorpion S. maurus palmatus); MTXₚ₁₁, synthetic maurotoxin with imposed standard (P11-like) disulfide bridging; MTXₚₛₛ, reduced or oxidized form of synthetic maurotoxin with a 2.6 dichioro-benzyl protecting group on the side-chain phenoxy ring of Tyr in position 32; HsTx1, toxin 1 from the scorpion Heterometrus spinifera; P11, P14, P17, toxins 1, 4, and 7, from the scorpion Pandinus imperator; HPLC, high pressure liquid chromatography; Fmoc, N-[(9-fluorenyl)methyloxycarbonyl]; SK and IK channels, small- and intermediate-conductance Ca2+-activated K⁺ channels, respectively; Kv channels, mammalian voltage-gated K⁺ channels; KV, a voltage-dependent K⁺ channel from A. pernix; Shaker B channel, insect voltage-gated K⁺ channels; TFMSA, trifluromethanesulfonic acid.
sumptive non-implication regarding ion channel recognition, one cannot rule out that they not only affect half-cystine pairings but also the spatial distribution of key toxin amino acid residues.

In the present work, we examined for the first time the sole contribution of the disulfide bridge arrangement on toxin pharmacology by maintaining unchanged the MTX primary structure. The aim was to produce and characterize an MTX variant adopting conventional P1-like disulfide bridges, referred to as MTX_P1 (Fig. 1A). For this purpose, we developed an innovative strategy for disulfide peptide synthesis based on a temporary chemical modification of the side chain of a trifunctional MTX amino acid residue (Tyr<sup>32</sup>) expected to guide the type of toxin half-cystine pairings (Fig. 1B). We focused on the Tyr<sup>32</sup> residue because formation of the short Cys<sup>31</sup>-Cys<sup>34</sup> (C7-C8) disulfide bridge (referred to as a 14-member disulfide ring) is very sensitive to local steric hindrance. Indeed, we have previously shown that replacement of the side-chain hydrogen atom of Gly<sup>23</sup> by a larger methyl group of Ala prevents the C7-C8 shown that replacement of the side-chain hydrogen atom of Gly<sup>23</sup> by a larger methyl group of Ala prevents the C7-C8 connection and forces the corresponding synthetic MTX analogue ([Ala<sup>33</sup>]-MTX) to adopt the conventional P1-like disulfide bridge arrangement of other α-KT66 members (18). This novel pattern of half-cystine pairings (C1-C5, C2-C6, C3-C7, and C4-C8) comprises the reorganization of two of the four disulfide bridges (C3-C7 and C4-C8, versus C3-C4 and C7-C8). The following approach was experimentally developed to produce MTX_P1 (Fig. 1B). (i) A classic stepwise solid-phase assembly of MTX peptide chain using a combination of Fmoc chemistry and t-butylium-side-chain protecting groups for trifunctional amino acid residues (19). In the case of Tyr<sup>32</sup>, a more acid-resistant 2,6 dichloro-benzyl group was used to protect its phenol ring. (ii) A trifluoroacetic acid treatment to remove all t-butylium-type protecting groups and to cleave the peptide from the resin. (iii) An oxidative folding of the Tyr<sup>32</sup>-protected MTX (MTX<sub>P1</sub>;<sup>32</sup>), and (iv) a final trifluoromethanesulfonic acid (TFMSA) treatment of the folded/oxidized MTX<sub>P1</sub>, to remove the Tyr<sup>32</sup> side-chain protecting group, thereby generating MTX_P1<sup>32</sup> (20). Using this procedure, we succeeded in the chemical production of MTX_P1 and demonstrate that its novel, but conventional, disulfide bridging is accompanied by marked differences in toxin properties.

**EXPERIMENTAL PROCEDURES**

**Materials**

N-α-Fmoc-<i>t</i>-amino acid, Fmoc-amide resin, and reagents used for peptide synthesis were obtained from PerkinElmer, except N-α-Fmoc-<i>t</i>-Tyr(2,6 dichloro-benzyl)-OH, which was from Fluka. Solvents were analytical-grade products and purchased from SDS. Enzymes (trypsin and chymotrypsin) were obtained from Roche Applied Science.

**Chemical Synthesis and Characterization of MTX<sub>P1</sub>**

The MTX<sub>P1</sub> variant was assembled by the solid-phase technique (19) using a peptide synthesizer (Model 433A; Applied Biosystems Inc.). Peptide chains were assembled stepwise on 0.35 milliequivalent of Fmoc-<i>N</i>-Fmoc-t-<i>N</i>-alkylamide/H<sub>2</sub>O at a flow rate of 5 ml/min (20). The folded/oxidized MTX<sub>P1</sub> peptide chain using a combination of Fmoc chemistry and t-butylium-type side-chain protecting groups for trifunctional amino acid residues (19). In the case of Tyr<sup>32</sup>, a more acid-resistant 2,6 dichloro-benzyl group was used to protect its phenol ring. (ii) A trifluoroacetic acid treatment to remove all t-butylium-type protecting groups and to cleave the peptide from the resin. (iii) An oxidative folding of the Tyr<sup>32</sup>-protected MTX (MTX<sub>P1</sub>;<sup>32</sup>), and (iv) a final trifluoromethanesulfonic acid (TFMSA) treatment of the folded/oxidized MTX<sub>P1</sub>, to remove the Tyr<sup>32</sup> side-chain protecting group, thereby generating MTX_P1<sup>32</sup> (20). Using this procedure, we succeeded in the chemical production of MTX_P1 and demonstrate that its novel, but conventional, disulfide bridging is accompanied by marked differences in toxin properties.

**FIG. 1. Half-cystine pairings of MTX, Pi1, and MTX_P1 and strategy for the chemical synthesis of MTX<sub>P1</sub>**

A, primary structures and corresponding half-cystine pairings of MTX, Pi1, and MTX_P1; B, strategy used for the chemical synthesis of MTX<sub>P1</sub>. The phenol ring of Tyr<sup>32</sup> from reduced MTX remains protected with the 2,6 dichloro-benzyl group after trifluoroacetic acid treatment (reduced MTX<sub>P1</sub>) of the MTX peptide resin, whereas the side-chain protecting groups (t-butylium-type denoted X) of other trifunctional amino acid residues are removed. The reduced MTX<sub>P1</sub>, folds/oxidizes to yield the oxidized MTX<sub>P1</sub>, with Pi1-like half-cystine pairings. Removal of the 2,6 dichloro-benzyl group by TFMSA treatment of the folded/oxidized MTX<sub>P1</sub>, generates MTX_P1.
Properties of MTX Variant with Pi1-like Disulfide Bridging

Assignment of Half-cystine Pairings of MTX Pi1 by Enzyme-based Cleavage and Edman Sequencing Analysis

MTX$_{pi1}$ (800 μg) was incubated with a mixture of trypsin and chymotrypsin at 10% (w/w) in 0.2 M Tris-HCl, pH 7.4, for 12 h at 37 °C. The resulting peptide fragments were then purified by reversed-phase HPLC (Chromolith RP18, 5 μm, 4.6 × 100 mm) with a 60-min linear gradient of 0.08% (v/v) trifluoroacetic acid/0–60% acetonitrile in 0.1% (v/v) trifluoroacetic acid/H$_2$O at a flow rate of 1 ml/min (λ = 230 nm) and freeze-dried prior to their analyses. These peptide fragments were hydrolyzed by acidolysis (6 N HCl/phenol), and their amino acid contents were determined (System 6300 amino acid analyzer; Beckman). The fragments were further characterized by mass spectrometry analysis (RP-DE Voyager; Perseptive Biosystems) and Edman sequencing using a gas-phase microsequencer (Applied Biosystems 470A). In standard HPLC conditions for analyzing phenylthiohydantoin (PTH) amino acid derivatives, diPTH-cystine elutes at a retention time of 9.8 min.

Circular Dichroism Analyses of MTX$_{pi1}$, MTX, and Pi1

Circular dichroism (CD) spectra were obtained on a Jasco J-810 spectropolarimeter equipped with a PTC-423S thermostat. A ratio of 2:20 was found between the positive CD band at 290.5 nm and the negative band at 192.5 nm. CD spectra were reported as the absorption coefficient (Δε) per amide. The far UV CD spectra were acquired at 20 °C in H$_2$O between 185 and 260 nm using a 0.1-cm path length cell. Data were collected twice at 0.6-nm intervals with a scan rate of 50 nm/min. As assessed by amino acid analysis, the concentration of MTX$_{pi1}$, MTX, or Pi1 was 40 nM.

Toxin Docking on Voltage-gated K$^+$ Channels

Atomic Coordinates—Atomic coordinates of MTX were obtained from the Swiss Protein Data base (Swiss-Prot www.expasy.ch) (number 1TXM).

Molecular Modeling—Molecular modeling of the S5-H5-S6 portions of rat K$_v$1.1, K$_v$1.2, K$_v$1.3, and Drosophila Shaker B channels was achieved on the basis of the crystal structure of the KcsA channel solved at a resolution of 3.2 Å (Swiss-Prot number 1BL8). The three-dimensional structural models of these channels were generated by using KcsA as a template and with the biopolymer homology modeling software of Swiss-model/Deep view 3.7 (Swiss-Prot, Switzerland). Amino acid sequence alignments between KcsA and K$_v$1.1, K$_v$1.2, K$_v$1.3, or Shaker B channels, which were generated by using CLUSTALW (V.1.82, www.ebi.ac.uk/clustalw/), showed that homologies are 69.8, 70.1, 69.1, and 65.6%, respectively. To avoid steric overlaps and clashes, modeled side chains and CO backbones of K$^+$ channels were subjected to energy refinement (until Δ$g$E < 0.05 kJ/mol·Å$^2$) using, successively, steepest-descent, conjugate gradient, and Newton-Raphson algorithms, with the consistent valence force-field as implemented in the INSIGHT II Discover3 module (1998 release, Molecular Simulations Inc., ACCEL RYS, San Diego, CA). Root mean square deviation values between the KcsA template CO backbone and the modeled K$_v$1.1, K$_v$1.2, K$_v$1.3, and Shaker B CO backbones were 0.48, 1.93, 0.32, and 1.68 Å, respectively.

A molecular model of Pi1 was obtained on the basis of the three-dimensional structure of MTX in solution (Swiss-Protein number 1TXM) by using the homology method of Swiss-Model/Deep view 3.7. Disulfide bridges were assigned using the Biopolymer module of InsightII. Similarly, this module was used to generate the molecular model of MTX$_{pi1}$. Molecular models were relaxed by 5,000 steps of 1 fs of dynamics simulation at 15 K, and the docking solutions from 0.05 kJ/mol·Å$^2$ using the algorithms and force field previously described for K$_v$1.1, K$_v$1.2, K$_v$1.3, and Shaker B channels. Amino acid sequence alignment between Pi1 and MTX (CLUSTALW) points to 88.2% sequence homology. Root mean square deviation values between template MTX CO backbone and the modeled Pi1 and MTX$_{pi1}$ CO backbones were 1.33 and 1.05 Å, respectively. Geometric quality of all models was evaluated using PROCHECK V3.5.4 (21, 22).

Protein Docking—Molecular interaction simulations were performed using the BIGGER program (biomolecular complex generation with global evaluation and ranking) (23). In the first step, a 1-Å three-dimensional matrix composed of small cubic cells, which represents the complex shape of each molecule, was generated. The translational interaction space was searched for each relative orientation of the two molecules by systematically shifting the probe matrix (toxin) to the target matrix (ion channel). 5,000 docking solutions were selected after probe rotation of 15° relative to the target, and this surface matching was repeated until a complete non-redundant search was achieved. The algorithm used by BIGGER performs a complete and systematic search for surface complementarities (both geometry complementarities and amino acid residue pairwise affinities are considered) between two potentially interacting molecules and enables an implicit treatment of molecular flexibility. In the second step, the 5,000 putative solutions

Fig. 2. Chemical synthesis of MTX$_{pi1}$. A, analytical C$_18$ reversed-phase HPLC elution profile of crude reduced MTX$_{pi1}$ after trifluoroacetic acid treatment. B, crude folded/oxidized MTX$_{pi1}$ after oxidative folding. C, MTX$_{pi1}$ after TFMSA treatment of folded/oxidized MTX$_{pi1}$. D, purified MTX$_{pi1}$.
Docking Solution Screening — The 15 best solutions were selected according to (i) the global score from BiGGER, (ii) toxin Lys residue (Lys23 for MTX and MTX Pi1, or Lys24 for Pi1) and β-sheet strand orientations toward the ion channel pore, and (iii) the best orientation, considering the electrostatic properties of both the toxin and the K+ channel. The GRASP software (24) was used to determine these electrostatic properties (GRASP; Howard Hughes Medical Institute, Columbia University, New York).

Structural Refinement of the Final Complexes — The screened docking solutions were minimized with a rigid-body method (Cα-locked) with steepest-descent algorithms using Deep-view V3.7 (until ΔE < 0.05 kJ/mol·Å⁻¹) with a GROMOS96 force field (25) to relieve possible steric clashes and overlaps. During structural refinement, a distance-dependent dielectric constant of 4 was used.

Docking Energy Calculations — Final docking energy of each best solution (ΔE toxin-channel + ΔE toxin) was obtained by subtracting the first complex energy (E toxin-channel) from the final complex energy (Cα-Cα distances locked) until ΔE < 0.05 kJ/mol·Å⁻¹ (GROMOS96 force field) (25), from the final complex energy (E toxin-channel) minimized under identical conditions.

Linear Regression — Linear regression was computed using the Prism software (GraphPad Prism version 3.0cx for MacOS X; GraphPad Software, San Diego, CA; www.graphpad.com).

Neurotoxicity of MTX Pi1 and MTX Tyr in Mice

The peptides were tested in vivo for toxicity by determining the LD₅₀ after intracerebroventricular injections into 20 g of C57/BL6 mice (animal testing agreement number 006573, delivered by the Ministère de l’Agriculture et de la Pêche). Groups of six mice per dose were injected with 5 µl of MTX Pi1 solution containing 0.1% (w/v) bovine serum albumin and 0.9% (w/v) sodium chloride.

Competitive Inhibition of ¹²⁵I-Apamin Binding onto Rat Brain Synaptosomes by MTX Pi1, MTX Tyr, and MTX

Close Interaction Analyses — Details of interactions were analyzed using the LIGPLOT program (26) on each best docking solution given by the screening method.

Properties of MTX Variant with Pi1-like Disulfide Bridging

Docking Solution Screening — The 15 best solutions were selected according to (i) the global score from BiGGER, (ii) toxin Lys residue (Lys for MTX and MTX Pi1, or Lys for Pi1) and β-sheet strand orientations toward the ion channel pore, and (iii) the best orientation, considering the electrostatic properties of both the toxin and the K+ channel. The GRASP software (24) was used to determine these electrostatic properties (GRASP; Howard Hughes Medical Institute, Columbia University, New York).

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Docking Energy Calculations — Final docking energy of each best solution (ΔE toxin-channel + ΔE toxin) was obtained by subtracting the sum of toxin energy alone (ΔE toxin) and ion channel energy alone (ΔE channel), after rigid body minimization (Cα-Cα distances locked) until ΔE < 0.05 kJ/mol·Å⁻¹ (GROMOS96 force field) (25), from the final complex energy (ΔE toxin-channel) minimized under identical conditions.

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Competitive Inhibition of ¹²⁵I-Apamin Binding onto Rat Brain Synaptosomes by MTX Pi1, MTX Tyr, and MTX

Rat brain synaptosomes were prepared as described by Gray and Whittaker (27). Aliquots of 50 µl of 0.1 nm ¹²⁵I-apamin were added to 400 µl of synaptosome suspension (0.4 mg protein/ml). Samples were incubated for 1 h at 4 °C with 50 µl of one of a series of concentrations of MTX Pi1 or MTX Tyr or MTX (10⁻³ M to 10⁻²⁴ M) in 500 µl of final
volume. The incubation buffer was 25 mM Tris-HCl, 10 mM KCl, pH 7.2. The samples were centrifuged, and the resulting pellets were washed three times in 1 ml of the same buffer. Bound radioactivity was determined by \( {\gamma} \) counting (Packard Crystal II). The values expressed are the means of triplicate experiments ± S.D. Nonspecific binding, less than 8% of the total binding, was determined in the presence of an excess (10 nM) of unlabeled apamin.

**Preparation and Electrophysiological Recordings of Xenopus Oocytes**

*Xenopus laevis* oocytes at stages V and VI were prepared for cRNA injection and electrophysiological recordings. The follicular cell layer was removed by enzymatic treatment with 2 mg/ml collagenase IA (Sigma) in classic Barth’s medium lacking external Ca\(^{2+}\). The cDNA
plasmids were linearized with SnaI (Shaker B), NotI (rat K,1,1), XhoI (rat K,1,2), and EcoRI (rat K,1,3) and transcribed with either T7 or SP6 RNA polymerase (mMessage mMachine kit; Ambion). The cells were microinjected 1–2 days later with 50 nl of cRNA (0.1–0.2 ml/g). Current records were then recorded at 20°C by standard two-microelectrode techniques using a voltage-clamp amplifier (GeneClamp 500; Axon Instruments) interfaced with a 16-bit AD/DA converter (Digidata 1200A; Axon Instruments). Electrodes filled with 140 mM KCl had an electric resistance of 0.5–1 MΩ. Voltage pulses were delivered every 15 s from a holding potential of −80 mV. Current records were sampled at 10 kHz and low-pass-filtered at 2 kHz using an eight-pole Bessel filter and stored on computer for subsequent analysis. The extracellular recording solution contained (in mM): 88 NaCl, 10 KCl, 2 MgCl₂, 0.5 CaCl₂, 0.5 niflumic acid, 5 HEPES, 0.1% bovine serum albumin, pH 7.4 (NaOH). Leak and capacitive currents were subtracted on-line by a P/4 protocol.

**RESULTS AND DISCUSSION**

**Solid-phase Synthesis and Physicochemical Characterization of MTX Tyr and MTX Pi1**—Stepwise assembly of MTX Tyr was achieved by means of Fmoc/t-butyI chemistry (19). For Tyr₁⁻², we used the more acid-resistant, but TFMSA-sensitive, 2,6-dichloro-benzyl side-chain protecting group that is not cleaved by trifluoroacetic acid treatment. A double coupling strategy was applied with Fmoc-amino acid hydroxybenzotriazole active esters. The yield of assembly ranged between 80 and 90%. Fig. 2 illustrates the elution profiles by C₁₈ reversed-phase HPLC of MTX Tyr and MTX Pi₁ at different steps of the synthesis: crude reduced MTX Tyr, after trifluoroacetic treatment (A), crude oxidized MTX Tyr, after oxidative folding (B), MTX Pi₁ resulting from TFMSA treatment of MTX Tyr (C), and purified MTX Pi₁ (D). These data suggest that the chemical strategy elaborated to synthesize MTX Pi₁ appears to be successful. However, a careful physicochemical characterization was required, especially to formally establish that MTX Pi₁ exhibits the Pi₁-like disulfide bridging.

First the relative molecular mass of purified MTX Pi₁ was verified by matrix-assisted laser desorption ionization–time of flight mass spectrometry analysis (Fig. 3A). An experimental $M_r$ (M+H)⁺ value of 3613.1 was obtained for MTX Pi₁, in close agreement with its deduced $M_r$ (M+H)⁺ of 3613.3. As expected, this experimental value also agrees with the experimental $M_r$ (M+H)⁺ of 3613.3 obtained for MTX (29). According to amino acid analysis after acidolysis of MTX Pi₁, the amino acid ratios were similar to the deduced values (Fig. 3B). The primary structure of MTX Pi₁ was further verified by Edman sequencing (data not shown). To establish the half-cystine pairings of MTX Pi₁, the folded/oxidized peptide was treated with a mixture of trypsin and chymotrypsin. As shown in Fig. 3C, the data demonstrate that, contrary to MTX, MTX Pi₁ exhibits half-cystine pairings between Cys³⁻⁴, Cys⁹⁻¹₀, Cys¹³⁻¹⁴, and Cys¹⁸⁻¹⁹ (which corresponds to the standard C₁⁻C₅, C₂⁻C₆, C₃⁻C₇, and C₄⁻C₈ Pi₁-like pairings). Thus, as expected, MTX Pi₁ differs from MTX by the two last disulfide bridges.
Properties of MTX Variant with Pi1-like Disulfide Bridging

(C3-C7 and C4-C8, instead of C3-C4 and C7-C8) and adopts a conventional pattern of disulfide bridging that is identical to those of other characterized H9251-KTx6 toxins (Fig. 3).

Structural Properties of MTX Pi1—

The CD spectrum of MTX Pi1 was recorded to assess its secondary structures and was compared with the CD spectra of MTX and Pi1 (Fig. 4A). Measurements were performed at a wavelength ranging from 185–260 nm. The data obtained correspond essentially to $\pi^*$ and n-$\pi^*$ transitions of the amide chromophores of the peptide backbones (30). The CD spectra show large negative contributions between 207 and 230 nm and large positive contributions around 190 nm, indicating the presence of both $\alpha$-helical and $\beta$-sheet structures. These data are consistent with peptide backbone folding according to $\alpha$-$\beta$ scaffolds (12) for MTX, MTX Pi1, and Pi1. However, the CD spectra analyses do not point to obvious structural changes between MTX Pi1 and MTX. For the sake of comparison with the three-dimensional structure of MTX (11), we therefore generated a computed molecular model of MTX Pi1. This model was obtained using MTX as a template; it was relaxed, minimized, and validated as described under “Experimental Procedures.” As shown in Fig. 4B, the Ca backbone of MTX Pi1 does not differ markedly from that of MTX despite the important differences in half-cystine pairings. In contrast, a detailed examination of the side chains of a number of trifunctional amino acid residues suggests some marked differences in their orientations (Fig. 4C). These structural changes may nevertheless be sufficient to significantly impact peptide pharmacology.

Pharmacology of MTX Pi1—

MTX Pi1 was tested in vivo for neurotoxicity by intracerebroventricular injections in C57/BL6 mice. It is lethal in mice, with an LD50 value of 90 ng/mouse. In comparison, the LD50 values of MTX (2) and Pi1 (31) are 80 and 200 ng per mouse, respectively. MTX Pi1 remains as fully active in vivo, indicating that both disulfide bridge patterns provide peptides of equipotent lethality. Interestingly, oxidized MTX Tyr, the intermediate reaction product that generates MTX Pi1 upon TFMSA treatment, is 9-fold less potent than MTX Pi1 for lethal activity in mice, with an LD50 value of 800 ng/mouse. This result suggests that the integrity of Tyr32 is key to expression of MTX Pi1 lethality.

To investigate the pharmacology of MTX Pi1, we first tested its ability to compete with $^{125}$I-apamin for binding onto SK channels of rat brain synaptosomes (Fig. 5). MTX Pi1 inhibits $^{125}$I-apamin binding with an IC50 value of 17.4 ± 5.6 nM, which is about 4-fold less potent than MTX, which exhibits an IC50 value of 4.4 ± 3.1 nM, in agreement with previous data (2). Therefore, the disulfide bridge pattern of the peptide (MTX-type versus Pi1-type) appears to mildly affect its binding onto rat brain apamin-sensitive SK channels. Additionally, the presence of the 2,6 dichloro-benzyl protecting group on Tyr32 sig-

![Chemical modification of Tyr32 alters MTX pharmacology.](http://www.jbc.org/Downloaded from UNIVERSITAETSBIBLIOTHEK Ulm on April 7, 2020)
Properties of MTX Variant with Pi1-like Disulfide Bridging

Fig. 8. Map detailing major molecular contacts between MTXPi1 and Shaker B channel. A, space-filling representation of the molecular model of MTXPi1. B, space-filling representation of the molecular model of Shaker B channel (S5-H5-S6 pore regions). The pore regions of each four α-subunits composing the ion channel are labeled A, B, C, and D. Identical color codes were used to highlight pairs of interacting amino acid residues in panels A and B. Numbers represent the positions of the specified amino acid residues within either MTXPi1 (A) or Shaker B channel (B) primary structure. The docking of MTXPi1 onto the Shaker B channel can be imagined by a 180° vertical rotation of MTXPi1, from left to right. Note that the scale of magnification between panels A and B is different.

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significantly decreased the ability of the peptide to compete with 125I-apamin for binding to SK channel (IC50 value of 2.6 ± 0.3 μM; 150-fold less potent).

Next, we tested the effects of MTXPi1 and MTX-Tyr onto Shaker B, rat K1.1, K1.2, and K1.3 expressed in Xenopus oocytes, because they are the regular targets of MTX (1, 2). As shown in Fig. 6A, MTXPi1 blocks Shaker B K+ outward currents with high affinity. The peptide starts to be active at 10 pM concentration and achieves the highest current block (97.7%) at about 10 nM. The effect of 10 nM MTXPi1 is readily reversible upon washout of the peptide (Fig. 6B). The effect of MTXPi1 is concentration-dependent with an IC50 value of current inhibition of 0.24 ± 0.12 nM (n = 63; Fig. 6C). This should be compared with the effect of MTX, which acts on Shaker B channels with an IC50 of 3.4 nM (9) in identical experimental conditions. These data indicate that the MTX peptide is ~14-fold more potent in binding onto Shaker B channels when reticulated with Pi1-like half-cystine pairings rather than with its wild-type pairings. For rat K1.2 channels, the extent of K+ current blockage by MTXPi1 is maximal with an IC50 value of 2.8 ± 2.1 nM. Compared with MTX, these values correspond to a 46-fold reduction in affinity but to an increase of about 30% in the extent of blockage (16). Altogether, the data obtained for Shaker B and K1.2 channels suggest that the change in disulfide bridging of the MTX peptide is accompanied, not only by modifications in affinity, but also by changes in the combined efficacy of ionic pore occlusion and K+ efflux by the peptide. This analysis is reinforced by examining the effect of MTXPi1 on rat K1.3 K+ currents (Fig. 6E). MTXPi1 interacts with K1.3 channels with an IC50 value of 102 ± 37 nM (n = 63), which represents a 3-fold increase in affinity as compared with MTX (16). Interestingly, MTXPi1 also blocks the K+ efflux to a greater extent (83 ± 4%) than MTX (~20%). A similar change in blocking efficacy toward K1.3 channel had already been observed with a three-disulfide-bridged MTX analog (16), suggesting that the peptide half-cystine pairing pattern may significantly affect ion channel pore occlusion. Finally, we also investigated the effect of MTXPi1 on rat K1.1 K+ currents (Fig. 6F) and found it to be mostly inactive, as reported for MTX (16).

To get some insight on the contribution of Tyr32 residue to MTXPi1 pharmacology, we also investigated the effects of folded/oxidized MTX-Tyr on the various voltage-gated K+ channels (Fig. 7). Interesting marked differences in the pharmacological properties of this peptide were observed, as compared with those of MTXPi1. Tyr32 appears to be key with regard to MTXPi1 affinity for Shaker B channel but not for the extent of K+ current blockage (Fig. 7, A and B). Indeed, with an IC50 value of 1,229 ± 41 nM (n = 70), the folded/oxidized MTX-Tyr is about 5,000-fold less potent than MTXPi1 for K+ channel interaction. For rat K1.2 channels, an inverted situation is observed (Fig. 7C). The IC50 value obtained for MTX-Tyr is grossly similar to that of MTXPi1 (6.5 ± 3.6 nM and 2.8 ± 2.1 nM, respectively), contrary to the extent of current blockage, which is markedly decreased from 100% to 34 ± 3% in the case of MTX-Tyr. These findings further support a key role of MTX Tyr32 residue for toxin effect on K1.2 channel, as reported previously (8). In contrast, the presence of a 2,6-dichloro-benzyl moiety on the Tyr32 phenol ring has no significant impact on rat K1.3 (Fig. 7D) or K1.1 (Fig. 7E) K+ channel pharmacology.

Docking of MTXPi1 onto Voltage-gated K+ Channels—We first performed a Blastp (V.2.2.5, us.expasy.org/tools/blast/) search against the whole Protein Data Bank to select the correct template to generate models of the S5-H5-S6 portions of rat K1.1, K1.2, K1.3, and Shaker B channels. The KcsA primary structure (Swiss-Prot number 1BL8) showed the best E-value score for all the voltage-gated K+ channels under consideration. In addition, CLUSTALW (V.1.82) amino acid sequence alignments indicate that KcsA channel is a premium template that presents sequence homologies of 69.8% (K1.1), 70.1% (K1.2), 69.1% (K1.3), and 66.5% (Shaker B). The three-dimensional structures of the molecular models generated were very similar to that of KcsA, with root mean square deviation values of 0.48 Å (K1.1), 1.93 Å (K1.2), 0.32 Å (K1.3), and 1.68 Å (Shaker B). The geometric quality of the models was assessed...
by the PROCHECK software (V.3.5.4). No amino acid residue was found to be in disallowed regions, thereby validating the structural properties of the models (data not shown). The three-dimensional structure of MTX (11) and the molecular models of both MTX Pi1 and Pi1 were used in docking experiments with the different models of voltage-gated K⁺ channels. Docking energies were calculated according to the procedures described under “Experimental Procedures.”

We first detailed the docking of MTX Pi1 on Shaker B channel as it exerts its highest affinity toward this K⁺ channel subtype (IC₅₀ value of 0.24 nM). Fig. 8 illustrates the amino acid residues of MTX Pi1 (Fig. 8A) that may interact with Shaker B channel residues (Fig. 8B), as identified according to docking simulation. It is worth noting that the Lys²³ and Tyr²⁸ residues of MTX Pi1 belong to the functional dyad that is reported to be crucial for toxin bioactivity (8, 9, 32).

Docking simulations suggest that MTX Pi1 and MTX possess similar overall interaction topologies. For example, the Lys⁷ and Lys²³ residues share the same interacting residues on Shaker B channel (the pair Thr⁴⁰⁶ and Val⁴⁰⁸ for Lys⁷, and Thr⁴⁰⁷ for Lys²³; data not shown for MTX). Interestingly, additional analyses show that MTX Pi1 possess specific molecular contacts (Asn²⁶ with Gly⁴⁰⁴ and Asp⁴⁰⁵) that are not observed in the MTX docking simulations. Moreover, MTX Pi1 seems to be more stabilized than MTX on Shaker B channel because of a greater number of molecular contacts with the outer loop domain (Glu³⁸⁰, Asn³⁸¹, and Ser³⁸²). One should note that Lys⁷⁷ of MTX Pi1 also interacts with Ser³⁷⁸ of Shaker B channel, whereas Lys²³ of MTX does not interact with any ion channel amino acid residue. This may reasonably explain the 14-fold difference in IC₅₀ values observed experimentally for MTX Pi1 and MTX. Next, we correlated the docking energies of MTX Pi1, MTX, and Pi1 on Shaker B channel with their experimentally observed IC₅₀ values (Fig. 9A). A high degree of correlation (r² = 0.97) was observed between docking energies and IC₅₀ values, which validates our overall molecular modeling approach. It also indicates that more detailed investigations of the interaction between MTX Pi1 and Shaker B channel will be permitted.

On K⁺,1.1 channel, MTX Pi1, MTX, and Pi1 are not significantly active. In agreement with these data, no satisfying docking simulations were obtained for these peptides. Thus, we next investigated the docking properties of the peptides on K⁺,1.2 channel (Fig. 9B). MTX Pi1 (IC₅₀ = 2.8 nM) is, respectively, 46- and 6-fold less active than MTX (IC₅₀ = 0.06 nM) and Pi1 (IC₅₀ = 0.44 nM) on K⁺,1.2. Docking simulations indicate that MTX Pi1, MTX, and Pi1 share basically a common interaction map with K⁺,1.2, although some subtle differences could be observed that may explain their distinct affinities. The Thr⁴, Lys⁷, and Asp²⁶ residues of both MTX and MTX Pi1 are in contact with identical amino acid residues, Gly⁷⁷ and Asp⁷⁸, of the K⁺,1.2 ion channel pore (data not shown). The major difference concerns Lys⁷⁷, which stabilizes MTX over the pore surface by interacting with Ser³⁸⁴. A previous study has also shown that Lys⁷⁷ of MTX (as well as Lys⁷⁷ and Lys⁸⁰) is crucial for K⁺,1.2 recognition (33). In contrast, according to our docking experiments, Lys⁷⁷ of MTX Pi1 does not interact with any K⁺,1.2 amino acid residue. This difference in Lys⁷⁷ behavior for its interaction with K⁺,1.2 may thus explain the 46-fold decrease in K⁺,1.2 affinity of MTX Pi1 over MTX. Similar docking experiments were performed for Pi1 (data not shown). As for Shaker B channel, the docking experiments on K⁺,1.2 channel indicate a very good correlation (r² = 0.99) between the docking energies of MTX Pi1, MTX, and Pi1, and the experimental IC₅₀ values thereof (Fig. 9B).

Docking simulations performed with the three peptides on rat K⁺,1.3 channel correlate well with the actual peptide pharmacologies. Relatively low-scoring interactions between MTX Pi1 and K⁺,1.3 channel or MTX and K⁺,1.3 channel were found (data not shown), consistent with their experimental IC₅₀ values. In addition, computed data on Pi1 docking show the existence of very few contacts between Pi1 and the K⁺,1.3 ion channel subtype, we considered an IC₅₀ value of 100 μM for Pi1 (31).

Fig. 9. Linear regressions showing the correlation between docking energies and experimental IC₅₀ values. A, docking of MTX Pi1, MTX, and Pi1 onto Shaker B channel. B, same as in panel A, but rat K⁺,1.2 channel. C, same as in panel A, but rat K⁺,1.3 channel. For this ion channel subtype, we considered an IC₅₀ value of 100 μM for Pi1 (31).
In this study, we generated molecular models of the various voltage-gated K⁺ channels using the KcsA structure as template. The structure of a novel K⁺ channel (KvAP) from *Aeropyrum pernix* has recently been described, after our own structural analyses were completed (34). Of note, this K⁺ channel is voltage-dependent, contrary to the KcsA channel. However, a careful comparison of the pore regions (S₅-H₇-S₆ segments) of KvAP and KcsA reveals an almost perfect superimposition of the α-carbon traces of both channels. In addition, the selectivity filter is essentially identical to that of KcsA. Despite these marked structural similarities, we also generated molecular models of the *Shaker* B, K₁.2, and K₁.3 channels using the KvAP channel as template instead of KcsA. In each case, models generated using either KcsA or KvAP as template were identical (data not shown), thereby validating the use of KcsA as template in our study.

**Concluding Remarks**—In the present work, we show that, by using a particular strategy of solid-phase peptide synthesis, one can act on the final half-cystine pairing pattern of a reticulated peptide without altering its chemical structure by either mutations or chemical modifications of specific amino acid residues, or both. Docking experiments ease the understanding of the side chains of certain amino acid residues. There-

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A Maurotoxin with Constrained Standard Disulfide Bridging: INNOVATIVE STRATEGY OF CHEMICAL SYNTHESIS, PHARMACOLOGY, AND DOCKING ON K+ CHANNELS

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