*Biochemistry*

**Supplementary Material**

# Distinct disulfide isomers of μ-conotoxins KIIIA and KIIIB block voltage-gated sodium channels

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**Table S1. Sequences of the M-4 and M-5 branches of the μ-conotoxin superfamily**

| Conotoxin | Sequence | Reference |
|---|---|---|
| **M-4 branch** | | |
| μ-GIIIA | RD**CC**TOOKK-**C**KDRQ**C**KOQR-**CC**A* | Cruz *et al.* (1985) (*1*) |
| μ-GIIIB | RD**CC**TOORK-**C**KDRR**C**KOMK-**CC**A* | Cruz *et al.* (1985) (*1*) |
| μ-TIIIA | RHG**CC**KGOKG-**C**SSRE**C**ROQH-**CC*** | Lewis *et al.* (2007) (*2*) |
| μ-PIIIA | ZRL**CC**GFOKS-**C**RSRQ**C**KOHR-**CC*** | Safo *et al.* (2000) (*3*) |
| μ-SxIIIA | R**CC**TGKKGS**C**SGRA**C**KNLK-**CC**A* | Walewska *et al.* (2008) (*4*) |
| **M-5 branch** | | |
| μ-KIIIA | **CC**-N----**C**SSKW**C**RDHSR**CC*** | Bulaj *et al.* (2005) (*5*) |
| μ-KIIIB | NG**CC**-N----**C**SSKW**C**RDHSR**CC*** | This work |
| μ-SIIIA | ZN**CC**-NG--G**C**SSKW**C**RDHAR**CC*** | Bulaj *et al.* (2005) (*5*) |
| μ-SmIIIA | ZR**CC**-NGRRG**C**SSRW**C**RDHSR**CC*** | West *et al.* (2002) (*6*) |
| μ-CnIIIC | ZG**CC**-NGPKG**C**SSKW**C**RDHAR**CC*** | Favreau *et al.* (2012) (*7*) |
| μ-BuIIIB | VGER**CC**KNGKRG**C**G-RW**C**RDHSR**CC*** | Holford *et al.* (2009) (*8*) |

**Table S2. Prepropeptide sequences of μ-conotoxins determined from cDNA clones. □ indicates putative cleavage site to produce the mature conotoxin sequence, which is underlined.**

| Conotoxin | Sequence | Ref. |
|---|---|---|
| μ-PIIIA | MMSKLGVLLTICLLLFPITALPMDGDQPADRLAERMQDNISSEEHPFEKR□QRLCCGFPKSCRSRQCKPHRCCGR | Corpuz *et al.* (2005) (*9*) |
| μ-SIIIA | MSKLGVLLTICLLLFPLTALPMDEDQPADQLEDRMQDDISSEQYPSFVRR□QKCCGEGSSCPKYFKNNFICGCC | Wang *et al.* (2006) (*10*) |
| μ-BuIIIB | MMSKLGVLLTICLLLFPLFALPQDGDQPADRPAERMQDDISSEQNPLLEKR□VGERCCKNGKRGCGRWCRDHSRCCGRR | Holford *et al.* (2009) (*8*) |
| μ-GIIIA | MSKLGVLLTICLLLFPLTALPMDGDEPANRPVERMQDNISSEQYPLFEKR□RDCCTPPKKCKDRQCKPQRCCAGR | Corpuz *et al.* (2005) (*9*) |
| μ-KIIIB | ----------KR□NGCCN---CSSKWCRDHSRCCGR | This work |

In addition to the two major disulfide isomers characterized in this paper, the biological activity and structure of the disulfide-deficient analog μ-KIIIA[C1A,C9A], which has the [C2-C15,C4-C16] disulfide connectivity, were characterized previously.[22] Intercysteine NOEs observed for the previously published structures of this analog and μ-KIIIA[22] are summarized in Table S3. For μ-KIIIA, disulfide connectivities could not be determined unambiguously except for the [C4-C16] connectivity, with an NOE observed between $C^{\alpha}H$ of Cys16 and $C^{\beta}H$ of Cys4. $C^{\beta}H$-$C^{\beta}H$ NOEs from Cys2 to Cys9 could be overlapped with sequential NOEs between Arg10 $C^{\alpha}H$ and Cys9 $C^{\beta}H$. In contrast, intercysteine NOEs were more clearly defined for the μ-KIIIA[C1A,C9A] analog, in which only two disulfide bonds were present. In addition to intercysteine NOEs observed between Cys4 and Cys16, $C^{\beta}H$-$C^{\beta}H$ NOEs between Cys2 and Cys15 were observed for this analog, thus defining its disulfide connectivity.

**Table S3. Intercysteine NOEs observed for μ-KIIIA and μ-KIIIA[C1A,C9A]. * indicates undetermined intensity due to overlap with other cross peaks. + in parentheses indicates intensity of the NOE.**

μ-KIIIA

| Disulfide | HA-HA | HA-HB | HB-HB | HN-HB | Antidiagnostic HN-HA |
|---|---|---|---|---|---|
| C1-C15 | - | - | HB2 1 – HB2 15 (*) | - | - |
| C2-C9 | - | - | HB2 2 – HB2 9 (*)<br>HB2 2 – HB3 9 (*) | - | - |
| C4-C16 | - | HA 16 – HB3 4 (+) | HB2 16 – HB2 4 (*)<br>HB2 16 – HB3 4 (*) | - | - |

μ-KIIIA[C1A,C9A]

| Disulfide | HA-HA | HA-HB | HB-HB | HN-HB | Antidiagnostic HN-HA |
|---|---|---|---|---|---|
| C2-C15 | - | HA 2 – HB2 15 (*)<br>HA 2 – HB3 15 (*) | HB2 15 – HB3 2 (++) | - | - |
| C4-C16 | - | HA 16-HB3 4 (+) | HB2 16 – HB2 4 (++)<br>HB2 16 – HB3 4 (+) | - | - |

**Table S4. Block of $Na_V1.2$, 1.4 and 1.7 by μ-KIIIA-P2.**[a]

| $Na_V$ subtypes | $k_{off}$ ($min^{-1}$) | $k_{on}$[b] ($\mu M \cdot min)^{-1}$ | $K_d$[c] (μM) |
|---|---|---|---|
| $Na_V1.2$ | 0.0044 ± 0.0023 | 0.019 ± 0.002 | 0.23 ± 0.12 |
| $Na_V1.4$ | 0.083 ± 0.026 | 0.1 ± 0.02 | 0.83 ± 0.31 |
| $Na_V1.7$ | 0.011 ± 0.001 | 0.007 ± 0.004 | 1.57 ± 0.91 |

[a] Values (mean ± S.D, n ≥ 3 oocytes) were obtained by two-electrode voltage clamp of *Xenopus* oocytes expressing rat $Na_V1$ channels as described in Methods and Materials. [b] From $[k_{obs} - k_{off}]$ / 10μM. [c] From $k_{off}/k_{on}$.

**Determination of disulfide connectivity of μ-KIIIB isomer.**

The $MS^2$ spectrum of μ-KIIIB-P2 shows an identical spectrum to that of peak1 and peak2 of μ-KIIIA (Figure S3), with the exception of the ions containing the N-terminal Asn-Gly. In a similar manner, only two foldamers (F11, F14) fit all the $MS^2$ fragment ions. Subsequent $MS^3$ fragmentation of 1100.5, which is identical in structure with 929.5 of μ-KIIIA (with the N-terminal extension), reveal the disulfide connectivity. Beside the N-terminal residue losses, giving rise to 986.3 and 929.3, ions at m/z 843.1 and 656.1 are observed, as in the $MS^3$ spectrum of 929.3 in μ-KIIIA-P1. As in the case of μ-KIIIA-P1, the ion 656.1 clarifies the unambiguous assignment of the disulfide connectivity. In this case, ambiguity arising through the relatively low intensity of 656.1 is eliminated by the $MS^4$ fragmentation of 843.1 that shows the presence of the same ion. The scheme of events is summarized in Figure S4. An identical $MS^2$ spectra for Peak-1 of μ-KIIIB (Figure S5), leaves again F11 and 14 as the two probable foldamers. As F11 has already been assigned to P2, the disulfide connectivity of μ-KIIIB-P1 is therefore assigned as F14.


**Figure S1.** 15 possible foldamers of μ-KIIIA-P1

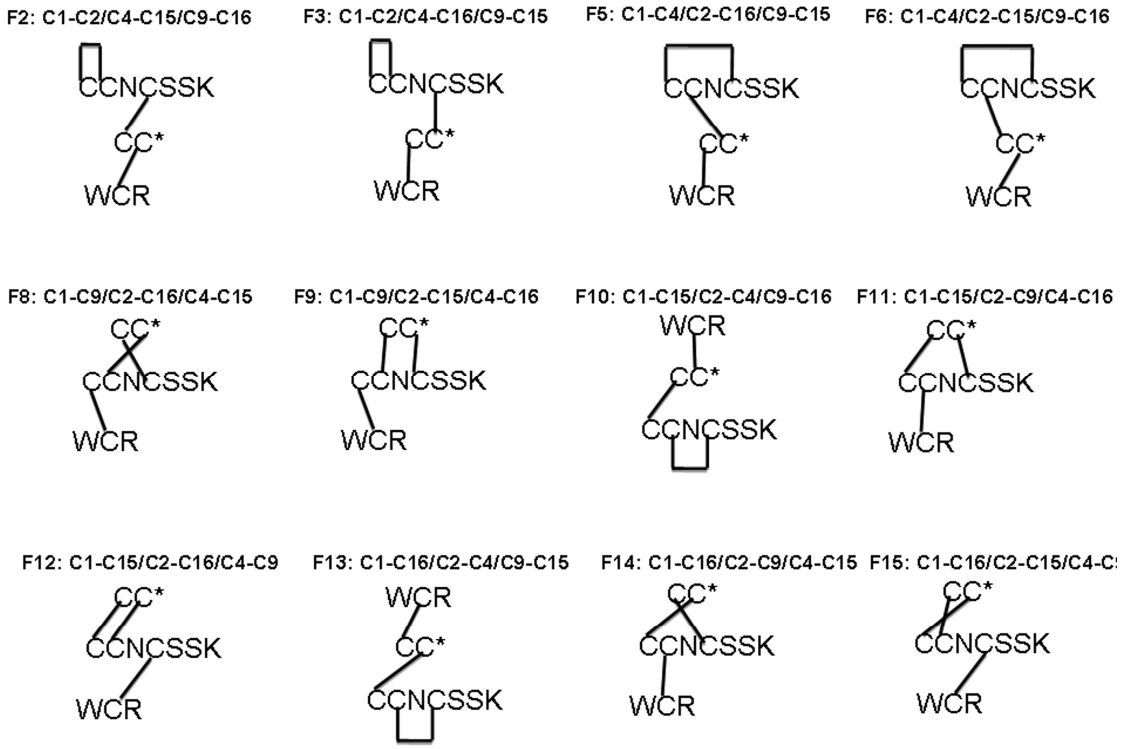


**Figure S2.** 12 possible foldamers of μ-KIIIA-P1 upon trypsin digestion

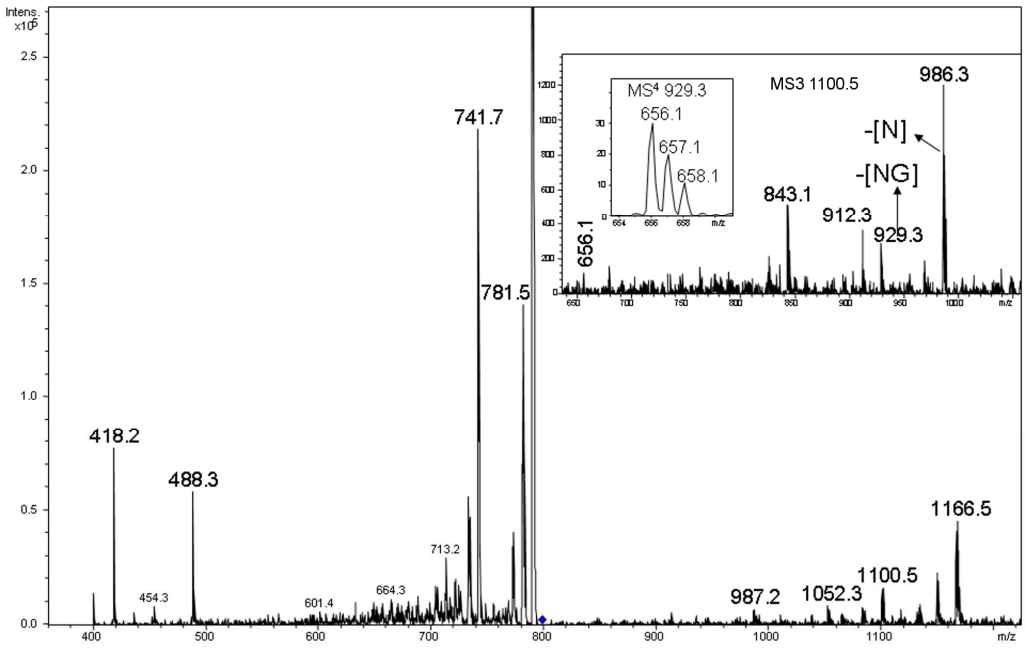


**Figure S3.** CID $MS^2$ spectrum of the peptide derived upon trypsin digestion of μ-KIIIB-P2. Inset shows the $MS^3$ spectra of 1101.5 and $MS^4$ of 843.1.

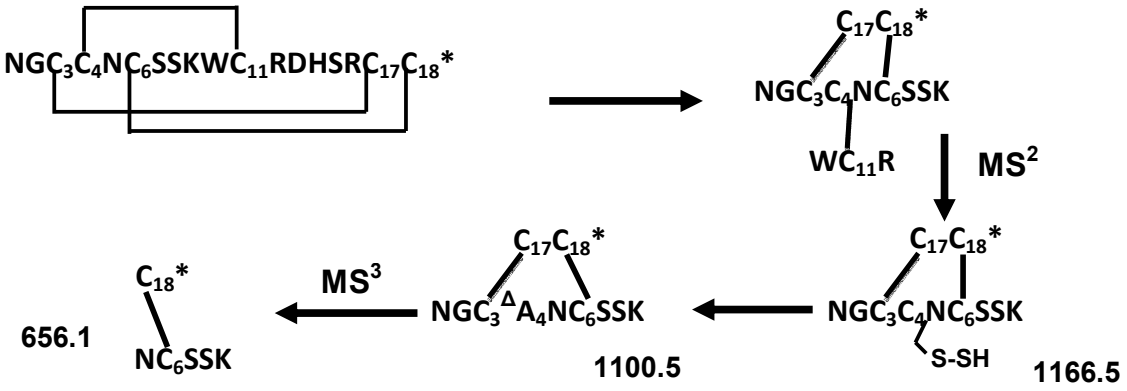


**Figure S4.** Assignments of the key $MS^n$ fragment ions of tryptic μ-KIIIB-P2.

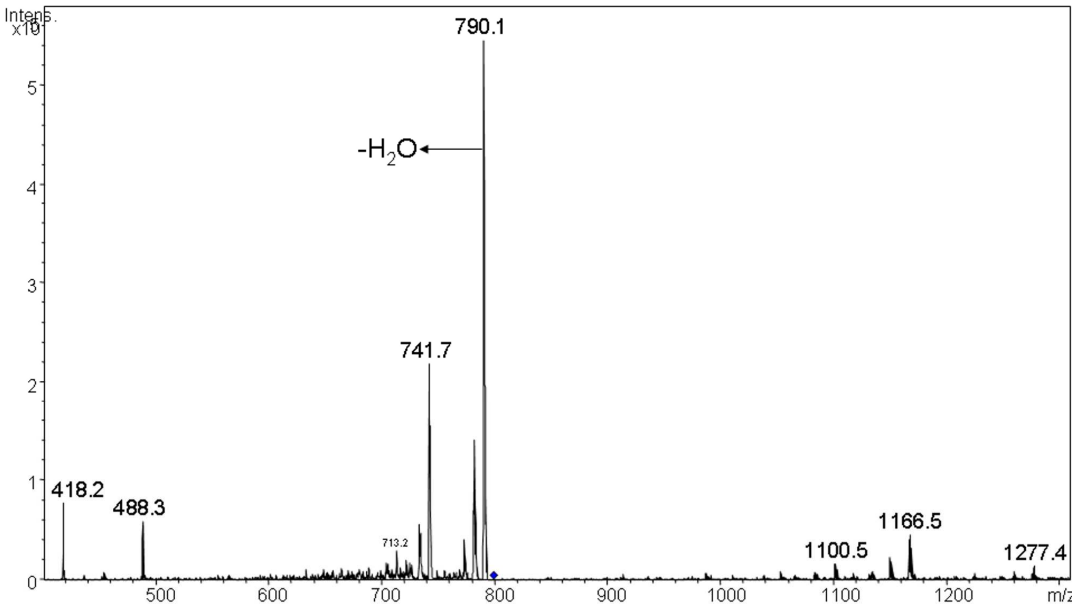


**Figure S5.** CID $MS^2$ spectrum of the peptide derived upon trypsin digestion of μ-KIIIB-P1.

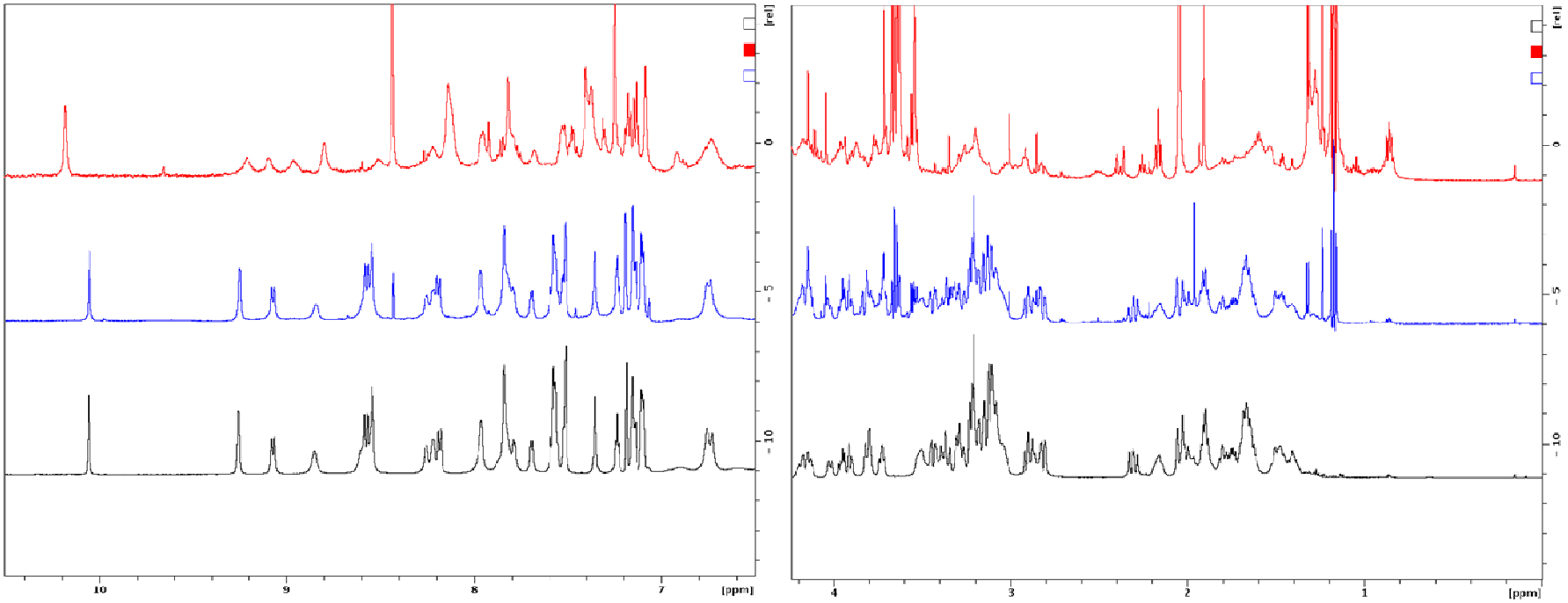


**Figure S6.** 1D $^{1}$H NMR spectra of μ-KIIIA-P1 (blue) and μ-KIIIA-P2 (red) recorded at 5 °C, pH 4.8 on a Bruker-DRX 600 spectrometer, compared with μ-KIIIA sample (black) for which solution structure was determined previously by Khoo *et al.* Left panel displays the amide region, right panel the aliphatic region.

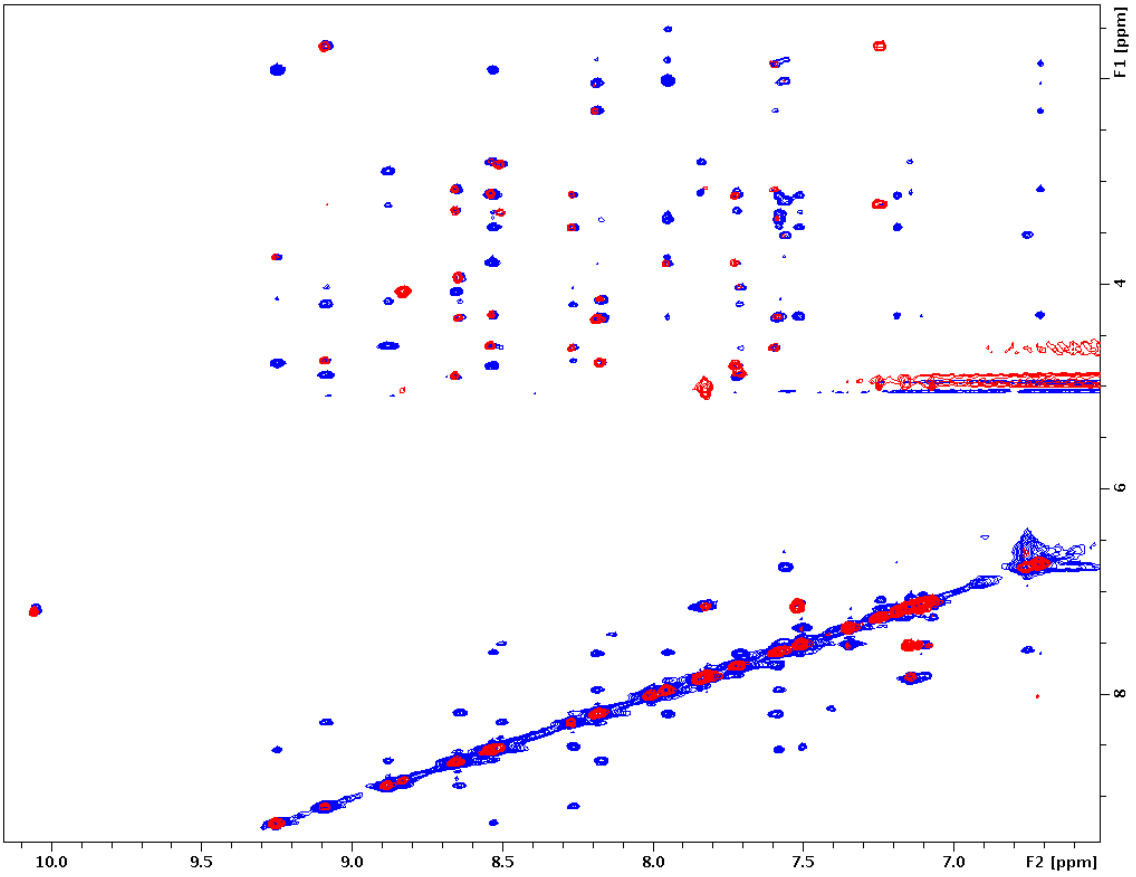


**Figure S7.** Amide and aromatic region of NOESY spectrum (blue) overlayed with TOCSY spectrum (red) for μ-KIIIB-P2 (at 5 °C and pH 4.8). NOESY (250 ms mixing time) and TOCSY (70 ms spin-lock time) spectra were acquired on a Bruker DRX-600 spectrometer.

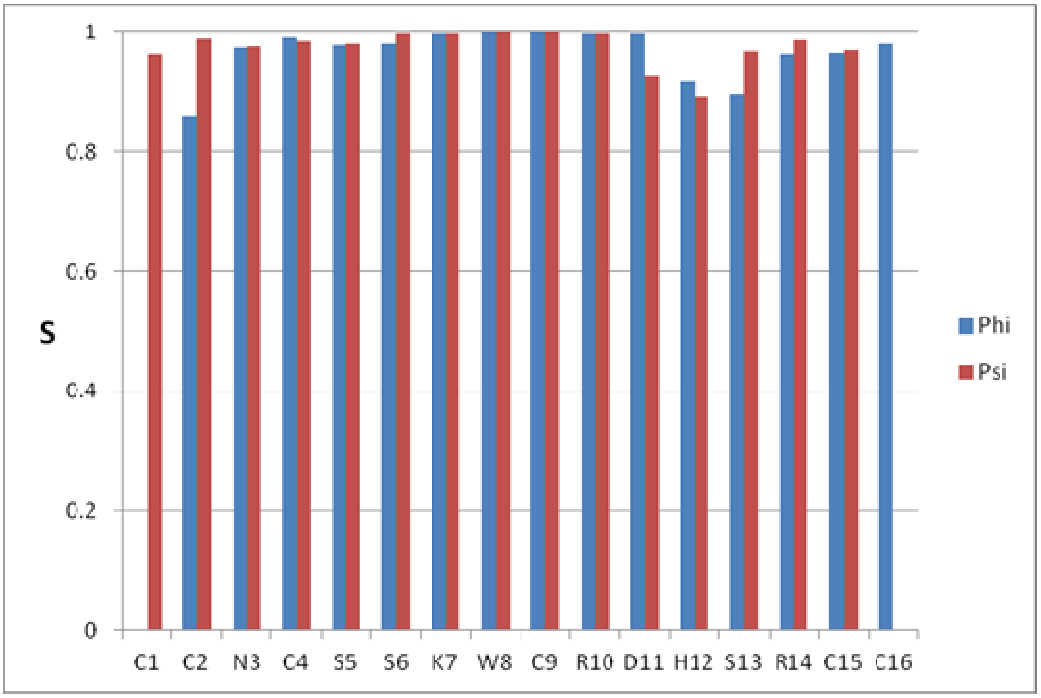


**Figure S8**. Angular order parameters (S) for the final 20 structures of μ-KIIIA[C1-C15,C2-C9,C4-C16] measured using MOLMOL for φ (blue) and ψ (red) backbone dihedral angles plotted as a function of residue.

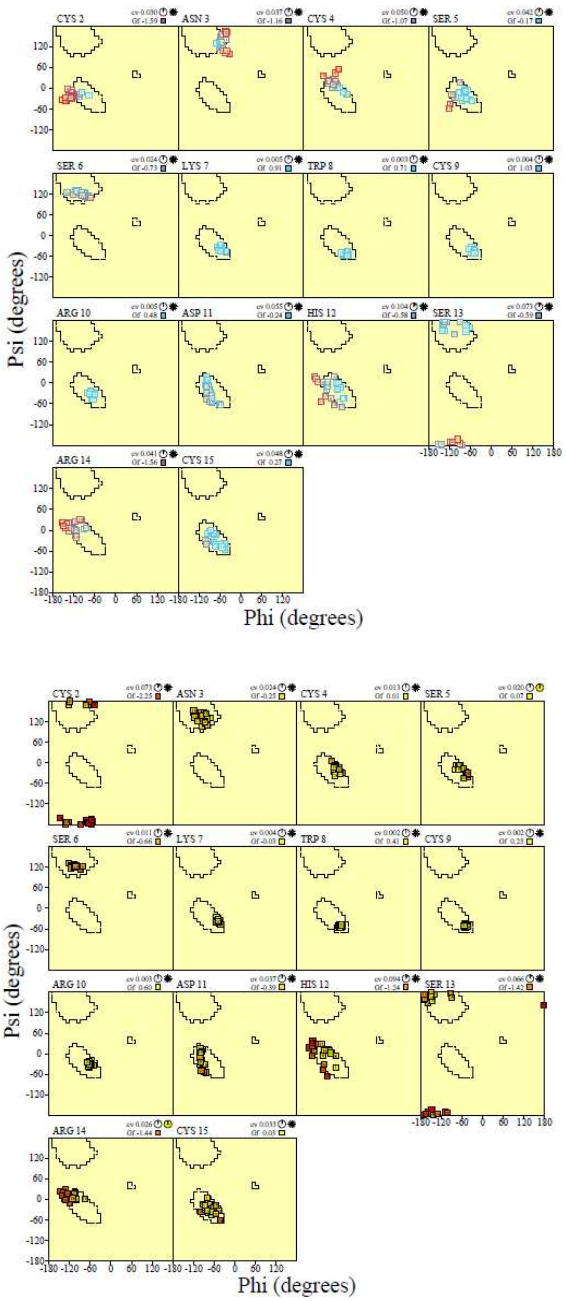


**Figure S9**. Residue Ramachandran plots of family of structures of μ-KIIIA[C1-C9,C2-C15,C4-C16] (top) and μ-KIIIA[C1-C15,C2-C9,C4-C16] (bottom)

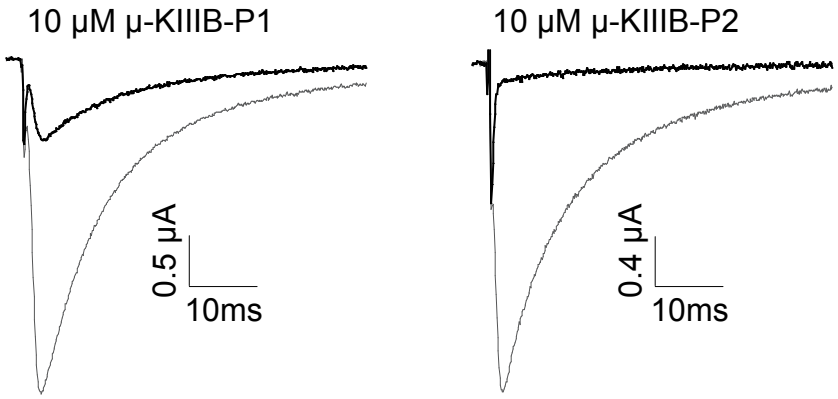


**Figure S10.** Representative sodium-current traces from voltage-clamped oocytes expressing rat $Na_V1.2$. µ-KIIIB-P1 (left) and µ-KIIIB-P2 (right) were each tested at a concentration of 10 µM. Each panel shows superimposed recordings before (control, *gray trace*) and after (*black trace*) 20-min exposure to the indicated peptide.

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