**Supporting information**

# Efficient enzymatic ligation of inhibitor cystine knot spider venom peptides: using sortase A to form double-knottins to probe voltage-gated sodium channel $Na_V1.7$

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**Figure S1.** Analytical HPLC traces obtained using 2%/min gradient 0–60% solvent B, with a flow rate of 0.3 mL/min for D1IA and gHwTx-IV, and 1%/min 0–60% solvent B, flow rate of 0.3mL/min for CcoTx-I and CcoTx-II (first 40 min of traces shown). Column is C18 analytical column (Grace Vydac, 150 x 2.1 mm, 5 µm; Thermo Fisher Scientific, Waltham, MA). Observed ESI-MS spectra and amide region of 1D $^{1}$H NMR spectra are also shown. D1Ia calculated mass = 3967.8; gHwTx-IV calculated mass = 4024.7; CcoTx-I calculated mass = 4044.6; CcoTx-II calculated mass = 4092.7.

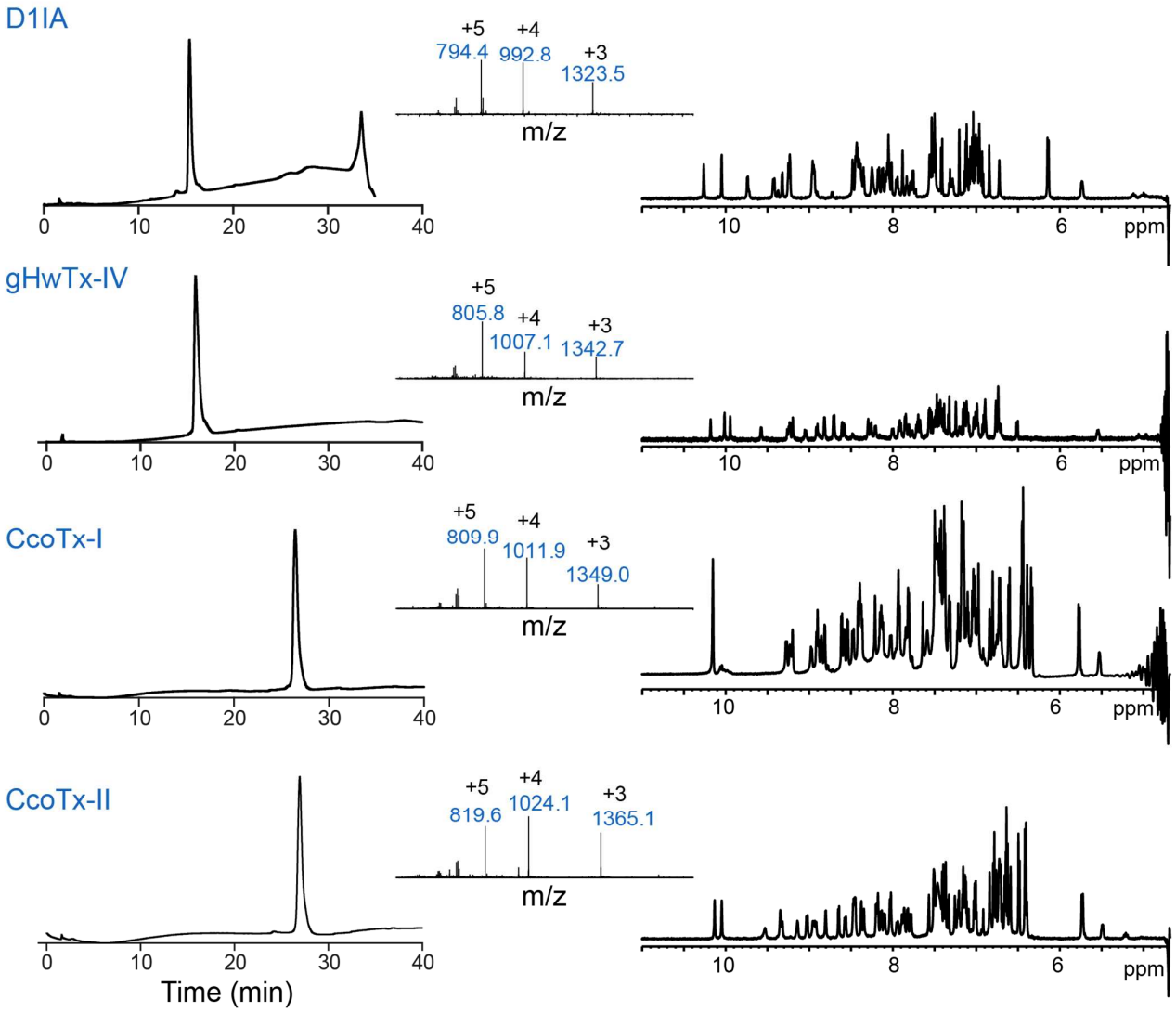


**Figure S2.** Analytical HPLC traces of oxidized monovalent peptides prior to ligation, obtained using 2%/min gradient 0–60% solvent B, with a flow rate of 0.3 mL/min for gHwTx-IV_GLPATGG, GGGG_gwTx-IV and D1Ia_GLPTGG, and a 1%/min gradient 0–60% solvent B, first 40 min shown for GGGG_D1Ia. Column is C18 analytical column (Grace Vydac, 150 x 2.1 mm, 5 μm; Thermo Fisher Scientific, Waltham, MA). Observed ESI-MS spectra and amide region of 1D $^{1}$H NMR spectra are also shown. gHwTx-IV_GLPATGG calculated mass = 4579.3; GGGG_gHwTx-IV calculated mass = 4253.0 D1Ia_GLPATGG calculated mass = 4522.3; GGGG_D1Ia calculated mass = 4196.0.

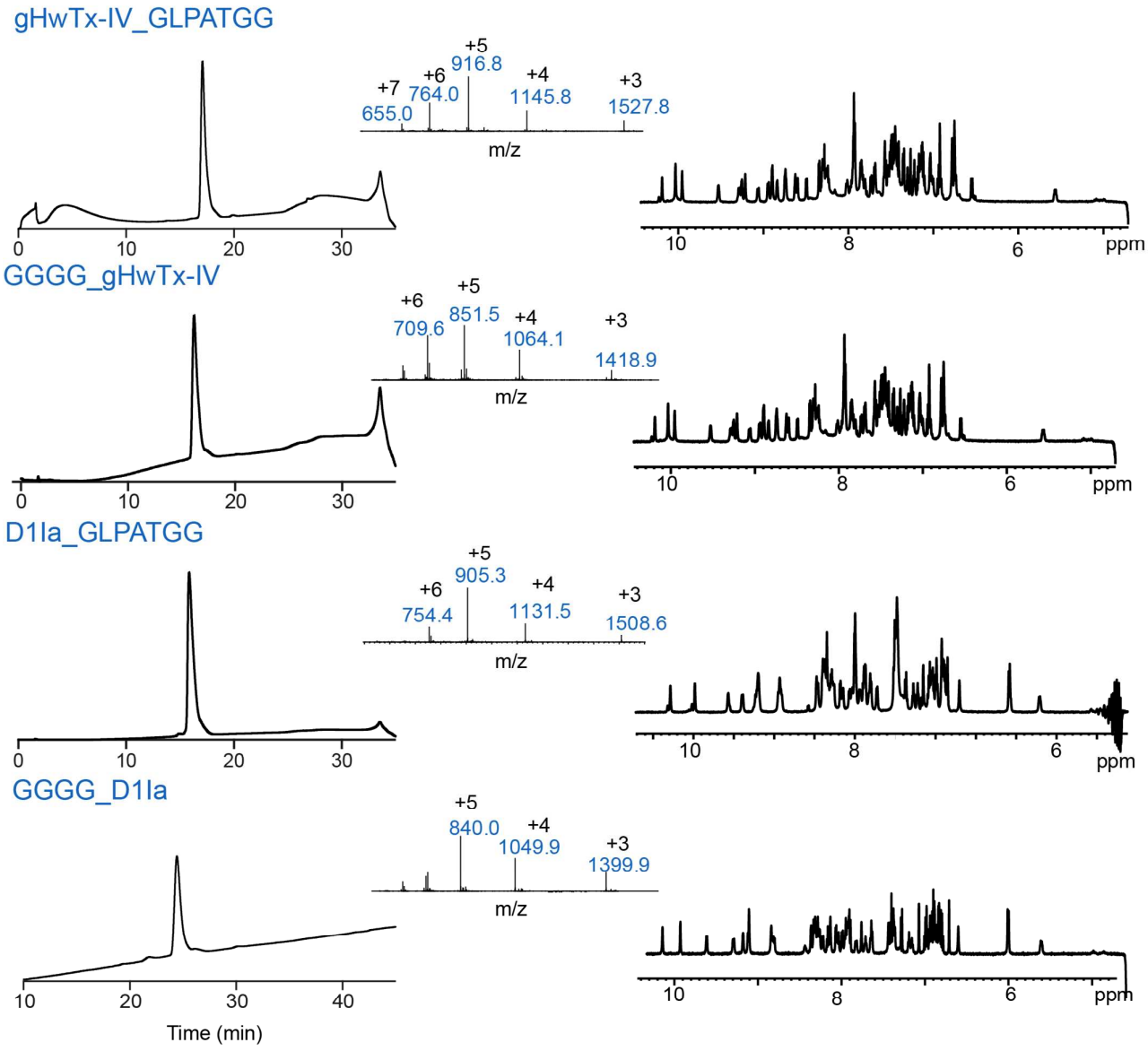


**Figure S3.** Analytical HPLC traces of ligated double-knottins obtained using 2%/min gradient 0–60% solvent B with a flow rate of 1 mL/min of DK02 and DK04, and a 1%/min gradient 0–60% solvent B for DK03 and DK05 (first 40 min shown). Column is C18 analytical column (Grace Vydac, 150 x 2.1 mm, 5 µm; Thermo Fisher Scientific, Waltham, MA). Observed ESI-MS spectra and amide region of 1D $^{1}$H NMR spectra are also shown. DK02 calculated mass = 8643.2; DK03 calculated mass = 8700.0; DK04 calculated mass = 8586.2; DK05 calculated mass = 8643.2.

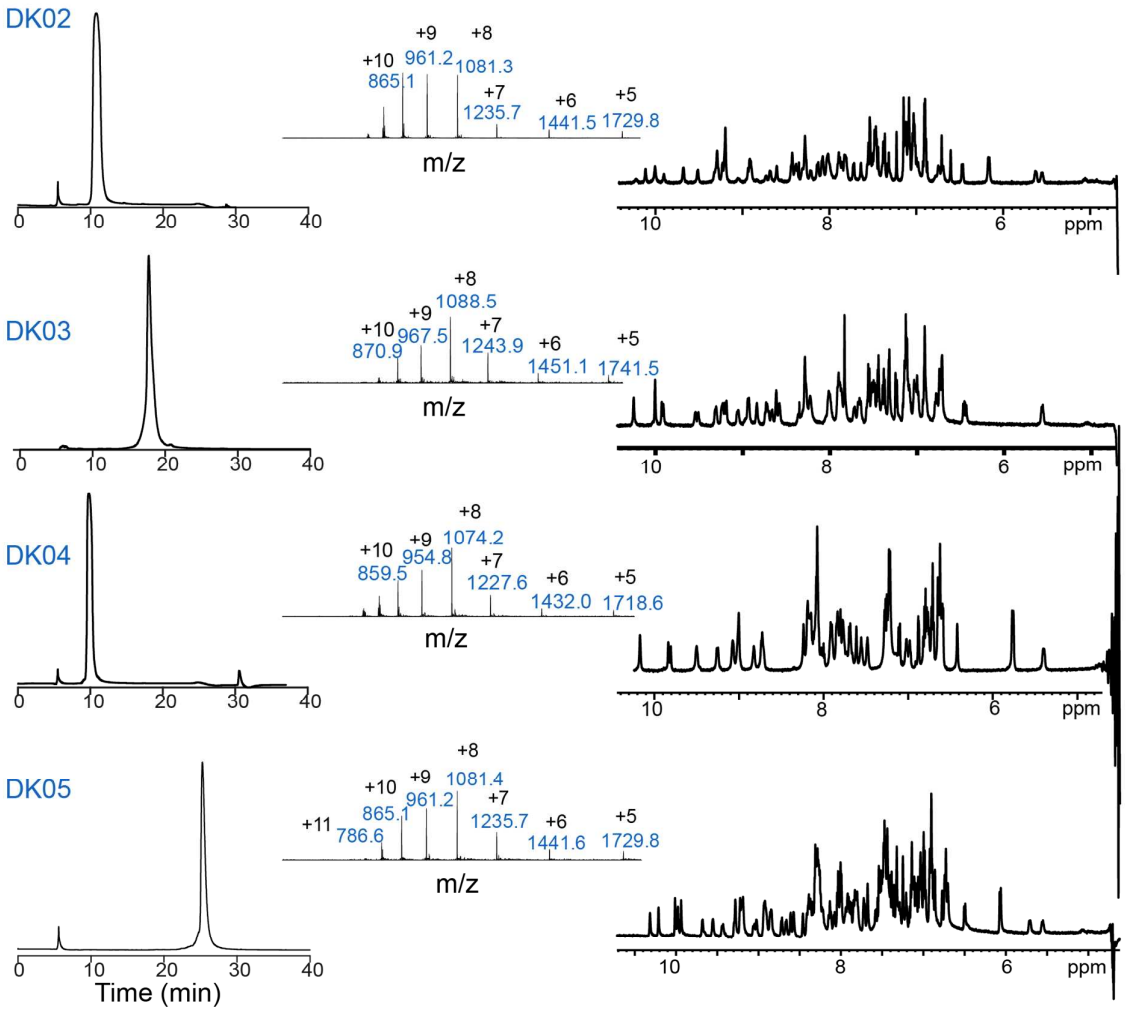
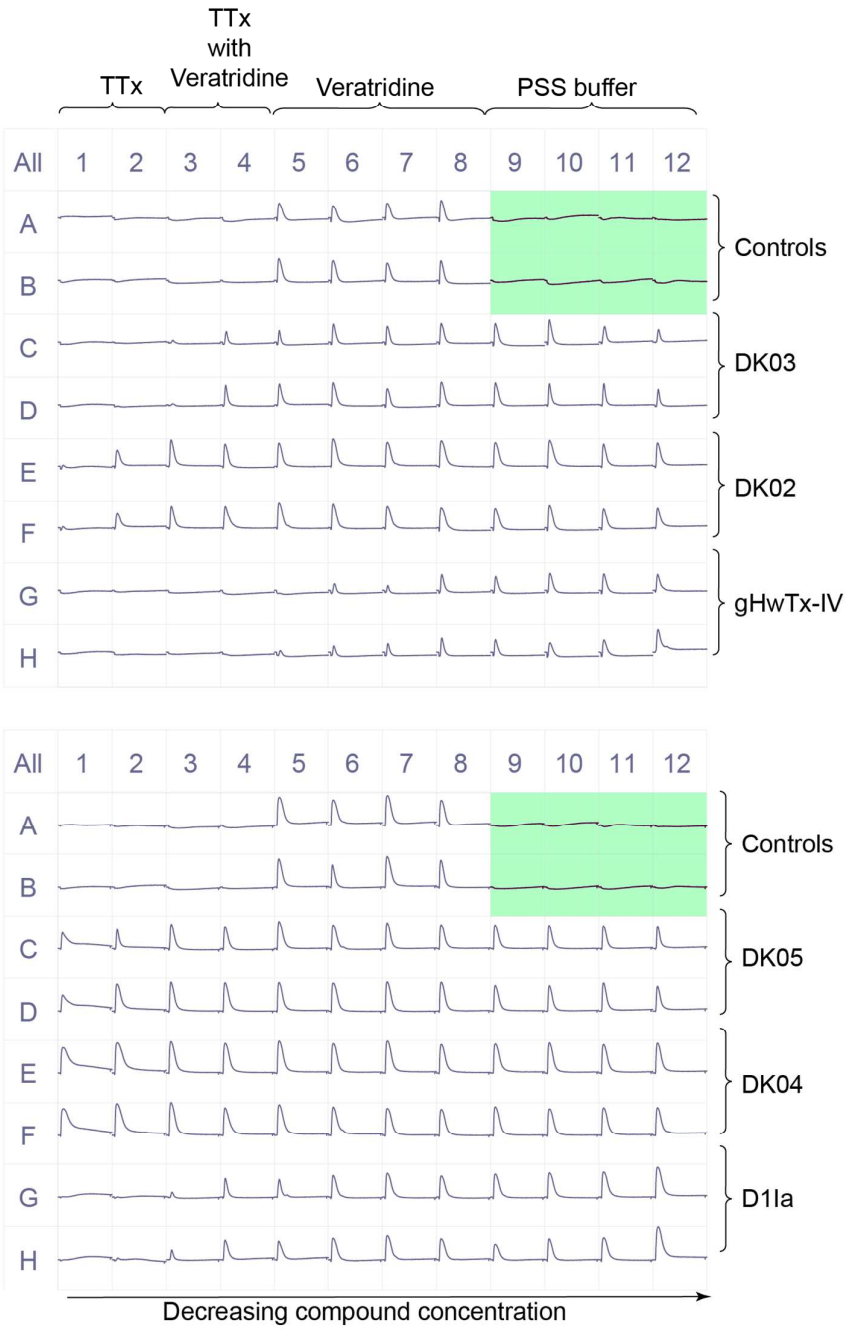


**Figure S4.** Example of raw data from a FLIPR$^{TETRA}$ assay. SH-SY5Y cells were incubated with scorpion toxin OD1 in PSS/BSA buffer and calcium-4 dye for 30 min prior to incubation of knottins for 15 min.[1] Veratridine was added to the cells and activity measured as the ability of a knottin to prevent veratridine activation. Influx of $Ca^{2+}$ into the cells is followed by fluorescence emission of the dye (excitation 470–495 nm and emission 515–575 nm) for 5 min. Tetrodotoxin (TTx) was used as a positive control.[1-2]



**References**

(1) Vetter, I.; Mozar, C. A.; Durek, T.; Wingerd, J. S.; Alewood, P. F.; Christie, M. J.; Lewis, R. J., Characterisation of $Na_V$ types endogenously expressed in human SH-SY5Y neuroblastoma cells. *Biochem. Pharmacol.* **2012,** *83* (11), 1562-1571.

(2) Agwa, A. J.; Lawrence, N.; Deplazes, E.; Cheneval, O.; Chen, R.; Craik, D. J.; Schroeder, C. I.; Henriques, S. T., Spider peptide toxin HwTx-IV engineered to bind to lipid membranes has an increased inhibitory potency at human voltage-gated sodium channel $hNa_V1.7$. *Biochim. Biophys. Acta* **2017,** *1859* (5), 835-844.