Electronic Supplementary Material (ESI) for RSC Medicinal Chemistry.

Supplementary Data for:

Posttranslational modifications of α-conotoxins: Sulfotyrosine and C-terminal amidation stabilise structures and increase acetylcholine receptor binding

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1. Mass spectra and analytical HPLC analyses of EpI, AnIB and PnIA variants.

ESI mass spectra were obtained in positive ion mode unless marked otherwise. Intensities were normalised to the highest intensity peak. Analytical RP-HPLC was carried out on a C18 analytical HPLC column (C18, 300 Å, 300 x 4.6 mm, 5 µm) using a 2%/min gradient of acetonitrile (0.045% TFA) in water (0.05% TFA). UV absorbance was detected at 214 nm (black traces) and 280 nm (red traces).

EpI[Y(SO$_3$)15Y]-OH

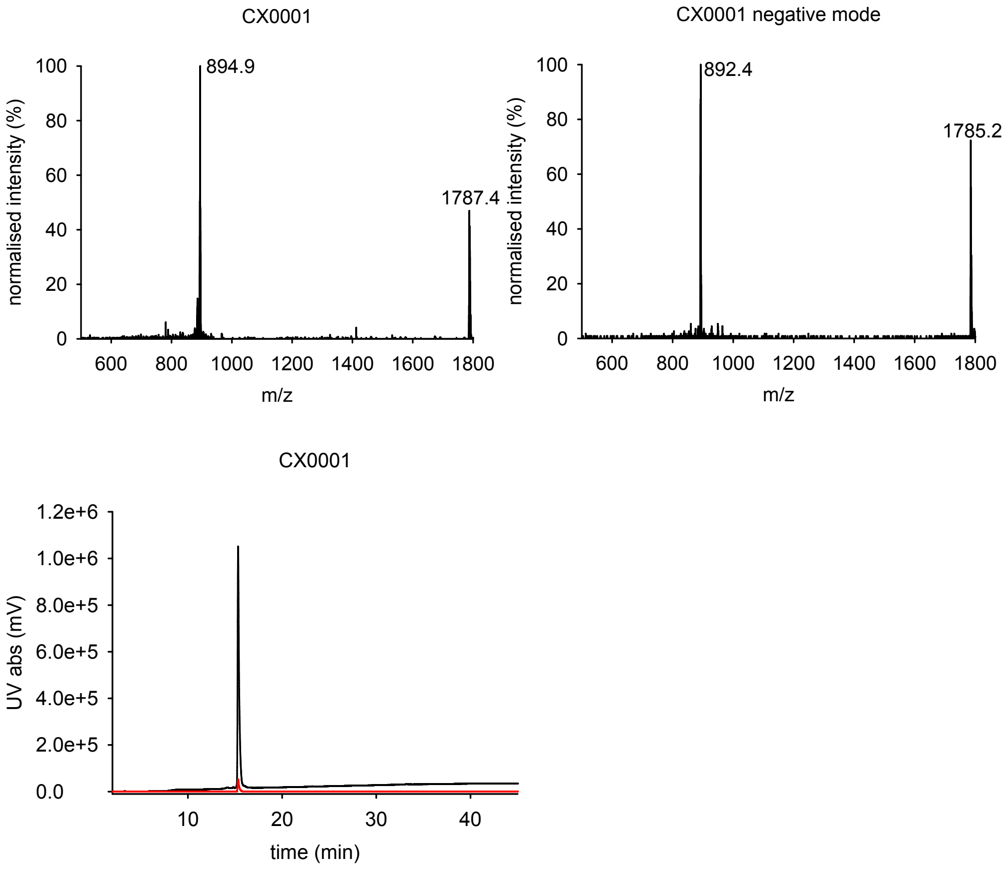


Figure S1a. *Mass spectra in positive and negative mode and analytical RP-HPLC trace for EpI[Y(SO$_3$)15Y]-OH. MW$_{calc}$: 1788.0, MW$_{obs}$: 1787.6.*

EpI[Y(SO_3)15Y]-NH_2

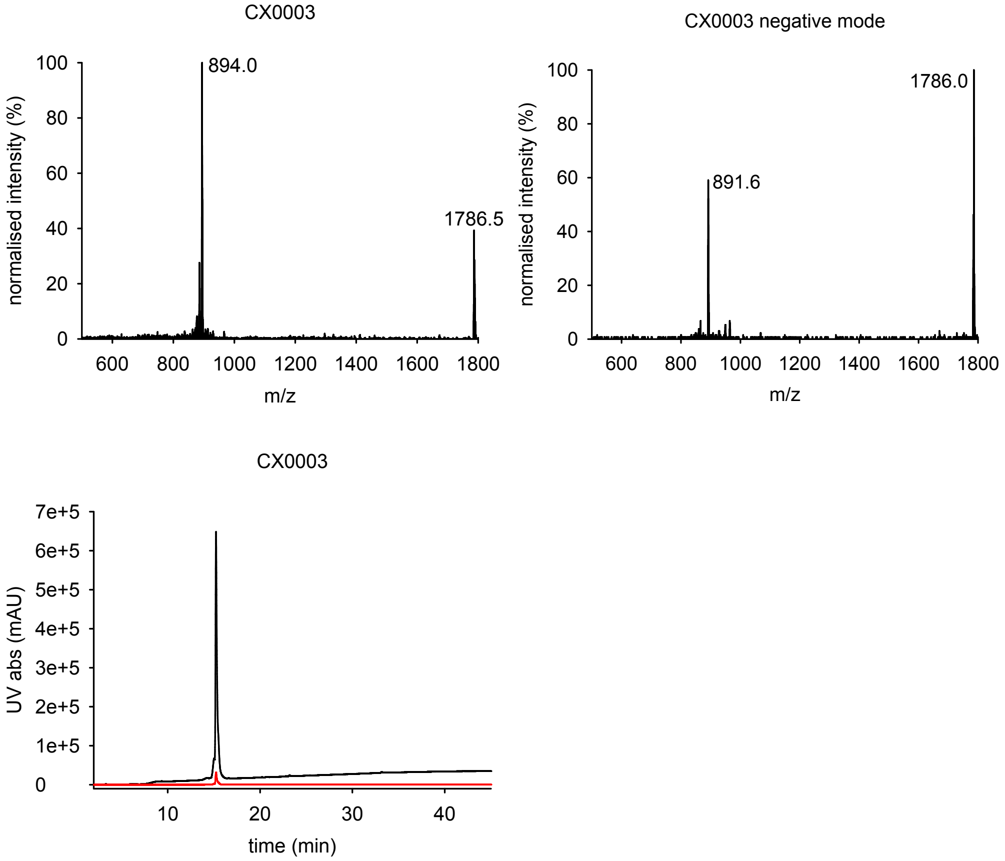
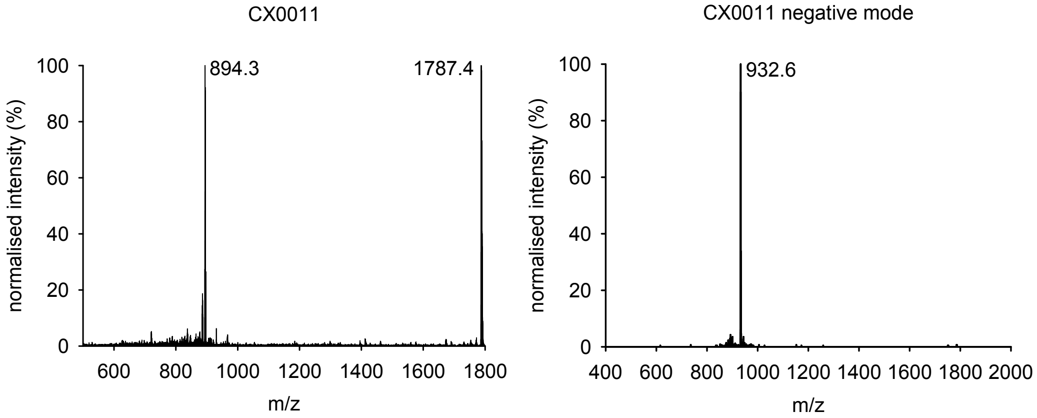


Figure S1b. *Mass spectra in positive and negative mode and analytical RP-HPLC trace for EpI[Y(SO_3)15Y]-NH_2. MW_{calc}: 1787.0, MW_{obs}: 1786.3.*

EpI-OH



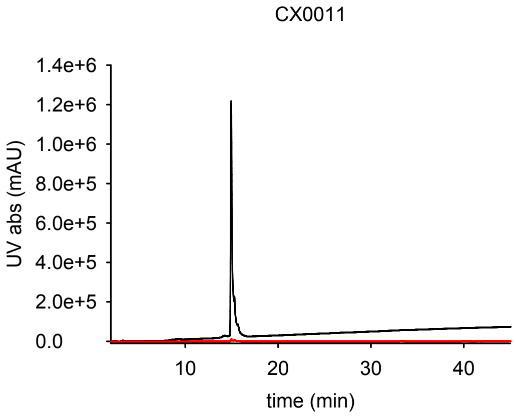


Figure S1c. *Mass spectra in positive and negative mode and analytical RP-HPLC trace for EpI-OH. MW*$_{calc}$*: 1867.0, MW*$_{obs}$*: 1787.0 (positive mode); 1867.2 (negative mode).*

EpI-NH$_2$

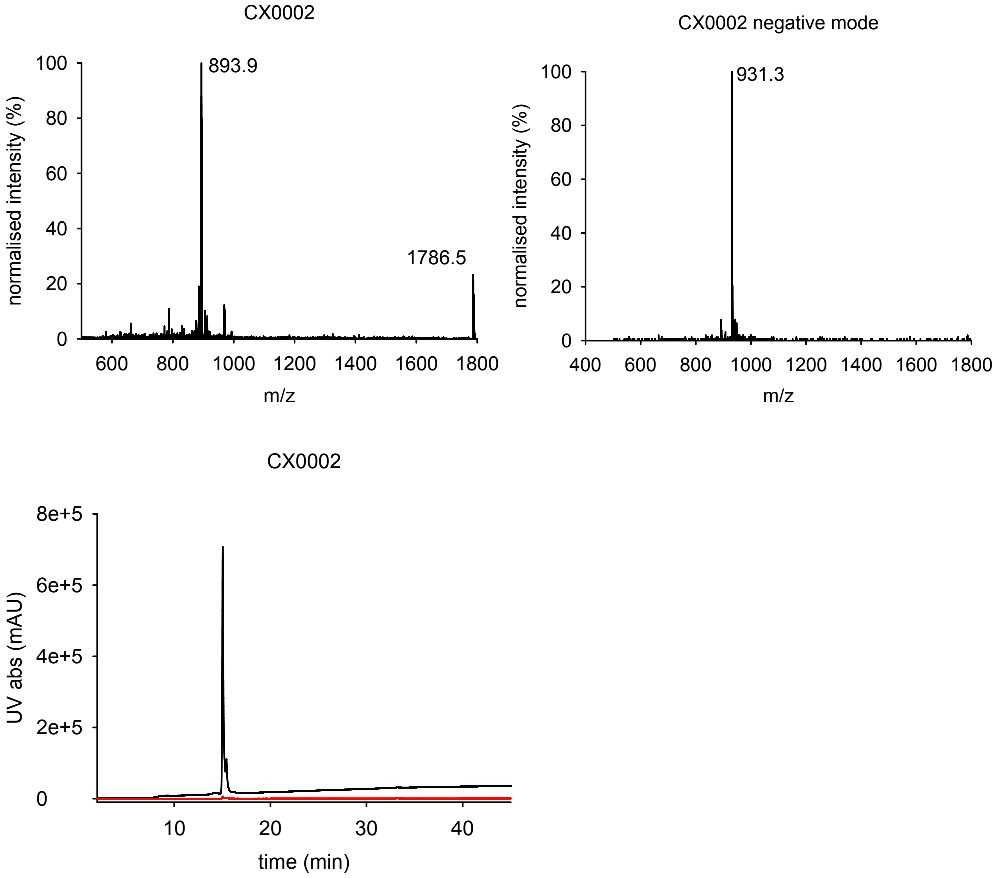


Figure S1d. *Mass spectra in positive and negative mode and analytical RP-HPLC trace for EpI-NH*$_2$*, MW*$_{calc}$*: 1866.0, MW*$_{obs}$*: 1786.2 (positive mode); 1864.6 (negative mode).*

AnIB[Y(SO$_3$)16Y]-OH

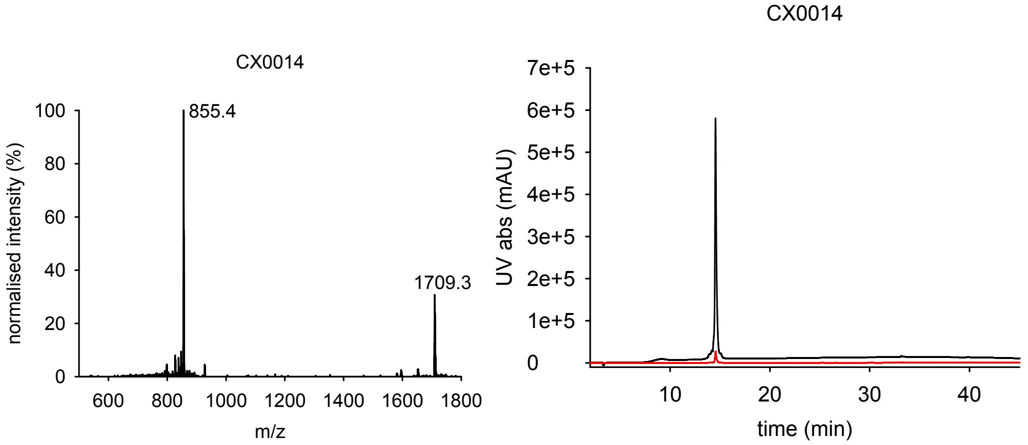


Figure S1e. *Mass spectrum and analytical RP-HPLC trace for AnIB[Y(SO$_3$)16Y]-OH. MW$_{calc}$: 1709.8, MW$_{obs}$: 1709.1.*

AnIB[Y(SO$_3$)16Y]-NH$_2$

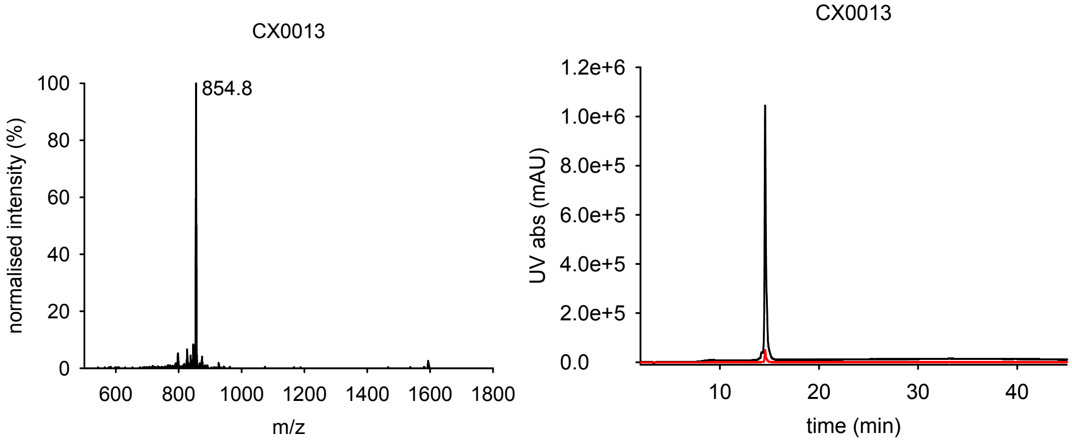


Figure S1f. *Mass spectrum and analytical RP-HPLC trace for AnIB[Y(SO$_3$)16Y]-NH$_2$. MW$_{calc}$: 1708.8, MW$_{obs}$: 1707.6.*

AnIB-OH

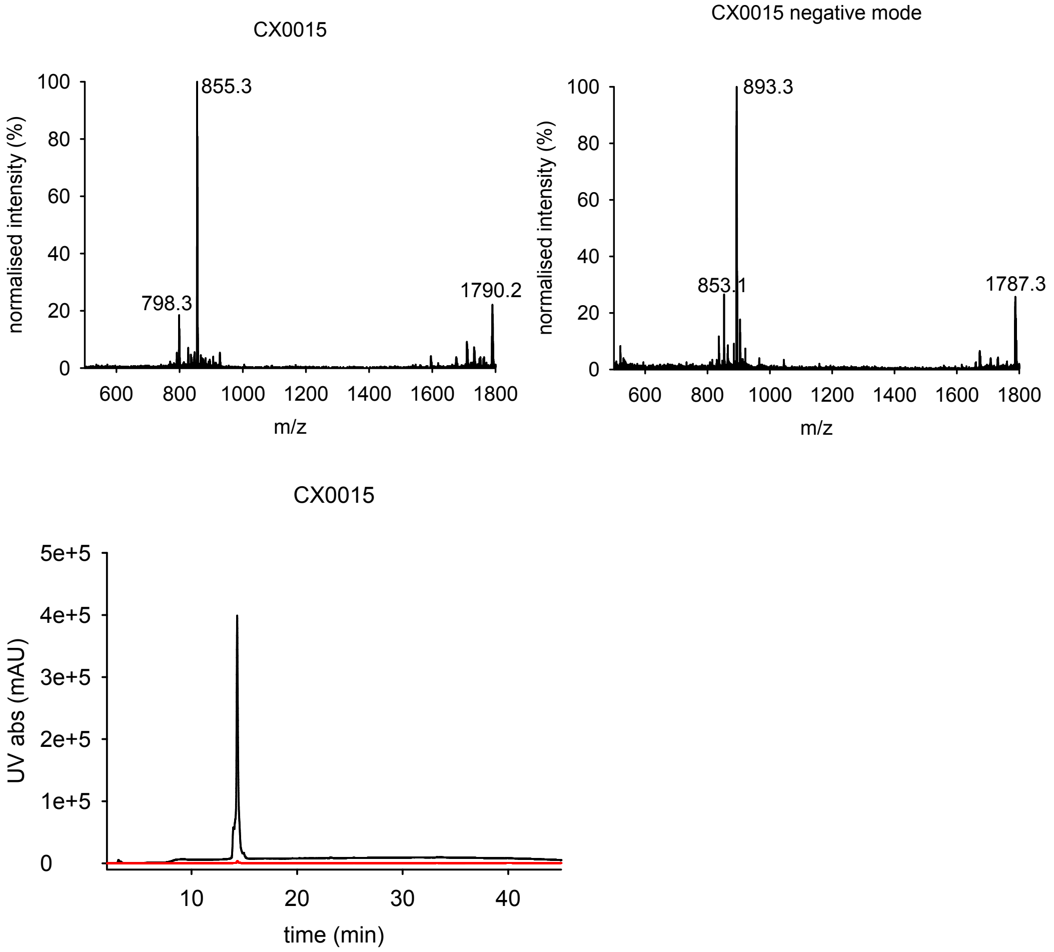


Figure S1g. *Mass spectra in positive and negative mode and analytical RP-HPLC trace for AnIB-OH.* MW_{calc}*: 1788.9,* MW_{obs}*: 1708.6 (positive mode); 1788.5 (negative mode).*

AnIB-NH$_2$

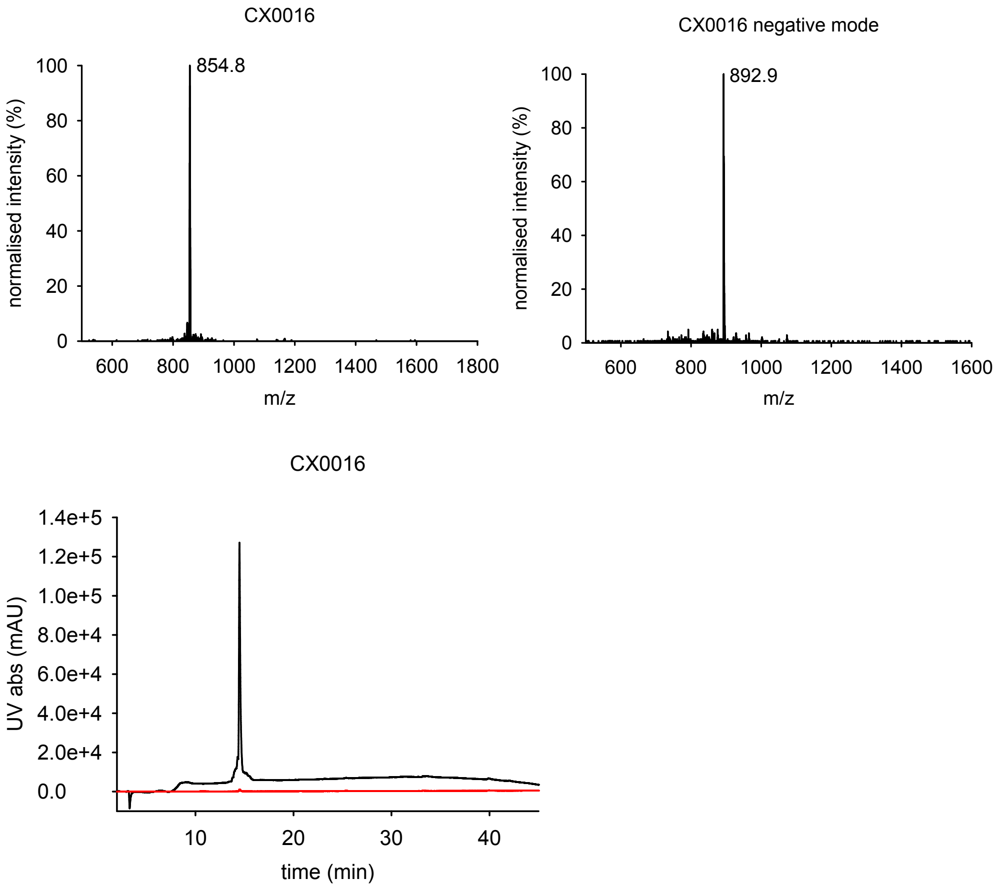


Figure S1h. *Mass spectra in positive and negative mode and analytical RP-HPLC trace for AnIB-NH$_2$. MW$_{calc}$: 1787.9, MW$_{obs}$: 1707.6 (positive mode); 1787.8 (negative mode).*

PnIA[Y(SO$_3$)15Y]-OH

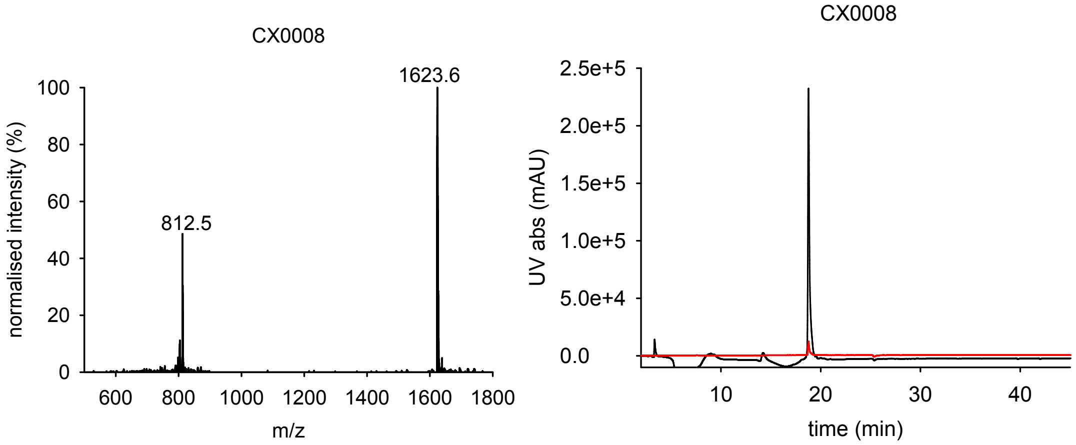


Figure S1i. *Mass spectrum and analytical RP-HPLC trace for PnIA[Y(SO$_3$)15Y]-OH. MW$_{calc}$: 1623.8, MW$_{obs}$: 1623.3.*

PnIA[Y(SO$_3$)15Y]-NH$_2$

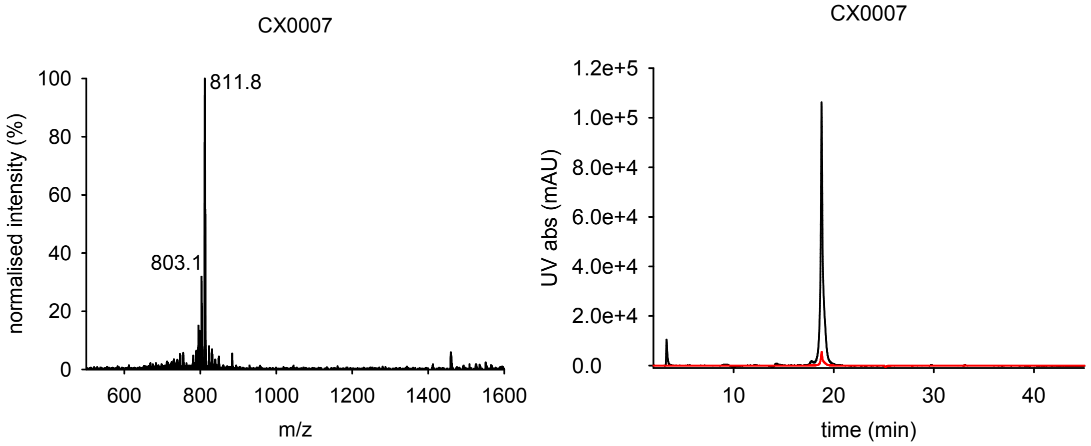


Figure S1j. *Mass spectrum and analytical RP-HPLC trace for PnIA[Y(SO$_3$)15Y]-NH$_2$. MW$_{calc}$: 1622.8, MW$_{obs}$: 1621.6.*

PnIA-OH

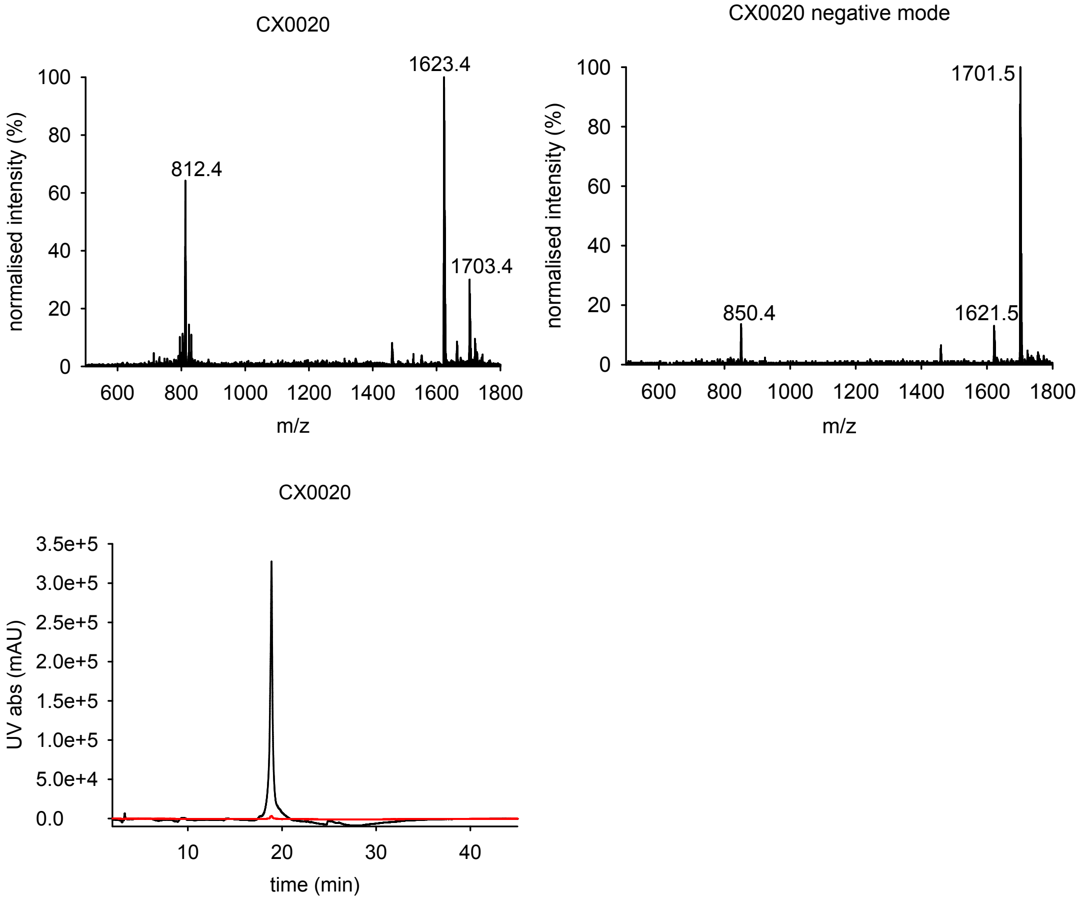


Figure S1k. *Mass spectra in positive and negative mode and analytical RP-HPLC trace for PnIA-OH. MW$_{calc}$: 1702.9, MW$_{obs}$: 1623.1 (positive mode); 1702.7 (negative mode).*

PnIA-NH$_2$

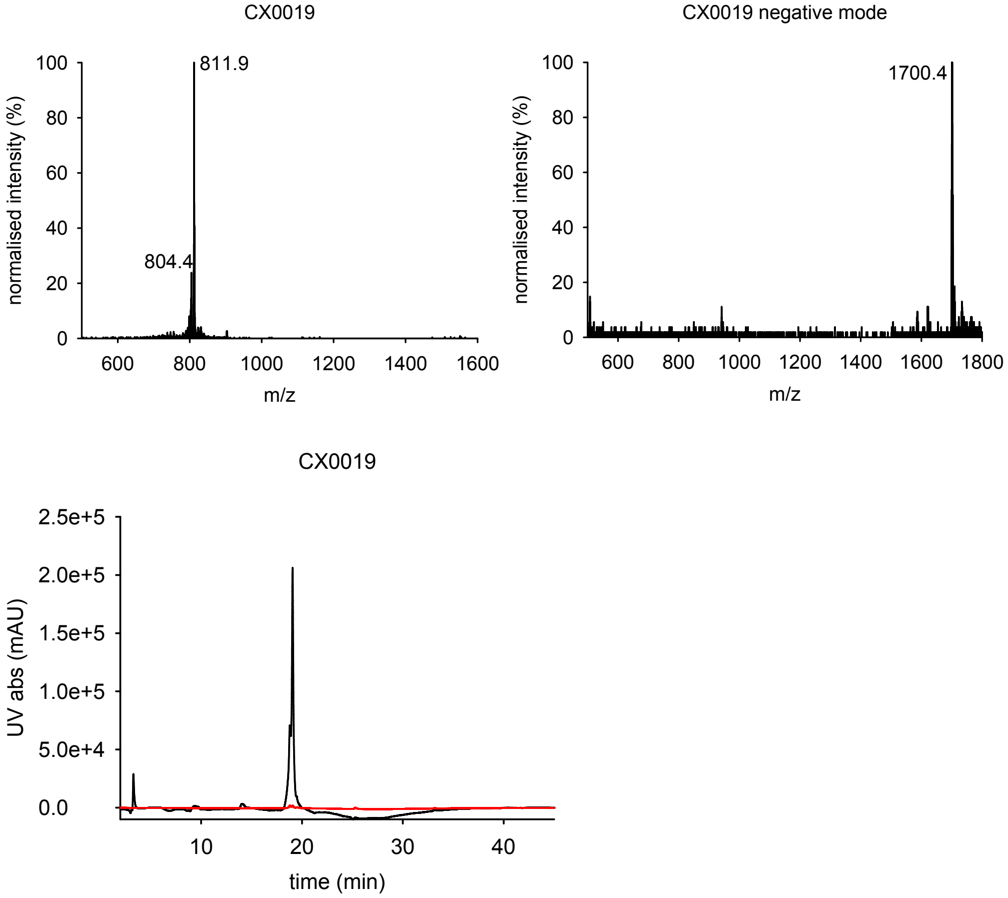


Figure S1l. *Mass spectra in positive and negative mode and analytical RP-HPLC trace for PnIA-NH$_2$. MW$_{calc}$: 1701.9, MW$_{obs}$: 1621.8 (positive mode); 1701.4 (negative mode).*

2. Binding of AnIB and PnIA variants to *Ac*-AChBP and *Ls*-AChBP.

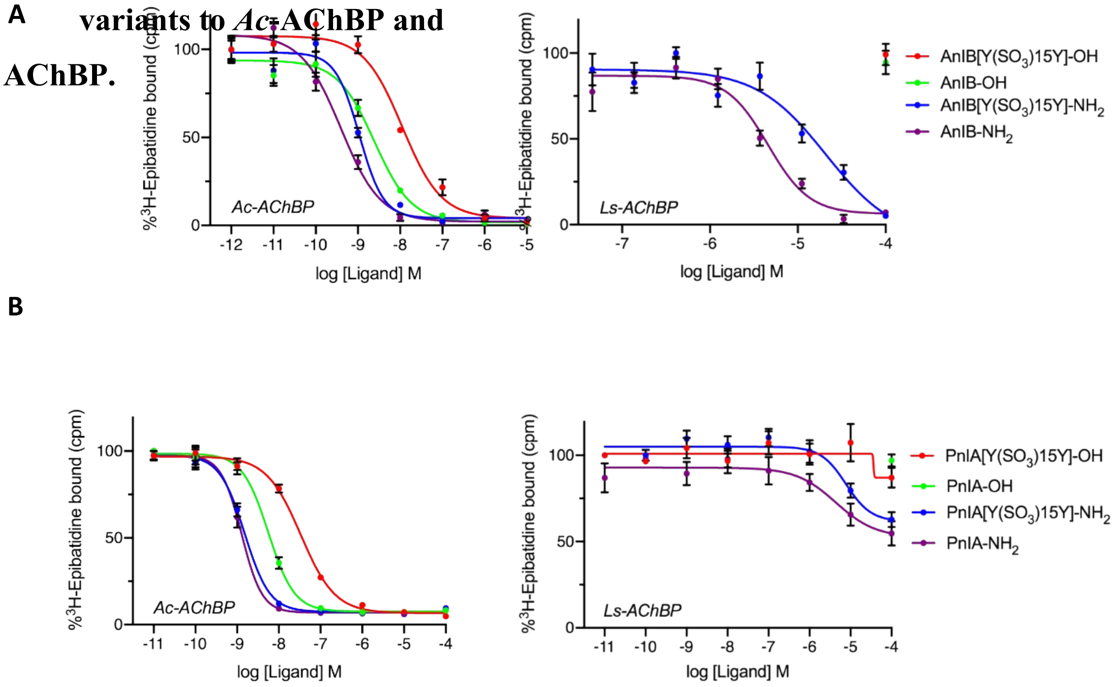


Figure S2. *Displacement of [^{3}H]-epibatidine from* Ac-*AChBP by (A) AnIB and (B) PnIA variants. Data represent mean ± SEM of triplicate data from three independent experiments.*

3. Secondary Hα NMR chemical shifts of PnIA[Y(SO$_3$)15Y]-NH$_2$ conformations.

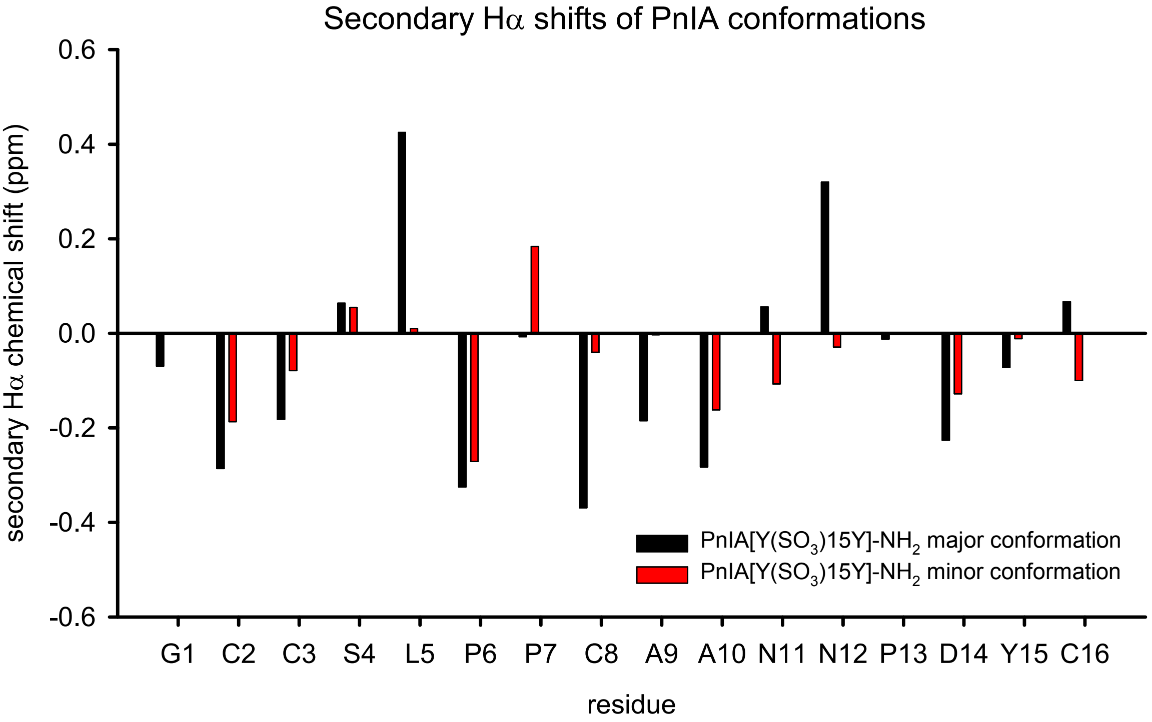


Figure S3. *Secondary Hα NMR chemical shifts of PnIA[Y(SO$_3$)15Y]-NH$_2$ conformations. Spectra were referenced to 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS) as an internal reference[1] and secondary Hα chemical shifts (ppm) were calculated as the difference between the measured chemical shift and the respective random coil shift from Whishart et al.[2]*

4. Structures of AnIB variants.

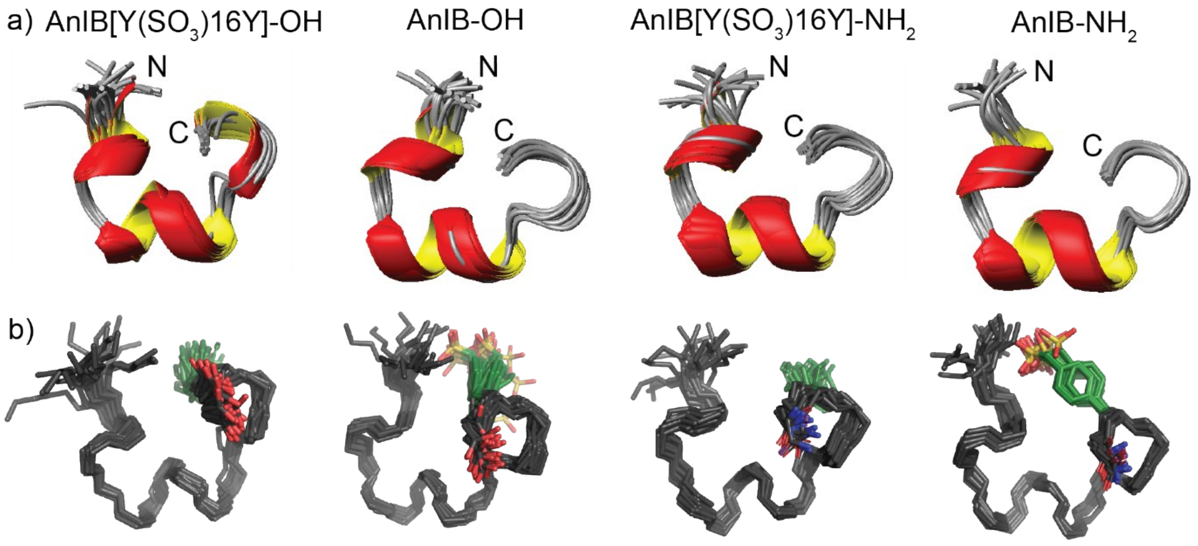


Figure S4. *Ensembles of 20 structures of AnIB variants with lowest energies and best MolProbity scores in (a) cartoon representation showing the α-helical regions (figures generated in MolMol) and (b) stick representation with backbone in grey, (sulfo)tyrosine in green and the C-terminal carboxylic acid/amide coloured by element type (figures generated in Pymol).*

5. Structure of PnIA-NH$_2$.

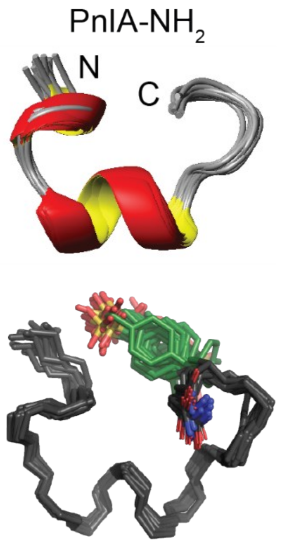


Figure S5. *Ensemble of 20 structures of PnIA-NH$_2$ with lowest energies and best MolProbity scores in (a) cartoon representation showing the α-helical regions (figures generated in MolMol) and (b) stick representation with backbone in grey, (sulfo)tyrosine in green and the C-terminal carboxylic acid/amide coloured by element type (figures generated in Pymol).*

Table S1. NMR structure calculation statistics for EpI variants

	EpI[Y(SO$_3$)15Y]-OH	**EpI-OH**	**EpI[Y(SO$_3$)15Y]-NH$_2$**	**EpI-NH$_2$**
Energies (kcal/mol)				
Overall	-586.49 ± 22.50	-570.14 ± 27.18	-536.68 ± 31.76	-517.00 ± 19.15
Bonds	4.87 ± 0.67	5.17 ± 0.92	4.22 ± 0.47	4.67 ± 0.55
Angles	16.51 ± 2.17	18.34 ± 5.81	12.88 ± 2.08	16.75 ± 2.52
Improper	5.24 ± 1.12	6.28 ± 1.93	5.27 ± 0.92	6.83 ± 2.09
Dihedral	68.62 ± 0.89	69.24 ± 1.49	68.96 ± 1.54	71.85 ± 1.23
Van der Waals	-52.34 ± 4.31	-49.69 ± 3.69	-55.11 ± 2.45	-52.80 ± 3.58
Electrostatic	-629.48 ± 22.58	-619.57 ± 33.41	-572.93 ± 31.66	-564.49 ± 20.65
NOE	0.030 ± 0.0070	0.033 ± 0.016	0.025 ± 0.0079	0.027 ± 0.010
cDih	0.071 ± 0.080	0.062 ± 0.094	0.0080 ± 0.021	0.16 ± 0.14
MolProbity Statistics				
Clashes (> 0.4 A/1000 atoms)	7.84 ± 3.37	7.48 ± 6.45	7.14 ± 4.35	6.56 ± 4.01
Poor rotamers	0	0	0	4.28 ± 3.59
Ramachandran Outliers (%)	0.36 ± 1.60	2.08 ± 3.70	0	0
Ramachandran Favoured (%)	99.29 ± 2.20	92.50 ± 3.73	99.64 ± 1.60	100
MolProbity score	1.44 ± 0.18	1.66 ± 0.50	1.33 ± 0.28	1.65 ± 0.40
MolProbity score percentile	95.30 ± 3.31	84.05 ± 14.14	96.50 ± 3.35	86.05 ± 13.43
Atomic RMSD (A)				
Mean global backbone	0.79 ± 0.34	1.23 ± 0.42	0.57 ± 0.18	0.62 ± 0.19
Mean global heavy	1.32 ± 0.31	1.83 ± 0.34	1.16 ± 0.19	1.38 ± 0.26
Experimental Restraints				
Distance restraints				
Short range (i-j < 2)	110	104	98	83
Medium range (i-j < 5)	39	37	39	37
Long range (i-j ≥ 5)	3	2	12	14

Hydrogen bond restraints	4	3	4	4
Total	156	146	153	138
Dihedral angle restraints				
phi	9	9	9	9
psi	11	11	11	11
chi1	5	5	4	4
Total	25	25	24	24

Table S2. NMR structure calculation statistics for AnIB variants

	AnIB[Y(SO$_3$)16Y]-OH	**AnIB-OH**	**AnIB[Y(SO$_3$)16Y]-NH$_2$**	**AnIB-NH$_2$**
Energies (kcal/mol)				
Overall	-511.93 ± 17.11	-515.24 ± 24.58	-538.22 ± 17.95	-619.69 ± 25.57
Bonds	4.20 ± 0.59	4.62 ± 0.43	3.78 ± 0.46	3.93 ± 0.40
Angles	11.85 ± 1.67	14.93 ± 1.82	9.62 ± 1.07	10.07 ± 1.53
Improper	5.66 ± 1.47	7.80 ± 2.88	4.52 ± 0.96	3.94 ± 0.73
Dihedral	71.39 ± 0.85	72.99 ± 0.99	71.83 ± 0.75	71.02 ± 0.69
Van der Waals	-52.39 ± 3.32	-53.39 ± 3.48	-54.39 ± 2.73	-59.32 ± 2.84
Electrostatic	-552.74 ± 19.30	-562.44 ± 25.81	-573.66 ± 18.43	-649.57 ± 25.69
NOE	0.0064 ± 0.0055	0.033 ± 0.0083	0.0099 ± 0.0043	0.010 ± 0.0045
cDih	0.095 ± 0.11	0.22 ± 0.29	0.066 ± 0.098	0.24 ± 0.14
MolProbity Statistics				
Clashes (> 0.4 A/1000 atoms)	3.38 ± 3.17	9.49 ± 5.10	3.62 ± 2.66	7.11 ± 3.61
Poor rotamers	0	0	0.83 ± 2.56	0
Ramachandran Outliers (%)	0	0.38 ± 1.72	0.33 ± 1.49	0.77 ± 2.37
Ramachandran Favoured (%)	96.33 ± 4.03	96.54 ± 3.93	90.67 ± 5.88	93.08 ± 5.52
MolProbity score	1.20 ± 0.46	1.67 ± 0.25	1.57 ± 0.41	1.71 ± 0.38
MolProbity score percentile	95.90 ± 5.34	88.20 ± 9.24	88.90 ± 8.97	85.20 ± 9.17

Atomic RMSD (A)				
Mean global backbone	1.28 ± 0.43	1.15 ± 0.43	1.25 ± 0.51	0.96 ± 0.38
Mean global heavy	1.44 ± 0.33	1.51 ± 0.36	1.30 ± 0.33	1.18 ± 0.25
Experimental Restraints				
Distance restraints				
Short range (i-j < 2)	75	65	81	98
Medium range (i-j < 5)	22	21	33	49
Long range (i-j ≥ 5)	4	10	9	11
Hydrogen bond restraints	0	0	0	0
Total	101	96	123	158
Dihedral angle restraints				
phi	12	11	9	10
psi	13	12	10	11
chi1	7	5	8	9
Total	32	28	27	30

Table S3. NMR structure calculation statistics for PnIA-NH2

	PnIA-NH$_2$
Energies (kcal/mol)	
Overall	-507.63 ± 37.48
Bonds	4.28 ± 0.50
Angles	17.67 ± 2.44
Improper	4.68 ± 1.36
Dihedral	64.66 ± 0.74
Van der Waals	-47.38 ± 3.05
Electrostatic	-551.74 ± 36.03
NOE	0.070 ± 0.0043
cDih	0.13 ± 0.10

MolProbity Statistics	
Clashes (> 0.4 A/1000 atoms)	15.21 ± 5.26
Poor rotamers	0
Ramachandran Outliers (%)	0
Ramachandran Favoured (%)	100
MolProbity score	1.66 ± 0.17
MolProbity score percentile	89.65 ± 4.74
Atomic RMSD (A)	
Mean global backbone	0.60 ± 0.19
Mean global heavy	1.23 ± 0.40
Experimental Restraints	
Distance restraints	
Short range (i-j < 2)	74
Medium range (i-j < 5)	36
Long range (i-j > 5)	8
Hydrogen bond restraints	4
Total	122
Dihedral angle restraints	
phi	9
psi	11
chi1	0
Total	20

6. Crystal structure of PnIA[A10L, D14K] in complex with *Ac*-AChBP (PDB: 2BR8).

Figure S6. *Cartoon structure of PnIA[A10L, D14K] (grey) in complex with Ac-AChBP (subunits coloured yellow, pink, green, cyan and magenta) showing binding of PnIA[A10L, D14K] at the interfaces of each of the five subunits (PDB: 2BR8)(top).*[3] *The central helical segment of PnIA[A10L, D14K] is mostly buried in the binding site while the N- and C-termini are exposed. A buffer sulfate ion that was observed in the crystal structure is shown as sticks and coloured by element, suggesting a possible position for the sulfotyrosine residue for native (sulfated and amidated) PnIA, potentially interacting with a disulfide bond (Cys188-Cys189) in the receptor (bottom).*

References

1. Wishart, D. S.; Bigam, C. G.; Yao, J.; Abildgaard, F.; Dyson, H. J.; Oldfield, E.; Markley, J. L.; Sykes, B. D., 1H, 13C and 15N chemical shift referencing in biomolecular NMR. *J. Biomol. NMR* **1995,** *6*, 135-140.
2. Wishart, D. S.; Bigam, C. G.; Holm, A.; Hodges, R. S.; Sykes, B. D., 1H, 13C and 15N random coil NMR chemical shifts of the common amino acids. I. Investigations of nearest-neighbor effects. *J. Biomol. NMR* **1995,** *5*, 67-81.
3. Celie, P. H., et al., Crystal structure of nicotinic acetylcholine receptor homolog AChBP in complex with an alpha-conotoxin PnIA variant. *Nat. Struct. Mol. Biol.* **2005,** *12*, 582-588.