Supporting Information

A fentanyl vaccine constructed upon opsonizing antibodies specific for the Galα1-3Gal epitope

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1. Immunoconjugation

Fentanyl-(α-Gal)-OVA double conjugate. A solution of Imject OVA (5 mg, Thermo Scientific) in PBS (2.5 mL) was dialyzed against borate buffer (0.5 M boric acid, 0.35 M KCl, pH adjusted to 9.0 with KOH) using a Slide-A-Lyzer Dialysis Cassette (10K MWCO, Thermo Scientific). α-Gal hapten[1,2] (2.5 mg) was added to the resulting solution, and additional borate buffer was added to make a total volume of 4 mL. Reaction was gently mixed with a rotator at rt for 24 h. The resulting solution was concentrated using an Amicon Ultra centrifugal filter (Merck Millipore) and dialyzed against MOPS buffer (100 mM 3-morpholinopropane-1-sulfonic acid, 900 mM NaCl, pH adjusted to 7.2 with NaOH). The volume of the protein conjugate solution was adjusted to 2.2 mL. Fentanyl hapten[3,4] (6 mg), Sulfo-NHS (19.8 mg, Sigma-Aldrich) and EDC·HCl (17.5 mg, Oakwood Products) were dissolved in DMF/H_2O (9:1, 270 μL). The reaction mixture was mixed at rt for 4 h before it was added to the above protein conjugate solution. After gentle mixing at 4 ℃ for 17 h, the reaction solution was dialyzed against PBS (Fisher Scientific) and the resulting solution was used for vaccine formulation and injection.

Fentanyl-OVA. Fentanyl hapten (8.1 mg), Sulfo-NHS (26.7 mg) and EDC·HCl (23.6 mg) were dissolved in DMF/H_2O (9:1, 360 μL). The reaction was mixed at rt for 4 h before it was added to the solution of Imject OVA (6 mg) in MOPS buffer (2.6 mL, 100 mM 3-morpholinopropane-1-sulfonic acid, 900 mM NaCl, pH adjusted to 7.2 with NaOH). After gentle mixing at 4 ℃ for 15 h, the reaction solution was dialyzed against PBS and the resulting solution was used for vaccine formulation and injection.

Fentanyl-BSA. Fentanyl hapten (1.2 mg), Sulfo-NHS (4.0 mg) and EDC·HCl (3.5 mg) were dissolved in DMF/H_2O (9:1, 60 μL). The reaction was mixed at rt for 3.5 h before it was added to the solution of Imject BSA (1 mg, Thermo Scientific) in PBS (0.5 mL). After gentle mixing at 4 ℃ for 16 h, the reaction solution was dialyzed against PBS and the resulting solution was used for ELISA.

2. MALDI-TOF Analysis of Protein Conjugates

The molecular weight of protein conjugates was determined by MALDI-TOF. The copy number of haptens on conjugates was calculated as:

$$\text{copy number}_{\alpha\text{-Gal}} = (MW_{\text{after conjugation}} - MW_{\text{before conjugation}})/(MW_{\alpha\text{-Gal hapten}} - MW_{\text{ethanol}})$$

$$\text{copy number}_{\text{fentanyl}} = (MW_{\text{after conjugation}} - MW_{\text{before conjugation}})/(MW_{\text{fentanyl hapten}} - MW_{\text{water}})$$

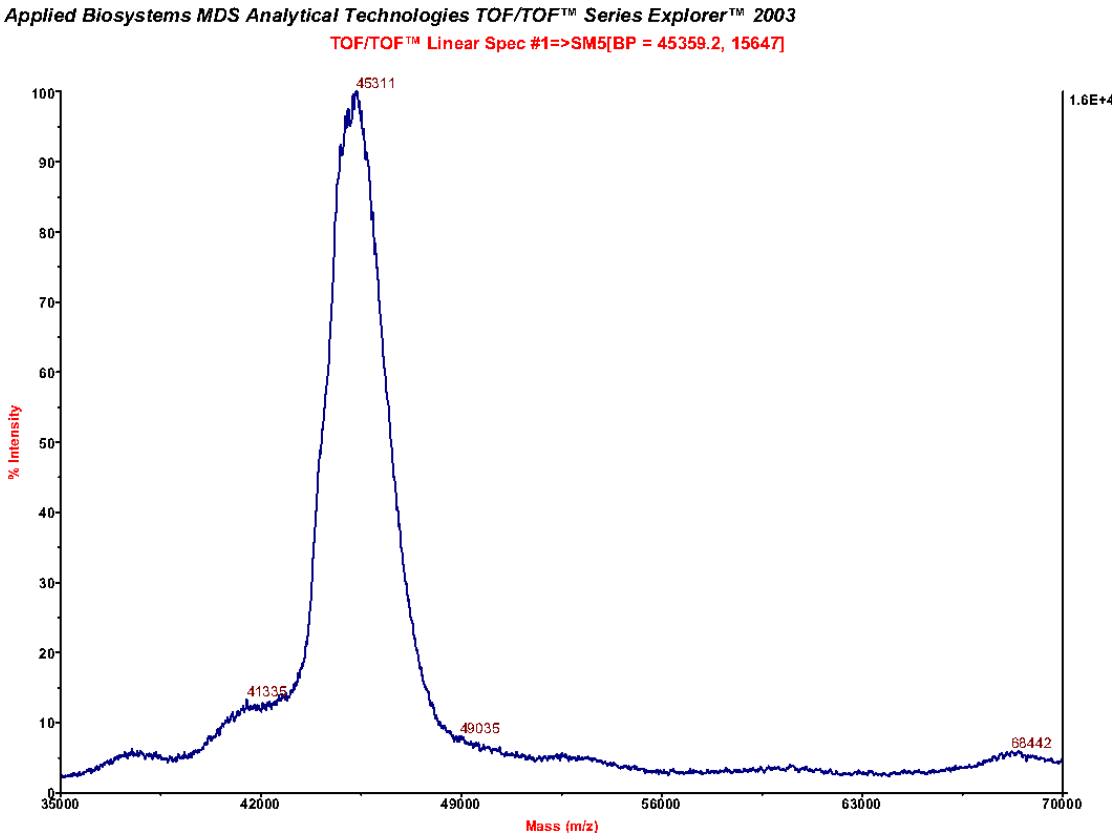

Figure S1. MALDI-TOF spectrum of (α-Gal)-OVA. MW=45311, copy number$_{a\text{-}Gal}$=1.4.

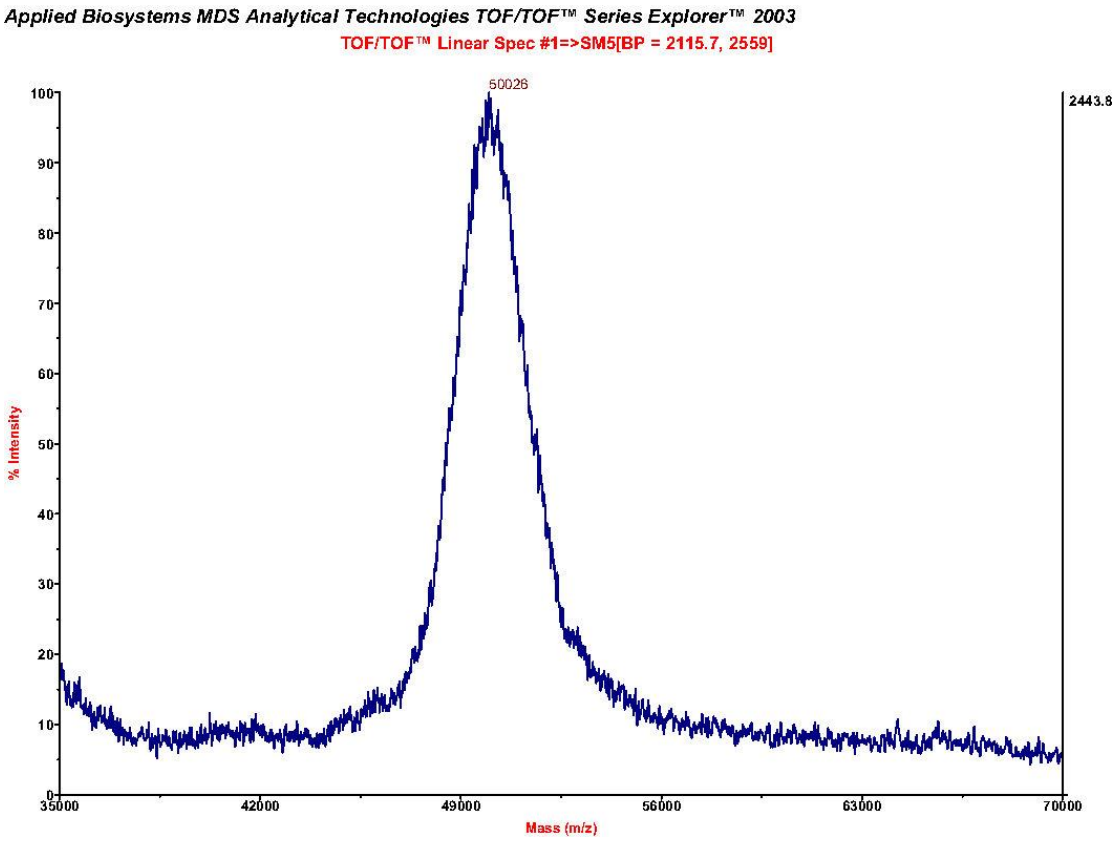

Figure S2. MALDI-TOF spectrum of fentanyl-(α-Gal)-OVA. MW=50026, copy number$_{fentanyl}$=12.5.

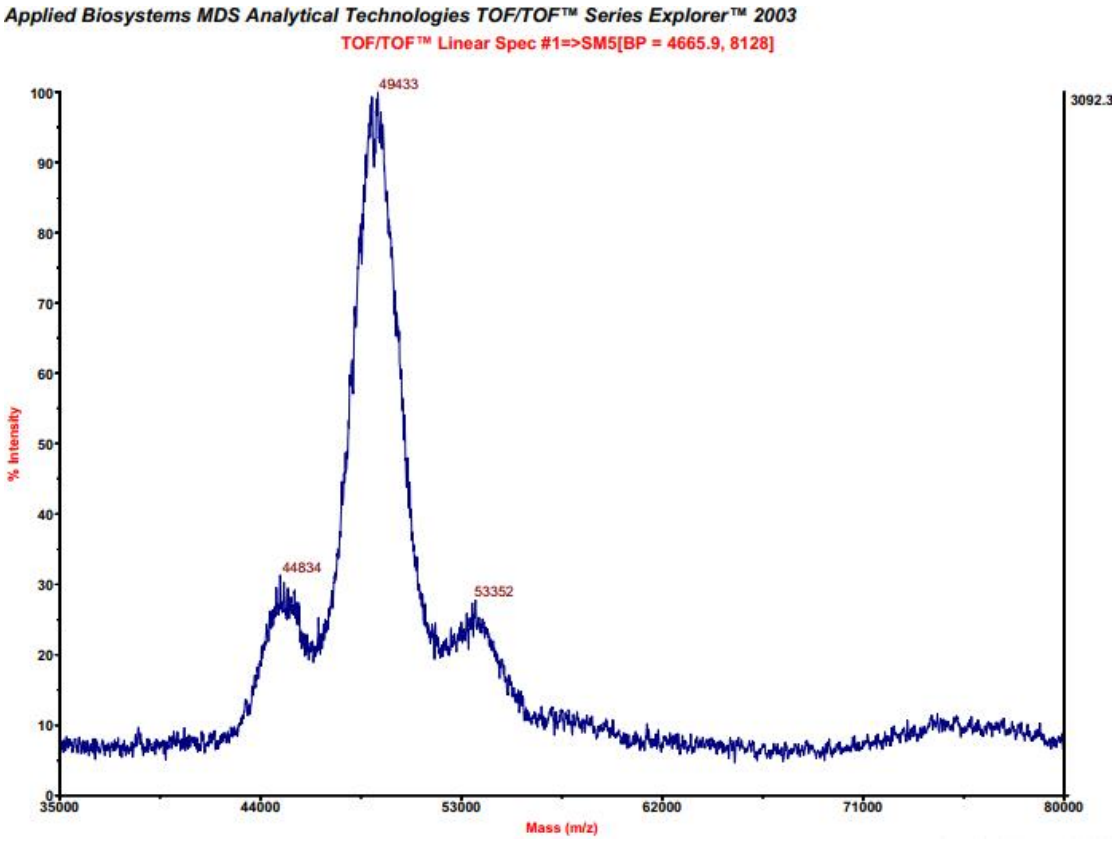

Figure S3. MALDI-TOF spectrum of fentanyl-OVA. MW=49433, copy number$_{fentanyl}$=13.6.

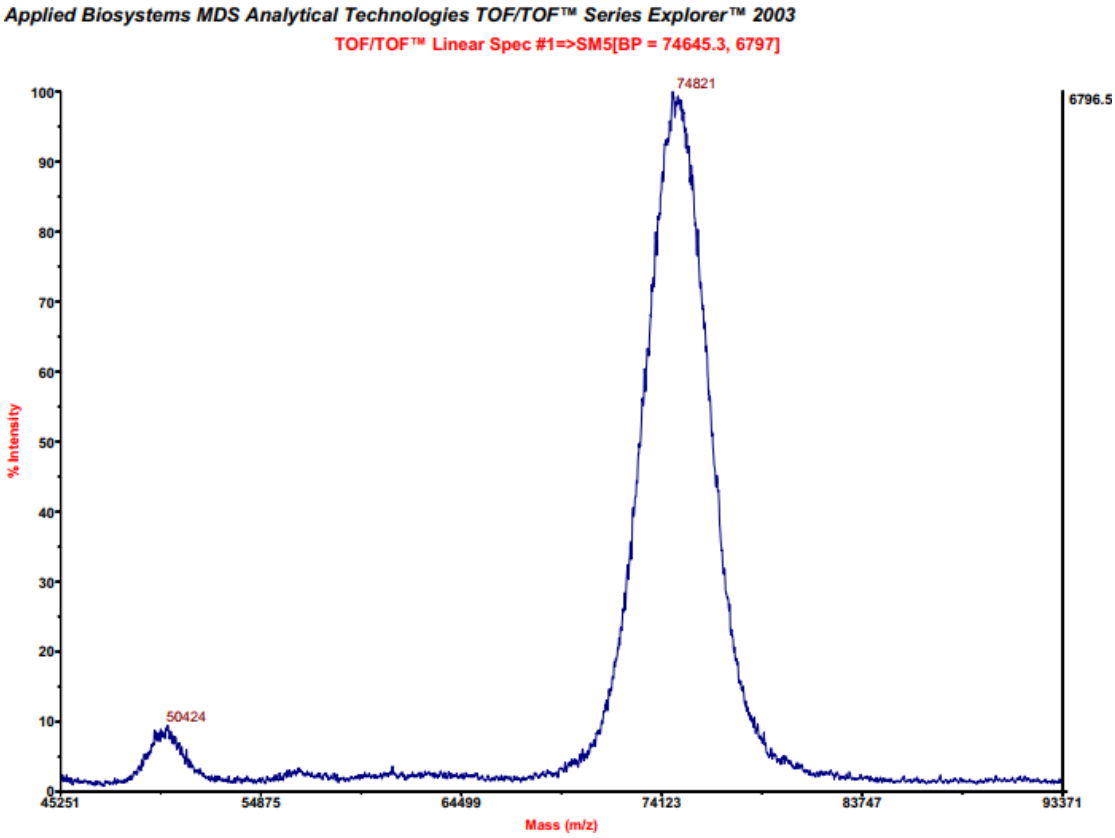

Figure S4. MALDI-TOF spectrum of fentanyl-BSA. MW=74821, copy number$_{fentanyl}$=22.7.

3. Induction of Anti-Gal Antibody Production in α1,3GalT Knockout Mice

Animal health was monitored by the scientists and veterinary staff of The Scripps Research Institute. Studies were carried out in compliance with the Scripps Institutional Animal Care and Use Committee (La Jolla, CA), and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice with disrupted α1,3galactosyltransferase (α1,3GalT) genes[5] were group-housed in an AAALAC-accredited vivarium with temperature/humidity-controlled rooms on a reverse light cycle (lights on: 9PM to 9AM). Experiment procedures were performed during the dark phase, generally between 2PM and 6PM. 11-24 week old mice were immunized with (α-Gal)-TT as previously described[1]. Mice were bled via retro-orbital sinus one week before fentanyl vaccine administration and sera were collected after centrifugation at 12,000 rpm. Midpoint anti-Gal IgG titers were determined from ELISA with (α-Gal)-BSA as the coating antigen. Sera from week 3 and week 5 were also analyzed to show the change of antibody level during the fentanyl vaccine immunization procedure (Table S1).

Table S1. Midpoint anti-Gal IgG titers shown as means ± SEM.

Bleed	Fent-Gal/Fent-Gal	Fent-Gal/Fent	Control
Week -1	39793 ± 6732	24153 ± 3269	52356 ± 7443
Week 3	30406 ± 9291	19352 ± 3198	39913 ± 5747
Week 5	20046 ± 5266	15697 ± 3889	28106 ± 3698

4. Fentanyl Vaccine Formulation and Administration

Fentanyl vaccine administration began two weeks after the final (α-Gal)-TT injection. On a per mouse basis, 50 μg of protein conjugate (fentanyl-(α-Gal)-OVA or fentanyl-OVA) in 50 μL of PBS was mixed with 50 μL of Alhydrogel (Invivogen) and injected intraperitoneally at weeks 0, 2, 4. The control group was injected with 50 μL of PBS mixed with 50 μL of Alhydrogel per mouse. Mice were bled via retro-orbital sinus at weeks 3 and 5.

5. ELISA Procedure for Midpoint Anti-Fent IgG Titer Determination

Corning 3690 Costar microplates were coated per well with 100 ng of fentanyl-BSA in 25 μL of PBS overnight at 37 ℃ to let the liquid evaporate. 5% skim milk in PBS was added (80 μL/well) to block unspecific binding for 1 h at rt. After shaking out the liquid, serum samples were added and serially diluted 1:1 in PBS containing 2% BSA across the 12 columns starting at 1:800. After incubation in a moist chamber at 37 ℃ for 1.5 h, plates were washed with water. Peroxidase-conjugated AffniPure Donkey Anti-Mouse IgG (H+L) (Jackson ImmunoResearch, 1:10000 in PBS containing 2% BSA, 25 μL/well) was added as the secondary antibody and the plates were incubated in a moist chamber at 37 ℃ for 1 h. After washing with water, Pierce TMB Substrate Kit (Thermo Scientific) was used to detect peroxidase activity (50 μL/well). After incubation at rt for 6 min, 2 M H_2SO_4 solution was added and plates were incubated at rt for another 20 min. The absorbance at 450 nm was measured using a SpectraMax M2e microplate reader (Molecular Devices). To calculate the midpoint titers, the absorbance data was normalized with the highest absorbance set as 100% and nonlinear regression curves were fitted using the log(inhibitor) vs. normalized response -- Variable slope equation in Prism 8 (GraphPad).

6. Surface Plasmon Resonance

SPR competitive binding assays were carried out as previously described.[6,7] Briefly, diluted serum samples were incubated with fentanyl of different concentrations and injected into a Biacore 3000 instrument (GE Healthcare) equipped with a research-grade CM5 sensor chip. Fentanyl-BSA was immobilized using the NHS/EDC coupling reaction to act as the ligand. IC_{50} values were calculated from the generated binding curves.

Table S2. SPR response (RU) from the competitive binding assays.

Fentanyl	Fent-Gal/Fent-Gal		Fent-Gal/Fent	
concentration (nM)	Week 3	Week 5	Week 3	Week 5
0.00	112.64	125.59	112.97	107.48
3.91	103.13	107.9	113.68	104.6
7.81	99.1	106.16	112.45	104.48
15.63	96.72	101.88	111.13	101.55
31.25	92.12	96.34	107.51	96.16
62.50	85.25	88.24	102.87	90.75
125.00	75.35	78.21	93.76	81.66
250.00	67.03	67.65	84.19	72.37
500.00	53.4	53.15	70.51	58.25
1000.00	38.22	37.49	52.89	41.6

7. Antinociception Assays

Mice were tested for cumulative response to fentanyl in hot plate and tail flick assays. Fentanyl citrate (Cayman Chemical) was used for its preferred solubility in water. The fentanyl citrate doses tested were 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.4 and 1.8 mg/kg. In the hot plate assay, mice were placed in an acrylic cylinder (14 cm diameter × 22 cm) on a 55 ℃ surface and measured for the latency to perform nociceptive responses including licking a hind paw, shaking/withdrawal of hind paw and jumping. Typical baseline was between 5 s and 15 s. 35 s was set as the cutoff time to prevent tissue damage. Mice were removed from the hot surface after nociceptive responses were observed or the cutoff time was reached. After the paws got cooled, the tail flick assay was carried out immediately using an IITC Life Science Tail Flick Analgesia Meter with mice lightly restrained in a small pouch. Time of withdrawal from a heated beam of light (with active intensity of 45%) was measured with an automatic cutoff time of 10 s. Typical baseline was 1-3 s. After the tail flick assay, mice were injected with fentanyl citrate solution intraperitoneally. The testing and injection procedures were repeated with intervals of 16 min until the cutoff time has been reached in both assays (i.e. full antinociception reached). For some of the mice, cutoff time was not reached with 1.8 mg/kg dose. In this case, testing was not continued to avoid putting too much liquid into the peritoneum. Drug effect was evaluated as %MPE, which is calculated as (test-baseline)/(cutoff-baseline) × 100. The response curves were fitted using the [Agonist] vs. normalized response -- Variable slope equation in Prism 8 (GraphPad) and ED_{50} values were calculated.

8. Blood-Brain Distribution Studies

Mice tested were injected with 0.2 mg/kg of fentanyl citrate. After 15 min, mice were fully anesthetized and decapitated. Trunk blood and brain were collected separately. The blood was

centrifuged to give the serum. The brain was mixed with 0.4-0.5 mL of PBS, homogenized using a bullet blender with beads added. To prepare the samples for LC-MS/MS analysis, 60 µL of serum/homogenized brain samples were added with 8 µL of the fentanyl-d5 internal standard (50 ng/mL in MeOH). The mixture was vortexed and 120 µL of 50 mM K_2CO_3 was added. After adding 420 µL of 7:3 hexane/ethyl acetate, the mixture was vortexed and centrifuged at 3000 rpm for 5 min. The top layer was collected and evaporated using GENEVAC. 68 µL of MeOH was added and the resulting samples were analyzed by LC-MS/MS to give the fentanyl concentrations.

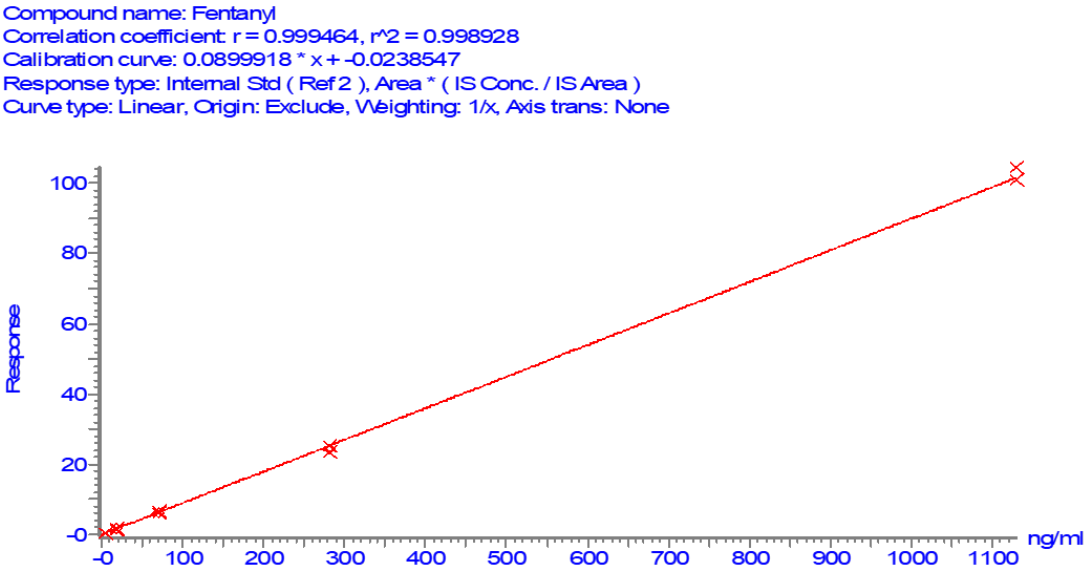

Figure S5. Fentanyl standard curve used to measure fentanyl concentrations in serum/brain samples.

9. References

1 K. Anraku, S. Sato, N. T. Jacob, L. M. Eubanks, B. A. Ellis and K. D. Janda, *Org. Biomol. Chem.*, 2017, **15**, 2979-2992.

2 N. T. Jacob, K. Anraku, A. Kimishima, B. Zhou, K. C. Collins, J. W. Lockner, B. A. Ellis and K. D. Janda, *Chem. Commun.*, 2017, **53**, 8156-8159.

3 P. T. Bremer, A. Kimishima, J. E. Schlosburg, B. Zhou, K. C. Collins and K. D. Janda, *Angew. Chem. Int. Ed.*, 2016, **55**, 3772-3775.

4 Y. Natori, C. S. Hwang, L. Lin, L. C. Smith, B. Zhou and K. D. Janda, *Beilstein J. Org. Chem.*, 2019, **15**, 1020-1031.

5 A. D. Thall, P. Malý and J. B. Lowe, *J. Biol. Chem.*, 1995, **270**, 21437-21440.

6 C. S. Hwang, L. C. Smith, Y. Natori, B. Ellis, B. Zhou and K. D. Janda, *ACS Chem. Neurosci.*, 2018, **9**, 1269-1275.

7 L. C. Smith, P. T. Bremer, C. S. Hwang, B. Zhou, B. Ellis, M. S. Hixon and K. D. Janda, *J. Am. Chem. Soc.*, 2019, **141**, 10489-10503.