A Sulfonated Supramolecular Host Based on Pillar[5]arene for Succinylcholine–Induced Neuromuscular Blockade Reversal

Supplementary information

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Materials and Methods

Materials and Instrumentation

Commercial reactants including Sch, Vec, Roc, Pan, dimethoxypillar[5]arene, and 1,3–propanesultone were purchased from Aladdin® (Shanghai), J&K Scientific® (Beijing), Sigma–Aldrich® and used without additional purification. Per–hydroxylated pillar[5]arene were synthesized based on the protocols reported [1]. ^{1}H NMR, ^{13}C NMR and 2D NOESY spectra were recorded by a JNM–ECZ400S/L1 instrument (JEOL, Japan), and were internally referenced to residual solvent signals (D_2O referenced at 4.79 ppm). ESI–MS spectra was measured by an LCMS–IT/TOF instrument (Shimadzu, Japan). Isothermal titration calorimetry (ITC) was performed using a MicroCal PEAQ–ITC instrument (Malvern Panalytical). Transmission electron microscopy (TEM) investigations were carried out on a H–7650B instrument (Hitachi, Japan). As for animal experiments, anesthesia for mice was conducted by R500 small animal anesthesia machine (RWD), and for rabbits was conducted by anesthesia instrument (Datex–Ohmeda). Open field test data were analyzed via EthoVision XT 11.5 software (Noldus). Rotarod test was performed using a RotaRod instrument (Ugo Basile, Italy). The TOF–ratio was recorded by HXD–IC028 TOF–Watch Monitor (Huaxiang Technology Development Co., Ltd. China). Mechanical ventilation for rabbits was conducted also by anesthesia instrument (Datex–Ohmeda). Electrocardiograms (ECG) were recorded by PowerLab 15T (ADInstruments) and analyzed by LabChart 7 (ADInstruments). KF–PRO–120 digital pathology slide scanner and K–Viewer software (KFBIO, China) were using to capture H&E color images.

Synthesis of SP[5]A

Per–hydroxylated pillar[5]arene (6.10 g, 10.0 mmol) and NaOH (8.20 g, 205 mmol) were dissolved in 350 mL of tetrahydrofuran (THF). The solution was stirred for 2 h at room temperature. Next, 1,3–propanesultone (26.9 g, 220 mmol) was slowly added to the mixture.

After reaction for 24 h at 45 ℃, the precipitate was collected by filtration, washed with THF for three times to afford the crude product as orange powder. For further purification, the crude product was then dissolved in an aqueous NaOH solution, precipitated with methanol, followed by filtration, and dried under vacuum to obtain sulfonated P[5]A sodium salt as yellow powder, referred to as SP[5]A (4.66 g, 2.27 mmol) with the yield of 22.7 %.

Animals and Cell Culture

6–week–old male C57BL/6J mice (weighing about 20.0 g) and 4–month–old male New Zealand rabbits (weighing about 2.50 kg) (Beijing Vital River Laboratory Animal Technology Co., Ltd., China) were maintained under SPF conditions in the animal facility of Tsinghua University. All animal experiments strictly complied with the standards of the Institutional Animal Care and Use Committee of Tsinghua University (protocol No. 21–YGC1.G24–1). Sch and SP[5]A were dissolved in PBS for *in vivo* injection. The L929 mouse fibroblast cell line was purchased from the American Type Culture Collection (ATCC, Rockeville, MD). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10 % fetal bovine serum, 1 % penicillin–streptomycin (penicillin 100 U/mL and streptomycin 100 mg/mL) in a humidified incubator at 37 ℃ with 5 % CO_2 humidified atmosphere.

Protocol for Behavioral Tests

Open Field Test

Male C57BL/6J mice weighing approximately 20.0 g were used for experiments. Sch (0.50 mg/kg) was administered intravenously via the tail vein, resulting in generalized fasciculations followed by paralysis. 30 s after Sch administration, mice were injected with either PBS or SP[5]A (2.84 mg/kg, equimolar to Sch) via the tail vein. Each mouse was placed into the center of the open field which is a white PVC box (L40* W40* H40 cm; illumination < 25 Lux) and was allowed to explore for 20 min after administration. HD cameras were used to record the

process of locomotor activity. Then the movies were analyzed through EthoVision XT 11.5 software (Noldus). Subsequently, the speed, movement time, and distance at different time period were measured.

RORR time assessment

Under normal conditions, mice exhibited a rapid righting reflex, transitioning quickly from a supine to a prone position. Sch–induced neuromuscular blockade led to muscle relaxation, preventing the mice from righting themselves. Male C57BL/6J mice weighing approximately 20.0 g were used for experiments. Sch (0.50 mg/kg) was administered intravenously via the tail vein, resulting in generalized fasciculations followed by paralysis. 30 s after Sch administration, mice were injected with either PBS or SP[5]A (2.84 mg/kg, equimolar to Sch) via the tail vein. The time taken for each mouse to regain its righting reflex following the administration of either PBS or SP[5]A was recorded, defined as the righting reflex recovery (RORR) time.

Rotarod Test

The rotor was set to 40 revolution per minute (RPM). C57BL/6J mice were trained on the rotor twice a day for one week, and each training time was 20 min. The mice were chosen that would not fall from the within 30 s. Sch (0.50 mg/kg) was administered intravenously via the tail vein, resulting in generalized fasciculations followed by paralysis. 30 s after Sch administration, mice were injected with either PBS or SP[5]A (2.84 mg/kg, equimolar to Sch) via the tail vein. The mice could not continuously move on the rotor for 30 s showed that the model of neuromuscular blockade was established successfully according to previously reported [2]. We subsequently measured the time it took for the mice to regain continuous motor activity on the rotor for 30 s, as well as the longest duration at various time points during which the mice could remain on the rotor without falling down after the PBS or SP[5]A administration.

Protocol for Neuromuscular Blockade Recovery

The protocol was improved according to the previous report [3]. Male New Zealand rabbit was anesthetized with 1 % propofol. The rabbit quadriceps femoris muscle was stimulate by the electrode patches which were placed on the shaved right leg. The acceleration sensor of the TOF–Watch Monitor could measure the twitch response of the quadriceps muscle. The transducer was attached to the tibia of the right leg to maximize swing amplitude, ensuring precise acceleration measurement. Once the TOF–Watch was set to Cal mode, continuous stimulation was applied to femoral nerve at 1 Hz, 10 mA until the twitch height stabilized. Afterwards, the TOF–Watch was changed to TOF mode and stimulated the femoral nerve at 2 Hz for 20 s until the T_1 stable with TOF–ratio > 90 %. Subsequently, rabbits were intravenously administered with Sch dissolved in PBS (0.50 mg/kg), immediately followed by endotracheal intubation with a tidal volume of 20 mL to facilitate mechanical ventilation and ensure regular breathing during the entirety of the experiment. Anesthesia was sustained using 3 % – 5 % sevoflurane. 1 min after the administration of Sch, either PBS or SP[5]A (2.84 mg/kg, equimolar to Sch) were injected at maximal muscle twitch inhibition (T_1 = 0). Then the effect of reversal *in vivo* was evaluated by measuring the period for TOF–recovery (> 90%) and spontaneous breathing recovery.

Serum Potassium Levels Assessment

The anesthesia procedure for rabbits was same as described above. In the control group, no drug treatment was administered. The SP[5]A group received only an injection of SP[5]A (2.84 mg/kg), while the Sch group and Sch + SP[5]A group were initial injected with Sch (0.50 mg/kg), followed by an injection of PBS or SP[5]A (2.84 mg/kg) after 1 min, respectively. Blood samples were collected from the rabbits before drug administration and at 2, 5, and 10 min post–administration. The blood samples were centrifuged at 3000 rpm for 20 min to separate the serum, and the relative serum potassium levels were determined.

Electrocardiography (ECG) Recording and Analysis

Male C57BL/6J mice were divided into the control group, Sch group and Sch + SP[5]A group. In the control group, no drug treatment was administered. The Sch group and Sch + SP[5]A group were initial injected with Sch (0.65 mg/kg), followed by an injection of PBS or SP[5]A (3.69 mg/kg, equimolar to Sch) after 30 s. After anesthetizing the mice with 3 % isoflurane inhalation, needle electrodes were inserted subcutaneously into two legs and right arm. The concentration of isoflurane was reduced to 1 % to provide consistent sedation, and ECG recording was commenced. ECG traces were recorded for about 5 min and analyzed using LabChart 7.

Rhabdomyolysis Assessment

Male C57BL/6J mice were divided into the control group, SP[5]A group, Sch group and Sch + SP[5]A group. The mice model of Sch–induced rhabdomyolysis was according to the previous report [4]. The control group administrated no drug. As for the Sch group and Sch + SP[5]A group, mice were administered Sch (1.60 mg/kg) intramuscularly into the right leg muscle, followed by a subsequent intramuscular injection of either PBS or SP[5]A (9.10 mg/kg, equimolar to Sch) after 1 min, while the mice of the SP[5]A group were only intramuscularly injected SP[5]A. 20 min after the administration, the muscle tissues from the injection site were collected for histopathological analysis via hematoxylin and eosin (H&E) staining, and blood samples were taken for serum biochemical assessment including creatine kinase (CK), urea, creatine (CREA), and uric acid (UA).

Cell Cytotoxicity Assessment

Cytotoxicity was assessed using the Cell Counting Kit–8 (CCK–8, Sigma–Aldrich) following the instructions. L929 cells were seeded in 96–well plates (8×10^3 cells per well in 200 μL complete growth medium). The plates were incubated for 24 h at 37 °C in a humidified atmosphere with 5 % CO_2 to allow cell adhesion. After the initial incubation, the medium was

replaced with fresh medium containing different concentrations of SP[5]A. The cells were then incubated for 24 h. Following treatment, 10 μL of the CCK–8 solution was added to each well, avoiding bubble formation to ensure accurate absorbance readings. The plates were further incubated at 37 °C for 4 h. Finally, the absorbance was measured at 450 nm using a microplate reader (Bio–Tek Instruments Inc., Vermont, USA) to quantify the number of viable cells.

Hematological and Histopathological Analysis for *In vivo* Biosafety Assessment

Male C57BL/6J mice were injected intravenously with either PBS and SP[5]A (1000 mg/kg). 48 h after the injection, mice were euthanized for blood samples collection. Liver function and kidney function levels were determined using serum obtained after centrifugation at 3000 rpm for 20 min. Meanwhile, representative organs such as heart, liver, spleen, lung, and kidney were gathered for tissue sectioning and evaluated via hematoxylin and eosin (H&E) staining.

Statistical Analysis

Graphical representation and statistical analysis of data was performed using GraphPad Prism 9.0 and OriginPro 2024b. Data were expressed as means ± SD and analyzed by unpaired *t*−test. The data were classified with *P* values and denoted by (*) for $P \leq 0.05$, (**) for $P \leq 0.01$, (***) for $P \leq 0.001$, and (****) for $P \leq 0.0001$.

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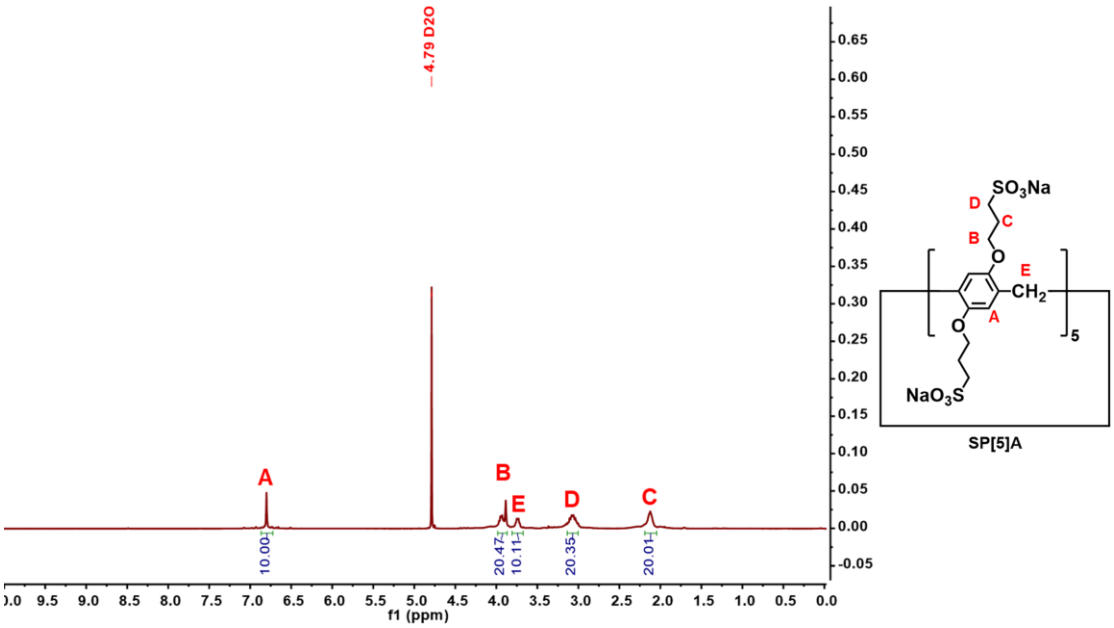

Figure. S1. ^{1}H NMR spectrum recorded (400 MHz, 298 K, RT, D_2O) for SP[5]A.

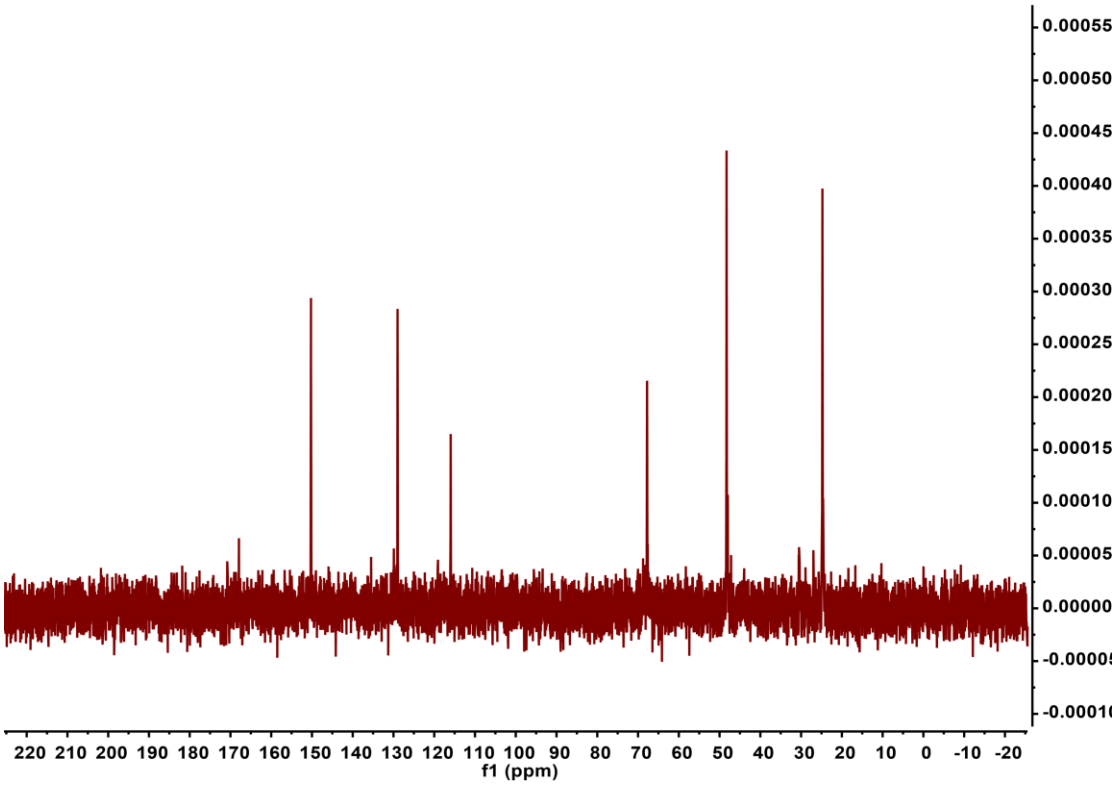

Figure. S2. ^{13}C NMR spectrum recorded (100 MHz, 298 K, RT, D_2O) for SP[5]A.

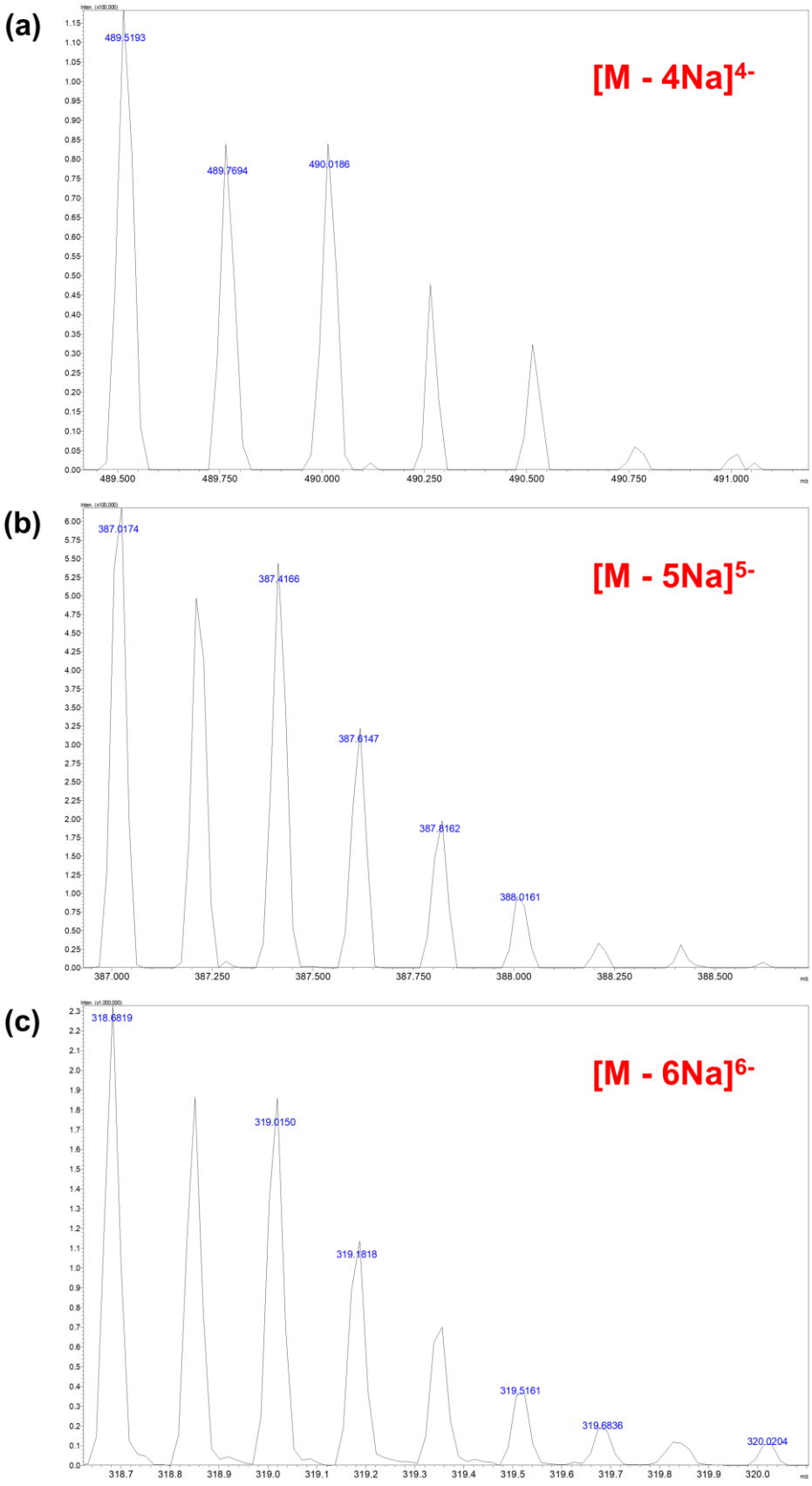

Figure. S3. ESI–MS spectra of SP[5]A. Experimental isotope pattern for $[M - 4Na]^{4-}$ **(a)**, $[M - 5Na]^{5-}$ **(b)** and $[M - 6Na]^{6-}$ **(c)**. Molecular formula: $C_{65}H_{80}Na_{10}O_{40}S_{10}$.

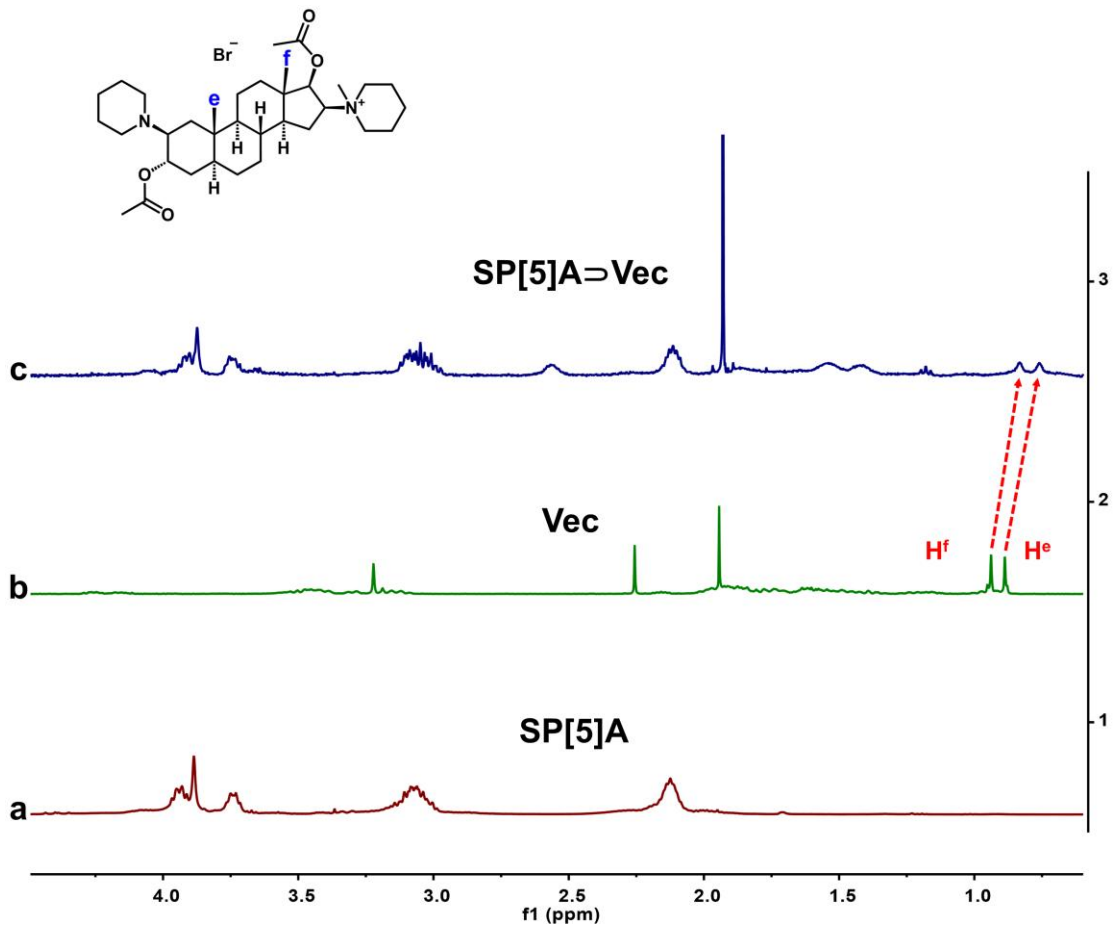

Figure. S4. ^{1}H NMR spectra recorded (400 MHz, 298 K, RT, D_2O) for **(a)** SP[5]A (2.5 mM), **(b)** Vec (2.5 mM), and **(c)** a mixture of Vec (2.5 mM) and SP[5]A (2.5 mM).

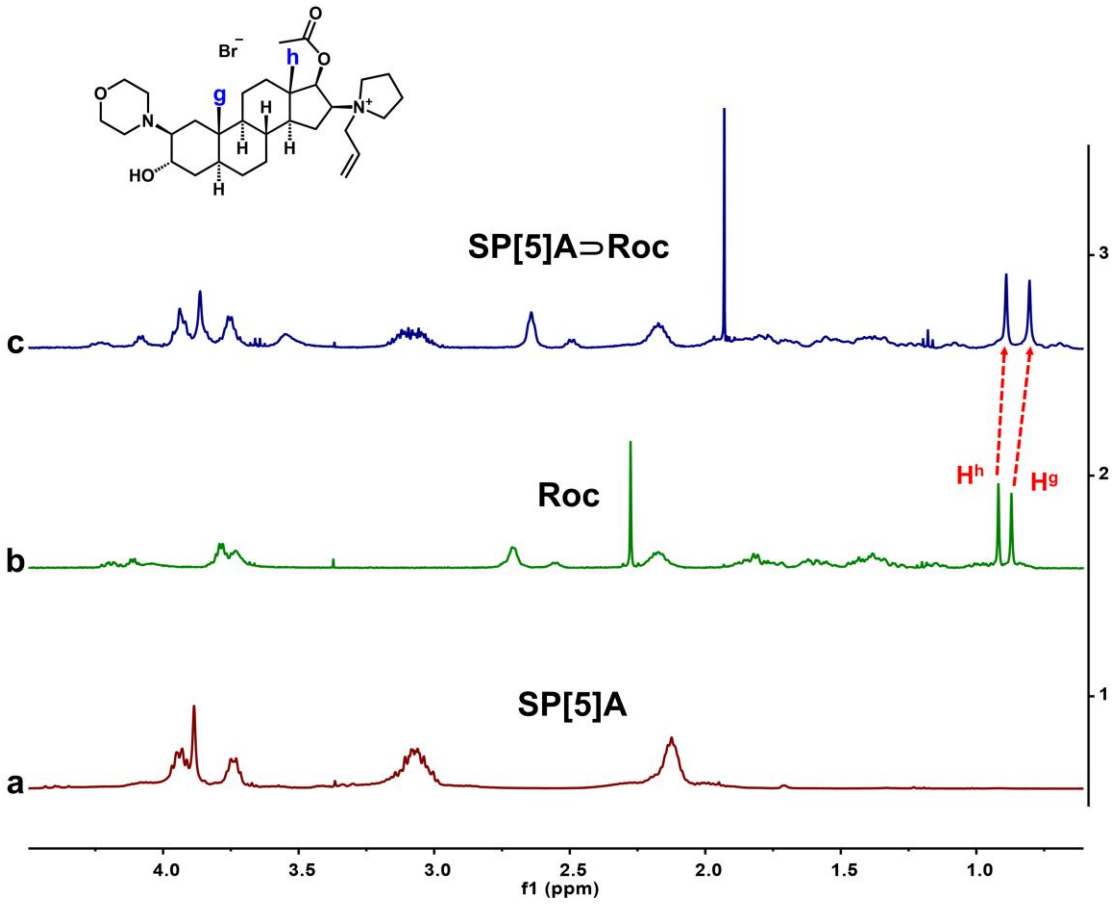

Figure. S5. ^{1}H NMR spectra recorded (400 MHz, 298 K, RT, D_2O) for **(a)** SP[5]A (2.5 mM), **(b)** Roc (2.5 mM), and **(c)** a mixture of Roc (2.5 mM) and SP[5]A (2.5 mM).

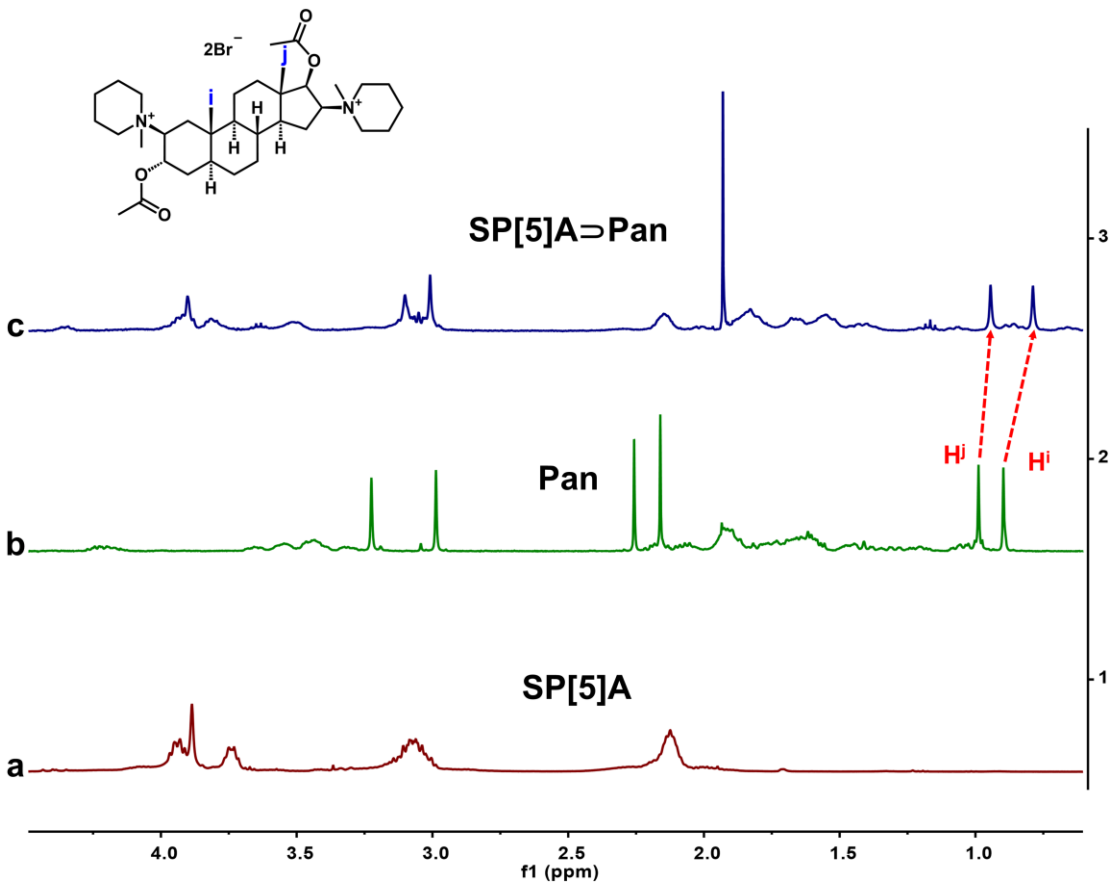

Figure S6. ^{1}H NMR spectra recorded (400 MHz, 298 K, RT, D_2O) for **(a)** SP[5]A (2.5 mM), **(b)** Pan (2.5 mM), and **(c)** a mixture of Pan (2.5 mM) and SP[5]A (2.5 mM).

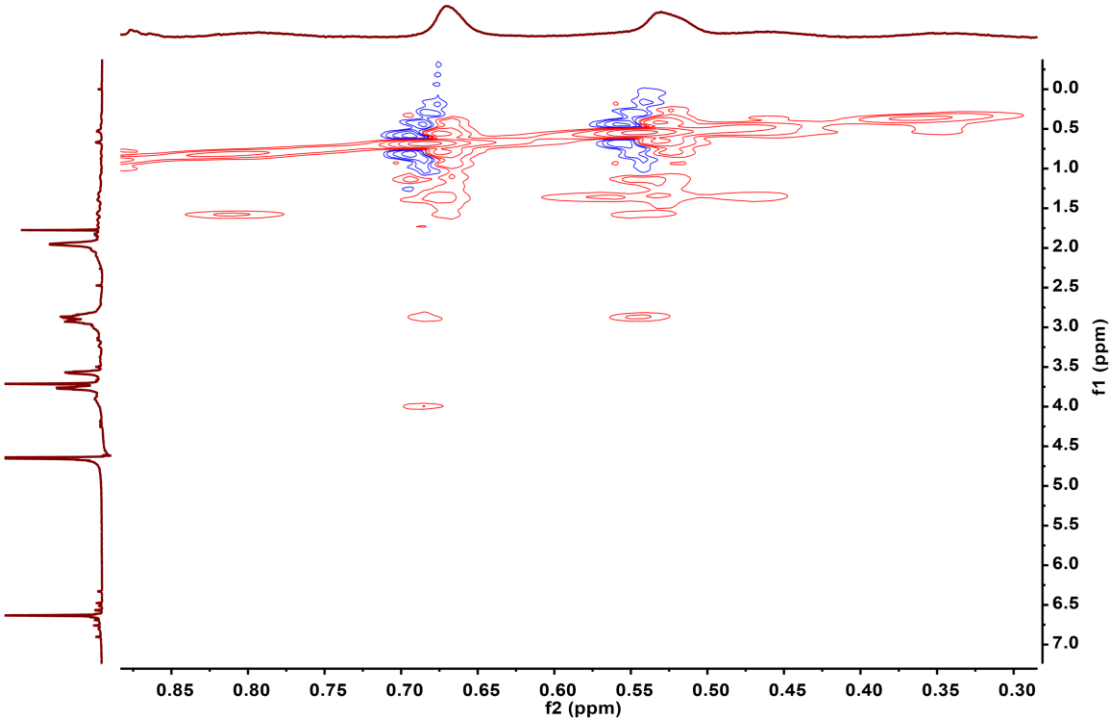

Figure S7. 2D NOESY NMR spectrum recorded (400 MHz, 298 K, RT, D_2O) for a mixture of Vec (2.5 mM) and SP[5]A (2.5 mM).

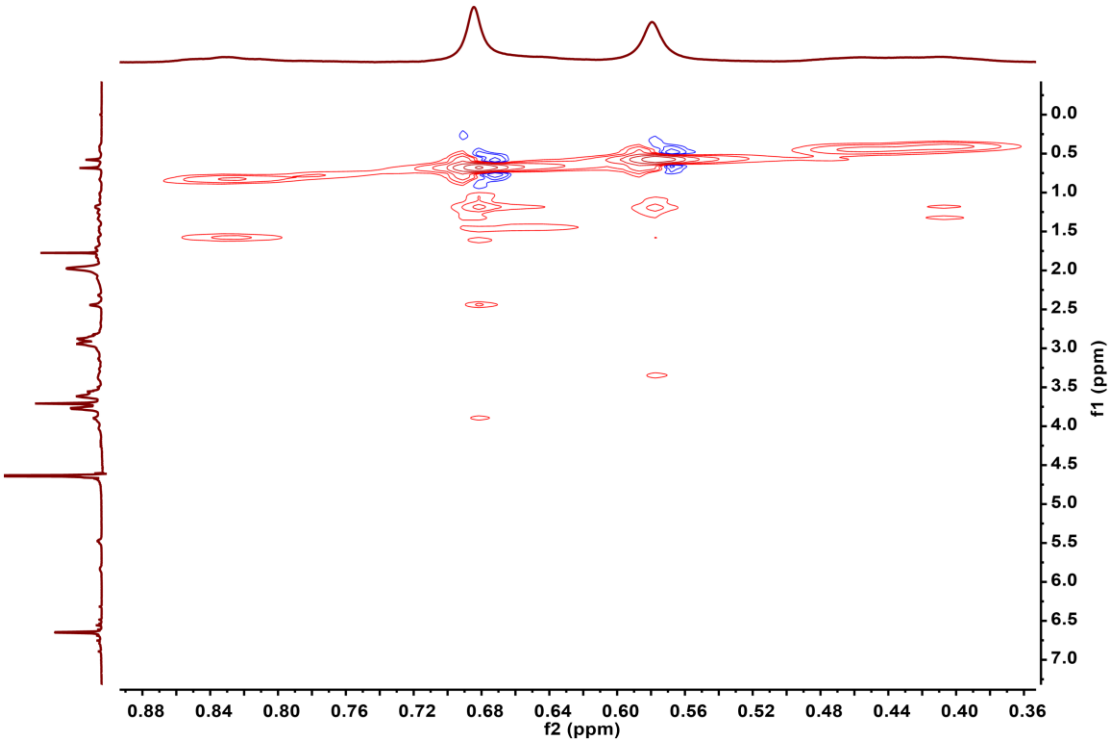

Figure S8. 2D NOESY NMR spectrum recorded (400 MHz, 298 K, RT, D_2O) for a mixture of Roc (2.5 mM) and SP[5]A (2.5 mM).

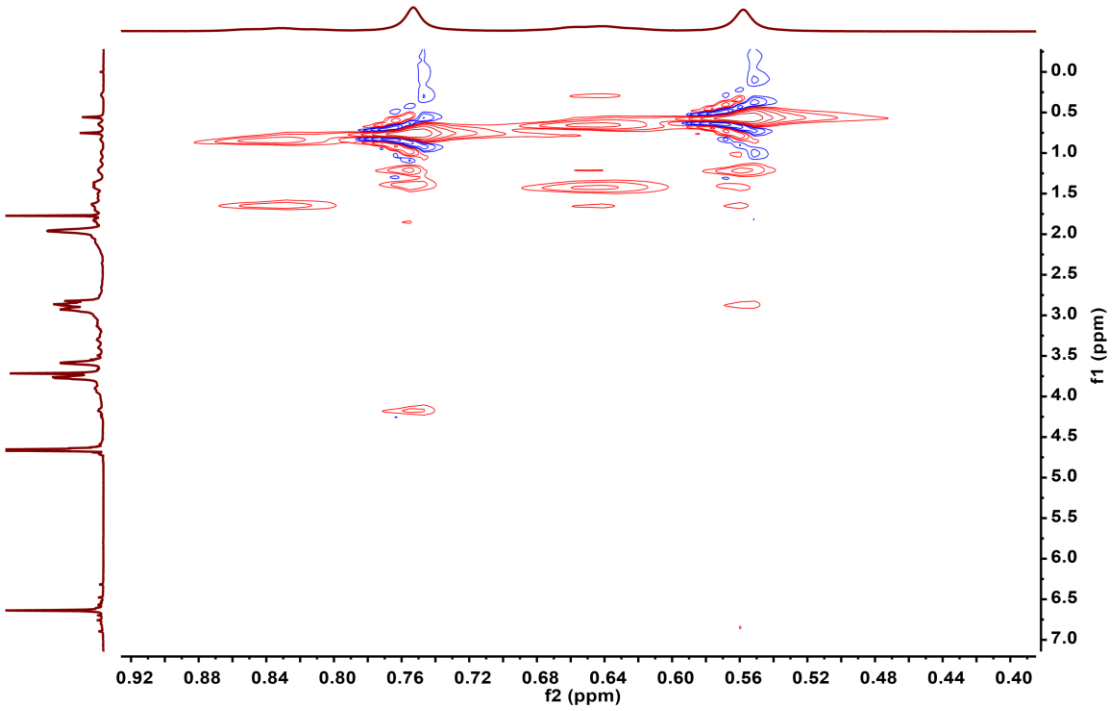

Figure S9. 2D NOESY NMR spectrum recorded (400 MHz, 298 K, RT, D_2O) for a mixture of Pan (2.5 mM) and SP[5]A (2.5 mM).

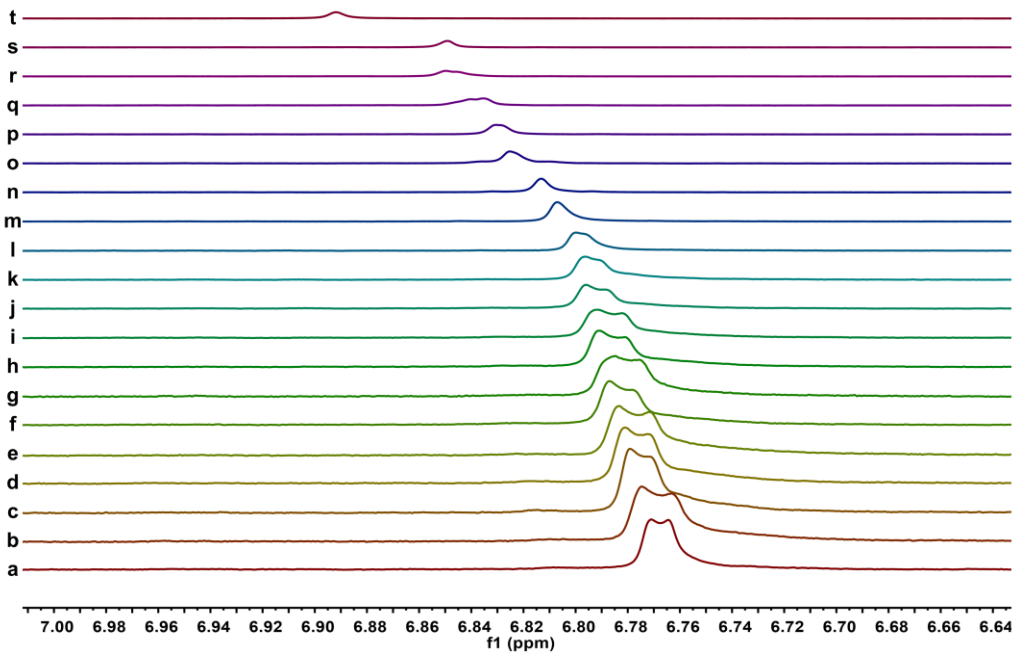

Figure S10. Partial ^{1}H NMR spectra (400 MHz, 298 K, RT, D_2O) of NMR titration between SP[5]A and Sch. The molar ratios of SP[5]A to Sch are: (a) 1: 0.050, (b) 1: 0.123, (c) 1: 0.244, (d) 1: 0.361, (e) 1: 0.476, (f) 1: 0.588, (g) 1: 0.698, (h) 1: 0.805, (i) 1: 0.909, (j) 1: 1.011, (k) 1: 1.111, (l) 1: 1.304, (m) 1: 1.667, (n) 1: 2, (o) 1: 2.727, (p) 1: 3.333, (q) 1: 5, (r) 1: 6, (s) 1: 7.333, (t) 1: 8.

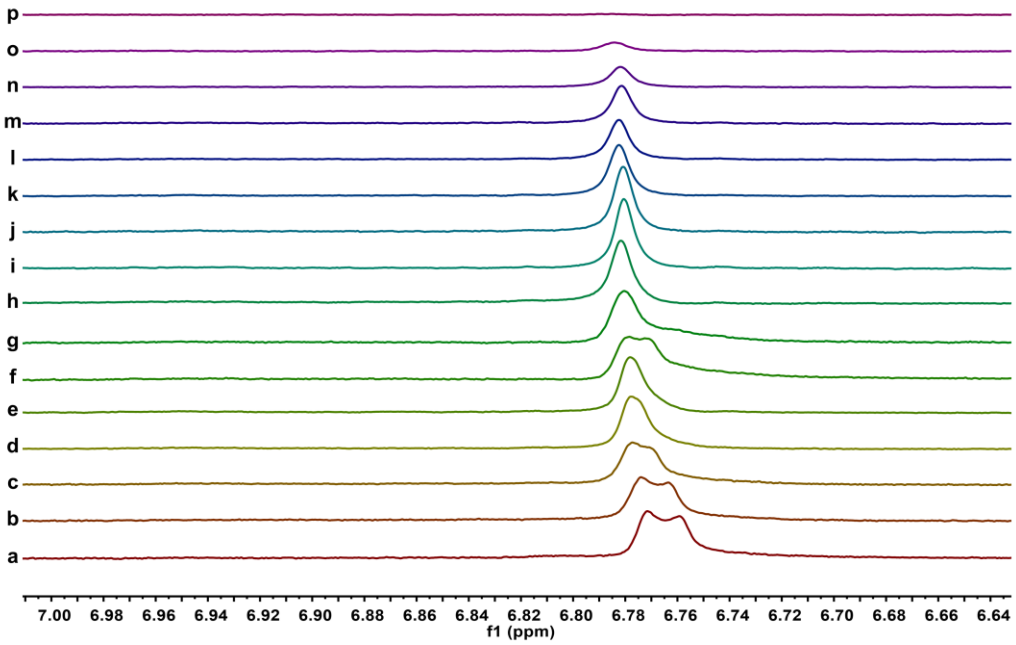

Figure S11. Partial ^{1}H NMR spectra (400 MHz, 298 K, RT, D_2O) of NMR titration between SP[5]A and Vec. The molar ratios of SP[5]A to Vec are: (a) 1: 0.050, (b) 1: 0.123, (c) 1: 0.244, (d) 1: 0.361, (e) 1: 0.476, (f) 1: 0.588, (g) 1: 0.698, (h) 1: 0.805, (i) 1: 0.909, (j) 1: 1.011, (k) 1: 1.111, (l) 1: 1.304, (m) 1: 1.667, (n) 1: 2, (o) 1: 2.727, (p) 1: 3.333.

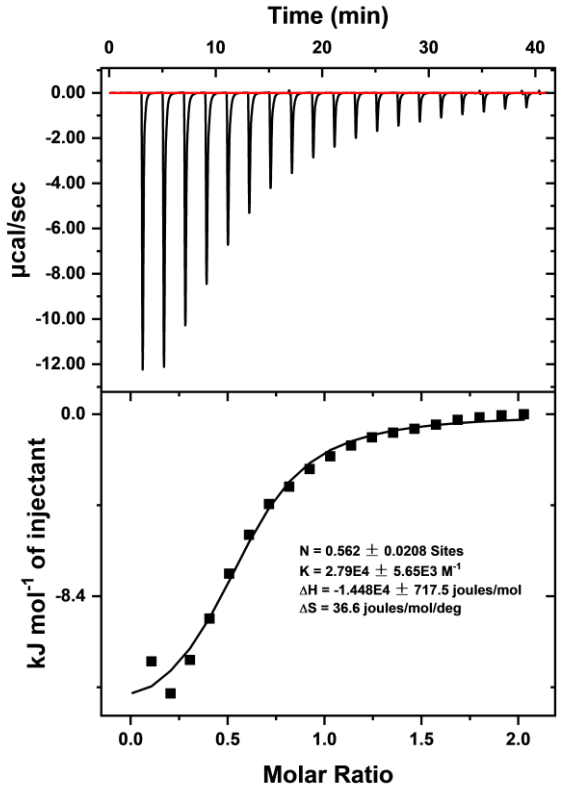

Figure S12. ITC titrations of Vec (2 mM) into SP[5]A (0.2 mM) in H_2O at 30 °C.

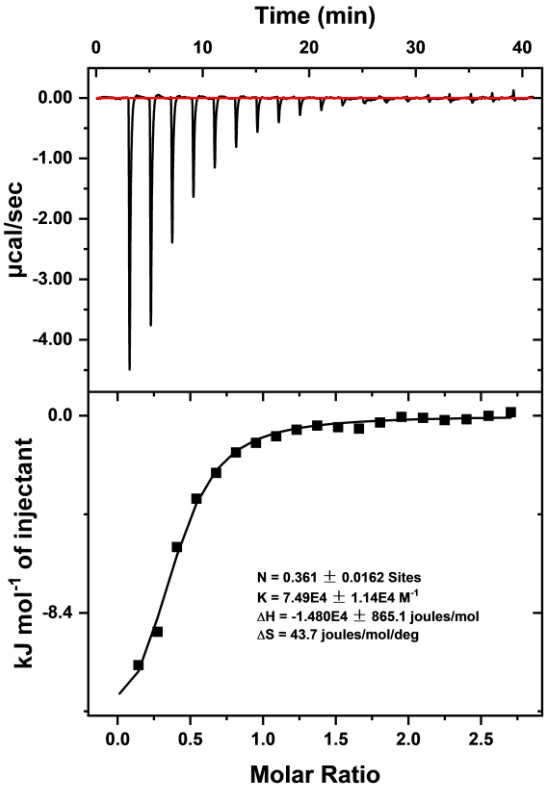

Figure S13. ITC titrations of Roc (2 mM) into SP[5]A (0.2 mM) in H_2O at 30 °C.

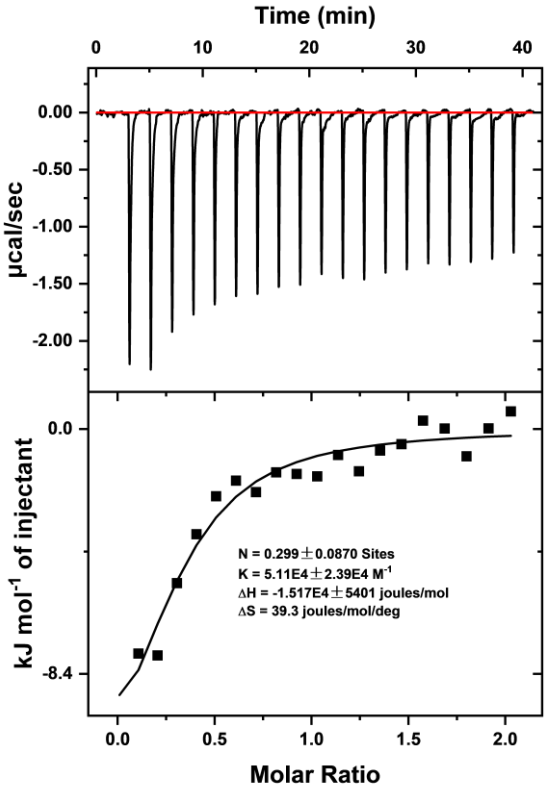

Figure S14. ITC titrations of Pan (2 mM) into SP[5]A (0.2 mM) in H_2O at 30 °C.

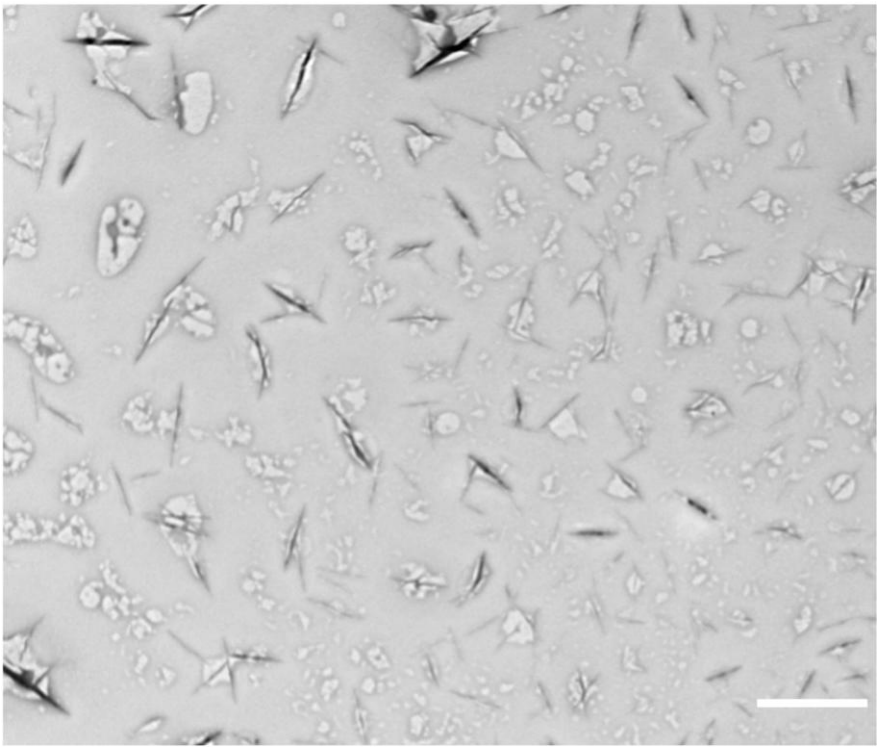

Figure S15. TEM image of SP[5]A (3mM)⊃Vec (3mM) complex. Scale bar = 2 μm.

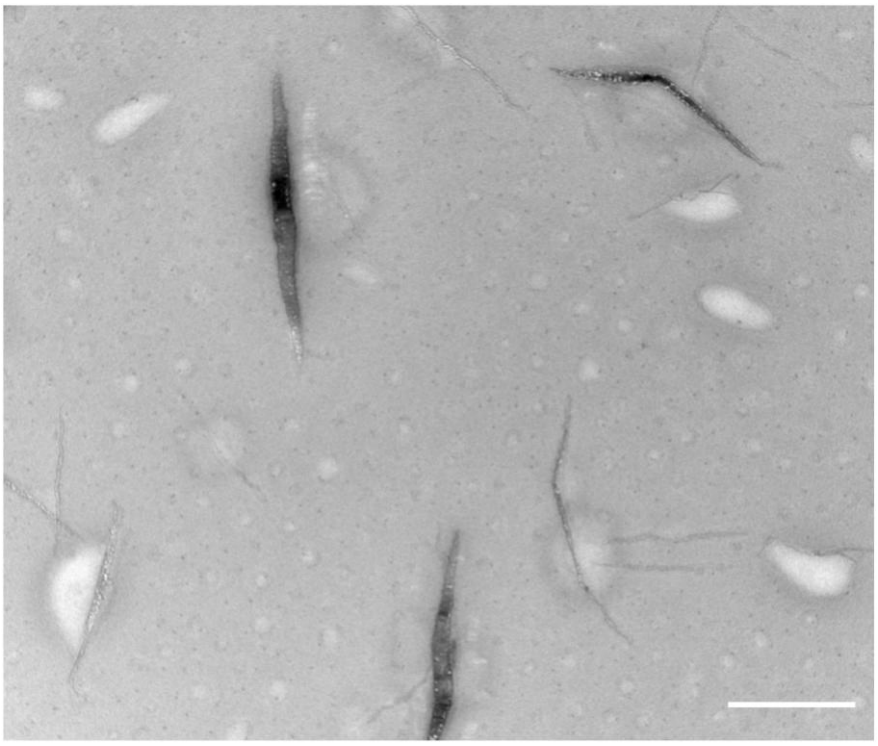

Figure S16. TEM image of SP[5]A (3mM)⊃Roc (3mM) complex. Scale bar = 500 nm.

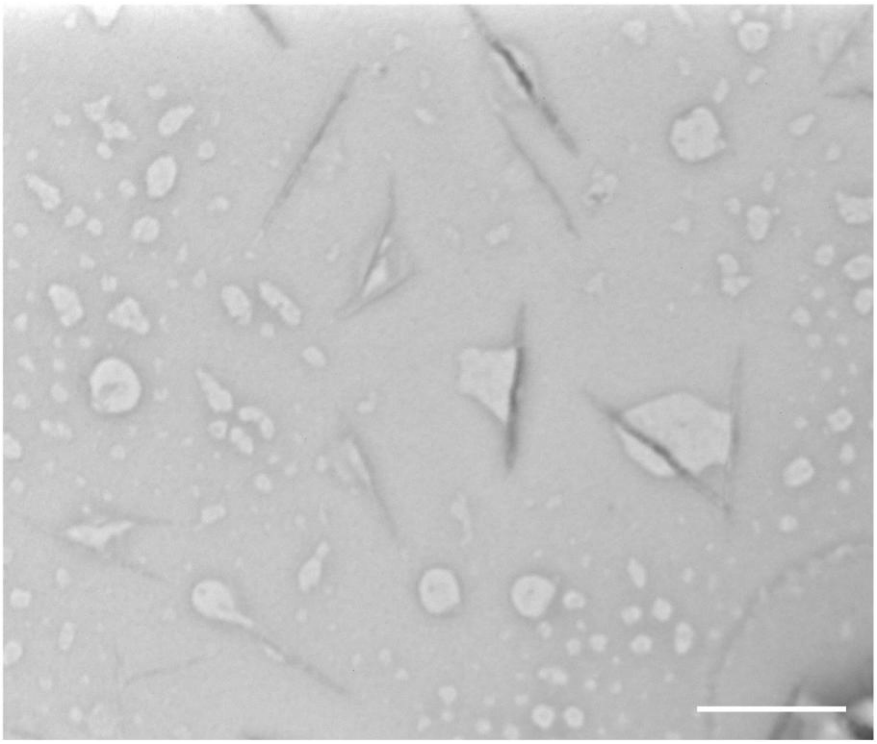

Figure S17. TEM image of SP[5]A (3mM)⊃Pan (3mM) complex. Scale bar = 1 μm.

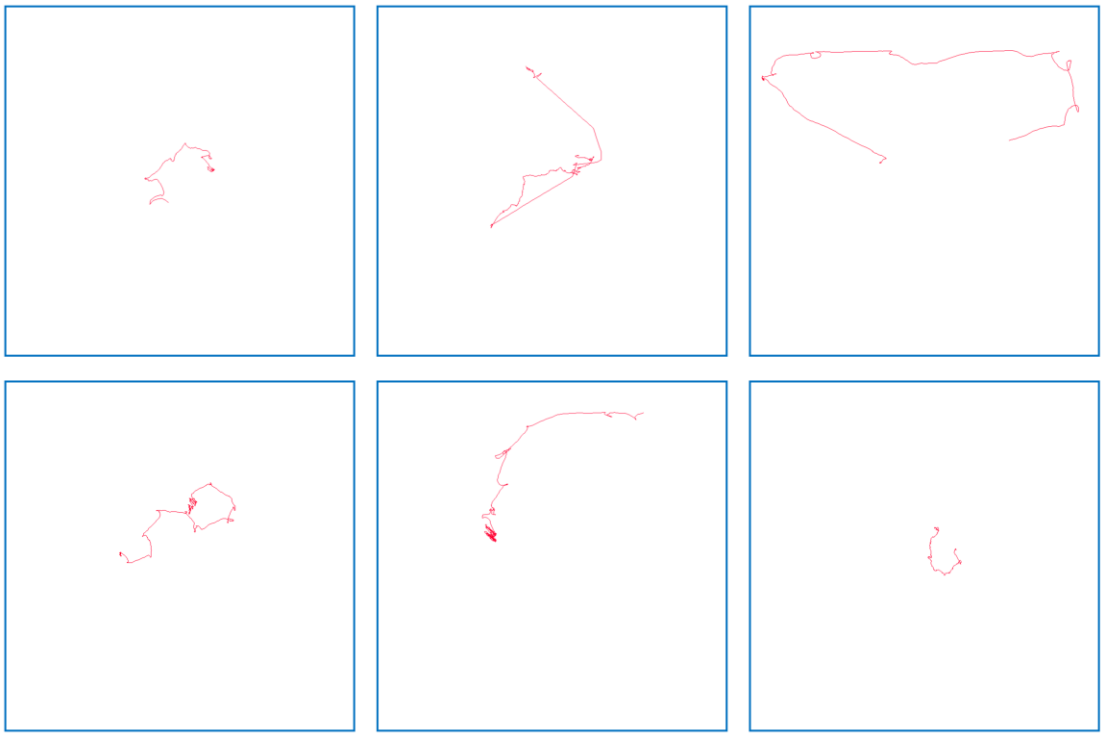

Figure S18. Detailed track sheets of the first minute in open field test for mice administrated Sch and PBS.

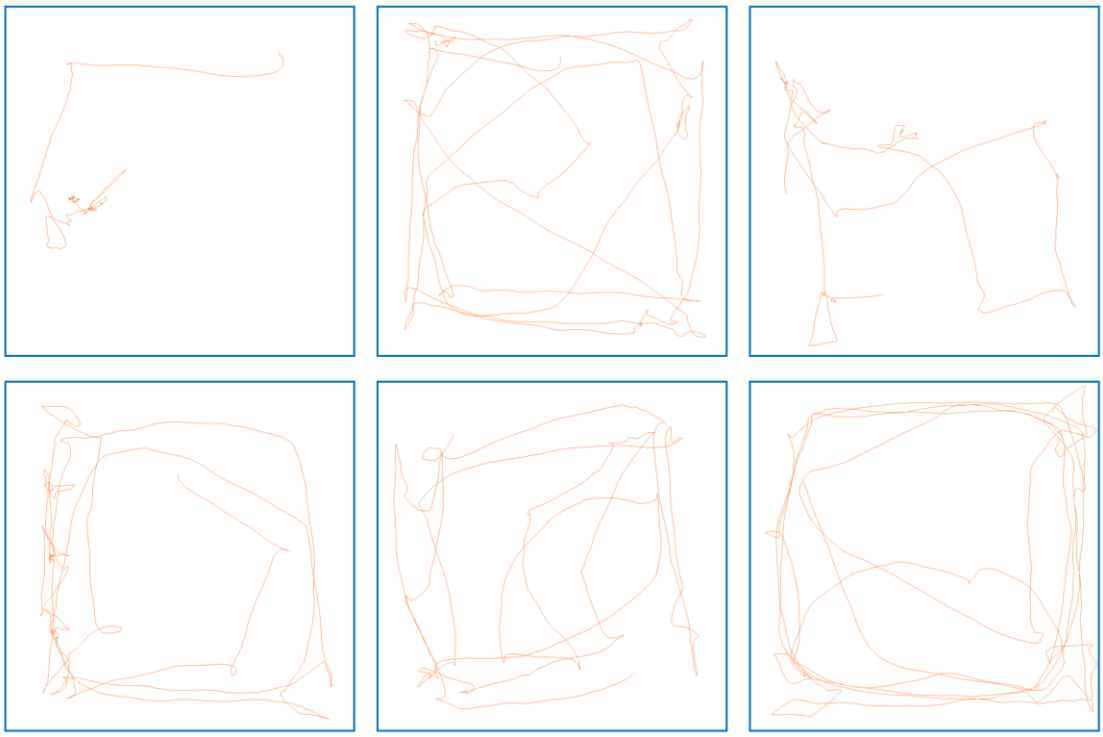

Figure S19. Detailed track sheets of the first minute in open field test for mice administrated Sch and SP[5]A.

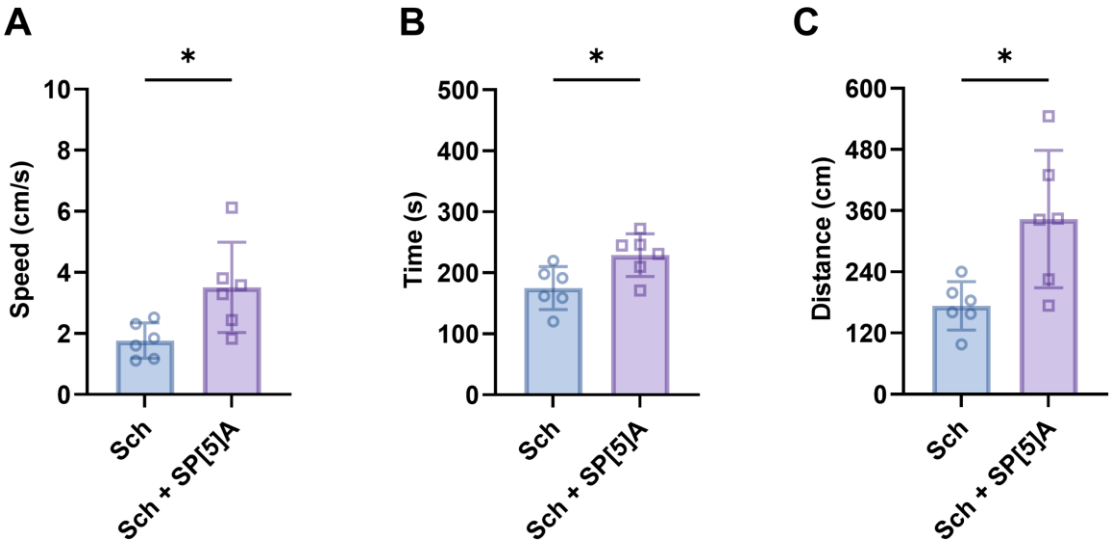

Figure S20. (**A** to **B**) Speed (**A**) and total movement duration (**B**) for the initial 5 min in open field test of mice treated with or without SP[5]A. (**C**) Distance for the most active 1–minute interval within the initial 5 min in open field test of mice treated with or without SP[5]A. $^{*}P \leq 0.05$ using unpaired *t*–test. Data are presented as means ± SD with n = 6.

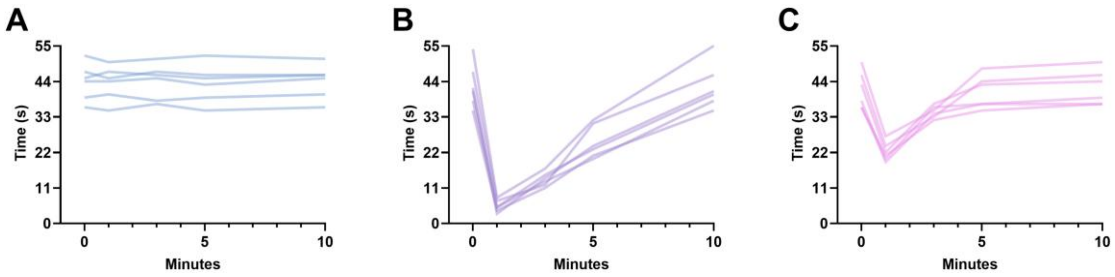

Figure S21. (**A** to **C**) Maximum continuous running time for each mouse in the control group (**A**), the Sch group (**B**), and the Sch + SP[5]A group (**C**) on the rotarod without falling within a 10–minute period (n = 6).

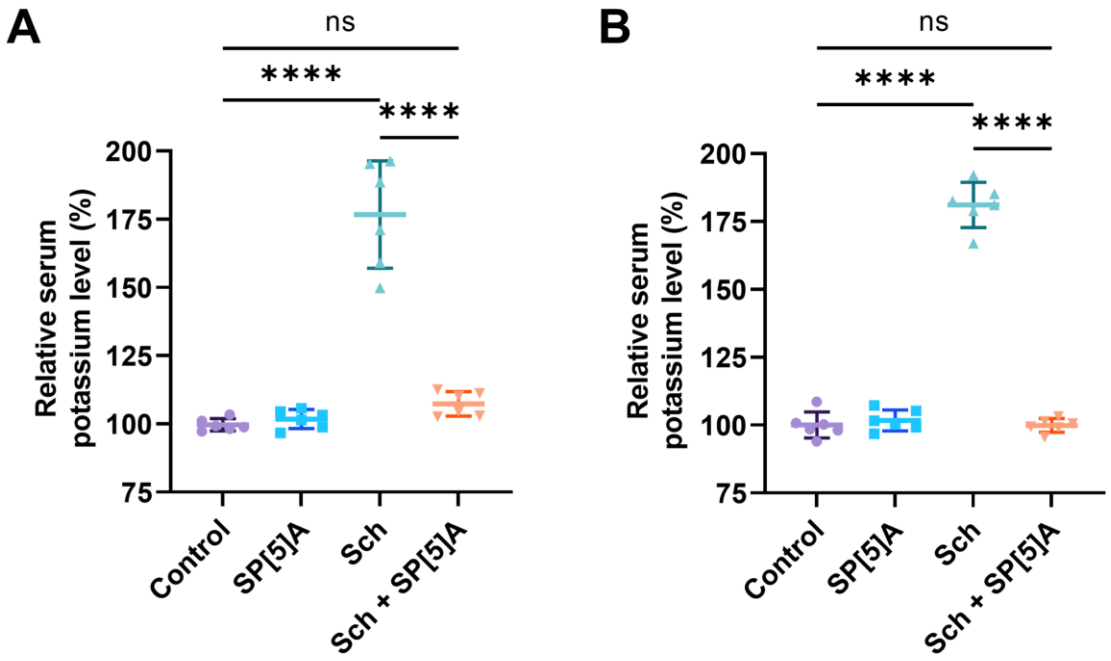

Figure S22. (**A** to **B**) Detailed serum potassium levels at 5 min (**A**), and 10 min (**B**) after different treatment. ns, not significant, and $^{****}P \leq 0.0001$ using unpaired *t*–test. Data are presented as means ± SD with n = 6.

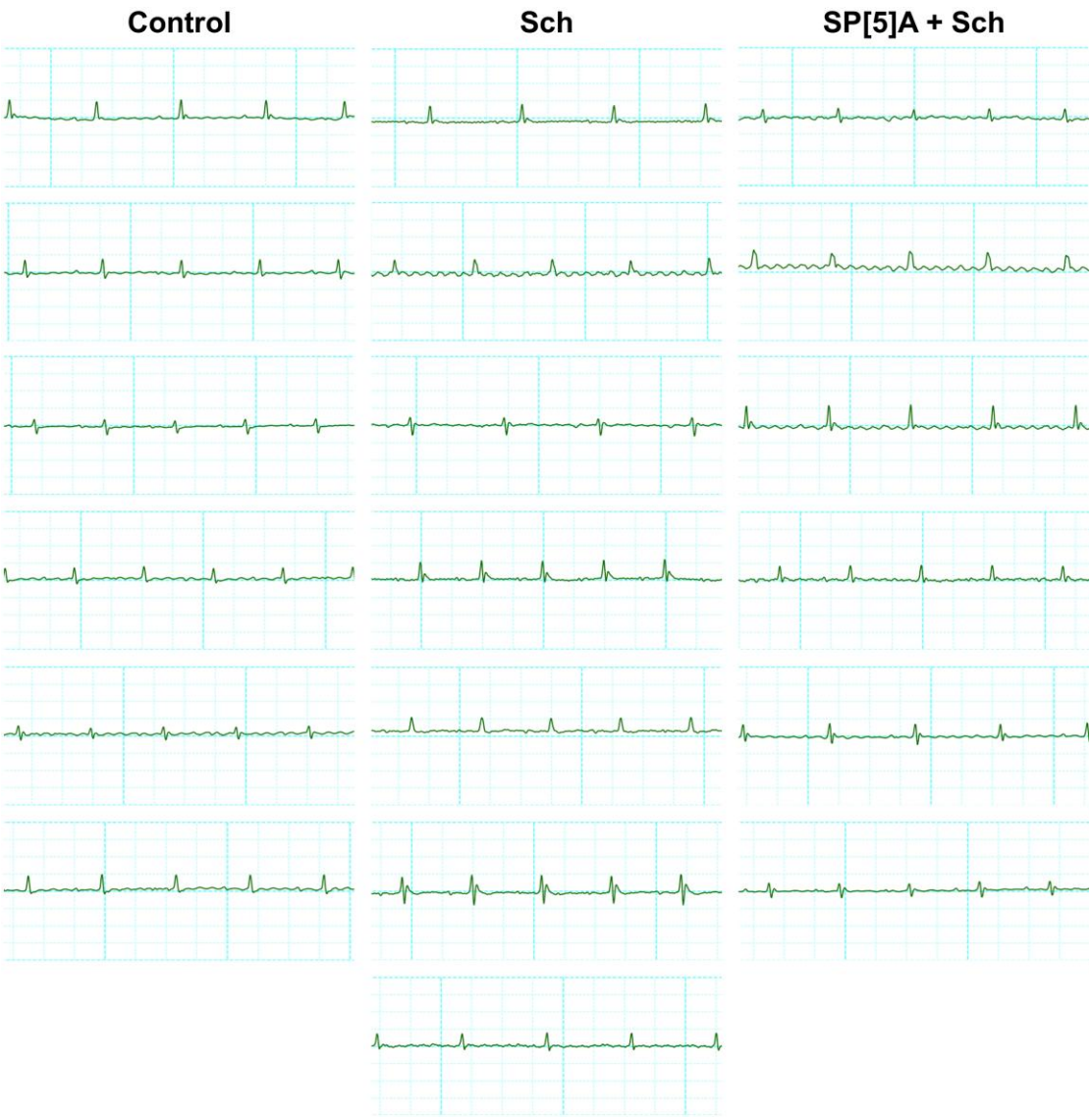

Figure S23. Detailed ECG recordings of mice in different groups before any injection.

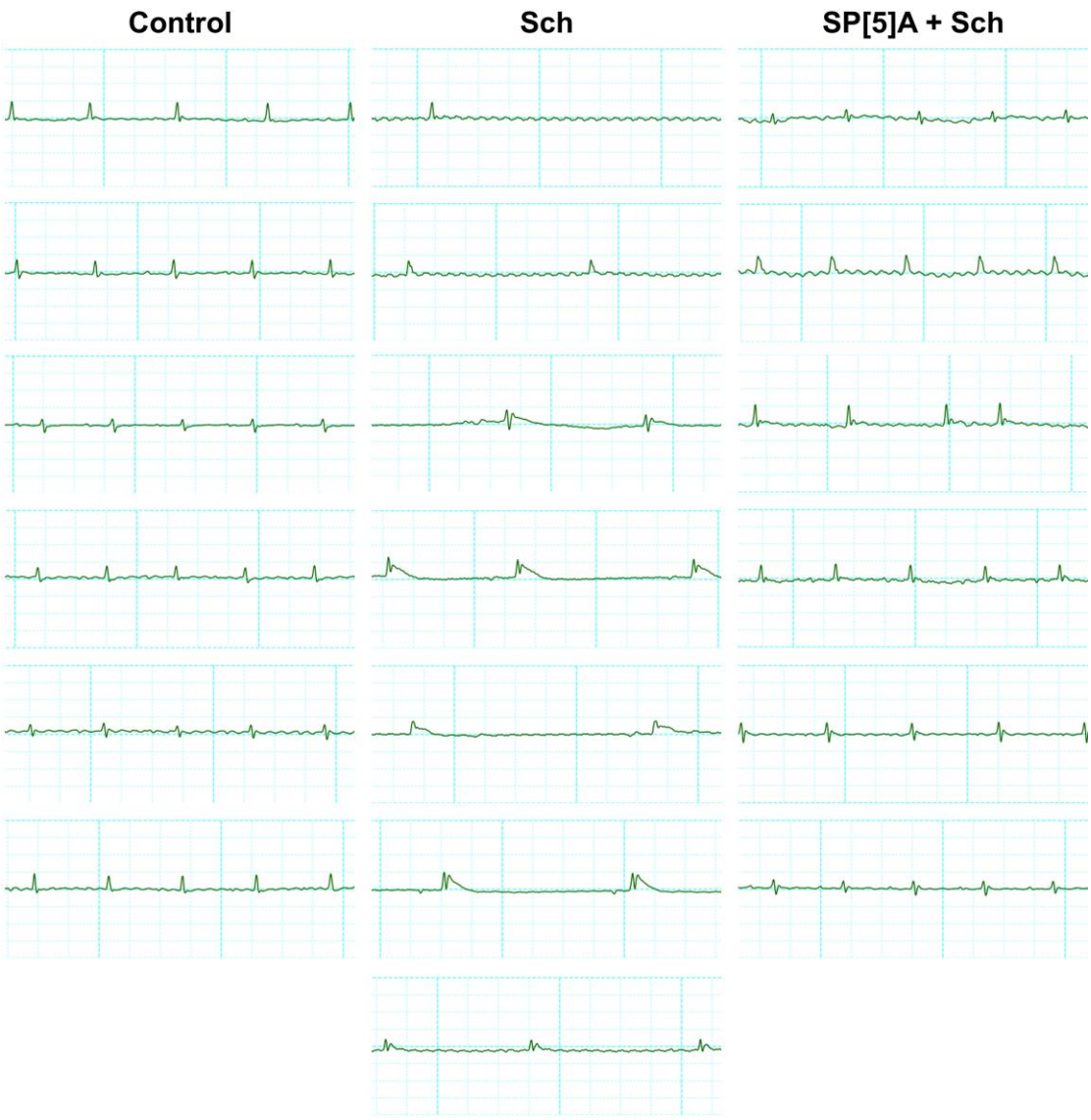

Figure S24. Detailed ECG recordings of mice in different groups at 20 s after the administration.

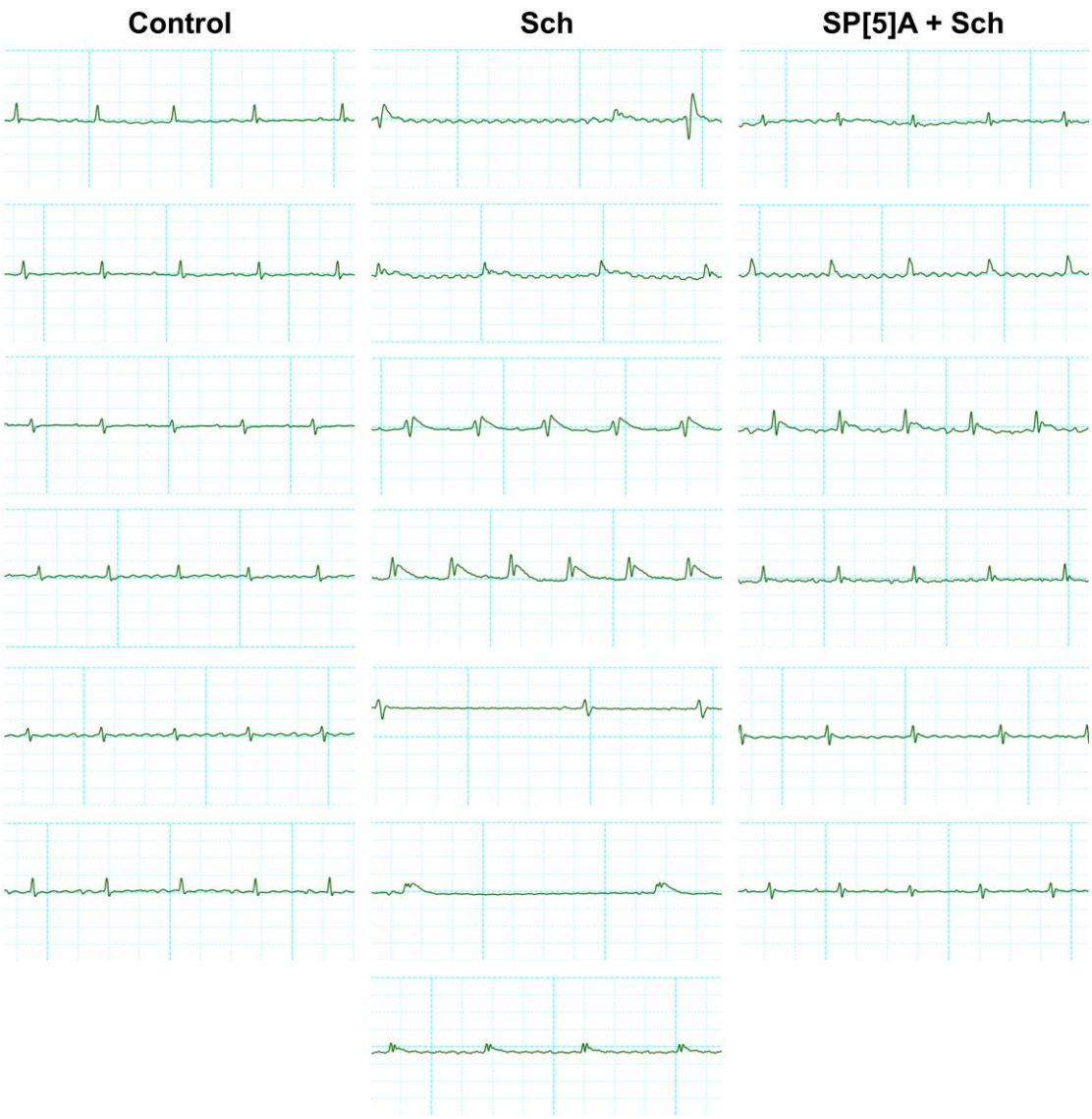

Figure S25. Detailed ECG recordings of mice in different groups at 40 s after the administration.

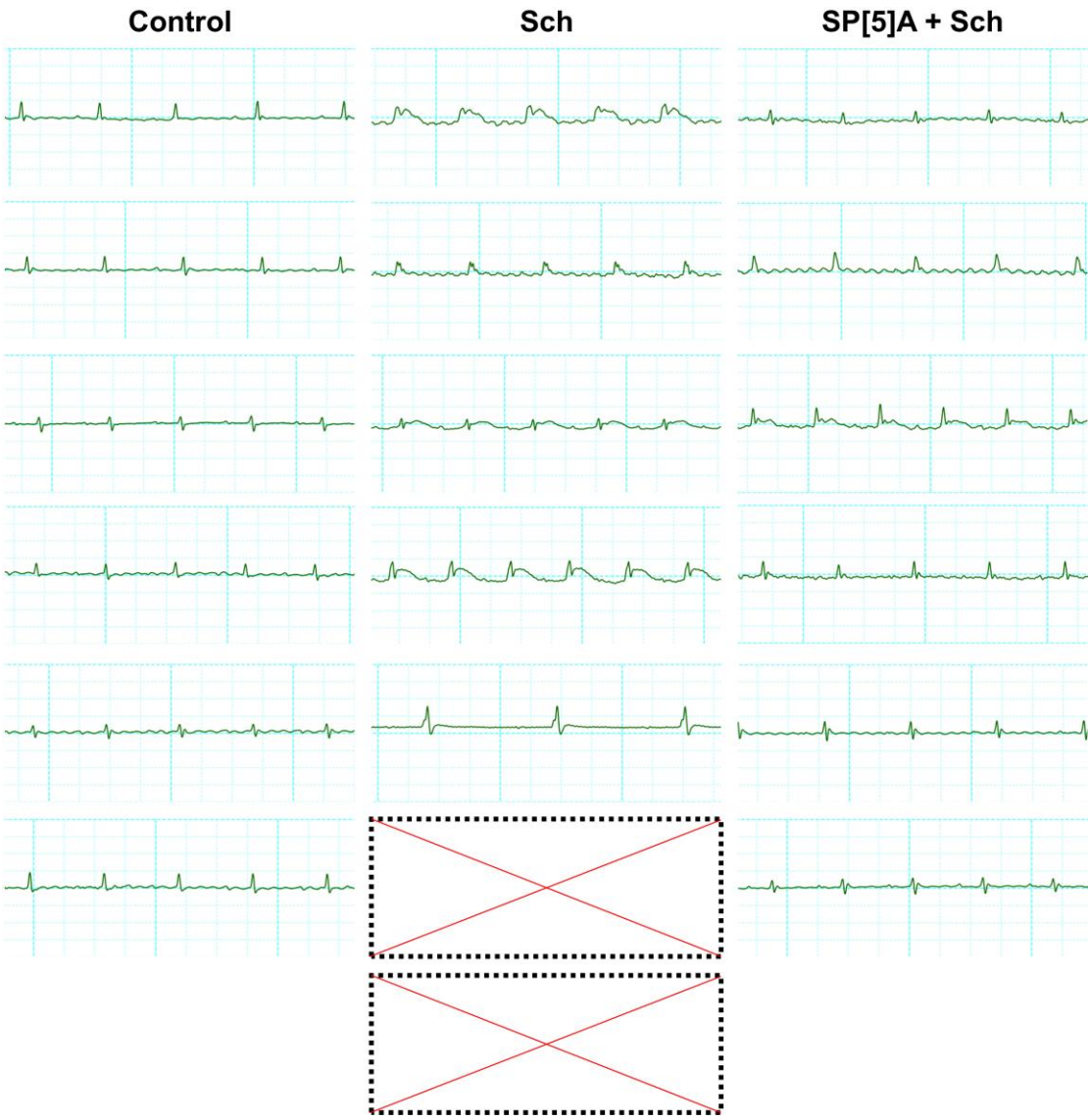

Figure S26. Detailed ECG recordings of mice in different groups at 1 min after the administration.

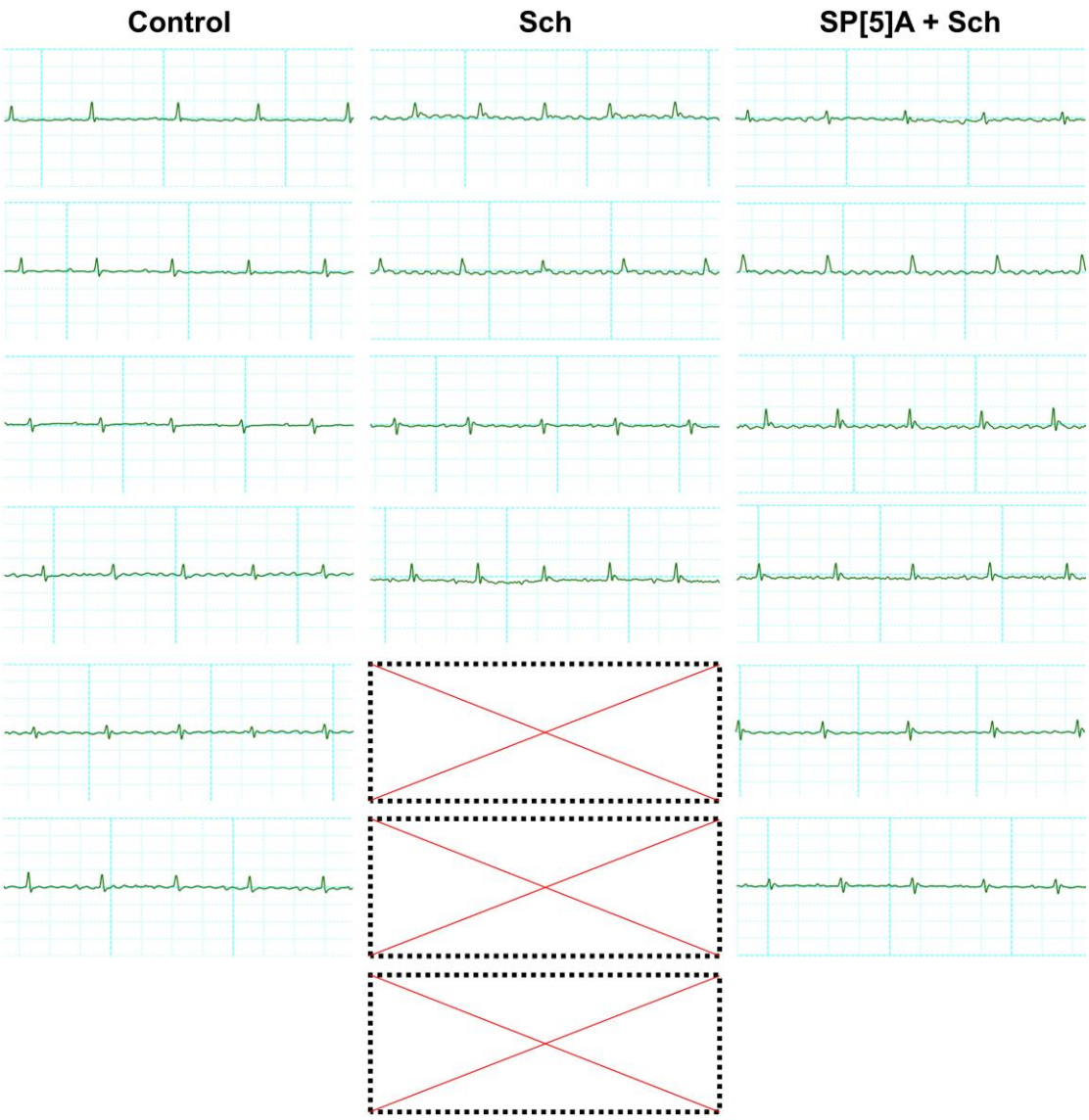

Figure S27. Detailed ECG recordings of mice in different groups at 3 min after the administration.

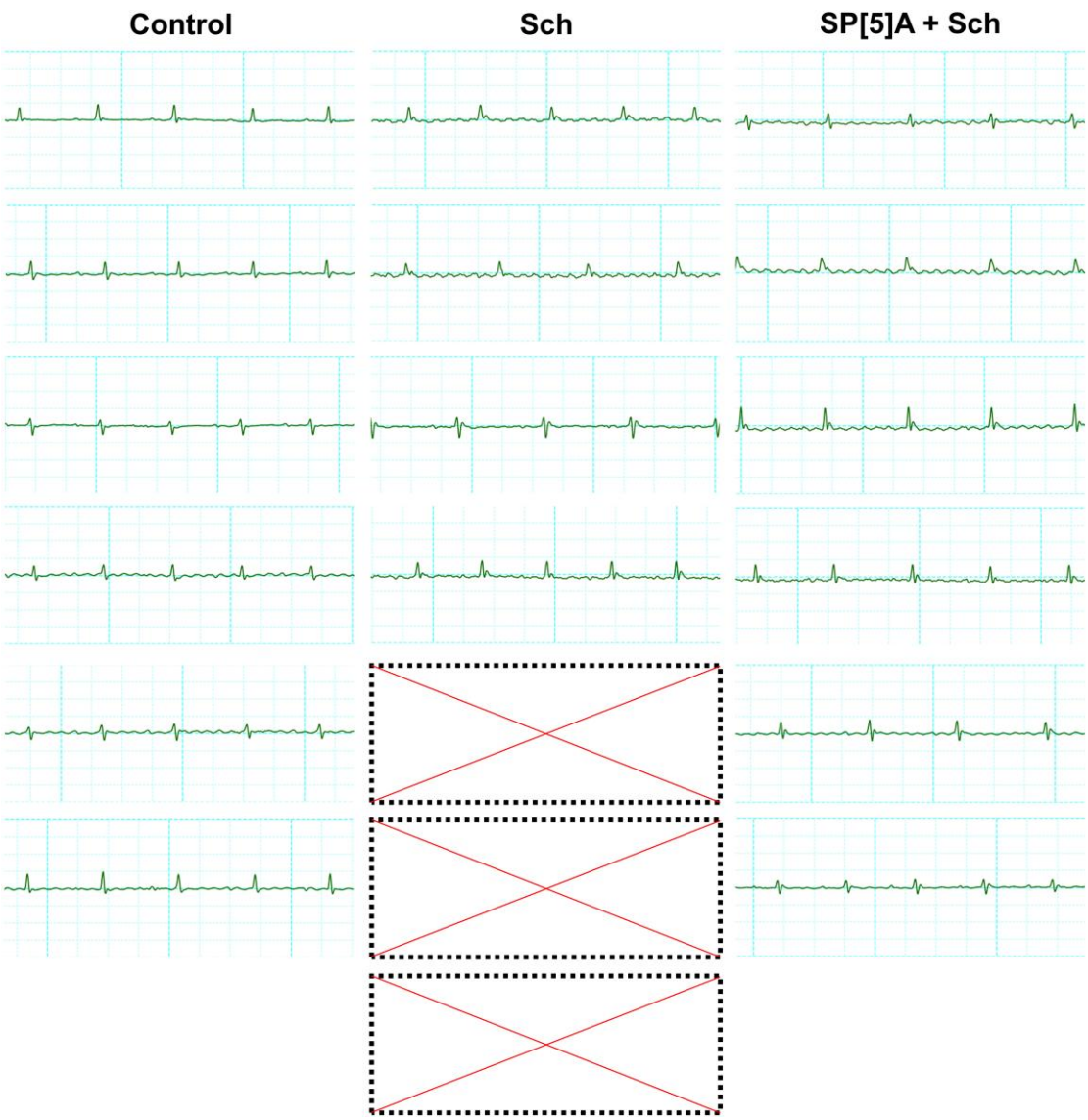

Figure S28. Detailed ECG recordings of mice in different groups at 5 min after the administration.

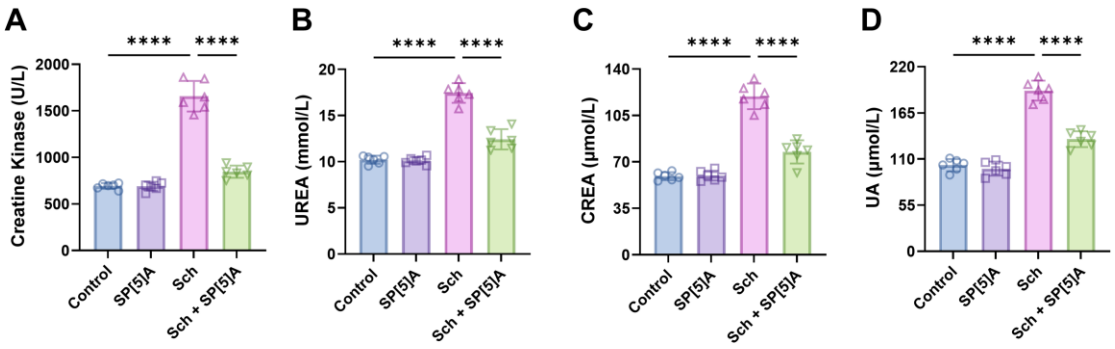

Figure S29. (**A** to **D**) Serum biochemical analysis included CK (**A**), UREA (**B**), CREA (**C**), and UA (**D**) of mice after different treatment. $^{****}P \leq 0.0001$ using unpaired *t*−test. Data are presented as means ± SD with n = 6.

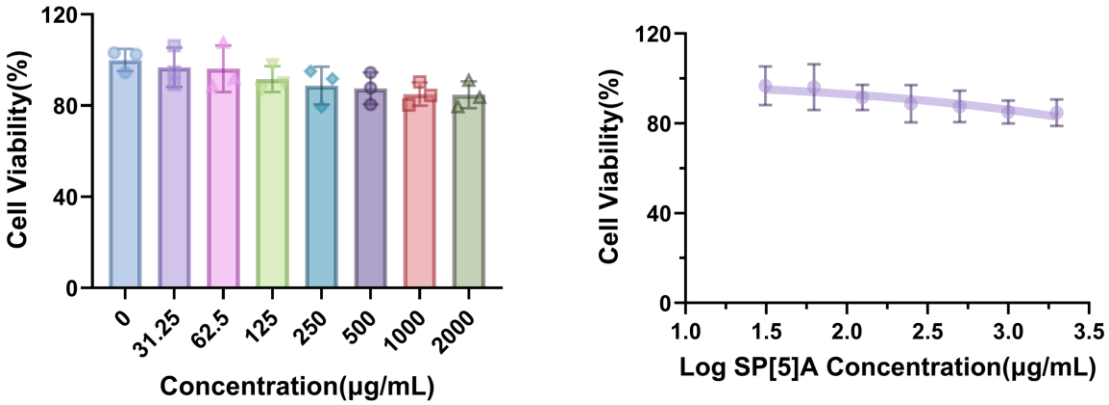

Figure S30. CCK–8 assay for mouse L929 cell viabilities under different concentrations of SP[5]A. Data are presented as means ± SD with n = 3.

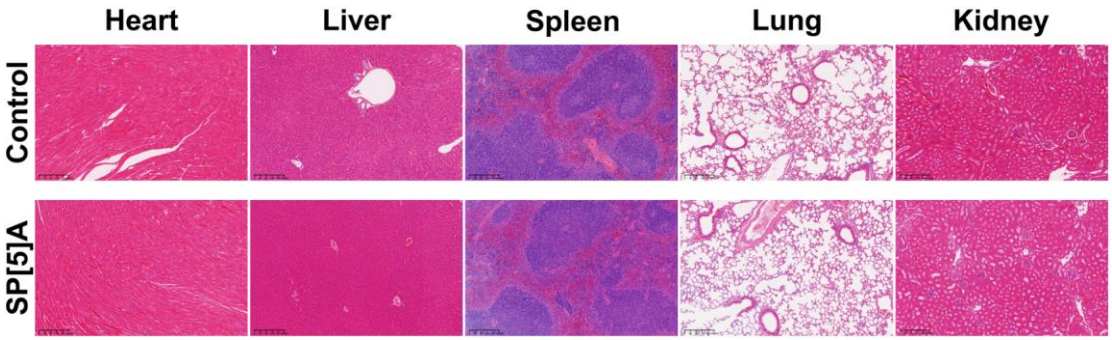

Figure S31. Representative pathological morphology of primary organs of mice after the injection of PBS or SP[5]A. Tissues were stained with hematoxylin and eosin (H&E).

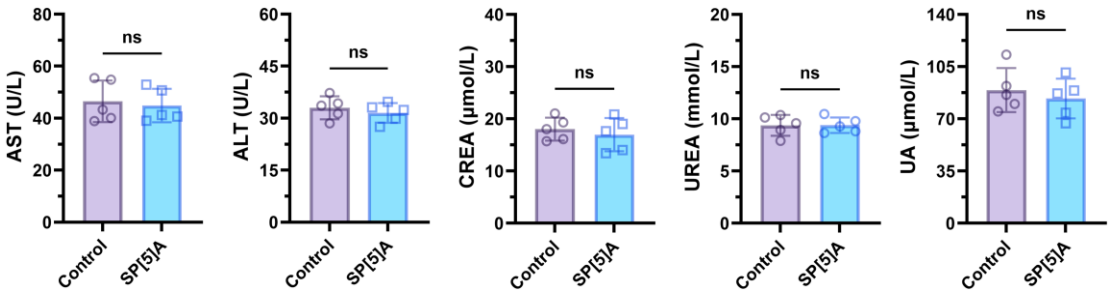

Figure S32. Quantitative serum biochemical analysis included aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine (CREA), urea, and uric acid (UA) of mice that administered with or without SP[5]A. Unpaired *t*–test was used. Data are presented as means ± SD with n = 5. ns, not significant.