Supplementary Information (SI) for ChemComm.
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Supplementary Information

# Nature Inspired Hybrid Molecular Design of Antiepileptic Molecule

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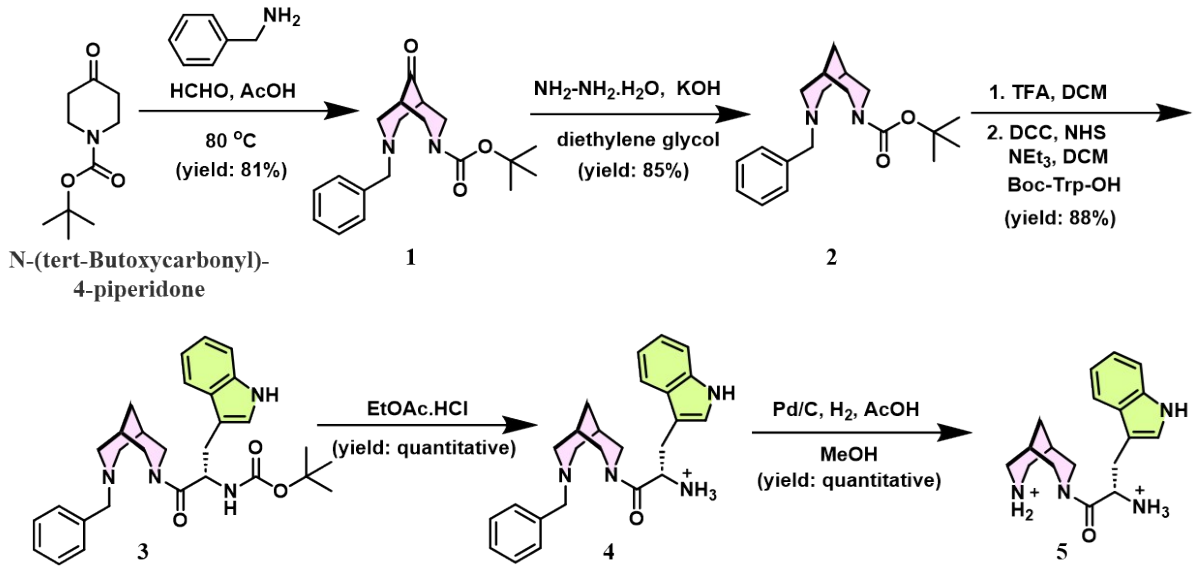
# 1. General Information

Tryptophan used was of L-configuration. Reactions were monitored by thin layer chromatography (TLC) and purifications were done using silica gel 100-200 mesh (SRL). TLC plates were purchased from Merck, India. Brucker-DPX-400 and Brucker-DPX-500 spectrometer were used for recording $^1$H NMR. Tetramethylsilane (TMS) was used as an internal standard. $^1$H NMR data are reported as s (singlet), d (doublet), br s (broad singlet), t (triplet) and m (multiplet), dd (double of doublet). Nicolet, Protégé 460 spectrometer was used for recording IR spectra with KBr pellets. Bruker MicrO-TOF-QII model High-resolution mass spectrometer (HRMS) was used for characterization.

# 2. Synthetic Procedure




**Scheme S1.** Synthesis of pseudopeptides **4** and **5.**

## 2.1. Synthetic Procedure of 3

To a well stirred and ice cooled solution of compound **2** (1000 mg, 3.16 mmol) in DCM (25 mL) was added TFA (5 mL), and the reaction mixture was stirred for 4h at room temperature. The reaction mixture was evaporated and used as such for further reaction. The obtained amine was added to the DCM solution of Boc-tryptophan (800 mg, 2.63 mmol), NHS (364 mg, 3.16 mmol), DCC (652 mg, 3.16 mmol) and $NEt_3$ (0.44 mL, 3.16 mmol). The resultant solution was stirred for 24h at room temperature. After completion of reaction, the reaction mixture was evaporated and the residue was dissolved in ethyl acetate and filtered. The filtrate was washed

with 0.2 N $H_2SO_4$, saturated aq. $NaHCO_3$ and water. The organic layer was collected and dried over anhyd. $Na_2SO_4$ and evaporated under vacuum to obtain the crude product, which was purified by silica gel column chromatography (Ethyl acetate/Hexane) to obtain **3** as semi-solid.

### 2.2. Synthetic Procedure of 4

An ice-cold solution of **3** (500 mg, 0.99 mmol, 1 equiv.) in ~40 mL of EtOAc·HCl was stirred for 3 h. The reaction was monitored by TLC and after completion, the reaction mixture was evaporated under a high vacuum with a KOH trap to afford the Boc-deprotected derivative **4**.

### 2.3. Synthetic Procedure of 5

To an ice-cold solution of **4** (300 mg, 0.74 mmol, 1 equiv.) in ~25 mL methanol, 10% w/w Pd/C catalyst (30 mg), acetic acid (0.3 mL) were added, and hydrogen gas was bubbled into the solution. The resulting solution was stirred for 4 h, after which it was filtered through a filter paper and the eluent solution was evaporated under a high vacuum to afford **5**.

## 3. Analytical data of pseudopeptides 3, 4 and 5

### 3.1. Compound 3

**Yield:** 88 %

**$^1$H NMR (500 MHz, $CDCl_3$):** δ 1.37 (s, minor), 1.42 (s, major, 9H), 1.46 (m, major+minor, 2H), 1.63 (m, major+minor, 1H), 1.80 (m, major+minor, 1H), 2.18 (d, major, *J* = 11 Hz, 1H), 2.30 (d, minor, *J* = 10 Hz), 2.52 (d, major+minor, *J* =13 Hz, 1H), 2.67 (d, major, *J* = 13.5 Hz, 1H), 2.79 (d, minor, *J* = 10.5 Hz), 2.90 (d, major, *J* = 11 Hz, 2H), 2.95 (d, minor, *J* = 11 Hz), 3.22 (m, major+minor, 2H), 3.39 (d, major+minor, *J* = 13.5 Hz, 1H), 3.50 (d, major+minor, *J* = 13 Hz, 1H), 3.73 (d, major, *J* = 13 Hz, 1H), 3.81 (d, minor, *J* = 12.5 Hz), 4.33 (d, minor, *J* = 13.5 Hz), 4.53 (d, major, *J* = 13.5 Hz, 1H), 5.03 (m, major+minor, 1H), 5.49 (d, minor, *J* = 9.5 Hz), 5.89 (d, major, *J* = 8 Hz, 1H), 7.05-7.35 (m, major+minor, 10H), 7.54 (d, minor, *J* = 7.5 Hz), 7.65 (d, major, *J* = 7.5 Hz, 1H), 8.07 (br s, minor), 8.17 (br s, major, 1H).

**$^{13}$C NMR (125 MHz, $CDCl_3$):** δ 25.0, 25.6, 28.3, 28.4, 28.8, 29.2, 29.5, 31.5, 46.6, 46.7, 49.8, 50.9, 58.2, 58.4, 58.5, 59.4, 63.4, 79.2, 111.0, 111.2, 111.4, 118.7, 119.0, 119.4, 121.9, 122.6, 122.9, 126.7, 128.2, 128.3, 128.7, 128.8, 136.0, 136.2, 138.0, 138.4, 155.2, 155.4, 170.2, 171.2.

**IR (KBr):** 3422, 2925, 1694, 1629, 1495, 1455, 1365, 1169 $cm^{-1}$.

**HRMS:** Calcd. for $C_{30}H_{39}N_4O_3$ m/z = 503.3022, found m/z = 503.3030.

## 3.2. Compound 4

**Yield:** quantitative

**$^1$H NMR (400 MHz, DMSO-*d*$_6$):** δ 1.21 (m, 2H), 1.64 (d, *J* = 14.0 Hz, 1H), 1.92 (m, 1H), 2.04 (m, 1H), 2.30 (d, *J* = 12.4 Hz, 1H), 2.88 (d, *J* = 12.4 Hz, 1H), 3.09 (m, 4H), 3.64 (d, *J* = 12.4 Hz, 1H), 3.80 (d, *J* = 11.2 Hz, 1H), 3.98 (m, 1H), 4.15 (d, *J* = 13.2 Hz, 1H), 4.48 (m, 2H), 6.95-7.10 (m, 2H), 6.97 (t, *J* = 7.2 Hz, 1H), 7.06 (t, *J* = 7.2 Hz, 1H), 7.20 (s, 1H), 7.30-7.45 (m, 5H), 7.71 (m, 2H), 8.35-8.70 (m, 3H), 11.02 (br s, 1H).

**$^{13}$C NMR (100 MHz, DMSO-*d*$_6$):** δ 33.8, 46.1, 47.9, 51.1, 54.3, 56.4, 61.6, 107.2, 112.1, 118.6, 119.2, 121.8, 125.3, 127.3, 129.2, 130.1, 132.2, 136.6, 171.6.

**IR (KBr):** 3431, 2925, 2858, 1717, 1642, 1456, 1376, 1234, 1106, 1045, 1016 cm$^{-1}$.

**HRMS:** Calcd. for $C_{25}H_{31}N_4O^+$ m/z = 403.2492, found m/z = 403.2504.

## 3.3. Compound 5

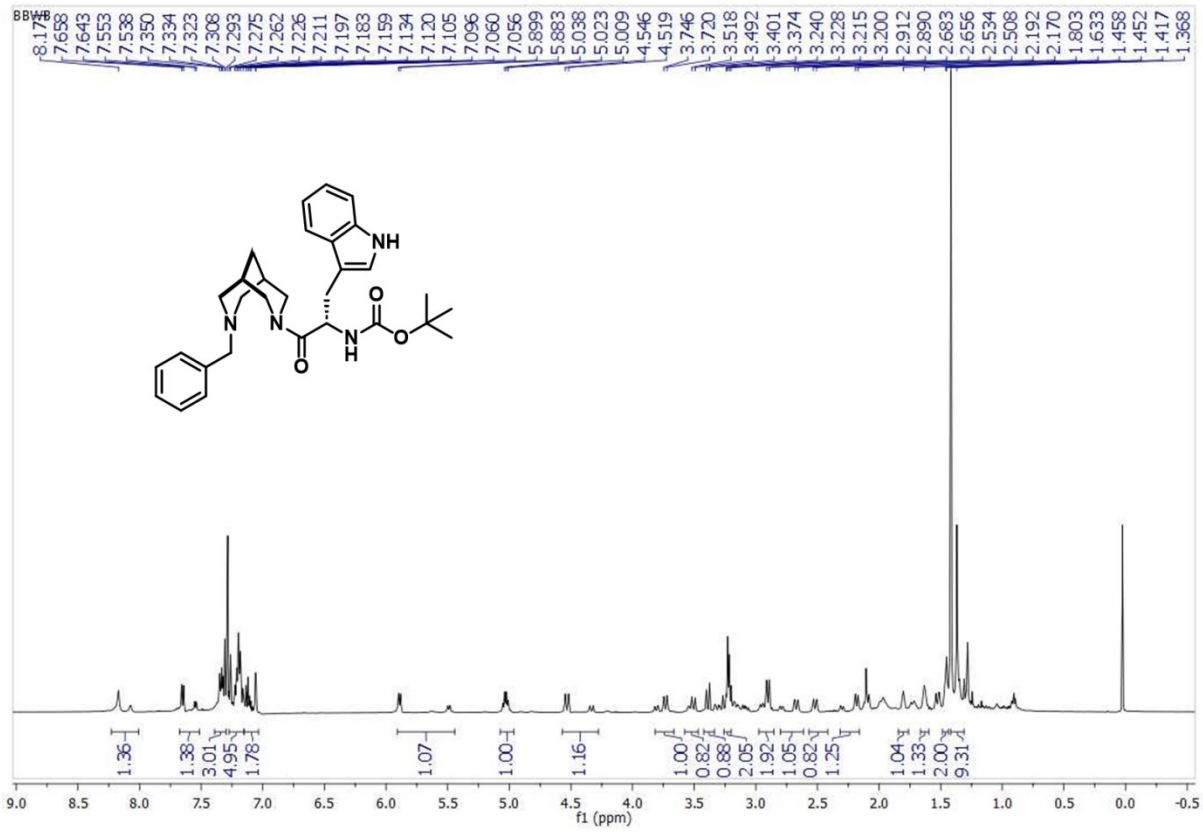
**Yield:** quantitative

**$^1$H NMR (400 MHz, DMSO-*d*$_6$):** δ 1.30 (d, *J* = 11.6 Hz, 1H), 1.72 (m, 2H), 1.97 (m, 1H), 2.39 (d, *J* = 12.4 Hz, 1H), 2.86-3.18 (m, 5H), 3.77 (d, *J* = 13.2 Hz, 1H), 4.32 (d, *J* = 13.2 Hz, 1H), 4.54 (br s, 1H), 6.98 (t, *J* = 7.2 Hz, 1H), 7.05 (t, *J* = 7.2 Hz, 1H), 7.19 (s, 1H), 7.34 (d, *J* = 8.4 Hz, 1H), 7.37 (d, *J* = 8.0 Hz, 1H), 8.44 (br s, 2H), 9.29 (br s, 1H), 11.07 (s, 1H).

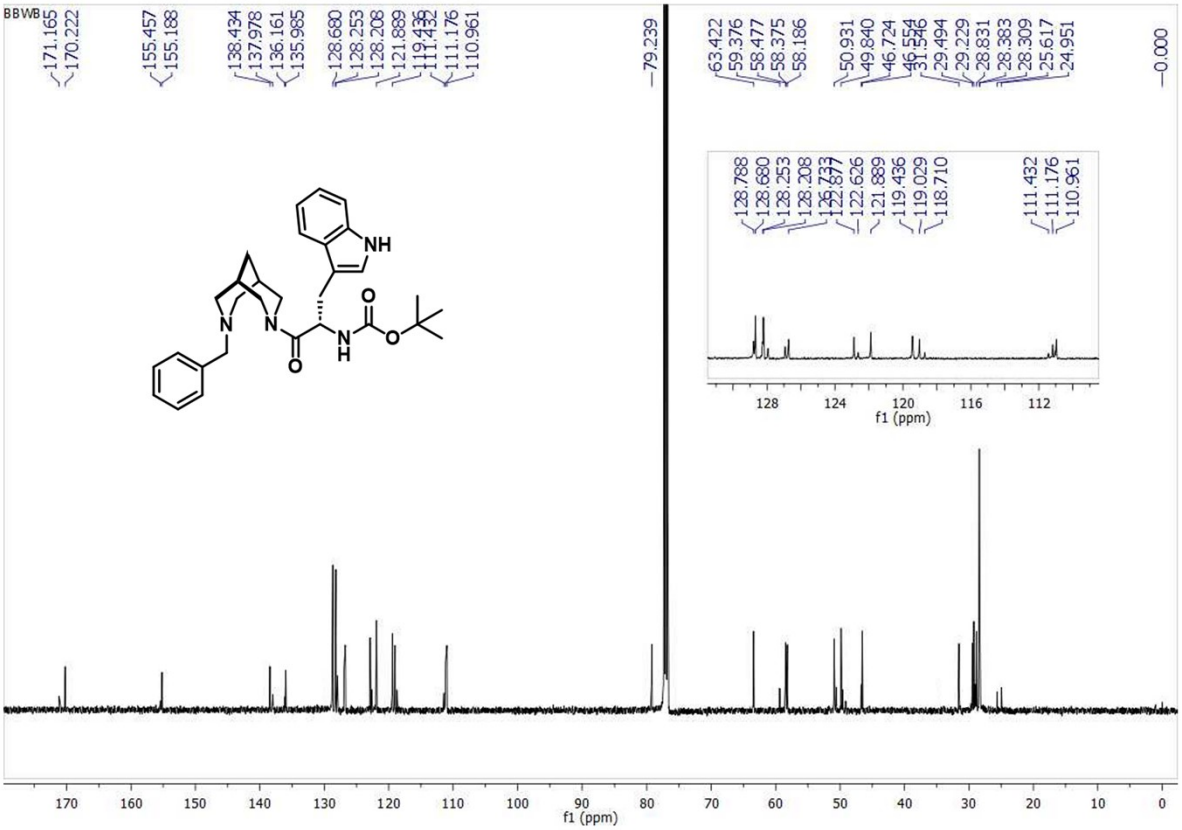
**$^{13}$C NMR (100 MHz, DMSO-*d*$_6$):** δ 21.6, 28.3, 34.7, 48.3, 50.6, 60.3, 107.2, 112.1, 118.6, 119.2, 121.8, 125.3, 127.4, 136.6, 172.5.

**IR (KBr):** 3427, 2930, 1645, 1457, 1382, 1237, 1104 cm$^{-1}$.

**HRMS:** Calcd. for $C_{18}H_{25}N_4O^+$ m/z = 313.2023, found m/z = 313.2041.




$^{1}$H NMR (500 MHz, $CDCl_3$) spectrum of **3**.




$^{13}$C NMR (125 MHz, $CDCl_3$) spectrum of **3**.

**Single Mass Analysis**
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
364 formula(e) evaluated with 2 results within limits (up to 10 closest results for each mass)
Elements Used:
C: 0-48 H: 0-50 N: 0-6 O: 0-5 Na: 0-1

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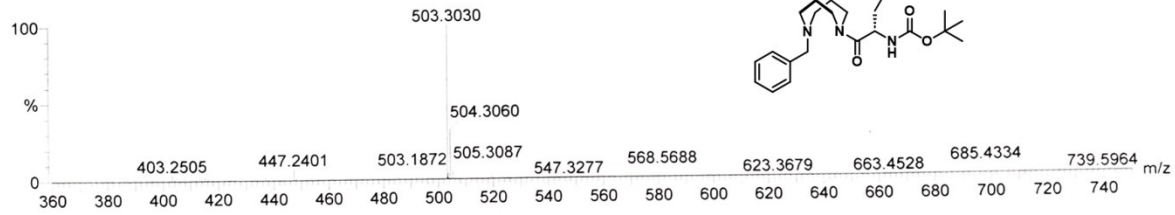
Capillary V 3, Cone V 40 , Desolvation Gas 800
ESI

30-Jul-2025

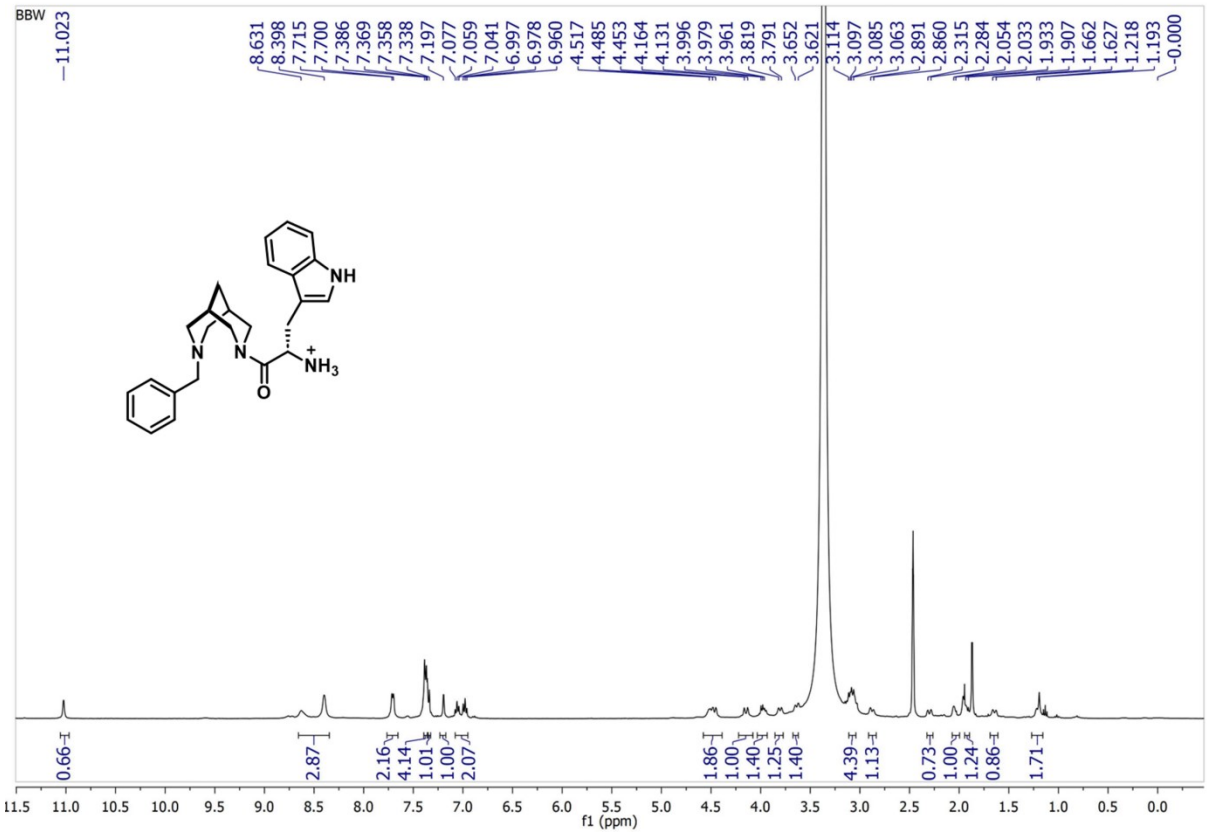
1: TOF MS ES+
1.22e+007



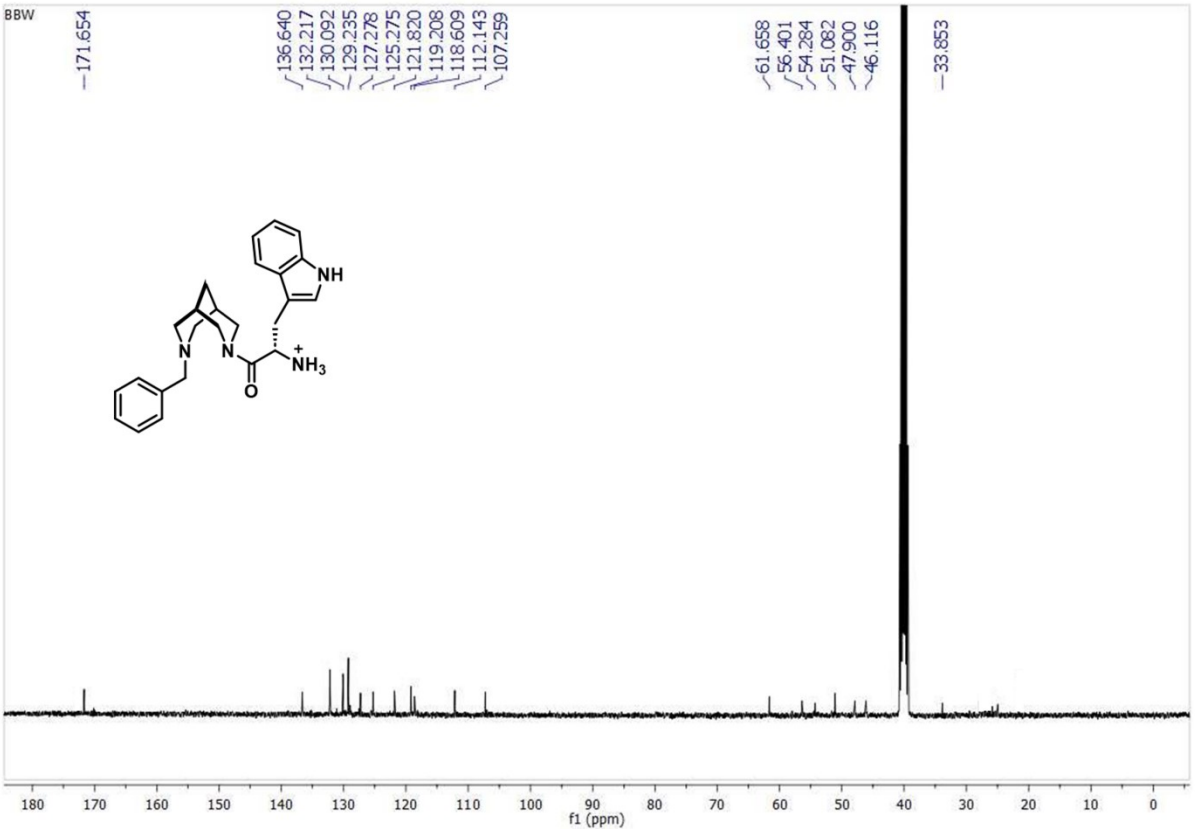




HRMS of **3**.



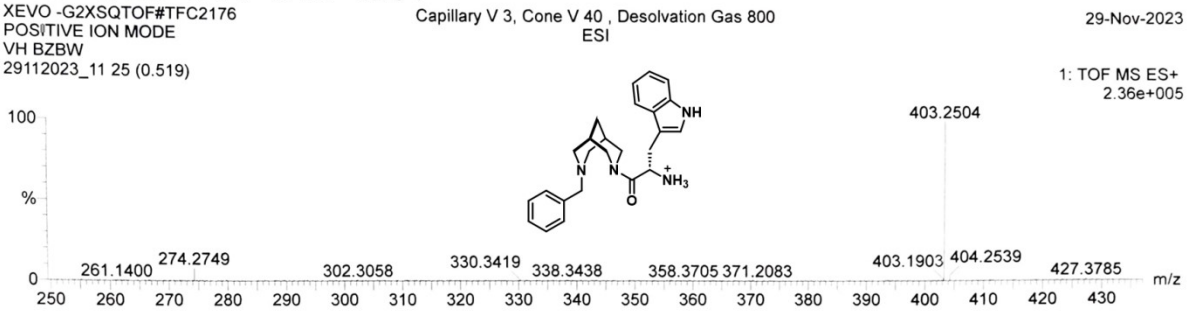

$^{1}$H NMR (400 MHz, DMSO-$d_6$) spectrum of **4**.



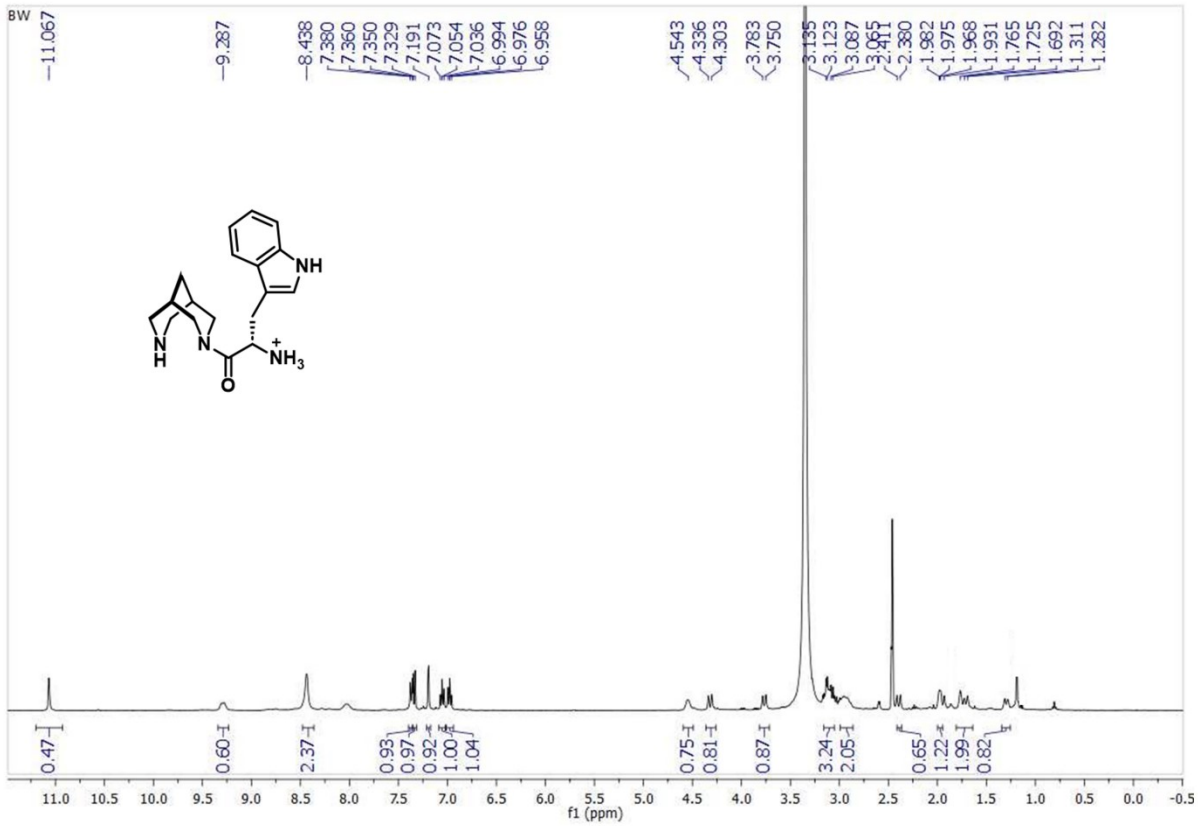

$^{13}$C NMR (100 MHz, DMSO-$d_6$) spectrum of **4**.

**Single Mass Analysis**
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

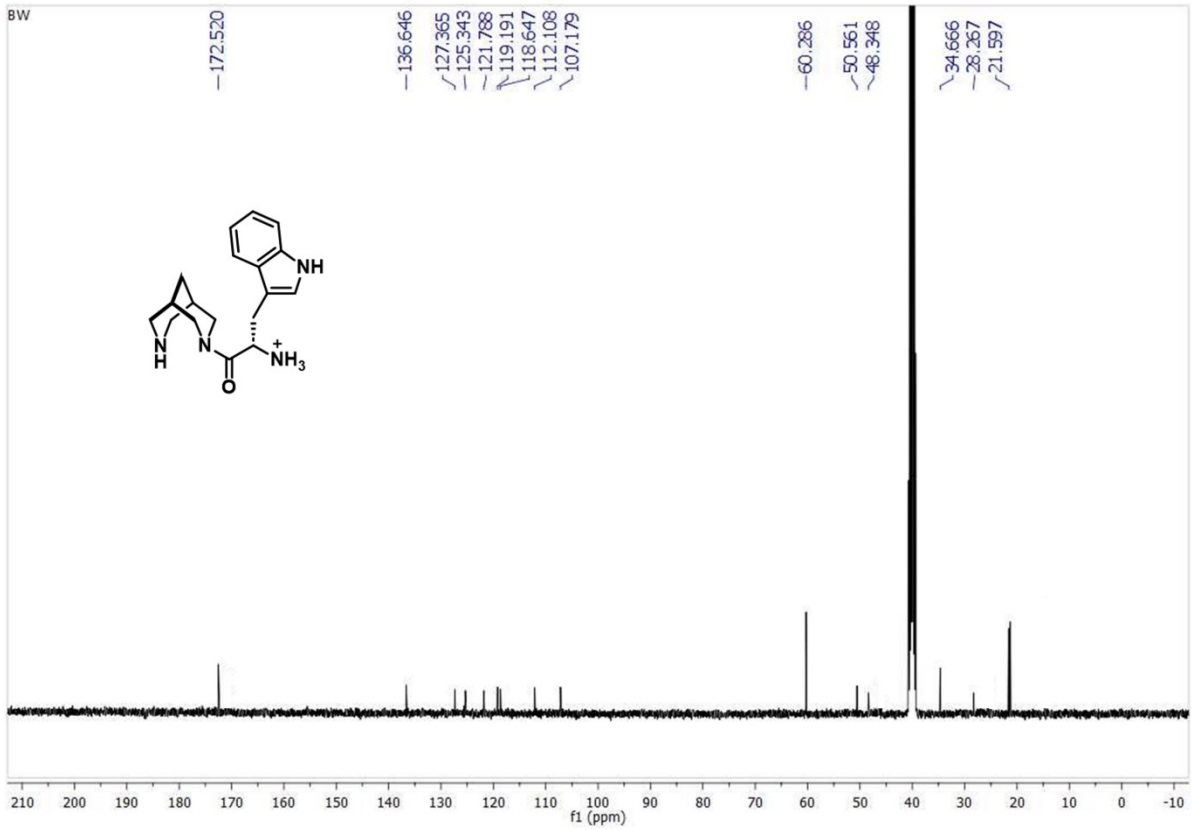
Monoisotopic Mass, Even Electron Ions
100 formula(e) evaluated with 1 results within limits (up to 10 closest results for each mass)
Elements Used:
C: 0-30 H: 0-35 N: 0-5 O: 0-2 Na: 0-1




HRMS of **4**.




$^{1}$H NMR (400 MHz, DMSO-$d_6$) spectrum of **5**.




$^{13}$C NMR (100 MHz, DMSO-$d_6$) spectrum of **5**.

**Single Mass Analysis**
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Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
745 formula(e) evaluated with 1 results within limits (up to 10 best isotopic matches for each mass)
Elements Used:
C: 0-65 H: 0-80 N: 0-10 O: 0-10 Na: 0-1

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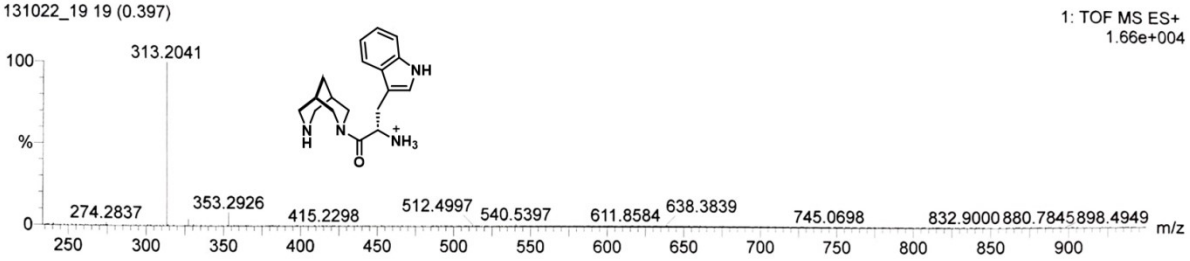
1.66e+004




HRMS of **5**.

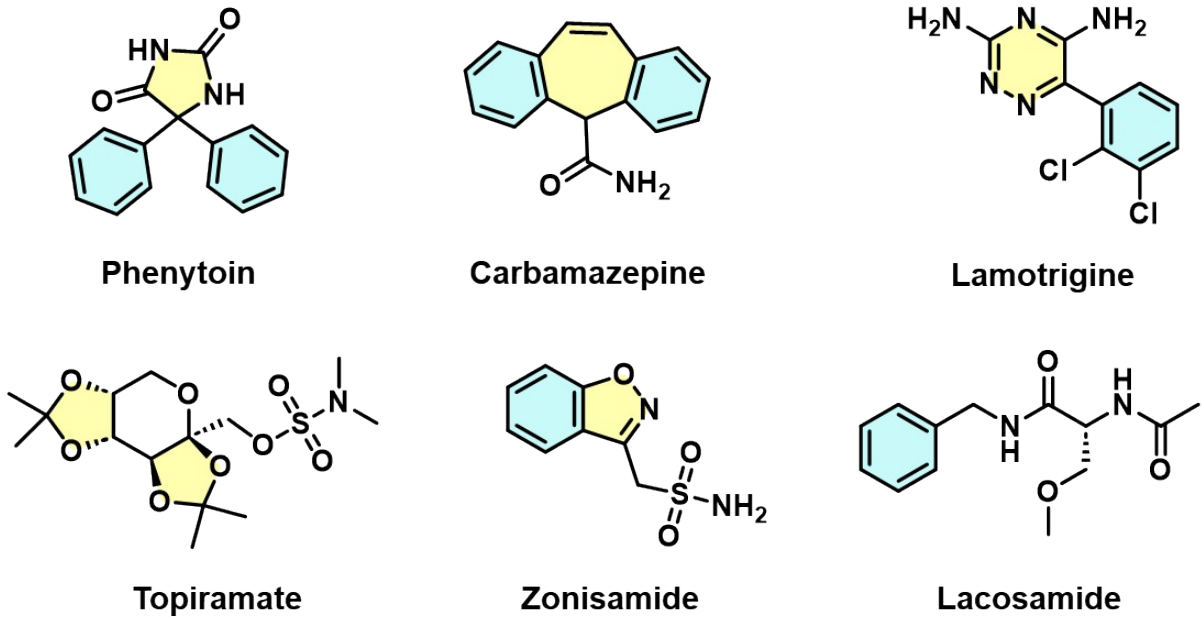



**Figure S1.** Chemical structures of drugs that act on sodium ($Na^+$) channels and reduce neuronal excitability.

**Table S1.** Mean values of frequency and amplitude from the Cornu Ammonis area 1 (CA1) and Dentate Gyrus (DG) regions of the hippocampus. For CA1, n = 13 slices; for DG, n = 10 slices.

| **Region** | **Normalized Frequency** | | **Normalized amplitude** | |
|---|---|---|---|---|
| | **Control (Mean ± SEM[a])** | **Compound 5 (20 µM) (Mean ± SEM)** | **Control (Mean ± SEM)** | **Compound 5 (20 µM) (Mean ± SEM)** |
| **CA1 n=13, N=9** | 1.00 ± 0.0 | 0.58 ± 0.08 P=0.0003 | 1.0 ± 0.0 | 0.71 ± 0.08 P=0.0061 |
| **DG n=10, N=7** | 1.00 ± 0.0 | 0.47 ± 0.10 P=0.0008 | 1.0 ± 0.0 | 0.61 ± 0.11 P=0.0068 |

[a] Standard Error of the Mean (SEM)

# 4. Methodology

## 4.1. Animals

All animal experiments were performed as per guidelines sanctioned by the Institutional Animal Ethics Committee (IAEC No. 120/IAEC/838, dated 09/06/22). The animals were maintained under normal environmental conditions with free access to food and water. All precautions were taken to minimise the number of animals used and to reduce the discomfort during the experiments.

### 4.2. Hippocampal slice preparations

Wistar rat (P12 – P28), was deeply anaesthetized (isoflurane), and quickly decapitated. The brain was rapidly removed and transferred into ice-cold ACSF (in mM): 125 NaCl, 25 $NaHCO_3$, 2.5 KCl, 1.25 $NaH_2PO_4$, 1 $MgCl_2$, 10 D-glucose, 2 $CaCl_2$ bubbled with 95% $O_2$ and 5% $CO_2$ (pH 7.4).[1] Cerebral hemispheres were separated using blade and glued to vibratome stage. Transverse hippocampal slices (400 μm) were prepared using Vibratome (Tedpella, USA). The slices were transferred to slice chamber and incubated for 30 min at 32 °C in ACSF bubbled carbogen. After 30 min of incubation the slices were kept at room temperature until used for the experiments.

### 4.3. Field recording experiments

The slices were transferred to the recording chamber of field recording setup continuously perfused with normal ACSF and bubbled with carbogen at a flow rate of 1.5 to 2.0 ml/min. After recording stable baseline, epileptiform events (EFEs) were induced with ACSF solution containing high potassium (7.5 mM K) and zero magnesium (HK-ACSF).[2] Once, epileptiform events were recorded for 10 minutes, HK-ACSF with drug was perfused to observe the effect.

### 4.4. Dorsal root ganglion (DRG) isolation protocol

Briefly, the rat was anesthetized with isoflurane and decapitated using sterile scissors. The vertebral column was removed from the dorsal side and a midline cut was made, Spinal cord was removed from the either side to access DRG. DRG could be visualized under the dissected microscope in the inter-vertebral foramen. DRGs were removed with sterile tweezers and collected in 15 ml tube with PBS (phosphate buffer saline) with antibiotic. Following three

washed with PBS to prevent contamination, DRGs were placed in 500 µl of dispase and 500 µl of 1.5 µg/µl collagenase at 37 °C for 30 minutes. Collagenase and dispase was replaced with Trypsin and the tube was placed at 37 °C for another 10 minutes. DRGs were then mechanically triturated using fire polished Pasteur pipette and again washed with PBS to stop the protease activity. PBS was replaced with DMEM and 10 % FBS. Cells were plated on poly-D-lysine (200 µg/ml) coated cover slip placed in 35 mm culture dish. The culture dish was stored in incubator (5% $CO_2$) until further use for patch clamp experiments.

### 4.5. $Na^+$ channel current protocol

$Na^+$ channel currents were recorded in small sized DRG neurons plated on 12 mm poly-D-lysine coated coverslip. Coverslip containing cells was placed in the recording chamber perfused with HEPES solution containing (in mM). Patch pipette with resistance of 3-6 MΩ was pulled using a PC-10 vertical puller (Narishige, Japan). Cesium based internal solution was used (in mM): 70 CsCl, 70 CsF, 6 NaCl, 10 EGTA, 10 HEPES, 3 Na-ATP (pH 7.4, adjusted with CsOH, 280-300 mOsm). 20 mM TEA-Cl and 30 µM $CdCl_2$ was added to the external solution to block potassium and Calcium currents respectively. $Na^+$ channel currents were studied under voltage clamp mode. Cell was held at -80 mV and step wise depolarization was performed from -80 mV to +70 mV for 40 ms.[3] All values were normalized to the maximum peak value of control current.

### 4.6. Potassium channel protocol

For recording potassium channels, a coverslip containing cells was placed in a recording chamber perfused with HEPES solution. Voltage clamp recordings were performed using a patch pipette (3-6 MΩ resistance) filled with potassium-based internal solution containing (in mM): 130 K-Gluconate, 5 NaCl, 1 EGTA, 5 Mg-ATP, 0.5 Na-GTP, 10 HEPES (pH 7.4, adjusted with KOH, 280-300 mOsmol l-1). 1 µM tetrodotoxin (TTX) and 50 µM $CdCl_2$ were added to block sodium and calcium channels, respectively, while recording $K^+$ currents. $K^+$ currents were recorded by holding the cell at -80 mV and then stepwise depolarization was done for 500 ms from -40 mV to +60 mV.

### 4.7. Analysis and statistics

For field recording experiments, the signals were acquired using an NI PCI 6221 data acquisition card and recorded with WinWCP V4.7.6 (Strathclyde Electrophysiology Software).

The data were filtered across a bandwidth from 1 Hz to 1 kHz, with a sampling frequency of 300 μs. To quantify the epileptiform activity, an online software program written in Python was developed to automatically analyze the number of events per slice, spike frequency, and peak amplitude (measured as the difference between the baseline and peak of the spike). For each slice, the average frequency and amplitude were calculated in a 1minute time window. The number of events per minute (events/min) and spike amplitude were measured in HK-ACSF following the treatment with pseudopeptide **5**. The mean values of these parameters (frequency and amplitude) per slice were used for statistical analysis. Event counts were normalized to the HK-ACSF baseline. Patch-clamp recordings were performed using a HEKA EPC 10 double patch-clamp amplifier, and data were acquired using PatchMaster software. The signals were filtered at 2.9 kHz and sampled at 10 kHz. The $Na^+$ and $K^+$ channel currents were analysed using Fitmaster (Germany). n represents the number of slices, and N represents the number of animals used. Results are presented as mean ± SEM, with statistical significance determined by a paired t-test: * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

### 4.8. Effect of compound 5 on cell viability- MTT assay

#### Method

Cell viability in all control and treatment groups was performed using MTT 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Hi-media). For this purpose, SH-SY5Y (10000 cells/ well) cells were seeded in 96 well plate. Compound **5** treatment was given at different concentrations (10 μM, 20 μM and 100 μM) for 24 hours and 48 hours. Post-treatment, media of the cultured plates were replaced with the MTT solution (0.5 mg/ml in serum-free- media) followed by incubation and solubilization of formazan crystal in DMSO. The absorbance was measured at 570 nm via a spectrophotometer (Tecan, 200pro).

#### Result

We assessed the toxicity of compound **5** in SH-SY5Y cells at different concentrations (10 μM, 20 μM and 100 μM) at 24 hours and 48 hours using MTT assay. We found that application compound **5** caused no-significant cytotoxic effect at either 24 hours and 48 hours in SH-SY5Y cells. A good cellular tolerance and maintenance of viability throughout the exposure period were observed.

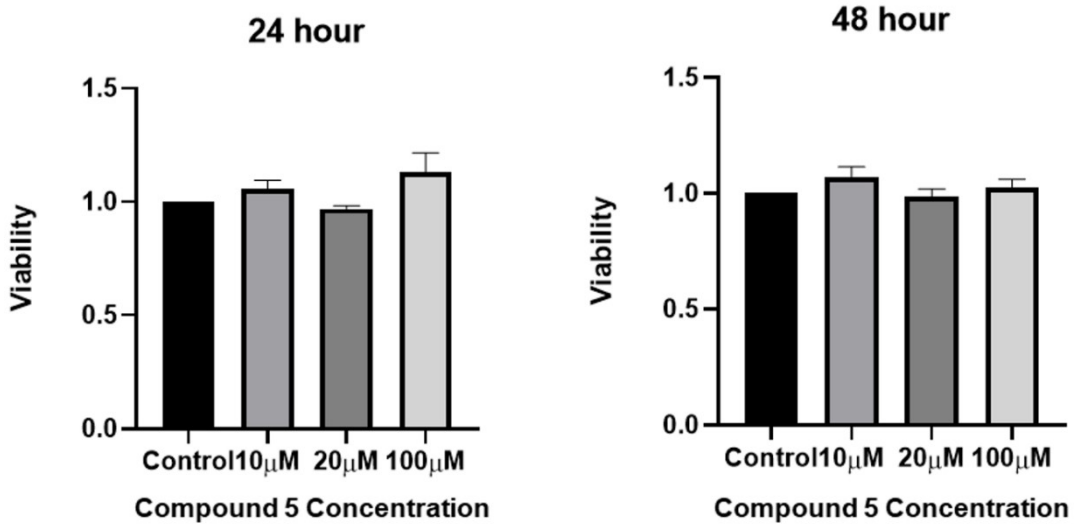



**Figure S2.** MTT assay to measure the cytotoxicity of compound **5** in SH-SY5Y cells. Cell viability at different concentrations (10 μM, 20 μM and 100 μM) at 24 hours and 48 hours are shown. *Data were presented as mean ± SEM, n = 3 per group.*

### *4.9* Effect of compound 4 on EFEs in hippocampus.

We investigated the effect of compound **4** (20μM) on EFEs induced in hippocampal synapses. After recording a control baseline, HK-ACSF was perfused to induce EFEs. After inducing the EFEs , compound **4** was perfused to evaluate the effect. Perfusion of the compound **4** does not significantly reduce the EFEs frequency (p=0.48, paired t- test, n=3 ) and amplitude (p=0.100, paired t-test, n=3), Fig S3.

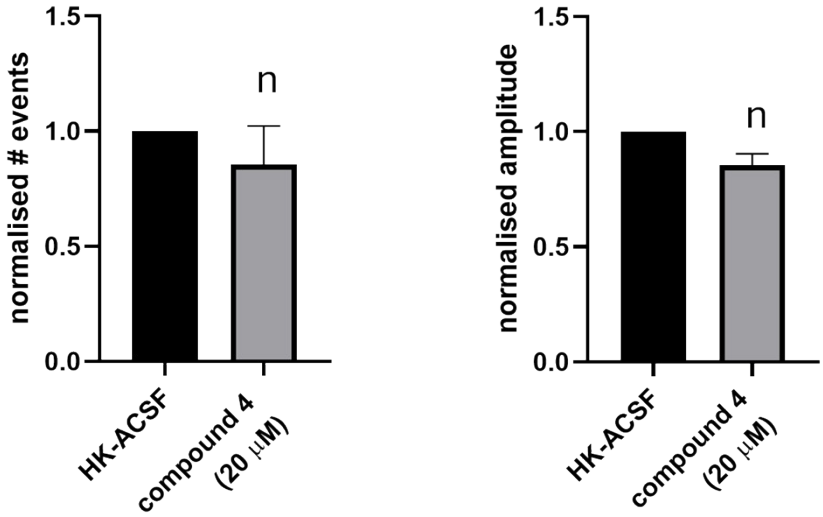



**Figure S3.** Effect of compound **4** (20μM) on EFEs in hippocampal synapses. Left shows the normalized number of events (frequency) and right shows the normalised peak amplitude. *Data were presented as mean ± SEM (n = 3).*

# 5. References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2. P. Punnakkal and D. Dominic, *Neuromol. Med.*, 2018, **20**, 90–96.

3. M. Roy and T. Narahashi, *J. Neurosci.*, 1992, **12**, 2104–2111.