Electronic Supplementary Material (ESI) for ChemComm.

Site-Specific Fluorescence Spectrum Detection and Characterization of hASIC1a Channels upon Toxin Mambalgin-1 binding in Live Mammalian Cells

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General

Chemical reagents were obtained from commercial sources (Energy-Chemical, J&K and TCI) and used as received. Organic solvent were obtained from Sinopharm Chemical Reagent Co., Ltd. The distillation fraction of petroleum ether used for flash chromatography is 60 to 90 ℃.Anhydrous toluene was dried with sodium by distillation according to standard experimental procedure. Chemical shifts (δ) are reported in ppm with TMS as internal standard.

Chemical Synthesis of Anap starting from 2-bromonaphthalene [1]

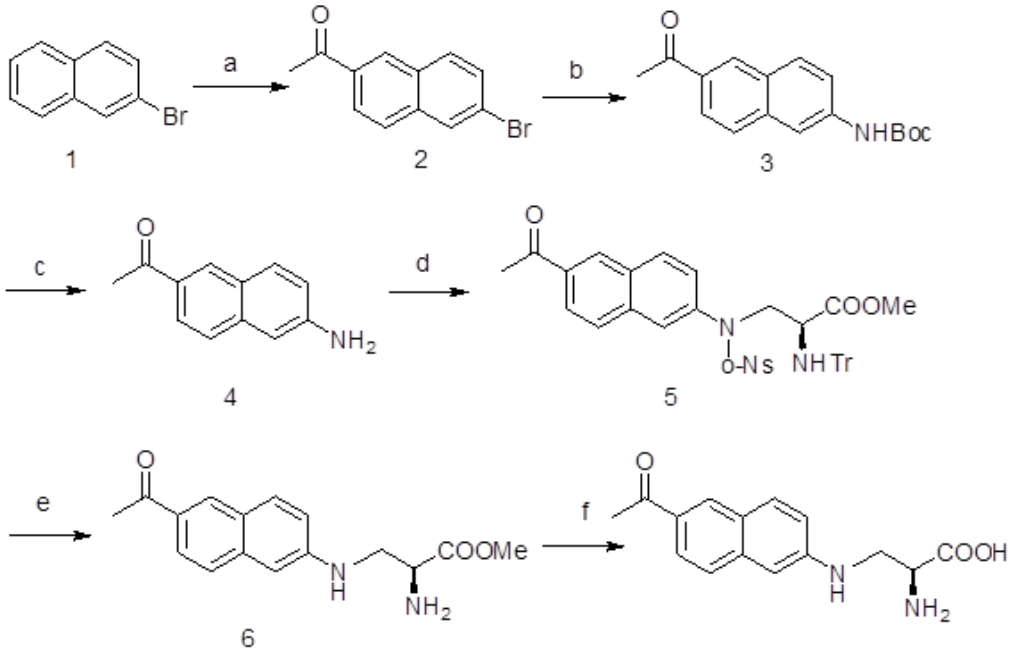


Figure S1. Synthesis of Anap starting from 2-bromonaphthalene. Reagents, conditions and yields are shown (a) AcCl, $AlCl_3$, $PhNO_2$, 100 °C, 5 h, 35 %; (b) Boc-NH_2, CuI, DMEDA, K_2CO_3, toluene, 110 °C, 30 h, 71 %; (c) TFA, DCM, rt, 3 h. 95 %; (d) i,o-NsCl, pyridine, DCM, rt, overnight; ii, N-Trt-Ser-OMe, DIAD, PPh_3, toluene, rt, overnight, 88 % over two steps; (e) i, TFA, DCM, H_2O, rt, 4 h; ii, PhSH, K_2CO_3, DMF, rt, 3 h, 73 % two steps; (f) 2 M HCl, 60 °C, 8 h, 97 %

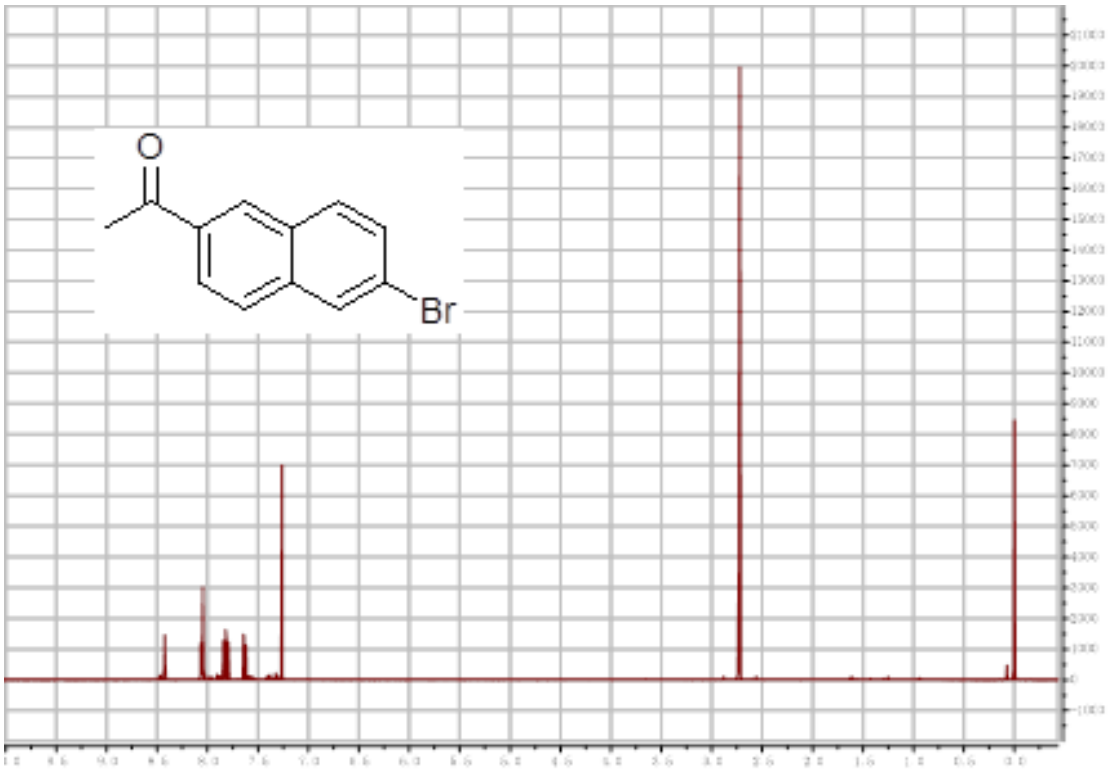
2-Acetyl-6-bromonaphthalene [2]

To a mixture of AcCl (1.44 mL, 20.4 mmol), anhydrous $AlCl_3$ (3.2 g, 24.0 mmol) and

26 mL $PhNO_2$ was added 1(5.0 g, 24.1 mmol). The mixture was stirred under nitrogen at 100℃ for 5 h and then cooled to 0℃. 20 mL concentrated HCl was added and stirred for another 30 mins. The two phases was separated and the aqueous phase was washed with EtOAc. The combined organic phase was washed with brine and dried over Na_2SO_4. The solution was concentrated and purified by flash chromatography (EtOAc/Petroleum ether = 1:12) affording compound 2 (1.76 g, 35 %) as white solid.

Resonance assignments, ^{1}H NMR (300 MHz, $CDCl_3$):
8.43 ppm (s, 1H), 8.08-8.02 ppm (m, 2H), 7.87-7.78 ppm (m, 2H), 7.64 ppm (dd, 1H), 2.72 ppm (s, 3H).

^{1}H NMR (solvent $CDCl_3$) of Compound 2 ($C_{12}H_9BrO$)

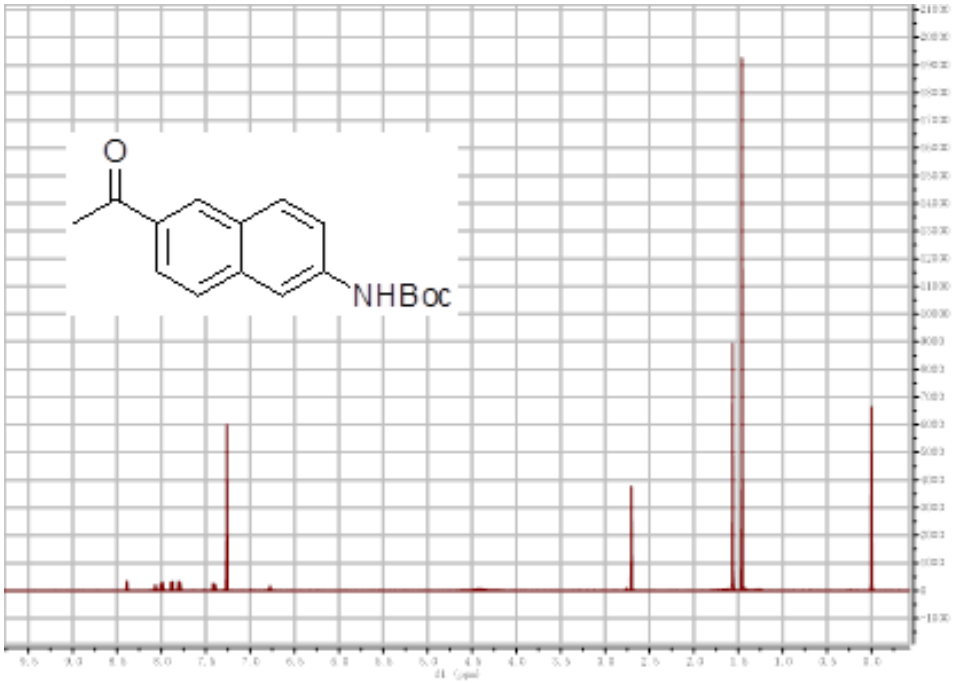


Tert-butyl-6-acetylnaphthalen-2-ylcarbamate [3]
To a solution of compound 2 (4.54 g, 18.3 mmol), Boc-NH_2 (2.51 g, 21.4 mmol), CuI (450 mg, 2.4 mmol) and K_2CO_3 (5.00 g, 36.2 mmol) in 36 mL toluene was added DMEDA (508 μL, 4.7 mmol). The reaction mixture was stirred under nitrogen at 110 ℃ for 30 h. After chilling to room temperature, the mixture was filtered and washed with EtOAc. The filtrate was concentrated and purified by flash chromatography (EtOAc/Petroleum ether = 1:6) affording compound 3 (3.76 g, 71 %) as a white solid.

Resonance assignments, ^{1}H NMR (300 MHz, $CDCl_3$):
8.39 ppm (s, 1H), 8.07 ppm (s, 1H), 8.01-7.98 ppm (dd 1H), 7.88 ppm (d 1H), 7.80

ppm (d 1H), 7.40 ppm (dd, 1H), 6.78 ppm (s, 1H), 2.76 ppm (s, 3H),1.56 ppm (s, 9H).

^{1}H NMR (solvent $CDCl_3$) of Compound 3 ($C_{17}H_{19}NO_3$)



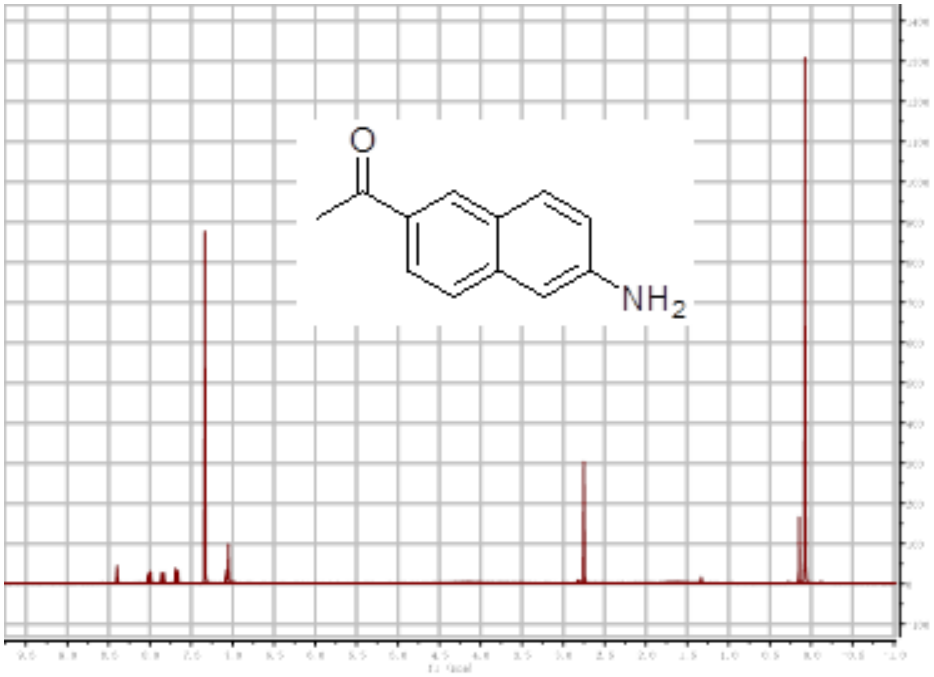
6-Acyl-2-naphthylamine [4]

Compound 3 (3.75 g, 13.1 mmol) was dissolved in 40 mL DCM and then cooled to 0 ℃.40 mL TFA was added dropwise. After stirred at room temperature for 3 h, the mixture was concentrated. The remaining sticky liquid was added 150 mL DCM and washed with saturated Na_2CO_3 aqueous solution and brine, dried over Na_2SO_4. The solvent was removed to give compound 4(2.31 g, 95 %) as a yellow solid.

Resonance assignments, ^{1}H NMR (300 MHz, $CDCl_3$):

8.40 ppm (s, 1H), 8.01 ppm (dd, 1H), 7.85 ppm (d, 1H), 7.67 ppm (d, 1H), 7.08-7.05 ppm (m, 2H), 2.75 ppm (s, 3H).

^{1}H NMR (solvent $CDCl_3$) of Compound 4 ($C_{12}H_{11}NO$)



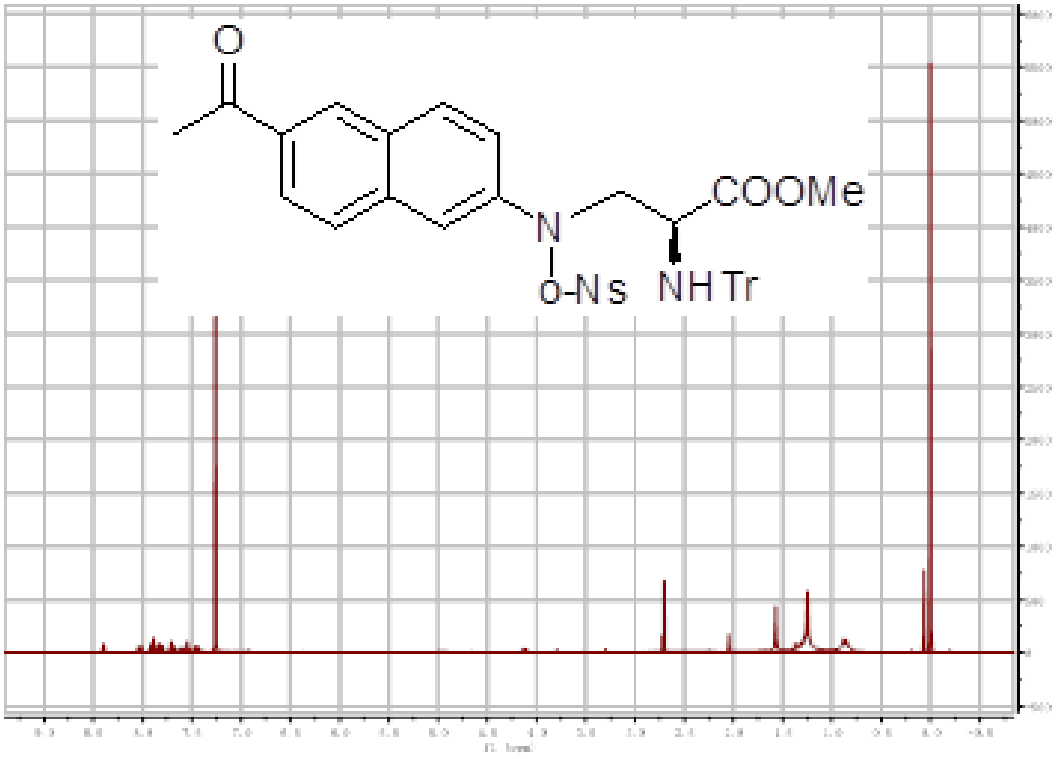
(S)-Methyl-3-(N-(6-Acetylnaphthalen-2-yl)-2-nitrophenyl-sulfonam--ido)-2-(tritylamino) propanoaten [5]

To an ice cold solution of 4 (1.89 g, 10 mmol) in 100 mL DCM was added pyridine (0.92 mL, 11.3 mmol) and o-NsCl (2.374 g, 10.7 mmol). The mixture was stirred at room temperature overnight. The mixture was washed with 1M HCl (70 mL), water , brine and dried over Na_2SO_4. The solution was concentrated to give a red solid which was dissolved in toluene (100 mL) and cooled on ice bath , N-Trt-Ser-OMe (7.37 g, 20.5 mmol), PPh_3 (5.35 g, 20.4 mmol) and DIAD (4.0 mL, 20.4 mmol) were added. The mixture was stirred at room temperature overnight. The solution was concentrated and purified by flash chromatography (EtOAc/Petroleum ether = 1:2) affording compound 5 (6.45 g, 88 %) as a red solid.

Resonance assignments, ^{1}H NMR (300 MHz, $CDCl_3$): (the high intensity peak at 7.27 were signal from solvent.)

8.39 ppm (s, 1H), 8.01 ppm (dd, 1H), 7.84 ppm (d, 1H), 7.66 ppm (d, 1H), 2.70 ppm (s, 3H), some other weak peaks from aromatic ring protons were not assigned.

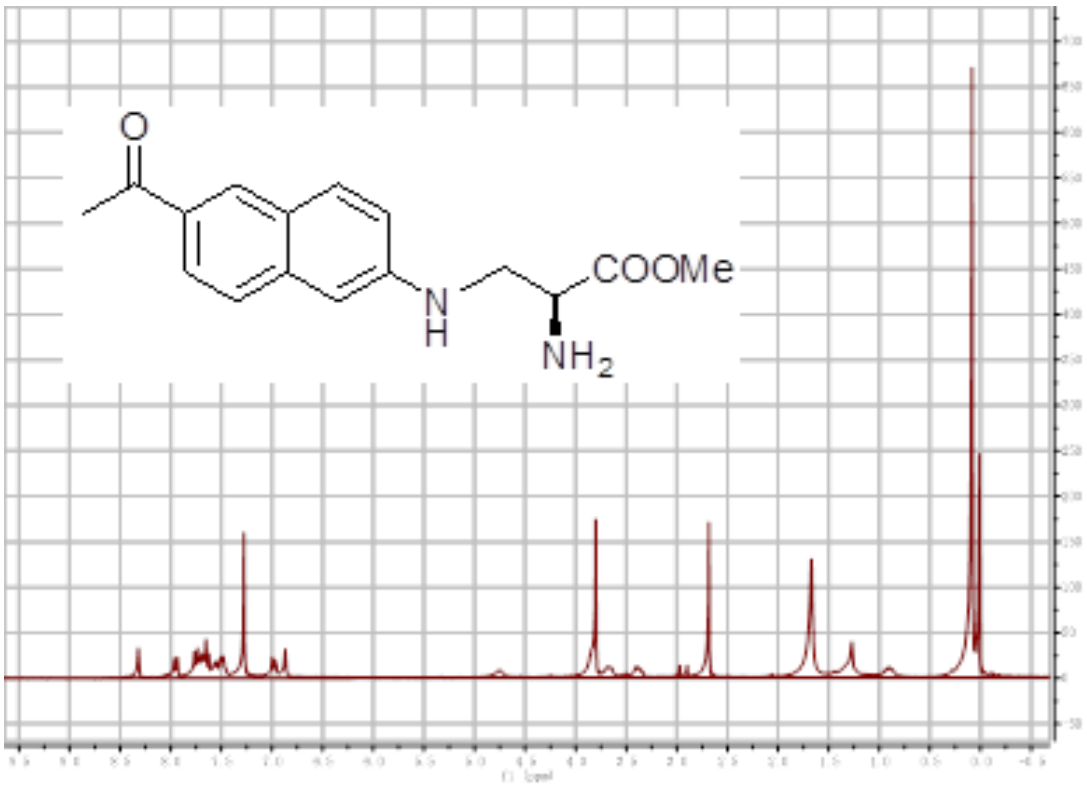
^{1}H NMR (solvent $CDCl_3$) of Compound 5 ($C_{41}H_{35}N_3O_7S$)



(S)-Methyl-3-(6-Acetylnaphthalen-2-ylamino)-2-amino–propanate [6]
To an ice cold solution of 5 (6.50 g, 9.1 mmol) in 70 mL DCM was added 10 mL TFA and 10 mL water. The mixture was stirred under room temperature for 4 h and solvent was removed under vacuum. The thick residue was dissolved in 45 mL DMF. Thiophenol (1.9 mL, 18.4 mmol) and K_2CO_3 (12.70 g, 919.0 mmol) were added to the solution. After stirred for 3 h, 350mL water was added and the mixture was extracted with EtOAc. The organic phase was washed with brine and dried over Na_2SO_4.The solution was concentrated and purified by flash chromatography (EtOAc/Petroleum ether = 2:1 then MeOH/DCM = 1:13) affording compound 6 (1.90 g, 73 %) as a yellow solid.

Resonance assignments, ^{1}H NMR (300 MHz, $CDCl_3$):
8.32 ppm (s, 1H), 7.94 ppm (dd, 1H), 7.77 ppm (d, 1H), 7.59 ppm (d, 1H), 6.98 ppm (dd, 1H), 6.87 ppm (d, 1H), 4.75 ppm (br, 1H), 3.80 ppm (s, 3H), 2.69 ppm (s, 3H)

^{1}H NMR (solvent $CDCl_3$) of Compound 6 ($C_{16}H_{19}N_2O_3$)

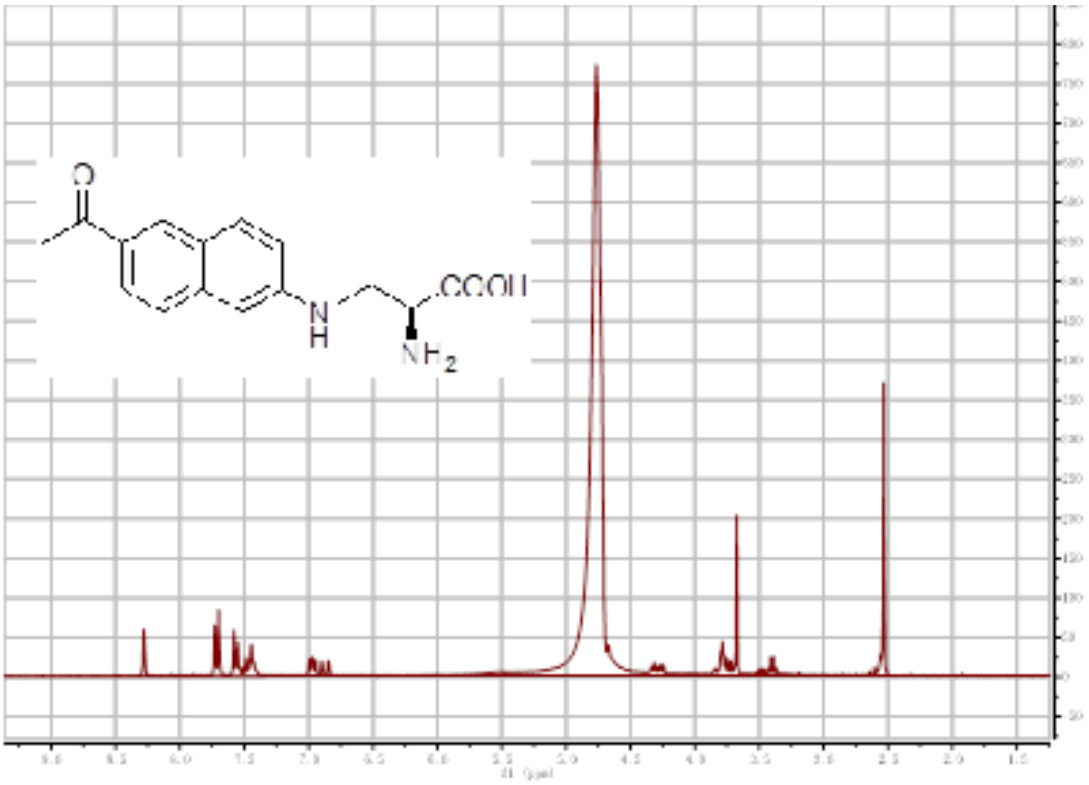


3-(6-acetylnaphthalen-2-ylamino)-2-aminopropanoic acid [Anap]

A mixture of compound 6 (1.88 g, 6.6 mmol) in 65 mL 2 M HCl was stirred at 60 ℃ for 8 h. The mixture was lyophilized to give a yellow solid which was washed with Et_2O and dried under vacuum affording L-Anap (1.74 g, 97 %) as a yellow solid.

Resonance assignments, ^{1}H NMR (300 MHz, $CDCl_3$):
8.28 ppm (s, 1H), 7.70 ppm (dd, 1H), 7.57 ppm (d, 1H), 7.44 ppm (d, 1H), 6.97 ppm (m, 1H), 6.87 ppm (d, 1H), 3.79 ppm (m, 2H), 3.68 ppm (m, 1H), 2.53 ppm (s, 3H)

^{1}H NMR (solvent D_2O) of Anap ($C_{15}H_{16}N_2O_3$)



Chemical Synthesis and Purification of the toxin Mambalgin-1

hASIC1a channel toxin mambalgin-1 was chemical synthesized as previous described (Pan *et al*, 2014 *Chem Commun (Camb)* **50**(44): 5837-5839).

Plasmid Construction

To construct the Anap-expression plasmid in the pcDNA3.1 vector, a special $tRNA_{CUA}^{EcLeu}$ and an Anap-specific aminoacyl-tRNA synthase (AnapRS) were required. A poly(A) tail was added to the 3'-terminus of special $tRNA_{CUA}^{EcLeu}$ to enhance its transcription and maturation in mammalian cells. A gene cluster containing eight tandem repeats of the H1 promoter $tRNA_{CUA}^{EcLeu}$ gene was used to further increase the expression level of the tRNA. The Anap-specific aminoacyl-tRNA synthetase (AnapRS) was expressed from a non-regulated CMV promoter.

After the sequences for the tRNA/AnapRS were determined, we have had the sequence synthesized by local service company (Shanghai Sangon, Shanghai, China).

Heterogeneous expression of hASIC1a-Anap on Chinese hamster ovary (CHO) cell membrane

Human source hASIC1a coding sequence in pcDNA3.1/Zeo (+) was prepared as described previously (Pan *et al.* 2014). Site-specific mutations in hASIC1a sequences were introduced by TAG stop codon and achieved through overlapping PCR. All primers were obtained from Sangon (Shanghai). Mutations were confirmed by restriction enzyme digest and DNA sequencing.

Table S1. Primers for mutation introduction in hASIC1a and further Anap incorporation

GENE	FORWARD PRIMER	REVERSE PRIMER
HASIC1a	5'-GGGGTACCATGGAACTGAAGGCCGAGG-3'	5'-CCCTCGAGTCAGCAGGTAAAGTCCTCGAAC-3'
HASIC1a-Y68TAG	5'-CAGTACTAGTTCCACTACCACCATGTCACCAAGCTC-3'	5'-GAGCTTGGTGACATGGTGGTAGTGGAACTAGTACTG-3'
HASIC1a-F69TAG	5'-CAGTACTACTAGCACTACCACCATGTCACCAAGCTC-3'	5'-GAGCTTGGTGACATGGTGGTAGTGCTAGTAGTACTG-3'
HASIC1a-T236TAG	5'-GGGGAGTAGGACGAGACGTCCTTCGAAGC-3'	5'-GCTTCGAAGGACGTCTCGTCCTACTCCCC-3'
HASIC1a-D347TAG	5'-GTACAAGGAGTGTGCATAGCCTGCTCTGGACTTC-3'	5'-GAAGTCCAGAGCAGGCTATGCACACTCCTTGTAC-3'

CHO cells were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum. For transfection, cell was transferred to 24-well flat bottom culture dish, and 0.8μg hASIC1a-TAG mutation and 0.8μg pAnap ($tRNA_{CUA}^{EcLeu}$ /AnapRS) plasmids in pcDNA3.1 vector for each well were applied with Lipofectamine 2000 (Invitrogen). After 5-6 h incubation, cells were transferred to poly-D-lysine (Sigma) coated slides for additional 24h culture with fresh media containing 50μM Anap.

To verify the expression of target proteins after incorporation of unnatural amino acids, green fluorescence protein (GFP) with site-specific Anap incorporation was first used. TAG stop codon was introduced into specific eGFP sequences through overlapping PCR. Mutations were confirmed by restriction enzyme digest and DNA sequencing. After transfection with normal EGFP plasmid or pcDNA3.1-EGFP-37TAG with/o pAnap, CHO cells were exchanged to fresh media in the presence or absence of 50μM Anap. Green fluorescence with the expression of GFP protein was only detected with the presence of all three factors: pcDNA3.1-EGFP-37TAG, pAnap and Anap.

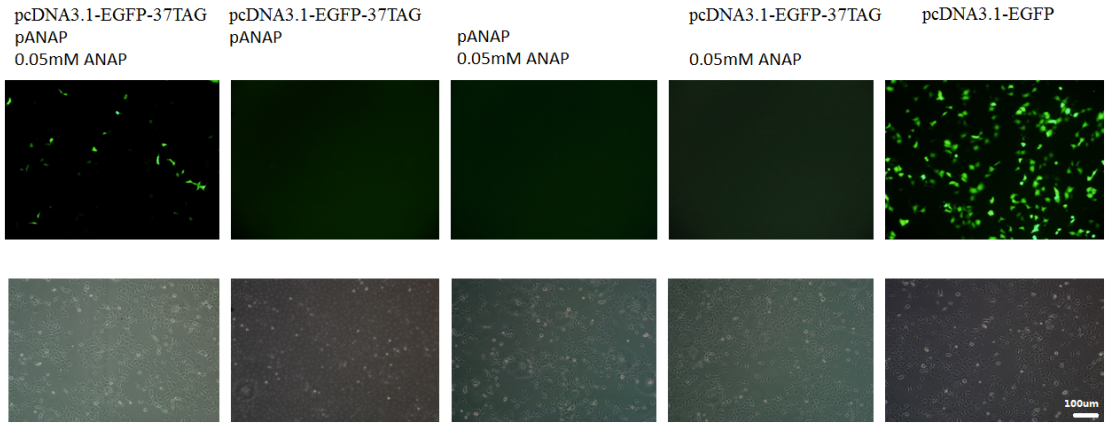


Figure S2. Fluorescence images of GFP proteins in CHO cells. The plasmids used for transfection was listed on top of each image. The upper images shows the green fluorescence of GFP proteins. The bottom images shows the bright field images of CHO cells. (bar size was shown at right corner of the final figure. All figures were in the same bar)

Double Photon Fluorescence Spectroscopy

After 24-h culture, cells were washed with PBS and cultured in fresh medium without Anap overnight. Cells were washed and exchanged medium (without Anap) every 2

hours for 3 times. Before live cell fluorescence imaging, cells were washed and flooded in bath solution (in mM): 150 NaCl, 4 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 10 HEPES (pH 7.4; 300 ~ 312 mOsm).

For Anap fluorescence measurement, a femtosecond pulse laser (MaiTai DeepSee, two photon) with wavelength tuning range from 690 nm to 1040 nm was used. The excitation light wavelength was selected by means of a sequence of prisms and a slit in the objective. The emitted fluorescence from the specimen is scanned and measured by mono-chromator and PMT. Emission fluorescence of membrane expressed hASIC1a-Anap channels were measured from 380nm to 550nm before and after application of Mambalgin-1.

Electrophysiology

Currents were recorded using the whole-cell patch-clamp technique. Patch electrodes, with resistances of 3-5 MΩ when filled with intracellular solution (in mM): 10 NaCl, 140 KCl, 2.0 CaCl2, 1.0 MgCl2, 10 HEPES, 5 EGTA (pH 7.33; 297 mOsm), were constructed from thick-walled borosilicated glass with filament (1.5 mm diameter; Sutter Instruments) on a four-stage puller (P-1000; Sutter, USA). Recordings were made using an EPC-10 amplifier (HEKA Electronic) and data acquisition was performed using PatchMaster software. Membrane potential was held at -70 mV in all experiments. Acid-induced currents were recorded by rapid exchanging of local solution from pH7.4 to acid pH through a Y-tube perfusion system. Toxins were applied 30s before pH dropping and persisted during low pH application. Channels were activated by acid perfusion every 2 min at least to allow for a complete recovery of the channels from desensitization. Recordings in which access resistance or capacitance changed by 10% during the experiment were excluded for data analysis.

To further confirm the expression of hASIC1a channels with incorporation of unnatural amino acid, no Anap was added to the culture media of CHO cells after transfection of pcDNA3.1-hASIC1a-68TAG and pAnap. No acid-sensing current was induced with application of acid, which suggested no expression of hASIC1a channels on the CHO cell membrane.

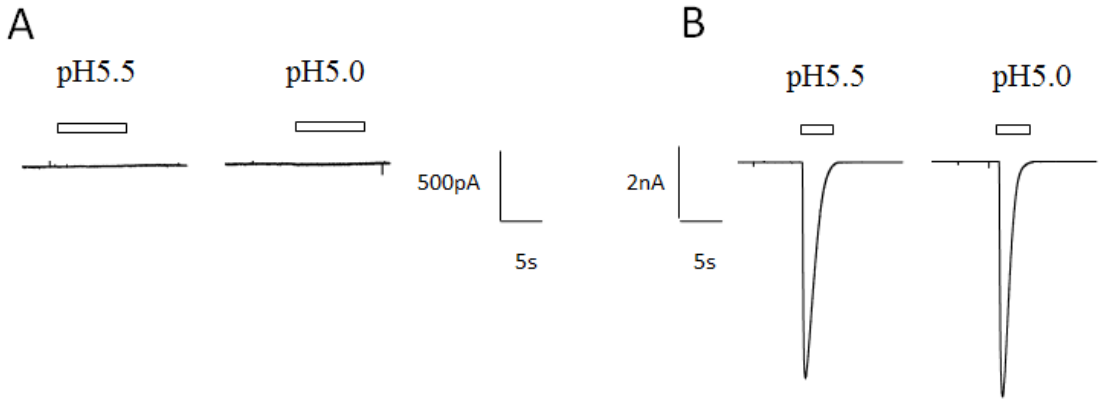


Figure S3. Traces of acid-induced currents in CHO cells transfected with pcDNA3.1-hASIC1a-68TAG and pAnap, (A) without Anap in the medium; (B) with 50 μM Anap in the medium.

Multiple fluorescence spectra curve were shown below for different cells, each of which were transferred with hASIC1a with mutant of Y68-Anap (Fig. S4), D347-Anap (Fig. S5), F69-Anap (Fig. S6) and T236-Anap (Fig. S7). The first two mutants (Y68-Anap, D347-Anap) demonstrated the obvious fluorescence spectra change upon toxin binding, while the two latter mutants (F69-Anap, T236-Anap) demonstrated almost unchanged fluorescence spectra with addition of 500 nM mambalgin-1.

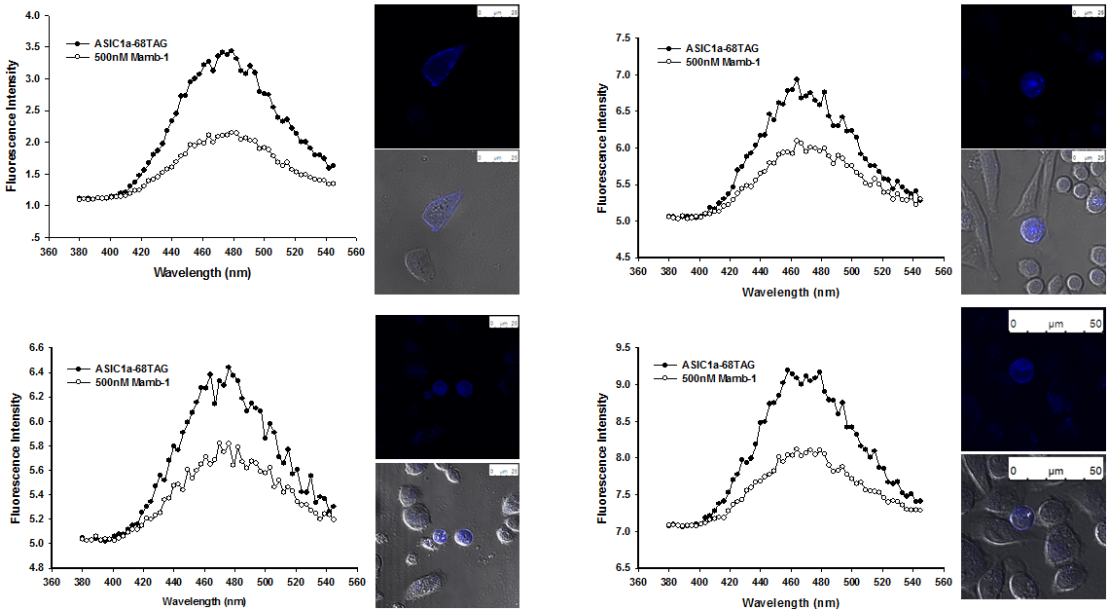


Figure S4. Additional fluorescence spectra of hASIC1a-Y68Anap in the absence or presence of 500 nM mambalgin-1, and a fluorescence image of the recording cell on the right (right, bar scale: 25 μm except the last one: 50 μm)

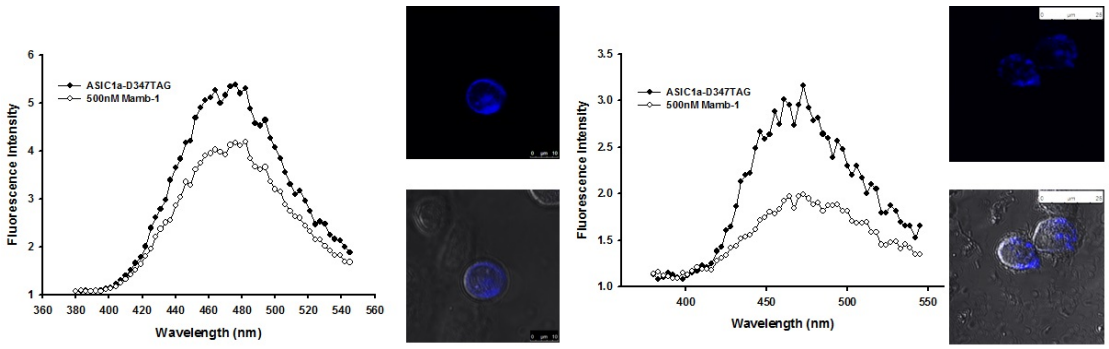


Figure S5. Additional fluorescence spectra of hASIC1a-D347Anap in the absence or presence of 500 nM mambalgin-1, and a fluorescence image of the recording cell on the right (left, bar scale: 10 μm, right, bar scale: 25 μm)

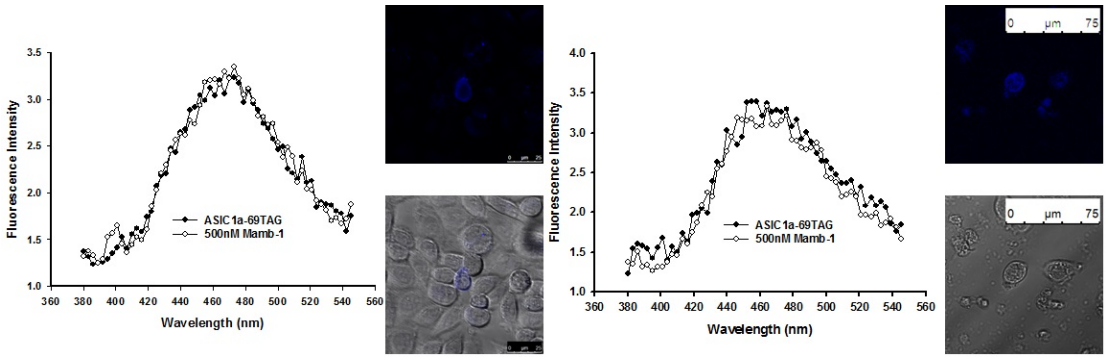


Figure S6. Additional fluorescence spectra of hASIC1a-F69Anap in the absence or presence of 500 nM mambalgin-1, and a fluorescence image of the recording cell on the right (left, bar scale: 25 µm, right, bar scale: 75 µm)

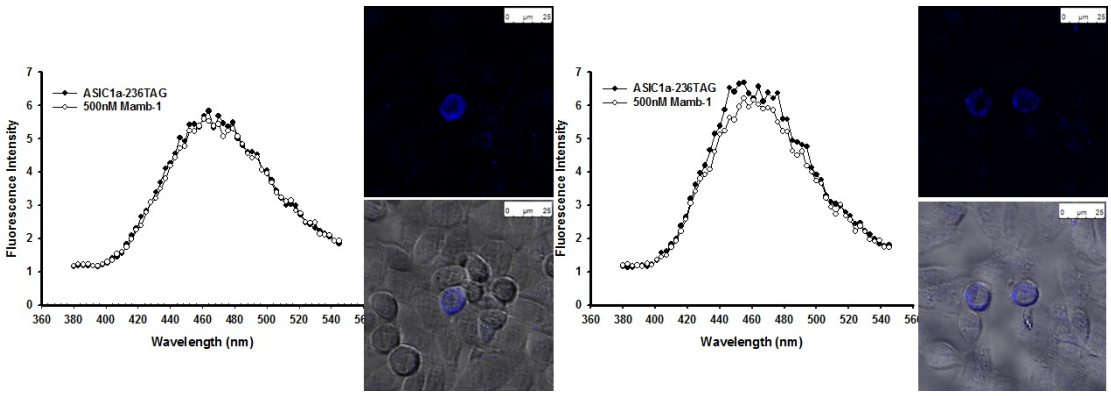


Figure S7. Additional fluorescence spectra of hASIC1a-T236Anap in the absence or presence of 500 nM mambalgin-1, and a fluorescence image of the recording cell on the right (bar scale: 25 µm)