Supporting Information

3-Difluoroalkyl Quaternary Oxindoles Inhibit Macrophage Pyroptosis by Blocking Inflammasome Recruitment of Caspase-1

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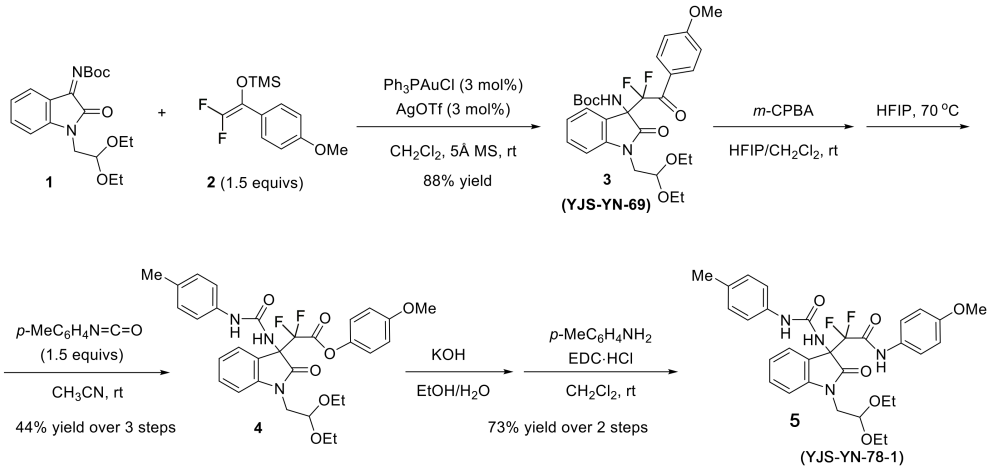
Materials and Methods

Chemical Screen

The Spectrum Collection compound library (2320 compounds, MicroSource Discovery Systems) and one collection (467 compounds) of lab made chemicals containing quaternary carbon stereocenters were used for screen. For IL-1β release screen, two thousand cells were seeded onto 96-well plates then primed with 1 μg/mL LPS for 4 hours. Then Nigericin and compounds (diluted to 10μM with DMSO) were added in triplicate. After 1 hour, IL-1β level in the medium was determined with ELISA kit (R&D Systems) according to the manufacturer's instructions.

Chemical Synthesis

The synthetic chemistry effort was concentrated on varying three aspects of the core (Figure 1B): the R_1 group (substituent of ketone), the R_2 group (C3-substitutent of oxindole) and the R_3 group (*N*-substituent of oxindole). All of the compounds tested in current study are prepared according to our previous works[1-2]. The purity of all tested compounds determined by ^{1}H NMR analysis was $\geqslant$95% (See the NMR and MS data in Supporting information). The synthetic route and characterization of compound **6** was shown in the following.



^{1}H NMR (400 MHz, $CDCl_3$): δ 8.89 (s, 1H), 7.38-7.31 (m, 4H), 7.14-7.03 (m, 8H), 6.97-6.95 (m, 2H), 4.76-4.73 (m, 1H), 4.04-3.99 (m, 1H), 3.79-3.65 (m, 3H), 3.60-3.48 (m, 2H), 2.32 (s, 3H), 2.23 (s, 3H), 1.12 (t, *J* = 6.8 Hz, 6H); ^{19}F NMR (376 MHz, $CDCl_3$): δ -111.33 (d, *J* = 262 Hz, 1F), -113.46 (d, *J* = 261 Hz, 1F); ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.39, 160.32 (t, *J* = 28 Hz), 153.49, 144.44, 136.06, 135.51, 132.92, 132.84, 130.17, 129.69, 129.37, 124.89, 123.94, 123.01, 121.32, 120.18, 113.50 (t, *J* = 260 Hz), 110.43, 100.70, 65.26 (t, *J* = 25 Hz), 63.62, 44.40, 20.99, 20.74, 15.28, 15.25. MS (EI): 580 (M^+, 2), 427 (14), 370 (4), 133 (33), 106 (81), 103 (100), 75 (31), 44 (32); HRMS (EI): Exact mass calcd for $C_{31}H_{34}N_4O_5F_2$ $[M]^+$: 580.2497, Found: 580.2499.

Cell culture and pyroptotic stimulations

J774A.1 and THP1 cell lines are purchased from American Type Culture Collection and cultured in RPIM 1640 medium with 10% FBS,100 U/mL penicillin, and 100 mg/mL streptomycin at 37 °C with 5% CO2. Bone marrow-derived macrophages from adult C57BL/6 mice were differentiated as reported previously. Briefly, mouse bone marrow cells were differentiated in RPIM 1640 medium supplemented with 10% FBS and 100 U/mL recombinant macrophage colony-stimulating factor (M-CSF) for 5-7 days. The

BMDMs were then cultured overnight in 24-well plates at 2 × 10^5 cells/well in 0.5 mL medium. For stimulations, cells were pre-primed with 1 μg/mL LPS for 4 hours followed by treatment of 5 μg/mL Nigericin, 5 mM ATP, 2 μg/mL Poly(dA:dT) plus 0.1% v/v Lipofectamine 3000 (Life Technologies) or Recombinant flagellin from S. typhimurium (InVivogen, tlrl-flic-10). Anthrax lethal factor and protective antigen were from List Biological Laboratories. The concentrations of lethal factor used for the experiments were 30 ng of PA/mL + 30 ng of LF/mL. For Neon electroporations, the Neon transfection system (Life Technologies) was used with 1720 Voltage, 10 Width, 2 Pulse settings and performed with 1 × 10^6 cells plus Flagellin 0.5 μg per electroporation condition and plated at 1 × 10^5 cells per 96-well for imaging and assays.

Measuring Cell Death by Propidium Iodide Uptake

Propidium Iodide Uptake was determined as previously described[3]. Treat the cells with a pyroptotic stimulus as previously described. Remove and save the medium, which contains dead and mitotic cells. Detach live cells using standard tissue culture techniques. Add the cells in medium and the cells from washes to the detached cells and harvest all cells by centrifugation at 500g for 5 min. Resuspend the harvested cells in PI-FACS buffer (Propidium iodide, Sigma-Aldrich 81845, 0.5 mg/mL in PBS). Incubate the cells at room temperature for 15 min in the dark. Measure cell death by flow cytometry as previously described[3].

ELISA assays

Serum samples were collected from C57BL/6 mice after treatment with LPS, saline and compound **6** for 24 hours. Serum concentrations of IL-1β and IL-18 mice were

determined by ELISA kit (R&D Systems) according to the manufacturer's instructions.

LDH and Alamar blue assay

After macrophages were treated with pyroptotic triggers and compound **6**, LDH release was measured with LDH assay kit (Abcam) according to the manufacturer's instructions. Briefly, absorbance was measured with microplate reader at a wavelength of 490 nm. Background optical absorbance was measured at 690 nm and was subtracted from primary measurements for each well. LDH content in the medium was calculated using a concurrent standard curve. The LDH concentrations in lysis group was assigned to 100% and the one in negative group was assigned to 0%.

Animals

C57BL/6J mice were obtained from the Model Animal Research Center (MARC) of Nanjing University (Nanjing, China). Animals were maintained in SPF grade laminar-flow housing apparatus under controlled temperature, humidity and 12-h light/dark regimen. The experimental animal facility has been accredited by AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International) and the IACUC (Institutional Animal Care and Use Committee) of Model Animal Research Center of Nanjing University approved all animal protocols used in this study. Zebrafish were housed and handled as per Nanjing University Laboratory Animal Services guidelines. All animal protocols were approved by IACUC of MARC and conducted in accordance with the MARC Guidelines for the Care and Use of Laboratory Animals.

Septic animal models

For generation of the LPS-induced mouse sepsis model, male C57BL/6J mice were randomly divided into three groups, designated “control” (n=5, only received 0.9% saline), “LPS” (n=5, received 5 mg/kg LPS alone) and “LPS+compound **6**” (n=5, received both compound **6** and 5 mg/kg LPS). male C57BL/6J mice were injected intraperitoneally with LPS (5 mg/kg of body weight) or control by PBS. To examine the effects of compound **6**, the mice were intraperitoneal injection with compound **6** (20 μg/g) for 1 h before LPS injection. Lung tissues were collected after LPS treatment 48 hours for quantification of gene expression and histology analysis.

Histology

After LPS treatment 24 hours, the superior lobe of the right lung was isolated and fixed in 4% paraformal-dehyde overnight followed by gradual dehydration. Then the lung tissues were embedded in paraffin and cut into 6μm sections. Hematoxylin and Eosin staining was performed as MARC SOP and the samples were imaged with Olympus BX51 inverted microscopy in brightfield**.**

Immunofluorescence

Immunofluorescence staining in cells was performed as previously reported. Briefly, the cells were fixed with 4% paraformaldehyde for 30 min, penetrated by 0.6% Triton X-100 for 1 h, and then blocked with goat serum. Subsequently, the cells were incubated with primary antibody against GSDMD (ab155233, Abcam) at 4 °C overnight, followed by incubation with Alexa Fluor-conjugated secondary antibody (Jackson ImmunoResearch) in the dark for 1 h. The nuclei were stained by 4′,6-diamidino-2-

phenylindole (DAPI) (Jackson ImmunoResearch) for 20 min. PI and FLICA staining was performed as according to the manufacturer's instructions (ICT097, Bio-rad). The fluorescent images were captured with Leica TCS SP5 confocal microscope.

Pyroptosome assay

Assay of the activity of the ASC pyroptosome was applied as the published method [4]. ASC pyroptosomes were purified from ASC–GFP expressing THP-1 cells.

Western Blotting

The Western Blot assay was conducted as previously described. Briefly, Cell lysates prepared in standard cell lysis buffer were separated in 10% SDS-polyacrylamide and proteins were transferred to PVDF membranes (Millipore). The membranes were subsequently blocked by 5% BSA dissolved in PBS for 1 h and probed with primary antibodies against caspase-1 (NBP1-45433, Novus Biologicals), GSDMD (ab155233, Abcam), Caspase-8 (1C12 Cell signaling), and secondary HRP-conjugated antibodies. β-tubulin and Histone (Santa Cruz Biotechnology) were used as an internal control. Western blotting bands were quantified using the Odyssey Infrared Imaging System (Tanon, China) by measuring band intensity (Area × OD) and developed using ECL detection reagent (Vazyme).

Caspase-1 activity assay

Caspase-1 activity was determined using the Caspase-1 Assay Kit (Fluorometric) from Abcam (ab39412) according to manufacturer’s instructions. Briefly, cells were lysed and the nuclei and organelles were removed by centrifugation at 20,000 g. 50 μ of total cytosolic protein was used to assess cytosolic caspase activity. Cell homogenates

were incubated up to 4 hours at 37°C with corresponding caspases substrate conjugated to the chromophore p-nitroanilide. Cleavage of substrate was quantified spectrophotometrically at 405 nm using plate reader (Bio-rad).

Inducible express of GSDM-NT in human cell

Human embryonic kidney (HEK) 293T cells and 293FT cells were obtained from ATCC and were cultured in Dulbecco's modified Eagle's medium (DMEM). Complementary DNA (cDNA) for human gasdermin D (GSDMD) N-terminal (1–275) was amplified from reverse-transcribed cDNA from THP-1 cells and inserted into a pTRIPZ vector. The plasmid harboring the GSDMD-NT gene was then cotransfected with the packing plasmids pSPAX2 and pMD2G into 293FT cells. The lentivirus particles were harvested, filtered, and concentrated by ultracentrifugation 48 h later and were used to infect 293T cells for another 48 h. 293T cells that are resistant to puromycin were selected.

Co-Immunoprecipitation (Co-IP)

The lysate of J774A.1 macrophage was immunoprecipitated overnights at 4 °C with 2 μg/mL anti-NLRP3 or anti-ASC antibodies, followed by incubation with pre-washed magnetic beads (Thermo Fisher) for 30 minutes in room temperature. Pellet beads using magnetic separation rack and wash with lysis buffer. Heat the sample to 95-100°C for 5 min and pellet beads using magnetic separation rack. The supernatant was analyzed by western blotting for studying interaction between inflammasome components.

Imaging of inflammasome assembly

pLEX-MCS-ASC-GFP plasmid was purchased from Addgene and the DNA sequence of ASC-GFP was cloned into pCMV backbone to generate pCMV-ASC-GFP plasmid. pCMV-ASC-GFP was transfected to BMDMs by using Neon Electroporation Transfection (ThermoFisher Cat. No. MPK5000S). BMDMs were primed with LPS and compound **6** as previously descripted, followed by Nigericin treatment and live imaging in biocytoculture system. All related images were captured with ZEISS LM780 laser scanning confocal microscope (Zeiss, German).

Detection of reactive oxygen species (ROS)

Intracellular ROS level was label by dichloro-dihydro-fluorescein diacetate (DCFH-DA) assays (Sigma). Briefly, cells were incubated with 50 μM DCFH-DA at 37°C for 30 min in darkness. Then, the cells were washed twice using cold PBS and harvested for fluorescence-activated cell sorting.

Molecular Docking Simulation

Molecular docking of compound **6** against Casapse-1 was carried out using Glide tool according to the specified instructions. In brief, for receptor protein, the crystal structure of pro-Caspase-1 (PDB ID: 2NN3) was obtained from protein data base and the polar hydrogen atoms and Kollman charges were assigned. For ligand, compound **6** partial charges were designated and non-polar hydrogen atoms were merged. All the torsion angles for compound **6** were set free to rotate through docking experiment. A grid map of pro-Caspase-1 was adjusted on whole protein structure to generate the grid map and to get the best conformational state of docking. The 100 number of runs were adjusted using docking experiments. All the docked complexes were further evaluated

on lowest binding energy (Kcal/mol) values. The two-dimensional graphical depiction of best docked complexes was accessed by ligand interaction diagram tool.

DARTS assay

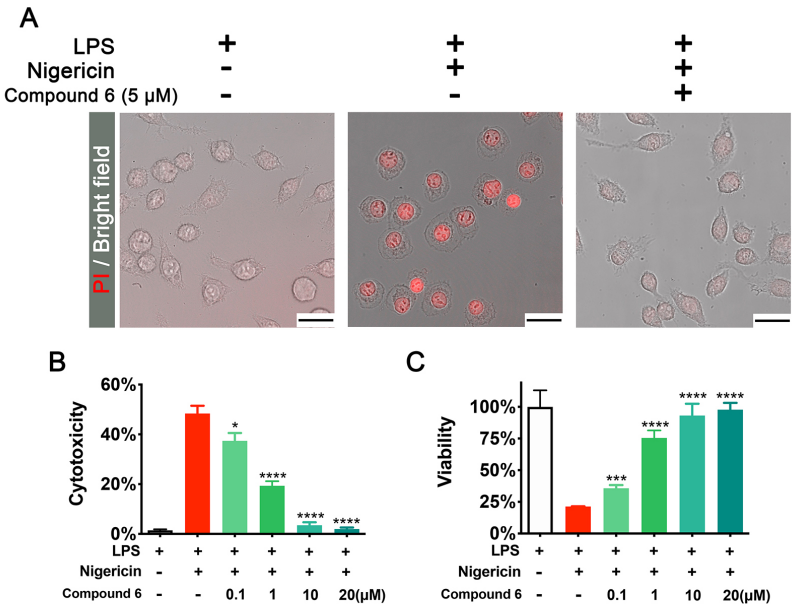
DARTS was performed as previously reported. Briefly, grow cells to approximately 80-85% confluence and lysate with lysis buffer (50 μL 20X protease inhibitor cocktail (Roche), 50 μL 1 M sodium fluoride, 100 μL 100 mM β-glycerophosphate, 100 μL 50 mM sodium pyrophosphate, and 10 μL 200 mM sodium orthovanadate with 690 μL M-PER reagent (M-PER, Thermo Scientific)). Centrifuge for 10 min at 18,000 X g at 4 °C. Incubate supernatant with compound **6** for 30 mins at room temperature with shaking with a thermomixer. Then add protease solutions to cell lysate and incubate at room temperature with protease of choice for 10 mins. Stop digestion by adding protease inhibitor cocktail and incubate on ice for 10 min. Add SDS-PAGE loading buffer and heat at 70 °C for 10 min for western blot preparation.

Statistical Analysis

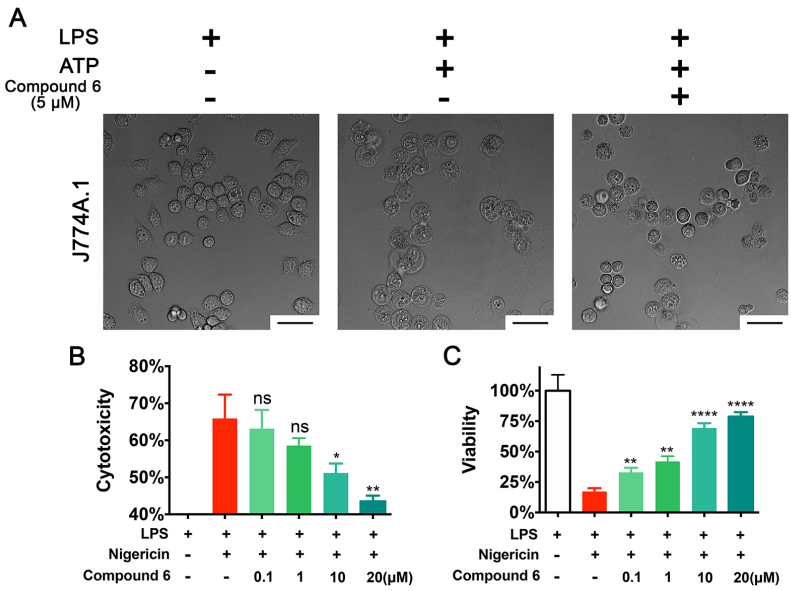
The data are expressed as mean ± SEM. GraphPad Prism 8.0 software was used to process the data. For comparisons between two groups, unpaired t-test was performed; for comparisons among multiple groups, One-way analysis of variance (ANOVA) test was performed. $P < 0.05$ was considered statistically significant.

1. Yu, J. S.; Zhou, J., A highly efficient Mukaiyama-Mannich reaction of N-Boc isatin ketimines and other active cyclic ketimines using difluoroenol silyl ethers catalyzed by Ph3PAuOTf. *Org Biomol Chem* **2015,** *13* (45), 10968-72.
2. Yu, J. S.; Liao, F. M.; Gao, W. M.; Liao, K.; Zuo, R. L.; Zhou, J., Michael Addition Catalyzed by Chiral Secondary Amine Phosphoramide Using Fluorinated Silyl Enol Ethers: Formation of Quaternary Carbon Stereocenters. *Angew Chem Int Ed Engl* **2015,** *54* (25), 7381-5.

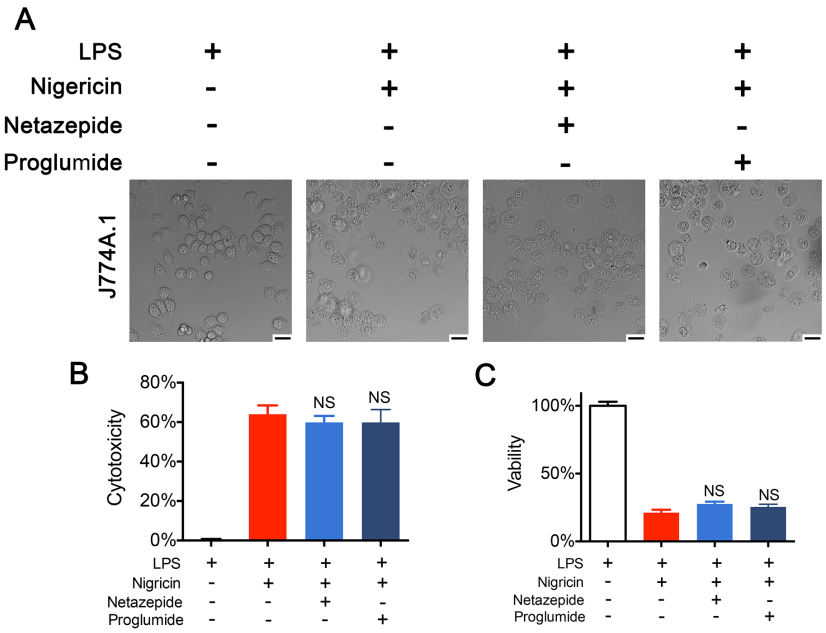
3. Crowley, L. C.; Scott, A. P.; Marfell, B. J.; Boughaba, J. A.; Chojnowski, G.; Waterhouse, N. J., Measuring Cell Death by Propidium Iodide Uptake and Flow Cytometry. *Cold Spring Harb Protoc* **2016,** *2016* (7).
4. Fernandes-Alnemri, T.; Alnemri, E. S., Assembly, purification, and assay of the activity of the ASC pyroptosome. *Methods in enzymology* **2008,** *442*, 251-70.



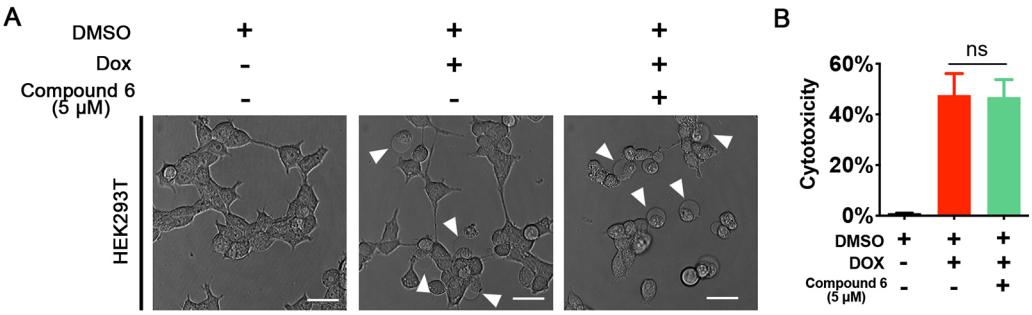
Supplement Figure 1. Compound 6 inhibits macrophage pyroptosis induced by Nigerincin. (A) Morphological analysis of compound **6** effect on pyroptotic inhibition in macrophages. PI staining labels perforated cells. The scale bar is 25 μm. **(B & C)** Quantification of cell death of J774A.1 macrophage with LDH and Alamar blue assay. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. Data expressed as mean ± SEM from n = 3 independent experiments.



Supplement Figure 2. Compound 6 inhibits macrophage pyroptosis induced by ATP. (A) Morphological analysis of J774A.1 cell. Compound **6** significantly reduces the number of cellular swelling and burst. Quantification of cell death of J774A.1 macrophage with LDH and Alamar blue assay. ns, not significant. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. Data expressed as mean ± SEM from n = 3 independent experiments.

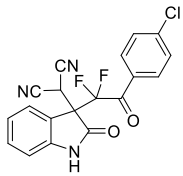


Supplement Figure 3. Blocking CCKB receptors with antagonists showed no pyroptosis inhibition effect. (A) Morphological analysis of J774A.1 cell with netazeptide or proglumide treatment. **(B & C)** Quantification of cell death of J774A.1 macrophage with LDH and Alamar blue assay. ns, not significant. Data expressed as mean ± SEM from n = 3 independent experiments.



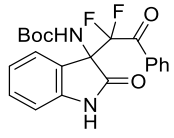
Supplement Figure 4. Compound 6 could not inhibit gasdermin-N domain driven cell death. (A) Morphological analysis of cell death. White triangles indicate the bursting cells. **(B)** Quantification of LDH level in cell culture medium. Data expressed as mean ± SEM from n = 3 independent experiments. A, scale bar=25μm. ns, not significant.

NMR and MS data for tested compound 6 analogues



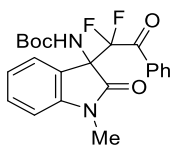
YJS-YH-67

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.74 (s, 1H), 7.95-7.93 (m, 2H), 7.68-7.66 (m, 1H), 7.46-7.42 (m, 3H), 7.19-7.15 (m, 1H), 7.05-7.04 (m, 1H), 4.94 (s, 1H); ^{19}F NMR (376 MHz, $CDCl_3$): δ -97.12 (d, *J* = 308 Hz, 1F), -98.74 (d, *J* = 308 Hz, 1F); ^{13}C NMR (100 MHz, $CDCl_3$): δ 184.39 (t, *J* = 30 Hz), 170.50 (d, *J* = 7 Hz), 142.41, 141.71, 132.06, 131.69 (t, *J* = 3 Hz), 129.36, 128.75, 125.80 (d, *J* = 3 Hz), 124.04, 120.58 (d, *J* = 4 Hz), 116.74 (dd, *J* = 270 Hz, *J* = 264 Hz), 111.45, 109.58, 108.71, 55.43 (t, *J* = 20 Hz), 26.89 (d, *J* = 8 Hz); IR (ATR): 3303, 2904, 1734, 1587, 1474, 1170, 1091, 753; HRMS (ESI): Exact mass calcd for $C_{19}H_{14}ClF_2N_4O_2$ $[M+NH_4]^+$: 403.0768, Found: 403.0769.



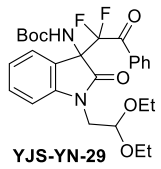
YJS-YN-27

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.72 (s, br, 1H), 7.89-7.88 (m, 2H), 7.59-7.55 (m, 1H), 7.42-7.34 (m, 3H), 7.28-7.25 (m, 1H), 7.01 (t, *J* = 7.6 Hz, 1H), 6.82-6.81 (m, 1H), 6.36 (s, 1H), 1.33 (s, 9H); ^{19}F NMR (376 MHz, $CDCl_3$): δ -106.71 (d, *J* = 276 Hz, 1F), -108.77 (d, *J* = 276 Hz, 1F); ^{13}C NMR (100 MHz, $CDCl_3$): δ 189.45 (t, *J* = 28 Hz), 172.85, 153.52, 142.32, 134.60, 132.91, 130.49, 130.18 (t, *J* = 3.6 Hz), 128.55, 125.39, 124.32, 122.84, 115.19 (t, *J* = 265 Hz), 110.62, 81.38, 65.97 (t, *J* = 25 Hz), 28.05. MS (EI): 402 (M^+, 4), 302 (1), 247 (4), 147 (100), 105 (23), 77 (20), 57 (15), 44 (13). HRMS (EI): Exact mass calcd for $C_{21}H_{20}N_2O_4F_2$ $[M]^+$: 402.1391, Found: 402.1395.



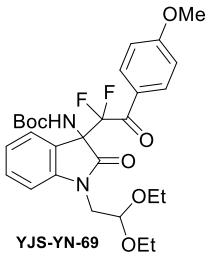
YJS-YM-90

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.82-7.80 (m, 2H), 7.60-7.56 (m, 1H), 7.42-7.33 (m, 4H), 7.03-7.00 (m, 1H), 6.84-6.82 (m, 1H), 6.18 (s, 1H), 3.21 (s, 3H), 1.27 (s, 9H); ^{19}F NMR (376 MHz, $CDCl_3$): δ -107.17 (d, *J* = 275 Hz, 1F), -108.29 (d, *J* = 275 Hz, 1F); ^{13}C NMR (100 MHz, $CDCl_3$): δ 188.83 (t, *J* = 28 Hz), 170.83, 153.35, 144.95, 134.54, 132.81, 130.56, 130.04 (t, *J* = 3.6 Hz), 128.52, 125.19, 123.81, 122.85, 115.43 (t, *J* = 265 Hz), 108.46, 80.98, 65.27 (t, *J* = 23 Hz), 28.03, 26.69. MS (EI): 416 (M^+, 6), 316 (2), 205 (3), 161 (100), 105 (14), 77 (16), 57 (9), 44 (6). HRMS (EI): Exact mass calcd for $C_{22}H_{22}N_2O_4F_2$ $[M]^+$: 416.1548, Found: 416.1551.



YJS-YN-29

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.83-7.81 (m, 2H), 7.62-7.58 (m, 1H), 7.43-7.39 (m, 2H), 7.34-7.30 (m, 2H), 7.10 (d, *J* = 8 Hz, 1H), 6.99 (t, *J* = 7.6 Hz, 1H), 6.20 (s, 1H), 4.73-4.70 (m, 1H), 4.04-4.00 (m, 1H), 3.81-3.49 (m, 5H), 1.29 (s, 9H), 1.18-1.12 (m, 6H); ^{19}F NMR (376 MHz, $CDCl_3$): δ -107.10 (d, *J* = 274 Hz, 1F), -108.54 (d, *J* = 274 Hz, 1F); ^{13}C NMR (100 MHz, $CDCl_3$): δ 189.13 (t, *J* = 28 Hz), 171.18, 153.29, 144.84, 134.48, 132.89, 130.17, 130.08 (t, *J* = 3.4 Hz), 128.47, 124.90, 123.47, 122.60, 115.42 (t, *J* = 265 Hz), 110.29, 101.12, 80.95, 65.06 (t, *J* = 23 Hz), 63.98, 63.85, 44.36, 28.03, 15.30, 15.26. MS (EI): 518 (M^+, 6), 217 (8), 149 (5), 105 (46), 103 (100), 84 (17), 77 (22), 75 (23), 57 (9), 44 (33). HRMS (EI): Exact mass calcd for $C_{27}H_{32}N_2O_6F_2$ $[M]^+$: 518.2228, Found: 518.2224.



YJS-YN-69

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.85-7.83 (m, 2H), 7.31-7.27 (m, 2H), 7.09-7.07 (m, 1H), 6.98-6.95 (m, 1H), 6.87-6.85 (m, 2H), 6.22 (s, 1H), 4.71-4.69 (m, 1H), 4.03-3.98 (m, 1H), 3.86 (s, 3H), 3.78-3.51 (m, 5H), 1.27 (s, 9H), 1.17-1.11 (m, 6H); ^{19}F NMR (376 MHz, $CDCl_3$): δ -106.80 (d, *J* = 273 Hz, 1F), -107.90 (d, *J* = 273 Hz, 1F); ^{13}C NMR (100 MHz, $CDCl_3$): δ 186.97 (t, *J* = 28 Hz), 171.29, 164.72, 153.32, 144.88, 132.91 (t, *J* = 4.2 Hz), 130.03, 125.64, 124.90, 123.72, 122.49, 115.72 (t, *J* = 265 Hz), 113.83, 110.17, 101.14, 80.84, 65.10 (t, *J* = 24 Hz), 63.94, 63.80, 55.58, 44.37, 28.02, 15.28, 15.27. MS (EI): 548 (M^+, 6), 402 (8), 217 (44), 135 (72), 103 (100), 92 (7), 77 (15), 75 (25), 44 (18). HRMS (EI): Exact mass calcd for $C_{28}H_{34}N_2O_7F_2$ $[M]^+$: 548.2334, Found: 548.2336.