Title: Discovery of Immunologically Inspired Small Molecules That Target the Viral Envelope Protein

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Supporting Information Table of Contents

Figure S1. Development and optimization of the competitive proximity luminescence assay ……,,,……S3

Figure S2. Quantification of antiviral IC_{90} values against DENV2 …….…….....S4

Figure S3. Affinity measurements for K786-9739 with sE and sE-M196V……S5

Figure S4. SAR studies of C200-5340 and K786-9739……..……...S6-7

Figure S5. Antiviral activity measured against representative dengue 1, 2, 3, and 4 and Zika viruses ……..……..……S8

Table S1. Initial assessment of antiviral activity …….……...…S9

Scheme S1. Synthetic of JW-068……....S10

REFERENCES for SUPPORTING INFORMATION……...……...S11

Figure S1. Development and optimization of the competitive proximity luminescence assay. a, ***left****,* Cross-titration of recombinant, soluble DENV2 prefusion E dimer (DENV2 sE_2) with **GNF2-biotin**. Titratable signal was observed at 200 and 300 nM **GNF2-biotin** with signal plateauing for both probe concentrations at 50-100 nM sE_2. ***right*****,** 10 μM of **3-110-22**, a known inhibitor of DENV2 E with K_D DENV2 sE_2 0.6 ± 0.2 μM[1], was used as a positive control to compete with 300 nM **GNF2-biotin** for binding to sE_2. Competition was observed at concentrations of sE_2 between 50 and 150 nM with optimal signal-to-background observed for 300 nM **GNF2-biotin** and 100 nM sE_2. **b,** Dose-response curves for positive control **3-110-22** (***left***) and negative control **JW-068** (***right***) in the competitive proximity luminescence assay using optimized conditions of 100 nM sE_2 and 300 nM **GNF2-biotin** in the presence of 0.3 or 0.6% DMSO. Data were fit by non-linear regression analysis. The signal-to-background and competition in the assay were the same at both DMSO concentrations tested. For our HTS and SAR experiments, the assay was performed with a constant concentration of 0.22% DMSO (vol/vol). **c,** The order of addition of reagents was varied to test the sequences depicted. For each set of reactions, we calculated the signal-to-background (S/B) in the absence of competitor (***left***) and the percent signal inhibition by 10 μM **3-110-22** (***right***). Approaches i and ii exhibited superior S/B; approach i was chosen for our experiments since addition of **GNF2-biotin** with the acceptor beads and protein was simpler than sequential addition of each of these reagents as in approach ii. **d,** Structure of **JW-068** and biolayer interferometry data showing that **JW-068** does not bind to DENV2 sE_2. Representative data are shown for *n* = 4 experiments. **3-110-22** was used as a positive control DENV2 sE_2.

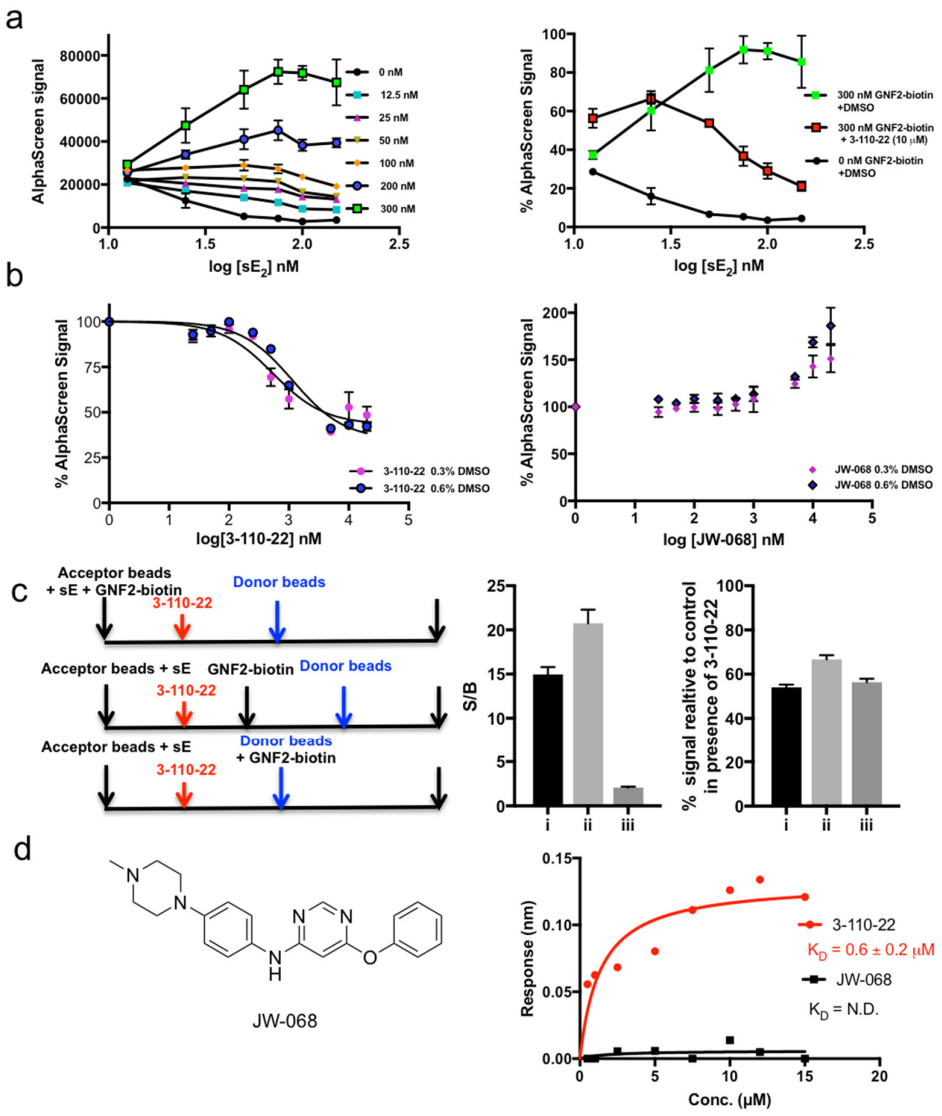


Figure S2. Quantification of antiviral IC_{90} values against DENV2. Antiviral activity against DENV2 was evaluated using the viral infectivity assay depicted in Figure 1d. Inhibitor-treatment was limited to a 45 minute pre-incubation with the viral inoculum and during the one-hour infection of cells. After this, all extracellular compound and virus were washed away, and the cells were overlaid with fresh medium. Single-cycle yield was quantified by viral plaque formation assay as a metric of successful viral entry 20-24 hours prior. IC_{90} values--defined as the concentration of inhibitor required to reduce single-cycle DENV2 viral yield by 10-fold in the infectivity assay – were determined by non-linear regression analysis of the data. IC_{90} values reported in Table 1 are the average of two or more independent experiments. Representative experiments are shown below.

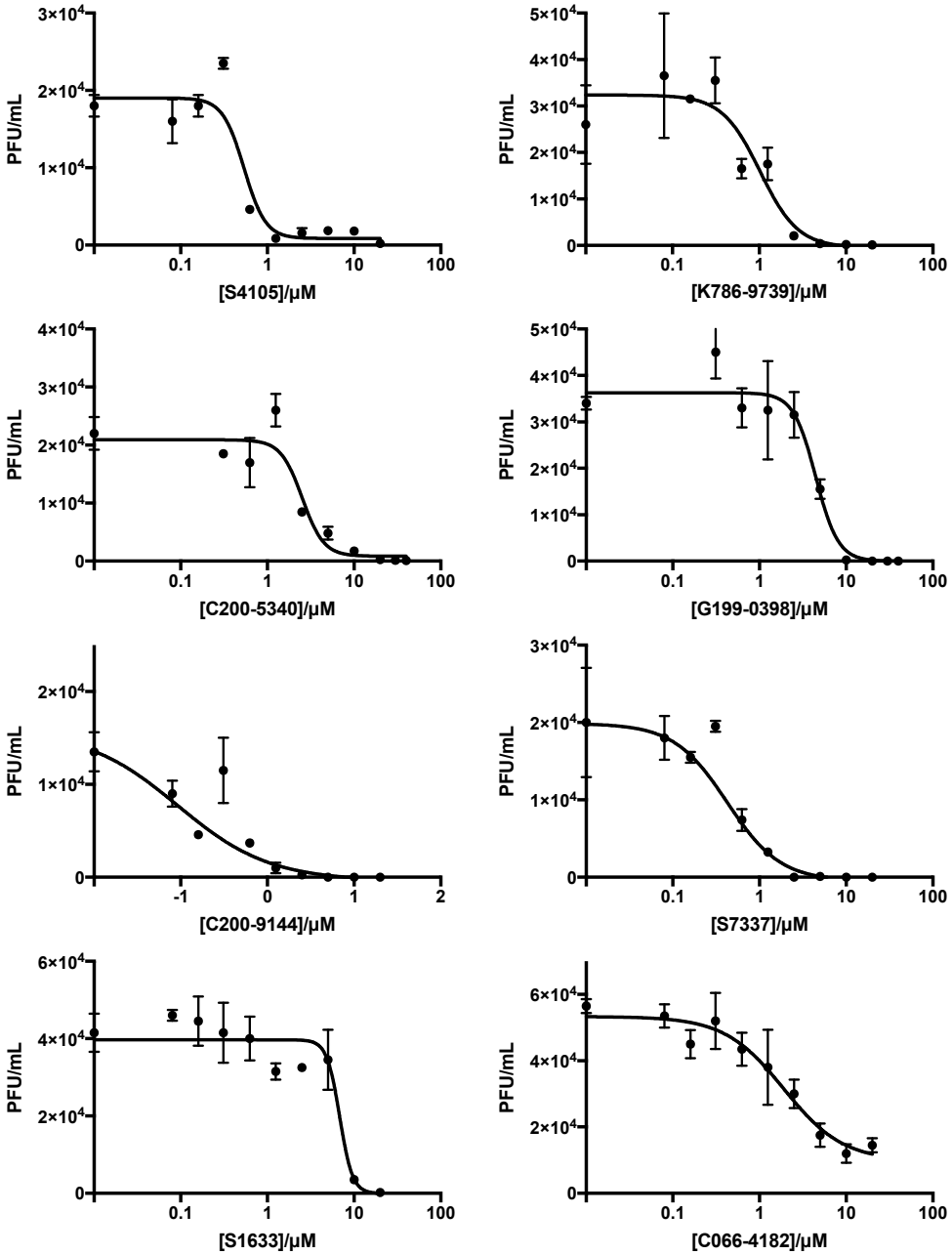


Figure S3. Affinity measurements for K786-9739 with recombinant, soluble E-WT and E-M196V dimer protein. K_D values for **K786-9739**'s interaction with DENV2 recombinant, soluble sE_2-WT (E-WT) and sE_2-M196V (E-M196V) proteins were determined by biolayer interferometry. Representative data for one experiment are shown with the average and standard deviation for $n \geqslant 2$ independent experiments presented in the figure legend.

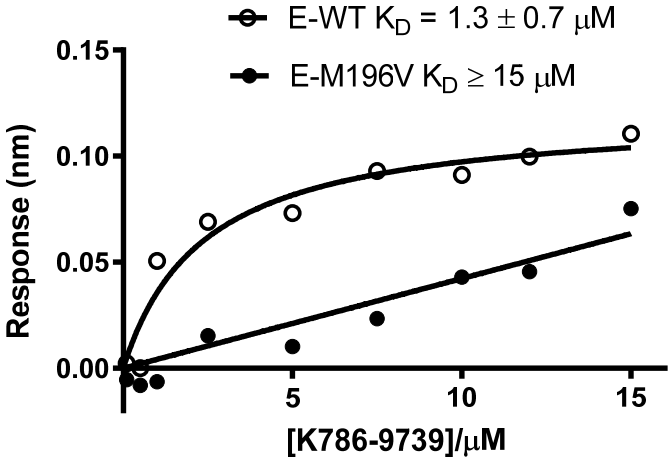


Figure S4 SAR studies of C200-5340 and K786-9739. The competitive proximity luminescence assay was performed with 300 nM **GNF2-biotin** and 200 nM recombinant DENV2 DI-DII protein in HEPES buffer (25 mM HEPES, 200 mM NaCl, pH 7.4) with 0.22% DMSO. IC_{50} values -- defined as the concentration of small molecule required to reduce luminescence in the assay by 50% -- were determined by nonlinear regression analysis. IC_{50} values for parental compounds identified in the HTS primary screen are in red and represent the average and standard deviation of 3 independent experiments. Analogs for each SAR study are shown in black; IC_{50} values represent the curve fit of duplicate samples. **a. C200-5340** study. **b. K786-9739** study.

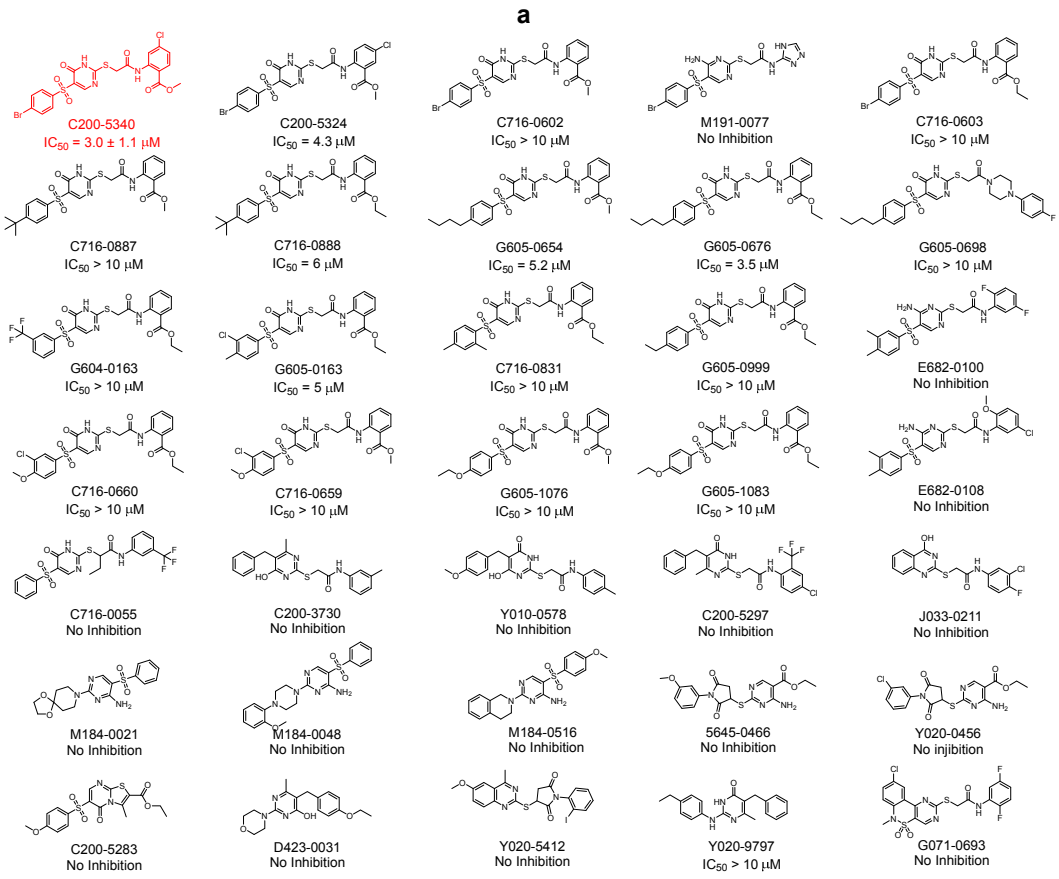


Figure S4 SAR studies of C200-5340 and K786-9739. Cont'd

b

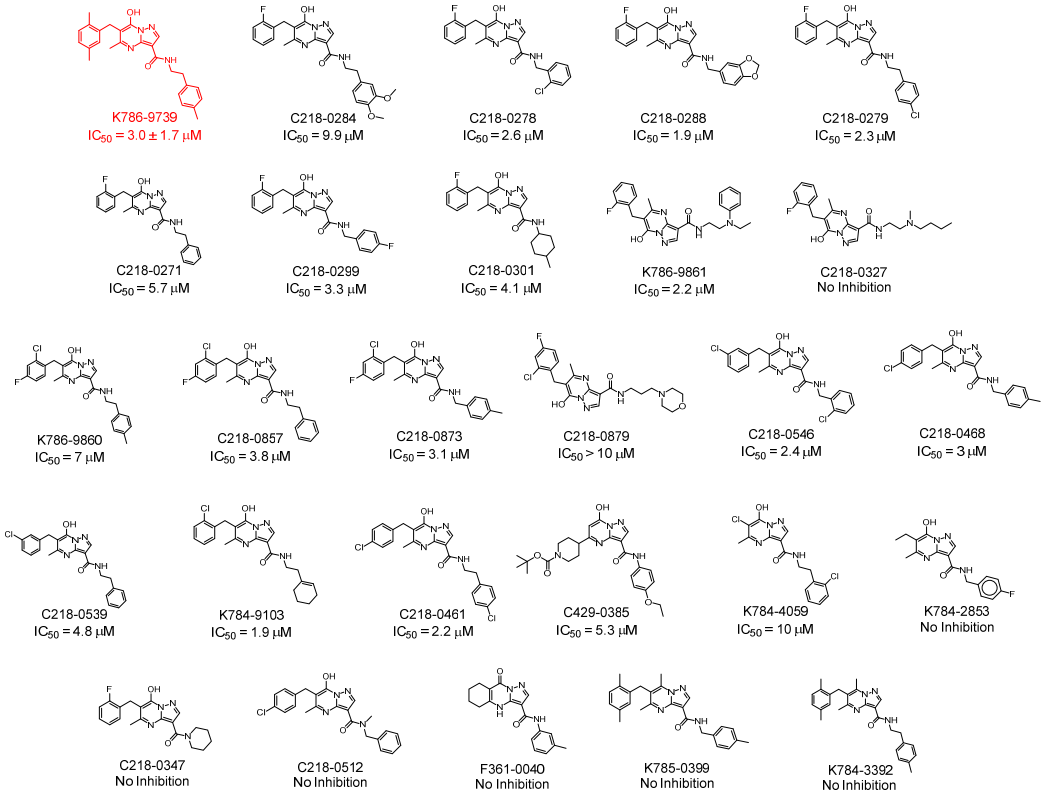


Figure S5. Antiviral activity measured against dengue 1, 2, 3, and 4 and Zika viruses. Antiviral activity was measured using the plaque reduction assay as outlined in Figure 1d. Briefly, inhibitor-treatment was limited to a 45 minute pre-incubation with the viral inoculum and during the one-hour infection of cells. After this, all extracellular compound and virus were washed away, and the cells were overlaid with carboxymethylcellulose-containing medium to allow formation of viral plaques. The percentage of plaque reduction was calculated as 100 – (number of plaques$_{\text{inhibitor-treated}}$ / number of plaques$_{\text{DMSO-treated}}$)*100 and is plotted versus the $\log_{10}$ concentration of inhibitor. $PRNT_{50}$ values, defined as the concentration to cause 50% reduction in the number of plaques, were determined by empirical analysis of data from two or more independent experiments and are reported in Table 4. Representative data are shown below.

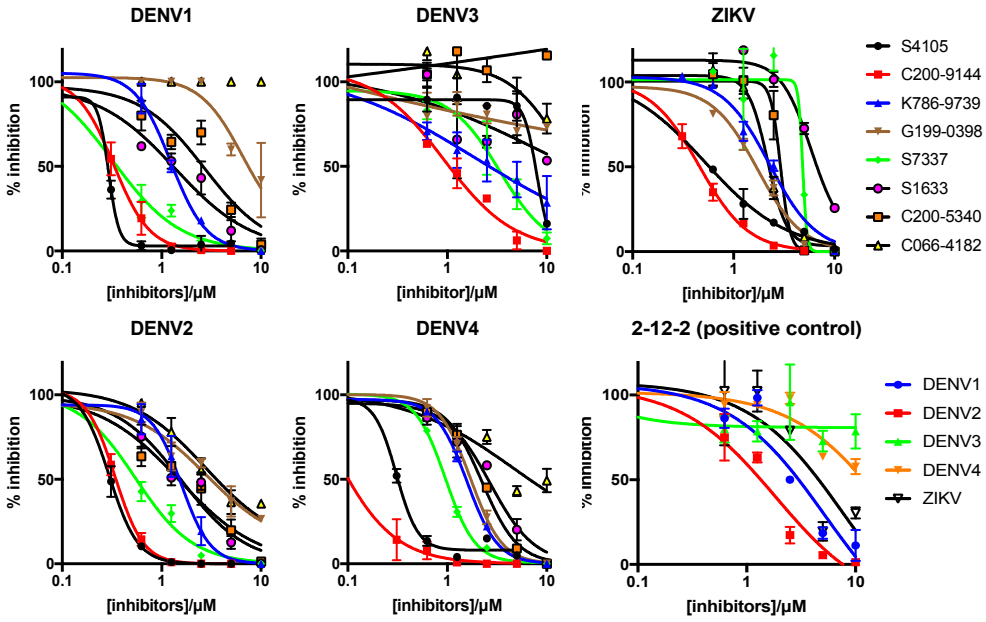
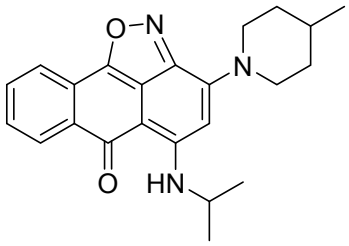


Table S1. Initial assessment of antiviral activity. The antiviral activity of compounds identified by HTS was initially assessed at two concentrations (3 and 10 μM) using the viral infectivity assay depicted in Figure 1d. Activity is reported as percent inhibition (% inhibition) defined as 100 – (single-cycle viral $yield_{nhibitor\text{-}treated}$ / single-cycle viral $yield_{DMSO\text{-}treated}$)*100. Values represent the average and standard deviation of 2 or more independent replicates. The eight compounds exhibiting the greatest antiviral activity were chosen for further characterization, including IC_{90} value determination (see Figure 1c for a flow chart of the screening progression). The structures of the four compounds not advanced for further study are shown here.



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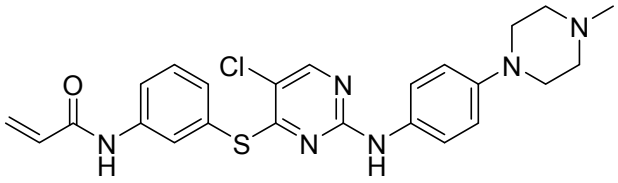
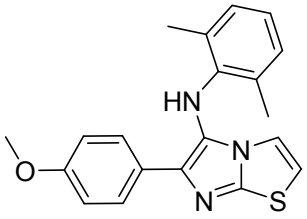


S1179



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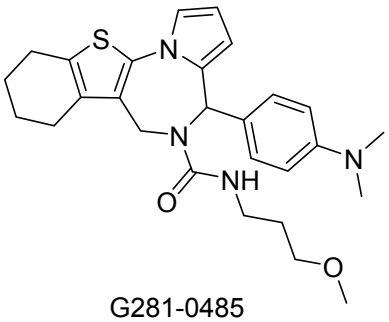
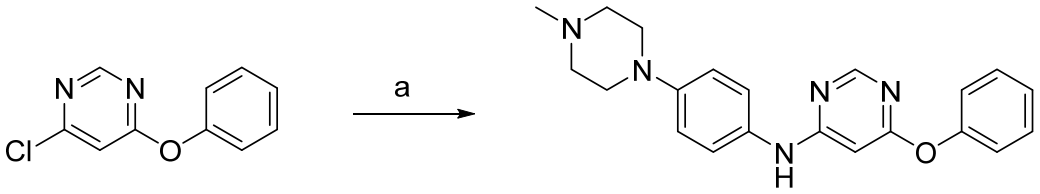


G281-0485

Compounds	% inhibition vs. DENV2 at 3 μM	% inhibition vs. DENV2 at 10 μM
C200-5340	40 ± 5	90 ± 5
G199-0398	60 ± 10	90 ± 5
G281-0485	65 ± 5	75 ± 2
K786-9739	≥ 99	≥ 99
S4105	60 ± 30	≥ 99
S1179	35 ± 20	80 ± 5
C066-4182	45 ± 5	80 ± 5
S1633	70 ± 10	90 ± 0
C620-0273	80 ± 10	90 ± 5
S7337	≥ 99	≥ 99
C200-9144	≥ 99	≥ 99
C239-0224	25 ± 15	70 ± 2

Scheme S1. Synthetic of JW-068. [a] Reagents and conditions: a) 4-(4-methylpiperazin-1-yl)aniline, TFA, 2-butanol, 110 °C, 4h.



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