# Supporting Information

## Bonavia et al. 10.1073/pnas.1017142108

### SI Text

**Cells and Viruses.** Respiratory syncytial virus (RSV) strains A-Long (VR-26) and B-Washington (RSV-9320 VR-955) and influenza virus strains A/PuertoRico/8/34 (VR-1469) and B/Lee/40 (VR-101) were obtained from ATCC. Influenza A/Udorn/72 and HIV-1 strains MDR52-52, br/92/020, SF162, JR-CSF, MDR807, and MDR769 were supplied by Southern Research Institute (SRI). Hepatitis C virus (HCV) 2a carrying the renilla-luciferase gene was obtained from Chiron. Dengue virus and yellow fever strains were supplied by Novartis Institutes for Tropical Diseases.

HEp-2, human epidermoid carcinoma of the larynx; HeLa, human cervical carcinoma; A549, human lung alveolar epithelial carcinoma; 293T, human embryonic kidney cells expressing the SV40 T antigen; MDCK, canine kidney epithelial; and Vero, African green monkey kidney epithelial cell lines, were obtained from ATCC. Human peripheral blood mononuclear cells were provided by SRI. Human cell lines Jurkat; Molt4; MT4; CEM SS, derived from acute T cell lymphoblastic leukemia; SupT1, derived from peripheral cell lymphoblastic lymphoma; and B cells Ramos and Raji, derived from Burkitt's lymphoma, were provided by Dr. Michael Daley (Oncology Department, Novartis Institutes for Biological Research, Cambridge, MA). Human primary bronchial epithelia cells (HBECs) were purchased from Lonza. Subgenomic genotype 1b replicon cell line with the firefly luciferase reporter, Huh-luc-neo-ET was obtained from Ralf Bartenshchalger (ReBlikon GmbH, Heidelberg, Germany). Huh7.5 cells were obtained from Charles Rice (Apath LLC, St Louis, MO).

Media for each cell line are described in the assays. The HBECs were grown in clear 12-well inserts (0.4 μM pore, Corning) to confluence in 1:1 mix of bronchial epithelial cell basal media (Lonza) and DMEM (Invitrogen) supplemented with bronchial epithelial growth media Singlequots of triiodothyronine(T3) (Lonza) and $5 \times 10^{-8}$ M *all-trans*-retinoic acid (Sigma). At confluence, the apical media is removed, and the cells were grown at air-liquid interphase (ALI) changing the basolateral media to differentiation media, which consists of growth media devoid of the T3 component. The cells were grown for 14 d in ALI, and polarization was determined by trans-well resistance measurement using a Millicell-ERS (Millipore) instrument before infection.

**RSV Quantification.** RSV infectious virus titers were determined by conventional plaque assay. Briefly, 10-fold dilutions of cell supernatants were used to inoculate HEp-2 cells before overlaying with EMEM (Lonza) with 1% Agarose (Lonza) and 1X Penicillin/Streptamicin/Glutamine (Invitrogen). Plaques were visualized 4 d later with neutral red (Sigma). Viral titers for RSV-A-Long were also detected by quantitative RT-PCR using Superscript III Platinum One-step Quantitative RT-PCR System (Invitrogen) amplifying the N gene of RSV using forward primer 5′-TTGGATCTGCAATCGCCA-3′, reverse primer 5′-CTTTTGATCTTGTTCACTTCTCCTTCT-3′, and probe 5′carboxyfluorescein-TGGCACTGCTGTATCTAAGGTCCTGCACT-tetramethylcarboxyrhodamine-3(FAM-TAMRA). The sequences for the primers and probes were first described in ref. 1. Analysis was performed on ABI Prism 7900HT (Applied Biosystems). For viral load quantitation in animals, RNA was extracted from the lung homogenates using the Qiagen RNeasy 96 kit (catalog no. 74181) carrier RNA (catalog no. 19073).

**$EC_{50}/CC_{50}$ Determination.** Cell cytotoxicity dose response curves (DRCs) were obtained parallel to virus quantification using CellTiter-Glo® Luminescent Cell Viability Assay (Promega) following the manufacturer instructions and read on a POLARstar OPTIMA (BMG Labtech). Cell media was supplemented with 1% DMSO to account for DMSO concentration after compound dilution. A nonlinear regression analysis was performed to find the 50% effect of cytotoxicity or efficacy (below) induced by each compound. Cell cytotoxicity ($CC_{50}$) curves were read at the same time of efficacy to match the time of cell incubation in the assay. For the T and B cell lines, the assay was performed at 72 h postplating in RPMI-1640 supplemented with 10% FBS.

DRC for RSV was performed in HEp-2 or A549 cells at multiplicity of infection (MOI) 0.3 in DMEM supplemented with 5% FBS and 1X penicillin/Streptamicin/glutamine in the presence of threefold serial dilutions of compounds. Cell cytotoxicity was determined by CellTiter-Glo® at 96 h postinoculation for RSV-A-Long and 120 h for RSV-B-Washington.

DRC for dengue and yellow fever virus was performed as previously described (2) using A549 cells at MOI 0.3 in the presence of twofold serial dilutions of compounds. Virus antigen production was quantified at 48 h by immunodetection using 4G2 antibody (mouse monoclonal against the envelope protein) and goat anti-mouse IgG conjugated with horse radish peroxidase as primary and secondary antibodies, respectively.

DRC for infectious HCV 2a virus was performed in Huh7.5 cells grown in DMEM supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acids (NEAs), and 10% FBS in presence of fivefold serial dilution of compound. Cells were inoculated with MOI 0.01, and luciferase was read at 72 h with renilla-luciferase system (Promega) following the manufacturer's recommendation. DRC in HCV replicon cells was performed in DMEM supplemented with 2 mM L-glutamine, 0.1 NEAs, and 10% FBS in presence of 3-fold serial dilution of compound (Cell cultures before assay were maintained with 0.25 mg/mL G418 (Invitrogen). Cell were read at 48 h postplating with firefly luciferase bright light plus (PerkinElmer) following the manufacturer's directions.

For adenosine and uridine rescue assays, the DRC method was a modification of the above DRC method for RSV in HEp-2 cells with the addition of 5 or 25 μM adenosine (Sigma) or uridine (Sigma) to the growth media.

DRC for influenza was performed in MDCK cells at MOI 0.005 in DMEM supplemented with 0.5 μg/mL L-(tosylamido-2-phenyl) ethyl chloromethyl ketone–treated trypsin and 1X penicillin/Streptamicin/glutamine in the presence of threefold serial dilutions of compounds. Cell cytotoxicity was determined by CellTiter-Glo® at 72 h postinoculation.

DRC for HIV was outsourced to SRI.

**Affinity Purification/Analysis.** Compound coupling and affinity purification was performed as described previously (3), and is illustrated for **6a** as follows. The tool compound was coupled to *N*-hydroxysuccinimide (NHS)-activated Sepharose 4 beads. HEp-2 cells infected with RSV-A-Long were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.5, 5% glycerol, 1.5 mM $MgCl_2$, 150 mM NaCl, 20 mM NaF, 1 mM $Na_3VO_4$, 1 mM dithiothreitol, 5 mM calyculin A, 0.8% Igepal-CA630, and a protease inhibitor cocktail). Cell lysates were preincubated with 20 mM **6b** (active), **6c** (inactive), or DMSO for 30 min, and incubated with **6a**-matrix for another 60 min. After extensive washes, bound material was eluted and resolved by SDS–polyacrylamide gel electrophoresis.

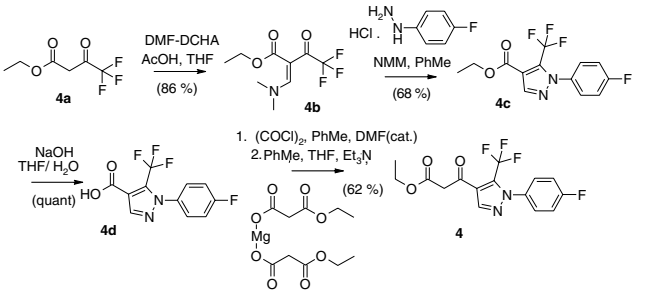
Gel lanes were cut into slices across the full separation range and subjected to in-gel tryptic digestion, followed by iTRAQ labelling (Applied Biosystems). Peptides extracted from DMSO-treated samples were labeled with iTRAQ reagent 116 and combined with extracts of compound-treated samples labeled with iTRAQ reagents 114 and 115, respectively. Liquid chromatography (LC)–tandem mass spectrometry was done using an Eksigent 1D1 HPLC system coupled to an LTQ Orbitrap mass spectrometer (Thermo-Finnigan). Tandem mass spectra were generated using pulsed-Q dissociation, enabling detection of iTRAQ reporter ions (4). Peptide mass and fragmentation data were used to query an in-house curated version of the IPI database using Mascot (Matrix Science). Protein identifications were validated using a decoy database. iTRAQ reporter ion-based quantification was performed with software developed in-house.

**Animal Model.** Female cotton rats (approximately 80 g, $n = 4$/group; Harlan Laboratories) were lightly anesthetized with isoflurane and infected intranasally with $10^5$ pfu RSV-Long in 100 μL of stabilization media (50% sucrose pH7.2/DMEM). Treatment was initiated 1 h postinoculation. Brequinar was formulated in sterile water, and series 1 was formulated in corn oil. Both compounds were administered orally twice a day up to 50 mg/kg (100 mg/kg per d). Control animals received vehicle only. At 4 and 7 d postinoculation, animals were euthanized; the lungs were harvested, weighed, and then homogenized in stabilization media. After homogenization, samples were frozen, thawed, and centrifuged at $537 \times g$ for 10 min at 4 °C. The supernatant was removed and frozen at −80 °C, prior to analysis. All animal studies were approved by the Institutional Animal Care and Use Committee of the Novartis Institutes for Bio-Medical Research Inc, Cambridge, MA. All animals were fed and watered ad libitum.

**Histopathology.** Spleen, thymus, small (duodenum, jejunum, ileum) and large intestine (cecum, colon, rectum), mesenteric lymph node, and bone marrow (in femur/tibia and sternum) were collected and fixed in 10% neutral buffered formalin. Tissues were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and then examined microscopically for histopathologic changes. Sections were evaluated using a semiquantitative scale (minimal to marked); minimal (+) change contained one or a few small foci; mild (++) change was composed of small to medium size foci; moderate (+++) change contained frequent and moderately sized foci, and marked (++++) change had extensive, confluent foci affecting most of the tissue. In these studies, only grades + through +++ were used.

**Synthesis of Tool Compounds.** Starting from commercially available ethyl trifluoroaceto acetate **1**, the pyrazole **3** is assembled via a Vilsmier-type condensation to generate activated intermediate **2** then reaction with an aryl hydrazine (5, 6). Subsequent conversion to the β-keto ester **4** is achieved via acylation-decarboxylation with potassium monomethylmalonate in 68–80% yield (7, 8). Conversion of a benzaldehyde to the corresponding chloroxime **5** (9) is accomplished in modest 60–80%, depending heavily on the substitution of the aromatic ring. Subsequent base catalyzed cycloaddition delivered the desired analogs **6a–c** in approximately 73–80% yield from **4**. The pyrrolidine scaffold was assembled from the *trans*-isomer of methyl 4-hydroxy-L-proline **7**. Reductive amination (10) affords the benzylic amine **8**, which is subjected to Mitsunobu inversion (11) to afford the *cis*-isomer **10**, via the ester **9**. Subsequent conversion of the hydroxyl to the corresponding mesylate **11** (12) facilitates displacement by mercaptobenzimidazole to generate the desired *trans*-isomer **12** through $S_N2$ inversion in 58–67% yield for the two steps. Hydrolysis to the acid **13** (>99% yield), facilitated peptide coupling to the desired amides **14a–c** in 66–90% yield (13).

**Scheme 1: General Method for Synthesis of 4.**




The 2-[1-Dimethylamino-meth-(Z)-ylidene]-4,4,4-triflouro-3-oxo-butyric acid ethyl ester **(4b)**. To a solution of ethyl trifluoroacetoacetate **(4a)** (6.35 mL, 43.5 mmol) in tetrahydrofuran (140 mL) was added acetic acid (5.0 mL, 87 mmol) and the reaction mix was cooled to 0 °C. Dimethylformamide-dicyclohexylacetal (87 mmol) was added dropwise, and the reaction mix was allowed to warm to room temperature and stirred for 2 h. The reaction was poured into ice water and extracted with ethyl acetate and washed with brine before the organic layer was dried over magnesium sulfate and concentrated in vacuo. The crude residue was purified by flash chromatography (0–50% EtOAc/heptanes) to provide **4b** (4.66 g, 86% yield) as a yellow solid.

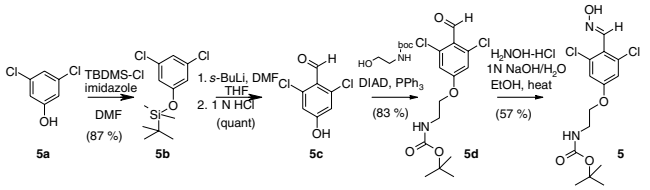
The 1-(4-Flouro-phenyl)-5-triflouromethyl-1H-pyrazole-4-carboxylic acid ethyl ester **(4c)**. To a solution of 2-[1-Dimethylamino-meth-(Z)-ylidene]-4,4,4-triflouro-3-oxo-butyric acid ethyl ester **(4b)** (4.66 g, 19.4 mmol) in toluene (100 mL) was added 4-flouro-phenylhydrazine hydrochloride (3.15 g, 19.4 mmol), followed by *N*-methylmorpholine (1.9 mL, 17.5 mmol), and reaction was stirred at room temperature for 2 h. The reaction was poured into water and extracted with ethyl acetate. The organic layer was washed with 10% citric acid solution, sodium bicarbonate solution, and brine, then dried over magnesium sulfate and concentrated in vacuo. The crude residue was purified by flash chromatography (0–20% EtOAc/heptanes) to provide **4c** (4.00 g, 68% yield) as a light creme-colored solid.

The 1-(4-Flouro-phenyl)-5-triflouromethyl-1H-pyrazole-4-carboxylic acid **(4d)**. To a solution of 1-(4-Flouro-phenyl)-5-triflouromethyl-1H-pyrazole-4-carboxylic acid ethyl ester **(4c)** (4.0 g, 13.2 mmol) in tetrahydrofuran (65 mL) was added a solution of lithium hydroxide (1N, 66 mL, 66 mmol) and stirred at room temperature for 24 h. The solvents were evaporated under reduced pressure, and the pH was adjusted to 4 by the addition of 1N HCl. The reaction mix was extracted with ethyl acetate; the organic layer was neutralized with aqueous sodium bicarbonate, dried over magnesium sulfate, and concentrated in vacuo to provide **4d** (2.7 g, >99% yield) as a white solid.

The 3-[1-(4-Flouro-phenyl)-5-triflouromethyl-1H-pyrazol-4-yl]-3-oxo-propionic acid methyl ester **(4)**. For reaction A: To a solution of 1-(4-Flouro-phenyl)-5-triflouromethyl-1H-pyrazole-4-carboxylic acid **(4d)** (2.7 g, 9.8 mmol) in dichloromethane (30 mL) was added $(COCl)_2$ (1.02 mL, 11.7 mmol), followed by a catalytic amount of dimethylformamide (one drop). The reaction was stirred at room temperature for 90 min, concentrated, and azeotroped with anhydrous toluene. For reaction B: To a solution of potassium ethylmalonate (1.4 g, 8.33 mmol) in toluene (30 mL) was added magnesium chloride (0.933 g, 9.8 mmol), tetrahydrofuran (15 mL) and triethylamine (2.9 mL, 20.6 mmol). This reaction mixture was warmed to 50 °C for 45 min, then cooled to room temperature and stirred for 15 min. The acyl chloride product (reaction A) was dissolved in toluene (30 mL) and was added to reaction B slowly via syringe. The reaction mix was stirred at room temperature for 2 h, and the mix was then poured into ice-cold 1N HCl. The organic layer was separated and washed with sodium bicarbonate and brine and dried over magnesium sulfate and concentrated in vacuo. The crude residue

was purified by flash chromatography (0–100% EtOAc/heptanes) to provide **4** (2.1 g, 62% yield) as a clear oil.

**Scheme 2: General Method for Ssynthesis of 5.**



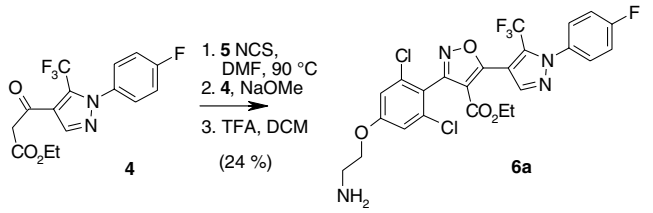

The *tert*-Butyl-(3,5-dichloro-phenoxy)-dimethyl-silane **(5b)**. To a solution of 3,5-dichlorophenol **(5a)** (4.90 g, 30.0 mmol) and imidazole (4.50 g, 66.0 mmol) in dimethylformamide (15 mL) was added tert-butyl-dimethylsilyl chloride (5.00 g, 33.0 mmol) at 0 °C, and the mixture was stirred at 0 °C for 20 min. Water was added at 0 °C, and the aqueous layer was extracted with ether. The organic layer was washed with brine, dried over sodium sulfate, and concentrated in vacuo. The residue was purified by flash column chromatography (0–50% EtOAc/Heptanes) to provide **5b** (7.09 g, 87% yield) as a colorless oil. $^1$H NMR (400 MHz, $CDCl_3$-*d*) δ ppm 6.75 (s, 1 H), 6.52 (s, 2 H), 0.76 (s, 9 H), 0.1 (s, 6 H); MS *m*/*z* 277 $[M + H]^+$.

The 2,6-Dichloro-4-hydroxy-benzaldehyde **(5c)**. To a solution of *tert*-Butyl-(3,5-dichloro-phenoxy)-dimethyl-silane **(5b)** (3.0 g, 11 mmol) in tetrahydrofuran (40 mL), *sec*-butyllithium (1.35 M solution in cyclohexanes, 8.3 mL, 11.2 mmol) was slowly added at −78 °C and the mixture was stirred at the same temperature for 30 min. To the mixture, dimethylformamide (1.3 mL, 16.5 mmol) was added at −78 °C, and the mixture was stirred at the same temperature for 90 min; 1N HCl was added at −78 °C, and the mixture was allowed to warm to room temperature. The aqueous layer was extracted with ethyl acetate. The organic layer was washed with sodium bicarbonate and brine, dried over sodium sulfate, and concentrated to provide **5c** (2.51 g, >99% yield) as a creme solid. $^1$H NMR (400 MHz, $CDCl_3$-*d*) δ ppm 10.29 (s, 1 H), 6.95 (s, 2 H); MS *m*/*z* 277 $[M + H]^+$.

The [2-(3,5-Dichloro-4-formyl-phenoxy)-ethyl]-carbamic acid *tert*-butyl ester **(5d)**. To a solution of 2,6-Dichloro-4-hydroxy-benzaldehyde **(5c)** (100 mg, 0.523 mmol) in anhydrous tetrahydrofuran (5 mL) was added triphenylphosphine (171 mg, 0.654 mmol), and the mixture was cooled to 0 °C. Boc ethanolamine (70 mg, 0.067 mmol) was then added to the mix, and (*E*)-diisopropyldiazene-1,2-dicarboxylate (DIAD) (76 mg, 0.375 mmol) was then added slowly to give a pale yellow solution, and the reaction was stirred at room temperature for 2 h, where solution becomes colorless. All solvents were removed under vacuum, and the crude residue was dissolved in ethyl acetate. The organic layer was washed with 1N NaOH, water and brine, dried over magnesium sulfate, and concentrated in vacuo. The crude residue was purified by chromatography (0–50% EtOAc/heptanes) to provide **5d** (120 mg, 83% yield).

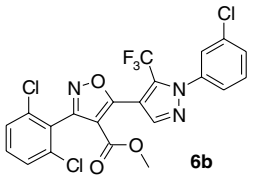
The {2-[3,5-Dichloro-4-(hydroxyimino-methyl)phenoxy]-ethyl}-carbamic acid tert-butyl ester (**5**). To a solution of hydroxylamine hydrocholoride (27.5 mg, 0.395 mmol) in sodium hydroxide solution (16 mg, 0.395 mmol) in water (2 mL) was added a solution of [2-(3,5-Dichloro-4-formyl-phenoxy)-ethyl]-carbamic acid *tert*-butyl ester **(5d)** (120 mg, 0.359 mmol) in ethanol (4 mL), and the mix was heated to 90 °C for 1 h. The solvents were evaporated under reduced pressure to leave a solid residue. The solid was washed with water and filtered with water and heptanes to provide **5** (72 mg, 57% yield) as a white solid.

**Scheme 3: General Method for Ssynthesis of Isoxazole/Pyrazole 6.**

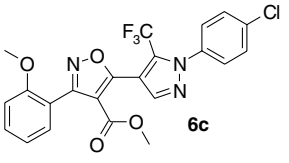



To a solution of oxime (**5**) (69 mg, 0.198 μmol) in dimethylformamide (1 mL) was added *N*-chlorosuccinimide (26.4 mg, 0.198 μmol), and the mixture was heated at 90 °C for 2 h. The flask was removed from heat and 3-[1-(4-Flouro-phenyl)-5-triflouromethyl-1H-pyrazol-4-yl]-3-oxo-propionic acid methyl ester (**4**) (0.068 g, 0.198 μmol) and sodium methoxide (25% wt in MeOH, 66 μL, 0.297 μmol) were added. The reaction was stirred at room temperature for 1 h. The reaction was quenched with 10% citric acid. The mixture was extracted with ethyl acetate, washed with aqueous sodium bicarbonate solution, dried over magnesium sulfate, and concentrated in vacuo. To the crude material (46 mg, 0.068 μmol) in dichloromethane (1.5 mL) was added TFA (1.5 mL), and the mixture was stirred at room temperature for 10 min. The solvents were concentrated under reduced pressure and azeotroped with dichloromethane. The crude residue was purified by reverse-phase chromatography (10–100% mobile phase, 0.1% TFA, over 20 min) to provide **6a** (117 mg, >99% yield). **6a**: $^1$H NMR (400 MHz, $CDCl_3$-*d*) δ ppm 8.21 (s, 1 H), 7.62–7.54 (m, 2 H), 7.33–7.19 (m, 2 H), 7.04 (s, 2 H), 4.17 (q, $J = 7.03$ Hz, 2 H), 4.11–4.02 (m, 2 H), 3.22–3.08 (m, 2 H), 1.09 (t, $J = 7.03$ Hz, 3 H); MS *m*/*z* 573 $(M + H)^+$; HRMS $(ES^+)$ for $C_{24}H_{18}N_4O_4F_4Cl_2$; calculated: $[M + H]^+ = 573.0719$; found: 573.0715.

The following compounds were prepared according to the preceeding general methods:

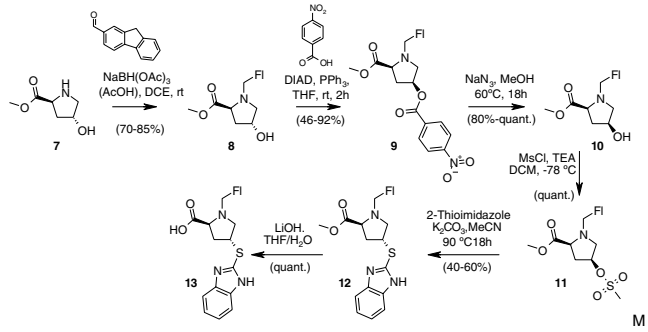



Compound **6b**:$^1$H NMR ($CDCl_3$, 400 MHz, δ 7.28) δ ppm 8.22 (s, 1H), 7.62–7.40 (m, 7H), 3.71 (s, 3H). HRMS: $[M + H]^+ =$ 515.9914, calculated = 515.9896. HPLC: $t_R = 12.95$ min, 100% purity.




Compound **6c**:$^1$H NMR ($CDCl_3$, 400 MHz, δ 7.28) δ ppm 8.24 (s, 1H), 7.61–7.52 (m, 6H), 7.15–7.11 (m, 1H), 7.03 (d, 1H, $J = 8$ Hz), 3.84 (s, 3H), 3.72 (s, 3H). HRMS: $[M + H]^+ =$ 478.0800, calculated = 478.0781. HPLC: $t_R = 12.38$ min, 100% purity.

**Scheme 9: Preparation of Proline Acid (13).**




Methyl (4*S*)-1-(9*H*-fluoren-2-ylmethyl)-4-hydroxy-D-prolinate (**8**). To a solution of methyl (4*S*)-4-hydroxy-D-prolinate (**7**) (1.20 g, 8.27 mmol) and 2-fluorenecarboxaldehyde (1.77 g, 9.09 mmol) in 1,2-dichloroethane (20 mL), a catalytic quantity of glacial acetic acid (47 μL, 0.83 mmol) was added, and the reaction mixture was stirred at room temperature for 10 min. Sodium triacetoxyborohydride (7.38 g, 33.10 mmol) was added, then the reaction mixture was stirred at room temperature for an additional 18 h. The reaction mixture was diluted with dichloromethane and was washed with saturated aqueous sodium bicarbonate solution followed by brine, then dried over sodium sulfate and concentrated in vacuo. The crude product was purified by flash chromatography (10–100% EtOAc/heptane) to obtain **8** as a creamy white solid (1.97 g, 74% yield). $^1$H NMR (400 mHz, $CDCl_3$): δ 7.77 (d, $J = 8.0$ Hz, 1H), 7.72 (d, $J = 8.0$ Hz, 1H), 7.58–7.48 (m, 2H), 7.40–7.26 (m, 3H), 4.48 (br s, 1H), 4.00 (m, 1H), 3.89 (s, 2H), 3.80–3.55 (m, 5H), 3.37 (m, 1H), 2.53 (m, 1H), 2.29 (m, 1H), 2.11 (m, 1H), 1.70–1.40 (m, 2H). LC/MS: $t_R$ 0.51 min; MS $m/z$ 324 $(M + H)^+$.

Methyl (4*R*)-1-(9*H*-fluoren-2-ylmethyl)-4-[(4-nitrobenzoyl) oxy]-D-prolinate (**9**). To a solution of methyl (4*S*)-1-(9*H*-fluoren-2-ylmethyl)-4-hydroxy-D-prolinate (**8**) (5.49 g, 16.98 mmol) in anhydrous THF (100 mL) cooled to 0 °C with an ice-water bath, triphenylphosphine (5.48 g, 18.67 mmol) and 4-nitrobenzoic acid (3.01 g, 18.67 mmol) were added. Under nitrogen, diisopropyl azocarboxylate (3.68 mL, 18.68 mmol) was added dropwise, and the reaction was warmed to room temperature and was stirred for 1 h. The reaction mixture was concentrated in vacuo. The dried residue was dissolved in diethyl ether and washed with water. The aqueous layer was back-extracted with diethyl ether, and the organic extracts were combined, washed with brine, dried over sodium sulfate, and concentrated in vacuo. The crude product was purified by a flash chromatography (8–100% EtOAc/heptane) to obtain the desired product **9** as a bright yellow solid (6.82 g, 85% yield). $^1$H NMR (400 MHz, $CDCl_3$): δ 8.36 (m, 1H), 8.15 (m, 1H), 7.89–7.81 (m, 2H), 7.40–7.27 (m, 3H), 5.38 (m, 1H), 4.06 (d, $J = 12$ Hz, 1H), 3.90 (s, 2H), 3.64 (d, $J = 12$ Hz, 1H), 3.47 (dd, $J = 8.0$, 8.0 Hz, 1H), 3.07 (d, $J = 8.0$ Hz, 1H), 2.85 (dd, $J = 12.0$, 4.0 Hz, 1H), 2.71 (m, 1H), 2.15 (m, 1H). LC/MS: $t_R$ 1.60 min; MS $m/z$ 473 $(M + H)^+$.

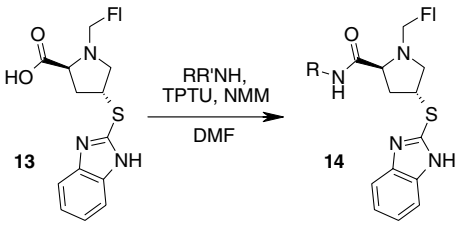
Methyl (4*R*)-1-(9*H*-fluoren-2-ylmethyl)-4-hydroxy-D-prolinate (**10**). To a solution of methyl (4*R*)-1-(9*H*-fluoren-2-ylmethyl)-4-[(4-nitrobenzoyl)oxy]-D-prolinate (**9**) (6.82 g, 14.43 mmol) in methanol (140 mL), sodium azide (12.34 g, 189.88 mmol) was added. Under nitrogen, the reaction was heated at 60 °C for 8 h. The reaction mixture was concentrated in vacuo. The dried residue was dissolved in ethyl acetate and washed with water. The aqueous layer was back-extracted with ethyl acetate, and the organic extracts were combined, washed with brine, dried over sodium sulfate, and concentrated in vacuo. The crude product was purified by a flash chromatography (14–100% EtOAc/heptane) to obtain the desired product **10** as an off-white solid (4.06 g, 87% yield). $^1$H NMR (400 MHz, $CDCl_3$): δ 7.76 (d, $J = 8.0$ Hz, 1H), 7.71 (d, $J = 8.0$ Hz, 1H), 7.53 (d, $J = 8.0$ Hz, 1H), 7.51 (s, 1H), 7.36 (dd, $J = 6.0$, 6.0 Hz, 1H), 7.33–7.26 (m, 2H), 4.26 (m, 1H), 7.95 (d, $J = 12$ Hz, 1H), 3.88 (s, 2H), 3.79 (d, $J = 12$ Hz, 1H), 3.62 (s, 3H), 3.39 (dd, $J = 12.0$, 4.0 Hz, 1H), 3.07 (dd, $J = 10.0$, 7.0 Hz, 2H), 2.67 (dd, $J = 12.0$, 4.0 Hz, 1H), 2.40 (ddd, $J = 16.0$, 8.0, 4.0 Hz, 1H), 1.97 (m, 1H). LC/MS: $t_R$ 0.84 min; MS $m/z$ 324 $(M + H)^+$.

Methyl (4*R*)-1-(9*H*-fluoren-2-ylmethyl)-4-[(methylsulfonyl) oxy]-D-prolinate (**11**). To a solution of methyl (4*R*)-1-(9*H*-fluoren-2-ylmethyl)-4-hydroxy-D-prolinate (**10**) (4.06 g, 12.55 mmol) and triethylamine (2.62 mL, 18.83 mmol) in anhydrous DCM (20 mL) cooled to −78 °C in a dry ice-acetone bath, methanesulfonyl chloride (1.17 mL, 15.07 mmol) was added dropwise. The reaction mixture was stirred at −78 °C for 15 min, resulting in the precipitation of an off-white solid. The reaction mixture was warmed up to room temperature, concentrated in vacuo, and diluted with dichloromethane. The organic phase was washed with water, and the aqueous layer was back-extracted with dichloromethane. The organic extracts were combined, washed with brine, dried over sodium sulfate, and concentrated in vacuo. The crude product was purified by flash chromatography (14–100% EtOAc/heptane) to obtain the desired product **11** as a white solid (4.90 g, 97% yield). $^1$H NMR (400 mHz, $CDCl_3$): δ 7.78 (d, $J = 8.0$ Hz, 1H), 7.74 (d, $J = 8.0$ Hz, 1H), 7.57–7.50 (m, 2H), 7.42–7.27 (m, 3H), 5.23 (br s, 1H), 4.14 (br s, 1H), 3.90 (s, 2H), 3.76 (s, 3H), 3.73 (br s, 1H), 3.39 (br s, 2H), 3.01 (s, 3H), 2.90–2.50 (m, 2H), 2.42 (br s, 1H), 1.52 (br s, 1H). LC/MS: $t_R$ 1.41 min; MS $m/z$ 402 $(M + H)^+$.

Methyl (4*S*)-4-(1*H*-benzimidazol-2-ylthio)-1-(9*H*-fluoren-2-ylmethyl)-D-prolinate (**12**). To a suspension of methyl (4*R*)-1-(9*H*-fluoren-2-ylmethyl)-4-[(methylsulfonyl)oxy]-D-prolinate (**11**) (4.90 g, 12.21 mmol) and potassium carbonate (3.37 g, 24.41 mmol) in a 1:1 mixture (120 mL) of acetonitrile and dioxane, 2-thiobenzimidazole (2.02 g, 13.43 mmol) was added. The reaction mixture was stirred at 90 °C for 18 h. The reaction mixture was cooled to room temperature, concentrated in vacuo, then diluted with ethyl acetate. The organic phase was washed with water once, and the aqueous layer was back-extracted with ethyl acetate. The organic extracts were combined, washed with brine, dried over sodium sulfate, and concentrated in vacuo. The crude product was purified by flash chromatography (14–100% EtOAc/heptane and 100% heptane to 44% EtOAc/heptane) to obtain the desired product **12** as a white solid (4.87 g, 88% yield). $^1$H NMR (400 mHz, $CDCl_3$): δ 7.78 (d, $J = 4.0$ Hz, 1H), 7.76 (d, $J = 4.0$ Hz, 1H), 7.58 (s, 1H), 7.56–7.45 (m, 3H), 7.37 (dd, $J = 8.0$, 8.0 Hz, 2H), 7.31 (m, 1H), 7.21 (m, 2H), 4.25 (m, 1H), 4.18–3.90 (m, 3H), 3.88 (s, 2H), 3.83 (m, 1H), 3.75 (s, 3H), 3.10 (m, 1H), 2.71 (m, 1H), 2.45 (s, 1H). LC/MS: $t_R$ 1.37 min; MS $m/z$ 456 $(M + H)^+$.

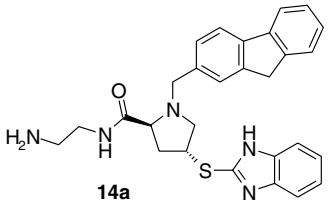
(4*S*)-4-(1*H*-Benzimidazol-2-ylthio)-1-(9*H*-fluoren-2-ylmethyl)-D-proline (**13**). To a solution of methyl (4*S*)-4-(1*H*-benzimidazol-2-ylthio)-1-(9*H*-fluoren-2-ylmethyl)-D-prolinate (**12**) (4.35 g, 11.84 mmol) in a mixture of THF (35 mL) and water (5 mL), lithium hydroxide (340 mg, 14.21 mmol) was added, and the reaction mixture was stirred at 60 °C for 18 h. The reaction was 80% complete. With additional lithium hydroxide (170 mg, 7.10 mmol), the reaction was heated at 60 °C for 6 h. The reaction mixture was concentrated in vacuo, and diluted with water (10 mL). The reaction mixture was washed successively with diethyl ether, ethyl acetate, and 10% methanol/dichloromethane. The aqueous layer was then concentrated in vacuo to obtain the desired acid **13** as a white solid (4.74 g, 90% yield). $^1$H NMR (400 mHz, $d_6$-DMSO): δ 7.38 (m, 2H), 7.34–7.15 (m, 5H), 7.15–7.25 (m, 2H), 4.22 (m, 1H), 4.11 (m, 1H), 3.55–3.35 (m, 2H), 3.06 (t, $J = 4$ Hz, 1H), 2.45 (m, 1H), 2.28 (m, 1H), 1.85 (m, 1H), 1.60 (s, 1H). LC/MS: $t_R$ 0.91 min; MS $m/z$ 354 $(M + H)^+$.

**Scheme 10: Preparation of Proline Amides (14) from Carboxylic Acid (13).**

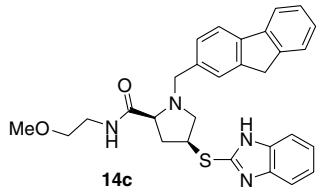



Compound **14b**: (4*S*)-4-(1*H*-benzimidazol-2-ylthio)-1-(9*H*-fluoren-2-ylmethyl)-*N*-(2-methoxyethyl)-D-prolinamide. To a solution of (4*S*)-4-(1*H*-Benzimidazol-2-ylthio)-1-(9*H*-fluoren-2-ylmethyl)-D-proline (**13**) (110 mg, 0.25 mmol) in *N*,*N*-dimethylacetamide (2 mL), 2-methoxyethylamine (26 μL, 0.30 mmol), *N*-methylmorpholine (33 μL, 0.30 mmol) followed by 2-(2-oxo-1(2H)-pyridyl)-1,1,3,3-tetramethyluroniumtetrafluorophosphate (89 mg, 0.30 mmol) were added, and the reaction mixture was stirred at room temperature for 18 h. The reaction was incomplete and was heated at 40 °C for an additional 18 h. The crude product was purified by a reverse-phase HPLC (30–90% organic mobile phase over 15 min) to obtain the desired product as a white solid (55 mg, 45% yield). Compound **14b**: $^1$H NMR (400 mHz, $CDCl_3$): δ 9.81 (br s, 1H), 7.76 (d, $J = 8.0$ Hz, 1H), 7.73–7.67 (m, 2H), 7.53 (d, $J = 8.0$ Hz, 1H), 7.46 (s, 1H), 7.40–7.26 (m, 4H), 7.21–7.14 (m, 2H), 4.24 (m, 1H), 3.99 (d, $J = 12.0$ Hz, 1H) 3.87 (s, 2H), 3.69–3.40 (m, 6H), 3.38 (s, 3H), 3.34 (m, 1H), 2.62 (m, 1H), 2.48 (m, 1H), 2.37 (m, 1H). HPLC: $t_R$ 8.19; MS $m/z$ 419 $(M + H)^+$ Chiral LC: >99% enantiomeric excess (ee).

The following compounds were prepared according to schemes 9/10:




Compound **14a**: $^1$H NMR (400 mHz, $CDCl_3$-*d*): δ 7.98 (s, 1 H), 7.62 (m, 1 H), 7.56–7.45 (m, 2 H), 7.42 (m, 1 H), 7.31–7.15 (m, 4 H), 7.06 (br.s, 2 H), 4.22 (m, 1 H), 3.69 (m, 3 H), 3.59–2.98 (m, 7 H), 2.48 (m, 2 H), 2.02 (m, 2 H). $m/z = 484.2$ [M + 1] (100), 485.2 [M + 2] (40), 486.2 [M + 3] (20); 100% UV purity at 220 nm; Chiral LC: >99% ee.




Compound **14c**:$^1$H NMR (400 mHz, $CDCl_3$-*d*): δ 7.75 (m, 2 H), 7.69 (d, $J = 8.0$ Hz, 1 H), 7.55–7.46 (m, 3 H), 7.36 (m, 1 H), 7.32–7.25 (m, 2 H), 7.19 (m, 2 H), 4.27 (m, 1 H), 4.00 (d, $J = 12.0$ Hz, 1 H), 3.85 (s, 2 H), 3.65–3.46 (m, 4 H), 3.44 (s, 3 H), 3.37 (m, 2 H), 3.21 (d, $J = 10$ Hz, 1 H), 3.02–2.93 (m, 1 H), 2.80–2.69 (m, 1 H), 2.08 (m, 1 H). $m/z = 499.3$ [M + 1] (100), 500.5 [M + 2] (30); 100% UV purity at 220 nm; Chiral LC: >99% ee.


1. Borg I, et al. (2003) Evaluation of a quantitative real-time PCR for the detection of respiratory syncytial virus in pulmonary diseases. *Eur Respir J* 21:944–951.
2. Yin Z, et al. (2009) An adenosine nucleoside inhibitor of dengue virus. *Proc Natl Acad Sci USA* 106:20435–20439.
3. Bantscheff M, et al. (2007) Quantitative chemical proteomics reveals mechanisms of action of clinical ABL kinase inhibitors. *Nat Biotechnol* 25:1035–1044.
4. Bantscheff M, et al. (2008) Robust and sensitive ITRAQ quantification on an LTQ orbitrap mass spectrometer. *Mol Cell Proteomics* 7:1702–1713.
5. Osei-gyimah P (2009) Method for synthesizing alkyl 4,4-dihalo-2-[(dialkylamino) methylene]-3-oxobutyrates. European Patent No. EP1000926..
6. Lui N, Brackemeyer T, Mueller P, Schneider M (2003) Method for producing 2-halogen-acyl-3-amino-acrylic acid and derivatives. World Intellectual Property Organization, WO/2003/051820.
7. Trend RM, Ramtohul YK, Stoltz BM (2005) Oxidative cyclizations in a nonpolar solvent using molecular oxygen and studies on the stereochemistry of oxypalladation. *J Am Chem Soc* 127:17778–17788.
8. Moyer MP, Feldman PL, Rapoport, Henry (1985) Intramolecular nitrogen-hydrogen, oxygen-hydrogen and sulfur-hydrogen insertion reactions. Synthesis of heterocycles from .alpha.-diazo. beta.-keto esters. *J Org Chem* 50:5223–5230.
9. Barbachyn MR, et al. (2002) Identification of phenylisoxazolines as novel and viable antibacterial agents active against gram-positive pathogens. *J Med Chem* 46:284–302.
10. Miyashiro J, et al. (2009) Synthesis and SAR of novel tricyclic quinoxalinone inhibitors of poly(ADP-ribose)polymerase-1 (PARP-1). *Bioorg Med Chem Lett* 19:4050–4054.
11. Swamy KCK, Kumar NNB, Balaraman E, Kumar KVPP (2009) Mitsunobu and related reactions: Advances and applications. *Chem Rev* 109:2551–2651.
12. Rosen T, Fesik SW, Chu DTW, Pernet AG (1988) Asymmetric synthesis of 2-substituted (4S)-4-aminopyrrolidines. SN2 displacement at the 4-position of the pyrrolidine moiety. *Synthesis* 40–44
13. Knorr R, Trzeciak A, Bannwarth W, Gillessen D (1991) 1,1,3,3-tetramethyluronium compounds as coupling reagents in peptide and protein chemistry *Proceedings of the Twenty-First European Peptide Symposium*, eds Giralt E, Andreu D (ESCOM, Leiden, The Netherlands), pp 62–64.