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SUPPLEMENTARY INFORMATION

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Fragment-derived inhibitors of human *N*-myristoyltransferase block capsid assembly and replication of the common cold virus

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Supplementary Information

Fragment-derived inhibitors of human *N*-myristoyltransferase block capsid assembly and replication of the common cold virus

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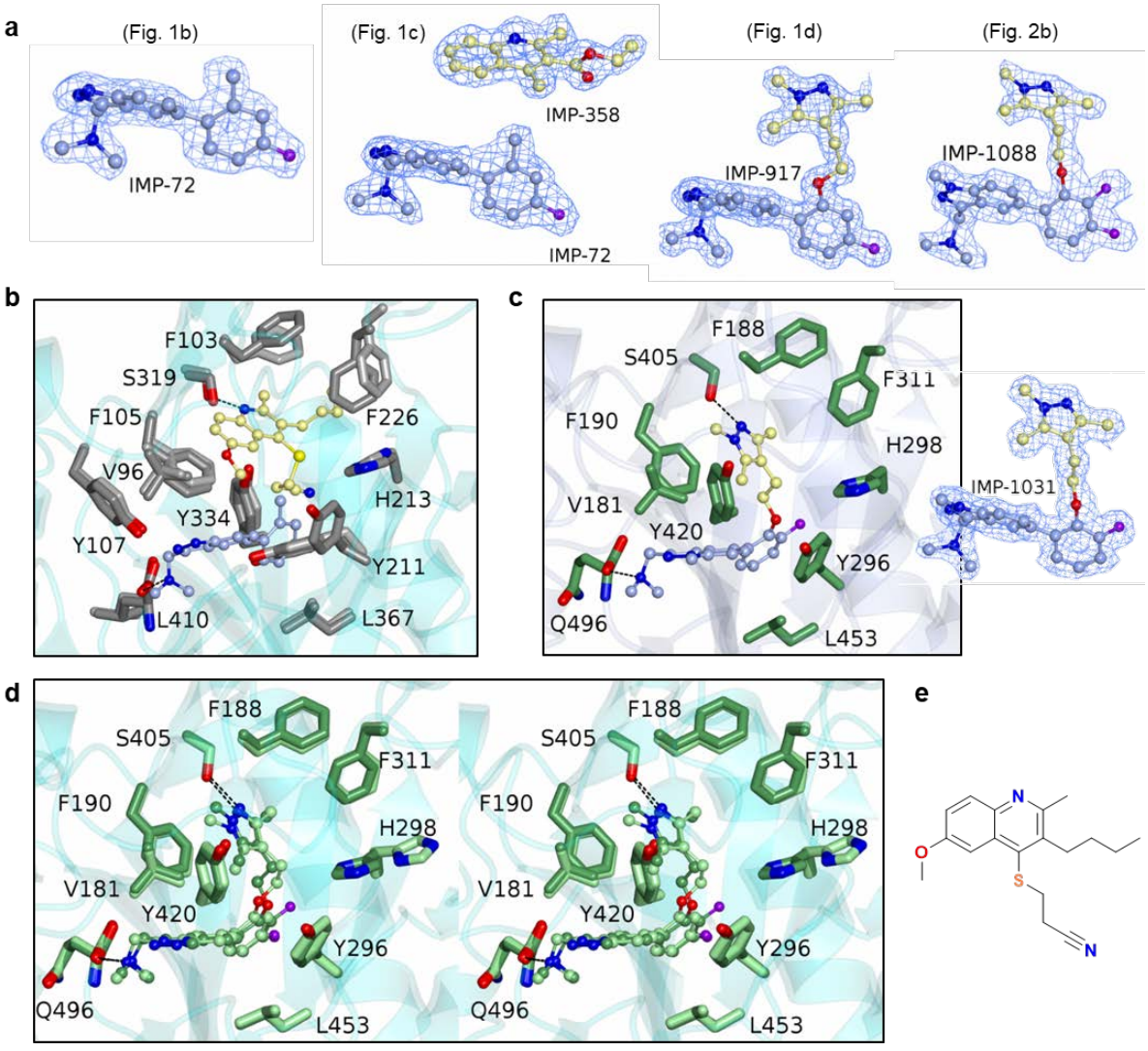
Supplementary Results

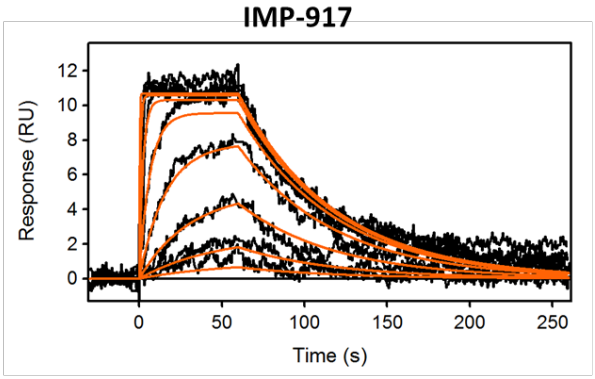
Supplementary Figure 1: Supplementary X-ray structure data. (**a**) $2F_{obs}$- F_{calc} electron density maps calculated using the respective refined coordinate sets, and displayed on the respective ligands determined by X-ray crystallography, colored as in main text, and displayed at the 1σ contour level. See also Supplementary Table 1. (**b**) Comparison of binding modes of IMP-72 (PDB: 5O48) and MRT00057965 (PDB: 4A95) in PvNMT, superimposed using CCP4MG, and colored by element: oxygen, red; nitrogen, blue; sulphur, yellow; fluorine, purple; carbon, ice blue (IMP-72), lemon (MRCT00057965). Protein residues are similarly colored in darker and lighter greys for the IMP-72 and MRCT00057965 complexes respectively. (**c**) Binding mode of IMP-1031 in HsNMT1 with Myr-CoA (PDB: 5O6J); inset to right shows electron density associated with IMP-1031. Carbon atoms of indazole and phenyl core groups of IMP-1031 are in ice blue, those of the pendant trimethylpyrazole are in lemon. (**d**) Stereo view of overlaid structures of HsNMT1:MyrCoA:IMP-917 and HsNMT1:MyrCoA:IMP-1088 complexes, with carbon atoms in light green and lawn green for IMP-917 and IMP-1088 structures, respectively. The change in linker orientation from in-plane



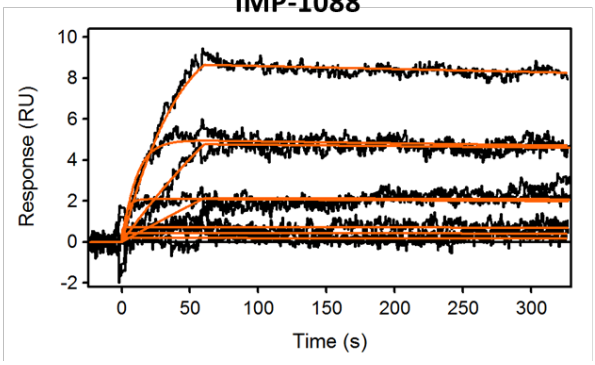
(IMP-917) to out-of-plane (IMP-1088) with respect to the phenyl ring causes a 0.6 Å displacement in the pocket, and concomitant reorientation of His298. Residue numbers are those of HsNMT1. (**e**) Structure of MRT00057965.

Supplementary Figure 2: Surface plasmon resonance analysis of the interaction between HsNMT1 and NMT inhibitors. Sensograms measured for (**a**) IMP-917 and (**b**) IMP-1088 were analyzed using a global fit (shown in orange) to a one-site kinetic model, and kinetic parameters (**c**) extracted. In the case of IMP-1088, R_{max} values were fitted locally for each concentration while keeping on- and off-rate fits global. The picomolar potency of IMP-1088 is driven primarily by a very low dissociation rate constant.

a



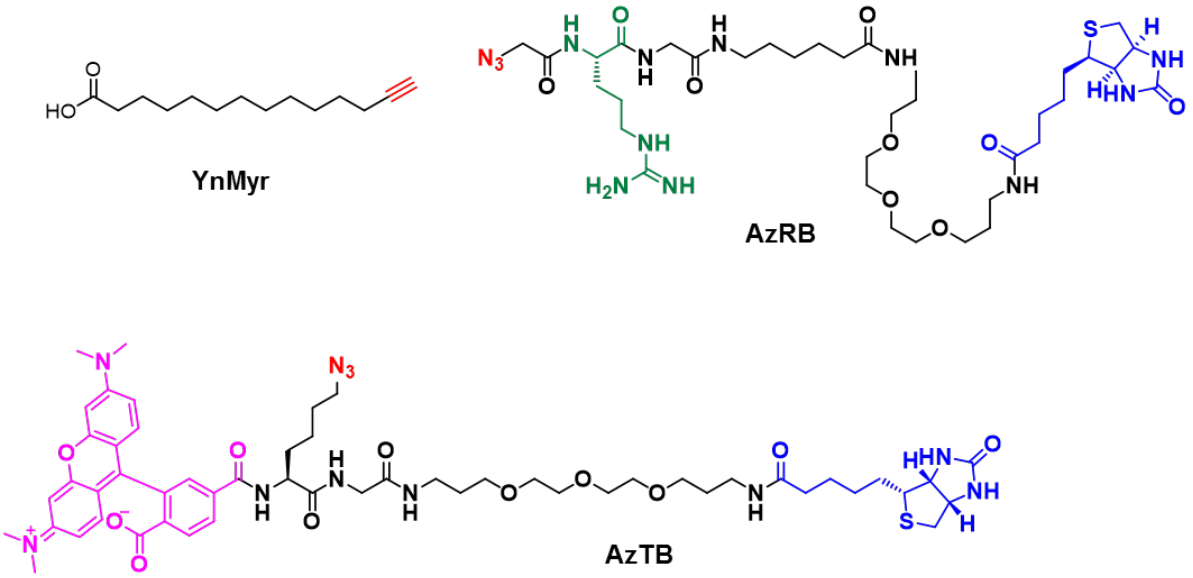
b



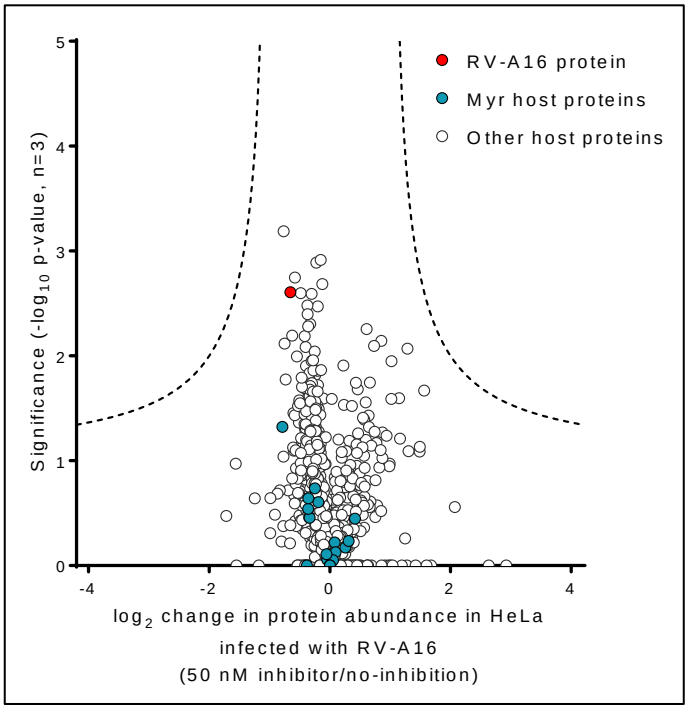
c

	IMP-917	IMP-1088
k_a [s^{-1}]	3.70 (± 0.01)*10^5	9.0 (± 0.2)*10^5
k_d [s^{-1}]	170 (±10)*10^{-4}	1.9 (± 0.1)*10^{-4}
K_D [nM]	46.0 (± 0.2)	≤0.210 (±0.010)
Residual value	0.5930	0.3201
IC_{50} [nM][a]	13.0	<1
[a] determined by biochemical NMT inhibition assay		

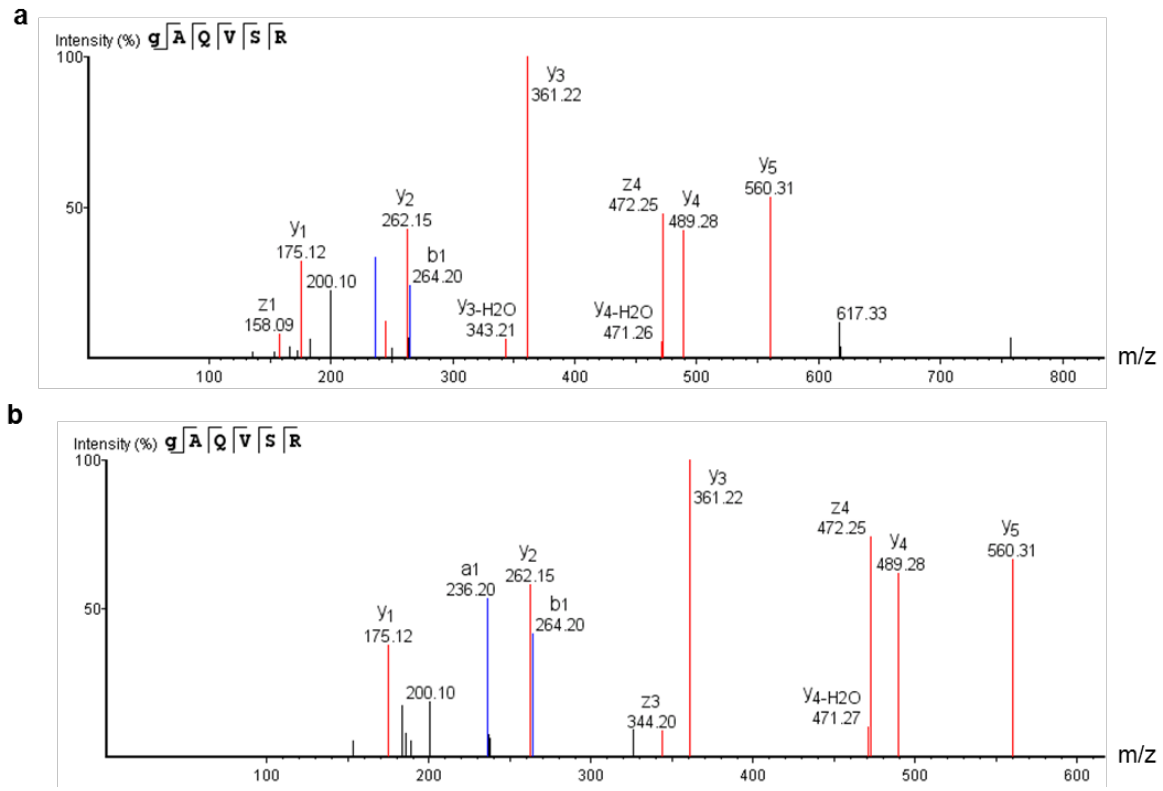
Supplementary Figure 3: Structures of chemical probes used in this study. Alkyne-tagged myristate analogue YnMyr, and capture reagents AzRB and AzTB.



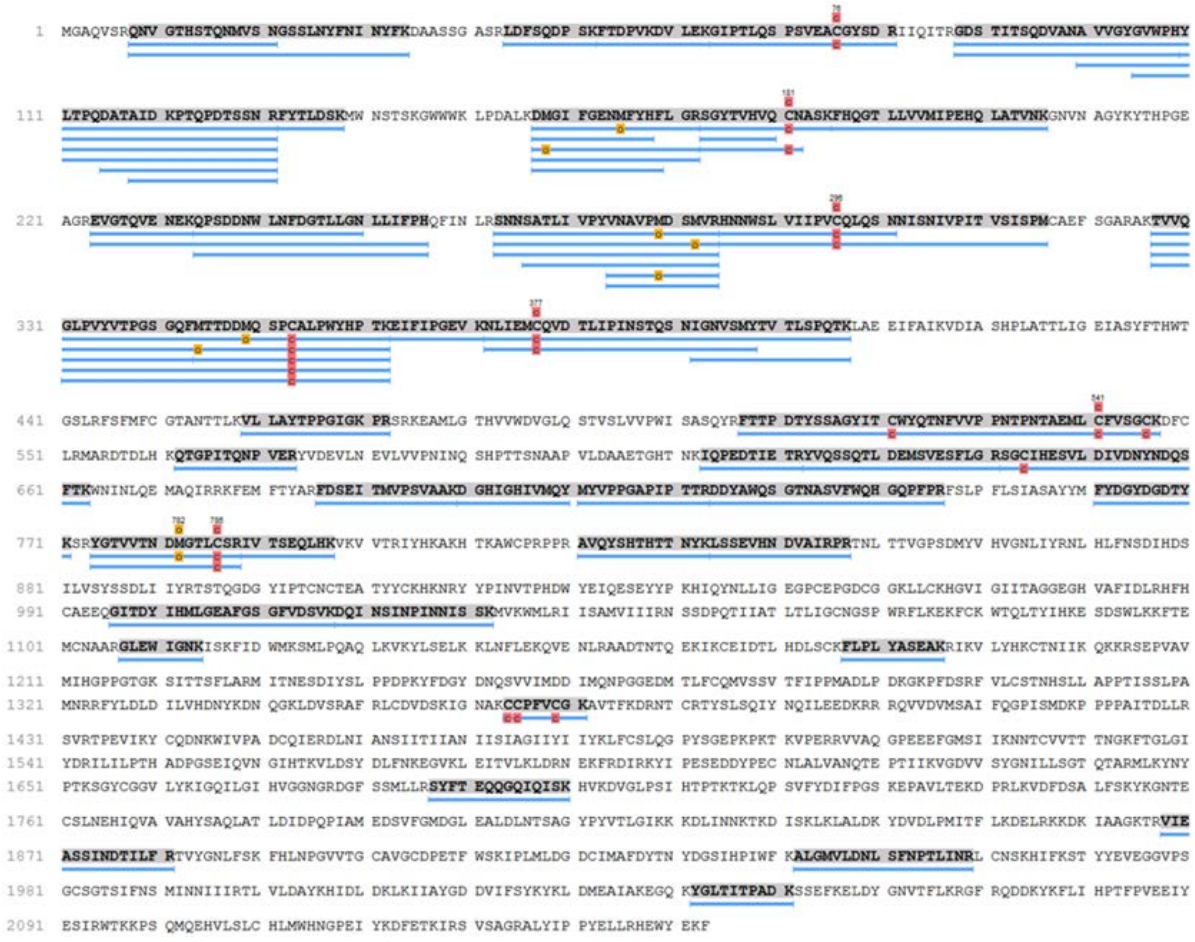
Supplementary Figure 4: Whole proteome analysis of rhinovirus RV-A16-infected HeLa cells with and without NMT inhibitor IMP-1088. Proteomes were analyzed at 6 hours post infection with rhinovirus RV-A16 (Multiplicity of Infection. MOI: 20), comparing vehicle (DMSO) treatment with IMP-1088 (50 nM). Quantification was performed by label-free protein quantification (LFQ) in MaxQuant,[1] and permutation-based false discovery rate (FDR) two sample *t*-tests were used to compare inhibition with control samples. A total of 1223 proteins were quantified, none of which show a significant change upon IMP-1088 treatment, indicating that changes observed in Fig. 3d are due to loss of myristoylation and not to changes in protein abundance. Note that a smaller number of myristoylated proteins are detectable at the whole proteome level due to lack of enrichment through YnMyr labeling. Dashed lines: *t*-test significance cut off; red: rhinovirus RV-A16 proteins; blue: known co-translationally myristoylated host proteins;[2] white: other host proteins.



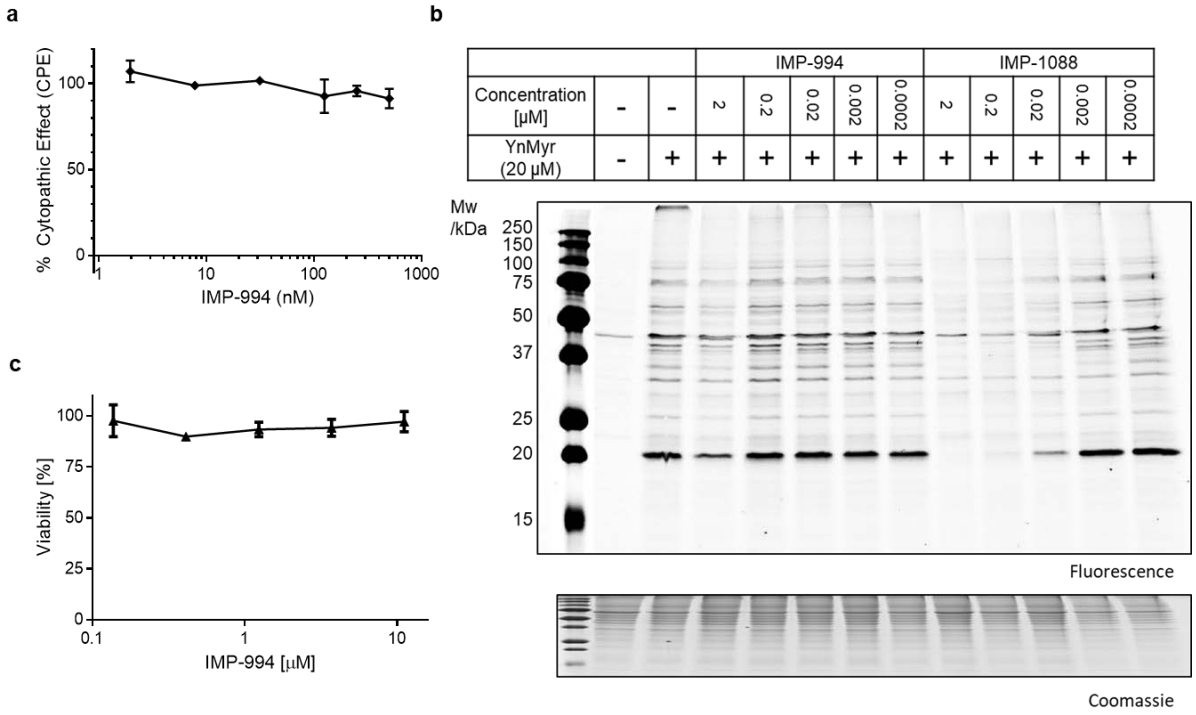
Supplementary Figure 5: MS/MS spectrum of rhinovirus RV-A16 polyprotein N-terminal peptide. Representative MS/MS spectra for the N-terminal YnMyr-tagged tryptic peptide derived from rhinovirus RV-A16 polyprotein; N-terminal tryptic peptide sequence is GAQVSR. Spectra shown in (**a**) and (**b**) are from two separate experiments, and are representative of multiple analyses.



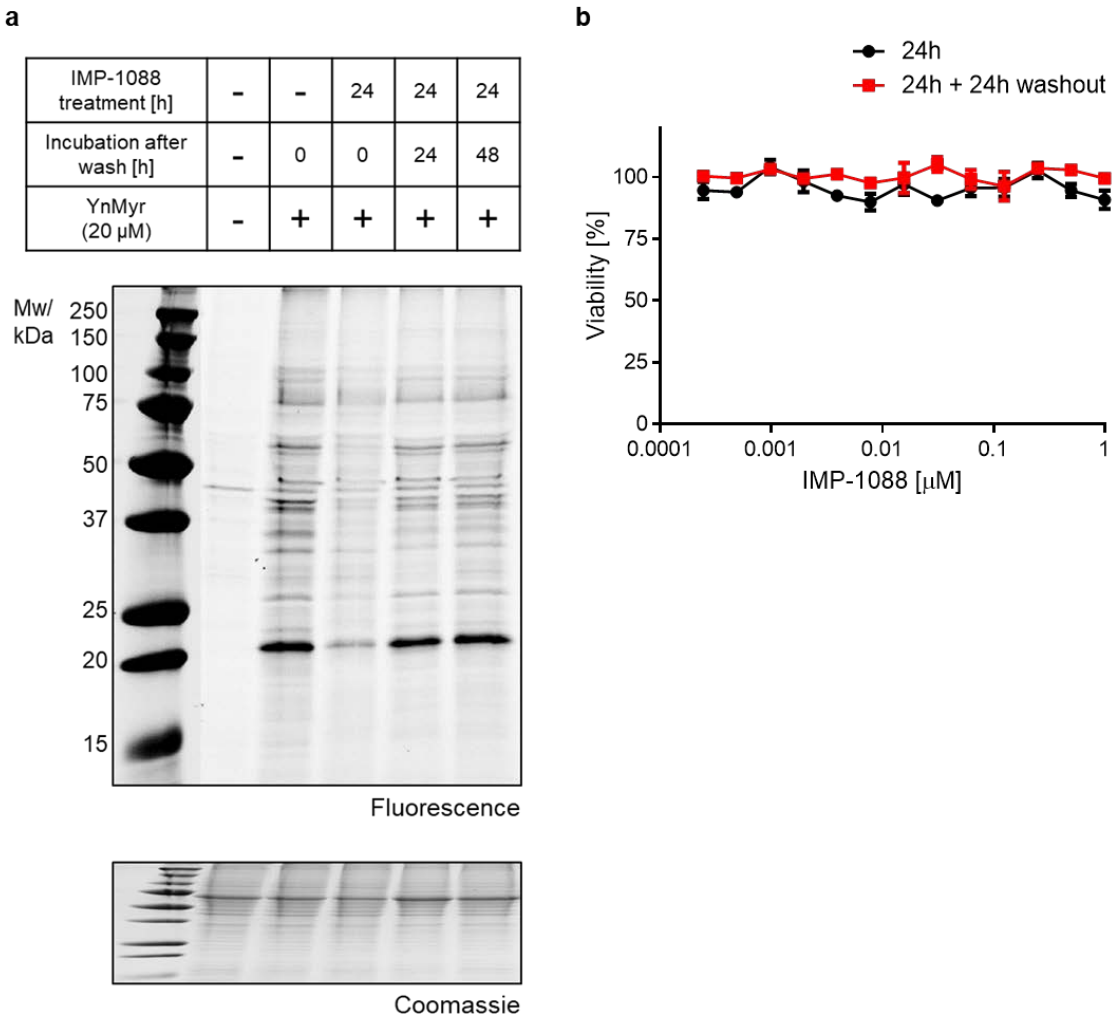
Supplementary Figure 6: Peptides identified by proteomic analysis of myristoylated proteins in rhinovirus RV-A16-infected HeLa cells labeled with YnMyr, mapped on the RV-A16 polyprotein sequence. Blue bars correspond to peptides identified by MS/MS analysis using PEAKS (see Supplementary Methods). Modified amino acids identified include C: Carbamidomethylation (+57.02 Da); O: Oxidation (Met, +15.99 Da).



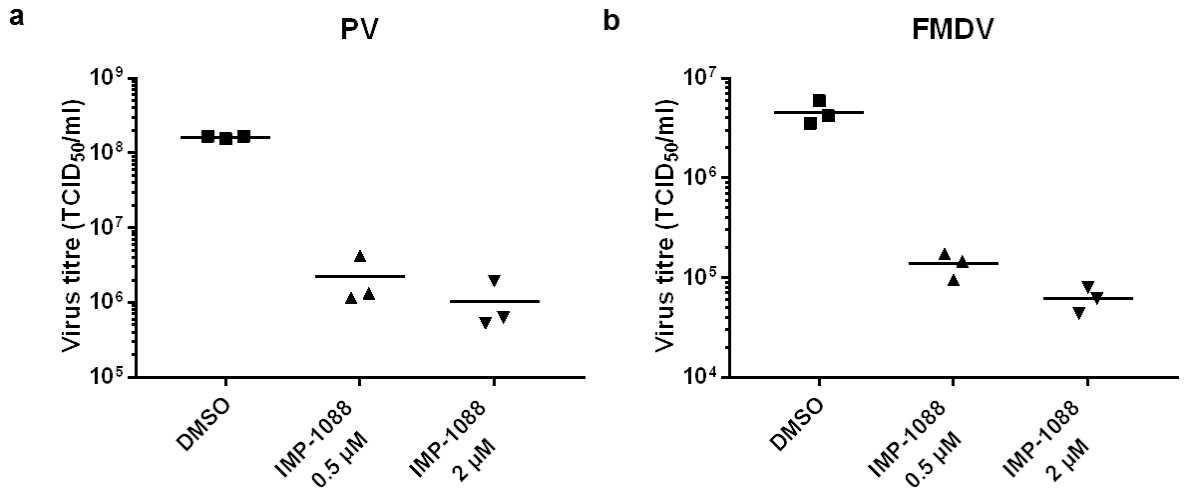
Supplementary Figure 7: In-cell antiviral, NMT activity and viability assays for IMP-994. (**a**) IMP-994 (HsNMT1 IC_{50} = 9 µM) has no impact on the cytopathic effect of infection of HeLa cells with RV-A16. (**b**) IMP-994 has negligible impact on NMT activity in HeLa cells. Cells were treated with the corresponding concentration of inhibitor or DMSO for 1 h, followed by YnMyr (20 µM), and cells incubated for 24 h. Lysates were subjected to ligation with AzTB, and analyzed by in-gel fluorescence. IMP-994 has no effect at sub-micromolar concentrations, whereas IMP-1088 substantially impacts NMT activity at 20 nM, in line with their respective NMT inhibitory activities. (**c**) IMP-994 has no impact on cell viability of uninfected HeLa cells, over a 72-hour exposure.



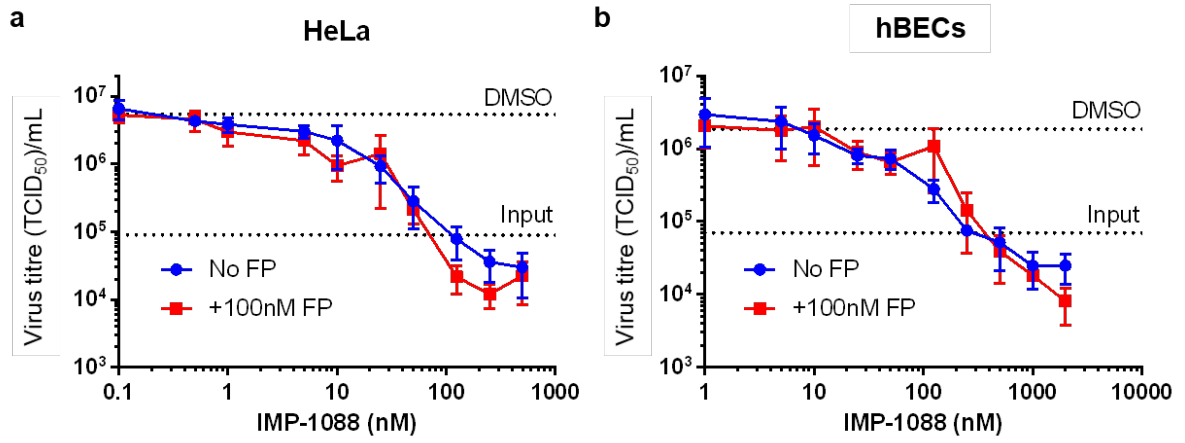
Supplementary Figure 8: Recovery of cells following wash-out of IMP-1088. **a**) HeLa cells were treated with 50 nM IMP-1088 for 1h, whereupon YnMyr (20 µM) was added and cells were incubated for 24h. Subsequently, cells were washed with DMEM to remove free IMP-1088, and cells incubated with fresh media containing YnMyr (20 µM) for a further 24-48h. Lysates were subjected to ligation with AzTB and analyzed by in-gel fluorescence and consistent gel loading confirmed by Coomassie stain. YnMyr labeling of myristoylated proteins in HeLa cells was significantly reduced following 24 hours inhibition, but had recovered 24 hours after IMP-1088 washout, and was maintained at 48h post-washout. **b**) HeLa cells were treated with varying concentrations of IMP-1088 (1 µM to 0.24 nM) for 24h, at which point cells were washed with DMEM and incubated with new media without inhibitor for a further 24h. Cell viability was quantified using MTS assay before (24 h) or 24h after (24h + 24h washout) media exchange, confirming no long-term effect on cell viability following IMP-1088 wash-out.



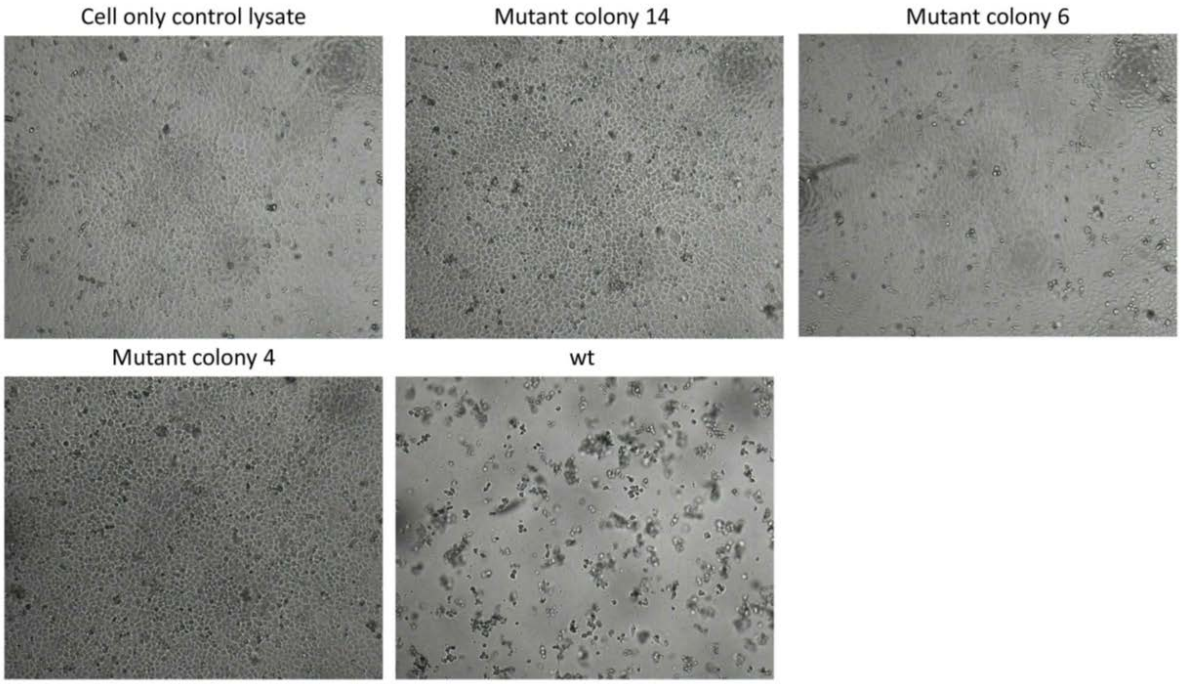
Supplementary Figure 9: IMP-1088 inhibits picornavirus infection. HeLa cells were infected with poliovirus (PV) (**a**) and BHK21 cells were infected with foot-and-mouth disease virus (FMDV) (**b**) at an MOI of 20. After 6h of replication in the presence of the indicated concentrations of IMP-1088 or the vehicle (DMSO), viral titers were determined by endpoint dilution assay ($TCID_{50}$: 50% Tissue Culture Infective Dose). The lines represent the mean of three replicates.



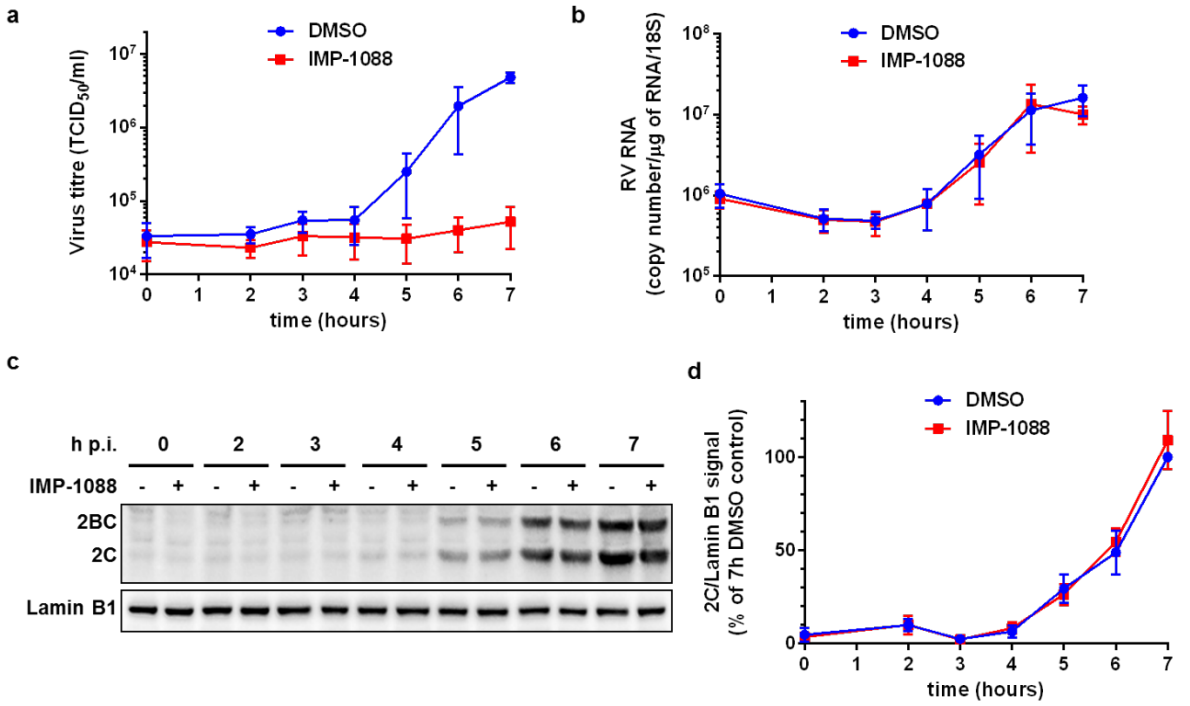
Supplementary Figure 10: **IMP-1088 is a potent inhibitor of RV infection in the presence of fluticasone propionate (FP), in HeLa cells and in human bronchial epithelial cells (hBECs)**. HeLa cells were infected with rhinovirus RV-A16 (MOI 20) for 6h (a) and primary human bronchial epithelial cells (hBECs) were infected with rhinovirus RV-A1 (MOI 5) for 7h (b), in the presence or absence of FP, and with the indicated concentrations of IMP-1088. Virus titers were determined by endpoint dilution assay. $TCID_{50}$: 50% Tissue culture Infective Dose, error bars: SEM, n=4.



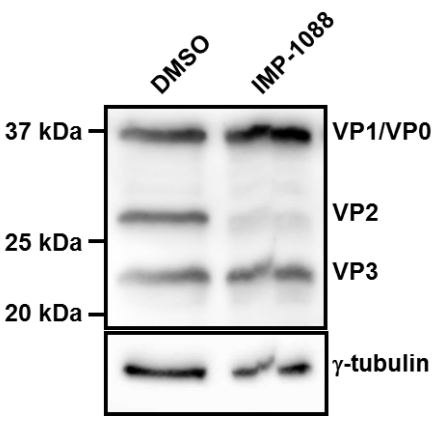
Supplementary Figure 11: RV-A16 with a non-functional myristoylation signal cannot be rescued from infectious copy plasmids. Production of infectious virus monitored by development of cytopathic effect (CPE) after transfection of BSR-T7/5 cells with RV-A16 infectious copy plasmids encoding either *wild-type* RV-A16 (wt) or myristoylation signal mutant (G2A) RV-A16 (mutant colonies 4, 6 or 14) or mock transfection (cell only control) and passage onto HeLa Ohio cells. Images were captured using a JuLi Cell Imager (Digital Bio) during the first passage on HeLa cells. Samples were passaged a further two times on HeLa cells with similar development of CPE in wt samples and no development of CPE in any of the mutant or control samples.



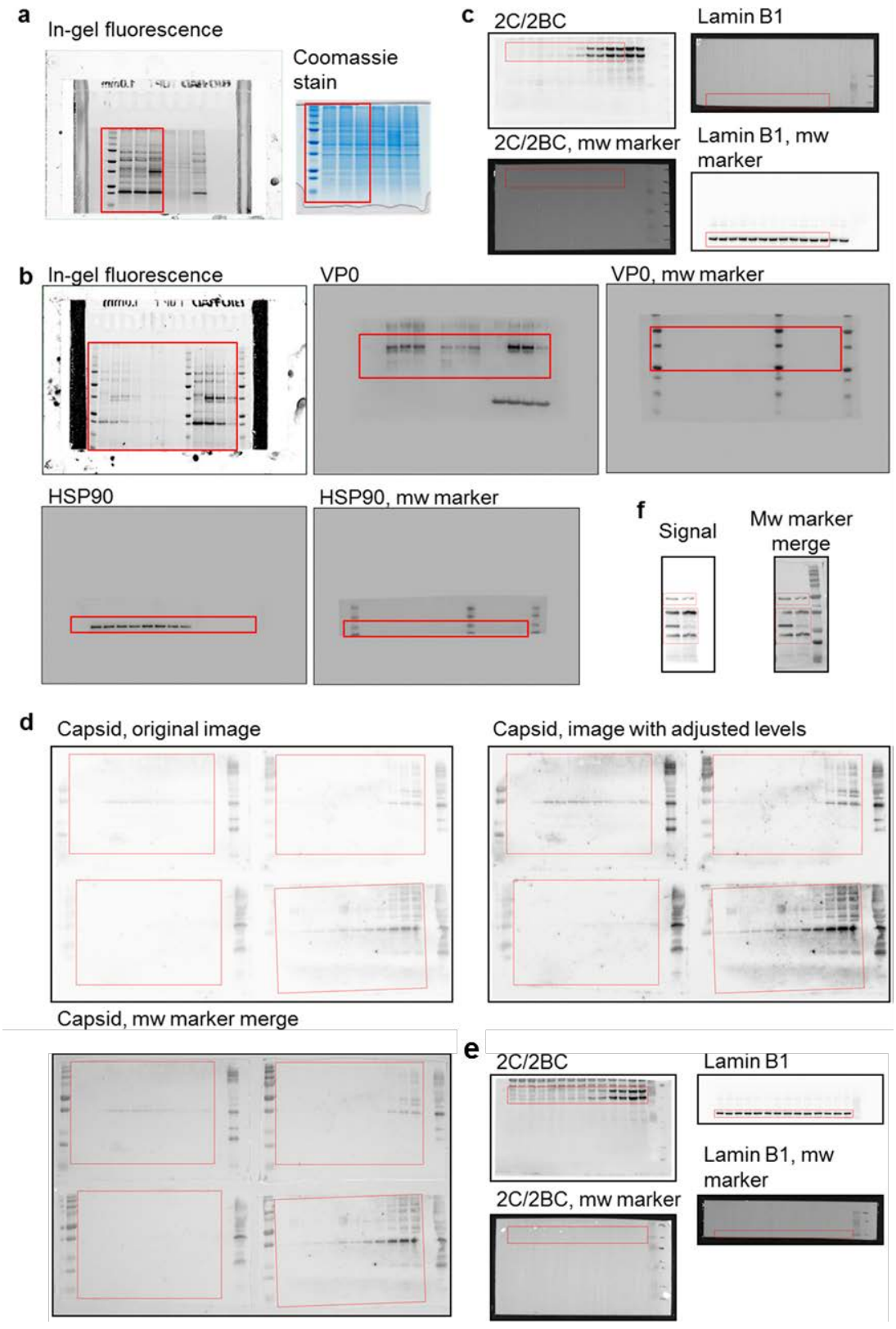
Supplementary Figure 12: IMP-1088 inhibits production of infectious rhinovirus particles in hBECs without inhibiting viral RNA or protein production. (**a**) to (**d**) Effect of IMP-1088 on rhinovirus replication kinetics in hBECs infected with RV-A1 (MOI 5) for 7 h in presence of IMP-1088 (500 nM) or DMSO (vehicle). At the indicated time points cell lysates were analyzed for virus titer by endpoint titration (a), viral RNA by quantitative PCR (b), or viral protein levels by Western blot with an antibody directed against rhinovirus nonstructural protein 2C ((c) and (d)). (c) Representative images of one of three independent repeats. (d) Quantification of signal corresponding to 2C protein, relative to Lamin B1 loading control, expressed as a percentage of 7 h DMSO control. Error bars: SEM, n=3.

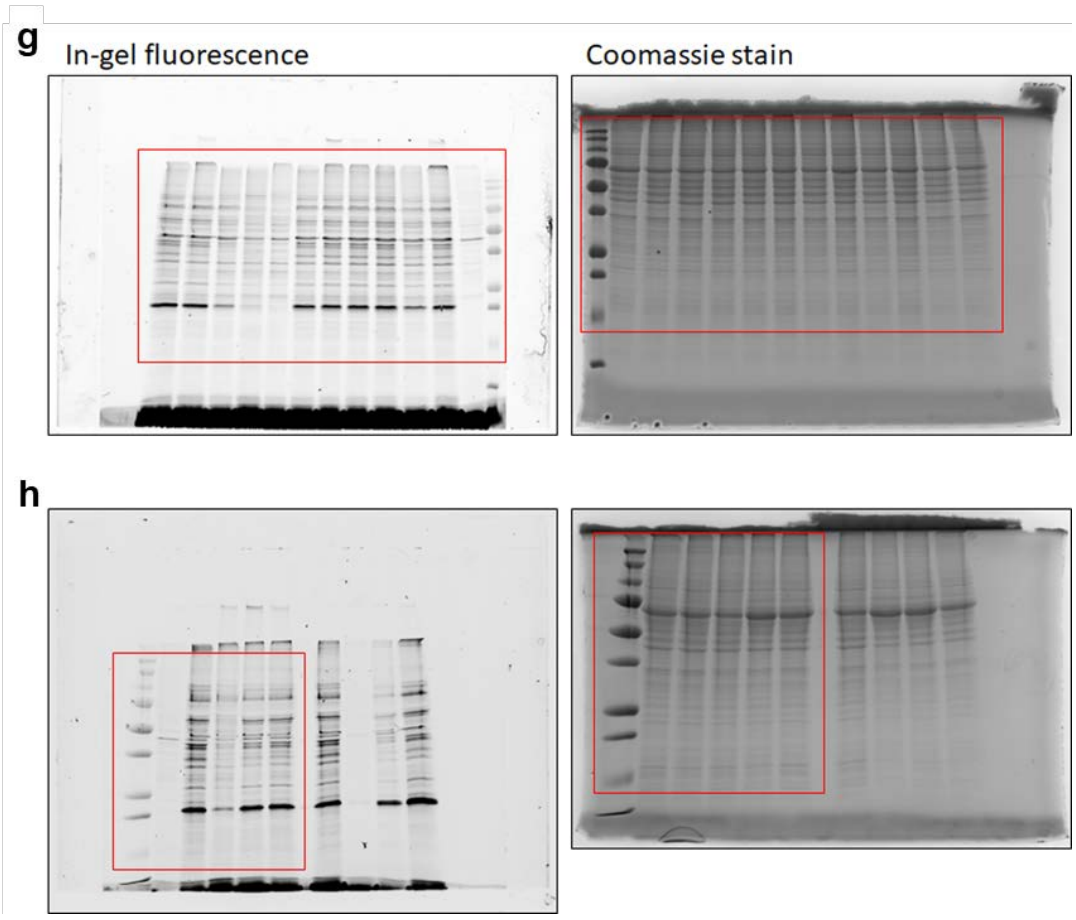


Supplementary Figure 13: NMT inhibition reduces the formation of VP2 protein in rhinovirus RV-A16-infected HeLa cells. HeLa cells were infected for 6h with rhinovirus RV-A16 (MOI 20) in the presence of IMP-1088 (500 nM) or DMSO (vehicle) and the resulting cell lysates were analyzed by Western blot with antibodies raised against whole RV-A16 capsids and anti-γ-tubulin as a loading control.



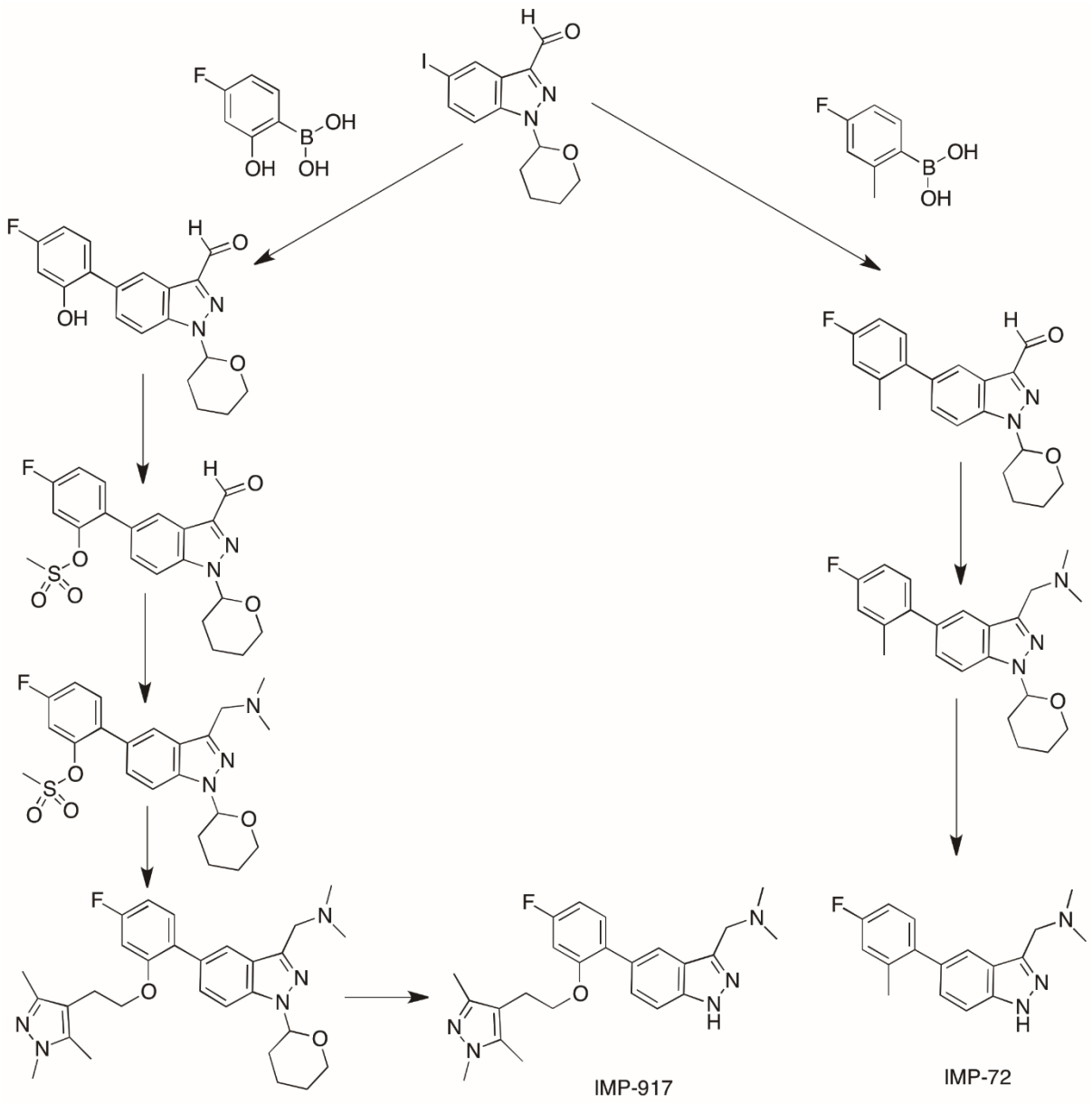
Supplementary Figure 14: Uncropped gel and blot images for main figures. (**a**) Figure panel 3c. (**b**) Figure panel 3e. (**c**) Figure panel 5c. (**d**) Figure panel 5e. (**e**) Supplementary Figure 8c. (**f**) Supplementary Figure 9. [Continued on following page] (**g**) Supplementary Figure 7. (**h**) Supplementary Figure 8.




g
In-gel fluorescence
Coomassie stain
h

Supplementary Figure 15: (**a**) synthesis of IMP-72 and IMP-917. (**b**) synthesis of IMP-994, IMP-1031 and IMP-1088. Please see Supplementary Methods for details of conditions and yields for each step.

a



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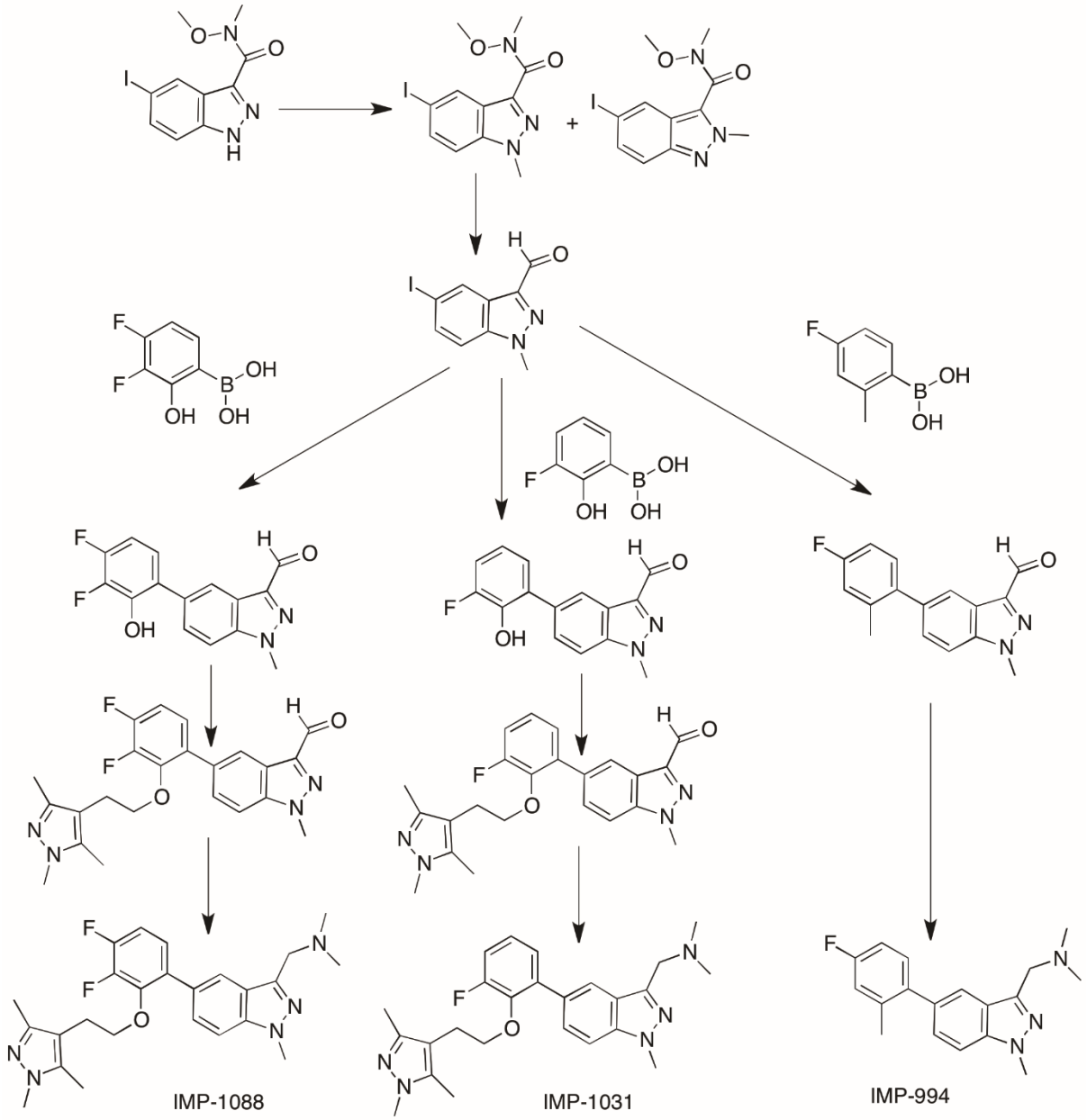


Supplementary Table 1. Crystallographic data for crystals of Plasmodium (Pv) and Human (Hs) NMT proteins in complex with co-factor and ligand.

	Pv:NHM:IMP-72	**Pv:NHM:IMP-72+IMP-358**	**Hs:MYA:IMP-917**	**Hs:MYA:IMP-1031**	**Hs:MYA:IMP-1088**
PDB accession	5O48	5O4V	5O6H	5O6J	5MU6
Data Collection					
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12$	$P2_12_12$	$P2_12_12$
Number of complexes in ASU	3	3	2	2	2
Unit cell *a*, *b*, *c* (Å)	57.56, 121.98, 178.25	57.56, 121.88, 179.05	79.14, 178.85, 58.46	79.92, 177.29, 58.30	78.73, 180.73, 58.99
Wavelength (Å) & beamline	0.9173, DLS i04-1	0.9795, DLS i04	0.9200, DLS i04-1	0.9795, DLS i04	0.9282, DLS i04-1
Resolution range (Å)	52-1.69 (1.72-1.69)	89-1.70 (1.73-1.70)	55-1.29 (1.31-1.29)	80-1.45 (1.47-1.45)	72-1.88 (1.93-1.88)
R_{merge}	0.134 (0.878)	0.171 (1.399)	0.062 (0.651)	0.067 (0.814)	0.254 (1.654)
R_{merge} in top intensity bin	0.055	0.067	0.025	0.028	0.044
R_{meas}	0.159 (1.036)	0.204 (1.671)	0.074 (0.885)	0.067 (0.814)	0.274 (1.808)
R_{pim}	0.083 (0.537)	0.111 (0.900)	0.039 (0.595)	0.043 (0.557)	0.101 (0.716)
Number of total reflections	941674 (48222)	865828 (43410)	1288618 (31879)	948412 (40936)	503120 (31793)
Number of unique reflections	139707 (6878)	139052 (6801)	206823 (9516)	145112 (6950)	69366 (5068)
Mean ([I]/SD[I])	9.7 (2.1)	6.4 (1.1)	14.7 (1.6)	15.2 (2.1)	7.8 (1.2)
$CC_{1/2}$	0.995 (0.650)	0.986 (0.360)	0.999 (0.697)	0.998 (0.766)	0.991 (0.501)
Completeness (%)	99.2 (99.9)	99.9 (100)	99.1 (93.1)	98.6 (96.6)	99.9 (99.9)
Multiplicity	6.7 (7.0)	6.2 (6.4)	6.2 (3.4)	6.5 (5.9)	7.3 (6.3)
Wilson plot B ($Å^2$)	7.5	12.8	9.7	12.6	13.1
Refinement					
Total number of atoms	11694	11521	7829	7681	6941
Protein	9882	9860	6559	6639	6267
Co-factor	192	192	126	126	126
Ligand	63	114	62	64	66
R_{work}	17.4	20.7	16.4	21.3	20.4
R_{free}	20.0	25.7	19.4	24.4	24.7
Number of reflections for R_{free}	7002	6874	10300	7227	3449
<B > ($Å^2$)	15.3	20.6	17.1	20.1	17.8
rms bond length deviation (Å)	0.026	0.018	0.028	0.024	0.008
rms angle deviation (°)	2.291	1.883	2.498	2.351	1.242

$R_{merge} = \Sigma(I_{hl} - < I_h >)/\Sigma < I_h >$

$R_{meas} = \Sigma\sqrt{(n_h/n_h - 1)}(I_{hl} - < I_h >)/\Sigma < I_h >$

$R_{pim} = \Sigma\sqrt{(1/n_h - 1)}(I_{hl} - < I_h >)/\Sigma < I_h >$

ASU, asymmetric unit; $CC_{1/2}$, half-data set correlation coefficient; MYA, myristoyl-CoA ; NHM, non-hydrolysable MYA analogue

Values in parentheses correspond to the highest resolution shell

Supplementary Methods

Synthesis of Chemical probes and Inhibitors

Chemical probes. YnMyr, AzTB and AzRB were prepared as described previously[3].

Compounds IMP-72, IMP-917, IMP-994, IMP-1031 and IMP-1088. See Supplementary Figure 15 for synthetic routes, experimental details and characterization data are presented below.

Compounds were analysed on an LC-MS system equipped with both an XBridge prep C18 5 µm, 19 × 100 mm OBD column and an XBridge C18 5 µm, 4.6 × 100 mm column over a gradient of methanol in water (5–98% over 12 minutes then 98% methanol for 3 minutes), both containing 0.1% formic acid. ^{1}H and ^{13}C NMR spectra were recorded on 400 MHz and 101 MHz respectively Bruker AV instruments at room temperature and were referenced to residual solvent signals. Data are presented as follows: chemical shift in ppm, integration, multiplicity (br = broad, app = apparent, s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet) and coupling constants in Hz. Mass spectra were obtained from the Mass Spectrometry Service of Department of Chemistry, Imperial College London. Note that IMP-358 is available commercially, and its synthesis is not detailed here.

IMP-72 Step 1. A solution of 5-iodo-1-(tetrahydro-2*H*-pyran-2-yl)-1*H*-indazole-3-carbaldehyde (356 mg, 1 mmol) was dissolved in DMF (5 mL) and treated with 4-fluoro-2-methylphenylboronic acid (154mg, 1mmol) and palladium acetate (3mg), followed by a solution of cesium carbonate (650mg, 2mmol) in water (5mL). The reaction mixture was heated under reflux for 1h, cooled to room temperature, and partitioned between EtOAc (20 mL) and water (20 mL). The organic phase was dried over Na_2SO_4, concentrated under reduced pressure and the crude product purified by flash column chromatography by elution with DCM to give 5-(4-fluoro-2-methylphenyl)-1-(tetrahydro-2H-pyran-2-yl)-1H-indazole-3-carbaldehyde as a colourless oil (340 mg, 100%). ^{1}H NMR (400 MHz, Chloroform-d) δ 10.30 (s, 1H), 8.24 (d, *J* = 2.2 Hz, 1H), 7.74 (d, *J* = 8.6 Hz, 1H), 7.44 (dd, *J* = 8.6, 1.7 Hz, 1H), 7.24 (dd, *J* = 8.4, 5.9 Hz, 1H), 7.02 (dd, *J* = 9.7, 2.7 Hz, 1H), 6.98 (td, *J* = 8.5, 2.8 Hz, 1H), 6.09 – 5.71 (m, 1H), 4.09 (ddd, *J* = 10.3, 4.0, 2.6 Hz, 1H), 3.90 – 3.76 (m, 1H), 2.64 (dtd, *J* = 11.4, 9.0, 5.1 Hz, 1H), 2.28 (s, 3H), 2.25 – 2.16 (m, 2H), 1.94 – 1.70 (m, 3H).

IMP-72 Step 2. A solution of 5-(4-fluoro-2-methylphenyl)-1-(tetrahydro-2H-pyran-2-yl)-1H-indazole-3-carbaldehyde (100 mg, 0.3 mmol) in DCE (4 mL) was treated with a solution of dimethylamine in THF (2M, 150µL, 0.3mmol). The solution was stirred at room temperature for 5min before addition of solid sodium triacetoxyborohydride (380 mg, 1.8 mmol). The reaction mixture was stirred at room temperature for 18h before being quenched with saturated sodium bicarbonate solution (5 mL). DCM (20 mL) was added and the layers were

separated. The organic extract was dried (Na_2SO_4) and concentrated under reduced pressure to give 1-(5-(4-fluoro-2-methylphenyl)-1-(tetrahydro-2*H*-pyran-2-yl)-1*H*-indazol-3-yl)-*N*,*N*-dimethylmethanamine as a colourless oil, which was taken on to the final step without further purification. ^{1}H NMR (400 MHz, Chloroform-d) δ 7.74 (d, *J* = 1.3 Hz, 1H), 7.62 (d, *J* = 8.5 Hz, 1H), 7.35 (dd, *J* = 8.7, 1.6 Hz, 1H), 7.24 (dd, *J* = 8.4, 6.0 Hz, 1H), 7.01 (dd, *J* = 10.0, 2.7 Hz, 1H), 6.96 (td, *J* = 8.5, 2.8 Hz, 1H), 5.75 (dd, *J* = 9.7, 2.6 Hz, 1H), 4.17 – 4.07 (m, 1H), 3.97 (d, *J* = 3.4 Hz, 2H), 3.85 – 3.78 (m, 1H), 2.68 – 2.57 (m, 1H), 2.40 (s, 6H), 2.27 (s, 3H), 2.22 – 2.15 (m, 1H), 2.11 (d, *J* = 3.0 Hz, 1H), 1.81 (td, *J* = 9.8, 7.9, 3.1 Hz, 2H), 1.74 – 1.64 (m, 1H).

IMP-72 Step 3. The THP protected indazole was dissolved in a 1:1 mixture of THF and methanol (2 mL), and treated with a solution of HCl in isopropanol (6 M, 1 mL). The reaction mixture was heated to 40^{o}C overnight. All volatiles were removed under reduced pressure and the residue was partitioned between EtOAc (20mL) and saturated sodium carbonate solution (10 mL). The crude product was purified by HPLC using gradient elution (50-98%) with methanol/water /0.1% formic acid to give 1-(5-(4-fluoro-2-methylphenyl)-1H-indazol-3-yl)-N,N-dimethylmethanamine formate salt (70.1 mg, yield over 2 steps 71%). ^{1}H NMR (400 MHz, Chloroform-d) δ 12.25 (s, 1H), 8.67 (s, 1H), 7.65 (d, J = 1.0 Hz, 1H), 7.54 (d, J = 8.7 Hz, 1H), 7.28 (d, J = 8.7 Hz, 1H), 7.18 (dd, J = 8.4, 6.0 Hz, 1H), 6.97 (dd, J = 9.8, 2.7 Hz, 1H), 6.91 (td, J = 8.4, 2.8 Hz, 1H), 4.43 (s, 2H), 2.70 (s, 6H), 2.22 (s, 3H); ^{13}C NMR (101 MHz, Chloroform-d) δ 168.56, 161.96 (d, J = 245.0 Hz), 140.48, 137.88 (d, J = 7.5 Hz), 137.70, 136.29, 134.52, 131.50 (d, J = 8.1 Hz), 128.77, 123.05, 119.41, 116.79 (d, J = 20.9 Hz), 112.57 (d, J = 20.8 Hz), 110.43, 52.29, 42.51, 20.68: ESI HRMS, found 284.1569 ($C_{17}H_{19}FN_3$, $[M + H]^+$, requires 284.1563).

IMP-917 Step 1. A solution of 5-iodo-1-(tetrahydro-2*H*-pyran-2-yl)-1*H*-indazole-3-carbaldehyde (570 mg, 1.6 mmol) was dissolved in dioxane (10mL) and treated with 4-fluoro-2-hydroxybenzene boronic acid (210mg, 2,0mmol) and tetrakis(triphenylphosphine) palladium(0) (20mg), followed by a solution of potassium phosphate (690 g, 3.2 mmol) in water (3 mL). The reaction mixture was heated under reflux for 2h, cooled to room temperature and evaporated under reduced pressure. The residue was partitioned between EtOAc (20 mL) and saturated sodium bicarbonate solution (20 mL). The organic phase was dried over Na_2SO_4, concentrated under reduced pressure and the crude product purified by flash column chromatography by elution with DCM/EtOAc (100:0, then 95:5) to give 5-(4-fluoro-2-hydroxyphenyl)-1-(tetrahydro-2*H*-pyran-2-yl)-1*H*-indazole-3-carbaldehyde as a colourless solid (480 mg, 90%); mp 171-173^{o}C. ^{1}H NMR (400 MHz, Chloroform-d) 10.22 (s, 1H), 8.35 (d, *J* = 1.5 Hz, 1H), 7.79 (d, *J* = 8.7 Hz, 1H), 7.59 – 7.52 (m, 1H), 7.27 – 7.20 (m, 1H), 6.79 – 6.69 (m, 2H), 5.88 (dd, *J* = 8.8, 2.7 Hz, 1H), 5.71 (d, *J* = 1.2 Hz, 1H), 4.19 – 3.96 (m, 1H), 3.82 (ddd, *J* = 11.5, 9.2, 3.4 Hz, 1H), 2.59 (dtd, *J* = 11.2, 8.9, 5.2 Hz, 1H), 2.19 (dddt, *J* = 20.8, 12.7,

5.8, 3.6 Hz, 2H), 1.92 – 1.63 (m, 3H). ^{13}C NMR (101 MHz, Chloroform-d) 187.21, 163.14 (d, *J* = 246.2 Hz), 153.83 (d, *J* = 12.0 Hz), 143.27, 140.15, 132.97, 131.58 (d, *J* = 10.1 Hz), 129.26, 123.96, 122.94, 122.50, 111.64, 107.86 (d, *J* = 21.4 Hz), 103.52 (d, *J* = 25.2 Hz), 86.39, 67.41, 29.18, 24.93, 21.94

IMP-917 Step 2. A solution of 5-(4-fluoro-2-hydroxyphenyl)-1-(tetrahydro-2*H*-pyran-2-yl)-1*H*-indazole-3-carbaldehyde (480 mg, 1.4 mmol) in THF (20 mL) was cooled to 0^{o}C and treated with triethylamine (418 µL, 3.0 mmol) followed by methanesulfonyl chloride (232 µL, 3.0 mmol). The reaction mixture was allowed to warm to room temperature and was stirred for 18h, before being quenched with saturated sodium bicarbonate solution (10 mL). EtOAc (50 mL) was added and the layers were separated. The aqueous phase was further extracted with EtOAc (20 mL) and the combined organic extracts were dried (Na_2SO_4) and concentrated under reduced pressure to give 5-fluoro-2-(3-formyl-1-(tetrahydro-2*H*-pyran-2-yl)-1*H*-indazol-5-yl)phenyl methanesulfonate as a colourless oil (585 mg, ~100%). ^{1}H NMR (400 MHz, Chloroform-d) 10.28 (s, 1H), 8.40 (d, *J* = 1.6 Hz, 1H), 7.79 (d, *J* = 8.7 Hz, 1H), 7.67 (dd, *J* = 8.6, 1.6 Hz, 1H), 7.52 (dd, *J* = 8.6, 6.2 Hz, 1H), 7.31 (dd, *J* = 8.9, 2.6 Hz, 1H), 7.17 (td, *J* = 8.2, 2.6 Hz, 1H), 5.89 (dd, *J* = 9.1, 2.6 Hz, 1H), 4.07 (dd, *J* = 8.8, 5.3 Hz, 1H), 3.83 (ddd, *J* = 12.6, 9.6, 3.3 Hz, 1H), 2.67 (s, 3H), 2.60 (dtd, *J* = 13.3, 9.3, 5.4 Hz, 1H), 2.21 (tt, *J* = 12.5, 4.0 Hz, 2H), 1.93 – 1.69 (m, 3H). ^{13}C NMR (101 MHz, Chloroform-d) 187.10, 161.90 (d, *J* = 250.0 Hz), 146.43 (d, *J* = 11.0 Hz), 143.36, 140.14, 132.59 (d, *J* = 9.4 Hz), 132.38, 130.73, 129.24, 122.46, 122.40, 114.81 (d, *J* = 20.9 Hz), 111.25 (d, *J* = 23.5 Hz), 86.29, 67.50, 38.06, 29.19, 24.89, 21.99.

IMP-917 Step 3. A solution of 5-fluoro-2-(3-formyl-1-(tetrahydro-2*H*-pyran-2-yl)-1*H*-indazol-5-yl)phenyl methanesulfonate (1.20 g, 3.3 mmol) in DCE (20 mL) was treated with a solution of dimethylamine in THF (2 M, 5.0 mL, 9.9 mmol), followed by glacial acetic acid (1200 µL, 19.8mmol). The solution was stirred at room temperature for 10min before addition of solid sodium triacetoxyborohydride (2.10 g, 9.9 mmol). The reaction mixture was stirred at room temperature for 18h before being quenched with sodium carbonate solution (2 M, 20 mL). DCM (20 mL) was added and the layers were separated. The aqueous phase was further extracted with DCM (10 mL) and the combined organic extracts were dried (Na_2SO_4) and concentrated under reduced pressure. The crude product was purified by column chromatography by elution with EtOAc/ diethylamine (95:5), then triturated with cyclohexane to give 2-(3-((dimethylamino)methyl)-1-(tetrahydro-2*H*-pyran-2-yl)-1*H*-indazol-5-yl)-5-fluorophenyl methanesulfonate as a colourless solid (880 mg, 60%). mp 148-150^{o}C. ^{1}H NMR (400 MHz, Chloroform-d) 8.00 (d, *J* = 1.3 Hz, 1H), 7.67 (d, *J* = 8.6 Hz, 1H), 7.56 – 7.51 (m, 1H), 7.52 – 7.46 (m, 1H), 7.34 – 7.27 (m, 1H), 7.16 (td, *J* = 8.2, 2.6 Hz, 1H), 5.73 (dd, *J* = 9.9, 2.6 Hz, 1H), 4.19 – 4.08 (m, 1H), 3.86 (d, *J* = 2.6 Hz, 2H), 3.80 (td, *J* = 10.9, 2.5 Hz, 1H), 2.63

(s, 3H), 2.60 – 2.55 (m, 1H), 2.33 (s, 6H), 2.19 (dd, *J* = 9.4, 4.6 Hz, 1H), 2.15 – 2.05 (m, 1H), 1.81 (ddd, *J* = 11.6, 7.7, 3.1 Hz, 2H), 1.75 – 1.64 (m, 1H).

IMP-917 Step 4. A solution of the appropriate 2-(3-((dimethylamino)methyl)-1-(tetrahydro-2*H*-pyran-2-yl)-1*H*-indazol-5-yl)-5-fluorophenyl methanesulfonate (97 mg, 0.21 mmol) in dry acetonitrile (1.5 mL) was transferred to a microwave vial and was treated with a solution of 2-(1,3,5-trimethyl-1H-pyrazol-4-yl)ethanol (65 mg, 0.42 mmol) followed by solid cesium carbonate (68mg, 0.21mmol). The vial was sealed vial and was heated under microwave irradiation to 140°C for 20min. The reaction mixture was partitioned between EtOAc (20 mL) and saturated sodium carbonate solution (10 mL). The organic phase was dried over Na_2SO_4, concentrated under reduced pressure and the crude product purified by flash column chromatography by elution with EtOAc followed by EtOAc /diethylamine (95:5) to give 1-(5-(4-fluoro-2-(2-(1,3,5-trimethyl-1*H*-pyrazol-4-yl)ethoxy)phenyl)-1-(tetrahydro-2*H*-pyran-2-yl)-1*H*-indazol-3-yl)-*N*,*N*-dimethylmethanamine, which was taken on to Step 5 without further purification ^{1}H NMR (400 MHz, Chloroform-d) 7.91 (d, *J* = 1.3 Hz, 1H), 7.56 (d, *J* = 8.8 Hz, 1H), 7.52 – 7.45 (m, 1H), 7.33 – 7.25 (m, 1H), 6.74 (td, *J* = 8.3, 2.5 Hz, 1H), 6.68 (dd, *J* = 11.0, 2.4 Hz, 1H), 5.71 (t, *J* = 2.8 Hz, 1H), 4.16 – 4.07 (m, 1H), 3.93 (t, *J* = 7.3 Hz, 2H), 3.82 – 3.74 (m, 1H), 3.72 (d, *J* = 1.3 Hz, 2H), 3.65 (s, 3H), 2.75 (t, *J* = 7.2 Hz, 2H), 2.57 (s, 1H), 2.32 (s, 6H), 2.18 (d, *J* = 8.0 Hz, 1H), 2.11 (s, 3H), 2.06 (d, *J* = 2.8 Hz, 1H), 1.90 (s, 3H), 1.79 (td, *J* = 9.3, 5.2 Hz, 2H), 1.72 – 1.64 (m, 1H).

IMP-917 Step 5. Crude 1-(5-(4-fluoro-2-(2-(1,3,5-trimethyl-1*H*-pyrazol-4-yl)ethoxy)phenyl)-1-(tetrahydro-2*H*-pyran-2-yl)-1*H*-indazol-3-yl)-*N*,*N*-dimethylmethanamine was dissolved in a 1:1 mixture of THF and methanol (2mL), and treated with a solution of HCl in isopropanol (6 M, 1 mL). The reaction mixture was heated to 40°C for 6h. All volatiles were removed under reduced pressure and the residue was partitioned between EtOAc (20 mL) and saturated sodium carbonate solution (10 mL). The crude product was purified by HPLC using gradient elution (5-98%) with methanol/water/0.1% formic acid to give 1-(5-(4-fluoro-2-(2-(1,3,5-trimethyl-1*H*-pyrazol-4-yl)ethoxy)phenyl)-1*H*-indazol-3-yl)-*N*,*N*-dimethylmethanamine formate salt as a colourless amorphous solid (19mg; yield over 2 steps 19%). LC-MS R_t = 11.1min, $[M + H]^+$ 422; ^{1}H NMR (400 MHz, methanol-d_4) δ 8.50 (s, 1H), 7.83 (s, 1H), 7.56 (d, *J* = 8.7 Hz, 1H), 7.43 (dd, *J* = 8.9, 1.6 Hz, 1H), 7.32 (dd, *J* = 8.4, 6.7 Hz, 1H), 6.89 (dd, *J* = 11.2, 2.4 Hz, 1H), 6.78 (td, *J* = 8.3, 2.4 Hz, 1H), 4.60 (s, 2H), 4.05 (t, *J* = 6.3 Hz, 2H), 3.57 (s, 3H), 2.87 (s, 6H), 2.74 (t, *J* = 6.3 Hz, 2H), 1.89 (s, 6H); ^{13}C NMR (101 MHz, methanol-d_4) δ 168.12, 163.04 (d, *J* = 244.1 Hz), 157.09 (d, *J* = 9.8 Hz), 145.57, 140.42, 137.69, 136.01, 132.09, 131.63 (d, *J* = 9.8 Hz), 129.42, 127.20, 122.47, 119.21, 112.23, 109.46, 106.66 (d, *J* = 21.7 Hz), 100.25 (d, *J* = 25.9 Hz), 68.49, 52.55, 42.23, 34.23, 22.94, 10.07, 7.85: ESI HRMS, found 422.2359 ($C_{24}H_{29}FN_5O$, $[M + H]^+$, requires 422.2356).

IMP-994, IMP-1031 and IMP-1088 Step 1. A suspension of 5-iodo-N-methoxy-N-methyl-1H-indazole-3-carboxamide (1.0 g, 3.0 mmol) in DCM (20 mL) was treated with a catalytic quantity of tetrabutylammomium bromide and a 50% solution of potassium hydroxide in water (20 mL). The biphasic mixture was cooled to 0°C before dropwise addition of iodomethane (204 μL, 4.4 mmol). The mixture was stirred vigorously and allowed to warm to room temperature overnight. The layers were separated and the aqueous layer re-extracted with DCM (10 mL). The organic phase was dried over Na_2SO_4, concentrated under reduced pressure and the crude product purified by flash column chromatography by elution with DCM/EtOAc (85:15, then 80:20) to separate the product regioisomers. The major product was eluted first and was assigned as 5-iodo-N-methoxy-N,1-dimethyl-1H-indazole-3-carboxamide by NMR (800 mg, 77%). mp 128-130 °C. ^{1}H NMR (400 MHz, Chloroform-d) δ 8.64 (d, *J* = 1.7 Hz, 1H), 7.68 (dd, *J* = 8.8, 1.5 Hz, 1H), 7.22 (d, *J* = 8.8 Hz, 1H), 4.13 (s, 3H), 3.86 (s, 3H), 3.54 (s, 3H); ^{13}C NMR (101 MHz, Chloroform-d) δ 162.88, 139.36, 135.62, 135.06, 131.63, 126.61, 110.81, 86.45, 61.72, 36.32, 34.81. Fractions containing the minor product were re-chromatographed to give a product that was assigned as 5-iodo-N-methoxy-N,2-dimethyl-2H-indazole-3-carboxamide by NMR. ^{1}H NMR (400 MHz, Chloroform-d) δ 8.10 (d, *J* = 1.7 Hz, 1H), 7.49 (dd, *J* = 9.1, 1.6 Hz, 1H), 7.45 (d, *J* = 9.0 Hz, 1H), 4.27 (s, 3H), 3.49 (s, 3H), 3.42 (s, 3H). ^{13}C NMR (101 MHz, Chloroform-d) δ 160.61, 145.89, 134.62, 129.75, 126.26, 123.29, 119.46, 88.31, 61.97, 40.32, 33.56.

IMP-994, IMP-1031 and IMP-1088 Step 2. A solution of 5-iodo-N-methoxy-N, 1-dimethyl-1H-indazole-3-carboxamide (800 mg, 2.3 mmol) in THF (5 mL) was cooled to 0°C before dropwise addition of a solution of lithium aluminium hydride in THF (2 M, 0.35 mL, 0.7 mmol). The reaction mixture was stirred for 30min then quenched by addition of EtOAc (1 mL), allowed to warm to room temperature then partitioned between EtOAc (20 mL) and potassium hydrogen sulphate solution (1 M, 20 ml). The organic phase was dried over Na_2SO_4 and concentrated under reduced pressure to give 5-iodo-1-methyl-1H-indazole-3-carbaldehyde as a pale yellow solid (600 mg, 91%) mp 134-136 °C. ^{1}H NMR (400 MHz, Chloroform-d) δ 10.14 (s, 1H), 8.61 (d, *J* = 1.5 Hz, 1H), 7.68 (dd, *J* = 8.9, 1.7 Hz, 1H), 7.21 (d, *J* = 8.9 Hz, 1H), 4.15 (s, 3H); ^{13}C NMR (101 MHz, Chloroform-d) δ 186.30, 141.69, 140.30, 135.90, 130.98, 123.96, 111.12, 88.37, 36.73

Step 3 (IMP-994). A solution of 5-iodo-1-methyl-1H-indazole-3-carbaldehyde (110 mg, 0.38 mmol) was dissolved in dioxane (5mL) and treated with 4-fluoro-2-methylphenylboronic acid (77 mg, 0.5 mmol) and tetrakis(triphenylphosphine) palladium(0) (10 mg), followed by a solution of potassium phosphate (166 mg, 0.75 mmol) in water (2 mL). The reaction mixture was heated under reflux for 4 h, cooled to room temperature and evaporated under reduced pressure. The residue was partitioned between EtOAc (20 mL) and water (20 mL). The

organic phase was dried over Na_2SO_4, concentrated under reduced pressure and the crude product purified by flash column chromatography by elution with DCM/ethylacetate (95:5) to give 5-(4-fluoro-2-methylphenyl)-1-methyl-1*H*-indazole-3-carbaldehyde as a colourless oil (81 mg, 79%). ^{1}H NMR (400 MHz , Chloroform-d, δ) 10.24 (d, *J* = 0.9 Hz, 1H), 8.26 – 8.19 (m, 1H), 7.53 (d, *J* = 8.6 Hz, 1H), 7.48 – 7.40 (m, 1H), 7.23 (dd, *J* = 8.4, 6.0 Hz, 1H), 7.00 (dd, *J* = 9.8, 2.8 Hz, 1H), 6.95 (td, *J* = 8.4, 2.8 Hz, 1H), 4.24 (s, 3H), 2.25 (s, 3H); ^{13}C NMR (101 MHz, Chloroform-d) δ 186.74, 162.11 (d, *J* = 245.9 Hz), 142.95, 140.49, 137.90 (d, *J* = 7.9 Hz), 137.32, 137.26, 131.53 (d, *J* = 7.9 Hz), 129.43, 122.27, 122.20, 116.82 (d, *J* = 20.9 Hz), 112.60 (d, *J* = 20.9 Hz), 109.10, 36.69, 20.67.

Step 3 (IMP-1088). A solution of 5-iodo-1-methyl-1H-indazole-3-carbaldehyde (1.0 g, 3.5 mmol) was dissolved in dioxane (10 mL) and treated with 3, 4-difluoro-2-hydroxybenzene boronic acid (880 mg, 5 mmol) and tetrakis(triphenylphosphine) palladium(0) (30 mg), followed by a solution of potassium phosphate (1.06 g, 5.0 mmol) in water (2 mL). The reaction mixture was heated under reflux for 1h, cooled to room temperature and evaporated under reduced pressure. The residue was partitioned between ethyl acetate/methanol (10:1, 100 mL) and brine (50 mL). The aqueous phase was re-extracted with ethyl acetate/methanol (10:1, 50 mL), and the combined organic extract was dried over Na_2SO_4, concentrated under reduced pressure and the crude product triturated from ethyl acetate to give 5-(3, 4-difluoro-2-hydroxyphenyl)-1-methyl-1H-indazole-3-carbaldehyde as an orange solid (483 mg, 48%) mp 216-218 °C. ^{1}H NMR (400 MHz, DMSO-d6) δ 10.36 (s, 1H), 10.16 (s, 1H), 8.25 (d, J = 1.5 Hz, 1H), 7.88 (d, J = 8.8 Hz, 1H), 7.69 (dd, J = 8.8, 1.6 Hz, 1H), 7.18 (ddd, J = 8.6, 6.1, 2.1 Hz, 1H), 7.05 – 6.91 (m, 1H), 4.25 (s, 3H); ^{13}C NMR (101 MHz, Chloroform-d) δ 187.15, 150.29 (dd, J = 245.0, 10.8 Hz), 142.79 (dd, J = 248.3, 11.3 Hz), 142.47 , 140.75 , 139.76 (d, J = 14.4 Hz), 133.44 , 129.52 , 127.17 , 125.88 – 124.97 (m), 121.70 , 121.40 , 111.08 , 107.71 (d, J = 16.7 Hz), 37.18 .

Step 3 (IMP-1031). A solution of 5-iodo-1-methyl-1H-indazole-3-carbaldehyde (600 mg, 1.75 mmol) was dissolved in dioxane (15 mL) and treated with 3-fluoro-2-hydroxybenzene boronic acid (355 mg, 2.3 mmol) and tetrakis(triphenylphosphine) palladium(0) (50 mg), followed by a solution of potassium phosphate (744 mg, 3.5 mmol) in water (4.5 mL). The reaction mixture was heated under reflux for 2h, cooled to room temperature and evaporated under reduced pressure. The residue was partitioned between ethyl acetate (20 mL) and saturated sodium bicarbonate solution (20 mL). The organic phase was dried over Na_2SO_4, concentrated under reduced pressure and the crude product triturated from EtOAc/hexane to give 5-(3-fluoro-2-hydroxyphenyl)-1-methyl-1H-indazole-3-carbaldehyde as an orange solid (388 mg, 82%). ^{1}H NMR (400 MHz, Chloroform-d_3) δ 10.26 (s, 1H), 8.49 (d, *J* = 2.0 Hz, 1H),

7.77 (dd, *J* = 8.8, 1.5 Hz, 1H), 7.57 (d, *J* = 8.8 Hz, 1H), 7.23 – 7.19 (m, 1H), 7.18 – 7.11 (m, 1H), 7.01 – 6.94 (m, 1H), 5.39 (d, *J* = 5.4 Hz, 1H), 4.25 (s, 3H).

Step 4 (IMP-994) A solution of 5-(4-fluoro-2-methylphenyl)-1-methyl-1H-indazole-3-carbaldehyde (27 mg, 0.1 mmol) in DCE (4mL) was treated with a solution of dimethylamine in THF (2 M, 150 µL, 0.3 mmol) followed by acetic acid (36 µL, 0.6 mmol. The solution was stirred at room temperature for 5min before addition of solid sodium triacetoxyborohydride (63mg, 0.3mmol). The reaction mixture was stirred at room temperature for 18h before being quenched with sodium carbonate solution (2 M, 5 mL). DCM (20 mL) was added and the layers were separated. The organic extract was dried (Na_2SO_4) and concentrated under reduced pressure. The crude product was purified by column chromatography by elution with EtOAc/diethylamine (95:5) to give 1-(5-(4-fluoro-2-methylphenyl)-1-methyl-1H-indazol-3-yl)-N,N-dimethylmethanamine as a colourless oil (19.0 mg, 63%). ^{1}H NMR (400 MHz, Chloroform-d) δ 7.72 (d, *J* = 1.7 Hz, 1H), 7.40 (d, *J* = 8.5 Hz, 1H), 7.33 (dd, *J* = 8.8, 1.5 Hz, 1H), 7.25 (dd, *J* = 8.4, 5.9 Hz, 1H), 7.00 (dd, *J* = 9.8, 2.8 Hz, 1H), 6.95 (td, *J* = 8.5, 2.8 Hz, 1H), 4.09 (s, 3H), 3.83 (s, 2H), 2.34 (s, 6H), 2.27 (s, 3H). ^{13}C NMR (101 MHz, Chloroform-d) δ 57.13, 155.18, 145.67, 140.22, 137.16, 136.71, 130.18, 128.35, 126.24, 123.94, 123.87, 123.55, 120.89, 115.63, 115.48, 111.38, 108.50, 54.62, 44.60, 44.44, 35.67, 35.59, 29.71, 24.44, 20.72, 11.45, 9.31, 1.03.

Step 4 (IMP-1088). A solution of 5-(3,4-difluoro-2-hydroxyphenyl)-1-methyl- 1H-indazole-3-carbaldehyde (374 mg; 1.30 mmol) and triphenylphosphine (679 mg; 2.59 mmol) in THF (7 mL) was treated with a solution of 2-(1,3,5-trimethyl-1H-pyrazol-4-yl)ethanol (399 mg, 2.59 mmol) in THF (5 mL). The reaction mixture was cooled to 0 °C before being treated with di-isopropyl azodicarboxylate (510 µL, 2.59 mmol). The reaction mixture was allowed to warm to room temperature, stirred overnight and concentrated under reduced pressure. The crude product was purified by column chromatography by gradient elution with hexane/IPA (80:20 to 70:30) to give 5-(3,4-difluoro-2-(2-(1,3,5-trimethyl-1H-pyrazol-4-yl)ethoxy)phenyl)-1-methyl-1H-indazole-3-carbaldehyde as a colorless solid (320 mg, 58%) mp 173-174 °C. ^{1}H NMR (400 MHz, Chloroform-d) δ 10.25 (s, 1H), 8.35 (d, J = 1.6 Hz, 1H), 7.59 (dd, J = 8.8, 1.5 Hz, 1H), 7.46 (d, J = 8.6 Hz, 1H), 7.12 (ddd, J = 8.3, 5.9, 2.2 Hz, 1H), 6.99 (td, J = 9.1, 7.2 Hz, 1H), 4.26 (s, 3H), 3.80 (t, J = 7.5 Hz, 2H), 3.60 (s, 3H), 2.60 (t, J = 7.5 Hz, 2H), 1.96 (s, 3H), 1.94 (s, 3H); ^{13}C NMR (101 MHz, Chloroform-d) δ 186.63, 150.88 (dd, J = 251.6, 13.4 Hz), 144.77 (dd, J = 248.3, 13.4 Hz), 143.07, 140.63, 136.86, 135.08, 133.31, 131.68, 131.30, 129.27, 124.89 (dd, J = 6.9, 3.5 Hz), 122.18, 111.64 (d, J = 17.3 Hz), 111.25 , 108.98 , 73.61 (d, J = 3.6 Hz), 36.73, 35.70, 24.38, 11.47, 9.37.

Step 4 (IMP-1031). A solution of 5-(3-fluoro-2-hydroxyphenyl)-1-methyl- 1H-indazole-3-carbaldehyde (350 mg; 1.30 mmol) and triphenylphosphine (679 mg; 2.59 mmol) in THF

(7 mL) was treated with a solution of 2-(1,3,5-trimethyl-1H-pyrazol-4-yl)ethanol (399 mg, 2.59 mmol) in THF (5 mL). The reaction mixture was cooled to 0 °C before being treated with di-isopropyl azodicarboxylate (510 µL, 2.59 mmol). The reaction mixture was allowed to warm to room temperature, stirred overnight and concentrated under reduced pressure. The crude product was purified by automated column chromatography by elution with hexane/IPA (gradient of 95:5 to 40:60) to give 5-(3,4-difluoro-2-(2-(1,3,5-trimethyl-1H-pyrazol-4-yl)ethoxy)phenyl)-1-methyl-1H-indazole-3-carbaldehyde as a colourless oil (490 mg, 93%) ^{1}H NMR (400 MHz, Chloroform-d) δ 10.27 (s, 1H), 8.40 (s, 1H), 7.68 – 7.60 (m, 1H), 7.47 (d, *J* = 8.8 Hz, 1H), 7.23 – 7.09 (m, 3H), 4.27 (s, 3H), 3.78 (t, *J* = 7.4 Hz, 2H), 3.60 (s, 3H), 2.60 (t, *J* = 7.4 Hz, 3H), 1.96 (s, 6H).

Step 5 (IMP-1088). 5-(3,4-difluoro-2-(2-(1,3,5-trimethyl-1H-pyrazol-4-yl)ethoxy)phenyl)-1-methyl-1H-indazole-3-carbaldehyde (160 mg, 0.38 mmol) was dissolved in THF (4 mL) and was reacted with dimethylamine solution (580 µL of a 2M solution in THF, 1.0 mmol) and acetic acid (114 µL, 2.0 mmol) followed by sodium acetoxyborohydride (211 mg, 1.0 mmol) and DCE (4 mL). The product was purified by SCX chromatography by elution with 2M NH_3 in methanol. Fractions containing the desired product were combined and evaporated under reduced pressure to give 1-(5-(3, 4-difluoro-2-(2-(1,3,5-trimethyl-1H-pyrazol-4-yl)ethoxy)phenyl)-1-methyl-1H-indazol-3-yl)-N,N-dimethylmethanamine (IMP-1088) as a colourless oil (170 mg, 99%). LC-MS R_t = 12.2 min (5-98% gradient), $[M + H]^+$ 454, ^{1}H NMR (400 MHz, Chloroform-d) δ 7.85 (s, 1H), 7.45 (d, J = 8.5 Hz, 1H), 7.31 (d, J = 8.8 Hz, 1H), 7.07 (ddd, J = 8.3, 5.7, 2.1 Hz, 1H), 6.91 (q, J = 8.6 Hz, 1H), 4.04 (s, 3H), 3.81 (s, 3H), 3.70 (t, J = 7.5 Hz, 2H), 3.53 (s, 3H), 2.55 (t, J = 7.6 Hz, 2H), 2.29 (s, 6H), 1.89 (s, 3H), 1.85 (s, 3H); ^{13}C NMR (101 MHz, Chloroform-d) δ 150.44 (dd, J = 248.7, 11.6 Hz), 144.70 (dd, J = 247.7, 13.4 Hz), 142.10, 140.21, 136.86, 132.44, 129.35, 129.19, 128.03, 125.29 – 124.41 (m), 123.34, 121.05, 111.50 (d, J = 17.1 Hz), 111.22, 108.55, 73.40 (d, J = 2.6 Hz , 55.54, 45.36, 35.49, 35.36, 24.33, 11.30, 9.18. ESI HRMS, found 454.2412 ($C_{25}H_{30}F_2N_5O$, $[M + H]^+$, requires 454.2418).

Step 5 (IMP-1031). 5-(3-fluoro-2-(2-(1,3,5-trimethyl-1H-pyrazol-4-yl)ethoxy)phenyl)-1-methyl-1H-indazole-3-carbaldehyde (100 mg, 0.24 mmol) was dissolved in THF (4 mL) and was reacted with dimethylamine solution (370 µL of a 2 M solution in THF, 0.74 mmol) and acetic acid (85 µL, 1.48 mmol) followed by sodium acetoxyborohydride (156 mg, 0.74 mmol) and DCE (4 mL). The product was purified by LC-MS by gradient elution with methanol/water/formic acid (20:80:0.1 to 98:2:0.1). Fractions containing the desired product were combined and evaporated under reduced pressure to give 5-(3-fluoro-2-(2-(1,3,5-trimethyl-1H-pyrazol-4-yl)ethoxy)phenyl)-1-methyl-1H-indazol-3-yl)-N,N-dimethylmethanamine (IMP-1031) as a colourless oil formate salt (40 mg, 33%). LC-MS R_t =

12.3 min (5-98%), $[M + H]^+$ 436. ^{1}H NMR (400 MHz, MeOD) δ 8.39 (s, 1H), 7.89 (d, *J* = 4.0 Hz, 1H), 7.62 – 7.51 (m, 2H), 7.19 (h, *J* = 5.1 Hz, 3H), 4.64 (s, 2H), 4.20 (s, 3H), 3.76 (t, *J* = 6.9 Hz, 2H), 3.49 (s, 3H), 2.93 (s, 6H), 2.59 (t, *J* = 6.8 Hz, 2H), 1.90 (s, 3H), 1.88 (s, 3H). ^{13}C NMR (101 MHz, MeOD) δ 164.38, 162.44, 141.50, 141.02, 139.51, 139.48, 139.43, 139.37, 135.24, 132.73, 132.66, 129.84, 124.48, 121.63, 117.61, 117.44, 113.45, 113.28, 110.20, 55.22, 45.05, 35.68, 20.80. ESI HRMS, found 436.2513 ($C_{25}H_{31}FN_5O$, $[M + H]^+$, requires 436.2513).

Crystallography and Biochemistry

Crystallization, X-ray data collection and structure resolution. PvNMT structures with bound ligands were obtained essentially as described previously[4]. HsNMT1 purified as previously described[2,5] was used for co-crystallization with ligands using the sitting-drop vapor diffusion method at 20 ˚C. Crystals of the IMP-1088 inhibitor complex grew in droplets formed by mixing 0.5 µL of protein solution (at 6.8 mg/mL, 0.3 mM of Myr-CoA and 0.5 mM of the ligand, in 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 1mM DTT, 5% (v/v) DMSO) and 0.5 µL of precipitant solution (23% (v/w) MME2K, 0.2M KBr, 100 mM sodium citrate pH 4.5, and 5% (v/v) glycerol). For the IMP-917 and IMP-1031 inhibitor complexes, protein was used at a concentration of 6.8 mg/mL and the precipitation solution was 17.5% PEG 3350, 0.1M sodium phosphate buffer (pH 4.6). Crystals were cryo-protected using precipitant solution supplemented with 10-20% glycerol, and flash cooled for subsequent data collection. X-ray data were collected at 100*K* using synchrotron radiation at Diamond Light Source (Harwell, UK). X-ray data collection and refinement statistics are summarized in Supplementary Table 1. The data sets were integrated with XDS,[6] and scaled and reduced using AIMLESS from the CCP4 package.[7] Crystals belonged to the $P2_12_12$ space group with two molecules of protein complex in the asymmetric unit. Structure resolution was accomplished using the molecular replacement method with the PHASER software[8] and the previously published crystallographic structure of HsNMT1 in complex with Myr-CoA as input model (PDB entry 4C2Y).[2] The initial model was subjected to alternate cycles of model building using COOT[9] and refinement using REFMAC5.[10] The structures consist of two molecules of NMT1 (chains A and B), each of them bound to one molecule of MyrCoA and one molecule of inhibitor, and were therefore refined using NCS restrictions. Overall, the resulting electron density allowed the entirety of both HsNMT1 molecules to be modeled with the exception of the 9 N-terminal residues of both A and B chains (NMT residues 109-114 and three residues of the N-terminal tag), and for the IMP-1088 complex residues 409-413 of chain B, which were flexible and thus not visible in the maps. Electron density maps of high quality were obtained for ligands, which allowed modeling both molecules of bound inhibitor (Supplementary Fig. 1). Difference

electron density maps were calculated using FFT software from the CCP4 package.[11] The geometry of the final models was validated using PROCHECK.[12] Figures were generated using PYMOL (DeLano Scientific LLC, http://pymol.sourceforge.net/) or CCP4MG[13].

NMT inhibition assays. IC_{50} values were determined for HsNMT1 and HsNMT2 using an assay based on fluorogenic detection of the CoASH produced during transfer of myristate from Myr-CoA to a model peptide, as described previously.[2,5] Note that the concentration of enzyme in the assay is ca. 5 nM, and thus IC_{50} values in the low nanomolar range and below cannot be determined with precision. IC_{50} values, where a value could be reliably determined, are reported as the mean from at least three independent assays; standard error in the mean was <10% in each case.

SPR Experiments. Sensorchip surfaces were prepared on a Biacore 3000 instrument (GE Healthcare). HsNMT1, purified as previously described[2,5], was captured at a flow rate of 5 µL/min on to a NTA sensor chip via His-tag interaction with Nickel at levels of ~10000 response units. No protein was immobilized on the first flow cell which was used as reference. NMT inhibitors were injected in a concentration series from 4.6 nM to 10 µM for a period of 60 s. Dissociation was measured for 300-600 s at a flow rate of 50 µL/min using the following assay running buffer: 10 mM Hepes, pH 7.4, 150 mM NaCl, 100 nM Myr-CoA, 3% DMSO and 0.05% Tween 20 at 10°C. The affinity (K_D) and kinetic binding parameters for the compounds were determined by global fitting the sensorgrams using a one-site kinetic model with Scrubber 2 software (BioLogic Software). IMP-1088 was injected from lowest to highest concentrations due to a slow off-rate. R_{max} for each concentration was fitted locally while keeping on-rate and off-rate fits global.

Cell Biology and Virology

Cells and viruses. The cervical epithelial HeLa Ohio (ECACC 93021013) and HeLa H1 (ATCC CRL-1958) cell lines were maintained in exponential growth in complete medium (high glucose DMEM supplemented with glutamine, 1% sodium bicarbonate, 25mM HEPES, and 10% fetal bovine serum). Human bronchial epithelial cells (hBECs) from normal donors (Lonza, CC2540) were cultured in bronchial epithelial cell growth medium according to manufacturer's recommendations (Lonza, CC-3170). The baby hamster kidney-21 (BHK21) cell line (Pirbright Institute Central Service Unit) was maintained in complete medium (GMEM supplemented with glutamine, 5% tryptose phosphate broth, 1% Penicillin/Streptomycin and 10% fetal bovine serum). Viral stocks of rhinovirus RV-A16, RV-A1, RV-A29 and RV-B14 (obtained from ATCC) were produced by infecting HeLa H1 cells. Viral stocks of poliovirus (PV) (type 1 Mahoney strain) were produced by infecting HeLa Ohio cells. Viral stocks of foot-

and-mouth disease virus (FMDV) type O1/Kaufbeuren (O1K) were produced by infecting BHK21 cells. Viral titers were determined by measuring the 50% tissue culture infective dose ($TCID_{50}$). The BSR-T7/5 cell line (a BHK-21 derived cell line stably expressing T7 RNA polymerase[14]) was maintained in high glucose DMEM supplemented with glutamine, 10% fetal bovine serum, 1% Penicillin/Streptomycin, 110 mg/L sodium pyruvate, and 0.4 mg/ml geneticin.

Multicycle replication assays. HeLa Ohio cells were seeded in 96-well plates and infected the day after at around 90% confluence with rhinovirus RV-A16 at an MOI of 0.5 in reduced-serum medium (2% fetal bovine serum), in the presence or absence of the indicated compounds or the quantity of DMSO vehicle corresponding to the maximal amount present in the experimental conditions. After 2 days of viral replication, the cell viability of infected cells and uninfected cells treated with the compounds was assessed by MTS assay using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega), according to the manufacturer's recommendation. Absorbance at 490nm was measured using a FLUOstar Omega 96-well plate reader. The percentage of CPE was calculated as follow: % CPE= 100 x A_{490} [(Uninfected compound-treated control - Infected compound-treated test sample) / (Uninfected compound-treated control - Infected DMSO-treated control)].

Viability assay after inhibitor washout in the absence of virus. HeLa cells were seeded in 96-well plates and treated the day after at approximately 90% confluency with varying concentrations of IMP-1088 (0.24 nM-1 µM), DMSO vehicle, or Puromycin (2µg/mL final concentration). After 24h, cells were washed with DMEM and fresh media was added. Cell viability was assessed 24h later by MTS assay as described above using an EnVision Xcite 2104 (PerkinElmer) plate reader. Cell viability was normalized to DMSO vehicle and Puromycin.

Tagging assay of HeLa cells in the absence of virus. To investigate the effect of NMT inhibitors on the *N*-myristoylation of proteins in HeLa cells, cells were seeded at 150,000 cells/dish in 60mm dishes. After 24h, cells were treated with varying concentrations of, respectively, IMP-994 and IMP-1088 (2 nM-2 µM final concentration), or DMSO vehicle. 1h later, YnMyr (20µM final concentration) or DMSO vehicle was added and the cells cultured for another 24h. Subsequently, cells were lysed and further modified with AzTB followed by SDS-PAGE gel electrophoresis as described below. To investigate the recovery of *N*-myristoylation after inhibitor washout, HeLa cells were seeded in 60mm dishes depending on the total incubation time (24h: 150,000 cells/dish; 48h: 75,000 cells/dish; 72h: 51,000 cells/dish). After 24h, cells were treated with IMP-1088 (50nM final concentration). 1h later, YnMyr (20µM final concentration) or DMSO vehicle was added and the cells were cultured for another 24h. Subsequently, cells were washed with DMEM and fresh media containing YnMyr (20 µM final

concentration) was added. After 0, 24 or 48h cells were lysed and further modified with AzTB followed by SDS-PAGE gel electrophoresis as described below.

Single-cycle replication assays

HeLa Ohio cells were seeded and pre-treated for 16 hours (or as otherwise indicated for time of addition experiments) with the indicated compounds or the quantity of DMSO vehicle corresponding to the maximal amount present in the experimental conditions. Cells were then infected at around 90% confluence with the indicated viruses at an MOI of 20 in complete medium, in the presence of the indicated compounds or the DMSO vehicle. After 1 hour of virus adsorption at room temperature, the unbound virus was washed away with PBS and viral replication was allowed to proceed at 37°C for 6 hours (or as otherwise indicated for replication kinetics experiments), in fresh medium containing the indicated compounds or the DMSO vehicle. For myristoylation analysis experiments, viral replication was carried out in the presence of 40 µM YnMyr and cells were lysed at the end of infection in PBS supplemented with 1% Triton X-100, 0.1% SDS and EDTA-free complete protease inhibitor (Roche Diagnostics). For viral titer determination, plates were freeze-thawed twice at the end of infection, scraped cells and medium were centrifuged at 10,000xg for 5 min at 4ºC to remove cell debris, and the supernatant containing the viral particles was used for viral titer determination. For analysis of cellular and viral proteins by Western blotting, cells were lysed in Laemmli sample buffer (50 µl per well of 24-well plate) and samples were incubated for 10 min at 100°C. For determination of the amount of viral RNA by quantitative real-time PCR (qRT-PCR), cells were lysed with RLT buffer (Qiagen) supplemented with β-Mercaptoethanol (350 µl per well of 24-well plate).

For PV and FMDV infection experiments, HeLa Ohio or BHK21 cells were respectively infected with poliovirus (PV) (type 1 Mahoney strain) or FMDV (type O1K) with an MOI of 20. After 1 hour of virus adsorption at 37°C, the unbound virus was washed away with PBS and viral replication was allowed to proceed at 37°C for 6 hours in fresh medium containing the indicated compounds or the DMSO vehicle. Plates were then frozen and viral titers were determined.

Human bronchial epithelial cells (hBECs) were seeded in 24-well plates in cell growth medium and the medium was replaced by basal medium (Lonza, CC-3171) 18 hours prior infection. Cells were then infected at around 90% confluence with RV-A1 at an MOI of 5 in basal medium, in the presence of the indicated compounds or the DMSO vehicle. Infections were then carried out as with HeLa cells, except that viral replication was allowed to proceed for 7 hours, as the replication kinetics seemed a bit slower than in HeLa cells.

Viral titer determination. HeLa Ohio cells were incubated in 96-well plates in reduced-serum medium (2% fetal bovine serum) supplemented with 1% Penicillin/Streptomycin with eight 10-fold dilutions of the virus samples in six replicates for 5 days. The presence of cytopathic effect (CPE) was assessed by microscopic observation and the 50% Tissue culture Infective Dose ($TCID_{50}$) was calculated with the Reed-Muench method.[15]

Mutagenesis of the RV-A16 myristoylation signal and virus recovery. Synthetic DNA (GeneART, ThermoFisher Scientific) incorporating a mutated myristoylation signal (ATG**GGC**GCTCAA changed to ATG**GCA**GCTCAA; G2A) at the start of the coding sequence for VP4, was inserted into a RV-A16 infectious copy plasmid[16] using *Sal*I and *Nde*I restriction sites and standard molecular biology techniques. Sanger sequencing was used to confirm the presence of the mutation in the plasmid. *Wild-type* and mutant plasmids were introduced into separate cultures of BSR-T7/5 cells by transfection (Lipofectamine 2000, ThermoFisher Scientific). Cultures were incubated at 37°C for 24 hours, freeze/thawed and recovered viruses in the lysates serially passaged on HeLa Ohio cells to observe virus induced cytopathic effect (CPE).

Analysis of 2C/2BC viral protein expression by Western blot. After infection, cell lysates were prepared as described above and the amount of DNA present in the cell lysates was determined with a NanoDrop instrument (Thermo Scientific), and an equivalent amount of DNA was loaded for each sample onto NuPAGE 4-12% Bis-Tris SDS-PAGE gels (Life Technologies). After separation by electrophoresis in NuPAGE MES SDS Running Buffer (Life Technologies), proteins were transferred to 0.45µm pore size PVDF membranes (Life Technologies) using a Mini Trans-Blot Cell (Bio-Rad) in Tris Glycine Transfer Buffer (Novex). Membranes were blocked in blocking buffer (Tris buffered saline (TBS) supplemented with 5% BSA and 0.1% Tween-20) for 1 hour at room temperature. The membranes were then incubated overnight at 4ºC with the following primary antibodies diluted in blocking buffer: rabbit serum directed against RV-A16 2C (1/15,000)[17] or rabbit anti-Lamin B1 (Proteintech 12987-1-AP, 1/10,000). After washes in blocking buffer, the membranes were incubated for 1 hour at room temperature with HRP-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch 111-035-144, 1/10,000), diluted in blocking buffer. Immobilized HRP was then detected with EZ-ECL (Geneflow) and images were acquired and quantified on a FUSION FX7 Advance image analyzer (Peqlab).

Quantitative real-time PCR (qRT-PCR). mRNA extraction was performed using the RNeasy Mini kit (Qiagen) following manufacturer's instructions. 1 µg of mRNA was reverse-transcribed for cDNA synthesis for 1 h at 37 ºC using the Omniscript RT kit (Qiagen) and random hexamers as primers (Promega). Quantification of the different mRNAs of interest was conducted by TaqMan assay using specific primers and probes (Invitrogen) as follows:

RV forward (50nM) 5'-GTGAAGAGCCSCRTGTGCT-3'

RV reverse (300nM) 5'-GCTSCAGGGTTAAGGTTAGCC-3'

RV probe (100nM) 5'-FAM-TGAGTCCTCCGGCCCCTGAATG- TAMRA-3'

18S forward (300nM) 5'- CGCCGCTAGAGGTGAAATTCT-3'

18S reverse (300nM) 5'- CATTCTTGGCAAATGCTTTCG-3'

18S probe (100nM) 5'-FAM-ACCGGCGCAAGACGGACCAGA-TAMRA-3'

Analysis was performed using the QuantiTect Probe PCR kit (Qiagen) and LightCycler® 480 System (Roche). For absolute quantification, each gene was normalized to the level of 18S and the exact number of copies of the gene of interest was calculated using a standard curve generated by amplification of plasmid DNA.

Sedimentation analysis. Concentrated lysates were prepared to analyze the sedimentation of rhinovirus capsid components. Four 150cm^2 plates of HeLa Ohio cells were grown to 90% confluency before infection with rhinovirus RV-A16 at MOI 20. After one hour virus adsorption at room temperature, cells were washed five times with D-PBS and the medium was replaced with 15mL of medium with DMSO as a mock treatment in 2 plates or IMP-1088 (500nM in DMSO) in the other two plates. The cells were incubated at 37 °C for six hours before all of the medium was removed and the cells were frozen at - 80 °C. Subsequently the cells were thawed and scraped into 3mL D-PBS and frozen at - 80 °C for a second time. The lysates were thawed for a second time on ice, Halt protease inhibitor and EDTA (1X, Thermo) and 1µl/mL Benzonase (≥250units, Sigma) were added to each lysate, which were incubated for 30 minutes on ice and the cell debris was pelleted at 2400xg for 10 minutes at 4 °C. The clarified lysate was aliquoted and frozen a further time at -80 °C. To separate virions and empty capsids from capsid intermediates, 5mL, 10-30% (w/v in PBS) sucrose density gradients (SDGs) were formed using a Gradient Master (Biocomp). A 100µl aliquot of each lysate was applied to the top of each SDG and the tubes were centrifuged at 286,794xg (RCF average) for 44 minutes at 8 °C. The samples were fractionated into 24 equal 240µl fractions using an Acura Manual 865 Repeat microdispenser (Socorex). To detect rhinovirus RV-A16 capsid components from the SDG, the fractions were analyzed by SDS-PAGE and Western blot using a sheep serum (S1688) specific for rhinovirus RV-A16. 10µl of each gradient fraction was denatured and reduced in Red Loading Buffer and DTT (1X final, both NEB) and heated at 96 °C for 3 minutes. The samples were then resolved through 12% Tris-Tricine gels and transferred to nitrocellulose membrane (0.45µM, GE Healthcare) using a Mini-Protean tetra cell (Biorad). Membranes were placed in blocking buffer (20mM Tris, 150mM NaCl pH7.6 with 0.1% tween-20 (TBS-T) with 1% BSA (Melford)) for one hour at room temperature followed by

incubation with S1688 serum (1/1000 in blocking buffer) overnight at 4 °C. The membranes were washed three times with TBS-T and then incubated with anti-sheep-HRP (Abcam, 1/2000 in blocking buffer) and washed three times with TBS-T. West Pico chemiluminescent substrate (Thermo) was added to the membrane and exposures of the membrane were collected and visualized using a G:Box Chemi XX6 (Syngene). The intensity of the rhinovirus VP1 protein band was quantitated to generate densitometry plots of relative band intensity using NIH ImageJ software (v1.50). Data were presented using Prism 7 software (GraphPad). Antisera against rhinovirus RV-A16 was raised in sheep immunized with purified, inactivated rhinovirus (Diagnostics Scotland).

Statistical analysis. All statistical analysis was performed using GraphPad Prism 7 software. Data are presented as mean ± standard error of the mean (SEM). For IC_{50} determination from single-cycle infection assays, the $TCID_{50}$/ml results were normalized and expressed as percentage of infected untreated control. For both multi-cycle and single-cycle replication assays, IC_{50} were determined by nonlinear regression with a log(inhibitor) vs. response (three parameters) equation. In Figure 3d, for each rhinovirus strain, the $\log_{10}(TCID_{50}/ml)$ values of the IMP-1088- treated condition were compared with the values of the DMSO control condition using a two-way ANOVA with Sidak's multiple comparisons test. Values from 3 independent experiments were analyzed. ****: $p<0.0001$. In Figure 3f, each IMP-1088-treated condition was compared to the DMSO control condition using one-way ANOVA with Dunnett's multiple comparisons test on $\log_{10}(TCID_{50}/ml)$. Values from 3 independent experiments were analyzed. **: $p=0.0013$, ****: $p<0.0001$.

Labeling and Proteomics

CuAAC ligation and in-gel fluorescence. Protein concentrations were adjusted to 2 mg/mL by addition of lysis buffer. A click mixture was prepared by mixing 1 µL capture reagent AzTB (10 mM in DMSO, final concentration 0.1 mM), 2 µL $CuSO_4$ (50 mM in H_2O, final concentration 1 mM), 2 µL tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (50 mM in H_2O, final concentration 1 mM) and 1 µL tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) (10 mM in DMSO, final concentration 0.1 mM). The mixture (6 µL per 100 µL of lysate) was added to each protein lysate and the reaction mixture was vortexed at room temperature for 1 h, then quenched by the addition of EDTA (10 mM final concentration). Subsequently, proteins were precipitated by addition of methanol, chloroform and ultrapure water (2:0.5:1 volume relative to the sample volume). Precipitates were isolated by centrifugation (14,000 x *g*, 10 min, RT) and washed twice with cold methanol. The pellets were air-dried and samples were re-suspended in 2% SDS/10 mM DTT in PBS (10 mg/mL). Once dissolved, the samples were diluted to 0.2% SDS by addition of PBS to a final concentration of 1 mg/mL. Typically, 10 µg proteins were loaded on a SDS-polyacrylamide electrophoresis gel. The fluorescence

of the gel was detected using a Typhoon imager (GE Healthcare) and the protein loading was checked by staining with Coomassie.

Sample preparation for MS-Based proteomics with enrichment of labeled proteins. Only low binding micro-centrifuge tubes (Eppendorf® Protein LoBind) were used. All solutions were prepared fresh. Protein lysates (300 µL-600 µg) were subjected to click chemistry as described above by using AzRB as capture reagent.[3] The samples were then added to 30 µL of pre-washed (0.2% SDS in PBS, 3 X 500 µL) NeutrAvidin® Agarose resin and gently vortex mixed for 2 hours at room temperature. The beads were pelleted (3,000 x *g*, 3 min) and the supernatant was removed. The beads were sequentially washed with 1% SDS in PBS (3 X 500 µL), 4M Urea in 50 mM ammonium bicarbonate (2 X 500 µL), and 50 mM ammonium bicarbonate (2 X 500 µL). For each wash step the beads were gently vortexed for 2 min followed by pelleting in a microcentrifuge (3,000 x *g*, 1 min). The beads were re-suspended in 50 mM ammonium bicarbonate (50 µL), DL-dithiothreitol (5 µL, 100 mM in 50 mM ammonium bicarbonate) was added and the beads were incubated at 55°C for 30 minutes in a shaker. The beads were washed with 50 mM ammonium bicarbonate (2 X 500 µL) and re-suspended in 50 mM ammonium bicarbonate (50 µL). Cysteines were alkylated by the addition of iodoacetamide (5 µL, 100 mM in 50 mM ammonium bicarbonate, at room temperature for 30 minutes in the dark). The beads were washed as before. Sequencing grade modified trypsin (Promega, 3 µL, 0.2 µg/µL in 50 mM ammonium bicarbonate) was added to the beads and the samples were incubated at 37°C overnight in a shaker. The beads were pelleted and the supernatant collected. The beads were washed with 60 µL 50mM ammonium bicarbonate and then with 1.5% trifluoroacetic acid in ultrapure water (60 µL) with gentle shaking for 10 minutes each time. The peptide solutions were stage-tipped according to a published protocol.[18] The peptides were eluted from the sorbent (SDB-XC poly(styrenedivinylbenzene) copolymer, from 3M) with 79% acetonitrile in water, dried in a Savant SPD1010 SpeedVac® Concentrator (Thermo Scientific) and stored at -80 °C until LC-MS/MS analysis. Peptides were reconstituted in 2% acetonitrile in water with 0.5% trifluoroacetic acid for LC-MS/MS analysis.

Sample preparation for MS-based proteomics for the whole proteome. Protein lysates (30 µg) were precipitated using the system MeOH:$CHCl_3$:H_2O described above. The pellet was washed with 10% water in methanol (200 µL X 2) and centrifuged at maximum speed and 16 °C for 10 minutes. The pellet was dissolved in 5 mM DTT in 50 mM ammonium bicarbonate (48 µL) and the solution incubated at 55°C for 30 minutes. Cysteines were alkylated by the addition of iodoacetamide (2.4 µL, 100 mM in 50 mM ammonium bicarbonate, at room temperature for 30 minutes in the dark). Sequencing grade modified trypsin (Promega, 5 µL, 0.2 µg/µL in 50 mM ammonium bicarbonate) was added to the solutions and the samples were incubated at 37°C overnight in a shaker. TFA was added to a final concentration of 0.5% (100

µL 0.75% trifluoroacetic acid in ultrapure water). The peptide solutions were stage-tipped according to a published protocol.[19] The peptides were eluted from the sorbent (SDB-XC poly(styrenedivinylbenzene copolymer, from 3M) with 79% acetonitrile in water, dried in a Savant SPD1010 SpeedVac® Concentrator (Thermo Scientific) and stored at -80 °C until LC-MS/MS analysis. Peptides were reconstituted in 2% acetonitrile in water with 0.5% trifluoroacetic acid for LC-MS/MS analysis.

nLC-MS/MS analysis. The analysis was performed using an EASY-Spray™ Acclaim PepMap RSLC column (50 cm × 75 µm inner diameter, Thermo Fisher Scientific) using a 2 h acetonitrile gradient in 0.1% aqueous formic acid at a flow rate of 250 nL/min. Easy nLC-1000 was coupled to a Q Exactive mass spectrometer via an easy-spray source (Thermo Fisher Scientific). The Q Exactive was operated in data-dependent mode with survey scans acquired at a resolution of 70,000 at m/z 200 (transient time 256 ms). Up to 10 of the most abundant isotope patterns with charge +2 or higher from the survey scan were selected with an isolation window of 2.0 m/z and fragmented by higher-energy collision dissociation (HCD) with normalized collision energies of 25. The maximum ion injection times for the survey scan and the MS/MS scans (acquired with a resolution of 17 500 at m/z 200) were 20 and 120 ms, respectively. The ion target value for MS was set to 10^6 and for MS/MS to 10^5, and the intensity threshold was set to 8.3×10^2.

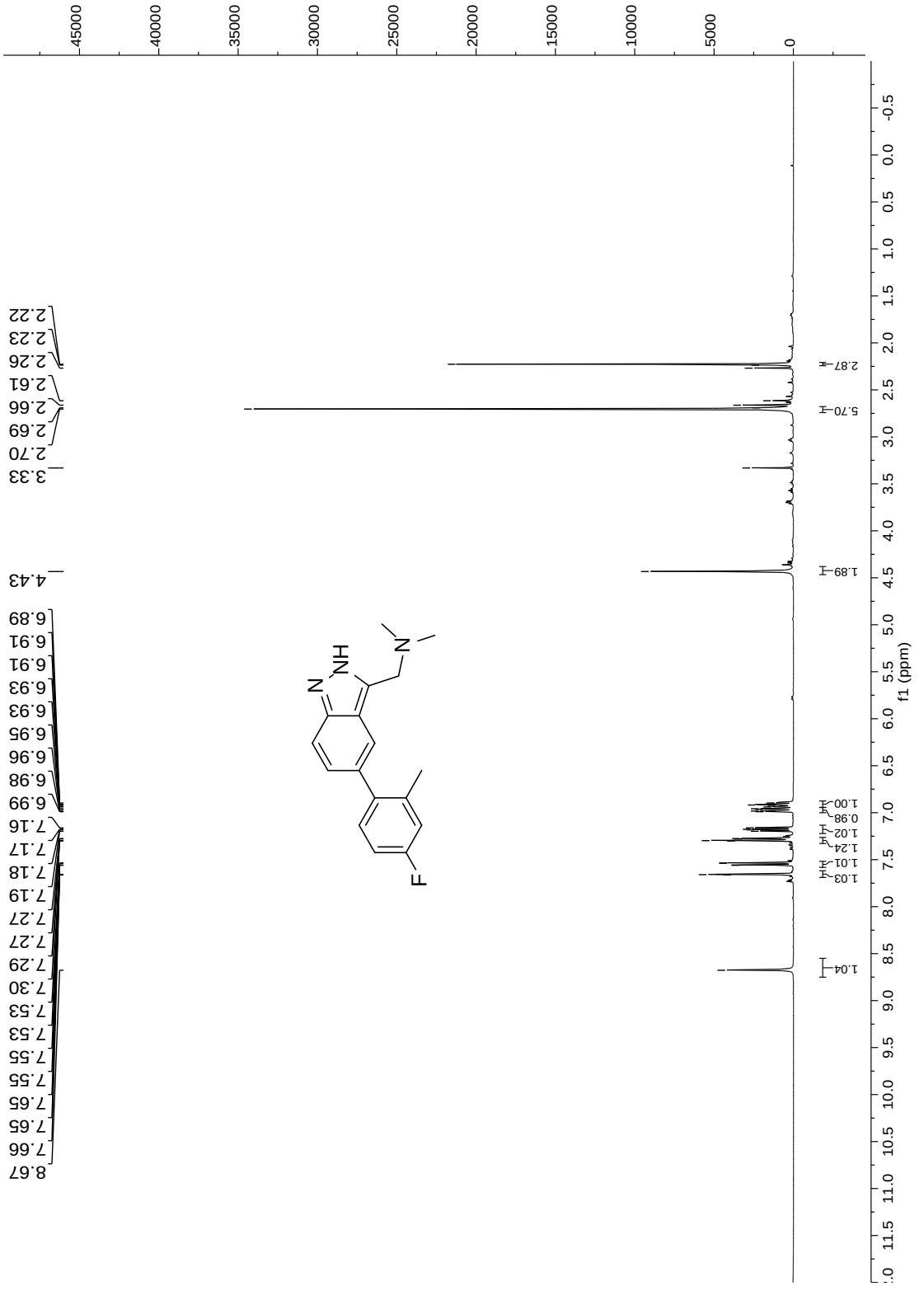
Proteomics data analysis. The data were processed with MaxQuant version 1.5.0.25 using the built-in Andromeda search engine and the built-in label-free quantification algorithm (MaxLFQ).[20] Peptides were identified from the MS/MS spectra searched against the human reference (with isoforms) proteome plus rhinovirus RV-A16 polyprotein (Uniprot, accessed April 2016). Cysteine carbamidomethylation was used as a fixed modification and methionine oxidation as variable modifications. 'Trypsin/P' was chosen as digestion mode enzyme. Up to two missed cleavages were allowed. The 'match between run' (time window 0.7 min) option was selected. 'Unique and razor peptides' were chosen for protein quantification. Other parameters were used as pre-set in the software. Processed data were further analysed using Perseus version 1.5.0.9, Microsoft Office Excel 2010 and GraphPad Prism version 5.03. MS data were further processed with PEAKS7.5,[21] which performs de novo peptide sequencing prior to database searches, in order to improve the accuracy of the results. The software also searches for common PTMs (PEAKS PTM) and point mutations (SPIDER). The data were searched against the same human reference (with isoforms) proteome plus rhinovirus RV-A16 polyprotein that were used in MaxQuant analyses. Trypsin (specific, up to three missed cleavages allowed) was selected for database searches, and no enzyme was chosen in de novo searches. The maximal mass error was set to 5 ppm for precursor ions and 0.01 Da for product ions. Carbamidomethylation was selected as a fixed modification, and methionine

oxidation as well as the lipid-derived adduct (+206.17 Da) to any amino acid at peptide N-terminus were set as variable modifications. The maximal number of modifications per peptide was set as three. The false discovery rate was set to 0.01 for peptides and minimum of 1 unique peptide per protein was required. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository[22] with the dataset identifier PXD005798.

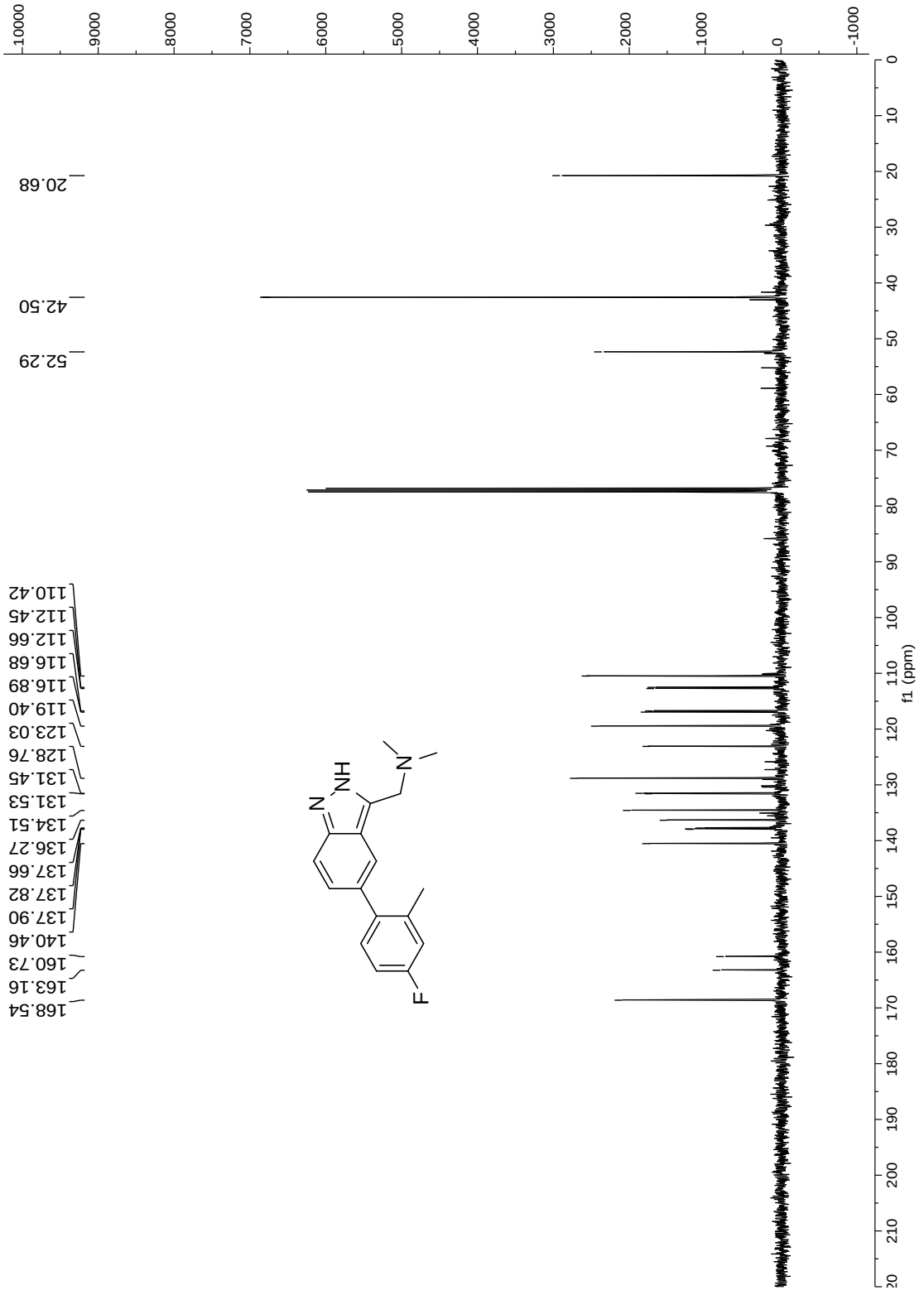
Protein enrichment for Western blot analysis. For enrichment of virus protein, 100 µg of protein lysate was labelled by CuAAC with AzTB as described above. After protein precipitation, the pellet was resuspended in 2% SDS, 10 mM DTT in PBS (25 µL, 4 mg/mL). Once the pellet was completely dissolved, 225 µL of PBS were added (final concentration 0.4 mg/mL, 0.2% SDS). 50 µL of this solution were then added to 20 µL of pre-washed (0.2% SDS in PBS, 3 X 300 µL) Dynabeads MyOne Streptavidin C1 and gently vortex mixed for 1.5 hours at room temperature. The supernatant was removed and the beads were washes with 0.2% SDS (3 X 300 µL). 12 µL of 0.2% SDS in PBS and 4 µL 4X SLB were added to the beads and the beads were boiled for 10 min. Samples were loaded on a SDS-polyacrylamide electrophoresis gel ("before pull down sample: 10 µL-0.4 µg proteins; supernatant: 10 µL-0.4 µg proteins; "pull down": 20 µg proteins.). Fluorescence in the gel was scanned using a Typhoon imager (GE Healthcare). Proteins were transferred to nitrocellulose membrane using a wet transfer setup, and the membrane washed with TBS-T (1 X TBS, 0.1% Tween-20), blocked (5% BSA in TBS-T), washed with TBS-T (3 X 5 minutes), incubated with the primary antibody for Rhinovirus 16 (QED Bioscience, 18758, 1/1,000 dilution) or HSP90 (Santa Cruz Biotechnology, sc-69703, 1/1,000 dilution) in blocking solution at 4 °C overnight, washed with TBS-T (4 X 5 minutes), incubated with the appropriate secondary antibody (Goat-α-mouse IgG (H+L) HRP, Advansta, R-05071-500, 1/10,000) in blocking solution for 1 hour at room temperature, washed with TBS-T (4 X 5 minutes) and developed with Luminata Crescendo Western HRP substrate (Millipore) on a Fujifilm LAS-3000 imager.

NMR Spectra for IMP-72, -917, -944, -1031 and -1088

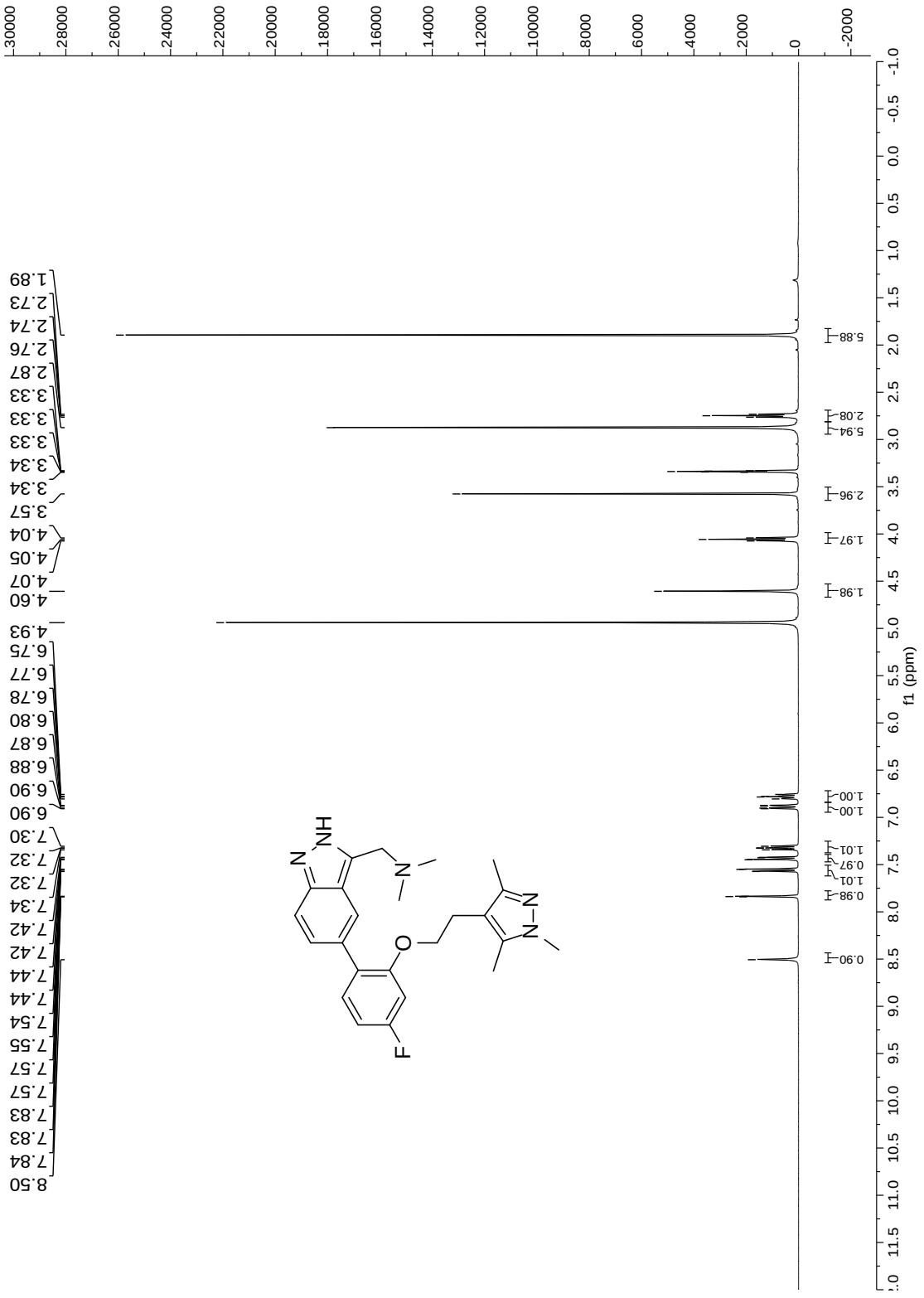
IMP-72: ^{1}H NMR (400 MHz, Chloroform-d)



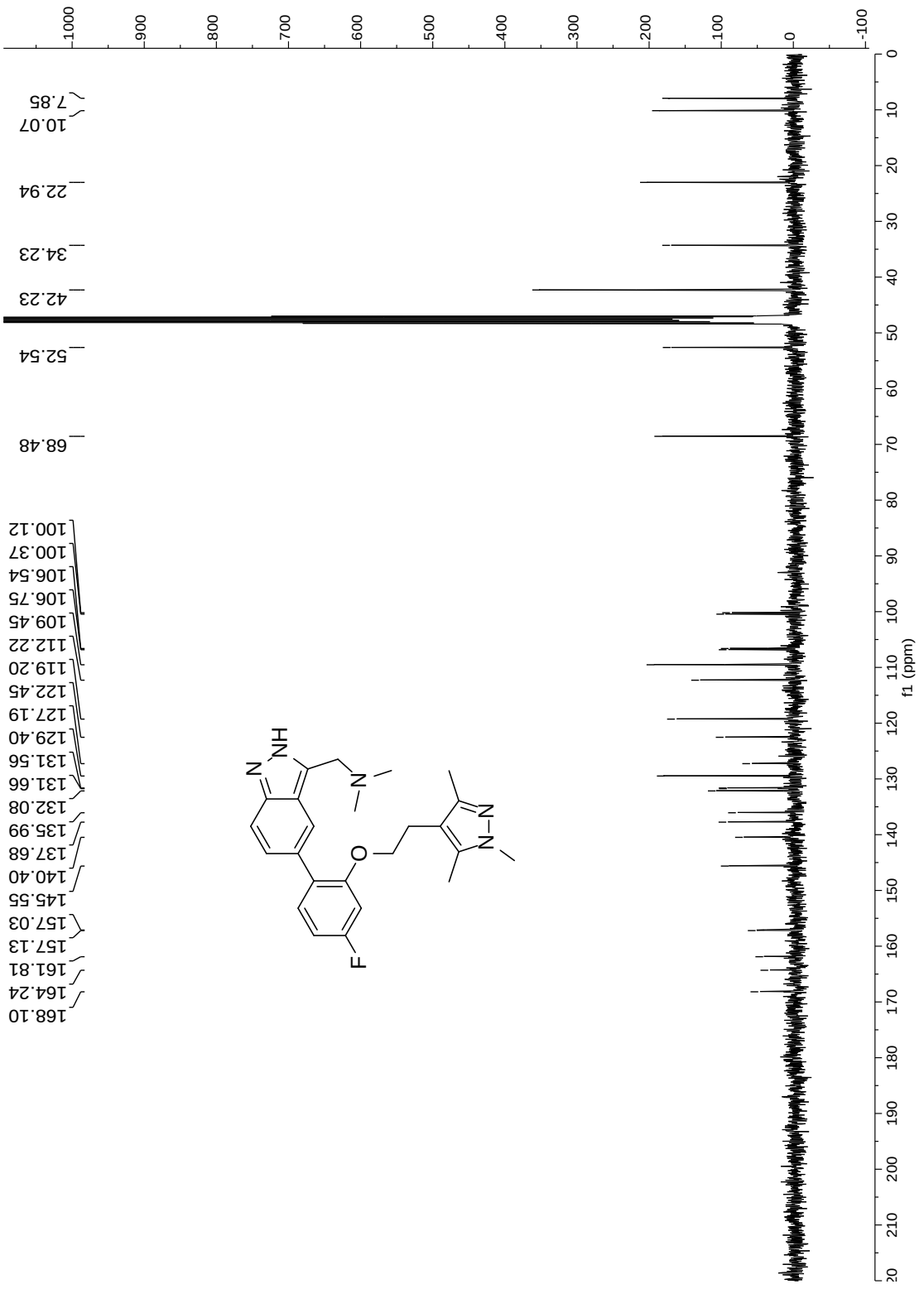
IMP-72: ^{13}C NMR (101 MHz, Chloroform-d)



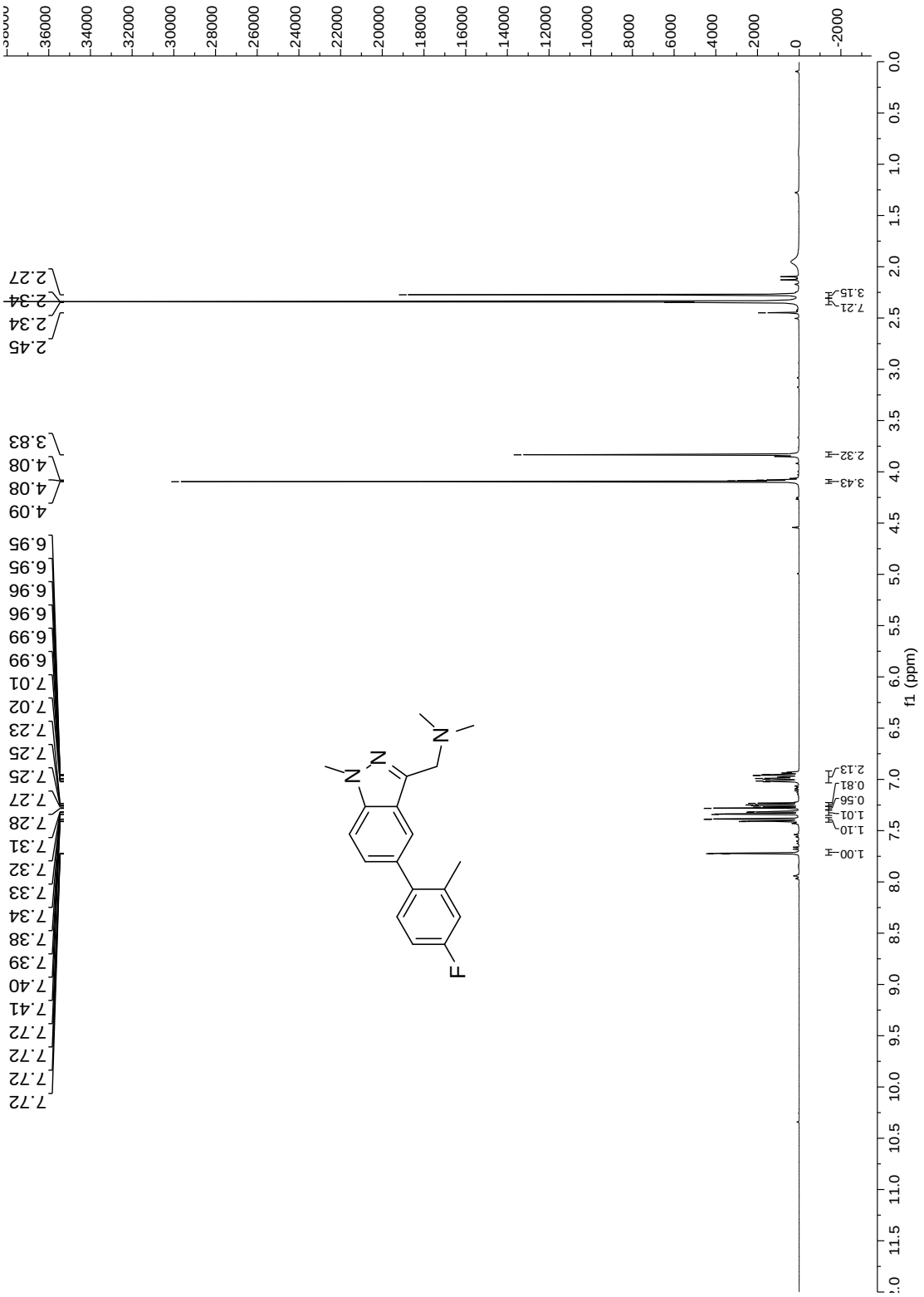
IMP-917: ^{1}H NMR (400 MHz, MeOD)



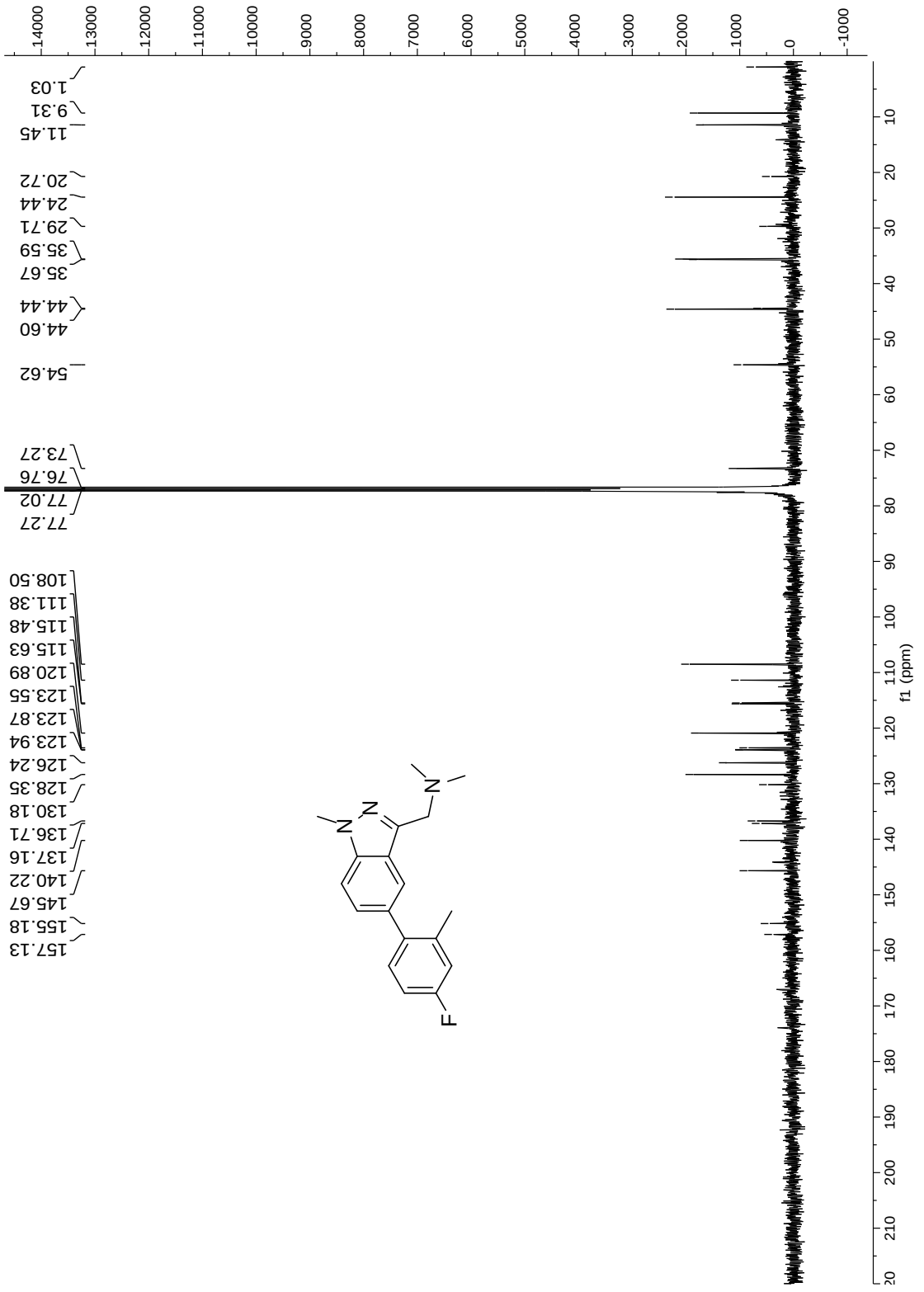
IMP-917: ^{13}C NMR (101 MHz, MeOD)



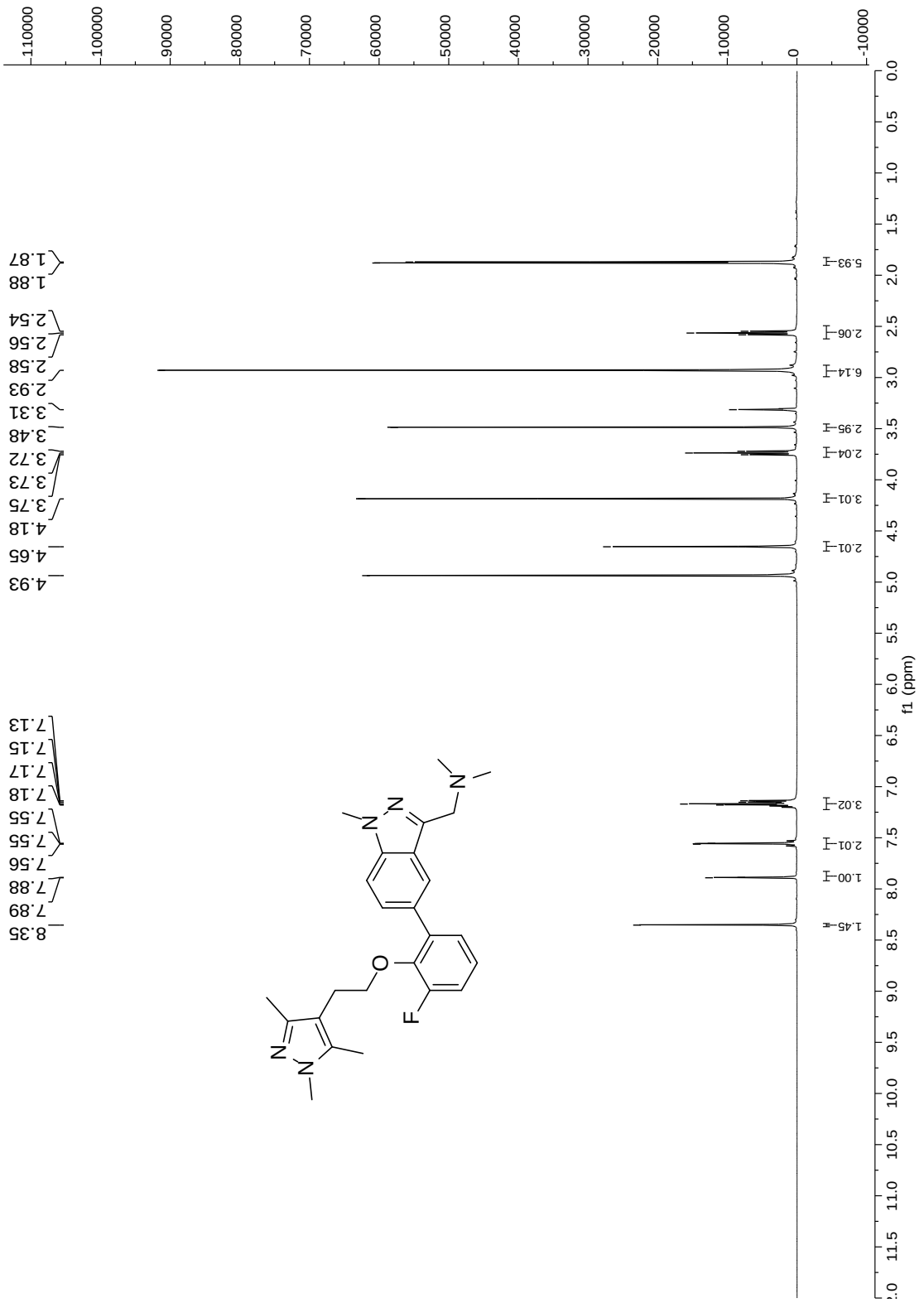
IMP-994: ^{1}H NMR (400 MHz, Chloroform-d)



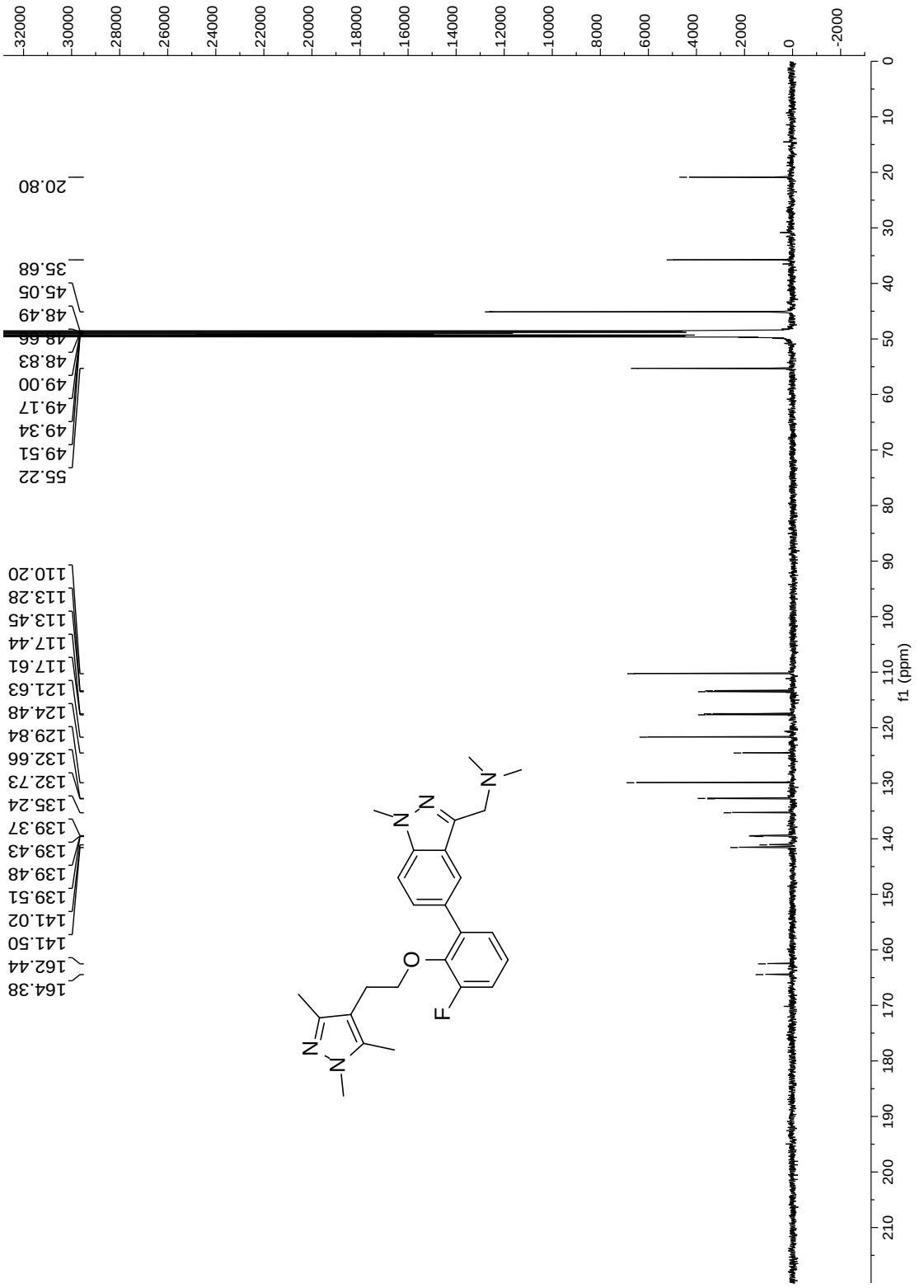
IMP-994: ^{13}C NMR (101 MHz, Chloroform-d)



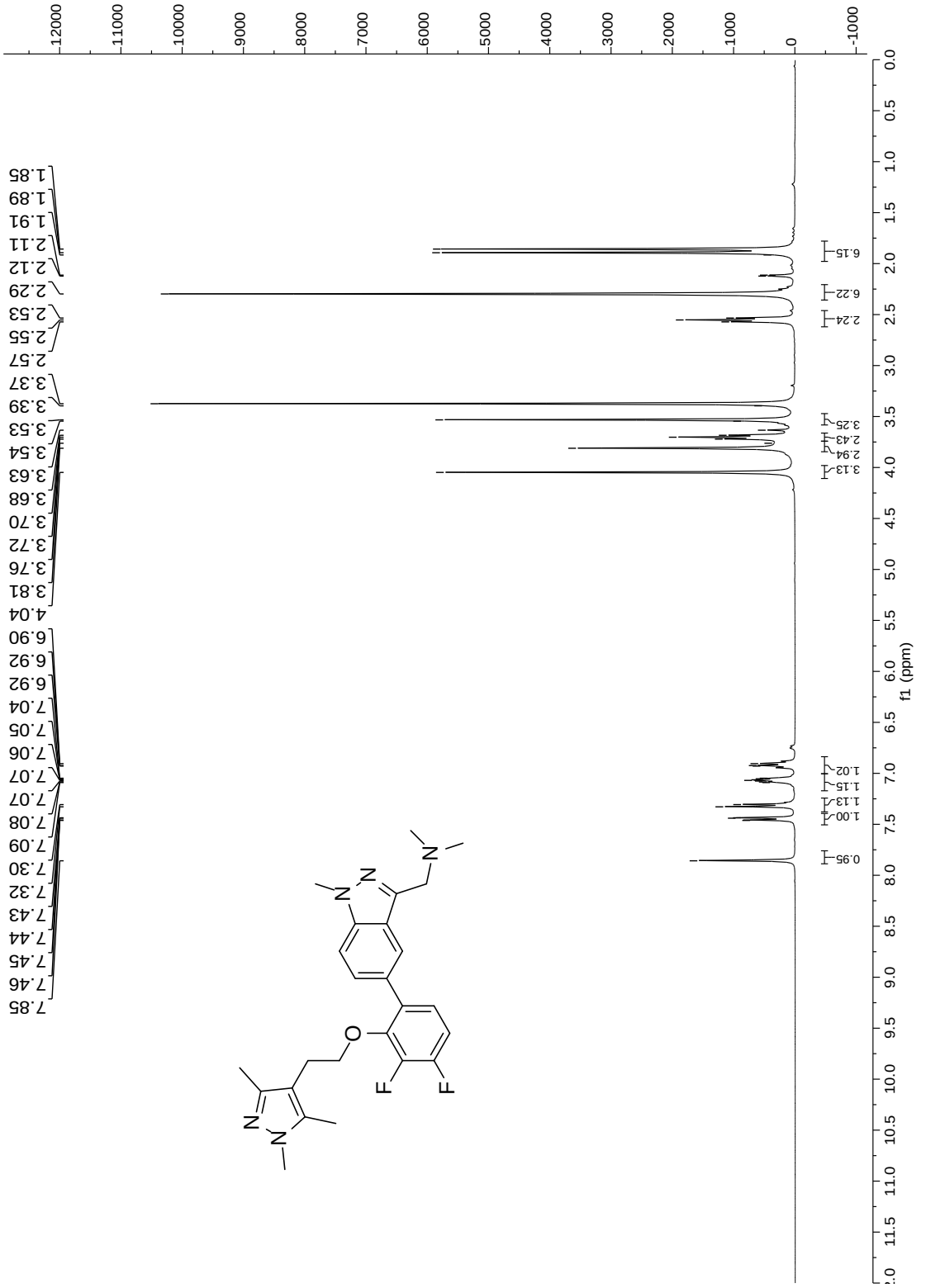
IMP-1031: ^{1}H NMR (400 MHz, MeOD)



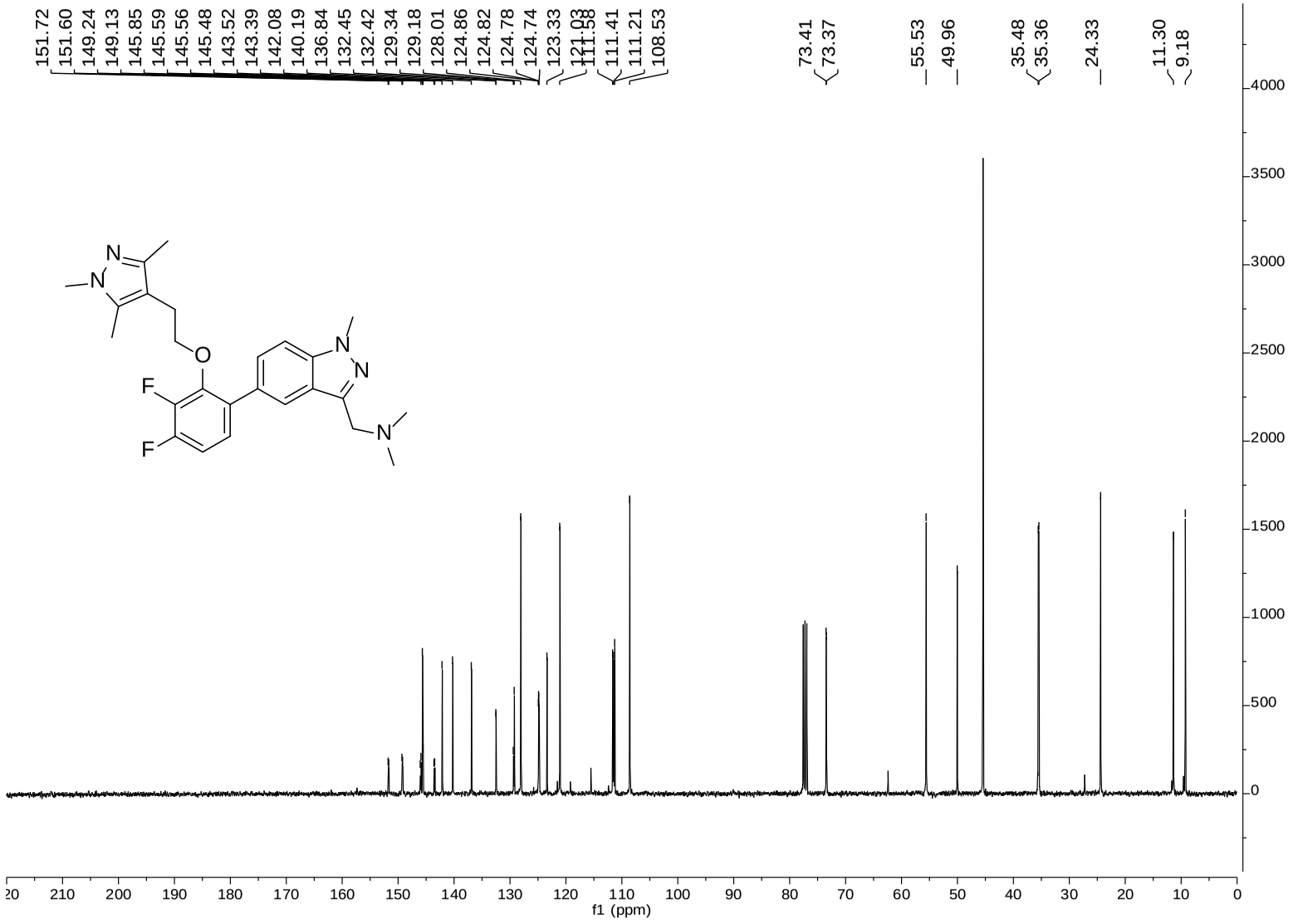
IMP-1031: ^{13}C NMR (101 MHz, MeOD)



IMP-1088: ^{1}H NMR (400 MHz, Chloroform-d)



IMP-1088: ^{13}C NMR (101 MHz, Chloroform-d)


151.72
151.60
149.24
149.13
145.85
145.59
145.56
145.48
143.52
143.39
142.08
140.19
136.84
132.45
132.42
129.34
129.18
128.01
124.86
124.82
124.78
124.74
123.33
121.03
111.58
111.41
111.21
108.53
73.41
73.37
55.53
49.96
35.48
35.36
24.33
11.30
9.18
N
N
O
F
F
N
N
N
4000
3500
3000
2500
2000
1500
1000
500
0
f1 (ppm)

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