Structure-Activity Relationships in Metal-Binding Pharmacophores for Influenza Endonuclease

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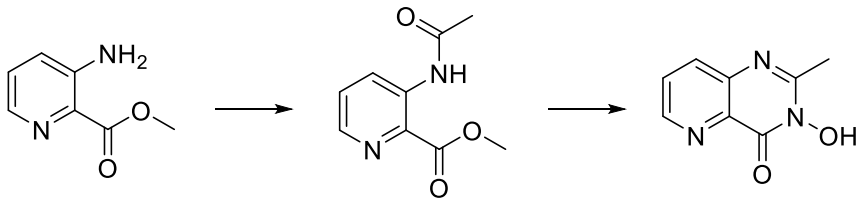
Supporting Information

Contents

Synthesis of Compounds

Scheme S1



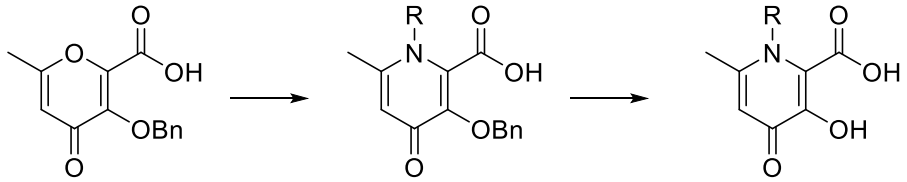
50 **6**

Methyl 3-acetamidopicolinate (50). Methyl 3-aminopicolinate (700 µl, 5.41 mmol) was stirred in acetic anhydride (5.1 mL, 54.1 mmol) neat for 4-6 hours at 55 °C. Upon completion of the acetylation reaction, as indicated by TLC analysis and ninhydrin staining, the reaction mixture was concentrated to dryness under high vacuum. The residual solids were taken up in DCM, washed once with saturated sodium bicarbonate, dried over magnesium sulfate, and concentrated to afford **50** as an off-white solid in 92% yield. ^{1}H NMR (400 MHz, CD_3OD): δ 8.72 (s, 1H), 8.46 (s, 1H), 7.92 (s, 1H), 3.74 (s, 3H), 2.28 (s, 3H). ESI-MS Experimental: 195.44. Calculated for $[C_9H_{11}N_2O_3]^+$: 195.20.

3-Hydroxy-2-methylpyrido[3,2-d]pyrimidin-4(3H)-one (6). Potassium hydroxide (1.6 g, 29.4 mmol) and hydroxylamine hydrochloride (1 g, 14.7 mmol) were taken up in methanol (10 mL) in a 35 mL microwave reaction vessel and were stirred at room temperature. This reaction was highly exothermic. After the hydrochloride salt had been neutralized and the temperature of the reaction cooled to <40 °C, methyl 3-acetamidopicolinate (950 mg, 4.9 mmol) was added. The reaction mixture was then irradiated at 120 °C for 15 min in a microwave reactor with stirring. Upon cooling to room temperature, acetic acid was used to acidify the mixture to pH ~4 and the mixture was concentrated to dryness under vacuum. The residual solids were then purified by C18

chromatography to afford 3-Hydroxy-2-methylpyrido[3,2-d]pyrimidin-4(3H)-one as a pink solid in 46% yield. ^{1}H NMR (400 MHz, CD_3OD): δ 8.74 (d, J = 1.5 Hz), 8.03 (dd, J = 8.4, 1.5 Hz), 7.76 (dd, J = 8.4, 4.3 Hz), 2.52 (s). ^{13}C NMR (126 MHz, DMSO-d_6): δ 157.49 (s), 155.72 (s), 149.04 (s), 143.32 (s), 138.08 (s), 135.49 (s), 128.56 (s), 20.89 (s). ESI-MS Experimental: 176.21. Calculated for $[C_8H_6N_3O_2]^-$: 177.16. HR-ESI-MS Experimental: 176.0466. Calculated for $[C_7H_5O_5]^-$: 176.0466. Δ = 0.0 ppm.

Scheme S2



51 **52: R = H** **53: R = Me** **54: R = OH** **17: R = H** **20: R = Me** **21: R = OH**

3-(Benzyloxy)-6-methyl-4-oxo-4H-pyran-2-carboxylic acid (51). Compound **51** was prepared according to literature procedure.[1]

3-(Benzyloxy)-6-methyl-4-oxo-1,4-dihydropyridine-2-carboxylic acid (52). 3-(Benzyloxy)-6-methyl-4-oxo-4H-pyran-2-carboxylic acid (200 mg, 0.77 mmol) was taken up in a 1:1 mixture of water (10 mL) and methanol (10 mL) in a sealable vessel. Ammonium hydroxide (30%) (328 µL, 2.3 mmol) was added and the vessel was sealed and heated to 75 ºC for 18-24 h. After this time, the reaction was cooled and concentrated under vacuum, diluted with 1M HCl and concentrated under vacuum to remove ammonium salts, and the resultant solids were purified by C18 column chromatography utilizing a MeOH/water system to afford 3-(benzyloxy)-6-methyl-4-oxo-1,4-dihydropyridine-2-carboxylic acid (122 mg, 0.47 mmol, 61% yield) as a white powder. ^{1}H NMR

(400 MHz, CD_3OD): δ 7.45 (t, J = 5.6 Hz, 4H), 7.31 (dd, J = 15.9, 6.3 Hz, 8H), 5.18 (s, 4H), 2.62 (s, 5H). ESI-MS Experimental: 259. Calculated for $[C_{14}H_{14}NO_4]^+$: 259.27.

3-(Benzyloxy)-1,6-dimethyl-4-oxo-1,4-dihydropyridine-2-carboxylic acid (53). 3-(Benzyloxy)-6-methyl-4-oxo-4H-pyran-2-carboxylic acid (200 mg, 0.77 mmol) was taken up in a 1:1 mixture of water (10 mL) and methanol (10 mL) in a sealable vessel. Methylamine (200 µL, 2.3 mmol) was added and the vessel was sealed and heated to 75 ºC for 18-24 h. After this time, the reaction was cooled and concentrated under vacuum, diluted with 1M HCl and concentrated under vacuum to remove methylammonium salts, and the resultant solids were purified by C18 column chromatography utilizing a MeOH/water system to afford 3-(benzyloxy)-1,6-dimethyl-4-oxo-1,4-dihydropyridine-2-carboxylic acid (100 mg, 0.366 mmol, 47 % yield) as a white solid. ^{1}H NMR (400 MHz, CD_3OD): δ 7.47 – 7.26 (m, 6H), 5.16 (s, 2H), 3.92 (s, 3H), 2.64 (s, 3H). ESI-MS Experimental: 274. Calculated for $[C_{15}H_{16}NO_4]^+$: 274.31.

3-(Benzyloxy)-1-hydroxy-6-methyl-4-oxo-1,4-dihydropyridine-2-carboxylic acid (54). 3-(Benzyloxy)-6-methyl-4-oxo-4H-pyran-2-carboxylic acid (200 mg, 0.769 mmol) was taken up in a 1:1 mixture of water (10 mL) and methanol (10 mL) in a sealable vessel. Hydroxylamine hydrochloride (160 mg, 2.3 mmol) was added and the vessel was sealed and heated to 75 ºC for 18-24 h. After this time, the reaction was cooled and concentrated under vacuum and the resultant solids were purified by C18 column chromatography utilizing a MeOH/water system to afford 3-(benzyloxy)-1-hydroxy-6-methyl-4-oxo-1,4-dihydropyridine-2-carboxylic acid (71.9 mg, 0.26 mmol, 34 % yield) as a white solid. ^{1}H NMR (400 MHz, CD_3OD): δ 7.45 (d, J = 6.7 Hz, 2H),

7.30 (s, 4H), 6.37 (s, 1H), 5.17 (s, 2H), 2.34 (s, 3H). ESI-MS Experimental: 276. Calculated for $[C_{14}H_{14}NO_5]^+$: 276.27.

General procedure for the deprotection of 17, 20-21

Benzyl protected pyridinone **17, 20-21** was taken up in a 5:5:1 mixture of concentrated HCl:HOAc:TFA and was stirred at room temperature for 48 h. After this time, acids were removed under high vacuum, the resultant solids were co-evaporated several times with methanol. The remaining solids were then purified by C18 chromatography eluting in a water/methanol system to yield the target compounds as off-white solids.

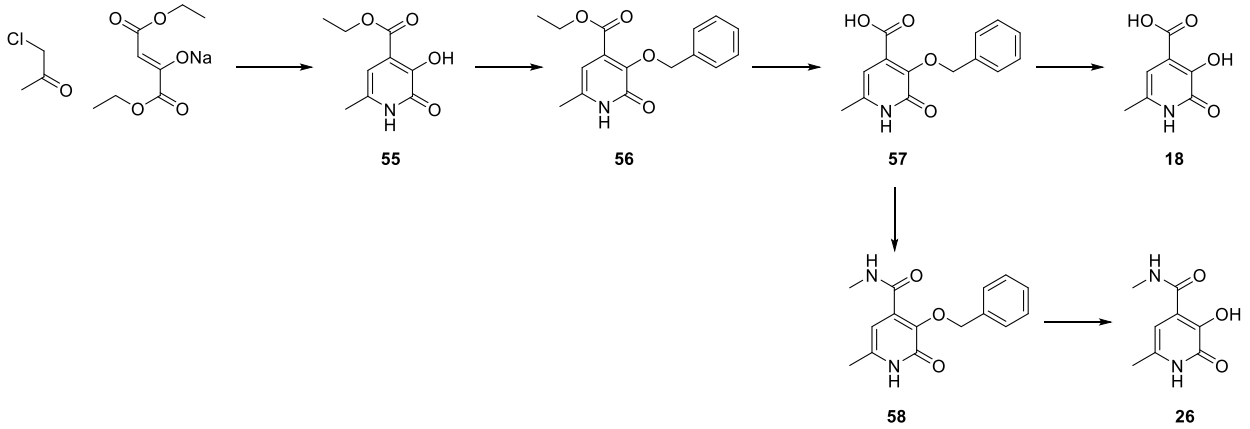
3-Hydroxy-6-methyl-4-oxo-1,4-dihydropyridine-2-carboxylic acid (17). Compound **17** was isolated as a white solid in 74 % yield, according to the general procedure. ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.92 (s, 1H), 2.48 – 2.38 (m, 3H). ^{13}C NMR (126 MHz, DMSO-d_6): δ 174.66 (s), 166.15 (s), 164.07 (s), 149.36 (s), 135.69 (s), 112.39 (s), 20.07 (d, J = 4.3 Hz). HR-ESI-MS Experimental: 168.0302. Calculated for $[C_7H_6NO_4]^-$: 168.0302. Δ = 0.0 ppm.

3-Hydroxy-1,6-dimethyl-4-oxo-1,4-dihydropyridine-2-carboxylic acid (20). Compound **20** was isolated as an off-white solid in 66 % yield, according to the general procedure. ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.98 (s, 1H), 4.13 (s, 3H), 2.48 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6): δ 164.61 (s), 162.31 (s), 152.62 (s), 148.08 (s), 129.44 (s), 113.00 (s), 20.89 (s). HR-ESI-MS Experimental: 182.0461. Calculated for $[C_8H_8NO_4]^-$: 182.0459. Δ = 1.1 ppm.

1,3-Dihydroxy-6-methyl-4-oxo-1,4-dihydropyridine-2-carboxylic acid (21). Compound **21** was isolated as an off-white solid in 57 % yield, according to the general procedure. ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.28 (s, 1H), 2.25 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6): δ 174.26 (s), 165.74 (s), 164.73 (s), 145.78 (s), 139.82 (s), 112.46 (d, J = 2.7 Hz), 20.00 (s). ESI-MS Experimental: 186.03 Calculated for $[C_7H_8NO_5]^+$: 186.15.

3-Hydroxy-6-methyl-4-thioxo-4H-pyran-2-carboxylic acid (22). 3-Hydroxy-6-methyl-4-oxo-4H-pyran-2-carboxylic acid (100 mg, 0.59 mmol) was dissolved in THF (15 mL) in a 50 mL round bottom flask. 1,1,1,3,3,3-Hexamethyldisiloxane (0.41 mL, 1.9 mmol) was added and stirred. Phosphorous pentasulfide (65.3 mg, 0.29 mmol) was added. The reaction was monitored by TLC and ferric chloride test. After one hour, the reaction mixture was evaporated under vacuum, and the residual solids were purified by silica column chromatography to afford **22** as a yellow solid, 51% yield. ^{1}H NMR (400 MHz, CD_3OD): δ 7.39 (s, 1H), 2.36 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6): δ 192.17 (s), 164.73 (s), 159.10 (s), 153.78 (s), 135.83 (s), 126.20 (d, J = 1.6 Hz), 19.28 (s). HR-ESI-MS Experimental: 184.9916. Calculated for $[C_7H_5O_4S]^-$: 184.9914. Δ = 1.1 ppm.

Scheme S3



Ethyl 3-hydroxy-6-methyl-2-oxo-1,2-dihydropyridine-4-carboxylate (55). Sodium diethyloxylacetate (1.14 g, 5.4 mmol) was dissolved in THF (30 mL). Chloroacetone (0.602 g, 6.51 mmol) was added to the stirring solution. Upon stirring for 10 min, saturated ammonia (32 mL, 16 mmol) solution was added and $AlCl_3 \cdot 6H_2O$ (0.13 g, 0.54 mmol) was then carefully added. The reaction was stirred under nitrogen for 5 d over which time the reaction turned a bright orange and an orange precipitate appeared. The resulting orange solid was filtered and taken up in 1M HCl and stirred for 30 min. The suspension was then filtered and rinsed with distilled water and crystalized from hot ethanol to afford **55** in 25-31% yield. ^{1}H NMR (400 MHz, CD_3OD): δ 6.21 (s, 1H), 4.31 (q, J = 7.0 Hz, 2H), 2.34 (s, 3H), 1.20 (dd, J = 15.5, 8.0 Hz, 3H). ESI-MS Experimental: 198. Calculated for $[C_9H_{12}NO_4]^+$: 198.20.

Ethyl 3-(benzyloxy)-6-methyl-2-oxo-1,2-dihydropyridine-4-carboxylate (56). Ethyl 3-hydroxy-6-methyl-2-oxo-1,2-dihydropyridine-4-carboxylate (1.0 g, 5.1 mmol) and potassium carbonate (0.77 g, 5.6 mmol) were dissolved in water (60 mL) with the aid of sonication. To this solution was added a solution of benzyl bromide (0.66 mL, 5.6 mmol) in CH_2Cl_2 (50 mL). Tetrabutylammonium chloride, hydrate (0.75 g, 2.5 mmol) was added as a phase transfer catalyst

for the reaction. The solution was stirred at 40 °C overnight, and when the reaction was complete by TLC, the two layers were separated. The aqueous layer was washed twice with CH_2Cl_2 and all organic portions were combined and dried, then concentrated under vacuum. The resulting solid was purified by column chromatography affording **56** in 62 % yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.56 – 7.48 (m, 2H), 7.32 (dt, J = 8.5, 6.9 Hz, 3H), 6.18 (s, 1H), 5.26 (s, 2H), 4.28 (q, J = 7.1 Hz, 2H), 2.34 (s, 3H), 1.27 (dd, J = 15.1, 8.0 Hz, 3H). ESI-MS Experimental: 288. Calculated for $[C_{16}H_{18}NO_4]^+$: 288.32.

3-(Benzyloxy)-6-methyl-2-oxo-1,2-dihydropyridine-4-carboxylic acid (57). Ethyl 3-(benzyloxy)-6-methyl-2-oxo-1,2-dihydropyridine-4-carboxylate was stirred in 60 mL of a 1:1 mixture of THF:4% KOH overnight under nitrogen, after which time organic solvents were evaporated under vacuum. The resulting aqueous solution was acidified to pH <2 with 4M HCl, resulting in **57** as a white precipitate which was collected by filtration in 94% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.52 (d, J = 7.3 Hz, 2H), 7.39 – 7.29 (m, 3H), 6.12 (s, 1H), 5.35 (s, 2H), 2.29 (s, 3H). ESI-MS Experimental: 260. Calculated for $[C_{14}H_{13}NO_4]^+$: 260.26.

3-Hydroxy-6-methyl-2-oxo-1,2-dihydropyridine-4-carboxylic acid (18). 3-(Benzyloxy)-6-methyl-2-oxo-1,2-dihydropyridine-4-carboxylic acid (**57**) was dissolved in a 5:5:1 mixture of hydrochloric acid, 37% (5 mL), acetic acid (5 mL), and TFA (1 mL). The mixture was stirred overnight at room temperature. Upon full deprotection as evidence by silica TLC and $FeCl_3$ stain analysis, the reaction was dried under vacuum and the residue purified by C18 chromatography eluting with a water-methanol system to yield **18** as a white solid in 95% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.39 (s, 1H), 2.21 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 170.22 (s),

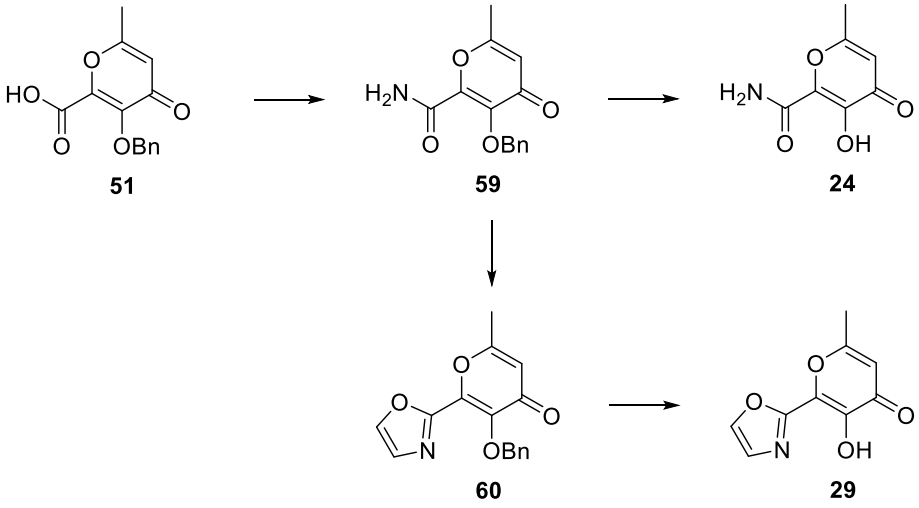
158.95 (s), 149.64 (s), 132.63 (s), 116.08 (s), 101.76 (s), 18.32 (s). HR-ESI-MS Experimental: 168.0303. Calculated for $[C_7H_6NO_4]^-$: 168.0302. Δ = 0.6 ppm.

3-(Benzyloxy)-N,6-dimethyl-2-oxo-1,2-dihydropyridine-4-carboxamide (58). 3-(Benzyloxy)-6-methyl-2-oxo-1,2-dihydropyridine-4-carboxylic acid (250 mg, 0.96 mmol) was dissolved and stirred in DMF (25 mL) in a round bottom flask. Triethylamine (0.20 mL, 1.4 mmol) and HATU (550 mg, 1.4 mmol) were added and stirred for 10 min. Methylamine (0.72 mL, 1.4 mmol) was then added and the reaction was stirred overnight under nitrogen at 55 ºC. Upon completion, all solvents were removed under vacuum and the residual solid was partitioned between ethyl acetate and saturated bicarbonate solution. The organic layer was separated, and the aqueous layer was washed once with ethyl acetate. The combined organics were dried and further purified by silica chromatography to yield the product as a white solid, 83% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.32 (d, J = 7.8 Hz, 5H), 6.24 (s, 1H), 5.21 (s, 2H), 2.74 (s, 3H), 2.22 (s, 3H). ESI-MS Experimental: 273. Calculated for $[C_{15}H_{17}N_2O_3]^+$: 273.30.

3-Hydroxy-N,6-dimethyl-2-oxo-1,2-dihydropyridine-4-carboxamide (26). 3-(Benzyloxy)-N,6-dimethyl-2-oxo-1,2-dihydropyridine-4-carboxamide) was dissolved in a 5:5:1 mixture of Hydrochloric Acid, 37% (5 mL), Acetic Acid (5 mL), and TFA (1 mL) and was stirred for 12 h under nitrogen at 40 ºC. Upon deprotection, all solvent acid was removed, and the resulting solid was co-evaporated exhaustively with methanol. Resultant solids were confirmed as pure **26** in quantitative yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.79 (s, 1H), 8.35 (d, J = 4.7 Hz, 1H), 6.22 (s, 1H), 2.76 (d, J = 4.7 Hz, 3H), 2.07 (d, J = 0.8 Hz, 3H). ^{13}C NMR (126 MHz, DMSO-d_6): δ

166.74 (s), 159.37 (s), 146.44 (s), 132.53 (s), 118.55 (s), 101.49 (d, J = 2.5 Hz), 26.57 (s), 18.48 (s). HR-ESI-MS Experimental: 181.0620. Calculated for $[C_8H_9N_2O_3]^-$: 181.0620. Δ = 0.0 ppm.

Scheme S4



3-(Benzyloxy)-6-methyl-4-oxo-4H-pyran-2-carboxamide (59). Compound **51** (500 mg) was dissolved and stirred in DMF (25 mL). Triethylamine (0.67 mL, 4.8 mmol) and HATU (887 mg, 2.3 mmol) were added and stirred for 10 min. Ammonium chloride (155 mg, 2.9 mmol) was then added and the reaction was stirred overnight under nitrogen at 60 ºC. Upon completion, all solvents were removed under vacuum and the residual solid was partitioned between ethyl acetate and saturated bicarbonate solution. The organic layer was separated, and the aqueous layer was washed once with ethyl acetate. The combined organics were dried and further purified by silica chromatography to yield the product as a white solid, 60% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.94 (d, J = 48.4 Hz, 2H), 7.54 – 7.05 (m, 5H), 6.35 (s, 1H), 5.15 (s, 2H), 2.26 (s, 3H). ESI-MS Experimental: 260. Calculated for $[C_{14}H_{14}NO_4]^+$: 260.26.

3-Hydroxy-6-methyl-4-oxo-4H-pyran-2-carboxamide (24). 3-(Benzyloxy)-6-methyl-4-oxo-4H-pyran-2-carboxamide (**59**) was dissolved in a 5:5:1 mixture of HCl (37%, 5 mL), HOAc (5 mL), and TFA (1 mL) and was stirred for 12 h under nitrogen at 25 ºC. Upon deprotection, all solvent acid was removed, and the resulting solid was co-evaporated exhaustively with methanol. Resultant solids were confirmed as pure **24** in quantitative yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.46 (s, 1H), 8.30 (s, 2H), 6.28 (s, 1H), 2.27 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6): δ 173.99 (s), 165.87 (s), 165.08 (s), 148.73 (s), 136.33 (s), 113.29 (d, J = 3.2 Hz), 19.75 (s). HR-ESI-MS Experimental: 168.0303. Calculated for $[C_7H_6NO_4]^-$: 168.0302. Δ = 0.6 ppm.

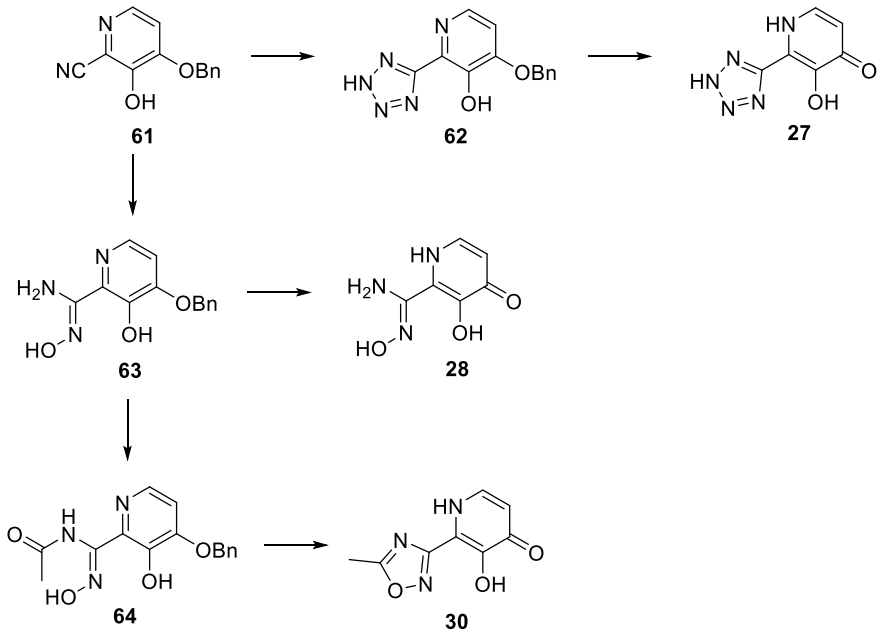
3-Hydroxy-N,6-dimethyl-4-oxo-4H-pyran-2-carboxamide (25). Compound **25** was prepared according to the same procedure as **24**, in 73% yield. ^{1}H NMR (400 MHz, CD_3OD): δ 6.37 (s, 1H), 2.94 (s, 3H), 2.40 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6): δ 174.09 (s), 165.09 (s), 163.90 (s), 147.98 (s), 136.71 (s), 113.29 (s), 26.32 (s), 19.82 (s). HR-ESI-MS Experimental: 182.0459. Calculated for $[C_8H_8NO_4]^-$: 182.0459. Δ = 0.0 ppm.

3-(Benzyloxy)-6-methyl-2-(oxazol-2-yl)-4H-pyran-4-one (60). 3-(Benzyloxy)-6-methyl-4-oxo-4H-pyran-2-carboxamide (125 mg, 0.48 mmol) was taken up in dioxane (1 mL) in a 10 mL microwave vessel with a stir bar. 2-Bromo-1,1-diethoxyethane (0.51 mL, 3.4 mmol) was added and the mixture was irradiated at 155 ºC for 65 min. Solvent was then removed under vacuum, and the resultant solids were take forward with no further purification.

3-Hydroxy-6-methyl-2-(oxazol-2-yl)-4H-pyran-4-one (29). 3-(Benzyloxy)-6-methyl-2-(oxazol-2-yl)-4H-pyran-4-one (**60**) was dissolved in a 5:5:1 mixture of HCl (37%, 5 mL), HOAc

(5 mL), and TFA (1 mL) and was stirred for 12 h under nitrogen at 45 ºC. Upon deprotection, all solvent acid was removed, and the resulting solid was co-evaporated several times with MeOH. Resultant solids were purified by C18 chromatography to yield **29** as an off-white solid in 30% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.08 (s, 1H), 8.34 (d, 1H), 7.50 (d, 1H), 6.37 (s, 1H), 2.33 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 178.96 (s), 170.59 (s), 159.28 (s), 149.35 (s), 145.98 (s), 139.78 (s), 133.88 (s), 116.54 (s), 24.60 (s). HR-ESI-MS Experimental: 216.0265. Calculated for $[C_9H_7NO_4Na]^+$: 216.0267. Δ = -0.9 ppm.

Scheme S5



4-(Benzyloxy)-2-(1H-tetrazol-5-yl)pyridin-3-ol (62). 4-(Benzyloxy)-3-hydroxypicolinonitrile (300 mg, 1.326 mmol) was dissolved in DMF (10 mL). Ammonium chloride (106 mg, 2.0 mmol) and sodium azide (129 mg, 2.0 mmol) were added and the mixture stirred at between 110-120 ºC for 3-4 h. The mostly-clear suspension turned opaque over this time. DMF was removed under vacuum (Explosion hazard: possible formation of azidic acid) and the residual was taken up in

water and sonicated. The mixture was put on ice with stirring and 4M HCl was added very carefully (only a few drops) to adjust the pH to acidic. The resultant solids were stirred on ice for 30 min and were isolated by filtration to afford 4-(benzyloxy)-2-(1H-tetrazol-5-yl)pyridin-3-ol (140 mg, 0.52 mmol, 39% yield) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.20 (d, J = 5.5 Hz, 1H), 7.51 (d, J = 7.2 Hz, 2H), 7.41 (t, J = 7.5 Hz, 2H), 7.38 – 7.33 (m, 2H), 5.34 (s, 2H). ESI-MS Experimental: 268.37. Calculated for $[C_{13}H_{10}N_5O_2]^-$: 268.24.

3-Hydroxy-2-(2H-tetrazol-5-yl)pyridin-4(1H)-one (27). Compound **27** was prepared according to the same procedure as **29**, to afford **27** as a light-pink solid in 58% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.87 (d, J = 6.4 Hz, 1H), 6.77 (d, J = 6.4 Hz, 1H). ^{13}C NMR (126 MHz, DMSO-d_6): δ 152.89 (s), 144.58 (s), 137.43 (s), 127.84 (s), 123.46 (s), 112.55 (s). HR-ESI-MS Experimental: 178.0368. Calculated for $[C_6H_4N_5O_2]^-$: 178.0370. Δ = -1.1 ppm.

4-(Benzyloxy)-N',3-dihydroxypicolinimidamide (63). Compound **61** (1g, 4.42 mmol), hydroxylamine hydrochloride (0.614 g, 8.8 mmol), and triethylamine (1.23 mL, 8.8 mmol) were taken up in ethanol (50 mL) and refluxed at 80 ºC for 4 h. At this time solvent was removed under vacuum, and the residual solids were taken up in 1M HCl. The aqueous solution was washed once with ethyl acetate, and the pH was adjusted to ~10 with 6M NaOH. A solid precipitate formed and was isolated by filtration to yield **63** as a white solid in 74% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.11 (s, 1H), 10.18 (s, 1H), 7.96 (d, J = 5.3 Hz, 1H), 7.45 (d, J = 6.7 Hz, 2H), 7.39 (t, J = 7.3 Hz, 3H), 7.11 (d, J = 5.3 Hz, 1H), 6.33 (s, 2H), 5.18 (s, 2H). ESI-MS Experimental: 260. Calculated for $[C_{13}H_{14}N_3O_3]^+$: 260.27.

N',3-Dihydroxy-4-oxo-1,4-dihydropyridine-2-carboximidamide (28). Compound **28** was prepared according to the same procedure as **29**, to afford **28** as a pink solid in 42% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.94 (s, 1H), 10.08 (s, 1H), 7.81 (d, 1H), 6.78 (d, 1H), 6.28 (s, 2H). ^{13}C NMR (126 MHz, DMSO-d_6): δ 154.17 (s), 152.86 (s), 144.10 (s), 140.42 (s), 132.23 (s), 112.40 (s). ESI- HR-ESI-MS Experimental: 168.0418. Calculated for $[C_6H_6N_3O_3]^-$: 168.0415. Δ = 1.8 ppm.

N-((4-(Benzyloxy)-3-hydroxypyridin-2-yl)(hydroxyimino)methyl)acetamide (64). To a 5 ºC solution of **63** (200 mg, 0.771 mmol) in dry THF (20 mL) stirring on ice was added acetic anhydride (0.073 mL, 0.77 mmol) dropwise. The solution stirred 30 min on ice and then further 1.5h at room temperature. Solvent was then evaporated under vacuum and the solid residue was purified by silica chromatography to give **64** in 83% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.73 (s, 1H), 8.04 (dd, J = 5.2, 1.1 Hz, 1H), 7.48 – 7.32 (m, 5H), 7.21 (d, J = 5.2 Hz, 1H), 5.22 (s, 2H), 2.16 (d, J = 1.3 Hz, 3H). ESI-MS Experimental: 302. Calculated for $[C_{15}H_{16}N_3O_4]^+$: 302.31.

3-Hydroxy-2-(5-methyl-1,2,4-oxadiazol-3-yl)pyridin-4(1H)-one (30). Compound **64** was dissolved in a 5:5:1 mixture of HCL (37%, 5 mL), HOAc (5 mL), and TFA (1 mL) and was stirred for 18 h under nitrogen at reflux (85 ºC). Upon completion, all solvent acid was removed, and the resulting solid was co-evaporated several times with methanol. Resultant solids were purified by C18 chromatography to yield **30** as a white solid in 41% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.34 (s, 1H), 7.87 (d, J = 7.0 Hz, 1H), 6.29 (d, J = 6.9 Hz, 1H), 2.17 (s, 3H). ^{13}C NMR (126

MHz, DMSO-d_6): δ 149.26 (s), 147.48 (s), 139.07 (s), 114.18 (s), 101.21 (s), 84.76 (s), 23.30 (s). HR-ESI-MS Experimental:192.0416. Calculated for $[C_8H_6N_3O_3]^-$: 192.0415. Δ = 0.5 ppm.

Protein Expression and Purification

Pandemic 2009 influenza H1N1 N-terminal PA endonuclease Δ52-64:Gly truncated construct was expressed from a pET-28a parent vector containing a kanamycin-resistance reporter gene, expression inducible by the Lac 1 operon. Endonuclease was expressed with an N-terminal, 8-histidine tag that was connected by a cleavable TEV protease site. This endonuclease construct expresses as inclusion bodies, and we were unable to optimize around this limitation. As such, large growth of cells were required. The transformation protocol was adapted from pET system manual (Novagen) using single competent BL21 cells. Briefly, 1µL of 25 ng/µL recombinant plasmid was used for transformation. Cells were mixed by gently mixing with plasmid and were heat shocked at 42 ºC for 30 sec followed by incubation on ice for 5 min. Outgrowth was plated on LB agarose plates containing 50 µg/mL kanamycin and incubated overnight at 37 ºC. One colony was scraped from the LB plate and added to 50 mL of SOC broth containing 50 µg/mL kanamycin and was incubated for 5 h at 37 ºC with shaking at 125 rpm. Glycerol stocks of this culture were prepared (0.9 mL cultured media + 0.1 mL 80% glycerol) and flash frozen for future expressions. 100 mL of SOC media containing 50 µg/mL kanamycin was combined with 1 mL frozen glycerol stock or 1 mL of the previously described 5 h growth and was incubated with shaking at 100-125 rpm overnight at 37 ºC as a starter culture. This starter culture was then equally divided into 9×1L batches of expression media (TB media with added 0.2% dextrose, 0.1 mM $MnCl_2$, and 0.1 mM $MgSO_4$) containing 50 µg/mL kanamycin. Cells were grown to the beginning of log phase (OD_{600} = between 0.6-0.8) at 37 ºC with shaking at 100-125 rpm. Expression of PA

endonuclease was then induced by addition of IPTG to a final concentration of 0.2 mM. The media was grown with shaking overnight at ~18 ºC. After approximately 18 h, the cells were harvested by centrifuging at 2000g for 30 min at 4 ºC. The resulting paste was stored at -80 ºC prior to lysis.

Cell paste was thawed on ice for 2 h and re-suspended in 25-35 mL of lysis buffer (20 mM Na_2PO_4, 500 mM NaCl, 25 mM imidazole, 1 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 0.2% Triton-X, pH=7.4) plus EDTA free protease inhibitor (~1:1 mixture of pellet and lysis buffer) and lysed by sonication - 5×25 sec pulses with 2 min rest periods on ice. To the cell lysates was added DNAse1 to a final concentration of 10-100 µg/mL, and the lysates were shaken at 125 rpm for 15-30 min on ice until the consistency of the lysate became free-flowing. Cell debris was then pelleted by centrifugation at 10000 rpm 35-45 min at 4 ºC. The supernatant was decanted from the pellet, and a HisTrap HP (Pharmacia) column was utilized to isolate His-tagged protein from the cell lysates according to the manufacturer's recommendations. Briefly, cell-free lysates from 4L of growth were loaded on 1×5mL column that had previously been charged with Ni ions. The column was then washed with binding buffer (20 mM Na_2PO_4, 500 mM NaCl, 25 mM imidazole, pH=7.4) until fraction absorbance reached a steady baseline. The protein was then eluted over a 45 min gradient at a flow rate of 4 mL/min, from 0-100% elution buffer (20 mM Na_2PO_4, 500 mM NaCl, 500 mM imidazole, pH=7.4). Pure target protein eluted between 40-60% elution buffer. SDS-PAGE analysis showed a band corresponding to endonuclease protein running at ~23kD with several small impurities.

Fractions containing endonuclease protein were combined in a 10K MWCO dialysis bag with 1000 units of TEV protease and were dialyzed against dialysis buffer (100 mM NaCl, 1 mM DTT, 1 mM $MnCl_2$, 20 mM Tris, 5% glycerol, pH=8.0) overnight with three buffer exchanges. The proteolytic cleavage of the protein is slow, and greatly benefits from the addition of excess

TEV protease. A white precipitate formed over time; however, while this precipitate contained some precipitated endonuclease, it is composed primarily of insoluble imidazole complexes. After buffer exchange, the solution was filtered through a 0.45 μm filter and was concentrated to 5-10 mg/mL employing a pressurized Amicon system and/or spin Amicon systems. The concentrated protein was then purified on a gel-permeation size exclusion column (GE Superdex 75, 10/300 GL) according to the manufacturer's recommendations in buffer (150 mM NaCl, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 20 mM HEPES, pH=7.5). A large peak corresponding to the cleaved endonuclease construct eluted at approximately 12 mL eluent. A small shoulder before the main peak was occasionally observed, which contained primarily uncleaved and/or unfolded endonuclease construct. Fractions containing pure cleaved product were combined and, depending on final concentration, concentrated to 3-5 mg/mL for crystallization experiments and storage. Stored protein was flash-frozen in liquid nitrogen and was kept at -80 ºC. This protein was viable for use in nuclease and DSF assays or for crystallography experiments up to one year after initial freezing.

Table S1. Cross-inhibition studies of compound **1** against PA_N and eight different metalloenzyme. Percent inhibition values from single concentration screens (200 μM) are shown.

Number	Compound	Endonuclease	HIV-IN 3′-Processing	HIV-IN Strand Transfer	hCAII	MMP-2	Glo-1	NDM-1	HDAC-6	Human Arginase 1	Human MetAP1
1		100% (IC_{50} = 43 ± 9 nM)	No Inhibition	37%	No Inhibition	11%	11%	13%	No Inhibition	8%	27%

Protein Crystallography

Crystals for soaking experiments (compound **3**) were obtained by utilizing a protein solution of 4-10 mg/mL in 150 mM NaCl, 20 mM HEPES pH 7.5, and 2 mM $MnCl_2$ and a precipitant solution of 25 % PEG 4000, 200 mM sodium acetate, and 100 mM Tris, pH=8.0. The protein and precipitant solutions were combined in a 2:1 ratio in hanging drop 24-well plates, with crystal forming within 3 days at 33-35 °C. MBPs were soaked in by incubating crystals in a 5-20 mM MBP precipitant solution. For co-crystallization experiments (compound **1**, **2**, and **33**), purified protein for crystallization was stored at 4-6 mg/mL at 4° C in buffer consisting of 150 mM NaCl, 20 mM HEPES at pH 7.5, 2 mM $MgCl_2$, and 2 mM $MnCl_2$. Co-crystallization experiments were set up in 24-well hanging drop plates with a 2:1 or 3:1 ratio of protein stock to reservoir solution. Reservoir solution consisted of 0.5 mM inhibitor, 25-40% PEG 4000, 100 mM Tris at pH 8.35, and 200-220 mM sodium acetate. Protein crystals grew by vapor diffusion at 33-35 °C in hanging drops. Colorless crystals with octahedral morphology appeared within 24 h and reached full size after 3-4 days with little to moderate protein precipitation observed. Crystals were typically 50 to 150 microns in each dimension. For both soaking and co-crystallization experiments, crystals were cryo-protected with reservoir solution supplemented with 10-20% ethylene glycol.

X-ray data collection and refinement

Crystals were screened for resolution and cryo-protection conditions on an in-house X-ray generator. For these experiments and for data collection of compound **3**, diffraction data were collected on a Bruker X8 Proteum diffractometer at 100 K, using a Bruker Microfocus Rotating Anode (MicroSar FR-592) X-ray generator with a Bruker APEX II CCD detector at wavelength 1.54178 Å. The data for the compound **2** structure were collected at the ALS 5.0.2 beamline using a double-crystal Si(111) monochromator set to a wavelength of 1.00 Å with a Pilatus3 6M 25 Hz detector. The datasets for compounds **1** and **33** were collected at the ALS 5.0.1 beamline using a single-crystal, cylindrically bent, Si(220) monochromator set to a wavelength of 0.977 Å with a Pilatus 6M 25 Hz detector. All data sets were integrated, merged, and scaled within iMosflm and the CCP4 software suite.[2] For all structures, phases were determined by molecular replacement against a previously published PA_N endonuclease structure (PDB ID: 4AWM)[3] using PHASER. Structures were refined with REFMAC5.[4] All figures were made in PyMOL.[5]

Table S2. X-ray crystallographic data collection and refinement statistics

	Compound **2**	Compound **3**	Compound **1**	Compound **33**
PDB ID	6DCY	6DCZ	6DZQ	6E0Q
Data Collection Statistics				
Space group	$P6_222$	$P6_222$	$P6_222$	$P6_222$
Cell dimensions a, b, c (Å); α, β, γ (°)	75.54, 75.54, 120.36; 90, 90, 120	75.84, 75.84, 121.30; 90, 90, 120	75.20, 75.20, 119.62; 90, 90, 120	75.09, 75.09, 119.67; 90, 90, 120
Resolution (Å)	37.77 – 2.08 (2.14 – 2.08)*	30.33 – 2.89 (3.07 – 2.89)*	44.05- 2.25 (2.32 – 2.25)*	44.03 – 2.35 (2.43 – 2.35)*
R_{merge}	0.083 (0.786)*	0.117 (0.485)*	0.063 (0.523)*	0.07 (0.517)
R_{pim}	0.021 (0.189)	0.056 (0.314)	0.014 (0.123)	0.016 (0.086)
$CC_{1/2}$	0.999 (0.910)	0.996 (0.848)	1.000 (0.978)	0.999 (0.978)
Wilson *B*-value $(Å)^2$	32.0	16.938	34.666	33.822
I/σ(I)	19.1 (3.4)*	12.5 (2.5)*	41.5 (8.5)*	40.3 (8.6)*
Completeness (%)	100.0 (100.0)*	95.7 (99.8)*	100.0 (100.0)*	100.0 (100.0)
No. unique reflections	12,849 (974)*	4826 (773)*	10,089 (899)*	8874 (846)*
Redundancy	16.9 (18.1)*	8.8 (5.4)*	35.3 (35.0)*	35.1 (36.8)
Refinement Statistics				
Resolution	37.77 – 2.08	28.89 – 2.89	44.05 – 2.25	44.03 – 2.35
R_{work}/R_{free}	0.225/0.271	0.200/0.276	0.205/0.253	0.200/0.258
Average B-factor $(Å)^2$	44.6	43.9	43.6	44.6
Ligand *B*-factor $(Å)^2$	47.2	35.4	37.4	40.0
R.m.s.d. bond lengths (Å)	0.010	0.008	0.009	0.009
R.m.s.d. bond angles(°)	1.42	1.28	1.31	1.40
Ramachandran outliers, allowed, and favored	0 (0%), 4 (2%), 166 (98%)	1 (1%), 10 (5%), 165 (94%)	0 (0%), 4 (2%), 170 (98%)	1 (1%), 5 (3%), 168 (96%)

* Metrics for highest resolution shell given in parentheses

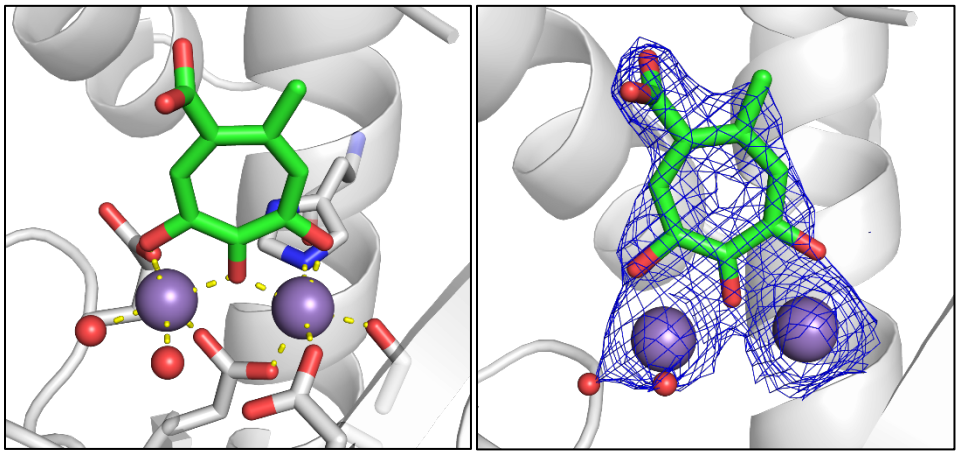
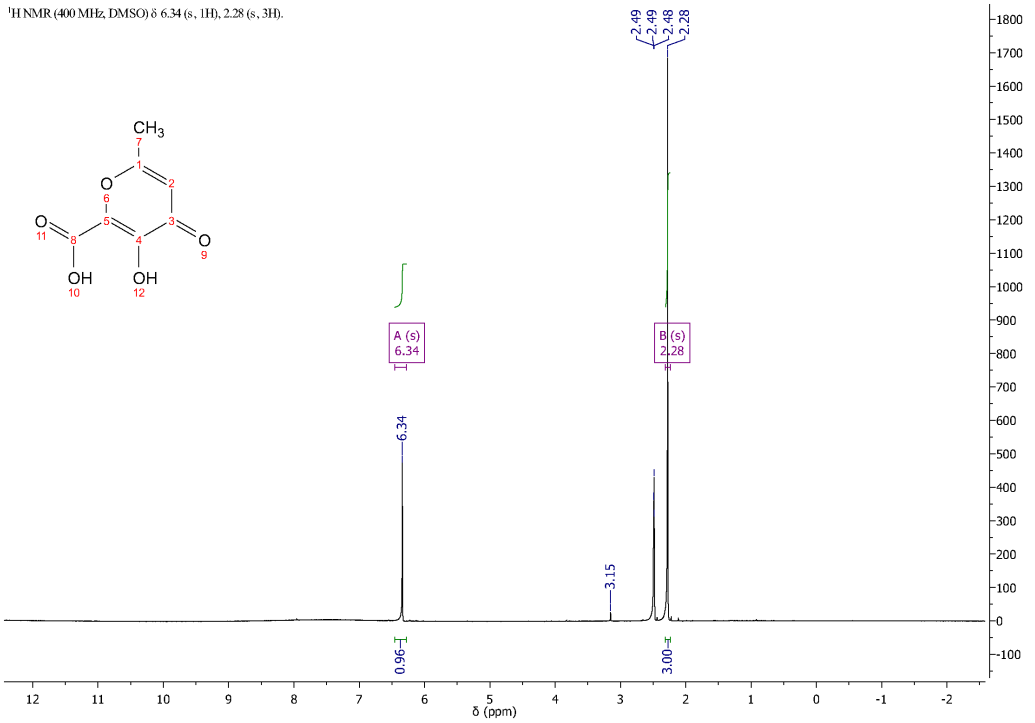
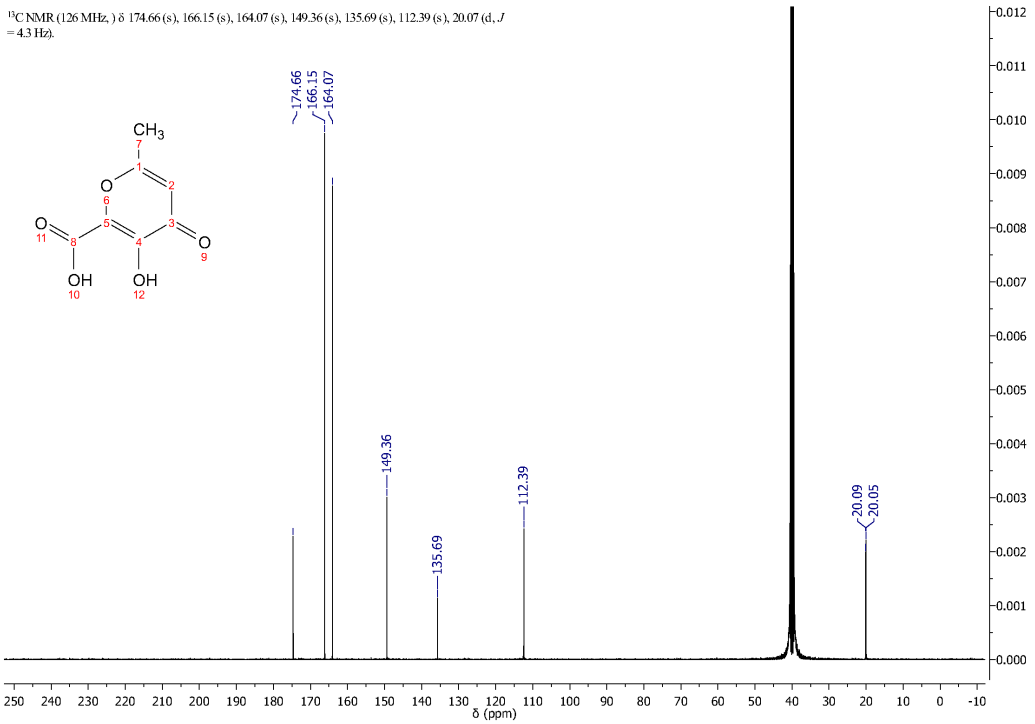


Figure S1. X-ray co-crystal structure of **33** bound to the active site metal centers of PA_N (PDB ID: 6E0Q). **33** was observed to bind similarly to **1** and **17**, with coordinating oxygen atoms replacing the three water molecules observed in the native structure. Bond distances for **33** to the metal ions are 2.1-2.2 Å. The carboxylic acid moiety was not observed to make any interactions with the protein active site or water network at this resolution, and it is inferred that the tight binding of this compound is due primarily to optimized metal coordinating interactions. Mn^{2+} ions are shown as purple spheres, water molecules as red spheres, dative bonds as yellow dashed lines, and electron density as blue mesh is the $2F_o$-F_c map contoured to 1σ.

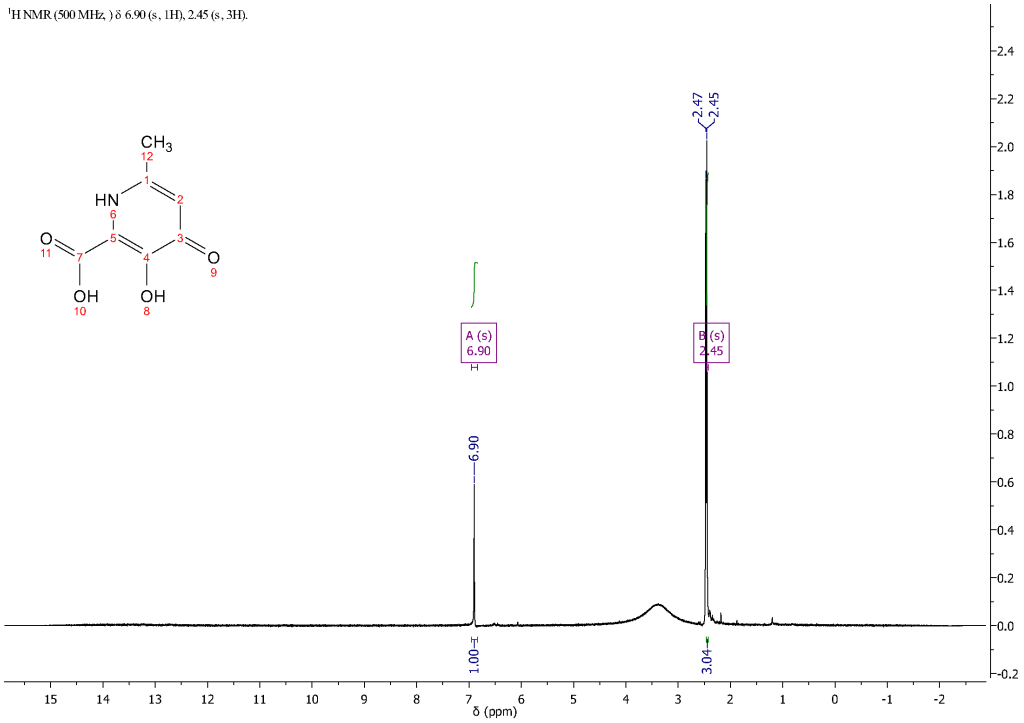
Representative NMR Spectra

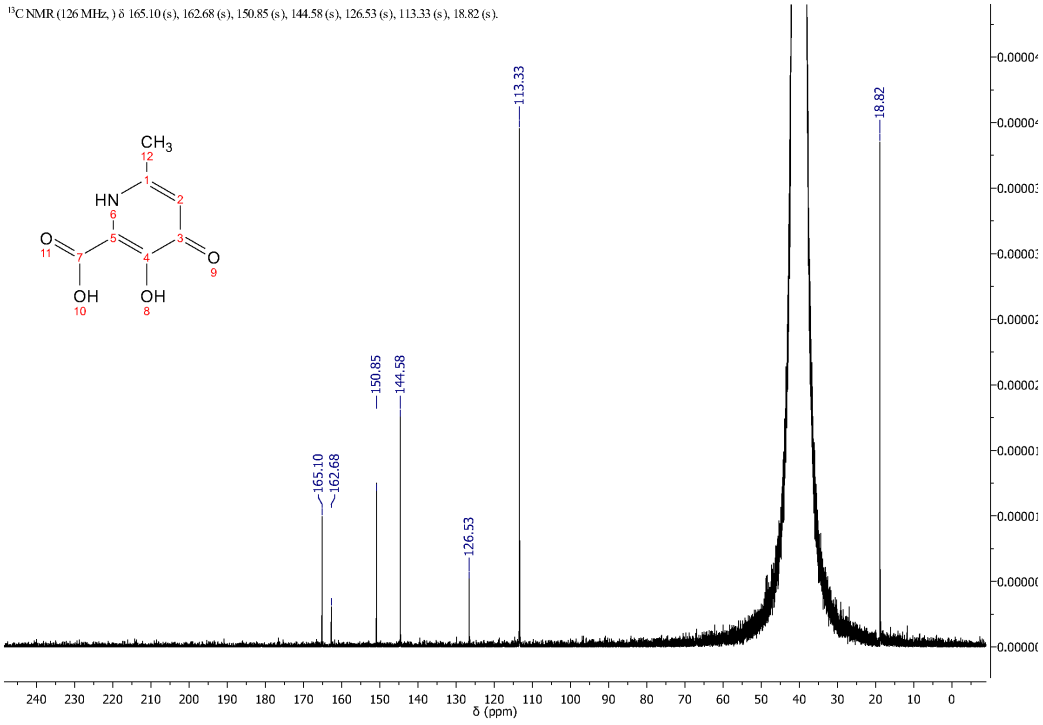
Compound **1**:



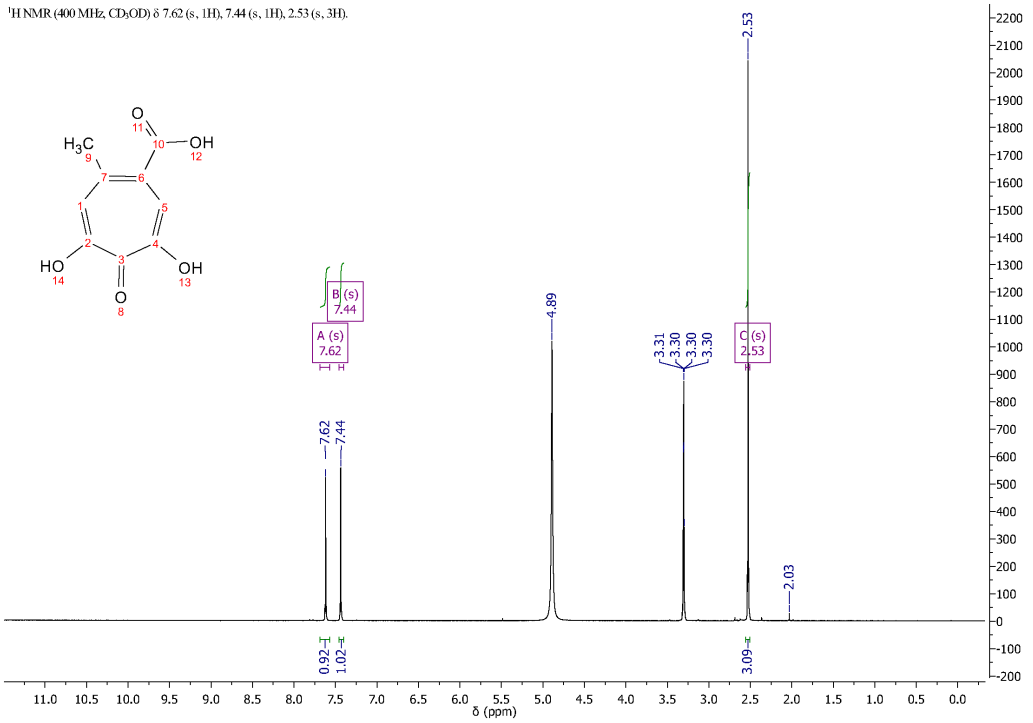


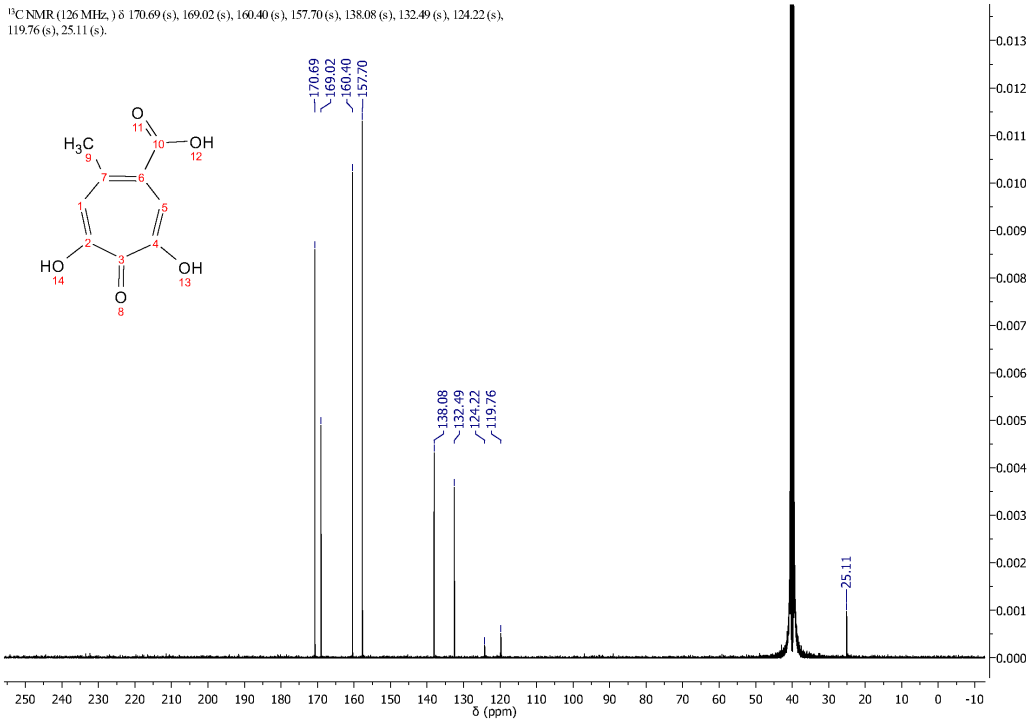
Compound **17**:


1H NMR (500 MHz,) δ 6.90 (s, 1H), 2.45 (s, 3H).
CH3
12
HN
6
OH
10
OH
8
O
9
O
11
A (s)
6.90
H
B (s)
2.45
6.90
2.47
2.45
1.00
3.04
δ (ppm)


13C NMR (126 MHz,) δ 165.10 (s), 162.68 (s), 150.85 (s), 144.58 (s), 126.53 (s), 113.33 (s), 18.82 (s).
CH3
12
HN
6
OH
10
OH
8
O
9
O
11
165.10
162.68
150.85
144.58
126.53
113.33
18.82
δ (ppm)

Compound **33**:


¹H NMR (400 MHz, CD₃OD) δ 7.62 (s, 1H), 7.44 (s, 1H), 2.53 (s, 3H).
B (s) 7.44
A (s) 7.62
7.62
7.44
4.89
3.31
3.30
3.30
3.30
2.53
2.03
0.92
1.02
3.09
δ (ppm)


¹³C NMR (126 MHz,) δ 170.69 (s), 169.02 (s), 160.40 (s), 157.70 (s), 138.08 (s), 132.49 (s), 124.22 (s), 119.76 (s), 25.11 (s).
170.69
169.02
160.40
157.70
138.08
132.49
124.22
119.76
25.11
δ (ppm)

Cell-based Activity and Toxicity

Antiviral activity and cytotoxicity assays for compounds **1, 3, 17, 18, 22, 27, 32,** and **33** against influenza A virus strain A/WSN/33 (H1N1) were performed as previously reported.[6] As expected based on the high ionizability and low molecular weight of these compounds,[7-8] none of these compounds showed appreciable cellular activity below 75 µM, and all compounds besides **32** and **33** were found to have CC_{50} values above 75 µM, with compounds **32** and **33** displaying CC_{50} = 12.5 µM and 6.3 µM, respectively. These data are shown in Figures S2 and S3.

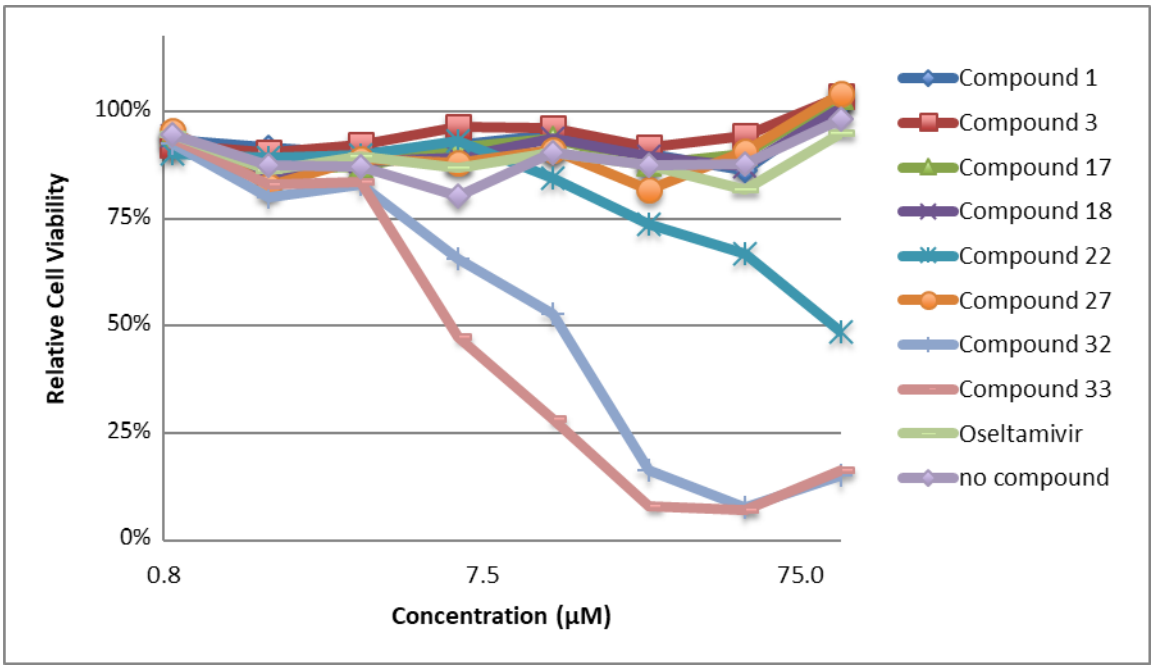


Figure S2. Cytotoxicity analysis of several MBP compounds.

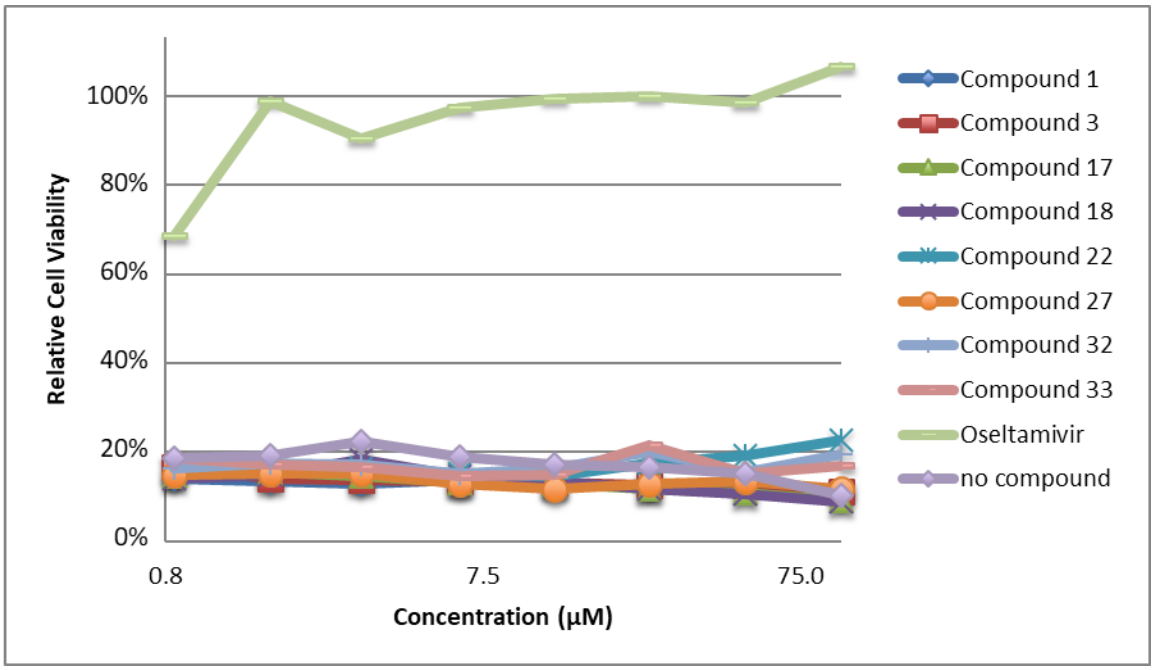
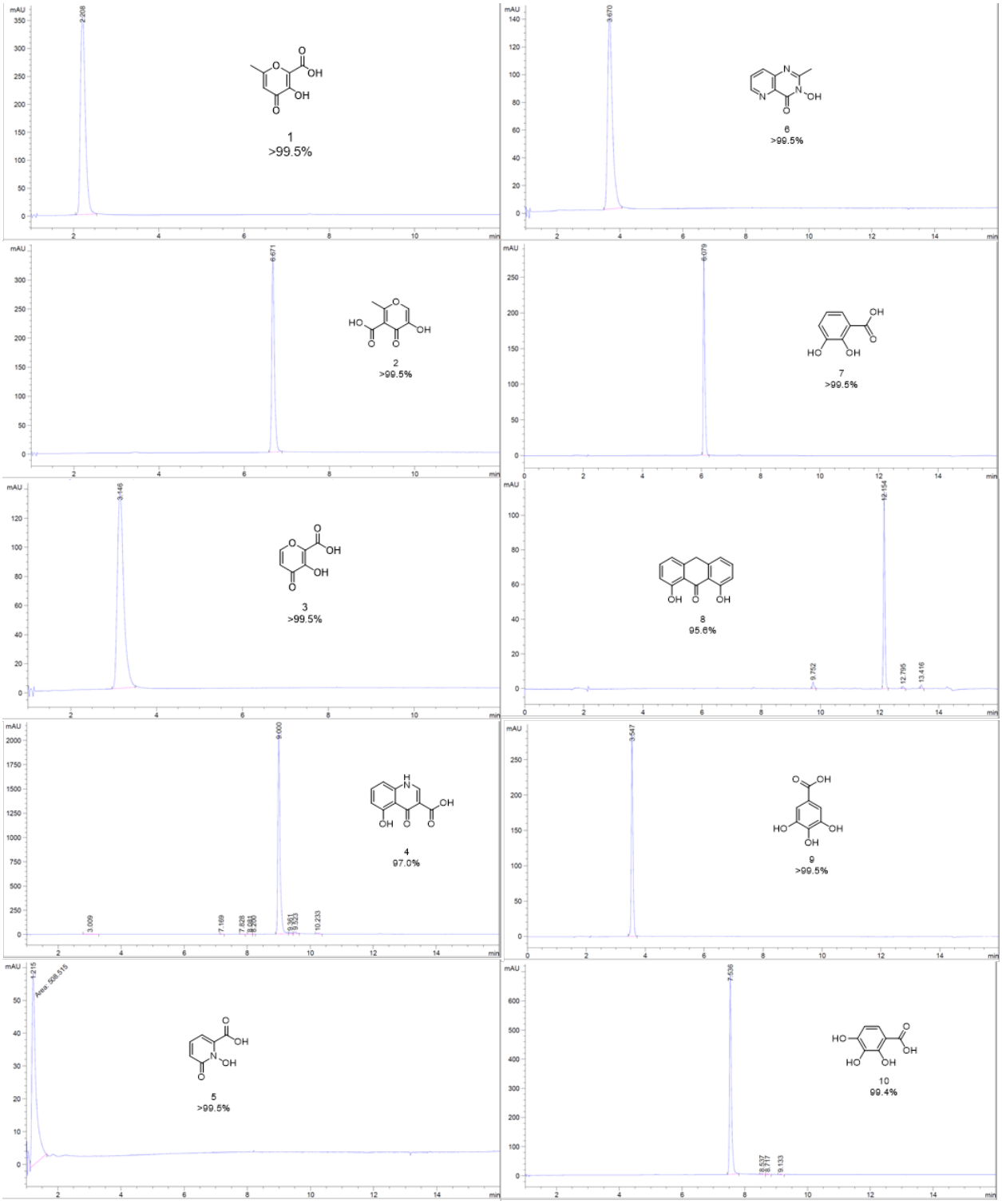
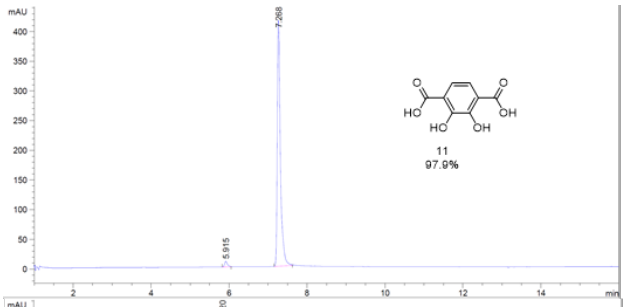


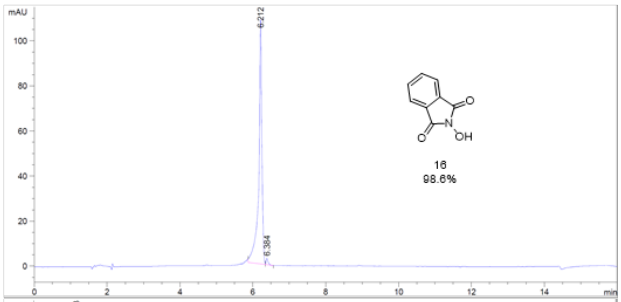
Figure S3. Cellular efficacy of several MBP compounds against influenza A virus, with oseltamivir as a control. As expected for small (MW<200) and highly ionizable fragments, these compounds showed no cellular efficacy at these concentrations, despite their high activity against PA_N in vitro.

HPLC Analysis of Assayed Compounds

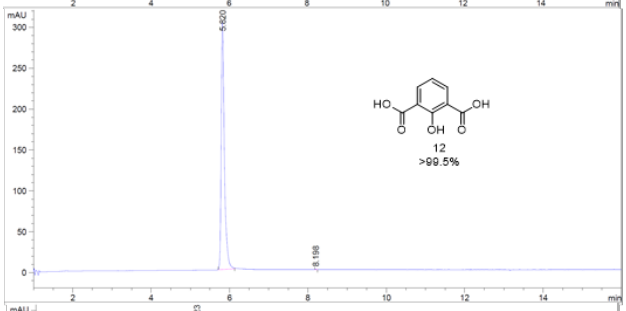




11
97.9%



16
98.6%



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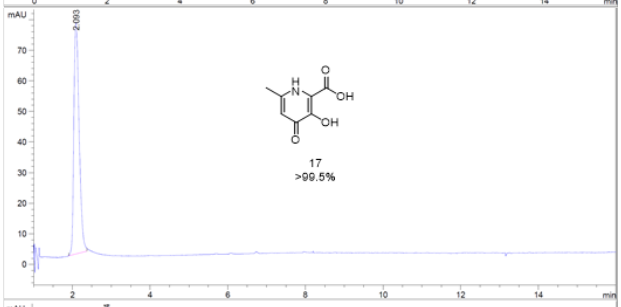
>99.5%



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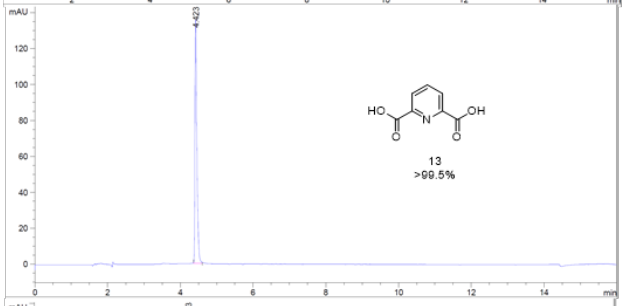

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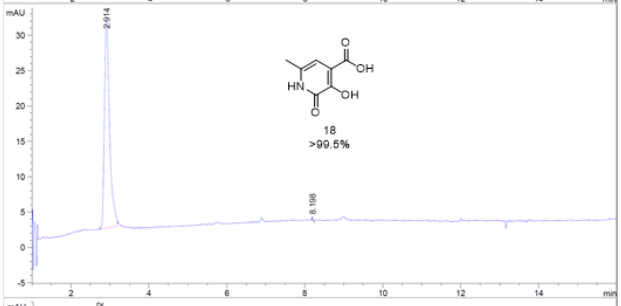

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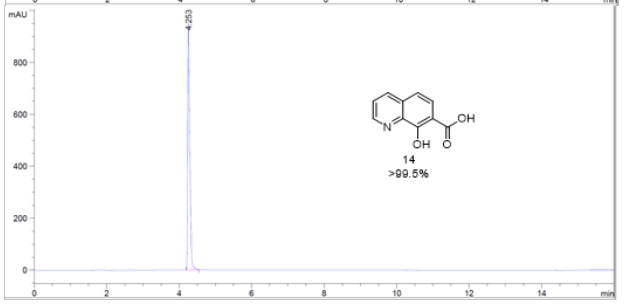

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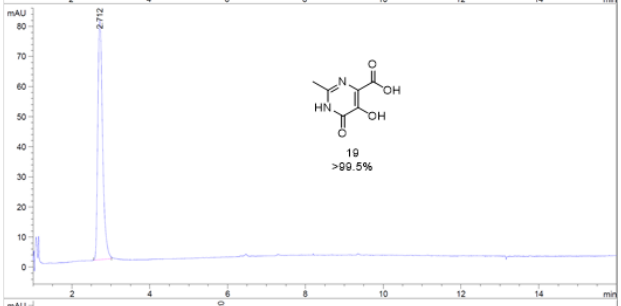

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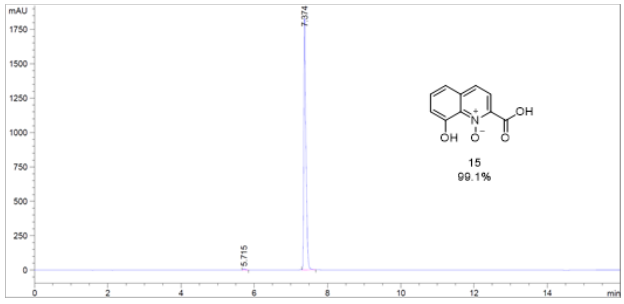

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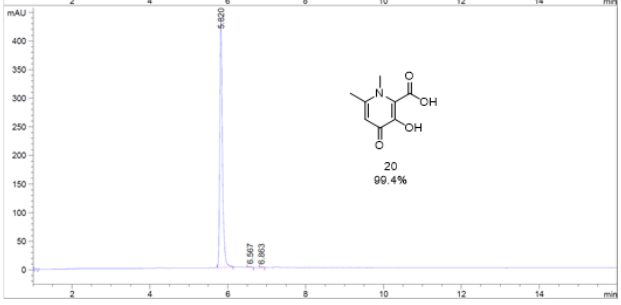

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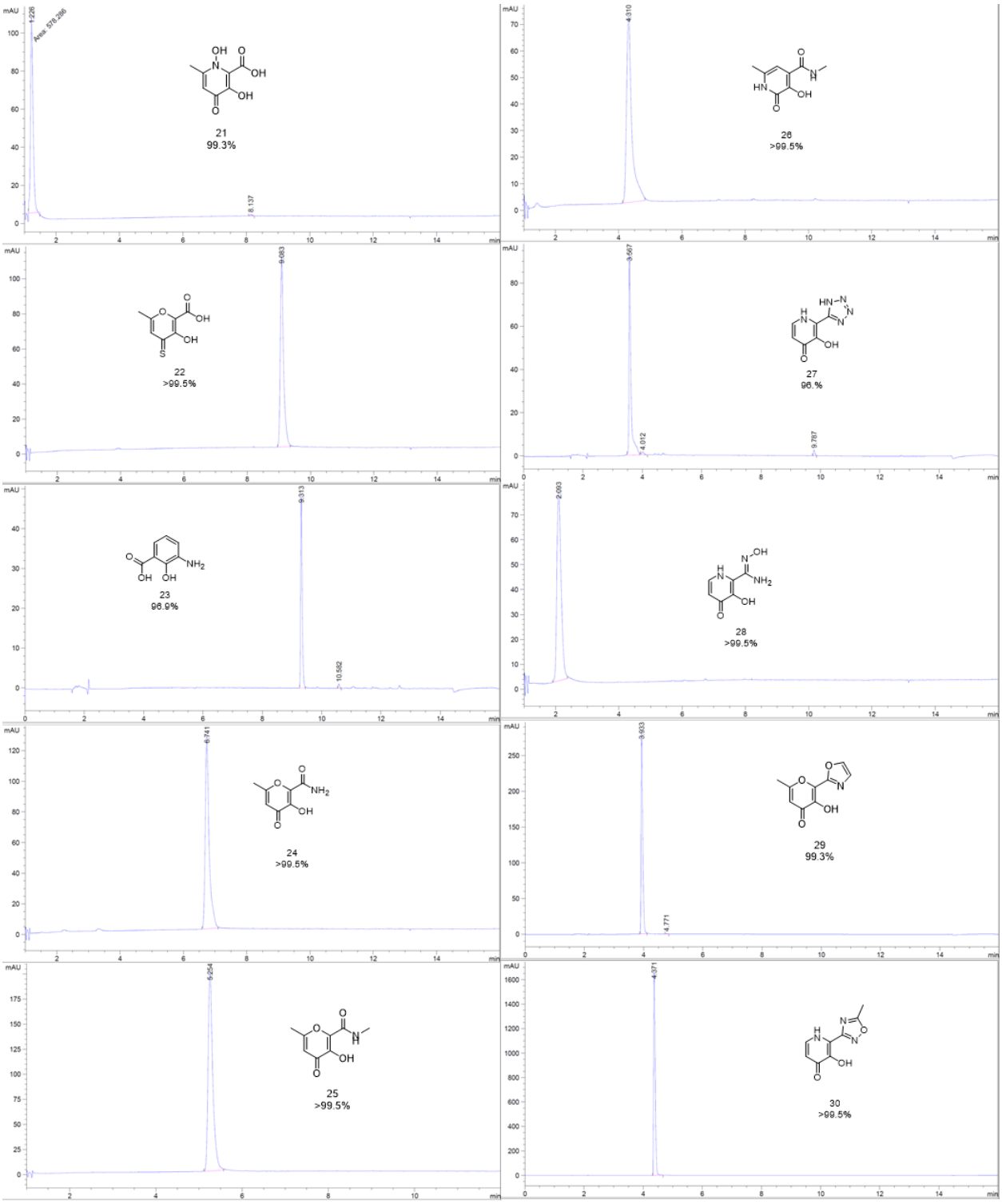
21
99.3%
26
>99.5%
22
>99.5%
27
96.%
23
96.9%
28
>99.5%
24
>99.5%
29
99.3%
25
>99.5%
30
>99.5%

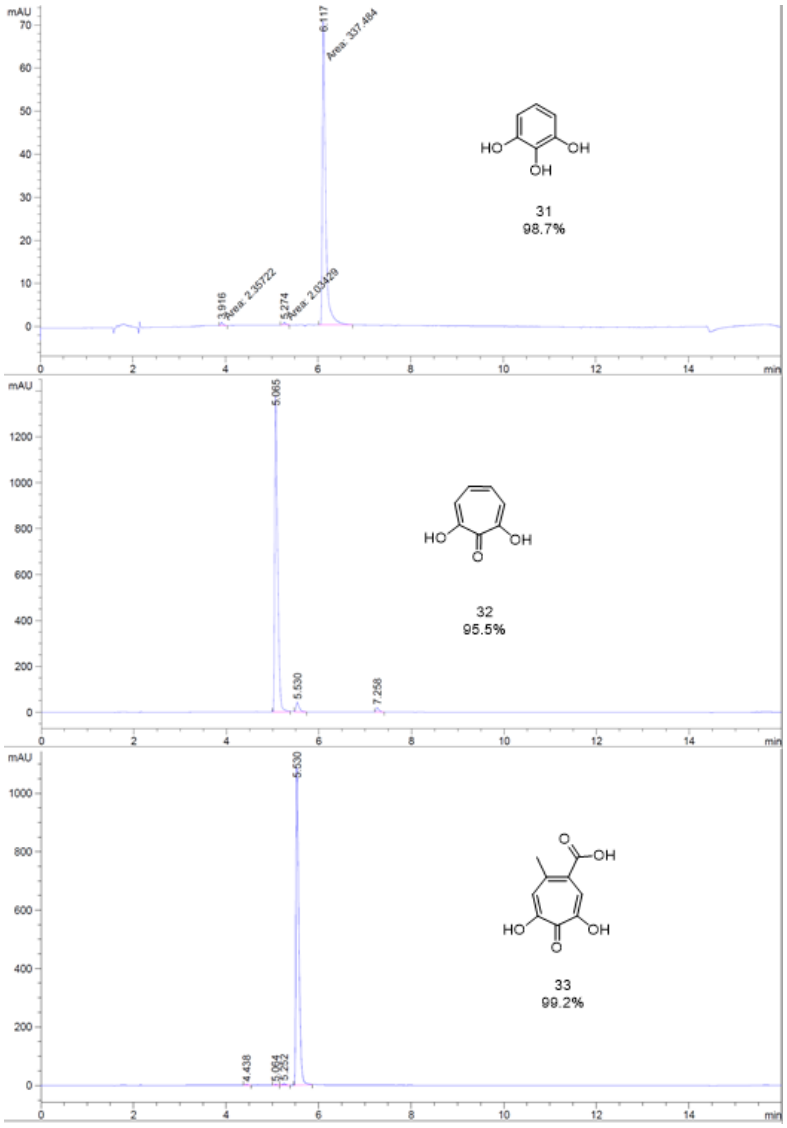
mAU
6.117
Area: 337.484
3.916
Area: 2.35722
5.274
Area: 2.03429
HO
OH
OH
31
98.7%
min
mAU
5.065
5.530
7.258
HO
OH
O
32
95.5%
min
mAU
6.530
4.438
5.064
5.252
OH
HO
OH
O
33
99.2%
min

Table S3. HPLC purity data for all assayed compounds.

Compound	Purity	**Compound**	Purity
1	>99.5%	**18**	>99.5%
2	>99.5%	**19**	>99.5%
3	>99.5%	**20**	99.4%
4	97.0%	**21**	99.3%
5	>99.5%	**22**	>99.5%
6	>99.5%	**23**	96.9%
7	>99.5%	**24**	>99.5%
8	95.6%	**25**	>99.5%
9	>99.5%	**26**	>99.5%
10	99.4%	**27**	96.0%
11	97.9%	**28**	>99.5%
12	>99.5%	**29**	99.3%
13	>99.5%	**30**	>99.5%
14	>99.5%	**31**	98.7%
15	99.1%	**32**	95.5%
16	98.6%	**33**	99.2%
17	>99.5%		

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