

Supporting Information

Novel indole-flutimide heterocycles with activity against influenza PA endonuclease and hepatitis C virus

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Experimental

Chemistry

General. Melting points were determined using a Büchi capillary apparatus and are uncorrected. The ¹H and ¹³C NMR spectra were obtained on either a Bruker MSL 400 (400 MHz ¹H; 100 MHz ¹³C) or Bruker 600 (600 MHz ¹H) spectrometer, using CDCl₃ or DMSO-*d*₆ as solvent. Chemical shifts are reported in δ (ppm) with the tetramethylsilane or solvent (DMSO-*d*₆) as internal standard. Splitting patterns are designated as s, singlet; d, doublet; dd, doublet of doublets; t, triplet; td, triplet of doublets; q, quartet; m, multiplet; bs, broad singlet. Coupling constants (*J*) are expressed in units of hertz (Hz). The spectra were recorded at 293 K (20 °C) unless otherwise specified. Carbon multiplicities were established by DEPT experiments. The 2D NMR experiments (HMQC, HMBC and COSY) were performed for the elucidation of the structures of the newly synthesized compounds. Analytical thin-layer chromatography (TLC) was conducted on precoated Merck silica gel 60 F254 plates (layer thickness 0.2 mm) with the spots visualized by iodine vapors and/or UV light. Column chromatography purification was carried out on silica gel 60 (0.040-0.063 mm). Elemental analyses (C, H, N) were performed by the Service Central de Microanalyse at CNRS (France), and were within $\pm 0.4\%$ of the theoretical values. Elemental analysis results for the tested compounds correspond to $>95\%$ purity. The commercial reagents were purchased from Alfa Aesar, Sigma-Aldrich, and Merck, and were used without further purification except for the benzyl bromoacetate. This reagent was purified by fractional distillation *in vacuo* prior to use. Organic solvents used were in the highest purity, and when necessary, were dried by the standard methods. Solvent abbreviations: THF, tetrahydrofuran; DMF, dimethylformamide; Et₂O, diethyl ether; MeOH, methanol; EtOH, ethanol; AcOEt, ethyl

acetate; DMSO, dimethylsulfoxide. Reagent abbreviations: DMAP, 4-(Dimethylamino)pyridine; DCC, *N,N'*-Dicyclohexylcarbodiimide; EDCI·HCl, N-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride; HOBt, 1-Hydroxybenzotriazole hydrate; DIEA, *N,N*-Diisopropylethylamine.

General procedure for the preparation of esters 2, 8 and 14.

A solution of (5-substituted-) 1*H*-indole-2-carboxylic acid (26.0 mmol), benzyl alcohol (32.5 mmol), and DMAP (5.2 mmol) in 162 mL of dichloromethane was treated with DCC (26.0 mmol) and stirred at room temperature for 3 h. The resulting mixture was filtered, concentrated *in vacuo*, taken up in 362 mL of ethyl acetate, and filtered. The solution was subsequently washed sequentially with 1 N HCl (2x30 mL), H₂O (2x30 mL), saturated aqueous solution of NaHCO₃ (2x50 mL), and brine (2x50 mL), dried over Na₂SO₄, and concentrated. The residue was purified by flash column chromatography on silica gel.

Benzyl 1*H*-indole-2-carboxylate (2)

It was prepared by reacting 1*H*-indole-2-carboxylic acid (**1**) with benzyl alcohol, following the general esterification procedure. Column chromatography on silica gel, using CH₂Cl₂ as an eluent, gave **2** (87% yield) as a pale yellow crystalline solid, of which the characteristics are consistent with the literature.¹

Benzyl 5-methoxy-1*H*-indole-2-carboxylate (8)

It was prepared by reacting 5-methoxy-1*H*-indole-2-carboxylic acid (**7**) with benzyl alcohol, following the general esterification procedure. Column chromatography on silica gel, using a mixture of eluents *n*-hexane/AcOEt (4:1), gave **8** (69% yield) as a pale yellow crystalline solid; mp 140-142 °C (AcOEt/Et₂O, *n*-pentane).

¹H NMR (400 MHz, CDCl₃) δ (ppm) 3.76 (s, 3H, OCH₃), 5.31 (s, 2H, OCH₂Ph), 6.91 (dd, 1H, *J*₁=8.9 Hz, *J*₂=2.4 Hz, *H*₆), 6.98 (d, 1H, *J*=2.2 Hz, *H*₄), 7.12 (d, 1H, *J*=2.1 Hz, *H*₃), 7.20 (dd, 1H,

$J_1=8.9$ Hz, $J_2=0.5$ Hz, H_7), 7.24-7.40 (complex m, 5H, $H_{2'}$, $H_{3'}$, $H_{4'}$, $H_{5'}$, $H_{6'}$), 8.96 (bs, 1H, NH); ^{13}C NMR (100 MHz, CDCl_3) δ (ppm) 55.6 (OCH_3), 66.6 (CH_2), 102.5 (C_4), 108.7 (C_3), 112.8 (C_7), 117.2 (C_6), 127.4 (C_2), 127.8 (C_3), 128.2 ($C_{2'}$, $C_{6'}$), 128.4 ($C_{4'}$), 128.6 ($C_{3'}$, $C_{5'}$), 132.3 (C_{7a}), 135.8 ($C_{1'}$), 154.7 (C_5), 161.8 ($\text{C}=\text{O}$). Anal. Calcd for $\text{C}_{17}\text{H}_{15}\text{NO}_3$: C, 72.58; H, 5.37; N, 4.98. Found C, 72.49; H, 5.48; N, 4.82.

Benzyl 5-fluoro-1*H*-indole-2-carboxylate (14)

It was prepared by reacting 5-fluoro-1*H*-indole-2-carboxylic acid (**13**) with benzyl alcohol, following the general esterification procedure. Column chromatography on silica gel, using a mixture of eluents *n*-hexane/AcOEt (4:1), gave **14** (70% yield) as a white crystalline solid; mp 151-153 °C (AcOEt/*n*-pentane).

^1H NMR (400 MHz, CDCl_3) δ (ppm) 5.41 (s, 2H, CH_2), 7.09 (td, 1H, $J_1=9.1$ Hz, $J_2=2.4$ Hz, H_6), 7.24 (d, 1H, $J=1.9$ Hz, H_3), 7.28-7.35 (m, 2H, H_4 , H_7), 7.35-7.51 (complex m, 5H, $H_{2'}$, $H_{3'}$, $H_{4'}$, $H_{5'}$, $H_{6'}$), 9.18 (bs, 1H, NH); ^{13}C NMR (100 MHz, CDCl_3) δ (ppm) 66.8 (CH_2), 106.5, 107.0 (d, $J_{\text{C-F}}=23.3$ Hz, C_4), 108.9, 109.0 (d, $J_{\text{C-F}}=5.5$ Hz, C_3), 112.7, 112.9 (d, $J_{\text{C-F}}=9.6$ Hz, C_7), 114.4, 114.9 (d, $J_{\text{C-F}}=27.0$ Hz, C_6), 127.5, 127.7 (d, $J_{\text{C-F}}=10.5$ Hz, C_{3a}), 128.3 ($C_{2'}$, $C_{6'}$), 128.5 ($C_{4'}$), 128.7 ($C_{3'}$, $C_{5'}$), 131.7 (C_2), 133.5 (C_{7a}), 135.6 ($C_{1'}$), 155.8, 160.5 (d, $J_{\text{C-F}}=236.8$ Hz, C_5), 161.6 ($\text{C}=\text{O}$). Anal. Calcd for $\text{C}_{16}\text{H}_{12}\text{FNO}_2$: C, 71.37; H, 4.49; N, 5.20. Found: C, 71.48; H, 4.57; N, 5.34.

General procedure for the preparation of diesters 3, 9 and 15.

Sodium hydride (9.78 mmol, 60% in mineral oil) was added portionwise to a stirred, ice-cold, solution of benzyl (5-substituted-) 1*H*-indole-2-carboxylate (8.89 mmol) in dry DMF (9 mL). After stirring at room temperature for 1 h under argon, ethyl bromoacetate (9.69 mmol), dissolved in dry DMF (3 mL) was added dropwise. Stirring was continued at rt for 24 h under argon, and the reaction mixture was then poured onto ice/water mixture (80 mL), and extracted with AcOEt (4x60 mL). The combined organic extracts were washed with brine

(3x25 mL), dried (Na₂SO₄), and evaporated *in vacuo*. The crude residue was purified by flash column chromatography on silica gel.

Benzyl 1-(2-ethoxy-2-oxoethyl)-1H-indole-2-carboxylate (3)

It was prepared by reacting benzyl ester **2** with ethyl-bromoacetate, following the general procedure for the preparation of diesters. Column chromatography on silica gel, using a mixture of eluents *n*-hexane/AcOEt (4:1), gave **3** (86% yield) as a colorless, clear, viscous oil.

¹H NMR (400 MHz, CDCl₃) δ (ppm) 1.24 (t, 3H, *J*=7.1 Hz, COOCH₂CH₃), 4.19 (q, 2H, *J*=7.1 Hz, COOCH₂CH₃), 5.32 (s, 2H, NCH₂COO), 5.35 (s, 2H, COOCH₂Ph), 7.18 (td, 1H, *J*₁=7.9 Hz, *J*₂=0.8 Hz, *H*₅), 7.29 (d, 1H, *J*=7.9 Hz, *H*₇), 7.32-7.48 (complex m, 7H, *H*₃, *H*₂', *H*₃', *H*₄', *H*₅', *H*₆' and *H*₆), 7.69 (d, 1H, *J*=8.0 Hz, *H*₄); ¹³C NMR (100 MHz, CDCl₃) δ (ppm) 14.2 (CH₂CH₃), 46.3 (NCH₂COO), 61.5 (COOCH₂CH₃), 66.4 (COOCH₂Ph), 109.7 (*C*₇), 111.7 (*C*₃), 121.2 (*C*₅), 123.0 (*C*₄), 125.7 (*C*₆), 126.2 (*C*₂), 127.4 (*C*_{3a}), 128.2 (*C*₃', *C*₅'), 128.3 (*C*₄'), 128.7 (*C*₂', *C*₆'), 136.0 (*C*₁'), 139.6 (*C*_{7a}), 162.0 (COOCH₂Ph), 168.9 (COOCH₂CH₃). HRMS/ESI⁺ (*m/z*): Calcd for C₂₁H₂₃NO₄: 353.1627; Found 353.1633.

Benzyl 1-(2-ethoxy-2-oxoethyl)-5-methoxy-1H-indole-2-carboxylate (9)

It was prepared by reacting benzyl ester **8** with ethyl-bromoacetate, following the general procedure for the preparation of diesters. Column chromatography on silica gel, using a mixture of eluents *n*-hexane/AcOEt (7:1, then 6:1), gave **9** (83%) as a colorless, viscous oil which was crystallized by adding *n*-pentane and cooling; mp 88-90 °C (Et₂O/*n*-pentane).

¹H NMR (400 MHz, CDCl₃) δ (ppm) 1.24 (t, 3H, *J*=7.1 Hz, COOCH₂CH₃), 3.84 (s, 3H, OCH₃), 4.19 (q, 2H, *J*=7.1 Hz, COOCH₂CH₃), 5.29 (s, 2H, NCH₂COO), 5.34 (s, 2H, COOCH₂Ph), 7.04 (dd, 1H, *J*₁=9.0 Hz, *J*₂=2.4 Hz, *H*₆), 7.07 (d, 1H, *J*=2.2 Hz, *H*₄), 7.19 (d, 1H, *J*=9.0 Hz, *H*₇), 7.31-7.48 (complex m, 5H, *H*₂', *H*₃', *H*₄', *H*₅', *H*₆'), 7.34 (d, 1H, *J*=0.6 Hz, *H*₃); ¹³C NMR (100 MHz, CDCl₃) δ (ppm) 14.2 (CH₂CH₃), 46.4 (NCH₂COO), 55.8 (CH₃O), 61.6 (COOCH₂CH₃), 66.3 (COOCH₂Ph),

103.1 (C_4), 110.7 (C_7), 111.1 (C_3), 117.2 (C_6), 126.5 (C_{3a}), 127.7 (C_2), 128.2 ($C_{2'}$, $C_{6'}$), 128.3 ($C_{4'}$), 128.7 ($C_{3'}$, $C_{5'}$), 135.2 (C_{7a}), 136.1 ($C_{1'}$), 155.1 (C_5), 161.9 (COOCH₂Ph), 169.0 (COOCH₂CH₃).

Anal. Calcd for C₂₁H₂₁NO₅: C, 68.65; H, 5.76; N, 3.81. Found C, 68.67; H, 5.89; N, 3.92.

Benzyl 1-(2-ethoxy-2-oxoethyl)-5-fluoro-1H-indole-2-carboxylate (15)

It was prepared by reacting benzyl ester **14** with ethyl-bromoacetate, following the general procedure for the preparation of diesters. Column chromatography on silica gel, using a mixture of eluents *n*-hexane/AcOEt (7:1, then 6:1), gave **15** (86% yield) as a colorless, viscous oil which was crystallized with cooling; mp 81-83 °C (Et₂O/*n*-pentane).

¹H NMR (400 MHz, CDCl₃) δ (ppm) 1.24 (t, 3H, $J=7.1$ Hz, COOCH₂CH₃), 4.19 (q, 2H, $J=7.1$ Hz, COOCH₂CH₃), 5.30 (s, 2H, NCH₂COO), 5.34 (s, 2H, COOCH₂Ph), 7.12 (td, 1H, $J_1=9.0$ Hz, $J_2=2.5$ Hz, H_6), 7.22 (dd, 1H, $J_1=9.1$ Hz, $J_2=4.2$ Hz, H_7), 7.32 (dd, 1H, $J_1=9.0$ Hz, $J_2=2.3$ Hz, H_4), 7.34-7.47 (complex m, 5H, $H_{2'}$, $H_{3'}$, $H_{4'}$, $H_{5'}$, $H_{6'}$), 7.36 (s, 1H, H_3); ¹³C NMR (100 MHz, CDCl₃) δ (ppm) 14.1 (CH₂CH₃), 46.3 (NCH₂COO), 61.6 (COOCH₂CH₃), 66.5 (COOCH₂Ph), 106.9, 107.3 (d, $J_{C-F}=23.3$ Hz, C_4), 110.5, 110.7 (d, $J_{C-F}=9.6$ Hz, C_7), 111.1, 111.3 (d, $J_{C-F}=5.3$ Hz, C_3), 114.3, 114.9 (d, $J_{C-F}=27.0$ Hz, C_6), 126.1, 126.3 (d, $J_{C-F}=10.2$ Hz, C_{3a}), 128.1 ($C_{2'}$, $C_{6'}$), 128.3 ($C_{4'}$), 128.6 ($C_{3'}$, $C_{5'}$), 130.4 (C_2), 135.7 ($C_{1'}$), 136.1 (C_{7a}), 156.0, 160.7 (d, $J_{C-F}=237.4$ Hz, C_5), 161.6 (COOCH₂Ph), 168.6 (COOCH₂CH₃). Anal. Calcd for C₂₀H₁₈FNO₄: C, 67.60; H, 5.11; N, 3.94. Found: C, 67.49; H, 5.14; N, 3.92.

General procedure for the preparation of acid esters 4, 10 and 16.

A solution of the respective benzyl 1-(2-ethoxy-2-oxoethyl)-1H-indole-2-carboxylate (1 mmol) in a mixture of absolute EtOH/AcOEt (2:1, 30 mL) was hydrogenated (Pd-C 10%, 45 mg) for 3 h, at room temperature and 50 psi pressure. The catalyst was filtered off, washed with hot EtOH (3x10 mL), and the combined filtrates were evaporated *in vacuo* to afford pure the respective acid esters.

1-(2-Ethoxy-2-oxoethyl)-1*H*-indole-2-carboxylic acid (4)

It was prepared by hydrogenolysis of diester **3** following the general procedure. Evaporation of the solvents gave **4** (96% yield) as a white crystalline solid; mp 189-191 °C (EtOH/Et₂O).

¹H NMR (400 MHz, CDCl₃) δ (ppm) 1.28 (t, 3H, *J*=7.1 Hz, COOCH₂CH₃), 4.24 (q, 2H, *J*=7.1 Hz, COOCH₂CH₃), 5.31 (s, 2H, NCH₂COO), 7.20 (t, 1H, *J*=7.5 Hz, *H*₅), 7.31 (d, 1H, *J*=8.4 Hz, *H*₇), 7.40 (td, 1H, *J*₁= 7.7 Hz, *J*₂=1.0 Hz, *H*₆), 7.54 (s, 1H, *H*₃), 7.33 (d, 1H, *J*=8.0 Hz, *H*₄), 8.83 (bs, 1H, COOH); ¹³C NMR (100 MHz, CDCl₃) δ (ppm) 14.3 (CH₂CH₃), 46.4 (NCH₂COOCH₂CH₃), 61.7 (COOCH₂CH₃), 109.9 (*C*₇), 113.7 (*C*₃), 121.5 (*C*₅), 123.3 (*C*₄), 126.3 (*C*_{3a}), 126.4 (*C*₆), 126.6 (*C*₂), 140.2 (*C*_{7a}), 167.1 (COOH), 169.0 (COOCH₂CH₃). Anal. Calcd for C₁₃H₁₃NO₄: C, 63.15; H, 5.30; N, 5.67. Found: C, 63.27; H, 5.63; N, 5.75.

1-(2-Ethoxy-2-oxoethyl)-5-methoxy-1*H*-indole-2-carboxylic acid (10)

It was prepared by hydrogenolysis of diester **9** following the general procedure. Evaporation of the solvents gave **10** (97% yield) as a white crystalline solid; mp 189-191 °C (AcOEt/*n*-pentane, Et₂O).

¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) 1.19 (t, 3H, *J*=7.1 Hz, COOCH₂CH₃), 3.77 (s, 3H, OCH₃), 4.12 (q, 2H, *J*=7.1 Hz, COOCH₂CH₃), 5.33 (s, 2H, NCH₂COO), 6.96 (dd, 1H, *J*₁=9.1 Hz, *J*₂=2.3 Hz, *H*₆), 7.15 (d, 1H, *J*=2.4 Hz, *H*₄), 7.18 (s, 1H, *H*₃), 7.52 (d, 1H, *J*=9.1 Hz, *H*₇), 12.94 (bs, 1H, OH); ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm) 14.1 (CH₂CH₃), 46.1 (NCH₂COO), 55.3 (OCH₃), 60.7 (COOCH₂CH₃), 102.4 (*C*₄), 109.6 (*C*₃), 111.7 (*C*₇), 116.0 (*C*₆), 125.8 (*C*_{3a}), 128.4 (*C*₂), 134.7 (*C*_{7a}), 154.3 (*C*₅), 162.8 (COOH), 169.1 (COOCH₂CH₃). Anal. Calcd for C₁₄H₁₅NO₅: C, 60.64; H, 5.45; N, 5.05. Found C, 60.59; H, 5.42; N, 5.08.

1-(2-Ethoxy-2-oxoethyl)-5-fluoro-1*H*-indole-2-carboxylic acid (16)

It was prepared by hydrogenolysis of diester **15** following the general procedure. Evaporation of the solvents gave **16** (93% yield) as a white crystalline solid; mp 178-180 °C (Et₂O/*n*-pentane).

¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) 1.19 (t, 3H, *J*=7.1 Hz, COOCH₂CH₃), 4.13 (q, 2H, *J*=7.1 Hz, COOCH₂CH₃), 5.39 (s, 2H, CH₂), 7.19 (td, 1H, *J*₁=9.2 Hz, *J*₂=2.3 Hz, H₆), 7.24 (s, 1H, H₃), 7.46 (dd, 1H, *J*₁=9.4 Hz, *J*₂=2.3 Hz, H₄), 7.64 (dd, 1H, *J*₁=9.1 Hz, *J*₂=4.3 Hz, H₇); ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm) 14.1 (COOCH₂CH₃), 46.3 (CH₂), 60.7 (CH₂CH₃), 106.1, 106.5 (d, *J*_{C-F}=23.2 Hz, C₄), 109.6, 109.7 (d, *J*_{C-F}=5.2 Hz, C₃), 112.1, 112.3 (d, *J*_{C-F}=9.6 Hz, C₇), 113.1, 113.7 (d, *J*_{C-F}=26.6 Hz, C₆), 125.4, 125.7 (d, *J*_{C-F}=10.6 Hz, C_{3a}), 130.2 (C₂), 135.9 (C_{7a}), 155.2 159.8 (d, *J*_{C-F}=234.3 Hz, C₅), 162.7 (COOH), 168.9 (COOCH₂CH₃). Anal. Calcd for C₁₃H₁₂FNO₄: C, 58.87; H, 4.56; N, 5.28. Found: C, 58.73; H, 4.55; N, 5.34.

General procedure for the preparation of *O*-benzyl hydroxamates **5, **11** and **17**.**

To a solution of the respective acid ester (1.14 mmol) in a mixture of CH₂Cl₂/DMF (4:1, 12 mL) were added sequentially EDCI·HCl (1.36 mmol), HOBT (1.36 mmol), DIEA (4.56 mmol) and *O*-benzyl hydroxylamine hydrochloride (1.37 mmol) and the mixture was stirred at 35 °C for 48 h under argon. The reaction mixture was concentrated under reduced pressure, poured onto ice/water mixture (40 mL), and extracted with AcOEt (4x40 mL). The combined organic extracts were washed with H₂O (3x40 mL), 10% aqueous solution of Na₂CO₃ (2x40 mL), brine (3x25 mL), dried (Na₂SO₄), and evaporated *in vacuo*. The residue was purified through flash column chromatography on silica gel.

2-(Benzyloxy)pyrazino[1,2-*a*]indole-1,3(2*H*,4*H*)-dione (5**)**

It was prepared by reacting the respective acid ester **4** with *O*-benzyl hydroxylamine hydrochloride following the general procedure for the preparation of *O*-benzyl hydroxamates. The residue was purified through flash column chromatography on silica gel,

using a mixture of eluents *n*-hexane/AcOEt 2:1 increased to AcOEt 100%, to afford **5** (67%) as a white crystalline solid; mp 219-221 °C (AcOEt/*n*-pentane).

¹H NMR (600 MHz, DMSO-*d*₆) δ (ppm) 5.09 (s, 2H, OCH₂Ph), 5.35 (s, 2H, H₄), 7.22 (t, 1H, *J*=7.4 Hz, H₈), 7.39-7.46 (complex m, 5H, H_{3'}, H_{4'}, H_{5'}, H₇, H₁₀), 7.59 (d, 2H, *J*=6.6 Hz, H_{2'}, H_{6'}), 7.61 (dd, 1H, *J*₁=8.4 Hz, *J*₂=0.5 Hz, H₆), 7.79 (d, 1H, *J*=8.0 Hz, H₉); ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm) 47.8 (C₄), 77.8 (OCH₂Ph), 106.7 (C₇), 111.2 (C₆), 121.4 (C₈), 122.7 (C₉), 125.48 (C_{10a}), 125.53 (C₁₀), 126.5 (C_{9a}), 128.4 (C_{3'}, C_{5'}), 128.9 (C_{4'}), 129.4 (C_{2'}, C_{6'}), 134.4 (C_{1'}), 136.6 (C_{5a}), 163.2 (C₁=O, C₃=O). Anal. Calcd for C₁₈H₁₄N₂O₃: C, 70.58; H, 4.61; N, 9.15. Found C, 70.49; H, 4.73; N, 9.33.

2-(Benzyloxy)-8-methoxypyrazino[1,2-*a*]indole-1,3(2*H*,4*H*)-dione (11)

It was prepared by reacting the respective acid ester **10** with *O*-benzyl hydroxylamine hydrochloride following the general procedure for the preparation of *O*-benzyl hydroxamates. The residue was purified through flash column chromatography on silica gel, using a mixture of eluents *n*-hexane/AcOEt (7:1) increased to AcOEt and finally AcOEt/MeOH (10:1), to give **11** (59%) as a white crystalline solid; mp 220-222 °C (THF/*n*-pentane).

¹H NMR (600 MHz, DMSO-*d*₆) δ (ppm) 3.79 (s, 3H, OCH₃), 5.08 (s, 2H, OCH₂Ph), 5.31 (s, 2H, H₄), 7.07 (dd, 1H, *J*₁=9.0 Hz, *J*₂=2.4 Hz, H₇), 7.23 (d, 1H, *J*=2.3 Hz, H₉), 7.30 (s, 1H, H₁₀), 7.37-7.48 (complex m, 3H, H_{3'}, H_{4'}, H_{5'}), 7.52 (d, 1H, *J*=9.1 Hz, H₆), 7.58 (m, 2H, H_{2'}, H_{6'}); ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm) 47.9 (C₄), 55.3 (OCH₃), 77.8 (OCH₂Ph), 102.5 (C₉), 106.2 (C₁₀), 112.2 (C₆), 117.2 (C₇), 125.6 (C_{10a}), 127.1 (C_{9a}), 128.4 (C_{3'}, C_{5'}), 128.9 (C_{4'}), 129.4 (C_{2'}, C_{6'}), 132.2 (C_{5a}), 134.5 (C_{1'}), 154.8 (C₈), 155.2 (C₁=O), 163.3 (C₃=O). Anal. Calcd for C₁₉H₁₆N₂O₄: C, 67.85; H, 4.80; N, 8.33. Found C, 67.92; H, 4.73; N, 8.45.

2-(Benzyloxy)-8-fluoropyrazino[1,2-*a*]indole-1,3(2*H*,4*H*)-dione (17)

It was prepared by reacting the respective acid ester **16** with *O*-benzyl hydroxylamine hydrochloride following the general procedure for the preparation of *O*-benzyl hydroxamates. The residue was purified through flash column chromatography on silica gel, using a mixture of eluents *n*-hexane/AcOEt (7:1 and then 2:1), to afford **17** (58%) as a white crystalline solid; mp 235-237 °C (THF/*n*-pentane).

¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) 5.09 (s, 2H, OCH₂Ph), 5.36 (s, 2H, H₄), 7.31 (t, 1H, J₁=9.2 Hz, J₂=2.4 Hz, H₇), 7.37 (s, 1H, H₁₀), 7.38-7.48 (complex m, 3H, H_{3'}, H_{4'}, H_{5'}), 7.52-7.62 (complex m, 3H, H₉, H_{2'}, H_{6'}), 7.67 (dd, 1H, J₁=9.1 Hz, J₂=4.5 Hz, H₆); ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm) 48.1 (C₄), 77.8 (OCH₂Ph), 106.4, 106.5 (d, J_{C-F}=5.3 Hz, C₁₀), 106.6, 107.1 (d, J_{C-F}=24.0 Hz, C₉), 112.8, 112.9 (d, J_{C-F}=9.6 Hz, C₆), 114.3, 114.8 (d, J_{C-F}=27.1 Hz, C₇), 126.6, 126.8 (d, J_{C-F}=10.8 Hz, C_{9a}), 127.1 (C_{10a}), 128.4 (C_{3'}, C_{5'}), 128.9 (C_{4'}), 129.4 (C_{2'}, C_{6'}), 133.5 (C_{5a}), 134.4 (C_{1'}), 155.2 (C₁=O), 155.5, 160.2 (d, J_{C-F}=235.3 Hz, C₈), 163.1 (C₃=O). Anal. Calcd for C₁₈H₁₃FN₂O₃: C, 66.66; H, 4.04; N, 8.64. Found: C, 66.62; H, 4.09; N, 8.56.

General procedure for the preparation of *N*-hydroxyimides **6, **12** and **18**.**

A solution of the appropriate *O*-benzyl hydroxamate (1 mmol) in a mixture of absolute EtOH/AcOEt (2:1, 100 mL), was hydrogenated (Pd-C 10%, 45 mg) for 3 h, at room temperature and 50 psi pressure. The catalyst was filtered off, washed with hot EtOH (3x20 mL), and the combined filtrates were evaporated *in vacuo*. Purification of the residue, using silica gel flash column chromatography, provided the pure *N*-hydroxyimides.

2-Hydroxypyrazino[1,2-*a*]indole-1,3(2*H*,4*H*)-dione (6**)**

It was prepared by hydrogenolysis of the corresponding diketopiperazine analogue **5** following the general procedure. After evaporation of the solvents the residue was purified by column chromatography on silica gel using a mixture of eluents AcOEt/MeOH (5:1), to

afford **6** (almost quantitative yield) as a pale yellow crystalline solid; mp 215-216 °C (dec, MeOH/Et₂O).

¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) 5.33 (s, 2H, *H*₄), 7.20 (td, 1H, *J*₁=7.8 Hz, *J*₂=0.6 Hz, *H*₈), 7.34 (s, 1H, *H*₁₀), 7.39 (td, 1H, *J*₁=7.7 Hz, *J*₂=1.0 Hz, *H*₇), 7.57 (dd, 1H, *J*₁=8.4 Hz, *J*₂=0.7 Hz, *H*₆), 7.76 (d, 1H, *J*=8.1 Hz, *H*₉), 10.64 (bs, 1H, OH); ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm) 47.4 (*C*₄), 106.2 (*C*₁₀), 111.2 (*C*₆), 121.3 (*C*₈), 122.6 (*C*₉), 125.3 (*C*₇), 125.6 (*C*_{10a}), 126.6 (*C*_{9a}), 136.6 (*C*_{5a}), 156.0 (*C*₁=O), 163.5 (*C*₃=O). Anal. Calcd for C₁₁H₈N₂O₃: C, 61.11; H, 3.73; N, 12.96. Found C, 61.15; H, 3.81; N, 12.92.

2-Hydroxy-8-methoxypyrazino[1,2-*a*]indole-1,3(2*H*,4*H*)-dione (12**)**

It was prepared by hydrogenolysis of the corresponding diketopiperazine analogue **11** following the general procedure. After evaporation of the solvents the residue was purified by column chromatography on silica gel using a mixture of eluents AcOEt/MeOH (5:1), to afford **12** (almost quantitative yield) as a pale yellow crystalline solid; Mp 228-230 °C (dec, AcOEt, MeOH/*n*-pentane).

¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) 3.78 (s, 3H, OCH₃), 5.29 (s, 2H, *H*₄), 7.04 (dd, 1H, *J*₁=9.0 Hz, *J*₂=1.8 Hz, *H*₇), 7.20 (d, 1H, *J*=1.5 Hz, *H*₉), 7.23 (s, 1H, *H*₁₀), 7.48 (d, 1H, *J*=9.1 Hz, *H*₆), 10.62 (bs, 1H, OH); ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm) 47.5 (*C*₄), 55.3 (OCH₃), 102.5 (*C*₉), 105.7 (*C*₁₀), 112.2 (*C*₆), 116.9 (*C*₇), 125.7 (*C*_{10a}), 127.1 (*C*_{9a}), 132.1 (*C*_{5a}), 154.8 (*C*₈), 155.9 (*C*₁=O), 163.5 (*C*₃=O). Anal. Calcd for C₁₂H₁₀N₂O₄: C, 58.54; H, 4.09; N, 11.38. Found C, 58.57; H, 4.11; N, 11.42.

8-fluoro-2-hydroxypyrazino[1,2-*a*]indole-1,3(2*H*,4*H*)-dione (18**)**

It was prepared by hydrogenolysis of the corresponding diketopiperazine analogue **17** following the general procedure. After evaporation of the solvents the residue was purified by column chromatography on silica gel, using a mixture of eluents AcOEt/MeOH (5:1), to

afford **18** (almost quantitative yield) as a pale yellow crystalline solid; Mp 205-207 °C (dec, AcOEt, MeOH/*n*-pentane).

¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) 5.34 (s, 2H, *H*₄), 7.28 (td, 1H, *J*₁=9.2 Hz, *J*₂=2.5 Hz, *H*₇), 7.30 (s, 1H, *H*₁₀), 7.53 (dd, 1H, *J*₁=9.6 Hz, *J*₂=2.5 Hz, *H*₉), 7.62 (dd, 1H, *J*₁=9.1 Hz, *J*₂=4.4 Hz, *H*₆), 10.70 (bs, 1H, OH); ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm) 47.4 (*C*₄), 105.9, 106.0 (d, *J*_{C-F}=5.0 Hz, *C*₁₀), 106.5, 107.0 (d, *J*_{C-F}=23.7 Hz, *C*₉), 112.7, 112.9 (d, *J*_{C-F}=9.7 Hz, *C*₆), 113.9, 114.5 (d, *J*_{C-F}=27.1 Hz, *C*₇), 126.7, 126.9 (d, *J*_{C-F}=10.7 Hz, *C*_{9a}), 127.2 (*C*_{10a}), 155.5, 160.2 (d, *J*_{C-F}=235.2 Hz, *C*₈), 155.8 (*C*₁=O), 163.4 (*C*₃=O). Anal. Calcd for C₁₁H₇FN₂O₃: C, 56.42; H, 3.01; N, 11.96. Found C, 56.45; H, 3.08; N, 11.92.

Synthesis of 2,8-dihydroxypyrazino[1,2-*a*]indole-1,3(2*H*,4*H*)-dione (**25**)

Compound **12** (2-hydroxy-8-methoxypyrazino[1,2-*a*]indole-1,3(2*H*,4*H*)-dione) (100 mg, 0.41 mmol) was suspended in dry CH₂Cl₂ (2 mL) and cooled at 0 °C. BBr₃ (1M in CH₂Cl₂, 1.35 mL, 1.35 mmol) was added dropwise and the mixture was stirred at rt, for 20 h under argon atmosphere. Ice-water (20 mL) was then added and CH₂Cl₂ was evaporated *in vacuo*. The residue was extracted with AcOEt (3x15 mL). The combined organic extracts were washed with water (2x10 mL), brine (2x10 mL), dried (Na₂SO₄), and evaporated *in vacuo* to afford **25** (93 mg, almost quantitative yield) as a pale yellow crystalline solid; mp >250 °C (dec, AcOEt, MeOH/*n*-pentane).

¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) 5.29 (s, 2H, *H*₄), 6.92 (dd, 1H, *J*₁=8.9 Hz, *J*₂=1.9 Hz, *H*₇), 7.00 (d, 1H, *J*=1.8 Hz, *H*₉), 7.14 (s, 1H, *H*₁₀), 7.33 (d, 1H, *J*=8.9 Hz, *H*₆), 9.18 (bs, 1H, C-OH), 10.58 (bs, 1H, N-OH); ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm) 47.4 (*C*₄), 105.0 (*C*₉), 105.2 (*C*₁₀), 111.8 (*C*₆), 116.9 (*C*₇), 125.6 (*C*_{10a}), 127.5 (*C*_{9a}), 131.7 (*C*_{5a}), 152.3 (*C*₈), 155.9 (*C*₁=O), 163.6 (*C*₃=O). Anal. Calcd for C₁₁H₈N₂O₄: C, 56.90; H, 3.47; N, 12.06. Found C, 56.82; H, 3.31; N, 12.10.

Synthesis of pyrazino[1,2-a]indole-1,3(2H,4H)-dione (**26**)

To a solution of **4** (1-(2-ethoxy-2-oxoethyl)-1*H*-indole-2-carboxylic acid) (600 mg, 2.43 mmol) in dry THF (7 mL) was added dropwise, under ice-cooling, a solution of thionyl chloride (524 mg, 4.40 mmol) in dry THF (0.6 mL). The mixture was stirred at 50 °C for 4 h and then at room temperature for another 2 h. The mixture was evaporated *in vacuo*, at low temperature (<35 °C) and the crude chloride was dissolved in dry THF (5 mL). To this solution was added, in one portion, a saturated solution of ammonia in dichloromethane (5 mL) and the mixture was stirred at 30 °C for 17 h. After removal of the solvents the crude residue was purified by flash column chromatography on silica gel, using a mixture of eluents *n*-hexane/THF (2:1), to afford pure the target compound **26** (410 mg, 84%) as a white crystalline solid; mp 239-241 °C (dec, THF/*n*-pentane).

¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm) 5.11 (s, 2H, *H*₄), 7.19 (td, 1H, *J*₁=7.8 Hz, *J*₂=0.6 Hz, *H*₈), 7.31 (s, 1H, *H*₁₀), 7.38 (td, 1H, *J*₁=7.7 Hz, *J*₂=1.0 Hz, *H*₇), 7.57 (dd, 1H, *J*₁=8.4 Hz, *J*₂=0.7 Hz, *H*₆), 7.76 (d, 1H, *J*=8.1 Hz, *H*₉), 11.69 (s, 1H, NH); ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm) 46.3 (*C*₄), 105.6 (*C*₁₀), 111.2 (*C*₆), 121.3 (*C*₈), 122.7 (*C*₉), 125.1 (*C*₇), 125.6 (*C*_{10a}), 126.7 (*C*_{9a}), 136.7 (*C*_{5a}), 159.0 (*C*₁=O), 168.2 (*C*₃=O). Anal. Calcd for C₁₁H₈N₂O₂: C, 66.00; H, 4.03; N, 13.99. Found: C, 66.13; H, 4.01; N, 13.81;

Experimental. Biological Assays.

Influenza PA endonuclease assay

The enzymatic influenza endonuclease assay was performed according to a previously published procedure.² Briefly, recombinant PA-Nter [residues 1-217 from the PA protein of influenza virus strain A/X-31] was expressed in *E. coli* and purified. One microgram of the enzyme was incubated with 1 μg (16.7 nM) of single-stranded circular DNA plasmid M13mp18 (Bayou Biolabs, Metairie, Louisiana) in the presence of the test compounds and at

a final volume of 25 μ L. The assay buffer contained 50 mM Tris-HCl pH 8, 100 mM NaCl, 10 mM β -mercaptoethanol and 1 mM $MnCl_2$. After 2 h incubation at 37°C, the reaction was stopped by heat inactivation (80°C, 20 min). Endonucleolytic digestion of the plasmid was visualized by gel electrophoresis on a 1% agarose gel with ethidium bromide staining and the amount of remaining intact plasmid was quantified by ImageQuant TL software (GE Healthcare). The percentage inhibition of PA endonuclease activity was plotted against the compound concentration on a semi-logarithmic plot, using GraphPad Prism software (GraphPad Software, La Jolla, CA). The 50% inhibitory concentrations (IC_{50}) were obtained by nonlinear least-squares regression analysis of the results from three independent experiments.

Cytotoxicity towards HEK293T cells

The compound cytotoxicity, expressed as MCC, was determined in HEK293T cells after 24 h incubation, using microscopic analysis of cell morphology.³

HCV Replication assays

Cell culture. Huh 5-2 stable cell line has been previously described in (25) and harbors a subgenomic reporter replicon of Con1 strain (genotype 1b). Specifically, it has been established upon transfection of Huh7-Lunet cells with the bicistronic RNA transcribed from pFK I389luc-ubi-neo/NS3-3'/5.1. This carries in the first cistron the firefly luciferase (luc) gene fused in frame with the neomycin gene (neo) under the translational control of the Con1 IRES and in the second cistron the Con1 NS3-3'NTR region. Cells were grown in high glucose (25 mM) Dulbecco's modified minimal essential medium (DMEM) (Invitrogen) supplemented with 2 mM L-glutamine, 0.1 mM non-essential amino acids, 100 U/ml penicillin, 100 μ g/ml streptomycin, 10% (v/v) fetal calf serum (referred to as complete DMEM) and 500 μ g/ml G418.

Anti-HCV assay: Anti-HCV assay in replicon cells was performed by seeding 1×10^4 cells per well in a 96-well flat bottom plate in 200 μ l complete DMEM supplemented with G418. Following incubation for 24 h at 37°C (5% CO₂), medium was removed and 2-fold serial dilutions in complete DMEM (without G418) of the test compounds were added in a total volume of 100 μ l. After 3 days of incubation at 37°C, cell culture medium was removed and luciferase activity was measured. Relative luminescence units were converted to percentage of treated with DMSO controls. The 50% effective concentration (EC₅₀) was defined as the concentration of compound that reduced the luciferase signal by 50%.

Measurement of median lethal concentration (LC₅₀) of the compounds. The LC₅₀ of the compounds in cells was determined by using the alamarBlue dye. It is a redox indicator that yields both a fluorescent signal and a colorimetric change from blue to red in response to the chemical reduction of growth medium, resulting from cell growth. Damaged and non-viable cells have lower innate metabolic activity, and generate a proportionally lower signal. AlamarBlue reduction is dependent on both the glycolytic and oxidative metabolism of glucose, which is important in the case of hepatocarcinoma cells as they produce energy mainly via glycolysis. Specifically, 10⁴ cells per well were seeded in 96-well flat bottom plates in total volume of 100 μ l complete DMEM. 24 h post-seeding, cells were incubated with the compounds for 72 hrs at 37°C (5% CO₂), alamarBlue (10 μ l/well) was added for a further 24 hrs and colorimetric changes were read at 550 nm with reference wavelength 620 nm using a plate photometer (MRX Dynatech Laboratories). Calculation of the compound concentration causing 50% cell death (LC₅₀) was performed using cells treated with DMSO as control sample. LC₅₀ values were determined by nonlinear regression analysis after converting the drug concentrations into log-X using Prism 5.0 software (GraphPad Software Inc.).

Luciferase and Bradford assays. Firefly luciferase activity in cell lysates was measured using the respective chemiluminescent assay kit (Promega), as manufacturer recommended, in a GloMax 20/20 single tube luminometer (Promega) for 10 s. Luciferase activities were normalized to the total protein amount determined using the Bradford assay reagent (Pierce).

Indirect immunofluorescence. Indirect immunofluorescence analysis of JFH1 NS5A was performed as described elsewhere (37). DNA was stained with propidium iodide (Sigma). Images were acquired with the Leica TCS-SP four-channel confocal microscope equipped with an argon ion laser and helium-neon laser.

Statistical analysis. In all diagrams, bars represent mean values of at least two independent experiments in triplicate. Error bars represent standard deviation. Only results subjected to statistical analysis using Student's t-test with $p \leq 0.05$ were considered as statistically significant and presented. Statistical calculations were carried out using Excel Microsoft Office®

Theoretical simulations

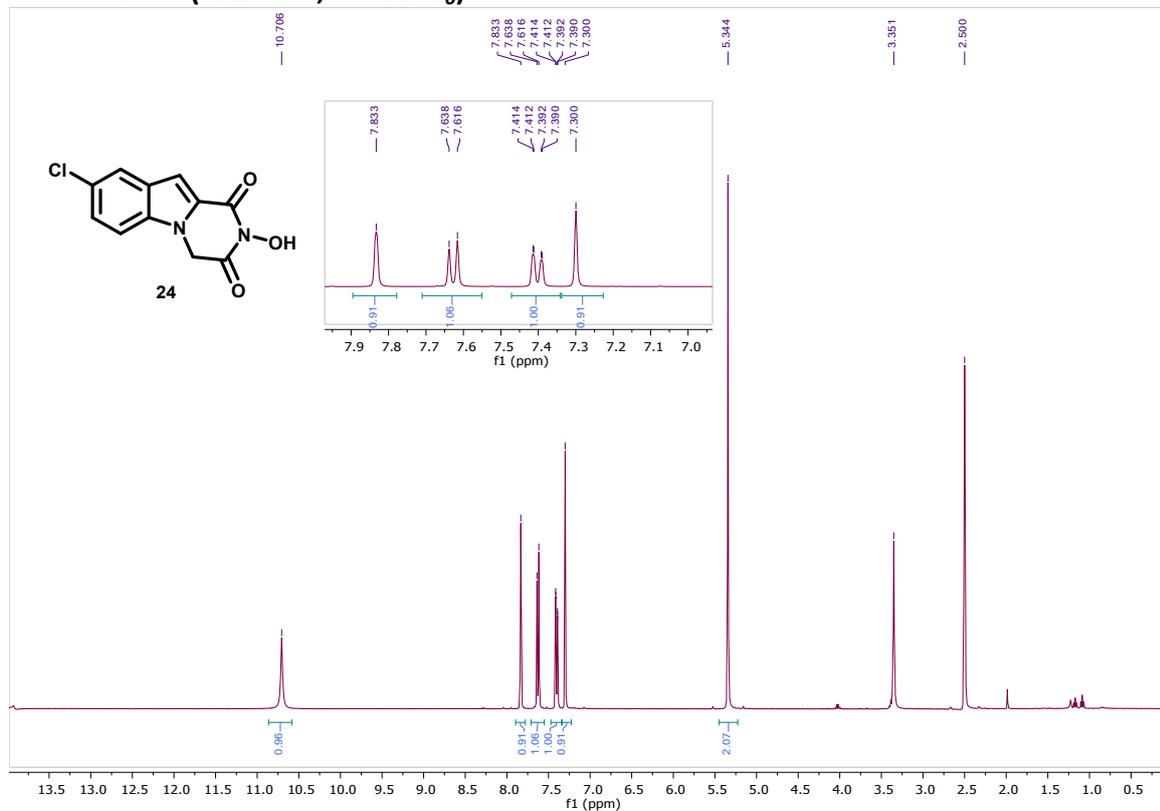
Docking calculations were performed using the Glide SP v. 6.6 sampling algorithm and the corresponding GScore SP5 scoring function (Schrodinger Inc.) with a rigid representation of the protein. The structures of PA endonuclease and HCV polymerase with pdb codes 4AWF, 4KIL, 4M5U and 1GX6, respectively, were downloaded from PDB. Protein preparation was performed by the corresponding routine as implemented in Maestro (Schrodinger Inc.). Prior to calculations, the designed molecules were prepared in terms of correct protonation states, tautomerism and stereoisomerism using the LigPrep routine (Schrodinger Inc.). The theoretical LogP and pK_a properties of the novel compounds were determined using Marvin and Calculator plugins (ChemAxon). Computational analysis of the PA endonuclease

solvation was performed using SZmap algorithm (Openeye Inc.). SZmap implements a semi-continuous solvation model for mapping the surface of the protein and identifies hydration sites of positive (unstable) and negative (stable) free energy. Characterization of water molecules according to their free energy permits rational design of high affinity ligands, which either displace unstable waters or replace stable waters by polar groups of similar capability for accommodating electrostatic interactions with the protein.

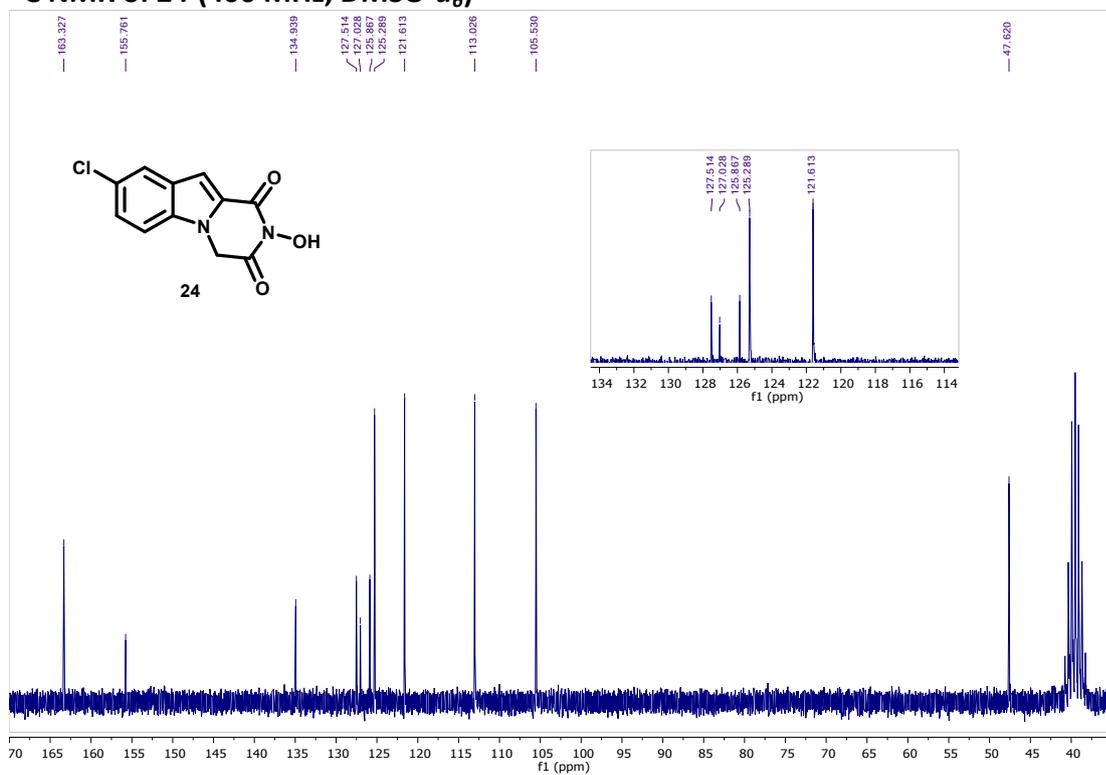
1. S. L. C. D. J. Kempf, *Journal of Organic Chemistry*, 1990 **55**, 1390-1394.
2. A. Stevaert, S. Nurra, N. Pala, M. Carcelli, D. Rogolino, C. Shepard, R. A. Domoaal, B. Kim, M. Alfonso-Prieto, S. A. Marras, M. Sechi and L. Naesens, *Mol. Pharmacol.*, 2015, **87**, 323-337.
3. A. Stevaert, R. Dallochio, A. Dessì, N. Pala, D. Rogolino, M. Sechi and L. Naesens, *J. Virol.*, 2013, **87**, 10524-10538.

IV Copies of NMR spectra

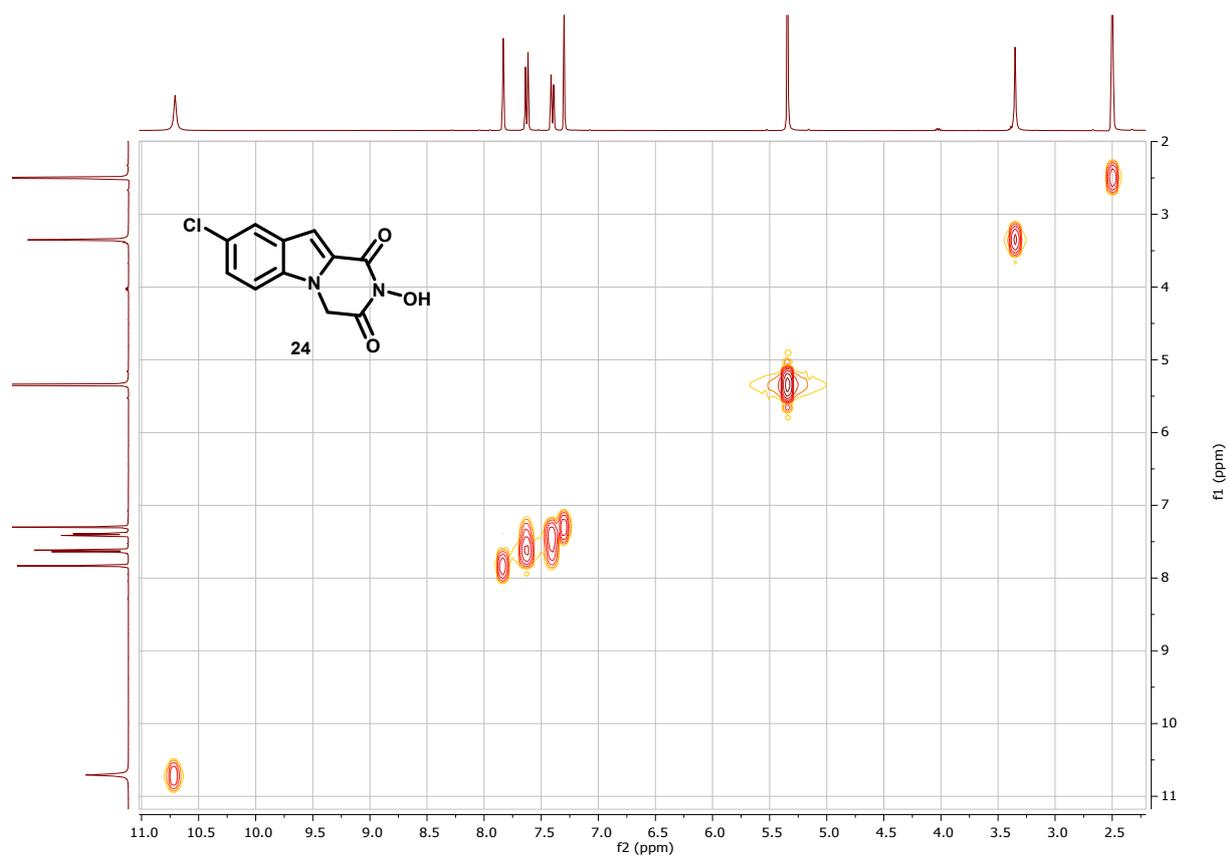
¹H NMR of 24 (400 MHz, DMSO-d₆)



¹³C NMR of 24 (400 MHz, DMSO-d₆)



COSY NMR of 24 (400 MHz, DMSO-*d*₆)



HSQC NMR of 24 (400 MHz, DMSO-*d*₆)

