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Synthesis and Antiviral Evaluation of New N-acylhydrazones Containing Glycine Residue

Baohe Tian[1], Meizi He[1], Zhiwu Tan[1], Shixing Tang[2], Indira Hewlett[2], Shuguang Chen[1], Yinxue Jin[1] and Ming Yang[1,*]

[1]State Key Laboratory of Natural and Biomimetic Drugs, Peking University Health Science Center, PO Box 261, Beijing 100191, China

[2]Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD 20892, USA

**Corresponding author: Prof Ming Yang, mingy@bjmu.edu.cn; yangm@bjmu.edu.cn*

N-acylhydrazones containing glycine residue 3a–j and 8a–h were synthesized as HIV-1 capsid protein assembly inhibitors. The structures of the novel N-acylhydrazone derivatives were characterized using different spectroscopic methods. Antiviral activity demonstrated that compound 8c bearing 4-methylphenyl moiety was the most active with low cytotoxicity.

Key words: antiviral activity, HIV-1 capsid assembly, N-acylhydrazone derivatives, synthesis

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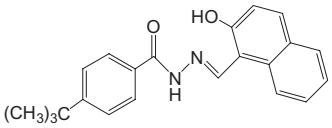
Human immunodeficiency virus type-1 (HIV-1) is the causative agent of acquired immunodeficiency syndrome (AIDS), which has become a major worldwide epidemic since 1981 (1). Over the past decade, extensive efforts have been made to discover a large number of molecules that can inhibit replication of HIV (2). Recently, major progress has been made in the treatment of HIV infection by the introduction of highly active antiretroviral therapy (HAART, a combination of nucleoside and non-nucleoside reverse transcriptase inhibitors, and/or protease inhibitors). Although HAART decreased the number of AIDS cases dramatically, it is not able to eradicate HIV-1 from patients. Because of drug-related side-effects, inconvenient dosing requirements, and/or the emergence of drug-resistant virus, there is an urgent need for the development of novel classes of anti-HIV agents targeting different steps of the viral life cycle (3–5).

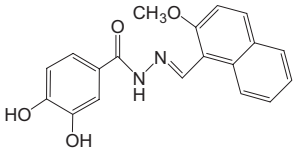
N-Acylhydrazones (NAHs), which include the fragment (–CO–NH–N=CH–), have attracted much attention in the last 20 years because of their biological properties as well as their chelating properties toward metal ions. Many of them have been reported to possess a broad spectrum of biological activities, such as antifungal (6–8), antimalarial (9), antitubercular (10), antiplatelet (11,12) activities, and have been studied extensively as potential therapeutic agents in a number of pharmaceutical contexts. Furthermore, recent studies have revealed that certain NAHs, such as N-(4-*tert*-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone (BBNH) and (E)-3,4-dihydroxy-N′-((2-methoxynaphthalen-1-yl)methylene)benzohydrazide (DHBNH), also displayed potential anti-HIV activities through inhibiting the RNA-dependent DNA polymerase and/or the RNase H (RNH) of HIV-1 reverse transcriptase (RT) (Figure 1) (13–15). These compounds represent a novel lead structure for the development of antiviral agents.

More recently, Peter, Jr has reported a series of small-molecule HIV inhibitors (16), and most of them contain a core structure of acylhydrazone moiety (Figure 2). These compounds were identified to bind to HIV-1 capsid protein (CA) and inhibit capsid assembly during viral maturation. HIV-1 CA plays important roles in the life cycle of HIV-1, and its proper assembly is critical to the viral infectivity (17–21). Thus, HIV-1 CA has become an attractive novel target of high priority for anti-HIV (22), and NAH derivatives deserve further studies as potential HIV-1 CA assembly inhibitors. In addition, CAP-1, one of phenylurea derivatives, has also been identified to bind to the N-terminal domain (NTD) of HIV-1 CA and inhibit capsid assembly during viral maturation (Figure 3) (23). Moreover, Kelly *et al.* have further determined the structure of the complex between CAP-1 and CA NTD (24), which may be used as a tool for drug screening.

In connection with our recent attempts to search for new HIV-1 CA assembly inhibitors (25–27), a series of acylhydrazone derivatives with new molecular structures were virtually screened for their ability to bind to HIV-1 CA using the docking technique. Molecular docking studies were performed using AutoDock 4 software (28) based on the X-ray crystal structure of HIV-1 CA NTD taken from the Protein Data Bank (PDB ID: 2JPR) (24). Among them, two molecules **3a** and **8a** containing glycine residue were found to be docked into the same binding site of the protein in the similar manner as CAP-1, especially in their binding models an aromatic ring inserted into the same hydrophobic pocket as that of CAP-1 in the complex between CAP-1 and HIV-1 CA (Figure 4). In comparison with the binding free energy (−10.58 kcal/mol) and the inhibition constant Ki (9.37 μM) of CAP-1, the low binding free energy (−11.05 and −12.41 kcal/mol, respectively) and inhibition constant Ki (7.31 and 6.80 μM, respectively) of compounds **3a** and **8a** suggested that they could tightly bind to HIV-1 CA and had good binding affinity with it in theory, because of hydrophobic forces, electrostatic interactions, and hydrogen-bond interactions. Moreover, the initial biological assay also showed that compounds **3a** and **8a** both had potent antiviral activities. Based on this preliminary result,



N-(4-*tert*-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone (BBNH)



(E)-3,4-dihydroxy-N'-((2-methoxynaphthalen-1-yl)methylene)benzohydrazide (DHBNH)

Figure 1: Structure of N-(4-*tert*-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone and (E)-3,4-dihydroxy-N′-((2-methoxynaphthalen-1-yl)methylene)benzohydrazide.

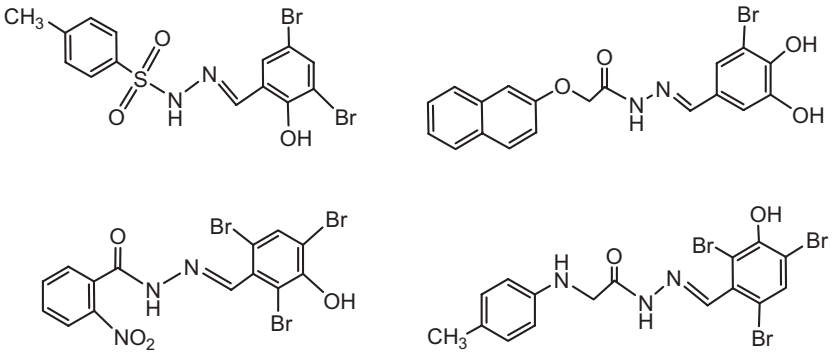


Figure 2: Some small-molecule HIV-1 capsid protein inhibitors.

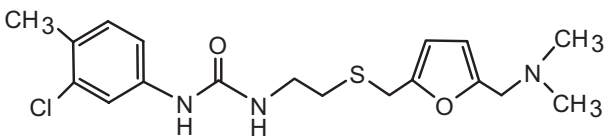


Figure 3: Structure of CAP-1.

to further improve their inhibitory activity other molecular modifications were made in which different kinds of substituents were introduced into the phenyl ring at the right end of the two molecules. Starting from the structures of **3a** and **8a**, we have synthesized their derivatives, and all the derivatives were tested for their antiviral activities. In addition, some representative derivatives were also assayed for their abilities to inhibit HIV-1 CA assembly *in vitro* using an ultraviolet spectrophotometry assay. Moreover, the structure–activity relationships (SAR) of this series of NAH compounds were investigated.

Experimental section

Chemistry

All solvents and reagents were purchased from commercial sources and used directly without purification. Progress of all the reactions was monitored by thin-layer chromatography (TLC) with silica gel GF254 plates, which were visualized by UV light. All melting points were determined with X-4 melting point apparatus and are uncorrected. Infrared spectra were recorded on a NEXUS-470 FTIR spectrophotometer (Thermo Nicolet Corporation, Madison, WI, USA) using KBr pallets. ^{1}H NMR spectroscopic analysis was performed on a Varian 300 MHz spectrometer using tetramethylsilane (TMS) as internal standard, and NMR chemical shifts were reported in δ ppm (parts per million) relative to internal standard. Splitting patterns were assigned as follows: s = singlet, d = doublet, t = triplet, and m = multiplet. Elemental analyses (C, H, N) were determined on a Vario EL III instrument (Elementar company, Hanau, Germany). Mass spectra were performed on a VG ZAB-HS mass spectrometer, and the values are reported in *m/z*. The reported chemical yields were not optimized.

Methyl N-(4-toluenesulfonyl)-glycinate (1)

Triethylamine (8.3 mL, 60 mmol), 4-toluenesulfonyl chloride (3.81 g, 20 mmol) and a catalytic amount of N,N-dimethylaminopyridine (DMAP) were added to a stirred suspension of methyl glycinate hydrochloride (2.51 g, 20 mmol) in CH_2Cl_2 (50 mL) in an ice bath. The reaction mixture was stirred at room temperature for 20 h while 2 N hydrochloric acid (80 mL) was added slowly. The organic phase was separated and washed with water (10 mL), dried with anhydrous Na_2SO_4 and evaporated in vacuo giving a residue, which was washed with a mixture of methanol and water (v/v: 1/10) and dried to afford the corresponding methyl ester **1** as a white solid. Yield: 84.4%; m.p. 91–93 °C.

N-(4-toluenesulfonyl)-glycinyl hydrazide (2)

Compound **1** (2.43 g, 10 mmol) was added in small portions to a stirred solution of 85% hydrazine hydrate (3 mL) in 5 mL ethanol.

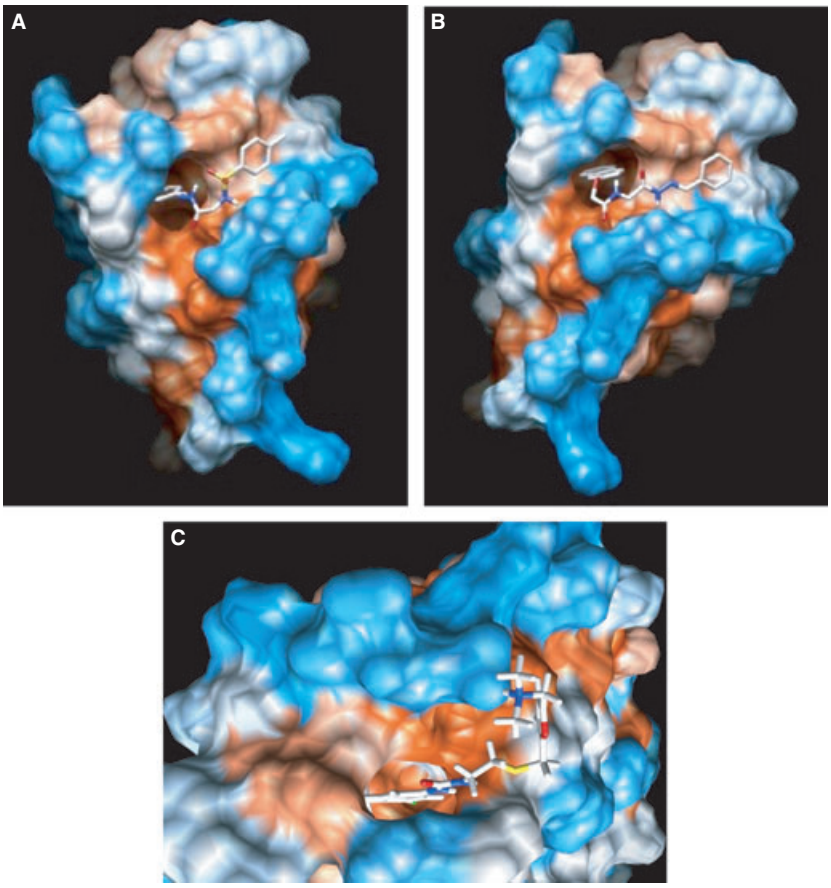


Figure 4: (A) Model of **3a** docked into HIV-1 capsid protein (CA) N-terminal domain (NTD). (B) Model of **8a** docked into HIV-1 CA NTD. (C) Model of CAP-1 docked into HIV-1 CA NTD.

The mixture was heated under reflux for 6 h. While cooling to room temperature, the resulting precipitate was filtered in vacuo, washed with cold water, and dried to give the corresponding hydrazide **2** as a white solid. Yield: 86.4%; m.p. 155–156 °C.

General procedures for the preparation of 3a, 3b, 3e, 3f, 3h, 3i (Method A)

To a magnetically stirred suspension of compound **2** (121.5 mg, 0.5 mmol) in anhydrous methanol (3 mL) was added the appropriate aldehyde/ketone (0.5 mmol). Shortly, the solution became homogeneous and within minutes the resulting hydrazone began to precipitate. After the mixture was stirred for 1–2 h more at room temperature, the precipitate was collected by filtration, washed with a small quantity of cold methanol and dried. Recrystallization of the reaction product from methanol gave the corresponding hydrazone.

Benzaldehyde N-(4-toluenesulfonyl)-glycinyl hydrazone (3a)

White crystalline solid; yield: 87.5%; m.p. 158–159 °C; ^{1}H NMR (DMSO-d_6): δ = 2.37 (s, 1H), 3.54, 4.03 (2d, 2H, J = 5.7 Hz), 7.36–7.44 (m, 5H), 7.60–7.73 (m, 4H), 7.82, 8.02 (2t, 1H, J = 5.7 Hz), 7.93, 8.15 (2s, 1H), and 11.38, 11.46 (2s, 1H); IR (KBr) ν: 3241, 3077, 2944, 2863, 1688, 1603, 1367, 1346, 1166 cm^{-1}; EI-MS (m/z): 331 (M^+); Anal. calcd for $C_{16}H_{17}N_3O_3S$: C 57.99, H 5.17, N 12.68; found C 57.70, H 5.13, N 12.43.

Acetophenone N-(4-toluenesulfonyl)-glycinyl hydrazone (3b)

White crystalline solid; yield: 83.4%; m.p. 154–155 °C; ^{1}H NMR (DMSO-d_6): δ = 2.20 (s, 3H), 2.37 (s, 3H), 3.70, 4.06 (2d, 2H, J = 5.7 Hz), 7.36–7.46 (m, 5H), 7.68–7.77 (m, 4H), 7.82, 8.00 (2t, 1H, J = 5.7 Hz), and 10.33, 10.73 (2s, 1H); IR (KBr) ν: 3245, 3194, 3086, 2924, 1686, 1596, 1376, 1332, 1167 cm^{-1}; EI-MS (m/z): 345 (M^+); Anal. calcd for $C_{17}H_{19}N_3O_3S$: C 59.11, H 5.54, N 12.17; found C 58.87, H 5.48, N 12.06.

2-Fluorobenzaldehyde N-(4-toluenesulfonyl)-glycinyl hydrazone (3e)

White crystalline solid; yield: 74.2%; m.p. 207–208 °C; ^{1}H NMR ($CDCl_3$): δ = 2.38 (s, 3H), 3.54, 4.03 (2s, 2H), 7.26–7.31 (t, 2H, J = 7.8 Hz), 7.37–7.42 (t, 2H, J = 7.8 Hz), 7.45–7.49 (m, 1H), 7.70–7.73 (m, 2H), 7.77–7.88 (m, 1H), and 8.14, 8.40 (2s, 1H); IR (KBr) ν: 3202, 3108, 2997, 2881, 1673, 1614, 1414, 1330, 1292, 1156 cm^{-1}; EI-MS (m/z): 349 (M^+); Anal. calcd for $C_{16}H_{16}FN_3O_3S$: C 55.00, H 4.62, N 12.03; found C 54.89, H 4.56, N 11.95.

4-Bromobenzaldehyde N-(4-toluenesulfonyl)-glycinyl hydrazone (3f)

White crystalline solid; yield: 89.3%; m.p. 213–214 °C; ^{1}H NMR (DMSO-d_6): δ = 2.37 (s, 3H), 3.53, 4.03 (2d, 2H, J = 3.0 Hz), 7.36–7.38 (m, 2H), 7.56–7.72 (m, 6H), 7.85, 8.04 (2t, 1H, J = 3.0 Hz),

7.90, 8.12 (2s, 1H), and 11.47, 11.53 (2s, 1H); IR (KBr) *v*: 3228, 3071, 2982, 2931, 1680, 1599, 1319, 1272, 1158 cm^{-1}; EI-MS (*m/z*): 409 ($M - 1^+$), 411 ($M + 1^+$); Anal. calcd for $C_{16}H_{16}BrN_3O_3S$: C 46.84, H 3.93, N 10.24; found C 46.90, H 3.94, N 10.33.

4-Nitrobenzaldehyde N-(4-toluenesulfonyl)-glycinyl hydrazone (3h)

Light yellow crystalline solid; yield: 91.5%; m.p. 249–250 °C; ^1H NMR (DMSO-d_6): δ = 2.38 (s, 3H), 3.58, 4.08 (2d, 2H, *J* = 4.8 Hz), 7.37–7.41 (m, 2H), 7.70–7.73 (m, 2H), 7.88–7.93 (m, 2H), 7.96, 8.03 (2s, 1H), 8.26–8.28 (m, 2H), 8.08, 8.29 (2t, 1H, *J* = 4.8 Hz), and 11.72, 11.77 (2s, 1H); IR (KBr) *v*: 3238, 3077, 2949, 1687, 1587, 1520, 1341, 1165 cm^{-1}; EI-MS (*m/z*): 376 (M^+); Anal. calcd for $C_{16}H_{16}N_4O_5S$: C 51.06, H 4.28, N 14.89; found C 50.90, H 4.23, N 14.62.

2-Furaldehyde N-(4-toluenesulfonyl)-glycinyl hydrazone (3i)

White crystalline solid; yield: 90.7%; m.p. 165–167 °C; ^1H NMR (DMSO-d_6): δ = 2.37 (s, 3H), 3.52, 3.94 (2d, 2H, *J* = 6.0 Hz), 6.60–6.63 (m, 1H), 6.86–6.90 (m, 1H), 7.37–7.41 (m, 2H), 7.69–7.73 (m, 2H), 7.81 (s, 1H), 7.83, 7.87 (2s, 1H), 8.04, 8.33 (2t, 1H, *J* = 6.0 Hz), and 11.36, 11.43 (2s, 1H); IR (KBr) *v*: 3275, 3135, 3085, 2944, 1681, 1599, 1439, 1376, 1308, 1149 cm^{-1}; EI-MS (*m/z*): 321 (M^+); Anal. calcd for $C_{14}H_{15}N_3O_4S$: C 52.33, H 4.70, N 13.08; found C 52.11, H 4.63, N 12.92.

General procedures for the preparation of 3c, 3d, 3g, 3j (Method B)

To a stirred mixture of compound **2** (121.5 mg, 0.5 mmol) in anhydrous methanol (3 mL) was added the appropriate aldehyde/ketone (0.5 mmol) and two drops of glacial acetic acid. The reaction mixture was heated at reflux for 3–8 h. After cooling, the precipitate was collected by vacuum filtration, washed with cold methanol and dried. Recrystallization of the reaction product from methanol gave the corresponding hydrazone.

4-Methylbenzaldehyde N-(4-toluenesulfonyl)-glycinyl hydrazone (3c)

White crystalline solid; yield: 72.6%; m.p. 185–186 °C; ^1H NMR (DMSO-d_6): δ = 2.32 (s, 3H), 2.36 (s, 3H), 3.51, 4.00 (2d, 2H, *J* = 2.7 Hz), 7.22–7.37 (m, 4H), 7.48–7.56 (m, 2H), 7.69–7.71 (m, 2H), 7.81, 8.00 (2t, 1H, *J* = 2.7 Hz), 7.88, 8.09 (2s, 1H), and 11.31, 11.39 (2s, 1H); IR (KBr) *v*: 3285, 3238, 3085, 2972, 2920, 2856, 1675, 1609, 1558, 1395, 1325, 1160 cm^{-1}; EI-MS (*m/z*): 345 (M^+); Anal. calcd for $C_{17}H_{19}N_3O_3S$: C 59.11, H 5.54, N 12.17; found C 58.96, H 5.51, N 12.13.

4-Methoxybenzaldehyde N-(4-toluenesulfonyl)-glycinyl hydrazone (3d)

White crystalline solid; yield: 78.4%; m.p. 184–185 °C; ^1H NMR (DMSO-d_6): δ = 2.37 (s, 3H), 3.79 (s, 3H), 3.51, 3.99 (2d, 2H, *J* = 5.7 Hz), 6.97–7.01 (m, 2H), 7.36–7.41 (m, 2H), 7.54–7.73 (m, 4H), 7.80, 8.00 (2t, 1H, *J* = 5.7 Hz), 7.87, 8.08 (2s, 1H), and 11.25, 11.34 (2s, 1H); IR (KBr) *v*: 3226, 3070, 2921, 2838, 1684, 1612, 1600, 1364, 1341, 1165 cm^{-1}; EI-MS (*m/z*): 361 (M^+); Anal. calcd for $C_{17}H_{19}N_3O_4S$: C 56.50, H 5.30, N 11.63; found C 56.62, H 5.30, N 11.66.

3-Methoxy-4-hydroxybenzaldehyde N-(4-toluenesulfonyl)-glycinyl hydrazone (3g)

White crystalline solid; yield: 74.8%; m.p. 168–170 °C; ^1H NMR (DMSO-d_6): δ = 2.31 (s, 3H), 3.79 (s, 3H), 3.52, 3.98 (2d, 2H, *J* = 5.1 Hz), 6.81 (d, 1H), 7.03 (t, 1H), 7.19 (d, 1H), 7.37 (t, 2H), 7.71 (d, 2H), 7.81, 8.02 (2t, 1H, *J* = 5.1 Hz), 9.48 (brs, 1H), and 11.14, 11.26 (2s, 1H); IR (KBr) *v*: 3228, 3071, 2982, 2931, 2835, 1680, 1599, 1319, 1272, 1158 cm^{-1}; EI-MS (*m/z*): 377 (M^+); Anal. calcd for $C_{17}H_{19}N_3O_5S$: C 54.10, H 5.07, N 11.13; found C 53.87, H 4.99, N 11.03.

3,4-Methylenedioxybenzaldehyde N-(4-toluenesulfonyl)-glycinyl hydrazone (3j)

White crystalline solid; yield: 67.9%; m.p. 202–204 °C; ^1H NMR (DMSO-d_6): δ = 2.38 (s, 3H), 3.51, 3.99 (2d, 2H, *J* = 6.0 Hz), 6.08 (s, 2H), 6.95–7.22 (m, 3H), 7.36–7.41 (m, 2H), 7.70–7.73 (m, 2H), 7.80, 8.00 (2t, 1H, *J* = 6.0 Hz), 7.82, 8.05 (2s, 1H), and 11.28, 11.36 (2s, 1H); IR (KBr) *v*: 3257, 3084, 2950, 2902, 1683, 1597, 1503, 1436, 1360, 1334, 1251, 1167, 1098 cm^{-1}; EI-MS (*m/z*): 375 (M^+); Anal. calcd for $C_{17}H_{17}N_3O_5S$: C 54.39, H 4.56, N 11.19; found C 54.49, H 4.60, N 11.32.

Ethyl 2-(2-naphthoxy) acetate (4)

A mixture of 2-naphthol (4.33 g, 30 mmol), ethyl 2-bromoacetate (3.3 mL, 30 mmol) and anhydrous K_2CO_3 (4.15 g, 30 mmol) in acetone (20 mL) was heated at reflux for 12 h. After cooling, inorganic salts were separated by filtration, washed with a small quantity of acetone, then the combined filtrate was evaporated in vacuo to dryness. The residue was washed with water, subsequently with a solution of petroleum ether/ethyl acetate (4:1) and dried to produce the corresponding ethyl 2-(2-naphthoxy) acetate **4** as a pink solid. Yield: 80%; m.p. 49 °C.

2-(2-Naphthoxy) acetyl hydrazide (5)

Compound **4** (4.6 g, 20 mmol) was added in small portions to a stirred solution of 85% hydrazine hydrate (6 mL) in 10 mL ethanol. The mixture was heated under reflux for 6 h. While cooling to room temperature, the resulting precipitate was filtered in vacuo, washed with cold water and dried to give the corresponding hydrazide **5** as a white solid. Yield: 92.6%; m.p. 174–175 °C.

Methyl 2-naphthyloxyacetamidoacetate (6)

Compound **5** (2.16 g, 10 mmol) was added to a stirred solution of HOAc (60 mL), 1 N HCl (30 mL) and water (250 mL), then to the resulted mixture in ice-salt bath a solution of $NaNO_2$ (0.69 g, 10 mmol) in cold water (30 mL) was added. After stirring for 15 min in ice-salt bath, the yellow solution was formed. The result-

ing azide was taken in cold ethyl acetate (300 mL), washed with 3% solution of $NaHCO_3$, washed with water, and finally dried (Na_2SO_4). A solution of methyl glycinate hydrochloride (1.26 g, 10 mmol) in ethyl acetate (200 mL) containing 2 mL of Et_3N was stirred at 0 °C for 30 min, filtered, and the filtrate was added to the azide solution. The mixture was kept at −5 °C for 12 h, then at room temperature for additional 12 h, followed by washing with 0.5 N HCl, water, 3% solution of $NaHCO_3$ and finally dried (Na_2SO_4). The solution was evaporated to dryness to give product **6** as a light yellow oil. Yield: 53.7%.

2-Naphthyloxyacetamidoacetohydrazide (7)

Compound **6** (1.37 g, 5 mmol) was added in small portions to a stirred solution of 85% hydrazine hydrate (2 mL) in 3 mL ethanol. The mixture was heated under reflux for 6 h. While cooling to room temperature, the resulting precipitate was filtered in vacuo, washed with cold water and dried to give the corresponding hydrazide **7** as a white solid. Yield: 81.7%; m.p. 179–180 °C; ^{1}H NMR (DMSO-d_6): δ = 3.76 (d, 2H, J = 5.7 Hz), 4.23 (s, 2H), 4.65 (s, 2H), 7.26–7.50 (m, 4H), 7.79–7.88 (m, 3H), 8.39 (t, 3H, J = 5.7 Hz), and 9.11 (s, 1H); EI-MS (m/z): 273 (M^+).

General procedures for the preparation of 8a, 8b, 8f, 8g

2-Naphthyloxyacetamidoacetohydrazones of benzaldehyde/acetophenone/4-bromobenzaldehyde/2-furaldehyde (**8a**, **8b**, **8f**, **8g**) were prepared according to the procedure in method A described earlier, starting from compound **7**.

Benzaldehyde 2-naphthyloxyacetamidoacetohydrazone (8a)

White crystalline solid; yield: 74.8%; m.p. 201–202 °C; ^{1}H NMR (DMSO-d_6): δ = 3.92, 4.35 (2d, 2H, J = 5.7 Hz), 4.70 (s, 2H), 7.23–7.50 (m, 7H), 7.68–8.00 (m, 5H), 8.05, 8.21 (2s, 1H), 8.36, 8.55 (2t, 1H, J = 5.7 Hz), and 11.48, 11.57 (2s, 1H); IR (KBr) v: 3347, 3204, 3065, 2916, 1672, 1631, 1540, 1217, 1183, 1070 cm^{-1}; EI-MS (m/z): 361 (M^+).

Acetophenone 2-naphthyloxyacetamidoacetohydrazone (8b)

White crystalline solid; yield: 70.5%; m.p. 221–222 °C; ^{1}H NMR (DMSO-d_6): δ = 2.26 (s, 3H), 4.04, 4.38 (2d, 2H, J = 5.7 Hz), 4.71 (s, 2H), 7.26–7.50 (m, 7H), 7.77–7.89 (m, 5H), 8.35, 8.51 (2t, 1H, J = 5.7 Hz), and 10.50, 10.81 (2s, 1H); IR (KBr) v: 3413, 3197, 3060, 2928, 1678, 1631, 1522, 1219, 1187, 1052 cm^{-1}; EI-MS (m/z): 375 (M^+); Anal. calcd for $C_{22}H_{21}N_3O_3$: C 70.38, H 5.64, N 11.19; found C 70.13, H 5.53, N 11.07.

4-Bromobenzaldehyde 2-naphthyloxyacetamidoacetohydrazone (8f)

White crystalline solid; yield: 78.3%; m.p. 219–220 °C; ^{1}H NMR (DMSO-d_6): δ = 3.92, 4.34 (2d, 2H, J = 4.8 Hz), 4.69 (s, 2H), 7.26–7.50 (m, 3H), 7.64 (s, 4H), 7.80–7.89 (m, 4H), 7.97, 8.18 (2s, 1H), 8.37, 8.56 (2t, 1H, J = 4.8 Hz), and 11.56, 11.64 (2s, 1H); IR (KBr) v: 3398, 3060, 2910, 2848, 1695, 1649, 1542, 1287, 1217, 1067 cm^{-1}; EI-MS (m/z): 439 ($M - 1^+$), 441 ($M + 1^+$); Anal. calcd for $C_{21}H_{18}BrN_3O_3$: C 57.29, H 4.12, N 9.54; found C 57.37, H 4.19, N 9.61.

2-Furaldehyde 2-naphthyloxyacetamidoacetohydrazone (8g)

Pink crystalline solid; yield: 72.1%; m.p. 205–206 °C; ^{1}H NMR (DMSO-d_6): δ = 3.90, 4.27 (2d, 2H, J = 5.7 Hz), 4.70 (s, 2H), 6.62 (d, 1H), 6.90 (d, 1H), 7.26–7.50 (m, 4H), 7.80–7.88 (m, 4H), 7.89, 8.10 (2s, 1H), 8.32, 8.54 (2t, 1H, J = 5.7 Hz), and 11.42, 11.51 (2s, 1H); IR (KBr) v: 3372, 3200, 3062, 2938, 1698, 1669, 1631, 1540, 1232, 1184, 1068 cm^{-1}; EI-MS (m/z): 351 (M^+); Anal. calcd for $C_{19}H_{17}N_3O_4$: C 64.95, H 4.88, N 11.96; found C 64.68, H 4.87, N 11.84.

General procedures for the preparation of 8c, 8d, 8e, 8h

2-Naphthyloxyacetamidoacetohydrazone of 4-methylbenzaldehyde/4-methoxybenzaldehyde/4-hydroxybenzaldehyde/3,4-methylenedioxybenzaldehyde (**8c**, **8d**, **8e**, **8h**) were prepared according to the procedure in method B described earlier, starting from compound **7**.

4-Methylbenzaldehyde 2-naphthyloxyacetamidoacetohydrazone (8c)

White crystalline solid; yield: 63.9%; m.p. 208–209 °C; ^{1}H NMR (DMSO-d_6): δ = 2.33 (s, 3H), 3.90, 4.33 (2d, 2H, J = 4.8 Hz), 4.69 (s, 2H), 7.23–7.59 (m, 8H), 7.80–7.89 (m, 3H), 7.96, 8.16 (2s, 1H), 8.36, 8.55 (2t, 1H, J = 4.8 Hz), and 11.42, 11.51 (2s, 1H); IR (KBr) v: 3398, 3086, 2973, 2913, 1691, 1654, 1542, 1218, 1185, 1062 cm^{-1}; EI-MS (m/z): 375 (M^+); Anal. calcd for $C_{22}H_{21}N_3O_3$: C 70.38, H 5.64, N 11.19; found C 70.17, H 5.53, N 11.02.

4-Methoxybenzaldehyde 2-naphthyloxyacetamidoacetohydrazone (8d)

White crystalline solid; yield: 67.2%; m.p. 187–188 °C; ^{1}H NMR (DMSO-d_6): δ = 3.79 (s, 3H), 3.90, 4.32 (2d, 2H, J = 5.4 Hz), 4.69 (s, 2H), 7.00 (t, 2H), 7.26–7.50 (m, 4H), 7.56 (d, 2H), 7.80–7.89 (m, 3H), 7.93, 8.14 (2s, 1H), 8.34, 8.53 (2t, 1H, J = 5.4 Hz), and 11.31, 11.45 (2s, 1H); IR (KBr) v: 3386, 3201, 3089, 2962, 2925, 1690, 1655, 1608, 1253, 1184, 1033 cm^{-1}; EI-MS (m/z): 391 (M^+); Anal. calcd for $C_{22}H_{21}N_3O_4$: C 67.51, H 5.41, N 10.74; found C 67.29, H 5.37, N 10.73.

4-Hydroxybenzaldehyde 2-naphthyloxyacetamidoacetohydrazone (8e)

White crystalline solid; yield: 61.0%; m.p. 152–154 °C; ^{1}H NMR (DMSO-d_6): δ = 3.89, 4.30 (2d, 2H, J = 6.0 Hz), 4.69 (s, 2H), 6.80–6.83 (m, 2H), 7.26–7.53 (m, 6H), 7.80–7.87 (m, 3H), 7.89, 8.09 (2s, 1H), 8.32, 8.51 (2t, 1H, J = 6.0 Hz), 9.91 (s, 1H), and 11.26, 11.36 (2s, 1H); IR (KBr) v: 3389, 3163, 3060, 2924, 1659, 1603, 1276, 1216, 1053 cm^{-1}; EI-MS (m/z): 377 (M^+); Anal. calcd for

$C_{21}H_{19}N_3O_4$: C 66.83, H 5.07, N 11.13; found C 67.14, H 5.14, N 11.20.

3,4-Methylenedioxybenzaldehyde 2-naphthyloxyacetamidoacetohydrazone (8h)

White crystalline solid; yield: 71.4%; m.p. 210–212 °C; ^{1}H NMR (DMSO-d_6): δ = 3.89, 4.31 (2d, 2H, J = 5.1 Hz), 4.69 (s, 2H), 6.08 (s, 2H), 6.95–6.99 (m, 1H), 7.10–7.13 (m, 1H), 7.26–7.50 (m, 5H), 7.80–7.86 (m, 3H), 7.89, 8.11 (2s, 1H), 8.36, 8.54 (2t, 1H, J = 5.1 Hz), and 11.38, 11.40 (2s, 1H); IR (KBr) ν: 3392, 3213, 3084, 2907, 1700, 1648, 1599, 1257, 1218, 1041 cm^{-1}; EI-MS (m/z): 405 (M^+); Anal. calcd for $C_{22}H_{19}N_3O_5$: C 65.18, H 4.72, N 10.37; found C 64.98, H 4.74, N 10.32.

Biological activity

Antiviral activity and cytotoxicity

Inhibition of Simian Immunodeficiency Virus-induced syncytium in CEM174 cell cultures was measured in a 96-well microplate containing 1×10^5 CEM cells/mL infected with 100 $TCID_{50}$ of Simian Immunodeficiency Virus (SIV) per well and containing appropriate dilutions of the tested compounds. After 5 days of incubation at 37 °C in 5% CO_2 containing humidified air, CEM giant (syncytium) cell formation was examined microscopically. The EC_{50} was defined as the compound concentration required to protect cells against the cytopathogenicity of SIV by 50%. The TC_{50} assay was performed in uninfected CEM174 cells under the same condition as above, and the value was determined as the concentration required to inhibit CEM cells proliferation by 50% in methyl thiazolyl tetrazolium reduction assay.

Purification of the recombinant CA

A 2-liter culture was grown to 37 °C in Lysogeny broth (LB), a nutritionally rich medium containing ampicillin (100 ug/mL) to an A_{600} of 0.8, isopropyl-β-D-thiogalactopyranoside was added to 0.2 mM and the incubation was changed to 30 °C for an additional 12 h. The cells were collected by centrifugation and resuspended in 80 mL lysis buffer (50 mM Mes, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM EDTA, 1 mM 1,4-Dithiothreitol). Cell lysis was carried on an Ultrasonic Disrupter, and the cell debris was separated by centrifugation. Then the CA was precipitated from the supernatant with 25% saturated ammonium sulfate; the precipitate was collected by centrifugation, resuspended in 12 mL dialysis buffer (50 mM Tris/HCl, 30 mM NaCl, 1 mM EDTA, 1 mM DTT) and then dialyzed against 300 mL of the same buffer overnight. Cellulose DE-52 was equilibrated with the dialysis buffer and the crude CA was loaded, eluted with the same buffer. Flow-through fractions were analyzed by denaturing SDS/polyacrylamide gel and the purified CA fraction was precipitated again with 50% saturated ammonium sulfate, collected by centrifugation, dialyzed against 300 mL H_2O for two times. Protein concentrations were determined by the Bradford method.

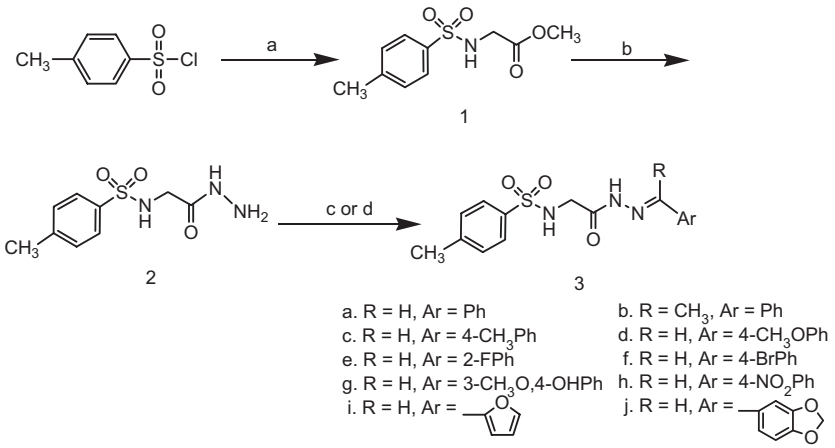
Ultraviolet spectrophotometry analysis

Ultraviolet spectrophotometry assay was performed at 350 nm on an Agilent 8453 spectrophotometer. A 1.0 μL of concentrated ligand in DMSO (1 mM) was added to a 75 μL aqueous solution (2 mL of 5 M NaCl mixed with 1 mL of 200 mM NaH_2PO_4, pH 8.0), and then added 25 μL CA (40 μM) to initiate the reaction. Spectral measurements were made every 10 seconds, following a short initial delay to allow sample equilibration. Relative assembly rates were estimated from initial slopes of the plots of absorbance versus time.

Results and discussion

Chemistry

The synthetic routes for the title compounds **3a–j** are described in Scheme 1. The 4-toluenesulfonyl chloride was first reacted with methyl glycinate hydrochloride in the presence of Et_3N, to give the key intermediate methyl N-(4-toluenesulfonyl)-glycinate **1** in good yield (29). This intermediate **1** was then converted to the corresponding hydrazide **2** by refluxing with hydrazine hydrate in ethanol.



Scheme 1: Synthesis of 4-toluenesulfonylhydrazone derivatives containing glycine residue. Reagents and conditions: (a) glycine methyl ester hydrochloride, Et_3N, CH_2Cl_2, 0 °C to rt, 20 h; (b) 85% $NH_2NH_2{\cdot}H_2O$, CH_3CH_2OH, reflux, 6 h; (c) RCOAr, anhydrous CH_3OH, rt, 1–2 h; (d) RCOAr, anhydrous CH_3OH, glacial acetic acid, reflux, 3–8 h.

The condensation of the hydrazide **2** with various aromatic aldehydes and ketones in methanol afforded the title compounds **3a–j**.

The title compounds **8a–h** were prepared according to the synthetic strategy outlined in Scheme 2. 2-Naphthol was first O-alkylated with ethyl 2-bromoacetate in the presence of anhydrous K_2CO_3 in acetone at refluxing temperature, to give ethyl 2-(2-naphthoxy) acetate **4**, which was reacted with hydrazine hydrate in ethanol to obtain the corresponding hydrazide **5** (30). The hydrazide **5** was then treated with aqueous $NaNO_2$ in HOAc and 1 N HCl at −5 °C to obtain the azide via the azide-coupling method, which was reacted directly with methyl glycinate hydrochloride in ethyl acetate in the presence of Et_3N at 0 °C for 30 min to give, after neutralization, the desired intermediate **6** (31). Treatment of **6** with hydrazine hydrate in ethanol under reflux afforded the corresponding hydrazide **7** as key intermediate, which underwent condensation with a variety of aromatic aldehydes and ketones, resulting in the formation of the corresponding acylhydrazone derivatives **8a–h** by a route analogous to that for **3a–j**.

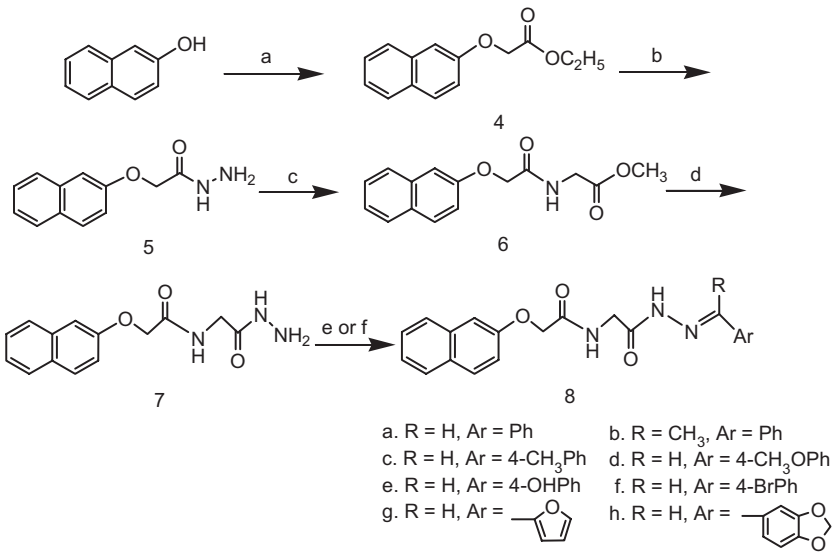
The purity of all the products was determined by TLC, melting points and elemental analysis. The physical and spectral properties of previously reported compounds, such as melting points, were in accord with literature values, and the structures of the novel compounds were confirmed by ^{1}H NMR, EI-MS and IR. According to the literature, this class of acyl hydrazones are in the form of E geometrical isomer for C=N double bond in DMSO-d_6, and the E geometrical isomer undergoes a rapid cis/trans amide equilibrium at room temperature (32,33), which is consistent with the analytical results of the ^{1}H NMR spectra of our synthesized derivatives, in which two sets of methylene, imine and amide protons signals of cis/trans amide conformers were observed. Furthermore, the molecular ion recorded in the MS was also in agreement with the molecular weight of the desired compounds.

Antiviral activity and cytotoxicity

The title compounds **3a–j** and **8a–h** were assayed for their antiviral activities using SIV-induced syncytium in CEM174 cells, and their cytotoxicities were also evaluated *in vitro* in uninfected CEM174 cells. Their EC_{50} (antiviral activity), TC_{50} (cytotoxicity), and therapeutic index (TI) are listed in Table 1. As shown in Table 1, each of the tested compounds possessed an EC_{50} value within the range from 0.47 μM to 20.2 μM, and a TC_{50} value ranging from 29.4 μM to more than 100 μM. Analyzing the activities of the synthesized compounds, the following SAR was observed.

In series **3a–j**, compared with the leading compound **3a** (EC_{50} = 1.5 μM), all other derivatives showed equivalent or decreased antiviral activities. Although the introduction of halogen groups (F in **3e**, Br in **3f**) into the phenyl ring at the right end resulted in approximately equal activities, their cytotoxicities were both significantly decreased with TC_{50} values of more than 100 μM. For example, **3e** and **3f** showed EC_{50} values of 1.7 and 2.1 μM, resulting in TI values of more than 58.8 and 47.6, respectively. Similarly, the substituents, such as methyl and methoxy groups on the phenyl ring in compounds **3c** and **3d**, caused slight decrease in antiviral activities, but both of them showed lower cytotoxicity with TC_{50} value of more than 100 μM. Only compound **3i** in which the phenyl ring in **3a** was replaced with the furyl ring displayed slightly higher activity (EC_{50} = 1.37 μM), but was more cytotoxic with a TC_{50} value of 31.6 μM when compared with **3a**.

The test results of the series **8a–h** indicated that all the derivatives with different substituted groups on the phenyl ring exhibited



Scheme 2: Synthesis of 2-naphthoxyacyl hydrazone derivatives containing glycine residue. Reagents and conditions: (a) ethyl 2-bromoacetate, K_2CO_3, acetone, reflux, 12 h; (b) 85%$NH_2NH_2{\cdot}H_2O$, CH_3CH_2OH; reflux, 6 h; (c) (1) $NaNO_2$, HCl, AcOH, H_2O; (2) glycine methyl ester hydrochloride, Et_3N, ethyl acetate, −5 °C to rt; (d) 85%$NH_2NH_2{\cdot}H_2O$, CH_3CH_2OH, reflux, 6 h; (e) RCOAr, anhydrous CH_3OH, rt, 1–2 h; (f) RCOAr, anhydrous CH_3OH, glacial acetic acid, reflux, 3–8 h.

Table 1: Antiviral activity and cytotoxicity of acylhydrazone derivatives on SIV-induced syncytium[a]

Compound	EC_{50} (μM)[b]	TC_{50} (μM)[c]	TI[d]
3a	1.5	83.5	55.7
3b	1.53	30.9	20.2
3c	2.5	>100	>40
3d	3.9	>100	>25.6
3e	1.7	>100	>58.8
3f	2.1	>100	>47.6
3g	20.2	53.8	2.7
3h	6.1	32.9	5.4
3i	1.37	31.6	23.1
3j	5.8	>100	>17.2
8a	2.25	77.0	34.2
8b	5.42	45.0	8.3
8c	0.47	>100	>212.8
8d	1.1	>100	>90.9
8e	4.2	29.4	7.0
8f	1.01	60.8	60.2
8g	5.33	>100	>18.8
8h	2	67.9	34.0

[a]This assay used the antiviral drug Indinavir at 1×10^{-6} M as positive control.
[b]Antiviral activity, concentration that inhibits viral replication by 50%.
[c]Cytotoxicity, concentration that is toxic to 50% of SIV-induced syncytium.
[d]Therapeutic index, TC_{50} value divided by EC_{50} value (TC_{50}/EC_{50}).

moderate to good antiviral activities with EC_{50} values in the range of 0.47–5.42 μM, in which the compound **8c** was found to be the most active. Compounds **8c** and **8d** (EC_{50} = 0.47 and 1.1 μM) in which methyl and methoxy groups were introduced into the phenyl ring were greater than the leading compound **8a** (EC_{50} = 2.25 μM). Furthermore, they possessed lower cytotoxicities with TC_{50} values of more than 100 μM, safer than compound **8a**. Bromosubstitution on the phenyl ring led to compound **8f** (EC_{50} = 1.01 μM), which showed over twofold higher inhibitory activity than **8a**, but its cytotoxicity was similar to **8a**. In addition, contrary to **3i**, the replacement with furyl ring in compound **8g**, gave rise to a significant decrease in both activity and toxicity (EC_{50} = 5.33 μM, TC_{50} > 100 μM).

These structure–activity relationship analyses indicated that the lipophilic groups on the phenyl ring were critical for the high antiviral activity, such as **3c**, **3e**, **3f**, **8e**, and **8f**, which might be explained by the increase in the molecular hydrophobicity that enhanced the hydrophobic interaction between the molecule and HIV-1 CA, thus resulting in better antiviral activity. Furthermore, the proper molecular size caused by the presence of these substituents could also contribute to their high affinity with the protein.

Inhibition of capsid assembly in vitro

Among all the title compounds, eight representative derivatives were selected to be further evaluated for their inhibitory effects on *in vitro* capsid assembly. In accordance with the literature (23), in our study the turbidity in direct relation to the assembly rate was monitored using ultraviolet spectrophotometry. The assembly rate indicates how strongly a molecule binds to the protein and inhibits capsid assembly. The assembly rate slowed when the molecule bound strongly because the strongly binding compound prevented or slowed the assembly. As shown in Table 2, dissolution of native HIV-1 CA into assembly buffer led to an increase in absorbance at an initial rate of 60.6 ± 12.1 mOD/min, while in the presence of CAP-1 the initial assembly rate decreased to 31.4 ± 1.2 mOD/min. The assembly rates in the presence of our synthesized compounds decreased to the range of 4.5 ± 0.1 to 28.7 ± 1.2 mOD/min. Especially, we found that in the presence of **8c** and **8d**, which showed high activities with EC_{50} values of 0.47 and 1.1 μM, the assembly rates decreased to 4.5 ± 0.1 mOD/min and 6.6 ± 0.1 mOD/min, respectively, but in the presence of **3j** and **8e**, which showed low activities with EC_{50} values of 5.8 and 4.2 μM, the assembly rates slightly decreased to 28.0 ± 0.7 and 28.7 ± 1.2 mOD/min, respectively. These results, in which the abilities to inhibit capsid assembly were consistent with the antiviral activities of these compounds, showed the CA-binding compounds could inhibit capsid assembly in varying degrees and hence exhibit different levels of antiviral activities.

Table 2: Effects of title compounds on capsid assembly

Compound	Assembly rate[a] (mOD/min)
None	60.6 ± 12.1
3a	22.2 ± 0.5
3c	8.7 ± 0.2
3j	28.0 ± 0.7
3e	26.1 ± 0.5
CAP-1	31.4 ± 1.2
8a	8.1 ± 0.2
8c	4.5 ± 0.1
8d	6.6 ± 0.1
8e	28.7 ± 1.2

[a]Each value is reported as mean ± SD (standard deviation) from two experiments in duplicate.

Conclusion

To summarize, we have designed and prepared a novel series of NAH derivatives containing glycine residue, most of which demonstrated potent antiviral activities. Among them, derivative **8c** showed the highest inhibitory activity with an EC_{50} value of 0.47 μM and TI value of more than 212.8. Ultraviolet spectrophotometry analysis confirmed that the abilities of the title compounds to inhibit capsid assembly showed a strong correlation with their antiviral activities. Therefore, this series should be evaluated as a suitable hit for the design of new anti-HIV agents, and further research in this area is in progress in our laboratory.

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