[c008]

Novel 2-Chloro-8-arylthiomethyldipyridodiazepinone Derivatives with Activity against HIV-1 Reverse Transcriptase

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ABSTRACT—Based on the molecular modeling analysis against Y181C HIV-1 RT, dipyridodiazepinone derivatives containing unsubstituted lactam nitrogen and 2-chloro-8-arylthiomethyl were synthesized *via* efficient route. Some of them were evaluated for their antiviral activity against HIV-1 RT subtype E and were found to exhibit virustatic activity comparable to some clinically used therapeutic agents.

INTRODUCTION

The introduction of antiretroviral therapy results in delayed progression of HIV-1. The majority of the existing therapy has targeted on the viral replication at reversed transcriptase (RT) and protease enzyme[1,2]. However, the emergence of drug resistance has been observed[3,4]. Therefore, new therapeutic agents are still needed. Recently, a new class of therapeutic agents has focused on inhibiting HIV entry into cells, CD4 binding, coreceptor binding and membrane fusion such as T-20[5]. A number of bioactive nucleoside-based compounds against HIV virus have been clinically used[6].

The dipyridodiazepinone nevirapine (**1**)[7] is a potent non-nucleoside inhibitor of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase and is approved as a therapeutic agent for the treatment of AIDS. On the basis of molecular modeling analysis against Y181C HIV-1 RT to modify nevirapine structure for higher antiviral activity (Table 1), it was shown that the dipyridodiazepinone derivatives containing unsubstituted lactam nitrogen and 2-chloro-8-arylthiomethyl (**T1** and **T2**) moiety, when compared with **68nv**[2] as reference, are effective inhibitors for this mutant enzyme. The result prompted us to develop an efficient synthetic route to prepare 2-chloro-5,11-dihydro-11-ethyl-8-(phenylthio)methyl-6*H*-dipyrido[3,2-*b*:2', 3'-*e*][1,4]diazepin-6-one (**T1**) and 2-chloro-5,11-dihydro-11-ethyl-8-(3-methoxy phenylthio)methyl-6*H*-dipyrido[3,2-*b*:2',3'-*e*][1,4]diazepin-6-one (**T2**) and to evaluate for their anti-HIV-1 activity as well as testifying our hypothesis.
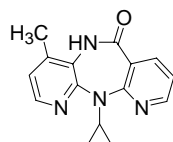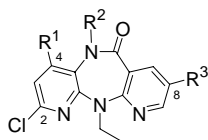

Nevirapine (**1**)

Table 1. Structure of the proposed compounds and calculated Y181C HIV-1-RT inhibitory affinities.

Cpds	R^1	R^2	R^3	Y181C log (1/C)			
				Expt.[a]	Calc.[b]	Calc.[c]	Calc.[d]
68nv	H	CH_3	CH_2SPh	8.0	7.83	7.77	7.79
T1	H	H	CH_2SPh		8.19	7.96	8.01
T2	H	H	$CH_2SPh(m\text{-}OCH_3)$		8.25	8.05	8.17
T3	H	CH_2OH	CH_2SPh		7.70	7.55	7.79
T4	CH_3CH_2	H	CH_2SPh		7.91	7.62	7.80
T5	OCH_3	H	$CH_2SPh(m\text{-}OCH_3)$		7.70	7.47	7.58

[a][ref. 8], [b]Calculated by CoMFA, [c]Calculated by CoMSIA, [d]Calculated by HQSAR [ref. 9].

RESULTS AND DISCUSSION

Chemistry

Synthesis of **T1** and **T2** was accomplished from commercially available 2-chloronicotinic acid (**2**) and 2,6-dichloro-3-nitropyridine (**3**). The intermediate aminopyridine **4** was prepared as shown in Scheme 1. Reaction of 2-chloronicotinyl chloride (**5**) and 3-amino-2,6-dichloropyridine (**6**), obtained from reduction of **3** provided pyridinecarboxamide **7**[8]. Treatment of **7** with ethylamine in xylene afforded not only the desired 2′- displacement but also competing substitutions of the 2- chloro and 6-chloro substituents as the significant side reactions. To improve the yield of aminopyridine **4**, it appears that ethylamine should be introduced before carboxamide formation. Thus, **2** was treated with ethylamine in sealed tube to give 2-(ethylamino)-3-pyridinecarboxylic acid (**8**) in quantitative yield. Then, acid chloride **9** from **8** was condensed with **6** to yield **4** in good yield. By this method, the increased and satisfied yield of **4** was obtained.
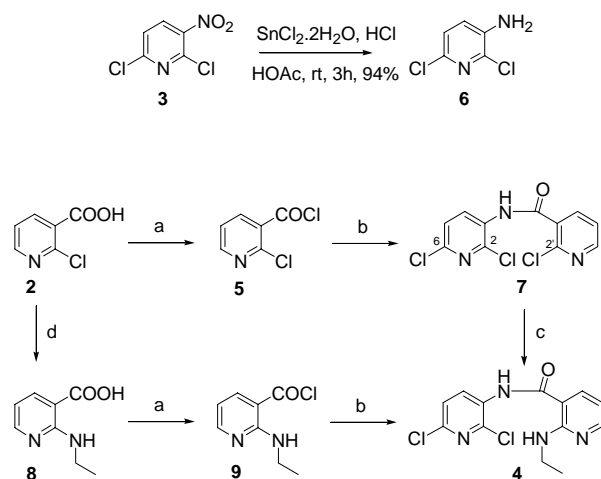
Scheme 1. Reagents and conditions: (a) (COCl)$_2$, benzene, DMF, rt, 1h; (b) **6**, dioxane, cyclohexane, pyridine, rt, 16h, 60%(from **5** to **7**) and 80%(from **9** to **4**); (c) EtNH$_2$, xylene, 120°C, 0.5h, 40%; (d) EtNH$_2$, 120°C, 4h, 99%
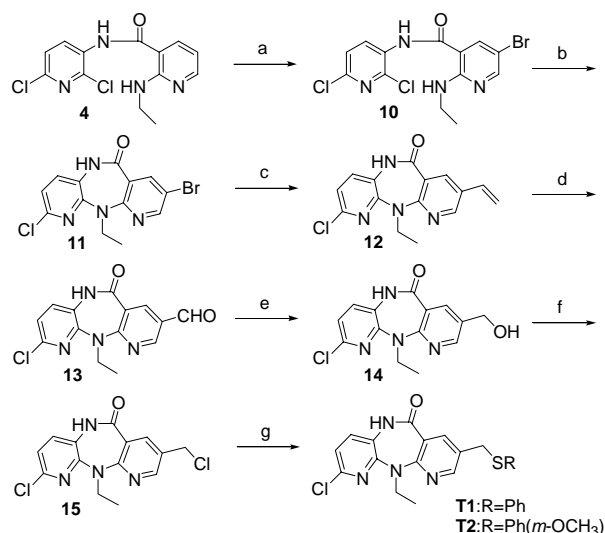
Scheme 2. Reagents and conditions: (a) Br_2, HOAc, KOAc, rt, 1h, 99%; (b) NaHMDS, py, 90 ^{o}C, 1h, 90%; (c) $CH_2CHSnBu_3$, $Pd(PPh_3)_4$, DMF, 90 ^{o}C, 1h, 75%; (d) O_3, CH_2Cl_2/MeOH, -78 ^{o}C then PPh_3, rt, 1h, 85%; (e) $NaBH_4$, THF, H_2O, rt, 0.5h, 93%; (f) $SOCl_2$, CH_2Cl_2, Et_3N, rt, 87%; (g) NaH, RSH, DMF, rt, 1h, 70% (**T1**) and 87% (**T2**)

The aminopyridine **4** was regioselectively brominated to afford *N*-(2,6-dichloro-3-pyridinyl)-5-bromo-2-ethylamino-3-pyridinecarboxamide (**10**) (Scheme 2). The azepinone ring was formed by treatment with sodium hexamethyldisilazane in pyridine to yield 8-bromo-2-chloro-5,11-dihydro-11-ethyl-6*H*-dipyrido[3,2-*b:*2',3'-*e*][1,4]diazepin-6-one (**11**). Such ring closure by using sodium hydride led to the undesired debrominated product. Coupling of **11** with vinyltributyltin in the presence of tetrakis(triphenylphosphine)palladium(0) provided 8-vinyl compound **12** which underwent ozonolysis to give aldehyde **13** in good yield. Reduction of **13** with $NaBH_4$ afforded alcohol **14** which was converted to the corresponding chloride **15** by treatment with thionyl chloride in dichloromethane. Reaction of **15** with thiophenolate and 3-methoxythiophenolate in DMF yielded **T1** and **T2**, respectively.

T1 and **T2** were synthesized from 2-chloronicotinic acid (**2**) and 2,6-dichloro-3-nitropyridine (**3**) in 10 steps with 26% and 32% overall yields, respectively.

Biological Testing

Compounds **T1** and **T2** together with some intermediates were evaluated for their virustatic and virucidal activities against HIV-1 subtype E (CRF01 AE). In addition, toxicity of the compounds, DMSO and cell controls were also included. Biological activity of **T1** and **T2** as well as of intermediates **14** and **15** was summarized in Table 2.

Table 2. Virustatic and virucidal activities at 50% effective concentration (EC_{50}) against HIV-1 subtype E.

Cpds	Virustatic EC_{50}, µg/ml		Virucidal EC_{50}, µg/ml	
	Day4	Day7	Day4	Day7
14	>10	>10	>10	>10
15	>10	>10	>10	>10
T1	< 1	1	>10	>10
T2	≤ 1	≤ 10	>10	>10

Compound **T1** exhibited virustatic activity at $EC_{50} \leq 1$ µg/ml for seven days. On the other hand, compound **T2** exhibited virustatic activity at $EC_{50} \leq 1$ μg/ml during the first four days but the activity decreased to $EC_{50} \leq 10$ μg/ml in the seventh day. Other intermediates did not show virustatic activity. This result suggested that thioaryl group could be involved in regulating virustatic activity. However, all compounds did not show virucidal activity.
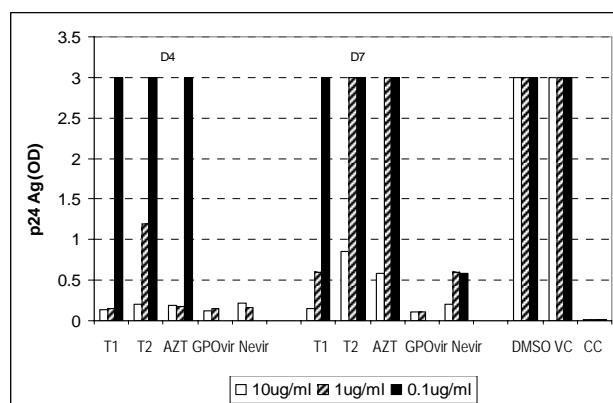

Figure 1. Virustatic activity of **T1** and **T2** observed in seven days. AZT = azidothymidine, GPOvir = analog of three RT inhibitors (nevirapine, lamivudine and stavudine), Nevir = nevirapine, DMSO = DMSO control, VC = virus control and CC = cell control.

Virustatic activity of **T1** and **T2** against HIV-1 subtype E observed in seven days was shown in Figure 1. **T1** exhibited comparable virustatic activity to that of AZT, GPOvir and nevirapine at concentration of 1 μg/ml in the fourth day. In the seventh day, **T1** exhibited comparable activity to that of nevirapine at concentration of 1 μg/ml and to that of GPOvir at 10 μg/ml but showed higher activity than AZT at 1 μg/ml. **T2** was less potent than **T1** (~6-fold) at concentration of 1 μg/ml.

Molecular Docking

In order to investigate the orientation and estimated binding energies of the inhibitors in the enzyme binding site, molecular docking by using Lamarckian genetic algorithm AutoDock 3.05 program[10] was used to dock **68nv**, **T1** and **T2** into the binding pocket of HIV-1 RT (pdb code 1KLM) and the results are compared with that of nevirapine as shown in Table 3. The grid size was set to 80x80x80. A grid spacing of 0.375 Å was used and the numbers of docking runs were set to 50. Superimposition of the binding pocket of HIV-1 RT with docked **68nv**, **T1** and **T2,** compared with nevirapine (green), is shown in Figure 2. The obtained results demonstrate clearly that **68nv** and both **T1** and **T2** oriented their structures in the HIV-1 RT binding site similar to nevirapine, however, the binding energies are much lower than that of nevirapine of about 4 kcal/mol. The explanation for this might be the fact that rotatable thio-phenyl side chain in the three compounds can interact with the residues in the binding site such as Lys103, Leu234, His235 and Tyr318. Furthermore, methoxy group attached in thio-phenyl side chain is also interact with Val106. This can significantly effect to the conformational change of the whole enzyme structure, and consequently reduce catalytic efficiency of the enzyme.

Table 3. The docked energy and free energy of binding (kcal/mol) of **T1** and **T2** as compared with nevirapine and **68nv**.

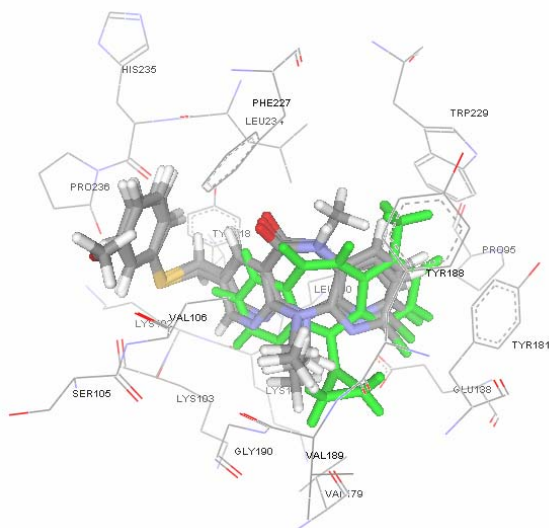| Cpds	Final Docked Energy (kcal/mol)	Free Energy of Binding (kcal/mol)
Nevirapine	-11.88	-11.24
68nv	-15.92	-15.03
T1	-15.58	-14.47
T2	-16.37	-14.82

Figure 2. The binding pocket of enzyme HIV-1 RT with **68nv**, **T1** and **T2** (atom type color) compared with nevirapine (green).

In summary, two dipyridodiazepinone derivatives, **T1** and **T2** were synthesized based on molecular modeling study and were found to exhibit virustatic activity against HIV-1 RT subtype E at 2.5 μM (1 μg/ml) and 23.5 μM (10 μg/ml), respectively.

EXPERIMENTAL PROCEDURES

Chemical methods

The ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini 2000 spectrophotometer referencing to solvent with chemical shift recorded as δ values in ppm. Coupling constants (J) are given in hertz. Infrared (IR) spectra were recorded in cm^{-1} on a Perkin Elmer 1760x FT-IR spectrometer. Mass spectra were obtained on a Finnigan Polaris GCQ mass spectrometer and accurate masses (HRMS) were obtained on a Bruker Micro TOF in ESI positive mode. Melting points (m.p.) were determined on a SMP3 melting point apparatus and are reported uncorrected in $^{\circ}$C. Column chromatography was performed on Scharlau silica gel 60 (70-230 mesh).

3-Amino-2,6-dichloropyridine (6). A solution of SnCl$_2$.2H$_2$O (970 mg, 4.3 mmol) in concentrated HCL (0.8 ml) was added dropwise to a stirred solution of 2,6-dichloro-3-nitropyridine (250 mg, 1.3 mmol) in acetic acid (2.6 ml) and the mixture was stirred for 3 hours at room temperature. Then the mixture was cooled in ice bath, and water was added. After stirring for an additional half an hour, the mixture was basified with aqueous 50% sodium hydroxide (pH 12). The reaction mixture was extracted with

CH_2Cl_2 and the combined organic layers were washed with water, dried (Na_2SO_4) and concentrated under reduced pressure to give **6** (200 mg, 94.8%) as white solid which could be used in the next step. Recrystallization from hexane/CH_2Cl_2 afforded needles; m.p. 121-122 ^{o}C. FTIR (KBr), ν_{max}: 3460, 3338, 1619, 1559, 1454, 1311, 1145, 720 cm^{-1}. ^{1}H NMR (CDCl$_3$, 200 MHz) δ: 7.06 (d, J=1.47 Hz, 2H). ^{13}C NMR (CDCl$_3$, 50 MHz) δ: 138.8, 137.3, 134.6, 125.1, 123.6 ppm. MS (EI), *m/z* (relative intensity): 162 (M$^+$, 100), 126 (20), 99 (10), 64 (30). HRMS (ESI-TOF) calcd. for $C_5H_5Cl_2N_2$ [M+H]$^+$ 162.9824; found: 162.9824.

2-(Ethylamino)-3-pyridine carboxylic acid (8). A stirred mixture of 2-chloronicotinic acid (**2**) (300 mg, 1.9 mmol), ethylamine (1.3 ml, 19.1 mmol) was heated at 120 ^{o}C in a sealed vessel for 4 hours. After cooling down and ethylamine was removed, the residue was purified by column chromatography (30% methanol/ethyl acetate) to give **8** (315 mg, 99.7%) as white solid; m.p. 178-180 ^{o}C. FTIR (KBr), ν_{max}: 3474, 3228, 1636, 1557, 1341, 1239 cm^{-1}. ^{1}H NMR (DMSO-d$_6$, 200 MHz) δ: 8.20 (dd, J=4.8, 1.8 Hz, 1H), 8.04 (dd, J=7.7, 1.8 Hz, 1H), 6.50 (dd, J=7.6, 5.1 Hz, 1H), 4.81 (br s, 1H), 3.43 (q, J=6.9 Hz, 2H), 1.15 (t, J=6.9 Hz, 3H). ^{13}C NMR (DMSO-d$_6$, 50 MHz) δ: 169.3, 158.4, 153.0, 140.1, 110.8, 106.9, 35.1, 15.0 ppm. MS (EI), *m/z* (relative intensity): 167 (M$^+$+1, 20), 151 (60), 133 (100), 122 (30), 93 (50), 78 (96). HRMS (ESI-TOF) calcd. for $C_8H_{11}N_2O_2$ [M+H]$^+$ 167.0815; found: 167.0812.

N-(2,6-Dichloro-3-pyridinyl)-2-ethylamino-3-pyridinecarboxamide (4). A stirred solution of **8** (315 mg, 1.9 mmol), in benzene (10 ml) was treated with oxalyl chloride (0.35 ml, 4.1 mmol) followed by a catalytic amount of *N,N*-dimethylformamide (2 drops) and the mixture was stirred at room temperature for 1 hour. Then the solvent was removed under reduced pressure to afford acid chloride 9 as yellow solid. This acid chloride was redissolved in 1,4-dioxane (10 ml) and added dropwise to a solution of 6 (200 mg, 1.2 mmol) in 1,4-dioxane (3 ml), cyclohexane (2 ml) and pyridine (0.2 ml, 2.4 mmol). After stirring at room temperature for 16 hours, the resultant precipitate was filtered. The solid was redissolved in CH_2Cl_2 and washed with saturated aqueous $NaHCO_3$. The organic layer was washed with water, dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by column chromatography (15% EtOAc/hexane) to yield **4** (308 mg, 80.7%) as pale yellow solid; m.p. 119-120 ^{o}C. FTIR (KBr), ν_{max}: 3431, 3351, 1660, 1572, 1504, 1301, 1235, 1122, 770 cm^{-1}. ^{1}H NMR (CDCl$_3$, 200 MHz) δ: 8.77 (d, J=7.3 Hz, 1H), 8.33 (d, J=4.4 Hz, 1H), 8.20 (s, 1H), 7.97 (br t, 1H), 7.74 (d, J=7.3 Hz, 1H), 7.34 (d, J=8.1 Hz, 1H), 6.60 (dd, J=7.3, 4.4 Hz, 1H), 3.61-3.48 (m, 2H), 1.29 (t, J=7.0 Hz, 3H). ^{13}C NMR (CDCl$_3$, 50 MHz) δ: 166.4, 158.1, 153.4, 143.5, 138.7, 135.3, 131.2, 130.9, 123.6, 110.5, 108.0, 35.8, 14.7 ppm. MS (EI), *m/z* (relative intensity): 310 (M$^+$, 20), 275 (25), 149 (35), 131 (100), 119 (20). HRMS (ESI-TOF) calcd. for $C_{13}H_{13}Cl_2N_4O$ [M+H]$^+$ 311.0461; found: 311.0464.

N-(2,6-Dichloro-3-pyridinyl)-5-bromo-2-ethylamino-3-pyridinecarboxamide (10). Br_2 (0.06 ml, 1.17 mmol) in acetic acid (1 ml) was added dropwise to a stirred solution of **4** (355 mg, 1.14 mmol), and potassium acetate (134 mg, 1.36 mmol) in acetic acid (15 ml). After 15 minutes, the reaction mixture was added water and the precipitate was collected by suction filtration and washed with water for several times to provide **10** (435 mg, 98.0%) as yellow solid; m.p. 193-194 ^{o}C. FTIR (KBr), ν_{max}:

3439, 3336, 1673, 1575, 1506, 1302, 1248, 787, 528 cm^{-1}. ^{1}H NMR (CDCl$_3$, 200 MHz) δ: 8.69 (d, J=8.8 Hz, 1H), 8.33 (d, J=2.2 Hz, 1H), 8.10 (br s, 1H), 7.89 (br t, 1H), 7.79 (d, J=2.2 Hz, 1H), 7.30 (d, J=8.1 Hz, 1H), 3.58-3.44 (m, 2H), 1.27 (t, J=7.3 Hz, 3H). ^{13}C NMR (CDCl$_3$, 50 MHz) δ: 165.4, 156.5, 154.2, 139.1, 137.4, 131.7, 130.7, 123.8, 109.6, 103.9, 36.1, 14.6 ppm. MS (EI), *m/z* (relative intensity): 388 (M$^+$, 60), 373 (15), 353 (20), 227 (70), 209 (60). HRMS (ESI-TOF) calcd. for C$_{13}$H$_{12}$BrCl$_2$N$_4$O [M+H]$^+$ 388.9566; found: 388.9579.

8-Bromo-2-chloro-5,11-dihydro-11-ethyl-6*H*-dipyrido[3,2-*b*:2',3'-*e*][1,4]diazepin-6-one (11). A solution of **10** (440 mg, 1.13 mmol) in pyridine (10 ml) was heated at 90°C under nitrogen atmosphere. Then the solution was added 0.6 M sodium bis(trimethylsilyl)amide in toluene (6.3 ml, 3.7 mmol) and the mixture was stirred at 90 ^{o}C for 15 minutes. After cooling down, the mixture was poured into ice water and stirred for additional 2 hours. The precipitate was filtered and washed with water to afford **11** (370 mg, 92.8%) as yellow solid; m.p. 263-264 ^{o}C. FTIR (KBr), ν$_{max}$: 3195, 3074, 2972, 1665, 1575, 1456, 1381, 1226, 687, 630 cm^{-1}. ^{1}H NMR (CDCl$_3$, 200 MHz) δ: 8.53 (br s, 1H), 8.49 (d, J=2.2 Hz, 1H), 8.24 (d, J=2.2 Hz, 1H), 7.27 (d, J=8.1 Hz, 1H), 7.06 (d, J=8.1 Hz, 1H), 4.81 (q, J=7.3 Hz, 2H), 1.25 (t, J=7.3 Hz, 3H). ^{13}C NMR (DMSO-d$_6$, 50 MHz) δ: 165.4, 156.8, 151.8, 150.5, 143.0, 142.6, 133.3, 126.3, 122.2, 120.4, 113.6, 41.5, 13.4 ppm. MS (EI), *m/z* (relative intensity): 352 (M$^+$, 80), 337 (42), 324 (75), 309 (20). HRMS (ESI-TOF) calcd. for C$_{13}$H$_{11}$BrClN$_4$O [M+H]$^+$ 352.9799; found: 352.9803.

2-Chloro-5,11-dihydro-11-ethyl-8-vinyl-6*H*-dipyrido[3,2-*b*:2',3'-*e*][1,4]diazepin-6-one (12). A solution of **11** (500 mg, 1.4 mmol) in *N,N*-dimethylformamide (7 ml) was treated with tetrakis(triphenylphosphine)palladium(0) (70 mg, 0.06 mmol) followed by vinyl tributyltin (1 ml, 3.42 mmol) and heated at 100 ^{o}C under nitrogen atmosphere for half an hour. After cooling down, the reaction mixture was poured into water and extracted with ethyl acetate. The combined organic layers were washed with 15% aqueous NH$_4$OH, brine and water, then dried (Na$_2$SO$_4$) and concentrated under reduced pressure. The residue was purified by column chromatography (30% EtOAc/hexane) to provide **12** (318 mg, 74.6%) as yellow solid; m.p. 201-202 ^{o}C. FTIR (KBr), ν$_{max}$: 3195, 2965, 1665, 1585, 1455, 1383, 1239, 688 cm^{-1}. ^{1}H NMR (CDCl$_3$, 200 MHz) δ: 9.37 (s, 1H), 8.46 (d, J=2.2 Hz, 1H), 8.20 (d, J=2.2 Hz, 1H), 7.35 (d, J=8.1 Hz, 1H), 7.04 (d, J=8.1 Hz, 1H), 6.66 (dd, J=17.6, 11.0 Hz, 1H), 5.78 (d, J=17.6 Hz, 1H), 5.35 (d, J=11.0 Hz, 1H), 4.22 (q, J=7.3 Hz, 2H), 1.27 (t, J=7.3 Hz, 3H). ^{13}C NMR (CDCl$_3$, 50 MHz) δ: 168.8, 157.9, 151.9, 150.1, 145.1, 137.7, 131.9, 131.8, 128.8, 125.0, 119.7, 119.6, 115.8, 42.1, 13.7 ppm. MS (EI), *m/z* (relative intensity): 300 (M$^+$, 100), 285 (35), 272 (92), 257 (35). HRMS (ESI-TOF) calcd. for C$_{15}$H$_{14}$ClN$_4$O [M+H]$^+$ 301.0851; found: 301.0855.

2-Chloro-5,11-dihydro-11-ethyl-8-formyl-6*H*-dipyrido[3,2-*b*:2',3'-*e*][1,4]diazepin -6-one (13). A −78°C cooled solution of 12 (343 mg, 1.14 mmol) in 1:1 dichloromethane-methanol (20 ml) was treated with O$_3$ for 30 minutes. After the completion of reaction, the solution was flew with O$_2$ for 5 minutes. Then the reaction mixture was added triphenylphosphine (598 mg, 2.28 mmol) and stirred for additional 1 hour at room temperature. The solvent was removed under reduced pressure and the residue was purified by column chromatography (30%EtOAc/hexane) to yield **13** (295 mg, 85.4%) as yellow solid; m.p. 216-217°C. FTIR (KBr), ν$_{max}$: 3203, 2939,

1702, 1674, 1596, 1456, 1354, 1231, 975, 692. ^{1}H NMR (CDCl$_3$, 200 MHz) δ: 10.02 (s, 1H), 9.53 (s, 1H), 8.60 (d, J=2.2 Hz, 1H), 8.64 (d, J=2.2 Hz, 1H), 7.41 (d, J=8.1 Hz, 1H), 7.13 (d, J=8.1 Hz, 1H), 4.34 (q, J=7.0 Hz, 2H), 1.32 (t, J=7.0 Hz, 3H). ^{13}C NMR (CDCl$_3$, 50 MHz) δ: 188.6, 168.1, 162.1, 153.8, 149.9, 145.3, 142.3, 132.3, 126.9, 125.0, 120.8, 118.8, 43.0, 13.7 ppm. MS (EI), *m/z* (relative intensity): 302 (M$^+$, 80), 287 (30), 274 (100), 260 (30), 245 (35). HRMS (ESI-TOF) calcd. for C$_{14}$H$_{12}$ClN$_4$O$_2$ [M+H]$^+$ 303.0643; found: 303.0645.

2-Chloro-5,11-dihydro-11-ethyl-8-hydroxymethyl-6*H*-dipyrido[3,2-*b*:2',3'-*e*][1,4] diazepin-6-one (14). A solution **13** (228 mg, 0.75 mmol) in tetrahydrofuran (12 ml) was added water (0.1 ml) followed by sodium borohydride (28.5 mg, 0.75 mmol). The mixture was stirred for half an hour, then diluted with water. Tetrahydrofuran was removed under reduced pressure and the precipitate was filtered and washed with water to yield **14** (214 mg, 93%) as white solid; m.p. 197-198°C. FTIR (KBr), ν_{max}: 3319, 3191, 2959, 1666, 1590, 1456, 1390, 1232, 1041 cm^{-1}. ^{1}H NMR (acetone-d$_6$, 200 MHz) δ: 9.50 (br s, 1H), 8.46 (d, J=2.2 Hz, 1H), 8.11 (d, J=2.2 Hz, 1H), 7.60 (d, J=8.1 Hz, 1H), 7.18 (d, J=8.1 Hz, 1H), 4.65 (d, J=5.13 Hz, 2H), 4.56 (t, J=5.86 Hz, 1H), 4.15 (q, J=7.3 Hz, 2H), 1.21 (t, J=7.3 Hz, 3H). ^{13}C NMR (acetone-d$_6$, 50 MHz) δ: 167.8, 158.7, 152.7, 151.0, 144.5, 140.3, 134.1, 133.4, 127.3, 121.2, 120.5, 61.5, 42.4, 14.0 ppm. MS (EI), *m/z* (relative intensity): 304 (M$^+$, 69), 289 (39), 276 (100), 261 (29), 247 (20), 164 (31). HRMS (ESI-TOF) calcd. for C$_{14}$H$_{14}$ClN$_4$O$_2$ [M+H]$^+$ 305.0800; found: 305.0805.

2-Chloro-5,11-dihydro-11-ethyl-8-chloromethyl-6*H*-dipyrido[3,2-*b*:2',3'-*e*][1,4] diazepin-6-one (15). A suspension of **14** (177 mg, 0.58 mmol) in dichloromethane (100 ml) was treated with thionyl chloride (0.3 ml) followed by triethylamine (1 ml). The reaction mixture was stirred at room temperature for 1 hour and the clear solution was obtained. Then the saturated aqueous NaHCO$_3$ was added and the mixture was extracted with CH$_2$Cl$_2$. The organic layer was washed with water, then dried (Na$_2$SO$_4$) and concentrated under reduced pressure. The residue was purified by column chromatography (20%EtOAc/hexane) to give **15** (163 mg, 87%) as pale yellow solid; m.p. 226-227°C. FTIR (KBr), ν_{max}: 3195, 2969, 1671, 1588, 1455, 1382, 1247, 699 cm^{-1}. ^{1}H NMR (CDCl$_3$, 200 MHz) δ: 9.15 (br s, 1H), 8.47 (d, J=2.9 Hz, 1H), 8.19 (d, J=2.9 Hz, 1H), 7.34 (d, J=8.1 Hz, 1H), 7.06 (d, J=8.1 Hz, 1H), 4.58 (s, 2H), 4.23 (q, J=7.3 Hz, 2H), 1.27 (t, J=7.3 Hz, 3H). ^{13}C NMR (CDCl$_3$, 50 MHz) δ: 168.3, 158.7, 151.7, 151.5, 145.2, 141.4, 131.9, 128.3, 125.0, 119.9, 119.6, 42.3, 42.2, 13.7 ppm. MS (EI), *m/z* (relative intensity): 322 (M$^+$, 54), 307 (31), 294 (62), 287 (37), 259 (100), 244 (28), 231 (21). HRMS (ESI-TOF) calcd. for C$_{14}$H$_{13}$Cl$_2$N$_4$O [M+H]$^+$ 323.0461; found: 323.0470.

2-Chloro-5,11-dihydro-11-ethyl-8-(phenylthio)-methyl-6*H*-dipyrido[3,2-*b*:2',3'-*e*] [1,4]diazepin-6-one (T1). A solution of thiophenol (0.1 ml, 0.97 mmol) in *N,N*-dimethylformamide (2 ml) was treated with 60%sodium hydride (65 mg, 1.6 mmol) under N$_2$ atmosphere. After 10 minutes, a solution of **15** (100 mg, 0.31 mmol) in *N,N*-dimethylformamide (3 ml) was added and the mixture was stirred at room temperature for 1 hour. The reaction was quenched by addition of water and the mixture was extracted with ethyl acetate. The organic layer was washed with water, then dried (Na$_2$SO$_4$) and concentrated under reduced pressure. The residue was purified by column chromatography (20%EtOAc/hexane) to give **T1** (85.5 mg, 70%) as yellow

solid; m.p. 188.5-189.5°C. FTIR (KBr), ν_{max}: 3182, 2965, 1665, 1585, 1455, 1383, 1239, 739, 688 cm^{-1}. ^{1}H NMR (CDCl$_3$, 200 MHz) δ: 9.33 (s, 1H), 8.29 (d, J=2.2 Hz, 1H), 8.09 (d, J=2.2 Hz, 1H), 7.34-7.20 (m, 6H), 7.04 (d, J=8.1 Hz, 1H), 4.18 (q, J=7.3 Hz, 2H), 4.05 (s, 2H), 1.23 (t, J=7.3 Hz, 3H). ^{13}C NMR (CDCl$_3$, 50 MHz) δ: 168.6, 157.8, 151.8, 145.1, 141.2, 134.9, 131.8, 130.6(2C), 129.1(2C), 128.6, 127.0(2C), 125.1, 119.7, 119.6, 42.0, 35.6, 13.6 ppm. MS (EI), *m/z* (relative intensity): 396 (M$^+$, 7), 287 (100), 259 (52), 231 (14). HRMS (ESI-TOF) calcd. for C$_{20}$H$_{18}$ClN$_4$OS [M+H]$^+$ 397.0884; found: 397..0894

2-Chloro-5,11-dihydro-11-ethyl-8-(3-methoxyphenylthio)-methyl-6*H*-dipyrido[3, 2-*b*:2',3'-*e*][1,4]diazepin-6-one (T2). A solution of 3-methoxythiophenol (0.14 ml, 1.14 mmol) in *N,N*-dimethylformamide (2 ml) was treated with 60%sodium hydride (76 mg, 1.88 mmol) under N$_2$ atmosphere. After 10 minutes, a solution of **15** (122 mg, 0.38 mmol) in *N,N*-dimethylformamide (3 ml) was added and the mixture was stirred at room temperature for 1 hour. The reaction was quenched by addition of water and the mixture was extracted with ethyl acetate. The organic layer was washed with water, then dried (Na$_2$SO$_4$) and concentrated under reduced pressure. The residue was recrystallized from hexane/EtOAc to afford **T2** (140 mg, 87%) as yellow crystals; m.p. 188-189°C. FTIR (KBr), ν_{max}: 3181, 2965, 1662, 1589, 1458, 1385, 1229, 1044, 767, 693 cm^{-1}. ^{1}H NMR (CDCl$_3$, 200 MHz) δ: 9.56 (br s, 1H), 8.30 (d, J=2.2 Hz, 1H), 8.08 (d, J=2.2 Hz, 1H), 7.33 (d, J=8.1 Hz, 1H), 7.18 (t, J=8.1 Hz, 1H), 7.04 (d, J=8.1 Hz, 1H), 6.90-6.72 (m, 3H), 4.06 (s, 2H), 3.73 (s, 3H), 1.23 (t, J=7.3 Hz, 3H). ^{13}C NMR (CDCl$_3$, 50 MHz) δ: 168.8, 159.9, 157.9, 151.8(2C), 145.1, 141.2, 136.2, 131.8, 129.9, 128.6, 125.2, 122.6, 119.7, 119.6, 115.8, 112.9, 55.3, 42.1, 35.4, 13.6 ppm. MS (EI), *m/z* (relative intensity): 426 (M$^+$, 6), 287 (100), 259 (57), 231 (15). HRMS (ESI-TOF) calcd. for C$_{21}$H$_{20}$ClN$_4$O$_2$S [M+H]$^+$ 427.0990; found: 427.0988.

Biological methods

Materials

1. DA5 (HIV-1 subtype E) cells were obtained from HIV-1 infected pregnant women. These viruses were X4 strain with the syncytium-inducing (SI) formation property, causing morphological changes (cytopathic effect) in infected cells.

2. White blood cells

- Peripheral blood mononuclear cells were obtained from blood donors and used for HIV virus isolation.

- H9 T-lymphoblastoid cell line obtained from Medical Research Centre, UK, was used to prepare the viral stock.

- C8166 T-lymphoblastoid cell line was used to test the activities of bioactive compounds in all experiments.

3. Bioactive compounds dissolved in 70-95% DMSO

4. Three anti-retroviral drugs (reverse transcriptase inhibitors) manufactured by the Government Pharmaceutical Organization (GPO), including ANTIVIR (100 mg AZT), GPO-vir (containing 200 mg Nevirapine, 150 mg Lamivudine, and 30 mg Stavudine), and NERAVIR (200 mg Nevirapine), were used as controls in all experiments.

Methods

All experiments were performed in duplicates.

Toxicity of bioactive compounds, anti-retroviral drugs, and DMSO against white blood cell culture

The solutions of the bioactive compounds and anti-retroviral drugs (diluted to 10, 1, and 0.1 µg/ml in the culture medium), as well as DMSO (diluted to 1%, 0.1%, and 0.01%), were dispensed into tissue culture plates in duplicates. C8166 cells were then added into each well and examined everyday for 7 days. Moreover, the cells were counted, stained with 1% tryphan blue in order to examine for cell viability under the microscope on day 0, day 4, and day 7, and compared with drug-free control cells.

Virustatic and virucidal tests

The viruses were used at 100 $TCID_{50}$ (50% tissue cell infectivity dose) level, which was determined by diluting the viruses in quadruplicates, and the potency of the viruses was calculated using Karber equation. Additionally, the following controls were used in all experiments.

1. Control viruses diluted to 100, 10, and 1 $TCID_{50}$ in order to verify that the viruses used in all experiments were at 100 $TCID_{50}$ level.

2. C8166 control cells were used for cell examination throughout the experiments

3. Bioactive control was used to investigate the effect of the compounds on the cells.

4. DMSO control was used to evaluate the effect of DMSO on virus proliferation in the cells.

5. Anti-retroviral drug controls (AZT, GPO-vir, and Nevirapine) were used to investigate the effect of the drugs on the cells.

Virustatic test

C8166 cells were incubated with the solutions of bioactive compounds for 30 minutes. The viruses were then added into each well at 100 $TCID_{50}$ level, and the samples were further incubated for 3 hours. After that, the cells were washed 4 times, and maintained for 7 days. The culture medium was changed on day 3, and the cells were examined for cytopathic effect on days 4 and 7. Additionally, the level of the p24 antigen in the culture medium was determined on day 7 using commercially-available ELISA kits in order to determine the EC_{50}, which is the concentration of each bioactive compound that inhibits the viruses by 50%.

Virucidal test

The viruses at 100 $TCID_{50}$ level were incubated with the solutions of bioactive compounds for 1 hour. C8166 cells were then added to each well, and the samples were further incubated for 1 hour. After that, the cells were washed 4 times, and maintained for 7 days. The culture medium was changed on day 3, and the cells were examined for cytopathic effect on days 4 and 7. Additionally, the level of the p24 antigen in the culture medium was also determined on day 7 using commercially-available ELISA kits.

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