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Synthesis and biological evaluation of deoxy-hematoxylin derivatives as a novel class of anti-HIV-1 agents

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ABSTRACT

SAR studies for the exploration a novel class of anti-human immunodeficiency virus type 1 (HIV-1) agents based on the hematoxylin structure (**1**) are described. The systematic deoxygenations of **1** including asymmetric synthesis were conducted to obtain a compound showing high potencies for inhibiting the nuclear import and viral replication as anti-HIV-1 agent. Among all, C-3-deoxygenated analog **16** exhibited most promising biological activities as anti-HIV-1 agent such as lower cytotoxicity (**16**:**1**; >80:40 μM), stronger inhibition of nuclear import (0.5:1.3 μM), and viral replication in HIV-1-infected TZM-bl cells (24.6:100 μM), human peripheral blood mononuclear cells (PMBCs) (30.1 μM: toxic). Different spectra of inhibitory activities against infected three healthy humans macrophages with high (donor A) and low (donor B and C) amounts of virus were also observed. Thus **16** showed 10-times stronger activity than **1** (**16**:**1**; 0.1:<1.0 μM) in the case of A, while **16** and **1** showed comparable activities in the cases of B and C (>0.01 and >0.001 μM). The comparison of the inhibition of viral p24 antigen production was clearly indicated that compound **16** is at least twofold more potent anti-viral activity than **1**. Thus, structures and actions of deoxy analogs particularly **16** could provide valuable information for the development of a novel class of anti-HIV-1 agents.

The human immunodeficiency virus type 1 (HIV-1) is the cause of acquired immunodeficiency syndrome (AIDS). The chemotherapy of AIDS is the most effective treatment, and there are currently four classes of HIV-1 inhibitors including integrase inhibitors, protease inhibitors (PI), nucleoside or non-nucleosides reverse transcriptase inhibitors (NRTI/NNRTI), entry or fusion inhibitors. Highly active antiretroviral therapy (HAART) using the combination of PI and NRTI/NNRTI in general has significantly changed the progression and outcome of the infection with HIV-1.[1] Although a large number of approved drugs are used in HAART, the emergence of the drug-resistance severely limits their clinical effectiveness.[2] Therefore, the development of new classes of drugs to combat HIV-1 infection is urgently needed.

The heartwood of *Hematoxylum campechianum* L. and *Caesalpinia sappan* L. has been used not only as mordant dye,[3] but also herbal medicine for treatment of diarrhea, dysentery, dyspepsia, leucorrhea, and diabetic complications.[4] The main component of *H. campechianum* and *C. sappan* are hematoxylin (**1**) and brazilin (**2**), respectively (Fig. 1), and the structures of these two compounds are almost identical, except the hematoxylin has an extra hydroxyl group at the C-4 position. The several biological activities of these compounds are reported, such as anti-inflammatory, inhibition of lens-aldose reductase, decrease of blood glucose level.[5] In addition, the inhibitory effect of **1** for HIV-1 integrase was reported by Merck & Co.,[6] and in the study, authors mentioned that the bis-catechol structure is essential for the inhibition of HIV-1 integrase. Furthermore, our co-author has recently found the novel anti-HIV-1 activity of **1** via the inhibition of the nuclear-import of HIV-1 preintegration complexes (PICs) by prohibiting the viral protein R (Vpr) and importin-α interaction.[7] These coincidental stimulative observations urged us to deepen the structure–activity relationships (SAR) of hematoxylin particularly focused on the catecholic hydroxyl groups. Here, we report the synthesis of deoxygenated hematoxylin analogs including asymmetric synthesis and the evaluation of their biological activities.

As shown in Figure 1, **1** has the four aromatic hydroxyl groups and one aliphatic hydroxyl group. In order to elucidate which hydroxyl group is essential for the activity of the inhibition of the nuclear-import, we planned the synthesis of a series of deoxygenated hematoxylin analogs. Although the deoxygenation of **2** at C-6a position was achieved by reduction of corresponding methyl

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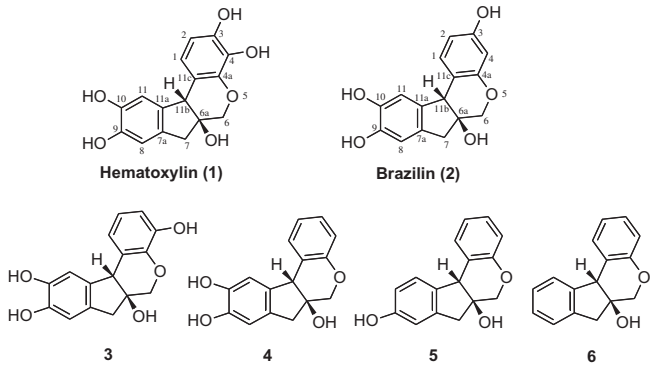
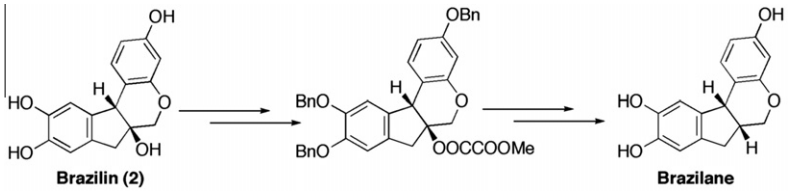


Figure 1. Structures of hematoxylin (**1**), brazilin (**2**), and their analogs (**3**–**6**).

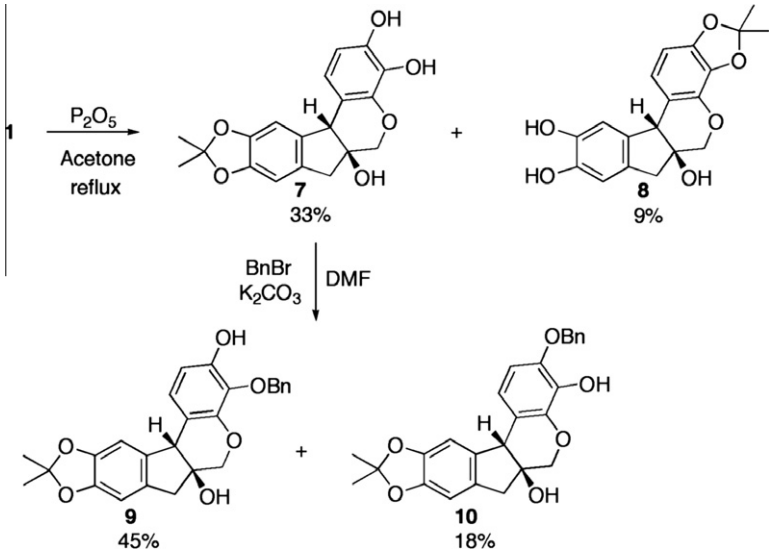
oxalate (Scheme 1),[8] there have been no reports of deoxygenation of the aromatic hydroxyl groups of **1** or **2** so far. Therefore, we planned systematic deoxygenation of aromatic hydroxyl groups on **1** as follows; here we used the nonselective reaction willing to give a mixture of the products in order to obtain possible various derivatives for screening of their bioactivities. First, the protection of the catechol moiety of **1** was conducted using acetone and phosphorus pentoxide under reflux for 1 h, giving acetonide **7** as a major product (Scheme 2). The acetonides **7** and **8** were separated with flash chromatogram, then mono-benzylation of the left catechol moiety of **7** with benzylbromide provided C-3- or C-4 *O*-benzyl products **9** and **10**. The determination of the benzylated position was confirmed with conversion from the **10** to the brazilin (**2**) as shown in Scheme 3. Treatment of **10** with trifluoromethanesulfonic anhydride (Tf_2O) in the presence of triethylamine afforded **11**. Deoxygenation of **11** with homogeneous Pd(0)-catalyzed reduction gave the C-4 deoxygenated product **12** in good yield.[9]

After deprotection of benzyl group of **12** with hydrogenation, hydrolysis of acetonide **13** gave brazilin (**2**), which was found to be identical with the naturally occurring brazilin (**2**). To the best of our knowledge, this is the first report of the conversion from hematoxylin (**1**) to brazilin (**2**). Likely, major benzylated product **9** was also converted to the corresponding tetraol analog **3** and triol analog **4** in a similar fashion (Scheme 4). Finally, diol analog **5** and monool analog **6** were synthesized asymmetrically as shown in Scheme 5.[10] Thus aldol condensation between 4-chromanone (**19**) and *m*-benzyloxybenzaldehyde in the presence of hydrochloric acid gave homoisoflavone **20**. After hydrogenation of **20** and reprotection of phenol, α-position of **22** was asymmetrically oxidized with (+)-[(8,8-dichlorocamphoryl)sulfonyl]oxaziridine (DCSO) to provide a-hydroxyl chromanone **23**. Then reduction of the a-hydroxy ketone and acid-catalyzed cyclization of **23** gave diol analog **5**, by which deoxygenation provided monool analog **6**.

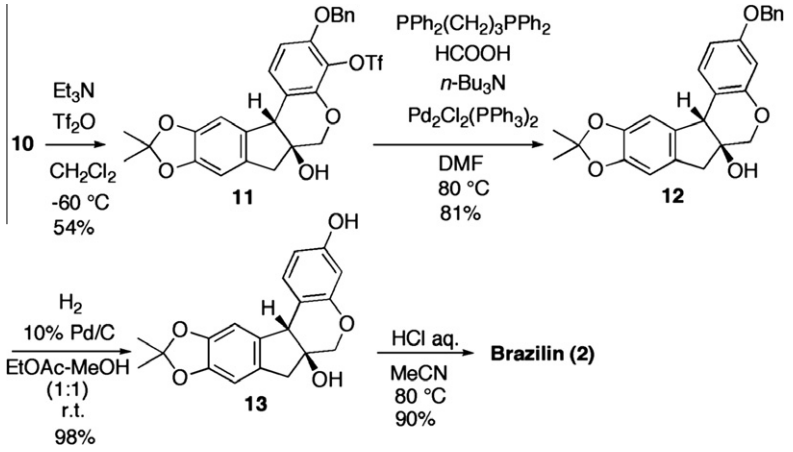
Cytotoxicity of analog **3**–**6** was measured by an in vitro tetrazolium-based colorimetric assay (MTT)[12] using the $CD4^+$ cell lines Molt4 as shown in Table 1. The cytotoxicity was increased successively by removal of hydroxyl groups at C-3- and C-4-positions (CC_{50} = 32 μM (**3**)–1.9 μM (**4**)). However, further removal of hydroxyl groups decreased suddenly the cytotoxicity (CC_{50} = 82 μM (**5**) to >100 μM (**6**)). Nextly, the nuclear import assays[13] with digitonin-permeabilized HeLa cells were performed. In all cases, effective inhibition of the nuclear import was observed. Interestingly, even monool analog **6** did not show drastic decrease of the inhibitory activity. Therefore, the existence of the phenolic hydroxyl groups, especially catechol group, does not seem to be essential for the inhibition of the nuclear import of HIV-1 PICs. Furthermore, inhibitory effect for the HIV-1 replication was evaluated using $CD4^+$–T cell-tropic HIV-1NL432-infected TZM-bl cells.[14] Moderate cytotoxic analog **5** had a higher inhibitory effect than **1** (IC_{50} = 67.6 μM (**5**) to >100 μM (**1**)), while tetraol **3** indicated the cytotoxicity for



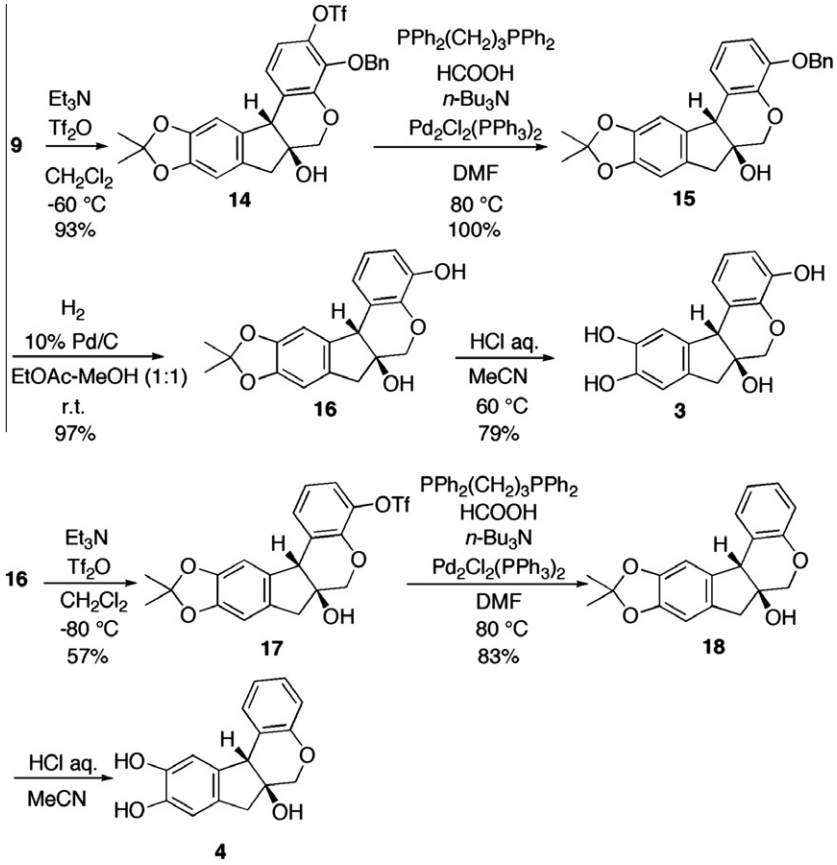
Scheme 1. Synthesis of brazilane.



Scheme 2. Selective protection of hematoxylin (**1**).

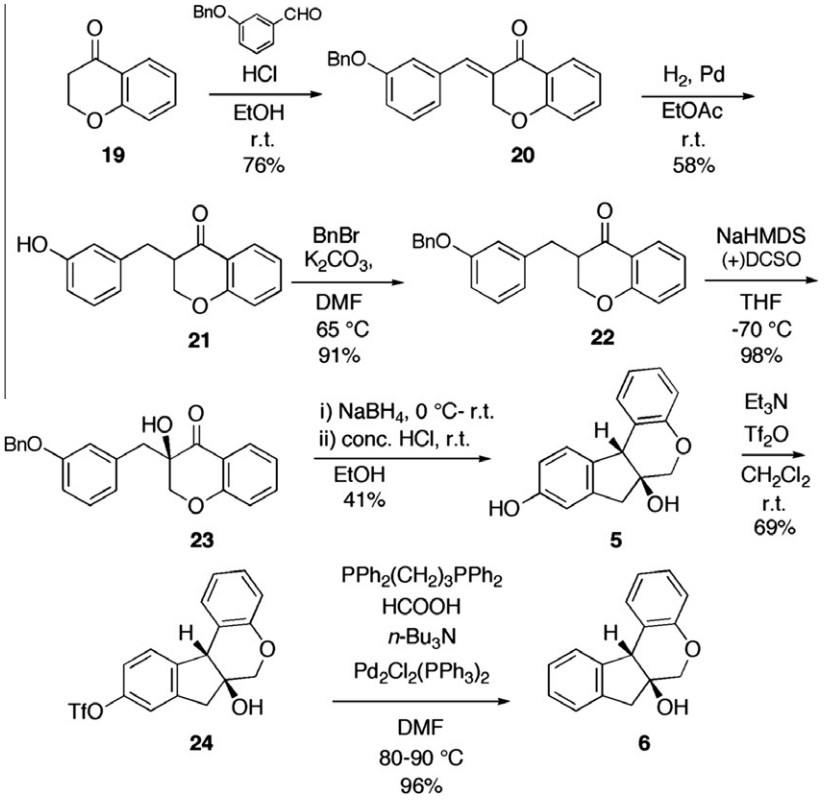


Scheme 3. Synthesis of brazilin (**2**).



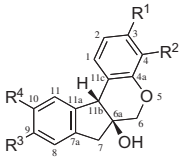
Scheme 4. Synthesis of deoxygenated hematoxylin analog **3** and **4**.

the TZM-bl cells. The most cytotoxic analog **4** also showed the cytotoxicity for the TZM-bl cells. On the other hand, the lowest cytotoxic analog **6** did not inhibit the infection. Therefore, further investigation with these analogs **4** and **6** was not performed. The replication inhibition of HIV-1NL432 in concanavalin A (Con A) and interleukin-2 (IL2) activated human peripheral blood mononuclear cells (PBMCs)[15] with diol **5** was studied to show a moderate inhibitory effect (IC_{50} = 25.1 μM). However, both compounds, **1** and **3** indicated the cytotoxicites for PBMCs. We also investigated the replication inhibition of these analogs in macrophage-tropic HIV-1NF-462-infected macrophages derived from $CD14^+$ cells,[16] where the diol **5** showed comparable inhibition activity to **1** (IC_{50} = 0.9 μM (**5**) to <1.0 μM (**1**)), while analog **3** exhibited better activity of 0.4 μM, which is about twice as active as analog **5**, suggesting that analog **3** has a potential for a new lead compound. From such structural information, we further examined the biological activities of C-9, C-10 hydroxy groups masked **15** and **16**, as analogs of **3**. As shown in Table 1, both compounds indicated lower cytotoxicities (>80 μM) and higher nuclear import inhibition activities (0.5 μM (**15**) and 0.8 μM (**16**)) than **1**. Furthermore, the compound **16** exhibited excellent viral replication inhibitory effects for TZM-bl, PBMC, and macrophage cells (24.6, 30.1, and 0.1 μM, respectively). On the other hand, compound **15** did not show any replication inhibition activity to those cells. The activity difference



Scheme 5. Asymmetric synthesis of deoxygenated hematoxylin analog **5** and **6**.

Table 1
Biological activity of deoxygenated analogs (**3–6**, **15** and **16**)[11]



Compound	R^1	R^2	R^3	R^4	CC_{50}[a] (μM)	Nucleor import IC_{50}[b] (μM)	Viral replication IC_{50}[b] (μM)				
							TZM-bl[c]	PBMCs[d]	Macrophages[e]		
									High	Low	
									A	B	C
Hematoxylin (**1**)	–OH	–OH	–OH	–OH	40	1.3	100.0	Toxic	<1.0	>0.01	>0.001
3	–H	–OH	–OH	–OH	32	0.9	Toxic	Toxic	0.4	NT	NT
4	–H	–H	–OH	–OH	1.9	1.9	Toxic	NT[f]	NT	NT	NT
5	–H	–H	–OH	–H	82	0.6	67.6	25.1	0.9	NT	NT
6	–H	–H	–H	–H	>100	2.7	—[g]	NT	NT	NT	NT
15	–H	–OBn	$–OC(CH_3)_2O–$		>80	0.8	—	NT	—	NT	NT
16	–H	–OH	$–OC(CH_3)_2O–$		>80	0.5	24.6	30.1	0.1	>0.01	>0.001

[a] CC_{50}: 50% cytotoxic concentration.
[b] IC_{50}: 50% inhibitory concentration.
[c] TZM-bl: a luciferase expressing reporter cell that possesses CD4 and both CXCR4 and CCR5 coreceptors.
[d] PBMCs: human peripheral blood mononuclear cells.
[e] Macrophages: terminal differentiated primary macrophages derived from three healthy donors, A, B and C were infected with HIV-1NF-462 viruses containing an amount of 4 ng (high) and 1 ng (low) of p24 antigen in the presence of compound for 8 days.
[f] NT: not test.
[g] —: no activity.

between **15** and **16** could be due to the alkylated and free hydroxyl groups at C-4-position, respectively. Thus the free hydroxyl group at C-4-position does seem to be essential for the viral replication inhibition activities. Finally, the inhibitory effect for HIV-1 infection of **1** and **16** were also observed with human macrophages derived from healthy donors A with a high amount of virus and B, and C with low amounts of virus (Table 1). According to high donor dependencies for the infection of macrophages, the compound **16** exhibited the donor dependent activities with a 50% inhibition concentration (IC_{50}) of 100 nM (donor A), <10 nM (donor B) and <1 nM (donor C), respectively. In the case of donor A, **16** showed apparently 10-times higher activity than **1**. Although **16** also

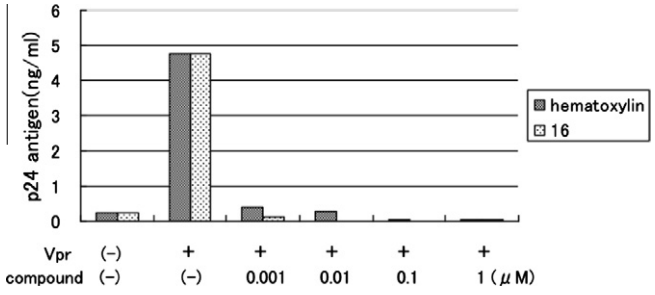


Figure 2. The effect of hematoxylin (**1**) and compound **16** on viral replication. Terminally differentiated primary macrophages derived from healthy donor C was infected with HIV-1 and then incubated in serial 10-fold dilutions of compounds ranging in concentration from 0 to 1 μM. The levels of virus in the culture supernatants were measured at 8 days after inoculation by p24 antigen ELISA. The data represent the average p24 value from two wells.

showed the comparable high activities in low amounts of virus infection conditions (donor B and C) to **1**, the comparison of the inhibition of viral p24 antigen production was clearly indicated that compound **16** is at least twofold more potent anti-viral activity than **1** (Fig. 2).[17]

In summary, a novel class of anti-HIV-1 agent based on deoxyhematoxylin derivatives was extensively developed. The present SAR studies suggested that hydroxyl group at C-4-position would be essential for the high viral replication inhibition activity and that free hydroxyl groups at C-9- and C-10-positions would increase the cytotoxicity. In addition, catecholic group is not necessary for inhibition of the nuclear import of HIV-1 PICs. The most potent compound was the C-3-deoxygenated analog **16**, which exhibited most promising biological activities as anti-HIV-1 agent such as lower cytotoxicity (**16**:**1**; >80:40 μM), stronger inhibition of nuclear import (0.5:1.3 μM), and viral replication in HIV-1-infected TZM-bl cells (24.6:100 μM), human peripheral blood mononuclear cells (PMBCs) (30.1 μM:toxic). In addition, different spectra of inhibitory activities against infected three healthy humans macrophages with high (donor A) and low (donor B and C) amounts of virus were also observed. In the case of A, **16** showed 10-times stronger activity than **1** (**16**:**1**; 0.1:<1.0 μM). On the other hand, **16** and **1** showed comparable activities in the cases of B and C (>0.01 and >0.001 μM). However, the comparison of the inhibition of viral p24 antigen production was clearly indicated that compound **16** is at least twofold more potent anti-viral activity than **1**. Thus, the present SAR studies could provide valuable information for the elaboration of novel class of anti-HIV-1 agents.

Acknowledgments

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11. Spectral data for compounds **2**–**6**, **15**, and **16**. Compound **2**: ^{1}H NMR (CD_3OD, 400 MHz) δ 7.18 (d, J = 8.3 Hz, 1H), 6.70 (s, 1H), 6.60 (s, 1H), 6.46 (dd, J = 8.3, 2.4 Hz, 1H), 6.30 (d, J = 2.4 Hz, 1H), 3.96 (s, 1H), 3.92 (d, J = 11.3 Hz, 1H), 3.68 (d, J = 11.3 Hz, 1H), 3.01 (d, J = 15.6 Hz, 1H), 2.76 (d, J = 15.6 Hz, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 157.71, 155.62, 145.55, 145.24, 137.40, 132.22, 131.33, 115.53, 112.89, 112.43, 109.96, 104.24, 78.05, 70.78, 50.95, 42.81. ESI-MS(−): 285 [M−H]; HRMS (ESI−): calcd for $C_{16}H_{13}O_5$ ($M-H^+$): 285.0763; found: 285.0755; $[\alpha]_{25}^{D}$: +84.8 (c = 0.41, CH_3OH); (+)−Brazilin (purchased from MP Biomedicals) $[\alpha]_{25}^{D}$: +69.5 (c = 0.41, CH_3OH). Compound **3**: ^{1}H NMR (CD_3OD, 400 MHz) δ 6.87 (br d, J = 7.6 Hz, 1H), 6.80 (dd, J = 7.6, 7.6 Hz, 1H), 6.74 (s, 1H), 6.67 (d, J = 7.6 Hz, 1H), 6.61 (s, 1H), 4.04 (s, 1H), 4.03 (d, J = 11.2 Hz, 1H), 3.74 (d, J = 11.2 Hz, 1H), 3.03 (d, J = 15.6 Hz, 1H), 2.80 (d, J = 15.6 Hz, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 146.72, 145.68, 145.25, 142.85, 136.83, 131.33, 125.00, 122.31, 121.87, 114.39, 112.86, 112.51, 78.09, 70.99, 51.51, 42.82; ESI-MS(−): 285 [M−H]$^-$; HRMS (ESI−): calcd for $C_{16}H_{13}O_5$ ($M-H^+$): 285.0763; found: 285.0758. Compound **4**: ^{1}H NMR (CD_3OD, 400 MHz) δ 7.36 (br d, J = 7.8 Hz, 1H), 7.09 (ddd, J = 7.8, 7.5, 1.5 Hz, 1H), 6.95 (ddd, J = 7.5, 7.3, 1.2 Hz, 1H), 6.82 (dd, J = 8.0, 1.2 Hz, 1H), 6.74 (s, 1H), 6.62 (s, 1H), 4.05 (br s, 1H), 3.96 (dd, J = 11.2, 1.5 Hz, 1H), 3.72 (d, J = 11.2 Hz, 1H), 3.04 (d, J = 15.6 Hz, 1H), 2.79 (d, J = 15.6 Hz, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 154.93, 145.76, 145.35, 136.87, 131.77, 131.45, 128.49, 124.38, 122.00, 117.92, 112.96, 112.44, 78.00, 70.84, 51.59, 42.90; ESI-MS(−): 269 [M−H]$^-$; HRMS (ESI−): calcd for $C_{16}H_{13}O_4$ ($M-H^+$): 269.0814; found: 269.0809. Compound **5**: ^{1}H NMR (CD_3CN, 400 MHz) δ 7.43 (dd, J = 8.0, 1.2 Hz, 1H), 7.16–7.12 (m, 2 H), 6.98 (ddd, J = 7.3, 7.3, 1.2 Hz, 1H), 6.93 (br s, 1H), 6.84 (dd, J = 8.8, 1.2 Hz, 1H), 6.65 (br s, 1H), 6.60 (dd, J = 8.3, 2.4 Hz, 1H), 4.08 (s, 1H), 3.95 (dd, J = 11.2, 1.2 Hz, 1H), 3.75 (d, J = 11.2 Hz, 1H), 3.62 (br s, 1H), 3.06 (d, J = 16.3 Hz, 1H), 2.92 (d, J = 16.3 Hz, 1H); ^{13}C NMR (CD_3CN, 100 MHz) δ 157.17, 154.37, 142.39, 136.58, 131.64, 128.38, 125.93, 124.31, 121.91, 117.54, 114.43, 112.83, 77.77, 70.45, 50.98, 42.78; ESI-MS(−): 253 [M−H]$^-$; HRMS (ESI−): calcd for $C_{16}H_{13}O_3$ ($M-H^+$): 253.0865; found: 253.0861. Compound **6**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.45 (br d, J = 7.3 Hz, 1H), 7.34–7.30 (m, 1H), 7.22–7.15 (m, 4 H), 7.05 (dd, J = 7.3, 1.2 Hz, 1H), 6.93 (dd, J = 8.3, 1.2 Hz, 1H), 4.25 (br s, 1H), 4.05 (dd, J = 11.2, 1.7 Hz, 1H), 3.81 (d, J = 11.2 Hz, 1H), 3.34 (d, J = 16.1 Hz, 1H), 2.97 (d, J = 16.1 Hz, 1H), 2.49 (s, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 154.98, 145.87, 141.01, 131.91, 128.68, 128.30, 127.88, 126.15, 125.44, 123.94, 122.15, 118.01, 77.88, 70.68, 52.15, 43.18; EI-MS(−): m/z 238 [M$^+$], 219; HRMS (EI+): calcd for $C_{16}H_{14}O_2$ (M^+): 238.0994; found: 238.0999. Compound **15**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.43–7.40 (m, 2 H, aromatic), 7.36–7.32 (m, 2 H, aromatic), 7.29–7.25 (m, 1H, aromatic), 6.95 (dd, J = 7.8, 1.2 Hz, 1H, aromatic), 6.89 (dd, J = 7.8, 7.8 Hz, 1H, aromatic), 6.77 (dd, J = 7.8, 1.2 Hz, 1H, aromatic), 6.70 (s, 1H, aromatic), 6.55 (s, 1H, aromatic), 5.10 (s, 2 H, CH_2Ph), 4.11 (d, J = 11 Hz 1H), 4.09 (s, 1H), 3.88 (d, J = 11 Hz 1H), 3.17 (d, J = 16 Hz 1H), 2.83 (d, J = 16 Hz 1H), 2.73 (br s, 1H), 1.64 (s, 3 H), 1.58 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 147.72, 146.72, 146.47, 143.49, 136.98, 136.02, 130.93, 128.45, 127.76, 127.20, 123.34, 122.80, 120.96, 117.74, 112.11, 105.50, 104.94, 70.94, 70.32, 50.70, 41.21, 25.69; EI-MS: m/z 416 [M$^+$], 325; HRMS (ESI+): calcd for $C_{26}H_{24}Na_1O_5$ ($M+Na^+$): 439.1521; found: 439.1538. Compound **16**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.95–6.89 (m, 2 H, aromatic), 6.82 (br dd, J = 2.7, 6.8 Hz, 1H, aromatic), 6.71 (s, 1H), 6.56 (s, 1H, aromatic), 4.12–4.08 (m, 2 H), 3.88 (d, J = 11 Hz, 1H), 3.19 (d, J = 16 Hz, 1H), 2.84 (d, J = 16 Hz, 1H), 2.53 (s, 1H), 1.65 (s, 3 H), 1.59 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 146.87, 146.64, 145.02, 140.48, 135.75, 130.66, 122.52, 121.79, 121.46, 117.90, 113.14, 105.49, 150.02, 77.68, 70.49, 50.56, 41.37, 25.74; ESI-MS(−): 325 [M−H]$^-$; HRMS (ESI−): calcd for $C_{19}H_{17}O_5$ ($M-H^+$): 325.1076; found: 325.1081.
12. MTT cell viability assay: Molt-4 cells were cultured in RPMI1640 supplemented with 10% FCS, penicillin and streptomycin. Molt-4 cells (1×10^5 cells/well) were cultured in 24 well plate at 37 °C for 2 days in the RPMI1640 containing serially diluted compounds ranging from 0 to 10 μM. MTT analysis was performed using a standard method as described previously.[18]
13. In vitro transport assay: The basic assay for nuclear import assay was performed as described elsewhere.[19] HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FCS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were plated onto glass coverslips 24 h before the assay and grown to 80% confluence. Cells were washed once in phosphate-buffered saline (PBS) and permeabilized with 40 μg/ml digitonin (Fluka) in transport buffer [TB: 20 mM HEPES–KOH [pH 7.3], 110 mM potassium acetate, 2 mM magnesium acetate, 5 mM sodium acetate and 1 mM dithiothreitol (DTT)] for 5 min at 4 °C. After washing three times with TB, in some case, the cells were further treated with 10 U/ml apyrase in TB at 30 °C for 5 min to delete the pool of nucleotide triphosphates. Nuclear import reaction was performed for 15 min at 28 °C. HeLa Cells were then washed three times with TB and fixed with 4%

formaldehyde in PBS. Coverslips were subsequently mounted in PBS containing 90% glycerol and examined by confocal laser scanning microscopy (FV 1000; Olimpus).

14. Viral infection of TZM-bl cells: TZM-bl cells were grown in DMEM containing 10% FCS. Cells (5×10^3 cells/well) in 96-well plates were cultured in the absence or presence of each chemical compound at various concentrations and then infected using a viral stock of the infectious molecular clone pNL432.[20] The viral titer of the stock virus was measured prior to infection in TZM-bl cells and adjusted to yield approximately 2×10^6 light units of firefly luciferase activity under these experimental conditions. Two days after infection, cells were harvested and lysed in lysis buffer (PicaGene ReporterLysis Buffer, Toyo B-Net, Tokyo), after which the expression of luciferase was quantified using the PicaGene luciferase assay system (Toyo B-Net, Tokyo) and a multimode microplate reader (Berthold Japan, Tokyo).
15. Viral infection of PBMCs: Human PBMCs were collected from three healthy donors and isolated on a Ficoll gradient (Immuno-Biological Laboratories) as previously described.[21] Freshly isolated PBMCs were suspended at a concentration of 1×10^6 cells/ml with RPMI 1640 supplemented with 10% FCS, 1 mM Sodium pyruvate, 400 unit/ml IL-2 (Shionogi Inc., Osaka, Japan), 50 nM 2-mercaptoethanol, and then stimulated with a concentration of 25 μg/ml Con A. After 6 days, Con A stimulated PBMC were cultured in the absence or presence of each chemical compound at various concentrations, and then infected using a viral stock of the infectious molecular clone pNL432 (a total of 2×10^6 light units of firefly luciferase activity) and subjected to Reverse Transcriptase Assay.[22]
16. Viral infection of macrophage: HIV-1 was introduced into 293T cells by transfection of macrophage-tropic pNF462 viruses encoding wild-type Vpr.[23] After filtration with a 0.45 μm Millipore filter, viral stocks were titrated by an enzyme-linked immunosorbent assay (ELISA) to determine the relative p24 values as described previously.[24] Monocytes were selected from PBMC using MACS CD14 MicroBeads (Miltenyi Biotec) and a MACS Separation column (Miltenyi Biotec) with a Quandro MACS Separation Unit (Miltenyi Biotec) as previously described.[25] Differentiated primary macrophages (2×10^5 cells/wells) were infected with HIV-1 (a total of 1 ng of p24) at 37 °C for 1 h. After washing three times with RPMI, the cells were cultured in RPMI containing serial 10-fold dilutions of the different compounds ranging in concentrations from 0 to 100 μM. Cell supernatants were collected 4 and 8 days after infection, and the p24 values were calculated by ELISA.
17. Compound **1** is also having inhibitory activity against HIV integrase,[6] therefore the observed results are including total activity of **1** against HIV virus.

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