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Synthesis and anti-HIV-1 activity of 4-[4-(4,6-bisphenylamino-[1,3,5]triazin-2-ylamino)-5-methoxy-2-methylphenylazo]-5-hydroxynaphthalene-2,7-disulfonic acid and its derivatives

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Abstract—A structure-based design approach has been used to optimize a lead HIV-1 entry inhibitor targeted to the envelope glycoprotein gp41. The docking study on this lead compound revealed important structural requirements that need to be preserved as well as structural non-requirements that could be eliminated to substantially reduce the molecular size of the lead compound. Based on the results from docking study, a limited number of analogues were designed and synthesized. This approach yielded a new analogue (compound **4**) that retained the anti-HIV-1 activity with reduced molecular size approaching towards more drug-like character.

1. Introduction

Recent data from UNAIDS indicate that over 42 million people are living with HIV/AIDS (AIDS Epidemic Update, December 2002, UNAIDS, Geneva, Switzerland; http://www.unaids.org/Unaids/EN/Resources/Publications). Despite the fact that potent anti-HIV drugs targeting protease and reverse transcriptase enzymes are available in many countries, more and more AIDS patients cannot use these drugs because of the serious side effects, the development of resistant strains to these drugs and the high cost of the regimens. After almost 20 years since the discovery of HIV, a new class of anti-HIV drug, fusion/entry inhibitor, T-20 (Fuzeon) has been approved recently by the US FDA. This is a remarkable development in AIDS drug discovery as this drug was not only very effective in clinical trials, but also a proof of the concept that in addition to the reverse transcriptase and protease, other critical sites of the virus could also be targeted for developing new classes of HIV inhibitors. Despite the success, it should be mentioned that T-20 is a peptide based drug and has several limitations since it is: (1) not orally available, (2) susceptible to proteolytic cleavage, and (3) very expensive.[1,2] Only a limited section of the HIV infected population can get the benefit of this drug. Therefore, discovery of non-peptidic HIV entry inhibitors as new anti-HIV drugs is urgently needed. Several groups have reported successful identification of HIV entry inhibitors towards that goal and have been published in several reviews.[3–10] Our laboratory initiated a systematic study in 1990s to identify HIV-1 entry inhibitors targeted to gp41,[11,12] the envelope glycoprotein transmembrane subunit, which has been shown to play an important role in virus fusion process.[13,14] The determination of X-ray crystal structure of the gp41 core in 1997[15,16] and identification of a deep cavity on the surface of the central coiled coil domain formed by three N-peptides (N36) paved the way to structure-based design of HIV-1 fusion inhibitors targeted to the cavity.[17,18] We hypothesized that any small molecule organic compounds that dock to this cavity may effectively block the interaction between the gp41 N- and C-terminal heptad repeats (NHR and CHR, respectively) to form the fusion-active six-helix bundle core structure,

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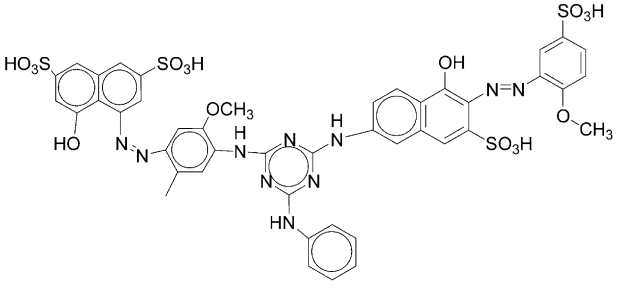
thereby preventing the fusion between the virus and the target cells. Based on this hypothesis, we have successfully identified an HIV-1 fusion inhibitor, ADS-J1 (Fig. 1), by computer-aided screening of a database consisting of 20,000 organic compounds using molecular docking technique as well as by a sandwich enzyme-linked immunosorbent assay (ELISA) using a conformation-specific monoclonal antibody (mAb), NC-1, which recognizes the gp41 six-helix bundle modeled by the N-peptide N36 and the C-peptide C34.[12,19,20] However, this compound is not an ideal candidate for development since it has a large molecular size ($M_r = 1089$ daltons). In the present study, we have analyzed the structure of ADS-J1 and identified a cluster of chemical groups that may not be required for the anti-HIV-1 activity. We thus designed several analogues of ADS-J1 by removing the structural non-requirements in order to identify a better lead compound with potential drug-like property.

1.1. Molecular modeling

The computer-aided docking strategy described previously[12] with the lead compound, ADS-J1, opened up a plethora of opportunities to understand the possible mechanism of molecular binding of this compound in the hydrophobic cavity and surrounding area. Although the molecule was much larger than traditional drug-like molecules, the compound showed remarkable inhibitory activities against a series of laboratory-adapted and clinical HIV-1 isolates. Based on the docking study, we have discovered that an important salt-bridge could be formed between one of the negatively charged groups (SO_3H) in ADS-J1 and a positively charged residues at the position of 574 (K574) in the peptide N36 (Fig. 2).[21] The mutation study confirmed the importance of this interaction. A similar ionic interaction was observed in the gp41 core crystal structure, where K574 forms a salt bridge with D632 of the C-peptide C34.[21,22] The docking study also revealed that certain portions of ADS-J1 were located outside the cavity region and making no noticeable contacts with the gp41 core structure. This provided us with the clue that the molecular size of ADS-J1 could be further reduced. Therefore, we decided to modify ADS-J1 by eliminating the areas which may not interact with any residues in the gp41 cavity or surrounding area. The objective was two-fold: (1) to reduce the molecular size of the lead compound to make it more drug-like and (2) to understand the structure–activity relationships.



ADS-J1

Figure 1. Chemical structure of ADS-J1.

In an attempt to corroborate the above findings we have initiated an in-depth study on the docking simulations of the newly designed compounds. This study provided us with some important understanding on the activity profile of the newly synthesized compounds and may shed some lights on designing new analogues with improved interactions, therefore, better activity. In addition, we have calculated the logP values of these analogue using the ClogP software (Biobyte Corporation, Claremont, CA, USA) to gain some insight on the influence of this important physico-chemical property on anti-HIV-1 activity (Table 1).

2. Chemistry

The synthesis of 4-[4-(4,6-Bisphenylamino-[1,3,5]triazin-2-ylamino)-5-methoxy-2-methylphenylazo]-5-hydroxynaphthalene-2,7-disulfonic acid (**4**) and two of its derivatives (**5** and **6**) was achieved via a series of chemical transformations (Scheme 1). Briefly, 4-(4-Amino-5-methoxy-2-methylphenylazo)-5-hydroxy-naphthalene-2,7-disulfo-nic acid, compound **2**, was obtained by treatment of a mixture of 4-amino-5-hydroxy-2,7-naphthalene disulfonic acid monosodium salt (**1**) with nitrous acid, followed by addition of 2-methoxy-5-methylaniline. Reaction of **2** with cyanuric chloride at −5 to 0 °C gave 4-[4-(4,6-dichloro-[1,3,5]triazin-2-ylamino)-5-methoxy-2-methylphenylazo]-5-hydroxy-naphthalene-2,7-disulfonic acid, disodium salt (**3**) in a moderate yield. The purification of **3** is problematic. Crystallization and silica gel chromatography were tried but failed to give pure **3**. The product was finally purified through chromatography on a column of Sephadex-LH-20 using 50% ethanol as the eluent. Reaction of intermediate **3** with aniline in the presence of DIPEA gave 4-[4-(4,6-bisphenylamino-[1,3,5]triazin-2-ylamino)-5-methoxy-2-methylphenylazo]-5-hydroxynaphthalene-2,7-disulfonic acid (**4**). Similarly, treatment of intermediate **3** with 4-aminopyridine and 4-amino-6-methoxypyrimidine in the presence of DIPEA afforded 4-{4-[4,6-bis-(pyridin-4-ylamino)-[1,3,5]triazin-2-ylamino]-5-methoxy-2-methylphenylazo}-5-hydroxynaphthalene-2,7-disulfonic acid disodium salt (**5**) and 4-{4-[4,6-bis-(6-methoxy-pyrimidin-4-ylamino)-[1,3,5]triazin-2-ylamino]-5-methoxy-2-methylphenylazo}-5-hydroxynaphthalene-2,7-disulfonic acid disodium salt (**6**), respectively, in moderate yield after chromatography on Sephadex LH-20 using 50% EtOH as the eluent. In both cases, crystallization and acidic precipitation failed to give pure material. In general, the purification of those products is difficult and very tedious, which requires repeat chromatography.

3. Biological assay and discussion

The inhibitory activities of the synthetic compounds (**4**–**6**) on HIV-1 mediated cell fusion, cytopathic effect (CPE) and gag protein p24 production as well as the gp41 six-helix bundle formation were determined as

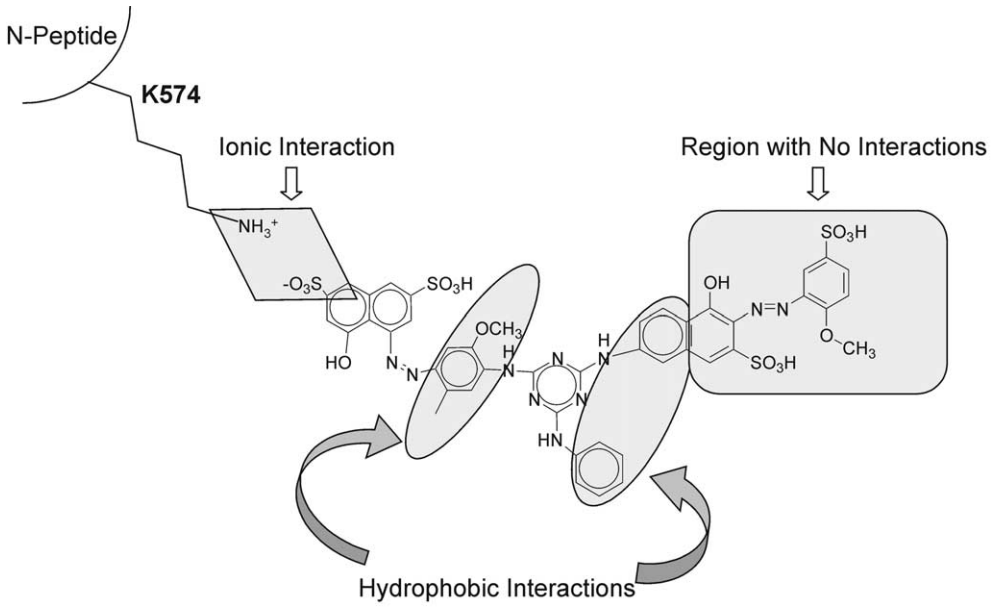


Figure 2. Schematics of the interaction sites of ADS-J1. The oblong areas indicate hydrophobic interaction sites; the square box area represents the no interactions region and amenable for modification and the angular square box shows the ionic interaction between the positively charged residue K574 in the N-peptide, N36, with the negatively charged group SO_3H of ADS-J1 forming a salt-bridge.

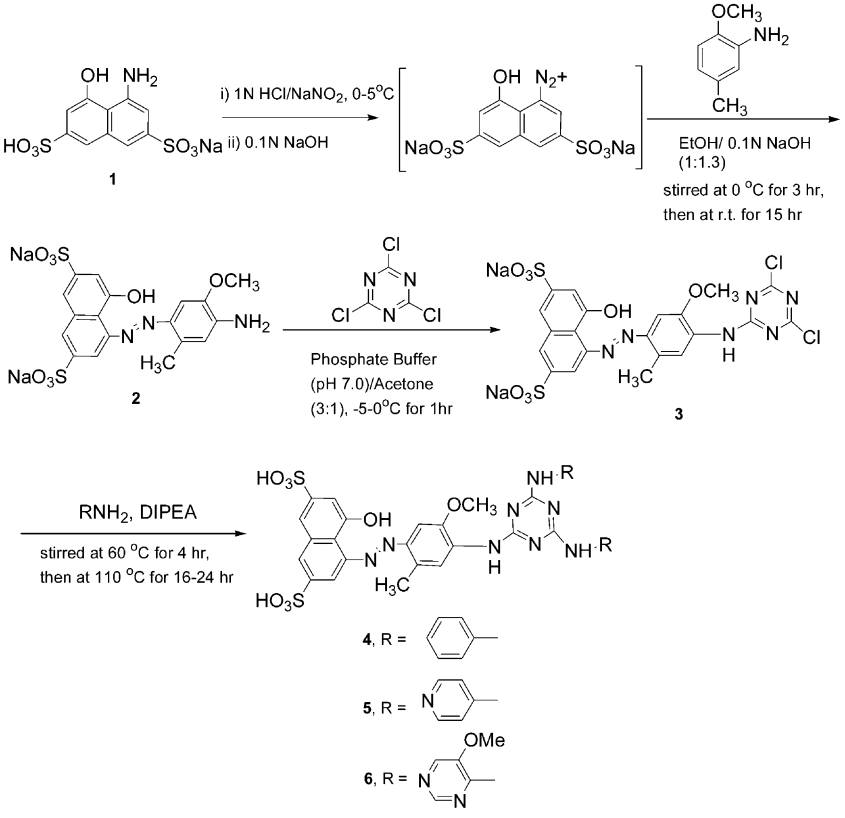
Table 1. Calculated logP values (ClogP) and inhibitory activity of ADS-J1 derivatives on CPE, p24 production, cell fusion, and the gp41 six-helix bundle formation

Compd	ClogP	IC_{50} (μM)[a] for inhibition of			
		CPE	p24 production	Cell fusion	Six-helix bundle formation
ADS-J1	−1.67	3.71±1.10	1.18±0.05	3.28±0.03	2.55±0.16
4	0.89	8.74±0.32	11.39±0.56	9.65±0.04	4.13±0.01
5	−2.11	28.52±4.74	15.51±1.70	35.74±3.23	59.17±15.47
6	−2.97	>80	>80	>80	>80

[a]Mean±S.D.

previously described.[12,19,20,23] The results were shown in Table 1. Compound **4** was first designed based on the docking study to eliminate the groups in ADS-J1 that may not participate in the interactions with the hydrophobic cavity or surrounding area. The compound showed comparable anti-HIV-1 activities as the parent compound ADS-J1 in CPE and cell fusion assays but had somewhat reduced activity in p24 production assay. Although there was no substantial improvement of activity, the molecular size of the compound became much smaller (M_r = 724 D) compared with ADS-J1 (M_r = 1089 D). This is a remarkable improvement. The modeling study also confirmed that even after pruning a large group from ADS-J1, compound **4** was able to form the salt-bridge and interacted with the hydrophobic groups. We wanted to further investigate whether the hydrophobic groups, for example, the phenyl groups, in the molecule are important for the anti-HIV-1 activity. We first replaced two phenyl groups attached to the triazine ring with two pyridinyl groups to reduce the hydrophobicity. The resulting compound (compound **5**) had a significantly reduced anti-HIV-1 activity (Table 1). The modeling study also confirmed that those two pyridyl groups were further away from the hydrophobic residues that made hydrophobic contacts in compound **4**. Subsequently, we replaced the two phenyl groups with two pyrimidinyl groups (compound **6**). This substitution led to the complete loss of the anti-HIV-1 activity. In consistent with the anti-HIV-1 activity, the compound **4** had similar inhibitory activity on the six-helix bundle formation as ADS-J1, while the compound **5** had much reduced activity and the compound **6** had no activity against the gp41 core formation. Close inspection of the docking poses of compound **6** showed that introduction of a bulky group (OCH_3) in the ortho position of the pyrimidinyl groups altered the conformation of the molecule substantially, thereby, having poor hydrophobic contacts in the hydrophobic cavity, although the compound was able to form the salt-bridge.

The above results suggest that besides the ionic interactions (salt-bridge formation), the active molecules bind to the hydrophobic cavity and surrounding area through hydrophobic interactions as indicated in Figure 2. The study also confirmed that although the original lead had higher molecular size compared to traditional drug-like molecules, it can be pruned to more acceptable structure by systemic molecular manipulations. The ClogP values have indicated that very low value of logP is detrimental for anti-HIV-1 activities. Further structure modifications are currently underway in order to remove some additional undesirable chemical groups and to increase the anti-HIV-1 potency.



Scheme 1.

4. Experimental

4.1. Molecular modeling

All molecular modeling studies were performed on a Silicon Graphics Octane R12000 workstation (sgi, Mountain View, CA, USA). The DOCK3.5 suit of software[24,25] (UCSF, CA, USA) was used for automated docking simulations. Sybyl 6.8 software Tripos Associates, Inc) was used for molecular visualization along with the CrystalEyes2 stereographic eye glasses (Stereographic Corp., San Rafel, CA, USA). Catalyst 4.7 (Accelrys, San Diego, CA, USA) was used for conformational study.

The automated docking was performed by following the method described previously.[12] In brief, 250 best conformations for each molecule were generated by using the ConFirm module within Catalyst 4.7 software. Gasteiger–Marsili charges[26,27] were assigned to these conformations and then converted to the DOCK35 formatted database for docking into the hydrophobic cavity of the gp41 core structure. One of the C-peptide of the core structure was removed to expose one of the three symmetrical hydrophobic cavities formed by the internal N-trimers (N-36).[28] Details of the protein preparation were described previously.[12] All docking poses were individually visualized for their interactions with the residues surrounding the cavity. Based on our earlier knowledge and in-depth mutation study we have considered only those docking poses, which formed the salt bridge between one of the negatively charged SO_3H groups and the positively charged lysine 574 (K574) residue and had hydrophobic interactions with the hydrophobic residues in the cavity.

logP values of all the compounds were calculated by Pomona College ClogP method available from Biobyte.

4.2. Chemistry

All reagents and solvents were purchased from Aldrich/Sigma, all of the highest grade available. ^{1}H NMR spectra were recorded with 300 MHz or Varian 500 MHz. The ESI-MS spectra were recorded on a Waters ZMD mass spectrometer. Gel filtration was carried out on a Saphedex-LH-20 column using 50% ethanol as the eluent.

4.2.1. 4-(4-Amino-5-methoxy-2-methylphenylazo)-5-hydroxy-naphthalene-2,7-disulfonic acid (2). A solution of 4-amino-5-hydroxy-2,7-naphthalene disulfonic acid monosodium salt **1** (1.36 g, 3.98 mmol) and $NaNO_2$ (276 mg, 400 mmol) in 0.1 N NaOH (10 mL) was added dropwise to 1 N HCl (5 mL) at 0–5 °C to generate the diazonium intermediate. The mixture was stirred for 30 min 0–5 °C, then added dropwise to a stirred solution of 2-methoxy-5-methylaniline (823.0 mg, 6.00 mmol) in 0.1N NaOH (40 mL) and ethanol (10 mL) at 0–5 °C. The resulting mixture was stirred at 0–5 °C for 2 h, and then at room temperature overnight (16 h). The mixture was lyophilized. The dried residue was mixed with water (100 mL), and washed with chloroform (3×40 mL) to remove un-reacted 2-methoxy-5-methylaniline. The

aqueous phase was lyophilized to give the azo-compound **2** (1.65 g); ^{1}H NMR (300 MHz, D_2O): δ 7.78 (s, 1H, naphthalene-H), 7.64 (s, 1H, naphthalene-H), 7.48 (s, 1H, naphthalene-H), 6.82 (s, 1H, naphthalene-H), 5.48 (s, 1H, tetra-substituted benzene-H), 5.06 (s, 1H, tetra-substituted benzene-H), 3.3.19 (s, 3H, OCH_3), 2.02 (s, 3H, CH_3); ESI-MS, 534.1 $(M+Na)^+$, 512.1 $(M+H)^+$.

4.2.2. 4-[4-(4,6-Dichloro-[1,3,5]triazin-2-ylamino)-5-methoxy-2-methylphenylazo]-5-hydroxy-naphthalene-2,7-disulfonic acid, disodium salt (3). To a stirred solution of **2** (270 mg, 0.55 mmol) in a phosphate buffer (15 mL, pH 7.0) was added cyanuric chloride (260 mg, 1.4 mmol) slowly in portions at −5 to 0 °C. The reaction mixture was stirred at the temperature for 1 h. Then the mixture was diluted with water, washed with $CHCl_3$ to remove excess cyanuric chloride. The aqueous phase was lyophilized, and the residue was chromatographed on a column of Sephadex LH-20 using 50% ethanol as the eluent to give compound **3** (235 mg); ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.29 (s, 1H, naphthalene-H), 8.11 (s 1H, naphthalene-H), 7.75 (s 1H, naphthalene-H), 7.64 (s, 1H, naphthalene-H), 7.26 (s, 1H, tetra-substituted benzene-H) 7.22 (s, 1H, tetra-substituted benzene-H), 5.50 (s, 2H, NH and OH), 3.99 (s, 3H, OCH_3), 2.73 (s, 3H, CH_3); FAB-MS: 659.1 $(M+H)^+$.

4.2.3. 4-[4-(4,6-Bisphenylamino-[1,3,5]triazin-2-ylamino)-5-methoxy-2-methylphenylazo]-5-hydroxynaphthalene-2,7-disulfonic acid (4). To a solution of compound **3** (50 mg, 75.82 μmol) and di-isopropyl ethylamine (1.5 mL) in DMSO (5 mL) was added aniline (40 mg, 429 μmol). The mixture was stirred at 60 °C for 4 h and then at 110 °C for 16 h. The solvent was removed under reduced pressure. The residue was suspended in 1 N HCl and the solid was collected by centrifugation. The solid was resuspended in 1 N HCl, and the precipitate was collected to give compound **4** (24 mg). ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.1 (s, 2H, SO_3H), 8.80 (s, 1H, naphthalene-OH), 8.28 (s 1H, naphthalene-H), 8.1 (s 1H, naphthalene-H), 7.8–7.6 (m, 5H, naphthalene-1H and monosubstituted benzene-4H), 7.4–7.27 (m, 4H, naphthalene-1H and monosubstituted benzene-3H), 7.26–7.05 (m, 5H, tetrasubstituted benzene-2H and monosubstituted benzene-3H), 3.95 (s, 3H, OCH_3), 2.75(s, 3H, CH_3); ESI-MS, 729.17 $(M+H)^+$.

4.2.4. 4-{4-[4,6-Bis-(pyridin-4-ylamino)-[1,3,5]triazin-2-ylamino]-5-methoxy-2-methylphenylazo}-5-hydroxynaphthalene-2,7-disulfonic acid, disodium salt (5). To a solution of compound **2** (50 mg, 75.85 μmol) and di-isopropyl ethylamine (1 mL) in DMSO (5 mL) was added 4-aminopyridine (28 mg, 296.8 μmol). The mixture was stirred at 60 °C for 3 h and then at 110 °C for 24 h. The solvent was removed under reduced pressure. The residue was suspended in 50% ethanol and the solid was collected by centrifugation. The residue was dissolved in water and was subject to gel filtration on a column of Sephadex LH-20 to give compound **5** (23 mg); ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.45 (s, 2H, naphthalene-2H), 8.2 (s 1H, naphthalene-H), 8.0 (bs 2H, pyridine-2H), 7.75 (s, 1H, naphthalene-H), 7.30–6.90(m, 4H, pyridine-2H and tetrasubstituted benzene-2H), 6.51–6.40 (s, 2H, pyridine-2H), 6.00 (s, 2H, pyridine-2H), 3.95 (s, 3H, OCH_3), 2.70 (s, 3H, CH_3)); ESI-MS, 776.12 $(M+H)^+$, 798.93 $(M+Na)^+$.

4.2.5. 4-{4-[4,6-Bis-(6-methoxy-pyrimidin-4-ylamino)-[1,3,5]triazin-2-ylamino]-5-methoxy-2-methylphenylazo}-5-hydroxynaphthalene-2,7-disulfonic acid, disodium salt (6). To a solution of compound 2 (28 mg, 42.48 μmol) and di-isopropyl ethylamine (1 mL) in DMSO (5 mL) was added 4-amino-6-methoxypyrimidine (40 mg, 319 μmol). The mixture was stirred at 60 °C for 3 h and then at 110 °C for 24 h. The solvent was removed under reduced pressure. The residue was washed thoroughly with acetone (3×10 mL) and then was subject to gel filtration on a column of Sephadex-LH-20 using 50% ethanol as the eluent to afford compound **6** (27 mg); ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.83 (s, 1H, naphthalene-H), 8.25 (s 1H, naphthalene-H), 8.15(s 1H, naphthalene-H), 8.08 (s, 2H, pyrimidine-2H), 7.74 (s, 1H, naphthalene-H), 7.24 (s, 1H, tetra-substituted benzene-H), 7.14 (s, 1H, tetra-substituted benzene-H), 6.61 (s, 2H, pyrimidine-2H), 3.97 (s, 3H, OCH_3), 3.76 (s, 6H, OCH_3), 2.77 (s, 3H, CH_3); ESI-MS (negative mode), 835.10 $(M-H)^-$.

4.3. HIV-1 mediated cell fusion assay[13]

Briefly, HIV-1_{IIIB}-infected H9 cells were labeled with a fluorescent reagent, 2′,7′-bis-(2-carboxyethyl)-5-and-6-carboxyfluorescein acetoxyethyl ester (BCECF-AM, Molecular Probes, Inc., Eugene, OR, USA) and incubated with MT-2 cells (ratio = 1:10) in a 96-well plate at 37 °C for 2 h in the presence of a test compound at graded concentrations. The fused and unfused BCECF-labeled HIV-1 infected cells were counted under an inverted fluorescence microscope (Zeiss, Germany) with an eyepiece micrometer disc.

4.4. CPE and p24 assays[13]

In brief, 1×10^4 MT-2 cells were infected with HIV-1_{IIIB} (100 $TCID_{50}$) in the presence a test compound at graded concentrations, followed by incubation at 37 °C overnight. The culture media were changed and cells were cultured for 4 days before collection of supernatants for measuring p24 antigen by ELISA.[12,19,20] On the sixth day post infection, an indicator XTT tetrazolium dye (PolySciences, Inc., Warrington, PA, USA) was added to the cells. After 4 h incubation, the soluble intracellular formazan was determined colorimetrically at 450 nm.

4.5. Sandwich ELISA for detecting the gp41 six-helix bundle[23]

Briefly, peptide N36 was pre-incubated with a test compound at the graded concentrations at 37 °C for 30 min, followed by addition of C34. After incubation for another 30 min, the mixture was added to wells of a 96-well polystyrene plate (Costar, Corning Inc., Corning, NY, USA) which were precoated with IgG purified from rabbit antisera directed against the N36/C34 complex. Then, the mAb NC-1 specific for the gp41 six-helix

bundle, biotin-labeled goat-anti-mouse IgG (Sigma Chemical Co., St. Louis, MO, USA), streptavidin-labeled horseradish peroxidase (Zymed, S. San Francisco, CA, USA), and the substrate 3,3′,5,5′-tetramethylbenzidine were added sequentially. Absorbance at 450 nm was read using an ELISA reader (Ultra 384, Tecan, Research Triangle Park, NC, USA).

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