1.0 mmol; 60% oil suspension) in DMF (1.7 $cm^3$) was added $CS_2$ (0.060 $cm^3$, 1.0 mmol) under an Ar atmosphere. After being stirred at 80 °C for 12 h, the mixture was concentrated. The residue was purified by flash chromatography over silica gel with *n*-hexane–EtOAc (8 : 2) to give the compound **24u** as a pale yellow solid (80.5 mg, 67%): mp 167 °C (from $CHCl_3$–*n*-hexane); IR (neat) $\nu_{max}/cm^{-1}$: 1624 (C=N); $\delta_H$ (400 MHz; $CDCl_3$; $Me_4Si$) 2.04–2.10 (2H, m, $CH_2$), 3.68 (2H, t, $J$ = 5.5 Hz, $CH_2$), 4.42 (2H, t, $J$ = 6.1 Hz, $CH_2$), 6.76 (1H, d, $J$ = 5.4 Hz, Ar) and 7.49 (1H, d, $J$ = 5.4 Hz, Ar). $\delta_C$ (100 MHz; $CDCl_3$) 21.5, 45.0, 48.5, 122.3, 128.4, 130.8, 131.0, 141.7 and 189.7; HRMS (FAB): *m/z* Calc. for $C_9H_9N_2S_3$ $[M + H]^+$ 240.9928; found: 240.9936.

**General procedure of *t*-BuNCS-mediated cyclization for *t*-Bu protected pyrimido[1,2-*c*][1,3]thiazin-6-imines 25–27, and 34: synthesis of *N*-(*tert*-butyl)-3,4-dihydro-9-nitro-2*H*,6*H*-pyrimido[1,2-*c*]-[1,3]benzothiazin-6-imine (25e).** To a mixture of 2-(2-fluoro-4-nitrophenyl)-1,4,5,6-tetrahydropyrimidine **21e** (2.0 g, 8.96 mmol) and NaH (716.8 mg, 17.92 mmol; 60% oil suspension) in DMF (29.8 $cm^3$) was added *t*-BuNCS (2.28 $cm^3$, 17.92 mmol) under an Ar atmosphere. After being stirred at −20 °C to rt for 2 days, EtOAc was added. The resulting solution was washed with sat. $NaHCO_3$, brine, and dried over $MgSO_4$. After concentration, the residue was purified by flash chromatography over aluminium oxide with *n*-hexane–EtOAc (10 : 0 to 9 : 1) to give compound **25e** as a pale yellow solid (1.77 g, 62%): mp 152–153 °C (from $CHCl_3$–*n*-hexane); IR (neat) $\nu_{max}/cm^{-1}$: 1604 (C=N), 1591 ($NO_2$), 1581 (C=N), 1523 ($NO_2$); $\delta_H$ (500 MHz; $CDCl_3$; $Me_4Si$) 1.39 (9H, s, 3 × $CH_3$), 1.91–1.96 (2H, m, $CH_2$), 3.66 (2H, t, $J$ = 5.2 Hz, $CH_2$), 3.88 (2H, t, $J$ = 5.7 Hz, $CH_2$), 7.97 (2H, dd, $J$ = 9.7, 2.3 Hz, Ar), 8.01 (2H, d, $J$ = 2.3 Hz, Ar) and 8.39 (1H, d, $J$ = 9.2 Hz, Ar). $\delta_C$ (125 MHz; $CDCl_3$) 21.7, 30.0, 45.3, 45.5, 54.5, 119.9, 120.3, 130.0, 131.1, 132.8, 136.1, 146.5 and 148.5; HRMS (FAB): *m/z* Calc. for $C_{15}H_{19}N_4O_2S$ $[M + H]^+$ 319.1229; found: 319.1229.

**General procedure of BrCN-mediated cyclization for pyrimido-[1,2-*c*][1,3]thiazin-6-imines 28 and 30: synthesis of 3,4-dihydro-9-methyl-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine (28g).** 3,4-Dihydro-9-methyl-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazine-6-thione **24g** (62.1 mg, 0.25 mmol) was suspended in a 0.1 M solution of NaOH in MeOH–$H_2O$ (9 : 1) (5 $cm^3$). After being stirred for 12 h under reflux, the mixture was concentrated. The residue was suspended in anhydrous EtOH (1 $cm^3$) and BrCN (53.0 mg, 0.50 mmol) was added under Ar atmosphere. After stirring for 2 h under reflux, the reaction mixture was quenched with 2 N NaOH. The whole was extracted with $CHCl_3$, and dried over $MgSO_4$. After concentration, the residue was purified by flash chromatography over aluminium oxide with *n*-hexane–EtOAc (9 : 1) to give the compound **28g** as colorless solid (39.2 mg, 68%): mp 121 °C (from $CHCl_3$–*n*-hexane); IR (neat) $\nu_{max}/cm^{-1}$: 1620 (C=N), 1569 (C=N); $\delta_H$ (500 MHz; $CDCl_3$; $Me_4Si$) 1.94–1.99 (2H, m, $CH_2$), 2.32 (3H, s, $CH_3$), 3.67 (2H, t, $J$ = 5.7 Hz, $CH_2$), 4.01 (2H, t, $J$ = 6.3 Hz, $CH_2$), 6.84 (1H, s, Ar), 7.02 (1H, d, $J$ = 8.6 Hz, Ar), 7.16 (1H, br s, NH) and 8.10 (1H, d, $J$ = 8.6 Hz, Ar). $\delta_C$ (125 MHz; $CDCl_3$) 21.1, 21.1, 43.8, 44.9, 123.6, 124.1, 127.4, 128.6, 128.8, 141.1, 146.6 and 153.6; HRMS (FAB): *m/z* Calc. for $C_{12}H_{14}N_3S$ $[M + H]^+$ 232.0908; found: 232.0912.

**General procedure of *tert*-butyl deprotection for pyrimido[1,2-*c*][1,3]benzothiazin-6-imines 28–30: synthesis of 3,4-dihydro-9-nitro-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine (28e).** TFA (1.5 $cm^3$) was added to a mixture of *N*-(*tert*-butyl)-3,4-dihydro-9-nitro-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine **25e** (47.8 mg, 0.15 mmol) in $CHCl_3$ and molecular sieves 4 Å (225 mg, powder, activated by heating with Bunsen burner). After being stirred under reflux for 1.5 h, the mixture was concentrated. To a mixture of this residue in $CHCl_3$ was added dropwise $Et_3N$ at 0 °C to adjust the pH to 8–9. The whole was extracted with EtOAc, and the extract was washed with sat. $NaHCO_3$, brine, and dried over $MgSO_4$. After concentration, the residue was purified by flash chromatography over aluminium oxide with *n*-hexane–EtOAc (19 : 1 to 1 : 1) to give compound **28e** as a pale yellow solid (24.9 mg, 63%): mp 170–172 °C (from $CHCl_3$–*n*-hexane); IR (neat) $\nu_{max}/cm^{-1}$: 1620 (C=N), 1587 ($NO_2$), 1568 (C=N), 1523 ($NO_2$); $\delta_H$ (400 MHz; $CDCl_3$; $Me_4Si$) 1.97–2.03 (2H, m, $CH_2$), 3.74 (2H, t, $J$ = 5.6 Hz, $CH_2$), 4.04 (2H, t, $J$ = 6.2 Hz, $CH_2$), 7.41 (1H, br s, NH), 7.93 (1H, d, $J$ = 2.2 Hz, Ar), 8.00 (1H, dd, $J$ = 9.0, 2.2 Hz, Ar) and 8.42 (1H, d, $J$ = 9.0 Hz, Ar). $\delta_C$ (100 MHz; $CDCl_3$) 20.8, 43.8, 45.2, 118.9, 120.5, 130.4, 130.8, 131.7, 145.1, 148.7 and 151.3; *Anal.* Calc. for $C_{11}H_{10}N_4O_2S$: C, 50.37; H, 3.84; N, 21.36. Found: C, 50.29; H, 4.03; N, 21.08%.

**General procedure of Suzuki–Miyaura cross coupling for 9-aryl pyrimido[1,2-*c*][1,3]thiazine derivatives: synthesis of *N*-(*tert*-butyl)-3,4-dihydro-9-phenyl-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine 25l.** To a solution of 9-bromo-*N*-(*tert*-butyl)-3,4-dihydro-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine **25k** (52.8 mg, 0.15 mmol) and phenylboronic acid (21.9 mg, 0.18 mmol) in a mixture of toluene (1.5 $cm^3$), EtOH (0.9 $cm^3$) and 1 M aq. $K_2CO_3$ (1.5 $cm^3$) was added $Pd(PPh_3)_4$ (6.9 mg, 4 mol%) and $PdCl_2(dppf)\cdot CH_2Cl_2$ (3.67 mg, 3 mol%). After being stirred at reflux for 1 h, the mixture was extracted with $CHCl_3$. The organic layers were dried over $MgSO_4$ and concentrated. The residue was purified by flash chromatography over aluminium oxide with *n*-hexane–EtOAc (10 : 0 to 9 : 1) to give the compound **25l** as colorless solid (44.8 mg, 85%): mp 122.5–124 °C (from $CHCl_3$–*n*-hexane); IR (neat) $\nu_{max}/cm^{-1}$: 1592 (C=N); $\delta_H$ (500 MHz; $CDCl_3$; $Me_4Si$) 1.40 (9H, s, 3 × $CH_3$), 1.90–1.95 (2H, m, $CH_2$), 3.64 (2H, t, $J$ = 5.4 Hz, $CH_2$), 3.89 (2H, t, $J$ = 6.0 Hz, $CH_2$), 7.33–7.37 (2H, m, Ar), 7.41–7.44 (3H, m, Ar), 7.58 (2H, d, $J$ = 6.9 Hz, Ar) and 8.25 (1H, d, $J$ = 8.6 Hz, Ar). $\delta_C$ (125 MHz; $CDCl_3$) 21.9, 30.0 (3C), 45.1, 45.4, 54.2, 122.7, 124.8, 126.5, 127.0 (2C), 128.0, 128.8 (2C), 128.9, 129.5, 138.3, 139.4, 142.9 and 147.7; HRMS (FAB): *m/z* Calc. for $C_{21}H_{24}N_3S$ $[M + H]^+$ 350.1691; found: 350.1683.

### Determination of anti-HIV activity

The sensitivity of three HIV-1 strains and two HIV-2 strains was determined by the MAGI assay.[32] The target cells (HeLa-CD4/CCR5-LTR/β-gal; 104 cells per well) were plated in 96 well flat microtiter culture plates. On the following day, the cells were inoculated with the HIV-1 (60 MAGI U per well, giving 60 blue


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cells after 48 h of incubation) and cultured in the presence of various concentrations of the drugs in fresh medium. Forty-eight hours after viral exposure, all the blue cells strained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were counted in each well. The activity of test compounds was determined as the concentration that blocked HIV-1 infection by 50% (50% effective concentration [$EC_{50}$]). $EC_{50}$ was determined by using the following formula:

$$EC_{50} = 10^{\wedge}[\log(A/B) \times (50 - C)/(D - C) + \log(B)],$$

wherein $A$: of the two points on the graph which bracket 50% inhibition, the higher concentration of the test compound, $B$: of the two points on the graph which bracket 50% inhibition, the lower concentration of the test compound, $C$: inhibitory activity (%) at the concentration $B$, $D$: inhibitory activity (%) at the concentration $A$.

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# Structure–activity relationship study of pyrimido[1,2-*c*][1,3]benzothiazin-6-imine derivatives for potent anti-HIV agents

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3,4-Dihydro-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine (PD 404182) is an antiretroviral agent with submicromolar inhibitory activity against human immunodeficiency virus-1 (HIV-1) and HIV-2 infection. In the current study, the structure–activity relationships of accessory groups at the 3- and 9-positions of pyrimido[1,2-*c*][1,3]benzothiazin-6-imine were investigated for the development of more potent anti-HIV agents. Several different derivatives containing a 9-aryl group were designed and synthesized using Suzuki–Miyaura cross-coupling and Ullmann coupling reactions. Modification of the *m*-methoxyphenyl or benzo[*d*][1,3]dioxol-5-yl group resulted in improved anti-HIV activity. In addition, the 2,4-diazaspiro[5.5]undec-2-ene-fused benzo[*e*][1,3]thiazine derivatives were designed and tested for their anti-HIV activities. The most potent 9-(benzo[*d*][1,3]dioxol-5-yl) derivative was two–threefold more effective against several strains of HIV-1 and HIV-2 than the parent compound, PD 404182.

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## 1. Introduction

Highly active antiretroviral therapy, involving the co-administration of nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), and/or protease inhibitors, is a standard treatment regimen for human immunodeficiency virus (HIV) infections. This regimen suppresses the replication of HIV and controls disease progression in HIV-infected patients.[1,2] Unfortunately, however, an increasing number of patients with HIV infection/AIDS have failed to respond to the current antiretroviral therapeutics because of serious problems including the emergence of drug-resistant HIV variants[3] and drug-related adverse effects.[4] With this in mind, there is therefore a continuous need to develop novel anti-HIV drugs that are more effective against drug-resistant viruses and produce fewer adverse effects. Recently, a series of extensive studies led to the development of a series of novel antiretrovirals with new mechanisms of action for anti-HIV therapy, including a fusion inhibitor (enfuvirtide),[5–7] an integrase inhibitor (raltegravir),[8,9] and a CC chemokine receptor type 5 (CCR5) antagonist (maraviroc).[10,11] CXC chemokine receptor type 4 (CXCR4) antagonists,[12–16] CD4 mimics,[17–20] gp41-binding peptides[21–23] and small molecules[24–26] represent promising alternative anti-HIV agents.

3,4-Dihydro-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine (PD 404182) (**1**) was previously reported as an antimicrobial agent that inhibited 3-deoxy-D-manno-octulosonic acid 8-phosphate synthase[27] and phosphopantetheinyl transferase (Fig. 1).[28,29] Following a recent random screening program using a multinuclear activation of a galactosidase indicator (MAGI) assay, compound **1** was identified as a new antiretroviral candidate with a high therapeutic index ($CC_{50}/EC_{50}$ >200). The MAGI assay allows for the inhibitory activity of an early-stage HIV infection, including inhibition of the virus attachment and membrane fusion to host cells, to be effectively evaluated.[30] Compound **1** showed a similar antiviral profile in HIV-1 infection to DS 5000[31] (adsorption inhibitor) and enfuvirtide (fusion inhibitor). The virucidal effects of compound **1** against the human hepatitis C virus, HIV, and simian immunodeficiency virus have also been reported.[32,33] The mechanism of action for compound **1**, however, has not yet been fully understood.

In our previous structure–activity relationship (SAR) study of compound **1**,[34] a number of PD 404182 derivatives were designed and synthesized according to a series of facile synthetic procedures,[35,36] in which the tricyclic heterocycles related to PD 404182 were easily obtained in a few steps from benzaldehydes via C–H functionalization or aromatic nucleophilic substitution.

*Abbreviations:* CCR5, CC chemokine receptor type 5; CXCR4, CXC chemokine receptor type 4; MAGI, Multinuclear activation of a galactosidase indicator; NNRTI, Non-nucleoside reverse transcriptase inhibitors; NRTI, Nucleoside reverse transcriptase inhibitors.

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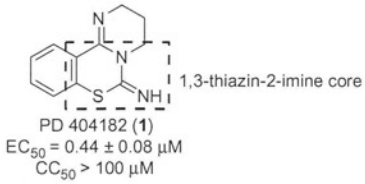



**Figure 1.** Structure of PD 404182.

The 6-6-6 fused pyrimido[1,2-*c*][1,3]benzothiazine scaffold and the heteroatom arrangement in the 1,3-thiazin-2-imine moiety are indispensable for the inhibitory activity of compound **1** against HIV infection (Fig. 1). Optimization studies indicated that the introduction of a hydrophobic group on the benzene ring and the cyclic amidine substructures effectively improved the antiviral activity by generating a potentially favorable interaction(s) with the target molecule(s). The most potent compounds identified were twofold more potent than PD 404182 and contained a phenyl group at 9-position of pyrimido[1,2-*c*][1,3]benzothiazine (compound **2**) or a geminal dimethyl group on the pyrimidine moiety (compound **3**) (Fig. 2).

In the current study, further structural optimization was conducted from the lead compounds **2** and **3** according to three approaches (Fig. 2), including the introduction of substituents on the 9-phenyl group (I), the substitution of the 9-phenyl group with fused arenes or heterocycles (II), and the modification of the cyclic amidine moiety (III). The anti-HIV profiles of the most potent derivative are also described.

## 2. Results and discussion

### 2.1. Synthesis of 9-aryl-3,4-dihydro-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine derivatives

The 9-aryl-3,4-dihydro-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine derivatives (**7** and **8**) were synthesized using a Suzuki–Miyaura cross-coupling reaction[37–39] of *N*-(*tert*-butyl)-protected bromide **4** with aryl boronic acid (pinacol ester) or by an Ullmann coupling[40] with pyrazole or imidazole (Scheme 1). Subsequent trifluoroacetic acid (TFA)-mediated deprotection of the *tert*-butyl groups afforded the desired biaryl-type derivatives.

### 2.2. Synthesis of spiropyrimidine-fused benzothiazinimine derivatives

The synthesis of the spiropyrimidine-fused derivatives started with the dialkylation of malononitrile with dihaloalkanes (**9**, **10**,

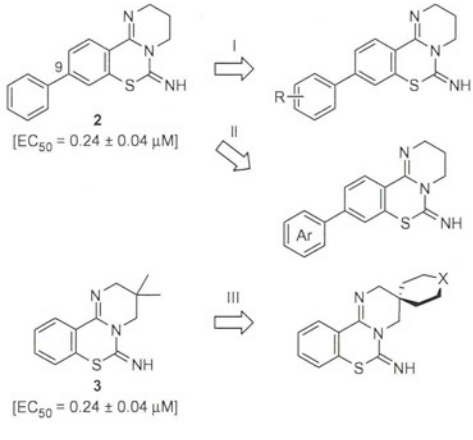
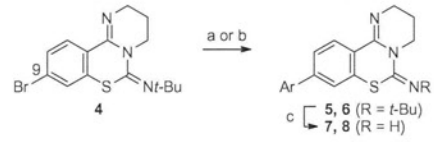
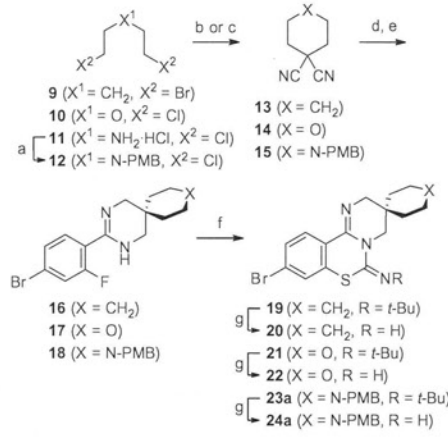



**Figure 2.** Strategy for the structural optimization of PD 404182 derivatives.




**Scheme 1.** Synthesis of 9-aryl-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine derivatives. Reagents and conditions: (a) R-B(OH)$_2$ or R-Bpin, Pd(PPh$_3$)$_4$, PdCl$_2$(dppf)·CH$_2$Cl$_2$, K$_2$CO$_3$, toluene or 1,4-dioxane, EtOH, H$_2$O, reflux, 29%-quant.; (b) pyrazole or imidazole, CuCl, K$_2$CO$_3$, acetylacetone, NMP, 130 °C, 51–71% (for **6n** or **6o**); (c) TFA, MS4Å, CHCl$_3$, (MeOH), reflux, 34–94%.

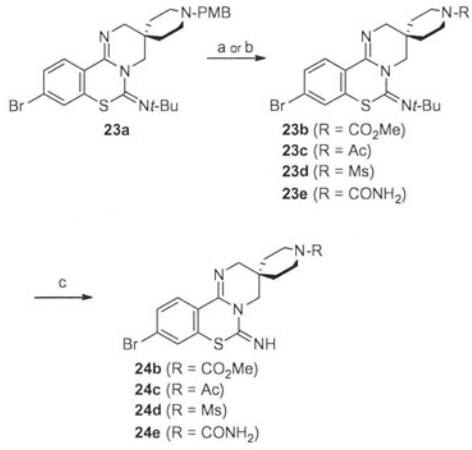



**Scheme 2.** Synthesis of spiropyrimidine-fused benzothiazinimine derivatives. Reagents and conditions: (a) (i) 4-methoxybenzoyl chloride, Et$_3$N, CH$_2$Cl$_2$, rt; (ii) LiAlH$_4$, Et$_2$O, rt, 75% (2 steps); (b) malononitrile, DBU, DMF, 50 °C, 8–60% (for **13** and **14**); (c) malononitrile, K$_2$CO$_3$, DMF, 65 °C, 85% (for **15**); (d) BH$_3$, THF, 0 °C to rt; (e) 4-bromo-2-fluorobenzaldehyde, I$_2$, K$_2$CO$_3$, *t*-BuOH, 70 °C, 11–62% [2 steps (d,e)]; (f) NaH, *t*-BuNCS DMF, rt −80 °C, 78–94%; (g) TFA, MS4Å, CHCl$_3$, reflux, 66–72%.

or **12**, Scheme 2). BH$_3$-mediated reduction of the alkylated malononitriles (**13–15**) followed by oxidative amidination[41] with 4-bromo-2-fluorobenzaldehyde gave the 2-phenyl-1,4,5,6-tetrahydropyrimidine derivatives (**16–18**). Subsequent exposure of compounds **16–18** to *tert*-butylisothiocyanate provided the tetracyclic compounds **19**, **21**, and **23a**. Deprotection of the *tert*-butyl groups in compounds **19**, **21**, and **23a** afforded the desired spiropyrimidine-fused benzothiazinimine derivatives (**20**, **22**, and **24a**). The substitution of the *p*-methoxybenzyl (PMB) group in compound **24a** was also attempted (Scheme 3). The treatment of compound **23a** with methyl chloroformate or acetyl chloride directly provided derivatives **23b** and **23c**, respectively. A two-step procedure, including the removal of the PMB group by treatment with 1-chloroethyl chloroformate followed by modification with mesyl chloride (MsCl) or trimethylsilyl isocyanate (TMSNCO) was used for the synthesis of the derivatives **23d** and **23e**, respectively, because the reaction of compound **23a** with MsCl and TMSNCO failed. Deprotection of the *tert*-butyl group in **23b–e** afforded the respective N-substituted derivatives **24b–e**.

### 2.3. Structure–activity relationships of 9-phenylpyrimido[1,2-*c*][1,3]benzothiazine derivatives

We initially examined substituent effects at the *para*-position of the 9-phenyl group of compound **2** (Table 1). The introduction of methoxycarbonyl (**7a**), cyano (**7b**), nitro (**7c**), and trifluoromethyl (**7d**) groups slightly reduced the anti-HIV activity (EC$_{50}$ = 0.44–0.81 μM), whereas a significant decrease in the anti-HIV activity was observed following the introduction of a carbamoyl group (**7e**) with hydrogen-bond donor/acceptor properties




**Scheme 3.** Synthesis of derivatives **24b–e** from **23a**. Reagents and conditions: (a) $ClCO_2Me$ or AcCl, $CH_2Cl_2$, 0 °C, 81–96% (for **23b** or **23c**); (b) (i) 1-chloroethyl chloroformate, $Et_3N$, $CH_2Cl_2$, 0 °C, then MeOH, reflux, (ii) MsCl or TMSNCO, ($Et_3N$), $CH_2Cl_2$, rt, 29–82% (2 steps, for **23d** or **23e**); (c) TFA, MS4Å, $CHCl_3$, reflux, 65–94%.

($EC_{50}$ = 8.71 μM). Compounds containing a hydrophobic group, including methoxy (**7f**, $EC_{50}$ = 0.24 μM), methylthio (**7g**, $EC_{50}$ = 0.20 μM), and trifluoromethoxy (**7h**, $EC_{50}$ = 0.38 μM) groups showed similar levels of anti-HIV activity to that of compound **2**. These results indicated that the hydrophobic and electron donating properties of these substituents had a positive impacts on improving the anti-HIV activity.

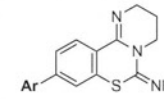
Similar SARs were observed following modifications at the *meta*-position of the 9-phenyl group (Table 1). For example, the addition of the electron-withdrawing methoxycarbonyl (**7i**), cyano (**7j**), and nitro (**7k**) groups resulted in a slight decrease in the anti-HIV activity ($EC_{50}$ = 0.39–1.26 μM), whereas the hydrophilic (1-hydroxy)ethyl (**7l**, $EC_{50}$ = 1.19 μM), acetamido (**7m**, $EC_{50}$ >10 μM), mesylamido (**7n**, $EC_{50}$ >10 μM), and hydroxy (**7o**, $EC_{50}$ = 2.62 μM) groups led to a reduction or loss in the levels of anti-HIV activity. In contrast, the introduction of a methoxy group (**7p**) at the *meta*-position of the 9-phenyl group improved the inhibitory activity ($EC_{50}$ = 0.15 μM). The more hydrophobic isopropoxy group (**7q**) maintained the anti-HIV activity of compound **2** ($EC_{50}$ = 0.32 μM), whereas the introduction of a phenyl group (**7r**) led to a decrease in the inhibitory activity ($EC_{50}$ = 1.35 μM).

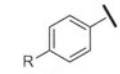
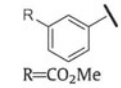
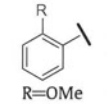
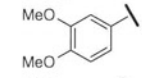
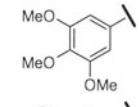
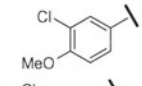
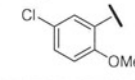
Similar anti-HIV activities to that of compound **2** were also exhibited by the *ortho*-methoxy (**7s**) and *ortho*-phenyl (**7t**) derivatives ($EC_{50}$ = 0.41 and 0.32 μM, respectively), suggesting that the twisted conformation of the biaryl axis in the 9-aryl-modified PD 404182 derivatives can be tolerated and can interact with the target molecule(s).

To develop more potent anti-HIV agents, several compounds were designed with bis- and tris-modifications on the 9-phenyl group of compound **2** (Table 1). The introduction of the 3,4-dimethoxy (**7u**, $EC_{50}$ = 0.27 μM) and 3,4,5-trimethoxy (**7v**, $EC_{50}$ = 0.25 μM) groups did not alter the bioactivity. The Cl-modified derivatives **7w** and **7x** exhibited similar levels of potency to compound **2** ($EC_{50}$ = 0.32 and 0.48 μM, respectively). Taken together, these results suggest that the hydrophobic property of the phenyl substituting group may provide the predominant contribution in any potential interaction with the target molecule(s).

We proceeded to investigate the impact of introducing a bicyclic aromatic group at the 9-position of the pyrimido[1,2-*c*][1,3]benzothiazine scaffold (Table 2). Modifications with a variety of 3,4-fused phenyl groups were investigated because the 2-naphthyl-modified analog (**8a**) exhibited slightly more potent

**Table 1**
Structure–activity relationships for biphenyl-type derivatives



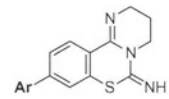

| Compound | Ar | $EC_{50}$[a] (μM) |
|---|---|---|
| |  | |
| **2** | R=H | 0.24 ± 0.04 |
| **7a** | R=$CO_2Me$ | 0.81 ± 0.29 |
| **7b** | R=CN | 0.44 ± 0.10 |
| **7c** | R=$NO_2$ | 0.46 ± 0.06 |
| **7d** | R=$CF_3$ | 0.55 ± 0.16 |
| **7e** | R=$CONH_2$ | 8.71 ± 0.82 |
| **7f** | R=OMe | 0.24 ± 0.04 |
| **7g** | R=SMe | 0.20 ± 0.06 |
| **7h** | R=$OCF_3$ | 0.38 ± 0.06 |
| |  | |
| **7i** | R=$CO_2Me$ | 0.39 ± 0.09 |
| **7j** | R=CN | 1.17 ± 0.27 |
| **7k** | R=$NO_2$ | 1.26 ± 0.13 |
| **7l** | R=$CH(OH)CH_3$ | 1.19 ± 0.19 |
| **7m** | R=NHAc | >10 |
| **7n** | R=NHMs | >10 |
| **7o** | R=OH | 2.62 ± 0.26 |
| **7p** | R=OMe | 0.15 ± 0.05 |
| **7q** | R=O*i*-Pr | 0.32 ± 0.10 |
| **7r** | R=Ph | 1.35 ± 0.26 |
| |  | |
| **7s** | R=OMe | 0.41 ± 0.10 |
| **7t** | R=Ph | 0.32 ± 0.12 |
| **7u** |  | 0.27 ± 0.04 |
| **7v** |  | 0.25 ± 0.03 |
| **7w** |  | 0.32 ± 0.04 |
| **7x** |  | 0.48 ± 0.06 |

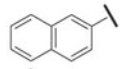
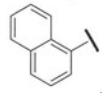
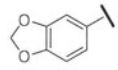
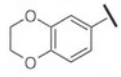
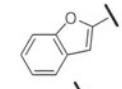
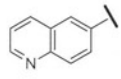
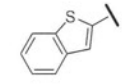
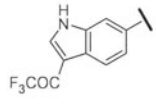
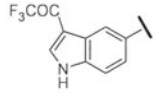
[a] $EC_{50}$ values represent the concentration of compound required to inhibit the HIV-1 infection by 50% and were obtained from three independent experiments.

anti-HIV activity ($EC_{50}$ = 0.20 μM) than that of the 1-naphthyl congener (**8b**, $EC_{50}$ = 0.39 μM). Compound **8c**, which contained a benzo[*d*][1,3]dioxol-5-yl group, displayed inhibitory activity twofold greater than that of compound **2** ($EC_{50}$ = 0.15 μM), whereas the 2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl derivative **8d** and quinolin-6-yl derivative **8e** exhibited less favorable effects ($EC_{50}$ = 0.26 μM and 0.25 μM, respectively). The introduction of trifluoroacetylindolyl groups (**8f** and **8g**) resulted in no anti-HIV activity, and the compounds also showed unexpected levels of cytotoxicity.

The substitution of the 9-phenyl group with a variety of different heterocyclic substructures was also investigated (Table 2). Pyridine substitution (**8h** and **8i**) led to a slight reduction in the anti-HIV activity ($EC_{50}$ = 0.45 μM and 0.54 μM, respectively), whereas the introduction of a furan (**8j**), benzofuran (**8k**), thiophene (**8l**), benzothiophene (**8m**), and pyrazole (**8n**) was well

**Table 2**
Structure–activity relationships for biaryl-type derivatives



| Compound | Ar | $EC_{50}$[a] (μM) | Compound | Ar | $EC_{50}$[a] (μM) |
|---|---|---|---|---|---|
| **8a** |  | 0.20 ± 0.06 | **8h** | | 0.45 ± 0.07 |
| **8b** |  | 0.39 ± 0.12 | **8i** | | 0.54 ± 0.04 |
| **8c** |  | 0.15 ± 0.03 | **8j** | | 0.26 ± 0.02 |
| **8d** |  | 0.26 ± 0.07 | **8k** |  | 0.20 ± 0.03 |
| **8e** |  | 0.25 ± 0.04 | **8l** | | 0.22 ± 0.07 |
| | | | **8m** |  | 0.26 ± 0.06 |
| **8f** |  | >1.00[b] | | | |
| | | | **8n** | | 0.42 ± 0.08 |
| **8g** |  | >1.00[b] | **8o** | | 5.12 ± 1.02 |

[a] $EC_{50}$ values represent the concentration of compound required to inhibit the HIV-1 infection by 50% and were obtained from three independent experiments.
[b] Cytotoxicity was observed at 10 μM.

tolerated and had little impact on the activity relative to compound **2** ($EC_{50}$ = 0.20–0.42 μM). It is worthy of note that the substitution of the 9-phenyl group with a basic imidazole moiety led to a significant reduction in the anti-HIV (**8o**, $EC_{50}$ = 5.12 μM).

Taken together, these data led to the identification of two highly potent compounds **7p** and **8c** ($EC_{50}$ = 0.15 μM), which contained *m*-methoxyphenyl and benzo[*d*][1,3]dioxol-5-yl groups, respectively. Furthermore, no cytotoxic effects were observed for these derivatives at 10 μM in the MAGI assay.

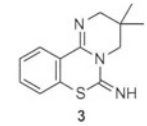
## 2.4. Structure–activity relationships of spiropyrimidine-fused benzothiazinimine derivatives

Several spiropyrimidine-fused derivatives were designed for the SAR study based on the geminal dimethylpyrimidine substructure **3** (Table 3).[42] Cyclohexane (**20**) and *N*-methoxycarbonylpiperidine (**24b**) derivatives exhibited the similar levels of anti-HIV activity to that of the parent dimethyl derivative **3**. In contrast, the tetrahydropyran (**22**) and *N*-(*p*-methoxybenzyl)piperidine (**24a**) derivatives exerted inhibitory activities that were five–sevenfold lower than that of the parent dimethyl derivative **3**. The *N*-acetyl- (**24c**), *N*-methanesulfonyl- (**24d**), and *N*-carbamoyl- (**24e**) piperidine derivatives also provided reduced levels of antiviral activity. With this in mind, the *N*-alkoxycarbonyl piperidine group was identified as a linkage for the introduction of additional functional group(s) to PD 404182 with potent anti-HIV activity (**24b**).

## 2.5. Anti-HIV profiles of the most potent derivative 8c

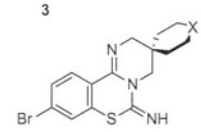
A time-of-drug addition study was carried out to further investigate the anti-HIV profile of the most potent derivative **8c** as an anti-HIV agent (Fig. 3). This assay has been used previously to

**Table 3**
Structure–activity relationships for spiropyrimidine-fused derivatives



$EC_{50}$ = 0.24 ± 0.04 μM

3



| Compound | X | $EC_{50}$[a] (μM) |
|---|---|---|
| **20** | $CH_2$ | 0.25 ± 0.01 |
| **22** | O | 1.73 ± 0.35 |
| **24a** | N-PMB | 1.45 ± 0.05 |
| **24b** | N-$CO_2$Me | 0.44 ± 0.02 |
| **24c** | N-Ac | 2.74 ± 0.15 |
| **24d** | N-Ms | 1.81 ± 0.43 |
| **24e** | N-$CONH_2$ | >10 |

[a] $EC_{50}$ values represent the concentration of compound required to inhibit the HIV-1 infection by 50% and were obtained from three independent experiments.

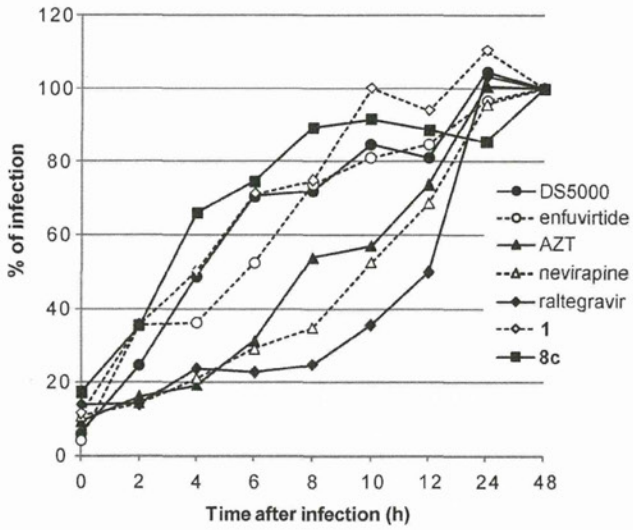



**Figure 3.** Time of drug addition profiles for infection by HIV-$1_{IIIB}$ strain of HeLa-CD4/CCR5-LTR/β-gal cells.

**Table 4**
*Anti-HIV activity of compounds* **1** *and* **8c** *against other HIV strains*

| Strains | $EC_{50}$[a] (μM) | |
|---|---|---|
| | **1**[34] | **8c** |
| HIV-$1_{NL4-3}$ | 0.38 ± 0.06 | 0.23 ± 0.09 |
| HIV-$1_{BaL}$ | 0.37 ± 0.06 | 0.13 ± 0.05 |
| HIV-$2_{EHO}$ | 0.31 ± 0.06 | 0.14 ± 0.02 |
| HIV-$2_{ROD}$ | 0.30 ± 0.06 | 0.10 ± 0.04 |

[a] $EC_{50}$ values represent the concentration of compound required to inhibit the HIV infection by 50% and were obtained from three independent experiments.

approximately determine which stage in the replication cycle of HIV-1 is inhibited by the compound. Two compounds (**1** and **8c**) were selected for testing in this assay together with five standard anti-HIV agents, including DS5000 (adsorption inhibitor),[31] enfuvirtide (fusion inhibitor),[6,7] AZT (NRTI),[43] nevirapine (NNRTI),[44,45] and raltegravir (integrase inhibitor).[8,9] The results revealed that the infection profile in the presence of compound **8c** was similar to that of DS5000 and enfuvirtide, suggesting that **8c** exerted its anti-HIV activity at the early stages of the viral infection, including the binding and fusion stage. This was similar to PD 404182, indicating that the bioactivity profile was not influenced by the newly appended functional group(s).

We also evaluated the antiviral activity of compounds **1** and **8c** against several HIV strains such as HIV-$1_{NL4-3}$, HIV-$1_{BaL}$, HIV-$2_{EHO}$, and HIV-$2_{ROD}$. This study enabled us to estimate the impact of the target molecules on the process of binding and fusion because these viruses have different susceptibilities[46] to different anti-HIV agents. These results implied that compounds **1** and **8c** exhibited their anti-HIV activity through different mechanisms from those of the known binding and fusion inhibitors including CCR5 antagonists, CXCR4 antagonists, and enfuvirtide. In addition, compound **8c** was two–threefold more effective against these HIV-1 and HIV-2 strains than PD 404182 (Table 4).

## 3. Conclusions

In conclusion, we have designed and synthesized a series of PD 404182 derivatives for the development of novel anti-HIV agents. The structural optimization study on the 9-position of pyrimido[1,2-*c*][1,3]benzothiazinimine identified two potent derivatives containing *m*-methoxyphenyl (**7p**) and benzo [*d*][1,3]dioxol-5-yl groups (**8c**) that exhibited threefold higher anti-HIV activity than that of PD 404182 (**1**). The common hydrophobic biaryl moiety is effective to improve the antiviral activity, providing potential interaction with the target molecule(s). In addition, we demonstrated that the most effective derivative, **8c**, inhibited viral infection against all of the HIV strains examined and acted at the early stage of the HIV infection. The design and synthesis of chemical probes based on these SAR data are being investigated to identify the target molecule(s).

## 4. Experimental

### 4.1. Synthesis

#### 4.1.1. General methods

$^1$H NMR spectra were recorded using a JEOL AL-400 or a JEOL ECA-500 spectrometer. Chemical shifts are reported in δ (ppm) relative to $Me_4Si$ ($CDCl_3$) or DMSO (DMSO-$d_6$) as internal standards. $^{13}$C NMR spectra were referenced to the residual solvent signal. Exact mass (HRMS) spectra were recorded on a JMS-HX/HX 110A mass spectrometer. Melting points were measured by a hot stage melting point apparatus (uncorrected). For flash chromatography, Wakogel C-300E (Wako) or aluminium oxide 90 standardized (Merck) were employed. For preparative TLC, TLC silica gel 60 $F_{254}$ (Merck) or TLC aluminium oxide 60 $F_{254}$ basic (Merck) were employed. For analytical HPLC, a Cosmosil 5C18-ARII column (4.6 × 250 mm, Nacalai Tesque, Inc., Kyoto, Japan) was employed with method A [a linear gradient of $CH_3CN$ containing 0.1% (v/v) TFA] or method B [a linear gradient of $CH_3CN$ containing 0.1% (v/v) $NH_3$] at a flow rate of 1 mL/min on a Shimadzu LC-10ADvp (Shimadzu Corp., Ltd., Kyoto, Japan), and eluting products were detected by UV at 254 nm. The purity of the compounds was determined by combustion analysis or HPLC analysis as >95%.

#### 4.1.2. General procedure of Suzuki–Miyaura cross coupling for 9-aryl pyrimido[1,2-*c*][1,3]thiazine derivatives 5 and 6: *N*-(*tert*-butyl)-3,4-dihydro-9-(4-methoxycarbonylphenyl)-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine 5a

To a solution of bromide **4** (52.8 mg, 0.15 mmol) and 4-(methoxycarbonyl)phenylboronic acid (32.4 mg, 0.18 mmol) in a mixture of toluene (1.5 mL), EtOH (0.9 mL) and 1 M aq $K_2CO_3$ (1.5 mL) was added $Pd(PPh_3)_4$ (6.9 mg, 4 mol %) and $PdCl_2(dppf)\cdot CH_2Cl_2$ (3.7 mg, 3 mol %). After being stirred under reflux for 1 h, the mixture was extracted with $CHCl_3$. The organic layers were dried over $MgSO_4$ and concentrated. The residue was purified by flash chromatography over aluminum oxide with *n*-hexane/EtOAc (10:0/9:1) to give the compound **5a** as colorless solid (47.3 mg, 77%): mp 201–202 °C (from $CHCl_3$–*n*-hexane); IR (neat) cm$^{-1}$: 1719 (C=O), 1593 (C=N); $^1$H NMR (400 MHz, $CDCl_3$) δ: 1.40 (s, 9H, 3 × $CH_3$), 1.90–1.96 (m, 2H, $CH_2$), 3.65 (t, *J* = 5.5 Hz, 2H, $CH_2$), 3.89 (t, *J* = 6.1 Hz, 2H, $CH_2$), 3.94 (s, 3H, $CH_3$), 7.36 (d, *J* = 1.7 Hz, 1H, Ar), 7.44 (dd, *J* = 8.5, 1.7 Hz, 1H, Ar), 7.65 (d, *J* = 8.2 Hz, 2H, Ar), 8.10 (d, *J* = 8.2 Hz, 2H, Ar), 8.28 (d, *J* = 8.5 Hz, 1H, Ar). $^{13}$C NMR (100 MHz, $CDCl_3$) δ: 21.9, 30.0 (3C), 45.2, 45.4, 52.1, 54.2, 123.0, 124.8, 127.0 (2C), 127.3, 129.1, 129.6, 129.8, 130.2 (2C), 138.0, 141.7, 143.8, 147.5, 166.8; HRMS (FAB): *m*/*z* calcd for $C_{23}H_{26}N_3O_2S$ $[M+H]^+$ 408.1746; found: 408.1748.

#### 4.1.3. *N*-(*tert*-Butyl)-3,4-dihydro-9-(1*H*-pyrazol-1-yl)-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine (6n)

To a solution of bromide **4** (52.8 mg, 0.15 mmol), pyrazole (12.3 mg, 0.18 mmol), CuCl (1.5 mg, 0.015 mmol) and $K_2CO_3$ (21.8 mg, 0.16 mol) in *N*-methylpyrrolidone (0.3 mL) was added

acetylacetone (3.8 μL, 0.038 mmol) under an Ar atmosphere. After being stirred at 130 °C for 19 h, EtOAc and brine were added. The organic layers were washed with $H_2O$, and dried over $MgSO_4$. After concentration, the residue was purified by flash chromatography over aluminum oxide with *n*-hexane/EtOAc (7:3) to give the title compound **6n** as colorless solid (39.8 mg, 71%): mp 132–133 °C (from $CHCl_3$–*n*-hexane); IR (neat) $cm^{-1}$: 1597 (C=N); $^1H$ NMR (400 MHz, $CDCl_3$) δ: 1.39 (s, 9H, 3 × $CH_3$), 1.90–1.96 (m, 2H, $CH_2$), 3.63 (t, *J* = 5.6 Hz, 2H, $CH_2$), 3.88 (t, *J* = 6.2 Hz, 2H, $CH_2$), 6.48 (dd, *J* = 2.7, 1.8 Hz, 1H, Ar), 7.47 (dd, *J* = 8.8, 2.2 Hz, 1H, Ar), 7.56 (d, *J* = 2.2 Hz, 1H, Ar), 7.73 (d, *J* = 1.8 Hz, 1H, Ar), 7.94 (d, *J* = 2.7 Hz, 1H, Ar), 8.28 (d, *J* = 8.8 Hz, 1H, Ar). $^{13}C$ NMR (100 MHz, $CDCl_3$) δ: 21.8, 30.0 (3C), 45.0, 45.4, 54.2, 108.2, 114.3, 115.9, 125.4, 126.7, 129.9, 130.8, 137.7, 141.0, 141.7, 147.3; HRMS (FAB): *m*/*z* calcd for $C_{18}H_{22}N_5S$ $[M+H]^+$ 340.1596; found: 340.1598.

#### 4.1.4. General procedure of *tert*-butyl deprotection for pyrimido[1,2-*c*][1,3]benzothiazin-6-imines (7, 8, 20, 22, and 24): 3,4-dihydro-9-(4-methoxycarbonylphenyl)-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine (7a)

TFA (2.0 mL) was added to a mixture of *N*-(*tert*-butyl)-protected pyrimido[1,2-*c*][1,3]benzothiazin-6-imine **5a** (38.4 mg, 0.094 mmol) in small amount of $CHCl_3$ and MS4Å (300 mg, powder, activated by heating with Bunsen burner). After being stirred under reflux for 1 h, the mixture was concentrated. To a stirring mixture of the residue in $CHCl_3$ was added dropwise $Et_3N$ at 0 °C to adjust pH to 8–9. The whole was extracted with EtOAc. The extract was washed with sat. $NaHCO_3$, brine, and dried over $MgSO_4$. After concentration, the residue was purified by flash chromatography over aluminum oxide with *n*-hexane/EtOAc (9:1/1:1) to give the title compound **7a** as colorless solid (27.3 mg, 83%): mp 185–186 °C (from $CHCl_3$–*n*-hexane); IR (neat) $cm^{-1}$: 1719 (C=O), 1619 (C=N), 1566 (C=N); $^1H$ NMR (500 MHz, $CDCl_3$) δ: 1.97–2.02 (m, 2H, $CH_2$), 3.71 (t, *J* = 5.7 Hz, 2H, $CH_2$), 3.94 (s, 3H, $CH_3$), 4.04 (t, *J* = 6.0 Hz, 2H, $CH_2$), 7.27 (d, *J* = 1.7 Hz, 1H, Ar), 7.46 (dd, *J* = 8.0, 1.7 Hz, 1H, Ar), 7.63 (d, *J* = 8.6 Hz, 2H, Ar), 8.10 (d, *J* = 8.6 Hz, 2H, Ar), 8.30 (d, *J* = 8.0 Hz, 1H, Ar). $^{13}C$ NMR (125 MHz, $CDCl_3$) δ: 21.0, 43.8, 45.0, 52.2, 122.0, 125.1, 126.2, 126.9 (2C), 129.5, 129.6, 129.7, 130.2 (2C), 142.1, 143.4, 146.2, 153.0, 166.7; HRMS (FAB): *m*/*z* calcd for $C_{19}H_{18}N_3O_2S$ $[M+H]^+$ 352.1120; found: 352.1119

#### 4.1.5. Bis(2-chloroethyl)-*N*-(4-methoxybenzyl)amine (12)

To a suspension of bis(2-chloroethyl)amine hydrochloride **11** (8.92 g, 50.0 mmol) in $CH_2Cl_2$ (300 mL) were added $Et_3N$ (2.89 mL, 100.0 mmol) and 4-methoxybenzoyl chloride (6.77 mL, 50.0 mmol). After being stirred at rt for 2 h, the reaction mixture was washed with 1 N HCl, satd $NaHCO_3$, brine, and dried over $MgSO_4$. After concentration, the residue was dissolved in anhydrous $Et_2O$ (250 mL) and $LiAlH_4$ (2.1 g, 55.0 mmol) was slowly added at 0 °C under an Ar atmosphere. After being stirred at rt overnight, the reaction mixture was quenched by addition of water, 2 N NaOH, and water. The mixture was dried over $MgSO_4$. After concentration, the residue was purified by flash chromatography over silica gel with *n*-hexane/EtOAc (19:1) to give the title compound **12** as colorless oil (9.88 g, 75%): $^1H$ NMR (400 MHz, $CDCl_3$) δ: 2.90 (t, *J* = 7.1 Hz, 4H, 2 × $CH_2$), 3.48 (t, *J* = 7.1 Hz, 4H, 2 × $CH_2$), 3.67 (s, 2H, $CH_2$), 3.80 (s, 3H, $CH_3$), 6.86 (d, *J* = 8.5 Hz, 2H, Ar), 7.24 (d, *J* = 8.5 Hz, 2H, Ar). $^{13}C$ NMR (100 MHz, $CDCl_3$) δ: 42.0 (2C), 55.2, 56.2 (2C), 58.6, 113.8 (2C), 129.7 (2C), 130.7, 158.9; LRMS (FAB): *m*/*z* $[M+H]^+$ 262.

#### 4.1.6. 1-(4-Methoxybenzyl)piperidine-4,4-dicarbonitrile (15)

To a solution of malononitrile (2.49 g, 37.7 mmol) in DMF (94.3 mL) was added $K_2CO_3$ (5.73 mg, 41.5 mmol). After being stirred at 65 °C for 2 h, a solution of chloride **12** (9.88 mg, 37.7 mmol) in DMF (37.7 mL) was added. After being stirred at same temperature for 5 h, EtOAc was added. The mixture was washed with 5% aq $NaHCO_3$, and dried over $MgSO_4$. After concentration, the residue was purified by flash chromatography over silica gel with *n*-hexane/EtOAc (2:1) to give the title compound **15** as yellow oil (8.13 g, 85%): IR (neat) $cm^{-1}$: 2248 (C≡N); $^1H$ NMR (400 MHz, $CDCl_3$) δ: 2.22 (t, *J* = 5.4 Hz, 4H, 2 × $CH_2$), 2.61 (br s, 4H, 2 × $CH_2$), 3.48 (s, 2H, $CH_2$), 3.80 (s, 3H, $CH_2$), 6.86 (d, *J* = 8.5 Hz, 2H, Ar), 7.19 (d, *J* = 8.8 Hz, 2H, Ar). $^{13}C$ NMR (100 MHz, $CDCl_3$) δ: 31.1, 34.1 (2C), 48.5 (2C), 55.2, 61.9, 113.8 (2C), 115.4 (2C), 129.2, 130.1 (2C), 159.0; HRMS (FAB): *m*/*z* calcd for $C_{15}H_{18}N_3O$ $[M+H]^+$ 256.1450; found: 256.1454.

#### 4.1.7. 3-(4-Bromo-2-fluorophenyl)-9-(4-methoxybenzyl)-2,4,9-triazaspiro[5.5]undec-2-ene (18)

To a solution of nitrile **15** (4.05 g, 15.9 mmol) in THF (39.8 mL) was added $BH_3$ in THF (79.5 mL, 79.5 mmol, 1.0 M) at 0 °C under an Ar atmosphere. The mixture was warmed to rt. After being stirred at 65 °C for 5 h, the reaction mixture was cooled to 0 °C, and 1 N HCl was added. After being stirred at rt for 1 h, the mixture was basified with 2 N NaOH. The whole was extracted with $CHCl_3$ and dried over $MgSO_4$. After concentration, the residue was dissolved in *t*-BuOH (159.0 mL) and 4-bromo-2-fluorobenzaldehyde (3.23 g, 15.9 mmol) was added. After being stirred at 70 °C for 30 min, $K_2CO_3$ (6.59 g, 47.7 mmol) and $I_2$ (5.05 g, 19.9 mmol) were added. After being stirred at same temperature for 3 h, the reaction mixture was quenched with sat. $Na_2SO_3$ until the iodine color almost disappeared. The reaction mixture was basified with 2 N NaOH. The whole was extracted with $CHCl_3$, and dried over $MgSO_4$. After concentration, the residue was purified by flash chromatography over aluminium oxide with EtOAc–MeOH (10:0/95:5) to give the title compound **18** as colorless solid (752 mg, 11%): mp 179–181 °C (from $CHCl_3$–*n*-hexane), IR (neat) $cm^{-1}$: 1630 (C=N); $^1H$ NMR (500 MHz, $CDCl_3$) δ: 1.45 (t, *J* = 5.4 Hz, 4H, 2 × $CH_2$), 2.35 (t, *J* = 5.4 Hz, 4H, 2 × $CH_2$), 3.16 (s, 4H, 2 × $CH_2$), 3.40 (s, 2H, $CH_2$), 3.73 (s, 3H, $CH_3$), 4.63 (s, 1H, NH), 6.78 (d, *J* = 8.6 Hz, 2H, Ar), 7.14–7.23 (m, 4H, Ar), 7.62 (t, *J* = 8.3 Hz, 1H, Ar). $^{13}C$ NMR (100 MHz, $CDCl_3$) δ: 27.3, 32.8 (2C), 49.1 (2C), 51.4 (2C), 55.2, 62.7, 113.5 (2C), 119.4 (d, *J* = 27.3 Hz), 122.7 (d, *J* = 12.4 Hz), 123.7 (d, *J* = 9.9 Hz), 127.8 (d, *J* = 3.3 Hz), 130.2, 130.3 (2C), 131.7 (d, *J* = 4.1 Hz), 150.3 (d, *J* = 1.7 Hz), 158.6, 159.7 (d, *J* = 251.6 Hz); $^{19}F$ NMR (500 MHz, $CDCl_3$) δ: −114.6. HRMS (FAB): *m*/*z* calcd for $C_{22}H_{26}BrFN_3O$ $[M+H]^+$ 446.1243; found: 446.1237.

#### 4.1.8. General procedure for *t*-BuNCS mediated cyclization: 9-bromo-*N*-(*tert*-butyl)-1′-(4-methoxybenzyl)-2*H*-spiro[benzo[*e*]pyrimido[1,2-*c*][1,3]thiazine-3,4′-piperidin]-6(4*H*)-imine (23a)

To a mixture of fluoride **18** (2.0 g, 4.48 mmol) and NaH (358.4 mg, 8.96 mmol; 60% oil suspension) in DMF (14.8 mL) was added *t*-BuNCS (1.14 mL, 8.96 mmol) under an Ar atmosphere. After being stirred at rt overnight, the reaction mixture was warmed to 60 °C. After being stirred at this temperature for 1 h, EtOAc was added. The resulting solution was washed with sat. $NaHCO_3$, brine, and dried over $MgSO_4$. After concentration, the residue was purified by flash chromatography over aluminum oxide with *n*-hexane/EtOAc (10:0/9:1) to give the title compound **23a** as colorless solid (2.28 g, 94%): mp 89–91 °C (from $CHCl_3$–*n*-hexane); IR (neat) $cm^{-1}$: 1577 (C=N); $^1H$ NMR (500 MHz, $CDCl_3$) δ: 1.37 (s, 9H, 3 × $CH_3$), 1.49–1.52 (m, 4H, 2 × $CH_2$), 2.40–2.46 (m, 4H, 2 × $CH_2$), 3.41 (s, 2H, $CH_2$), 3.47 (s, 2H, $CH_2$), 3.75 (s, 2H, $CH_2$), 3.80 (s, 3H, $CH_3$), 6.85 (d, *J* = 8.6 Hz, 2H, Ar), 7.22 (d, *J* = 8.6 Hz, 2H, Ar), 7.28–7.31 (m, 2H, Ar), 8.03 (d, *J* = 8.6 Hz, 1H, Ar). $^{13}C$ NMR (100 MHz, $CDCl_3$) δ: 29.7, 29.9 (3C), 32.6 (2C), 49.2 (2C), 51.6, 54.3, 55.2, 55.5, 62.7, 113.6 (2C), 124.5, 126.3, 126.8, 129.2, 130.0, 130.1, 130.4 (2C), 130.9, 137.5, 146.3, 158.7; HRMS

(FAB): *m/z* calcd for $C_{27}H_{34}BrN_4OS$ $[M+H]^+$ 541.1637; found: 541.1633.

#### 4.1.9. 9-Bromo-*N*-(*tert*-butyl)-1′-(methoxycarbonyl)-2*H*-spiro[benzo[*e*]pyrimido[1,2-*c*][1,3]thiazine-3,4'-piperidin]-6(4*H*)-imine (23b)

To the solution of *N*-(4-methoxybenzyl)piperidine **23a** (40.6 mg, 0.075 mmol) in $CH_2Cl_2$ (0.38 mL) was added methyl chloroformate (86.4 μL, 1.13 mmol) at 0 °C under an Ar atmosphere. After being stirred at same temperature for 30 min, the reaction mixture was concentrated. The residue was purified by flash chromatography over silica gel with *n*-hexane/EtOAc (1:1) to give compound **23b** as a colorless solid (29.2 mg, 81%): mp 157–158 °C (from *n*-hexane); IR (neat) $cm^{-1}$: 1699 (C=O), 1577 (C=N); $^1H$ NMR (400 MHz, $CDCl_3$) δ: 1.37 (s, 9H, 3 × $CH_3$), 1.46 (t, *J* = 5.6 Hz, 4H, 2 × $CH_2$), 3.44 (br s, 4H, 2 × $CH_2$), 3.56 (br s, 2H, $CH_2$), 3.70 (s, 3H, $CH_3$), 3.81 (s, 2H, $CH_2$), 7.29–7.33 (m, 2H, Ar), 8.05 (d, *J* = 8.5 Hz, 1H, Ar). $^{13}C$ NMR (100 MHz, $CDCl_3$) δ: 29.9 (3C), 30.1, 32.2 (2C), 39.9 (2C), 50.8, 52.5, 54.3, 55.2, 124.7, 126.1, 126.8, 129.3, 130.0, 130.9, 137.7, 146.3, 155.9; HRMS (FAB): *m/z* calcd for $C_{21}H_{28}BrN_4O_2S$ $[M+H]^+$ 479.1116; found: 479.1115.

#### 4.1.10. 9-Bromo-*N*-(*tert*-butyl)-1′-(methanesulfonyl)-2*H*-spiro[benzo[*e*]pyrimido[1,2-*c*][1,3]thiazine-3,4'-piperidin]-6(4*H*)-imine (23d)

To the solution of *N*-(4-methoxybenzyl)piperidine **23a** (54.2 mg, 0.10 mmol) in $CH_2Cl_2$ (0.5 mL) were added $Et_3N$ (28.9 μL, 0.20 mmol) and 1-chloroethyl chloroformate (21.8 μL, 0.20 mmol) at 0 °C under an Ar atmosphere. After being stirred at same temperature for 30 min, the reaction mixture was concentrated. The residue was dissolved in MeOH (2.0 mL). After being stirred under reflux for 10 min, the reaction mixture was concentrated. The residue was dissolved in $CHCl_3$, and was washed with sat. $NaHCO_3$, brine, and dried over $MgSO_4$. After concentration, the residue was dissolved in $CH_2Cl_2$ (1.0 mL) and $Et_3N$ (28.9 μL, 0.20 mmol) and methanesulfonyl chloride (15.5 μL, 0.20 mmol) was added at rt under an Ar atmosphere. After being stirred at rt for 10 min, the reaction mixture was washed with sat. $NaHCO_3$, brine, and dried over $MgSO_4$. After concentration, the residue was purified by flash chromatography over aluminum oxide with *n*-hexane/EtOAc (6:4) to give compound **23d** as a colorless solid (40.9 mg, 82%): mp 177 °C (from $CHCl_3$–*n*-hexane); IR (neat) $cm^{-1}$: 1577 (C=N), 1331 ($NSO_2$), 1155 ($NSO_2$); $^1H$ NMR (400 MHz, $CDCl_3$) δ: 1.38 (s, 9H, 3 × $CH_3$), 1.62 (t, *J* = 5.5 Hz, 4H, 2 × $CH_2$), 2.80 (s, 3H, $CH_3$), 3.21–3.27 (m, 2H, $CH_2$), 3.31–3.37 (m, 2H, $CH_2$), 3.46 (s, 2H, $CH_2$), 3.84 (s, 2H, $CH_2$), 7.29–7.33 (m, 2H, Ar), 8.05 (d, *J* = 8.5 Hz, 1H, Ar). $^{13}C$ NMR (100 MHz, $CDCl_3$) δ: 29.8, 29.9 (3C), 32.0 (2C), 34.7, 42.0 (2C), 50.1, 54.4, 55.1, 124.8, 125.9, 126.9, 129.4, 130.0, 130.8, 137.9, 146.3; HRMS (FAB): *m/z* calcd for $C_{20}H_{28}BrN_4O_2S_2$ $[M+H]^+$ 499.0837; found: 499.0840.

### 4.2. Determination of anti-HIV activity

The sensitivity of three HIV-1 strains and two HIV-2 strains was determined by the MAGI assay. The target cells (HeLa-CD4/CCR5-LTR/β-gal; $10^4$ cells/well) were plated in 96-well flat microtiter culture plates. On the following day, the cells were inoculated with the HIV-1 (60 MAGI U/well, giving 60 blue cells after 48 h of incubation) and cultured in the presence of various concentrations of the test compounds in fresh medium. Forty-eight hours after viral exposure, all the blue cells stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were counted in each well. The activity of test compounds was determined as the concentration that blocked HIV-1 infection by 50% (50% effective concentration [$EC_{50}$]). $EC_{50}$ was determined by using the following formula:

$$EC_{50} = 10\hat{}[\log(A/B) \times (50 - C)/(D - C) + \log(B)], \quad (1)$$

wherein

A: of the two points on the graph which bracket 50% inhibition, the higher concentration of the test compound,
B: of the two points on the graph which bracket 50% inhibition, the lower concentration of the test compound,
C: inhibitory activity (%) at the concentration B,
D: inhibitory activity (%) at the concentration A.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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.08.030.

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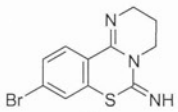
42. Because a 9-brominated derivative **25** exhibited comparable anti-HIV activity with compound **2** in our previous SAR study,[34] we employed compound **25** as a lead.




**25**

$EC_{50}$ = 0.25 ± 0.09 μM

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# Design and synthesis of biotin- or alkyne-conjugated photoaffinity probes for studying the target molecules of PD 404182

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ABSTRACT

To investigate the mechanism of action of the potent antiviral compound PD 404182, three novel photoaffinity probes equipped with a biotin or alkyne indicator were designed and synthesized based on previous structure–activity relationship studies. These probes retained the potent anti-HIV activity of the original pyrimidobenzothiazine derivatives. In photoaffinity labeling studies using HIV-1-infected H9 cells (H9IIIB), eight potential proteins were observed to bind PD 404182.

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## 1. Introduction

3,4-Dihydro-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine (PD 404182) (**1**)[1–3] is a potent antiviral agent against the human immunodeficiency virus (HIV) and the hepatitis C virus (HCV) (Fig. 1).[4,5] In structure–activity relationship (SAR) studies[5,6] of compound **1** using a series of facile synthetic procedures,[7,8] we identified several derivatives **2–4** that exhibited two- or three-fold more potent anti-HIV activity than compound **1**. The comparative time of drug addition study using standard anti-HIV agents demonstrated that compound **1** showed a similar antiviral profile against HIV-$1_{IIIB}$ infection with that of DS 5000 (adsorption inhibitor)[9] and enfuvirtide (fusion inhibitor),[10] indicating that compound **1** impaired virus replication at the early-stage of HIV infection.[5] Additionally, the antiviral activities of compound **1** against multiple HIV clades suggest that the target molecule of compound **1** is not chemokine receptors (CC chemokine receptor type 5[11] or CXC chemokine receptor type 4[12]).[5] Recently, the virucidal effects of compound **1** against HCV, HIV and the simian immunodeficiency virus have also been reported.[13] However, the mode of action and mechanism of antiviral activity of compound **1** has not yet been fully elucidated.

Photoaffinity labeling is an efficient approach to identify the target protein(s) of biologically active molecules.[14] In modern drug discovery, there have been a number of successful examples that have determined the target molecules and identified the binding site through the formation of a covalent bond between the ligand and the specific protein.[15] In general, photoaffinity probes contain three functional groups: a bioactive scaffold, a photoreactive group and an indicator group. A biotin-tag is widely employed as an indicator because biotinylated proteins can be detected and isolated by several immunological methods or through a biotin-avidin interaction.[16] A terminal alkyne is an alternative indicator for Huisgen cycloaddition-mediated conjugation with various azide-modified reporters, such as fluorescent-azide and biotin-azide after the crosslinking reaction onto the target protein(s).[17]

In this article, the design and synthesis of biotin- or alkyne-conjugated photoaffinity probes based on previous SAR studies, and its application for photoaffinity labeling studies are described.

## 2. Results and discussion

### 2.1. Design of biotin- or alkyne-conjugated photoaffinity probes from PD 404182

Trifunctional probes for the target protein(s) of compound **1** and the derivatives were designed on the basis of our previous SAR investigations.[5,6] In our previous study, the introduction of a hydrophobic group on the benzene ring and the cyclic amidine

*Abbreviations:* MAGI, multinuclear activation of a galactosidase indicator.

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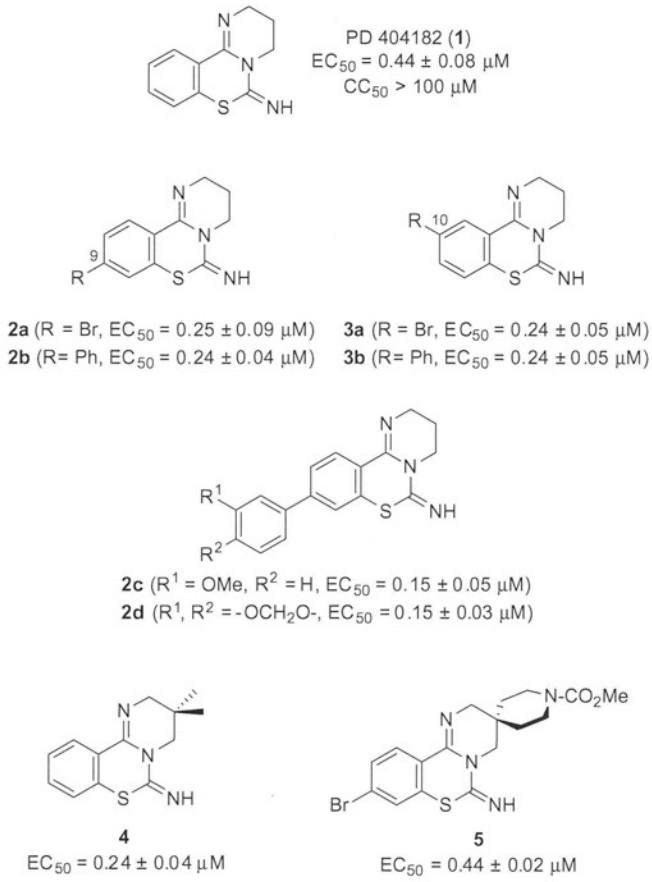



**Figure 1.** Structures and anti-HIV activity of PD 404182 and the derivatives **2–5**.

substructures effectively improved antiviral activity (compounds **2–4**, Fig. 1). We expected that these moieties would potentially take part in a favorable interaction(s) with the target molecule(s), and the incorporation of a hydrophobic and photoreactive benzophenone group on the pyrimidobenzothiazine scaffold would be tolerated. Additionally, the *N*-alkoxycarbonyl piperidine group onto the amidine substructure of **1** reproduced potent anti-HIV activity (compound **5**), indicating that this part could be used as a linkage position for the addition of functional groups.

With this in mind, we designed three photoaffinity probes. Compound **6** was modified with indicator biotin via a photoreactive benzophenone group onto the benzene ring substructure (Fig. 2). Compound **7** equips the biotin and benzophenone groups on the right-part amidine moiety. The biotin moiety is conjugated with benzophenone via a polyethylene glycol (PEG) linker as the spacer. Compound **8** is an alkyne-containing derivative.

### 2.2. Synthesis of biotin-conjugated probe 6

Synthesis of the probe **6** started with the preparation of benzophenone boronic acid pinacol ester **11** (Scheme 1). Condensation of *p*-(hydroxymethyl)benzoic acid **9** and *N,O*-dimethylhydroxylamine followed by TBDPS protection of a primary hydroxy group gave an amide **10**. Subsequent nucleophilic addition of an in situ-generated organolithium compound easily provided the desired boronate **11**.[18]

We next assembled the components to synthesize the biotin-conjugated probe **6** (Scheme 1). Alkylation of compound **2a** with *p*-methoxybenzyl (PMB) bromide followed by Suzuki–Miyaura cross coupling with compound **11** afforded a benzophenone-conjugated pyrimidobenzothiazine **13**. Desilylation of **13** and the subsequent reaction with *p*-nitrophenyl chloroformate afforded the carbonate **16**. The biotin moiety was incorporated by reaction of **16** with biotin-PEG-$NH_2$ (**15**), which was prepared by catalytic hydrogenation of azide **14**.[19] TFA-mediated deprotection of the PMB group in compound **17** provided the desired probe **6**.

### 2.3. Synthesis of biotin-conjugated probe 7

Synthesis of the biotin-conjugated probe **7** is outlined in Scheme 2. PMB protection of compound **18**[6] followed by selective removal of the PMB group on the piperidine ring provided compound **20**. Separately, the synthesis of biotin-benzophenone adduct **23** started from 4-(*tert*-butyldiphenylsilyloxy)methyl-4′-(hydroxymethyl)benzophenone **21**.[20] The treatment of **21** with

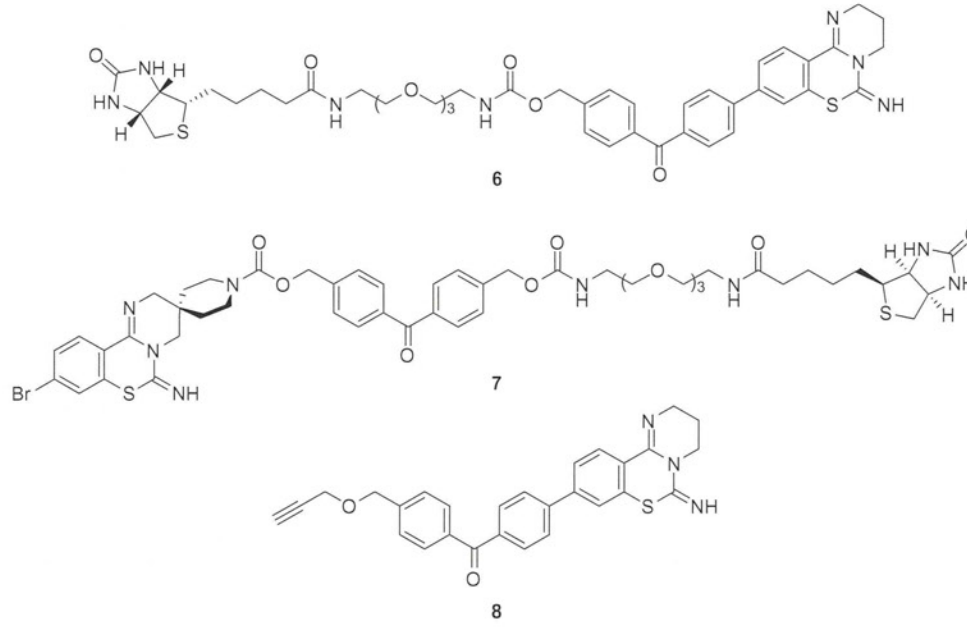
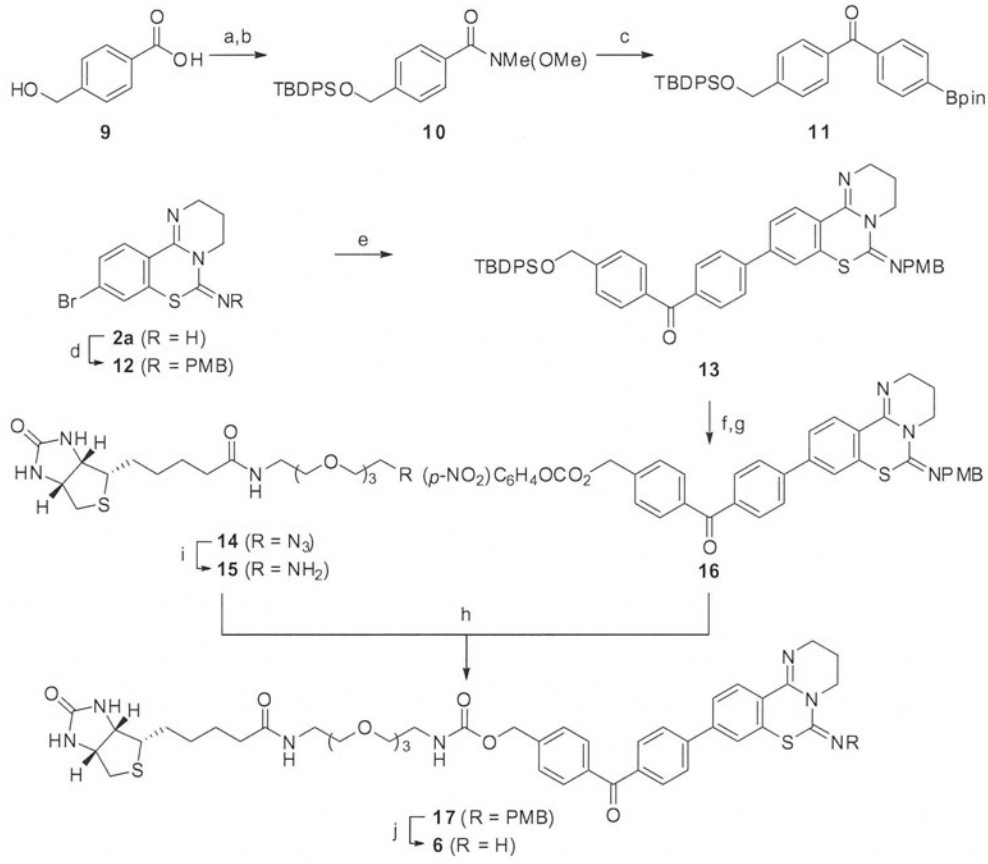



**Figure 2.** Structures of photoaffinity probes **6–8**.




**Scheme 1.** Synthesis of biotin-conjugated probe **6**. Reagents and conditions: (a) HNMe(OMe)·HCl, EDC·HCl, HOBt·$H_2O$, $Et_3N$, DMF, rt; (b) TBDPSCl, $Et_3N$, DMAP, $CH_2Cl_2$, rt, 49% [2 steps (a,b)]; (c) 2-(4-bromophenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane, *t*-BuLi, THF, pentane, −78 to rt, 83%; (d) *t*-BuOK, DMF, 0 °C, then PMBBr, rt, 98%; (e) **11**, $Pd(PPh_3)_4$, $PdCl_2(dppf)\cdot CH_2Cl_2$, $K_2CO_3$, toluene, EtOH, $H_2O$, reflux, 96%; (f) TBAF, THF, rt; (g) *p*-nitrophenyl chloroformate, pyridine, $CH_2Cl_2$, reflux; (h) $Et_3N$, DMF, rt to 40 °C, 46% [3 steps (f–h)]; (i) $H_2$, 10% Pd-C, MeOH, rt; (j) MS4Å, TFA, $CHCl_3$, rt, 35%.

chloroformate furnished a carbonate **22**. Biotin-PEG-$NH_2$ **15** was successfully conjugated onto **22** to give the biotin-benzophenone adduct **23**. Desilylation of **23**, treatment with *p*-nitrophenyl chloroformate and coupling with **20** provided biotin/benzophenone-conjugated **26**. PMB deprotection of **26** afforded the desired probe **7**.

### 2.4. Synthesis of alkyne-containing probe 8

We next investigated the synthesis of alkyne-containing probe **8** (Scheme 3). Suzuki–Miyaura cross coupling of compound **27**[5] with boronate **11** gave compound **28**. Subsequent modifications including desilylation, propargylation, and removal of the *tert*-butyl group provided the expected alkyne-conjugated probe **8**.

### 2.5. Anti-HIV activity of biotin- or alkyne-conjugated probes

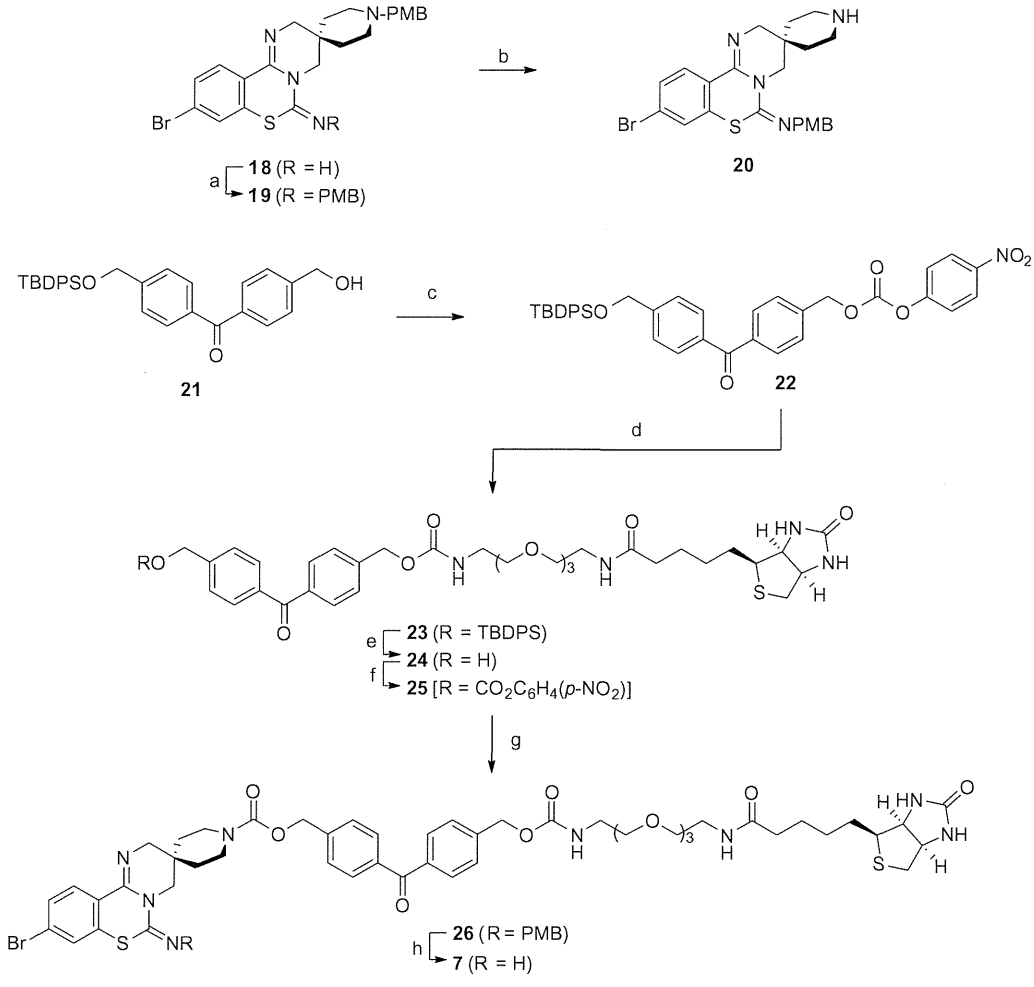
The antiviral activities of probes **6–8** against HIV-$1_{IIIB}$ were measured by multinuclear activation of a galactosidase indicator (MAGI) assay. In this assay, the inhibitory activity against HIV infection at the early stage, including virus attachment and membrane fusion to host cells, can be evaluated.[21] Both biotin-conjugated probes **6** and **7** showed potent anti-HIV activity with $EC_{50}$ values of 6.87 and 5.11 μM, respectively (Table 1). These activities were slightly lower than that of compound **1**; however, the incorporation of large functional groups including benzophenone, the PEG linker and the biotinyl reporter was largely tolerated. Alkyne-conjugated probe **8** potently inhibited HIV infection ($EC_{50}$ = 0.64 μM). These probes **6–8** represent promising tools for the identification of the target molecule(s) of compound **1** and the derivatives.

### 2.6. Photoaffinity labeling experiment using biotin-conjugated probes for HIV-1-infected H9 cells

Probes **6** and **7** were applied to the experiment for target identification of compound **1** and the derivatives. After HIV-1-infected H9 cells (H9IIIB) were incubated with a probe (**6** or **7**) for 1 h, the cells were exposed to UV–vis light (>300 nm) for 1 min. After cell lysis, the biotinylated proteins were captured with NeutrAvidin agarose beads. The whole was subjected to separation by SDS–PAGE followed by Western blot analysis.

Eight bands of 95, 80, 75, 70, 60, 55, 48 and 40 kDa proteins were observed from the cell samples incubated with probe **6** (Lane A, Fig. 3). These bands were competed by unlabeled compound **3a**, suggesting that the labeling was PD 404182-specific (Lane C). In contrast, these bands, with the exception of the 70 and 40 kDa bands, were not detected in the cells incubated with probe **7** (Lane B). This observation indicated that the potential target proteins did not fully interact with the benzophenone group on the right-part amidine moiety in the pyrimidobenzothiazine scaffold of **7**.

This preliminary experiment demonstrated that the synthesized probe **6** could be useful for the identification of the target protein(s) of compound **1**. Efforts of the crosslinking experiments using alkyne-conjugated probe **8** are also currently in progress.




**Scheme 2.** Synthesis of biotin-conjugated probe **7**. Reagents and conditions: (a) *t*-BuOK, DMF, 0 °C, then PMBBr, rt, 81%; (b) 1-chloroethyl chloroformate, $Et_3N$, $CH_2Cl_2$, 0 °C, then MeOH, reflux; (c) 4-nitrophenyl chloroformate, pyridine, $CH_2Cl_2$, reflux; (d) **15**, $Et_3N$, DMF, rt, quant. [2 steps (c,d)]; (e) HF-pyridine, THF, 0 °C to rt, 73%; (f) 4-nitrophenyl chloroformate, pyridine, $CH_2Cl_2$, reflux, 80%; (g) **20**, $Et_3N$, DMF, rt; (h) MS4Å, TFA, $CHCl_3$, rt, 36% [2 steps (g,h)].

## 3. Conclusions

In conclusion, we have designed and synthesized novel photoaffinity probes of antiviral PD 404182 with photoreactive benzophenone, and biotin or alkyne indicators. The probes exhibited equipotent or slightly less potent anti-HIV activities when compared with the activity of the parent compound **1**. Preliminary photoaffinity labeling experiments suggest that these probes could be useful in the identification of a potential target protein(s), the binding site on the target protein(s) and the mechanism(s) of action of PD 404182 derivatives.

## 4. Experimental

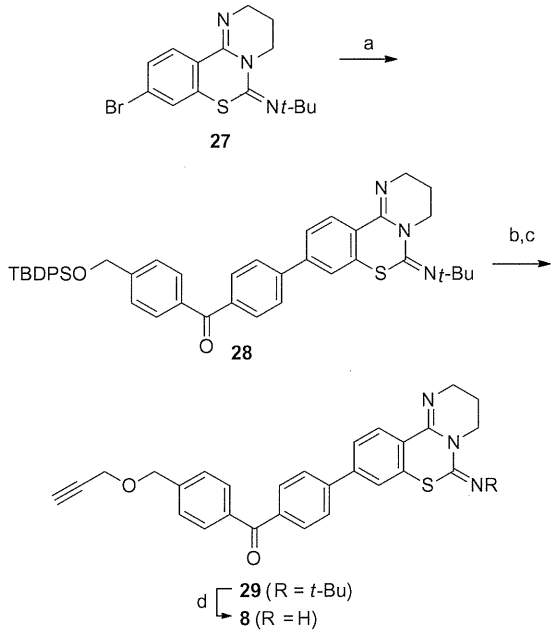
### 4.1. Synthesis

#### 4.1.1. General methods

$^1$H NMR spectra were recorded using a JEOL AL-400 or a JEOL ECA-500 spectrometer. Chemical shifts are reported in $\delta$ (ppm) relative to $Me_4Si$ ($CDCl_3$) or DMSO (DMSO-$d_6$) as internal standards. $^{13}$C NMR spectra were referenced to the residual solvent signal. Exact mass (HRMS) spectra were recorded on a JMS-HX/HX 110A mass spectrometer. Melting points were measured by a hot stage melting point apparatus (uncorrected). For flash chromatography, Wakogel C-300E (Wako) or aluminum oxide 90 standardized (Merck) were employed. For preparative TLC, TLC silica gel 60 $F_{254}$ (Merck) or TLC aluminum oxide 60 $F_{254}$ basic (Merck) were employed. For analytical HPLC, a Cosmosil 5C18-ARII column (4.6 × 250 mm, Nacalai Tesque, Inc., Kyoto, Japan) was employed with a linear gradient of $CH_3CN$ containing 0.1% (v/v) $NH_3$ at a flow rate of 1 mL/min on a Shimadzu LC-10ADvp (Shimadzu Corp., Ltd, Kyoto, Japan), and eluting products were detected by UV at 254 nm. Preparative HPLC was performed using a COSMOSIL 5C18-ARII column (20 × 250 mm, Nacalai Tesque Inc.) with a linear gradient of MeCN containing 0.1% (v/v) $NH_3$ at a flow rate of 8 mL/min on Shimadzu LC-6AD (Shimadzu corporation, Ltd). The purity of the compounds **6–8** was determined by HPLC analysis as >95%.

#### 4.1.2. 4-[(*tert*-Butyldiphenylsilyloxy)methyl]-*N*-methoxy-*N*-methylbenzamide (10)

To a mixture of 4-(hydroxymethyl)benzoic acid **9** (4.6 g, 30.0 mmol), *N,O*-dimethylhydroxylamine hydrochloride (14.6 g, 150.0 mmol), $Et_3N$ (21.7 mL, 150.0 mmol) in DMF (300 mL) were added EDC·HCl (11.5 g, 60.0 mmol) and HOBt·$H_2O$ (9.2 g, 60.0 mmol). After being stirred at rt overnight, solvent was evaporated. The residue was dissolved in EtOAc, and washed with 1 N HCl, satd $NaHCO_3$, brine, and dried over $MgSO_4$. The filtrate was




**Scheme 3.** Synthesis of alkyne-conjugated probe **8**. Reagents and conditions: (a) **11**, $Pd(PPh_3)_4$, $PdCl_2(dppf)\cdot CH_2Cl_2$, $K_2CO_3$, toluene, EtOH, $H_2O$, reflux, 71%; (b) TBAF, THF, rt; (c) NaH, THF, propargyl bromide, 0 °C to rt, 60% [2 steps (b,c)]; (d) MS4Å, TFA, $CHCl_3$, reflux, 92%.

**Table 1**
Anti-HIV activities of the probes **6–8**

| Compound | $EC_{50}$[a] (μM) |
|---|---|
| PD 404182[5] | 0.44 ± 0.08 |
| **6** | 6.87 ± 2.22 |
| **7** | 5.11 ± 1.31 |
| **8** | 0.64 ± 0.06 |

[a] $EC_{50}$ values represent the concentration of compound required to inhibit the HIV-1 infection by 50%, and were obtained from three independent experiments.

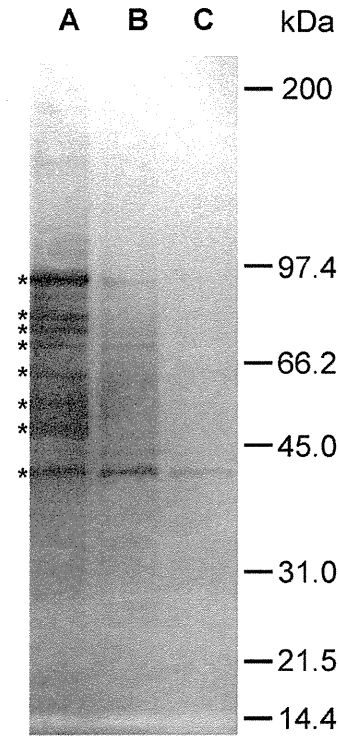



**Figure 3.** Western blot analysis of the photolabeled proteins with biotin-conjugated probes **6** and **7**. H9IIIB cells were incubated with (A) 20 μM probe **6**, (B) 20 μM probe **7**, and (C) 20 μM probe **6** and 40 μM compound **3a**. The cells were exposed to UV light for 1 min and were lysed. The resulting photolabeled proteins were captured onto NeutrAvidin-agarose and the whole was subjected to SDS–PAGE. The resulting gel was analyzed by Western blotting with streptavidin–HRP.

concentrated to give crude Weinreb amide (4.05 g, ca. 20.7 mmol). To the mixture of the Weinreb amide, a solution of $Et_3N$ (8.98 mL, 62.1 mmol) and DMAP (252.9 mg, 2.1 mmol) in $CH_2Cl_2$ (138 mL) was slowly added TBDPSCl (5.83 mL, 22.8 mmol). After being stirred at rt for 3 h, the reaction mixture was quenched with water. After concentration, the residue was dissolved in EtOAc. The mixture was washed with satd $NaHCO_3$, brine, and dried over $MgSO_4$. After concentration, the residue was purified by flash column chromatography over silica gel with *n*-hexane/EtOAc (3:1) to give the title compound **10** as colorless oil (6.98 g, 49%): IR (neat) $cm^{-1}$: 1644 (C=O); $^1H$ NMR (400 MHz, $CDCl_3$) δ: 1.10 (s, 9H, 3 × $CH_3$), 3.36 (s, 3H, $CH_3$), 3.57 (s, 3H, $CH_3$), 4.80 (s, 2H, $CH_2$), 7.36–7.43 (m, 8H, Ar), 7.65–7.70 (m, 6H, Ar); $^{13}C$ NMR (100 MHz, $CDCl_3$) δ: 19.3, 26.8 (3C), 33.8, 61.0, 65.2, 125.4 (2C), 127.7 (4C), 128.2 (2C), 129.8 (2C), 132.6, 133.3 (2C), 135.5 (4C), 143.8, 169.9; HRMS (FAB): *m/z* calcd for $C_{26}H_{32}NO_3Si$ $[M+H]^+$ 434.2152; found: 434.2160.

#### 4.1.3. 4-[(*tert*-Butyldiphenylsilyloxy)methyl]-4′-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzophenone (11)

To a solution of 1,4-dibromobenzene (3.13 g, 13.3 mmol) and 2-isopropoxy-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (2.80 mL, 13.8 mmol) in anhydrous THF (60 mL) was added *t*-BuLi (19.4 mL, 1.55 M in pentane, 30.0 mmol) dropwise over 3 min at −78 °C under an Ar atmosphere. After being stirred at −78 °C for 30 min, additional *t*-BuLi (19.4 mL, 1.55 M in pentane, 30.0 mmol) was added dropwise over 3 min. After being stirred at the same temperature for additional 20 min, compound **10** (3.25 g, 7.5 mmol) was added. The reaction mixture was warmed to rt over 1 h and quenched with satd $NH_4Cl$. The whole was extracted with EtOAc and the extract was dried over $MgSO_4$. After concentration, the residue was purified by silica gel chromatography with *n*-hexane/EtOAc (9:1) to give the title compound **11** as yellow oil (3.60 g, 83%): IR (neat) $cm^{-1}$: 1659 (C=O); $^1H$ NMR (400 MHz, $CDCl_3$) δ: 1.11 (s, 9H, 3 × $CH_3$), 1.37 (s, 12H, 4 × $CH_3$), 4.85 (s, 2H, $CH_2$), 7.37–7.46 (m, 8H, Ar), 7.69 (d, *J* = 6.6 Hz, 4H, Ar), 7.75–7.80 (m, 4H, Ar), 7.92 (d, *J* = 8.0 Hz, 2H, Ar); $^{13}C$ NMR (100 MHz, $CDCl_3$) δ: 19.3, 24.8 (4C), 26.8 (3C), 65.2, 84.2 (2C), 125.6 (2C), 127.8 (4C), 128.9 (2C), 129.8 (2C), 130.2 (2C), 133.2 (2C), 134.5 (2C), 134.8, 135.5 (4C), 136.2, 140.0, 146.0, 196.6; HRMS (FAB): *m/z* calcd for $C_{36}H_{42}BO_4Si$ $[M+H]^+$ 577.2945; found: 577.2949.

#### 4.1.4. 9-Bromo-3,4-dihydro-*N*-(*p*-methoxybenzyl)-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine (12)

To the flask containing **2a** (740.4 mg, 2.50 mmol) and *t*-BuOK (561.1 mg, 5.00 mmol) was added DMF (10.0 mL) at 0 °C under an Ar atmosphere. After being stirred at the same temperature for 30 min, PMB-Br (729.0 μL, 5.00 mmol) was added. After being stirred at rt for 1 h, the reaction mixture was quenched with $H_2O$. The whole was extracted with EtOAc, and washed with satd $NaHCO_3$, brine, and dried over $MgSO_4$. After concentration, the residue was purified by flash column chromatography over aluminum oxide with *n*-hexane/EtOAc (3:1) to give the title compound **12** as pale yellow amorphous (1.02 g, 98%): IR (neat) $cm^{-1}$: 1661 (C=N), 1510 (C=N); $^1H$ NMR (400 MHz, $CDCl_3$) δ: 1.97-2.03 (m, 2H), 3.64 (t, *J* = 5.7 Hz, 2H, $CH_2$), 3.80-3.84 (m, 5H, $OCH_3$, $CH_2$), 4.14 (s, 2H, $CH_2$), 6.86 (d, *J* = 8.5 Hz, 2H, Ar), 7.21-7.27 (m, 3H, Ar), 7.38 (dd,

$J$ = 8.2, 1.8 Hz, 1H, Ar), 7.43 (d, $J$ = 1.8 Hz, 1H, Ar); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$: 19.8, 38.7, 44.3, 47.7, 55.3, 111.9, 114.1 (2C), 124.8, 127.9, 129.5, 130.2, 130.3 (2C), 132.6, 133.4, 138.7, 147.6, 159.1; HRMS (FAB): $m/z$ calcd for $C_{19}H_{19}N_3OS$ $[M+H]^+$ 416.0432; found: 416.0431.

#### 4.1.5. 9-{4-[4-(*tert*-Butyldiphenylsilyloxy)methyl]-benzoylphenyl}-3,4-dihydro-*N*-(*p*-methoxybenzyl)-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine (13)

$Pd(PPh_3)_4$ (32.8 mg, 4 mol %) and $PdCl_2(dppf)\cdot CH_2Cl_2$ (17.4 mg, 3 mol %) were added to a solution of **12** (296.2 mg, 0.71 mmol) and **11** (409.4 mg, 0.71 mmol) in toluene (7.1 mL)-EtOH (4.3 mL)-1 M aq $K_2CO_3$ (7.1 mL). After being stirred at reflux for 1 h, the mixture was extracted with $CHCl_3$. The extract was dried over $MgSO_4$ and concentrated. The residue was purified by flash chromatography over aluminum oxide with *n*-hexane/EtOAc (1:0 to 9:1) to give the title compound **13** as pale yellow amorphous (536.2 mg, 96%): IR (neat) cm$^{-1}$: 1658 (C=O), 1607 (C=N), 1511 (C=N); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$: 1.12 (s, 9H, 3 × $CH_3$), 2.03–2.08 (m, 2H), 3.70 (t, $J$ = 5.5 Hz, 2H, $CH_2$), 3.77 (s, 3H, $CH_3$), 3.88 (t, $J$ = 5.9 Hz, 2H, $CH_2$), 4.19 (s, 2H, $CH_2$), 4.86 (s, 2H, $CH_2$), 6.84 (d, $J$ = 8.5 Hz, 2H, Ar), 7.28 (m, 1H, Ar), 7.38–7.56 (m, 14H, Ar), 7.71 (dd, $J$ = 7.6, 1.2 Hz, 4H, Ar), 7.81 (d, $J$ = 8.0 Hz, 2H, Ar), 7.86 (d, $J$ = 8.0 Hz, 2H, Ar); $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$: 19.3, 19.8, 26.8 (3C), 39.0, 44.3, 47.7, 55.2, 65.1, 112.2, 113.9 (2C), 125.6 (2C), 125.7, 127.0 (2C), 127.7 (4C), 128.7, 129.5, 129.8 (2C), 130.1 (2C), 130.2, 130.3 (2C), 130.5 (2C), 133.1 (2C), 135.0, 135.5 (4C), 136.2, 136.4, 137.0, 142.1, 143.5, 146.0, 148.2, 158.9, 195.8; HRMS (FAB): $m/z$ calcd for $C_{49}H_{48}N_3O_3SSi$ $[M+H]^+$ 786.3186; found: 786.3178.

#### 4.1.6. *N*-(2-[2-{2-(2-Aminoethoxy)ethoxy}ethoxy]ethyl)-5-[(3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl]pentanamide (15)

To the solution of **14** (116.0 mg, 0.26 mmol) in MeOH (2.0 mL) was added 10% Pd-C (wetted with ca. 55% water, 160.0 mg). After being stirred at rt overnight under $H_2$ atmosphere, the mixture was filtered through a celite pad and concentrated. The crude product was used for the next step without further purification.

#### 4.1.7. 4-(4-{6-[(4-Methoxybenzyl)imino]-2,3,4,6-tetrahydrobenzo[*e*]pyrimido[1,2-*c*][1,3]thiazin-9-yl}benzoyl)benzyl {13-oxo-17-[(3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl]-3,6,9-trioxa-12-azaheptadecyl}carbamate (17)

To a solution of **13** (157.2 mg, 0.20 mmol) in THF (2.0 mL) was added TBAF in THF (1 M, 0.50 mL, 0.50 mmol). After being stirred at rt overnight, the reaction mixture was quenched with satd $NH_4Cl$. The whole was extracted with $CHCl_3$ and dried over $MgSO_4$. After concentration, the residue was subjected to flash column chromatography over aluminum oxide with *n*-hexane/EtOAc (5:1–0:1) to give the desilylated compound. To a solution of the resulting compound in $CH_2Cl_2$ (6.0 mL) were added *p*-nitrophenyl chloroformate (60.5 mg, 0.30 mmol) and pyridine (64.6 μL, 0.8 mmol). After being stirred under reflux for 1 h, additional *p*-nitrophenyl chloroformate (12.0 mg, 0.06 mmol) was added. After being stirred under reflux for additional 30 min, the reaction mixture was washed with brine, and dried over $MgSO_4$. After concentration, the solution of resulting residue (crude **16**) in DMF (2.0 mL) was added to the solution of **15** (ca. 0.26 mmol) and $Et_3N$ (86.7 μL) in DMF (3.0 mL). After being stirred at rt for 8 h, the reaction mixture was stirred at 40 °C overnight. After concentration, the residue was purified by flash column chromatography over aluminum oxide with $CHCl_3$/MeOH (1:0–95:5) followed by flash column chromatography over silica gel with $CHCl_3$/MeOH (1:0–9:1) to give the title compound **17** as pale yellow amorphous (90.6 mg, 46%): IR (neat) cm$^{-1}$: 1699 (C=O), 1656 (C=O), 1607 (C=N), 1511 (C=N); $^1$H NMR (500 MHz, $CDCl_3$) $\delta$: 1.39–1.45 (m, 2H, $CH_2$), 1.57–1.74 (m, 4H, 2 × $CH_2$), 2.03–2.08 (m, 2H, $CH_2$), 2.20 (t, $J$ = 6.9 Hz, 2H, $CH_2$), 2.70 (d, $J$ = 12.6 Hz, 1H, CH), 2.87 (dd, $J$ = 12.6, 4.6 Hz, 1H, CH), 3.12 (d, $J$ = 11.7, 4.6 Hz, 1H, CH), 3.40–3.43 (m, 4H, 2 × $CH_2$), 3.54–3.71 (m, 14H, 7 × $CH_2$), 3.77 (s, 3H, $CH_3$), 3.88 (t, $J$ = 6.0 Hz, 2H, $CH_2$), 4.19 (s, 2H, $CH_2$), 4.26–4.29 (m, 1H, CH), 4.45–4.47 (m, 1H, CH), 5.17 (s, 1H, NH), 5.20 (s, 2H, $CH_2$), 5.65 (s, 1H, NH), 6.07 (s, 1H, NH), 6.48 (s, 1H, NH), 6.84 (d, $J$ = 8.0 Hz, 2H, Ar), 7.26–7.28 (m, 2H, Ar), 7.44–7.62 (m, 7H, Ar), 7.81 (d, $J$ = 8.0 Hz, 2H, Ar), 7.85 (d, $J$ = 8.0 Hz, 2H, Ar); $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$: 19.8, 25.5, 28.0, 28.1, 35.9, 39.0, 39.1, 40.4, 40.9, 44.3, 47.7, 55.2, 55.5, 60.1, 61.7, 65.8, 69.9, 69.9, 70.0, 70.2, 70.3 (2C), 112.2, 114.0 (2C), 125.7, 127.1 (2C), 127.4 (2C), 127.6, 128.6, 129.5, 130.2 (2C), 130.3 (2C), 130.6 (2C), 135.0, 136.5, 136.7, 137.1, 141.4, 142.0, 143.7, 148.2, 156.3, 158.9, 163.9, 173.2, 195.7; HRMS (FAB): $m/z$ calcd for $C_{52}H_{62}N_7O_9S_2$ $[M+H]^+$ 992.4050; found: 992.4050.

#### 4.1.8. 4-[4-(6-Imino-2,3,4,6-tetrahydrobenzo[*e*]pyrimido[1,2-*c*][1,3]thiazin-9-yl)benzoyl]benzyl {13-oxo-17-[(3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl]-3,6,9-trioxa-12-azaheptadecyl}carbamate (6)

TFA (2.0 mL) was added to a mixture of **17** (62.9 mg, 0.063 mmol) in small amount of $CHCl_3$ (1 or 2 drops) and molecular sieves 4 Å (300 mg, powder, activated by heating). After being stirred at rt for 4 h, $Et_3N$ was added dropwise to the stirring mixture at 0 °C to adjust pH to 8–9. The whole was extracted with $CHCl_3$, and washed with satd $NaHCO_3$, brine, and dried over $MgSO_4$. After concentration, the residue was purified by flash chromatography over aluminum oxide with $CHCl_3$/MeOH (1:0–95:5) followed by preparative HPLC to give the title compound **6** as colorless solid (19.3 mg, 35%): IR (neat) cm$^{-1}$: 1699 (C=O), 1654 (C=O), 1621 (C=O), 1601 (C=N), 1574 (C=N); $^1$H NMR (500 MHz, $CDCl_3$) $\delta$: 1.39–1.44 (m, 2H, $CH_2$), 1.60–1.76 (m, 4H, 2 × $CH_2$), 1.99–2.04 (m, 2H, $CH_2$), 2.20 (t, $J$ = 7.4 Hz, 2H, $CH_2$), 2.71 (d, $J$ = 12.6 Hz, 1H, CH), 2.88 (dd, $J$ = 12.6, 5.0 Hz, 1H, CH), 3.11 (d, $J$ = 11.7, 5.0 Hz, 1H, CH), 3.40–3.43 (m, 4H, 2 × $CH_2$), 3.54–3.63 (m, 12H, 6 × $CH_2$), 3.73 (t, $J$ = 5.4 Hz, 2H, $CH_2$), 4.06 (t, $J$ = 6.0 Hz, 2H, $CH_2$), 4.28 (t, $J$ = 6.0 Hz, 1H, CH), 4.47 (t, $J$ = 6.0 Hz, 1H, CH), 5.20 (s, 2H, $CH_2$), 5.44 (s, 1H, NH), 5.73 (s, 1H, NH), 6.37 (s, 1H, NH), 6.66 (s, 1H, NH), 7.32 (s, 1H, Ar), 7.48 (d, $J$ = 8.0 Hz, 2H, Ar), 7.52 (d, $J$ = 8.6 Hz, 1H, Ar), 7.69 (d, $J$ = 8.0 Hz, 2H, Ar), 7.81 (d, $J$ = 8.0 Hz, 2H, Ar), 7.88 (d, $J$ = 8.0 Hz, 2H, Ar), 8.36 (d, $J$ = 8.6 Hz, 1H, Ar); $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$: 20.8, 25.6, 28.0, 28.2, 35.9, 39.0, 40.4, 40.9, 43.9, 44.7, 51.2, 55.6, 60.1, 61.7, 65.7, 69.9, 70.0, 70.1, 70.3 (2C), 122.0, 125.2, 125.8, 126.9 (2C), 127.4 (2C), 129.6, 129.7, 130.2 (2C), 130.7 (2C), 137.0, 141.5, 142.2, 142.9, 144.8, 146.6, 152.9, 156.3, 164.1, 173.3, 195.6; HRMS (FAB): $m/z$ calcd for $C_{44}H_{54}N_7O_8S_2$ $[M+H]^+$ 872.3475; found: 872.3481.

#### 4.1.9. *N*-[9-Bromo-1′-(4-methoxybenzyl)-2*H*-spiro(benzo[*e*]-pyrimido[1,2-*c*][1,3]thiazine-3,4′-piperidin)-6(4*H*)-ylidene]-1-(4-methoxyphenyl)methanamine (19)

By a procedure identical with that described for synthesis of **12** from **2a**, the imine **18** (274.3 mg, 0.57 mmol) was converted into **19** as colorless amorphous (275.1 mg, 81%): IR (neat) cm$^{-1}$: 1668 (C=N), 1510 (C=N); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$: 1.61–1.64 (m, 4H, 2 × $CH_2$), 2.36–2.42 (m, 2H, $CH_2$), 2.45–2.51 (m, 2H, $CH_2$), 3.45 (s, 2H, $CH_2$), 3.47 (s, 2H, $CH_2$), 3.55 (s, 2H, $CH_2$), 3.80 (s, 3H, $CH_3$), 3.81 (s, 3H, $CH_3$), 4.12 (s, 2H, $CH_2$), 6.82-6.87 (m, 4H, Ar), 7.19–7.23 (m, 5H, Ar), 7.38 (dd, $J$ = 8.2, 1.8 Hz, 1H, Ar), 7.44 (d, $J$ = 2.0 Hz, 1H, Ar); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$: 28.2, 32.4 (2C), 39.1, 48.7 (2C), 54.6, 55.2, 55.3, 55.4, 62.6, 111.9, 113.7 (2C), 113.9, 114.1 (2C), 124.8, 128.0, 129.7, 130.0, 130.2 (4C), 133.4, 133.4, 138.6, 147.1, 158.8, 159.1; HRMS (FAB): $m/z$ calcd for $C_{31}H_{34}BrN_4O_2S$ $[M+H]^+$ 605.1586; found: 605.1585.

**4.1.10. *N*-[9-Bromo-2*H*-spiro(benzo[*e*]pyrimido[1,2-*c*][1,3]-thiazine-3,4'-piperidin)-6(4*H*)-ylidene]-1-(4-methoxyphenyl)-methanamine (20)**

To a solution of **19** (60.6 mg, 0.10 mmol) in $CH_2Cl_2$ (0.5 mL) were added $Et_3N$ (28.9 μL, 0.20 mmol) and 1-chloroethyl chloroformate (21.8 μL, 0.20 mmol) at 0 °C under an Ar atmosphere. After being stirred at the same temperature for 30 min, the reaction mixture was concentrated. The residue was dissolved in MeOH (2.0 mL). After being stirred under reflux for 10 min, the reaction mixture was concentrated. The residue was dissolved in $CHCl_3$, and was washed with satd $NaHCO_3$, brine, and dried over $MgSO_4$. After concentration, the crude product was used for the next step without further purification.

**4.1.11. 4-[4-(*tert*-Butyldiphenylsilyloxymethyl)benzoyl]benzyl {13-oxo-17-[(3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl]-3,6,9-trioxa-12-azaheptadecyl}carbamate (23)**

To a solution of **21**[20] (240.3 mg, 0.50 mmol) in $CH_2Cl_2$ (15.0 mL) were added *p*-nitrophenyl chloroformate (151.2 mg, 0.75 mmol) and pyridine (161.4 μL, 2.00 mmol). After being stirred under reflux for 1 h, the reaction mixture was washed with brine, and dried over $MgSO_4$. After concentration, the solution of the resulting residue in DMF (7.5 mL) was added to a mixture of **15** (ca. 0.20 mmol) and $Et_3N$ (216.8 μL) in DMF (5.0 mL). After being stirred at rt overnight, the mixture was concentrated. The residue was purified by flash column chromatography over silica gel with $CHCl_3$/MeOH (1:0–95:5) to give the title compound **23** as colorless amorphous (471.5 mg, quant.): IR (neat) $cm^{-1}$: 1700 (C=O), 1656 (C=O), 1609 (C=O); $^1H$ NMR (400 MHz, $CDCl_3$) δ: 1.12 (s, 9H, 3 × $CH_3$), 1.39–1.46 (m, 2H, $CH_2$), 1.61–1.76 (m, 4H, 2 × $CH_2$), 2.19–2.23 (m, 2H, $CH_2$), 2.69–2.76 (m, 1H, CH), 2.85–2.90 (m, 1H, CH), 3.09–3.15 (m, 1H, CH), 3.39–3.43 (m, 4H, 2 × $CH_2$), 3.54–3.66 (m, 12H, 6 × $CH_2$), 4.26–4.33 (m, 1H, CH), 4.45–4.51 (m, 1H, CH), 4.85 (s, 2H, $CH_2$), 5.19 (s, 2H, $CH_2$), 5.54 (br s, 1H, NH), 5.68 (br s, 1H, NH), 6.55 (br s, 1H, NH), 6.72 (br s, 1H, NH), 7.36–7.48 (m, 10H, Ar), 7.69 (d, *J* = 7.6 Hz, 2H, Ar), 7.70 (d, *J* = 7.6 Hz, 2H, Ar), 7.77 (d, *J* = 5.5 Hz, 2H, Ar), 7.79 (d, *J* = 5.5 Hz, 2H, Ar); $^{13}C$ NMR (100 MHz, $CDCl_3$) δ: 19.3, 25.5, 26.8 (3C), 28.1, 28.2, 35.9, 39.1, 40.5, 40.9, 55.5, 60.1, 61.7, 65.1, 65.8, 69.9, 70.0, 70.0, 70.2, 70.4 (2C), 125.6 (2C), 127.4 (2C), 127.8 (4C), 129.8 (2C), 130.1 (2C), 130.2 (2C), 133.2 (2C), 135.5 (4C), 136.1, 137.4, 141.2, 146.0, 156.3 163.9, 173.2, 196.0; HRMS (FAB): *m/z* calcd for $C_{50}H_{65}N_4O_9SSi$ $[M+H]^+$ 925.4242; found: 925.4246.

**4.1.12. 4-[4-(Hydroxymethyl)benzoyl]benzyl {13-oxo-17-[(3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl]-3,6,9-trioxa-12-azaheptadecyl}carbamate (24)**

To a solution of **23** (383.0 mg, 0.41 mmol) in THF (8.2 mL) was added HF-pyridine (617.7 μL) at 0 °C. After being stirred at rt overnight, the reaction was quenched with satd $NaHCO_3$. The whole was extracted with $CHCl_3$, and washed with water and brine, and dried over $MgSO_4$. After concentration, the residue was purified by preparative TLC over silica gel with $CHCl_3$/MeOH (85:15) to give the title compound **24** as colorless oil (204.2 mg, 73%): IR (neat) $cm^{-1}$: 1696 (C=O), 1650 (C=O), 1609 (C=O); $^1H$ NMR (400 MHz, $CDCl_3$) δ: 1.34–1.41 (m, 2H, $CH_2$), 1.55–1.73 (m, 4H, 2 × $CH_2$), 2.07 (br s, 1H, OH), 2.16 (t, *J* = 7.4 Hz, 2H, $CH_2$), 2.68 (d, *J* = 12.9 Hz, 1H, CH), 2.85 (dd, *J* = 12.9, 4.9 Hz, 1H, CH), 3.08 (dd, *J* = 11.8, 7.4 Hz 1H, CH), 3.37–3.42 (m, 4H, 2 × $CH_2$), 3.51–3.64 (m, 12H, 6 × $CH_2$), 4.23 (t, *J* = 6.2 Hz, 1H, CH), 4.43 (t, *J* = 6.2 Hz, 1H, CH), 4.78 (s, 2H, $CH_2$), 5.18 (s, 2H, $CH_2$), 5.51 (br s, 1H, NH), 5.82 (br s, 1H, NH), 6.34 (br s, 1H, NH), 6.75 (br s, 1H, NH), 7.45 (d, *J* = 8.3 Hz, 2H, Ar), 7.48 (d, *J* = 8.3 Hz, 2H, Ar), 7.76 (d, *J* = 8.0 Hz, 2H, Ar), 7.77 (d, *J* = 8.0 Hz, 2H, Ar); $^{13}C$ NMR (125 MHz, $CDCl_3$) δ: 25.5, 28.0, 28.2, 35.8, 39.1, 40.4, 40.9, 55.6, 60.2, 61.8, 64.2, 65.7, 69.9, 69.9 (2C), 70.1, 70.3 (2C), 126.4 (2C), 127.3 (2C), 130.2 (2C), 130.2 (2C), 136.2, 137.1, 141.3, 146.4, 156.4, 164.1, 173.5, 196.0; HRMS (FAB): *m/z* calcd for $C_{34}H_{47}N_4O_9S$ $[M+H]^+$ 687.3064; found: 687.3058.

**4.1.13. 4-(4-{[(4-Nitrophenoxy)carbonyloxy]methyl}benzoyl)-benzyl 13-oxo-17-[(3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl]-3,6,9-trioxa-12-azaheptadecylcarbamate (25)**

To a solution of **24** (28.2 mg, 0.04 mmol) in $CH_2Cl_2$ (1.2 mL) were added *p*-nitrophenyl chloroformate (24.8 mg, 0.12 mmol) and pyridine (13.2 μL, 0.16 mmol). After being stirred under reflux for 1 h, the reaction mixture was washed with brine, and dried over $MgSO_4$. After concentration, the residue was purified by preparative TLC over aluminum oxide with $CHCl_3$/MeOH (9:1) to give the title compound **25** as colorless amorphous (27.9 mg, 80%): IR (neat) $cm^{-1}$: 1768 (C=O), 1698 (C=O), 1656 (C=O), 1612 (C=O); $^1H$ NMR (400 MHz, $CDCl_3$) δ: 1.38–1.45 (m, 2H, $CH_2$), 1.59–1.76 (m, 4H, 2 × $CH_2$), 2.20 (t, *J* = 7.4 Hz, 2H, $CH_2$), 2.72 (d, *J* = 12.7 Hz, 1H, CH), 2.88 (dd, *J* = 12.7, 4.9 Hz, 1H, CH), 3.12 (dd, *J* = 11.8, 7.4 Hz, 1H, CH), 3.38–3.44 (m, 4H, 2 × $CH_2$), 3.55–3.63 (m, 12H, 6 × $CH_2$), 4.28 (t, *J* = 6.0 Hz, 1H, CH), 4.47 (t, *J* = 6.0 Hz, 1H, CH), 5.19 (s, 2H, $CH_2$), 5.38 (s, 2H, $CH_2$), 5.52 (br s, 1H, NH), 5.69 (br s, 1H, NH), 6.44 (br s, 1H, NH), 6.66 (br s, 1H, NH), 7.41 (d, *J* = 9.3 Hz, 2H, Ar), 7.47 (d, *J* = 8.0 Hz, 2H, Ar), 7.56 (d, *J* = 8.0 Hz, 2H, Ar), 7.79 (d, *J* = 8.0 Hz, 2H, Ar), 7.84 (d, *J* = 8.0 Hz, 2H, Ar), 8.29 (d, *J* = 9.3 Hz, 2H, Ar); $^{13}C$ NMR ($CDCl_3$, 100 MHz) δ: 25.5, 28.1, 28.2, 35.9, 39.1, 40.5, 40.9, 55.5, 60.2, 61.8, 65.8, 69.9, 70.0, 70.0 (2C), 70.2, 70.4 (2C), 121.7 (2C), 125.3 (2C), 127.5 (2C), 128.1 (2C), 130.2 (2C), 130.4 (2C), 136.8, 137.9, 138.6, 141.7, 145.5, 152.4, 155.4, 156.3, 163.9, 173.3, 195.5; HRMS (FAB): *m/z* calcd for $C_{41}H_{50}N_5O_{13}S$ $[M+H]^+$ 852.3126; found: 852.3127.

**4.1.14. 4-(4-{3,17-Dioxo-21-[(3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl]-2,7,10,13-tetraoxa-4,16-diazahenicosyl}benzoyl)benzyl 9-bromo-6-imino-4,6-dihydro-2*H*-spiro(benzo[*e*]pyrimido[1,2-*c*][1,3]thiazine-3,4'-piperidine)-1'-carboxylate (7)**

To a solution of **20** (ca. 0.027 mmol) in DMF (0.4 mL) were added $Et_3N$ (11.7 μL, 0.081 mmol) and the solution of **25** (23.3 mg, 0.027 mmol) in DMF (0.4 mL) at rt. After being stirred at the same temperature for 1 h, the reaction mixture was concentrated. The residue was subjected to preparative TLC over silica gel with $CHCl_3$/MeOH (9:1) to give crude imine **26**. By a procedure identical with that described for synthesis of **6** from **17**, the crude **26** was converted into **7** as a colorless amorphous (10.4 mg, 36%): IR (neat) $cm^{-1}$: 1699 (C=O), 1655 (C=O), 1612 (C=O), 1573 (C=N); $^1H$ NMR (400 MHz, $CDCl_3$) δ: 1.39–1.46 (m, 2H, $CH_2$), 1.53 (d, *J* = 5.6 Hz, 4H, 2 × $CH_2$), 1.61–1.72 (m, 4H, 2 × $CH_2$), 2.20 (t, *J* = 7.3 Hz, 2H, $CH_2$), 2.71 (d, *J* = 12.7 Hz, 1H, CH), 2.89 (dd, *J* = 12.7, 4.9 Hz, 1H, CH), 3.12 (d, *J* = 12.1, 7.3 Hz, 1H, CH), 3.39–3.44 (m, 4H, 2 × $CH_2$), 3.53–3.63 (m, 18H, 9 × $CH_2$), 3.93 (s, 2H, $CH_2$), 4.28 (t, *J* = 5.7 Hz, 1H, CH), 4.47 (t, *J* = 6.5 Hz, 1H, CH), 5.14 (s, 1H, NH), 5.19 (s, 2H, $CH_2$), 5.22 (s, 2H, $CH_2$), 5.68 (s, 1H, NH), 6.01 (s, 1H, NH), 6.52 (s, 1H, NH), 7.22 (d, *J* = 2.0 Hz, 1H, Ar), 7.34 (dd, *J* = 8.8, 2.0 Hz, 1H, Ar), 7.45 (d, *J* = 8.0 Hz, 2H, Ar), 7.46 (d, *J* = 8.0 Hz, 2H, Ar), 7.79 (m, 4H, Ar), 8.10 (d, *J* = 8.8 Hz, 1H, Ar); $^{13}C$ NMR (100 MHz, $CDCl_3$) δ: 25.5, 28.1, 28.1, 29.6, 32.2 (2C), 35.8, 39.1, 39.9 (2C), 40.5, 40.9, 49.9, 54.6, 55.4, 60.1, 61.8, 65.8, 66.4, 69.9, 70.0 (2C), 70.2, 70.4 (2C), 125.0, 125.3, 126.0, 127.3 (2C), 127.4 (2C), 129.6, 130.2 (2C), 130.3 (2C), 130.4, 130.6, 137.0, 137.1, 141.4, 141.5, 145.1, 152.6, 155.0, 156.3, 163.8, 173.3, 195.7; HRMS (FAB): *m/z* calcd for $C_{50}H_{62}BrN_8O_{10}S_2$ $[M+H]^+$ 1077.3214; found: 1077.3213.

#### 4.1.15. *N*-(*tert*-Butyl)-9-{4-[4-(*tert*-butyldiphenylsilyloxy)-methyl]benzoylphenyl}-3,4-dihydro-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine (28)

Compound **27** (2.17 g, 6.17 mmol) was subjected to the general cross-coupling procedure as described for the synthesis of **13** to give the title compound **28** as colorless solid (3.16 g, 71%): mp 152–153 °C (from $CHCl_3$/*n*-hexane): IR (neat) $cm^{-1}$: 1656 (C=O), 1623 (C=N), 1593 (C=N); $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$: 1.12 (s, 9H, 3 × $CH_3$), 1.41 (s, 9H, 3 × $CH_3$), 1.91-1.97 (m, 2H), 3.65 (t, $J$ = 5.4 Hz, 2H, $CH_2$), 3.90 (t, $J$ = 6.2 Hz, 2H, $CH_2$), 4.86 (s, 2H, $CH_2$), 7.37-7.48 (m, 10H, Ar), 7.69-7.71 (m, 6H, Ar), 7.81 (d, $J$ = 8.3 Hz, 2H, Ar), 7.88 (d, $J$ = 8.3 Hz, 2H, Ar), 8.30 (d, $J$ = 8.5 Hz, 1H, Ar); $^{13}C$ NMR (100 MHz, $CDCl_3$) $\delta$: 19.3, 21.9, 26.8 (3C), 30.0 (3C), 45.2, 45.5, 54.2, 65.2, 123.0, 124.9, 125.7 (2C), 126.9 (2C), 127.4, 127.8 (4C), 129.1, 129.8 (2C), 129.9, 130.2 (2C), 130.7 (2C), 133.2 (2C), 135.5 (4C), 136.2, 137.2, 138.0, 141.7, 143.2, 146.1, 147.6, 195.9; HRMS (FAB): *m*/*z* calcd for $C_{45}H_{48}N_3O_2SSi$ $[M+H]^+$ 722.3237; found: 722.3244.

#### 4.1.16. *N*-(*tert*-Butyl)-3,4-dihydro-9-[4-(4-propargyloxymethyl)-benzoylphenyl]-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine (29)

To a solution of **28** (200.0 mg, 0.28 mmol) in THF (2.8 mL) was added TBAF in THF (1 M, 0.55 mL, 0.55 mmol). After being stirred at rt for 2 h, the reaction mixture was quenched with satd $NH_4Cl$. The whole was extracted with EtOAc, and washed with brine, and dried over $MgSO_4$. The filtrate was concentrated. To the solution of the resulting residue in THF (2.8 mL) was added NaH (22.8 mg, 0.55 mmol, 60% oil suspension) at 0 °C. After being stirred at the same temperature for 30 min, propargyl bromide (31.5 μL, 0.42 mmol) was added dropwise. After being stirred at rt overnight, the reaction was quenched with water. The whole was extracted with EtOAc, and washed with brine, and dried over $MgSO_4$. After concentration, the residue was purified by flash column chromatography over aluminum oxide with *n*-hexane/EtOAc (5:1) to give the title compound **29** as colorless solid (87.2 mg, 60%): mp 133–135 °C (from $CHCl_3$/*n*-hexane): IR (neat) $cm^{-1}$: 1656 (C=O), 1620 (C=N), 1593 (C=N); $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$: 1.41 (s, 9H, 3 × $CH_3$), 1.91–1.97 (m, 2H), 2.50 (t, $J$ = 2.3 Hz, 1H, CH), 3.65 (t, $J$ = 5.5 Hz, 2H, $CH_2$), 3.90 (t, $J$ = 6.1 Hz, 2H, $CH_2$), 4.25 (d, $J$ = 2.3 Hz, 2H, $CH_2$), 4.71 (s, 2H, $CH_2$), 7.39 (d, $J$ = 1.7 Hz, 1H, Ar), 7.46–7.50 (m, 3H, Ar), 7.70 (d, $J$ = 8.0 Hz, 2H, Ar), 7.82 (d, $J$ = 8.0 Hz, 2H, Ar), 7.87 (d, $J$ = 8.0 Hz, 2H, Ar), 8.30 (d, $J$ = 8.3 Hz, 1H, Ar); $^{13}C$ NMR (100 MHz, $CDCl_3$) $\delta$: 21.9, 30.0 (3C), 45.2, 45.4, 54.2, 57.6, 70.9, 75.0, 79.3, 123.0, 124.8, 126.9 (2C), 127.3, 127.5 (2C), 129.1, 129.9, 130.2 (2C), 130.7 (2C), 136.9, 137.0, 137.9, 141.6, 142.2, 143.4, 147.5, 195.7; HRMS (FAB): *m*/*z* calcd for $C_{32}H_{32}N_3O_2S$ $[M+H]^+$ 522.2215; found: 522.2207.

#### 4.1.17. 3,4-Dihydro-9-[4-(4-propargyloxymethyl)-benzoylphenyl]-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine (8)

Using a procedure identical with that described for synthesis of **6** from **17**, the imine **29** (42.8 mg, 0.08 mmol) was allowed to react under reflux for 1 h with TFA (2.0 mL) and MS4Å (300 mg). Purification by flash chromatography over aluminum oxide with *n*-hexane/EtOAc (9:1 to 1:1) gave the title compound **8** as colorless solid (35.4 mg, 92%): mp 159–160 °C (from $CHCl_3$/*n*-hexane): IR (neat) $cm^{-1}$: 1654 (C=O), 1619 (C=N), 1573 (C=N); $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$: 1.96–2.04 (m, 2H), 2.50 (t, $J$ = 2.4 Hz, 1H, CH), 3.72 (t, $J$ = 5.6 Hz, 2H, $CH_2$), 4.05 (t, $J$ = 6.1 Hz, 2H, $CH_2$), 4.25 (d, $J$ = 2.4 Hz, 2H, $CH_2$), 4.71 (s, 2H, $CH_2$), 7.26–7.31 (m, 2H, Ar, NH), 7.48–7.51 (m, 3H, Ar), 7.67–7.89 (m, 6H, Ar), 8.33 (d, $J$ = 8.5 Hz, 1H, Ar); $^{13}C$ NMR (100 MHz, $CDCl_3$) $\delta$: 21.0, 43.8, 45.0, 57.6, 70.9, 75.0, 79.3, 122.0, 125.1, 126.3, 126.9 (2C), 127.5 (2C), 129.6, 129.7, 130.2 (2C), 130.7 (2C), 137.0, 137.1, 142.2, 142.3, 143.0, 146.2, 153.0, 195.7; HRMS (FAB): *m*/*z* calcd for $C_{28}H_{24}N_3O_2S$ $[M+H]^+$ 466.1589; found: 466.1589.

### 4.2. Determination of anti-HIV activity

The sensitivity of HIV-$1_{IIIB}$ strain was determined by the MAGI assay. The target cells (HeLa-CD4/CCR5-LTR/β-gal; $10^4$ cells/well) were plated in 96-well flat microtiter culture plates. On the following day, the cells were inoculated with the HIV-1 (60 MAGI U/well, giving 60 blue cells after 48 h of incubation) and cultured in the presence of various concentrations of the test compounds in fresh medium. Forty-eight hours after viral exposure, all the blue cells stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were counted in each well. The activity of test compounds was determined as the concentration that blocked HIV-1 infection by 50% (50% effective concentration [$EC_{50}$]). $EC_{50}$ was determined by using the following formula:

$$EC_{50} = 10^{\wedge}[\log(A/B) \times (50 - C)/(D - C) + \log(B)]$$

wherein

A: of the two points on the graph which bracket 50% inhibition, the higher concentration of the test compound,

B: of the two points on the graph which bracket 50% inhibition, the lower concentration of the test compound,

C: inhibitory activity (%) at the concentration B,

D: inhibitory activity (%) at the concentration A.

### 4.3. Photoaffinity labeling experiments using HIV-1-infected H9 cells (H9IIIB)

1 μL of probe **6** or **7** (10 mM solution in DMSO) was added to H9 cells chronically infected with HIV-1 (H9IIIB) in D-MEM with 10% fetal bovine serum (500 μL, 0.5 × $10^6$ cells). For the competitive evaluation (Fig. 3, lane C), 2 μL of compound **3a** (10 mM solution in DMSO) was also added. The cells were incubated at 37 °C for 1 h. Then the cells were photolabeled by irradiation by UV (MUV-202U, Moritex Co., Japan) at room temperature for 1 min at a distance of 3 cm through a longpass filter (LU0300, Asahi spectra Co.). The mixture was centrifuged at 200 × g for 5 min and the supernatant was removed. The cells were washed with PBS once and were lysed in RIPA buffer containing 1% protease inhibitor cocktail (Nacalai Tesque, Inc., Japan) at 4 °C for 30 min. After centrifugation at 16500 × g for 15 min, the supernatant was used for the next experiment.

NeutrAvidin-agarose beads (50 μL, Thermo), which were equilibrated with RIPA buffer, were treated with the supernatant containing 180 μg of proteins and were incubated at 4 °C for 1 h. The beads were then centrifuged at 9100 × g for 30 sec and washed with RIPA buffer (repeated three times). After heating the beads at 95 °C for 5 min in sample buffer [50 mM Tris–HCl (pH 8.0), 2% SDS, 0.1% BPB, 10% glycerol, 2% β-ME], the supernatants were subjected to SDS–PAGE electrophoresis (SuperSep™Ace, 5–20%, Wako) and the separated proteins were transferred onto a PVDF membrane. The membrane was blocked with Blocking One (Nacalai Tesque, Inc.) at room temperature for 1 h, and was then incubated with a streptavidin–HRP conjugate (Invitrogen; 1:5000 in PBS with 0.1% Tween) at 4 °C overnight. The membrane was treated with Chemi-Lumi One L (Nacalai Tesque, Inc.). Biotinylated proteins were detected by Image Quant LAS 4000mini (GE Healthcare).

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.01.016.

## References and notes


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