Bioorganic & Medicinal Chemistry 21 (2013) 2079–2087

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

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

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ARTICLE INFO

Article history:
Received 12 December 2012
Revised 5 January 2013
Accepted 5 January 2013
Available online 16 January 2013

Keywords:
Anti-HIV agents
PD 404182
Photoaffinity labeling
Pyrimidobenzothiazine

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.

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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http://dx.doi.org/10.1016/j.bmc.2013.01.016

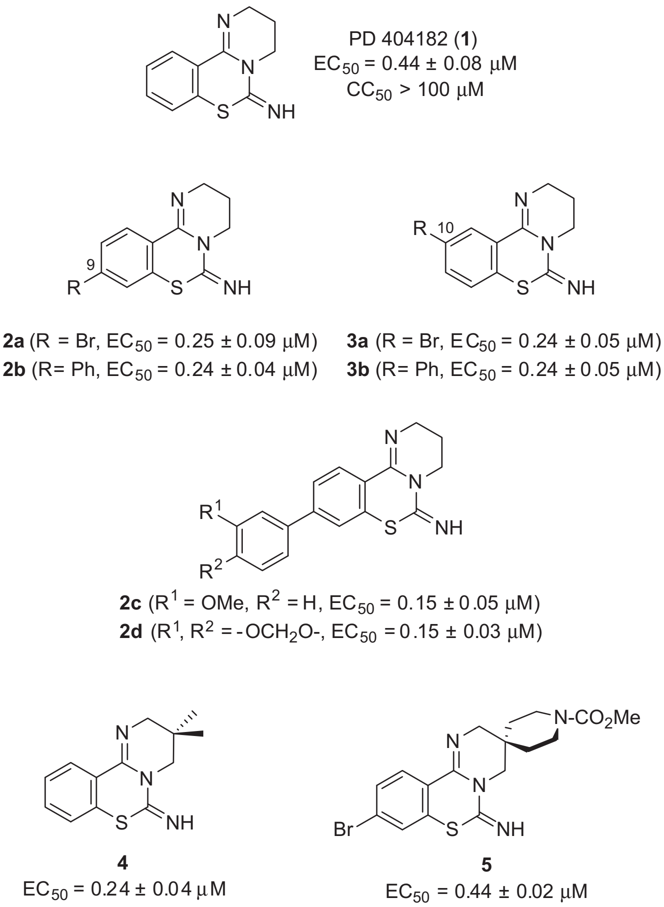


Figure 1. Structures and anti-HIV activity of PD 404182 and the derivatives **2–5**.

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

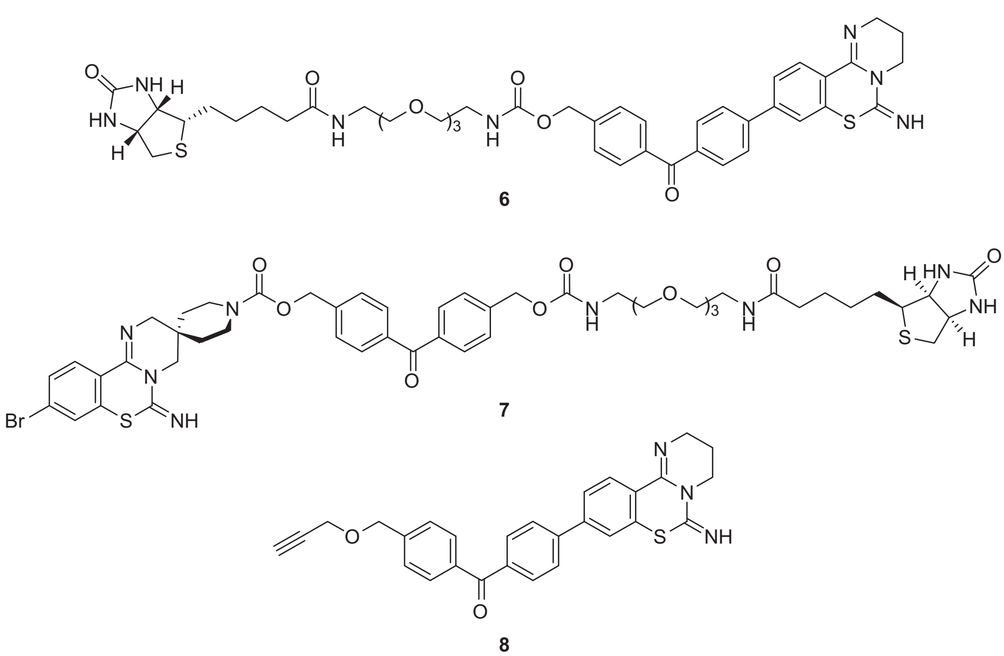
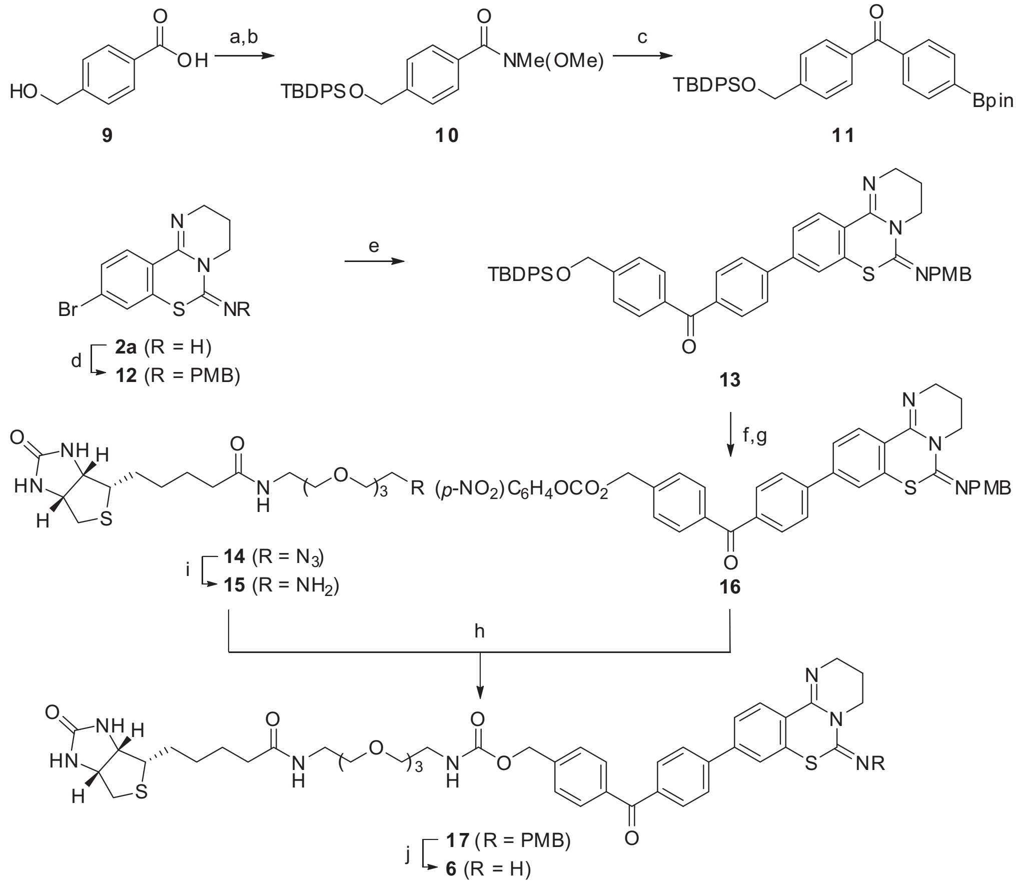


Figure 2. Structures of photoaffinity probes **6–8**.



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**, $Pd(PPh_3)_4$, $PdCl_2(dppf)·CH_2Cl_2$, 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

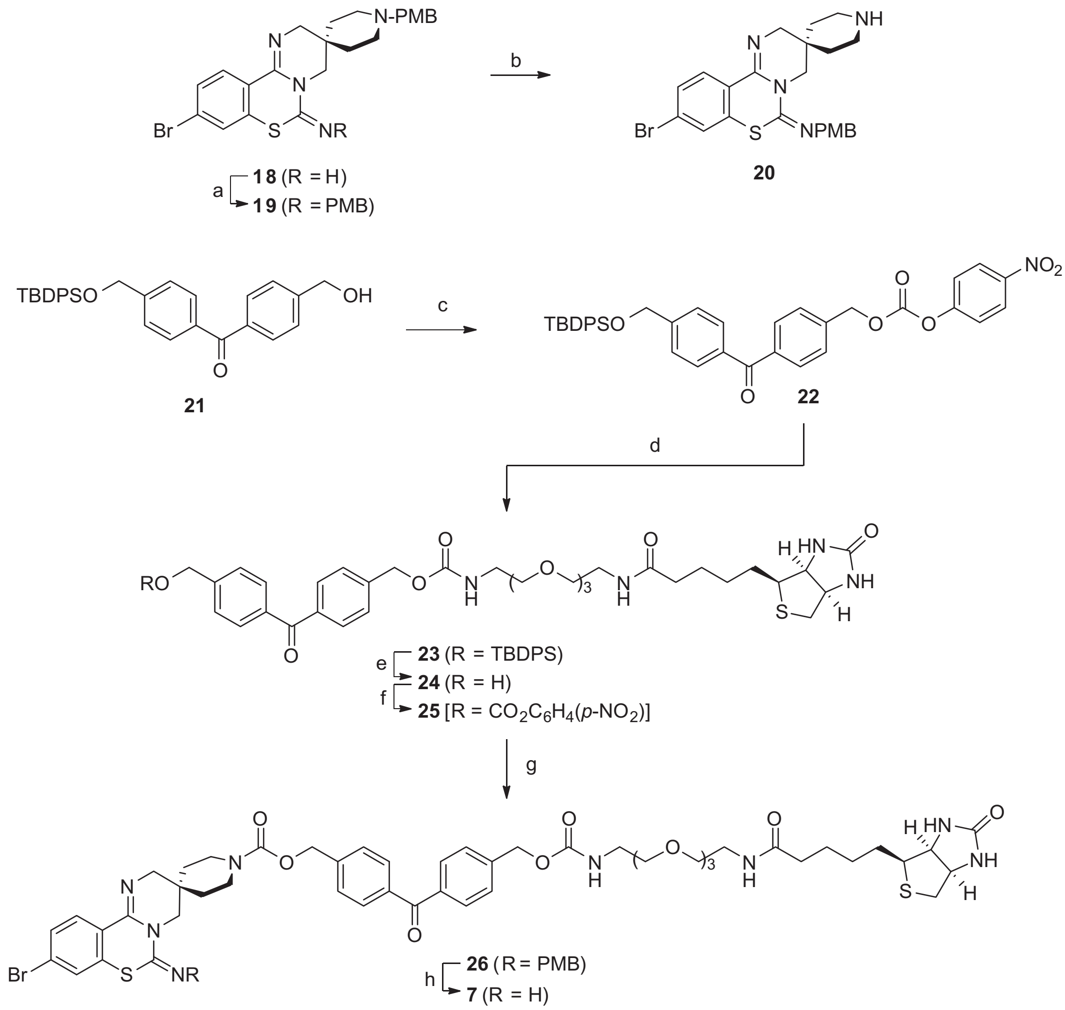
The antiviral activities of probes **6**–**8** against HIV-1_{IIIB} were measured by multinuclear activation of a galactosidase indicator (MAGI) assay. In this assay, the inhibitory activity against HIV infection at the early stage, including virus attachment and membrane fusion to host cells, can be 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

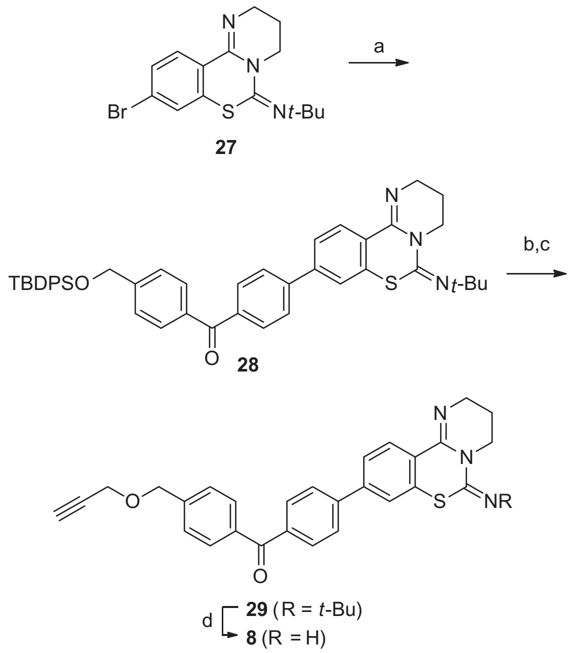
4.1. Synthesis

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 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 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%.

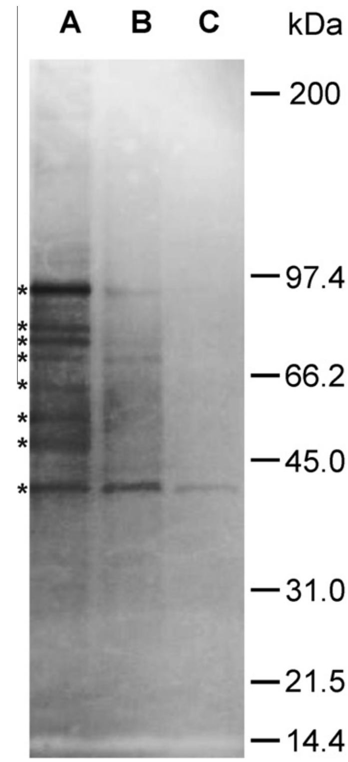


Figure 3. Western blot analysis of the photolabeled proteins with biotin-conjugated probes **6** and **7**. H9IIIB cells were incubated with (A) 20 μM probe **6**, (B) 20 μM probe **7**, and (C) 20 μM probe **6** and 40 μM compound **3a**. The cells were exposed to UV light for 1 min and were lysed. 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.

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.

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, $CDCl_3$) δ: 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, $CDCl_3$) δ: 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, $CDCl_3$) δ: 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-tetra-hydrobenzo[*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, $CDCl_3$) δ: 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, $CDCl_3$) δ: 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, $CDCl_3$) δ: 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, $CDCl_3$) δ: 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, $CDCl_3$) δ: 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, $CDCl_3$) δ: 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); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 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, $CDCl_3$) δ: 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); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 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, $CDCl_3$) δ: 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); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 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, $CDCl_3$) δ: 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).

Acknowledgments

This work was supported by Grants-in-Aid for Scientific Research and Platform for Drug Discovery, Informatics, and Structural Life Science from MEXT; and Health and Labor Science Research

Grants (Research on HIV/AIDS, Japan). T.M. is grateful for JSPS Research Fellowships for Young Scientists.

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

1. PD 404182 (**1**) was previously reported to exhibit antimicrobial activity by inhibition of 3-deoxy-D-manno-octulosonic acid 8-phosphate synthase[2] or phosphopantetheinyl transferase.[3]
2. Birck, M. R.; Holler, T. P.; Woodard, R. W. *J. Am. Chem. Soc.* **2000**, *122*, 9334.
3. (a) Duckworth, B. P.; Aldrich, C. C. *Anal. Biochem.* **2010**, *403*, 13; (b) Foley, T. L.; Yasgar, A.; Garcia, C. J.; Jadhav, A.; Simeonov, A.; Burkart, M. D. *Org. Biomol. Chem.* **2010**, *8*, 4601.
4. Chockalingam, K.; Simeon, R. L.; Rice, C. M.; Chen, Z. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 3764.
5. Mizuhara, T.; Oishi, S.; Ohno, H.; Shimura, K.; Matsuoka, M.; Fujii, N. *Org. Biomol. Chem.* **2012**, *10*, 6792.
6. Mizuhara, T.; Oishi, S.; Ohno, H.; Shimura, K.; Matsuoka, M.; Fujii, N. *Bioorg. Med. Chem.* **2012**, *20*, 6434.
7. Mizuhara, T.; Inuki, S.; Oishi, S.; Fujii, N.; Ohno, H. *Chem. Commun.* **2009**, 3413.
8. Mizuhara, T.; Oishi, S.; Fujii, N.; Ohno, H. *J. Org. Chem.* **2010**, *75*, 265.
9. Baba, M.; Scgols, D.; Pauwels, R.; Nakashima, H.; De Clercq, E. *J. Acquir. Immune Defic. Syndr.* **1990**, *3*, 493.
10. (a) Kilby, J. M.; Eron, J. J. *N. Engl. J. Med.* **2003**, *348*, 2228; (b) Lalezari, J. P.; Henry, K.; O'Hearn, M.; Montaner, J. S.; Piliero, P. J.; Trottier, B.; Walmsley, S.; Cohen, C.; Kuritzkes, D. R.; Eron, J. J., Jr.; Chung, J.; DeMasi, R.; Donatacci, L.; Drobnes, C.; Delehanty, J.; Salgo, M. *N. Engl. J. Med.* **2003**, *348*, 2175; (c) Matthews, T.; Salgo, M.; Greenberg, M.; Chung, J.; DeMasi, R.; Bolognesi, D. *Nat. Rev. Drug Discov.* **2004**, *3*, 215.
11. (a) Dorr, P.; Westby, M.; Dobbs, S.; Griffin, P.; Irvine, B.; Macartney, M.; Mori, J.; Rickett, G.; Smith-Burchnell, C.; Napier, C.; Webster, R.; Armour, D.; Price, D.; Stammen, B.; Wood, A.; Perros, M. *Antimicrob. Agents Chemother.* **2005**, *49*, 4721; (b) Fätkenheuer, G.; Pozniak, A. L.; Johnson, M. A.; Plettenberg, A.; Staszewski, S.; Hoepelman, A. I. M.; Saag, M. S.; Goebel, F. D.; Rockstroh, J. K.; Dezube, B. J.; Jenkins, T. M.; Medhurst, C.; Sullivan, J. F.; Ridgway, C.; Abel, S.; James, I. T.; Youle, M.; Van Der Ryst, E. *Nat. Med.* **2005**, *11*, 1170; (c) Skerlj, R.; Bridger, G.; Zhou, Y.; Bourque, E.; McEachern, E.; Langille, J.; Harwig, C.; Veale, D.; Yang, W.; Li, T.; Zhu, Y.; Bey, M.; Baird, I.; Satori, M.; Metz, M.; Mosi, R.; Nelson, K.; Bodart, V.; Wong, R.; Fricker, S.; Huskens, D.; Schols, D. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 6950.
12. (a) Bridger, G. J.; Skerlj, R. T.; Thornton, D.; Padmanabhan, S.; Martellucci, S. A.; Henson, G. W.; Abrams, M. J.; Yamamoto, N.; De Vreese, K.; Pauwels, R.; De Clercq, E. *J. Med. Chem.* **1995**, *38*, 366; (b) Bridger, G. J.; Skerlj, R. T.; Hernandez-Abad, P. E.; Bogucki, D. E.; Wang, Z.; Zhou, Y.; Nan, S.; Boehringer, E. M.; Wilson, T.; Crawford, J.; Metz, M.; Hatse, S.; Princen, K.; De Clercq, E.; Schols, D. *J. Med. Chem.* **2010**, *53*, 1250; (c) Fujii, N.; Oishi, S.; Hiramatsu, K.; Araki, T.; Ueda, S.; Tamamura, H.; Otaka, A.; Kusano, S.; Terakubo, S.; Nakashima, H.; Broach, J. A.; Trent, J. O.; Wang, Z.-X.; Peiper, S. C. *Angew. Chem., Int. Ed.* **2003**, *42*, 3251; (d) Ueda, S.; Oishi, S.; Wang, Z.-X.; Araki, T.; Tamamura, H.; Cluzeau, J.; Ohno, H.; Kusano, S.; Nakashima, H.; Trent, J. O.; Peiper, S. C.; Fujii, N. *J. Med. Chem.* **2007**, *50*, 192; (e) Inokuchi, E.; Oishi, S.; Kubo, T.; Ohno, H.; Shimura, K.; Matsuoka, M.; Fujii, N. *ACS Med. Chem. Lett.* **2011**, *2*, 477.
13. Chamoun, A. M.; Chockalingam, K.; Bobardt, M.; Simeon, R.; Chang, J.; Gallay, P.; Chen, Z. *Antimicrob. Agents Chemother.* **2012**, *56*, 672.
14. (a) Dorman, G.; Prestwich, G. D. *Biochemistry* **1994**, *33*, 5661; (b) Kotzyba-Hibert, F.; Kapfer, I.; Goeldner, M. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 1296; (c) Fleming, S. A. *Tetrahedron* **1995**, *51*, 12479; (d) Tomohiro, T.; Hashimoto, M.; Hatanaka, Y. *Chem. Rec.* **2005**, *5*, 385.
15. (a) Drake, R. R.; Neamati, N.; Hong, H.; Pilon, A. A.; Sunthankar, P.; Hume, S. D.; Milne, G. W. A.; Pommier, Y. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 4170; (b) Lin, W.; Li, K.; Doughty, M. B. *Bioorg. Med. Chem.* **2002**, *10*, 4131; (c) Al-Mawsawi, L. Q.; Fikkert, V.; Dayam, R.; Witvrouw, M.; Burke, T. R., Jr.; Borchers, C. H.; Neamati, N. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 10080.
16. (a) Hofmann, K.; Kiso, Y. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 3516; (b) Hatanaka, Y.; Hashimoto, M.; Kanaoka, Y. *Bioorg. Med. Chem.* **1994**, *2*, 1367; (c) Kinoshita, T.; Cano-Delgado, A.; Seto, H.; Hiranuma, S.; Fujioka, S.; Yoshida, S.; Chory, J. *Nature* **2005**, *433*, 167; (d) Kotake, Y.; Sagane, K.; Owa, T.; Mimori-Kiyosue, Y.; Shimizu, H.; Uesugi, M.; Ishihama, Y.; Iwata, M.; Mizui, Y. *Nat. Chem. Biol.* **2007**, *3*, 570.
17. For examples of alkyne-conjugated photoaffinity probes with benzophenone see: (a) Ballell, L.; Alink, K. J.; Slijper, M.; Versluis, C.; Liskamp, R. M.; Pieters, R. J. *ChemBioChem* **2005**, *6*, 291; (b) Sieber, S. A.; Niessen, S.; Hoover, H. S.; Cravatt, B. F. *Nat. Chem. Biol.* **2006**, *2*, 274; (c) Salisbury, C. M.; Cravatt, B. F. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 1171; (d) Kalesh, K. A.; Sim, D. S.; Wang, J.; Liu, K.; Lin, Q.; Yao, S. Q. *Chem. Commun.* **2010**, *46*, 1118; (e) Eirich, J.; Orth, R.; Sieber, S. A. *J. Am. Chem. Soc.* **2011**, *133*, 12144.
18. Jiang, Q.; Ryan, M.; Zhichkin, P. *J. Org. Chem.* **2007**, *72*, 6618.
19. Fusz, S.; Srivatsan, S. G.; Ackermann, D.; Famulok, M. *J. Org. Chem.* **2008**, *73*, 5069.
20. Denholm, A. A.; George, M. H.; Hailes, H. C.; Tiffin, P. J.; Widdowson, D. A. *J. Chem. Soc., Perkin Trans. 1* **1995**, *5*, 541.
21. Watanabe, K.; Negi, S.; Sugiura, Y.; Kiriyama, A.; Honbo, A.; Iga, K.; Kodama, E. N.; Naitoh, T.; Matsuoka, M.; Kano, K. *Chem. Asian J.* **2010**, *5*, 825.