form of C34 as an antigen peptide producing structure-specific antibodies. We have performed comparative studies of antisera isolated from mice immunized with the C34 trimer in binding affinity for the C34 trimer and for the C34 monomer.

2. Materials and methods

2.1. Immunization and sample collection

Six-week-old male BALB/c mice, purchased from Sankyo Laboratory Service Corp. (Tokyo, Japan), were maintained in an animal facility under specific pathogen-free conditions. The experimental protocol used was approved by the ethical review committee of Tokyo Medical and Dental University. Freund incomplete adjuvant and PBS were purchased from Wako Pure Chemical Industries (Osaka, Japan). DMSO (endotoxin free) was purchased from Sigma–Aldrich (St. Louis, MO).

All mice were bled one week before immunization. One hundred micrograms of antigen (C34 monomer C34REG) was dissolved in PBS (50 μL) and DMSO (1 μL). The antigen C34 trimer triC34e (100 μg) was dissolved in PBS (50 μL). This solution was mixed with Freund incomplete adjuvant (50 μL) and the mixture was injected subcutaneously under anesthesia on days 0, 7, 14, 21 and 28. Mice were bled on days 5, 12, 19, 26 and 33. Serum was separated by centrifugation (1500 rpm) at 4 °C for 10 min, and inactivated at 56 °C for 30 min. Sera were stored at −80 °C before use.

2.2. Serum titer ELISA

Tween-20 (polyoxyethylene (20) sorbitan monolaurate) and hydrogen peroxide (30%) were purchased from Wako Pure Chemical Industries (Osaka, Japan). 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) was purchased from Sigma-Aldrich. Anti-mouse IgG (H+L)(goat)-HRP was purchased from EMD Chemicals (San Diego, CA). Ninety-six well microplates were coated with 25 μL of a synthetic peptide in a 10 μg/mL solution in PBS at 4 °C overnight. The coated plates were washed 10 times with deionized water and blocked with 150 μL of blocking buffer (0.02% PBST, PBS with 0.02% Tween 20, containing 5% skim milk) at 37 °C for 1 h. The plates were washed with deionized water 10 times. Mice sera were diluted in 0.02% PBST with 1% skim milk, and 50 μL of twofold serial dilutions of sera from 1/200 to 1/409600 were added to the wells and allowed to incubate at 37 °C for 2 h. The plates were again washed 10 times with deionized water. HRP-conjugated anti-mouse IgG, diluted 1:2000 in 0.02% PBST (25 μL), was added to each well. After incubation for 45 min, the plates were washed 10 times and 25 μL of HRP substrate, prepared by dissolving ABTS (10 mg) in 200 μL of HRP staining buffer—a mixture of 0.5 M citrate buffer (pH 4.0, 1 mL), H_2O_2 (3 μL), and H_2O (8.8 mL)—was added. After 30 min incubation, the reaction was stopped by addition of 25 μL/well 0.5 M H_2SO_4, and optical densities at 405 nm were recorded.

2.3. Virus preparation

For virus preparation, 293FT cells in a 60 mm dish were transfected with 10 μg of the pNL4-3 construct by the calcium phosphate method. The supernatant was collected 48 h after transfection, passed through a 0.45 μm filter, and stored at −80 °C as the virus stock.

2.4. Neutralizing assay (P24 assay)

For viral neutralizing assay, the NL4-3 virus (5 ng of p24) was bound to MT-4 cells (5×10^4 cells/200 μL) by spinoculation at 2100 g for 20 min at 4 °C. After removal by washing out of unbound virus, cells were resuspended with 200 μL of medium containing 10 μL sera from immunized or pre-immunized mice and were cultured. Half of the culture medium was changed every 2 or 3 days. At 7 days after infection, the level of p24 in the culture supernatant was determined by the p24 ELISA kit (PerkinElmer, MA).[17]

3. Results and discussion

In the C34 trimer, triC34e, which was previously synthesized,[16] the triplet repeat of arginine and glutamic acid (RERERE) was added to the C-terminal end of the C34 sequence to increase solubility in buffer solution, and glycine was fused to the C-terminus (Fig. 1A and B). The C3-symmetric template with three hydrophilic branches of equal length was adopted to assemble three peptide strands. As a control peptide, which corresponds to the monomeric form of C34, C34REG having RERERE and Gly in the C-terminus was used (Fig. 1C).[16]

To investigate whether antibodies are efficiently produced, mice were immunized with C34REG and triC34e and the increase in the titer in 5 weeks' immunization was observed (Fig. 2). Titers and specificity of antisera isolated from mice immunized with C34REG or triC34e were evaluated by serum titer ELISA against coated synthetic antigens. In each case, the increase in antibody production was observed as time passed. The most active antiserum for each antigen was utilized for the evaluation of binding activity by ELISA (Fig. 3). The C34REG-induced antibody showed approximately 1.2 times higher antibody titer against the coated C34REG than against the coated triC34e; the serum dilutions at 50% bound are 1.06×10^{-3} and 1.30×10^{-3}, respectively (Fig. 3A and B). The triC34e-induced antibody showed approximately 23 times higher titer against the coated triC34e than against the coated C34REG; the 50% bound serum dilutions are 3.15×10^{-4} and 7.30×10^{-3}, respectively (Fig. 3A and B). C34REG-induced or triC34e-induced antibody did not show any significant binding titer against an unrelated control peptide (Fig. 3C and D). Although purified monoclonal antibodies were not used for this evaluation, the antibodies produced exploited specific affinity for each antigen of the monomer or the trimer. These results suggest the synthesis of structure-involving antigens leads to the production of antibodies with structural specificity.

It is important to know if the antisera produced have inhibitory activity against HIV-1 infection. Accordingly, the inhibitory activity of the antisera was assessed by p24 assays utilizing the antisera bled from three mice that showed antibody production for each antigen (Fig. 4). The experiments using HIV-1 was performed in the biosafety level 3 laboratory #5 in the National Institute of Infectious Diseases. Sera from mice immunized with the monomer C34REG and the trimer triC34e antigens contained antiviral activities compared to control sera. Any significant difference of inhibitory effects was not observed between the sera isolated from C34REG-immunized mice and those from triC34e-immunized mice. The synthetic C34 trimeric antigen induces antibodies with a structural preference, but the levels of neutralization activity of sera from mice immunized with the C34 trimer were similar with those of sera from the C34 monomer-immunized mice. This suggests that antibodies with structural specificity against the gp41-C34-derived region do not always have more potent neutralization activity. The difference of recognition mechanism of two types of antibodies might cause different neutralizing mechanism although their levels of neutralization activity are almost the same. This result is not consistent with the data of the synthetic antigen molecules derived from N36, in which the N36 trimer-specific antibodies showed higher neutralization activity against HIV-1 infection than the N36 monomer-specific antibodies.[8] In any case,

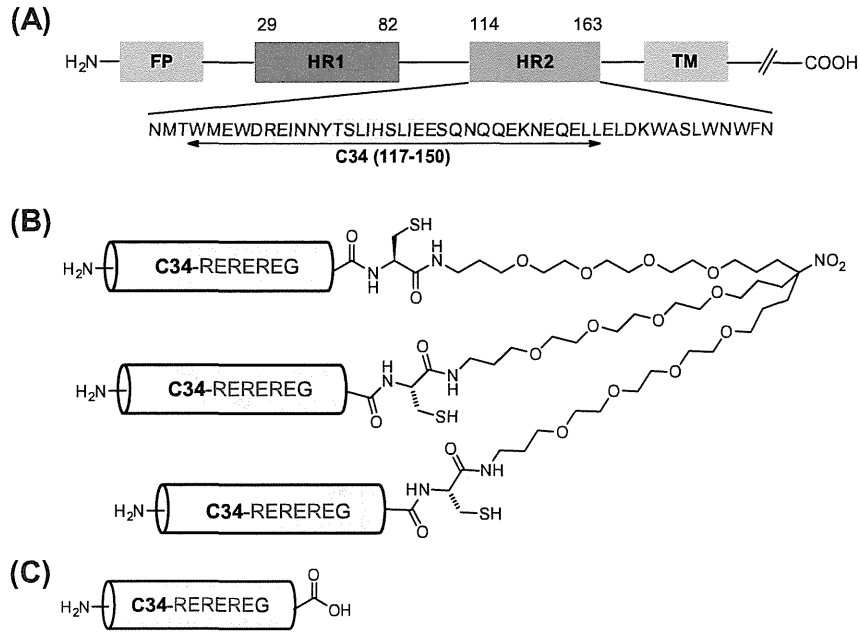


Figure 1. The sequence of C34 in gp41 (NL4-3) (A). FP and TM represent hydrophobic fusion peptide and transmembrane domain, respectively. Structures of C34-derived peptides, the C34 trimer with a C3-symmetric linker, triC34e (B), and the C34 monomer, C34REG (C).

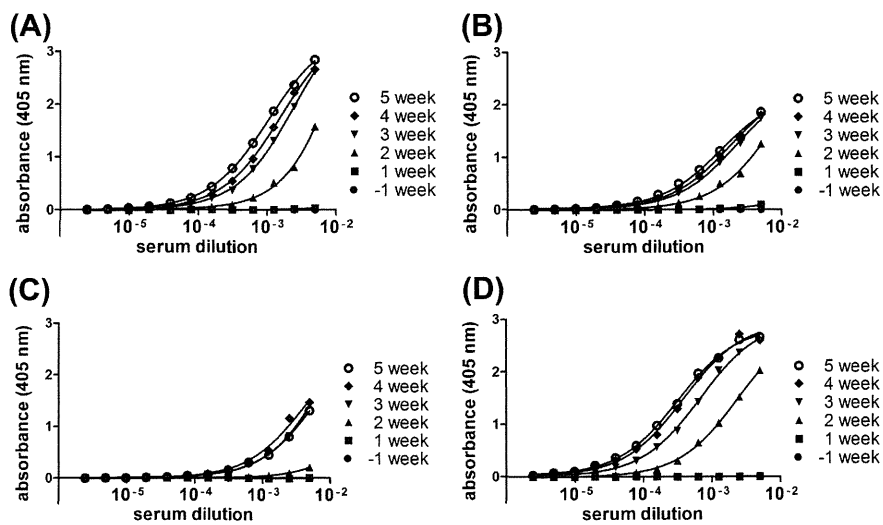


Figure 2. Results of serum titer ELISA of antisera collected during immunization (from one week before start to five weeks after immunization start) to determine the immunogenicity of designed antigens. The titers were evaluated as followings; antiserum against C34REG binding to C34REG (A); antiserum against C34REG binding to triC34e (B); antiserum against triC34e binding to C34REG (C); antiserum against triC34e binding to triC34e (D).

the synthetic C34 trimeric antigen induces antibodies with a structural preference and potent neutralization activity. In case antibodies bind to the gp41 C-terminal HR2 region and suppress membrane fusion, they may recognize the primary amino acid sequence of the C34 region or its structural conformation, because the C34 region is located outside in the formation of a six-helical bundle. It is suggested that suppressant potencies of these types of antibodies are almost similar. In addition, the action of these antibodies might be orthogonal and supplementally effective.

Recently, broadly active and potent neutralization antibodies, PG-9 and PG-16, were isolated from sera of HIV-1 infected individuals.[3] The antibodies can neutralize ~80% of HIV-1 isolates across all clades with approximately one order of magnitude higher potency than those of broad neutralizing mAbs reported previously. It is interesting that the epitopes for these mAbs are quaternary, and preferentially displayed on Env trimers, as expressed on the surface of virions and transfected cells. These results suggest that there may be production mechanisms for antibodies recognizing epitope structures.[18–20] The sera obtained from immunization of the C34 trimer antigen have structural specificity and neutralization activity. Thus, our trimer antigens, including the N36 trimer,[8] could work efficiently as a new class of HIV-1 vaccines.

Concerning inhibitory activity of these C-region peptides against HIV-1 entry, the potency of triC34e is one hundred times higher than that of C34REG.[16] It indicates that a trimeric form is critical as the active structure of the inhibitor, although as vaccines

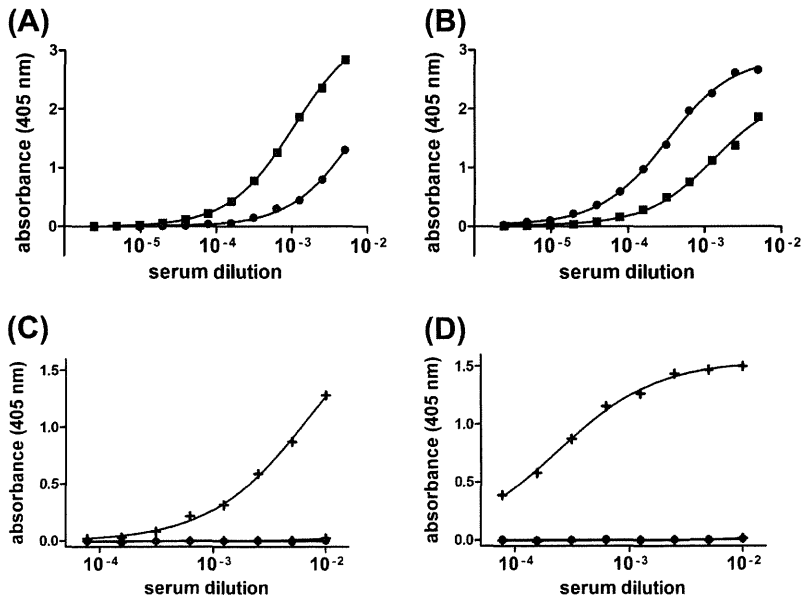


Figure 3. Serum titers of the antibodies produced by the fifth immunization of the C34REG antigen and the fourth immunization of the triC34e antigen. These titers were evaluated against ELISA templates of C34REG (monomer) (A) and triC34e (trimer) (B), using sera obtained from a C34REG-immunized mouse (■) and a triC34e-immunized mouse (●) as each representative. Titers of C34REG-induced antibodies were evaluated against ELISA templates of C34REG (+) and an unrelated control peptide (●) (C), and titers of triC34e-induced antibodies were evaluated against ELISA templates of triC34e (+) and an unrelated control peptide (●) (D). Unrelated control peptide: CH_3CO-GELDKWEKIRLRPGGGC(CH_2CONH_2)-NH_2.

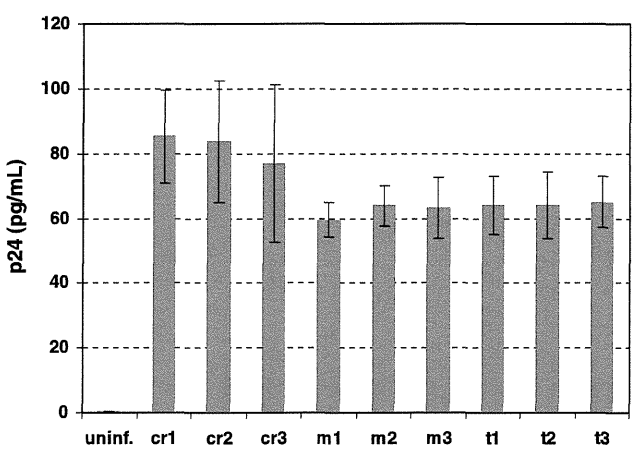


Figure 4. Determination of neutralization activity of the antibodies produced by immunization of C34REG and triC34e antigens. Inhibition of HIV-1 (NL4-3 strain) infection by produced antibodies was evaluated by the p24 assay in MT-4 cells. Y-axis shows the amount of p24 production. Uninf means uninfected cells. Pre-immunization sera (−1 week) were used as controls (cr1-3). C34REG- and triC34e-immunization sera (5 weeks) were used (m1-3 and t1-3, respectively). Experiments were conducted in triplicate. Error bars show standard error of mean.

there is no significant difference in neutralization activity of induced antibodies between the monomer and the trimer.

The exposed timing of epitopes of the helical region trimers is limited in the fusion step,[21] and carbohydrates are not included in the amino acid residues of the regions. The effectiveness of the vaccine design based on the gp41 helical regions is supported by the critical advantages cited above. Our developed N36 and C34 trimer-form specific antibodies might have the above properties. The designs of antigens and inhibitors targeting the dynamic supramolecular mechanism of HIV-1 fusion will be useful for future studies on AIDS vaccines and inhibitors.

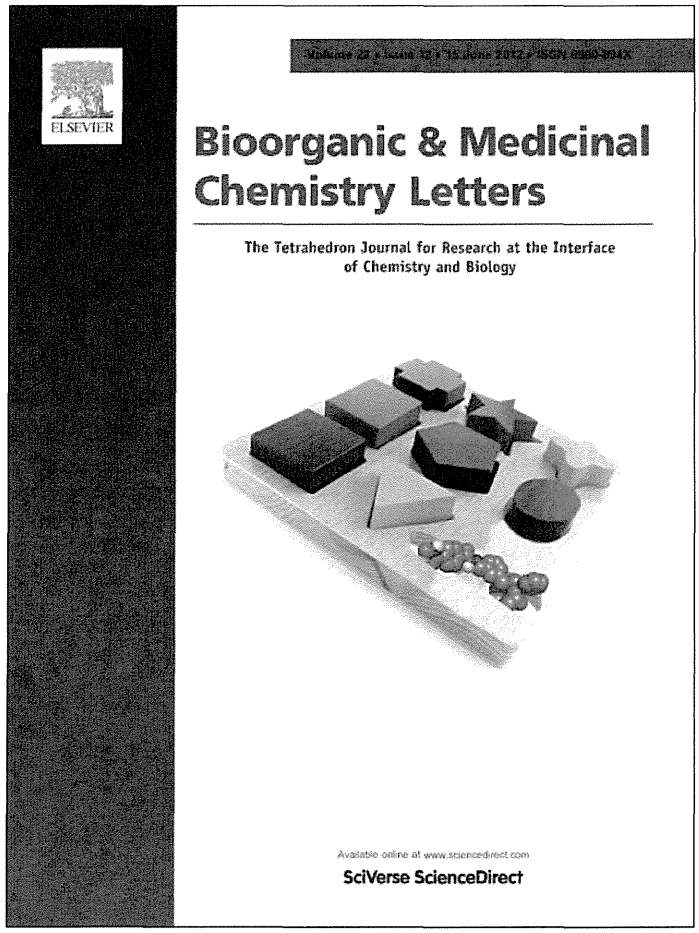
Acknowledgments

This work was supported in part by Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and Health and Labour Sciences Research Grants from Japanese Ministry of Health, Labour, and Welfare. C.H. was supported by JSPS Research Fellowships for Young Scientists.

References and notes

1. Cabezas, E.; Wang, M.; Parren, P. W.; Stanfield, R. L.; Satterthwait, A. C. *Biochemistry* **2000**, *39*, 14377.
2. Burton, D. R.; Barbas, C. F. I. I. I.; Persson, M. A.; Koenig, S.; Chanock, R. M.; Lerner, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 10134.
3. Walker, L. M.; Phogat, S. K.; Chan-Hui, P.-Y.; Wagner, D.; Phung, P.; Goss, J. L.; Wrin, T.; Simek, M. D.; Fling, S.; Mitcham, J. L.; Lehrman, J. K.; Priddy, F. H.; Olsen, O. A.; Frey, S. M.; Hammond, P. W.; Kaminsky, S.; Zamb, T.; Moyle, M.; Koff, W. C.; Poignard, P.; Burton, D. R. *Science* **2009**, *326*, 285.
4. De Rosny, E.; Vassell, R.; Wingfield, R. T.; Wild, C. T.; Weiss, C. D. *J. Virol.* **2001**, *75*, 8859.
5. Tam, J. P.; Yu, Q. *Org. Lett.* **2002**, *4*, 4167.
6. Xu, W.; Taylor, J. W. *Chem. Biol. Drug Des.* **2007**, *70*, 319.
7. Louis, J. M.; Nesheiwat, I.; Chang, L.; Clore, G. M.; Bewlet, C. A. *J. Biol. Chem.* **2003**, *278*, 20278.
8. Nakahara, T.; Nomura, W.; Ohba, K.; Ohya, A.; Tanaka, T.; Hashimoto, C.; Narumi, T.; Murakami, T.; Yamamoto, N.; Tamamura, H. *Bioconjugate Chem.* **2010**, *21*, 709.
9. Jiang, S.; Lin, K.; Strick, N.; Neurath, A. R. *Nature* **1993**, *365*, 113.
10. Wild, C. T.; Shugars, D. C.; Greenwell, T. K.; McDanal, C. B.; Matthews, T. J. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 9770.
11. Kilby, J. M.; Hopkins, S.; Venetta, T. M.; DiMassimo, B.; Cloud, G. A.; Lee, J. Y.; Alldredge, L.; Hunter, E.; Lambert, D.; Bolognesi, D.; Matthews, T.; Johnson, M. R.; Nowak, M. A.; Shaw, G. M.; Saag, M. S. *Nat. Med.* **1998**, *4*, 1302.
12. Kilby, J. M.; Eron, J. J. *N. Eng. J. Med.* **2003**, *348*, 2228.
13. 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. Eng. J. Med.* **2003**, *348*, 2175.
14. Liu, S.; Jing, W.; Cheng, B.; Lu, H.; Sun, J.; Yan, X.; Niu, J.; Farmar, J.; Wu, S.; Jiang, S. *J. Biol. Chem.* **2007**, *282*, 9612.
15. Chan, D. C.; Fass, D.; Berger, J. M.; Kim, P. S. *Cell* **1997**, *89*, 263.
16. Nomura, W.; Hashimoto, C.; Ohya, A.; Miyauchi, K.; Urano, E.; Tanaka, T.; Narumi, T.; Nakahara, T.; Komano, J. A.; Yamamoto, N. *ChemMedChem* **2012**, *7*, 205.

17. Ohba, K.; Ryo, A.; Dewan, M. Z.; Nishi, M.; Naito, T.; Qi, X.; Inagaki, Y.; Nagashima, Y.; Tanaka, Y.; Okamoto, T.; Terashima, K.; Yamamoto, N. *J. Immunol.* **2009**, *183*, 524.
18. Pejchal, R.; Walker, L. M.; Stanfield, R. L.; Phogat, S. K.; Koff, W. C.; Poignard, P.; Burton, D. R.; Wilson, I. A. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 11483.
19. Doores, K. J.; Burton, D. R. *J. Virol.* **2010**, *84*, 10510.
20. Walker, L. M.; Simek, M. D.; Priddy, F.; Gach, J. S.; Wagner, D.; Zwick, M. B.; Phogat, S. K.; Poignard, P.; Burton, D. R. *PLoS Pathog.* **2010**, *6*, e1001028.
[21]. Zwick, M. B.; Saphire, E. O.; Burton, D. R. *Nat. Med.* **2004**, *10*, 133–134.



Bioorganic & Medicinal Chemistry Letters 22 (2012) 4169–4172

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Pharmacophore-based small molecule CXCR4 ligands

Tetsuo Narumi [a], Tomohiro Tanaka [a], Chie Hashimoto [a], Wataru Nomura [a], Haruo Aikawa [a], Akira Sohma [a], Kyoko Itotani [a], Miyako Kawamata [b], Tsutomu Murakami [b], Naoki Yamamoto [c], Hirokazu Tamamura [a,*]

[a] Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Chiyoda-ku, Tokyo 101-0062, Japan
[b] AIDS Research Center, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo 162-8640, Japan
[c] Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117597, Singapore

ARTICLE INFO

Article history:
Received 16 March 2012
Revised 4 April 2012
Accepted 7 April 2012
Available online 20 April 2012

Keywords:
HIV entry inhibitors
Chemokine receptor
AIDS
Low molecular weight CXCR4 ligands

ABSTRACT

Low molecular weight CXCR4 ligands were developed based on the peptide T140, which has previously been identified as a potent CXCR4 antagonist. Some compounds with naphthyl, fluorobenzyl and pyridyl moieties as pharmacophore groups in the molecule showed significant CXCR4-binding activity and anti-HIV activity. Structure–activity relationships were studied and characteristics of each of these three moieties necessary for CXCR4 binding were defined. In this way, CXCR4 ligands with two types of recognition modes for CXCR4 have been found.

The chemokine receptor CXCR4 is classified into a family of G protein-coupled receptors (GPCRs), and transduces signals of its endogenous ligand, CXCL12/stromal cell-derived factor-1 (SDF-1).[1] The CXCR4-CXCL12 axis plays a physiological role in chemotaxis,[2] angiogenesis[3] and neurogenesis[4] in embryonic stages. The CXCR4 receptor is linked to many disorders including HIV infection/AIDS,[5] metastasis of cancer cells,[6] leukemia cell progression,[7] rheumatoid arthritis.[8] Since CXCR4 is an important drug target in these diseases, it is thought that effective agents directed to this receptor may be useful leads for therapeutic agents. To date, we and others have developed several potent CXCR4 antagonists. A highly potent antagonist, T140, a 14-mer peptide with a disulfide bridge, and its downsized analogue, FC131, with a cyclic pentapeptide scaffold, and several other related compounds have been reported.[9] Based on T140 and FC131, small-sized linear anti-HIV agents such as ST34 (**1**) have been developed (Fig. 1).[10] AMD3100,[11] KRH-1636,[12] Dpa–Zn complex (**2**)[13] and other azamacrocyclic compounds such as **3**,[14] which like **1**, contain benzylamine and electron-deficient aromatic groups, have also been reported as nonpeptidic antagonists. Compound **1** possesses significant anti-HIV activity but does not have high CXCR4 binding affinity. In the present study, more effective linear CXCR4 antagonists derived from compound **1** have been examined, and structure–activity relationship studies of these compounds have been performed.

Initially, three segments of compound **1** were selected for structural modification to support the design of new synthetic compounds: replacement of the 4-trifluoromethylbenzoyl group (Fig. 2, R^1), modification of the stereochemistry of the 1-naphthylethylamine moiety (R^2) and introduction of pyridine moieties on the nitrogen atom (R^3). In a previous study of T140 analogues, 4-fluorobenzoyl was found to be superior to 4-trifluoromethylbenzoyl as an N-terminal moiety. Thus, 4-fluorobenzyl, 4-fluorobenzoyl and 4-fluorophenylethyl groups were used as substitutes for the 4-trifluoromethylbenzoyl group (R^1) in **1**. The (*R*)-1-naphthylethylamine moiety in **1** is also present in KRH-1636 where it has the (*S*)-stereochemistry and thus both the (*R*) and (*S*)-stereoisomers were investigated in the present study. Several CXCR4 antagonists such as KRH-1636,[12] Dpa-Zn complex (**2**)[13] and Dpa-cyclam compound (**3**),[14] contain pyridyl rings. Thus, 2, 3, or 4-pyridylmethyl and 2, 3, or 4-pyridylethyl groups were introduced on the nitrogen atom of the 4-aminomethylbenzoyl group (R^3). With these modifications, a total of $3 \times 2 \times 6 = 36$ compounds (**12–47**) were designed (Fig. 2).

The synthesis of the structural fragment, Unit 1 is shown in Scheme 1. N-nosylation of 4-amino-methylbenzoic acid (**4**) with 2-nitrobenzenesulfonyl chloride and subsequent esterification gave the *t*-butyl ester **5**. Introduction of an R^3 moiety by means of a Mitsunobu reaction followed by removal of the Ns group yielded amines **6A–F**. Introduction of either 4-fluorobenzyl or 4-fluorophenylethyl groups by reductive amination of **6A–F** produced amines **7Ai–Fi** or **7Aiii–Fiii**, respectively. Conversion of **6A–F** to the appropriate amide (**7Aii-Fii**), and subsequent deprotection of the *tert*-butyl group yielded Unit 1, **8Ai–Fiii**.

* Corresponding author.
E-mail address: tamamura.mr@tmd.ac.jp (H. Tamamura).

0960-894X/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmcl.2012.04.032

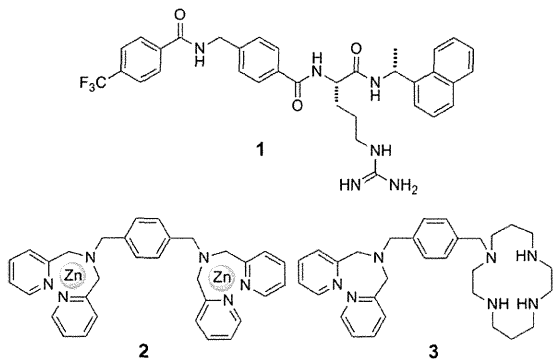


Figure 1. The structures of **1** (ST34), Dpa-Zn complex (**2**) and Dpa-cyclam compound (**3**).

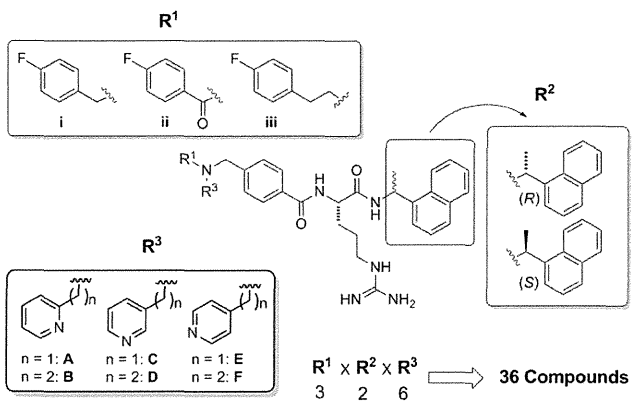
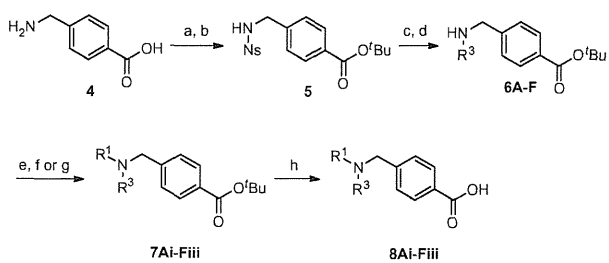


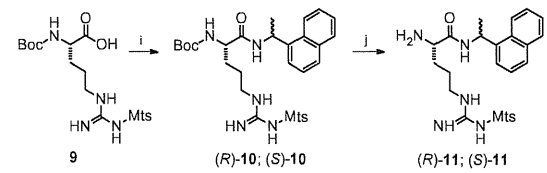
Figure 2. The structures of substituents for three parts of compound **1** in the design of new compounds.



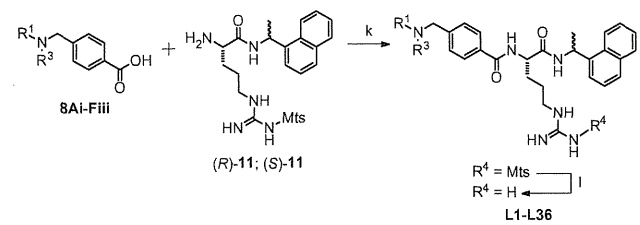
Scheme 1. The synthetic scheme of Unit 1, compounds **8Ai–Fiii**. Reagents and conditions and yields: (a) NsCl, Et_3N, THF/H_2O (1/1); (b) isobutene, THF/H_2SO_4 (10/1), 39% (2 steps); (c) PPh_3, DEAD, R^3OH, THF; (d) PhSH, K_2CO_3, DMF, 42–92% (2 steps); (e) $NaBH(OAc)_3$, 4-fluorobenzaldehyde, CH_2Cl_2; (f) $NaBH(OAc)_3$, (4-fluorophenyl)acetaldehyde, CH_2Cl_2; g) 4-fluorobenzoyl chloride, Et_3N, CH_2Cl_2, 51-94%; (h) TFA then 4 M HCl/EtOAc, quantitative; The structures of R^1 and R^3 are shown in Fig. 2 as i–iii and **A–F**, respectively. Ns = 2-nitrobenzenesulfonyl, ^{t}Bu = *tert*-butyl, DEAD = diethyl azodicarboxylate.

The synthesis of Unit 2 is shown in Scheme 2. Condensation of Boc-Arg(Mts)-OH (**9**) and (*R*)-1-naphthylethylamine or its (*S*) isomer produced amides (*R*)-**10** or (*S*)-**10**. Removal of the Boc group gave Unit 2, (*R*)-**11** and (*S*)-**11**.

Compounds **12–47** were synthesized by amide condensation of Unit 1, **8Ai–Fiii**, with Unit 2, (*R*)-**11** and (*S*)-**11**, and subsequent deprotection of the Mts group, as shown in Scheme 3.[15] All the synthetic compounds were purified by preparative reverse phase HPLC. In cases where peaks derived from side products appeared around the target peaks on the HPLC profile, the precise analysis was accomplished, giving rise to lower yields (Scheme 3, 1).



Scheme 2. Synthetic schemes of Unit 2, compounds (***R/S*)-11**. Reagents and conditions: (i) EDCI·HCl, $HOBt·H_2O$, Et_3N, (*R/S*)-(+/−)-1-(1-naphthyl)ethylamine, CH_2Cl_2, 83–97%; (j) TFA then 4 M HCl/EtOAc, quantitative; EDCI·HCl = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, $HOBt·H_2O$ = 1-hydroxybenzotriazol monohydrate, Mts = 2,4,6-trimethylphenylsulfonyl, Boc = *tert*-butoxycarbonyl.



Scheme 3. Synthetic schemes of compounds **12–47**. Reagents and conditions: (k) EDCI·HCl, $HOBt·H_2O$, Et_3N, DMF, 36-95%; (l) TMSBr, *m*-cresol, 1,2-ethanedithiol, thioanisole, TFA, 4–54%. The structures of R^1 and R^3 are shown in Figure 2 as i–iii and **A–F**, respectively.

The CXCR4-binding activity of synthetic compounds was assessed in terms of the inhibition of [^{125}I]-CXCL12 binding to Jurkat cells, which express CXCR4.[16] The percent inhibition of all the compounds at 10 μM is shown in Table 1. Several of the compounds showed significant binding affinity. In general, compounds in which the 1-naphthylethylamine moiety (R^2) has the (*S*)-stereochemistry, as in KRH-1636, are more potent than the (*R*)-stereoisomers. Ten compounds (**26–28, 30, 33, 36, 39, 44, 45** and **47**, Table 1) were found to induce at least 30% inhibition and compounds **26**, **27** and **33**, which have a pyridyl group with a nitrogen atom at the β-position, showed more than 60% inhibition. It is noteworthy that compounds **26** and **27** in which R^2 is a (*R*)-1-naphthylethylamine moiety, are both more potent than the corresponding (*S*)-stereoisomers **44** and **45**. Compounds **26, 27** and **33**, have a 4-fluorobenzyl or 4-fluorophenylethyl group, which rather than an amide, is a reductive alkyl type (R^1). As can be seen from Table 1, there is a tendency for compounds with a pyridyl group with a nitrogen atom at the β-position (R^3: C or D), to be more potent in terms of CXCR4-binding activity than the corresponding compounds, which have a pyridyl group with a nitrogen atom at the α- or γ- position (R^3: A, B, E or F), and those with a reductive alkyl 4-fluorobenzyl or 4-fluorophenylethyl group (R^1: i or iii), to be more potent in CXCR4-binding activity than the corresponding compounds, with a 4-fluorobenzoyl group (R^1: ii).

Compounds were next evaluated for anti-HIV activity and cytotoxicity. CXCR4 is the major co-receptor for the entry of T-cell line-tropic (X4-) HIV-1.[5] Accordingly, inhibitory activity against X4-HIV-1 (NL4-3 strain)-induced cytopathogenicity in MT-4 cells (anti-HIV activity), and reduction of the viability in MT-4 cells (cytotoxicity) were assessed[16] and are shown in Table 1. Compounds **26** and **33–35** showed significant anti-HIV activity with EC_{50} values in the micromolar range. Compounds **26** and **33** showed both potent CXCR4-binding activity (79% and 60% inhibition at 10 μM, respectively) and anti-HIV activity (EC_{50} = 11 and 13 μM, respectively), the two activities being highly correlated. Compounds **34** and **35** have significant anti-HIV activity with EC_{50} values of 8 and 10 μM, respectively, which is higher than CXCR4-binding activities, which are 16% and 20% inhibition at 10 μM, respectively. Compound **27**, which showed relatively high CXCR4-binding activity (69% inhibition at 10 μM), failed to show

Table 1
CXCR4-binding activity, anti-HIV activity and cytotoxicity of compounds **12–47**

Compd no.	R^1 [a]	R^2 [b]	R^3 [c]	Inhibition[d] (%)	EC_{50}[e] (μM)	CC_{50}[f] (μM)	Compd no.	R^1 [a]	R^2 [b]	R^3 [c]	Inhibition[d] (%)	EC_{50}[e] (μM)	CC_{50}[f] (μM)
12	i	(*R*)	A	0	>20	35	**30**	i	(*S*)	A	30 ± 1.1	>4	11
13	i	(*R*)	B	4 ± 1.7	>4	23	**31**	i	(*S*)	B	25 ± 3.3	>20	24
14	i	(*R*)	C	6 ± 0.7	>20	37	**32**	i	(*S*)	C	27 ± 1.7	>20	41
15	i	(*R*)	D	24 ± 1.7	n.d.	n.d.	**33**	i	(*S*)	D	60 ± 1.5	13	65
16	i	(*R*)	E	12 ± 3.0	>20	39	**34**	i	(*S*)	E	16 ± 1.2	8	44
17	i	(*R*)	F	16 ± 2.2	n.d.	n.d.	**35**	i	(*S*)	F	20 ± 1.3	10	44
18	ii	(*R*)	A	3 ± 0.9	>20	38	**36**	ii	(*S*)	A	36 ± 1.8	>20	37
19	ii	(*R*)	B	6 ± 3.9	>20	41	**37**	ii	(*S*)	B	0	>20	43
20	ii	(*R*)	C	11 ± 0.8	>20	45	**38**	ii	(*S*)	C	14 ± 1.4	>20	57
21	ii	(*R*)	D	22 ± 4.1	n.d.	n.d.	**39**	ii	(*S*)	D	32 ± 8.4	n.d.	n.d.
22	ii	(*R*)	E	6 ± 2.7	>20	45	**40**	ii	(*S*)	E	13 ± 15	>20	51
23	ii	(*R*)	F	12 ± 1.9	n.d.	n.d.	**41**	ii	(*S*)	F	25 ± 13	>20	47
24	iii	(*R*)	A	15 ± 2.1	n.d.	n.d.	**42**	iii	(*S*)	A	16 ± 5.1	>4	9.9
25	iii	(*R*)	B	13 ± 0.6	>20	27	**43**	iii	(*S*)	B	23 ± 14	>4	13
26	iii	(*R*)	C	79 ± 14	11	47	**44**	iii	(*S*)	C	36 ± 13	n.d.	n.d.
27	iii	(*R*)	D	69 ± 5.0	>11	11	**45**	iii	(*S*)	D	35 ± 5.2	n.d.	n.d.
28	iii	(*R*)	E	44 ± 5.4	n.d.	n.d.	**46**	iii	(*S*)	E	26 ± 23	n.d.	n.d.
29	iii	(*R*)	F	0	n.d.	n.d.	**47**	iii	(*S*)	F	51 ± 6.6	n.d.	n.d.
KRH-1636				100	0.33	80	FC131				100	0.16	>10
AMD3100				n.d.	0.062	55	1 (ST34)				n.d.	7.4	66
AZT				n.d.	0.058	100							

[a,c] The structures of R^1 and R^3 are shown in Fig. 2 as i–iii and **A–F**, respectively.
[b] The absolute configuration in stereochemistry of R^2 shown in Fig. 2 is described.
[d] CXCR4-binding activity was assessed based on the inhibition of the [^{125}I]-CXCL12 binding to Jurkat cells. Inhibition percentages of all the compounds at 10 μM were calculated relative to the inhibition percentage by T140 (100%).
[e] EC_{50} values are the concentrations for 50% protection from X4-HIV-1 (NL4-3 strain)-induced cytopathogenicity in MT-4 cells.
[f] CC_{50} values are the concentrations for 50% reduction of the viability of MT-4 cells. All data are the mean values from at least three independent experiments.

significant anti-HIV activity at concentrations below 11 μM because of high cytotoxicity (CC_{50} = 11 μM). With the exception of **27**, **30**, **42** and **43**, the tested compounds showed no significant cytotoxicity (CC_{50} >20 μM, Table 1). On the other hand, compounds **26**, **27**, **33**, **34** and **35** at concentrations below 100 μM failed to show significant protective activity against macrophage-tropic (R5-) HIV-1 (NL(AD8) strain)-induced cytopathogenicity in PM-1/CCR5, whereas the EC_{50} of the CCR5 antagonist SCH-D[17] in this assay was 0.055 μM (data not shown). Since instead of CXCR4, R5-HIV-1 strains use the chemokine receptor CCR5, a member of the GPCR family, as the major co-receptor for their entry, this suggests that these compounds do not bind to CCR5. Thus, compounds **26**, **27**, **33**, **34** or **35** have highly selective affinity for CXCR4. Compounds **34** and **35**, which have significant anti-HIV activity, have a pyridyl group with a nitrogen atom at the γ-position, in contrast to compounds **26**, **27** and **33** which also show CXCR4-binding activity, but have a pyridyl group with a nitrogen atom at the β-position. Furthermore, compounds **34** and **35** have R^1 = 4-fluorobenzyl and R^2 = (*S*)-1-naphthylethylamine. A possible explanation of these observations is that compounds **34** and **35** compete with HIV-1 in binding to CXCR4 while compounds **26** and **33** compete with HIV-1 and CXCL12. Compound **27** does not compete with HIV-1 because of its high cytotoxicity. This suggests that the CXCR4 binding site used by compounds **34** and **35** differs slightly from that used by compounds **26**, **27** and **33**.

Low molecular weight CXCR4 ligands with two types of recognition modes for CXCR4 have been obtained in this study: one causes competition with HIV-1 on CXCR4 whereas the other causes competition with HIV-1 and CXCL12. These compounds have selective affinity for CXCR4 because they do not significantly bind to CCR5. Further structural modification studies of these CXCR4 ligands are the subject of an ongoing project.

Acknowledgements

This work was supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, Japan Human Science Foundation, and Health and Labour Sciences Research Grants from Japanese Ministry of Health, Labor, and Welfare. T.T. and C.H. are grateful for the JSPS Research Fellowships for Young Scientists.

References and notes

1. (a) Nagasawa, T.; Kikutani, H.; Kishimoto, T. *Proc. Natl. Acad. Sci. U. S. A.* **1994**, *91*, 2305; (b) Bleul, C. C.; Farzan, M.; Choe, H.; Parolin, C.; Clark-Lewis, I.; Sodroski, J.; Springer, T. A. *Nature* **1996**, *382*, 829; (c) Oberlin, E.; Amara, A.; Bachelerie, F.; Bessia, C.; Virelizier, J. L.; Arenzana-Seisdedos, F.; Schwartz, O.; Heard, J. M.; Clark-Lewis, I.; Legler, D. L.; Loetscher, M.; Baggiolini, M.; Moser, B. *Nature* **1996**, *382*, 833; (d) Tashiro, K.; Tada, H.; Heilker, R.; Shirozu, M.; Nakano, T.; Honjo, T. *Science* **1993**, *261*, 600.
2. Bleul, C. C.; Fuhlbrigge, R. C.; Casanovas, J. M.; Aiuti, A.; Springer, T. A. *J. Exp. Med.* **1996**, *2*, 1101.
3. (a) Tachibana, K.; Hirota, S.; Iizasa, H.; Yoshida, H.; Kawabata, K.; Kataoka, Y.; Kitamura, Y.; Matsushima, K.; Yoshida, N.; Nishikawa, S.; Kishimoto, T.; Nagasawa, T. *Nature* **1998**, *393*, 591; (b) Nagasawa, T.; Hirota, S.; Tachibana, K.; Takakura, N.; Nishikawa, S.; Kitamura, Y.; Yoshida, N.; Kikutani, H.; Kishimoto, T. *Nature* **1996**, *382*, 635.
4. (a) Zhu, Y.; Yu, Y.; Zhang, X. C.; Nagasawa, T.; Wu, J. Y.; Rao, Y. *Nat. Neurosci.* **2002**, *5*, 719; (b) Stumm, R. K.; Zhou, C.; Ara, T.; Lazarini, F.; Dubois-Dalcq, M.; Nagasawa, T.; Hollt, V.; Schulz, S. *J. Neurosci.* **2003**, *23*, 5123.
5. (a) Deng, H. K.; Liu, R.; Ellmeier, W.; Choe, S.; Unutmaz, D.; Burkhart, M.; Marzio, P. D.; Marmon, S.; Sutton, R. E.; Hill, C. M.; Davis, C. B.; Peiper, S. C.; Schall, T. J.; Littman, D. R.; Landau, N. R. *Nature* **1996**, *381*, 661; (b) Feng, Y.; Broder, C. C.; Kennedy, P. E.; Berger, E. A. *Science* **1996**, *272*, 872.
6. (a) Koshiba, T.; Hosotani, R.; Miyamoto, Y.; Ida, J.; Tsuji, S.; Nakajima, S.; Kawaguchi, M.; Kobayashi, H.; Doi, R.; Hori, T.; Fujii, N.; Imamura, M. *Clin. Cancer. Res.* **2000**, *6*, 3530; (b) Müller, A.; Homey, B.; Soto, H.; Ge, N.; Catron, D.; Buchanan, M. E.; McClanahan, T.; Murphy, E.; Yuan, W.; Wagner, S. N.; Barrera, J. L.; Mohar, A.; Verastegui, E.; Zlotnik, A. *Nature* **2001**, *410*, 50; (c) Tamamura, H.; Hori, A.; Kanzaki, N.; Hiramatsu, K.; Mizumoto, M.; Nakashima, H.; Yamamoto, N.; Otaka, A.; Fujii, N. *FEBS Lett.* **2003**, *550*, 79.
7. (a) Tsukada, N.; Burger, J. A.; Zvaifler, N. J.; Kipps, T. J. *Blood* **2002**, *99*, 1030; (b) Juarez, J.; Bradstock, K. F.; Gottlieb, D. J.; Bendall, L. J. *Leukemia* **2003**, *17*, 1294.
8. (a) Nanki, T.; Hayashida, K.; El-Gabalawy, H. S.; Suson, S.; Shi, K.; Girschick, H. J.; Yavuz, S.; Lipsky, P. E. . *J. Immunol.* **2000**, *165*, 6590; (b) Tamamura, H.; Fujisawa, M.; Hiramatsu, K.; Mizumoto, M.; Nakashima, H.; Yamamoto, N.; Otaka, A.; Fujii, N. *FEBS Lett.* **2004**, *569*, 99.
9. (a) Murakami, T.; Nakajima, T.; Koyanagi, Y.; Tachibana, K.; Fujii, N.; Tamamura, H.; Toshida, N.; Waki, M.; Matsumoto, A.; Yoshie, O.; Kishimoto, T.; Yamamoto, N.; Nagasawa, T. *J. Exp. Med.* **1997**, *186*, 1389; (b) Tamamura, H.; Xu, Y.; Hattori, T.; Zhang, X.; Arakaki, R.; Kanbara, K.; Omagari, A.; Otaka, A.; Ibuka, T.; Yamamoto, N.; Nakashima, H.; Fujii, N. *Biochem. Biophys. Res.*

Commun. **1998**, *253*, 877; (c) Tamamura, H.; Omagari, A.; Oishi, S.; Kanamoto, T.; Yamamoto, N.; Peiper, S. C.; Nakashima, H.; Otaka, A.; Fujii, N. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2633; (d) 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.; Peiper, S. C. *Angew. Chem., Int. Ed.* **2003**, *42*, 3251; (e) Tamamura, H.; Hiramatsu, K.; Ueda, S.; Wang, Z.; Kusano, S.; Terakubo, S.; Trent, J. O.; Peiper, S. C.; Yamamoto, N.; Nakashima, H.; Otaka, A.; Fujii, N. *J. Med. Chem.* **2005**, *48*, 380; (f) Tamamura, H.; Araki, T.; Ueda, S.; Wang, Z.; Oishi, S.; Esaka, A.; Trent, J. O.; Nakashima, H.; Yamamoto, N.; Peiper, S. C.; Otaka, A.; Fujii, N. *J. Med. Chem.* **2005**, *48*, 3280; (g) Hashimoto, C.; Tanaka, T.; Narumi, T.; Nomura, W.; Tamamura, H. *Expert Opin. Drug Disc.* **2011**, *6*, 1067.

10. Tamamura, H.; Tsutsumi, H.; Masuno, H.; Mizokami, S.; Hiramatsu, K.; Wang, Z.; Trent, J. O.; Nakashima, H.; Yamamoto, N.; Peiper, S. C.; Fujii, N. *Org. Biomol. Chem.* **2006**, *4*, 2354.
11. Schols, D.; Struyf, S.; Van Damme, J.; Este, J. A.; Henson, G.; DeClarcq, E. *J. Exp. Med.* **1997**, *186*, 1383.
12. Ichiyama, K.; Yokoyama-Kumakura, S.; Tanaka, Y.; Tanaka, R.; Hirose, K.; Bannai, K.; Edamatsu, T.; Yanaka, M.; Niitani, Y.; Miyano-Kurosaki, N.; Takaku, H.; Koyanagi, Y.; Yamamoto, N. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 4185.
13. Tamamura, H.; Ojida, A.; Ogawa, T.; Tsutsumi, H.; Masuno, H.; Nakashima, H.; Yamamoto, N.; Hamachi, I.; Fujii, N. *J. Med. Chem.* **2006**, *49*, 3412.
14. Tanaka, T.; Narumi, T.; Ozaki, T.; Sohma, A.; Ohashi, N.; Hashimoto, C.; Itotani, K.; Nomura, W.; Murakami, T.; Yamamoto, N.; Tamamura, H. *ChemMedChem* **2011**, *6*, 834.
15. *For example, the synthesis of compound* **30**: To a stirred solution of **8Ai** (176 mg, 0.415 mmol, HCl salt) in DMF (4 mL) were added EDCI·HCl (104 mg, 0.454 mmol), HOBt·H_2O (58.4 mg, 0.381 mmol), Et_3N (301 μL, 2.16 mmol) and (***S***)-**11** (320 mg, 0.657 mmol, HCl salt) at 0 °C. The mixture was stirred at room temperature for 43 h. The reaction mixture was diluted with $CHCl_3$ and washed with saturated citric acid, saturated $NaHCO_3$ and brine, and dried over $MgSO_4$. Concentration under reduced pressure followed by flash column chromatography over silica gel with $CHCl_3$/MeOH (20/1) gave the condensation product (175 mg, 0.208 mmol, 50% yield) as white powder. To this compound were added *m*-cresol (75.0 μL, 0.714 mmol), 1,2-ethanedithiol (225 μL, 2.68 mmol), thioanisole (225 μL, 1.91 mmol), TFA (3 mL) and bromotrimethylsilane (495 μL, 3.82 mmol) with stirring at 0 °C, and the stirring was continued at room temperature for 3.5 h under N_2. The reaction mixture was concentrated under reduced pressure, followed by addition of Et_2O to precipitate the product. After washing with Et_2O, the crude product was purified by preparative HPLC and lyophilized to give the compound **30** (15.6 mg, 0.0236 mmol, 13%) as white powder. ^{1}H NMR δ_H (400 MHz; DMSO-d_6) 1.49 (m, 2H), 1.51 (d, J = 7.2 Hz, 3H), 1.80–1.62 (m, 2H), 3.07 (dd, J = 6.4, 12.8 Hz, 2H), 3.85 (s, 2H), 3.91 (s, 4H), 4.54 (m, 1H), 5.72 (m, 1H), 7.13 (t, J = 8.8 Hz, 2H), 7.40 (m, 1H), 7.60–7.45 (m, 10H), 7.75–7.95 (m, 5H), 8.10 (m, 1H), 8.40 (d, J = 8.0 Hz, 1H), 8.58 (m, 1H), 8.65 (d, J = 7.6 Hz, 1H); LRMS (ESI), m/z calcd for $C_{39}H_{42}FN_7O_2$ $(MH)^+$ 660.34, found 660.31.
16. Tanaka, T.; Tsutsumi, H.; Nomura, W.; Tanabe, Y.; Ohashi, N.; Esaka, A.; Ochiai, C.; Sato, J.; Itotani, K.; Murakami, T.; Ohba, K.; Yamamoto, N.; Fujii, N.; Tamamura, H. *Org. Biomol. Chem.* **2008**, *6*, 4374.
17. Tagat, J. R.; McCombie, S. W.; Nazareno, D.; Labroli, M. A.; Xiao, Y.; Steensma, R. W.; Strizki, J. M.; Baroudy, B. M.; Cox, K.; Lachowicz, J.; Varty, G.; Watkins, R. *J. Med. Chem.* **2004**, *47*, 2405.

Bioorganic & Medicinal Chemistry 21 (2013) 2518–2526

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

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

CD4 mimics as HIV entry inhibitors: Lead optimization studies of the aromatic substituents

Tetsuo Narumi [a], Hiroshi Arai [a], Kazuhisa Yoshimura [b,c], Shigeyoshi Harada [b,c], Yuki Hirota [a], Nami Ohashi [a], Chie Hashimoto [a], Wataru Nomura [a], Shuzo Matsushita [b], Hirokazu Tamamura [a,*]

[a] Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Chiyoda-ku, Tokyo 101-0062, Japan
[b] Center for AIDS Research, Kumamoto University, Kumamoto 860-0811, Japan
[c] AIDS Research Center, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo 162-8640, Japan

ARTICLE INFO

Article history:
Received 22 January 2013
Revised 25 February 2013
Accepted 26 February 2013
Available online 7 March 2013

Keywords:
CD4 mimicry
Conformational change in gp120
HIV entry inhibitor
Envelope protein opener

ABSTRACT

Several CD4 mimics have been reported as HIV-1 entry inhibitors that can intervene in the interaction between a viral envelope glycoprotein gp120 and a cell surface protein CD4. Our previous SAR studies led to a finding of a highly potent analogue **3** with bulky hydrophobic groups on a piperidine moiety. In the present study, the aromatic ring of **3** was modified systematically in an attempt to improve its antiviral activity and CD4 mimicry which induces the conformational changes in gp120 that can render the envelope more sensitive to neutralizing antibodies. Biological assays of the synthetic compounds revealed that the introduction of a fluorine group as a *meta*-substituent of the aromatic ring caused an increase of anti-HIV activity and an enhancement of a CD4 mimicry, and led to a novel compound **13a** that showed twice as potent anti-HIV activity compared to **3** and a substantial increase in a CD4 mimicry even at lower concentrations.

1. Introduction

The first step of HIV entry into host cells is the interaction of a viral envelope glycoprotein gp120 with the cell surface protein CD4.[1] Such a viral attachment process is an attractive target for the development of the drugs to prevent the HIV-1 infection of its target cells.[2] Several small molecules including BMS-806,[3] IC-9564[4] and NBDs[5] have been identified that inhibit the viral attachment process by binding to gp120. Recently, we and others have been exploring the potentials of NBDs-derived CD4 mimics as a novel class of HIV entry inhibitors (Fig. 1).[6–8]

Small molecular CD4 mimics identified by an HIV syncytium formation assay showed potent cell fusion and virus cell fusion inhibitory activity against several HIV-1 laboratory and primary isolates.[5] Furthermore, the interaction of CD4 mimics with a highly conserved and functionally important pocket on gp120, known as the 'Phe43 cavity', induces conformational changes in gp120,[9] a process which occurs with unfavorable binding entropy, leading to a favorable enthalpy change similar to those caused by binding of the soluble CD4 binding to gp120. These unique properties render CD4 mimics valuable not only for the development of entry inhibitors, but which also, when combined with neutralizing antibodies function as envelope protein openers-putatively, stimulants.[10]

The structure of the complex formed by NBD-556 (**1**) bound to the gp120 core from an HIV-1 clade C strain (C1086) was recently determined by X-ray analysis (PDB: 3TGS).[11] As expected with molecular modeling by us[8a] and others,[6a] NBD-556 binds with Phe43 cavity with its *p*-chlorophenyl ring inserted into the cavity, and in addition multiple contacts were observed, with Trp112, Val255, Phe382, Ile424, Asn425, Trp427, Gly473, and Val430 of gp120 were observed (Fig. 2). However, no obvious interaction with Arg59 of CD4 was observed, although the salt bridge formation between Arg59 of CD4 and Asp368 of gp120 is a critical interaction of the viral attachment.[12] Based on this binding model, several potent compounds were recently identified.[6c,7]

Prior to those studies, we performed structure–activity relationship (SAR) studies based on the modification of the piperidine moiety of CD4 mimics to interact with Val430 and/or Asp368. These resulted in the discovery of a potent compound **3** which has bulky hydrophobic groups on its piperidine ring, and shows significant anti-HIV activity and lower cytotoxicity than other known CD4 mimics.[8c] Our study of the docking of **3** into the Phe43 cavity of gp120 suggests that the cyclohexyl group of **3** can interact hydrophobically with the isopropyl group of Val430.

We hypothesized that the optimization of the aromatic ring of **3** would lead to an increase of antiviral activity and CD4 mimicry, the latter inducing the conformational changes in gp120. Here, we de-

* Corresponding author. Tel.: +81 3 5280 8036; fax: +81 3 5280 8039.
E-mail address: tamamura.mr@tmd.ac.jp (H. Tamamura).

0968-0896/$ - see front matter © 2013 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.bmc.2013.02.041

scribe the systematic modification of the aromatic ring of **3** for further optimization to evaluate substituent effects on anti-HIV activity, cytotoxicity and CD4 mimicry.

2. Results and discussion

The co-crystal structure of **1** with the gp120 core revealed that the aromatic group of **1** binds to gp120 by several aromatic–aromatic and hydrophobic interactions (Fig. 2). In particular, hydrophobic space surrounded by the hydrophobic amino acid residues Trp112, Val255, Phe382, and Ile424 is likely to be affected by substituents at the *meta*- and *para*-positions of the aromatic ring, and consequently we decided to investigate substituents at these positions (Fig. 3).

Initially, we selected a chlorine or a methyl group to serve as the *para*-substituent of the aromatic group because CD4 mimic compounds such as **1** (NBD-556) with a *p*-chloro substituent, and because **3** showed significant anti-HIV activity compared to other substituents. Further, CD4 mimic structures such as **2** with a *p*-methyl substituent also showed potent anti-HIV activity and exhibits lower cytotoxicity than those with the *p*-chlorophenyl derivatives.[8a] Next, we chose several halogens including F, Cl and Br, to be the *meta*-substituent on the aromatic group since previous SAR studies revealed that the introduction of an appropriate group with an electron-withdrawing ability at the *meta*-position leads to an increase of binding affinity and antiviral activity.[6a] Furthermore, to investigate whether electron withdrawal and hydrophobicity of the *meta*-position are appropriate, the CD4 mimics with a *meta*-methyl substituent, which has electron-donating properties and is similar in size to bromine, were also synthesized. Finally, two piperidine scaffolds (the 2,2,6,6-tetramethylpiperidine **A** and the dicyclohexylpiperidine **B**) were combined with these aromatics via the oxalamide linker.

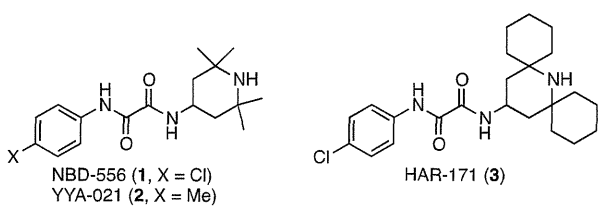


Figure 1. Structures of NBD-556 (**1**), YYA-021 (**2**) and HAR-171 (**3**).

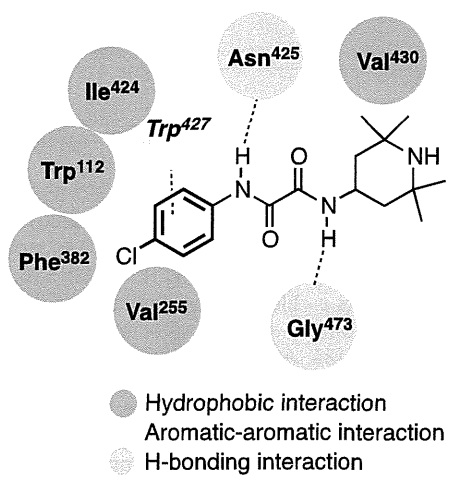


Figure 2. Major interactions between NBD-556 and Phe43 cavity of gp120.

2.1. Chemistry

The syntheses of novel compounds are depicted in Schemes 1 and 2. Starting from the appropriate aniline with *m*- and *p*-substituents, coupling with ethyl chloroglyoxylate in the presence of Et_3N gave the corresponding amidoesters **6a–c** and **7a–c**. Subsequently, microwave-assisted aminolysis[13] of **6a–c** and **7a–c** with commercially available 4-amino-2,2,6,6-tetramethylpiperidines afforded the desired compounds **8a–c** and **9a–c** (Scheme 1). A series of CD4 mimics with two cyclohexyl groups **13a–c** and **14a–c** were prepared from 2,2,6,6-tetramethylpiperidin-4-one **10** by the method previously reported,[8c] with slight modification (Scheme 2). Briefly, treatment of **10** with cyclohexanone in the presence of ammonium chloride gave a 2,6-substituted piperidin-4-one **11** via Grob fragmentation followed by intramolecular cyclization.[14] Reductive amination with *p*-methoxybenzyl amine, acidic treatment with TMSBr/TFA, and oxidative cleavage of *p*-methoxybenzyl group with cerium(IV) ammonium nitrates (CAN) furnished the corresponding 4-aminopiperidines (**12**) with higher yields and less burdensome purifications than the previous method. Finally, coupling of **12** with the corresponding esters **6a–c** and **7a–c** under microwave irradiation provided the desired compounds **13a–c** and **14a–c**.

2.2. Biological evaluation

The anti-HIV activity of the synthetic compounds was evaluated against an R5 primary isolate YTA strain. IC_{50} values were determined by the WST-8 method as the concentrations of the compounds that conferred 50% protection against HIV-1-induced cytopathogenicity in PM1/CCR5 cells. Cytotoxicity of the compounds based on the viability of mock-infected PM1/CCR5 cells was also evaluated using the WST-8 method. The assay results for compounds **8a–c** and **13a–c** with a *p*-chlorophenyl group are shown in Table 1. The parent compound **1** and compound **8a**,[6a] known as JRC-II-191, showed significant anti-HIV activities (IC_{50}

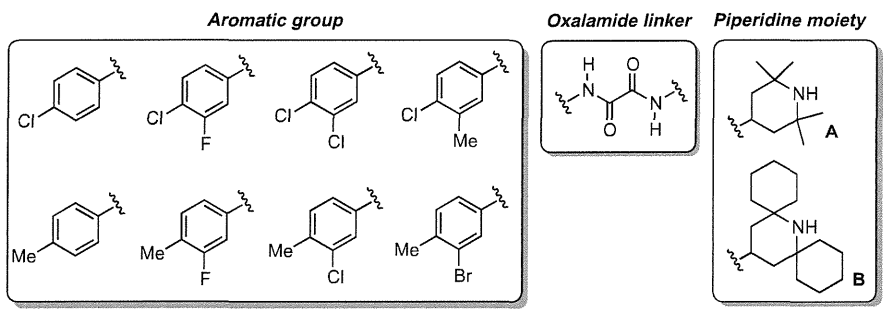
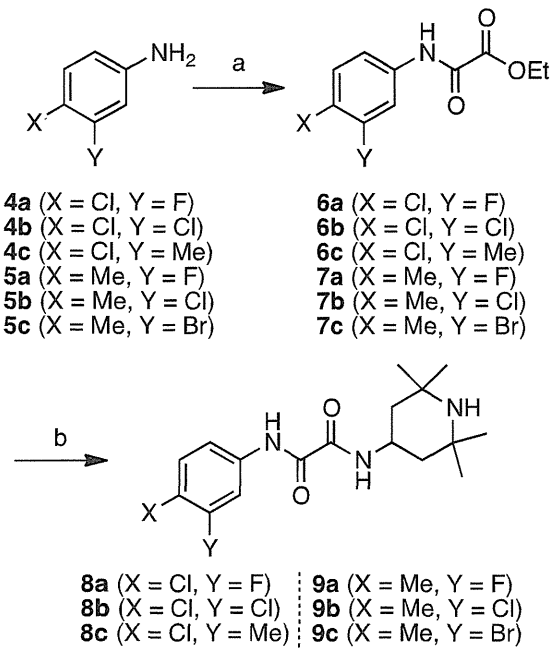
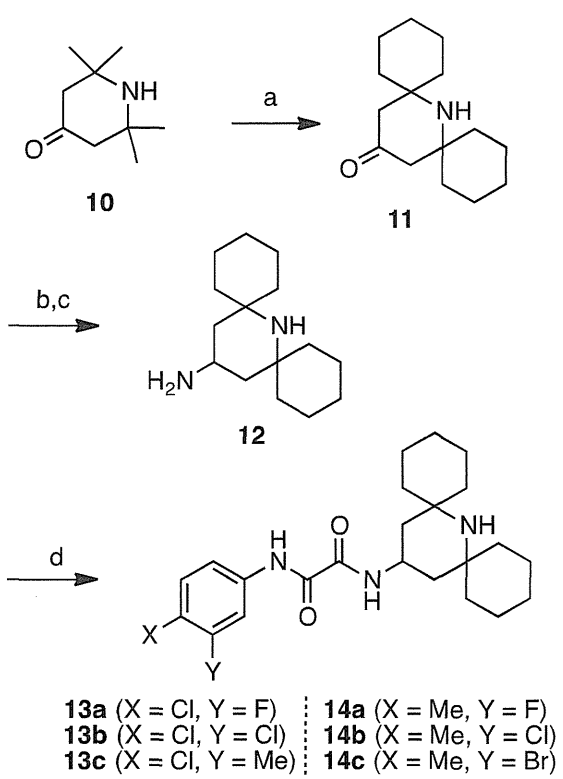


Figure 3. The structures of scaffolds in the design of novel CD4 mimics.



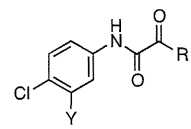
Scheme 1. Reagents and conditions: (a) ethyl chloroglyoxylate, Et_3N, THF; (b) 4-amino-2,2,6,6-tetramethylpiperidine, Et_3N, EtOH, 150 °C, microwave.

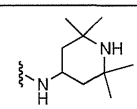
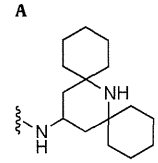


Scheme 2. Reagents and conditions: (a) cyclohexanone, NH_4Cl, DMSO, 60 °C; (b) *p*-methoxybenzylamine, $NaBH_3CN$, MeOH, then 1 M TMSBr in TFA; (c) CAN, CH_3CN/H_2O (v:v = 2:1); (d) **6** or **7**, Et_3N, EtOH, 150 °C, microwave.

of **1** = 0.61 μM and IC_{50} of **8a** = 0.32 μM). Compound **8b**[6a] having a *m,p*-dichlorophenyl group and compound **8c**[6a] (JRC-II-193) having a *p*-chloro-*m*-tolyl group showed moderate anti-HIV activity (IC_{50} of **8b** = 4.1 μM and IC_{50} of **8c** = 3.3 μM) but their potency was

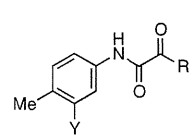
Table 1
Anti-HIV activity and cytotoxicity of compounds **8a–c** and **13a–c** containing a *p*-chlorophenyl group[a]

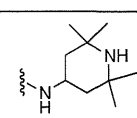
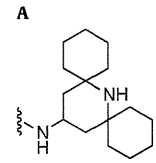


Compd	R	Y	IC_{50}[b] (μM) YTA48P	CC_{50}[c] (μM)
1		H	0.61	110
8a	**A**	F	0.32	94
8b	**A**	Cl	4.1	36
8c	**A**	Me	3.3	38
3		H	0.43	120
13a	**B**	F	0.23	11
13b	**B**	Cl	0.62	11
13c	**B**	Me	2.6	15

[a] All data are the mean values from three of more independent experiments.
[b] IC_{50} values of the multi-round assay are based on the inhibition of HIV-1-induced cytopathogenicity in PM1/CCR5 cells.
[c] CC_{50} values are based on the reduction of the viability of mock-infected PM1/CCR5 cells.

Table 2
Anti-HIV activity and cytotoxicity of compounds **9a–c** and **14a–c** containing a *p*-tolyl group[a]



Compd	R	Y	IC_{50}[b] (μM) YTA48P	CC_{50}[c] (μM)
2		H	9.0	260
9a	**A**	F	2.8	110
9b	**A**	Cl	3.2	62
9c	**A**	Br	>10	32
14a		F	0.54	91
14b	**B**	Cl	6.2	11
14c	**B**	Br	3.2	11

[a] All data are the mean values from three of more independent experiments.
[b] IC_{50} values of the multi-round assay are based on the inhibition of HIV-1-induced cytopathogenicity in PM1/CCR5 cells.
[c] CC_{50} values are based on the reduction of the viability of mock-infected PM1/CCR5 cells.

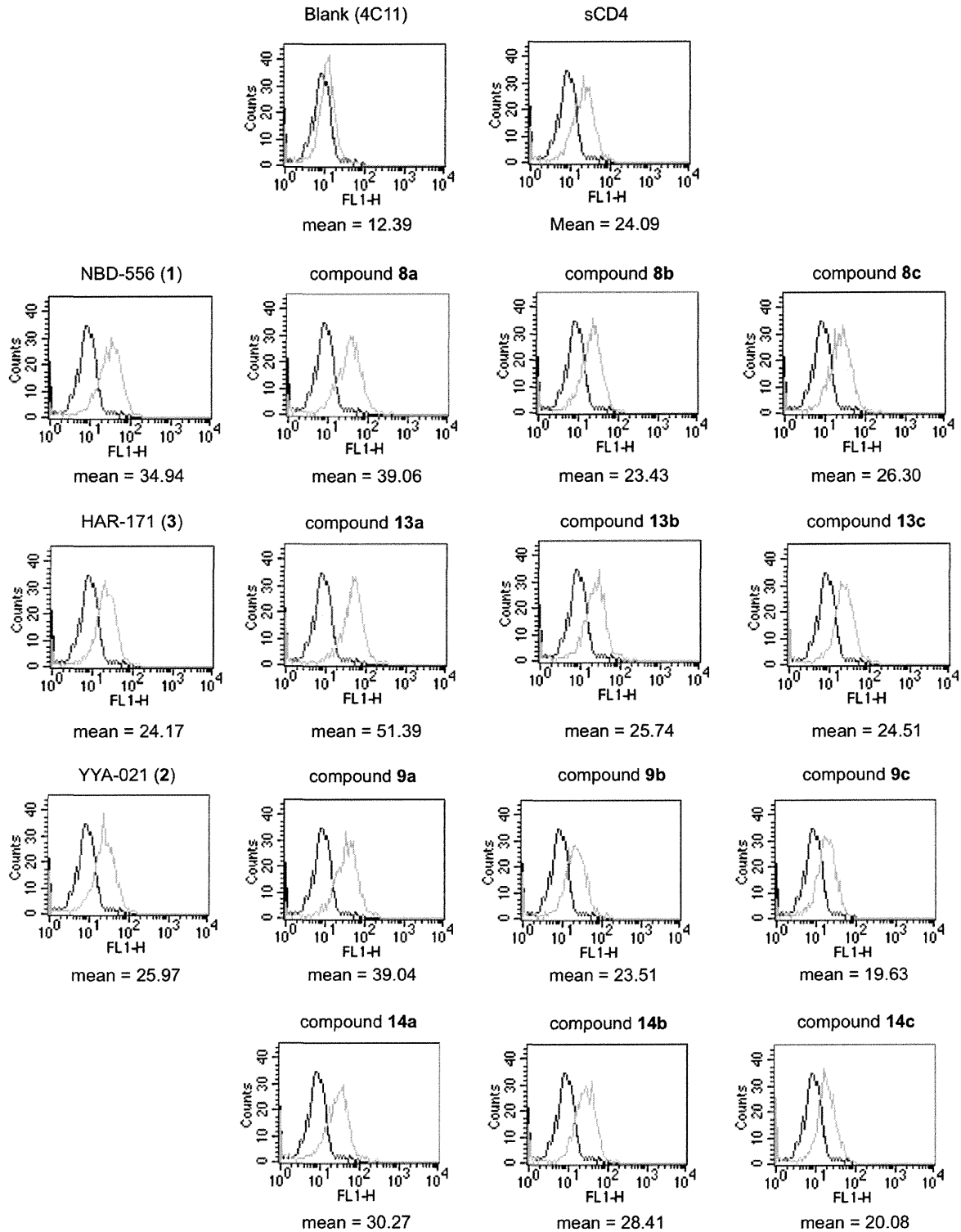


Figure 4. FACS analysis of synthetic compounds **8**, **9**, **13** and **14**.

approximately 10-fold lower than that of compound **8a**. The cytotoxicity of **8b** and **8c** is relatively stronger than that of **8a** (CC_{50} of **8b** = 36 μM and CC_{50} of **8c** = 38 μM). Compounds **13a–c** with hydrophobic cyclohexyl groups in the piperidine moiety showed more potent anti-HIV activity than the corresponding compounds **8a–c**, confirming the contribution of the bulky hydrophobic group(s) to an increase of antiviral activity. Our lead compound **3** showed significant anti-HIV activity comparable to that of compound **8a** (IC_{50} = 0.43 μM) but, consistent with previous results, exhibited lower cytotoxicity. In particular, compound **13a** with a *m*-fluoro-*p*-chlorophenyl group exhibited the highest anti-HIV activity. The IC_{50} value of **13a** was 0.23 μM, whose potency was

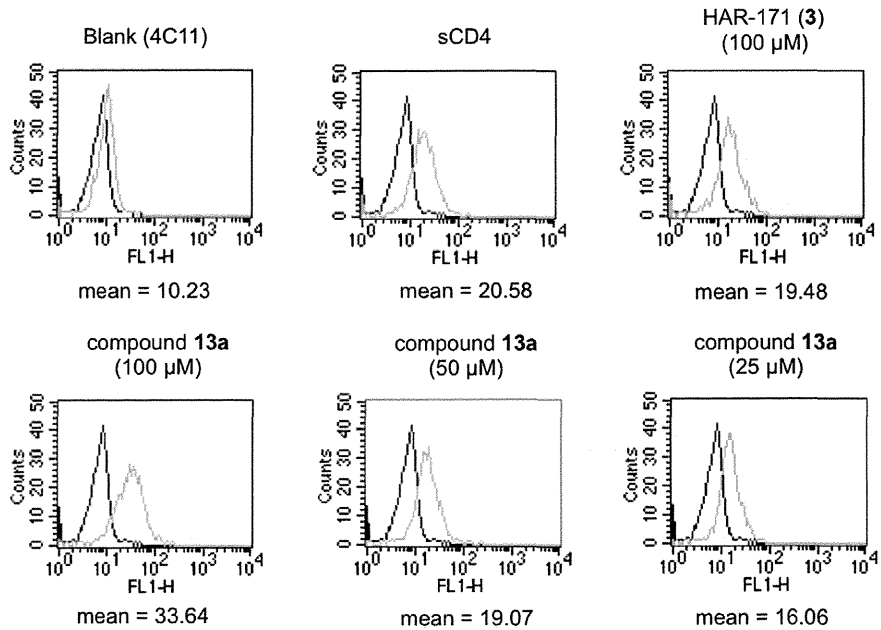


Figure 5. FACS analysis of **3** and **13a** in different concentrations.

approximately twice as high as that of compound **3**. Notably, compound **13b** with a *m,p*-dichlorophenyl group showed 7-fold more potent anti-HIV activity than the corresponding compound **8b**. Compound **13c**, which has a *p*-chloro-*m*-tolyl group, showed potent anti-HIV activity comparable to that of the corresponding compound **8c** and an increase of cytotoxicity (CC_{50} = 15 μM). We observed a tendency for compounds **13a–c** with both hydrophobic cyclohexyl groups and a *m,p*-disubstituted phenyl group to exhibit higher cytotoxicity than the corresponding tetramethyl-type compounds **8a–c**. No clear reason for an increase of cytotoxicity in the *m,p*-disubstituted phenyl group-containing compounds is apparent.

Assay results for the compounds **9a–c** and **14a–c** with a *p*-tolyl group are shown in Table 2. As expected, replacement of the *p*-chloro substituent with a *p*-methyl group resulted in somewhat reduction of anti-HIV activity. Compound **2**, YYA-021 has significant anti-HIV activity (IC_{50} = 9.0 μM) and exhibits the lowest cytotoxicity among all of the compounds tested (CC_{50} = 260 μM). These results are consistent with our previous SAR studies involving the aromatic ring. Introduction of a fluorine at the *meta*-position of the *p*-tolyl group, e.g. in compound **9a** and **14a**, improved the antiviral activity, as observed with **8a** and **13a** and a similar tendency was observed for compound **9b** with a *m*-chloro-*p*-tolyl group. In particular, compound **14a** with cyclohexyl groups and a *m*-fluoro-*p*-tolyl group showed slightly higher anti-HIV activity than the parent compound **1**. Among the compounds with *m*-bromo-*p*-tolyl groups, it was found that compound **9c**, with a 2,2,6,6-tetramethylpiperidine group, showed no anti-HIV activity at a concentration below 10 μM, whereas compound **14c** with hydrophobic cyclohexyl groups attached to the piperidine moiety, showed moderate activity (IC_{50} = 3.2 μM), indicating that the hydrophobic modification of piperidine ring can contribute to an increase in anti-HIV activity.

All the synthetic compounds were evaluated for their CD4 mimicry on the conformational changes in gp120 by fluorescence activated cell sorting (FACS) analysis, and the results are shown in Figure 4. The profile of binding of a CD4-induced (CD4i) monoclonal antibody (4C11) to the Env-expressing cell surface pretreated with the synthetic compounds was assessed in terms of the mean fluorescence intensity (MFI). The increase in binding affinity for 4C11 (by the pretreatment with synthetic compounds) suggests that those compounds can reflect the CD4 mimicry as a consequence of the conformational changes in gp120. Our previous studies disclosed that the profiles of the binding to the cell surface pretreated with **1**, **2**, or **3** were similar to those observed in pretreatment with soluble CD4, indicating that these compounds offer a significant enhancement of binding affinity for 4C11.[8] As shown in Figure 4, similar results were obtained with those compounds in this FACS analysis (MFI of **1**, **2**, and **3** = 34.94, 25.97, and 24.17, respectively). A notable increase in binding affinity for 4C11 was observed in essentially all the synthetic compounds. The compounds **8a**, **9a**, **13a** and **14a** with a *meta*-fluorine in the aromatic ring, showed significant anti-HIV activity, and produced a substantial increase in binding affinity for 4C11. These results suggested that the introduction of a fluorine group at the meta position of the aromatic ring is significant not only for the increase of anti-HIV activity, but also for the enhancement of a CD4 mimicry. In particular, a remarkable improvement in binding affinity for 4C11 was observed with **13a** (MFI = 51.39) which has twofold more potent anti-HIV activity than the lead compound **3** (HAR-171), and is the most active compound in terms of both anti-HIV activity and the CD4 mimicry resulting from the conformational change in gp120. The profiles of pretreatment of the cell surface with compounds **8b** and **13b** having a *m,p*-dichlorophenyl group, compounds **8c** and **13c** having a *p*-chloro-*m*-tolyl group, and compounds **9b** and **14b** with a *m*-chloro-*p*-tolyl group were similar to results obtained for **3**, suggesting that these compounds produced slightly lower enhancement compared to those of compounds **8a**, **9a**, **13a** and **14a** but significant levels of binding affinity for 4C11. On the other hand, pretreatment with compounds **9c**, which failed to show significant anti-HIV activity and **14c**, which had moderate anti-HIV activity resulted in a slight decrease of binding affinity for 4C11, suggesting that the introduction of a Br group at the *meta*-position of *p*-tolyl group is not advantageous to a CD4 mimicry, possibly due to the steric hindrance caused by the two bulky substituents. These results are consistent with previous observations that a limited size and electron-withdrawing ability of the aromatic substituents are required for potent anti-HIV activity and CD4 mimicry.[8a]

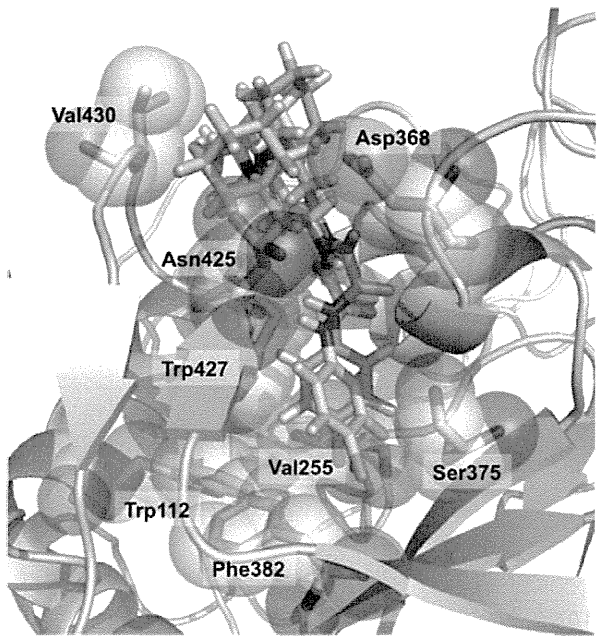


Figure 6. The modeled structure of **13a** (yellow carbon atoms) in the complex with the Phe43 cavity in gp120 (3TGS) overlaid with the modeled structure of **3** (green carbon atoms).

Since **13a** showed higher CD4 mimicry than the other compounds tested, the effect of the solution concentration of **13a** on the binding affinity for 4C11 was investigated. As shown in Figure 5, pretreatment of the cell surface with a 100 μM solution of **13a** produced a higher increase in the binding affinity for 4C11 than pretreatment with the same concentration of compound **3**. Interestingly, the profile pretreated with a 50 μM solution of **13a** was similar to that with a 100 μM of compound **3**, and even with a 25 μM solution of **13a** a potent enhancement of the binding affinity for 4C11 was observed: MFI of **13a** at concentrations of 50 μM and 25 μM = 19.07 and 16.06, respectively. This observation suggests that **13a** could serve as a novel lead compound for the development of envelope protein openers for the use combined with neutralizing antibodies because of its effectiveness at low concentrations.

The substantial increase in the CD4 mimicry of **13a** even at a low concentration is not easily explained because HAR-171 (**3**) and **13a** would be expected to form the similar binding modes with gp120. A probable contribution of **13a** is suggested by modeling studies docked into the Phe43 cavity in gp120 (3TGS) in which the depth and direction of the aromatic ring of **13a** is slightly different from those in compound **3** (Fig. 6), leading to the possible formation of appropriate interactions with the hydrophobic amino acid residues such as Val255 and Phe382, and therefore explaining the increased potency observed in the anti-HIV activity and CD4 mimicry of **13a**.

3. Conclusion

CD4 mimics are attractive agents not only for the development of a novel class of HIV entry inhibitors but also as possible cooperating agents for the neutralizing antibodies—that is, envelope protein openers. In the present study, a structure–activity relationship study of a series of CD4 mimic compounds was performed with a view to improving the biological activity of HAR-171 (**3**), which was identified in our previous studies as a promising lead compound with anti-HIV activity, cytotoxicity and CD4 mimicry resulting from the conformational change in gp120. Systematic modification of the *meta*- and *para*-substituents of the aromatic ring of **3** led to some potent compounds. In particular, **13a**, which has a bulky hydrophobic group on its piperidine ring and a *m*-fluoro-*p*-chlorophenyl group, demonstrated twofold more potent anti-HIV activity and much higher CD4 mimicry than **2** following the conformational changes in gp120, although the cytotoxicity of **13a** is relatively high. Further structural modification studies of the aromatic ring and the oxalamide linker to improve pharmaceutical profiles will be the subject of future reports.

4. Experimentals

^{1}H NMR and ^{13}C NMR spectra were recorded using a Bruker Avance III spectrometer. Chemical shifts are reported in δ (ppm) relative to Me_4Si (in $CDCl_3$) as internal standard. Low- and high-resolution mass spectra were recorded on a Bruker Daltonics microTOF focus in the positive and negative detection mode. For flash chromatography, silica gel 60 N (Kanto Chemical Co., Inc.) was employed. Microwave reactions were performed in Biotage Microwave Reaction Kit (sealed vials) in an Initiator™ (Biotage). The wattage was automatically adjusted to maintain the desired temperature for the desired period of time.

4.1. Chemistry

4.1.1. Ethyl 2-((4-chloro-3-fluorophenyl)amino)-2-oxoacetate (6a)

To a stirred solution of 3-fluoroaniline (1.11 g, 10.0 mmol) in $CHCl_3$ (30.0 mL) was added dropwise *N*-chlorosuccinimide (NCS) in $CHCl_3$ (20.0 mL) at 0 °C. The mixture was stirred at 0 °C for 42 h. After the reaction mixture was concentrated under reduced pressure, the residue was dissolved in Et_2O. The mixture was washed with water, and dried over $MgSO_4$. Concentration under reduced pressure followed by flash chromatography over silica gel with EtOAc/*n*-hexane gave 4-chloro-3-fluoroaniline (259.4 g, 18% yield) as crystalline solids. To a stirred solution of the above aniline (259.4 mg, 1.78 mmol) in THF (8.9 mL) were added at 0 °C ethyl chloroglyoxylate (237.3 μL, 2.14 mmol) and Et_3N (296.6 μL, 2.14 mmol). The mixture was stirred at room temperature for 12 h. After the precipitate was filtrated off, the filtrate solution was concentrated under reduced pressure. The residue was dissolved in EtOAc, and washed with 1.0 M HCl, saturated $NaHCO_3$ and brine, then dried over $MgSO_4$. Concentration under reduced pressure to provide the title compound **6a** (435.2 mg, 99% yield) as brown crystals, which was used without further purification.

^{1}H NMR (500 MHz, $CDCl_3$) δ 1.44 (t, J = 7.50 Hz, 3H), 4.43 (q, J = 7.50 Hz, 2H), 7.24–7.25 (m, 1H), 7.35–7.40 (m, 1H), 7.70–7.75 (m, 1H), 8.93 (br, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 13.0, 64.1, 108.5 (d, J = 26.3 Hz), 115.9 (d, J = 3.75 Hz), 117.3 (d, J = 18.8 Hz), 130.9 (d, J = 10.0 Hz), 135.9, 153.9, 158.1 (d, J = 246.3 Hz), 160.5; HRMS (ESI), *m/z* calcd for $C_{10}H_{10}ClFNO_3$ (MH^-) 244.0182, found 244.0183.

4.1.2. Ethyl 2-((3,4-dichlorophenyl)amino)-2-oxoacetate (6b)

To a stirred solution of 3,4-dichloroaniline **4b** (1.94 g, 12.0 mmol) in THF (20.0 mL) were added ethyl chloroglyoxylate (1.11 mL, 10.0 mmol) and Et_3N (15.2 mL, 11.0 mmol) at 0 °C. The mixture was stirred at room temperature for 6 h. After the precipitate was filtrated off, the filtrate solution was concentrated under reduced pressure. The residue was dissolved in EtOAc, and washed with 1.0 M HCl, saturated $NaHCO_3$ and brine, then dried over $MgSO_4$. Concentration under reduced pressure to provide the title compound **6b** (1.58 g, 95% yield) as white powder, which was used without further purification.

^{1}H NMR (500 MHz, $CDCl_3$) δ 1.44 (t, J = 7.00 Hz, 3H), 4.43 (q, J = 7.00 Hz, 2H), 7.44 (d, J = 8.50 Hz, 1H), 7.49–7.51 (m, 1H), 7.87, 2.35 (d, J = 2.50 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.0, 64.0, 119.0, 121.5, 129.0, 130.8, 133.2, 135.7, 153.9, 160.5; HRMS (ESI), m/z calcd for $C_{10}H_{10}Cl_2NO_3$ (MH^+) 262.0038, found 262.0031.

4.1.3. Ethyl 2-((4-chloro-3-methylphenyl)amino)-2-oxoacetate (6c)

By use of a procedure similar to that described for the preparation of compound **6b**, the aniline **4c** (3.34 g, 24.0 mmol) was converted into the title compound **6c** (4.63 g, 96% yield) as white powder.

^{1}H NMR (500 MHz, $CDCl_3$) δ 1.43 (t, J = 7.00 Hz, 3H), 2.38 (s, 3H), 4.42 (q, J = 7.00 Hz, 2H), 7.33 (d, J = 8.50 Hz, 1H), 7.43–7.46 (m, 1H), 7.51–7.54 (m, 1H), 8.82 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.0, 20.2, 63.8, 118.5, 122.0, 129.7, 130.9, 134.8, 137.1, 153.8, 160.9; HRMS (ESI), m/z calcd for $C_{11}H_{13}ClNO_3$ (MH^+) 242.0578, found 242.0568.

4.1.4. Ethyl 2-((3-fluoro-4-methylphenyl)amino)-2-oxoacetate (7a)

By use of a procedure similar to that described for the preparation of compound **6b**, the aniline **5a** (3.00 g, 24.0 mmol) was converted into the title compound **7a** (4.24 g, 94% yield) as white powder.

^{1}H NMR (500 MHz, $CDCl_3$) δ 1.43 (t, J = 7.20 Hz, 3H), 2.25 (s, 3H), 4.42 (q, J = 6.80 Hz, 2H), 7.12–7.21 (m, 2H), 7.48–7.56 (m, 1H), 8.83 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.2 (2C), 63.8, 107.1 (d, J = 27.5 Hz), 115.0 (d, J = 10.0 Hz), 122.3 (d, J = 17.5 Hz), 131.6 (d, J = 6.25 Hz), 135.3 (d, J = 13.8 Hz), 153.8, 160.8, 161.1 (d, J = 243.8 Hz); HRMS (ESI), m/z calcd for $C_{11}H_{13}FNO_3$ (MH^+) 226.0879, found 226.0878.

4.1.5. Ethyl 2-((3-chloro-4-methylphenyl)amino)-2-oxoacetate (7b)

By use of a procedure similar to that described for the preparation of compound **6b**, the aniline **5b** (3.40 g, 24.0 mmol) was converted into the title compound **7b** (5.19 g, 94% yield) as white powder.

^{1}H NMR (500 MHz, $CDCl_3$) δ 1.43 (t, J = 7.00 Hz, 3H), 2.35 (s, 3H), 4.42 (q, J = 7.00 Hz, 2H), 7.22 (d, J = 8.50 Hz, 1H), 7.41–7.43 (m, 1H), 7.71 (d, J = 2.00 Hz, 1H), 8.83 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.0, 20.0, 63.8, 118.0, 120.3, 131.2, 133.3, 134.7, 135.0, 153.8, 160.8; HRMS (ESI), m/z calcd for $C_{11}H_{13}ClNO_3$ (MH^+) 242.0584, found 242.0573.

4.1.6. Ethyl 2-((3-bromo-4-methylphenyl)amino)-2-oxoacetate (7c)

By use of a procedure similar to that described for the preparation of compound **6b**, the aniline **5c** (4.47 g, 27.0 mmol) was converted into the title compound **7c** (6.24 g, 96% yield) as white powder.

^{1}H NMR (500 MHz, $CDCl_3$) δ 1.43 (t, J = 7.00 Hz, 3H), 2.38 (s, 3H), 4.42 (q, J = 7.00 Hz, 2H), 7.23 (t, J = 8.50 Hz, 1H), 7.48–7.53 (m, 1H), 7.83–7.90 (m, 1H), 8.80 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.0, 22.4, 63.9, 118.7, 123.4, 125.0, 131.0, 135.0, 135.2, 153.7, 160.8; HRMS (ESI), m/z calcd for $C_{11}H_{13}BrNO_3$ (MH^+) 286.0079, found 286.0068.

4.1.7. N^1-(4-Chloro-3-fluorophenyl)-N^2-(2,2,6,6-tetramethylpiperidin-4-yl)oxalamide (8a)

To a solution of compound **6a** (70.0 mg, 0.286) in EtOH (2.9 mL) were added Et_3N (0.200 mL, 1.45 mmol) and 2,2,6,6-tetramethylpiperidin-4-amine (0.150 mL, 0.870 mmol). The reaction mixture was stirred for 3 h at 150 °C under microwave irradiation. After being concentrated in vacuo, the residue was extracted with $CHCl_3$, and washed with saturated $NaHCO_3$ and brine, then dried over $MgSO_4$. Concentration under reduced pressure to provide the title compound **8a** (34.6 mg, 34% yield) as white powder.

^{1}H NMR (500 MHz, $CDCl_3$) δ 0.99–1.50 (m, 15H), 1.92 (dd, J = 3.50, 9.00 Hz, 2H), 4.20–4.32 (m, 1H), 7.21–7.25 (m, 1H), 7.34–7.41 (m, 1H), 7.69–7.73 (m 1H), 9.31 (br, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 28.4, 34.8, 43.8, 44.5, 51.0, 108.3 (d, J = 26.3 Hz), 115.8 (d, J = 3.75 Hz), 117.1 (d, J = 17.5 Hz), 130.8, 136.2 (d, J = 8.75 Hz), 157.6, 158.1 (d, J = 247.5 Hz), 158.4; HRMS (ESI), m/z calcd for $C_{17}H_{24}ClFN_3O_2$ (MH^+) 356.1536, found 356.1548.

4.1.8. N^1-(3,4-Dichlorophenyl)-N^2-(2,2,6,6-tetramethylpiperidin-4-yl)oxalamide (8b)

By use of a procedure similar to that described for the preparation of compound **8a**, the compound **6b** (261.0 mg, 1.00 mmol) was converted into the title compound **8b** (520.0 mg, 70% yield) as white powder.

^{1}H NMR (500 MHz, $CDCl_3$) δ 1.07 (t, J = 12.0 Hz, 2H), 1.16 (s, 6H), 1.28 (s, 6H), 1.90–1.93 (m, 2H), 4.20–4.32 (m, 1H), 7.26 (m, 1H), 7.40–7.48 (m, 2H), 7.88 (s, 1H), 9.33 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 28.5 (2C), 34.9 (2C), 43.8, 44.6 (2C), 50.9 (2C), 119.0, 121.4, 128.7, 130.8, 133.1, 135.8, 157.7, 158.5; HRMS (ESI), m/z calcd for $C_{17}H_{22}Cl_2N_3O_2$ (MH^-) 370.1095, found 370.1105.

4.1.9. N^1-(4-Chloro-3-methylphenyl)-N^2-(2,2,6,6-tetramethylpiperidin-4-yl)oxalamide (8c)

By use of a procedure similar to that described for the preparation of compound **8a**, the compound **6c** (482.0 mg, 2.00 mmol) was converted into the title compound **8c** (364.0 mg, 49% yield) as white powder.

^{1}H NMR (500 MHz, $CDCl_3$) δ 1.07 (t, J = 12.0 Hz, 2H), 1.15 (s, 6H), 1.28 (s, 6H), 1.86–1.94 (m, 2H), 4.15–4.31 (m, 1H), 7.21–7.24 (m, 1H), 7.32–7.38 (m, 2H), 7.74 (s, 1H), 9.24 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 19.6, 28.5 (2C), 34.9 (2C), 43.7, 44.7 (2C), 50.9 (2C), 117.9, 120.2, 131.2, 133.1, 134.7, 135.1, 157.5, 158.8; HRMS (ESI), m/z calcd for $C_{18}H_{25}ClN_3O_2$ (MH^-) 350.1641, found 350.1656.

4.1.10. N^1-(3-Fluoro-4-methylphenyl)-N^2-(2,2,6,6-tetramethylpiperidin-4-yl)oxalamide (9a)

By use of a procedure similar to that described for the preparation of compound **8a**, the compound **7a** (225.0 mg, 1.00 mmol) was converted into the title compound **9a** (161.0 mg, 48% yield) as white powder.

^{1}H NMR (500 MHz, $CDCl_3$) δ 1.07 (t, J = 12.5 Hz, 2H), 1.15 (s, 6H), 1.28 (s, 6H), 1.92 (dd, J = 12.5, 3.50 Hz, 2H), 2.26 (s, 3H), 4.12–4.32 (m, 1H), 7.12–7.20 (m, 2H), 7.30–7.37 (m, 1H), 7.48–7.54 (m, 1H), 9.27 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.2, 28.5 (2C), 34.9 (2C), 43.7, 44.7 (2C), 50.9 (2C), 107.1 (d, J = 26.3 Hz), 115.0, 121.8 (d, J = 17.5 Hz) 131.6, 135.4, (d, J = 15.0 Hz), 157.5, 158.8, 161.1 (d, J = 242.5 Hz); HRMS (ESI), m/z calcd for $C_{18}H_{25}FN_3O_2$ (MH^-) 334.1936, found 334.1942.

4.1.11. N^1-(3-Chloro-4-methylphenyl)-N^2-(2,2,6,6-tetramethylpiperidin-4-yl)oxalamide (9b)

By use of a procedure similar to that described for the preparation of compound **8a**, the compound **7b** (482.0 mg, 1.00 mmol) was converted into the title compound **9b** (448.0 mg, 48% yield) as white powder.

^{1}H NMR (500 MHz, $CDCl_3$) δ 1.09 (t, J = 12.5 Hz, 3H), 1.18 (s, 6H), 1.30 (s, 6H), 1.93–1.95 (m, 2H), 2.41 (s, 3H), 4.20–4.34 (m, 1H), 7.30–7.37 (m, 2H), 7.44–7.46 (m, 1H), 7.53 (d, J = 2.50 Hz, 1H), 9.25 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 20.3, 28.5 (2C), 34.9 (2C), 43.7, 44.7 (2C), 50.9 (2C), 118.5, 122.0, 130.0, 130.7, 134.8, 137.1, 157.5, 158.8; HRMS (ESI), m/z calcd for $C_{18}H_{25}ClN_3O_2$ (MH^-) 350.1641, found 350.1636.

4.1.12. N^1-(3-Bromo-4-methylphenyl)-N^2-(2,2,6,6-tetramethylpiperidin-4-yl)oxalamide (9c)

By use of a procedure similar to that described for the preparation of compound **8a**, the compound **7c** (285.0 mg, 1.00 mmol) was converted into the title compound **9c** (157.0 mg, 40% yield) as white powder.

^{1}H NMR (500 MHz, $CDCl_3$) δ 1.07 (t, J = 12.5 Hz, 3H), 1.15 (s, 6H), 1.28 (s, 6H), 1.91 (dd, J = 8.00, 4.00 Hz, 2H), 2.38 (s, 3H), 3.70–3.75 (m, 1H), 7.22 (d, J = 8.50 Hz, 1H), 7.30–7.37 (m, 1H), 7.43–7.45 (m, 1H), 7.90 (d, J = 2.50 Hz, 1H), 9.25 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 22.4, 28.5 (2C), 34.9 (2C), 43.7, 44.7 (2C), 50.9 (2C), 118.6, 123.4, 125.0, 131.0, 134.9 (2C), 157.5, 158.8; HRMS (ESI), m/z calcd for $C_{18}H_{25}BrN_3O_2$ (MH^-) 394.1136, found 394.1158.

4.1.13. Amine (12)

The compound **11** was prepared according to the reported procedure.[14] To a stirred solution of piridone **11** (247.8 mg, 1.05 mmol) in MeOH (2.10 mL) was added p-methoxybenzylamine (0.41 mL, 3.15 mmol). After being stirred at room temperature for 23 h, sodium cyanoborohydride was added and stirred at room temperature for 48 h. The reaction mixture was poured into saturated $NaHCO_3$ and extracted with EtOAc, then dried over $MgSO_4$. After concentration under reduced pressure, the residue was treated with 1 M TMS in THF (4.8 mL). The mixture was stirred at 0 °C for 14 h. Concentration under reduced pressure followed by short chromatography with $CHCl_3$/MeOH gave the PMB-protected amine. To a solution of the above amine (584.0 mg, 1.64 mmol) in CH_3CN/H_2O (13.1 mL, v:v = 2:1) was added CAN (2.74 g, 8.2 mmol). The mixture was stirred at room temperature for 14 h. The reaction mixture was diluted with 0.5 M HCl and washed with CH_2Cl_2. The water layer was alkalized and extracted with EtOAc, then dried over Na_2SO_4. Concentration under reduced pressure followed by flash chromatography over silica gel with EtOAc–EtOH (4:1) to gave the title compound **12** (175.5 mg, 71% yield) as a yellow oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ 1.15–1.85 (m, 24H), 2.95–3.05 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 22.2 (2C), 22.8 (2C), 26.2 (2C), 37.3 (2C), 42.3 (2C), 43.6 (2C), 47.0, 53.2 (2C); HRMS (ESI), m/z calcd for $C_{15}H_{29}N_2$ (MH^+) 237.2325, found 237.2321.

4.1.14. N^1-((4-Chloro-3-fluorophenyl)-N^2-(2,6-dicyclohexylpiperidin-4-yl)oxalamide (13a)

By use of a procedure similar to that described for the preparation of compound **8a**, the compound **6a** (36.8 mg, 0.150 mmol) was converted into the title compound **13a** (7.6 mg, 12% yield) as yellow powder.

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.71–2.28 (m, 24H), 2.03–2.20 (m, 2H), 4.02–4.16 (m, 1H), 7.13–7.18 (m, 1H), 7.27–7.33 (m, 1H), 7.62–7.66 (m, 1H), 9.25 (br, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.1, 22.0 (2C), 22.6 (2C), 25.8 (2C), 29.3, 29.7 (2C), 31.9, 70.5, 108.3 (d, J = 26.3 Hz), 115.8, 117.1 (d, J = 18.8 Hz), 130.8, 136.2 (d, J = 10.0 Hz), 157.6, 158.1 (d, J = 247.5 Hz), 158.6; HRMS (ESI), m/z calcd for $C_{23}H_{32}ClFN_3O_2$ (MH^+) 436.2162, found 436.2156.

4.1.15. N^1-(4-Chlorophenyl)-N^2-(2,6-dicyclohexylpiperidin-4-yl)oxalamide (13b)

By use of a procedure similar to that described for the preparation of compound **8a**, the compound **6b** (31.3 mg, 0.120 mmol) was converted into the title compound **13b** (28.0 mg, 52% yield) as white powder.

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.96 (t, J = 12.5 Hz, 2H), 1.10–1.84 (br, 20H), 2.05–2.19 (m, 2H), 4.08–4.21 (m, 1H), 7.23–7.33 (br, 1H), 7.39–7.46 (m, 2H), 7.88 (t, J = 1.00 Hz, 1H), 9.34 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.1, 22.1 (2C), 22.7 (2C), 26.1 (2C), 31.6, 37.2 (2C), 42.6, 43.0, 43.6, 52.6 (2C), 119.0, 121.4, 128.7, 130.8, 133.1, 135.8, 157.7, 158.5; HRMS (ESI), m/z calcd for $C_{23}H_{32}Cl_2N_3O_2$ (MH^+) 452.1872, found 452.1865.

4.1.16. N^1-((4-Chloro-3-methylphenyl)-N^2-(2,6-dicyclohexylpiperidin-4-yl)oxalamide (13c)

By use of a procedure similar to that described for the preparation of compound **8a**, the compound **6c** (121.0 mg, 0.500 mmol) was converted into the title compound **13c** (15.1 mg, 7% yield) as white powder.

^{1}H NMR (500 MHz, $CDCl_3$) δ 0.87–1.88 (br, 22H), 2.09–2.20 (m, 2H), 2.38 (s, 3H), 4.09–4.22 (m, 1H), 7.32–7.33 (m, 1H), 7.41–7.43 (m, 1H), 7.51 (d, J = 2.00 Hz, 1H), 7.73 (m, 1H), 9.24 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 20.2, 22.1 (2C), 22.7 (2C), 26.0 (2C), 29.7, 37.0, 42.3 (2C), 42.8 (2C), 43.4, 52.9 (2C), 118.4, 122.0, 130.0, 130.6, 134.8, 137.1, 157.5, 158.9; HRMS (ESI), m/z calcd for $C_{24}H_{35}ClN_3O_2$ (MH^+) 430.2267, found 430.2264.

4.1.17. N^1-(3-Fluoro-4-methylphenyl)-N^2-(2,6-dicyclohexylpiperidin-4-yl)oxalamide (14a)

By use of a procedure similar to that described for the preparation of compound **8a**, the compound **7a** (225.0 mg, 1.00 mmol) was converted into the title compound **14a** (27.5 mg, 7% yield) as white powder.

^{1}H NMR (500 MHz, $CDCl_3$) δ 0.971 (t, J = 12.5 Hz, 2H), 1.18–1.86 (m, 20H), 2.13–2.16 (m, 2H), 2.26 (s, 3H), 4.09–4.21 (m, 1H), 7.13–7.18 (m, 2H), 7.33 (d, J = 8.00 Hz, 1H), 7.50–7.53 (m, 1H), 9.27 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.2, 22.2 (2C), 22.8 (2C), 26.1 (2C), 37.2 (2C), 42.2 (2C), 43.3 (2C), 43.5, 52.6 (m, 2C), 107.0 (d, J = 27.5 Hz), 115.0 (d, J = 3.75 Hz), 121.8 (d, J = 17.5 Hz), 131.6 (d, J = 6.25 Hz), 135.4 (d, J = 10.0 Hz), 157.5, 158.9, 161.3 (d, J = 242.5 Hz); HRMS (ESI), m/z calcd for $C_{24}H_{33}FN_3O_2$ (MH^-) 414.2554, found 414.2562.

4.1.18. N^1-(3-Chloro-4-methylphenyl)-N^2-(2,6-dicyclohexylpiperidin-4-yl)oxalamide (14b)

By use of a procedure similar to that described for the preparation of compound **8a**, the compound **7b** (120.5 mg, 0.500 mmol) was converted into the title compound **14b** (12.9 mg, 6% yield) as white powder.

^{1}H NMR (500 MHz, $CDCl_3$) δ 0.973 (t, J = 12.5 Hz, 2H), 1.18–1.86 (br, 20H), 2.11–2.19 (m, 2H), 2.35 (s, 3H), 4.09–4.21 (m, 1H), 7.20–7.22 (m, 1H), 7.30–7.32 (m, 1H), 7.35–7.37 (d, J = 2.50 Hz, 1H), 7.73 (m, 1H), 9.22 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 19.6, 22.1 (2C), 22.7 (2C), 26.0 (2C), 29.7, 37.0, 42.1 (2C), 42.7 (2C), 43.2, 53.3 (2C), 118.0, 120.3, 131.2, 133.0, 134.7, 135.1, 157.5, 158.8; HRMS (ESI), m/z calcd for $C_{24}H_{33}ClN_3O_2$ (MH^+) 430.2267, found 430.2257.

4.1.19. N^1-(3-Bromo-4-methylphenyl)-N^2-(2,6-dicyclohexylpiperidin-4-yl)oxalamide (14c)

By use of a procedure similar to that described for the preparation of compound **8a**, the compound **7c** (142.0 mg, 0.500 mmol) was converted into the title compound **14c** (11.5 mg, 5% yield) as white powder.

^{1}H NMR (500 MHz, $CDCl_3$) δ 0.67–2.07 (br, 22H), 2.28 (br, 2H), 2.38 (s, 3H), 4.09–4.21 (m, 1H), 7.22 (d, J = 8.00 Hz, 1H), 7.28–7.38 (br, 1H), 7.43 (dd, J = 4.50, 2.50 Hz, 1H), 7.90 (d, J = 2.50 Hz, 1H), 9.21 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.1, 22.1 (2C), 22.4 (2C), 22.7 (2C), 25.9, 30.0, 31.6, 36.9 (2C), 42.7 (3C), 52.7, 52.9, 118.6, 123.4, 125.0, 131.0, 134.9, 135.1, 157.4, 158.8; HRMS (ESI), m/z calcd for $C_{24}H_{33}BrN_3O_2$ (MH^+) 474.1762, found 474.1746.

4.2. Antiviral assay and cytotoxicity assay

Anti-HIV activity and cytotoxicity measurements in PM1/CCR5 cells (Yoshimura et al., 2010) were based on viability of cells that

had been infected or not infected with 100 TCID50 of an R5 primary isolate YTA48P exposed to various concentrations of the test compound. After the PM1/CCR5 cells were incubated at 37 °C for 7 days. The 50% inhibitory concentration (IC_{50}) values and the 50% cytotoxic concentration (CC_{50}) were then determined using the Cell Counting Kit-8 assay (Dojindo Laboratories). All assays were performed in duplicate or triplicate.

4.3. FACS analysis

JR-FL (R5, Sub B) chronically infected PM1 cells were pre-incubated with 0.5 μg/mL of sCD4 or 100 μM of a CD4 mimic for 15 min, and then incubated with an anti-HIV-1 mAb, 4C11, at 4 °C for 15 min. The cells were washed with PBS, and fluorescein isothiocyanate (FITC)-conjugated mouse anti-human IgG antibody was used for antibody-staining. Flow cytometry data for the binding of 4C11 (green lines) to the Env-expressing cell surface in the presence of a CD4 mimic are shown among gated PM1 cells along with a control antibody (anti-human CD19: black lines). Data are representative of the results from a minimum of two independent experiments. The number at the bottom of each graph shows the mean fluorescence intensity (MFI) of the antibody 4C11.

4.4. Molecular modeling

Dockings of compounds **3** and **13a** were performed using Molecular Operating Environment modeling package (MOE 2008. 10, Canada), into the crystal structure of gp120 (PDB, entry 3TGS).

Acknowledgements

This work was supported in part by Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and Health and Labour Sciences Research Grants from Japanese Ministry of Health, Labor, and Welfare. We are grateful to Professor Yoshio Hayashi and Dr. Fumika Yakushiji, Tokyo University of Pharmacy and Life Sciences for their assistance in the molecular modelings.

Supplementary data

Supplementary data (NMR charts of compounds) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.02.041.

References and notes

1. Chan, D. C.; Kim, P. S. *Cell* **1998**, *93*, 681.
2. (a) Kadow, J.; Wang, H.-G.; Lin, P.-F. *Curr. Opin. Investig. Dugs* **2006**, *7*, 721; (b) Repik, A.; Clapham, P. R. *Structure* **2008**, *16*, 1603.
3. Holz-Smith, S.; Sun, I. C.; Jin, L.; Matthews, T. J.; Lee, K. H.; Chen, C. H. *Antimicrob. Agents Chemother.* **2001**, *45*, 60.
4. Lin, P.-F.; Blair, W.; Wang, T.; Spicer, T.; Guo, Q.; Zhou, N.; Gong, Y.-F.; Wang, H.-F. H.; Rose, R.; Yamanaka, G.; Robinson, B.; Li, C.-B.; Fridell, R.; Deminie, C.; Demers, G.; Yang, Z.; Zadjura, L.; Meanwell, N.; Colonno, R. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 11013.
5. Zhao, Q.; Ma, L.; Jiang, S.; Lu, H.; Liu, S.; He, Y.; Strick, N.; Neamati, N.; Debnath, A. K. *Virology* **2005**, *339*, 213.
6. (a) Madani, N.; Schön, A.; Princiotto, A. M.; LaLonde, J. M.; Courter, J. R.; Soeta, T.; Ng, D.; Wang, L.; Brower, E. T.; Xiang, S.-H.; Do Kwon, Y.; Huang, C.-C.; Wyatt, R.; Kwong, P. D.; Freire, E.; Smith, A. B., III; Sodroski, J. *Structure* **2008**, *16*, 1689; (b) LaLonde, J. M.; Elban, M. A.; Courter, J. R.; Sugawara, A.; Soeta, T.; Madani, N.; Princiotto, A. M.; Kwon, Y. D.; Kwong, P. D.; Schön, A.; Freire, E.; Sodroski, J.; Smith, A. B., III *Bioorg. Med. Chem. Lett.* **2011**, *20*, 354; (c) LaLonde, J. M.; Kwon, Y. D.; Jones, D. M.; Sun, A. W.; Courter, J. R.; Soeta, T.; Kobayashi, T.; Princiotto, A. M.; Wu, X.; Schön, A.; Freire, E.; Kwong, P. D.; Mascola, J. R.; Sodroski, J.; Madani, N.; Smith, A. B., III *J. Med. Chem.* **2012**, *55*, 4382.
7. Curreli, F.; Choudhury, S.; Pyatkin, I.; Zagorodnikov, V. P.; Bulay, A. K.; Altieri, A.; Kwon, Y. D.; Kwon, P. D.; Debnath, A. K. *J. Med. Chem.* **2012**, *55*, 4764.
8. (a) Yamada, Y.; Ochiai, C.; Yoshimura, K.; Tanaka, T.; Ohashi, N.; Narumi, T.; Nomura, W.; Harada, S.; Matsushita, S.; Tamamura, H. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 354; (b) Narumi, T.; Ochiai, C.; Yoshimura, K.; Harada, S.; Tanaka, T.; Nomura, W.; Arai, H.; Ozaki, T.; Ohashi, N.; Matsushita, S.; Tamamura, H. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 5853; (c) Narumi, T.; Arai, H.; Yoshimura, K.; Harada, S.; Nomura, W.; Matsushita, S.; Tamamura, H. *Bioorg. Med. Chem.* **2011**, *19*, 6735.
9. (a) Schön, A.; Madani, N.; Klein, J. C.; Hubicki, A.; Ng, D.; Yang, X.; Smith, A. B., III; Sodroski, J.; Freire, E. *Biochemistry* **2006**, *45*, 10973; (b) Schön, A.; Lam, S. Y.; Freire, E. *Future Med. Chem.* **2011**, *3*, 1129.
10. Yoshimura, K.; Harada, S.; Shibata, J.; Hatada, M.; Yamada, Y.; Ochiai, C.; Tamamura, H.; Matsushita, S. *J. Virol.* **2010**, *84*, 7558.
11. Kwon, Y. D.; Finzi, A.; Wu, X.; Dogo-Isonagie, C.; Lee, L. K.; Moore, L. R.; Schmidt, S. D.; Stuckey, J.; Yang, Y.; Zhou, T.; Zhu, J.; Vicic, D. A.; Debnath, A. K.; Shapiro, L.; Bewley, C. A.; Mascola, J. R.; Sodroski, J. G.; Kwong, P. D. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 5663.
12. (a) Kwong, P. D.; Wyatt, R.; Robinson, J.; Sweet, R. W.; Sodroski, J.; Hendrickson, W. A. *Nature* **1998**, *393*, 648; (b) Kwong, P. D.; Wyatt, R.; Mcajeed, S.; Robinson, J.; Sweet, R. W.; Sodroski, J.; Hendrickson, W. A. *Structure* **2000**, *8*, 1329.
13. McFarland, C.; Vicic, D. A.; Debnath, A. K. *Synthesis* **2006**, 807.
14. Sakai, K.; Yamada, K.; Yamasaki, T.; Kinoshita, Y.; Mito, F.; Utsumi, H. *Tetrahedron* **2010**, *66*, 2311.