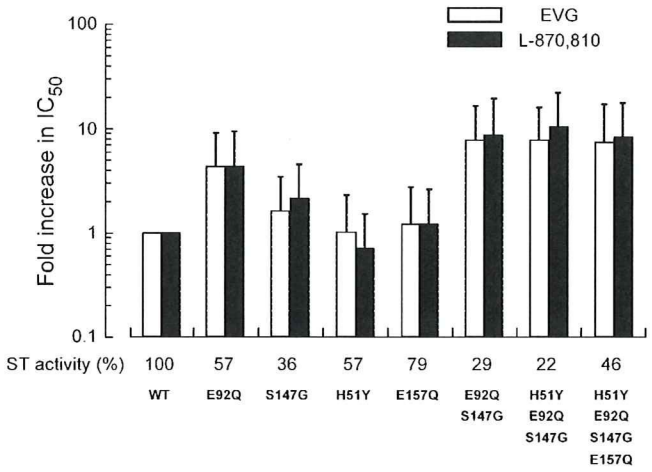


FIG. 4. Effect of EVG-selected mutations on IN strand transfer activity and on the inhibition of strand transfer by IN inhibitors. The strand transfer activities of recombinant IN enzymes carrying EVG-selected mutations were determined using an oligonucleotide-based strand transfer assay. Strand transfer (ST) activity of IN mutants was compared to that of the wild type (WT); results are shown as percentages of wild-type activity. The effect of IN inhibitors on strand transfer was also determined for wild-type and mutant IN enzymes; results are expressed as the increase (*n*-fold) in IC_{50} values of inhibitors relative to those of the wild type.

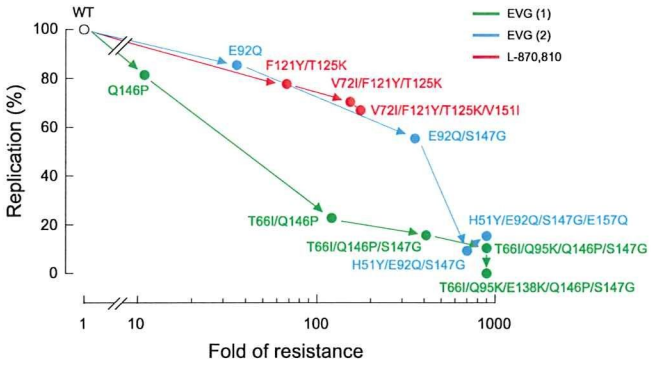


FIG. 5. Replication kinetics of EVG- and L-870,810-resistant viral variants. The replication kinetics of wild-type and IN inhibitor-resistant viral variants were determined by p24 ELISA. The relationship of replication capacity and change (*n*-fold) in susceptibility (shown in Table 3) is depicted. Variants are plotted according to the observed order of their emergence during selection experiments in vitro. Replication kinetics of EVG-selected mutants derived from the two independent selection experiments (shown in Fig. 3) are plotted in different colors. WT, wild type.

bility to EVG at the level of inhibition of strand transfer, consistent with its identification as a primary EVG resistance mutation in the virological analyses.

Replication kinetics of IN inhibitor-resistant variants. The effects of IN mutations on the replication kinetics of HIV-1 variants were assessed by comparing their levels of p24 production in culture supernatants to that of wild-type virus (Fig. 5). At day 5 postinfection, levels of p24 production by the HIV-1_{E92Q} and HIV-1_{Q146P} variants were 86% and 82% of HIV-1_{WT} levels, respectively. These variants showed high-level (36-fold) or moderate (11-fold) resistance to EVG (Table 3), whereas the replication levels of both were similar to those of the wild type. However, the introduction of additional EVG resistance mutations further decreased p24 production, which is indicative of a decline in the levels of viral replication. In particular, HIV-$1_{T66I/Q146P/S147G}$, HIV-$1_{T66I/Q95K/Q146P/S147G}$, HIV-$1_{T66I/Q95K/E138K/Q146P/S147G}$, HIV-$1_{H51Y/E92Q/S147G/}$, and HIV-$1_{H51Y/E92Q/S147G/E157Q}$ all showed significantly reduced levels of p24 production (less than 20% of wild-type levels by day 5 in all cases). Thus, there was an inverse correlation between the levels of EVG resistance and the viral replication capacity; that is, as resistance to EVG increased, viral replication decreased. Interestingly, viral variants carrying L-870,810-selected mutations had more moderate reductions in replication capacity, even in the case of the HIV-$1_{V72I/F121Y/T125K/V151I}$ variant that had high-level resistance to both L-870,810 and EVG (68% of wild-type levels). These results indicate that mutations associated with resistance to IN inhibitors can have various effects on viral replication capacity. The reduced replication capacity of EVG-resistant variants was not rescued in the presence of the inhibitor (data not shown), as was observed previously for NFV-resistant variants in the presence of NFV (35). Thus, the reduced replication capacity of IN inhibitor-resistant variants may present a barrier to their emergence in vivo.

Antiviral effect of IN inhibitors on retroviruses. The antiviral activity of EVG against other retroviruses, including MLV and SIV, was assessed. EVG and L-870,810 inhibited the integration of the HIV-based vector used as a positive control for the luciferase assay (EC_{50} values of 0.8 and 5.0 nM, respectively), as observed in the MAGI assay with HIV-1_{IIIB} (Fig. 6). EVG and L-870,810 suppressed the replication of MLV infection (EC_{50} values of 5.8 and 22 nM, respectively) as well as that of the primate retrovirus SIV (0.5 and 3.2 nM, respectively), indicating that IN inhibitors have antiviral activity against a broad range of retroviruses.

DISCUSSION

The data described here show that EVG inhibits HIV replication by specifically blocking the strand transfer reaction mediated by IN, as demonstrated by the intracellular accumulation of 2-LTR DNA products, a signature of nonproductive integration. Furthermore, EVG directly blocked the production of strand transfer products in an in vitro strand transfer assay. Confirming that EVG is a bona fide IN inhibitor, we selected EVG-resistant viral variants in vitro and demonstrated that the resulting viral variants had acquired multiple mutations in the IN coding region and had simultaneously acquired reduced phenotypic susceptibility to EVG. HIV-1 molecular clones carrying the EVG-selected IN mutations had an EVG-resistant phenotype and in many cases also had reduced susceptibility to another IN inhibitor, L-870,810. These data provide formal proof that the observed IN mutations are indeed EVG resistance mutations and that EVG is an IN inhibitor.

Among the IN mutations observed to be selected by EVG, two mutations, T66I and E92Q, appeared to provide the major contribution to EVG resistance. Both of these individual mutations resulted in >30-fold reduced susceptibility to EVG. The T66I mutation conferred cross-resistance to S-1360 and

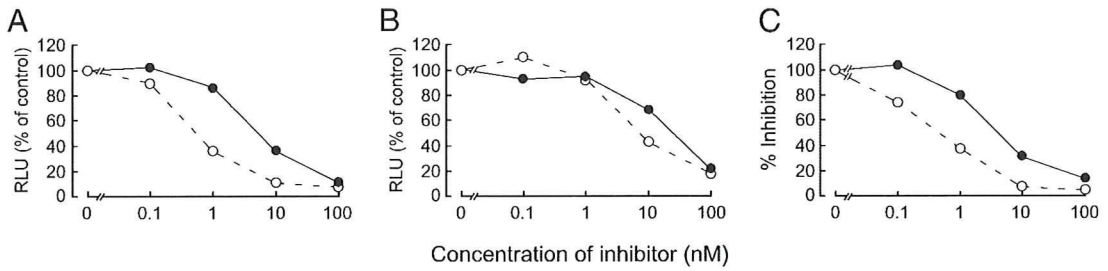


FIG. 6. Effect of IN inhibitors on retroviruses. Antiviral activities of EVG (open circles with dashed lines) and L-870,810 (closed circles with solid lines) against HIV-based (A) or MLV-based (B) vectors harboring the luciferase gene were determined by measuring luciferase activity at 48 h posttransduction. Results are expressed as percentages of relative luciferase units (RLU) compared to those of the no-inhibitor control. (C) Anti-SIV activity was determined using the MAGI assay. These results shown are one representative assay from three independent experiments.

L-731,988 (Table 3) and was also previously observed in an independent EVG selection by Jones et al. (26). The E92Q mutation, when introduced into a recombinant IN enzyme, also reduced the susceptibility of the resulting mutant IN enzyme to EVG, as measured by the reduced EVG inhibition of the in vitro strand transfer assay (Fig. 4). The other IN mutations identified, including H51Y, Q95K, E138K, Q146P, S147G, and E157Q, individually resulted in lower changes (*n*-fold) in EVG susceptibility (1.0- to 11.0-fold) but, when added to either the T66I or the E92Q mutation, further increased resistance to EVG to various degrees relative to either mutation alone. Interestingly, the accumulation of these EVG-selected IN mutations resulted in a significant attenuation of viral replication kinetics. Thus, the emergence of resistance to IN inhibitors may be associated with reductions in viral fitness, which may provide a barrier to the emergence of these mutations in vivo or be associated with lower viral loads if they do emerge.

Of the three HIV enzymes PR, RT, and IN, the structure and mechanism of IN are the least well understood, and despite extensive efforts, the structure of the complete IN enzyme remains to be determined. Only partial two-domain crystal structures of the IN apoenzyme are available, and no structure showing full-length IN bound to its viral cDNA substrate has been published. During integration in vivo, IN functions in the preintegration complex, which also includes RT and the viral DNA (2, 3). Some limited evidence suggests that RT interacts with the active site of IN (39). IN has also been proposed to function with several cellular factors including IN interactor 1 (Ini1) (27) and lens-epithelium-derived growth factor (LEDGF/p75) (7). In the context of these associated cellular factors, IN may retain a different conformation compared to that of the recombinant enzyme alone. This may be one of the reasons that only moderate EVG resistance was observed in the oligonucleotide-based strand transfer assay compared to a cell-based antiviral assay.

Alignment of several IN CCD structures deposited in the Protein Data Bank indicates that there are two regions with poorly defined or disordered structures, including residues 47 to 56 and 140 to 152 (Fig. 7; see Fig. S1 in the supplemental material). Of these two disordered regions, residues 140 to 152 have been implicated as a flexible loop involved in viral cDNA binding (20, 21, 53). Although the precise structural details are unknown, the flexible loop has been proposed to adopt different conformations in the presence or absence of the viral cDNA (12). Notably, several of the EVG-selected mutations that we observed are located on or adjacent to this proposed flexible loop, including E138K, Q146P, and S147G. The flexible loop is important for the catalytic activity of IN (21, 32), and as shown in Fig. 4, the introduction of mutations in these residues, especially S147G, drastically reduced the catalytic activity of IN. Previously published data also demonstrated that another mutation at codon 147 (S147I) resulted in HIV-1 that was highly replication defective, including effects on viral DNA synthesis (47). Indeed, S147 is highly conserved among various retroviruses (see Fig. S2 in the supplemental material), highlighting the importance of the loop for IN function. It is possible that IN inhibitor resistance mutants may have additional pleiotropic effects on processes in viral replication other

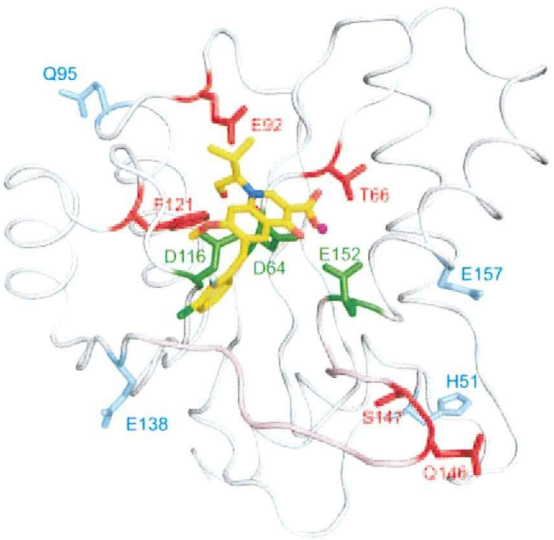


FIG. 7. Location of IN mutations associated with resistance to EVG. EVG in complex with the HIV-1 IN CCD is shown along with the catalytic triad residues (D64, D116, and E152) (green) and a magnesium ion (magenta). Amino acid residues conferring resistance to EVG as primary mutations (T66, E92, F121, Q146, and S147) or as secondary mutations (H51, Q95, E138, and E157) are shown in red and cyan, respectively. The flexible loop (residues 140 to 152) is shown in pink.

than integration; in particular, RT and IN were previously suggested to interact functionally (25).

Recently, an in silico docking simulation of HIV IN with several IN inhibitors including EVG was reported (44). Notably, that author showed that in the best-fit model for EVG docked to IN, the isobutyl substituent on the quionolone moiety of EVG orients directly towards IN residue E92. Interestingly, the hydroxyl component of the isobutyl on the quinolone replaces a water molecule that is coordinated by residue E92 between the two catalytic residues D64 and E152. This docking structure may provide insight into the mechanism of IN inhibition by EVG and provides a starting point for understanding the mechanism of EVG resistance mediated by the E92Q substitution. However, it is uncertain whether this docking simulation represents the precise binding mode of EVG with IN in vivo. Therefore, to accurately assess the binding mode of IN inhibitors with IN, available structural data need to be supplemented by a variety of other approaches. In this study, a virological approach and an enzymatic approach were integrated to characterize the mechanism of action, antiviral activity, and resistance profile of EVG in vitro.

As shown in Fig. 7, primary EVG resistance mutations are located around the catalytic triad of the CCD of IN and are surrounded by the secondary mutations. Among the residues affected by primary mutations, E92 and F121 are located close to EVG on the model and might interact with the IN inhibitor. However, the mechanism by which these mutations interact with the IN inhibitor or with the viral cDNA to mediate resistance is currently unclear. Recently, clinical isolate data from patients experiencing virologic failure in ongoing phase III studies of another IN inhibitor, raltegravir, were reported; E92Q was among the mutations noted to develop in these raltegravir failure patients, usually in combination with another IN mutation, N155H (11, 48). These preliminary clinical data and the data presented here with L-870,810, indicate that the E92Q mutation may be able to mediate resistance and potential cross-resistance to multiple IN inhibitors including EVG and raltegravir. Consistent with the data described here, site-directed mutant HIV carrying the E92Q mutation has been confirmed to show resistance to EVG and to have low-level (approximately sixfold) reduced susceptibility to raltegravir (26).

Several of the IN residues affected by primary mutations observed in EVG-selected variants including T66, E92, and S147 are absolutely conserved among the retroviruses tested (HIV-1, HIV-2, SIV, and MLV) and in retroviruses from multiple mammalian species (see Fig. S2 in the supplemental material). The significant conservation of mammalian retroviral IN CCDs at both the level of sequence homology and structure of the active site was demonstrated by the ability of EVG to inhibit HIV, SIV, and MLV IN activity. This suggests that EVG, and probably other IN inhibitors, binds to a conformationally conserved region of all retroviral INs; the binding of EVG and other IN inhibitors to IN is also likely to involve the catalytic magnesium ion. Taken together, these results suggest that several distinct mechanisms may contribute to IN inhibitor resistance, including conformational changes in the structure of IN that affect the binding of the IN inhibitor, charge effects, steric hindrance, loss of stabilizing binding interactions, or, possibly, alterations in magnesium binding.

A similar reduction in viral replication capacity as a result of drug resistance mutations was previously reported for NRTI resistance mutations (K65R, L74V, and M184V) (45, 55) and for PI resistance mutations (D30N) (49). Mutations that act to compensate for some of the loss of viral replication resulting from drug resistance, for example, GAG processing mutants, have also been described (18, 36, 52). At least one of the EVG secondary mutations, E157Q, may have an analogous role, as it partially restored strand transfer activity that was attenuated by other EVG-selected mutations and also further enhanced resistance to EVG (Fig. 4). Some secondary IN mutations might act to compensate for the altered conformation of IN resulting from the structural effects of primary resistance mutations. The E138K mutation may be such an example, as on its own, it showed no effect on susceptibility to either EVG or L-870,810. The clinical implications of the reduction in fitness resulting from the selection of EVG-resistant mutations are not yet understood.

In conclusion, EVG is a potent inhibitor of the HIV IN enzyme that acts by blocking the strand transfer reaction and is effective not only against HIV but also against other retroviruses. Moreover, the emergence of viral variants that were highly resistant to EVG was associated with significant reductions in viral replication in vitro. These results indicate that EVG should be highly effective for the treatment of HIV-1-infected patients, including those who have had virologic failure of their highly active antiretroviral therapy due to the emergence of HIV-1 drug resistance to approved antiretroviral drugs.

ACKNOWLEDGMENTS

We thank Shinjiro Hino for technical advice and Mieko Ikeuchi for technical assistance. We appreciate Damian McColl for critically reading and commenting on the manuscript.

This work was supported in part by a grant for the Promotion of AIDS Research from the Ministry of Health and Welfare of Japan (E.K. and M.M.), a grant for Research for Health Science Focusing on Drug Innovation from the Japan Health Science Foundation (E.K. and M.M.), and a grant from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (E.K.). K.S. is supported by the 21st Century COE Program of the Ministry of Education, Culture, Sports, Science, and Technology.

REFERENCES

1. **Asante-Appiah, E., and A. M. Skalka.** 1997. Molecular mechanisms in retrovirus DNA integration. Antivir. Res. **36**:139–156.
2. **Brown, P. O., B. Bowerman, H. E. Varmus, and J. M. Bishop.** 1987. Correct integration of retroviral DNA in vitro. Cell **49**:347–356.
3. **Bukrinsky, M. I., N. Sharova, T. L. McDonald, T. Pushkarskaya, W. G. Tarpley, and M. Stevenson.** 1993. Association of integrase, matrix, and reverse transcriptase antigens of human immunodeficiency virus type 1 with viral nucleic acids following acute infection. Proc. Natl. Acad. Sci. USA **90**:6125–6129.
4. **Butler, S. L., M. S. Hansen, and F. D. Bushman.** 2001. A quantitative assay for HIV DNA integration in vivo. Nat. Med. **7**:631–634.
5. **Chackerian, B., E. M. Long, P. A. Luciw, and J. Overbaugh.** 1997. Human immunodeficiency virus type 1 coreceptors participate in postentry stages in the virus replication cycle and function in simian immunodeficiency virus infection. J. Virol. **71**:3932–3939.
6. **Chakrabarti, L. A., K. J. Metzner, T. Ivanovic, H. Cheng, J. Louis-Virelizier, R. I. Connor, and C. Cheng-Mayer.** 2003. A truncated form of Nef selected during pathogenic reversion of simian immunodeficiency virus SIVmac239Δnef increases viral replication. J. Virol. **77**:1245–1256.
7. **Cherepanov, P., G. Maertens, P. Proost, B. Devreese, J. Van Beeumen, Y. Engelborghs, E. De Clercq, and Z. Debyser.** 2003. HIV-1 integrase forms stable tetramers and associates with LEDGF/p75 protein in human cells. J. Biol. Chem. **278**:372–381.
8. **Chow, S. A.** 1997. In vitro assays for activities of retroviral integrase. Methods **12**:306–317.

9. **Clavel, F., and A. J. Hance.** 2004. HIV drug resistance. N. Engl. J. Med. **350:**1023–1035.
10. **Condra, J. H., W. A. Schleif, O. M. Blahy, L. J. Gabryelski, D. J. Graham, J. C. Quintero, A. Rhodes, H. L. Robbins, E. Roth, M. Shivaprakash, D. Titus, T. Yang, H. Tepplert, K. E. Squires, P. J. Deutsch, and E. A. Emini.** 1995. In vivo emergence of HIV-1 variants resistant to multiple protease inhibitors. Nature **374:**569–571.
11. **Cooper, D., J. Gatell, J. Rockstroh, C. Katlama, P. Yeni, A. Lazzarin, J. Chen, R. Isaacs, H. Teppler, and B. Nguyen for the BENCHMRK-1 Study Group.** 2007. Abstr. 14th Conf. Retrovir. Opportun. Infect., abstr. 105aLB.
12. **De Luca, L., G. Vistoli, A. Pedretti, M. L. Barreca, and A. Chimirri.** 2005. Molecular dynamics studies of the full-length integrase-DNA complex. Biochem. Biophys. Res. Commun. **336:**1010–1016.
13. **Dyda, F., A. B. Hickman, T. M. Jenkins, A. Engelman, R. Craigie, and D. R. Davies.** 1994. Crystal structure of the catalytic domain of HIV-1 integrase: similarity to other polynucleotidyl transferases. Science **266:**1981–1986.
14. **Engelman, A., K. Mizuuchi, and R. Craigie.** 1991. HIV-1 DNA integration: mechanism of viral DNA cleavage and DNA strand transfer. Cell **67:**1211–1221.
15. **Fan, J., E. Kodama, Y. Koh, M. Nakao, and M. Matsuoka.** 2005. Halogenated thymidine analogues restore the expression of silenced genes without demethylation. Cancer Res. **65:**6927–6933.
16. **Fikkert, V., A. Hombrouck, B. Van Remoortel, M. De Maeyer, C. Pannecouque, E. De Clercq, Z. Debyser, and M. Witvrouw.** 2004. Multiple mutations in human immunodeficiency virus-1 integrase confer resistance to the clinical trial drug S-1360. AIDS **18:**2019–2028.
17. **Fikkert, V., B. Van Maele, J. Vercammen, A. Hantson, B. Van Remoortel, M. Michiels, C. Gurnari, C. Pannecouque, M. De Maeyer, Y. Engelborghs, E. De Clercq, Z. Debyser, and M. Witvrouw.** 2003. Development of resistance against diketo derivatives of human immunodeficiency virus type 1 by progressive accumulation of integrase mutations. J. Virol. **77:**11459–11470.
18. **Gatanaga, H., Y. Suzuki, H. Tsang, K. Yoshimura, M. F. Kavlick, K. Nagashima, R. J. Gorelick, S. Mardy, C. Tang, M. F. Summers, and H. Mitsuya.** 2002. Amino acid substitutions in Gag protein at non-cleavage sites are indispensable for the development of a high multitude of HIV-1 resistance against protease inhibitors. J. Biol. Chem. **277:**5952–5961.
19. **Goldgur, Y., R. Craigie, G. H. Cohen, T. Fujiwara, T. Yoshinaga, T. Fujishita, H. Sugimoto, T. Endo, H. Murai, and D. R. Davies.** 1999. Structure of the HIV-1 integrase catalytic domain complexed with an inhibitor: a platform for antiviral drug design. Proc. Natl. Acad. Sci. USA **96:**13040–13043.
20. **Goldgur, Y., F. Dyda, A. B. Hickman, T. M. Jenkins, R. Craigie, and D. R. Davies.** 1998. Three new structures of the core domain of HIV-1 integrase: an active site that binds magnesium. Proc. Natl. Acad. Sci. USA **95:**9150–9154.
21. **Greenwald, J., V. Le, S. L. Butler, F. D. Bushman, and S. Choe.** 1999. The mobility of an HIV-1 integrase active site loop is correlated with catalytic activity. Biochemistry **38:**8892–8898.
22. **Grinsztejn, B., B. Y. Nguyen, C. Katlama, J. M. Gatell, A. Lazzarin, D. Vittecoq, C. J. Gonzalez, J. Chen, C. M. Harvey, and R. D. Isaacs.** 2007. Safety and efficacy of the HIV-1 integrase inhibitor raltegravir (MK-0518) in treatment-experienced patients with multidrug-resistant virus: a phase II randomised controlled trial. Lancet **369:**1261–1269.
23. **Hazuda, D. J., N. J. Anthony, R. P. Gomez, S. M. Jolly, J. S. Wai, L. Zhuang, T. E. Fisher, M. Embrey, J. P. Guare, Jr., M. S. Egbertson, J. P. Vacca, J. R. Huff, P. J. Felock, M. V. Witmer, K. A. Stillmock, R. Danovich, J. Grobler, M. D. Miller, A. S. Espeseth, L. Jin, I. W. Chen, J. H. Lin, K. Kassahun, J. D. Ellis, B. K. Wong, W. Xu, P. G. Pearson, W. A. Schleif, R. Cortese, E. Emini, V. Summa, M. K. Holloway, and S. D. Young.** 2004. A naphthyridine carboxamide provides evidence for discordant resistance between mechanistically identical inhibitors of HIV-1 integrase. Proc. Natl. Acad. Sci. USA **101:**11233–11238.
24. **Hazuda, D. J., P. Felock, M. Witmer, A. Wolfe, K. Stillmock, J. A. Grobler, A. Espeseth, L. Gabryelski, W. Schleif, C. Blau, and M. D. Miller.** 2000. Inhibitors of strand transfer that prevent integration and inhibit HIV-1 replication in cells. Science **287:**646–650.
25. **Hehl, E. A., P. Joshi, G. V. Kalpana, and V. R. Prasad.** 2004. Interaction between human immunodeficiency virus type 1 reverse transcriptase and integrase proteins. J. Virol. **78:**5056–5067.
26. **Jones, G., R. Ledford, F. Yu, M. Miller, M. Tsiang, and D. McColl.** 2007. Abstr. 14th Conf. Retrovir. Opportun. Infect., abstr. 627.
27. **Kalpana, G. V., S. Marmon, W. Wang, G. R. Crabtree, and S. P. Goff.** 1994. Binding and stimulation of HIV-1 integrase by a human homolog of yeast transcription factor SNF5. Science **266:**2002–2006.
28. **Kaufmann, G. R., and D. A. Cooper.** 2000. Antiretroviral therapy of HIV-1 infection: established treatment strategies and new therapeutic options. Curr. Opin. Microbiol. **3:**508–514.
29. **Kehlenbeck, S., U. Betz, A. Birkmann, B. Fast, A. H. Goller, K. Henninger, T. Lowinger, D. Marrero, A. Paessens, D. Paulsen, V. Pevzner, R. Schohe-Loop, H. Tsujishita, R. Welker, J. Kreuter, H. Rubsamen-Waigmann, and F. Dittmer.** 2006. Dihydroxythiophenes are novel potent inhibitors of human immunodeficiency virus integrase with a diketo acid-like pharmacophore. J. Virol. **80:**6883–6894.
30. **Kodama, E. I., S. Kohgo, K. Kitano, H. Machida, H. Gatanaga, S. Shigeta, M. Matsuoka, H. Ohrui, and H. Mitsuya.** 2001. 4′-Ethynyl nucleoside analogs: potent inhibitors of multidrug-resistant human immunodeficiency virus variants in vitro. Antimicrob. Agents Chemother. **45:**1539–1546.
31. **LaFemina, R. L., C. L. Schneider, H. L. Robbins, P. L. Callahan, K. LeGrow, E. Roth, W. A. Schleif, and E. A. Emini.** 1992. Requirement of active human immunodeficiency virus type 1 integrase enzyme for productive infection of human T-lymphoid cells. J. Virol. **66:**7414–7419.
32. **Lee, M. C., J. Deng, J. M. Briggs, and Y. Duan.** 2005. Large-scale conformational dynamics of the HIV-1 integrase core domain and its catalytic loop mutants. Biophys. J. **88:**3133–3146.
33. **Little, S., G. Drusano, R. Schooley, D. Haas, P. Kumar, S. Hammer, D. McMahon, K. Squires, R. Asfour, D. Richman, J. Chen, A. Saah, R. Leavitt, D. Hazuda, B. Y. Nguyen, and the Protocol 004 Study Team.** 2005. Abstr. 12th Conf. Retrovir. Opportun. Infect., abstr. 161.
34. **Los Alamos National Laboratory Theoretical Biology and Biophysics Group T-10.** 2001. HIV sequence compendium, 2001. Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, NM.
35. **Matsuoka-Aizawa, S., H. Sato, A. Hachiya, K. Tsuchiya, Y. Takebe, H. Gatanaga, S. Kimura, and S. Oka.** 2003. Isolation and molecular characterization of a nelfinavir (NFV)-resistant human immunodeficiency virus type 1 that exhibits NFV-dependent enhancement of replication. J. Virol. **77:**318–327.
36. **Myint, L., M. Matsuda, Z. Matsuda, Y. Yokomaku, T. Chiba, A. Okano, K. Yamada, and W. Sugiura.** 2004. Gag non-cleavage site mutations contribute to full recovery of viral fitness in protease inhibitor-resistant human immunodeficiency virus type 1. Antimicrob. Agents Chemother. **48:**444–452.
37. **Nameki, D., E. Kodama, M. Ikeuchi, N. Mabuchi, A. Otaka, H. Tamamura, M. Ohno, N. Fujii, and M. Matsuoka.** 2005. Mutations conferring resistance to human immunodeficiency virus type 1 fusion inhibitors are restricted by gp41 and Rev-responsive element functions. J. Virol. **79:**764–770.
38. **Neuveut, C., and K. T. Jeang.** 1996. Recombinant human immunodeficiency virus type 1 genomes with *tat* unconstrained by overlapping reading frames reveal residues in Tat important for replication in tissue culture. J. Virol. **70:**5572–5581.
39. **Oz Gleenberg, I., O. Avidan, Y. Goldgur, A. Herschhorn, and A. Hizi.** 2005. Peptides derived from the reverse transcriptase of human immunodeficiency virus type 1 as novel inhibitors of the viral integrase. J. Biol. Chem. **280:**21987–21996.
40. **Palella, F. J., Jr., K. M. Delaney, A. C. Moorman, M. O. Loveless, J. Fuhrer, G. A. Satten, D. J. Aschman, S. D. Holmberg, et al.** 1998. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. N. Engl. J. Med. **338:**853–860.
41. **Pommier, Y., A. A. Johnson, and C. Marchand.** 2005. Integrase inhibitors to treat HIV/AIDS. Nat. Rev. Drug Discov. **4:**236–248.
42. **Reinke, R., D. J. Lee, and W. E. Robinson, Jr.** 2002. Inhibition of human immunodeficiency virus type 1 isolates by the integrase inhibitor L-731,988, a diketo acid. Antimicrob. Agents Chemother. **46:**3301–3303.
43. **Sato, M., T. Motomura, H. Aramaki, T. Matsuda, M. Yamashita, Y. Ito, H. Kawakami, Y. Matsuzaki, W. Watanabe, K. Yamataka, S. Ikeda, E. Kodama, M. Matsuoka, and H. Shinkai.** 2006. Novel HIV-1 integrase inhibitors derived from quinolone antibiotics. J. Med. Chem. **49:**1506–1508.
44. **Savarino, A.** 2007. In-silico docking of HIV-1 integrase inhibitors reveals a novel drug type acting on an enzyme/DNA reaction intermediate. Retrovirology **4:**21.
45. **Sharma, P. L., and C. S. Crumpacker.** 1997. Attenuated replication of human immunodeficiency virus type 1 with a didanosine-selected reverse transcriptase mutation. J. Virol. **71:**8846–8851.
46. **Shibata, R., M. Kawamura, H. Sakai, M. Hayami, A. Ishimoto, and A. Adachi.** 1991. Generation of a chimeric human and simian immunodeficiency virus infectious to monkey peripheral blood mononuclear cells. J. Virol. **65:**3514–3520.
47. **Shin, C. G., B. Taddeo, W. A. Haseltine, and C. M. Farnet.** 1994. Genetic analysis of the human immunodeficiency virus type 1 integrase protein. J. Virol. **68:**1633–1642.
48. **Steigbigel, R., P. Kumar, J. Eron, M. Schechter, M. Markowitz, M. Loufty, J. Zhao, R. Isaacs, B. Nguyen, H. Teppler, and the BENCHMRK-2 Study Group.** 2007. Abstr. 14th Conf. Retrovir. Opportun. Infect., abstr. 105bLB.
49. **Sugiura, W., Z. Matsuda, Y. Yokomaku, K. Hertogs, B. Larder, T. Oishi, A. Okano, T. Shiino, M. Tatsumi, M. Matsuda, H. Abumi, N. Takata, S. Shirahata, K. Yamada, H. Yoshikura, and Y. Nagai.** 2002. Interference between D30N and L90M in selection and development of protease inhibitor-resistant human immunodeficiency virus type 1. Antimicrob. Agents Chemother. **46:**708–715.
50. **Tisdale, M., R. E. Myers, B. Maschera, N. R. Parry, N. M. Oliver, and E. D. Blair.** 1995. Cross-resistance analysis of human immunodeficiency virus type 1 variants individually selected for resistance to five different protease inhibitors. Antimicrob. Agents Chemother. **39:**1704–1710.
51. **Toossi, Z., H. Mayanja-Kizza, J. Baseke, P. Peters, M. Wu, A. Abraha, H. Aung, A. Okwera, C. Hirsch, and E. Arts.** 2005. Inhibition of human immu-

nodeficiency virus-1 (HIV-1) by beta-chemokine analogues in mononuclear cells from HIV-1-infected patients with active tuberculosis. Clin. Exp. Immunol. **142:**327–332.

52. **Verheyen, J., E. Litau, T. Sing, M. Daumer, M. Balduin, M. Oette, G. Fatkenheuer, J. K. Rockstroh, U. Schuldenzucker, D. Hoffmann, H. Pfister, and R. Kaiser.** 2006. Compensatory mutations at the HIV cleavage sites p7/p1 and p1/p6-gag in therapy-naive and therapy-experienced patients. Antivir. Ther. **11:**879–887.
53. **Wang, J. Y., H. Ling, W. Yang, and R. Craigie.** 2001. Structure of a two-domain fragment of HIV-1 integrase: implications for domain organization in the intact protein. EMBO J. **20:**7333–7343.
54. **Weiner, M. P., G. L. Costa, W. Schoettlin, J. Cline, E. Mathur, and J. C. Bauer.** 1994. Site-directed mutagenesis of double-stranded DNA by the polymerase chain reaction. Gene **151:**119–123.
55. **White, K. L., N. A. Margot, T. Wrin, C. J. Petropoulos, M. D. Miller, and L. K. Naeger.** 2002. Molecular mechanisms of resistance to human immunodeficiency virus type 1 with reverse transcriptase mutations K65R and K65R+M184V and their effects on enzyme function and viral replication capacity. Antimicrob. Agents Chemother. **46:**3437–3446.
56. **Zolopa, A., M. Mullen, D. Berger, P. Ruane, T. Hawkins, L. Zhong, S. Chuck, J. Enejosa, B. Kearney, and A. Cheng.** 2007. Abstr. 14th Confer. Retrovir. Opportun. Infect., abstr. 143LB.

研究成果の刊行に関する一覧表（H19-21 年度）

研究代表者：京都大学ウイルス研究所　松岡　雅雄
研究分担者：京都大学大学院薬学研究科　藤井　信孝
大石　真也

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
Wang Z-X., Tamamura H., Frilot N., Broach J., Fujii N., Peiper S.C.	Screening and characterization of cyclic pentapeptide CXCR4 antagonists/inverse agonists using a pheromone responsive reporter gene in Saccharomyces cerevisiae: utility of G protein coupled receptor constitutively active mutants.	Moser, Bernhard; Letts, Gordon L.; Neote, Kuldeep.	Chemokine Biology--Basic Research and Clinical Application	Birkhaeuser Verlag	Basel,	2007	61-77

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Narumi T., Hayashi R., Tomita K., Kobayashi K., Tanahara N., Ohno H., Naito T., Kodama E., Matsuoka M., Oishi S., Fujii N.	Synthesis and biological evaluation of selective CXCR4 antagonists containing alkene dipeptide isosteres.	Org. Biomol. Chem.	8(3)	616-621	2010
Mizuhara T., Oishi S., Fujii N., Ohno H.	Efficient synthesis of pyrimido[1,2-c][1,3] benzothiazin-6-imines and related tricyclic heterocycles by SNAr-type C-S, C-N, or C-O bond formation with heterocumulenes.	J. Org. Chem.	75(1)	265-268	2010
Nishikawa H., Nakamura S., Kodama E., Ito S., Kajiwara K., Izumi K., Sakagami Y., Oishi S., Ohkubo T., Kobayashi Y., Otaka A., Fujii N., Matsuoka M.	Electrostatically constrained α-helical peptide inhibitors replication of HIV-1 resistant to enfuvirtide.	Int. J. Biochem. Cell Biol.	41(4)	891-899	2009
Mizukoshi F., Baba F., Goto Y., Setoguchi A., Fujino Y., Ohno K., Oishi S., Kodera Y., Fujii N., Tsujimoto H.	Antiviral activity of membrane fusion inhibitors that target gp40 of the feline immunodeficiency virus envelope protein.	J. Vet. Med. Sci.	71(1)	121-124	2009
Izumi K., Kodama E., Shimura K., Sakagami Y., Watanabe K., Ito S., Watabe T., Terakawa Y., Nishikawa H., Sarafianos S. G., Kitaura K., Oishi S., Fujii N., Matsuoka M.	Design of peptide- based inhibitors of HIV-1 strains resistant to T-20.	J. Biol. Chem.	284(8)	4914-4920	2009

Watabe T., Terakawa Y., Watanabe K., Ohno H., Nakano H., Nakatsu T., Kato H., Izumi K., Kodama E., Matsuoka M., Kitaura K., Oishi S., Fujii N.	X-ray crystallographic study of an HIV-1 fusion inhibitor with the gp41 S138A substitution.	J. Mol. Biol.	392(3)	657-665	2009
Mizuhara T., Inuki S., Oishi S., Fujii N., Ohno H.	Cu(II)-mediated oxidative intermolecular ortho C-H functionalisation using tetrahydropyrimidine as the directing group.	Chem. Commun.	(23)	3413-3415	2009
Oishi S., Kodera Y., Nishikawa H., Kamitani H., Watabe T., Ohno H., Tochikura T., Shimane K., Kodama E., Matsuoka M., Mizukoshi F., Tsujimoto H., Fujii N.	Design and synthesis of membrane fusion inhibitors against the feline immunodeficiency virus.	Bioorg. Med. Chem.	17(14)	4916-4920	2009
Oishi S., Kamitani H., Kodera Y., Watanabe K., Kobayashi K., Narumi T., Tomita K., Ohno H., Naito T., Kodama E., Matsuoka M., Fujii N.	Peptide bond mimicry by (E)-alkene and (Z)-fluoroalkene peptide isosteres: synthesis and bioevaluation of alpha-helical anti-HIV peptide analogues.	Org. Biomol. Chem.	7(14)	2872-2877	2009
Evans B.J., Wang Z., Broach J.R., Oishi S., Fujii N., Peiper S.C.	Expression of CXCR4, a G-protein-coupled receptor for CXCL12 in yeast identification of new-generation inverse agonists.	Methods Enzymol.	460	399-412	2009
Tanaka T., Nomura W., Narumi T., Esaka A., Oishi S., Ohashi N., Itotani K., Evans B.J., Wang Z.X., Peiper S.C., Fujii N., Tamamura H.	Structure-activity relationship study on artificial CXCR4 ligands possessing the cyclic pentapeptide scaffold: the exploration of amino acid residues of pentapeptides by substitutions of several aromatic amino acids.	Org. Biomol. Chem.	7(18)	3805-3809	2009
Tanaka M., Kajiwara K., Tokiwa R., Watanabe K., Ohno H., Tsutsumi H., Hata Y., Izumi K., Kodama E., Matsuoka M., Oishi S., Fujii N.	Bioorganic synthesis of end-capped anti-HIV peptides by simultaneous cyanocysteine-mediated cleavages of recombinant proteins.	Bioorg. Med. Chem.	17(21)	7487-7492	2009
Kajiwara K., Watanabe K., Tokiwa R., Kurose T., Ohno H., Tsutsumi H., Hata Y., Izumi K., Kodama E., Matsuoka M., Oishi S., Fujii N.	Bioorganic synthesis of a recombinant HIV-1 fusion inhibitor, SC35EK, with an N-terminal pyroglutamate capping group from recombinant protein.	Bioorg. Med. Chem.	17(23)	7964-7970	2009
Ujike M., Nishikawa H., Otaka A., Yamamoto N., Yamamoto N., Matsuoka M., Kodama E., Fujii N., Taguchi F.	Heptad repeat-derived peptides block protease-mediated direct entry from the cell surface of severe acute respiratory syndrome coronavirus but not entry via the endosomal pathway pathway.	J. Virol.	82(1)	588-592	2008
Oishi S., Ito S., Nishikawa H., Watanabe K., Tanaka M., Ohno H., Izumi K., Sakagami Y., Kodama E., Matsuoka M., Fujii N.,	Design of a novel HIV-1 fusion inhibitor that displays a minimal interface for binding affinity.	J. Med. Chem.	51(3)	388-391	2008
Oishi S., Masuda R., Evans B., Ueda S., Goto Y., Ohno H., Hirasawa A., Tsujimoto G., Wang Z., Peiper S.C., Naito T., Kodama E., Matsuoka M., Fujii N.	Synthesis and application of fluorescein- and botin-labeled molecular probes for chemokine receptor CXCR4	ChemBioChem.	9	1154-1158	2008

Ohta Y., Oishi S., Fujii N., Ohno H.	Facile synthesis of 3-(aminomethyl)isoquinolines by copper-catalysed domino four-component coupling and cyclisation.	Chem. Commun.	7	835-837	2008
Ohno H., Okano A., Kosaka S., Tsukamoto K., Ohata M., Ishihara K., Maeda H., Tanaka T., Fujii N.	Direct construction of bicyclic heterocycles by palladium-catalyzed domino cyclization of propargyl bromides.	Org. Lett.	10(6)	1171-1174	2008
Liapi A., Pritchett J., Jones O., Fujii N., Parnavelas J.G., Nadarajah B.	Stromal-derived factor 1 signalling regulates radial and tangential migration in the developing cerebral cortex.	Dev. Neurosci.	30 (1-3)	117-131	2008
Kasiyanov A., Fujii N., Tamamura H., Xiong H.	Modulation of network-driven, GABA-mediated giant depolarizing potentials by SDF-1α in the developing hippocampus.	Dev. Neurosci.	30(4)	285-292	2008
Driessen W.H., Fujii N., Tamamura H., Sullivan S.M.	Development of peptide-targeted lipoplexes to CXCR4-expressing rat glioma cells and rat proliferating endothelial cells.	Mol. Ther.	16(3)	516-524	2008
Narumi T., Tomita K., Inokuchi E., Kobayashi K., Oishi S., Ohno H., Fujii N.	Diastereoselective synthesis of highly functionalized fluoroalkene dipeptide isosteres and its application to Fmoc-based solid phase synthesis of a cyclic pentapeptide mimetic.	Tetrahedron	64(19)	4332-4346	2008
Inokuchi E., Narumi T., Niida A., Kobayashi K., Tomita K., Oishi S., Ohno H., Fujii N.	Efficient synthesis of trifluoromethyl and related trisubstituted alkene dipeptide isosteres by palladium-catalyzed carbonylation of amino acid-derived allylic carbonates.	J. Org. Chem.	73(10)	3942-3945	2008
Nakata H., Steinberg S.M., Koh Y., Maeda K., Takaoka Y., Tamamura H., Fujii N., Mitsuya H.	Potent synergistic anti-HIV effects using combinations of a CCR5.	Antimicrob. Agents. Chemother.	52(6)	2111-2119	2008
Nishikawa H., Kodama E., Sakakibara A., Fukudome A., Izumi K., Oishi S., Fujii N., Matsuoka M.	Novel screening systems for HIV-1 fusion mediated by two extra-virion heptad repeats of gp41	Antivir. Res.	80(1)	71-76	2008
Ueda S., Kato M., Inuki S., Ohno H., Evans B., Wang Z., Peiper S.C., Izumi K., Kodama E., Matsuoka M., Nagasawa H., Oishi S., Fujii N.	Identification of novel nonpeptide CXCR4 antagonists by ligand-based design approach.	Bioorg. Med. Chem. Lett.	18(14)	4124-4129	2008
Mikami S., Nakase H., Yamamoto S., Takeda Y., Yoshino T., Kasahara K., Ueno S., Uza N., Oishi S., Fujii N., Nagasawa T., Chiba T.	Blockade of CXCL12/CXCR4 axis ameliorates murine experimental colitis.	J. Pharmacol. Exp. Ther.	327(2)	383-392	2008
Nishikawa H., Oishi S., Fujita M., Watanabe K., Tokiwa R., Ohno H., Kodama E., Izumi K., Kajiwara K., Naitoh T., Matsuoka M., Otaka A., Fujii N.	Identification of minimal sequence for HIV-1 fusion inhibitors.	Bioorg. Med. Chem.	16(20)	9184-9187	2008

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.	Structure-activity relationship study of CXCR4 antagonists bearing the cyclic pentapeptide scaffold: identification of the new pharmacophore.	Org. Biomol. Chem.	6(23)	4374-4377	2008
Tamamura H., Tsutsumi H., Nomura W., Tanaka T., Fujii N.	A future perspective on the development of chemokine receptor CXCR4 antagonists.	Expert Opin. Drug Discov.	3(10)	1155-1166	2008
Oishi S., Narumi T., Ohno H., Otaka A., Fujii N.	Synthesis of highly functionalized alkene dipeptide isosteres and its application to the structure-activity relationship study on bioactive peptides.	J. Synth. Org. Chem. Jpn.	66(9)	846-857	2008
Sasaki Y., Fujii N., Otaka A.	Development of copper-mediated allylation of γ-activated-α,β-unsaturated lactam toward peptide mimetic synthesis.	Tetrahedron Lett.	48(18)	3221-3224	2007
Watanabe T., Ueda S., Inuki S., Oishi S., Fujii N., Ohno H.	One-pot synthesis of carbazoles by palladium-catalyzed N-arylation and oxidative coupling.	Chem. Commun.	43	4516-4518	2007
Watanabe T., Oishi S., Fujii N., Ohno H.	Gold-catalyzed hydroarylation of allenes: a highly regioselective carbon-carbon bond formation producing six-membered rings.	Org. Lett.	9(23)	4821-4824	2007
Ohno H., Iuchi M., Fujii N., Tanaka T.	Zipper-mode double C-H activation: palladium-catalyzed direct construction of highly-fused heterocyclic systems.	Org. Lett.	9(23)	4813-4815	2007
Tamamura H., Tanaka T., Tsutsumi H., Nemoto K., Mizokami S., Ohashi N., Oishi S., Fujii N.	Versatile use of acid-catalyzed ring-opening of β-aziridinyl-α,β enoates to stereoselective synthesis of peptidomimetics.	Tetrahedron	63(37)	9243-9254	2007
Narumi T., Tomita K., Inokuchi E., Kobayashi K., Oishi S., Ohno H., Fujii N.	Facile synthesis of fluoroalkenes by palladium-catalyzed reductive defluorination of allylic gem-difluorides.	Org. Lett.	9(17)	3465-3468	2007
Ohno H., Aso A., Kadoh Y., Fujii N., Tanaka T.	Heck-type cyclization of oxime ethers: stereoselective carbon-carbon bond formation with aryl halides to produce heterocyclic oximes.	Angew. Chem. Int. Ed.	46(33)	6325-6328	2007
Kubonishi S., Kikuchi T., Yamaguchi S., Tamamura H., Fujii N., Watanabe T., Arenzana-Seisdedos F., Ikeda K., Matsui T., Tanimoto M., Katayama Y.	Rapid hematopoietic progenitor mobilization by sulfated colominic acid.	Biochem. Biophys. Res. Commun.	355(4)	970-975	2007
Cluzeau J., Oishi S., Ohno H., Wang Z., Evans B., Peiper S.C., Fujii N.	Design and synthesis of all diastereomers of cyclic pseudo-dipeptides as mimics of cyclic CXCR4 pentapeptide antagonists.	Org. Biomol. Chem.	5(12)	1915-1923	2007
Ohno H., Mizutani T., Kadoh Y., Aso A., Miyamura K., Fujii N., Tanaka T.	A highly regio- and stereoselective formation of bicyclo[4.2.0]oct-5-ene derivatives through thermal intramolecular [2 + 2] cycloaddition of allenes.	J. Org. Chem.	72(12)	4378-4389	2007
Ohno H., Ohta Y., Oishi S., Fujii N.	Direct synthesis of 2-(aminomethyl)indoles through copper(I)-catalyzed domino three-component coupling and cyclization reactions.	Angew. Chem. Int. Ed.	46(13)	2295-2298	2007

Hamaguchi H., Kosaka S., Ohno H., Fujii N., Tanaka T.	Bromoallenes as allyl dication equivalents in the presence or absence of palladium(0): direct construction of bicyclic sulfamides containing five- to eight-membered rings by tandem cyclization of bromoallenes.	Chem-Eur. J.	13(6)	1692-1708	2007
Tsutsumi H., Tamamura H., Fujii N.	Inhibitors of the chemokine receptor CXCR4:chemotherapy of AIDS, metastatic cancer, leukemia and rheumatoid arthritis.	Lett. Drug Design Discov.	4(1)	20-26	2007
大石真也、藤井信孝	ペプチドをリードとした創薬	蛋白質 核酸 酵素 増刊 ケミカルバイオロジー 長野哲雄・長田裕之・菊地和也・上杉志成編集	52	1696-1701	2007
大石真也、藤井信孝	ケミカルバイオロジーを基盤とするペプチド創薬	遺伝子医学 MOOK8 ペプチドと創薬 寒川賢治・南野直人編集		87-91	2007

JOC Note
pubs.acs.org/joc

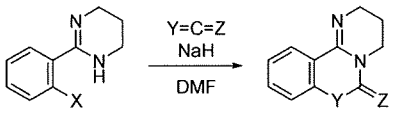
Efficient Synthesis of Pyrimido[1,2-*c*][1,3]benzothiazin-6-imines and Related Tricyclic Heterocycles by S_NAr-Type C−S, C−N, or C−O Bond Formation with Heterocumulenes

Tsukasa Mizuhara, Shinya Oishi, Nobutaka Fujii,* and Hiroaki Ohno*

Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan

nfujii@pharm.kyoto-u.ac.jp; hohno@pharm.kyoto-u.ac.jp

Received October 30, 2009



A simple and practical synthetic method of pyrimido[1,2-*c*]-[1,3]benzothiazin-6-imines and related tricyclic heterocycles has been developed. Treatment of 2-(2-haloaryl)-tetrahydropyrimidines with NaH and a heterocumulene such as carbon disulfide, isothiocyanates, and isocyanates in DMF provides the desired cyclization products through a regioselective S_NAr-type reaction. This method provides direct access to PD 404182 and related compounds.

The pyrimidobenzothiazine derivative PD 404182 (**1**) was recently discovered to be an antibiotic agent (Figure 1).[1,2]

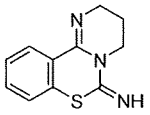


FIGURE 1. Structure of PD 404182.

This compound inhibits 3-deoxy-D-*manno*-octulosonic acid 8-phosphate (KDO 8-P) synthase, which catalyzes the condensation of phosphoenolpyruvate and arabinose 5-phosphate in the first committed step in the synthesis of KDO (an integral part of the lipopolysaccharide layer in Gram-negative bacteria). PD 404182 is considered to be an important lead in the development of structurally novel antibiotics effective against multidrug-resistant bacteria.[2b] Extensive study of the structure−activity relationship (SAR) of PD 404182 has not been carried out, presumably due to the lack of an efficient synthetic method suitable for lead optimization, as well as the cost of commercially available PD 404182.[3]

To develop a reliable, short-step synthetic method of tricyclic heterocycles related to PD 404182, we planned a novel strategy based on carbon (sp^2)−heteroatom bond formation using 2-(2-haloaryl)tetrahydropyrimidine derivatives. The carbon−heteroatom bond formation reaction is becoming a powerful methodology for construction of various heterocycles, providing several biologically active compounds.[4] The nucleophilic aromatic substitution (S_NAr) reaction is a well-established transition metal-free[5,6] carbon−heteroatom bond formation reaction.[7,8] In general, the S_NAr reaction requires harsh conditions (> 100 °C) and/or sufficiently activated aromatic rings by powerful electron-withdrawing group(s) (e.g., nitro). We describe a direct synthesis of tricyclic heterocycles related to PD 404182 by a regioselective S_NAr-type reaction of tetrahydropyrimidine-substituted haloarenes with heterocumulene in the absence of additional electron-withdrawing groups.[9] The efficient short-step synthesis of PD 404182 is also presented.

(1) Birck, M. R.; Holler, T. P.; Woodard, R. W. *J. Am. Chem. Soc.* **2000**, *122*, 9334–9335.

(2) (a) Golebiowski, A.; Klopfenstein, S. R.; Portlock, D. E. *Curr. Opin. Chem. Biol.* **2001**, *5*, 273–284. (b) Sansom, C. *Drug Discov. Today* **2001**, *6*, 499–500.

(3) $76.20/2 mg, Sigma-Aldrich.

(4) For reviews on transition metal-catalyzed carbon−heteroatom bond formation, see: (a) Hartwig, J. F. *Synlett* **1997**, 329–340. (b) Baranano, G. M.; Hartwig, J. F. *Curr. Org. Chem.* **1997**, *1*, 287–305. (c) Hartwig, J. F. *Angew. Chem., Int. Ed.* **1998**, *37*, 2046–2067. (d) Hartwig, J. F. *Acc. Chem. Res.* **1998**, *31*, 852–860. (e) Wolfe, J. P.; Wagaw, S.; Marcoux, J. F.; Buchwald, S. L. *Acc. Chem. Res.* **1998**, *31*, 805–818. (f) Hartwig, J. F. *Pure Appl. Chem.* **1999**, *71*, 1417–1423. (g) Yang, B. Y.; Buchwald, S. L. *J. Organomet. Chem.* **1999**, *576*, 125–146. (h) Hassan, J.; Sevingnon, M.; Gozzi, C.; Shulz, E.; Lemaire, M. *Chem. Rev.* **2002**, *102*, 1359–1469. (i) Muci, A. R.; Buchwald, S. L. *Top. Curr. Chem.* **2002**, *219*, 131–209. (j) Littke, A. F.; Fu, C. C. *Angew. Chem., Int. Ed.* **2002**, *41*, 4177–4211. (k) Ley, S. V.; Thomas, A. W. *Angew. Chem., Int. Ed.* **2003**, *42*, 5400–5449.

(5) Separation of the transition metal catalyst sometimes can be problematic during the synthesis of pharmaceuticals and fine chemicals because of their residual toxicity. For recent examples on transition metal-free carbon−heteroatom bond formation via benzyne intermediate, see: (a) Shi, L.; Wang, M.; Fan, C.-A.; Zhang, F.-M.; Tu, Y.-Q. *Org. Lett.* **2003**, *5*, 3515–3517. (b) Liu, Z.; Larock, R. C. *Org. Lett.* **2003**, *5*, 4673–4675. (c) Narayan, S.; Seelhammer, T.; Gawley, R. E. *Tetrahedron Lett.* **2004**, *45*, 757–759. (d) Liu, Z.; Larock, R. C. *Org. Lett.* **2004**, *6*, 99–102. (e) Liu, Z.; Larock, R. *J. Org. Chem.* **2006**, *71*, 3198–3209. (f) Bolliger, J. L.; Frech, C. M. *Tetrahedron* **2009**, *65*, 1180–1187.

(6) (a) Carroll, M. A.; Wood, R. A. *Tetrahedron* **2007**, *63*, 11349–11354. (b) Rey, V.; Soria-Castro, S. M.; Arguello, J. E.; Penenory, A. B. *Tetrahedron Lett.* **2009**, *50*, 4720–4723.

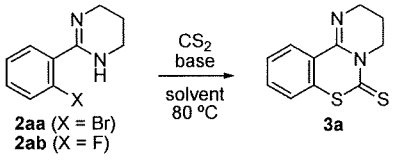
(7) For reviews on nucleophilic aromatic substitution reaction, see: (a) Bunnet, J. F.; Zahler, R. E. *Chem. Rev.* **1951**, *49*, 273–412. (b) Buncel, E.; Dust, J. M.; Terrier, F. *Chem. Rev.* **1995**, *95*, 2261−2280 and references cited therein.

(8) For recent examples on nucleophilic aromatic substitution reaction, see: (a) Annulli, A.; Mencarelli, P.; Stegel, F. *J. Org. Chem.* **1984**, *49*, 4065–4067. (b) Gorvin, J. H. *J. Chem. Soc., Perkin Trans. 1* **1988**, 1331–1335. (c) Raeppel, S.; Raeppel, F.; Suffert, J. *Synlett* **1998**, 794–796. (d) Ratz, A. M.; Weigel, L. O. *Tetrahedron Lett.* **1999**, *40*, 2239–2242. (e) Rogers, J. F.; Green, D. M. *Tetrahedron Lett.* **2002**, *43*, 3585–3587. (f) Grecian, S. A.; Hadida, S.; Warren, S. D. *Tetrahedron Lett.* **2005**, *46*, 4683–4685. (g) Barbero, N.; SanMartin, R.; Domínguez, E. *Tetrahedron* **2009**, *65*, 5729–5732.

(9) For related reactions of electron-deficient haloarenes with carbon disulfide, see: (a) D'Amico, J. J.; Tung, C. C.; Dahl, W. E.; Dahm, D. J. *J. Org. Chem.* **1976**, *41*, 3564–3568. (b) Leymarie-Beljean, M.; Pays, M.; Richer, J.-C. *J. Heterocycl. Chem.* **1980**, *17*, 1175–1179. (c) Anderson-McKay, J. E.; Liepa, A. J. *Aust. J. Chem.* **1987**, *40*, 1179–1190. (d) Easmon, J.; Heinisch, G.; Hofmann, J.; Langer, T.; Grunicke, H. H.; Fink, J.; Pürstinger, G. *Eur. J. Med. Chem.* **1997**, *32*, 397–408. (e) Kitson, T. M. *Bioorg. Chem.* **2000**, *27*, 73–88. (f) Kobayashi, K.; Komatsu, T.; Konishi, H. *Heterocycles* **2009**, *78*, 2559−2564. For a copper-catalyzed reaction with carbon disulfide, see: (g) Murru, S.; Ghosh, H.; Sahoo, S. K.; Patel, B. K. *Org. Lett.* **2009**, *11*, 4254–4257.

DOI: 10.1021/jo902327n Published on Web 12/08/2009

TABLE 1. Optimization of Reaction Conditions with CS_2[a]



entry	X	base (equiv)	solvent	time (h)	yield (%)[b]
1	Br	NaH (5)	MeCN	4	trace
2	Br	NaH (5)	THF	4	trace
3	Br	NaH (5)	DMF	6	75
4	Br	NaH (2)	DMF	12	88
5	Br	none	DMF	12	12
6	Br	Et_3N (2)	DMF	12	trace
7	Br	KH (2)	DMF	6	trace
8	Br	NaO*t*-Bu (2)	DMF	6	27
9	F	NaH (2)	DMF	12	86

[a]All reactions were carried out at 80 °C with 2 or 5 equiv of CS_2 (corresponding to the base loading). [b]Isolated yields.

Initial experiments were carried out with bromoarene **2aa**, which can be readily obtained by oxidative amidination[10] of 2-bromobenzaldehyde with propanediamine, and carbon disulfide as a heterocumulene (Table 1). Exposure of **2aa** with sodium hydride (5.0 equiv) and carbon disulfide (5.0 equiv) in acetonitrile or THF afforded only a trace amount of desired compound **3a** (entries 1 and 2). The desired reaction was efficiently promoted in DMF to give **3a** in 75% yield (entry 3). A decreasing amount of sodium hydride and carbon disulfide (2.0 equiv) slightly improved the yield of **3a** (88%) under the reaction for 12 h (entry 4).[11] The reaction in the absence of sodium hydride provided a yield of **3a** of only 12%. We next screened several bases such as triethylamine, potassium hydride[12] and sodium *tert*-butoxide (entries 6–8): sodium hydride was the most effective (entry 4). The fluoride **2ab** gave a comparable result with the bromide **2aa** to afford **3a** in 86% yield under optimized conditions (entry 9).

With knowledge of the optimized conditions, we examined the reaction of several substituted substrates (Table 2). Substrates **2b–d** having a methoxy, methyl, or fluoro group at the 4-position provided the corresponding cyclized products **3b–d** in good-to-excellent yields (76–95%, entries 1–3). Whereas the reaction of **2e** bearing the 4-nitro group at 80 °C resulted in formation of a complex mixture, the reaction at room temperature gave the cyclization product **3e** in 73% yield (entry 4). A methoxy group on the 5-position considerably diminished the reactivity, affording **3f** in only 17% yield (entry 5). This was presumably due to increased electron density at the carbon substituted by a bromine atom. In the case of **2g** bearing a 5-nitro group, the corresponding product **3g** was obtained by the reaction at room temperature (entry 6), similarly to **2e** (entry 4). Pyridine derivatives **4** and **6** showed different reactivity depending on the position of the nitrogen atom: the 2-bromopyridine derivative **6** gave a better result (71%, entry 8) than the 3-bromopyridine derivative **4** (18%, entry 7). The

TABLE 2. Reaction of Substituted 2-(2-Halophenyl)-1,4,5,6-tetrahydropyrimidines[a]

entry	substrate	product	yield (%)[b]
1	**2b** (R = OMe, X = F)	**3b** (R = OMe)	95
2	**2c** (R = Me, X = Br)	**3c** (R = Me)	88
3	**2d** (R = F, X = Br)	**3d** (R = F)	76
4	**2e** (R = NO_2, X = F)	**3e** (R = NO_2)	–[c] (73)[d]
5	**2f** (R = OMe)	**3f** (R = OMe)	17
6	**2g** (R = NO_2)	**3g** (R = NO_2)	–[c] (57)[d]
7	**4**	**5**	18
8	**6**	**7**	71
9	**8**	**9**	quant.

[a]Unless otherwise stated, reactions were carried out with CS_2 (2.0 equiv) and NaH (2.0 equiv) in DMF at 80 °C for 12 h. [b]Isolated yields. [c]A complex mixture formed. [d]Yields in parentheses indicate those of the reactions at rt.

naphthalene derivative **8** afforded the tetracyclic compound **9** in quantitative yield (entry 9).

To further expand our methodology for construction of other heterocyclic frameworks, we investigated the reaction using isothiocyanates or isocyanates[13,14] as heterocumulene (Table 3). When benzylisothiocyanate was employed, the reaction of **2aa** or **2ab** efficiently proceeded to give the corresponding *N*-arylated product **10** in 82% and 97% yields, respectively (entries 1 and 2). The reaction with *tert*-butylisothiocyanate exclusively furnished an *S*-arylated product **11** as a single isomer (entry 3). These results indicate

(10) Ishihara, M.; Togo, H. *Tetrahedron* **2007**, *63*, 1474–1480.
(11) Larger amounts of unidentified byproduct were formed when using 5 equiv of NaH than in the reaction with 2 equiv of NaH (entry 4).
(12) A reason for the significant countercation effect (NaH vs. KH) on the reactivity is unclear.
(13) For related reactions of electron-deficient (haloaryl)isothiocyanates, see: (a) Muthusamy, S.; Paramasivam, R.; Ramakrishnan, V. T. *J. Heterocycl. Chem.* **1991**, *28*, 759–763. (b) Zambounis, J. S.; Christen, E.; Pfeiffer, J.; Ribs, G. *J. Am. Chem. Soc.* **1994**, *116*, 925–931. (c) Huang, S.; Connolly, P. J. *Tetrahedron Lett.* **2004**, *45*, 9373–9375.
(14) For related transition metal-catalyzed reactions, see: (a) Ferraccioli, R.; Carenzi, D. *Synthesis* **2003**, 1383–1386. (b) Benedí, C.; Bravo, F.; Uriz, P.; Fernández, E.; Claver, C.; Castillón, S. *Tetrahedron Lett.* **2003**, *44*, 6073–6077. (c) Yang, D.; Liu, H.; Yang, H.; Fu, H.; Hu, L.; Jiang, Y.; Zhao, Y. *Adv. Synth. Catal.* **2009**, *351*, 1999–2004. (d) Murru, S.; Mondal, P.; Yella, R.; Patel, B. K. *Eur. J. Org. Chem.* **2009**, 5406–5413. (e) Qiu, J.-W.; Zhang, X.-G.; Tang, R.-Y.; Zhong, P.; Li, J.-H. *Adv. Synth. Catal.* **2009**, *351*, 2319–2323. (f) Shen, G.; Lv, X.; Bao, W. *Eur. J. Org. Chem.* **2009**, 5897–5901.

TABLE 3. Reaction with Isothiocyanates or Isocyanates[a]

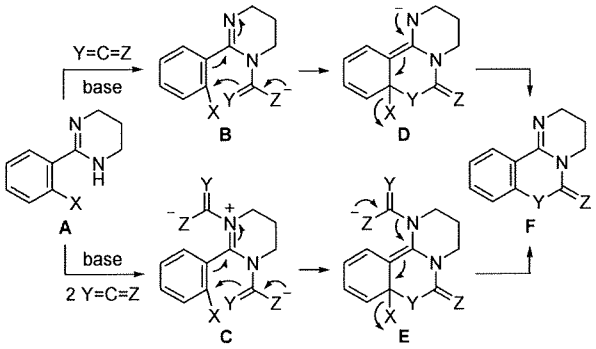
entry	substrate	R-NCX	product	yield (%)[b]
1	**2aa** (X = Br)	BnNCS	**10**	82
2	**2ab** (X = F)			97
3[c]	**2ab**	*t*-BuNCS	**11**	62[d,e]
4	**2ab**	BnNCO	**12**	quant.
5[c]	**2ab**	*t*-BuNCO	**13** (Y = N*t*-Bu, Z = O) **14** (Y = O, Z = N*t*-Bu)	54 18[e]
6	**2ab**	PhNCO	**15**	quant.
7	**16**	*t*-BuNCS	**17**	49[e]

[a]Unless otherwise stated, reactions were carried out with R-NCX (2.0 equiv) and NaH (2.0 equiv) in DMF at rt for 2−3 h. [b]Isolated yields. [c]These reactions were carried out at 80 °C. [d]A trace amount of regioisomeric *N*-arylation product was also formed. [e]Isolated as a single isomer.

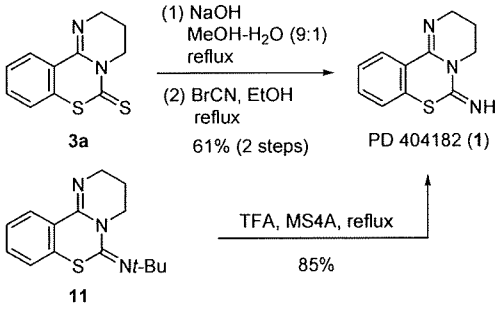
that the regioselectivity of the reaction can be perfectly switched by changing a substituent on the nitrogen atom. As expected, the reaction of **2ab** with benzylisocyanate provided an *N*-arylated product **12** in quantitative yield (entry 4) as in the case with isothiocyanate (entries 1 and 2). Interestingly, *tert*-butylisocyanate showed moderate selectivity to mainly afford an *N*-arylation product **13** (54%), formed by the arylation at the more bulky position, as well as an *O*-arylation product **14** (18%, entry 5). Phenylisocyanate also provided an *N*-arylated product **15** (entry 6). The 2-phenylimidazoline derivative **16** (a 5-membered-ring amidine congener) also provided the corresponding *S*-arylated product **17** in a slightly decreased yield (49%, entry 7).

This reaction would proceed via nucleophilic addition of the amidine moiety to heterocumulene followed by an intramolecular S_NAr reaction of the resulting adducts such as **B** (Scheme 1). Nonactivated aromatic rings efficiently reacted under relatively mild conditions, so two molecules of

SCHEME 1. Proposed Reaction Mechanisms



SCHEME 2. Synthesis of PD 404182



the heterocumulene may be involved in the reaction to form the intermediate **C** in which the amidine moiety can be a more powerful electron-withdrawing group suitable for the S_NAr-type reaction. The regioselectivity in the nucleophilic attack on the aromatic ring (Y vs. Z) is controlled by a subtle balance of inherent nucleophilicity and steric hindrance of these functionalities.

We finally focused on the synthesis of PD 404182 (**1**) (Scheme 2). Hydrolysis of the carbamodithioate derivative **3a** followed by treatment with cyanogen bromide[15] readily afforded the desired compound **1**. The same compound was also obtained in a single step by heating compound **11** in trifluoroacetic acid in the presence of molecular sieves.

In conclusion, we developed a simple and practical synthetic method for tricyclic heteroarenes related to PD 404182. This reaction provides divergent access to several related heterocycles under mild conditions without a powerful activating group. Further investigations including SAR study of these derivatives are currently underway.

Experimental Section

General Procedure for Synthesis of 3,4-Dihydro-2*H*-pyrimido-[1,2-*c*][1,3]benzothiazine-6-thione (3a) (Table 1, Entry 4). To a mixture of **2aa** (59.8 mg, 0.25 mmol) and NaH (20.0 mg, 0.50 mmol; 60% oil suspension) in DMF (0.83 mL) was added carbon disulfide (30.5 μL, 0.50 mmol) under an Ar atmosphere. After being stirred at 80 °C for 12 h, the mixture was concentrated in vacuo. The residue was purified by flash chromatography over silica gel with *n*-hexane−EtOAc (9:1) to give compound **3a** as a pale-yellow solid (51.4 mg, 88%): mp 139−141 °C (from $CHCl_3$−*n*-hexane); IR (neat) (cm^{-1}) 1624 (C=N); ^1H NMR (400 MHz, $CDCl_3$) δ 2.01−2.07 (m, 2H,

(15) Peter, S.; Gerhard, S. *Ger. Offen.* DE2811131, 1979.

CH_2), 3.76 (t, J = 5.6 Hz, 2H, CH_2), 4.45 (t, J = 6.2 Hz, 2H, CH_2), 7.03 (dd, J = 7.8, 1.5 Hz, 1H, Ar), 7.28−7.33 (m, 1H, Ar), 7.41 (ddd, J = 8.0, 7.6, 1.5 Hz, 1H, Ar), 8.20 (dd, J = 8.0, 1.2 Hz, 1H, Ar); ^{13}C NMR (125 MHz, $CDCl_3$) δ 21.6, 45.5, 48.6, 121.6, 126.5, 127.5, 128.9, 131.1, 131.8, 144.2, 189.8. Anal. Calcd for $C_{11}H_{10}N_2S_2$: C, 56.38; H, 4.30; N, 11.95. Found: C, 56.23; H, 4.44; N, 11.85.

Acknowledgment. This work was supported by a Grant-in-Aid for Encouragement of Young Scientists (A) (H.O.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO), and Targeted Proteins Research Program.

Supporting Information Available: Experimental procedures, full characterization, and ^{1}H and ^{13}C NMR charts of substrates and cyclization products. This material is available free of charge via the Internet at http://pubs.acs.org.

Cu(II)-mediated oxidative intermolecular *ortho* C–H functionalisation using tetrahydropyrimidine as the directing group†

Tsukasa Mizuhara, Shinsuke Inuki, Shinya Oishi, Nobutaka Fujii* and Hiroaki Ohno*

Received (in Cambridge, UK) 19th March 2009, Accepted 6th April 2009
First published as an Advance Article on the web 7th May 2009
DOI: 10.1039/b905586j

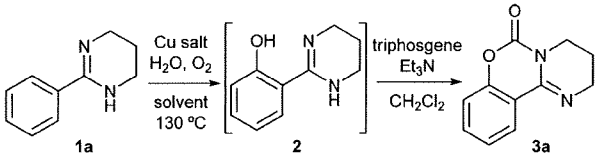
Tetrahydropyrimidine works efficiently as a directing group in Cu(II)-mediated oxidative aromatic C–H functionalisation for the selective introduction of oxygen or nitrogen to the *ortho*-position.

Transition metal-catalysed C–H functionalisation has received considerable attention in recent years. Notable progress has been made especially with Pd, Ru, Rh, and Pt catalysts, which allow atom-economical transformations using simple substrates. Directing group assisted intra/intermolecular C–H functionalisation[1] is considered to be one of the most promising approaches as a new carbon–carbon[1] or carbon–heteroatom bond[2,3] is selectively formed at a non-functionalised position proximal to the directing group. Recent research in this area has revealed that nitrogen-containing functional groups such as pyridines,[4] imines,[5] oximes (and ethers),[6] oxazolines[7] and amidines[8] as well as oxygen-containing functional groups such as amides,[9] esters,[10] ketones,[11] carboxylic acids[12] and phenols[13] effectively act as directing groups for regioselective C–H functionalisation. These functional groups and their equivalents are involved in biologically active natural and synthetic compounds while directing group-assisted C–H functionalisation serves as a powerful tool for the synthesis and modification of these molecules.

As part our ongoing program directed toward the development of C–H functionalisation reactions for the efficient construction of heterocyclic frameworks,[14] we designed an experiment for the oxidative introduction of a heteroatom by aromatic C–H functionalisation with the assistance of an *ortho*-tetrahydropyrimidinyl group. This group can be considered as a promising drug-like structure as well as a synthetic equivalent of a carboxylic group. Reinaud and co-workers previously reported a copper-catalysed *ortho*-hydroxylation reaction of benzoic acid using carboxyl as a directing group.[15] Yu *et al.*[16a] and Chatani *et al.*[16b] independently reported copper-mediated oxidative intermolecular C–H functionalisation using a pyridine moiety as the directing group. The latter reactions provide efficient access to functionalised pyridines with a biaryl structure by simply treating the biaryl substrates with a copper salt for several hours. More recently, copper-catalysed syntheses of benzimidazoles[17a] and benzoxazoles[17b] by oxidative intramolecular C–H functionalisation using amidine or amide as the directing/nucleophilic group were reported. Herein, we describe copper-mediated oxidative intermolecular C–H functionalisation using tetrahydropyrimidine as the directing group. In most cases, the reaction is complete within 1 h to provide 2-(tetrahydropyrimidinyl)phenol and aniline derivatives by the *ortho*-selective introduction of an oxygen or nitrogen atom.[18]

We initially investigated the reaction conditions for this C–H hydroxylation (Table 1). In the presence of H_2O (1.0 equiv.), treatment of 2-phenyl-1,4,5,6-tetrahydropyrimidine (**1a**) with CuO, $Cu(OH)_2$, $Cu(OTf)_2$ or $Cu(tfa)_2$ (1.0 equiv.) in DMF at 130 °C under an oxygen atmosphere led to the recovery of unchanged starting material and the desired C–H oxidation did not occur (entries 1–4). Using $Cu(OAc)_2$,[16a] however, led to the formation of the desired *ortho*-hydroxylated compound **2** (*ca.* 69% yield) although the isolation of **2** in its pure form was extremely difficult because of its basicity. We then attempted to isolate **3a** as the protected form: after the disappearance of **1a** (monitored by TLC), the reaction mixture was evaporated *in vacuo* and treated with triphosgene

Table 1 Optimisation of reaction conditions for C–H hydroxylation[a]



Entry	Cu salt (equiv.)	Solvent	Time/min	Yield (%)[b]
1	CuO (1.0)	DMF	20	N.r.[e]
2	$Cu(OH)_2$ (1.0)	DMF	20	N.r.[e]
3	$Cu(OTf)_2$ (1.0)	DMF	20	N.r.[e]
4	$Cu(tfa)_2$ (1.0)	DMF	20	N.r.[e]
5	$Cu(OAc)_2$ (1.0)	DMF	20	61
6	$Cu(OAc)_2$ (1.0)	Acetonitrile	60	11
7	$Cu(OAc)_2$ (1.0)	Dioxane	60	11
8	$Cu(OAc)_2$ (0.2)	DMF	60	30
9	$Cu(OAc)_2$ (2.0)	DMF	15	27
10[c]	$Cu(OAc)_2$ (1.0)	DMF	20	70
11[cd]	$Cu(OAc)_2$ (1.0)	DMF	20	56

[a] After completion of C–H hydroxylation (monitored by TLC), the reaction mixture was evaporated and treated with triphosgene (1.05 equiv.) and Et_3N (4.0 equiv.) in CH_2Cl_2 at 0 °C to rt for 1 h. [b] Isolated yields. [c] After completion of C–H hydroxylation (monitored by TLC), the reaction mixture was treated with TMEDA (4.0 equiv.) at 130 °C for 1 min. In this case, TMEDA (additional 4.0 equiv.) was used for the next step instead of Et_3N. [d] Reaction was carried out under air. [e] No reaction. Abbreviation: TMEDA = *N*,*N*,*N*′,*N*′-tetramethylethylenediamine.

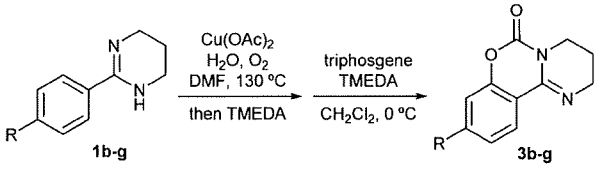
Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto, 606-8501, Japan.
E-mail: hohno@pharm.kyoto-u.ac.jp; Fax: +81 75 753 4570; Tel: +81 75 753 4571

† Electronic supplementary information (ESI) available: Experimental details and NMR spectral data. See DOI: 10.1039/b905586j

(1.05 equiv.) and triethylamine (4.0 equiv.) in CH_2Cl_2 to afford pure **3a** in a yield of 61% (entry 5). When acetonitrile or dioxane was used as the solvent instead of DMF, yields of **3a** decreased considerably (11%, entries 6 and 7). Lowering the loading of $Cu(OAc)_2$ to 0.2 equiv. also resulted in a decreased yield for **3a** (30%, entry 8), which indicates low catalyst efficiency. When using 2.0 equiv. of $Cu(OAc)_2$, the yield also decreased and this was contrary to our expectation (27%, entry 9). Considering that the *ortho*-hydroxylated product **2** may form a complex with the copper salt, we further optimised the reaction conditions including the carbonylation procedure. Initially, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TMEDA) was added as a bidentate ligand to the oxidative C–H functionalisation reaction mixture and this resulted in the complete inhibition of the desired transformation. Similarly, use of TMEDA instead of triethylamine as the base for carbonylation did not improve the yield of **3a**. On the other hand, treatment with TMEDA (4.0 equiv.) at 130 °C for 1 min after the C–H hydroxylation followed by the carbonylation using additional TMEDA (4.0 equiv.) increased the yield to 70% (entry 10). The reaction under air resulted in a decreased yield (56%, entry 11), which indicates that molecular oxygen participates in the re-oxidation of the copper catalyst.

Using the optimised conditions (Table 1, entry 10), we examined the reaction of several substituted substrates (Table 2). Substitution with electron-donating groups such as methoxy (**1b**, entry 1) or methyl groups (**1c**, entry 2) was tolerated to afford the desired products **3b** and **3c** in 64% and 61% yields, respectively. The chemoselectivity of this reaction was evaluated by a reaction where aryl bromide **1d** was used and the desired product **3d** was obtained in a 45% yield (entry 3). Methoxycarbonyl (entry 4) and trifluoromethyl groups (entry 5) had a relatively small effect on the reactivity of these substrates and the use of the highly electron-deficient arene **1g** bearing a nitro group decreased the yield considerably (19%, entry 6). These results indicate that this reaction is sensitive to the presence of electron-withdrawing groups on the aromatic ring. In all cases, reactions without TMEDA gave less favourable results.

Table 2 Cu-catalysed C–H hydroxylation of 4-substituted 2-phenyl-1,4,5,6-tetrahydropyrimidines[a]



Entry	Substrate (R)	Product	Yield (%)[b]
1	**1b** (OMe)	**3b**	64 (53)
2	**1c** (Me)	**3c**	61 (54)
3	**1d** (Br)	**3d**	45 (37)
4	**1e** (CO_2Me)	**3e**	46 (43)
5	**1f** (CF_3)	**3f**	43 (38)
6	**1g** (NO_2)	**3g**	19 (16)

[a] These reactions were carried out using the optimised procedure (Table 1, entry 10). [b] Isolated yields. Yields in parentheses indicate those of the reactions containing Et_3N (as shown in Table 1, entry 5).

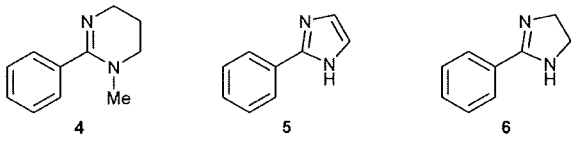
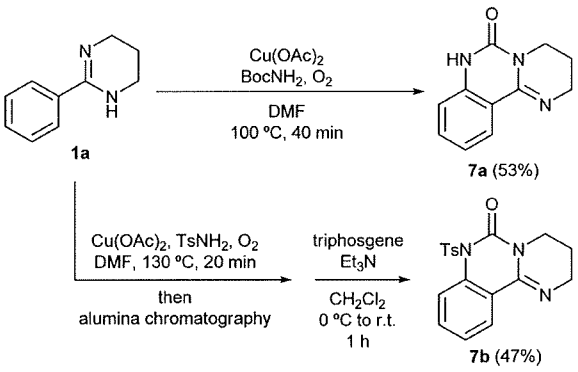


Fig. 1 Various amidine analogues.



Scheme 1 C–H amidation with $BocNH_2$ and $TsNH_2$.

Next, we investigated the ability of other amidine analogues to function as directing groups (Fig. 1). The reaction of the N-methylated analogue **4** and 2-phenylimidazole **5** did not produce the desired *ortho*-hydroxylated products under standard reaction conditions and the starting materials were recovered. Interestingly, the five-membered ring amidine in **6** was not effective as a directing group either. These results suggest that subtle differences in the intermediate formed by a copper salt and a directing group strongly affect the reactivity of the substrates.

Finally, we investigated C–H amidation (Scheme 1). We found that the reaction of amidine **1a** with $Cu(OAc)_2$ (1.0 equiv.) and *tert*-butyl carbamate (3.0 equiv.) in DMF at 100 °C for 40 min directly afforded the tricyclic aniline derivative (**7a**) in 53% yield. This reaction occurred by cyclisation involving the elimination of *tert*-butoxide. *p*-Toluenesulfonamide also reacted with **1a** under identical conditions to afford **7b** in 47% yield after alumina column chromatography[19] followed by treatment with triphosgene–Et_3N.

Although the exact mechanism of the *ortho* C–H oxidation is unclear, a single electron transfer (SET) pathway *via* a radical-cation intermediate[16a] is supported by the fact that the presence of an electron-withdrawing group on the benzene ring considerably decreased the product yields. The observed *ortho*-selectivity can be attributed to an intramolecular transfer of the coordinating group on the copper atom.

In conclusion, we have developed a copper-mediated oxidative *ortho* C–H functionalisation using tetrahydropyrimidine as a directing group. This reaction applies to 2-phenyl-1,4,5,6-tetrahydropyrimidines having an electron-donating or a weak electron-withdrawing group and affords the corresponding phenol derivatives within 1 h. Use of *tert*-butyl carbamate or tosylamide instead of H_2O promotes the introduction of a nitrogen functionality to give aniline derivatives. As far as we are aware, this is the first example of an oxidative

intermolecular C–H functionalisation using an amidine moiety as the directing group. Further studies that include an investigation of the exact reaction mechanism and the application to synthesis of biologically-active compounds are now in progress.

This work was supported by a Grant-in-Aid for Encouragement of Young Scientists (A) (H.O.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and Targeted Proteins Research Program, and the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO). S.I. is grateful to Research Fellowships of the Japan Society for the Promotion of Science (JSPS) for Young Scientists.

Notes and references

1 For recent reviews on transition metal-catalysed directed C–H activations, see: (*a*) G. Dyker, *Angew. Chem., Int. Ed.*, 1999, **38**, 1698; (*b*) V. Ritleng, C. Sirlin and M. Pfeffer, *Chem. Rev.*, 2002, **102**, 1731; (*c*) D. Alberico, M. E. Scott and M. Lautens, *Chem. Rev.*, 2007, **107**, 174. For recent examples, see: (*d*) H. Kawai, Y. Kobayashi, S. Oi and Y. Inoue, *Chem. Commun.*, 2008, 1464; (*e*) M. Tobisu, I. Hyodo, M. Onoe and N. Chatani, *Chem. Commun.*, 2008, 6013.

2 For recent examples of intramolecular reactions, see: (*a*) B. D. Dangel, J. A. Johnson and D. Sames, *J. Am. Chem. Soc.*, 2001, **123**, 8149; (*b*) K. Inamoto, T. Saito, M. Katsuno, T. Sakamoto and K. Hiroya, *Org. Lett.*, 2007, **9**, 2931; (*c*) K. Inamoto, C. Hasegawa, K. Hiroya and T. Doi, *Org. Lett.*, 2008, **10**, 5147; (*d*) M. Wasa and J.-Q. Yu, *J. Am. Chem. Soc.*, 2008, **130**, 14058; (*e*) K. Inamoto, Y. Arai, K. Hiroya and T. Doi, *Chem. Commun.*, 2008, 5529.

3 For recent examples of intermolecular reactions, see: (*a*) H. Chen, S. Schlecht, T. C. Semple and J. F. Hartwig, *Science*, 2000, **287**, 1995; (*b*) A. R. Dick, K. L. Hull and M. S. Sanford, *J. Am. Chem. Soc.*, 2004, **126**, 2300; (*c*) W. C. P. Tsang, N. Zheng and S. L. Buchwald, *J. Am. Chem. Soc.*, 2005, **127**, 14560; (*d*) D. Kalyani, A. R. Dick, W. Q. Anani and M. S. Sanford, *Org. Lett.*, 2006, **8**, 2523; (*e*) B. V. S. Reddy, L. R. Reddy and E. J. Corey, *Org. Lett.*, 2006, **8**, 3391; (*f*) X. Wan, Z. Ma, B. Li, K. Zhang, S. Cao, S. Zhang and Z. Shi, *J. Am. Chem. Soc.*, 2006, **128**, 7416; (*g*) D.-H. Wang, X.-S. Hao, D.-F. Wu and J.-Q. Yu, *Org. Lett.*, 2006, **8**, 3387; (*h*) J.-Q. Yu, R. Giri and X. Chen, *Org. Biomol. Chem.*, 2006, **4**, 4041 and references therein; (*i*) J.-J. Li, T.-S. Mei and J.-Q. Yu, *Angew. Chem., Int. Ed.*, 2008, **47**, 6452; (*j*) L. V. Desai, K. J. Stowers and M. S. Sanford, *J. Am. Chem. Soc.*, 2008, **130**, 13285.

4 For representative examples, see: (*a*) R. F. Jordan and D. F. Taylor, *J. Am. Chem. Soc.*, 1989, **111**, 778; (*b*) Y.-G. Lim, Y. H. Kim and J.-B. Kang, *J. Chem. Soc., Chem. Commun.*, 1994, 2267; (*c*) N. Chatani, Y. Ie, F. Kakiuchi and S. Murai, *J. Org. Chem.*, 1997, **62**, 2604; (*d*) N. Chatani, T. Asaumi, S. Yorimitsu, T. Ikeda, F. Kakiuchi and S. Murai, *J. Am. Chem. Soc.*, 2001, **123**, 10935; (*e*) V. G. Zaitsev, D. Shabashov and O. Daugulis, *J. Am. Chem. Soc.*, 2005, **127**, 13154; (*f*) A. M. Berman, J. C. Lewis, R. G. Bergman and J. A. Ellman, *J. Am. Chem. Soc.*, 2008, **130**, 14926, see also, ref. 16; (*g*) X. Zhao, E. Dimitrijevič and V. M. Dong, *J. Am. Chem. Soc.*, 2009, **131**, 3466.

5 For representative examples, see: (*a*) N. A. Williams, Y. Uchimura and M. Tanaka, *J. Chem. Soc., Chem. Commun.*, 1995, 1129; (*b*) F. Kakiuchi, M. Yamauchi, N. Chatani and S. Murai, *Chem. Lett.*, 1996, 111; (*c*) S. Oi, Y. Ogino, S. Fukita and Y. Inoue, *Org. Lett.*, 2002, **4**, 1783; (*d*) K. Ueura, T. Satoh and M. Miura, *Org. Lett.*, 2005, **7**, 2229; (*e*) L. Ackermann, *Org. Lett.*, 2005, **7**, 3123; (*f*) S. J. Pastine, D. V. Gribkov and D. Sames, *J. Am. Chem. Soc.*, 2006, **128**, 14220.

6 (*a*) L. V. Desai, K. L. Hull and M. S. Sanford, *J. Am. Chem. Soc.*, 2004, **126**, 9542; (*b*) L. V. Desai, H. A. Malik and M. S. Sanford, *Org. Lett.*, 2006, **8**, 1141; (*c*) H.-Y. Thu, W.-Y. Yu and C.-M. Che, *J. Am. Chem. Soc.*, 2006, **128**, 9048; (*d*) K. Parthasarathy, M. Jeganmohan and C.-H. Cheng, *Org. Lett.*, 2008, **10**, 325.

7 (*a*) F. Kakiuchi, T. Sato, M. Yamauchi, N. Chatani and S. Murai, *Chem. Lett.*, 1999, 19; (*b*) X. Chen, J.-J. Li, X.-S. Hao, C. E. Goodhue and J.-Q. Yu, *J. Am. Chem. Soc.*, 2006, **128**, 78.

8 S. Oi, E. Aizawa, Y. Ogino and Y. Inoue, *J. Org. Chem.*, 2005, **70**, 3113.

9 For representative examples, see: (*a*) Y. Kametani, T. Satoh, M. Miura and M. Nomura, *Tetrahedron Lett.*, 2000, **41**, 2655; (*b*) M. D. K. Boele, G. P. F. van Strijdonck, A. H. M. de Vries, P. C. J. Kamer, J. G. de Vries and P. W. N. M. van Leeuwen, *J. Am. Chem. Soc.*, 2002, **124**, 1586; (*c*) O. Daugulis and V. G. Zaitsev, *Angew. Chem., Int. Ed.*, 2005, **44**, 4046; (*d*) D. Kalyani, N. R. Deprez, L. V. Desai and M. S. Sanford, *J. Am. Chem. Soc.*, 2005, **127**, 7330; (*e*) R. Ferraccioli, D. Carenzi, E. Motti and M. Catellani, *J. Am. Chem. Soc.*, 2006, **128**, 722; (*f*) Z. Shi, B. Li, X. Wan, J. Cheng, Z. Fang, B. Cao, C. Qin and Y. Wang, *Angew. Chem., Int. Ed.*, 2007, **46**, 5554; (*g*) S. Yang, B. Li, X. Wan and Z. Shi, *J. Am. Chem. Soc.*, 2007, **129**, 6066; (*h*) C. E. Houlden, C. D. Bailey, J. G. Ford, M. R. Gagné, G. C. Lloyd-Jones and K. I. Booker-Milburn, *J. Am. Chem. Soc.*, 2008, **130**, 10066; (*i*) Y.-X. Jia and E. P. Kündig, *Angew. Chem., Int. Ed.*, 2009, **48**, 1636.

10 For representative examples, see: (*a*) B. M. Trost, K. Imi and I. W. Davies, *J. Am. Chem. Soc.*, 1995, **117**, 5371; (*b*) F. Kakiuchi, Y. Tanaka, T. Sato, N. Chatani and S. Murai, *Chem. Lett.*, 1995, 679; (*c*) K. Kashiwagi, R. Sugise, T. Shimakawa, T. Matuura, M. Shirai, F. Kakiuchi and S. Murai, *Organometallics*, 1997, **16**, 2233.

11 For representative examples, see: (*a*) S. Murai, F. Kakiuchi, S. Sekine, Y. Tanaka, A. Kamatani, M. Sonoda and N. Chatani, *Nature*, 1993, **366**, 529; (*b*) M. Sonoda, F. Kakiuchi, N. Chatani and S. Murai, *J. Organomet. Chem.*, 1995, **504**, 151; (*c*) F. Kakiuchi, Y. Yamamoto, N. Chatani and S. Murai, *Chem. Lett.*, 1995, 681; (*d*) M. Sonoda, F. Kakiuchi, A. Kamatani, N. Chatani and S. Murai, *Chem. Lett.*, 1996, 109; (*e*) F. Kakiuchi, M. Yamauchi, N. Chatani and S. Murai, *Chem. Lett.*, 1996, 111; (*f*) M. Sonoda, F. Kakiuchi, N. Chatani and S. Murai, *Bull. Chem. Soc. Jpn.*, 1997, **70**, 3117.

12 (*a*) M. Miura, T. Tsuda, T. Satoh, S. Pivsa-Art and M. Nomura, *J. Org. Chem.*, 1998, **63**, 5211; (*b*) R. Giri, N. Maugel, J.-J. Li, D.-H. Wang, S. P. Breazzano, L. B. Saunders and J.-Q. Yu, *J. Am. Chem. Soc.*, 2007, **129**, 3510; (*c*) H. A. Chiong, Q.-N. Pham and O. Daugulis, *J. Am. Chem. Soc.*, 2007, **129**, 9879; (*d*) R. Giri and J.-Q. Yu, *J. Am. Chem. Soc.*, 2008, **130**, 14082, see also, ref. 15.

13 For representative examples, see: (*a*) L. N. Lewis and J. F. Smith, *J. Am. Chem. Soc.*, 1986, **108**, 2728; (*b*) T. Satoh, T. Itaya, M. Miura and M. Nomura, *Chem. Lett.*, 1996, 823; (*c*) M. Miura, T. Tsuda, T. Satoh and M. Nomura, *Chem. Lett.*, 1997, 1103; (*d*) T. Satoh, Y. Kawamura, M. Miura and M. Nomura, *Angew. Chem., Int. Ed. Engl.*, 1997, **36**, 1740; (*e*) K. Kokubo, K. Matsumasa, M. Miura and M. Nomura, *J. Org. Chem.*, 1997, **62**, 4564; (*f*) M. Miura, T. Tsuda, T. Satoh, S. Pivsa-Art and M. Nomura, *J. Org. Chem.*, 1998, **63**, 5211.

14 (*a*) H. Ohno, K. Miyamura, Y. Takeoka and T. Tanaka, *Angew. Chem., Int. Ed.*, 2003, **42**, 2647; (*b*) H. Ohno, M. Yamamoto, M. Iuchi and T. Tanaka, *Angew. Chem., Int. Ed.*, 2005, **44**, 5103; (*c*) T. Watanabe, S. Ueda, S. Inuki, S. Oishi, N. Fujii and H. Ohno, *Chem. Commun.*, 2007, 4516; (*d*) H. Ohno, M. Iuchi, N. Fujii and T. Tanaka, *Org. Lett.*, 2007, **9**, 4813; (*e*) T. Watanabe, S. Oishi, N. Fujii and H. Ohno, *Org. Lett.*, 2007, **9**, 4821; (*f*) T. Watanabe, S. Oishi, N. Fujii and H. Ohno, *Org. Lett.*, 2008, **10**, 1759.

15 O. Reinaud, P. Capdevielle and M. Maumy, *Synthesis*, 1990, 612.

16 (*a*) X. Chen, X.-S. Hao, C. E. Goodhue and J.-Q. Yu, *J. Am. Chem. Soc.*, 2006, **128**, 6790; (*b*) T. Uemura, S. Imoto and N. Chatani, *Chem. Lett.*, 2006, **35**, 842.

17 (*a*) G. Brasche and S. L. Buchwald, *Angew. Chem., Int. Ed.*, 2008, **47**, 1932; (*b*) S. Ueda and H. Nagasawa, *Angew. Chem., Int. Ed.*, 2008, **47**, 6411.

18 For a related copper-catalysed C–H arylation using an oxazoline or imidazoline as the directing group, see: H.-Q. Do, R. M. Kashif Khan and O. Daugulis, *J. Am. Chem. Soc.*, 2008, **130**, 15185.

19 Because the separation of **7a** and the by-product **3a** was difficult, separation by alumina column chromatography was necessary before carbonylation.

CHEMBIOCHEM

DOI: 10.1002/cbic.200700761

Synthesis and Application of Fluorescein- and Biotin-Labeled Molecular Probes for the Chemokine Receptor CXCR4

Shinya Oishi,*[a] Ryo Masuda,[a] Barry Evans,[b] Satoshi Ueda,[a] Yukiko Goto,[a] Hiroaki Ohno,[a] Akira Hirasawa,[a] Gozoh Tsujimoto,[a] Zixuan Wang,[b] Stephen C. Peiper,[b] Takeshi Naito,[c] Eiichi Kodama,[c] Masao Matsuoka,[c] and Nobutaka Fujii*[a]

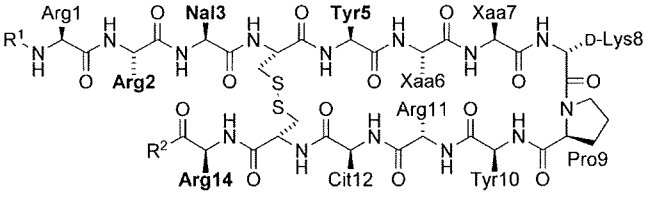
The design, synthesis, and bioevaluation of fluorescence- and biotin-labeled CXCR4 antagonists are described. The modification of D-Lys8 at an ε-amino group in the peptide antagonist Ac-TZ14011 derived from polyphemusin II had no significant influence on the potent binding of the peptide to the CXCR4 receptor. The application of the labeled peptides in flow cytometry and confocal microscopy studies demonstrated the selectivity of their binding to the CXCR4 receptor, but not to CXCR7, which was recently reported to be another receptor for stromal cell-derived factor 1 (SDF-1)/CXCL12.

Introduction

The CXC chemokine receptor 4 (CXCR4) is a G-protein-coupled cell-surface receptor that was identified previously as a coreceptor for infection by the T-cell-line-tropic (X4) human immunodeficiency virus type 1 (HIV-1).[1,2] Stromal cell-derived factor 1 (SDF-1)/CXCL12 is a homeostatic chemokine that regulates a number of physiological and pathologic processes through its interaction with and activation of CXCR4. SDF-1 secreted in bone-marrow stromal cells supports the retention of hematopoietic stem cells (HSCs), progenitor cells, and B-cell precursors in the hematopoietic microenvironment.[3] SDF-1 expression is implicated in the survival, growth, and development of CXCR4-expressing cells, including normal and malignant B lymphocytes, hematopoietic progenitors, and carcinoma cells.[3,4] It has also been demonstrated that concentration gradients of SDF-1 promote the homing of HSCs to bone marrow, the recruitment of progenitor cells to sites of ischemic tissue damage, and the metastasis of CXCR4-expressing neoplastic cells to target organs.[4,5]

Recently, CXCR7 (RDC1, CCX-CKR2) was reported to be another receptor for SDF-1.[6,7] CXCR7 promotes cell survival, growth, and adhesion in vitro and in vivo.[7,8] Furthermore, the expression pattern of CXCR7 is complementary to that of CXCR4 in the migrating primordium.[9,10] Therefore, the SDF-1–CXCR7 axis, like SDF-1–CXCR4, is relevant to the control processes of cell growth, migration, and recruitment. To investigate the distribution and localization of two binding partners of SDF-1, CXCR4 and CXCR7, both in vitro and in vivo, it would be useful to have access to selective and specific fluorescence- and otherwise-labeled ligands for these receptors.

To date, several CXCR4-receptor probes have been prepared and applied both in vitro[11–14] and in vivo.[15] Fluorescein-labeled SDF-1 was utilized to detect the CXCR4-dependent internalization of SDF-1 by stromal bone-marrow cells.[11] This labeled agonist was useful for evaluating the mechanism of receptor activation. We developed a potential radiopharmaceutical agent based on the polyphemusin II derived CXCR4 antagonist T140 (Scheme 1). Thus, [^{111}In]–diethylenetriaminepenta-



	R^1	R^2	Xaa6	Xaa7
T140	H	OH	Arg	Lys
Ac-TZ14011 (**1**)	Ac	NH_2	Cit	Arg

Scheme 1. Structure of the selective CXCR4 antagonists T140, which was used to design probe Ac-TZ14011 (**1**). Bold type indicates the pharmacophore residues.

[a] *Dr. S. Oishi, R. Masuda, Dr. S. Ueda, Y. Goto, Dr. H. Ohno, Dr. A. Hirasawa, Prof. Dr. G. Tsujimoto, Prof. Dr. N. Fujii*
Graduate School of Pharmaceutical Sciences, Kyoto University
Sakyo-ku, Kyoto 606-8501 (Japan)
Fax: (+81) 75-753-4570
E-mail: soishi@pharm.kyoto-u.ac.jp
nfujii@pharm.kyoto-u.ac.jp

[b] *B. Evans, Dr. Z. Wang, Prof. Dr. S. C. Peiper*
Department of Pathology, Medical College of Georgia
Georgia 30912 (USA)

[c] *T. Naito, Dr. E. Kodama, Prof. Dr. M. Matsuoka*
Institute for Virus Research, Kyoto University
Sakyo-ku, Kyoto 606-8507 (Japan)

Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

acetic acid (DTPA) labeled Ac-TZ14011 was designed for the in vivo imaging of CXCR4-expressing tumors.[15] Rhodamine-conjugated azamacrocycle antagonists were also developed; however, the small molecules were taken up into the cells by a potential active-transport process.[13]

On the basis of our previous research on peptide-based CXCR4 antagonists,[16] we conducted an extensive structure–activity-relationship analysis of labeled ligands with CXCR4 receptors expressed on the cell surface. Herein, we report the design of the labeled antagonists and their application in in vitro experiments, including flow cytometry. The selectivity of the ligand for CXCR4 versus CXCR7 was also investigated by confocal microscopy.

Results and Discussion

Peptide design and synthesis

Previous alanine-scanning experiments identified four indispensable pharmacophore residues of T140, which are located peripheral to the disulfide bridge.[17] On the other hand, modification of the N and C termini or β-turn region with several types of functional groups or peptidomimetics did not lead to a decrease in activity.[16] For example, arylacyl functional groups, such as fluorobenzoyl, at the N terminus of T140 analogues enhanced anti-HIV activity.[18] On the basis of these precedent structure–activity-relationship studies on T140 derivatives, we designed two types of potential labeled CXCR4 ligands (Tables 1 and 2). The functional groups for labeling were attached with an appropriate spacer by acylation to the α-amino group of the N-terminal Arg1 residue or on the ε-amino group of D-Lys8. To identify appropriate fluorophores that did not affect peptide binding affinity to CXCR4, carboxyfluorescein and Alexa Fluor 488, which have a similar fluorescence spectrum, were used for fluorescence labeling. The different functional groups on the fluorescent section of peptides could have an effect on the affinities of the peptides for CXCR4. The Alexa Fluor 488 dye, which contains an amino group, an imino group, and two sulfonate groups on a xanthene structure, exhibited greater photostability and pH insensitivity.

Peptide resins were constructed manually by standard Fmoc-based solid-phase peptide synthesis (SPPS) by using *N,N'*-diisopropylcarbodiimide (DIC)/1-hydroxybenzotriazole (HOBt). Fluorescein or acetyl modification at the N-terminal α-amino group of peptides **1**–**4** was carried out on the resin by using carboxyfluorescein/DIC/HOBt or Ac_2O/pyridine, respectively. For the preparation of peptides **6**–**8** and **10** with either a fluorescein label or a 6-aminocaproic acid (Acp) linker combined with a fluorescein, biotin, or Alexa Fluor 488 label on the ε-amino group of D-Lys8, a 4-methyltrityl (Mtt) group was used for side-chain protection (Scheme 2). After the removal of the Mtt group on peptide **11** with 1,1,1,3,3,3-hexafluoropropan-2-ol (HFIP), an Acp linker and/or labeling groups were attached to the peptide resin **12** by a standard protocol to afford the labeled protected peptide resins **13**–**16**. Treatment of the protected peptide resins with TFA/1,2-ethanedithiol (EDT)/H_2O (95:2.5:2.5) followed by air oxidation in aqueous solution yielded the expected peptides **1**–**4**, **6**–**8**, and **17**.

Labeling with Alexa Fluor 488 was performed with the activated succinimidyl ester, which is commercially available. The

Table 1. Sequences and biological activity of labeled T140 analogues.

R-Arg-Arg-Nal-Cys-Tyr-Cit-Arg-D-**Xaa**-Pro-Tyr-Arg-Cit-Cys-Arg-NH_2

Peptide	R	D-Xaa	IC_{50} [nM][a]
1	Ac	D-Lys	5.2±0.1
2	Ac	D-Glu	6.7±2.6
3	fluorescein	D-Lys	24±0.3
4	fluorescein	D-Glu	199±26
5	Alexa Fluor 488	D-Glu	5700±769

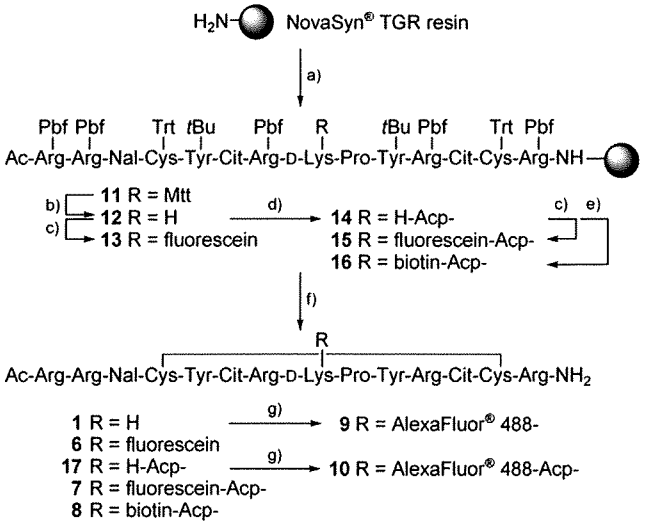
[a] IC_{50} values for the peptides are based on the inhibition of [^{125}I]SDF-1 binding to CHO cells that were transfected with CXCR4.

Table 2. Sequences and biological activity of Ac-TZ14011 analogues.

Ac-Arg-Arg-Nal-Cys-Tyr-Cit-Arg-D-**Lys**(R)-Pro-Tyr-Arg-Cit-Cys-Arg-NH_2

Peptide	R	IC_{50} [nM][a]
1	H (amine)	5.2±0.1
6	fluorescein	16±0.8
7	fluorescein-Acp-	26±2.4
8	biotin-Acp-	11±0.1
9	Alexa Fluor 488	8.1±3.5
10	Alexa Fluor 488-Acp-	267±19

[a] IC_{50} values for the peptides are based on the inhibition of [^{125}I]SDF-1 binding to CHO cells that were transfected with CXCR4.



Scheme 2. Synthesis of D-Lys8-labeled CXCR4 antagonists: a) Fmoc-based peptide synthesis; b) CH_2Cl_2/HFIP/TFE/TES (65:20:10:5); c) carboxyfluorescein, DIC, HOBt; d) Fmoc-Acp-OH, DIC, HOBt, then 20% piperidine/DMF; e) biotin, DIC, HOBt; f) TFA/EDT/H_2O (95:2.5:2.5), then NH_4OH; g) Alexa Fluor 488–OSu, iPr_2NEt. DMF: *N,N*-dimethylformamide; Fmoc: 9-fluorenylmethoxycarbonyl; Pbf: 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; TFE: 2,2,2-trifluoroethanol; TES: triethylsilane; Trt: triphenylmethyl (trityl).

precursor peptides (e.g., **1**, **17**) were modified in DMF to provide the expected peptides **5**, **9**, and **10** with a single Alexa Fluor 488 dye moiety.[19]

Biological evaluation of fluorescein- and biotin-labeled peptides

The CXCR4-antagonistic activity of peptides **1**–**10** was evaluated with respect to the inhibition of [^{125}I]SDF-1 binding to CXCR4 Chinese hamster ovary (CHO) cell transfectants. The replacement of D-Lys8 in the parent peptide **1** with D-glutamic acid had no significant effect on the bioactivity of the peptide (IC_{50}(**1**) = 5.2 nM; IC_{50}(**2**) = 6.7 nM; Table 1); this is consistent with the results of previous Glu-scanning experiments of a related peptide.[20] This result suggested that the modification of the β turn $i+1$ position of the peptides with a functional group for labeling would be possible. Fluorescein modification of the N terminus of peptides **1** and **2** led to a slight and significant decrease in inhibitory activity (IC_{50}(**3**) = 24 nM; IC_{50}(**4**) = 199 nM), respectively. Although the substituted benzoyl and pyridinecarbonyl groups at the N terminus of the peptide improved its bioactivity,[18] an additional xanthene or carboxyl group might be unfavorable to ligand binding with CXCR4. The Alexa Fluor 488 labeled peptide **5** showed a significant decrease in inhibitory activity (IC_{50}(**5**) = 5.7 μM); this indicates that the N terminus is inappropriate for fluorescence labeling.

Modification of the ε-amino group of D-Lys8 in the parent peptide **1** was another promising approach to the creation of labeled CXCR4 antagonists (Table 2). The fluorescein-modified peptides **6** and **7** exhibited slightly decreased bioactivity but retained significant binding affinity for CXCR4 (IC_{50}(**6**) = 16 nM; IC_{50}(**7**) = 26 nM). The biotin-labeled peptide **8** containing an Acp spacer, which would be helpful for the simultaneous binding of **8** with CXCR4 and avidins, was also a potent inhibitor (IC_{50}(**8**) = 11 nM). Thus, the presence of a functional group at this position for labeling, with or without an Acp spacer, did not appear to influence the bioactivity of the peptide. We concluded that the D-Lys8 residue in the β-turn region might be unimportant for direct molecular recognition by CXCR4; consequently, this position was considered to be more appropriate for labeling. The Alexa Fluor 488 labeled peptide **9** without an Acp linker showed nearly equipotent inhibitory activity to that of the parent peptide **1** (IC_{50}(**9**) = 8.1 nM). In contrast, significantly lower bioactivity was observed for peptide **10**, which contains an Acp linker (IC_{50}(**10**) = 267 nM). This result implies that the modified xanthene structure of Alexa Fluor 488 might cause some unfavorable interactions with the receptor, contrary to our expectations. The two potent labeled peptides **6** and **9** were used for further experiments.

Application of fluorescence-labeled peptides to flow cytometry and confocal microscopy studies

The applicability of the fluorescence-labeled CXCR4 antagonists **6** and **9** to in vitro experiments was investigated (Figure 1A and B). CHO cells that expressed high levels of the CXCR4 receptor and CXCR4-negative control cells were incubated with peptide **6** or **9** (200 nM), and the resulting mixtures were analyzed by flow cytometry. The CXCR4-expressing cells bound the fluorescent ligand, but the cells that did not express CXCR4 were not stained. The binding of peptides **6** and **9** was inhibited by competition with the unlabeled specific CXCR4 antagonist T140 (200 nM). This result supports the specificity of the fluorescent ligands for CXCR4. With the fluorescent probe **6**, lymphocytes derived from mouse spleen were identified by light scatter gating and analyzed for binding of the fluorescent antagonists (Figure 1C). Peptide **6** bound to CXCR4-expressing lymphocytes, and the staining was inhibited competitively by the addition of unlabeled T140 (200 nM). Peptide **6** was also utilized for the detection of chemotactic cells in a transmigration assay with CXCL12 (Figure 1D). Whereas a low percentage of the cells in the top well of a chemotaxis chamber were positive, the cells which passed through 3-μm pores in response to the CXCL12 chemotactic gradient were all stained positively with peptide **6**.

The probing ability of **6** and **9** for CXCR4 was also verified by confocal microscopy studies on CXCR4-expressing HEK293 cells (Figure 2). The cell surface of CXCR4-positive cells was stained with peptides **6** and **9** in a dose-dependent manner (Figure 2A; see also the Supporting Information). This result is in contrast to a previous report that a rhodamine-labeled azamacrocycle localizes in the cytoplasm by nonspecific uptake.[13] Staining was not observed with CXCR4-negative control cells; this suggests that receptor recognition of these fluorescent peptides is specific to CXCR4 (Figure 2C). Furthermore, CXCR7-expressing HEK293 cells were not stained by the fluorescent peptides **6** and **9** (Figure 2B); this indicates that these T140 derivatives are selective inhibitors of the CXCR4 receptor.

Conclusions

In the current study the effects of labeling a peptide at different positions with various functionalities with a view to retaining indispensable interactions with the CXCR4 receptor was investigated. Fluorescein, biotin, and Alexa Fluor 488 moieties on the D-Lys8 ε-amino group of the parent peptide were appropriate labels. The resulting labeled peptides exhibited specific and high affinity for the CXCR4 receptor, but not for the CXCR7 receptor. The labeled peptides could be useful as selective molecular probes for the CXCR4 receptor in future in vitro and/or in vivo experiments.

Experimental Section

General procedure for peptide synthesis: Protected peptide resins were constructed manually by standard Fmoc-based SPPS on NovaSyn TGR resin (192 mg, 0.05 mmol) by using DIC (39 μL, 0.25 mmol) in combination with HOBt (38 mg, 0.25 mmol). The side chains Tyr, Glu, Lys, Cys, and Arg were protected with *t*Bu, *t*Bu ester, Boc, Trt, and Pbf groups, respectively. For the preparation of peptides **6**–**8** and **10**, the Mtt group was used to protect the D-Lys8 side chain. The N-terminal α-amino group was acetylated by treatment of the resin with Ac_2O (24 μL, 0.25 mmol) and pyridine (40 μL, 0.10 mmol). Biotin (61 mg, 0.25 mmol) or carboxyfluorescein (94 mg, 0.25 mmol) was coupled to the peptide by using DIC

