domain of Tn3 resolvase (clones 1 and 2). The second group of variants has semirigid linkers (clones 3–13). The third group has flexible linker sequences composed of Gly-Ser sequences (clones 14–19). In the clones of the third group, the first two amino acids of the zinc finger domain, Tyr and Lys, are substituted with Phe and Glu, respectively. The recombination efficiencies were determined in the *E. coli*-based assay (Figure 3B). The results indicate two important phenomena. (1) The variant with a 12-amino acid linker was the most efficient (clone 7, Figure 3A), suggesting that there is an optimal linker length. (2) The variants with linkers composed of only Gly-Ser sequences were most efficient (clones 15–17, Figure 3A), indicating that ZFRs with flexible linkers tended to recombine most efficiently.

ZFR-Catalyzed Recombination in Mammalian Cells. To evaluate the recombination efficiency of ZFR variants in mammalian cells, we constructed a reporter cell line from Flp-In-CHO-K1 containing a cassette that encodes EGFP driven by a CMV promoter flanked by target sites (Figure 4). As each cell contains a single copy of the reporter gene, the recombination efficiency can be calculated from the proportion of cells with or without EGFP fluorescence. Additionally, the expression of ZFR was monitored by the expression of DsRed; this gene was placed downstream of the ZFR gene via a IRES sequence. The genes encoding ZFRs utilized in this study were amplified from a pAra plasmid shown in Figure 2A. Thus, the sequences of clones are the same as those utilized in experiments in *E. coli*.

With this reporter system, recombination efficiencies could be evaluated 48 h after transfection. Reported procedures involving retroviral-based transduction, selection, and evaluation take nearly 10 days.[8] The fluorescence intensity of cells was detected by FACS analysis (Figure S4 of the Supporting Information). The cells with recombinant genes were those that were EGFP-negative and DsRed-positive. The recombination efficiencies depended on the number of finger modules and on the linker lengths (Figure 5). As in *E. coli*, the five-finger proteins were the most efficient in recombination. The optimal linker length was six residues, which is different from that in *E. coli*. Additionally, recombination in mammalian cells was not as efficient as that in *E. coli*.

■ DISCUSSION

This study demonstrated that ZFR recombinases can be designed to specifically target sites in *E. coli* and mammalian cells and that recombination efficiency depends on the affinity of the ZFP for the DNA target and on the length of the linker between the DNA binding domain and the recombinase domain. The ZFR with five fingers had the highest recombination efficiency in both *E. coli* and CHO-K1 cells. The DNA binding affinity of this particular ZFP was saturated when the DNA binding domain had more than five fingers. The association and dissociation with DNA binding depend on the number of finger modules.[33] It is possible that the ZFR with five fingers was the most efficient recombination because the balance of association with dissociation and turnover was optimal. Guo et al. have also reported that four and five ZF domains are optimal for activity of ZFN.[34] On the basis of our data, the apparent K_d values of the four-, five-, and six-finger proteins derived from this particular ZFP were similar. The dependence on the number of finger modules was common in both *E. coli* and mammalian cells, but the recombination efficiency was lower in mammalian cells. In CHO-K1 cells, DNA is sequestered in chromatin structures. Additionally, the circular form of plasmid DNA could enhance recombination in the bacterial cells.

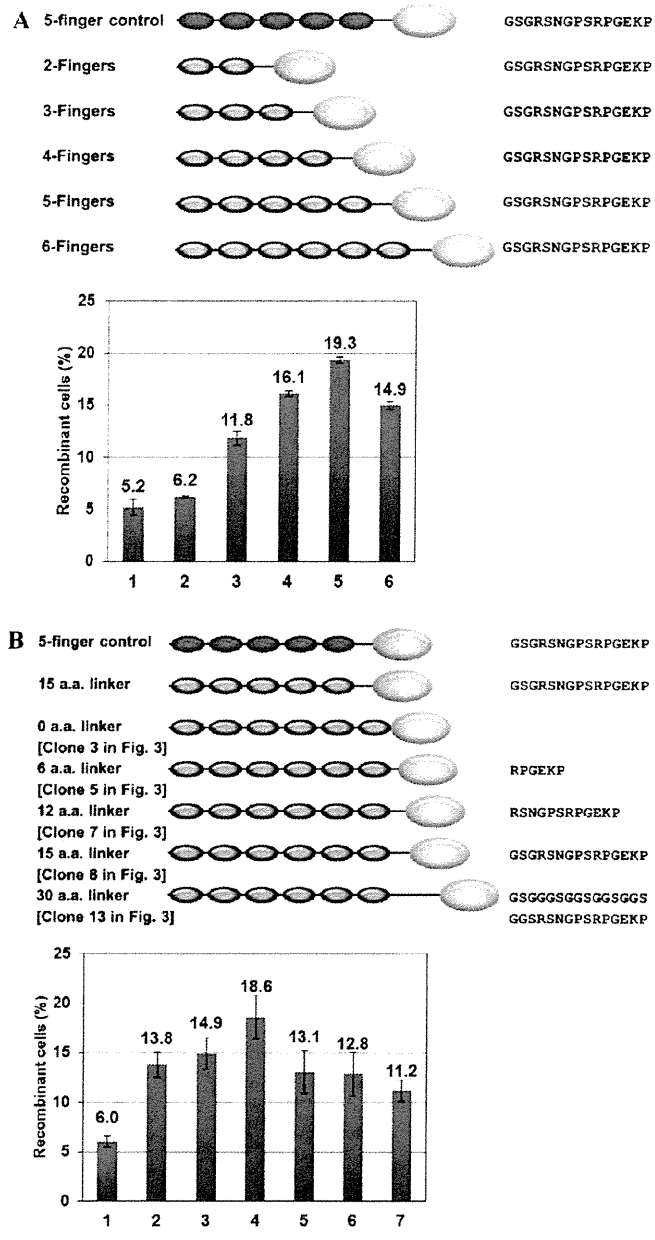

Figure 5. Recombination efficiency of ZFRs containing various numbers of fingers (A) and with various linkers (B) in mammalian cells. The top cartoons represent ZFR constructs utilized in the analyses. Green, blue, and yellow spheres represent zinc finger modules without sequence specificity, zinc finger modules with sequence specificity, and the Tn3 catalytic domain, respectively. Letters at the right of the cartoons are the linker sequences of the constructs. (A) Dependence on the number of fingers of ZFRs. The columns are as follows: column 1, five-finger control (nonspecific DNA binding); column 2, two fingers; column 3, three fingers; column 4, four fingers; column 5, five fingers; column 6, six fingers and different linker lengths. (B) Dependence on linker length. The columns are as follows: column 1, nontarget five-finger control with 15 amino acids; column 2, targeted five-finger ZFR with 15-amino acid linker; columns 3–7, targeted six-finger ZFRs with linker lengths of 0, 6, 12, 15, and 30 amino acids, respectively. The error bars show the SEM of three or more independent experimental results.

Recombination efficiency was dependent on the linker between the zinc finger domain and the recombinase domain. ZFRs with the shortest linkers had a very low efficiency of

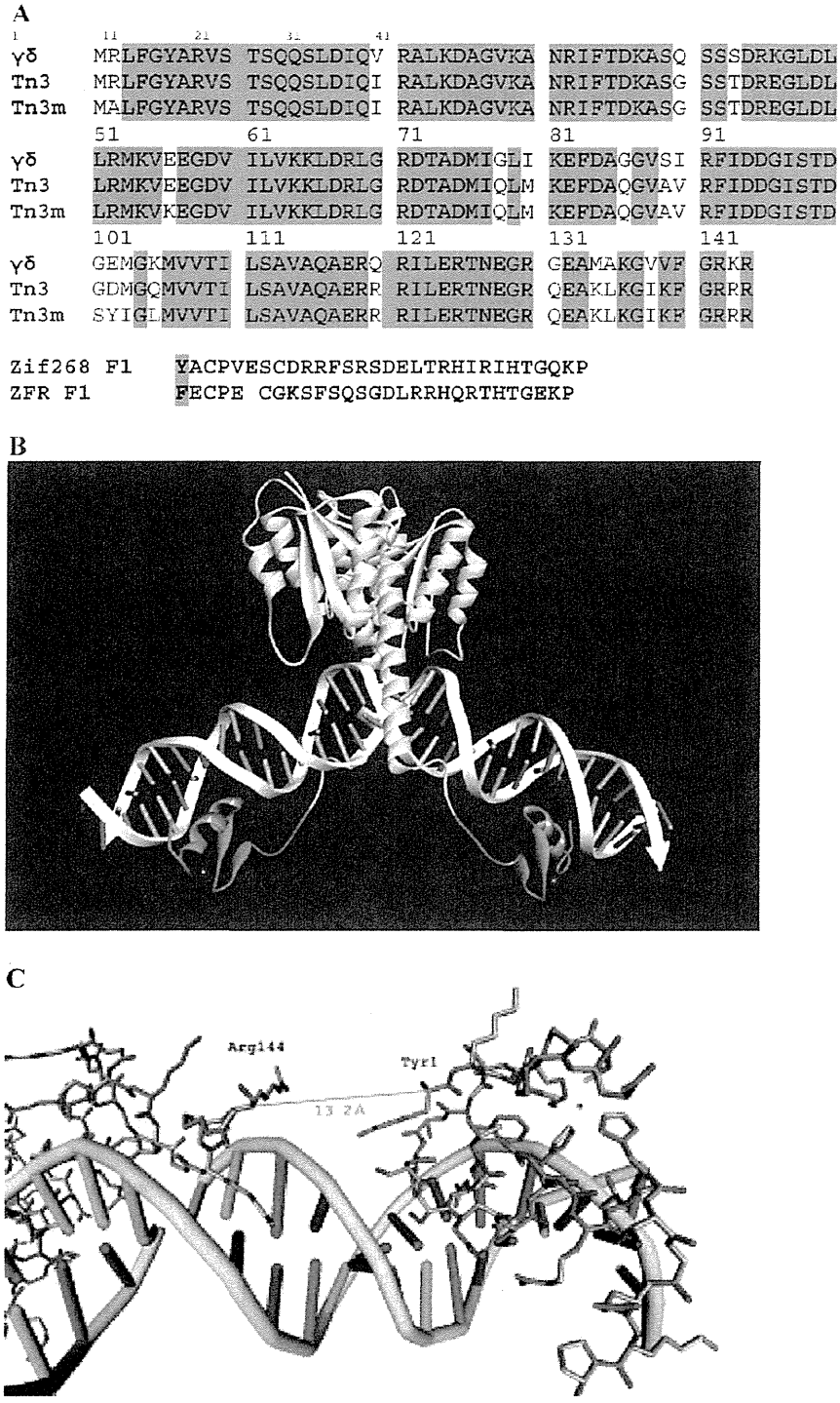

Figure 6. Representative result of molecular modeling of the resolvase domain and the first zinc finger module separated by a six-amino acid linker sequence. (A) Sequence alignment of resolvases *γδ* and Tn3 and the Tn3 hyperactivated mutant (Tn3m) (top), the first finger of zif268, and ZFR. Conserved residues are highlighted in red, and amino acid substitutions in the hyperactive mutant are highlighted in yellow. The N-terminal aromatic amino acids of zinc fingers are highlighted in blue. (B) The yellow ribbon indicates *γδ* resolvase, the red ribbon the six-amino acid linker, the green ribbon the N-terminal zinc finger domain, and the gray ribbon the zinc ion. (C) Distances between C*α* atoms of Arg144 and tyrosine (Tyr) at the N-terminus of zif268. The N-terminal amino acid of the zinc finger domain is phenylalanine (Phe) in ZFRs utilized in this study.

recombination in both bacterial and mammalian cells. Second, the length of linkers based on the original sequences was critical. Proteins with linkers containing 12 amino acid residues were the most efficient in recombination. In the Gly-Ser linker variants, the recombination efficiency reached a maximum at six amino acids. This result indicates that both the length and the flexibility of the linker are important.

A molecular modeling study was performed in an attempt to assess the reasons for the differences in recombination efficiency among the linker mutants. In the modeling of the ZFR complex with target DNA, the linker length of six amino acids was optimal for the DNA binding of ZFR when the linker sequence was flexible (Figure 6A). When the domains were modeled bound to the target sequence, the distance between

the Cα atom of Arg144 in the γδ resolvase (Figure S5 of the Supporting Information) and that of Tyr at the N-terminus of the zinc finger domain is ~13.2 Å (Figure 6B,C). In polypeptides in the extended conformation, the distance between Cα atoms of sequential amino acids is 3.8 Å. Thus, a linker consisting of three amino acids (clone 4 or clone 14) should allow the protein to bind to both DNA regions, although these ZFRs had very low recombination efficiencies. In the complex with DNA, the amino groups at positions 145 and 146 of the main chain in γδ resolvase interact with the phosphate backbone of DNA and amino acids of these positions are involved in the folding of the catalytic domain (Figure S5 of the Supporting Information). In the case of clone 4, the Lys-Pro residues at the C-terminus of linker residues are involved in the folding of the zinc finger domain. Thus, these amino acids are considered to be members of both domains, not of the linker sequences. With this reasoning, the six and nine amino acids in the linkers for clones 4 and 5, respectively, are shorter than the theoretically optimal length. Moreover, in the sequences of the six- and nine-amino acid linkers, the amino acid at position 146 is Pro, which could disrupt the interaction with DNA phosphate, thus lowering the recombination efficiency. Consistent with these estimations, the Gly-Ser linker with six or nine amino acids (clones 15 and 16, respectively) showed the best recombination ratio. This evidence indicates that the residues at the C-terminus of the catalytic domain and the N-terminus of the zinc finger domain are involved in domain folding because Lys-Pro residues at the N-terminus of the zinc finger domains are not included in these clones. Variants around this optimal linker length, especially those with 12 and 15 amino acids, had similar recombination efficiencies. These results show that the flexibility of the linker is not necessary when the linker length is optimal. In mammalian cells, the variant with a linker of six amino acids (clone 5) showed the best recombination and the zero-amino acid linker (clone 3) showed better recombination than the variants with longer linkers of more than 12 amino acids. The reason for this effect is unclear, but it could be due to differences in the structures of target sites on the plasmid DNA compared to the genomic DNA. Additionally, the distances between the binding sites in these systems are different. In the genomic target, the binding sites are separated by sequences of more than 2500 bp.

In this study, a newly developed recombination system allowed measurement of recombination efficiencies of ZFRs in *E. coli* and in mammalian cells. In mammalian cells, recombination with genomic targets was evaluated within 48 h of the transient expression of recombinases. Artificial enzymes such as ZFN and ZFR have been studied mainly by using viral vector systems to deliver their genes into mammalian genomes. In a report describing utilization of the retrovirus vectors for gene delivery, the recombination efficiency was as high as ~18%.[8] In our study, we also observed up to 18% recombination in cells. This system could be utilized in future studies to evaluate function of ZFRs on specific targets.

■ ASSOCIATED CONTENT

Supporting Information

Details of subcloning, experimental results of plasmid digestion and sequencing, results of FACS analyses, and a description of key interactions in γδ resolvase. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: nomura.mr@tmd.ac.jp or tamamura.mr@tmd.ac.jp. Phone: +81-3-5280-8036. Fax: +81-3-5280-8039.

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Notes

The authors declare no competing financial interest.

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Pharmacophore-based small molecule CXCR4 ligands

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

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

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ABSTRACT

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

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

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

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

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

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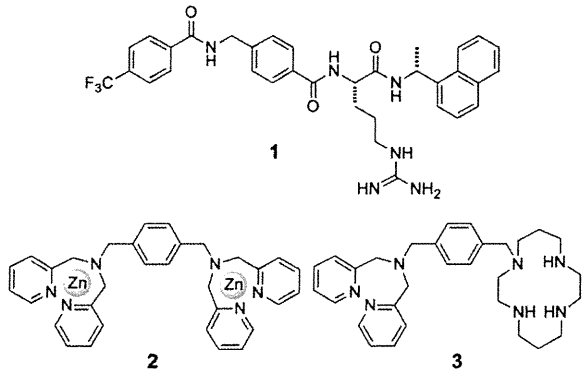


Figure 1. The structures of **1** (ST34), Dpa-Zn complex (**2**) and Dpa-cyclam compound (**3**).

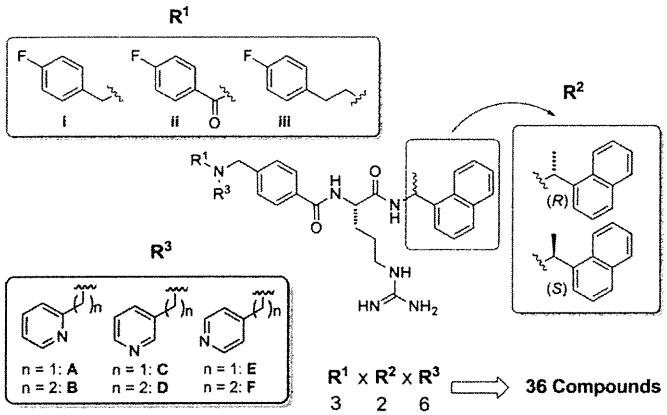
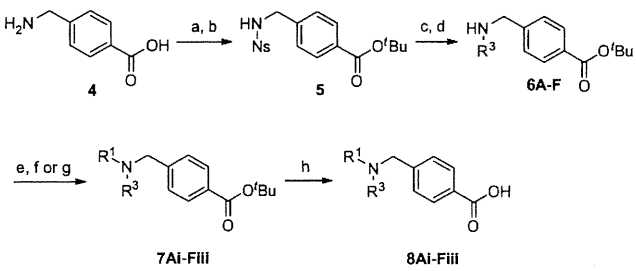


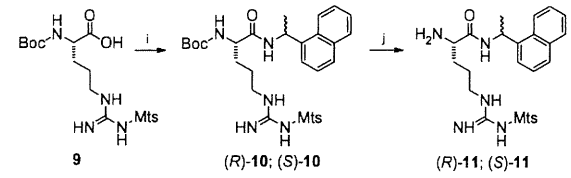
Figure 2. The structures of substituents for three parts of compound **1** in the design of new compounds.



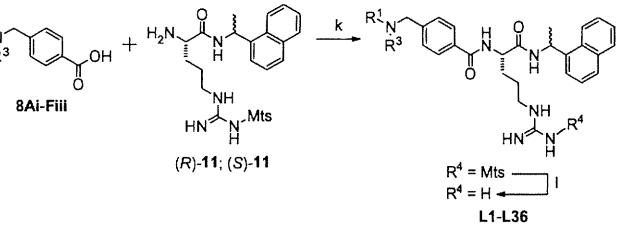
Scheme 1. The synthetic scheme of Unit 1, compounds **8Ai–Fiii**. Reagents and conditions and yields: (a) NsCl, Et_3N, THF/H_2O (1/1); (b) isobutene, THF/H_2SO_4 (10/1), 39% (2 steps); (c) PPh_3, DEAD, R^3OH, THF; (d) PhSH, K_2CO_3, DMF, 42–92% (2 steps); (e) $NaBH(OAc)_3$, 4-fluorobenzaldehyde, CH_2Cl_2;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

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

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



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



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

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

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

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

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

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

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

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

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15. *For example, the synthesis of compound* ***30***: To a stirred solution of **8Ai** (176 mg, 0.415 mmol, HCl salt) in DMF (4 mL) were added EDCI·HCl (104 mg, 0.454 mmol), HOBt·H_2O (58.4 mg, 0.381 mmol), Et_3N (301 μL, 2.16 mmol) and ***(S)*-11** (320 mg, 0.657 mmol, HCl salt) at 0 °C. The mixture was stirred at room temperature for 43 h. The reaction mixture was diluted with $CHCl_3$ and washed with saturated citric acid, saturated $NaHCO_3$ and brine, and dried over $MgSO_4$. Concentration under reduced pressure followed by flash column chromatography over silica gel with $CHCl_3$/MeOH (20/1) gave the condensation product (175 mg, 0.208 mmol, 50% yield) as white powder. To this compound were added *m*-cresol (75.0 μL, 0.714 mmol), 1,2-ethanedithiol (225 μL, 2.68 mmol), thioanisole (225 μL, 1.91 mmol), TFA (3 mL) and bromotrimethylsilane (495 μL, 3.82 mmol) with stirring at 0 °C, and the stirring was continued at room temperature for 3.5 h under N_2. The reaction mixture was concentrated under reduced pressure, followed by addition of Et_2O to precipitate the product. After washing with Et_2O, the crude product was purified by preparative HPLC and lyophilized to give the compound **30** (15.6 mg, 0.0236 mmol, 13%) as white powder. ^{1}H NMR δ_H (400 MHz; DMSO-d_6) 1.49 (m, 2H), 1.51 (d, J = 7.2 Hz, 3H), 1.80–1.62 (m, 2H), 3.07 (dd, J = 6.4, 12.8 Hz, 2H), 3.85 (s, 2H), 3.91 (s, 4H), 4.54 (m, 1H), 5.72 (m, 1H), 7.13 (t, J = 8.8 Hz, 2H), 7.40 (m, 1H), 7.60–7.45 (m, 10H), 7.75–7.95 (m, 5H), 8.10 (m, 1H), 8.40 (d, J = 8.0 Hz, 1H), 8.58 (m, 1H), 8.65 (d, J = 7.6 Hz, 1H); LRMS (ESI), m/z calcd for $C_{39}H_{42}FN_7O_2$ $(MH)^+$ 660.34, found 660.31.
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Stereoselective Formation of Trisubstituted (*Z*)-Chloroalkenes Adjacent to a Tertiary Carbon Stereogenic Center by Organocuprate-Mediated Reduction/Alkylation

Tetsuo Narumi, Takuya Kobayakawa, Haruo Aikawa, Shunsuke Seike, and Hirokazu Tamamura*

Department of Medicinal Chemistry, Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, 2-3-10 Kandasurugadai, Chiyoda-ku, Tokyo 101-0062, Japan

tamamura.mr@tmd.ac.jp

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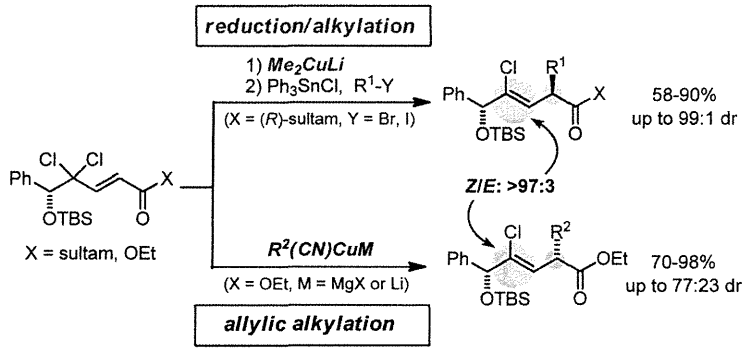
ABSTRACT



A robust and efficient method for the synthesis of trisubstituted (*Z*)-chloroalkenes is described. A one-pot reaction of γ,γ-dichloro-α,β-enoyl sultams involving organocuprate-mediated reduction/asymmetric alkylation affords α-chiral (*Z*)-chloroalkene derivatives in moderate to high yields with excellent diastereoselectivity, and allylic alkylation of internal allylic *gem*-dichlorides is also demonstrated. This study provides the first examples of the use of allylic *gem*-dichlorides adjacent to the chiral center for novel 1,4-asymmetric induction.

Stereoselective formation of functionalized alkenes is a challenging task in organic synthesis, and construction of halogenated alkenes while controlling the geometry of double bonds is of particular interest.[1] Among various halogenated alkenes, chloroalkenes have attracted considerable interest in recent years,[2–6] not only because of their potential as synthetically valuable intermediates[7] but also because of their importance as structural components of natural products.[8]

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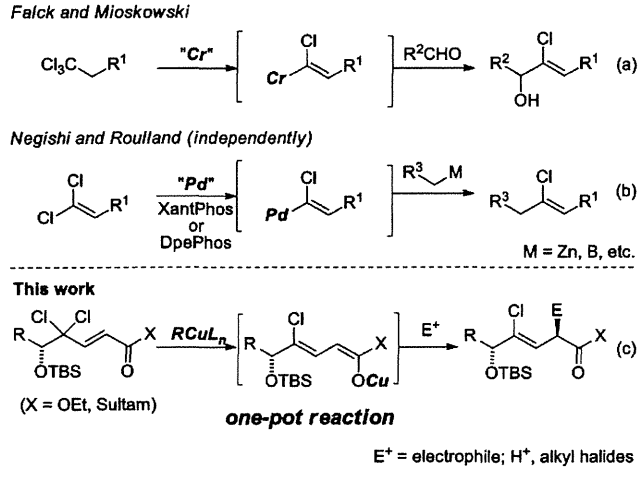
Despite the utility and importance of chloroalkenes, however, reactions leading to the stereoselective formation of trisubstituted (*Z*)-chloroalkenes are still limited.[5,6] Falck and Mioskowski reported that the reaction of $CrCl_2$ with 1,1,1-trichloroalkanes leads to the formation of (*E*)-chlorovinylidene chromium carbenoids, which can react with aldehydes to afford (*Z*)-chlorinated allylic alcohols (Scheme 1a).[5a] An alternative method is the Pd-catalyzed cross-coupling of 1,1-dichloro-1-alkenes with organometallic reagents.[6] In particular, Pd-catalyzed couplings with large bite angle bisphosphines such as Xantphos and DPEphos allow the selective formation of (*Z*)-chloroalkenes while avoiding the formation of bis-substituted products as has been described independently by Negishi[6a] and by Roulland[6b–d] (Scheme 1b). While these protocols have found widespread utility for the synthesis of these important structures, the development of efficient systems for stereoselective and divergent synthesis of trisubstituted (*Z*)-chloroalkenes bearing various functionalities remains challenging.

As part of a program aimed at development of novel approaches to chloroalkenes, we envisioned that the organocuprate-mediated reduction[9] of γ,γ-dichloro-α,β-unsaturated carbonyl compounds would permit an efficient access to (*Z*)-chlorinated dienolate intermediates, which can be trapped with an appropriate electrophile, providing trisubstituted (*Z*)-chloroalkenes (Scheme 1c).

Scheme 1. Synthesis of Trisubstituted (*Z*)-Chloroalkenes



In this paper, we describe the stereoselective formation of trisubstituted (*Z*)-chloroalkenes utilizing the organocuprate-mediated reduction/asymmetric alkylation of γ,γ-dichloro-α,β-enoyl sultam. This is a one-pot reaction which provides in high yield the synthetically valuable compounds containing a (*Z*)-chloroalkene flanking two stereogenic centers, the α-chiral-β,γ-unsaturated carbonyl motif, and a chiral allylic alcohol. In addition, we report the first allylic alkylation of *internal* allylic *gem*-dichlorides that provides an alternative method for the diastereoselective synthesis *via* 1,4-asymmetric induction of these important structural motifs.

We prepared sultam **1** and enoate **2** from chiral α,α-dichloro-β-hydroxyester,[10] reported by Imashiro and Kuroda, as suitable substrates for reaction development (Figure 1). At the onset of our studies, it was unclear if the reaction of those substrates with organocuprates would entail reduction, generating the dienolate intermediate. In order to estimate the electron-accepting ability, our investigation started with measurement of the reduction potentials (E_{Red}). The reduction potentials of sultam **1** and enoate **2** were -1.50 and -1.65 V, respectively. Based on these results and House's observation that α,β-unsaturated carbonyl compounds with reduction potentials between *ca.* -2.4 V and *ca.* -1.1 V can react with organocuprates such as Me_2CuLi to give the conjugate addition products,[11] these substrates were expected to promote both the single-electron transfer reduction and the allylic alkylation.

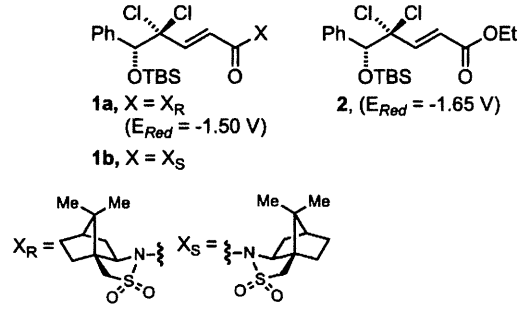


Figure 1. Substrates for organocuprate-mediated reduction and their reduction potentials (E_{Red}).

In order to control the reaction products, the reactivity of sultam **1a** with organocuprates was examined (Table 1),

(6) For selected examples of Pd-catalyzed cross-coupling, see: Cross-coupling with organozincs: (a) Tan, Z.; Negishi, E. *Angew. Chem., Int. Ed.* **2006**, *45*, 762. Cross-coupling with organoborans: (b) Guinchard, X.; Bugaut, X.; Cook, C.; Roulland, E. *Chem.—Eur. J.* **2009**, *15*, 5793. (c) Roulland, E. *Angew Chem. Int. Ed.* **2008**, *47*, 3762. (d) Liron, F.; Fosse, C.; Pernolet, A.; Roulland, E. *J. Org. Chem.* **2007**, *72*, 2220. Cross-coupling with other organometallics, see ref 1a.

(7) (a) Geary, L. M.; Hultin, P. G. *J. Org. Chem.* **2010**, *75*, 6354. (b) Bell, M.; Poulsen, T. B.; Jørgensen, K. A. *J. Org. Chem.* **2007**, *72*, 3053. (c) Jones, G. B.; Wright, J. M.; Plourde, G. W., II; Hynd, G.; Huber, R. S.; Mathews, J. E. *J. Am. Chem. Soc.* **2000**, *122*, 1937. (d) Alami, M.; Gueugnot, S.; Domingues, E.; Linstrumelle, G. *Tetrahedron* **1995**, *51*, 1209.

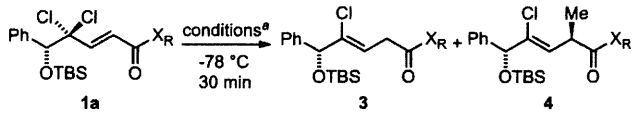
(8) For a recent example of the natural product bearing chloroalkene motif: Ando, H.; Ueoka, R.; Okada, S.; Fujita, T.; Iwashita, T.; Imai, T.; Yokoyama, T.; Matsumoto, Y.; van Soest, R. W. M.; Matsunaga, S. *J. Nat. Prod.* **2010**, *73*, 1947 and also ref 1a.

(9) For selected examples of organocuprate-mediated reduction, see: (a) Narumi, T.; Niida, A.; Tomita, K.; Oishi, S.; Otaka, A.; Ohno, H.; Fujii, N. *Chem. Commun.* **2006**, 4720. (b) Meyers, A. I.; Snyder, L. *J. Org. Chem.* **1992**, *57*, 3814. (c) Fujii, N.; Habashita, H.; Shigemori, N.; Otaka, A.; Ibuka, T.; Tanaka, M.; Yamamoto, Y. *Tetrahedron lett.* **1991**, *32*, 4969. (d) Takano, S.; Sekiguchi, Y.; Ogasawara, K. *J. Chem. Soc., Chem. Commun.* **1988**, 449 and references cited therein.

(10) Imashiro, R.; Kuroda, T. *J. Org. Chem.* **2003**, *68*, 974. For details of the preparation of sultam **1** and enoate **2**, see the Supporting Information.

(11) House, H. O.; Umen, M. J. *J. Org. Chem.* **1973**, *38*, 2417.

Table 1. Reactivity of Sultam **1a** with Organocuprates



entry	reagents	additives[b]	*Z*/*E* of **3**[c]	**3**+**4**, yield (%)[d]	**3**/**4**[c]
1	Me_2CuLi	–	>97:3	81	77:23
2	Me_2CuLi[e]	–	>97:3	93	>97:3
3	*n*-Bu_2CuLi[e]	–	>97:3	99	90:10
4	MeCu(CN)Li	–	>97:3	99	61:39[f]
5	Me_2CuLi[e]	TMSCl	>97:3	83	83:17
6	Me_2CuLi[e]	$BF_3 \cdot OEt_2$	>97:3	91	31:69
7	Me_2CuLi[e]	HMPA	>97:3	76	>97:3
8	$Me_2Cu(CN)Li_2$	–	>97:3	99	>97:3

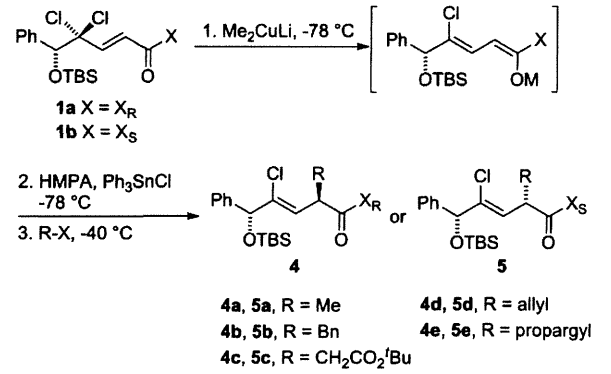
[a] All reactions were carried out on a 0.1 mmol scale with 4 equiv of organocuprates in the presence of Li salts. [b] 4 equiv. [c] Determined by ^1H NMR. [d] Yields of isolated products. [e] Higher order cuprates (*ca.* 0.4 equiv) were contained. [f] Diastereomeric ratio (dr) = 97:3.

and as expected, exposure of **1a** to Me_2CuLi followed by protic workup afforded a mixture of the reduced compound **3** and the α-alkylated product **4** in high yield (81%, entry 1). Significantly, excellent *Z*-selectivity was observed.[12] The use of a 2.4:1 MeLi·LiBr/CuI mixture enabled selective reduction, providing pure reduced compound **3** in excellent yield (93%, entry 2). Changing the methyl group of alkyl ligands to an *n*-butyl group resulted in decreased selectivity (entry 3). Although the reaction with lower order cyanocuprate or Me_2CuLi with TMSCl did not furnish better selectivity, addition of $BF_3 \cdot OEt_2$ led to the preferable formation of α-alkylated product **4** (entries 4–6). In contrast, the reaction with HMPA provided excellent selectivity to the reduction but a decreased yield (76%, entry 7). The best result was obtained with higher order cyanocuprate, derived from CuCN·2LiCl and 2 equiv of MeLi·LiBr, which gave **3** in excellent yield and selectivity (entry 8). Having identified higher order cyanocuprate as the preferred reducing agent, we selected the Gilman reagent (Me_2CuLi) as an optimal reducing agent because of the sufficient reactivity and selectivity to reduction.

The optimized reduction condition in Table 1 (entry 2) was applied to the one-pot reduction/asymmetric alkylation (Table 2). Previous studies have revealed that the transmetalation from Cu and/or Li dienolate intermediates to the more reactive Sn dienolate intermediates is critical for smooth alkylation.[9a] A variety of alkyl halides were allowed to react with the (*Z*)-chlorinated dienolate intermediate to provide chloroalkenes **4a**–**4e** flanking two stereogenic centers in moderate to high yield with >97% *Z*-selectivity. HPLC analysis showed that all the reactions

(12) A NOESY cross-peak was observed between the olefinic proton and the allylic stereogenic center, suggesting that the geometry of the double bond was defined as shown; see the Supporting Information.

Table 2. One-Pot Reduction/Asymmetric Alkylation of (*R*)-Sultam **1a** and (*S*)-Sultam **1b**[a]



entry	substrate	R–X	**4** or **5**, yield (%)[b]	dr (%)[c]
1	**1a**	MeI	**4a**, 58	97:3
2[d]	**1a**	BnBr	**4b**, 83	99:1
3	**1a**	$BrCH_2CO_2{}^tBu$	**4c**, 90	97:3
4	**1a**	allylBr	**4d**, 81	97:3
5[d]	**1a**	propargylBr	**4e**, 82	>95:5[e]
6	**1b**	MeI	**5a**, 60	99:1
7	**1b**	BnBr	**5b**, 86	95:5
8	**1b**	$BrCH_2CO_2{}^tBu$	**5c**, 57	97:3
9	**1b**	allylBr	**5d**, 70	97:3
10[d]	**1b**	propargylBr	**5e**, 46	>95:5[e]

[a] All reactions were carried out with 4 equiv of organocuprates, 16 equiv of HMPA, 2 equiv of Ph_3SnCl, and 8 equiv of alkyl halide. [b] Yields of isolated products. [c] Determined by HPLC. [d] At −30 °C. [e] Determined by ^1H NMR.

proceeded with excellent diastereoselectivity. The reactions with methyl iodide and bezyl bromide provided the corresponding α-alkylated products **4a** and **4b** in 58% and 83% yields, respectively (entries 1 and 2), and the absolute configuration of **4a** was confirmed by single-crystal X-ray analysis (Figure 2). Importantly, this strategy is amenable to the introduction of functional groups such as ester, allyl, and propargyl groups suitable for further transformation. Treatment of dienolate with *tert*-butyl bromoacetate and allyl bromide afforded the desired (*Z*)-chloroalkenes **4c** and **4d** with ester and allyl functionality, in high yields (entries 3 and 4). Propargyl bromide also gave the corresponding (*Z*)-chloroalkene **4e** in moderate yield (entry 5). In addition, this one-pot strategy can be applied to (*S*)-sultam **1b**, providing the corresponding chloroalkenes **5a**–**5e** in moderate to high yields (entries 6–10).

Finally, allylic alkylation of allylic *gem*-dichlorides was examined. Recently, Feringa reported that terminal allylic *gem*-dichlorides undergo Cu-catalyzed asymmetric allylic alkylation with Grignard reagents affording (*Z*)-chloroalkenes bearing an allylic stereogenic center with excellent regio- and enantioselectivity.[4a] Guided by this work, we attempted Cu-catalyzed S_N2'-type alkylation with γ,γ-dichloro-α,β-enoate **2**, but these conditions did not work for enoate **2**, possibly due to the lower reactivity of the

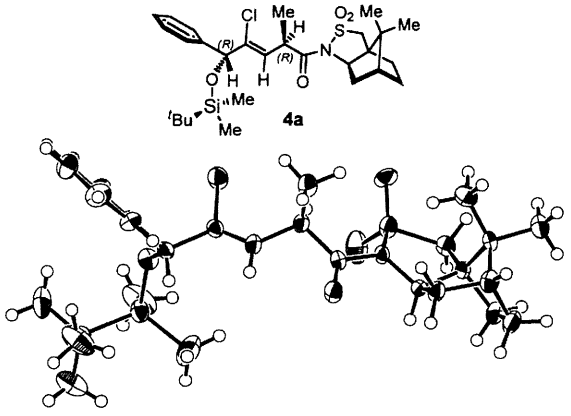
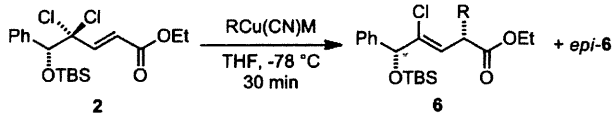


Figure 2. ORTEP representation of **4a**.

Table 3. Diastereoselective Allylic Alkylation of γ,γ-Dichloro-α,β-enoate **2** by 1,4-Asymmetric Induction[a]



entry	RCu(CN)M	Z/E[b]	**6**, yield (%)[c]	dr[d]
1	MeCu(CN)Li	>97:3	**6a**, 98	74:26
2	EtCu(CN)MgBr	>97:3	**6b**, 96	66:34
3	BnCu(CN)MgCl	>97:3	**6c**, 70	77:23
4	^{i}BuCu(CN)MgCl	>97:3	**6d**, 95	69:31

[a] All reactions were carried out on a 0.2 mmol scale with 4 equiv of organocuprates in the presence of Li salts. [b] Determined by ^{1}H NMR. [c] Yields of isolated products. [d] Determined by HPLC.

internal allylic system (see Supporting Information). Attention was therefore turned to the organocuprate-mediated allylic alkylation. As presented in Table 1 (entry 4), the lower order cyanocuprate (MeCu(CN)Li) promotes the allylic alkylation preferably to provide the α-methylated product **4a** with excellent diastereoselectivity (dr = 97:3). Extensive experimentation with MeCu(CN)Li revealed that the electron transfer from organocuprates competes significantly with allylic alkylation of the sultam **1a**, and the exclusive formation of **4a** was not realized. During the course of our studies on the allylic alkylation, we considered that the chiral center at C5 adjacent to the allylic *gem*-dichloride might induce the diastereoselectivity without chiral auxiliaries.

This hypothesis was tested with the enoate **2**. As shown in Table 3, treatment of **2** with MeCu(CN)Li afforded the α-methylated β,γ-enoate **6a** in 98% yield as a 74:26 mixture of diastereomers with excellent *Z*-selectivity. The major isomer was the (2*S*)-isomer, identified by the correlation with the same compound **6a**, prepared from the corresponding (*S*)-sultam-derived compound **5a**.[13] Similar results were obtained using EtCu(CN)MgBr, BnCu(CN)-MgCl, and *i*BuCu(CN)MgCl affording the corresponding α-alkylated chloroalkenes **6b**–**d** in high yields with similar selectivities (entries 2–4). Although the observed diastereoselectivity has not been rationalized,[14] these results suggest that stereochemistry at C2 can be controlled by the chiral center at C5 *via* 1,4-asymmetric induction.

In conclusion, we have described a one-pot organocuprate-mediated reduction/asymmetric alkylation of γ,γ-dichloro-α,β-unsaturated carbonyl compounds. This protocol allows not only the exclusive formation of trisubstituted (*Z*)-chloroalkenes in high yields but also the construction of an α-stereogenic center with excellent diastereoselectivity. The resulting products are notable for their high functionality and can perform as a potentially useful intermediate for this important class of molecules. In addition, we have identified a unique reactivity of substrates containing an allylic *gem*-dichloride system with organocuprates. These findings have proven to be useful for the development of novel reactions based on these classes of molecules. Efforts to elucidate the origin of novel 1,4-asymmetric induction and to extend this work to the diastereoselective synthesis of peptidomimetics with a chloroalkene moiety are currently in progress.

Acknowledgment. This research was supported in part by a Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and a grant from SENSHIN Medical Research Foundation. We are grateful to Prof. Shigeru Nishiyama and Dr. Tsuyoshi Saito (Keio University) for their assistance in the measurement of the reduction potentials.

Supporting Information Available. Representative procedures, characterization data, cif file of compound **4a**, and copies of NMR and HPLC spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

(13) See the Supporting Information for details.

(14) At the present stage of our understanding, the steric repulsions between the olefinic proton at C3 and the Ph group at C5 may destabilize the reactive conformer, which would lead to the (2*R*)-isomer. DFT calculations also suggest that the reactive conformer to the (2*S*)-isomer is favored by 4.42 kJ/mol over the conformer to the (2*R*)-isomer. See the Supporting Information.

The authors declare no competing financial interest.

—Review—

ケミカルバイオロジーを基盤とした抗 HIV 剤の創製

玉村啓和

Development of Anti-HIV Agents Based on Chemical Biology

Hirokazu Tamamura

Department of Medicinal Chemistry, Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University; Kanda-Surugadai 2-3-10, Chiyoda-ku, Tokyo 101-0062, Japan.

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Recently, highly active anti-retroviral therapy (HAART), which involves a combinational use of reverse transcriptase inhibitors and HIV protease inhibitors, has brought us a great success in the clinical treatment of AIDS patients. However, HAART has several serious clinical problems. These drawbacks encouraged us to find novel drugs and increase repertoires of anti-HIV agents with various action mechanisms. The recent disclosing of the dynamic supramolecular mechanism in HIV-entry has provided potentials to find a new type of drugs. To date, we have synthesized HIV-entry inhibitors, especially coreceptor CXCR4 antagonists. In addition, CD4 mimics in consideration of synergic effects with other entry inhibitors or neutralizing antibodies have been developed. The development of the above anti-HIV agents is based on the concept of reverse chemical genomics, in which target molecules are fixed. On the other hand, based on the concept of forward chemical genomics, in which active compounds are searched according to the screening of random libraries, effective peptide leads such as integrase inhibitors derived from fragment peptides of HIV-1 Vpr have been discovered. As such, from a point of view on chemical biology, anti-HIV leads have been found utilizing reverse and forward chemical genomics. Furthermore, antibody-based therapy or AIDS vaccine is still thought to be a promising treatment. Thus, peptidic antigen molecules based on artificial remodeling of the dynamic structures of a surface protein gp41 in HIV fusion have been developed. The present chemical biology approaches would be essential for discovery of anti-HIV agents in consideration of cocktail therapy of AIDS.

Key words——anti-human immunodeficiency virus (HIV) agent; AIDS vaccine; chemical biology; CXCR4 antagonist; CD4 mimic; integrase inhibitor

1. はじめに

1980 年頃サンフランシスコやニューヨークにおいて，これまで知られていなかった進行性の呼吸器障害により死亡する例が相次いで報告された．患者らは体内の CD4 陽性リンパ球が急速に減少又は消失し，免疫不全状態を呈しており，この疾患は後天性免疫不全症候群（acquired immunodeficiency syndrome, AIDS）と名付けられた．この疾患はすぐに全世界に広がり，原因ウイルスであるヒト免疫不全ウイルス（human immunodeficiency virus, HIV）が単離された．HIV は空気感染ではなく主に性的感染，血液感染，母子感染の 3 つの経路により感染し，現在世界で 4000 万人以上の HIV 感染者がおり，多くの発展途上国においては今もなお感染者数が増加している．さらに，多くの先進国では感染者数が減少しているのに対し，日本では増加傾向にある．[1,2]

大きく分けて HIV には，HIV-1 及び HIV-2 が存在し，HIV-1 は西半球，ヨーロッパ，アジア，アフリカ中央部・南部・東部で多くみられ，HIV-2 はアフリカ西部で多くみられる．HIV-2 に比べ HIV-1 は感染例が多く，感染力も強いため，抗 HIV 薬やエイズワクチン開発は主に HIV-1 を標的として行われている．

HIV-1 は，直径約 110 nm の RNA 型エンベロープウイルス（レトロウイルス）で，約 9500 塩基からなる 2 コピーの RNA ゲノムや逆転写酵素，インテグラーゼなどを含む核（キャプシド，capsid）と，それを取り囲む球状エンベロープによって構成され

東京医科歯科大学生体材料工学研究所（〒101-0062 東京都千代田区神田駿河台 2-3-10）
e-mail: tamamura.mr@tmd.ac.jp

本総説は，日本薬学会第 131 年会シンポジウム S05 で発表したものを中心に記述したものである．

る．ウイルス粒子の外側を構成するエンベロープには，外に突き出している糖タンパク質 gp120 とその内側に存在する脂質二重膜を貫通する糖タンパク質 gp41 からなるスパイクがある．この gp120 と gp41 はヘテロダイマーを形成し，さらにヘテロダイマーを形成したタンパク質がホモ三量体を形成して HIV 膜上に十数個存在している［Fig. 1(A)］．これまでの研究により HIV-1 の宿主細胞への侵入から新ウイルスの出芽までの一連の複製サイクル（ライフサイクル）が詳細に解明されてきた．このサイクルは数ステップに分けることができ，ウイルスの宿主細胞への吸着・膜融合，RNA ゲノムの逆転写，ウイルス DNA の宿主 DNA への組込み（インテグレーション）によるプロウイルス DNA の形成と複製，ウイルス構成タンパク質のプロセッシング，ウイルス粒子の構築，出芽，ウイルスの成熟化の過程を経て増殖していく［Fig. 1(B)］.[3,4]

これまで，上述のような HIV のライフサイクルを各ステップで阻害するような薬剤の研究・開発が進められてきた．主に HIV 固有の酵素をターゲットとして，ウイルスのライフサイクルの異なる作用点に働くものがある．代表的な抗 HIV 薬として核酸系若しくは非核酸系逆転写酵素阻害剤，プロテアーゼ阻害剤，インテグラーゼ阻害剤が開発され，臨床において用いられている．また，侵入過程を阻害する膜融合阻害剤，CCR5 阻害剤も FDA で認可されている.[5] しかし，HIV-1 は変異を起こし易いため，単剤療法では薬剤耐性があらわれ薬剤の効果がなくなってしまう．そのため HIV 感染症の治療には，薬剤の耐性変異が重ならないように数種の薬剤を組み合わせた多剤併用療法を行っている．これは，highly active anti-retroviral therapy（HAART）と呼ばれ，主に逆転写酵素阻害剤，プロテアーゼ阻害剤，インテグラーゼ阻害剤の中から 2，3 剤の組み合わせで用いられる.[6] HAART は有効な治療法として成果を挙げているが，長期投与による高額な治療費，重篤な副作用，耐性ウイルスの出現などの問題点もある．そのため，これまでの薬剤とは異なる作用機序を持つ新たな薬剤の開発が望まれており，創薬化学者には実際に使用できる薬剤のレパートリーを増やすことが求められている．われわれは以前からコレセプター CXCR4 阻害剤を中心に抗 HIV 剤を創製しており，最近ではケミカルバイオ

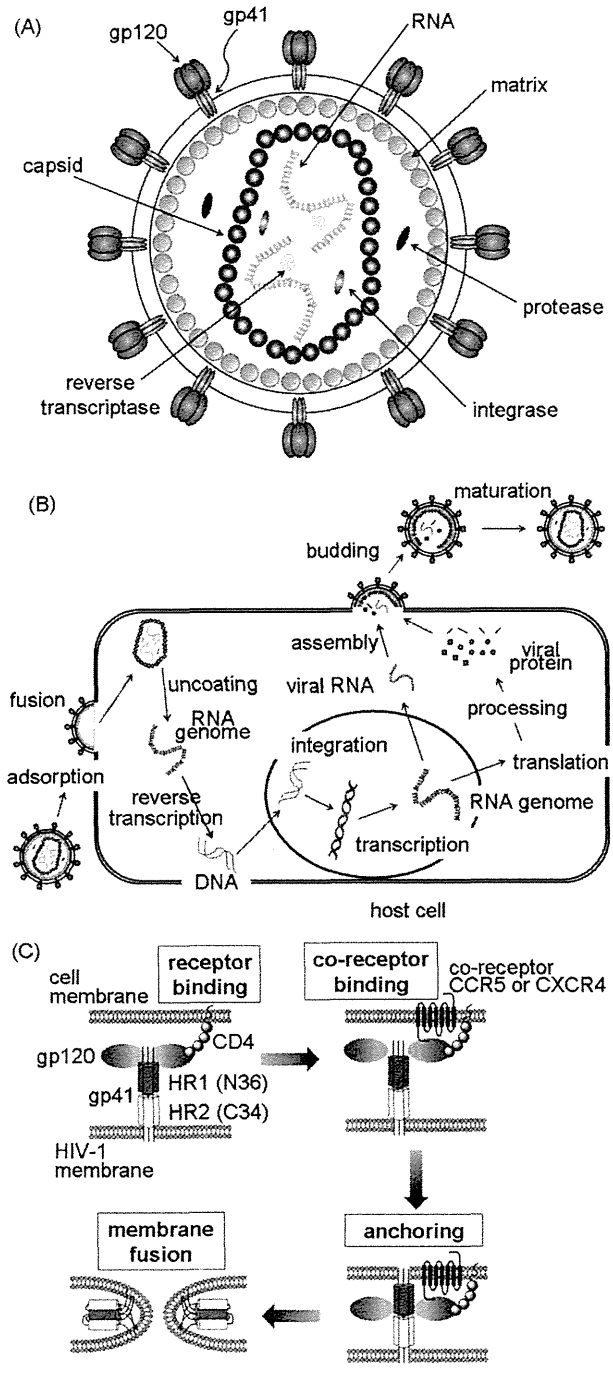

Fig. 1. (A) Structure of HIV Virion, (B) Replication Cycle of HIV, (C) Mechanism of HIV-1 Entry and Fusion

玉村啓和

1964 年生まれ．京都大学大学院薬学研究科修士課程在学中に助手に着任，博士（薬学）．京都大学大学院薬学研究科助教授を経て，2005 年より東京医科歯科大学生体材料工学研究所教授．1999–2000 年 米国国立衛生研究所/米国国立がん研究所へ留学．専門はペプチド・天然物を基盤とした創薬化学，ケミカルバイオロジー．

ロジー的手法を駆使して，吸着阻害剤 CD4 ミミック，膜融合阻害剤，インテグラーゼ阻害剤，HIV の侵入の膜融合機構をターゲットとした人工設計型抗原分子を創製している．本論文では，その研究内容について報告する．

2. コレセプター CXCR4 阻害剤

HIV の宿主細胞への吸着・膜融合に関して，まず，HIV エンベロープタンパク質 gp120 が細胞表面上の第一受容体である糖タンパク質 CD4 に結合し，gp120 のコンフォメーション変化の後に，gp120 は第二受容体（コレセプター）である CCR5 あるいは CXCR4 に結合する．[7,8] CCR5 と CXCR4 は 7 回膜貫通 G タンパク質共役型受容体（7TM-GPCR）に属するケモカイン受容体である．CCR5 は HIV 感染の初期に主流になるウイルス株マクロファージ指向性 HIV-1（R5-HIV-1）が主に使用するコレセプターであり，CXCR4 は HIV 感染の後期に主流になるウイルス株 T 細胞指向性 HIV-1（X4-HIV-1）が主に使用するコレセプターである．gp120 の CD4，CCR5 あるいは CXCR4 に対する結合より，gp120 と非共有結合的にヘテロダイマーを形成している gp41 の N 末端側が露出され，gp41 に存在する膜挿入ペプチドが標的細胞の細胞膜にアンカリングする．アンカリングの後，三量体の gp41 の N 末端側の helix 領域である HR1（NHR, N-region）領域（N36 配列を含む領域）と C 末端側 helix 領域である HR2（CHR, C-region）領域（C34 配列を含む領域）が逆平行に結合し，六量体を形成することで HIV の膜と標的細胞の膜が近づき膜融合を引き起こす［Fig. 1(C)］.[9]

まず，玉村の以前の所属機関である京都大学大学院薬学研究科の藤井信孝教授の下で，コレセプター CXCR4 阻害剤の創製に取り組んだ．コレセプター CXCR4 を抗エイズ薬のターゲットとすることは，もう 1 つの主要なコレプターである CCR5 の阻害剤 Maraviroc（Pfizer Inc.）[10]が臨床使用されたことより妥当であると考えられ，CXCR4 阻害剤も早急な開発と安全性の確認が期待される．HIV のコレセプター指向性を考えると，HIV 感染直後の前期からエイズを発症する後期に移行するに従って，CCR5 指向性の株から CXCR4 指向性の株が主流になっていく．このことから，CCR5 阻害剤だけでなく，片手落ちにならないように CXCR4 阻害剤も必要と思われる．1989 年頃から，カブトガニの血球由来の防御ペプチド polyphemusin の構造活性相関研究を精力的に行い，T22 という 18 残基からなる侵入阻害ペプチドを見い出した．[11]CXCR4 がコレセプターとして同定された後，T22 は CXCR4 アンタゴニストであることが証明され，[12] 構造最適化により 14 残基からなる強力な CXCR4 アンタゴニスト活性を示す T140 を見い出した．[13] その生体内安定性を向上させた T140 誘導体は現在，臨床試験（phase II）中である．[14,15] また，T140 のファルマコフォアのアミノ酸残基を基にした環状ペンタペプチドライブラリーを構築し，この中から T140 と同等のアンタゴニスト活性を有する誘導体 FC131 の創出に成功した［Fig. 2(A)］.[16]

これらのリード化合物を基にさらに低分子量のペプチドミミックも見い出しており，[17] さらに，非ペプチド性の CXCR4 アンタゴニストである二核亜鉛錯体[18]やその誘導体[19]を創出している［Fig. 2(B)］. CXCR4 が HIV のコレセプターであることが報告されてから数年後に，CXCR4 とその内因性リガンド CXCL12 の相互作用が，種々の固形がんの転移や血液がんの進行，関節リウマチの炎症等に大きく係わっていることが明らかにされた．[20] それに伴い，T140 誘導体等ががん転移阻害活性，白血病の進行の阻害活性，抗関節リウマチ作用を有することを明らかにした．[20–22] また，他の研究者からも AMD3100（Genzyme Corp.）や KRH-1636（Kureha Chemical & Daiichi Sankyo Co., Ltd.）等種々の CXCR4 アンタゴニストが報告されているが，誌面の関係上，総説を参照されたい．[23] 生理的には，CXCR4 は CXCL12 との相互作用により，胎生時の血管形成や心形成，造血，神経形成において progenitor cell の遊走や活性化等の重要な作用を示すことから，CXCR4 アンタゴニストの副作用を十分検討する必要がある．以上より CXCL12/CXCR4 情報伝達系の制御は，HIV 感染症，がん転移，白血病，関節リウマチ等の多くの疾病に対する創薬戦略として有望であり，言い方を変えれば，CXCR4 は multiple 創薬ターゲットとして魅力的である．現在，立体配座固定化テンプレートを活用し，より有用な創薬リードへ導くよう進めている．

3. 吸着阻害剤 CD4 ミミック

HIV は細胞侵入時に，エンベロープタンパク質

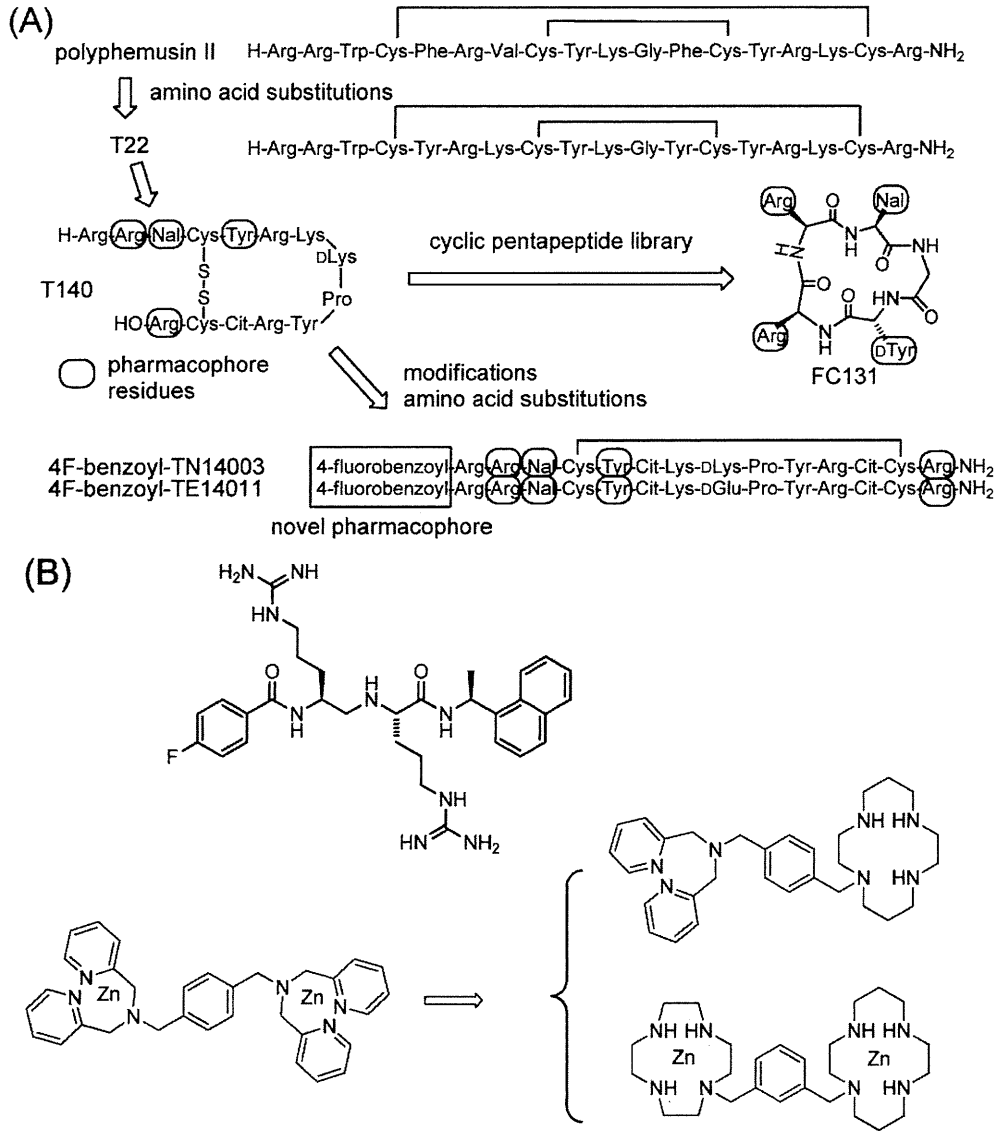

Fig. 2. (A) Development of CXCR4 Antagonists Based on Horseshoe Crab Peptides, Nal=L-3-(2-naphthyl)alanine, Cit=L-citrulline, (B) Structures of a Peptide Mimic CXCR4 Antagonist with Low Molecular Weight (upper), a Dipicolylamine (Dpa)-Zinc(II) Complex (lower left) and Its Derivatives (lower right)

gp120が細胞表面上の第一受容体CD4に結合し，gp120のコンフォメーション変化が生じ，gp120-CD4-コレセプター（非共有結合複合体）の形成，gp41と宿主細胞膜の相互作用を経て膜融合する（Fig. 1）．NBD-556はHIV-1の複合体形成阻害スクリーニングにより見い出されたHIV侵入阻害剤である．[24] また，NBD-556（Fig. 3）は可溶性CD4と同様にgp120と相互作用することによりgp120の構造変化を促すことから，低分子型CD4ミミックとして注目されている．これまでに可溶性CD4とgp120の共結晶構造が明らかにされ，CD4のPhe^{43}の側鎖がgp120の特徴的な空洞（Phe-43 cavity）に入り込む形で相互作用することが明らかにされている．[25] われわれは，可溶性CD4とgp120の共結晶構造を基にした分子モデリング解析（Flex-SIS module of SYBYL 7.1）を行い，Phe^{43}だけでなくArg^{59}もgp120と相互作用することを示し，[26] NBD-556のアニリン部位がPhe^{43}の側鎖と，テトラメチルピペリジン環部位がArg^{59}の側鎖と対応するようにgp120と相互作用することが示唆された（Fig. 3）．さらに，NBD-556のクロロアニリン部位はCD4に比べ，Phe-43 cavityに6.5 Å程度深く入り，gp120の芳香族アミノ酸（Trp^{447}, Phe^{382}, Trp^{112}）と，リンカーであるオキサミド構造は水素

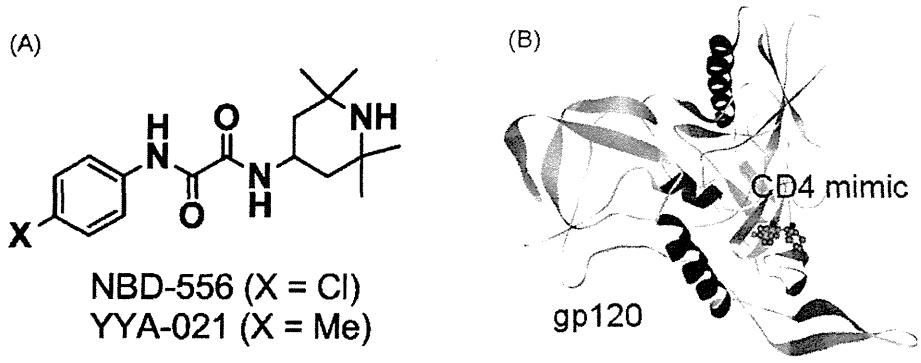

Fig. 3. (A) Structures of CD4 Mimics NBD-556 and YYA-021, (B) Docking Model of gp120 and NBD-556

結合供与体として gp120 と相互作用していることも示唆されている.[25] すなわち，この CD4 ミミックは適切なリンカーを用いることにより，CD4 のペプチド二本鎖上の 2 つの部位を含む広範囲な領域をミミックしている．われわれは Phe-43 cavity 周辺の構造的及び静電的要求を明らかにする目的で，NBD-556 の芳香環パラ位に関する構造活性相関研究を行った.[26] これにより，芳香環パラ位にある程度嵩高くかつそれほど電子供与性の強くない官能基を有する誘導体（YYA-021 等）が顕著な抗 HIV 活性及び gp120 の構造変化能を有することを明らかにした（Fig. 3）．また，この構造変化により CCR5/CXCR4 などのコレセプター結合領域や CD4-induced site を認識する中和抗体の結合能が上昇することも併せて明らかにしている.[27] さらに，これら CD4 ミミックは前章で述べた CXCR4 アンタゴニスト T140 と併用すると，顕著な相乗効果を示し,[26] CD4 ミミック-T140 誘導体のハイブリッド化合物の創製にも成功している.[28] このように CD ミミックは中和抗体やコレセプター阻害剤との併用により，さらに有用性が上がると思われる．

一般にタンパク質-タンパク質間の相互作用では，相互作用領域が連続した一次構造（アミノ酸配列）で存在するのではなく，高次構造の中で不連続に存在することが少なくない．この場合，相互作用領域に係わる官能基を適当なリンカーで架橋した低分子化合物により，分子量が数百倍という元の親タンパク質の機能を模倣することも可能である．この HIV 侵入阻害剤 CD4 ミミックはその一例であろう．

4. インテグラーゼ阻害剤

これまで臨床で使用された抗エイズ薬や上述のわれわれが創製した抗 HIV 剤はすべて標的分子設定型のリバースケミカルジェネティクス的手法により創出されたものである．例えば，逆転写酵素阻害剤やプロテアーゼ阻害剤を開発する場合は，それぞれの酵素（標的分子）に結合するように分子設計されている．CXCR4 アンタゴニストや CD4 ミミックを創製した際は，CXCR4 や gp120（標的分子）に結合するようにデザインした．次に，創薬候補品等有用なリード化合物を創出するケミカルバイオロジー的手法として，このようなリバースケミカルジェネティクス的手法とは方向性が正反対になるフォワードケミカルジェネティクス的手法に着目した．すなわち，ランダムライブラリーから抗 HIV 活性を指標にスクリーニングするというフォワードケミカルジェネティクス的手法を用い，有用な抗 HIV 剤のリード化合物を見い出そうとした．

まず，HIV 構成タンパク質の中に HIV 自身の複製を阻害するものが存在するだろうという仮説の下に，上述のランダムライブラリーのソースを HIV-1 の遺伝子産物であるタンパク質（Gag・Pol・Env・Vpu・Vpr・Rev・Tat）由来のアミノ酸配列を基にしたオーバーラッピングペプチドライブラリー（アミノ酸 10–17 残基）とした．このライブラリーを用いて，簡便に評価できるインテグラーゼ（IN）阻害活性（細胞内で阻害活性を評価する系ではない）のスクリーニングを行った結果，アクセサリータンパク質である Vpr 由来の 3 個の部分ペプチドに抗 IN 活性が見い出された．これらの部分ペプチドには共通して LQQLLF 配列が含まれていた（Fig. 4）.[29] LQQLLF 配列が阻害活性の発現に重要なモチーフであると考えられ，また，細胞内で活性を発現させるため，LQQLLF モチーフを中心に数種類のペプチドを合成し，細胞膜透過性モチーフで

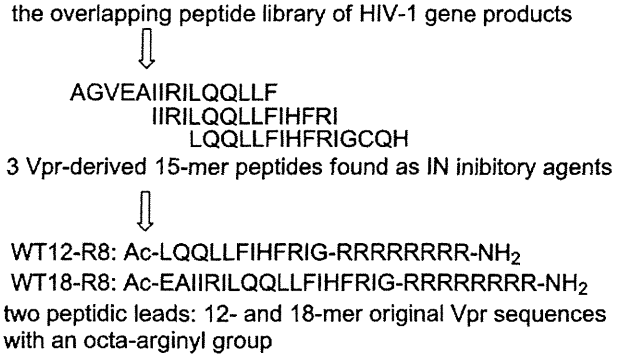

Fig. 4. Development of Vpr-derived IN Inhibitors

あるocta-Arg（RRRRRRRR）をC末端側に付与した．その結果，抗IN活性だけでなく細胞レベルでの抗HIV-1活性ともに強力なペプチドWT18-R8（Ac-EAIIRILQQLLFIHFRIG-RRRRRRRR-NH_2）及びWT12-R8 (Ac-LQQLLFIHFRIG-RRRRRRRR-NH_2）を同定した（Fig. 4）．LQQLLFモチーフはこのVpr中のαヘリックス構造部分に存在していることから，αヘリックス構造が阻害活性の発現に重要であると考えられる.[30]感染の前期過程において，VprもINも酵素や鋳型RNA及び様々な細胞内宿主因子を含むプレインテグレーション複合体（Pre-Integration Complex, PIC）と呼ばれる複合体の中に存在し，VprはINと相互作用することでHIV自身のオートインテグレーションを制御している（BAFのような作用）かもしれない．いずれにしても，HIV構成タンパク質の中にHIV自身の複製を阻害するものが存在する可能性がある．また，PIC中でVprは溶液中とは異なるコンフォメーションをとり，LQQLLFモチーフを含むαヘリックス構造部分が外側に提示され，INと相互作用して，INの作用をマスクしているのかもしれない．これは，大きなタンパク質の中に秘められた神秘的な活性が部分ペプチドに存在する，すなわち，親タンパク質の作用とは異なる作用が部分ペプチドに存在する，といういわゆるクリプタイド（＝cryptic peptide）の概念にも関係すると思われる.[31]このIN阻害ペプチドはさらに構造を最適化することにより，Raltegravir（Merck Sharp & Dohme Corp.）[32]とは違うアロステリック機構を持つ新たな抗IN剤の創出として期待できる．

現在同様に，ランダムライブラリーから抗HIV活性を指標にスクリーニングするというフォワードケミカルジェネティクス的手法を用い，HIV-1 matrix protein（MA）由来の部分ペプチドが抗HIV活性を示すことを見い出しており，本法を用いることにより，有用な抗HIV剤のリード化合物を見い出すことができる可能性がある．

5. 膜融合機構をターゲットとしたワクチンと膜融合阻害剤

これまで臨床で使用できる多くの抗HIV薬が開発され，多剤併用療法（HAART）は大きな効果を上げてきた．さらに，広範囲のウイルス株に対して中和活性を持つワクチンを開発することによって，特に最近感染者が増加しているアフリカ，アジア等の発展途上国でAIDS及びHIV感染症で苦しむ患者のQOLの改善や治療における選択肢の拡大に貢献できると考えられる．HIVワクチンの開発においてはこれまでの通常の感染症に対して有効である弱毒化ワクチン，生ワクチンといった方法はHIVの易変異性ゆえに危険視されたこともあり使うことができない．さらに，通常抗体誘導を行う際には，ウイルス粒子表面のタンパク質における部分配列を合成しその配列特異的な抗体を誘導しているが，誘導された抗体はそのアミノ酸配列に特異的に結合するものの，中和標的の立体構造に対しての特異性や結合活性は概して低い．変異の激しいHIVを標的とする場合，アミノ酸の一次配列だけではなく，立体構造を認識して結合する中和抗体を誘導することが望ましいと考えられ，これまでとは違ったワクチン開発が必要となっている．そこで，立体構造を保持した状態の抗原分子を用いることによって，立体構造に対しても特異的な抗体を誘導することができれば，HIVに対してもより高い中和活性を有する抗体が誘導できるのではないかと考えた．

HIVは先に述べたように，gp120-CD4-コレセプター（非共有結合複合体）の形成後，ホモ三量体のgp41が宿主細胞膜にアンカリングし，HR1領域とHR2領域の会合により6ヘリックスバンドル（六量体）を形成して宿主細胞膜と膜融合する（Fig. 1）．これまでにこの膜融合過程を標的とした創薬研究が広く行われ，Enfurvirtide（fuzeon / T20）（Roche/Trimeris）が膜融合阻害剤としてFDAから認可されている.[33] EnfurvirtideはHR2（CHR, C-region）領域の部分ペプチドであり，HR2ミメティックとしてHR1領域に相互作用することで，

6ヘリックスバンドル形成を阻害する．また，HR1及びHR2ヘリックス領域はHIV株間で高く保存されていることから，gp41のアミノ酸配列を認識する中和抗体（2F5及び4E10）が見い出されている．[34] さらに，近年ではgp41に対する特異性向上を目的として，その立体構造を模倣したgp41ミメティックの創製研究が行われ，HR1又はHR2ヘリックス領域を3価型テンプレートで束ねたgp41ミメティックが複数合成され，阻害剤や人工抗原分子として応用されている．[35-37] しかし，これらgp41ミメティックは非等価なテンプレートの構造上，ヘリックス領域の位相にずれが生じ，天然の三量体構造を再構築しているとは言いがたい．われわれは天然型のgp41が有する等価な三量体構造を模倣するために，ヘリックス領域を等価に配置可能なC3対称性テンプレートを新たにデザインし，3本のHR1（NHR, N-region）領域由来ペプチド（N36）をN端側から集積させたgp41ミメティック（N36三量体）を創製した（Fig. 5）．[38] 合成したN36三量体のCDスペクトル測定により，三量体は単量体に比べ高いαヘリックス性を有していることが明らかになり，C3対称性テンプレート上でのN36三量体構造の形成が示唆された．また，HR2（CHR, C-region）領域由来ペプチド（C34）と混合させるとαヘリックス性が上昇したことから，このgp41ミメティックは天然の構造を高く反映していると考えられる．合成したN36三量体をマウスに免疫した結果，得られた血清はN36単量体よりもN36三量体に対する抗体価が約30倍高く，三量体構造を特異的に認識する抗体が誘導されていることが示唆された．さらに，N36三量体の免疫によって得られた血清は，単量体の免疫に比べ，強い中和活性がみられたことから，三量体構造を特異的に認識する抗体の有用性が示唆された．われわれが合成したN36三量体ミミックはC3対称性テンプレートを用いることにより，天然の三量体構造を再構築することができ，これを抗原分子として使用することにより天然の高次構造を特異的に認識する抗体が誘導されたと考えられる．なお，このN36三量体ミミックは，阻害剤としてもN36の量論比に相当する抗HIV活性を有していた．また，HR1領域ペプチドの対になっているHR2領域ペプチド（C34）の三量体ミミックに関しても，別のC3対称性テンプレートを合成し，3本のC34をC端側から集積させたgp41ミメティック（C34三量体）を創製した．同様に，C34三量体ミミックは単量体よりも三量体

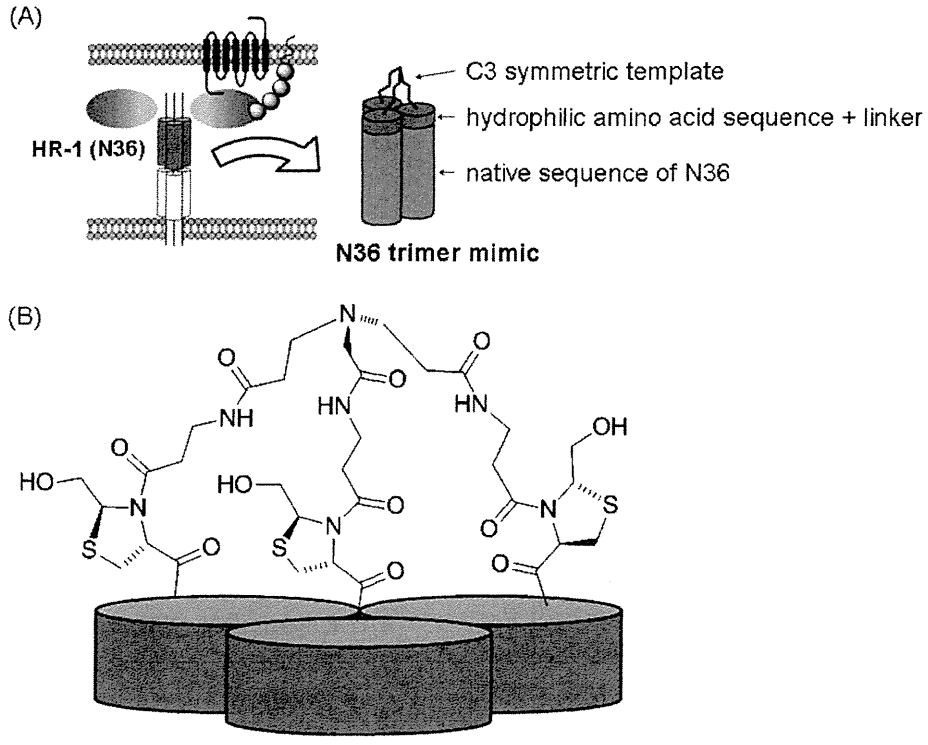

Fig. 5. (A) Development of an N36 Trimer Mimic Using a C3-Symmetric Template, (B) Structure of the C3-Symmetric Template

構造を特異的に認識する抗体の誘導，及び，中和抗体の誘導において優れていることが示唆された．なお，このC34三量体ミミックは，阻害剤としては単量体よりも100倍強い抗HIV活性を有しており，現在，この理由を解明中である．

第3章のCD4ミミックは，タンパク質-タンパク質間（gp120-CD4間）の相互作用の領域に係わる官能基を適当なリンカーで架橋した低分子化合物であり，元の親タンパク質の機能を模倣したものである．本章のgp41ミメティックは，タンパク質-タンパク質間の相互作用に係わる部分ペプチドを適当なテンプレート上に配置することで，タンパク質の高次構造そのものを再構築したものである．このケミカルバイオロジー的手法は規則的な二次構造の組み合わせで構成されるタンパク質を模倣する場合に有効である．

これらのgp41三量体ミメティックは今後実験動物レベルを上げていくことにより，立体構造を保持した抗原分子の有効性を示すことができると考えられる．ワクチンに関しては，gp41以外にも宿主細胞側のタンパク質CXCR4の細胞外領域を標的とした人工抗原分子を創製し，免疫により抗体誘導を確認している．宿主側のタンパク質を抗原分子とすることは，常識的ではないが，広範囲のウイルス株に対して有効性が期待でき，耐性出現の危険性も少ないという利点もある．

6. おわりに

毎年種々の抗HIV薬が開発され，薬を飲み続ければAIDSは死なない病のようになってきた．しかし，一生投薬を続ける必要があり，いずれの抗HIV薬も根治に至るものではない．そのため，臨床で使用できる薬剤のレパートリーを増やすことが求められている．われわれは以前からHIV感染のコレセプターであるCXCR4の阻害剤を中心に，標的分子設定型のリバースケミカルジェネティクス的手法により抗HIV剤を創製してきた．最近さらに抗HIV剤のターゲットを増やし，HIV侵入の動的超分子機構をターゲットとしたCD4ミミックを創製した．このCD4ミミックはCXCR4阻害剤や中和抗体等との併用において，相乗効果を示した．さらに，ランダムライブラリーから抗HIV活性を指標にスクリーニングするというフォワードケミカルジェネティクス的手法を用い，リード化合物を見い出した．すなわち，インテグラーゼ阻害活性を有するVprの部分ペプチドを見い出した．このようにケミカルバイオロジー的方法も取り入れ，いろいろな観点からリード化合物を探索し，種々の抗HIV剤の創製を行っている．また，最近再度注目されてきたエイズワクチンに関しても，HIV侵入の動的超分子機構をターゲットとしてテンプレート等ケミカルバイオロジーの概念を用い，人工設計型抗原分子を創製している．阻害剤及びワクチンの両方に力を入れており，カクテル療法を視野に入れた抗AIDS薬の創製を考えている．

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