the canonical mechanisms of cross-desensitization between GPCRs (i.e., cross-phosphorylation; data not shown) and down-modulation.

Finally, Akt activity being essential to cell survival, it is likely that the inhibition of its phosphorylation by the KiSS-1 receptor would result in apoptosis, either by itself or in conjunction with other proapoptotic signals. The signaling of the GPR54 and other Gq-coupled GPCRs includes both proapoptotic and antiapoptotic events. Depending on the cellular environment and the subtypes of signaling molecules involved, one or the other may prevail. However, very recently (33), new information showed that the signaling of the GPR54 in breast tumor cells could specifically promote the expression of an array of proapoptotic genes, suggesting that it may cross the line between metastasis suppressors and tumor suppressors.

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Development of Anti-HIV Agents Targeting Dynamic Supramolecular Mechanism: Entry and Fusion Inhibitors Based on CXCR4/CCR5 Antagonists and gp41-C34-Remodeling Peptides

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Abstract: A molecular mechanism involved both in HIV-entry and -fusion steps has been disclosed in detail: The interaction of an HIV envelope protein, gp120, with chemokine receptors, CXCR4 and CCR5, which were identified as major co-receptors in association with CD4, triggers conformational changes in the gp120-gp41 (another envelope protein) complex, and subsequently forms the trimer-of-hairpins structure of gp41 followed by virus-cell membrane fusion. The elucidation of the above dynamic supramolecular mechanism in HIV-entry and -fusion has provided insights into new type of drugs that can block HIV infection. Based on this, we have developed not only coreceptor antagonists (1) but also fusion inhibitors (2). (1) Potent CXCR4 antagonists, T22 and T140, have been developed through the structure-activity relationship studies on tachyplesins and polyphemusins that are horseshoe crabs' antimicrobial peptides. T22, which was initially found to bind gp120 and CD4, and T140 selectively suppress T-cell line-tropic HIV-1 (X4-HIV-1) entry based on their specific binding to CXCR4. Furthermore, molecular-size reduction of T140 using cyclic pentapeptide templates brought us to find low molecular weight CXCR4 antagonists, such as FC131. (2) Artificial remodeling of a gp41 fragment, C34, has led to development of strong inhibitors of HIV-fusion into cells. These fusion inhibitors effectively block the formation of the trimer-of-hairpins structure of gp41. HIV-entry/fusion inhibitors such as CXCR4 antagonists and C34 analogs would improve the clinical chemotherapy of AIDS and HIV-infected patients. This review article focuses on our recent research on the development of the above two types of inhibitors, including comparative studies with several CXCR4 antagonists besides T22/T140-related compounds and other fusion inhibitors such as Fuzeon (T-20).

Keywords: AIDS, chemokine, low molecular weight CXCR4 antagonist, X4-HIV-1 entry, cancer metastasis, rheumatoid arthritis, fusion inhibitor, artificial remodeling.

INTRODUCTION

Highly active anti-retroviral therapy (HAART), which utilizes a combination of HIV protease inhibitors and reverse transcriptase inhibitors, has brought us a great success and hope in the clinical treatment of HIV-infected patients [48]. However, HAART involves serious clinical problems such as the emergence of multi-drug resistant (MDR) HIV-1 strains, significant side effects, nonetheless high costs, etc. These drawbacks encouraged us to develop brand-new drugs with novel action mechanisms, such as HIV-entry and -fusion inhibitors. Recently, the molecular mechanism concerning the HIV-1 replication has been elucidated in detail, especially for a dynamic supramolecular mechanism relevant to HIV entry/fusion steps: At first, an HIV envelope protein gp120 interacts with a cell surface protein CD4, which leads to a conformational change in gp120 followed by subsequent binding of gp120 to the second cellular receptors, such as CCR5 [1,12,17,20,21] and CXCR4 [22]. These are the major co-receptors for the entry of macrophage-tropic (R5-) and T cell line-tropic (X4-) HIV-1, respectively, whereas these play a physiologically important role as the receptors for endogenous ligands, chemokines. Next, the interaction of gp120 with CCR5 or CXCR4 triggers penetration of another envelope protein gp41 to the cell membrane from the *N*-terminus end and formation of the gp41 trimer-of-hairpins structure in the middle region, which causes fusion of HIV/cell-membranes and results in completion of the infection [11]. Elucidation of the above dynamic molecular machinary drove many researchers to develop effective inhibitors blocking HIV-entry/fusion steps targeting the second receptors, CCR5 and CXCR4, and the dynamic process involving the gp41 structure change.

This article reviews our recent approaches into the development of CXCR4 antagonists and gp41-fragment-remodeling peptides targeting the dynamic supramolecular mechanism.

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1. CXCR4 and CCR5 Antagonists

1-1. Biostable Lead Compounds Derived from T140

R5-HIV-I strains, which use CCR5 as a co-receptor, constitute majority in the early stage of HIV infection,

whereas X4-HIV-1 strains, which use CXCR4 as a co-receptor, are major species in the late stage of HIV infection and AIDS. Our research has focused on drug discovery targeting CXCR4. Tachyplesins and polyphemusins are 17-mer and 18-mer antimicrobial peptides contained in horseshoe crabs, respectively. Structure-activity relationship (SAR) studies on these peptides led to the discovery of an 18-mer peptide, T22 ([$Tyr^{5,12}$, Lys^{7}]-polyphemusin II) that was initially found to interact with gp120 and CD4 [89], which were not the real targets for the expression of the activity, and its downsized 14-mer peptide, T140 [26,45,79,91] (Fig. (**1**)). T22 and T140 proved to strongly block an X4-HIV-1 entry through their specific binding to CXCR4 [52,53,101]. Four residues in T140, Arg^{2}, L-3-(2-naphthyl)alanine $(Nal)^{3}$, Tyr^{5} and Arg^{14}, are indispensable for high potency (Fig. (**1**)) [88], and T140 forms an antiparallel β-sheet structure that is maintained by a disulfide bridge between Cys^{4} and Cys^{13} and connected by a type II' β-turn with Lys^{7}-D-Lys^{8}-Pro^{9}-Tyr^{10} at the i – (i+3) site [90]. Examination of biostability *in vitro* disclosed that T140 is not stable in mouse serum or in rat liver homogenate due to degradative deletion of Arg^{1}, Arg^{2}, Nal^{3} and Arg^{14} from *N*-/*C*-terminus, which causes drastic diminution in the T140 activity [81,87]. *N*-Terminal benzoylation and *C*-terminal amidation of T140 analogs suppressed their biodegradations leading to development of novel effective compounds, which showed increased bio-stability and even higher CXCR4-antagonistic activity. Through intensive SAR studies on *N*-terminal benzoylation, we found that an aromatic ring having an electron-withdrawing substituent, such as a *p*-fluorobenzoyl moiety, at the *N*-terminus constitutes a novel pharmacophore for strong anti-HIV activity [83]. 4F-benzoyl-TN14003 and 4F-benzoyl-TE14011 are biostable analogs, which have two orders of magnitude higher anti-HIV activity than T140 (Fig. (**1**)). Both peptides are promising lead compounds of 14-mer peptides for clinical development.

1-2. Low Molecular Weight CXCR4 Antagonists Based on Cyclic Pentapeptides

The four indispensable residues of T140, Arg^{2}, Nal^{3}, Tyr^{5} and Arg^{14}, are shown to be in close proximity to each other in the spatial structure by conformational analysis (Fig. (**1**)) [90]. Thus, the pharmacophore-guided approach based on these four residues might lead to the development of low molecular weight CXCR4 antagonists. Cyclic pentapeptides have been utilized as conformationally restricted templates disposing functional groups in medicinal chemistry [28,31,32,61,73,98], e.g. in efficient discovery of bioactive lead compounds such as integrin antagonists [31,32,98] and endothelin antagonists [28,73]. Thus, the library of cyclic pentapeptides containing these four residues of T140 (Arg^{2}, Nal^{3}, Tyr^{5} and Arg^{14}) was constructed. Initially, we devised possible library (total 192 compounds) using two L/D-Arg, L/D-Nal, L/D-Tyr and Gly (a spacer) to dispose indispensable functional groups of the T140 side-chain in space. Utilization of two focused libraries consisting of conformation-based and sequence-based libraries (total 60 compounds) led to rapid and efficient discovery of a hit compound, FC131, which has strong CXCR4-antagonistic activity comparable to that of T140 [27] (Fig. (**1**)). NMR and simulated annealing molecular dynamics (SA-MD) analysis of FC131 showed a backbone structure with a nearly symmetrical pentagonal shape. The pharmacophore-guided approach using cyclic pentapeptide templates proved to be useful for the lead discovery of low molecular weight CXCR4 antagonists.

We also wish to advance a research project to develop non-peptidic CXCR4 antagonists. Initially, we tried to investigate contributions of each amide bond in FC131 to the biological activity in order to develop FC131-derived pseudopeptides, in which the peptide character is reduced to access more drug-like structures. The practical utility of (*E*)-alkene dipeptide isosteres (EADIs) and reduced amide-type dipeptide isosteres (RADIs) has been intensively investigated in their introduction into biologically active peptides (Fig. (**2**)) [14,25,34,38,82,86,100]. Backbone replacements of amide bonds in peptides by EADIs and RADIs provide information on the contributions of the corresponding amide bonds to biological activity toward development of peptide-lead drugs. Thus, to identify the biological importance of these amide bond in FC131, EADIs and RADIs of Arg-Nal and Nal-Gly were synthesized

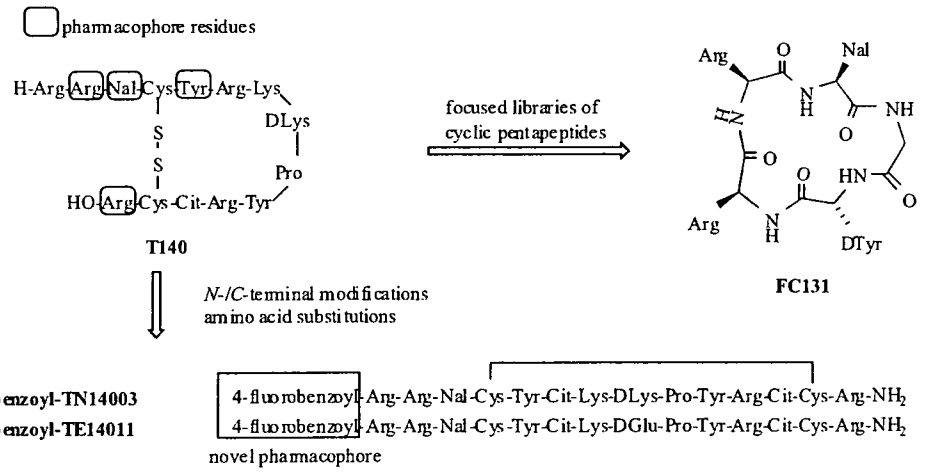


Fig. (1). Development of bio-stable T140 analogs and a low molecular weight CXCR4 antagonist, FC131, based on cyclic pentapeptide templates. Cit = L-citrulline.

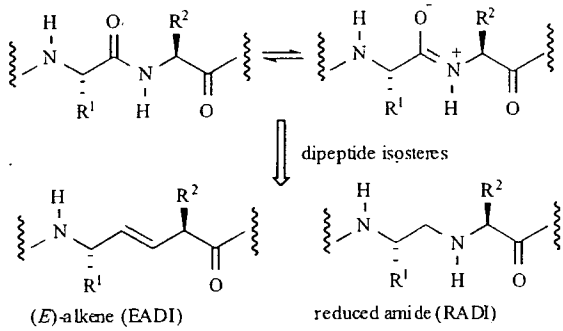


Fig. (2). Structures of (*E*)-alkene dipeptide isosteres (EADIs) and reduced amide-type dipeptide isosteres (RADIs).

[84], since the amide bonds between Arg^2 and Nal^3 and between Nal^3 and Cys^4 in T140 were cleaved by treatment with rat liver homogenates [81,87]. (Arg-L/D-Nal)-type EADIs, **5** and **8**, were synthesized by the combination of analogs, in which the above isosteres were introduced, were prepared by the synthetic strategy of cyclic pentapeptides as reported in the previous paper (Table 1) [27]. (*E*)-Alkene substitutions, which can fix amide bonds in a plane form, cause the conformational restriction of the backbones. NMR and SA-MD analysis showed that the parent peptide, FC131, and these EADI-introduced pseudopeptides have nearly equal distances between any two β-carbons in all of the side chains: these compounds maintain similar dispositions of pharmacophores, suggesting that the biological differences among these compounds are derived only from the (*E*)-alkene–amide bond unit replacement. Substitutions of (Arg-L/D-Nal)-type EADIs for Arg-Nal caused a remarkable decrease in anti-HIV activity (Table 1, FC13110 and FC13414): The amide bonds of the Arg-L/D-Nal sequences were necessary for high potency, suggesting that either a deletion of the hydrogen bonding interaction with CXCR4 by the EADI introduction or an increase in hydrophobicity might be unsuitable. An (Arg-Nal)-type RADI-containing FC131 analog did not show significant

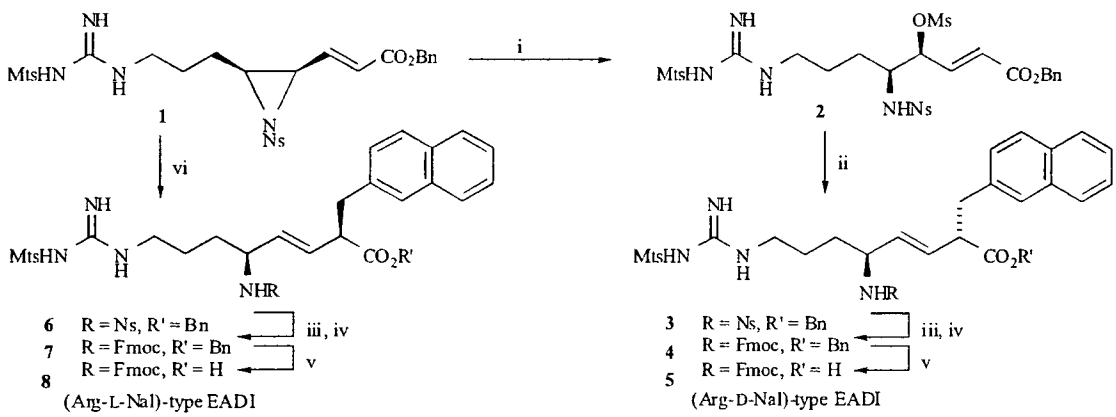


Fig. (3). Synthesis of (Arg-L/D-Nal)-type EADIs by the combination of stereoselective aziridinyl ring-opening reactions and organozinc-copper-mediated *anti*-S_N2' reactions toward a single substrate of γ,δ-*cis*-γ,δ-epimino (*E*)-α,β-enoate **1**. Mts = 2,4,6-trimethylphenylsulfonyl; Ns = 2-nitrobenzenesulfonyl. *Reagents:* (i) MsOH; (ii) 2-naphthylmethylCu(CN)ZnBr·BF_3; (iii) PhSH, K_2CO_3; (iv) Fmoc-OSu, Et_3N; (v) thioanisole, TFA; (vi) 2-naphthylmethylCu(CN)ZnBr·2LiCl.

stereoselective aziridinyl ring-opening reactions and organozinc-copper-mediated *anti*-S_N2' reactions toward a single substrate of γ,δ-*cis*-γ,δ-epimino (*E*)-α,β-enoate **1** (Fig. (**3**)) [92,93]. A (Nal-Gly)-type EADI **11** was also synthesized by the samarium diiodide (SmI_2)-induced reduction of a γ-acetoxy-α,β-enoate **9** (Fig. (**4**)) [36,58]. RADIs of Arg-Nal and Nal-Gly were prepared by a standard method of reductive amination. Then, several FC131 anti-HIV activity (FC13126), suggesting that the planar nature of the amide bond is critical to maintain the pentagonal shape conformation for high anti-HIV activity. As in the case of Arg-Nal, an importance of the amide bond of the Nal-Gly sequence was indicated (FC13122 and FC13130). These results will provide useful information for the design of non-peptide CXCR4 antagonists derived from FC131.

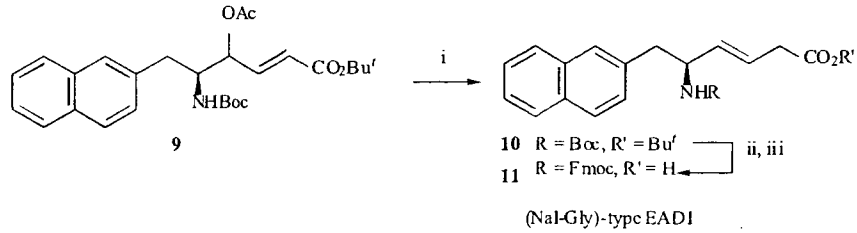
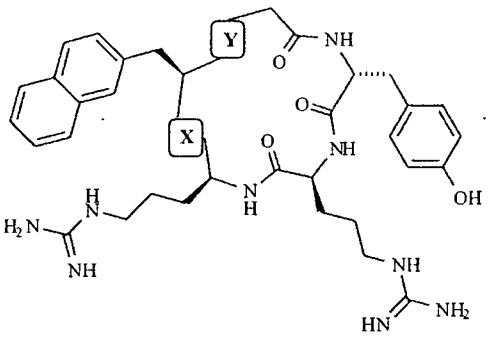


Fig. (4). Synthesis of a (Nal-Gly)-type EADI **11** by the samarium diiodide (SmI_2)-induced reduction of a γ-acetoxy-α,β-enoate **9**. *Reagents:* (i) SmI_2, ^{t}BuOH; (ii) anisole, TFA; (iii) Fmoc-OSu, Et_3N.

Table 1. Anti-HIV Activity of FC131 Analogs Containing Dipeptide Isosteres



Compd.	Sequence (cyclo)	X	Y	EC_{50} (μM)
FC131	(-D-Tyr-Arg-Arg-Nal-Gly-)	-CO-NH-	-CO-NH-	0.073
FC13110	(-D-Tyr-Arg-Arg-Nal-Gly-)	-CH=CH-	-CO-NH-	2.4
FC13126	(-D-Tyr-Arg-Arg-Nal-Gly-)	$-CH_2$-NH-	-CO-NH-	> 100
FC13122	(-D-Tyr-Arg-Arg-Nal-Gly-)	-CO-NH-	-CH=CH-	2.4
FC13130	(-D-Tyr-Arg-Arg-Nal-Gly-)	-CO-NH-	$-CH_2$-NH-	0.98
FC134	(-D-Tyr-Arg-Arg-D-Nal-Gly-)	-CO-NH-	-CO-NH-	1.9
FC13414	(-D-Tyr-Arg-Arg-D-Nal-Gly-)	-CH=CH-	-CO-NH-	9.1

1-3. Low Molecular Weight CXCR4 Antagonists Based on Structural Tuning of Cyclic Tetrapeptide-scaffolds

The cyclic pentapeptide, FC131, has a Gly residue as a spacer for cyclization. To diminish the ring size, the Nal-Gly sequence of FC131 was replaced by 4-amino-5-naphthalen-2-yl-pentanoic acid (γ-Nal), 4-amino-5-naphthalen-2-yl-pent-2-enoic acid (γ-(*E*)-Nal), etc. Among these γ-amino acid-containing cyclic tetrapeptides, an analog with substitution of γ-Nal for Nal-Gly, FC151, showed high CXCR4-antagonistic activity (IC_{50} = 54 nM) (Fig. (**5**)). This suggests that the Gly residue and the amide bond of the Nal-Gly sequence are not necessary for high activity. On the other hand, to optimize the ring structures of FC131-derived compounds, the utility of templates different from that of cyclic pentapeptides was investigated. Since the four essential amino acid residues of T140 are disposed in close vicinity each other due to the disulfide bridge and cyclic peptides having the Arg-Arg-Nal sequence, such as FC131, showed high CXCR4-antagonistic activity, we designed and prepared disulfide-bridged cyclic peptide libraries involving the *N*-3-guanidinopropanoyl-L/D-Cys(S-)-L/D-Arg-L/D-Nal-L/D-Cys(S-)-NH_2 (or –tyramine) sequence (total 32 compounds). Among these compounds, FC205 [*N*-3-guanidinopropanoyl-Cys(S-)-Arg-Nal-D-Cys(S-)-NH_2] and FC225 [*N*-3-guanidinopropanoyl-Cys(S-)-Arg-Nal-D-Cys(S-)-tyramine] exhibited significant CXCR4-antagonistic activity (IC_{50} = 690 and 530 nM, respectively) (Fig. (**6**)).

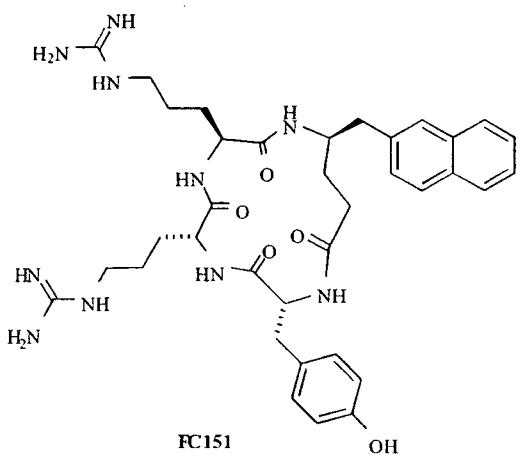


Fig. (5). Structure of a γ-Nal-containing cyclic tetrapeptide, FC151.

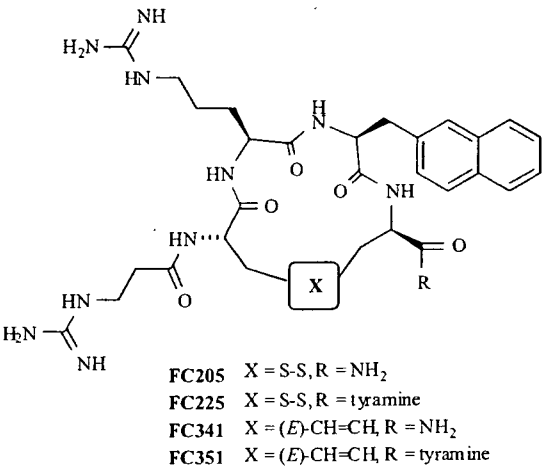


Fig. (6). Structures of cyclic analogs of FC131 that were bridged by a disulfide or an olefin.

FC205 and FC225 have a common combination of chiralities of composed amino acids, suggesting that these compounds form similar conformations. Furthermore, cyclic analogs that were bridged by an olefin instead of a disulfide in FC205 and FC225 were synthesized. These olefin-bridged peptides, FC341 and FC351, showed moderate CXCR4-antagonistic activity, which is lower (IC_{50} = 1-10 μM) compared to that of FC205 and FC225 (Fig. (**6**)). Exploratory studies on further downsizing and reduction of peptide character, including the discovery of useful scaffolds besides cyclic tetra- and pentapeptides, are now in progress. In addition, we have also developed other small-sized CXCR4 antagonists involving the novel pharmacophore such as a *p*-fluorobenzoyl moiety (data will be published).

1-4. Anti-cancer-metastasis and Anti-cancer Cell Progression Activities of CXCR4 Antagonists

CXCR4 is a seven transmembrane (7TM) GPCR, which transduces signals of its endogenous ligand, stromal cell-derived factor-1 (SDF-1)/CXCL12 [6,54,56,94]. The interaction of CXCL12 and CXCR4 plays an important role in the migration of progenitor cells during embryologic development of the cardiovascular, hemopoietic and central nervous systems. Recently, this interaction has been shown to be relevant to several problematic diseases, such as cancer cell metastasis/progression [5,7,29,37,40,41,49,50,51,62-64,67-69,77,78,85,96] and rheumatoid arthritis (RA) [55], in addition to HIV infection. Malignant cells from at least 23 different types of cancer express CXCR4 [3]: e.g. B cell chronic lymphocytic leukemia (CLL) [96], pre-B acute lymphoblastic leukemia (ALL) [37], non-Hodgkin lymphoma [5], multiple myeloma [64], pancreatic cancer [41,49], prostate cancer [77], breast cancer [50,85], ovarian cancer [68,69], neuroblastoma [29], kidney cancer [67], small cell lung cancer (SCLC) [7,40], melanoma [51,62,78] and brain cancer [63]. A. Müller *et al.* reported that CXCR4 is highly expressed on the surface of human breast cancer cells, while CXCL12 is highly expressed in lymph nodes, bone marrow, lung and liver, which constitute the common metastasis destinations of breast cancer [50]. Pulmonary metastasis of breast cancer cells was significantly inhibited by neutralizing anti-CXCR4 antibody [50] and 4F-benzoyl-TN14003 [85] in SCID mice, suggesting that the suppression of CXCL12/CXCR4 interaction may represent a novel therapeutic strategy against breast cancer metastasis that involves this ligand-receptor system. Furthermore, another biostable T140 analog, 4F-benzoyl-TE14011, significantly suppressed pulmonary metastasis of melanoma cells by using a sustained drug release formulation of biodegradable poly D,L-lactic acid (PLA) microcapsules [78]. In addition, T140 analogs exhibited significant inhibitory effects on the progression of pancreatic cancer cells [41,49], small cell lung cancer cells [7], pre-B ALL cells [37], CLL B cells, etc. These results suggest that CXCR4 antagonists, especially inverse agonists that have no CXCL12-like agonistic activity, have the potential of promising agents for cancer chemotherapy.

1-5. Anti-rheumatoid Arthritis (RA) Activity of CXCR4 Antagonists

Rheumatoid arthritis (RA) is an annoying disorder, which is mainly caused by the $CD4^+$ memory T cell accumulation in the inflamed synovium. T. Nanki *et al.* found that CXCL12 which is released in the RA synovium stimulates migration of the memory T cells that highly express CXCR4, and thereby inhibits T cell apoptosis [55]. This suggests that the CXCL12/CXCR4 interaction plays an important role in T cell accumulation in the RA synovium. 4F-benzoyl-TN14003 significantly suppressed the delayed-type hypersensitivity (DTH) response induced by sheep red blood cells (SRBC) and collagen-induced arthritis (CIA), which represent *in vivo* mouse models of this pathology [80]. These findings suggest that the CXCL12/CXCR4 axis may become a useful therapeutic target for RA chemotherapy, and that CXCR4 antagonists also have great promise as anti-RA agents. Actually, P. Matthys *et al.* reported the first paper to show that a CXCR4 antagonist, AMD3100, which is described in the next section, had anti-RA activity [46].

1-6. Other CXCR4 Antagonists

In 1997, three CXCR4 antagonists, the bicyclam AMD3100 (AnorMED, Inc.) [65] (Fig. (7)), T22 [52] and ALX40-4C (Ac-[D-Arg]$_9$-NH_2; NPS Allelix) [19], were incidentally reported at the same time. These compounds have a common character: high basicity. The amino acid residues in CXCR4 used for interaction with T140 and AMD3100 were comparatively studied using Ala-scanning mutagenesis and computation docking simulation analyzed by J. O. Trent *et al.* [95]. Critical residues for both T140 and AMD3100 bindings mainly exist in the second extracellular loop (ECL2) of CXCR4, but these are slightly different, indicating that the mechanisms of these antagonists are different. In association with AMD3100, an *N*-pyridinylmethylene cyclam (monocyclam) AMD3465 (AnorMED, Inc.) [16], a non-cyclam AMD8665 (AnorMED, Inc.) [70] and AMD070 (AnorMED, Inc.) [97] were found as new CXCR4 antagonists [41] (Fig. (7)). Twin functional agents based on AMD3100 and galactosylceramide (GalCer) analog conjugates were reported [15]. Another low molecular weight CXCR4 antagonist, KRH-1636 (Kureha Chemical & Sankyo), which might be derived from intensive modification of the *N*-terminal tripeptide of T140, Arg-Arg-Nal, was reported as an orally bioavailable agent [35]. AMD3465, AMD8665 and KRH-1636 have a 4-[[[pyridin-2-yl-methyl]amino]methyl]phenyl group as a common substructure unit, which might be a critical pharmacophore. Distamycin analogs, such as NSC651016 [33], and a flavonoid compound, ampelopsin [42], were found to be CXCR4 antagonists that have different structures. Several Arg-mimetic conjugates, CGP64222, R3G and NeoR, were also reported as cationic CXCR4 antagonists [8,9,13]. These low molecular weight antagonists including our lead compounds seem to be promising agents for chemotherapy of AIDS, cancer, RA, etc.

1-7. Advantageous Characters of T140-derived CXCR4 Antagonists

The emergence of MDR HIV-1 variants is one of the most serious problems in the clinical treatment of HIV-1-infectious and AIDS patients. H. Nakashima *et al.* found that T140 showed remarkable delaying effect against the generation of drug-resistant strains *in vitro* [39]. The difficulty of the generation of drug-resistant HIV-1 strains might be a great advantage of T140-derived compounds.

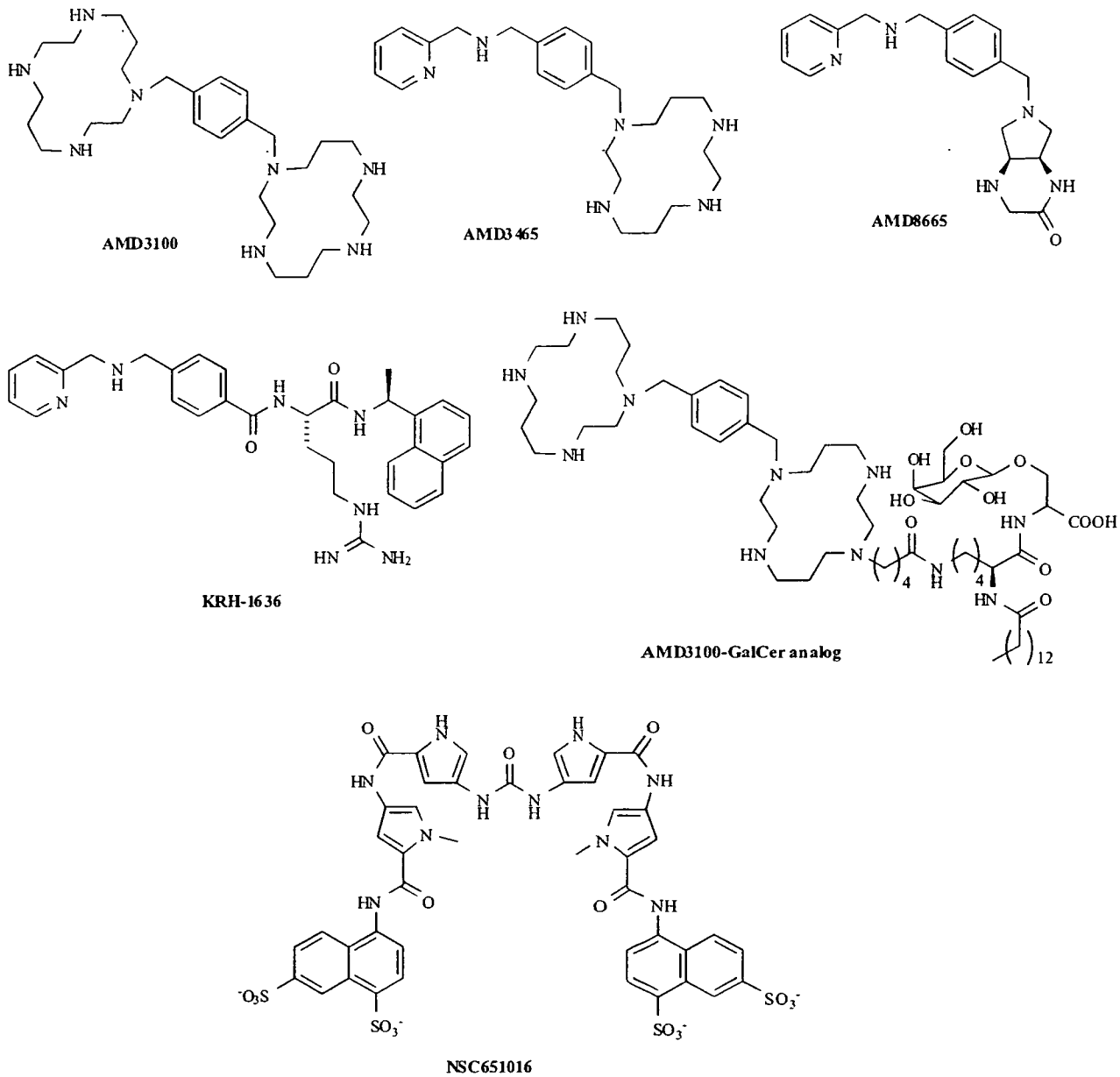


Fig. (7). Other CXCR4 antagonists.

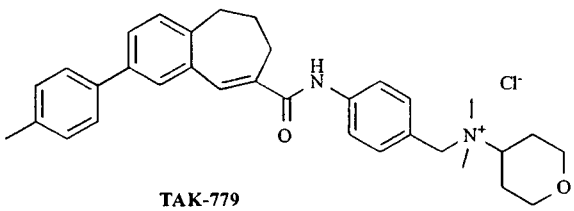
Antagonists are normally classified into inverse agonists that show no agonistic activity and partial agonists that show weak agonistic activity. Partial agonists of CXCR4 have CXCL12-like agonistic activity through CXCR4 and might activate cancer cells and memory T cells that highly express CXCR4. Especially, in terms of cancer and RA chemotherapy, inverse agonists have a clinical advantage, since they do not show any activating effects on CXCR4. S. C. Peiper et al. revealed that T140 is an inverse agonist, whereas AMD3100 and ALX40-4C are partial agonists, based on evidences that T140 treatment of CXCR4 wild type and constitutively active mutant (CAM), which were coupled to the pheromone response pathway in yeast, reduced autonomous signaling, while AMD3100 or ALX40-4C treatment induced the partial G protein activation in a dose-dependent manner [102]. This difference of the actions of AMD3100 and T140 toward CXCR4 might be caused by the difference of binding sites of these agents on CXCR4 (Section 1-6).

Cell adhesion-mediated drug resistance (CAM-DR) represents one of the serious problems in a clinical use of several anti-cancer drugs. T140 analogs showed significant effects overcoming CAM-DR in *in vitro* CLL, ALL and SCLC experiments [7,37].

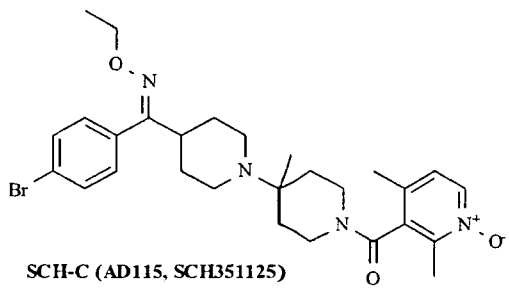
1-8. CCR5 Antagonists

Several chemokine antagonists against another co-receptor CCR5, which is used by primary HIV (R5-HIV-1) strains, have been developed. The validity of this research for development of CCR5 antagonists is based on the

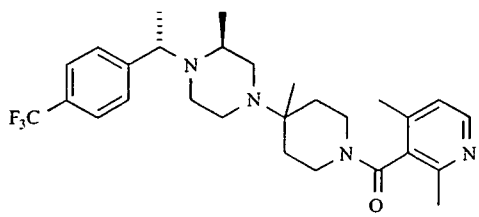
finding that individuals who have the CCR5 32 deletion mutation are healthy and strongly protected from HIV-1 infection [4]. CCR5 antagonists that have been reported to date are follows (Fig. (**8**)): a quaternary ammonium anilide, TAK779 [2], its orally bioavailable derivative, TAK220 (Takeda), a piperidino-piperidine, SCH-C (AD115, SCH351125) [59,74], piperidinopiperazine series AD101 (SCH350581), AD114 (SCH350634) [76], SCH-D (SCH417690) [75] (Schering-Plough), a spiro-diketopiperazine, AK602/ONO-4128/GW873140 (Ono & GlaxoSmithKline) [44], a 2-phenyl-4-(piperidin-1-yl)butanamine compound, a 1,3,4-trisubstituted pyrrolidine, MRK-1, its cyclopentane analog, CMPD 167 (Merck) [24], UK-427,857 (Pfizer) [59], AMD887 (AnorMED, Inc.) [66], synthetic RANTES analogs, AOP-, NNY-, and PSC-RANTES [30], etc. Individuals who have this CCR5 mutation completely lack functional CCR5 but are healthy. Since it suggests that blocking the function of CCR5 might not have any significant side-effects, CCR5 antagonists are thought to be useful as anti-HIV agents. However, CCR5 antagonists cannot suppress the emergence of the more pathogenic HIV-1 (X4-HIV-1) strains that contribute to the accelerated decrease in $CD4^+$ T cells. In a combinational use with the above CCR5 antagonists and CXCR4 antagonists, no viral replication with any HIV-1 strains was observed *in vitro* [66]. CCR5 antagonists also have the potential of promising agents for AIDS chemotherapy, especially in their combinational use.



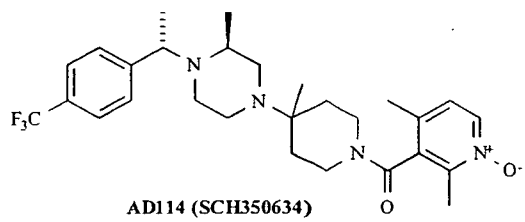
TAK-779



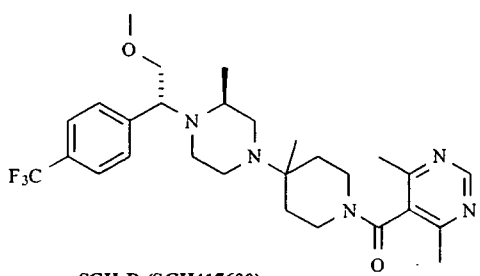
SCH-C (AD115, SCH351125)



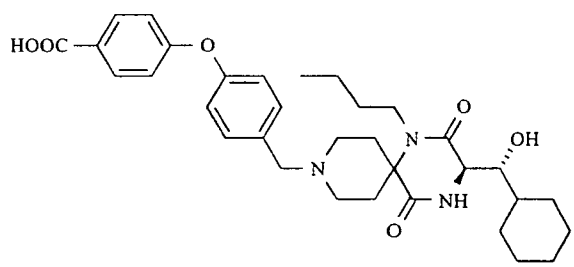
AD101 (SCH350581)



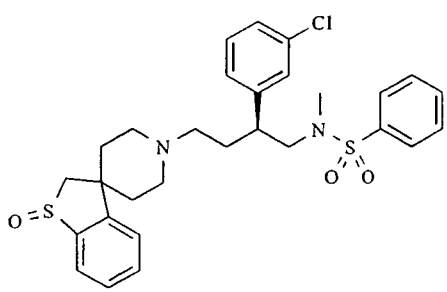
AD114 (SCH350634)



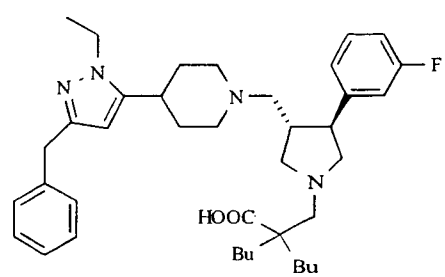
SCH-D (SCH417690)



AK602/ONO-4128/GW873140

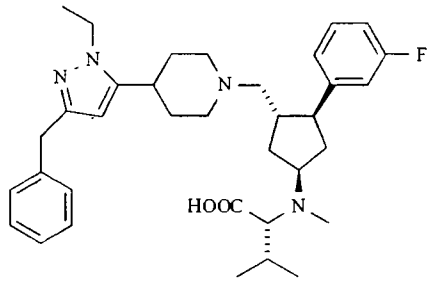


2-phenyl-4-(piperidin-1-yl) butanamine compound

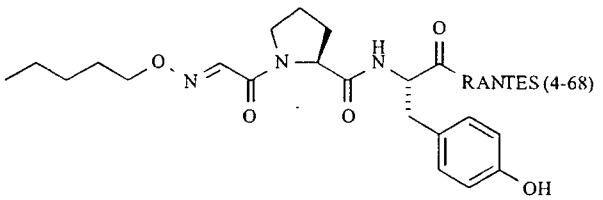


MRK-1

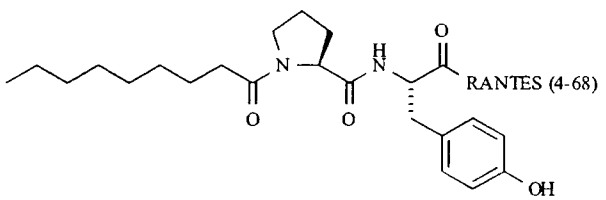
(Fig. 8). contd.....



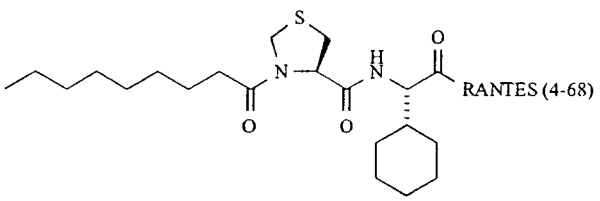
CMPD 167



AOP-RANTES



NNY-RANTES



PSC-RANTES

Fig. (8). CCR5 antagonists.

2. Fusion Inhibitors Targeting the Dynamic Supramolecular Mechanism

2-1. Gp41-Fragment-remodeling Peptides

The binding of gp120 to CCR5/CXCR4 triggers the formation of the trimer-of-hairpins structure of gp41 and the subsequent fusion of HIV/cell membranes, as described in the introduction. Thus, a dynamic supramolecular mechanism involving membrane fusion becomes rational targets for inhibitors against HIV-1 replication. The trimer-of-hairpins structure of gp41 is formed as a bundle of six α-helices, which involve antiparallel packing by both inner three-stranded coiled coils derived from the gp41 *N*-terminal helical region and the outer three-stranded coiled coils derived from the *C*-terminal helical region (Fig. (**9**)) [11]. The subdomain is composed of two peptides, N51 and C43, which are *N*-region 51 residue and *C*-region 43 residue peptides, respectively [43]. According to previous papers, several *C*-peptides derived from *C*-terminal helical region inhibited bundle formation of six α-helices and thereby HIV-1 infection. A *C*-peptide, C34, which has the native sequence of a gp41 fragment, exhibited potent inhibitory activity against HIV-1 fusion [10]. However, C34 has a defect in solubility in aqueous media. Thus, we developed highly soluble C34 analogs (SC peptides) by artificial remodeling of C34 (Fig. (**9**)) [57]. In the helical wheel diagram of C34, the amino acid residues at *a*, *d*, and *e* positions, which are essential for interaction with the inner coiled-coil strand formed by an *N*-region peptide (N36), were maintained without any substitutions, whereas non-

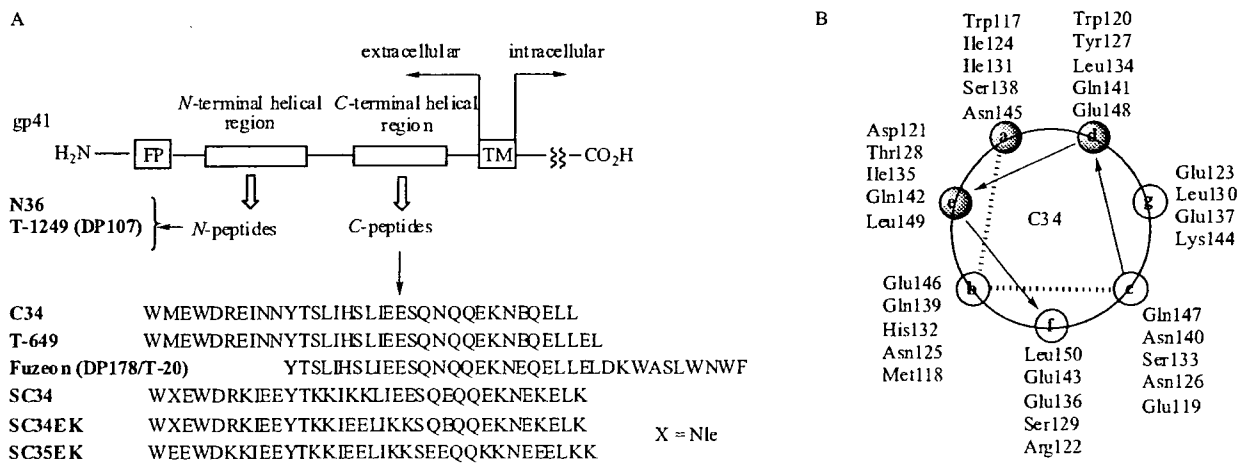


Fig. (**9**). A: Schematic representation of gp41 and sequences of *C*-peptides. FP = Fusion peptide; TM = transmembrane domain; B: Helical wheel representation of C34. Residues are numbered based on HIV-1 NL4-3 gp41.

conserved residues at *b*, *c*, *f*, and *g* positions, which are located in solvent-accessible region, were replaced by Glu or Lys. Several side-chain ion pairs of Glu-Lys formed between i and i+4 positions is thought to enhance solubility and α-helicity of C34 analogs (Fig. (**10**)). The aqueous solubility of SC peptides, SC34, SC34EK and SC35EK, was increased by more than 3 orders of magnitude, compared to that of C34. Analytical ultracentrifugation sedimentation of the N36/SC peptide complexes indicated that each N36/SC peptide forms a six-molecule complex consisting of three molecules each of N36 and SC peptide. Comparison of melting temperatures of the complexes based on the changes in $[\theta]_{222}$ of CD analysis as a function of temperature revealed that stabilities of the N36/SC peptide complexes were remarkably increased, compared to that of the N36/C34 complex. The six-helix bundle structures of the N36/SC peptide complexes were confirmed by X-ray analysis. Anti-HIV activities of these SC peptides were superior or comparable to that of C34, and ten-fold stronger than that of Fuzeon (DP178, T-20, Trimeris & Roche) [99] in the multinuclear activation of the galactosidase indicator assay (MAGI assay). Furthermore, SC peptides were active even against a Fuzeon-resistant strain. As a result, highly soluble and potent fusion inhibitors, SC34, SC34EK and SC35EK, have been developed by the remodeling of C34 based on introduction of Glu-Lys pairs into the solvent-accessible surface of the six-helix bundle. Studies on a further increase in helicity and anti-HIV activity, downsizing and reduction of peptide character are now in progress.

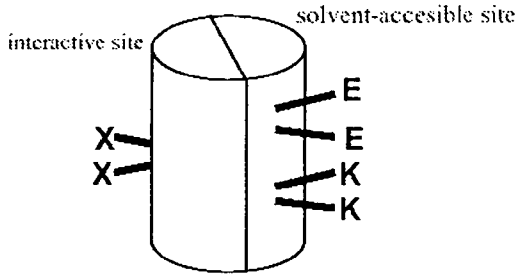


Fig. (10). Formation of side-chain ion pairs of Glu-Lys between i and i+4 positions and appropriate disposition of X-residues by α-helix formation.

2-2. Other Fusion Inhibitors

Approval of FDA to clinical use of Fuzeon in March, 2003, has brought us a great hope toward fusion inhibitors as a new class of anti-HIV drugs against MDR HIV-1 strains. C34, T-649 [18], Fuzeon, SC peptides are all 34—36-mer peptides derived from *C*-terminal helical region of gp41, as described in the above section. T-1249 (DP107, Trimeris & Roche) [47], which is a 38-mer peptide derived from *N*-terminal helical region of gp41, is Trimeris & Roche's second inhibitor active against Fuzeon-resistant isolates. Several researchers have tried to discover small non-peptide inhibitors that block gp41 activation [60,71,72]. Membrane fusion is a valid target for inhibition of an HIV-1 entry due to clinical use of Fuzeon. However, low molecular weight inhibitors, which are highly potent and really useful, have not yet been discovered. S. C. Harrison, S. L. Schreiber et al. identified non-natural binding elements that contribute to the formation of a stable complex with the inner coiled-coil strand and to inhibition of membrane fusion using a biased combinatorial chemistry library [23]. Researches on development of useful fusion inhibitors (small organic compounds) are ongoing in many laboratories.

CONCLUSION

The recent researches on the development of anti-HIV agents are assorted into two orthogonal approaches in general terms: 1) the improvement of conventional drugs, such as reverse transcriptase inhibitors and protease inhibitors, which are classified in known drug categories; and 2) the discovery of new drugs with novel action mechanisms. This review article has focused on the latter issues (2). CXCR4 antagonists derived from T140 including its low molecular weight analogs were developed as HIV co-receptor inhibitors. Furthermore, since the CXCL12/CXCR4 system is involved in progression and metastasis of several types of cancer cells and migration of the memory T cells, these CXCR4 antagonists are also useful compounds for cancer and RA chemotherapy. In association to the appearance of Fuzeon, highly soluble and potent fusion inhibitors, a series of SC peptides, have been developed by the remodeling of C34 based on introduction of Glu-Lys pairs. These therapeutic candidates that block the early stage of the HIV replication would be idealistic in the complement of HAART.

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ABBREVIATIONS

HIV = Human immunodeficiency virus

X4-HIV-1 = T cell line-tropic HIV-1
HAART = Highly active anti-retroviral therapy
MDR = Multi-drug resistant
R5-HIV-1 = Macrophage-tropic HIV-1
AIDS = Acquired immunodeficiency syndrome
SAR = Structure-activity relationship
T22 = [Tyr^{5,12}, Lys^{7}]-Polyphemusin II
Nal = L-3-(2-Naphthyl)alanine
SA-MD = Simulated annealing molecular dynamics
EADI = (*E*)-Alkene dipeptide isostere
RADI = Reduced amide-type dipeptide isostere
7TM GPCR = 7-Transmembrane segment G-protein-coupled receptor
SDF-1 = Stromal cell-derived factor-1 = CXCL12
RA = Rheumatoid arthritis
CLL = Chronic lymphocytic leukemia
ALL = Acute lymphoblastic leukemia
SCLC = Small cell lung cancer
SCID = Severe combined immunodeficient
PLA = Poly D,L-lactic acid
DTH = Delayed-type hypersensitivity
SRBC = Sheep red blood cells
CIA = Collagen-induced arthritis
ECL = Extracellular loop
GalCer = Galactosylceramide
CAM = Constitutively active mutant
CAM-DR = Cell adhesion-mediated drug resistance
RANTES = Regulated on activation, normal T cell expressed and secreted
CD = Circular dichroism
MAGI = Multinuclear activation of the galactosidase indicator
FDA = Food and Drug Administration

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Facile access to (*Z*)-alkene-containing diketopiperazine mimetics utilizing organocopper-mediated *anti*-S_N2' reactions

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Abstract—Regio- and stereoselective *anti*-S_N2' alkylation of γ-phosphoryloxy-α,β-unsaturated-δ-lactams with organocopper reagents allowing the preparation of *N*-alkylated-α,δ-substituted-β,γ-unsaturated-δ-lactams as highly functionalized diketopiperazine mimetics is presented.

2,5-Diketopiperazine **1** is the smallest possible cyclic peptide consisting of two α-amino acid residues. This highly constrained scaffold is seen in large numbers of biologically active compounds and serves as a privileged structure in medicinal chemistry.[1] Recently, we engaged in the development of synthetic methodologies for the preparation of (*E*)-alkene dipeptide isosteres (EADIs) as potential *trans*-peptide bond mimetics[2] along with their application to biologically active peptides.[3] On the basis of our research into EADIs, we envisioned that incorporation of (*Z*)-alkene units structurally similar to the *cis*-amide bonds in 2,5-diketopiperazines would provide diketopiperazine mimetics **2** as a novel promising scaffold for drug discovery (Fig. 1). This type of mimetic

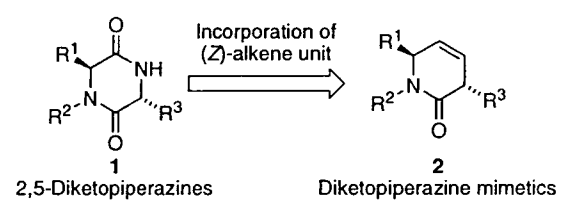

Figure 1. Diketopiperazine mimetics possessing substituted (*Z*)-alkenes as *cis*-amide bond.

Keywords: Organocopper; *anti*-S_N2' reaction; Phosphate; Peptidomimetic.
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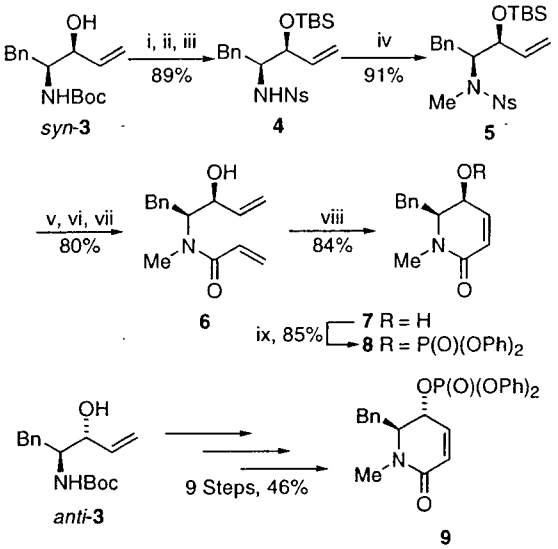
would be able to dissolve well in various media by preventing the formation of hydrogen-bonding networks that would otherwise result from the two peptide bonds. Pioneering studies were recently reported by Guibé and co-workers[4] and Knight et al.[5] for the preparation of similar structures that led to (*Z*)-alkene dipeptide isosteres and ergot alkaloids, respectively. However, stereoselective incorporation of divergent α-substituents into a common key intermediate has yet to be reported.

Our synthetic approach toward EADIs utilizes organocopper-mediated *anti*-S_N2' reaction of acyclic α,β-enoates possessing leaving groups at the γ-position. Proper choice of organocopper reagents allows a common substrate to be converted to various α-alkylated products. Development of a facile and efficient synthetic method toward functionalized diketopiperazine mimetics such as **2** is strongly desirable, since this class of compounds is of medicinal and synthetic value. In this letter, novel organocopper-mediated synthetic protocols are presented for highly functionalized diketopiperazine mimetics possessing a wide variety of α-substituents. These are discussed from the viewpoint of choice of the cyclic substrates and reagents.

The synthesis of requisite substrates for organocopper reactions is summarized in Scheme 1. The easily obtainable *N*-Boc allyl alcohol derivatives[6] *syn*-**3** and *anti*-**3** were chosen as starting materials. Conversion of the *N*-protecting group of *syn*-**3** to *N*-Ns (Ns = 2-nitrobenzenesulfonyl) followed by *O*-derivatization



Scheme 1. Synthesis of key substrates. Reagents: (i) 4 M HCl–dioxane; (ii) Ns-Cl, 2,4,6-collidine, $CHCl_3$; (iii) TBS-OTf, 2,6-lutidine, CH_2Cl_2; (iv) K_2CO_3, MeI, DMF; (v) $HSCH_2CO_2H$, LiOH, DMF; (vi) CH_2=CHCOCl, Et_3N, CH_2Cl_2; (vii) TBAF, THF; (viii) Grubbs' catalyst second generation, CH_2Cl_2; (ix) $(PhO)_2P(O)Cl$, pyridine, CH_2Cl_2. Abbreviations: Ns: 2-nitrobenzenesulfonyl; TBS: *tert*-butyldimethylsilyl.

with TBS group gave *N*-Ns amide derivative **4**. Treatment of **4** with MeI in the presence of K_2CO_3 afforded the *N*-Me sulfonamide **5**.[7] After removal of the Ns group by treatment with a thiol under basic conditions, the resulting secondary amine was acylated with acryloyl chloride followed by *O*-TBS deprotection with TBAF to afford acrylamide **6**. Ring-closing metathesis reaction of **6** with Grubbs' second-generation catalyst[8] proceeded smoothly at room temperature to yield γ-hydroxy-α,β-unsaturated-δ-lactam **7**. Although the activation of γ-hydroxy units in the acyclic enoates with the methanesulfonyl (Ms) group has afforded satisfactory results in organocopper-mediated synthesis of EADIs, attempted *O*-methanesulfonylation of **7** failed to afford any desired product due to its instability during purification over silica gel. Furthermore, *O*-acetylated derivatives proved to be inadequate for the subsequent copper-mediated α-alkylation, even though the acetylated compounds could be obtained in high yield. After extensive survey of γ-activation methodologies, we found that lactam γ-phosphoryloxy functionality[9–11] was suitable as a leaving group in terms of its stability and reactivity. Reaction of **7** with diphenylphosphoryl chloride in the presence of pyridine yielded the requisite key intermediate **8** in satisfactory isolated yield. The corresponding diastereomer **9** was also synthesized from *anti*-**3** by a sequence of reactions identical to those used for the preparation of **8**.

Next, we investigated α-alkylation of phosphate **8** with organocopper reagents (Fig. 2 and Table 1).[12] Reaction in THF of **8** with organocopper reagent prepared from equimolar amounts of MeMgCl and CuI in the presence

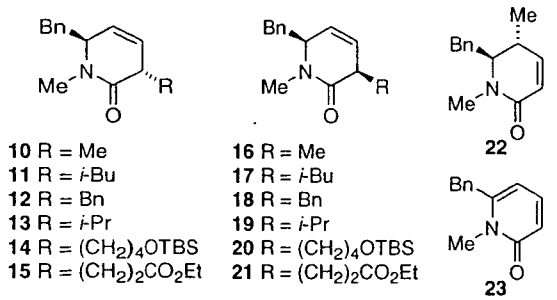


Figure 2. Structures of compounds obtained from the reaction of phosphates **8** and **9** with organocopper reagents.

Table 1. Organocopper-mediated reactions of phosphates **8** and **9**

Entry	Substrate	Reagent (2 equiv)[a,b]	Product(s) (%)[c]
1	**8**	MeCuI·MgCl·2LiCl	**10** (93)
2	**8**	MeCuI·MgCl	**10** (24), **22** (40)
3	**8**	MeCu·LiI·LiBr	**10** (83)
4	**8**	*i*-BuCu·2LiI·2LiCl	**11** (82)
5	**8**	BnCuI·MgCl·2LiCl	**12** (80)[d]
6	**8**	*i*-PrCuI·MgCl·2LiCl[e]	**23** (62)
7	**8**	*i*-PrCu(CN)·MgCl·2LiCl	**13** (81)[d]
8	**8**	$TBSOCH_2(CH_2)_2CH_2Cu{\cdot}2LiI{\cdot}2LiCl$	**14** (80)
9	**8**	$BrZnCu(CN){\cdot}CH_2CH_2CO_2Et{\cdot}2LiCl$[f]	**15** (80)
10	**9**	MeCuI·MgCl·2LiCl	**16** (73)
11	**9**	*i*-BuCuI·MgCl·2LiCl	**17** (81)
12	**9**	BnCuI·MgCl·2LiCl	**18** (85)
13	**9**	*i*-PrCu(CN)·MgCl·2LiCl	**19** (89)
14	**9**	$TBSOCH_2(CH_2)_2CH_2Cu{\cdot}2LiI{\cdot}2LiCl$	**20** (81)
15	**9**	$BrZnCu(CN){\cdot}CH_2CH_2CO_2Et{\cdot}2LiCl$[f]	**21** (79)

[a] Reaction condition (−78 °C, 20 min) was used except for entries 6, 9, and 15.
[b] THF or mixed solvent consisting of THF and Et_2O (or Et_2O–pentane) was used.
[c] Isolated yield.
[d] Small amount of S_N2 product was isolated.
[e] Reaction at −78 °C for 20 min, then at 0 °C for 40 min.
[f] Reaction at 0 °C for 60 min.

of LiCl proceeded at −78 °C in *anti*-S_N2' manner with high regio- and stereoselectivity to yield the desired α-alkylated mimetic **10** in high chemical yield (Table 1, entry 1). In contrast, organocopper-mediated reaction in the absence of LiCl afforded a mixture of *anti*-S_N2'-(**10**, 24%) and S_N2-product (**22**, 40%) (entry 2), which indicated the critical involvement of the Li salt in high α-selectivity. Whereas the role of the Li salt in affecting reaction regioselectivity is not well understood, we speculate that structural changes of the reagent/substrate complex induced by the Li salt were responsible for the observed high regioselectivity. MeCu·LiI·LiBr in THF–Et_2O derived from an equimolar mixture of MeLi·LiBr and CuI was also a useful reagent for *anti*-S_N2' alkylation of **8** (entry 3). Encouraged by these results, we examined the synthesis of other α-functionalized diketopiperazine mimetics utilizing several kinds of organocoppers prepared from equimolar amounts of organometallic reagent and copper(I) salt in the pres-

ence of Li salt. Treatment of **8** with *i*-BuCu·2LiI·2LiCl and BnCuI·MgCl·2LiCl gave the corresponding *anti*-S_N2' alkylation products **11** and **12** in reasonable yields, respectively (entries 4 and 5). Reaction of *i*-PrCuI·MgCl·2LiCl did not proceed at −78 °C, but gave predominantly the pyridinone derivative **23** at room temperature. This was probably due to the poor nucleophilicity of the reagent attributable to its steric bulkiness (entry 6). On the other hand, use of the cyanocuprate reagent, *i*-PrCu(CN)·MgCl·2LiCl, drastically improved the yield of desired *anti*-S_N2' alkylation product **13** (entry 7).

Introduction of functional groups amenable to further chemical manipulation was examined next. α-Alkylation of **8** with an *O*-TBS-protected hydroxybutyl group was possible by the use of $TBSOCH_2(CH_2)_2CH_2Cu{\cdot}2LiI{\cdot}2LiCl$ (entry 8). Copper–zinc mixed reagents possessing functional groups have shown synthetic usefulness through the application to various types of activated allylic compounds.[13] Recently, Knochel et al. reported that cyclic allylic phosphonates were alkylated in *anti*-S_N2' fashion by the action of functionalized copper–zinc reagents.[10] Independently, we also found that the reaction of **8** with a copper–zinc mixed reagent ($BrZnCu(CN)CH_2CH_2CO_2Et{\cdot}2LiCl$) proceeded unequivocally in *anti*-S_N2' manner to yield α-substituted compound **15** possessing ester functionality (entry 9).[14] Furthermore, diastereomeric **9** was also alkylated in *anti*-S_N2' manner with various organocopper reagents to yield functionalized diketopiperazine mimetics (entries 10–15).

The absolute configurations of diketopiperazine mimetics **12** or **16** were unambiguously determined to be 3,6-*trans* (3*S*,6*S*) or 3,6-*cis* (3*R*,6*S*) by X-ray analyses.[15] Based on these results, relative configuration of the corresponding diastereomer **10** (vs **16**) or **18** (vs **12**) was assigned as 3,6-*cis* or 3,6-*trans*. ^1H NMR measurements of these diastereomeric pairs indicated that the α-protons of 3,6-*trans* compounds (**10** and **12**) appeared ca. 0.6 ppm upfield from the corresponding α-protons of the 3,6-*cis* isomers. These *trans*- and *cis*-isomers were derived from 5,6-*cis*-**8** and 5,6-*trans*-phosphate **9**, respectively, indicating that the organocopper-mediated S_N2' reactions proceeded in high *anti*-selectivity. Other S_N2'-products resulting from 5,6-*cis*-phosphate **8** also exhibited upfield chemical shifts of α-protons as compared to those of the corresponding 5,6-*trans*-derived compounds. Based on these results and the high level of stereoselectivity observed in organocopper-mediated *anti*-S_N2' reactions, compounds (**11**, **13–15**) and (**17**, **19–21**) were assigned 3,6-*trans*- and 3,6-*cis*-configurations, respectively.

In summary, reported herein are new and practical synthetic methodologies for preparation of functionalized diketopiperazine mimetics **2** containing (*Z*)-alkene units. Of note are the use of organocopper-mediated *anti*-S_N2' reactions to γ-phosphoryloxy-α,β-unsaturated-δ-lactams, which proceed with high regio- and stereoselectivities. Unequivocal access to various diastereomerically pure α-substituted mimetics is possible depending on the choice of organocopper reagents. Diversity of substituents at the 1- or 6-positions of the ring can also be assured by the selection of *N*-alkylating reagents or starting amino acids. Enhancement of α-selectivity in the organocopper-mediated reaction is attributable to the addition of Li salt, even though the basis for this effect is not well understood. Investigating the effects of Li salts and biological evaluation of these mimetics, including the conversion to linear (*Z*)-alkene-type dipeptide isosteres as a counterpart to EADIs, will be presented in due course.

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Supplementary data

Supplementary data associated with this article can be found, in the online version at doi:10.1016/j.tetlet.2005.04.057.

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12. Representative procedure for organocopper-mediated *anti*-S_N2' reaction of γ-phosphoryloxy-α,β-unsaturated-δ-lactams: To a stirred solution of CuI (37.3 mg, 0.196 mmol) and LiCl (16.6 mg, 0.392 mmol) in dry THF (0.75 mL) was added MeMgCl in THF (3.0 M, 0.0653 mL, 0.196 mmol) under argon at −78 °C, and the mixture was stirred for 10 min at 0 °C. To the solution of organocopper reagent was added dropwise a solution of the phosphate **8** (44.1 mg, 0.0981 mmol) in dry THF (0.75 mL) at −78 °C, and the mixture was stirred for 20 min. The reaction was quenched with a 1:1 saturated NH_4Cl–28%NH_4OH solution (2 mL). The mixture was extracted with Et_2O, and then the extract was washed with water, and dried over $MgSO_4$. Concentration under reduced pressure followed by flash chromatography over silica gel with *n*-hexane–EtOAc (1:1) yielded mimetic **10** (19.6 mg, 0.0910 mmol, 93%) as colorless oil.
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QSAR & Combinatorial Science QCS

A simple, Automated Quasi-4D-QSAR, Quasi-multi Way PLS Approach to Develop Highly Predictive QSAR Models for Highly Flexible CXCR4 Inhibitor Cyclic Pentapeptide Ligands Using Scripted Common Molecular Modeling Tools

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Abstract

A methodology for developing highly predictive ($r^2 > 0.9$) 3D-QSAR models ($q^2 > 0.7$) based on sixteen flexible CXCR4 cyclic pentapeptide inhibitors is reported. The effective automated use of common molecular modeling tools such as Macromodel and Sybyl is demonstrated. The recently developed multi-way Partial Least Square (PLS) approach for discovering the bioactive conformers and alignment was used in a quasi-multi-way PLS approach. Twenty-five conformers for each compound were generated by Monte Carlo conformational searches and alignments (seventy five in total) were based on the templates from the three most active compound conformers. These were aligned in Sybyl Molecular Databases and Sybyl Molecular Spreadsheets. All repetitive tasks were automated by use of simple Unix shell, python and Sybyl Programming Language (SPL) scripts. This efficient protocol furnished three 3D-QSAR models with q^2 values of 0.714, 0.734 and 0.657 and predictive r^2 values of 0.951, 0.990, and 0.956 respectively. The best 3D-QSAR model predicted the biological activities of nine test compounds from all activity ranges within 0.5 log units.

1 Introduction

The human chemokine receptor CXCR4 is the stromal cell-derived factor (SDF-1α) chemokine receptor. Several diseases have been reported to be linked to CXCR4 such as AIDS [1, 2], cancer metastasis and progression [3], and rheumatoid arthritis [4]. Masuda *et al.*[5] reported an eighteen-residue peptide T22, to be a CXCR4 inhibitor. T140, a fourteen residue peptide, was reported by Tamamura *et al.* [6] as a more potent CXCR4 inhibitor. Fujii *et al.* [7] have recently employed orthogonal combination of conformations with sequence-based libraries for the discovery of potent cyclic pentapeptides as CXCR4 inhibitors. The biological data reported by Fujii *et al.* [7] is used in this QSAR study.

The Quantitative Structure Activity Relationship (QSAR) is among the most widely used techniques in rational drug design. Following the pioneering work of Hansch *et al.* [8] in 2D-QSAR, several techniques like Comparative Molecular Field analysis (CoMFA) [9], Molecular Shape Analysis MSA [10], distance geometry [11], molecular similarity matrices [12], Comparative Molecular Similarity Index Analysis (CoMSIA) [13], Condensed-phase Optimized Molecular Potentials for Atomistic Simulation Studies (COMPASS) [14], and Hypothetical Active Site Lattice (HASL) [15] have been developed for three dimensional QSAR (3D-QSAR). Among these techniques, CoMFA has been widely used [16] as it provides for the visual display of electrostatics and steric fields of the regions important for biological activity. However, CoMFA models have been reported to be very sensitive to the chosen bioactive conformations [17], selection of alignment rules [18], spatial orientation and grid sizes [19]. The issue of the choice of the bioactive conformation has been addressed with techniques such as conformational averaging (conformational ensembles) [20], employing several conformers in a multi-way data array [21], multi-conformational ligand representation [22], tensor decomposition [23], and three-way-PLS analysis [24]. The second important issue of alignment rule selection has been addressed by the Field Based Similarity Searching (FBSS) program for automated alignment [25], automated alignment using

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the GOLD program [26], multiple orientation ligand representation [22], use of docking algorithms and protocols for aiding in alignment selection [27], use of Generalized Procrustes Analysis (GPA) for consensus molecular alignment [28], use of local structure analysis (molecular footprints) for partial molecular alignment [29] and cross-validated R^2 guided region selection (q^2-GRS) for CoMFA [19]. The development of 3D-QSAR models for highly flexible ligands is challenging [30]. The choice of the active conformer and the selection of the alignment rule may be guided by available experimental data like the X-ray crystal structure of ligand-receptor complexes or the solution NMR structure of active analogs.

The cyclic pentapeptide CXCR4 inhibitors [7] are highly flexible, with hundreds of possible conformers within a few kcal/mol range of the global minimum. There are many reports on sophisticated techniques to handle the problem of choosing the active conformation, such as conformation ensembles and multi-way data arrays. There are also several ways to handle the problem of alignment in CoMFA based 3D-QSAR such as using FBSS or GOLD. However, to our knowledge, a simple, efficient method, employing regular molecular modeling tools, to develop CoMFA based 3D-QSAR models for highly flexible ligands has not been reported. We therefore, developed the methodology reported here to provide such a method.

2 Material and Methods

All computation work was done on Silicon Graphics Octane workstations and an Origin 2000 server running the IRIX 6.5 operating system. The molecular modeling software used were Macromodel version 7.0 [31] and Sybyl version 6.9.1 [32]. Python programs were run using Python version 2.3 on a Dell PC running the Windows XP operating system.

2.1 Data Set

We chose a subset of twenty five cyclic pentapeptide inhibitors of CXCR4 whose IC_{50} values were determined by displacement binding of [^{125}I]SDF-1 to CHO transfectants stably expressing CXCR4 [7]. In brief, the CXCR4 transfectants were incubated with [^{125}I]SDF-1 (0.15 nM) on a shaker at 4 °C for one hour in the presence and absence of the cyclic pentapeptides. The unbound isotope was separated by centrifugation and counted. The inhibitory ability of the cyclic pentapeptides was analyzed in triplicate at 0.01, 0.10, 1.0, and 10.0 μM concentrations. The specific IC_{50} values were determined by Scatchard analysis [7].

The cyclic pentapeptide sequences and their respective bioactivities are presented in Table 1. We divided this set into a training set of sixteen compounds and test set of nine compounds. The training set comprises of the cyclic peptide sequence Tyr-Arg-Arg-Nal-Gly. The training set had the unnatural amino acid Nal (NapthylAlanine) placed in all possible positions around the ring.

The NMR structure of the most active compound FC131 has been reported by Fujii *et al.* [7], and it was made available to us by Trent *et al.* [33]. In the absence of the X-ray crystal structure of the ligand-receptor complex, the use of the NMR based structure for the bioactive conformation, has been reported [34]. We, therefore, used the NMR structure of FC131 as the template, by keeping the cyclic pentapeptide backbone identical for all of the training and test data set molecules.

2.2 Conformer selection

The Maxwell-Boltzmann distribution gives the population of various conformers at any given temperature. This has been used for conformer population ratio studies of epothilones [35]. For highly flexible molecules, like the cyclic pentapeptides, there are hundreds of possible conformers within a 5 kcal/mol energy range of the global minima. We chose the twenty five lowest energy conformers of every training set compound for the QSAR study. These conformers were within 3–5 kcal/mol energy range of the respective global minimas (see Table 1).

2.3 Molecular structure building and conformational search

The structures of all the cyclopentapeptides were built in Macromodel and minimized using the AMBER* force field [36]. The Polak–Ribiere conjugate gradient method [37] was employed with a gradient convergence criteria of 0.01 kcal/Å-mol. The conformation of the central core cyclic pentapeptide ring was preserved, by employing positional constraint of 239.23 kcal/mol-Å on the fifteen backbone ring atoms. The conformational search was performed in Macromodel using the Monte Carlo Multiple Minimum method [38] (10,000 steps, 11.96 kcal/mol energy window, with subsequent of minimization of 10,000 steps to ensure convergence). Water solvation was simulated using the Generalized Born (GB/SA) implicit solvation method [39]. All the backbone bonds of the of the central pentapeptide ring (N-Cα and Cα-C) were fixed with a torsion constraint of 2,392.34 kcal/mol-Å.

Macromodel creates a single output file containing the conformations, starting with the global minimum conformer, followed by the rest of the conformers in the order of ascending energy. The individual data files of the conformers (twenty five each) can be created in Macromodel, by sequentially reading the conformers and then saving each of them. However, since we would have had to repeat this process (16×25) four hundred times, we automated this task by using a python script [40]. All the data files were converted into mol2 files using BABEL, a file format conversion utility [41]. The repetitive task of converting four hundred files was performed using a Unix shell script [42].