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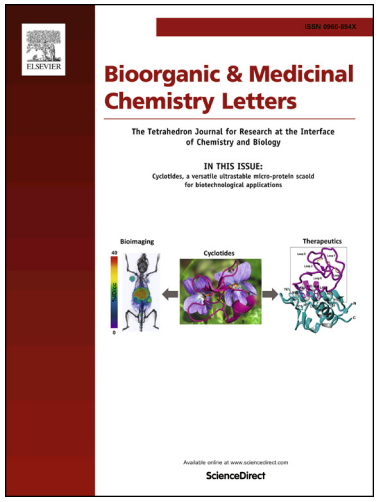

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Head-to-tail macrocyclization of cysteine-free peptides using an *o*-aminoanilide linker

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ABSTRACT. A head-to-tail macrocyclization protocol for the preparation of cysteine-free cyclic peptides was investigated. The *o*-aminoanilide linker constructed in the peptide sequence by a standard Fmoc-based peptide synthesis procedure was subjected to nitrite-mediated activation under acidic conditions toward *N*-acyl benzotriazole as the active ester species. The subsequent cyclization smoothly proceeded by neutralization in the presence of additives such as 1-hydroxybenzotriazole (HOBt) and 1-hydroxy-7-azabenzotriazole (HOAt) to afford the expected cyclic pentapeptide, a CXCR4 antagonist. The cyclization efficiencies were dependent on the precursor open-chain sequence. The application of this step-wise activation-cyclization protocol to microflow reaction systems is also described.

Keywords: chemokine; CXCR4 antagonist; cyclic peptide; macrocyclization: microflow reaction

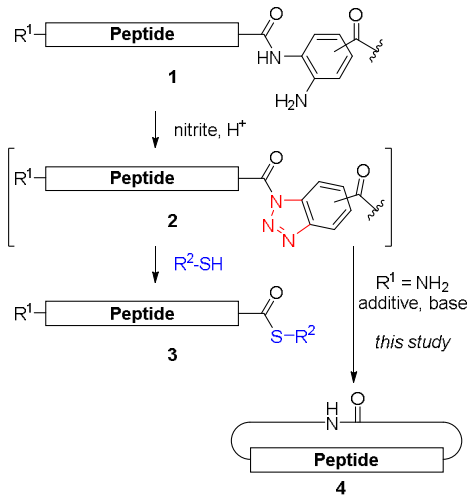
Abbreviations: CXCR4, CXC chemokine receptor 4; Dbz, 3,4-diaminobenzoic acid; DPPA, diphenylphosphoryl azide; Nal, 3-(2-naphthyl)-L-alanine; NCL, native chemical ligation; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; SDF-1, stromal cell-derived factor 1; SPPS, solid-phase peptide synthesis

Cyclic peptides are attractive scaffolds that provide great chemical diversity arising from their various macrocyclic structures and the combination of their component amino acids.[1,2] A number of approved drugs as well as promising clinical candidates with head-to-tail cyclic peptide structures have been developed through medicinal chemistry efforts.[3] For the facile preparation of cyclic peptides by solid-phase approaches, several characteristic linkers have been reported, which are converted into active ester species at the C-terminus of the precursor open-chain peptides. For example, Kaiser oxime resin was employed for the synthesis of side-chain bridged and head-to-tail cyclic peptides via a Boc strategy of solid-phase peptide synthesis (SPPS).[4] Sulfamylbutyryl linker, compatible with the basic conditions of Fmoc-SPPS, is subjected to activation by treatment with iodoacetonitrile or (trimethylsilyl)diazomethane.[5] The subsequent nucleophilic addition of the N-terminal amino group led to formation of the head-to-tail cyclic peptides.[6] This safety-catch linker has also been employed for the preparation of peptide thioester segments[7,8] for native chemical ligation (NCL) in protein synthesis.[9,10]

The 3,4-diaminobenzoic acid (Dbz) linker (*o*-aminoanilide linker) is another useful active ester precursor (Scheme 1).[11] The Dbz linker is activated by treatment with *p*-nitrophenyl chloroformate to form an *N*-acyl benzimidazolone intermediate, which can be converted into the peptide thioester in the presence of an appropriate thiol. By taking advantage of similar activation principles, a number of protocols for peptide thioester preparations have also been developed.[12,13] Recently, an alternative activation protocol of Dbz linker **1** was reported, in which $NaNO_2$-mediated triazole formation provides a reactive *N*-acyl benzotriazole **2**.[14] Treatment of **2** with a thiol affords a peptide thioester **3** as an active species for NCL. A poly(arginine) solubilizing tag can be conjugated at the C-terminus of peptide segments via the Dbz linker to improve the solubility during protein synthesis and then be converted into the C-terminal acid by hydrolysis.[15] We envisioned that the cyclization would proceed even without a Cys group to yield cyclic peptide **4** when the N-terminal amino group is located in close proximity to the C-terminal *N*-acyl benzotriazole intermediate **2**. In this study, we

investigated a Cys-free macrocyclization protocol using a C-terminal Dbz linker for cyclic peptide synthesis.

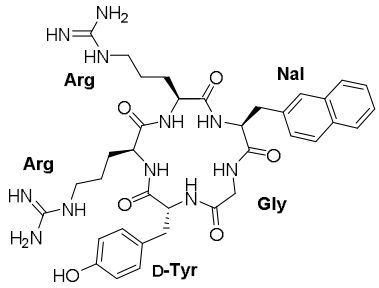
Scheme 1. Application of a C-terminal Dbz linker for the preparation of thioesters and cyclic peptides.



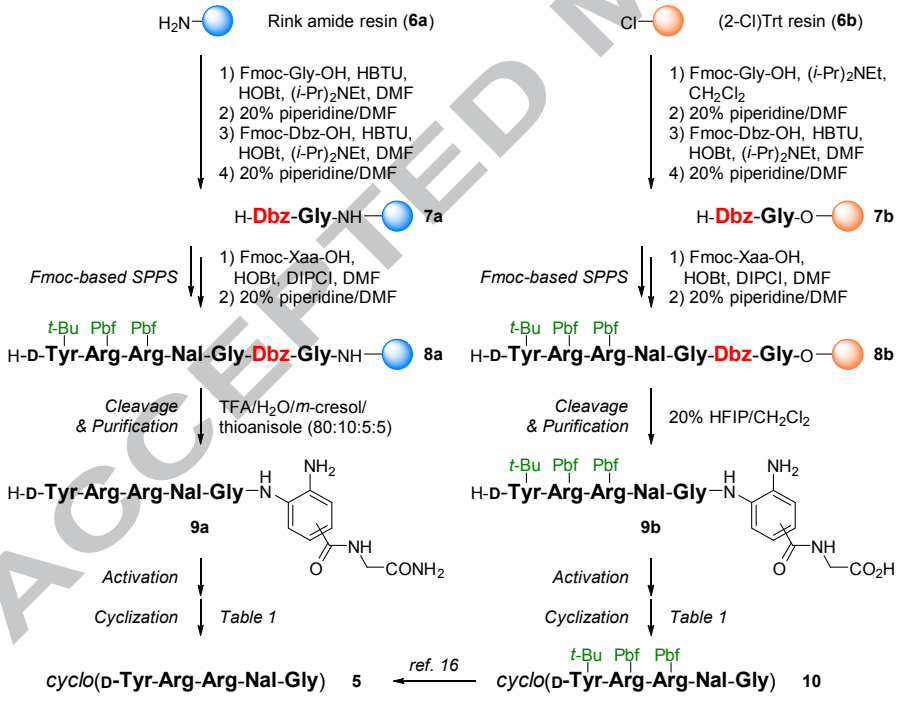
As a model peptide, we chose the cyclic pentapeptide, FC131 (**5**, Figure 1), which exhibits highly potent antagonistic activity against stromal cell-derived factor 1 (SDF-1) binding to CXC chemokine receptor 4 (CXCR4).[16] FC131 and its derivatives also inhibit CXCR4-mediated HIV-1 infection to show potent anti-HIV activity.[16,17] Initially, we synthesized two linear peptide substrates **9a,b**, in which Gly carboxamide was subjected to activation by nitrite-mediated benzotriazole formation of the Dbz linker. The cyclization at the Gly-D-Tyr peptide bond in FC131 is advantageous in that no epimers are generated and steric hindrance derived from the side-chain can be avoided during the activation–cyclization process (Scheme 2). For the preparation of peptide **9a** without employing protecting groups for the side-chain functional groups [H-D-Tyr-Arg-Arg-Nal-Gly-Dbz-Gly-NH_2; Nal: 3-(2-naphthyl)-L-alanine], the Dbz linker was loaded on the Rink amide resin **6a** via a Gly linker. The peptide sequence was constructed using standard Fmoc-based solid-phase synthesis (SPPS). The final deprotection and cleavage from resin **8a** with a cocktail of TFA/H_2O/*m*-

cresol/thioanisole provided peptide **9a**. The side-chain protected peptide **9b** having a Dbz linker [H-D-Tyr(*t*-Bu)-Arg(Pbf)-Arg(Pbf)-Nal-Gly-Dbz-Gly-OH] was also synthesized by Fmoc-based SPPS on the (2-Cl)Trt resin **6b** as well as cleavage from resin **8b** by treatment with 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP)/CH_2Cl_2 (2:8) at room temperature.

Figure 1. Structure of FC131 (**5**).

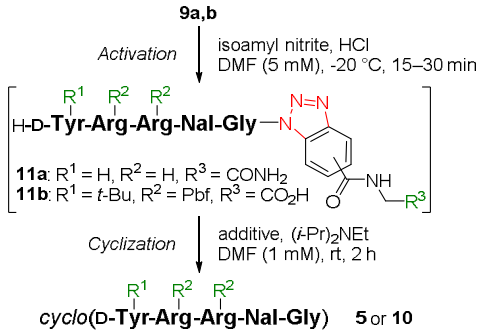


Scheme 2. Syntheses of unprotected and protected FC131 (**5** and **10**).



Next, the key activation–cyclization processes of **9a,b** were investigated (Table 1). When linear unprotected peptide **9a** was treated with $NaNO_2$ in diluted aq. HCl by the standard protocol for Dbz activation[14] followed by cyclization of the intermediate **11a** under basic conditions, only hydrolyzed product was obtained (data not shown). The same reaction using isoamyl nitrite in DMF gave the expected cyclic peptide **5** in 27% yield (entry 1). To improve the cyclization efficiency, we attempted the conversion of *N*-acyl benzotriazole into an alternative active ester form by addition of auxiliary reagents. Using a catalytic quantity of 1-hydroxybenzotriazole (HOBt, 10 mol%), the product yield of **5** was significantly improved (entry 2). The use of stoichiometric amounts of HOBt was more effective (entry 3). The best result for the preparation of unprotected cyclic peptide **5** was obtained with 1-hydroxy-7-azabenzotriazole (HOAt, entry 4). In the case of the protected peptide substrate **9b**, activation and the macrocyclization process resulted in satisfactory yield of **10** even in the absence of auxiliary reagents (entry 5). Because the choice of base for the cyclization of **11b** had a significant effect on the product yield (Supplementary Table S2), *N*,*N*-diisopropylethylamine ((*i*-Pr)$_2$NEt) was employed for further experiments. The cyclization efficiency was not improved in the presence of catalytic and stoichiometric amounts of HOBt (entries 6 and 7), while the addition of HOAt slightly increased the product yield (entry 8). These results suggested that the ability of the *N*-acyl benzotriazole to be converted to an active ester species was similar to that of the HOBt ester and slightly less compared with that of the HOAt ester.

Table 1. Cyclization of the unprotected and protected peptides **9a,b** with a C-terminal Dbz linker.



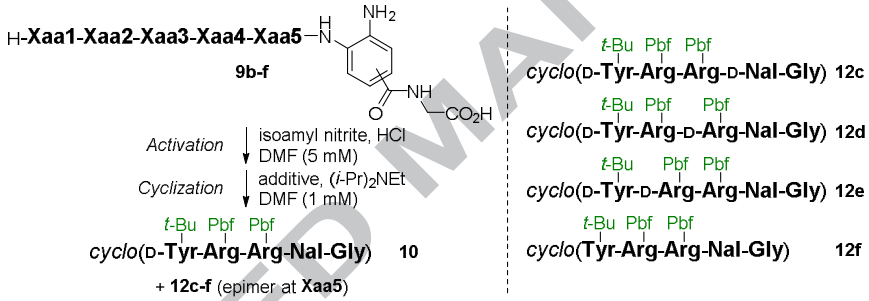
entry	compound	additive (eq.)	yield (%)[a]
1	**9a**	-	27
2	**9a**	HOBt (0.1)	49
3	**9a**	HOBt (1.5)	57
4	**9a**	HOAt (1.5)	71
5	**9b**	-	77
6	**9b**	HOBt (0.1)	79
7	**9b**	HOBt (1.5)	77
8	**9b**	HOAt (1.5)	91

[a]HPLC yield.

We next investigated the nitrite-mediated activation–cyclization process at the different cyclization sites for the preparation of protected cyclic peptide **10** (Table 2). Favorable conformation(s) of the linear precursor for macrocyclization may improve the product yield, while the efficiency of acylation might be decreased due to the presence of the side-chain of the C-terminal amino acid. We synthesized four additional protected pentapeptides **9c–f** by the identical procedure for the synthesis of **9b** on (2-Cl)Trt resin. Cyclization of peptide **9c** at the Nal–Gly peptide bond smoothly proceeded in the presence or absence of auxiliary reagents to provide the cyclic peptide **10** in high yields (83–98%) with low epimerization. In contrast, no or significantly less product **10** was obtained by the activation–cyclization processes from **9d–f** in the absence of any auxiliary reagents (10% yield from **9d**; no product from **9e**; 39% yield from **9f**). In the case of cyclization from **9d** at the Arg(Pbf)–Nal peptide bond, epimerized product **12d** was preferentially obtained over **10**. These results suggested that the cyclization efficiencies were highly dependent on

the peptide sequence and therefore, its conformations. For the linear substrates **9d–f**, the auxiliary reagents were effective at improving the cyclization yield. In the Dbz linker-mediated cyclization from **9d** and **9f**, even catalytic amounts of HOBt (10 mol%) significantly increased the yield of **10**. In particular, addition of stoichiometric amounts of HOAt facilitated the efficiency of macrocyclization from all linear substrates **9b–f** (68% yield from **9d**; 42% yield from **9e**; 80% yield from **9f**) with minimal levels of byproduct epimer formation. The yields exceeded those from diphenyl phosphoryl azide (DPPA)-mediated macrocyclization of linear pentapeptide substrates, which is one of the conventional protocols for cyclic peptide synthesis (Supplementary Table S3).[18]

Table 2. Cyclization of protected peptides at alternative peptide bonds.



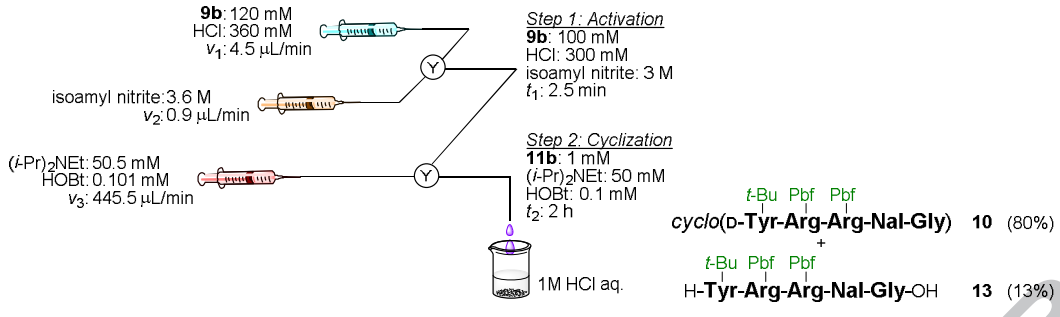
compound	sequence [**Xaa1**-**Xaa2**-**Xaa3**-**Xaa4**-**Xaa5**]	yield (**10** / **12c-f**; %)[a] additive (eq.)			
		none	HOBt (0.1)	HOBt (1.5)	HOAt (1.5)
9b	D-Tyr(*t*-Bu)-Arg(Pbf)-Arg(Pbf)-Nal-Gly	77 / -	79 / -	77 / -	91 / -
9c	Gly-D-Tyr(*t*-Bu)-Arg(Pbf)-Arg(Pbf)-Nal	83 / 2	90 / 1	96 / 1	98 / 1
9d	Nal-Gly-D-Tyr(*t*-Bu)-Arg(Pbf)-Arg(Pbf)	10 / 14	47 / 6	59 / 7	68 / 5
9e	Arg(Pbf)-Nal-Gly-D-Tyr(*t*-Bu)-Arg(Pbf)	-	-	37 / 0	42 / 0
9f	Arg(Pbf)-Arg(Pbf)-Nal-Gly-D-Tyr(*t*-Bu)	39 / 0	67 / 0	76 / 0	80 / 0

[a]HPLC yield.

During the course of our investigation, the on-resin cyclization of a protected peptide with a C-terminal Dbz linker was reported.[19] The 14-residue sunflower trypsin inhibitor-1 (SFTI-1) was obtained in 42% yield via on-resin cyclization, final deprotection and intramolecular disulfide bond formation.[19] With reference to this approach, we assessed the synthesis of the cyclic pentapeptide via on-resin cyclization of the linear protected peptide on polyethylene glycol–polystyrene (PEG–PS) resin. However, FC131 (**5**) was obtained in only 11% yield with concomitant formation of a cyclic decapeptide (6% yield, cyclic dimer peptide) (Supplementary Scheme S1). This indicated that lower loading rate of the *N*-acyl benzotriazole intermediate on solid support or high dilution in solution-phase before the macrocyclization step under basic conditions would be needed to obtain the expected monomeric cyclic pentapeptide in good yield.

On the basis of these insights, we designed a microflow reactor using DMF as the solvent for the synthesis of cyclic pentapeptide **10** via the activation–cyclization process using the Dbz linker-containing substrate **9b** (Figure 2). Two Y-shape mixers were connected with tubing. To the first mixer, substrate **9b** and isoamyl nitrite in DMF were introduced, after which the activation of the Dbz-linker occurred within 2.5 min to form *N*-acyl benzotriazole intermediate **11b** (step 1). In the second mixer, a mixture of **11b** was diluted with a 100-fold volume of DMF containing excess (*i*-Pr)$_2$NEt and catalytic HOBt (10 mol%). The cyclization process continued in the tubing for two hours (step 2) and the mixture was poured into an HCl solution to quench the reaction. The protected cyclic peptide **10** was obtained in satisfactory yield (80%). Recently, there have been a number of synthetic processes for peptide materials using microflow reactors.[20] This microflow-based approach using a Dbz-linker-containing linear peptide should be applicable to the synthesis of a variety of cyclic peptides even on a large scale.

Figure 2. Dbz linker-mediated macrocyclization using a microflow reaction system.



In summary, we established a Dbz activation-cyclization protocol for the synthesis of the cyclic peptide, FC131, a CXCR4 antagonist that does not contain a Cys residue. Both side chain-protected and unprotected linear substrates were subjected to macrocyclization via nitrite-mediated acyl benzotriazole formation in the presence of HOAt and HOBt as additives to afford the expected cyclic peptide in moderate to good yields. This nitrite-mediated protocol has an advantage over a safety-catch sulfonamide linker in that the peptide C-terminus can be activated under mild acidic conditions without protection of the N-terminal amino group. The investigations using a series of linear substrates demonstrated that the efficiency primarily depended on the macrocyclization site. The best result involved the macrocyclization at the Nal-Gly peptide bond with minimal epimerization. The stepwise activation–cyclization protocol using a continuous microflow reaction system process also gave a satisfactory result comparable with that obtained from the batch-wise process under the corresponding conditions. The established protocol should be applicable to the synthesis of a variety of cyclic pentapeptides including natural products and receptor ligands and be adaptable to large scale preparations.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at #####.

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