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Efficient Macrocyclization for Cyclicpeptide Using Solid-Phase Reaction

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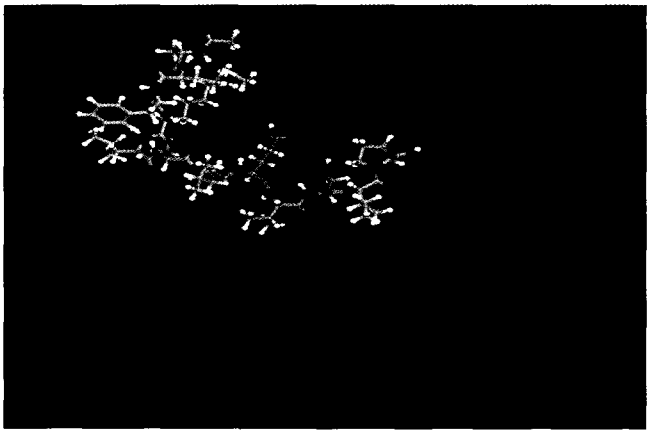
Cyclicpeptides are important targets in peptide synthesis because of their interesting biological properties. Constraining highly flexible linear peptides by cyclization is one of the mostly widely used approaches to define the bioactive conformation of peptides. Cyclic peptides often have increased receptor affinity and metabolic stability over their linear counterparts. We carried out virtual screening experiment *via* docking in order to understand the interaction between HLE-Human Leukocyte Elastase and ligand peptide and to identify the sequence that can be a target in various ligand peptides. We made cyclic peptides as a target base on Met-Ile-Phe sequence having affinity for ligand and receptor active site docking. There are three ways to cyclize certain sequences of amino acids such as Met-Ile-Phe-Gly-Ile. First is head-to-tail cyclization method, linking between *N*-terminal and *C*-terminal. Second method utilizes amino acid side chain such as thiol functional group in Cys, making a thioether bond. The last one includes an application of resin-substituted amino acids in solid phase reaction. Among the three methods, solid phase reaction showed the greatest yield. Macrocyclization of Fmoc-Met-Ile-Phe-Gly-Ile-OBn after cleavage of Fmoc protection in solution phase was carried out to give macrocyclic compound **5** in about 7% yield. In the contrast with solution phase reaction, solid phase reaction for macrocyclization of Met-Ile-Phe-Gly-Ile-Asp-Tentagel in normal concentrated condition gave macrocyclic compound **7** in more than 35% yield.

Key words: Macrocyclization, Cyclicpeptide, Solid-phase, Combinatorial chemistry, Human Leukocyte Elastase

INTRODUCTION

Cyclic peptides (Coxon *et al.*, 1988; Qian *et al.*, 1990; Schmidt *et al.*, 1991; Shioiri *et al.*, 1972) are important targets in peptide synthesis because of their interesting biological properties. Constraining highly flexible linear peptides by cyclization is one of the mostly widely used approaches to define the bioactive conformation of peptides. Cyclic peptides often have increased receptor affinity and metabolic stability over their linear counterparts.

We carried out virtual screening experiment via docking in order to understand the interaction between HLE-Human Leukocyte Elastase and ligand peptide and to identify the sequence that can be a target in various ligand peptides. We made cyclic peptides as a target base on Met-Ile-Phe sequence having affinity for ligand and receptor active site



Docking model

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docking.

Among the three ways of cyclization, that is sidechain-to-head, sidechain-to-backbone, and head-to-tail, the latter has been investigated extensively. Two general approaches are used for synthesis. Classical solution-phase linear peptide cyclization under high dilution conditions, and resin based cyclization. The cyclic peptide in solution-phase (Herzig *et al.*, 1986) was synthesized by cyclization after stepwise linear peptide synthesis (Graf *et al.*, 1996; Reichwein *et al.*, 2000). This synthetic procedure presents some disadvantages, such as the necessity to isolate the desired peptide from the excess reagents, which leads in some cases to a considerable loss of the product. Furthermore, cyclization in solution requires very dilute concentrations of the linear peptide to minimize the formation of the cyclodimer and oligomers.

In the early 1990's, a new strategy has been introduced for peptide synthesis on the solid-phase, based on the attachment of the amino acid side chain to the solid support. In the past few years, interest in solid-phase synthesis techniques has increased significantly, especially in conjunction with their application to combinatorial chemistry (Dolle *et al.*, 1999; Gordon *et al.*, 1996; Kobayashi *et al.*, 1999). Libraries of peptides and organic molecules have proven to be powerful tools for facilitating the discovery process of new lead and therapeutic compounds. Linear peptide libraries have emerged as a powerful approach for new drug lead discovery. Futhermore, conformationally constrained cyclic peptides and related macrocycles represent better prospects in view of their reduced degrees of freedom and improved chemical and bicchemical stabilities.

MATERIALS AND METHODS

Materials

Reactions were generally carried out under a positive pressure of dried nitrogen gas; for moisture sensitive reactions the glassware was flame-dried under a stream of dried nitrogen gas. Dried nitrogen gas was supplied by Shin Yang Co., dried through Silica gel, blue (Shinyo Pure Chemicals Co. LTD., practical grade) and inlet to the reaction vessel by the silicon tubing connected a stainless steel needle. Air and moisture sensitive liquid reagents were transferred by disposable syringe. and were introduced into reaction vessels through Suba Seal white-rubber septa (Brown *et al.*, 1975) (William Freeman LTD.) Solid reagents were added either in a nitrogen filled glove bags or under a stream of dried nitrogen gas. All reactions were stirred with a Teflon covered magnetic stirring bar.

Anhydrous solvents were distilled before use. They were typically purchased from Oriental Chemical Ind (extra pure grade). Dichloromethane were distilled from calcium hydride (Sigma) powder under a stream of dried argon gas (Shin Yang Co.). Triethylamine was distilled from calcium hydride (Sigma) powder. Anhydrous *N*-methylmorphorine and diethylamine was purchased from Aldrich(extra pure grade).

The solvents (dichloromethane, hexane, ethyl acetate, methanol) for flash chromato graphy were purchased from Duk San Pure Chemical. Co. LTD. and Sam Chun Pure Chemical. Co. LTD. These solvents were re-distilled before use. Flash chromatography following the method of Still (Still *et al.*, 1978) employed Merck silica gel (Kieselgel 60, 200-400 mesh). Analytical thin-layer chromatography (TLC) was performed with commercial silica gel glass plates (Sigma-Aldrich, 250 μm layer tickness, 5-17 μm particle size, 60 Å pore size, fluorescent indicator). Compounds were visualized using Ultra-Violet illumination (UVP Ind., Mineral light Lamp, Multiband UV-254/366 nm).

Removal of solvents *in vacuo* refers to evaporation with a Buchii rotary evaporator (R-114) using a aspirator. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Varian Gemini 2000 instrument (200 MHz). Chemical shifts are reported in parts per million (ppm) downfield from the internal standard, tetramethylsilane. Coupling constants are reported in hertz (Hz). Carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on a Varian Gemini 2000 instrument (50MHz), fully decoupled and chemical shifts are reported in parts per million (ppm) downfield that are determined relative to the carbon signals of the solvent ($CDCl_3$, 77.0 ppm).

Infrared spectra were recorded in 5mm path length potassium bromide cells or potassium bromide disk on JASCO FT/IR-430. All drawing chemical structures were generated by CS ChemDraw Ultra Version 5.

Synthetic procedures

Preparation of Fmoc-Gly-Ile-OBn(1) Fmoc-Gly-OH (1000 mg, 3.36 mmol),1-hydroxybenzotriazole (682 mg, 5.05 mmol) and *N*-methylmorphorine (0.74 ml, 6.32 mmol) are dissolved in 10% DMF/dichloromethane (30 ml), the solution is stirred and cooled in an ice-water bath while EDCI (709 mg, 3.7 mmol) is added. Stirring is continued for one hour at 0°C and then Ile-OBn (774 mg, 3.36 mmol) is added. The solution is stirred overnight at room temperature. The reaction solution is diluted with ethyl acetate. The mixture of ethyl acetate (300 ml) and of a saturated solution of $NaHCO_3$ in water (150 ml) is added to the residue and the organic phase extracted with a 10%solution of citric acid in water (200 ml), again with saturated $NaHCO_3$ (150 ml) and water (150 ml). The solution is dried over anhydrous Na_2SO_4 filtered and evaporated to dryness *in vacuo*. The crude dipeptide derivative is purified by recrystallization with hexane and ethyl acetate system in 88% yield.

^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.78 (d, J = 7.6 Hz, 2H),

7.6 (d J = 7.6 Hz, 2H), 7.36 (m, 10H), 6.4 (d, J = 8.2 Hz, 2H), 5.15 (d, J = 5.0 Hz, 2H), 4.68 (m, 1H), 4.42 (d, J = 7.0 Hz, 3H), 4.2 (m, 2H), 3.9 (br s, 3H), 3.15 (m, 3H), 0.84 (m, 9H).

Preparation of Fmoc-Phe-Gly-Ile-OBn(2) To a soution of Fmoc-Gly-Ile-OBn (1520 mg, 3 mmol) in acetonitril (30 ml), diethylamine (30 ml) is added and the reaction is allowed to proceed at room temperature for 3 hours. The diethylamine and the solvent are evaporated in vacuo at a bath temperature not exeeding 30°C.

Fmoc-Phe-OH (1162 mg, 3 mmol), 1-hydroxybenzotriazole (608 mg, 4.5 mmol) and *N*-methylmorphorine (0.65 ml, 6 mmol) are dissolved in 10% DMF/dichloromethane (20 ml), the solution is stirred and cooled in an ice-water bath while EDCI (633 mg, 3.3 mmol)is added. Stirring is continued for one hour at 0°C and then H-Gly-Ile-OBn (1217 mg, 3 mmol) is added. The solution is stirred overnight at room temperature. The reaction solution is diluted with ethyl acetate. The mixture of ethyl acetate (200 ml) and of a saturated solution of $NaHCO_3$ in water (100 ml) is added to the residue and the organic phase extracted with a 10%solution of citric acid in water (100 ml), again with saturated $NaHCO_3$ (100 ml) and water (100 ml). The solution is dried over anhydrous Na_2SO_4 filtered and evaporated to dryness in vacuo. The crude tripeptide derivative is purified by recrystallization with hexane and ethyl acetate system in 70% yield.

^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.74 (d, J = 6.6 Hz, 2H), 7.58 (d, J = 6.6 Hz, 2H), 7.36 (m, 10H), 6.4 (br s, 2H), 5.15 (d, J = 5.2 Hz, 2H), 4.6 (m, 2H), 4.2 (d, J = 6.2 Hz, 2H), 3.73 (m, 2H), 3.15 (m, 2H), 1.25 (m, 4H), 0.86 (m, 9H).

Preparation of Fmoc-Ile-Phe-Gly-Ile-OBn(3) To a soution of Fmoc-Phe-Gly-Ile-OBn (820 mg, 1.8 mmol) in acetonitril (20 ml), diethyl amine (20 ml) is added and the reaction is allowed to proceed at room temperature for 3 hours. The diethylamine and the solvent are evaporated in vacuo at a bath temperature not exeeding 30°C.

Fmoc-Ile-OH (636 mg, 1.8 mmol), 1-hydroxybenzotriazole (365 mg 2.7 mmol) and *N*-methylmorphorine (0.3 ml, 3.6 mmol) are dissolved in 10% DMF/dichloromethane (10 ml), the solution is stirred and cooled in an ice-water bath while EDCI (380 mg, 1.98 mmol) is added. Stirring is continued for one hour at 0°C and then H-Phe-Gly-Ile-OBn (710 mg, 1.8 mmol) is added. The solution is stirred overnight at room temperature. The reaction solution is diluted with ethyl acetate. The mixture of ethyl acetate (150 ml) and of a saturated solution of $NaHCO_3$ in water (70 ml) is added to the residue and the organic phase extracted with a 10%solution of citric acid in water (100 ml), again with saturated $NaHCO_3$ (70 ml) and water (70 ml). The solution is dried over anhydrous Na_2SO_4 filtered and evaporated to dryness *in vacuo*. The crude tetrapeptide derivative is purified by recrystallization with hexane and ethyl acetate system in 65% yield.

^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.8 (d, J = 6.6 Hz, 2H), 7.58 (d, J = 6.6 Hz, 2H), 7.36 (m, 10H), 6.85 (m, 3H), 6.5 (d, J = 5.8 Hz, 1H), 5.12 (m, 3H), 4.75 (m, 3H), 4.45 (m, 3H), 3.9 (m, 4H), 3.15 (m, 1H), 2.95 (m, 1H), 1.15 (s, 4H), 0.82 (m, 18H).

Preparation of Fmoc-Met-Ile-Phe-Gly-Ile-OBn(4) To a soution of Fmoc-Ile-Phe-Gly-Ile-OBn (700 mg, 0.9 mmol) in acetonitril (15 ml), diethylamine (15 ml) is added and the reaction is allowed to proceed at room temperature for 3 hours. The diethylamine and the solvent are evaporated in vacuo at a bath temperature not exeeding 30°C.

Fmoc-Met-OH (334 mg, 0.9 mmol), 1-hydroxybenzotriazole (182 mg, 1.35 mmol) and N-methylmorphorine (0.19 ml, 1.8 mmol) are dissolved in 10% DMF/dichloromethane (15 ml), the solution is stirred and cooled in an ice-water bath while EDCI (190 mg, 0.99 mmol) is added. Stirring is continued for one hour at 0 and then H-Ile-Phe-Gly-Ile-OBn (615 mg, 0.9 mmol) is added. The solution is stirred overnight at room temperature. The reaction solution is diluted with ethyl acetate. The mixture of ethyl acetate (100 ml) and of a saturated solution of $NaHCO_3$ in water (50 ml) is added to the residue and the organic phase extracted with a 10%solution of citric acid in water (50 ml), again with saturated $NaHCO_3$ (50 ml) and water (50 ml). The solution is dried over anhydrous Na_2SO_4, filtered and evaporated to dryness in vacuo. The crude pentapeptide derivative is purified by recrystallization with hexane and ethyl acetate system in 55% yield.

^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.8 (d, J = 7.0 Hz, 2H), 7.58 (d, J = 7.0 Hz, 2H), 7.36 (m, 10H), 7.17 (br s, 5H), 6.85 (m, 3H), 5.12 (q, J = 12.7 Hz, 2H), 4.65 (m, 2H), 4.2 (m, 4H), 3.9 (m, 1H), 3.4 (m, 1H), 2.55 (t, J = 7.0 Hz, 2H), 2.15 (s, 3H), 1.22 (s, 6H), 0.82 (m, 12H).

Preparation of cyclo-(Met-Ile-Phe-Gly-Ile)(5) To a linear peptide of Fmoc-Met-Ile-Phe-Gly-Ile-OBn (100 mg, 0.125 mmol), the Fmoc group and benzyl group were removed with 0.2 N NaOH (10 ml) and the reaction is allowed to proceed at room temperature for 3 hours. The reaction mixture was extracted with dichlormethane and the solvent evaporated *in vacuo* at a bath temperature not exeeding 30°C.

The deprotected linear peptide of HN-Met-Ile-Phe-Gly-Ile-OH and HATU (48 mg, 0.125 mmol) were stirred in DMF/dichloromethane (10 ml) at 0°C. HOBT (17 mg, 0.125 mmol) and DIPEA (0.02 ml, 0.375 mmol) were added dropwise to the solution. The solution is stirred overnight at room temperature. After the reaction, all volatiles were

removed *in vacuo*. The peptide cyclo-(Met-Ile-Phe-Gly-Ile) was isolated with 5% dichlromethane/methanol silicagel column system to yield (5 mg, 7%).

^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.78 (d, J = 5.8 Hz, 2H), 7.6 (d, J = 5.8 Hz, 2H), 7.36 (m, 3H), 4.2 (m, 3H), 3.6 (m, 2H), 3.15 (s, 1H), 2.25 (m, 1H), 2.17 (s, 3H), 0.84 (m, 18H).

Preparation of Fmoc-Asp(CO-OCH$_2$-resin)OtBu(6) Tentagel's OH resin (1000 mg, 0.3 mmol) was swollen in 10 ml of DMF for 15 min. Fmoc-Asp(OtBu)-OH (617 mg, 1.5 mmol) and triphenylphosphine (393 mg, 1.5 mmol) were dissolved in 5 ml of DMF and added to the swollen resin. DEAD (0.24 ml, 1.5 ml) was diluted to 2 ml with DMF and added dropwise to the mixture at room temperature. The reaction was run overnight. The mixture was then removed, and the resin was washed with three times each with DMF and methanol, and finally with diethyl ether and then dried *in vacuo*.

Preparation of cyclo-(Met-Ile-Phe-Gly-Ile-Asp)(7) After chain assembly of the linear peptide from Ile and to Met, Fmoc protecting group and *t*Bu protecting group were removed with 20% piperidine (15 ml) and 5% TFA (15 ml) respectively. The resin (0.04 mmol) in DMF (10 ml) was then cooled to 0°C, and HATU (76 mg, 0.2 mmol) and HOBT (27 mg, 0.2 mmol) was added. Next, DIPEA (0.07 ml, 0.4 mmol) and NMO (0.02 ml, 0.2 mmol) was added dropwise, and the reaction was monitored by ninhydrin test. The ninhydrin test was negative, indicating the cyclization reaction was complete. The polymer material was filtered, washed and dried.

The resin obtained was treated with 10ml of liquid HF in the presence of anisole (0.5 ml) and dimethyl sulfide (0.2 ml) for 1 h at room temperature. After the evaporation of HF, the resin was washed three times with diethyl ether (30 ml), dried and extracted with 50% AcOH(20 ml). The extract was diluted with water and freeze dried.

^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.78 (d, J = 5.8 Hz, 2H), 7.6 (d, J = 5.8 Hz, 2H), 4.65 (m, 2H), 4.2 (m, 4H), 3.9 (m, 1H), 3.4 (m, 1H), 2.25 (m, 1H), 2.1 (s, 3H), 0.88 (m, 18H).

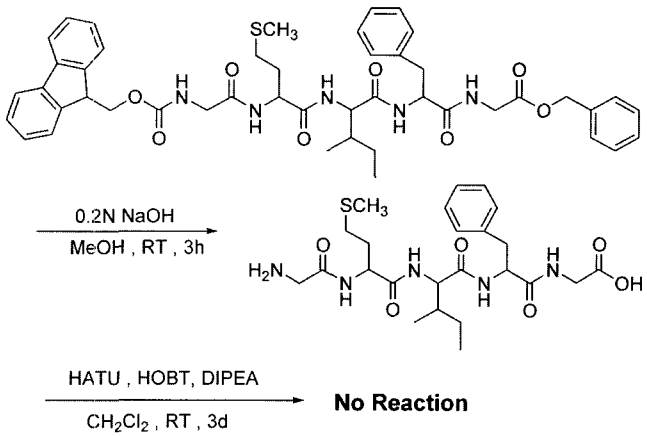
RESULT AND DISCUSSION

First of all, the cyclic peptide was synthesized by cyclization in solution-phase (Herzig *et al.*, 1986) after stepwise linear peptide synthesis. This synthetic procedure presents some disadvantages, such as the necessity to isolate the desired peptide from the excess reagents, which leads in some cases to a considerable loss of the product. Macrocyclization in solution requires very diluted concentrations of the linear peptide to minimize the formation of the dimer and oligomers. Particularly, the direct macrocyclization between *N*-terminal and *C*-terminal did not give satisfactory yield. A different method for the synthesis of cyclic peptide libraries has been developed where the key cyclization step involves reaction between a *C*-terminal cysteine side chain and an *N*-terminal bromoacyl group (Njoroge *et al.*, 1990).

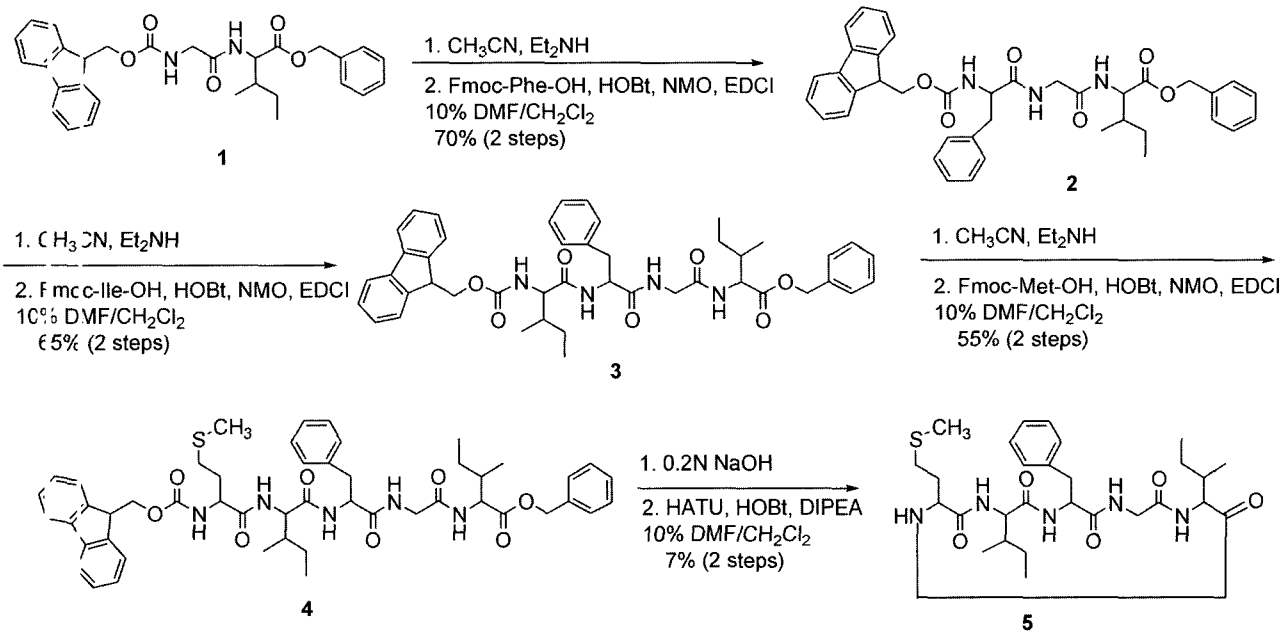
As peptide macrocyclization relies on intramolecular ring closure, the final deprotected linear precursor is allowed to react under diluted conditions. Deprotection of Fmoc involves the use of strongly basic and nucleophilic piperidine, which makes any activated ester approach incompatible. In fact, diketopiperazine formation is one of the most common problems facing peptide acid synthesis. We inversigated the utility of solid-phase reaction to overcome the difficulty of general macrocyclization. It could be not necessary to carry out the macrocyclization in high-diluted condition.

The array of Met-Ile-Phe as a designed core was extended with glycine in both sides resulting in Gly-Met-Ile-Phe-Gly. Macrocyclization of this peptide, which was synthesized by known peptide synthetic method, was not successful in any concentration (Scheme 1).

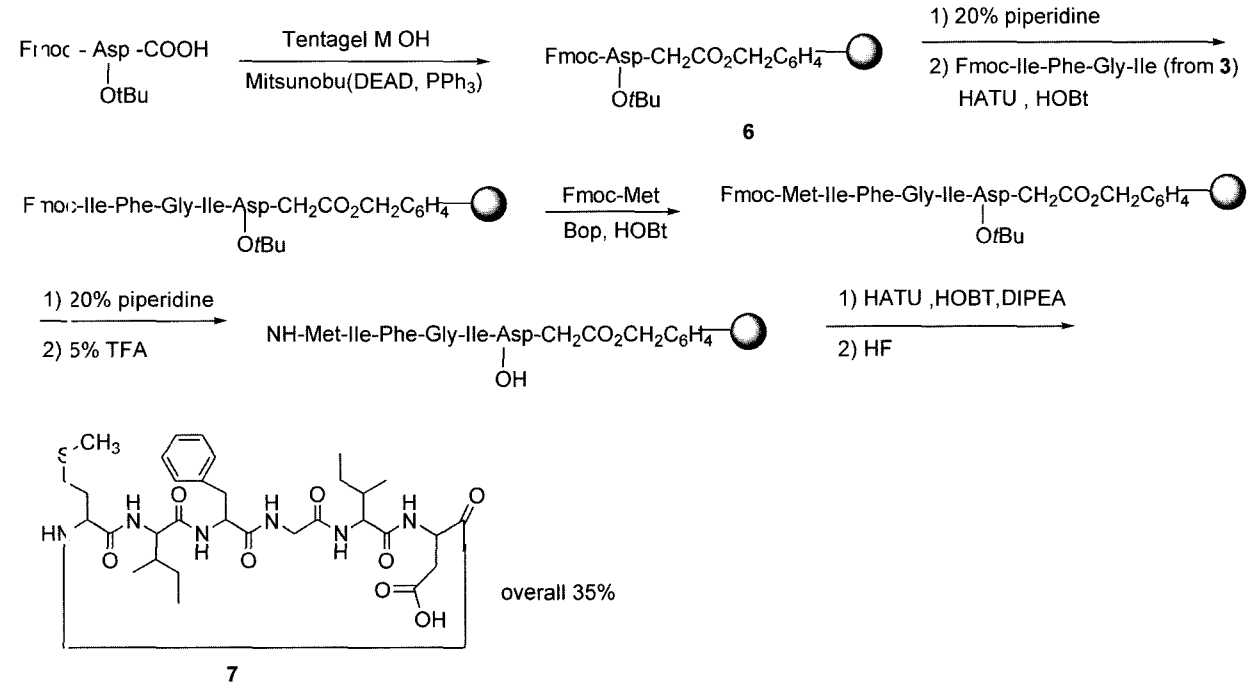
Other linear peptides, such as Gly-Met-Ile-Phe-Ile and Ile-Met-Ile-Phe-Gly-Gly, were applied to carry out macrocyclization reaction as well. These trial did not give any detectable macrocyclization product. Finally, peptide Met-Ile-Phe-Gly-Ile was macrocyclized to give compound **5** in 7% yield. The synthetic scheme for the preparation of the linear peptides in solution phase is shown in Scheme 2. This fact showed that macrocyclization reactin was not easy in our peptide in solution-phase. We decided to utilize solid-phase reaction for macrocyclization. Compound **1** was prepared by the condensation of Fmoc-Gly-OH and Ile-OBn in 88% yield. Regular procedure from compound **2** led to compound **4** in overall about 20% yields, as shown in Scheme 2.



Scheme 1. Macrocyclization of Gly-Met-Ile-Phe-Gly



Scheme 2 Macrocyclization of peptide Met-Ile-Phe-Gly-Ile in solution phase (HOBT: 1-Hydroxybenzotriazole, EDCI: 1-(3-Dimethylaminopropyl)-3-ethylca bodiimide, HATU: O-(7-Azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate)



Scheme 3. Macrocyclization of peptide in solid-phase

Macrocyclization of compound **4** after cleavage of Fmoc group was carried out under several conditions. The macrocyclization between amino group and carboxylic acid moiety was done in the treatment with HATU, HOBT, and DIPEA to give macrocyclic compound **5** in about 7% yield.

Eventually, we applied this macrocyclization to a solid phase reaction. Benzyl group of compound **3** (Fmoc-Ile-Phe-Gly-Ile-OBn) was deprotected by hydrogenation. The condensation of deprotected compound **3** and Asp-Tentagel-OH gave compound **6**. This was identified by the positive reaction of the Ninhydrin test, which showed the existence of amino group. As shown in Scheme 3, Met-residue was attached by normal condition followed by the

macrocyclizaton to give macrocyclic compound **7** in overall 35% yield. We believed that macrocyclization reaction itself gave product in more than 50% yield. Furthermore, this solid phase reaction was carried out in quite concentrated condition (0.35 M) rather than high dilution condition (5×10^{-5}), which was applied to macrocyclization normally.

We tried to analyze the chiral variation using Marfey's reagent (Mavromoustakos *et al.*, 1999). Liquid chromatography (LC) analysis showed the existence of minor shoulders (less than 1%) as most major peaks, possibly due to diastereotopic contamination (Kistaludy *et al.*, 1979).

Conclusionally, macrocyclization in solid phase reaction was carried out more efficiently and in proper concentration, comparing with the corresponding solution phase reaction. The investigation of biological activity is still in progress.

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