Synthetic Study of Membrane Protein Based on Ligation Chemistry

（ライゲーション法を用いた膜蛋白質の合成化学的研究）

Takeshi Sato

Graduate School of Science

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Abbreviations

Acm	Acetamidemethyl
Boc	*t*-butoxycarbonyl
BocOSu	*N*-*t*-butoxycarbonyoxysuccinimide
*t*Bu	*t*-butyl
Bu	*n*-butyl
Bzl	benzyl
*c*Hep	cycloheptyl
*c*Hex	cyclohexyl
Cl-Z	2-chlorobenzyloxycarbonyl
CLEAR	cross-linked ethoxylate acrylate resin
DCC	cyclohexylcarbodiimide
DCM	dichloromethane
DIEA	*N,N*-diisopropylethylamine
DMSO	dimethylsulfoxide
DMF	dimethylformamide
DTT	dithiothreitol
Fmoc	9-fluorenylmethoxycarbonyl
HOBt	1-hydroxybenzotriazole
HBTU	*O*-(benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate
HOObt	3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine
$HOCH_2$-Pam,	4-(hydroxymethyl)phenylacetoamidomethyl
MALDI-TOF,	matrix assisted laser desorption ionization time-of-flight
NMP,	1-methylpyrolidin-2-one
ORL1	Opioid receptor like 1
Ph	phenyl
RP-HPLC	reversed-phase high performance liquid chromatography
Tos	*p*-tluenesulfonyl
TFA	Trifluoroacetic acid
Trt	triphenylmethyl

General introduction

General introduction

In recent statistical analyses of the genomes of several organisms, 30% of all open reading frames were found to encode helix-bundle membrane proteins [1]. These membrane proteins play crucial roles in numerous processes associated with biological membranes including ion / molecule transport, communication, and energy transduction, which implies that they perform key functions in regulating the physiological state of the cell. This is especially true for receptors and ion channels that control second messenger concentrations in the cytosol. These properties indicate that they would be suitable targets for pharmaceutical drugs, as evidenced by the fact that more than two-thirds of today's drugs target these two protein classes [2]. However, knowledge of the structure and function of these membrane proteins remains to be uncovered. The scarcity is due to the well-known fact that the procedures for characterizing such proteins are not as straightforward as for their soluble counterparts. One of the most serious problems is the preparation of a purified sample in a large quantity. The tools of molecular biology expression represent a potentially major technique for their preparation. However, membrane proteins are normally expressed at very low levels. The use of an efficient heterologous expression system is an important approach for the preparation of such proteins. However, the level expression of many membrane proteins is typically low, often due to toxicity, heterogeneous post-translational modifications, instability, and partial proteolysis [3]. As an alternative approach for obtaining membrane proteins, chemical synthesis represents a viable alternative. Peptide ligation is a promising technique for the synthesis of membrane proteins, especially these with multiple transmembrane regions.

Chemistry has always played an important role in the study of biological processes, and recent progress in the field of peptide synthesis has extended the size limits of proteins that can be chemically synthesized [4, 5]. Using the solid phase method, peptide building blocks of about 30 amino acid residues long can be synthesized routinely. The synthesized peptide building blocks are then condensed by a ligation strategy to give a larger polypeptide. However, while ligation techniques have been applied successfully to water-soluble proteins, their application to membrane proteins, especially those with multiple transmembrane regions, is extremely limited. The first problem involves the synthesis of large building blocks. The transmembrane region is

generally rich in β-branched amino acids, which introduces difficulties during the chain assembly of the peptide [5]. The hydrophobic nature of the peptide or the building block is source of another general problem. Solubilization and purification of such peptides is always a challenge.

In this theme, the chemical synthesis of membrane proteins using peptide ligation methods is described. Difficulties during the synthesis, in the purification steps or in the ligation step, are revealed and solved by the introduction of new techniques.

Peptide synthesis by ligation strategy

Recent advances in the field of peptide ligation chemistry have extended the size of proteins that can be chemically synthesized. Based on the solid phase method, introduced by R.B. Merrifield [6], a peptide building block of about 30 amino acid residues can be rapidly and simply synthesized. These building blocks are then condensed by ligation methods. Thus far, all of the ligation methods currently used have adopted peptide C-terminal thiocarboxylic acid or thioesters as builiding blocks, and conceptually, are classified into two groups.

The first involves the selective activation of a carbonyl group at the C-terminal amino acid residue, which was initially proposed by Blake *et.al.* [7]. They prepared a peptide with a thiocarboxylic acid at the C-terminal by the solid phase method and then carried out segment condensation with an amino component in the presence of silver ions (**Fig. 1**). In this strategy, a protecting group for the side-chain carboxyl group is not required, since the thiocarboxyl group is selectively activated by the silver ions. Only the side-chain amino group requires protection. This strategy was successfully applied to the synthesis of α-inhibitin[8], and other polypeptides [9], and provided one of the basic strategies for the present ligation chemistry.

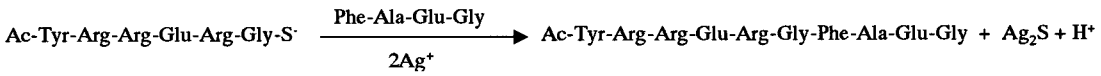
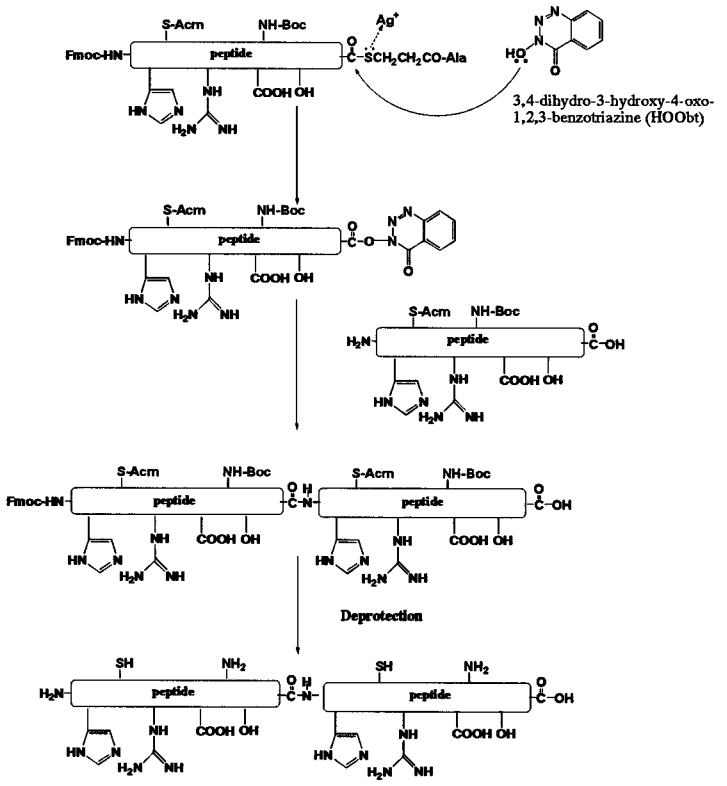


Fig.1 Segment condensation by the thiocaboxyl segment condensation strategy

However, three main problems remain for the method to be generally used. The first one is an unstableness of thiocarboxylic acid, which is easily decomposed by oxidation or hydrolysis. The second is the high nucleophilicity of the thiol moiety of the thiocarboxyl group. This high nucleophilicity causes side reactions when side-chain amino group protection is performed with a reagent such as *N*-(*t*-butoxycarbonyloxy)succinimide (Boc-OSu). The last is that no route is available for the preparation of cystein containing polypeptides using this thiocarboxyl segment condensation strategy.

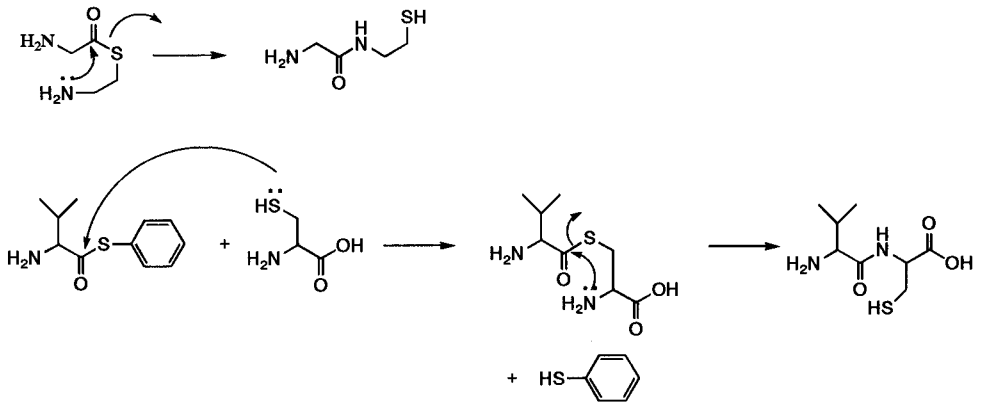
These problems were overcome by Hojo and Aimoto by an introduction of new ligation strategy called the thioester method [10]. In this thioester method, *S*-alkyl thioester of a partially protected segment is used as a building block. Although the interaction between the sulfur atom of the thioester and a silver ion is weaker than that of the sulfur atom in the thiocarboxyl group and a silver ion, the thioester is still selectively activated by a silver ion. Thus, no protection is required for the side-chain



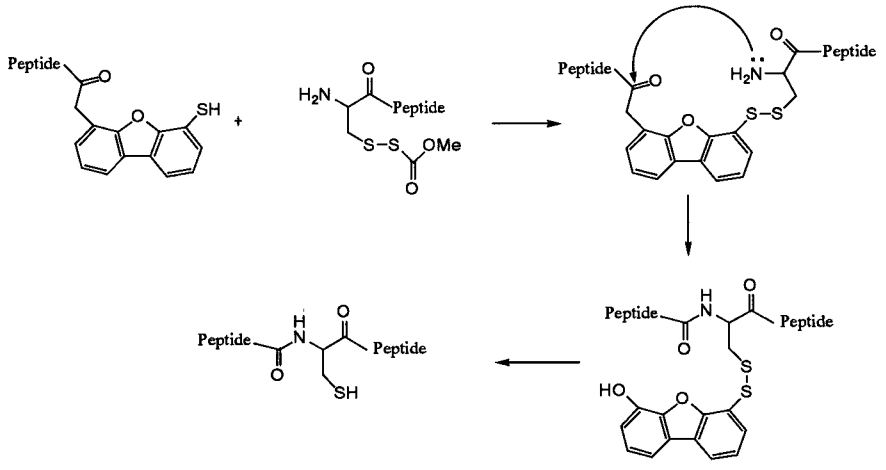
Scheme 1 Reaction mechanism of the thioester method

carboxyl group during segment condensation. Furthermore, the thioester is not as strongly nucleophilic as the thiocarboxyl group and, hence, various types of protecting groups can be introduced to the side-chain amino groups in the peptide thioester.

The second ligation method, involving a chemoselective capture and an intramolecular acyl rearrangement, is based on two findings reported by Wieland and Kemp. In 1953, Wieland *et.al.* [12] reported that *S*-glycylcysteamine underwent a spontaneous intramolecular S-N acyl rearrangement to form *N*-glycylcysteamine and furthermore, an intramolecular thioester exchange reaction easily took place between valine thiophenyl ester and cysteine to give N-valylcystein (**Scheme 2**). In 1981, Kemp *et.al.* developed a thiol capture method, in which a peptide bond is formed via a disulfide exchange reaction followed by an intramolecular O-N acyl rearrangement (**Scheme 3**)[13].

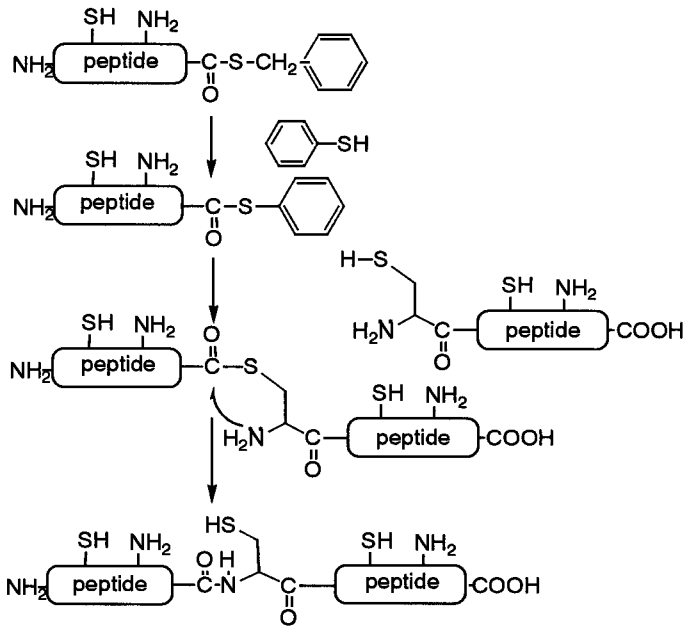


Scheme 2 Peptide bond formation reactions via a thioeaters exchange reaction, followed by an S-N acyl rearrangement



Scheme 3 Prior thiol capture ligation by Kemp

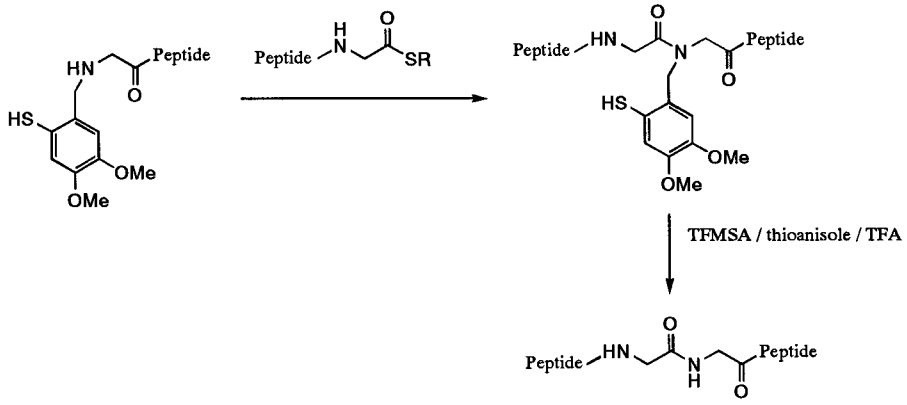
The concept of these discoveries by the two groups was applied to establish a peptide ligation method by two groups [14]. In the reaction, referred as native chemical ligation (NCL) by Kent, an intramolecular thioester exchange reaction initially occurs between a peptide C-terminal thioester and the N-terminal Cys peptide, followed by peptide bond formation reaction via an S-N acyl rearrangement to give the product (**Scheme 4**). No protecting group is required for the peptide segment in this reaction. The addition of thiophenol to the native chemical ligation reaction mixture generates a more reactive thiophenyl thioester through thiol exchange from the carboxymethyl thioester as well as from the benzyl thioester [15].



Scheme 4 Reaction mechanism of the native chemical ligation

As can be seen from the reaction mechanism, the amino acid sequence of the coupling site is restricted to Xaa-Cys, where the Cys is the N-terminal amino acid of the C-terminal building block and Xxx is an amino acid bearing an α-thioester at the C-terminal of the N-terminal building block. Thus, NCL does not allow the ligation of an unmodified polypeptide sequence that is devoid of suitably located cysteine residues. Recently, several groups have introduced auxiliaries for the ligation of peptides that permit the condensation to proceed without the need for a cysteine residue at the condesation site. Three scaffolds of removable auxiliaries have been reported to date:

the 2-mercaptoethoxy group [16], methoxy groups-attached 2-mercaptobenzyl group [17] and 1-phenyl-2-mercaptoethyl groups [18]. The concept of the ligation strategy in which an auxiliary is involved is summarized in **Scheme 5**. The development of the method is currently on going.



Scheme 5 4,5-Dimethoxy-2-mercaptobenzylamine involved in the condensation of a peptide with peptide thioester [17]

Concerning the theme

In this theme, the author describes the chemical synthesis of a membrane protein. While a number of water-soluble proteins have been synthesized using the ligation method, no reports on the synthesis of a membrane protein with multiple transmembrane regions using ligation method had been reported at the time this research was started. Details on the synthesis of membrane proteins remain to be uncovered. The research began with synthesis of a membrane protein with two transmembrane regions, F_1F_0 ATP synthase subunit c (Chapter I). The experiment was conducted as an initial trial to determine whether the synthesis of a membrane protein with multiple transmembrane regions was feasible. During the synthesis, conditions for the purification of a peptide with a transmembrane region were investigated. Based on the information obtained through the synthesis of F_1F_0 ATP synthase subunit c, further experiments were performed to establish a basis for the synthesis of a polytopic membrane protein. For this purpose, G protein coupled receptor (GPCR, **Fig. 2**) was chosen as the next object to be investigated. GPCRs constitute the largest single

family of signal transduction molecules. These receptors are found in virtually every eukaryotic cell type from a single cell microorganism such as yeast to human. GPCRs bind diverse signaling molecules including biogenic amines, sugars, peptides, and proteins that initiate numerous cellular processes including sexual conjugation, vision, pain perception, growth, blood pressure, and carbohydrate and fat metabolism. Indeed, these receptors are the target of >50% of the current therapeutic agents on the market [19]. These GPCRs would be difficult to obtain using genetic strategies. The experiments described here were not aimed at the total synthesis of GPCR itself, but investigations of chemical synthesis of the receptor would contribute to research of this field. The Opioid Receptor Like 1 [20] was selected as a target.

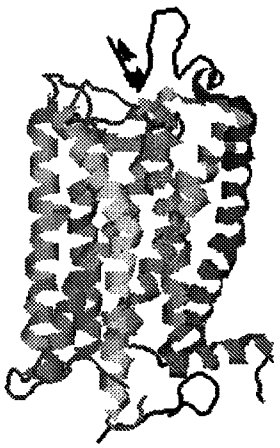


Fig. 2 Model structure of GPCR, bovine rhodopsin[21].

Prior to the synthesis, a preparative experiment was performed in which two ligation methods, the thioester method and NCL method were combined for the synthesis of a single polypeptide (Chapter II). These results led to development of a strategy for the synthesis of ORL1.

The first step in the synthesis of ORL1 began with an examination of the synthesis of ORL1(288-380). This portion of the protein contains the seventh transmembrane domain and the C-terminal intracellular domain. During the synthesis, a technique for the preparation of a building block containing the transmembrane region was introduced. The ligation reaction was also investigated in detail, to define optimum conditions for the synthesis of this portion of the protein.(Chapter III and IV).

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Chapter I

Synthesis of F_1F_0 ATP synthase subunit c

Introduction

The synthesis of a two transmembrane protein by the thioester method [1, 2] is described as an initial step in the synthesis of a multiple transmembrane protein. In addition to the synthesis, the RP-HPLC purification of the transmembrane peptide was examined. RP-HPLC is widely used for the purification of chemically synthesized peptides, but difficulties are frequently encountered during the RP-HPLC purification of membrane peptides due to their insolubility in the mobile phases, and irreversible adsorption to the column. Suitable purification conditions must be found for preparing membrane peptides in high purity. As a target, F_1F_0 ATP synthase subunit c (Sub.c, **Fig. 1**) was chosen. Sub.c is thought to consist of two membrane-spanning α-helices connected by a polar loop[3].

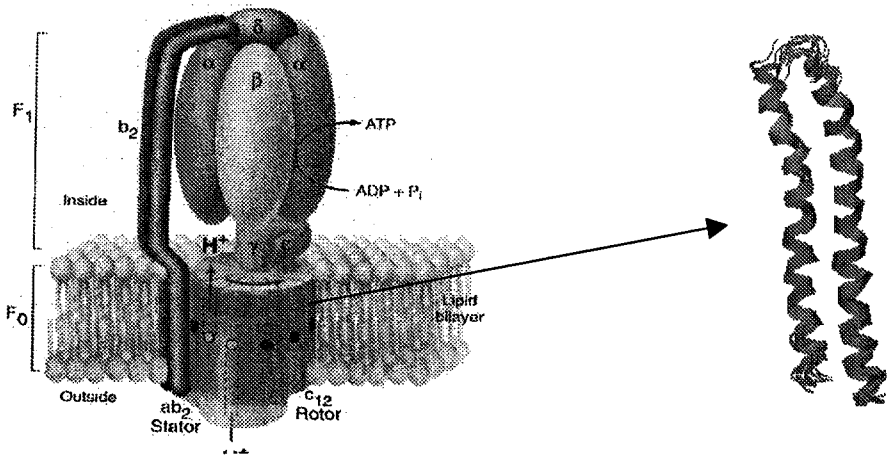


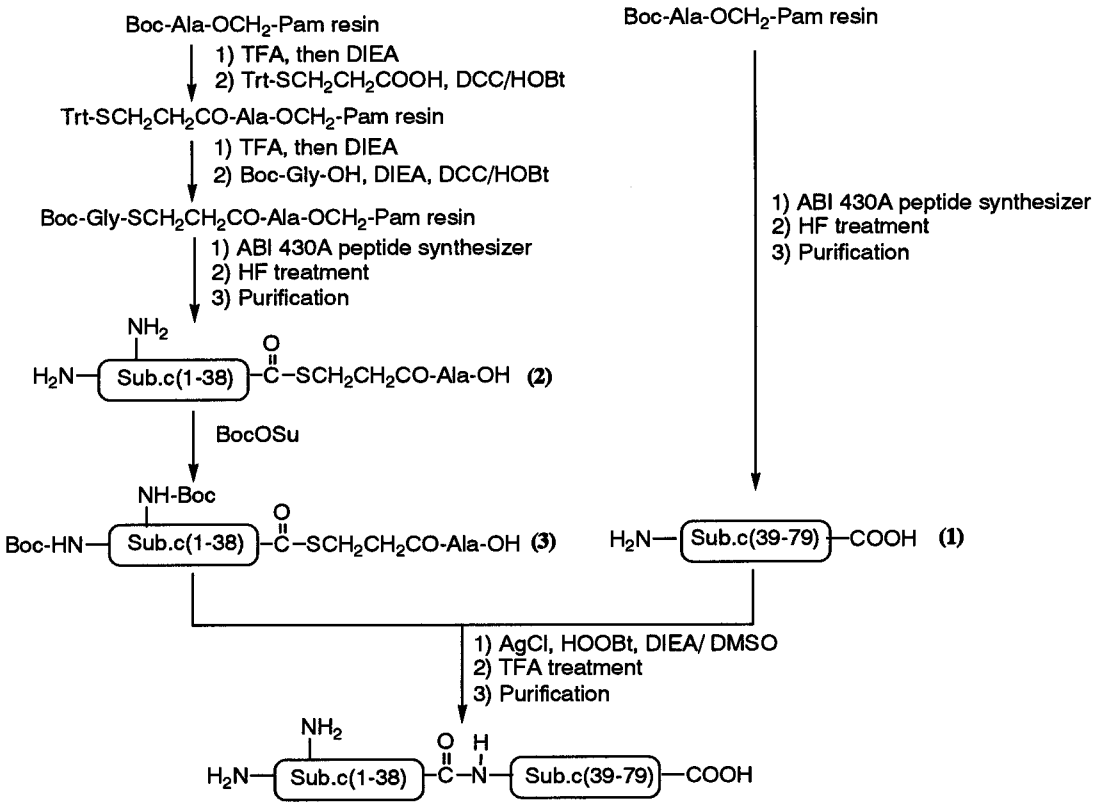
Fig. 1 Model structure of F_1F_0 ATP synthase (on the left) and subunit c (on the right) [3].

Results and discussion

The amino acid sequence of Sub.c is shown in **Fig.2**, and strategy for the synthesis is shown in **Scheme 1**. In order to make a length of the building block almost equal, the coupling site was chosen in the loop region (between the transmembrane domains). A glycine residue was chosen as the C-teminal amino acid residue of the N-terminal building block to avoid epimerization.

Met-Glu-Asn-Leu-Asn-Met-Asp-**Leu-Leu-Tyr-**[10]
Met-Ala-Ala-Ala-Val-Met-Met-Gly-Leu-Ala-[20]
Ala-Ile-Gly-Ala-Ala-Ile-Gly-Ile-Gly-Ile-[30]
Leu-Gly-Gly-Lys-Phe-Leu-Glu-Gly↓Ala-Ala-[40]
Arg-Gln-Pro-Asp-**Leu-Ile-Pro-Leu-Leu-Arg-**[50]
Thr-Gln-Phe-Phe-Ile-Val-Met-Gly-Leu-Val-[60]
Asp-Ala-Ile-Pro-Met-Ile-Ala-Val-Gly-Leu-[70]
Gly-Leu-Tyr-Val-Met-Phe-Ala-Val-Ala[79]-OH

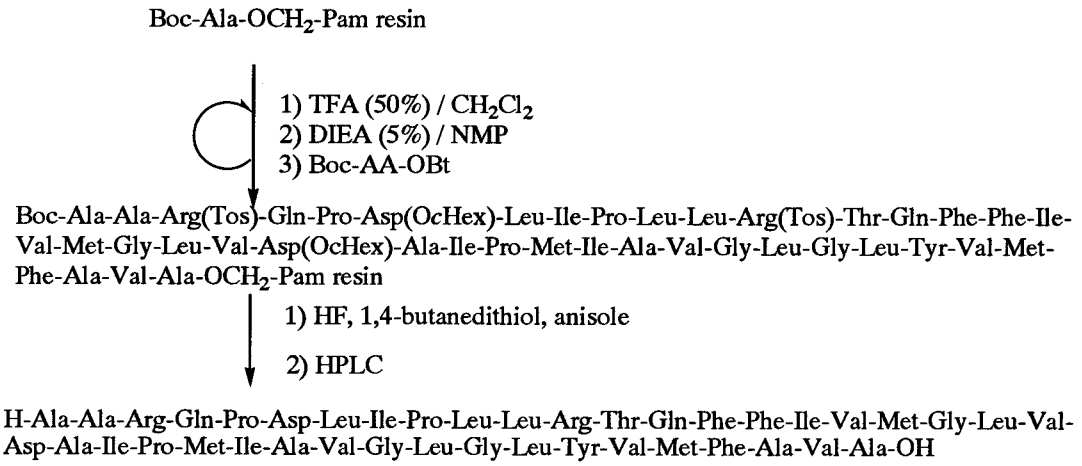
Fig.2 Amino acid sequence of F_1F_0 ATP synthase subunit c. Putative transmembrane regions are shown in bold and an arrow shows a coupling site.



Scheme 1 Strategy for the synthesis of Subunit c

Preparation of Sub.c(39-79) (1)

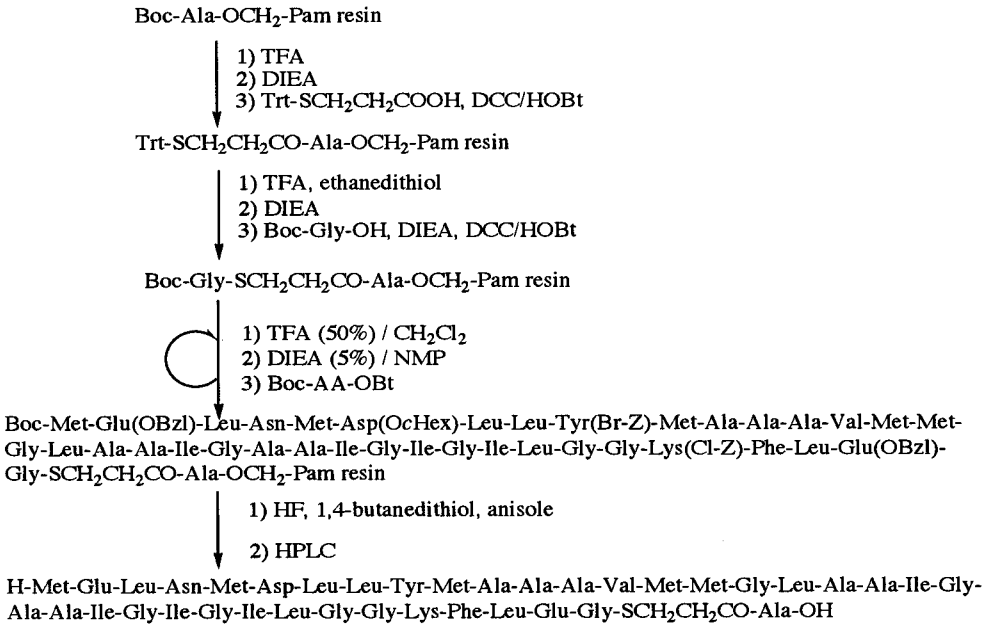
The preparation of peptide **1** is summarized in **Scheme 2**. Starting from Boc-Ala-OCH_2-Pam resin, peptide **1** was synthesized using automated peptide synthesizer in Boc chemistry. The peptide component was cleaved from the resin by treatment with HF [4, 5]. Purification was then performed by means of RP-HPLC. Details of the purification are discussed in a later section.



Scheme 2 Synthetic scheme of peptide **1**

Preparation of Sub.c(1-38)-SCH_2CH_2CO-Ala (2)

The preparation of peptide thioester **2** is summarized in **Scheme 3**. Starting from Boc-Ala-OCH_2-Pam resin, Boc-Gly-SCH_2CH_2CO-Ala-OCH_2-Pam resin was manually synthesized using the procedure reported by Kawakami et.al [6]. The rest of The elongation process was performed on an automated peptide synthesizer in Boc chemistry. The peptide component was cleaved from the resin by treatment with HF. The purification was then performed by means of RP-HPLC. Detaisl of the purification are discussed in a later section.



Scheme 3 Synthetic scheme of peptide **2**

Solubility check

Since RP-HPLC is one of the simplest analytical and preparative methods, it is frequently used in the purification of transmembrane peptides. However, a combination of an ODS column with an eluent of 0.1% TFA aqueous acetonitrile, the most common system for the purification of peptides, does not even elute such a peptide due to strong hydrophobic interactions between the peptide and column.

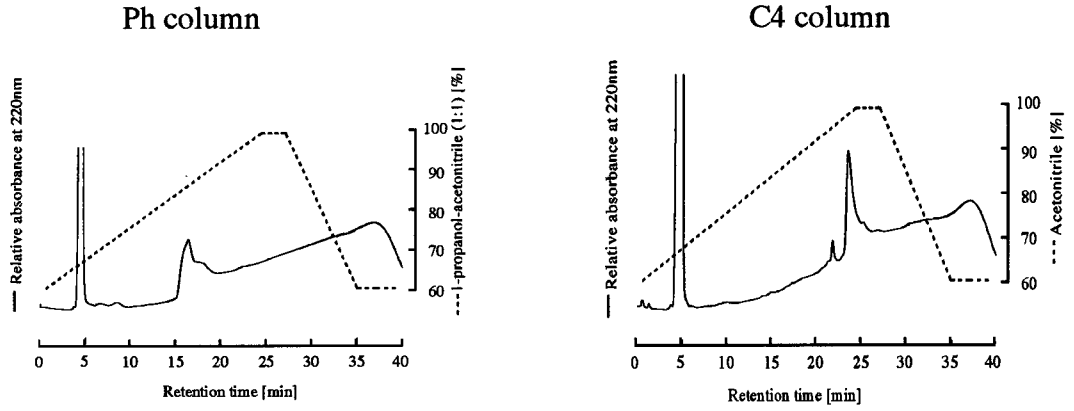


Fig. 3 RP-HPLC elution profile of peptide **1**. Column : Cosmosil 5Ph AR-300 (4.6 x 150 mm)(on the left), Cosmosil 5C4 AR-300 (4.6 x 150 mm)(on the right); linear gradient (25 min) from 0.1% TFA water to 0.1% acetonitrile

In a trial for the purification of peptide **1** using Ph or C4 columns, which is frequently employed for the separation of hydrophobic peptides, a peptide component could be successfully eluted(**Fig. 3**), but separation was not achieved. Therefore, in a search for an RP-HPLC purification system, especially an eluent system, for the transmembrane peptide, the first objective was to prepare a molecularly dispersed solution of the crude transmembrane peptide with no detergent being present. A number of alcohols, including 1-propanol, 2-propanol, 1-butanol, 2-butanol, 2-methyl-2-propanol, 1-pentanol, and 1-hexanol were examined. Each alcohol was mixed with acetonitrile and water (alcohol : acetonitrile : water = 2 : 2 : 1) and the crude powdered Sub.c(39-79) was added to the solution. The mixture was kept at room temperature and the amount of peptide component in the supernatant was determined by amino acid analysis. The amount of Gly in the supernatant of each mixture is shown in **Table 1**. Trifluoroethanol (TFE) had the highest solubility and 1-propanol was found to have a relatively higher ability to dissolve peptide **1**, in crude powder form.

Table 1 Solubilities of crude peptide **1** in the mixtures of acetonitorile, water, and alcohols (2:1:2). Solubilities were determined from the amount of Gly contained in supernatant.

Alcohol	Solubility [nmol / ml]
1-Propanol	98
2-Propanol	93
1-Butanol	93
2-Butanol	80
TFE	150

RP-HPLC conditions

Based on the data from a solubility check, a mixture containing 1-propanol was tested for use as a component of an RP-HPLC eluent system. TFE, showing the best ability to dissolve the crude powder of peptide **1**, was not effective for the separation. Since the use of formic acid in an eluent system is also known to be effective for the

purification of hydrophobic peptides [7, 8], we examined eluents containing formic acid. Ph and C4 columns were employed for the evaluation of those eluents. Superior resolution and recovery was obtained using the Ph column, and, therefore, the elution profiles shown in this paper are limited to that of a Ph column. Conditions were evaluated by a comparison of the yield of peptide **1**. The conditions and results are summarized in **Table 2**.

A relatively good resolution and recovery was obtained by employing a linear gradient of formic acid-water (2:3) and formic acid-1-propanol (4:1) as the eluent(**Fig. 4**). The desired peptide component had a retention time of 14 min (peak A) with a yield of 33%. For comparison, a linear gradient of

Table 2 The comparison of eluents. The numbers represent the yields based on Ala on resin. Column: Cosmosil 5Ph AR-300 (4.6x150 mm) and Cosmosil 5C4 AR 300 (4.6x150 mm). Sample: Crude powder of sub.c (39-79)

Eluent system		Column	
Solution A	Solution B	Phenyl	C4
Formic acid/ water (2:3)	Formic acid/1-propanol (4:1)	33.1%	27.8%
Formic acid/ water (2:3)	Formic acid/2-propanol (4:1)	30.7%	20.1%
Water containing 0.1%TFA	1-propanol/ acetonitrile containing 0.1%TFA	8.1%	7.0%
Water containing 0.1%TFA	Acetonitrile containing 0.1%TFA	——	5.2%

formic acid-water (2:3) and formic acid-2-propanol (4:1) was examined as an eluent system(**Fig. 5**), which was employed by Bollhagen *et.al.* for the purification of a transmembrane peptide[6]. No significant difference was observed in the resolution, but the yield (31%) was slightly lower than that of with 1-propanol and the retention time of the desired peptide was shifted behind compared to the system described above.

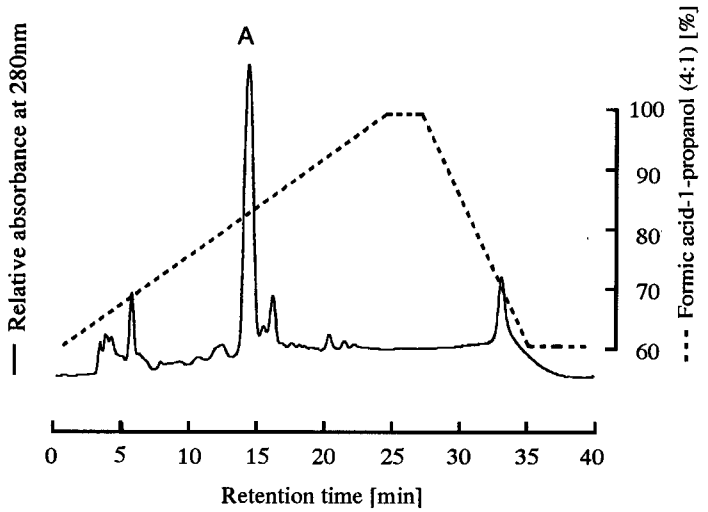


Fig. 4 RP-HPLC elution profile of peptide **2-1** in different eluent systems. Column : Cosmosil 5Ph AR-300 (4.6x150 mm); Eluent A : formic acid –water (2 : 3); Eluent B : formic acid –1-propanol(4 : 1); Flow rate : 0.65 ml / min.

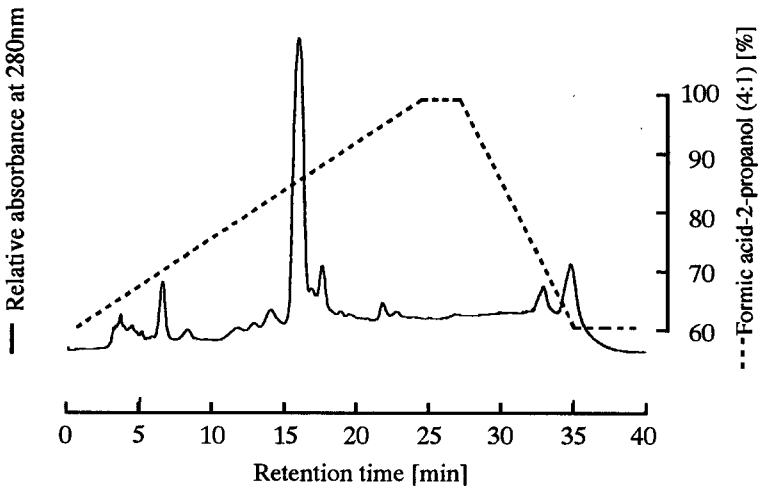


Fig. 5 RP-HPLC elution profile of peptide **2-1** in different eluent systems. Column : Cosmosil 5Ph AR-300 (4.6x150 mm); Eluent A : formic acid –water (2 : 3); Eluent B : formic acid –2-propanol(4 : 1); Flow rate : 0.65 ml / min.

A linear gradient of 0.1% TFA in water and 0.1% TFA in 1-propanol-acetonirile (1 : 1) also showed good separation (**Fig. 6**). Such a mixture would be applicable to the purification of hydrophobic peptides or peptides with protecting groups that are cleavable by acids.

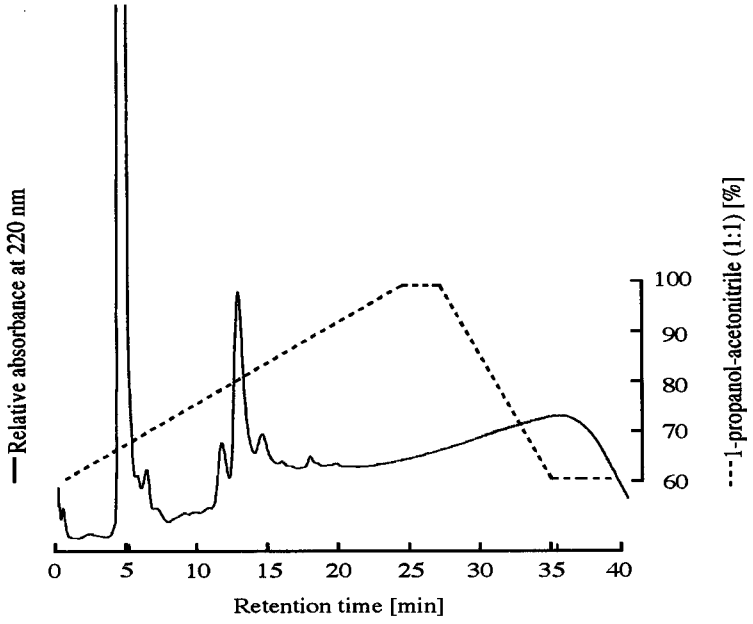


Fig. 6 RP-HPLC elution profile of peptide **1** in different eluent systems. Column : Cosmosil 5Ph AR-300 (4.6x150 mm); Eluent A : 0.1% TFA water; Eluent B : 0.1% TFA 1-propanol-acetonitrile (1 : 1); Flow rate : 0.5 ml / min.

Preparation of building blocks

Purification of the building blocks was performed by RP-HPLC on Cosmosil 5Ph AR-300 column (10 x 250 mm) with a linear gradient of formic acid-water (2:3) and formic acid–1-propanol (4:1). The semi-preparative RP-HPLC profile for peptide **1** is almost the same as that of an analytical sample (**Fig. 4**). For injection to the column, the crude powder was dissolved in TFA. The major peak at 14 min contained the desired peptide **1**, and the mass number and the amino acid composition was in good agreement with the calculated values. Peptide **1** was obtained in a yield of 15% based on the Ala residue content on the resin. The mass number of the content in the16 min peak was larger than that of peptide **1** by 98 Da, suggesting that it had been trifluoroacetylated. This peak became higher as the time of the crude peptide to be

dissolved in TFA increased. This side reaction could be avoided by making the time for dissolving the peptide in TFA shorter. **Fig. 7** shows the RP-HPLC profile of the peptide, peptide thioester **2**. The peak at 19 min contained peptide **2**, and the mass number and amino acid composition were in good agreement with the calculated values. The yield was 11% based on the Gly residue on the resin.

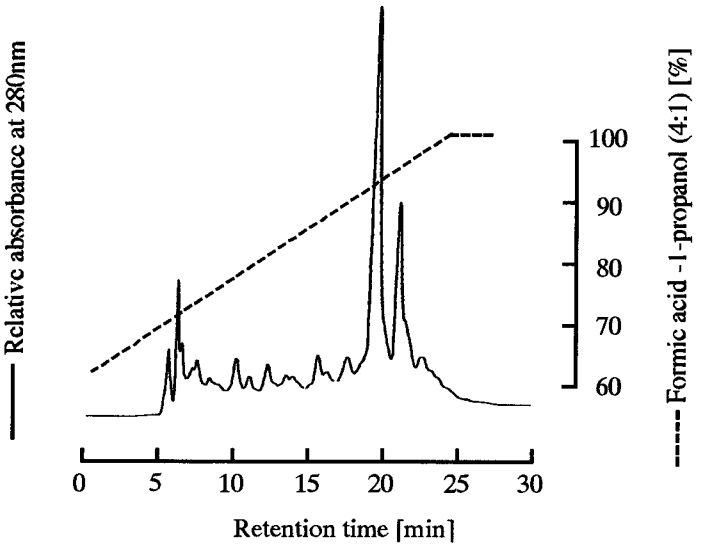


Fig.7 RP-HPLC elution profile of peptide **2** in different eluent systems. Column : Cosmosil 5Ph AR-300 (4.6x150 mm); Eluent A : formic acid –water (2 : 3); Eluent B : formic acid –2-propanol(4 : 1); Flow rate : 0.65 ml / min.

Peptide **2** was treated with BocOSu in DMSO to give the partially protected peptide, Boc-[Lys(Boc)^{34}]-sub.c(1-38)-SCH_2CH_2CO-Ala (**3**). To monitor the reaction, RP-HPLC, using 1-propanol and acetonitrile as the eluent was employed instead of the system using formic acid, since the Boc group is removed under acidic conditions.

Condensation by the thioester method

The condensation of peptides **1** and **3** was carried out in the presence of silver chloride, HOOBt, and DIEA in DMSO. The reaction mixture was stirred for 48 h at room temperature, and then treated with TFA to remove Boc groups. The RP-HPLC profile of the TFA-treated reaction mixture is shown in **Fig. 8**. Three main sharp peaks at (**A**) 16 min, (**B**) 21 min, and (**C**) 23 min were collected and checked by mass analysis.

The mass number of the material corresponding to peak **C** was in good agreement with the calculated value of the desired product (MS found for *m/z* : 8256.8; calcd for $[M+H]^+$:8257). The amino acid composition was also in good agreement with the calculated value ($Asp_{4.9}Thr_{0.8}Glu_{3.9}Pro_{2.4}Gly_{10.2}Ala_{13}Val_{5.1}Met_{7.2}Ile_{7.5}Leu_{12.1}Tyr_{1.4}Phe_{4.0}Arg_{1.7}$).

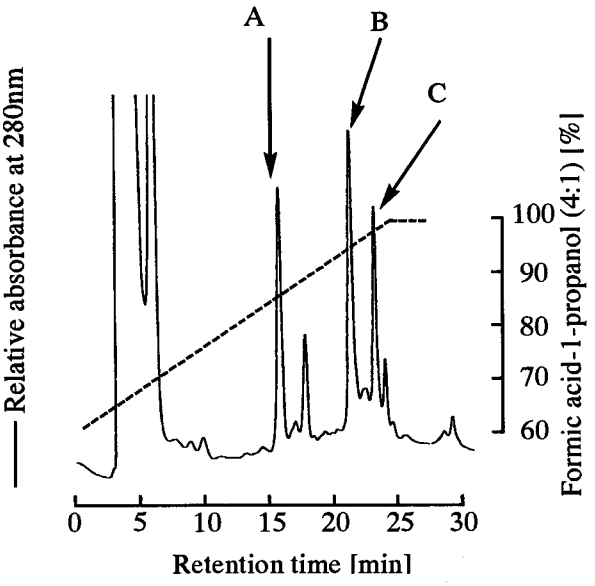


Fig. 8 RP-HPLC profile of the TFA treated reaction mixture. Condensation by the thioester method was carried out in DMSO at room temperature for 48 h. Column, Cosmosil 5Ph AR300, 4.6x150mm; liner gradient (25 min) from formic acid-water (2:3) to formic acid -1-propanol (4:1). Peaks A, B, and C were found to be the C-terminal building block, the thioester hydrolyzed N-terminal building block, and the desired product, respectively.

The yield of the condensation was 16% based on the C-terminal building block. The condensation was also performed at 50 °C and the yield was improved to 23%.

A mixture of chloroform and methanol (2 : 1), which as typically used for the sample preparation of membrane proteins, was also examined as a solvent for the condensation. The RP-HPLC profile of the TFA-treated reaction mixture is shown in **Fig. 9**. The mass number and the amino acid composition of the content of peak **D** was in good agreement with the calculated values of the desired product (MS found for *m/z* : 8257.3). The yield was found to be 26%, based on the C-terminal building

block. The content of peak **E** had a mass number by 14 units in excess of what would have been expected for the thioester hydrolyzed N-terminal building block.

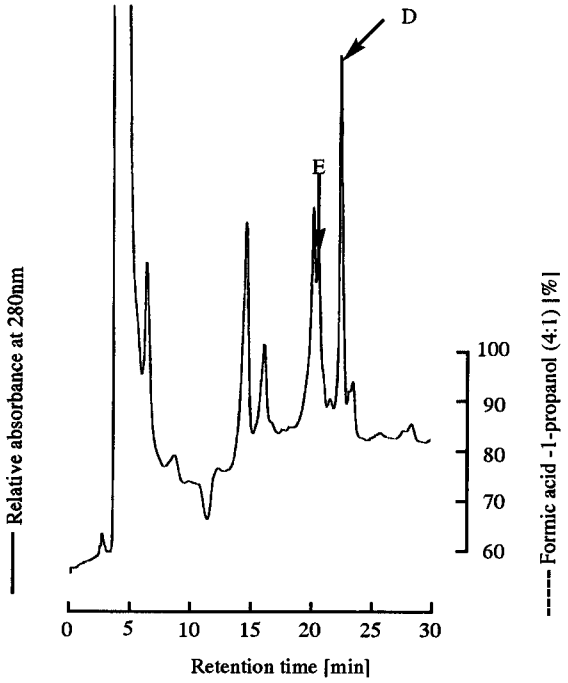


Fig. 9 RP-HPLC profile of the TFA treated reaction mixture. Condensation by the thioester method was carried out in chloroform-methanol(2:1) for 48 h at rt. Column, Cosmosil 5Ph AR300, 4.6x150mm; liner gradient (25 min) from formic acid-water (2:3) to formic acid -1-propanol (4:1). The content of peak D was found to be the desired product. The content of peak E had a larger mass number by 14 units than the expected thioester hydrolyzed N-terminal building block.

This side reaction can be explained by a formation of a methyl ester from to the methanol in the solvent. Considering the fact that the thioester is a type of active ester and methanol, a primary alcohol, has a high reactivity for the esterification of a carboxyl acid, this side reaction appears to be unavoidable in this solvent system. Therefore, 2-propanol, instead of methanol, was examined as a component of the solvent mixture. The RP-HPLC profile of the TFA-treated reaction mixture is shown in **Fig. 10**. The mass number and the amino acid composition of the content of peak

H was in good agreement with the calculated values of the desired product. Peaks **F** and **G** were characterized as peptide **1** and peptide building block **3** respectively. Esterification could be avoided when this secondary alcohol was used, but the yield of the condensation reaction decreased to14%. Other secondary or tertiary alcohols were not tested, the data from the solubility experiment indicated that the ability of these alcohols to dissolve the transmembrane peptide was low. In the view of their high solubility, HFIP [9] and phenol [10] were also examined. Using HFIP, however, the condensation reaction, for unknown reasons, failed to proceed, and the reactants did not dissolve in phenol.

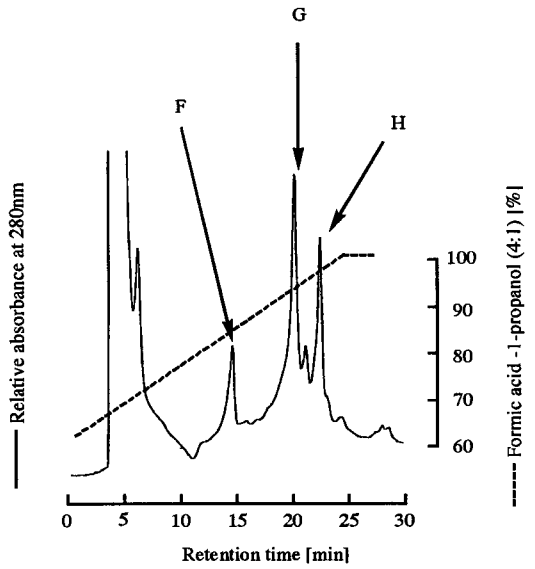


Fig. 10 RP-HPLC profile of a TFA treated reaction mixture . Condensation by the thioester method was carried out in chloroform-2-propanol(2:1) for 48 h at rt. Column, Cosmosil 5Ph AR300, 4.6x150mm; liner gradient (25 min) from formic acid-water (2:3) to formic acid -1-propanol (4:1). The esterification mentioned in the text could be avoided in this system.

Conclusion

The main goal of this experiment was to synthesize a membrane protein, with two transmembrane regions, by means of the thioester method. As a result, Sub.c was successfully synthesized. The effective conditions for the separation of a transmembrane peptide by RP-HPLC were also found. Since a number of membrane proteins are thought to contain multiple transmembrane regions, the results described here should be of general use in research on membrane proteins.

Material and method

General

Boc-Ala-OCH_2-Pam resin was purchased from Applied Biosystems, Inc. (Foster City, CA). Amino acid derivatives were purchased from the Peptide Institute Inc. (Osaka, Japan). Peptide chain elongation was carried out by using a peptide synthesizer 430 A (Applied Biosystems, Inc.) according to the protocol of the system software version 1.40 NMP/ *t*-Boc on a 0.5 mmol scale with end capping by acetic anhydride. The first ten amino acids were coupled in single coupling protocol and the remainder in a double coupling protocol. Ether, used for the precipitation and washing of peptides, was peroxide-free (Nacalai Tesque, Kyoto, Japan). DMSO, used for the condensation, was silylation grade (Pierce, Rockford, IL). The amino acid compositions of peptides were analyzed using an L-8500 amino acid analyzer (Hitachi Ltd., Tokyo, Japan) after hydrolysis with constant boiling point HCl for amino acid analysis (Nacalai Tesque) at 110°C for 48 h in an evacuated sealed tube. The peptide mass number was determined by MALDI-TOF mass spectrometry using a Voyager™DE (PerSeptive Biosystems, Inc., Framingham, MA). The matrix used was sinapinic acid and peptides were dissolved in a mixture of formic acid and trifluoroethanol.

Synthesis of Sub.c(39-79) (1)

Starting from the Boc-Ala-OCH_2-Pam resin (0.77 mmol/g, 0.65 g), a protected peptide resin corresponding to the sequence of Sub.c(39-79), Boc-Ala-Ala-Arg(Tos)-Gln-Pro-Asp(O*c*Hex)-Leu-Ile-Pro-Leu-Leu-Arg(Tos)-Thr-Gln-Phe-Phe-Ile-Val-Met-Gly-Leu-Val-Asp(O*c*Hex)-Ala-Ile-Pro-Met-Ile-Ala-Val-Gly-Leu-Gly-Leu-Tyr-Val-Met-Phe-Ala-Val-Ala-OCH_2-Pam resin was obtained (2.9 g). The protected peptide resin (300 mg) was treated with a mixture of anhydrous HF (8.5 ml), anisole (0.75 ml), and 1,4-butanedithiol (0.75 ml) by stirring at 0 °C for 90 min [**4, 5**]. After evaporation of the HF under reduced pressure, ether was added to the mixture, and the resulting precipitate was washed with ether three times and then dissolved in TFA. The solution was passed through a glass filter and precipitated by the addition of cold ether. The precipitate was washed with ether, and mixed with 50% aqueous acetonitrile and freeze-dried to give the crude powder (189 mg).

Synthesis Sub.c(1-38)-SCH_2CH_2CO-Ala (2)

Boc-Ala-OCH_2-Pam resin (0.77 mmol / g, 1.2 g) was treated with 50% TFA / CH_2Cl_2 solution (1 x 5 min and 1 x 20 min), washed with CH_2Cl_2 (3 x 1 min). Following a wash with NMP for 0.5 min, the resin was treated with 5% DIEA in NMP (3 x 1min), and then washed with NMP (3 x 1 min). Trt-$SCH_2CH_2CO_2H$ (1.3 g, 3.6 mmol), HOBt-H_2O (0.54 g, 3.6 mmol), and DCC (0.72 g, 3.6 mmol) in NMP (10 ml) was mixed for 30 min and the resulting solution was added to the resin. The suspension was shaken for 1 h, followed by a ninhydrin assay to measure the residual free amine content on the resin, and washed with NMP (3 x 1 min). The resin was treated with a NMP solution containing 10% acetic anhydride and 5% DIEA (1 x 10 min) and then washed with NMP (5 x 1 min). The resulting resin was treated with TFA containing 5% ethanedithiol (1 x 5 min, 1 x 10 min, 1 x 20 min, and 1 x 30 min) and then washed with CH_2CH_2 (3 x 1 min). Following a wash with NMP for 0.5 min, the resin was treated with 5% DIEA in NMP (3 x 1min), and then washed with NMP (3 x 1 min). A solution of Boc-Gly (0.69 g, 3.6 mmol), HOBt-H_2O (0.54 g, 3.6 mmol), and DCC (0.72 g, 3.6 mmol) in NMP (10 ml) was stirred for 1 h. This solution was added to the resin and mixed for 14 h, followed by a ninhydrin assay to measure the residual free amine content on the resin, and washed with NMP (3 x 1 min). The resin was treated with a NMP solution containing 10% acetic anhydride and 5% DIEA (1 x 10 min), washed with NMP (5 x 1 min) and then with methanol (5 x 1 min), and dried in reduced pressure to give Boc-Gly-SCH_2CH_2CO-Ala-OCH_2-Pam resin (0.71 g, 0.50 mmol). Starting with the Boc-Gly-SCH_2CH_2CO-Ala-OCH_2-Pam resin (0.71 g, 0.50 mmol). 1.7 g of a protected peptide resin corresponding to the sequence of Sub.c(1-38), Boc-Met-Glu(OBzl)-Leu-Asn-Met-Asp(O*c*Hex)-Leu-Leu-Tyr(Br-Z)-Met-Ala-Ala-Ala-Val-Met-Met-Gly-Leu-Ala-Ala-Ile-Gly-Ala-Ala-Ile-Gly-Ile-Gly-Ile-Leu-Gly-Gly-Lys(Cl-Z)-Phe-Leu-Glu(OBzl)-Gly-

SCH_2CH_2CO-Ala-OCH_2-Pam resin was obtained. This peptide resin (300 mg) was treated with mixture of anhydrous HF (8.5 ml), anisole (0.75 ml), and 1.4-butanedithiol (0.75 ml) with stirring at 0 °C for 90 min. After evaporation of the HF under reduced pressure, ether was added to the mixture, the resulting precipitate was washed with ether three times and then dissolved in TFA. The solution was passed through a glass filter and the material precipitated by the addition of cold ether. After the precipitate had been washed with ether, it was mixed with 50% of aqueous acetonitrile and freeze-

dried to give the crude powder (158 mg).

Solubility check of the crude peptide

Several alcohols, acetonitrile, and water were mixed in a ratio of 4:4:2. The crude powder of peptide **1** was added to the solvent (200 μl) at room temperature and the suspension allowed to stand for 2 days. The supernatant (50 μl) was removed, evaporated under reduced pressure, suspended in a mixture of acetonitrile and water, and freeze-dried. The amount of peptide was determined by amino acid analysis.

Evaluating RP-HPLC condition

Analytical RP-HPLC was performed on Cosmosil columns (4.6x150 mm, 5C4AR-300, 5TMS, 5Ph, and 5PhAR-300, Nacalai Tesque). 1-Propanol for RP-HPLC was HPLC grade (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and formic acid was chromatography grade (Nacalai Tesque). The crude powder of peptide **1** (2.0 mg) was dissolved in TFA (50 μl), and the solution (5 μl) injected into the column. Peaks were collected and freeze-dried prior to amino acid analysis. Each condition was evaluated by the isolated yield as determined by amino acid analysis.

Purification of peptide 1 and peptide thioester 2

Purification of these segments was performed on a Cosmosil 5PhAR-300 column (10 x 250 mm, Nacalai Tesque), and a linear gradient of formic acid / water (2:3) and formic acid / 1-propanol (4:1) at a flow rate of 3.0 ml/min was employed.

For peptide **1**, 12 mg of crude powder was applied to the column to give purified peptide **1** (5.7 mg, 4.7μmol, 15% yield based on the Ala content of the starting resin): MS (MALDI TOF) found: *m/z* 4433.4. Calcd for $[M+H]^+$ 4433.4 (average). Amino acid analysis: $Asp_{2.1}Thr_{1.0}Glu_{2.2}Pro_{3.4}Gly_{3.4}Ala_6Val_{5.0}Met_{2.9}Ile_{4.5}Leu_{6.7}$ $Tyr_{0.8}$ $Phe_{2.9}Arg_{2.1}$.

For peptide **2**, 6 mg of crude powder was loaded on the column to give purified peptide **2** (1.7 mg, 0.36 μmol, 11% yield based on Gly on the resin): MS (MALDI TOF) found: *m/z* 4001.3. Calcd for $[M+H]^+$ 4001.9 (average). Amino acid analysis: $Asp_{2.8}Glu_{1.9}Gly_{7.0}Ala_8Val_{1.0}Met_{4.6}Ile_{4.1}Leu_{6.1}Phe_{1.0}Lys_{1.0}$.

Preparation of Boc-[Lys(Boc)^{34}]-Sub.c(1-38)-SCH_2CH_2CO-Ala (3)

Peptide **2-2** (8.0 mg, 1.7 μmol) and BocOSu (4.3 mg, 20 μmol) were dissolved in DMSO. DIEA was added to the solution followed by stirring for 1 h, and the reaction was monitored by RP-HPLC [Cosomosil 5PhAR300 (4.6 x 150 mm, Nacalai Tesque), using a linear increasing gradient of 1-propanol–acetonitrile (1:1) in 0.1% aqueous TFA at flow rate of 0.5 ml / min]. The DMSO was removed by evaporation under reduced pressure, and the reaction mixture was washed with ether. The precipitate was suspended in aqueous acetonitrile and freeze-dried to give partially protected peptide **3**, Boc-[Lys^{34}(Boc)]-sub.c(1-38)-SCH_2CH_2CO-Ala-OH (7.2 mg, 1.7 μmol).

Condensation by the thioester method

Peptide **1** (2.3 mg, 0.52 μmol), peptide **3** (2.2 mg, 0.52 μmol), HOOBt (2.6 mg, 16 μmol) was dissolved in DMSO (400 μl). DIEA (1.8μl, 10 μmol) and AgCl (0.3 mg, 2.1 μmol) were then added to the solution followed by stirring for 48 h. DMSO was removed under reduced pressure, and residue was suspended in aqueous acetonitrile and freeze-dried. The reaction mixture was dissolved in TFA containing 5 % water, stirred for 90 min, and then purified by RP-HPLC [Cosmosil 5PhAR-300 (4.6 x 150 mm, Nacalai Tesque), using a linear gradient of formic acid / 1-propanol (4:1) over formic acid / water (2:3) at a flow rate of 0.65 ml/min] to give Sub.c (85 nmol) and the isolated yield based on the C terminal building block was determined to be 16%: MS (MALDI TOF) found: *m/z* 8256.1. Calcd for $[M+H]^+$ 8257.1 (average). Amino acid analysis: $Asp_{4.9}Thr_{0.8}Glu_{3.9}Pro_{2.4}Gly_{10.2}Ala_{13}Val_{5.1}Met_{7.2}Ile_{7.5}Leu_{12.1}Tyr_{1.4}Phe_{4.0}$ $Arg_{1.7}$.

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Chapter II

Use of thiosulfonate for the protection of thiol groups in peptide ligation by the thioester method

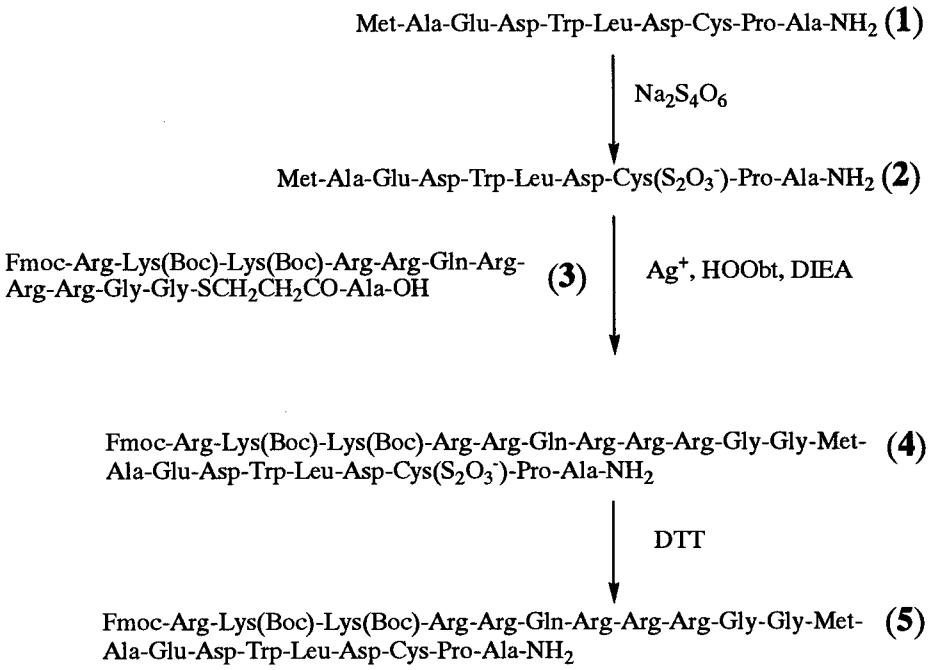
Introduction

The chemical synthesis of a large protein frequently requires multiple ligation steps. Moreover, in many cases, different ligation methodologies must be employed in the synthesis of a single protein. In this chapter, the author describes a technique that enables a combination of two established ligation methodologies, the thioester method[1] and the native chemical ligation method[2,3], for a synthesis of a single polypeptide.

The thioester method is an established methodology that is used in the synthesis of various types of polypeptides[4]. The use of the thioester method requires a partially protected peptide thioester and a peptide in which side chain amino groups and -SH groups are protected with a t-butyloxycarbonyl (Boc) group and acetamidomethyl (Acm) group[5], respectively. While the introduction of Boc groups is a simple technique, using a reagent such as *N-t*-butyloxycarbonyloxysuccinimide, it is difficult to introduce Acm groups or methylbenzyl groups to free -SH groups on a peptide. In the preparation of a building block for the thioester method, Cys is introduced into the peptide using Cys(Acm) at the peptide chain elongation reaction on a resin. There is, however, no established method for masking a free -SH group on a peptide. The application of the native chemical ligation method to the preparation of a building block for the thioester method is problematic. When a polypeptide is synthesized using the native chemical ligation method, the product contains at least one free –SH group of Cys due to the reaction mechanism involved. To overcome this problem, thiosulfonate ($S_2O_3^-$) was chosen as an –SH protecting group. Thiosulfonate was utilized to chemically modify a protein [6] and introduced to the quantitative recovery of cysteine in the amino acid analysis of proteins [7]. This thiosulfonate can be introduced to a free –SH on a peptide by simply mixing the peptide and sodium tetrathionate ($Na_2S_4O_6$) in a solvent, which can then be removed by treatment with dithiothreitol (DTT). In this chapter, the stability of thiosulfonate, as a –SH protecting group on a building block during a condensation reaction, in the presence of silver chloride, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine(HOObt), and diisopropylethylamine(DIEA) is described, in an attempt to devise a strategy for the synthesis of a single polypeptide by a combination of the native chemical ligation method and the thioester method.

Results and discussion

The experimental protocol is summarized in **Scheme 1.**



Scheme 1 Scheme for the experiment

For this purpose, a cysteine containing peptide, representing a peptide synthesized by means of the native chemical ligation method, in which the amino acid sequence was Met-Ala-Glu-Asp-Trp-Leu-Asp-Cys-Pro-Ala-NH_2(**1**) was prepared. Thiosulfonate was introduced to the free –SH group on peptide **1** by mixing the peptide and sodium tetrathionate in DMSO in the presence of DIEA. **Fig. 1** shows the RP-HPLC elution profile of the reaction mixture (120 min after the addition of sodium tetrathionate). The peak corresponding to the desired peptide component, Met-Ala-Glu-Asp-Trp-Leu-Asp-Cys($S_2O_3^-$)-Pro-Ala-NH_2 (**2**) appeared at 20 min (shown by an arrow). The content of the 25 min peak was determined to be a dimer of peptide**1**. Since dimerization occured in this system, the maximum isolated yield was 44%.

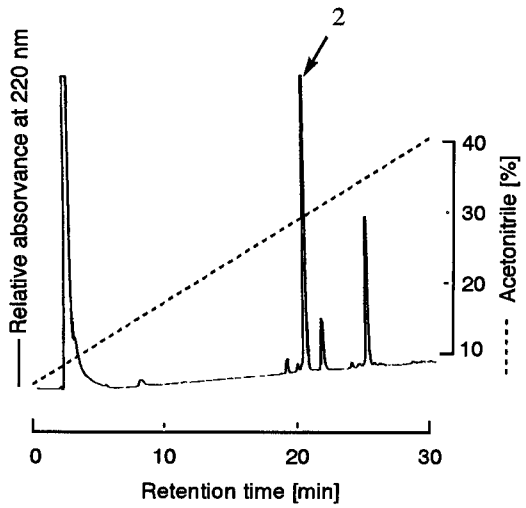


Fig. 1 RP-HPLC elution profile of a reaction mixture in which thiosulfonate is introduced to peptide **1**. Column: Cosmosil 5C18 AR-300 (4.6x150 mm), eluent: 0.1% TFA aq.acetonitrile, 1.0 ml / min.

For the condensation reaction with peptide building block, a peptide thioester, Fmoc-Arg-Lys(Boc)-Lys(Boc)-Arg-Arg-Gln-Arg-Arg-Arg-Gly-Gly-SCH_2CH_2CO-Ala-OH (**3**) was prepared as an N-terminal building block using a procedure originally reported by Kawakami et.al.[8] The coupling reaction of building blocks **2** and **3** by the thioester method was examined. **Fig. 2** shows the RP-HPLC elution profiles of the reaction mixtures at the reaction times of 0 min and 120 min. The retention of the desired product, Fmoc-Arg-Lys(Boc)-Lys(Boc)-Arg-Arg-Gln-Arg-Arg-Arg-Gly-Gly-Met-Ala-Glu-Asp-Trp-Leu-Asp-Cys($S_2O_3^-$)-Pro-Ala-NH_2 (**4**), was 35 min and the coupling yield, based on building block **2**, was 60%. As shown in the elution profile, the coupling reaction proceeded without any significant side reactions, indicating that thiosulfonate is stable under the conditions used in the thioester method. The removal of the N-terminal Fmoc from peptide **4** for further synthesis was also examined. The deprotection was successfully performed to give the product (**5**) by treatment with 3% piperidine / DMSO (v /v) for 30 min (**Fig. 1**).

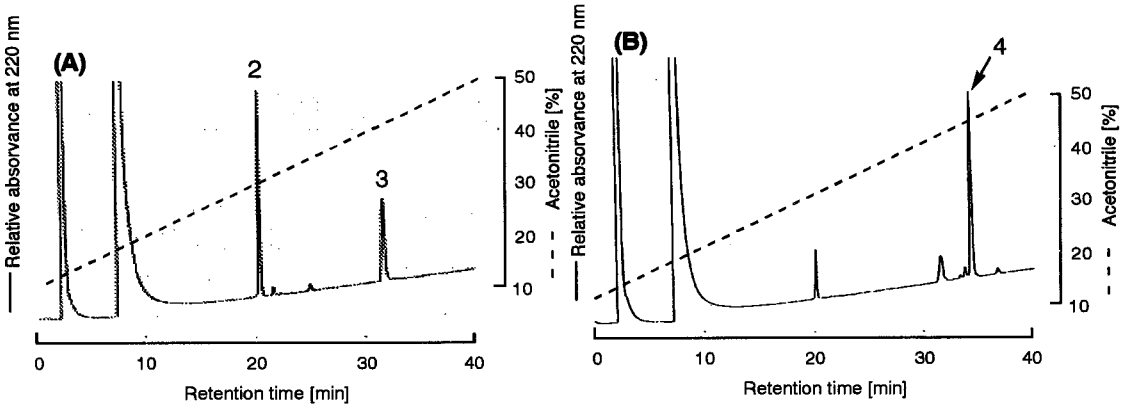


Fig. 2 RP-HPLC elution profiles of the coupling reaction mixtures. (A) Elution profile of the starting materials (B) Elution profile of the coupling mixture at a reaction time of 120 min. The desired product is indicated by an arrow. Column: Cosmosil 5C18 AR-300 (4.6x150 mm), eluent: 0.1% TFA aq.acetonitrile, 1.0 ml / min.

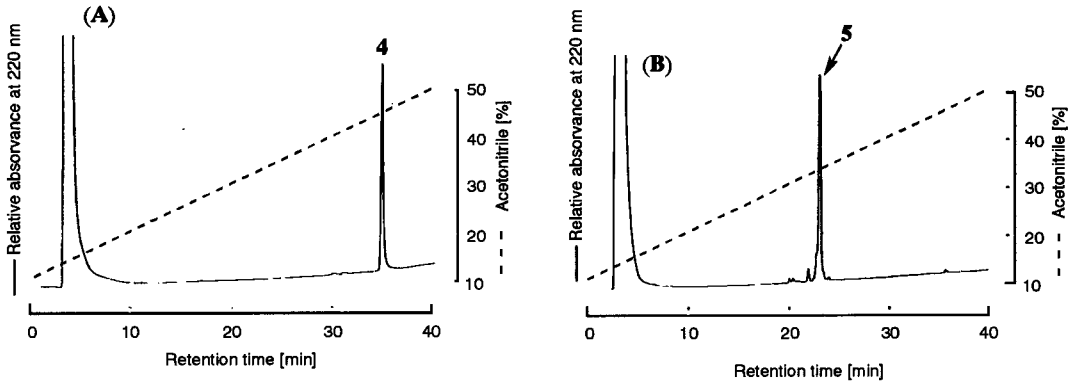
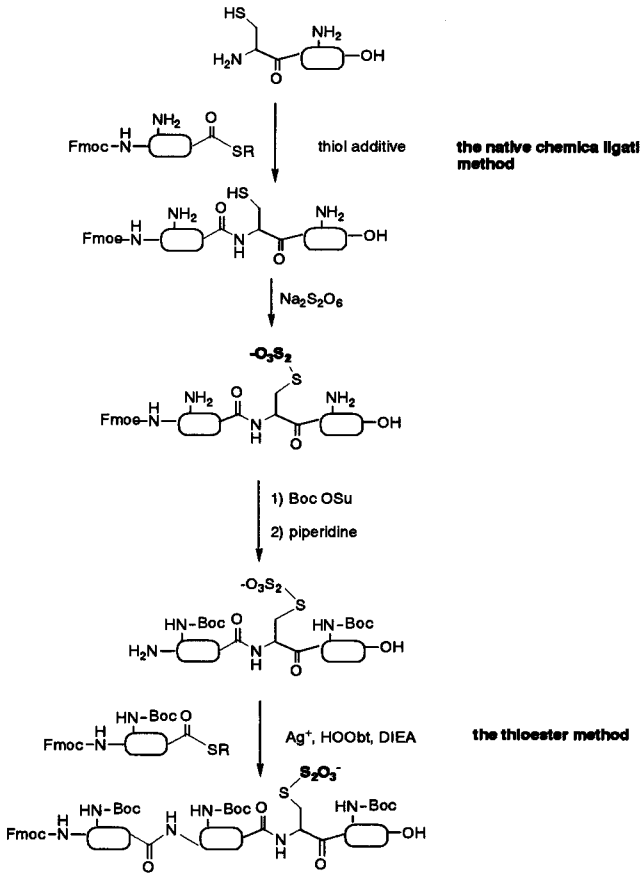


Fig. 3 RP-HPLC elution profiles of the removal of Fmoc from peptide **4**. (A) Elution profile of the starting material (**4**). (B) Elution profile of the reaction mixture at a reaction time of 30 min. The desired product is indicated by an arrow. Column: Cosmosil 5C18 AR-300 (4.6x150 mm), eluent: 0.1% TFA aq.acetonitrile, 1.0 ml / min.

Conclusion

As a result of this experiment, it appears likely that thiosulfonate can reliably be used as a protecting group for an -SH group in peptide ligation using the thioester method. Furthermore, a strategy for the synthesis of a single polypeptide by a combination of the native chemical ligation method and the thioester method is proposed in **Scheme 2**. The combination of these two techniques eliminates the restriction associated with amino acid sequence specificity at the site of peptide ligation in the synthesis of the larger polypeptide.



Scheme 2 Strategy for the synthesis of a single polypeptide by a combination of the native chemical ligation method and the thioester method.

Material and method

General

Boc-Ala-OCH_2-Pam resin was purchased from Applied Biosystems, Inc. (Foster City, CA). Amino acid derivatives were purchased from the Peptide Institute Inc. (Osaka, Japan). Peptide chain elongation for the synthesis of peptide thioester **1** was carried out by using a peptide synthesizer 433 A (Applied Biosystems, Inc.) according to the protocol of *FastMoc* program on a 0.25 mmol scale with end capping by acetic anhydride. Peptide chain elongation for the synthesis of peptide thioester **3** was carried out by using a peptide synthesizer 433 A (Applied Biosystems, Inc.) according to the *t*-Boc HOBt / DCC protocol on a 0.5 mmol scale with end capping by acetic anhydride. Ether, used for the precipitation and washing of peptides, was peroxide-free (Nacalai Tesque, Kyoto, Japan). DMSO, used in the condensation reaction, was silylation grade (Pierce, Rockford, IL). The amino acid compositions of peptides were analyzed using an L-8500 amino acid analyzer (Hitachi Ltd., Tokyo, Japan) after hydrolysis with constant boiling point HCl for amino acid analysis (Nacalai Tesque) at 110°C for 24 h in an evacuated sealed tube. The peptide mass number was determined by MALDI-TOF mass spectrometry using a Voyager^{TM}DE (PerSeptive Biosystems, Inc., Framingham, MA).

Synthesis of Met-Ala-Glu-Asp-Trp-Leu-Asp-Cys($S_2O_3^-$)-Pro-Ala-NH_2 (2)

Peptide **1** (1.4 μmol) and $Na_2S_4O_6$ were mixed in 3% DIEA in DMSO for 120 min at room temperature to give the desired product peptide **2** in a yield of 44%. Peptide **2**: MS (MALDI-TOF) found 1260.0, calcd 1261.3 (M+H)$^+$.

Synthesis of Fmoc-Arg-Lys(Boc)-Lys(Boc)-Arg-Arg-Gln-Arg-Arg-Arg-Gly-Gly-Met-Ala-Glu-Asp-Trp-Leu-Asp-Cys($S_2O_3^-$)-Pro-Ala-NH_2 (4)

Peptide **2** (0.51 μmol), Peptide **3** (0.56 μmol), AgCl (1.7 μmol), HOObt (16.8 μmol), and DIEA (11 μmol) was mixed and stirred in DMSO (400 μl) at room temperature. Peptide **4** : MS (MALDI-TOF) found 3116.1, calcd 3119.0 (M+H)$^+$; Amino acid analysis: $Asp_{2.2}Glu_{2.5}Pro_{1.0}Gly_{2.0}Ala_2Cys_{0.6}Met_{0.9}Leu_{1.0}Trp_{nd}Arg_{5.3}$

Synthesis of Arg-Lys(Boc)-Lys(Boc)-Arg-Arg-Gln-Arg-Arg-Arg-Gly-Gly-Met-Ala-Glu-Asp-Trp-Leu-Asp-Cys($S_2O_3^-$)-Pro-Ala-NH_2 (5)

Peptide **4** was dissolved in 3% piperidine / DMSO (v /v) for 30 min to give the product (**5**). Peptide **5** : MS (MALDI-TOF) found 2897.9, calcd 2896.8 ($M+H)^+$

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Chapter III

Synthesis of ORL1(288-370)

Introduction

Through the synthesis of F_1F_0 ATP synthase subunit c, effective conditions for the transmembrane peptide purification were defined, and the thioester method was also found to be effective for the synthesis of a membrane protein. However, from the synthetic point of view, the size and difficulty associated with handling a peptide with transmembrane region(s) continue to be challenging. Therefore, the goal of the experiments described in this thesis was to establish a basis for the synthesis of a polytopic membrane protein through the synthesis of a G protein coupled receptor (GPCR), Opioid Receptor Like 1 (ORL1)[1]. GPCR is considered to be composed of seven transmembrane regions and bind diverse signaling molecules including biogenic amines, sugars, peptides, and proteins that initiate numerous cellular processes including sexual conjugation, vision, pain perception, growth, blood pressure, and carbohydrate and fat metabolism. The amino acid sequence of ORL1 is shown in **Fig.1**.

MEPLFPAPFWEVIYGSHLQGNLSLLSPNHSLLPPHLLLNASHGAFLPLGL

KVT**IVGLYLAVCVGGLLGNCLVMYVIL**RHTKMK**TATNIYIFNLALADTLV**[100]

LLTLPFQGTDILLGFWPFGNALCK**TVIAIDYYNMFTSTFTLTAMSV**DRYV

AICHPIRALDVRTSSKA**QAVNVAIWALASVVGVPVAIMGSA**QVEDEEIEC[200]

LVEIPTPQDYWGPVF**AICIFLFSFIVPVLVISVCYSLM**IRRLRGVRLLSG

SREKDRNLRR**ITRLVLVVVAVFVGCWTPVQVFVLA**QGLGVQPSSETAVAI[300]

LR**FCTALGYVNSCLNPILYAFL**DENFKACFRKFCCASALRRDVQVSDRVR

SIAKDVALACKTSETVPRPA

Fig. 1 Amino acid sequence of ORL1. The putative transmembrane regions are shown in bold. The target fragment, ORL1(251-370) is shown within the square.

To begin an examination of the synthesis, the work was focused a portion of the protein that contains the sixth and the seventh transmembrane regions and the intracellular domain at the C-tarminal, ORL1(251-370) (**Fig. 2**). Judging from the sequence, the

synthesis of ORL1(251-370) requires different ligation steps. The strategy proposed in the previous chapter plays a crucial role in the synthesis. Based on the strategy, the author started the examination of the synthesis of ORL1(288-370) according to the scheme shown below (**Scheme 1**).

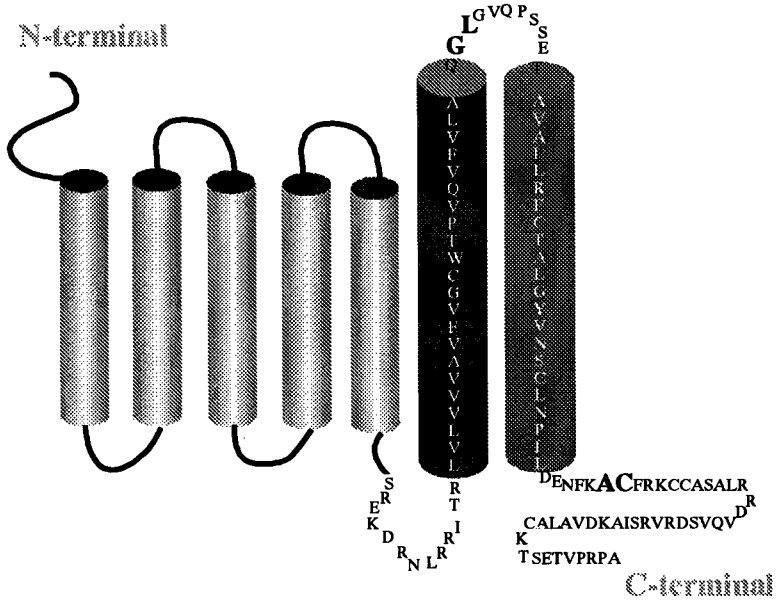
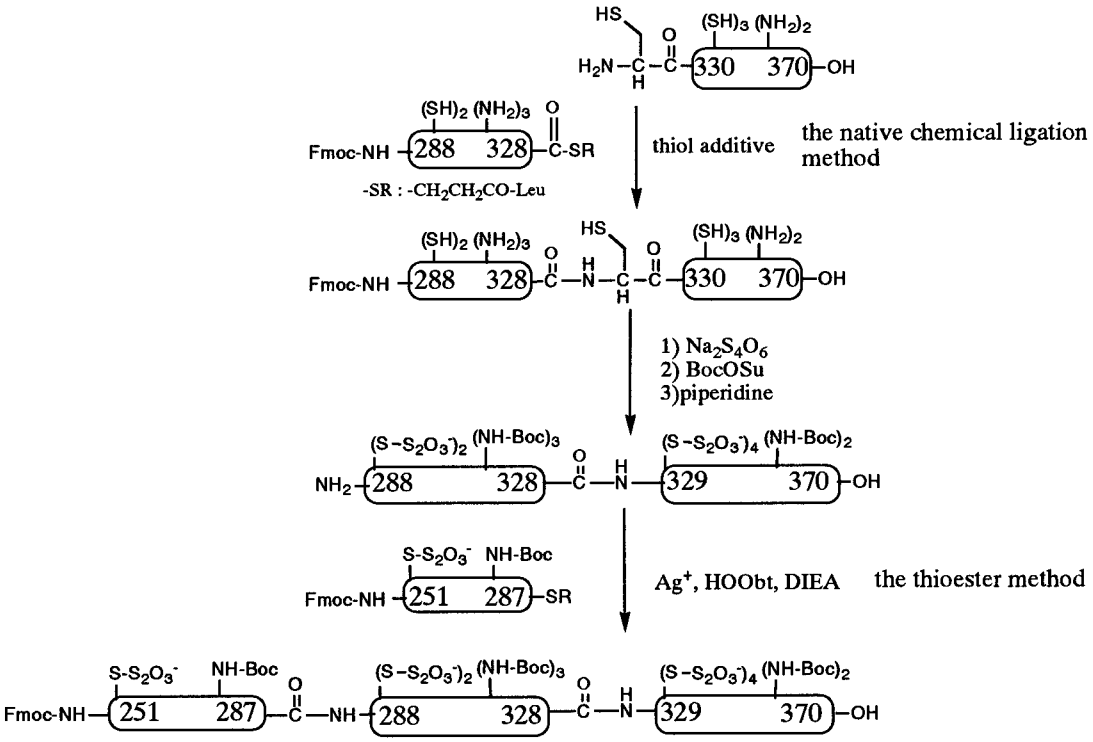


Fig. 2 Schematic representation of ORL1(251-370).



Scheme 1 Synthetic scheme of ORL1(251-370)

One of the problems that can be easily imagined during the synthesis is the difficulty associated with solubilizing an extremely hydrophobic building block under conditions that is suitable for ligation, especially, in the case in which the ligation is performed in an aqueous buffer. Searching for a solubilizing reagent such as a detergent is a general way to solve the problem. Indeed, two groups have reported the synthesis of a membrane protein via the native chemical ligation method using additives to dissolve the building blocks. The first group used trifluoroethanol (TFE) as a solvent for building blocks. They performed the native chemical ligation method in an aqueous solution containing 30% TFE to synthesize Influenza A virus M2[2]. The other group examined additives such as TFE, guanidium chloride, urea and SDS for their abilities to dissolve the hydrophobic building blocks. As a result, they synthesized the potassium channel of two transmembrane protein via the native chemical ligation method using an aqueous solution containing SDS (1%)[3]. However, the problem involving the use of a detergent is that there is no best detergent for all of the cases. Indeed, the author's first trial in the coupling reaction of the 7th transmembrane region and the C-terminal intracellular region failed. The building block containing the transmembrane region was not soluble to an aqueous solution even in the presence of a detergent such as SDS (This is discussed below.). Another technique that can be applied to handle a transmembrane peptide is the introduction of a sequence of lysines (Lys-tag, **Fig. 3**) at the terminus of the peptide itself. This Lys-tag was introduced by Deber *et.al.* and has continuously contributed to elucidating information on the function of transmembrane region itself[4].

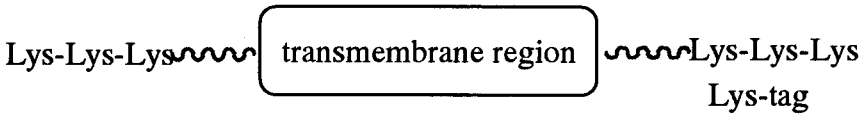


Fig. 3 Schematic representation of a peptide with a Lys-tag

In this chapter, the author describes experiments that must provide general information on the synthesis of a membrane protein using the native chemical ligation method through the synthesis of ORL1(288-370) (**Fig.4**). The experiment began with the introduction of a new design of a building block for the synthesis of membrane

protein via a ligation strategy. A sequence of arginines (Arg-tag) is introduced to a thioester moiety of the building block containing a transmembrane region to enhance its solubility based on the proposal made by Deber *et.al.* Detailed investigation was also made to optimize the conditions, especially the use of the detergent and the selection a thiol additive, for the synthesis of ORL1(288-370) by native chemical ligation method

Leu-Gly-Val-Gln-Pro-Ser-Ser-Glu-Thr-**Ala-Val-Ala-Ile-Leu-Arg-**

Phe-Cys-Thr-Ala-Leu-Gly-Tyr-Val-Asn-Ser-Cys-Leu-Asn-Pro-Ile-

Leu-Tyr-Ala-Phe-Leu-Asp-Glu-Asn-Phe-Lys-Ala↓Cys-Phe-Arg-Lys-

Phe-Cys-Cys-Ala-Ser-Ala-Leu-Arg-Arg-Asp-Val-Gln-Val-Ser-Asp-

Arg-Val-Arg-Ser-Ile-Ala-Lys-Asp-Val-Ala-Leu-Ala-Cys-Lys-Thr-

Ser-Glu-Thr-Val-Pro-Arg-Pro-Ala

Fig. 4 Amino acid sequence of ORL1(288-370). The transmembrane region is shown in bold and the coupling site is shown by an arrow.

Results and discussion

Introduction of Arg-tag to a building block for peptide ligation

As mentioned in the introduction, some strategies must be developed for the transmembrane-containing building block to obtain a higher solubility in solvents, especially in an aqueous solution for successful ligation by the native chemical ligation method. For this purpose, the author designed a peptide thioester building block with a sequence of arginines (Arg-tag) at the end of the C-terminal of the building block (**Fig. 5**). The amino acids comprising the tag moiety should be Arg instead of Lys since the side chain of Arg does not require any protecting group even during the coupling reaction using the thioester method. The Arg-tag must serve as a cation core that leads to the production of a molecularly dispersed solution.

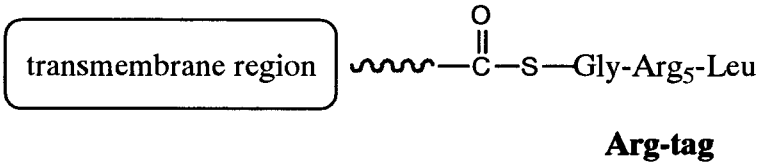
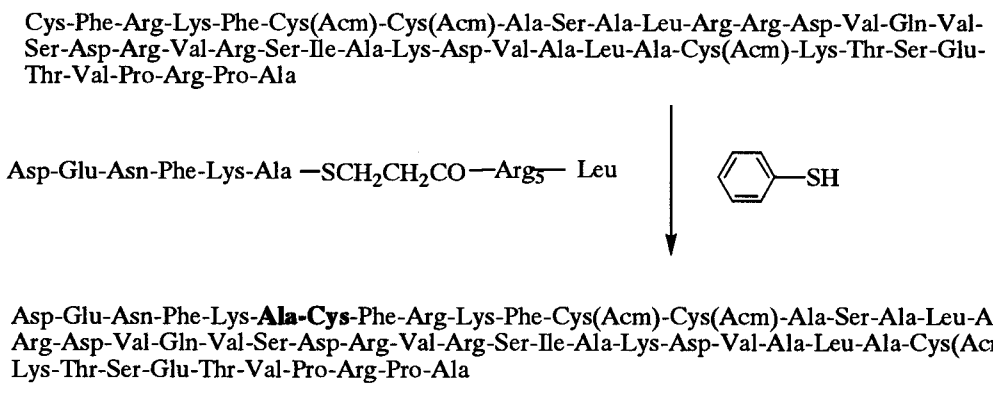


Fig. 5 Schematic representation of Arg-tag containing peptide thioester

To examine the effect of the Arg-tag on the ligation reaction, a model experiment involving the ligation of a peptide thioester, Asp-Glu-Asn-Phe-Lys-Ala-SCH_2CH_2CO-Gly-Arg_5-Leu (**1**) and [Cys(Acm)^{334,335,336}]ORL1(329-370) (**2**) was performed as summarized in **Scheme 2**. The coupling reaction was carried out via native chemical ligation method in a ligation buffer containing 100 mM sodium phosphate, 6 M guanidine hydrochloride, and 1% thiophenol at a pH of 7.0 (standard conditions for a ligation via the native chemical liagtion method).

In **Fig.4B** RP-HPLC elution profile of the reaction mixture at the reaction time of 4 h is shown. All of the peaks that appeared on the profile were collected and the contents of each were checked by mass analysis. The mass number and the amino acid composition of the material corresponding to peak **a** were in good agreement with the calculated value of the desired product. The content of peak **b** was characterized as Arg-tag moiety, $HSCH_2CH_2CO$-Gly-Arg_5-Leu (MS found for *m/z* : 1016.5; calcd for $[M+H]^+$:1016). It can be concluded that the addition of the Arg-tag has no serious effect on the reactivity of a peptide thioester during a ligation using the native chemical ligation method.



Scheme 4-2 Model synthesis of [Cys(Acm)^{334,335,336}]ORL1(323-370)

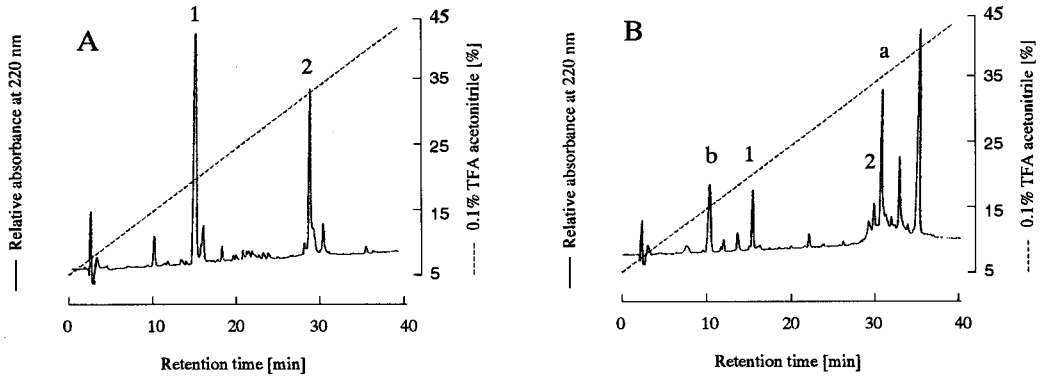


Fig. 6 RP-HPLC elution profiles of the reaction mixture of the ligation of peptides **1** and **2**. A : Profile of the reaction time of 0 h. B : Profile of the reaction time of 4 h.

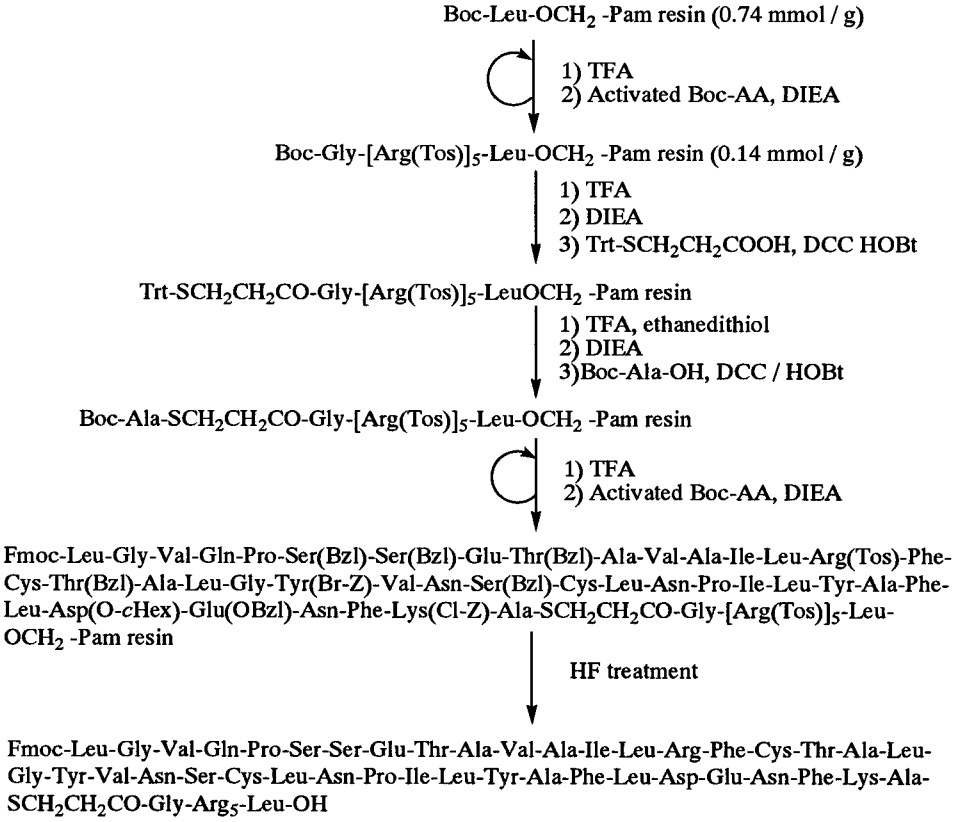
Preparation of Fmoc-ORL1(288-328)-SCH_2CH_2CO-Gly-Arg_5-Leu (3)

Starting from Boc-Leu-OCH_2-Pam resin, Fmoc-ORL1(288-328)-SCH_2CH_2CO--Arg-tag **(3)** was synthesized in two different ways, a manual synthesis using *in situ* neutralization protocol [5] (**Scheme 3**) and a machine-assisted synthesis (**Scheme 4**). Whether in the manual or in machine-assisted synthesis, it is important to start with a low loading capacity for the synthesis of a long peptide in a step-wise fashion. The adjustment of the capacity was performed at the introduction of Gly to $[Arg(Tos)]_5$-Leu-Pam resin to a value of 0.14 mmol / g. *In situ* neutralization protocol for a manual synthesis introduced by Kent's group[5] is an effective, rapid and simple. The protocol this method is summarized in **Table 1**.

Table1 In situ neutralization protocol for manual synthesis

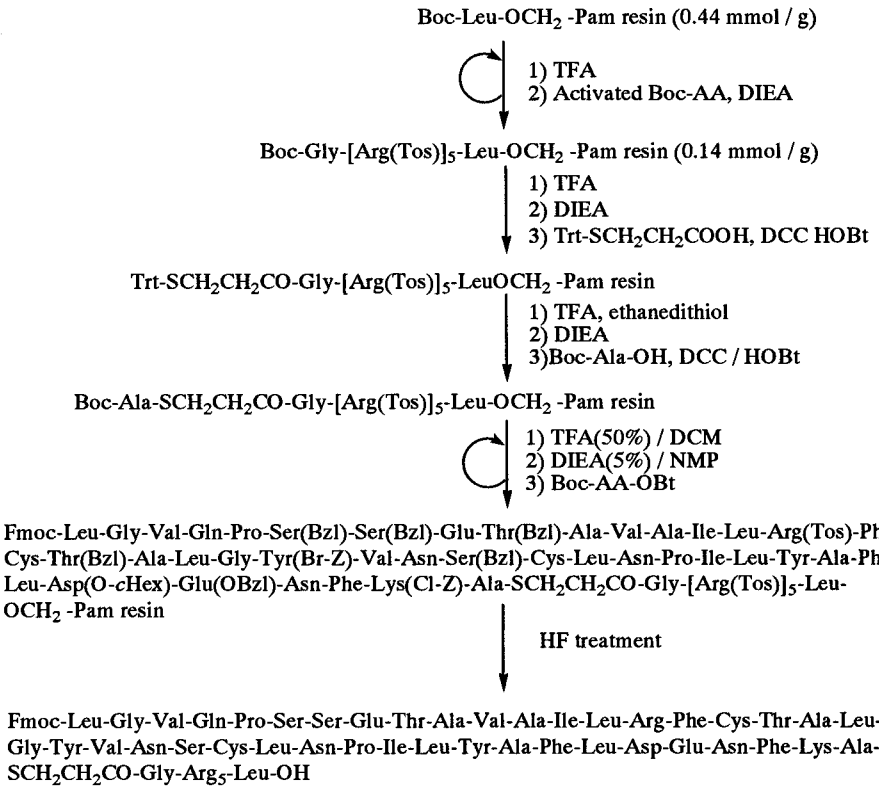
Synthesis cycle	Component	Time and mode
Deprotection	100% TFA	Two 1.5 min shake
Wash	DMF	1-min flow wash
Coupling	Activated Boc-AA	10-min shake
	DIEA	Take resin sample for ninhydrin test
Wash	DMF	1-min flow wash

The N^{α}-Boc group was removed by treatment with 100% TFA, and the peptide-resin salt was rinsed with a single flow wash with DMF. The activated Boc-amino acid was added with a sufficient excess amount of DIEA to neutralize the peptide-resin salt. Coupling was performed for 10 min, followed by a single flow wash with DMF prior to the next cycle. In the synthesis of peptide **3**, a minor modification was made to the above protocol. After the coupling cycle, following the DMF flow wash, a capping cycle by acetic anhydride was introduced. Activation of the Boc-amino acid was carried out with *N*-[1H-(benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminimum hexafluorophosphate *N*-oxide (HBTU) and diisopropylethylamine (DIEA). The synthetic scheme for the synthesis of peptide **3** is shown in **Scheme 3**. As shown in **Scheme 3**, the introduction of Ala-SCH_2CH_2CO- was carried out in the procedure described in Chapter I.



Scheme 3 Synthesis of the peptide **3** using *in situ* neutralization protocol for manual synthesis

The machine-assisted synthesis of peptide **3** is shown in **Scheme 4**. Preparation of Boc-Ala-SCH_2CH_2CO-Gly-[Arg(Tos)]$_5$-Leu-Pam resin was manually performed following the same procedure shown in the scheme, and the reminder of the elongation process was performed on the automated peptide synthesizer.



Scheme 4 Machine-assisted synthesis of peptide **3**

The purification of peptide **3** was performed by RP-HPLC on Cosmosil 5Ph AR-300 (10 x 250 mm) column with a linear gradient of formic acid-water (2:3) and formic acid–1-propanol (4:1). For the injection to the column, the crude powder was dissolved in TFA. An analytical RP-HPLC profile of peptide **3** synthesized manually by *in situ* neutralization protocol is shown in **Fig. 7**. The major peak at 17 min (shown by an arrow) contained the desired peptide **3**, and the mass number and amino acid composition were in good agreement with the calculated values. Peptide **3** was obtained in a yield of 2.1% based on the Leu residue on the resin.

An analytical RP-HPLC profile of peptide **3** synthesized by an automated peptide

synthesizer is shown in **Fig. 8.** The major peak at 17 min (shown by an arrow) contained the desired peptide **3**, and the mass number and amino acid composition were in good agreement with the calculated values. Peptide **3** was obtained in a yield of 1% based on the Leu residue on the resin.

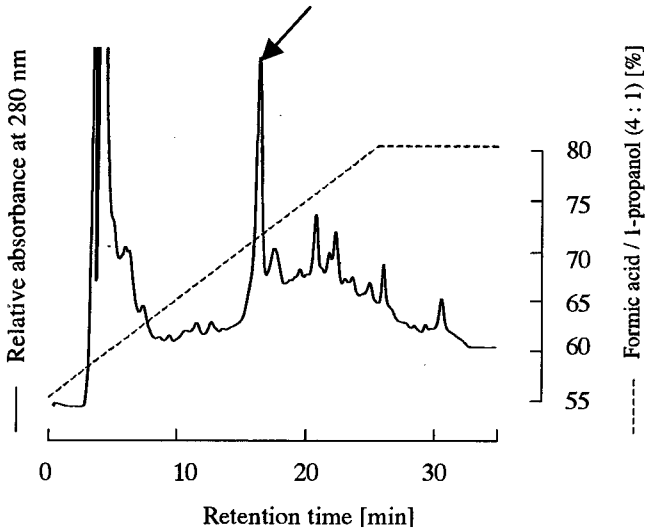


Fig.7 RP-HPLC elution profile of crude peptide **3** synthesized manually using *in situ* neutralization protocol. Column : Cosmosil 5Ph AR-300, 4.6 x 150 mm; linear gradient (25 min) from formic acid-water (2 : 3) to formic acid-1-propanol (4 : 1). Flow rate : 0.65 ml / min.

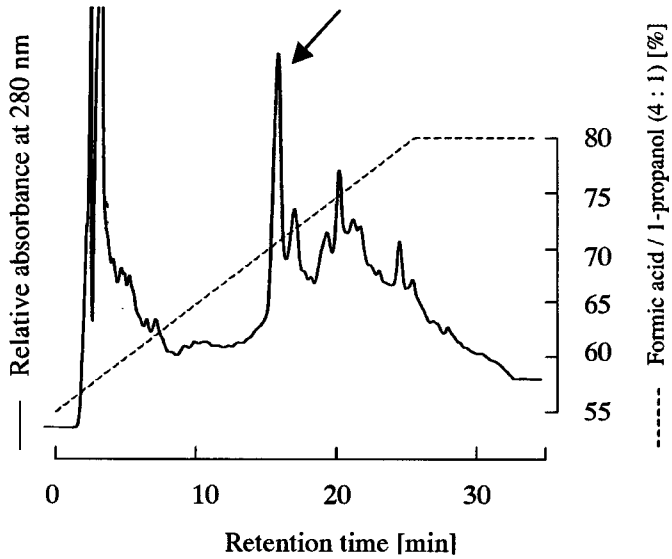


Fig.8 RP-HPLC elution profile of crude peptide **3** synthesized by automated peptide synthesizer. Column: Cosmosil 5Ph AR-300, 4.6 x 150 mm; linear gradient (25 min) from formic acid-water (2 : 3) to formic acid-1-propanol (4 : 1). Flow rate : 0.65 ml / min.

For a comparison, a peptide thioester building block without Arg-tag (Fmoc-ORL1(288-328)-SCH_2CH_2CO-Leu **4**)was prepared. Starting from Boc-Leu-OCH_2-Pam resin, Boc-Ala-SCH_2CH_2CO-Leu-OCH_2-Pam resin was manually synthesized as it was described in Chapter I, and the rest of the elongation was then performed on an automated peptide synthesizer. Purification of peptide **4** was performed by RP-HPLC on Cosmosil 5Ph AR-300 column (10 x 250 mm) with a linear gradient of formic acid-water (2:3) and formic acid–1-propanol (4:1). An analytical RP-HPLC profile of peptide **4** is shown in **Fig.9**. For injection to the column, the crude powder was dissolved in TFA. Judging from a result of the mass and the amino acid analysis, the main peak at 13 min contained a desired peptide **4**. The yield was extremely low. However, the most serious problem for this peptide thioester **4** was its insolubility to an aqueous solution. No detergents or organic solvents that are proper for its solubilization were able to dissolve the peptide thioester **4** for a further ligation step.

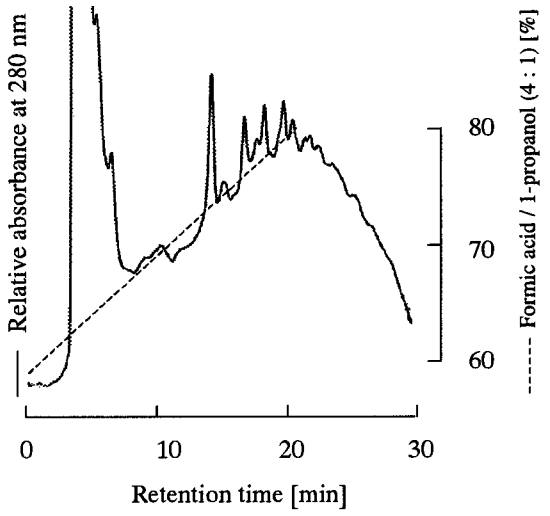


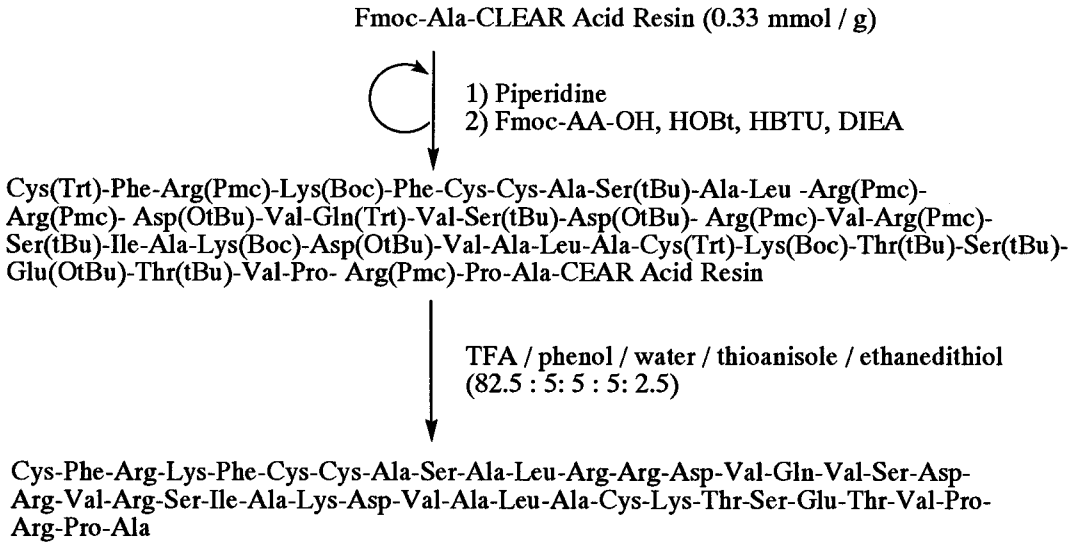
Fig. 9 RP-HPLC elution profile of crude peptide **4**. Column: Cosmosil 5Ph AR-300, 4.6 x 150 mm; linear gradient (25 min) from formic acid-water (2 : 3) to formic acid-1-propanol (4 : 1). Flow rate : 0.65 ml / min.

An Arg-tag was introduced to the thioester moiety of a peptide thioester containing a transmembrane region for the purpose of forming a cation core in the system. The effects of this cation core resulted in an improved resolution on the RP-HPLC purification profile of Arg-tag introduced building block (**Fig.7 and 8**) and an enhanced solubility of the building block in a detergent containing aqueous buffer. The author

concludes that the introduction of Arg-tag represents an effective approach for the preparation of building blocks for the synthesis of a membrane protein.

Preparation of ORL1(329-370) (*5*)

Starting from Fmoc-Ala-CLEAR Acid resin, ORL1(329-370) was synthesized as summarized in **Scheme 5**. After the elongation, the resulting peptide resin was treated with a mixture of TFA / phenol / water / thioanisole / ethanedithiol (82.5 : 5: 5 : 5: 2.5 (v / v), reagent K) to give a crude powder. Purification was performed by RP-HPLC using an ODS column. The RP-HPLC elution profile of ORL1(329-370) is shown in **Fig.10**. Judging from the result of the mass and the amino acid analysis, the main peak at 19 min contained a desired peptide. The yield was 5%.



Scheme 5 Synthetic scheme of peptide **5**.

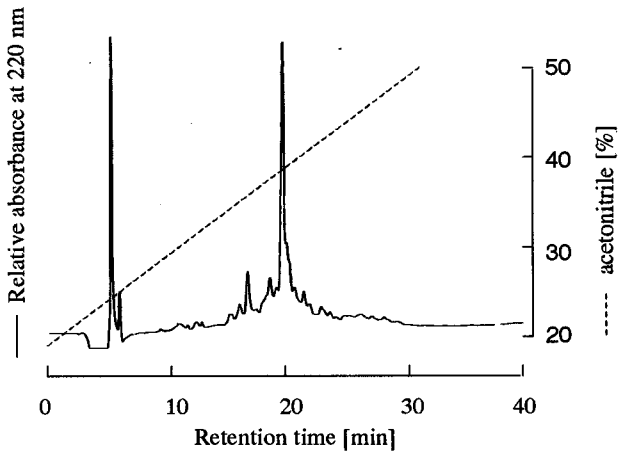
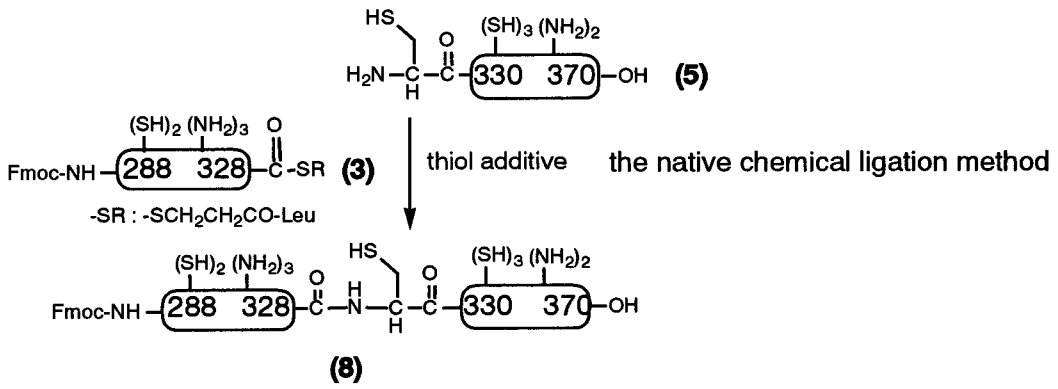


Fig. 10 RP-HPLC elution profile peptide **5**. Column : Cosmosil 5C18 AR-300 column(4.6 x 150 mm); eluent : 0.1% TFA aq. acetonitrile; Flow rate 1.0 ml / min.

Synthesis of Fmoc-ORL1(288-370) by the native chemical ligation method

The synthesis of Fmoc-ORL1(288-370) by means of native chemical ligation method was examined (**Scheme 6**). Considering the very hydrophobic nature of peptide **3**, reaction conditions must be investigated in order for the ligation reaction to proceed efficiently.



Scheme 6 Synthesis of Fmoc-ORL1(288-370)

As described in the introduction, Muir's group found that in the presence of 1% SDS, hydrophobic building blocks remained soluble throughout the course of the reaction, whereas the building blocks were found to slowly precipitate when other conditions such as 50% TFE, 30% TFE + 4.8 M guanidium chloride and so on were used [3].

Therefore, the first choice for the reaction condition investigation was to employ a aqueous solution containing 1% SDS. Equal amounts of peptide **3** and **5** were dissolved in a ligation buffer (pH 7.2) containing 100 mM sodium phosphate, thiophenol 1% (v/v), 34 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), and 1% SDS. TCEP was added to the ligation buffer to minimize disulfide formation [6].

All of the reactants were soluble at the time reaction was started (a precipitate began to appear in the course of time). The RP-HPLC profile of the reaction mixture is shown in **Fig.11**. All of the peaks that appeared on the profiles were collected and the content of each fraction was characterized by mass analysis. As shown in **Fig. 11A**(reaction time of 0 h), peak **a** was found to be peptide **3,** whereas peptide **5**, C-tail, was not detectable at a wavelength of 280 nm. The origin of the peaks at retention times of 13 and 17 min was from thiophenol. The content of the 30 min peak could not be characterized by MS. The RP-HPLC elution profile of the reaction mixture is shown in **Fig. 11B**. The molecular mass of the desired product was not found in any of the peaks that appeared on the profile.

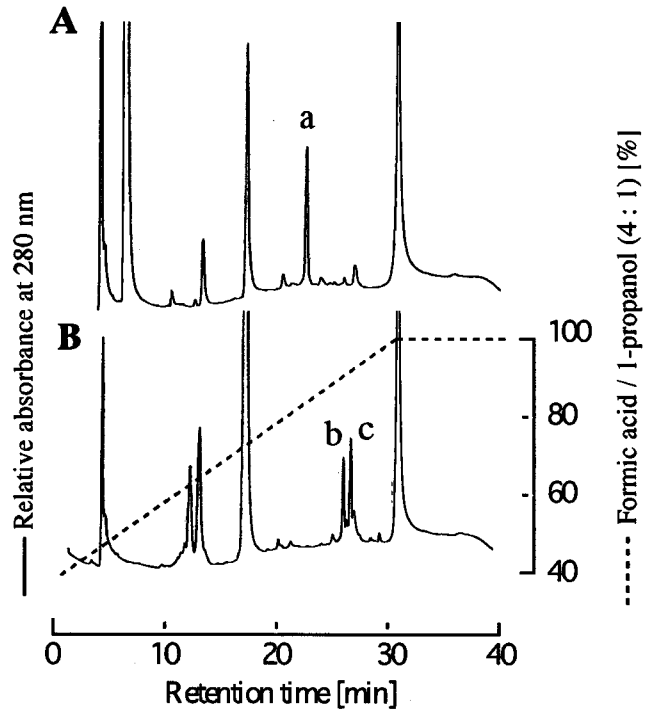


Fig. 11 RP-HPLC elution profile of ligation mixture. The ligation was carried out in an aqueous solution containing 100 mM sodium phosphate, thiophenol 34 mM,TCEP 1% thiophenol, and SDS 1%. A : Profile at a reaction time of 0 h. B : profile at the reaction time of 24 h.

The molecular mass found for peaks **b** and **c** were 5930.2 (**6**) and 4760.0 (**7**) respectively. The putative structures of the content in each peak are shown in **Fig. 12**.

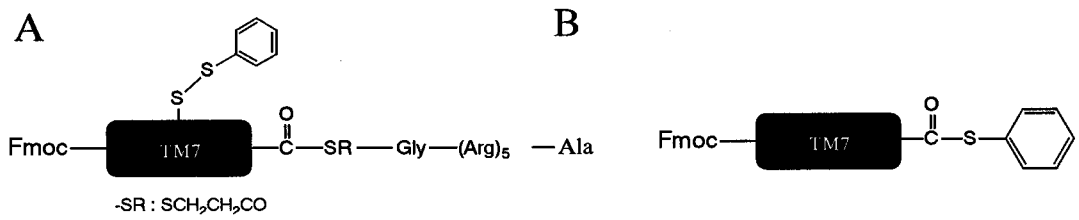


Fig. 12 The putative molecular structures of compound **6** (A) and **7**(B).

The putative structure shown in **Fig. 12A** involves a formation of a disulfide bonds although the reaction was carried out in the presence of TCEP. The result showed that the product could not be obtained using this previously reported condition for the synthesis of a membrane protein via the native chemical ligation method. To obtain the product and to determine what was happening in the first trial, the author examined the reaction conditions as listed in **Table 2**.

Table 2 Conditions for the synthesis of Fmoc-ORL1(288-370)

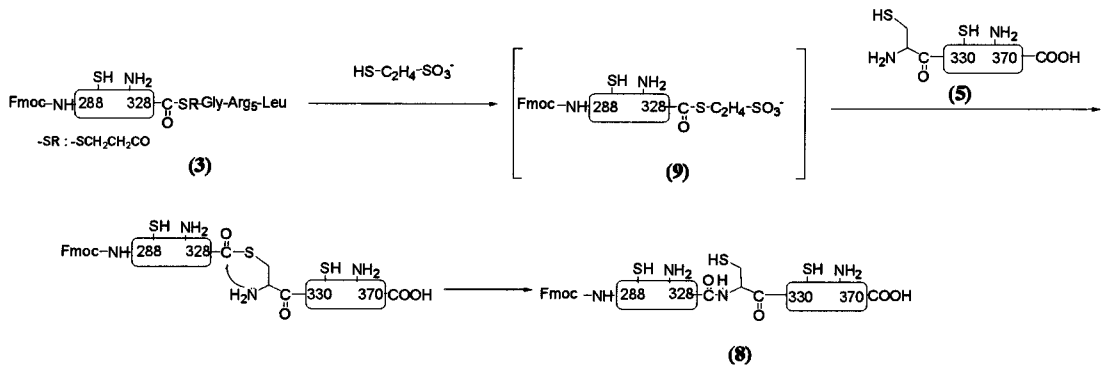
Exp.	Detergent	CMC [mM]	Concentration [mM]	Thiol	pH	Other additives
1	SDS	8	35 (1%)	thiophenol	7.2	TCEP
2	SDS	8	35	MESA	7.2	TCEP
3	SDS	8	7	thiophenol	7.2	TCEP
4	SDS	8	7	MESA	7.2	TCEP
5	HTMA	1	0.9	MESA	7.2	TCEP

MESA : 2-mercaptoethanesulfonic acid
HTMA : Hexadecyltrimethylammonium bromide

The conditions used in Exp.1 was the first trial for the synthesis mentioned above. 2-mercaptoethanesulfonic acid (MESA) was originally employed as an alternative thiol additive for native chemical ligation methods because of its non-harmful and ordorless characteristics. However, in this case, MESA was introduced as a thiol additive for a

different reason that will be discussed later in this chapter. The concentration of SDS was changed to below the critical micelle concentration (CMC) to observe the effect of micelle on the synthesis. Hexamethyltrimethylammonium bromide (HTMA) was employed to examine the effect of charge on the synthesis. Results are mentioned below.

In Exp.2, a thiol additive was changed to MESA. The reaction scheme involving MESA as a thiol additive is summarized in **Scheme 7**.



Scheme 7 Reaction mechanism for the native chemical ligation method using MESA as a thiol additive.

The RP-HPLC elution profile of the ligation reaction mixture is shown in **Fig. 13**. **Fig. 13A** and **B** show the profiles of a reaction mixture at the start and at the reaction time of 48 h. All of the peaks that appeared on the profile are collected, and content of each peak were characterized by MS analysis. Peak **a** was characterized as the starting material peptide **3** (molecular mass found : 5714; calcd for $[M+H]^+$: 5711.6). From peak **b**, two mass values were found, as shown in **Fig. 14**. The values 5714.2 and 9314.4 correspond to the molecular mass of peptide **3** and the desired product Fmoc-ORL1(288-370) (**8**; Calcd for $[M+H]^+$: 9310.8) respectively. Peak **c** was characterized as an intermediate peptide thioester, Fmoc-ORL1(288-328)-$SCH_2CH_2SO_3^-$(**9**; found for m/z : 4794.9; calcd for $[M+H]^+$:4795.5). The contents of the other peaks could not be characterized because the mass values could not be obtained. The results indicate that by using MESA as a thiol additive, the ligation reaction proceededs to give the product **8**. However, as shown in **Fig. 13,** and **14**, detectable amounts of peptide thioester **3** remained in the reaction mixture even at a reaction time of 48 h.

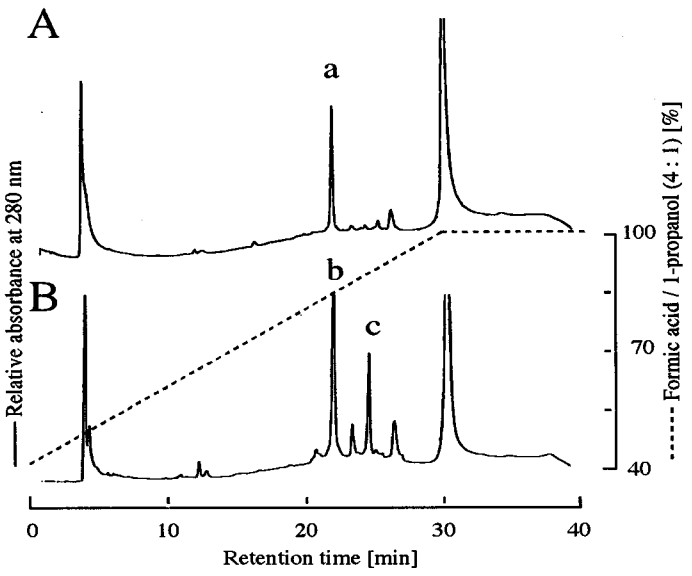


Fig. 13 RP-HPLC elution profile of the ligation mixture of Exp.2. A: profile at a reaction time of 0 h; B: profile at a reaction time of 48 h. Column: Cosmosil 5C4 AR 300 (4.6 x 150 mm); Linear gradient from formic acid-water (2 : 3) to formic acid -1-propanol (4 :1); Flow rate : 0.65 ml / min.

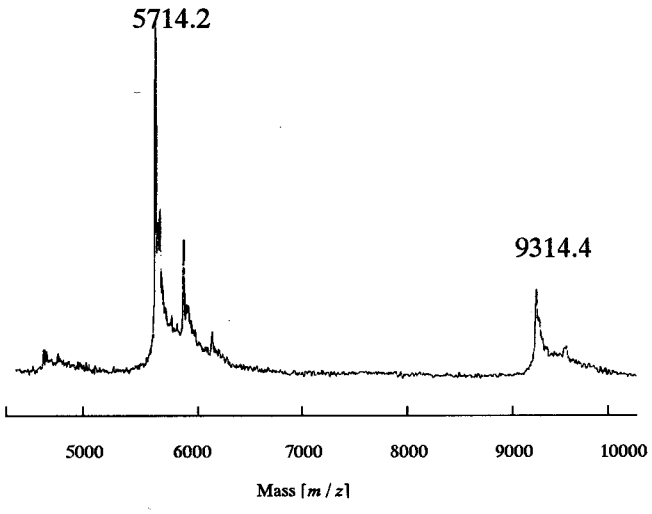


Fig. 14 Result of the mass analysis of peak **b**.

An improvement in the ligation reaction to give the product **8** can be explained by the speculation to be mentioned below (**Fig.15**). In Exp.1, when the ligation reaction is started, peptide **3** is solubilized by the effect of SDS micelles (Stage I). After the thioester exchange by thiophenol, the entire molecule is covered with an SDS micelle (Stage II). Thus no further reaction can occur since the active thioester is inside the micelle.

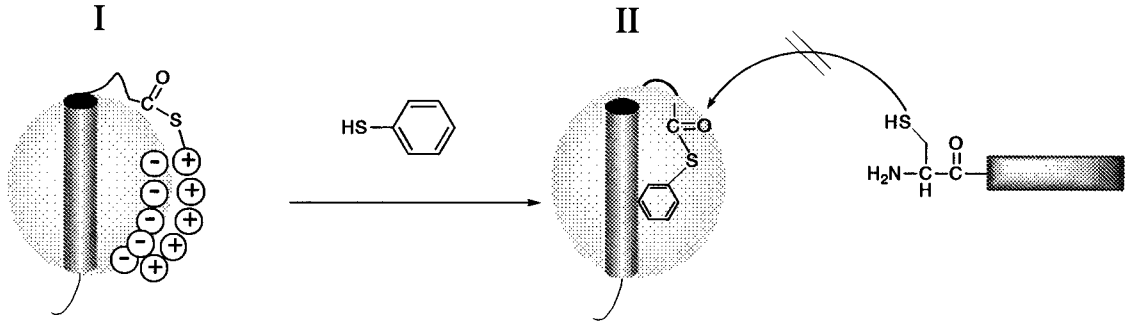


Fig.15 Schematic representation of the ligation reaction in presence of thiophenol and 1% SDS.

In Exp.2, on the other hand, the thioester moiety of peptide thioester intermediate **9**, must be located outside of detergent micelle because of the negative charge conferred by sulfonate group(**Fig. 16**). Thus the next thioester exchange by the thiol group on N-terminal cystein of peptide **5** occurs to give the product **8** via S-N acyl shift.

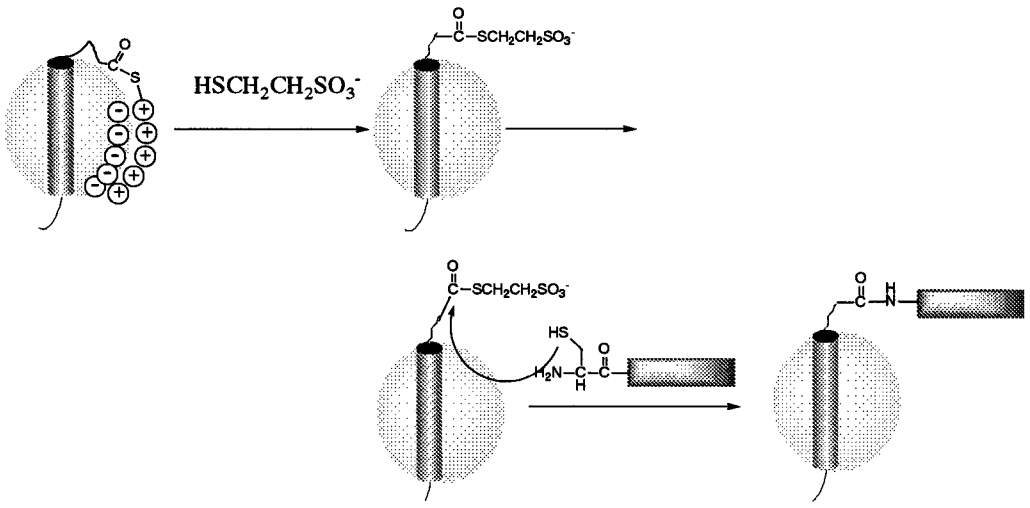


Fig. 16 Putative reaction mechanism in Exp.2

However, the fact that peptide thioesters **3** and **9** are present in the reaction mixture at the reaction time of 48 h is the next problem to overcome. The use of a detergent is an important factor that must be considered.

In Exp. 3 and 4, the ligation reactions were carried out at SDS concentrations below CMC (7 mM). For a comparison with previous experiments, thiophenol (Exp.3) and MESA (Exp.4) were employed as thiol additives in each condition. In Exp. 3, the ligation reaction was carried out in a ligation buffer (pH 7.2) containing 100 mM sodium phosphate, thiophenol 1% (v/v), 34 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), and 7 mM SDS. The RP-HPLC elution profile of the ligation reaction mixture of Exp. 3 is shown in **Fig. 17**. **Fig. 17A** and **B** show the profile of the reaction mixture at the start and at a reaction time of 24 h. All of the peaks that appeared on the profile were collected, and the content of each peak was determined by mass analysis.

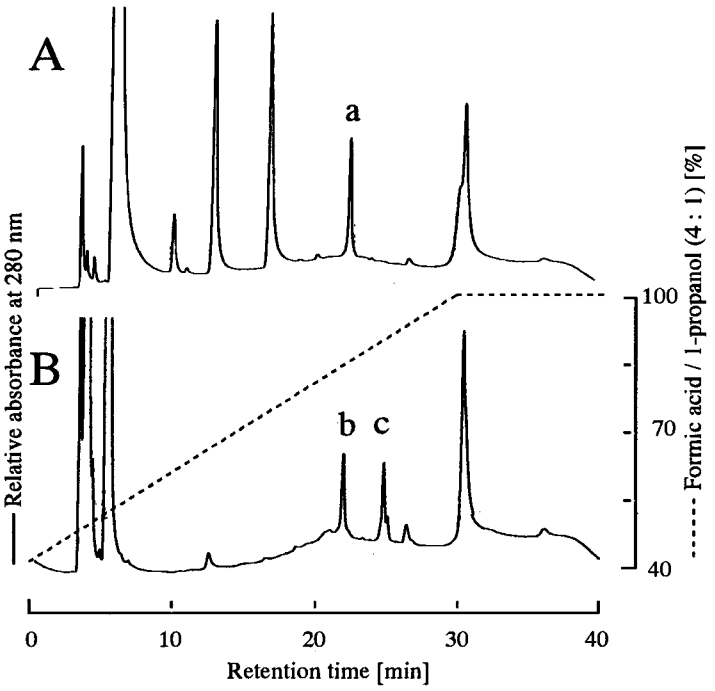


Fig. 17 RP-HPLC elution profile of the ligation mixture of Exp.3. A: profile at a reaction time of 0 h; B: profile at a reaction time of 24 h. Column: Cosmosil 5C4 AR 300 (4.6 x 150 mm); Linear gradient from formic acid-water (2 : 3) to formic acid -1-propanol (4 :1); Flow rate : 0.65 ml / min.

The content of peak **a** was characterized as the starting material peptide **3** (molecular mass found : 5713; calcd for $[M+H]^+$: 5711.6). From peak **b**, a single value 9293.6 was found, which corresponds to the desired product Fmoc-ORL1(288-370) (**8**; Calcd for $[M+H]^+$: 9310.8), whereas this value was not found in Exp.1. Peak **c** was characterized as a thiophenyl ester intermediate **7**.

In the case of Exp.4, mentioned above, the conditions, except for the thiol additive, were the same. MESA (60 mM) was employed as the thiol additive. The RP-HPLC elution profile of the ligation reaction mixture of Exp. 4 is shown in **Fig. 18**. **Fig. 16A** and **B** show the profiles of the reaction mixture at the reaction times of 3h and 24 h respectively. All of the peaks that appeared on the profile were collected, and each peak was characterized by mass analysis.

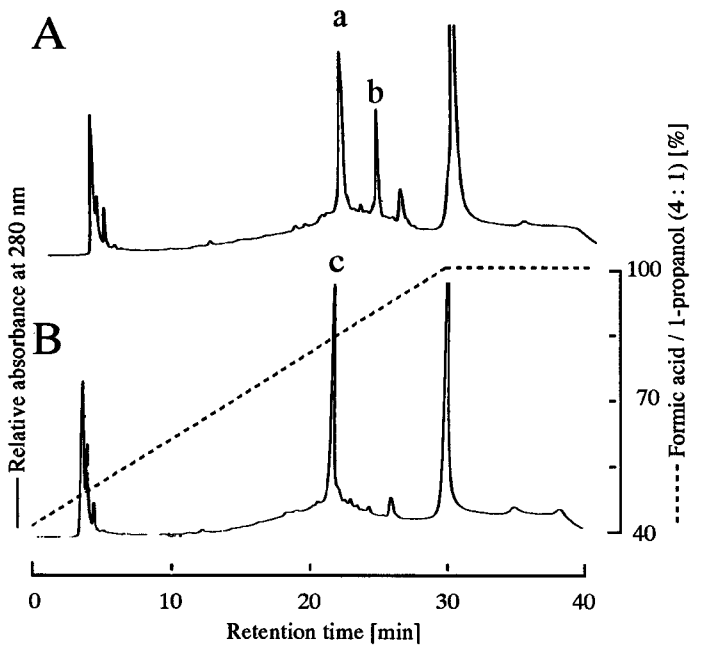


Fig. 18 RP-HPLC elution profile of the ligation mixture of Exp.4. A: profile at the reaction time of 3 h; B: profile at the reaction time of 24 h. Column: Cosmosil 5C4 AR 300 (4.6 x 150 mm); Linear gradient from formic acid-water (2 : 3) to formic acid -1-propanol (4 :1); Flow rate : 0.65 ml / min.

From peak **a**, two mass values were found (**Fig. 19A**), 5718.4 and 9320.1, which corresponds to the starting material peptide **3** and the product **8** respectively. Peak **b** was characterized as peptide thioester intermediate **9** (Found for *m* /*z* :4801.3). From peak **c**, only one mass value, 9304.1 was found, which corresponds to the molecular mass of the desired product. As shown in **Fig.18B**, the peak corresponding to the peptide thioester intermediate disappeared completely.

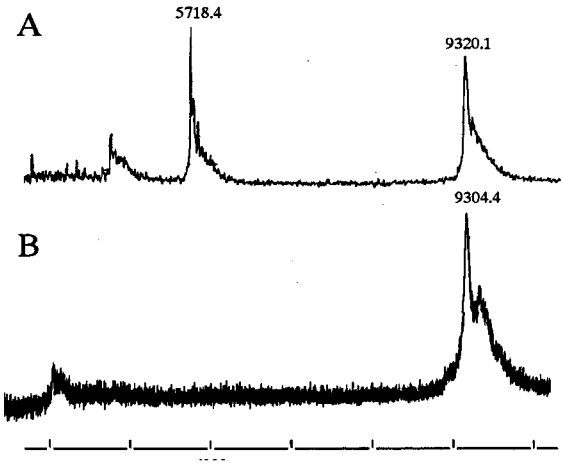


Fig. 19 Mass spectra of peaks **a** and **c** on Fig. 18. A: peak **a**; B : peak **c**

By setting the SDS concentration at 7 mM, the ligation reaction proceeded to give the desired product. Especially, a remarkable result was obtained in Exp.4, in which SDS concentration of 7mM and MESA were used as a thiol additive. The ligation reaction was complete within 24 h, whereas in Exp.2 and 3, the reaction was not completed, even after 48 h. It is difficult to provide a reasonable explanation for the extremely slow reaction rate observed in Exp.3. There is a possibility that the thioester moiety, the site of the reaction, of peptide thiophenyl ester **3** is covered with SDS molecules.

The author performed another experiment to examine the effect of the charge of the detergent on the ligation reaction (Exp. 5). The conditions were the same as those used in previous experiments but hexadecyltrimethylammonium bromide (HDTA) was employed as a detergent at a concentration of 0.9 mM (CMC:1 mM). **Fig. 20A**, **B**, and **C** show profiles of a reaction mixture at the start, a reaction time of 3 h and 48 h, respectively. All of the peaks that appeared on the profile were collected, and then content of each peak was determined by MS analysis.

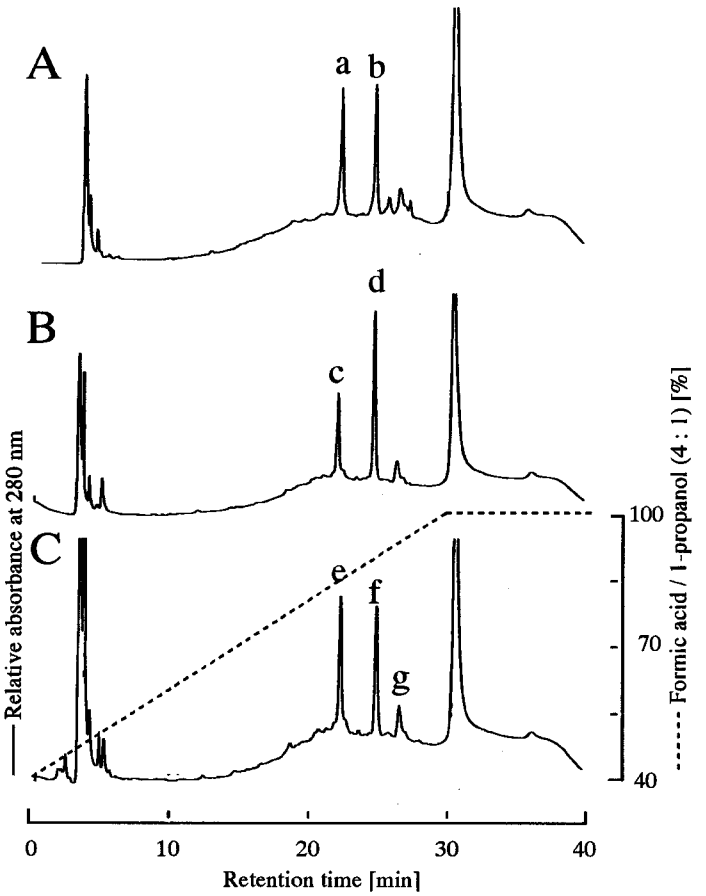


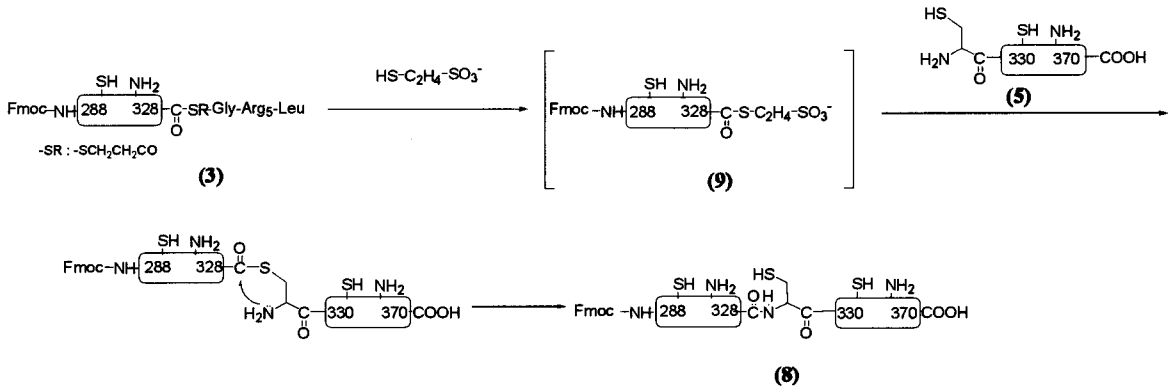
Fig. 20 RP-HPLC elution profile of the ligation mixture of Exp.4. A: profile at the reaction time of 0 h; B: profile at the reaction time of 3 h. C : at the reaction time of 48 h. Column Cosmosil 5C4 AR 300 (4.6 x 150 mm); Linear gradient from formic acid-water (2 : 3) to formic acid - 1-propanol (4 :1); Flow rate : 0.65 ml / min.

Peak **a** was characterized as the starting material, peptide **3**. Peaks **b**, **d**, and **f** were characterized as peptide thioester intermediate **8**. In peak **c**, the mass value corresponding to the desired product was found (molecular mass found : 9302.6). Other peptide components were not observed in peak **c**. In peak **g**, the value of 4672.2 was found, which corresponds to the value of the thioester hydrolyzed peptide **3** (Calcd for $[M+H]^+$:4673.4). By changing the charge on the detergent molecule from negative (SDS) to positive (HDTA), the conditions, conversion of peptide thioester **3** to peptide

thioester intermediate **8** was complete in less than 3 h. However, this rapid conversion did not accelerate the rate of the overall reaction. One explanation for this is that the negative charge on the sulfonate group attracts HDTA molecules so that the amine component, peptide **5** is not able to contact with the peptide thioester **8** for the reaction to proceed efficiently.

Isolation of Fmoc-ORL1(288-370) (8)

Using the optimized conditions of Exp. 4 in the previous section, the author synthesized peptide **8** for use in further synthesis reactions (**Scheme 8**). In the preparation of peptide **8**, the reaction mixture was treated with DTT before being loaded on the column. This treatment does not change the shape the profile. The content of peak **c** in **Fig. 18B** was collected and subjected to amino acid analysis for the characterization. Due to the extremely hydrophobic nature of peptide **8**, the hydrolysis of the peptide was performed using the conditions reported by Tsugita *et.al* [7].



Scheme 8 Synthetic scheme for the preparation of peptide **8**.

The hydrolysis was carried out using a mixture of concentrated hydrochloride (33%) and TFA (2 :1) in an evacuated sealed tube at 166 °C for 50 min. The amino acid composition obtained in the analysis was as follows: $Asp_{4.3(7)}Thr_{1.9(4)}Ser_{1.4(7)}Glu_{4.0(5)}$ $Pro_{nd(4)}Gly_{(2)}Ala_{4.8(11)}Val_{5.4(8)}Ile_{2.0(3)}Leu_{4.7(8)}Tyr_{nd(2)}Phe_{3.6(5)}Lys_{1.4(4)}Arg_{3.3(7)}$. The calculated value is shown in parenthesis. It is obvious that the composition is not in agreement with the calculated composition. The possibility that the peptide component obtained from the peak **c** on the profile of **Fig. 18B** was a mixture can not be excluded. Further purification procedure, size exclusion chromatography, was applied for the peptide component(s). For the purification, a mixture of formic acid and water (2 : 3) was

used as the eluent. For loading the sample on the column, it was dissolved in a mixture of formic acid and water (2 :3, the eluent). The profile of the peptide component(s) is shown in **Fig. 21**.

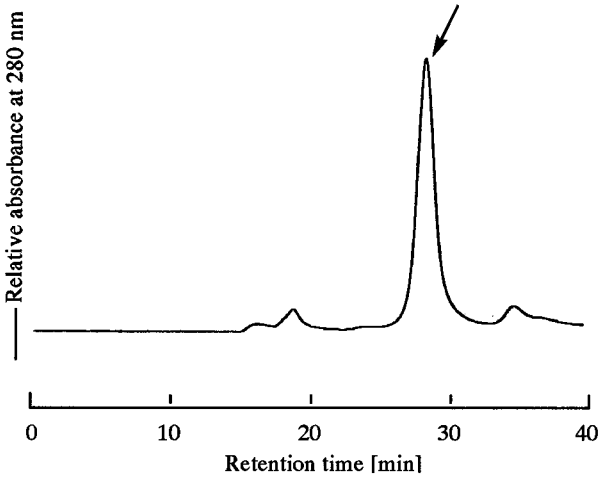


Fig. 21 Elution profile of the content of the peak **c** on Fig **18B**. Column : Superdex 75 10 / 300 GL(10 x 300 mm); eluent : formic acid-water (2 : 3); flow rate : 0.4 ml / min.

The peak with a retention time of 28 min (arrow) was collected and subjected to the amino acid analysis. The amino acid composition of the material in the peak was as follows : $Asp_{6.8(7)}Thr_{3.1(4)}Ser_{4.0(7)}Glu_{4.8(5)}Pro_{3.3(4)}Gly_{(2)}Ala_{11.2(11)}Val_{7.1(8)}$ $Ile_{2.8(3)}Leu_{8.2(8)}Tyr_{1.7(2)}$ $Phe_{5.1(5)}Lys_{3.4(4)}Arg_{6.7(7)}$. The composition is in agreement with the calculated value, and the result indicates that this purification procedure, using size exclusion chromatography is required for the isolation of peptide **8**.

The coupling yield for the synthesis of peptide **8** was calculated to be 25.8% based on peptide **3**.

Conclusion

Arg-tag, playing a role as a cation core, contributes to creating a dispersed solution, which results in a better resolution in the RP-HPLC purification and enhancing the solubility of the building block itself. The course of the experiments to optimize the conditions for the synthesis of the membrane protein by the native chemical ligation

method revealed some important factors that affect the synthesis. The key is the combination of thiol additive and detergent used. In this research, a combination of 2-mercaptoethane sulfonic acid and SDS at a concentration of 7 mM was found to be the most effective condition for the ligation reaction to proceed. Repulsion between the negative charge on the sulfonate of MESA involving the peptide thioester and SDS must have kept the thioester moiety bare out in the solution to be attacked by an amino component peptide building block (**Fig. 16**). The findings in this chapter will contribute to the further synthesis of ORL1 or membrane proteins.

Material and method

General

Boc-Leu-OCH_2-Pam resin was purchased from Applied Biosystems, Inc. (Foster City, CA). Amino acid derivatives were purchased from the Peptide Institute Inc. (Osaka, Japan).). Ether, used for washing of peptides, was peroxide-free (Nacalai Tesque, Kyoto, Japan). The amino acid compositions of peptides, except for peptide **5**, were analyzed using an L-8500 amino acid analyzer (Hitachi Ltd., Tokyo, Japan) after hydrolysis with a mixture HCl (35%) and trifluoroacetic acid (2 :1) at 166°C for 50 min [4-6] in an evacuated sealed tube. Hydrolysis of peptide 5 was carried out with constant boiling point hydrochloride for 24 h at 110°C. The peptide mass number was determined by MALDI-TOF mass spectrometry using a Voyager™DE (PerSeptive Biosystems, Inc., Framingham, MA).

Model experiment for introduction of Arg-tag

Peptide thioester **1** (1.0 mg, 0.7 μmol) and peptide **2** (0.3 mg, 0.06 μmol) were dissolved in a buffer containing 100 mM sodium phosphate, 6 M guanidine hydrochloride, and 1% thiophenol at pH of 7.0. The reaction was monitored by RP-HPLC [Cosmosil 5C18 AR-300 (4.6 x 150 mm), using a linear gradient of 0.1% TFA acetonitrile over 0.1% TFA water at a flow rate of 1.0 ml / min]. The results of the mass analysis and the amino acid analysis were as follow: MS found for *m/z* : 5576.9; calcd for $[M+H]^+$:5574.4; $Asp_{4.9}Thr_{2.1}Ser_{3.7}Glu_{3.0}Pro_{nd}Ala_{6.6}Val_{5.2}Ile_{1.0}Leu_{2.4}Phe_{3.2}Lys_{3.6}Arg_{5.8}$.

Synthesis of Boc-Gly-[Arg(Tos)]$_5$-Leu-OCH_2-Pam resin

Starting with the Boc-Leu-OCH_2-Pam resin (450 mg, 0.74 mmol / g), synthesis of Boc-[Arg(Tos)]$_5$-Leu-OCH_2-Pam resin, was performed manually in stepwise fashion by solid-phase method using *in situ* neutralization protocol in Boc chemistry [4]. Each synthetic cycle consisted of N^{α}-Boc removal by a 1- to 2-min treatment with neat TFA, 1- to 2- min flow wash with DMF, a 10- to 20-min coupling time with 1.2 mmol of preactivated Boc-amino acid in the presence of excess DIEA, and a second DMF wash. N^{α}-Boc-amino acids(1.0 mmol) were preactivated for 2 min with 1.0 mmol of HBTU in the presence of DIEA (1.4 mmol). After each coupling step, yields were determined by measuring the residual free amine content on the peptide-resin using a quantitative

ninhydrin assay

Synthesis of Boc-Ala-SCH_2CH_2CO-Gly-$(Arg)_5$-Leu-OCH_2-Pam resin

Boc-Gly-[Arg(Tos)]$_5$-Leu-OCH_2-Pam resin was treated with a 50% TFA / CH_2Cl_2 solution (1 x 5 min and 1 x 20 min), washed with CH_2Cl_2 (3 x 1 min). Following a wash with NMP for 0.5 min, the resin was treated with 5% DIEA in NMP (3 x 1min), and then washed with NMP (3 x 1 min). Trt-$SCH_2CH_2CO_2H$ (0.35 g, 1.0 mmol), HOBt-H_2O (0.15 g, 1.0 mmol), and DCC (0.20 g, 1.0 mmol) in NMP (10 ml) was mixed for 30 min and the resulting solution was added to the resin. The suspension was shaken for 1 h, followed by a ninhydrin assay measuring the residual free amine on the resin, and washed with NMP (3 x 1 min). The resin was treated with a NMP solution containing 10% acetic anhydride and 5% DIEA (1 x 10 min) and then washed with NMP (5 x 1 min). The resulting resin was treated with TFA containing 5% ethanedithiol (1 x 5 min, 1 x 10 min, 1 x 20 min, and 1 x 30 min) and then washed with CH_2CH_2 (3 x 1 min). Following a wash with NMP for 0.5 min, the resin was treated with 5% DIEA in NMP (3 x 1min), and then washed with NMP (3 x 1 min). A solution of Boc-Ala (0.19 g, 1.0 mmol), HOBt-H_2O (0.15 g, 1.0 mmol), and DCC (0.20 g, 1.0 mmol)in NMP (10 ml) was stirred for 1 h. This solution was added to the resin and the suspension mixed for 14 h, followed by a ninhydrin assay to measure the residual free amine content on the resin, and washed with NMP (3 x 1 min). The resin was treated with a NMP solution containing 10% acetic anhydride and 5% DIEA (1 x 10 min), washed with NMP (5 x 1 min) wash to give the Boc-Ala-SCH_2CH_2CO-Gly-$(Arg)_5$-Leu-OCH_2-Pam resin. (0.98 g, Ala content in the resin found to be 0.15 mmol / g).

Fmoc-ORL1(288-328)-SCH_2CH_2CO-Gly-Arg_5 -Leu-OH

Starting with the Boc-Ala-SCH_2CH_2CO-Gly-$(Arg)_5$-Leu-OCH_2-Pam resin, the synthesis of Fmoc-Leu-Gly-Val-Gln-Pro-Ser(Bzl)-Ser(Bzl)-Glu(Obzl)-Thr(Bzl)-Ala-Val-Ala-Ile-Leu-Arg(Tos)-Phe-Cys-Thr(Bzl)-Ala-Leu-Gly-Tyr(Br-Z)-Val-Asn-Ser(Bzl)-Cys-Leu-Asn-Pro-Ile-Leu-Tyr(Br-Z)-Ala-Phe-Leu-Asp(O*c*Hex)-Glu(OBzl)-Asn-Phe-Lys(Cl-Z)-Ala-SCH_2CH_2CO-Leu-OCH_2-Pam resin, was performed manually in stepwise fashion by solid-phase method using *in situ* neutralization protocol in Boc chemistry. Each synthetic cycle consisted of N^{α}-Boc removal by a 1- to 2-min treatment with neat TFA, a 1- to 2- min flow wash by DMF, a 10- to 20-min coupling time with 1.2 mmol of

preactivated Boc-amino acid in the presence of excess DIEA, and a second NMP wash. N^{α}-Boc-amino acids(1.0 mmol), except for N^{α}-Boc-Asn, were preactivated for 2 min with 1.0 mmol of HBTU (0.5 M in DMF) in the presence of DIEA (1.4 mmol). Preactivation of N^{α}-Boc-Asn was performed with HOBt (1.0 mmol) and DCC (1.0 mmol) for 30 min followed by the addition of DIEA (1.4 mmol) before coupling. After each coupling step, yields were determined by measuring residual free amine content on the peptide-resin with a quantitative ninhydrin assay. After coupling of the Gln residues, a DCM flow wash was used before and after deprotection by using TFA, to prevent possible high temperature (TFA/ DMF)-catalyzed pyrrolidone formation. The amount of fully protected peptide resin obtained was 1.31 g.

This fully protected peptide resin (300 mg) was deprotected and cleaved from the resin by treatment with a mixture of anhydrous HF (8.5 ml), anisole (0.75 ml) and 1,4-butanedithiol (0.75 ml) with stirring at 0 °C for 90 min. After evaporation of the HF under reduced pressure, ether was added to the mixture, the resulting precipitate was washed three times with ether and dissolved in TFA. This solution was passed through a glass filter and the product precipitated by the addition of cold ether. After washing the precipitate with ether, it was mixed with 50% aqueous acetonitrile and freeze-dried to give a crude powder (130 mg).

Purification was performed by RP-HPLC to give the purified Fmoc-ORL1(288-328)-SCH_2CH_2CO-Gly-Arg_5 -Leu-OH in the yield of 2.1%. MALDI TOF : found for *m* / *z* : 5710.8 (calcd for [M+H]+: 5711.6: amino acid analysis : $Asp_{4.0}Thr_{1.6}Ser_{1.9}Glu_{3.3}Pro_{nd}Gly_3Ala_{5.1}Cys_{nd}Val_{3.0}Ile_{1.8}Leu_{7.0}Tyr_{1.3}Phe_{3.5}Lys_{0.9}Arg_{5.6}$.

Synthesis of Boc-[Arg(Tos)]$_5$-Leu-OCH_2-Pam resin

Starting with the Boc-Leu-OCH_2-Pam resin (2.0 g, 0.44 mmol / g), synthesis of Boc-[Arg(Tos)]$_5$-Leu-OCH_2-Pam resin, was performed manually in a stepwise fashion by a solid-phase method using *in situ* neutralization protocol in Boc chemistry. Each synthetic cycle consisted of N^{α}-Boc removal by a 1- to 2-min treatment with neat TFA, a 1- to 2- min flow wash by NMP, a 10- to 20-min coupling time with 1.2 mmol of preactivated Boc-amino acid in the presence of excess DIEA, and a second NMP wash. N^{α}-Boc-amino acids(1.0 mmol) were preactivated for 2 min with 1.0 mmol of HBTU in the presence of DIEA (3.0 mmol). After each coupling step, yields were determined by measuring residual free amine on the peptide-resin using a quantitative ninhydrin

assay. The amount of obtained fully protected peptide resin was 3.6 g.

Synthesis of Boc-Ala-SCH_2CH_2CO-Gly-[Arg(Tos)]$_5$-Leu-OCH_2-Pam resin

Boc-[Arg(Tos)]$_5$-Leu-OCH_2-Pam resin (1.0 g) was treated with 50% TFA / CH_2Cl_2 solution (1 x 5 min and 1 x 20 min), washed with CH_2Cl_2 (3 x 1 min). Following a wash with NMP for 0.5 min, the resin was treated with 5% DIEA in NMP (3 x 1min), and then washed with NMP (3 x 1 min). A solution of Boc-Gly (0.31 g, 1.8 mmol), HOBt-H_2O (0.27 g, 1.8 mmol), and DCC (0.36 g, 1.8 mmol) in NMP (10 ml) was stirred for 1 h. This solution was added to the resin and the suspension mixed for 30 min, followed by a ninhydrin assay to measure the residual free amine content on the resin, and washed with NMP (3 x 1 min). The resin was treated with a NMP solution containing 10% acetic anhydride and 5% DIEA (1 x 10 min), washed with NMP (5 x 1 min). Trt-$SCH_2CH_2CO_2H$ (0.66 g, 1.8 mmol), HOBt-H_2O (0.27 g, 1.8 mmol), and DCC (0.36 g, 1.8 mmol) in NMP (10 ml) was mixed for 30 min and the resulting solution was added to the resin. The suspension was shaken for 1 h, followed by a ninhydrin assay to measure the residual free amine content on the resin, and washed with NMP (3 x 1 min). The resin was treated with a NMP solution containing 10% acetic anhydride and 5% DIEA (1 x 10 min) and then washed with NMP (5 x 1 min). Resulting resin was treated with TFA containing 5% ethanedithiol (1 x 5 min, 1 x 10 min, 1 x 20 min, and 1 x 30 min) and then washed with CH_2CH_2 (3 x 1 min). Following a wash with NMP for 0.5 min, the resin was treated with 5% DIEA in NMP (3 x 1min), and then washed with NMP (3 x 1 min). A solution of Boc-Ala (0.34 g, 1.8 mmol), HOBt-H_2O (0.27 g, 1.8 mmol), and DCC (0.36 g, 1.8 mmol) in NMP (10 ml) was stirred for 1 h. This solution was added to the resin and the suspension mixed for 14 h, followed by a ninhydrin assay to measure the residual free amine content on the resin, and washed with NMP (3 x 1 min). The resin was treated with a NMP solution containing 10% acetic anhydride and 5% DIEA (1 x 10 min), washed with NMP (5 x 1 min) and then with methanol (5 x 1 min), and dried under reduced pressure to give the Boc-Ala-SCH_2CH_2CO-Gly-[Arg(Tos)]$_5$-Leu-OCH_2-Pam resin (1.0 g, Ala : 0.23 mmol / g).

Fmoc-ORL1(288-328)-SCH_2CH_2CO-Gly-Arg$_5$-Leu-OH

Starting from Boc-Ala-SCH_2CH_2CO-Gly-[Arg(Tos)]$_5$Leu-OCH_2-Pam resin (0.8 g, Ala :

0.23 mmol / g), a synthesis of a protected peptide resin corresponding to the sequence of ORL1(288-328) was performed on a peptide synthesizer ABI 433A (Applied Biosystems, Inc.) according to the protocol of *t*-Boc HOBt / DCC on a 0.5 mmol scale with end capping by acetic anhydride. The first ten amino acids were coupled in a single coupling protocol and the remainder in a double coupling protocol. The amount of resulting protected peptide resin, Fmoc-Leu-Gly-Val-Gln-Pro-Ser(Bzl)-Ser(Bzl)-Glu(OBzl)-Thr(Bzl)-Ala-Val-Ala-Ile-Leu-Arg(Tos)-Phe-Cys-Thr(Bzl)-Ala-Leu-Gly-Tyr(Br-Z)-Val-Asn-Ser(Bzl)-Cys-Leu-Asn-Pro-Ile-Leu-Tyr(Br-Z)-Ala-Phe-Leu-Asp(OcHex)-Glu(OBzl)-Asn-Phe-Lys(Cl-Z)-Ala-SCH_2CH_2CO-Leu-OCH_2-Pam resin, was 1.20 g. The protected peptide resin (300 mg) was treated with a mixture of anhydrous HF (8.5 ml), anisole (0.75 ml), and 1,4-butanedithiol (0.75 ml) by stirring at 0 °C for 90 min. After evaporation of the HF under reduced pressure, ether was added to the mixture, and the resulting precipitate was washed with ether three times and then dissolved in TFA. The solution was passed through a glass filter and precipitated by the addition of cold ether. After washing the precipitate with ether, it was mixed with 50% aqueous acetonitrile and freeze-dried to give a crude powder of Fmoc-Leu-Gly-Val-Gln-Pro-Ser-Ser-Glu-Thr-Ala-Val-Ala-Ile-Leu-Arg-Phe-Cys-Thr-Ala-Leu-Gly-Tyr-Val-Asn-Ser-Cys-Leu-Asn-Pro-Ile-Leu-Tyr-Ala-Phe-Leu-Asp-Glu-Asn-Phe-Lys-Ala-SCH_2CH_2CO-Leu (159 mg). Purification was performed by RP-HPLC to give the purified Fmoc-ORL1(288-328)-SCH_2CH_2CO-Gly-Arg_5 -Leu-OH in the yield of 1.0%. MALDI TOF : found for *m / z* : 5710.8 (calcd for [M+H]+: 5711.6: amino acid analysis : $Asp_{4.0}Thr_{1.8}Ser_{2.0}Glu_{3.0}Pro_{nd}Gly_{2.9}Ala_5Cys_{nd}Val_{3.0}Ile_{2.0}Leu_{7.1}Phe_{3.5}Lys_{1.0}Arg_{5.8}$.

Synthesis of ORL1(329-370) (5)

Fmoc-Ala-CLEAR Acid Resin and amino acid derivatives were purchased from the Peptide Institute Inc. (Osaka, Japan).). Ether, used for washing the peptides, was peroxide-free (Nacalai Tesque, Kyoto, Japan). Peptide chain elongation was carried out by using a peptide synthesizer 433 A (Applied Biosystems, Inc.) according to the protocol of *FastMoc 0.25 mmol MonPrevPk* with end capping by acetic anhydride. The first 25 amino acids were coupled in single coupling protocol and the remainder in a double coupling protocol. The amino acid compositions of peptides were analyzed using an L-8500 amino acid analyzer (Hitachi Ltd., Tokyo, Japan) after hydrolysis with constant boiling point HCl for amino acid analysis (Nacalai Tesque) at 110°C for 48 h in

an evacuated sealed tube. The peptide mass number was determined by MALDI-TOF mass spectrometry using a Voyager™DE (PerSeptive Biosystems, Inc., Framingham, MA).

Starting with Fmoc-Ala-CLEAR Acid Resin (0.33 mmol / g, 0.80 g), a protected peptide resin corresponding to the sequence of peptide **5**, Cys(Trt)-Phe-Arg(Pmc)-Lys(Boc)-Phe-Cys(Trt)-Cys(Trt)-Ala-Ser(^{t}Bu)-Ala-Leu-Arg(Pmc)-Arg(Pmc)-Asp(O^{t}Bu)-Val-Gln(Trt)-Val-Ser(^{t}Bu)-Asp(O^{t}Bu)-Arg(Pmc)-Val-Arg(Pmc)-Ser(^{t}Bu)-Ile-Ala-Lys(Boc)-Asp(O^{t}Bu)-Val-Ala-Leu-Ala-Cys(Trt)-Lys(Boc)-Thr(^{t}Bu)-Ser(^{t}Bu)-Glu(OtBu)-Thr(tBu)-Val-Pro- Arg(Pmc)-Pro-Ala-CEAR Acid Resin was obtained (1.3 g). This peptide resin (100 mg) was treated with mixture of TFA, phenol, water, thioanisole, and ethanedithiol (82.5 : 5 : 5 : 5 : 2.5 (v / v), Reagent K) with stirring for 150 min. Ether was added to the reaction mixture followed by stirring for 20 min. The resulting precipitate was washed with ether three times and then dissolved in a mixture of water and acetonitrile. The solution was passed through the disposable ODS column and then freeze-dried to give a crude powder (21 mg). Purification of peptide **5** was performed on a Cosmosil 5C18ARII column (10 x 250 mm, Nacalai Tesque), and a linear garadient of 0.1% TFA acetinitrile over 0.1% aqueous TFA at a flow rate of 2.5 ml / min was employed. 27 mg of crude powder was applied to the column to give the purified peptide (4.6 mg) in a yield of 10%. [MS (MALDI TOF) found: m / z 4656.8. Calcd for $[M+H]^+$ 4656.5 (average). Amino acid analysis : $Asp_{3.0}Thr_{2.3}Ser_{3.5}Glu_{2.2}Pro_{1.5}Ala_6Cys_{nd}Val_{4.6}Ile_{1.0}Leu_{2.2}Phe_{1.9}Lys_{2.8}Arg_{5.6}$]

Synthesis of Fmoc-ORL1(288-370) via native chemical ligation

Peptide **3** (0.3 mg, 53 nmol) and **5** (0.3 mg 64 nmol) were dissolved in a ligation buffer (pH 7.2) containing 100 mM sodium phosphate, 34 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 60 mM thiol, and a detergent. As a thiol additive, 2-mercaptoethane sulfonic acid and thiophenol was used. For a detergent, SDS (35 mM and 7 mM) and hexadecyltrimethylammoniun bromide (0.9 mM) were used. The reaction was monitored by RP-HPLC [Cosmosil 5C4 AR-300 column (4.6 x 150 mm), using a linear gradient of formic acid and 1-propanol (4 : 1) over formic acid and water (2 : 3) at the flow rate of 0.65 ml / min]. Size exclusion chromatography was performed with Superdex 75 10 / 300 GL (10 x 300 mm) and a mixture of formic acid-water (2 : 3) was employed as the eluent at flow rate of 0.4 ml / min.

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Chapter IV

Synthesis of ORL(251-370)

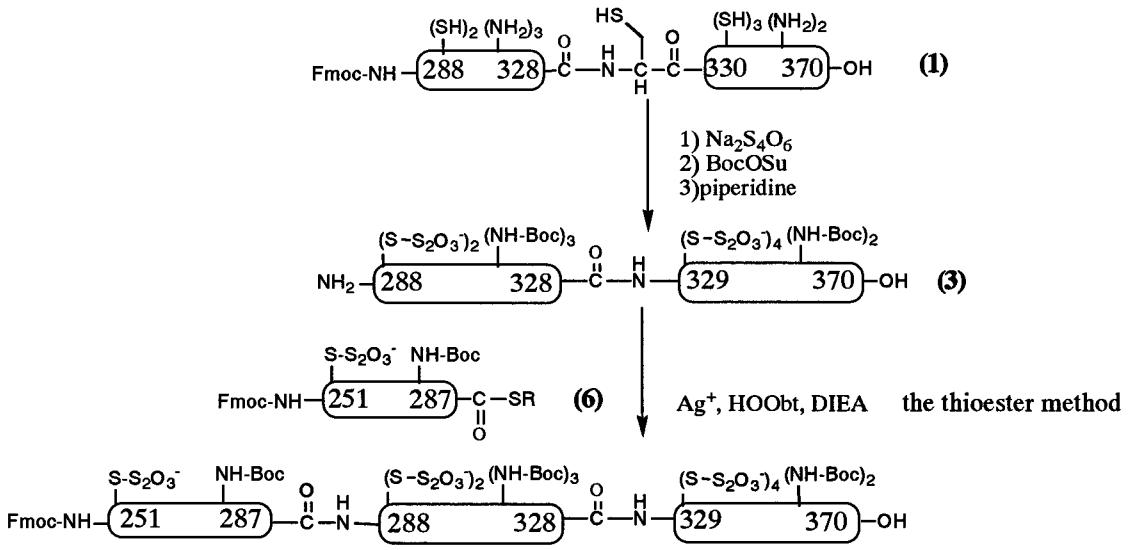
Introduction

The synthesis of ORL1(251-370) (**Fig. 1**) by the condensation of ORL1(251-287) and ORL1(288-370) via the thioester method is describe in this chapter. The synthesis was performed using the procedure outlined in **Scheme 1**. Through the experiment, the author evaluates the synthesis by the combination of different ligation chemistries, the thioester method and the native chemical ligation method.

Ser-Arg-Glu-Lys-Asp-Arg-Asn-Leu-Arg-Arg-**Ile-Thr-Arg-Leu-Val-Leu-Val-Val-Val-Ala-Val-Phe-Val-Gly-Cys-Trp-Thr-Pro-Val-Gln-Val-Phe-Val-Leu**-Ala-Gln-Gly^{287}↓Leu^{288}-Gly-Val-Gln-Pro-Ser-Ser-Glu-Thr-**Ala-Val-Ala-Ile-Leu-Arg-Phe-Cys-Thr-Ala-Leu-Gly-Tyr-Val-Asn-Ser-Cys-Leu-Asn-Pro-Ile-Leu-Tyr-Ala-Phe-Leu**-Asp-Glu-Asn-Phe-Lys-Ala-Cys-Phe-Arg-Lys-Phe-Cys-Cys-Ala-Ser-Ala-Leu-Arg-Arg-Asp-Val-Gln-Val-Ser-Asp-Arg-Val-Arg-Ser-Ile-Ala-Lys-Asp-Val-Ala-Leu-Ala-Cys-Lys-Thr-Ser-Glu-Thr-Val-Pro-Arg-Pro-Ala^{370}

Fig. 1 Amino acid sequence of ORL1(251-370). The transmembrane region is shown in bold and the coupling site is indicated by an arrow.

The experiment began with the preparation of Fmoc-[Cys($S_2O_3^-$)^{275,304,313,329,334,335,360}, Lys(Boc)^{327,332,354,361}]ORL1(288-370) a segment of which was synthesized via the native chemical ligation method described in the previous chapter. Another peptide thioester, Fmoc-ORL1(251-287)-SCH_2CH_2CO-Ala-Arg_5-Leu, was also prepared as a building block. The ligation reaction for the synthesis of ORL1(251-370) via the thioester method was then examined.



Scheme 1 Synthesis of ORL1(251-370)

Results and discussion

Preparation of [Cys $(S_2O_3^-)^{275,304,313,329,334,335,360}$, Lys $(Boc)^{327,332,354,361}$]ORL1(288-370)

The introduction of a thiosulfonate group to thiol groups on Fmoc-ORL1(288-370)(**1**) was performed using the procedure described in the chapter II with minor modifications. In chapter II, the introduction of thiosulfonate group to a thiol group was performed on a model peptide in DMSO in the presence of sodium tetrathionate. Under the conditions used, however, it was not possible to fully protect the thiol groups on peptide **1**. The possibility exists for an inter- or intramolecular disulfide bond formation between the thiol groups on peptide **1**. Instead of using DMSO as solvent, a buffer containing 0.1 M sodium phosphate and 6 M guanidine hydrochloride with sodium tetrathionate at pH 7.1 was used. The pH must be kept below 7.3, or the same phenomenon that occurred in the case of using DMSO results. Peptide **1** was soluble in the buffer. The introduction of the thiosulfonate was monitored by RP-HPLC. **Fig. 2A** and **B** show RP-HPLC elution profiles of the reaction mixture at reaction times of 0 min and 180 min respectively. Peak **a** corresponds to the starting material peptide **1**, whereas peak **b** the desired product, Fmoc-[Cys$(S_2O_3^-)^{275,304,313,329,334,335,360}$]ORL1(288-370) (**2**).

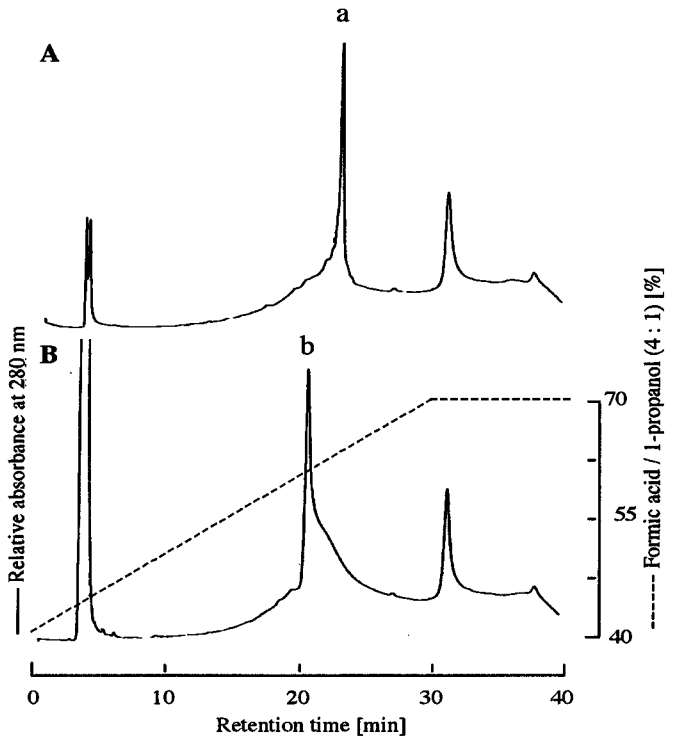
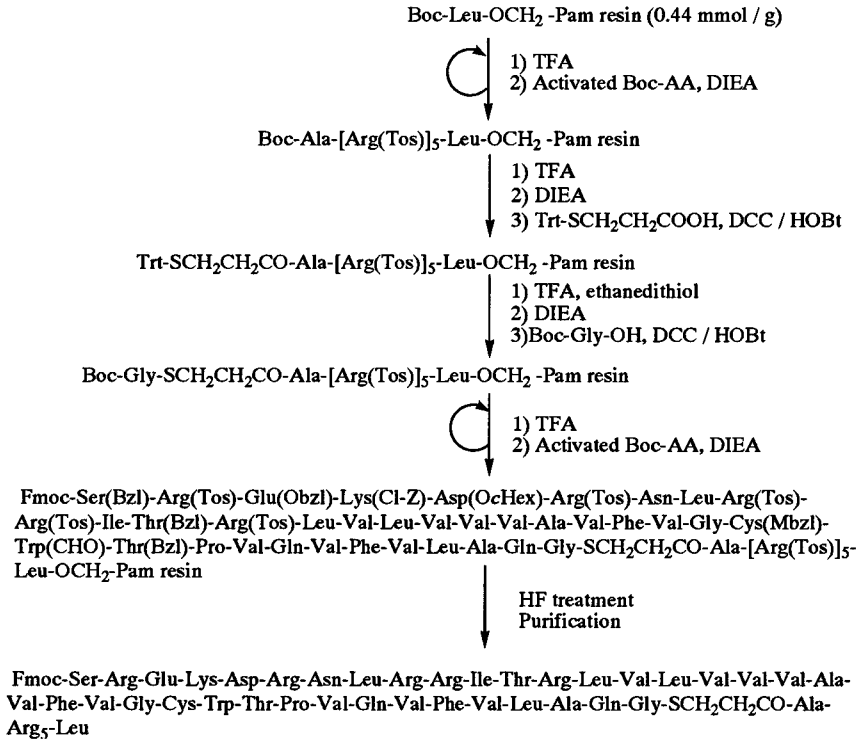


Fig. 2 RP-HPLC elution profile of the reaction mixture used to introduce a thiosulfonate to peptide 5 at a reaction time of 0 min (A) and 180 min (B). Column: Cosmosil 5Ph AR 300 (4.6 x 150 mm); linear gradient from formic acid-water (2 : 3) to formic acid -1-propanol (4 :1); Flow rate : 0.65 ml / min.

The introduction of Boc groups to amino groups on the side chain on peptide **2** was performed by mixing peptide thioester **2** and BocOSu in DMSO in the presence of DIEA (1%). However, the use of RP-HPLC to monitor the reaction and the purification failed because the product failed to elute from the column. Therefore, the rest of the process for the preparation was performed without a purification step. The Fmoc group was removed by piprodine treatment for 30 min, and the solvent was removed by evaporation under reduced pressure. To the residual solid, water containing TFA (0.1%) was added to remove piperidine from the residual solid and to precipitate the peptide component. This operation was performed for 3 times, and the precipitate was dissolved in a mixture of acetonitrile and water and then freeze-dried to give peptide component (**3**). Although the molecular mass and the amino acid composition were not measured, the peptide was used for further synthesis.

Preparation of Fmoc-[Cys($S_2O_3^-$)^{275}Lys(Boc)^{254}]ORL1(251-287)-SCH_2CH_2CO-Ala-Arg_5-Leu

Starting from a Boc-Leu-OCH_2-Pam resin, Fmoc-ORL1(251-287)-SCH_2CH_2CO-Ala-Arg_5-Leu (**4**) was synthesized by manual synthesis using *in situ* neutralization protocol [1]. The scheme for the synthesis of peptide **4** is shown in **Scheme 2**. As shown in **Scheme 2**, the introduction of Gly-SCH_2CH_2CO- was carried out using a the procedure reported by Kawakami *et.al.*[2]. Purification of peptide **4** was performed by RP-HPLC on a Cosmosil 5Ph AR-300 (10 x 250 mm) column using a linear gradient of formic acid-water (2:3) and formic acid–1-propanol (4:1). For the injection to the column, the crude powder was dissolved in acetonitrile. In **Fig. 3**, an analytical RP-HPLC profile of peptide **4** is shown. The major peak at 17 min (shown by an arrow) contained the desired peptide **4**.



Scheme 2 Synthesis of peptide building block **4** using *in situ* neutralization protocol.

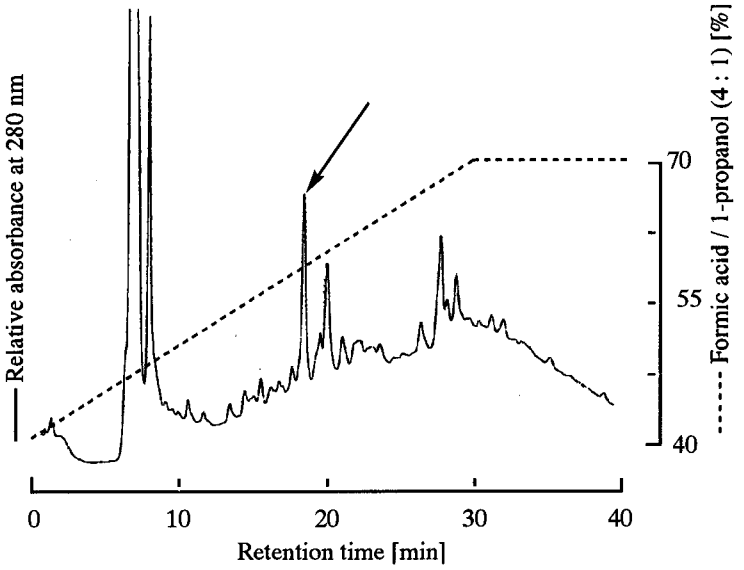


Fig. 3 RP-HPLC elution profile of crude peptide thioester **4**. Column Cosmosil 5Ph AR 300 (4.6 x 150 mm); linear gradient from formic acid-water (2 : 3) to formic acid -1-propanol (4 :1); Flow rate : 0.65 ml / min.

For the introduction of the thiosulfonate group, peptide thioester **4** was treated with $Na_2S_4O_6 \cdot 2H_2O$ in an aqueous buffer (pH 7.1) containing 0.1 M sodium phosphate and 6 M guanidium chloride to give Fmoc-[Cys($S_2O_3^-$)^{275}] ORL1(251-287)-SCH_2CH_2CO-Ala-Arg_5-Leu (**5**). The other peaks with retention times of 15 and 19 min were found to be byproducts resulting from impurities that could not be separated in the previous purification.

The introduction of a Boc group to the amino group on the side chain of peptide **5** was carried out by stirring the peptide thioester and BocOSu in DMSO in the presence of DIEA (1%). After a1 h reaction, the desired peptide thioester building block, Fmoc-[Cys($S_2O_3^-$)^{275}Lys(Boc)^{254}]ORL1(251-287)-SCH_2CH_2CO-Ala-Arg_5-Leu (**6**) was obtained. The reaction was monitored by RP-HPLC using 1-propanol and acetonitrile as the eluent instead of formic acid, since the Boc group is removed under acidic conditions.

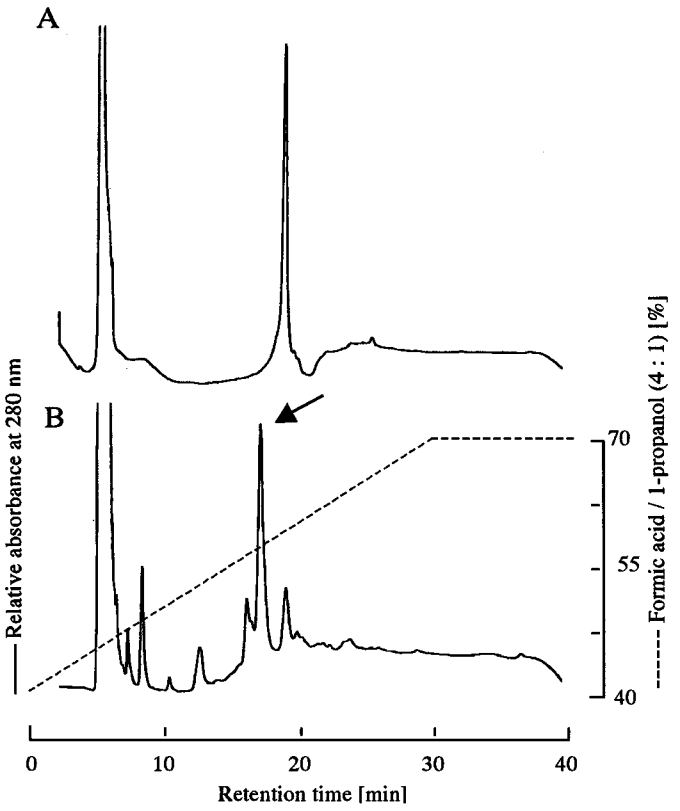


Fig. 4 RP-HPLC elution profile of the reaction mixture for the introduction of thiosulfonate to peptide. A : the profile at the reaction time of 0 h; B : the profile at the reaction time of 2 h. Column Cosmosil 5Ph AR 300 (4.6 x 150 mm); linear gradient from formic acid-water (2 : 3) to formic acid -1-propanol (4 :1); Flow rate : 0.65 ml / min.

Condensation by the thioester method

The condensation of peptides **3** and **6** was carried out in the presence of silver chloride, HOObt, and DIEA in DMSO. Peptide **3** was used in this reaction without purification by size exclusion chromatography. The reaction mixture was stirred for 24 h at room temperature and then subjected to analyses by SDS-PAGE and size exclusion chromatography. The results of the analyses are shown in **Fig. 5 and 6.** The SDS-PAGE results are shown in **Fig. 5**. Each band (a, b, and c) appeared on the gel can be characterized as follows: **a**, Fmoc-[Cys($S_2O_3^-$)^{275,275,304,313,329,334,335,360},Lys(Boc)^{254,327,332,354,361}]ORL1(250-370) (**7**, product calculated molecular mass : 14803.3); **b**,

peptide **3** (C terminal building block); **c**, peptide **6** (N-terminal building block). The size exclusion chromatography results are shown in **Fig. 6**. Peak **d** and **e** were collected for amino acid analysis.

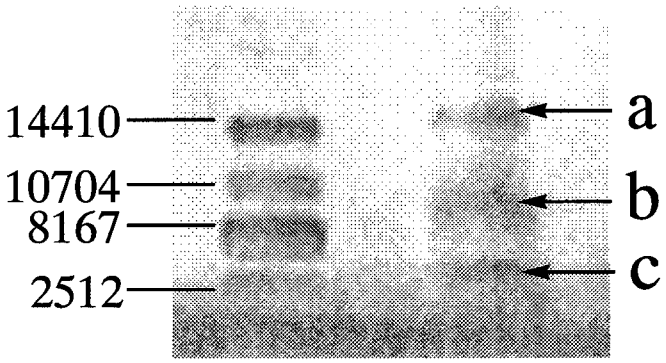


Fig. 5 SDS-PAGE gel showing the progress of the ligation reaction.

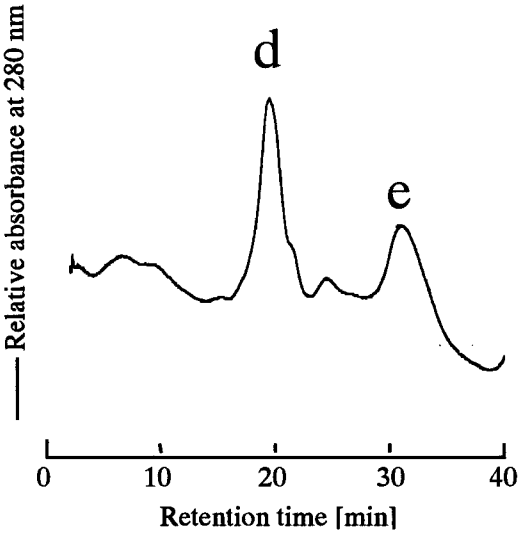


Fig. 6 Elution profile of the coupling reaction mixture after treatment with TFA. Column : Superdex 75 10 / 300 GL (10 x 300 mm); eluent : formic acid-water (2 : 3); flow rate : 0.4 ml / min.

Hydrolysis of the samples were carried out with a mixture of concentrated hydrochloride (33%) and TFA (2 :1) in an evacuated sealed tube at 166 °C for 50 min[3]. The result of the amino acid analysis is shown in **Table 1**.

Table 1 Result of amino acid analysis of the contents obtained in peak **d** and **e** on **Fig. 6**.

Peak	Amino acid composition
d	$Asp_{3.9}Thr_{1.8}Ser_{2.8}Glu_{2.8}\ Pro_{nd}Gly_{2}Ala_{4.2}Cys_{nd}Val_{4.2}Ile_{1.9}Leu_{4.9}Tyr_{nd}Phe_{2.9}Lys_{1.2}Arg_{3.4}$
e	$Asp_{8.2}Thr_{4.2}Ser_{7.8}\ Glu_{6.8}Pro_{nd}Gly_{4}Ala_{13.3}Cys_{nd}Val_{15.8}Ile_{2.7}Leu_{6.5}Tyr_{2.1}Phe_{9.0}Lys_{4.8}Trp_{nd}Arg_{14.3}$

The amino acid composition of the material peak **e** agreed with that of the desired product, peptide **7**. This result, together with the result from **Fig. 5**, indicates that the condensation reaction proceeded to give the product peptide **7**. The yield of the coupling reaction was found to be 9.3% based on peptide **6**. The amino acid composition for peak **d** agreed with the composition of the compound obtained from peak **c** in **Fig. 18B** from the chapter III. This compound, based on the finding in the previous chapter, is a mixture that contains ORL1(288-370). Since peptide **3** was applied to the reaction system without purification by size exclusion chromatography, the presence of the peak **d** in the profile is reasonable. It is noteworthy that, judging from the retention time of the profile from the size exclusion chromatography, the actual size of the desired product peptide **7** in the eluent system is somewhat smaller than that of the reactant, ORL1(288-370) (retention time of 28 min). One possible explanation for the phenomenon is oligomer formation. ORL1(288-370) may form an oligomer (homo-trimer or tetramer) that elutes faster than peptide **7** from the column.

Peptide **7** can be deprotected for further structure or function study of the protein, or can be used in a further synthesis.

Conclusion

Ligation by means of the thioester method described in this chapter permitted the synthesis of the peptide component corresponding to the sequence of ORL1(251-370).

During the synthesis of ORL1(251-370), Arg-tag was introduced to enhance the

solubility of the building block containing the transmembrane region in an aqueous solution, and the reaction conditions of the native chemical ligation method were optimized for use in the preparation of a membrane protein. The synthesis can also be used as the first synthesis of a polypeptide using different ligation chemistries, *i.e.*, the thioester method and the native chemical ligation method. However, it must be stated, based on the results here, that a standard technique for the preparation of a small peptide, such as RP-HPLC for the purification, is not always effective for the preparation of a relatively large polypeptide with a transmembrane region, whereas it works well for a soluble polypeptide of the same size. Furthermore, as the size of the reactant becomes larger, site specific or chemoselective ligation becomes more difficult. A strategy must be developed for the coupling site out to be used in the solvent.

To conclude this thesis, the techniques for handling transmembrane peptide or peptide building block and for the synthesis of a membrane protein as large as 14 kDa via the ligation method were successfully established. However, it was clearly shown that other techniques such as new auxiliary or ligation reactions on the lipid bilayer will be required for the total synthesis of larger membrane protein such as the G protein coupled receptor.

Material and merthod

General

Boc-Leu-OCH_2-Pam resin was purchased from Applied Biosystems, Inc. (Foster City, CA). Amino acid derivatives were purchased from the Peptide Institute Inc. (Osaka, Japan).). Ether, used for washing of peptides, was peroxide-free (Nacalai Tesque, Kyoto, Japan). The amino acid compositions of peptides were analyzed using an L-8500 amino acid analyzer (Hitachi Ltd., Tokyo, Japan) after hydrolysis with a mixture of concentrated hydrochloride and TFA (2 :1) for 50 min in an evacuated sealed tube. The peptide mass number was determined by MALDI-TOF mass spectrometry using a Voyager^{TM}DE (PerSeptive Biosystems, Inc., Framingham, MA). Polyacrylamide gel for SDS-PAGE was purchased from ATTO CORPORATION (Tokyou, Japan).

Preparation of Fmoc-ORL1(288-370) (1)

Fmoc–ORL1(288-328)-SCH_2CH_2CO-Gly-Arg_5-Leu (1.2 mg, 46 nmol) and ORL1(329-370) (1.2 mg, 70 nmol)were dissolved in a ligation buffer (pH 7.2) containing 100 mM sodium phosphate, 34 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 60 mM 2-mercaptoethane sulfonic acid, and 7 mM SDS. The mixture was stirred for 24 h at room temperature. The mixture was treated with DTT, and then loaded on the column for purification. The purification was performed by RP-HPLC (Column: Cosmosil 5C4 AR 300 (4.6 x 150 mm); A linear gradient from formic acid-water (2 : 3) to formic acid -1-propanol (4 :1); Flow rate : 0.65 ml / min.) to give the peptide component (1) (0.8 mg)

Preparation of Fmoc-[Cys($S_2O_3^-$)^{275,304,313,329,334,335,360}]-ORL1(288-370) (2)

Peptide **1** (0.8 mg) and $Na_2S_4O_6 \cdot 2H_2O$ (2.6 mg, 8.6 μmol) were dissolved in the aqueous buffer (pH 7.1) containing 0.1 M sodium phosphate and 6 M guanidine hydrochloride with sodium tetrathionate. The progress of the reaction was monitored and purified by RP-HPLC. The characterization of the desired product was performed by MS (MALDI TOF found for *m / z*: found : 9997.4; calcd for $[M+H]^+$: 9982.6).

Preparation of [Cys($S_2O_3^-$)^{275,304,313,329,334,335,360}, Lys(Boc)^{327,332,354,361}]ORL1(288-370)(3)

The introduction of Boc groups to amino groups to the side chain of peptide **2** was performed by mixing peptide thioester **2** and BocOSu (0.9 mg, 4.3 μmol) in DMSO in the presence of DIEA (1%). Removal of Fmoc group was accomplished by treatment with piperidine. The reaction was carried out for 30 min, and the solvent was removed by evaporation under reduced pressure. To the resulting material, a mixture of water and ethanol (1 :1) containing 0.1% TFA was added to remove the residual piperidine and to precipitate the peptide component. This operation was performed 3 times, and the precipitate was dissolved in a mixture of acetonitrile and water and freeze-dried to give peptide component **3** as a powder (0.2 mg)

Synthesis of Boc-Ala-[Arg(Tos)]$_5$-Leu-OCH$_2$-Pam resin

Starting with the Boc-Leu-OCH$_2$-Pam resin (352 mg, 0.44 mmol / g), the synthesis of Boc-[Arg(Tos)]$_5$-Leu-OCH$_2$-Pam resin, was performed manually in a stepwise fashion by the solid-phase method using *in situ* neutralization protocol in Boc chemistry. Each synthetic cycle consisted of N^{α}-Boc removal by a 1- to 2-min treatment with neat TFA, a 1- to 2- min flow wash with DMF, a 10- to 20-min coupling time with 1.2 mmol of preactivated Boc-amino acid in the presence of excess DIEA, and a second DMF wash. N^{α}-Boc-amino acids(1.0 mmol) were preactivated for 2 min with 1.0 mmol of HBTU in the presence of DIEA (3.0 mmol). After each coupling step, yields were determined by measuring the residual free amine on the peptide-resin with the quantitative ninhydrin assay.

Synthesis of Boc-Gly-SCH$_2$CH$_2$CO-Ala-[Arg(Tos)]$_5$-Leu-OCH$_2$-Pam resin

Boc-Ala-[Arg(Tos)]$_5$-Leu-OCH$_2$-Pam resin was treated with 50% TFA / CH_2Cl_2 solution (1 x 5 min and 1 x 20 min), washed with CH_2Cl_2 (3 x 1 min). Following a wash with NMP for 0.5 min, the resin was treated with 5% DIEA in NMP (3 x 1min), and then washed with NMP (3 x 1 min). Trt-SCH$_2$CH$_2$CO$_2$H (0.35 g, 1.0 mmol), HOBt-H$_2$O (0.15 g, 1.0 mmol), and DCC (0.20 g, 1.0 mmol) in NMP (10 ml) was mixed for 30 min and the resulting solution was added to the resin. The suspension was shaken for 1 h, followed by a ninhydrin assay to measure the residual free amine on the resin, and washed with NMP (3 x 1 min). The resin was treated with a NMP solution containing 10% acetic anhydride and 5% DIEA (1 x 10 min) and then washed with NMP (5 x 1 min). The resulting resin was treated with TFA containing 5% ethanedithiol (1 x 5 min,

1 x 10 min, 1 x 20 min, and 1 x 30 min) and then washed with CH_2CH_2 (3 x 1 min). Following a wash with NMP for 0.5 min, the resin was treated with 5% DIEA in NMP (3 x 1min), and then washed with NMP (3 x 1 min). A solution of Boc-Gly(0.18 g, 1.0 mmol), HOBt-H_2O (0.15 g, 1.0 mmol), and DCC (0.20 g, 1.0 mmol)in NMP (10 ml) was stirred for 1 h. This solution was added to the resin and mixed for 14 h, followed by a ninhydrin assay to measure the residual free amine on the resin, and then washed with NMP (3 x 1 min). The resin was treated with a NMP solution containing 10% acetic anhydride and 5% DIEA (1 x 10 min), washed with NMP (5 x 1 min) wash to give the Boc-Gly-SCH_2CH_2CO-Ala-$[Arg(Tos)]_5$-Leu-OCH_2-Pam resin.

Synthesis of Fmoc-ORL1(251-287)-SCH_2CH_2CO-Ala-Arg_5-Leu (4)

Starting with the Boc-Gly-SCH_2CH_2CO-Ala-$[Arg(Tos)]_5$-Leu-OCH_2-Pam resin, synthesis of Fmoc-Ser(Bzl)-Arg(Tos)-Glu(Obzl)-Lys(Cl-Z)-Asp(O*c*Hex)-Arg(Tos)-Asn-Leu-Arg(Tos)-Arg(Tos)-Ile-Thr(Bzl)-Arg(Tos)-Leu-Val-Leu-Val-Val-Val-Ala-Val-Phe-Val-Gly-Cys(Mbzl)-Trp(CHO)-Thr(Bzl)-Pro-Val-Gln-Val-Phe-Val-Leu-Ala-Gln-Gly-SCH_2CH_2CO-Ala-$[Arg(Tos)]_5$-Leu-OCH_2-Pam resin, was performed manually in a stepwise fashion by the solid-phase method using *in situ* neutralization protocol in Boc chemistry. Each synthetic cycle consisted of N^{α}-Boc removal by a 1- to 2-min treatment with neat TFA, a 1- to 2- min flow wash by DMF, a 10- to 20-min coupling time with 1.2 mmol of preactivated Boc-amino acid in the presence of excess DIEA, and a second DMF wash. N^{α}-Boc-amino acids (1.0 mmol), except for N^{α}-Boc-Asn, were preactivated for 2 min with 1.0 mmol of HBTU (0.5 M in DMF) in the presence of DIEA (3.0 mmol). Preactivation of N^{α}-Boc-Asn was performed with HOBt (1.0 mmol) and DCC (1.0 mmol) for 30 min followed by the addition of DIEA (3.0 mmol) before coupling. After each coupling step, yields were determined by measuring the residual free amine on the peptide-resin with the quantitative ninhydrin assay. After the coupling of Gln residues, a DCM flow wash was used before and after deprotection with TFA, to prevent possible high temperature (TFA/ DMF)-catalyzed pyrrolidone formation. The amount of obtained fully protected peptide resin was 0.89 g.

This fully protected peptide resin (300 mg) was deprotected and cleaved from the resin by a treatment with a mixture of anhydrous HF (8.5 ml), anisole (0.75 ml) and 1,4-butanedithiol (0.75 ml) with stirring at 0 °C for 90 min. After evaporation of the HF under reduced pressure, ether was added to the mixture, the resulting precipitate was

washed with ether three times and then dissolved in TFA. This solution was passed through a glass filter and precipitated by the addition of cold ether. After the precipitate had been washed with ether, it was mixed with 50% aqueous acetonitrile and freeze-dried to give the crude powder of Fmoc-Ser-Arg-Glu-Lys-Asp-Arg-Asn-Leu-Arg-Arg-Ile-Thr-Arg-Leu-Val-Leu-Val-Val-Val-Ala-Val-Phe-Val-Gly-Cys-Trp-Thr-Pro-Val-Gln-Val-Phe-Val-Leu-Ala-Gln-Gly-SCH_2CH_2CO-Ala-Arg_5-Leu (166 mg). Purification was performed by RP-HPLC to give the purified Fmoc-ORL1(251-287)-SCH_2CH_2CO-Ala-Arg_5-Leu in the yield of 1.9% (MS found : 5501.1 calcd for $[M+H]^+$: 5501.6; amino acid anaysis : $Asp_{1.7}Thr_{1.4}Ser_{0.6}Glu_{3.0}Pro_{nd}Gly_2Ala_{2.6}Cys_{nd}Val_{7.9}Ile_{1.0}Leu_{5.4}$ $Phe_{2.3}Lys_{0.7}Trp_{nd}Arg_{8.9}$).

Synthesis of Fmoc-[Cys($S_2O_3^-$)^{275}Lys(Boc) ^{254}]ORL1(251-287)-SCH_2CH_2CO-Ala-Arg_5-Leu

Peptide **4** (0.8 mg, 0.14 μmol) and $Na_2S_4O_6$ •$2H_2O$ (0.4 mg, 1.4 μmol)were dissolved in an aqueous buffer (pH 7.1) containing 0.1 M sodium phosphate and 6 M guanidium chloride, and stirred for 1 h to give Fmoc-[Cys^{275}($S_2O_3^-$)]ORL1(251-287)-SCH_2CH_2CO-Ala-Arg_5-Leu. The reaction was monitored by RP-HPLC [Cosomosil 5PhAR300 (4.6 x 150 mm, Nacalai Tesque), using a linear increasing gradient of formic acid-1-propanol (4 : 1) over formic acid-water (2 : 3) at a flow rate of 0.65 ml / min]. Purification of the desired peptide thioester building block was performed by RP-HPLC under the same condition. The desired product was characterized by MS (MALDI TOF found for *m* / *z*: 5613.0; calcd for $[M+H]^+$: 5613.7).

The peptide thioester and BocOSu (0.4 mg, 40 nmol) were dissolved in DMSO. DIEA (1%, *v* /*v*) was added to the solution followed by stirring for 1 h, while monitoring the reaction by RP-HPLC [Cosomosil 5PhAR300 (4.6 x 150 mm, Nacalai Tesque), using a linear increasing gradient of 1-propanol–acetonitrile (1:1) in 0.1% aqueous TFA at flow rate of 1.0 ml / min]. The DMSO was removed by evaporation under reduced pressure, and the reaction mixture was washed with ether. The precipitate was suspended in aqueous acetonitrile and freeze-dried to give the partially protected peptide thioester, Fmoc-[Cys($S_2O_3^-$)^{275}Lys(Boc)^{254}]ORL1(251-287)-SCH_2CH_2CO-Ala-Arg_5-Leu (30 nmol, MS found : 5714.4; calcd for $[M+H]^+$: 5714.8; amino acid composition : $Asp_{1.7}Thr_{1.4}Ser_{0.6}Glu_{3.0}Pro_{nd}Gly_2Ala_{2.6}Cys_{nd}Val_{7.9}Ile_{1.0}Leu_{5.4}$ $Phe_{2.3}Lys_{0.7}Trp_{nd}Arg_{8.9}$).

Condesation by the thioester method

Peptide **3** (0.2 mg), peptide **6** (0.1 mg, 4.0 nmol), HOObt (2.6 mg, 16 μmol) was dissolved in DMSO (400 μl). DIEA (1.8μl, 10 μmol) and AgCl (0.3 mg, 2.1 μmol) were then added to the solution followed by stirring for 48 h. The progress of the reaction was monitored by SDS-PAGE. The reaction solution (40 μl) was sampled and the solvent removed under reduced pressure. The residual solid was dissolved in the loading buffer (pH 8.0, 50 mM Tris•Cl, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and applied to the gel. The purification was performed by size exclusion chromatography. DMSO was removed from the reaction mixture under reduced pressure, and the residual solid was dissolved in a mixture of formic acid and water (2 :3) and then loaded on the column. The chromatography was performed with a column, Superdex 75 10 / 300 GL (10 x 300 mm)column, and a mixture of formic acid-water (2 : 3) was employed as the eluent at a flow rate of 0.4 ml / min.

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Publications

Takeshi Sato, Toru Kawakami, Kenichi Akaji, Hiroki Konishi, Koji Mochizuki, Toshimichi, Fujiwara, Hideo, Akutsu and Saburo Aimoto. Synthesis of a membrane protein with two transmembrane regions. *J.Peptide Sci.*, 2002, **8**, 172-180.

Takeshi Sato and Saburo Aimoto. Use of thiosulfonate for the protection of thiol groups in peptide ligation by the thioester method. *Tetrahedron Letters*, 2003, **44**, 8085-8087.

Presentation

Oral presentation

Takeshi Sato, Toru Kawakami, Kenichi Akaji, Koji Mochizuki, Toshimichi Fujiwara, Hideo Akutsu, and Saburo Aimoto; Synthesis of a Membrane Protein with Two Transmembrane Regions, F_1F_0 ATP synthase subunit c; 79th Spring Meeting of The Chemical Society of Japan, Konan University, Kobe, Japan, March, 2001

Sato, T., Saito, Y., Kawakami, T., Aimoto, S. Methodological studies for membrane protein synthesis. Focused on peptide segment preparation and segment condensation.The 5th Australian Peptide conference, Daydream Island, Australia, October, 2003

Poster presentation

Takeshi Sato, Toru Kawakami, Kenichi Akaji, Koji Mochizuki, Toshimichi Fujiwara, Hideo Akutsu, and Saburo Aimoto; Synthesis of transmembrane protein, F_1F_0 ATP synthase subunit c by the thioester method; 36th Japanese Peptide Symposium, Kyoto, October, 1999.

Takeshi Sato, Yasuhiro Saito and Saburo Aimoto; Chemical synthesis of a membrane protein; 18th American Peptide Symposium, Boston, USA, July, 2003.

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