공학박사학위논문

Synthesis of *o*-Nitrobenzyl Amine/Alcohol Derivatives for Solid-phase Organic Synthesis and Cell Membrane Protein Isolation

고체상 유기 합성과 세포막 단백질의 분리를 위한 오쏘-나이트로벤질 아민/알코올 유도체들의 합성

2014년 8월

서울대학교 대학원

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ABSTRACT

Synthesis of *o*-Nitrobenzyl Amine/Alcohol Derivatives for Solid-phase Organic Synthesis and Cell Membrane Protein Isolation

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Photochemistry is one of the unique and useful subdiscipline of chemistry. Photoreactive molecules, the core compounds in photochemistry, are undergone chemical reactions by irradiation of light, while remaining stable under various conditions such as acidic or basic reaction conditions. With such characteristic features, photoreactive molecules have been used as a linker for solid-phase organic synthesis or a photolabile protecting groups. Furthermore, the light with a wavelength above 315 nm does not give severe damages to biomolecules, and thus, the related photochemistry has been applied to chemical biology field.

In this thesis, *o*-nitrobenzyl amine/alcohol derivatives which can absorb

UVA light (365 nm) well are synthesized for solid-phase organic synthesis and isolation cell membrane proteins. In the first part, a novel photocleavable linker which contains *o*-nitrobenzyl amine moiety was effectively synthesized in six steps with 33 % of synthetic yield, without any complex purification steps. Synthesized photocleavable linker could absorb UVA light with wavelength of 330 to 370 nm well, and showed similar or better photocleavage kinetics compared with established photolinkers. Based on these results, synthesized photocleavable linker was successfully applied to solid-phase organic synthesis. Leu-enkephalin amide (H-YGGFL-NH_2) was synthesized by using photolinker-coupled polymer supports, with high purity. Acyl-phenylhydrazone of peptide *C*-terminus was oxidized by photo-oxidation of photocleavable linker, and peptide acid and ester were synthesized by addition of nucleophiles to acyl-phenyldiazene. Glycopeptides-immobilized polymer supports which can be applied to bioassays were prepared by imine formation reaction between glycans with peptides which coupled with polymer supports via photocleavable linker, and the glycopeptides were analyzed by mass spectroscopy analysis after photocleavage. The synthesized peptides on the photocleavable linker coupled polymer supports were analyzed by laser desorption-ionization mass spectroscopy method without using any additional cleavage steps and matrices.

In the second part of thesis, isolation method of the cell membrane protein by using *o*-nitrobenzyl alcohol moiety containing linkers is described. For the isolation of cell membrane protein, linkers which consisted of amine catchable part, photoreactive part, hydrophilic spacer, and tethering part for

immobilization were synthesized. As functional group for tethering, azide which can undergo the copper assisted azide-alkyne cycloaddition reaction and biotin which has high affinity with streptavidin were selected. The reactivity of synthesized molecules toward amine and alkyne was confirmed by a model reaction with amino acids and 4-pentynoic acid in solution phase. Copper assisted azide-alkyne reaction underwent between azide labeled lysine with 4-pentynoic acid in solution-phase; however, it did not undergo between azide-labeled bovine serum albumin with 4-pentynoic coupled polymer supports. Instead of azide-containing molecule, biotin-containing molecule was used for the isolation of proteins. Bovine serum albumin was labeled with biotin-containing molecule, and this labeled protein was bound with streptavidin which immobilized on the beads. And also, cell membrane proteins of *Escherichia coli* which were labeled with biotin-containing molecule were bound with streptavidin-coated beads. Bound proteins were released from the beads by irradiation of UVA light. Isolated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and compared with the results of authentic proteins.

Keywords: Photochemistry, *o*-Nitrobenzyl amine/alcohol derivatives, Solid-phase organic synthesis, Cell membrane protein

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ACA	aminocarproic acid
AcOH	acetic acid
ANP	3-amino-3-(2-nitrophenyl)propionic acid
BOP	benzotriazol-1-yloxy-tris(dimethylamino) phosphonium hexafluorophsphate
BSA	bovine serum albumin
CB[7]	cucurbit[7]ril
CHCA	alpha-cyano-4-hydroxycinnamic acid
2-CTC	2-Chlorotrityl chloride
CuAAC	copper assisted azide-alkyne cycloaddition
DCM	dichloromethane
DI water	deionized water
DIPEA	*N*,*N'*-diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMF	*N*,*N'*-dimethylformaide
DMSO	dimethylsulfoxide
DODT	3,6-dioxa-1,8-octanedithiol
DOLDI	direct on-bead laser desorption/ionization
DSC	*N*,*N'*-disuccinimidyl carbonate
E. coli	*Escherichia coli*
ESI	electrospray ionization
EtOAc	ethyl acetate
EtOH	ethanol
FITC	fluorescein isothiocyanate
Fmoc	9-fluorenylmethyloxycarbonyl
FT	Fourier transform
HBA	hydrazinobenzoic acid
HOBt	*N*-hydroxybenzotriazole
HOMO	highest occupied molecular orbital
HPLC	high-performance liquid chromatography
HRMS	high-resolution mass spectroscopy

IR	infrared
LC	liquid chromatography
LUMO	lowest unoccupied molecular orbital
MALDI	matrix-assisted laser desorption/ionization
MeCN	methyl cyanide
MeOH	methanol
MS	mass spectroscopy
NMR	nuclear magnetic resonance
NVOC	6-nitrovertryloxycarbonyl
OBOC	one-bead one-compound
Osu	*O*-succinimide
PBS	phosphate buffered saline
PBST	Tween 20 in phosphate buffered saline
PCA	photocleavable amino acid
PLL	photolabile linker
PNA	peptide nucleic acid
ProFiM	protein fishing molecule
Rbf	round-bottom flask
RBITC	rhodamine b isothiocyanate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SiNPs	silica nanoparticles
SPPS	solid-phase peptide synthesis
TFA	trifluoroacetic acid
TFAA	trifluoroacetic anhydride
THAP	2',4',6'-trihydroxyacetophenone
THF	tetrahyrdofuran
THPTA	tris(3-hydroxypropyltriazolymethyl)amine
TIPS	triisopropylsilane
TLC	thin layer chromatography
TOF	time of flight
UV	ultraviolet
UVA	ultraviolet A
Vis	visible

Synthesis of *o*-Nitrobenzyl Amine/Alcohol Derivatives for Solid-phase Organic Synthesis and Cell Membrane Protein Isolation

I. Introduction

I. 1. Basics of Photochemistry

I. 1. 1. Principles of photochemistry

In general chemical reactions, activation energies for the corresponding chemical reactions are essential. Several energy sources are used for providing the activation energies. Some chemical reactions can be processed with light as an energy source, and these reactions are called "photochemical reaction" which was named after their energy source, and the study on photochemical reactions is called "photochemistry".[1]

Light is a kind of electromagnetic wave, and it has a certain energy level which corresponds to its wavelength (Figure 1, a).[2] Light can be classified into three main categories according to its wavelength; i) ultraviolet (UV) light (light with a wavelength of 100 to 400 nm), ii) visible light (light with a wavelength 400 to 700 nm), and iii) infrared (IR) light (light with a wavelength 700 to 2500 nm) (Figure 1, b). Because the energy of the waves increase as the wavelength is decrease, UV light has the highest energy among them meanwhile IR light has the lowest. Light can give its energy to molecule which has a certain molecular orbital (MO) energy level that is suitable to receive the energy of the light. Energy levels of MO are determined by the chemical structure of molecules, especially the degree of conjugation. Energy gap between the highest occupied

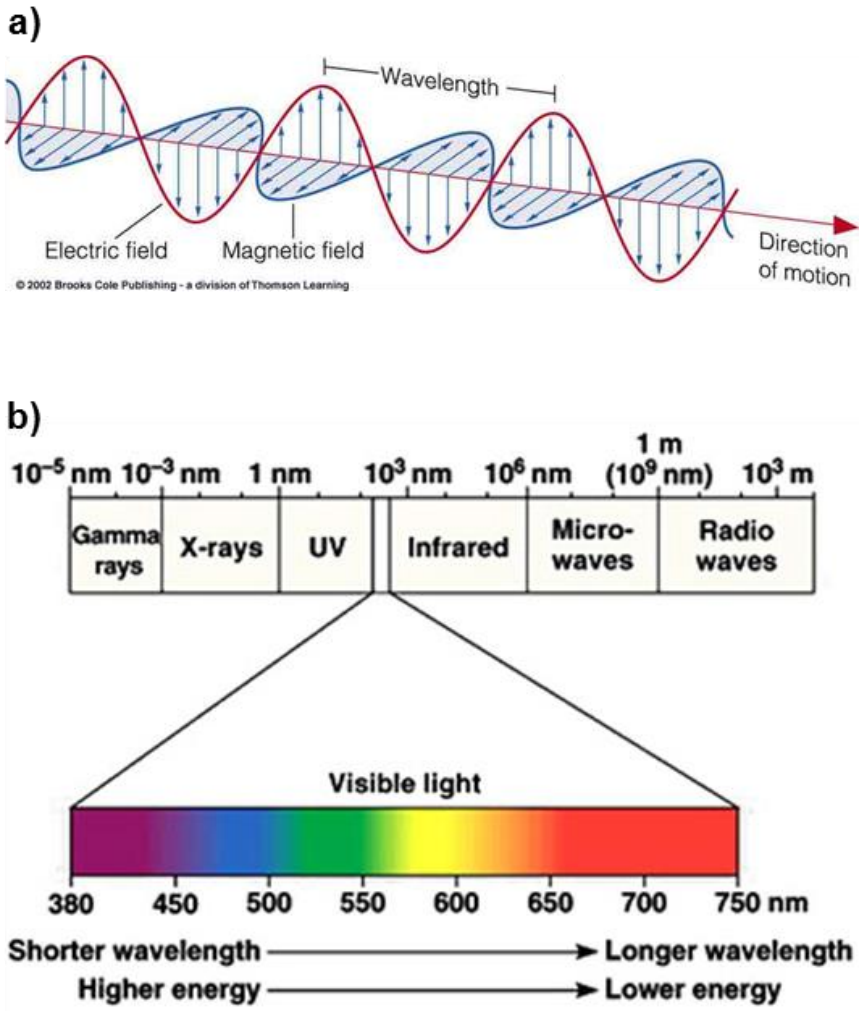


Figure 1. a) Shape of electromagnetic wave and b) spectrum of light**.**

molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) is decreased as the degree of conjugation is increased. For this reason, highly conjugated molecules can absorb light of long wavelengths, on the other hands, molecules has no conjugation structures like alkanes cannot absorb light with a wavelength of above 200 nm (Figure 2. a). For example, buta-1,3-diene which has the simplest conjugated system only can absorb the energy of light with a wavelength of around 217 nm or shorter, whereas fluorescein isothiocyanate (FITC), one of the most famous fluorescent dye, can absorb the energy of light with a wavelength of 495 nm (Figure 2. b).[3] When molecules absorb the energy of light, an electron which located at HOMO is transferred to LUMO. The overall electron configuration of this state is called "excited state", compared with "ground state" which is the original electron configuration of molecule (Figure 2).

Because the excited molecules are very reactive and unstable, they rapidly try to emit their excess energy in two ways. First one is transfer of excess energy to the surrounding system by the emission of electromagnetic wave which contains the excess energy of excited molecule (Figure 3. a). According to the energy level of excited molecule, the kinetic of energy transfer and the energy of electromagnetic are various. When plenty of vibrational and rotational energy levels exist in the excited molecule, energy transfer occurs step by step and the energy of

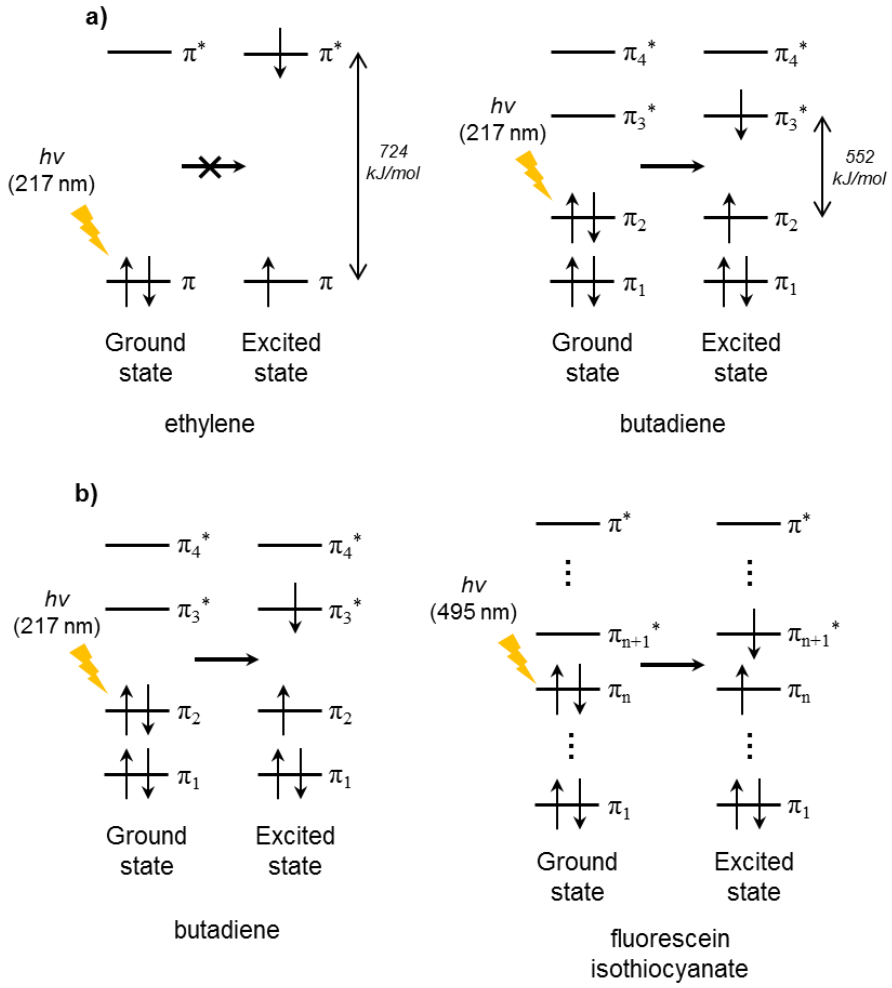


Figure 2. Electronic transiton of ethylene, butadiene, and FITC. a) Ethylene cannot be excited by irradiation the light with wavelength of 217 nm (left), meanwhile butadiene can be (right). b) butadiene can be excited by only UV irradiation (217 nm, left), meanwhile FITC can be excited by even visible light (495 nm, right).

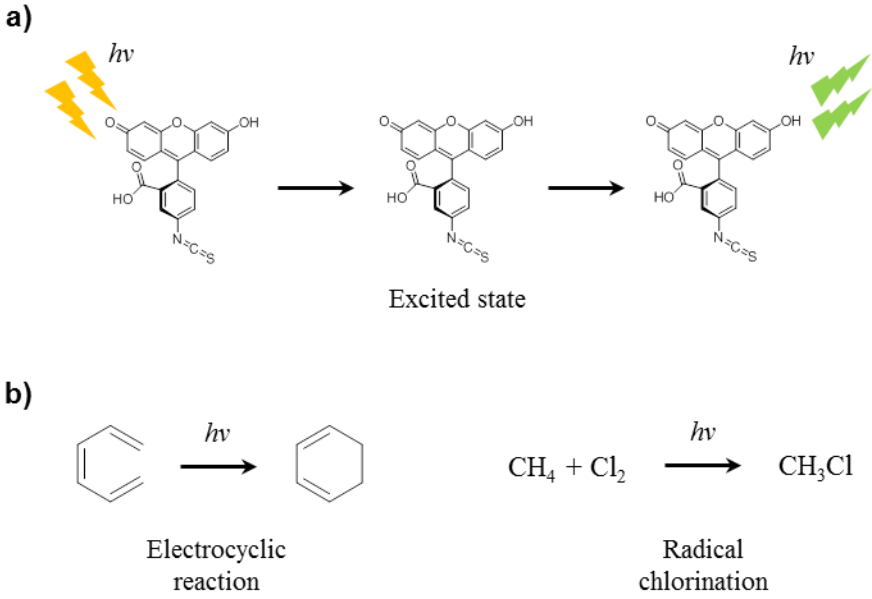


Figure 3. Two ways for emission the excess energy of excited molecule; a) transfer of excess energy to external system through emission of electromagnetic wave, b) transfer of excess energy through intra- (left) or inter- (right) molecular reactions.

electromagnetic wave is not too much because the energy gap of vibrational and rotational energy level is narrow. On the other hand, when only a few vibrational and rotational energy levels or only electronic energy level which has broad energy gap exist in the energy level of excited molecule, the energy transferal occurs directly and the energy of emitted electromagnetic wave is quite high. In the both cases, the energy level and the structure of molecules is almost the same as the unirradiated molecules after the energy transfer occurs. A typical molecule which follows this mechanism is called a fluorophore. According to their MO energy levels, fluorophores are excited by specific light source, emit their characteristic fluorescence, and are restored to the original molecular structure.

Second one is the transfer of excess energy through intra- or inter-molecular reaction (Figure 3. b). Due to their excess energy, the excited molecules can undergo the chemical reactions before the energy relaxation. Electrocyclic ring closure and ring opening reactions are typical intramolecular reactions, and radical halogenation which is initiated by UV light irradiation is a kind of intermolecular reactions. By undergoing the chemical reactions, the excess energy of excited molecules is transferred to the surrounding system in the form of heat of reaction.

I. 1. 2. Photoreactive molecules

Actually every molecules can absorb the light with a wavelength of 10 nm or shorter due to the HOMO-LUMO energy gap of them.[4] In spite of this theoretical background, actual photochemical reaction by irradiation of light with a wavelength of shorter than 200 nm is hardly undergone without special vacuum apparatus, because most of the light energy transferred to the nearby molecules during irradiation; nitrogen and oxygen molecules in the air.[5, 6] Because the photochemical reactions under this condition are not too practical, general definition of "photoreactive molecules" in photochemistry field is the molecule which can absorb the light with a wavelength of longer than at least 200 nm. The simplest photoreactive molecule is buta-1,3-diene which can absorb the energy of light with a wavelength of 217 nm. Benzene ring which has six pi electrons in a cyclic conjugated system can absorb light energy well with a wavelength of around 240 to 260 nm.[3] Due to this property, benzene ring derivatives also can absorb the energy of light with a wavelength of 240 nm or longer and undergo the photochemical reaction. Highly conjugated molecules also can absorb light with a wavelength around 250 nm as mentioned molecules. Moreover, they can absorb the long-wavelength (around 365 nm) UV light, visible light, and even the IR light if the HOMO-LUMO energy gap of the molecules is smaller than the energy of the corresponding light. These lights with a long-wavelength such as visible and IR light have not enough energy for breaking pi bond; therefore the photochemical reactions cannot occur. Instead, the visible lights are

emitted from the excited molecules along with the mechanism which explained at the previous section. With this property, FITC, rhodamine b isothiocyanate (RBITC), or Alexa fluor family of fluorescent dyes which have high degree of conjugations are widely used as fluorochromes. They can emit the characteristic visible lights after the excitation by irradiation of light with corresponding wavelength (Figure 4 and Table 1).[7, 8]

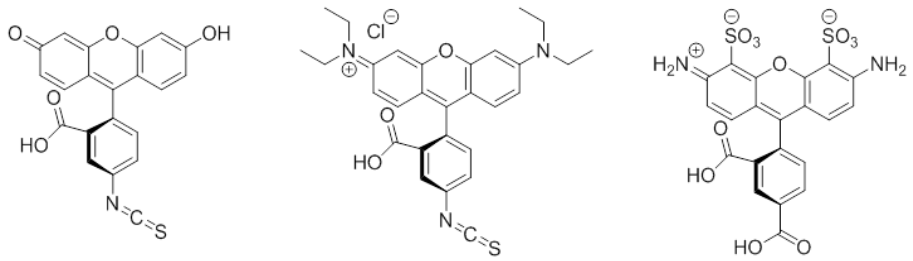


Figure 4. Structure of representable fluorephores, FITC, RBITC, and Alexa Fluor® 488.

Table 1. Specification of Common Fluorochromes

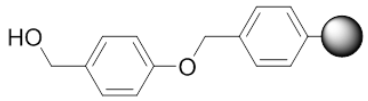
Fluorochrome	Fluorescence emission color	λ_{max} for excitation (nm)	Excitation laser line (nm)	λ_{max} for emission (nm)
Alexa Fluor® 405	Blue	401	360,405,407	421
Pacific Blue®	Blue	410	360,405,407	455
Alexa Fluor® 488	Green	495	488	519
FITC	Green	494	488	519
Phycoerythrin (PE)	Yellow	496, 546	488,532	578
PE-Texas Red®	Orange	496, 546	488,532	615
Texas Red®	Orange	595	595	615
Allophycocyanin (APC)	Red	650	595,633,635,647	660
Alexa Fluor® 647	Red	650	595,633,635,647	668
PE-Cy5	Red	496,546	488,532	667
Peridinin chlorophyll protein (PerCP)	Red	482	488,532	678
PerCP-Cy5.5	Far Red	482	488,532	695
PE-Cy7	Infrared	496,546	488,532	785
APC-Cy7	Infrared	650	595,633,635,647	785

I. 2. Photoreactive Linkers and Protecting Groups

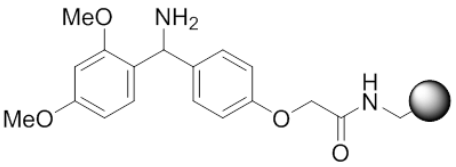
I. 2. 1. Photoreactive linkers

In the solid-phase peptide synthesis (SPPS), synthesized peptides are usually connected with solid supports via "linkers" which involves the releasing of synthesized peptides. Because the peptides must be conjugated with solid supports during synthesizing, linkers must be stable in the synthesis conditions. And also, synthesized peptides must be released without any unwanted modification. Therefore, linkers must be rearranged in specific conditions which do not harmful to the synthesized peptides.

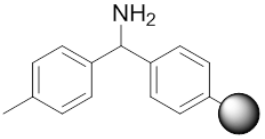
Various linkers or linker coupled polymer supports are developed and commonly used in the SPPS field such as Wang linker,[9] Rink amide linker,[10] 4-methylbenzhydrylamine (MBHA) linker,[11] trityl linker[12] and so on. These linkers are very useful for the mass production of peptides (Figure 5). Most of these linkers are acid-labile linkers so the synthesized peptides are released from the solid supports in the side chain deprotection step of 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry. This releasing of peptides is unavoidable, so it is impossible to prepare the peptides-conjugated solid supports with these linkers. Special linkers such as safety-catch linker[13, 14] was developed to overcome this problem, however,



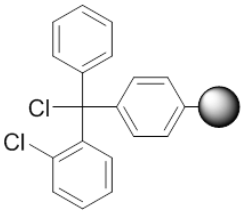
Wang linker coupled polymer support



Rink amide linker coupled polymer support



4-Methylbenzhydrylamine linker coupled polymer support



2-chlorotrityl chloride linker coupled polymer support

Figure 5. Commonly used linker-coupled polymer supports.

the synthesized peptides sometimes be damaged in the release condition of these linkers. For that, photoreactive linkers are supposed as alternative linkers. Because they are stable in acidic and basic conditions, and rearranged in the mild condition, "unwanted releasing" and "damage during releasing" problems can be overcome with them.

The first attempt to SPPS with "photoreactive linker" was reported by Rich *et al*. in 1973.[15] They introduce nitro groups to Merrifield resin[16] by simple nitration, and synthesize the peptide. Synthesized peptide was released from the resin by photolytic cleavage well. However, the photolytic cleavage yield was too low in case of longer peptide due to poor swelling properties which was originated from numerous polar nitro groups.[17] To overcome this problem, researchers used the poly(ethylene glycol) (PEG) as soluble supports.[18-20] Although the swelling problem was overcome with the soluble supports, isolation of the supports was too uncomfortable compared with in the case of SPPS. Eventually, researchers had started to find new method which can control the degree of nitration, and realized that the degree of nitration can be controlled by using "photoreactive linker" as building block, rather than direct nitration of the supports. Based on this enlightenment, two representable photoreactive linkers were reported in 1995; one is 4-[4-(1-Fmoc-aminoethyl)-2-methoxy-5-nitrophen-oxy]butanoic acid (Fmoc-photolabile linker, Fmoc-PLL) which reported by Holmes *et al.*,[21] and another one is *N*-Fmoc-3-

amino-3-(2-nitrophenyl)propionic acid (Fmoc-ANP linker) which reported by Geysen *et al*. (Figure 6. a).[22]

Fmoc-PLL was synthesized from acetovanillone with seven steps. Synthesized peptides are released as peptide amide form while PLL is converted to nitrosoacetophenone by light irradiation. Fmoc-PLL has two electron donating groups on its benzene ring, so it can absorb not only UV light but also UVA light which has longer wavelength (around 365 nm) well compared with Fmoc-ANP linker which has no electron donating groups. This property is a great advantage for bioapplications because UVA light is less harmful to biomolecules than UV light. In spite of this merit, however, Fmoc-PLL has a critical drawback. In the synthesis of Fmoc-PLL, reductive amination of ketoxime to amine underwent too slowly (Figure 6. b), and to top it off, the synthetic yield was too low without autoclave apparatus.

Fmoc-ANP was synthesized from 2-nitrobenzaldehyde, with only two simple reaction steps. Benzaldehyde moiety of 2-nitrobenzaldehyde is converted into 3-amino-3-phenyl moiety via Perkin-type condensation with malonic acid and ammonium acetate. This condensation reaction underwent fast, especially compared with reductive amination of Fmoc-PLL. As like Fmoc-PLL, synthesized peptides are released as peptide amide form and ANP is converted to nitrosoacetophenone by light irradiation. However, it can hardly absorb the UVA light (365 nm) because

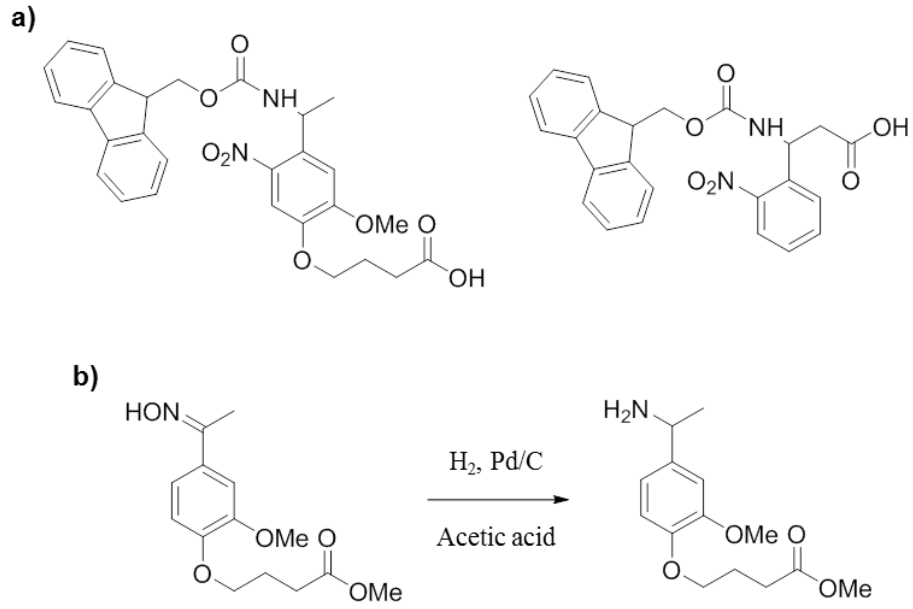


Figure 6. a) Representable established photolinkers; Fmoc-PLL (left) and Fmoc-ANP linker (right). b) Reductive amination step of Fmoc-PLL synthesis.

it has no electron donating groups. When the photolytic cleavage of Fmoc-Arg(Tos)-ANP resin was started with UVA light, only less than 20 % of Fmoc-Arg(Tos)-NH_2 was released after 15 minutes, and 50 % of Fmoc-Arg(Tos)-NH_2 was barely released after 3 hours.[22]

I. 2. 2. Photoreactive protecting groups

Protecting groups are important constituent in organic synthesis, especially for the synthesis of complex organic compound. As like linkers in SPPS, protecting groups must be stable in the reaction condition, and must be removed without any unwanted modification of synthesizing molecule. The specificity of removal condition of protecting group is important, because side reaction occurs when the unintended protecting groups are removed. In this point of view, photoreactive protecting groups are very useful in organic synthesis because they are orthogonal with acid or base labile protecting groups. For this purpose, various kinds of photoreactive protecting groups, for example, *o*-nitrobenzyl alcohol derivatives,[23-25] benzophenone derivatives,[26] benzyl alcohol derivatives,[27-30] fluorenecarboxylates,[31] cinnamyl esters,[32] vinylsilanes,[33] and so on, have been developed by many researchers (Figure 7).

Among the various photoreactive protecting groups, *o*-nitrobenzyl alcohol derivatives are commonly used. The most popular photoreactive

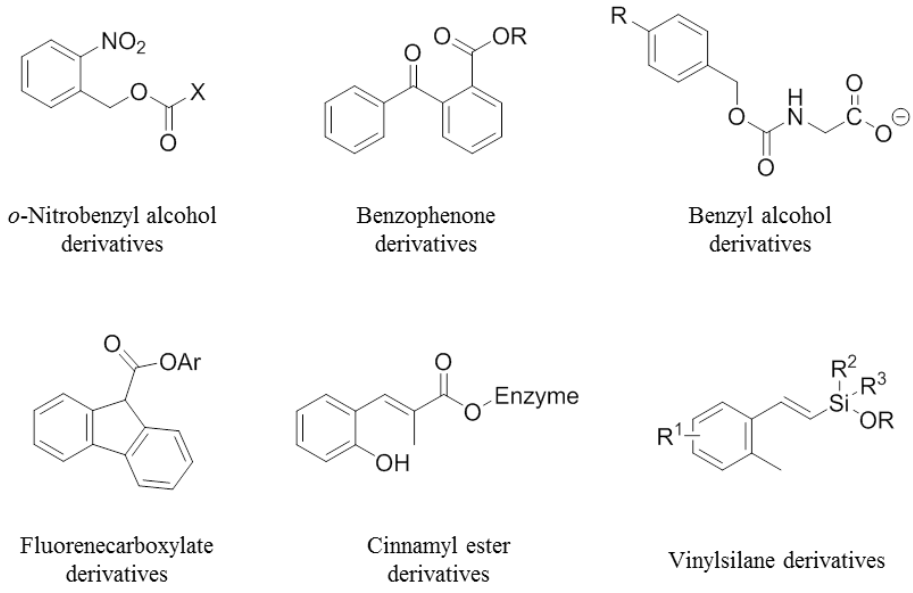
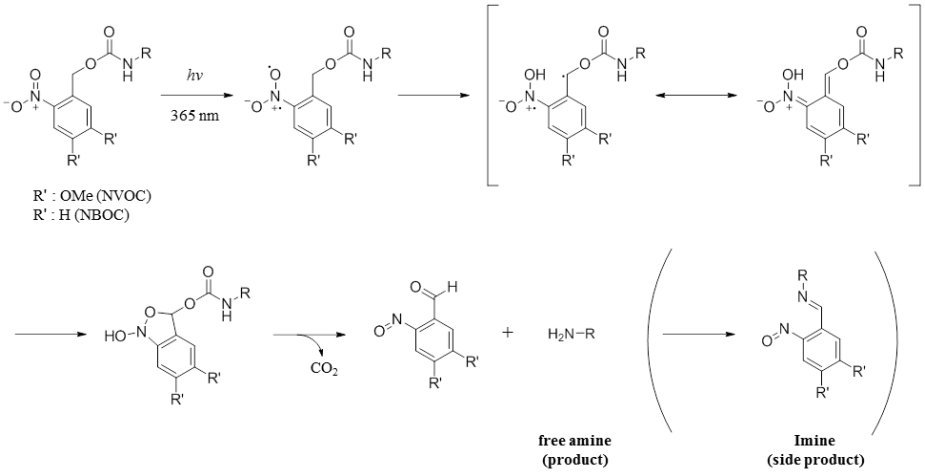


Figure 7. Commonly used photoreactive protecting groups.

protecting group is 6-nitrovertroyloxycarbonyl (NVOC) group, which is reported by Woodward *et al*.[23] NVOC commonly used for the protection of amino group, especially α-amino group of amino acid. In the deprotection step, the removal mechanism of NVOC is similar with it of PLL linker because it has almost same chemical structure with PLL linker. As shown at Scheme 1, NVOC is converted to highly reactive diradical species by UVA light (365 nm) exposure. Protected amino group is deprotected by radical rearrangement of excited NVOC, and as the side product, *o*-nitrosobenzaldehyde and carbon dioxide are generated. Nitrobenzyloxycarbonyl (NBOC)[25] group which has no electron donating groups on its benzene ring is also used as photoreactive protecting groups. NBOC and ANP linker are similar in the way that NVOC and PLL linker are, therefore, NBOC can hardly absorb the UVA light (365 nm) due to the same reason with ANP linker. NVOC and NBOC have been used for the protection of not only for amino group of amino acids, but also other functional groups such as imidazole group in histidine, phosphate group in nucleotide monomers, hydroxyl groups in carbohydrates, and even ketones.

When NVOC or NBOC are removed from the protected amino groups, however, a critical side reaction occurs due to the side product. Deprotected amino groups can easily react with benzaldehyde derivatives which are the side product of NVOC and NBOC, and be converted into imine (Scheme 1). To suppress this side reaction, hydrazide derivatives

such as semicarbazide hydrochloride are added into the reaction solution as benzaldehyde scavenger. And also, avoiding the optimal pH for imine formation (around pH 4.5 to 5.5)[34] with diluted sulfuric acid can be another solution.[23]



Scheme 1. Deprotection process of NVOC and NBOC.

I. 3. Applications of Photochemistry

I. 3. 1. Photochemistry in solid-phase peptide synthesis

Since the SPPS method was invented by R. B. Merrifield in 1963, it has been used in the various research fields such as deoxyribonucleic acid/ribonucleic acid (DNA/RNA) synthesis, enzyme substrate screening, bioactive molecule synthesis, and so on.[16] In the early stage of SPPS, the desired peptides were synthesized on polymer supports and cleaved before their biological activity test or identification of their sequences.[35, 36] Synthesis and cleavage of the peptides have been successful with acid/base labile protecting groups and proper use of linkers. Naturally, photochemistry could not attract the researchers' attention at early stage of SPPS. However, the merits of photochemistry was started to receive attention recently.

Lam *et al.* reported preparation of one-bead one-compound (OBOC) peptide library with just "split-and-mix" the solid supports during synthesizing the peptides (Figure 8) and on-bead assay with it in 1991.[37] A numerous kind of peptides candidates (# of amino acid candidates to the power # of synthesis cycle, theoretically) could be synthesized easily, and the polymer supports which contain targets' peptide substrates could be screened quickly among the candidates. However, compared with the ease of screening the polymer supports which contain the target peptides,

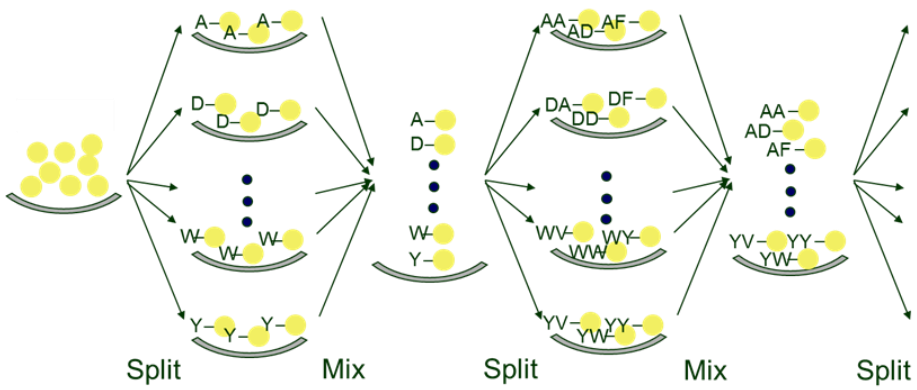


Figure 8. Preparation of OBOC peptide library with "split-and-mix" method.

identification of screened peptides was too difficult in this method. Because the synthesized peptides must be immobilized on the polymer supports till the end of on-bead assays, common linkers such as acid/base labile linkers could not be used. Alternatively, Edman degradation method or small molecule tagging method were used for the identification of screened peptides, but these methods required long analysis time or additional reaction step.[38-42] To overcome these problems, Kim *et al.* prepared an OBOC peptide library with Fmoc-PLL coupled polymer supports (Figure 9).[43] Due to the robustness of Fmoc-PLL in acidic and basic conditions, peptides were not swept away while they were synthesized. After on-bead assay and screening the target polymer supports, peptides were released from the polymer supports by UVA light exposure and identified. Compared with previously described method, screened peptides were identified very quickly because the photocleavage time was very short (10 to 30 minutes). The peptide substrates and unknown protein substrates for tyrosine kinases ($p60^{c\text{-}src}$ and ZAP-70) were screened with prepared OBOC peptide library. This Fmoc-PLL used OBOC peptide library was also applied for the screening of peptide substrates for other kinases such as Brk,[44] protein kinase A, and Yak1.[45, 46]

Photolabile protecting groups have not been used commonly in SPPS, but it is certain that they are useful in synthesizing peptides on a chip by using optical system. Unlike the case of general chemical reaction in a

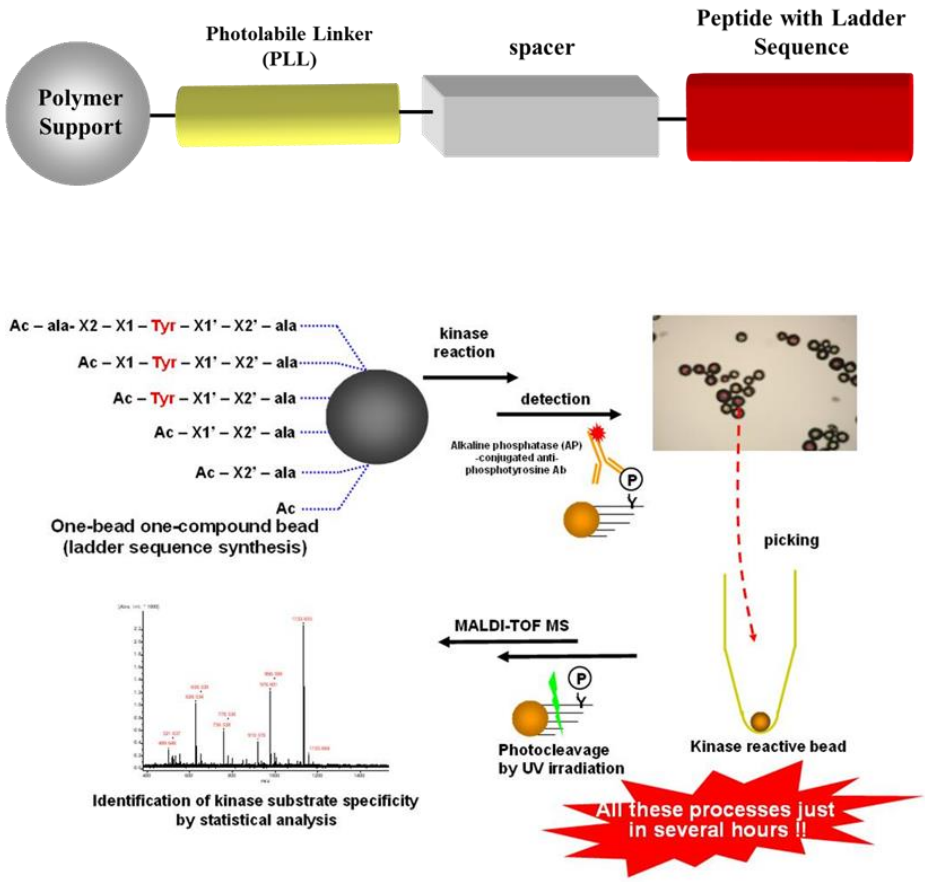


Figure 9. Outline of prepared OBOC peptide library (above) and method for screening of tyrosine kinase substrate (below).

flask, reaction volume on a chip can be reduced to microliter-size and the chip can be integrated very easily for the photochemical reaction, which is controlled by UV irradiation. Shin *et al.* and Liu *et al.* prepared peptide and peptide nucleic acid (PNA) microarrays on a slide glass using automatic micromirror array system (Figure 10).[47, 48] In this micromirror array system, the specific micro-sized area on the NVOC coated slide glass was irradiated by UVA light, and the NVOC groups on the exposed area were removed.[23] Then, the next NVOC-protected amino acids or PNA monomers were treated to the slide glass, they were coupled to the deprotected amino groups on the UV exposed area (Figure 11. a). Peptide and PNA microarrays were thus prepared through repetition of the deprotection and coupling process, and some model biological experiments were performed with the prepared microarrays (Figure 11. b-d).

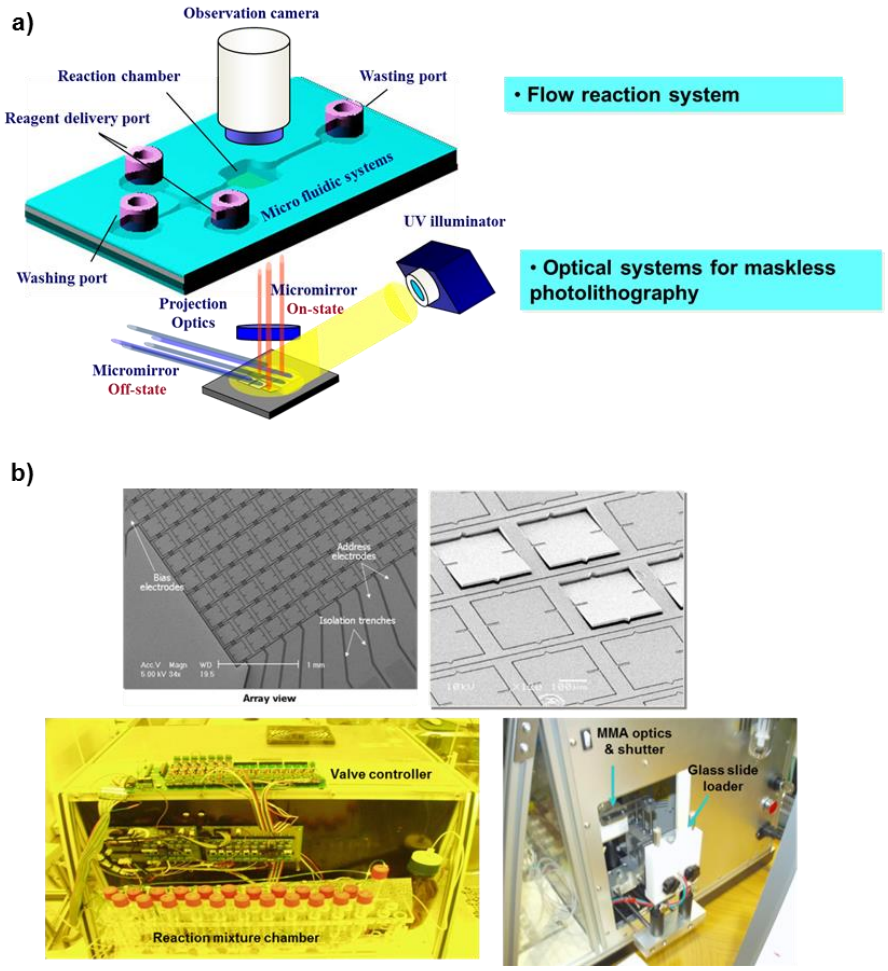


Figure 10. Automatic peptide/PNA microarray fabrication system; a) overview of reaction chamber, b) images of micromirror array system.

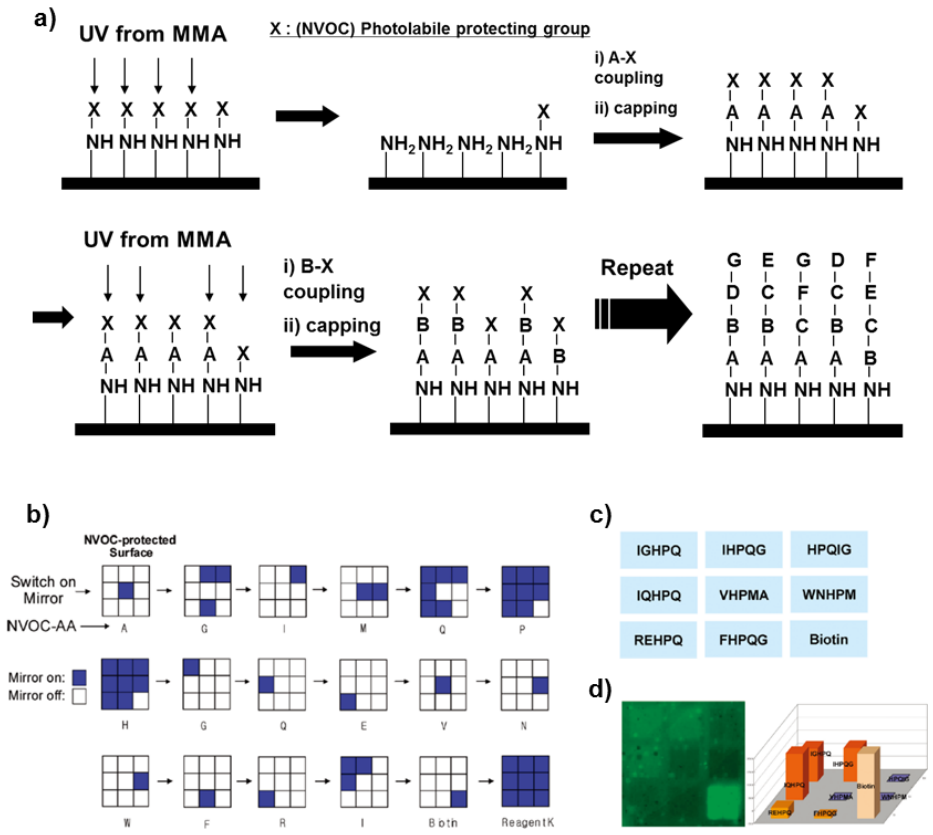


Figure 11. Fabrication of peptide microarray and its bioapplication; a) selective deprotection and coupling with micromirror array system, b) mask design and NVOC-amino acid coupling, c) fabricated peptide microarray, d) fluorescence image of peptide microarray after Cy3-streptavidin binding and quantitative analysis of fluorescence.

I. 3. 2. Photochemistry in chemical biology

Most biomolecules can be damaged by harsh external conditions, for example strong acidic or basic condition. Therefore, the reaction involving biomolecules must be performed under the physiological conditions. Considering this, it is not strange that the photochemistry has been applied to the chemical biology field. Among various applications, photoconjugation is one of the representative photochemical reactions in chemical biology. The literal meaning of photoconjugation is the conjugation reaction mediated by light irradiation, but in the chemical biology field, the meaning of photoconjugation is restricted to the conjugation reaction between the biomolecules and others. This photoconjugation is usually performed via four representative reactions; radical addition,[49] nitrene addition,[50-52] carbene addition,[53-56] and oxime ligation.[57] In most of the radical addition for photoconjugation, benzophenone has been used as the radical generator. According to Dorman *et al.*, C=O double bond of benzophenone is broken by n-π^* transition which is caused by absorption of UVA light. As result, benzophenone is converted to the diradicaloid triplet state (Figure 12. a).[49] This diradicaloid triplet state is very reactive due to the electron-deficient oxygen n-orbital, so it abstracts the hydrogen from the C-H bond of nearby molecules. The remaining radicals after hydrogen extraction are recombined and as a result, new covalent bond is formed. This

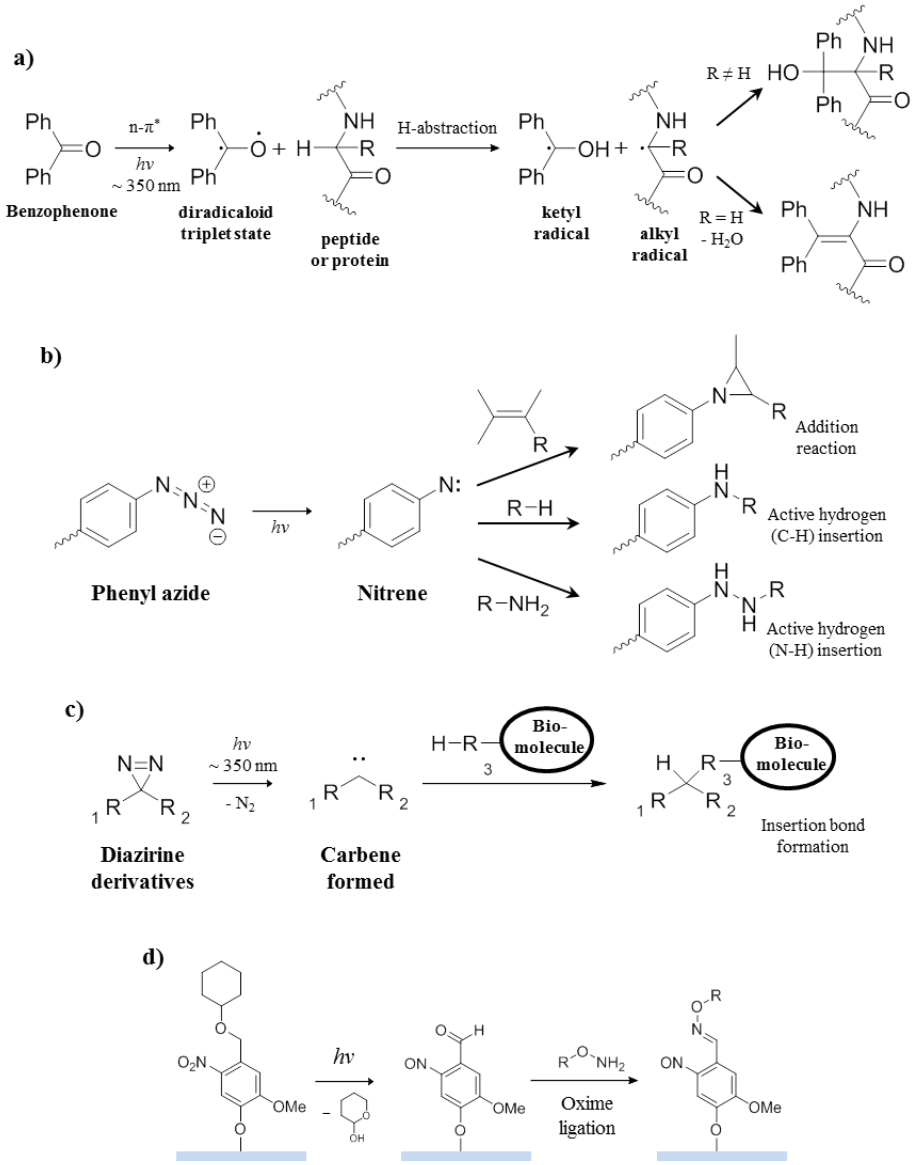


Figure 12. Photoconjugation reactions in the chemical biology by using a) benzophenone, b) aryl azides, c) diazirine derivatives, and d) oxime ligation method.

photoconjugation with benzophenone is favored due to its mild excitation condition, and various applications have been reported.

For the nitrene addition reaction, aryl azide derivatives are used as the nitrene source. When the light with a wavelength of around 250 to 350 nm irradiates aryl azide derivatives, the azide groups (of aryl azide) are converted into nitrene with the loss of nitrogen gas (Figure 12. b).[52] The generated nitrenes can be reacted with nearby molecules and make new covalent bonds via addition reaction, active hydrogen (C-H or N-H) insertion, and so on. Phenyl azide (unsubstituted aryl azide) is not suitable for the photoconjugation because it is converted into nitrene under UV light with short-wavelength (around 250 to 280 nm) which can damage biomolecules. However, nitrophenyl azide can be converted into nitrene under UVA light with long-wavelength (around 350 to 400 nm), and is suitable for photoconjugation.

Carbenes for the conjugation reaction are originated from diazirine derivatives. When the light with a wavelength of around 350 nm is irradiated to aryl or alkyl diazirine derivatives, diazirine groups are converted into nitrogen gas, and as a result, carbenes are formed (Figure 12. c).[56] Generated carbenes can be reacted with nearby molecules via simple insertion, without any side reactions such as ring expansion and self-dimerization which can occur in the previous photoconjugation method. Furthermore, diazirine moiety has better photo-stability and less HOMO-LUMO energy gap than phenyl azide groups. Therefore, the

diazirine derivatives are more suitable for photoconjugation than phenyl azide derivatives.

Recently, photoconjugation via oxime ligation method was reported by Pauloehrl *et al*. (Figure 12. d).[57] They introduced *o*-nitrobenzyl acetal on a solid support, and converted them into benzaldehyde by selective UVA light irradiation. After the UV exposure, aminooxy-functionalized molecules were immobilized onto the UV irradiated site via oxime ligation. This result is notable because the suggested immobilization method has selectivity. Benzaldehydes are reacted with amine-like functional groups such as amino-, aminooxy-, and hydrazino groups. Therefore, only specific biomolecules which have these functional groups can be conjugated, whereas "any" organic molecules could be conjugated with the previously mentioned photoconjugation method.

Not only these conjugation reaction, detachment reaction and photo-triggered reaction can be performed with photochemistry, and a number of papers on these topics have been reported. In detachment reaction, biomolecules are bound to their receptors which are conjugated to the solid supports via photolinkers. The biomolecule-bounded receptors are released from the solid supports, by site-specific UV light irradiation (Figure 13. a). Wang *et al*. reported the releasing method of biomolecules which are immobilized on a glass surface via photolinker with two-photon excitation. Immobilized biomolecules were released by irradiation of light with a wavelength of 750 nm which is less harmful to the biomolecules.[58]

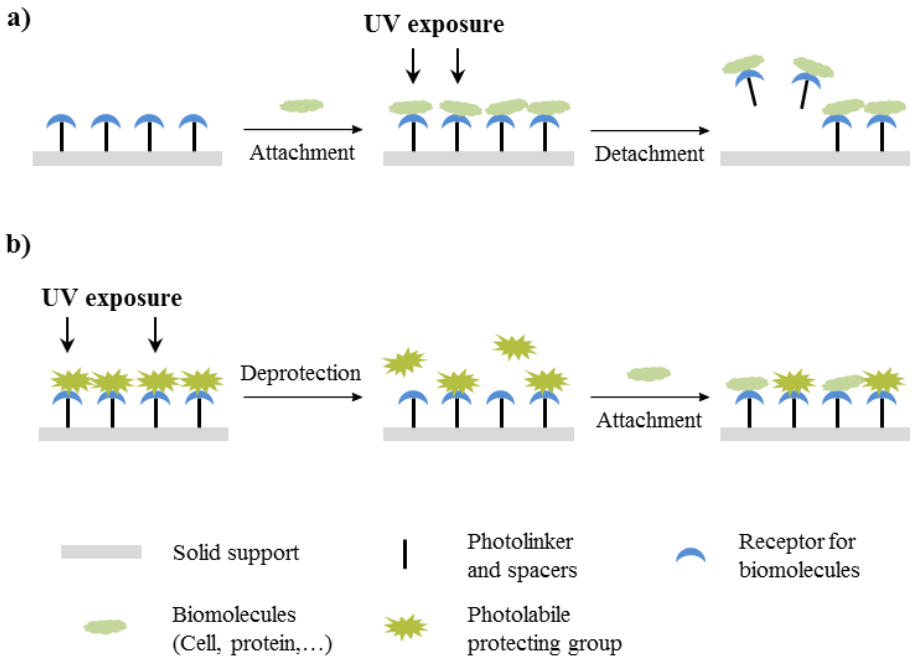


Figure 13. Overall concepts of another photochemical reactions in chemical biology; a) detachment reaction, b) photo-triggered reaction.

Wirkner *et al.* and Shin *et al.* introduced the binding motives of cells such as RGD-derivative peptides or antibodies onto a glass surface via photolinker.[59, 60] Cells were bound to the specific site of glass surface where the binding motives were selectively introduced. After that, cells were released from the glass surface (with RGD peptides) by UV light irradiation.

In photo-triggered reaction, crucial functional groups which plays important role in biological reactions are protected with photoreactive protecting group and selectively deprotected by UV light exposure (Figure 13. b). Petersen *et al.* and Ohmuro-Matsuyama *et al.* reported on the "caged RGD cyclic peptides" in which the side chain of aspartic acid was protected with photolabile protecting group immobilized on a glass surface.[61, 62] Target cells could not recognize the side chain-protected caged RGD cyclic peptides. Therefore, cells were only attached to the uncaged (deprotected) cyclic RGD peptides after UV irradiation. One step further, Salierno *et al.* reported on the cell migration assays with the same glass slide.[63] They exposed UV light to specific small areas of the glass surface step by step, and observed the cell migration process. Cells were only attached to the irradiated site, and as the nearby area was irradiated, attached cells gradually migrated toward the newly deprotected area slowly.

I. 4. Research Objectives

In this thesis, synthesis of UVA light sensitive *o*-nitrobenzyl amine/alcohol derivatives and their applications to the SPOS and cell membrane protein isolation are described. In the first part, we synthesized facile photocleavable linker which the merits of established photolinkers (Fmoc-PLL and Fmoc-ANP linker) are combined. Newly synthesized photolinker could absorb UVA light like Fmoc-PLL. Also it was synthesized rapidly and efficiently with six synthetic steps including the Perkin-type condensation reaction like Fmoc-ANP linker. To prove the usefulness of synthesized photocleavable linker, some solid-phase organic reactions were demonstrated. Peptide amides, acids, and methyl ester were synthesized with photocleavable linker coupled resins. Also, glycans were immobilized to the resins via photocleavable linker. And we tried to analysis these synthesized molecules using matrix-assisted laser desorption/ionization -time of flight mass spectroscopy (MALDI-TOF MS) analysis method without using matrices and additional photocleavage steps.

In the second part, we designed and synthesized the molecules for isolation the cell membrane protein. Synthesized molecules were consisted of amine catchable part, photoreactive part, hydrophilic spacer, and tethering part for immobilization. At first, we tried to confirm the reactivity of synthesized molecules toward amine, alkyne, and streptavidin

with model system. After that we tried to capture-and-release the model proteins using the synthesized molecule and also tried to isolation the membrane proteins of *Escherichia coli* (*E. coli*) using synthesized molecules.

II. Experimental Section

II. 1. General

II. 1. 1. Materials

HiCore® (loading level: 0.3 mmol/g and 0.37 mmol/g),[64] 2-chlorotrityl chloride (2-CTC) resin (loading level: 1.26 mmol/g and 1.43 mmol/g), Fmoc-*O*-succinimide (Fmoc-Osu), Fmoc-amino acids and their derivatives (except Fmoc-4-hydrazinobenzoic acid, Fmoc-Lys(N_3)-OH, and Fmoc-Lys(biotin)-OH), benzotriazol-1-yloxy-tris(dimethylamino)-phosphonium hexafluorophosphate (BOP), and *N*-hydroxybenzotriazole (HOBt) anhydrous were purchased from BeadTech Inc. (Korea). Veratraldehyde, malonic acid, ammonium acetate, acetyl chloride, methyl alcohol (MeOH), trifluoroacetic anhydride (TFAA), *N*,*N*′-diisopropylethylamine (DIPEA), ninhydrin, phenol, potassium cyanide (KCN), trifluoroacetic acid (TFA), triisopropylsilane (TIPS), α-cyano-4-hydroxycinnamic acid (CHCA), sodium cyanoborohydride ($NaBH_3CN$), acetovanillone, ethyl 4-bromobutyrate, sodium borohydride ($NaBH_4$), succinic anhydride, *N*,*N*′-disuccinimidyl carbonate (DSC), *N*-acetylglycine, L-lysine, 4-pentynoic acid, tris(3-hydroxypropyltriazolylmethyl)amine (THPTA), (+)-sodium L-ascorbate, lactose, 3'-sialyllactose, 4-dimethylamino-pyridine (DMAP), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich. Deuterium solvents for nuclear magnetic resonance (NMR) spectroscopy were

purchased from Cambridge Isotope Laboratories (USA). Sodium iodide (NaI) and potassium iodide (KI) were purchased from Junsei (Japan). Ethyl alcohol (EtOH), *N*,*N*′-dimethylformamide (DMF), pyridine, magnesium sulfate anhydrous ($MgSO_4$), 70 % of aqueous nitric acid solution, sodium hydroxide (NaOH), sodium bicarbonate ($NaHCO_3$), 12 *N* of aqueous hydrochloric acid (HCl) solution, dichloromethane (DCM), ethyl acetate (EtOAc), potassium carbonate (K_2CO_3), *n*-hexane, tetrahydrofuran (THF), acetic acid (AcOH), piperidine, acetonitrile (methyl cyanide; MeCN), acetic anhydride (Ac_2O), sodium chloride (NaCl), potassium chloride (KCl), and copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) were purchased from Daejung Chemicals (Korea). 3,6-dioxa-1,8-octanedithiol (DODT) and 1,13-diamino-4,7,10-trioxatridecane were purchased from Alfa Aesar. Fmoc-Lys(N_3)-OH was purchased from Aapptec (USA). Fmoc-Lys(biotin)-OH was purchased from Novabiochem (Germany). Sphero™ coated fluorescent nanospheres (streptavidin coated, 0.1 % w/v, 0.4-0.6 μm of diameter) were purchased from Spherotech Inc. (USA). Bugbuster™ was purchased from Novagen (USA).[65] Fmoc-4-hydrazinobenzoic acid (Fmoc-4-HBA) was purchased from Bachem (Switzerland). Hyclone® phosphate buffered saline (PBS, pH 7.4) was purchased from Theromo Scientific (USA). Fmoc-PLL was purchased from Advanced Chemtech (USA).

II. 1. 2. Apparatus

Avance 400 MHz Fourier transform-nuclear magnetic resonance (FT-NMR) spectrometer was used for ^{1}H- and ^{13}C-NMR analysis (Bruker Daltonics, Germany). Mecasys OPTIZEN 2120UV was used for UV-Vis absorbancy measurement (Mecasys, Korea). Spot light source LIGHTNINGCURE2000 L8222-01 was used as UV source (4.5 W/cm^2 at a point 10 mm away from the output end; Hamamatsu, Japan). Voyager-DETM STR Biospectrometry Workstation and TOF/TOF™ 5800 system were used for MALDI-TOF MS analysis (AB Sciex, USA). Thermo Finnigan LCQ liquid chromatography/mass spectroscopy (LC/MS) was used for high-performance liquid chromatography and mass spectroscopy (Thermo Fisher Scientific, USA). UVT series 4-LC was used for visualization during thin layer chromatography (4 W lamp, 254 nm, 265 μW/cm^2 at a point 15 cm away from the lamp) and UVA source (4 W lamp, 365 nm, 350 μW/cm^2 at a point 15 cm away from the lamp, Dong Seo Science, Korea). Thermomixer comfort was used for incubating the microtubes (Eppendorf, Germany).

II. 1. 3. Ninhydrin Color Test (Kaiser Test)[66]

A few mg of resins were picked up and put into a test tube. Kaiser test solutions (KCN solution; 0.014 mol/L KCN in pyridine, ninhydrin /EtOH

solution (5 %, w/v), phenol/EtOH solution (40 %, v/v), 2~3 drops of each) were added into the test tube. Then, the resin mixture contained test tube was heated at 100 ℃ for 5 minutes. After heating, the resin which contains the primary amine groups gives a color change into deep dark blue, while the resin which does not contain any primary amine groups gives no color change (yellow color). This test was usually performed to confirm the completion of coupling reaction.

II. 1. 4. Fmoc Quantitation[67]

The resins which contained Fmoc group were weighed exactly, put into a reaction vial. A 20 % (v/v) piperidine/DMF (3 mL) was poured into the vial, and the vial was agitated at room temperature for 50 minutes. The reaction solution was collected after the reaction, and diluted 100-fold with DMF. The baseline of absorbance at 290 nm was measured with DMF. The absorbance of diluted solution at 290 nm was measured. The loading level of resins was calculated by using the following formula:

$$\text{Loading level (mmol/g)} = (55.206 \times A_{290} - 1.0223) / wt$$

A_{290} means absorbance of sample solution at 290 nm, and *wt* means overall weight of resins which were treated with 20 % (v/v)

piperidine/DMF. Generally, the measurement of absorbancy is repeated for about 2 or 3 times repeated for accurate data.

II. 2. Synthesis and Applications of Fmoc-3-amino-3-(4,5-dimethoxy-2-nitrophenyl)propionic Acid (Fmoc-PCA Linker)

II. 2. 1. Synthesis of Fmoc-PCA linker

Fmoc-3-amino-3-(4,5-dimethoxy-2-nitrophenyl)propionic acid (Fmoc-photocleavable linker, Fmoc-PCA linker) was synthesized by the following steps.[68]

Synthesis of 3-amino-3-(3,4-dimethoxyphenyl)propionic acid

Well-crushed veratraldehyde (2.5 g, 15.05 mmol), ammonium acetate (4.64 g, 60.18 mmol), and malonic acid (6.26 g, 60.18 mmol) were dissolved in 150 mL of EtOH. The reaction solution was refluxed for 18 hours. After the reaction, white solids were formed and separated by filtration. This white compound was gently washed with cold EtOH three or more times till the filtrate became colorless, and dried *in vacuo*. After drying, 3-amino-3-(3,4-dimethoxyphenyl)propionic acid was obtained as a white powder (2.046 g, 60 % yield). ^{1}H NMR (400 MHz, D_2O/K_2CO_3): δ 7.04-6.99 (m, 3 H), 4.55 (t, *J* = 7.1 Hz, 1 H), 3.83 (s, 3 H), 3.81 (s, 3 H), 2.86 (dd, *J* = 7.9, 9.1 Hz, 1 H), 2.75 (dd, *J* = 6.8, 9.9 Hz, 1 H). ^{13}C NMR (100 MHz, D_2O/NaOH): δ 183.00, 150.78, 149.84, 140.76, 121.70, 114.64,

112.88, 58.58, 58.52, 55.47, 49.80 ppm. m.p.: 213-216 ℃. Calculated mass: 225.24. ESI-HRMS (negative mode): m/z = 224.0928.

Synthesis of 1-(3,4-dimethoxyphenyl)-3-methoxy-3-oxopropan-1-aminium chloride

Dried 3-amino-3-(3,4-dimethoxyphenyl)propionic acid (1.6 g, 7.10 mmol) was suspended in 150 mL of anhydrous MeOH, and acetyl chloride (1.5 mL, 21.37 mmol) was added into this suspension. The reaction mixture was stirred and refluxed for 3 hours, and the solvent was evaporated by reduced pressure and dried *in vacuo*. After drying, 1-(3,4-dimethoxyphenyl)-3-methoxy-3-oxopropan-1-aminium chloride was obtained as white salt form (1.860 g, 95 % yield). ^{1}H NMR (400 MHz, CD_3OD): δ 7.52-6.86 (m, 3 H), 4.66 (t, *J* = 7.1 Hz, 1 H), 3.84 (s, 3 H), 3.80 (s, 3 H), 3.65 (s, 3 H), 3.17 (dd, *J* = 7.9, 9.1 Hz, 1 H), 3.01 (dd, *J* = 6.8, 9.9 Hz, 1 H). ^{13}C NMR (100 MHz, CD_3OD): δ 171.86, 151.38, 150.97, 129.88, 121.37, 113.17, 112.22, 56.87, 56.64, 53.12, 52.88, 39.42 ppm. m.p.: 183-185 ℃. Calculated mass: 275.73. ESI-HRMS (positive mode): m/z = 240.1229.

Synthesis of methyl 3-(3,4-dimethoxyphenyl)-3-(2,2,2-trifluoroacetamido)propanoate

Methyl 1-(3,4-dimethoxyphenyl)-3-methoxy-3-oxopropan-1-aminium chloride (1.860 g, 6.75 mmol) was dissolved into 80 mL of pyridine. The reaction solution was cooled to 0 ℃ with an ice bath, and TFAA (1.2 mL, 8.50 mmol) was added to the vigorously stirred solution. After 1 hour, the reaction solution was poured into a separation funnel with EtOAc. This solution was acidified with 6 *N* HCl aqueous solution until the pH of solution was reached to about 2-3, and the resulting two layers were partitioned. The aqueous layer was drained out, and the remaining organic layer was washed with 0.5 *N* of aqueous $NaHCO_3$ solution, collected and dried by using $MgSO_4$. The dried organic layer was filtrated and evaporated by reduced pressure. As a result, methyl 3-(3,4-dimethoxyphenyl)-3-(2,2,2-trifluoro-acetamido)propanoate was obtained as yellow crystal forms (1.921 g, 85 % yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.01-6.86 (m, 3 H), 5.24 (q, *J* = 7.7 Hz, 1 H), 3.76 (s, 3 H), 3.74 (s, 3 H), 3.60 (s, 3 H), 2.99 (dd, *J* = 9.3, 9.6 Hz, 1 H), 2.87 (dd, *J* = 6.1, 5.4 Hz, 1 H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 170.45, 148.84, 148.41, 132.91, 118.61, 111.73, 110.50, 55.51, 51.50, 50.06 ppm. m.p.: 85-86 ℃. Calculated mass: 335.28. ESI-HRMS (positive mode): m/z = 336.1055.

Synthesis methyl 3-(4,5-dimethoxy-2-nitrophenyl)-3-(2,2,2-trifluoroacetamido)propanoate

Nitration of methyl 3-(3,4-dimethoxyphenyl)-3-(2,2,2-trifluoro-acetamido)propanoate was performed as follows. About 20 mL of 70 % nitric acid aqueous solution was stirred in an ice bath, and to this solution, methyl 3-(3,4-dimethoxyphenyl)-3-(2,2,2-trifluoro-acetamido)propan-oate (1.921 g, 5.73 mmol) was added slowly. The solution was stirred in a dark place for 2 hours, and the resulting orange-colored reaction solution was quenched by pouring ca. 200 mL of cold water (about ten or more times of the volume of used nitric acid) to the reaction solution. A light yellow solid appeared from the solution by chilling at 4 ℃ for 15 minutes. The solid was collected by filtration, and washed with water at least three times. The washed product was dissolved into DCM, and dried with $MgSO_4$. The organic layer was filtered and evaporated by reduced pressure, yielding methyl 3-(4,5-dimethoxy-2-nitrophenyl)-3-(2,2,2-trifluoro-acetamido)propanoate as ivory powder form (1.844 g, 85 % yield) after drying *in vacuo*. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.57 (s, 1 H), 7.28 (s, 1 H), 5.24 (q, J = 7.7 Hz, 1 H), 3.76 (s, 3 H), 3.74 (s, 3 H), 3.60 (s, 3 H), 2.99 (dd, J = 9.3, 9.6 Hz, 1 H), 2.87 (dd, J = 6.1, 5.4 Hz, 1 H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.99, 153.28, 147.89, 140.15, 129.90, 109.98, 107.63, 56.27, 56.09, 39.93, 39.51 ppm. m.p.: 167-169 ℃. Calculated mass: 380.27. ESI-HRMS (positive mode): m/z = 381.0859.

Synthesis of Fmoc-PCA linker

Methyl 3-(4,5-dimethoxy-2-nitrophenyl)-3-(2,2,2-trifluoroacetamido)-propanoate (1.844 g, 4.85 mmol) was dissolved in 150 mL of 0.1 *N* of aqueous NaOH solution. The solution was stirred and refluxed for 5 hours, and cooled to room temperature. After that, the pH of the solution was adjusted to about 7 by adding 6 *N* HCl. Then, the color of solution was changed to yellow. The resulting solution was stirred in an ice bath, followed by addition of 1.2 mL of DIPEA. After that, 1.083 g of Fmoc-Osu in 50 mL of THF was added slowly into the reaction mixture. The reaction mixture was stirred for 1 hour in an ice bath, and overnight at room temperature. When the reaction was ended, THF was evaporated from the reaction mixture by reduced pressure, and the remaining aqueous solution was slowly acidified to pH 2-3 with 6 *N* of HCl. Product was precipitated during acidification, and separated by centrifugation. Fmoc-PCA linker was obtained as bright ivory-colored powder form after freeze drying (1.942 g, 81 % yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.38 (s, 1 H), 8.12 (d, J = 8.0 Hz, 1 H), 7.88-7.24 (m, 10 H), 5.57 (q, J = 7.4 Hz, 1 H), 4.27 (d, J = 5.8 Hz, 2 H), 4.18 (t, J = 6.7 Hz, 1 H), 3.87 (s, 3 H), 3.85 (s, 3 H), 2.70 (d, J = 6.6 Hz, 2 H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 171.37, 155.28, 153.17, 147.38, 143.93, 143.54, 140.73, 140.69, 139.91, 133.08, 127.61, 126.98, 126.90, 125.04, 120.14, 120.10, 109.90, 107.31, 65.41, 56.20, 56.04, 47.56, 46.62 ppm. m.p.: 210-215 ℃. Calculated mass: 492.48. ESI-HRMS (positive mode): m/z = 493.1613.

II. 2. 2. Applications of Fmoc-PCA linker

Preparation of Acetylated PCA linker for the measurement of UV-Vis absorbency of PCA linker

Fmoc-PCA linker (63.38 mg, 0.13 mmol) and DIPEA (44.84 μl, 0.26 mmol) were dissolved into 3 mL of DCM, and this reaction solution was added to 2-CTC resin (loading level: 1.43 mmol/g, 30 mg). Coupling reaction was kept for 2 hours, and the resins were washed with DCM and MeOH three times each. Then, Fmoc group was removed by treating with 20 % (v/v) piperidine/DMF for 1 hour, and the resins were washed with DMF, DCM, and MeOH three times each. Acetic anhydride (300 μl) and DIPEA (300 μl) dissolved in 2.4 mL of DMF were added to the resins, and the reaction was kept for 1 hour at room temperature. The resins were washed with DMF, DCM, and MeOH three times each, and dried *in vacuo*. The dried resins were treated with 3 mL of 2 % (v/v) TFA/DCM mixture for 30 minutes, and the reaction solution was collected. Collected solution was evaporated with reduced pressure, and dissolved in 1 mL of DMF. The resulting solution was 100-fold diluted in DMF, and the UV-Vis absorbance of solution was measured with Mecasys OPTIZEN 2120UV.

Photocleavage of Fmoc-Phe-NH_2

HiCore (loading level: 0.3 mmol/g, 100 mg) was swollen in DMF for 10 minutes, and the solvent was drained out. Fmoc-PCA linker was coupled to the resins with BOP coupling method (2 equiv. of Fmoc-PCA linker, BOP, and HOBt, 4 equiv. of DIPEA in DMF) for 2 hours, and the resins were washed with DMF, DCM, and MeOH three times each. The Fmoc group was removed by treating with 20 % (v/v) piperidine/DMF for 1 hour, and the resins were washed with DMF, DCM, and MeOH three times each. Then, Fmoc-Phe-OH was coupled to the resins with BOP/HOBt reagents and the resins were washed by the same manner. After washing and drying, ca. 10 mg of Fmoc-Phe-PCA-HiCore was exactly weighed and transferred into 2 mL microtube each, and DMF (1 mL) was added. The resins were irradiated by UVA light (365 nm, about 4.5 W/cm^2) for a certain period of time (1 to 20 minutes). Photocleavage products were analyzed by HPLC, and the amount of Fmoc-Phe-NH_2 was calculated with a standard curve.

Synthesis of peptide amide with Fmoc-PCA linker coupled resins

HiCore (0.3 mmol/g, 100 mg) was swollen in DMF for 10 minutes, and the solvent was drained out. Fmoc-PCA linker was coupled to the resins with the BOP coupling method for 2 hours, and the resins were washed with DMF, DCM, and MeOH three times each. Then, Fmoc group was removed by treating with 20 % (v/v) piperidine/DMF for 1 hour, and the

resins were washed with DMF, DCM, and MeOH three times each. After that, the reaction mixture of the resulting resins and 4 equiv. of Fmoc-amino acids (sequentially, leucine, phenylalanine, glycine, glycine, and tyrosine), BOP, HOBt, and 8 equiv. of DIPEA in DMF were shaken for 2 hours. After the each coupling reaction, the resins were washed with DMF, DCM, and MeOH about three times. The Fmoc group was removed by the same manner following the coupling step, and the resins were washed with DMF, DCM, and MeOH three times each. Whole peptide sequences were synthesized by repetition of the coupling and deprotection reactions. After the whole peptide sequences were coupled on the resins, the side chain protecting groups were removed by using the cleavage cocktail (TFA : DODT : H_2O : TIPS = 94 : 2.5 : 2.5 : 1, volume ratio) for 1 hour. The resins were washed with TFA, DCM, and MeOH three times each. About 10 mg of resulting resin was transferred into 2 mL microtube, and 1 mL of MeOH was added. UVA light (365 nm, about 4.5 W/cm^2) was irradiated to the resins for 20 minutes. The released peptide in MeOH was analyzed by HPLC and MALDI-TOF MS. As a control, the same peptide was synthesized by using Fmoc-PLL instead of Fmoc-PCA linker.

Synthesis of peptide acid and peptide methyl ester with phenylhydrazine-PCA coupled resins

Fmoc-PCA linker was coupled to HiCore by the same method as previously described. Fmoc group was removed with 20 % (v/v) piperidine/DMF for 1 hour, and the resins were washed with DMF, DCM, and MeOH three times each. After that, Fmoc-4-HBA was coupled to the resin with BOP coupling method (2 equiv. of Fmoc-4-HBA, BOP, and HOBt, 4 equiv. of DIPEA in DMF) for 2 hours. The resins were washed with DMF, DCM, and MeOH three times each. Fmoc group was removed with 20 % (v/v) piperidine/DMF for 1 hour, and the resins were washed with DMF, DCM, and MeOH three times each. Then, 4 equiv. of Fmoc-amino acids (sequentially Fmoc-6-aminocaproic acid (Fmoc-ε-ACA), Fmoc-3-aminopropionic acid (Fmoc-β-Ala-OH), Fmoc-ε-ACA, and Fmoc-β-Ala-OH), BOP, HOBt, and 8 equiv. of DIPEA in DMF was added to the resins and the coupling reaction was kept for 2 hours. The resins were washed with DMF, DCM, and MeOH three times each. After the each coupling step, Fmoc group was removed by treating with 20 % (v/v) piperidine/DMF for 1 hour, and the resins were washed with same manner. After the whole coupling steps, Fmoc-β-Ala-ε-ACA-β-Ala-ε-ACA-HBA-PCA-HiCore (Fmoc-$\beta\varepsilon\beta\varepsilon$-HBA-PCA-HiCore) was prepared, and dried *in vacuo*. For the photocleavage of the synthesized peptide from the resins, dried resins (~ 10 mg) were weighed into two microtubes each, and 100 μl of MeOH and DI water were added to each microtubes. Resins were irradiated by UVA light (365 nm) for 10 minutes, and the supernatant

solutions were collected. The released compounds were analyzed by ESI-MS.

Preparation of H-*β*-Ala-*ε*-ACA-*β*-Ala-*ε*-ACA-PCA-HiCore for glycan immobilization

H-*β*-Ala-*ε*-ACA-*β*-Ala-*ε*-ACA-HBA-PCA-HiCore (H-*βεβε*-PCA-HiCore) was prepared by using Fmoc chemistry and BOP coupling method as previously described. In the coupling step, 3 equiv. of Fmoc building blocks (sequentially Fmoc-PCA linker, Fmoc-*ε*-ACA, Fmoc-*β*-Ala-OH, Fmoc-*ε*-ACA, and Fmoc-*β*-Ala-OH), BOP (49.09 mg, 0.11 mmol), HOBt (15.00 mg, 0.11 mmol), and 6 equiv. of DIPEA (38.67 μl, 0.22 mmol) were dissolved in 3 mL of DMF. The reaction solution was added to HiCore (100 mg, 0.37 mmol/g, 0.037 mmol) for 2 hours, and resins were washed with DMF, DCM, and MeOH three times each. After the each coupling step, Fmoc group was removed by treating with 20 % (v/v) piperidine/DMF for 30 minutes, and resins were washed by the same method. H-*βεβε*-PCA-HiCore was prepared by repetition of coupling and deprotection steps. The resulting resins were dried *in vacuo* for further modification or glycan immobilization.

Immobilization of lactose and 3′-sialyllactose to prepared resins

Glycans (lactose and 3'-sialyllactose) were immobilized onto amine-functionalized HiCore (H-*βεβε*-PCA-HiCore) via Schiff base formation.[69] For immobilization of glycans to amine-functionalized HiCore, 5 mg of glycans (lactose or 3'-sialyllactose) and 10 mg of sodium cyanoborohydride ($NaCNBH_3$) were dissolved in 100 μl of 5:2 (v/v) DMSO/AcOH. This solution was added into microtube which 5 mg of H-*βεβε*-PCA-HiCore were contained, and the reaction was kept for 5 hours at 65 ℃ with gentle shaking (1000 rpm). After this immobilization step, resins were washed with DI water at least five times. The immobilized glycopeptides were released from the resins by UVA light irradiation, and the released glycopeptides were analyzed by ESI-MS.

Preparation of additive solutions for direct on-bead laser desorption/ionization-time of flight (DOLDI-TOF) method

Silica nanoparticles (SiNPs, 100-200 nm of diameter), a matrix substitute, was prepared by Stöber method,[70] and dispersed in EtOH (20 mg/mL). As ion source of DOLDI system, 1 ml of 100 mM salt solutions (NaCl, NaI, KCl, and KI in DI water) were prepared. For the conventional matrix solution for peptides, more than 10 mg of CHCA was dissolved in 100 μl of TA30 solvent (0.1 % of TFA in acetonitrile/water (v/v, 30:70)).

Loading of peptide-anchored resins onto MALDI plate

Small amount of H-YGGFL-PCA-HiCore (less than 0.1 mg) was transferred to a microtube. The resins were swollen in 10 μl of SiNPs suspension (20 mg of SiNPs in 1 mL of EtOH) for 30 minutes, and washed. After that, the resins were suspended in 10 μl of 100 mM sodium chloride solution, and 1 μl of resin-contained mixture was deposited onto a MALDI plate. As a positive control of on-bead analysis, same resins without SiNPs treatment were suspended by 10 μl of CHCA solution, and 1 μl of resin-contained mixture was deposited. As negative control of on-bead analysis, the resins were loaded onto the plate by the same manners as the positive control, except DI water was used instead of matrix solution. After deposition, the remained solvents were rapidly dried *in vacuo*, with blocking the external UV light.

Analysis of peptide with DOLDI-TOF method

H-YGGFL-PCA-HiCore was loaded on a MALDI plate, and set on Voyager-DETM STR Biospectrometry Workstation, and the samples were analyzed. The instrument setting was as follows; reflector mode, delayed extraction, positive method, accelerating voltage: 20000 V, grid voltage 65 %, extraction delay time: 180 nsec. Raw data was analyzed by using Data Explorer 4.0.0.0.

Analysis of peptide with DOLDI-TOF method by using various salt additives

Small amount of H-YGGFL-PCA-HiCore (less than 1 mg) was swollen in 10 μl of SiNPs suspension (20 mg of SiNPs in 1 mL of EtOH) for 30 minutes, and washed. Washed resins were divided into four, and were suspended with four kind of 100 mM salt solution (NaCl, NaI, KCl, and KI). After that, four kinds of resin-contained suspensions (1 μl) were deposited onto a MALDI plate. Remaining solvents were dried rapidly dried *in vacuo*, with blocking the UV light. The resin loaded MALDI plate was set on Voyager-DETM STR Biospectrometry Workstation, and samples were analyzed. The instrument setting was as following; reflector mode, delayed extraction, positive method, accelerating voltage: 20000 V, grid voltage 65 %, extraction delay time: 180 nsec. Raw data was analyzed by using Data Explorer 4.0.0.0.

II. 3. Synthesis and Application of Protein Fishing Molecule (ProFiM)

II. 3. 1. Synthesis of ProFiM

ProFiM, which consisted of fishing hook (photoreactive moiety), fishing rod (azide group or biotin), and fishing gut (hydrophilic spacer) was synthesized with following synthetic steps.

Synthesis of ethyl 4-(4-acetyl-2-methoxyphenoxy)butanoate

Acetovanillone (3.50 g, 21.06 mmol, 1.05 equiv.) and K_2CO_3 (4.37 g, 31.59 mmol, 1.58 equiv.) were added into 100 mL round-bottom flask (Rbf), and dissolved in 15 mL of DMF. Ethyl 4-bromobutyrate (2.86 mL, 20.01 mmol, 1 equiv.) was added into Rbf, and stirred for 18 hours at room temperature. Then, DI water was added into Rbf for dissolving the remaining white slurry, and the whole solution was transferred into separation funnel. EtOAc was added for extraction, and water layer was drained out. The organic layer was washed with brine at least three times. The resulting organic layer was dried with $MgSO_4$, and evaporated. The desired product was colorless oil, and slowly solidified while drying *in vacuo* (5.413 g, 19.31 mmol, 96.5 % yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.57-7.52 (m, 2 H), 6.90 (d, J = 8.3 Hz, 1 H), 4.18-4.12 (m, 4 H), 3.92

(s, 3 H), 2.57-2.53 (m, 5 H), 2.19 (q, *J* = 6.8 Hz, 2 H), 1.26 (t, *J* = 7.1 Hz, 3 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 196.82, 173.06, 152.73, 149.38, 130.60, 123.27, 111.40, 110.59, 67.90, 60.55, 56.06, 30.66, 26.27, 24.40, 14.29.

Synthesis of ethyl 4-(4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy)butanoate

Ethyl 4-(4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy)butanoate was synthesized via nitration and reduction of ethyl 4-(4-acetyl-2-methoxyphenoxy)butanoate. The nitration step was performed according to the reported procedures.[71] About 20 mL of 70 % of aqueous nitric acid solution was poured into 250 mL Rbf and stirred at -2 ℃. Well-crushed ethyl 4-(4-acetyl-2-methoxyphenoxy)butanoate (3.00 g, 10.70 mmol) was carefully added into the Rbf, and the Rbf was wrapped to protect from external UV light (from this step, the reaction system were protected from light). The reaction was kept for 3 hours at room temperature, and then the reaction solution was poured into Erlenmeyer flask which contains about 180 mL of chilled water. The flask was placed in a refrigerator (4 ℃) for 15 minutes to sediment the product (because the synthesized compound can be hydrolyzed under this condition, precipitation time was kept as short as possible). After that, the precipitate was filtered and washed with water twice. The precipitate was dissolved in DCM. The organic layer was

dried with $MgSO_4$, accumulated into 250 mL Rbf, and evaporated, and then, bright ivory colored compound was appeared. The compound was dissolved in 150 mL of 1:1 (v/v) EtOH/THF, and placed in an ice bath. To this solution, $NaBH_4$ (809.56 mg, 21.40 mmol, 2 equiv.) was carefully added and stirred. After 3 hours, the reaction solvent was evaporated under reduced pressure, and the residue was dissolved in EtOAc and water for the extraction. The aqueous layer was extracted with EtOAc, and the organic layer was washed with water twice. The organic layer was dried with $MgSO_4$, evaporated, and dried *in vacuo*. The crude product was purified with column chromatography on silica gel using 2:1 (v/v) hexane/EtOAc (R_f = 0.14). Bright-ivory colored powder was obtained after purification (2.063 g, 6.30 mmol, 58.9 % yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.54 (s, 1 H), 7.36 (s, 1 H), 5.25 (qr, *J* = 6.2 Hz, 1 H), 4.19-4.10 (m, 4 H), 3.98 (s, 3 H), 2.54 (t, *J* = 7.2 Hz, 2 H), 2.19 (q, *J* = 6.9 Hz, 2 H), 1.56 (d, *J* = 6.3 Hz, 3 H), 1.27 (t, *J* = 7.2 Hz, 3 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.09, 154.16, 146.87, 139.42, 137.35, 109.09, 108.79, 68.35, 65.67, 60.62, 56.36, 30.69, 24.45, 24.35, 14.24.

Synthesis of 4-(4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy)-butanoic acid

Ethyl 4-(4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy)butanoate (1.860 g, 5.68 mmol, 1 equiv.) was dissolved in 80 mL of DCM, and

NaOH (909 mg, 22.73 mmol, 4 equiv.) in EtOH (10 mL) was added. The reaction was kept for 18 hours at room temperature, and the resulting solvent was evaporated. The remaining residues were dissolved in water, transferred into separation funnel and washed with DCM twice. The aqueous layer was transferred into centrifuge tube, and acidified with 2 *N* of HCl. The light-lemon colored product was precipitated when the pH reached around 2, and the desired product was obtained by centrifugation and freeze-drying (1.540 g, 5.15 mmol, 90.6 % yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.54 (s, 1 H), 7.36 (s, 1 H), 5.53 (d, J = 6.2 Hz, 1 H), 5.25 (qr, J = 6.2 Hz, 1 H), 4.06 (t, J = 6.4 Hz, 3 H), 3.90 (s, 3 H), 2.39 (t, J = 7.2 Hz, 2 H), 1.95 (q, J = 6.9 Hz, 2 H), 1.36 (d, J = 6.3 Hz, 3 H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 173.94, 153.44, 146.19, 138.91, 138.00, 109.17, 108.50, 67.93, 63.87, 56.06, 29.94, 25.12, 24.03.

Synthesis of ProFiM-azide and ProFiM-biotin

ProFiM-azide and ProFiM-biotin were synthesized with conventional solid-phase peptide synthesis method. At first, 2-CTC resin (100 mg, loading level: 1.26 mmol/g, 0.126 mmol) was swollen in DCM for 10 minutes, and filtered. Then, 0.252 mmol of Fmoc-Lys(N_3)-OH (99.39 mg, for ProFiM-azide) or Fmoc-Lys(biotin)-OH (149.87 mg, for ProFiM-biotin) was dissolved in 3 mL of DCM, and DIPEA (87.79 μl, 0.504 mmol) was added. The solution was added to 2-CTC resin, and the reaction

mixture was shaken for 2 hours at room temperature. The resin was washed with DCM and MeOH three times each. The Fmoc group was removed by treating with 3 mL of 20 % (v/v) piperidine/DMF for 30 minutes at room temperature, and the resin was washed with the same manner as above. Fmoc-4,7,10-trioxa-1,13-tridecanediamine succinimic acid[72] (Fmoc-Ttds-OH, 86.80 mg, 0.246 mmol), BOP (108.62 mg, 0.246 mmol), HOBt (33.19 mg, 0.246 mmol), and DIPEA (85.56 μl, 0.491 mmol), were dissolved in 3 mL of DMF, and the reaction solution was treated with the resin. The reaction was kept for 2 hours at room temperature, and the resin was washed with the same manner as above. Deprotection of amino group and washing step was also performed with the same manner as above. After washing, 4-(4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy)butanoic acid (73.50 mg, 0.246 mmol), BOP (108.62 mg, 0.246 mmol), HOBt (33.19 mg, 0.246 mmol), and DIPEA (85.56 μl, 0.491 mmol), were dissolved in 3 mL of DMF, and the reaction solution was treated with resin. The reaction mixture was shaken for 2 hours at room temperature. After the reaction, resin was washed with the same manner as above, and dried *in vacuo*. DSC (314.58 mg, 1.228 mmol) and DMAP (15.02 mg, 0.123 mmol) were dissolved in 3 mL of DMF, and the reaction solution was treated with the resin for 4 hours at room temperature. And then, resin was washed with DMF and DCM, and dried *in vacuo*. Dried resin was swollen in 2.85 mL of DCM, and 150 μl of TFA was added. The resin was incubated for 30 minutes at room temperature,

and filtrated. The filtrate was evaporated to obtain a light-yellow oils; ProFiM-azide ($[M+H]^+$: 897.38), and ProFiM-biotin ($[M+H]^+$: 1097.47). Synthesized crude ProFiM molecules were dissolved in 200 μl of DMSO (about 1 g/mL), and stored at freezer (under - 20 ℃) before usage.

II. 3. 2. Applications of ProFiM for isolation of cell membrane proteins

Reactivity test of ProFiM-azide toward amine and alkyne with model amino acids

N-acetylglycine (1 mg) and L-lysine (1 mg) were dissolved in 99 μl of DI water respectively, and ProFiM-azide solution (1 g/mL in DMSO, 1 μl, 1.12 μmol) was added into each solution. The mixtures were gently shaken for 30 minutes at room temperature. After that, 4-pentynoic acid (0.11 mg, 1.12 μmol), 50 μl of pre-mixed $CuSO_4 \cdot 5H_2O$ (0.28 mg, 1.12 μmol) and THPTA[73] (2.42 mg, 5.58 μmol) in DI water, and 50 μl of (+)-sodium-L-ascorbate (2.21 mg, 11.16 μmol) in DI water were added into the each reaction mixture. The mixtures were gently shaken for 2 hours at room temperature (under 900 rpm). Each mixture was analyzed with ESI-MS.

Preparation of alkyne-coupled polymer support for click chemistry

Fmoc-ε-ACA (38.40 mg, 87 μmol), BOP (38.48 mg, 87 μmol), HOBt (11.76 mg, 87 μmol), and DIPEA (30.31 μl, 17.4 μmol) were dissolved into 3 mL of DMF, and the reaction solution was treated with Tentagel resin (100 mg, 0.29 mmol/g, 29 μmol) for 2 hours. After coupling step, the resin was washed with DMF, DCM, and MeOH three times each. The resin was treated with 3 mL of 20 % (v/v) piperidine/DMF for 30 minutes to remove the Fmoc group, and the resin was washed by the same way as above. Fmoc-*β*-Ala-OH (27.09 mg, 87 μmol) was coupled the resin as above, and the whole coupling steps were repeated to prepare H-*βεβε*-Tentagel. To the H-*βεβε*-Tentagel, 4-pentynoic acid (8.53 mg, 87 μmol) was coupled by BOP coupling method, and washed with DMF, DCM, and MeOH. The washed resin was dried and stored *in vacuo* before usage.

Capture-and-release performance of ProFiM-azide with model protein

BSA (2 mg) was dissolved in 99 μl of PBS (pH 7.4). After that, 1 μl of diluted ProFiM-azide solution (1 g/mL in DMSO) was added and well-mixed with BSA solution. Conjugation of BSA and ProFiM-azide was performed for 30 minutes at room temperature with gentle shaking (under 900 rpm). After conjugation, 10 μl of PBS which contained $CuSO_4$ (5.57 μg, 2.23 nmol) and THPTA (96.99 μg, 11.16 nmol), and 10 μl of (+)-sodium-L-ascorbate solution (44.22 μg, 0.22 μmol, dissolved into PBS,

prepared just before the reaction) were added into ProFiM solution, and 2 mg of alkyne-coupled Tentagel was also added. The reaction mixture was shaken for 2 hours at room temperature, and the resin was washed with DI water about five times. The washed resin was transferred into transparent microtube with 10 μl of DI water. For photocleavage of BSA, the resin was irradiated by UVA light (365 nm) for 10 minutes. The supernatant solution was collected, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Capture-and-release performance of ProFiM-biotin with model protein

BSA (2 mg) was dissolved in 99 μl of PBS (pH 7.4). After that, 1 μl of diluted ProFiM-biotin solution (1 g/mL in DMSO) was added and well-mixed with BSA solution. Conjugation of BSA and ProFiM-biotin was performed for 30 minutes at room temperature with gentle shaking (under 900 rpm). After that, 2 mg of streptavidin-coated beads were added into the reaction mixture. The reaction was kept for 2 hours at room temperature, and the beads were washed with 0.1 % (v/v) Tween 20/PBS (PBST) and DI water at least five times. Washed beads were transferred into transparent microtube with 10 μl of DI water. For photocleavage of BSA, beads were irradiated by UVA light with wavelength of 365 nm for

10 minutes, and supernatant solution was collected by centrifugation, and analyzed by SDS-PAGE.

Isolation of outer cell membrane proteins of *E. coli* with ProFiM-biotin

ProFiM-biotin solution (1 g/mL in DMSO, 1 μl) was added into 1 ml of *E. coli* suspended PBS solution, and incubated for 30 minutes with gentle shaking (under 900 rpm). After the reaction, decantation supernatant solution and resuspension of *E. coli* with 10 μM of lysine in PBS was repeated at least three times. Washed *E. coli* was lysed with Bugbuster™, and the cell debris were removed by centrifugation (4 °C, 16000 rpm, 30 minutes) after cell lysis. After that, 2 mg of streptavidin-coated beads was added into the cell lysate mixture, and the whole reaction mixture was shaken gently at room temperature for 2 hours. The beads were washed by centrifugation (19000 rpm, 5 minutes) and suspended with 0.1 % (v/v) PBST and DI water at least five times. After washing, beads were suspended in 10 μl of DI water, and irradiated by UVA light with wavelength of 365 nm for 10 minutes. After irradiation, supernatant solution was separated from the beads by centrifugation, collected, and analyzed with SDS-PAGE.

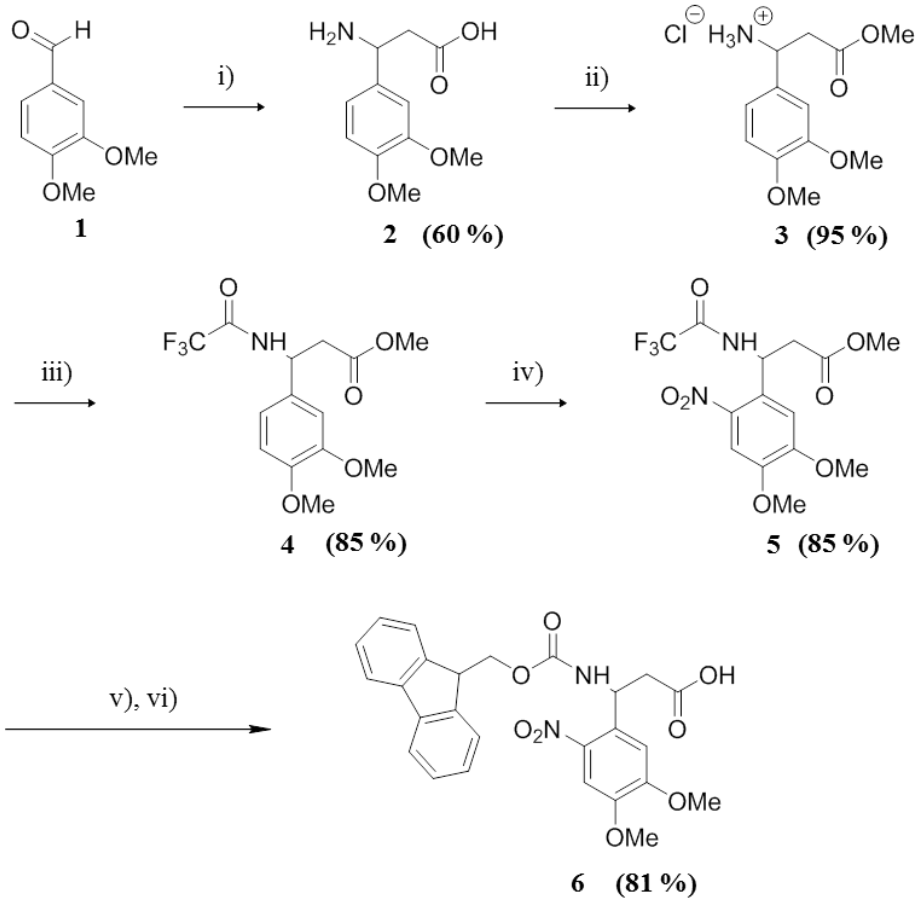
III. Results and Discussion

III. 1. Synthesis and Applications of Fmoc-PCA Linker

In spite of their usefulness, Fmoc-PLL and Fmoc-ANP linker have not been used widely due to their drawbacks which are mentioned in Chapter I. 2. 1. Their drawbacks make them inconvenient for utilizing in SPPS. We thought that the drawbacks could be overcome by the merit of opponent linker. Based on this idea, we tried to synthesize novel photolinker from electron donating group substituted benzene ring via Perkin-type condensation reaction.

III. 1. 1. Synthesis of Fmoc-PCA linker

The photolinker was synthesized from veratraldehyde (compound 1, scheme 2) which has two electron donating groups (methoxy group) on its benzene ring.[68] Actually 6-nitroveratraldehyde also can be used as a starting material. However, 6-nitroveratraldehyde is too expensive despite of low purity. Furthermore, more labor, reaction time, and expensive reagents are not required for the nitration of benzene ring. Considering these, veratraldehyde was selected as a starting material. Veratraldehyde was converted to 3-amino-3-(3,4-dimethoxy-phenyl)propionic acid (compound 2) via Perkin-type condensation reaction. The non-nitrated starting material has another merit at this stage. According to Tan *et al*., Perkin-type condensation reaction was accelerated by electron donating



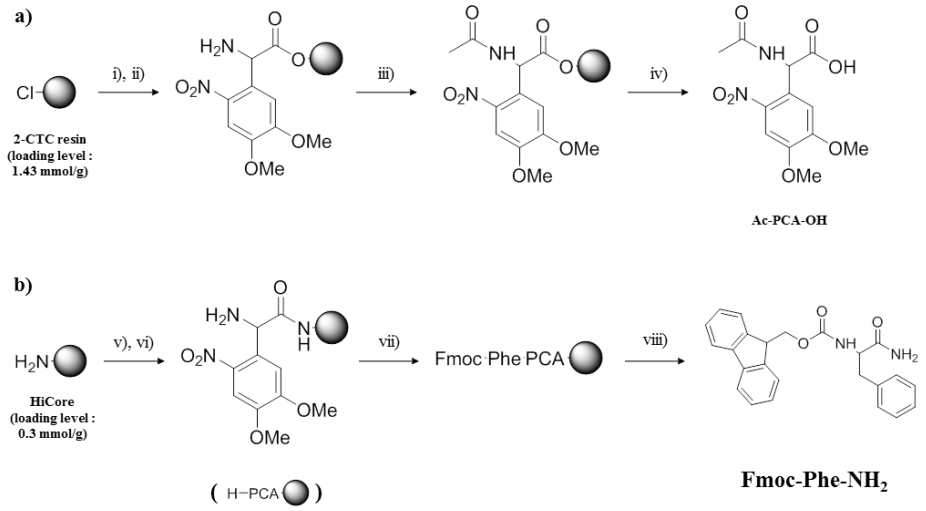
Scheme 2. Overall synthetic pathway of Fmoc-PCA linker. i) malonic acid, NH_4OAc in EtOH, reflux, ii) acetyl chloride in MeOH, reflux, iii) TFAA in pyridine, iv) 70% nitric acid solution, 0 ℃. v) 0.1 *N* NaOH solution, reflux, vi) Fmoc-Osu, DIPEA in THF, 0 ℃ and then room temperature.

groups such as methoxy group, and hindered by electron withdrawing groups such as nitro group.[74] For this reason, the theatrical synthetic yield of 3-amino-3-(3,4-dimethoxy-phenyl)propionic acid (60 %) was higher than that of 3-amino-3-(2-nitrophenyl)propionic acid. After the condensation reaction, two functional groups of compound 2 (carboxylic acid and amino group) were protected by using esterification and amidation before the nitration of benzene ring (95 % and 85 %, respectively).[21] Nitration of methyl 3-(3,4-dimethoxy phenyl)-3-(2,2,2-trifluoroacetamido)propanoate (compound 4) was performed as previously established nitration method, and the nitro group was introduced only at the 6-position.[21, 71] This site-selectivity was originated from the electron donating effect and the steric hindrance by methoxy group at 3-position, as the case of Fmoc-PLL synthesis. After trifluoroacetyl group and methyl ester group were removed by saponification, Fmoc protecting group was introduced to the free amino group by the Schötten-Baumann condition.[75, 76] After these steps, Fmoc-3-amino-3-(4,5-dimethoxy-2-nitrophenyl) propionic acid (compound 6, Fmoc-PCA linker, abbreviated from **p**hoto-**c**leavable **a**mino acid linker) was synthesized in 33 % overall yield. This synthetic strategy is notable that any chromatographic separation was not required during all the reaction steps.

III. 1. 2. UV-Vis absorption spectra and photocleavage kinetics of Fmoc-PCA Linker

As a preliminary test, the UV-Vis absorption spectra and photocleavage yield of Fmoc-PCA linker were characterized. Before the confirmation of UV-Vis absorption spectra of Fmoc-PCA linker, Fmoc group must be removed because it can absorb the UV light well. Therefore, acetylated PCA linker (Ac-PCA-OH) which has no Fmoc group was prepared as alternative of Fmoc-PCA linker (Scheme 3. a). In the UV-Vis absorption spectra of Ac-PCA-OH, light with the wavelength of 260 to 400 nm was absorbed well by Ac-PCA-OH (Figure 14. a). And significantly, λ_{max} of Ac-PCA-OH was about 350 nm. From this result, we could conclude that Fmoc-PCA linker might be rearranged by UVA light irradiation, as like Fmoc-PLL. In addition, Ac-PCA-OH did not absorb the light with the wavelength of above 410 nm. Therefore, it was certain that Fmoc-PCA linker might be stable in general fluorescent lighting.

The photocleavage yield of Fmoc-PCA linker was estimated by measuring the amount of released Fmoc-Phe-NH_2 from the resins, as model reaction. For this purpose, Fmoc-PCA linker and Fmoc-Phe-OH were serially coupled to HiCore (Scheme 3. b) by BOP coupling method. HiCore has been proved to be an ideal polymer supports for solid-phase photochemical reaction.[64] Synthesized Fmoc- Phe-NH_2 was released by irradiation of UVA light (365 nm), and the amount of released Fmoc-Phe-



Scheme 3. a) Preparation of Ac-PCA-OH. i) Fmoc-PCA linker, DIPEA in DCM, ii) 20 % (v/v) piperidine/DMF, iii) acetic anhydride, DIPEA in DMF, and iv) 2 % (v/v) TFA/DCM. b) Preparation of Fmoc-Phe-NH_2. v) Fmoc-PCA linker, BOP, HOBt, DIPEA in DMF, vi) 20 % (v/v) piperidine/DMF, vii) Fmoc-Phe-OH, BOP, HOBt, DIPEA in DMF, and viii) UVA light irradiation.

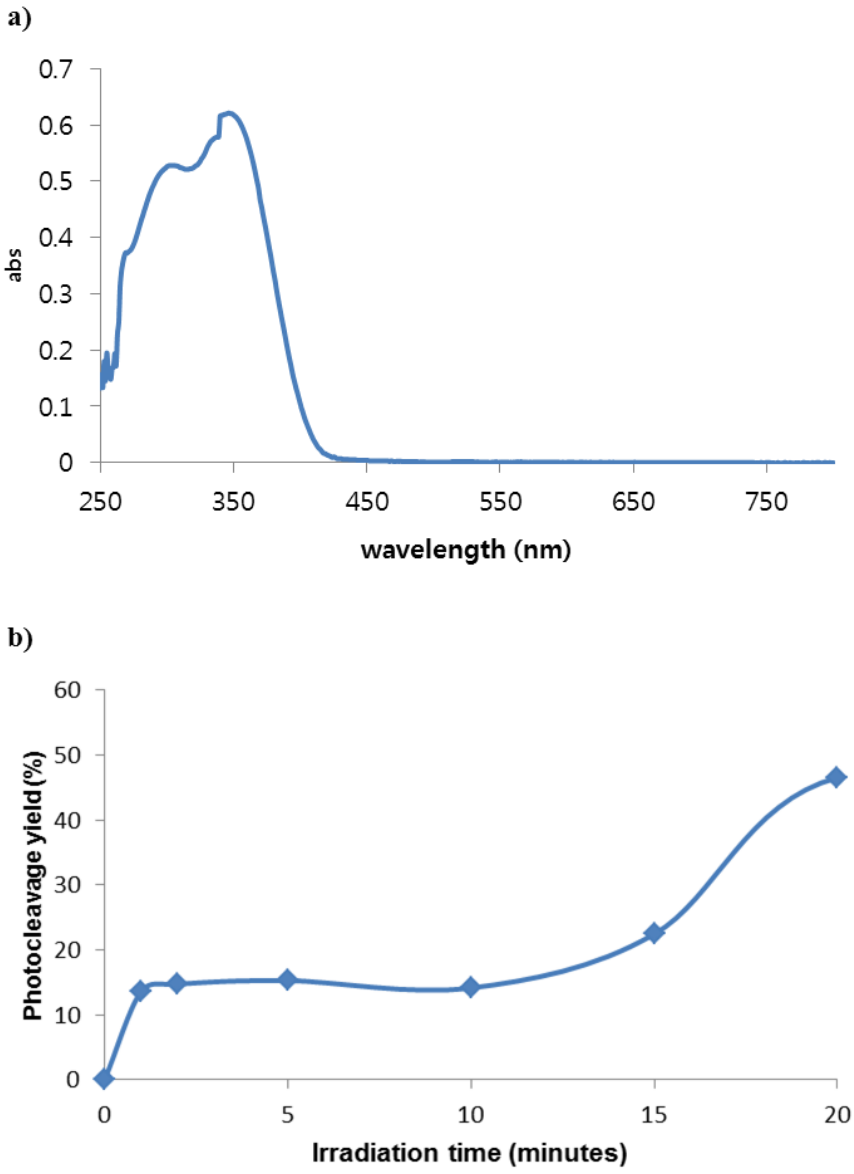


Figure 14. a) UV-Vis absorption spectra of Ac-PCA-OH. λ_{max} of Ac-PCA-OH was around 350 nm. b) Photocleavage kinetic profile of Fmoc-PCA linker.

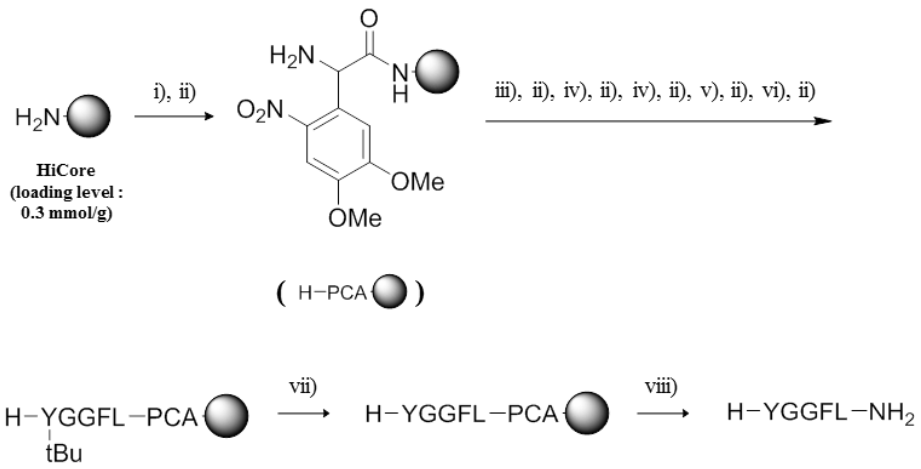
NH_2 was measured with own calibration curve. The photocleavage yield of Fmoc-Phe-NH_2 increased over time, and reached ca. 50 % in 20 minutes (Figure 14. b). This result was comparable to those with Fmoc-PLL and Fmoc-ANP linker.[22, 64] The photocleavage efficiency of Fmoc-PCA linker with UVA light was similar or better compared to it of Fmoc-PLL and Fmoc-ANP linker. From this result, we could confirm that Fmoc-PCA linker could be used as the alternative photolinkers for on-bead bioassays.

III. 1. 3. Synthesis of peptide amide with Fmoc-PCA linker coupled resins

In general SPPS, peptides are synthesized on a linker-coupled resins with Fmoc or Boc chemistry. After coupling all the amino acid building blocks to the resins, side-chain protection groups are removed with cleavage cocktail. In the case of commonly used Rink amide resin,[77] not only the global deprotection but also releasing of the synthesized peptide occurs during this step. Although the released peptides are purified by sedimentation and centrifugation, it is certain that the impurities originated from the side-chain protecting groups might exist in the crude product. Being different from Rink amide resin, however, the synthesized peptides still remain on the resins even after the global deprotection step when the photolabile linker was used. And also, the impurities originated from the side-chain protecting groups could be easily removed by simple filtration.

Therefore, synthesized peptides with photolabile linkers showed high purity without complex separation step such as sedimentation.

To confirm the performance of Fmoc-PCA linker in solid-phase peptide amide synthesis, leu-enkephalin amide (H-YGGFL-NH_2)[78] was synthesized on the Fmoc-PCA linker coupled HiCore with Fmoc chemistry (Scheme 4). After the global deprotection with cleavage cocktail, leu-enkephalin amide was released from the resins by UV exposure. The released product was analyzed with HPLC (Figure 15) and MALDI-TOF MS analysis (Figure 16). HPLC analysis of the released product showed a single peak at around 18.5 minutes, which was identical to the authentic leu-enkephalin amide, and there was no significant side product. The MALDI-TOF MS analysis of the released product showed $[M+H]^+$: 555.62 and $[M+Na]^+$: 577.56. Overall, these results proved that the peptides could be synthesized with high purity with Fmoc-PCA linker. And this result also meant that Fmoc-PCA linker was stable at both Fmoc-chemistry and global deprotection conditions including piperidine or TFA treatment, as expected.



Scheme 4. Synthetic scheme of H-YGGFL-NH_2. i) Fmoc-PCA linker, BOP, HOBt, DIPEA in DMF, ii) 20 % (v/v) piperidine/DMF, iii) Fmoc-Leu-OH, BOP, HOBt, DIPEA in DMF, iv) Fmoc-Gly-OH, BOP, HOBt, DIPEA in DMF, v) Fmoc-Phe-OH, BOP, HOBt, DIPEA in DMF, vi) Fmoc-Tyr(tBu)-OH, BOP, HOBt, DIPEA in DMF, vii) TFA/DODT/water/TIPS (94 : 2.5 : 2.5 : 1, volume ratio), and viii) UVA light irradiation.

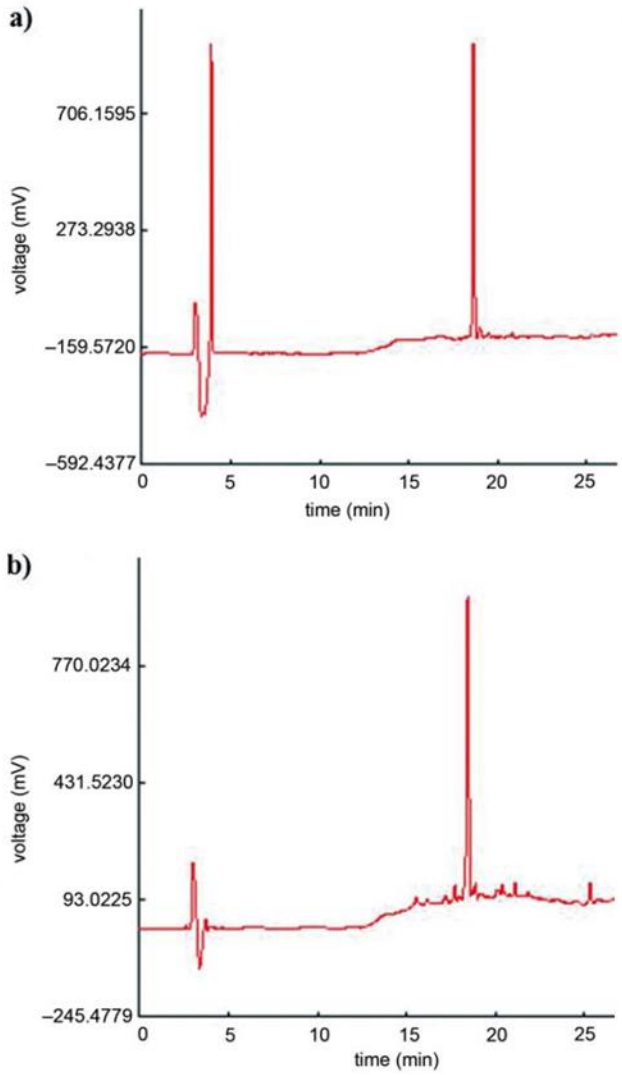


Figure 15. HPLC analysis of a) authentic H-YGGFL-NH_2 and b) synthesized H-YGGFL-NH_2 by using the Fmoc-PCA linker.

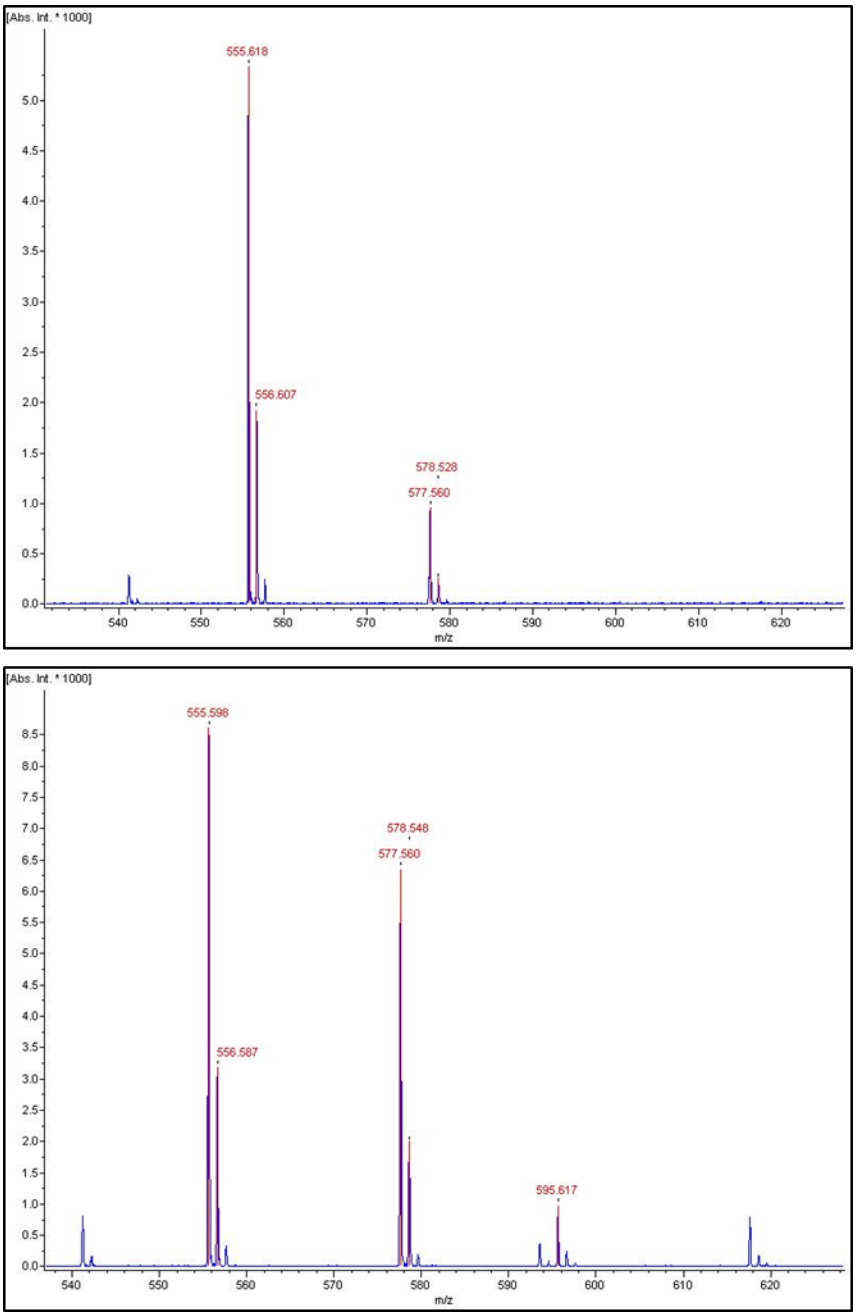
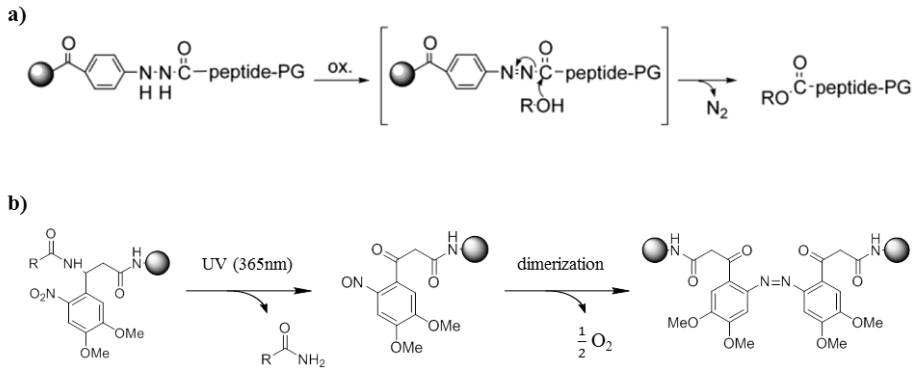


Figure 16. MALDI-TOF MS analysis of synthesized H-YGGFL-NH_2 ($[M+H]^+$: 555.62, $[M+Na]^+$: 577.56), using Fmoc-PCA linker (up) and Fmoc-PLL (below).

III. 1. 4. Synthesis of peptide acid and peptide methyl ester with phenylhydrazine-PCA coupled resins

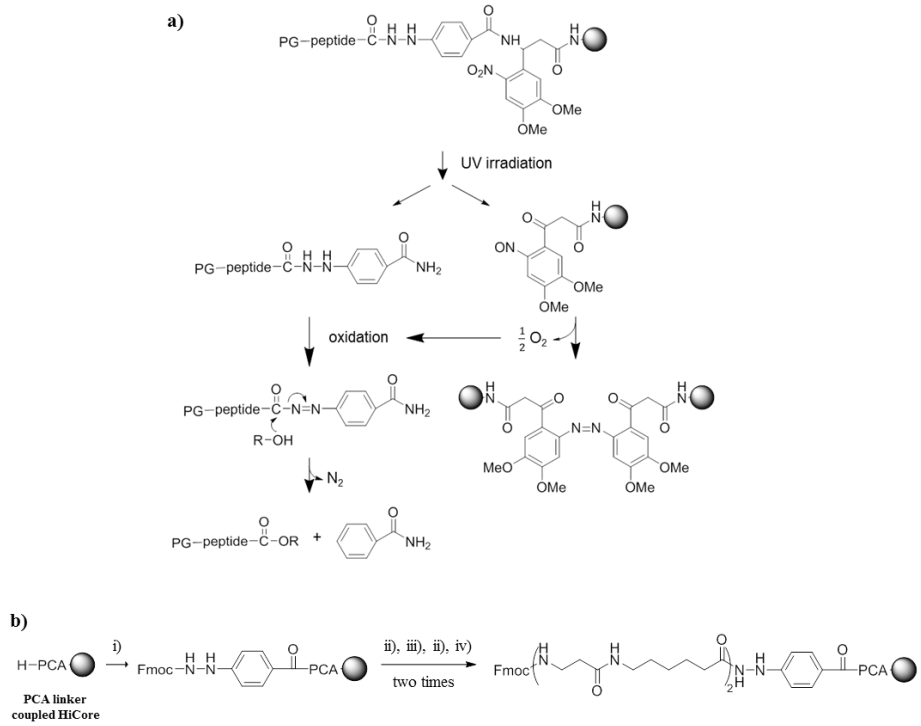
Not only peptide acid and peptide amide, but also peptide esters are needed for some biological applications.[79, 80] In spite of their needs, however, synthesis of peptide esters is quite cumbersome because the original peptides can be modified during esterification conditions. Thus, not only C-terminus but also side chains of acidic amino acids (Asp and Glu) could undergo the esterification reaction. To overcome these problems and synthesize peptide ester from the resins conveniently, the linkers which can release the synthesized peptides as an ester form under the basic conditions (tertiary base/MeOH) was used.[81, 82] This releasing condition is a little harsh, therefore, Peters *et al.* proposed a facile method for the synthesis of peptide ester with aryl hydrazine linker which was originally introduced by Wieland *et al.* (Scheme 5. a).[83, 84] According to his report, phenylhydrazine group has a similar nucleophilicity as the amino group so it could be reacted with HOBt-activated ester, affording acyl-phenylhydrazone bond. Because the acyl-phenylhydrazone bond is stable under base and acid conditions, the synthesized peptides are not released during the conventional SPPS condition such as Fmoc or Boc chemistry. Alternatively, the synthesized peptides are only released from the resins by oxidation and nucleophilic addition reaction. When acyl-phenylhydrazone bond is oxidized to acyl-phenyldiazene bond, the



Scheme 5. a) Preparation of peptide acid and peptide ester via oxidation of acyl-phenylhydrazide moiety. b) Dimerization of nitrosobenzyl derivatives.

resulting acyl-phenyldiazene bond is decomposed into benzene ring and nitrogen gas when the nucleophiles such as water or alcohols attack the carbonyl group. After cleavage step, peptide acids can be obtained when water is used as a nucleophile, and peptide esters can be obtained when alcohols are used as a nucleophile. This method is one of the efficient synthetic methods for the peptide ester under mild condition. However, it still has some drawbacks such as longer oxidation time (more than 2 hours) and the product can be contaminated by metal impurities which come from the oxidizing agent. Because it is certain that the above mentioned problems of hydrazine linker could be overcome if the acyl-phenylhydrazone bond is oxidized rapidly without using any metal oxidizing agent, we tried to find out the oxidation condition without using metal oxidizing agent.

While looking for the mild oxidation condition, we noticed an interesting chemistry in the overall photochemical reaction of Fmoc-PCA linker. According to the radical rearrangement mechanism of PCA linker, nitrophenyl group of Fmoc-PCA linker is converted into nitrosophenyl group which can be easily dimerized with another nearby nitrosophenyl group after the photocleavage (Scheme 5. b).[23] Because the oxygen gas is generated during this dimerization reaction, we expected that the acyl-phenylhydrazone bond could be oxidized by the oxygen molecule (Scheme 6. a).



Scheme 6. a) Proposed pathway for peptide acid and ester formation with phenylhydrazine-PCA conjugated linker. b) Preparation of Fmoc-*βεβε*-HBA-PCA-HiCore, i) Fmoc-4-HBA, BOP, HOBt, DIPEA in DMF, ii) 20% (v/v) piperidine/DMF, iii) Fmoc-*ε*-ACA, BOP, HOBt, DIPEA in DMF, iv) Fmoc-*β*-Ala-OH, BOP, HOBt, DIPEA in DMF.

To confirm our hypothesis, a model peptide sequence was synthesized on the phenylhydrazine linker and Fmoc-PCA linker coupled HiCore (Fmoc-*βεβε*-HBA-PCA-HiCore, Scheme 6. b). The resulting HiCore was swollen in water and MeOH each, and UV was irradiated to the each resins after swelling. The collected supernatants were analyzed by ESI-MS, and the result is shown in Figure 17. In both of the two cases, the peak which corresponds to Fmoc-*βεβε*-HBA-NH_2 ($[M+H]^+$: 742.3) was observed. It was certain that Fmoc-*βεβε*-HBA-NH_2 was originated from the photocleavage of PCA linker. Interestingly, only the peak which corresponds to Fmoc-*βεβε*-OMe ($[M+H]^+$: 623.3) was observed when MeOH was used as a nucleophile, and Fmoc-*βεβε*-OH ($[M+H]^+$: 608.5) was observed as major product when water was used a nucleophile. From these results, it is certain that the acyl-phenylhydrazone bond of Fmoc-*βεβε*-HBA-NH_2 proceeded the proposed reactions (oxidation and nucleophilic addition reaction) by single photocleavage reaction without using any additional oxidizing agent. With the merits of short reaction time and no additives, this facile synthetic method for peptide ester can substitute the previously reported peptide ester synthesis method.

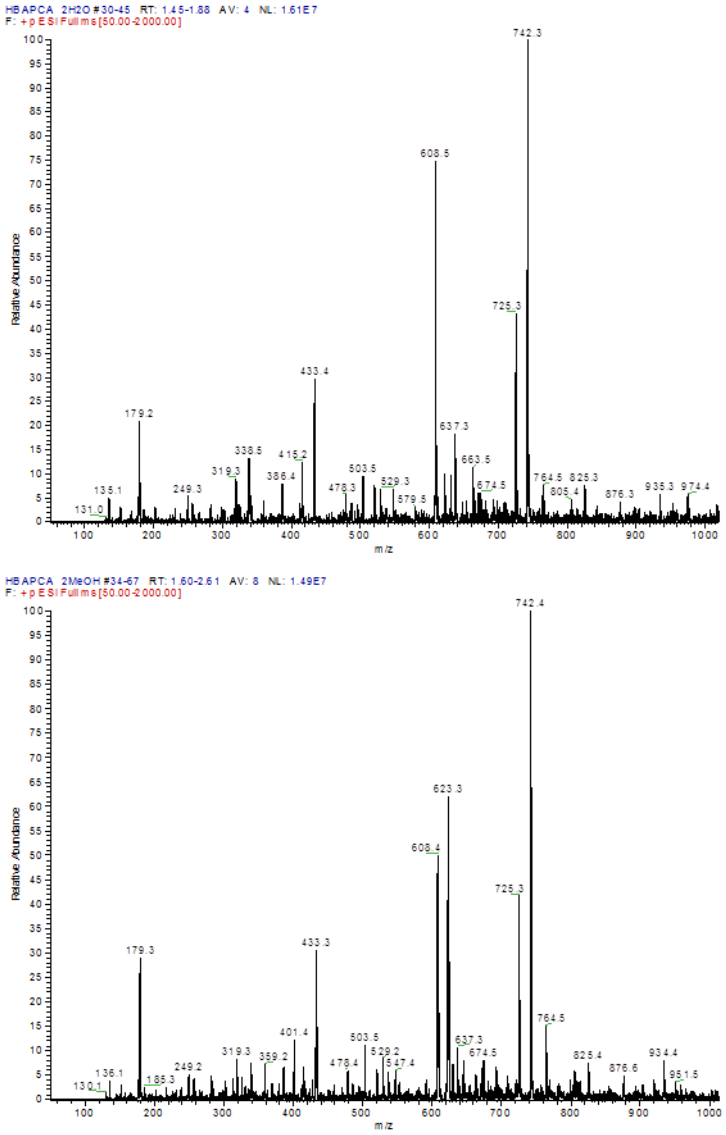
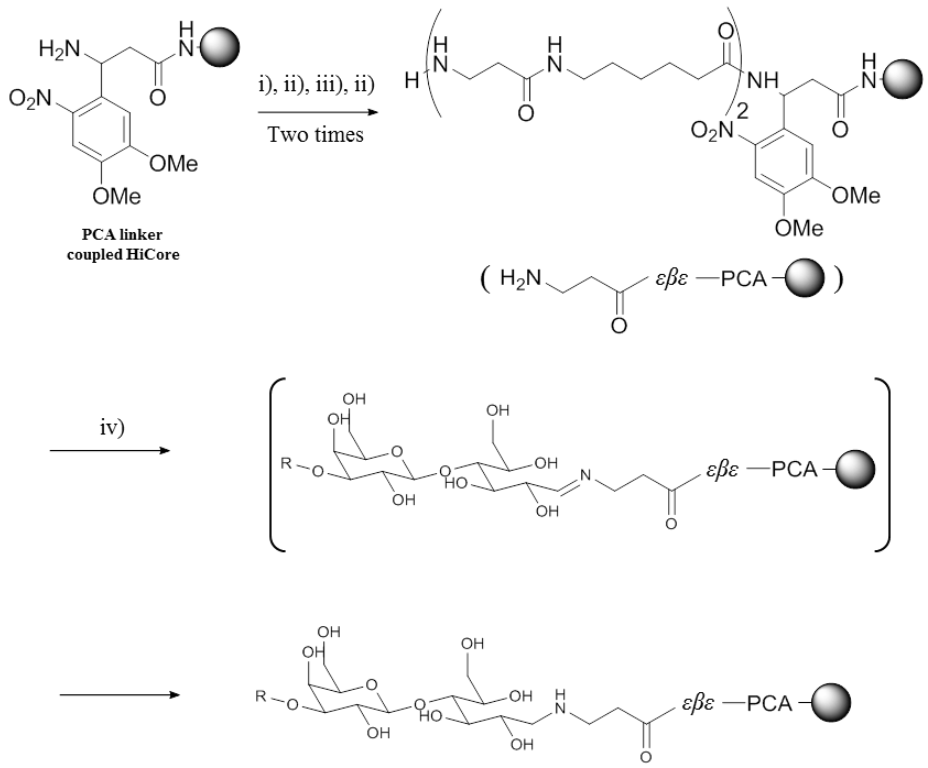


Figure 17. ESI-MS analysis of released compounds from phenylhydrazine-PCA linker coupled HiCore, in the aqueous media (above) and in the methanol media (below).

III. 1. 5. Immobilization of glycans onto PCA linker coupled resins for on-bead assays

Not only peptides, but also glycopeptides have been synthesized or immobilized on resins for the on-bead screening.[85] Because some glycoproteins such as membrane proteins play an important role in the metabolism of living cells, the need for of immobilization and on-bead assays of glycopeptides (or proteins) is keep increasing nowadays.[86, 87]

To immobilize glycans, H-*βεβε*-PCA-HiCore resin was prepared with Fmoc chemistry. The non-natural peptide spacer (*βεβε*) was often used for enhancing the accessibility of the synthesized molecules toward enzymes or antibodies by reducing the steric hindrance caused by polymer backbone. In Scheme 7, immobilization reaction of lactose and 3'-sialyllactose to amine-functionalized HiCore (H-*βεβε*-PCA-HiCore) is described. The reducing ends of glycans can be converted into aldehyde group, and this aldehyde group can be conjugated with the amino group on the resin via Schiff base formation. The Schiff base (imine), however, can be easily hydrolyzed and the immobilized glycan is released from the resins under the Schiff base formation condition, because the equilibrium constant of the corresponding reaction is nearly 1. To prevent the reverse reaction (hydrolysis of Schiff base), $NaCNBH_3$ which is one of the typical mild reducing agent was added to the reaction mixture. Because of its reducing property, carbon-nitrogen double bond of Schiff base was



Scheme 7. Preparation of glycopeptide coupled PCA-HiCore. i) Fmoc-*ε*-ACA, BOP, HOBt, DIPEA in DMF, ii) 20 % (v/v) piperidine/DMF, iii) Fmoc-*β*-Ala-OH, BOP, HOBt, DIPEA in DMF, iv) lactose or 3'-sialyllactose, $NaCNBH_3$ in 5:2 (v/v) DMSO/AcOH (R = H for lactose, R = sialyl group for 3'-sialyllactose).

converted to single bond. Due to this reduction reaction, carbon atom which was located next to nitrogen atom lost its electrophilicity, and the hydrolysis of Schiff base did not occurred.

Immobilized glycopeptides were released from the resins by the same way as the peptides were released, and analyzed by ESI MS. As shown in Figure 18, lactose and 3'-sialyllactose conjugated glycopeptides appeared at their estimated molecular mass ($[M+H]^+$: 712.87 for lactose-$\beta\varepsilon\beta\varepsilon$-$NH_2$, $[M+H]^+$: 1003.40 for 3'-sialyllactose-$\beta\varepsilon\beta\varepsilon$-$NH_2$). Therefore, we can conclude that the glycans were successfully immobilized onto the resins. With these glycan-immobilized resins, further studies for the screening of glycan-binding proteins can be possible.

III. 1. 6. Mass analysis of peptides with Direct On-bead Laser Desorption/Ionization method

According to the principle of MALDI-TOF MS analysis, matrices which are mixed with analytes at the sample loading stage are necessary (Figure 19. above).[88] Because of the laser's high energy, laser-absorbed matrices are vaporized and driven into TOF mass spectrometer. At the same time, the analytes, which are placed onto the MALDI-TOF sample plate, were also driven into TOF mass spectrometer and analyzed. Due to this reason, the mass spectra of added matrices appear very strongly. This fact sometimes makes confusion when the mass spectra of the analytes are

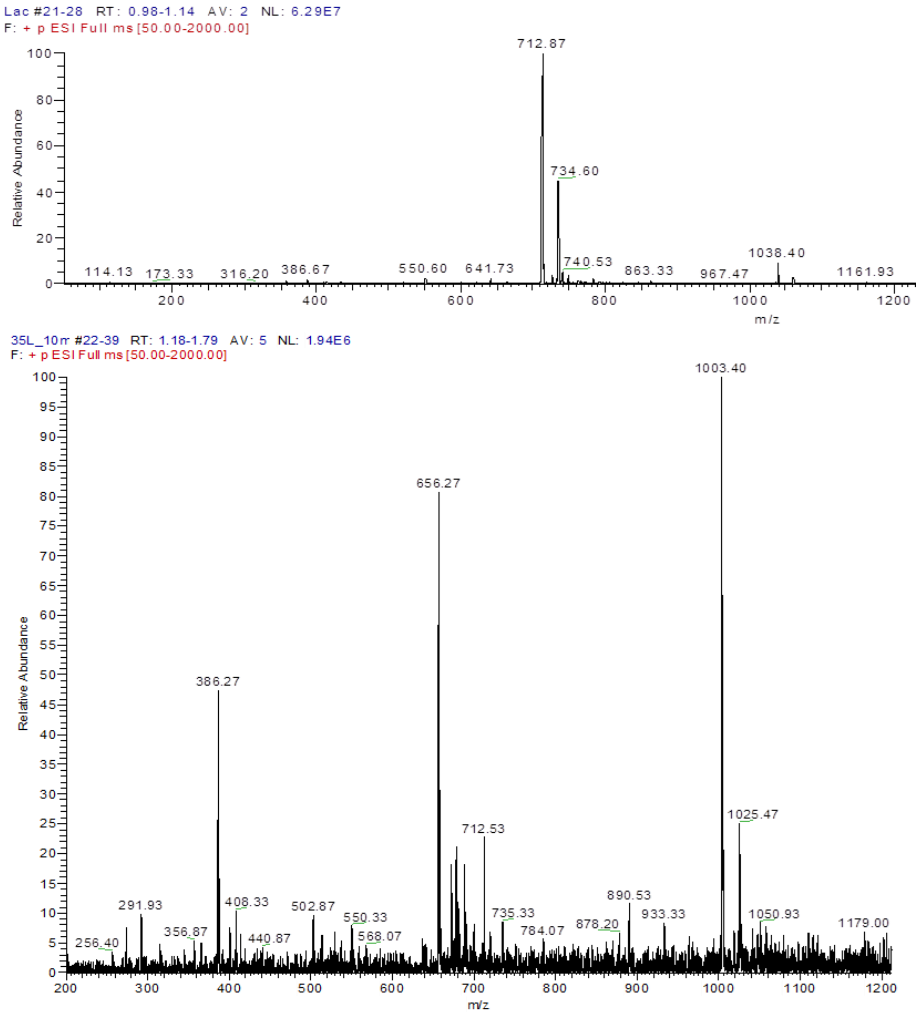


Figure 18. ESI-MS analysis of lactose-$\beta\varepsilon\beta\varepsilon$-$NH_2$ ($[M+H]^+$: 712.87, above) and 3'-sialyllactose-$\beta\varepsilon\beta\varepsilon$-$NH_2$ ($[M+H]^+$: 1003.40, below).

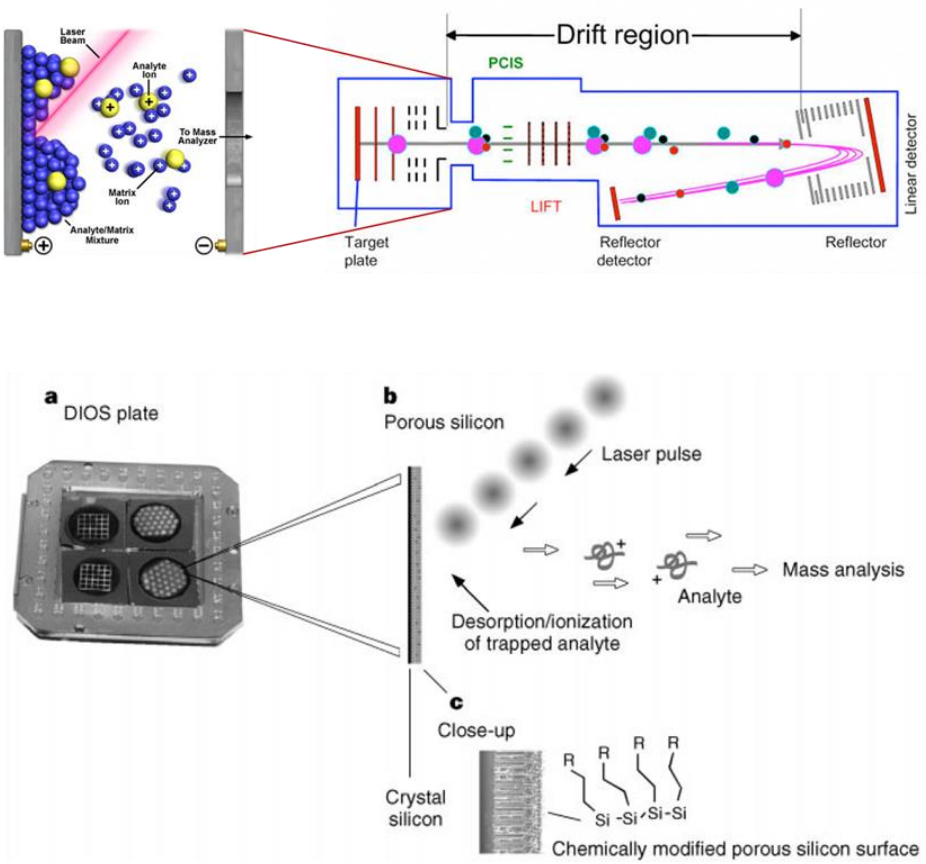


Figure 19. The principle of MALDI-TOF analysis (above) and desorption-ionization mass spectrometry on porous silicon (below).

similar to the added matrices. To overcome this problem, many researchers who want to analyze small molecules without any confusions have tried to analyze the analytes without organic matrices.[89] As an alternative additive, silica nanostructures have been applied (Figure 19. below).[90-94] Because silica has a good electrical conductivity, it can absorb the laser energy and transfer the energy to the analytes very efficiently. Furthermore, silica can be easily fabricated as nanostructure such as nanorod or nanoparticles, which enhances the efficiency of laser absorption. Although there are such merits, the carbon-carbon single bond or carbon-nitrogen single bond cannot be cleaved by the leaser energy in the presence of silica nanostructure. Thus, the analytes which are conjugated on the resin cannot be analyzed without being released. It means that "additional releasing step" is required for the on-bead mass analysis of the screened analytes. Because this "additional step" is inconvenient and sometimes the analytes can be damaged during this step, we tried to find a facile mass analysis method, in which the "additional step" could be omitted. Along the way, we have noticed that the nitrogen laser of 337 nm is commonly used in the MALDI-TOF apparatus. According to the UV-Vis absorption spectra of PCA linker in the previous chapter, PCA linker could absorb not only UVA light of 365 nm but also UVA light of 337 nm. Therefore, we expected that synthesized molecules could be released by irradiation of nitrogen laser.

The on-bead photocleavage by nitrogen laser was tested as a preliminary study. Leu-enkephalin amide was synthesized on the PCA linker coupled HiCore as described at Chapter III. 1. 3 (Scheme 4). Before the sample resins were loaded onto the MALDI-TOF plate, they were swollen in the SiNPs/EtOH solution for embedding of SiNPs.[95] We expected that the embedded SiNPs enhance the on-bead cleavage efficiency by absorbing and transferal the energy of laser although the absence of matrix. Differ from the matrix, however, SiNPs cannot ionize the analytes because they have not any acidic sites. Therefore in this model experiment, sodium chloride which is the most common salt was selected as the ion source. SiNPs-embedded resins were loaded onto the MALDI-TOF plate with sodium chloride solution, and dried rapidly. This rapid drying of sample should be critical points, because the large crystals of sodium chloride were formed when the solvent was dried very slowly. When the crystals were formed, analyte-sodium ion adducts could not be formed well because most of sodium ions were used for forming the crystals. Moreover, the laser irradiation to the resins was inhibited by reflection effect from these crystals.

The result of mass analysis was shown in Figure 20. When the CHCA was added as an additive, mass spectra of target peptides appeared ($[M+Na]^+$: 578.43, $[M+K]^+$: 594.58). At the same times, mass spectra of CHCA also appeared at 189.12, 145.05, and so on. The desired mass spectra were also observed in the presence of SiNPs and sodium chloride

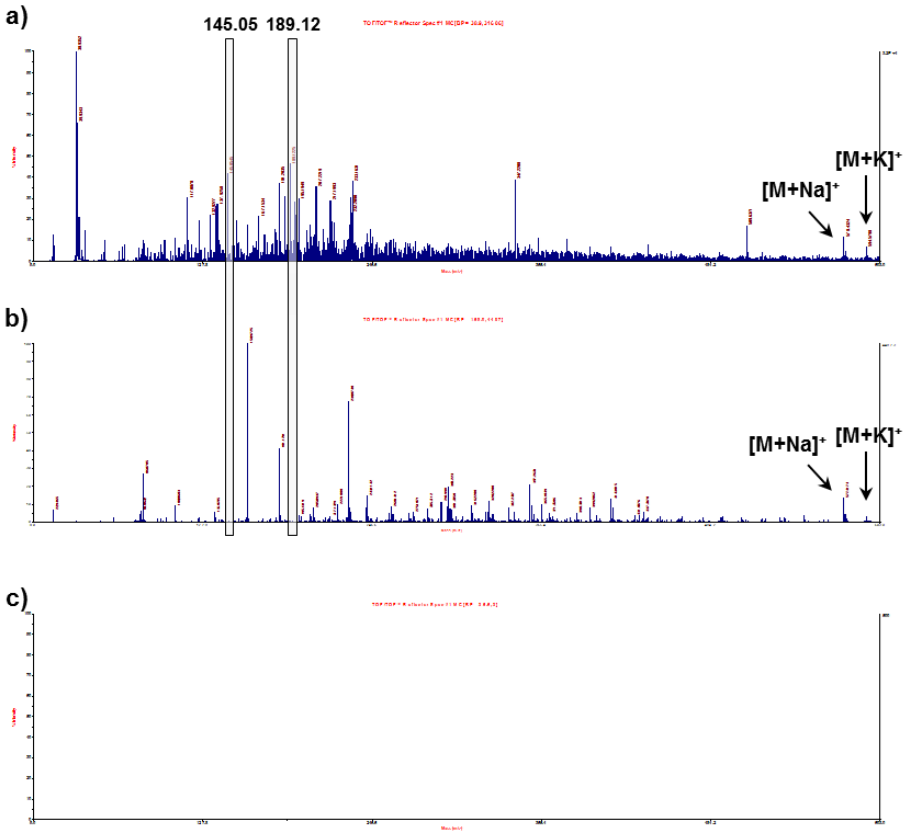


Figure 20. DOLDI-TOF MS analysis result of Leu-enkephalin amide with various conditions: a) analysis with CHCA, b) analysis with SiNPs/NaCl, and c) analysis without any additives. Mass peaks for CHCA, $[M+Na]^+$, and $[M+K]^+$ were written.

([M+Na]$^+$: 577.42). On the other hand, they were not observed when the laser absorbent (SiNPs) and ion sources (NaCl) were not added. From these results, we ascertained that the on-bead photocleavage with nitrogen laser occurred successfully in the presence of laser absorbent and ion source. Because on-bead analytes could be directly analyzed with laser absorption, we named this facile analysis method as DOLDI-TOF mass analysis method after the initial of "**D**irect **O**n-bead **L**aser **A**bsorption/**I**onization **T**ime-**O**f-**F**light".

During the DOLDI-TOF mass analysis, we found a very interesting result. Compared with the mass peaks of MALDI-TOF, there was only one major mass peak observed by DOLDI-TOF method. It was probably caused by the ion sources. Because the amount of ion sources was in excess compared with other ions existed in the solvent, the analytes were ionized as only one kind of salt-adduct. Therefore, only the sodium ion adducts were detected in DOLDI-TOF spectra, whereas proton and potassium ion adducts were also observed in MALDI-TOF spectra. This result showed a possibility of quantitative analysis of analytes with DOLDI-TOF method, without using any complex tagging method.

To confirm the effect of ion sources in the DOLDI-TOF method, 100 mM of salt solutions which are commonly used in biological fields were prepared. The ion sources were added to the peptide loaded resin, and the mass analysis results were shown in Figure 21. As previously shown, mass

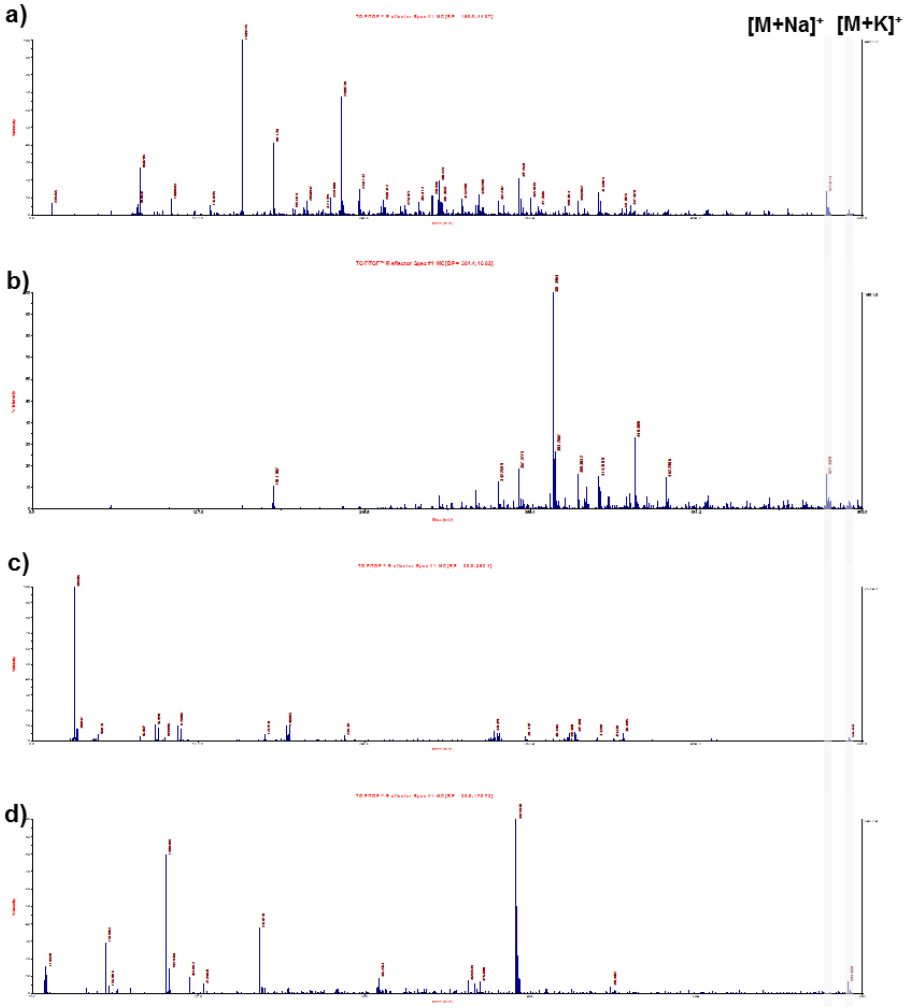


Figure 21. DOLDI-TOF MS analysis result of Leu-enkephalin amide with various salts. a) NaCl, b) NaI, c) KCl, and d) KI.

spectra of the target peptides appeared as one major peak which corresponds to the ion-adducts form. Thus, $[M+K]^+$ was observed when potassium salts were added, and $[M+Na]^+$ was observed when sodium salts were added. From this result, we concluded that the commonly used salts could be adapted to the DOLDI-TOF method, and potential possibility for quantitative analysis was also shown.

III. 2. Synthesis and Application of Protein Fishing Molecule (ProFiM)

A cell, the fundamental unit of living organism, usually interacts with extracellular environment through cell membrane. The cell membrane is consisted of phospholipid bilayer and quite large amount of proteins which are embedded in the bilayer. These proteins are so called "cell membrane protein". Membrane proteins are one of most important part of cell because they have responsibility for various biological activities of the cell. For example, they can bind with various numbers of proteins,[96] and also act as an identification protein.[97] Furthermore, they are associated with surface recognition,[98] signal transporting,[99] and have enzyme activity.[100] Because of these roles and importance, there had been many trials for isolation the membrane proteins to study their biological properties. Gradient centrifugation,[101] aqueous biphasic partitioning,[102, 103] and purification with monoclonal antibody were used for isolation the membrane proteins.[104] But the membrane proteins could not be isolated with high yield and purity because each trial had their own limitations. Recently, a powerful method using the host-guest interaction between bead-bound cucurbit[7]uril (CB[7]) and ferrocene-derivative labeled membrane protein was reported by Lee *et al*.[105] Although membrane proteins were isolated with high purity and yield by this method, a critical problem still remained. Not only with this method but also with other

tagging methods, the tagging compounds were usually bound to the isolated protein via amide bond and remained even after isolation. Because of these tagging compounds, the isolated membrane proteins lost their positive charge, and turned into more hindered structure compared with the untagged ones. Therefore, the biological activity of the isolated proteins is different from the natural ones. Furthermore, the isolated membrane proteins could not be identified by mass analysis because some of their lysine residues were inevitably modified. These problems might limit the identification and the use of the membrane protein. Thus, the tagging compounds must be removed without any traces after isolation (Figure 22). To archive this goal, we tried to synthesize a novel linker for isolation of the plasma membrane protein, which we named as ProFiM (named after **Pro**tein **fi**shing **m**olecule).

III. 2. 1. Synthesis of ProFiM

First of all, we designed the molecular structure of ProFiM by considering the following issues. Basically, ProFiM must be conjugated to the target proteins under the mild condition which does not damage the cell or the target proteins, and released from the target proteins easily. These two qualifications could be satisfied by using activated hydroxyl group containing photolabile linker which has similar molecular structure with NVOC group (Figure 23. a and b). It is possible that the hydroxyl

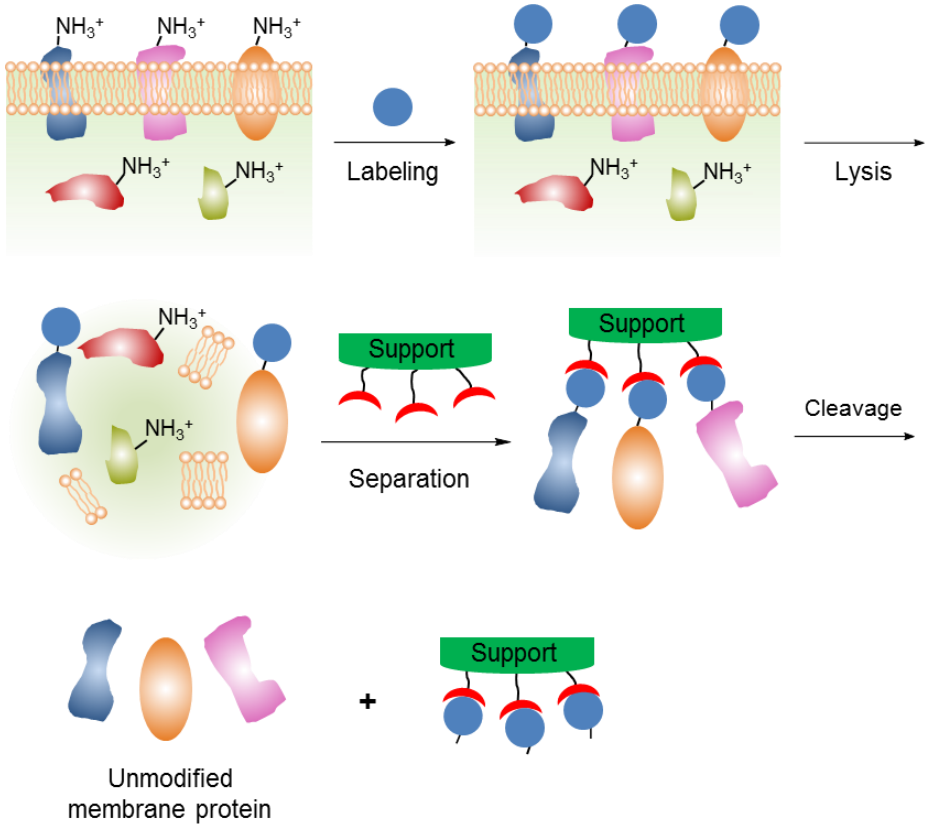


Figure 22. Overall concept for isolation of the cell membrane protein.

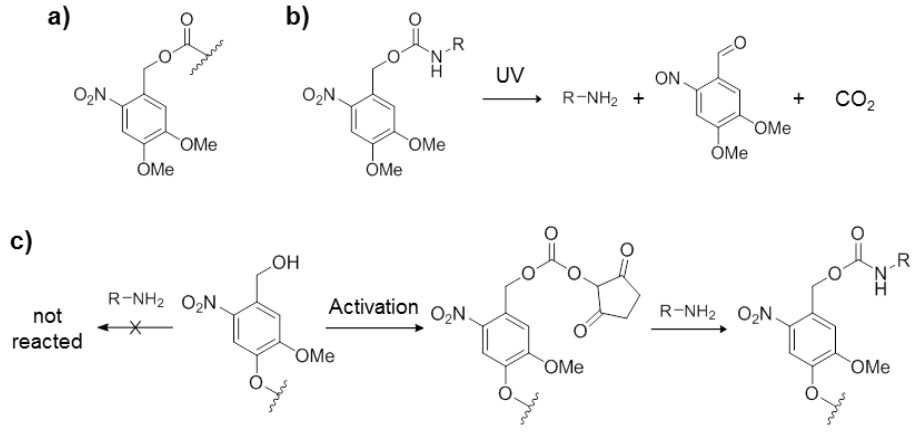


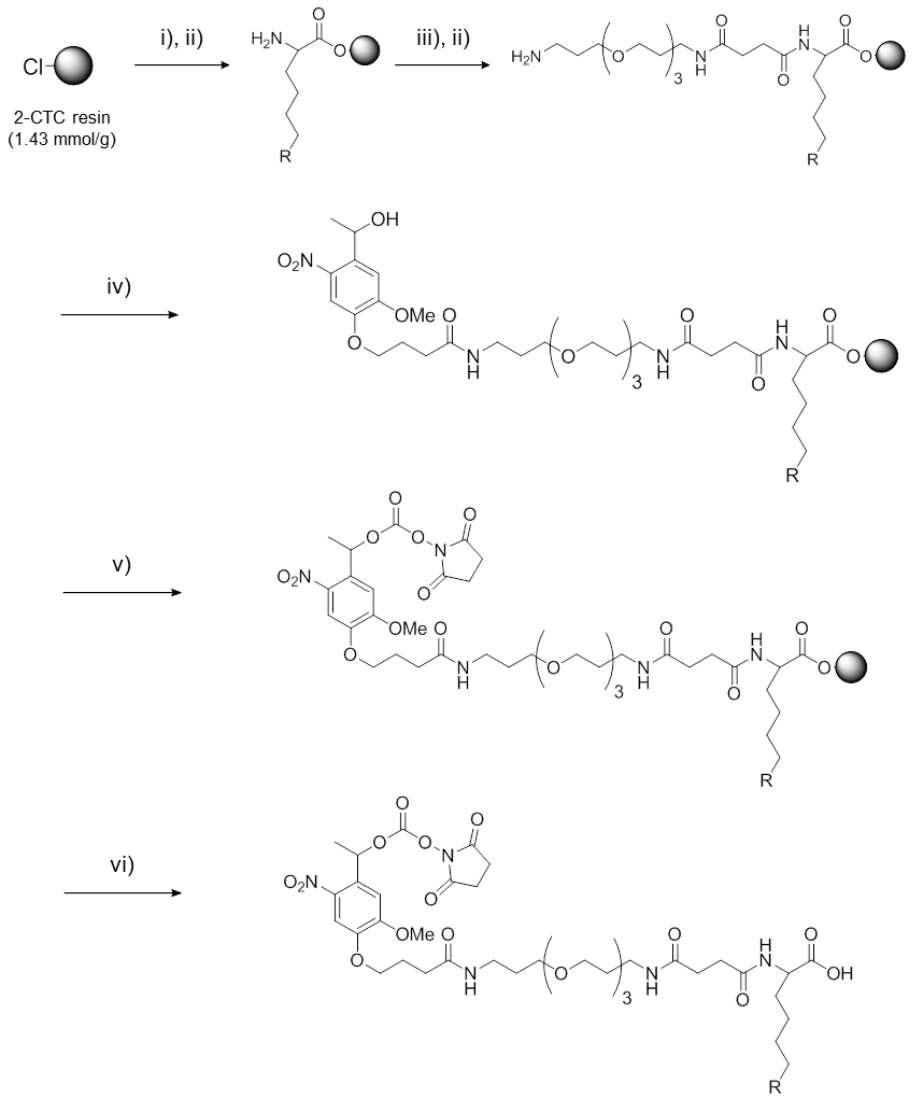
Figure 23. a) Structure of NVOC, b) deprotection of NVOC-protected amine, and c) strategy for conjugation of amino group to *o*-nitrobenzyl alcohol.

group containing photolabile linker[106] could act as NVOC group when it is conjugated with amino group via carbamate bond. However, the reactivity of *o*-nitrobenzyl alcohol moiety is too low to synthesize. To make a carbamate bond, *o*-nitrobenzyl alcohol moiety must be converted to an active carbonate by treating with DSC (Figure 23. c).[107] This active carbonate can be reacted with nucleophiles such as amino or hydroxy group which are rich in proteins under the physiological condition.[108] Among the nucleophiles, amino group has stronger nucleophilicity than others. Therefore, most of the active carbonate would be reacted with amino group and form a carbamate bond. The carbamate bond is quite stable under the physiological condition, however, can be converted into free amino group by intramolecular radical rearrangement when the nearby nitrophenyl motif absorbs UVA light as NVOC.[109] This hydroxyl group containing photolabile linker which has two electron donating groups can absorb UVA light (365 nm) which is less harmful to cell or proteins, and the target proteins are not damaged during releasing process. Next, the linker must have a binding site for target proteins on solid supports. By using solid supports, unlabeled (unwanted) proteins can be washed out easily and the captured-and-released target proteins can be isolated without any impurities. The binding site must not be reacted with cells or proteins during labeling step and should remain stable until the target proteins are released. To satisfy these requirements, azide group and biotin were chosen. Azide group can undergo the copper-catalyzed azide-

alkyne (CuAAC) reaction with alkynes, and biotin can bind to streptavidin specifically. Because these two molecules can't react with other molecules which are located at the cell surface, they can serve as a suitable binding site of the linker molecule. Thus, capturing site and binding site were connected with hydrophilic flexible spacer which contains oligoethylene glycol,[110] to increase the accessibility and hydrophilicity of the linker molecules.

The designed linker molecules were synthesized by solid-phase organic synthesis method (Scheme 8). Considering the mild conditions for the synthesis of the linker molecules, 2-CTC resin which can release the target compound as a free acid form was selected as the polymer support, instead of commonly used Rink amide resin. During the synthesis step, hydroxyl group containing photolabile linker was used without any protection of the hydroxyl group, because the hydroxyl group of the photolabile linker has poor reactivity due to the steric hindrance under BOP/HOBt coupling condition.[106]

ESI-MS analysis of the crude products which obtained after cleavage from the resins, not only showed the desired products (ProFiM-azide; $[M+H]^+$: 897.47, ProFiM-biotin; $[M+H]^+$: 1097.47) but also non-activated form of products (ProFiM-azide; $[M+H]^+$: 756.93, ProFiM-biotin; $[M+H]^+$: 956.46) (Figure 24). Although they were not activated, the non-activated form of ProFiM-azide and ProFiM-biotin could be immobilized to the resins. And then, the immobilization of target protein-conjugated



Scheme 8. Synthetic scheme of ProFiM-azide (R = azide) and ProFiM-biotin (R = biotin amide). i) Fmoc-Lys(N_3)-OH (for ProFiM-azide) or Fmoc-Lys(biotin)-OH (for ProFiM-biotin), DIPEA in DCM, ii) 20 % (v/v) piperidine/DMF, iii) Fmoc-Ttds-OH, BOP, HOBt, DIPEA in DMF, iv) photoreactive linker, BOP, HOBt, DIPEA in DMF, v) DSC, DMAP in DMF, and vi) 5 % (v/v) TFA/DCM.

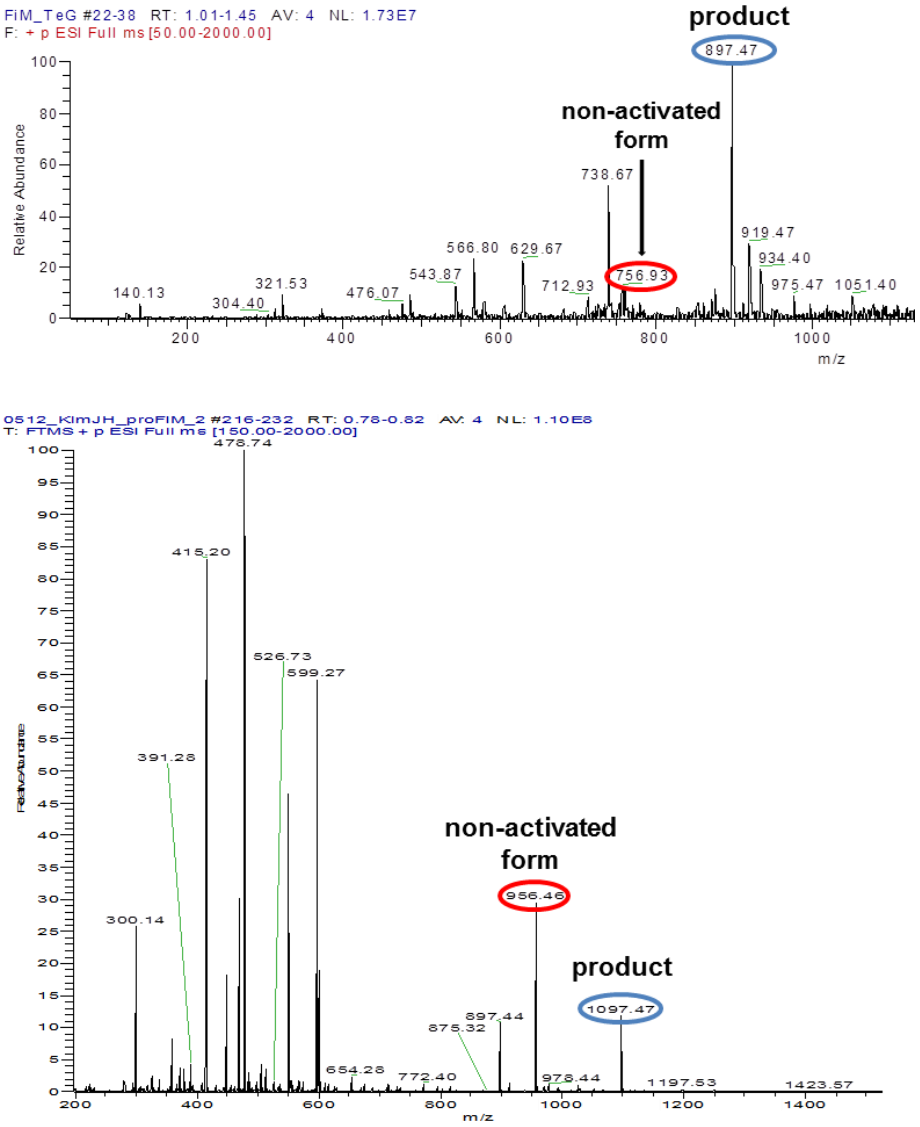
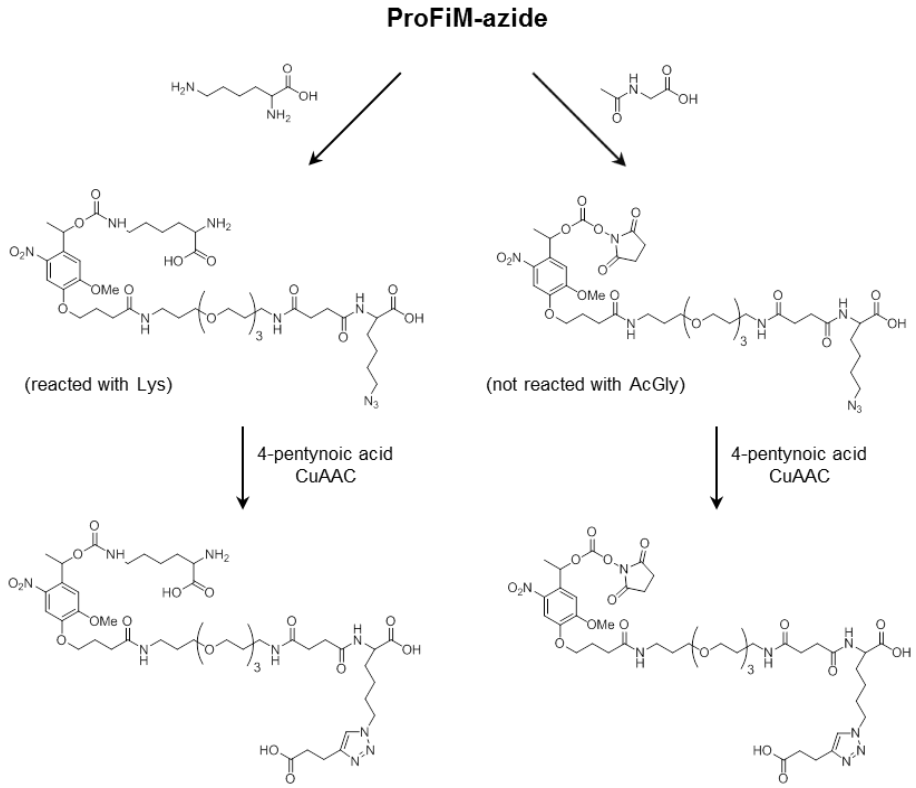


Figure 24. ESI-MS analysis of ProFiM-azide (above) and ProFiM-biotin (below).

ProFiMs could be inhibited. Because of this reason, the side products must be removed from the crude product. However, the separation of the side products was too difficult due to the following reasons; i) both two molecules are quite hydrophilic, and very long separation time is needed for traditional column chromatography with organic solvents, ii) the difference of their hydrophilicity was not large, and thus, separation of the two molecules cannot be successful with single trial, iii) the activated carbonate group could be reacted with water during purification steps. Therefore, the yield of desired product could be decreased as the separation time with liquid chromatography is increased. However, the binding of ProFiM-labeled proteins will not be interfered by the non-activated ProFiM if the non-activated ProFiM is washed out after the labeling step. Due to these reasons, crude ProFiM molecules were used without additional separation steps.

III. 2. 2. Reactivity test of ProFiM-azide toward amine and alkyne

Scheme 9 shows the overall strategy for the reactivity test of the synthesized ProFiM-azide with model amino acids and alkyne in solution phase. To confirm the reactivity toward amino group of ProFiM-azide, lysine which has two amino groups and *N*-acetylglycine were selected as model amino acids for conjugation reaction. Figure 25 shows the results



Scheme 9. Overall startagy for the reactivity test of synthesized ProFiM-azide with lysine, acetylglycine, and 4-pentynoic acid in solution phase.

of ESI-MS analysis after the conjugation step. Lysine conjugated ProFiM-azide ($[M+H]^+$: 928.46) was observed from lysine-ProFiM-azide mixture (Figure 25, a). On the other hand, only non-activated ProFiM-azide ($[M+H]^+$: 756.37) was observed from the *N*-acetylglycine-ProFiM-azide mixture (Figure 25, b). These results revealed that the only lysine (amine containing molecule) was reacted with ProFiM. From these results, the reactivity of ProFiM-azide toward free amino group was proven. As the next step, copper-catalyzed azide-alkyne cycloaddition (CuAAC)[111-113] reaction between alkyne and (lysine conjugated) ProFiM-azide was tried to confirm the reactivity of ProFiM-azide toward alkyne. CuAAC reaction is a kind of 1,3-dipolar cycloaddition, and triazole ring is formed after the reaction. For the CuAAC reaction, Cu(I) catalyst and ligands for chelating the copper catalyst are required. In our case, the mixture of $CuSO_4 \cdot 5H_2O$ and (+)-sodium-$_L$-ascorbate was used as the source of Cu(I) catalyst. The Cu(II) from $CuSO_4$ can be directly reduced to Cu(I) by $_L$-ascorbate, and could catalyze 1,3-dipolar cycloaddition. As the ligand of this system, we chose THPTA[73] among the known ligands such as tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA),[114, 115] because Liu *et al.* reported that THPTA has high water solubility and low biological toxicity than other ligands. These reagents and 4-pentynoic acid were added into lysine conjugated ProFiM-azide mixture or *N*-acetylglycine-ProFiM azide mixture, and each mixture was analyzed by ESI-MS (Figure 25. c and d). As expected, both of lysine conjugated ProFiM-azide and non-activated

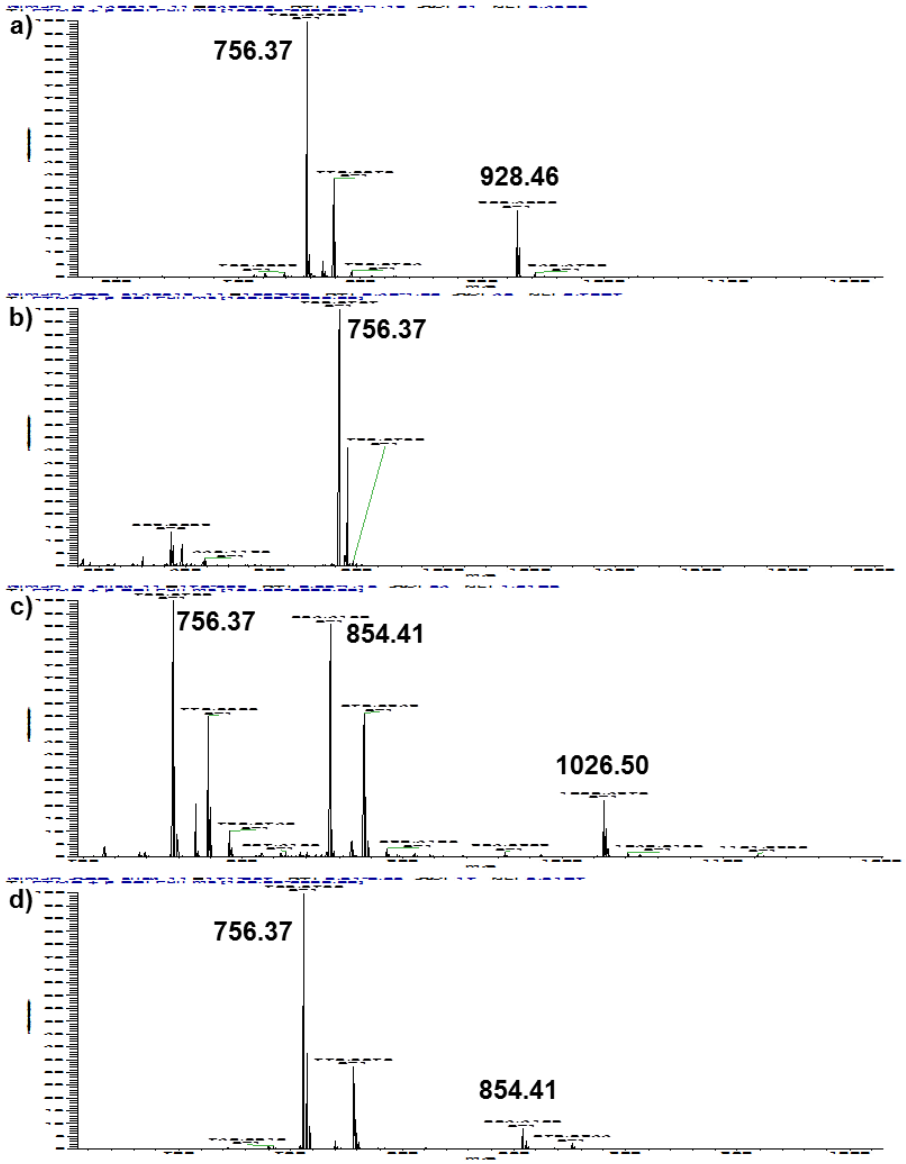
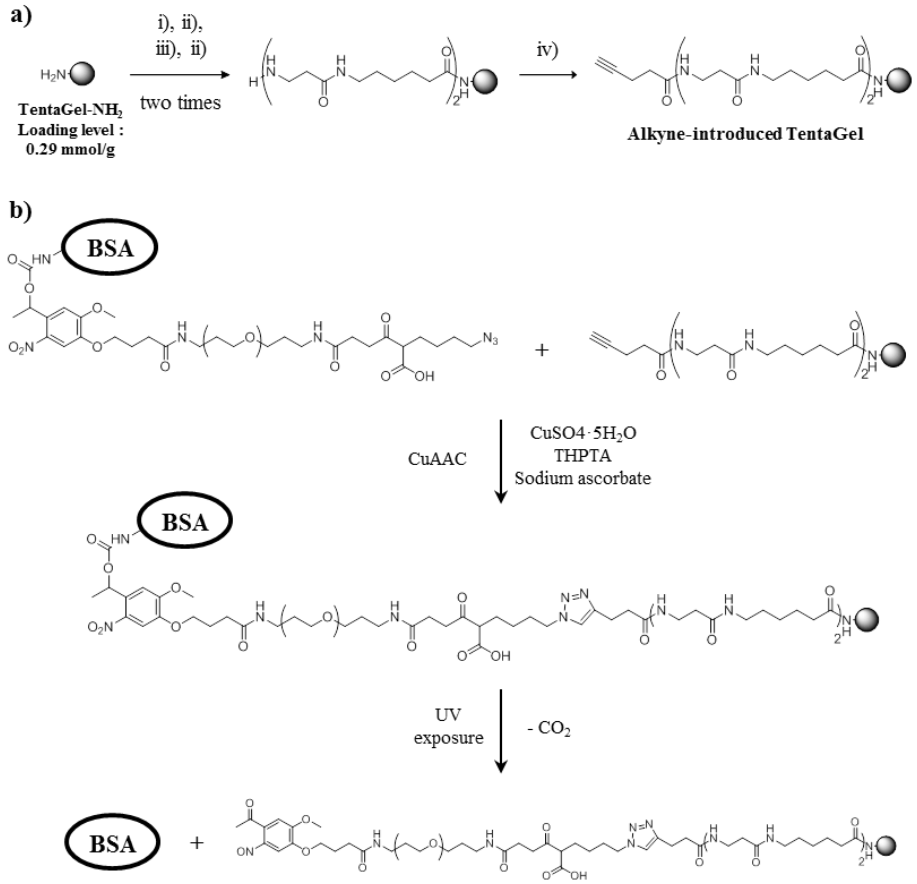


Figure 25. ESI-MS analysis of a) lysine and ProFiM-azide mixture, b) *N*-acetylglycine and ProFiM-azide mixture, c) lysine, ProFiM-azide, and reagents for CuAAC mixture, and d) *N*-acetylglycine, ProFiM-azide, and reagents for CuAAC mixture.

ProFiM-azide were undergone CuAAC reaction and the mass peaks of the resulting product appeared ($[M+H]^+$: 1026.50 and $[M+H]^+$: 854.41). Thus, we proved ProFiM-azide could be reacted with amine and alkyne, via carbamate bond formation and CuAAC reaction.

III. 2. 3. Capture-and-release performance of ProFiM-azide and ProFiM-biotin with model protein

For confirmation of protein capture-and-release ability of ProFiM-azide, BSA was selected as a model protein. When BSA was treated with ProFiM-azide, BSA and ProFiM-azide was connected via carbamate bond as the case of model reaction with amino acid. After the conjugation step, $CuSO_4 \cdot 5H_2O$ and THPTA, a ligand for copper catalyst, were added to the reaction mixture for CuAAC reaction. As the alkyne source, alkyne-coupled resins were prepared (Scheme 10. a). Rigid spacer ($\beta\varepsilon\beta\varepsilon$) was introduced to Tentagel™-NH_2 resin for reducing the steric hindrance. Then, 4-pentynoic acid was coupled to the resins for the introduction of alkyne functionality. Releasement of BSA from the resin was confirmed by SDS-PAGE after photocleavage (Scheme 10. b). In the first trial, CuAAC reaction was performed for 2 hours which is twice longer than reported ones.[116, 117] Unexpectedly, however, no protein was released from the resin, which was proved by no significant band in SDS- PAGE (datas were not shown). These results indicate that the immobilization of BSA



Scheme 10. a) Preparation of alkyne-introduced resins, i) Fmoc-*ε*-ACA, BOP, HOBt, DIPEA in DMF, ii) 20 % (v/v) piperidine/DMF, iii) Fmoc-*β*-Ala-OH, BOP, HOBt, DIPEA in DMF, iv) 4-pentynoic acid, BOP, HOBt, DIPEA in DMF. b) Overall scheme for immobilization-and-releasement of BSA.

did not occur under the experimental condition. We assumed that the CuAAC reaction was too slow on the resin, which has different environment from solution phase. The immobilization of ProFiM-azide labeled BSA via CuAAC reaction was tried again with longer reaction time. Regardless of our efforts, BSA was not immobilized via CuAAC reaction even after 3 days of reaction. This unexpected result might come from the problems of the 'mobility of molecules' and 'steric hindrance' problems on solid support. According to the already known CuAAC reaction mechanism, copper catalyst must approach and coordinate to alkyne motif.[118] At the same time, the azide group must approach to the copper-coordinated alkyne during the CuAAC process. In our system, however, the chances for the coordination of copper catalyst to alkyne and azide group approach to alkyne would be too low due to the low mobility of immobilized alkyne. Furthermore, most of alkyne and azide groups would be screened off by resins and protein. Thus, efficient collision could not be occurred even though the copper catalysts were coordinated to alkynes successfully.

As an alternative approach for the immobilization of labeled protein, the same experiment was performed with ProFiM-biotin and streptavidin-coated beads. After the immobilization of ProFiM-biotin labeled BSA and photocleavage, the supernatant solution was analyzed with SDS-PAGE. As shown in Figure 26. a, the supernatant solution (lane 3) showed a band corresponding to BSA (lane 2). In conclusion, BSA could be captured

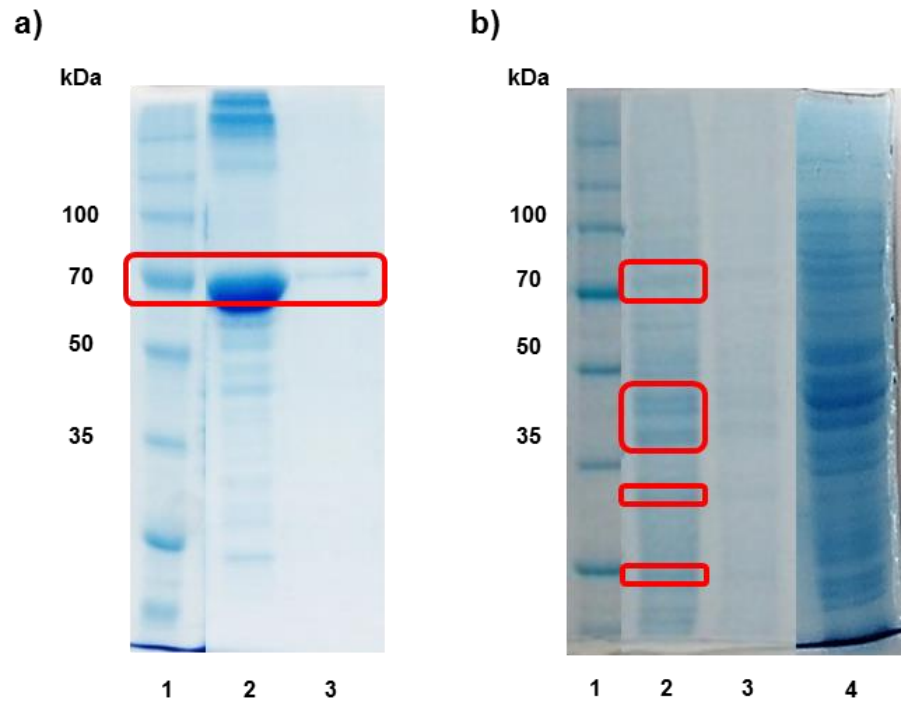


Figure 26. SDS-PAGE result of isolated proteins; a) size marker (lane 1), authentic BSA in PBS solution (lane 2), and isolated BSA (lane 3). b) Size marker (lane 1), isolated proteins (lane 2), supernatant before photocleavage (lane 3), and whole lysates of *E. coli* (lane 4). Proteins were stained by using coomassie blue. Significant bands were indicated in the red boxes.

once and released from the support after photocleavage. The reason why immobilization was successful with ProFiM-biotin, and not with ProFiM-azide can be found from the strong binding affinity between biotin and streptavidin. Although the mobility of solid supports might be still low, streptavidin can capture the ProFiM-biotin labeled proteins more frequently due to their high binding affinity.

III. 2. 4. Isolation of cell membrane proteins of *E. coli* with ProFiM-biotin

Encouraged with the previous result of model test, isolation of cell membrane proteins of *E. coli* was tried with ProFiM-biotin. Being different from the previous experiment which using the model protein, cell lysate contains various kind of amine-containing molecules. Therefore, it is important to prevent the reaction between ProFiM-biotin and unwanted compound (cytosolic proteins, in this case). For reducing the amount of unreacted ProFiM-biotin, the conjugation reaction was performed with enough time. However, the unreacted ProFiM-biotin still existed even though the reaction time was enough for the conjugation reaction. Therefore, ProFiM-biotin treated *E. coli* was washed with lysine solution to block the left over active carbonate groups before the lysis. Then, the whole lysates supernatant solution was treated with streptavidin-coated bead for immobilization of proteins. After the immobilization step,

supernatant solution after photocleavage was analyzed by SDS-PAGE. As shown in the Figure 26. b, some specific bands appeared with strong signal in the photocleaved supernatant solution (lane 2), while no significant band were observed in the supernatant solution before photocleavage (lane 3). From these results, we could conclude that the target membrane proteins were captured once and released after photocleavage without any significant nonspecific adsorption. Based on the case of NVOC, it was certain that the lysine which ProFiM-biotin was coupled might be unmodified after releasing. This characteristic was not noticed from in the isolation of known cell membrane proteins. However, it will be a great advantage for the identification of isolated unknown membrane protein with MS/MS analysis method. Because the amino groups of lysine were not modified, isolated proteins with ProFiM-biotin might be decomposed into smaller peptide fragments by trypsin digestion, compared with the lysine-modified proteins which were isolated with general labeling method. Furthermore, desired molecules such as fluorophores can be labeled to the isolated proteins via amino group of lysine. We expected that this property may be useful for the further study about isolated proteins.

IV. Conclusions

In this thesis, novel photoreactive molecules which can absorb the UVA light with wavelength of around 330 to 370 nm were synthesized, and applied them to several chemical and biological experiments.

In the first part, novel photolinker for solid-phase peptide synthesis which named as Fmoc-PCA linker was synthesized. Fmoc-PCA linker has the merits that combine the advantage of both established photolinkers. Fmoc-PCA linker could be synthesized more efficiently than Fmoc-PLL, and rearranged under milder conditions than Fmoc-ANP linker. The photocleavage efficiency of Fmoc-PCA linker was almost identical to Fmoc-PLL. Peptide amide, acid, and methyl ester were synthesized efficiently by using Fmoc-PCA linker. In addition, Fmoc-PCA linker could be adapted for the preparation of glycan immobilized resins. Moreover, a facile mass analysis method without using matrices and any additional cleavage step was developed with Fmoc-PCA linker. Pre-ionized target molecules were released from the resins by the rearrangement of PCA linker under nitrogen laser (337 nm) irradiation, and analyzed by TOF method. From these results, we proved that Fmoc-PCA linker can be applied to various biological experiments as photolinkers. The Fmoc-PCA linker is expected to be used as the alternative photocleavable linkers.

In the second part, ProFiMs which contains photoreactive moiety were synthesized for the isolation of cell membrane proteins without any modification. ProFiMs are consisted of *N*-succinimidyl-activated

carbonate group which could react with amino group containing molecules such as lysine or proteins, and azide or biotins which could undergo the specific reactions with alkyne or streptavidin. Amino group capturing ability of ProFiM-azide was confirmed by the reaction with lysine solution and CuAAC reaction was also processed well with 4-pentynoic acid in solution phase. However, CuAAC reaction did not proceed well with alkyne immobilized resins. Instead, the membrane proteins of *E. coli* were isolated by using ProFiM-biotin and streptavidin coated beads. Being different from other protein isolation method with protein labeling, unmodified proteins could be isolated by utilizing this system. This unmodified protein isolation method will open a new avenue for the isolation and identification of unknown cell membrane proteins.

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요 약

광화학은 독특하고 유용한 성질을 지닌 화학의 한 분야이다. 광화학의 핵심 물질인 광 반응성 물질은 산 혹은 염기 조건에서 안정한 반면, 빛의 노광에 의해 화학 반응을 일으킨다. 이러한 특수한 성질을 활용하여 광 반응성 물질들은 고체상 유기합성의 연결체 혹은 광 분해성 보호기로 활용되어왔다. 또한 광화학은, 315 나노미터 이상의 파장을 지닌 빛은 생체 물질들에 심각한 손상을 주지 않는다는 점을 통해 화학 생물학에 적용되고 있다.

본 학위 논문은 고체상 유기 합성과 세포막 단백질의 분리를 위한 365 나노미터 파장대의 자외선을 잘 흡수하는 오쏘-나이트로벤질 아민/알코올 유도체들의 합성에 대해 서술하고 있다. 첫 번째로, 오쏘-나이트로벤질 아민 기능기가 포함되어 광 분해가 가능한 새로운 연결체를 합성하고, 고체상 유기 합성에 적용하였다. 새로이 합성된 광 분해성 연결체는 복잡한 반응 단계 없이 여섯 단계만에 33 %의 수율로 합성되었다. 합성된 광 분해성 연결체는 330에서 370 나노미터의 파장을 가진 자외선을 잘 흡수하였고, 기존 광 분해성 연결체들과 비슷하거나 더 나은 광 분해능을 보였다. 이러한 성질들을 토대로, 합성된 광 분해성 연결체를 고체상 유기합성에 적용시켰다. 광 분해성 연결체가 결합된 고분자 지지체를 사용하여 류신엔케팔린 아미드를 높은 순도로 합성하였다. 광 분해성 연결체의 광 산화 효과를 통한 펩타이드의 카르복실 말단의 아실-페닐하이드라존의 산화와 친핵성 첨가반응을 통해 카복시산 및 에스터 형태의 펩타이드를 합성하였다. 생물 검정에 적용이 가능한 글리코펩타이드가 결합된 고분자 지지체를 글리칸과 광 분해성 연결체를 통해 고분자 지지체에 결합된 펩타이드간의 이민 형성 반응을 이용하여 제작하였고, 합성된 글리코펩타이드는 광 분해 후 질량분석을 통해 확인할 수 있었다. 또한 광 분해성 연결체에 의해 고분자 지지체에 결합되어있는 펩타이드를 매트릭스와 별도의 광 분해과정 없이 고분자 지지체 위에 결합된 상태로 레이저 흡수-이온화 질량 분석을 통해 분석하였다.

두 번째로, 오쏘-나이트로벤질 알코올을 포함하고 있는 물질을 사용한

세포막 단백질의 분리를 시도하였다. 세포막 단백질의 분리를 위해 아민기와 반응 할 수 있는 부분, 광 반응 부분, 친수성 스페이서 부분, 그리고 고분자 지지체와 결합되는 부분으로 구성된 물질을 합성하였다. 고분자 지지체와 결합되는 기능기로는 구리 보조 아자이드-알카인 고리첨가반응을 할 수 있는 아자이드기와 스트렙타비딘과 강한 결합을 형성하는 바이오틴을 선택하였다. 아자이드기를 도입하여 합성된 물질과 아민기, 알카인기와의 반응성은 아미노산과 4-펜타이노익산을 이용한 액체상에서의 시험 반응을 통해 확인할 수 있었다. 아자이드가 도입된 라이신과 4-펜타이노익산간의 구리 보조 아자이드-알카인 고리첨가반응은 액체상에서 잘 진행되었으나, 아자이드기가 도입된 소 혈청 단백질과 4-펜타이노익산이 결합된 고분자 지지체간에서는 해당 반응이 일어나지 않았다. 이에 아자이드 대신 바이오틴을 도입시킨 물질을 소 혈청 단백질과 결합시켰고, 해당 단백질을 스트렙타비딘이 고정화 된 입자에 결합시켰다. 또한 이 바이오틴을 도입시킨 물질을 대장균의 세포막 단백질과 결합 시킨 뒤, 해당 단백질들을 스트렙타비딘이 고정화된 입자에 결합시켰다. 결합된 단백질들은 자외선 노광을 통해 입자로부터 떨어져 나왔다. 이와 같은 방법을 통해 얻어진 단백질들을 소듐 도데실설파이트 폴리아크릴아미드 겔 전기 영동법을 통해 분석하였고, 이 결과를 해당 단백질 대조군의 결과와 비교하였다.

주요어 : 광화학, 오쏘-나이트로벤질 아민/알코올 유도체, 고체상 유기 합성, 세포막 단백질

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