Ph.D. Dissertation

Development of 4-fluorophenyl 3-nitro-2-pyridinesulfenate as a new Npys protecting reagent and its application to efficient disulfide formation

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ABBREVIATIONS

Ac: acetyl

Acm: acetamidomethyl

Ala: alanine

Arg (R): arginine

Asn (N): asparagine

Asp (D): aspartic acid

aq.: aqueous solution

Bn (Bzl): benzyl

Boc: *tert*-butoxycarbonyl

*t*Bu: tertiary butyl

Cs_2CO_3: cesium carbonate

Cys: cysteine

1,2-DCE: 1,2-dichloroethane

DBU: 1,8-diazabicyclo[5.4.0]undec-7-ene

DCM: dichloromethane

DIPCI: diisopropylcarbodiimide

DIPEA: *N*,*N*-diisopropylethylamine

DMF: *N*,*N*'-dimethylformamide

DMSO: dimethylsulfoxide

DTT: dithiothreitol

EDT: 1,2-ethanedithiol

equiv.: equivalent

Et: ethyl

Fmoc: 9-fluorenylmethyloxycarbonyl

Gln (E): glutamic acid

Glu (Q): glutamine

Gly (G): glycine

HATU: 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate

His (H): histidine

HOAt: 1-hydroxy-7-azabenzotriazole

HOBt: 1-hydroxybenzotriazole

HPLC: high-performance liquid chromatography

HRMS: high-resolution mass spectrometry

Ile (I): isoleucine

IR: infrared spectroscopy

Leu (L): leucine

Lys (K): lysine

Me: methyl

Met (M): methionine

Mmt: 4-methoxytrityl

mp: melting point

MS: mass

Mts: mesitylenesulfonyl

NMR: nuclear magnetic resonance

Npys: 3-nitro-2-pyridinesulfenyl

Npys-Cl: 3-nitro-2-pyridinesulfenyl chloride

Npys-OMe: methyl 3-nitro-2-pyridinesulfenate

Npys-OPh: Phenyl 3-nitro-2-pyridinesulfenate

Npys-OPh(*p*F): 4-fluorophenyl 3-nitro-2-pyridinesulfenate

Npys-OPh(*p*MeO): 4-methoxyphenyl 3-nitro-2-pyridinesulfenate

Pbf: *N*-ω-(2,2,4,6,7-pentamethyldihydrobezofuran-5-sulfonyl)

PEG: polyethylene glycol

Ph: phenyl

Phe (F): phenylalanine

*i*Pr: isopropyl

Pro (P): proline

quant.: quantitative yield

rt: room temperature

Ser (S): serine

S*t*Bu: tertiary butylthio

TEA (Et_3N): triethylamine

TFA: trifluoroacetic acid

Thr (T): threonine

THF: tetrahydrofuran

TIPS: triisopropylsilane

Tmob: 2,4,6-trimethoxybenzyl

TMSOTf: trimethylsilyl trifluoromethanesulfonate

Trp (W): tryptophan

Trt: triphenylmethyl

Tyr (Y): tyrosine

Val (V): valine

UV: ultraviolet

PROLOGUE

In organic chemistry, protecting groups are very important for the synthesis of functional molecules such as drugs, peptides and proteins. Protecting groups have been developed to carry out a selective reaction at one reactive site on molecules with multifunctional groups. Thus, other reactive sites are temporarily blocked with protecting groups generally to prevent undesired side reactions. Based on the concept of protection and subsequent deprotection, protecting groups become one of the most important factors in the synthetic organic chemistry [1-3]. In particular, in the field of peptide and protein synthesis, the various amino acids are used as building blocks, which are condensed with each other via amide bonds. In this process, functional groups on each amino acid which are not involved in the formation of the amide bond are generally protected by a protecting group.

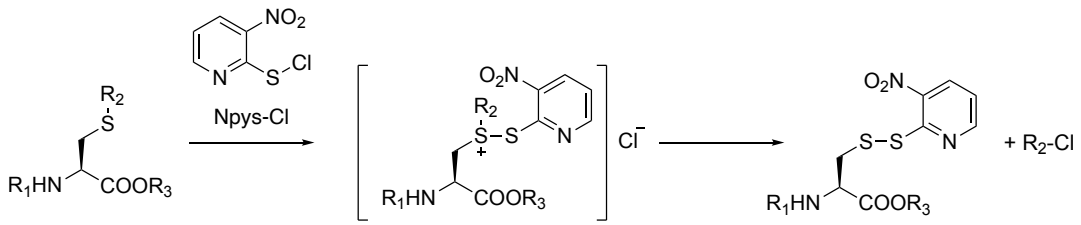
Nowadays, there are a large number of protecting groups available for the different functional groups of amino acids and with the versatile strategies for their selective removal, peptide chemistry has been able to successfully cope with the chemical synthesis of complex peptides and even proteins. Within the 20 proteinogenic amino acids, there are functional side chains such as amino, hydroxy, thiol, indole, imidazole, carbamoyl, and carboxylic acid [4]. Therefore, tremendous efforts have been made to develop building blocks of amino acids with suitable protecting groups that are resistant to certain deprotection conditions but can be readily removed under other conditions. Thanks to the development of these amino acid building blocks, Solid-Phase Peptide Synthesis (SPPS) techniques were successfully initiated by Merrifield et al. in the 1960s [5]. SPPS has great advantages in terms of automations because it only needed to repeat a simple cycle and the methods based on the chemistry of protecting groups such as tertiary butyloxycarbonyl (Boc) and 9-fluorenylmethoxycarbonyl (Fmoc) have been established [6] and have greatly contributed to the chemical synthesis of peptides and proteins [7].

Cysteine (Cys), found in peptides and proteins, is a unique proteinogenic amino acid, characterized by a thiol (SH) side chain which allows to form a disulfide bond between two cysteines. And it is known that the disulfide bond is particularly important in restricting certain peptide and protein conformations to produce their biology functions with proper biophysical and proteolytic properties [8, 9]. In the chemical synthesis of disulfide-containing peptides, Vigneaud, who was awarded the 1955 Nobel prize of Chemistry, succeeded in 1953 the first chemical synthesis of the hormone oxytocin, one-disulfide containing peptide of therapeutic interest [10, 11]. Moreover, insulin

containing one intrachain and two interchain disulfide bonds is secreted by the pancreas to lower blood sugar and has been a critical drug for treating Type I diabetes, over the last 100 years. The solid-phase chemical synthesis of insulin (bovine) was firstly reported by Marglin and Merrifield in 1966 [12].

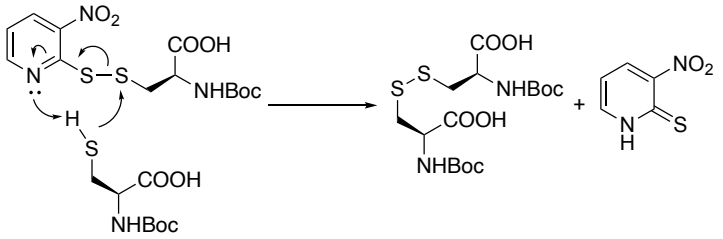
On the other hand, disulfide bond is also a valuable linking group to construct hybrid molecules which is important in recent bioconjugate chemistry [13]. Since hybrid molecules are capable of expressing bifunctional effects and therefore have the potential to effectively regulate the life system in a coordinated manner [14]. Two thiol-containing components in peptides, proteins, polymers, sugars, nucleic acids and drugs, etc. derived from different bioactive molecules can selectively be conjugated via the disulfide bond. Such hybrid molecules have been extensively used in recent years in the life science field, including drug discovery [15].

To chemically construct such disulfide bonds in peptides, proteins or hybrid molecules, many thiol-protecting groups for the Cys side chains have been developed such as: benzyl (Bzl, Bn) [16] trityl (Trt) [17], acetamidomethyl (Acm) [18], methylbenzyl (MeBn) [19], *tert*-butyl (*t*-Bu) [20], *tert*-butylsulphenyl (S*t*-Bu) [21], pyridinesulfenyl (Pys) [22] and 3-nitro-2-pyridinesulfenyl (Npys) [23] etc. Among these thiol protecting groups, the Npys group which was developed by Matsueda and Aiba in 1978 would be the most valuable. The first reported synthetic Npys and the first example of a stable pyridinesulfenyl halide was Npys chloride (commonly abbreviated as Npys-Cl) [24]. The S-Npys of cysteine derivatives (Cys(Npys)) could be easily prepared by reaction of the protected thiol group with Npys-Cl (**Scheme 1**) [25]. Through the elimination of the original protected group, Cys(Npys) derivatives could be obtained in high yields.



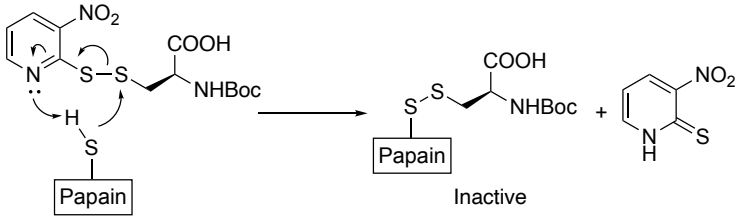
Scheme 1. S-protecting group-Npys exchange reaction

After obtaining the S-Npys, one of the most successful applications of the Npys protective group has been reported in the regioselective formation of disulfide bonds. In the case of cysteine side chain protection, the S-Npys group acts as an active disulfide to react with another unprotected thiol group and generates a disulfide bond. In 1981, the synthesis of $(Boc\text{-}Cys\text{-}OH)_2$ was reported by Matsueda *et al.* (**Scheme 2**) [26].



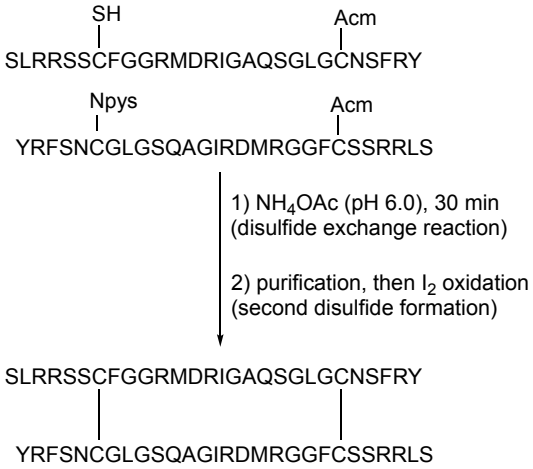
Scheme 2. Synthesis of $(Boc\text{-}Cys\text{-}OH)_2$

Moreover, this unique chemistry of the Npys group has been widely applied to various research fields. Using Boc-Cys(Npys)-OH, the SH group of the active site Cys of papain was rapidly modified, leading to a complete loss of activity because of the formation of papain-S-S-(Boc-Cys-OH) (**Scheme 3**). Full enzymic activity was restored after treatment with reducing reagent tributylphosphine, due to the disulfide bond cleavage. As the results, the Npys group increased the choice of the protecting groups to mask the thiol function and moreover gave a unique concept of inactivation and reactivation on the enzyme function.



Scheme 3. Inactivation of papain according to Matsueda *et al*. [26]

In addition, in peptide chemistry, the Npys group was utilized for the syntheses of disulfide peptide analogues. For example, Chino *et al.* [27] synthesized the antiparallel dimer of α-human atrial natriuretic peptides using a two-step strategy involving an Npys-mediated disulfide bond formation and followed by an iodine oxidation between two suitably protected peptides to form the second disulfide bond (**Scheme 4**). Afterwards, other groups used the same concept to apply the synthesis of various kinds of cyclic peptides. For example, Ruiz-Gayo *et al.* used the same strategy to obtain antiparallel dimers of uteroglobin-like peptides [28]. Simmonds and colleagues synthesized fragments of ω-conotoxin peptides [29], which are neurotoxin peptides contained in the venom of marine snails, by coupling a Cys(Npys)-protected pentapeptide to an unprotected thiol peptide in degassed water (pH 6.0) over 24 h in the same concept.



Scheme 4. Synthesis of an antiparallel dimer of α-human atrial natriuretic peptide

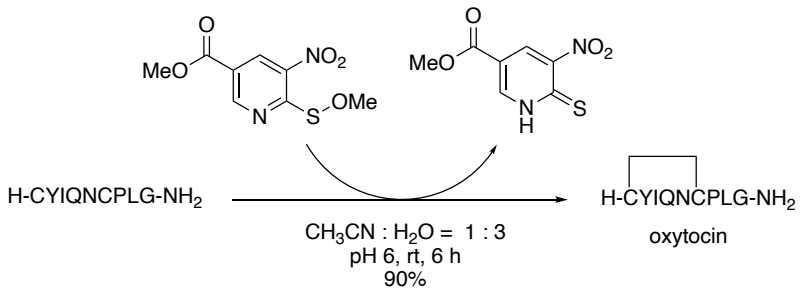
However, to the best of our knowledge, Npys-Cl is the only reagent used for 3-nitro-2-pyridine (Npy)-sulfenylation of the functional groups so far. However, the sulfenyl chloride has some obvious defects, such as instability with light or moisture, even at low temperatures and its tendency to dimerize, resulting in a disulfide precipitate, Npys dimer in various solvents (**Table 1**) [30]. These are the major drawbacks in the Npys-based chemistry.

Table 1. Npys-Cl stability according to Pugh *et al*. [30]

Solvent	Disulfide precipitate (%) [a] after 2 h	Disulfide precipitate (%) [a] after 20 h
DCM	0	0
DCM/TEA	0	7
DMF	0	0
DMF/TEA	19	40
DMF/2 M NaOH	71	74
DMSO	32	49
DMSO/TEA	33	39
Dioxane	0	0
Dioxane/TEA	100	100
Dioxane/2 M NaOH	42	51
2 M NaOH	57	57

a: Calculated for the conversion of Npys-Cl to dimer.

Recently, Hayashi group had found that methyl 3-nitro-2-pyridinesulfenate (Npys-OMe) derivative which was obtained as a stable crystalline, could act as a mild oxidizing reagent. This compound can oxidize the reduced-form oxytocin with two unprotected thiols to oxytocin containing one disulfide bond in acetonitrile / water (1:3, pH 6) at room temperature (**Scheme 5**) [31, 32].



Scheme 5. Synthesis of oxytocin from reduced form with Npys-OMe derivative

3-Nitro-2-pyridinesulfenates (Npys-ORs) are an attractive new category of the Npys reagents to replace Npys-Cl. However, their chemical characterizations were not well-known so far. In this thesis study, I newly found that Npy-sulfenates would be act as an Npys reagent and among them, 4-fluorophenyl 3-nitro-2-pyridinesulfenate (Npys-OPh(*p*F)) was a particularly useful one. Based on this discovery, I further applied this compound to i) the Npy-sulfenylation on amino, hydroxyl and thiol groups, ii) the synthesis of disulfide peptide of oxytocin from two peptide fragments and disulfide-linked peptide-glycoconjugate, and iii) total synthesis of disulfide-linked protein analogue.

In Chapter 1, to develop practical Npys reagents, various Npys-OR derivatives were synthesized and evaluated their ability in Npy-sulfenylation to functional groups of amino acid derivatives. Among synthetic Npys-OR derivatives, it was found that Npys-OPh(*p*F) is superior to Npys-Cl with regards to storing and handling. Npy-sulfenates were prepared from the parent compound Npys-Cl, using a straightforward method under mild reaction conditions. In terms of preparation efficiency, Npys-OPh(*p*F) was superior to other sulfenates and in terms of reactivity, Npys-OPh(*p*F) can react similarly to Npys-Cl with amino, hydroxy and thiol groups that are involved in various amino acids under basic conditions.

In Chapter 2, to efficiently construct disulfide-linked hybrid molecules, Npys-ORs mediated Solid-Phase Disulfide Ligation (SPDSL) were developed. Firstly, the Npy-sulfenylation to Fmoc-Cys(*t*-Bu)-OH with a variety of Npys-ORs was examined under acid conditions. Using Npys-OPh(*p*F), a satisfactory yield of Cys(Npys) was obtained with 97%. From Chapter 1, Npys-OPh(*p*F) has good storage and solution stability, compared to the conventional Npys-Cl. Secondly, from these advantages, Npys-OPh(*p*F) was chosen as a representative compound and its chemistry was applied to the SPDSL strategy. Namely, Npys-OPh(*p*F) resin was successfully developed and utilized for the asymmetric disulfide bond formation such as the synthesis of the intermediate disulfide peptide of oxytocin from two thiol peptide fragments and disulfide-linked peptide-glycoconjugate.

The intrinsic structures of proteins that have been developed during evolution have not always been optimized for their functions to an ideal and perfect state. Therefore, it may be possible to diversify the functions of proteins by converting the unique amide bonds of proteins to other chemical bonds. For example, replacing the amide bond in the main chain with a disulfide bond may be a valuable strategy leading to the design of more functional proteins and this would be the first example to our knowledge. Hence, in Chapter 3, the first total chemical synthesis of partially disulfide-linked

human immunodeficiency virus type 1 protease (HIV-1 PR) analogue was designed and accomplished. A designed sequence of HIV-1 PR composed of 115aa residues with an additional water-solubilizing moiety at the C-terminal was divided into three fragment peptides. Each terminal in the fragment peptide was changed to the Cys or 3-mercaptopropionic moiety to perform the disulfide ligation. After preparing three fragment peptides by SPPS, the first disulfide ligation was performed between N-terminal fragment with Cys(Npys) at its C-terminus and the central fragment with an unprotected SH at its N-terminus. A residual Cys(*t*Bu) at the C-terminal of the resultant fragment was then converted into Cys(Npys) using Npys-OPh(*p*F) in solution, followed by the directly disulfide ligation with the C-terminal fragment with an unprotected SH at its N-terminus to give the complete peptide sequence by the final deprotection of the Mts protecting group at Trp42. As a result, the total synthesis of partially disulfide-linked HIV-1 PR analogue was obtained with an isolated yield of 11% over only 8 steps from the fragment condensation.

The details of results and discussions were shown below.

Chapter 1: Preparation and stability evaluation of 3-nitro-2-pyridinesulfenate analogues as protecting reagents

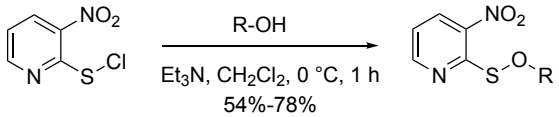
1. Introduction

1.1. Npys group acting as a protecting group

Applications of the Npys-Cl to synthetic chemistry were widely investigated. It had been demonstrated that the Npys group could selectively protect amine (-NH_2), hydroxy (-OH), and thiol (-SH) groups, respectively [33] (Protections of amino, hydroxy and thiol groups are introduced in Chapters 1 and 2, respectively.). In 1980s, Matsueda *et. al.* reported that in the presence of a base, the Npys-Cl could react with amines or alcohols to afford Npy sulfenamides and sulfenates, respectively.

The synthesis of the Npy sulfenamides (N-Npys) was generally investigated by reacting Npys-Cl with the desired amino components under basic conditions. For examples: a collection of N- and side chain Npys-protected amino acids were synthesized by i) Matsueda *et. al.* (Boc-Lys(Npys)-OH [24]), ii) Rosen *et. al.* (Npys-Lys(Npys)-OH [34]), iii) Pugh *et. al.* (Boc-Lys(Npys)-OH) [30]) and iv) Rajagopalan *et. al.* (Boc-Lys(Npys)-OH, an acid form and *tert*-butylammonium salt [35]).

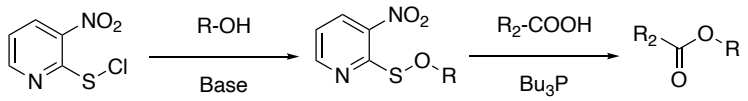
In 1981, Matsueda and Kaiser investigated the synthesis of various Npy sulfenates (O-Npys) in great details. As shown in **Scheme 6**, it was reported that various Npy sulfenates were obtained by coupling Npys-Cl to various alcohols involving from methanol to 4-(hydroxymethyl)phenylacetic acid. The reaction involved equimolar amounts of Npys-Cl, trimethylamine and alcohols in dichloromethane for 1 h at 0 °C. This reaction was also proved to be selective for alcohols over carboxyl groups. The Npys-Cl readily reacted with alcohols to produce Npy sulfenates and a series of Npy sulfenates were synthesized, with yields ranging from 54% to 78%.



Scheme 6. Synthesis of Npy sulfenates by Matsueda and Kaiser [36]

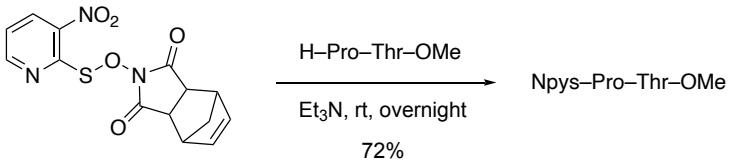
1.2. Applications of 3-nitro-2-pyrinesulfenate (Npys-OR) derivatives

Based on the reaction in **Scheme 6**, Matsueda and Mukaiyama *et al*. [37, 38] further reported applications of the Npys group. As shown in **Scheme 7**, in the presence of tertiary phosphine, Npy sulfenates could react with carboxylic acid to afford esters. The extent of racemization for this reaction was determined to be less than 1%. The reaction proceeded under SPPS conditions, and the reaction rate could be controlled by selecting appropriate activation levels by tuning number of equivalents of phosphine.



Scheme 7. O-Npys mediated ester synthesis

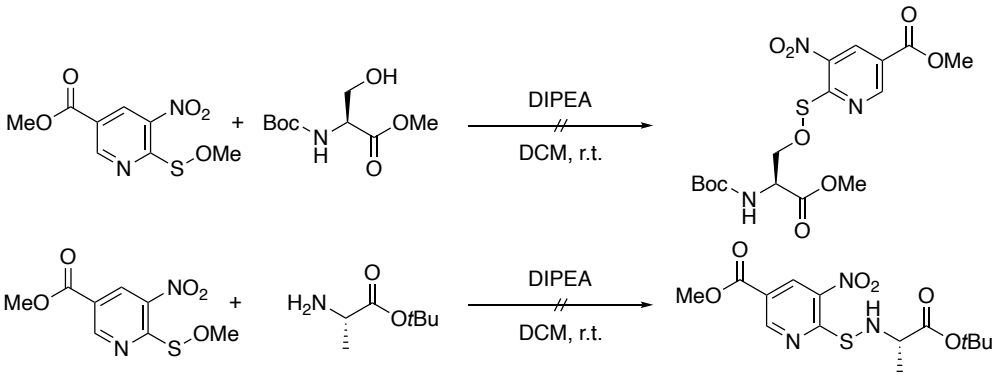
Moreover, synthesis of various Npy sulfenates (Npys-ORs) was originally reported by Matsueda *et. al.* in 1980s. Among the applications of these Npys-ORs, it is attractive that N-(3'-nitro-2'-pyridinesulfenyloxy)-5-norbornene-2.3-dicarboximide (Npys-NDC) which was prepared in high yield, was frequently used as a mild and selective Npys-protecting agent for amino groups even in the presence of hydroxy functions. The reaction of equimolar amounts of this Npys-OR, dipeptide H-Pro-Thr-OMe and Et_3N overnight at room temperature gave a corresponding protected dipeptide Npys-Pro-Thr-OMe in 72% yield (**Scheme 8**).



Scheme 8. Synthesis of Npys-Pro-Thr-OMe

On the other hand, to the best of our knowledge, Npys-Cl is almost the only reagent which was used for Npy-sulfenylation on -NH_2 and -OH so far except for the above mentioned Npys-NDC. However, the sulfenyl chloride has some obvious drawbacks, such as instability issues with light or moisture and its tendency to oxidize with itself to form Npys dimer, even at low temperatures [30]. With an aim to solve those issues, I thought that 3-nitro-2-pyridinesulfenates (Npys-ORs) which can be easily prepared from Npys-Cl would be attractive compounds as stable Npys derivatives.

As mentioned in Prologue, various alkoxy 3-nitro-2-pyridinesulfente derivatives (Npys-ORs) were developed in Hayashi group. Among these derivatives, the Npys-OMe derivative was chosen to determine the effectiveness as a Npy-sulfenylation reagent on -NH_2 and -OH. The stability of Npys-OMe derivative was also good, because it has been already found that Npys-OMe was obtained as a stable crystalline [31]. However, as shown in **Scheme 9**, because of the low reactivity of Npys-OMe derivative in Npy-sulfenylation, it might be inadequate for the Npys protection of amino and hydroxy groups. Accordingly, more reactive Npys phenoxides were designed for this purpose rather than alkoxides.



Scheme 9. Coupling of Npys-OMe derivative with H-Ala-O*t*Bu and Boc-Ser-OMe

In the Chapter 1, a new Npys protecting reagent based on the phenoxy sulfenates was described. The reagent should meet the following criteria: i) mildly prepared from available compounds, ii) reactive to amino, hydroxy and thiol groups for the Npy-sulfenylation, and iii) stable under storage conditions and solvents. For details, in the first part, we investigated the syntheses of various Npys-OR derivatives. Then, by using the obtained Npys-ORs, the protecting reactions to various functional groups commonly encountered in peptide chemistry were conducted. Finally, the stability of the selected protecting reagent Npys-OPh(*p*F) was compared to that of conventional reagent Npys-Cl.

2. Results and discussions:

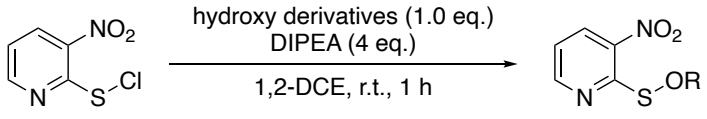
2.1. Synthesis of phenyl 3-nitro-2-pyridinesulfenates

As presented in the introduction of this Chapter, Npy sulfenates can be easily prepared under basic conditions. Thus, a series of Npys phenoxides was synthesized by coupling various phenols (1 equivalent) with Npys-Cl (1 equivalent) in the presence of *N*,*N*-diisopropylethylamine (DIPEA, 4 equivalents) under 1,2-DCE. The detailed results are shown in **Table 2**.

In entry 1, through the reaction of Npys-Cl with phenol, phenyl 3-nitro-2-pyridinesulfenate (Npys-OPh, **3**) was firstly synthesized with a yield in 57%. In entries 2-7, a series of *para*-substituted phenols was also investigated. 4-Fluorophenol (**1**, **Table 2**, entry 4) and 4-methoxyphenol (**2**, **Table 2**, entry 6) afforded the corresponding Npys phenoxides in yields of 66% and 68%, respectively, which have higher yields than the unsubstituted phenol. Moreover, the corresponding Npys phenoxides of 4-chlorophenol (**4**) in **Table 2** (entry 3), 4-acetoxyphenol (**5**), in **Table 2** (entry 5) and 4-methylphenol (**6**) in **Table 2** (entry 7) were synthesized with the lower yields in 50%, 32% and 53%, respectively.

In contrast, in **Table 2** (entry 2), no corresponding Npys phenoxide was obtained using 4-nitrophenol. Because the strong electron withdrawing character of the nitrophenyl ring weakened the resulting S-O bond, thus compromising the formation of its Npys phenoxide. On the other hand, using other aromatic alcohols with strong electron withdrawing substituents, such as pentafluorophenol and 1-hydroxybenzotriazole (HOBt) in **Table 2**, (entries 8 and 9), a similar result of no corresponding compound was also observed.

Table 2. Synthesis of 3-nitro-2-pyridinesulfenates (Npys-ORs)



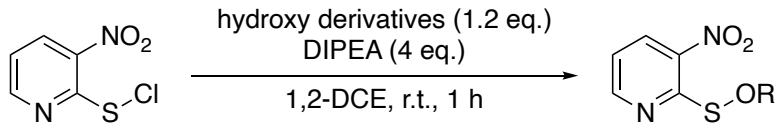
Entry	Hydroxy derivatives	Yield (%)[a]
1	phenol	57
2	4-nitrophenol	0
3	4-chlorophenol	50
4	4-fluorophenol	66[b]
5	4-acetoxyphenol	32
6	4-methoxyphenol	68
7	4-methylphenol	53
8	pentafluorophenol	0
9	1-hydroxybenzotriazole	0

a: isolated yield.
b: isolated yield when using 2 eq. of DIPEA was 65% [36].

Then, in **Table 2** (entry 6) and **Table 3** (entry 4), the reaction of both 1.0 and 1.2 equivalents of 4-methoxyphenol, gave the same yields in 68%. In contrast, by the use of 1.2 equivalents of 4-fluorophenol in **Table 3** (entry 1), the yield of Npys-OPh(*p*F) (**2**) was improved from 66% to 73%.

Finally, orientations of the substituted groups in the case of 4-fluorophenol and 4-methoxyphenyl, were also optimized. In **Table 3** (entries 1-3), the effects of the substitution position of a fluorine atom on the phenyl ring (*para*-(**1**), *ortho*-(**7**), *meta*-(**8**)) or in **Table 3** (entries 4-6), a methoxy group (*para*-(**2**), *ortho*-(**9**), *meta*-(**10**)) were compared. As a result, in **Table 3** (entry 1), the *para*-substituent afforded a higher synthetic yield than other substituents.

Table 3. Reactions between Npys-Cl and *para-, ortho-, meta-,* substituted phenols



Entry	Hydroxy derivatives	Yield (%)[a]
1	4-fluorophenol	73
2	2-fluorophenol	54
3	3-fluorophenol	40
4	4-methoxyphenol	68
5	2-methoxyphenol	22
6	3-methoxyphenol	20

a: isolated yield

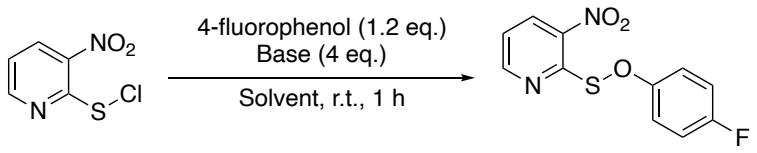
2.2. Optimizations of the reaction conditions in the synthesis of Npys-OPh(*p*F)

Next, the preparation of Npys-OPh(*p*F) (**1**) under various reaction conditions was investigated in great details. As shown in **Table 4**, 1.2 equivalents of 4-fluorophenol as a substrate was used to optimize this reaction.

First, as shown in **Table 4** (entry 1), the synthesis of Npys-OPh(*p*F) (**1**) virtually did not proceed, in the absence of a base, because most of Npys-Cl was decomposed to the Npys dimer during the reaction, which is easy detected by the appearance of its insoluble precipitate in various solvents. Next, as shown in **Table 4** (entries 2-5), effect of several non-nucleophilic bases on the synthesis of **1** was also investigated. It was found that this kind of non-nucleophilic base was essential for the formation of the desired **1**. In particular, DIPEA promoted the reaction favorably, as opposed to Et_3N, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), or cesium carbonate (Cs_2CO_3). Then, as shown in **Table 4** (entries 2 and 6-8), the solvents using in this reaction were also screened. 1,2-DCE and DCM gave better yields comparing with THF and DMF.

Moreover, through the previous reports of syntheses of Npys derivatives (shown in **Scheme 6**), the previous syntheses of Npys-ORs were performed at 0 ºC and under anhydrous conditions, using Npys-Cl as the starting compound. In contrast, based on the condition optimizations, without particular precautions, Npys phenoxides could be synthesized at room temperature and under atmospheric conditions because of the addition of a non-nucleophilic base. In a nutshell, I established a convenient preparation protocol for Npys derivatives using newly developed Npys-OPh(*p*F) (**1**).

Table 4. Optimizations of base and solvent in the synthesis of Npys-OPh(*p*F)



Entry	Base	Solvent	Yield (%)[a]
1	None	1,2-DCE	0
2	DIPEA	1,2-DCE	73
3	Et_3N	1,2-DCE	60
4	DBU	1,2-DCE	0
5	Cs_2CO_3	DMF[b]	0
6	DIPEA	DCM	70
7	DIPEA	THF	54
8	DIPEA	DMF	6

a: isolated yield

b: Cs_2CO_3 is not soluble in 1,2-DCE and DMF was used.

2.3. Use of Npys phenoxides as protecting reagents

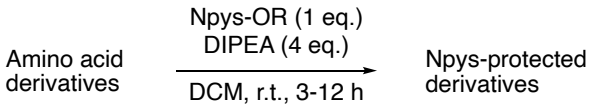
Next, the reactivity of phenoxy sulfenates in the Npy-sulfenylation was assessed toward various functional groups of amino acids such as -NH_2, -OH and -SH groups using Npys-OPh(*p*F) (**1**) and Npys-OPh(*p*MeO) (**2**), in the presence of DIPEA in DCM (**Table 5**).

As shown in **Table 5**, Npys-OPh(*p*F) (**1**) successfully introduced an Npys group to the α-amino group of HCl·H-Ala-O*t*Bu, and the corresponding product, Npys-Ala-O*t*Bu (**11**) was obtained with a yield of 91% in 3 h (**Table 5**, entry 1) at room temperature. Npys-OPh(*p*MeO) (**2**) also protected the amino group of HCl·H-Ala-O-*t*Bu with an 82% yield, but required a longer reaction time (6 h) than compound **1** (**Table 5**, entry 2).

Npys-Leu-O*t*Bu (**12**) was also obtained using **1** with a yield of 87%, which is comparable to that of the alanine, suggesting that **1** could protect an α-amino group independently of the bulk of the side chain (**Table 5**, entry 3). Next, the protections of side chains of several amino acids using **1** were evaluated. The ε-amino group of Fmoc-Lys-OH·HCl was protected and desired product, Fmoc-Lys(Npys)-OH (**13**) was prepared with a yield of 85% (**Table 5**, entry 4). Moreover, the less nucleophilic hydroxy groups in Boc-Ser-OMe and Z-Tyr-OMe were also protected, giving the corresponding Boc-Ser(Npys)-OMe (**14**) and Z-Tyr(Npys)-OMe (**15**), in yields of 30% and 44%, respectively (**Table 5**, entries 5 and 6).

On the other hand, the carboxy group of Fmoc-Gly-OH was failed to react with **1** (**Table 5**, entry 7). This was consistent with the selective introduction of an Npys group to the ε-amino group of Fmoc-Lys-OH·HCl in the presence of an unprotected carboxy group (**Table 5**, entry 4). Thus, **1** has the ability to protect amino and hydroxy groups with Npys, a reaction commonly encountered in peptide syntheses. Moreover, the reaction with the unprotected thiol group of Z-Cys-OEt gave the protected compound, Z-Cys(Npys)-OEt (**16**) in 14% yield (entry 8), but (Z-Cys-OEt)$_2$ (**17**), which is a cystine derivative derived from Z-Cys-OEt, was also recovered from the reaction in 33% yield (**Table 5**).

Table 5. Npy-sulfenylation of amino acid derivatives using Npy sulfenates



Entry	Npys-OR	Amino acid derivative	Npys-protected derivative	Yield (%)[a]
1	Npys-OPh(*p*F) (**1**)	HCl·H-Ala-O*t*Bu	Npys-Ala-O*t*Bu (**11**)	91
2	Npys-OPh(*p*MeO) (**2**)	HCl·H-Ala-O*t*Bu	Npys-Ala-O*t*Bu (**11**)	82[b]
3	Npys-OPh(*p*F) (**1**)	HCl·H-Leu-O*t*Bu	Npys-Leu-O*t*Bu (**12**)	87
4	Npys-OPh(*p*F) (**1**)	Fmoc-Lys-OH·HCl	Fmoc-Lys(Npys)-OH (**13**)	85[c]
5	Npys-OPh(*p*F) (**1**)	Boc-Ser-OMe	Boc-Ser(Npys)-OMe (**14**)	30[d]
6	Npys-OPh(*p*F) (**1**)	Z-Tyr-OMe	Z-Tyr(Npys)-OMe (**15**)	44[e]
7	Npys-OPh(*p*F) (**1**)	Fmoc-Gly-OH	Fmoc-Gly-O(Npys)	0
8	Npys-OPh(*p*F) (**1**)	Z-Cys-OEt	Z-Cys(Npys)-OEt (**16**)	14[f]

a: isolated yield after 3 h unless otherwise noted.
b: NMR yield after 6 h (75% and 76% after 3 and 12 h, respectively).
c: isolated yield after 6 h.
d: isolated yield when using 2 eq. of Npys-OPh(*p*F) (**1**).
e: isolated yield of 43% after 6 h.
f: $(Z\text{-Cys-OEt})_2$ (**17**) was also isolated with a yield of 33%.

2.4. Stability of 4-fluorophenyl 3-nitro-2-pyridinesulfenyl (Npys-OPh(*p*F)) (1)

Finally, to demonstrate the usefulness of Npys-OPh(*p*F) (**1**), the stability was examined under both solution and storage conditions. The stability of **1** in dimethylsulfoxide (DMSO) was examined. Since the previous literature reported that Npys-Cl was unstable in DMSO, 49% of Npys-Cl decomposed to Npys dimer during 20 h [30]. In contrast, **1** was stable for more than 20 h. These results indicated that **1** possesses much higher stability during storage and in the solvent than Npys-Cl (**Table 6**).

Table 6. Stability of Npys-Cl and Npys-OPh(*p*F) (**1**) in DMSO at room temperature

Compound	Precipitate (%)[a]	
	2 h	20 h
Npys-Cl	32[b]	49[b]
Npys-OPh(*p*F) (**1**)	0	0

[a] The percentage of Npys derivatives formed Npys dimer as a precipitate.
[b] See reference. [30]

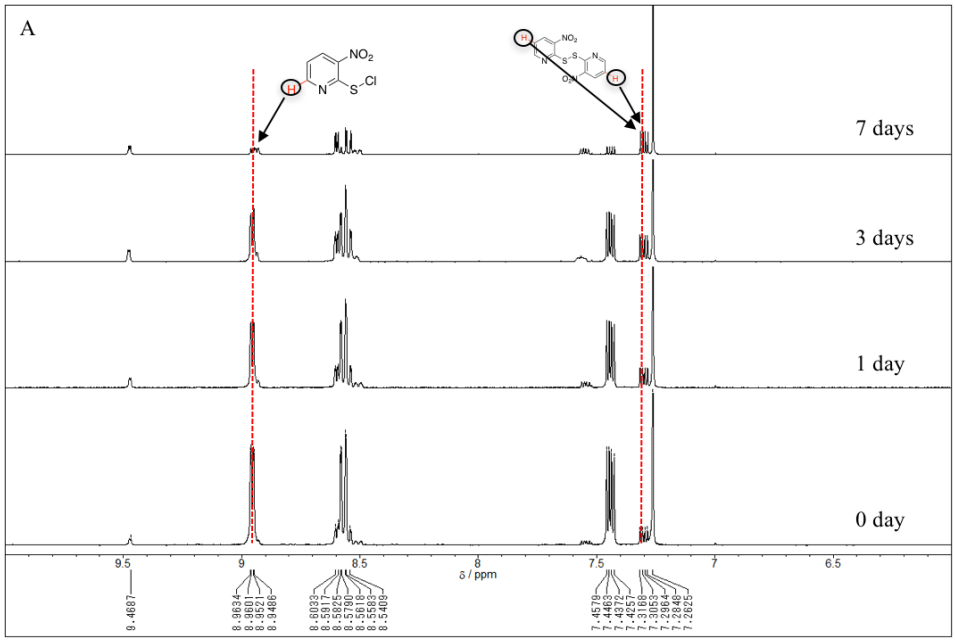
Moreover, in the previous reports, the conventional protecting reagent Npys-Cl was reported to be stable for several months at 4 ºC in a closed container [8]. However, in this study, NMR analysis showed that 25% of Npys-Cl was converted to the Npys dimer after 3 weeks under these conditions. In contrast, no decomposition of the new reagent (**1**) was observed after 2 months at 4 ºC. Moreover, **1** was stable at room temperature under shelf conditions for one day, after which it gradually decomposed to 58% of its starting amount after 7 days while Npys-Cl decomposed to 72% of its starting amount (**Table 7**).

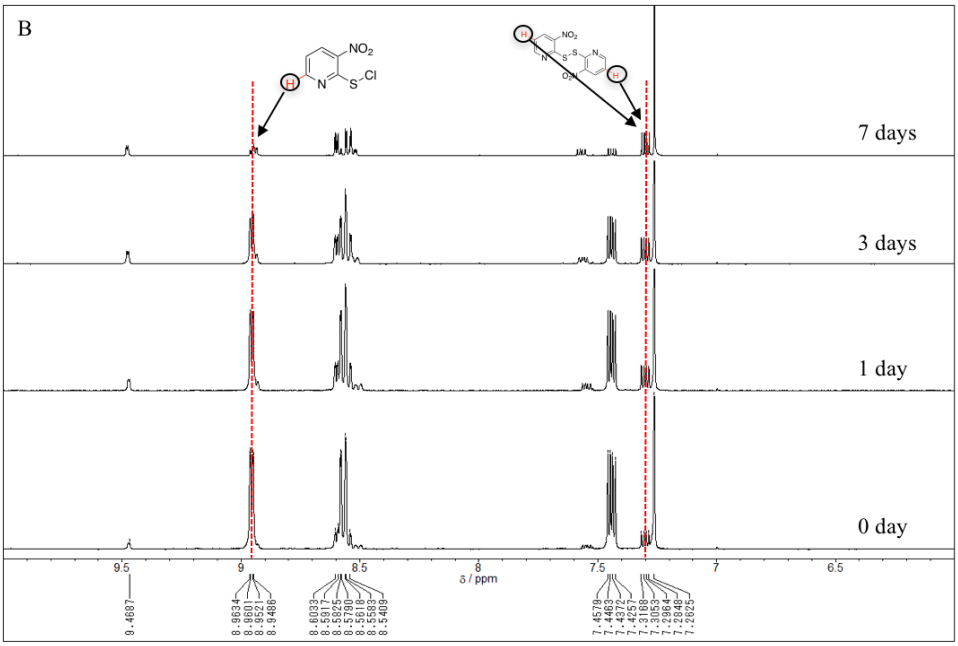
Table 7. Stability of Npys-Cl and Npys-OPh(*p*F) (**1**) under shelf and light-shielded conditions at room temperature.

Compound	Yield of Npys dimer (%)[a]			
	0 day	1 day	3 days	7 days
	Under shelf condition			
Npys-Cl[b]	25	35	49	72
Npys-OPh(*p*F) (**1**)	0	0	26	58
	Under light-shielded condition			
Npys-Cl[b]	25	37	49	71
Npys-OPh(*p*F) (**1**)	0	0	26	47

[a] The NMR yield calculated by the formula shown in experimental part.
[b] Stability test of Npys-Cl at room temperature was performed using Npys-Cl stored in a refrigerator at 4 ºC for three weeks.

^{1}H NMR charts of Npys-Cl and Npys-OPh(*p*F) (**1**) which were stored under shelf and light-shielded conditions at room temperature for 0, 1, 3 and 7 days, were shown in **Figure 1**. The peaks of Npys-Cl, Npys-OPh(*p*F) (**1**) and Npys dimer which is the product of the decomposition of compounds Npys-Cl and **1**, were determined.





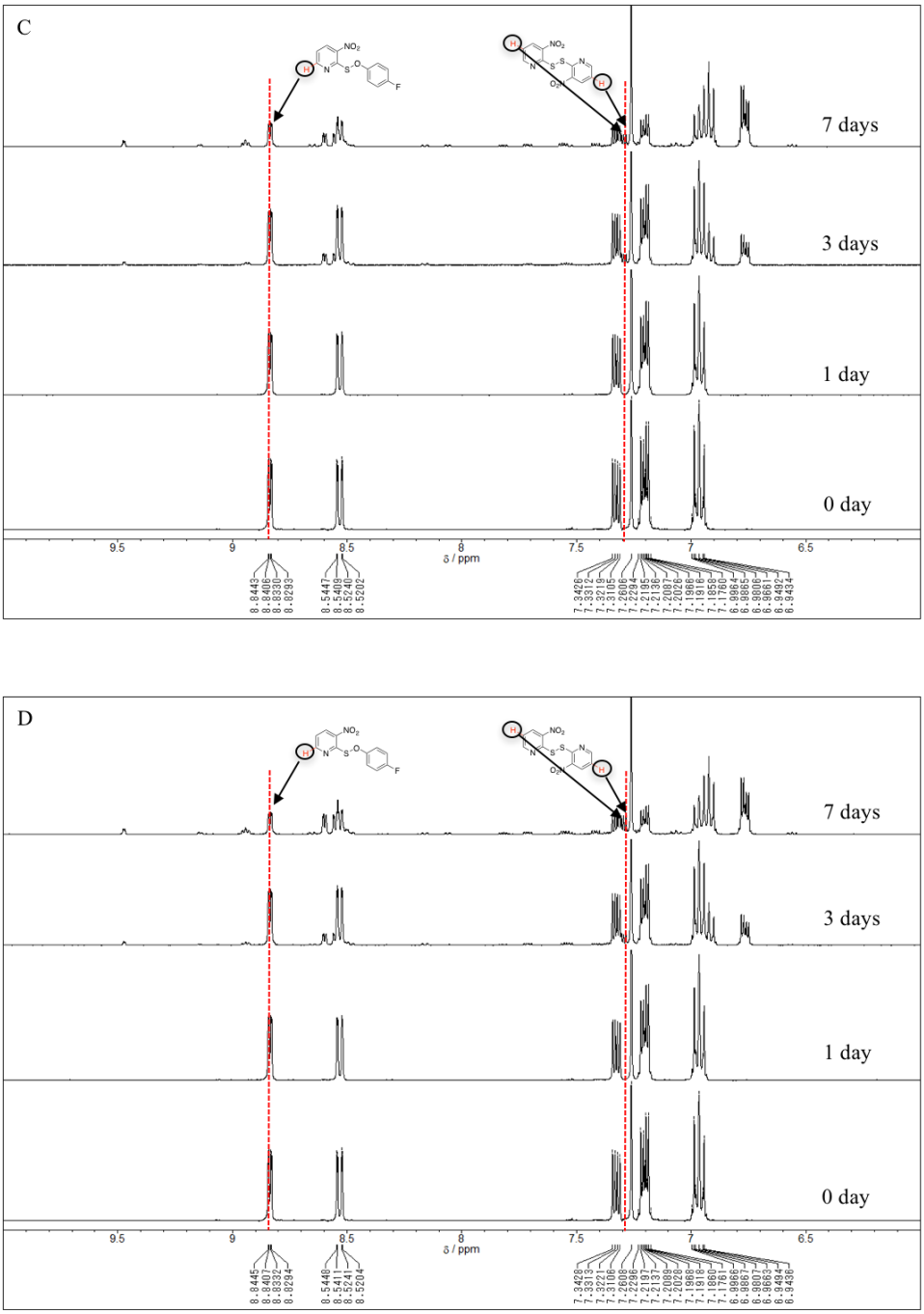


Figure 1. ^{1}H NMR spectra (400 MHz, $CDCl_3$) of Npys-Cl and Npys-OPh(*p*F) (**1**) under shelf and lighted-shielded conditions, respectively. A: Npys-Cl stored under shelf conditions. B: Npys-Cl stored under light-shielded conditions. C: Npys-OPh(*p*F) stored under shelf conditions. D: Npys-OPh(*p*F) stored under light-shielded conditions.

Conclusion

In Chapter 1, a new Npy-sulfenylation reagent Npys-OPh(*p*F) (**1**) was developed. This reagent can be prepared from its parent compound Npys-Cl with a straightforward method under mild reaction conditions. This new reagent can protect amino, hydroxy and thiol groups in various amino acids under basic conditions. The reagent is more stable to storage and handling than the conventional protecting reagent Npys-Cl, and therefore is more practically useful than Npys-Cl, making it a reagent of choice for Npys-based chemistry.

Chapter 2. Use of solid-supported 4-fluorophenyl 3-nitro-2-pyridinesulfenate in the construction of disulfide-linked hybrid molecules

1. Introduction

1.1. Importance of disulfide bonds

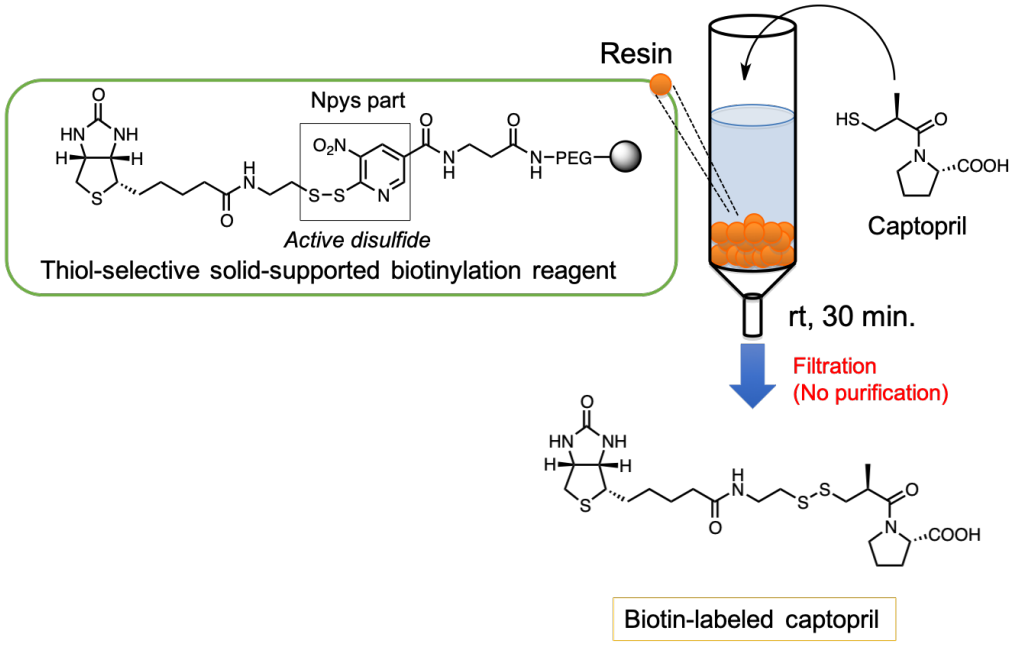
Disulfide bonds are important covalent bonds that not only cross-link two cysteine (Cys) residues in peptides and proteins to define conformations and express biological functions, but also conjugates two individual thiol-containing components to form hybrid molecules [39]. In the former, disulfide bonds give the rigid and stable tertiary structures of peptides and proteins. The resultant conformational restrictions and their maintenance have a profound effect on their biology activity through enhanced biophysical and proteolytic stability [40-44]. Interchain disulfide linkages are also critical for defining the topological feature of peptides and proteins such as the insulin super family composed of two-chain heterodimer [45] and hair collagen composed of multi-chain [46]. In the latter, in recent drug discovery, a disulfide hybrid-molecule that combines two SH-containing components with different pharmacological properties has been reported in antibody-drug and peptide-drug conjugates. It is also beneficial when conducting the drug screening using de-novo designed multi-disulfide peptides library with different folding [47].

As mentioned in Prologue, and until now, the chemical synthesis of disulfide bonds had been always employed by air [48], DMSO [49] and I_2 [50] oxidation. However, many problems associated with the formation of disulfide bonds using these methods had been reported, for instance, long reaction time, overoxidation and formation of different conformers or polymers [51]. Along with the development of the thiol-protecting groups, Acm, Trt, MeBn, *t*-Bu, S*t*-Bu, Pys and Npys groups were used for protection of cysteine residues, they have been applied to the regioselective disulfide bond formation.

1.2. Solid-phase disulfide ligation (SPDSL) strategy

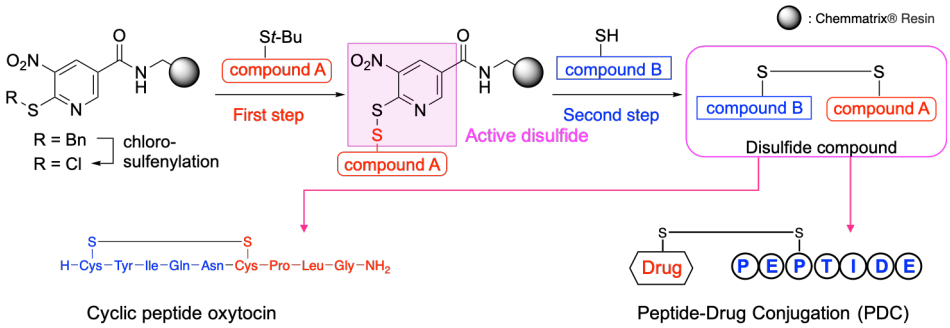
Inspired by the chemistry of the Npys group which was mentioned in the Prologue, Hayashi group had developed a thiol-selective solid-supported biotinylation reagent [52]. This solid-supported reagent is consistent of three parts, i.e., a resin with a polyethyleneglycol linker, an Npys group which acts as the disulfide bond activator and a biotin which is attached to the Npys group through the active disulfide bond.

As shown in **Scheme 10**, the SH-containing captopril can be selectively biotinylated by this solid-supported reagent. Namely, biotin can be efficiently transferred from the resin to captopril by forming a new disulfide bond and the biotin-labeled captopril can be easily obtained in good purity by a simple filtration without additional purification steps.



Scheme 10. Biotinylation of captopril using a solid-phase Npys-biotinylated reagent

By taking advantages of the disulfide exchange reaction based on the solid-supported Npys group, Hayashi group had also developed an efficient platform for the disulfide transformation, i.e. one-pot Solid-Phase Disulfide Ligation (SPDSL) strategy using Npys-Cl resin as a key intermediate [53]. This solid-support strategy is consisting of two reactions, i.e, (i) *t*-Bu protected Cys-containing component (compound A) is firstly loaded onto the Npys resin via an active disulfide, then (ii) the resulting resin is readily transferred to another unprotected SH-containing component (compound B) by a disulfide exchange reaction. This results in a selective formation and release of new disulfide compound from the resin. Furthermore, this SPDSL strategy has been successfully applied to the syntheses of cyclic peptide oxytocin [53] and peptide-drug conjugate (PDC) consisting of hydrophilic and hydrophobic components [13] (**Scheme 11**).



Scheme 11. Solid-phase disulfide ligation (SPDSL) strategy [53]

The SPDSL strategy is becoming a facile platform for the preparation of disulfide bonds. However, as discussed in Chapter 1, Npys-Cl has obvious disadvantages: it is unstable to light and moisture and prone to dimerization even at low temperatures. Due to this physicochemical issue, laborious chlorination of the Npys resin (chlorosulfenylation in **Scheme 11**) is actually an essential process for the SPDSL. Furthermore, the preparation of the unstable Npys-Cl resin must be performed just before the use. On the other hand, as mentioned in Chapter 1, various alkyl and aryl 3-nitro-2-pyridinesulfenes have recently been developed, among which Npys-OPh(*p*F) has better stability and handling properties than the conventional Npys-Cl.

Therefore, in Chapter 2, development of a new solid-support disulfide ligation reagent based on the Npys-OR is described. The new reagent must meet the following criteria: 1) As a surrogate of Npys-Cl resin, Npys-OR resin is reactive to protect Cys residues and forms active disulfides without any activation step. 2) Npys-OR resin can be produced from available solid-support under mild conditions and needs to function in the preparation of disulfide peptides or conjugates from two thiol components. 3) The solid-supported resin is more stable under storage conditions than the Npys-Cl resin.

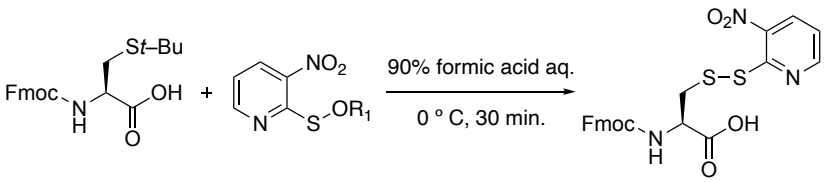
To the best of our knowledge, the detailed features of Npys-OR derivatives have not been investigated. Therefore, I firstly screened the reactivity of various Npys-ORs toward protected Cys derivatives under various reaction conditions. Then, I evaluated the ability of Npys-OR resin as a SPDSL agent by preparing an intermediate disulfide peptide leading to formal synthesis of oxytocin and disulfide linked peptide-glycoconjugate. Finally, I examined the physicochemical stability of the new Npys resin compared to the conventional Npys-Cl resin.

2. Results and discussions:

2.1. Synthesis of Fmoc-Cys(Npys)-OH using Npy sulfenates

To develop a new solid-supported Npys agent which is applicable to the SPDSL strategy, the reaction of Npys-OR derivatives with S-protected cysteine derivatives was studied. Namely, as a model experiment, Fmoc-Cys(Npys)-OH bearing an active disulfide was synthesized by coupling Fmoc-Cys(*t*-Bu)-OH with a series of Npys-ORs under 90% formic acid aq. at 0 ºC for 30 min [54]. The detailed results are shown in **Table 8**. In entries 1 and 2, when Npys alkoxides, Npys-OMe and Npys-OBn [31], were reacted with Fmoc-Cys(*t*-Bu)-OH, the corresponding Cys(Npys) derivative was obtained in moderate yields of 57% and 59%, respectively. These results indicated that alkoxides with pKa values of ~15 [55] are less reactive than Npys-Cl. In contrast, a series of Npys phenoxides such as phenyl (pKa = 10, entry 3: 88%), 4-methoxyphenyl (pKa = 10.2, entry 4: 82%), 4-methylphenyl (pKa = 10.1, entry 5: 86%) afforded Fmoc-Cys(Npys)-OH in higher yields. Especially, in entries 6 and 7, when using 4-fluorophenol (pKa = 9.89, Npys-OPh(*p*F)) and 4-chlorophenol (pKa = 9.41), a satisfactory yield of Fmoc-Cys(Npys)-OH was obtained with 96% and 98%, respectively. Moreover, in Chapter 1, it is indicated that Npys-OPh(*p*F) can be synthesized in good yield from its parent compound, Npys-Cl and has better stability in storage both in solid state and in solution than the Npys-Cl. Therefore, from advantages of high reactivity with Cys(*t*-Bu) and physicochemical stability, Npys-OPh(*p*F) was chosen as a representative compound for the further optimization of reaction conditions.

Table 8. Synthesis of Fmoc-Cys(Npys)-OH by the reaction of Fmoc-Cys(*t*-Bu)-OH with Npys-OR

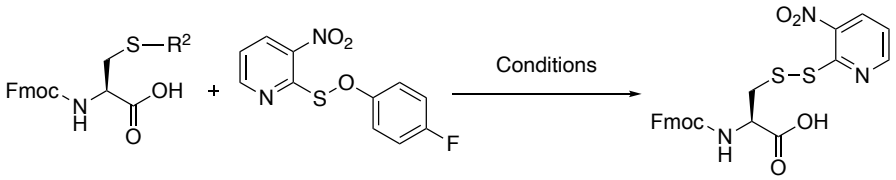


Entry	R[1*]	Isolated yield (%)
1	Methyl	57
2	Benzyl	59
3	Phenyl	88
4	4-Methoxyphenyl	82
5	4-Methylphenyl	86
6	4-Fluorophenyl (Npys-OPh(*p*F))	96
7	4-Chlorophenyl	98

*: Fmoc-Cys(Npys)-OH was also synthesized in a yield of 97% with Npys-Cl in 90% HCOOH aq. at 0 ºC for 30 min.

To optimize the reaction conditions in the conversion to Cys(Npys) by Npys-OPh(*p*F), the reaction solvents were firstly examined. As shown in **Table 9**, the conversion of Cys(*t*-Bu) to Cys(Npys) in DCM gave the moderate yield of 66%, which is much lower than 96% of yield under 90% HCOOH aq. (entries 1 and 2). The use of glacial acetic acid also gave a high yield with 97% (entry 3). In contrast, under the basic condition with DIPEA, desired product was not obtained (entry 4). These results suggested that the conversion to Cys(Npys) was efficient and preferable under acidic conditions. Next, under acidic conditions, the reactivity of Npys-OPh(*p*F) toward other orthogonal thiol-protecting groups such as Acm, 4-methoxybenzyl (4-MeOBn), Trt, Bn and S*t*-Bu groups were also examined. Among these protecting groups, Acm and 4-MeOBn gave the corresponding Cys(Npys) in high yield of 95 and 98% (entries 5 and 6). In contrast, when Trt and Bn were used the yields were lower 50 and 30%, respectively. S*t*-Bu protected cysteine was inadequate for this exchange reaction under acidic conditions (entry 9). On the other hand, in the reaction with unprotected Cys, Cys(Npys) was produced at low yield of 26% together with the production of homodimer (Fmoc-Cys-OH)$_2$ (Fmoc-cystine, entry 10). From these results, it is indicated that Npys-OPh(*p*F) is an efficient agent for the formation of active disulfide from several protected cysteines under acidic conditions, suggesting that Npys-OPh(*p*F) could be a superior surrogate of Npys-Cl.

Table 9. Conversion of the thiol-protecting groups into the Npys group



Entry	R^2	Conditions	Isolated yield (%)
1	*t*-Bu	DCM, 0 ºC, 1 h to r.t., 12 h	66
2[a]	*t*-Bu	90% HCOOH aq., 0 ºC, 30 min	96
3	*t*-Bu	CH_3COOH, r.t., 30 min.	97
4	*t*-Bu	DIPEA (2 eq.) DCM, 0 ºC, 1 h to r.t., 12 h	N.O.
5	Acm	90% HCOOH aq., 0 ºC, 30 min	95
6	4-MeOBn	90% HCOOH aq., 0 ºC, 30 min	98
7	Trt	90% HCOOH aq.: CH_2Cl_2 = 1 : 1 0 °C, 30 min	50
8	Bn	90% HCOOH aq.: CH_2Cl_2 = 5 : 1 0 °C, 30 min	30
9	S*t*-Bu	90% HCOOH aq., 0 °C, 30 min	N.O.
10	H	90% HCOOH aq., 0 ºC, 30 min	26[b]

N.O.: not obtained

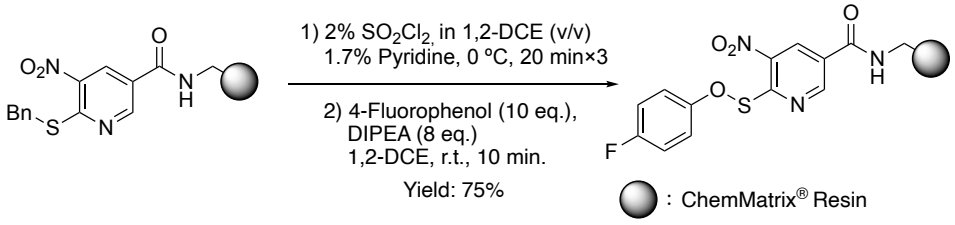
a: Same as **Table 8**, Entry 6

b: $(Fmoc\text{-}Cys\text{-}OH)_2$ was also isolated with a yield of 18%.

2.2. Synthesis of solid-supported Npys-OPh(*p*F)

To apply the chemistry of Npys-OPh(*p*F) to the SPDSL strategy, a solid-supported form of this compound was synthesized. As a solid-support, a polyethylene glycol ChemMatrix® resin, which swells well in both organic and aqueous solutions, was chosen.

As shown in **Scheme 12**, Npys-Cl resin was prepared from the corresponding Npys-Bn resin by chlorosulfenylation with 2% (v/v) SO_2Cl_2/1,2-DCE in the presence of pyridine [53]. After washing the resulting Npys-Cl resin with 1,2-DCE, the mixture of 4-fluorophenol and DIPEA in 1,2-DCE was immediately added to the resin. This solid-phase reaction was examined by changing the preparation conditions (see in experimental part), and calculated the loading value of 4-fluorophenol from the content of fluorine on resin through the elemental analysis. As a result, just in 10 min reaction with 4-fluorophenol (8 eq.) and DIPEA (10 eq.), Npys-OPh(*p*F) resin (**18**) can be easily obtained with the substitution yield of 75%.



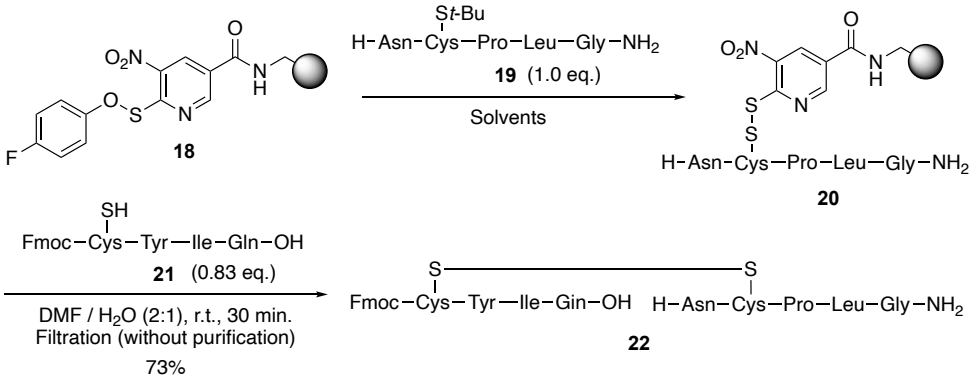
Scheme 12. Synthesis of Npys-OPh(*p*F) resin

2.3. Synthesis of disulfide bond in oxytocin and disulfide-linked peptide-glycoconjugate

To examine the ability of new Npys-OPh(*p*F) resin **18** in the SPDSL strategy, a disulfide peptide consisted of two peptide fragments derived from oxytocin was synthesized. C-terminal, Cys-protected pentapeptide, H-Asn-Cys(*t*-Bu)-Pro-Leu-Gly-NH_2 and N-terminal, Cys-unprotected tetrapeptide, Fmoc-Cys-Tyr-Ile-Gln-OH were prepared by standard Fmoc-based SPPS method, respectively [53]. Then, as depicted in **Scheme 13**, the Npys-OPh(*p*F) resin was mixed with Cys(*t*-Bu) containing peptide **19** in various solvents and its loading yield onto the resin was calculated by the HPLC analysis.

The results were shown in Table 10. According to the previous synthetic procedure using 90% formic acid aq. with Npys-Cl resin [55], the Npys-OPh(*p*F) resin **18** was also treated under the same reaction condition (entries 1-3). The peptide-loaded resin was obtained with a good yield of 93% in the use of 7.5 equivalents of resin by shaking for overnight (entry 3). This result indicated that resin **18** can load the peptide via an active disulfide without any pre-activation step. The reaction was further improved by the use of acetic acid as a solvent, which gave a similar peptide-loading yield of 91% in spite of the decreased quantity (3.7 equivalents) of the resin used. Conversely, under non-acidic conditions such as CH_3CN : H_2O (1:1) and DMF, the loading yields were very low of 0 and 33%, respectively (entries 5 and 6). In the presence of LiCl, the loading yield was drastically increased compared to the case without LiCl. This is probably because the lithium salt not only changes the conformation of the peptide by complexing with the oxygen atom of the amide bond in the organic solvent, but also swells the polystyrene resin, allowing the reaction between the peptide and the Npys-resin to be carried out efficiently [56]. In particular, in 0.4 M LiCl/acetic acid (entry 8), the peptide was almost completely loaded onto the 3.7 equivalents of resin at 1 h. As shown in **Figure 2**, the peak of peptide **19** was almost disappeared in 1 h and the peak of 4-fluorophenol was clearly appeared.

Next, the peptide-attached on the resulting resin **20** was transferred into N-terminal Cys-unprotected tetrapeptide **21**. After shaking the mixture of resin **20** and peptide **21** (0.83 eq.) for 30 min in DMF : H_2O (2 : 1), the peak of peptide **21** was completely disappeared and the single peak of desired disulfide peptide **22** was appeared in HPLC analysis. By removing the resin by simple filtration, the desired disulfide peptide **22** was obtained in 73% yield with high purity (94%) (**Figure 3**).



Scheme 13. Synthesis of disulfide bond in oxytocin via SPDSL by Npys-OPh(*p*F) resin **18**

Table 10. Loading yields of peptide **19** onto Npys-OPh(*p*F) resin **18**

Entry	Solvent	Resin **18** (eq.)	Loading yield of **19** (%)[a]		
			1 h	3 h	overnight
1	90% formic acid aq.	2.2	0	16	N.T.
2	90% formic acid aq.	3.7	12	33	N.T.
3	90% formic acid aq.	7.5	17	41	93
4	acetic acid	3.7	24	43	91
5	CH_3CN : H_2O (1:1)	3.7	0	0	0
6	DMF	3.7	0	0	33
7	0.4 M LiCl/90% formic acid aq.	3.7	55	60	74
8	0.4 M LiCl/acetic acid	3.7	99	N.T	N.T.

N.T.: not tested.

a. Loading yield (%) = [1 – (HPLC peak area of residual peptide **19** / HPLC peak area of original peptide **19**)] x 100.

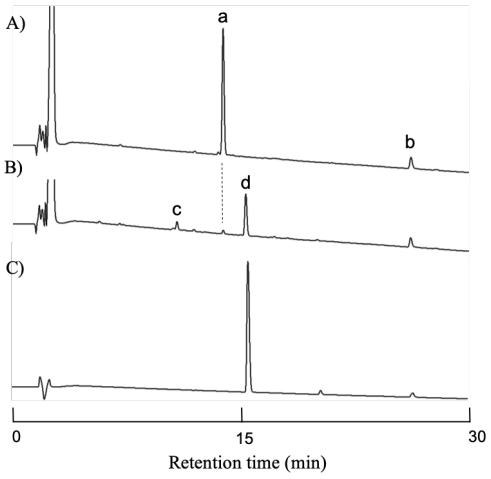


Figure 2. HPLC analysis of the reaction mixture (**Table 10**, Entry 8): A) 0 h, B) 1 h, C) 4-fluorophenol, a: peptide **19**, b: original impurity peak, c: H-Asn-Cys-Pro-Leu-Gly-NH_2, d: 4-fluorophenol. HPLC conditions are a linear gradient starting from 5% CH_3CN in 0.1% aqueous TFA to 65% CH_3CN in 0.1% aqueous TFA over 30 min at a flow rate of 1.0 mL/min and detection at 230 nm.

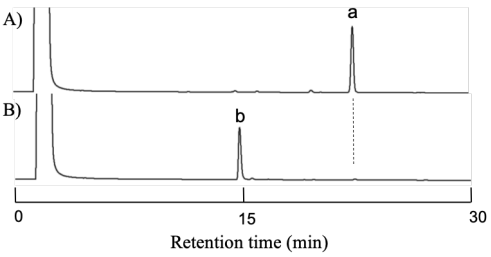
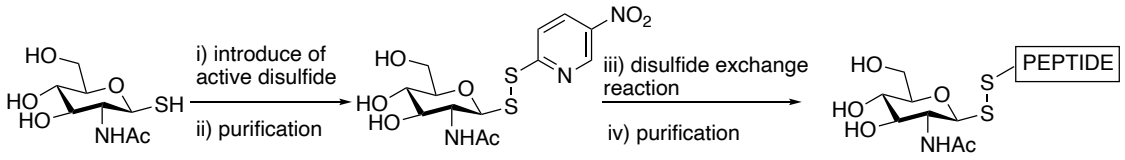


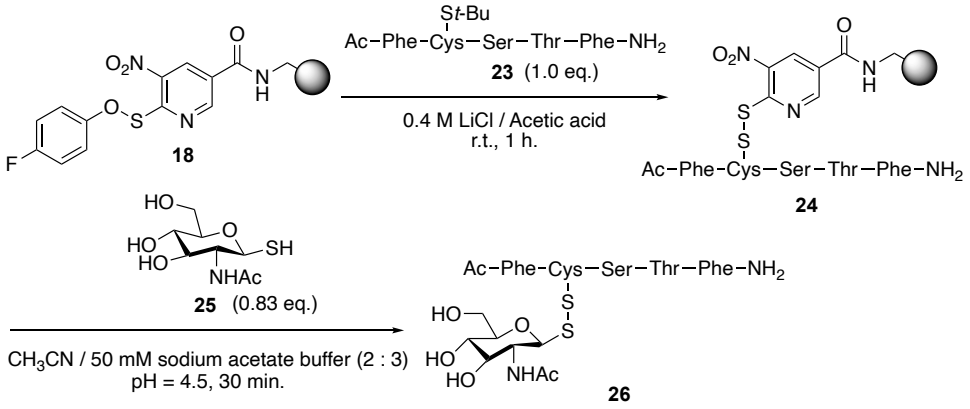
Figure 3. HPLC analysis of the reaction mixture (reaction between peptide-attached resin **20** and peptide **21**): A) 0 h, B) 30 min, a: peptide **21**, b: peptide **22**. HPLC conditions are a linear gradient starting from 25% CH_3CN in 0.1% aqueous TFA to 55% CH_3CN in 0.1% aqueous TFA over 30 min at a flow rate 1.0 mL / min and detection at 230 nm.

To further examine the ability of the Npys-OPh(*p*F) resin, the synthesis of an asymmetric disulfide-linked peptide-glycoconjugate between the Cys-containing peptide and glycosyl thiol was conducted. Previously, a glycoconjugate in which a pentapeptide (Ac-Phe-Cys-Ser-Thr-Phe-NH_2) derived from human IgG2 sequence and an *N*-acetylglucosamine derivative are linked by a disulfide bond has been reported [57]. This pentapeptide of a human IgG2 sequence with Cys in place of Asn-297 was developed as a structural mimetic of the natural asparagine glycosylate. However, as shown in **Scheme 14**, this synthesis needed cumbersome solution-phase synthesis including the preparation of activated thioglycoside, the formation of disulfide between Cys-containing pentapeptide and thioglycoside, and some purification steps.



Scheme 14. Synthesis of disulfide linked peptide-glycoconjugate

On the contrary, using almost the same two components of the pentapeptide with a protected Cys residue, Ac-Phe-Cys(*t*-Bu)-Ser-Thr-Phe-NH_2 (**23**), and 2-acetamido-2-deoxy-1-thio-β-D-glucose (**25**), a disulfide linked glycoconjugate (**26**) was successfully synthesized by the newly developed Npys-OPh(*p*F) resin (**18**). As shown in **Scheme 15**, the peptide **23** was loaded onto the 3.7 equivalents of Npys-OPh(*p*F) resin in 1 h under 0.4 M LiCl/acetic acid. In the HPLC analysis shown in **Figure 4**, the peak of peptide **23** was completely disappeared. Then, the resulting peptide-attached on the resin **24** was transferred into 2-acetamido-2-deoxy-1-thio-β-D-glucose **25**. After shaking the resin **24** with glycosyl thiol **25** for 30 min in CH_3CN: 50 mM sodium acetate buffer (2:3, pH = 4.5), the disulfide-linked peptide-glycoconjugate **26** was produced in the HPLC analysis (**Figure 4**) and obtained with an isolated yield of 47%. These results indicated that the Npys-OPh(*p*F) resin can be used to synthesize disulfide-linked conjugates not only with peptides but also with other molecules like sugars.



Scheme 15. Synthesis of disulfide-linked glycoconjugate **26** on Npys-OPh(*p*F) resin **18**

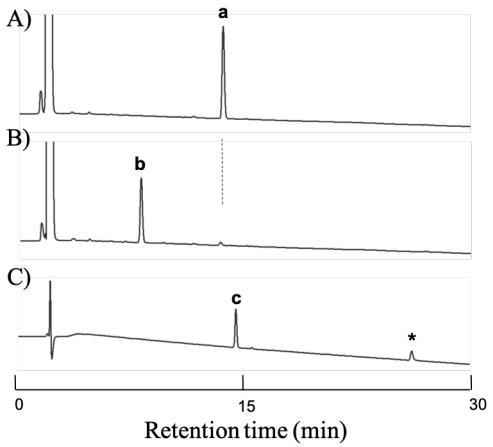


Figure 4. HPLC analysis of the reaction mixture and HPLC analysis of purified glycoconjugate (**26**). A) 0 h, B) 1 h, C) purified glycoconjugate (**26**), a: peptide **23**, b: 4-fluorophenol, c: purified glycoconjugate **26**, *original impurity peak. HPLC purity: 96%. HPLC conditions are a linear gradient starting from 25% CH_3CN in 0.1% aqueous TFA to 55% CH_3CN in 0.1% aqueous TFA over 30 min at a flow rate of 1.0 mL/min and detection at 230 nm.

2.4. Stability of solid-supported Npys-OPh(*p*F)

Finally, to demonstrate the usefulness of the Npys-OPh(*p*F) resin (**18**), its stability was examined by measuring the loading yield of peptide **19** to the resin stored under various conditions. In the previous reports, the conventional Npys-Cl resin must be used instantly after chlorination of Npys-Bn resin [53]. In fact, in the case of the Npys-Cl resin, the peptide fragments could no longer be supported on the resin after only one day of storage at room temperature. On the other hand, Npys-OPh(*p*F) resin (**18**) maintained its stability after one day at room temperature and gradually decomposed in one week. Surprisingly, the Npys-OPh(*p*F) resin was stable under -20 ºC more than 3 months. These results indicated that Npys-OPh(*p*F) resin possesses much higher stability under suitable storage conditions than the conventional Npys-Cl resin.

Table 11. Stability of Npys-Cl resin and Npys-OPh(*p*F) resin **18**

Entry	Storage condition	Time	Loading yield of peptide **19** (%)[a]	
			Npys-Cl Resin	Npys-OPh(*p*F) Resin **18**
1	Room temperature	1 day	0	97
2		3 days	0	63
3		7 days	0	22
4	4 ºC	30 days	0	36
5	-20 ºC	1 day	0	98
6		30 days	0	99
7		100 days	0	98

a Loading yield (%) = [1–(HPLC peak area of residual peptide **19** / HPLC peak area of original peptide **19**)] x 100.

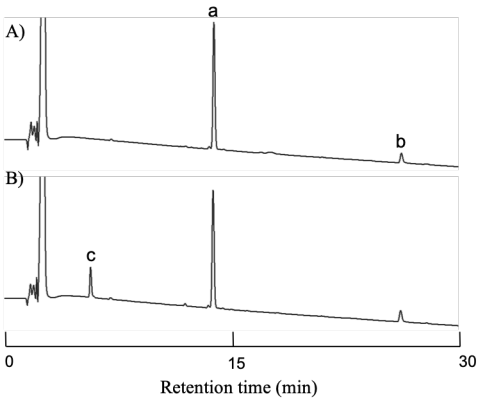


Figure 5. HPLC analysis of the reaction mixture (using resin **18** in Entry 1, **Table 11**). A) 0 h, B) 1 h, a: peptide **19**, b: original impurity peak, c: non-peptide peak. HPLC conditions are a linear gradient starting from 5% CH_3CN in 0.1% aqueous TFA to 65% CH_3CN in 0.1% aqueous TFA over 30 min at a flow rate 1.0 mL/min and detection at 230 nm.

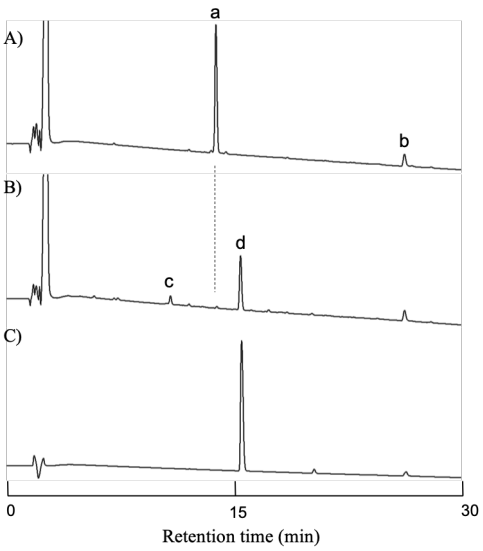


Figure 6. HPLC analysis of the reaction mixture (using resin **18** in Entry 7, **Table 11**). A) 0 h, B) 1 h, C) 4-fluorophenol, a: peptide **19**, b: original impurity peak, c: H-Asn-Cys-Pro-Leu-Gly-NH_2, d: 4-fluorophenol. HPLC conditions are a linear gradient starting from 5% CH_3CN in 0.1% aqueous TFA to 65% CH_3CN in 0.1% aqueous TFA over 30 min at a flow rate 1.0 mL/min and detection at 230 nm.

Conclusion

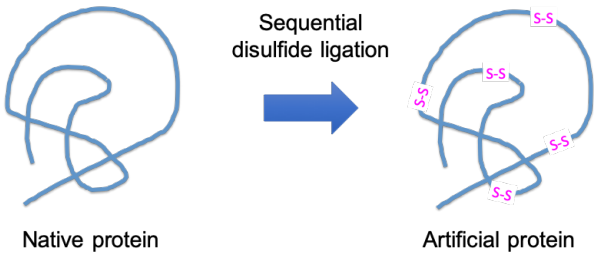
In Chapter 2, a new Npys-mediated solid-support agent, Npys-OPh(*p*F) resin **18**, was successfully developed, based on the screening in the conversion of the protected cysteine to the Cys(Npys) in solution-phase. The resin **18** can be prepared from the Npys-Cl resin with the straightforward method in a short time. In SPDSL strategy, without laborious activation step, the Npys-OPh(*p*F) resin **18** was applicable to the efficient synthesis of the disulfide bond in oxytocin and disulfide-linked peptide-glycoconjugate. Moreover, the agent is more stable to storage than the conventional Npys-Cl resin. As a useful and stable SPDSL agent, this resin **18** can contribute to the preparation of disulfide compounds.

Chapter 3. Modular chemical synthesis of the human immunodeficiency virus type 1 protease (HIV-1 PR) analogue via serial disulfide-bond formation

1. Introduction

1.1. Conversion of amide bonds into disulfide bonds in protein

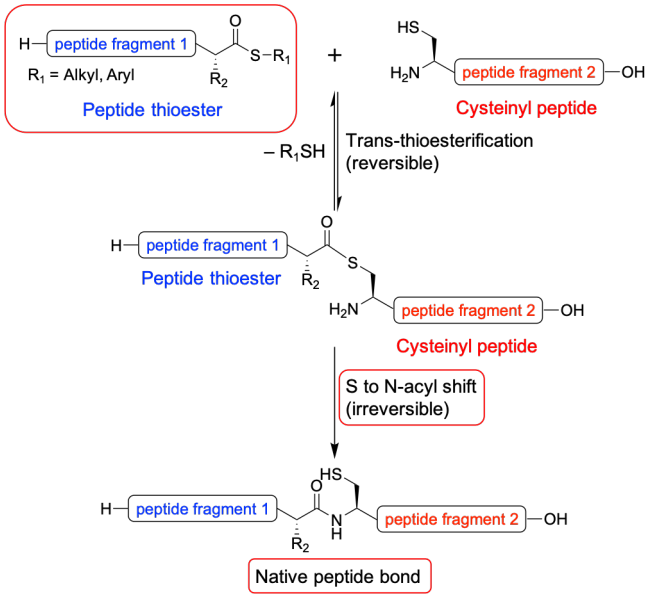
As mentioned in Chapter 2, disulfide bonds are one of the useful covalent bonds for preparing hybrid molecules and also important in maintaining the secondary and tertiary structures of peptides and proteins. On the other hand, since the inherent structure of a protein is not always optimized for its function, converting the chemical structure of the main chain of a protein to a different form may be worthwhile to diversify important aspects of protein functions. This would also be useful for elucidating protein functions and for screening inhibitors or antagonists in drug discovery [58]. As a potential bioisostere of amide bonds (**Scheme 16**), I thought that disulfide bonds are interesting because they can be easily constructed by mild oxidation, although they have the disadvantages of less ability to form hydrogen bonds and to restrict conformation than amide bonds. For example, it is possible that certain amide bonds in proteins, especially those at non-hydrogen bonding positions, can be converted to disulfide bonds without loss of biological activity.



Scheme 16. Conversion of amide bonds into disulfide bonds in protein

On the other hand, now a day, chemical synthesis of proteins becomes popular, whose peptide chains are generally constructed by the fragment condensation using an innovative native chemical ligation (NCL) method (**Scheme 17**) [59]. However, there are still some disadvantages of NCL, such as the difficulty in synthesizing C-terminal thioesters and the C-terminal residues cause a decrease in reaction rate and side reactions [60]. Therefore, the conversion of amide bonds to disulfide bonds may facilitate the assembly of the entire peptide chain. To the best of our knowledge, attempts to convert the protein backbone into more flexible multiple disulfide bonds by the precise design have not been

well investigated so far.



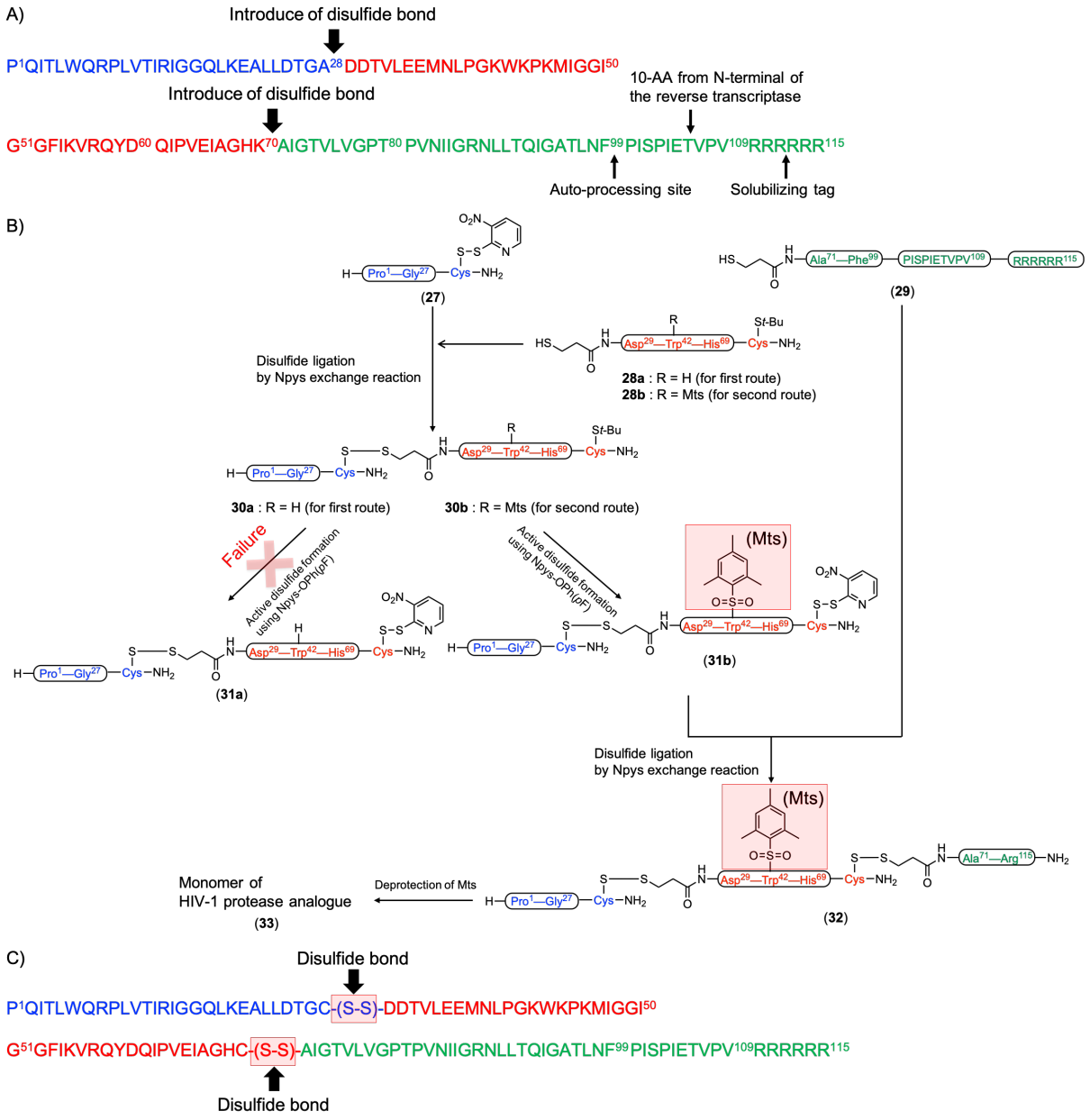
Scheme 17. Native Chemical Ligation (NCL) strategy

In this Chapter, I attempted the total chemical synthesis of a human immunodeficiency virus type 1 protease (22 kDa, abbreviated as HIV-1 PR) analogue, in which two amide bonds of the main chain in the sequence are converted to disulfide bonds. HIV-1 PR is a retrovirus-encoded aspartyl protease consisting of two identical 99-residue polypeptide monomers (11 kDa) that associate by non-covalent forces to form an active enzyme molecule with a single catalytic site (Asp25 and Asp25') [60]. As one of the most attractive targets for the drug development against AIDS, HIV-1 PR has been well studied. The chemical synthesis of various HIV-1 PR analogues contributes to elucidate the details of the proteolytic mechanism of HIV-1 PR and efficacy of inhibitors [61].

In the synthesis of the above mentioned partially disulfide-linked HIV-1 PR analogue, I planned to use Npys-OPh(*p*F) as an efficient disulfide forming agent to condense three peptide fragments by disulfide bonds. This method involves the regiospecific formation of each cysteine pair to correctly build two disulfide cross-links in the main chain of the HIV-1 PR analogue.

2. Results and discussions:

2.1. Initial synthetic plan (Route 1)



Scheme 18. A) The amino acid sequence of the HIV-1 protease monomer precursor (with 10-AA from N-terminal of reverse transcriptase and 6-Arg solubilizing tag. B) Synthetic route for disulfide-linked HIV-1 PR analogue. C) Amino acid sequence of partially disulfide-linked HIV-1 PR analogue (115aa).

To synthesize a partially disulfide-linked HIV-1 PR analogue consisting of 115aa residues, the whole sequence was divided into three peptide fragments [62]. Their sequences are as shown in **Scheme 18**-A: (Pro1–Gly27)-Cys(Npys)28-NH_2 (**27**), SH-(Asp29–His70)-Cys(*t*-Bu)71-NH_2 (**28a**, for first route), SH-(Asp29–Trp42(Mts)–His70)-Cys(*t*-Bu)71-NH_2 (**28b**, for second route) and SH-(Ala71–Phe99)-auto-processing tag-$(Arg)_6$-NH_2 (**29**). In the construction of the whole sequence, I planned to condense the three fragment peptides by disulfide ligations twice as shown in **Scheme 18**-B) and these fragment peptides **27**-**29** were synthesized with the automated Fmoc-SPPS method using the PRELUDE® peptide synthesizer (details in Experimental Part).

To introduce the thiol groups required for the correct disulfide bond formation among fragment peptides, Ala28 and Lys70 on the C-terminal of fragments **27** and **28** were replaced to Cys28 and Cys70, respectively, and 3-mercaptopropionyl groups were introduced to α-amino groups of Asp29 and Ala71. On the other hand, Cys67 and Cys95 on fragments **28** and **29** were replaced to Ala to prevent the undesired disulfide bridge formation, since these Cys to Ala replacements are known to have no effect on the catalytic activity and kinetic parameters similar to the wild HIV-1 PR have been reported [63]. Moreover, in order to improve the poor solubility of fragment **29** and polypeptide intermediates SH-(Ala71–Phe99), synthesized from fragment **29**, a solubilizing moiety containing six residues of Arg (6-Arg) was introduced at the C-terminus of peptide fragment **29** [62]. However, this solubilizing moiety must be removed after assembly of the full-length monomeric polypeptide chain of the HIV-1 PR analogue to give a mature 99-residue product. Therefore, on the basis of the previously reported auto-proteolytic processing, which is a native mechanism of HIV-1 PR maturation in the viral replication [62], the 10 amino acid sequence (PISPIETVPV) from the C-terminal of reverse transcriptase adjacent to the HIV-1 PR sequence on the genome were inserted between the fragment 29 and the solubilizing moiety. After the 115-residue polypeptide is assembled, if the polypeptide is properly folded under physiological conditions to form an active protease, it was expected that an automated processing similar to that occurring *in vivo* can remove the C-terminal insert. This results in the production of a homodimer HIV-1PR analogue with 2 x 99 residues [62, 63].

For the synthesis of HIV-1 PR analogue (Route 1) as depicted in **Scheme 18**-B, the disulfide ligation between Npys-containing fragment **27** and fragment **28a** with an unprotected SH group at the N-terminus was initially performed in CH_3CN/0.1 M sodium acetate buffer (pH = 4.5) for 24 h at room temperature. In the reaction, intense yellow color of thiopyridone appeared as soon as the two reactants were mixed. This indicated that elimination of the Npys group from fragment **27** and the ligation with fragments **28a** is progressed. Then, the reaction mixture was purified by reversed phase HPLC to give the fragment **30a** with an isolated yield of 32%.

Next, the *t*-Bu protected side chain of Cys in the resultant fragment **30a** was attempted to convert to corresponding active Npys disulfide (Cys(Npys)) by the addition of Npys-OPh(*p*F) under acidic conditions (AcOH) at room temperature. However, a severe side reaction occurred in a very short time (less than 1 min) after the start of the reaction. In HPLC analysis and mass measurement, an Npys-adducted fragment **31a-bp1** (Npy-sulfenylation) was detected as a byproduct and the desired fragment peptide **31a** was not produced (See **Figure 7**, **Table 12**). Moreover, it was indicated that *t*-Bu protected Cys in **31a-bp1** was no modified from the mass measurement, suggesting that the other amino acid residue in fragment **30a** reacted with Npys-OPh(*p*F). During reaction for 3 h, fragment **30a** was completely consumed, resulted in the production of fragment **31a-bp1** with additional two-Npys-adduct (**31a-bp2**). To obtain the desired fragment peptide **31a**, the reaction under different conditions were examined, however, it was difficult to find the suitable conditions without the side reaction.

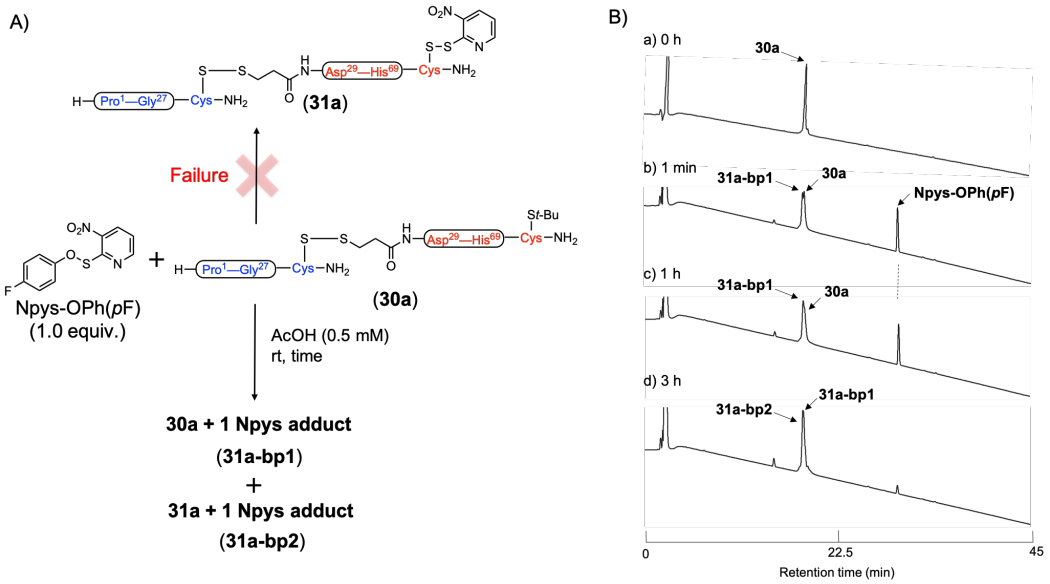


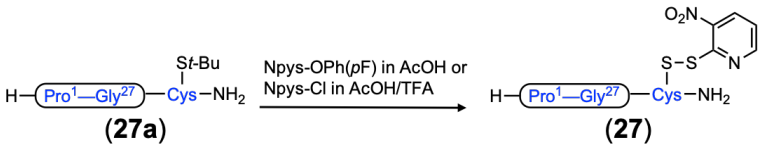
Figure 7. A) Side reaction on fragment **30a**. B) HPLC analysis of Npy-sulfenylation reaction on fragment **30a**

Table 12. The formation of active disulfide on fragment **30a**

Entry	Solvent	Equiv. of Npys-OPh(*p*F)	Observed peptide			
			1 min	1 h	3 h	24 h
1	AcOH	1.0	**30a** and **31a-bp1**	**30a** and **31a-bp1**	**31a-bp1** and **31a-bp2**	**31a-bp1** and **31a-bp2**
2	AcOH	2.0	**31a-bp1**	**31a-bp1**	**31a-bp1** and **31a-bp2**	**31a-bp2**[a]
3	90% AcOH aq.	1.0	**31a-bp1**	**31a-bp1**	**31a-bp1** and **31a-bp2**	**31a-bp1** and **31a-bp2**

[a] The mass spectra of **31a-bp2** with an additional Npys group was also observed.

To determine the amino acid residue suffering the side reaction of the Npys addition, fragment **27a** and fragment **34** ((Asp29–His70)-Cys71(*t*-Bu)-NH_2, a derivative of fragment **28a** without the 3-mercaptopropionyl group at the N-terminus) were used to react with Npys-OPh(*p*F) under the same reaction condition in acetic acid, respectively. As shown in **Schemes 19** and **20** and **Tables 13** and **14**, this side reaction was only observed in the fragment **34**, yielding two fragments **34a** ((Asp29–His70)-Cys71(*t*-Bu)-NH_2 + 1 Npys adduct) and **34b** ((Asp29–His70)-Cys71(Npys)-NH_2 + 1 Npys adduct), but not on the fragment **27a**.



Scheme 19. Npy-sulfenylation on peptide fragment **27a**

Table 13. Npy-sulfenylation on fragment **27a**

Entry	Compound	Solvent	Eq.	Observed peptide			
				1 min	1 h	3 h	24 h
1	Npys-OPh(*p*F)	AcOH	1.0	**27a**	**27a**	**27** and **27a**	**27** and **27a**
2[a]	Npys-OPh(*p*F)	AcOH	2.0	**27a**	**27** and **27a**	**27** and **27a**	**27** and **27a**
3	Npys-Cl	AcOH/TFA[1)]	1.0	**27** and **27a**	**27** and **27a**	**27** and **27a**	**27** and **27a**

[a] HPLC of Entry 2 were shown below.

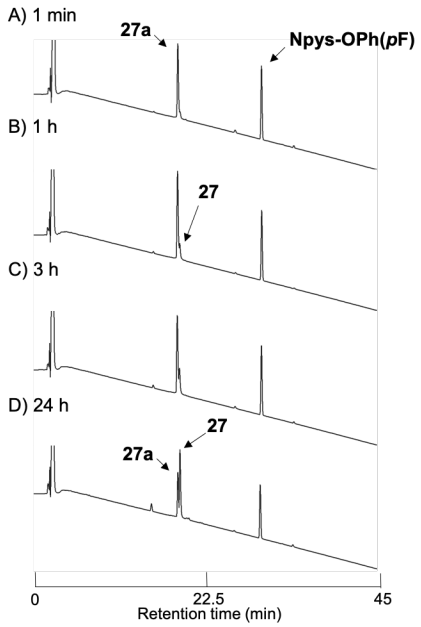
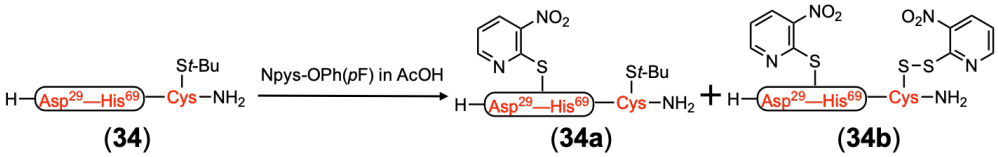


Figure 8. HPLC of Npy-sulfenylation on fragment **27a** (Entry 2 in **Table 12**). HPLC conditions are a linear gradient starting from 5% CH_3CN in 0.1% aqueous TFA to 95% CH_3CN in 0.1% aqueous TFA over 45 min at a flow rate 1.0 mL / min and detection at 214 nm.



Scheme 20. Npy-sulfenylation on peptide fragment **34**

Table 14. Npy-sulfenylation on fragment **34**

Entry	Eq.	Observed peptide				
		1 min	1 h	3 h	6 h	24 h
1[a]	1.0	**34a**	**34a** and **34b**	**34a** and **34b**	**34a** and **34b**	**34a** and **34b**
2	5.0	**34a** and **34b**	**34a** and **34b**	**34a** and **34b**[b]	**34a** and **34b**[b]	**34a** and **34b**[b]

[a] HPLC of Entry 1 were shown below.
[b] After 3 h reaction to 24 h, the ESI-MS showed **34b** and **34b** with an additional Npys group.

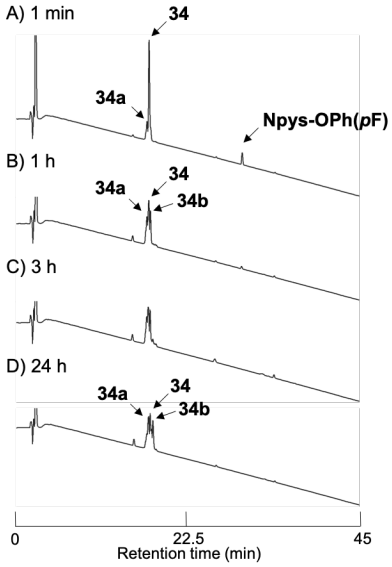


Figure 9. HPLC of Npy-sulfenylation on fragment **34**. HPLC conditions are a linear gradient starting from 5% CH_3CN in 0.1% aqueous TFA to 95% CH_3CN in 0.1% aqueous TFA over 45 min at a flow rate 1.0 mL / min and detection at 214 nm.

2.2. Enzymatic digestion of fragment 34 and Npy-sulfenylation fragment, 34b

Next, to further determine the amino acid residue suffering the Npy-sulfenylation, the enzymatic digestion of fragments **34** and **34b** were performed. An endoproteinase, Lys-C was used to cleavage the C-terminal of Lys at the positions 41, 45 and 55 on both fragments and the resulting crude solution was analyzed by MALDI-TOF MS. As a result, an Npys adduct of the digested peptide fragment WKPK (42-45) with the mass spectrum of calc. 711.36; found. 711.60 was detected in the digested fragment **34b** (**Figure 10**-B), suggesting that either Trp, Lys or Pro has been modified by Npy-sulfenylation. Since it is previously reported that Npys-Cl can react to the indole group at side chain of Trp under acidic conditions [64], the Npy-sulfenylation was suspected to occur at Trp42 in fragment **34b**. Therefore, to avoid the Npy-sulfenylation, I planned to introduce a mesitylenesulfonyl (Mts) group to Trp42 as a protecting group, which is generally used in the Fmoc-based SPPS.

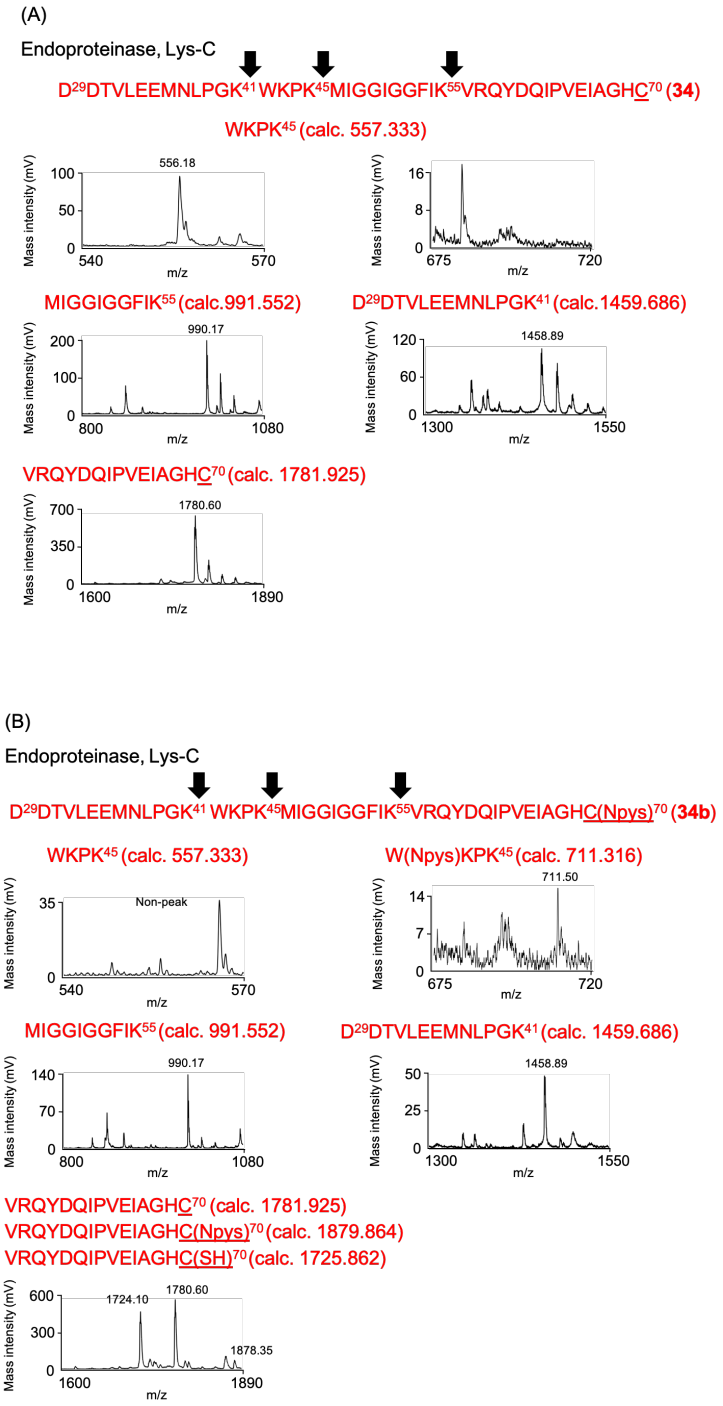


Figure 10. MALDI-TOF MS spectra of digestion of fragments **34** (A) and **34b** (B)

2.3. Second synthetic plan (Routes 2)

As shown in **Figure 11**, based on the aforementioned results, I planned a second synthetic route (Route 2) using a new fragment peptide **28b** with Mts-protected Trp42 instead of the fragment **28a**. Namely, fragment **27** was mixed with fragment **28b** to synthesize the fragment peptide **30b** under the same reaction condition as that used in Route 1. As shown in **Figure 11**-B), the chemo-selective disulfide ligation between these two fragments proceed without any competing side reaction and fragment **30b** was efficiently formed. Purification of the reaction mixture with reversed-phase HPLC resulted in the desired fragment **30b** with an isolated yield of 66%.

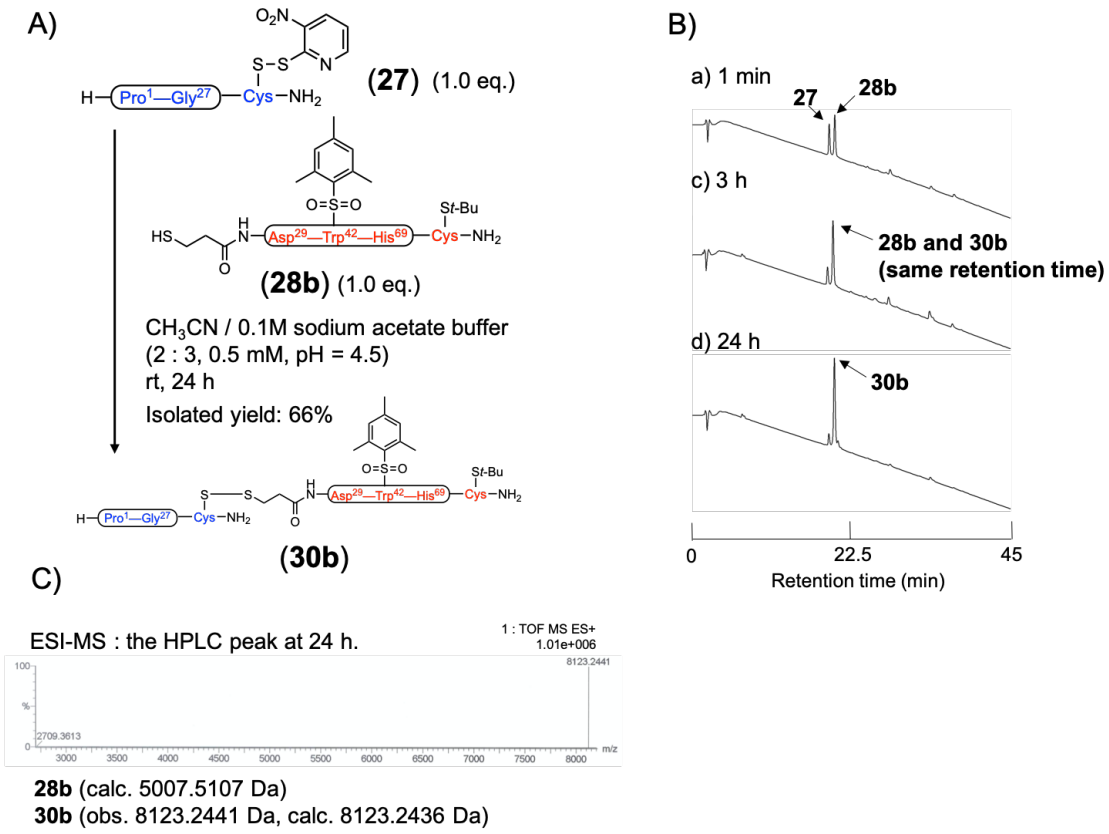
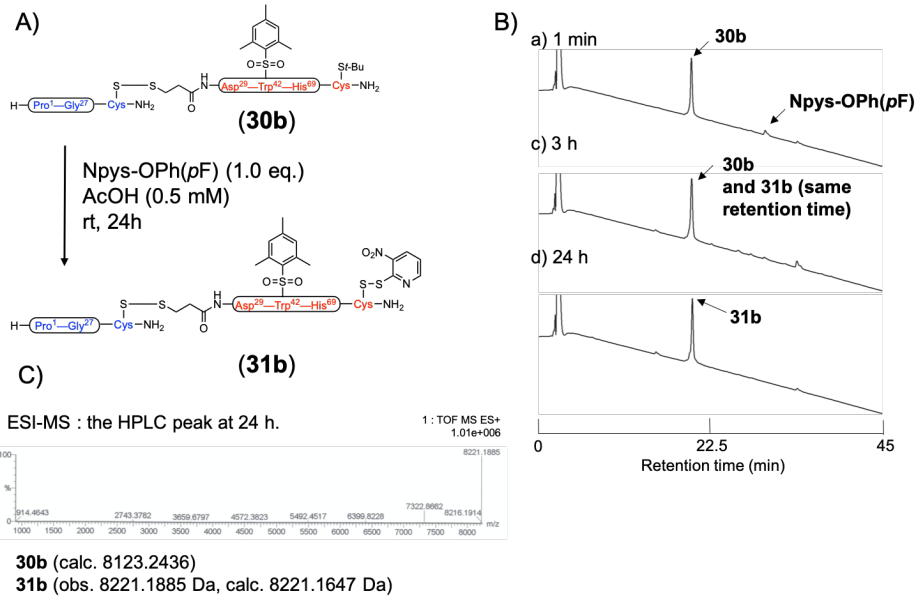


Figure 11. A) Synthetic scheme for fragment **30b**, B) HPLC charts of the reaction, C) ESI-MS spectrum of 24 h reaction. The spectrum gave an observed mass of 8123.2441 Da (calcd 8123.2436 Da, average isotopes, not obtained fragment **28b**). HPLC conditions are a linear gradient starting from 5% CH_3CN in 0.1% aqueous TFA to 95% CH_3CN in 0.1% aqueous TFA over 45 min at a flow rate 1.0 mL / min and detection at 214 nm.

Next, the fragment peptide **30b** in AcOH (0.5 mM solution) was mixed with 1.0 eq. of Npys-OPh(*p*F) at room temperature to introduce the Npys group at Cys. As shown in **Figure 12**, it was difficult to detect the reaction in HPLC analysis since the peak eluted at 19 min was not changed. This is because both fragments **30b** and **31b** were co-eluted at the same retention time. Therefore, the reaction was checked by the ESI-MS analysis. As a result, the reaction was efficiently proceeded and only fragment **31b** was detected after 24 h (**Figure 12**-C)). These results indicated that the reaction using fragment with protected side chain of Trp42 was successfully proceeded without byproduct observed in the Route 1. This would be additional evidence that Npys-OPh(*p*F) selectively modifies Trp42 in the fragment peptide **28a**.



30b (calc. 8123.2436)
31b (obs. 8221.1885 Da, calc. 8221.1647 Da)

Figure 12. A) synthetic scheme for fragment **31b**, B) HPLC of Npy-sulfenylation reaction on fragment **30b**, C) ESI-MS spectrum of 24 h reaction. The spectrum gave an observed mass of 8221.1885 Da (calcd 8221.1647 Da, average isotopes, not obtained fragment **30b**). HPLC conditions are a linear gradient starting from 5% CH_3CN in 0.1% aqueous TFA to 95% CH_3CN in 0.1% aqueous TFA over 45 min at a flow rate 1.0 mL / min and detection at 214 nm.

Then, the Npys-containing fragment peptide **31b** without further purification was directly ligated to the unprotected thiol-containing fragment peptide **29** in CH_3CN/0.1 M sodium acetate buffer (pH = 4.5, 2:3, peptide solution; 0.5 mM) for 5 h at room temperature to form the second disulfide bond. It is worth to note that the first disulfide bond was stably maintained in the presence of the unprotected thiol group in the fragment peptide **29** during the second disulfide exchange reaction. A good disulfide ligation yield of 38% in the synthesis of the fragment peptide **32** from fragment peptide **31b** was obtained after HPLC purification.

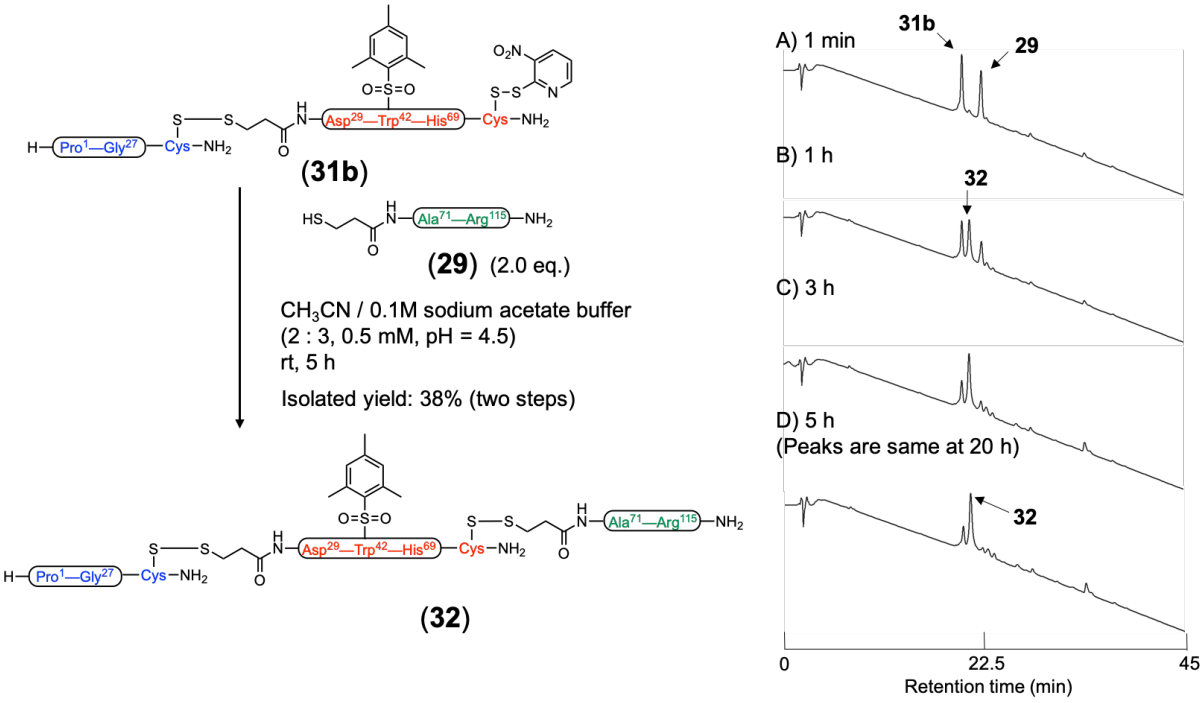


Figure 13. Synthetic scheme and HPLC charts of fragment **32.** HPLC conditions are a linear gradient starting from 5% CH_3CN in 0.1% aqueous TFA to 95% CH_3CN in 0.1% aqueous TFA over 45 min at a flow rate 1.0 mL / min and detection at 214 nm.

These results show that the two disulfide ligations proceed in a regioselective manner without the formation of the wrong disulfide bridging, resulting in entire structure of Mts-protected HIV-1 PR analogue (**32**).

Finally, the Mts group of fragment **32** was deprotected by treatment with 1 M trimethylsilyl trifluoromethanesulfonate (TMSOTf)/TFA in the presence of diphenyl sulfide (Ph-S-Ph) and *m*-cresol at 0 °C for 2 h [65]. The crude HIV-1 PR analogue was precipitated by the addition of cold ether to the reaction mixture, and purified by preparative HPLC. As a result, a partially disulfide-linked HIV-1 PR analogue **33** was obtained in a yield of 44% from fragment **32**. Therefore, the total isolated yield of **33** over 8 steps was 11 % with the purity of 96 % (see HPLC chart in **Figure 20**).

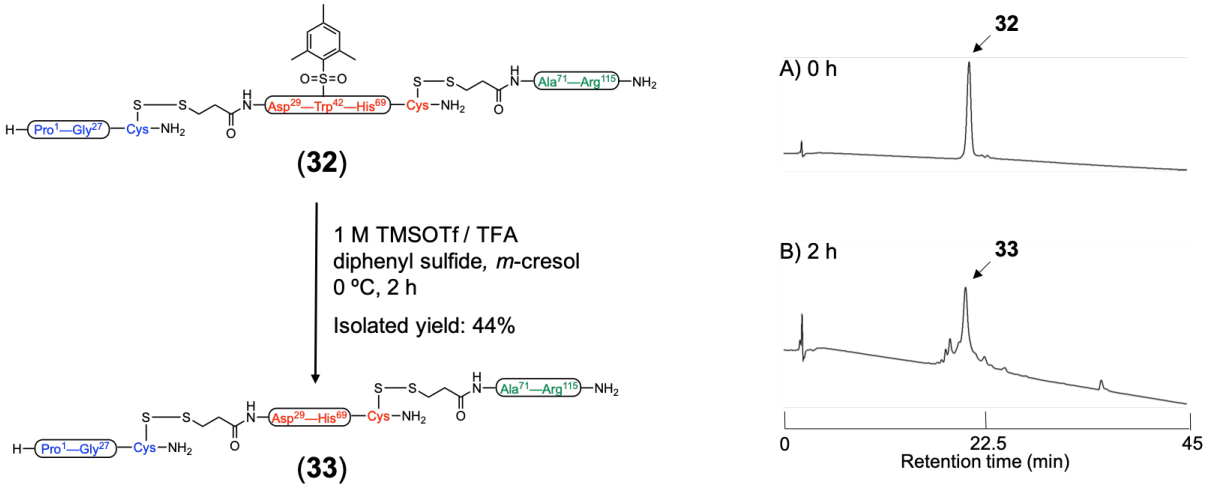


Figure 14. HPLC analysis of deprotection of polypeptide **32**. A) 0 h (before reaction) B) 2 h. HPLC conditions are a linear gradient starting from 5% CH_3CN in 0.1% aqueous TFA to 95% CH_3CN in 0.1% aqueous TFA over 45 min at a flow rate 1.0 mL / min and detection at 214 nm.

Further studies for folding the resulting HIV-1 PR analogue **33** to the dimer form followed by auto-processing to cleave the water-solubilizing moiety, and finally measuring its HIV-1 protease activity are remaining as the near future issues.

Conclusion

In Chapter 3, it is indicated that Npys-OPh(*p*F)-based disulfide ligation could be applied to the synthesis of the partially disulfide-linked artificial protein (11 kDa). Namely, the first total chemical synthesis of the double disulfide-linked HIV-1 protease monomer analogue (**33**) was achieved. A modular synthesis of active disulfide using Npys-OPh(*p*F) and Npys-based disulfide exchange reaction gave synthetic analogue **33** in good yields with high-purity. This study might expand the applicability of Npys-based chemistry to the synthesis of various proteins. Finally, the correct folding and full activity of this synthetic partially disulfide-linked HIV-1 protease monomer analogue would be determined in the near future.

CONCLUSIONS

This Ph.D. dissertation is entitled "Development of 4-fluorophenyl 3-nitro-2-pyridinesulfenate as a new Npys protecting reagent and its application to efficient disulfide formation" and describes the development of new 3-nitro-2-pyridinesulfenate (Npys-OR) reagents which is a stable surrogate of the Npys-Cl and its resin in solution- and solid-phase synthesis.

The field encompassing peptide and protein synthesis has increased quickly over the last 50 years, thanks to the development of solid-phase peptide synthesis techniques and the improvement and diversification of protecting groups for the functional group in amino acid derivatives. Among protecting groups, the 3-nitro-2-pyridinesulfenyl (Npys) acts as a protective group for amine, hydroxy and thiol groups. In the protection of the thiol group, the Npys group acts as an active disulfide to react with another unprotected thiol group and generates a disulfide bond.

Since disulfide bonds in many functional proteins and peptides play a critical role in maintaining their three-dimensional structures and biological activities, a unique chemistry of Npys has been widely applied to the preparation of disulfide bonds in these molecules.

However, Npys-Cl is the only reagent used for 3-nitro-2-pyridine (Npy)-sulfenylation of the functional group, so far, and this sulfenyl chloride has some obvious defects, such as instability issues with light or moisture, even at low temperatures. Namely, this is one of the major drawbacks in the Npys chemistry. Based on such a background, it is necessary to develop a new Npys reagent as the replacement for the conventional Npys-Cl. To solve this issue, I focused on a series of 3-nitro-2-pyridine (Npy) sulfenates.

In this dissertation, development of a new Npys reagent focused on Npy sulfenates and its application to the disulfide formation are described in three chapters as follows.

In Chapter 1, a new Npys-based reagent, Npys-OPh(*p*F) has been developed in replacement of its parent compound, Npys-Cl. The reactivity of Npys-OPh(*p*F) to functional groups in amino acid derivatives was investigated under various reaction conditions, and it found that Npys-OPh(*p*F) can protect amine, hydroxy and thiol groups. Moreover, Npys-OPh(*p*F) was improved physicochemical stability in comparison to Npys-Cl. Therefore, it is indicated that Npys-OPh(*p*F) is more convenient

than Npys-Cl, thus making Npys-OPh(*p*F) a reagent of choice for the Npys-based chemistry.

In Chapter 2, as a new Npys-mediated solid-support agent, Npys-OPh(*p*F) resin has been developed. This new agent can be prepared from the Npys-Cl resin with a straightforward method in a short time. In the SPDSL strategy, Npys-OPh(*p*F) resin was applicable to the disulfide bond formation in the synthesis of oxytocin and disulfide-linked peptide-glycoconjugate. Moreover, the resin can be stored more stably than the conventional Npys-Cl resin. Thanks to this property, Npys-OPh(*p*F) resin overcame a laborious activation step required in the use of Npys-Cl resin. As a useful and stable SPDSL agent, Npys-OPh(*p*F) resin can contribute to the preparation of a variety of disulfide-linked conjugates useful for peptide science, chemical biology and drug development.

In Chapter 3, the chemistry of Npys-OPh(*p*F) was applied to the modular chemical synthesis of a partially disulfide-linked human immunodeficiency virus type 1 protease (HIV-1 PR) analogue via fragment couplings using serial disulfide-bond formation. A modular synthesis of the active disulfide using Npys-OPh(*p*F) and disulfide exchange reactions gave the above-mentioned synthetic HIV-1 PR analogue in good yield with high-purity. These results indicate that Npys-based disulfide ligation may be applied to the synthesis of a variety of proteins.

In summary, novel Npys reagents, Npys-OPh(*p*F) and its resin form are expected to contribute to the further drug development, organic chemistry, and peptide science.

EXPERIMENTAL PARTS

1. Materials and methods

Solvents and reagents were purchased from Kanto Chemical Co., Inc., Kokusan Chemical Co., Ltd., NACALAI TESQUE, INC., Tokyo Chemical Industry Co., Ltd., Wako Pure Chemical Industries, Ltd., and Watanabe Chemical Industries, Ltd.

Melting points were measured with a Yanaco MP-500D melting point apparatuses.

IR spectra were recorded on JASCO FT/IR 4100 spectrometer.

^{1}H NMR spectra were measured in $CDCl_3$, MeOD or D_2O solution using Bruker AVANCE-III (400 MHz) spectrophotometer and referenced to TMS (0.00 ppm) and TSP-d_4, (0.00 ppm).

^{13}C NMR spectra were measured in $CDCl_3$ solution using Bruker AVANCE-III (100 MHz) and Bruker Biospin AV-600 (150 MHz) spectrophotometers and $CDCl_3$ (77.05 ppm) as a reference. Peak multiplicities are reported with the following abbreviations are used: s, singlet; d, doublet; t, triplet; m, multiplet.

Mass spectra were obtained on Waters MICRO MASS LCT-premier and a JEOL JMS-700.

Column chromatography was performed on silica gel 60N (spherical, neutral) (40-50 μm).

Thin layer chromatography (TLC) was performed on precoated plates (0.25 mm, silica gel Merk Kieselgel $60F_{254}$),

Compounds were visualized with UV light, phosphomolybdic acid stain, and ninhydrin stain.

Preparative thin-layer chromatography was performed on precoated plates (2 mm, PLC Silica gel 60 F_{254}).

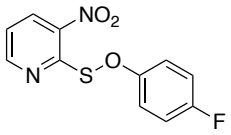
Preparative HPLC was performed using a C18 reversed-phase column (19 x 150 mm; SunFire™

Prep C18 OBD™ 5 μm) with a binary solvent system.

Analytical HPLC was performed using a C18 reversed-phase column (4.6 x 150 mm; SunFire™ C18 5 μm) with a binary solvent system.

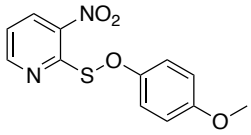
Chapter 1

4-Fluorophenyl 3-nitro-2-pyridinesulfenate (Npys-OPh(*p*F), **1**)



4-Fluorophenol (29.4 mg, 0.26 mmol, 1.0 equiv.) and *N*, *N*-diisopropylethylamine (DIPEA, 174 μL, 1.04 mmol, 4.0 equiv.) were added to a solution of 3-nitro-2-pyridinesulfenyl chloride (Npys-Cl, 50 mg, 0.26 mmol, 1.0 equiv.) dissolved in dry 1,2-dichloroethane (1,2-DCE, 0.8 mL) at room temperature. After stirring for 1 h at this temperature, the mixture was diluted further in $CHCl_3$ (10 mL). The organic layer was washed with 5% citric acid aq., H_2O and brine. The combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure. The resulting residue was purified by flash column chromatography (hexane : ethyl acetate = 5 : 1), to give **1** (46 mg, 0.17 mmol, 66%) as a yellow solid; m.p. 93.3-94.5 ºC; IR (KBr) 1494, 1323, 800 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.84 (dd, J = 1.6 and 4.6 Hz, 1H), 8.53 (dd, J = 1.6 and 8.3 Hz, 1H), 7.40-7.27 (m, 1H), 7.26-7.15 (m, 2H), 7.02-6.90 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 163.3, 158.8 (d, J_{CF} = 241 Hz), 155.4 (d, J_{CF} = 2.2 Hz), 155.0, 137.5, 133.1, 120.1, 118.4 (d, J_{CF} = 8.2 Hz, 2 carbons), 115.7 (d, J_{CF} = 24 Hz, 2 carbons); HRMS (ES+) *m/z* calcd for $C_{11}H_8N_2O_3SF$ $[M+H]^+$ 267.0240, found 267.0237.
With 1.2 equivalents of 4-fluorophenol, **1** was synthesized in 73% yield.

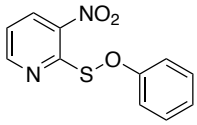
4-Methoxyphenyl 3-nitro-2-pyridinesulfenate (Npys-OPh(*p*MeO), **2**)



Compound **2** was prepared in the same manner as described for compound **1** using Npys-Cl (50 mg, 0.26 mmol), DIPEA (0.17 mL, 1.04 mmol) and 4-methoxyphenol (32.5 mg, 0.26 mmol).

Yield, 50 mg, 0.18 mmol, 68%; a yellow solid; m.p. 109.6-110.4 ºC; IR (KBr) 1500, 1327, 825 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.85 (dd, J = 1.6 and 4.5 Hz, 1H), 8.52 (dd, J = 1.6 and 8.3 Hz, 1H), 7.32-7.29 (m, 1H), 7.19-7.16 (m, 2H), 6.82-6.78 (m, 2H), 3.76 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 163.8, 155.8, 154.9, 153.5, 137.4, 133.1, 119.9, 118.3 (2 carbons), 114.3 (2 carbons), 55.7; HRMS (ES+) *m/z* calcd for $C_{12}H_{11}N_2O_4S$ $[M+H]^+$ 279.0440, found 279.0433.
Both 1.0 and 1.2 equivalents of 4-methoxyphenol gave **2** in same yields of 68%.

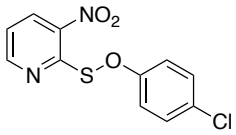
Phenyl 3-nitro-2-pyridinesulfenate (**3**)



Compound **3** was prepared in the same manner as described for compound **1** using Npys-Cl (50 mg, 0.26 mmol), DIPEA (0.17 mL, 1.04 mmol) and phenol (28 μL, 0.31 mmol).

Yield, 37 mg, 0.15 mmol, 57%; a yellow solid; m.p. 84.8-85.7 ºC; IR (KBr) 1512, 1324, 846 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.82 (dd, *J* = 1.6 and 4.6 Hz, 1H), 8.53 (dd, *J* = 1.6 and 8.3 Hz, 1H), 7.33-7.28 (m, 3H), 7.26-7.23 (m, 2H), 7.09-7.06 (m, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 163.5, 159.3, 155.0, 137.6, 133.1, 129.4 (2 carbons), 123.5, 119.9, 116.9 (2 carbons); HRMS (ES+) *m/z* calcd for $C_{11}H_9N_2O_3S$ $[M+H]^+$ 249.0334, found 249.0332.

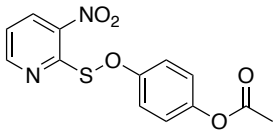
4-Chlorophenyl 3-nitro-2-pyridinesulfenate (**4**)



Compound **4** was prepared in the same manner as described for compound **1** using Npys-Cl (50 mg, 0.26 mmol), DIPEA (0.17 mL, 1.04 mmol) and 4-chlorophenol (25 μL, 0.26 mmol).

Yield, 37 mg, 0.13 mmol, 50%; a yellow solid; m.p. 108.6-109.5 ºC; IR (KBr) 1510, 1324, 810 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.81 (dd, *J* = 4.5 and 1.5 Hz, 1H), 8.54 (dd, *J* = 8.3 and 1.5 Hz, 1H), 7.36-7.27 (m, 1H), 7.25-7.22 (m, 2H), 7.19-7.13 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 163.0, 157.9, 155.0, 137.6, 133.1, 129.2 (2 carbons), 128.4, 120.1, 118.3 (2 carbons); HRMS (ES+) *m/z* calcd for $C_{11}H_8N_2O_3SCl$ $[M+H]^+$ 282.9944, found 282.9951.

4-Acetoxyphenyl 3-nitro-2-pyridinesulfenate (**5**)

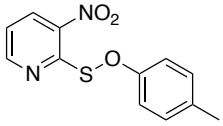


Compound **5** was prepared in the same manner as described for compound **1** using Npys-Cl (50 mg, 0.26 mmol), DIPEA (0.17 mL, 1.04 mmol) and 4-hydroxyphenyl acetate (40 mg, 0.26 mmol).

Yield, 25.6 mg, 83 μmol, 32%; a yellow solid; m.p. 106.7-107.8 ºC; IR (KBr) 1741, 1496, 1324, 835 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.83 (dd, *J* = 1.6 and 4.5 Hz, 1H), 8.52 (dd, *J* = 1.6 and 8.3 Hz, 1H), 7.34-7.30 (m, 1H), 7.25-7.20 (m, 2H), 7.01-6.97 (m, 2H), 2.28 (s, 3H); ^{13}C NMR ($CDCl_3$,

100 MHz) δ 169.6, 163.2, 156.8, 155.0, 146.3, 137.6, 133.1, 122.2 (2 carbons), 120.0, 117.8 (2 carbons), 21.0; HRMS (ES+) *m/z* calcd for $C_{13}H_{11}N_2O_5S$ $[M+H]^+$ 307.0389, found 307.0386.

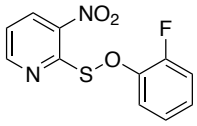
4-Methylphenyl 3-nitro-2-pyridinesulfenate (**6**)



Compound **6** was prepared in the same manner as described for compound **1** using Npys-Cl (50 mg, 0.26 mmol), DIPEA (0.17 mL, 1.04 mmol) and 4-methylphenol (27 μL, 0.26 mmol).

Yield, 36.3 mg, 0.14 mmol, 53%; a yellow solid; m.p. 108.5-109.4 ºC; IR (KBr) 1499, 1234, 853 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.82 (dd, J = 1.6 and 4.6 Hz, 1H), 8.51 (dd, J = 1.6 and 8.3 Hz, 1H), 7.32-7.27 (m, 1H), 7.15-7.12 (m, 2H), 7.09-7.05 (m, 2H), 2.29 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 163.7, 157.2, 155.0, 137.5, 133.01, 132.97, 129.8 (2 carbons), 119.8, 116.8 (2 carbons), 20.6; HRMS (ES+) *m/z* calcd for $C_{12}H_{11}N_2O_3S$ $[M+H]^+$ 263.0490, found 263.0495.

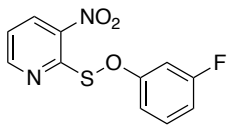
2-Fluorophenyl 3-nitro-2-pyridinesulfenate (**7**)



Compound **7** was prepared in the same manner as described for compound **1** using Npys-Cl (50 mg, 0.26 mmol), DIPEA (0.17 mL, 1.04 mmol) and 2-fluorophenol (29 μL, 0.31 mmol).

Yield, 38 mg, 0.14 mmol, 54%; a yellow solid; m.p. 138.7-139.7 ºC; IR (KBr) 1492, 1324, 790 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.87 (d, J = 4.5 Hz, 1H), 8.53 (d, J = 8.3 Hz, 1H), 7.38-7.31 (m, 2H), 7.16-7.11 (m, 1H), 7.06-6.98 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 163.0, 155.1, 153.1 (d, J_{CF} = 248 Hz), 147.3 (d, J_{CF} = 11 Hz), 137.4, 133.0, 124.13 (d, J_{CF} = 15 Hz), 124.12 (d, J_{CF} = 4.4 Hz), 120.1, 118.6, 116.6 (d, J_{CF} = 19 Hz); HRMS (ES+) *m/z* calcd for $C_{11}H_8N_2O_3SF$ $[M+H]^+$ 267.0240, found 267.0242.

3-Fluorophenyl 3-nitro-2-pyridinesulfenate (**8**)

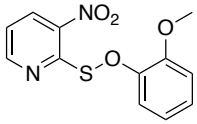


Compound **8** was prepared in the same manner as described for compound **1** using Npys-Cl (50

mg, 0.26 mmol), DIPEA (0.17 mL, 1.04 mmol) and 3-fluorophenol (29 μL, 0.31 mmol).

Yield, 28 mg, 0.10 mmol, 40%; a yellow solid; m.p. 78.5-79.9 ºC; IR (KBr) 1512, 1322, 798 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.82 (dd, J = 1.0 and 4.5 Hz, 1H), 8.54 (dd, J = 1.1 and 8.3 Hz, 1H), 7.35-7.32 (m, 1H), 7.25-7.21 (m, 1H), 7.05-7.02 (m, 1H), 6.99-6.95 (m, 1H), 6.80-7.75 (m, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 163.2 (d, J_{CF} = 247 Hz), 162.8, 160.5 (d, J_{CF} = 11 Hz), 155.0, 137.6, 133.1, 130.1 (d, J_{CF} = 9.9 Hz), 120.1, 112.6 (d, J_{CF} = 2.9 Hz), 110.3 (d, J_{CF} = 21 Hz), 104.7 (d, J_{CF} = 26 Hz); HRMS (ES+) *m/z* calcd for $C_{11}H_8N_2O_3SF$ $[M+H]^+$ 267.0240, found 267.0237.

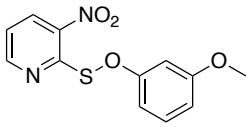
2-Methoxylphenyl 3-nitro-2-pyridinesulfenate (**9**)



Compound **9** was prepared in the same manner as described for compound **1** using Npys-Cl (50 mg, 0.26 mmol), DIPEA (0.17 mL, 1.04 mmol) and 2-methoxyphenol (34 μL, 0.31 mmol).

Yield, 18 mg, 65 μmol, 22%; a yellow solid; m.p. 123.7-124.8 ºC; IR (KBr) 1498, 1327, 852 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.87 (dd, J = 1.5 and 4.5 Hz, 1H), 8.50 (dd, J = 1.5 and 8.2 Hz, 1H), 7.35-7.28 (m, 2H), 7.07-7.03 (m, 1H), 6.96-6.94 (m, 1H), 6.85-6.81 (m, 1H), 3.96 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 163.8, 155.0, 150.3, 148.7, 137.4, 133.0, 124.3, 120.4, 119.8, 117.8, 112.3, 56.1; HRMS (ES+) *m/z* calcd for $C_{12}H_{11}N_2O_4S$ $[M+H]^+$ 279.0440, found 279.0431.

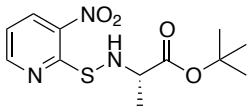
3-Methoxylphenyl 3-nitro-2-pyridinesulfenate (**10**)



Compound **10** was prepared in the same manner as described for compound **1** using Npys-Cl (50 mg, 0.26 mmol), DIPEA (0.17 mL, 1.04 mmol) and 3-methoxyphenol (35 μL, 0.31 mmol).

Yield, 14.2 mg, 0.051 mmol, 20%; a yellow solid; m.p. 81.4-82.4 ºC; IR (KBr) 1487, 1326, 940 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.82 (dd, J = 1.5 and 4.5 Hz, 1H), 8.52 (dd, J = 1.5 and 8.3 Hz, 1H), 7.32-7.28 (m, 1H), 7.20-7.16 (m, 1H), 6.86-6.81 (m, 2H), 6.64-6.61 (m, 1H), 3.78 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 163.4, 160.6, 160.4, 155.0, 137.6, 133.0, 129.8, 119.9, 109.0, 108.9, 103.3, 55.4; HRMS (ES+) *m/z* calcd for $C_{12}H_{11}N_2O_4S$ $[M+H]^+$ 279.0440, found 279.0432.

(*S*)-*tert*-Butyl 2-(((3-nitropyridin-2-yl)thio)amino)propanoate (Npys-Ala-O*t*Bu, **11**)

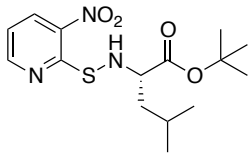


Npys-OPh(*p*F) (**1**, 46 mg, 0.17 mmol, 1.0 equiv.) was added to a solution of HCl·H-Ala-O*t*Bu (31.4 mg, 0.17 mmol, 1.0 equiv.) in dichloromethane (DCM, 1 mL) at room temperature. To this solution was then added DIPEA (112 μL, 0.68 mmol 4.0 equiv.). After stirring for 3 h at this temperature, the mixture was diluted further in $CHCl_3$ (10 mL). The organic layer was washed with 5% citric acid aq., H_2O and brine. The combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure. The resulting residue was purified by flash column chromatography (hexane : ethyl acetate = 5 : 1) to give **11** (48 mg, 0.16 mmol, 91%) as a yellow solid.

^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.74 (dd, *J* = 1.6 and 4.6 Hz, 1H), 8.51 (dd, *J* = 1.6 and 8.2 Hz, 1H), 7.26-7.22 (m, 1H), 4.25 (s, 1H), 3.55-3.53 (m, 1H), 1.51 (s, 9H), 1.41 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.0, 164.2, 153.5, 140.3, 133.6, 119.3, 81.5, 58.9, 28.1 (3 carbons), 18.6; HRMS (ES+) *m/z* calcd for $C_{12}H_{17}N_3O_4NaS$ $[M+Na]^+$ 322.0837, found 322.0834.

Compound **11** was also prepared in the same manner as described using Npys-OPh(*p*MeO) (**2**, 50 mg, 0.18 mmol), DIPEA (120 μL, 0.72 mmol) and HCl·H-Ala-O*t*Bu (34 mg, 0.18 mmol). After stirring for 3, 6 and 12 h, **11** was obtained in NMR yields of 75, 82 and 76%, respectively.

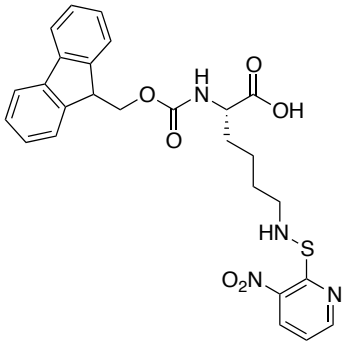
(*S*)-*tert*-Butyl 4-methyl-2-(((3-nitropyridin-2-yl)thio)amino)pentanoate (Npys-Leu-O*t*Bu, **12**)



Compound **12** was prepared in the same manner as described for compound **11** using Npys-OPh(*p*F) (**1**, 50 mg, 0.19 mmol), DIPEA (95 μL, 0.56 mmol) and HCl·H-Leu-O*t*Bu (42 mg, 0.19 mmol).

Yield, 56 mg, 0.16 mmol, 87%; a yellow solid; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.71 (dd, *J* = 1.6 and 4.5 Hz, 1H), 8.50 (dd, *J* = 1.6 and 8.2 Hz, 1H), 7.26-7.22 (m, 1H). 6.91-6.77 (m, 1H), 4.05 (br s, 1H), 3.46-3.43 (m, 1H), 1.93-1.90 (m, 1H), 1.63-1.51 (m, 2H), 1.51 (s, 9H), 0.98-0.94 (m, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 173.2, 164.7, 153.3, 140.1, 133.6, 116.1, 81.3, 63.5, 43.5, 28.1 (3 carbons), 24.7, 22.9, 22.4; HRMS (ES+) *m/z* calcd for $C_{15}H_{23}N_3O_4NaS$ $[M+Na]^+$ 364.1307, found 364.1308.

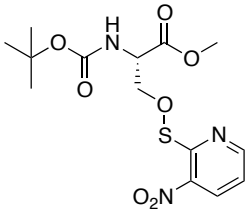
(*S*)-2-((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-6-(((3-nitropyridin-2-yl)thio)amino)hexanoic acid (Fmoc-Lys(Npys)-OH, **13**)



Compound **13** was prepared in the same manner as described for compound **11** using Npys-OPh(*p*F) (**1**, 30 mg, 0.11 mmol), DIPEA (74 μL, 0.45 mmol) and Fmoc-Lys-OH·HCl (45.6 mg, 0.11 mmol).

Yield, 50 mg, 95.7 μmol, 85%; a yellow solid; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.74 (d, J = 3.4 Hz, 1H), 8.48 (dd, J = 1.5 and 8.2 Hz, 1H), 7.74 (d, J = 7.5 Hz, 2H), 7.62-7.50 (m, 2H), 7.42-7.35 (m, 2H), 7.32-7.27 (m, 2H), 7.22-7.18 (m, 1H), 5.39 (d, J = 8.1 Hz, 1H), 4.58-4.30 (m, 3H), 4.21 (t, J = 6.8 Hz, 1H), 2.96-2.93 (m, 3H), 1.93-1.40 (m, 6H); HRMS (ES+) *m*/*z* calcd for $C_{26}H_{26}N_4O_6NaS$ $[M+Na]^+$ 545.1471, found 545.1476.

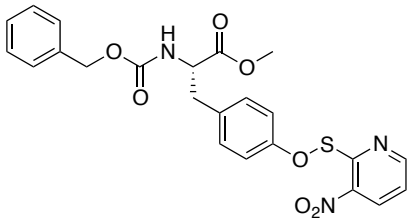
(*S*)-Methyl 2-((*tert*-butoxycarbonyl)amino)-3-(((3-nitropyridin-2-yl)thio)oxy)propanoate (Boc-Ser(Npys)-OMe, **14**)



Compound **14** was prepared in the same manner as described for compound **11** using Npys-OPh(*p*F) (**1**, 58 mg, 0.22 mmol), DIPEA (75 μL, 0.44 mmol) and Boc-Ser-OMe (25 mg, 0.11 mmol).

Yield, 13 mg, 35 μmol, 30%; a yellow solid; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.93 (d, J = 3.1 Hz, 1H), 8.55 (dd, J = 1.6 and 8.4 Hz, 1H), 7.40-7.35 (m, 2H), 4.58 (dd, J = 3.4 and 9.6 Hz, 1H), 4.45-4.42 (m, 1H), 4.09 (dd, J = 2.9 and 9.6 Hz, 1H) 3.81 (s, 3H), 1.52 (s, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.3, 164.4, 155.7, 154.3, 137.5, 133.6, 119.9, 79.9, 78.2, 54.9, 52.7, 28.5 (3 carbons); HRMS (ES+) *m*/*z* calcd for $C_{14}H_{19}N_3O_7NaS$ $[M+Na]^+$ 396.0841, found 396.0841.

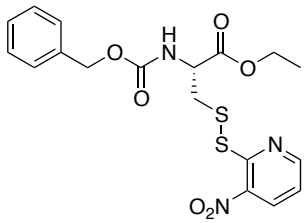
(*S*)-Methyl 2-(((benzyloxy)carbonyl)amino)-3-(4-(((3-nitropyridin-2-yl)thio)oxy)phenyl)propanoate (Z-Tyr(Npys)-OMe, **15**)



Compound **15** was prepared in the same manner as described for compound **11** using Npys-OPh(*p*F) (**1**, 20 mg, 0.075 mmol), DIPEA (50 μL, 0.3 mmol) and Z-Tyr-OMe (24.7 mg, 0.075 mmol).

Yield, 16 mg, 0.033 mmol, 44%; a yellow solid; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.81 (d, J = 3.5 Hz, 1H), 8.52 (dd, J = 1.4 and 8.3 Hz, 1H), 7.35-7.26 (m, 6H), 7.14 (d, J = 8.6 Hz, 2H), 7.00 (d, J = 8.6 Hz, 2H), 5.19 (d, J = 7.9 Hz, 1H), 5.12-5.05 (m, 2H), 4.65-4.60 (m, 1H), 3.71 (s, 3H), 3.12-3.02 (m, 2H); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 171.9, 163.4, 158.5, 155.6, 155.0, 137.6, 136.2, 133.0, 130.8, 130.5, 130.2 (2 carbons), 128.6 (2 carbons), 128.3, 128.1, 119.9, 117.2 (2 carbons), 67.0, 54.8, 52.4, 37.4; HRMS (ES+) *m*/*z* calcd for $C_{23}H_{22}N_3O_7S$ $[M+H]^+$ 484.1178, found 484.1181.

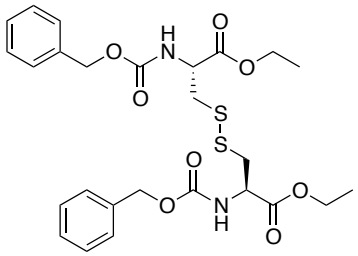
(*R*)-Ethyl 2-(((benzyloxy)carbonyl)amino)-3-((3-nitropyridin-2-yl)disulfanyl)propanoate (Z-Cys(Npys)-OEt, **16**)



Compound **16** was prepared in the same manner as described for compound **11** using Npys-OPh(*p*F) (**1**, 30 mg, 0.11 mmol), DIPEA (74 μL, 0.45 mmol) and Z-Cys-OEt (28 mg, 0.11 mmol).

Yield, 7 mg, 0.016 mmol, 14%; a yellow oil; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.79 (d, J = 4.1 Hz, 1H), 8.47 (d, J = 8.2 Hz, 1H), 7.50-7.27 (m, 5H), 7.25-7.20 (m, 1H), 6.88 (d, J = 7.5 Hz, 1H), 5.12 (s, 2H), 4.65-4.62 (m, 1H), 4.18 (q, J = 7.1 Hz, 2H), 3.57 (dd, J = 4.9 and 14 Hz, 1H), 3.25 (dd, J = 4.7 and 14 Hz, 1H), 1.26 (t, J = 7.2 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.4, 157.2, 156.1, 154.0, 142.8, 136.4, 134.1, 128.7 (2 carbons), 128.4 (2 carbons), 128.3, 121.2, 67.3, 62.1, 53.4, 42.0, 14.3; HRMS (ES+) *m*/*z* calcd for $C_{18}H_{20}N_3O_6S_2$ $[M+H]^+$ 438.0794, found 438.0792.

(2*R*,2'*R*)-Diethyl 3,3'-disulfanediylbis(2-(((benzyloxy)carbonyl)amino)propanoate) ((Z-Cys-OEt)$_2$, **17**)



Compound **17** was prepared in the same manner as described for compound **11** using Npys-OPh(*p*F) (**1**, 30 mg, 0.11 mmol), DIPEA (74 μL, 0.45 mmol) and Z-Cys-OEt (28 mg, 0.11 mmol).

Yield, 21 mg, 0.037 mmol, 33%; a white solid; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.35-7.30 (m, 10H), 5.69 (d, J = 7.4 Hz, 2H), 5.11 (s, 4H), 4.70-4.55 (m, 2H), 4.32-4.15 (m, 4H), 3.25-3.00 (m, 4H), 1.28 (t, J = 7.1 Hz, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.4 (2 carbons), 155.9 (2 carbons), 136.3 (2 carbons), 128.7 (4 carbons), 128.4 (2 carbons), 128.3 (4 carbons), 67.3 (2 carbons), 62.2 (2 carbons), 53.6 (2 carbons), 41.5 (2 carbons), 14.3 (2 carbons); HRMS (ES+) *m*/*z* calcd for $C_{26}H_{33}N_2O_8S_2$ $[M+H]^+$ 565.1678, found 565.1677.

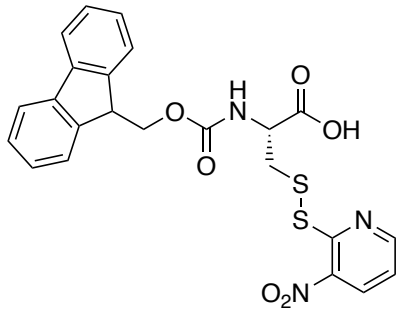
Stability experiment

Npys-Cl (5 mg) and Npys-OPh(*p*F) (**1**) (5 mg) were stored under shelf and light-shielded conditions at room temperature for 0, 1, 3 and 7 days. The compound was dissolved in $CDCl_3$ (1 mL). Then ^1H NMR charts were measured. After that, the yield of Npys dimer, which is the product of decomposition of compounds Npys-Cl and Npys-OPh(*p*F) (**1**), was determined and calculated with the following formula.

Formula: the yield of Npys dimer (%) = 2 x peak area of Npys dimer / (2 x peak area of Npys dimer + peak area of Npys derivatives)

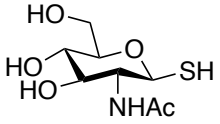
Chapter 2

N-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-*S*-((3-nitropyridin-2-yl)thio)-*L*-cysteine, Fmoc-Cys(Npys)-OH



In a typical experiment, 4-fluorophenyl 3-nitro-2-pyridinesulfenate (Npys-OPh(*p*F), 40.0 mg, 0.15 mmol, 1.2 equiv.) was added to a solution of Fmoc-Cys(*t*-Bu)-OH (50.0 mg, 0.13mmol, 1.0 equiv.) in 4 mL of 90% HCOOH with 10% H_2O and the mixture was stirred at 0 ºC for 30 min. The solvent was removed by lyophilization. And the residue was purified by silica gel preparative layer chromatography with $CHCl_3$: MeOH = 3 : 1 to give the desired product (59.7 mg, 0.12mmol, 96% yield) as yellow solid. ^{1}H NMR (MeOD, 400 MHz) δ 8.71 (d, *J* = 4.14 Hz, 1H), 8.53 (d, *J* = 8.16 Hz, 1H); 7.78 (d, *J* = 7.47 Hz, 2H), 7.67 (d, *J* = 5.77 Hz, 2H), 7.37 (t, *J* = 14.24 Hz, 3H), 7.30-7.29 (m, 2H), 4.43-4.39 (m, 1H), 4.33 (t, *J* = 16.56 Hz, 2H), 4.22 (d, *J* = 13.12 Hz, 1H), 3.37-3.34 (m, 1H), 3.29-3.17 (m, 1H); HRMS (ES+) *m/z* calcd for $C_{23}H_{19}N_3O_6NaS_2$ $[M+Na]^+$ 520.0613, found 520.0615.

1-Thio-2-acetamido-2-deoxy-β-D-glucopyranose (**25**)



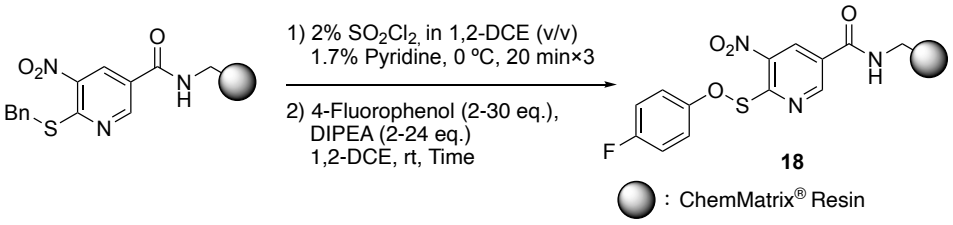
Compound **25** was synthesized by the previously reported method [57] with minor modifications. NaOMe (26.66 mg, 0.494 mmol) was added to a solution of 1-thioacetyl-3,4,6-tri-*O*-acetyl-2-deoxy-2-acetamido-β-D-glucopyranoside (50 mg, 0.123 mmol) in MeOH. After stirring overnight at room temperature, the reaction was quenched with Amberlyst® 15 ion-exchange resin to acidify it to pH 2-3. After the filtration to remove the resin, the solvent was removed in *vacuo*. **25** was obtained as a white solid (25.69 mg, 0.434 mmol, 88% yield) without further purification. ^{1}H NMR (400 MHz, D_2O) δ 4.70-4.67 (m, 1H), 3.91-3.88 (m, 1H), 3.78-3.73 (m, 2H), 3.50-3.48 (m, 3H), 2.06 (s, 3H).

3. Synthesis of Npys-OPh(*p*F) Resin (18)

6-Benzylthio-5-nitronicotinyl (Npys-Bn) resin was synthesized by the previously reported method [53]. Npys-Bn Resin (1 eq.) was added to a solution of 2% (v/v) SO_2Cl_2 in 1,2-DCE in the presence of 1.7 % pyridine (v/v) at 0 ºC. After vortex stirring for 20 min at 0 ºC, the solution was filtered under a gentle stream of nitrogen (The above steps are triplications). Then, the resin was sequentially washed with ice-chilled CH_2Cl_2 five times and ice-chilled 1,2-DCE thrice under a gentle stream of nitrogen to give the Npys-Cl resin. Next, the Npys-Cl resin was instantly mixed with 4-fluoropheol (2-30 eq.) and *N*, *N*-diisopropylethylamine (DIPEA, 2-24 eq.) in the presence of 1,2-DCE and stirred by vortex mixing for 10 min followed by filtration. The resin was sequentially washed with CH_2Cl_2 (x 5) and MeOH (x 3). The resin was dried in *vacuo* to obtain the resin **18**.

In a typical experiment (**Table 15**, Entry 2), Npys-Bn Resin (30.0 mg, 15.9 μmol) was added to a solution of 2% SO_2Cl_2 in 1,2-DCE (736 μL) in the presence of pyridine (12.98 μL, 160.7 μmol). After obtaining the Npys-Cl resin, it was instantly mixed with 4-fluoropheol (17.82 mg, 160.0 μmol) and *N*, *N*-diisopropylethylamine (DIPEA, 22.16 μL, 127.2 μmol) in the presence of 1,2-DCE (1.8 mL). Then, the resin was dried in *vacuo* to obtain the resin **18** (28.42 mg, 11.3 μmol, yield: 75%). Elemental analysis of resin **18** indicated that the formula was $C_{196.62}H_{354.44}N_6O_{91.11}S_2F_2$ (Calcd: C, 54.08; H, 8.20; N, 1.93; S, 1.47; F, 0.87; Found: C, 52.12; H, 7.82; N, 2.19; S, 1.11; F, 0.65)

Table 15. Evaluation of fluorine content in synthesized Npys-OPh(*p*F) resin



Entry	DIPEA	4-Fluorophenol	Time	Conversion[a]
1	2 eq.	2 eq.	10 min	34%
2	8 eq.	10 eq.	10 min	75%
3	24 eq.	30 eq.	10 min	64%
4	8 eq.	10 eq.	1 h	54%

a. Conversions were calculated from the content of fluorine atom on resin **18**.

Synthesis of the peptide fragment

H-Asn-Cys(*t*-Bu)-Pro-Leu-Gly-NH_2 (19)

Peptide **19** was prepared in the same manner as previous reported [53] using Fmoc-Rink amide resin (500 mg, 0.29 mmol). The crude was purified by reversed-phase HPLC to give peptide **19** (53 mg, 78.9 μmol, 27% yield). HRMS (ES+) *m/z* calcd for $C_{24}H_{44}N_7O_6S$ $[M+H]^+$ 558.3074, found 558.3076.

Fmoc-Cys-Tyr-Ile-Gln-OH (21).

Peptide **21** was prepared in the same manner as previous reported [53] using Wang-resin (500 mg, 0.355 mmol). The crude was purified by reversed-phase HPLC to give peptide **21** (50 mg, 66.8 μmol, 19% yield). HRMS (ES+) *m/z* calcd for $C_{38}H_{46}N_5O_9S$ $[M+H]^+$ 748.3016, found 748.3016.

Ac-Phe-Cys(*t*-Bu)-Ser-Thr-Phe-NH_2 (23)

A 20% piperidine/DMF solution was added to the Fmoc-Rink amide resin (300 mg, 0.135 mmol) in a reaction vessel. After removing the 20% piperidine/DMF solution, the peptide chains were elongated by treatment with Fmoc-amino acid (3 eq.), *N*, *N*′-diisopropylcarbodiimide (DIPCI, 3 eq.), and 1-hydroxybenzotriazole (HOBt, 3 eq.) for 2–3 h. These reactions were repeated to lengthen the desired peptide. Then, acetic anhydride (Ac_2O, 5 eq.) with DIPEA (5 eq.) in DMF was added to the resins and reacted for 30 min at room temperature. The synthesized resins were treated with TFA: 1,3-dimethoxybenzene (DMB): H_2O (40: 1: 2, 8 ml) for 3 h. The crude products were precipitated with Et_2O and washed twice. After drying, the residual solids were purified by reversed-phase HPLC to give the peptide **23** (36.3 mg, 0.052 mmol, 39%). HRMS (ES+) *m/z* calcd for $C_{34}H_{48}N_6O_8S$ $[M+H]^+$ 701.3333, found 701.3334.

Solid-phase disulfide ligation

Synthesis of intermediate disulfide peptide 22 of oxytocin

A solution of H-Asn-Cys(*t*-Bu)-Pro-Leu-Gly-NH_2 (**19**) (0.95 mg, 1.42 μmol, 1.0 eq.) in 0.4 M lithium chloride (LiCl, 4.8 mg) / AcOH (284 μL) was added to Npys-OPh(*p*F) resin **18** (9.9 mg, 5.25 μmol, 3.7 eq.) at room temperature. After vortex stirring for 1 h at room temperature, the reaction mixture was detected and filtered. Resulting peptide-resin **20** was washed with H_2O ten times.

Then, a solution of Fmoc-Cys-Tyr-Ile-Gln-OH (**21**) (0.88 mg, 1.18 μmol, 0.83 eq.) in DMF/H_2O (2:1, 787 μL) was added to the peptide-resin **20** at room temperature. After vortex stirring for 30 min at room temperature, the mixture was filtered and the resin was washed with DMF five times. The filtrate and DMF used for the wash were collected and condensed in *vacuo* to give disulfide peptide **22** (1.07 mg, 0.86 μmol, 73% yield, HPLC purity: 94%). HRMS (ES+) *m*/*z* calcd for $C_{58}H_{79}N_{12}O_{15}S_2$ $[M+H]^+$ 1247.5229, found 1247.5226.

Synthesis of disulfide linked peptide-glycoconjugate 26

Glycoconjugate **26** was prepared in the same manner as described for disulfide peptide **22** using Npys-OPh(*p*F) resin. Ac-Phe-Cys(*t*-Bu)-Ser-Thr-Phe-NH_2 (**23**) (1.53 mg, 2.18 μmol, 1.0 eq.) in 0.4 M LiCl (7.4 mg) / AcOH (436 μL) was added to Npys-OPh(*p*F) resin (20 mg, 8.07 μmol, 3.7 eq.). Resulting peptide-resin was washed with H_2O ten times.

Then, a solution of **25** (0.43 mg, 1.81 μmol, 0.83 eq.) in CH_3CN: 50 mM sodium acetate buffer (2:3, pH = 4.5, 1.2 mL) was added to the peptide-resin. After vortex stirring for 30 min at room temperature, the end point of the reaction was confirmed by thin-layer chromatography. HPLC purification was used to collect the purity and condensed in *vacuo* to give disulfide linked glycoconjugate **26**. (0.9 mg, 0.85 μmol, 47% yield). HRMS (ES+) *m*/*z* calcd for $C_{58}H_{79}N_{12}O_{15}S_2$ $[M+H]^+$ 880.3221, found 880.3224.

Chapter 3

Synthesis of fragment 27 by Npy-sulfenylation on fragment 27a on resin

Peptide **27** (40 μmol) on resin was obtained on automated peptide synthesizer (PRELUDE®). Then, Npys-OPh(*p*F) (10.7mg, 1.0 equiv.) under 0.4 M LiCl/AcOH was added directly on peptide synthesizer PRELUDE®. After reaction for 2 h, the resin with peptide fragment **27a** was washed with DMF and MeOH (automated program) and dried in vacuo. To analyze the crude peptide, the peptide-resin **27a** was treated with TFA cocktail (TFA/water/TIPS, 95:2.5:2.5). The crude peptide was purified by reversed-phase HPLC to give fragment **27** (21.8 mg, 6.7 μmol, 17% yield). HRMS (ESI) *m/z* calcd for $C_{145}H_{239}N_{42}O_{40}S_2$ $[M+H]^+$ 3272.7004, found 3272.7422.

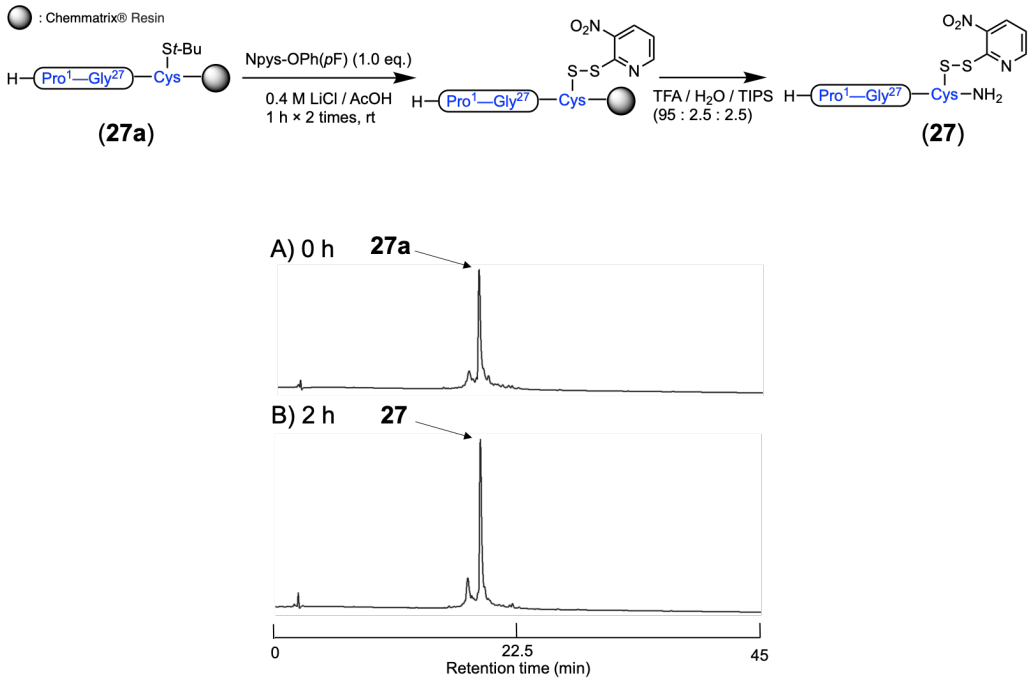


Figure 15. HPLC analysis of the Npy-sulfenylation reaction on fragment **27**. A) 0 h (before reaction) B) 2 h. HPLC conditions are a linear gradient starting from 5% CH_3CN in 0.1% aqueous TFA to 95% CH_3CN in 0.1% aqueous TFA over 45 min at a flow rate 1.0 mL / min and detection at 214 nm.

Synthesis of three peptide fragments 28a, 28b and 29

Peptide fragments were synthesized by (9-fluorenylmethyloxycarbonyl, Fmoc-based solid-phase peptide synthesis) Fmoc-SPPS on PRELUDE® automated peptide synthesizer. After setting the H-Rink amide Chemmatrix resin in a reaction vessel, DMF (10 min x 3) was added to swell. After 30 min, the solvent was removed. Then, the coupling steps were carried out by N_2 bubbling (10 min x 2) using the Fmoc-amino acid (5.0 equiv.)/DMF, 1-hydroxy-7-azabenzotriazole (HOAt) (10.0 equiv.) and *O*-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluoro-phosphate (HATU) (10.0 equiv.)/DMF, and *N,N*-diisopropylethylamine (DIEA) (20.0 equiv.)/DMF, respectively. Double coupling could be carried out when needed (for high sterically hindered amino acids right after proline). The resin and solutions were mixed by N_2 bubbling (30 min). By repeating Fmoc-deprotection and coupling reaction steps, the peptide-resins with protecting groups were obtained. After washing the resin with DMF and MeOH, the cleavage and deprotection steps was carried out by TFA cocktail 8 mL for 3.0 h. Then the TFA solutions were concentrated by pure N_2. The crude peptide was then precipitated with cold Et_2O and then centrifuged (two times). Finally, the crude peptide was dissolved in H_2O (0.1% TFA) /CH_3CN and purified by preparative RP-HPLC.

Peptide fragment **28a:**

(SH)-DD[30] TVLEEMNLPG[40] KWKPKMIGGI[50] GGFIKVRQYD[60] QIPVEIAGHC(*t*-Bu)[70] (**28a**)

Fragment **28a** was prepared in the above-mentioned automated peptide synthesizer (PRELUDE®) using H-Rink-amide Chemmatrix resin (81 mg, 40 μmol) and TFA cocktail (TFA/water/TIPS/EDT, 94:2.5:1:2.5). The crude was purified by reversed-phase HPLC to give fragment (28.5 mg, 5.9 μmol, 15% yield). HRMS (ESI) *m/z* calcd for $C_{217}H_{347}N_{56}O_{60}S_4$ $[M+H]^+$ 4825.4706, found 4825.4697.

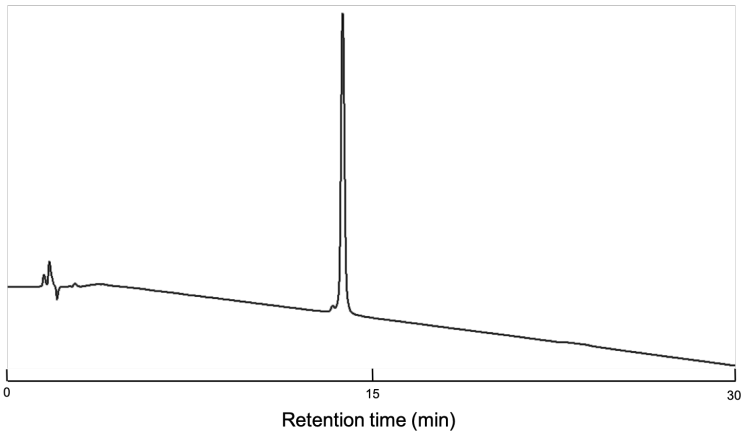


Figure 16. HPLC analysis of the purity of fragment **28a**. HPLC purity is 99%. HPLC conditions are a linear gradient starting from 5% CH_3CN in 0.1% aqueous TFA to 65% CH_3CN in 0.1% aqueous TFA over 30 min at a flow rate 1.0 mL / min and detection at 214 nm.

Peptide fragment **28b:**

(SH)-DD^{30} TVLEEMNLPG^{40} KW(Mts)KPKMIGGI^{50} GGFIKVRQYD^{60} QIPVEIAGHC(*t*-Bu)^{70} (**28b**)

Fragment **28b** was prepared in the above-mentioned automated peptide synthesizer (PRELUDE®) using H-Rink-amide Chemmatrix resin (89 mg, 40 µmol) and TFA cocktail (TFA/water/TIPS/EDT, 94:2.5:1:2.5). The crude was purified by reversed-phase HPLC to give fragment (32.3 mg, 6.5 µmol, 16% yield). HRMS (ESI) *m/z* calcd for $C_{226}H_{357}N_{56}O_{62}S_5$ $[M+H]^+$ 5007.5107, found 5007.5122.

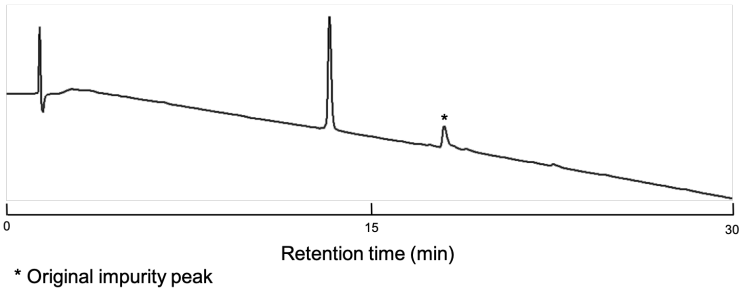


Figure 17. HPLC analysis of the purity of fragment **28b**. HPLC purity is 96%. HPLC conditions are a linear gradient starting from 5% CH_3CN in 0.1% aqueous TFA to 95% CH_3CN in 0.1% aqueous TFA over 45 min at a flow rate 1.0 mL / min and detection at 214 nm.

Peptide fragment **29:**

(SH)-AIGTVLVGPT^{80} PVNIIGRNLL^{90} TQIGATLNF^{99} PISPIETVPV^{109} RRRRRR^{115} (**29**)

Fragment **29** was prepared in the above-mentioned automated peptide synthesizer (PRELUDE®) using H-Rink-amide Chemmatrix resin (89 mg, 40 μmol) and TFA cocktail (TFA/water/TIPS/EDT, 94:2.5:1:2.5). The crude was purified by reversed-phase HPLC to give fragment (22.0 mg, 4.4 μmol, 11% yield). HRMS (ESI) *m/z* calcd for $C_{226}H_{357}N_{56}O_{62}S_5$ $[M+H]^+$ 5018.9158, found 5018.9141.

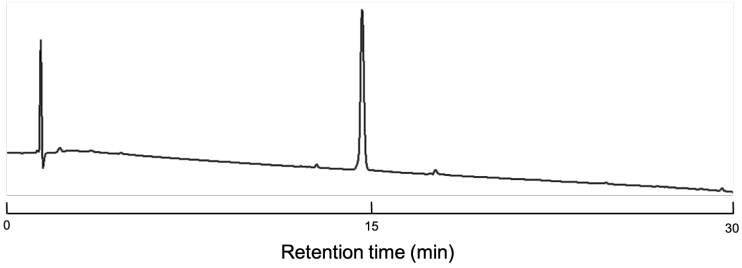
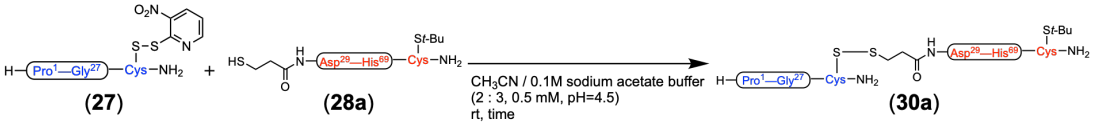


Figure 18. HPLC analysis of the purity of fragment **29**. HPLC purity is 97%. HPLC conditions are a linear gradient starting from 5% CH_3CN in 0.1% aqueous TFA to 95% CH_3CN in 0.1% aqueous TFA over 45 min at a flow rate 1.0 mL / min and detection at 214 nm.

Disulfide ligation between fragments 27 and 28a

After obtaining the purity of peptide fragment **27** (6.0 mg, 1.57 μmol, 1.0 equiv.), peptide fragment **28a** (8.5 mg, 1.57 μmol, 1.0 equiv.) were added in CH_3CN/0.1 M sodium acetate buffer (pH = 4.5) (3.2 mL, 0.5 mM) for a measured time. An aliquot of the peptide solution (5 μL) was diluted with H_2O (45 μL). Then 20 μL of the reaction solution, which was strictly defined with injection loop volume, was analyzed by reversed-phase HPLC with detection by UV spectroscopy (214 nm). After reaction of 24 h, the crude peptide was purified by reversed-phase HPLC to give fragment **30a** (4.0 mg, 0.5 μmol, 32% yield). HRMS (ESI) *m/z* calcd for $C_{357}H_{582}N_{96}O_{98}S_5$ $[M+H]^+$ 7941.2034, found 7941.0679.



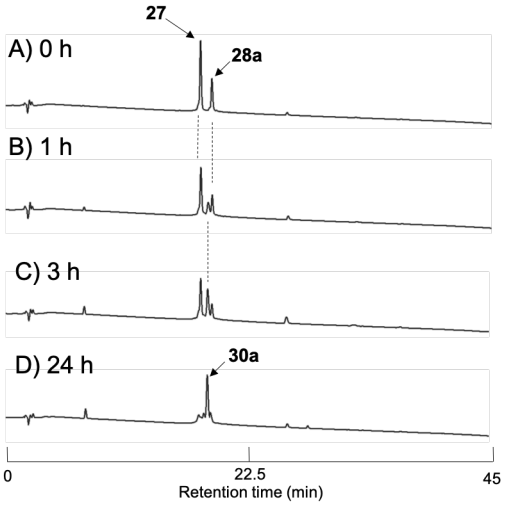


Figure 19. HPLC analysis of the disulfide ligation between fragments **27** and **28a**. A) 0 h (before reaction) B) 2 h C) 3 h D) 24 h. HPLC conditions are a linear gradient starting from 5% CH_3CN in 0.1% aqueous TFA to 95% CH_3CN in 0.1% aqueous TFA over 45 min at a flow rate 1.0 mL / min and detection at 214 nm.

Enzymatic digestion of fragments 34 and 34b

Peptide solution (50 μM) of fragments **34** and **34b** was incubated in Lys-C solution (20 μg/mL) in 10 mM PBS buffer (pH 7.4) at 37 °C for 4 h, respectively. De-salted with ZipTip U-C18 (Millipore Co.), analyzed using MALDI-TOF MS.

Disulfide ligation between fragments 27 and 28b

After obtaining the purity of peptide fragment **27** (51.4 mg, 13.4 μmol, 1.0 equiv.), peptide fragment **28b** (74.57 mg, 13.4 μmol, 1.0 equiv) were added in CH_3CN/0.1 M sodium acetate buffer (pH = 4.5) (26.8 mL, 0.5 mM) for a measured time. An aliquot of the peptide solution (5 μL) was diluted with H_2O (45 μL). Then 20 μL of the reaction solution, which was strictly defined with injection loop volume, was analyzed by reversed-phase HPLC with detection by UV spectroscopy (214 nm). After reaction of 24 h, the crude peptide was purified by reversed-phase HPLC to give fragment **30b** (72.26 mg, 8.90 μmol, 66% yield). HRMS (ESI) *m/z* calcd for $C_{366}H_{591}N_{96}O_{100}S_6$ $[M+H]^+$ 8123.2436, found 8123.2441.

Disulfide ligation between fragments 29 and 30b

Fragment **30b** (14.8 mg, 1.60 μmol, 1.0 equiv.) was reacted with Npys-OPh(*p*F) (0.425 mg, 1.60 μmol, 1.0 equiv.) in acetic acid to obtain Npys-containing fragment **31b**. After stirred for 24 h, the reaction solvent was removed by lyophilization, the residue was used in the next step without further purification. Peptide fragment **29** (18.6 mg, 3.2 μmol, 2.0 equiv.) were directly added in CH_3CN/0.1 M sodium acetate buffer (pH = 4.5) (3.2 mL, 0.5 mM) at rt. After reaction of 5 h and lyophilization, the crude peptide was purified by reversed-phase HPLC to give polypeptide **32** (8.02 mg, 0.61 μmol, 38% yield). HRMS (ESI) *m/z* calcd for $C_{585}H_{966}N_{167}O_{158}S_7$ $[M+H]^+$ 13083.0733, found 13083.1133.

Deprotection of polypeptide 32 to form monomer of HIV-1 protease analogue 33

The resulting polypeptide **32** (7.72 mg, 0.52 μmol) was treated with 1 M trimethylsilyl trifluoromethanesulfonate (TMSOTf)/TFA (2 mL) in the presence of diphenyl sulfide (Ph-S-Ph, 480 μL) and *m*-cresol (148 μL) at 0 °C for 2 h [65]. Then, ice-chilled Et_2O was added. The precipitate was collected by centrifugation and dried in vacuo. The crude was purified by reversed-phase HPLC to give monomer of HIV-1 protease analogue **33** (3.35 mg, 0.23 μmol, 44%) as a white solid. HMRS (ESI) *m/z* calcd for $C_{576}H_{956}N_{167}O_{156}S_6$ $[M+H]^+$ 12901.0332, found 12901.0625.

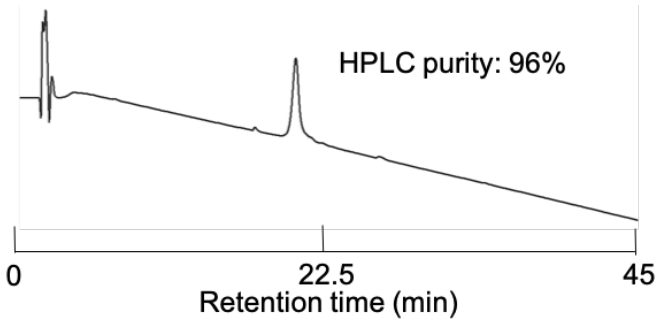


Figure 20. HPLC analysis of the purity of polypeptide **33**. HPLC conditions are a linear gradient starting from 5% CH_3CN in 0.1% aqueous TFA to 95% CH_3CN in 0.1% aqueous TFA over 45 min at a flow rate 1.0 mL / min and detection at 214 nm.

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LIST OF PUBLICATIONS

This list is up to date at the present time (January 2022).

Publications in journals as research papers:

1) **Yan Cui**, Cédric Rentier, Akihiro Taguchi, Kentaro Takayama, Atsuhiko Taniguchi, Yoshio Hayashi, 4-Fluorophenyl 3-nitro-2-pyridinesulfenate as a practical protecting agent for amino acids, *J. Pep. Sci.*, **24**, e3070 (2018).

2) **Yan Cui**, Akihiro Taguchi, Kiyotaka Kobayashi, Hayate Shida, Kentaro Takayama, Atsuhiko Taniguchi, Yoshio Hayashi, Use of solid-supported 4-fluorophenyl 3-nitro-2-pyridinesulfenate in the construction of disulfide- linked hybrid molecules, *Org. Biomol. Chem.*, **18**, 7094-7097 (2020).

Reference papers:

1) Kiyotaka Kobayashi, Akihiro Taguchi, **Yan Cui**, Hayate Shida, Kyohei Muguruma, Kentaro Takayama, Atsuhiko Taniguchi, Yoshio Hayashi, "On-resin" disulfide peptide synthesis with methyl 3-nitro-2-pyridinesulfenate, *Eur. J. Org. Chem.*, **2021**, 956-963 (2020).

2) Akihiro Taguchi, Kiyotaka Kobayashi, **Yan Cui**, Kentaro Takayama, Atsuhiko Taniguchi, Yoshio Hayashi, Disulfide-driven cyclic peptide synthesis of human endothelin-2 with a solid-supported Npys-Cl, *J. Org. Chem.*, **85**, 1495-1503 (2020).

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