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

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To construct disulfide-linked hybrid molecules systematically and efficiently, we established a more practical solid-phase disulfide ligation (SPDSL) system with enhanced utility. The group Npys-OPh(*p*F) shows reactivity similar to that of Npys-Cl, but it is more stable. An efficient synthesis of the cyclic peptide oxytocin and a peptide–sugar conjugate was accomplished as models. These results indicate that the Npys-OPh(*p*F) resin functions as a common synthetic platform in SPDSL.

The disulfide bond is an important bond which stabilizes three-dimensional structures and thus maintains the biological functions of many peptides and proteins. It is also important in the construction of valuable hybrid molecules.[1] Two thiol-containing units in bioactive molecules such as peptides, proteins, sugars, nucleic acids or drugs can be selectively connected by a disulfide link, and since the resultant hybrid molecules express bifunctional actions, they can highly regulate the biological systems in a coordinated manner. Hybrid molecules have been used extensively in recent life science research programs, including drug discovery.[2]

There is however no integrated system built on the basis of a common synthetic platform for the efficient construction of disulfide-linked hybrid molecules. Thus, we focused on some properties of the thiol protection afforded by the 3-nitro-2-pyridinesulfenyl (Npys) group. This protective group was originally developed by Matsueda and Aiba in 1978 and their first reagent for protection was Npys chloride (Npys-Cl).[3] Npys-protected cysteine (Cys(Npys)) is prepared with Npys-Cl and it is highly reactive to unprotected thiol groups, with which it forms the corresponding disulfide product.[4] Focusing on the chemoselectivity of the Npys group, we developed a one-pot solid-phase disulfide ligation (SPDSL) system. This system uses the Npys-Cl resin as a key chemical (Fig. 1)[5] and involves the two-step reactions shown in Fig. 1B. The two steps include the following: (1) the *t*-Bu protected Cys-containing derivative A is loaded onto the Npys-Cl resin (**1**) *via* an active disulfide bond, and (2) component A on the resin is readily transferred to another unprotected SH-containing component B by a rapid and selective disulfide exchange reaction, resulting in the formation of a disulfide conjugate (A-S-S-B). This strategy overcomes drawbacks in the usual solution strategy such as tedious purifications. This SPDSL strategy has been successfully applied to disulfide-driven cyclic peptide synthesis (DdCPS) as a means of synthesizing cyclic disulfide peptides.[5] In this DdCPS, after the preparation of the disulfide peptide, the entropically advantageous intramolecular amide bond formation can be achieved with the proximity effect of each reaction point, and affords a promising method for the synthesis of cyclic multi-disulfide peptides.[5b] This SPDSL strategy has also achieved an efficient synthesis of peptide–drug conjugates.[6] However, a major drawback of the system is that the Npys-Cl resin (**1**) is unstable in the presence of light or moisture and tends to dimerize even at low temperatures.[7] Consequently, it must be prepared from its Bn form immediately prior to use.

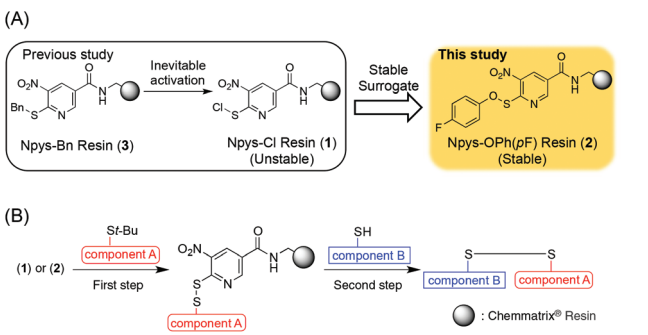

Fig. 1 Development of the novel Npys-OPh(*p*F) resin (**2**) to replace the unstable Npys-Cl resin (**1**) (A) and a schematic illustration of the Npys-mediated solid-phase disulfide ligation (SPDSL) strategy (B).

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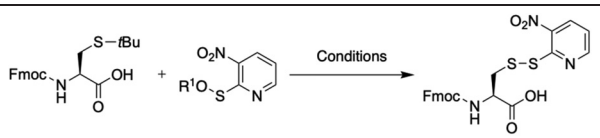
Since we had recently synthesized various alkyl and aryl 3-nitro-2-pyridinesulfenates (Npys-OR) in order to develop a new oxidative agent for the intramolecular disulfide formation,[8] we examined Npys-OR, which can be adapted to the SPDSL strategy in place of Npys-Cl. It was found that the 4-fluoro-phenoxide of Npys, *i.e.*, Npys-OPh(*p*F), can serve as a potential surrogate in the solution reaction. The corresponding Npys-OPh(*p*F) resin (**2**) was synthesized and its ability as a surrogate was evaluated by its use in the synthesis of the mono-cyclic disulfide peptide and a peptide–monosaccharide conjugate with a disulfide linkage. Its physicochemical stability was compared to that of the conventional Npys-Cl resin (**1**).

In the development of a new solid-supported Npys agent applicable to the SPDSL strategy, the interaction of Npys derivatives and S-protected cysteine derivatives is critical. Accordingly, we evaluated the behavior of several newly developed Npys-OR compounds[9] in the conversion reaction in a solution of Fmoc-Cys(*t*-Bu)-OH to an active disulfide-containing Fmoc-Cys(Npys)-OH. When Npys-Cl (1.2 eq.) was used, the desired compound was obtained in 97% yield under the aforementioned reaction conditions with a 90% aqueous HCOOH solution as the solvent. When Npys-OMe (1.2 eq., entry 1), a mild disulfide bond forming agent for unprotected Cys-containing peptides,[8] was used, a moderate yield (57%) was obtained at room temperature (rt) as can be seen in Table 1. Npys-OBn (1.2 eq., entry 2) gave a similar yield (59%) at 0 °C. These results indicate that alkoxides with a pK_a value of ~15 (ref. 10) are less reactive than Npys-Cl. With the use of a better leaving group, *e.g.* phenoxide (entry 3, Npys-OPh, pK_a = 10 (ref. 10)), the yield was improved to 88%. Accordingly, to adjust the reactivity of the phenoxide further, compounds with both electron-donating and electron-withdrawing groups at the *p*-position of the phenyl ring were examined. Introduction of electron-donating methyl or methoxy groups (entries 4 and 5) at this position led to slightly decreased yields (86% and 82%, respectively). This is probably due to the slight increase of the pK_a values of both leaving groups (pK_a: 10.1 and 10.2,[10] respectively) and the reduction of the electrophilic nature of the sulfenate sulfur atom by increased electron donation from the substituted phenyl ring. Conversely, when an electron-withdrawing chlorine or fluorine atom was introduced at the *p*-position (entries 6 and 7; pK_a of *p*-chlorophenol and *p*-fluorophenol = 9.41 and 9.89 (ref. 10) respectively), the yields improved, attaining values similar to those with Npys-Cl (98 and 96%, respectively). Based on these results, we selected *p*-fluorophenyl 3-nitro-2-pyridinesulfenate (Npys-OPh(*p*F)) as a replacement for Npys-Cl.

In an effort to understand the effect of the solvent on the yield of the active disulfide, we examined the reaction in other solvent systems. In glacial acetic acid under anhydrous conditions, the reaction proceeded at rt, giving the product with a good yield (97%, entry 8). However, in dichloromethane (DCM), the reaction proceeded relatively slowly, giving a 66% yield (entry 9) and none of the desired product was obtained under basic conditions with *N*,*N*-diisopropylethylamine (DIPEA) in DCM (entry 10). These results suggest that the reaction proceeds under neutral or acidic conditions and is promoted in the presence of an acid. To further understand the reactivity of Npys-OPh(*p*F) with thiols and other thiol-protecting groups, several Fmoc-Cys(R)-OH derivatives were reacted with Npys-OPh(*p*F) under the formic acidic conditions defined above (Table S1, ESI†). As a result, the thiols protected by *t*-Bu, Acm or 4-MeOBn are suitable for the formation of an active disulfide. Finally, we chose the combination of Npys-OPh(*p*F) and Cys(*t*-Bu) to realize SPDSL in the next experiment, which was performed on a solid-phase system.

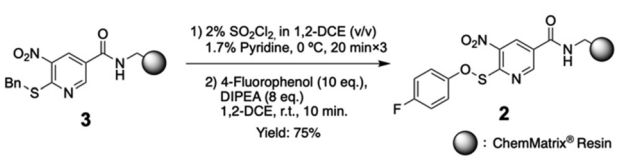
To apply the chemistry of Npys-OPh(*p*F) to the SPDSL strategy, we synthesized a solid-supported form of the compound. As a solid support, a polyethylene glycol-based ChemMatrix® resin,[11] which swells in both organic and aqueous solutions, was chosen. As shown in Scheme 1, the ChemMatrix® resin with 3-nitro-2-benzylthiopyridine-5-carboamine (**3**) was converted to the corresponding Npys-Cl resin by treatment with 2% (v/v) SO_2Cl_2/1,2-DCE in the presence of pyridine.[5] The subsequent reaction with 4-fluorophenol, producing the Npys-OPh (*p*F) resin, was examined. The conversion efficiency was determined from the level of fluorine contained in the resin as determined by elemental analysis. As shown in Table S2 (in

Table 1 Synthesis of Fmoc-Cys(Npys)-OH from Fmoc-Cys(*t*-Bu)-OH and Npys-OR



		Conditions			
Entry	R^{1} [a,b]	Solvent	Temp. (°C)	Time (h)	Isolated yield (%)
1	Me	90% HCOOH aq.	rt	0.5	57
2	Bn	90% HCOOH aq.	0	0.5	59
3	Ph	90% HCOOH aq.	0	0.5	88
4	4-Me-Ph	90% HCOOH aq.	0	0.5	86
5	4-MeO-Ph	90% HCOOH aq.	0	0.5	82
6	4-Cl-Ph	90% HCOOH aq.	0	0.5	98
7	4-F-Ph	90% HCOOH aq.	0	0.5	96
8	4-F-Ph	CH_3COOH	rt	0.5	97
9	4-F-Ph	DCM	0 to rt[c]	12	66
10	4-F-Ph	DCM, DIPEA (2 eq.)	0 to rt[c]	12	N.O.[d]

[a] The amount of Npys-OR is 1.2 equivalents. [b] Fmoc-Cys(Npys)-OH was also synthesized in a yield of 97% with Npys-Cl in 90% HCOOH aq. at 0 °C for 30 min. [c] Reaction conditions: at 0 °C for 1 h and then at rt for 12 h. [d] N.O.: not obtained.



Scheme 1 Synthesis of the Npys-OPh(*p*F) resin (**2**).

the ESI†), the best conversion efficiency of 75% was obtained by a 10 min treatment with 4-fluorophenol (10 eq.) at rt in the presence of DIPEA (8 eq.) as a base in 1,2-dichloroethane (1,2-DCE), reaction conditions similar to those used in the solution-phase synthesis.[9] In this way, the Npys-OPh(*p*F) resin is easily prepared from available solid supports.

To confirm that the Npys-OPh(*p*F) resin is compatible with the SPDSL strategy, a model study was carried out in the synthesis of oxytocin. The resin was used in the synthesis of a disulfide peptide consisting of two oxytocin-derived fragments, the C-terminal Cys-protected 5-mer, H-Asn-Cys(*t*-Bu)-Pro-Leu-Gly-NH_2 (**4**), and the N-terminal Cys-free 4-mer, Fmoc-Cys-Tyr-Ile-Gln-OH (**5**), which are intermediates in the synthesis of oxytocin.[5a] As shown in Scheme 2, the resin was mixed with the Cys(*t*-Bu) peptide (**4**), vortexed for 1–24 h and then washed with CH_3CN and H_2O. The completion of the loading was indicated by the disappearance of the peptide (**4**) from the solution, as monitored by HPLC analysis. As shown in Table 2, 90% aqueous formic acid (entries 1–3) was first used, based on the result from a model study performed in solution (Table 1). With 2.2 eq. of resin, a low loading yield (16%) was observed in a 3 h reaction (entry 1), indicating that the Npys-OPh(*p*F) resin can load the peptide *via* an active disulfide. To optimize the reaction conditions, the amount of resin used was studied (entries 2 and 3) and a high peptide loading yield (93%) was achieved with 7.5 eq. of resin and a vortex time of 24 h (entry 3). The reaction was further improved by the use of acetic acid as a solvent which gave a similar peptide-loading yield (91%) in spite of the decreased quantity (3.7 eq.) of the resin. Under non-acidic conditions, *e.g.* $CH_3CN : H_2O$ (1 : 1) and DMF (entries 5 and 6), the loading was unsatisfactory with yields of 0% and 33%, respectively, but upon the addition of LiCl (entries 7 and 8), the compounds were completely loaded onto 3.7 eq. of resin in 1 h (HPLC chart, see Fig. S1, ESI†).

Since it has been reported that Li salts in an organic solvent can not only change the conformation of a peptide by complexation with an oxygen atom of each amide bond but also can swell the polystyrene resin,[12] these effects might be also involved in the reaction between the peptide and poly-

Table 2 Loading yields of peptide **4** onto the Npys-OPh(*p*F) resin

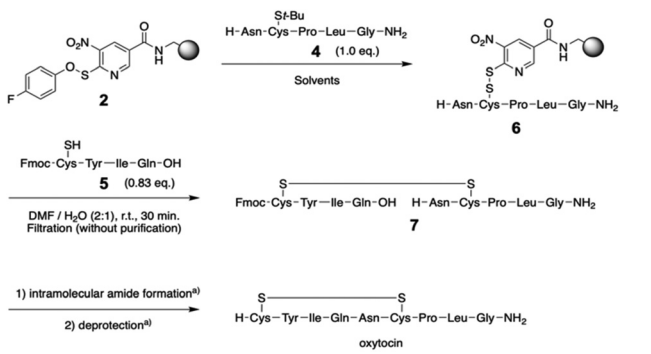
Entry	Solvent	Resin **2** (eq.)	Loading yield of **4**[a] (%) 1 h	3 h	24 h
1	90% formic acid aq.	2.2	0	16	N.T.
2	90% formic acid aq.	3.7	12	33	N.T.
3	90% formic acid aq.	7.5	17	41	93
4	Acetic acid	3.7	24	43	91
5	$CH_3CN : H_2O$ (1 : 1)	3.7	0	0	0
6	DMF	3.7	0	0	33
7	0.4 M LiCl/90% formic acid aq.	3.7	55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

[a] Loading yield (%) = [1 − (HPLC peak area of residual peptide **4**/HPLC peak area of original peptide **4**)] × 100. N.T.: not tested.

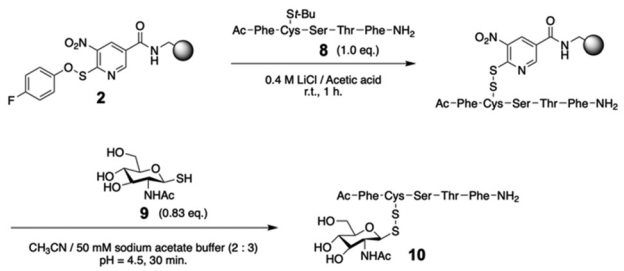
ethylene glycol-based Npys-ChemMatrix resin. Thus, an Npys-OPh(*p*F) moiety can react efficiently with S-protected Cys-containing peptides under acidic conditions on the resin, resulting in the formation of a solid-supported Npys-based active disulfide.

To construct a disulfide peptide, the resulting resin-bound C-terminal peptide fragment was transferred to another Cys-containing N-terminal fragment (**5**), whose N-terminal α-amino group was attached to an Fmoc-protecting group, by vortexing the peptide-resin (**6**) with peptide **5** in DMF : H_2O (2 : 1) for 30 min at rt. The result was complete consumption of peptide **5** and appearance of a single new peak, the desired disulfide peptide (**7**), as observed in the HPLC analysis of the reaction solution (for the HPLC chart, see Fig. S2, ESI†). After removal of the resin by filtration, and with no further purification steps, the disulfide peptide with 94% purity was obtained from the filtrate with an isolated yield of 73%. This result is a formal total synthesis of oxytocin.[5a] As previously reported,[5a] this disulfide peptide can be converted to oxytocin through intramolecular amide formation and subsequent Fmoc deprotection.

To demonstrate the broad versatility of the reaction, the synthesis of an Npys-OPh(*p*F) resin sugar hybrid molecule was conducted. A reported disulfide linked glycopeptide, in which a pentapeptide of a human IgG2 sequence and an *N*-acetylglucosamine derivative are linked by a disulfide bond, was chosen. This compound was developed as a structural mimetic of the natural asparagine glycosylate.[13] A cumbersome solution-phase synthesis of this compound was previously accomplished and included the preparation of activated thioglycoside, and the formation of a disulfide bond between Cys-containing pentapeptide and thioglycoside. This synthetic method of a glycoconjugate required three purification steps. However, as shown in Scheme 3, our Npys-OPh (*p*F) resin-mediated SPDSL of the pentapeptide (Ac-Phe-Cys(*t*-Bu)-Ser-Thr-Phe-NH_2) (**8**) formed a human IgG2 sequence with Cys in place of Asn-297. A subsequent reaction with 2-acetamido-2-deoxy-1-thio-β-D-glucose (**9**) afforded a disulfide-



Scheme 2 Synthesis of a disulfide bond in oxytocin *via* SPDSL with the Npys-OPh(*p*F) resin (**2**). [a] With the final intramolecular amide formation and Fmoc deprotection, oxytocin was successfully obtained.[5a]



Scheme 3 Synthesis of disulfide-linked glycoconjugate **10** *via* SPDSL with the Npys-OPh(*p*F) resin (**2**).

linked glycoconjugate (**10**) with 96% purity and an isolated yield of 47%. In this case, only one final HPLC purification was necessary (see the ESI and Fig. S3 and S4† for details). This result indicates that our SPDSL strategy using the Npys-OPh(*p*F) resin can simplify the procedure, creating a disulfide-linked conjugate not only between peptides but also between sugars, and presumably, drugs, polynucleotides and proteins.

Finally, to demonstrate the usefulness of the Npys-OPh(*p*F) resin, we investigated its long-term stability. As shown in Table S3,† using the Npys-Cl resin after storage for 1 day at rt, the loading of the peptide fragment failed. By contrast, the Npys-OPh(*p*F) resin was largely unchanged after storage for one day at rt, and gradually decomposed in one week (Table S3, Fig. S5 and S6†). However, the Npys-OPh(*p*F) resin was stable at −20 °C for more than 3 months (Table S3†). These results indicate that the stored Npys-OPh(*p*F) resin is more stable than the conventional Npys-Cl resin and the preparation of the Npys-OPh(*p*F) resin immediately prior to its use is unnecessary. This stands in contrast to the behavior of the Npys-Cl resin.

To summarize, we have developed the Npys-OPh(*p*F) resin (**2**), a new Npys-mediated solid-support agent. This new agent (**2**) can be prepared from the ChemMatrix® resin and 3-nitro-2-benzylthio-pyridine-5-carboamine (**3**) with a straightforward method in a short time. In the SPDSL strategy, the Npys-OPh (*p*F) resin is applicable to disulfide bond formation in, for example, the synthesis of oxytocin and disulfide-linked glycoconjugates. Moreover, the agent is more stable upon storage than the conventional Npys-Cl resin (**1**) and as a result, the Npys-OPh(*p*F) resin obviates a laborious activation step necessary when using the Npys-Cl resin. As a useful and stable SPDSL agent, the Npys-OPh(*p*F) resin can contribute to the preparation of a variety of disulfide-linked conjugations useful in peptide science, chemical biology and drug development.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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