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Preparation of Protein Conjugates via Homobifunctional Diselenoester Cross-Linker

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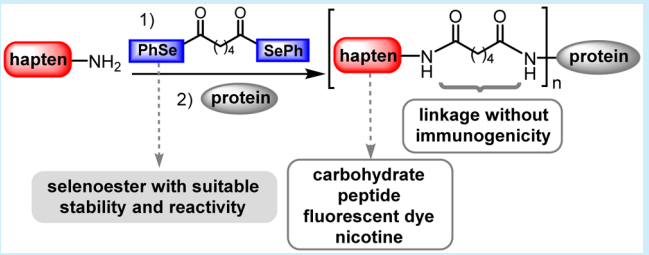
Supporting Information

ABSTRACT: Adipic acid diselenoester was developed as an efficient cross-linker for covalent protein conjugation with a variety of small molecular haptens, including mono- and disaccharides, peptide, fluorescence dye, and nicotine. Compared to the counterparts of *N*-hydroxysuccinimide (NHS) and *p*-nitrophenyl (PNP) linkers, the diselenoester linker demonstrates improved balance between reactivity and stability and coupling of haptens to proteins under mild conditions with high incorporation efficiency.

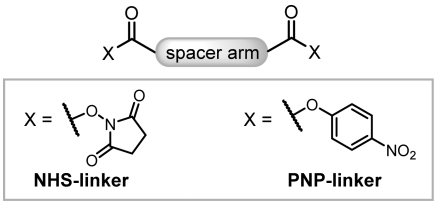


Protein conjugates are widely utilized for synthetic vaccines,[1] therapeutic proteins,[2] antibody−drug conjugates (ADCs),[3] protein immobilization,[4] and generation of highly specific monoclonal antibodies for the purpose of immunochemical analysis, diagnostic tests, and therapeutic treatment, etc.[5]

One of the most popular and readily available tools for preparation of protein conjugates is perhaps the cross-linker. Although the gamut of protein conjugation techniques using the cross-linker has grown considerably over the past decade,[6] an ideal linker that fulfills the following demands remained to be explored. First, the conjugation should be performed under mild conditions to prevent denaturation of protein. Second, high efficiency of conjugation is usually required. In terms of synthetic vaccines, the loading levels of haptens should reach a desired range on carrier proteins to generate effective antibody titers[7] as well as to capture antibodies used in enzyme-linked immunosorbent assay (ELISA) analysis.[8] Meanwhile, some molecular haptens, such as complex oligosaccharides, need challenging multistep syntheses. Thus, it is preferred to consume a minimum amount of precious haptens to meet the demand of loading level. Third, a linker using an aliphatic straight chain as the backbone would be desirable from an immunological point of view, since linkers that contain motifs of heterocycles, such as triazole, squaramide, and 4-(maleimidomethyl)cyclohexane-1-carboxylaten proved to be immunodominant,[9] leading to a reduced immune response to the haptens targeted.

Homobifunctional cross-linkers that symmetrically possess same reactive groups at opposite ends are one of most useful protocols for conjugation.[10] *N*-Hydroxysuccinimide-associated linker (NHS-linker, Scheme 1) stands out as a typical example. However, a common issue involved is the fact that after one end of the NHS-linker reacts with hapten the resulting intermediate of activated ester on the other end lacks adequate stability to withstand purification techniques, tending to hydrolyze and degrade rapidly prior to the next step of cross-linking with protein, resulting in a low level of incorporation.[11] It is worth noting that it that NHS-esters occasionally underwent ring-opening reactions in cases of high steric congestion of the ester carbonyl group or the incoming nucleophile, giving rise to a mixed succinohydroxamic anhydride.[12] Surprisingly, several attempts to prepare amide using NHS-ester suffered from ring-opening as a major reaction, yielding more than 50% side products.[12f,c,g]

Scheme 1. Typical Homobifunctional Cross-Linkers



In 2004, adipic acid *p*-nitrophenyl diester linker (a typical PNP-linker, Scheme 1) was developed by Bundle and co-workers;[13] it exhibits improved stability and permits efficient coupling with free amino groups present in the target proteins. It has been broadly employed for the preparation of neoglycoproteins.[11,14] Unfortunately, compared to NHS-linker, PNP-linker generally resulted in lower hapten loading.[11b] Therefore, an ideal cross-linker possessing optimal balance between stability and reactivity remained to be explored.

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As part of our ongoing investigations of efficient conjugate chemistry for glyco- and protein conjugates,[15] we report herein adipic acid diselenoester **4**, a practical homobifunctional cross-linker, which contains $PhSe^-$ as leaving groups and has improved the balance between reactivity and stability. The design of **4** was inspired by recent reports on selenoester used in peptide ligation.[16] According to our studies, **4** can achieve high efficiency of incorporation with a wide range of small molecules. The intermediate of half ester from **4** allows purification by chromatography column on silica gel. Notably, the backbone of adipic acid was chosen to avoid immunogenicity against the linker.

Our initial studies focused on the reaction progress of 2-phenylethan-1-amine (**2**) with three activated ester **1a**, **1b**, and **1c**, respectively (Scheme 1), under the mixed solvent/buffer conditions [3:1 v/v 10 mM phosphate-buffered saline (PBS) buffer (pH 7.5)/DMF] (Figure 1). The concentrations of

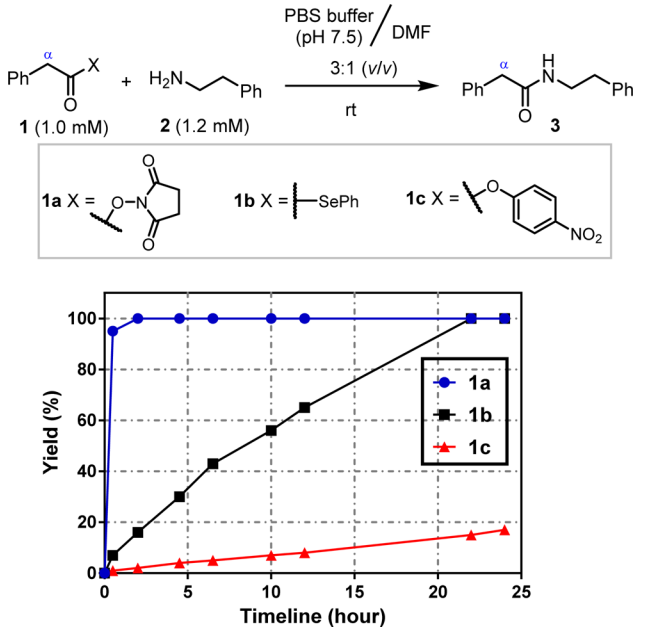
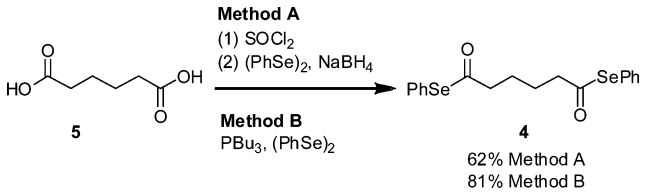


Figure 1. Reaction curves (yields of **3** vs time) of amide bond formation between **1a**–**c** and 2-phenylethan-1-amine (**2**). The yields were estimated by calculations of the analytical HPLC integrations of **1** and **3**.

selenoester and amine were set to be 1.0 and 1.2 mM, respectively, because these concentrations allow reactions be monitored by analytical HPLC and mimic the concentration of lysine residue in proteins to the greatest extent. As shown in Figure 1, as expected, coupling using NHS-ester **1a** proceeded in a quantitative manner within 0.5 h, showing its remarkable reactivity. However, to our surprise, the yield of PNP-ester **1c** is less than 20% after 24 h. The 24-h reaction of selenoester **1b** provided **3** in more than 90% yield. As a result, the order of reactivity of the three active esters is NHS-ester **1a** > selenoester **1b** > PNP-ester **1c**. On the other hand, in the absence of amine **2**, 5 h incubation of three active esters (approximately 10 mM concentration) under the mixed solvent/buffer conditions [1:1 v/v 10 mM PBS buffer (pH 7.5)/DMF] led to 71%, 58%, and 54% (estimated by ^{1}H NMR integrations of α-positions of esters **1a**–**c** and 2-phenylacetic acid; see the Supporting Information) hydrolysis of **1a**, **1b**, and **1c**, respectively. Consequently, in comparison to NHS- and PNP-ester, selenoester demonstrates optimal balance between reactivity and stability.

According to the results noted above, we chose to synthesize adipic acid diselenoester **4** as the cross-linker in our subsequent conjugation studies. As illustrated in Scheme 2, **4** was prepared

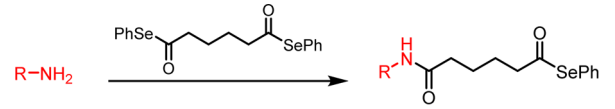
Scheme 2. Synthesis of Homobifunctional Cross-Linker 4

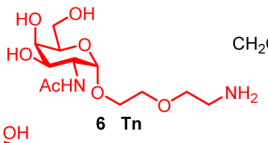
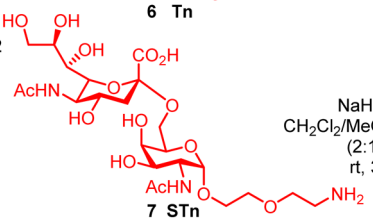
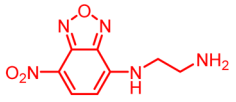
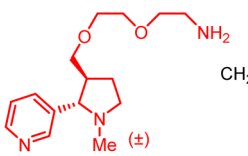


by reduction of diphenyl diselenide with sodium borohydride in THF/MeOH (10:3), followed by reaction with adipoyl chloride derived from adipic acid **5** (treated with thionyl chloride).[17] Alternatively, one-step protocol of treatment of **5** with diphenyl diselenide in the presence of PBu_3 afforded **4** in 81% yield. Remarkably, **4** was stable toward purificationby flash chromatography column on silica gel.

With cross-linker **4** in hand, we embarked on the attachment of **4** to various haptens to prepare half selenoesters (Table 1). Substrate amines of Tn antigen **6**, STn antigen **7**, and nicotine derivative **10** were prepared from the corresponding azides by

Table 1. Synthesis of Half Selenoesters 11–15



entry	$R-NH_2$	conditions	product (yield/%)[a]
1	 **6 Tn**	CH_2Cl_2/MeOH/CH_3CN (2:1:1) rt, 2 h	**11** (67)
2	 **7 STn**	$NaHCO_3$ CH_2Cl_2/MeOH/CH_3CN (2:1:1) rt, 3 h	**12** (48)
3	H_2N-STTSAPTT-COOH **8 MUC5AC**	*i*-Pr_2NEt CH_2Cl_2/MeOH (1:4) rt, 0.5 h	**13** (90)
4	 **9 NBD**	Et_3N DMF/CH_2Cl_2 (1:3) rt, 15min	**14** (61)
5	 **10 nicotine**	CH_2Cl_2/MeOH/CH_3CN (2:1:1) rt, 2 h	**15** (70)

[a]Isolated yields.

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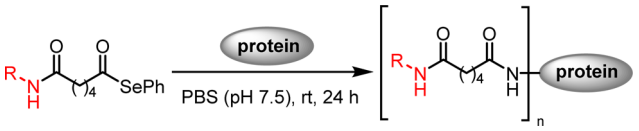
Table 2. Summary of the Loading Efficiency[a]



entry	half selenoester	product	molar ratio of half selenoester to protein	hapten incorporated per protein	hapten incorporation efficiency[b] (%)	loading[c] (%)
1	**11**	Tn−BSA (**16a**)	10:1	n = 8.3 (5[d], 5[e])	83	14
2	**11**	Tn−BSA (**16b**)	20:1	n = 14.6 (10[d], 11[e])	73	25
3	**11**	Tn−BSA (**16c**)	30:1	n = 19.4 (15[d], 13[e])	65	33
4	**12**	STn−BSA (**17**)	30:1	n = 11.6 (4[e])	39	20
5	**13**	MUC5AC−BSA (**18**)	50:1	n = 17.9	36	30
6	**14**	NBD−BSA (**19**)	20:1	n = 14.0	70	24
7	**15**	nicotine−BSA (**20**)	30:1	n = 17.2	59	29
8	**11**	Tn−OVA (**21a**)	10:1	n = 2.0	20	10
9	**11**	Tn−OVA (**21b**)	20:1	n = 4.4	22	22

[a]The concentration of protein is 0.1 mM. [b]The percentage of hapten incorporated over hapten (half selenoester) used. [c]The percentage of lysine in protein incorporated by hapten. Only 30−35 out of the 59 lysine residues of BSA are usually accessible for coupling with haptens;[20] the maximum loading percentage of BSA is 50−59%. There are 20 lysine residues in OVA. [d]Values reported in ref 13 using adipic acid *p*-nitrophenyl diester linker. [e]Value measured experimentally by ourselves using adipic acid *p*-nitrophenyl diester linker.

hydrogenation using Pd/C. Peptide MUC5AC (**8**), a potential target for immunotherapy for pancreatic cancer, was synthesized via linear solid-phase peptide synthesis (see Supporting Information for the preparation of substrates **6**, **7**, **8**, and **10**). The preparation of 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) ethylenediamine **9**, which contains a popular fluorescent moiety, followed the reported procedure.[18] To avoid dimerization, 5 equiv of **4** was used in each entry. In entries 2−4, base had to be added to speed up the reactions. With regard to preparation of **12**, inorganic base $NaHCO_3$ was applied because the initial trial to add trimethylamine led to difficult purification where neither chromatography on silica gel nor size-exclusion column (LH-20) was able to remove this organic base. It is noteworthy that side product (mainly PhSeSePh) together with an excess of **4** are nonpolar as well as insoluble in water and are readily separated from highly hydrophilic half selenoesters of carbohydrate and peptide (such as **11**−**13**) by extraction. For instance, separation of **12** from the residue of reaction through extraction displayed sufficient purity based on the analysis of ^{1}H and ^{13}C NMR spectra. The stability of half selenoester **11** toward hydrolysis in the different pHs was researched. For 8 h incubation in 10 mM PBS, no hydrolysis was observed at pH 6.5 and 7.0; 50% of **11** hydrolyzed at pH 7.5; more than 95% of **11** hydrolyzed at pH 8.0. Owing to the high stabilities, the half selenoesters allow purification by either silica gel chromatography (including **11**, **14**, and **15**) or reversed-phase HPLC (including **13**). In addition, the solution of half selenoester **12** in D_2O was kept in an NMR tube for a week, and no hydrolysis was detected.

The half esters **11**−**15** were then coupled to the protein bovine serum albumin (BSA) by 24 h incubation in 10 mM PBS buffer (pH 7.5) at ambient temperature. The BSA conjugates **16**−**20** were obtained as white powders after dialysis against deionized water followed by freeze-drying. The average level of incorporation was calculated from the increase of the molecular weight of the BSA as determined by MALDI-TOF MS,[19] with sinapinic acid as the matrix. Table 2 summarizes the results obtained for the preparation of protein conjugates **16**−**20**. In comparison with PNP-linker used to prepare Tn−BSA (**16**), **4** displayed improved efficacy of loading on the basis of the values reported[13] and those measured experimentally by our own (Table 2, entries 1−3): on average, 3, 4, and 5 more Tn haptens per BSA were introduced when half selenoester/protein ratios of 10:1, 20:1, and 30:1 were employed, respectively (Figure 2). In particular, the preparation of

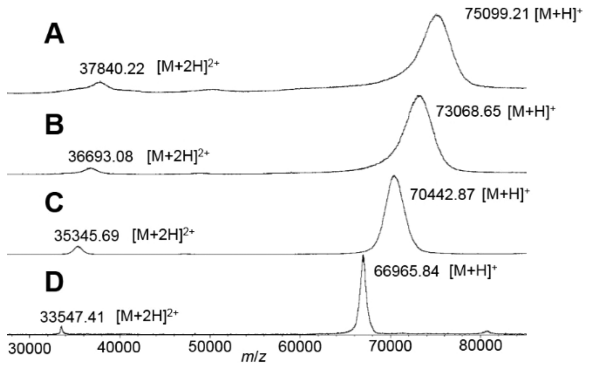


Figure 2. MALDI-TOF spectra of Tn−BSA conjugate **16** formed from different molar ratios of **11**/BSA: (A) molar ratio of **11**/BSA = 30:1, n = 19.4; (B) molar ratio of **11**/BSA = 20:1, n = 14.6; (C) molar ratio of **11**/BSA = 10:1, n = 8.3; (D) BSA calibration standard.

STn−BSA (**12**, Table 2, entry 4) using PNP-linker resulted in less STn loaded than when **4** was employed. Similar to the investigation by Bundle et al.,[13] increasing the amount of half selenoesters per BSA had only a moderate effect on the efficiency of hapten incorporation. We also investigated the conjugation of half selenoester **11** with ovalbumin (OVA), another frequently used carrier protein in synthetic vaccines (Table 2, entry 8 and 9). In the presence of nonglycosylated peptides ionization of glycopeptides (such as OVA) was strongly suppressed,[21] making it challenging to identify the peaks of OVA and Tn−OVA in MALDI-TOF spectra. To our delight, gel electrophoresis of OVA and **21b** showed two well-defined bands (see the Supporting Information). Although this electrophoresis analysis of conjugation cannot give definite information about loading, it narrows down the range of molecular weight in MALDI-TOF spectra and ensures the homogeneity of the conjugate.

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In conclusion, adipic acid diselenoester **4**, a practical homobifunctional cross-linker featuring an improved balance between reactivity and stability, is presented for covalent protein conjugation with high efficacy under mild conditions. The scope of utility of **4** is exemplified by the diversity of small molecular haptens, including monosaccharide (Tn antigen), disaccharide (STn antigen), peptide (MUC5AC), fluorescent dye (NBD), and nicotine. The intermediates of activated selenoesters are of high stability and can be purified by chromatography.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b02568.

Detailed experimental procedures, NMR spectra, and MALDI-TOF MS spectra (PDF)

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Notes

The authors declare no competing financial interest.

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