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Polyfluorophenyl Ester-Terminated Homobifunctional Cross-Linkers for Protein Conjugation

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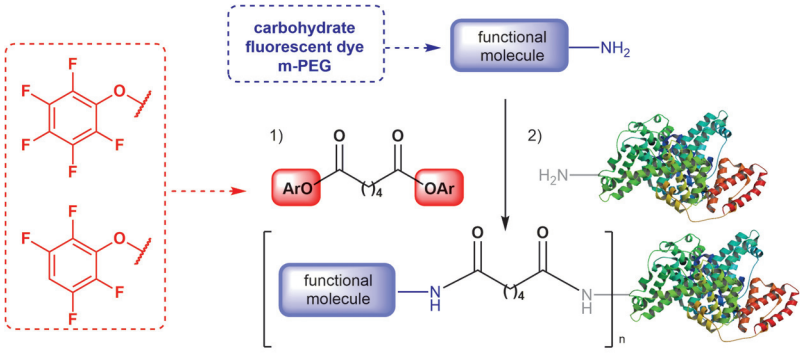

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Abstract Along with *N*-hydroxysuccinimidyl, *p*-nitrophenyl, and phenylseleno esters, tetra- and penta-fluorophenyl esters were comparatively evaluated in term of their reactivity and hydrolytic stability. Their homobifunctional cross-linkers were prepared to conjugate proteins with small molecules, including carbohydrates, fluorescent dyes, and poly(ethylene glycol) monomethyl ether. The conjugations proceeded under mild conditions, affording the corresponding protein conjugates with good efficiency.

Key words protein conjugation, cross-linking, carbohydrates, dyes, polyethylene glycol, linking group

Protein conjugation has a variety of applications in life-science research and assay development.[1] The desirable level of conjugation of small molecules with proteins depends on the particular application. For the preparation of a semi-synthetic vaccine,[2] a high degree of conjugation is needed to obtain multivalency of the hapten and to ensure sufficient immunogenicity, whereas for conjugation with an antibody or an enzyme, a low to moderate degree of conjugation might be optimal to permit retention of the biological activity of the protein. However, a high efficacy of incorporation is always desirable, especially in cases where the small molecule is precious, such as complex oligosaccharides derived from challenging multistep syntheses. Therefore, choosing an effective protein-conjugation strategy is important.[3] Because of their operational and structural simplicity, homobifunctional cross-linkers stand out as valuable tools for bioconjugation practitioners.[4]

The most important component of a homobifunctional cross-linker is its terminal reactive functional group, which establishes the method and mechanism for conjugation. Given the surface availability of amino groups in many proteins, and because lysine is one of most popular handles for conjugation, various activated esters have been synthesized and employed for a wide range of amide-bond-forming conjugations. Homobifunctional *N*-hydroxysuccinimidyl (NHS) esters are widely used, and some are commercially available. Unfortunately, NHS esters have a short half-life, of the order of hours at physiological pH values, which occasionally results in lower conjugation efficiencies.[4] In addition, for homobifunctional cross-linkers, a two-step conjugation procedure is generally required for effective hetero-conjugation without the formation of a homodimer or polymer. But the half-NHS ester, the first-step intermediate, tends to hydrolyze and to degrade rapidly during purification.[5]

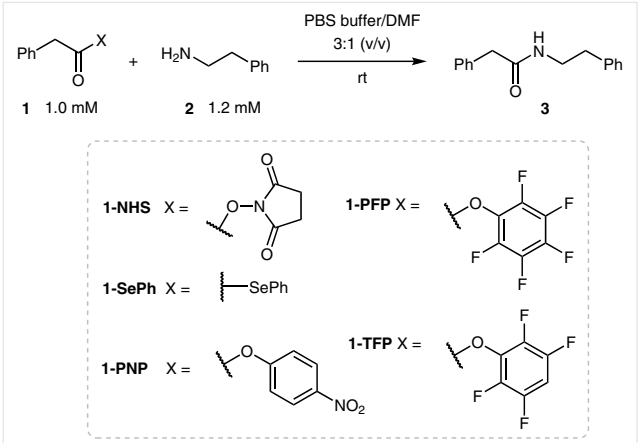
To increase the stability of half-esters, Bundle and co-workers developed a homobifunctional *p*-nitrophenyl (PNP) ester that has been frequently used in syntheses of neoglycoproteins.[6] However, the PNP ester is sometimes too unreactive to achieve a high degree of conjugation. We have described a homobifunctional phenylseleno ester (SePh ester) cross-linker[7] that demonstrates greater hydrolytic stability and greater reactivity, permitting highly efficient

conjugation with carbohydrates or peptides. However, due to its poor solubility in aqueous system, the SePh ester is not suitable for conjugation of hydrophobic molecules, such as aflatoxin B1, a highly toxic contaminant in agricultural commodities.[8] Therefore, there is no 'universal' activated ester that is capable of efficiently conjugating all types of small molecules with proteins. More activated esters need to be comprehensively studied to meet the demands of conjugation in various situations.

As part of a research program toward efficient conjugate chemistry for glycoconjugates and protein conjugates[7,9] and their applications in fully synthetic vaccines,[10] we report the use of homobifunctional cross-linkers terminated by a 2,3,5,6-tetrafluorophenyl (TFP) or a pentafluorophenyl (PFP) ester. Our interest in polyfluorophenyl esters arose from the successful application of TFP and PFP esters in peptide[11] and glycopeptide[12] syntheses. It has been reported that TFP esters are as reactive as NHS esters, but demonstrate greater hydrolytic stability.[13] In addition, Huang's group reported that pentafluorophenol (PFP-OH), the by-product of conjugation, is less nucleophilic than NHS and that PFP-OH does not interfere with the desired product, which is sensitive to nucleophilic attack.[14] In spite of these advantages, however, TFP and PFP esters have been rarely used in protein conjugation, particularly in cross-linkers.

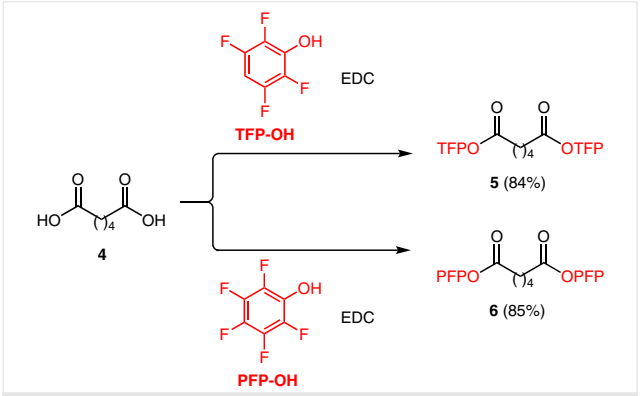
We began our investigation by comparing the reactivities of TFP and PFP esters to those of an NHS ester, a PNP ester, and an SePh ester at various pH values. As shown in Scheme 1, 1-**NHS**, **1-SePh**, **1-PNP**, **1-TFP**, and **1-PFP** were chosen as model compounds, and their reactions with (2-phenylethyl)amine (**2**) under mixed solvent/buffer conditions [3:1 v/v 10 mM phosphate-buffered saline (PBS)/DMF] were examined. **1-TFP** and **1-PFP** showed a greater reactivity than **1-SePh** or **1-PNP**, but less than that of **1-NHS** (Figure 1). **1-PFP** was slightly more reactive than **1-TFP**. On the other hand, the hydrolytic stabilities were also evaluated (see Supporting Information). The order of stability of the five activated esters was **1-NHS** > **1-PFP** > **1-TFP** > **1-SePh** > **1-PNP**.

Collectively, in contrast to NHS and PNP esters, TFP and PFP esters showed a better balance of reactivity and stability, displaying a potential for use in protein conjugation.



Scheme 1 Reaction between phenethylamine (**2**) and the activated esters **1-NHS**, **1-SePh**, **1-PNP**, **1-PFP**, and **1-TFP**

Consequently, we prepared the corresponding cross-linkers **5**[15] and **6**[16] (Scheme 2). An adipic acid backbone was chosen as a spacer because of its nonimmunogenic character.[17]



Scheme 2 Preparation of homobifunctional cross-linkers **5** and **6**

Next, we attempted to connect **5** and **6** to various small molecules to prepare the half-esters **11**–**14** (Table 1). Four substrate amines [Tn antigen (**7**),[7] 3-(acetylamino)-3-deoxy-D-glucopyranosylamine (*N*-GluNAc; **8**),[9a] *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)ethane-1,2-diamine (NBD; **9**),[7]

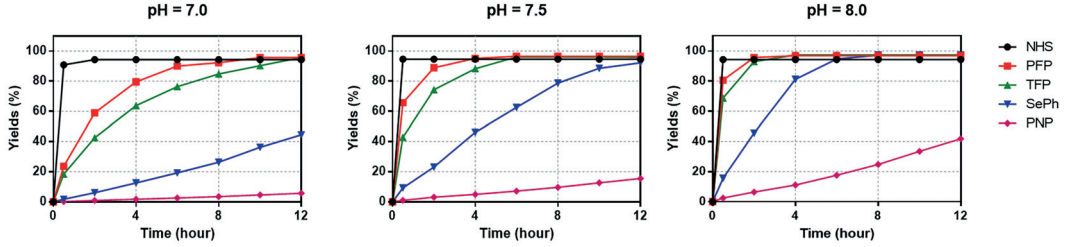
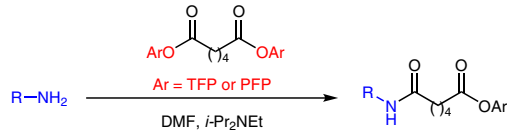
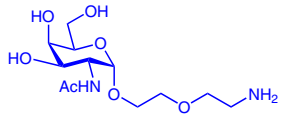
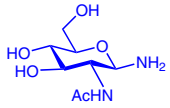
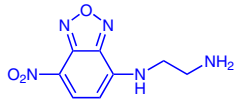
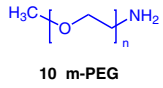


Figure 1 Reaction curves (yields of **3** versus time) of amide bond formation between (2-phenylethyl)amine (**2**) and the activated esters **1-NHS**, **1-SePh**, **1-PNP**, **1-PFP**, and **1-TFP** at various pH values. The yields were estimated by calculation of the analytic HPLC integrations for **1** and **3**. Because of hydrolysis, the highest yield of **3** from **1-NHS** was slightly lower than those from **1-TFP** or **1-PFP**.

Table 1 Synthesis of Half-Esters **11–14**



Entry	R–NH_2	Product	Yield[a] (%)
1	 **7 Tn**	**11-TFP** **11-PFP**	50 47
2	 **8 *N*-GluNAc**	**12-TFP** **12-PFP**	46 52
3	 **9 NBD**	**13-TFP** **13-PFP**	75 74
4	 **10 m-PEG**	**14-TFP** **14-PFP**	78 80

[a] Isolated yield.

and poly(ethylene glycol) monomethyl ether (m-PEG; **10**)[18]] were prepared according to the known procedures and treated with five equivalents of cross-linker **5** or **6** in dry DMF, to give the corresponding half-esters **11–14** in moderate to good yields. The half-esters were purified by either silica gel chromatograph or trituration with Et_2O (see Supporting Information). The moderate yields of the carbohydrate substrates (Table 2, entries 1 and 2) were mainly due to the hydrolysis of esters during purification.

The purified half-esters **11–14** were then coupled with bovine serum albumin (BSA) and ovalbumin (OVA) by incubation for 24 hours in buffer.[19] The average degree of incorporation was calculated from the increase in the molecular weight of the BSA and OVA, as determined by MALDI-TOF MS,[20] with sinapinic acid as the matrix. TFP linker **5** and PFP linker **6** showed a good efficiency, comparable to that of PNP linker but inferior to that of SePh linker, in the preparation of neoglycoprotein Tn-BSA (Table 2, entries 1–3 and 6–8). With regards to PEGylation,[21] the TFP ester performed better than NHS ester (entry 15).[22,23] Unexpectedly, the PFP linker was less effective than SePh linker in the conjugation of hydrophobic NBD derivatives to BSA (entry 13), even though 40% DMF was employed in the PBS buffer.

In summary, the reactivities and stabilities of five types of activated ester were evaluated. Homobifunctional cross-linkers terminated by tetra- or pentafluorophenyl esters showed good efficiency in the preparation of protein conjugates. Under similar conditions, pentafluorophenyl esters, such as **11-PFP** (**15f**) and **11-PFP** (**15g**), showed better incorporation efficiencies than tetrafluorophenyl esters, such as **11-TFP** (**15a**), **11-TFP** (**15b**), **12-PFP** (**16b**), or **12-TFP** (**16a**). The information gained in this study should be helpful in considering the types of cross-linkers that might meet the demands of protein conjugation in various situations.

Funding Information

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Supporting Information

Supporting information for this article is available online at https://doi.org/10.1055/s-0036-1590974.

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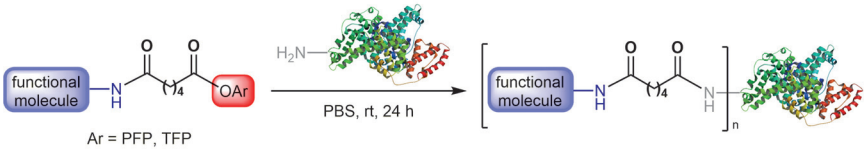
Table 2 Summary of the Loading Efficiency[a]



Entry	Half-ester	Product	pH[b]	Molar ratio[c]	*n*[d]	Efficiency[e] (%)	Loading[f] (%)
1	**11-TFP**	Tn-BSA (**15a**)	7.5	30:1	11.6 (19.4[g], 13[h])	39	20
2	**11-TFP**	Tn-BSA (**15b**)	7.5	20:1	8.1 (14.6[g], 11[h])	41	14
3	**11-TFP**	Tn-BSA (**15c**)	7.5	10:1	6.6 (8.3[g], 5[h])	66	11
4	**11-TFP**	Tn-BSA (**15d**)	7.0	20:1	8.7	43	15
5	**11-TFP**	Tn-BSA (**15e**)	8.0	20:1	8.2	41	14
6	**11-PFP**	Tn-BSA (**15f**)	7.5	30:1	13.3 (19.4[g], 13[h])	44	22
7	**11-PFP**	Tn-BSA (**15g**)	7.5	20:1	9.0 (14.6[g], 11[h])	45	15
8	**11-PFP**	Tn-BSA (**15h**)	7.5	10:1	5.5 (8.3[g], 5[h])	55	9
9	**11-PFP**	Tn-BSA (**15i**)	7.0	20:1	8.3	41	14
10	**11-PFP**	Tn-BSA (**15j**)	8.0	20:1	7.8	39	13
11	**12-TFP**	*N*-GluNAc-BSA (**16a**)	7.5	20:1	7.1	35	12
12	**12-PFP**	*N*-GluNAc-BSA (**16b**)	7.5	20:1	9.8	49	17
13	**13-TFP**	NBD-BSA (**17**)	7.5	20:1	13.3	66	22
14	**13-PFP**	NBD-BSA (**18a**)	7.5	20:1	12.4[i] (14[g])	62	21
15	**13-PFP**	NBD-BSA (**18b**)	7.5	40:1	20.4[i]	51	34
16	**14-TFP**	m-PEG-BSA (**19a**)	7.5	20:1	8.9	44, (21[j])	15
17	**14-TFP**	m-PEG-BSA (**19b**)	7.5	40:1	14.6	36	24
18	**14-PFP**	m-PEG-BSA (**20a**)	7.5	20:1	9.2	46	16
19	**14-PFP**	m-PEG-BSA (**20b**)	7.5	40:1	14.9	37	25
20	**11-TFP**	sTn-OVA (**21a**)	7.5	20:1	3.4 (4.4[g])	17	17
21	**11-PFP**	Tn-OVA (**21b**)	7.5	20:1	2.9 (4.4[g])	14	14
22	**12-TFP**	*N*-GluNAc-OVA (**22a**)	7.5	20:1	2.2	11	11
23	**12-PFP**	*N*-GluNAc-OVA (**22b**)	7.5	20:1	3.7	18	18
24	**14-TFP**	m-PEG -OVA (**23a**)	7.5	20:1	3.8	19	19
25	**14-TFP**	m-PEG -OVA (**23b**)	7.5	40:1	5.8	14	29
26	**14-PFP**	m-PEG-OVA (**24a**)	7.5	20:1	3.3	16	16
27	**14-PFP**	m-PEG-OVA (**24b**)	7.5	40:1	6.0	15	30

[a] Protein concentration 0.1 mM.
[b] pH of PBS.
[c] Molar ratio of half-ester to protein.
[d] Number of small molecules incorporated per protein molecule.
[e] The percentage of small molecules incorporated over the half-ester used.
[f] The percentage of lysine in the protein incorporating the small molecule. Only 30–35 of the 59 lysine residues of BSA are usually accessible to coupling by small molecules; the maximum loading percentage of BSA is 50–59%. There are 20 lysine residues in OVA.
[g] Value obtained by using adipic acid phenylselenyl diester cross-linker.[7]
[h] Value obtained by using adipic acid *p*-nitrophenyl diester cross-linker.[7]
[i] The half-esters were dissolved in DMF (0.8 mL) and added to PBS (1.2 mL).
[j] Value obtained by using an NHS ester.[21] The degree of PEGylation was determined by the trinitrobenzenesulfonic acid method.

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(15) **Bis(2,3,5,6-tetrafluorophenyl) Adipate (5); Typical Procedure**
Adipic acid (1.00 g, 6.84mmol) and EDC (3.15 g, 16.8 mmol) were dissolved in dry CH_2Cl_2 (50 mL), and the solution was allowed cool to 0 °C in an ice bath. 2,3,5,6-tetrafluorophenol (2.50 g, 15.1 mmol) was added, and the solution was warmed to rt and stirred for 6 h. After removal of the solvent under reduced pressure, the crude product was purified by flash column chromatography [silica gel, PE–CH_2Cl_2 (1:1)] to give a white solid; yield: 2.54 g (84%); mp 99.1–99.3 °C.
^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.01 (t, *J* = 8.8 Hz, 2 H), 2.77 (s, 4 H), 1.94 (d, *J* = 4.8 Hz, 4 H). ^{19}F NMR (376 MHz, $CDCl_3$): δ = –138.40 to –140.13 (m), –152.79 to –153.62 (m). ^{13}C NMR (101 MHz, $CDCl_3$): δ = 168.90, 145.97 (dtd, *J* = 248.5, 11.9, 4.2 Hz), 140.53 (dddd, *J* = 250.4, 15.3, 4.8, 2.3 Hz), 129.80–129.23 (m), 103.19 (t, *J* = 22.8 Hz), 32.87, 23.84.

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(19) **Protein Conjugates of Half-Esters; General Procedure**
BSA or OVA (10 mg) was dissolved in PBS (pH 7.5, 2 mL), and the appropriate half-ester **11**–**14** was dissolved in DMF (100 µL). The ester solution was slowly injected into the protein solution and the mixture was left for 24 h at rt. The aqueous phase was then collected, diluted with deionized water, and dialyzed against five changes of deionized water. The solution was lyophilized to afford the protein conjugate as a white solid.

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