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# Efficient formation of heterodimers from peptides and proteins using unsymmetrical polyfluorophenyl esters of dicarboxylic acids

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**An efficient method for the heteroconjugation of biomolecules carrying free amino groups was reported previously, where mixed polyfluorophenyl diesters of dicarboxylic acids with varied aliphatic chain length were shown to be efficient reagents for the conjugation of a variety of model biomolecules. The concept was based on the differential reactivity of the esters towards amines. The concept has now been further optimized, and a 2,6-difluorophenyl-pentafluorophenyl diester combination has been demonstrated to be the most efficient, both with respect to selectivity and to reaction rate. A pentafluorophenyl ester reacts faster with an amino group and requires a weaker base than a 2,6-difluorophenyl ester that requires a stronger base and longer reaction time. With the use of this combination of esters, we obtained considerably shortened reaction times compared with those reported previously, yet still retaining the desired selectivity in heteroconjugation. The increased reactivity of the bifunctional reagent allowed the construction of sophisticated peptide heteroconjugates from peptides, carbohydrates and proteins, showing a wide scope of applicability in the field of assembling functional bioconjugates.** 

*Supporting information can be found in the online version of this article.*

**Keywords:** conjugation; bioconjugation; linker; heteroconjugation; polyfluorophenyl esters

## Introduction

In recent years, bioconjugation has developed to become an independent and rapidly developing field of research [1]. The combination of two functional biomolecules, to form a heteroconjugate with the properties of both of its individual components, has many applications in biotechnology and biomedicine [2]. Immobilizations on solid support are ubiquitous in chip-based *in vitro* diagnostics and other bioanalytical applications. The incorporation of fluorophores, radiolabels and other reporter groups in proteins and peptides selectively and in high yields is a prerequisite for molecular imaging. The applicability of efficient conjugation techniques is vast and ranges from fundamental biological and biochemical research to biomedicinal applications, including medical imaging and therapy [3]. It is fair to say that bioconjugation constitutes an indispensable interface between synthetic organic chemistry and biology.

Bifunctional linkers are the fundaments of bioconjugate chemistry and are essential molecular tools in biotechnology [4]. Most of the commercially available bifunctional reagents are designed to form linkages using two different and orthogonal functional groups that are abundant in biomolecules. Orthogonality between the reactive ends of the reagent ensures the formation of heteroconjugate products in two separate steps under mild conditions.

It is often desirable to form a linkage between two molecules using the same functional group, such as the primary amino group of the side chain of lysine. The best known reagents for amino group conjugations are based on the reactivity of imidodiesters [5] or *N*-hydroxysuccinimide diesters [6]. Although widely used due to the availability of a variety of linker sizes, reagents of these types are not practical for heteroconjugate formation because the reactivity is the same for both esters and a statistical distribution of homoconjugates and heteroconjugates is obtained. Diethylsquarate [7] or thiophosgene [8], on the other hand, can be used to link two different molecules carrying amino groups to form heteroconjugates in high yields because the reactions of the first and the second amino group occur at different rates and provide selectivity in heteroconjugation. They react within minutes with the first amino group that allows for successful formation and separation of the monoconjugate, which can subsequently be reacted with the second amino group at a considerable slower rate. These reagents are very efficient but provide no variation in linker size, which limits their usefulness when the possibility to vary the distance between the two heteroconjugate subunits is of interest, especially when the heteroconjugate is designed to interact simultaneously with two different biomolecular targets.

Recently, we reported on a very simple, robust and reliable technology to form heteroconjugates from biomolecules carrying

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free primary amino groups [9]. The strategy was based on aliphatic dicarboxylic acid diesters where two esters were formed from phenols with different pKa values and therefore with different reactivities toward amino groups. A diester where the reactivity of each of the two esters is different towards primary amino groups provides an opportunity to react the diester with two biomolecules on different time scales. One can separate the initially formed monoconjugate and react it under controlled conditions with a second biomolecule to form a heteroconjugate in high yield. We have shown experimentally that an *o*-fluorophenyl ester and a pentafluorophenyl ester separated by a linear aliphatic chain is a convenient bifunctional reagent that allows for the construction of conjugates from amino group containing species such as peptides and carbohydrates. The concept provides an opportunity to conveniently vary the distance between the biomolecular components by selecting different diacids that are abundantly available from commercial suppliers. To enhance the difference in reaction rate between the esters, we performed each reaction step under conditions that differed in basicity. As the reaction is typically performed in dry DMSO, no problems with solubility of the reagents or hydrolysis of the esters were observed. Although this technology turned out to give pure conjugates, it suffered from a very significant drawback. The time required to complete the entire conjugation procedure was long. Although the pentafluorophenyl ester reacted momentarily, the reaction of the *o*-fluorophenyl ester with the amine from the second biomolecule sometimes required between 4 and 6 days to reach completion. This led on occasion to decomposition of fragile biomolecules under the reaction conditions.

Here, we wish to report on an improved method for bioconjugation, where the difference between the pKa values of the phenols used for diester formation is smaller. The polyfluorophenyl esters were systematically varied and investigated with regards to reactivity under the reaction conditions, because a variety of pKa values is accessible simply by varying the number and position of fluorine atoms in the aromatic ring. A pair of polyfluorophenyl esters was identified, which provides both specificity and short conjugation times. Demonstrated reaction examples include the formation of peptide-peptide conjugates as well as peptide-protein conjugates.

## Materials and Methods

### Abbreviations used in the Paper

AcOEt, ethyl acetate; DCM, dichloromethane; DIC, *N,N'*-diisopropylcarbodiimide; DIPEA, *N,N'*-diisopropylethylamine; DMF, *N,N*-dimetyloformamid; DMSO, dimethyl sulfoxide; equiv, equivalents; HCTU, (2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate); HEPES, hydroxyethyl piperazineethanesulfonic acid; PyBOP, (Benzotriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate; HSA, human serum albumin; py, pyridine; NMP, *N*-Methyl-2-pyrrolidone; TFA, trifluoroacetic acid; Tfa, trifluoracetyl; TIS, triisopropylsilane.

### General Methods

All reagents and solvents were purchased from commercial sources and were used without further purification. TLC was performed on 60-$F_{254}$ silica and 60-$F_{254}$ aluminum oxide plates (Merck, Darmstadt, Germany), and spots were visualized with UV light ($\lambda = 254$ nm) unless otherwise stated. Sep-Pak C-18 cartridges (0.7 g) purchased from Waters Corporation, Milford, MA, USA. NMR spectra were recorded on a Varian Unity INOVA ($^1$H at 499.9 MHz) (Varian Inc., Palo Alto, CA, USA) or a Varian Unity ($^{13}$C at 100.6 MHz and $^{19}$F at 376.3 MHz) spectrometer. Chemical shifts were referenced directly via the internal standards: $^1$H NMR spectra were referenced to the TMS signal ($^1$H $\delta$ 0.0), $^{13}$C NMR spectra were referenced to the middle deuterochloroform signal ($^{13}$C $\delta$ 77.0), $^{19}$F NMR spectra were referenced to $CCl_3F$ indirectly via the lock signal. All the peptides and peptide conjugates were characterized by MALDI-TOF mass spectrometry, on an Applied Biosystems Voyager-DE PRO instrument (Applied Biosystems, Foster City, CA, USA), which was calibrated to Perkin Elmer Seqazyme Peptide Mass Standards Kit Calmix 2 and Calmix 3 (PerkinElmer, San Jose, CA, USA). Electrospray mass spectra (ESI-MS) for direct-infused dilute methanol solutions were recorded in both positive and negative ion modes using a Perkin-Elmer SCIEX API 150-EX mass spectrometer. High-resolution mass spectrometry (HRMS) characterization of the diesters was carried out on an Apex-Qe Ultra 7T instrument (Bruker Daltonics Inc., Billerica, MA, USA) using ESI in the positive ion mode. The instrument was calibrated with the Tunemix mixture (Bruker Daltonics). The mass accuracy was approximately 2–3 ppm. The instrumental parameters were as follows: scan range, 200–1200 m/z; drying gas, nitrogen; temperature of drying gas, 200 °C; potential between spray needle and orifice, set at 4.5 kV; source accumulation time, 0.5 s; and ion accumulation time, 0.5 s. The samples for MS experiments (~0.05 mg) were dissolved in the methanol containing $10^{-4}$ M NaCl. Melting points were measured on Stuart SMP10 Melting Point Apparatus (Beacon Road, Stone, Staffordshire, UK) and were uncorrected. IR experiments performed on Perkin Elmer Spectrum-100 FT-IR spectrometer equipped with an attenuated total reflectance (ATR) accessory. Analytical HPLC was performed using a Hypersil C-18 Gold column, 170-Å pore size, 5-Å particle size, 250 × 4.6 mm diameter, eluting with a 30–70% gradient of acetonitrile in water containing 0.1% of TFA over 40 min at a flow rate of 1.2 ml/min. HPLC purifications of the peptides were performed by HPLC semi-preparative Hypersil C-18 column (Runcom, UK) 250 × 20 mm, 5-μm particle size eluting with a 35–75% gradient of acetonitrile in water containing 0.1% of TFA over 40 min at a flow rate of 10 ml/min.

All reagents and gels used for gel electrophoresis and gel staining were from Invitrogen (Life Technologies, Grand Island, NY, USA). SDS-PAGE electrophoresis was conducted using NuPAGE® Novex 4–12% Bis-Tris Gel 1.5 mm, which was developed in 3-(N-morpholino)propanesulfonic acid running buffer. All gels were stained using Colloidal Blue Staining Kit.

### Peptide Synthesis

Peptides were synthesized using automated solid-phase methodology on an Applied Biosystems 433A peptide synthesizer, employing standard Fmoc/$^t$Bu strategy using the FastMoc synthesis program. The syntheses were performed on 0.1-mmol scale, and $H_2N$-RinkAmide-ChemMatrix (PCAS BioMatrix Inc., Saint-Jean-sur-Richelieu, QC, Canada) resin with a loading of 0.46 mmol g$^{-1}$ was used as the solid support. All the coupling steps were conducted with a HCTU/DIPEA (Iris Biotech GmbH, Marktredwitz, Germany and Peptides International Inc. Louisville, Kentucky, USA) activation cocktail. Fmoc deprotection was achieved by piperidine/NMP (2/8 v/v) treatment. All reagents used in the peptide synthesis were prepared according to the manufacturer's protocols. The side chains of the amino acids

  

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(Iris Biotech GmbH and Pepnet Inc.) were protected by base-stable groups: *tert*-butyl ester (Asp, Glu), trityl (His, Asn, Gln) and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Arg). Peptide cleavage from the solid support was achieved by treatment with the cleaving cocktail (TFA/TIS/$H_2O$ : 95/2.5/2.5 v/v) Purifications of the peptides were performed by HPLC.

### Synthesis of the Peptide 3

A resin containing peptide (0.1 mmol) with a free *N*-terminus, obtained directly from the peptide synthesizer, was immersed in the NMP solution (5 ml) of 7-methoxycoumarin-3-carboxylic acid (88 mg, 0.4 mmol, 4 equiv.), PyBOP (208 mg, 0.4 mmol, 4 equiv.) and DIPEA (104 mg, 140 µl, 0.8 mmol, 8 equiv.). The reaction mixture was gently agitated for 2 h, and then the resin was filtered off, washed five times with NMP and twice with DCM. The peptide was cleaved from the resin and purified by HPLC.

### 2,4-difluorophenyl Pentafluorophenyl Adipate (2a)

The compound was prepared according to the procedure described previously [9]. In short, pyridine (5 mmol) in $CH_3CN$ (10 ml) was added drop wise to a solution of the adipoyl chloride (1.83 g,10 mmol, 2 equiv) in $CH_3CN$ (50 ml) at 0 °C with vigorous stirring. The reaction mixture was allowed to attain rt (25 °C) and a solution of 2,4-difluorophenol (650 mg,5 mmol, 1 equiv) in $CH_3CN$ (5 ml) was added and stirring was continued for 3 h. $CH_3CN$ was removed under reduced pressure, and the residue was dissolved in AcOEt (100 ml) and washed with 1 N HCl (100 ml). The crude solution of the ester was dried over anhydrous $MgSO_4$ and was concentrated. The obtained monoesters were purified by flash chromatography (silica gel), eluting firstly with DCM to remove diester byproduct, and later, the mobile phase was changed to 30% AcOEt in DCM. Obtained monoester **1a** (yield: 920 mg, 72%) was taken directly to the next stage without further characterization. The monoester **1a** (258 mg, 1 mmol) were treated with pentafluorophenol (370 mg, 2 mmol, 2 equiv) and DIC (250 mg, 2 mmol, 2 equiv) in a mixture of $CH_3CN$ and pyridine (9 : 1, 10 ml) for 2 h at rt. The reaction mixture was concentrated, and the residue was subjected to column chromatography (silica gel) using $CH_2Cl_2$ as eluent to obtain the active diesters **2a** as white crystalline solid. Yield: 438 mg, 98%; m.p. 42–44 °C, IR (ATR): 2965, 2890, 1788, 1763, 1618, 1518, 1505, 1381, 1243, 1094, 986, 856, 737; $^1$H NMR (500 MHz, CDCl3) $\delta$ ppm 7.11 (m, 1H), 6.91 (m, 2H), 2.75 (m, 2H), 2.68 (m, 4H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ ppm 170.4, 168.9, 124.2, 124.1, 111.3, 111.1, 105.3, 105.1, 104.8, 33.3, 32.9, 24.0; $^{19}$F NMR (376 MHz, $CDCl_3$) $\delta$ ppm −113.1 (m), 124.0 (m), −153.3 (m), −158.6 (m), −162.8 (m); HRMS: calculated for $C_{18}H_{11}F_7O_4Na$: 447.0443, observed: 447.0433.

### 2,6-difluorophenyl Pentafluorophenyl Adipate (2b)

Prepared analogously to the compound **2a**; white solid, yield: 424 mg, 95%; m.p. 41–43 °C, IR (ATR): 2943, 2882, 1770, 1516, 1603, 1516, 1384, 1292, 1246, 1100, 986, 782; $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ ppm 7.17 (m, 1H), 6.97 (m, 2H), 2.74 (m, 2H), 1.93 (m, 4H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ ppm 169.6, 168.9, 156.9, 153.6, 142.8, 141.1, 139.5, 137.8, 136.2, 126.9, 125.93, 112.9–111.1 (m), 32.9, 23.9; $^{19}$F NMR (376 MHz, $CDCl_3$) $\delta$ ppm −126.8 (m), −153.3 (m), −158.6 (m), −162.9 (m); HRMS: calculated for $C_{18}H_{11}F_7O_4Na$: 447.0443, observed: 447.0432.

### 2,4,5-trifluorophenyl Pentafluorophenyl Adipate (2c)

Prepared analogously to the compound **2a**; white solid, yield: 424 mg, 96%; m.p. 43 °C, IR (ATR) 3085, 2926, 1773, 1517, 1409, 1369, 1288, 1209, 1119, 988, 854; $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ ppm 7.02 (m, 1H), 2.75 (m, 2H), 2.68 (m, 2H), 1.90 (m, 4H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ ppm 169.9, 168.9, 112.7, 112.5, 106.4, 106.2, 105.9, 33.2, 32.9, 24.0, 23.9; $^{19}$F NMR (376 MHz, $CDCl_3$) $\delta$ ppm −129.8 (m), −136.6 (m), −140.7 (m), −153.3 (m), −158.6 (m), −162.8 (m); HRMS: calculated for $C_{18}H_{10}F_8O_4Na$: 465.0349, observed: 465.0345.

### 2,4,6-trifluorophenyl Pentafluorophenyl Adipate (2d)

Prepared analogously to the compound **2a**; white solid, yield: 427 mg, 96%; m.p 43–44 °C; IR (ATR) 2956, 2886, 1791, 1763, 1516, 1469, 1456, 1381, 1250, 1212, 1097, 985, 916, 839, 737; $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ ppm 6.76 (m, 2H), 2.74 (m, 4H), 1.92 (m, 4H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ ppm 169.6, 168.9, 160–153 (m), 142.4–136 (m), 101.1, 100.8, 100.6, 32.9, 23.9; $^{19}$F NMR (376 MHz, $CDCl_3$) $\delta$ ppm −110.8 (m), −123.5 (m), −153.2 (m), −158.4 (m), −162.7 (m); HRMS: calculated for $C_{18}H_{10}F_8O_4Na$: 465.0349, observed: 465.0342.

### 2,6-difluorophenyl Pentafluorophenyl Suberate (2e)

Prepared analogously to the compound **2a**; white solid, yield: 466 mg, 98%; m.p. 40–42 °C; IR (ATR) 2939, 2863, 1772, 1517, 1449, 1416, 1365, 1289, 1243, 1202, 1096, 1037, 984, 856, 769; $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ ppm 7.17 (m, 1H), 6.98 (m, 2H), 2.68 (m, 4H), 1.83 (m, 4H), 1.51 (m, 4H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ ppm 170.1, 169.4, 156.5, 154.0, 144–139 (m), 127.2, 126.3, 126.2, 126.1, 112.1, 112.0, 111.9, 111.8, 33.4, 33.1, 28.4, 24.6, 24.5; $^{19}$F NMR (376 MHz, $CDCl_3$) $\delta$ ppm −126.8 (m), −153.2 (m), −158.5 (m), −162.8 (m); HRMS: calculated for $C_{20}H_{15}F_7NaO_4$: 475.0756, observed: 475.0750.

### General Procedure for the Synthesis of Peptide-linker Conjugates 4a–e

Synthesis of the peptide derivative **4a**: The dodecapeptide, **3** (7.5 mg, 5 µmol) was dissolved in anhydrous DMSO (1 ml), and to the peptide solution thus obtained, a solution of the active diester **2a** (4 mg, 10 µmol) pyridine (0.12 ml) was added. The reaction was followed by reverse phase analytical HPLC and was stirred until the peptide **3** reacted completely. The reaction times varied from 45 to 70 min. DMF (200 µl) was added to the reaction mixture, and the peptide products were precipitated with diethyl ether and were centrifuged. The residue was redissolved in DMF (1 ml) and precipitated again using diethyl ether. The obtained crude product **4a** was then analyzed by analytical HPLC, indicating sufficient purity. Therefore, the obtained peptide was dissolved in $CH_3CN/H_2O$ (1 : 9 v/v), lyophilized and used for further purposes, without any extra purification; 7.4 mg (84%). MALDI-MS: expected for $[M+H]^+$: 1778.22, observed: 1778.43.

Likewise, peptide conjugates **4b**, **4c**, **4d**, **4e** were synthesized from peptide **3** and diesters **2b**, **2c**, **2d**, **2e**, respectively.

**4b**: Yield: 6.6 mg (74%), MALDI-MS: expected for $[M+H]^+$: 1778.22, observed: 1778.69.

**4c**: Yield: 7.1 mg (80%) including impurities, MALDI-MS after analytical HPLC: expected for $[M+H]^+$: 1796.43, observed: 1796.15.



**4d**: Yield: 7.7 mg (85%) including impurities, MALDI-MS after analytical HPLC: expected for $[M+H]^+$: 1796.43, observed: 1796.55.

**4e**: Yield: 8 mg (90%), MALDI-MS: expected for $[M+H]^+$: 1806.54, observed: 1806.62.

### General Procedure for the Synthesis of the Peptide-peptide Conjugate 6a and 6e

Synthesis of peptide conjugate **6a**: The peptide **5** (3 mg, 0.6 μmol) was dissolved in anhydrous DMSO (1 ml), and a solution of the peptide derivative **4a** (or **4b**) (1.5 mg, 1.2 μmol, 2 equiv.), pyridine (0.2 ml) and DIPEA (20 μl) in DMSO (0.5 μl) was added. The stirred reaction was followed by reverse phase analytical HPLC, and stirring was continued until **5** reacted completely. The reaction mixture was diluted with anhydrous diethyl ether and centrifuged. The residue was dissolved in TFA (1 ml) and precipitated again using diethyl ether. The crude product **6a** was then purified by semi-preparative reversed-phase HPLC. The purified fractions were analyzed by MALDI-TOF mass spectrometry and were pooled together, concentrated and lyophilized. The isolated yield of the functionalized peptide **6a** was 3.3 mg (84%). MALDI-MS: expected for $[M+H]^+$: 6635.05, observed: 6635.13.

Likewise, peptide conjugate **6e** was obtained from peptide **5** and peptide derivative **6e** using analogous procedure. Yield: 3.5 mg (88%). MALDI-MS: expected for $[M+H]^+$: 6661.11, observed: 6661.15.

### Synthesis of the Peptide-peptide Conjugate 8a and 8b

These compounds were prepared in 80% and 70% yield from peptides **7a** of **7b**, respectively, and **4b** using essentially the same procedure as for compound **6a**, but with twice the molar amount of peptide **4b**. The reaction time for **8a** was approximately 36 h and for **8b** 60 h. MALDI-MS: expected for **8a** $[M+H]^+$: 8520.79, observed: 8520.76; expected for **8b** $[M+H]^+$: 8215.23, observed: 8215.15.

### Synthesis of the Trisaccharide Derivative 10

A solution of 2-(*p*-aminophenyl)ethyl 2-O-(α-L-fucopyranosyl)-3-O-(α-D-galactopyranosyl)-β-D-galactopyranoside [9] (60 mg, 0.1 mmol) was dissolved in dry DMSO (0.5 ml) containing pyridine (0.05 ml), and then **2b** (63 mg, 0.15 mmol) was added. After 30 min, TLC (AcOEt/AcOH/MeOH/$H_20$, 12 : 3 : 3 : 2; detection with 5% sulfuric acid + heating) showed appearance of a faster $R_f$ 0.5 spot and disappearance of the starting sugar $R_f$ 0.1 spot. After 5 h, all starting materials had disappeared and the mixture was diluted with $H_20$/AcOH (98 : 2, 3 ml), washed with AcOEt-ether 1 : 1, the aq. phase was evaporated to approximately ½ volume, and was applied to a Sep-Pack Plus (C-18 cartridges, Waters), which was initially washed with $CH_3CN$ and later equilibrated with water. The cartridge was washed first with water, then the mobile phase with the increasing concentration of $CH_3CN$ (10% steps, up to 50%) gave TLC-pure fractions containing **10**, which were pooled, concentrated to half volume and then lyophilized. Yield: 59 mg, 70%. $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ ppm 7.41 (d, $J$ = 8.79, 2H), 7.28 (m, $J$ = 8.79, 3H), 7.07 (m, 2H), 5.24 (m, 1H), 4.62 (d, $J$ = 7.64, 1H), 4.28 (m, 3H), 4.21 (m, 1H), 4.07 (m, 1H), 3.98 (m, 2H), 3.77 (m, 10H), 3.38 (m, 1H), 3.16 (m, 1H), 3.00 (m, 1H), 2.88 (m, 1H), 2.75 (m, 2H), 2.46 (s, 2H), 1.74 (s, 4H), 0.96 (s, 3H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ ppm 173.7, 171.8, 156.3, 154.5, 153.8, 144.1, 136.1, 135.3, 129.2, 127.2, 126.9, 121.4, 112.4, 112.2, 101.5, 98.9, 93.8, 77.3, 74.8, 72.9, 72.3, 71.6, 70.3, 69.9, 69.8, 69.5, 68.6, 68.3, 66.8, 63.9, 61.8, 61.2, 39.3, 36.4, 34.7, 33.0, 24.9, 24.3, 15.8; 112.0, 111.9, 111.8, 33.4, 33.1, 28.4, 24.6, 24.5; $^{19}$F NMR (376 MHz, $CDCl_3$) $\delta$ ppm −127.4. ESI-MS: expected for $[M+H]^+$: 847.3, observed: 847.3. Expected for $[M+Na]^+$ 870.3, observed: 870.2.

### Synthesis of the Sugar-peptide Conjugate 11

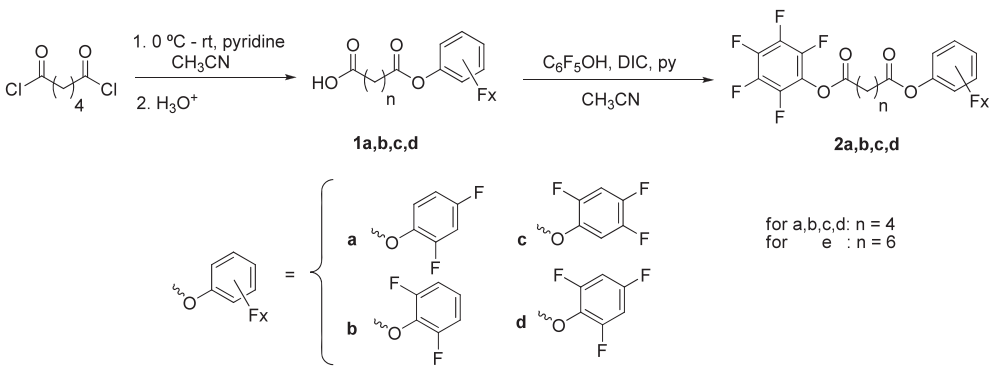
This compound was prepared in 90% yield from peptides **10** and **3** using essentially the same coupling procedure as for compound **6a** in 24 h. MALDI-MS: expected for $[M+H]^+$: 2255.79, observed: 2255.67.

### Synthesis of the Peptide Derivative 13

This compound was prepared in 85% yield from **12** and linker **2b** using essentially the same coupling procedure as for compound **4b** in 3 h. MALDI-MS: Expected for $[M+H]^+$: 5330.48, Observed: 5330.36.

### Conjugation of 13 to HSA

Lyophilized HSA (1 mg) was suspended in the HEPES buffer pH = 8 (1.5 ml, 50 mM), and the suspension was stirred until a clear solution was obtained. To the solution, derivative **13** (1 mg, 0.2 μmol, 10 equiv.) was added and the reaction mixture was stirred at rt. After 24 h, another portion of the peptide **13** was added (1 mg, 0.2 μmol, 10 equiv.). After 48 h of total reaction time, the reaction mixture was diluted with water to a total volume of 10 ml and processed by means of ultrafiltration (cut-off = 30 K Da, 4400 rpm) until the volume of the upper liquid reached 1 ml. The protein-containing




**Scheme 1.** The synthesis of mixed diesters (linker candidates).

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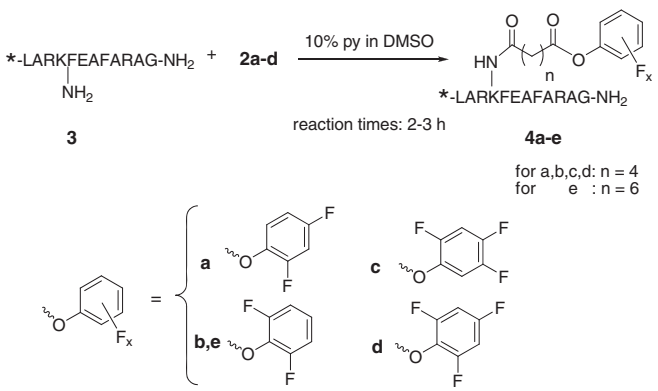
solution was diluted up to 10 ml and ultrafiltrated again down to the volume of 1 ml. This procedure was repeated ten times; after which, the filtrate did not show any fluorescent properties. The obtained protein solution was analyzed by SDS-PAGE. To determine the relative level of HSA functionalization, we have compared the total protein concentration contained in the retentate with the concentration of the functionalized protein determined by the UV absorption of the coumarin ($\varepsilon = 22000\ M^{-1}\ cm^{-1}$). The concentration of the modified species obtained from the Lambert–Beer formula gave the concentration of 16.34 μM. The sample used for UV analysis was diluted four times and subjected to the analysis of total protein concentration by a certified Amino Acid Analysis Center, Uppsala, basing on mean concentration of tyrosine, threonine and serine, as these amino acids do not occur in the peptide used for conjugation. The concentration value thus obtained was 3.3 μM.

## Results and Discussion

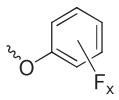
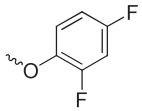
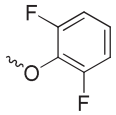
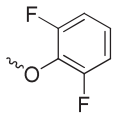
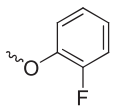
We previously identified pentafluorophenyl esters as very fast and reliable amidation reagents for the first step of the heteroconjugation reaction and concluded based on those investigations that no improvement was necessary because a pentafluorophenyl ester provides an essentially instantaneous reaction with a lysine side chain in the presence of base. Attention was instead focused on the slow reaction of the *o*-fluorophenyl ester. Four polyfluorophenyl esters formed from phenols with pKa values in the range from 8.7 to 7.5 were prepared to identify candidates for incorporation in the diester for improved reactivity in the second amidation reaction. Mixed adipic acid diesters (**1a–d)** were synthesized according to the procedure published previously [9] (Scheme 1). The adipic acid dichloride was reacted with a polyfluorophenyl ester (1 equiv.) in the presence of pyridine (1 equiv.) to give the monoester after aqueous workup. Monoesters obtained according to this procedure were further reacted with pentafluorophenol in a carbodiimide mediated reaction to form the mixed diesters **2a–d**.

All four diesters were reacted with the α-helical dodecapeptide **3** having one lysine at position 4 (Scheme 2). At this stage, it was desired that only the pentafluorophenyl ester would react with the amino group, whereas the other ester should remain intact. The reaction was performed in 10% pyridine in DMSO.

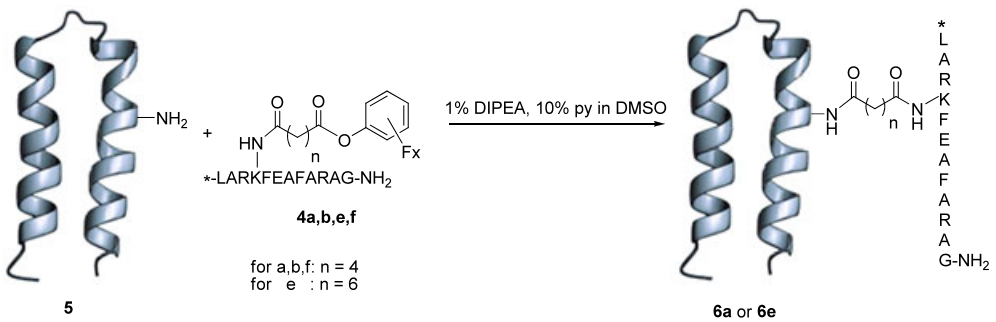



**Scheme 2.** The reaction between the linker candidates **1a–d** and dodecapeptide **3**; * = 7-methoxycoumarin-3-carbonyl.

**Table 1.** Reaction times and yields for the reactions between peptide **5** and peptide derivatives functionalized with polyfluorophenyl esters

| Peptide derivative | | $(CH_2)_n$ | Reaction time[a]/ yield[b] |
|---|---|---|---|
| |  | | |
| **4a** |  | 4 | 60 h/80% |
| **4b** |  | 4 | 36 h/85% |
| **4e** |  | 6 | <48 h/80% |
| **4f** |  | 4 | Approx. 50% Conversion after 72 h |

[a]The reaction mixture was sampled every 12 h, and the obtained aliquots were analyzed by HPLC and MALDI-MS.
[b]Weight obtained after lyophilization.




Sequence of the peptide **5**:

Ac-NAADJEARIK(Tfa)HLRERJKARGPRDC(Acm)AQJAEQLARAFERFARAG-$NH_2$

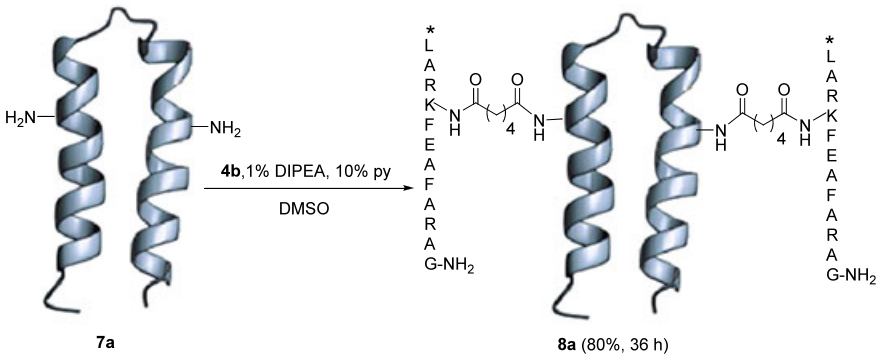
**Scheme 3.** Conjugation of 42-residue polypeptide **5** to peptide derivatives formed from reacting dodecapeptide **3** with bifunctional reagents.



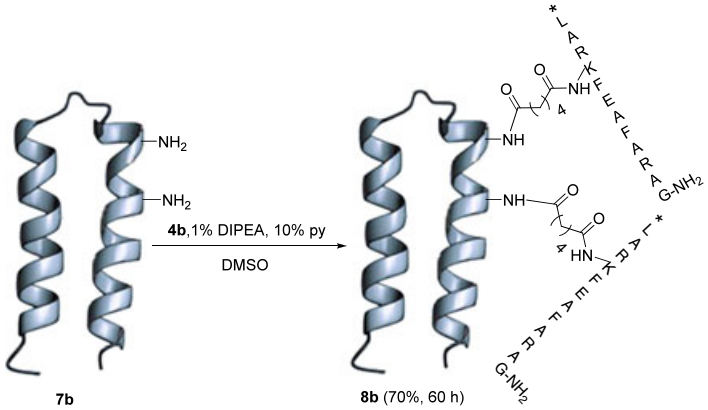
To facilitate identification of the reaction products, the dodecapeptide **3** was *N*-terminated with the fluorophore 7-methoxycoumarin-3-carbonyl.

In all cases, the reactions between diesters **2a–d** and the peptide **3** were completed within 2–3 h. However, only experiments where compounds **2a** and **2b** were used gave pure functionalized peptide




Sequence of the peptide **7a**:

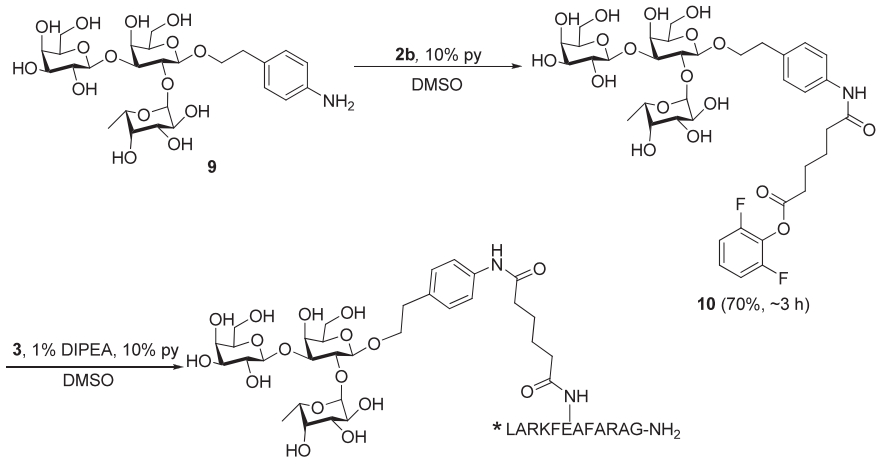
Ac-NEADLEAKIRHLAEK(dansyl)LEARGPEDC(Acm)EQLAEQLKRAFARK(Tfa)G-OH




Sequence of the peptide **7b**:

Ac-NAADJEAAIRHLAERJAARGPVDAKQJKEQLARAFEAFK(*)RAG-NH2

**Scheme 4.** Reactions of 42-residue polypeptides **7a** and **7b** to form triple conjugates **8a** and **8b**. The peptide **7a** holds four lysine residues where Lys-15 is modified by incorporation of a dansyl fluorophore (dansyl = 5-(dimethylamino)naphthalene-1-sulfonyl), and the side chain of Lys-41 is protected by a Tfa (trifluoracetyl) group, whereas Lys-8 and Lys-35 remain free for conjugation. The side chain of the cysteine residue Cys-24 is protected by an Acm (acetylaminomethyl) group. The peptide **7b** holds three lysine residues where Lys-39 is functionalized with a coumarin fluorophore (coumarin = 7-methoxycoumarin-3-carbonyl), whereas Lys-25 and Lys-28 remain free for conjugation.




**Scheme 5.** The synthesis of the carbohydrate-peptide conjugate **11**.



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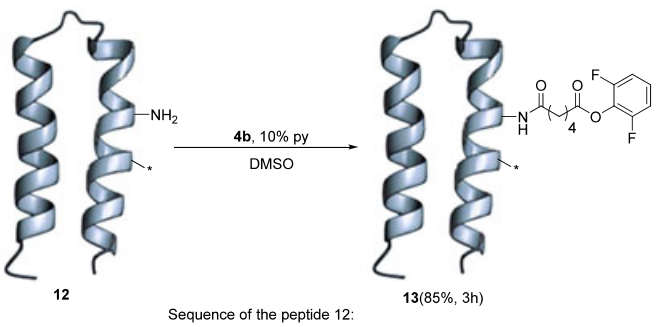
derivatives, **4a** and **4b**, respectively. The reactions with the trifluorophenyl esters **2c** and **2d** gave rise to significant amounts of the peptide homoconjugate formed from two copies of **3** and the linker reagent. The results showed that it was possible to selectively form monoamides from pentafluorophenyl esters in the presence of 2,4-difluorophenyl and 2,6-difluorophenyl esters. Both difluorophenyl esters could therefore be used in a bifunctional reagent where the functionalized peptide derivative formed in the initial reaction with the pentafluorophenyl ester was to be reacted with a second biomolecule in the presence of 1% DIPEA to form a heteroconjugate in high yield. A bifunctional linker thus prepared would provide not only the required selectivity but also an improved reaction rate compared with that of the previously published 2-fluorophenyl pentafluorophenyl diester reagent [9]. Because the linker candidates **2c** and **2d** led to the formation of homoconjugates, they were excluded from further investigations.

To evaluate the performance of the 2,6-difluorophenyl and 2,4-difluorophenyl esters with respect to the difference in rate of amidation in comparison to that of the pentafluorphenyl ester, we investigated the conjugation of the peptide derivatives **4a, 4b** and **4e** to a 42-residue peptide **5** [10,11] (Scheme 3). The polypeptide was previously designed to fold into a helix-loop-helix motif and shown to form a helix-loop-helix dimer in aqueous solution [12]. The 42-residue *N*-acetylated polypeptide **5** has a single lysine residue with a free amino group at position 17. The experiment was performed in DMSO in the presence of 10% pyridine and 1% DIPEA, and the results are summarized in Table 1. For comparison, we also conjugated the peptide derivative **4f** [9] carrying the *o*-fluorophenyl ester to the 42-residue polypeptide **5**.



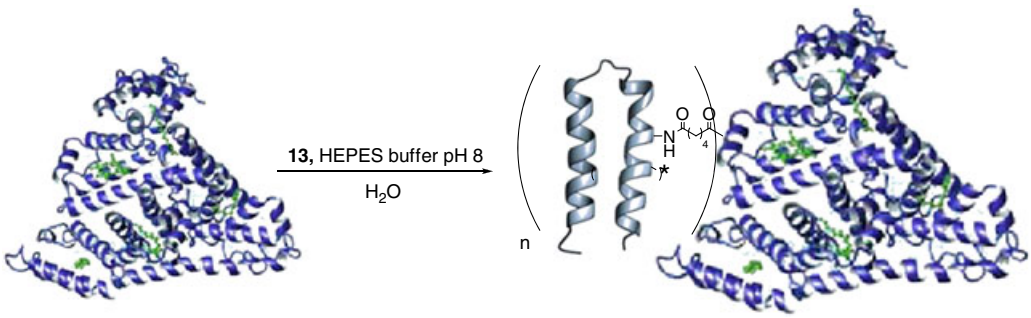

**Scheme 6.** The synthesis of the peptide derivative **13**. The *N*-acetylated peptide **12** carries two lysine residues, where Lys-10 was derivatized with the coumarin fluorophore (coumarin = 7-methoxycoumarin-3-carbonyl), whereas Lys-17 is free and available for functionalization.

The functionalized peptide derivatives formed in the reaction between the dodecapeptide **3** and the bifunctional reagents equipped with 2,4-difluorophenyl or 2,6-difluorophenyl esters (**4a** and **4b**) reacted with **5** at a significantly increased reaction rate in comparison to that observed in the reaction between **5** and the dodecapeptide carrying an *o*-fluorophenyl ester (**4f**). Under the reaction conditions, **4f** did not reach more than 50% conversion into the heteroconjucate even after 72 h. This suggests that the use of the 2,6-difluorophenyl ester increases the reaction rate by at least a factor of four without loss of selectivity. We also successfully synthesized the peptide derivative **4e** from peptide **3** and **2e** and the 2,6-difluorophenyl ester equipped with a longer aliphatic chain to prove that an extension of the linker does not affect the reaction time significantly.

The improved methodology was next examined in the assembly of more sophisticated peptide constructs, and two units of peptide **4b** were conjugated to the two 42-residue *N*-acetylated helical peptides **7a** and **7b**, each possessing two lysines with free side-chain amino groups (Scheme 4). In polypeptide **7a**, each lysine was situated on a separate helix well separated in the sequence. The conjugation went smoothly and reached completion in approximately 36 h, giving peptide conjugate **8a**. In polypeptide **7b**, two lysine residues were positioned in the same helical segment and in close spatial proximity. In this case, it was suspected that after successful reaction with the first **4b** unit, access to the second free lysine might become difficult. Despite the complexity of this model system, we were able to successfully obtain the doubly ligated peptide conjugate **8b** in 70% yield in 60 h.

The optimized methodology turned out to function well in the case of constructing neoglycoconjugates. A derivative of the blood group B antigenic determinant, **9**, equipped with a free amino group was reacted under mildly basic conditions with the linker **2b** to give the 2,6-difluorophenyl ester **10** in 70% yield. The activated trisaccharide **10** was then conjugated to the peptide **3** in the presence of 1% DIPEA, resulting in the formation of neoglycoconjugate **11** in very good (90%) yield (Scheme 5). The astonishingly short reaction time was probably due to the absence of sterical interferences between the reagents.

From the perspective of utilizing the current linker methodology for protein functionalization, HSA was selected as a model protein. The choice of this particular protein was not casual, as it illustrates numerous advantages of the present technology. It is one of the most abundant proteins in human blood; it is used as a vehicle for drug delivery and has pharmacokinetic




**Scheme 7.** Conjugation of HSA with the peptide derivative **13**. A mixture of HSA conjugates was obtained because of the fact that the functionalizing reagent does discriminate between the lysine residues on the surface of the HSA protein.



implications [13]. Thus, if the linker technology was to prove successful in the functionalization of HSA, new opportunities in drug delivery could be opened. HSA is a well-characterized and investigated protein frequently used in conjugation reactions. It is also cheap and readily available from commercial sources. For conjugation reactions of proteins, DMSO cannot be used, and this investigation was performed using HEPES buffer at pH 8 as the solvent. At this pH, a compromise had to be found between protein conjugation that requires unprotonated amino groups and undesired active ester hydrolysis. For conjugation to HSA, peptide **12** was selected and reacted with **2b** in 10% pyridine in DMSO to give compound **13** (Scheme 6). After isolation, the peptide derivative was added to the HSA solution in HEPES buffer at pH 8 (Scheme 7).

The reaction was discontinued after 48 h, and the protein-peptide conjugate purified by ultrafiltration, where unreacted **13** equipped with coumarin was removed by repeated filtrations until no fluorescence, was observed in the filtrate (Figure 1). The retentate showed intense fluorescence apparently due to the presence of **13** equipped with coumarin and conjugated to HSA, which was retained by the filter because of the high molecular weight of the peptide-protein conjugate.

The supernatant was analyzed by SDS-PAGE followed by coomassie staining (Figure 2), and several bands were observed. Apparently, several copies of **13** were incorporated to form an array of conjugates with a range of molecular weights. The bifunctional reagent does not show selectivity towards the 58 lysine residues, most of which are equally distributed on the surface of the protein, making them available to react with other molecules in the system [14]. Therefore, it is not possible to obtain labeled conjugates with a well-defined number of polypeptides. Nevertheless, the separated protein conjugates showed fluorescent properties due to the coumarin fluorophore, thus showing the efficiency of the reagent also in aqueous environment. A quantitative comparison between the total protein concentration as measured by quantitative amino acid analysis and the concentration of the fluorescent label as determined from the absorbance measured by UV spectroscopy resulted in an approximate 1 : 2 ratio, which indicated that on the average, every HSA molecule was functionalized with five peptide **13** units.

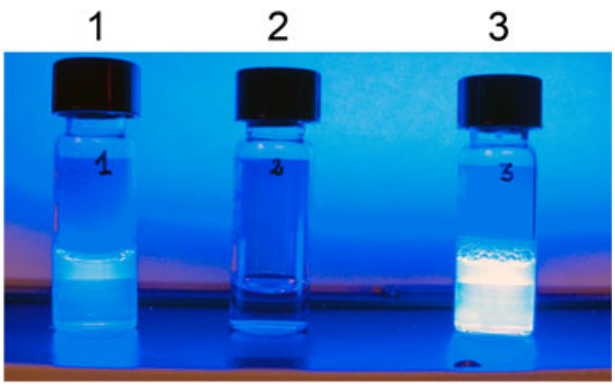



**Figure 1.** Illustration of functionalization of human serum albumin, showing filtrates at different stage of ultrafiltration and supernatant after filtration, irradiated with UV light at 366 nm. Vial 1, filtrate after first ultrafiltration cycle. Vial 2, filtrate after sixth ultrafiltration cycle. Vial 3, retentate after sixth ultrafiltration cycle showing strong fluroescence emission due to peptide-protein conjugate.

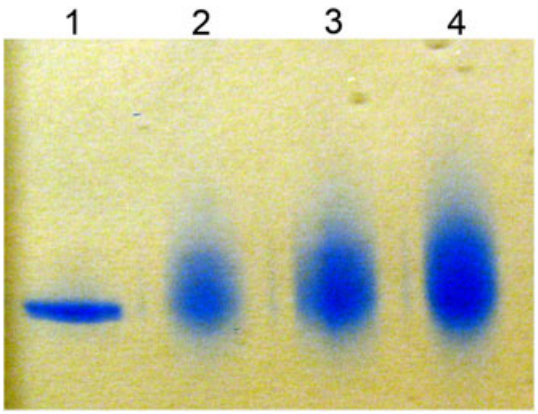



**Figure 2.** SDS-PAGE analysis of HSA functionalized with the peptide derivative **13**. Lane 1, HSA reference. Lane 2, HSA functionalized with **13** (500 fold diluted reaction mixture). Lane 3, HSA functionalized with **13** (100 fold diluted reaction mixture). Lane 4, HSA functionalized with **13** (10 fold diluted reaction mixture).

## Conclusion

In summary, a bifunctional reagent with improved properties over those previously reported has been developed for the covalent linkage of biomolecules with free amino groups including peptides, proteins and carbohydrates. The optimal bifunctional reagent for the conjugation of biomolecules where variation of distance between subunits is required is a diester formed from aliphatic dicarboxylic acids. An evaluation of polyfluorophenyl ester pairs showed that a 2,6-difluorophenyl ester 'in concerto' with a pentafluorophenyl ester, provides both selectivity and optimal reaction times for the assembly of heteroconjugates. The time needed to prepare the complete conjugate proved to be approximately four times shorter compared with the time needed using the reagents reported previously [9], which is of considerable importance in the conjugation of sensitive biomolecules. The new linker methodology proved to be efficient in constructing a variety of conjugates between peptides and peptides, between peptides and carbohydrates as well as between peptides and proteins, demonstrating that the scope of the applicability is wide. The results suggest that this linker technology has the capacity to significantly influence biotechnological and biochemical research whenever construction of bifunctional conjugates is required. It is envisioned that the bifunctional linkers reported here will find their optimal use in the heteroconjugation of proteins using lysine side chains, a reaction for which currently few alternatives exist, if access to spacers of variable size is required.

### Acknowledgement

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