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Synthesis and characterization of a 'fluorous' (fluorinated alkyl) affinity reagent that labels primary amine groups in proteins/peptides

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Strong non-covalent interactions such as biotin-avidin affinity play critical roles in protein/peptide purification. A new type of 'fluorous' (fluorinated alkyl) affinity approach has gained popularity due especially to its low level of non-specific binding to proteins/peptides. We have developed a novel water-soluble fluorous labeling reagent that is reactive (via an active sulfo-*N*-hydroxylsuccinimidyl ester group) to primary amine groups in proteins/peptides. After fluorous affinity purification, the bulky fluorous tag moiety and the long oligoethylene glycol (OEG) spacer of this labeling reagent can be trimmed via the cleavage of an acid labile linker. Upon collision-induced dissociation, the labeled peptide ion yields a characteristic fragment that can be retrieved from the residual portion of the fluorous affinity tag, and this fragment ion can serve as a marker to indicate that the relevant peptide has been successfully labeled. As a proof of principle, the newly synthesized fluorous labeling reagent was evaluated for peptide/protein labeling ability in phosphate-buffered saline (PBS). Results show that both the aqueous environment protein/peptide labeling and the affinity enrichment/separation process were highly efficient. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: fluorinated alkyl tag; primary amine labeling; flourous affinity tag; protein/peptide labeling; acid cleavable tag

Introduction

Proteins and peptides are present in living organisms at widely different levels. Given the complexity of biological samples, extraction and fractionation of proteins/peptides of interest can be very challenging. It is usually necessary to enrich and/or purify native proteins and peptides for their further characterization. Conjugating an affinity tag to protein/peptide is an attractive approach to protein/peptide enrichment and purification. Biotin is almost exclusively chosen as the affinity tag moiety in protein/peptide conjugating reagents due to the biotin–avidin interaction, which is one of the strongest non-covalent interactions in nature with a dissociation constant (K_d) of 10^{-15}. For affinity purification purposes, however, this well-known biotin–avidin interaction also has several limitations: First, some proteins/peptides may non-specifically bind to avidin;[1] second, low recovery of the biotin-tagged molecules in affinity purification is common because of the incomplete elution of biotinylated molecules from avidin resins;[2,3] third, the biotin moiety tends to reduce the ability to distinguish different peptides based upon hydrophobicity of the tagged peptides and thus compromises the separation of these peptides on reversed-phased liquid chromatography (LC) columns[4] and fourth, fragments originating from the biotin moiety can complicate the mass spectral interpretation of biotin-tagged peptides.[5] Incorporation of a cleavable linker between the biotin moiety and the peptide reactive group allows the removal of the biotin moiety, and this approach has been employed in cleavable ICAT reagents[4,6] that target the free thiol group in proteins/peptides.[7]

The substantial non-covalent interaction between fluorinated alkyl groups (makes the fluorine-fluorine interaction an alternative approach to biotin–avidin for affinity-based binding and separation. Fluorine–fluorine ('fluorous affinity') based binding and separation was first demonstrated in the fluorous biphasic catalysis technique.[8] Since then, fluorous affinity methods were successfully utilized in a wide spectrum of organic syntheses.[9] Recently, fluorous affinity techniques were introduced to proteomics,[10] metabolomics,[11] microarrays for probing small biomolecules[12–14] and fabrication of 'clickable' fluorous thin films.[15] No water-soluble fluorous affinity-based labeling reagent has been developed for primary amine group, an important conjugation target that exists in almost all proteins/peptides in the form of lysine side chains and unmodified *N*-termini.

Flourous affinity is characterized by strong non-covalent interactions between the fluorous affinity tag (which can be conjugated to molecules of interest) and a fluorous resin. The interactions between fluorous resins and proteins/peptides are generally weak, and the non-specific binding of protein/peptides to the fluorous resin, if any, can be easily disrupted. Compared to the biotin group, which is rather small (244 Da), fluorous tags are usually larger (448 Da for a commonly used tag $C_8F_{17}CH_2CH_3$). The strength of fluorous affinity increases with the size (i.e. the number of fluorine atoms) of the fluorous tag.[16] Moreover, fluorous compounds are usually insoluble in water due to the hydrophobicity of the fluorous tag moiety. This main caveat limits the application of fluorous compounds in biological systems when the physiological environment needs to be maintained [e.g. in phosphate-buffered saline (PBS)], and this

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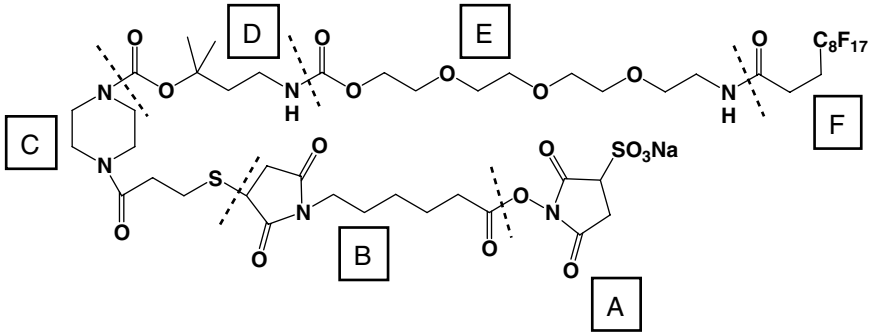

Figure 1. The structure of the synthesized fluorous labeling reagent sulfo-NHS-$(OEG)_3$-perfluorooctane. Building block A is the leaving group when the labeling reagent conjugates protein/peptide via a primary amine group. The tertiary carbamate group in blocks C and D is acid labile.

hydrophobicity may cause the fluorous tag conjugated proteins to aggregate. Incorporation of a polar, hydrophilic spacer between the fluorinated alkyl moiety and the protein/peptide reactive group will increase the solubility of the reagent to various extents and thereby alleviate aggregation of labeled proteins. However, the enlarged fluorous tag moiety significantly increases the sizes of labeled peptides and may further complicate their fragmentation patterns in tandem mass spectrometry experiments. To avoid this problem, it can be helpful to trim fluorinated alkyl and spacer moieties from labeled peptides before LC–MS analysis. Trimming part of the detergent/matrix molecule that is useful in sample preparation, but may otherwise interfere with MS analysis, was shown as an effective strategy to increase signal-to-noise ratios in a MALDI-MS study of membrane proteins.[17]

In this study, we have developed a novel water-soluble fluorous labeling reagent (sulfo-NHS-$(OEG)_3$-perfluorooctane in Fig. 1) that includes a protein/peptide reactive group (sulfo-*N*-hydroxylsuccinimidyl ester in blocks A and B, Fig. 1),[18] an acid labile linker (a tertiary carbamate group), an oligoethylene glycol $(OEG)_3$ spacer and a perfluorinated alkane moiety (C_8F_{17}). Efficiency in labeling peptide/protein was demonstrated in PBS. Fluorous affinity enrichment of labeled peptide/protein and cleavage of the acid labile linker were also tested as part of characterization of this novel labeling reagent.

Experimental

Materials

3-Hydroxy-3-methylbutyronitrile, (fluorenylmethoxycarbonyloxy) succinimide (Fmoc-OSu), $LiAlH_4$, pyridine, *N*,*N*-tetrabutylammonium fluoride (TBAF), trifluoroacetic acid (TFA), ammonium formate, bovine serum albumin (BSA), DL-dithiothreitol (DTT), iodoacetamide (IA) and 3,3′-disulfanediyldipropanoic acid were purchased from Sigma (St. Louis, MO). Diisopropylethylamine (DiEA) and *p*-nitrophenylchloroformate were purchased from Acros (Raritan, NJ). ACTH (4–11) was purchased from American Peptide Company, Inc. (Sunnyvale, CA). Sequencing grade modified trypsin was purchased from Promega (Madison, WI). Fluorous silica beads (5 μm) and 4,4,5,5,6,6,7,7,8,8,9,9,10,10,11,11,11-heptadecafluoroundecanoic acid were purchased from Fluorous Technologies, Inc. (Pittsburg, PA). A cartridge (fitting the cartridge holder in c-ICAT kit, Applied Biosystems, Foster City, CA) packed with fluorous silica beads was manufactured under 400 PSI with a bed volume of 100 μl by Optimize Technologies (Oregon City, OR). 2-{2-[2-(2-Amino-ethoxy)-ethoxy]-ethoxy}-ethanol and [*N*-(ε-maleimidocaproyloxy) sulfosuccinimide ester] (sulfo-EMCS) were purchased from Molecular Bioscience (Boulder, CO). *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDCI) was purchased from Advanced ChemTech (Louisville, KY). 1-Hydroxybenzotriazole, anhydrous (HOBt) was purchased from Chem-Impex International, Inc. (Wood Dale, IL). *N*,*N*′-disuccinimidyl carbonate and 1-Boc-piperazine were purchased from Oakwood Products, Inc. (West Columbia, SC), tris(2-carboxyethyl) phosphine hydrochloride (TCEP·HCl) was purchased from EMD Chemicals, Inc. (Gibbstown, NJ). All chemicals were used as received without further purification.

Synthesis of sulfo-NHS-$(OEG)_3$-perfluorooctane (Scheme 1)

4,4,5,5,6,6,7,7,8,8,9,9,10,10,11,11,11-Heptadecafluoro-N-(2-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)ethyl)undecanamide (1)

EDCI (0.64 g, 3.3 mM, 1.1 eq.) and HOBt (0.68 g, 4.5 mM, 1.5 eq.) were added to a stirred mixture of 2-(2-(2-(2-aminoethoxy) ethoxy)ethoxy)ethanol (0.62 g, 3.2 mM, 1.05 eq.) and 4,4,5,5,6,6,7,7,8,8,9,9,10,10,11,11,11-heptadecafluoroundecanoic acid (1.50 g, 3.0 mM, 1.0 eq.) in 20 ml anhydrous dichloromethane (CH_2Cl_2, Sigma) at room temperature. After 16 h stirring, the solution was diluted with CH_2Cl_2, washed by saturated sodium bicarbonate and extracted by CH_2Cl_2. The combined organic layer was washed by brine, dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was purified by flash column chromatography (SiO_2, eluent: 9 : 1 CH_2Cl_2/MeOH) as pale brown oil **1** (1.56 g, yield: 78%). ^1H NMR (400 MHz, $CDCl_3$) δ 2.49 (m, 2H), 2.58 (m, 2H), 3.49 (t, $J = 5.0$ Hz, 2H), 3.56 (t, $J = 5.1$ Hz, 2H), 3.67 (m, 4H), 3.71 (m, 4H), 3.81 (t, $J = 6.0$ Hz, 2H), 4.45 (t, $J = 6.0$ Hz, 2H), 6.26 (s, 1H). ESI-MS: $(M + H)^+ = m/z$ 668.1 (calculated MW of $C_{19}H_{22}F_{15}NO_5$: 667.4 Da).

16,16,17,17,18,18,19,19,20,20,21,21,22,22,23,23,23-heptadecafluoro-13-oxo-3,6,9-trioxa-12-azatricosyl 4-nitrobenzoate (2)

4-Nitrophenyl chloroformate (0.72 g, 3.6 mM, 1.5 eq.) in 5 ml anhydrous CH_2Cl_2 was slowly added to a solution of **1** (1.4 g, 2.4 mM, 1.0 eq.) and DiEA (0.93 g, 7.2 mM, 3.0 eq.) in 15 ml anhydrous CH_2Cl_2 at 4 °C. The mixture was stirred for 16 h while gradually warmed to room temperature. The reaction was quenched by adding ice-cold 0.5 N HCl, followed by CH_2Cl_2 extraction. The organic layer was washed by H_2O, brine, dried over $MgSO_4$ and concentrated *in vacuo*. The crude product was purified by flash column chromatography (SiO_2, eluent: 1 : 1 hexane/ethylacetate) as pale yellow waxy solid **2** (1.80 g, yield:

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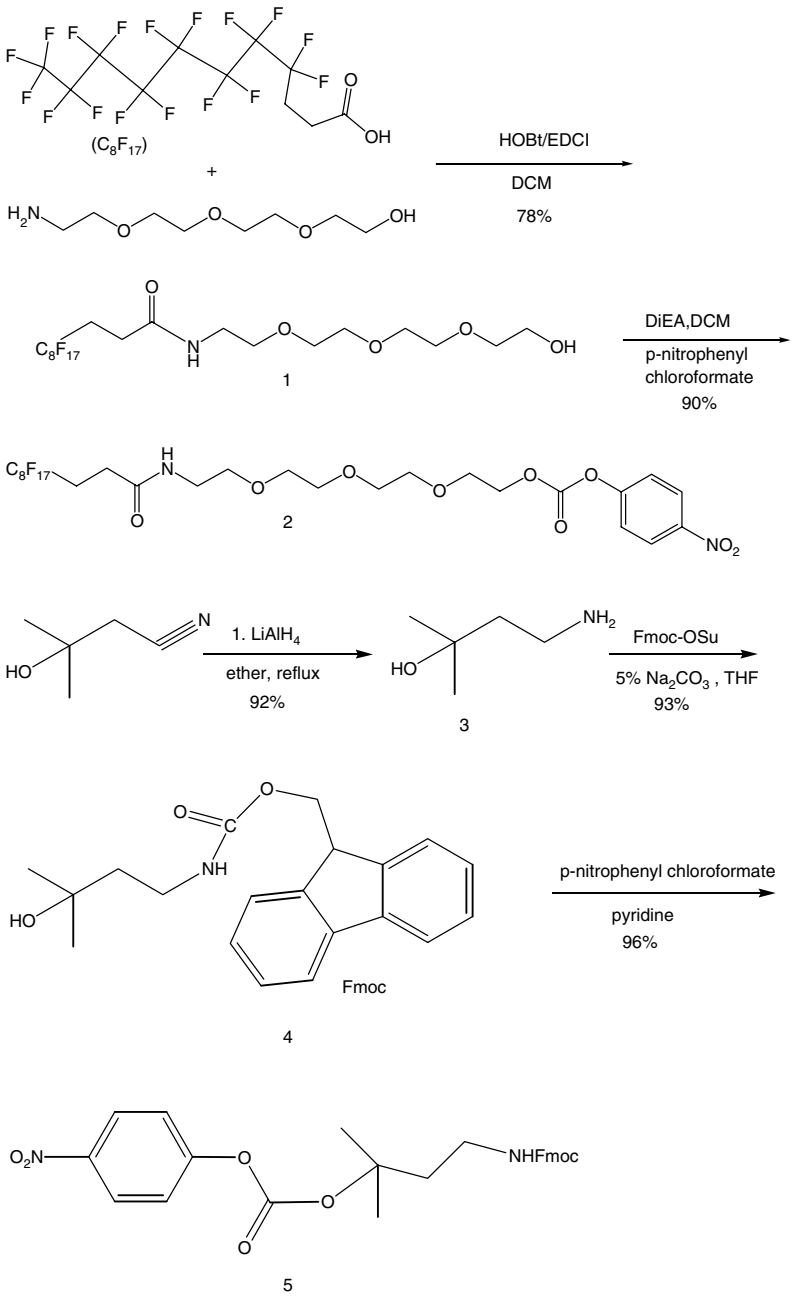
90%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.46 (m, 2H), 2.49 (m, 2H), 3.47 (t, $J = 5.0$ Hz, 2H), 3.57 (t, $J = 5.0$ Hz, 2H), 3.65 (m, 4H), 3.70 (m, 4H), 3.81 (t, $J = 6.0$ Hz, 2H), 4.44 (t, $J = 6.0$ Hz, 2H), 6.25 (s, 1H), 7.38 (d, $J = 9.2$ Hz, 2H), 8.28 (d, $J = 9.2$ Hz, 2H). ESI-MS: $(M + H)^+ = m/z$ 817.1 (calculated MW of $C_{26}H_{25}F_{17}N_2O_8$: 816.5 Da).

4-Amino-2-methyl-butane-2-ol (3)

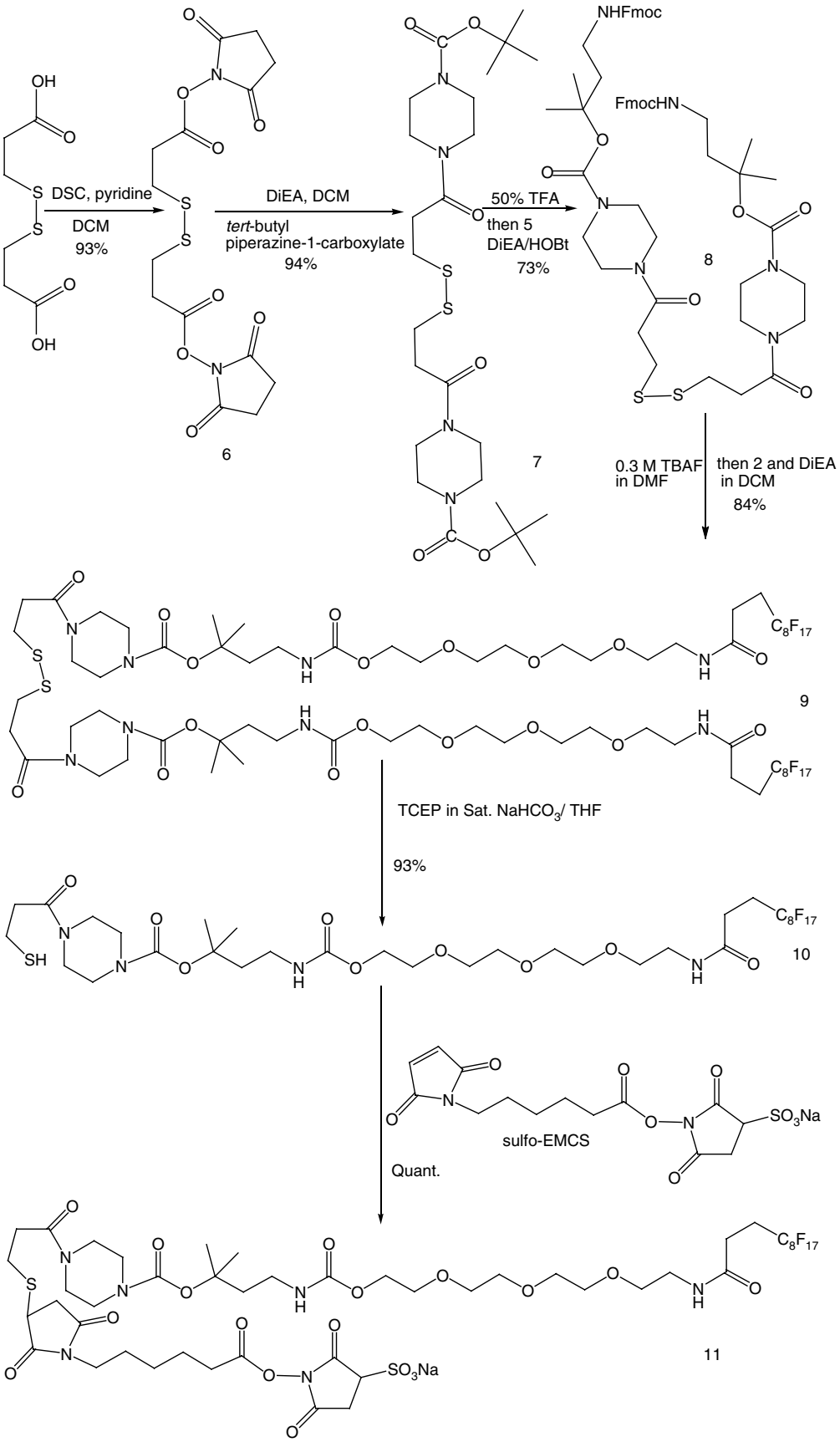
To a vigorously stirred solution of $LiAlH_4$ (2.8 g, 74 mM, 5.0 eq.) in 140 ml ice-cold dry ether, 2-methylpent-4-yn-2-ol (1.45 g, 14.8 mM, 1.0 eq.) in 50 ml dry ether was slowly added. After stirring for 1 h at room temperature, the mixture was refluxed for 10 h. After cooling, 15 ml ice-cold 10% NaOH was slowly added under stirring to quench the reaction. After an additional 2 h stirring, precipitates were filtered and washed thoroughly with 70 ml THF three times. After the removal of THF *in vacuo*, 80 ml of 15% NaOH was added to the residue followed by extraction with 90 ml of CH_2Cl_2 three times. The organic phases were combined, washed by brine and dried over $MgSO_4$ overnight. Solvents were evaporated to afford product as pale yellow oil **3** (1.40 g, yield: 92%), which was used for the next step without further purification.

(3-Hydroxy-3-methyl-butyl)-carbamic acid 9H-fluoren-9-ylmethyl ester (4)

Compound **3** (0.60 g, 5.8 mM, 1.0 eq.) was taken up in 20 ml of 5% Na_2CO_3 and cooled in an ice-water slurry bath. Fmoc-Osu (2.16 g, 6.4 mM, 1.1 eq.) in 25 ml of THF was slowly added to the mixture. After stirring for 16 h, THF was removed *in vacuo* and



Scheme 1. Synthesis of sulfo-NHS-$(OEG)_3$-perfluorooctane.



Scheme 1. (*Continued*).

the aqueous phase was extracted by 70 ml of CH_2Cl_2 three times. The extracts were combined, washed by brine, dried over $MgSO_4$ and evaporated to dryness. Crude product was purified by flash column chromatography (SiO_2, eluent: 6 : 1 CH_2Cl_2/ethylacetate) as white solid **4** (1.75 g, yield: 93%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.27 (s, 6H), 1.71 (t, J = 6.8 Hz, 2H), 3.64 (t, J = 6.8 Hz, 2H), 4.22 (t, J = 6.8 Hz, 1H), 4.38 (d, J = 6.8 Hz, 2H), 5.37 (s, 1H), 7.31 (t, J = 7.2 Hz, 2H), 7.40 (t, J = 7.2 Hz, 2H), 7.60 (d, J = 7.2 Hz, 2H), 7.76 (d, J = 7.2 Hz, 2H). ESI-MS: $(M + H)^+ = m/z$ 326.2 (calculated MW of $C_{20}H_{23}NO_3$: 325.4 Da).

Carbonic acid 3-(9H-fluoren-9-ylmethoxycarbonylamino)-1, 1-dimethyl-propyl ester 4-nitro-phenyl ester (5)

To a solution of **4** (0.76 g, 2.3 mM, 1.0 eq.) and pyridine (5 ml) in 25 ml of CH_2Cl_2 was slowly added 4-nitrophenyl chloroformate (0.94 g, 4.6 mM, 2.0 eq.) in 9 ml of CH_2Cl_2 at 0 °C. The mixture was stirred for 8 h at room temperature and poured into 90 ml of cold 0.5 N HCl. CH_2Cl_2 (300 ml) was used three times for extraction and the combined extracts were washed by brine, dried over $MgSO_4$ before evaporation to dryness. Crude product was purified by flash column chromatography (SiO_2, eluent: 5 : 1 hexane/ethylacetate) as colorless to light yellow oil **5** (1.10 g, yield: 96%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.60 (s, 6H), 2.07 (t, J = 7.6 Hz, 2H), 3.40 (t, J = 7.6 Hz, 2H), 4.22 (t, J = 6.8 Hz, 1H), 4.42 (d, J = 6.8 Hz, 2H), 4.93 (s, 1H), 7.28 (t, J = 7.2 Hz, 2H), 7.23 (t, J = 7.2 Hz, 2H), 7.35 (d, J = 8.4 Hz, 2H), 7.40 (t, J = 7.2 Hz, 2H), 7.58 (d, J = 7.2 Hz, 2H), 7.76 (d, J = 7.2 Hz, 2H), 8.24 (d, J = 8.4 Hz, 2H). ESI-MS: $(M + H)^+ = m/z$ 491.2 (calculated MW of $C_{27}H_{26}N_2O_7$: 490.5 Da).

Bis(2,5-dioxopyrrolidin-1-yl) 3,3′-disulfanediyldipropanoate (6)

To a solution of 3,3′-disulfanediyldipropanoic acid (50 mg, 0.24 mM, 1.0 eq.) and pyridine (0.11 ml, 6.0 eq.) in 3 ml of CH_2Cl_2 was added *N,N′*-disuccinimidyl carbonate (182 mg, 0.72 mM, 3.0 eq.). The mixture was stirred at room temperature overnight and then diluted by 10 ml of CH_2Cl_2 before pouring into 10 ml of cold 0.2 N HCl. An additional 50 ml of CH_2Cl_2 was used for extractions and the extracts were washed by brine, dried over $MgSO_4$ before evaporation to dryness. Crude product was purified by flash column chromatography (SiO_2, eluent: 2 : 3 hexane/ethylacetate) as white solid **6** (89 mg, yield 93%). ESI-MS: $(M + H)^+ = m/z$ 405.5 (calculated MW of $C_{14}H_{16}N_2O_8S_2$: 404.4 Da).

Di-tert-butyl 4,4′-(3,3′-disulfanediylbis(propanoyl))bis(piperazine-1-carboxylate) (7)

To a solution of **6** (0.5 g, 1.23 mM, 1.0 eq.) and DiEA (0.47 ml) in 11 ml CH_2Cl_2 was slowly added *tert*-butyl piperazine-1-carboxylate (0.51 g, 2.72 mM, 2.2 eq.) in 5 ml of CH_2Cl_2 at room temperature. After 3 h stirring, solvents were evaporated and crude product was recrystallized by hexane/ethylacetate as white solid **7** (0.62 g, yield: 94%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.47 (s, 18H), 2.76 (t, J = 6.8 Hz, 4H), 2.97 (t, J = 6.8 Hz, 4H), 3.46 (m, 8H), 3.48 (t, J = 4.9 Hz, 4H), 3.60 (t, J = 4.9 Hz, 4H). ESI-MS: $(M + H)^+ = m/z$ 547.4 (calculated MW of $C_{24}H_{42}N_4O_6S_2$: 546.7 Da).

Bis(4-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-2-methylbutan-2-yl)4,4′-(3,3′-disulfanediylbis(propanoyl))bis(piperazine-1-carboxylate) (8)

Compound **7** (0.5 g, 0.91 mM, 1.0 eq.) was taken up by 2.5 ml of 50% TFA in CH_2Cl_2 under stirring at room temperature. After 1 h of cleavage reaction, solvents were evaporated under reduced pressure and the resulting residue was mixed with 6 ml of CH_2Cl_2, 1.7 ml of DiEA and HOBt (27.6 mg, 0.20 eq.). To this mixture was added compound **5** (0.98 g, 2.0 mM, 2.2 eq.) in 5 ml of CH_2Cl_2. After stirring for 16 h, 30 ml of cold 0.5 N HCl was added to quench the reaction and 200 ml of CH_2Cl_2 was then used for three extractions. The extracts were washed by brine, dried by $MgSO_4$ and evaporated to dryness. Crude product was purified by flash column chromatography (SiO_2, eluent: ethylacetate) as white solid **8** (0.7 g, yield: 73%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.49 (s, 12H), 2.30 (t, J = 6.8 Hz, 4H), 2.74 (t, J = 7.0 Hz, 4H), 2.95 (t, J = 7.0 Hz, 4H), 3.28 (t, J = 6.8 Hz, 4H), 3.41 (m, 4H), 3.44 (m, 8H), 3.57 (m, 4H), 4.20 (t, J = 6.4 Hz, 2H), 4.40 (d, J = 6.4 Hz, 4H), 4.80 (s, 2H), 7.31 (t, J = 7.6 Hz, 4H), 7.40 (t, J = 7.6 Hz, 4H), 7.58 (d, J = 7.6 Hz, 4H), 7.62 (d, J = 7.6 Hz, 4H). ESI-MS: $(M + H)^+ = m/z$ 1049.9 (calculated MW of $C_{56}H_{68}N_6O_{10}S_2$: 1049.3 Da).

23,23,24,24,25,25,26,26,27,27,28,28,29,29,30,30,30-Heptadecafluoro-2-methyl-6,20-dioxo-7,10,13,16-tetraoxa-5,19-diazatriacontan-2-yl4-(3-((3-(4-(24,24,25,25,26,26,27,27,28,28,29,29,30,30,31,31,31-heptadecafluoro-3,3-dimethyl-7,21-dioxo-2,8,11,14,17-pentaoxa-6,20-diazahentriacontan-1-oyl)piperazin-1-yl)-3-oxopropyl)disulfanyl)propanoyl)piperazine-1-carboxylate (9)

To a solution of **8** (84 mg, 0.8 mM, 1.0 eq.) in 0.7 ml anhydrous DMF was added 66 mg TBAF. After 2 h, TLC showed complete de-protection and compound **2** (135 mg, 0.16 mM, 2.0 eq.) in 4 ml anhydrous CH_2Cl_2 was slowly added at room temperature, followed by the addition of DiEA (0.03 ml). The mixture was stirred overnight, and then the reaction was quenched by adding 10 ml of ice-cold 0.2 N HCl. The separated aqueous phase was further extracted by using 60 ml of CH_2Cl_2 three times. The extracts were washed by brine, dried by $MgSO_4$ and evaporated to dryness. Crude product was purified by flash column chromatography (SiO_2, eluent: 13 : 1 CH_2Cl_2/MeOH) as pale oil **9** (135 mg, yield: 84%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.47 (s, 12H), 2.00 (t, J = 7.2 Hz, 4H), 2.47 (m, 4H), 2.51 (m, 4H), 2.76 (t, J = 7.1 Hz, 4H), 2.95 (t, J = 7.0 Hz, 4H), 3.25 (q, J = 7.2 Hz, 4H), 3.40 (m, 16H), 3.56 (t, J = 5.0 Hz, 4H), 3.60 (m, 24H), 4.19 (t, J = 5.0 Hz, 4H), 4.99 (s, 2H), 6.63 (s, 2H). ESI-MS: $(M + H)^+ = m/z$ 1992.5 (calculated MW of $C_{66}H_{88}F_{34}N_8O_{18}S_2$: 1991.5 Da).

23,23,24,24,25,25,26,26,27,27,28,28,29,29,30,30,30-Heptadecafluoro-2-methyl-6,20-dioxo-7,10,13,16-tetraoxa-5,19-diazatriacontan-2-yl 4-(3-mercaptopropanoyl)piperazine-1-carboxylate (10)

A volume of 0.6 ml of TCEP·HCl (65 mg, 0.23 mM, 4.0 eq.) in saturated $NaHCO_3$ was added to 3 ml of THF solution of compound **9** (113 mg, 0.057 mM, 1.0 eq.). After stirring for 9 h, solvents were evaporated under reduced pressure. The crude product was purified by flash column chromatography (SiO_2, eluent: 20 : 1 CH_2Cl_2/MeOH) as pale brown oil **10** (52 mg, yield: 93%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.47 (s, 6H), 1.73 (t, J = 8.0 Hz, 1H), 2.00 (t, J = 7.2 Hz, 2H), 2.49 (m, 2H), 2.52 (m, 2H), 2.65 (t, J = 6.8 Hz, 2H), 2.81 (q, J = 7.2 Hz, 2H), 3.24 (q, J = 7.2 Hz, 2H), 3.40 (m, 8H), 3.56 (t, J = 5.2 Hz, 2H), 3.63 (m, 12H), 4.19 (t, J = 5.2 Hz, 2H), 4.99 (s, 1H), 6.63 (s, 1H). ESI-MS: $(M + H)^+ = m/z$ 997.3 (calculated MW of $C_{33}H_{45}F_{17}N_4O_9S$: 996.8 Da).

Sodium 1-((6-(3-((3-(4-(24,24,25,25,26,26,27,27,28,28,29,29,30,30,31,31,31-heptadecafluoro-3,3-dimethyl-7,21-dioxo-2,8,11,14,17-pentaoxa-6,20-diazahentriacontan-1-oyl)piperazin-1-yl)-3-oxopropyl)thio)-2,5-dioxopyrrolidin-1-yl)hexanoyl)oxy)-2,5-dioxopyrrolidine-3-sulfonate(sulfo-NHS-(OEG)$_3$-perfluorooctane) (11)

To a solution of **10** (10 mg, 24 mM, 1.0 eq.) in anhydrous DMF was added sulfo-EMCS (sodium salt, 25.5 mg, 25.6 mM, 1.05 eq.) and the mixture was stirred at room temperature for 1 h and then for an additional 3 h at 40 °C. Solvents were removed under reduced pressure and final product **11** was obtained as pale yellow waxy solid. ^{1}H NMR (400 MHz, DMSO-d$_6$) δ 1.30 (q, J = 7.2 Hz, 2H), 1.38 (s, 6H), 1.49 (q, J = 7.2 Hz, 2H), 1.60 (q, J = 7.2 Hz, 2H), 1.84 (t, J = 7.6 Hz, 2H), 2.41 (m, 4H), 2.44 (t, J = 7.2 Hz, 2H), 2.62 (t, J = 6.8 Hz, 2H), 2.70 (m, 2H), 2.84 (m, 2H), 3.02 (m, 2H), 3.20 (q, J = 6.0 Hz, 2H), 3.30–3.51 (m, 24H), 4.02 (m, 4H), 7.18 (t, J = 5.6 Hz, 1H), 8.12 (t, J = 5.6 Hz, 1H). Negative ion ESI-MS: $(M-H)^- = m/z$ 1383.2 (calculated MW of $C_{47}H_{60}F_{17}N_6NaO_{18}S_2$: 1407.1 Da).

Labeling of ACTH (4–11) using sulfo-NHS-(OEG)$_3$-perfluorooctane

A volume of 10 µl of 1 mg/ml ACTH (4–11) was diluted in 120 µl of PBS 8.0 and the solution was added to a 1.5-ml vial containing 0.038 mg labeling reagent (sulfo-NHS-(OEG)$_3$-perfluorooctane, see structure in Fig. 1). The labeling proceeded at room temperature for 1 h, and then excess labeling reagent was consumed by reacting with approximate 0.5 mg polymer-bound tris(2-aminoethyl)-amine for 1 h. After centrifugation, the polymer beads were washed by 100 µl of 80% MeOH and 100% MeOH twice, and supernatants were combined. A volume of 1 µl of the collected supernatant was resuspended in 100 µl of 2.5% FA in MeOH for direct ESI-MS analysis. The rest of the supernatant was vacuum dried and resuspended in 50 µl of TFA for cleavage. The cleavage reaction was completed after 1 h at 37 °C. After the removal of the TFA under reduced pressure, residues were dissolved in 100 µl of 50% MeOH containing 2.5% FA for ESI-MS analysis.

Labeling of BSA using sulfo-NHS-(OEG)$_3$-perfluorooctane and affinity purification of the labeled tryptic BSA peptides

A volume of 10 µl of 10 mg/ml BSA stock solution was diluted in 35 µl PBS buffer (pH 8.0) and the solution was transferred to a vial containing 0.26 mg fluorous reagent, followed by a brief vortex. The mixture was incubated for 2 h at room temperature while shaking. The excess labeling reagent was consumed by mixing with 15 µl of 50 mm tris–HCl for about 1 h. The labeled BSA was then subjected to in-solution trypsin digestion. Briefly, 80 µl of 8.0 M urea was added to the labeling mixture, followed by the addition of 1.7 µl of 200 mM DTT in 50 mM ammonium bicarbonate for 1 h reduction at 45 °C. A volume of 6 µl of 200 mM IA in 50 mM ammonium bicarbonate was added for 1 h alkylation at room temperature in the dark, and the excess IA was consumed by incubation with 6 µl of DTT for 1 h at room temperature. The mixture was diluted by adding 1 ml of 50 mM ammonium bicarbonate before trypsin (6 µg) digestion at 37 °C for 15 h. The digestion was stopped by adding 5% FA until colorless precipitate was observed (pH 3.0, hydrolysis by-product of the labeling reagent); the mixture was then separated by centrifugation and the precipitates were further washed by 200 µl of 5% FA. Combined supernatant was evaporated to dryness and one-sixth of the sample was resuspended in 450 µl of buffer A (20 mM ammonium formate, 0.1 M acetic acid in 60% MeOH) for fluorous affinity purification. Tryptic BSA mixture was loaded onto a cartridge packed with fluorous silica gel beads at 10 µl/min by syringe pump. After washing with buffer A (3 ml), the retained modified peptides were eluted by 2 ml of buffer B (20 mM ammonium formate, 0.1 M acetic acid in 100% MeOH) at a flow rate of 10 µl/min. The eluate was dried under vacuum and resuspended in 95% TFA with 5% anisole for incubation at 37 °C for 2 h. After removal of solvents, cleaved peptides were resuspended in 50 µl of 5% FA, 2% ACN and 6 µl was injected for LC–MS analysis.

LC–MS/MS analysis and database searching

LC–ESI MS/MS was performed on a Finnigan LTQ™-ion trap mass spectrometer (Thermo Electron, San Jose, CA). Peptide sample (8 µl) was first loaded onto a C$_{18}$ trapping column and washed with 3% acetonitrile and 0.1% formic acid for 60 min for desalting, then the peptides were eluted onto a reversed-phase C$_{18}$ analytical column (PicoFrit Column:75 µm ID, 15 µm tip ID, packed with 5 µm BioBasic C$_{18}$, 10 cm length, New Objective, Woburn, MA) by a 60-min gradient made of A buffer (0.1% formic acid/97% water/3% acetonitrile, v/v/v) and B buffer (0.1% formic acid/3% acetonitrile/97% water, v/v/v) at a flow rate of 200–500 nl/min. Separated peptides were analyzed under the data-dependent acquisition mode controlled by Xcalibur, 2.2 version (Thermo Electron). After a survey scan in the mass range m/z 300–2000, the seven most intense precursor ions were selected and subjected to fragmentation by collision-induced dissociation (CID). The normalized collision energy was set at 35% with activation Q value being 0.25 and dynamic exclusion of 100 s. The acquired raw data were processed by BioWorks software, version 3.3 (Thermo Electron). The parameters for SEQUEST database searching were set as follows: differential mass increase of 57.02 Da on cysteinyl residue, 15.99 Da on methionine and 367.02 Da on lysine. The number of missed cleavage sites was set to three. The search results were filtered by cross-correlation score (XCorr), i.e. 2.0 for singly charged peptide ions, 2.5 for doubly charged peptide ions and 3.0 for the triply charged ions. MS/MS spectra for detected modified peptides were manually examined to ensure the quality of identifications.

Results and Discussion

The fluorous labeling reagent, sulfo-NHS-(OEG)$_3$-perfluorooctane, can be divided into six building blocks (Fig. 1). The synthesis of this labeling reagent was based on a retrosynthetic analysis shown in Fig. 2. The target product, sulfo-NHS-(OEG)$_3$-perfluorooctane, can be obtained through a highly efficient coupling reaction between **1** (sulfo-EMCS, blocks A and B in Fig. 1) and a thiol compound **2** (blocks C–F in Fig. 1), which can be derived from the cleavage of the disulfide bond in its dimer, compound **3**. Compound **3** can be synthesized through formation of a carbamate using **4** and carbonate compound **5** after the removal of the Fmoc group with TBAF in DMF. The acid labile linker in compound **4** can be constructed through carbonate compound **7**[19] and piperazine derivative **6**, which can be easily prepared from dithiobis(succinimidyl propionate) **9** and 1-Boc-piperazine **10**. Compound **5** can be prepared from hydroxyl compound **8** by reacting with *p*-nitrophenylchloroformate. The (OEG)$_3$ spacer in compound **8** can be introduced by the incorporation of (OEG)$_3$-mono-amine **12** to fluorous compound **11** via amide bond

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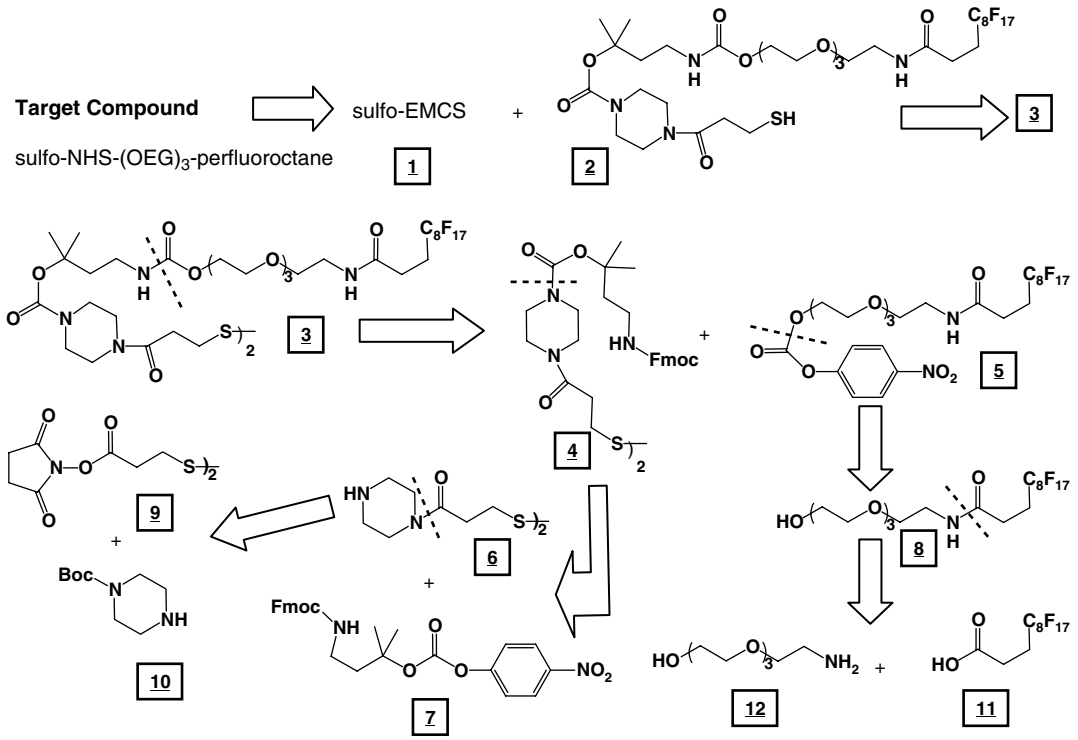


Figure 2. Retrosynthetic analysis of sulfo-NHS-$(OEG)_3$-perfluorooctane.

formation. Experimental procedures for compiling those building blocks are detailed in Scheme 1.

The fluorous labeling reagent was synthesized via a 10-step reaction (Scheme 1) with an overall yield of 21%. This newly synthesized sulfo-NHS-$(OEG)_3$-perfluorooctane was characterized by negative ion electrospray mass spectrometry and by NMR. Dissolved in 50% MeOH, the labeling reagent was detected by negative ion mass spectrometry as shown in Fig. 3 (bottom panel). Upon CID, the molecular anion [sulfo-NHS-$(OEG)_3$-perfluorooctane – H]$^-$ at *m/z* 1383.3 yielded two major product ions at *m/z* 605.0 and 560.9 (Fig. 3, middle panel) corresponding to the product ion from the cleavage at the tertiary C–O bond in block D (Fig. 1) and subsequent loss of a CO_2 Further fragmentation of the product ion at *m/z* 605.0 yielded the fragment ion at *m/z* 560.9 (Fig. 3, top panel) after the neutral loss of CO_2. The product ion at *m/z* 560.9 in the MS/MS spectrum of *m/z* 1383.3 and the ion at *m/z* 560.9 in the MS/MS/MS spectrum of *m/z* 605.0 were the same, both ions yielded *m/z* 387.0 (spectrum not shown) upon CID, corresponding to the formation of sulfo-EMCS as the result of thiol ether bond (between blocks B and C in Fig. 1) cleavage. This mass spectral evidence supports the structure of newly synthesized sulfo-NHS-$(OEG)_3$-perfluorooctane as shown in Fig. 1. It appears that the bonds that link the different building blocks are also the favored sites of cleavage upon fragmentation of the molecule.

Sulfo-NHS-$(OEG)_3$-perfluorooctane was employed to label a small peptide ACTH (4–11) (NH_2-M-E-H-F-R-W-G-K) in PBS at room temperature (Scheme 2). After 1 h reaction time, the free amine groups (*N*-terminal and lysine side chain) in ACTH (4–11) were almost completely tagged by the labeling reagent (approximately 29% of the peptides were singly tagged at either *N*-terminal or lysine side chain, 68% were doubly tagged at both *N*-terminal and lysine side chain and 3% were untagged). The bulky part (corresponding to building blocks D–F in Fig. 1) of the labeling reagent was trimmed from the labeled ACTH peptide upon treatment with TFA as expected, leaving a small tag of 367.2 Da (net mass increase to the amine group in the peptide by adding building blocks B and C in Fig. 1). The cleavage of the tertiary carbamate group (in block C and D, Fig. 1) by strong acid is quantitative,[20] as further evidenced by the fact that no ACTH peptide with uncleaved sulfo-NHS-$(OEG)_3$-perfluorooctane tag could be found on the ESI-MS spectrum of the labeling reaction mixture after acid cleavage.

The positive ion ESI-MS/MS spectrum of the labeled ACTH peptide (after TFA cleavage) is shown in Fig. 4. Most of the *b* and *y* ions can be observed. Interestingly, a fragment ion at *m/z* 368.1 (which corresponds to the residual of sulfo-NHS-$(OEG)_3$-perfluorooctane cleaved from the amide bonds formed during the labeling reaction, its structure is shown in Scheme 2) also appeared on the MS/MS spectrum; and this ion could serve as a marker of the labeling and fluorous tag moiety removal processes. The newly synthesized fluorous labeling reagent indeed efficiently modified the free amine groups in the peptide; and the fluorous tag moiety could be easily trimmed. The residual of sulfo-NHS-$(OEG)_3$-perfluorooctane (building blocks B and C in Fig. 1) after acid cleavage did not interfere with the fragmentation of the labeled peptide, which provided an informative decomposition pattern for unambiguous identification of the peptide.

To further demonstrate the potential of the sulfo-NHS-$(OEG)_3$-perfluorooctane in protein/peptide modification, it was employed to label BSA in PBS. No precipitation was observed throughout the labeling reaction, which indicated that the labeling can be performed in aqueous solution without introducing any organic solvent to the reaction mixture. After trypsin digestion, the tagged peptides were easily enriched through fluorous affinity purification. After TFA cleavage as detailed above, the tagged peptides were analyzed by LC–MS/MS. About 95% of the

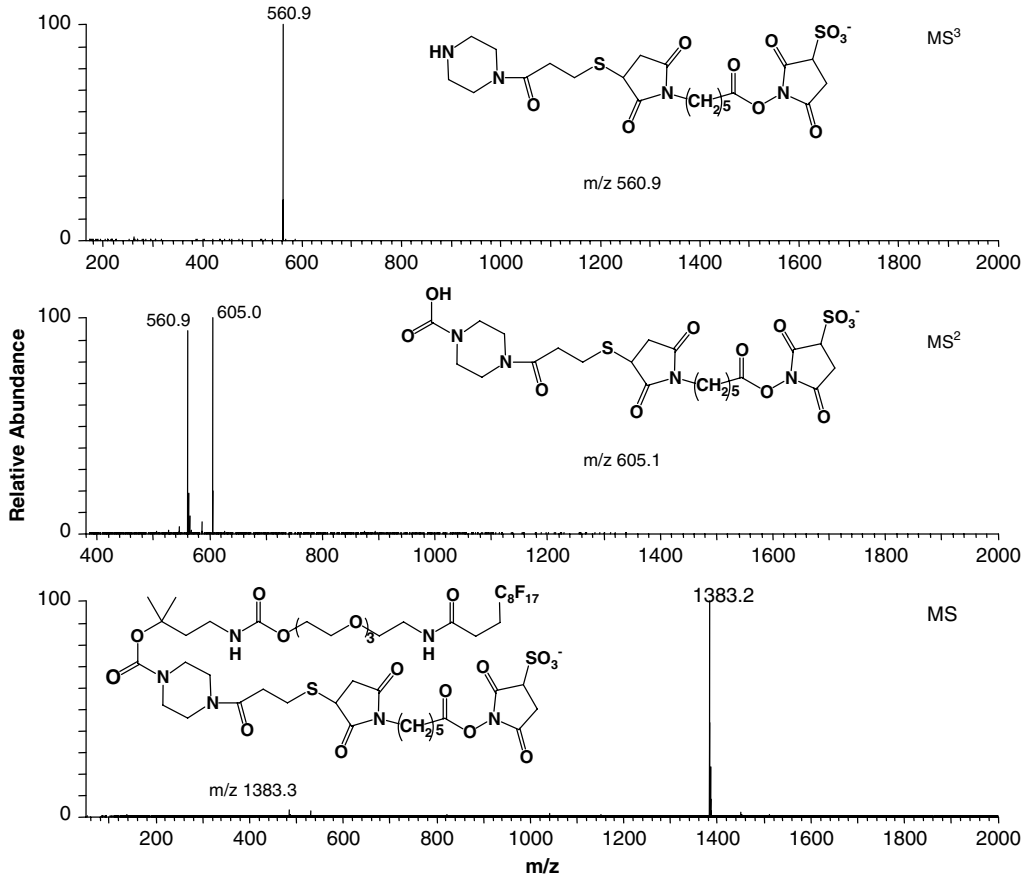
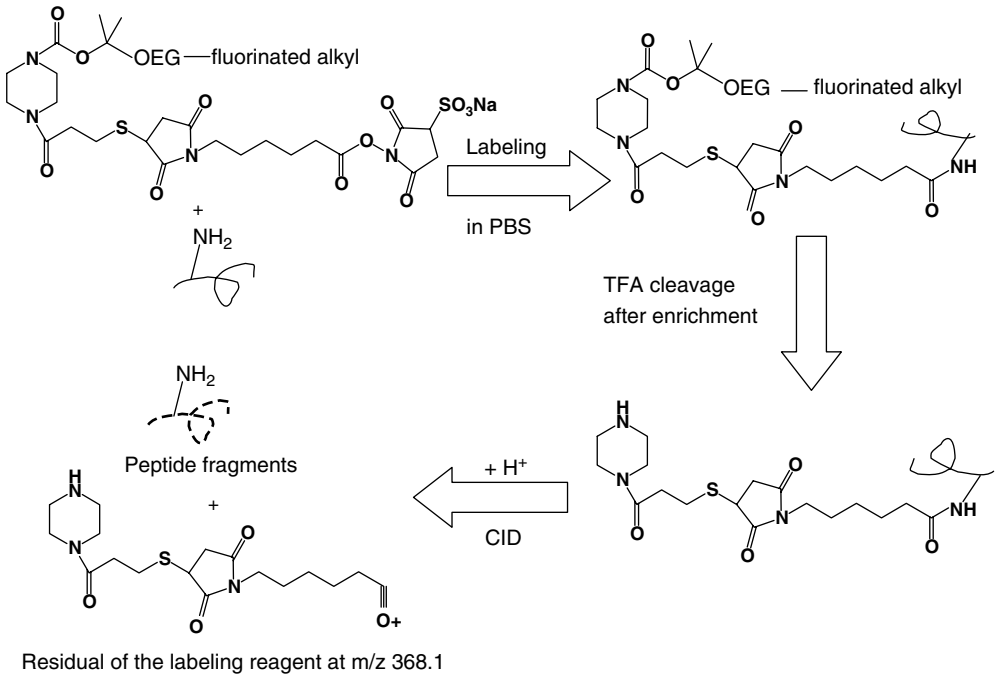


Figure 3. Negative ion electrospray mass spectrometry analysis of sulfo-NHS-$(OEG)_3$-perfluorooctane and its fragmentation pattern. (Bottom panel) MS spectrum of the labeling reagent. Singly charged anion at *m/z* 1383.3 corresponds to deprotonated sulfo-NHS-$(OEG)_3$-perfluorooctane. (Middle panel) MS^2 spectrum of precursor ion at *m/z* 1383.3. Product ion at *m/z* 605.0 corresponds to cleavage of the precursor ion at the tertiary C–O bond in building block D in Fig. 1. (Top panel) MS^3 spectrum of ion at *m/z* 605.0. Upon CID, *m/z* 605.0 undergoes neutral loss of CO_2 to yield product ion at *m/z* 560.9.

identified peptides were tagged (Table 1). This indicates that affinity purification based on fluorine–fluorine interaction was indeed highly effective in enriching labeled peptides; non-specific binding was thus minor. The sulfo-NHS-$(OEG)_3$-perfluorooctane tagged peptides had longer retention times on the reversed-phase C_{18} column due to the increased hydrophobicity introduced by the fluorous alkyl chain. Once the fluorous tag moiety was trimmed, the elution profile of tagged peptide was similar to that of the unmodified peptides in reversed-phased LC separation. Fifteen out of 16 tryptic peptides (Table 1) were identified as tagged peptides in a single LC–MS/MS experiment followed by database searching against BSA sequence using SEQUEST algorithm. All these tagged tryptic peptides contain at least one labeled lysine residue in the middle of their sequences, as trypsin does not cleave the protein at labeled lysine residues. Most of the tryptic peptides in Table 1 have a C-terminal arginine. If the BSA was fully unfolded to allow all lysine residues to be labeled at their free amino group, then only peptides with C-terminal arginine (Arg-C) would be produced by trypsin digestion, and in this case trypsin would serve as an Arg-C protease. As shown in Fig. 5, the residual fluorous tag had little influence on the fragmentation of the labeled BSA peptides. In addition to the *b* and *y* ions that verify the peptide sequence, a characteristic ion at *m/z* 368.1 (structure in Scheme 2) appeared on the spectrum to confirm that this peptide segment of BSA was indeed labeled by the fluorous reagent.

Unlike ICAT reagents[4,6,7] that target free thiols (e.g. side chain of unmodified cysteine residue), sulfo-NHS-$(OEG)_3$-perfluorooctane targets primary amine groups (e.g. unmodified side chain of the lysine residue) which is about threefold more abundant than the cysteine residue in living organisms.[21] Moreover, conjugating sulfo-NHS ester (sulfo-*N*-hydroxylsuccinimidyl ester) to a primary amine is a robust reaction that has been widely employed in many biological fields.[22–27] The overall size of sulfo-NHS-$(OEG)_3$-perfluorooctane is large, the reactive group (sulfo-NHS ester) and the bulky fluorous tag moiety are linked by a long spacer of OEG and alkyl chain, and this long spacer minimizes the steric effect imposed by the fluorous tag moiety on the reactive sulfo-NHS ester. Moreover, incorporation of the OEG linker increases sulfo-NHS-$(OEG)_3$-perfluorooctane's solubility in aqueous environments, which is critical in labeling proteins and peptides, especially those located on living cell surfaces.

It is well documented that negatively charged sulfonate groups (SO_3^-) in a molecule impede diffusion of such a molecule into the cytosol because of the barrier of the non-polar lipid bilayer.[18] Moreover, the OEG spacer and fluorous tag moiety in sulfo-NHS-$(OEG)_3$-perfluorooctane may strengthen the molecule's membrane impermeability due to a combination of OEG spacer's hydrophilicity and perfluorocarbons' lipophobicity.[28] These properties of sulfo-NHS-$(OEG)_3$-perfluorooctane make this reagent desirable for labeling live mammalian and bacterial cell surfaces. Notably, it lacks certain limitations noted above caused by the



Scheme 2. Labeling of protein/peptide using sulfo-NHS-$(OEG)_3$-perfluorooctane.

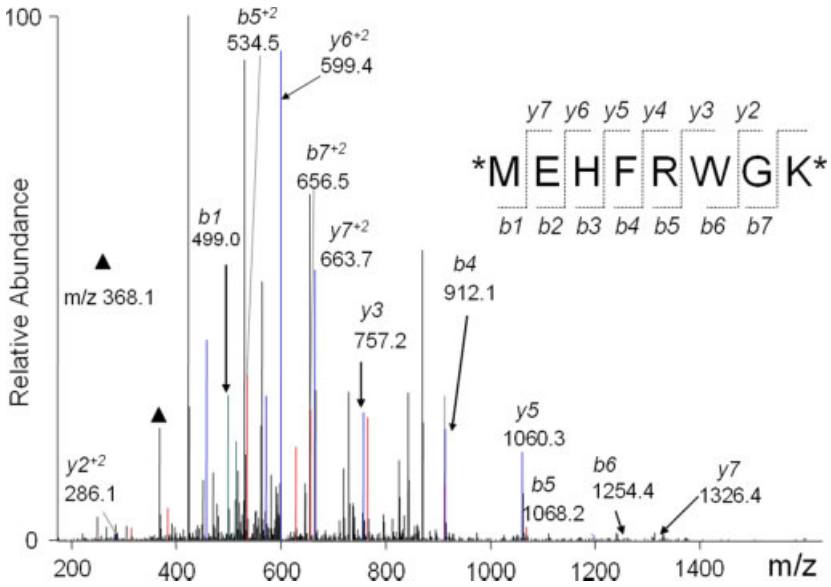


Figure 4. Positive ion MS/MS of peptide ACTH (4–11) labeled by sulfo-NHS-$(OEG)_3$-perfluorooctane and then treated with trifluoroacetic acid to trim the fluorinated alkyl moiety. Approximately 10 μg of the labeled ACTH was analyzed by ESI-MS using direct injection. A triply charged precursor ion at *m/z* 609.5 was isolated and fragmented upon CID to generate this MS^2 spectrum. *N*-terminus and the lysine side chain of the peptide were labeled. (▲) The unique fragment ion at *m/z* 368.1 corresponds to the residual of the labeling reagent. The series of *b* and *y* ions confirm the sequence of the peptide. Fragment ion mass error tolerance: 0.4 Da.

biotin moiety in sulfo-NHS–biotin (Pierce, Rockford, IL). The relatively large size, its hydrophilicity and its lipophobicity also make sulfo-NHS-$(OEG)_3$-perfluorooctane suitable to label fungal cell surfaces, and less likely to penetrate cell walls when it is compared to other smaller sulfo-NHS esters, such as sulfo-NHS–LC–biotin and sulfo-NHS–SS–biotin (Pierce). Thus, there should be much less ambiguity that sulfo-NHS-$(OEG)_3$-perfluorooctane labeled peptide segments are indeed exposed portions of fungal cell wall proteins.

In conclusion, a novel fluorous labeling reagent sulfo-NHS-$(OEG)_3$-perfluorooctane has been synthesized through 11 steps of simple chemical reactions with an overall yield of 21%. Sulfo-NHS-$(OEG)_3$-perfluorooctane reacts with free amine groups from peptides/proteins through an active sulfo-*N*-hydroxylsuccinimidyl ester group. The labeling reaction proceeded efficiently in PBS buffer without the presence of any organic solvent, which must be added into the reaction mixture in the case where water-insoluble fluorous labeling reagents are employed. More importantly, a homogeneous solution of reaction mixture was observed throughout the labeling process, which indicated that the fluorous labeling reagent was not only soluble

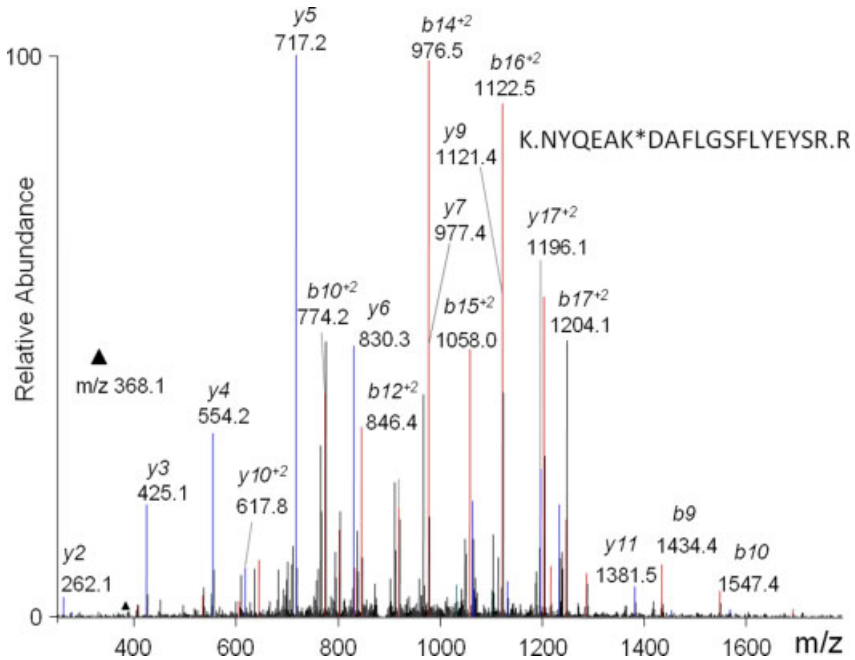


Figure 5. Positive ion collision-induced dissociation MS/MS of a BSA tryptic peptide tagged by labeling reagent sulfo-NHS-$(OEG)_3$-perfluorooctane at lysine residue (K*). After fluorous affinity enrichment and treatment with trifluoroacetic acid, labeled peptides derived from 2 μg of BSA were loaded on the HPLC column for LC–MS/MS analysis. The doubly charged precursor ion at *m/z* 890.7 yielded series of *b* and *y* ions that confirm the sequence of the peptide. (▲) The unique fragment ion at *m/z* 368.1 corresponds to the residual of the labeling reagent.

Table 1. Identified tryptic peptides derived from sulfo-NHS-(OEG)3-perfluorooctane labeled bovine serum albumin (BSA)[a]

Sequence of detected peptide	XCorr[b]
K.NYQEAK*DAFLGSFLYEYSR.R	6.22
R.ADLAK*YIC@DNQDTISSK.L	6.14
K.GLVLIAFSQYLQQC@PFDEHVK.L	5.80
R.ADLAK*YIC@DNQDTISSK*LK.E	5.55
R.LAK*EYEATLEEC@C@AK.D	5.16
K.EAC@FAVEGPK*LVVSTQTALA	4.86
K.FWGK*YLYEIAR.R	4.43
R.K*VPQVSTPTLVEVSR.S	4.32
K.SLHTLFGDELC@K*VASLR.E	4.28
R.ETYGDMADC@C@EK*QEPER.N	4.17
K.VTK*C@C@TESLVNR.R	3.79
R.ETYGDMADC@CEK*QEPER.N	3.67
R.ETYGDM#ADC@CEK*QEPER.N	3.56
R.ALK*AWSVAR.L	3.39
R.C@ASIQK*FGER.A	3.02
R.C@C@TK*PESER.M	2.64

*Lysine residue was modified by fluorous affinity tag, # methionine was oxidized and @cysteine was modified by iodoacetamide.
[a] Bulky perfluorooctane moiety and OEG linker of the labeling reagent were trimmed by acid cleavage, which resulted in a net mass increase of 367.1 Da on labeled lysine residue.
[b] Cross-correlation score determined by searching peptide tandem mass spectra against BSA sequence by the use of SEQUEST algorithm.

in water, but also, due to the incorporation of a hydrophilic $(OEG)_3$ linker, did not cause any aggregation of the labeled proteins. Fluorous affinity purification was performed to isolate the tagged tryptic peptides of BSA; the majority of peptides eluting from the fluorous affinity column were identified by LC–MS analysis as labeled peptides. Once the bulky fluorous tag moiety and the OEG linker were trimmed by acid cleavage, a small residue (from the fluorous labeling reagent) still attached to the peptides had minimal influence on the elution profile on C_{18} chromatography and on MS/MS fragmentation pattern of the labeled peptides. All of these results indicate that labeling proteins/peptides by the newly synthesized sulfo-NHS-$(OEG)_3$-perfluorooctane followed by affinity separation/enrichment can be readily incorporated into most LC–MS-based proteomic approaches. The combination of its size, solubility in water, hydrophilicity of its spacer moiety and lipophobicity of its fluorinated alkyl moiety makes this novel sulfo-*N*-hydroxylsuccinimidyl ester very suitable for labeling living cell surface proteins/peptides.

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