Synthesis of a New Organic Linker Molecule for Protein Immobilization

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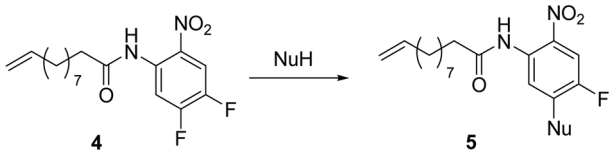
Recent biological and medicinal experimental techniques for diagnosis and drug discovery utilize microarrays of proteins immobilized on solid substrates.[1] Such protein "chips" can have proteins on surface attached covalently[2,3] or non-covalently[4] between proteins and linker molecules frequently by chemisorption of the film components to the substrate from solution.[5] For the well defined systems, organic monolayer films have formed versatile model study. The arrays of proteins attached to a solid surface have a great potential for detecting interactions of protein-ligand, protein-protein, and antibody-antigen.

Among several self-assembling systems, organosulfur compounds including dialkyl sulfides,[6] dialkyl disulfides,[7] and thiols[8] have been investigated intensively on gold. It has been recognized that self-assembled monolayers (SAM) using organosufur compounds on gold are advantageous over other SAM systems because of higher structural order by densely packing long chains. In addition, versatile functionalization of terminal group at the monolayer surface suggests ideal model systems for immobilization and interfacial interaction. Several functional groups including anhydride,[9] succinimidyl ester,[10] maleimide,[11] and phosphinothioester[12] have been applied for the chemoselective binding of proteins.

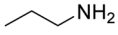
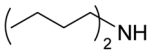
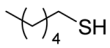
Here, we report a new potential substrate for the covalent and specific immobilization of nucleophilic molecules. The new organic linker, disulfide **1**, has 4, 5-difluoro-2-nitroaniline amide moiety on the head and a long carbon chain with a triazole group. We envisioned that the compound would be prepared *via* 1,3-dipolar cycloaddition of azide **2** and alkyne **3**,[13] and hoped that a Meisenheimer complex[14] of 4,5-difluoro-2-nitroaniline amide moiety would serve for a covalent attachment of amine or thiol moieties, and eventually proteins on the surface (Scheme 1).

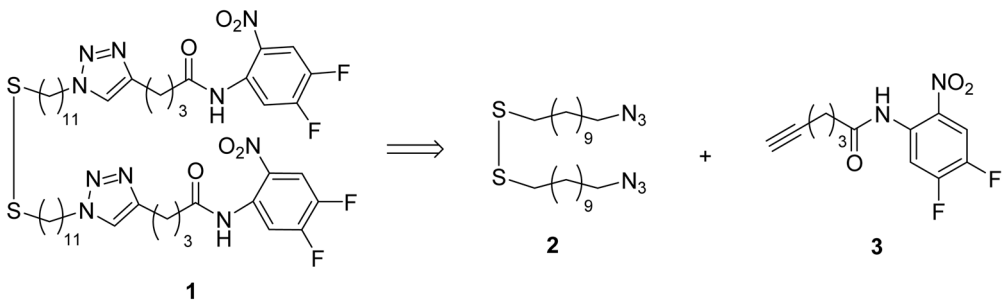
Prior to the synthesis of the molecule, we wanted to evaluate the reactivity of the substrate **1** toward nucleophilic aromatic substitution. Therefore, we first prepared a amide **4** from 4,5-difluoro-2-nitroaniline and 10-undecenoyl chloride and have tried the substitution reaction with various amines and a thiol as a model study (Table 1). The reaction was carried out in THF/water at room temperature. Primary and secondary amines reacted with the nitroaniline in minutes to afford good yields. Cyclic amines provided better than non-cyclic secondary amines, and thiol provided the best yield in the shortest reaction time as expected. On the basis of the result, we anticipate the functional groups in proteins, amine or thiol, would show the similar reaction pattern toward the

Table 1. Nucleophilic aromatic substitution reaction in THF/water

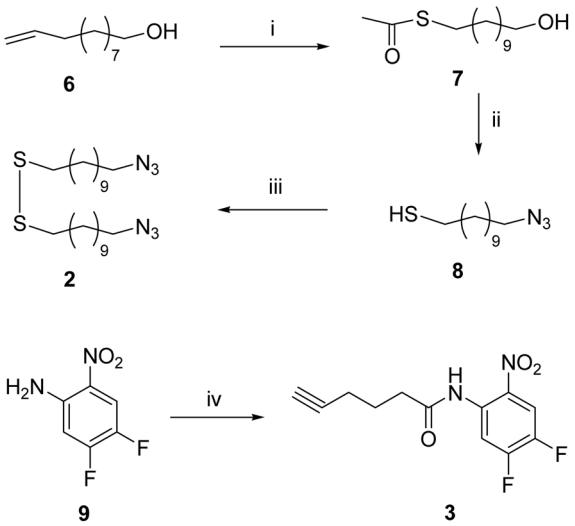


4 **5**

entry	NuH	temp	Time (h)		Yield (%)
1		rt	1	**a**	98
2		rt	0.5	**b**	97
3		rt	0.25	**c**	96
4		rt	2	**d**	96
5		rt	1	**e**	98
6		rt	0.25	**f**	94



Scheme 1.



Scheme 2. Reagents and conditions; (i) CH_3COSH, AIBN, *hv*, 56%; (ii) a) MsCl, TEA, CH_2Cl_2; b) NaN_3, HMPA, 40 °C, 70%; c) NaOH, MeOH/H_2O, 47%; (iii) I_2, O_2, 15%; (iv) 5-Hexynoyl chloride, NaH, THF, 20%.

moiety on gold surface, though with low reactivity.

For the preparation of compound **1**, we first treated 10-undecen-1-ol **6** with thioacetic acid in the presence AIBN under U.V. lamp (350 nm) to obtain thioacetate **7** in 56% yield.[15] Compound **7** was converted to azathiol **8** via a three step sequence, mesylation (quantitative yield), substitution with NaN_3 (70%), and hydrolysis (47%). Oxidative coupling of aza-thiol **8** using I_2 in the presence of O_2 afforded aza-disulfide **2** in 15% yield.[16] Coupling partner compound **3** was readily prepared by treatment of **9** with 5-hexynoyl chloride in 20% yield (Scheme 2).

Coupling reaction to **1** was carried out by Huisgen 1,3-dipolar cycloaddition. In particular, the solution of compound **2** and **3** in a 1:1 mixture of water and *tert*-butyl alcohol in the presence of $CuSO_4$ and sodium ascorbate was stirred under N_2 to yield **1** in 35% yield.[13]

In conclusion, we synthesized a new potential organic linker molecule **1** for protein immobilization on gold surface. Study of formation of self-assembled monolayers of this molecule on gold surface and the reaction with peptides or proteins is under study and the results will be disclosed in due course.

Experimental Section

A representative procedure for the nucleophilic aromatic substitution reaction of **4**.

To a solution of **4** (80 mg, 0.24 mmol) in THF/H_2O (0.5 mL/0.5 mL) was added propyl amine (30 mg, 0.47 mmol). The mixture was stirred at room temperature under N_2 for 1 hr and concentrated. The crude mixture was purified by silica-gel column chromatography to afford **5a** (87 mg, 98%).

[4-Fluoro-2-nitro-5-(propyl amino)-phenyl]-10-undecenamide (5a): ^{1}H-NMR (400 MHz, $CDCl_3$) δ 11.07 (s, 1H), 8.21 (d, 1H, *J* = 8.4 Hz), 7.89 (d, 1H, *J* = 12 Hz), 5.75-5.85 (m, 1H), 4.91-5.00 (m, 2H), 4.84 (s, 1H), 3.28 (q, 2H, *J* = 7.2 Hz), 2.48 (t, 2H, *J* = 7.6 Hz), 2.03 (q, 2H, *J* = 7.2 Hz), 1.70-1.77 (m, 4H), 1.31-1.41 (10H), 1.03 (t, 3H, *J* = 7.2 Hz).

[4-Fluoro-2-nitro-5-(dimethylamino)-phenyl]-10-undecenamide (5b): ^{1}H-NMR (400 MHz, $CDCl_3$) δ 10.92 (s, 1H), 8.27 (d, 1H, *J* = 8.8 Hz), 7.88 (d, 1H, *J* = 15.2 Hz), 5.81 (m, 1H), 4.96 (m, 2H), 3.16 (s, 3H), 3.15 (s, 3H), 2.47 (t, 2H, *J* = 7.6 Hz), 2.03 (q, 2H, *J* = 7.6 Hz), 1.74 (q, 2H, *J* = 7.6 Hz), 1.25-1.39 (10H).

(4-Fluoro-2-nitro-5-pyrolidinyl-phenyl)-10-undecenamide (5c): ^{1}H-NMR (400 MHz, $CDCl_3$) δ 11.03 (s, 1H), 8.11 (d, 1H, *J* = 8.8 Hz), 7.87 (d, 1H, *J* = 14.8 Hz), 5.79 (m, 1H), 4.93 (m, 2H), 3.62 (bs, 4H), 2.46 (t, 2H, *J* = 7.6 Hz), 2.00 (bs, 6H), 1.74 (q, 2H, *J* = 7.6 Hz), 1.26-1.39 (10H).

[4-Fluoro-2-nitro-5-(di-*n*-butyl amino)-phenyl]-10-undecenamide (5d): ^{1}H-NMR (400 MHz, $CDCl_3$) δ 10.95 (s, 1H), 8.29 (d, 1H, *J* = 8.8 Hz), 7.86 (d, 1H, *J* = 15.6 Hz), 5.80 (m, 1H), 4.95 (m, 2H), 3.42 (t, 4H, *J* = 7.2 Hz), 2.45 (t, 2H, *J* = 7.6 Hz), 2.03 (q, 2H, *J* = 7.2 Hz), 1.74 (q, 2H, *J* = 7.6 Hz), 1.64 (q, 4H, *J* = 7.6 Hz), 1.31-1.40 (14H), 0.96 (t, 6H, *J* = 7.6 Hz).

[4-Fluoro-2-nitro-5-(1-heptane thio)-phenyl]-10-undecenamide (5e): ^{1}H-NMR (400 MHz, $CDCl_3$) δ 10.55 (s, 1H), 8.84 (d, 1H, *J* = 7.2 Hz), 7.87 (d, 1H, *J* = 10 Hz), 5.79 (m, 1H), 4.99 (m, 2H), 2.96-3.07 (t, 2H, *J* = 7.2 Hz), 2.46-2.70 (m, 4H), 2.04 (q, 2H, *J* = 6.4 Hz), 1.81-1.43 (10H), 1.29-1.39 (10H), 0.88 (t, 3H, *J* = 6.8 Hz).

(4-Fluoro-2-nitro-5-piperidinyl-phenyl)-10-undecenamide (5f): ^{1}H-NMR (400 MHz, $CDCl_3$) δ 10.81 (s, 1H), 8.42 (d, 1H, *J* = 8.8 Hz), 7.88 (d, 1H, *J* = 14 Hz), 5.78 (m, 1H), 4.96 (m, 2H), 3.38 (t, 4H, *J* = 5.2 Hz), 2.47 (t, 2H, *J* = 7.2 Hz), 2.03 (q, 2H, *J* = 7.2 Hz), 1.66-1.78 (8H), 1.29-1.41 (10H).

11-Hydroxyundecyl ethanethioate (7): To a solution of 10-undecen-1-ol (500 mg, 2.94 mmol) and AIBN (241 mg, 0.147 mmol) in 3 mL of THF was added thioacetic acid (394 mg, 5.87 mmol). The solution was stirred for 14 hr under UV lamp and 2 mL of water was added to the solution. The mixture was extracted with methylene chloride (3 mL × 3), and the organic layer was dried over $MgSO_4$. Filtration was followed by concentration and purification on silica-gel chromatography to afford 405 mg of **7** in 56% yield. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 3.64 (t, 2H, *J* = 6.8 Hz), 2.86 (t, 2H, *J* = 7.2 Hz), 2.32 (s, 3H), 1.53-1.60 (m, 4H), 1.27-1.36 (14H); Mass (EI, m/z) 246 (M^+).

11-Azidoundecane-1-thiol (8): A solution of **7**, methanesulfonyl chloride (810 mg, 3.29 mmol), and triethylamine (668 mg, 6.58 mmol) in 3 mL of methylene chloride was stirred at room temperature for 2 hr. The reaction mixture was quenched with water (3 mL) and extracted with methlene chloride (3 mL × 3). The organic layer was dried over $MgSO_4$, filtered, and concentrated to afford 1.06 g of crude mesylate. Without purification, the crude product was treated with sodium azide (690 mg, 10.6 mmol) in HMPA (4 mL) at 40 °C for 2 hr. Water (5 mL) was added to the solution and the mixture was extracted with ether (3 mL × 3). The organic layer was dried over MgSO4, filtered, and

concentrated. The crude product was purified by column chromatography to afford the corresponding azide compound (672 mg, 70%). The azide (281 mg, 1.04 mmol) was dissolved in 3 mL of methanol containing NaOH (166 mg, 4.15 mmol). The mixture was stirred at room temperature for 2 hr. The mixture was diluted with methylene chloride (5 mL) and washed with saturated NH_4Cl. The organic layer was dried over $MgSO_4$, filtered, and concentrated. The crude product was purified by silica-gel column chromatography (hexane:ethyl acetate = 20:1) to afford compound **8** (112 mg, 47%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 3.25 (t, 2H, J = 7.2 Hz), 2.52 (q, 2H, J = 7.6 Hz), 1.56-1.62 (m, 4H), 1.28-1.35 (14H); Mass (EI, m/z) 229 (M^+).

1,2-Bis(11-azidoundecyl)disulfide (2): To a solution of thiol **8** (193 mg, 0.842 mmol) in 2 mL of THF was added I_2 (64 mg, 0.253 mmol). O_2 was bubbled through the solution for 4 hr. The mixture was concentrated and purified by silica-gel column chromatography to yield compound **2** (57 mg, 15%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 3.26 (t, 4H, J = 6.8 Hz), 2.68 (t, 4H, J = 7.6 Hz), 1.56-1.69 (m, 8H), 1.25-1.38 (28H); HRMS *m/z* (M^+) Calcd for $C_{22}H_{44}N_6S_2$: 456.3069. Found 456.3065.

***N*-(4',5'-Difluoro-2'-nitrophenyl)hex-5-ynamide (3):** To a solution of 4,5-difluoro-2-nitroaniline (1.17 g, 6.69 mmol) in THF was added NaH (161 mg, 6.69 mmol) at 0 °C. The mixture was stirred at room temperature for 1 hr. To the resulting solution was added 5-hexynoyl chloride (585 mg, 4.46 mmol), and the solution was stirred at room temperature for 4 hr. Water was added to quench the reaction, and the mixture was extracted with methylene chloride (3 mL × 3). The organic layer was washed with brine, dried over $MgSO_4$, and concentrated. The crude product was purified by silica-gel column chromatography to afford compound **3** (179 mg, 15%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 10.50 (s, 1H), 8.84 (m, 1H), 8.12 (m, 1H), 2.67 (t, 2H, J = 7.2 Hz), 2.35 (dt, 2H, J = 6.8 Hz, J = 2.8 Hz), 2.03 (t, 1H, J = 2.4 Hz), 1.98 (m, 2H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 172.50, 155.51, 146.30, 136.70, 131.92, 119.50, 112.10, 83.70, 71.25, 35.40, 25.50, 20.10; HRMS *m/z* (M^+) Calcd for $C_{12}H_{10}F_2N_2O_3$: 268.0659. Found: 268.0650.

1,2,3-Triazole disulfide (1): To a solution of **2** (300 mg, 0.658 mmol) and **3** (353 mg, 1.32 mmol) in 4 mL of *tert*-butyl alcohol, water, and DMF (1:1:2 mixture), were added sodium ascorbate (26 mg, 0.132 mmol) and copper sulfate (3 mg, 0.013 mmol). The solution was stirred at room temperature for 15 hr under nitrogen atmosphere. Water was added and the mixture was extracted with methylene chloride (3 mL × 3). The organic layer was dried over $MgSO_4$, filtered, and concentrated. The crude product was purified by silica-gel column chromatography to afford compound **1** (220 mg, 35%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 10.47 (s, 2H), 8.82 (m, 1H), 8.09 (m, 2H), 6.97 (d, 2H, J = 8.4 Hz), 4.31 (t, 4H, J = 7.2 Hz), 2.85 (t, 4H, J = 7.6 Hz), 2.67 (t, 4H, J = 7.6 Hz), 2.59 (t, 4H, J = 7.6 Hz), 2.14 (t, 4H, J = 7.6 Hz), 1.23-1.32 (28H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 175.21 (2 C), 156.82 (2 C), 145.42 (2 C), 142.11 (2 C), 136.43 (2 C), 132.02 (2 C), 124.45 (2 C), 112.32 (2 C), 110.21 (2 C), 51.24 (2 C), 38.79 (2 C), 34.33 (2 C), 31.48 (2 C), 26.11-29.89 (20 C); Mass (EI, m/z) 496 (1/2 M^+).

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