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Summary **α-lipoic acid (LA, racemate) is commonly used as an antioxidant supplement but its bioavailability is not yet fully understood. Naturally occurring LA (R-isomer, R-LA) exists usually in the protein bound form through acid-amide bond at ε-amino group of lysine residues, typically in the pyruvate dehydrogenase complex. The bound R-LA functions as a critical cofactor in the enzyme involved in acyl-transfer reaction. It is, however, not known whether the protein bound R-LA functions as an actual active physiological species or as the antioxidant stock that is released occasionally dependent on the oxidative stress state. In the present study, we addressed the role of lipoamidase as a regulator of free LA level in physiological antioxidant defense or aging. To measure the functional variation of lipoamidase activity under physiological condition, we prepared a new fluorescent lipoamidase substrate derived from lipoyllysine.**
***Key Words*: α-lipoic acid, antioxidant, fluorescent probe**

Introduction

Antioxidant activity of LA and dihydrolipoic acid (DHLA) has affected much attention [*1-3*]. LA can directly scavenge various reactive oxygen species (ROS) at the concentration range of 0.05-1 mM. In addition to direct scavenging of various radical species, a couple of LA and DHLA is considered to take part in regenerating oxidized antioxidants, such as glutathione (GSH), vitamin E and C [*4-6*], thus LA can regulate the redox state of the cell by controlling the concentration of GSH revel. Anti-aging ability of LA can be also expected, because the oxidative stress has been thought to connect with aging. On the contrary, Lee and coworkers reported that the significant life extension can be seen only in the case of caloric restriction [*7*]. Nevertheless, continuing oral uptake of antioxidant such as LA or in some cases R-LA seems to be enough useful to recover or maintain functions of mitochondria, brain, and decrease RNA oxidation in nerve tissue [*8-10*]. One of the benefits of LA is its low toxicity toward a human body. For example, recent research results demonstrated that LA can regulate the metabolic function [*11, 12*], which suggests possible usage in weight-loss supplement. As can be seen, free LA is actually antioxidant, but in fact LA exists in a protein bound form in a human body. The real active form of LA *in vivo* as an antioxidant is not fully clarified, therefore, it is important to investigate the role of protein bound LA on the occasion of the oxidative stress. We focused our attention on the lipoamidase activity on the oxidative stress state, in other words, we anticipated the hydrolysis of amide bond between LA and lysine residue under the stress. To prove this hypothesis, it is necessary to synthesize a novel probe to detect this hydrolytic cleavage [*13*]. In this paper, we synthesized a new fluorescent lipoyllysine derivative to investigate physiological action of lipoamidase on the lipoyllysine segment in protein.

Materials and Methods

Chemicals

Copper (II) carbonate, *N*-hydroxysuccinimide (NHS), *N,N'*-dicyclohexylcarbodiimide (DCC), L-lysine

monohydrochloride, 9-fluorenylmethyloxycarbonyl (Fmoc) chloride, trifluoroacetic acid (TFA) were purchased from Nacalai Tesque Co. (Kyoto, Japan). DL-thioctic acid (α-lipoic acid, racemate) was purchased from Kanto Chemical Co. (Tokyo, Japan). 5-dimethylaminonaphthalene-1-sulfonyl (Dansyl) chloride was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). *N*α-(*tert*-butoxycarbonyl)-L-lysine was purchased from Sigma-Aldrich Japan Co. (Tokyo, Japan). All of these chemicals were used without further purification. The reaction was monitored by using TLC aluminum sheets (Silica Gel 60 F_{254}) provided by MERCK Japan Co. (Tokyo, Japan). Open column chromatography was performed on silica gel 60 (70-230 mesh) provided by Nacalai Tesque Co. (Kyoto, Japan). Water was purified by using ADVANTEC PWE-500 and PWU-100. NMR spectra were measured in $CDCl_3$ by using JEOL GSX-500 and chemical shifts were represented against TMS as an internal standard.

*Synthesis of succinimidyl lipoate (SL **1**)*

Succinimidyl lipoate (SL) **1** was synthesized by the modified method of Katzhendler and coworkers (181 mg, 0.60 mmol, 59 %) [*14*].

*Complexation of lysine to cuprous ion (L_2Cu **2**)*

L-Lysine monohydrochloride (1.20 g, 6.60 mmol) and $CuCO_3$ (2.00 g, 16.2 mmol) were stirred in 75 mL of water at 100 ^oC for 2 h. The blue solution was filtered and the insoluble solid was washed with 25 mL of water. The clear blue solution of lysine-copper complex **2** was used without further purification in the next step.

*Substitution of fluorenylmethyloxycarbonyl (Fmoc) group on the ε-amino group (FL_2Cu **3**)*

$NaHCO_3$ (129 mg, 1.54 mmol) was added to the blue solution of **2** (15.4 mL) followed by dropping Fmoc chloride (516 mg, 2.00 mmol) solution in dioxane (15 mL) at r.t. Bluish white powder was immediately precipitated and the solution was stirred for 8 h. The formed precipitate was filtered and washed with acetone then water carefully, until the color of filtrate disappeared. The obtained precipitate of **3** (706 mg, 1.23 mmol, 61 %) is hardly soluble in commonly used organic solvents.

*Synthesis of Fmoc-lysine (FL **4**) : Decomposing Cu complex*

To the suspended solution of **3** (286 mg, 0.36 mmol) in water (2.3 mL) was added EDTA-2Na solution (1M, 2.2 mL) and the mixture was stirred at 100 ^oC for 15 min. After the addition, the solution color gradually turned to greenish blue. The white precipitate produced in a reaction was filtered and washed with water until the color of filtrate disappeared. Fmoc-lysine **4** was collected as a white powder (235 mg, 0.64 mmol, 90 %).

*Synthesis of α-dansyl-ε-Fmoc-lysine (DFL **5**)*

Dansylation reaction was carried out according to the method of Gros and Labouesse [*15*]. After the reaction, the mixture was concentrated *in vacuo* and the crude reaction mixture was loaded on SiO_2 column chromatography (ethyl acetate to ethyl acetate/ethanol/water = 85/10/5). After evaporating the solvent, DFL **5** was obtained as a yellow viscous solid (97 mg, 0.16 mmol, 54 %).

*Producing α-dansyl-lysine (DL **6**) : Removing Fmoc group*

The compound **5** (91 mg, 0.15 mmol) and pyrrolidine (90 μL) was stirred for 24 h in CH_2Cl_2 at r.t. The obtained yellowish white precipitate **6** was filtered and washed with 10 mL of CH_2Cl_2 (33 mg, 86 μmol, 57 %).

*Synthesis of α-dansyl-lipoyllysine (DLL **7**) : Coupling of DL and SL*

The compounds **6** (7.6 mg, 20 μmol) and **1** (6.1 mg, 20 μmol) was dissolved in dioxane (1.5 mL) and 0.1M $NaHCO_3$ (1.5 mL). The mixture was stirred for 6 h at r.t. The solvent was evaporated and the crude product was purified by column chromatography on SiO_2 (ethyl acetate to ethyl acetate:ethanol:water = 75:20:5). α-Dansyl-lipoyllysine was obtained in 95 % yield (11.0 mg, 19 μmol) as a yellowish-white powder.

Yield : 95 %. ^{1}H NMR ($CDCl_3$): δ 1.11 (br, 2H), 1.38 (br, 2H), 1.61 (br, 6H), 1.86 (dddd, 1H, *J* = 12.4, 7.2, 7.2, 6.4 Hz), 2.11 (m, 2H), 2.42 (dddd, 1H, *J* = 12.4, 6.4, 6.4, 5.2 Hz), 2.70 (s, 2H), 2.89, (s, 6H), 2.94 (br, 2H), 3.09 (ddd, 1H, *J* = 17.6, 7.2, 6.4 Hz), 3.14 (ddd, 1H, *J* = 17.6, 6.4, 5.2 Hz), 3.53 (dtd, 1H, *J* = 7.2, 6.8, 6.4 Hz), 3.85 (br, 1H), 5.93 (br, 1H), 6.12 (br, 1H), 7.20 (d, 1H, *J* = 7.3 Hz), 7.50 (dd, 1H, *J* = 7.3, 7.3 Hz), 7.55 (dd, 1H, *J* = 8.5, 8.5 Hz), 8.23 (d, 1H, *J* = 7.3 Hz), 8.35 (d, 1H, *J* = 8.5 Hz), 8.52 (d, 1H, *J* = 8.5 Hz). ^{13}C NMR ($CDCl_3$): δ 21.6, 25.4, 28.3, 28.8, 31.9, 34.5, 36.3, 38.4, 39.0, 40.2, 45.5, 55.5, 56.4, 115.5, 119.5, 123.3, 128.4, 129.5, 129.6 (overlapped signals), 130.5, 134.8, 151.2, 174.2, 174.5. HRMS (FAB+) Calc. for $C_{26}H_{38}S_3O_5N_3$: m/z 568.1974; Found: 568.1976 (MH^+).

Results

<Insert Fig. 1>

DLL **7** was prepared by two methods depicted in Fig. 1 and 2. α-Lipoic acid was first activated by DCC and NHS (Fig. 1). The activated acid was then reacted with α-*t*-Boc-lysine. After the removal of *t*-Boc group by the treatment of TFA, the resultant α-lipoyllysine was found to be hardly soluble in common organic solvents. In sodium bicarbonate solution (0.1 M) to which it was slightly soluble, the dansylation reaction preceded a little to give a trace amount of product.

<Insert Fig. 2>

As shown in Fig. 2, synthetic methodology was modified to improve the product yield. Amino acid group of L-lysine was first protected by chelating to cuprous ion. Free ε-amino group of lysine was then protected by Fmoc group, because Fmoc group can be easily removed in a moderate basic condition. After decomposition of Cu chelate complex, α-amino group of L-lysine was dansylated. By the treatment of pyrrolidine, Fmoc group was smoothly removed and the fluorescent α-dansyl-lysine was obtained. At the same time, activated lipoic acid was synthesized in the same manner as shown in Fig. 1. Finally, the obtained two segments (DL and SL) were coupled to achieve the synthesis of DLL **7** in a moderate yield. The structure of **7** was confirmed by using ^{1}H, ^{13}C NMR and mass spectra.

In the ^{1}H NMR spectrum, the aliphatic signals of lipoic acid and lysine segments were appeared from 1.11 ppm to 3.85 ppm. The aromatic protons derived from dansyl group were assigned to the six signals from 7.20 ppm to 8.52 ppm. Two kinds of amide protons were assigned to the signals at 5.93 and 6.12 ppm, indicating the successful amidation reaction between lysine-lipoic acid, and lipoic acid-dansyl group, respectively. The molecular ion peak appeared at 568.1976 (MH^+) in high resolution mass spectrum (HRMS) also supported the proposed structure.

<Insert Fig. 3>

According to the UV-Vis absorption spectrum (Fig. 3), the absorption maximum was appeared at 340 nm (ε = 4500 L/mol·cm), which is almost same as that of typical dansyl amino acid. The green fluorescence of **7** with its

maximum at 500 nm was detected even at low concentration (10 nmol/L).

Discussion

In the first synthetic pathway in Fig. 1, we failed to obtain the enough amount of the desired product due to the low solubility of the intermediate (lipoyllysine) in water and common organic solvents. It only dissolves in strong acidic solution like TFA. We thus designed and devised a new synthetic pathway drawn in Fig. 2. Fortunately, all of the intermediates in Fig. 2 were soluble in either water or organic solvents. We chose Fmoc group to protect ε-amino group, because it dissociates in a moderate basic condition. If we use the ε-protecting group which dissociate in an acidic condition, the dimethylamino group in a dansyl fluorophore will be easily protonated to lose fluorescent character. Another advantage of this method is that prospective metabolized compound is obtained as an intermediate, which is required as a reference in a bioassay. It is also advantageous that the coupling reaction of lipoyl domain was carried out in the final step. Disulfide bond of LA is quite vulnerable to UV light and heat, therefore, careful and mild reaction conditions are required to protect 1,2-dithiorane ring. Instability of the disulfide bond often becomes critical problem in this research field. In fact, the insoluble gelatin-like colorless solid was obtained when the solvent was evaporated off. This might be the polymerized product caused by the cleavage of disulfide bond.

<Insert Fig. 4>

From the aspect of steric hindrance, we checked the bulkiness of this fluorescent group. According to the result of computer simulation carried out using the MOPAC program (PM3 optimized), it was suggested that dansyl group and amide moiety has enough distance not to interact each other (Fig. 4). As in the UV-Vis absorption spectrum of **7**, molar absorption coefficient of dithiorane ring was not decreased at all. These data indicate that it brings high absolute luminescent intensity and thus is applicable for bioassay of lipoamidase *in vivo*. Lipoamidase activity in tissue is now allowed to be determined using this newly synthesized lipo-amide substrate.

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Captions

Fig. 1. Synthetic pathway of α-dansyl lipoyllysine.

Fig. 2. Another synthetic pathway of α-dansyl lipoyllysine.

Fig. 3. UV-Vis absorption (solid) and fluorescence spectra (dotted) of 7 in dioxane at r.t. The concentrations were 100 μmol/L and 100 nmol/L, respectively. Fluorescence spectrum was measured on the excitation at the absorption maximum (340 nm).

Fig. 4. Result of calculation of 7 by MOPAC program (PM3).

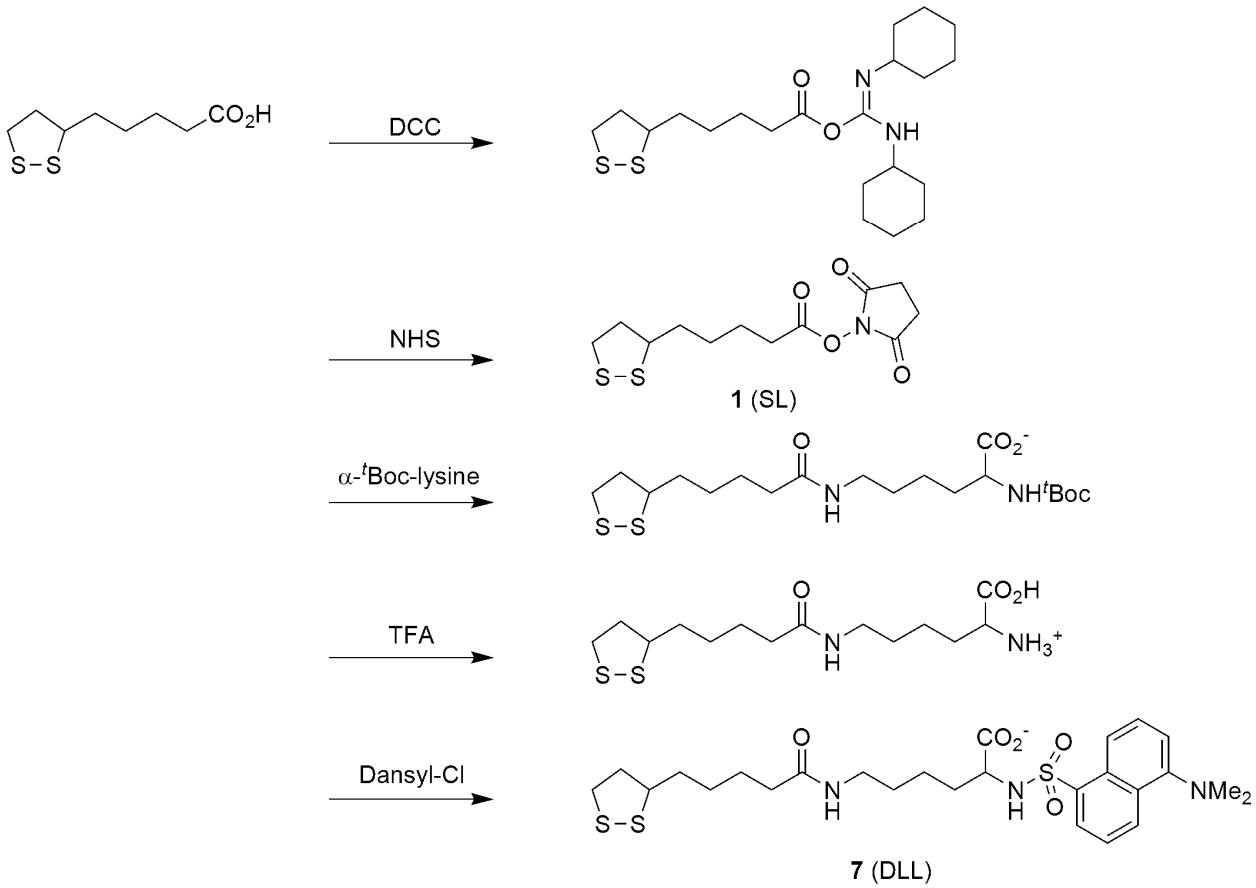
CO2H
S–S
DCC
O
N
O
NH
NHS
1 (SL)
α-tBoc-lysine
CO2-
NHtBoc
TFA
CO2H
NH3+
Dansyl-Cl
NMe2
7 (DLL)

Fig1

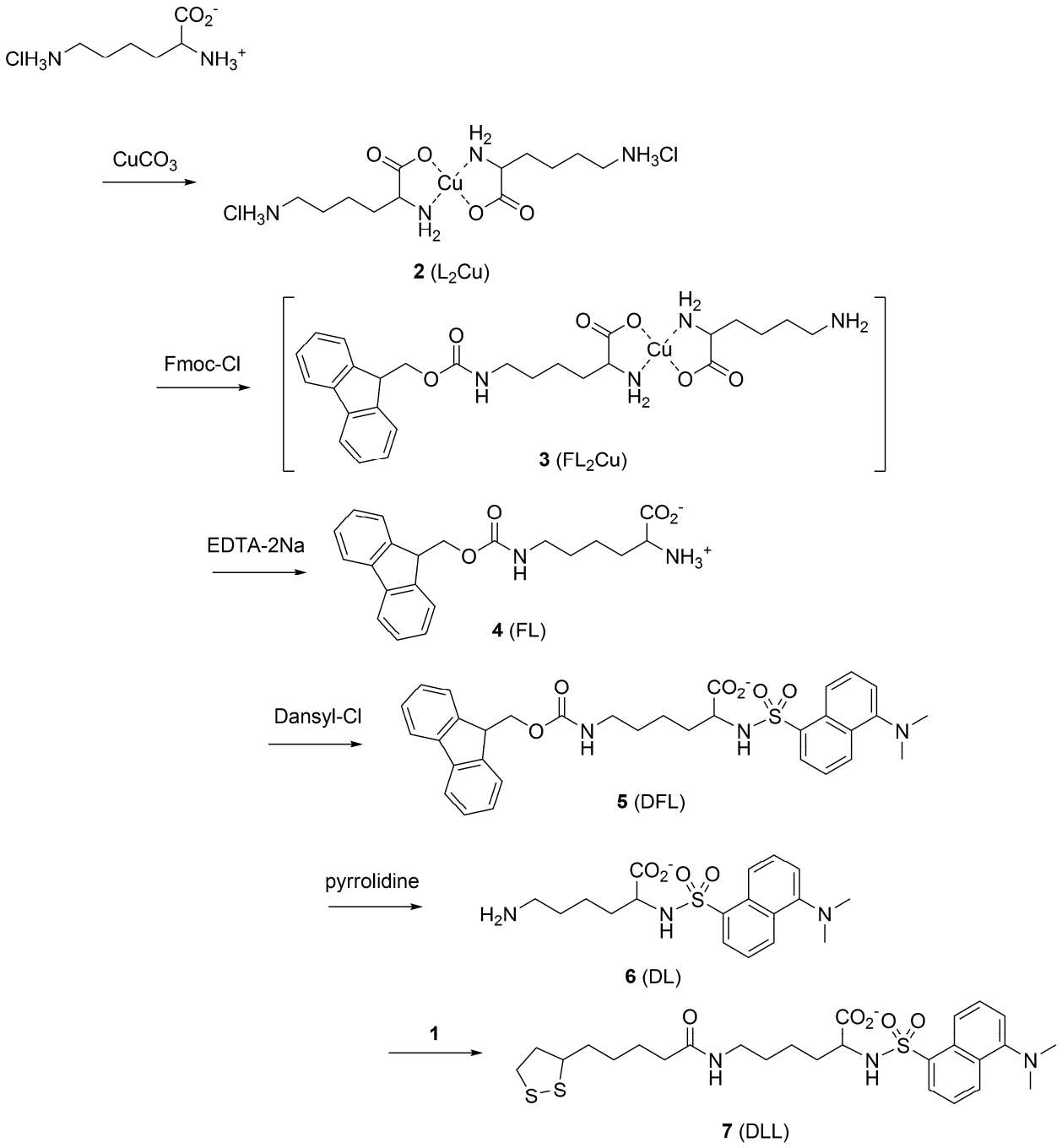
CuCO3
2 (L2Cu)
Fmoc-Cl
3 (FL2Cu)
EDTA-2Na
4 (FL)
Dansyl-Cl
5 (DFL)
pyrrolidine
6 (DL)
1
7 (DLL)

Fig2

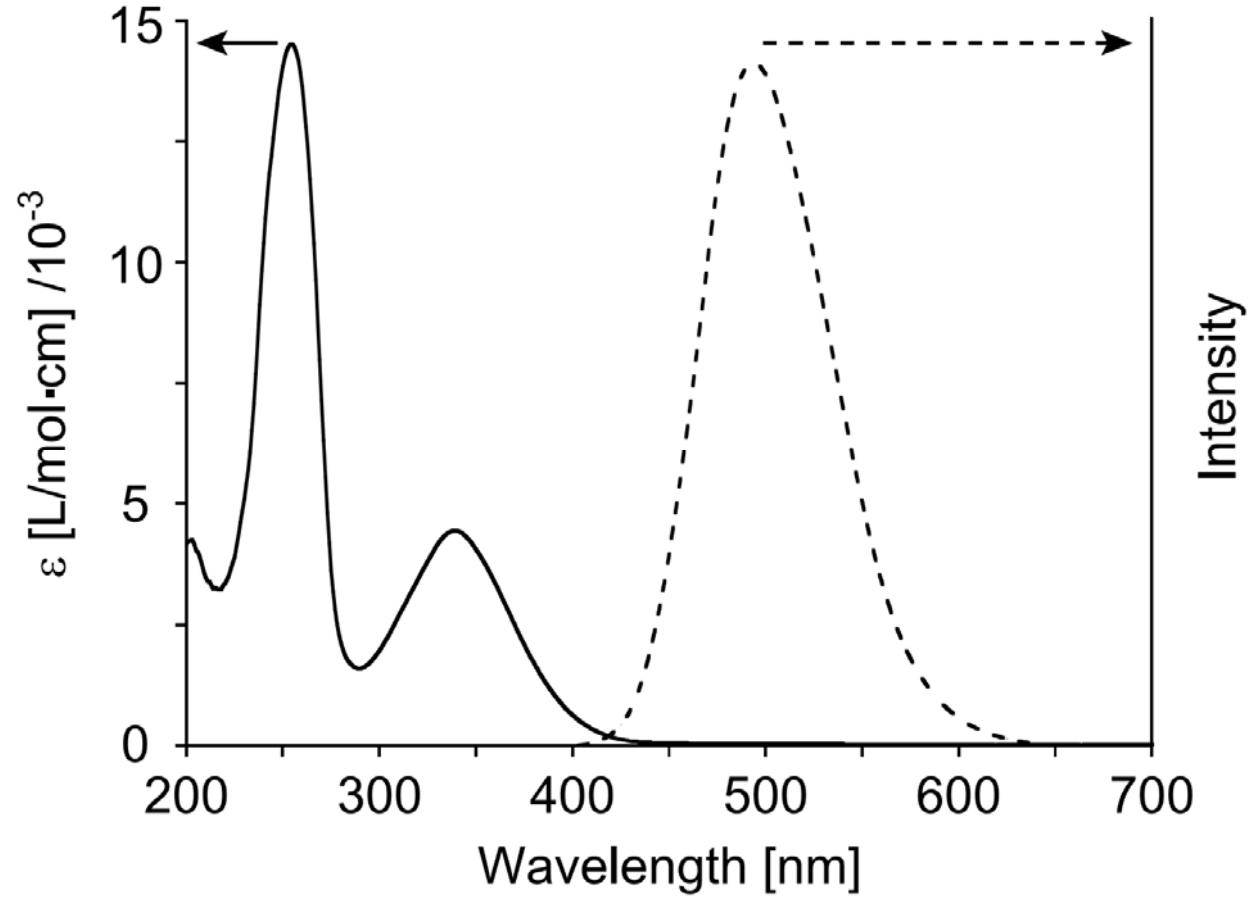
15
10
5
0
ε [L/mol·cm] /10-3
200
300
400
500
600
700
Wavelength [nm]
Intensity

Fig3

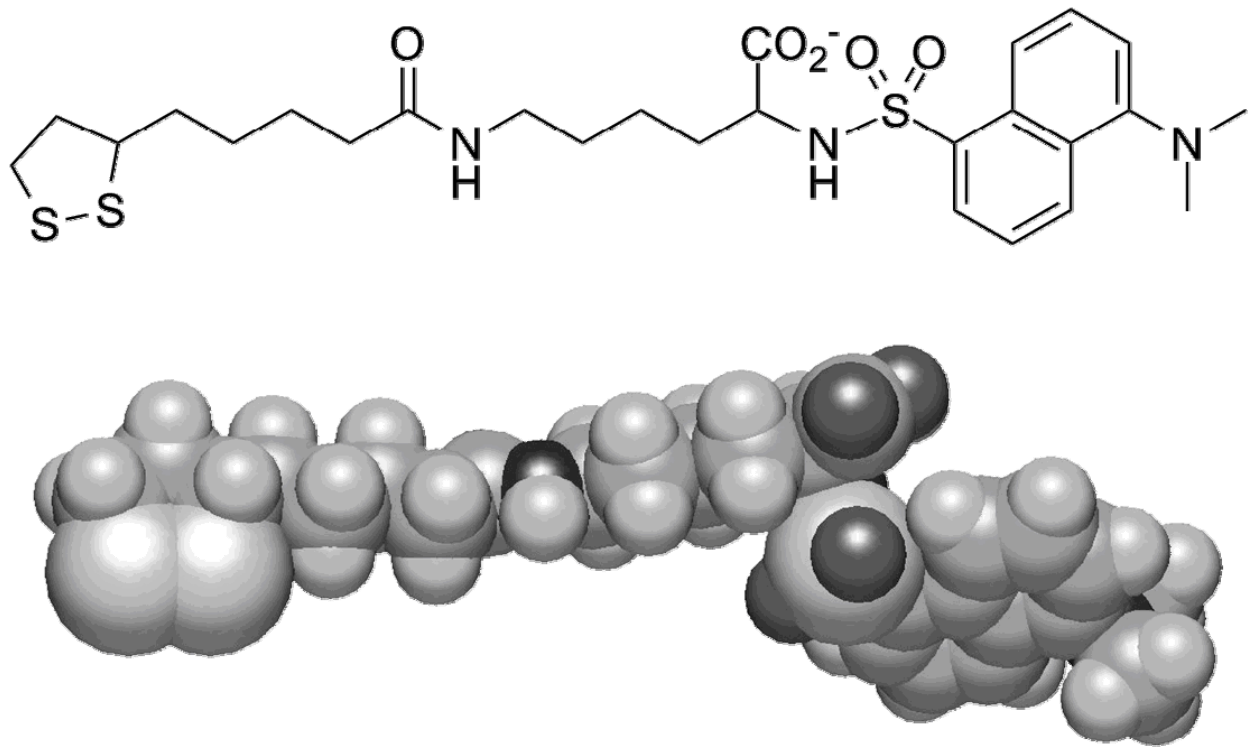

Fig4