ABSTRACT

Mechanistic Studies of Photochemical Protein Modification using 1,8-Naphthalimides

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Certain 4-alkylamino-1,8-naphthalimides have been used as photosensitizers for photochemical protein crosslinking. In my research various types of 4-amino and *N*-substituents naphthalimides have been synthesized and studied for application to protein crosslinking and tissue bonding. Ribonuclease A (RNase A) was used as a model protein in the protein for crosslinking studies. The photochemical crosslinking experiments were analyzed using SDS-PAGE electrophoresis and western blot detection. Biotinylated naphthalimides were also synthesized and it was demonstrated that while they could catalyze photochemical protein crosslinking, they were not incorporated into the crosslinked proteins. It was shown previously that tyrosine and histidine residues were involved in protein crosslinking through type I and type II mechanisms. Therefore, suspect amino acids such as tyramine, tryptamine, L-tryptophan, and dopamine were combined with biotin in order to allow us to detect their incorporation into the photooxidized protein. It was also shown that the biotin-tyramine compound and biotin-LC-hydrazide can serve as inhibitors of photochemical protein crosslinking. These studies serve to allow us to better understand mechanism of photochemical protein crosslinking.

Mechanistic Studies of Photochemical Protein Modification using 1,8-Naphthalimides

by

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A Thesis

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LIST OF ABBREVIATIONS

Boc	*tert*-butoxycarbonyl
BOP	Benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium
DMF	*N*,*N*-dimethylformamide
DMSO	dimethylsulfoxide
HRP	horseradish peroxidase
Met	methionine
PBS	phosphate buffered saline, pH 7.4
RNase A	ribonuclease A
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBS	tris-buffered saline
TEA	triethylamine
TFA	trifluoroacetic acid
TLC	thin layer chromatography

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There are many people who have helped me make the past such a meaningful time. Most of all, I would like to express my gratitude to my research mentor Dr. Robert Kane who have always assisted me and given me many chances at Baylor. Also, thank you to the members of my thesis committee, Charles M. Garner and Kenneth T. Park, whose have helped me become a better scientist. Thank you to all of the members in my lab group, especially, Jose, Drew, Gabriella, Brittany, and Thai, for supporting each other and providing each other guidance. In addition, I would also like to express thanks to Dr. Kap Duk Lee in the Dongguk University in Korea for allowing me receive such an outstanding educational opportunity in Baylor. Furthermore, I would like to express my sincere gratitude to Department of Chemistry and Biochemistry at Baylor for every care.

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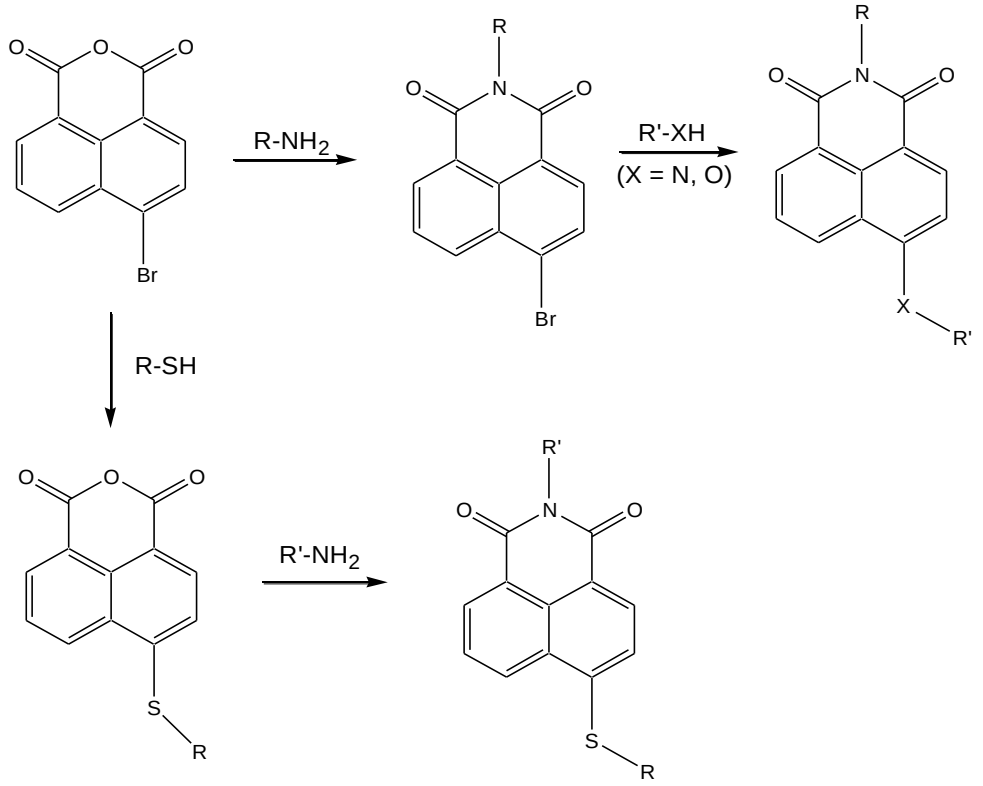
CHAPTER ONE

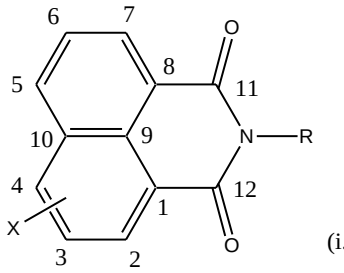
Introduction

A wide variety of 4-substituted 1, 8-naphthalimide derivatives (figure 1) are readily available from the commercially bromoanhydride via a combination of anhydride condensation and nucleophilic aromatic substitution reactions. The general reactions are shown in Scheme 1.



Scheme 1. General reactions of 4-bromo-1,8-naphthalic anhydride

These compounds have been studied as enzyme inhibitors[1,6], photo-sensitizers[2], antitumor drugs[3], chromophores[4], and protein and tissue cross-linkers[5]. For example,



R=alkylamino, amine group
X=H, electron donor or withdrawing group
(i.e. halogen, alkyl group, nitrogen, sulfur, and oxygen)

Figure 1. General structure and numbering of substituted-1,8-naphthalimides

Braña *et al*. investigated the synthesis of numerous naphthalimide derivatives and tested their biological activity as anticancer and antitumor reagents. In particular, two monomeric naphthalimides (amonafide and mitonafide, Figure 2) were tested for biological activity using HeLa cell lines[6], and in phase I and phase II clinical trials. While these compounds showed activity against solid tumors, they also showed significant neurotoxicity.[7,8] Substituted naphthalimides with nitrogen atoms at carbon 4 were found to have a higher cytotoxic activity than compounds substituted with a carbon, sulfur, or oxygen atoms. This group also studied the antitumor activity of other types of naphthalimides such as disubstituted naphthalimides and bisnaphthalimides (i.e. elinafide).[6]

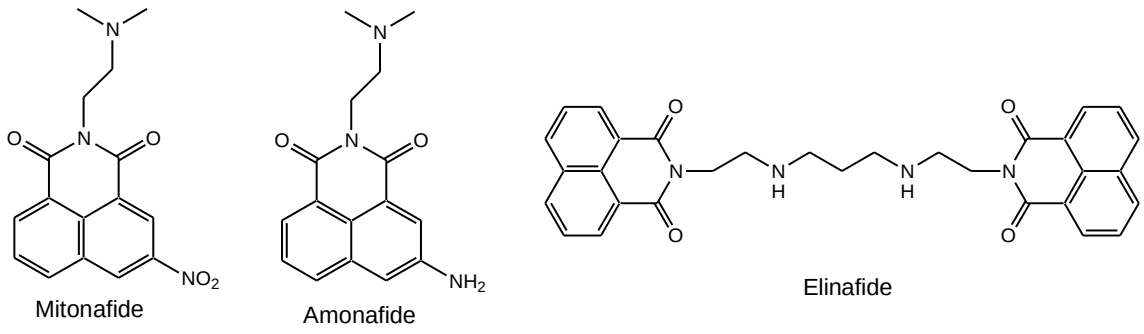


Figure 2. Structures of mitonafide, amonafide, and elinafide

Many interesting applications of naphthalimides are due to their photochemical activity. Particulary, 4-amino-1,8-naphthalimides are well known as chromophores and fluorophores[4]. When a naphthalimide is substituted at the carbon-4 position with amino or alkylamino substituents, they absorb visible light ($\lambda_{max} \approx$ 435~445nm), due to excitation to the S$\pi \rightarrow \pi^*$ state.[9,10,11] The fluorescence quantum yield of these N-substituted 1,8-naphthalimide compounds is also higher than non-substituted compounds.[11,12]

Nitrogen substituted 1,8-naphthalimides include both an electron-donating and an electron-withdrawing group, That are arranged so that they dramatically affect the naphthalimide's π-system. When photochemical excitation occurs, significant change separation occurs. The partial positive (+δ) charge is localized on the 4-nitrogen and the partial negative (–δ) charge is localized on the imide. When a 4-alkylamino-1,8-naphthalimide is substituted with an aliphatic amine on the C-4 substituent, an electron of a distal amine was found to transfer to the electron deficient component, thereby quenching the fluorescence.[13] When the amine group at the end tail is protonated or complexed with a metal ion, the fluorescence is not quenched.[14]

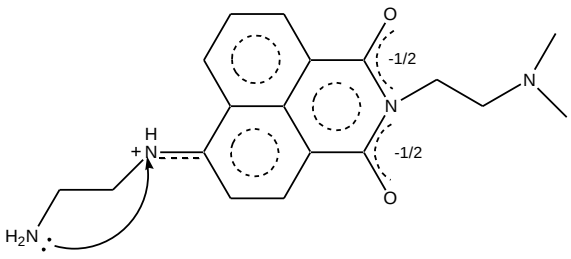


Figure 3. The resonance structure of separated charges

Based on the 'on and off' characteristic of this type of compound, a photo-switching naphthalimide has been studied as part of a poly (propyleneimine) dendrimer (D1). Interaction of 1,8-naphthalimide and the attached *N*,*N*-dimethylamino group allows electron transfer, and therefore the fluorescence emission is normally quenched

(OFF-state). However, when metal cations (i.e. Zn^{2+}, Ni^{2+}, Pb^{2+}, Co^{2+}, Cu^{2+}, and Ag^{+}) complex with nitrogen on *N*,*N*-diethylamino group, the electron donor potential is reduced, therefore allowing fluorescence (ON-state).[15]

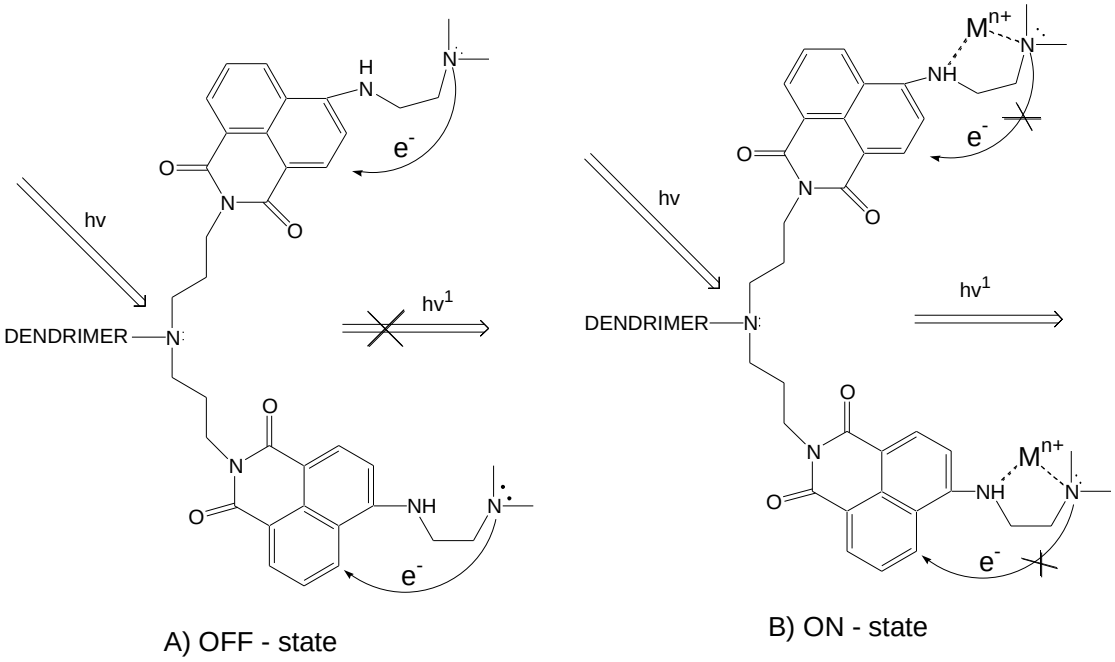
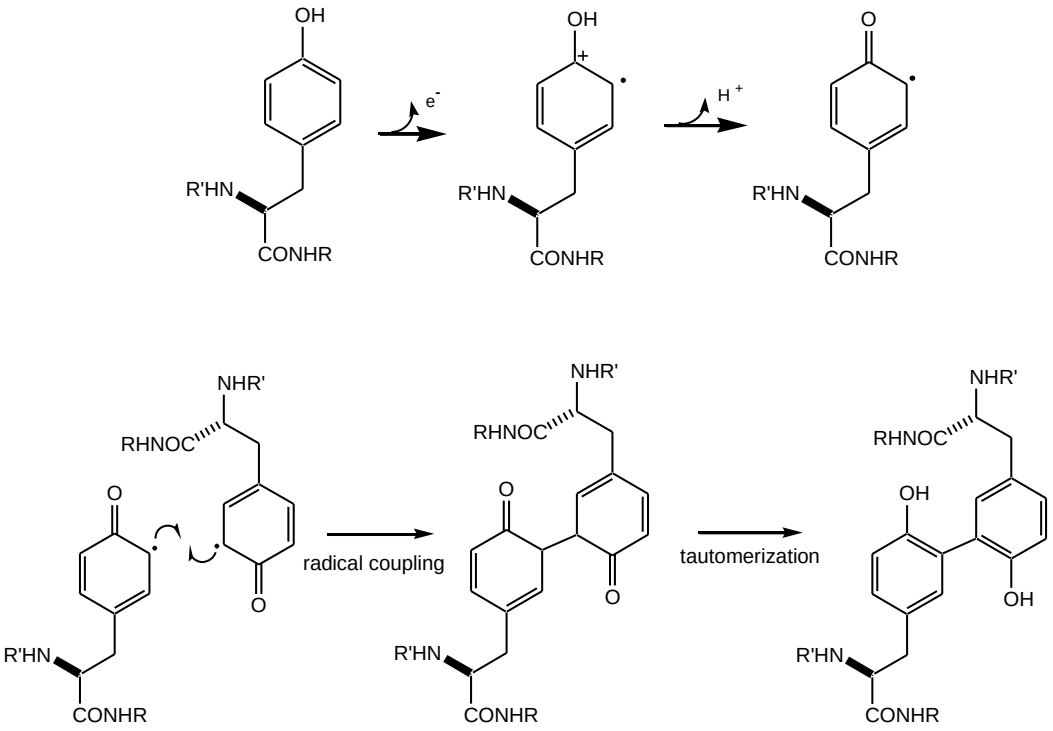


Figure 4. Scheme of Fluorophore-spacer-receptor ON-OFF supramolecular system

The ability of certain naphthalimides to photochemically crosslink proteins has been of interest in our lab for some time. Photochemical protein crosslinking is well established to occur by a variety of mechanisms. The actual mechanisms of photochemical protein cross-linking are not easily identified because of the wide variety of possible reactive species and conformations found in proteins. For these reasons, photochemical cross-linking mechanisms have been often studied using model systems such as amino acids or small peptide sequences instead of whole proteins.

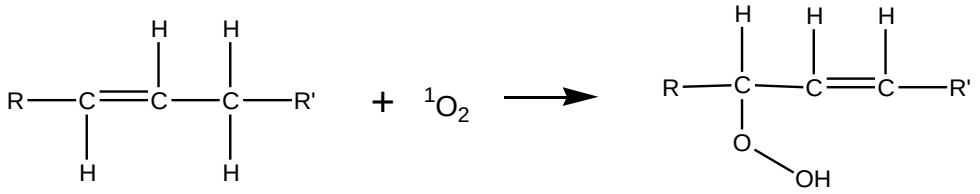
Photochemical oxidation has been organized in two general categories, type I and type II, both of which have been shown to accelerate cross-linking of proteins which contain oxidizable side chains. For example, dityrosine can be cross-linked by the

combination of two tyrosine radicals. This reaction involving free radical production is called the type I photosensitized reaction. A proposed mechanism for this dimerization of tyrosine is shown in Scheme 2.[16]

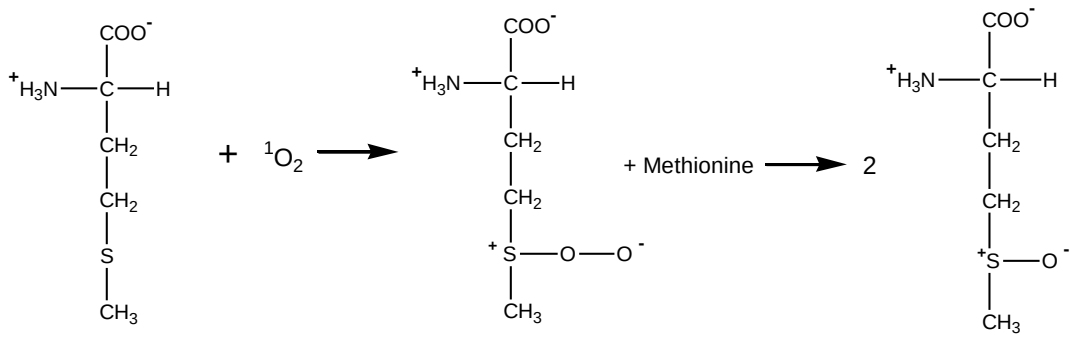


Scheme 2. A possible mechanism for the production of dityrosine by photo-oxidation

Some photosensitizers absorb photons efficiently and generate singlet oxygen ($^1O_2$) from triplet oxygen ($^3O_2$) effectively. This reaction is called the type II photosensitized reaction. These types of compounds have typically high molar extinction coefficients, a high quantum yield of triplet state, and a long excited-state lifetime. Singlet oxygen ($^1O_2$) reacts fast because it is more electrophilic than oxygen in ground energy state, and it is able to oxidize many biomoelcules. Two examples of reactions of singlet oxygen are shown in Figure 5.[17]



(a) The addition of singlet oxygen to an olefin



(b) The addition of singlet oxygen an organic sulfide; resulting in two molecules of oxidized methionine

Figure 5. Characteristic reactions of singlet oxygen ($^1O_2$)

Woods *et al*. synthesized numerous of 4-substituted-1,8-naphthalimides and investigated their relative abilities to photochemical cross-link the protein RNase A.[18] In that work it was demonstrated that non-substituted naphthalimides were the most active in photochemical RNase A crosslinking (Figure 6). Follow-up studies suggest that dityrosine formation is an important part of this cross-linking.[18]

One good type I photosensitizer used in protein cross-linkings is rose bengal (Figure 7). Rose bengal was tested for the photochemical crosslinking of the small protein, RNase A, and demonstrated 90% of intermolecular cross-linking after 90 minutes irradiation.[19] This crosslinking appears to involve histidine residues. In a model study, the photochemical crosslinking of *N*-benzoyl-L-histidine with rose bengal was demonstrated by Shen *et al*., forming the dimer product as shown in Figure 8.[20]

Figure 6. RNase A cross-linking rates for different types of 1,8-naphthalimides

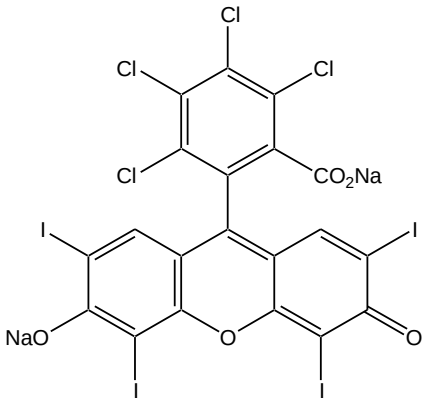


Figure 7. The structure of Rose Bengal

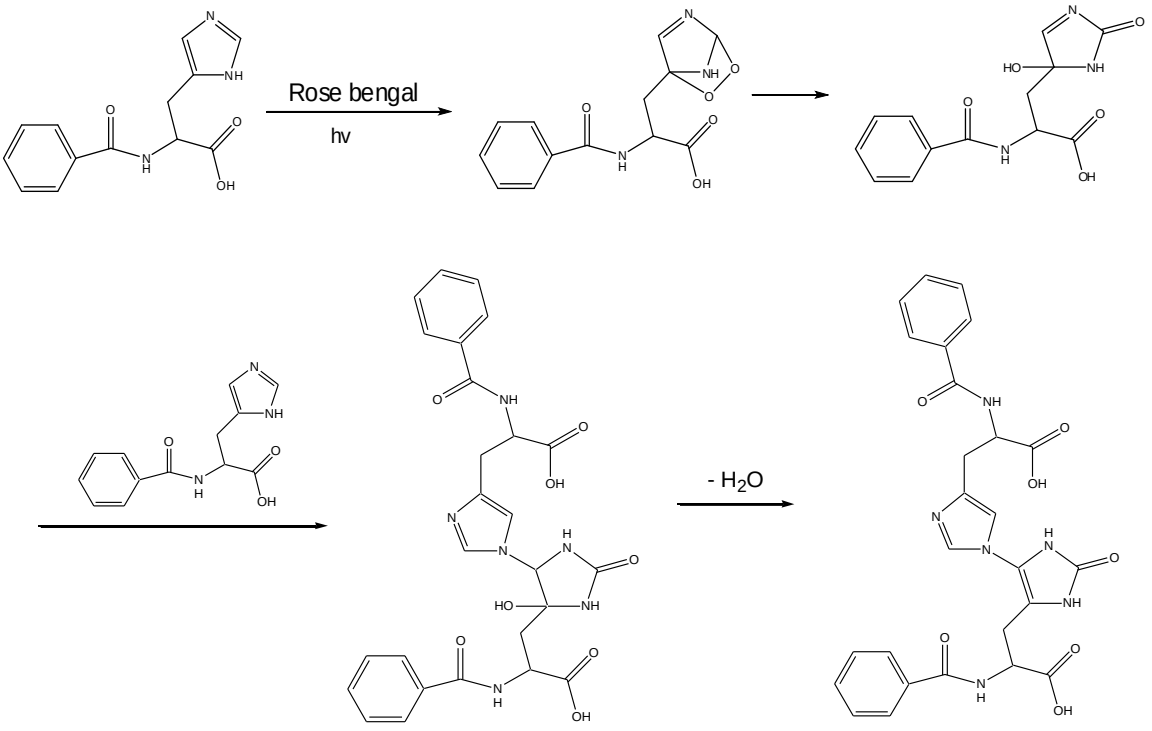


Figure 8. The cross-linked product of N-benzoyl-L-histidine by photooxidation (type I)

Protein cross-linking may potentially be predictive of tissue bonding or adhesion, as protein-rich surfaces commonly make the sites of tissue bonding. In fact, photochemical tissue bonding (PTB) has been investigated in surgical procedures such as repair of nerve, blood vessels, and cornea.[21] One example of photochemical tissue bonding involves *N,N'*-bis(3-diazopyruvoyl)-2,2'-(ethylenedioxy)bis(ethylamine) (DPD) (Figure 9). DPD was photo-activated in the presence of collagen type I molecule and generated a firm bond to the tissues through solid gelatin strips and achilles tendon.[22]

Judy *et al*. reported the synthesis and tissue-bonding abilities of two bisnaphthalimides, a brominated bis-naphthalimide and a more hydrophilic bisnaphthalimide polyether (Figure 10). These bisnaphthalimides were demonstrated to photochemically catalyze tissue adhesion, especially in tissues rich in collagen such as

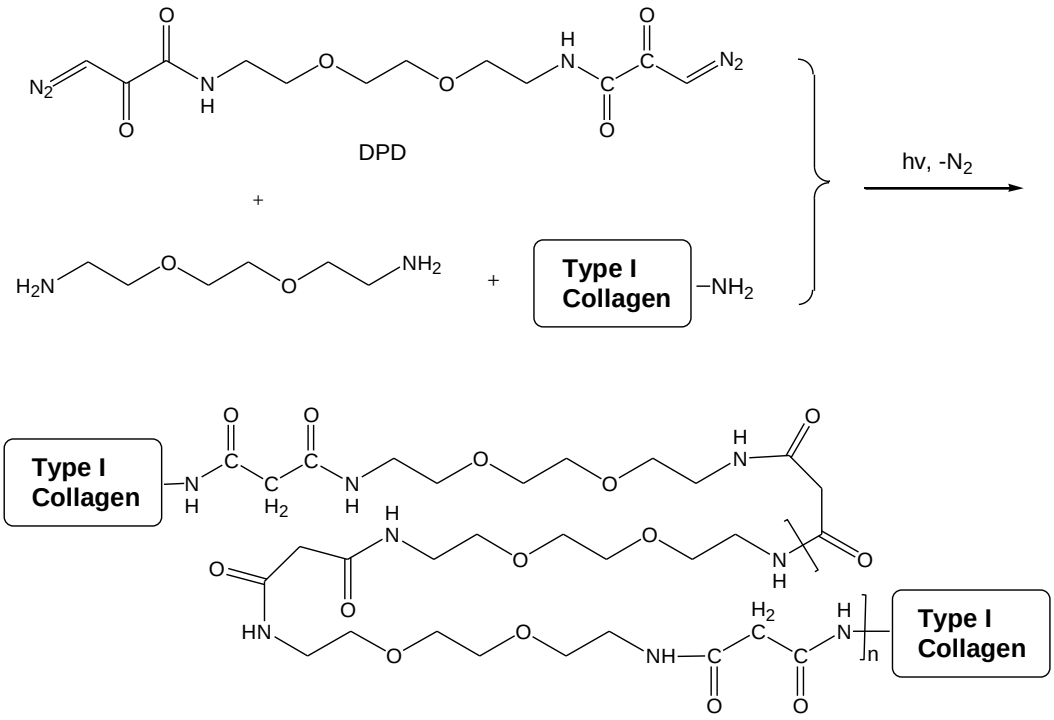


Figure 9. The reaction of DPD and type I collagen by photo-activation

cornea, skin, and meniscus. Such an approach to tissue bonding may present a potential solution to problems (i.e. weak bond, tissue damage) associated with traditional adhesion methods, which includede the use of polymeric glues as well as other approaches such as thermal tissue welding.[23-28]

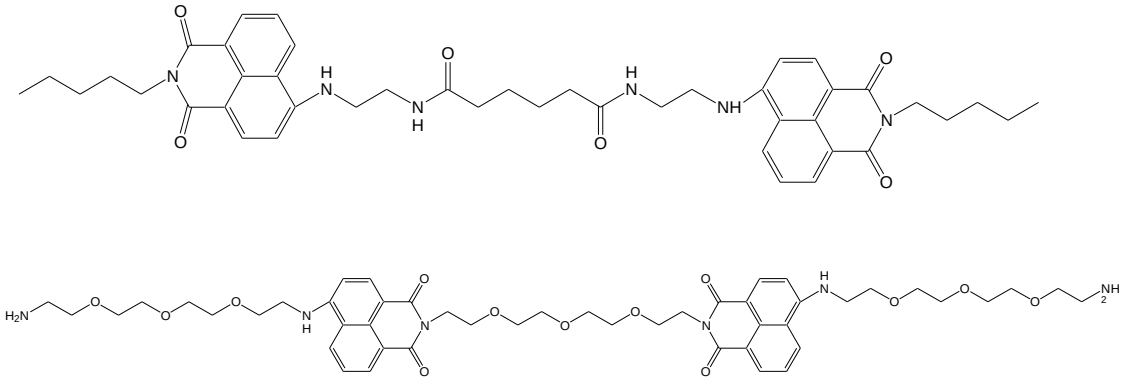


Figure 10. Structures of *bis*-naphthalimides

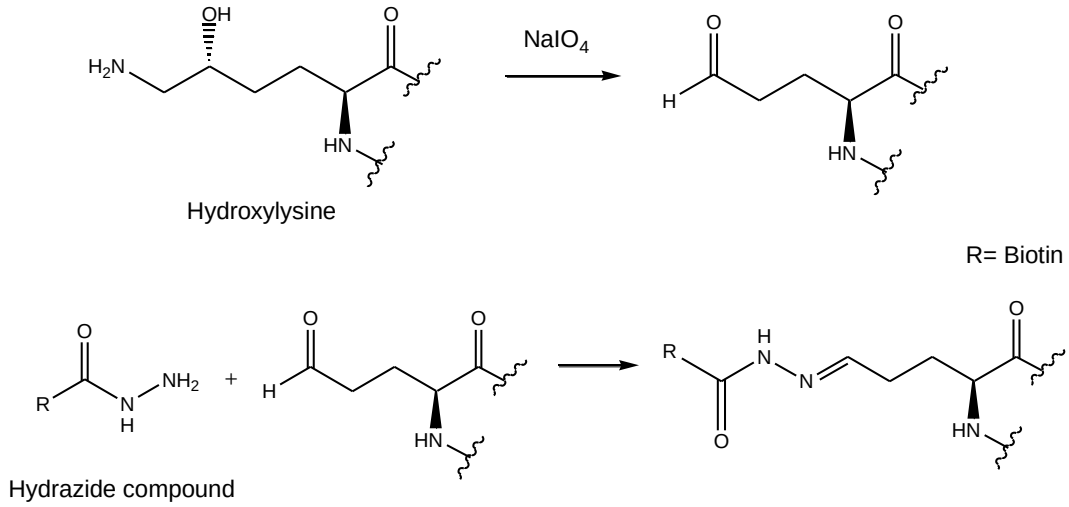
Following up the pioneering work of Judy and Matthews *et al.*, our group has studied the photochemical bonding of bovine meniscus using a variety of substituted 4-amino-1,8-naphthalimides.[29] While these experiments demonstrate the viability of this approach to tissue bonding, the large standard deviations found in these physical measurements make systematic structure-activity studies challenging and extremely time consuming.

In order to identity a model system that is more predictive than the simple solution-phase protein crosslinking experiments, but that is less difficult than tissue adhesion experiments, our group has began to explore the conjugation of soluble proteins to intact fresh tissue samples. Previously, proteins have been covalently attached to simple solid substrates such as glass, gold, silica, and organic polymers.[30,31]

Compounds are routinely immobilized to solid and tissue surfaces by physical and chemical methods, including physical adsorption, specific surface interactions[32], and covalent bonds. Example of physical adsorption includes biochemical techniques such as dot blotting (i.e. western blotting). An example of a non-covalent specific method is the utility of the vitamin biotin and streptavidin, due to their strong and selective. Surfaces can be precoated with streptavidin as the tag and are them able to non-covalently capture biotinylated proteins.[32] An example of covalent bonding is the use of aldehyde groups on glass surfaces which can capture primary amine groups on protein via the formation of a Schiff's base.[33] To detect all of these surface modifications, specific and sensitive detection techniques are used such as enzyme-linked colorimetric[34], fluorescent[35,36], chemiluminescent, or luminescent.[37,38]

In preliminary experiments in our lab, tissue samples were oxidized using alkaline periodate. Hydroxylysine, a non-standard amino acid found in collagen is oxidized to an aldehyde under these conditions (Scheme 3).[39] The resulting aldehyde-rich tissue surface can be reacted with a hydrazide derivative of biotin (Biotin-LC-hydrazide) that reacts rapidly and selectively with the aldehyde groups. Detection with

a chemiluminescent system based on avidin-horseradish peroxidase revealed that biotin had been covalently linked to the tissue only after oxidation. These experiments led us to extend these experiments to analyze photochemical tissue oxidation by naphthalimides.



Scheme 3. The scheme of oxidation of diols using sodium periodate, and reaction with biotin-hydrzide

CHAPTER TWO

Materials and Methods

Reagents and Materials

Methylene chloride, ethyl acetate, and methaol were obtained from commercial sources and were used after distillation. Anhydrous ethanol and DMF were used as received. Ribonuclease A was obtained from USB Company. Polyacrylamide gels (Gradipore brand 4-20% Tris-glycine iGels) were obtained from VWR. Phosphate buffered saline (PBS) solution was used 1× concentration. Solutions for Western blots were obtained from Thermal Fisher Scientific Inc. All other reagents were provided from Sigma-Aldrich, VWR, and Alfa Aesar and were used as received. Fresh bovine knee meniscal tissue and bovine pericardium were harvested and supplied by a local slaughterhouse within a few hours of the death of the animal.

Instrumentation

Bruker Icon NMRs (300 MHz and 500 MHz) were used for ^{1}H NMR and ^{13}C NMR analysis. A NanoDrop® ND-1000 spectrometer was used for all UV-vis absorbance measurements. Accurate mass determinations were performed by Alejandro Ramirez in the Baylor University mass spectroscopy center. Mass spectroscopy was measured by using LCQ, using the Thermo Finnigan LCQ Classic, Prospec from VG Prospec micromass, and Orbitrap with the Thermoscientific LTQ Orbitrap Discovery. An EXFO Nanocure 100W mercury arc lamp system was used as a light source in photochemical protein crosslinking and tissue modification studies.

For electrophoresis and Western blots, a Bio-Rad Mini-Protean 3 cell electrophoresis system was used with a VWR 105 power supply. For Western blots, a Bio-Rad

trans-blot® SD was used to transfer from gel to membrane and an Ultra Quant system was used for imaging.

UV-vis Measurements

All monomeric naphthalimides were diluted with DMSO to 10 different concentrations of 0.0001 mM, 0.0002 mM, 0.0003 mM, 0.0004 mM, 0.0005 mM, 0.0006 mM, 0.0007 mM, 0.0008 mM, 0.0009 mM, 0.001 mM. The absorption of each solution was measured at the λ_{max} of the naphthalimide compounds. The plot of the absorption versus concentration was drawn for all naphthalimides, and each extinction coefficient was calculated.

Synthetic Procedures

4-Bromo-1,8-Naphthalimide (compound **9**), 1,8-naphthalimide (compound **24**), and 4-ethylsulfanyl-1,8-naphthalic anhydride were synthesized by Dr. Jeremy Woods.[40] All other compounds were synthesized by procedures that follow. 1,8-naphthalimides were customarily converted to HCl salts as described below. The resulting salts were dissolved in PBS solution, and the PBS buffered solutions were used in gel and tissue experiments.

Synthesis of 1,8-Naphthalimides

2-(2-Amino-ethyl)-benzo[de]isoquinoline-1,3-dione (Compound **1**, Figure 11). To a 25 mL suspension of 1,8-naphthalic anhydride (500 mg, 2.51 mmol) in ethanol was

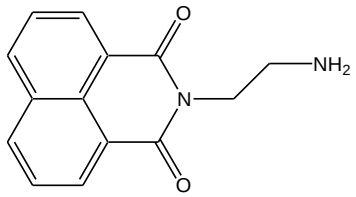


Figure 11. Structure of compound **1**

added 5 equivalents of diethylendiamine (0.840 mL, 12.5 mmol). The mixture was stirred for 2 hours at room temperature in the dark. After checking the TLC, the solvent was removed using a rotary evaporator. 30 mL DI-water was added to the flask and then extracted with CH_2Cl_2 (30 mL × 3 times). The residual water in methylene chloride layer was removed by magnesium sulfate. The solution was filtered and the solvent evaporated under vacuum. The residue was then purified by flash column chromatography (CH_2Cl_2 : MeOH = 90 : 10). Compound **1** was obtained as light-yellow colored powder in 52% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.06 (t, 2H, *J* = 6 Hz), 4.27 (t, 2H, *J* = 6 Hz), 7.7 (d, 2H, *J* = 9 Hz), 8.20 (dd, 2H, *J* = 9, 6 Hz), 8.59 (d, 2H, *J* = 6 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 40.55, 43.17, 122.60, 126.95, 128.23, 131.35, 131.61, 134.01, 164.50; DEI-HRMS m/z 240.0893 (MH^+) ($C_{14}H_{12}N_2O_2$ requires 240.0899, Δ = -2.5 ppm); λ_{max} = 350 nm

(1,3-Dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-carbamic acid tert-butyl ester (Compound **2**, Figure 12) A suspension of 1,8-naphthalic anhydride (500 mg, 2.52 mmol) was prepared in 30mL ethanol in a 100 mL round bottom flask. 1.2 equivalents *tert*-butyl carbazate (400 mg, 3.02 mmol) was added and the mixture was refluxed overnight. After reaction the solvent was removed using a rotary evaporator, and flash

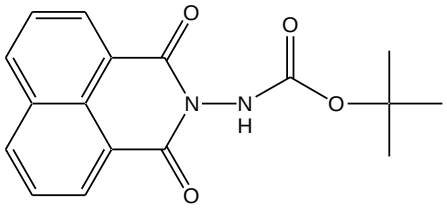


Figure 12. Structure of compound **2**

column chromatography was performed on the residue (CH_2Cl_2 : MeOH = 96 : 4). The final product was dried under vacuum and characterized by ^{1}H NMR, ^{13} C NMR and

mass spectroscopy. The compound **2** was obtained as a white solid in 44% yield. ^{1}H NMR (300 MHz, acetone-d_6) δ 1.31 (s, 9H), 7.90 (dd, 2H, J = 9, 6 Hz), 8.47 (d, 2H, J = 9 Hz), 8.58 (d, 2H, J = 6 Hz), 8.71 (s, broad NH); DEI-HRMS m/z 212.0585 (M-tBOC) ($C_{12}H_8N_2O_2$ requires 212.0586, Δ = -0.5 ppm)

2-Amino-benzo[de]isoquinoline-1,3-dione (Compound **3**, Figure 13) Compound **2** (310 mg, 0.99 mmol) was dissolved in a 10 mL : 10 mL mixture of CH_2Cl_2 : TFA. The solution was stirred for 1.5 hours at room temperature. After this time, 20 mL of

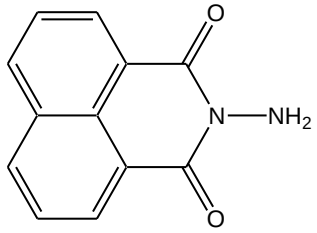


Figure 13. Structure of compound **3**

DI water was added to the reaction flask, and the solution was made pH 8~9 with ammonium hydroxide. The solution was extracted with CH_2Cl_2 (25 mL × 3 times) and the organic layer was dried over magnesium sulfate and filtered. Removal of the solvent under reduced pressure afforded pure product as a yellow solid in 89% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.47 (s, NH_2), 7.69 (dd, 2H, J = 8.2, 7.4 Hz), 8.16 (d, 2H, J = 8.4 Hz), 8.56 (d, 2H, J = 7.3 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 121.91, 126.80, 127.05, 131.55, 131.59, 134.47, 160.96; DEI-HRMS m/z 212.0576 (M) ($C_{12}H_8N_2O_2$ requires 212.0586, Δ = -4.7ppm); λ_{max} = 352 nm

4-Ethylsulfanyl-1,8-naphthalic anhydride (Compound **4**, Figure 14) 4-Bromo-1,8-naphthalic anhydride (1.0 g, 3.6 mmol) and 1.9 equivalents anhydrous potassium carbonate (0.945 g, 6.84 mmol) were mixed in a 50 mL anhydrous DMF. 4 equivalents

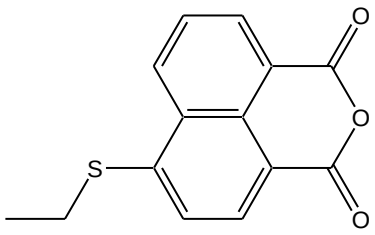


Figure 14. Structure of compound **4**

of ethanethiol (1.07 mL, 0.895 g, 14.4 mmol) were added to DMF solution. This mixture was stirred at room temperature in the dark for 19 hours under nitrogen gas. The solution was poured onto 300 mL ice to give a yellow suspension, and was stirred for 2 hours. A yellow solid was obtained by filtration, which was recrystallization from ethanol to give the pure product in 76% yield. ^{1}H NMR (300 MHz, acetone-d_6) δ 1.49 (t, 3H, J = 7.3 Hz), 3.21 (q, 2H, J = 7.4 Hz), 7.52 (d, 1H, J = 8.0 Hz), 7.75 (dd, 1H), 8.45 (d, 1H), 8.59 (dd, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 13.35, 26.21, 114.46, 119.27, 122.25, 127.00, 129.46, 130.50, 131.45, 132.95, 133.71, 148.09, 160.60, 160.79; DEI-HRMS m/z 258.0349 (M) ($C_{14}H_{10}O_3S$ requires 258.0351, Δ = -0.6 ppm)

2-(2-Amino-ethyl)-6-ethylsulfanyl-benzo[de]isoquinoline-1,3-dione (Compound **5**, Figure 15) 4-Ethylsulfanyl-1,8-naphthalic anhydride (0.25 g, 0.96 mmol) was dissolved in 50 mL ethanol in a 100 mL round bottom flask. 10 equivalents ethylenediamine (0.643 mL, 9.60 mmol) was added to the suspension, and it was stirred at room temperature under nitrogen gas in the dark for 16 hours. The ethanol was removed under reduced pressure. 30 mL of DI water was added to the flask, and the mixture extracted with

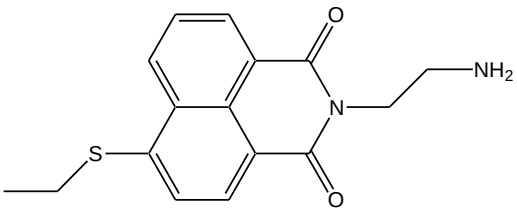


Figure 15. Structure of compound **5**

CH_2Cl_2 (30 mL × 3 times). The CH_2Cl_2 layer was dried over magnesium sulfate and the solution filtered. The organic layer evaporated and the residue submitted to flash column chromatography (CH_2Cl_2 : MeOH = 95 : 5). Compound **5** was obtained as yellow colored powder in 85% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.47 (t, 3H, *J* = 6.6 Hz), 3.05 (t, 2H, *J* = 6.6 Hz), 3.19 (q, 2H, *J* = 7.4 Hz), 4.26 (t, 2H, *J* = 6 Hz), 7.51 (d, 1H, *J* = 8.0 Hz), 7.72 (dd, 1H, *J* = 8.4, 7.4 Hz), 8.45 (d, 1H, *J* = 7.9 Hz), 8.53 (d, 1H, *J* =8.5 Hz), 8.59 (d, 1H, *J* = 7.3 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 13.54, 26.39, 40.56, 43.13, 118.93, 122.67, 123.00, 126.56, 128.41, 129.56, 130.20,130.90, 131.62, 145.44, 164.36; DEI-HRMS m/z 300.0926 (M) ($C_{16}H_{16}N_2O_2S$ requires 300.0932, Δ = -2.0 ppm); λ_{max} = 395 nm

(6-Ethylsulfanyl-1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-carbamic acid tert-butyl ester (Compound **6**, Figure 16) To a suspension of 4-ethylsulfanyl-1,8-naphthalic anhydride (0.60 g, 2.32 mmol) in 40 mL ethanol was added 2 equivalents *tert*-butyl carbazate (0.461 g, 3.49 mmol). The suspension was refluxed in the dark for 16 hours, after which all solid was dissolved. The ethanol was removed under reduced pressure

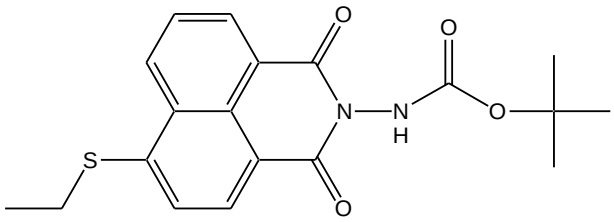


Figure 16. Structure of compound **6**

and the crude product was purified by flash column chromatography (CH_2Cl_2:MeOH = 100:0, 96:4). The pure compound **6** was obtained a light yellow colored solid in 89 % yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.50 (t, 3H, *J* = 7.4 Hz), 2.16 (s, 9H), 3.20 (q, 2H, *J* = 7.4 Hz), 7.53 (d, 1H, *J* = 7.9 Hz), 7.75 (dd, 1H, *J* = 8.3, 7.5 Hz), 8.50 (d, 1H, *J* = 7.9

Hz), 8.60 (d, 1H, *J* = 8.5 Hz), 8.65 (d, 1H, *J* = 7.3 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 13.45, 26.33, 28.12, 82.54, 118.43, 122.41, 122.69, 126.59, 128.28, 129.59, 130.78, 131.60, 132.37, 146.56, 154.18, 162.47, 162.51; DEI-HRMS m/z 272.0605 (M-tBOC) ($C_{14}H_{12}N_2O_2S\cdot+$ requires 272.0614, Δ = -3.3 ppm)

2-Amino-6-ethylsulfanyl-benzo[de]isoquinoline-1,3-dione (Compound **7**, Figure 17) Compound **7** (0.70 g, 1.87 mmol) was dissolved in a 10 mL :10 mL mixture of CH_2Cl_2 : TFA. The solution was stirred for 1.5 hours at room temperature. After this time, 20 mL of DI water was added to the reaction flask, and the solution was made pH 8~9 with ammonium hydroxide. The solution was extracted with CH_2Cl_2 (25 mL × 3 times) and the organic layer was dried over magnesium sulfate and filtered. Remove of

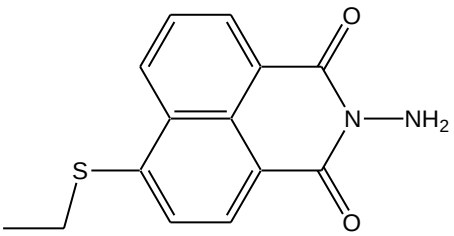


Figure 17. Structure of the compound **7**

the solvent under reduced pressure provided the pure product as a yellow colored solid in 99% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.48 (t, 3H, *J* = 7.4 Hz), 3.19 (q, 2H, *J* = 6 Hz), 5.65 (br, NH_2), 7.49 (d, 1H, *J* = 7.9 Hz), 7.73 (dd, 1H, *J* = 8.3, 7.4 Hz), 8.44 (d, 1H, *J* = 7.9 Hz), 8.56 (d, 1H, *J* = 8.5 Hz), 8.61 (d, 1H, *J* =8.2 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 13.36, 13.44, 26.31, 117.67, 121.99, 122.18, 122.39, 126.69, 127.13, 129.45, 130.82, 131.43, 131.97, 132.14, 132.77, 147.07, 161.12, 161.23; DEI-HRMS m/z 272.0621 (M) ($C_{14}H_{12}N_2O_2S$ requires 272.0619, Δ = 0.7 ppm); λ_{max} = 400 nm

Attempted synthesis of *5-Hydrazinocarbonyl-pentanoic acid (6-ethylsulfanyl-1,3-dioxo-1H,3H-benzo[de]isoq-uinolin-2-yl)-amide* (Compound **8**, Figure 18) 4-ethylsulfanyl-1,8-naphthalic anhydride (0.25 g, 0.96 mmol) was dissolved in 35 mL ethanol in a 100 mL round bottom flask. 5 equivalents adipic dihydrazide (0.84 g, 4.84 mmol) was added to the suspension, and the mixture was refluxed under nitrogen gas in the dark for 16 hours. The ethanol was removed under reduced pressure, and a methylene chloride and methanol mixture was added to the flask and then filtered. The organic layer was evaporated and the residue purified by flash column chromatography (CH_2Cl_2 : MeOH = 92 : 8, 90 : 10). A product was obtained as a light-yellow colored powder in 34 % yield. Unfortunately, the NMR and mass spectra did not correspond to the desired compound. No further characterization was performed.

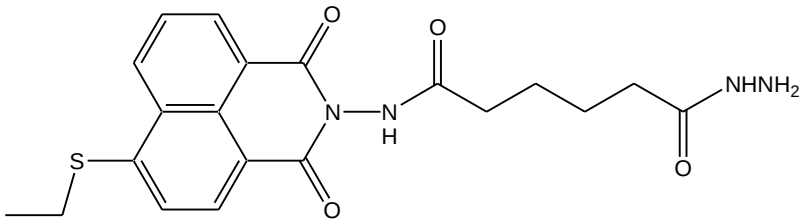


Figure 18. Structure of compound **8**

^{1}H NMR (300 MHz, DMSO) δ 1.43 (t, 3H, *J* = 7.3 Hz),1.60 (m, 4H), 2.07 (t, 2H, *J* = 6.5Hz), 2.35 (t, 2H, *J* = 6.5 Hz), 3.29 (q, 2H, *J* = 7.3 Hz), 3.34(s, 6H), 4.18 (br, NH_2), 7.78 (d, 1H, *J* = 8.1 Hz), 7.91(dd, 1H, *J* = 8.4, 7.5 Hz), 8.41(d, 1H, *J* = 8.0 Hz), 8.58 (dd, 2H, *J* = 8.5, 6.7 Hz), 8.96 (br, NH), 10.63 (br, NH); ^{13}C NMR (75 MHz, DMSO) δ 13.32, 24.67, 24.76, 25.16, 32.95, 33.20, 117.84, 122.40, 122.77, 127.37, 127.41, 128.71, 130.23, 131.10, 131.73, 145.65, 161.36, 161.42, 170.94, 171.39; m/z 454.87

6-Bromo-2-(2-diethylamino-ethyl)-benzo[de]isoquinoline-1,3-dione (compound **9**, Figure 19) In a 100 mL round bottom flask, 4-bromo-1,8-naphthalic anhydride (1.00 g,

3.61 mmol) and 1.1 equivalents *N*,*N*-diethylethylenediamine (0.588 mL, 3.97 mmol) were suspended in 50 mL ethanol. This solution was stirred at room temperature in the dark for 1 hour at which point the reaction solution was dissolved clearly. The solvent was removed by reduced pressure, and the crude product was purified by flash column

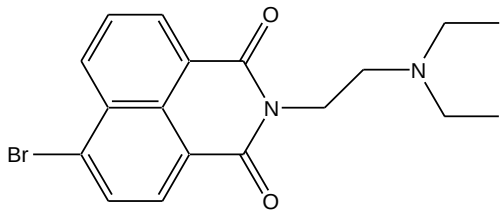


Figure 19. Structure of compound **9**

chromatography (CH_2Cl_2 : MeOH = 96 : 4). The pure compound **9** was obtained as a yellow colored solid in 80 % yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.07 (t, 6H, *J* = 6 Hz), 2.67 (q, 4H, *J* = 6 Hz), 2.76 (t, 2H, *J* = 6 Hz), 4.27 (t, 2H, *J* = 6 Hz), 7.83 (dd, 1H, *J* = 9, 6 Hz), 8.02 (d, 1H, *J* = 6 Hz), 8.39 (d, 1H, *J* = 6 Hz), 8.54 (d, 1H, *J* = 9 Hz), 8.63 (d, 1H, *J* = 6 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.18, 38.14, 47.60, 49.79, 122.24, 123.11, 128.10, 129.03, 130.28, 130.66, 131.12, 131.21, 132.02, 133.29, 163.62; DEI-HRMS *m/z* 374.0628 (M) ($C_{18}H_{19}N_2O_2Br$ requires 374.0630, Δ = -0.5 ppm); λ_{max} = 348 nm

6-(2-Amino-ethylamino)-2-(2-diethylamino-ethyl)-benzo[de]isoquinoline-1,3-dione (Compound **10**, Figure 20) Compound **9** (0.240 g, 0.637 mmol) was dissolved in 10 mL DMSO in a sealed tube. 3.1 equivalents triethylamine (0.270 mL, 1.97 mmol) and 2 equivalents ethylenediamine (0.100 mL, 1.274 mmol) also were added in the sealed tube. The solution was heated to 150 ºC in an oil bath for 8 hours. This solution was then stirred at room temperature for an additional 13 hours. The solvent was removed under reduced pressure, and the crude product was purified by flash column chromatography (CH_2Cl_2 : MeOH = 83 : 17, 80 : 20). The compound **10** was

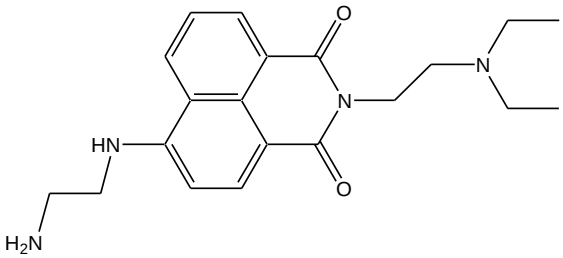


Figure 20. Structure of compound **10**

obtained as an orange colored solid in 53 % yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.09(t, 6H, *J* = 7.1 Hz), 2.67 (q, 4H, *J* = 7.1 Hz), 2.77 (m, 2H), 3.16 (t, 2H, *J* = 6.2 Hz), 3.39 (q, 2H, *J* = 5.0 Hz), 4.25 (m, 2H), 6.67 (d, 1H, *J* = 8.5 Hz), 7.60 (dd, 1H, *J* = 8.4, 7.4 Hz), 8.16 (d, 1H, *J* = 8.5 Hz), 8.41 (d, 1H, *J* = 8.4 Hz), 8.54 (d, 1H, *J* = 7.4 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.23, 37.63, 40.14, 44.85, 47.66, 49.90, 104.42, 110.21, 120.48, 123.02, 124.68, 126.31, 129.83, 131.09, 134.46, 149.72, 164.12, 164.70; DEI-HRMS *m/z* 354.2048 (M) ($C_{20}H_{26}N_4O_2$ requires 354.2056, Δ = -2.3 ppm); λ_{max} = 444 nm

Attempted synthesis of *5-Hydrazinocarbonyl-pentanoic acid (6-bromo-1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-amide* (Compound **11**, Figure 21) To a 50 mL round bottom flask, 4-bromo-1,8-naphthalic anhydride (0.30 g, 1.08 mmol) and 2 equivalents adipic hydrazide (0.377 g, 2.16 mmol) were dissolved in 30 mL ethanol. The mixture

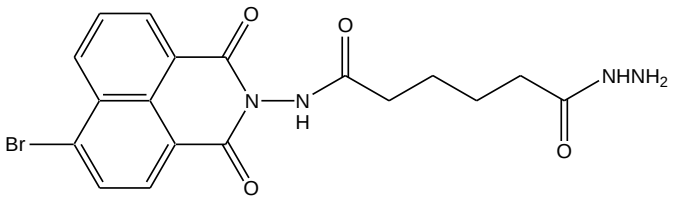


Figure 21. Structure of compound **11**

was refluxed for 16 hours. The solvent was removed under reduced pressure and the crude product was dissolved in 30 mL of DI water. This solution was extracted with CH_2Cl_2 (3 × 20 mL), dried over magnesium sulfate filtered, and concentrated under

reduced pressure. The crude product was purified by flash column chromatography (CH_2Cl_2 : MeOH = 92 : 8, 90 : 10). A product was obtained as a light yellow colored solid in 36 % yield. Unfortunately, the NMR and mass spectra did not correspond to the desired compound. No further characterization was performed. ^{1}H NMR (300 MHz, DMSO) δ 1.58 (t, 4H), 2.04 (t, 2H, *J* = 6.5 Hz), 2.34 (t, 2H, *J* = 6.4 Hz), 3.31 (s, 3H), 4.17 (s, NH), 8.02 (dd, 1H, *J* = 8.5, 7.4 Hz), 8.24 (d, 1H, *J* = 7.9 Hz), 8.36 (d, 1H, *J* = 7.9 Hz), 8.59 (d, 1H, *J* = 8.5 Hz), 8.60 (d, 1H, *J*= 7.4 Hz), 8.94 (s, NH), 10.68 (s, NH); ^{13}C NMR (75 MHz, DMSO) δ 24.66, 24.75, 32.91, 33.19, 38.66, 110.08, 121.68, 122.46, 128.16, 129.05, 129.99, 130.05, 131.62, 131.66, 132.35, 133.42, 161.08, 161.12, 170.96, 171.38; m/z 478.87; λ_{max} = 358 nm

Synthesis of Biotinylated Naphthalimide Derivatives

5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-6-yl)-pentanoic acid [2-(1,3-diozo-1H,3H-benzo[de]isoquinolin-2-yl)-ethyl]-amide (Compound **12**, Figure 22) In a 100

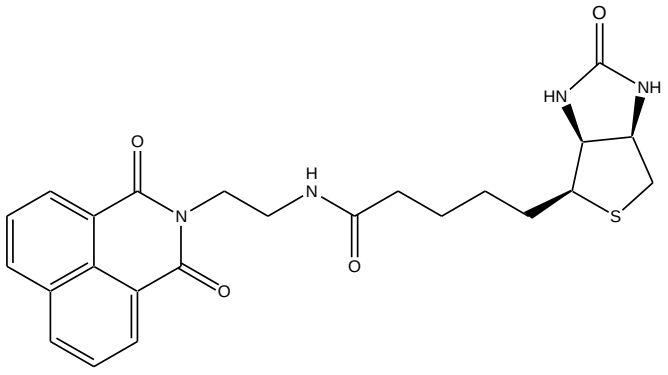


Figure 22. Structure of compound **12**

ml round bottom flask, D-(+) biotin (0.296 g, 1.22 mmol) and 1.1 equivalents BOP (0.594 g, 1.34 mmol) were dissolved in 20 ml of DMF. This solution was stirred at 0°C using an ice bath. Compound **1** (0.30 g, 1.22 mmol) and triethylamine (0.67 mL, 3.78

mmol) were dissolved in 10 mL of DMF in a 30 mL Erlenmeyer flask. Using a syringe, the compound **1** solution was transferred to the biotin solution drop wise. The mixture was stirred overnight at ambient temperature, and then the solvent was evaporated under reduced pressure. After evaporation, white colored precipitated crude product was remained. 40 mL of ethyl acetate was added to the crude product in round bottom flask, and crude product was stored at -5 ^{o}C for 16 hours. The solid product was then filtered from the ethyl acetate, which dissolved the residual starting material and reagents. Ethyl acetate layer and solid product were separated by filtration. The white colored filtrate was dried under vacuum, and characterized by ^{1}H NMR, ^{13}C NMR and mass spectroscopy, and provided a yield of 71 %. ^{1}H NMR (500 MHz, DMSO) δ 1.17 (m, 2H), 1.34 (m, 3H), 1.50 (m, 1H), 1.92 (t, 2H, *J* = 7.5 Hz), 2.54 (d, 1H, *J* = 12.5 Hz), 2.78 (dd, 1H, *J* = 12.5, 5 Hz), 2.96 (q, 1H, *J* = 8.5 Hz), 3.37 (q, 2H, *J* = 5 Hz), 4.02 (t, 1H), 4.12 (t, 2H, *J* = 5 Hz), 4.27 (t, 1H, *J* = 7.5 Hz), 6.32 (s, NH), 6.36 (s, NH), 7.86 (t, 2H, *J* = 7.5 Hz), 8.43 (d, 2H, *J* = 8 Hz), 8.47 (d, 2H, *J* = 7.5 Hz); ^{13}C NMR (75 MHz, DMSO) δ 25.10, 27.93, 28.05, 35.21, 36.27, 55.30, 59.14, 60.91, 122.26, 127.14, 127.49, 130.55, 131.26, 134.11, 162.65, 163.61, 172.21; m/z 467.27 (M) ($C_{24}H_{26}N_4O_4S$ requires 467.17)

5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-6-yl)-pentanoic acid [2-(6-ethylsulfanyl-1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-ethyl]-amide (Compound **13**, Figure 23) In a 100 ml round bottom flask, D-(+) biotin (0.160 g, 0.664 mmol) and 1.1 equivalents BOP (0.323 g, 0.730 mmol) were dissolved in 20 ml of DMF. This solution was stirred at 0°C using an ice bath. Compound **5** (0.20 g, 0.664 mmol) and triethylamine (0.290 mL, 2.058 mmol) were dissolved in 10 mL of DMF in a 30 mL Erlenmeyer flask. Using a syringe, the compound **5** solution was transferred to the biotin solution drop wise. The mixture was stirred overnight at ambient temperature, and then the solvent was evaporated under vacuum. After evaporation, white colored precipitated crude product was remained. 40 mL ethyl acetate was added to the crude

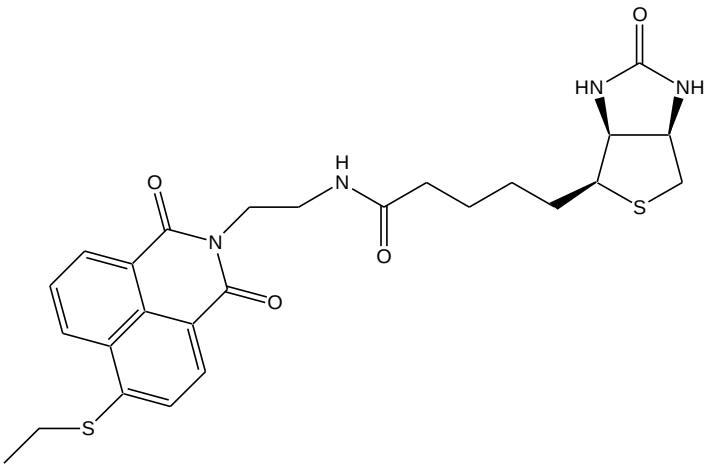


Figure 23. Structure of compound **13**

product in round bottom flask, and crude product was stored at -5 ^{o}C for 16 hours. The solid product was then filtered from the ethyl acetate, which dissolved the residual starting material and reagents. Ethyl acetate layer and solid product were separated by filtration. The white colored filtrate was dried under vacuum and characterized by ^{1}H NMR, ^{13}C NMR and mass spectroscopy, and provided a yield of 80 %. ^{1}H NMR (300 MHz, DMSO) δ 1.17 (t, 2H, *J* = 7.2 Hz), 1.38 (t, 3H, *J* = 7.2 Hz), 1.50 (m, 1H), 1.92 (t, 2H, *J* = 7.2 Hz), 2.56 (d, 1H, *J* = 12.3 Hz), 2.79 (dd, 1H, *J* = 12.7, 5.1 Hz), 2.96 (m, 1H), 3.33 (m, 4H), 4.04 (t, 1H, *J* = 6.9 Hz), 4.11 (t, 2H, *J* = 5.7 Hz), 4.29 (t, 1H, *J* = 7.4 Hz), 6.35 (s, NH), 6.39 (s, NH), 7.74 (d, 1H, *J* = 8.0 Hz), 7.88 (t, 2H, *J* = 7.7 Hz), 8.36 (d, 1H, *J* = 7.9 Hz), 8.51 (d, 2H, *J* = 8.4 Hz); ^{13}C NMR (75 MHz, DMSO) δ 8.61, 13.41, 25.13, 25.18, 27.93, 28.04, 35.21, 36.26, 45.73, 55.30, 59.15, 60.91, 118.56, 122.72, 122.91, 127.11, 127.71, 128.59, 129.39, 130.35, 130.07, 144.22, 162.64, 163.34, 163.39, 172.19; *m/z* 527.13 (MH^{+}) ($C_{26}H_{30}N_4O_4S_2$ requires 526.17); λ_{max} = 400 nm

5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-6-yl)-pentanoic acid (6-ethylsulfanyl-1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-amide (Compound **14**, Figure 24) In a 100 ml round bottom flask, D-(+) biotin (0.220 g, 0.915 mmol) and 1.1 equivalents BOP

(0.445 g, 1.00 mmol) were dissolved in 20 ml of DMF. This solution was stirred at 0°C using an ice bath. Compound **7** (0.250 g, 0.915 mmol) and triethylamine (0.397 mL, 2.836 mmol) were dissolved in 10 mL of DMF in a 30 mL Erlenmeyer flask. Using a

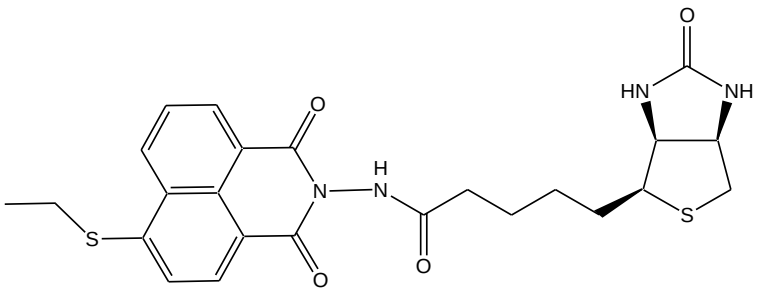


Figure 24. Structure of compound **14**

syringe, the compound **7** solution was transferred to the biotin solution drop wise. The mixture was stirred overnight at ambient temperature, and then the solvent was evaporated under reduced pressure. After evaporation, precipitated crude product was remained. 40 mL of ethyl acetate was added to the crude product in round bottom flask, and crude product was stored at -5 ^{o}C for 16 hours. The solid product was then filtered from the ethyl acetate, which dissolved the residual starting material and reagents. Ethyl acetate layer and solid product were separated by filtration. The light-yellow colored filtrate was dried under vacuum, and characterized by ^{1}H NMR, ^{13}C NMR and mass spectroscopy and provided a yield of 68 %. ^{1}H NMR (300 MHz, DMSO) δ 1.17 (t, 2H, *J* = 7.1 Hz), 1.40 (m, 3H), 1.65 (m, 3H), 2.36 (t, 2H, *J* = 7.1 Hz), 2.60 (m, 1H), 2.89 (dd, 1H, *J* = 12.5, 5.0 Hz), 3.20 (m, 2H), 4.21 (m, 1H), 4.32 (m, 1H), 6.38 (s, NH), 6.45 (s, NH), 7.79 (d, 1H, *J* = 8.1 Hz), 7.93 (t, 1H, *J* = 8.3 Hz), 8.42 (d, 1H, *J* = 8.0 Hz), 8.58 (d, 2H, *J* = 8.3 Hz), 10.63 (s, NH); ^{13}C NMR (75MHz, DMSO) δ 13.32, 25.00, 25.16, 27.95, 28.00, 32.93, 55.37, 59.18, 61.01, 117.84, 122.40, 122.77, 127.38, 128.72, 130.25, 131.13, 131.75, 145.66, 161.38, 161.45, 162.68, 171.03; *m/z* 499.07 (MH^{+}) ($C_{24}H_{26}N_4O_4S_2$ requires 498.14)

5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-6-yl)-pentanoic acid {2-[2-(2-diethylamino-ethyl)-1,3-dioxo-2,3-dihydro-1H-benzo[de]isoquinolin-6-ylamino]-ethyl}-amide (Compound **15**, Figure 25) In a 50 ml round bottom flask, D-(+) biotin (0.047 g, 0.196 mmol) and 1.1 equivalents BOP (0.095 g, 0.215 mmol) were dissolved in 10 ml of DMF. This solution was stirred at 0°C using an ice bath. Compound **10** (0.250 g, 0.915 mmol) and triethylamine (0.10 mL, 0.607 mmol) were dissolved in 5 mL of DMF in a 10 mL Erlenmeyer flask. Using a syringe, the compound **10** solution was transferred to the biotin solution drop wise. The mixture was stirred overnight at ambient temperature, and then the solvent was evaporated under reduced pressure. After evaporation, precipitated crude product was remained. 20 mL of ethyl acetate was added to the crude product in round bottom flask, and crude product was stored at -5 ^{o}C for 16 hours. The solid product was then filtered from the ethyl acetate, which dissolved the residual starting material and reagents. Ethyl acetate layer and solid product were separated by filtration. The filtrate was dried under vacuum and characterized by ^{1}H NMR, ^{13}C NMR and mass spectroscopy and provided a yield of 88%.

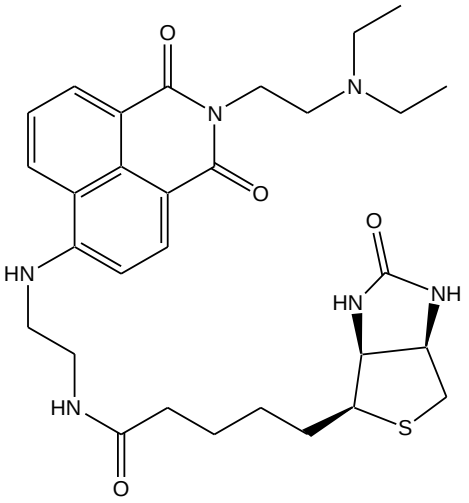


Figure 25. Structure of compound **15**

^{1}H NMR (300 MHz, DMSO) δ 1.24 (t, 6H, *J* = 6.5 Hz), 1.26 (m, 2H), 1.40(m, 1H), 1.50 (m, 4H), 2.08 (t, 2H, *J* = 7.5 Hz), 2.75 (dd, 1H), 2.96 (m, 1H), 3.08 (m, 1H), 3.20 (m,

2H), 3.25 (m, 4H), 3.40 (m, 2H), 3.42 (m, 2H), 4.24 (m, 1H), 4.32 (m, 2H), 6.33 (s, NH), 6.37 (s, NH), 6.86 (d, 1H, *J* = 8.5 Hz), 7.71 (t, 1H, *J* = 8.0 Hz), 7.92 (s, NH), 8.10 (s, NH), 8.28 (d, 1H, *J* = 8.0 Hz), 8.45 (d, 1H, *J* = 7.5 Hz), 8.61 (d, 1H, *J* = 8.5 Hz); ^{13}C NMR (75 MHz, DMSO) δ 14.05, 20.73, 25.18, 27.96, 28.08, 35.17, 36.44, 37.32, 42.96, 46.65, 48.26, 55.32, 59.14, 59.72, 60.93, 103.79, 107.50, 120.07, 121.77, 124.45, 128.69, 129.60, 130.90, 134.46, 150.92, 162.64, 163.07, 164.09, 173.08; *m/z* 364.33 (MH^+) ($C_{30}H_{40}N_6O_4S_1$ requires 363.16)

Attempted synthesis of *6-Oxo-6-{N'-[5-(2-oxo-hexahydro-thieno[3,4-d]imidazol-6-yl)-pentanoyl]-hydrazino}-hexanoic acid(6-ethylsulfanyl-1,3-dioxo-1H,3H-benzo[de] isoquinolin-2-yl)-amide* (Compound **16**, Figure 26) In a 50 ml round bottom flask, D-(+) biotin (0.040 mg, 0.169 mmol) and 1.1 equivalents BOP (0.082 mg, 0.186 mmol) were dissolved in 10 ml of DMF. This solution was stirred at 0°C using an ice bath. Compound **8** (0.070 mg, 0.169 mmol) and triethylamine (0.10 mL, 0.524 mmol) were dissolved in 5 mL of DMF in a 10 mL Erlenmeyer flask. Using a syringe, the compound **11** solution was transferred to the biotin solution drop-wise. The mixture was stirred overnight at ambient temperature, and then the solvent was evaporated under

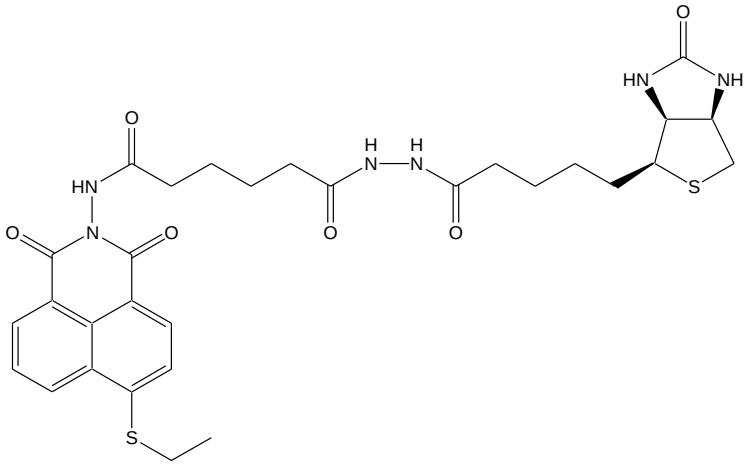


Figure 26. Structure of compound **16**

vacuum. After evaporation, precipitated crude product was remained. 20 mL of ethyl acetate was added to the crude product in round bottom flask, and crude product was stored at -5 ^{o}C for 16 hours. The solid product was then filtered from the ethyl acetate, which dissolved the residual starting material and reagents. Ethyl acetate layer and solid product were separated by filtration. The yellow colored filtrate was dried under vacuum, and characterized by ^{1}H NMR and provided a yield of 92%. No further characterization was performed. ^{1}H NMR (300 MHz, DMSO) δ 1.17 (t, 2H, *J* = 7.2 Hz), 1.38 (t, 3H, *J* = 7.4 Hz), 1.56 (m, 3H), 1.64 (m, 4H), 2.13 (m, 4H), 2.37 (m, 2H), 2.81 (dd, 1H, *J* = 5.04 Hz), 3.10 (m, 2H), 3.30 (m, 1H), 4.15 (m, 1H), 4.30 (m, 1H), 6.37 (br, NH), 6.43 (br, NH), 7.79 (d, 1H, *J* = 7.0 Hz), 7.92 (dd, 1H, *J* = 8.3, 7.5 Hz), 8.42 (d, 1H, *J* = 7.5 Hz), 8.58 (t, 2H, *J* = 7.2 Hz), 9.71 (s, NH), 10.63 (s, NH)

Biotin-Amino acids Compounds

5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoic acid [2-(4-hydroxy-phenyl)-ethyl]-amide (Compound **17**, Figure 27) In a 100 ml round bottom flask, D-(+) biotin (0.350 g, 1.457 mmol) and BOP (0.710 g, 1.602 mmol) were dissolved in 20 ml of DMF. This solution was stirred at 0°C using an ice bath. Tyramine (0.200 g, 1.457 mmol) and triethylamine (0.63 mL, 4.516 mmol) then were dissolved in 10 mL of DMF in a 30 mL Erlenmeyer flask. Using a syringe, the tyramine solution was transferred to the biotin solution drop wise. The mixture was stirred overnight at ambient temperature, and then the solvent was evaporated under reduced pressure. After evaporation,

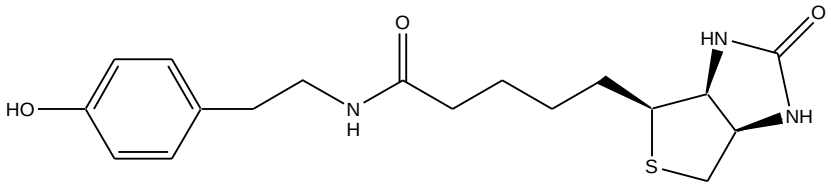


Figure 27. Structure of compound **17**

precipitated crude product was remained. 40 mL of ethyl acetate was added to the crude product in round bottom flask, and crude product was stored at -5 ^{o}C for 16 hours. The solid product was then filtered from the ethyl acetate, which dissolved the residual starting material and reagents. Ethyl acetate layer and solid product were separated by filtration. The filtrate was re-crystallized from hot ethanol. The final product was dried under vacuum and characterized by ^{1}H NMR, ^{13}C NMR and mass spectroscopy. The biotin-tyramine compound was obtained as a white-light pink colored powder in 36 % yield. ^{1}H NMR (300 MHz, DMSO) δ 1.25(m, 2H), 1.44 (m, 3H), 1.56 (m, 1H), 2.01 (t, 2H, *J* = 7.2 Hz), 2.54 (m, 3H), 2.80 (dd, 1H, *J*= 12.5, 5.1 Hz), 3.06 (m, 1H), 3.42 (m, 1H), 4.11 (m, 1H), 4.29 (m, 1H), 6.35 (s, NH), 6.41 (s, NH), 6.65 (d, 2H, *J* = 8.4 Hz), 6.95 (d, 2H, *J* = 8.4 Hz), 7.79 (t, 1H, *J* = 5.4 Hz), 9.15(s, NH); ^{13}C NMR (75 MHz, DMSO) δ 18.53, 25.26, 27.99, 28.14, 34.38, 35.17, 55.39, 55.99, 59.16, 60.99, 115.01, 129.40, 129.52, 155.54, 162.67, 171.81; *m/z* 364.33 (MH^{+}) ($C_{18}H_{25}N_3O_3S$ requires 363.16)

5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoic acid [2-(1H-indol-3-yl)-ethyl]-amide (Compound **18**, Figure 28) In a 100 ml round bottom flask, D-(+) biotin (200 mg, 0.830 mmol) and BOP (400 mg, 0.910 mmol) were dissolved in 20 ml of DMF. This solution was stirred at 0 °C using an ice bath. Tryptamine (130 mg, 0.830 mmol) and triethylamine (0.360 mL, 2.560 mmol) were dissolved in 10 mL of DMF in a 30 mL Erlenmeyer flask. Using a syringe, the tryptamine solution was transferred to the biotin solution drop-wise. The mixture was stirred overnight at ambient

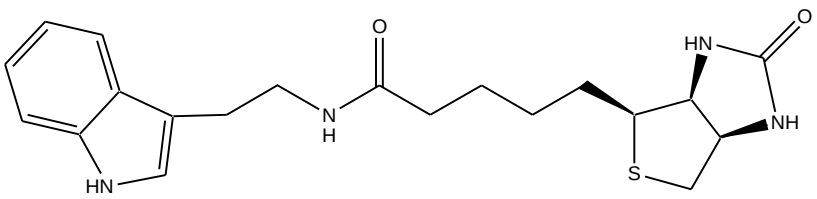


Figure 28. Structure of compound **18**

temperature, and then the solvent was evaporated under reduced pressure. After evaporation, precipitated crude product was remained. 40 mL of ethyl acetate was added to the crude product in round bottom flask, and crude product was stored at -5 ^{o}C for 16 hours. The solid product was then filtered from the ethyl acetate, which dissolved the residual starting material and reagents. Ethyl acetate layer and solid product were separated by filtration. The filtrate was dried under vacuum, and characterized by ^{1}H NMR and provided a yield of 90 %. ^{1}H NMR (300 MHz, DMSO) δ 1.29 (m, 2H), 1.50 (m, 3H), 1.60 (m, 1H), 2.05 (t, 2H, *J* = 7.2 Hz), 2.54 (d, 2H, *J* = 12.9 Hz), 2.59 (s, 1H), 2.81 (m, 3H), 3.05 (m, 3H), 4.11 (m, 1H), 4.30 (m, 1H), 6.35 (s, NH), 6.42 (s, NH), 6.98 (t, 1H, *J* = 7.5 Hz), 7.05 (t, 1H, *J* = 7.1 Hz), 7.13 (s, 1H), 7.32 (d, 1H, *J* = 8 Hz), 7.52 (d, 1H, *J* = 7.7 Hz), 7.90 (t, 1H, *J* = 5.6 Hz), 10.79 (s, NH); *m/z* 387.20 (MH^{+}) ($C_{20}H_{26}N_4O_2S_1$ requires 389.51)

3-(1H-Indol-3-yl)-2-[5-(2-oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoylamino]-propionic acid methyl ester (Compound **19**, Figure 29) In a 100 ml round bottom flask, D-(+) biotin (200 mg, 0.829 mmol) and 1.1 equivalents of BOP (400 mg, 0.911 mmol) were dissolved in 20 mL of DMF. This solution was stirred at 0°C using ice bath. L-tryptophan methyl ester hydrochloride (210 mg, 0.829 mmol) and triethylamine (0.360 mL, 2.560 mmol) were dissolved in 10 mL of DMF in a 30 mL Erlenmeyer flask. Using a syringe, the L-tryptophan methyl ester solution was

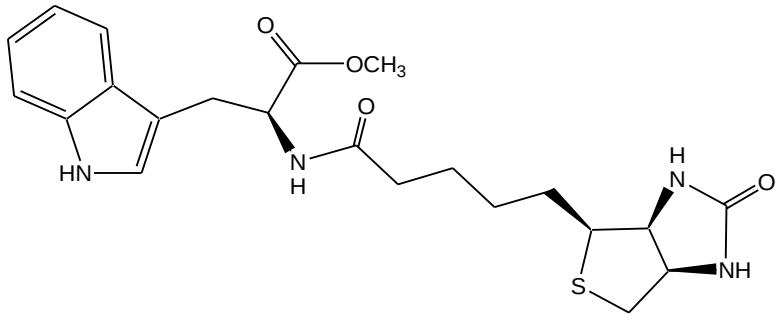


Figure 29. Structure of compound **19**

transferred to the biotin solution drop wise. The mixture was stirred overnight at ambient temperature, and then the solvent was evaporated under reduced pressure. The crude product was purified by flash column chromatography (MeOH : Ethyl acetate = 6 : 94, 8 : 92). Fractions containing the desired product were combined and the solvent evaporated under vacuum. The structure was confirmed by ^{1}H NMR, ^{13}C NMR and mass spectroscopy. The final product was obtained as a clear white colored solid in 63% yield. ^{1}H NMR (300 MHz, DMSO) δ 1.22 (m, 2H), 1.42 (m, 3H), 1.53 (m, 1H), 2.05 (t, 2H, *J* = 5.7 Hz), 2.78 (m, 1H), 3.01 (d, 1H, *J* = 8.7Hz), 3.08 (d, 1H, *J* = 5.7 Hz), 3.54 (s, 3H), 4.07 (m, 1H), 4.26 (m, 1H), 4.41 (m, 1H), 6.33 (s, NH), 6.36 (s, NH), 6.96 (t, 1H, *J* = 7.5 Hz), 7.03(t, 1H, 7.8 Hz), 7.11 (s, NH), 7.30 (d, 1H, *J* = 7.8 Hz), 7.45 (d, 1H, *J* = 7.8 Hz), 8.20 (d, 1H, *J* = 7.5 Hz), 10.82 (s, NH); ^{13}C NMR (75MHz, DMSO) δ 25.05, 27.04, 27.92, 34.67, 36.40, 36.44, 51.72, 52.94, 55.33, 59.15, 60.94, 109.54, 111.37, 117.96, 118.35, 120.91, 123.57, 127.03, 136.05, 162.66, 172.19, 172.55 ;m/z 445.27 (MH^{+}) ($C_{22}H_{28}N_4O_4S$ requires 444.56)

5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoic acid [2-(3,4-dihydroxy-phenyl)-ethyl]-amide (Compound **20**, Figure 31) In a 100 ml round bottom flask, D-(+) biotin (200 mg, 0.829 mmol) and 1.1 equivalents of BOP (400 mg, 0.911 mmol) were dissolved in 20 mL of DMF. This solution was stirred at 0°C using ice bath. Dopamine (0.127 g, 0.829 mmol) and triethylamine (0.360 mL, 2.560 mmol) were dissolved in 10 mL of DMF in a 30 mL Erlenmeyer flask. Using a syringe, the

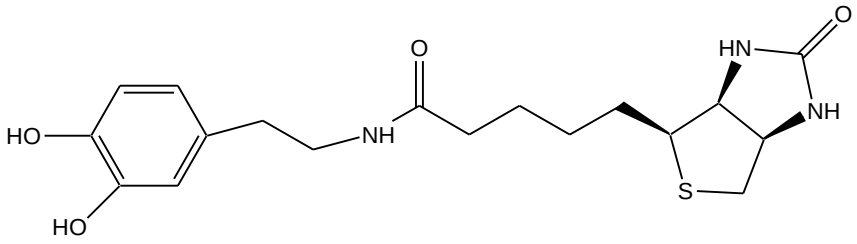


Figure 30. Structure of compound **20**

dopamine solution was transferred to the biotin solution drop wise. The mixture was stirred overnight at ambient temperature, and then the solvent was evaporated under reduced pressure. After evaporation, light-white colored liquid state crude product was remained in the round flask. 40 mL of ethyl acetate poured to the crude product, and ethyl acetate layer and white colored precipitate were filtrated and purified by column chromatography. The structure was confirmed by ^{1}H NMR, ^{13}C NMR and mass spectroscopy. The final product was obtained as a white colored solid in 32% yield. ^{1}H MNR (300 MHz, D_2O) δ 1.13 (m, 2H), 1.23 (t, 2H, *J* = 7.2 Hz), 1.46 (m, 3H), 1.50 (m, 1H), 2.13 (t, 2H, *J* = 7.2 Hz), 2.69 (t, 2H, *J* = 8.1 Hz), 2.98 (dd, 1H, *J* = 13.0, 5.1 Hz), 3.15 (m, 1H), 3.42 (t, 2H, *J* = 6 Hz), 4.32 (m, 1H), 4.57 (m, 1H), 6.68 (d, 1H, *J* = 8.4 Hz), 6.76 (s, NH), 6.82 (d, 1H, *J* = 8.1 Hz), 7.44 (m, 1H), 7.70 (m, OH), 7.80 (m, OH); ^{13}C NMR (75 MHz, D_2O) δ 13.22, 20.49, 25.13, 35.40, 39.94, 61.71, 111.65, 116.72, 125.43, 126.32, 142.25, 143.76, 165.34, 176.55; DEI-HRMS *m/z* 402.1456 (MNa^+) ($C_{18}H_{25}N_3O_4SNa$ requires 402.1465, Δ = -2.2 ppm)

Photochemical Protein Crosslinking

A 15 µl sample of 0.235 mM RNase A in PBS and same volume of 0.50 mM sensitizer (naphthalimide) also in PBS were transfer to microcentrifuge tubes (1.5 mL), along with any other additives such as biotinylated compounds. The solutions were irradiated by Xenon lamp (using 320-500nm filter, 550 mW, 2.82 W/cm^2) or Mercury lamp (using 400-500nm filter, 2800 mW) for 5 min with the distance between solution and light part maintained at 5~7 mm with the tube in water bath to prevent overheating. The resulting protein samples were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE Analysis

To all samples 15 µl of 3 × SDS-tris-HCl loading buffer was added. This buffer included bromophenol blue for tracking and DTT as a reducing agent. The resulting solutions were heated at 100 ºC for 5 min in a boiling water bath, and then were cooled on ice. 10 µl of each sample was loaded onto a 4-20% polyacrylamide gel. SDS running buffer was used and the electrophoresis was carried out at 155 V for 50 minutes. After the electrophoresis, the gels were stained with fast type of Coomassie blue R-250 solution for 1 hour and destained by washing several times with a solution of 85:5:10 DI-water/glycerol/acetic acid. In the case of biotinylated naphthalimides, the gels were also analyzed by Western blotting.

Western Blotting Analysis

After the separation by protein by SDS-PAGE, the gel was briefly rinsed in DI-water for 3 minutes and then equilibrated in freshly prepared Towbin buffer for 5 minutes. Four pieces of extra thick blotting papers were soaked in Towbin buffer, and 2 pieces of blotting papers were placed on the anode, followed by a pre-wetted nitrocellulose membrane. The gel was placed on the membrane and all bubbles were carefully removed, followed by 2 more pieces of blotting papers. Finally, the cathode was carefully placed on the stack, and the blotting unit was connected to a power supply for 25 minutes (100 mA, 40 V). The nitrocellulose membrane was then removed from the transfer apparatus and blocked in SuperBlock blocking buffer in TBS, which contains a proprietary protein in Tris-buffered saline containing Kathon anti-microbial agent, with Tween 20 reagent for 1 hour at room temperature with shaking. The membrane was then washed with washing buffer with agitation for ≥ 5 minutes (× 6 times), after which the HRP-avidin conjugate solution was added and shaking continued for 1 hour at room temperature. The membrane was then washed by suspending it in wash buffer and agitating for ≥ 5 minutes (× 6 times). A working solution was then freshly prepared by

mixing equal part of the stable peroxide solution and the luminol/enhancer solution to give 0.1 ml working solution per cm^2 of membrane. The membrane was then shaken with the solution for 2.5 minutes. The membrane was removed from working solution and placed it under the camera in a dark chamber. The membrane was exposed for 60 seconds in the chamber. The signals from the decomposition of luminol were captured by the camera using the Ultra Quant program.

The Stability of Biotin and 1,8-Naphthlimide during Irradiation

In order to confirm the stability of biotin with naphthalimide during the photochemical cross-linking reaction, we performed experiments examines the ^1H NMR of biotin treated under oxidizing conditions. D-(+) biotin (6 mg) was dissolved in D_2O and $NaIO_4$ was added to the biotin solution. The ^1H NMR spectra was recorded after 15minutes and 30 minutes. Similarly, D-(+) biotin (6 mg) was dissolved in D_2O and H_2O_2 was added to the biotin solution. After 15 minutes and 30 minutes, the ^1H NMR spectra was recorded. In addition, methionine was reacted with $NaIO_4$ and H_2O_2 (1:1 ratio, respectively) to provide additional examples of oxidation of a sulfide.

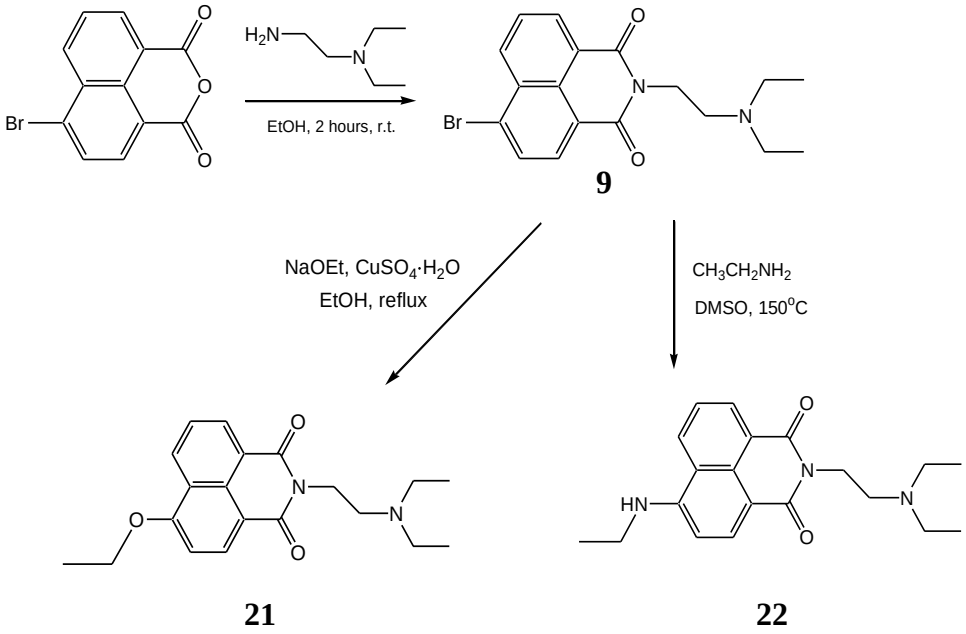
The ^1H NMR spectra of these oxidized compounds were compared with ^1H NMR spectra of naphthalimide and biotin reaction products. Briefly, four samples were prepared in DMSO-$_{d6}$: naphthalimide **10**, D (+)-biotin, and an equimolar solution of compound **10** and D-(+) biotin, and the biotinylated naphthalimide compound **15**. ^1H NMR spectra were acquired for each of the four samples after 0, 5, 15, and 30 minutes of irradiation (Mercury lamp, 2800 mW).

CHAPTER THREE

Results and Discussion

Synthesis of Various Types Naphthalimide Compounds

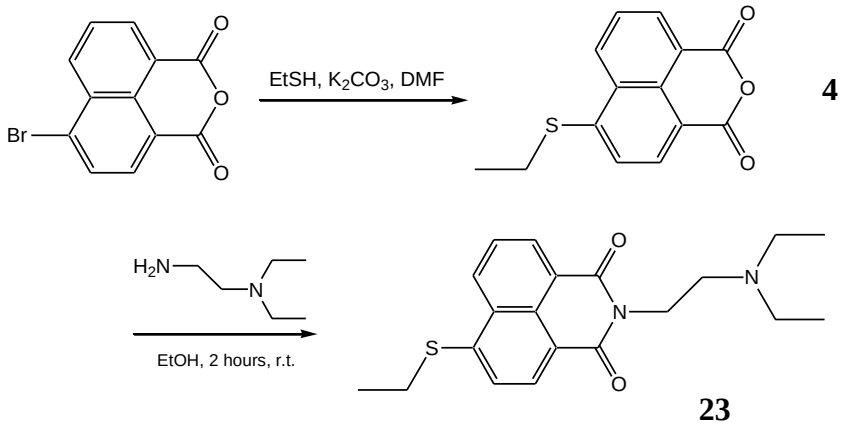
The monomeric 4-bromo-1,8-naphthlaimide compounds were synthesized using 4-bromo-1,8-naphthalic anhydride as a common starting material. The starting material was reacted with *N,N*'-diethylethylenediamine by condensation reaction. The second step is the nucleophilic aromatic substitution of the 4-bromo substituted with another amine or alkoxy group onto the 4-position. The synthetic methodology for compounds **9**, **21**, **22**, **23**, **24** was taken from Dr. Woods' dissertation.[40] These compounds were used as sensitizers for protein crosslinking assay. According to Figure 6, the unsubstituted 1,8-naphthlaimide (compound **24**) was demonstrated to have a higher crosslinking rate than other 1,8-naphthalimides.



Scheme 4. Synthesis of compounds **21** and **22**

4-Ethylsulfanyl-1,8-Naphthalimides

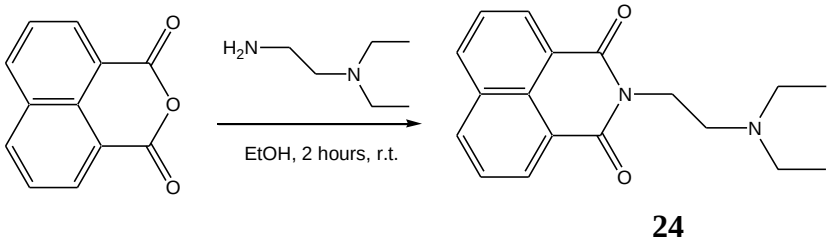
4-Ethylsulfanyl-1,8-naphthalimide was prepared by nucleophilic aromatic substitution followed by the condensation of the primary amine. Fortunately, the anhydride intermediate was easy to crystallize by cold water and isolate by filtration. This synthetic route is shown in Scheme 5.



Scheme 5. Synthesis of compounds **4** and **23**

Unsubstituted 1,8-Naphthalimide

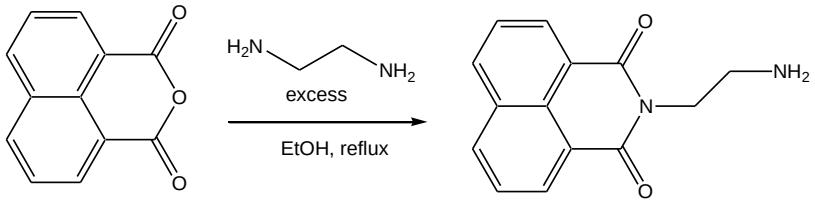
Compound **24** was prepared by condensation of the primary amine onto 1,8-naphthalic anhydride.



Scheme 6. Synthesis of compound **24**

Similarly, compound **1** was synthesized by condensation of 1,8-naphthalic anhydride with excess ethylenediamine to avoid *bis*-naphthalimide products. This

reaction is shown in Scheme 7. Compound **3** was prepared according to the methodology in Scheme 8. In the first step, *tert*-butyl carbazate was condensed with 1,8-naphthalic anhydride, after which the BOC protecting group was removed strong acid in methylene chloride.



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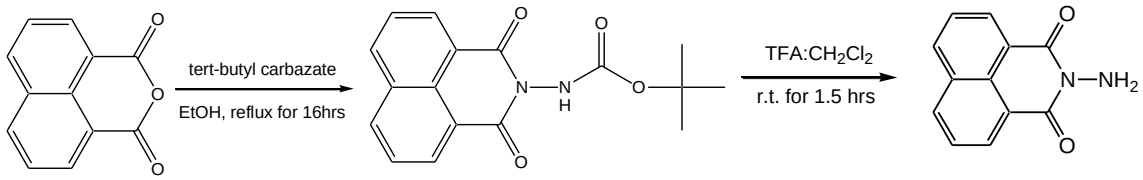
Scheme 7. Synthesis of compound **1**



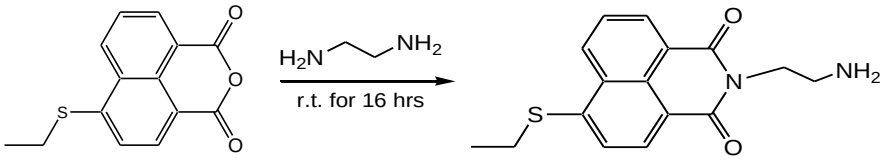
2 **3**

Scheme 8. Synthesis of compound **2** and **3**

4-Ethylsulfanyl-1,8-Naphthalimide Derivatives

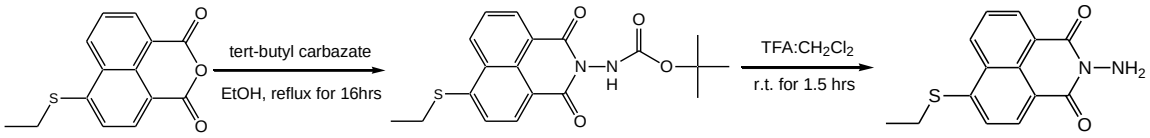
The syntheses of the 4-ethylsulfanyl-1,8-naphthalaimides derivatives **4**, **7**, and **8** were synthesized in the same manner as the 4-ethylsulfanyl derivatives in Scheme 5. Compound **5** was prepared by condensation of excess ethylenediamine onto 4-ethylsulfanyl-1,8-naphthalic anhydride. Compounds **7** and **8** were synthesized in the same manner as Scheme 8. In the synthesis of compound **7**, 4-ethylsulfanyl-1,8-naphthalic anhydride reacted with *tert*-butyl carbazate. The free amine was revealed by treatment with a strong acid and methylene chloride solution. Compound **8** was synthesized by reaction of adipic acid dihydrazide with excess amounts of 4-ethylsulfanyl-1, 8-naphthalic anhydride (compound **4**). However, according to ^{1}H

NMR, ^{13}C NMR and mass spectroscopy, it is assumed that the product compound **8** involves -$COCH_3$ group on the other hydrazide. Those synthetic routes are shown in Scheme 9, Scheme 10 and Scheme 11.



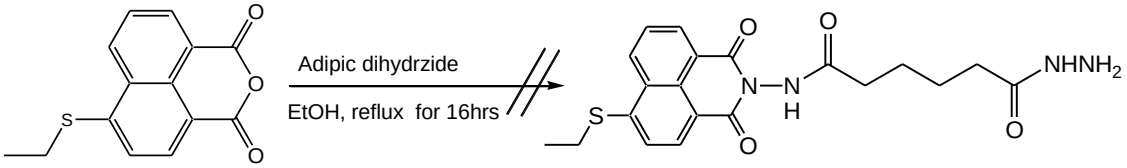
4 **5**

Scheme 9. Synthesis of compound **5**



4 **6** **7**

Scheme 10. Synthesis of compound **6** and **7**



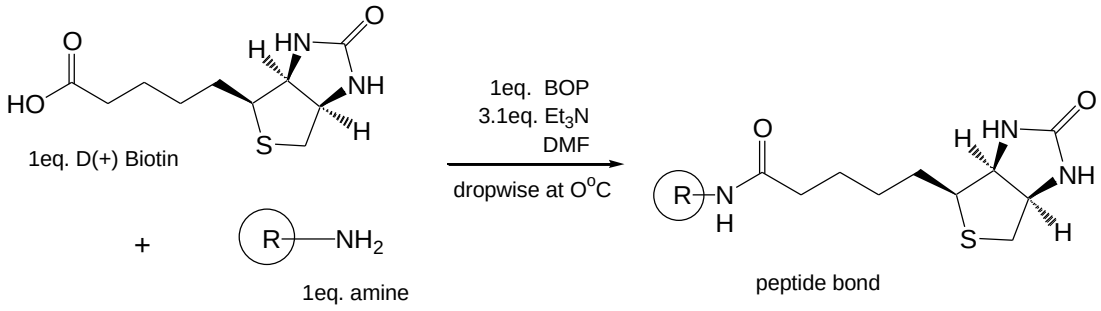
4 **8**

Scheme 11. Unsuccessful synthesis of compound **8**

Synthesis of Biotinylated Compounds

Biotin-avidin chemistry has been studied as a useful research tool because of the extraordinary affinity of avidin for biotin (Ka = 10^{15} M^{-1}). Pierce, a commercial company, has suggested guidelines to pick suitable biotinylated reagents for various purposes and applications. In addition, they provided various methods of detection such as Western blotting and chemiluminescence.[41]

The biotinylated naphthalimide and biotinylated amino acid compounds were synthesized by condensation reaction using a BOP reagent as a catalyst. This reaction produces a peptide bond between two molecules when a carboxyl group of biotin reacts with an amine (or hydrazide) of the other molecule. The BOP reagent is commonly used for biotin activation in peptide coupling. This reaction is shown in Scheme 12.

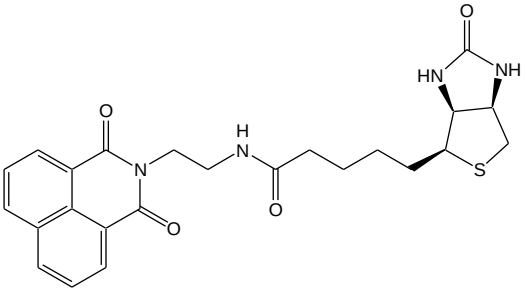


Scheme 12. The general scheme of peptide bond formation

Biotinylated naphthalimide derivatives were designed in order to investigate the potential of the naphthalimide photosensitizers to attach covalently directly to the protein. Compounds **12-15** (figure 31) were synthesized by condensation of D-(+) biotin and naphthalimides **1**, **5**, **7**, **10**, respectively. After reaction, the products were separated in two steps. In the first step, DMF was removed completely under reduced pressure. The second step, ethyl acetate was added into crude products, which were then allowed to precipitate. The products were obtained as pure solids and their structures were confirmed by ^{1}H NMR, ^{13}C NMR, and mass spectroscopy.

The involvement of amino acids, and especially tyrosine, in protein crosslinking has been demonstrated in previous experimentation. The preparation of biotinylated amino acids was expected to help us to understand the mechanisms involved in the photochemical protein crosslinkings mediated by naphthalimides. These compounds could also potentially help us discover new protein modification strategies.

Compounds **17**, **18**, **19**, and **20** were synthesized by a similar method to the one shown in Scheme 12. The reaction is the condensation of D-(+) biotin and amines such as tyramine, tryptamine, L-tryptophan, and dopamine. These biotinylated amino acid derivatives were then used for protein cross-linking assay.



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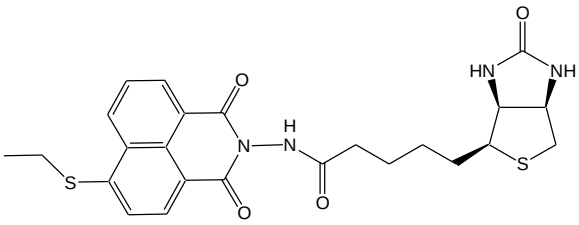

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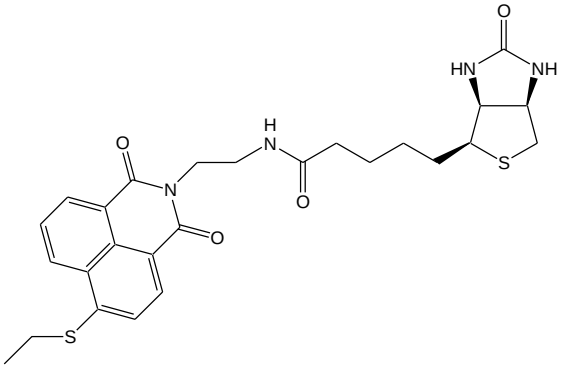

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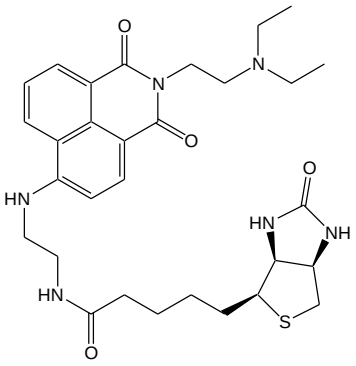
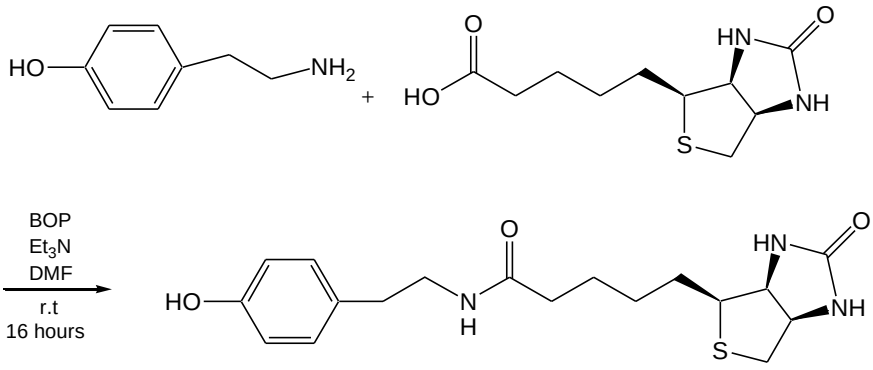
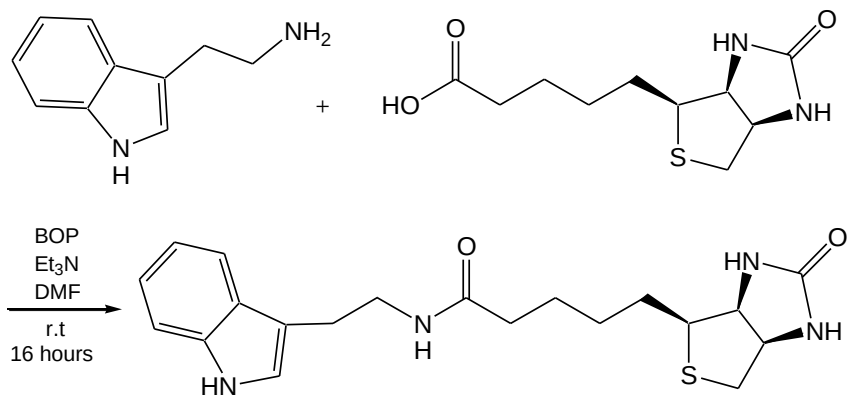


15

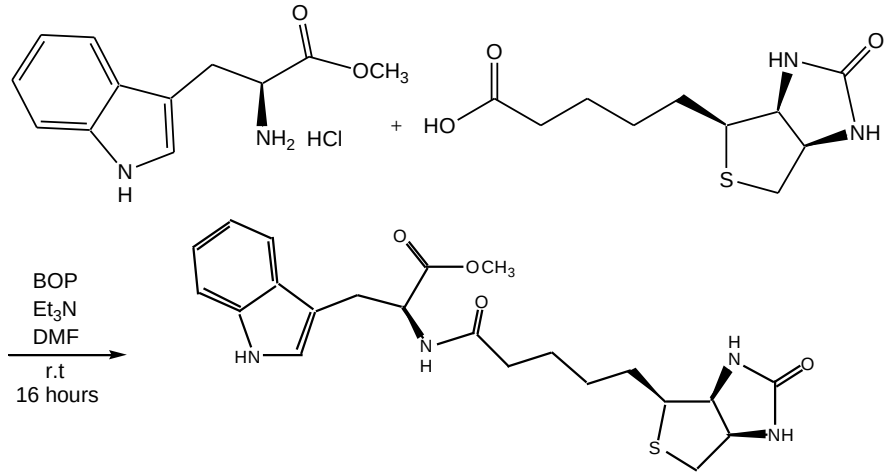
Figure 31. Structures of compounds **12 13**, **14**, and **15**



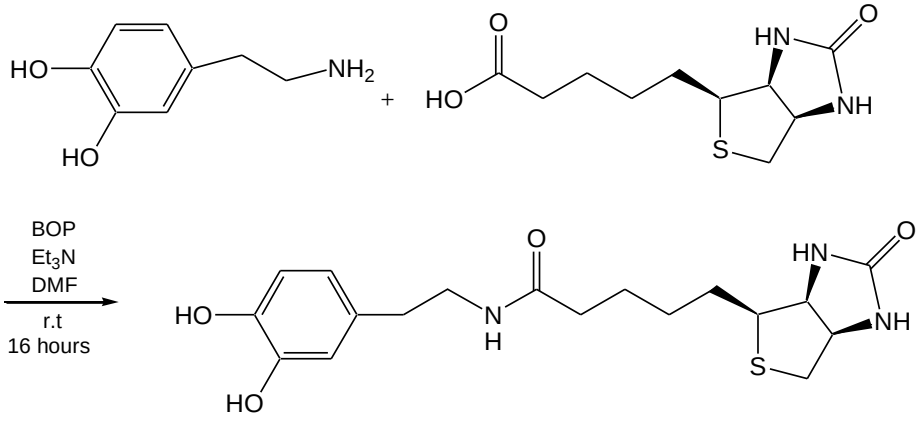
Scheme 13. Synthetic scheme of compound **17**



Scheme 14. Synthetic scheme of compound **18**



Scheme 15. Synthetic scheme of compound **19**



Scheme 16. Synthetic scheme of compound **20**

UV-vis Measurement

1,8-Naphthalimide compounds absorb light in the long UV to visible region of the spectrum. They have relatively long-wavelength λ_{max} values because their structures involve an extended conjugated system. In addition, depending on substituted groups such as amino, ethoxy, and sulfanyl, their λ_{max} values can be made shorter or longer. Because absorbance is related to concentrations of compounds, proper concentration is very important in producing a linear graph. Each compound was carefully prepared in concentrations from 0.0001 mM ~ 0.001 mM in DMSO.

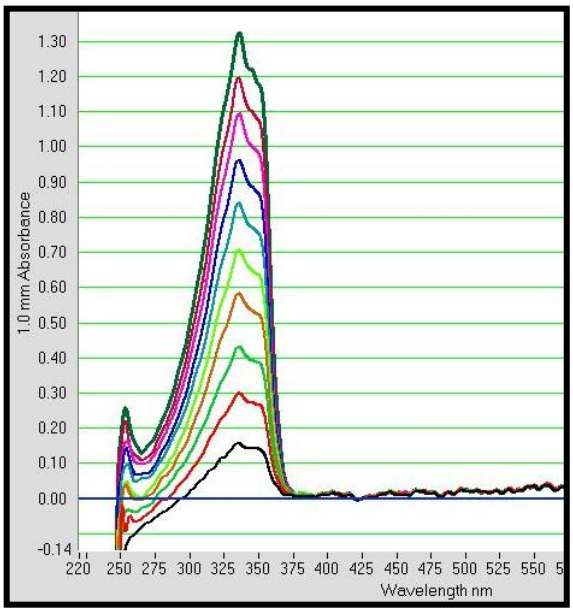
Table 1. λ_{max} values and extinction coefficients for 4-substituted-1,8- naphthalimides derivatives

Naphthalimide compounds	λ_{max} (nm)	Extinction coefficient (mM^{-1} cm^{-1})
1	335	9.37×10^6 at 350 nm
3	352	1.14×10^7at 352 nm
5	395	1.47×10^7 at 395 nm
7	397	9.37×10^6 at 400 nm
10	444	1.44×10^7 at 444 nm
13	400	9.25×10^6 at 400 nm

Woods's dissertation[40] reported some λ_{max} values of several 4-substituted-1,8-naphthalimide derivatives. According to those results, the 4-bromo and 4-unsubstituted derivatives have the shortest λ_{max} values. Because bromine is electron-withdrawing, it does not help toward extending the conjugated system. Also, the 4-unsubstituted derivative has no groups that extend the conjugated system. In some cases, it was observed that the effective protein crosslinking agents had lower wavelength λ_{max}.

Nitrogen, oxygen, and sulfur have a lone pair which can affect an extended conjugation system. This effect can reduce the energy difference of $\pi \rightarrow \pi^*$ transition

excited energy, causing a blue shift to occur. In this case, 4-arylsulfanyl and 4-alkylsulfanyl, which involve a sulfur group, have the highest λ_{max} values. But there are no clear correlations between λ_{max} values and the amount of protein cross-linking. Table 1 represents some wavelength values (λ_{max}) for 4-substituted-1,8- naphthalimide derivatives. Compound **1** containing a primary amine such as *N*-ethylamine group has a 335nm value. And compound **5** containing 4-alkylsulfany and *N*-ethylamine shows a higher λ_{max} value of 395nm. Because a sulfur atom can cause the conjugated system to increase by electron withdrawal, compound **5** absorbs light of longer wavelength values.



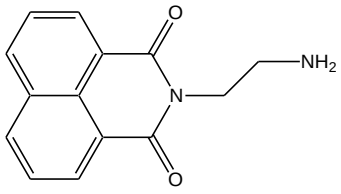
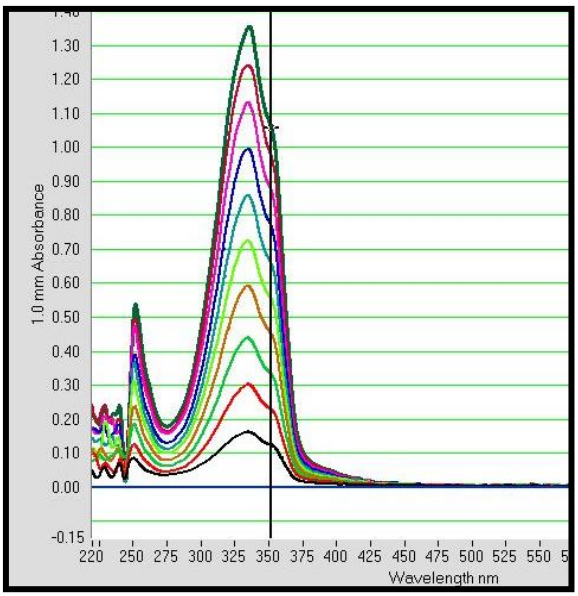


Figure 32. A structure and an *UV-vis* spectrum of compound **1**



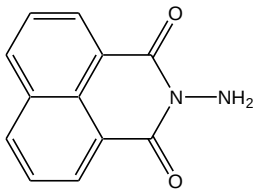
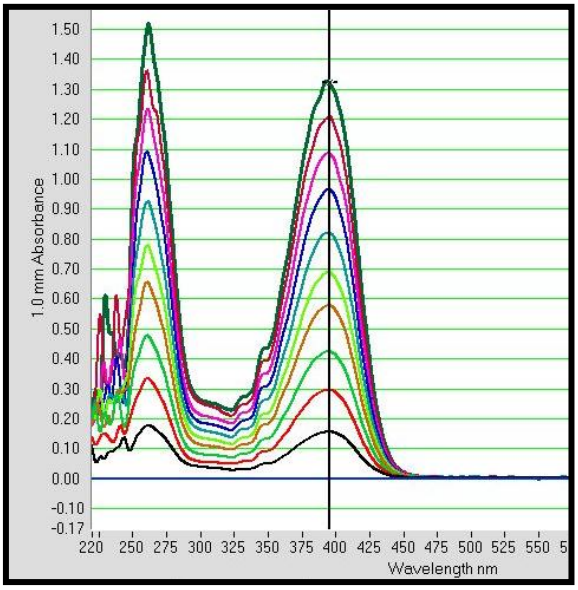


Figure 33. A structure and an *UV-vis* spectrum of compound **3**



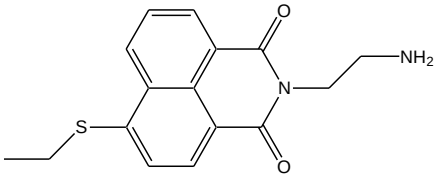


Figure 34. A structure and an *UV-vis* spectrum of compound **5**

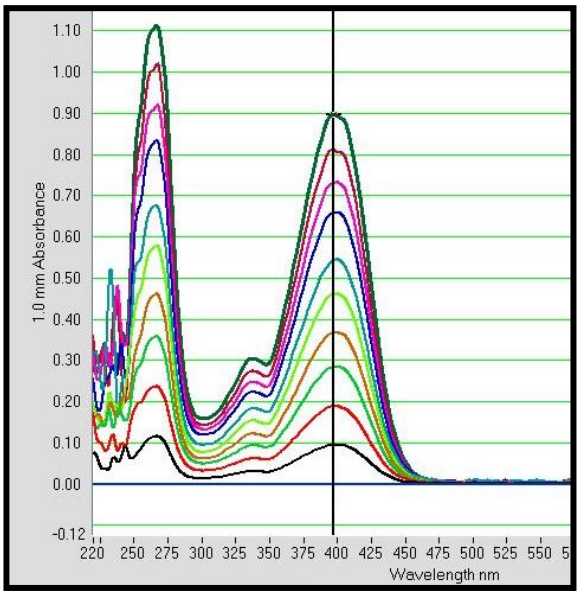


Figure 35. A structure and an *UV-vis* spectrum of compound **7**

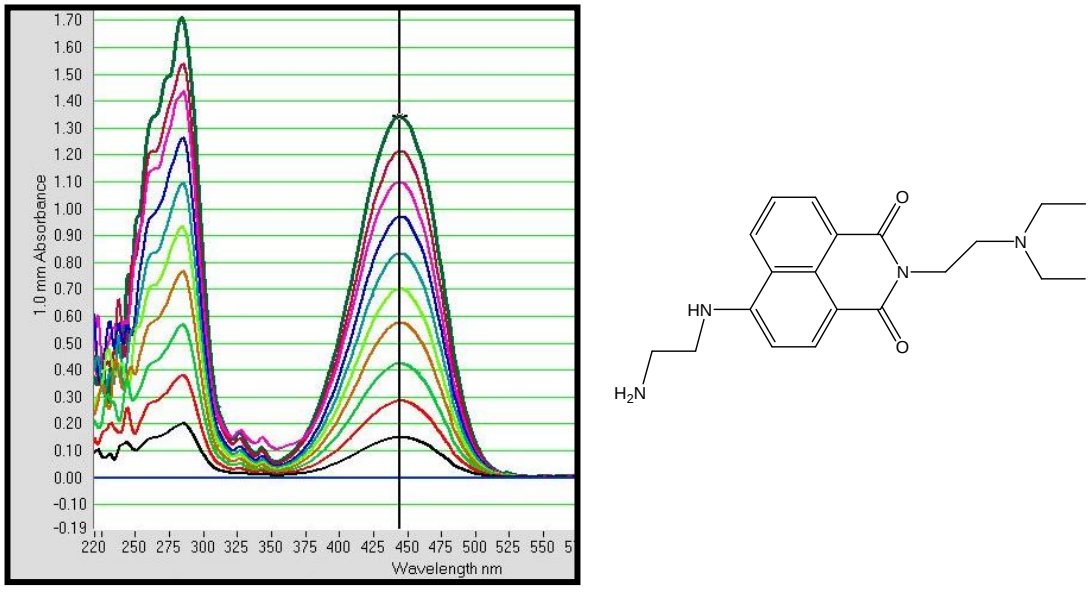


Figure 36. A structure and an *UV-vis* spectrum of compound **10**

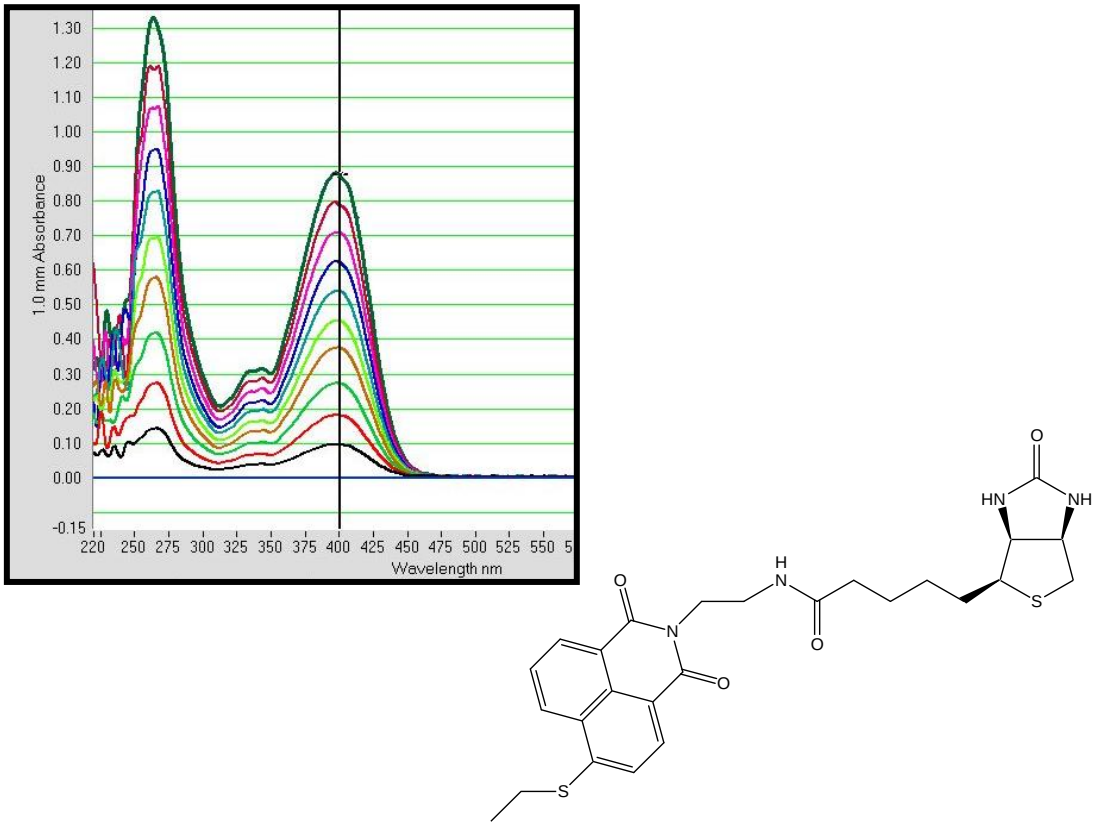


Figure 37. A structure and an *UV-vis* spectrum of compound **13**

Photochemical Cross-Linking of RNase A by 1,8-Naphthalimide Compounds

The studies of photochemical protein crosslinking with various naphthalimides as sensitizers were investigated. A *bis*-4-alkylamino-1,8-naphthalimide [42] had previously been used bond human meniscal and articular cartilage with laser irradiation. These tissue surfaces consist of numerous organic molecules such as collagen and other proteins and proteoglycins. In order to better understand the mechanism of the photochemical protein crosslinking reaction, our group has performed photochemical crosslinking reactions using RNase A with different types of naphthalimide derivatives. Because the RNase A is a relatively small (~13.7 kD) protein, stable, and well characterized, this protein has been used broadly in studies of photochemical protein cross-linking.[20] Cheng *et al.*[43] has demonstrated that RNase A was crosslinked by monomeric and dimeric 1,8-naphthalimides in presence of the light. The resulting

protein mixtures were analyzed by SDS-PAGE gel electrophoresis and the gels stained with coomassie blue R-250 solution. This technique was used to evaluate the extent of protein dimerization, trimerization, and oligomerization.

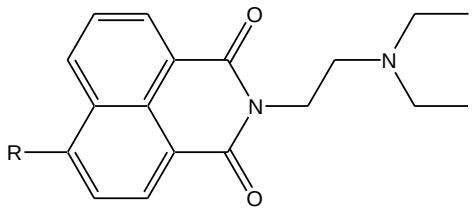
RNase A includes amino acids such as lysine and histidine, which are potential sites for photochemical protein crosslinking. In our experiments, the unsubstituted naphthalimide **24** was shown to be derivative in the most efficient derivative in the photochemical crosslinking of RNase A. Therefore, naphthalimide **24** was employed in photochemical protein crosslinking experiments in this paper. Figure 38 shows the result of photochemical protein crosslinking with monomeric 1,8-naphthalimides derivatives. The unsubstituted naphthalimide and 4-sulfanyl-1,8-naphthalimide were observed to produce crosslinked protein, while the other 4-substituted-1,8-naphthalimide derivatives were not active in this assay. The multiple bands in case 1 indicate a dimeric, trimeric, tetrameric, and mulitmeric proteins were formed. The 4-alkysulfayl-1,8-naphthalimide treated sample (lane 3) produced dimeric and trimeric protein. The control lane 6 shows that RNase A is not crosslinked by light in the absence of the naphthalimide photosensitizers.

Photochemical Cross-Linking of RNase A by Biotinylated-1,8-Naphthalimide Compounds

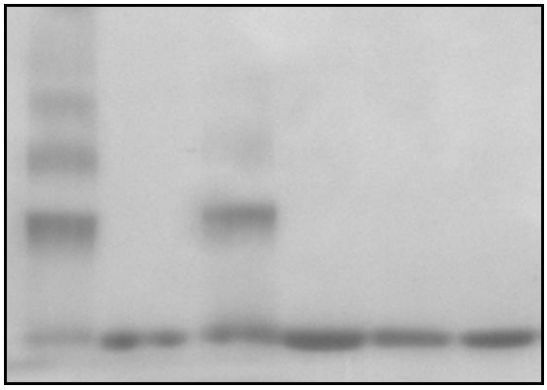
Biotinylated 1,8-naphthalimide derivatives synthesized in this work were examined for their ability to catalyze the crosslinking of RNase A using the same procedure as described in Figure 38. Biotinylated naphthalimides **12**,**13**,**14**,**15**, and **16** were used in this experiment.

In this experiment, synthesized biotinylated-1,8-naphthalimide derivatives were used to examined to determine their ability to cross-link RNase A under irradiation. Figure 39-(a) shows results that are similar to the previous experiments. Compound **12**, the 4-unsbstituted naphthalimide, demonstrated the most cross-linking activity, while

(a)



(b)



lane	:	1	2	3	4	5	6
naphthalimide compound	:	24	9	23	21	22	-
R	:	(-H)	(-Br)	(-SR)	(-OR)	(-NR)	
minutes light	:	5	5	5	5	5	5

Figure 38. (a) The structure of naphthalimide derivatives. (b) Crosslinkng of RNase A with naphthalimide derivatives. Protein concentrations were 0.235 mM, Naphthalimides concentrations were 0.05 mM. Irradiation was with a xenon lamp at 550 mW (2.82 W/cm^2) for 5 minutes

compound **16**, which contains 4-alkylsulfanyl substituted, shows activity slightly weaker than compound **12**. Compound **13**, **14**, and **15** were capable of cross-linking but less effective than compounds **12** and **16**. Figure 39-(b) shows a western blot after SDS-PAGE electrophoresis with detection of biotin residues. It is possible for naphthalimide to bond with amino acids on the protein RNase A. If this occurs, the biotin maker can be detected by chemiluminescence. Signals were not detected in the western blot. Therefore, naphthalimides did not react with amino acids on RNase A during photochemical protein crosslinking.

Effects of RNase A Crosslinking by Biotin-Amino Acids derivatives

In order to investigate the effects of amino acids in photochemical crosslinking, our research group previously studied the ability of amino acids to inhibit the photochemical protein crosslinking reaction. Each of the 20 standard amino acids was included in the RNase A crosslinking reaction (using compound **24**) and the resulting photochemical crosslinking was compared with the control band no added amino acids. It was observed that tyrosine, tryptophan, and cystein inhibited the photochemical reaction. Compound **17**, biotin-tyramine, was expected to also cause inhibition of crosslinking, as tyramine and tyrosine have the same functional group (phenol) which causes run to be antioxidants. It is known that tyrosine can form dityrosine by a type I photo-oxidation mechanism.[16] Figure 40-(a) shows the inhibition of the photochemical cross-linking of RNase A containing biotin-tyramine with naphthalimide sensitization (Coomassie staining). Lane 6 and 7 serve as controls lacking biotin-tyramine and naphthalimide **24**, respectively. Lane 8 represents the biotin-tyramine added after irradiation. Samples in 1-5 were treated with 1, 0.5, 0.25, 0.1, 0.05 mM concentrations of biotin-tyramine, respectively. As the concentrations of the biotin-tyramine were increased, the inhibitory effects were observed to increase. Figure 40-(b) shows a western blot after SDS-PAGE electrophoresis with detection of biotin residues.

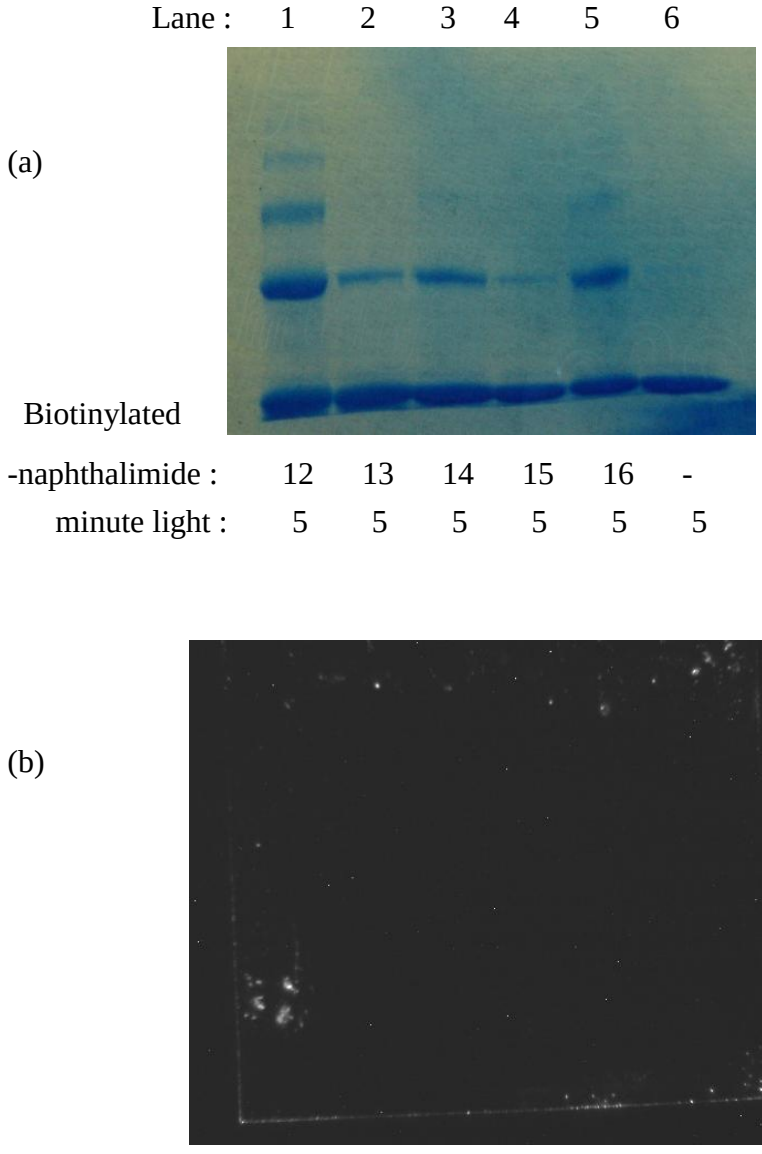


Figure 39. (a) Crosslinking of RNase A and biotinylated-1,8-naphthalimide derivatives. (b) Western blot. Conditions; Protein concentration was 0.235 mM, concentration of biotinylated naphthalimide was 0.5 mM. Irradiation was with mercury lamp (2800 mW) for 5 minutes

It is possible for tyramine to form covalent bonds with amino acids on the protein RNase A. If that occurs, the biotin marker can be detected by chemiluminescence. If the signal intensity is very strong, such as the sample in lane 1, it means that there is a large amount of biotin-derivatives attached on the RNase A. This experiment demonstrates that the biotin-tyramine compound inhibits the naphthalimide-mediated photochemical crosslinking of RNase A in a dose dependant manner only when present during the photolysis. The chemiluminescent analysis of the incorporation of biotin-tyramine into RNase A demonstrated that biotin-tyramine covalently bonds to RNase A if it is present during photochemical oxidation using naphthalimide sensitizers.

Effects of RNase A Crosslinking by Biotin-LC-Hydrazide

Biotin-LC-hydrazide is a commercially available biotin reagent containing a hydrazide group capable of bonding directly with electrophilic residues such as aldehydes, ketones, and activated carboxylate groups, produces a hydrazone bond. This compound's inhibitory effect on photochemical protein crosslinking and incorporation into photochemically acid acted RNase A was investigated. The experiments were identical to the biotin-tyramine experiments and the results are presented in Figure 41. This experiment demonstrated that this compound inhibited naphthalimide-mediated protein crosslinking in a concentration-dependant fashion, and that the compound was incorporated into the protein when present during irradiation.

The Stability of Biotin and 1,8-Naphthalimide during Irradiation

In these biotin-detection experiments it is critical to make certain that the label (biotin) is not destroyed during the photochemical crosslinkings reaction. Accordingly, we investigated the changes to biotin upon treatment with naphthalimide and light by ^{1}H NMR. One very likely reaction is the oxidation of the thio-ether residue in the biotin.

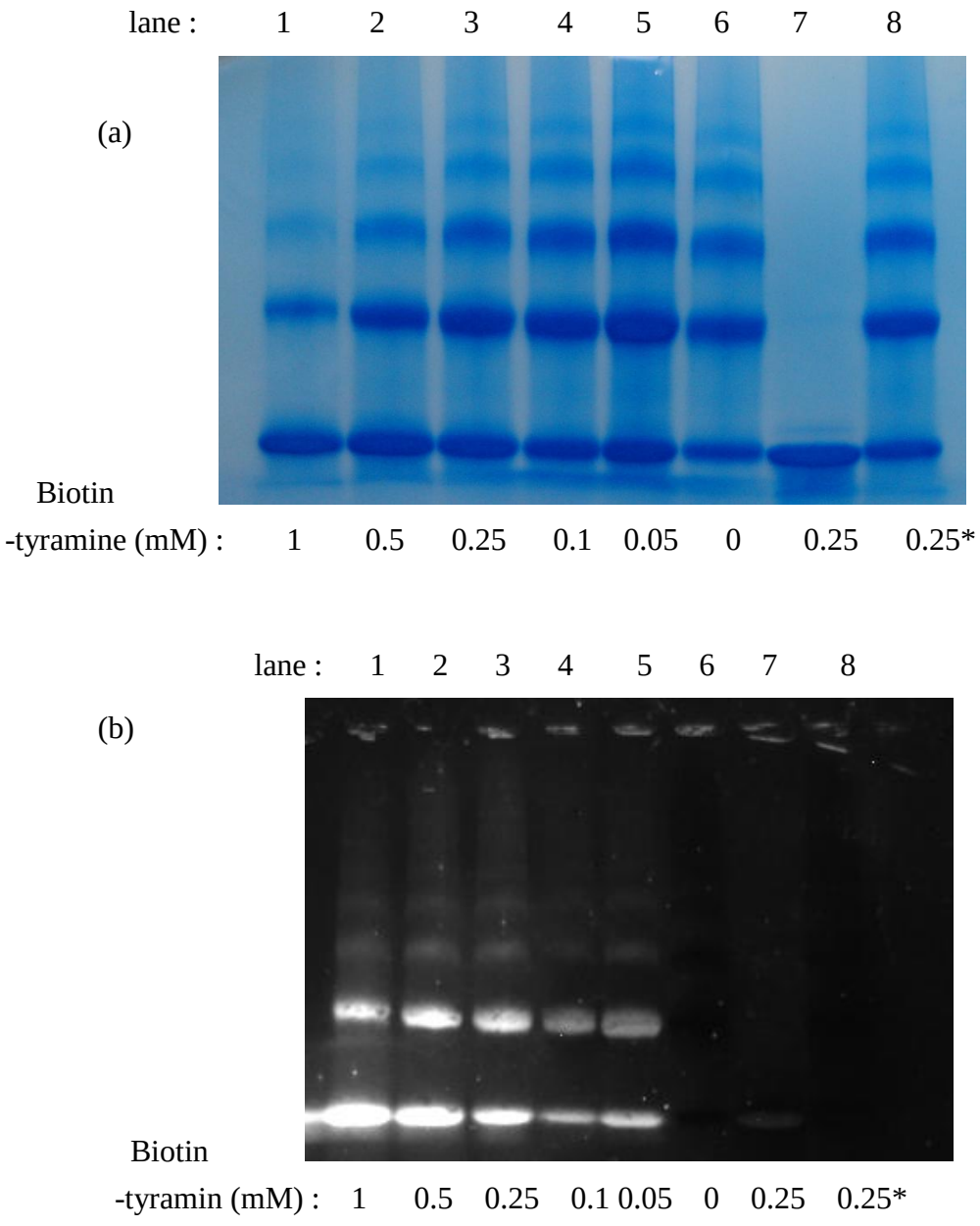


Figure 40. Inhibitory effect of biotin-tyramine on RNase A crosslinking by naphthalimide **24**. (a) Coomassie stained gel. (b) Western blot. Conditions; RNase A protein concentration was 0.235 mM, concentrations of biotin–tyramine were 1, 0.5, 0.25, 0.1, 0.05 mM. Naphthalimide **24** was 0.5 mM. Irradiation was with a mercury lamp at 2800 mW for 5 minutes. In lane 8, the protein was treated with 0.25 mM biotin-tyramine after irradiation

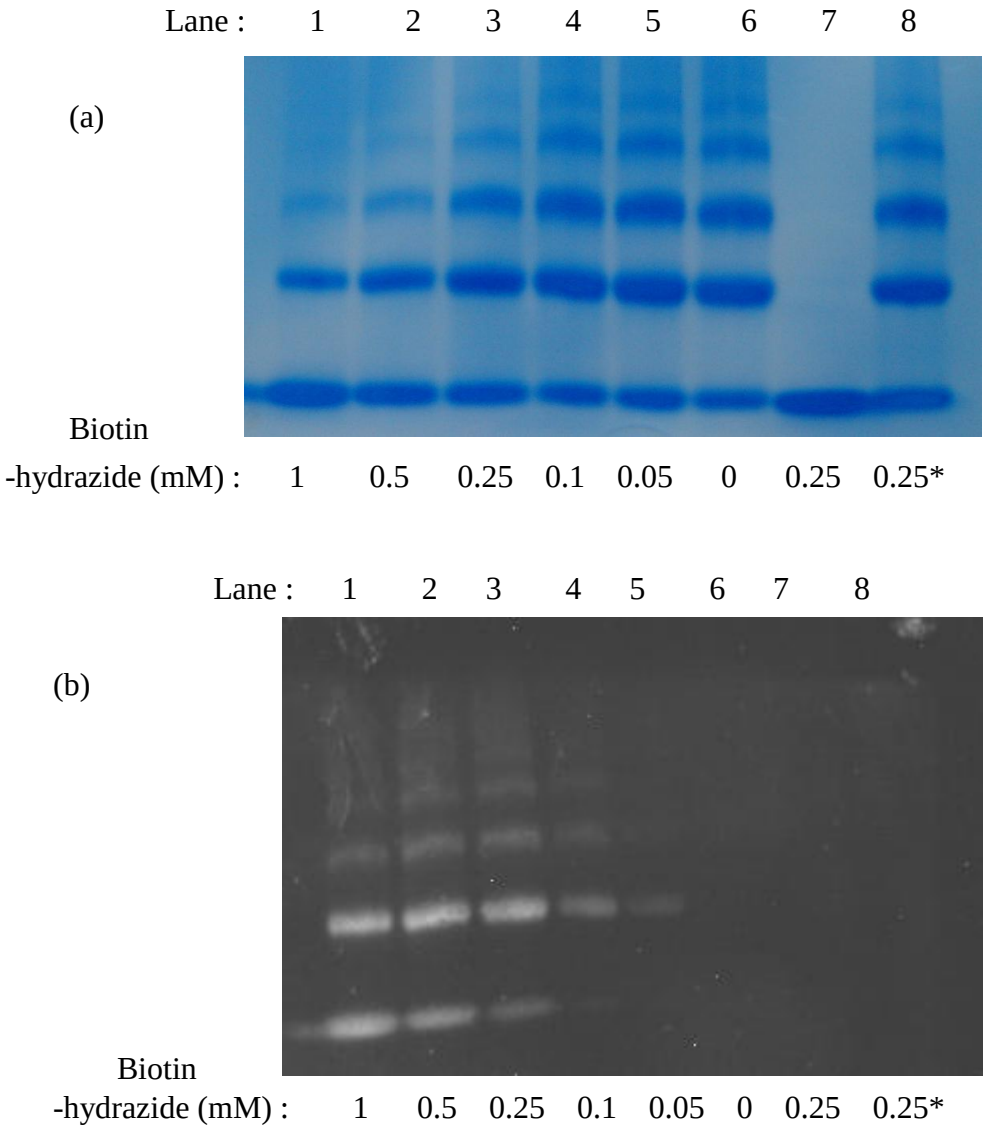


Figure 41. Inhibitory effect of biotin-LC-hydrazide on RNase A crosslinking by naphthalimide **24** (a) Coomassie stained gel. (b) Western blot. Conditions; RNase A 0.235 mM; biotin–LC-hydrazide 1, 0.5, 0.25, 0.1, 0.05 mM. Naphthalimide **24** was 0.5 mM. Irradiation was with a mercury lamp at 2800 mW for 5 minutes. In lane 8, the protein was treated with 0.25 mM biotin-hydrazide after irradiation

To create reference compounds, biotin was reacted with two oxidizing reagents, sodium periodate ($NaIO_4$) and hydrogen peroxide (H_2O_2). Biotin reacts with a moderate oxidizing reagent such as sodium periodate to produces a sulfur oxide (sulfoxide), whereas biotin reacts to with very a strong oxidizing reagent such as hydrogen peroxide produce a sulfur dioxide (sulfone). In Figure 42, these structures of the different oxidized products of biotin are shown. In figure 42-(b), when sulfur was converted to sulfur the sulfoxide, the H_2 and H_3 protons were deshielded from δ 2.92 ppm to δ 3.20 ppm and from δ 2.75 ppm to δ 3.62 ppm. Figure 42-(c) shows that ^{1}H NMR spectrum of biotin oxidized to the sulfone. Due to the complexity of the ^{1}H NMR spectra of protons on carbon next to sulfur in D-(+) biotin, the less complicated molecule methionine was also used in this study. Two additional examples were obtained by following the previous procedure, only with methionine, which was treated with sodium periodate ($NaIO_4$) and hydrogen peroxide (H_2O_2). The results of this experiment are presented in Figure 43, which contains the ^{1}H NMR of the various methionine oxidation products.

In order to investigate the effects of photochemical irradiation of the naphthalimide photosensitizers, ^{1}H NMR spectra of the compounds after irradiation were obtained. The ^{1}H NMR of naphthalimide **10**, biotin, a physical mixture of compound **10** and biotin, and the biotinylated naphthalimide **15** were acquired biotin and after irradiation. Figure 44 shows the result of a 30 minute irradiation of biotin (Figure 44-a), compound **10** (Figure 44-b), a physical mixture of compound **10** and biotin (Figure 44-c), and the biotinylated naphthalimide **15**. It is clear from the ^{1}H NMR spectra that the biotin residue survives the photochemical treatment in each example, thus giving us confidence that biotin is not destroyed in the naphthalimide-mediated photochemical reaction.

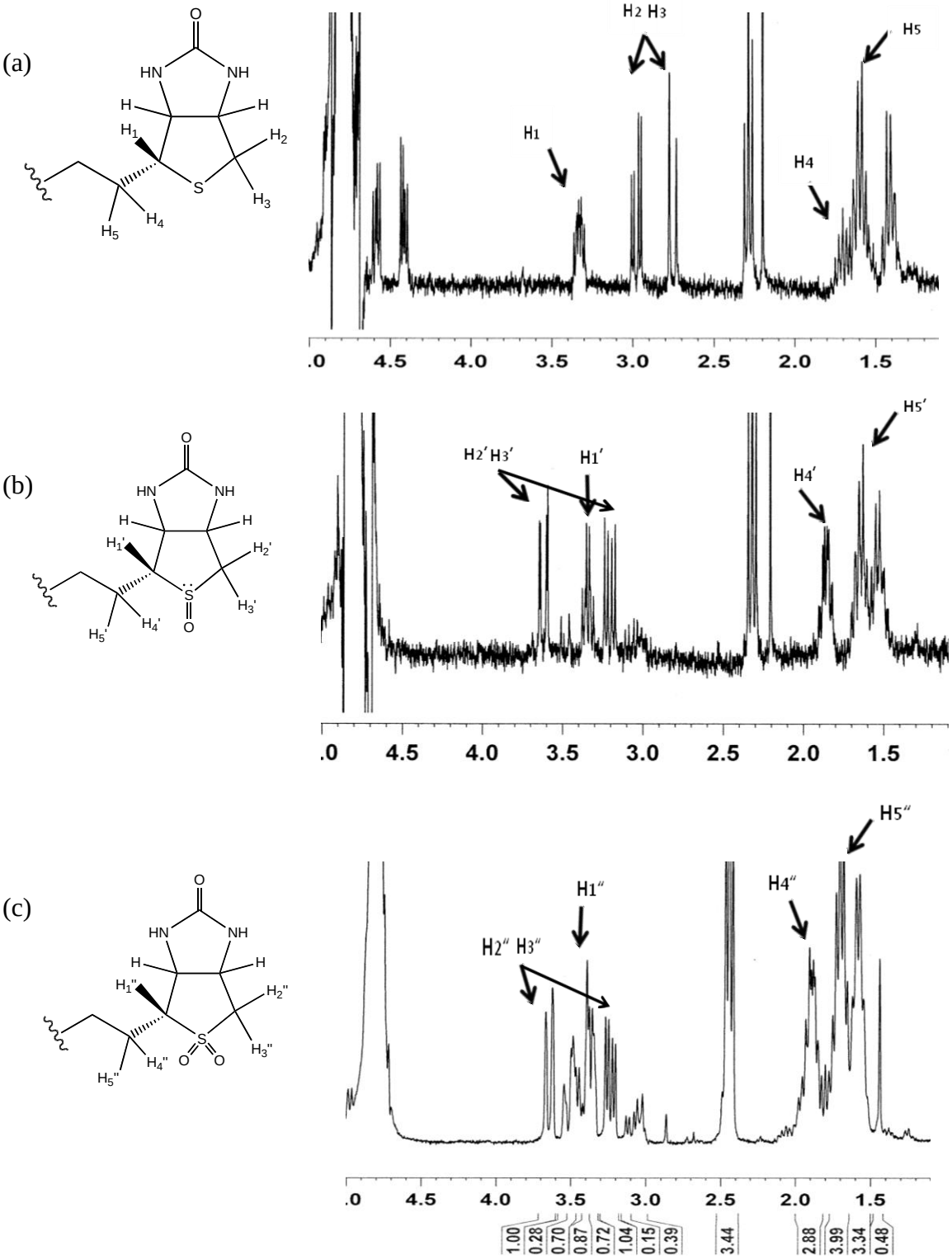
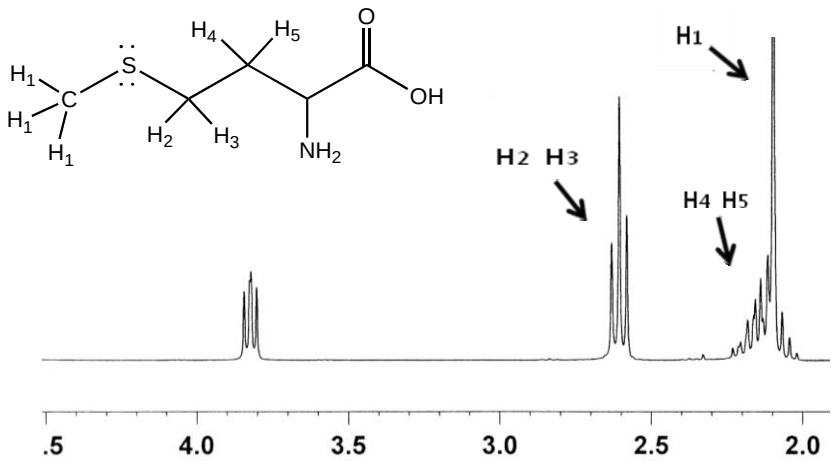
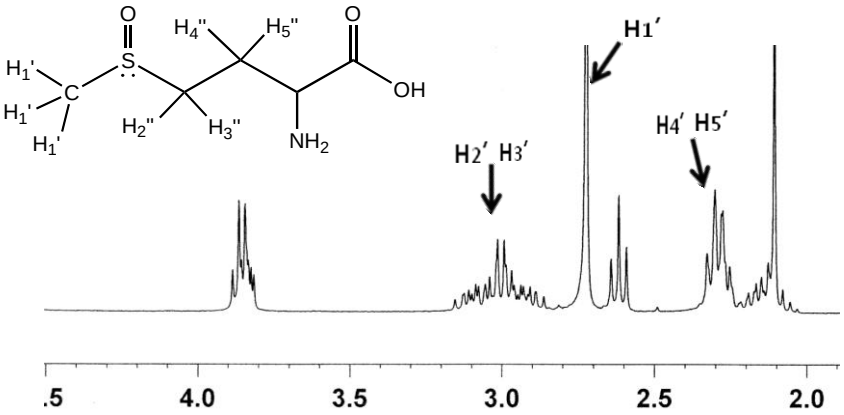


Figure 42. The ^{1}H NMR in D_2O of biotin treated with $NaIO_4$ and H_2O_2, respectively

(a)



(b)



(c)

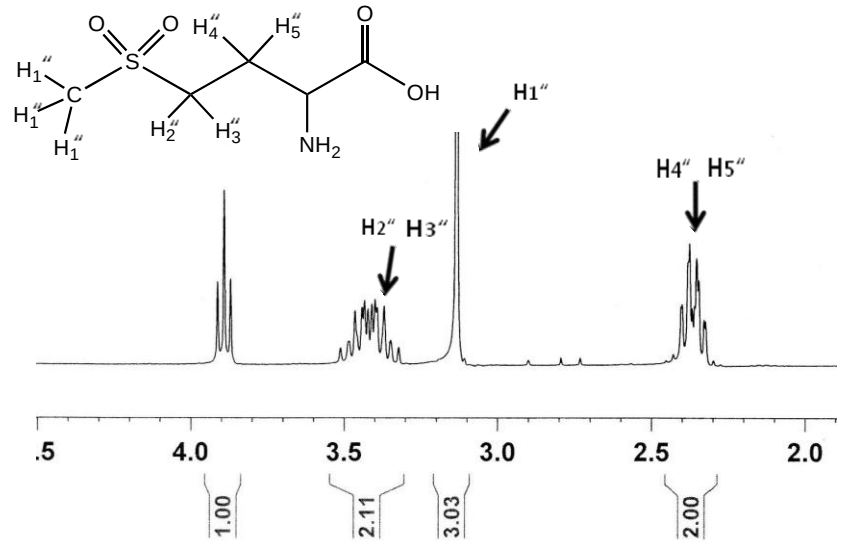
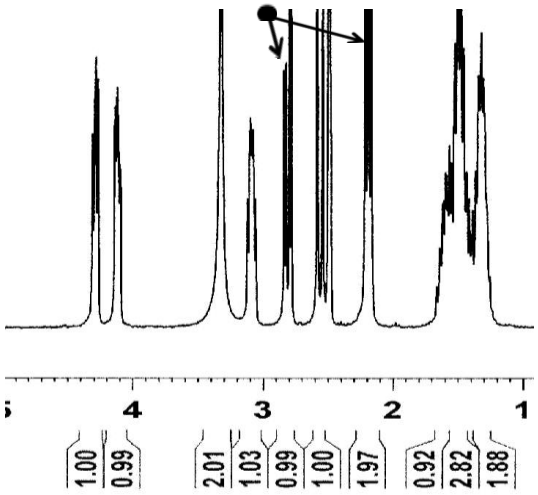
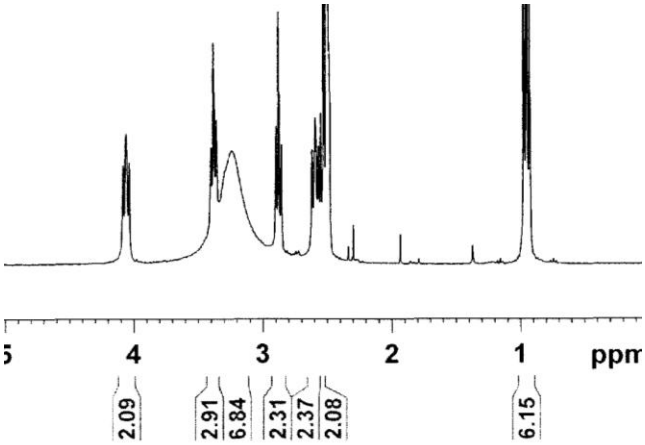


Figure 43. The ^{1}H NMR in D_2O of methionine treated with $NaIO_4$ and H_2O_2, respectively

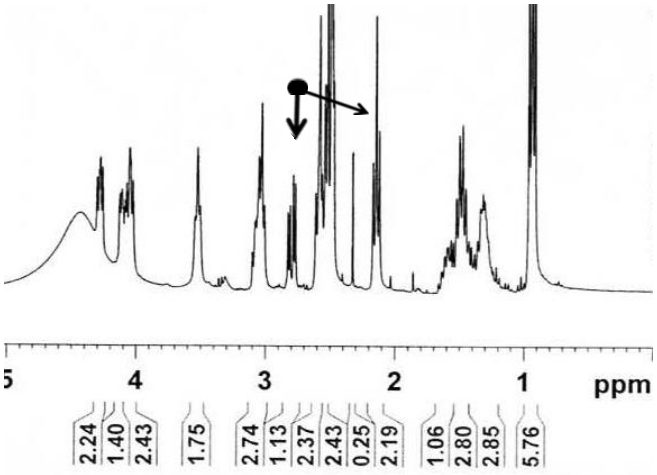
(a)


4
3
2
1
1.00
0.99
2.01
1.03
0.99
1.00
1.97
0.92
2.82
1.88

(b)


4
3
2
1
ppm
2.09
2.91
6.84
2.31
2.37
2.08
6.15

(c)



(d)

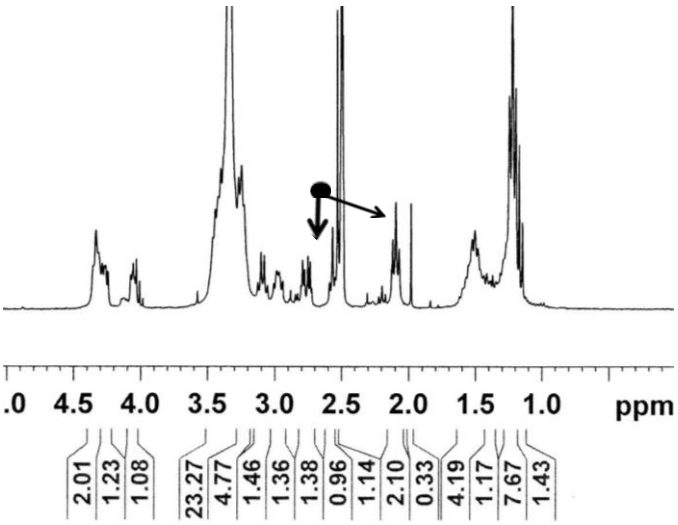


Figure 44. The ^{1}H NMR in DMSO of (a) Biotin with irradiation for 30 minutes, (b) Naphthalimide **10** with irradiation for 30 minutes (c) Biotin+Naphthalimide **10** with irradiation for 30minutes (d) Biotinylated-naphthalimide **15** with irradiation for 30 minutes

CHAPTER FOUR

Conclusions and Future Directions

A variety of 1, 8-naphthalimide derivatives and biotinylated-naphthalimides derivatives have been synthesized. Biotinylated-naphthalimide **12-15** were used for photochemical RNase A crosslinking assay and western blot. The experiment observed positive results for photochemical crosslinking. No signal was detected in western blot. These results indicate that naphthalimides were capable of crosslinking but did not bond with RNase A during the photochemical crosslinking reaction.

Several biotin-conjugated amino acids derivatives were synthesized and used in photochemical protein crosslinking. Biotin-tyramine was shown relative to crosslinking reaction under irradiation and inhibition effects for photochemical RNase A corsslinking. The western blot detected biotin residues, which means biotin-tyramine might attach to RNase A directly. The mechanism of photochemical crosslinking was not fully understood, however, this study supports the understanding of crosslinking mechanism.

Future studies should involve the investigation of photochemical protein crosslinking with synthesized 1, 8-naphthalimide derivatives and biotin-amino acids derivatives such as biotin-dopamine and biotin-tryptophan. In addition, research on tissue surface bonding with irradiation has been studied.[24,28,42] The mechanism of tissue surface bonding is not fully understood but is presently being studied because of complexity and variety of possible mechanisms. It is possible to understand the mechanistic study of tissue bonding using synthesized 1, 8-naphthalimide derivatives and biotin-conjugated amino acids derivatives.

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