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Synthesis and Photochemical Protein Crosslinking Studies of Hydrophilic Naphthalimides

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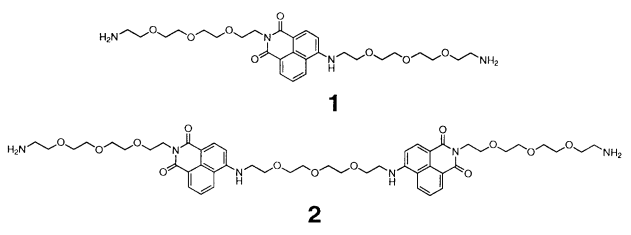
Abstract—A mixture of 4-alkylamino-1,8-naphthalimides has previously been reported to exhibit potential utility as a photochemical tissue-bonding reagent. In order to determine which constituents of the mixture were responsible for the observed tissue bonding and to facilitate study of the mechanism, we have synthesized each of the primary constituents of the mixture. Each naphthalimide synthesized has been demonstrated to photochemically crosslink proteins.

4-Alkylamino-1,8-naphthalimides are of recent interest due to their interesting photochemical properties. For example, the formation of relatively long-lived charge separated species has been demonstrated for certain derivatives,[1] and recently it has been reported that electron transfer within these compounds has a directional bias.[2,3] Of particular interest to our group, a mixture of 4-alkylamino-1,8-naphthalimides, precipitated from the mixture produced upon solvolytic condensation of 1,11-diamino-3,6,9-trioxadodecane with 4-chloro-1,8-naphthoic anhydride, has been demonstrated to photochemically crosslink proteins (in vitro) and bond collagenous tissues (in vivo).[4–7] LC–MS analysis has revealed that this mixture is primarily composed of monomeric (**1**) and dimeric naphthalimides (**2**–**4**).[8] Distinct from the more widely studied laser initiated thermal tissue coagulation (welding), photochemical tissue bonding using these compounds appears to occur via an athermal mechanism, as little localized heating is observed upon irradiation of dye-coated tissues. However, the precise mechanism of the tissue bonding is unknown. Accordingly, we have synthesized the monomer **1** and the three isomeric dimers (**2**–**4**) in order to carefully characterize each component's contribution to tissue bonding. We have now evaluated the ability of these compounds to photochemically crosslink proteins, and are currently performing studies aimed at clarifying the molecular mechanism of the protein crosslinking previously observed.

Each of the syntheses begins with diamine **5**, which we synthesized according to Kern.[9] Diamine **5** is initially monoprotected as the *t*-BOC carbamate **6** in reasonable yield, which allows for efficient subsequent reactions with a single terminus of the diamine and also makes the purification of intermediate products much more



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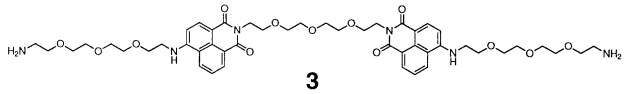

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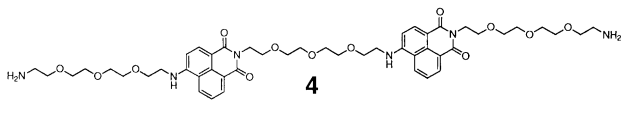

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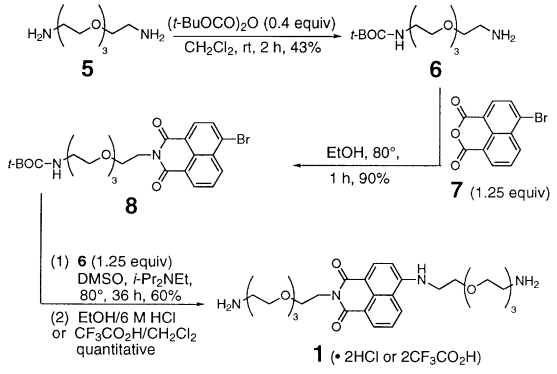
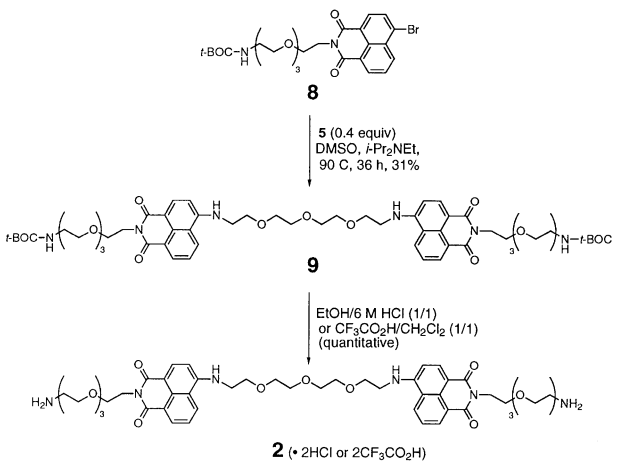
tractable. This monoprotected diamine **6** efficiently condenses with 4-bromo-1,8-naphthalic anhydride (**7**) in hot ethanol to afford the 4-bromo-1,8-naphthalimide derivative **8** in good yield after silica gel flash column chromatography (Scheme 1).[10]

Condensation of naphthalimide **8** with 1.25 equiv of diamine **6** in hot DMSO with Hünig's base affords a diprotected diamine (60% after purification by medium pressure silica gel chromatography). Deprotection of the diprotected product with either ethanol/aqueous HCl or 50% trifluoroacetic acid (TFA) in dichloromethane afforded the respective acid-addition salts of monomeric naphthalimide **1** in quantitative yield.

The displacement of the bromine of **8** by the amine nucleophile significantly limited the ultimate yield of this synthesis. In fact, this transformation was the primary obstacle to the efficient construction of each of the four target compounds. Accordingly, we explored a number of variations on the standard condensation protocol. For example, copper salts, at a variety of oxidation states, have been reported to catalyze the amination of aryl halides.[11,12] Unfortunately, we have been unable to identify a copper salt which enhances yields or increases reaction rates in these particular systems. The Pd(II) catalyzed aryl halide amination chemistry as described by Buchwald[13,14] and others[15,16] was also explored. Once again, improvements in yield or reaction rate were not forthcoming. Finally, a wide range of solvents (including *N*-methylpyrrolidinone[17]) was surveyed before DMSO was identified as the optimal solvent for these syntheses. 2,2,2-Trifluoroethanol is also an excellent solvent for these transformations.



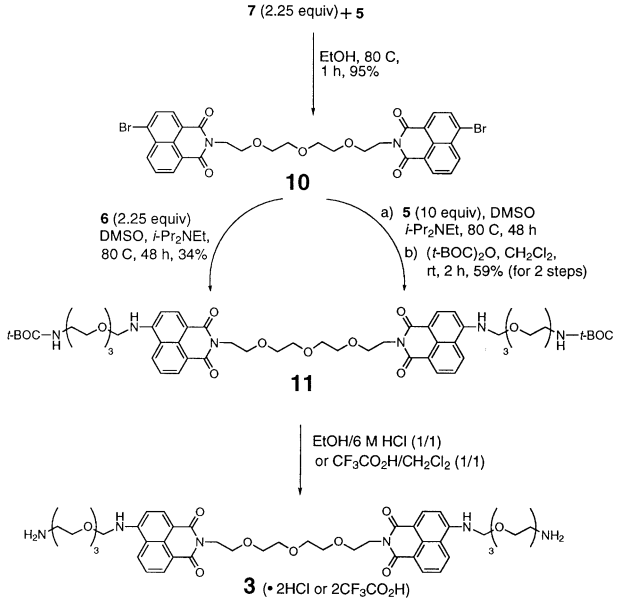
Scheme 1. Synthesis of naphthalimide **1**.



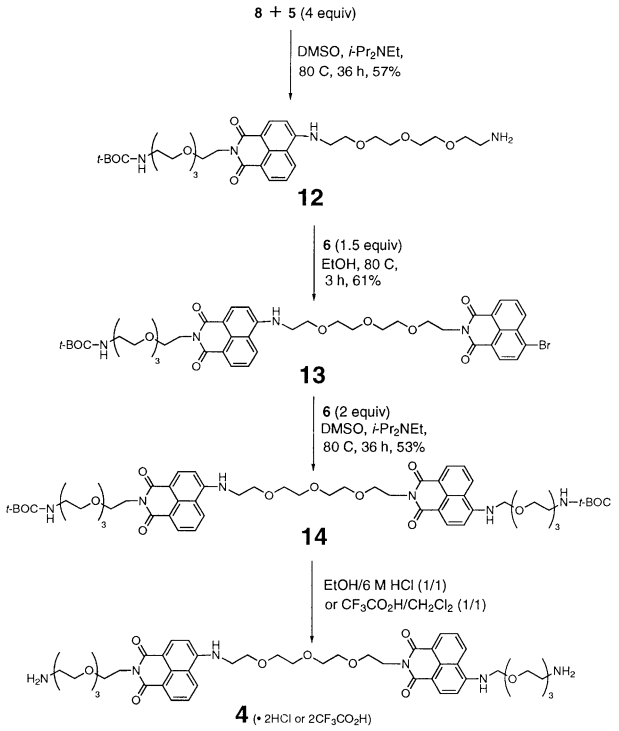
Scheme 2. Synthesis of naphthalimide **2**.

The synthesis of dimer **2** begins with the condensation of naphthalimide **8** with 0.4 equiv of diamine **5** in hot DMSO with Hünig's base, affording the diprotected dimeric naphthalimide **9**, which is best purified by preparative silica gel TLC (Scheme 2). Although the monoprotected monomeric compound **12** was found to be the major byproduct in this reaction, we have been unable to drive this reaction to completion. In any event, deprotection of compound **9** with either ethanol/aqueous HCl or 50% trifluoroacetic acid (TFA) in dichloromethane afforded the respective acid-addition salts of naphthalimide **2** in quantitative yield.

The synthesis of dimer **3** begins with the condensation of excess naphthoic anhydride **7** with diamine **5** in hot ethanol, affording dibromide **10** in good yield after flash column chromatography (Scheme 3). This compound is then condensed with excess monoprotected diamine **6** in hot DMSO with Hünig's base to afford the diprotected dimer **11** in poor yield after flash column purification. Although the primary impurity in this reaction is also a monocondensation product, we have as yet been unable to increase the yield of the desired product by either adding additional equivalents of **6** or by running the reaction for an extended time. Higher yields of **11** were available by condensing dibromide **10** with 10 equiv of diamine **5**, followed by *t*-BOC protection of the crude reaction mixture and column chromatography. Acidic deprotection of **11** quantitatively converts it to the water soluble salts **3**.



Scheme 3. Synthesis of naphthalimide **3**.

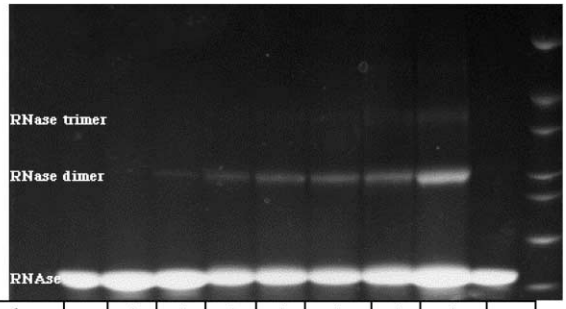


Scheme 4. Synthesis of naphthalimide **4**.

The final target, dimer **4**, was synthesized by condensation of bromonaphthalimide **8** with excess diamine **5** so as to afford the monoprotected diamine **12** (Scheme 4). Reaction of **12** with anhydride **7** provided bromo dimer **13**, which afforded the diprotected dimer **14** after condensation with excess **6**. The prep-TLC purified dimer **14** was then deprotected to yield the diamine bis-naphthalimide **4**.

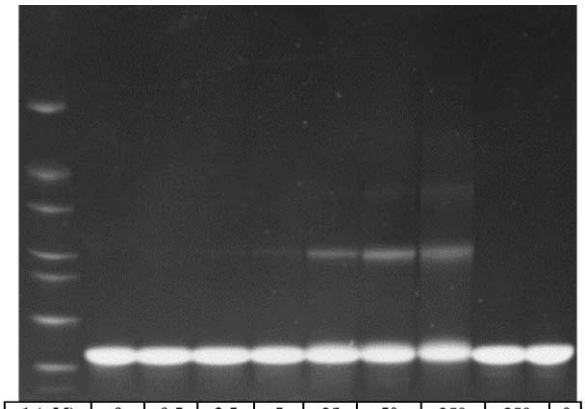
With these four pure compounds in hand we explored their ability to serve as photochemical protein crosslinking agents. We used RNase A as a model protein in these experiments, as this low-MW soluble protein has been used as a model protein in previous photochemical protein crosslinking experiments.[18] We initially looked at the ability of monomeric naphthalimide **1** to crosslink RNase A after exposure to various doses of light. Buffered solutions containing RNase A (0.2 mM) and compound **1** (0.1 mM) were exposed for various times to light from a xenon arc lamp (power: 540 mW; irradiance: 7.63 W/cm^2; filtered to emit 400–600 nm). The resulting protein solutions were thoroughly denatured and reduced (5 min @ 100 °C in SDS loading buffer containing 2-mercaptoethanol) and examined using SDS–PAGE with Coomassie staining (Fig. 1). It is clear from this experiment that the monomeric naphthalimide **1** photochemically crosslinks soluble RNase A in a light-dependent manner.

We also explored the dependence of the photochemical crosslinking on naphthalimide **1** concentration. Buffered solutions containing RNase A (0.2 mM) and various concentrations of compound **1** (0.5–250 μM) were exposed for 11 min. As before, the resulting protein solutions were examined using SDS–PAGE (Fig. 2). It



1	-	+	+	+	+	+	+	+	-
hν (min)	0	0	1	3	5	7	9	11	11
% xlinked	0	0	0.5	2.3	3.5	4.3	7.0	16.8	0

Figure 1. Light dependence of photochemical protein crosslinking by naphthalimide **1**. RNase A (0.2 mM); naphthalimide **1** (0.1 mM); light (min); % crosslinked by densitometry of dimer and trimer bands.

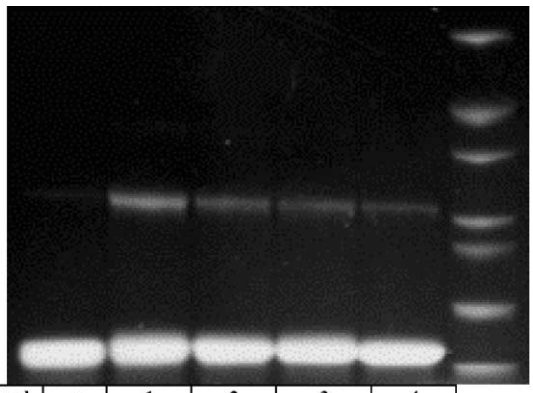


1 (μM)	0	0.5	2.5	5	25	50	250	250	0
hν	+	+	+	+	+	+	+	-	-
% xlinked	0	0	0.7	1.3	8.3	16.8	18.2	0	0

Figure 2. Dependence of photochemical protein crosslinking on naphthalimide concentration. RNase (0.2 mM); compound **1** (μM); light (11 min).

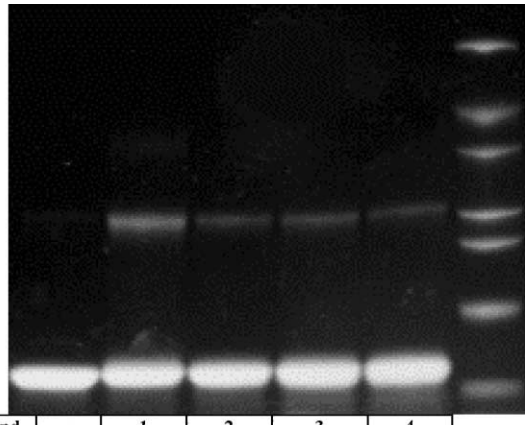
is clear from this experiment that photochemical protein crosslinking by naphthalimide **1** is dependent on the concentration of **1**. Similar results are seen for the other naphthalimides (data not shown).

Finally, we compared the relative abilities of the four naphthalimides to photochemically crosslink RNase. We first looked at the relative abilities of equimolar concentrations of the four compounds to potentiate the crosslinking of RNase (Fig. 3). In this assay, the monomeric naphthalimide (**1**) is by far the most effective crosslinker, with dimer **4** appearing to be the least effective. This was somewhat suprising, as the *chromaphore* concentration of a solution of monomer **1** is significantly lower than that of an equimolar concentration of a dimer. Accordingly, we repeated the experiment with the absorbance (A_{453}) of the naphthalimide solutions normalized (Fig. 4). This experiment reconfirmed the superiority of monomer **1** as a crosslinking agent, and under these conditions differences between the crosslinking abilities of the three dimers are very modest.



compd	-	1	2	3	4
% xlinked	0.6*	10.2	5.1	4.0	2.5

Figure 3. Dependence of photochemical protein crosslinking on naphthalimide structure. RNase (0.2 mM); 11 min light; compounds (63 μM). *Unmodified RNase sample had a small amount of dimer.



compd	-	1	2	3	4
conc. (μM)	-	63	58	43	50
% xlinked	0.4	9.3	3.2	3.4	2.5

Figure 4. Dependence of photochemical protein crosslinking on naphthalimide structure. RNase (0.2 mM); 11 min light; compounds (normalized to give equivalent absorbance at λ_{453}: **1**, 63 μM; **2**, 58 μM; **3**, 43 μM; **4**, 50 μM).

In conclusion, we have demonstrated the synthesis of the four primary constituents of a mixture able to photochemically bond collagenous tissues. Each of the naphthalimides is able to crosslink a model soluble protein (RNase A) in a dose and light dependent manner. In a side-by-side comparison, the monomeric naphthalimide **1** appears to be the most effective protein crosslinking agent. We are presently performing studies aimed at determining the mechanism of protein crosslinking by these compounds, and we are also exploring the abilities of these compounds to bond biological tissues.

Acknowledgements

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