MARCH/APRIL 1998
Volume 9, Number 2

Bioconjugate Chemistry

ARTICLES

Preparation and Photoactivation of Caged Fluorophores and Caged Proteins Using a New Class of Heterobifunctional, Photocleavable Cross-Linking Reagents

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The design, synthesis, and spectroscopic and chemical properties of four members of a new class of heterobifunctional photocleavable (caged) cross-linking reagents were described. One of the two reactive groups of the cross-linker reacted with amino groups to form the corresponding photolabile carbamates. Amino group containing compounds or proteins caged with these reagents can be coupled through the thiol reactive oxirane group of the cross-linker to a different biomolecule or to a thiol-derivatized surface. The 3,4-dimethoxy-6-nitrophenyl photoisomerization group of the reagent was physically and chemically isolated from the cross-linking functionality, and the high extinction coefficient and red-shifted action spectrum of this chromophore make it suitable for photoactivation applications of caged compounds on surfaces or in living cells. The bifunctional, photocleavable cross-linking reagents were used to prepare a thiol reactive caged rhodamine 110. The new reagents and conjugation procedures described may be used as part of a general procedure to cage the activity of proteins by physically masking binding sites.

INTRODUCTION

Light-directed activation of caged compounds has emerged as a powerful technique for generating concentration jumps of biomolecules in complex molecular environments (*1*) and has provided important information on the reaction mechanism of protein activity in muscle and in living cells (*1*, *2*). Functional groups of molecules that have been protected with caged compounds include the primary amine (*3*), primary and secondary alcohol (*4*), phosphate (*5*), ketone (*6*), thiol (*7*, *13*), and carboxylic acid (*8*). Irradiation of functional groups protected with the 2-nitrophenyl-based caged reagents with near-ultraviolet light leads to a photoisomerization reaction that cleaves the bond linking the protected functionality of interest to the caged group. Caged reagents have been used to prepare inactive yet photoactivatable enzyme substrates (*5*), receptor ligands (*9*, *10*), and fluorescent probes (*11*, *12*). In addition, caged reagents capable of labeling amino and thiol groups have been used to inhibit the activity of proteins (caged proteins) by modifying one or more essential amino acid residues (*7*, *13*−*15*, *26*). These caged groups can be efficiently removed from the caged protein with concomitant recovery of its activity following irradiation of the conjugate with near-ultraviolet light.

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[⊗] Abstract published in *Advance ACS Abstracts,* December 15, 1997.

S1043-1802(97)00147-X CCC: $15.00
Published on Web 03/16/1998

In addition to these monofunctional caged reagents, heterobifunctional photocleavable reagents have been described to protect amino or thiol groups of small molecules (*25*) and proteins (*7*, *15*) as their corresponding photolabile carbamates or thioethers, respectively (*7*, *15*, *25*). The second reactive group of the cross-linking reagent may be used to attach the caged compound to a different biomolecule; for example, an antibody molecule for targeting the caged compound to a specific site (*15*), or a derivatized surface, or a fluorescent dye for imaging or for probing protein−protein interactions (*7*, *25*).

The few photocleavable cross-linking reagents described in the literature all harbor this second reactive group at the 4-position of the 2-nitrophenyl ring (*7*, *15*, *25*). In this study, we designed a new class of reagents in which the second reactive group was introduced off the benzylic carbon atom. The photocleavable cross-linking reagents were designed with the following operational considerations in mind. (1) Caged reagents should react with the functional group of interest in aqueous solution and at a slightly alkaline pH. The second reactive group, an epoxide, is stable in the absence of free thiols and can then be reacted in a second step with thiol functions. (2) The photoactivation reaction should exhibit a reasonable quantum yield and an action spectrum in the near-ultraviolet wavelength region (340−400 nm) to avoid interference with other biomolecules. (3) The caged group should exhibit a high molar absorptivity for photoactivation reactions performed on optically thin samples. (4) The photoproducts of the photoactivation reaction should not be toxic or reactive with other functional groups. The new reagents described in this report fulfill most of the requirements listed above. Suitable examples are provided to demonstrate the functional group selectivity of the two reactive groups. Members of this new class of cross-linking reagents were used for the preparation and photoactivation of caged fluorophores and caged proteins.

MATERIALS AND METHODS

3,4-Dimethoxy-6-nitrobenzaldehyde, di-*N*-succinimidylcarbonate (DSC), vinyl magnesium bromide, *m*-chloroperbenzoic acid (MCPBA), 4-(dimethylamino)pyridine (DMAP), and 4,6-diphenylthieno[3,4-*d*][1,3]dioxol-2-on-5,5-dioxide (TDO) were purchased from Aldrich. Bovine serum albumin (BSA), polylysine (average molecular weight of 40 000 kDa), and 2-iminothiolane were purchased from Sigma. Tetramethylrhodamine iodoacetamide (IATMR) was a gift from J. Corrie (NIMR, Mill Hill, London). Acrylodan and aminodextran were purchased from Molecular Probes (Eugene, OR). Rhodamine 110 was purchased from Lambda Physik (Göttingen, Germany). All other reagents were of the highest quality available and obtained from Sigma or Aldrich unless stated otherwise. G-Actin was prepared according to Marriott (*14*).

The NMR spectra were measured on a 500 MHz Bruker instrument; mass spectrophotometry was carried out on a Finnigan MAT 900 or Finnigan HSQ 30 instrument. Infrared spectra were recorded on a Perkin-Elmer 1760 X FT-IR spectrophotometer. Absorption spectra were recorded on a Hewlett-Packard 82152 diode array spectrophotometer. Fluorescence spectroscopy was performed on an SLM-AB2 fluorometer (Sopra, Buttlelborn, Germany). Light-directed photoactivation of caged compounds was performed essentially as described by Marriott (*14*).

Syntheses. All syntheses were conducted in a darkened room or protected from room light with aluminum foil.

*Preparation of 1-Hydroxy-1-(3,4-dimethoxy-6-nitrophenyl)-2-propene (**I**).* 3,4-Dimethoxy-6-nitrobenzaldehyde (1.88 g, 8.95 mmol) freshly recrystallized out of toluene, was dissolved in 50 mL of dry THF under an argon atmosphere. The yellow solution was cooled to −70 °C and 10.7 mL of a 1 M solution of vinylmagnesium bromide in THF added dropwise. The deep red solution was stirred for 3 h and slowly warmed to 20 °C and then 40 mL of a saturated NH_4Cl solution added dropwise. The red solution was extracted three times with ethyl acetate and the organic phase washed five times with saturated NaCl and dried over $MgSO_4$ and the solvent removed. The red oily residue (2.44 g) was taken up in a little ethyl acetate and eluted through a silica gel column developed in 3:1 hexane/ethyl acetate. The solvent was removed from the product fractions producing an orange-red powder. Yield: 1.53 g (6.33 mmol, 72%). MW: 239.23 ($C_{11}H_{13}NO_5$). Mp: 101−102 °C. ^{1}H-NMR ($CDCl_3$, in ppm): 2.63 (s, broad, 1H), 3.94 (s, 3H), 3.96 (s, 3H), 5.24 (dd, J = 11 Hz, 2 Hz, 1H), 5.42 (dd, J = 18 Hz, 2 Hz, 1H), 5.92 (d, J = 6 Hz, 1H), 6.07 (ddd, J = 18 Hz, 11 Hz, 6 Hz, 1H), 7.20 (s, 1H), 7.57 (s, 1H). MS (70 eV) *m*/*z* (%): 239 (42) M^+, 162 (100) $C_9H_8NO_2^+$.

*Preparation of 1-(3,4-Dimethoxy-6-nitrophenyl)-2,3-epoxypropyl Hydroxide (**II**).* *m*-Chloroperoxybenzoic acid (MCPBA, technical grade, about 80% pure) was washed three times with phosphate buffer at pH 7.5 and dried. Purified MCPBA (1.311 g, 7.60 mmol) was dissolved in 20 mL of CH_2Cl_2 and slowly dropped into a precooled solution of 1.80 g (7.52 mmol) of **I** in 15 mL of CH_2Cl_2. The bright yellow solution was stirred for 48 h at room temperature. The white precipitate was filtered and washed with CH_2Cl_2 and then 70 mL of a saturated solution of $NaHCO_3$ added to the solution which was stirred for 30 min. The organic phase was washed seven times with a saturated solution of $NaHCO_3$, then three times with a saturated solution of NaCl. The organic phase was dried over $MgSO_4$ and the solvent removed. The yellow, oily residue (1.90 g) was recrystallized out of ethyl acetate/hexane. Yield: 1.55 g (6.10 mmol, 81%, orange needles). MW: 255.23 ($C_{11}H_{13}NO_6$). Mp: 118−121 °C. ^{1}H-NMR ($CDCl_3$, in ppm): isomer a, 2.62 (s, 1H), 2.71 (dd, J = 5 Hz, 2 Hz, 1H), 2.90 (dd, J = 5 Hz, 2 Hz, 1H), 3.32 (ddd, J = 5 Hz, 5 Hz, 2 Hz, 1H), 3.96 (s, 3H), 3.98 (s, 3H), 5.57 (dd, J = 5 Hz, 2 Hz, 1H), 7.23 (s, 1H), 7.66 (s, 1H); isomer b, 2.63 (s, 1H), 2.82 (dd, J = 5 Hz, 2 Hz, 1H), 3.02 (dd, J = 5 Hz, 2 Hz, 1H), 3.62 (ddd, J = 5 Hz, 5 Hz, 2 Hz, 1H), 3.96 (s, 3H), 4.02 (s, 3H), 5.76 (dd, J = 5 Hz, 2 Hz, 1H), 7.28 (s, 1H), 7.67 (s, 1H). MS (70 eV) *m*/*z* (%): 255 (35) M^+, 164 (100) $C_9H_{10}NO_2^+$. FAB-MS *m*/*z*: 256.1 MH^+MS.

*Photolytic Isomerization of **II**.* A 100 μM solution of **II** dissolved in methanol was irradiated in a quartz cuvette with ultraviolet light (350 and 420 nm) according to Marriott (*14*). The absorption spectrum of the sample was recorded at different irradiation times.

*Preparation of 1-(3,4-dimethoxy-6-nitrophenyl)-2,3-epoxypropyl chloroformate (**III**).* In a well-ventilated hood, 566 mg of **II** (2.22 mmol) dissolved in 10 mL of water free dioxane in a dried 50 mL flask was treated with 0.18 mL of water free pyridine (2.25 mmol) together with 0.26 mL of diphosgene (2.20 mmol) which was slowly dropped into the yellow solution. The reaction mixture was stirred for 2 h at room temperature, the precipitate filtered off, the solvent removed, and the flask left for 1 h under a high vacuum to remove excess diphosgene. The

oily orange-brown residue was taken up in a little ethyl acetate and eluted through a silica gel column developed in 3:1 hexane/ethyl acetate. The solvent was removed from the product fractions yielding a yellow powder. Yield: 620 mg (1.95 mmol, 88%). MW: 317.68 ($C_{12}H_{12}NO_7Cl$). Mp: 178−181 °C. ^{1}H-NMR ($CDCl_3$, in ppm): isomer a, 3.57 (dd, J = 12 Hz, 4 Hz, 1H), 3.76 (dd, J = 12 Hz, 4 Hz, 1H), 3.94 (s, 3H), 3.97 (s, 3H), 5.52−5.56 (m, 1H), 6.32 (d, J = 8 Hz, 1H), 7.20 (s, 1H), 7.71 (s, 1H); isomer b, 3.92 (s, 3H), 3.96 (s, 3H), 4.00 (dd, J = 12 Hz, 2 Hz, 1H), 4.19 (dd, J = 12 Hz, 3 Hz, 1H), 4.66−4.68 (m, 1H), 6.13 (d, 3 Hz, 1H), 7.00 (s, 1H), 7.73 (s, 1H). MS (70 eV) m/z (%): 317 (82) M^+, 136 (100) $C_8H_8O_2^+$.

*Preparation of 4-[1-(3,4-Dimethoxy-6-nitrophenyl)-2,3-epoxypropyl-1-oxycarbonyloxy]-3-oxo-2,5-diphenyl-2,3-dihydrothiophen-1,1-dioxide (**IV**).* **II** (500 mg, 1.959 mmol) dissolved in 4 mL of water free THF was treated with 672 mg of freshly recrystallized, 4,6-diphenylthieno[3,4-*d*][1,3]dioxol-2-on-5,5-dioxide (2.062 mmol). The orange solution was refluxed for 4 h in the absence of base and the solvent evaporated to give an orange residue that was recrystallized out of dry toluene. Yield: 186.7 mg (0.321 mmol, 82%). MW: 581.56 ($C_{28}H_{23}NO_{11}$). Mp: 176−180 °C. ^{1}H-NMR (in $CDCl_3$, in ppm): isomer a, 2.35 (dd, J = 4 Hz, 3 Hz, 1H), 3.06 (dd, J = 4 Hz, 3 Hz, 1H), 3.53 (ddd, J = 4 Hz, 4 Hz, 2 Hz, 1H), 3.90 (s, 3H), 3.95 (s, 3H), 5.14 (s, 1H), 6.62 (d, J = 4 Hz, 1H), 7.01 (s, 1H), 7.27−7.32 (m, 4H), 7.43−7.48 (m, 4H), 7.60 (s, 1H), 7.92−7.97 (m, 2H); isomer b, 2.39 (dd, J = 4 Hz, 3 Hz, 1H), 3.08 (dd, J = 4 Hz, 3 Hz, 1H), 3.64 (ddd, J = 4 Hz, 4 Hz, 2 Hz, 3H), 3.92 (s, 3H), 3.97 (s, 3H), 5.15 (s, 1H), 6.67 (d, J = 4 Hz, 1H), 7.07 (s, 1H), 7.27−7.32 (m, 4H), 7.43−7.48 (m, 4H), 7.63 (s, 1H), 7.92−7.97 (m, 2H); isomer c, 2.83 (ddd, J = 4 Hz, 4 Hz, 2 Hz, 1H), 3.48 (ddd, J = 4 Hz, 4 Hz, 2 Hz, 1H), 3.64 (ddd, J = 4 Hz, 4 Hz, 2 Hz, 3H), 3.96 (s, 3H), 3.98 (s, 3H), 5.16 (s, 1H), 6.78 (d, J = 3 Hz, 1H), 7.08 (s, 1H), 7.43−7.48 (m, 4H), 7.52−7.57 (m, 8H), 7.65 (s, 1H), 7.92−7.97 (m, 2H); isomer d, 2.92 (ddd, J = 4 Hz, 4 Hz, 2 Hz, 1H), 3.48 (ddd, J = 4 Hz, 4 Hz, 2 Hz, 1H), 3.64 (ddd, J = 4 Hz, 4 Hz, 2 Hz, 3H), 3.96 (s, 3H), 3.99 (s, 3H), 5.18 (s, 1H), 6.81 (d, J = 3 Hz, 1H), 7.14 (s, 1H), 7.43−7.48 (m, 4H), 7.52−7.57 (m, 8H), 7.67 (s, 1H), 7.92−7.97 (m, 2H). FAB-MS m/z: 582.4 MH^+.

*Preparation of 1-(3,4-Dimethoxy-6-nitrophenyl)-2,3-epoxypropyl Carbonate (**V**).* **IV** (150 mg, 0.258 mmol) dissolved in 0.5 mL of water free THF was treated with 65.8 mg of **II** (0.258 mmol) and 30 μL of *N*-ethyldiisopropylamine (0.258 mmol). The flask was subjected to bath sonification for 2 min, and the orange solid, which precipitated out of solution, was left for 1 h at room temperature. This precipitate was filtered off and the solvent evaporated to give an orange residue that was crystallized out of toluene/hexane as an orange powder. Yield: 106 mg (0.198 mmol, 77%). MW: 536.2 ($C_{23}H_{24}N_2O_7$). Mp: 115−120 °C. ^{1}H-NMR ($CDCl_3$, in ppm): isomer a, 2.38 (dd, J = 6 Hz, 3 Hz, 1H), 2.77 (ddd, J = 6 Hz, 6 Hz, 3 Hz, 1H), 2.98 (dd, J = 6 Hz, 3 Hz, 1H), 3.92 (s, 3H), 6.61 (d, J = 3 Hz, 1H), 7.00 (s, 1H), 7.58 (s, 1H); isomer b, 2.38 (dd, J = 6 Hz, 3 Hz, 1H), 2.80 (ddd, J = 6 Hz, 6 Hz, 3 Hz, 1H), 2.98 (dd, J = 6 Hz, 3 Hz, 1H), 3.93 (s, 3H), 6.64 (d, J = 3 Hz, 1H), 7.02 (s, 1H), 7.62 (s, 1H); isomer c, 2.41 (dd, J = 6 Hz, 3 Hz, 1H), 2.87 (ddd, J = 6 Hz, 6 Hz, 3 Hz, 1H), 3.55 (dd, J = 6 Hz, 3 Hz, 1H), 3.95 (s, 3H), 6.67 (d, J = 3 Hz, 1H), 7.05 (s, 1H), 7.63 (s, 1H); isomer d, 2.41 (dd, J = 6 Hz, 3 Hz, 1H), 3.48 (ddd, J = 6 Hz, 6 Hz, 3 Hz, 1H), 3.55 (dd, J = 6 Hz, 3 Hz, 1H), 3.95 (s, 3H), 6.72 (d, J = 3 Hz, 1H), 7.11 (s, 1H), 7.72 (s, 1H). MS (70 eV) m/z (%): 536 (20) M^+, 238 (100) $C_{11}H_{12}NO_5^+$.

*Preparation of N-1-[(3,4-Dimethoxy-6-nitrophenyl)-2,3-epoxypropyloxycarbonyl]-4-(N,N-dimethylamino)pyridinium 3-Oxo-2,5-diphenyl-2,3-dihydrothiophen-1,1-dioxide-4-hydroxide (**VIa**).* **IV** (150 mg, 0.258 mmol) dissolved in 0.4 mL of dry THF was treated with a solution of 35 mg of DMAP (0.285 mmol) in 0.1 mL of dry THF. After a 2 min sonification treatment, an orange precipitate formed that was left to stand for 1 h at room temperature and centrifuged, and the orange residue was washed with THF. Yield: 166 mg (0.237 mmol, 92%). MW: 704.7 (cation, 404.4; cation, $C_{19}H_{22}N_3O_7$). Mp: 85 °C (dec). ^{1}H-NMR ($CDCl_3$, in ppm): isomer a, 2.84 (dd, J = 5 Hz, 2 Hz, 1H), 3.02 (dd, J = 5 Hz, 2 Hz, 1H), 3.25 (s, 6H), 3.58 (ddd, J = 5 Hz, 5 Hz, 2 Hz, 1H), 3.93 (s, 3H), 4.04 (s, 3H), 6.63 (d, J = 4 Hz, 1H), 6.70 (d, J = 7 Hz, 2H), 7.05 (s, 1H), 7.64 (s, 1H), 8.12 (d, J = 7 Hz, 2H); isomer b, 2.89 (dd, J = 5 Hz, 2 Hz, 1H), 3.11 (dd, J = 5 Hz, 2 Hz, 1H), 3.25 (s, 6H), 3.63 (ddd, J = 5 Hz, 5 Hz, 2 Hz, 1H), 3.96 (s, 3H), 4.06 (s, 3H), 6.72 (d, J = 7 Hz, 2H), 6.75 (d, J = 3 Hz, 1H), 7.09 (s, 1H), 7.66 (s, 1H), 8.14 (d, J = 7 Hz, 2H); counterion, 4.91 (s, 1H), 7.10−7.40 (m, 8H), 7.95−8.30 (m, 2H). FAB-MS m/z: 404.5 M^+ (cation).

*Preparation of N-1-[(3,4-Dimethoxy-6-nitrophenyl)-2,3-epoxypropyloxycarbonyl]-4-(N,N-dimethylamino)pyridinium Succinimide-N-hydroxide (**VIb**).* **VII** (150 mg, 0.378 mmol) dissolved in 0.4 mL of dry THF was treated with a solution of 51 mg of DMAP (0.42 mmol) in 0.1 mL of dry THF. After a 2 min sonification treatment, an orange precipitate formed that was left to stand for 1 h at room temperature and centrifuged, and the orange residue was washed with THF. Yield: 180 mg (0.34 mmol, 90%). MW: 704.7 (cation, 404.4; cation, $C_{19}H_{22}N_3O_7$). Mp: 88 °C (dec). ^{1}H-NMR ($CDCl_3$, in ppm): isomer a, 2.84 (dd, J = 5 Hz, 2 Hz, 1H), 3.02 (dd, J = 5 Hz, 2 Hz, 1H), 3.25 (s, 6H), 3.58 (ddd, J = 5 Hz, 5 Hz, 2 Hz, 1H), 3.93 (s, 3H), 4.04 (s, 3H), 6.63 (d, J = 4 Hz, 1H), 6.70 (d, J = 7 Hz, 2H), 7.05 (s, 1H), 7.64 (s, 1H), 8.12 (d, J = 7 Hz, 2H); isomer b, 2.89 (dd, J = 5 Hz, 2 Hz, 1H), 3.11 (dd, J = 5 Hz, 2 Hz, 1H), 3.25 (s, 6H), 3.63 (ddd, J = 5 Hz, 5 Hz, 2 Hz, 1H), 3.96 (s, 3H), 4.06 (s, 3H), 6.72 (d, J = 7 Hz, 2H), 6.75 (d, J = 3 Hz, 1H), 7.09 (s, 1H), 7.66 (s, 1H), 8.14 (d, J = 7 Hz, 2H); counterion, 2.79 (s, 8H). FAB-MS m/z: 404.5 M^+ (cation).

*Preparation of 1-(3,4-Dimethoxy-6-nitrophenyl)-2,3-epoxypropylsuccinimidyl Carbonate (**VII**).* **II** (100 mg, 0.392 mmol) dissolved in 2 mL of acetonitrile was treated with 110 mg (0.431 mmol) of di-*N*-succinimidyl carbonate. *N*-Ethyldiisopropylamine (70 μL, 0.431 mmol) was added and the reaction mixture stirred for 5 h at room temperature. After evaporation of the solvent, the residue was dissolved in ethyl acetate and washed three times with 20% citric acid, sodium bicarbonate solution, and saturated sodium chloride. The organic phase was dried over magnesium sulfate, the solvent evaporated, and the yellow residue recrystallized out of chloroform/hexane producing yellow-orange crystals. Yield: 138 mg (0.348 mmol, 89%). MW: 396.32 ($C_{16}H_{16}N_2O_{10}$). ^{1}H-NMR ($CDCl_3$, in ppm): isomer a, 2.79 (s, 4H), 2.82 (dd, J = 5 Hz, 2 Hz, 1H), 3.02 (dd, J = 5 Hz, 2 Hz, 1H), 3.56 (ddd, J = 5 Hz, 5 Hz, 2 Hz, 1H), 3.95 (s, 3H), 4.04 (s, 3H), 6.66 (d, J = 4 Hz, 1H), 7.05 (s, 1H), 7.64 (s, 1H); isomer b, 2.79 (s, 4H), 2.90 (dd, J = 5 Hz, 2 Hz, 1H), 3.10 (dd, J = 5 Hz, 2 Hz, 1H), 3.63 (ddd, J = 5 Hz, 5 Hz, 2 Hz, 1H), 3.96 (s, 3H), 4.06 (s, 3H), 6.77 (d, J = 3 Hz, 1H), 7.09 (s, 1H), 7.67 (s, 1H). MS (70 eV) m/z (%): 396 (51) M^+, 238 (100) $C_{11}H_{12}NO_5^+$. FAB-MS m/z: 397.4 MH^+.

*Preparation of Bis[1-(3,4-dimethoxy-6-nitrophenyl)-2,3-epoxypropyl]-N,N′-rhodamine Carbonate (**VIII**).* **IV** (100 mg, 0.180 mmol) dissolved in 0.5 mL of water free THF was treated with 40 μL of *N*-ethyldiisopropylamine (0.350

mmol) and 32 mg of rhodamine 110 hydrochloride (0.086 mmol). An orange precipitate developed over a 2 min sonification treatment. The reaction mixture was left to stand for 4 h at room temperature and centrifuged, and the solvent was evaporated and the orange residue taken up in 5 mL of ethyl acetate. Successive washing of this organic solution with saturated $NaHCO_3$, 20% citric acid, and saturated NaCl was used to remove the sulfonate. The organic phase was then dried over $MgSO_4$, the solvent removed, and the product recrystallized from the residue out of toluene. Yield: 58 mg (0.065 mmol, 75%). MW: 892.5 ($C_{44}H_{36}N_4O_{17}$). Mp: 188−193 °C. ^{1}H-NMR ($CDCl_3$, in ppm): isomer a, 2.35 (dd, J = 4 Hz, 3 Hz, 1H), 3.06 (dd, J = 4 Hz, 3 Hz, 1H), 3.53 (ddd, J = 4 Hz, 4 Hz, 2 Hz, 1H), 3.90 (s, 3H), 3.95 (s, 3H), 6.62 (d, J = 4 Hz, 1H), 7.01 (s, 1H), 6.68 (d, 8 Hz, 1H), 7.10 (s, 1H), 7.15 (d, 8 Hz, 1H), 7.17 (d, 7 Hz, 1H), 7.19 (dd, J = 7 Hz, 7 Hz, 1H), 7.30 (dd, J = 7 Hz, 7 Hz, 1H), 7.60 (s, 1H), 8.10 (d, J = 7 Hz, 1H); isomer b, 2.39 (dd, J = 4 Hz, 3 Hz, 1H), 3.08 (dd, J = 4 Hz, 3 Hz, 1H), 3.64 (ddd, J = 4 Hz, 4 Hz, 2 Hz, 3H), 3.92 (s, 3H), 3.97 (s, 3H), 6.65 (d, J = 4 Hz, 1H), 6.68 (d, J = 8 Hz, 1H), 7.07 (s, 1H), 7.10 (s, 1H), 7.15 (d, J = 8 Hz, 1H), 7.17 (d, J = 7 Hz, 1H), 7.19 (dd, J = 7 Hz, 7 Hz, 1H), 7.30 (dd, J = 7 Hz, 7 Hz, 1H), 7.63 (s, 1H), 8.10 (d, J = 7 Hz, 1H); isomer c, 2.83 (ddd, J = 4 Hz, 4 Hz, 2 Hz, 1H), 3.48 (ddd, J = 4 Hz, 4 Hz, 2 Hz, 1H), 3.64 (ddd, J = 4 Hz, 4 Hz, 2 Hz, 3H), 3.96 (s, 3H), 3.98 (s, 3H), 6.68 (d, J = 8 Hz, 1H), 6.78 (d, J = 3 Hz, 1H), 7.08 (s, 1H), 7.10 (s, 1H), 7.15 (d, J = 8 Hz, 1H), 7.17 (d, J = 7 Hz, 1H), 7.19 (dd, J = 7 Hz, 7 Hz, 1H), 7.30 (dd, J = 7 Hz, 7 Hz, 1H), 7.65 (s, 1H), 8.10 (d, J = 7 Hz, 1H); isomer d, 2.92 (ddd, J = 4 Hz, 4 Hz, 2 Hz, 1H), 3.48 (ddd, J = 4 Hz, 4 Hz, 2 Hz, 1H), 3.64 (ddd, J = 4 Hz, 4 Hz, 2 Hz, 3H), 3.96 (s, 3H), 3.99 (s, 3H), 6.81 (d, J = 3 Hz, 1H), 6.68 (d, J = 8 Hz, 1H), 7.10 (s, 1H), 7.14 (s, 1H), 7.15 (d, J = 8 Hz, 1H), 7.17 (d, J = 7 Hz, 1H), 7.19 (dd, J = 7 Hz, 7 Hz, 1H), 7.30 (dd, J = 7 Hz, 7 Hz, 1H), 7.67 (s, 1H), 8.10 (d, J = 7 Hz, 1H). FAB-MS m/z: 893.3 MH^+.

Reaction of 2-Iminothiolane with BSA and Aminodextran. BSA (4 mg) dissolved in 1 mL of 50 mM borate buffer at pH 8.5 was treated with 40 μL of a freshly prepared solution of 3.5 mg of 2-iminothiolane in 250 μL of distilled water. The solution was left at room temperature for 1 h and then centrifuged for 10 min at 10 000 rpm. The protein conjugate was dialyzed overnight against two changes of phosphate buffer at pH 7.5, previously degassed and saturated with nitrogen gas to prevent oxidation of the thiol groups. After dialysis, the thiolated BSA conjugate was centrifuged at 14 000 rpm at 4 °C. A similar labeling procedure was used to prepare the thiolated dextran from aminodextran.

Determination of the Free Thiol Content of BSA. A 10 mM stock solution of Acrylodan (15 μL) (the thiol reactive form of the fluorescent dye Prodan) dissolved in DMF was added to 300 μl of a 60 μM solution of iminothiolane−BSA at pH 8.0. The reaction mixture was left for 2 h at room temperature, and the solution was centrifuged for 10 min at 10 000 rpm and dialyzed for 2 days against phosphate buffer at pH 7.5 and 4 °C with three changes of buffer. The protein conjugate was centrifuged for 20 min at 14 000 rpm at 4 °C before recording its absorption spectrum. The concentration of Prodan in the conjugate was calculated from the absorption value at 385 nm using an extinction coefficient of 18 500 M^{-1} cm^{-1} (*24*).

Reaction of II with Thiolated BSA. A 60 μM solution of thiolated BSA(500 μL) in borate buffer at pH 9.0 was treated with 20 μL of a 20 mM stock solution of compound **II** dissolved in acetonitrile. The solution turned yellow and was left to stand for 2 h. The BSA conjugate was centrifuged for 10 min at 10 000 rpm at 4 °C and the solution dialyzed for 2 days against phosphate buffer at pH 7.5 and 4 °C with three changes of buffer. The conjugate was then centrifuged for 20 min at 14 000 rpm at 4 °C and the absorption spectrum recorded. The labeling ratio of **II**:BSA was determined using an extinction coefficient for **II** of 5000 M^{-1} cm^{-1} at 350 nm, no losses in BSA were assumed to occur during the workup of the conjugate.

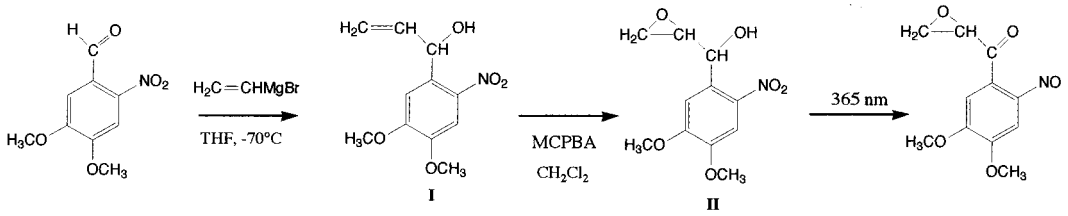
Coupling of G-Actin with Tetramethylrhodamine−Iodoacetamide. Rabbit muscle G-actin, purified according to Marriott (*14*), in G-buffer (2 mM Tris, 0.2 mM $CaCl_2$, and 0.2 mM ATP at pH 8.0) containing 0.5 mM DTT was treated with 13 μL of a 10 mM stock solution of IATMR dissolved in DMF and the reaction mixture left for 2 h at room temperature. The protein was centrifuged for 30 min at 100000*g* at 4 °C and dialyzed overnight against two changes of G-buffer without DTT at 4 °C. After a clear spin at 100000*g* for 1 h, the polymerization was initiated by addition of 2 mM $MgCl_2$ and 0.1 M KCl to the supernatant and the mixture left for 2 h at room temperature. Ultracentrifugation at 100000*g* for 1 h and resuspension of the F-actin pellet in G-buffer was followed by overnight dialysis against G-buffer without DTT. The labeled G-actin solution was clarified by centrifugation (100000*g* for 1 h) and absorption spectrometry used to calculate a labeling ratio of 0.4 fluorophore per actin monomer using an extinction coefficient for TMR of 96 500 M^{-1} cm^{-1} at 550 nm (*26*).

Labeling of TMR−G-Actin with VI. A 30 μM solution of TMR-labeled G-actin (800 μL) in G-buffer without DTT was mixed with 20 μL of a freshly prepared 20 mM stock solution of **VI** in acetonitrile. After a 45 min reaction at room temperature in the dark, the protein was centrifuged for 20 min at 14 000 rpm at 4 °C and dialyzed for 16 h at 4 °C against nitrogen-purged G-buffer without DTT with two buffer changes. The protein was clarified by ultracentrifugation at 100000*g* for 1 h and the absorption spectrum recorded. The protein labeling ratio was calculated using an extinction coefficient for **VI** of 5000 M^{-1} cm^{-1} at 350 nm and 3400 M^{-1} cm^{-1} at 290 nm (*14*).

Reaction of the TMR−G-Actin Conjugate of VI with Thiolated Dextran. Aminodextran labeled with 2-iminothiolane as described earlier was added to a 20 μM solution of TMR-labeled G-actin in a borate-based G-buffer at pH 9.5. After a 1 h reaction, the complex was centrifuged for 20 min at 14 000 rpm at 4 °C and dialyzed overnight against two changes of Tris-based G-buffer with 1 mM DTT. The fluorescent actin-dextran complex was clarified by centrifugation for 20 min at 14 000 rpm at 4 °C.

Reaction of VIII with G-Actin. Two milliliters of 25 μM F-actin was depolymerized during dialysis over 2 days at 4 °C against nitrogen-purged G-buffer without DTT. The G-actin solution was clarified by centrifugation for 1 h at 100000*g* at 4 °C. A 20 mM stock solution of **VIII** (20 μL) was added to 500 μL of the G-actin solution after adjusting its pH to 9.0. After a 1 h reaction at room temperature, the actin conjugate was clarified by centrifugation for 30 min at 10 000 rpm and dialyzed for 2 days against G-buffer (pH 8.0) containing 1 mM DTT. After a clear spin for 1 h at 100000*g* at 4 °C, the labeling ratio of **VIII**:G-actin was determined by absorption spectroscopy using an extinction coefficient for **VIII** of 5000 M^{-1} cm^{-1} at 350 nm and 3400 M^{-1} cm^{-1} at 290 nm (*14*).

Scheme 1



RESULTS AND DISCUSSION

The Grignard reaction of vinylmagnesium bromide on 3,4-dimethoxy-6-nitrobenzaldehyde created a hydroxyl and vinyl group off the benzylic carbon which provided the starting point for the synthesis of the two different reactive groups of the cross-linking reagent (Scheme 1). The first group, an activated carbamate or carbonate, was used to react with and cage an amino group of the compound of interest to its corresponding photolabile carbamate, while the second group, an oxirane, was used to alkylate thiol groups and served to cross-link the caged amino compound to a different thiol-containing biomolecule. This synthetic approach also allowed the physical and chemical separation of the two nucleophilic reactive groups from the photoisomerization functionality. This design feature permitted the use of the 3,4-dimethoxy-6-nitrophenyl group, which has a high molar absorptivity and a red-shifted action spectrum in the 340−420 nm wavelength region. These spectroscopic properties are important for applications of caged compounds in living cells or on surface monolayer (*27*). Since the photocleavable cross-linking reagents introduced by Senter et al. (*15*), Marriott et al. (*7*), and Olejnik et al. (*25*) incorporate the second reactive group at the 4-position of the nitrophenyl group, no other substitutions of the nitrophenyl ring are possible, and correspondingly, these reagents exhibit a low molar absorptivity and blue-shifted action spectra.

Reactivity of the Oxirane Group with Thiols. Treatment of compound **I** with MCPBA generated the epoxy alcohol, compound **II**, in excellent yield (Scheme 1). Although the benzylic proton was resolved for each isomer of compound **II** using NMR spectroscopy, it was not possible to physically isolate the diastereomers. To show that the oxirane group of compound **II** reacted specifically with thiol groups, a qualitative analysis of the reactions of compound **II** with 2-mercaptoethanol was performed under various conditions of pH and solvent composition. In summary, these results showed alkylation of mercaptoethanol with compound **II** occurred in aqueous solutions at a pH value of 8.0−9.5 or in organic solvents under slightly acidic or neutral conditions. Compound **II** did not react with primary amines under these conditions (data not shown). Investigations were also made on the reactions of the oxirane group of compound **II** with reduced thiol groups of native BSA, containing a single reduced thiol group, and a 2-iminothiolane conjugate of BSA containing several reactive thiol groups. Dialysis of these reaction mixtures removed excess compound **II**, and allowed an absorption spectrophotometric analysis-based calculation of the labeling ratio of **II**:BSA for each conjugate. Native BSA was found to harbor a single molecule of **II** per BSA molecule, while the iminothiolane−BSA was labeled with six molecules of **II** per BSA (Figure 1). An independent determination of the number of reactive thiol groups in the iminothiolane−BSA conjugate using the thiol labeling reagent Acrylodan (*24*) also showed there were six free thiol groups (data not shown). Evidently at this slightly basic pH, the oxirane group was able to react selectively with reduced thiol groups of proteins.

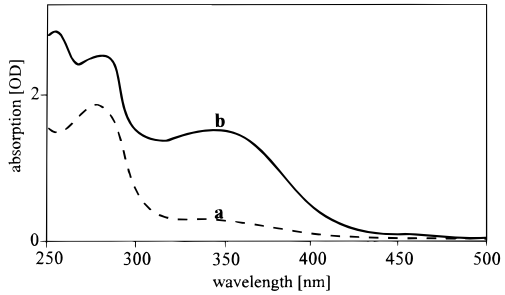


Figure 1. Absorption spectra of (a) native BSA labeled (59 μM) with compound **II** and (b) iminothiolane-labeled BSA (59 μM) labeled with compound **II**.

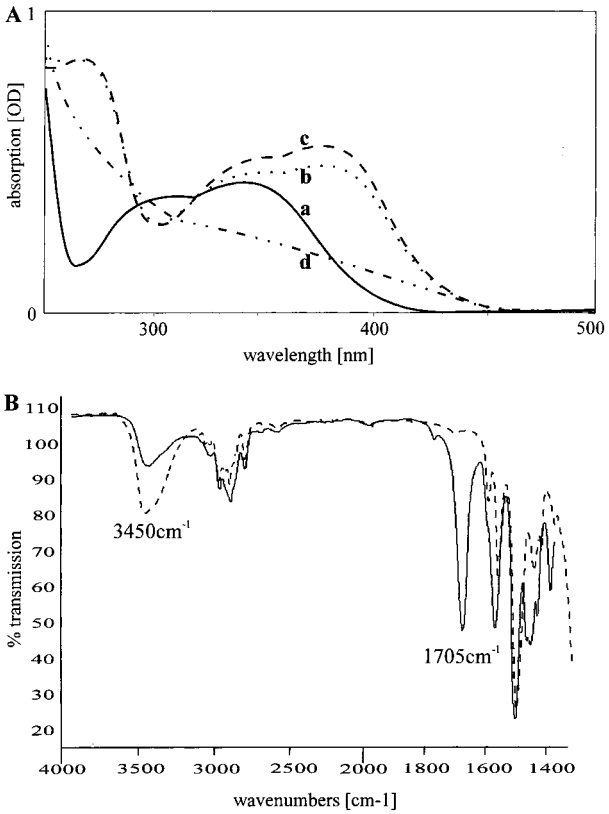
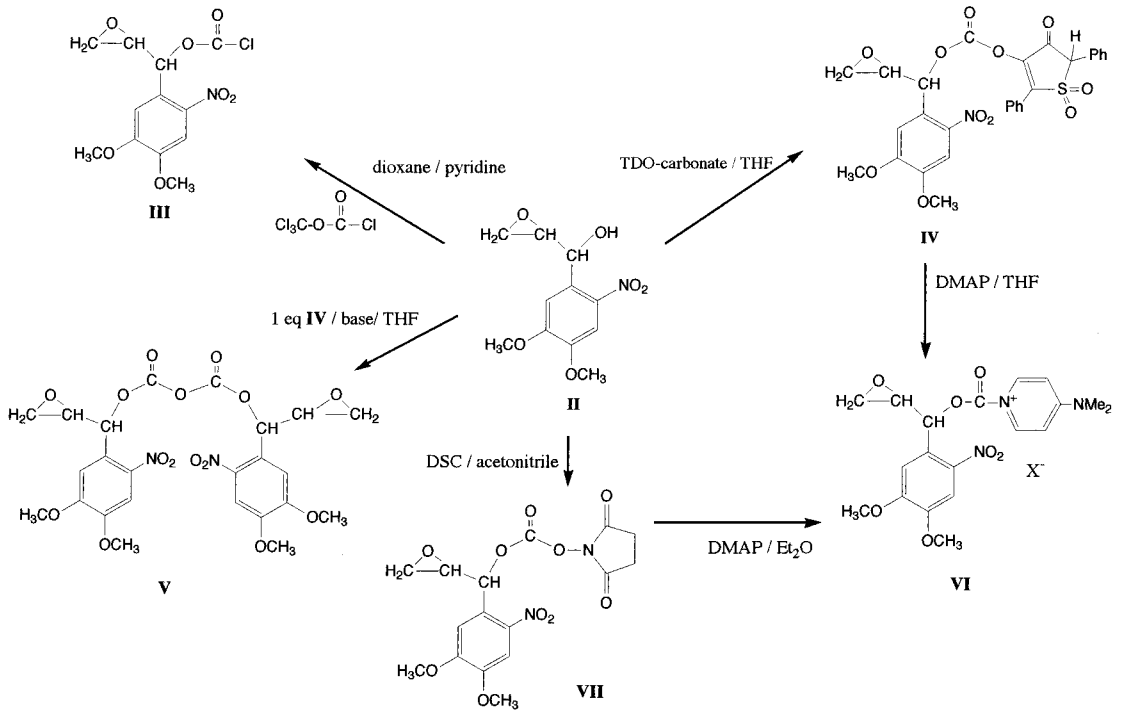


Figure 2. (A) Absorption spectra of **II** in ethanol (a) before irradiation with 365 nm light and (b) after irradiation for 30 s, (c) 90 s, and (d) 900 s. (B) Infrared spectra of **II** before (dashed line) and after a 90 s irradiation with near-ultraviolet light (solid line).

Spectral Properties of II. The absorption spectrum of a defined concentration of an ethanolic solution of compound **II** (Figure 2A) showed that the chromophore had a broad n−π* transition centered at 350 nm and an extinction coefficient at this wavelength of 5000 M^{-1} cm^{-1}. Excitation of this ethanolic solution with near-ultraviolet light (340−420 nm) led to a dose-dependent loss of the 350 nm band, while two new absorption

Scheme 2



transitions evolved with maxima at 268 and 378 nm (Figure 2A). These absorption bands most probably belong to the 3,4-dimethoxy-6-nitrosoacetophenone photoproduct. Evidence that this reaction occurred via a photoisomerization reaction of the nitrophenyl group was provided by an analysis of the infrared spectra of a film of **II** recorded before and after near-ultraviolet irradiation (Figure 2B) which showed a loss of the hydroxyl absorption band at 3450 cm^{-1} of compound **II** and a gain of a ketone absorption band at 1705 cm^{-1}. These results are consistent with the reaction pathway presented in Scheme 1.

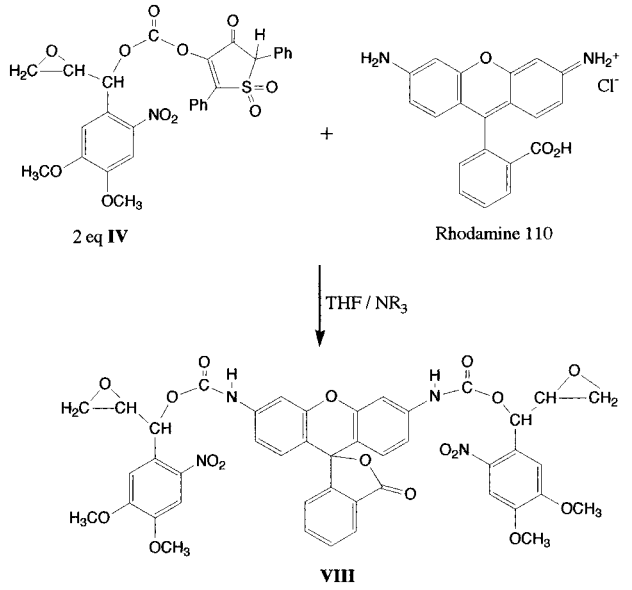
Preparation of Photocleavable, Cross-Linking Reagents Based on Compound II. Compound **II** proved to be a key intermediate in the synthesis of the four cross-linking reagents described in this report (Scheme 2). On the basis of earlier, successful studies in which NVOC-Cl was used to cage amino groups (*14*, *15*, *20*), we decided to prepare compound **III**, the corresponding chloroformate of **II**, by activation of its benzylic hydroxyl group using diphosgene. Analysis of the reaction showed the presence of two major products which, after purification over silica gel and NMR analysis, proved to be two isomers at the benzylic carbon. Isomer **IIIa** was crystallized out of 2:1 hexane/ethyl acetate. The isomers exhibited identical absorption and infrared spectra, and they could not be indistinguished by mass spectrophotometry. The photoisomerization rates of **IIIa** and **IIIb** were found to be similar as judged by a qualitative TLC analysis of the amount of product that formed upon irradiation of each isomer with 365 nm light (data not shown). Compound **III** reacted poorly with the amino groups of polylysine or G-actin in bicarbonate buffer at pH 8.5. Furthermore, although the unsubstituted chloroformate, NVOC-Cl, has been shown to react with amino groups in both organic and aqueous solvents (*3*, *14*), this was not the case with compound **III**, and presumably, the substituent on the benzylic carbon atom deactivated the chloroformate group. These findings prompted the syntheses of more activated carbonates and carbamates of compound **II** that could be used to protect amino groups in aqueous or organic solvents.

Steglich's reagent (TDO) (*17*) was chosen to make a more activated carbonate of compound **II**. This reaction proceeded smoothly in THF under reflux to produce compound **IV** in high yield. The two isomers of compound **IV** were not resolved using the purification scheme described in Materials and Methods. However, the photoisomerization rates of the isomers of compound **IV** should not differ to any significant extent given the similarity of the photoisomerization rates found for the isomers of compound **III**.

An interesting, competing side reaction was discovered in the synthesis of compound **IV**. This involved the reaction of compound **IV** with compound **II**, accelerated by the addition of catalytic amounts of base, which yielded the symmetrical carbonate **V**, a further, thiol reactive, homobifunctional, photocleavable cross-linking reagent (Scheme 2).

Photocleavable, Cross-Linking Reagents and the Preparation of a Thiol Reactive Caged Rhodamine 110. Compound **IV** was shown to react efficiently with amino groups (e.g. rhodamine 110, glycine and polylysine, data not shown) in organic solvent and in aqueous solution at pH 8.5 to produce the corresponding carbamate. Amidation of the two aromatic amino groups of the highly fluorescent rhodamine 110 fluorophore has been shown to form the nonfluorescent, lactone form of the dye which lacks a visible absorption band and fluorescence emission (*18*). Similarly, compound **IV** was shown to react with rhodamine 110 to produce a nonfluorescent, bis-substituted carbamate (**VIII**, Scheme 3). Irradiation of the nonfluorescent (caged) rhodamine 110 with near-ultraviolet light cleaved the two carbamate bonds and liberated free rhodamine 110 with concomitant recovery of its visible absorption band and intense fluorescence emission centered at 523 nm (Figure 3A,B). This reaction was quite rapid since the photoactivation of a 500 μL

Scheme 3



sample of 50 μM solution of caged rhodamine 110 in the presence of 5 mM DTT was complete within 240 s (Figure 3A,B).

One of the oxirane groups of compound **VII** was used to label a thiol group on a cysteine residue of G-actin in a reaction conducted in aqueous buffer at pH 8.5. A labeling ratio of 1:1 for compound **VIII**:G-actin was calculated using absorption spectroscopy, suggesting the caged fluorophore was covalently attached to cysteine-374 (*24*). This caged fluorescent G-actin conjugate was able to polymerize under physiological conditions as seen by a comparison of the absorption spectra of the supernatant and pellet fractions of a high-speed centrifugation run, which showed the pellet fraction contained the majority of the photoactivated actin (Figure 4). Irradiation of the nonfluorescent G-actin conjugate with near-ultraviolet light cleaved the carbamate bond that linked the caged groups to rhodamine 110 and liberated the highly fluorescent rhodamine 110, and a polymerization competent G-actin that harbored the 3,4-dimethoxy-6-nitrosophenylketone-based photoproduct (Figure 4).

Photocleavable, Cross-Linking Reagents with Improved Water Solubility and Amino Group Reactivity. To improve upon the water solubility of heterobifunctional photocleavable cross-linking reagents, and to increase their reactivity toward the ϵ-amino group of lysine residues of proteins, compound **VI**, the DMAP salt of compound **IV** was prepared via two synthetic routes outlined in Scheme 2. In the first approach, the succinimide carbonate of **II** was prepared in a reaction of **II** with di-*N*-succinimidyl carbonate in acetonitrile which produced the crystalline product **VII** in high yield. Treatment of compound **VII** in THF with DMAP led to the precipitation of the DMAP salt of compound **VI**. In the second synthetic approach, compound **VI** was prepared in a single-step reaction of compound **IV** with DMAP as described in Materials and Methods. Compound **VI**, a highly activated, water soluble carbamate reacted rapidly with amino groups of proteins at a pH between 8 and 9 (Scheme 4). Compound **VI** was found to hydrolyze in water, and consequently, its reactions with proteins should be performed using the reactants at a relatively high concentration. Compound **VI** can be added to protein labeling reactions as a solid to reduce any risk of organic solvent-based protein denaturation.

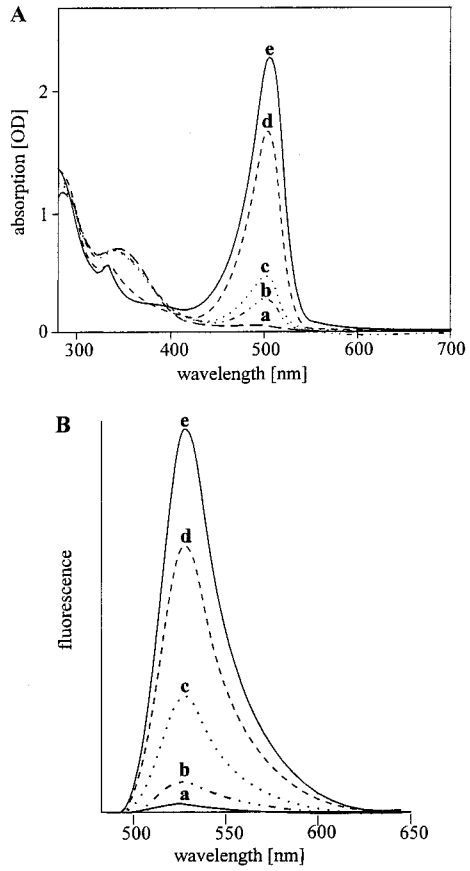


Figure 3. (A) Absorption spectra of a 50 μM solution of caged rhodamine 110 in EtOH/H_2O (10:1) containing 2 mM DTT before irradiation (a) and after near-ultraviolet irradiation (340−400 nm) for the following times: (b) 5 s, (c) 10 s, (d) 180 s, and (e) 240 s. (B) Fluorescence emission spectra of caged rhodamine 110 with excitation at 488 nm of a 15 μM solution of caged rhodamine 110 in EtOH/H_2O (10:1) before irradiation (a) and after near-ultraviolet irradiation (340−400 nm) for the following times: (b) 5 s, (c) 20 s, (d) 60 s, and (e) 240 s.

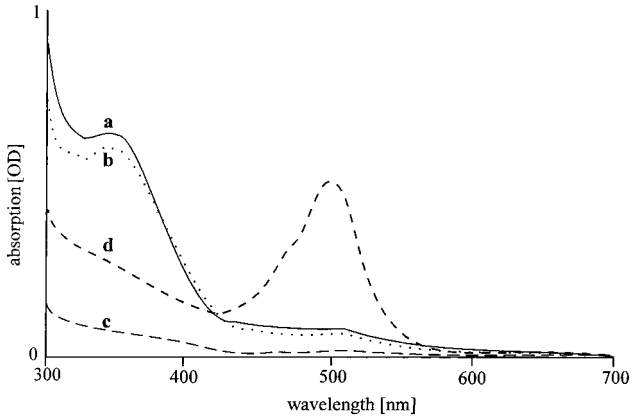
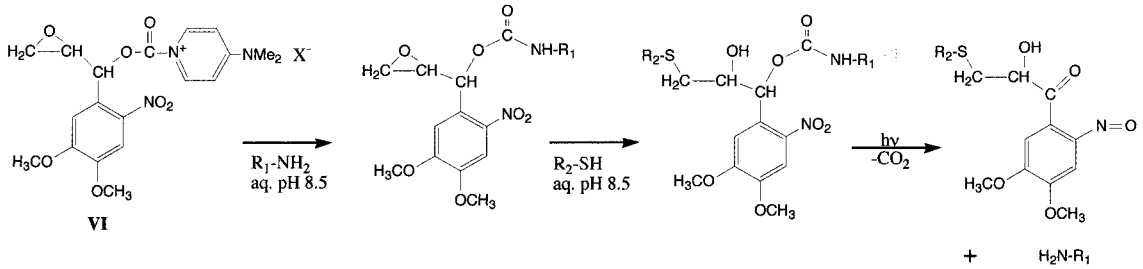


Figure 4. (a) Absorption spectrum of G-actin-labeled caged rhodamine 110 before irradiation with near-ultraviolet light in G-buffer containing 2 mM DTT. The G-actin was treated with $MgCl_2$ and KCl to 2 and 100 mM, respectively, and after 2 h, the protein was centrifuged at 100000*g* for 60 min. (b) Absorption spectrum of the pellet fraction of this centrifugation run after resuspension in an equal volume of F-buffer. (c) Absorption spectrum of the supernatant fraction of this centrifugation run. (d) Absorption spectrum of the resuspended pellet fraction (from part b) after irradiation with near-ultraviolet light for 10 min in the presence of 5 mM DTT.

It should be noted however that in organic solvents and in the presence of nucleophiles DMAP-activated salts such as **VI** undergo esterification or even reaction to

Scheme 4



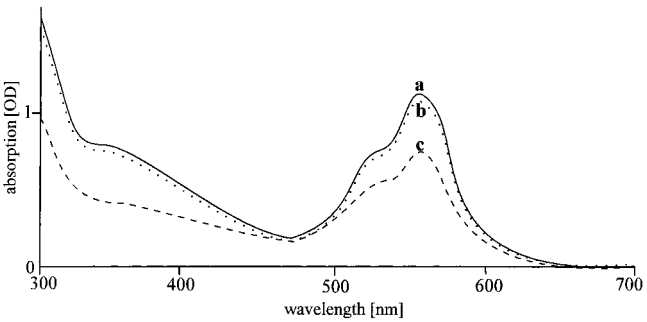


Figure 5. (a) Absorption spectra of the IATMR-labeled G-actin−dextran complex in G-buffer containing 2 mM DTT. After addition of $MgCl_2$ and KCl to 2 and 100 mM, respectively, and 2 h at room temperature, the sample was centrifuged at 100000*g* for 60 min. (b) Absorption spectrum of the supernatant fraction. No pellet fraction was found after the centrifugation; i.e. the actin was caged. The supernatant fraction was then irradiated with near-ultraviolet light for 10 minutes, and after 2 h at room temperature, the sample was centrifuged at 100000*g* for 60 min. (c) Absorption spectrum of the pellet fraction resuspended in an equal volume of F-buffer.

isocyanates (*21*), and therefore, stock solutions of **VI** made in acetonitrile should be prepared just before the protein labeling reaction.

Application of Photocleavable, Cross-Linking Reagents for the Preparation of Caged G-Actin. Compound **VI** was used as part of a new labeling procedure to prepare caged G-actin, i.e. a G-actin conjugate that does not polymerize under physiological conditions (*14*). An average of 3.5 lysine residues of TMR-labeled G-actin were protected as their corresponding photolabile carbamates with compound **VI**. One or more of the oxirane groups of this fluorescent G-actin conjugate of compound **IV** were then cross-linked to a thiolated dextran. Sixty minutes after addition of physiological salt to this caged G-actin cross-linked complex, the sample was subjected to high-speed centrifugation and the absorption spectra of the supernatant and resuspended pellet fractions were recorded (Figure 5). These data showed the majority of the fluorescently labeled G-actin conjugate was contained in the supernatant fraction and was, consequently, polymerization incompetent (Figure 5). A 500 μL sample of this caged G-actin complex in F-buffer was exposed to near-ultraviolet light for 10 min in the presence of 5 mM DTT to photocleave the cross-link between G-actin and the dextran, and then left for 60 min at room temperature to allow for polymerization. After high-speed centrifugation of the sample, absorption spectroscopic analysis now showed the majority of the actin was in the resuspended pellet fraction; i.e. the photoactivated actin had polymerized into F-actin filaments (Figure 5). The inability of the G-actin cross-linked dextran complex to polymerize in physiological salt probably arose from a physical masking of the actin binding site on the caged G-actin by the flexible, cross-linked dextran molecule. This mode of inhibition was evidently relieved after the light-directed dissociation of the carbohydrate polymer. Importantly, the photocleavage reaction released native, fully functional G-actin, while the photoproduct remained covalently attached to the thiolated dextran. This new approach to caging the activity of G-actin required the modification of fewer lysine residues compared to the NVOC-Cl method (*14*).

An interesting parallel to the caged G-actin complex described above has been reported by Mann and Vanamann (*26*). These authors demonstrated that, although a succinmidyl D-biotin conjugate of calmodulin could still activate the enzyme phosphodiesterase, this activity was completely blocked when the calmodulin conjugate was pretreated with avidin. Presumably, avidin, like the dextran in G-actin, physically masked the PDE binding site of calmodulin. Given the importance of specific interactions in protein function, targeted disruption of protein binding sites using the photocleavable reagents and the dextran conjugation methods described herein could be used as part of a general approach to caging the activity of a wide range of proteins. Native, active proteins would then be liberated from these caged dextran cross-linked complexes upon illumination with near-ultraviolet light.

SUMMARY

In this study, we designed, synthesized, and characterized four members of a new class of heterobifunctional photocleavable cross-linking reagents. Biomolecules or fluorophores caged with these reagents can be covalently cross-linked to a second thiol-containing protein, ligand, or derivatized surface through the oxirane moiety. The cross-linking reaction may be used for the following applications: (1) to prepare a caged protein by masking an active site with a thiolated dextran, (2) to target the caged compound to a specific site using a thiolated antibody (*15*), (3) to attach the caged compound to a thiol-derivatized surface, and (4) to render the caged compound fluorescent in order to image or to quantify the yield of the photoactivation reaction.

The activated carbonate and carbamate groups of two of the reagents described in this report can be used to protect almost any amino group as a photolabile carbamate in either water or organic solvent. The order of reactivity toward amino groups of the different activated groups in water was found to be R-DMAP$^+$ > R-TDO > R-OSu > R-Cl. Compound **IV**, the TDO carbonate of compound **II**, was used to protect amino groups in organic solvents, while compound **VI**, the DMAP salt of compound **IV**, was the most water soluble and reactive toward amino groups. The physical and chemical separation of the cross-linking functionality from the photoisomerization functionality in these reagents allowed the introduction of one or more methoxy groups into the nitrobenzyl ring which increased its absorption cross section and shifted its action spectrum to a lower energy.

One of the new bifunctional, photocleavable cross-linking reagents, compound **IV**, was used to prepare a thiol reactive, nonfluorescent (caged) rhodamine 110. Irradiation of this caged fluorescent actin conjugate with near-ultraviolet light generated free, highly fluorescent rhodamine 110 and a polymerization competent G-actin conjugate. Compound **V**, a water soluble photocleavable cross-linking reagent, was used as part of a new approach to caging the activity of G-actin by exploiting the ability of a dextran molecule cross-linked to G-actin to physically mask its actin binding site. Photocleavage of the G-actin−dextran cross-linked complex in F-buffer with near-ultraviolet light liberated native G-actin which polymerized into filaments and released the dextran conjugate of the 3,4-dimethoxy-6-nitrosoketone-based photoproduct. The new photocleavable, cross-linking reagents and conjugation methods described in this article can form the basis of a new, general approach to caging the activity of proteins.

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BC970147O