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Multifunctional Cross-Linking Reagents. I. Synthesis and Properties of Novel Photoactivable, Thiol-Directed Fluorescent Reagents[1)]

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Bifunctional photo-activable fluorescent thiol reagents of a new type were synthesized. A maleimide group was bonded to an azidocoumarin group *via* a methylene chain as a spacer. Reagents of this type react first with a cysteine residue of a protein through the maleimide group, and then form another bond with an amino acid side chain of the protein upon irradiation with light, through a nitrene group formed from the azide. Although the reagent is non-fluorescent, the products are highly fluorescent. The fluorescence characteristics of model compounds of these reagents are also described.

Keywords——cross-linking reaction; heterobifunctional reagent; all-or-none fluorescent reagent; nitrene from azide; 7-aminocoumarin; 7-azidocoumarin; maleimide

Studies on bio-macromolecular organizations are fundamental to an understanding of biological phenomena. For example, to elucidate the molecular organization of membranes, studies of long-term protein-protein interactions within membranes are necessary.[2)] The use of cross-linking reagents is a general technique for systematic structural investigation of such organizations, since the only direct chemical method for study of the protein interaction is based on the elucidation of the location of inter-chain chemical bonds.[3)]

The most versatile method of cross-linking is to use "bifunctional" reagents, which have two reactive groups capable of reacting with, and forming bridges between, the side chains of amino acids in the proteins.[2,3)] Bifunctional reagents carrying two identical reactive groups (X), so-called homobifunctional reagents (**1**) (Chart 1), are commonly used. However, in the course of studying various biological systems it has become clear that the utility of

X-[A]-X (**1**) X-[A]-Y (**2**)

X,Y : functional groups

A : spacer part of the molecules

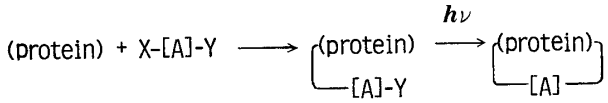


Chart 1

conventional homobifunctional reagents is limited due to several inherent problems: random collisional cross-linking, long reaction time, difficulty in controlling the reaction, and non-selective cross-linking. It is therefore more desirable to use "heterobifunctional" reagents (**2**), where the two reactive groups (X, Y) are sufficiently different to permit well-controlled sequential reactions of each group in turn. Among several heterobifunctional reagents, a very promising new group consists of reagents in which one of the reactive functions is a relatively unreactive group which can be photoactivated to give a highly reactive photolysis product. With such reagents, a well-defined sequence of reaction steps can be achieved by carrying out the first step in the dark and the second one in the presence of activating light (Chart 1).

Arylnitrenes can be generated from arylazides and have a short life time and high reactivity. Their reaction is nonselective and does not require a specific reactive group in proteins, and this broad reactivity is advantageous when cross-linking is the primary purpose.[4)]

Fluorescence spectroscopy is among the most sensitive, versatile, and potentially informative methods available for studying the conformation and dynamics of macromolecules.[5)] In many cases, an "extrinsic" fluorophore can be introduced into a protein either by covalent coupling or by reversible interaction.[5a)] The use of such extrinsic fluorophores is particularly valuable when a reagent itself is non-fluorescent and becomes fluorescent on reaction with a substrate, since this kind of "all-or-none" reagent is applicable to studies of biomacromolecules both as a tracer and reporter. We have confirmed that "all-or-none" fluorescent reagents, directed to thiol groups in proteins, are extremely useful in very broad areas of biomedical research.[5a,6)]

The approach introduced in this paper combines crosslinking achieved by a heterobifunctional method, involving a photoactivable group, with the probing and reporting power of the fluorescence techniques. To make the first of the two bifunctional groups reasonably selective, we adopted the maleimide group, which is directed to thiol groups in proteins among other nucleophiles. As the second (photoactivable) group, the azidocoumarin group (a precursor of a reactive arylnitrene) was adopted.

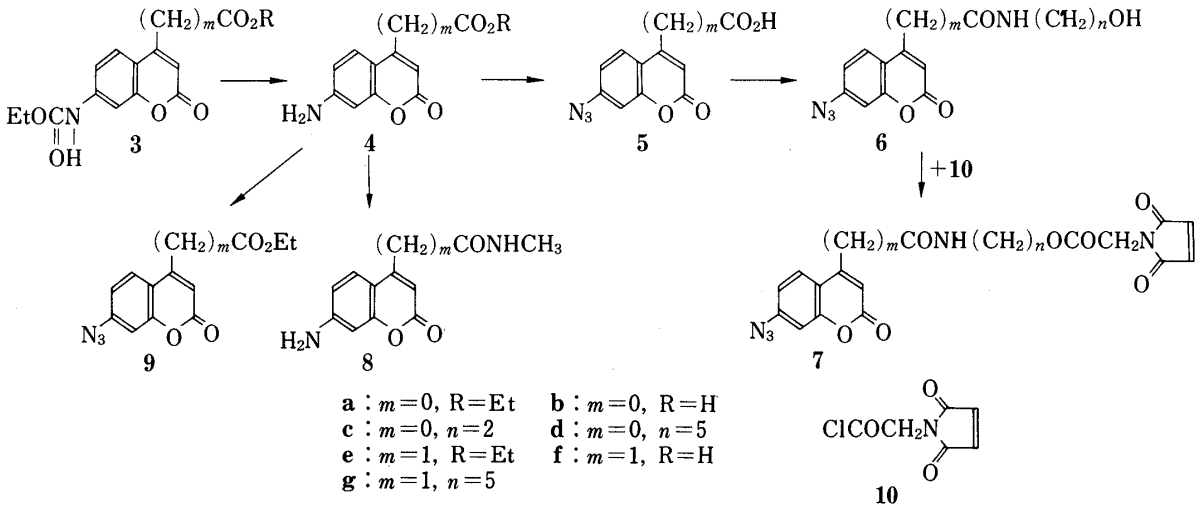
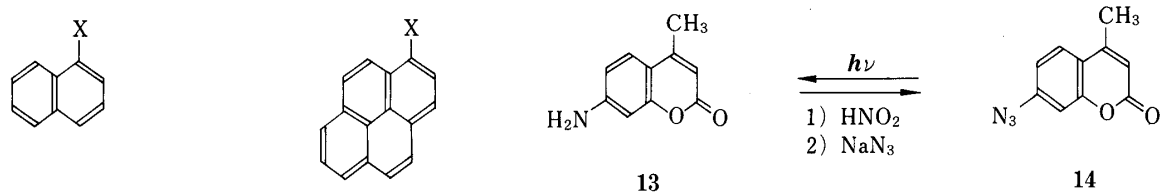


Chart 2

The schemes used to synthesize these reagents are presented in Chart 2. Esters of 7-aminocoumarin-4-carboxylic acid (**4a**) and -4-acetic acid (**4e**) were the key starting compounds, and they were converted into the azido derivatives (**5**), which were then transformed into the amides of amino alcohols, spacer molecules. These amido alcohols (**6**) were coupled with maleoylglycyl chloride (**10**) to produce the heterobifunctional reagents (**7**).



11 12 13 14

a : X=NH_2
b : X=N_3

Chart 3

$$Ar-N_3 + (protein) \xrightarrow{h\nu} Ar-NH-(protein)$$

Chart 4

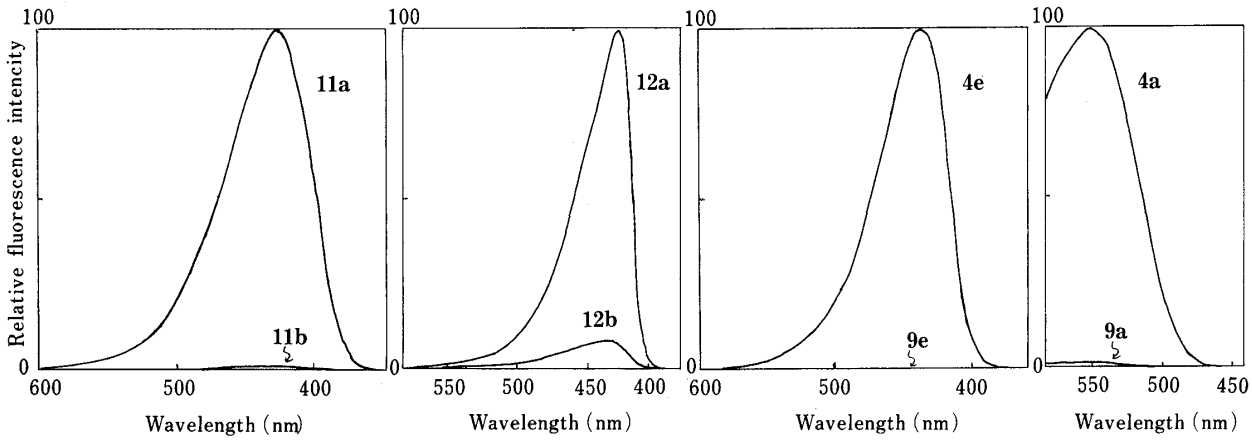


Fig. 1. Comparison of Fluorescence Intensities of Aromatic Amines and Azides

11a (2.81×10^{-5} M) and **11b** (1.25×10^{-5} M) were excited at 332 nm, **12a** (6.32×10^{-6} M) and **12b** (3.96×10^{-5} M) were excited at 362 nm, **4e** (7.32×10^{-6} M) and **9e** (7.14×10^{-6} M) were excited at 364 nm, and **4a** (1.09×10^{-5} M) and **9a** (1.06×10^{-5} M) were excited at 389 nm. The solvent was ethanol, and the optical density of all compounds was around 0.1 at the wavelength of excitation.

The fluorescence spectra of the aminocoumarins (**4**) and azidocoumarins (**9**) are compared in Fig. 1. For comparison, fluorescence spectra of pairs of aromatic amines (**11**) and azides (**12**) are also shown (Fig. 1). Aromatic amines have strong fluorescence maxima, and the excitation spectra of aminocoumarins show their maxima at 364 nm for **4e**, and 389 nm for **4a**. In contrast, all the aromatic azides show very weak fluorescence. Rather efficient intersystem crossing to the triplet may be the reason for this quenching.[7]

Since arylazides have been commonly used for photolabeling in biological studies, it is rather surprising that there is little information on the chemistry of the photoproducts of these azides with biomacromolecules.[4] In an attempt to elucidate the nature of these photoproducts, 7-azido-4-methylcoumarin (**14**), prepared from the amine (**13**), was selected as the simplest model. Photolysis of **14** was examined in methanol and cyclohexane solutions. None of the desired insertion products in which the coumarin ring is covalently bound to solvent molecules were isolated, but the amine (**13**), which is apparently formed through hydrogen abstraction from the solvent by the intermediate nitrene,[8] was obtained in low yield. However, we tentatively assumed that the amino compounds are the primary photoproducts in the photolabeling of proteins with azide reagents (Chart 4).[8]

Since fluorescence spectra are generally affected by the environment, the fluorescence characteristics of 7-aminocoumarin derivatives, which are plausible model compounds for the photoproducts from these new reagents, were examined. The excitation and emission maxima, relative fluorescence intensity and quantum yield are summarized in Table I. The model

TABLE I. Fluorescence Characteristics and Quantum Yields of 7-Aminocoumarin Derivatives in Ethanol

Amine	Maximal emission (nm)	Maximal excitation (nm)	Relative fluorescence intensity	Quantum yield
4a	555	389	6	—
8a	530	374	18	—
4e	441	364	100	0.83[a]

a) Calculated by the method of Parker and Rees,[9] based on a value of 0.55 for quinine sulfate.

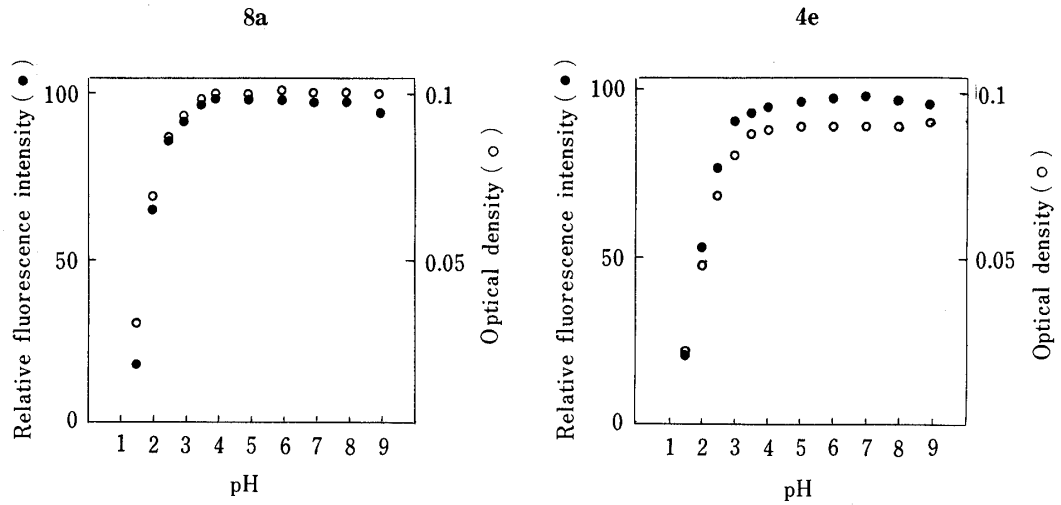


Fig. 2. pH-Dependence of the Absorption and Fluorescence Intensity Values of **8a** and **4e**

8a (8.00×10^{-6} M) in buffer containing 1% ethanol, **4e** (5.61×10^{-6} M) in buffer containing 1% ethanol at 25 °C. 0.1 M H_3PO_4–KH_2PO_4 for pH 1.5—4.0, 0.1 M citric acid–Na_2HPO_4 for pH 3.0—7.0 and 0.1 M KH_2PO_4–$Na_2B_4O_7$ for pH 6.0—8.0.

compound **4e** is highly fluorescent, though it has a nonconjugated ester substituent at the 4-position of the coumarin ring. Conjugation of an ester or amide group with coumarin at the 4-position (**4a** and **8a**) decreases the fluorescence intensity, but it is advantageous that the excitation and emission maxima are both in the long wavelength region.

Figure 2 shows the pH dependence of the absorption and fluorescence intensity of **8a** and **4e**. The constant fluorescence intensity over the pH range of 4—9 suggests that the fluorophore can be used over a wide operational pH range. At lower pH, the fluorescence intensity decreases with decrease of pH value; this phenomenon can be explained on the basis of protonation at the 7-amino group of the coumarin ring.

The wavelength of maximal emission (cm^{-1}) of the aminocoumarin (**4a** or **4e**) fluorescence changes monotonically as a function of polarity in ethanol–water mixtures in terms of Kosower's Z value (Fig. 3). The wavelength decreases from a value of 18 ($k\,cm^{-1}$) in ethanol to 17.3 ($k\,cm^{-1}$) in water. In Fig. 4, the correlation of the wavelength of maximal emission of aminocoumarins (**4a** and **4e**) with solvent viscosity is shown to be small. The effect of varying the solvent viscosity on the maximal wavelength was measured by using a mixed aqueous solution of sucrose and Ficoll, a high molecular weight of polymer of sucrose.[10] On the basis of these effects of solvents we conclude that fluorophores related to **8** should be useful as reporters for hydrophobic regions of biopolymers.

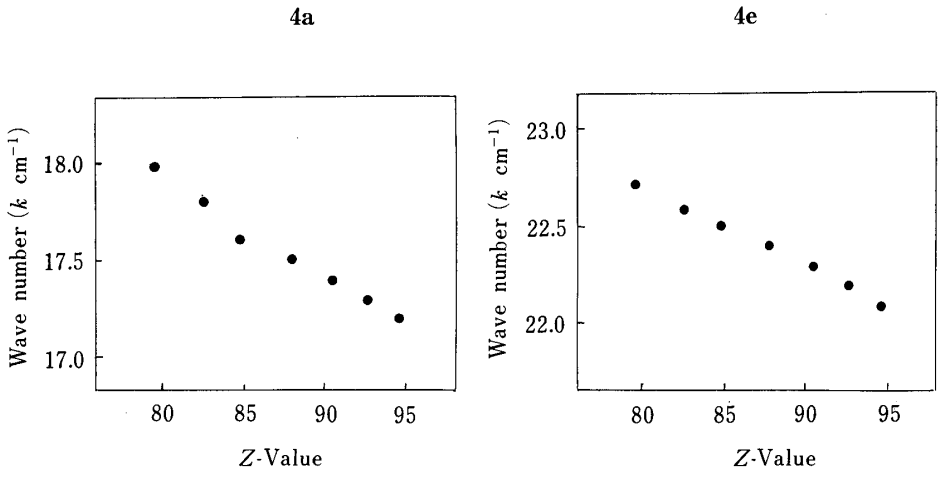


Fig. 3. Polarity Effect on the Wavelengths of Maximal Emission of **4a** and **4e**

4a (8.00×10^{-6} M), **4e** (5.61×10^{-6} M) at 25 °C. Kosower's "Z" value 79.6 (100% EtOH), 82.5 (90% EtOH), 84.8 (80% EtOH), 87.9 (60% EtOH), 90.5 (40% EtOH), 92.6 (20% EtOH) and 94.6 (100% H_2O).

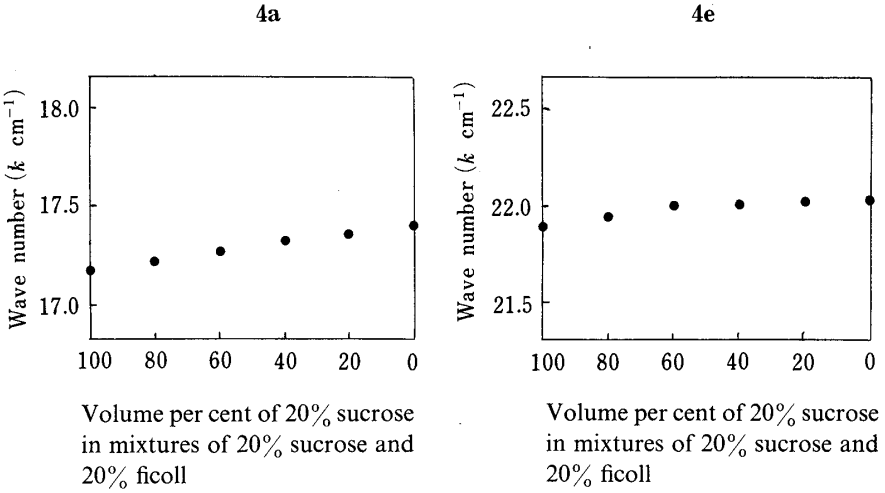


Fig. 4. Viscosity Effect on the Wavelengths of Maximal Emission of **4a** and **4e**

4a (8.00×10^{-6} M), **4e** (5.61×10^{-6} M) at 25 °C containing 1% ethanol.

If the photolysis of the aromatic azides leads to corresponding amino derivatives such as **8**, the above general spectral behavior has an interesting implication. Although the reagents themselves are nonfluorescent, the reaction products formed on irradiation may become fluorescent. Such "all-or-none" properties are expected to be useful in the application of these heterobifunctional reagents. In 1978 Leonard *et al.* reported a fluorescent photoaffinity labeling reagent, 8-azido-1,N^6-ethenoadenosine-3′,5′-cyclic monophosphate.[11] The present work confirms that aromatic azides generally can be used as "all-or-none" fluorescent photolabeling groups. Application of **7** in studies of the structures and functions of proteins containing thiol groups will be reported elsewhere.

Experimental

All melting points are uncorrected. Infrared (IR) spectra were recorded on a JASCO IRA-1 spectrometer in Nujol mulls. Ultraviolet (UV) spectra were measured with a Hitachi 200-10 spectrophotometer. Nuclear magnetic resonance (NMR) spectra were obtained with JEOL JNM-FX 200 and Hitachi R-24A spectrometers. Chemical shifts

were recorded in ppm units using tetramethylsilane as an internal reference. Fluorescence spectra were measured with a Hitachi 650-60 spectrophotofluorometer.

Ethyl 7-Aminocoumarin-4-carboxylate (4a)——A mixture of distilled diethyl oxalacetate (11.4 g, 60.6 mmol) and *m*-aminophenol (5.30 g, 48.6 mmol) was stirred in an oil bath at 100 °C for 1 h, then allowed to cool. Ether was added to the reaction mixture to give a viscous solid, which was triturated with ether and collected by filtration. Recrystallization from ethanol gives **4a** orange needles of mp 195.0—195.5 °C (dec.), 3.1 g, 27%. UV λ_{max}^{EtOH} nm (ε): 391.5 (1.23×10^4). IR ν_{max}^{Nujol} cm^{-1}: 3440 and 3350 (NH), 1730 (lactone), 1700 (ester). NMR (DMSO-d_6) δ: 1.35 (3H, t, J=8 Hz, $OCH_2C\underline{H}_3$), 4.35 (2H, q, J=8 Hz, $OC\underline{H}_2CH_3$), 6.25 (1H, s, 3-H), 6.45 (1H, d, J=2 Hz, 8-H), 6.55 (1H, dd, J=9, 2 Hz, 6-H), 7.65 (1H, d, J=9 Hz, 5-H). *Anal.* Calcd for $C_{12}H_{11}NO_4$: C, 61.80; H, 4.75; N, 6.01. Found: C, 62.08; H, 4.65; N, 6.14.

7-Aminocoumarin-4-carboxylic Acid (4b)——A solution of **4a** (2.4 g, 10 mmol) in methanol (70 ml) was treated with 2 N potassium hydroxide (6.2 ml). The mixture was refluxed for 3 h, then allowed to cool, and water was added. The pH of the resulting solution was adjusted to 2 with conc. hydrochloric acid until the 7-aminocoumarin-4-carboxylic acid (**4b**) had precipitated. After being collected by suction, the solid was dried, and recrystallized from ethanol to provide **4b** as red needles of mp 247—248 °C (dec.); 1.8 g, 86%. IR ν_{max}^{Nujol} cm^{-1}: 3440 and 3340 (NH), 1690 (lactone), 1630 (carboxylic acid). NMR (DMSO-d_6) δ: 6.25 (1H, s, 3-H), 6.45 (1H, s, 8-H), 6.55 (1H, dd, 6-H), 7.75 (1H, d, 5-H). *Anal.* Calcd for $C_{10}H_7NO_4 \cdot 1/4EtOH$: C, 58.19; H, 3.95; N, 6.46. Found: C, 58.29; H, 4.05; N, 6.18.

7-Azidocoumarin-4-carboxylic Acid (5b)——Conc. sulfuric acid (9.6 ml) was added to a suspension of **4b** (5.02 g, 24.5 mmol) in glacial acetic acid (24 ml) at 0 °C. Next, a solution of sodium nitrite (1.8 g, 26 mmol) in water (12 ml) was added, and after the mixture become homogeneous, urea (622 mg) was added. In the dark, a solution of sodium azide (1.8 g, 25 mmol) in water (12 ml) was added dropwise to the solution and the whole was stirred at 0 °C for 1 h. A precipitated solid was collected by filtration, and **5b** was obtained as dark yellow needles of mp 192 °C (dec.) by recrystallization from ethanol; 4.17 g, 74%. IR ν_{max}^{Nujol} cm^{-1}: 2100 (azide), 1730 (lactone), 1685 (carboxylic acid).

2-(7-Azidocoumarin-4-carboxamido)ethanol (6c)——In the dark, a solution of **5b** (500 mg, 2.2 mmol) and triethylamine (250 mg, 2.5 mmol) in ab. THF (10 ml) was cooled to −15 °C and isobutyloxycarbonyl chloride (0.3 ml, 2.3 mmol) was added. After 15 min, a solution of ethanolamine (134 mg) in THF (10 ml) was added, and the reaction mixture was stirred at −15 °C for 2 h, then overnight at room temperature. After the precipitate had been removed by filtration and the filtrate evaporated, the residue was again dissolved in ethyl acetate. The organic solution was washed with water, 0.5 M citric acid solution, water, sat. sodium bicarbonate solution, water and sat. sodium chloride solution. The organic solution was dried over anhyd. magnesium sulfate. The solvent was removed by evaporation, and **6c** was obtained as pale yellow needles of mp 167.5—168.5 °C (dec.) by recrystallization of the residue from acetonitrile; 380 mg, 63%. IR ν_{max}^{Nujol} cm^{-1}: 2120 (azide), 1730 (lactone), 1650 (amide). NMR (DMSO-d_6) δ: 3.40 (4H, m, $NHC\underline{H}_2C\underline{H}_2O$), 4.70 (1H, t, J=8 Hz, OH), 6.45 (1H, s, 3-H), 7.06 (1H, dd, J=8, 2 Hz, 6-H), 7.12 (1H, d, J=2 Hz, 8-H), 7.72 (1H, d, J=8 Hz, 5-H), 8.75 (1H, t, J=6 Hz, amide). *Anal.* Calcd for $C_{12}H_{10}N_4O_4$: C, 52.58; H, 3.68; N, 20.43. Found: C, 52.42; H, 3.55; N, 20.51.

5-(7-Azidocoumarin-4-carboxamido)pentanol (6d)——Preparation of **6d** was carried out essentially as described for **6c**, except for the use of 5-aminopentanol instead of ethanolamine. **6d** was obtained as pale yellow needles of mp 135—136 °C (dec.) by recrystallization from acetonitrile; 64%. IR ν_{max}^{Nujol} cm^{-1}: 2120 (azide), 1730 (lactone), 1650 (amide). NMR (DMSO-d_6) δ: 1.35 (6H, m, $NHCH_2C\underline{H}_2C\underline{H}_2C\underline{H}_2CH_2O$), 3.21 (4H, m, $NHC\underline{H}_2CH_2CH_2CH_2C\underline{H}_2O$), 4.22 (1H, t, J=8 Hz, OH), 6.35 (1H, s, 3-H), 7.00 (1H, dd, J=8, 2 Hz, 6-H), 7.10 (1H, s, 8-H), 7.65 (1H, d, J=8 Hz, 5-H), 8.60 (1H, t, J=6 Hz, amide). *Anal.* Calcd for $C_{15}H_{16}N_4O_4$: C, 56.96; H, 5.10; N, 17.71. Found: C, 56.86; H, 5.07; N, 17.65.

***N*-[2-(7-Azidocoumarin-4-carboxamido)ethoxycarbonylmethyl]maleimide (7c)**——Triethylamine (220 mg, 2.1 mmol) and 4-(*N*, *N*-dimethylamino)pyridine (26 mg, 0.21 mmol) were added to a solution of **6c** (590 mg, 2.1 mmol) in ab. THF (26 ml). A solution of maleoylglycyl chloride (**10**)[12] (375 mg, 2.1 mmol) in THF (13 ml) was then added dropwise at 0 °C. The mixture was stirred at 0 °C for 2 h, then at room temperature for 2 h. After the precipitate had been removed by filtration and the filtrate evaporated, the residue was applied immediately to a column (silica gel). **7c** was obtained as colorless crystals of mp 159 °C (dec.) by recrystallization from acetone–petroleum ether; 196 mg, 22%. IR ν_{max}^{KBr} cm^{-1}: 2120 (azide), 1710 (imide, lactone, ester), 1650 (amide). NMR (DMSO-d_6) δ: 3.55 (2H, q, J=6 Hz, $NHC\underline{H}_2CH_2O$), 4.27 (2H, t, J=6 Hz, $NHCH_2C\underline{H}_2O$), 4.29 (2H, s, $COCH_2N$), 6.48 (1H, s, 3-H), 7.13 (2H, s, maleimide), 7.17 (1H, dd, J=9, 2 Hz, 6-H), 7.24 (1H, d, J=2 Hz, 8-H), 7.76 (1H, d, J=9 Hz, 5-H), 9.05 (1H, t, J=6 Hz, amide). *Anal.* Calcd for $C_{18}H_{13}N_5O_7$: C, 52.56; H, 3.19; N, 17.03. Found: C, 52.70; H, 3.22; N, 16.91.

***N*-[5-(7-Azidocoumarin-4-carboxamido)-pentyloxycarbonylmethyl]maleimide (7d)**——Preparation of **7d** was carried out essentially as described for **7c**, except for the use of **6d** instead of **6c**. **7d** was obtained as colorless crystals of mp 99.5 °C (dec.) by recrystallization from acetone–petroleum ether; 29%. IR ν_{max}^{KBr} cm^{-1}: 2120 (azide), 1710 (imide, lactone, ester), 1645 (amide). NMR (DMSO-d_6): 1.50 (6H, m, $NHCH_2C\underline{H}_2C\underline{H}_2C\underline{H}_2CH_2O$), 3.28 (2H, q, J=6 Hz, $NHC\underline{H}_2CH_2$), 4.10 (2H, t, J=6 Hz, $CH_2C\underline{H}_2O$), 4.26 (2H, s, $COCH_2N$), 6.49 (1H, s, 3-H), 7.15 (2H, s, maleimide), 7.16 (1H, dd, J=8, 2 Hz, 6-H), 7.23 (1H, d, J=2 Hz, 8-H), 7.75 (1H, d, J=8 Hz, 5-H), 8.88 (1H, t, J=6 Hz, amide). *Anal.* Calcd for $C_{21}H_{19}N_5O_7$: C, 55.63; H, 4.22; N, 15.45. Found: C, 55.82; H, 4.35; N, 15.21.

Ethyl 7-Ethoxycarbonylaminocoumarin-4-acetate (3e)——Diethyl acetonedicarboxylate (33.3 g, 165 mmol) and

m-ethoxycarbonylaminophenol (25.0 g, 138 mmol) were added to 75% sulfuric acid (101 ml). The mixture was stirred for 3 h at 25 °C, then poured into ice-water (300 ml) and the precipitate was collected by filtration. The cake was washed with 2% sodium carbonate (600 ml), then washed with water until the washings were neutral, and dried *in vacuo*. The residual solid was recrystallized from acetonitrile to give colorless needles of mp 175—176 °C, 18.1 g, 41%. IR ν_{max}^{Nujol} cm^{-1}: 3300 (NH), 1710 (lactone, urethane, ester). NMR (DMSO-d_6) δ: 1.33 (3H, t, J=8 Hz, $OCH_2C\underline{H}_3$), 1.99 (3H, t, J=8 Hz, $OCH_2C\underline{H}_3$), 3.94 (2H, s, CH_2CO), 4.18 (4H, m, $OC\underline{H}_2CH_3$), 6.32 (1H, s, 3-H), 7.48 (3H, m, 5, 6, 8-H), 10.00 (1H, s, NH). *Anal.* Calcd for $C_{16}H_{17}NO_6$: C, 60.18; H, 5.37; N, 4.39. Found: C, 60.11; H, 5.34; N, 4.43.

7-Aminocoumarin-4-acetic Acid (4f)——Conc. sulfuric acid (17 ml) was added to a suspension of **3e** (17.0 g, 53.3 mmol) in glacial acetic acid (17 ml), and the mixture was refluxed for 5 h at 120 °C. After cooling, the mixture was poured into ice-water (170 ml). The pH of the solution was adjusted to 2 and the mixture of **4f** and **4e** was collected by filtration. Isolation of **4f** from **4e** was carried out by extraction with satd. $NaHCO_3$ aq., and **4f** was obtained as pale yellow leaflets of mp 224—225.5 °C (dec.) from ethanol; 6.05 g, 51.8%. IR ν_{max}^{Nujol} cm^{-1}: 3480 and 3380 (NH), 1720 (lactone), 1660. NMR (DMSO-d_6): 3.70 (2H, s, CH_2CO), 5.92 (1H, s, 3-H), 6.40 (1H, d, J=2 Hz, 8-H), 6.50 (1H, dd, J=9, 2 Hz, 6-H), 7.30 (1H, d, J=9 Hz, 5-H). *Anal.* Calcd for $C_{11}H_9NO_4$: C, 60.27; H, 4.14; N, 6.39. Found: C, 60.12; H, 4.18; N, 6.40. **4e** was obtained as orange needles of mp 143—145 °C (dec.) from ethanol; 1.60 g, 12.2%. IR ν_{max}^{Nujol} cm^{-1}: 1720 (lactone, ester), 1685, 1655. NMR (DMSO-d_6) δ: 1.15 (3H, t, J=7 Hz, $OCH_2C\underline{H}_3$), 3.75 (2H, s, CH_2CO), 4.02 (2H, q, J=7 Hz, $OC\underline{H}_2CH_3$), 5.89 (1H, s, 3-H), 5.95 (2H, br s, NH_2), 6.36 (1H, d, J=3 Hz, 8-H), 6.45 (1H, dd, J=9, 3 Hz, 6-H), 7.20 (1H, d, J=9 Hz, 5-H). *Anal.* Calcd for $C_{13}H_{13}NO_4$: C, 63.15; H, 5.30; N, 5.67. Found: C, 63.15; H, 5.12; N, 5.59.

7-Azidocoumarin-4-acetic Acid (5f)——Preparation of (**5f**) was carried out as described for **5b**, except for the use of **4f** instead of **4b**. **5f** was obtained as pale yellow needles of mp 172.5 °C (dec.) from ethanol; 65%. IR ν_{max}^{Nujol} cm^{-1}: 2110 and 2090 (azide), 1720 (lactone), 1690 (carboxylic acid). NMR (DMSO-d_6) δ: 4.83 (2H, s, CH_2CO), 6.35 (1H, s, 3-H), 7.00 (1H, dd, J=9, 2 Hz, 6-H), 7.06 (1H, d, J=2 Hz, 8-H), 7.65 (1H, d, J=9 Hz, 5-H). *Anal.* Calcd. for $C_{11}H_7N_3O_4$: C, 53.88; H, 2.88; N, 17.14. Found: C, 53.96; H, 2.87; N, 17.13.

5-(7-Azidocoumarin-4-acetamido)pentanol (6g)——In the dark, a solution of 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate (482 mg, 1.14 mmol) and 1-hydroxybenzotriazole (308 mg, 2.28 mmol) in dist. acetonitrile (20 ml) was added to a solution of **5f** (280 mg, 1.14 mmol) and 5-aminopentanol (117 mg, 1.14 mmol) in dist acetonitrile (35 ml) at 0 °C. After being stirred for 1 h at 0 °C, the mixture was stirred overnight at room temperature. The solution was evaporated and the residue was dissolved in ethyl acetate. The organic solution was washed with water, 0.5 M citric acid solution, water, sat. sodium bicarbonate solution, and sat. sodium chloride solution. The organic solution was dried over anhyd. magnesium sulfate, and the solvent was removed by evaporation. **6g** was obtained as pale yellow needles of mp 157.5—159.5 °C (dec.) from dioxane; 163 mg, 43%. IR ν_{max}^{Nujol} cm^{-1}: 3270 (NH), 2120 and 2100 (azide), 1730 (lactone), 1640 (amide). NMR (DMSO-d_6) δ: 1.30 (6H, m, $NHCH_2C\underline{H}_2C\underline{H}_2C\underline{H}_2CH_2O$), 3.04 (2H, m, $NHC\underline{H}_2CH_2$), 3.26 (2H, m, $CH_2C\underline{H}_2O$), 3.86 (2H, s, CH_2CO), 6.32 (1H, s, 3-H), 7.00 (1H, dd, J=9, 2 Hz, 6-H), 7.10 (1H, d, J=2 Hz, 8-H), 7.71 (1H, d, J=9 Hz, 5-H), 8.10 (1H, t, J=8 Hz, amide).

***N*-[5-(7-Azidocoumarin-4-acetamido)pentyloxycarbonylmethyl]maleimide (7g)**——Triethylamine (86 mg, 0.85 mmol) and 4-(*N*, *N*-dimethylamino)pyridine (10 mg, 0.0085 mmol) were added to a stirred solution of **6g** (280 mg, 0.85 mmol) in ab. THF (50 ml). A solution of maleoylglycyl chloride[9] (147 mg, 0.85 mmol) in ab. THF (5 ml) was added dropwise to the above solution at −40 °C. The mixture was allowed to warm gradually to room temperature over 3 h, and was stirred at room temperature for 2 h. The precipitate was removed by filtration, and the filtrate was evaporated at room temperature. The residue was passed through a silica gel column (15 g) with a mixture of ethyl acetate with benzene (1 : 1). **7g** was obtained as colorless crystals of mp 134.5 °C (dec.) from acetone–*n*-pentane; 138 mg, 34%. IR ν_{max}^{KBr} cm^{-1}: 2120 (azide), 1720 (lactone, imide, ester), 1635 (amide). NMR (DMSO-d_6) δ: 1.36 (6H, m $NHCH_2C\underline{H}_2C\underline{H}_2C\underline{H}_2CH_2O$), 3.06 (2H, q, J=5 Hz, $NHC\underline{H}_2CH_2$), 3.69 (2H, s, CH_2CO), 4.05 (2H, t, J=6 Hz, $CH_2C\underline{H}_2O$), 4.25 (2H, s, $COCH_2N$), 6.37 (1H, s, 3-H), 7.14 (2H, s, maleimide), 7.15 (2H, m, 6-H, 8-H), 7.77 (1H, d, J=8 Hz, 5-H), 8.18 (1H, t, J=5 Hz, amide). *Anal.* Calcd for $C_{22}H_{21}N_5O_7$: C, 56.53; H, 4.53; N, 14.98. Found: C, 56.58; H, 4.60; N, 14.86.

7-Aminocoumarin-4-carboxylic Acid Methylamide (8a)——A solution of **4a** (167 mg, 0.71 mmol) in dist. ethanol (50 ml) was saturated with a monomethylamine at 0 °C in a sealed tube. The solution was stored for three days at room temperature, then excess methylamine and ehtanol were removed by evaporation. **8a** was obtained as yellow needles of mp 295—296 °C (dec.) from methanol; 53 mg, 35%. IR ν_{max}^{Nujol} cm^{-1}: 3430 and 3340 (NH), 1700 (lactone), 1640 (amide). NMR (DMSO-d_6) δ: 2.76 (3H, d, J=5 Hz, $NHCH_3$), 5.98 (1H, s, 3-H), 6.30 (2H, br s, NH_2), 6.42 (1H, d, J=2 Hz, 8-H), 6.54 (1H, dd, J=9, 2 Hz, 6-H), 7.36 (1H, d, J=9 Hz, 5-H), 8.67 (1H, q, J=5 Hz, amide). UV λ_{max}^{EtOH} nm (ε): 373 (1.26×10^4). *Anal.* Calcd for $C_{11}H_{10}N_2O_3$: C, 60.54; H, 4.62; N, 12.84. Found: C, 60.33; H, 4.72; N, 12.88.

Ethyl 7-Azidocoumarin-4-carboxylate (9a)——Preparation of **9a** was carried out as described for **5b**, except for the use of **4a** (1.17 g, 5.0 mmol) instead of **4b**. **9a** was obtained as pale yellow plates of mp 98.5—99.5 °C (dec.) from ethanol; 880 mg, 68%. IR ν_{max}^{Nujol} cm^{-1}: 2120 and 2100 (azide), 1710 (lactone, ester). UV λ_{max}^{EtOH} nm (ε): 341 (1.38×10^4). NMR (DMSO-d_6) δ: 1.34 (3H, t, J=7 Hz, $OCH_2C\underline{H}_3$), 4.36 (2H, q, J=7 Hz, $OC\underline{H}_2CH_3$), 6.69 (1H, s, 3-H), 7.00

(1H, dd, J=9, 2 Hz, 6-H), 7.04 (1H, d, J=2 Hz, 8-H), 8.00 (1H, d, J=9 Hz, 5-H). *Anal.* Calcd for $C_{12}H_9N_3O_4$: C, 55.60; H, 3.50; N, 16.21. Found: C, 55.79; H, 3.44; N, 16.07.

Ethyl 7-Azidocoumarin-4-acetate (9e)——Preparation of **9e** was carried out as described for **5b**, except for the use of **4e** (1.24 g, 5.0 mmol) instead of **4b**. **9e** was obtained as pale yellow needles of mp 127.5—128.5 °C (dec.) from ethanol; 915 mg, 67%. IR ν_{max}^{Nujol} cm^{-1}: 2120 and 2100 (azide), 1720 (lactone, ester). UV λ_{max}^{EtOH} nm (ε): 328.5 (1.81×10^4). NMR (DMSO-d_6) δ: 1.20 (3H, t, J=7 Hz, $OCH_2\underline{CH_3}$), 3.95 (2H, s, CH_2CO), 4.12 (2H, q, J=7 Hz, $OC\underline{H_2}CH_3$), 6.38 (1H, s, 3-H), 7.02 (1H, dd, J=9, 2 Hz, 6-H), 7.08 (1H, d, J=2 Hz, 8-H), 7.62 (1H, d, J=9 Hz, 5-H). *Anal.* Calcd for $C_{13}H_{11}N_3O_4$: C, 57.14; H, 4.06; N, 15.38. Found: C, 57.31; H, 3.97; N, 15.19.

7-Azido-4-methylcoumarin (14)——A stirred suspension of 175 mg (1.0 mmol) of 7-amino-4-methylcoumarin **13**[13] and 0.4 ml (7 mmol) of conc. H_2SO_4 in 1 ml AcOH was treated with 76 mg (1.1 mmol) of $NaNO_2$ in 0.5 ml of H_2O under cooling with ice-water. The mixture was stirred for 3 min, then 25 mg (0.4 mmol) of NaN_3 in 0.5 ml water was added dropwise, and the whole was stirred under cooling for a further 2 h. Then 2 ml of cold water was added. The precipitate was collected by filtration with suction, washed with cold-water until neutral, and dried *in vacuo*. The crude product was passed through a short column of silica gel (4 g) and the CH_2Cl_2 eluate was monitored by thin-layer chromatography (TLC). Recrystallization of the product from CH_2Cl_2–*n*-hexane gave yellow needles, 160 mg (80% yeild), mp 116—118 °C. IR ν_{max}^{Nujol} cm^{-1}: 2120 (N_3), 1720 (C=O).NMR ($CDCl_3$) δ: 2.42 (3H, s, C_4–CH_3), 6.22 (1H, s, C_3–H), 6.93 (1H, d, J=2 Hz, C_8–H), 6.95 (1H, dd, J=9, 2 Hz, C_6–H), 7.60 (1H, d, J=9 Hz, C_5–H). Mass spectra (MS) m/z: 201 (M^+), 173 (base). *Anal.* Calcd for $C_{10}H_7N_3O_2$: C, 59.70; H, 3.51; N, 20.89. Found: C, 59.93; H, 3.44; N, 20.78.

Photolysis of 14——(i) In Methanol: A solution of **14** (201 mg, 1.0 mmol) in MeOH (50 ml) was added through a Microfeeder to 450 ml of MeOH, under irradiation with a 100 W high pressure Hg arc through a Pyrex filter in a nitrogen atmosphere. The reaction mixture was concentrated *in vacuo* and the residure was purified by silica gel PLC (20 × 20 cm) with CH_2Cl_2–AcOEt (5 : 1). Recrystallization of the product from EtOH gave 8 mg (5%) of **13** (mp 215—216 °C), which was identical with an authentic sample on the basis of mixed mp determination and IR comparison.

(ii) In Cyclohexane: A solution of **14** (201 mg, 1.0 mmol) in 50 ml of EtOH was added to 450 ml of cyclohexane under irradiation as described above, and the mixture was worked up similarly. Recrystallization of the product from EtOH gave 24 mg (14%) of **13**.

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References and Notes

1) Organic Fluorescent Reagents. X. For Part IX, see: E. Sato, M. Miyakawa and Y. Kanaoka, *Chem. Pharm. Bull.*, **32**, 336 (1984).
2) K. Peters and F. M. Richards, *Ann. Rev. Biochem.*, **46**, 523 (1977).
3) *a*) H. Fasold, J. Klappenberger, C. Meyer and H. Renold, *Angew. Chem. Int. Ed. Engl.*, **10**, 795 (1971); *b*) F. Wold, *Methods Enzymol.*, **25**, 623 (1972); *c*) T. H. Ji, *Biochim. Biophys. Acta*, **559**, 39 (1979).
4) *a*) H. Bayley, J. R. Knowles, *Methods Enzymol.*, **46**, 69 (1977); *b*) H. Nakayama, M. Nozawa and Y. Kanaoka, *Chem. Pharm. Bull.*, **27**, 2775 (1979).
5) *a*) Y. Kanaoka, *Angew. Chem. Int. Ed. Engl.*, **16**, 137 (1977); *b*) Y. Kanaoka and T. Sekine, *Chemistry and Biology*, **21**, 233 (1983).
6) Y. Kanaoka, *Yakugaku Zasshi*, **100**, 973 (1980).
7) *a*) A. Reiser and R. Marley, *Trans. Faraday Soc.*, **64**, 1806 (1968); *b*) G. Dreyfuss, K. Schwartz, E. R. Blout, J. R. Barrio, F-T. Liu and N. J. Leonard, *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 1199 (1978).
8) E. F. V. Scriven, "Reactive Intermediates," Vol. 2, ed. by R. A. Abramovitch, Plenum Press, New York, 1982, pp. 1—54.
9) C. P. Parker and W. T. Rees, *Analyst*, **85**, 587 (1960).
10) *a*) G. M. Edelman and W. O. McClure, *Acc. Chem. Res.*, **1**, 65 (1968); *b*) W. O. McClure and G. M. Edelman, *Biochemistry*, **5**, 1908 (1966).
11) G. Dreyfuss, K. Schwartz, E. R. Blout, J. Barrio, F.-T. Liu and N. J. Leonard, *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 1199 (1978).
12) L. Paul, A. Dittmer and C. Rusch, *Chem. Ber.*, **100**, 2757 (1967).
13) T. Sekine, H. Itakura, T. Namihisa, T. Takahashi, H. Nakayama and Y. Kanaoka, *Chem. Pharm. Bull.*, **29**, 3286 (1981).