Chem. Pharm. Bull. 27(11)2775—2780(1979)

UDC 547.583.7.04 : 541.14.04

Photo-Affinity Labeling. II.[1] Photolysis of Amido Derivatives of Nitrophenyl Azides[2]

Hitoshi Nakayama, Masayuki Nozawa, and Yuichi Kanaoka

Faculty of Pharmaceutical Sciences, Hokkaido University[3]

(Received May 16, 1979)

Photolysis of some amido derivatives of nitrophenyl azides was studied. In most cases, the products isolated were azo compounds and primary amines formed *via* triplet nitrenes. However, an anthranil, an intramolecular insertion product of nitrene into the adjacent amido group, was a major product in the case of *o*-amidonitrophenyl azide. A quenching study using isoprene showed that anthranil is formed from a singlet nitrene. The reaction modes of the nitrophenyl azides and the applicability of the compounds as potential photoaffinity labels are discussed.

Keywords——photo-affinity; nitrophenyl azides; nitrene; insertion; anthranil; multiplicity

Affinity labels have found widespread application for covalently marking the active sites of enzymes and the binding sites of proteins.[4] In addition, their potential usefulness in the isolation and identification of biochemically important receptor-site macromolecules has been recognized.[5,6] Although Westheimer *et al.* first reported the use of photolytically generated carbenes to probe the structure of the active site of chymotrypsin in 1962,[7] it is only recently that photo-affinity labeling has begun to find substantial applications in biological studies.[8,9] The use of photogenerated reagents for labeling biological active sites has two main advantages over conventional reagents for the chemical modification of biopolymers.[8,9] First, photo-affinity probes are inert until photolysis, permitting control experiments to be done before photolysis to ensure labeling of the desired active site. Second, many photo-affinity probes can insert themselves into carbon-hydrogen bonds and, therefore, do not require the presence of particular reactive functional groups at the binding site. We have been investigating photo-affinity labeling[1] in an attempt to make this technique more general and to determine what factors must be taken into account in using the method. We report here some of our initial studies in this area, dealing with the photolysis of some amido derivatives of nitrophenyl azides as model compounds of photosensitive groups.

The chemistry of aryl nitrenes[10] and the photochemistry of azido compounds[11] have recently been reviewed. In particular, the problem of aryl azide photoaffinity probes has been discussed by Knowles.[8,9] Aryl azides are stable in the absence of light, but give highly reactive nitrene on photolysis. In general, nitrenes are more selective than carbenes, the

1) Part I: H. Nakayama and Y. Kanaoka, *FEBS Letters*, **37**, 200 (1973).
2) Photoinduced Reactions. XL. Part XXXIX: Y. Kanaoka, H. Okajima, and Y. Hatanaka, *J. Org. Chem.*, **44**, 1749 (1979).
3) Location: *Kita 12, Nishi 6, Kita-ku, Sapporo 060, Japan.*
4) For example, see: B.R. Baker, "Design of Active-Site Directed Irreversible Enzyme Inhibitors," J. Wiley, New York, 1967.
5) S.J. Singer, *Advan. Protein Chem.*, **22**, 1 (1967).
6) E. Shaw, *Physiol. Rev.*, **50**, 244 (1970).
7) *a*) A. Singh, E.R. Thornton, and F.H. Westheimer, *J. Biol. Chem.*, **237**, PC3006 (1962); *b*) J. Shafer, P. Baronowsky, R. Laursen, F. Finn, and F.H. Westheimer, *ibid.*, **241**, 421 (1966).
8) J.R. Knowles, *Accounts Chem. Res.*, **5**, 155 (1972).
9) For a recent leading review see: H. Bayley and J.R. Knowles, in "The Enzymes," Vol. 46, ed. by W.B. Jakoby and M. Wilchek, Academic Press, New York, 1977, p. 69.

NII-Electronic Library Service

photogenerated species initially used by Westheimer *et al.*[7] If a nitrene is generated *in situ* at a binding locus, direct insertion, abstraction-coupling or addition reactions will then result in covalent attachment of the label to the site. As representative examples, aryl azides containing a nitro group have recently successfully employed for the labeling of antibodies[12] and red cell membranes.[13] The nitro group was, according to Knowles,[8,9] selected for two reasons. First, the substitution shifts λ_{max} into the visible region, away from the ultraviolet absorption of proteins. Second, a nitro group will increase the reactivity of the nitrene. In view of this work, it is rather surprising that there is so far little information on the chemistry of the photo-products of these nitrophenyl azides,[14] and no chemical studies have been reported on the nature of the photoreactions involved in these biological applications.[15]

p-Nitrophenyl azide **3a** was selected first as the simplest model. In our original design of site-specific photo-labeling reagents,[15b] the moieties **1** and **2**, which contain nitro, azido and carbonyl groups, were included in amino acid or peptide substrates as photo-sensitive precursor groups. The Y groups in **1** and **2** must be suitably site-specific for the target enzymes. In the present work the amides (**3b** and **3c**) were selected as model compounds, since reagents designed for proteolytic enzymes such as chymotrypsin should contain appropriate amino acids or peptides in which the Y groups are therefore suitably substituted amino groups.[15] Compounds **3b** and **3c** were synthesized by the usual methods from the corresponding acids, **1a** and **2a**, *via* the azides, **1b** and **2b**, respectively (Chart 1).

Experimental[16]

4-Nitrophenyl Azide (**3a**)——Pale yellow needles from EtOH, 75%, mp 71—72° (dec.) (lit.,[17] mp 74°).

5-Azido-2-nitrobenzoic Acid (**1b**)——Reddish-yellow needles from benzene, 70%, mp 164.5—166° (dec.) (lit.,[15a] mp 165—166°).

2-Azido-4-nitrobenzoic Acid (**2b**)——Pale yellow plates from benzene, 65%, mp 148—150° (dec.). IR ν_{max}^{Nujol} cm^{-1}: 2160 (N_3). *Anal.* Calcd. for $C_7H_4O_4$: C, 40.39; H, 1.94; N, 26.92. Found: C, 40.47; H, 1.93; N, 27.01.

5-Azido-2-nitrobenzamide (**3b**)——**1b** (3.0 g) was converted into the acid chloride with PCl_5 (5.0 g) in ether as usual, followed by treatment in an NH_3-saturated solution of tetrahydrofuran to give the amide (**3b**), 47%: yellow needles from EtOH, mp 169—169.5° (dec.). IR ν_{max}^{Nujol} cm^{-1}: 3480—3220 (NH), 2180 (N_3), 1670 ($CONH_2$). *Anal.* Calcd. for $C_7H_5N_5O_3$: C, 40.58; H, 2.43; N, 33.81. Found: C, 40.84; H, 2.41; N, 33.72.

2-Azido-4-nitrobenzamide (**3c**)——**2b** (3.0 g) was converted into the amido (**3c**) as described above, 40%: pale yellow needles from AcOEt, mp 176—176.5° (dec.). IR ν_{max}^{Nujol} cm^{-1}: 3420—3170 (NH), 2200 (N_3), 1675 (CONH). *Anal.* Calcd. for $C_7H_5N_5O_3$: C, 40.58; H, 2.43; N, 33.81. Found: C, 40.41; H, 2.29; N, 33.84.

Photolysis of 3a——(i) In Benzene: A solution of **3a** (1.33 g) in benzene (400 ml) was irradiated for 6 hr in an atmosphere of N_2. The reaction mixture was concentrated *in vacuo* and the residue was chromatographed on alumina. Elution with benzene–ether (10: 1 v/v) yielded the strating material (65%) and 4,4′-dinitroazobenzene (**4a**: 304 mg, 28%). **4a**: mp 225—227° (dec.). (lit.,[18] mp 222—223°).

(ii) In Methanol: **3a** was irradiated as described above for 4 hr. The reaction residue was dissolved

10) P.A. Smith in "Nitrenes," ed. by W. Lwowski, Interscience Publishers, New York, 1970, p. 99.
11) A. Reiser and H.M. Wagner, "The Chemistry of the Azido Group," ed. S. Patai, Interscience Publishers, London, New York, 1971, p. 441; R.A. Abramovitch and E.P. Kyba, *ibid.*, p. 221.
12) *a*) G.W.J. Fleet, J.R. Knowles, and R.R. Porter, *Biochem. J.*, **128**, 499 (1972); *b*) R.A.G. Smith and J.R. Knowles, *ibid.*, **141**, 51 (1974).
13) H. Kiefer, J. Lindstrom, E.S. Lennox, and S.J. Singer, *Proc. Natl. Acad. Sci. U.S.*, **67**, 1688 (1970).
14) *a*) L. Hormer, A. Christmann, and A. Gross, *Chem. Ber.*, **96**, 399 (1963); *b*) R.A. Odum and A.M. Aaronson, *J. Am. Chem. Soc.*, **91**, 5689 (1969); *c*) R. Purvis, R.K Smalley, W.A. Strachan, and H. Suschitzky, *J. Chem. Soc., Perkin I.*, **1978**, 191.
15) *a*) R.E. Galarcy, L.C. Craig, J.M. Jamieson, and M.P. Printz, *J. Biol. Chem.*, **249**, 3510 (1974); *b*) H. Nakayama and Y. Kanaoka, *Seikagaku*, **47**, 723 (1975).
16) Melting points are uncorrected. Spectra were measured on a Jasco IR-A-1 spectrophotometer, a Shimadzu UV-200 spectrophotometer, a Hitachi RMU-5E mass spectrometer, and a Hitachi R24A NMR spectrometer, using tetramethylsilane as an internal standard. The photolysis apparatus consisted of an Ushio UM-102 100-watt high-pressure mercury lamp in a water-jacketed Pyrex immersion well. Solutions were flushed with nitrogen for 30 min before photolysis.
17) E. Noelting and O. Michel, *Chem. Ber.*, **26**, 86 (1893).
18) R.A. Abramovitch, S.R. Challand, and E.F.V. Scriben, *J. Org. Chem.*, **37**, 2705 (1972).

NII-Electronic Library Service

in benzene at 45° and undissolved material was removed by filtration. The filtrate was concentrated *in vacuo* and the residue was chromatographed on alumina. Elution with benzene gave **3a** (21%) and **4a** (48%), then elution with benzene–ether (10: 1 5: 1 v/v) gave *p*-nitroaniline (**5a**: 45 mg or 4%), mp 143—145°.

(iii) In CH_3CN in the Presence of N,N-Dimethylaniline (0.2 M): A solution of **3a** (1.33 g) and N,N-dimethylaniline (9.6 g) in CH_3CN (400 ml) was irradiated for 3 hr and worked up as described above. Elution successively with petrol. ether, benzene–ether (10: 1 v/v) and benzene–ether (5: 1), gave N,N-dimethylaniline (816 mg), **3a** (3%) and **4a** (27%), respectively. The subsequent fraction eluted with benzene–ether (2: 1) gave **5a** (15%) after alumina TLC (benzene).

Photolysis of 3b in MeOH——A solution of **3b** (828 mg) in MeOH (400 ml) was irradiated for 90 min. Precipitates were collected by filtration to give 3,3′-dicarbamoyl-4,4′-dinitroazobenzene **4b** (275 mg, 39%), mp>300°. *Anal.* Calcd. for $C_{14}H_{10}N_6O_6$: C, 46.93; H, 2.81; N, 23.46. Found: C, 46.45; H, 2.80; N, 23.31. UV λ_{max}^{MeOH} nm (ε); 262 (20800), 310 (16300); IR ν_{max}^{Nujol} cm^{-1}: 3380 and 3180 (NH), 1660 (C=O), 1625 (C=C), 1520 and 1350 (NO_2); MS *m/e*: 358 (M^+). The filtrate was concentrated *in vacuo* and the residue was purified by TLC (silica gel; benzene: AcOEt: EtOH=3: 3: 1) to give, in addition to **3b** (12%), 5-amino-2-nitrobenzamide **5b** (52 mg, 7%) as yellow needles of mp 217—225° (dec.) from AcOEt. MS *m/e*: 181 (M^+). **5b** was converted by treatment with Ac_2O as usual into an acetamido derivative which formed pale yellow needles of mp 240—242° (dec.) from AcOEt. IR ν_{max}^{Nujol} cm^{-1}: 3480—3220 (NH), 1680 (C=O). The acetamido compound was shown to be identical (mixed mp) with an authentic sample of 5-acetamido-2-nitrobenzamide prepared from **1a** through its acid chloride.

Photolysis of 3c in MeOH——A solution of **3c** (828 mg) in MeOH (400 ml) was irradiated as described for **3b**. On removal of the solvent *in vacuo* (bath temp. 45°) the residue was treated with AcOEt. The undissolved portion was collected and recrystallized from DMF–H_2O to give 2,2′-dicarbamoyl-5,5′-dinitrozaobenzene **4c** as fine pale orange needles of mp 298—301° (dec.), 70 mg, 10%. *Anal.* Calcd. for $C_{14}H_{10}N_6O_6$: C, 46.93; H, 2.81; N, 23.46. Found: C, 46.54; H, 3.01; N, 23.29. UV λ_{max}^{MeOH} nm (ε): 329 (17700). The AcOEt solution was concentrated *in vacuo* and the residue was chromatographed on alumina with ether. The eluate was further purified by TLC (alumina) to yield **3c** (4%), 2-amino-4-nitrobenzamide **5c** (7%), and **6** (15%). **5c** was obtained as pale yellow prisms of mp 221—223° (dec.) from AcOEt (lit.,[19] mp 225°). 3-Amino-6-nitro-2,1-benzisoxazole **6** was obtained as dark red prisms of mp 197—197.5° (dec.) from acetone; 107 mg, 15%. *Anal.* Calcd. for $C_7H_5N_3O_4$: C, 46.93; H, 2.81; N, 23.46. Found: C, 47.31; H, 2.83; N, 23.07. UV λ_{max}^{MeOH} nm (ε): 232 (14500), 272 (12700), 313 (6600), 435 (3100); IR ν_{max}^{Nujol} cm^{-1}: 3380 and 3320 (NH), 1660 (C=N), 1620 (C=C), 1520 and 1340 (NO_2); NMR (DMSO-d_6) δ: 7.15 (1H, dd, J=2 Hz, 10 Hz, 5-H), 7.75 (1H, d, J= 10 Hz, 4-H), 7.90 (1H, d, J=2 Hz, 7-H), 8.60 (2H, s, 3-NH_2); MS *m/e*: 181 (M^++2), 179 (M^+).

2,4-Diacetamidobenzamide (9)——(i) From **6**: A solution of **6** (50 mg) in MeOH (100 ml) was hydrogenated with Pd-charcoal as a catalyst for 7 hr. On removal of the solvent *in vacuo* the residue (8; MS *m/e*: 151 (M^+)) was reacted with Ac_2O as usual to give 2,4-diacetamidobenzamide **9**; colorless needles of mp 263—267° (dec.) from EtOH. IR ν_{max}^{Nujol} cm^{-1}: 3400, 3200 (NH), 1695, 1670 ($CONH_2$). MS *m/e*: 235 (M^+).

(ii) Form **2a**: **2a** was transformed by treatment with Ac_2O as usual into 2-acetamido-4-nitrobenzoic acid; pale yellow needles from AcOEt, mp 214.5—215.5° (dec.) (lit.,[20] mp 215°). This amide (1.0 g) was converted into the corresponding acid chloride with PCl_5 (1.1 g) in tetrahydrofuran (50 ml) as usual, and the chloride was treated with NH_3 in tetrahydrofuran to give 2-acetamido-4-nitrobenzamide; pale yellow needles from EtOH, mp 206—209° (dec.) (lit.,[21] mp 218—223°). A solution of the amide (100 mg) in EtOH (20 ml) was hydrogenated with Pd-charcoal (50 mg) as a catalyst at room temp. 2-Acetamido-4-aminobenzamide was obtained as colorless needles from EtOH, mp 193—196°; IR ν_{max}^{Nujol} cm^{-1}: 3390—3200 (NH), 1680, 1620 (CONH). The amine was acetylated with Ac_2O to give **9**; colorless needles of mp 263—267° (dec.) from EtOH; MS *m/e*: 235 (M^+). This sample was shown to be identical with **9** obtained from **6** by spectral comparison (IR, MS) and mixed mp 263—266° (dec.).

Quenching Studies——A 10 mM solution (10 mg/5 ml) of **3c** in MeOH was irradiated for 15 min in the presence or absence of 0.4 M isoprene. After evaporation to dryness *in vacuo* the residue was dissolved in AcOEt (2 ml) and a small amount of the undissolved portion (azo compound, **4c**) was filtered off. A 5 μl aliquot of the filtrate was applied to a 20×20 cm thin-layer plate (Merck DC-fertigplatten, aluminumoxid 60F 254, type E) and developed with ether. Each well-separated spot (the *Rf* values of **6**, **5c** and **3c** were 0.67, 0.47 and 0.37, respectively) was monitored with a Shimadzu CS-900 chromatoscanner at 260 nm and determined on the basis of the calibration line of a corresponding authentic sample under similar conditions.

Results and Discussion

Photolysis of the nitrophenyl azides gave the azo compound **4** and the primary amine **5** (Chart 1). In the case of *o*-amidonitrophenyl azide **3c**, an anthranil (2,1-benzisoxazole)

19) M. Julia, N. Hurion, and H.D. Tam, *Chem. Ther.*, **5**, 343 (1970).
20) H.L. Wheeler and B. Barnes, *Am. Chem. J.*, **20**, 217 (1898).
21) M.T. Bogert and F. Steiner, *J. Am. Chem. Soc.*, **27**, 1330 (1905).

NII-Electronic Library Service

Table I. Photoproducts Obtained from the Nitrophenyl Azides (3)[a]

Azide	Conditions		Yield (%)		
	Solvent (mM)	Time (hr)	Recovered	4	5
3a	Methanol (20)	4	**3a** (21)	**4a** (48)	**5a** (4)
3a	Benzene (20)	6	**3a** (65)	**4a** (28)	—
3a	Acetonitrile-Dimethylaniline	3	**3a** (3)	**4a** (27)	**5a** (15)
3b	Methanol (10)	1.5	**3b** (12)	**4b** (39)	**5b** (7)
3c[b]	Methanol (10)	1.5	**3c** (4)	**4c** (10)	**5c** (7)

a) A 100 W high-pressure mercury lamp in a Pyrex immersion well was used.
b) In addition to **4c** and **5c**, **6** was isolated in 15% yield.

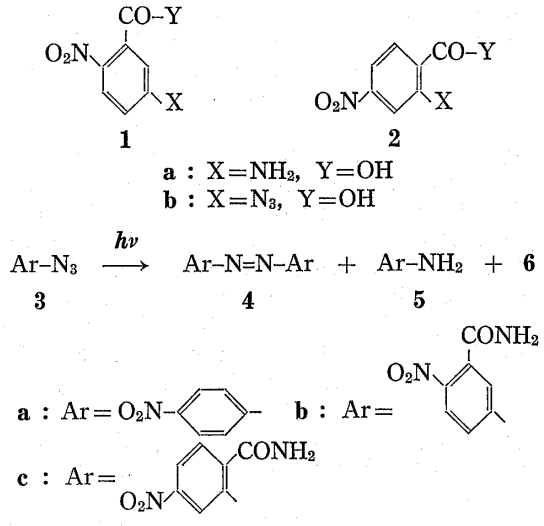

Chart 1

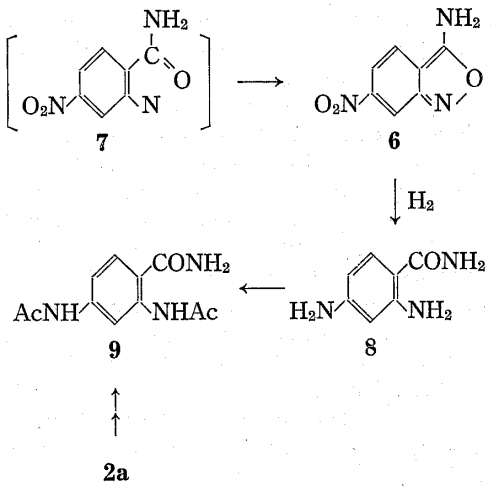

Chart 2

derivative **6** was obtained as a major product in addition to **4c** and **5c**. Except in this case, the azo compound **4** was a major product in all the reactions of nitrophenyl azides with yields of 10—48%. Although the primary amine **5** was not obtained in benzene, in polar solvents such as methanol and N,N-dimethylaniline-acetonitrile, **5** was obtained and its yield increased as the nucleophilicity of the solvent increased. The results of photolyses are given in Table I. The structure of **6** was supported by analytical and spectroscopic data, and confirmed by its chemical reactions. The mass spectrum gave a molecular ion peak (M^+, 179) and also a noticeable (M^++2) ion peak, which suggests the formation of a hydrogen-abstraction product under the conditions of measurement. The aromatic region of the NMR spectrum shows a pattern very similar to that of 6-nitroanthranil previously reported.[22] Compound **6** was reduced with ease to diaminobenzamide **8** followed by acetylation to form diacetamidobenzamide **9**, which was identical with an authentic sample obtained from **2a** (Chart 2).

The reactions generally open to a nitrene are shown in Chart 3.[10,11] For aryl nitrenes, simple intermolecular insertion reactions are rather uncommon. Instead, an internal bond reorganization is dominant, eventually leading to ring-expanded products (azepines) in the presence of nucleophilic trapping agents such as amines.[10,11] The reason for this may be that aryl nitrenes are generally not sufficiently electron deficient to promote typical nitrene reactivity.[23] It should be possible to increase the electrophilic character of the aryl nitrene

22) A-U-Rahman and A.J. Boulton, *Tetrahedron, Supplement*, **7**, 49 (1966).
23) R.A. Abramovitch, "Organic Reactive Intermediates," ed. by S.P. McManus, Academic Press, New York, 1973, p. 126.

NII-Electronic Library Service

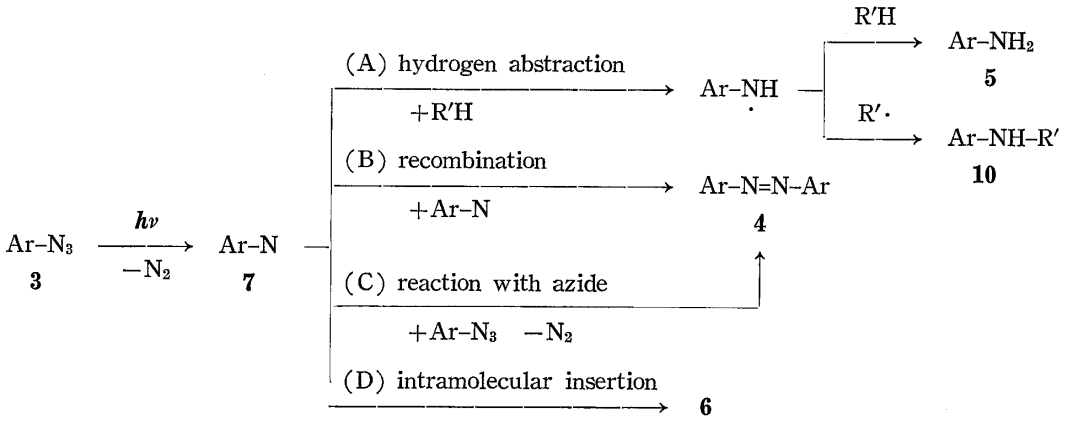

Chart 3

by the introduction of electron-withdrawing substituents such as a nitro group into the aromatic ring, leading to more facile intermolecular substitution. Abramovitch *et al.*, indeed, have confirmed this by thermolysis of aryl azides containing a nitro substituent.[18]

In the photolysis of nitrophenyl azides, however, neither intermolecular substitution nor the formation of azepines was observed. Photolytic products isolated were the azo compound **4** and primary amine **5**, *via* triplet nitrenes, except for the anthranil derivative **6**. The contrast between the modes of reaction in thermolysis and photolysis of nitrophenyl azides suggests a marked difference in the nature of the intermediate nitrenes formed under the two reaction conditions. In the thermolysis of azides, singlet nitrenes are generated predominantly and subsequently drop to the triplet nitrene ground state.[23] Introduction of a nitro group into the aromatic ring can increase the electrophilic character of the singlet aryl nitrene.[18] In general, singlet aryl azides excited by irradiation may be converted in part into triplet azides by an intersystem crossing process (pathway a), and then both of the azides generate nitrenes (pathways b and c) (Chart 4). The introduced nitro group apparently promotes the intersystem crossing of singlet to triplet azides (pathway d), and consequently increases the fraction of triplet nitrenes.[14c] The finding that most of the photolytic products isolated were from triplet nitrenes in this work can be explained in terms of the multiplicities of the intermediate nitrenes, as summarized in Chart 4.

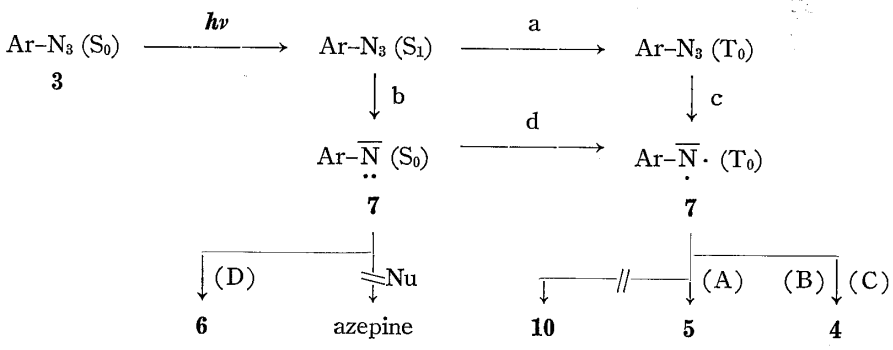

Chart 4

The isolation of an anthranil derivative **6** on photolysis of **3c** is interesting, since as far as we know, there is only one report on the photochemical formation of anthranils; *i.e.*, 3-phenylanthranil from 2-azidobenzophenone.[24] However, no information is available about the multiplicity of the nitrene involved. A quenching study of the formation of **6** using isoprene as a triplet quencher was therefore undertaken; the results of irradiation of **3c** in

24) P.A.S. Smith, B.B. Brown, R.K. Putney, and R.F. Reinish, *J. Am. Chem. Soc.*, **75**, 6355 (1953).

NII-Electronic Library Service

Table II. Photolysis of 2-Azido-4-nitrobenzamide (**3c**) in the Presence and Absence of Isoprene[a)]

Concentration of isoprene (M)	Yield (%)[b)]		
	Recovered	**6**	**5c**
0	25	21	14
0.2	27	38	9

a) A 10 mM solution of **3c** was photolyzed for 15 min with a 100 W high-pressure mercury lamp in a Pyrex immersion well.
b) Azo compound **4c** was not determined in this experiment.

the absence and presence of isoprene are shown in Table II. A significant increase in the formation of **6** and a slight decrease in that of **5** observed on addition of the quencher indicate that **6** is formed from a singlet nitrene **7** (Chart 2, 4).

Aryl nitrenes with adjacent sites of unsaturation are capable of cyclization and internal bond reorganization.[10,11)] Thus aryl azides which have an appropriate substituent *ortho* to the azide group may undergo intramolecular reactions *via* singlet nitrenes. In fact, in photolyses of *o*-azidobenzoic acid derivatives, the formation of azepines has been reported.[14c,25)] In contrast, 2-carbamoylphenyl azide containing a nitro substituent **3c** gave no azepine, but instead formed anthranil **6** by cyclization with the neighboring amido carbonyl. The introduction of a nitro group, an electron-withdrawing substituent, probably prevents azepine formation by decreasing the electron density on the aromatic ring, at which the electrophilic nitrene attacks, while increasing the electrophilicity of the singlet nitrene renders attack on the adjacent amido oxygen more likely.

In the present work, we did not isolate any intermolecular insertion products. While direct intermolecular insertion reactions of aryl nitrenes are rare, insertion products are common in intramolecular reactions. The observed formation of anthranil by intramolecular insertion into the amide bond may indicate that the nitrophenyl nitrenes react intramolecularly with peptide amide bonds in close proximity in proteins. If a nitrene is generated *in situ* at a binding site of a biological macromolecule, such an "intramolecular" reaction with a neighboring position might occur efficiently. We have preliminarily observed the photolabeling of the binding site of chymotrypsin employing these photosensitive groups,[15b)] while Craig *et al.* reported the labeling of peptide hormone binding sites with some aryl azides containing a nitro group.[15a)] Studies of further applications of reagents containing these photosensitive groups to biological macromolecules are now in progress.

Acknowledgement We wish to thank the Japan Society for the Promotion of Science and the Foundation for the Promotion of Research on Medicinal Resources for financial support.

25) a) M.F.G. Stevens, A.C. Mair, and J. Reisch, *Photochem. Photobiol.*, **13**, 441 (1971); b) A.C. Mair and M.F.G. Stevens, *J. Chem. Soc.* (*C*), **1971**, 2317.

NII-Electronic Library Service