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Photocross-Linking of Nucleic Acids to Associated Proteins

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ABSTRACT: Photocross-linking is a useful technique for the partial definition of the nucleic acid-protein interface of nucleoprotein complexes. It can be accomplished by one or two photon excitations of wild-type nucleoprotein complexes or by one photon excitation of nucleoprotein complexes bearing one or more substitutions with photoreactive chromophores. Chromophores that have been incorporated into nucleic acids for this purpose include aryl azides, 5-azidouracil, 8-azidoadenine, 8-azidoguanine, 4-thiouracil, 5-bromouracil, 5-iodouracil, and 5-iodocytosine. The various techniques and chromophores are described and compared, with attention to the photochemical mechanism.

KEY WORDS: azide, aryl azide, 8-azidoadenosine, 8-azidoguanosine, 5-azidouridine, 5-bromo-2′-deoxyuridine, 5-bromouracil, 5-bromouridine, 5-iodocytidine, 5-iodo-2′-deoxyuridine, 5-iodouracil, 5-iodouridine, nucleoprotein, photocrosslinking, 4-thiouracil, 4-thiouridine, 4-thiothymidine, two-photon excitation.

I. INTRODUCTION

For many years researchers have utilized photocross-linking for investigating specific interactions between nucleic acids and proteins. This technique involves the formation of a covalent bond(s) between the nucleic acid and protein resulting from UV excitation of a photoreactive group in the nucleoprotein complex. This can be accomplished by irradiating the wild-type complex or by irradiating a photoreactive group incorporated into the nucleic acid or protein. Identification of the covalent bond(s) that forms helps to define the interface between the nucleic acid and the protein, providing a better understanding of the interactions that occur in the nucleoprotein complex. Even though the chromophore can exist in either the nucleic acid or the protein, this review focuses mainly on chromophores in the nucleic acid.

Obtaining a significant photocross-linking yield (>10%) is important for ensuring that the cross-linking relates to a specific nucleoprotein interaction and for providing adequate material for characterization. Knowing the photochemical properties of the cross-linking chromophore, such as the absorption characteristics and cross-linking mechanism, should improve the experimental design and consequently the cross-linking yield. To illustrate variability, UV spec-

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tra of several popular chromophores are compared in Figures 1, 2, and 3. An understanding of the cross-linking mechanism may even allow prediction of the orientation of the cross-linking groups within the nucleoprotein complex. For example, with halogenated pyrimidines as photoreactive sites, a correlation of crystal structural data with cross-linking yields using the halogenated pyrimidines suggests that high yields occur when the chromophore of the nucleic acid exists in a π-stacking arrangement with the chromophore in the protein (Valegård et al., 1994; Meisenheimer et al., 1996; Oubridge et al., 1994; Stump and Hall, 1995).

This review provides general information regarding several popular chromophores that are used to photocross-link nucleic acids to proteins. The chromophores discussed include, nonsubstituted nucleic acids, 4-thiouracil, various aryl azides and azido nucleosides, and halogenated pyrimidines. Brief descriptions of each chromophore explaining its use, mechanism, selectivity, where to purchase or find information on its synthesis, and the advantages and disadvantages follow. In addition, a table of general information on various light sources that are available is provided. Because our area of expertise lies in the photocross-linking itself, the isolation and characterization of the cross-link is only mentioned briefly.

II. DESCRIPTION

A. Nonsubstituted Nucleic Acids

1. Photocross-Linking of Nonsubstituted Nucleic Acids by Single Photon Excitation

Many researchers have taken advantage of the photoreactivity of the wild-type nucleic bases themselves to study the interactions between a nucleic acid and a protein and have avoided making chemical substitutions. Without the substitution of photoactive analogs in the nucleic acid, structural perturbations to the nucleoprotein complex are eliminated. Some DNA-protein systems that have been studied are the cross-linking of fd phage DNA to fd gene 5 protein (Paridiso et al., 1979), *Eschericha coli.* DNA to bovine serum albumin (BSA) (Smith 1964), DNA to *E. coli* RNA polymerase (Harrison, et al., 1982), DNA to DNA polymerase (Markowitz, 1972), $(T_4G_4)_2$ to *O. nova* telomere-binding protein (Hicke et al., 1994), and T_8 to human A1 hnRNP (Merrill et al., 1988). RNA systems have been researched as well; examples are RNA to aminoacyl synthetase (Budowsky and Abdurashidova 1989), tRNA to ribosomal complexes (Abdurashidova et al., 1991), and 23S RNA to the *E. coli* 50 S ribosomal protein S7 (Williams and Konigsberg 1991). Typical photocross-linking yields are 5 to 20%; Merrill and co-workers (1988) have reported a yield as high as 85%.

2. Mechanism of Photocrosslinking

The optimum excitation wavelength range for all the nucleic bases is between 250 and 270 nm. When a nucleic base is excited by a single photon, it is promoted to the first excited singlet state (S_1). From S_1, which has a lifetime of 10 ps, the excited base can either react, relax back to ground state, or intersystem cross to the first excited triplet state (T_1 lifetime = 1 μs) (Budowsky et al., 1986). T_1 can also react or relax to ground state (Budowsky and Abdurashidova, 1989). The photocross-linking of a nucleic base to an amino acid can occur from either S_1 or T_1. Because the S_1 state has a very short lifetime, cross-linking

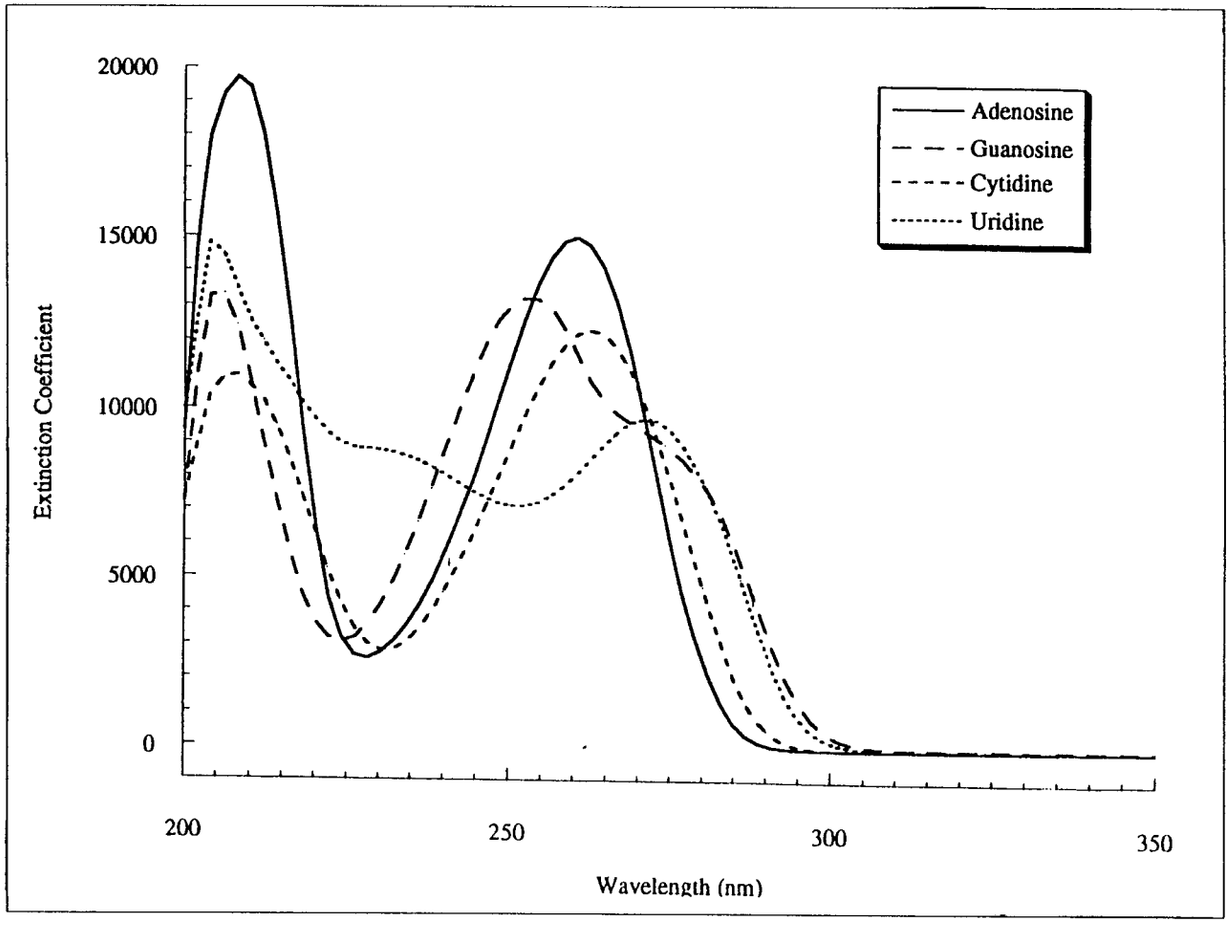


FIGURE 1. UV spectra of the nucleosides adenosine, guanosine, cytidine, and uridine. Units for extinction coefficient are l/mol · cm.

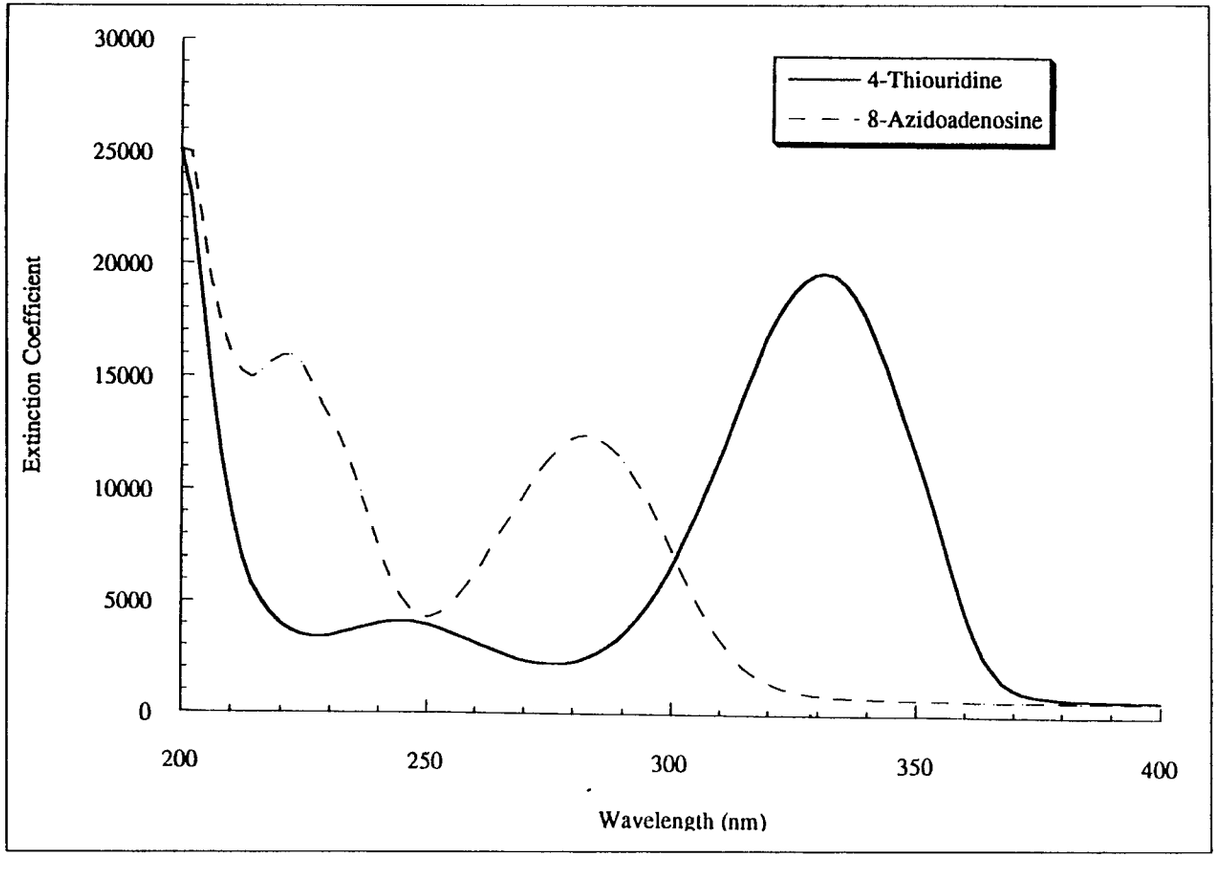


FIGURE 2. UV spectra of 4-thiouridine and 8-azidoadenosine. Units for extinction coefficient are l/mol · cm.

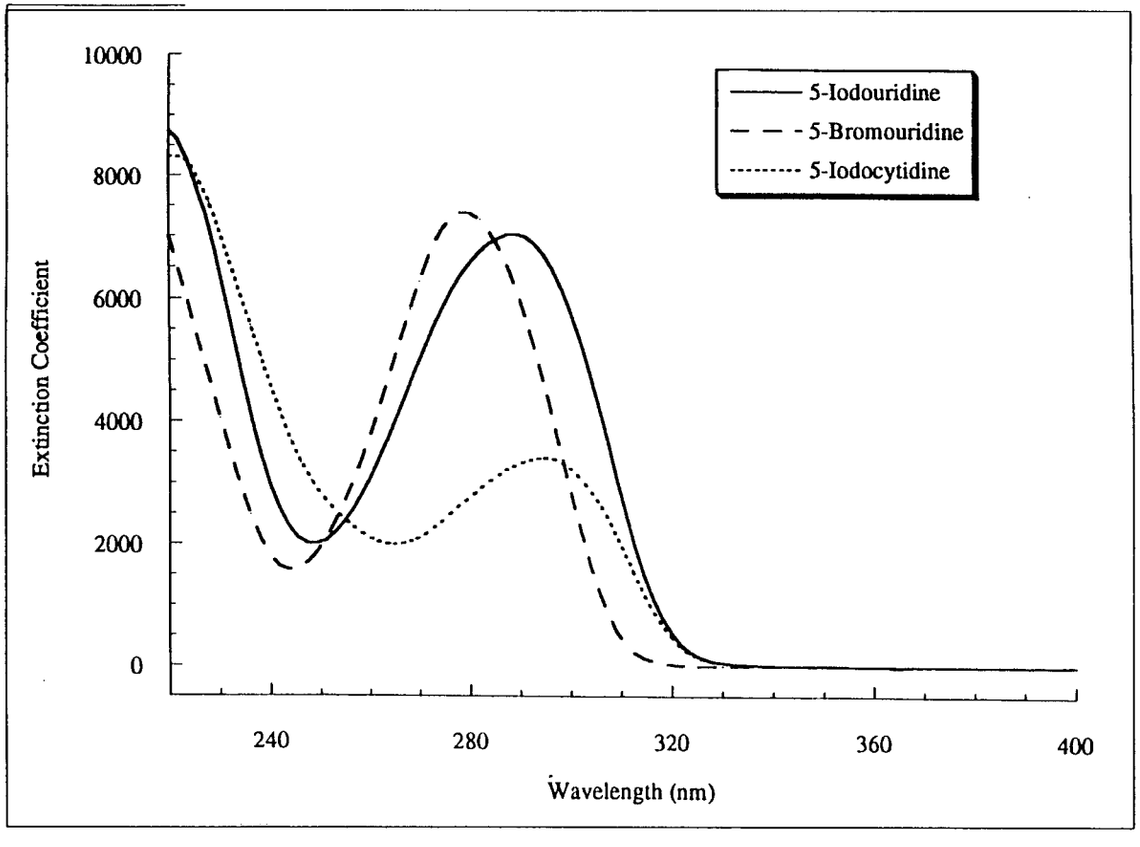


FIGURE 3. UV spectra of 5-iodouridine, 5-bromouridine, and 5-iodocytidine. Units for extinction coefficient are l/mol · cm.

from this state must involve groups in contact at the time of excitation (Budowsky et al., 1986), assuming there are no long-lived reaction intermediates. Cross-linking via the T_1 state, which still has a relatively short lifetime, probably also involves groups in proximity at the time of excitation.

Several mechanisms have been postulated for wild-type nucleoprotein photocrosslinking primarily as a function of the amino acid residue. In this regard, amino acid residues can function as hydrogen atom donors, electron donors, or nucleophiles. Mechanistic conclusions are often based upon the results of model studies; most of these have been performed with pyrimidine bases and nucleosides (Saito et al., 1983a; Smith, 1970; Jellinek and Johns, 1970). A free radical mechanism with the excited base abstracting a hydrogen atom from a neighboring amino acid residue to produce a pyrimidinyl radical followed by radical combination is one of the most common mechanisms (Williams and Konigsberg, 1991). It is consistent with the results of a model study of the reaction of uridine with cysteine by Johns and Jellineck (1970). They concluded that an excited triplet uracil abstracts hydrogens from cysteines to form dihydrouridine. The resulting thiol radicals then react with ground-state uracil to form the 5-substituted product, as shown in Figure 4. Smith (1970) studied a similar reaction of cysteine with thymine and proposed a thiol radical addition to the analogous 5-position. In contrast, the photoaddition of lysine to thymidine (Saito et al., 1983b), and threonine and serine analogs to 5-methylcytosine (Shaw and Shetlar, 1989), appear to involve a nucleophilic acyl substitution-type reaction mechanism, as shown in Figure 5. Shetlar and coworkers (1992) also looked at the reaction between thymidine and an analog of tyrosine. They suggested an electron transfer

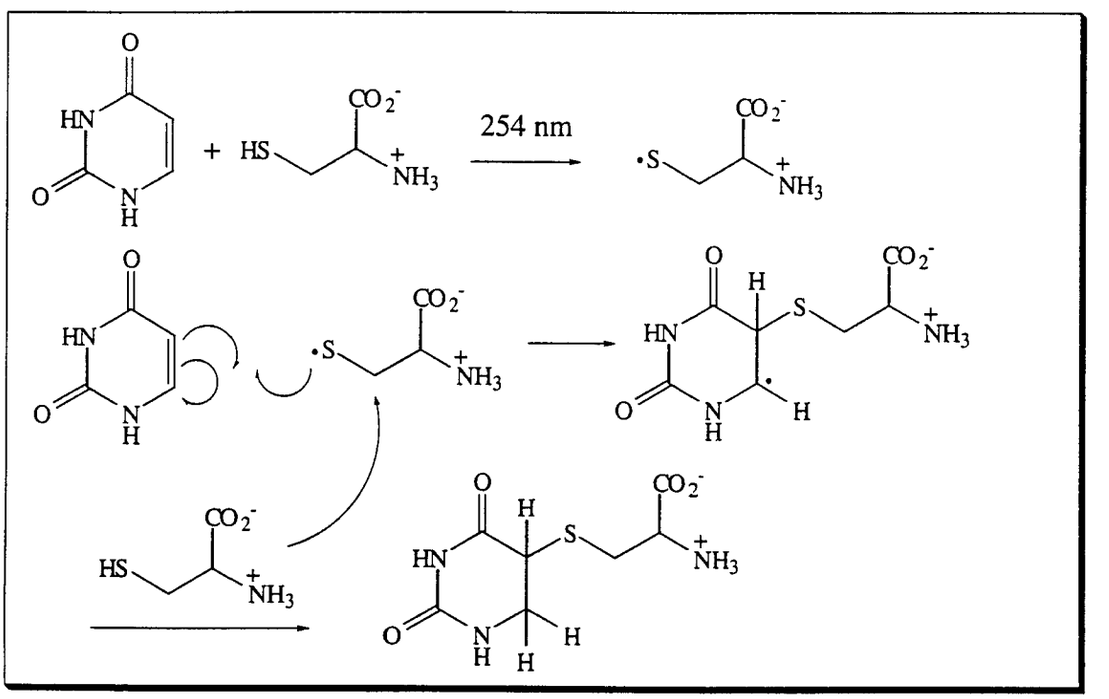


FIGURE 4. Proposed mechanism for the photoaddition of L-cysteine to uracil.

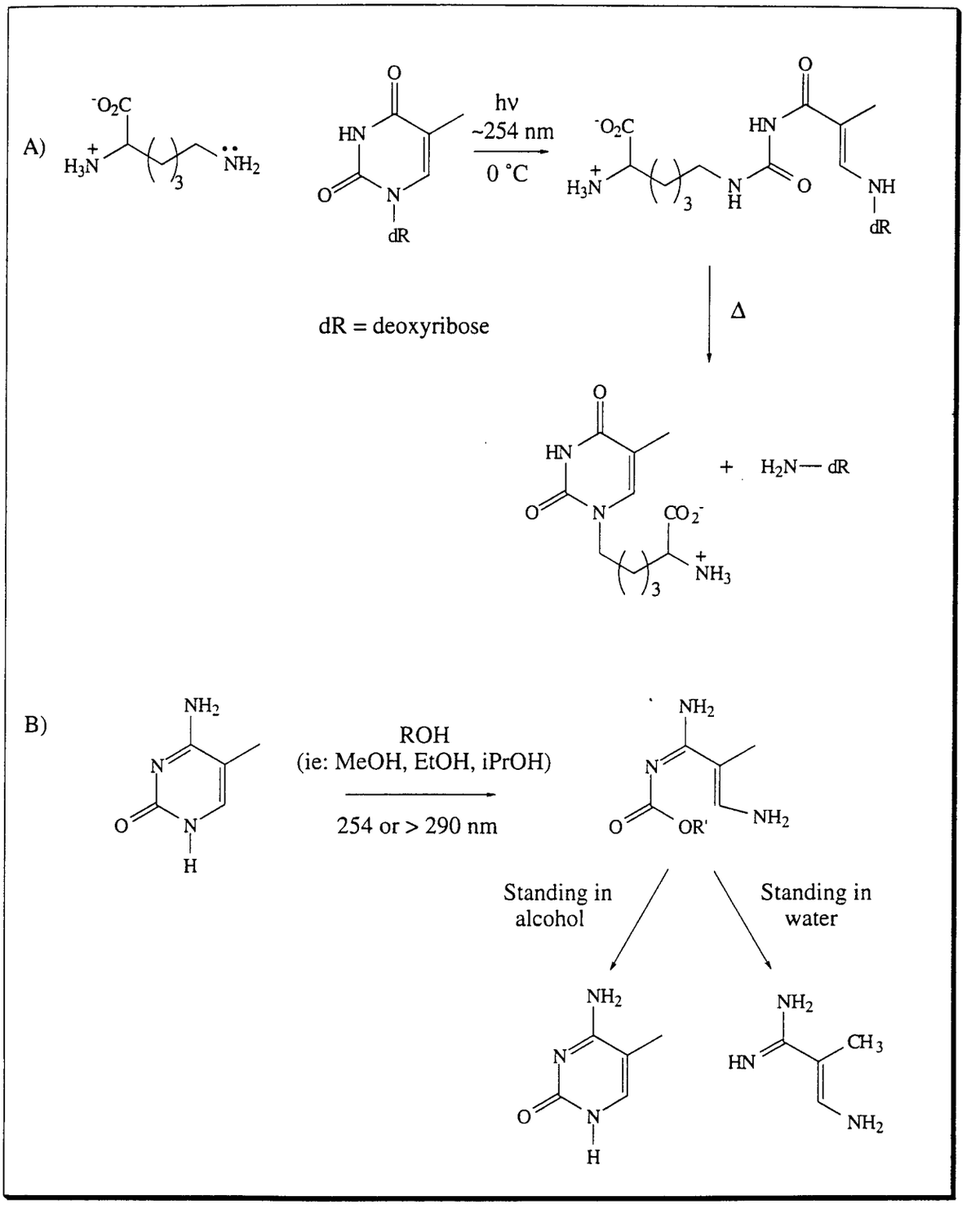


FIGURE 5. (A) Saito's mechanism for the photoaddition of lysine to thymidine; (B) Shetlar's mechanism for the photaddition of threonine and serine analogs to 5-methyl cytosine.

mechanism where coupling is initiated by an excited tyrosine donating an electron to the pyrimidine (Shaw et al., 1992) (Figure 6). Although these model studies give some mechanistic insights, the pathway may be somewhat different in the macromolecular system. Of particular importance is the restriction on the diffusion of a photochemically formed geminate radical or radical ion pair. A nucleoprotein complex for which some cross-links have been characterized to both a pyrimidine and a purine is a model for the telomere of the ciliated protozoan *Oxytricha nova*. The model was single-stranded $(T_4G_4)_2$ bound to *Oxytricha* telomere-binding protein. Cross-links from thymidines of the DNA to a histidine and a tyrosine of the binding protein and from a deoxyguanosine of the DNA to a tyrosine of the binding protein were assigned based on similar cross-links with DNAs bearing single 5-bromodeoxyuridine substitutions (*vide infra*) (Hicke et al., 1994).

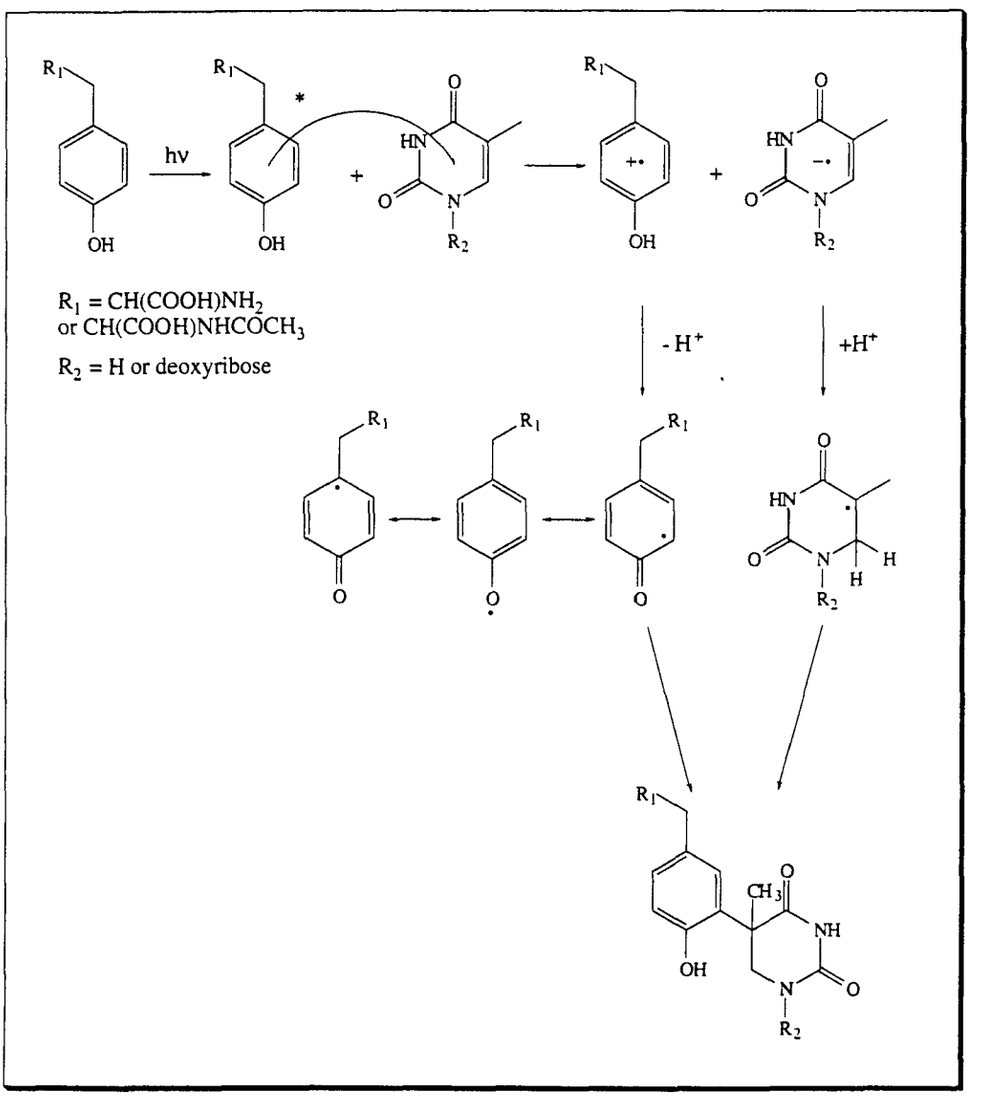


FIGURE 6. Shetlar's electron transfer mechanism for the addition of tyrosine to thymine or thymidine.

Single-strand breaks of the nucleic acid can also occur, although mainly during high-intensity irradiation (Kovalsky and Budowsky, 1990; Görner, 1994). This is discussed in the following section on two photon excitation. The population of only the lower excited states, S_1 and T_1, will minimize single strand breaks. To avoid two-photon excitation, Budowski and Abdurashidova (1989) suggest irradiating with an intensity of 10^{18} photons per centimeter per second (10^8 W/m^2) or lower at 260 nm.

3. Selectivity of Photocross-Linking

After excitation of the nucleic bases, a reaction can take place with numerous amino acid residues. In fact, Williams and Konigsberg (1991) stated that because little is known about which amino acids cross-link in the macromolecular system, all 20 amino acids should be considered when cross-linking is observed. Some of the amino acids, and the systems in which each is known to cross-link, are listed in Table 1.

As mentioned earlier, some model experiments have been performed to test the reactivity of various amino acids with the bases themselves (Shetlar et al., 1984; Saito et al., 1983b). Shetlar and co-workers measured the quantum yields of the photoaddition of 20 amino acids (proline excluded) to calf thymus DNA. The amino acids most reactive toward the DNA were Cys, Lys, Phe, Trp, and Tyr, whereas His, Glu, and Asp were moderately reactive. The least reactive amino acids were Arg, Leu, and cystine. Even though some of the amino acids did not seem as reactive in these model studies, they also state that none of the 20 amino acids can be ruled out as potential cross-linkers.

4. Advantages/Disadvantages of Using Nonsubstituted Nucleic Acids for Photocross-Linking

The advantages are

1. No unnatural nucleotides or amino acids need to be incorporated; therefore, no structural perturbations to the nucleoprotein complex are created.

TABLE 1
Examples of Some Amino Acids Responsible for Photocross-Linking in Various DNA/RNA Systems

Nucleic Acid	Protein	Cross-linked amino acid	Ref.
fd phage DNA	Gene V	Cys	Paridiso et al., 1979
Oligo p(T)	Rat DNA polymerase β	Ser	Dong et al., 1994
		His	Prasad et al., 1993
T_8	*E. coli* SSB	Phe	Merrill et al., 1984
DNA	Histones H1 and H3	Lys	Saito and Sugiyama, 1990; Kurochkina and Kolomijtseva, 1992
(T_4G_4)	*O. nova* telomere-binding protein	Tyr, His	Hicke et. al., 1994
23S RNA	*E. coli* 50S ribosomal protein S7	Tyr	Williams and Konigsberg, 1991
16S RNA	*E. coli* 50S ribosomal protein S7	Met	Williams and Konigsberg, 1991

2. Photocross-links can occur with a number of amino acid residues.
3. The chromophores (nucleic bases) require no additional synthesis.
4. Reported yields of cross-linking have been anywhere from ~5 to 20%, with some yields reported as high as 85% (Williams and Konigsberg, 1991).

Some of the disadvantages are

1. Irradiation is usually carried out at wavelengths below 300 nm, making absorption by other chromophores in the complex possible. This can lead to photodamage to the system, lowering the cross-linking yield and/or complicating the characterization of the cross-link.
2. The mechanism of cross-linking in the macromolecular system is still poorly understood, making predictions more difficult.
3. Long irradiation times may be required to achieve a reasonable yield of cross-linked nucleoprotein.
4. Sequencing of both the nucleic acid and protein components are required for identification of the cross-linking site.

5. Photocross-Linking of Nonsubstituted Nucleic Acids Using Two Photon Excitation

Single-photon excitation promotes a nucleic base from S_0 to S_1; intersystem crossing to T_1 can follow. The absorption of an additional photon by the excited nucleic base promotes the base from S_1 or T_1 to a higher excited state, H_S and H_T, respectively (Kovalsky, and Budowsky, 1990). This kind of excitation is called biphotonic excitation (Simic, 1993) and is achieved with high-intensity pulsed lasers emitting 10^9 W/m^2 with nanosecond pulses or 10^{12} W/m^2 with picosecond pulses (Kovalsky et al., 1990). RNA-protein cross-linking yields obtained with the 30S subunit of *E. coli* ribosomes were compared using low- and high-intensity irradiations (Budowsky et al., 1986). The RNA-protein cross-linking yield with high-intensity irradiation was 50% with several 10-ns pulses from a 266-nm Nd-YAG laser. The yield was 20 to 100 times larger than those yields achieved with low-intensity irradiation.

6. Mechanism of Photocross-Linking

Like single-photon excitation, little is known about the mechanism of cross-linking nucleic bases to amino acid residues in proteins by biphotonic excitation. When a nucleic base absorbs an additional photon, the base is promoted to higher electronic energy levels, H_S or H_T. Although with picosecond pulses at 248 nm or 266 nm, the second photon is mainly absorbed by the base in S_1 (Simic, 1993). The energy of these higher electronic levels are ~ 6 to 10 eV, whereas the gas phase ionization potentials of the nucleic bases are ≥ 8 eV (Simic, 1993). As a result, two-photon absorption can cause ionization of the nucleic base to form cation radicals (Budowsky et al., 1986). The ionization of nucleic bases in DNA or RNA is the most likely cause of single-strand breaks. Because the purines possess the lowest ionization potentials with guanine at 8.24 eV, most single-strand breaks occur at the 3′ phosphodiester bond of the nucleotide bearing the purine (Croke et al., 1988). Kovalsky and co-workers (1990) suggested that a single-strand break might be a result of "local peculiarities" of the tertiary structure of the nucleic acid. They also offered that single-strand breaks can be affected by buffer conditions and ligands, so an alteration in these conditions may affect their distribution in the nucleic acid.

7. Selectivity of Photocross-Linking

The amino acid residues reactive in nucleic acid-protein photocross-linking with

single photon excitation should also be considered good candidates with two-photon excitation.

8. Advantages/Disadvantages of Using Two Photon Excitation for Nucleic Acid-Protein Photocross-Linking

Some of the advantages of using two photon excitation are

1. A relatively high yield of crosslink (~50%) can be obtained with one or two short picosecond pulses.
2. The experiment is completed in a very short period of time, which can be necessary with unstable systems.
3. Two-photon excitation offers all of the advantages of single-photon excitation of nonsubstituted nucleic acids (i.e., no incorporation of nonnatural nucleic bases, no additional synthesis required, etc.)

The disadvantages of using two photon excitation are mostly similar to the disadvantages of single photon excitation

1. The mechanism of photocross-linking is still not proven, so predictions of cross-linking sites are unreliable.
2. Irradiation is usually carried out at wavelengths < 300 nm, where other chromophores in the complex can absorb, resulting in possible photodamage.
3. Two-photon absorption can result in an increase in alkali-sensitive strand breaks compared with single-photon excitation.

B. 4-Thiouridine-Substituted Nucleic Acids

1. Photocross-Linking of 4-Thiouridine-Substituted Nucleic Acids

4-Thiouridine's (4-SU) structure is similar to uridine's, with only the 4-keto oxygen replaced by a sulfur atom (Figure 7). In

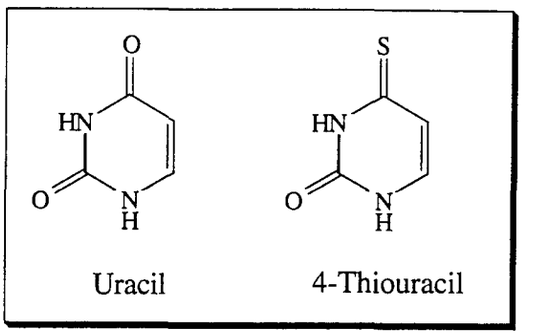


FIGURE 7. Comparison of uracil and 4-thiouracil chromophores.

comparing the Van der Waals radii, the sulfur is only 0.45 Å larger than oxygen (Favre, 1990). This small variation in structure and 4-SU's high photoreactivity with amino acid residues and nucleic bases make 4-thiouracil an excellent chromophore to study nucleic acid-protein and nucleic acid-nucleic acid interactions. In addition, the substitution with sulfur at the 4-position centers the main π-π^* absorption band around 330 nm, compared with uracil's π-π^* absorption band centered at 260 nm (Favre, 1990). The ability to excite 4-SU at wavelengths above 300 nm can aid in minimizing unwanted additional photochemistry or photodamage to the system.

Many RNA-protein and RNA-RNA interactions have been investigated using 4-SU incorporation to induce photocross-linking. Some examples as well as cross-linking yields (if reported) include $tRNA^{Lys}$ with the reverse transcriptase of human immunodeficiency virus type 1 (9.8%) (Mishima, and Steitz, 1995), pre-tRNA with tRNA ligase (~90%) (Tanner et al., 1988), RNA-protein interactions in monkey kidney cells (CV1) (20%) (Favre et al., 1986), pre-mRNA substrates with U5 snRNP before the initial step in splicing (Wyatt et al., 1992), RNA-protein interactions in domains 1 and 2 of the *E. coli* ribosomal subunit (<10%) (Hajnsdorf et al., 1989), and snRNAs with pre-mRNA substrates (Newman et al., 1995; Kim, and Abelson, 1996).

2. Mechanism of Photocross-Linking

4-SU appears to be indiscriminate when cross-linking to an amino acid in a protein due to its high photoreactivity (Favre, 1990); however, the mechanism of 4-SU photocrosslinking is not well understood. Even though most of the amino acids with which 4-SU cross-links in macromolecular systems have not been identified, model mechanistic studies demonstrated its reactivity with alcohols and amines (Figure 8) (Favre, 1990). Previous work on the coupling of 4-SU to cytidine suggests the formation of a radical intermediate (Milder and Kliger, 1985). The postulated mechanism of 4-SU coupling to alcohols and amines is formation of the 4-SU triplet, hydrogen abstraction from the alcohol or amine to form a 4-SU radical intermediate, followed by radical combination (Favre, 1990). 4-SU has also been shown to photocouple to alkenes via 2 + 2 cycloaddition (Favre and Dubreuil, 1991). The triplet was implicated from quenching experiments (Milder and Kliger, 1985), including one with molecular oxygen (Favre, 1990). Milder and Kliger (1985) also determined the quantum yield of intersystem crossing of 4-SU to be 0.9 ± 0.1, further suggesting that the majority of the photochemistry takes place in the triplet manifold.

3. Synthesis

The incorporation of 4-SU into RNA can be accomplished a number of ways. For example, an RNA can be specifically labeled with 4-SU by joining two *in vitro* RNA transcripts, where one of the transcripts contains 4-thioUpG. In the presence of a bridging oligodeoxynucleotide, these

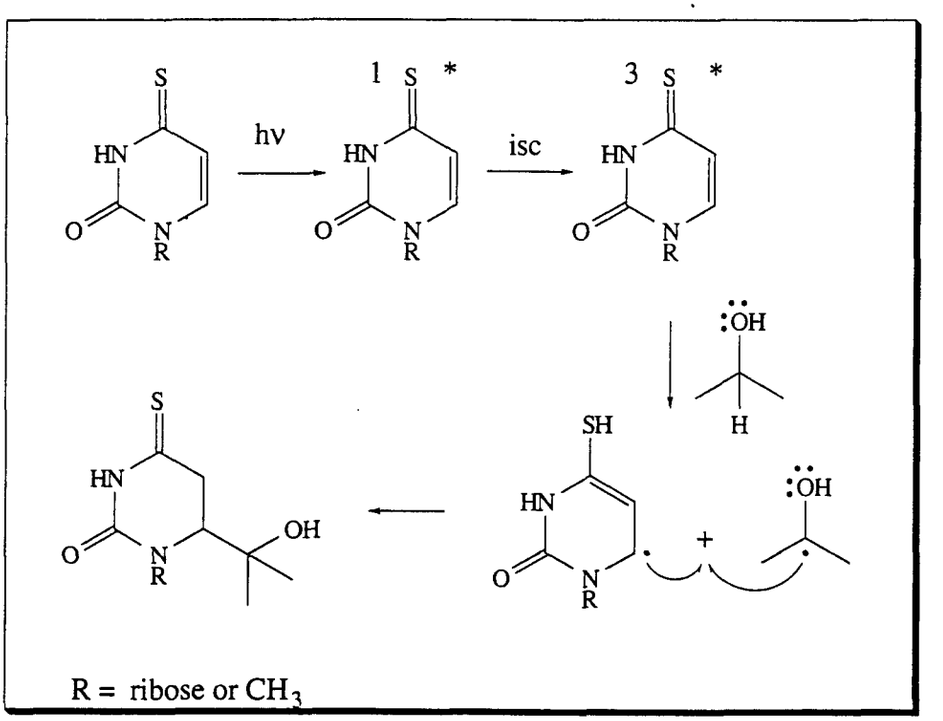


FIGURE 8. Photoaddition of a hydrogen atom donor (alcohol or amine) to either 4-SU or a 4-thiouracil-substituted derivative.

two RNA transcripts can be joined using T4 DNA ligase (Sontheimer, 1994; Wyatt, et al., 1992). *In vitro* transcription of a 4-SU-labeled RNA can be carried out using T7 RNA polymerase and 4-SUTP in place of UTP (Debreuil et al., 1991). The disadvantage of this technique is that the 4-SUTP is not commercially available and must be synthesized. 4-Thiouridine can also be incorporated site specifically in RNA by solid-state synthesis using O^4-triazolouridine phosphoramidite, which is readily prepared from uridine phosphoramidite (Shah et al., 1994). After RNA synthesis, the O^4-triazolouridine is transformed to 4-thiouridine with thiolacetic acid. The synthesis of 4-SdU-substituted DNA can be achieved on a DNA synthesizer using 4-*S*-(pivaloyloxymethyl)thiodeoxyuridine phosphoramidite (Vitorino Dos Santos et al., 1993). Similarly, 4-thiothymidine can be incorporated into DNA either enzymatically using Klenow fragment of DNA polymerase I (Favre, 1990) or chemically (Xu et al., 1992). Other synthetic techniques include the thiation of uracil with Lawesson's reagent (Dzik et al., 1987) or phosphorus pentasulfide (Fox et al., 1959) and an amino-thiol exchange using hydrogen sulfide, where the 4-amino group of cytidine is replaced by sulfur. The latter method can be used with nucleosides, oligonucleotides, and polynucleotides (Favre, 1990). Table 2 lists sources and references for the use of the 4-thiouracil chromophore.

4. Advantages/Disadvantages of Using 4-SU in Nucleic Acid-Protein Photocross-Linking

The advantages of using 4-SU as a chromophore for photocrosslinking are

1. By only replacing the oxygen of uridine with sulfur, little structural perturbation will occur in the nucleoprotein complex.
2. 4-SU has similar base pairing properties to uridine.
3. 4-SU is stable in the absence of photoactivation.
4. Its high photoreactivity suggests that it will cross-link with many possible amino acid residues at the nucleic acid-protein interface.

TABLE 2
Table of Sources and References for the Use of 4-Thiopyrimidine Chromophores

Reagent	Source	Ref.
4-SU	Sigma Chemical Co., St. Louis, MO	Heihoff et al., 1990; Favre et al., 1993, 1986; Milder and Kliger, 1985; Fox et al., 1959
4-S-UpG	Sigma Chemical Co., St. Louis, MO	Wyatt et al., 1992; Sontheimer and Steitz, 1993; Sontheimer, 1994; Mishima and Steitz, 1995
4-S-TTP	Amersham Life Sciences, Inc., Arlington Heights, IL; US Biochemicals, Cleveland, OH	Favre, 1990
4-S-T phosphoramidite	Glen Research, Sterling, VA	
4-S-UTP	Synthesis	Debreuil et al., 1991
4-S-(pivaloyloxymethyl)thiodeoxyuridine phosphoramidite	Synthesis	Clivio et al., 1992; Dos Santos et al., 1993

5. 4-SU is photoactivated at longer wavelengths (>320 nm), which allows selective excitation, minimizing unwanted additional photochemistry.
6. 4-SU is naturally occurring in *E. coli* tRNA.
7. Photocross-linking yields of usually ≥10% can be obtained; a yield as high as 90% has been reported (Tanner et al., 1988).

Some of the disadvantages of using 4-SU are

1. Little is known about the mechanism of cross-linking in the macromolecular system, making prediction of cross-linked sites unreliable.
2. High reactivity with nucleic bases can lower cross-linking yields to associated proteins.
3. Most forms of 4-thiouracil need to be synthesized prior to nucleic acid incorporation.
4. When using 4-thioUpG to label RNA at a specific site, the 4-SU must always be next to a guanosine that may interrupt the native binding conformation.

C. Azido Aryl- and Azido-Substituted Nucleic Acids

Photocross-Linking of Azido Aryl- and Azido-Substituted Nucleic Acids

Azides are a popular choice for studying nucleic acid-protein interactions due to the highly reactive nitrene generated after irradiation with long wavelength UV light. Alkyl, aryl, and nitroaryl azides are all capable of generating the nitrene; however, the aryl and nitroaryl azides are more effective as photocross-linking agents. This is explained by the aryl and nitroaryl azide's ability to absorb light ≥300 nm and their excellent stability in the dark (Bayley and Knowles, 1977). The alkyl azides only have reasonable stability in the dark, can rearrange intramolecularly after photolysis, and absorb only at wavelengths below 300 nm (Bayley and Knowles, 1977). Therefore, the information covered on azides focuses on the aryl azides and their use in the photocross-linking of nucleoprotein complexes.

Aryl azides have been used in a number of ways to study the interactions of proteins and nucleic acids via photocross-linking. For example, a guanosine residue of mRNA was modified with 2,4-dinitro-5-fluorophenyl azide and cross-linked to *E. coli* threonyl tRNA synthetase, with a greater than 50% yield (Zenkova et al., 1995). 4-Azidophenylacyl bromide was covalently attached to a phosphorothioate residue on the DNA backbone and cross-linked to the active site of TAQ1 restriction endonuclease, with photocross-linking yields of 15 to 20% (Mayer and Barany, 1995). 4-Azidophenylacyl bromide can also be used to modify exposed cysteine residues on a protein to enhance photoreactivity. Dumoulin et al. (1993) used this technique to study the recognition helix of Lex A repressor and how it is positioned in the major groove of DNA.

Nucleotide bases bearing a directly attached azide functionality have also been employed as photoaffinity probes. The 8-azido analog of adenine and the 5-azido analog of pyrimidines have been utilized to explore the nucleotide binding domain of M_r 40K in the chaperonin proteins Gro EL and Gro ES (Martin et al., 1993) and the interaction of DNA with calf thymus terminal transferase (27 to 40% yield) (Farrar et al., 1991), respectively. 2,8-Diazido-ATP was used to investigate the nucleotide-binding sites of the subunits of F_1-ATP synthase (Schäfer et al., 1995). Some examples of

photoactivatable azides along with references can be found in Table 3.

2. Mechanism of Photocross-Linking

The irradiation of an azide with long-wavelength UV light produces a nitrene that can react nonspecifically and rapidly with molecules in close range (Hanna et al., 1993). The nitrene can react through either the singlet or triplet state (Bayley and Staros, 1984). Singlet nitrenes are known to react by insertion into O-H, N-H, and sometimes C-H bonds. In addition, the singlet nitrene can also form a benzazirine intermediate that can react with nucleophilic groups such as thiols (Figure 9). Triplet nitrenes are known to react with C-H bonds and also with molecular oxygen to generate azoxy and nitro compounds (Figure 10). When the aryl azide is substituted with electron-withdrawing groups, the reactivity of the nitrene is greatly increased. One advantage of using aryl azides is the high-reaction quantum yield. Even with 254 nm excitation, the chemistry of the photoactivated azide is often complete before any other light-sensitive chromophores can be affected (Bayley and Knowles, 1977). Although it may seem desirable to create such a highly reactive species after photoactivation, the nitrene may react with molecules such as water, co-solvents, or buffers such as Tris instead of the desired substrate, which can lead to lower cross-linking yields.

In addition to azides reacting photochemically with solvent molecules or water, they are also rapidly reduced to their corresponding amines by dithiothreitol (DTT) (Bayley and Staros, 1984). If a reducing agent is needed, in conjunction with aryl azide photocross-linking, the use of monothiols, as opposed to dithiols, is preferred (Czarnecki et al., 1979). If it is necessary that DTT be used, the reaction should be carried out at a pH less than 7. Czarnecki et al. (1979) found that if the pH was above 7, the 8-azidoadenosine analogs lost their photoactivity.

The mechanism of cross-linking of the 8-azidopurine and 5-azidopyrimidine analogs is similar to that of aryl azides, where a highly reactive nitrene is generated after photoactivation. The advantage of using the 8-azidopurines and 5-azidopyrimidines is that they are not as bulky as other azido chromophores, causing less structural perturbations to the complex. However, if the azide is positioned ortho to a ring nitrogen atom, such as in 2-azidoadenosine, isomerization to the tetrazole can occur, resulting in extremely low photocross-linking reactivity (Figure 11) (Bayley and Knowles, 1977).

3. Selectivity of Photocross-Linking

Azides are not selective in their reactions due to the high reactivity of the intermediate nitrene generated (Hanna et al., 1993). In fact, the nitrene does not require specific amino acids in the binding site for successful cross-linking (Czarnecki et al., 1979). Some of the amino acids that are known to react with aryl nitrenes are mentioned in Table 4. Other amino acids are suspected to react but most likely form unstable complexes that are never detected (Bayley and Staros, 1984). These amino acids include Asp, Met, Glu, Asn, Gln, peptide bonds, and possibly cystine. Although this high reactivity seems advantageous, the bulkiness of most aryl azide chromophores can disrupt the native conformation of the nucleoprotein complex, resulting in nonspecific binding (Czarnecki et al., 1979) and uninformative cross-linking.

TABLE 3
Sources and References for the Synthesis of Azido Chromophores

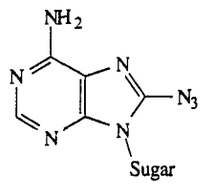
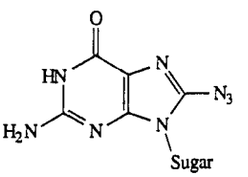
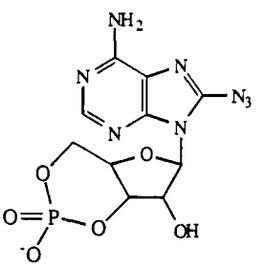
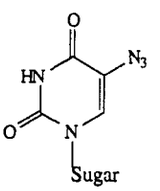
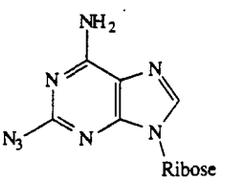
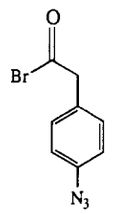
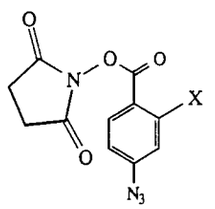
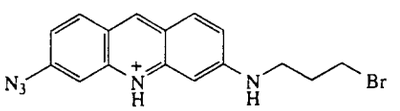
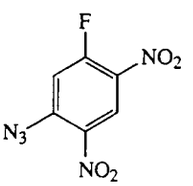
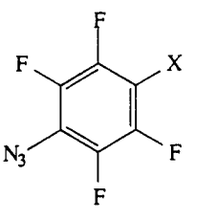
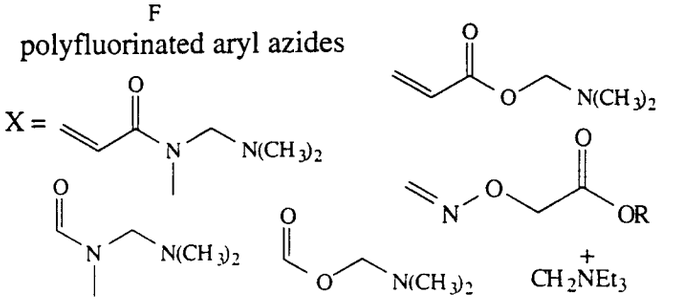
Chromophore	Source	Ref.
 8-azidoadenosine 8-azido-2'-deoxyadenosine	Sigma Chemical Co., St. Louis, MO; Research Products International, Inc., Mt. Prospect, IL (for triphosphates); ICN, Costa Mesa, CA (for radioisotope)	Czarnecki et al., 1979; Evans et al., 1989; Martin et al., 1993; Salvucci et al., 1992; Woody et al., 1984; Valenzuela et al., 1991; Knoll et al., 1992;
 8-azidoguanosine 8-azido-2'-deoxyguanosine	Sigma Chemical Co., St. Louis, MO; Research Products International, Inc. Mt. Prospect, IL (for triphosphate)	Czarnecki et al., 1979 Wower et al., 1994
 8-azido cyclic adenosine monophosphate	Sigma Chemical Co., St. Louis, MO	Eppler, et al., 1982
 5-azidouridine 5-azido-2'-deoxyuridine	Research Products International, Inc., Mt. Prospect, IL (for triphosphate)	Evans et al., 1986; Evans and Haley, 1987; Farrar et al., 1991; Lee et al., 1991; Hammond et al., 1992; Wower et al., 1994
 2-azidoadenosine	Research Products International, Inc., Mt. Prospect, IL (for triphosphate)	Macfarlane et al., 1982; Wower et al., 1994

TABLE 3
Sources and References for the Synthesis of Azido Chromophores

Chromophore	Source	Ref.
 p-azidophenacyl bromide	Sigma Chemical Co., St. Louis, MO	Hanna et al., 1993; He et al., 1995; Yang and Nash, 1994; Praseuth et al., 1988
 X=H, 4-azidobenzoic acid N-hydroxysuccinimide ester X=NO_2, N-(4-azido-2-nitro benzoyloxy)succinimide	Sigma Chemical Co. St. Louis, MO	Bartholomew et al., 1995; Lannutti et al., 1996 Capson et al., 1995
 3-azido-6-(3-bromopropylamino)-acridine		Le Doan et al., 1987
 2,4-dinitro-5-fluorophenylazide		Zenkova et al., 1995
 polyfluorinated aryl azides		Soundararajan et al., 1993 Wjatschesslaw et al., 1995
		

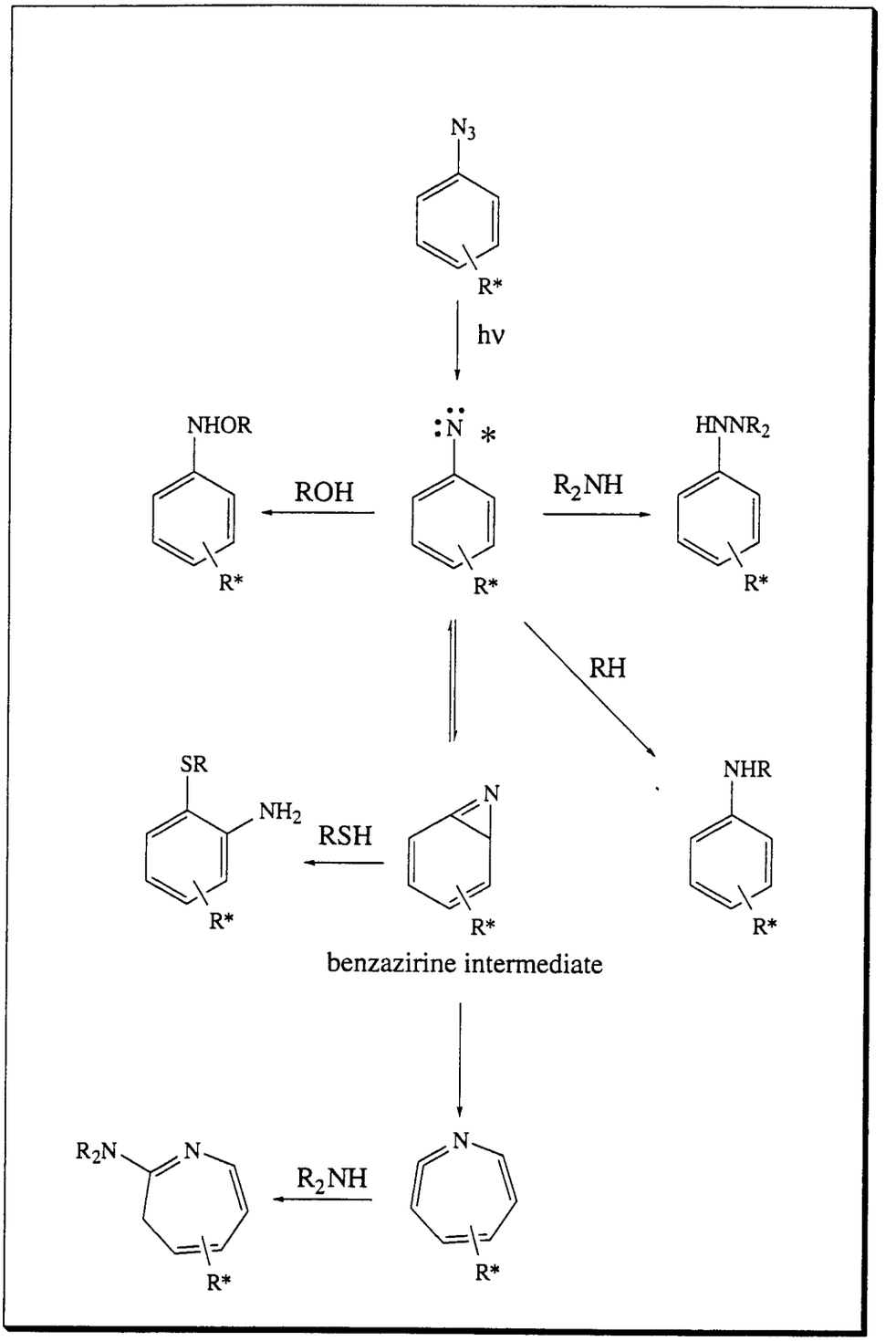


FIGURE 9. Reaction of singlet aryl nitrenes. R*, electron-withdrawing group.

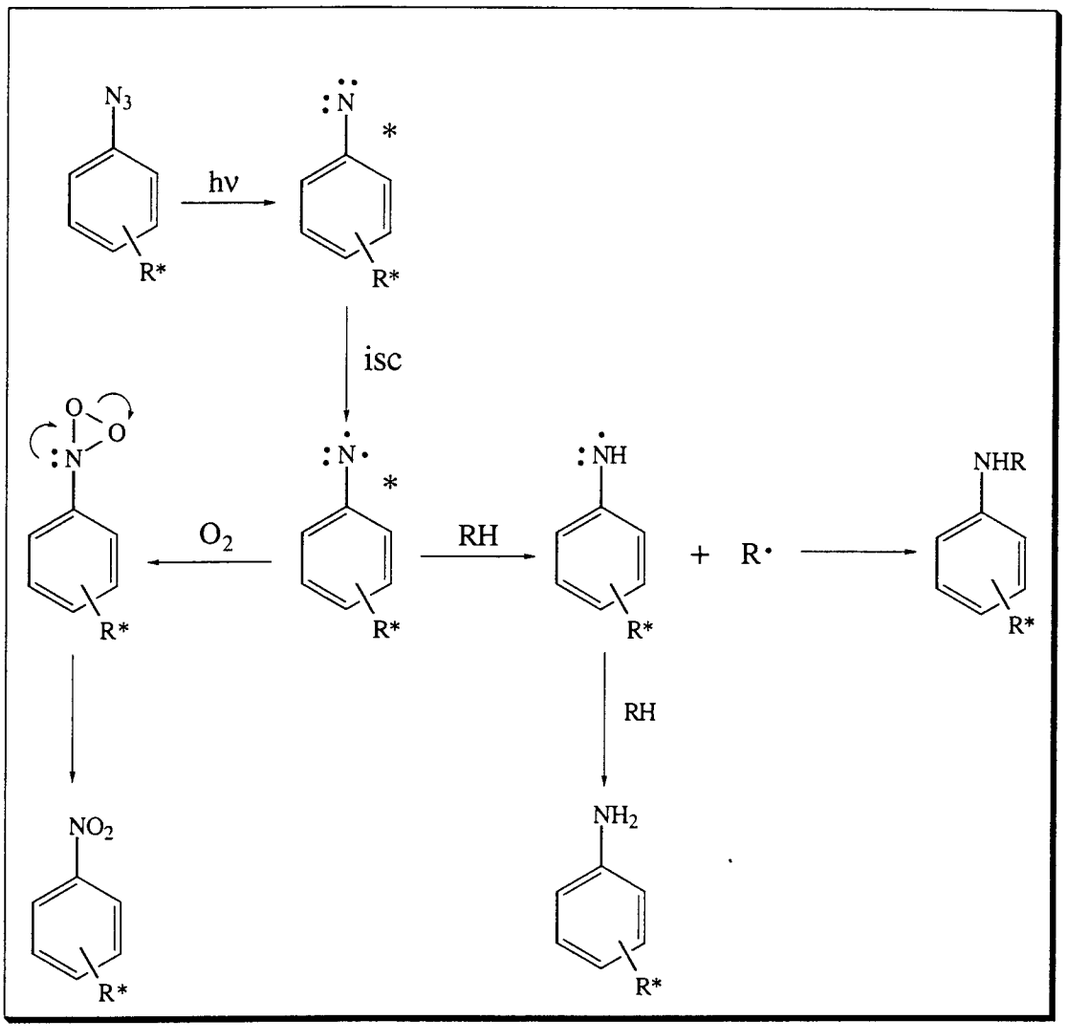


FIGURE 10. Reactions of triplet aryl nitrenes. R*, electron-withdrawing group.

4. Synthesis

Most of the aryl azides must be synthesized before incorporation into nucleic acids or proteins. Because a number of azido chromophores exist, the references for their syntheses, or where they can be purchased, are summarized in Table 3. Various methods can be used to incorporate the azido chromophores into DNA, RNA, or protein. These methods include incorporating the azide using enzymes (Hanna et al., 1993; Lee et al., 1991; Bartholomew et al., 1995) or solid-state synthesis (Capson et al., 1995), tethering the aryl azide to the backbone of a modified DNA or RNA containing a phosphorothioate (Yang and Nash, 1994; Mayer and Barany, 1995), or covalently attaching the azide to an exposed cysteine on the corresponding protein (Dumoulin et al., 1993; Pendergrast et al., 1992). To modify the nucleic acid or protein, the azido-containing reagent should have the photoactivatable azide on one end of the molecule and a nonphotoactivatable electrophilic group on the other end, which can attach to a nucleophilic site on the nucleic acid or protein (Bayley and Staros, 1984).

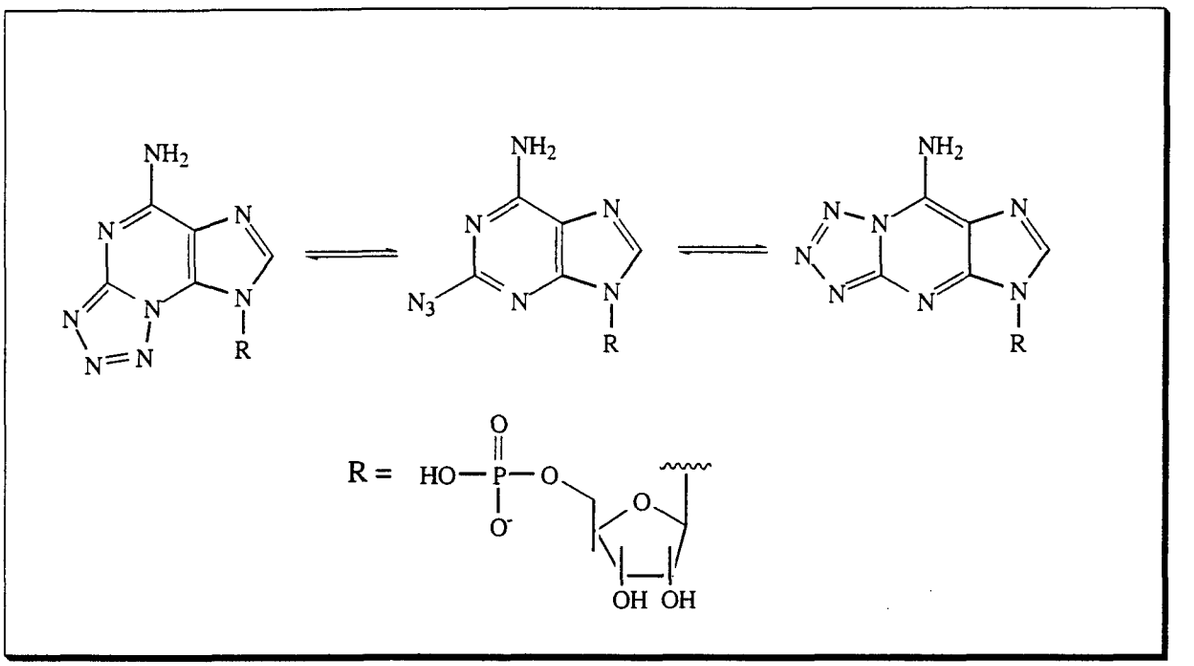


FIGURE 11. An example of the equilibrium that can exist between 2-azidoadenosine and its corresponding tetrazole (Bayley and Staros, 1984).

TABLE 4
Table of Amino Acid Residues or Functional Groups Found in Proteins That Are Known to Photocross-Link with Aryl Azides (Bayley and Staros, 1984)

Aromatic groups	Alkyl groups	Nitrogen bearing	Hydroxyl groups	Thiol groups
Phe	Ala	Lys	Ser	Cys
Tyr	Gly	His	Thr	
Trp	Leu	N-terminus	Tyr	
His	Ile			
	Val			

5. Advantages/Disadvantages of Using Azides for Nucleic Acid-Protein Photocross-Linking

Some advantages of using azides for nucleic acid-protein photocrosslinking are

1. The aryl azide produces a highly reactive nitrene after irradiation that can react with most amino acid residues.
2. Aryl azides can absorb at wavelengths ≥300 nm.
3. Photocross-linking yields can be ≥50%.
4. Aryl azides have excellent stability in the dark.
5. The azide can be attached to the nucleotide or to a phosphorothioate backbone of either RNA or DNA.
6. The long tether of some aryl azides allows exploration of the binding site's molecular environment.

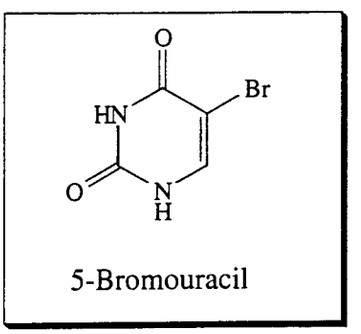
The disadvantages are

1. The highly reactive nitrene generated after photolysis can react with solvent or water molecules lowering cross-linking yields.
2. The long tether of most aryl azides may cause structural perturbations to the nucleoprotein complex.
3. Most aryl azides must be synthesized prior to incorporation into, or modification of, nucleic acids. Aryl azides are rapidly reduced to amines by DTT. 8-Azidopurine triphosphates are not substrates for some polymerase enzymes.

D. 5-Bromouridine-Substituted Nucleic Acids

1. Photocross-Linking of Bromouridine-Substituted Nucleic Acids

The substitution of 5-bromouridine (BrU) for uridine in RNA and 5-bromo-2′-deoxyuridine (BrdU) for thymidine in DNA has been used over the last 20 years to investigate nucleoprotein interactions via photocross-linking. Bromouracil (Figure 12) is a popular choice among chromophores because excitation can be achieved using



5-Bromouracil

FIGURE 12. The 5-bromouracil chromophore.

long wavelength ultraviolet light (>300 nm) (Hicke et al., 1994). Furthermore, the Van der Waals radius of bromine (1.95 Å) is similar to the radius of a methyl group (2.0 Å), making bromouracil comparable to thymine in size. As a consequence, bromouracil-substituted DNAs and in some cases RNAs (Gott et al., 1991) show specific binding to proteins with affinities similar to those of the wild-type nucleic acids.

Nucleoprotein complexes that have been studied using bromouracil substitution include the lac repressor protein and lac operator (Ogata and Gilbert, 1977), the binding site of the specificity (S) subunit of the restriction enzyme EcoK1 (Chen et al., 1995), major chromosomal protein (MC1) and its DNA (Katouzian-Safadi et al., 1991), the α and β-chains of telomere- binding protein and *Oxytricha nova* telomeric DNA (Hicke et al., 1994), and the R17 bacteriophage coat protein and its associated viral RNA hairpin (Gott et al., 1991), to name a few. Examples of nucleoprotein photocross-linking yields obtained with BrU-substituted RNA or DNA are summarized in Table 5.

2. Mechanism of Photocross-Linking

The mechanism for photoreaction of the bromouracil chromophore has been studied in great detail by various research groups using photoreduction to uracil and photocoupling to amino acid derivatives as mechanistic probes (Campbell et al., 1974; Ito et al., 1980; Swanson et al., 1981; Dietz et al., 1987b). Excitation at 254 nm produces the π,π* singlet state that undergoes carbon-bromine bond homolysis to generate the 5-uracilyl radical. Although some cross-linking to associated proteins likely occurs through C-Br bond homolysis, the uracilyl

TABLE 5
Examples of Photocross-Linking Yields Obtained with BrU Substituted Nucleic Acids

BrU-substituted nucleic acid	Associated protein	Photo-cross-linking yields (%)	Ref.
DNA	MC1	20	Katouzian-Safadi et al., 1991
O. nova telomeric DNA	Telomere-binding protein	25	Hicke et al., 1994
R17 viral RNA	R17 bacteriophage coat protein	45	Gott et al., 1991
DNA	GCN4	2	Blatter et al., 1992
pre-tRNA	tRNA ligase	50	Tanner et al., 1988

breaks of the nucleic acid (Hutchinson and Köhnlein, 1980; Cook and Greenberg, 1996).

Additional investigations have revealed a higher level of complexity. Swanson and co-workers (1981) reported that the UV spectrum of bromouracil, taken in 2-propanol solvent, contains an intense absorption at 276 nm (ε 7640 M^{-1} cm^{-1}) with a weak shoulder at 300 nm (ε 338 M^{-1} cm^{-1}) corresponding to π-π^* and n-π^* transitions, respectively. A difference in reactivity as a function of excitation wavelength was apparent from deuterium incorporation during photoreduction, as shown in Figure 13 (Swanson et al., 1981; Dietz et al., 1987). This experiment together with sensitization and quenching experiments indicated that the $^1\pi,\pi^*$ state undergoes C-Br bond homolysis and intersystem crossing to the triplet state and that $^1n,\pi^*$ state undergoes only intersystem crossing to the triplet state. The triplet then reacts exclusively by single-electron transfer. The lowest energy triplet state has a π,π^* configuration and lies 74 kcal/mol above ground state (Rothman and Kearns, 1967).

The photocoupling of BrU to various amino acid derivatives and a peptide linkage have been reported (Ito et al., 1980; Dietz et al., 1987; Dietz and Koch, 1987; Dietz and Koch, 1989). Ito and co-workers (1980) showed that bromouracil coupled to tryptophan derivatives (Figure 14) and established that the reaction was sensitized by the triplet sensitizer acetone and quenched by the triplet quencher 1,3-pentadiene. They proposed that coupling was initiated by single-electron transfer from the tryptophan to triplet bromouracil. Dietz and co-workers (1987) used 308-nm monochromatic light to couple tryptophan, histidine, and tyrosine derivatives and a peptide bond to bromouracil. They postulated that the coupling occurred via the bromouracil triplet state formed by intersystem crossing of the $^1n,\pi^*$ state. Electron transfer-initiated coupling requires having amino acid residues with low ionization potentials nearby that are willing to donate an electron to the excited base. The gas phase ionization potentials of some amino acids, or similar structures, known to cross-link with bromouracil are Trp (7.9 eV) (Amouyal et al., 1979), Tyr (8.52 eV) (Franklin et al., 1969), His (8.96 eV) (Ramsey, 1979), Cys (9.28 eV), and Cys-Cys (8.46 eV) (Franklin et al., 1969).

A recent paper by Norris and co-workers (1996b) suggests a further refinement on the electron transfer mechanism when tyrosine is the electron donor. They propose, based on quantum yield measurements as a function of excitation wavelength, that coupling of bromouracil to tyrosine occurs by electron transfer through excitation of

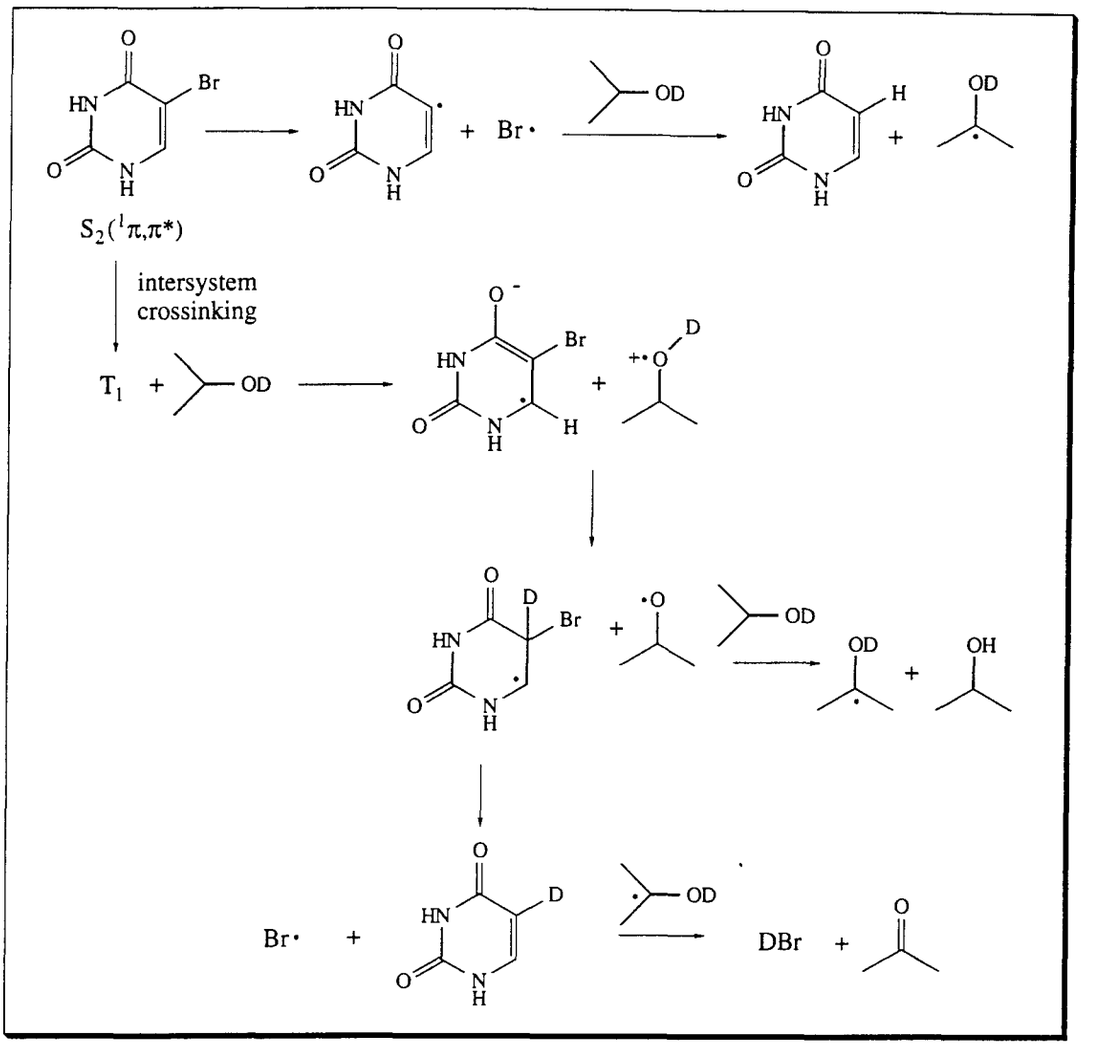


FIGURE 13. *Mechanism of photoreduction of 5-bromouracil as indicated by deuterium incorporation.*

the tyrosine rather than the bromouridine. The mechanism (Figure 15) shows the 5-uridinyl radical adding to another molecule of tyrosine that is most probable in the model studies because the derivative of tyrosine was in excess. However, in a nucleoprotein complex the 5-uridinyl radical likely combines with the adjacent tyrosine radical cation held in proximity by the macromolecular structure. Because tryptophan also absorbs at wavelengths above 300 nm and is known to eject electrons after excitation (Creed, 1984), it probably reacts with the bromouracil chromophore in a parallel manner. In contrast, histidine that is transparent at 308 nm probably reacts as originally proposed (Dietz and Koch, 1987) via electron transfer from the imidazole to triplet bromouracil. In the model reactions, whether reaction occurs predominantly through excitation of the amino acid chromophore or the bromouracil chromophore is probably a complex function of the respective absorption properties, excited state lifetimes, and excited state reactivities. In a macromolecular system, especially if the reactive π-systems bear a stacked geometry, the actual chromophore that is excited

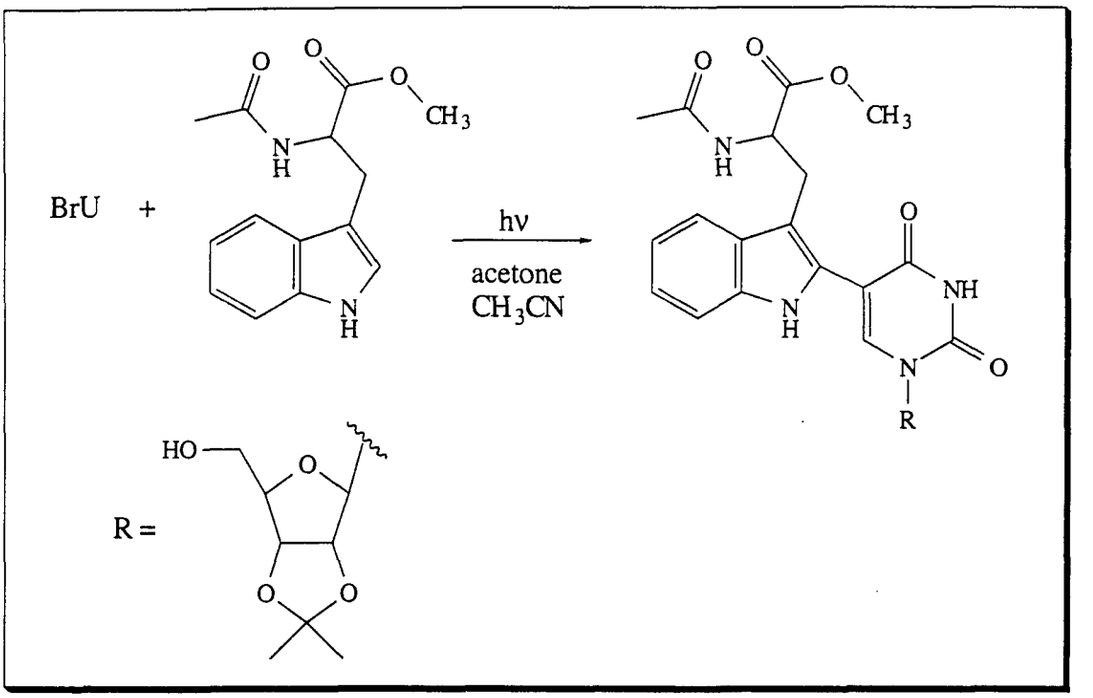


FIGURE 14. Photocoupling of an analog of BrU and *N*-acetyltryptophan methyl ester.

becomes ambiguous, and UV light might cause a direct charge transfer between the electron donor and acceptor.

3. Selectivity of Photocross-Linking

As mentioned previously, the bromouracil chromophore has been shown to cross-link with various amino acid derivatives. In addition to the aromatic amino acids (Dietz and Koch, 1987), BrU has been shown to cross-link to cystine (Dietz and Koch, 1989), a peptide linkage (Dietz et al., 1987), and even alanine (Blatter et al., 1992) in small molecule or macromolecular systems. Table 6 summarizes amino acids thought to be reactive with the bromouracil chromophore. The structures for photoproducts formed from the reactions of BrU with some aromatic amino acid derivatives mentioned previously are shown in Figure 16.

4. Synthesis

The brominated uracil derivatives can all be purchased commercially, leaving no need for synthesis. The triphosphates of 5-bromouridine (Gott et al., 1991) and 5-bromo-2′-deoxyuridine (Khalili et al., 1988) can be incorporated enzymatically with no reported problems. Furthermore, the phosphoramidite of 5-bromo-2′-deoxyuridine can be incorporated synthetically using a DNA synthesizer (Hicke et al., 1994). Table 7 lists commercial sources for bromouridine, bromouridine triphosphate, and 5-bromodeoxyuridine phosphoramidite.

5. Advantages/Disadvantages of Using Bromouridine Substituted Nucleic Acids in Photocross-Linking

Bromouridine has several advantages

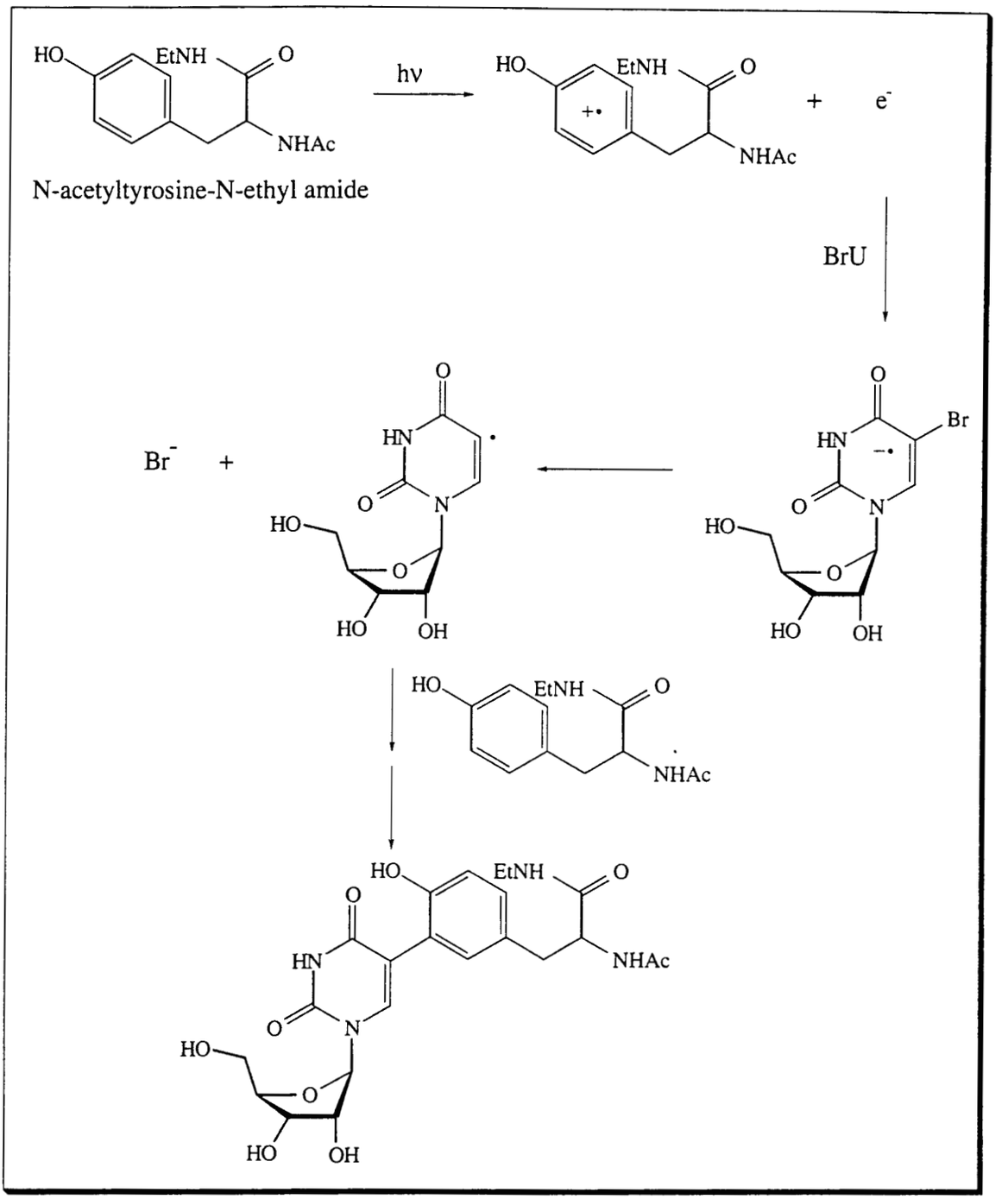


FIGURE 15. Mechanism for photocoupling of a tyrosine derivative to 5-bromouridine.

1. The bromouracil chromophore can be excited at wavelengths above 300 nm, thereby minimizing protein photodamage.
2. Photocross-linking yields have been reported as high as 70% (Katouzian-Safadi and Charlier, 1994).
3. Bromouracil is similar in size to thymine, thereby not disturbing significantly the native structure of the complexes involving DNA.
4. Many mechanistic studies have been carried out using BrU and various amino

TABLE 6
Examples of Some Amino Acids That Have Been Shown to Photocross-Link with Bromouracil, Bromouridine, or 2'-Deoxybromouridine

Amino acids found to cross-link to BrU	Ref.
Tyr	Willis et al., 1994; Liu et al., 1994; Allen et al., 1991; Dietz and Koch, 1987; Hicke et al., 1994
Trp	Dietz and Koch, 1987; Ito et al., 1980;
His	Katouzian-Safadi and Charlier, 1994; Dietz and Koch, 1987; Hicke et al., 1994
Cystine	Dietz and Koch, 1989
Lys	Weintraub, 1973
Ala	Blatter et al., 1992

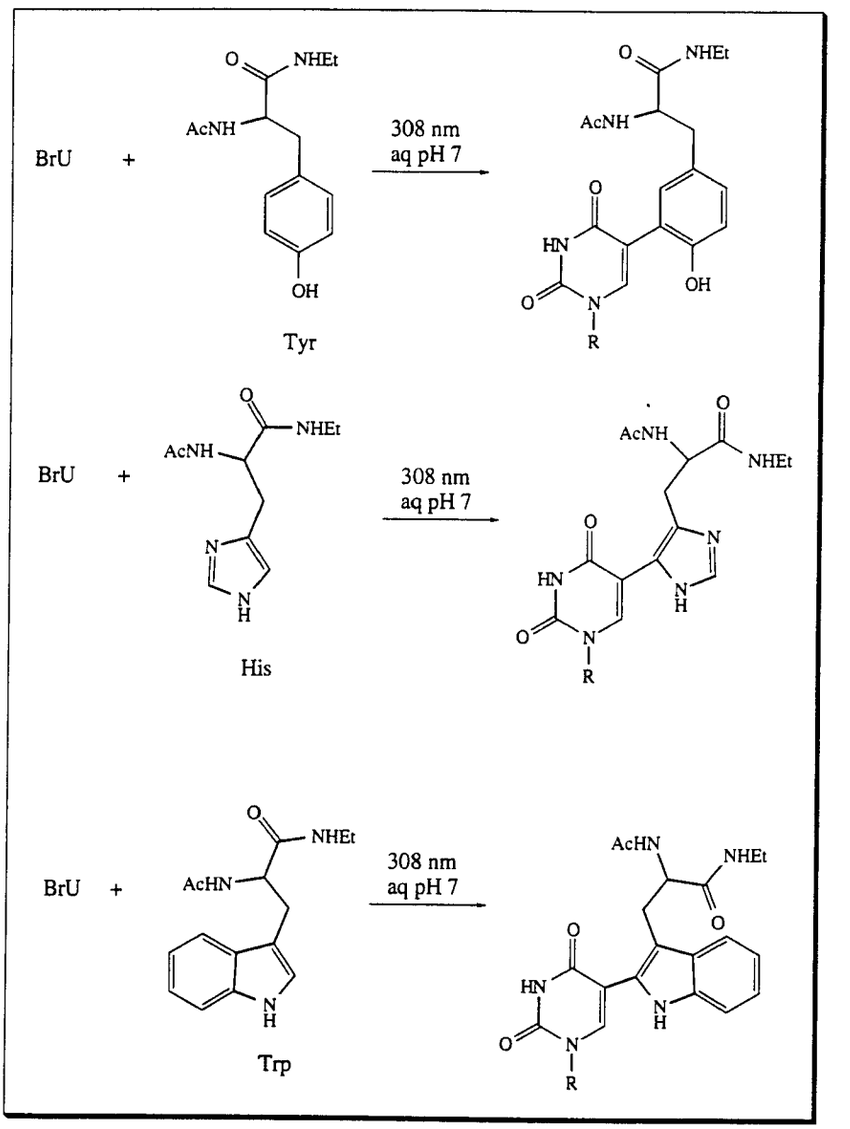


FIGURE 16. Products of photocoupling 5-bromouracil and/or 5-bromouridine to various aromatic amino acid derivatives.

TABLE 7
Table of Sources for the Purchase of 5-Bromouracil Derivatives

Chromophore	Source	Ref.
5-Bromouridine 5-Bromo-2′-deoxyuridine	Sigma Chemical Co., St. Louis, MO	Norris et al., 1996; Shetlar et al., 1991
5-Bromouridine triphosphate	Sigma Chemical Co., St. Louis, MO	Willis et al., 1994; Gott et al., 1991
5-Bromo-2′-deoxyuridine phosphoramidite	Glen Research, Sterling, VA; Cruachem, Inc., Dulles, VA	Hicke et al., 1994

acid derivatives, suggesting a plausible pathway for photocross-linking in the macromolecular system.
5. The chromophore can be synthetically or enzymatically incorporated into DNA or RNA. Solid-state synthesis allows specific single substitution.

Some of the disadvantages of using bromouracil in photocrosslinking nucleoprotein complexes are

1. If the vinyl radical is produced by excitation at shorter wavelengths (i.e., 254 nm), single-strand breaks can occur (Hutchinson and Köhnlein, 1980; Cook and Greenberg, 1996), which would lower cross-linking yields.
2. Lower yields are obtained when compared with the other halopyrimidines (5-iodouridine and 5-iodocytidine).
3. For reasonable cross-linking yields, the bromouracil chromophore must be near an oxidizable amino acid residue, basically an aromatic or heteroaromatic one or one bearing a sulfur atom.
4. Irradiations in the region of 300 to 315 nm still lead to some protein damage due to absorption of other chromophores in the nucleoprotein complex.

E. 5-Iodouridine and 5-Iodocytidine-Substituted Nucleic Acids

1. Photocross-Linking of 5-Iodouridine and 5-Iodocytidine-Substituted Nucleic Acids

Bromouridine has been the most popular of the halopyrimidines used in photocross-linking studies of nucleoprotein complexes. However, irradiations just above 300 nm can still cause unwanted photodamage from excitation of other light-absorbing chromophores in the complex. Substitution of uridine for 5-iodouridine (IU), and cytidine for 5-iodocytidine (IC) (Figure 17), can help to minimize this problem in part through more intense absorption at longer wavelengths. Although iodine is larger than bromine, it is only slightly larger than a methyl group with a Van der Waals radius of 2.15 Å. At least some single substitutions in both DNA and RNA do not perturb the specific protein-binding constant (Willis et al., 1993). Multiple substitutions in RNA, however, can cause a significant increase in the K_d (Meisenheimer et al., 1996; Jensen et al., 1995).

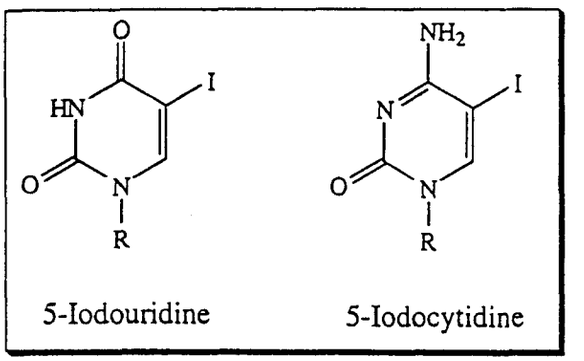


FIGURE 17. 5-Iodouridine and 5-iodocytidine chromophores.

Recently, both IU and IC have been used in nucleoprotein photocross-linking. Photocross-linking of an RNA hairpin to the human U1A snRNP protein (Stump and Hall, 1995), a viral RNA to its associated R17 bacteriophage coat protein (Willis et al., 1993), *O. nova* telomeric DNA to telomere-binding protein (Willis et al., 1993), and *E. coli* RecA protein to single-stranded DNA (Malkov and Cameriniotero, 1995) were all carried out using IU substitution. IC photocross-linking was also tested in the MS2 bacteriophage system, where a viral RNA hairpin was photocross-linked to the MS2 coat protein (Meisenheimer et al., 1996). The percent yields of photocross-linking for the various systems are shown in Table 8. IU has also been used as a photocross-linking tool in a new methodology called "photoSELEX", which is a form of "covalent SELEX" (Jensen et al., 1995). SELEX is an *in vitro* selection of nucleic acid ligands for target molecules from a large combinatorial library and stands for systematic evolution of ligands by exponential enrichment (Tuerk and Gold, 1990). PhotoSELEX identifies nucleic acids that will bind with high affinity to a target protein and photocross-link to the target in high yield from a large combinatorial library of nucleic acids bearing photoreactive groups.

2. Mechanism of Photocross-Linking

The mechanisms of photocross-linking for IU and IC to an amino acid appear to be similar. Early mechanistic studies with IU and IC suggested bond homolysis of the carbon-iodine bond to form the uridin-5-yl or cytidin-5-yl radical, respectively, as the initial photochemical events (Rahn, 1992). Recent model photochemical reactions of IU and IC with a tyrosine derivative with 325-nm monochromatic excitation showed adduct formation and reduction to uridine and cytidine, respectively (Norris et al., 1996; Meisenheimer et al., 1996). Quantum yield measurements as a function of tyrosine concentration have indicated C-I bond homolysis as the primary photochemical event (Norris, C. et al., 1996). Adduct formation is proposed to result from addition of the uridin-5-yl or cytidin-5-yl radical to the π-system of tyrosine and reduction by hydrogen atom abstraction from tyrosine. The disadvantage of generating this reactive 5-yl radical in a nucleoprotein complex is that hydrogen atom abstraction can occur at neighboring sugar residues, causing possible strand breakage of the nucleic acid (Rahn and Sellin, 1983; Wood et al., 1991; Sugiyama et al., 1993).

Although the model studies indicate C-I homolytic bond cleavage as the initial

TABLE 8
Photocross-Linking Yields Obtained Using IU- and IC-Substituted Nucleic Acids

Nucleic Acid	Protein	Yield (%)	Ref.
IdU substituted telomeric DNA	Telomere-binding protein (Tyr and His)	70	Willis et al., 1993
IU substituted viral RNA hairpin coat	R17 bacteriophage protein (Tyr)	94	Willis et al., 1993
IU substituted RNA	Human U1A snRNP protein (Tyr and Phe)	58	Stump and Hall, 1995
IU substituted RNA	Human immunodeficiency virus type I Rev protein	40	Jensen et al., 1995
IU substituted single stranded DNA	*E. coli* Rec A protein (Met, Phe)	19	Malkov, and Cameriniotero, 1995
IC substituted viral RNA hairpin	MS2 bacteriophage coat protein (Tyr)	75–95	Meisenheimer et al., 1996

photochemical process, the mechanism might logically depend on the local environment. Interestingly, the nucleoprotein complexes that undergo high-yield photocross-linking, where the crystal structure is known, show a π-stacking arrangement between the pyrimidine and the aromatic amino acid residue at the cross-linking site (Meisenheimer et al., 1996; Stump and Hall, 1995). This π-stacking environment is ideal for an electron transfer process, which could occur if an oxidizable amino acid residue is stacked with a halopyrimidine. The resulting radical anion/radical cation pair can then easily go on to form a cross-link, possibly with little or no attendant hydrogen atom abstraction. A further observation that points to a photoelectron transfer mechanism is the lack of reactivity of iodinated bases, which do not exist in a π-stacking geometry with an aromatic amino acid residue (Meisenheimer et al., 1996).

Photocross-linking of the *O. nova* telomere has been studied with wild-type DNA, BrdU-substituted DNA (Hicke et al., 1994), and IdU-substituted DNA (Willis et al., 1993). With wild-type DNA, four cross-linking bands were observed by polyacrylamide gel electrophoresis, three to the α-chain (Tyr142, Tyr239, and His292) and one to the β-chain (Tyr134) of the DNA binding protein. The yield of each cross-link to the α-chain was enhanced by a specific BrdU or IdU substitution. Interestingly, the same primary cross-links were observed in all three experiments; the only difference was the yield of primary vs. secondary cross-linking. With IdU substitution, the yield was very high and almost no secondary cross-linking was observed. BrdU substitution produced an intermediate result. This observation together with the bromouridine-tyrosine (Figure 15) (Norris et al., 1996) and thymidine-tyrosine (Figure 6) model studies (Shaw et al., 1992) suggests that all three cross-linking reactions occur because of an initial photoelectron transfer probably within a nucleoside-amino acid residue π-complex. The yield of primary cross-links is then a function of the respective quantum yields and competitive photoreactions of other chromophores in the nucleoprotein complex. Yields with IdU substitution are particularly high because of several favorable factors, including longer-wavelength absorption and the excellent leaving group reactivity of iodide.

3. Selectivity of Photocross-Linking

The halopyrimidines, IU and IC, are known to cross-link to some of the same

amino acids as bromouridine. IU (Norris et al., 1996) and IC (Meisenheimer et al., 1996) have both been shown to couple to tyrosine in model reactions. Spectroscopic characterization of the photoadducts established cross-linking from the 5-position of uridine and the 5-position of cytidine to the phenolic ring of tyrosine, ortho to its hydroxyl group (Figure 18). The product generated has the same regiochemistry as the product isolated from the reaction of bromouracil with tyrosine. IU has also been shown to couple to the ortho, meta, and para positions of phenylalanine in a model reaction (Norris et al., 1996). In macromolecular systems, cross-linking of an IU or IdU residue to tyrosine (Willis et al., 1993; Willis et al., 1994), histidine (Willis et al., 1993), phenylalanine (Stump and Hall, 1995; Malkov and Cameriniotero, 1995) and methionine residues (Malkov and Cameriniotero, 1995) and cross-linking of an IC residue to a tyrosine residue (Meisenheimer et al., 1996) have all been demonstrated either directly or indirectly. Direct identification was by Edman sequencing and indirect identification was by comparison with the corresponding BrU or BrdU-induced cross-link identified by Edman sequencing.

4. Synthesis

5-Iodouridine and 5-iodocytidine can be purchased commercially (Table 9) in suitable forms for enzymatic (Willis et al., 1993; Meisenheimer et al., 1996) or solid-state synthetic (Willis et al., 1993) incorporation into nucleic acids.

5. Advantages/Disadvantages of Using IU- and IC-Substituted Nucleic Acids for Photocross-Linking

Several advantages to IU and IC for nucleoprotein photocross-linking are

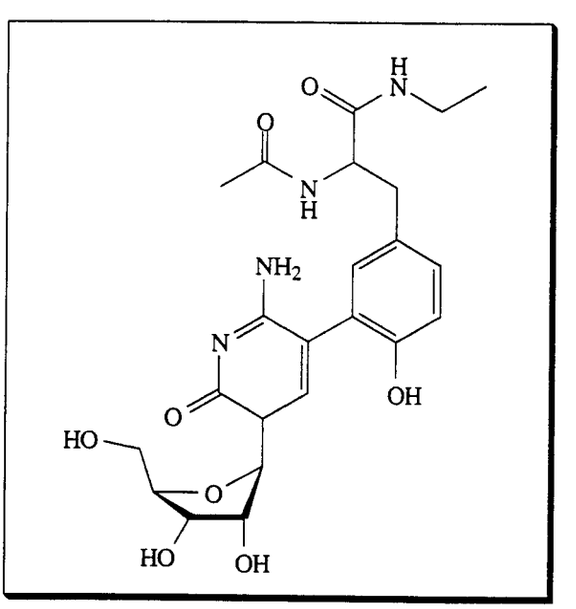


FIGURE 18. Adduct regiochemistry obtained when IC is irradiated in the presence of *N*-acetyltyrosine *N*-ethyl amide. The same regiochemistry is achieved with IU.

TABLE 9
Table of Sources for the Purchase of 5-Iodouracil and 5-Iodocytosine Derivatives

Reagent	Source	Ref.
5-Iodouridine/5-iodocytidine; 5-iodo-2′-deoxyuridine/5-iodo-2′-deoxycytidine	Sigma Chemical Co., St. Louis, MO	Norris et al., 1996; Meisenheimer et al., 1996
5-Iodouridine triphosphate; 5-Iodocytidine triphosphate	Sigma Chemical Co., St. Louis, MO	Willis et al., 1993; Jensen et al., 1995; Meisenheimer et al., 1996; Stump and Hall, 1995
5-Iodo-2′-deoxyuridine phosphoramidite; 5-iodo-2′-deoxycytidine phosphoramidite	Glen Research, Sterling, VA	Willis et al., 1993; Malkov and Cameriniotero, 1995

1. Both IUTP and ICTP can be purchased commercially.
2. High photocross-linking yields, even as high as 70 to 90%, can be obtained (Table 8).
3. IU and IC can be easily incorporated enzymatically or synthetically into nucleic acids.
4. Irradiations can be carried out at wavelengths > 300 nm (i.e., 325 nm) minimizing unwanted photodamage to the system.

Two potential concerns are

1. The size of the iodine may cause structural perturbation in the nucleoprotein complex, especially with multiple substitution. High-yield cross-linking can still be obtained provided the protein concentration is set significantly above the dissociation constant.
2. Although IU and IC will probably photocross-link with the same amino acid residues as BrU, reactivity with only Tyr, His, Met, and Phe have been implicated.

III. LIGHT SOURCES

Several different light sources can be used for achieving nucleoprotein photocross-linking. Use of one chromophore over another may be a function of the available light source (Table 10). For example, if nonsubstituted nucleic acids are used, a light source that emits below 300 nm (i.e., low-pressure, mercury phosphorescence lamp, 254 nm) is useful. If 4-SU is used, then light sources that emit above 300 nm (i.e., medium- or high-pressure mercury or mercury-xenon lamps or black lamps) would be appropriate. However, most of the light sources, excluding lasers, that emit above 300 nm, have broad emission band widths that tail to 200 nm. Even mercury phosphorescence lamps coated with a phosphor to shift the emission from 254 nm to 300, 302, 312, or 350 nm still emit some light at 254 nm. Therefore, filters are needed to block out the shorter wavelength light. This is important when performing photochemical experiments using substituted, as opposed to nonsubstituted, nucleic acids. The use of filters can minimize absorption of light by the wild-type nucleic bases and amino acid residues. Because absorption by filter materials such as polystyrene and Pyrex is a function of their thickness, observing the UV absorption of the filter with a spectrometer is recommended prior to use.

TABLE 10
Ultraviolet Light Sources

UV light source	λ_{max} (nm)	Comments	Source
Rayonet Photochemical Reactor®	254/ 300/350	Narrow band width/ broad band width	Southern New England Ultraviolet Co., Branford, CT
Transilluminator, low-pressure mercury or germicidal lamps	254/ 302/312	Narrow band width/ broad band width	Spectronics Corp., Westbury, NY; Fisher Scientific, Pittsburgh, PA; VWR, Inc., Denver, CO
Stratalinker®	302/312	Broad band width	Stratagene, La Jolla, CA
Medium-pressure mercury lamp	Continuum plus 313/366	Broad band width	Ace Glass, Vineland, NJ; Oriel Corp., Stratford, CT
High-pressure mercury and mercury/xenon lamps	Continuum plus 313/366	Broad band width	Oriel Corp., Stratford, CT
Excimer lasers: krypton fluoride/xenon chloride/xenon fluoride	248/308/351	Monochromatic; modest price	Lambda Physik, Acton, MA; Lumonics, Inc., Ontario, Canada; MPB Technologies, Dorval, Quebec, Canada
Neodymium (Nd) YAG laser	266/355	Monochromatic 3rd/4th harmonics	Continuum, Inc., Santa Clara, CA; Spectra-Physics, Mt. View, CA
Nd-YAG pumped dye laser	Tunable in the region 300–400 nm	Narrow band pass	Continuum, Inc., Santa Clara, CA
Nd-YAG pumped optical parametric oscillator (OPO)	Tunable in the region 300–350 nm	Narrow band pass	Opotek, Inc., Carlsbad, CA
Helium cadmium laser (HeCd)	325	Monochromatic	Omnichrome Chino, CA; Liconix, Inc., Santa Clara, CA
Nitrogen laser	337	Monochromatic	Laser Photonics, Inc., Orlando, FL; Laser Science, Inc., Newton, MA

Useful filters and their sources are summarized in Table 11. Lasers, although the more expensive choice, are superior light sources because their monochromicity permits more specific excitation of the desired chromophore. Table 10 lists various light sources, their wavelength at maximum emission, and where to find information regarding each source.

IV. ISOLATION OF THE CROSS-LINKED NUCLEOPROTEIN AND ITS CHARACTERIZATION

As mentioned previously, this section only provides general information on the isolation and characterization of nucleoprotein cross-links. For an excellent review on this subject see Williams and Konigsberg (1991). The isolation and characterization of cross-linked nucleoprotein involves several steps. First, the cross-linked material is digested with trypsin or other specific proteinase. Following enzymatic digestion, the cross-linked segment is isolated by either anion exchange HPLC (Williams and Konigsberg, 1991) or gel electrophoresis followed by electroblotting onto a membrane (Stump and Hall, 1995). Currently, the peptide is most commonly sequenced by Edman degradation. Recent developments in electrospray mass spectrometry (Valaskovic et al., 1996) bode well for the future of this technique in characterizing cross-linking sites. Mass spectral sequencing also has the potential for providing structural information about the molecular nature of the actual cross-link. At present, the cross-linking bond is assumed to be that predicted from model reactions. With any sequencing methodology, a high photocross-linking yield will be beneficial because of the significant material losses during isolation.

V. SUMMARY

Nucleoprotein photocross-linking is a useful structural tool for establishing contacts at the nucleic acid-protein interface. Knowledge of these contacts may facilitate subsequent crystallographic or NMR structure determinations. High-yield, high-specificity cross-linking may also prove to be

TABLE 11
Filters

Type of Filter	Use	Source
Polystyrene (petri dish)	Reduces transmission of wavelengths below 300 nm	Fisher Scientific, Pittsburgh, PA
Pyrex®	Reduces transmission of wavelengths below 300 nm	Corning Glass, Inc., Corning, N.Y.
Interference	Isolates narrow UV spectral region	Oriel Corp., Stratford, CT
Neutral density	Attenuates incident beam	Oriel Corp., Stratford, CT
Monochromators	Narrows bandwidth to as little as 10 nm	Oriel Corp., Stratford, CT

useful for the discovery of new proteins postulated to bind to specific nucleic acid sequences. Cross-linking has been achieved through one- and two-photon excitation of wild-type complexes and one- photon excitation of complexes bearing substitutions with photoreactive chromophores such as aryl azides, azidonucleosides, thiopyrimidines, and halopyrimidines. Examples of high cross-linking yields with each of these techniques have been reported; however, for each technique, the yield probably is a function of the local environment of the excited chromophore and its cross-linking mechanism. Success in cross-linking may require some trial and error because the local environment of the reactive chromophore or chromophores cannot be predicted easily.

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