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Unraveling the Photochemistry of the 5-Azido-2′-deoxyuridine Photoaffinity Label

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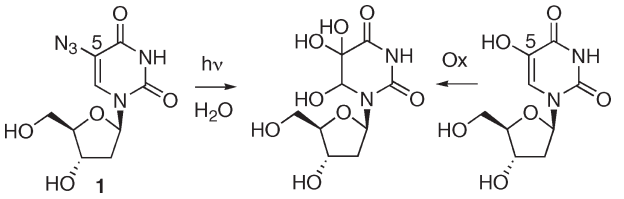
Received July 17, 2009



UV irradiation of 5-azido-2′-deoxyuridine in water provides up to seven products. All likely result from a pivotal azirene, formed by the intramolecular rearrangement of the initially formed nitrene, that undergoes nucleophilic addition at its C5 position. This study strongly suggests that only nucleophilic amino acid residues in close proximity are cross-linkable in photolabeling experiments by using the 5-azidouracil photophore.

5-Azidouracil nucleotides have long been used to isolate and characterize proteins containing either a uracil/thymine or DNA binding site through photocross-linking formation.[1,2] Conjugated to sugars, 5-azidonucleotides have also been employed to study numerous sugar transferase proteins.[3,4] More importantly, isolation and sequencing of photolabeled peptides resulting from the digestion of cross-linked proteins have allowed the mapping of peptide domains involved in binding sites.[2a,b,d,e,4a,4b,4d] Regretfully, the amino acid responsible for cross-linking has never been identified even though such information is crucial to dissect the atomic interactions and reconstruct the structural topography of the binding site. Understanding the photochemical behavior of the 5-azidouracil moiety would be particularly useful to decipher this important question as well as to understand its mechanism of photocross-linking that still remains unknown. It is generally assumed that the photochemistry of azidonucleotides is mostly similar to that of aryl azides and consequently that amino acid residues in their immediate vicinity are indiscriminately cross-linked by the UV-generated short-lived nitrene intermediates.[1,2b,2f,3a,4c] The recent discovery that the photoreactive intermediate of 8-azidoadenosine was not its corresponding nitrene but a diazaquinodimethane derivative[5] and our experience in the photochemistry of azidonucleosides[6] prompted us to investigate the photochemistry of 5-azido-2′-deoxyuridine **1**.[7]

Herein, we demonstrate that in aqueous solution, **1** photochemically reacts with water to provide a complex mixture of unstable products which are all likely to derive from a common azirene precursor. The photochemical behavior of **1** in methanol fully supports our reactive scheme.

Exposure of an aqueous solution of **1** (1.8 mM) to UV light ($\lambda > 290$ nm) led to the formation of up to seven products (**2a**, **b**, **3a**,**b**, **4**, **5a**,**b**) depending on the pH of the solution (Scheme 1). At pH ca. 4, an equimolar mixture of two products (**2a**,**b**) (S5, Supporting Information) was quantitatively formed. These photoproducts were identified as the diastereomeric nucleoside derivatives of hydrated isodialuric acid by comparison of their ^{1}H NMR spectrum with those of authentic standards prepared by oxidation of 5-hydroxy-2′-deoxyuridine[8] with use of Na_2IrCl_6 (S6, Supporting Information).[9] Irradiation of an aqueous solution of **1** at pH 6−7 led to the formation of five additional products (**3a**, **b**, **4**, **5a**,**b**) (S7, Supporting Information) that could be isolated by HPLC. Their respective distribution evolved upon standing in aqueous solution concomitantly to the disappearance of **2**. These products were identical with those formed when the pH of the aqueous solution of **2a**,**b** was raised from 4 to 6−7 (S8, Supporting Information). Consequently, **3**−**5** clearly resulted from the transformation of **2**.

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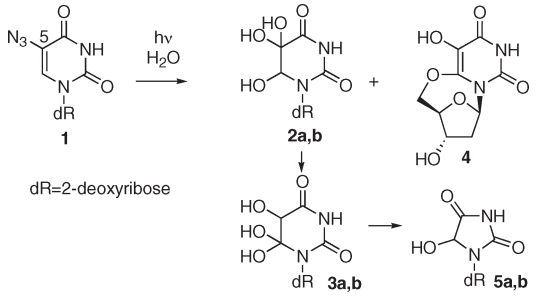
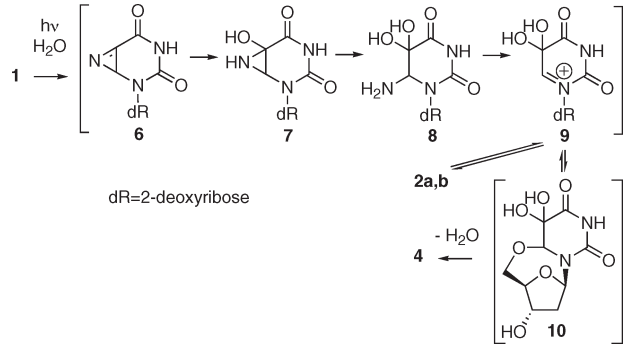
DOI: 10.1021/jo901544a Published on Web 08/04/2009

SCHEME 1



SCHEME 2

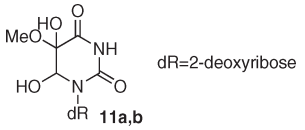


Interestingly, isodialuric acid nucleosides **2a**,**b** are known to lead to the corresponding dialuric and 5-hydroxyhydantoin derivatives.[8] Analysis of ^{13}C NMR literature data[8] allowed the identification of **3a**,**b** and **5a**,**b** as the diastereomeric nucleoside derivatives of dialuric acid and 5-hydroxyhydantoin, respectively.

Compound **4** has not been previously reported. Its O^6-5′ cyclic structure (HRMS (ESI, $(M + Na)^+$) calcd for $C_9H_{10}N_2O_6Na$ 265.0437, found 265.0446) was supported by the characteristic H5′/H5″ signals (δ 3.96, d, $J = 12.7$ Hz and δ 4.60, d, $J = 12.7$ Hz)[10a] and H1′ signal (δ 6.78, dd, $J = 8.5$, 2.2 Hz).[10] Further evidence was brought by the NMR LR coupling between C6 (δ 148.9) attributed to its ^3J coupling with H1′ and H5′/H5″. The remaining quaternary carbons at δ 124.3, 150.2, and 164.8, were attributed to C5, C2, and C4, respectively. Whereas oxidation of 5-hydroxypyrimidine derivatives has been well studied,[8,9] formation of cyclonucleosides in this context has never been reported.

A possible mechanism accounting for the formation of the isodialuric acid derivatives **2a**,**b** and the cyclic nucleoside **4** is outlined in Scheme 2. Photochemical excitation of **1** likely gives rise to the nitrene singlet that inserts into the C5−C6 double bond leading to the unstable azirene **6**. Nucleophilic addition of water at position C5 of **6** gives rise to the aziridine intermediate **7**, which undergoes water induced nucleophilic ring-opening at the C5 position to provide the unstable 5,5-dihydroxy-6-aminodihydrouracil **8**. This cascade of reaction is supported by the similar photoreactivity in H_2O of a 6-azidouracil derivative and the chemical reactivity of its photoproduct.[11] Namely, it has been proposed that photoexcitation of an aqueous solution of 6-azido-1,3,5-trimethyluracil gives rise to the corresponding azirene that is hydrolyzed to the unstable 5-amino-6,6-dihydroxy-1,3,5-trimethydihydrouracil. Then, in our case, **8** equilibrates with the iminium **9**, which undergoes either major hydration to provide **2** or minor intramolecular C5′ hydroxyl addition to transiently provide **10** that subsequently dehydrates to give **4**. The 1/1 ratio of the photochemical formation of isomers **2** at pH 4 indicates that water addition at C6 of iminium **9** is not stereoselective. Interestingly, a similar water addition/intramolecular 5′-hydroxyl addition competition at C6 of a zwitterionic intermediate has been observed in the course of the 254 nm irradiation of 2′-deoxyuridine.[10b] In our case, the presence of OH groups at C5 likely drives a dehydration reaction that precludes isolation of **10**.

Then, we reasoned that if this suggested mechanism was correct, irradiation of **1** in MeOH should provide O^5-methyl analogues of **2**. This should impede the α-hydroxyketone isomerization reaction and the subsequent ring contraction reaction. Pleasingly, exposure of a methanolic solution of **1** to UV light led most exclusively to compounds **11** as two diastereomers (S3,18, Supporting Information). Traces of **4** were also isolated. Structural analysis was carried out on the major and most stable isomer **11a** (HRMS (ESI, $(M + Na)^+$) calcd for $C_{10}H_{16}N_2O_8Na$ 315.0804, found 315.0805). The major difference between the NMR data of **11a** and **2** was the presence of a OCH_3 signal (δH 3.47; δC 58.9). The presence of a methine at C6 (δH 4.73; δC 89.9) and a $^5OCH_3$ was attested by a ^3J H6−C1′ and a ^3J $^5OCH_3$−C6 coupling, respectively.



It is likely that MeOH adds to **6** to provide a methoxyaziridine that during HPLC purification undergoes hydrolysis then C6-amine elimination/water addition to provide **11**. The presence of only two diastereomers suggests that either some steps could be diastereospecific or that initial formation of the four possible diastereomers of **11** could be followed by ring chain tautomerism at N1−C6 with formation of the more stable cis or trans diastereomers as observed for 6-hydroxylated-5,6-dihydropyrimidine derivatives.[12] Formation of **4** in MeOH can be explained either by acetal hydrolysis of the O^5-methyl analogue of **10** or methanol elimination.

This work definitively elucidates the reported self-photodestruction of the 5-azidouracil photolabel[1a,13] that likely originates from an intramolecular rearrangement of the initially produced nitrene. Also, the instability of some photolabeled peptides[4a] can be explained by a reversible cross-link formation at position 6 of iminium **9**. More importantly, our results seriously question the general

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expectation that any amino acid residue close to the 5-azidouracil moiety is cross-linkable. Instead, our results support the idea that photoexcitation of the 5-azidouracil moiety leads to an azirene intermediate that cross-links only to nearby nucleophilic amino acid residues. Interestingly, detailed examination of protein/DNA (or protein/pyrimidine nucleotide) complexes studied by X-ray crystallography[14−16] and photoaffinity labeling[2b,d,e] consistently reveals the presence of a nucleophilic amino acid (Arg, Lys, Tyr) among the closest residues to the C5 position of the pyrimidine moiety. Finally, since **1** can also generate DNA oxidation products, our results widely open a new opportunity for this kind of probe.

Experimental Section

General. ^{1}H Chemical shifts (δ) are reported in ppm relative to residual HOD peak set at 4.8 ppm in D_2O. ^{13}C chemical shifts are reported in ppm relative to an external capillary standard of dioxane (δ 67.8).

UV Irradiation. Irradiation experiments were performed with a Pyrex-filtered light emitted by a 150 W high-pressure Hg lamp and HPLC grade solvents. Solutions were degazed under nitrogen for 30 min prior to UV exposure. For small scale irradiation experiments, aqueous solutions of **1** (5 mg in 10 mL) were exposed for 3 min to the UV light then lyophilized. Acetic acid was used to acidify the aqueous solution for irradiation experiments performed at pH 4. Increasing the pH of the acidic irradiated solutions to 6−7 was achieved with K_2CO_3. For semipreparative scale irradiation, a solution of **1** (30 mg in 60 mL) was exposed for 5 min to the UV light then lyophilized (aqueous solutions) or evaporated (methanolic solutions).

HPLC Purification. The aqueous photolysate of **1** (95 mg) was purified on an Atlantis Prep T3 (5 μm, 250 × 10 mm) column with 0.05 M aqueous ammonium acetate as eluent for 10 min then a multistep gradient of CH_3CN in 0.05 M aqueous ammonium acetate (0% to 1% in 5 min, 1% to 20% in 5 min, isocratic in 20% for 5 min, then 20% to 0% in 5 min) at a flow rate of 4 mL/min. Peaks at 4.7 (**3a**), 5.2 (**3b**), 8.9 (**5a**), 9.9 (**5b**), 25.1 (**4**), and 29.1 min (**1**) were collected and lyophilized. The methanolic photolysate of **1** (90 mg) was purified on a SunFire Prep C18 (5 μm, 250 × 10 mm) column with use of a multistep gradient of CH_3CN in 0.05 M aqueous ammonium acetate (1% to 2.5% in 22.5 min, 2.5% to 20% in 5 min, isocratic in 20% for 5 min, then 20% to 1% in 5 min) at a flow rate of 4 mL/min. Peaks at 8.3 (**11a**), 9.9 (**11b**), 12.0 (**4**), and 30.6 min (**1**) were collected and lyophilized.

5-Hydroxy-*O*^{6},5′-cyclo-2′-deoxyuridine (4): HRMS (ESI) *m*/*z* calcd for $C_9H_{10}N_2O_6Na$ $[M + Na]^+$ 265.0437, found 265.0446. UV (λ max, H_2O) 266 nm. ^{1}H NMR (500 MHz, D_2O) δ 6.78 (dd, J = 8.5, 2.1 Hz, 1H), 4.72 (dd, J = 6.6 Hz, 1H), 4.61 (dd, J = 12.7, 1.2 Hz 1H), 4.42 (br s, 1H), 3.96 (d, J = 12.7 Hz, 1H), 2.58 (ddd, J = 15.3, 6.6, 2.1 Hz, 1H), 2.36 (dd, J = 15.3, 8.5 Hz, 1H). ^{13}C NMR (125.7 MHz, D_2O) δ 164.8, 150.2, 148.9, 124.3, 87.3, 86.5, 78.7, 74.2, 42.3.

***N*^{1}-(2-Deoxy-β-D-*erythro*-pentofuranosyl)-*O*^{5}-methylisodialuric acid (11a, major isomer):** HRMS (ESI) *m*/*z* calcd for $C_{10}H_{16}N_2O_8Na$ $[M + Na]^+$ 315.0804, found 315.0805). ^{1}H NMR (500 MHz, D_2O) δ 6.05 (dd, J = 8.1, 6.2 Hz, 1H), 4,74 (s, 1H), 4.34 (ddd, J = 6.8, 3.5, 3.2 Hz, 1H), 3.93 (ddd, J = 5.5, 4.0, 3.5 Hz, 1H), 3.76 (dd, J = 12.1, 4.0 Hz, 1H), 3.71 (dd, J = 12.1, 5.5 Hz, 1H), 3.47 (s, 3H), 2.31 (ddd, J = 13.9, 8.1, 6.8 Hz, 1H), 2.15 (ddd, J = 13.9, 6.2, 3.2 Hz, 1H). ^{13}C NMR (125.7 MHz, D_2O) δ 170.9, 153.5, 89.9, 87.3, 86.9, 86.4, 71.9, 62.9, 58.9, 38.6.

***N*^{1}-(2-Deoxy-β-D-*erythro*-pentofuranosyl)-*O*^{5}-methylisodialuric acid (11b, minor isomer):** ^{1}H NMR (300 MHz, D_2O) δ 6.05 (dd, J = 8.1, 6.3 Hz, 1H), 4.93 (s, 1H), 4.42 (dt, J = 6.5, 3.0 Hz, 1H), 3.91 (ddd, J = 4.9, 3.6, 3.0 Hz, 1H), 3.80 (dd, J = 12.3, 3.6 Hz, 1H), 3.72 (dd, J = 12.3, 4.9 Hz, 1H), 3.55 (s, 3H), 2.31 (ddd, J = 13.9, 8.1, 6.5 Hz, 1H), 2.20 (ddd, J = 13.9, 6.3, 3.0 Hz, 1H).

Acknowledgment. ICSN/CNRS and Region Champagne−Ardenne are acknowledged for a doctoral fellowship to S.G. and financial support, respectively. We thank J. Tomasina for preliminary studies.

Supporting Information Available: Copies of HPLC chromatograms of the photolysis of **1**, NMR spectra of **1**, the photolysis of **1**, **2**−**5**, and **11**. This material is available free of charge via the Internet at http://pubs.acs.org.

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