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The Photochemistry of Thymine in Frozen Aqueous Solution: Trimeric and Minor Dimeric Products

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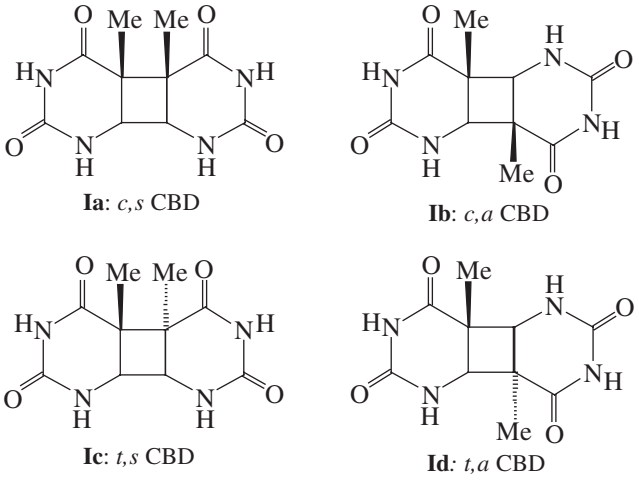
ABSTRACT

Early work identified three compounds, namely the *c,s* cyclobutane dimer, the so-called (6-4) photoproduct (5-hydroxy-6-4′-(5-methylpyrimidin-2′-one)-5,6-dihydrothymine) and a trimer hydrate, as products formed upon UV irradiation of thymine in frozen aqueous solution. More recent work has shown that an (α-4) product, namely α-4′-(5′-methylpyrimidine-2′-one)-thymine, is a likely product formed under these reaction conditions. During a thorough reinvestigation of the photochemistry of Thy in ice at −78.5°C, we found that a variety of other products could be detected. In addition to the *c,s* dimer, the other three known cyclobutane dimers, namely the *c,a*, *t,s* and *t,a* forms, are produced, although in considerably smaller amounts. The so-called "spore product" of thymine (5,6-dihydro-5-(α-thyminyl)thymine) is likewise formed. Two other dimers have been identified as minor products; one of these has been determined to be 5-(thymin-3-yl)-5,6-dihydrothymine and the other has been tentatively assigned to be a (5-4) adduct (6-hydroxy-5-4′-(5-methylpyrimidin-2′-one)-5,6-dihydrothymine). Compounds with the behavior expected of true trimeric compounds have been isolated *via* HPLC and characterized by mass spectrometry and photochemical behavior. One of these materials, putatively containing an oxetane ring, decomposes thermally to a secondary trimeric product that is then converted into the known trimer hydrate.

INTRODUCTION

The field of study dealing with the photochemistry of nucleic acids and their components has developed immensely over the past 50 years or so, with a large variety of nucleobase and nucleoside photoproducts being discovered (for reviews see, *e.g.* [1–4]). Among the first photoreactions studied were those undergone by thymine (Thy) in frozen aqueous solution. This particular system was particularly intriguing as a system for investigation, as photolysis with 254 nm light from a germicidal lamp leads to a high percentage conversion of parent Thy into a compound that could be reconverted, after thawing, almost completely back to Thy by irradiation with the same light source. The photoreversible product of this reaction was first isolated and identified as a Thy dimer by Beukers and Berends (5) and Wang (6), although its unequivocal structural identification as the *c,s* cyclobutane dimer (CBD) was accomplished somewhat later (7–9). (See Scheme 1 for the structures of the four possible CBDs of Thy.)



Scheme 1.

In addition to the *c,s* CBD, evidence for two other products was noted early on by Smith (10). One was a compound that absorbed light in the region above 300 nm. The structure of this compound was later shown to correspond to the so-called thymine (6-4) adduct (5-hydroxy-6-4′-(5-methylpyrimidin-2′-one)-5,6-dihydrothymine) (11); one of the enantiomers of this compound is shown as **II** in Scheme 2. The other product appeared to be trimeric in character, being split by irradiation with 254 nm light to form the Thy *c,s* CBD (**Ia**) and Thy. Gunther and Prusoff (12) confirmed this latter result. This trimeric compound was not characterized further. However, the structure of a thermal secondary product, a trimer hydrate, has been chemically and structurally characterized *via* NMR and X-ray diffraction (13–15). The stereoconfiguration of this product is shown as **III** in Scheme 2; the numbering system is that employed by Flippen and Karle (14). Using this numbering, the configuration of this compound can be described as 14R,25R,26R. This product was split by UV light to form the (4,6) adduct **II** and Thy.

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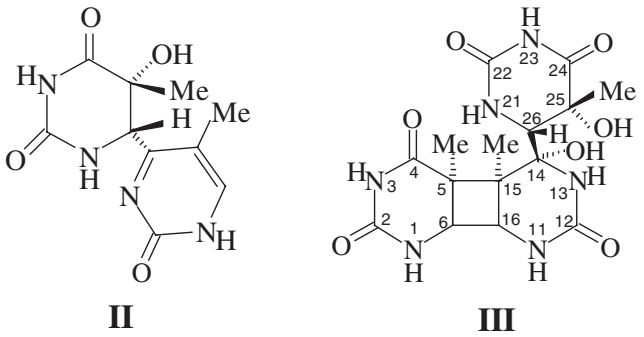


II **III**

Scheme 2.

Until recently, there were no reports of other photoproducts being formed upon irradiation of Thy in ice. In particular, there was no evidence in the literature indicating that other CBDs of Thy are produced. Both Fisher and Johns (16), in their comprehensive review of the literature on the cyclobutane photodimers of nucleic acid components, and Varghese (17), in his extensive review on the photochemistry of nucleic acids, indicate unequivocally that the *c,s* dimer is the only CBD formed by Thy *via* irradiation in the frozen aqueous state. Indeed, the study of the photochemistry of Thy in frozen aqueous solution was dormant for many years, probably because the apparent advanced state of knowledge concerning this system suggested that there was little novel information remaining to be discovered. However, in 2012 a so-called "α-4" product was identified as a likely component of reaction mixtures resulting from irradiation of Thy in ice (see Results and Discussion). We present findings here indicating that, in addition to the products mentioned above, a variety of other photoproducts are formed in this system. In the following paragraphs, we describe the results of our efforts to identify some of those products that are present immediately after irradiation of Thy in frozen solution at dry ice temperature. It should be noted that after concentration of irradiated solutions by rotatory evaporation, a variety of secondary thermal reaction products appear. Although we have characterized a few of these compounds, no effort has been made to identify the remainder of these secondary products.

MATERIALS AND METHODS

General aspects. Thymine was obtained from Aldrich (Milwaukee, WI). HPLC solvents were from Fisher (Fair Lawn, NJ), whereas NMR solvents were from Aldrich. Preparative separations were done on a Shiseido Capcell UG120 10 × 250 mm column (5 μm particle size [Yokohama, Japan]) (Column A), whereas analytical HPLC utilized a Capcell UG120 4.6 × 150 mm column (5 μm particle size) (Column B). A Varian Microsorb Phenyl column (4.6 × 50 mm, 5 μm particle size) (Column C) was utilized for some final repurifications, whereas several analyses were done with Column D, a Microsorb Amino column (4.6 × 150 mm column, 5 μm particle size). The HPLC set-up used was a Rainin binary gradient pumping system (Emeryville, CA) coupled to a Hewlett-Packard 1040A diode array detector (Palo Alto, CA). NMR spectra were run at 600 MHz on a Varian INOVA NMR spectrometer (Palo Alto, CA). The following instruments were used to obtain mass spectra: an Applied Biosystems 4700 mass spectrometer (Foster City, CA) operating in the reflector mode, a Waters Micromass ZQ4000 instrument (Beverly, MA) and a Sciex API300 triple quadrupole electrospray instrument (Toronto, ON, Canada).

Irradiation methods. Irradiations using light mainly at 254 nm were done with Spectronics BLE 1T-155 lamps (Westbury, NY); irradiations with light centered at 312 nm were done with Spectronics BLE 1T-158 lamps. In preparative irradiations at short wavelengths, the 1T155 lamps were unfiltered; studies on a smaller scale, using a Vycor filtered portion of the lamp surface, showed that there was no significant difference between samples irradiated with the unfiltered output of these lamps and those irradiated with filtered lamp output.

Photochemical irradiations were done at 254 nm on batches of frozen aqueous Thy solution. Portions to be irradiated (usually 250 mL or 500 mL) were placed in a 13 × 9 inch nonstick baking pan (Bradshaw International, Rancho Cucamonga, CA) and then frozen on dry ice slabs (−78.5°C). During the freezing process, a Pyrex baking dish (4 quart, 15 × 10 inches; Corning Incorporated, Corning, NY) was placed on top of the pan in which the solution was contained; this helped avoid frosting of the surface of the solution. During each irradiation, four lamps (contained in two Spectroline X-Series lamp housings [Spectronics, Westbury, NY]) were placed on top of the pan containing the frozen solution. The fluence at the surface of the frozen aqueous layer in the pans averaged about 25 J m^{-2} s^{-1}, as measured using a Spectronics DM-254N Ultraviolet Meter (Spectronics).

After irradiation was completed, the pan containing the irradiated solid solution was floated in a second 13 × 9 inch baking pan, filled halfway with hot tap water until thawing occurred. Immediately after the last bit of frozen solution had disappeared, the solution was transferred into a flask and refrigerated. (Alternatively, in some cases, the thawed solution was immediately refrozen for further irradiation.)

Preparation of authentic samples of thymine cyclobutane dimers. For the studies described below, we required an authentic sample of each of the Thy CBDs. Acetone photosensitization was used for preparation of these samples. The applicable protocols used for making and isolating these compounds are described in Appendix S1 (Section A1).

Identification of potentially interesting photoproducts via HPLC *analysis of a freshly irradiated frozen solution of Thy.* For purposes of identification of products of potential interest, prior to workup for preparative separation, a 2 mL sample (of a 500 mL solution [2 mM] that had been freshly irradiated for 60 min with 254 nm light on dry ice and then thawed [as described above]) was injected on a semipreparative Capcell reverse phase column (Column A). Doubly distilled water and methanol were the eluents and the flow rate was 4.0 mL min^{-1}. The following gradient (termed Gradient A) was used: 0 min: 1.5% MeOH; 5 min: 1.5% MeOH; 11 min: 20% MeOH; 15 min: 20% MeOH; 15 min 15 s: 1.5% MeOH; 20 min: 1.5% MeOH. The HPLC traces at three wavelengths, resulting from this chromatographic run, are shown in Fig. 1. Numbers within the trace identify the peaks corresponding to substances that are of interest in the present work. The chemical identity (or identities) of the compounds eluting within each peak are indicated in the legend to Fig. 1 in cases where these substances have been identified; details of the establishment of these identities are given in Results and Discussion.

Protocols for irradiation of frozen thymine solutions on a preparative scale and their workup for preparative HPLC. For isolation of the various photoproducts of interest, irradiations were done using two different concentrations of Thy. For most irradiations, 500 mL batches of frozen Thy at a concentration of 2 mM were irradiated for 60 min. Under these conditions, about 88% of the parent Thy is converted into products. Typically, the resultant solution was concentrated 100-fold *via* rotatory evaporation of the irradiated solution at 40°C and then filtered. For a number of photoproducts, discussed below, such preparations provided enough material for characterization. However, for some photoproducts produced in small yields, we used an alternative procedure to obtain sufficient amounts for structural characterization. In this case, 10 L of solution was irradiated and concentrated 250-fold in several stages, using rotatory evaporation at 40°C. After each stage, the concentrate was chilled by placing it on ice and then filtered using a Millipore HAWP 0.45 micro filter; this eliminated much of the *c,s* CBD, which is overwhelmingly the major product. The resulting 40 mL of concentrate was refrigerated until it was subjected to preparative chromatography. The relatively long times required to carry out the irradiation of 10 L of parent solution (*ca* 3 days), as well as the amount of time required for rotatory evaporation of the resultant 10 L of irradiated solution, does present some disadvantages. Indeed, one of the compounds produced (that corresponding to peak **7** in Fig. 1) is lost in significant amounts (through decomposition) when this irradiation/workup protocol is employed. In addition, much of the product found in peak **12** (which is trimeric in nature) decomposes during the time required for workup. This compound, however, was produced in sufficient yield that a single irradiation of a batch of 500 mL for 60 min sufficed for producing enough of this product for our purposes. As this compound decomposes upon standing to form the product eluting in peak **13**, similar runs were used to obtain this compound.

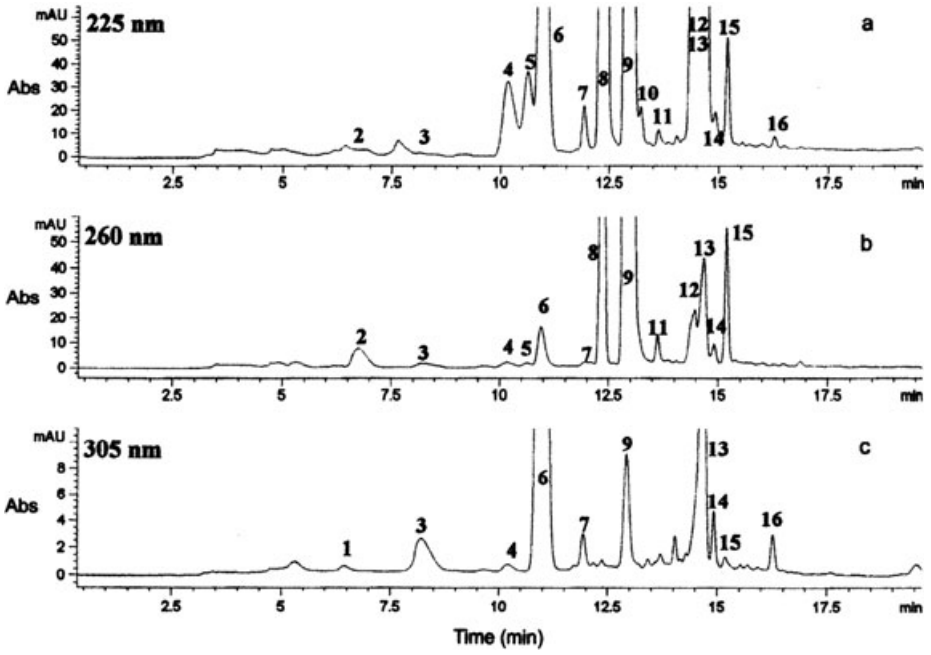


Figure 1. The HPLC traces at 225, 260 and 305 nm for an injection of 2 mL of thymine solution, freshly irradiated at 254 nm on dry ice for 60 min, thawed, filtered and chromatographed as described in the body of the article. The identity of the compound(s) eluting within each peak is as follows: **2**: 5-hydroxymethyluracil (**IV**); **3**: 5-formyluracil (**V**); **4**: the *c,a* thymine cyclobutane dimer (**Ib**); **5**: trimer hydrate isomer (**III**); **6**: 6–4 photoadduct (**II**); **7**: compound with an absorption spectrum similar to **II**; **8**: thymine *c,s* cyclobutane dimer (**Ia**) + dihydrothymine; **9**: thymine + *t,a* thymine cyclobutane dimer (**Id**) + putative isomer of **III**; **10**: thymine *t,s* cyclobutane dimer (**Ic**); **11**: a compound with UV spectral similarities to **15** and identified as **VIII**; **12**: thymine trimer; **13**: thymine trimer decomposition product; **14**: **IX** (compound with double maxima in absorption spectra); **15**: the thymine "spore product" (**X**); **16:** decomposition product arising from material in peak **7**. Peak **1** has an absorption spectrum with some similarity to that of **II**, but was produced in an insufficient amount for structural characterization.

To isolate sufficient amounts of the compound corresponding to peak **7** for characterization, it was desirable that the amount of time between irradiation and chromatography be kept at a minimum. Of particular concern was minimization of the amount of time that the irradiated solution spent in contact with the elevated temperatures used in rotatory evaporation. In this situation, 1.5 L of 20 mM aqueous Thy solution was irradiated in the frozen state in 250 mL batches. After 45 min irradiation time, the solution was thawed, stirred and refrozen; it was then irradiated for an additional 45 min. Under these conditions, about 50% of the parent Thy was, on average, converted into products. The resulting solution was filtered to remove Thy *c,s* CBD precipitate suspended in the solution and then concentrated 250-fold in stages as described above. After filtration, the resulting 6 mL of solution was chromatographed as soon as feasible. It should be noted that this protocol is not one of choice for production of the compound corresponding to peak **12**, as the amount present in irradiated solution is relatively much smaller (about 30-fold), as compared with the amount produced when 2 mM solution is irradiated for 60 min to the higher extent of conversion of 88%. (This figure is based on comparison of the integrated area for peak **12** obtained from an HPLC chromatogram run at 225 nm when 200 μL of freshly irradiated 20 mM solution was injected to the analogous area obtained for peak **12** when 2 000 μL of 2 mM solution that had been freshly irradiated for 60 min was injected.)

RESULTS AND DISCUSSION

Introductory remarks

In the early parts of this section, we describe the separation, purification and characterization of the various photoproducts formed when Thy is irradiated in the frozen state as a 2 mM solution (conditions under which considerable amounts of trimeric products are formed) and as a 20 mM solution (conditions under which the amounts of trimeric products produced are small). We then provide detailed discussions of certain aspects of photoinduced trimer formation and trimer thermal decomposition. Finally, we briefly discuss the observation of non-CBD products formed when solutions of Thy are irradiated in liquid solution.

First pass separation of the various stable products produced when a large volume (10 L) of aqueous thymine (2 mM) is irradiated in frozen solution at dry ice temperature

For separation of the 40 mL of concentrated solution, prepared as described in Materials and Methods, a gradient was used in which water and methanol were the eluents; the flow rate was 4 mL min^{-1}. Two milliliter volumes of concentrate were injected on the column; each volume was filtered immediately prior to injection using Costar Spin-× 0.22 μM nylon centrifuge tube filters (Corning Inc.). A number of gradients were tried in our studies, but the most effective in achieving both a good first pass separation and in eluting all peaks from the column was that termed Gradient A, described in Materials and Methods. Using this gradient, a number of peaks, including peaks **1**, **2** and **3**, eluted between 3 and 9.4 min and were collected as a group (F1). A second fraction (F2), eluting between 9.4 and 12.1 min, contained the following peaks: **4** (the putative *c,a* CBD), **5** (the known trimer hydrate (**III**)), **6** (the 6-4 adduct **II**) and peak **7**, which contains a compound that has an absorption spectrum very similar to that of **II**. The *c,s* CBD (peak **8**) eluted between 12.1 and 12.5 min (F3), followed by a fraction eluting between 12.5 and 13.2 min (F4) that was predominately Thy (peak **9**). Fraction F5, eluting over the time range between 13.2 and 14.4 min, contained putative *t,s* Thy CBD (peak **10**) followed closely by a

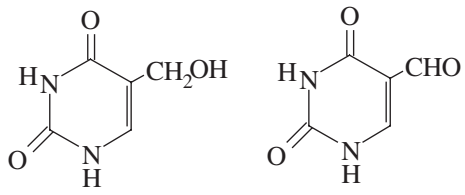
compound with an absorption maximum at about 262 nm (peak **11**). Between 14.4 and 16.8 min a number of peaks eluted and were collected as a group (F6); the dominant peaks corresponded to two compounds, one with twin absorption maxima at 265 and 307 nm (peak **14**) and the other being a compound with an absorption maximum at about 262 nm (peak **15**). The final fraction (F7) contained a variety of compounds present in small amounts, including the compound corresponding to peak **16**, which was found to be a thermal decomposition product that arose from the material contained in peak **7**. (The putative identities of the Thy CBD peaks in the above chromatogram were established through comparison of their retention times with those of authentic samples; see Appendix A1.) (Note that the retention times seen in the preparative separation of a concentrated solution, as described immediately above, do not necessarily correspond to those given in Fig. 1, which displays the chromatogram obtained when unconcentrated freshly irradiated solution was injected.)

Two peaks shown in Fig. 1 are missing from the above listing, namely peaks **12** and **13**. These peaks correspond to putative Thy trimer(s) (peak **12**) and primary thermal decomposition product(s) (peak **13**) arising from the trimer(s) in peak **12**. Both of these compounds were unstable over the period of time (a week or more) required to produce and workup 10 L of irradiated solutions for use in preparative chromatography, decomposing to form trimer hydrate(s). These compounds were obtained by an alternative method described later.

5-hydroxymethyluracil and 5-formyluracil are found in F1

It was found that F1 contained at least 13 components. Two compounds, termed PF_{11} and PF_{12}, (denoted as 2 and 3 in Fig. 1) were dominant components of this mixture and were isolated for characterization. The details of the workup of this fraction will be found in Section A2 in Appendix S1. Compounds PF_{11} and PF_{12} were identified as 5-hydroxymethyluracil (**IV**) and 5-formyluracil (**V**) respectively (see Scheme 3). These identifications were based on (1) the identity of their HPLC retention times and UV spectra with those of authentic samples, (2) the observation that, in each case, coinjection of purified compound and authentic compound gave a single HPLC peak and (3) in the case of **V**, the material had the expected molecular mass, as measured by electrospray mass spectrometry in the negative ion mode. (Here, and in the analogous discussions given below, we will denote a particular fraction by the notation Fx and the components of interest contained within that fraction as PF_{xi}. Thus, PF_{11} and PF_{12} are the two components of primary interest that eluted in the collected fraction F1.)

It cannot be concluded that these two compounds are photoreaction products; they may simply be, at least in part, impurities in the parent Thy. Examination of unirradiated 2 mM solutions of Thy *via* HPLC, using the same conditions employed for production of the HPLC chromatogram displayed in Fig. 1, indicated that small amounts of these compounds were present. Indeed, T. Douki (private communication) has indicated that traces of oxidation products are always present in commercial samples of nucleobases and nucleosides, as determined by sensitive analytical techniques. Future studies, to determine whether **IV** and **V** are formed *via* photochemical processes should utilize stringently purified samples of Thy.



Scheme 3.

So as to get an idea of the importance of these compounds, as compared with products definitely produced by photochemical reaction, isolated "yields" of **IV** and **V** were determined. This was done by comparison of the UV absorbance of diluted samples of the compound of interest to samples of authentic material of known concentration. The value for **IV**, based on consumed Thy, was about 0.01%; the corresponding value for **V** was about 0.006%.

Table S1, found in the Supporting Information, may be useful in following the discussion in the following paragraphs. This table displays, for each collected fraction (*e.g.* F1), the peaks collected in that fraction (*e.g.* peaks 2 and 3), the corresponding products in that fraction (along with their product designations [*e.g.* PF_{11} and PF_{12}]) and estimated yields of each product.

Identification of the products PF_{21}, PF_{221}, PF_{222} and PF_{25}

Purified samples of products PF_{21}, PF_{221}, PF_{222} and PF_{25} were isolated from F2, as described in Section A2 in Appendix S1. The product contained in F21 (PF_{21}), corresponding to peak **4** in Fig. 1, was quite pure; UV irradiation of this compound resulted in its complete conversion into Thy, as determined by HPLC. Coinjection with an authentic sample of *c,a* Thy CBD resulted in a single HPLC peak. The absorption spectrum, as measured by the diode array HPLC detector, was superimposable on that of authentic *c,a* Thy CBD. Consequently, PF_{21} can be identified as the *c,a* CBD (**Ib**) of Thy. Its isolated yield, based on percentage of the consumed parent Thy, was determined by measuring the absorbance of a diluted sample of purified **Ib** after complete conversion into Thy by UV irradiation; using the known ε_{max} for Thy (7 800), this yield was calculated to be about 0.5%.

The product PF_{221}, corresponding to peak **5** in Fig. 1, displayed photochemical behavior characteristic of that expected for a trimer hydrate (*e.g.* **III**); irradiation at 254 nm produced a mixture of Thy and **II**. Further discussion of trimer hydrates, which are not primary photoproducts, is given in a later section of this article. The identification of PF_{222}, corresponding to peak 6, as the (6-4) adduct **II** was established by the following observations: (1) its absorption spectrum was identical to that published by Varghese and Wang (11), having a λ_{max} at 316 nm; (2) treating with acid accompanied by heating converted it into a compound with an absorption spectrum identical to the characterized decomposition product of **II**, namely 6-(5-methylpyrimidin-2-one)thymine (Thy(6-4)m^{5}Pyo) (11) it had the expected molecular mass of **II**, as determined by mass spectrometry.

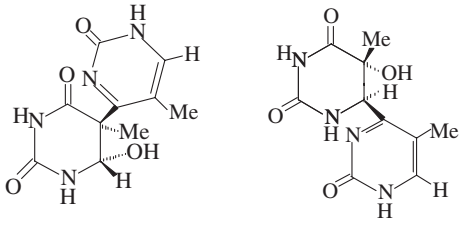
Measurement of the absorbance at 316 nm in unfractionated F22 (due to **II**) and use of the known ε_{max} of 4 940 of this compound (11) were used to estimate that its isolated yield, based on consumed Thy, was about 0.2%. (The residual trimer hydrate accompanying **II** in F22 does not absorb light at 316 nm.)

The final compound of interest in F2, namely PF_{25}, corresponds to Peak **7** and consists primarily of a compound with an

absorption spectrum profile similar to that of **II**. Assuming that ε_{max} for this compound is the same as that of **II**, its isolated yield was evaluated to be about 0.005% (about 40-fold less than the yield of **II**). We found that a high percentage of PF_{25} (about 74%) decomposed upon standing for 64 h at room temperature and, indeed, a significant amount was decomposed after standing for a week in the refrigerator at 4°C. Therefore, for the purposes of using a shorter time scale for isolating a sufficient amount of pure PF_{25} for mass spectrometric and proton NMR investigation, we used an alternative procedure for preparation and purification of this compound; this protocol is described in Appendix A3a. From this procedure about 185 μg of PF_{25} was obtained.

The freshly purified PF_{25} had the following spectral properties: UV (in H_2O): λ_{max} = 312 nm, λ_{min} = 248 nm; MALDI MS: [M + 1] at 253.07; ES MS, run in the negative ion mode: [M − 1] at 251.08. These data indicate that PF_{25} is dimeric in character. However, because of the instability of this compound, we were unsuccessful in obtaining an NMR spectrum attributable to a pure compound; using d_6-DMSO as a solvent, the NMR spectrum contained a number of extra peaks. We were, however, able to tentatively identify those belonging to the PF_{25} *via* their peak size and using chemical shifts observed for the Thy (6-4) adduct (11) and 1-methylthymine (1-MeT) (6-4) and (5-4) adducts (18). The assignment of these peaks is given in Appendix 3a. Several possibilities can be envisioned for the structural assignment of PF_{25}. The first possibility is that it is a (5-4)-type adduct, one enantiomer of which shown in Scheme 4 as **VI**. If indeed PF_{25} is a (5-4) adduct, then an alternative structure, in which the hydroxyl and methyl groups are *trans* to one another, cannot be ruled out. Still another possibility is that PF_{25} is a (6-4) adduct similar to **II**, but in which the hydroxyl and 5-methylpyrimidin-2-one group are *trans* to one another (**VII** in Scheme 4). Two (5-4) adducts and a (6-4) adduct have been identified as products when 1-MeT is irradiated in frozen aqueous solution, whereas a uracil (6-4) adduct, in which the hydroxyl and pyrimidin-2-one group are *trans* to one another, has been characterized (19). Although the NMR evidence is not conclusive, examinations of the thermal reactivity and the acid decomposition behavior of PF_{25} suggest that it should be tentatively identified as **VI** or its *trans* isomer (see above). The details of these studies are provided in A3a; we will merely summarize the results of these studies here. (Also described in Appendix A3a are results from photochemical experiments designed to convert the (6-4) adduct **II** to its Dewar valence isomer.)

As remarked above, PF_{25} is unstable upon standing. HPLC of an aqueous solution of this compound that had stood for 64 h at room temperature showed that three significant products were formed, one of which had an absorption spectra similar to that of the parent PF_{25}. This latter product had the same elution time as the material collected in fraction 7 (corresponding to peak 16 in Fig. 1). Heating of PF_{25} for 30 s at 100°C produced about the same degree of decomposition and the same products. Heating PF_{25} for 10 min in 0.5 M HCl, followed by acid removal by rotatory evaporation, did not result in production of Thy(6-4)m^5Pyo, the dehydration product of **II** formed when it is similarly heated (11). Instead, one of the main peaks appearing displayed a UV absorption profile similar to that of the parent, but with an elution time corresponding to peak 16. (It can be noted that heating of the uracil analog of **VII** produces Ura(6-4)Pyo [19].) We made no attempt to identify the thermal reaction products of PF_{25}; however, it can be noted that the initially formed (5-4) adduct of 1-methylthymine, which appears to have the hydroxyl and 2′-pyrimidone ring *trans* to one another, decomposes on standing to form a second later eluting (5-4) photoproduct (20) in which these two groups are *cis* with respect to one another. This suggests the possibility that PF_{25} and the compound eluting in peak 16 are a corresponding pair of (5-4) isomers. Future structural studies (*e.g.* NMR and X-ray diffraction studies), aimed at definitive determination of the structure of the apparently more stable isomeric decomposition product corresponding to peak 16, could be helpful in proving or disproving the structure (**VI**) proposed for the putatively less stable PF_{25}.



VI **VII**

Scheme 4.

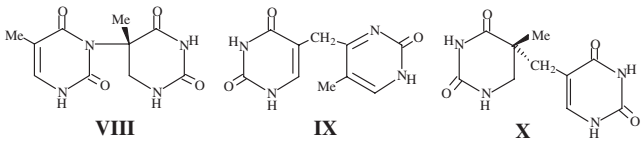
Isolation and characterization of the products contained in F3

This fraction contains mainly the well-known "ice dimer" of Thy, namely the *c,s* CBD; as pointed out above, this is the dominant product produced when Thy is irradiated in frozen aqueous solution. We looked carefully at F3, however, to see if other products might coelute with it. After reduction in F3 to 3.5 mL and filtration, the concentrate was rechromatographed on Column A; the eluent was 88% H_2O/12% MeOH flowing at a rate of 4 mL min^{-1}. Components characteristic of F2 eluted prior to the *c,s* CBD, which came off the column at 6.0 min. However, a peak centered at 6.8 min followed the *c,s* CBD peak; the material (PF_{33}) corresponding to this peak was collected and repurified. This material was identified as dihydrothymine (TH_2) through the superimposability of its absorption spectrum on the spectrum of authentic TH_2 and the fact that PF_{33} coeluted with authentic TH_2 when coinjected on the above column. However, similar to the cases of 5-hydroxymethyluracil and 5-formyluracil (see above), this compound may be an impurity in the parent Thy used for irradiation; a very small peak with a retention time and an absorption spectrum similar to that of TH_2 was observed in the HPLC of an unirradiated 2 mM Thy solution.

Isolation and characterization of the products contained in F4

Thy is the predominant product found in this fraction. However, several minor components also elute in F4. The fraction was reduced in volume and rechromatographed in 1 mL batches on the Capcell Column A using 88% H_2O/12% MeOH at 4 mL min^{-1} as eluent. Five peaks eluted prior to Thy and were collected. The first three small peaks corresponded to materials that eluted in F2 and F3. Two larger peaks, eluting at 6.6 and 7.1 min were collected for detailed study and termed F44 and F45; Thy eluted between 7.3 and 7.8 min. Fraction F44 contained a single compound, PF_{44}, that was identified as **Id**, the *t,a*

CBD of Thy. Three pieces of evidence supported this identification: (1) Irradiation of PF_{44} with 254 nm light-produced Thy as the sole product. (2) PF_{44} has the same retention time as the *t,a* CBD of Thy; when an authentic sample of the *t,a* Thy CBD was coinjected with PF_{44}, they coeluted as a single peak. (3) When the UV spectra of the authentic dimer sample and of PF_{44} were run with the diode array detector, utilizing the peaks produced when they were injected separately, they were superimposable. After repurification of the material in F45 to remove residual Thy, a similar approach was taken with the resultant PF_{45}. Once again, photoreversal evidence, HPLC evidence and UV spectral data identified this compound as the *t,s* Thy CBD (**Ic**). (It should be noted that elution orders can be switched on Capcell UG120 columns by varying the solvent compositions used for elution. Thus, whereas the Thy *t,s* CBD elutes after Thy when Gradient A is used, it elutes prior to Thy under the isocratic elution conditions described immediately above.)



VIII **IX** **X**

Scheme 5.

Characterization of the materials contained in F5

As described in Appendix A2c, this fraction was shown to contain parent Thy, *t,s* Thy CBD (**Ic**) and another product, PF_{53}, whose identification we discuss here; the isolation and purification of the latter product are described in Appendix 2c. The UV absorption spectrum of purified PF_{53} had a maximum at 266 nm and a minimum at 238 nm with a ratio of A_{268}/A_{238} of 2.5. The MALDI mass spectrum indicated that PF_{53} had a molecular mass for $[M + 1]$ of 252.94, whereas electrospray MS in the negative ion mode gave a molecular mass of $[M - 1] = 251.08$. These molecular mass measurements indicate that PF_{53} is dimeric in nature and, in particular, mandate that the number of hydrogens present in the two parent reacting thymines must be the same in the final dimeric product. The 600 MHz proton NMR spectrum, run in d_6-DMSO, displayed the following peaks: δ (ppm): 1.67 (3H, C**H**3), 1.80 (3H, C**H**3), 3.02 (1H, dd, J = 11.8 Hz and 4.2 Hz, C6**H**a), 3.74 (1H, d, each member slightly broadened, J = 11.8 Hz, C6**H**b), 7.61 (1H, s, C**H**6′), N**H** (10.19, 11.36 [broad]). Based on the above data and the discussion given below, compound PF_{53} can likely be identified as 5-(thymin-3-yl)-5,6-dihydrothymine (**VIII** in Scheme 5). Using an approximate value for ε_{266} = 8 000 (that of the spore product; see below), the isolated yield of PF_{53} can be estimated to be about 0.01%.

The pattern of proton NMR peaks displayed by PF_{53} is quite similar to the pattern observed by Shaw and Cadet (21) for the pyrimidine ring protons of the corresponding thymidine nucleoside products, namely the 5R* and 5S* diastereomers of (5-thymidin-3-yl)-5,6-dihydrothymidine. One point of particular interest is the presence of two methyl groups in PF_{53}, one with a chemical shift values characteristic of that of attachment to an unsaturated double bond and the second displaying a chemical shift higher than would be expected for simple attachment to a saturated C–C linkage; this is in line with the chemical shifts observed by Shaw and Cadet for the similar nucleoside products in which the saturated C5 is attached to nitrogen. Secondly, the C6 protons give a widely split AB system with one component centered at δ3.02 and the other at δ3.74. Similar widely split AB systems were observed for the two diastereomers of the corresponding thymidine adducts. We were only able to observe two of the three expected NH protons; one of these may have broadened to the extent of being unobservable.

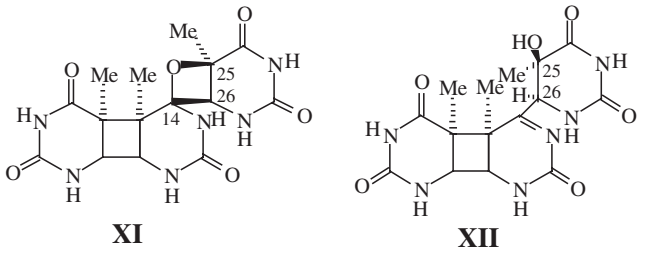
Characterization of PF_{64} and PF_{65}, the main photoproducts contained in F6

As described in Appendix A2d, we isolated and purified two products from F6. These were termed PF_{64} and PF_{65}. The evidence leading to identification of PF_{64} as likely being an α-4 conjugate, namely α-4′-(5′-methylpyrimidine-2′-one)-thymine (**IX** in Scheme 5), has been given previously (18). The yield of this compound is quite small, being estimated to be about 0.02%. Spectroscopic evidence indicates that compound PF_{65} is the Thy spore product, namely 5,6-dihydro-5-(α-thyminyl)thymine, one enantiomer of which is shown as **X** in Scheme 5. The MALDI mass spectrum shows a peak for $[M + H^+]$ of 253.1, corresponding to that expected for a dimeric substance. The electrospray MS, run in the negative ion mode, yields a molecular mass for $[M - H]$ of 251.1; this is again consistent with the molecular mass expected for a dimer. The UV spectrum shows a single absorption maximum at 266 nm and a minimum at 238 nm. The ratio of A_{266}/A_{238} was 2.49. The 600 MHz proton NMR spectrum, run in d_6-DMSO, displayed the following peaks: δ (ppm): 0. 99 (3H, C**H**3), 2.34 (1H, *d*, J = 14 Hz, C5C**H**), 2.54 (1H, *d*, J = 14 Hz, C5C**H**), 2.88 (1H, *d*, J = 12 Hz, components slightly doubled, C6**H**), 3.03 (1H, *d*, J = 12 Hz, components slightly doubled, C6**H**), 7.16 (1H, *s*, C'6**H**), 7.46 (1H, *s*, N**H**), 9.90 (1H, *s*, N**H**), 11.61 (1H, *s*, very broad, N**H**). The NMR spectral data are essentially identical to those published for a chemically synthesized sample of 5,6-dihydro-5-(α-thyminyl)thymine (22). As the mass spectral and UV data are also those expected of this adduct, PF_{65} can be identified as (5,6-dihydro-5-(α-thyminyl) thymine (**X**). Using the value ε_{266} = 8000 (22), the approximate isolated yield of this compound was calculated to be 0.07%.

Isolation and partial characterization of the thymine trimer and a decomposition product

Wang (15), in his initial study of the thymine trimer hydrate (**III**), proposed that an initially formed trimeric species, namely the oxetane **XI** (see Scheme 6), reacts to form the final product **III** *via* a water-mediated process. Wang indicated that **III** can lose water upon standing in 2*N* HCl at room temperature and suggested that the product had the structure shown as **XII** (15) (which also has the molecular mass of a Thy trimer). Based on field ionization mass spectrometric evidence, it was proposed that **III** likewise loses water at elevated temperature to form **XII** (23). However, evidence indicating that **XI** and **XII** are stable enough to be isolated as products has not been published. (Structures **XI** and **XII** are drawn with the same stereochemical configurations given in [15].) It should be noted, however, that alternate diastereomeric forms of both **XI** (which has the stereochemistry 14S,25R,26R) and **XII** (25R,26S) can be drawn; each of these compounds would contain a component derived from the *c,s* CBD.)

We describe results here showing that a compound, namely PG_{12}, with properties expected of **XI** (and/or similar compounds) can be detected in freshly irradiated frozen solutions of Thy and suggesting that **XII** (and/or related compounds) may be a product lying on the pathway leading from PG_{12} to **III**. (Preliminary results suggest that both **III** and a diastereomer of **III** may be present in solutions of PG_{12} after standing overnight; see Appendix A3b.)



Scheme 6.

As can be seen in Fig. 1, two overlapping peaks (**12** and **13**), centered at about 14.5 min, appeared in the HPLC chromatogram of frozen aqueous 2 mM Thy that had been freshly irradiated at 254 nm on dry ice. The broadness and the suggestion of structure displayed by peak **12** in Fig. 1 suggest that the envelope of this peak could encompass more than one diastereomer of trimer PG_{12}. However, strong evidence to support this assertion is not yet available.

The first of these peaks, corresponding to the compound denoted as PG_{12}, was considerably diminished in area when freshly prepared photoreaction mixtures were allowed to stand for extended periods of time, either at room temperature or in a cold room; concomitantly, the area of peak **13** increased. Faster loss of the area for peak **12** was observed when fresh photoreaction mixture was concentrated by rotatory evaporation at 40°C. In this latter case, this loss of PG_{12} results from two processes occurring simultaneously, namely precipitation (this compound is quite insoluble in water) and decomposition to form material eluting in peak **13** (PG_{13}). We isolated sufficient amounts of PG_{12} and PG_{13} for mass spectral analysis from workup of 500 mL of solution that had been irradiated on dry ice for 60 min, immediately concentrated 100-fold at 40°C and then processed as described above for the "first pass" separation of irradiated Thy reaction mixtures. Only the materials corresponding to peaks **12** and **13** were collected in these separations.

As expected, the compound PG_{13} is also thermally labile. It likewise decomposes to form **III** (and a putative diastereomer) upon standing. Further details are given in Appendix A3b.

UV spectra of PG_{12} and PG_{13} were obtained using the spectral capture feature of the HPLC diode array detector; overlaid spectra of these two compounds are displayed in Fig. S1. It is interesting to note that the UV spectrum of PG_{12} is similar to that of **III**, whereas the spectrum of PG_{13} contains a region of relatively low absorbance extending out to about 340 nm. Evidence that this latter feature is intrinsic to PG_{13}, and not due to a coeluting impurity, is given below.

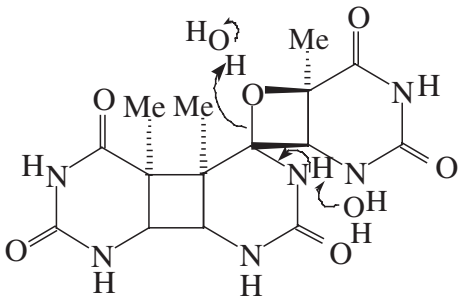
Electrospray mass spectrometry of freshly purified PG_{12}, run in the negative ion mode, yielded a molecular mass of 377.1 for the $[M - 1]$ peak. This indicates that the molecular mass of PG_{12} is 378.1, corresponding to that expected for a compound containing three Thy. The electrospray mass spectrum of freshly repurified PG_{13}, run in the positive ion mode, gave a protonated molecular mass of 379.1, corresponding to the mass expected for a trimer of Thy.

Due to the insolubility and instability of PG_{12} and the instability of PG_{13}, we were unsuccessful in obtaining NMR spectra of these two substances. Because of this lack of NMR data for PG_{12} and PG_{13}, we are unable to provide definitive structures of these compounds. However, we have obtained photochemical data suggesting that PG_{12} is an oxetane and that, in contrast, PG_{13} is not. When PG_{12} is irradiated with 254 nm light in solution, HPLC analysis indicates that there are three products formed, namely **II** ((6–4) adduct), the *c,s* CBD of Thy (**Ia**), and Thy. (A quantitative discussion of this decomposition process and its implications is given in Appendix A4.) However, similar irradiation of PG_{13} in aqueous solution yields only **II** and Thy; this latter behavior is similar to that observed when **III** is irradiated under the same conditions. Compounds with structures similar to those shown as **XI** in Scheme 6 would be expected to display the same photochemical characteristics as PG_{12}; splitting the oxetane ring in such compounds would yield Thy and *c,s* Thy CBD (**Ia**), whereas splitting of the cyclobutane ring would give Thy and **II**. It can be noted that the photochemical splitting of PG_{12} to Thy and the Thy *c,s* CBD is similar to the photochemical behavior for one of the compounds detected by Smith (10) in his very early work on the photochemistry of Thy in the frozen state

Likewise, we can only speculate about possible structures of PG_{13}. One feature of particular interest is the broad weak absorption at λ greater than 260 nm in the UV spectrum of PG_{13} shown in Fig. S1. The possibility that this tail is an artifact, perhaps due to coeluting impurity, can be discounted by the following observation. When identical solutions of PG_{13} were irradiated, either at 254 nm or with wavelengths greater than 300 nm (obtained by placing a series of 5 × 1 mm thick Schott WG-305 cutoff filters (Fish-Schurman, New Rochelle, NY) in front of the cuvette, with light from a BLE 1T-158 lamp), the same splitting of PG_{13} to Thy and **II** occurred; the only difference was the time required for complete reaction (4 min at 254 and 16 min at the longer wavelengths). Based on the molecular masses and UV spectra of PG_{12} and PG_{13}, along with knowledge that the reaction sequence $PG_{12} \rightarrow PG_{13} \rightarrow$ **III** occurs, it can be hypothesized that PG_{12} corresponds to a structure similar to that of oxetane **XI** and that PG_{13} has structure similar in nature to **XII**.

Can the proposed structure **XII** account for the UV spectrum of PG_{13} at $\lambda > 260$ nm? Examination of **XII** shows that it contains a ring that can be regarded structurally as being analogous to substituted 2-cyclohexen-1-ones. Indeed, the absorption spectrum of PG_{13} has a strong resemblance to the corresponding spectra of such cyclohexenones, which display a $n-\pi^*$ transition in the region above 260 nm (see, *e.g.* [24]; the spectra numbered 2025 (2-cyclohexen-1-one) and 2027 (3,5-dimethyl-2-cyclohexen-1-one) are particularly relevant). Thus, the UV spectrum of PG_{13} is indeed consistent with the structure given by **XII**. As noted above, Wang (15) assigned the structure of the reaction product formed *via* treatment of **III** with 2 M HCl (his structure **VII**) to be **XII**. He indicated that this product displayed an absorption maximum at 285 nm and that irradiation in water-produced Thy and **II** as products. These observations

suggest, but do not prove, that Wang's product and PG_{13} are the same compound.



Scheme 7.

Assuming that the primary reaction product PG_{12} indeed has a structure given by **XI** and PG_{13} is described by **XII**, a hypothetical reaction sequence can be proposed describing the thermal decomposition of **XI**. First, the oxetane ring in **XI** undergoes water-mediated ring opening (displayed in Scheme 7) and forms **XII**. In the second step of this sequence, addition of water to the $N = C$ bond in **XII** would lead to **III**. (Other diastereomers of **XI** and **XII** could also be considered as reactive species in such a hypothetical process.)

Quantitative aspects of the production of cyclobutane dimers and the trimer

A quantitative assay of the amounts of each of the four CBDs, the trimer PG_{12} and the (6-4) adduct was carried out on a solution of Thy (1.86 mM) that had been freshly irradiated for 64 min (exposure of 96 kJ m^{-2}) at dry ice temperature (as opposed to the isolated yields provided for some of the products in previous sections). The details of this study are provided in Section A4 in Appendix S1. Based on the original Thy concentration, the following are the percentages of Thy present as parent compound and each product: Thy: 11.9%, **Ia**: 76.7%; **Ib**: 1.3%; **Ic**: 0.1%; **Id**: 0.07%; **II**: 0.7%; trimer: 6.3%. The sum of these percentages is 97.1%, in reasonable agreement with that expected from mass balance. The corresponding yields of product, based on consumed Thy, are as follows: **Ia**: 87.0%; **Ib**: 1.5%; **Ic**: 0.11%; **Id**: 0.08%; **II**: 0.8%; trimer: 7.1%. Our result for the trimer PG_{12} can be compared with that of Rahn and Hosszu (25), who determined that around 10% of the Thy was converted into trimer hydrate (presumably **III**, see Appendix A4) when an 0.79 mM solution of Thy was irradiated at liquid nitrogen temperature (−196°C). As a by-product of our efforts to measure the amount of trimer formed, we were able to determine that about 61% of the trimer was decomposed by extensive irradiation with 254 nm light to form three Thy whereas about 39% was photolyzed to form Thy and **II** (Appendix A4).

We also carried out experiments to determine the lowest exposure at which various CBDs and trimeric product could be detected. (The details of these studies will be found in Appendix A4.) These experiments were carried out on frozen solutions of Thy (1.86 mM) using the irradiation conditions described in Materials and Methods and analytical protocols described in Appendix A4. In brief, using 25 μL injections of unconcentrated photoreaction mixture, we found that both the *c,a* CBD (**Ib**) and the trimer PG_{12} could be detected after 8 s irradiation time (an exposure of 200 J m^{-2}). Using the same size of injection, exposures of about 24 kJ m^{-2} (16 min) were required before the *t,s* (**Ic**) and *t,a* CBDs (**Id**) could be detected. Using larger injection volumes (250 and 300 μL respectively), both **Ib** and the trimer could be detected after 1 s of irradiation time. These results are consistent with our observations that the earlier two compounds are very minor products, whereas the yields of **Ib** and PG_{12} are considerably more substantial.

Photochemical formation of the Thy (6-4) and spore products in fluid aqueous solution

Early work by Fisher and Johns (26) indicated that, in part, the four cyclobutane dimers of Thy are produced in fluid solution by a mechanism involving the excited singlet state of the nucleobase; kinetic studies showed that these singlet-state reactions took place in Thy aggregates in the fluid solution. We carried out studies to explore the possibility that the Thy (6-4) adduct (**II**) and spore photoproduct (**X**) could be similarly produced *via* photoreaction in the liquid state. Indeed, our results indicate that these dimeric products are produced, along with putative 5-(thymin-3-yl)-5,6-dihydrothymine (**VIII**). These protocols for these experiments and the results are discussed in detail in Appendix A5.

Concluding Remarks

In this study, we have shown that the photochemistry of Thy in frozen aqueous solution is considerably more complex than previously thought, based on work published before 2012. This earlier work indicated that only the *c,s* CBD (**Ia**), a (6-4) adduct (**II**) and a trimer hydrate (**III**) were formed as products in this system (16). In this work, we observed that each of these compounds was indeed present as a significant photoproduct. However, the present work, using modern HPLC techniques, also establishes that a variety of other dimeric and trimeric products can be isolated as well. In particular, it has been found that the three other CBDs are formed, with the *c,a* CBD being produced in significant yield. The compound 5,6–dihydro-5-(α-thyminyl) thymine (**X**) (commonly termed the thymine spore adduct) and 5-(thymin-3-yl)-5,6-dihydrothymine (**VIII**) are also minor dimeric products. Two compounds, having molecular masses identifying them as true trimeric forms of Thy, are also produced. One appears to be an oxetane (**XI**), a secondary photoproduct originally proposed by Wang to act as a precursor to formation of trimer hydrate **III**; the other, a thermal decomposition product of **XI** with proposed structure **XII**, is an intermediate in the reaction of **XI** to form **III**. In addition to these results, evidence for the presence of a likely α-4 conjugate of Thy (**IX**) in irradiated frozen solution has been recently presented (18). Our results indicate that the aggregates of Thy, produced when aqueous solutions of this compound are frozen, may not be as ordered as previously thought.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. UV spectra of two thymine trimmers PG_{12} and PG_{13} (putatively **XI** and **XII**).

Table S1. Summary of the peaks collected in each fraction, identities of those compounds contained in each fraction and the corresponding product designations and estimated yields of each product.

Appendix S1. Contains protocols for the preparation of authentic samples of the four cyclobutane dimers of Thy and for the isolation of photoproducts from various chromatographic fractions obtained from the "first pass" separation of the photoreaction mixture resulting from irradiation of Thy in the frozen state. It also describes the results from NMR and various chemical studies on the product PF_{25} and contains a discussion of certain quantitative aspects of the production of the four Thy cyclobutane dimers, the (6–4) adduct (**II**) and the Thy trimer PG_{12}. Also included are the protocols used to detect Thy (6–4) and spore products in UV-irradiated fluid aqueous solutions of Thy, along with detailed results from these studies.

REFERENCES

1. Cadet, J. and P. Vigny (1990) The photochemistry of nucleic acids. In *Bioorganic Photochemistry*, **Vol. 1** (Edited by H. Morrison), pp. 1–272. John Wiley and Sons, New York.
2. Davies, R. J. H. (1995) Ultraviolet radiation damage in DNA. *Biochem. Soc. Trans.* **23**, 407–418.
3. Cadet, J., M. Berger, T. Douki, B. Morin, S. Raoul, J. Ravanat and S. Spinelli (1997) Effects of UV and visible radiation on DNA- final base damage. *Biolog. Chem.* **378**, 1275–1286.
4. Ravanat, J.-L., T. Douki and J. Cadet (2001) Direct and indirect effects of UV radiation on DNA and its components. *J. Photochem. Photobiol., B* **63**, 88–102.
5. Beukers, R. and W. Berends (1960) Isolation and identification of the irradiation product of thymine. *Biochim. Biophys. Acta* **41**, 550–551.
6. Wang, S. Y. (1960) Reversible behavior of the ultra-violet irradiated deoxyribonucleic acid and its apurinic acid. *Nature* **188**, 844–846.
7. Blackburn, G. M. and R. J. H. Davies (1965) The structure of thymine photo-dimer. *Chem. Commun.* 215.
8. Weinblum, D. and H. E. Johns (1966) Isolation and characterization of the irradiation products of thymine. *Biochim. Biophys. Acta* **114**, 450–459.
9. Anet, R. (1965) The photoproduct of thymine: an NMR study. *Tetrahedron Lett.* **371**, 3–3716.
10. Smith, K. C. (1963) Photochemical reactions of thymine, uracil, uridine, cytosine and bromouracil in frozen solution and in dried films. *Photochem. Photobiol.* **2**, 503–517.
11. Varghese, A. J. and S. Y. Wang (1968) Thymine-thymine adduct as a photoproduct of thymine. *Science* **160**, 186–187.
12. Gunther, H. L. and W. H. Prusoff (1967) Photosensitivity of 6-azothymine and its effect on the formation of photoproducts of thymine and cytosine. *Biochim. Biophys. Acta* **149**, 361–368.
13. Flippen, J. L., I. L. Karle and S. Y. Wang (1970) Crystal and molecular structure of a thymine phototrimer. *Science* **169**, 1084–1085.
14. Flippen, J. L. and I. L. Karle (1971) The crystal structure of a thymine trimer, $C_{15}H_{20}N_6O_7{\cdot}H_2O$, a photoproduct of thymine. *J. Amer. Chem. Soc.* **93**, 2762–2768.
15. Wang, S. Y. (1971) Thymine phototrimer. *J. Amer. Chem. Soc.* **93**, 2768–2771.
16. Fisher, G. J. and H. E. Johns (1976) Pyrimidine photodimers. In *Photochemistry and Photobiology of Nucleic Acids*, **Vol. 1** (Edited by S. Y. Wang), pp. 225–294. Academic Press, New York.
17. Varghese, A. J. (1972) Photochemistry of nucleic acids and their constituents. *Photophysiology* **7**, 208–274.
18. Shetlar, M. D. and J. Chung (2012) The (α-4) photoconjugates of 5-methylcytosine, 1,5-dimethylcytosine, 1-methylthymine and thymidine. *Photochem. Photobiol.* **88**, 336–343.
19. Shetlar, M. D. and V. J. Basus (2011) The photochemistry of uracil: a reinvestigation. *Photochem. Photobiol.* **87**, 82–102.
20. Shetlar, M. D. and J. Chung (2011) The (5-4) and (6-4) adducts of 1-methylthymine and their Dewar valence isomers. *Photochem. Photobiol.* **87**, 802–817.
21. Shaw, A. A. and J. Cadet (1990) Radical combination processes under the direct effects of gamma radiation on thymidine. *J. Chem. Soc. Perkin Trans.* **2** 2063–2070.
22. Bergstrom, D. E. and K. F. Rash (1979) Synthesis of 5,6-dihydro-5-(α-thyminyl)thymine. *J. Org. Chem.* **44**, 1414–1417.
23. Fensalau, C., S. Y. Wang and P. Brown (1970) Field ionization mass spectrometry of photopolymers of thymine. *Tetrahedron* **26**, 5923–5927.
24. Simon, W. W., ed. (1979) *Sadtler Handbook of Ultraviolet Spectra*. Sadtler Research Laboratories, Philadelphia, PA.
25. Rahn, R. O. and J. L. Hosszu (1969) Photochemical studies of thymine in ice. *Photochem. Photobiol.* **10**, 131–137.
26. Fisher, G. J. and H. E. Johns (1970) Ultraviolet photochemistry of thymine in aqueous solution. *Photochem. Photobiol.* **11**, 429–444.