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Examining the base stacking interaction in a dinucleotide context via reversible cyclobutane dimer analogue formation under UV irradiation

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Abstract

Substituted tolyl groups are considered as close isosteres of the thymine (T) residue. They can be recognized by DNA polymerases as if they were thymine. Although these toluene derivatives are relatively inert toward radical additions, our recent finding suggests that the dinucleotide analogue TpTo (To = 2'-deoxy-1-(3-tolyl)-D-ribofuranose) supports an *ortho* photocycloaddition reaction upon UV irradiation, producing two cyclobutane pyrimidine dimer (CPD) analogues **2** and **3**. Our report here further shows that formation of these CPD species is reversible under UVC irradiation, resembling the photochemical property of the CPD species formed between two Ts. Analyzing the stability of these CPD analogues suggests that one (**2**) is more stable than the other (**3**). The TpTo conformer responsible for **2** formation is also more stable than that responsible for **3** formation, as indicated by the Gibbs free energy change calculated from the constructed Bordwell thermodynamic cycle. These different stabilities are not due to the varying photochemical properties, as proved by quantum yields determined from the corresponding photoreactions. Instead, they are ascribed to the different stacking interaction between the T and the To rings both in the TpTo dinucleotide as well as in the formed CPD analogues. Factors contributing to the ring stacking interactions are also discussed. Our proof-of-concept approach suggests that a carefully designed Bordwell cycle coupled with reversible CPD formations under UV irradiation can be very useful in studying DNA base interactions.

Introduction

Toluene and its derivatives like 2,4-difluorotoluene are considered as close steric analogues of thymine (T). They possess similar size and shape to T,¹⁻³ and can be readily incorporated into DNA by polymerases as if they were T.³⁻⁶ Thymine is known for its rich photochemistry and is the most UV sensitive nucleobase.⁷ Contrastingly, these aromatic analogues possess rather different molecular orbital diagrams from those of T, and are suggested to be relatively inert under UV irradiation.⁷⁻⁹ Thus, they are commonly used as solvents for radical chemistry, including many [2 + 2] photocycloaddition reactions.¹⁰⁻¹²

However, in the presence of an alkene, benzene and its derivatives are known to support three different modes of photocycloaddition: the [2 + 2] or *ortho*, the [3 + 2] or *meta*, and the

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[4 + 2] or *para* photocycloaddition.⁹ In particular, the *meta* cycloaddition to aromatics is well documented and has been used during the syntheses of a number of natural products.^{13–15} The *ortho* and *para* photocycloadditions occur relatively rarely and have not received much attention in organic synthesis.⁹ Concerted *ortho* and *para* photocycloadditions of olefins are forbidden to occur from the first excited-state, but they are formally allowed from the second excited-state of benzene.⁹

The regioselectivity of the *ortho* photocycloaddition is implied to be dependent on the electronic properties of the two reaction partners.¹⁶ The *ortho* addition usually prevails when a high degree of charge transfer is involved in the exciplex.¹⁶ Nucleobases are known for their rich redox properties,¹⁷ the electron transfer between a thymine and a tolyl moiety during photo-excitation is likely, which may account for the observed [2 + 2] cycloaddition in dinucleotide TpTo (**1**) upon UVC irradiation, resulting in two pyrimidine cyclobutane dimer (CPD) analogues **2** and **3** (Scheme 1).¹⁸ The stacked structure in the dinucleotide framework likely plays an essential role in minimizing the entropic effect and providing the required template to enable the putative electron transfer between the T and To residues and the subsequent photoreaction. Furthermore, the excited C5=C6 bond on the T ring may also play a role in facilitating the photoreaction. Under photo-excitation, the thymine C5=C6 is known to exhibit a di-radical-like character, conducting an H-abstraction reaction during spore photoproduct formation in the UV irradiated TpT sequence.^{19, 20}

Also surprising are the chemical structures of the two CPD analogues **2** and **3**. Unlike the commonly observed CPD species where two pyrimidine C5=C6 bonds cyclize, the C5=C6 bond on the T ring reacts with either the C4=C5 or the C3=C4 on the To moiety to form **2** and **3** respectively. Thymine dimerization reaction has been suggested to occur within a femtosecond; such an ultrafast reaction rate implies that the original DNA conformation largely controls the structure of the resulting thymine photoproducts.^{21–24} If the T by To substitution does not drastically slow down the photoreaction, structures of the photoproduct will still reflect the original TpTo conformation. Therefore, at least in the dinucleotide context, the TpTo stacking structure is likely different from that of the TpT, as reflected by the structures of **2** and **3**. In B-DNA, the two adjacent Ts rotate ~ 36° against each other. Assuming a similar rotation exists in the dinucleotide context, the hydrophobic To moiety must result in an additional ~ 30° rotation on top of the rotation already present in a natural dinucleotide species. As a consequence, instead of the C5=C6 bond, the C4=C5 or the C3=C4 bond in the To ring is involved in the *ortho* photocycloaddition.

The fact that both the C3=C4 and the C4=C5 bonds of the To ring are reactive suggests that either side of the ring can stack on the thymine residue. These two bonds are symmetric against the C1–C4 vector on the tolyl ring; similar stacking structures in the corresponding conformers of **1** are expected before they dimerize into CPDs under UV irradiation. However, at 77K, the yield of **2** nearly doubles that of **3** (7.8% vs 4.6%),¹⁸ suggesting either the conformer population of **1**, the photoproduct stability, or the photo-reactivity of the involving species is different. We thus wonder what accounts for the enhanced production of **2** over its isomer **3**.

Results and Discussion

Photoreactions of regular CPD species are reversible, with the photo-equilibria dependent on the wavelength used.^{25, 26} For instance, thymine absorbs around 267 nm while the T<>T does not have an obvious UV absorption beyond 230 nm.²⁵ Thus, a shorter wavelength such as 225 nm favours the reverse reaction; irradiation of the TpT solution only leads to 2.5% of T<>T. In contrast, the T<>T yield increases to 95% upon irradiation under the 289 nm UV light as TpT is well excited under this wavelength while T<>T is not.²⁶ To determine the

optimal wavelength for our TpTo photochemical studies, we recorded the UV absorption for compounds **1**, **2** and **3**. **1** absorbs strongly at 269 nm, with an extinction coefficient of $8600 \text{ M}^{-1}\text{cm}^{-1}$ (Figure 1). Different from the formation of T<>T, which results in the disappearance of UV absorption above 230 nm due to loss of conjugation between the C5=C6 and the C4=O bonds,²⁶ the T<>To isomers **2** and **3** retain two conjugated C=C bonds on the To moiety, thus still exhibiting a weak absorption around 270 nm. The extinction coefficients for both **2** and **3** were found to be $1300 \text{ M}^{-1} \text{ cm}^{-1}$ at 270 nm, roughly one sixth of that for TpTo. The UV spectra of **2** and **3** are almost indistinguishable, indicating they possess very similar electronic structures.

These observations suggest that **1**, **2** and **3** absorb similarly throughout the UV-Vis region; it is very difficult to identify a wavelength which favours the formation of T<>To. We therefore chose the 254 nm UV irradiation to study the T<>To reverse reactions. Irradiating **2** in a frozen 1 : 1 glycerol/water glass at 77K for 15 minutes resulted in the formation of **1** in 38% yield (Figure 2A). No other photoproducts such as **3** were detected, suggesting the occurrence of a strictly reverse reaction between **1** and **2**. If the photoreaction is conducted at an elevated temperature such as $-78 \text{ }^\circ\text{C}$, other products presumably due to the decomposition of **2** can be observed. This reaction reaches completion within the first minute, as demonstrated by the unchanged yield of **1** after a prolonged UV irradiation. Irradiating **3** under the same conditions produced **1** in 47% yield (Figure 2B). Again, the reaction is completed within the first minute; no other product was observed.

Photoreaction of **1** at 77K produces **2** and **3** with both yields less than 10%.¹⁸ In contrast, starting from either **2** or **3**, the 254 nm UV irradiation leaves more than half of the T<>To species un-reacted. The observation can be rationalized by the fact that in the former reaction, only a very small portion of **1** are correctly aligned to facilitate the T<>To formation; the vast majority of **1** molecules adopt random conformations which only lead to rapid thermal quenching after photo-excitation. In contrast, starting from a T<>To at 77K, the resulting **1** is trapped at the reactive conformation, which can readily dimerize back to the original T<>To species. Similar observations were made in the TpT/T<>T photoreaction.^{27, 28} Formations of **2** and **3** require different TpTo conformations. The trapped conformation in resulting **1** also means it was unable to adopt the conformation suitable to form the other CPD isomer. Thawing the ice should release this conformational trap, thus allowing production of both T<>To isomers after UV irradiation.

Indeed, applying the irradiation-thaw-freeze cycle to **2** led to the emergence of **3** (Figure 3), and repeating the cycle multiple times resulted in a gradual increase of **3**. After 6 cycles, a mixture of **1**, **2** and **3** was obtained with the ratios among these three species very close to those produced from the irradiation of **1**.¹⁸ These ratios would be the same should enough cycles be conducted. Similar results were also obtained from the photoreaction of **3**.²⁹

Such observations imply that these three species can reach “photochemical equilibrium” under the 254 nm UV light. Since only TpTo molecules with a certain conformation can dimerize into **2** or **3**, we designate the conformation favouring **2** formation as conformation **a**, and that favouring **3** formation as conformation **b**. A Bordwell thermodynamic cycle shown in Scheme 2 is thus constructed for this TpTo photoreaction. From the Bordwell cycle, the photo-equilibrium constant ($K_{eq} 1$) for the **a** \rightleftharpoons **b** conformational change can be calculated using the equilibrium constants of the other three reactions. At 77K, irradiation of **1** produces **2** at 7.8% yield and **3** at 4.6% yield. The $K_{eq} 3$ for the **2** \rightleftharpoons **3** conversion is thus determined to be 0.59. The equilibrium constants $K_{eq} 2$ and $K_{eq} 4$ are determined by respective reverse reactions at 77K. The 38% yield of **1** (**a** in Scheme 2) from the photo-reverse reaction of **2** suggests $K_{eq} 2$ to be 1.63 (62/38); while the 47% yield of **1** (**b**) from

the reverse reaction of **3** suggests K_{eq4} to be 0.89 (47/53). Thus, K_{eq1} for the **a** \rightleftharpoons **b** conversion can be calculated by the equation below:

$$K_{eq1} = K_{eq2} \cdot K_{eq3} \cdot K_{eq4} = 1.63 \times 0.59 \times 0.89 = 0.85$$

This K_{eq} suggests that the conformer **a**, which enables the formation of **2**, out-populates the conformer **b** by 15%. Using a temperature of 77K, the K_{eq} of 0.85 corresponds to a ΔG of 0.11 kJ/mole for the **a** \rightleftharpoons **b** conversion. Thus, the higher yield of **2** seems to be due to the enhanced stability of **a**, suggesting more **1** molecules are available for the formation of **2**.

In order for such a hypothesis to be valid, both **2** and **3** as well as conformers **a** and **b** should possess the same photochemical properties. We thus determined the quantum yields (ϕ) for the formation and reversion of **2** or **3** via a modified literature procedure using TpT as the internal standard.²² Since TpT and TpTo exhibits different UV absorption at 254 nm, their HPLC signal integrations were calibrated using correspondent extinction coefficients. The resulting CPD species were calibrated as well. The T \rightleftharpoons T and T \rightleftharpoons To formations were monitored against reaction time and plotted in Figure 4. Using the $\phi_{T\rightarrow T}$ and $\phi_{T\rightarrow To}$ determined previously as references,^{22, 26} the ϕ s associated with the formation and reversion of **2** or **3** at 77K were obtained (Table 1). Detailed description for the quantum yield determination is available in the supporting information.

The ϕ s for the two reverse processes (ϕ_{-2} and ϕ_{-3}) were found to be equal, although both of which are slightly bigger than unity. This may be ascribed to the slight inaccuracy for the $\phi_{T\rightarrow T}$ determined during the T \rightleftharpoons T reverse process, which is used as the basis for our calculation. Moreover, the $\phi_{T\rightarrow T}$ shown in Table 1 was determined at ambient temperature, which may be different from the value at 77K. At 80 K, the $\phi_{T\rightarrow T}$ was indicated to be unity;²⁷ our work here shows that it may also be overestimated. Despite the uncertainty for these absolute values, it is probably safe to state that the ϕ s for the reversion of **2** and **3** are slightly bigger than $\phi_{T\rightarrow T}$. The reduced stability for these T \rightleftharpoons To complexes relative to TpT under UV light may result from the enhanced stability of the aromatic To ring comparing with the thymine residue. ϕ_{-2} and ϕ_{-3} cannot be accurately determined at 298K due to the rapid decays of **2** and **3** in aqueous solution.²⁹

The ϕ for **2** formation (ϕ_2) is higher than $\phi_{T\rightarrow T}$; while ϕ_3 is similar to $\phi_{T\rightarrow T}$. As the To ring is likely to be more inert than T toward UV excitation, the higher quantum yield observed in **2** formation likely results from the enhanced stacking interaction between the T and the To rings in aqueous solution.³⁰ Moreover, the ratio between ϕ_2 and ϕ_3 is found to be 1.7, which is identical to the ratio between the chemical yields of **2** and **3** obtained from the **1** photoreaction. All these data suggest that the photochemical properties are same for these T \rightleftharpoons To isomers in both forward and backward reactions; their different chemical yields are due to the different populations of conformers **a** and **b**.

What is responsible for the enhanced stability of conformer **a**? The T and To rings likely stack similarly in **a** and **b** as suggested by the fact that the two reactive bonds on the To ring are symmetric against the C1–C4 vector. Comparing the structures of **2** and **3** suggests that the major difference between these two species is the position of the methyl moiety on the To ring (CH₃-To). As the photoreaction is conducted in an aqueous solution, a direct exposure of the hydrophobic methyl group to water is not favourable energetically. The thymine methyl group are similarly exposed to water in **2** and **3** (Scheme 2). However, the CH₃-To interacts with three neighbouring carbon-based H atoms in **2** as reflected by the 2D ROESY spectrum,¹⁸ suggesting it is located in a hydrophobic pocket. This pocket consequently decreases the surface exposure of CH₃-To to water, making **2** relatively more stable. As the DNA conformation determines the outcome of the photoreaction,^{21–24} a retrospective argument may also be correct given no big post-reaction conformational

change occurs. Thus, the hydrophobic pocket around CH₃-To is likely also present in **a**, which results in its enhanced stability over **b**.

Moreover, as shown in Scheme 2, the CH₃-To in **b** adopts an eclipsed position with the hydrophilic C4=O moiety on the thymine ring. Therefore, the interaction between these two groups is repulsive by nature. The rise per base pair is found to be ~ 2.56 Å in A-DNA and 3.37 Å in B-DNA;³¹ while NMR³² and X-ray crystallographic studies³³ of the 2,4-difluorotoluene containing duplex oligonucleotides reveal no obvious structural change comparing with thymine containing oligomers. Although the distance between the T and the To rings in the dinucleotide TpTo is not known, its enhanced ring stacking interaction in aqueous solution suggests the distance to be within the range of 2.56 Å – 3.37 Å, if it is not shorter than 2.56 Å. The Van der Waals radius is determined to be 2.0 Å for the –CH₃ group and 1.5 Å for an oxygen atom,³⁴ suggesting the CH₃-To and C4=O must interact in **b**. This repulsive interaction also contributes to the reduced stability of **b**.

Conclusions

In summary, after constructing a Bordwell thermodynamic cycle via CPD photoreactions, we provide convincing evidence that not only is the CPD analogue **2** more stable than its counterpart **3**, but also the TpTo conformer **a**, which is responsible for the formation of **2**, is more stable than its counterpart **b**. Our approach is different from a traditional Bordwell cycle as the equilibrium is driven and maintained by UV irradiation. This work is of significance as it serves as a proof-of-concept example to demonstrate the feasibility of using a Bordwell cycle constructed via DNA photochemistry to understand the fundamental physical properties of DNA. It was recently suggested that it is the base stacking interaction, but not the H-bonding interaction between complementary strands that stabilizes the duplex structure.³⁵ Although our work is conducted using a toluene based thymine analogue, given the similar photo-reactivity occurring in natural di-pyrimidine steps, this methodology can be readily applied to a natural system in revealing the essential thermodynamic factors affecting DNA stability. For instance, in TpT, rotation of the glycosidic bond of the 3'-end thymine residue results in the formation of the *cis-syn* CPD or the *trans-syn* II CPD.³⁶ Although formation of the (6–4) photoproduct is also expected, its formation will not alter the photo-equilibrium between these CPDs. A similar Bordwell cycle can thus be built. Comparing the stability of these CPDs should provide a direct experimental measurement on the energy gain associated with the thymine stacking interaction.

Experimental section

Synthesis of **1**, photo-syntheses and characterizations of **2** and **3**

These experiments were conducted as described in our previous publication.¹⁸

Photo-reverse reaction of **2** and **3** in glycerol/water (1 : 1) glass at 77K

Irradiation of **2** or **3** (1 mM concentration) was conducted in glycerol/water (1 : 1) glass in an EPR tube at 77K. To ensure the formation of a transparent glass, an equal volume of glycerol was added to the aqueous solution of **2** or **3** before freezing. The resulting glass was then exposed to the 254 nm UV light in liquid N₂. After photoreaction, the EPR tube was taken out of liquid N₂, the glass was subsequently warmed to room temperature and analyzed by HPLC using a program described in our previous work.¹⁸ The photoreaction was also conducted with the glycerol/water glass in a 10-cm Petri dish in liquid N₂. Identical results were obtained from these Petri dish reactions.

Photoreaction of **2** or **3** after multiple irradiation-thaw-freeze cycles

The photo-reverse reactions of **2** and **3** were carried out using experimental conditions described above. 200 μL of the glycerol/water (1 : 1) solution of **2** or **3** was placed into an EPR tube and frozen at 77K by immersing the tube into liquid N_2 . After irradiation under 254 nm UV light at 77K for 15 minutes, the resulting glass was allowed to warm to ambient temperature, 10 μL of the resulting solution was extracted and saved for later HPLC analysis. The remaining solution was re-frozen in liquid N_2 and irradiated for another 15 minutes. Such an irradiation-thaw-freeze cycle was repeated at least 7 times.

Quantum yield determination

Quantum yields for formations and reversions of **2** and **3** were determined using a modified literature protocol where dinucleotide TpT was added as an internal standard.^{22, 23} Briefly, 0.33 mM TpT and TpTo were co-dissolved in glycerol/water (1:1) solution at pH 7. 0.5 ml of this solution was transferred to a Petri dish and irradiated by a UVC lamp at ambient temperature with the lamp 9.75 inches above the sample plate. At a given reaction time, 40 μL of the reaction solution was extracted and analyzed by HPLC. Similarly, 60 μL of the solution was placed on a Petri dish, flash frozen in liquid N_2 to form a transparent glass, and irradiated by UV light. After a certain reaction time, the glass was warmed to ambient temperature, 40 μL of the resulting solution extracted and analyzed by HPLC. The quantum yields for the **2/3** formation and reversion processes were calculated by comparing the amount of resulting T<>T with that of T<>To using formulas shown in literature.²² Details about the quantum yield calculation are available in the supporting information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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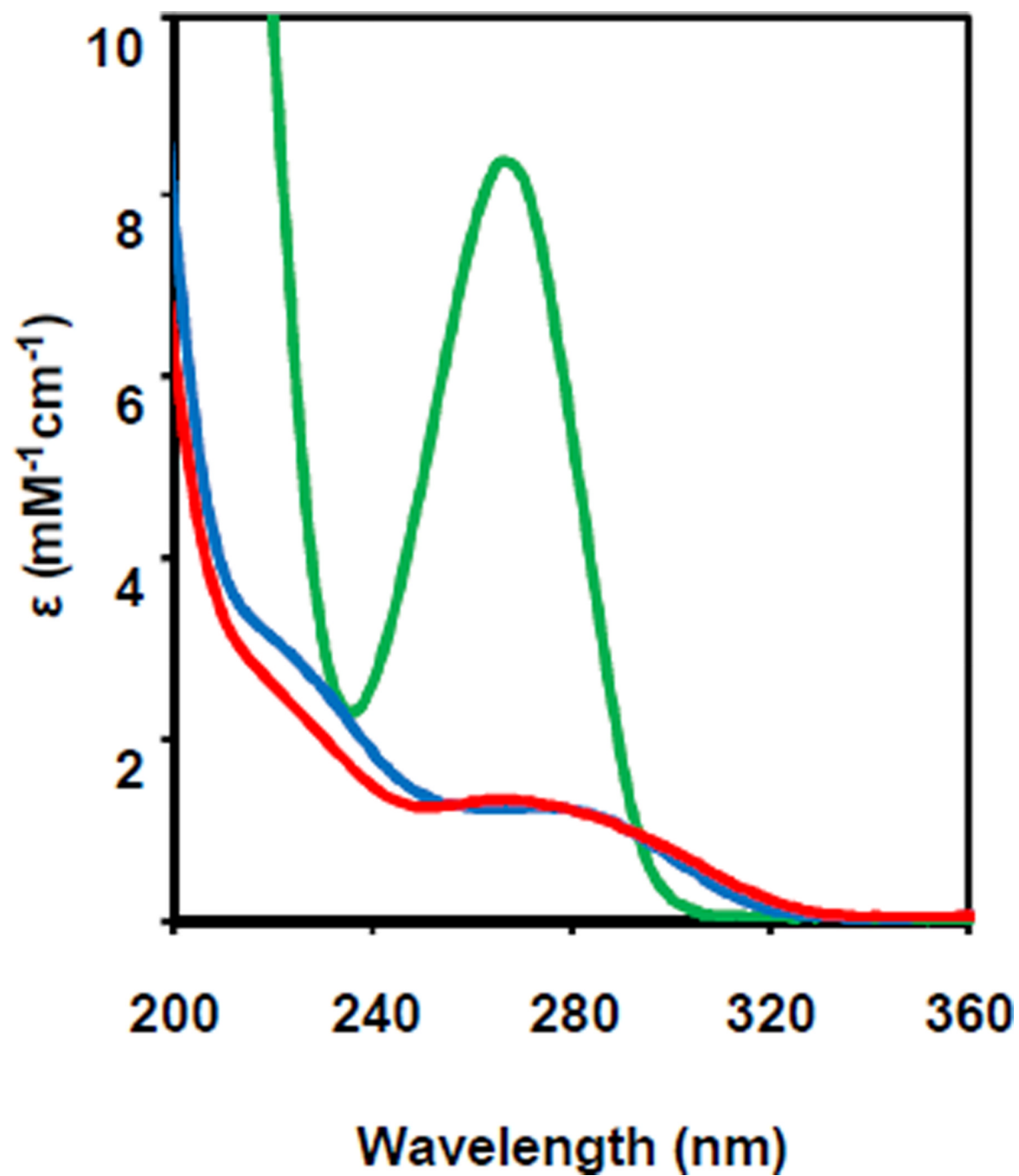


Figure 1. UV-vis spectra of TpTo (**1** green line), its photo-dimerization products **2** (blue line) and **3** (red line).

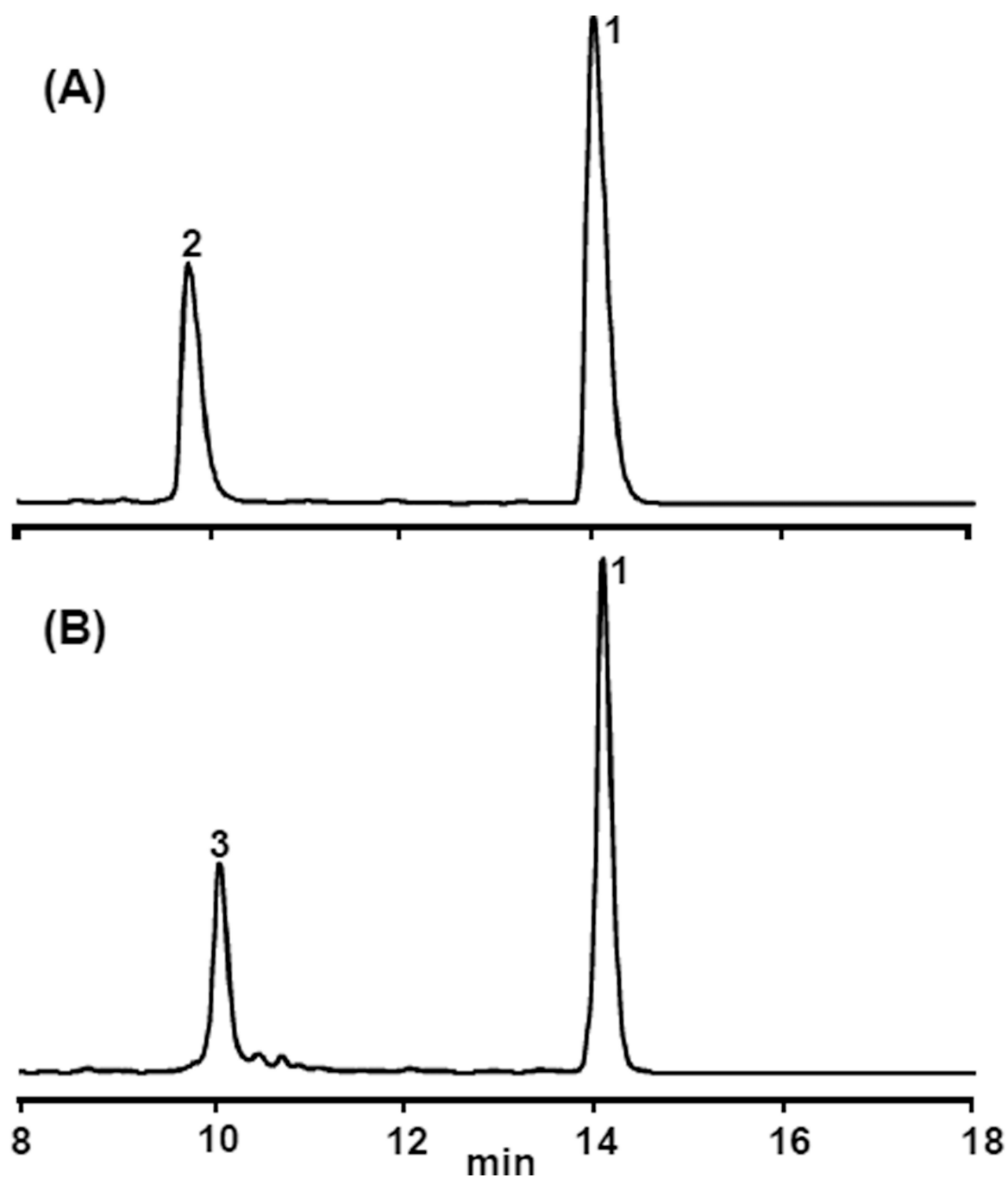


Figure 2. The HPLC chromatograph of **2** (A) and **3** (B) photoreaction in a frozen 1 : 1 glycerol/water glass at 77K under 254 nm UV light for 15 minutes. 38% of **2** and 47% of **3** were converted back to **1** when reactions reached the “photostationary” state respectively.

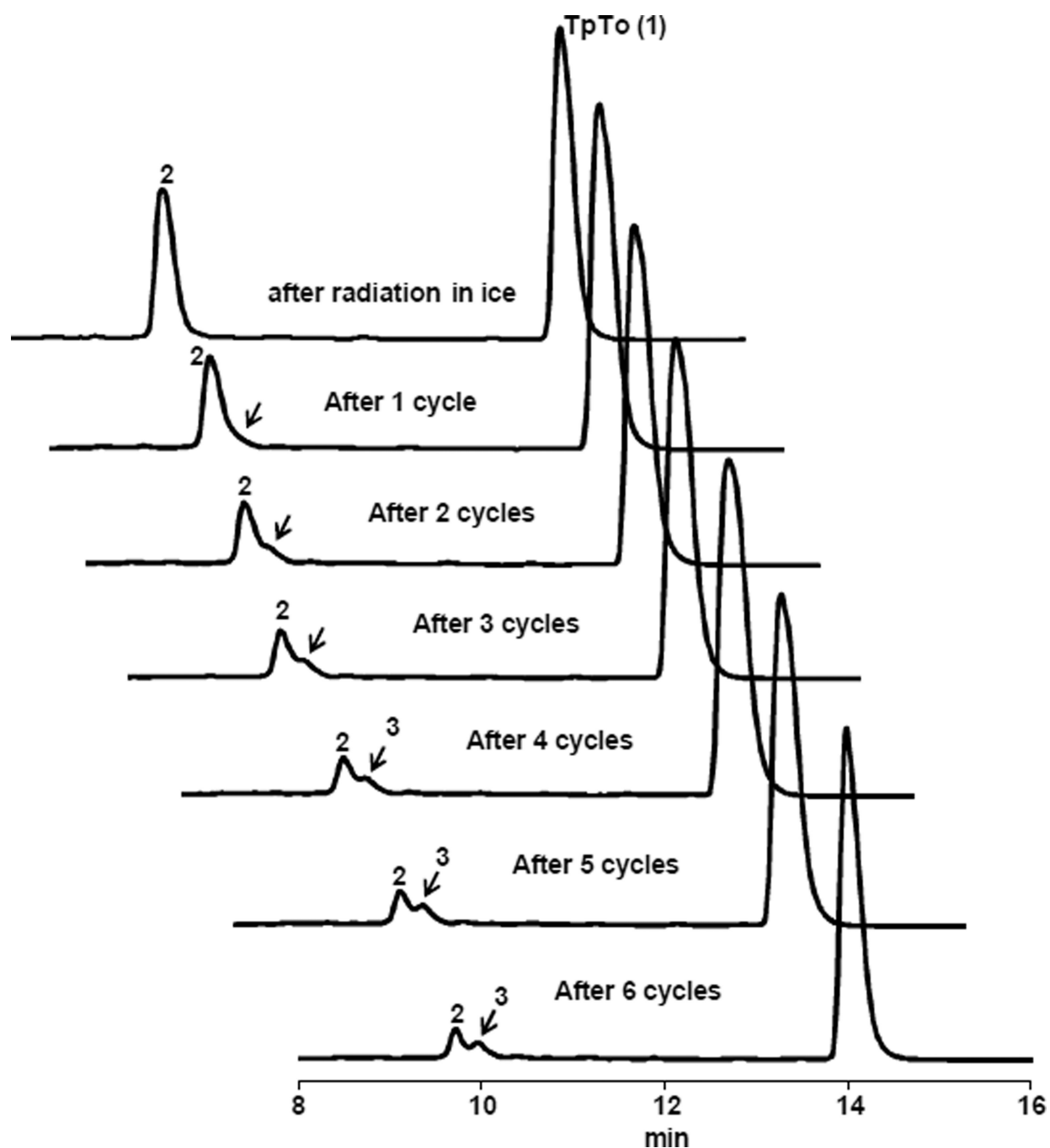


Figure 3.

The HPLC chromatograph describing the photoreaction of **2** after multiple irradiation-thaw-freeze cycles. For each cycle, the photoreaction was conducted in a frozen 1 : 1 glycerol/water glass at 77K under 254 nm UV light for 15 minutes. No **3** was found before such a cycle started due to the conformational trap at 77K. The conformational restraint in the resulting **1** was released after thawing the ice; **3** was then observed after the subsequent UV irradiation. After 6 cycles, the ratios among compounds **1/2/3** approached the ratios at the “photostationary” state obtained directly from the photoreaction of **1** at 77K.¹⁸

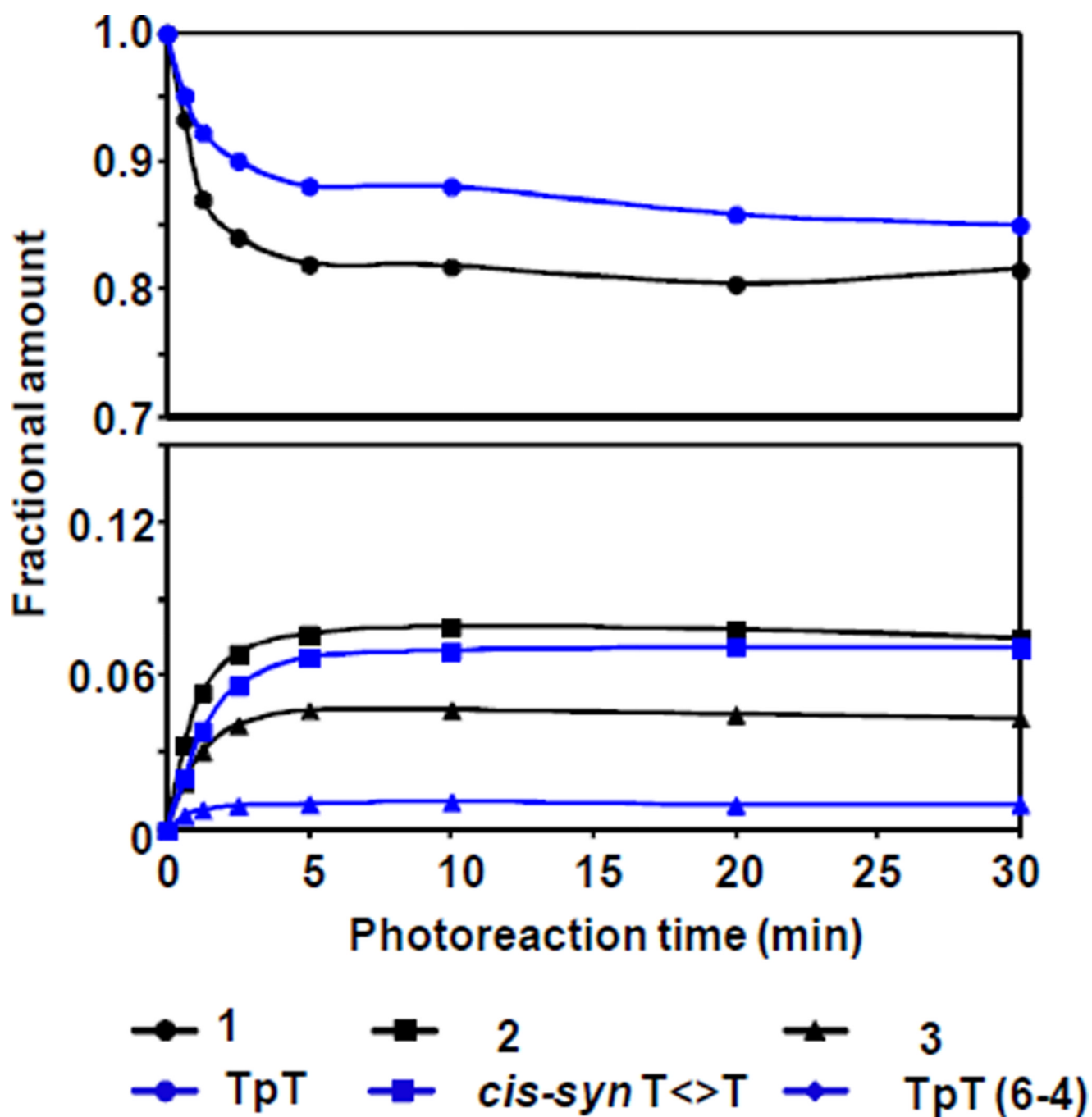
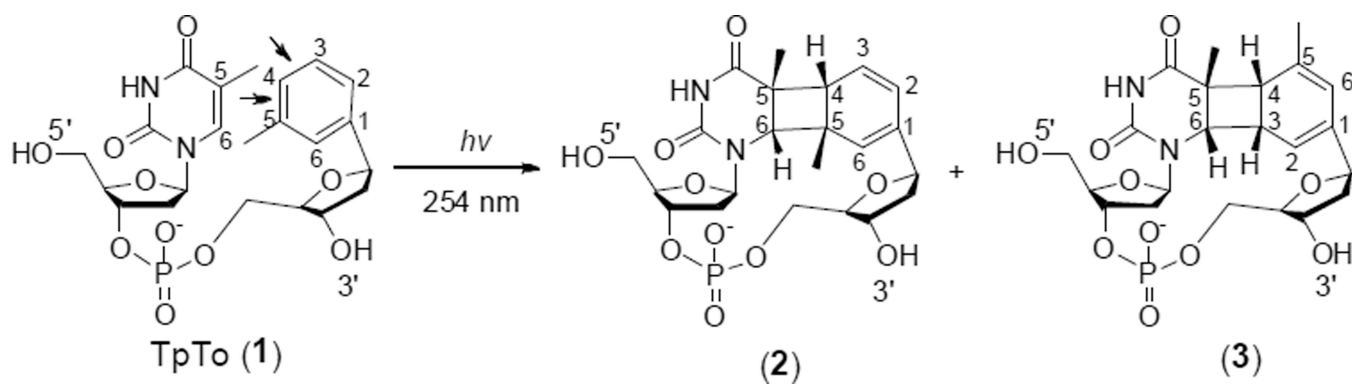
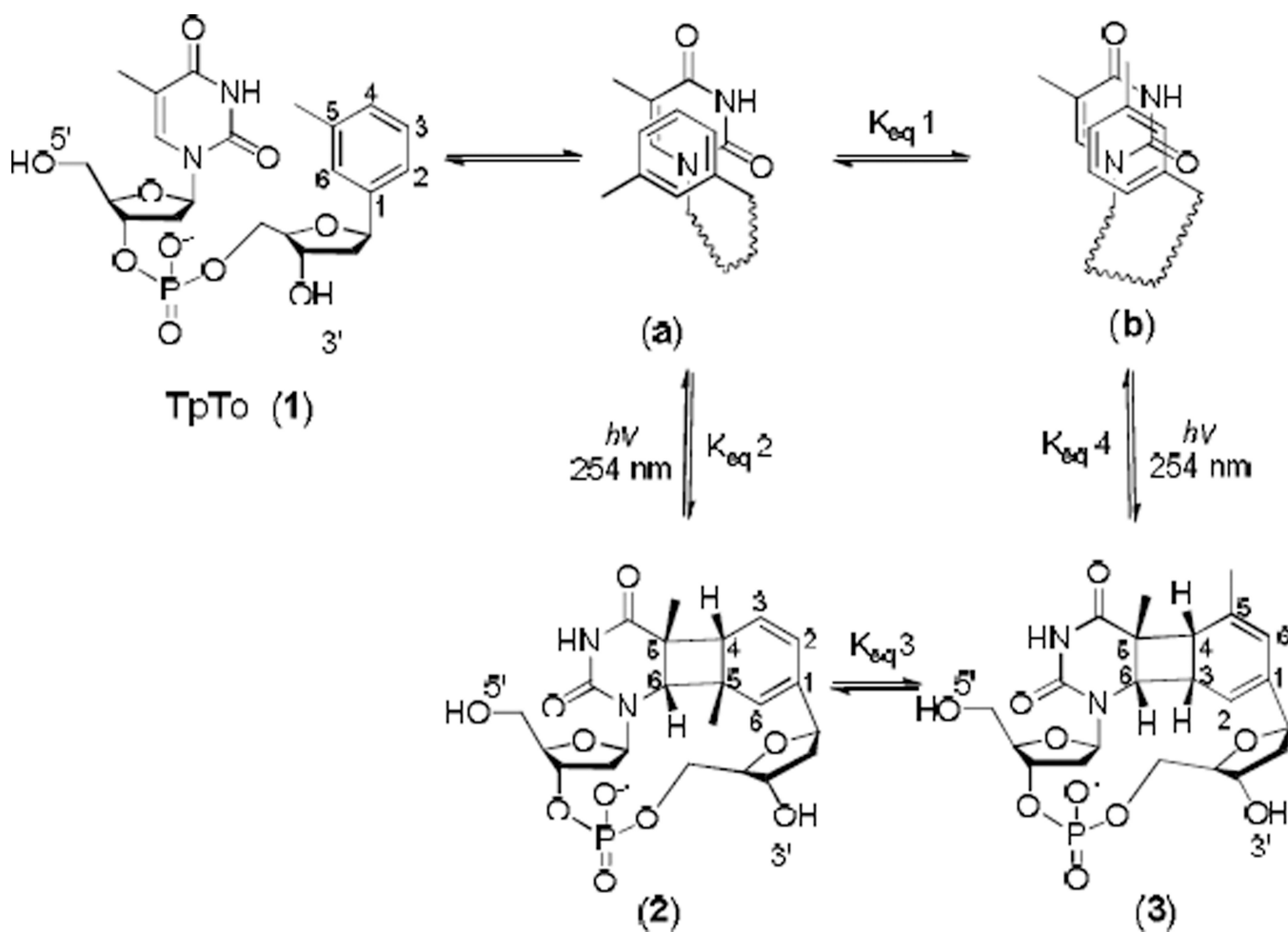


Figure 4. Fractional amount of TpT/TpTo as well as of their respective photoproducts as the function of irradiation time in a frozen 1 : 1 glycerol/water glass at 77K.

**Scheme 1.**

UV photoreaction of dinucleotide TpTo to produce 2 and 3.

**Scheme 2.**

The Bordwell thermodynamic cycle constructed for the photoreaction of **1**. The species **a** and **b** denote the two specific conformations of **1** which result in the formation of **2** and **3** respectively upon UVC irradiation.

Table 1

Quantum yield (ϕ) determined for the formation and reversion of **2** or **3** at 77K using dinucleotide TpT as an internal standard as well as $\phi_{T \rightarrow T}$ of 0.011 and $\phi_{T \rightarrow T}^-$ of 0.75 as references.

2	$\phi_{\mathbf{2}}$	3	$\phi_{\mathbf{3}}$
$(1.97 \pm 0.1) \times 10^{-2}$	1.16 ± 0.1	$(1.15 \pm 0.1) \times 10^{-2}$	1.16 ± 0.1