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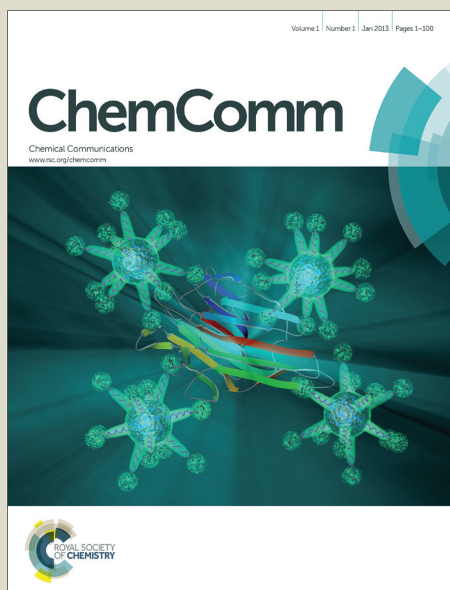

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COMMUNICATION

Triple helix conformation-specific blinking of Cy3 in DNA

Cite this: DOI: 10.1039/x0xx00000x

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Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

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We report that Cy3 undergoes triple helix conformation-specific blinking in DNA. Blinking patterns were affected by the stabilization of the Hoogsteen base-pair, suggesting that not only the presence but also the fluctuating behaviour of the triple helix can be monitored by the changes in the Cy3 blinking patterns.

To track biological events at the single-molecule level, one approach would be to develop a detection strategy that specifically relies on phenomena that become pronounced during the observation of single molecules. Among such phenomena, in our recent studies we have focused on the fluctuating emissions between bright ''on'' and dark "off" states of fluorescent molecules, or so-called "blinking".[1-3] During the repetitive cycles of excitation of the S_0 ground state to the singlet excited state (S_1) and subsequent emissions that allow a return to S_0 (on-state), fluorescent molecules occasionally become non-fluorescent (off-state). Switching between the on-state and off-state of a fluorescent molecule results in a blinking of the fluorescence. Potential transient dark off-states that may cause such blinking are i) the triplet state resulting from the intersystem crossing, ii) the reduced or oxidized state triggered by electron transfer, iii) formation of a non-fluorescent complex between a fluorescent molecule and a quencher, iv) intramolecular spirocyclization, and v) the isomerized state formed by photo-triggered *trans-cis* isomerization.[4-6] The biological events and/or the changes in the surrounding local microenvironment that modulate these processes would be detectable at the single-molecule level by monitoring of the fluorescence blinking.[4-6]

Based on the synthesis and observation of DNA site-specifically modified with a fluorescent molecule, we have shown that the charge-separation, charge-transfer, and charge-recombination dynamics in DNA can be measured by monitoring the blinking.[1] In this case, the blinking was caused by the successive reduction and re-oxidation cycles of the fluorescent molecule ATTO 655. Based on the fact that the charge-transfer dynamics in DNA is strongly affected by the DNA sequence,[7] we demonstrated that single-nucleotide differences in DNA that modulate the charge-recombination kinetics can be detected by monitoring the blinking of the fluorescence.[2] Recently, we reported that blinking triggered by formation of the triplet state of R6G provides information regarding the solvent accessibility of the fluorescent molecule.[3] Since the triplet quenching reaction is affected by the accessibility of a fluorescent molecule to molecular oxygen, the more it is exposed to a solvent, the faster its triplet excited state is quenched by molecular oxygen. We showed that the transition between the hairpin and double helix conformation can be tracked by monitoring the blinking caused by the triplet formation.[3] In this study, we focus on the blinking of Cy3 caused by *cis-trans* isomerization in the context of DNA.

Cyanine dyes such as Cy3 are widely used as a fluorescent probe to investigate various biological phenomena. Cy3 was one of the most common fluorescent molecules of choice during the early stages of single-molecule spectroscopy.[5] It is known to undergo *trans-cis* photo-isomerization and successive *cis-trans* back thermal-isomerization by rotation around the C–C bonds of the poly-methine chain, and these dynamics have been well studied by transient absorption spectroscopy.[8] This *cis-trans* isomerization causes the blinking of the fluorescence. The photo-physical properties of Cy3 conjugated to DNA have also been investigated.[9,10] Based on the molecular dynamic simulations, Spiriti *et al*. indicated that *cis-trans* isomerization of the Cy3 attached to the 5'-end of DNA is modulated in a sequence-dependent manner.[11] The structure of DNA has a remarkable conformational heterogeneity, which has been suggested to play an important role in a number of gene expression processes.[12] Since local DNA conformations may exist rarely and transiently, the development of single-molecule-level probing

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† Electronic Supplementary Information (ESI) available: Experimental procedures, and melting temperature (T_m) measurements for Cy3-modified DNA. See DOI: 10.1039/c000000x/

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methods that allow to investigate when and how they appear is highly desired. In addition to a canonical right-handed B-form conformation, DNA can adopt A-form, left-handed Z-form, triple helix, and quadruplex conformations.[12] Herein, we focus on the fact that the size of the Cy3 is just about the same as the width of the triple helix (Fig. 1). Since the *cis-trans* isomerization efficiency is considered to be strongly dependent on the steric effects that impact the rotation of the molecule,[5] we hypothesized that Cy3 may exhibit a triple helix-specific blinking. Herein, we synthesized DNA site-specifically modified with Cy3 and investigated how the blinking of Cy3 is affected by the DNA conformation—i.e., single strand, double helix, and triple helix. Our results showed that Cy3 shows a characteristic blinking pattern in the context of triple helix DNA, exhibiting a longer duration of the off-time (τ_{OFF}) and on time (τ_{ON}) compared to the other two DNA conformations.

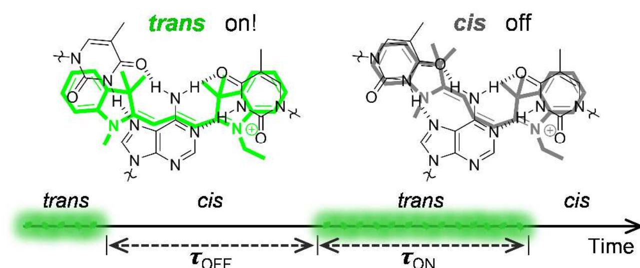

Fig. 1. Suggested configurations of T:A:T base-pair in triple helix, Cy3 in *trans* (cyan) and *cis* (blue) conformations, and schematic representation of blinking caused by *trans-cis* isomerization of Cy3.

In order to bury a fluorescent molecule in the π-stacks in the context of a triple helical structure, Cy3 was attached to DNA using an aminolinker (X).[1-3] To ensure the triple helix formation, the sequence was designed based on the intramolecular triple helix-forming sequence well-studied by Marky and co-workers, with a few base alterations in the loop region (Fig. 2).[13] Cy3 was placed in the context of a double helix, and a single-strand conformation by adding adequate complementary strands. We also synthesized DNA in which some As were replaced with A-derivatives having an additional amino group to stabilize specifically the Watson-Crick base-pairing (diaminopurine (D)) or the Hoogsteen base-pairing (8-aminoadenine (8-amA)).

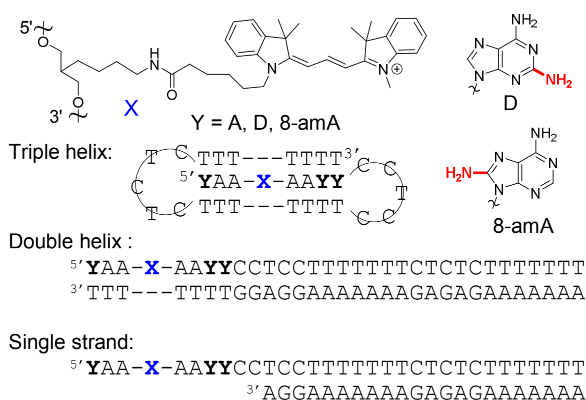

Fig. 2. Chemical structures of Cy3 modified aminolinker X, diaminopurine (D), 8-aminoadenine (8-amA), and DNA sequences used in this study.

Blinking was monitored by utilizing fluorescent correlation spectroscopy (FCS), and the τ_{OFF} and τ_{ON} values were obtained by fitting the data using the following autocorrelation equation (1),[6,10,14]

$$G(\tau)=1+\left(\frac{1}{N}\right)\left(\frac{1}{1+\tau/\tau_D}\right)\left(\frac{1}{1+(1/S^2)(\tau/\tau_D)}\right)^{\frac{1}{2}}\left(1+\left(\frac{\tau_{Off}}{\tau_{On}}\right)\exp\left(-\tau\left(\frac{1}{\tau_{Off}}+\frac{1}{\tau_{On}}\right)\right)\right) \quad (1)$$

where N is the number of molecules within the sample volume element, τ_D is the translational diffusion time, $S = \omega_1/\omega_o$ is the radius of the measurement area ω_0 and half of its axial length ω_1. τ_{OFF} corresponds to the lifetime of the *cis*-state, and τ_{ON} reflects the *trans-cis* photo-isomerization rate. FCS was measured in the presence of either 7.5% or 15% (v/v) of PEG 20,000 to increase the viscosity and the τ_D value so as to facilitate the separation of the diffusion and the blinking dynamics. Representative FCS curves are shown in Figure 3, and the τ_{OFF} and τ_D values and τ_{ON} and count rate per particle (CPP) are summarized in Figure 4A and 4B, respectively. In addition to a significant increase in τ_D values, τ_{ON} and τ_{OFF} values also moderately increased as the amount of PEG in the solution increased. Since the *cis-trans* isomerization rate decreseas to some extent with the increase in the viscosity of the solution, we can conclude that τ_{ON} and τ_{OFF} reflects the blinking caused by the usual *cis-trans* isomerization of Cy3 reported in the literature.[5,8,10] Interestingly, the highest τ_{OFF} was observed for the triple helix conformation, suggesting that back thermal isomerization proceeds more slowly than the other two conformations. At the same time, the duration of τ_{ON} in the triple helix was also the longest among the three conformations. These results show that photo-isomerization also proceeds relatively slowly in the triple helix, which was consistent with the finding that this conformation had the highest CPP.

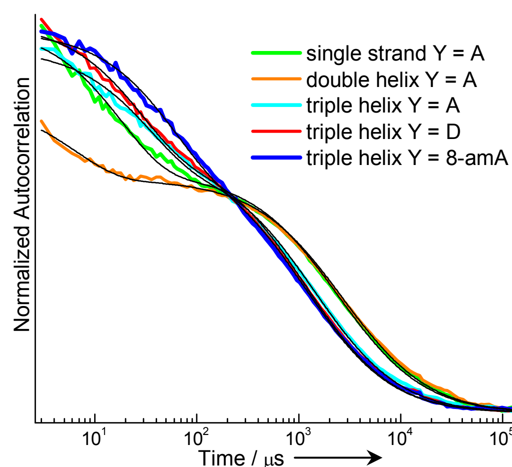

Fig. 3. Representative FCS time traces. The sample aqueous solution contained 4 nM Cy3 modified-DNA (with 8 nM of complementary strand for single strand and double helix), 100 mM NaCl, 10 mM $MgCl_2$, 7.5% PEG-20,000 in 10 mM Na phosphate buffer (pH 7.0). The smoothed black curves superimposed on the experimental data correspond to the theoretical fitting curves.

Table 1. Melting temperatures (T_m) for dissociation of triple helix and double helix.

entry	T_m (ºC)		
	Y = A	Y = D	Y = 8-amA
double helix PEG 7.5%	64.3 ± 0.1	64.7 ± 0.1	62.4 ± 0.1
double helix PEG 15%	66.6 ± 0.1	66.7 ± 0.1	64.0 ± 0.2
triple helix PEG 7.5%	41.5 ± 0.1	42.9 ± 0.2	45.2 ± 0.1
triple helix PEG 15%	42.1 ± 0.2	43.2 ± 0.1	45.2 ± 0.1

The same DNA concentrations and buffer conditions were used as were used for FCS measurements shown in Figure 3.

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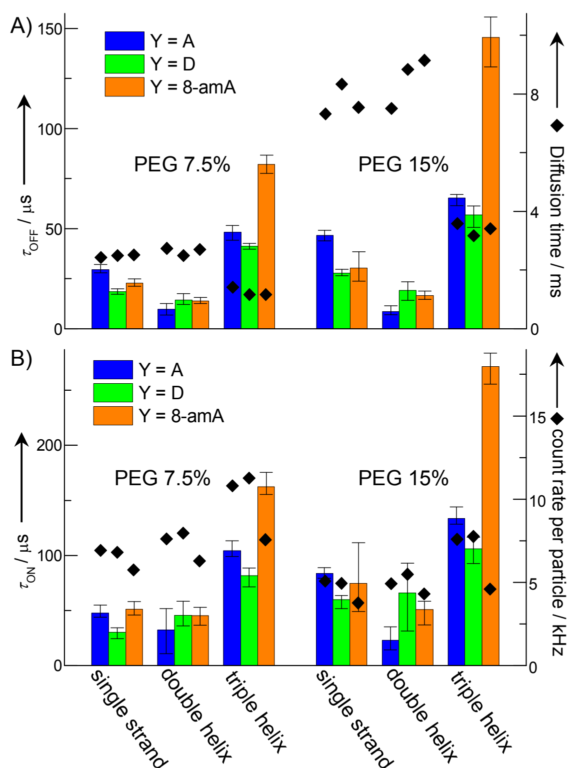

Fig. 4. Bar graphs summarizing (A) τ_{OFF} and τ_{D} (♦), (B) τ_{ON} and count rate (♦).

To further confirm that the increase in the τ_{OFF} and τ_{ON} values were specific to the triple helix conformation of DNA, we replaced some As with Ds and 8-amAs (Figure 5). D is a derivative of adenine which is well-established to stabilize the Watson-Click base-pair. We previously reported the synthesis and thermodynamic properties of 8-amA-containing DNA, demonstrating that the amino group at the adenine C8 position stabilizes the Hoogsteen base-pair in the triple helix conformation while slightly destabilizing the Watson-Click base-pair.[15] Because the fluorescence intensity of Cy3 was higher in the context of the triple helix or double helix than in the single strand conformation, we determined the melting temperature of DNA (T_m) by measuring the fluorescence melting curve analysis (Table 1, Fig. S1). Cooperative single-step melting behavior was observed for both the double helix and triple helix conformations, which was consistent with the report by Marky and co-workers.[13] The Ds slightly stabilized both the triple helix and double helix conformation. 8-amAs stabilized the triple helix conformation, but caused a slight destabilization of the double helix. Of special interest, the replacement of adenine with 8-amAs caused a significant increase in the τ_{OFF} and τ_{ON} values in the triple helix conformation (Fig. 4). In sharp contrast, even though the Ds slightly increased the thermal stability of the entire triple helix conformation, the replacement of adenines with Ds resulted in a slight decrease in the τ_{OFF} and τ_{ON} values in the triple helix conformation. Since the FCS experiments were conducted at room temperature (25 °C), which is far below the T_m, these results indicate that the relatively weak Hoogsteen base-pair fluctuates more than the Watson-Crick base pair, and that the stability or the fluctuating nature of the Hoogsteen base-pair plays a key role in the triple helix-specific blinking. While 2-amino group locates at the minor groove, 8-amino group sticks out to the major groove. It may also be possible that the increase of the steric bulk in the major groove caused by 8-amino substitution affect the *cis-trans* isomerization rate to some extent.

Under ensemble conditions, fluorescence intensity of 8-amA modified DNA was about two-fold higher in triple helix than in double helix (Fig. S1). On the other hand, CPP appeared to be only modestly affected by DNA conformation which can be mainly explained by the long τ_{OFF} in triple helix conformation. These results show that blinking could significantly affect the brightness of the fluorescent molecule when observed at the single-molecule level.

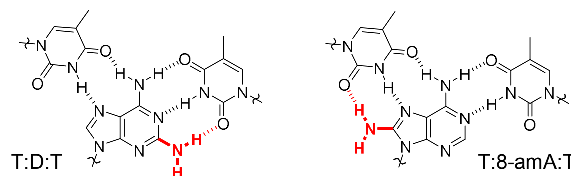

Fig. 5. Chemical structures of T:D:T and T:8-amA:T base-pairs in triple helix.

While the isomerization of Cy3 proceeded slightly faster in the double helix than in the single strand conformation in the present system, Asanuma and co-workers,[16] and Ginger and co-workers[17] reported that *trans-cis* photo-isomerization of azobenzene, which is smaller in size than Cy3, proceeds less efficiently when it is incorporated in the double helix conformation than in the single strand. When Cy3 was replaced with the larger Cy5 in the present system, triple helix conformation-specific blinking was not observed (data not shown). These results suggest that the triple helix-specific blinking of Cy3 originates from its molecular size, which is just about the width of the triple helix conformation.

Conclusions

In summary, we incorporated Cy3 into DNA with a single strand, double helix, or triple helix conformation and investigated the differences in blinking behavior by FCS. The τ_{ON} and τ_{OFF} values of the blinking, which reflect the inverse of the *trans-cis* photo-isomerization rate and the *cis-trans* back isomerization rate, respectively, were highest in the triple helix conformation. These values were further increased in a triple helix conformation-specific manner by stabilizing or reducing the fluctuation of the Hoogsteen base-pair by adding an additional amino group at the C8 position of adenine. These results suggest that Cy3 can be used not only to track the presence but also to report the fluctuating behavior of the triple helix conformation, the extent of which can be monitored by the changes in τ_{ON} and τ_{OFF} values. Cy3 is widely used in single-molecule FRET experiments to study the conformational change of nucleic acids.[18] Our results suggest that changes in blinking patterns originated by *cis-trans* isomerization may be taken into account during the analysis. The general strategy demonstrated herein, namely, using DNA to control the blinking of Cy3, may also aid in regulating the blinking of various cyanine dyes.

We thank Prof. T. Majima for kind assistant, and Dr. N. Kato of Olympus for assistance with the data analysis. This work has been partly supported by a Grant-in-Aid for Scientific Research (Innovative Areas ''Nanomedicine Molecular Science'' (No. 2306) and Projects 23240074, 24350084 and 23655155) from the MEXT of Japanese Government, and the SENTAN project from Japan Science and Technology Agency.

1 K. Kawai, E. Matsutani, A. Maruyama and T. Majima, *J. Am. Chem. Soc.*, 2011, **133**, 15568.
2 K. Kawai, T. Majima and A. Maruyama, *ChemBioChem*, 2013, **14**, 1430.

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3 K. Kawai, T. Koshimo, A. Maruyama and T. Majima, *Chem. Commun.*, 2014, **50**, 10478.
4 J. Widengren, U. Mets and R. Rigler, *J. Phys. Chem.*, 1995, **99**, 13368; T. Ha and P. Tinnefeld, *Annu. Rev. Phys. Chem.*, 2012, **63**, 595; S. van de Linde and M. Sauer, *Chem. Soc. Rev.*, 2014, **43**, 1076; J. Xu, J. Chang, Q. Yan, T. Dertinger, M. P. Bruchez and S. Weiss, *J. Phys. Chem. Lett.*, 2013, **4**, 2138; S.-n. Uno, M. Kamiya, T. Yoshihara, K. Sugawara, K. Okabe, M. C. Tarhan, H. Fujita, T. Funatsu, Y. Okada, S. Tobita and Y. Urano, *Nat. Chem.*, 2014, **6**, 681; P. Holzmeister, A. Gietl and P. Tinnefeld, *Angew. Chem., Int. Ed.*, 2014, **53**, 5685; H.-C. Yeh, C. M. Puleo, Y.-P. Ho, V. J. Bailey, T. C. Lim, K. Liu and T.-H. Wang, *Biophys. J.*, 2008, **95**, 729.
5 M. Levitus and S. Ranjit, *Q. Rev. Biophys.*, 2011, **44**, 123.
6 H.-S. Shin, A. Okamoto, Y. Sako, S. W. Kim, S. Y. Kim and C.-G. Pack, *J. Phys. Chem. A*, 2013, **117**, 27.
7 K. Kawai, H. Kodera, Y. Osakada and T. Majima, *Nat. Chem.*, 2009, **1**, 156; K. Kawai, M. Hayashi and T. Majima, *J. Am. Chem. Soc.*, 2012, **134**, 4806; K. Kawai and T. Majima, *Acc. Chem. Res.*, 2013, **46**, 2616.
8 K. Jia, Y. Wan, A. Xia, S. Li, F. Gong and G. Yang, *J. Phys. Chem. A*, 2007, **111**, 1593.
9 M. E. Sanborn, B. K. Connolly, K. Gurunathan and M. Levitus, *J. Phys. Chem. B*, 2007, **111**, 11064; B. J. Harvey, C. Perez and M. Levitus, *Photochem. Photobiol. Sci.*, 2009, **8**, 1105; E. M. S. Stennett, N. Ma, A. van der Vaart and M. Levitus, *J. Phys. Chem. B*, 2014, **118**, 152; A. Smith Darren, P. Holliger and C. Flors, *J Phys Chem B*, 2012, **116**, 10290.
10 I. H. Stein, S. Capone, J. H. Smit, F. Baumann, T. Cordes and P. Tinnefeld, *ChemPhysChem*, 2012, **13**, 931.
11 J. Spiriti, J. K. Binder, M. Levitus and A. van der Vaart, *Biophys. J.*, 2011, **100**, 1049.
12 R. R. Sinden, C. E. Pearson, V. N. Potaman and D. W. Ussery, *Adv. Genome Biol.*, 1998, **5A**, 1; H. Sugiyama, *Bull. Chem. Soc. Jpn.*, 2007, **80**, 823.
13 A. M. Soto, D. Rentzeperis, R. Shikiya, M. Alonso and L. A. Marky, *Biochemistry*, 2006, **45**, 3051; H.-T. Lee, C. Carr, H. Siebler, L. Waters, I. Khutsishvili, F. Iseka, B. Domack, C. M. Olsen and L. A. Marky, *Methods Enzymol.*, 2011, **492**, 1.
14 G. Bonnet, O. Krichevsky and A. Libchaber, *Proc. Natl. Acad. Sci. U. S. A.*, 1998, **95**, 8602; J. Kim, S. Doose, H. Neuweiler and M. Sauer, *Nucleic Acids Res.*, 2006, **34**, 2516; M. Schuettpelz, J. C. Schoening, S. Doose, H. Neuweiler, E. Peters, D. Staiger and M. Sauer, *J. Am. Chem. Soc.*, 2008, **130**, 9507; T. Kaji, S. Ito, S. Iwai and H. Miyasaka, *J. Phys. Chem. B*, 2009, **113**, 13917; W. Al-Soufi, B. Reija, M. Novo, S. Felekyan, R. Kuehnemuth and C. A. M. Seidel, *J. Am. Chem. Soc.*, 2005, **127**, 8775.
15 K. Kawai, I. Saito and H. Sugiyama, *Tetrahedron Lett.*, 1998, **39**, 5221.
16 X. Liang, H. Asanuma and M. Komiyama, *J. Am. Chem. Soc.*, 2002, **124**, 1877; H. Asanuma, X. Liang, H. Nishioka, D. Matsunaga, M. Liu and M. Komiyama, *Nat. Protoc.*, 2007, **2**, 203.
17 Y. Yan, X. Wang, J. I. L. Chen and D. S. Ginger, *J. Am. Chem. Soc.*, 2013, **135**, 8382.
18 Z. Xie, N. Srividya, T. R. Sosnick, T. Pan and N. F. Scherer, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 534; A. Y. Kobitski, A. Nierth, M. Helm, A. Jaeschke and G. U. Nienhaus, *Nucleic Acids Res.*, 2007, **35**, 2047; S. K. Sarkar, N. M. Andoy, J. J. Benitez, P. R. Chen, J. S. Kong, C. He and P. Chen, *J. Am. Chem. Soc.*, 2007, **129**, 12461; T. Fessl and D. M. J. Lilley, *Biophys. J.*, 2013, **105**, 2175.

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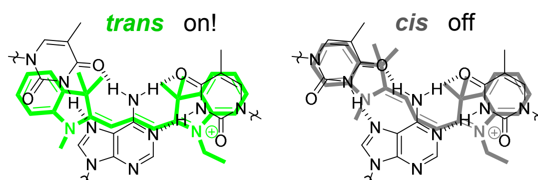

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