A Highly Fluorescent DNA Base Analogue that Forms Watson−Crick Base Pairs with Guanine

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Received July 14, 2000

Fluorescent DNA base analogues such as, for example, 2-aminopurine (2-AP), 1,N^6-ethenoadenine and 3-methyl-isoxanthopterin have received much attention as fluorescent markers for detecting nucleic acids and monitoring changes in their structure and dynamics in various contexts.[1] There is also an increased need of fluorescent chromophores that may be incorporated into nucleic acids, with minimal disturbance of structure, for single-molecule and advanced dynamics studies and in connection with transmembrane uptake mechanism studies. Two crucial properties of such compounds are their ability to form specific base pairs with the natural bases and to have a sufficient fluorescence quantum yield also when incorporated into oligonucleotides. These requirements are not fully met for the DNA base analogues mentioned above. The most extensively used fluorescent base analogue, 2-AP,[2] forms stable base pairs with thymine, but it can also form moderately stable base pairing with cytosine.[3] Furthermore, although the fluorescence quantum yield of 2-AP itself in uncomplexed form is high (68%),[2] incorporation into a single- or double-stranded oligonucleotide reduces it by approximately a 100-fold.

We can here report that the *t*ricyclic *c*ytosine base analogue, 3,5-diaza-4-oxophenothiazine, tC (Figure 1), besides being a very good cytosine analogue, has a previously unrecognized, desirably high fluorescence quantum yield even after incorporation into single- and double-stranded systems. The tricyclic ring system of tC increases the base stacking, and it has previously been shown that it increases the melting temperature of both DNA−RNA−,[4] DNA−peptide nucleic acid (PNA)−[5] and PNA−PNA-duplexes[5] and discriminates well between G and A targets.[4] It has also been shown that the phenoxazine analogue of tC, when incorporated into short oligonucleotides, improves cell permeation.[6] We will here present the striking fluorescence quantum yield properties, the fluorescence anisotropy, fluorescence lifetimes, absorption, and induced circular dichroism of this chromophore when incorporated into nucleic acid context, and also demonstrate the great advantage of using it for fluorescence energy transfer (FRET) distance probing.

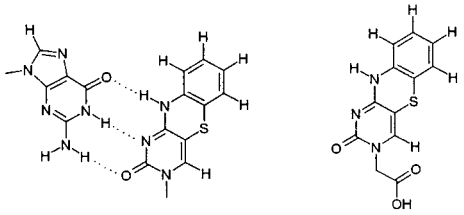

Figure 1. Structure of free tC to the right and G-tC base pair to the left.

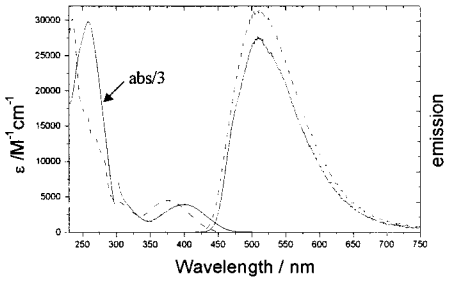

Figure 2. Isotropic absorption spectra (left) and emission spectra (right) of free tC (dashed curves) and tC-PNA (solid curves). Measurements performed at 25 °C in a 50 mM sodium phosphate buffer (pH 7.5).

The fluorescence emission of free tC (Figure 1) (synthesis according to Roth et al.[7]), of tC incorporated into a decamer of PNA (H-GTAGAT(tC)ACT-Lys-NH_2), tC-PNA, and its complementary DNA[8] were measured using a SPEX fluorolog 3 spectrofluorimeter. The shape of the emission of nonbase paired tC in the different environments is not significantly changed upon using different buffers, salt concentrations, or temperatures[9] and has a maximum at ∼505 nm (Figure 2). Quantum yields of fluorescence (ϕ_f) were determined relative to the quantum yield of 9, 10-diphenylanthracene in ethanol ($\phi_f = 0.95$).[10] The amazing finding is that the high quantum yield ($\phi_f = 0.20$) of free tC, is preserved also for tC in tC-PNA, and tC-PNA−DNA-duplex at all tested conditions. In Table 1 representative values for the quantum yields are shown. These properties are as far as we know unique for a DNA base analogue. As the quantum yield of tC could depend on the surrounding bases, it would be interesting to change the neighboring bases of tC. So far only a preliminary study on a single-stranded decamer of PNA (H-G(tC)AGA(tC)-GA(tC)G-Lys-NH_2) have been done, showing a quantum yield slightly lower than 0.20.[11] Excitation anisotropy[12] measurements of the tC-PNA and the tC-PNA−DNA-duplex (performed on a SPEX fluorolog τ2 spectrofluorimeter) showed a small increase in anisotropy upon hybridization (see Supporting Information, S1), which is good evidence that the oligomers actually form a duplex. The small change is in fair agreement with an earlier study of the change in anisotropy depending on molecular weight of oligonucleotides.[13]

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(1) (a) Holmén, A.; Nordén, B.; Albinsson, B. *J. Am. Chem. Soc.* **1997**, *119*, 3114−3121. (b) Holmén, A.; Albinsson, B.; Nordén, B. *J. Phys. Chem.* **1994**, *98*, 13460−13469. (c) Nordlund, T. M.; Andersson, S.; Nilsson, L.; Rigler, R.; Gräslund, A.; McLaughlin, L. W. *Biochemistry* **1989**, *28*, 9095−9103. (d) Hawkins, M. E.; Pfleiderer, W.; Balis, F. M.; Porter, D.; Knutson, J. R. *Anal. Biochem.* **1997**, *244*, 86−95.

(2) Ward, D. C.; Reich, E.; Stryer, L. *J. Biol. Chem.* **1969**, *244*, 1228−1237.

(3) Freese, E. *J. Mol. Biol.* **1959**, *1*, 87−105.

(4) Lin, K.; Jones, R. J.; Matteucci, M. *J. Am. Chem. Soc.* **1995**, *117*, 3873−3874.

(5) Eldrup, A. B.; Nielsen, B. B.; Haaima, G.; Rasmussen, H.; Kastrup, J. S.; Christensen, C.; Nielsen, P. E. *Eur. J. Org. Chem.* **2001**, in press.

(6) Flanagan, W. M.; Wagner, R. W.; Grant, D.; Lin, K. Y.; Matteucci, M. D. *Nature Biotechnol.* **1999**, *17*, 48−52.

(7) Roth, B.; Schloemer, L. A. *J. Am. Chem. Soc.* **1963**, *28*, 2659−2672.

(8) The DNA was purchased from MedProbe AS, Oslo.

(9) Temperatures: 15, 25, 35 °C. Buffers: Na^+ citrate buffer (pH 4), Na^+ phosphate buffer (pH 7.5), glycine−NaOH buffer (pH 10). Total Na^+ concentration: 10, 50, 100 mM. The concentrations of oligomers, here as throughout this work, were between 1 and 25 μM. The bandwidth for all fluorescence measurements both for excitation and emission was 1 nm. The excitation wavelengths were at the maximum of the lowest-energy band of free tC (375 nm) and of the tC-PNA (400 nm).

(10) Morris, J. V.; Mahaney, M. A.; Huber, J. R. *J. Phys. Chem.* **1976**, *80*, 969−974.

(11) The decrease in quantum yield ($\phi_f \approx 0.15$) of the decamer with 3 tCs probably depends on difficulties in solving the oligo.

(12) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*; Plenum Press: New York, 1983; pp 112−150.

(13) Murakami, A.; Nakaura, M.; Nakatsuji, Y.; Nagahara, S.; Tran-Cong, Q.; Makino, K. *Nucleic Acids Res.* **1991**, *19*, 4097−4102.

10.1021/ja0025797 CCC: $20.00
Published on Web 02/16/2001

Table 1. Fluorescence Properties of free tC, tC in tC-PNA, and in tC-PNA−DNA Duplex

	free tC	tC-PNA	tC-PNA−DNA
ϕ_f[a]	0.20	0.22	0.21
τ[a]/ns	3.7	6.2	5.9

[a] Measured in 50 mM sodium phosphate buffer (pH 7.5) and at 25 °C.

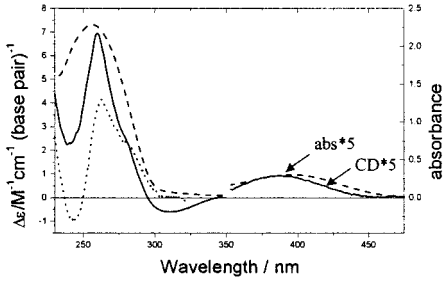

Figure 3. Circular dichroism (solid curve) and absorption (dashed curve) spectra of tC-PNA hybridized to complementary DNA. Experiments performed in 50 mM sodium phosphate buffer (pH 7.5) at 25 °C. The tC-PNA and the complementary DNA were left to hybridize at 25 °C for at least 30 min before recording the CD-spectrum. For comparison, a CD spectrum of the corresponding PNA−DNA duplex without tC is also inserted (dotted curve, data from Tomac et al.[16]).

The time-resolved fluorescence measurements were performed on the SPEX τ2 fluorimeter using the phase shift and modulation technique.[14] The lifetimes for free tC, tC-PNA, and tC-PNA−DNA are presented in Table 1.

The isotropic absorption measurements (Figure 2) of the free tC, tC-PNA, and tC-PNA−DNA-duplex were performed on a Cary 4B spectrophotometer.[15] A desirable property of the tC-base is that it has its lowest-energy absorption peak, which according to preliminary results is directed along the long-axis of tC, at 375 nm ($\epsilon \approx 4500$ cm^{-1} M^{-1}), thus well separated from the nucleobase absorption at 260 nm (Figure 2). This allows the tC-base to be selectively excited. In the tC-PNA, and upon hybridization between tC-PNA and complementary DNA this band is red-shifted (400 nm) and reduced in intensity ($\epsilon \approx 4000$ cm^{-1} M^{-1}, data for the duplex not shown). The red-shift and hypochromicity give further support that the tC base is indeed stacked between the nucleobases.

Circular dichroism (CD) measurements for tC-PNA−DNA (performed on a Jasco J-720 spectropolarimeter), show resemblance (Figure 3) for the DNA-region, with the CD-spectra of PNA−DNA hybrids previously reported by Tomac et al.[16] Thus, tC does not seem to distort the duplex conformation significantly. The positive CD signal, at the lowest transition of tC, and the negative peak at ∼310 nm also originating from tC (Figure 3), are results from the chromophore being in the chiral environment of the PNA−DNA helix.

For the FRET[17] measurements an HPLC-purified carboxytetramethylrhodamine labeled decamer of DNA,[18] Rh-DNA, complementary to the tC-PNA, was used.[19] The calculations of the decrease in donor emission show (see Supporting Information, S2) that the maximum energy transfer efficiency is ∼60% and that this value is reached at an approximate Rh-DNA:tC-PNA ratio of 1, as expected. κ^2 can be approximated to $^1/_3$, assuming that the plane of tC and then also the transition moment of its lowest transition is fixed at 90° to the vector connecting it with the transition moment of rhodamine and that rhodamine has random orientation.[20] Combined with the overlap integral between tC and rhodamine, which was calculated to be 2.3×10^{-13} cm^6/mmole, this gives $R_0 \approx 40$ Å. With an energy-transfer efficiency of ∼60%, as in the present case, the distance between the chromophores would be 37 Å. The estimated distance, using a model of a PNA−DNA duplex,[21] is 33 Å.

In conclusion, the preserved and high quantum yields of tC in single- and double-stranded systems are to our knowledge unique. This DNA base analogue, like 2-AP, has an absorption band separated from the normal DNA band at 260 nm and can therefore be selectively excited. The emission from tC has a maximum at ∼505 nm and therefore has a good overlap with for example the rhodamine excitation spectrum. Combined with the rigid and well-defined geometry of tC in a duplex, these properties suggest that tC with advantage could be used in energy-transfer systems. By contrast to most earlier studies, in which a random orientation of donor and acceptor moieties have been assumed, the firm stacking of tC between nucleobases may allow a refined quantitative treatment for accurately measuring distances within the molecular system. The fluorescence results support that tC (donor) and rhodamine (acceptor) is indeed an excellent pair for energy-transfer experiments. Providing the preserved, high-fluorescence quantum yield of tC is also present when tC is incorporated in DNA, it will be valuable as a fluorescent marker of DNA, for example for following DNA in electrophoretic gel or single-molecule manipulation experiments or for studying uptake of oligomers into cells or liposomes. In such studies, the fact that tC forms stable base pairs with G and minimally distorts the duplex, is a major advantage compared to having dangling molecules such as for example fluorescein and rhodamine tethered to the oligomers. One could also envisage using tC for studying DNA dynamics on the nanosecond time scale.

Supporting Information Available: Excitation anisotropy tC-PNA and tC-PNA−DNA-duplex; emission titration (FRET) of tC-PNA with Rh-DNA; structure of the Rh-DNA (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA0025797

(14) Lakowicz, J. R.; Laczko, G.; Cherek, H.; Gratton, E.; Limkemann, M. *Biophys. J.* **1984**, *46*, 463−477.

(15) The extinction coefficient (ϵ_{260}) at 80 °C for the decamer of DNA was 100700 cm^{-1} M^{-1}, and for the tC-PNA we obtained $\epsilon_{260} \approx 120000$ cm^{-1} M^{-1}. Hybridization between the strands was performed by mixing the solutions at 80 °C and slow-cooling the sample to 25 °C.

(16) Tomac, S.; Sarkar, M.; Ratilainen, T.; Wittung, P.; Nielsen, P. E.; Nordén, B.; Gräslund, A. *J. Am. Chem. Soc.* **1996**, *118*, 5544−5552.

(17) Förster, T. *Ann. Phys.* **1948**, *2*, 55−75.

(18) Rhodamine-labeled DNA was purchased from Life Technologies Inc.

(19) Extinction coefficient for the decamer of Rh-DNA was 144000 cm^{-1} M^{-1} at 260 nm and 82000 cm^{-1} M^{-1} at 555 nm (rhodamine peak). Experiments performed on the SPEX 3 fluorimeter using a bandwidth of 1 nm for both for the excitation and emission. As $\epsilon_{rhodamine}/\epsilon_{tC}$ has a minimum (∼0.6) at 393 nm, this wavelength was used to excite the system. Titration of Rh-DNA to the tC-PNA was followed by at least 15 min at 25 °C for hybridization. Emission spectra were recorded and compared with the same spectra of pure tC-PNA and Rh-DNA.

(20) Dale, R. E.; Eisinger, J. In *Biochemical Fluorescence: Concepts*; Chen, F., Edelhoch, H., Eds.; Marcel Dekker: New York, 1975; pp 115−284.

(21) Ratilainen, T.; Holmén, A.; Tuite, E.; Haaima, G.; Christensen, L.; Nielsen, P. E.; Nordén, B. *Biochemistry* **1998**, *37*, 12331−12342.