

# Structure–Function Relationships of Phenoxazine Nucleosides for Identification of Mismatches in Duplex DNA by Fluorescence Spectroscopy

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The effects of the flanking sequence on the mismatch-detection capabilities of the fluorescent nucleoside phenoxazine ( $\text{tC}^{\text{O}}$ ) were examined in a systematic fashion, and compared to the previously reported fluorescent, phenoxazine-based nucleoside  $\zeta^{\text{f}}$ . We see some similarities for the two fluorescent nucleosides, for example, the emission intensity of the C-mismatched duplex is always the highest, and a three-peak pattern in the spectrum emerges when the fluorosides are base-paired with A. However, phenoxazine was only able to distinguish a mismatch from the fully base-paired duplex in 11 out of 16 flanking sequences, and was able to identify each of the mismatches in six of those sequences. Therefore,  $\text{tC}^{\text{O}}$  shows poorer discrimination of mismatches than was previously re-

ported for  $\zeta^{\text{f}}$ , which could be used to identify all base-pairing partners in all immediately flanking sequences, albeit in some cases by using mercuric ions to selectively quench the emission of the T-mismatched duplex. The mercuric titration might resolve the overlap of fluorescence curves of  $\text{tC}^{\text{O}}$  in some flanking sequences, but not for 5'-d(C $\text{tC}^{\text{O}}$ G) and 5'-d(T $\text{tC}^{\text{O}}$ A) due to overlap of A-mismatch and G-match fluorescence curves. A pH titration was performed on  $\zeta^{\text{f}}$ ,  $\text{tC}^{\text{O}}$  and a N5-methylated derivative of  $\text{tC}^{\text{O}}$ , which showed that the emergence of the three-peak pattern is associated with the de-protonation of N5 in the fluorosides. We also show that neither the  $\alpha$ - nor  $\beta$ -anomer of the phenothiazine nucleoside ( $\text{tC}$ ) was able to detect a mismatch in any of the flanking sequences examined.

## Introduction

Fluorescent base analogues have gained considerable interest as probes in various biological systems, for example to detect single nucleotide polymorphisms (SNPs). SNPs are single-nucleotide variations<sup>[1]</sup> that, if located inside a protein-encoding region, can have a direct effect on the structure and function of the protein in question.<sup>[2]</sup> SNPs have consequently been linked to various diseases and disorders.<sup>[3]</sup> Therefore, methods for rapid screening of SNPs are desirable because they enable the identification of disease-causing genes. There are currently several methods of SNP typing available, most of which rely on hybridisation in that the probe sequence binds to the wild-type sequence, but not to the sequence containing a mismatch (SNP).<sup>[4]</sup> Hybridisation assays, some of which use an enzymatic step, to cleave,<sup>[5]</sup> digest<sup>[6]</sup> or elongate<sup>[7]</sup> the wild-type sequence, require very specific conditions, in particular the temperature must be carefully selected and controlled.<sup>[8]</sup> The use of enzymes prevents ultrahigh-throughput assays because the enzymatic step is often the time-controlling factor. Therefore a fluorescent probe that is able to directly identify its base-pairing partner, either by a change in its emission intensity or emissive colour would be a powerful tool in assisting high-throughput SNP typing.

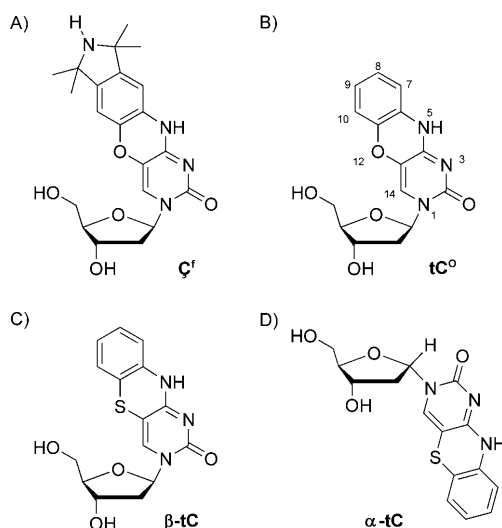
A fluorescent probe that enables direct detection of mismatches in duplex DNA would avoid cumbersome hybridisation protocols because the probe strand would always anneal to the target strand, regardless of whether it is fully complementary or not. Such fluorescent probes need to fulfill several criteria: an appreciable emission intensity, an excitation wavelength separated from the natural absorbance of DNA, an

emission maximum in the visible or near-infrared region and variation in fluorescence depending on its base-pairing partner. Several such probes, termed “base-discriminating fluorescent nucleosides” (BDFs) by Saito and co-workers, have been reported.<sup>[9]</sup> Most of these compounds are, however, limited to the detection of a single mismatch, that is, they can distinguish between a fully complementary sequence and one SNP allele.<sup>[10]</sup> It would be more advantageous if the fluorescent probe, in addition to detecting a mismatch, would also be able to directly identify the SNP in question. Furthermore, flanking bases should not quench the emission of the probe to a discernable degree, as is the case for some BDFs.<sup>[11]</sup>

Recently, we reported a fluorescent nucleoside,  $\zeta^{\text{f}}$  (Scheme 1 A), which, after incorporation into duplex DNA, was able to uniquely identify each of the four DNA bases in duplex DNA.<sup>[12]</sup> Upon further examination, it became evident that there was a high degree of variance in accordance with the immediately flanking bases and for some flanking sequences overlap of fluorescence curves prevented identification of each mismatch.<sup>[13]</sup> The spectral overlap was resolved by titrating mercuric ions into solution, which selectively quenched the emission intensity of the T-mismatch duplex.<sup>[13]</sup> Thus, all mis-

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Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/cbic.201000478>.



**Scheme 1.** The four structurally related fluorescent nucleosides that were incorporated into duplex DNA in this study. A)  $\text{C}^f$ , B)  $\text{tC}^0$  (phenoxazine), the numbering system used throughout this article is shown (2, 4, 6, 11 and 13 omitted for clarity), C)  $\beta\text{-tC}$  ( $\beta$ -anomer of phenothiazine) and D)  $\alpha\text{-tC}$  ( $\alpha$ -anomer of phenothiazine).

matches could be uniquely identified. Because there is substantial synthetic work involved in the production of  $\text{C}^f$ ,<sup>[14]</sup> we were interested in determining if the phenoxazine<sup>[15]</sup> moiety ( $\text{tC}^0$ ; Scheme 1 B), upon which  $\text{C}^f$  was based, would show similar results. The assessment of  $\text{tC}^0$  as a SNP probe would furthermore provide valuable information about the importance of the structure of  $\text{C}^f$  for mismatch detection, more precisely the tetramethylpyrrolidine ring (TMP ring) that is fused to the phenoxazine scaffold. The TMP ring is not conjugated into the  $\pi$  system of the chromophore, nor does it adversely interfere with the structure of DNA based on the CD spectra and  $\Delta T_m$  values.<sup>[12]</sup> Therefore, one might expect the two fluorosides to respond similarly to their microenvironment in duplex DNA. However, the only flanking sequence ever tested by using phenoxazine, 5'-d(AtC<sup>0</sup>G), shows that it is unable to detect a mismatch,<sup>[16]</sup> whereas  $\text{C}^f$  not only detects a mismatch in this flanking sequence, but it identifies each of the 3 mismatches.<sup>[13]</sup> Given the large flanking sequence variation of  $\text{C}^f$  fluorescence, a systematic flanking study of  $\text{tC}^0$  was required for the analysis of its structure–function relationship with regards to mismatch detection.

Another interesting derivative of phenoxazine is phenothiazine ( $\text{tC}$ ; Scheme 1 C, D), in which a single oxygen atom (O12 in Scheme 1 B) has been replaced with a sulfur atom. Like phenoxazine and  $\text{C}^f$ , phenothiazine does not perturb DNA duplexes.<sup>[17]</sup> It has been used as a redox-active probe,<sup>[18]</sup> to measure distances in DNA by FRET<sup>[19]</sup> and as a PNA-nucleoside.<sup>[20]</sup> However the mismatch-detection capabilities of phenothiazine have never been documented, even though some features of  $\text{tC}$  emission make it a promising candidate for SNP detection; the emission of phenothiazine is red-shifted by approximately 50 nm in comparison to both  $\text{C}^f$  and  $\text{tC}^0$ <sup>[17c,20]</sup> and, unlike most fluorescent nucleosides, a flanking G/C base pair does not quench the  $\text{tC}$ -emission, in duplex DNA.<sup>[17b]</sup>

We show that the flanking sequence has a profound effect on the mismatch-detection capabilities of phenoxazine ( $\text{tC}^0$ ). There is a large variation in the emission of  $\text{tC}^0$  between flanking sequences. The fluorescent nucleoside is able to detect a mismatch in a number of sequences, but is not as good a SNP probe as the previously described  $\text{C}^f$ , primarily because the three-peak pattern in the A-mismatched  $\text{tC}^0$  duplexes is also observed to some extent in the fully base-paired duplex. Finally we show that both phenothiazine ( $\text{tC}$ ) anomers are unsuitable for SNP detection.

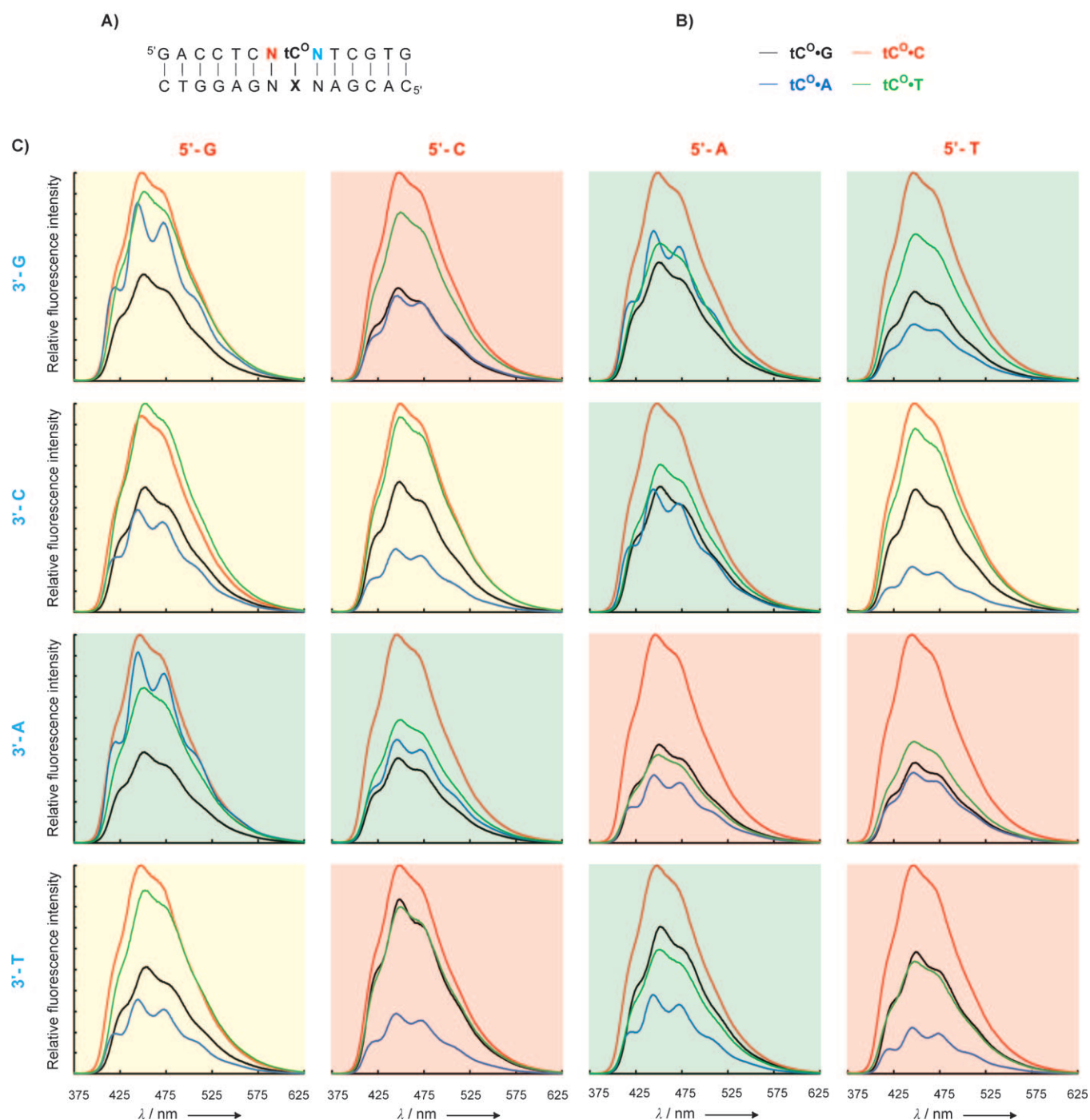
## Results and Discussion

### Photophysical properties of $\text{tC}^0$ -modified oligomers

Phenoxazine was incorporated into a 14-mer oligodeoxynucleotide (ODN) sequence (Figure 1 A) that was previously used to study flanking-sequence dependence on the fluorescence of  $\text{C}^f$  in DNA duplexes,<sup>[13]</sup> after preparation of the  $\text{tC}^0$ -phosphoramidite.<sup>[15]</sup> Each of the 16 phenoxazine-labelled ODNs was annealed to its complementary strand (G opposite  $\text{tC}^0$ ) as well as the three possible mismatches (A, C and T opposite  $\text{tC}^0$ ; Figure 1 B). The  $T_m$  values of the duplexes containing the flanking sequence 5'-d(GtC<sup>0</sup>A) were measured, which showed similar results as for  $\text{C}^f$ , that is, that all duplexes are stable at 20 °C (Supporting Information). The emission of phenoxazine in single-stranded DNA was of comparable intensity to that of the mismatches (data not shown). The relative fluorescence intensities of the 64  $\text{tC}^0$ -duplexes at 20 °C are shown in Figure 1 C.

First, there is a high degree of variance in the emission intensities of the duplexes, depending on the flanking sequence (Figure 1 C), which was also the case for  $\text{C}^f$ .<sup>[13]</sup> The mismatch-detection capabilities of phenoxazine is clearly highly dependent on the immediate flanking sequence because some sequences are better equipped to identify the mismatches, for example 5'-(TtC<sup>0</sup>G). As we have previously done for  $\text{C}^f$ ,<sup>[13]</sup> the flanking sequences were grouped into three distinct categories depending on the spectral overlap. Spectra were considered to overlap if the difference in the area under the corrected emission curves was less than 15%, except when the shape of the spectrum (especially for the A-mismatch) could be used for identification. The first category (Figure 1 C, green background), in which  $\text{tC}^0$  was able to detect and identify a mismatch, included six out of the 16 possible flanking sequences. The second class contained five flanking sequences (Figure 1 C, yellow background), in which the phenoxazine nucleoside was able to detect a mismatch, but was unable to identify the mismatched base. The third category held the remaining flanking sequences, in which  $\text{tC}^0$  was unable to detect whether or not a mismatch had occurred (Figure 1 C, red background).

The A-mismatched duplexes had a three-peak pattern, with peaks arising at approximately 425, 450 and 475 nm. An additional shoulder could be seen at approximately 520 nm (Figure 1 C, blue lines). This three-peak pattern for A-mismatches could also be observed when  $\text{C}^f$  was used as a fluorescent probe; this enabled the unambiguous identification of the A-



**Figure 1.** A) The DNA sequence used for the flanking study in which N is G, C, A or T and X is tC<sup>0</sup>'s base-pairing partner. B) The colour codes of the emission spectra for the different tC<sup>0</sup> base pairs. C) The relative emission intensity of all 64 tC<sup>0</sup> duplexes. The nucleotides flanking the 5'- and 3'-side of tC<sup>0</sup> are red and blue, respectively and change from G, C, A and T horizontally (5'-flanking) and in the same order vertically for the 3'-flanking side. The sequences that can readily distinguish between all base-pairing partners have a green background, whereas those that are only able to identify a mismatch from the fully base-paired duplex have a yellow background. The sequences in which tC<sup>0</sup> is unable to distinguish between the fully base-paired G and one of the mismatches have a red background. Each panel has been normalised separately by defining the emission intensity of the most fluorescent duplex as 1.00. Panels are highlighted green if the difference in area under the corrected emission curves exceeds 15% for all duplexes, except where it is possible to use the three-peak pattern to identify the A-mismatched duplexes. Panels are highlighted yellow if the difference in area under the corrected emission curves of the C-mismatched and T-mismatched duplexes does not exceed 15%. Panels are highlighted red if any mismatched duplex does not exceed 15% difference from that of the fully base-paired duplex, except in flanking sequences where it is possible to use the three-peak pattern of A-mismatched duplexes to distinguish it from the fully base-paired duplex.

mismatch.<sup>[13]</sup> In contrast to tC<sup>f</sup>, this peak pattern was not only observed for A-mismatches, but also in the fully base-paired tC<sup>0</sup>-duplexes (Figure 1 C, black lines). Therefore, the three-peak

pattern could not be used for unambiguous identification of A-mismatches by using tC<sup>0</sup>, in particular for the sequences 5'-d(CtC<sup>0</sup>G) and 5'-d(TtC<sup>0</sup>A), in which the emission intensities of

the fully base-paired duplex and the A-mismatch are similar, as well as the three-peak pattern. Hence, the fully base-paired and A-mismatched duplexes cannot be distinguished from one another in these two sequences.

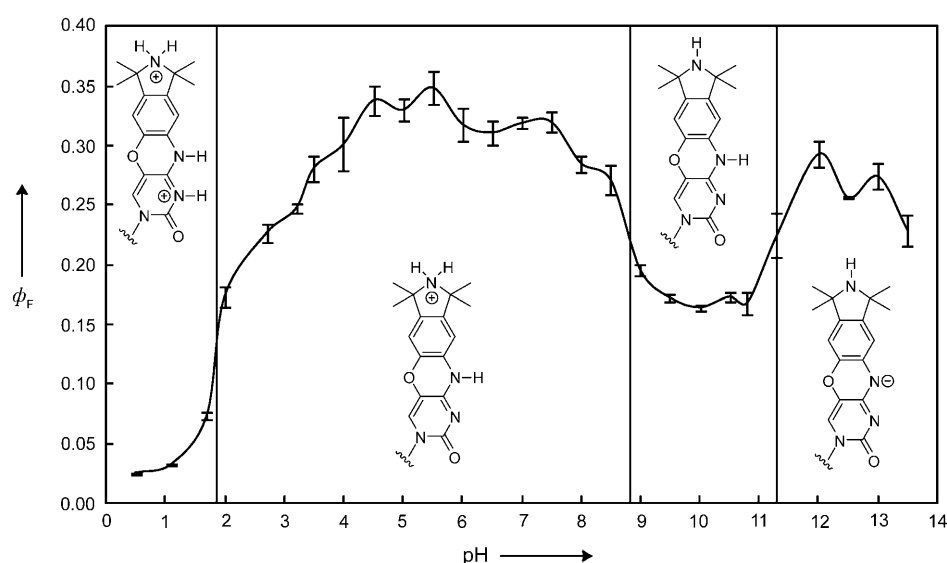
In spite of the large variation in the fluorescence of nucleosides  $\zeta^f$  and  $\text{tC}^0$  when placed in different structural contexts (mismatches and flanking sequence variation), there are not many clear trends, and this makes it difficult to identify the causes. Plausible explanations include different exposure of the fluorescent nucleoside to the solvent and electronic interactions within the helical stack. For example, the high fluorescence for the  $\text{tC}^0$ -C-mismatch in all the sequence contexts might be explained by the fact that the  $\text{tC}^0$ -C base pair (or the  $\zeta^f$ -C base pair) is the weakest of all the  $\text{tC}^0$  base pairs and might, therefore, have a tendency to unstack. In fact, increasing the temperature increases the fluorescence of the  $\zeta^f$ -C base pair more than the other  $\zeta^f$  base pairs.<sup>[21]</sup> However, addition of KI, which is a known solution quencher of fluorescence, had a similar effect on all the  $\zeta^f$  base pairs,<sup>[21]</sup> indicating that  $\zeta^f$  was equally exposed to the solvent, regardless of base-pairing partner. Thus, clear-cut explanations are not available at this point, but high-resolution data of select sequences would be valuable for providing the detailed structural contexts.

### Comparison of $\text{tC}^0$ and $\zeta^f$ -modified oligomers for fluorescent detection of mismatches

Because  $\text{tC}^0$  and  $\zeta^f$  are very similar in structure, it is not surprising that they have some common traits, both in the emission intensities and the shapes of the spectra. As discussed above, the A-mismatched phenoxazine-duplex always displayed a three-peak pattern, even though the intensity of the pattern was not as pronounced as for  $\zeta^f$ .<sup>[13]</sup> Another common feature of  $\text{tC}^0$  and  $\zeta^f$ , is that the emission intensity of the C-mismatched duplexes was consistently higher than that of the fully base-paired duplex. However, in spite of these similarities,  $\zeta^f$  was a better probe of mismatches than  $\text{tC}^0$ ;  $\zeta^f$  was able to detect a mismatch in 13 (compared with 11 for  $\text{tC}^0$ ) flanking sequences and identify its base-pairing partner in 10 (opposed to 6 for  $\text{tC}^0$ ) of those sequences.<sup>[13]</sup> Furthermore, and most importantly, all overlap issues of  $\zeta^f$  could be resolved by selectively quenching the emission of the T-mismatched duplex with mercuric ions. This  $\text{Hg}^{2+}$  quenching can be utilised in the case of 5'-d(CtC<sup>0</sup>T), 5'-d(AtC<sup>0</sup>A) and 5'-d(TtC<sup>0</sup>T), because it is the T-mismatched duplex that overlaps with the fully base-paired duplex. However, this method cannot be used to resolve the overlap issues of phenoxazine in the flanking se-

quences 5'-d(CtC<sup>0</sup>G) and 5'-d(TtC<sup>0</sup>A) in which the G-matched and the A-mismatched duplexes overlap, because the A-mismatched and G-matched duplexes were quenched to a similar extent, regardless of flanking sequence, upon addition of mercuric ions to  $\zeta^f$ -labelled duplexes.<sup>[13]</sup>

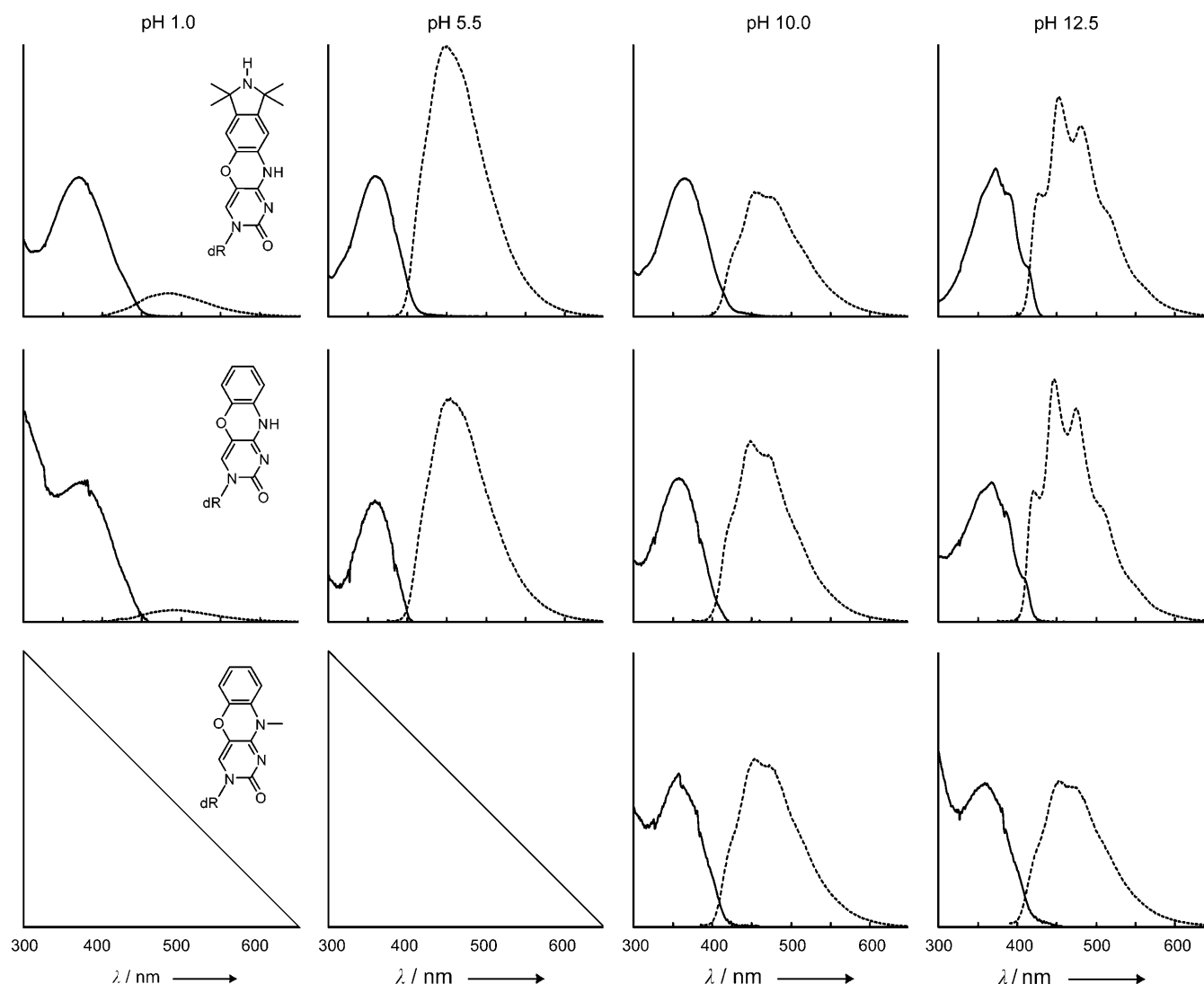
The difference in the mismatch-identification capability, of the two fluorescent nucleosides presumably lies in the tetramethylpyrrolidine ring, which is the only structural difference between  $\text{tC}^0$  and  $\zeta^f$ . The TMP ring has a secondary amine, which is protonated under mild conditions (physiological pH). Therefore, to further study the effect of protonation on fluorescence, the fluorescence of  $\zeta^f$  was examined at different pH values (Figure 2), which showed that the emission of  $\zeta^f$  is highly dependent on pH.



**Figure 2.** The quantum yield of  $\zeta^f$  as a function of pH. The four regions, attributed to different protonation states (see inserts) are visually estimated from the change in quantum yield.

Figure 2 has been subdivided into four regions, according to the quantum yield of  $\zeta^f$ . Each of these regions of varying quantum yield is likely associated with different protonation states of the nucleoside. At highly acidic pH (<2.0), the pyrrolidine ring and the N3 atom of  $\zeta^f$  are presumably both protonated, which clearly causes a dramatic reduction in fluorescence emission. From acidic to slightly alkaline pH (2.0–9.0), at which only the pyrrolidine ring of the nucleoside is expected to be protonated,  $\zeta^f$  shows the maximum emission of all fluorescent nucleosides measured. In the pH range from 9–11, there is a drop in the quantum yield and subsequently, the emission intensity, which is likely associated with the neutral form of  $\zeta^f$ . At alkaline pH (>11.0), the quantum yield rises again, and the emission spectra become structured (Figure 3, top).

At pH 7.0, the positive charge on the TMP ring of  $\zeta^f$  is probably causing an electron-withdrawing inductive effect, which alters the polarisation of the chromophore and thereby changes the emission properties of  $\zeta^f$ , relative to  $\text{tC}^0$ . The effect of the TMP ring on the pH profile was investigated by determining the effect of pH on the emission of  $\text{tC}^0$  at selected pH values (Figure 3, middle). The emission intensity of  $\text{tC}^0$  was



**Figure 3.** The normalised absorption (—) and emission (----) profiles of  $\zeta^f$ ,  $tC^\circ$  and  $N5\text{-CH}_3\text{-}tC^\circ$  (**3**; top to bottom) at pH 1.0, 5.5, 10.0 and 12.5 (left to right). Due to poor solubility, the spectra for  $N5\text{-CH}_3\text{-}tC^\circ$  (**3**) at pH 1.0 and 5.5, are omitted. All spectra have been normalised to the same scale (0.05 a.u. absorbance at 365.5 nm)

nearly nonexistent at pH 1.0, as was the case for  $\zeta^f$ , and exhibited high emission at pH 5.5. In contrast to  $\zeta^f$ , however, the emission intensity of  $tC^\circ$  at pH 10.0 is similar to that at pH 5.5; this is consistent with the fact that there is no difference in polarisation of the  $tC^\circ$  structure at these pH values.

At alkaline pH, the quantum yield of  $tC^\circ$  increased, and the spectrum became structured, as was observed for  $\zeta^f$  (Figure 3, middle). Interestingly, the three-peak pattern that emerged for  $\zeta^f$  and  $tC^\circ$  at high pH (Figure 3) is identical to the three-peak pattern observed for the A-mismatched duplexes (Figure 1C) and is attributed to the deprotonation of N5 of the fluorescent nucleosides (Figure 2). To confirm this hypothesis, we prepared an N5-methylated derivative of phenoxazine (**3**; Scheme 2) and determined the effect of pH on its fluorescence. Due to lower solubility of the methylated derivative of phenoxazine, absolute ethanol was used as a co-solvent (30%) in the buffer systems. Even with the cosolvent, the solubility at pH 1.0 and 5.5, was still too low for accurate quantum yield determinations.

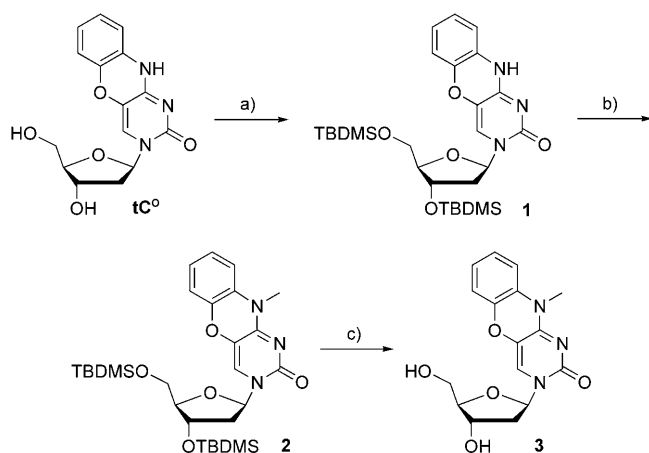
The N5-methylated phenoxazine does not exhibit the three-peak pattern at alkaline pH values (Figure 3, bottom), nor does the emission intensity increase, which is in agreement with our hypothesis that the de-protonation of N5 of both  $\zeta^f$  and  $tC^\circ$  at alkaline pH causes the three-peak pattern.

The emergence of the three-peak pattern in both alkaline medium and in the A-mismatched duplex for  $\zeta^f$  and  $tC^\circ$ , suggests a similar polarisation of the nucleosides. Therefore, the three-peak pattern for the A-mismatch might originate from a tautomer that would stabilise the mismatch pair between  $\zeta^f$  or  $tC^\circ$  and A and, in effect, remove the proton from the N5 atom of the fluorescent nucleoside. However, more detailed structure studies are required to confirm this hypothesis.

#### Photophysical properties of $tC$ -modified oligomers

As previously discussed, the phenothiazine nucleoside ( $tC$ ) is similar to that of phenoxazine ( $tC^\circ$ ), except that a sulfur atom

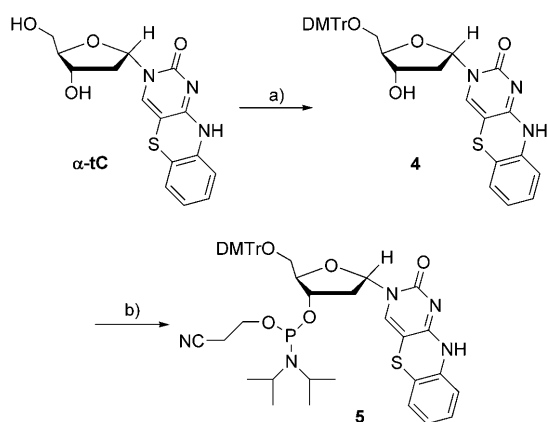




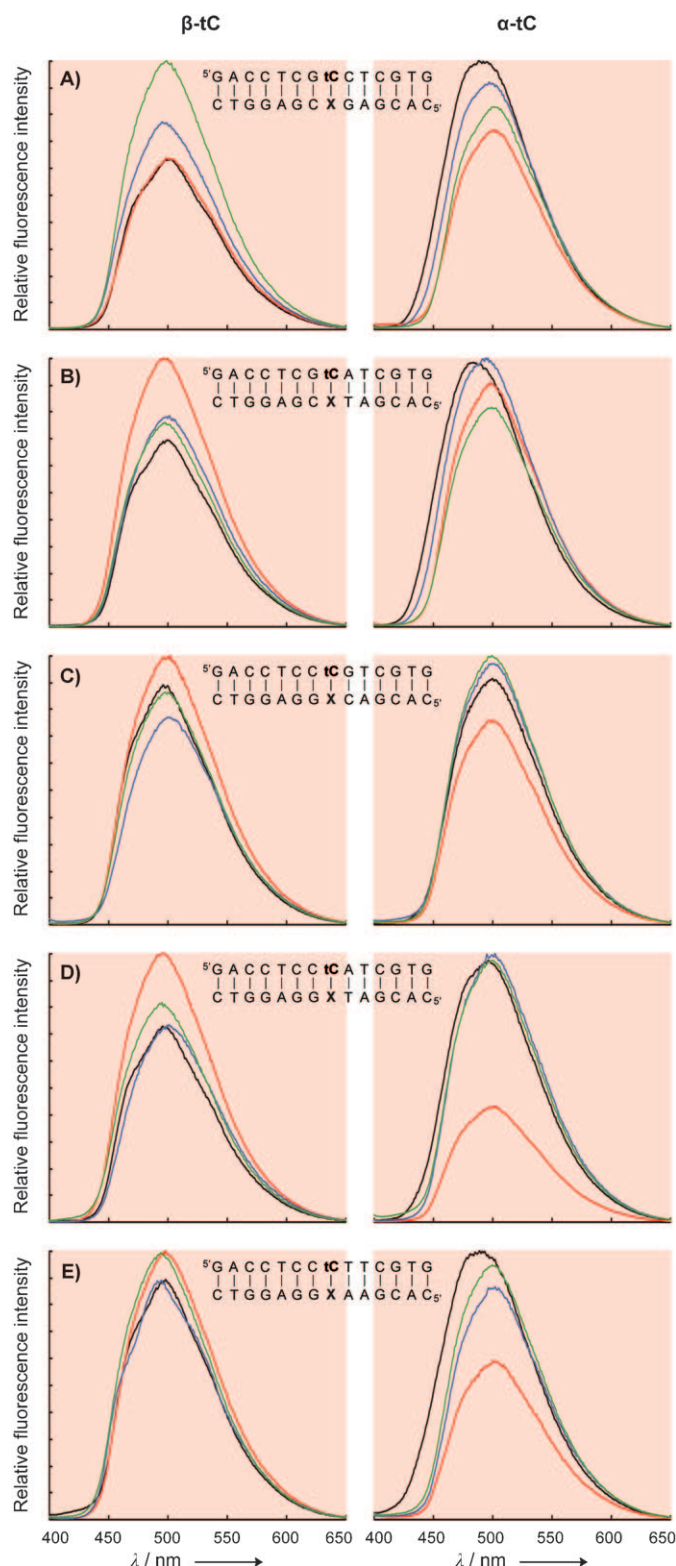
**Scheme 2.** Synthesis of the fluorescent nucleoside *N*5-CH<sub>3</sub>-**tC**<sup>0</sup> (**3**) from the phenoxazine nucleoside (**tC**<sup>0</sup>). a) TBDMS-Cl, imidazole, DMF (90%); b) CH<sub>3</sub>I, K<sub>2</sub>CO<sub>3</sub>, DMF (62%); c) *tert*-butyl ammonium fluoride, THF, (84%).

is substituted for a single oxygen atom (O12). Despite the similarity between phenoxazine and phenothiazine, the synthetic schemes for the fluorosides utilise two different techniques.<sup>[15,22]</sup> Because the synthesis of phenothiazine yields both the  $\alpha$ - and  $\beta$ -anomers,<sup>[22]</sup> they were separated during synthesis and both incorporated into oligonucleotides. The synthesis of the phosphoramidite for  $\beta$ -phenothiazine has already been reported,<sup>[22]</sup> and the tritylation and phosphitylation of the  $\alpha$ -anomer was carried out in a similar manner (Scheme 3).

Initially, five sequences were chosen for incorporations of **tC** because they encompass the different types of mismatch detection in phenoxazine duplexes, that is, duplexes that can detect and identify a mismatch (5'-d(CXA) and 5'-d(GXA)), a duplex that can detect a mismatch (5'-d(GXC)), and finally duplexes that are unable to detect mismatches (5'-d(CXG) and 5'-d(CXT); Figure 4). Both anomers of phenothiazine, incorporated into duplex DNA, exhibited markedly lower emission intensities than **C**<sup>f</sup> and **tC**<sup>0</sup>-modified DNAs (Table S1 in the Supporting Information).



**Scheme 3.** Synthesis of phosphoramidite **5** from fluorescent nucleoside  $\alpha$ -**tC**. a) DMTrCl, DMAP, pyridine; b) NC(CH<sub>2</sub>)<sub>2</sub>OP(N(*i*Pr)<sub>2</sub>)<sub>2</sub>, DIPAT (81% over two steps).



**Figure 4.** The relative emission intensity of the sequences tested by using the  $\beta$ - (left) and  $\alpha$ -anomer (right) of phenothiazine at 20 °C. A) 5'-d(GtCC), B) 5'-d(GtCA), C) 5'-d(CtCG), D) 5'-d(CtCA), E) 5'-d(CtCT). The difference in emission intensity of the fully base-paired duplex (black line) and at least one of the mismatched duplex, is always under 15%. Each panel has been normalised separately by defining the emission intensity of the most fluorescent duplex as 1.00. The colour codes used are; **tC**-G (black), **tC**-C (red), **tC**-A (blue) and **tC**-T (green).

Similar fluorescence results were obtained for both the  $\alpha$ - and  $\beta$ -anomers. In all flanking sequences, the fully base-paired duplex and the mismatched duplexes show similar emission intensities, that is, according to our previously used classification system, all phenothiazine-flanking sequences are red. Furthermore, the three-peak pattern cannot be seen in any phenothiazine-labelled duplex. Thus, the introduction of a sulfur atom has a detrimental effect on mismatch detection. Because phenothiazine was unable to discriminate between mismatches, only the initial subset of five flanking sequences out of 16 possible was examined. It has been reported that neighbouring bases have a negligible effect on the emission of the  $\beta$ -anomer of phenothiazine.<sup>[17b]</sup> Unfortunately, the same is true for the opposing nucleobase, that is, it is unable to distinguish between its base-pairing partners.

$\beta$ -Phenothiazine was expected to interact similarly with its base-pairing partner as phenoxazine because NMR spectroscopy studies have shown that  $\beta$ -tC is incorporated into the duplex and forms a stable base pair with G.<sup>[17a]</sup> In contrast, the  $\alpha$ -anomer was not expected to be paired within the duplex due to the geometry at the anomeric linkage. A better discrimination of the opposite base was observed by using the  $\alpha$ -anomer in two sequences, 5'-d(GtCC) and 5'-d(CtCT) (Figure 4). The difference is not sufficient to distinguish the mismatches from the fully base-paired duplex, as decided by the 15% criteria. However, it was unexpected that a greater difference in fluorescence would be observed for the  $\alpha$ -anomer, because the  $T_m$  data shows that the fully base-paired and all-mismatched duplexes have the same thermal stability (Table S2), which indicates similar structural context. Interestingly, the  $\alpha$ -anomer mismatches have the highest thermal stability of all mismatches, which suggests that the fluorescent nucleoside has some form of stabilising effect on the duplex. At this time, the nature of this effect is unclear. The  $T_m$  values for the  $\beta$ -anomer show variance similar to the tC<sup>0</sup>- and  $\zeta^f$ -labelled duplexes (Supporting Information), which is in agreement with the fact that the  $\beta$ -anomer is involved in base-pairing interactions within the duplex.

## Conclusions

The mismatch-detection capabilities of  $\alpha$ - and  $\beta$ -anomers of phenothiazine (tC) were investigated. Neither anomer of tC is suitable for SNP detection because they were unable to detect mismatches in any of the tested sequences. In contrast, phenoxazine (tC<sup>0</sup>) was able to detect mismatches for some flanking sequences. However, tC<sup>0</sup> was not as good of a mismatch probe as the previously reported  $\zeta^f$ , which was able to identify all mismatches in any flanking sequence. In six flanking sequences, tC<sup>0</sup> is able to detect and identify its base-pairing partner, whereas in five flanking sequences it is only able to detect a mismatch. In the remaining five flanking sequences, the fluorescent nucleoside is unable to distinguish between the fully base-paired and a mismatched duplex. Furthermore, the three-peak pattern that was previously exclusively seen with A-mismatches can be seen in the fully base-paired duplex, which further complicates identification of base-pairing

partner. In two immediately flanking sequences, 5'-d(CtC<sup>0</sup>G) and 5'-d(TtC<sup>0</sup>A), the appearance of the three-peak pattern in the fully base-paired duplex makes it impossible to discriminate between the A-mismatched and the fully base-paired duplex. As a consequence, the previous strategy of adding mercuric ions to resolve spectral overlap for  $\zeta^f$  will likely not work, because Hg<sup>2+</sup> ions selectively only reduce the emission of the T-mismatched duplexes. Therefore, the TMP ring of  $\zeta^f$  increases the mismatch-detection capabilities of the phenoxazine scaffold, presumably through polarisation of the chromophore by the aliphatic amine, which is protonated at physiological pH. Given the large differences in the fluorescent properties of these structurally related nucleosides, it is of interest to explore the effects of other substituents on the photophysical properties of phenoxazine.

## Experimental Section

**Materials:** Chemicals were obtained from commercial suppliers (Sigma-Aldrich and Acros) and were used without further purification. Solvents were stored over activated 3 Å molecular sieves. CH<sub>3</sub>CN, CH<sub>2</sub>Cl<sub>2</sub> and pyridine were always freshly distilled over CaH<sub>2</sub> prior to use. H<sub>2</sub>O was purified on a Barnstead EASYpure RoDi system. NMR spectra were recorded on a Bruker Avance 400 MHz instrument. Chemical shifts are reported in ppm relative to residual solvent peaks (CDCl<sub>3</sub>: 7.26 ppm for <sup>1</sup>H and 77.0 ppm for <sup>13</sup>C; CD<sub>3</sub>OD: 4.84 and 3.31 ppm for <sup>1</sup>H and 49.05 ppm for <sup>13</sup>C; 85% aq. H<sub>3</sub>PO<sub>4</sub> (external standard): 0.00 ppm for <sup>31</sup>P). <sup>1</sup>H NMR spectroscopy coupling constants are reported in Hz and refer to apparent multiplicities. ESI mass spectra were obtained on a Bruker micrOTOF-Q mass spectrometer. Analytical thin-layer chromatography was performed on glass backed TLC extra-hard layer plates (Kieselgel 60 F254, 0.25 mm, Silicycle). Visualisation was achieved by UV and p-anisaldehyde staining. Silica-gel chromatography was performed by using ultrapure flash silica gel from Silicycle (0.040–0.063 mm, 60 Å).

**Synthesis of TBDMS-protected phenoxazine (1):** (Scheme 2) *tert*-butyldimethylsilyl (TBDMS) chloride (0.406 g, 2.7 mmol) was added to a mixture of phenoxazine (0.100 g, 0.27 mmol) and imidazole (0.110 g, 1.61 mmol) in anhyd. dimethylformamide (DMF; 1.2 mL). The reaction was stirred at 22 °C for 8 h, after which the mixture was poured over H<sub>2</sub>O (5 mL) and extracted with EtOAc (3 × 15 mL). The organic extracts were combined and successively washed with H<sub>2</sub>O (3 × 5 mL) and brine (10 mL), dried over anhyd. Na<sub>2</sub>SO<sub>4</sub>, and the solvent was removed in vacuo. The product was purified by column chromatography by using acetone/petroleum ether (1:4) as an eluent to yield the protected phenoxazine (0.128 g, 90%) as a colourless oil. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  = 0.08 (s, 6H; Si-CH<sub>3</sub>), 0.15 (s, 3H; Si-CH<sub>3</sub>), 0.18 (s, 3H; Si-CH<sub>3</sub>), 0.89 (s, 9H; Si-C(CH<sub>3</sub>)<sub>3</sub>), 0.97 (s, 9H; Si-C(CH<sub>3</sub>)<sub>3</sub>), 2.07–2.13 (m, 1H; 2'H), 2.34–2.40 (m, 1H; 2'H), 3.18 (dd,  $J$  = 2.4, 11.3 Hz, 5'H), 3.89–3.91 (m, 1H; 5'H), 3.95 (dd,  $J$  = 2.4, 11.3 Hz, 1H; 3'H), 4.40–4.44 (m, 1H; 4'H), 6.32 (t,  $J$  = 6.5 Hz, 1H; 1'H), 6.65 (dd,  $J$  = 1.3, 7.9 Hz, 1H; ArH), 6.83 (ddd,  $J$  = 1.3, 7.9 Hz, 1H; ArH), 7.56 (s, 1H; H14), 7.62 ppm (d,  $J$  = 7.9 Hz, 1H; ArH); <sup>13</sup>C NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  = −5.53, −4.53, −3.56, 18.00, 18.54, 25.67, 25.72, 26.78, 41.91, 62.34, 70.83, 85.65, 87.53, 114.28, 123.96, 134.19, 126.84, 127.99, 142.67, 153.68, 154.92 ppm; HR-ESI-MS:  $m/z$ : calcd for C<sub>27</sub>H<sub>43</sub>N<sub>3</sub>O<sub>5</sub>Si<sub>2</sub>: 568.2639 [ $M$ +Na]<sup>+</sup>, found: 568.2636.

**N5-Methylation of TBDMS-protected phenoxazine (2):** (Scheme 2) MeI (0.002 mL, 0.034 mmol) was added to a mixture of

**1** (0.012 g, 0.022 mmol) and  $K_2CO_3$  (0.06 g, 0.045 mmol) in dry DMF (0.5 mL). The reaction mixture was stirred at 22 °C for 6 h, poured over  $H_2O$  (3 mL) and extracted with EtOAc (3 × 10 mL). The combined organic extracts were successively washed with  $H_2O$  (3 × 3 mL) and brine (10 mL), dried over anhyd.  $Na_2SO_4$ , and the solvent was removed in vacuo. The product was purified through column chromatography, by using acetone/petroleum ether (2:98) as an eluent to furnish the product (0.008 g, 62%) as an off-white solid.  $^1H$  NMR (400 MHz,  $CDCl_3$ ):  $\delta$  = 0.08 (s, 6H; Si- $CH_3$ ), 0.14 (s, 3H; Si- $CH_3$ ), 0.15 (s, 3H; Si- $CH_3$ ), 0.89 (s, 9H; Si- $C(CH_3)_3$ ), 0.96 (s, 9H; Si- $C(CH_3)_3$ ), 2.0–2.06 (m, 1H; 2'H), 2.15–2.20 (m, 1H; 2'H), 3.36 (s, 3H; N- $CH_3$ ), 3.75 (dd,  $J$  = 2.7, 11.3 Hz, 5'H), 3.86–3.88 (m, 2H; 3'H, 5'H), 4.39–4.42 (m, 1H; 4'H), 6.34 (t,  $J$  = 6.5 Hz, 1H; 1'H), 6.58–6.61 (m, 1H; ArH), 6.84–6.87 (m, 3H; Ha, ArH), 6.99–7.01 ppm (m, 1H; ArH);  $^{13}C$  NMR (400 MHz,  $CDCl_3$ ):  $\delta$  = –5.53, –4.84, –4.63, 17.98, 18.54, 25.73, 26.02, 28.69, 40.73, 62.83, 71.78, 85.10, 87.42, 109.64, 113.92, 123.51, 125.61, 128.09, 129.60, 133.70, 144.33, 149.34, 149.80 ppm; HR-ESI-MS  $[M+Na]^+$ :  $m/z$ : calcd for  $C_{28}H_{45}N_3O_5Si_2$ : 582.2795, found: 582.2782.

**Deprotection of N5-methylated-TBDMS-protected phenoxazine (3):** (Scheme 2) A solution of **2** (0.008 g, 0.014 mmol) in THF (0.5 mL) was treated with a solution of 1 M tetrabutylammonium fluoride (0.031 mL, 0.031 mmol). The reaction mixture was stirred at 22 °C for 5 h, after which, the solvent was removed in vacuo. The product was purified by preparative TLC by using acetone/petroleum ether (1:1) as the mobile phase, yielding N5-methyl-phenoxazine (0.004 g, 84%) as an off-white solid.  $^1H$  NMR (400 MHz,  $CD_3OD$ ):  $\delta$  = 2.16–2.18 (m, 2H; 2'H), 3.33 (s, 3H; N-Me), 3.72–3.76 (m, 2H; 5'H), 3.86–3.88 (m, 1H; 3'H), 4.36–4.37 (m, 1H; 4'H), 6.30 (t,  $J$  = 6.7 Hz, 1H; 1'H), 6.66 (d,  $J$  = 7.5 Hz, 1H; ArH), 6.85–6.89 (m, 2H; ArH), 6.97–6.99 (m, 1H; ArH), 7.00 ppm (s, 1H; H14);  $^{13}C$  NMR (400 MHz,  $CD_3OD$ ):  $\delta$  = 29.21, 40.44, 63.05, 72.48, 86.69, 111.72, 115.18, 124.70, 126.55, 127.00, 145.72, 151.05 ppm; HR-ESI-MS  $[M+Na]^+$ :  $m/z$ : calcd for  $C_{16}H_{17}N_3O_5$ : 354.1066, found: 345.1053.

**Tritylation of  $\alpha$ -phenothiazine (4):** (Scheme 3)  $\alpha$ -phenothiazine (0.110 g, 0.329 mmol), 4,4'-dimethoxytrityl chloride (DMTrCl; 0.167 g, 0.490 mmol) and 4-dimethylaminopyridine (DMAP; 2 mg, 0.001 mmol) were placed in a previously flame-dried round-bottomed flask and kept under vacuum overnight. Pyridine (4 mL) was added, and the reaction mixture stirred for 80 min, after which, a small amount of  $CH_3OH$  (3–5 drops) was added to quench the reaction. The solvent was removed in vacuo. The product was purified by column chromatography by using an eluent gradient of  $CH_2Cl_2$ ,  $CH_3OH$  and  $Et_3N$  (99:0:1 → 94:5:1). The product was associated with  $Et_3N$ , which was not removed, to stabilise the DMT group.  $^1H$  NMR (400 MHz,  $CDCl_3$ ):  $\delta$  = 2.45–2.53 (m, 2H), 3.14–3.21 (m, 2H), 3.79 (s, 6H), 4.43–4.49 (m, 2H), 6.07 (t,  $J$  = 6.5 Hz, 1H), 6.07–6.86 (m, 7H), 7.21–7.29 (m, 9H), 7.32 (s, 1H), 7.39–7.45 ppm (m, 1H);  $^{13}C$  NMR (400 MHz,  $CDCl_3$  + 10%  $[D_6]DMSO$ ):  $\delta$  = 45.39, 54.66, 62.90, 70.59, 85.93, 86.12, 112.73, 123.46, 126.25, 126.62, 127.44, 127.50, 127.66, 128.46, 129.43, 129.54, 133.43, 135.01, 135.27, 135.75, 143.99, 154.46, 157.97 ppm; HR-ESI-MS:  $m/z$ : calcd for  $C_{36}H_{33}N_3O_6S$ : 658.1988  $[M+Na]^+$ , found: 658.1936.

**Phosphitylation of DMTr-protected  $\alpha$ -phenothiazine (5):** Diisopropylamine tetrazolide (DIPAT; 0.067 g, 0.390 mmol) and DMTr-protected phenoxazine (0.2 g with  $Et_3N$ ) were dissolved in dry pyridine (5 mL) and the solvent evaporated in vacuo (see Scheme 3). This was performed twice. The reaction mixture was dissolved in dry  $CH_2Cl_2$  (3 mL) and 2-cyanoethyl- $N,N,N'$ -tetraisopropyl-phosphordiamidite (0.136 mL, 0.429 mmol) was added. The reaction mixture was stirred at 22 °C for 90 min. After completion, the mixture was diluted with  $CH_2Cl_2$  (75 mL), washed with sat. aq  $NaHCO_3$

(3 × 20 mL) and brine (25 mL), dried over anhyd.  $Na_2SO_4$ , and the solvent was subsequently removed in vacuo. Further purification was achieved by repetitive precipitation: the product was dissolved in a minimum amount of dry  $CH_2Cl_2$  (about 1 mL), an excess of cold  $n$ -hexane (about 100 mL) was added, and the liquid was decanted. This procedure was repeated twice, to furnish the product as a white solid (0.230 g, 81%).  $^{31}P$  NMR (400 MHz,  $CDCl_3$ ):  $\delta$  = 148.97, 149.41 ppm; HR-ESI-MS  $[M+Na]^+$ :  $m/z$ : calcd for  $C_{45}H_{50}N_5O_7PS$ : 858.3066, found: 858.3078.

**Synthesis and purification of modified ODNs:** The fluorescent phosphoramidites were used in automated oligodeoxynucleotide (ODN) synthesis to prepare the fluorescent ODNs, as previously reported for  $\zeta^f$ .<sup>[13]</sup> Syntheses of the modified ODNs was performed by following standard protocols of automated DNA synthesis, except for the incorporation of the modified phosphoramidite, for which the coupling time was extended (from 60 + 30 s to 300 + 300 s). Subsequent purification was obtained by 20% denaturing polyacrylamide gel electrophoresis (DPAGE). The identity of the ODNs was confirmed by MALDI-TOF mass spectrometry by using a Bruker autoflex III (Table S3) the samples were spotted on a MTP AnchorChip 400/384 TF, by using 3-hydroxycypicolinic acid as the matrix.

**Fluorescence measurements:** Fluorescence spectra of all modified nucleosides and duplexes were recorded on a SPEX FluoroMax spectrofluorometer, equipped with a constant temperature controller, by using a cell with spectral range 170–2200 nm (Spectrocell corporation, Orelan, PA, USA). The excitation wavelength was set to 365.5 nm for  $\zeta^f$  and  $tc^0$ , and 390 nm for both anomers of  $tc$ . Slits width were set to 1.000 nm for quantum yield determination. Emission spectra of nucleosides were measured by using 5–15  $\mu$ m solutions, and the concentrations of the duplexes were between 5–10  $\mu$ m. Nucleoside solutions were measured at the appropriate pH value by using the following solutions; 50 mM oxalate buffer (pH 0.50–2.70), 50 mM citrate buffer (pH 3.20–6.00), 50 mM phosphate buffer (pH 6.50–8.00), 50 mM Tris buffer (pH 8.50–9.00), 50 mM glycine/NaOH buffer (pH 9.50–10.50), dilute NaOH (pH 10.80–13.50). Due to the insolubility of the N-methylated derivative of phenoxazine in buffers, a 30% EtOH/buffer solution was used, when determining the quantum yield of the nucleoside. The duplex DNA samples were buffered to pH 7.00 by using a phosphate buffer (10 mM  $Na_2HPO_4$ , 100 mM NaCl, 0.1 mM  $Na_2EDTA$ ), and the samples annealed prior to fluorescence measurements, as described in the  $T_m$  measurements. The fluorescence quantum yields were measured (Tables S1 and S4) by the relative method<sup>[23]</sup> by using anthracene ( $\Phi_f$  = 0.27) in abs. EtOH as standard.<sup>[24]</sup>

**$T_m$  measurements:** All measurements were performed in phosphate buffer (10 mM  $Na_2HPO_4$ , 100 mM NaCl, 0.1 mM  $Na_2EDTA$ , pH 7.00). Concentrations of duplexes were 2.5  $\mu$ M, with the non-fluorescent complementary strand in 25% excess. Before the  $T_m$  measurements all samples were annealed by using a MJ Research Peltier Thermal Cycler (PTC)-200 DNA Engine, as follows: Heated to 90 °C for 2 min, gradually cooled to 60 °C over 5 min, to 40 °C over 10 min, to 22 °C over 15 min, then cooled to 4 °C. The samples were subsequently degassed by bubbling argon through the solutions. Absorbance of the samples was then measured at 260 nm as a function of temperature by using a UV/vis spectrometer (Perkin-Elmer Lambda 25) equipped with a Peltier Temperature Programmer. The temperature was scanned from 20–90 °C with a heating rate of 1 °C min<sup>–1</sup> and each duplex measured in triplicate.



## Acknowledgements

We thank the Science Institute of the University of Iceland for financial support.

**Keywords:** base discrimination • flanking sequences • fluorescent probes • nucleosides • single nucleotide polymorphism

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Received: August 15, 2010

Published online on February 14, 2011