Pergamon

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Tetrahedron Letters 44 (2003) 8571–8575

TETRAHEDRON LETTERS

Synthesis and characterisation of novel 3′-*O*- and 5′-*O*- modified azobenzene-thymidine phosphoramidites and their oligonucleotide conjugates as colorimeter DNA probes and FRET quenchers

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Received 20 August 2003; revised 1 September 2003; accepted 16 September 2003

Abstract—The synthesis of two new 'first generation' azobenzene based thymidine phosphoramidites **1** and **2** having the chromophore (DABCYL) covalently incorporated as an ester in the 3′-*O*- and 5′-*O*-positions of the deoxyribose, and the incorporation of these molecules into 16-mer Chronic Myeloid Leukaemia (CML) antisense oligonucleotides, giving **7** and **8**, respectively, is described. These compounds were designed as highly coloured probes, and to participate in a Fluorescence Resonance Energy Transfer (FRET) mechanism in the design of novel molecular beacons.

The design, synthesis, and biological evaluation of modified nucleotides and oligonucleotides has attracted substantial interest in recent years.[1] Such systems are promising candidates for the development of novel nucleic acid targeting therapeutic drugs for the antisense approach,[2] in homogeneous DNA sequence techniques,[3] as tools for gene-sequencing,[4] as molecular probes,[5] and in particular as molecular beacons.[6] Recently, Komiyama et al. conjugated simple azobenzene units into short oligonucleotides as side chains. Further they have incorporated these within the strands using prochiral diol[7] or *D*- or *L*-threoninol[8] based spacers, which were used to gain 'external control' (*cis–trans* isomerisation) over duplex and triplex DNA formation [demonstrated by melting temperature (T_m) experiments].[8] Even though these compounds are highly desirable, such modifications alter the local oligonucleotide backbone structure as they lack both the deoxyribose unit and the nucleic acid bases.[9] Furthermore, these azobenzene dyes only absorb strongly in the UV, a drawback for their use in molecular probes or as fluorescent quenchers.[10] We are particularly interested in the development of metal based nucleic acid targeting molecules. To this end we have developed several ruthenium-based antisense oligonucleotide conjugates capable of achieving site specific photosensitised cleavage of the *bcr-abl* signature sequence of the Philadelphia chromosome associated with Chronic Myeloid Leukaemia (CML),[11] as well as lanthanide based ribonuclease mimics for phosphodiester cleavage of mRNA.[12] In addition, we have recently made several highly coloured azobenzene based chemosensors for Na^+ and K^+, where distinct colour changes occurred upon ion recognition (which takes place through modulation of the Internal Charge Transfer, ICT, mechanism of the molecule).[13]

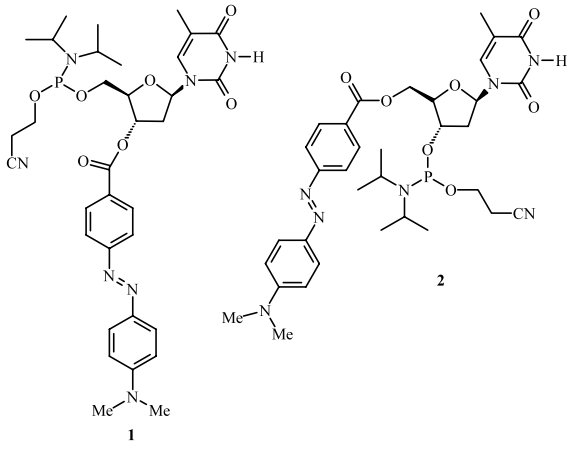

Figure 1. The structures of the two phosphoramidites **1** and **2**.

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doi:10.1016/j.tetlet.2003.09.115

Herein, we describe the synthesis of two nucleoside phosphoramidites **1** and **2** (Fig. 1) functionalised with the well known DABCYL (4-[4-dimethylaminophenylazo] benzoic acid, Fig. 2)[14] chromophore covalently incorporated as an ester at the 3′-*O*- and 5′-*O*-positions. We also describe the incorporation of **1** and **2** into a 16-mer CML antisense oligonucleotide at the 5′-terminus (i.e. giving rise to '*back to front*' derivative as in the case of **1**, or the more conventional '*front to front*' analogue **2**) as potential molecular probes for nucleic acids, and some preliminary analysis of these conjugates using melting temperature analysis and CD spectroscopy.

The reason for this particular design employed for **1** and **2** is threefold. Firstly, the DABCYL chromophore (which has very low fluorescence quantum yield)[13] is able to participate in Fluorescence Resonance Energy Transfer (FRET) as an excited state quencher, which is important in developing novel molecular beacons. Secondly, we predict that even though the two molecules might exert some slight influence on the structure of the oligonucleotides ('*back to front*' versus '*front to front*') they should not perturb double helix formation and therefore not alter greatly their physical properties such as the melting temperatures (T_m).[7,8] Thirdly, these compounds, being strongly absorbing in the visible region can be used for visual detection or colorimetric probes for oligonucleotides.[14] To the best of our knowledge these are the first examples of strongly visually absorbing azobenzene based oligonucleotide conjugates directly incorporated into the ribose unit of a thymidine nucleoside.

The synthesis of **1** and **2** is shown in Scheme 1. DABCYL was synthesised in accordance with published procedure.[15] Our initial synthetic strategy was to use the *N*-succinimide activated ester of DABCYL **3** and to incorporate it into either a 3′-*O*- and 5′-*O*-protected form of thymidine **4**. However, this initial attempt was unsuccessful. Therefore, a new approach was adopted for the synthesis of **1**. Thymidine **4** was first protected as its 5′-*O*-tritylthymidine, followed by coupling to DABCYL (in its acid form), using EDCl and DMAP in anhydrous THF at room temperature for 48 h. The intermediate was purified using silica flash column chromatography (CH_2Cl_2:EtOAc, 70:30) and isolated in 63% yield as a red coloured residue which crystallised upon standing. This was followed by the deprotection of the 5′-*O*-trityl group using 80% acetic acid under reflux for 30 min. The resulting thymidine–DABCYL conjugate **5** was purified by flash column chromatography (CH_2Cl_2:EtOAc, 70:30) and isolated in 55% yield as an orange coloured powder which when recrystallised from CH_3CN provided red crystals suitable for X-ray crystallographic analysis.[16,†] The structure of **5** (Fig. 3) clearly shows the azobenzene in its expected

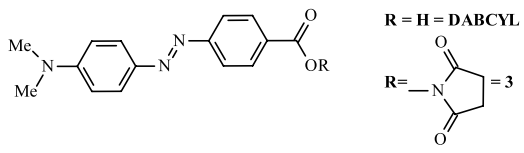
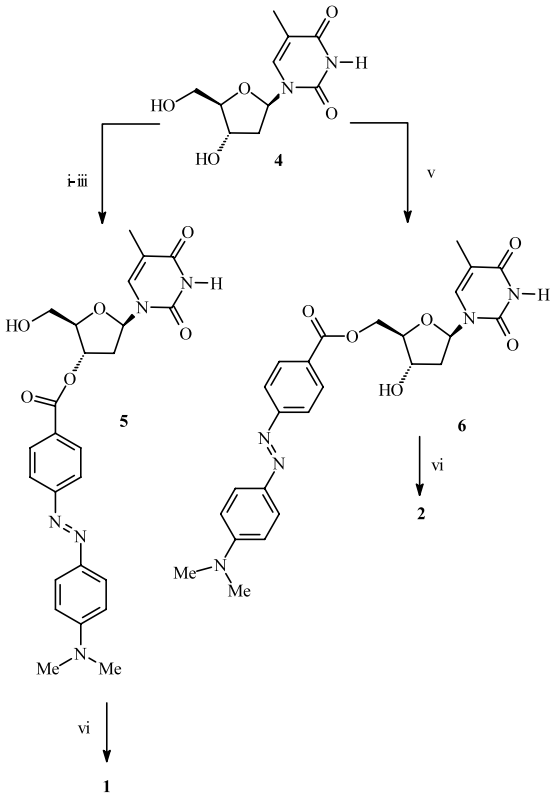


Figure 2. DABCYL (**R** = **H**) and the corresponding succinimide ester **3**.



Scheme 1. Synthesis of **1** and **2**. (i) Triphenylmethyl (trityl) chloride, pyridine, 30 min; (ii) DABCYL, EDCl, DMAP, THF, rt, 48 h; (iii) 80% AcOH, reflux 30 min; (vi) 2-cyanoethyldiisopropyl-chlorophosphoramidite, DIPEA, CH_2Cl_2, 30 min; (v) DABCYL, EDCl, DMAP, THF; (vi) 2-cyanoethyldiisopropyl-chlorophosphoramidite, DIPEA, CH_2Cl_2, 30 min.

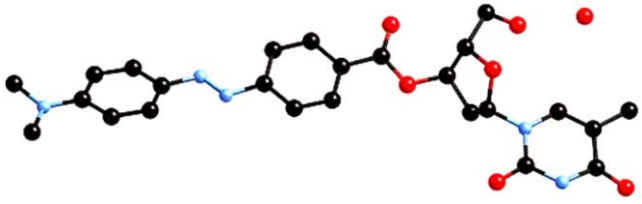


Figure 3. The X-ray crystal structure of **5** showing the azobenzene chromophore in its *trans*-configuration. A water molecule can also be seen hydrogen bonding to the 5′ hydroxy group. Hydrogen atoms have been omitted for clarity.

† Data were collected a Bruker-AXS SMART diffractometer using the SAINT-NT16a software with graphite monochromated Mo-Kα radiation. A crystal was mounted on to the diffractometer at low temperature under nitrogen at ca. 120 K. The structures were solved using direct methods and refined with the SHELXTL version 516b and the non-hydrogen atoms were refined with anisotropic thermal parameters. Additional material available from the Cambridge Crystallographic Data Centre (CCDC No. 220683) comprises relevant tables of atomic coordinates, bond lengths and angles, and thermal parameters.

trans configuration with the angle between the aromatic rings measured as 21°. The crystals contained a single water molecule in the unit cell, which were hydrogen bonded to themselves and the 5′-hydroxyl group of **5** forming an infinite hydrogen-bonded chain along the *b*-axis. These chains are packed together such that they form an interdigitated array that is held together by a combination of *edge-to-face* and π–π interactions in the *a*–*c* plane forming intricate three-dimensional hydrogen bonded structure.

Phosphoramidite **1** was formed using a standard method by stirring **5** at 0°C with 2-cyanoethyldiisopropylchloro-phosphoramidite in CH_2Cl_2 in the presence of DIPEA, for 30 min.[17] The resulting mixture was then allowed to warm to room temperature and stirred for a further 30 min. Subsequently, **1** was purified by flash column chromatography (CH_2Cl_2:EtOAc, 50:50) and isolated in 73% yield. The purity of this compound was verified by reverse phase HPLC (C18), in CH_3CN, which showed two peaks after 3.1 and 3.8 min. respectively in 40:60 ratio (ESI) which were assigned to the two diastereoisomers of **1**. The existence of such isomers in phosphoramidite chemistry is well known, caused by the introduction of the phosphorus moiety.[18] This was also confirmed by ^{1}H NMR (ESI) and ^{31}P NMR which showed two peaks at 149.8 and 149.4 ppm. The ESMS of **1** showed the presence of a peak at 694.3093 (calcd for $C_{34}H_{45}N_7O_7P$: 694.3118) for $M+H^+$ and a peak at 716.3 for the $M+Na^+$.

As in the case of **1**, we initially attempted to make **2** by protecting the 3′-*O* hydroxy group. Several different methods were employed, including the use of acetyl, benzyl or tertbutylsilyl ether, followed by coupling to DABCYL. Unfortunately, the removal of the protecting group proved troublesome and often led to the decomposition of the product. Because of these drawbacks, we attempted to add the DABCYL moiety directly to **4**, without any protection, on the basis that the 5′-hydroxy group should be more reactive than the 3′-hydroxy group, and this should give the desired product in higher yield. This was indeed found to be the case, and the 5′-*O*- functionalised azobenzene **6** was obtained in 30% yield after purification by flash column chromatography (EtOAc:DIPEA, 199:1). The structure of **6** was also determined by X-ray crystallography, and showed the *trans* isomer as previously seen for **5**.

In an analogous fashion to **1**, compound **2** was formed by reacting **6** with 2-cyanoethyldiisopropylchlorophosphoramidite in DCM in the presence of DIPEA, yielding **2** in 80% yield as a red-coloured residue, which was characterised using conventional methods. Both the ^{1}H and ^{31}P NMR showed the presence of two diastereoisomers and the ESMS showed two peaks for $M+H^+$ (found: 694.3062) and $M+Na^+$, respectively, indicating that **2** was successfully formed.

The two phosphoramidites **1** and **2** were incorporated into a 16–mer oligonucleotide (shown below) using standard solid phase phosphoramidite chemistry (200 nmol scale) on an automated DNA synthesiser (Beckman 1000M). The chosen strand is one we have previously investigated in our antisense programme for targeting CML.[11] This yielded two antisense strands where our modified thymidine oligonucleotides [marked as either (**1**) or (**2**) in **7** and **8**, respectively] would give rise to two different conformational environments within these strands.

5′-(**1**)-TCTTCCTTATTGATGG-3′ **7**

5′-(**2**)-TCTTCCTTATTGATGG-3′ **8**

The preparation of **7** and **8** involved dissolving the precursors **1** and **2** in a 50:50 mixture of dry CH_3CN:pyridine and incorporating these in a large excess onto the 16-mer to ensure efficient coupling. These were removed from the solid support using concentrated ammonia (with no noticeable ester hydrolysis of the DABCYL esters under these conditions), and purified by semi-preparative reverse phase HPLC (C18), using gradient elution (20%B→60%B over 15 min where B = CH_3CN, and A = 0.1M TEAA, pH 6.9), which enabled the isolation of **7** or **8** after 10 min. The UV–vis spectra of the peak eluting at 10.8 min showed absorption bands at 260 nm, characteristic of DNA, and a second absorption at 459 nm corresponding to the DABCYL chromophore. The integration of the absorption bands were in the correct ratio expected for the additive UV–vis spectra of the 17-mer and DABCYL, indicating that **7** and **8** had been successfully synthesised (Fig. 4).

In order to investigate the effect of **7** and **8** after incorporation into a double stranded DNA, these modified 17-mer oligonucleotides were annealed to a 34-mer (5′-TGACCATCAATAAGGAAGAAGCCTTCAGCGGCC-′3)[11] target CML oligonucleotide at 80°C over 30 min. The T_m of these double stranded oligonucleotides were then measured and determined using a derivative plot, to be 52.0°C. Importantly, when the T_m was measured experimentally for the same 17-mer-34-mer duplex lacking the azobenzene moiety, it was determined to be 51.0°C. This result strongly sug-

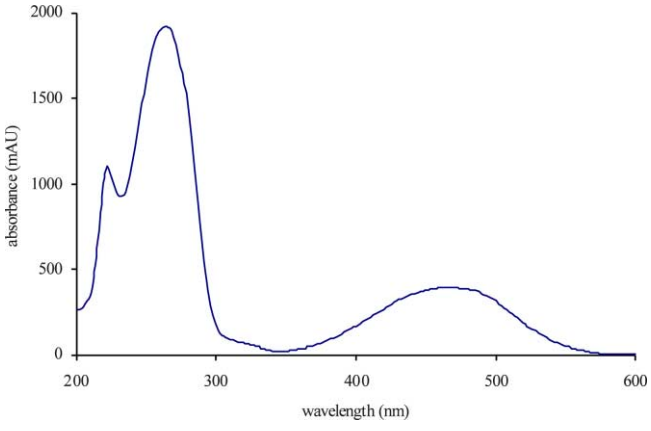


Figure 4. The UV–vis spectra of **7** showing the absorption of the DABCYL chromophore between 350 and 580 nm, and the absorption of the DNA oligonucleotides component at ca. 260 nm.

gests that the incorporation of the azobenzene chromophore does not adversely affect the stability of the double helix, indicating that this does not impose significant physical changes on the hybridised strand. This is important for the application of **7** or **8** as probes or quenchers in molecular beacons. Furthermore, even though the CD spectra of **7** or **8** were somewhat different prior to duplex formation, only minor changes were observed to that of the non-functionalised 17-mer, after annealing with the 34-mer target, again indicating only small conformational effect.

We also investigated the possibilities of **5** and **6** undergoing *trans–cis* photo isomerisation. However, we were unable to record this process at room temperature in solution. This is not unexpected, as it is known that the *trans–cis* photo isomerisation for Methyl Red is very rapid in solution. However, the *trans-cis* isomerisation can for be observed in polymeric matrixes or in the presence of cyclodextrins.[19] Currently we are evaluating the ability of **7** and **8** to undergo such isomerisation after hybridisation to the above 34-mer CML strand.

In summary, we have developed two novel azobenzene conjugated oligonucleotides by coupling DABCYL into thymidine at the 3′-*O*- and 5′-*O*-hydroxy moieties. To the best of our knowledge these are the first examples of such modified and highly coloured azobenzene–thymidine conjugates, where the dye is directly attached to the sugar unit. Currently we are evaluating the properties of **7** and **8** as novel DNA probes for genetic analysis as well components of molecular beacons. We are also undertaking the incorporation of the DABCYL into thymidine via the amide linkage. This will be the subject of future publications.

Characterisation of 1 and 2

1: Obtained in yield of 73% (0.086 g, 0.12 mmol) as a red coloured solid; mp decomposition; δ_H (400 MHz, $CDCl_3$) 8.16 (2H, d, $J=8.04$ Hz, Ar), 7.91 (4H, m, Ar), 7.67 (1H, s, -N-*CH*=C(CH_3)), 6.77 (2H, d, $J=7.52$ Hz, Ar), 6.50 (1H, m, *C(1)H*), 5.60 (1H, d, $J=6.0$, *C(3)H*), 4.38 (1H, m, *C(4)H*), 4.12 (2H, m, -P-O-CH_2-CH_2), 3.95 (3H, m, -P-O-CH_2-CH_2), 3.65 (2H, m, *C(5)H*$_2$), 3.12 (6H, s, -N(CH_3)$_2$), 2.68 (2H, m, -N(*CH*(CH_3)$_2$)$_2$), 2.35 (2H, m, *C(2)H*$_2$), 1.98 (3H, s, -N-CH=C(CH_3)), 1.25 (12H, m, -N(CH(CH_3)$_2$)$_2$); δ_C (100 MHz, d_6-DMSO) 165.8, 163.6, 156.3, 152.9, 150.4, 143.6, 135.5, 130.6, 128.9, 125.5, 122.0, 117.4, 111.4, 85.0, 84.6, 75.9, 64.2, 63.4, 58.7, 43.3, 38.0, 37.7, 24.7, 20.5, 14.1; δ_P (162 MHz, d_6-DMSO) 149.8, 149.4; m/z 694.1 (M+H); m/z found: 694.3093 ([M+H]$^+$ calculated for $C_{34}H_{45}N_7O_7P$: 694.3118).

2: Obtained in a yield of 80% (0.132 g, 0.19 mmol) as a red coloured solid; mp decomposition; δ_H (400 MHz, d_6-acetone) 8.22 (2H, d, $J=8.04$ Hz, Ar), 7.91 (4H, m, Ar), 7.48 (1H, s, -N-*CH*=C(CH_3)), 6.89 (2H, d, $J=7.52$ Hz, Ar), 6.37 (1H, m, *C(1)H*), 4.87 (1H, d, $J=6.0$ Hz, *C(3)H*), 4.65 (2H, m, *C(5)H*$_2$), 4.42 (1H, m, *C(4)H*), 3.89 (2H, m, -P-O-CH_2-CH_2), 3.71 (2H, m, -P-O-CH_2-CH_2), 3.15 (6H, s, -N(CH_3)$_2$), 2.80 (2H, m, -N(*CH*-(CH_3)$_2$)$_2$), 2.53 (2H, m, *C(2)H*$_2$), 1.69 (3H, s, -N-CH=C(CH_3)-), 1.24 (12H, m, -N(CH(CH_3)$_2$)$_2$); δ_C (100 MHz, d_6-acetone) 165.9, 163.8, 156.6, 153.9, 150.8, 143.9, 136.1, 131.1, 130.3, 125.9, 122.5, 118.7, 112.0, 110.8, 85.4, 84.1, 83.7, 74.1, 64.7, 60.2, 59.0, 43.6, 39.2, 24.5, 20.5, 14.1; δ_P (162 MHz, d_6-acetone) 149.5, 149.3; m/z 694 (M+H); (m/z found: 694.3062, [M+H]$^+$ calculated for $C_{34}H_{45}N_7O_7P$: 694.3118).

Acknowledgements

This work was supported by the Kinerton Ltd., Trinity College Dublin, and the Higher Education Authority in Ireland through the HEA 1999 Research Programme on Advanced Materials. AO'B would like to thank Enterprise Ireland for a PhD studentship. We especially thank Professor R. Jeremy H. Davies and Clarke S. Stevenson, Department of Biology and Biochemistry, Queen's University of Belfast and Drs. John O'Brien, Frederic Pfeffer and Susan Quinn (TCD) for their help and support, and Dr. Hazel M. Moncrieff for helpful discussion.

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