A microfluidic platform to synthesise a G-quadruplex binding ligand†

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An aromatic triarylpyridine chromophore promotes π-stacking interactions with the terminal G-tetrad in quadruplex DNA, stabilizing the structure and presenting a pathway towards cancer treatment by inhibition of telomerase. An interesting parent compound in this class is the dimethylamino functionalised 4′-aryl-2,6-bis(4-aminophenyl)pyridine. However, access to this compound using traditional batch synthetic methodology is limited, due to thermodynamic and kinetic constraints. A novel approach to the synthesis of this compound has been developed, involving dynamic thin films, overcoming a series of competing reactions, effectively controlling chemical reactivity and selectivity.

Introduction

Anticancer agents that target DNA are some of the most effective agents in clinical use and have been reported to significantly increase the survival of cancer patients when used in combination with drugs that have different mechanisms of action.[1] Consequently, there has been much effort in identifying cancer-specific molecular targets as new generation therapeutics. Guanine-rich sequences that can potentially form quadruplexes occur in the promoter region of certain oncogenes and at the 3′-terminus of telomeric DNA, hence making the G-quadruplex a potentially attractive target for selective anti-cancer therapy and drug development.[2–10] G-quadruplex structures can be stabilized by specific ligands in a new approach to cancer treatment, aimed at inhibition of telomerase, an enzyme involved in telomere maintenance and cell immortality.[11–18] Triarylpyridines are a class of G-quadruplex binding ligands, which do not bind to duplex DNA.[19] The aromatic chromophore promotes π-stacking interactions with the terminal G-tetrad thereby stabilizing the quadruplex structure. The dimethylamino functionalised 4′-aryl-2,6-bis(4-aminophenyl)pyridine is an important parent compound to access this class of compounds, however, the direct synthesis of this target is not possible using traditional batch methodology due to a series of competing reactions. Here we show a synthesis route to access this compound involving dynamic thin films, overcoming a series of competing reactions. Furthermore, we demonstrate the high G-quadruplex binding and stabilizing properties of the parent dimethylamino functionalised 4′-aryl-2,6-bis(4-aminophenyl)pyridine using molecular dynamics simulation and the G-quadruplex stabilising property of its derivative using Fluorescence Resonance Energy Transfer (FRET).

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Experimental

Materials and methods

All chemicals (*p*-aminoacetophenone, *p*-dimethylamino-benzaldehyde, NaOH, propan-1-ol, PEG300) were purchased from Aldrich and were used without further purification. A Protensive 100 series spinning disc processor (SDP) (detailed below) was used with integrated feed pumps to direct the reactants onto the rotating disc. The solutions were delivered onto the disc surface using feed jets integrated into continuous flow gear pumps (MicroPumps). A grooved steel disc with 100 mm diameter was used, which was manufactured from 316 stainless steel with the grooved disc having 80 concentric engineered grooves equally spaced at a depth of 0.6 mm. The disc rotation was varied from 300–2500 rpm. The samples were collected from the outlet for analysis. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded on a Bruker AV500 instrument in 5 mm NMR tubes. Samples were recorded in DMSO-d_6 solution in ppm (δ) and referenced to the internal residual partially-deuterated DMSO septet at 2.50 ppm (^{1}H NMR) and 39.52 ppm (^{13}C NMR). Compound **4** was recorded in $CDCl_3$ solution in ppm (δ) and referenced to the internal residual partially-deuterated $CDCl_3$ singlet at 7.26 ppm (^{1}H NMR) and 77.16 ppm (^{13}C NMR). Compound **8** was recorded in MeOD solution in ppm (δ) and referenced to the internal residual partially-deuterated multiplet at 3.31 ppm (^{1}H NMR) and 49.00 ppm (^{13}C NMR).

Detailed experimental procedures can be obtained from the ESI section.†

Results and discussion

Molecular dynamics simulation

Amine substituents of triarylpyridine provide active sites for hydrogen bonding and cation dipole interactions with the sugar-phosphate backbone and the loops of the G-quadruplex, with replacement of amine by oxygen centred moieties resulting in a detectable loss of binding and stabilising properties.[19] We envisioned that an amino functionalised 2,4,6-triarylpyridine with a dimethylamino group, with multiple polarising and protonation sites, would provide enhanced binding and

stabilising properties. To validate this we examined the interaction of dimethylamino functionalised 4′-aryl-2,6-bis(4-aminophenyl)pyridine with the *Oxytrichia nova* telomeric DNA quadruplex d(GGGGTTTTGGGG) using molecular dynamics simulations (Fig. 1A). The compound (Fig. 1A) was positioned within the quadruplex structure following the crystal structure of an acridine complex.[20] As shown in Fig. 1C & 1D, the compound is found to remain bound between the upper Guanine tetrad and the connecting loop for the duration of the 10 ns simulation. In this position there is a very large contact area between the extended aromatic regions of the compound and the guanine nucleotides. The strength of the attractive interaction between the compound and the DNA is highlighted by a calculated 29 kcal/mol free energy change upon removing it to the surrounding aqueous medium. The presence of the compound appears to have a stabilizing effect on the quadruplex structure as a whole within our simulations. To demonstrate this we heated a smaller version of our quadruplex system over 12 ns of simulation. Without the triarylpyridine present the quadruplex structure begins to melt at around 320K, but it remains stable above 350K with the compound bound (Fig. 1B). Indeed our calculations establish the dimethylamino functionalised 4′-aryl-2,6-bis(4-aminophenyl)pyridine, as an important parent compound en route to a family of functionalised G-quadruplex binding ligands.

Protocol to access the parent dimethylamino functionalised triarylpyridine

Recently we reported the 'one-pot' synthesis of triarylpyridines in polyethylene glycol (PEG) in developing a more benign approach towards molecular drug discovery.[21] An important intermediate in this reaction is a 1,5-diketone, which in the presence of ammonium acetate undergoes cyclization and aerial oxidation to form the triarylpyridine. Attempts to synthesize one of the 1,5-diketone intermediates, notably, compound **5** (Scheme 1), involving sequential Claisen-Schmidt condensation and Michael addition reactions, and hence the corresponding triarylpyridine, **6** (Scheme 1), using a traditional batch methodology approach was unsuccessful. The major product generated was a Schiff base adduct of the Claisen Schmidt condensation product, compound **4** (Scheme 1). Competing reactions are often encountered in chemical synthesis, and the ability to control chemical reactivity and selectivity is at the core of tackling difficult synthetic problems.[22,23] In the present case the synthesis of **5**, was unsuccessful in a batch process even at high temperature (140 °C) and in a microwave reactor (see supporting information). The anisotropic thermal environment associated with batch flask processing coupled with poor mass and heat transfer favours the formation of **4**, which precipitates from solution,

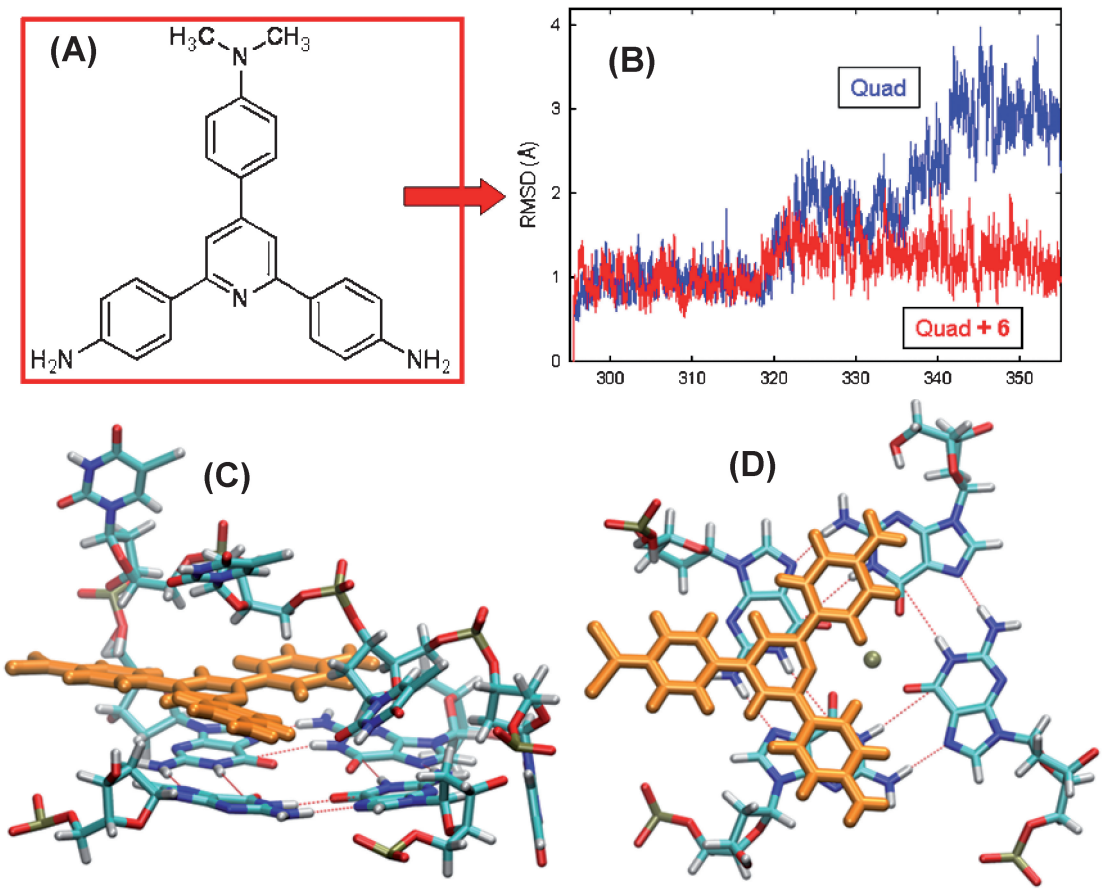
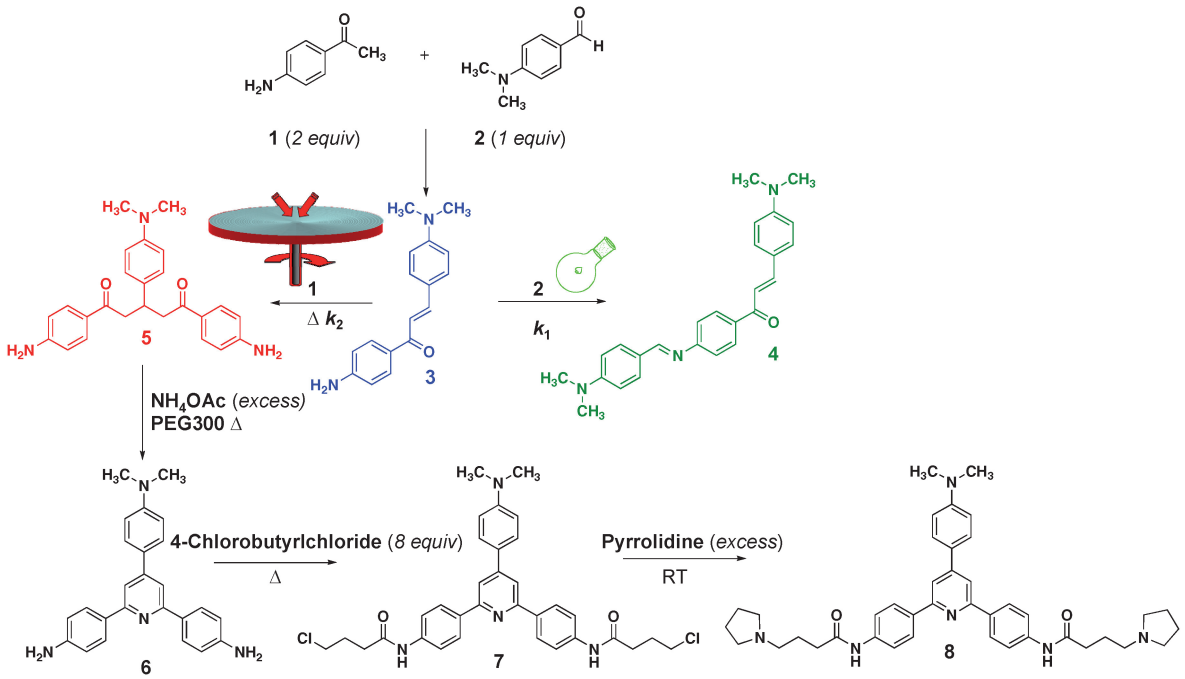


Fig. 1 (A): Structural formula of the dimethylamino functionalised 4′-aryl-2,6-bis(4-aminophenyl)pyridine. (B): Stabilisation of the *Oxytrichia nova* telemeric DNA sequence by the compound as indicated by the root mean square deviation of the guanine nucleotides plotted against temperature with and without the compound present in molecular dynamics simulations. (C and D): Average location of compound binding between the upper Guanine tetrad and the connecting loop as viewed from beside and above the plane of the Guanine tetrad.



Scheme 1 Consecutive and concurrent reactions leading to the formation of the dimethylamino functionalised 4′-aryl-2,6-bis(4-aminophenyl)pyridine, **6**; k_1 and k_2 represent the rate constants for the formation of the Schiff base adduct of the Claisen Schmidt condensation product, **4**, and the 1,5-diketone, **5**, respectively.

thereby limiting the formation of **5**. It is therefore evident that in order to access the 1,5-diketone intermediate, it is of paramount importance to shut down the formation of the Schiff base adduct of the Claisen Schmidt condensation product, compound **4**. A common route often used to overcome this problem is a series of functional group protection and deprotection synthetic steps, which is inefficient, waste generating and time consuming.

In general two chemical principles govern selectivity in synthesis: kinetics and thermodynamics. In the present case, the kinetic selectivity (increasing the reaction rate $k_2 \ggg k_1$) for the formation of compound **5**, is driven by overcoming the thermodynamic free energy barrier. This is normally difficult due to the inability to access uniformly high temperatures in a flask. Process intensification, however, alleviates the obstacles of the relaxed fluid dynamic regime associated with conventional batch methodology in chemical synthesis.[24] The SDP is a prototype of an intensified module offering operating conditions with rapid heat and mass transfer under continuous flow conditions with residence times reduced to seconds rather than minutes or hours.[24] Thin highly sheared films (25–200 μm) are generated on the rapidly rotating disc surface contributing to many influential chemical processing characteristics, such as: high surface area to volume ratio between the film and the disc surface, very high heat and mass transfer rates between the film/disc, intense mixing environment and uniform heat transfer throughout the entire reaction mixture, in contrast to limited heat conduction and convection in a batch reactor. High heat transfer rates allow the use of higher operating temperatures resulting in faster reaction rates. The thickness of the film is considered to be so small, and mixing within the film so intense, that temperature variations across the height of the film are assumed to be negligible. Average shear rates, as high as 13000 s^{-1} can be achieved on the disc surface in comparison to 300 s^{-1} in a conventional batch reactor.[25–32] Indeed, SDP has been successfully used for performing polymerisation (both free radical, cationic and condensation) reactions,[28,30] precipitation reactions with a narrow particle size distribution[32] and for performing reactions with intrinsically fast kinetics. These include a phase-transfer-catalysed Darzens' reaction,[26] and the re-arrangement of α-pinene oxide to campholenic aldehyde.[27]

We believe that the active micromixing in the thin films over a rapidly rotating spinning disc at elevated temperatures, will overcome the associated kinetic and thermodynamic constraints in the synthesis of **5** (Scheme 1).

A series of multiple pass experiments were performed on a 10 cm diameter spinning disc at various feed rates, disc speeds and temperatures (see supporting information) with **5** forming on the disc following optimization of the above mentioned parameters to maximize conversion (>45%). It is indeed evident that reducing the disc speed decreases the formation of the 1,5-diketone, thereby selectively forming the Claisen Schmidt product, **3**, and increasing the disc speed maximises the formation of **5** (Fig. 2A & 2B). Furthermore the 1,5-diketone obtained can be readily cyclised to give the triarylpyridine **6** (94% yield) (Scheme 1), as a high efficacy parent compound for G-quadruplex binding and stabilisation (see supporting information).

G-quadruplex stabilization efficacy of the aminolysed derivative

The parent triarylpyridine, **6**, is further modified by appending side chains to provide distinct quadruplex interaction features. In particlular long tail amine substituents have been reported to provide hydrogen bonding and cation dipole interactions with the sugar-phosphate backbone and loops. In order to validate

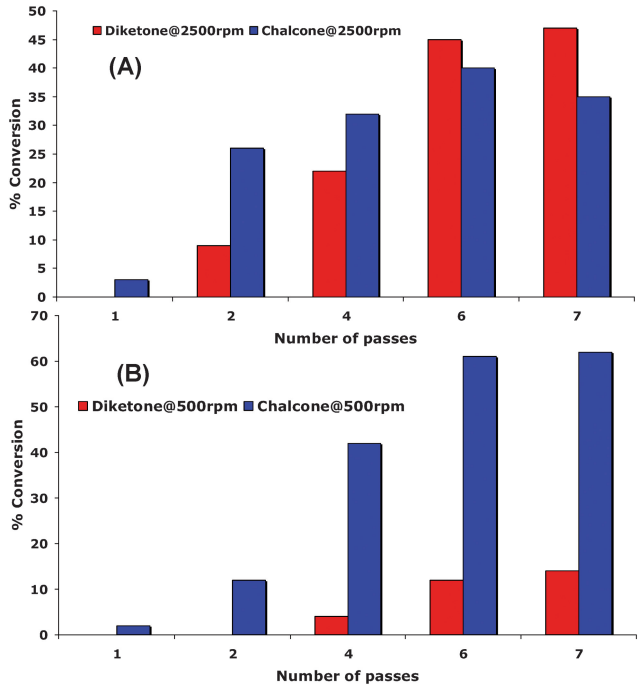


Fig. 2 Chemical selectivity for the formation of the 1,5-diketone and the Claisen Schmidt chalcone at disc speeds of (A) 2500 rpm and (B) 500 rpm at a feed rate of 0.5 ml/sec and temperature 140 °C.

the G-quadruplex stabilising efficiency in the present case, the parent compound was further acylated (compound **7**) and aminolysed (compound **8**), with the synthetic procedures described in the ESI (Scheme 1). Guanine-rich sequences that can potentially form quadruplexes occur in the promoter region of certain oncogenes, such as c-myc, K-ras and c-kit, and at the 3′-terminus of telomeric DNA.[2–10] Herein, the stabilisation potential (ΔT_m) of compound **8** was analysed using FRET with a dual-labeled c-kit quadruplex, 5′-FAM-d(GGG CGG GCG CGA GGG AGG GG)-TAMRA-3′. Indeed the compound exhibited a ΔT_m of 7.2 °C, (FRET $\Delta T_m \pm 1$ °C). The stabilisation potential is herein comparable to the triarylpyridines previously reported, and is higher than the previously reported non-dimethylamino functionalised analogue.[19] These results validate our claim that the 4′-aryl-2,6-bis(4-aminophenyl)pyridine is an important parent compound for the synthesis of potent G-quadruplex binding ligands *via* further modifications.

Conclusions

In conclusion, we show a synthesis route to access the parent 4′-aryl-2,6-bis(4-aminophenyl)pyridine involving dynamic thin films, overcoming a series of competing reactions, effectively controlling chemical reactivity and selectivity. In addition, we demonstrate using molecular dynamics simulation the high G-quadruplex binding and stabilizing properties of the parent dimethylamino functionalised 4′-aryl-2,6-bis(4-aminophenyl)pyridine. This control in chemical reactivity and selectivity offers an exciting approach in the selective synthesis of functionalized molecules which are otherwise not possible using traditional batch methodology, where alternative strategies involving multiple step syntheses would be required. The new approach will allow a more direct access to therapeutic molecules where there are thermodynamic and kinetic constraints in chemical reactions using traditional batch methodology.

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