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Designed DNA probes from the neocarzinostatin family: Impact of glycosyl linkage stereochemistry on bulge base binding

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ABSTRACT

Bulged sites in DNA and RNA have become targets for rational drug design due to their suspected involvement in a number of key biomolecular processes. A lead compound, derived from the enediyne natural product NCS-chrom has been used to inform chemical synthesis of a family of designed probes of DNA bulges, one of which shows 80 nM affinity for a two base bulged target. Key contributors to binding of these spirocyclic compounds have been studied in order to correlate affinity and specificity with structural features. Herein, we demonstrate that the glycosyl linkage stereochemistry of the pendant aminofucosyl group plays a pivotal role in binding, and coupled with insight obtained with various bulged targets, will allow rational design of second generation ligands.

1. Introduction

Bulged structures in nucleic acids are suspected to be contributors in a variety of diseased states.[1] They have been proposed as intermediates in a number of biological processes,[2] as binding motifs for regulatory proteins, and as essential elements in naturally occurring antisense RNA's.[3] In the case of HIV-1, one of the gene regulatory proteins, *Tat*, binds to a three base pair bulge within a hairpin stem-loop RNA conformation termed TAR (trans-activation response region). Binding then results in the control of gene expression via formation of an antitermination complex.[4] Bulged targets have also been implicated in the etiology of at least 12 human neurodegenerative genetic diseases. In these cases genetic variations in the lengths of $(CTG)_n{\cdot}(CAG)_n$, $(CGG)_n{\cdot}(CCG)_n$, or $(GAA)_n{\cdot}(TTC)_n$ so called 'triplet repeats' in genomic DNA have been attributed to reiterative synthesis due to slippage and bulge formation in the newly formed DNA strand Figure 1.[5]

We have established a program to design and evaluate synthetic bulge binders modeled on congeners of the enediyne natural product neocarzinostatin chromophore (NCS-chrom). One of its degradation products **1** recognizes unique bulged nucleic acid sequences with high affinity and selectivity,[6] its prism shaped spirocyclic skeleton conferring affinity for 2-base bulged targets. However, the lack of availability of this agent coupled with its instability (half-life ~5 h at Ph 8.2)[7] prompted us to develop simplified structural mimics. The most refined class of agents we have identified to date is exemplified by spiro-eneone **2**, which is available from a convergent synthesis involving an unprecedented intramolecular spiro-aldol cyclization.[8] A number of aminoglycosylated derivatives of this template have been examined for bulge-binding affinity, the most promising being α-aminofucosylated anomer **3** which mimics **1** and shows high (80 nM) affinity for a specific 2 base (TG) DNA bulged sequence. This is the highest affinity yet recorded for a synthetic bulged binder, stereochemistry evidently playing a key role as its right handed helical stereoisomer **4** shows weaker affinity yet mirrors the binding preference of **3** against a panel of sequences.[9] In an effort to understand the contribution of various stereochemical elements to binding we became interested in assessing the corresponding β-aminofucosylated analogs of **3** and **4** and set about their preparation.

2. Results and discussion

The racemic spiroalkene template **5** is available via a convergent synthesis described previously.[8] Derivatization to form α-*N*-methyl fucosylated chimeras is easily accomplished through trichloroacetimidate coupling, stereochemical control is a function of neighboring group participation.[10] In order to access the corresponding β-*N*-methyl fucosylated analogs however necessitated

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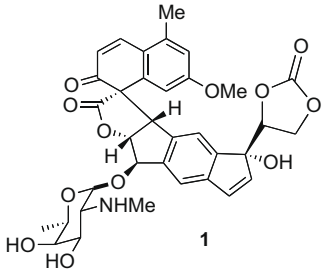
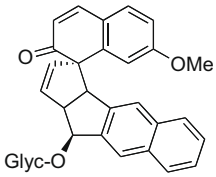


1



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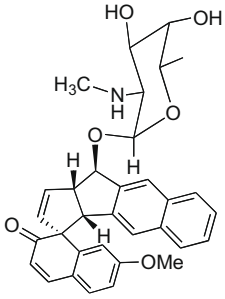

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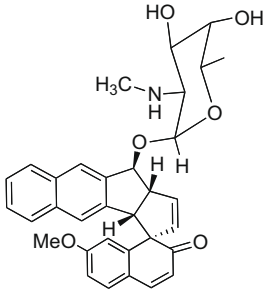


4

preparation of Fmoc capped *N*-methyl trichloroacetimidate **6** from the precursor aminolactol (Scheme 1). Glycosylation efficiency and selectivity for beta glycosylation was examined under a variety of conditions (Table 1), the optimal promoter being boron trifluoride in the presence of molecular sieves at −30 °C. Coupling followed by Fmoc deprotection gave a mixture of diastereomeric adducts **7** and **8** with *exclusive* β-geometry, desilylation giving desired products **9** (having a right handed twist), and **10** (with a left handed twist).

2.1. Bulged binding assay

Compounds **9** and **10** were screened against a panel of eight carefully selected synthetic oligos, using **1**, **3** and **4** as reference points (Chart 1 and Table 1).[11] Fluorescence changes were used to measure the dissociation constants and a range of duplex, hairpin, one, two and three base bulged sequences were used as substrates. Consistent with expectations, no apparent binding was observed to duplex control sequence (entry 1) and binding of all four synthetic agents mirrored **1** against an unbulged hairpin (entry 2). Moderate affinity for the β-glycosylated analogs were obtained against the duplex sequence with a TG bulge (entry 3), outperforming both the α-linked analogs and **1**, and presumably reflecting unique binding interactions in this (thermodynamically

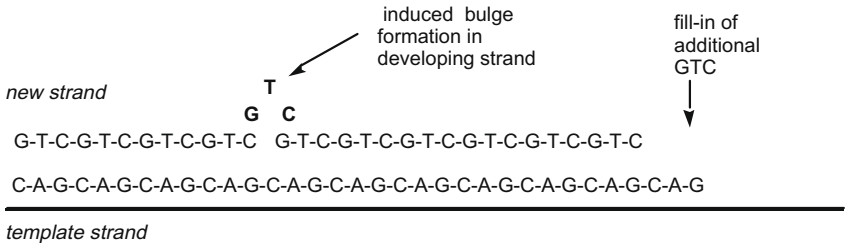
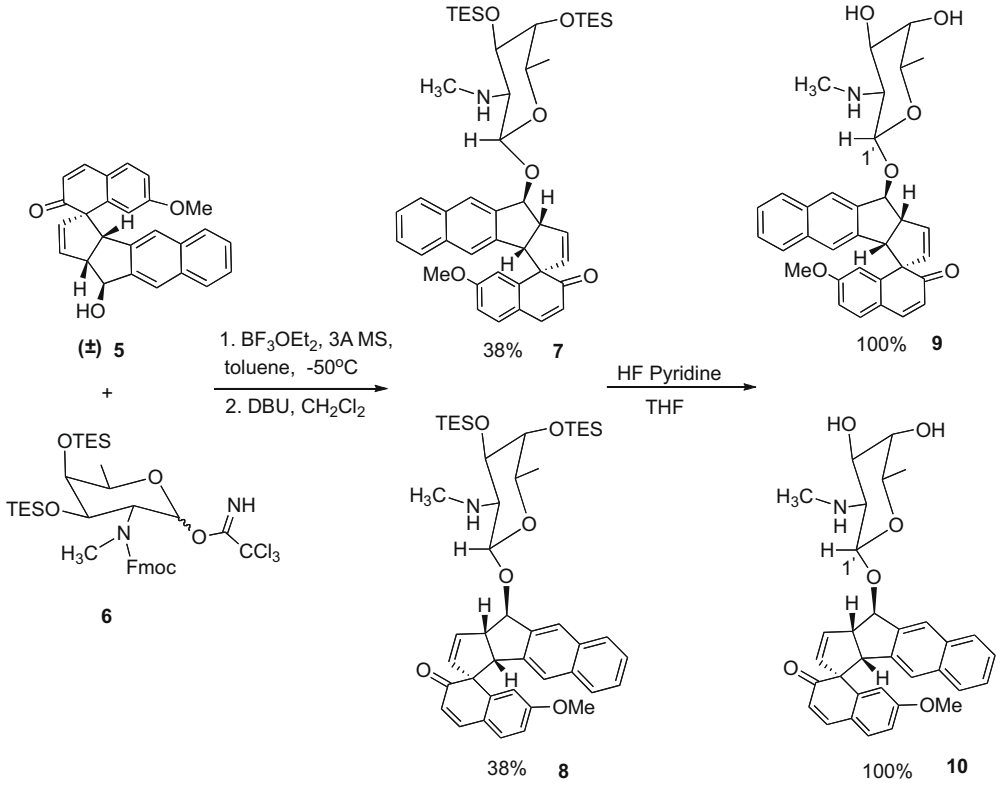


Figure 1. Formation of bulge in CAG rich region—triplet expansion.



Scheme 1. Synthesis of β-fucosylated NCSi-gb mimics **9** and **10**

Table 1
Aminoglycosylation of **5** with Fmoc tricholoracetimidate **6**

Entry	Lewis acid	Equivalents of LA	Solvent	Temperature (°C)	% (**7**+**8**)
1	TfOH	2	$PhCH_3$	−50	10
2	TfOH	2	$PhCH_3$	−30	22
3	TfOH	4	$PhCH_3$	−50	<5
4	TfOH	4	$PhCH_3$	−30	<5
5	$BF_3{\cdot}OEt_2$	2	$PhCH_3$	−50	20
6	$BF_3{\cdot}OEt_3$	2	$PhCH_3$	−40	20
7	$BF_3{\cdot}OEt_3$	2	$PhCH_3$	−30	30
8	$BF_3{\cdot}OEt_3$	2	$PhCH_3$	−20	20
9	$BF_3{\cdot}OEt_3$	4	$PhCH_3$	−50	30
10	$BF_3{\cdot}OEt_3$	4	$PhCH_3$	−40	50
11	$BF_3{\cdot}OEt_3$	4	$PhCH_3$	−30	76
12	$BF_3{\cdot}OEt_3$	4	$PhCH_3$	−20	60

unstable) complex.[7] The hairpin stabilized variant of this sequence however showed pronounced affinity for **1** and it is α-mimics (entry 4), and differences between **9** and **10** were also revealed. Those differences are accentuated against a single base (G) bulge (entry 5), analog **9** outperforming **1**, **3** and **10** and comparable to **4**. In the remaining substrates (entries 6–8) analogs **9** and **10** were broadly comparable to **3** and **4**, with the highest affinity for any β-analog (190 nM, entry 8) suggesting that glycosyl linkage stereochemistry is not the principal factor driving affinity for bulges. This finding correlates with functional studies of mixed α/β[8] and α[9] glycosylated bulged binders in DNA slippage assays.[8,9]

Closer examination of dissociation constant trends allows correlation to be made between structural features of DNA bulge binders and various bulged DNA microenvironments. Duplex DNA with two base bulges show a preference for β-analogs, but seem to show only a slight preference for left-handed twists. This contrasts the preference for right handed alpha conformers we have seen previously.[8,9] In hairpin DNA, two-base bulges have shown preference for the alpha, right handed natural product—so it is no surprise that the best synthetic bulge binder is the right handed alpha anomer.

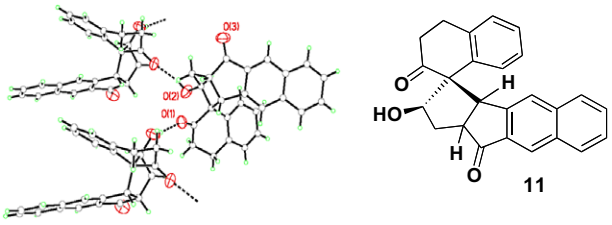


Figure 3. ORTEP of core spirocycle **11**.

Single bulge DNA does not appear to have an overwhelming preference for either alpha nor beta glycosylated binder, but did show preference for a left handed turn explaining the poor binding observed with the (right handed) natural product **1**. Three base bulges on hairpin DNA, although bound most efficiently to the natural product compared to the developed synthetic analogs, show a preference for the left handed alpha and beta synthetic products over their right handed counterparts.

3. Rationalization for binding

Molecular volume analysis of **1** (right handed geometry) compared to **3** and **10** (both left handed) suggests the contribution of the wedge shaped template itself may be instrumental in establishing efficient recognition of the prism shaped pocket of the TG bulge (Fig. 2).[12,13] This is further supported by comparison of the ORTEP for the basic spirocyclic structure **11** (Fig. 3),[14] where the (offset) arenes seem ideally suited to occupy the floor of the bulged cavity, the appended (glycosyl) elements serving to impart secondary, sequence selectivity.

Closer scrutiny of the binding preferences for the agents (Table 2) reveals subtle features of note: (1) in all cases, affinity for bulges follows the order 2 > 3 > 1 base bulge; (2) where sub-micromolar affinity is observed, the left handed templates (**3** and **10**) have

Entry	Sequence Code	Conformation
1	DA12:DAc12	5'-GTCCGATGCGTG 3'-CAGGCTACGCAC
2	HT3AT	5'-GTCCGATGCGTG T 3'-CAGGCTACGCAC T T
3	DA12:BA14	5'-GTCCGATGCGTG 3'-CAGGCTACGCAC TG
4	HT3AGTT	5'-GTCCGATGCGTG T 3'-CAGGCTACGCAC T T TG
5	HT3AGT	5'-GTCCGATGCGTG T 3'-CAGGCTACGCAC T T G
6	HT3AGCTT	5'-GTCCGATGCGTG T 3'-CAGGCTACGCAC T T TG C
7	HT3AGTA	5'-GTCCGTTGCGTG T 3'-CAGGCAACGCAC T T TG
8	HT4AGTT	5'-GTCCGATGCGTG T T 3'-CAGGCTACGCAC T T TG

Chart 1. Oligonucleotides used in binding studies.

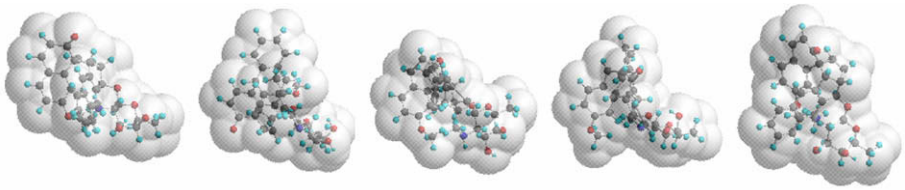


Figure 2. Molecular volume analysis of **1**, **3**, **4**, **9** and **10** [MM2].

Table 2
Oligonucleotide binding for synthetic α/β anomeric aminoglycosides*

Entry	Sequence code	1	3	4	9	10
1	DA12:DAc12	307	NAB	NAB	NAB	NAB
2	HT3AT	10	10	13	9.2	8.2
3	DA12:BA14	2.18	0.6	0.8	0.43	0.4
4	HT3AGTT	0.033	0.08	0.13	1.6	0.2
5	HT3AGT	20.6	8.4	4.1	5.5	14
6	HT3AGCTT	0.71	2.1	1.7	1.95	1.8
7	HT3AGTA	12.5	0.22	1.4	0.44	0.39
8	HT4AGTT	0.026	0.11	0.24	1.5	0.19

* Dissociation constants (μM) of DNA binding by the drugs determined via emission spectra (λ_{exc} 360 nm; λ_{emm} 480 nm) at 5 °C. NAB = no apparent binding.

greater affinity than right handed (**4** and **9**) viz. entries 3, 4, 7, 8; (3) within the left handed series (**3** and **10**) the α-glycosidic derivative shows the highest affinity (80 nM). It is also interesting to note that the synthetic agents outperform the naturally derived **1** for affinity to the single base bulge (entry 5) but that in this case it is the *right* handed (**4** and **9**) molecules which show greater affinity. Taken together, these data imply that there exist numerous structural subtleties which can offer potential for the design of agents which might target specific bulged motifs of genomic interest. Variability in the helical twist of the platform, the nature of appended aminosugars, and the stereochemistry of the linkage all play a contributing role to binding, providing logical opportunity for refinement. As the data confirms that α-linked aminofucosyl congeners have highest overall affinity for bulged targets, a logical refinement will be to investigate other related α-linked aminosugars of both natural and synthetic origin. These findings amplify patterns observed previously using α/β aminoglycosylated substrates against *RNA* targets, where clear preferences for α derivatives against 2 base RNA bulges were established.[15]

4. Summary

Bulges in nucleic acids have become targets for rational drug design. Affinity for these sites differs substantially from traditional (e.g., duplex) targets, the design criteria calling for (i) a wedge shaped template that engages in base-drug π−π stacking in the floor of the bulge (ii) defined helical twist geometry in the template, as exemplified by spirocyclic arenes (iii) a pendant aminosugar group with stereochemically defined linkage to the bulge binder template. Binding is influenced by flanking sequence surrounding the bulge, with highest affinities for synthetic agents ranging from 80 nM for a left handed α-glycosyl linked analog to 200 nM for its β-counterpart. Since the naturally derived lead compound (**1**) has a *right handed* twist, it suggests that myriad binding interactions are responsible for affinity to the prism-shaped cavity of the bulged environment. Combined these data may prove useful in the development of compounds which incapacitate bulged sequences in biological systems, as targets of specific interest become identified.[16]

5. Experimental

NMR spectra were obtained on either a Varian Mercury 300 (300 MHz) or a Varian Inova 500 (500 MHz) spectrometer. Mass spectra were obtained either on a Micromass LCT mass spectrometer (Harvard University Mass Spectrometry Facility) or a Finnigan LTQ-FT mass spectrometer (Northeastern University). Analytical thin layer chromatography (TLC) was performed using silica gel 60 F524 precoated plates (Scientific Adsorbents, Inc.). Preparative thin layer chromatography was carried out with Silica Gel GF (Analtech, Inc.). Flash chromatography was performed using silica gel 60 (230–400 Mesh, Whatman Inc.). All reactions were carried out under anhydrous, inert atmosphere (nitrogen or argon) with dry, freshly distilled solvents unless otherwise noted.

5.1. *N*-Fmoc-*N*-methylaminolactol

To a solution of compound 6-methyl-3-methylamino-4,5-bis-triethylsilanyloxy-tetrahydro-pyran-2-ol (92 mg, 0.21 mmol)[10] in THF and water (2 mL, 2:1) was added K_2CO_3 (63 mg, 0.46 mmol) and Fmoc-Cl (59 mg, 0.21 mmol) sequentially. The resultant solution was stirred at room temperature for 30 min then water (1 mL) added and the aqueous layer extracted with ether (3 × 50 mL). The organic layer was dried ($MgSO_4$) and concentrated in vacuo. The resulting residue was purified by silica gel chromatography (hexanes:ethyl acetate = 6:1–7:3) to afford the title compound (122 mg, 95%) as white solid (mp 87–91 °C); R_f 0.28 (hexanes:ethyl acetate = 7:3); ^{1}H NMR (500 MHz, $CDCl_3$): (mixture of rotamers) 0.4–0.65 (m, 12H), 0.8–1.1 (m, 18H), 1.2 (d, 3H), 2.4 (s, 1H), 2.8 (s,3H), 3.2 (m, 1H), 3.4 (t, 1H), 3.6 (t, 1H), 4.2 (d, 2H), 4.7 (t, 1H), 5.2 (d, 1H), 5.4 (s, 1H), 7.3 (t,2H), 7.4 (t, 2H), 7.6 (d, 2H), 7.8 (d, 2H); ^{13}C NMR (300 MHz, $CDCl_3$): 4.1, 5.9, 17.5, 32.8, 46.5, 59.5, 60.1, 65.4, 69.1, 73.3, 91.2, 120.1, 126.5, 127.9, 128.9, 140.1, 141.9, 155.7; HRMS (ESI), *m*/*z* $(M+Na)^+$: calcd 628.3490, obsd 628.3498.

5.1.1. Glycosides 7 and 8

A solution of *N*-fmoc-*N*-methylaminolactol (122 mg, 0.195 mmol, 1.0 equiv) in freshly distilled dichloromethane (1 mL) was added to a flame dried 10 mL round bottom flask precharged with NaH (95%, 4 mg, 0.156 mmol, 0.8 equiv). The resultant suspension was stirred at 0 °C for 10 min, and then trichloroacetonitrile (563 mg, 3.9 mmol, 20 equiv) added. The mixture was warmed to room temperature and stirred for a further 30 min then the contents filtered through a layer of Celite®. The filtrate was concentrated in vacuo to afford a white solid (crude mass 140 mg). Glycosyl acceptor **5** (10 mg, 0.026 mmol),[9] anhydrous toluene (1.5 mL) and 3 Å molecular sieves (60 mg) were added, and the mixture was stirred at room temperature for 30 min then cooled to −30 °C. A solution of boron trifluoride etherate (10 μL, 0.078 mmol) in toluene (300 μL) was added in 6 portions at 10 min intervals. After addition, the reaction was stirred at −30 °C for 1 h and then quenched by addition of sodium bicarbonate (25 mg). The reaction mixture was filtered through a plug of cotton wool, and the filtrate concentrated in vacuo to dryness. DBU (16 μL, 0.104 mmol) was added followed by dry CH_2Cl_2 (3.2 mL) and the resulting solution stirred at room temperature for 1 h. Phosphate buffer (pH 5.5, 0.2 M, 0.41 mL) was then added, followed by additional CH_2Cl_2 (10 mL). The organic layer was separated, washed with brine (2.5 mL) and condensed in vacuo. The crude oil was purified by preparative TLC (hexanes:ethyl acetate = 3:1) to afford compound **7** (7.60 mg, 38% over two steps) and **8** (7.59 mg, 38% over two steps) both obtained as yellow syrups.

Compound **7**: R_f 0.13 (hexanes:ethyl acetate = 1:3); ^{1}H NMR (500 MHz, $CDCl_3$): *δ 0.62–0.72* (m, 12H), 0.94–1.06 (m, 18H), 1.34 (d, *J* = 6 Hz, 3H), 2.02 (m, 1H), 2.83 (s, 3H), 3.17 (s, 3H), 3.63–3.81 (m, 3H), 4.11 (d, *J* = 7.5 Hz, 2H), 4.16 (dd, *J* = 10.5, 2.5 Hz, 1H), 4.49 (d, *J* = 8 Hz, 1H), 5.41 (s, 1H), 5.47 (dd, *J* = 8, 2 Hz, 1H), 5.81 (s, 1H), 6.22 (d, *J* = 9.5 Hz, 1H), 6.46–6.51 (m, 3H), 7.15 (d, *J* = 8.5 Hz, 1H), 7.28–7.35 (m, 4H), 7.53 (d, *J* = 9.5 Hz, 1H), 7.83 (d, *J* = 5 Hz, 1H), 8.32 (s, 1H).

Compound **8**: R_f 0.32 (hexanes:ethyl acetate = 1:3); ^{1}H NMR (300 MHz, $CDCl_3$): *δ 0.62–0.72* (m, 12H), 0.94–1.06 (m, 18H), 1.29 (d, *J* = 6 Hz, 3H), 2.53 (s, 3H), 2.79 (m, 1H), 3.19 (s, 3H), 3.55–3.66 (m, 4H), 4.03 (dd, *J* = 11, 2.5 Hz, 1H), 4.48 (d, *J* = 8 Hz, 1H), 5.43 (d, *J* = 2 Hz, 1H), 5.47 (dd, *J* = 8, 2 Hz, 1H), 5.81 (d, *J* = 2 Hz, 1H),

6.22 (d, J = 10 Hz, 1H), 6.44 (s, 1H), 6.53 (dd, J = 11, 3 Hz, 2H), 7.19 (d, J = 8 Hz, 1H), 7.24–7.34 (m, 4H), 7.53 (d, J = 10 Hz, 1H), 7.70 (d, J = 7 Hz, 1H), 7.93 (s, 1H).

5.1.2. Glycoside 9

To a solution of glycoside **7** (7.6 mg, 0.01 mmol) in tetrahydrofuran (0.7 mL) was added hydrogen fluoride-pyridine complex (152 μL). The resultant suspension was stirred at room temperature for 1 h then quenched by addition of sodium bicarbonate (200 mg). The mixture was filtered through a plug of Celite® and concentrated in vacuo. The residue was purified by preparative TLC (ethyl acetate:methanol:water = 9:2:1) to furnish glycoside **9** (5.3 mg, 100%) as a colorless oil; Rf 0.48 (ethyl acetate:methanol:water = 9:2:1); ^{1}H NMR (500 MHz, CD_3OD): δ 1.45 (d, J = 6 Hz, 3H), 2.54 (s, 3H), 3.04–3.08 (m, 1H), 3.17 (s, 3H), 3.62–3.67 (m, 3H), 4.02 (dd, J = 10.5, 2.5 Hz, 1H), 4.45 (d, J = 8 Hz, 1H), 4.60 (s, 1H), 4.95 (d, J = 8 Hz, 1H), 5.41 (d, J = 2 Hz, 1H), 5.45 (dd, J = 8, 2 Hz, 1H), 5.80 (d, J = 2.5 Hz, 1H), 6.19 (d, J = 9.5 Hz, 1H), 6.43 (s, 1H), 6.49 (dd, J = 7.5, 2.5 Hz, 1H), 6.65 (dd, J = 10.5, 2.5 Hz, 1H), 7.34–7.40 (m, 4H), 7.75 (d, J = 10 Hz, 1H), 7.82 (d, J = 7.5 Hz, 1H), 7.91 (s, 1H) ; HRMS (ESI), m/z $(M+H)^+$: calcd 540.2381, obsd 540.2386.

5.1.3. Glycoside 10

With identical procedure to that used in the synthesis of **9**, glycoside **8** (7 mg, 0.009 mmol) furnished glycoside **10** (4.8 mg, 100%) as a colorless oil; R_f 0.55; (ethyl acetate:methanol:water = 9:2:1); ^{1}H NMR (500 MHz, CD_3OD): δ 1.37 (d, J = 6 Hz, 3H), 2.62 (s, 3H), 2.82–2.86 (m, 1H), 3.20 (s, 3H), 3.64–3.71 (m, 3H), 3.98 (dd, J = 10, 2.5 Hz, 1H), 4.48 (d, J = 7 Hz, 1H), 4.60 (s, 1H), 4.80 (d, J = 8.5 Hz, 1H), 5.48 (dd, J = 8, 2 Hz, 1H), 5.53 (d, J = 2.5 Hz, 1H), 5.86 (d, J = 2.5 Hz, 1H), 6.19 (d, J = 9.5 Hz, 1H), 6.43 (s, 1H), 6.49 (dd, J = 8, 2.5 Hz, 1H), 6.64 (dd, J = 11.5, 2.5 Hz, 1H), 7.31–7.37 (m, 4H), 7.74 (d, J = 7 Hz, 1H), 7.75 (d, J = 10 Hz, 1H), 7.90 (s, 1H); HRMS (ESI), m/z $(M+H)^+$: calcd 540.2381, obsd 540.2379.

5.1.4. Binding and functional assays

Fluorescence quenching studies were conducted using a SPEX fluoromax-2 at 4 °C in phosphate buffer (10 mmol, pH 7.0). Emission spectra were obtained in the range 400–600 nm upon excitation at 385 nm. Emission readings at 490 nm were imported into binding calculations. Dissociation constants (k_d) were derived from curve fitting (Kaleidagraph).[14,17] DNA slippage synthesis was measured using the primer/template oligomers of thymidylate (20-mer) and deoxyadenylate (30-mer) in a reaction catalyzed by the Klenow fragment of DNA polymerase 1.[18,8,9]

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