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Molecular probes of DNA bulges: Functional assay and spectroscopic analysis

Graham B. Jones,[a,*] Yiqing Lin,[a] Ziwei Xiao,[b] Lizzy Kappen[b] and Irving H. Goldberg[b]

[a]*Bioorganic and Medicinal Chemistry Laboratories, Department of Chemistry and Chemical Biology, Northeastern University, Boston, MA 02115, USA*
[b]*Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02215, USA*

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Abstract—Bulged structures in DNA and RNA have been linked to biomolecular processes involved in numerous diseases, thus probes with affinity for these nucleic acid targets would be of considerable utility to chemical biologists. Herein, we report guided chemical synthesis of small molecules capable of binding to DNA bulges by virtue of their unique (spirocyclic) geometry. The agents, modeled on a natural product congener, show pronounced selectivity for specific bulged motifs and are able to enhance slipped DNA synthesis, a hallmark functional assay of bulge binding. Significantly, bulge-agent complexes demonstrate characteristic fluorescent signatures depending on bulge and flanking sequence in the oligo. It is anticipated that these signature patterns can be harnessed as molecular probes of bulged hotspots in DNA and RNA.

1. Introduction

Nucleic acids can have richly diverse structures, including hairpins, knots, pseudoknots, triple helices, loops, helical junctions, and bulges.[1] Though poorly understood, bulged structures in nucleic acids are of general biological significance.[2] They have been proposed as intermediates in a multitude of biological processes,[3] as binding motifs for regulatory proteins, and as essential elements in naturally occurring antisense RNAs.[4] 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 anti-termination complex.[5] 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 (Fig. 1).[6] Despite these opportunities, relatively few attempts have been made to prepare compounds with affinity for bulges. This is in stark contrast to agents which target DNA duplexes, where factors governing affinity and selectivity are now well appreciated.[1]

Our interest in these targets stems from prior work on congeners of the enediyne natural product neocarzinostatin chromophore (NCS-chrom). Degradation product **1** recognizes unique bulged nucleic acid sequences with high affinity and selectivity,[7] its prism-shaped spirocyclic skeleton conferring affinity for two-base bulged targets. However, the lack of availability of this agent coupled with its instability (half-life ~5 h at pH 8.2)[8] led to a search for synthetic bulged binders, exemplified by the thermodynamically stable spiro-enone **2**, available from a convergent synthesis via keto-aldehyde **3** which undergoes spiro-aldol cyclization.[9] Simple β-aminoglucose conjugates of **2** show sub-micromolar affinity for two-base bulges and possess desirable fluorescent properties to allow rapid binding assays with synthetic oligo' substrates.[9] Though encouraging, we were particularly interested to emulate the *N*-methyl fucosylated system present in **1**, and specifically its unusual α-glycosidic linkage, which is reasoned to contribute to its potency.

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* Corresponding author. Tel.: +1 617 373 8619; fax: +1 617 373 8795; e-mail: gr.jones@neu.edu

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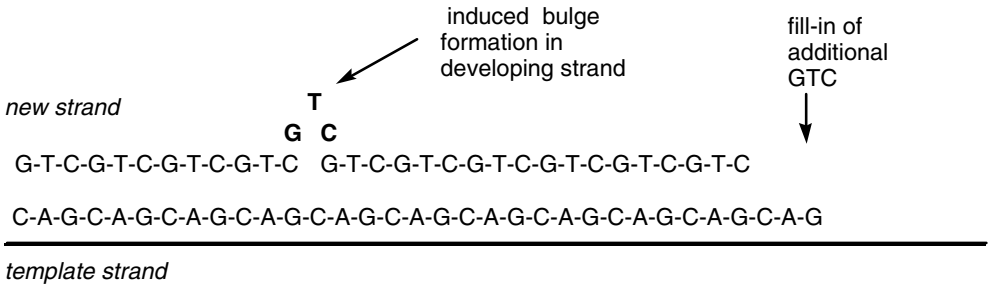
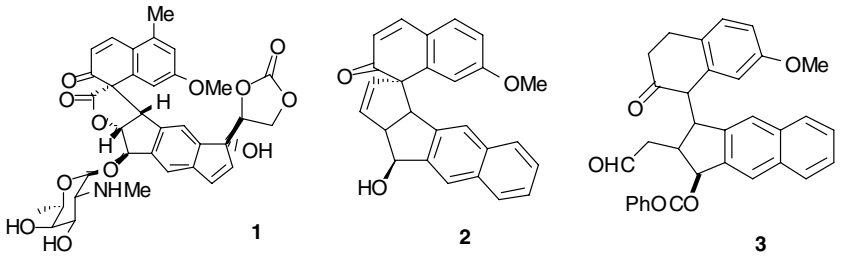


Figure 1. Formation of bulge in CAG rich region—triplet expansion.



2. Results and discussion

With quantities of **2** in hand a synthesis of an *N*-methyl fucosyl sugar was initiated from β-aminoglucose. Following orthogonal protection the primary alcohol (**4**) was revealed and converted to the corresponding iodide **6**, followed by silylation to give **7** (Fig. 2). Radical induced de-iodination was followed by double deprotection to give amino alcohol **10**. The choice of **10** and the intended glycosylation methodology was influenced by Myers, who has reported the exclusive α stereochemistry obtained for *N*-methyl fucosyl derivatives to be a function of neighboring group participation.[10] Conversion to trichloroacetimidate **11** then allowed glycosylation selectivity to be probed with **2** under various conditions. It should be noted that the route to **11** eliminates a number of potentially hazardous steps from the published synthesis.[11] Lewis acid mediated coupling in the presence of molecular sieves was followed by desilylation and gave a mixture of diastereomeric adducts **13a** and **13b**. In both cases, exclusive α geometry is observed, conjugate **13a** having a left-handed twist, and **13b** a right-handed twist.

2.1. Bulged binding assay

Compounds **13a** and **13b** were screened against a panel of 17 synthetic oligos, using **1** as a reference point (Fig. 3). Fluorescence changes were used to measure the dissociation constants and the results are presented in Table 1. The dissociation constant of **13a** with the TG containing bulge sequence HT3AGTT (entry 3), at 0.08 μM, is the highest affinity yet recorded for any synthetic bulge binder and suggests the important contribution of the α-glycoside linkage for tight binding. However, it is important to recognize that in contrast to **1**, spirocycle **13a** has a left-handed geometry yet shows slightly stronger affinity to the two-base bulged DNA than the right-handed diastereomer **13b**. The binding differences of **13a** or **13b** to DNA duplexes with a two-base bulge, with a one-base bulge, with a three-base bulge or without a bulge (entries 1–3, 5, 6, and 13) indicate that the two-base bulge is the much preferred binding site. The affinity differences between **13a** or **13b** and a two-base bulge against a one-base bulge or a three-base bulge resemble the preference for **1**. A switch from a pyrimidine to a purine base 3′ to the bulge resulted in marked reduction in affinity for **1** (entries 7–9 vs 3). The right-handed **13b** showed similar but reduced sequence requirement, while **13a** lost much of this requirement (entries 7 and 9). During the course of analysis, unexpected fluorescence phenomena were observed depending on sequence and agent in question and prompted closer scrutiny. In the case of **13a** and **13b**, both fluorescence quenching and fluorescence stimulation were observed. Quenching occurred when GC base pairs are next to the bulge and stimulation was observed for those oligonucleotides with AT base pairs next to the bulge. It is possible that, for the bulged DNA with AT base pairs at both sides of the bulge, the bulge is more flexible than those bulges with GC base pairs next to the bulge, and this difference in bulge flexibility results in different binding structures of the bulge–drug complexes, hence the difference in fluorescence change. To test this hypothesis, additional oligonucleotides (entries 14–17) were prepared and their binding with **13a** or **13b** studied. Results are consistent with this proposal. In all cases where GC base pairs were present on both side of the bulge (entries 11, 12, 16, and 17), fluorescence quenching was observed. For those oligonucleotides with one AT base pair and one GC base pair next to the bulge, either fluorescence stimulation or quenching resulted. To confirm that binding profiles of the synthetic agents were similar to that of lead compound **1**, competition experiments between **1** and **13a** or **13b** for binding to the bulged structure HT3AGTT (entry 3, Fig. 3) were conducted. Fluorescence quenching between the oligo and **1** was reversed upon addition of **13a/13b**. The concentration of **13a/13b** at 50% reversal was consistent with the dissociation constants measured (data not shown). These data suggest that a high degree of molecular recognition is

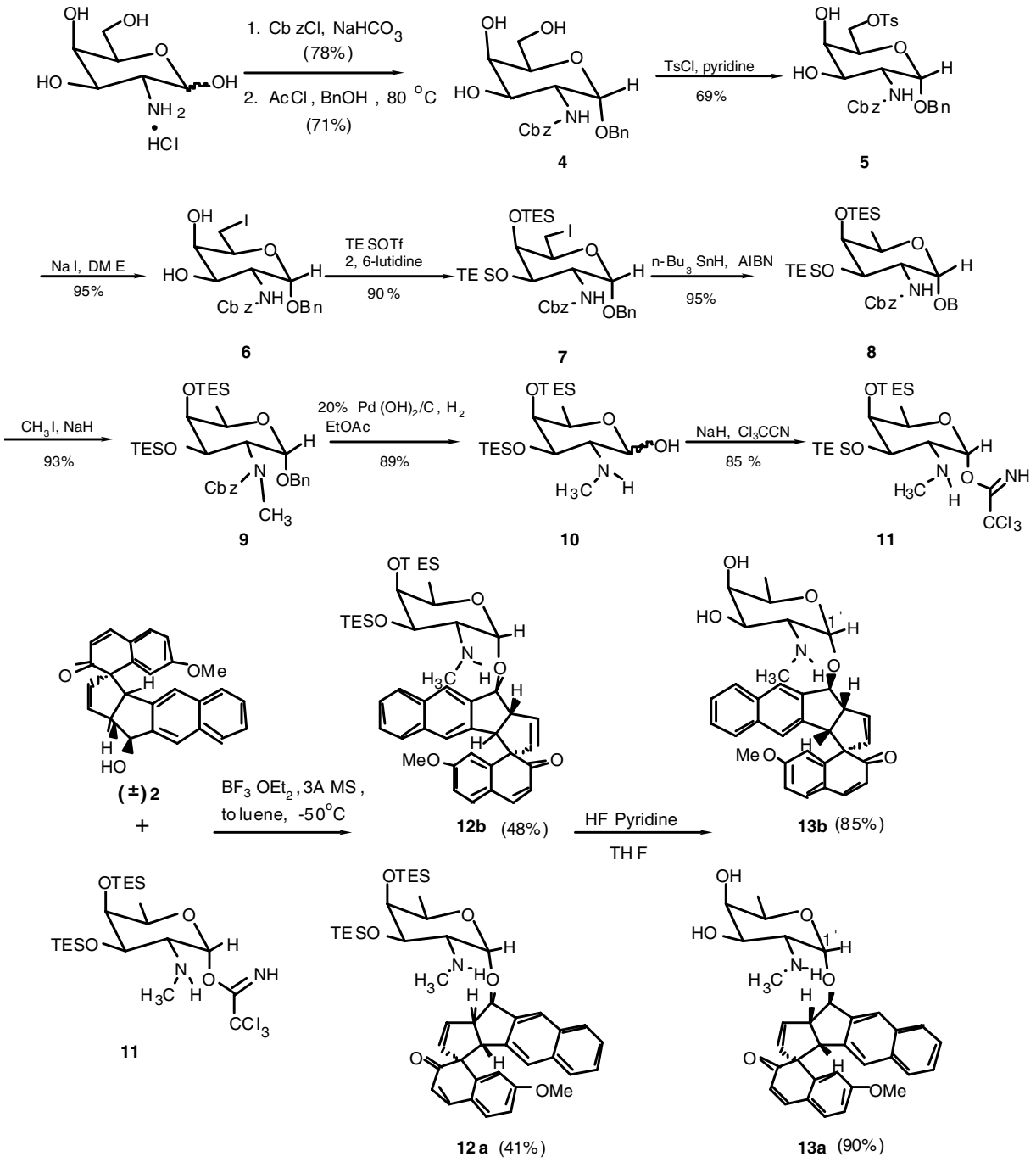


Figure 2. Preparation of α-fucosylated Spiro-alkene anomers.

attained in the oligo-spirocycle binding pocket, and invites analysis using NOESY methodology which has proven useful in this class of agents,[12] and of special significance in the comparison of stereoisomer pairs.[13]

2.2. Functional assay of DNA slippage

Due to their desirable bulge binding properties, we also conducted a functional assay on both **13a** and **13b** which assess ability to induce slipped DNA synthesis.[14] Specifically, compounds were assayed using *Escherichia coli* DNA polymerase I (Klenow fragment) and T_{20}/A_{30} repeats as primers/templates. The annealed mixture of T_{20}/A_{30} (2 μM) in 50 mM Tris–HCl, pH 7.5, was supplied with 5 mM magnesium chloride, 4 mM dithiothreitol, 1 mM dATP, 0.5 mM TTP, and 1.3 μCi [α-^{32}P]TTP before starting synthesis by the addition of enzyme. The test compounds were present from the beginning of the reaction. After incubation at room temperature for 5 h, the amount of ^{32}P TMP incorporated into acid-precipitable DNA products was determined. Table 2 shows that both **13a** and **13b** enhance synthesis relative to control, with **13a** the more effective. To further verify, the reaction products were analyzed on DNA sequencing gels in experiments using 5′-^{32}P-end labeled T9 as primer on A_{30} template. This assay further confirmed that the products obtained in the presence of the test compounds were much longer

Entry	Sequence Code	Conformation
1	DA12:DAc12	5'-GTCCGATGCGTG 3'-CAGGCTACGCAC
2	DA12:BA14	5'-GTCCGATGCGTG 3'-CAGGCTACGCAC TG
3	HT3AGTT	5'-GTCCGATGCGTG T 3'-CAGGCTACGCAC T TG
4	HT4AGTT	5'-GTCCGATGCGTG TT 3'-CAGGCTACGCAC TT TG
5	HT3AGT	5'-GTCCGATGCGTG T 3'-CAGGCTACGCAC T G
6	HT3AGCTT	5'-GTCCGATGCGTG T 3'-CAGGCTACGCAC T TG C
7	HT3AGTA	5'-GTCCGTTGCGTG T 3'-CAGGCAACGCAC T TG
8	HT3TGTA	5'-GTCCGTAGCGTG T 3'-CAGGCATCGCAC T TG
9	HT3GGTA	5'-GTCCGTCGCGTG T 3'-CAGGCAGCGCAC T TG
10	HT3GGTT	5'-GTCCGACGCGTG T 3'-CAGGCTGCGCAC T TG
11	HT3GGTC	5'-GTCCGGCGCGTG T 3'-CAGGCCGCGCAC T TG
12	HT3CGTC	5'-GTCCGGGGCGTG T 3'-CAGGCCCCGCAC T TG
13	HT3AT	5'-GTCCGATGCGTG T 3'-CAGGCTACGCAC T
14	HT3CGTA	5'-GTCCGTGGCGTG T 3'-CAGGCACCGCAC T TG
15	HT3AGTC	5'-GTCCGGTGCGTG T 3'-CAGGCCACGCAC T TG
16	HT3GATC	5'-GTCCGGCGCGTG T 3'-CAGGCCGCGCAC T TA
17	HT3GTTG	5'-GTCCGCCGCGTG T 3'-CAGGCGGCGCAC T TT

Figure 3. Oligonucleotides used in binding studies.

Table 1. Dissociation constants (μM) of DNA binding (Fig. 3)

Entry	Sequence code	**1**	**13a**	**13b**
1	DA12:DAc12	307	NAB	NAB
2	DA12:BA14	2.18	**0.6 (0.3)**	**0.8 (0.3)**
3	HT3AGTT	0.033	**0.08 (0.02)**	**0.13 (0.03)**
4	HT4AGTT	0.026	**0.11 (0.01)**	**0.24 (0.04)**
5	HT3AGT	20.6	**8.4 (1.4)**	**4.1 (0.3)**
6	HT3AGCTT	0.71	**2.1 (0.4)**	**1.7 (0.1)**
7	HT3AGTA	12.5	**0.22 (0.03)**	**1.4 (0.2)**
8	HT3TGTA	22.2	**1.13 (0.04)**	**0.55 (0.34)**
9	HT3GGTA	12.9	0.29 (0.07)	**1.96 (0.33)**
10	HT3GGTT	0.064	0.14 (0.03)	0.11 (0.11)
11	HT3GGTC	0.416	0.10 (0.004)	0.16 (0.06)
12	HT3CGTC	0.5	0.15 (0.03)	0.17 (0.02)
13	HT3AT	10	**10 (3)**	**13 (8)**
14	HT3CGTA		**0.11 (0.01)**	**0.16 (0.03)**
15	HT3AGTC		0.29 (0.09)	**0.26 (0.09)**
16	HT3GATC		0.24 (0.08)	0.15 (0.17)
17	HT3GTTG		0.16 (0.04)	0.3 (0.1)

Dissociation constants (μM) of DNA binding (Fig. 3) by the drugs determined via emission spectra (λ_{exc} 360 nm; λ_{emm} 480 nm for **13a** and **13b**) at 5 °C. Values in parentheses represent standard deviations. Stimulation of drug fluorescence is indicated in bold whereas quenching is shown in standard type. NAB, no apparent binding.

than those in the control reactions lacking them, suggesting that they can induce slipped synthesis by stabilizing bulged transient intermediates.

3. Summary

Bulges in DNA and RNA have been linked to biomolecular processes involved in numerous diseased states. Synthetic agents capable of selectively binding to such targets hold promise as probes of bulge structures and potentially as therapeutics. The synthetic agents produced herein demonstrate considerable specificity for two-base DNA bulges and are able to enhance slipped DNA synthesis in functional assays. Taken with data obtained for the natural product NCSi-gb, specific structural features have been identified which contribute to efficient molecular recognition, and paves the way for rational drug design.

Table 2. Effect of compounds (**13a** and **13b**) on DNA slippage synthesis

	^{32}P incorporated (CPM)	Stimulation
Control	2359	—
Compound **13a** (40 μM)	75040	31.8
Compound **13b** (40 μM)	37027	15.7

Additionally, spectroscopic fingerprints of bulge–oligo complexes have been identified, which may lead to the development of diagnostic probes of nucleic acid microstructures.[15] With an efficient route to nanomolar bulge binders developed, it is now a realistic possibility to develop analogs which can incapacitate or ameliorate the impact of bulged sequences in biological systems.

4. Experimental procedures

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 precotated 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.

4.1. Toluene-4-sulfonyloxy-6-benzyloxy-5-benzyloxycarbonylamino-3,4-dihydroxy- tetrahydro-pyran-2-ylmethyl ester (5)

A flame dried 25-mL round-bottomed flask was charged with compound **4**[16] (460 mg, 1.14 mmol) and anhydrous pyridine (8.5 mL). The solution was cooled to 0 °C, and 4-dimethylaminopyridine (8 mg, 0.065 mmol) and *p*-toluenesulfonyl chloride (270 mg, 1.42 mmol) were added sequentially. The reaction mixture was allowed to warm to room temperature and was stirred for 12 h. The reaction mixture was diluted with ethyl acetate (100 mL), and washed with 3 N aqueous hydrochloric acid (3× 10 mL) and saturated sodium chloride (20 mL). The organic layer was dried over magnesium sulfate and concentrated. The crude product was purified by silica gel chromatography (hexanes/ethyl acetate = 1:1) to afford **5** (439 mg, 69%) as a white solid. TLC (hexanes/ethyl acetate = 1:2): R_f 0.53; ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.46 (s, 3H), 2.81 (br s, 1H), 3.65 (br s, 1H), 3.80 (d, J = 10 Hz, 1H), 3.91 (s, 1H), 4.00–4.08 (m, 2H), 4.22 (t, J = 10 Hz, 1H), 4.28 (dd, J = 10, 5 Hz, 1H), 4.42 (d, J = 11.5 Hz, 1H), 4.68 (d, J = 11.5 Hz, 1H), 4.90 (d, J = 4 Hz, 1H), 5.07–5.17 (m, 3H), 7.27–7.39 (m, 12H), 7.82 (d, J = 8.5 Hz, 2H); HRMS (ESI), m/z $(M+H)^+$: calcd 558.1798, obsd 558.1818.

4.2. (2-Benzyloxy-4, 5-dihydroxy-6-iodomethyl-tetrahydro-pyran-3yl)-carbamic acid benzyl ester (6)

To a solution of compound **5** (360 mg, 0.65 mmol) in freshly distilled 1,2-dimethoxyethane (DME, 7 mL) was added sodium iodide (970 mg, 6.5 mmol). The resultant solution was heated to 85 °C for 12 h. After cooling to room temperature, the reaction mixture was diluted with ethyl acetate (150 mL) and washed with brine (50 mL). The organic layer was dried over magnesium sulfate and concentrated. The crude product was purified by silica gel chromatography (hexanes/EtOAc = 1:1 through 1:2) to afford **6** (315 mg, 95%) as a white solid.

TLC (hexanes/ethyl acetate = 1:2): R_f 0.71; ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.22 (s, 1H), 3.37–3.39 (m, 1H), 3.81–3.84 (m, 1H), 3.98 (t, J = 7 Hz, 1H), 4.06–4.09 (m, 2H), 4.52 (d, J = 12 Hz, 1H), 4.84 (d, J = 11 Hz, 1H), 4.95 (d, J = 4 Hz, 1H), 5.08–5.12 (m, 2H), 5.22 (d, J = 9.5 Hz, 1H), 7.39 (m, 10H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 3.49, 51.74, 67.73, 69.76, 69.90, 71.14, 71.47, 96.89, 128.43, 128.50, 128.60, 128.83, 136.10, 136.91, 158.03; HRMS (ESI), m/z $(M+Na)^+$: calcd 536.0546, obsd 556.0562.

4.3. (2-Benzyloxy-6-iodomethyl-4,5-bis-triethylsilanyloxy-tetrahydro-pyran-3-yl)-carbamic acid benzyl ester (7)

To a precooled (−78 °C) solution of compound **6** (300 mg, 0.59 mmol) in freshly distilled dichloromethane (25 mL) were added 2,6-lutidine (417 μL, 3.51 mmol) and triethylsilyl trifluoromethanesulfonate (535 μL, 2.34 mmol). The resultant solution was held at −78 °C for 30 min and then allowed to warm to 0 °C for 4 h. Excess triethylsilyl trifluoromethanesulfonate was quenched with methanol (0.5 mL) and the solution condensed in vacuo. The resultant residue was purified by silica gel chromatography (hexanes/EtOAc = 20:1) to afford **7** (390 mg, 90%) as a colorless oil. TLC (hexanes/ethyl acetate = 9:1): R_f 0.47; ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.60–0.80 (m, 12H), 0.93–1.05 (m, 18H), 3.23–3.27 (m, 2H), 3.79 (d, J = 10 Hz, 1H), 3.90 (t, J = 7 Hz, 1H), 4.09 (s, 1H), 4.29 (t, J = 18 Hz, 1H), 4.56 (d, J = 12 H, 1H), 4.81 (d, J = 11.5 Hz, 2H), 4.90 (d, J = 3.5 Hz, 1H), 5.11 (s, 2H), 7.34–7.40 (m, 10H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 4.43, 5.17, 5.53, 7.10, 7.26, 51.00, 67.02, 69.67, 71.25, 73.03, 73.33, 97.70, 128.17, 128.28, 128.35, 128.47, 128.74, 136.73, 137.46, 155.97; HRMS (ESI), m/z $(M+Na)^+$: calcd 742.2456, obsd 742.2551.

4.4. (2-Benzyloxy-6-methyl-4,5-bis-triethylsilanyloxy-tetrahydro-pyran-3-yl)-carbamic acid benzyl ester (8)

To a solution of compound **7** (390 mg, 0.53 mmol) in freshly distilled 1,2-dimethoxyethane (10 mL) were added tributyltin hydride (426 μL, 1.58 mmol) and 2,2′-azobisisobutyronitrile (14 mg, 0.104 mmol). The solution was deoxygenated and then heated to 85 °C for 4 h. Upon cooling to room temperature, the reaction mixture was diluted with a mixture of EtOAc/hexanes (1:2, 150 mL), washed with water (50 mL) and then brine (50 mL). The organic layer was dried over magne-

sium sulfate and concentrated in vacuo. Purification of the residue by flash chromatography (100% hexanes through 96% hexanes/4% EtOAc) afforded **8** (307 mg, 95%) as a colorless oil. TLC (hexanes/ethyl acetate = 9:1): R_f 0.42; ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.62–0.76 (m, 12H), 0.94–1.06 (m, 18H), 3.71–3.73 (m, 1H), 3.81–3.89 (m, 2H), 4.32 (dt, J = 11, 7 Hz, 1H), 4.50 (d, J = 12 Hz, 1H), 4.70 (d, J = 12.5 Hz, 1H), 4.83 (d, J = 10.5 Hz, 1H), 4.88–4.91 (m, 1H), 5.11 (d, J = 2.5 Hz, 2H), 7.24–7.41 (m, 10H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 5.20, 5.51, 7.11, 7.22, 17.20, 51.16, 66.91, 68.14, 69.48, 71.51, 75.10, 98.26, 127.95, 128.28, 128.45, 128.66, 136.85, 137.98, 156.02; HRMS (ESI), *m/z* $(M+Na)^+$: calcd 638.3309, obsd 638.3300.

4.5. (2-Benzyloxy-6-methyl-4,5-bis-triethylsilanyloxy-tetrahydro-pyran-3-yl)-methyl-carbamic acid benzyl ester (9)

To a solution of **8** (304 mg, 0.49 mmol) in tetrahydrofuran (6 mL) was added sodium hydride (60% dispersion in mineral oil, 200 mg, 4.9 mmol), followed by iodomethane (0.93 mL, 14.7 mmol). The resultant suspension was heated to 40 °C for 30 min and diluted with EtOAc/hexanes (1:2, 120 mL). The organic layer was washed with phosphate buffer (0.5 M, pH 7.0, 2 × 25 mL), dried over magnesium sulfate, and concentrated in vacuo. The residue was purified by flash chromatography (hexanes/EtOAc = 20:1) to afford **9** (290 mg, 93%) as a colorless oil. TLC (hexanes/ethyl acetate = 9:1): R_f 0.42; ^{1}H NMR (500 MHz, $CDCl_3$): *N*-diastereomeric mixture (rotamers) as shown in SI; ^{13}C NMR (75 MHz, $CDCl_3$): *N*-diastereomeric mixture (rotamers) as shown in SI; HRMS (ESI), *m/z* $(M+Na)^+$: calcd 652.3466, obsd 652.3451.

4.6. 6-Methyl-3-methylamino-4,5-bis-triethylsilanyloxy-tetrahydro-pyran-2-ol (10)

To a solution of **9** (290 mg, 0.46 mmol) in ethyl acetate (10 mL) was added palladium hydroxide on activated carbon (300 mg, 20% w/w Pd). The flask was flushed with argon, evacuated, then a hydrogen atmosphere introduced. The solution was stirred at room temperature for 12 h and then filtered through a plug of Celite®. The filtrate was concentrated and the crude product was purified by flash chromatography (hexanes/EtOAc = 7:3 with v/v 4% triethylamine) to afford **10** (166 mg, 89%) as a colorless oil. TLC (hexanes/ethyl acetate = 7:3 with v/v 4% triethylamine): R_f 0.26; ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.63–0.71 (m, 12H), 0.96–1.01 (m, 18H), 1.19 (d, J = 7 Hz, 3H), 2.41 (s, 3H), 2.89 (dd, J = 10, 3 Hz, 1H), 3.60 (d, J = 1 Hz, 1H), 3.74 (dd, J = 9.5, 2.5 Hz, 1H), 4.01 (q, J = 6.5 Hz, 1H), 5.26 (d, J = 3.5 Hz, 1H). Similar with the data reported;[2] HRMS (ESI), *m/z* $(M+Na)^+$: calcd 406.2809, obsd 406.2815.

4.7. Glycosides 12a and 12b

A heterogeneous mixture of **2** (15 mg, 0.039 mmol), trichloroacetimidate **11**[17] (90 mg, 0.16 mmol), and 3 Å molecular sieves (80 mg) in anhydrous toluene (2 mL) was stirred at room temperature for 30 min then cooled to −50 °C. A solution of boron trifluoride etherate (10 μL, 0.078 mmol) in toluene (300 μL) was added in six portions at 10 min intervals. The mixture was stirred at −50 °C for 1 h then solid sodium bicarbonate (25 mg) added. The contents were filtered through a plug of cotton wool and the filtrate concentrated in vacuo then purified by flash chromatography (hexanes/EtOAc = 4:1) to afford **12a** (12.5 mg, 41%) and **12b** (14.5 mg, 48%) as colorless oils.

Compound **12a**: TLC (hexanes/ethyl acetate = 1:3): R_f 0.38; ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.59–0.77 (m, 12H), 0.91–1.03 (m, 18H), 1.30 (d, J = 6.5 Hz, 3H), 2.30 (s, 3H), 2.88 (dd, J = 9, 3.5 Hz, 1H), 3.19 (s, 3H), 3.69–3.72 (m, 2H), 3.85–3.88 (m, 1H), 3.97–4.06 (m, 1H), 4.51 (d, J = 7.5 Hz, 1H), 5.18 (d, J = 3 Hz, 1H), 5.31 (d, J = 5 Hz, 1H), 5.52 (dd, J = 6, 2.5 Hz, 1H), 5.90 (d, J = 3 Hz, 1H), 6.26 (d, J = 9.5 Hz, 1H), 6.48 (s, 1H), 6.54 (dd, J = 6, 2.5, 1H), 6.58 (dd, J = 8.5, 2.5 Hz, 1H), 7.23 (d, J = 8 Hz, 1H), 7.30–7.39 (m, 3H), 7.57(d, J = 10 Hz, 1H), 7.73 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 5.48, 7.26, 17.44, 29.92, 35.14, 55.14, 59.21, 59.35, 68.32, 69.73, 72.74, 75.39, 84.08, 98.12, 113.22, 116.16, 122.20, 123.58, 124.21, 125.61, 125.74, 127.90, 130.61, 131.88, 133.09, 133.70, 137.73, 139.78, 141.55, 143.76, 145.81, 150.73, 160.60, 203.17; HRMS (ESI), *m/z* $(M+H)^+$: calcd 768.4198, obsd 768.4181.

Compound **12b**: TLC (hexanes/ethyl acetate = 4:1): R_f 0.24; ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.55–0.72 (m, 12H), 0.86–1.00 (m, 18H), 1.25 (d, J = 6 Hz, 3H), 2.43 (s, 3H), 2.88 (dd, J = 10, 3.5 Hz, 1H), 3.18 (s, 3H), 3.61–3.65 (m, 2H), 3.85–3.92 (m, 2H), 4.52 (d, J = 8.5 Hz, 1 H), 5.26 (d, J = 3.5 Hz, 1H), 5.29 (d, J = 2.5 Hz, 1H), 5.50 (dd, J = 6, 2.5 Hz, 1H), 5.87 (d, J = 2 Hz, 1H), 6.24 (d, J = 10 Hz, 1H), 6.41 (s, 1H), 6.46 (dd, J = 5.5, 3.5 Hz, 1H), 6.57 (dd, J = 8.5, 6 Hz, 1H), 7.22 (d, J = 8 Hz, 1H), 7.29–7.36 (m, 3H), 7.55 (d, J = 10 Hz, 1H), 7.68 (s, 1H), 7.70 (d, J = 7.5 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 5.51, 7.25, 17.35, 29.90, 35.19, 55.16, 58.73, 59.29, 68.05, 69.48, 72.52, 75.30, 83.57, 97.32, 113.46, 116.13, 122.19, 123.62, 124.61, 125.59, 125.79, 127.91, 130.58, 132.13, 133.24, 133.70, 137.89, 140.22, 141.61, 143.71, 145.71, 150.73, 160.61, 202.94; HRMS (ESI), *m/z* $(M+H)^+$: calcd 768.4198, obsd 768.4184.

4.8. Glycosides 13a and 13b

To a solution of glycoside **12a** (12.5 mg, 0.022 mmol) in tetrahydrofuran (1.4 mL) was added hydrogen fluoride–pyridine complex (280 μL). The resultant suspension was stirred at room temperature for 1 h, then solid sodium bicarbonate (500 mg) added. The mixture was filtered through a plug of Celite® and concentrated in vacuo. The residue was purified by flash chromatography (EtOAc/MeOH/H_2O = 9:2:1) to furnish glycoside **13a** (7.6 mg, 90%) as a colorless oil. Similar procedure (from **12b**) produced glycoside **13b** (8.3 mg, 85%).

Compound **13a**: TLC (ethyl acetate/methanol/water = 9:2:1): R_f 0.55; ^{1}H NMR (500 MHz, CD_3OD): δ 1.27 (d, J = 6.5 Hz, 3H), 2.75 (s, 3H), 3.16 (s, 3H), 3.64–3.67 (m, 2H), 3.84 (dd, J = 10.5, 2.5 Hz, 1H), 3.92–3.96 (m, 2H), 4.51 (d, J = 7 Hz, 1H), 5.43 (s, 1H), 5.48 (dd, J = 6, 2.5 Hz, 1H), 5.50 (d, J = 3.5 Hz, 1H), 6.19 (d, J = 10 Hz, 1H), 6.31 (s, 1H), 6.46 (dd, J = 6, 2.5 Hz, 1H), 6.72 (dd, J = 8.5, 2.5 Hz, 1H), 7.33–7.43 (m, 5H), 7.76 (d, J = 10 Hz, 1H), 7.80 (d, J = 8 Hz, 1H), 7.89 (s, 1H); HRMS (ESI), m/z $(M+H)^+$: calcd 540.2386, obsd 540.2388.

Compound **13b**: TLC (ethyl acetate/methanol/water = 9:2:1): R_f 0.55; ^{1}H NMR (500 MHz, CD_3OD): δ 1.39 (d, J = 6.5 Hz, 3H), 2.30 (s, 3H), 3.15 (s, 3H), 3.25–3.29 (m, 1H), 3.64–3.67 (m, 1H), 3.75 (d, J = 2.5 Hz, 1H), 3.89–3.94 (m, 2H), 4.18 (q, J = 7 Hz, 1H), 4.51 (d, J = 7.5 Hz, 1H), 5.36 (s, 1H), 5.42 (d, J = 4 Hz, 1H), 5.48 (dd, J = 6, 3 Hz, 1H), 5.76 (d, J = 2.5 Hz, 1H), 6.20 (d, J = 10 Hz, 1H), 6.31 (s, 1H), 6.47 (dd, J = 6, 2.5 Hz, 1H), 6.74 (dd, J = 8, 2.5 Hz, 1H), 7.32–7.45 (m, 5H), 7.77 (d, J = 9.5 Hz, 1H), 7.83 (d, J = 8 Hz, 1H), 7.94 (s, 1H); HRMS (ESI), m/z $(M+H)^+$: calcd 540.2386, obsd 540.2365.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.10.052.

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