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Stability and Selectivity of Unnatural DNA with Five-Membered-Ring Nucleobase Analogues

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Abstract: In an effort to develop an orthogonal third base pair for the storage of genetic information, thiophene and furan heterocycles have been examined as nucleobase analogues. The stability of the unnatural bases was evaluated in duplex DNA paired opposite other unnatural bases as well as opposite the natural bases. Several unnatural base pairs are identified that are both reasonably stable and strongly selective against mispairing with native bases. These results expand the potential nucleobase analogues with which the genetic alphabet may be expanded to include five-membered-ring heterocycles.

Introduction

In an attempt to expand the genetic alphabet, and eventually the genetic code, we have evaluated a variety of unnatural and predominantly hydrophobic nucleobase analogues.[1−6] The stability and selectivity required for the storage of genetic information is based on the complementary pairing of purine and pyrimidine derivatives with appropriate hydrogen bonding patterns.[7] However, it is not clear that hydrogen bonding is the only interaction capable of generating the forces required for stable information storage and retrieval. We have been interested in the use of hydrophobic bases to circumvent the hydrogen bonding patterns of natural base pairs.[8] Hydrophobic base pairs are expected to favor unnatural pairing between two unnatural bases, both in duplex DNA and during DNA replication, due to the forced desolvation that would be required during their mispairing with natural bases. In addition to expanding the genetic alphabet, additional stable and selective base pairs would also facilitate hybridization or encoding experiments in cases where natural sequences cross-hybridize, or where increased information storage is desirable.[9−16] Previous efforts to increase the number of bases available for information storage in DNA have relied on N- or C-glycosidic nucleosides with purine- or pyrimidine-like nucleobases with unique patterns of H-bond donors and acceptors.[17−21] However, such an approach, based solely on purine- and pyrimidine-like nucleobase analogues, severely limits the available design strategies. Furthermore, a strategy based on purine or pyrimidine modification is limited by the existence of relatively stable base tautomers that can mispair with native bases and thereby reduce selectivity.[22−24]

Recent reports that H-bonds are not an absolute requirement for stable duplex formation[1−5] or efficient replication[6] point to new design strategies based on the use of hydrophobic scaffolds with little or no similarity to the natural bases. We have recently reported the synthesis and characterization of a variety of unnatural nucleosides that possess hydrophobic groups instead of purine or pyrimidine bases.[1−6] Unnatural nucleobase analogues with indole, phenyl, naphthyl, and isocarbostyryl scaffolds have been previously characterized, and a variety have been identified that pair in duplex DNA with a stability and selectivity comparable to or greater than that of natural base pairs. Isocarbostyryl (**ICS**) and several derivatives including methyl isocarbostyryl (**MICS**), 4-methylisocarbostyryl (**4MICS**), and propynyl methyl isocarbostyryl (**PIM**) have been exten-

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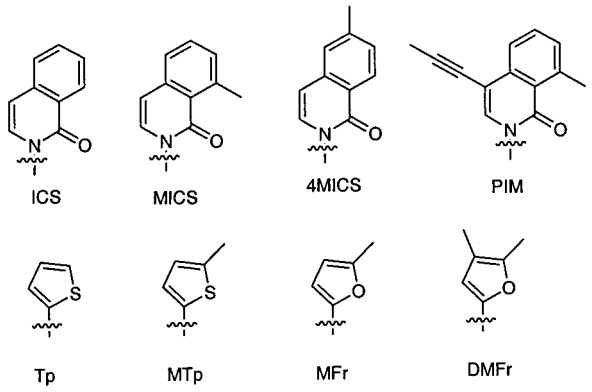
Chart 1



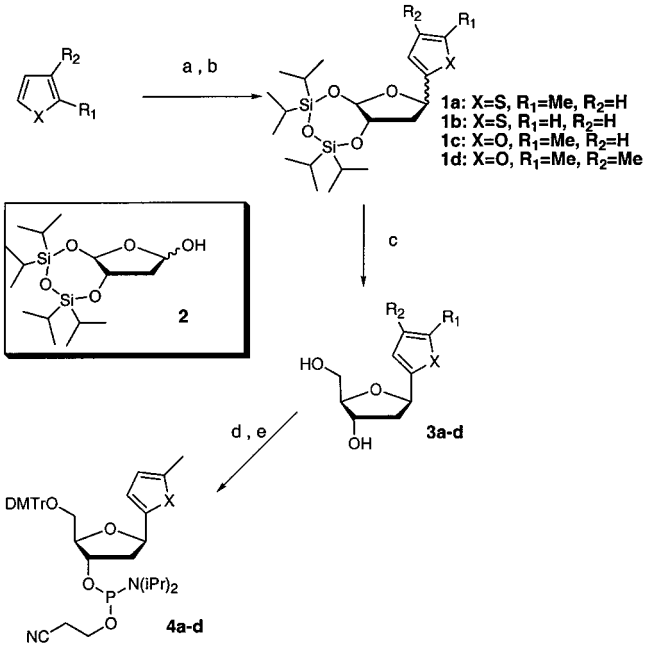
sively examined, as have 7-azaindole (**7AI**) and several of its derivatives (Chart 1). Each of these nucleobase analogues has a large hydrophobic surface that is capable of packing with flanking bases. However, such large nucleobase analogues may result in duplex distortion, especially when several unnatural pairs are contiguous. A sequence dependence of this kind could potentially limit the utility of these unnatural bases.

To further explore alternative nucleobase scaffolds, we have been interested in examining the ability of five-membered-ring nucleobase analogues to form stable and selective unnatural base pairs in duplex DNA. While structural data are not yet available, comparison of the stability of duplex DNA containing the smaller unnatural bases with that of DNA containing the larger **ICS** and **7AI** analogues is expected to help define the relative contribution of hydrophobic packing and potential duplex distortion. Unnatural base pairs formed between two five-membered-ring analogues, or between a five- and a six-membered-ring analogue, are expected to optimize inter- and intrastrand base packing, while minimizing potentially deleterious duplex distortions. We synthesized four unnatural nucleosides, containing either a thiophene or a furan heterocyclic ring, and incorporated these base analogues into DNA (Chart 1). The synthesis and thermodynamic characterization of each nucleoside analogue are reported herein. Thermodynamic stability is assayed by determining the melting temperature (T_m) of duplex DNA containing the unnatural bases paired opposite native bases, defined as a mispair, or opposite other five- or six-membered-ring nucleobase analogues, defined as correct pairs.

Materials and Methods

Synthesis of the four C-glycosides followed literature procedures (Scheme 1).[25] Briefly, the furan or thiophene was ortholithiated with *n*-butyllithium and added to 2-deoxy-3,5-*O*-(tetraisopropyldisiloxane-1,3-diyl)-D-erythropentofuranose.[25,26] This gave the corresponding diol in moderate yield as a diastereomeric mixture which was cyclized under acidic conditions and deprotected. The nucleosides **3a**−**d** were converted to trityl-protected phosphoramidites by literature methods.[27] An Applied Biosystems Inc. 392 DNA/RNA synthesizer was used to synthesize the oligonucleotides 5′-GCGATG**X**GTAGCG-3′ and 5′-CGCTAC**Y**CATCGC-3′, containing either a nucleoside analogue or a natural base at positions **X** and **Y**. The T_m of the duplexes was measured in 10 mM PIPES (pH 7) with 100 mM NaCl and 10 mM $MgCl_2$ on a Cary 300 Bio UV−visible spectrophotometer.

Scheme 1 [a]



[a] Conditions: (a) *n*-BuLi, −20 °C, then **2**, THF; (b) *p*-TsOH, CH_2Cl_2, 4 Å molecular sieves; (c) TBAF, THF; (d) DMTrCl, pyridine; (e) $CNC_2H_4OPClN(iPr)_2$, $EtN(iPr)_2$, CH_2Cl_2.

Table 1. T_m Values for Duplex Containing **Tp** and **MTp**[a]

5′-dGCGTACXCATGCG
3′-dCGCATGYGTACGC

X	Y	T_m (°C)	X	Y	T_m (°C)
Tp	**Tp**	51.3	**MTp**	**MTp**	52.1
	MTp	51.6		**DMFr**	51.2
	MFr	50.1		**ICS**	54.8
	DMFr	49.4		**MICS**	57.1
	ICS	54.2		**PIM**	54.8
	MICS	56.4		**4MICS**	56.2
	PIM	55.2		**7AI**	53.2
	4MICS	55.4		A	48.2
	7AI	52.5		T	46.5
	A	48.5		C	43.2
	T	46.5		G	47.5
	C	42.3			
	G	47.2			

[a] See text for experimental details.

Results and Discussion

Mispairs between Five-Membered-Ring Analogues and Natural Bases. Duplex melting temperatures are reported in Tables 1−3. Fully complementary natural oligonucleotides formed stable duplexes with T_m's ranging from 58.7 to 61.8 °C. Duplexes containing a single mispair among the natural bases were less stable, with T_m's ranging from 44.8 °C (dC:dC mispair) to 55.4 °C (dG:dA). Duplexes containing the five-membered-ring base analogues opposite native bases were also destabilized. The observed T_m values ranged from 42.2 to 49.1 °C, where the higher melt temperatures correspond to mispairs between the unnatural base and adenine. This is consistent with the large size and hydrophobicity of dA.

The nature of the heteroatom, presumably disposed in the minor groove of the duplex, also affected duplex stability.

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Table 2. T_m Values for Duplex Containing **MFr** and **DMFr**[a]

5′-dGCGTAC**X**CATGCG
3′-dCGCATG**Y**GTACGC

X	Y	T_m (°C)	X	Y	T_m (°C)
MFr	**MFr**	50.2	**DMFr**	**DMFr**	51.7
	MTp	50.4		**ICS**	52.5
	DMFr	49.5		**MICS**	54.1
	ICS	53.5		**PIM**	54.2
	MICS	55.9		**4MICS**	54.1
	PIM	54.9		**7AI**	52.7
	4MICS	55.4		A	49.1
	7AI	52.6		T	48.8
	A	47.5		C	46.4
	T	47.2		G	44.8
	C	42.2			
	G	44.3			

[a] See text for experimental details.

Table 3. T_m Values for Duplex with Alternate Sequence Context[a]

5′-dGCGTAC**X**CATGCG
3′-dCGCATG**Y**GTACGC

X	Y	T_m (°C)
ICS	**MFr**	54.2
	MTp	55.2
MICS	**MFr**	55.4
	MTp	56.9

[a] See text for experimental details.

The methylthiophenes were more stable than the methylfurans, except when opposite dT, where **MFr**:dT was 0.7 °C more stable than **MTp**:dT. The generally increased stability of mispairs with the thiophene may result from favorable interstrand or intrastrand packing due to the greater size or polarizability of sulfur relative to oxygen. Alternatively, a more favorable H-bond between water and oxygen, relative to water and sulfur, may stabilize the furan-containing oligonucleotide in the single-stranded state.

Sterics also appeared to play an important role in determining the stability of the mispairs formed between a five-membered-ring analogue and a native base. The mispairs formed between **Tp** and either of the purines were more stable than those formed with pyrimidines, presumably due to the reduced surface area of dT and dC. Addition of a methyl group to the thiophene ring resulted in little change in duplex stability, with the exception of the mispair with dC; **MTp**:dC melted 0.9 °C higher than **Tp**:dC. Duplex stability was significantly more sensitive to the addition of a second methyl group to the furan ring. The **DMFr** mispair formed with dA, dT, or dG was 0.5 to 1.6 °C more stable than the corresponding mispairs with **MFr**. The mispair with dC was again the most stabilized by the addition of the methyl group, with **DMFr**:dC being 4.2 °C more stable than **MFr**:dC. Overall, the most stable mispairs were formed between **DMFr** and dA, which is again likely due to the greater size and hydrophobicity of adenine relative to the other natural nucleobases.

Base Pairs between Five-Membered-Ring Analogues. There was no obvious trend between stability and methyl substitution or the nature of the heteroatom. The unnatural base pairs formed between the five-membered rings were somewhat more stable than mispairs with native bases, with observed T_m values of 49.4−52.1 °C. The **Tp**:**Tp** self-pair melted at 51.3 °C, while the mispairs with native bases were 2.8−9.0 °C less stable. **MTp** was even more selective, with the **MTp**:**MTp** self-pair melting at 52.1 °C, which is 3.9−8.9 °C more stable than the mispairs. While the stability of these hydrophobic self-pairs is compromised relative to that of natural Watson−Crick pairs, the selectivity is comparable (Table 1). This could be useful in hybridization experiments requiring a lower melting duplex, but where selectivity must remain high (see below).

Five-Membered-Ring Analogues Opposite ICS Derivatives. Pairing the five-membered rings opposite hydrophobic bases of larger size resulted in a further increase in base pair stability. Opposite the **ICS** derivatives (**ICS**, **MICS**, **4MICS**, and **PIM**), the stabilities of the resulting duplex ranged from 52.5 to 57.1 °C. The stability of the unnatural pair generally increased with the size of the **ICS** analogue (**ICS** to **MICS** or **4MICS**). As an exception, the stabilities were decreased when the five-membered-ring analogues were paired opposite **PIM**. The origin of the destabilizing effect of the remote propynyl substituent is not clear at this time.

Similar to the mispairs discussed above, pairs with the **ICS** derivatives were generally more stable with the sulfur-containing heterocycles, except opposite **PIM**, where the minor groove atom had little effect. Opposite the increased bulk of the **ICS** derivatives, base pair stability was again sensitive to methyl substitution of the heterocycle. The additional methyl group of **MTp**, relative to **Tp**, resulted in a small increase in stability, except opposite the largest unnatural base, **PIM**, where the increased bulk destabilized the base pair by 0.4 °C. Addition of a second methyl group to the furan (**MFr** versus **DMFr**) destabilized the pair by 0.7−1.8 °C. Apparently, the greater size of the **ICS** derivatives results in a duplex environment that is less accommodating of the added methyl group.

The **MTp**:**MICS** pair was the most stable unnatural pair found in this study, with T_m = 57.1 °C. This pair is only marginally less stable than a dA:dT in the same sequence context (T_m = 59.2 °C). Moreover, **MTp** paired opposite **MICS** is significantly more selective against mispairing with a native base (by a minimum of 8.9 °C) than the native bases are against mispairs with themselves (a minimum of only 3.8 °C).

Five-Membered-Ring Analogues Opposite 7AI. The unnatural base **7AI** showed little thermal selectivity opposite any of the five-membered-ring analogues. Each pair melted between 52.5 and 53.2 °C. This range of stabilities is somewhat reduced relative to that for natural pairs, but like the pairs with **ICS** and its analogues, the **7AI** pairs are strongly selective against mispairing with natural bases. For example, both the **MFr**:**7AI** and **MTp**:**7AI** pairs are at least 5 °C selective against mispairing.

Use of Unnatural Bases To Modify Hybridization Properties of Oligonucleotides. Large differences in the melting temperatures between dA:dT- and dG:dC-rich duplexes is one of the biggest obstacles to hybridization experiments.[10,14,15,28,29] Approaches to equalizing duplex stabilities at an optimal value have involved the addition of tetramethylammonium[30] or betain[31] salts, or the incorporation of modified[32] or "universal" bases that show little selectivity in pairing with natural

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Chart 2

dCGCATG**T**GTACGG dGCGTAC**A**CATGCC	dCGCATG**A**GTACGG dGCGTAC**G**CATGCC
59.2 °C	55.4 °C
dCGCATG**T**GT**MTp**CGG dGCGTAC**A**CA**MTp**GCC	dCGCATG**A**GT**MTp**CGG dGCGTAC**G**CA**MTp**GCC
51.2 °C	46.2 °C

bases.[11,28,33−36] In addition to large stability differences, the melting temperature of a duplex may not be strongly sensitive to the presence of a mismatch, especially for more stable duplexes. For example, the T_m difference between a perfectly matched duplex and one containing a single mismatch may be as small as 0.5 °C.[11] The limited sensitivity to mismatches constitutes another fundamental limitation to hybridization technologies.[11]

The five-membered-ring nucleobases described herein provide an additional method to tailor duplex stability. By modifying stability without compromising selectivity, the analogues might prove useful in mispair detection. Because highly stable (e.g., dG:dC-rich) duplexes are generally less sensitive to mispairing, incorporation of the destabilizing but selective analogues should result in increased sensitivity to mispairs. To examine the potential of the unnatural base pairs in this context, a reasonably stable mispair was examined in the context of a fully native duplex as well as in a duplex containing a **MTp** self-pair (Chart 2). The fully native duplex showed a 3.8 °C T_m difference between a dA:dT pair and a dA:dG mispair. In a duplex containg the **MTp** self-pair, the same mispair was destabilized by 5.0 °C. In this case, the presence of the unnatural base pair renders the T_m more sensitive to mispairing elsewhere in the duplex.

Shape and H-bond complementarity are not absolute requirements for unnatural DNA base pairs. It is possible to use hydrophobic interactions to optimize nucleic acid properties such as duplex stability, enzymatic replication, or sensitivity of the duplex to mispairs. It is therefore important to examine nucleobase analogues based on ring structures other than the natural purines and pyrimidines. Herein, we have reported the characterization of duplex stability of DNA containing furan and thiophene nucleobase analogues. The measured T_m's demonstrate that, when packed opposite one another, the smaller ring structures are of insufficient size for efficient intra- and interstrand hydrophobic packing within duplex DNA. However, when paired opposite larger hydrophobic ring structures, reasonably stable hydrophobic pairs are formed. For example, the **MTp**:**MICS** unnatural base pair is only marginally less stable than a dA:dT pair in the same sequence context. Moreover, as described above, the ability of five-membered-ring analogues to alter duplex stability and sensitivity to mispairing may also prove useful for hybridization experiments. We are currently examining a variety of furan and thiophene derivatives that could result in unnatural base pairs with improved thermodynamic or kinetic properties.

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Experimental Section

Oligonucleotide concentrations were determined spectrophotometrically with extinction coefficients calculated using the Biopolymer Calculator (http://paris.chem.yale.edu/extinct.html). Extinction coefficients of the unnatural nucleosides were also determined: d**Tp**, $\epsilon_{235} = 6.8 \times 10^3\ M^{-1}\ cm^{-1}$; d**MTp**, $\epsilon_{256} = 6.3 \times 10^2\ M^{-1}\ cm^{-1}$; d**MFr**, $\epsilon_{233} = 1.4 \times 10^4\ M^{-1}\ cm^{-1}$; d**DMFr**, $\epsilon_{234} = 1.3 \times 10^4\ M^{-1}\ cm^{-1}$. The melting experiments were carried out with 3 μM duplex in 10 mM PIPES (pH 7), 10 mM $MgCl_2$, and 100 mM NaCl, using a Cary 300 Bio UV−vis spectrophotometer. The heating rate was 0.5 °C/min between 16 and 80 °C. Melting temperatures were obtained from the derivative method utilizing the Cary Win UV thermal application software. The experimental error of the melting temperatures is approximately ±0.5 °C.

Representative Procedure: Synthesis of Methyl Thiophene Nucleoside 3a. To a stirred mixture of 2-deoxy-3,5-*O*-(tetraisopropyldisiloxane-1,3-diyl)-D-erythropentofuranose (**2**; 756 mg, 2 mmol) and anhydrous THF (15 mL) was added a solution of 2-methyl-5-lithiothiophene (6 mmol) in THF (10 mL) dropwise under argon at −20 °C. The resulting solution was stirred for 1 h and then quenched with saturated aqueous NH_4Cl. After multiple extractions with CH_2Cl_2, the organic layer was washed with brine, dried with Na_2SO_4, filtered, and concentrated. Silica gel column chromotography (25% ethyl acetate in hexane) afforded a mixture of the corresponding 2-[(1*R*/*S*)-2-deoxy-3,5-*O*-(tetraisopropyldisiloxane-1,3-diyl)-D-ribofuranosyl]-5-methylthiophene. A solution of 2-[(1*R*/*S*)-2-deoxy-3,5-*O*-(tetraisopropyldisiloxane-1,3-diyl)-D-ribofuranonosyl]-5-methylthiophene in CH_2Cl_2 (20 mL) was stirred with TsOH (30 mg) and molecular sieves 4 Å (10 g) for 1 h. After addition of solid $NaHCO_3$, the mixture was filtered and concentrated. Silica gel column chromotography (20% ethyl acetate in hexane) afforded a mixture of 2-[2-deoxy-3,5-*O*-tetraisopropyldisiloxane-1,3-diyl)-α/β-D-ribofuranosyl]-5-methylthiophene (**1a**) as a colorless oil. The mixture **1a** was dissolved in THF (10 mL), and 4 mL (4 mmol) of a 1 M solution of *tert*-butylammonium fluoride (4 mL, 4 mmol) in THF was added. After 1 h the mixture was quenched with saturated aqueous NH_4Cl and extracted three times with ethyl acetate. The combined organics were washed with brine, dried over Na_2SO_4, filtered, and concentrated. The crude product was purified via silica gel column chromatography (12% methanol and 45% ethyl acetate in hexane), and the α and β anomers were separated by preparative HPLC (Varian Dynamax-100 Å Si column; isocratic separation with 10% 2-propanol in hexane; 20 mL/min) to yield 68 mg (16% yield over three steps) of **2a**β (stereochemical assignment was confirmed by means of ^{1}H−NOESY NMR): ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.79 (1H, d, J = 3.0 Hz), 6.59 (1H, d, J = 3.0), 5.31 (1H, dd, J = 9.8, 5.9 Hz), 4.37 (1H, m), 3.93 (1H, m), 3.59−3.71 (2H, m), 2.49 (3H, s), 2.24 (1H, ddd, J = 13.5, 5.9, 2.2 Hz) 2.13 (1H, ddd, J = 13.5, 9.8, 6.2 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 125.0, 124.8, 87.2, 76.2, 73.7, 63.2, 44.1, 15.4; HRMS calcd for $C_{10}H_{14}O_3SNa$ (MNa^+) 237.0556, found 237.0560.

Methylthiophene Phosphoramidite 4a. To a solution of nucleoside **3a**β (58 mg, 0.27 mmol) in pyridine (3 mL) was added DMTr-Cl (117 mg, 0.352 mmol) in four portions over 5 min. After being stirred at room temperature for 30 min, the reaction mixture was diluted with ethyl acetate (20 mL) and brine (10 mL). The aqueous layer was additionaly extracted three times with ethyl acetate. The combined organics were dried over Na_2SO_4, filtered, concentrated, and purified by silica gel column chromatography (50% ethyl acetate in hexane) to afford the tritylated nucleoside (107 mg). To a solution of the tritylated nucleoside in CH_2Cl_2 (3 mL) at 0 °C were added diisopropylethylamine (250 μL, 1.794 mmol) and 2-cyanoethyl diisopropyl aminochlorophosphoramidite (105 μL, 0.471 mmol). After 15 min, the reaction mixture was partitioned between CH_2Cl_2 (20 mL) and saturated aqueous $NaHCO_3$ (20 mL). The layers were separated, and the aqueous layer was extracted with 2 × 20 mL of CH_2Cl_2. The combined organics were dried over Na_2SO_4, filtered, and concentrated. Purification by silica

gel column chromatography (30% ethyl acetate in 2% triethylamine/hexane) afforded phosphoramidite **4a** (113 mg, 58.4% over two steps): ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.45−7.59 (2H, m), 7.33−7.40 (4H, m), 7.18−7.31 (3H, m), 6.78−6.84 (5H, m), 6.60 (1H, m), 5.31 (1H, dd, J = 10.3, 5.3 Hz), 4.52 (1H, m), 4.17 (1H, m), 3.78 (3H, s), 3.77 (3H, s), 3.65−3.84 (2H, m), 3.55−3.62 (2H, m), 3.29 (0.5H, dd, J = 9.9, 4.7 Hz), 3.24 (0.5H, dd, J = 9.9, 4.7 Hz), 3.18 (0.5H, dd, J = 9.9, 4.4 Hz), 3.16 (0.5H, dd, J = 9.9, 4.4 Hz), 2.60 (1H, t, J = 6.3 Hz) 2.46 (3H, s), 2.45 (1H, t, J = 6.3 Hz), 2.40 (0.5H, m), 2.31 (0.5H, m), 2.13−2.21 (1H, m), 1.18 (3H, d, J = 6.9 Hz), 1.17 (3H, d, J = 6.9 Hz), 1.15 (3H, d, J = 6.9 Hz), 1.06 (3H, d, J = 7.0 Hz); ESMS calcd for $C_{40}H_{49}N_2O_6PSNa$ (MNa^+) 739, found 739.

Thiophene Nucleoside 3bβ: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.28 (1H, d, J = 3.0 Hz) 7.01 (1H, m), 6.97 (1H, d, J = 3.0), 5.43 (1H, dd, J = 9.7, 5.7 Hz), 4.47 (1H, m), 3.99 (1H, m), 3.65−3.82 (2H, m), 2.32 (1H, ddd, J = 13.2, 5.7, 2.1 Hz)), 2.21 (1H, ddd, J = 13.2, 9.7, 6.2 Hz); HRMS calcd for $C_9H_{12}O_3SNa$ (MNa^+) 223.0399, found 223.0403.

Thiophene Phosphoramidite 4b: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45−7.59 (2H, m), 7.33−7.40 (4H, m), 7.18−7.31 (4H, m), 7.05 (1H, m), 6.98 (0.5H, dd, J = 3.5, 1.2 Hz), 6.78−6.84 (4H, m), 5.42 (1H, dd, J = 10.6, 5.0 Hz), 4.53 (1H, m), 4.20 (1H, m), 3.79 (3H, s), 3.78 (3H, s), 3.68−3.82 (2H, m), 3.54 (2H, m), 3.32 (0.5H, dd, J = 10.0, 4.4 Hz), 3.29 (0.5H, dd, J = 10.0, 4.4 Hz), 3.17 (0.5H, dd, J = 10.0, 4.1 Hz), 3.16 (0.5H, dd, J = 10.0, 4.1 Hz), 2.61 (1H, t, J = 6.3 Hz) 2.47 (0.5H, m), 2.46 (1H, t, J = 6.4 Hz), 2.31 (0.5H, m), 2.15−2.22 (1H, m), 1.18 (3H, d, J = 6.7 Hz), 1.17 (3H, d, J = 6.9 Hz), 1.15 (3H, d, J = 6.9 Hz), 1.06 (3H, d, J = 6.8 Hz);^{13}C NMR (125 MHz, $CDCl_3$) δ 158.3, 152.3, 151.5, 145.0, 136.2, 136.1, 130.2, 130.1, 129.1, 128.3, 127.8, 127.7, 126.6, 114.4, 113.0, 86.0, 76.2, 75.8, 73.7, 73.6, 64.0, 58.4, 55.2, 55.1, 43.3, 43.2, 38.6, 24.6, 24.5, 20.3, 20.2; ESMS calcd for $C_{39}H_{47}N_2O_6PNa$ (MNa^+) 725, found 725.

Methylfuran Nucleoside 3cβ: ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.20 (1H, d, J = 2.9 Hz), 5.91 (1H, d, J = 2.9), 5.14 (1H, dd, J = 9.9, 6.2 Hz), 4.50 (1H, m), 3.98 (1H, m), 3.78 (1H, m), 3.68 (1H, m), 2.42 (1H, ddd, J = 13.2, 9.9, 6.2 Hz), 2.28 (3H, s), 2.12 (1H, ddd, J = 13.2, 6.2, 2.2 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 149.9, 145.7, 114.6, 111.4, 87.1, 73.5, 72.8, 63.2, 39.6, 11.6; HRMS calcd for $C_{10}H_{14}O_4Na$ (MNa^+) 221.0784, found 221.0782.

Methylfuran Phosphoramidite 4c: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45−7.59 (2H, m), 7.33−7.40 (4H, m), 7.18−7.31 (3H, m), 6.78−6.84 (5H, m), 6.20 (1H, m), 5.91 (1H, m), 5.11 (1H, dd, J = 10.4, 5.3 Hz), 4.55 (1H, m), 4.18 (1H, m), 3.79 (3H, s), 3.78 (3H, s), 3.68−3.82 (2H, m), 3.54−3.64 (2H, m), 3.25 (0.5H, dd, J = 9.9, 4.8 Hz), 3.20 (0.5H, dd, J = 9.9, 5.1 Hz), 3.15 (0.5H, dd, J = 9.9, 4.4 Hz), 3.13 (0.5H, dd, J = 9.9, 4.4 Hz), 2.60 (1H, t, J = 6.3 Hz), 2.45 (1H, t, J = 6.3 Hz), 2.40−2.45 (1H, m) 2.25 (0.5H, m), 2.22 (3H, s), 2.18 (0.5H, m), 1.19 (3H, d, J = 6.8 Hz), 1.17 (3H, d, J = 6.9 Hz), 1.15 (3H, d, J = 6.9 Hz), 1.10 (3H, d, J = 7.0 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) 158.3, 152.3, 151.5, 145.0, 136.2, 136.1, 130.2, 130.1, 129.1, 128.3, 127.8, 127.7, 126.6, 114.4, 113.0, 108.6, 106.1, 85.7, 85.4, 76.2, 75.8, 73.7, 73.6, 64.3, 58.4, 58.3, 55.2, 55.1, 43.3, 43.2, 38.6, 24.6, 24.5, 20.3, 20.2, 13.6; ESMS calcd for $C_{40}H_{49}N_2O_7PNa$ (MNa^+) 723, found 723.

Dimethylfuran Nucleoside 3dβ: ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.08 (1H, s), 5.08 (1H, dd, J = 9.9, 5.9 Hz), 4.44 (1H, m), 3.94 (1H, m), 3.74 (1H, m), 3.66 (1H, dd, J = 11.7, 4.8 Hz), 2.37 (1H, ddd, J = 13.3, 9.9, 6.2 Hz), 2.17 (3H, s), 2.08 (1H, ddd, J = 13.3, 5.9, 2.2 Hz), 1.90 (3H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ 149.9, 147.8, 114.6, 111.4, 87.1, 73.4, 72.8, 63.2, 39.7, 11.4, 9.7; HRMS calcd for $C_{11}H_{16}O_4Na$ (MNa^+) 235.0941, found 235.0947.

Dimethylfuran Phosphoramidite 4d: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45−7.59 (2H, m), 7.33−7.40 (4H, m), 7.18−7.31 (3H, m), 6.78−6.84 (5H, m), 6.09 (1H, s), 5.06 (1H, dd, J = 10.7, 5.3 Hz), 4.53 (1H, m), 4.17 (1H, m), 3.79 (3H, s), 3.78 (3H, s), 3.68−3.82 (2H, m), 3.54−3.64 (2H, m), 3.22 (0.5 H, dd, J = 10.0, 5.3 Hz), 3.19 (0.5H, dd, J = 10.9, 5.3 Hz), 3.14 (0.5H, dd, J = 10.0, 4.1 Hz), 3.12 (0.5H, dd, J = 10.9, 4.1 Hz), 2.60 (1H, t, J = 6.4 Hz), 2.46 (1H, t, J = 6.4 Hz), 2.35−2.43 (1H, m), 2.21 (0.5H, m), 2.14 (0.5H, m), 2.12 (3H, s),1.90 (3H, s), 1.19 (3H, d, J = 6.8 Hz), 1.17 (3H, d, J = 6.9 Hz), 1.16 (3H, d, J = 6.9 Hz), 1.10 (3H, d, J = 7.0 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 158.3, 150.1, 147.8, 145.0, 136.2, 136.1, 130.2, 130.1, 129.1, 128.3, 127.8, 127.7, 126.6, 114.4, 113.1, 113.0, 111.1, 86.0, 85.6, 76.3, 75.9, 73.7, 73.6, 64.2, 58.4, 55.2, 43.2, 43.1, 38.5, 24.6, 24.5, 11.4, 9.8; ESMS calcd for $C_{41}H_{51}N_2O_7PNa$ (MNa^+) 737, found 737.

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