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Synthesis and characterization of a DNA analogue stabilized by mercapto C-nucleoside induced disulfide bonding

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Abstract—A redox-active nucleobase analogue of a nucleotide was synthesized and incorporated into DNA using phosphoramidite chemistry. An analogue-containing oligonucleotide in the absence of a reducing reagent formed a stable duplex with a substantially higher melting temperature compared to that of a standard DNA duplex of the same length.

The strategy of replacing the natural bases of DNA with analogues has allowed the possibility of adding new functions to the molecule. Efforts to expand the genetic alphabet have been made with respect to artificial gene control as well as the development of functionalized biopolymers.[1] Recently, investigations into duplex formation, which depend on metal-mediated base pairing[2] and interbase hydrophobic interactions,[3] were reported. We are interested in increasing the information content of DNA using unnatural nucleic acids, where the pairing of the base analogues is driven by reversible covalent bonding instead of hydrogen bonding. Thiol groups can be easily oxidized into disulfides, and disulfide bonds can be broken into thiol groups by reducing agents. There are several papers in the literature describing the application of disulfide cross-linked oligonucleotides for supplying a conformational lock.[4] However, there are very few reports describing nonnatural DNA molecules constructed in which base pairing is controlled by the redox environment.[5] Among nonnatural DNA molecules, C-nucleotides are very interesting for their variety of biological activity. C-nucleosides such as showdomycine,[6] have a broad spectrum of biological activity and have stimulated considerable interest as potential antitumor, anti-bacterial and anti-cancer agents.[7] We report here the synthesis of a novel thiophenol-bearing β-C-nucleoside **3** and the influence of disulfide base pairing on the thermal stability of the DNA duplex (Fig. 1).

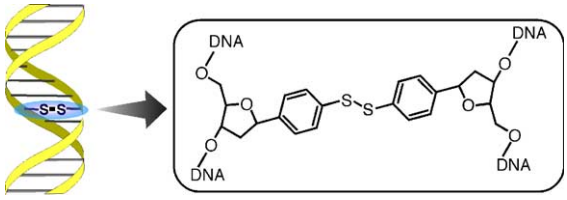

Figure 1. Schematic representation of a DNA duplex induced by the formation of disulfide bonding.

The synthetic route to mercaptophenyl β-C-nucleoside **3** is shown in Scheme 1. We first tried the coupling reaction of alkylation using the benzyl protected thiophenol (1.5 equiv) and 3,5-ditoluoyl-1-α/β-methoxy-2-deoxy-D-ribose[8] **1** (1 equiv) in the presence of a Lewis acid (2 equiv $SnCl_4$) in CH_2Cl_2 at −15 °C under an Ar atmosphere. Although the yield of the Friedel–Crafts alkylation was high (~65%), the *S*-benzyl group of the product could not be deprotected with strong acids, Birch reduction, etc. The effects of another protective groups for thiophenol were investigated (Table 1). Each thiophenol protected by *S*-*p*-nitrobenzyl, *S*-acetoamidemethyl, *S*-cyanoethyl and *S*-benzoyl group did not react with compound **1** under the same condition (benzyl protected thiophenol). The *p*-methoxybenzyl protected thiophenol produced **9**β and **9**α. The free thiol group attacked the anomeric carbon (1′-position) of compound **1** for cleavage of the protective group by the

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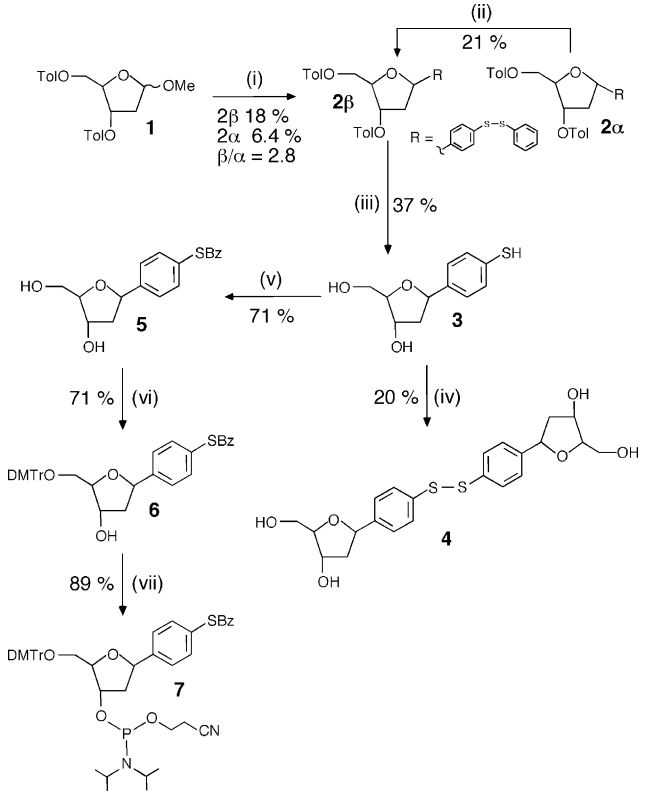


Scheme 1. Synthetic route of target mercapto-nucleoside **3** and **7**. (i) Diphenyldisulfide, $SnCl_4$ (2 equiv) in CH_2Cl_2 at −15 °C; (ii) 10%-TsOH, TFA in CH_2Cl_2 at 0 °C; (iii) $LiAlH_4$ in THF at 4 °C; (iv) 1%-H_2O_2 in MeOH at rt; (v) BzCl, DIPEA in THF at 4 °C; (vi) DMTrCl in pyridine at rt; (vii) DIPEA, 2-cyanoethyl-*N*,*N*′-diisopropyl-chlorophosphoramidite in CH_2Cl_2 at rt.

Lewis acid. Therefore, as the starting material for the synthesis of mercaptophenyl β-C-nucleoside **3**, the readily available diphenyldisulfide was used. The diphenyldisulfide was coupled with compound **1** in the presence of $SnCl_4$ (2 equiv). The β-anomer **2** was separated by column chromatography and recrystallization to obtain the production 18% yield (α-anomaer 6.4%).[9,10] It was found that **2**α was converted to **2**β in the presence of 10%-TsOH in TFA and CH_2Cl_2 (1:4) at 0 °C to afford the β isomer (**2**β 21%) and α-isomer (**2**α 11%).[11,12] The three protected groups, two toluoyl groups and a mercaptophenyl group, were removed in one step using $LiAlH_4$ (37%).[13] The stereochemistry at the anomeric position of **3** was identified by ^{1}H NMR, ^{1}H–^{1}H COSY and ^{1}H NOE experiments. The β anomer had a 1′-resonance which appeared as a nearly evenly spaced doublet of doublets (J = 5.4 and 10.7 Hz). Similar coupling constants have been reported for related β-C-nucleosides.[12] Irradiation of the 1′H gave a NOE on 4′H (5.1%) and 2′Hα (3.0%). Similarly irradiation on 2′Hβ gave a NOE on 3′H (7.1%), and irradiation on 2′Hα yielded enhancements on 1′H (9.1%).

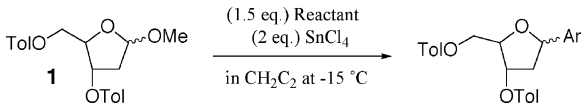
The regioselectivity of the electrophilic substitution reaction was identified by the multiplicity of the phenyl protons of compound **3** in the ^{1}H NMR spectrum. Two protons of the phenyl group of the base analogue showed two doublet signals; δ 7.23 and 7.25 ppm. The coupling constant (J) values were 12.2 and 11.8 Hz, respectively, and both signals were coupled. Thus the phenyl group at the position *para* to the **S**–**S** moiety of the diphenyldisulfide attacks at the 1′β position on the ribose, so that the thiol group is located in the *p*-position towards the C-nucleoside **3**. The mercaptophenyl β-C-nucleoside **3** was further S-benzoylated (71%), 5′-O-dimethoxytritylated (71%) and 3′-O-phosphitylated (89%) to give 2-cyanoethyl phosphoramidite **7**.[14] Having obtained compound **7**, the oligonucleotides containing the mercaptophenyl β-C-nucleoside were readily prepared by standard automated DNA synthetic methods. The *S*-benzoyl group was cleaved by a 27% ammonia solution at 55 °C for 2 h with the cleavage of oligonucleotide from the solid phase.

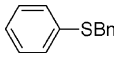
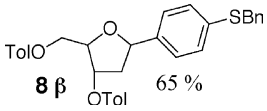
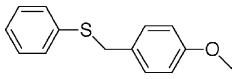
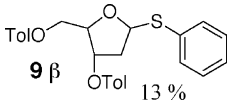
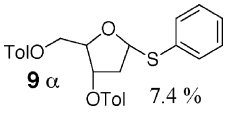
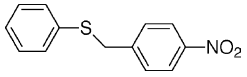
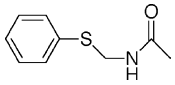
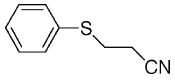
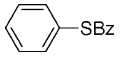
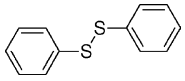
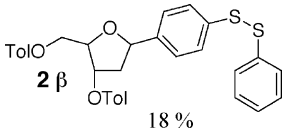
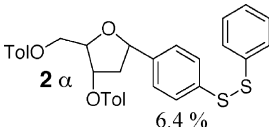
Nucleoside **3** was allowed to dimerize (in 1%-H_2O_2, MeOH) and then analyzed by TLC. The R_f value (0.14) of the dimer **4** is lower than the R_f value (0.34) of monomer **3** ($CHCl_3$–MeOH 4:1). After 30 min, the spot corresponding to monomer **3** disappeared on the TLC, and solvent was then removed under reduced pressure. The crude product was chromatographed and recrystallized from H_2O to afford **4**.[15] The ^{1}H NMR spectra support the formation of a disulfide dimer by the downfield shift observed for the phenyl protons (two doublets) of the base analogue in the dimer **4** (δ 0.13, 0.20 ppm, respectively, compared with those of the monomer). The chemical shifts of the phenyl protons can be rationalized in terms of expanding the π-conjugation from the analogue base unit to the disulfide bonding. The electron impact (EI) mass spectrum also provided clear evidence for the formation of dimer **4** by oxidation (m/z 450).

We tested the stability and pairing ability of mercaptonucleoside **3** (**S**) in the non-self-complementary 15 mer duplexes by thermal denaturation (Fig. 2).[16] The thermal denaturation experiments of the corresponding duplex were determined by UV-melting curve analysis and the corresponding T_m-data are summarized in Table 2.[17] In the presence of mercaptoethanol, the melting temperature of duplex **III**·**IV** (33 °C) was similar to that of the mismatch duplex **I**·**IV** (32 °C). Thus, under reducing conditions, the presence of the two base analogues in **III**·**IV** destabilizes the duplex structure to approximately the same extent as a mismatch base pair. The presence of mercaptoethanol had little influence on the melting temperature of duplexes **I**·**IV** and **I**·**VI**.

In the absence of a reducing reagent, the melting temperature of the duplex **III**·**IV** was 73 °C. The elevated stabilization of the duplex in the absence of a reducing reagent was significantly greater than for the natural duplex **I**·**II** (ΔT_m 29 °C). This result suggests that the matching **S**–**S** base pair was formed by air oxidation. The melting temperature of duplex **III**·**IV** was higher than duplex **I**·**II** since the disulfide base pair interacts more strongly (i.e. covalently) in comparison to the natural A–T base pair (i.e. hydrogen bonding). Duplex **V**·**VI** contains each **S** nucleoside at the penultimate base

Table 1. Coupling reaction of the compound **1** and the protected thiophenol by Friedel–Crafts alkylation



Reactant	Products/%	
	β-form	α-form
	 **8 β** 65 %	Trace
	 **9 β** 13 %	 **9 α** 7.4 %
	No reaction	
	No reaction	
	No reaction	
	No reaction	
	 **2 β** 18 %	 **2 α** 6.4 %

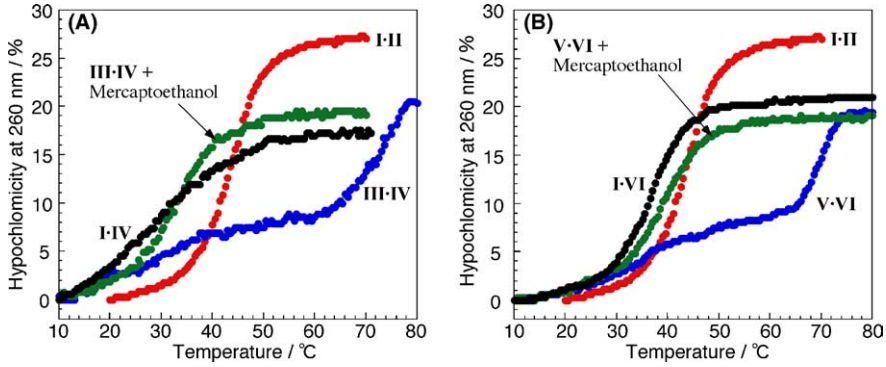


Figure 2. Melting curves for duplexes induced by the formation of disulfide bonding. (A) **S** nucleoside at the center position. (B) **S** nucleoside at the penultimate position. See conditions in Table 2.

position. This duplex also displayed a high melting temperature in the absence of mercaptoethanol (70 °C). The mismatch sequence **I·VI** had a similar T_m to **V·VI** in the presence mercaptoethanol (37, 39 °C, respectively).

In summary, we have demonstrated a synthetic route to a novel mercapto C-nucleoside and characterized a disulfide base pair incorporated into an oligonucleotide. The disulfide base pair is formed inside the duplex and significantly increases the thermal stability.

Table 2. Sequence information and effects of T_m of DNA including mercapto-nucleoside **3** (**S**)

DNA	Sequences of duplex	Additive	T_m/°C
I	5′-CACATTAATGTTGTA	a	44
II	3′-GTGTAATTACAACAT	b	43
III	5′-CACATTA**S**TGTTGTA	a	73
IV	3′-GTGTAAT**S**ACAACAT	b	33
I	5′-CACATTAATGTTGTA	a	32
IV	3′-GTGTAAT**S**ACAACAT	b	32
V	5′-C**S**CATTAATGTTGTA	a	70
VI	3′-G**S**GTAATTACAACAT	b	39
I	5′-CACATTAATGTTGTA	a	37
VI	3′-G**S**GTAATTACAACAT	b	38

[Mercaptoethanol]: a = 0 μM, b = 75 μM. [DNA] = 15 μM/base pair in 10 mM Na–phosphate buffer, 100 mM NaCl, pH 7.0, 1 °C/min.

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9. Selected data for compound **2**. **2**β: ^{1}H NMR ($CDCl_3$): δ 2.20 (1H, m), 2.39 (3H, s), 2.43 (3H, s), 2.49 (1H, J = 9.3, 14.1 Hz, dd), 4.52 (1H, m), 4.63 (2H, m), 5.21 (1H, J = 5.1, 11.2 Hz, dd), 5.59 (1H, J = 6.4 Hz, d), 7.27 (9H, m), 7.46 (4H, m), 7.91 (2H, J = 8.3 Hz, d), 7.97 (2H, J = 8.3 Hz, d). ^{13}C NMR ($CDCl_3$): δ 21.7, 21.7, 41.7, 64.7, 80.3, 83.0, 126.7, 126.9, 127.0, 127.2, 127.5, 127.6, 127.7, 127.8, 129.2, 129.5, 129.7, 129.7, 136.6, 136.9, 143.9, 144.2, 166.1, 166.4; FABMS m/e 571 $(M+H)^+$; Anal. Calcd for $C_{33}H_{30}O_5S_2$: C, 69.45; H, 5.30; N, 0.00; S, 11.24. Found: C, 69.45; H, 5.26; N, 0.00; S, 11.18. **2**α: ^{1}H NMR ($CDCl_3$): δ 2.27 (1H, m), 2.39 (3H, s), 2.40 (3H, s), 2.91 (1H, m), 4.55 (2H, m), 4.66 (1H, m), 5.34 (1H, J = 6.6 Hz, t), 5.58 (1H, m), 7.24–8.00 (17H, m). ^{13}C NMR ($CDCl_3$): δ 21.6, 40.2, 64.4, 76.1, 79.6, 82.1, 126.1, 126.4, 126.8, 126.9, 127.3, 127.5, 128.7, 128.8, 128.8, 129.3, 129.4, 135.7, 136.7, 141.5, 143.5, 143.6, 165.6, 165.9; FABMS m/e 571 $(M+H)^+$.
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13. $LiAlH_4$ (0.524 g, 13.8 mmol) was carefully added to a solution of the bis-toluoylester **2** (1.58 g, 2.76 mmol) in dry THF (50 mL). The reaction mixture was stirred at 4 °C for 1 h and then quenched by a 1 N H_2SO_4 aqueous solution (3 mL). The residue was poured into 50 mL of 1 N HCl, and then extracted with 50 mL of CH_2Cl_2 (eight times), dried over anhydrous $MgSO_4$, and evaporated. The crude product was chromatographed with $CHCl_3$–MeOH (9:1) and then recrystallized from MeOH to afford the mercaptophenyl nucleoside (**3**) as colorless needles (0.231 g, 37%). ^{1}H NMR (CD_3OD): δ 1.90 (1H, m), 2.15 (1H, J = 2.4, 5.1, 5.1 Hz, ddd), 3.65 (2H, m), 3.91 (1H, J = 3.9, 10.7 Hz, td), 4.29 (1H, br), 5.05 (1H, J = 5.4, 10.7 Hz, dd), 7.23 (1H, J = 12.2 Hz, d), 7.25 (1H, J = 11.8 Hz, d); ^{13}C NMR (CD_3OD): δ 44.9, 64.1, 74.5, 81.3, 89.2, 128.0, 129.9, 130.5 132.2, 140.4; EIMS m/e 226 $[M]^+$; Anal. Calcd for $C_{11}H_{14}O_3S$: C, 58.38; H, 6.24; N, 0.00; S, 14.17. Found: C, 58.25; H, 6.18; N, 0.00; S, 14.18. ε_{260}: 7080 cm^{-1} M^{-1} in 100 mM NaCl and 10 mM Na-phosphate, pH 7.0.
14. Compound **6** (270 mg, 0.426 mmol) was dissolved in 3 mL of dry CH_2Cl_2 and purged with Ar for 2 min. To the stirred solution was added *N*,*N*′-diisopropylethylamine (110 mg, 0.852 mmol) and 2-cyanoethyl-*N*,*N*′-diisopropylchlorophosphoramidite (121 mg, 0.511 mmol). The reaction mixture was stirred under Ar while protected from light for 1 h. The reaction mixture was added to 30 mL of CH_2Cl_2 and washed twice with 30 mL of H_2O. The organic layer was dried over $MgSO_4$, filtered and evaporated. The crude compound was chromatographed with 1% trimethylamine in hexane–EtOAc (3:1) to obtain **7** as a colorless solid (276 mg, 89%). ^{1}H NMR ($CDCl_3$): δ 1.12–1.31 (14H, m), 1.65 (1H, br), 2.04 (1H, m), 2.38 (1H, m), 2.46 (1H, J = 6.3 Hz, t), 2.62 (1H, J = 6.5 Hz, t), 3.31 (2H, m), 3.78 (6H, s), 4.27 (1H, br), 4.53 (1H, br), 5.22 (1H, J = 4.6, 9.6 Hz, dd), 6.84 (4H, J = 5.0, 9.0 Hz, dd), 7.19–7.66 (16H, m), 8.04 (2H, J = 8.3 Hz, d); FABMS m/e 833 $[M+H]^+$; HRMS (FAB) calcd for $C_{48}H_{54}N_2O_7PS$ 833.3389, found 833,3376.
15. Selected data for dimer **4**. ^{1}H NMR (CD_3OD): δ 1.90 (1H, m), 2.17 (1H, J = 5.4, 13.2 Hz, dd), 3.64 (2H, J = 4.9 Hz, d), 3.92 (1H, br), 4.29 (1H, br), 5.09 (1H, J = 5.4, 10.7 Hz, dd), 7.36 (1H, J = 8.3 Hz, d), 7.45 (1H, J = 7.8 Hz, d); EIMS m/e 450 $[M]^+$; HRMS (FAB) calcd for $C_{22}H_{27}O_6S_2$ 451.1249, found 451.1251.
16. All T_m values of the duplexes (15 μM/base pair) were measured in 100 mM NaCl and 10 mM Na-phosphate, pH 7.0. The absorbances of the duplexes were monitored at 260 nm from 10 to 90 °C using a heating rate of 1 °C/min.
17. Maldi-Tof MS data for oligonucleotides and duplexes. **III**: $[(M-H)^-]$ calcd 4537.8, found 4536.4. **IV**: $[(M-H)^-]$ calcd 4555.8, found 4552.8. **III**·**IV**: $[(M-H)^-]$ calcd 9092.6, found 9093.6. **V**: $[(M-H)^-]$ calcd 4537.8, found 4537.2. **VI**: $[(M-H)^-]$ calcd 4555.8, found 4555.1. **V**·**VI**: $[(M-H)^-]$ calcd 9092.6, found 9091.2.