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Synthesis and ESR studies of nitronyl nitroxide-tethered oligodeoxynucleotides

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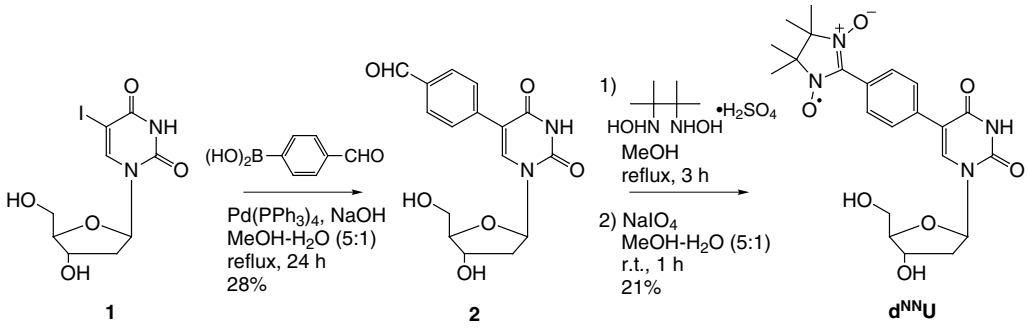
Abstract—We report on the development of a nucleoside labeled with a nitronyl nitroxide group, ^{NN}U, and the synthesis of oligodeoxynucleotides containing ^{NN}U. The spin signals of ^{NN}U-containing oligodeoxynucleotides varied with the degree of hybridization of the complementary strand and the distance between nitronyl nitroxide spins.

Recent progress in spin labeling techniques makes ESR a promising technique for investigating many biological systems. In particular, site-directed spin labeling (SDSL) is emerging as a powerful technique for exploring the structure and dynamics of biomolecules.[1] The SDSL technique of the labeled biomolecules provides much information on the dynamics of the nitroxide group, the collision rate between a nitroxide group and a freely diffusing paramagnetic agent, and the distance between a nitroxide group and another paramagnetic species fixed in the biomolecular structure. Many nucleosides labeled with nitroxide radicals, such as 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO)[2] and 2,2,5,5-tetramethylpyrrolidine 1-oxyl,[3] have been developed to analyze DNA structures and DNA–protein interactions. However, there is still no precedent for nucleosides labeled with a nitronyl nitroxide group, which are known to be a key structure of the nitric oxide (NO) scavenger, 2-phenyl-4,4,5,5-tetramethylimidazoline-3-oxide 1-oxyl (PTIO).[4] When nitronyl nitroxides are incorporated into selected DNA sites, then they are expected to be a new powerful SDSL method, as well as being an NO trap. Here, we report on the development of a novel nucleoside labeled with a nitronyl nitroxide group, ^{NN}U, and the synthesis of oligodeoxynucleotides (ODNs) containing ^{NN}U. The change in



Scheme 1.

Keywords: Pyrene; Oligodeoxynucleotide; Fluorescence; Quadruplex.
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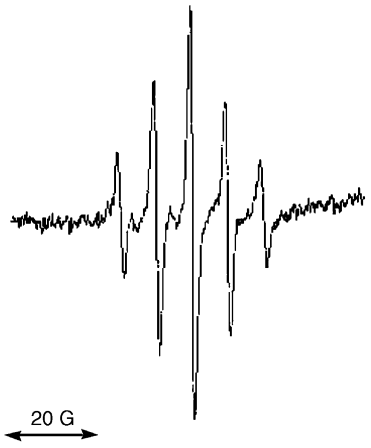
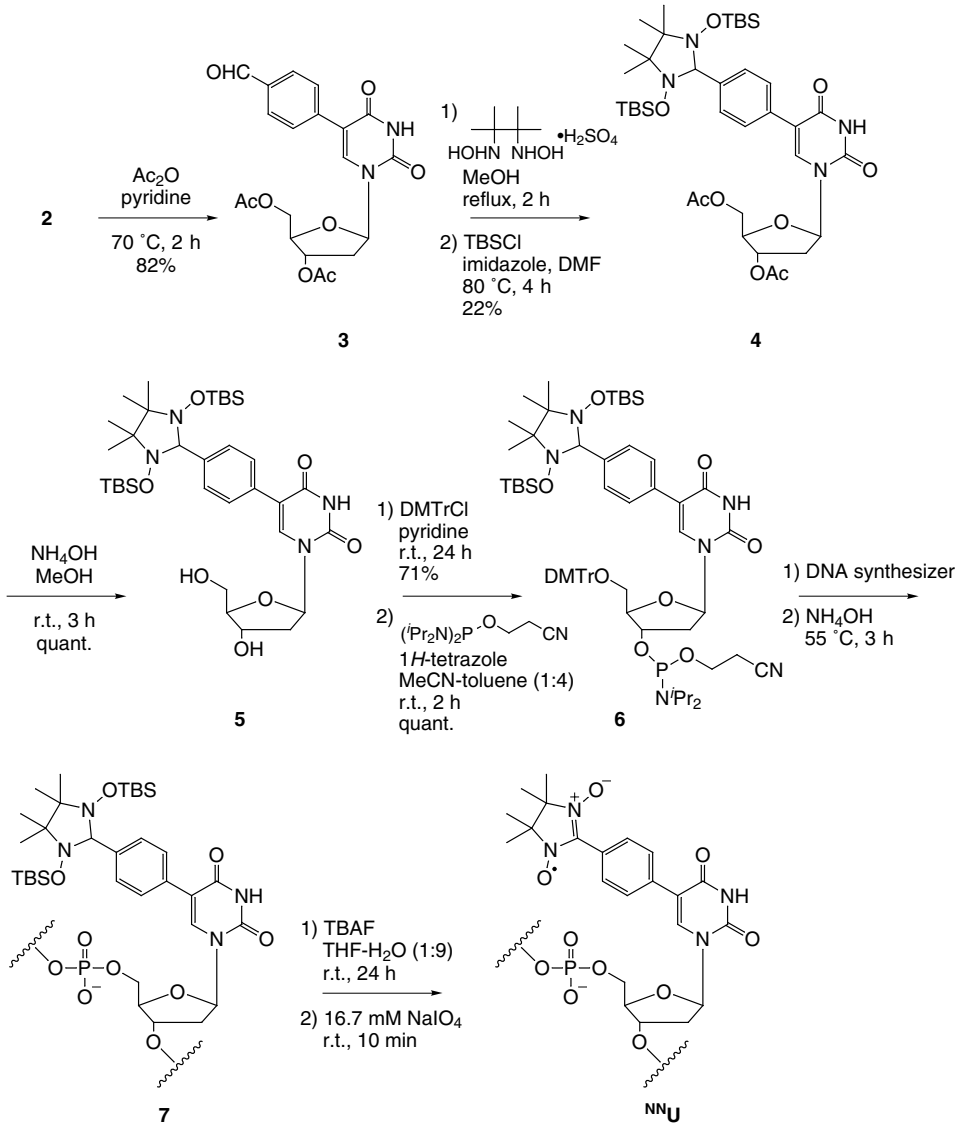


Figure 1. ESR spectrum of $d^{NN}U$. A solution of $d^{NN}U$ (100 μM) in 50 mM sodium phosphate and 0.1 M sodium chloride (pH 7.0) was examined at 27 °C. The ESR spectrum was obtained using a Bruker EMX spectrometer, in the X-band, with a modulation amplitude of 2.0 G, a time constant of 20 ms, and a microwave power of 1 mW. The sample holder was a glass capillary tube. g = 2.0065, and a_N = 8.11 G.

the spin signals of ^{NN}U-containing ODNs caused by the hybridization with the complementary strand and the distance between adjacent nitronyl nitroxide spins have been investigated.

We synthesized ^{NN}U from 5-iodo-2′-deoxyuridine **1** (Scheme 1). The Suzuki–Miyaura coupling of **1** with 4-formylphenylboronic acid yielded the aldehyde-possessing nucleoside **2**. The aldehyde **2** was then treated with 2,3-bis(hydroxyamino)-2,3-dimethylbutane, and subsequently oxidized with sodium periodate to give the ^{NN}U deoxynucleoside ($d^{NN}U$).[5]

The ESR spectrum of $d^{NN}U$ was measured, and is shown in Figure 1. The ESR spectrum was recorded at 27 °C on a Bruker EMX spectrometer operating in the X-band using a glass capillary tube. The ESR data shows that the ESR spectrum of $d^{NN}U$ exhibits a characteristic five-line signal, with a typical intensity ratio of 1:2:3:2:1 (a_N = 8.11 G), and is close to that of PTIO (a_N = 8.2 G).[4a]



Scheme 2.

Table 1. ^{NN}U-containing ODN duplexes used in this study

	Sequences
ODN1/ODN1′	5′-d(CGCAAT^{NN}UTAACGC)-3′ 3′-d(GCGTTA A ATTGCG)-5′
ODN0/ODN1′	5′-d(CGCAATATAACGC)-3′ 3′-d(GCGTTAAATTGCG)-5′
ODN2(0)/ODN2(0)′	5′-d(CGCAAT^{NN}U^{NN}UAACGC)-3′ 3′-d(GCGTTA A A TTGCG)-5′
ODN2(1)/ODN2(1)′	5′-d(CGCAA^{NN}UA^{NN}UAACGC)-3′ 3′-d(GCGTT A T A TTGCG)-5′
ODN2(2)/ODN2(2)′	5′-d(CGCAA^{NN}UAC^{NN}UAACGC)-3′ 3′-d(GCGTT A TG A TTGCG)-5′
ODN2(3)/ODN2(3)′	5′-d(CGCAA^{NN}UACG^{NN}UAACGC)-3′ 3′-d(GCGTT A TGC A TTGCG)-5′
ODN2(4)/ODN2(4)′	5′-d(CGCAA^{NN}UACGT^{NN}UAACGC)-3′ 3′-d(GCGTT A TGCA A TTGCG)-5′
ODN2(8)/ODN2(8)′	5′-d(CGCAA^{NN}UACGTACGT^{NN}UAACGC)-3′ 3′-d(GCGTT A TGCATGCA A TTGCG)-5′

Direct incorporation of d^{NN}U into the oligodeoxynucleotides (ODN) using the conventional phosphoramidite method was found to be very difficult, because the preparation of the corresponding phosphoramidite was prohibited by the high oxidizing ability of the nitronyl nitroxide group. Therefore, we chose a synthetic route in which the oxidation to nitronyl nitroxide was carried out after the DNA synthesis step. The synthetic route to obtain ^{NN}U-containing ODN is shown in Scheme 2. The two hydroxy groups of the aldehyde **2** were protected using acetyl groups. The aldehyde **3** was treated with 2,3-bis(hydroxyamino)-2,3-dimethylbutane, and the two hydroxylamine groups of the resulting compound were immediately protected using *tert*-butyldimethylsilyl groups, to give compound **4**. The hydrolysis of **4** afforded the ^{NN}U precursor **5**,[6] which was protected by a 4,4′-dimethoxytrityl group, and quantitatively converted to cyanoethyl phosphoramidite **6**. The amidite **6** was employed in a conventional solid phase synthesis of the ODNs. After ODN synthesis, deprotection was conducted using aqueous ammonia at 55 °C for 3 h to give the ODN **7**. The silyl group of **7** was removed using tetrabutylammonium fluoride, and then oxidized with sodium periodate to quantitatively yield ^{NN}U-containing ODNs. The crude ^{NN}U-containing ODNs were then purified using reversed phase HPLC. The composition of the ODNs was determined using MALDI-TOF mass spectrometry.[7] A summary of the ODNs synthesized is shown in Table 1.

Before the ESR studies on the ^{NN}U-containing ODNs were carried out, we examined the structure and structural stability of the 13-mer ODN duplex ODN1/ODN1′ containing an ^{NN}U base using CD spectroscopy and melting temperature (T_m) measurements. In the CD spectrum of ODN1/ODN1′, a negative peak at 253 nm and a positive peak at 277 nm were observed. This spectrum was close to that observed for the natural duplex ODN0/ODN1′, indicating that the ^{NN}U-containing ODN maintains a B-DNA duplex. In the T_m measurements of ODN1/ODN1′, a sigmoidal curve at the A_{254} change was observed, and the value of T_m was determined to be 46.0 °C from the first derivative of the sigmoidal curve. This value of T_m is 4.8 °C lower than that observed for ODN0/ODN1′ (50.8 °C).

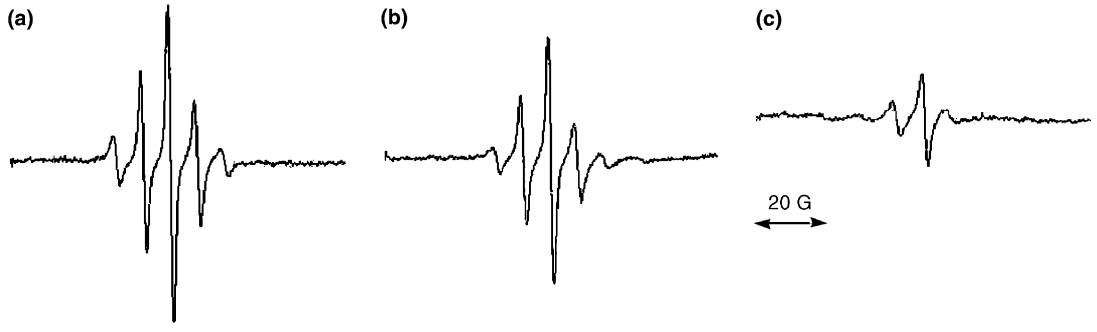


Figure 2. ESR spectra of the ^{NN}U-containing ODNs. A solution of the duplexes (100 μM) in 50 mM sodium phosphate and 0.1 M sodium chloride (pH 7.0) was examined at 27 °C. The ESR spectrum was measured using a Bruker EMX spectrometer, in the X-band, with a modulation amplitude of 2.0 G, a time constant of 20 ms, and a microwave power of 1 mW. The sample holder was a glass capillary tube. (a) Single-stranded ODN1; (b) ODN1/ODN1′; and (c) ODN2(0)/ODN2(0)′.

Next, an ESR spectral analysis of the single-stranded ODN1 and the duplex ODN1/ODN1′ was carried out, and the results are shown in Figure 2a and b. The ESR spectrum of the single-stranded ODN1 exhibited the characteristic five-line pattern, with a typical hyperfine coupling constant of a_N = 8.21 G, whereas the value of a_N for the spin probe in ODN1/ODN1′ (8.25 G) was slightly higher than that of the single-stranded state. This result reflects the increased micropolarity at the binding site of the nitronyl nitroxide group in the major groove of the duplex. In addition, the duplex formation caused the line broadening. This line broadening is attributed to the motional restriction of the ^{NN}U spin label within duplex DNA.[2f,8]

Using our synthetic protocol for an ^{NN}U-containing ODN, we prepared a doubly ^{NN}U-labeled ODN ODN2(0), which contained an ^{NN}U^{NN}U sequence. In the ESR spectrum of ODN2(0) hybridized with a complementary strand ODN2(0)′, the signal intensity was observed to be much lower than that of ODN1/ODN1′ in spite of this duplex having two spin labels (Fig. 2c). The remarkable line broadening of ODN2(0)/ODN2(0)′ is assumed to originate from the spin–spin interaction and motional restriction between the labeled side chains due to the close proximity of the spin labels within the duplex.[9,10]

We also prepared other doubly ^{NN}U-labeled ODNs with different distances between two nitronyl nitroxide groups (Table 1), and measured the resulting ESR signals. The ESR signal intensities observed for each duplex are shown in Figure 3. As the distance between the two ^{NN}U sites increased, the signal intensities gradually increased. In particular, when the distance between the spins was separated over two base pairs, then the signal intensity increased markedly. When the distance between the spins was separated over four base pairs, then the interaction between two ^{NN}U spin labels was eliminated. The change in ESR signal intensities of doubly ^{NN}U-labeled ODNs will be a useful tool for monitoring spatial proximity at room temperature.

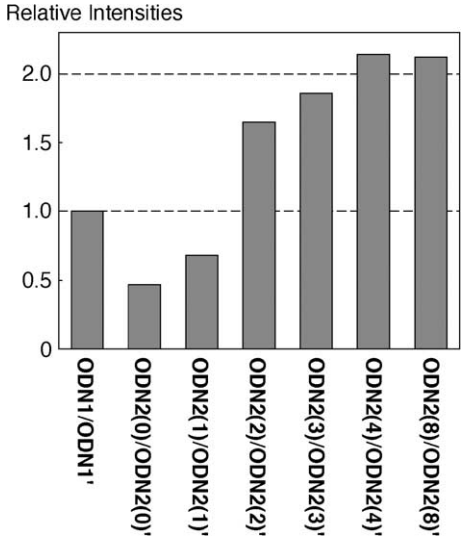


Figure 3. Comparison of the signal intensities obtained from the ESR spectra of ^{NN}U-containing duplexes. A solution of the duplexes (100 μM) in 50 mM sodium phosphate and 0.1 M sodium chloride (pH 7.0) was examined at 27 °C. The ESR spectrum was performed using a Bruker EMX spectrometer, in the X-band, with a modulation amplitude of 2.0 G, a time constant of 20 ms, and a microwave power of 1 mW. The sample holder was a glass capillary tube. The ESR peak intensity of ODN1/ODN1′ was normalized to 1.0.

In conclusion, we have designed a nucleoside labeled with a nitronyl nitroxide group, d^{NN}U, and have synthesized ODNs containing one or two ^{NN}Us groups. The spin signals from the ^{NN}U-containing ODNs varied because of the degree of hybridization of the complementary strand and the distance between the nitronyl nitroxide spins. Therefore, ^{NN}U spin labels will be useful as a powerful SDSL tool for exploring the structure and dynamics of nucleic acids.

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5. Spectroscopic data for $d^{NN}U$: blue foam; R_f 0.27 ($CHCl_3$–MeOH = 20:1); λ_{max} [50 mM sodium phosphate, pH = 7.0, 0.1 M NaCl/nm (ε)] 293.4 (18180), 570.0 (750); FABMS, *m/e* 461 $[(M+2H)^+]$; HRMS calcd for $C_{22}H_{29}N_4O_7$ $[(M+2H)^+]$ 461.2036, found 461.2036; ESR (50 mM sodium phosphate, pH = 7.0, 0.1 M NaCl) $a_{N(1)} = a_{N(3)} = 8.11$ G.
6. Spectroscopic data for **5**: white foam; ^1H NMR ($CDCl_3$, 400 MHz) δ 11.56 (s, 1H), 7.64 (s, 1H), 7.43 (d, $J = 7.7$ Hz, 2H), 7.37 (d, $J = 7.7$ Hz, 2H), 7.28–7.16 (m, 9H), 6.83 (d, $J = 8.7$ Hz, 4H), 6.18 (t, $J = 6.6$ Hz, 1H), 5.34 (d, $J = 4.2$ Hz, 1H), 4.48 (s, 1H), 4.22–4.12 (m, 1H), 3.92–3.87 (m, 1H), 3.74–3.64 (m, 8H), 2.31 (ddd, $J = 6.6$, 6.9, 13.3 Hz, 1H), 2.17 (ddd, $J = 3.9$, 6.6, 13.3 Hz, 1H), 1.11 (s, 12H), 0.74 (s, 9H), 0.73 (s, 9H), −0.09 (s, 3H), −0.12 (s, 3H); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 170.3, 161.9, 149.7, 144.7, 139.4, 136.8, 135.5, 135.4, 132.4, 129.9, 129.6, 129.6, 127.7, 127.6, 126.5, 113.1, 113.0, 93.4, 85.6, 85.5, 84.9, 70.5, 67.5, 67.5, 63.7, 54.9, 39.5, 26.0, 24.1, 17.5, 17.0, −4.0, −5.2; FABMS, *m/e* 993 $[(M+H)^+]$; HRMS calcd for $C_{55}H_{77}N_4O_9Si_2$ $[(M+H)^+]$ 993.5229, found 993.5220.
7. MALDI-TOF mass, ODN1, $[M-OH]^-$ calcd 4118.82, found 4118.59; ODN2(0), $[M-O_2H]^-$, calcd 4320.06, found 4320.19; ODN2(1), $[M-O_2H]^-$, calcd 4329.08, found 4329.34; ODN2(2), $[M-O_2H]^-$, calcd 4618.26, found 4617.80; ODN2(3), $[M-O_2H]^-$, calcd 4947.46, found 4946.91; ODN2(4), $[M-O_2H]^-$, calcd 5251.66, found 5251.83; ODN2(8), $[M-O_2H]^-$, calcd 6487.45, found 6487.82.
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