JOC The Journal of Organic Chemistry

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4,4-Difluoro-4-bora-3a,4a-diaza-*s*-indacene as a Bright Fluorescent Label for DNA

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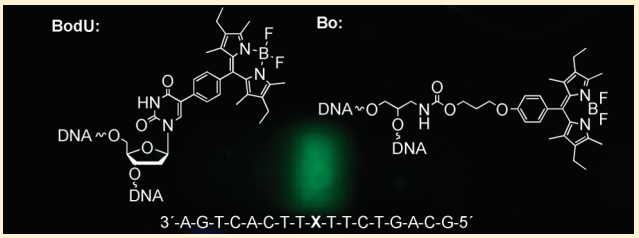
Supporting Information

ABSTRACT: 4,4-Difluoro-4-bora-3a,4a-diaza-*s*-indacene (BODIPY) as a fluorescent label can be incorporated into DNA by two conceptually different ways: the non-nucleosidic DNA base surrogate **Bo** exhibits high brightness but no preferential base-pairing properties, whereas the BODIPY-modified uridine **BodU** has reduced quantum yields but shows preferred Watson−Crick base pairing with adenine.



4,4-Difluoro-4-bora-3a,4a-diaza-*s*-indacene (BODIPY) represents an excellent fluorescent label in chemical biology.[1] The BODIPYs combine several advantages, mainly nanosecond lifetimes, fluorescence with sharp and narrow peaks, high quantum yields,[2,3] reasonable stability under physiological conditions,[4] and the possibility for synthetic tuning.[5−9] BODIPYs are mainly used for protein labeling and attached using a linker with a reactive anchor group at the end.[10,11] In a similar approach, DNA can be labeled with BODIPY at the 5′-end; however, terminal labeling limits the versatile applicability.[10,12] Two years ago we presented the synthesis and optical properties of a variety of BODIPY-modified 2′-deoxyuridines.[13] Moreover, we could incorporate one of these labels into oligonucleotides and show that the DNA polymerase-catalyzed nucleotide incorporation opposite to BODIPY-modified 2′-deoxyuridine (**BodU**) in templates follows Watson−Crick selectivity and gets bypassed for further elongation.[14] In a synthetically completely different approach, chromophores can be incorporated as DNA base surrogates to promote intercalation of the dye.[15,16] Herein, we compare both synthetic approaches, DNA base modifications and DNA base substitutions, for the BODIPY dye. We present the optical properties of **BodU**-modified DNA and compare it with BODIPY as DNA base surrogate (**Bo**). For the latter modification the synthetic procedure is reported as well.

The synthesis of **BodU** and its synthetic incorporation into oligonucleotides using automated phosphoramidite chemistry followed our published procedures.[13,14] For the **Bo**-type modification (*S*)-3-amino-1,2-propanediol was used as an acyclic linker and substitute for the 2′-deoxyriboside between the phosphodiester bridges. This substitute provides high chemical stability and conformational flexibility for the chromophore to intercalate. The synthesis of the corresponding **Bo**-type phosphoramidite **1** started with the assembly of the chromophore (Scheme 1). The dipyrromethane was formed from the aldehyde **2** and 3-ethyl-2,4-dimethylpyrrole (**3**) by condensation under acidic conditions. Subsequently, the intermediate compound was oxidized to the dipyrromethene with *p*-chloranil and treated with $BF_3 \cdot OEt_2$, yielding the BODIPY derivative **4** (64%). The chromophore **4** was tethered to the DMT-protected (*S*)-3-amino-1,2-propanediol **5** via a carbamate function, which facilitated the synthesis of the corresponding DNA building block, because it is not necessary to protect this linker functionality. Treatment of **4** and **5** with 1,1′-carbonyldiimidazole gave **6** in good yield (79%). The synthesis of the phosphoramidite **1** as the **Bo**-type DNA building block and its incorporation into oligonucleotides were accomplished by standard procedures.[15]

For both types of DNA modifications, **BodU** and **Bo**, two representative modified oligonucleotides, **DNA1**/**DNA2** and **DNA3**/**DNA4**, were prepared (Chart 1). The corresponding DNA double strands expose the chromophore to two different variations in the neighborhood: (i) the dye was placed either in an A-T environment (**DNA1** and **DNA3**) or next to G-C base pairs (**DNA2** and **DNA4**) and (ii) within each duplex set the base opposite to the chromophore site was varied (e.g., **DNA1Y** with Y = C, T, A, G).

To study the influence of the **BodU** and **Bo** modifications on the DNA double helix, we determined the thermal stability of all modified duplexes by measuring the melting temperatures (T_m) at 260 nm and comparing them with published values of the corresponding unmodified duplexes (Table 1).[16] For the **Bo**-type modification, three major results can be drawn from these data: (i) the melting temperatures of all modified duplexes lie in the fairly narrow ranges of 55−56 °C (**DNA1Y**) and 62−64 °C (**DNA2Y**), (ii) all modified duplexes show a strong destabilization by 4.7−6.8 °C compared to the unmodified duplexes,[16] and (iii) **Bo** as a DNA base substitution does not exhibit any preferred pairing with the "counterbase". On the other hand, the **BodU** modification induces also strong destabilization, between 3.5 and 12.5 °C; however, the destabilizing effect is

Received: December 20, 2010
Published: March 07, 2011

Scheme 1. Synthesis of the Bo-phosphoramidite 1

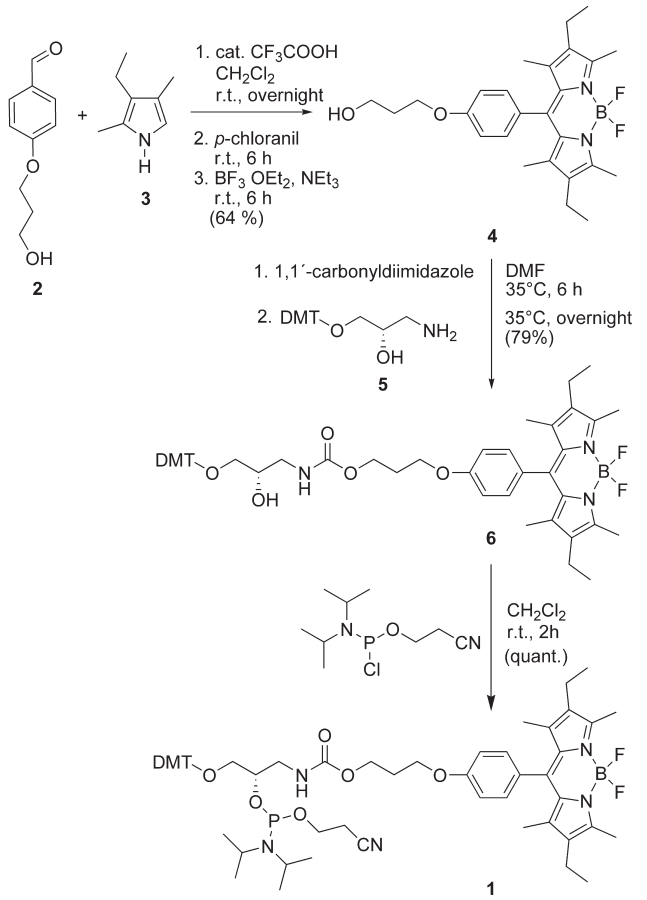
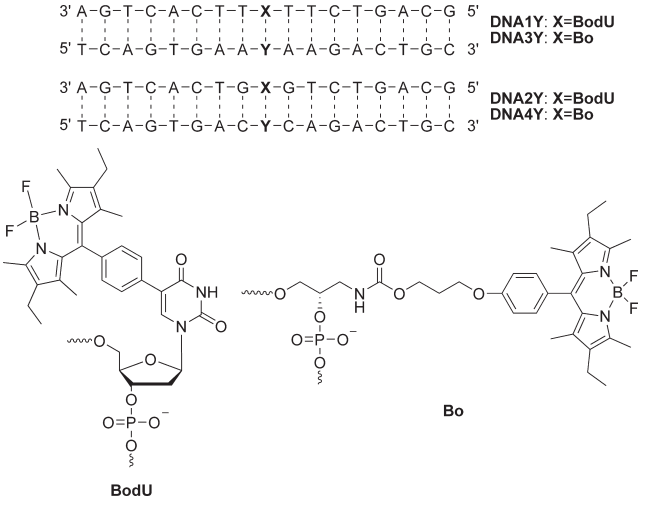


Chart 1. Structure of BodU and Bo Modifications in Oligonucleotides and Sequences of DNA1Y−DNA4Y (Y = C, T, A, G)



the lowest with A as the complementary base (−5.0 °C in **DNA1A** and −3.5 °C in **DNA2A**). This result indicates that the 2′-deoxyuridine of **BodU** maintains its preferred binding to adenine despite the BODIPY modification. This supports our results from primer extension experiments previously published.[14]

The quantum yields of the fluorescence of both types of BODIPY modifications in single-stranded oligonucleotides are remarkably high and lie within the ranges of 39−46% (**BodU**) and 80−90% (**Bo**). Such high quantum yields are typical for this chromophore and make it, as mentioned in the introductory part, an excellent fluorescent label. The shapes of the fluorescence spectra are also very similar. The situation changes dramatically if the **BodU**-modified single strands **DNA1** and **DNA2** are hybridized with the corresponding counterstrands (Figure 1). As a result, the fluorescence is quenched significantly and quantum yields between <0.01 and 0.06 are obtained in the double strands **DNA1Y** and **DNA2Y**. The lowest quantum yields are obtained with the duplexes that exhibit the highest T_m values, which could give a hint as to what causes the dramatic fluorescence quenching. In the case of a Watson−Crick-type base pairing of **BodU** with adenine the BODIPY-phenyl moiety is possibly twisted much more significantly due to steric interactions with the DNA groove. The charge transfer contribution in the system is thereby enhanced and the emission is reduced (similar to a TICT state).[17] In DNA duplexes with the wrong counterbases (C, T, and G) some conformational flexibility allows regaining some fluorescence, and the quantum yields are slightly higher. The UV/vis absorption spectra support this interpretation. The spectra of the single strands **DNA1** and **DNA2** show the typical BODIPY sharp peak at 527 nm, whereas the absorption of the corresponding duplexes exhibits significantly flattened peaks, indicating a changed structural scenario (see Supporting Information).

In contrast to the **BodU**-modified double strands, the fluorescence intensities of the **Bo**-modified duplexes, **DNA3Y** and **DNA4Y**, are maintained much more efficiently (Figure 1). The quantum yields lie in the applicable range of 0.46−0.69. Since the **Bo** chromophore does not exhibit any preferred base in the complementary strand, the quantum yields are quite similar within each duplex set. A small amount of quenching is observed both in the single strand **DNA4** and in the duplexes **DNA4Y**, which is probably due to stacking interactions with the adjacent guanines. For biological applications, however, the brightness of a dye is much more meaningful than the quantum yield. Therefore, we calculated the brightness of the **Bo** label in DNA and compared it to the well-known fluorescein label, representatively for **DNA3**. The **Bo**-labeled oligonucleotides exhibit a brightness of about 55,000 M^{-1} cm^{-1} in the single strand and 44,000 M^{-1} cm^{-1} in the double strand **DNA3A**, whereas the corresponding fluorescein-labeled DNA shows only a brightness of approximately 20,000 M^{-1} cm^{-1} in the single strand and 18,000 M^{-1} cm^{-1} in the double strand. This is a remarkable result, since it shows that the BODIPY dye is a much brighter fluorescent label in DNA than the routinely applied fluorescein, at least in the given sequence.

In conclusion, we have shown that BODIPY as a fluorescent label can be incorporated into DNA by two conceptually different ways: the BODIPY-modified uridine **BodU** exhibits preferred Watson−Crick base pairing with adenine in the counterstrand but shows dramatically reduced quantum yields, whereas the non-nucleosidic DNA base surrogate **Bo** exhibits high quantum yields but no preferential base-pairing properties. Most importantly, the **Bo** label in **DNA3A** has significantly higher brightness compared to that of standard fluorescein, which makes the BODIPY label a useful tool for chemical bioanalytics. Further investigations may focus on

dx.doi.org/10.1021/jo102519k |J. Org. Chem. 2011, 76, 2301–2304

Table 1. Melting Temperatures (T_m), Quantum Yields (Φ), and Brightness (B) of DNA1Y−DNA4Y

	T_m (°C)[a]	ΔT_m (°C)[a,b]	Φ[c]	B[d]
		BodU		
DNA1C	50.0	−12.5	0.05	1280
DNA1T	50.0	−12.5	0.03	720
DNA1A	57.5	−5.0	<0.01	120
DNA1G	50.5	−12.0	0.01	250
DNA2C	60.5	−7.5	0.06	2350
DNA2T	59.3	−8.7	0.04	1650
DNA2A	64.5	−3.5	0.02	850
DNA2G	60.5	−7.5	0.03	1330
		Bo		
DNA3C	55.7	−6.8	0.69	36400
DNA3T	55.8	−6.7	0.65	35600
DNA3A	55.8	−6.7	0.69	38200
DNA3G	55.7	−6.8	0.68	38400
DNA4C	63.3	−4.7	0.47	22100
DNA4T	62.3	−5.7	0.49	22800
DNA4A	62.1	−5.9	0.47	22000
DNA4G	62.0	−6.0	0.46	23400

[a] Conditions: 2.5 μM DNA, λ 260 nm, 20−90 °C, interval 0.7 °C/min, 2.5 μM duplex in 10 mM Na−P_i buffer, 250 mM NaCl, pH 7.0. [b] In comparison to the unmodified references: T_m = 62.5 °C for **DNA1A**/**DNA3A** and T_m = 68.0 °C for **DNA2A**/**DNA4A**, each with T instead of **BodU** or **Bo**, respectively.[16] [c] For **DNA3** and **DNA3A** by photon counting; for all others indirectly by using **DNA3A** as a standard (λ_{ex} 510 nm). All quantum yields were also supported by measurements using fluorescein as a standard. [d] $B = \Phi \cdot \varepsilon$.

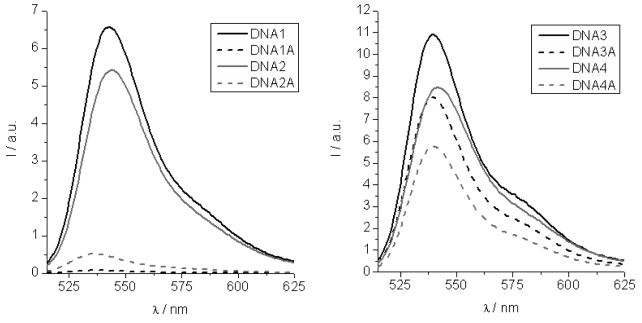


Figure 1. Fluorescence of **BodU**-modified **DNA1Y**/**DNA2Y** (left) and **Bo**-modified **DNA3Y**/**DNA4Y** (right). Conditions: 2.5 μM in Na−P_i buffer at pH 7, 250 mM NaCl, 20 °C, excitation at 510 nm. All other optical spectra are shown in the Supporting Information.

non-natural counterbases to the **Bo** unit, such as pyrene and perylene.

EXPERIMENTAL SECTION

Synthesis of 4. 4-(3-Hydroxypropoxy)benzaldehyde (**2**; 2.40 g, 13.2 mmol) was dissolved in absolute CH_2Cl_2 (120 mL) under argon. 3-Ethyl-2,4-dimethylpyrrole (**3**; 4.06 g, 33 mmol) was added, and the mixture was stirred at room temperature for 15 min. Trifluoroacetic acid (0.2 mL) was added, and the solution was stirred at room temperature overnight. *p*-Chloranil (3.25 g, 13.2 mmol) was added, and the mixture was stirred for another 6 h. Triethylamine (31.3 mL, 224 mmol) was added to the deep red solution. After 30 min $BF_3 \cdot OEt_2$ (31.5 mL, 251 mmol) was added and the mixture was stirred for another 6 h. The reaction mixture was extracted three times with H_2O (50 mL). The organic phase was dried over Na_2SO_4, and the solvent was removed. After purification by chromatography on silica gel (CH_2Cl_2/acetone 20/1) **4** was obtained as a red solid in 64% yield. ^{1}H NMR (CD_2Cl_2, 600 MHz): δ 7.18 (d, 2H, J = 8.7 Hz), 7.03 (d, 2H, J = 8.7 Hz), 4.17 (t, 2H, J = 6.1 Hz), 3.85 (t, 2H, J = 5.9 Hz), 2.49 (s, 6H), 2.33 (q, 4H, J = 7.6 Hz), 2.06 (tt, 2H, J_1 = 6.1 Hz, J_2 = 5.9 Hz), 1.36 (s, 6H), 0.99 (t, 6H, J = 7.6 Hz). ^{13}C NMR (CD_2Cl_2, 150 MHz): δ 159.9, 153.8, 141.0, 139.1, 133.2, 131.5, 129.9, 128.1, 115.4, 66.0, 60.3, 32.5, 17.3, 14.8, 12.6, 12.0. ^{19}F NMR (CD_2Cl_2, 300 MHz): δ −145.4 (d), −145.7 (d). HRMS (EI-MS): calcd for $C_{26}H_{33}BF_2N_2O_2$ [M^+] 454.2603, found 454.2610.

Synthesis of 6. To a solution of **4** (100 mg, 0.22 mmol) in absolute DMF (5 mL) was added 1,1′-carbonyldiimidazole (54.0 mg, 0.33 mmol), and the mixture was stirred for at 35 °C for 6 h under argon. DMT-protected (*S*)-3-amino-1,2-propanediol (**5**; 175 mg, 0.44 mmol) was added, and the solution was stirred at 35 °C overnight. The solvent was removed, and the product was purified by chromatography on silica gel (CH_2Cl_2/acetone 15/1), yielding 79% of **6** as a red foam. ^{1}H NMR (CD_2Cl_2, 400 MHz): δ 7.43 (m, 2H), 7.35−7.20 (m, 7H), 7.18 (d, 2H, J = 8.5 Hz)), 7.01 (d, 2H, J = 8.5 Hz), 6.85 (m, 4H), 5.03 (m, 1H), 4.24 (t, 2H, J = 6.3 Hz), 4.09 (t, 2H, J = 6.3 Hz), 3.85 (m, 1H), 3.78 (s, 6H), 3.37 (m, 1H), 3.20−3.10 (m, 3H), 2.49 (s, 6H), 2.32 (q, 4H, J = 7.7 Hz), 2.12 (tt, 2H, J_1 = 6.3 Hz, J_2 = 6.3 Hz), 1.36 (s, 6H), 0.99 (t, 6H, J = 7.7 Hz). ^{13}C NMR (CD_2Cl_2, 150 MHz): δ 159.8, 159.1, 157.5, 153.8, 145.3, 141.0, 139.1, 136.2, 133.2, 131.5, 130.4, 129.9, 128.4, 128.3, 128.2, 128.1, 127.2, 115.4, 113.5, 86.6, 70.7, 65.4, 65.1, 62.1, 56.6, 44.6, 29.4, 17.4, 14.8, 12.6, 12.0. ^{19}F NMR (CD_2Cl_2, 300 MHz): δ −145.4 (d), −145.7 (d). HRMS (EI-MS): calcd for $C_{51}H_{58}BF_2N_3O_7$ [M^+] 873.4336, found 873.4327.

Synthesis of 1. **6** (0.19 g, 0.21 mmol) was dissolved in absolute CH_2Cl_2 (3 mL) under argon. EtN(*i*Pr)$_2$ (73 μL, 0.42 mmol) and 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (85 μL, 0.38 mmol) were added, and the solution was stirred at room temperature for 2 h. The organic phase was washed with saturated aqueous $NaHCO_3$ and dried over Na_2SO_4. The solvent was removed, and the product was dried under vacuum, yielding **1** as a red solid. The product was used immediately for oligonucleotide synthesis. ^{31}P NMR ($CDCl_3$, 121 MHz): δ 150.1, 149.8.

Synthesis of DNA1−DNA4. Oligonucleotides were prepared on a synthesizer using standard phosphoramidite chemistry. For the **Bo** and **BodU** phosphoramidites the coupling time was enhanced from 96 to 500 s. Commercially available reagents and CPG (1 μmol) were used. After preparation the trityl-off oligonucleotide was cleaved off the resin and deprotected by treatment with concentrated NH_4OH at 45 °C for 12 h. The oligonucleotide was dried and purified by HPLC on an RP-C18 column using the following conditions: A = NH_4OAc buffer (50 mM; pH 6.5); B = MeCN. The oligonucleotides were lyophilized and quantified by their absorbance at 260 nm on a photometer. Duplexes were formed by heating to 90 °C (15 min) followed by slow cooling. UV/vis (ε_{260} (M^{-1} cm^{-1})/yield (%)): 158 900/32.4 (**DNA1**), 164 100/41.8 (**DNA2**), 151 200/12.4 (**DNA3**), 156 00/12.5 (**DNA4**). ESI-MS (m/z): M^+ calcd for **DNA1** 5513, found 1837.9 $[M/3]^{3-}$, 1378.2 $[M/4]^{4-}$; M^+ calcd for **DNA2** 5563, found 1854.7 $[M/3]^{3-}$, 1390.8 $[M/4]^{4+}$; M^+ calcd for **DNA3** 5478, found 1825.7 $[M/3]^{3-}$, 1368.9 $[M/4]^{4+}$; M^+ calcd for **DNA4** 5528, found 1842.2 $[M/3]^{3-}$, 1381.4 $[M/4]^{4+}$.

ASSOCIATED CONTENT

Supporting Information. Text and figures giving experimental procedures, data and images of ^{1}H/^{13}C/^{19}F NMR spectra for **4** and **6**, the ^{31}P NMR spectrum for **1**, MS analysis for **4** and **6**, HPLC analysis of **DNA1**−**DNA4**, and absorption/emission

spectra of **DNA1**−**DNA4** and **DNA1Y**−**DNA4Y**. This material is available free of charge via the Internet at http://pubs.acs.org.

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ACKNOWLEDGMENT

Financial support by the Deutsche Forschungsgemeinschaft and the University of Regensburg is gratefully acknowledged. T. E. is grateful for a elite fellowship of the State of Bavaria. We thank Hartmut Yersin and his group for photon-counting measurements.

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