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5(6)-Carboxyfluorescein Revisited: New Protecting Group, Separation of Isomers, and their Spectral Properties on Oligonucleotides

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Pentafluorophenyl esters of 5- and 6-carboxyfluorescein-3′,6′-*O*-dipivalate can be easily separated in multigram quantities by column chromatography. The individual isomers were converted into stable phosphoramidites suitable for oligonucleotide synthesis. The use of the cyclohexylcarbonyl (Chc) protecting group instead of pivaloyl (Piv) facilitates the separation of isomers. The fluorescence spectra of 5- and 6-carboxyfluoresceins on oligonucleotides were compared.

INTRODUCTION

Carboxy derivatives of fluorescein (*1*) are widely used for labeling of biomolecules (*2*), e.g., in DNA biotechnology applications (*3*−*7*). The mixture of 5- and 6-isomers of carboxyfluorescein **1** can be easily obtained by condensation of 1,2,4-benzenetricarboxylic anhydride and resorcinol (*1*, *8*). However, the individual isomers are highly preferred as labels because of physicochemical and spectral homogeneity of labeled species. The separation of 5- and 6-isomers of fluorescein derivatives was reported by fractional crystallization (*9*−*15*) or extensive chromatography (*16*−*18*). The fluorescence of fluorescein is determined by the xanthene moiety, but can be finely modulated by various substituents in the benzene ring (*19*). Thus, it was of interest to compare the spectral properties of individual regioisomers of carboxyfluorescein when attached to an oligonucleotide. Surprisingly, we were unable to find any literature precedent for this.

The pivaloyl group is a convenient protection for fluorescein hydroxyls in phosphoramidite reagents for the synthesis of labeled oligonucleotides (*17*, *20*−*23*). Oligonucleotides containing carboxyfluorescein as an amide derivative (FAM) in the 5′-terminal position are the most useful conjugates applicable as DNA probes or PCR primers (the nonmodified 3′ terminus is suitable for elongation by DNA polymerases). There is no need to introduce multiple FAMs into an oligonucleotide because fluorescein is prone to self-quenching (*8*). Although both 5- and 6-FAM phosphoramidites for 5′-terminal labeling are commercially available (*24*, *25*), they are (a) rather expensive and (b) derived from primary alcohols, thus reducing their stability during storage as acetonitrile solutions in a DNA synthesizer (*26*).

EXPERIMENTAL PROCEDURES

General. Reagents and solvents obtained from commercial suppliers were used without further purification. CH_2Cl_2 was used freshly distilled from CaH_2. HPLC-grade DMF was distilled under reduced pressure and stored over 4 Å molecular sieves under argon. Other solvents were used as received. *N*,*N*-Diisopropylamino-2-cyanoethoxychlorophosphine was prepared as described (*27*, *28*).

500 MHz ^{1}H, 125.7 MHz ^{13}C, and 202.4 MHz ^{31}P NMR spectra were recorded on a Bruker DRX-500 spectrometer and referenced to $CDCl_3$ (7.26 ppm for ^{1}H and 77.1 ppm for ^{13}C), DMSO-d_6 (2.50 ppm for ^{1}H and 39.5 ppm for ^{13}C) (*29*), and 85% aq H_3PO_4 (0.00 ppm for ^{31}P). 564.4 MHz ^{19}F NMR spectra were recorded on a Varian Unity NMR spectrometer and referenced to $PhCF_3$ as the internal standard (−63.72 ppm for ^{19}F). ^{1}H−^{13}C gradient-selected HMQC and HMBC spectra were obtained by using 2048 (t_2) × 256 (t_1) complex point data sets, zero-filled to 2048 (F_2) × 1024 (F_1) points. The spectral widths were 13 and 200 ppm for ^{1}H and ^{13}C dimensions, respectively. HMBC spectra were measured with a 50 ms delay for the evolution of long-range couplings. ^{1}H NMR coupling constants are reported in hertz (Hz) and refer to apparent multiplicities. High-resolution mass spectra (HRMS) were recorded in positive-ion mode using an IonSpec FT ISR mass spectrometer (MALDI) or PE SCIEX QSTAR pulsar mass spectrometer (ESI). Melting points were determined using a Boetius heating table and are uncorrected. Analytical thin-layer chromatography was performed on Kieselgel 60 F_{254} precoated aluminum plates (Merck); spots were visualized under UV light (254 nm). Silica gel column chromatography was performed using Merck Kieselgel 60 0.040−0.063 mm. The oligonucleotide synthesis was carried out on a BiosSet ASM-700 instrument on a 200 nmol scale using standard manufacturer's protocols. Oligonucleotides were isolated using electrophoresis in 20% denaturing (7 M urea) PAGE in Tris−borate buffer, pH 8.3, and desalted by gel filtration on a Sephadex G-25 column in saltless buffer. MALDI-TOF mass spectra of oligonucleotide conjugates were recorded in positive-ion mode on a Bruker Ultraflex spectrometer. UV absorption spectra were recorded using a Varian Cary-500 spectrophotometer in a 0.1 M carbonate buffer pH 8.3−9.5. Fluorescence studies in the same buffer were performed using a large-aperture apparatus described earlier (*30*). The fluorescence quantum yields Φ_f were measured by the relative method using a standard of fluorescein in 0.1 M NaOH ($\Phi_f = 0.95 \pm 0.03$) (*31*). The measurements were performed at an angle of 90° to the exciting light beam. The optical density of the solutions in the 1 cm quartz cell at the excitation wavelength

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did not exceed 0.1. The values of Φ_f were corrected with the refractive index of the solvent; the measured refractive indexes of 0.1 M NaOH and carbonate buffer were 1.3335 and 1.3340 at 20 °C, respectively. Fluorescence lifetimes were determined by the method of time-correlated single-photon counting using a PRA 3000 nanosecond lifetime fluorometer. The parameters of the fluorescence decays were analyzed using T900 software (Edinburgh Instruments). The samples used in quantum yield and fluorescence lifetime measurements were not degassed.

Cyclohexanecarboxylic anhydride (*32*) was prepared as follows. A mixture of cyclohexanecarboxylic acid (1 equiv) and $SOCl_2$ (1.5 equiv) was heated for 4 h at 60 °C, then evaporated and distilled under reduced pressure to give cyclohexanecarbonyl chloride (91%, bp 71−73 °C/10 Torr). The chloride (50.7 g, 0.346 mol) was dissolved in benzene (350 mL); pyridine (56 mL, 0.69 mol) and cyclohexanecarboxylic acid (44.9 g, 0.35 mmol) were added subsequently; and the mixture was stirred for 2.5 h at 60 °C, then cooled, filtered, and evaporated. The residue was distilled *in vacuo* to afford Chc_2O as a colorless liquid (68.8 g, 91%, bp 125−135 °C/1 Torr). ^{1}H NMR ($CDCl_3$) δ = 2.38 (tt, 2H, $J_{a,a}$ = 11.2 Hz, $J_{a,e}$ = 3.7 Hz, H-1*a*), 1.94 (m, 4H, H-2*e*,6*e*), 1.76 (m, 4H, H-3*e*,5*e*), 1.63 (m, 2H, H-4*e*), 1.46 (m, 4H, H-2*a*,6*a*), 1.25 (m, 6H, H-3*a*,4*a*,5*a*). ^{13}C NMR ($CDCl_3$) δ = 171.9 (2C, *C*O), 44.0 (2C, C1), 28.4 (4C, C2,6), 25.6 (2C, C4), 25.2 (4C, C3,5).

5(6)-Carboxy-3′,6′-*O*-diacylfluoresceins, Pentafluorophenyl Esters (4a−d); General Procedure. To the magnetically stirred, ice-cooled solution of 5(6)-carboxyfluorescein **1** (20.00 g, 53.2 mmol) in DMF (100 mL) was added dropwise diisopropylethylamine (36.3 mL, 213 mmol) followed by trimethylacetic or cyclohexylcarbonic anhydride (117 mmol). The reaction solution was stirred at room temperature for 72 h, protected from light. The solvent was rotary-evaporated, and residue was dissolved in CH_2Cl_2 (170 mL) and EtOAc (350 mL), washed with 1 M phosphate buffer (3 × 300 mL, pH 7.0), and dried over $MgSO_4$. After evaporation of solvents, the remaining residue was dried in high vacuum overnight. Then, it was dissolved in EtOAc (400 mL), ice-cooled, and pentafluorophenol (11.75 g, 63.9 mmol) in EtOAc (50 mL), followed by dropwise addition of the solution of DCC (13.15 g, 63.8 mmol) in EtOAc (150 mL) within 1 h. After 2 h, the cooling was removed, and the mixture was stirred overnight at room temperature. The precipitate of dicyclohexylurea was filtered off, and the solution was concentrated to dryness. The residue was purified by column chromatography on silica gel eluting with $CHCl_3$; desired fractions were combined and evaporated to dryness. The resultant foam was dissolved in toluene (55 mL), and hexane (400 mL) was added. After crystallization at −20 °C overnight, the precipitate formed was filtered off and dried *in vacuo* to give the mixture of isomers as a white crystalline solid (25.28 g, 67% of **4a** + **4c** or 28.73 g, 71% of **4b** + **4d**). The solids were separated by chromatography on a silica gel column (850 g) with a step gradient of EtOAc in toluene (toluene → 0.1% EtOAc → 1% EtOAc in toluene) as the mobile phase to afford pure individual isomers **4a** (15.57 g, 62%) and **4c** (9.55 g, 38%) or **4b** (17.09 g, 59%) and **4d** (11.40 g, 40%) as white foams.

3′,6′-*O*-Bis(2,2-dimethyl-1-oxopropyl)-3-oxo-spiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthene]-5-carboxylic acid, Pentafluorophenyl Ester (4a). R_f 0.12 (toluene); mp 233−234 °C (toluene−hexane). ^{1}H NMR ($CDCl_3$) δ = 8.88 (br. s, 1H, H-4), 8.47 (dd, 1H, $J_{6,7}$ = 8.0 Hz, $^4J_{4,6}$ = 1.5 Hz, H-6), 7.37 (d, 1H, $J_{6,7}$ = 8.0 Hz, H-7), 7.10 (d, 2H, $J_{3',4'} = J_{5',6'}$ = 1.8 Hz, H-4′,5′), 6.87−6.79 (m, 4H, H-1′,2′,7′,8′), 1.37 (s, 18H, C*H*$_3$). ^{13}C NMR ($CDCl_3$) δ = 176.5 (2C, *C*OBu^t), 167.6 (C3), 161.2 (*C*$O_2C_6F_5$), 158.4 (C7a), 153.1 (2C, C4a′,10a′), 151.6 (2C, C3′,6′), 141.3 (m, 2C, $^1J_{CF}$ = 253 Hz, $^2J_{CF}$ = 12 Hz, C2″,6″), ∼140 (m, $^1J_{CF}$ ∼ 240 Hz, C4″), ∼138 (m, 2C, $^1J_{CF}$ ∼ 240 Hz, C3″,5″), 137.1 (C6), 129.4 (C5), 128.7 (2C, C1′,8′), 128.2 (C4), 127.2 (C3a), ∼125 (m, C1″), 125.1 (C7), 118.1 (2C, C2′,7′), 115.2 (2C, C8a′, 9a′), 110.7 (2C, C4′,5′), 82.2 (C1), 39.3 (2C, *C*CH$_3$), 27.2 (6C, *C*H$_3$). ^{19}F NMR ($CDCl_3$) δ = −11.51 (d, 2F, $J_{2'',3''} = J_{5'',6''}$ = 18.3 Hz, F-2″,6″), −16.11 (t, 1F, $J_{3'',4''} = J_{4'',5''}$ = 21.4 Hz, F-4″), −20.89 (m, 2F, F-3″,5″). HRMS (MALDI+): *m*/*z* [M + H]$^+$ calcd for $C_{37}H_{28}F_5O_9^+$ 711.1648; found 711.1642.

3′,6′-*O*-Bis(cyclohexylcarbonyl)-3-oxo-spiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthene]-5-carboxylic acid, Pentafluorophenyl Ester (4b). R_f 0.14 (toluene); mp 147−148 °C (toluene−hexane). ^{1}H NMR ($CDCl_3$) δ 8.88 (m, 1H, H-4), 8.47 (dd, 1H, $J_{6,7}$ = 8.0 Hz, $^4J_{4,6}$ = 1.4 Hz, H-6), 7.37 (d, 1H, $J_{6,7}$ = 8.0 Hz, H-7), 7.11 (m, 2H, H-4′,5′), 6.83 (m, 4H, H-1′,2′,7′,8′), 2.57 (tt, 2H, $J_{a,a}$ = 11.2 Hz, $J_{a,e}$ = 3.6 Hz, H-1*a*‴), 2.05 (m, 4H, H-2*e*‴,6*e*‴), 1.82 (m, 4H, H-3*e*‴,5*e*‴), 1.70 (m, 2H, H-4*e*‴), 1.59 (m, 4H, H-2*a*‴,6*a*‴), 1.33 (m, 6H, H-3*a*‴,4*a*‴,5*a*‴). ^{13}C NMR ($CDCl_3$) δ 174.0 (2C, *C*OCH), 167.6 (C3), 161.2 (*C*$O_2C_6F_5$), 158.4 (C7a), 152.8 (2C, C4a′,10a′), 151.6 (2C, C3′,6′), ∼141.5 (m, 2C, $^1J_{CF}$ ∼ 247 Hz, *C*2″,6″), ∼140 (m, $^1J_{CF}$ ∼ 240 Hz, C4″), ∼138 (m, 2C, $^1J_{CF}$ ∼ 240 Hz, C3″,5″), 137.1 (C6), 129.4 (C5), 128.8 (2C, C1′,8′), 128.2 (C4), 127.2 (C3a), 125.4 (m, C1″), 125.1 (C7), 118.2 (2C, *C*2′,7′), 115.2 (2C, C8a′,9a′), 110.8 (2C, C4′,5′), 82.2 (C1), 43.3 (2C, C1‴), 29.0 (4C, *C*2‴,6‴), 25.7 (2C, C4‴), 25.4 (4C, C3‴,5‴). ^{19}F NMR ($CDCl_3$) δ = −11.52 (d, 2F, $J_{2'',3''} = J_{5'',6''}$ = 18.3 Hz, F-2″,6″), −16.12 (t, 1F, $J_{3'',4''} = J_{4'',5''}$ = 21.4 Hz, F-4″), −20.90 (m, 2F, F-3″,5″). HRMS (MALDI+): *m*/*z* [M + Na]$^+$ calcd for $C_{41}H_{31}F_5NaO_9^+$ 785.1780; found 785.1799.

3′,6′-*O*-Bis(2,2-dimethyl-1-oxopropyl)-3-oxo-spiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthene]-6-carboxylic acid, Pentafluorophenyl Ester (4c). R_f 0.05 (toluene); mp 202−203 °C (toluene−hexane). ^{1}H NMR ($CDCl_3$): δ = 8.45 (dd, 1H, $J_{4,5}$ = 8.0 Hz, $^4J_{5,7}$ = 1.4 Hz, H-5), 8.21 (d, 1H, $J_{4,5}$ = 8.0 Hz, H-4), 7.96 (br. s, 1H, H-7), 7.10 (br. s, 2H, H-4′,5′), 6.84 (m, 4H, H-1′,2′,7′,8′), 1.36 (s, 18H, C*H*$_3$). ^{13}C NMR ($CDCl_3$) δ = 176.5 (2C, *C*OBu^t), 167.7 (C3), 161.2 (*C*$O_2C_6F_5$), 153.6 (C7a), 153.0 (2C, C4a′,10a′), 151.6 (2C, C3′,6′), 141.2 (m, 2C, $^1J_{CF}$ = 257 Hz, *C*2″,6″), 140.0 (m, $^1J_{CF}$ = 240 Hz, C4″), 138.0 (m, 2C, $^1J_{CF}$ = 241 Hz, C3″,5″), 133.4 (C6), 132.3 (C5), 131.0 (C3a), 128.8 (2C, C1′,8′), 126.4 (C7), 125.9 (C4), 124.8 (m, C1″), 118.1 (2C, *C*2′,7′), 115.2 (2C, C8a′,9a′), 110.7 (2C, C4′,5′), 82.3 (C1), 39.3 (2C, *C*CH$_3$), 27.1 (6C, *C*H$_3$). ^{19}F NMR ($CDCl_3$) δ = −11.15 (d, 2F, $J_{2'',3''} = J_{5'',6''}$ = 18.3 Hz, F-2″,6″), −16.09 (t, 1F, $J_{3'',4''} = J_{4'',5''}$ = 21.9 Hz, F-4″), −21.00 (m, 2F, F-3″,5″). HRMS (MALDI+): *m*/*z* [M + H]$^+$ calcd for $C_{37}H_{28}F_5O_9^+$ 711.1648; found 711.1655.

3′,6′-*O*-Bis(cyclohexylcarbonyl)-3-oxo-spiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthene]-6-carboxylic acid, Pentafluorophenyl Ester (4d). R_f 0.05 (toluene); mp 124−126 °C (toluene−hexane). ^{1}H NMR ($CDCl_3$) δ 8.45 (dd, 1H, $J_{4,5}$ = 8.0 Hz, $^4J_{5,7}$ = 1.2 Hz, H-5), 8.20 (d, 1H, $J_{4,5}$ = 8.0 Hz, H-4), 7.96 (br. s, 1H, H-7), 7.11 (br. s, 2H, H-4′,5′), 6.84 (m, 4H, H-1′,2′,7′,8′), 2.57 (tt, 2H, $J_{a,a}$ = 11.2 Hz, $J_{a,e}$ = 3.6 Hz, H-1*a*‴), 2.05 (m, 4H, H-2*e*‴,6*e*‴), 1.82 (m, 4H, H-3*e*‴,5*e*‴), 1.69 (m, 2H, H-4*e*‴), 1.59 (m, 4H, H-2*a*‴,6*a*‴), 1.33 (m, 6H, H-3*a*‴,4*a*‴, 5*a*‴). ^{13}C NMR ($CDCl_3$) δ 174.0 (2C, *C*OCH), 167.7 (C3), 161.2 (*C*$O_2C_6F_5$), 153.6 (C7a), 152.8 (2C, C4a′,10a′), 151.6 (2C, C3′,6′), ∼141.5 (m, 2C, $^1J_{CF}$ ∼ 249 Hz, *C*2″,6″), ∼140 (m, $^1J_{CF}$ = 240 Hz, C4″), ∼138 (m, 2C, $^1J_{CF}$ = 241 Hz, C3″,5″), 133.4 (C6), 132.3 (C5), 131.1 (C3a), 128.8 (2C, C1′,8′), 126.5 (C7), 126.0 (C4), 125.4 (m, C1″), 118.2 (2C, *C*2′,7′), 115.2 (2C, C8a′,9a′), 110.7 (2C, C4′,5′), 82.4 (C1), 43.3 (2C, C1‴), 29.0 (4C, *C*2‴,6‴), 25.7 (2C, C4‴), 25.4 (4C, C3‴,5‴). ^{19}F NMR ($CDCl_3$) δ = −11.16 (d, 2F, $J_{2'',3''} = J_{5'',6''}$ = 18.3 Hz, F-2″,6″), −16.10 (t, 1F, $J_{3'',4''} = J_{4'',5''}$ = 22.0 Hz, F-4″), −20.98 (m, 2F,

F-3″,5″). HRMS (MALDI+): *m/z* [M + H]$^+$ calcd for $C_{41}H_{32}F_5O_9^+$ 763.1961; found 763.1979.

Amides 5a−d; General Procedure. To a solution of activated ester **4a−d** (5.0 mmol) in CH_2Cl_2 (60 mL), *trans*-4-aminocyclohexanol hydrochloride (0.76 g, 5.0 mmol) and DIEA (1.74 mL, 10.0 mmol) were added. The suspension was diluted with DMF (60 mL) and stirred overnight at room temperature to give a homogeneous solution. The latter was diluted with $CHCl_3$ (200 mL), washed with water (4 × 200 mL), dried over Na_2SO_4, and evaporated to dryness. The residue was purified by chromatography on a silica gel column (100 g) with a step gradient of MeOH (0 → 5%) in toluene as the mobile phase to give amides **5a−d** as white amorphous foams.

3′,6′-*O*-Bis(2,2-dimethyl-1-oxopropyl)-5-(4-hydroxycyclohexylaminocarbonyl)-3-oxo-spiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthene] (5a). Yield 3.17 g (quant.) R_f 0.30 (MeOH−toluene 15:85 v/v). ^{1}H NMR (DMSO-d_6): δ = 8.57 (d, 1H, $J_{4'',NH}$ = 7.9 Hz, N*H*), 8.55 (br. s, 1H, H-4), 8.28 (d, 1H, $J_{6,7}$ = 8.2 Hz, H-6), 7.48 (d, 1H, $J_{6,7}$ = 8.2 Hz, H-7), 7.27 (s, 2H, H-4′,5′), 6.93 (s, 4H, H-1′,2′,7′,8′), 4.56 (d, 1H, J = 4.0 Hz, O*H*), 3.78 (m, 1H, H-4″), 3.42 (m, 1H, H-1″), 1.92−1.80 (m, 4H, H-2*e*″,3*e*″,5*e*″,6*e*″), 1.48−1.20 (m, 22H, H-2*a*″,3*a*″, 5*a*″,6*a*″, C*H*$_3$). ^{13}C NMR (DMSO-d_6) δ = 175.8 (2C, *C*OBu^t), 167.8 (C3), 163.5 (*C*ONH), 154.0 (C7a), 152.3 (2C, C4a′,10a′), 150.7 (2C, C3′,6′), 136.7 (C6), 135.0 (C5), 129.1 (2C, C1′,8′), 125.6 (C3a), 124.1 (C4), 123.6 (C7), 118.4 (2C, *C*2′,7′), 115.6 (2C, C8a′,9a′), 110.2 (2C, C4′,5′), 81.0 (C1), 68.2 (C1″), 48.1 (C4″), 38.5 (2C, *C*CH$_3$), 34.0 (2C, *C*2″,6″), 30.0 (2C, C3″,5″), 26.5 (6C, *C*H$_3$). HRMS (MALDI+): *m/z* [M + H]$^+$ calcd for $C_{37}H_{40}NNaO_9^+$ 642.2698; found 642.2709.

3′,6′-*O*-Bis(cyclohexylcarbonyl)-5-(4-hydroxycyclohexylaminocarbonyl)-3-oxo-spiro[isobenzofuran-1(3*H*),9′-[9*H*] xanthene] (5b). Yield 3.42 g (quant). R_f 0.34 (MeOH−toluene 1:9 v/v). ^{1}H NMR (DMSO-d_6) δ 8.57 (d, 1H, $J_{4'',NH}$ = 7.8 Hz, N*H*), 8.55 (br. s, 1H, H-4), 8.28 (dd, 1H, $J_{6,7}$ = 8.0 Hz, $^4J_{4,6}$ = 1.4 Hz, H-6), 7.49 (d, 1H, $J_{6,7}$ = 8.0 Hz, H-7), 7.26 (br. s, 2H, H-4′,5′), 6.92 (s, 4H, H-1′,2′,7′,8′), 4.56 (d, 1H, J = 4.3 Hz, O*H*), 3.79 (m, 1H, H-4″), 3.42 (m, 1H, H-1″), 2.61 (tt, 2H, $J_{a,a}$ = 11.0 Hz, $J_{a,e}$ = 3.6 Hz, H-1*a*‴), 1.99 (m, 4H, H-2*e*‴,6*e*‴), 1.87 (m, 4H, H-2*e*‴,3*e*‴,5*e*″,6*e*″), 1.72 (m, 4H, H-3*e*‴,5*e*‴), 1.61 (m, 2H, H-4*e*‴), 1.55−1.18 (m, 14H, H-2*a*″,3*a*″,5*a*″,6*a*″,2*a*‴, 3*a*‴,4*a*‴,5*a*‴,6*a*‴). ^{13}C NMR (DMSO-d_6) δ 173.2 (2C, *C*OCH), 167.8 (C3), 163.5 (*C*ONH), 153.9 (C7a), 152.1 (2C, C4a′,10a′), 150.7 (2C, C3′,6′), 136.7 (C6), 135.0 (C5), 129.1 (2C, C1′,8′), 125.7 (C3a), 124.1 (C4), 123.6 (C7), 118.4 (2C, *C*2′,7′), 115.5 (2C, C8a′,9a′), 110.2 (2C, C4′,5′), 81.1 (C1), 68.2 (C1″), 48.1 (C4″), 41.9 (2C, C1‴), 34.0 (2C, *C*2″,6″), 30.0 (2C, C3″,5″), 28.2 (4C, *C*2‴,6‴), 25.1 (2C, C4‴), 24.5 (4C, C3‴,5‴). HRMS (MALDI+): *m/z* [M + Na]$^+$ calcd for $C_{41}H_{43}NNaO_9^+$ 716.2830; found 716.2808.

3′,6′-*O*-Bis(2,2-dimethyl-1-oxopropyl)-6-(4-hydroxycyclohexylaminocarbonyl)-3-oxo-spiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthene] (5c). Yield 3.06 g (95%). R_f 0.24 (MeOH−toluene 15:85 v/v). ^{1}H NMR (DMSO-d_6) δ = 8.37 (d, 1H, $J_{4'',NH}$ = 7.8 Hz, N*H*), 8.22 (d, 1H, $J_{4,5}$ = 8.2 Hz, H-5), 8.14 (d, 1H, $J_{4,5}$ = 8.2 Hz, H-4), 7.79 (s, 1H, H-7), 7.28 (s, 2H, H-4′,5′), 6.94 (s, 4H, H-1′,2′,7′,8′), 4.51 (br. s, 1H, O*H*), 3.67 (m, 1H, H-4″), 3.32 (m, 1H, H-1″), 1.83−1.72 (m, 4H, H-2*e*″,3*e*″,5*e*″,6*e*″), 1.31 (s, 18H, C*H*$_3$), 1.30−1.14 (m, 4H, H-2*a*″,3*a*″,5*a*″,6*a*″). ^{13}C NMR (DMSO-d_6) δ = 175.8 (2C, *C*OBu^t), 167.6 (C3), 163.4 (NH*C*O), 152.3 (2C, C4a′,10a′), 152.1 (C7a), 150.7 (2C, C3′,6′), 141.2 (C6), 130.0 (C5), 129.3 (2C, C1′,8′), 127.3 (C3a), 125.1 (C4), 122.0 (C7), 118.4 (2C, *C*2′,7′), 115.6 (2C, C8a′,9a′), 110.2 (2C, C4′,5′), 81.1 (C1), 68.1 (C1″), 48.1 (C4″), 38.5 (2C, *C*CH$_3$), 34.0 (2C, *C*2″,6″), 29.9 (2C, C3″,5″), 26.5 (6C, *C*H$_3$). HRMS (MALDI+): *m/z* [M + Na]$^+$ calcd for $C_{37}H_{39}NNaO_9^+$ 664.2517; found 664.2498.

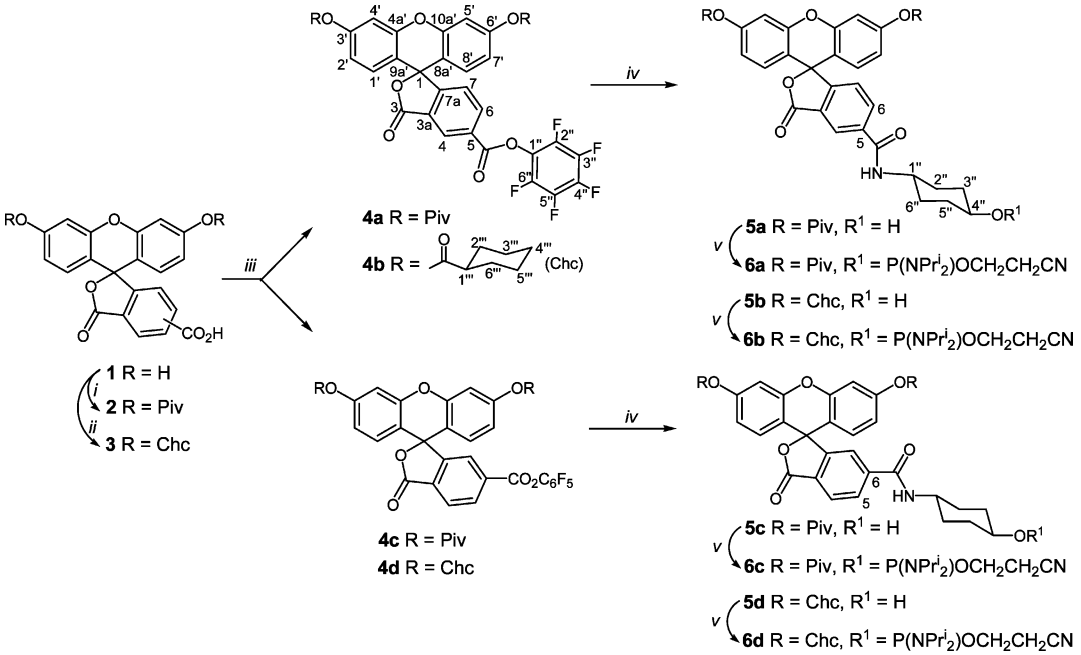
3′,6′-*O*-Bis(cyclohexylcarbonyl)-6-(4-hydroxycyclohexylaminocarbonyl)-3-oxo-spiro[isobenzofuran-1(3*H*),9′-[9*H*]-xanthene] (5d). Yield 3.26 g (94%). R_f 0.20 (MeOH−toluene 1:9 v/v). ^{1}H NMR (DMSO-d_6) δ 8.38 (d, 1H, $J_{4'',NH}$ = 7.6 Hz, N*H*), 8.22 (dd, 1H, $J_{4,5}$ = 8.1 Hz, $^4J_{5,7}$ = 1.0 Hz, H-5), 8.14 (d, 1H, $J_{4,5}$ = 8.1 Hz, H-4), 7.81 (s, 1H, H-7), 7.28 (s, 2H, H-4′,5′), 6.93 (s, 4H, H-1′,2′,7′,8′), 4.51 (d, 1H, J = 4.4 Hz, O*H*), 3.67 (m, 1H, H-4″), 3.29 (m, 1H, H-1″), 2.61 (tt, 2H, $J_{a,a}$ = 11.0 Hz, $J_{a,e}$ = 3.6 Hz, H-1*a*‴), 1.97 (m, 4H, H-2*e*‴,6*e*‴), 1.84−1.65 (m, 8H, H-2*e*″,3*e*″,5*e*″,6*e*″,3*e*‴,5*e*‴), 1.61 (m, 2H, H-4*e*‴), 1.54−1.43 (m, 4H), 1.38−1.14 (m, 10H) (H-2*a*″, 3*a*″,5*a*″,6*a*″,2*a*‴,3*a*‴,4*a*‴,5*a*‴,6*a*‴). ^{13}C NMR (DMSO-d_6) δ 173.2 (2C, *C*OCH), 167.6 (C3), 163.4 (NH*C*O), 152.1 (2C, C4a′,10a′), 152.0 (C7a), 150.7 (2C, C3′,6′), 141.1 (C6), 130.0 (C5), 129.2 (2C, C1′,8′), 127.4 (C3a), 125.1 (C4), 122.0 (C7), 118.5 (2C, *C*2′,7′), 115.6 (2C, C8a′,9a′), 110.2 (2C, C4′,5′), 81.1 (C1), 68.1 (C1″), 48.1 (C4″), 41.9 (2C, C1‴), 34.0 (2C, *C*2″,6″), 29.9 (2C, C3″,5″), 28.2 (4C, *C*2‴,6‴), 25.1 (2C, C4‴), 24.5 (4C, C3‴,5‴). HRMS (MALDI+): *m/z* [M + Na]$^+$ calcd for $C_{41}H_{43}NNaO_9^+$ 716.2830; found 716.2817.

Phosphoramidites 6a−d; General Procedure. Amide **5a−d** (4.0 mmol) was evaporated with dry CH_2Cl_2 (2 × 50 mL), dissolved in dry CH_2Cl_2 (50 mL) and DIEA (4.4 mmol, 0.765 mL), followed by addition of *N*,*N*-diisopropylamino-2-cyanoethoxychlorophosphine (1.04 g, 4.4 mmol), and the mixture was stirred under argon for 1 h. After conversion of the starting compound was complete (monitoring by TLC, Me_2CO−toluene 3:7 + 1% pyridine v/v/v), the mixture was washed with 5% $NaHCO_3$ (50 mL) and sat NaCl (60 mL). The organic layer was dried over Na_2SO_4 during 1 h, evaporated to dryness, and the residue was purified by chromatography on a silica gel column (100 g) with 5% acetone in toluene + 1% pyridine (v/v/v) as the mobile phase. Fractions containing product were combined and evaporated. The resulting phosphoramidite was dissolved in dry toluene (25 mL) and precipitated into hexane (350 mL). The obtained compound was filtered, dissolved in CH_2Cl_2, and evaporated to dryness to afford compounds **6a−d** as white amorphous solids.

3′,6′-*O*-Bis(2,2-dimethyl-1-oxopropyl)-5-[4-(*N*,*N*-diisopropylamino-2-cyanoethoxyphosphinyloxy)cyclohexylaminocarbonyl]-3-oxo-spiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthene] (6a). Yield 2.60 g (77%). R_f 0.68 (Me_2CO−toluene 3:7 + 1% pyridine v/v/v). ^{1}H NMR (DMSO-d_6) δ 8.58 = (d, 1H, $J_{4'',NH}$ = 7.6 Hz, N*H*), 8.55 (br. s, 1H, H-4), 8.28 (dd, 1H, $J_{6,7}$ = 7.9 Hz, $^4J_{4,6}$ = 1.2 Hz, H-6), 7.49 (d, 1H, $J_{6,7}$ = 7.9 Hz, H-7), 7.27 (s, 2H, H-4′,5′), 6.93 (s, 4H, H-1′,2′,7′,8′), 3.87−3.65 (m, 4H, H-1″,4″, POC*H*$_2$), 3.63−3.52 (m, 2H, PNC*H*), 2.77 (t, 2H, J = 6.0 Hz, C*H*$_2$CN), 2.04 (m, 1H), 1.96 (m, 1H), 1.90 (m, 2H) (H-2*e*″,3*e*″,5*e*″,6*e*″), 1.47 (m, 4H, H-2*a*″,3*a*″,5*a*″,6*a*″), 1.31 (m, 18H, CC*H*$_3$), 1.15 (d, 12H, J = 6.7 Hz, CHC*H*$_3$). ^{13}C NMR (DMSO-d_6) δ = 175.8 (2C, *C*OBu^t), 167.8 (C3), 163.6 (*C*ONH), 154.0 (C7a), 152.3 (2C, C4a′,10a′), 150.7 (2C, C3′,6′), 136.7 (C6), 135.0 (C5), 129.1 (2C, C1′,8′), 125.6 (C3a), 124.1 (C4), 123.6 (C7), 118.9 (*C*N), 118.4 (2C, *C*2′,7′), 115.6 (2C, C8a′,9a′), 110.2 (2C, C4′,5′), 81.0 (C1), 71.7 (d, $^2J_{P,C}$ = 19.2 Hz, C1″), 57.8 (d, $^2J_{P,C}$ = 19.2 Hz, POC*H*$_2$), 47.6 (C4″), 42.4 (d, $^2J_{P,C}$ = 13.0 Hz, PNC*H*), 38.5 (2C, *C*CH$_3$), 32.6, 32.5 (*C*2″,6″), 29.7 (2C, C3″,5″), 26.5 (6C, C*C*H$_3$), 24.2 (d, 2C, $^3J_{P,C}$ = 8.0 Hz), 24.1 (d, 2C, $^3J_{P,C}$ = 8.0 Hz) (CH*C*H$_3$), 19.7 (d, $^3J_{P,C}$ = 7.4 Hz, *C*H$_2$CN). ^{31}P NMR (DMSO-d_6) δ = 144.96. HRMS (ESI+): *m/z* [M + Na]$^+$ calcd for $C_{46}H_{56}N_3NaO_{10}P^+$ 864.3595; found 864.3556.

3′,6′-*O*-Bis(cyclohexylcarbonyl)-5-[4-(*N*,*N*-diisopropylamino-2-cyanoethoxyphosphinyloxy)cyclohexylaminocarbonyl]-3-oxo-spiro[isobenzofuran-1(3H),9′-[9H]xanthene] (6b). Yield 2.73 g (76%). R_f 0.69 (Me_2CO−toluene 3:7 + 1% pyridine v/v/v). ^{1}H NMR (DMSO-d_6) δ 8.58 (d, 1H, $J_{4'',NH}$ = 7.8 Hz,

Scheme 1[a]



[a] Reagents and conditions: *i*, Piv_2O, DIEA, DMF, 72 h; *ii*, Chc_2O, DIEA, DMF, 72 h; *iii*, C_6F_5OH, DCC, EtOAc, overnight; *iv*, *trans*-4-aminocyclohexanol, DIEA, CH_2Cl_2, DMF, overnight; *v*, $Pr^i_2N(Cl)POCH_2CH_2CN$, CH_2Cl_2, 1 h.

N*H*), 8.55 (br. s, 1H, H-4), 8.28 (dd, 1H, $J_{6,7}$ = 8.1 Hz, $^4J_{4,6}$ = 1.2 Hz, H-6), 7.49 (d, 1H, $J_{6,7}$ = 8.1 Hz, H-7), 7.26 (br. s, 2H, H-4′,5′), 6.92 (s, 4H, H-1′,2′,7′,8′), 3.88−3.67 (m, 4H, H-1″,4″, POCH_2), 3.63−3.54 (m, 2H, C*H*CH$_3$), 2.77 (t, 2H, J = 6.0 Hz, CH_2CN), 2.61 (tt, 2H, $J_{a,a}$ = 11.0 Hz, $J_{a,e}$ = 3.6 Hz, H-1*a*‴), 2.07−1.88 (m, 8H, H-2*e*″,3*e*″,5*e*″,6*e*″,2*e*‴,6*e*‴), 1.72 (m, 4H, H-3*e*‴,5*e*‴), 1.60 (m, 2H, H-4*e*‴), 1.54−1.21 (m, 14H, H-2*a*″,3*a*″,5*a*″,6*a*″,2*a*‴,3*a*‴,4*a*‴,5*a*‴,6*a*‴), 1.15 (d, 12H, J = 6.7 Hz, CH_3). ^{13}C NMR (DMSO-d_6) δ 173.2 (2C, *C*OCH), 167.7 (C3), 163.6 (*C*ONH), 153.9 (C7a), 152.1 (2C, C4a′,10a′), 150.7 (2C, C3′,6′), 136.7 (C6), 135.0 (C5), 129.1 (2C, C1′,8′), 125.7 (C3a), 124.1 (C4), 123.6 (C7), 118.9 (*C*N), 118.4 (2C, *C*2′,7′), 115.5 (2C, C8a′,9a′), 110.2 (2C, C4′,5′), 81.0 (C1), 71.7 (d, $^2J_{P,C}$ = 17.8 Hz, C1″), 57.8 (d, $^2J_{P,C}$ = 17.8 Hz, PO*C*H$_2$), 47.6 (C4″), 42.4 (d, $^2J_{P,C}$ = 12.6 Hz, PN*C*H), 41.9 (2C, C1‴), 32.6, 32.5 (*C*2″,6″), 29.7 (2C, C3″,5″), 28.2 (4C, *C*2‴,6‴), 25.1 (2C, C4‴), 24.5 (4C, C3‴,5‴), 24.2 (d, 2C, $^3J_{P,C}$ = 7.4 Hz), 24.1 (d, 2C, $^3J_{P,C}$ = 7.4 Hz) (CH*C*H$_3$), 19.7 (d, $^3J_{P,C}$ = 6.9 Hz, *C*H$_2$-CN). ^{31}P NMR (DMSO-d_6) δ 144.97. HRMS (ESI+): *m*/*z* [M + Na]$^+$ calcd for $C_{50}H_{60}N_3NaO_{10}P^+$ 916.3908; found 916.3892.

3′,6′-*O*-Bis(2,2-dimethyl-1-oxopropyl)-6-[4-(*N*, *N*-diisopropylamino-2-cyanoethoxyphosphinyloxy)cyclohexylaminocarbonyl]-3-oxo-spiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthene] (6c). Yield 2.51 g (74%). R_f 0.60 (Me$_2$CO−toluene 3:7 + 1% pyridine v/v/v). ^{1}H NMR (DMSO-d_6) δ = 8.39 (d, 1H, $J_{4'',NH}$ = 7.8 Hz, N*H*), 8.22 (d, 1H, $J_{4,5}$ = 8.0 Hz, H-5), 8.14 (d, 1H, $J_{4,5}$ = 8.0 Hz, H-4), 7.79 (s, 1H, H-7), 7.28 (s, 2H, H-4′,5′), 6.94 (s, 4H, H-1′,2′,7′,8′), 3.76−3.60 (m, 4H, H-1″,4″, POCH_2), 3.58−3.50 (m, 2H, PNC*H*), 2.74 (t, 2H, J = 6.0 Hz, CH_2CN), 1.95 (m, 1H), 1.87 (m, 1H), 1.81 (m, 2H) (H-2*e*″,3*e*″,5*e*″,6*e*″), 1.42−1.29 (m, 22H, H-2*a*″,3*a*″,5*a*″,6*a*″, CCH_3), 1.12 (m, 12H, CHCH_3). ^{13}C NMR (DMSO-d_6) δ = 175.8 (2C, *C*OBu^t), 167.6 (C3), 163.4 (NH*C*O), 152.3 (2C, C4a′,10a′), 152.1 (C7a), 150.7 (2C, C3′,6′), 141.1 (C6), 129.9 (C5), 129.2 (2C, C1′,8′), 127.3 (C3a), 125.1 (C4), 122.0 (C7), 118.8 (*C*N), 118.4 (2C, *C*2′,7′), 115.6 (2C, C8a′,9a′), 110.2 (2C, C4′,5′), 81.1 (C1), 71.85 (d, $^2J_{P,C}$ = 17.8 Hz, C1″), 57.8 (d, $^2J_{P,C}$ = 17.8 Hz, PO*C*H$_2$), 47.6 (C4″), 42.3 (d, $^2J_{P,C}$ = 12.6 Hz, PN*C*H), 38.5 (2C, *C*CH$_3$), 32.6 (d, $^3J_{P,C}$ = 3.4 Hz), 32.5 (d, $^3J_{P,C}$ = 3.4 Hz) (*C*2″,6″), 29.7, 29.6 (C3″,5″), 26.71 (6C, C*C*H$_3$), 24.2 (d, 2C, $^3J_{P,C}$ = 7.4 Hz), 24.1 (d, 2C, $^3J_{P,C}$ = 6.9 Hz) (CH*C*H$_3$), 19.7 (d, $^3J_{P,C}$ = 6.9 Hz, *C*H$_2$CN). ^{31}P NMR (DMSO-d_6) δ = 144.88. HRMS (ESI+): *m*/*z* [M + Na]$^+$ calcd for $C_{46}H_{56}N_3NaO_{10}P^+$ 864.3595; found 864.3565.

3′,6′-*O*-Bis(cyclohexylcarbonyl)-6-[4-(*N*,*N*-diisopropylamino-2-cyanoethoxyphosphinyloxy)cyclohexylaminocarbonyl]-3-oxo-spiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthene] (6d). Yield 2.84 g (79%). R_f 0.62 (Me$_2$CO−toluene 3:7 + 1% pyridine v/v/v). ^{1}H NMR (DMSO-d_6) δ 8.39 (d, 1H, $J_{4'',NH}$ = 7.6 Hz, N*H*), 8.22 (d, 1H, $J_{4,5}$ = 8.0 Hz, H-5), 8.14 (d, 1H, $J_{4,5}$ = 8.0 Hz, H-4), 7.80 (s, 1H, H-7), 7.28 (s, 2H, H-4′,5′), 6.93 (s, 4H, H-1′,2′,7′,8′), 3.75−3.60 (m, 4H, H-1″,4″, POCH_2), 3.59−3.50 (m, 2H, C*H*CH$_3$), 2.74 (t, 2H, J = 5.8 Hz, CH_2CN), 2.62 (tt, 2H, $J_{a,a}$ = 11.0 Hz, $J_{a,e}$ = 3.6 Hz, H-1*a*‴), 2.02−1.68 (m, 12H, H-2*e*″,3*e*″,5*e*″,6*e*″,2*e*‴,3*e*‴,5*e*‴,6*e*‴), 1.61 (m, 2H, H-4*e*‴), 1.52−1.19 (m, 14H, H-2*a*″,3*a*″,5*a*″,6*a*″,2*a*‴,3*a*‴, 4*a*‴,5*a*‴,6*a*‴), 1.12 (d, 6H, J = 6.4 Hz), 1.11 (d, 6H, J = 6.4 Hz) (CH_3). ^{13}C NMR (DMSO-d_6) δ 173.2 (2C, *C*OCH), 167.6 (C3), 163.4 (NH*C*O), 152.1 (2C, C4a′,10a′), 152.0 (C7a), 150.7 (2C, C3′,6′), 141.1 (C6), 130.0 (C5), 129.2 (2C, C1′,8′), 127.4 (C3a), 125.1 (C4), 122.0 (C7), 118.8 (*C*N), 118.5 (2C, *C*2′,7′), 115.6 (2C, C8a′,9a′), 110.2 (2C, C4′,5′), 81.1 (C1), 71.7 (d, $^2J_{P,C}$ = 17.8 Hz, C1″), 57.8 (d, $^2J_{P,C}$ = 17.8 Hz, PO*C*H$_2$), 47.6 (C4″), 42.3 (d, $^2J_{P,C}$ = 12.6 Hz, PN*C*H), 41.9 (2C, C1‴), 32.6 (d, $^2J_{P,C}$ = 4.0 Hz), 32.5 (d, $^2J_{P,C}$ = 4.0 Hz) (*C*2″,6″), 29.7, 29.6 (C3″,5″), 28.2 (4C, *C*2‴,6‴), 25.1 (2C, C4‴), 24.5 (4C, C3‴,5‴), 24.2 (d, 2C, $^3J_{P,C}$ = 7.4 Hz), 24.1 (d, 2C, $^3J_{P,C}$ = 6.9 Hz) (CH*C*H$_3$), 19.7 (d, $^3J_{P,C}$ = 6.9 Hz, *C*H$_2$CN). ^{31}P NMR (DMSO-d_6) δ 144.89. HRMS (ESI+): *m*/*z* [M + Na]$^+$ calcd for $C_{50}H_{60}N_3NaO_{10}P^+$ 916.3908; found 916.3886.

RESULTS AND DISCUSSION

Our aim was to prepare stable and inexpensive 5- and 6-FAM phosphoramidites and to study the fluorescence of the regioisomers on an oligonucleotide. The synthesis of phosphoramidite reagents is given in Scheme 1. First, we found that 5(6)-carboxyfluorescein-3′,6′-*O*-dipivalate **2** (*17*) can be converted into pentafluorophenyl (Pfp) ester (similar as described for isobutyryl derivatives (*8*)) and the resulting mixture (**4a** + **4c**) is easily resolved by column chromatography on silica gel in

Chart 1. Structures of 5′-(5-FAM) and 5′-(6-FAM) Oligonucleotide Conjugates

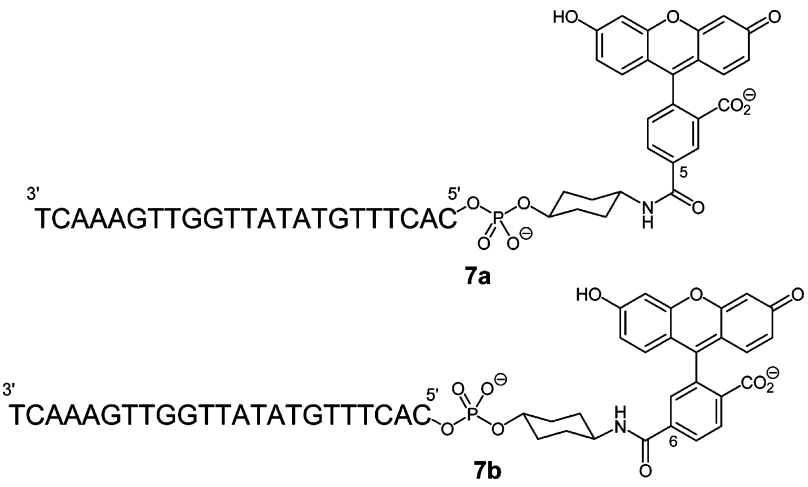


Table 1. Spectroscopic (λ^{abs}_{max}, λ^{abs}_{max}, $\Delta\nu^{fl}_{1/2h}$)[a] and Photophysical (Φ_f, τ_f, k_f)[b] Characteristics of Fluorescent Oligonucleotide Conjugates 7a (5-FAM) and 7b (6-FAM) at Various pH (20 °C)

	data for conjugates **7a**/**7b**					
pH	λ^{abs}_{max}, nm	λ^{fl}_{max}, nm	$\Delta\nu^{fl}_{1/2h}$,[c] cm^{-1}	$\Phi_f \pm 0.05$	$\tau_f \pm 0.02$, ns	$k_f \times 10^{-8} \pm 0.1$, s^{-1}
8.35	494/494	521/518	1990/1440	0.58/0.48	4.22/4.21	1.4/1.1
8.50	494/494	521/517	1710/1430	0.48/0.53	4.19/4.18	1.1/1.3
9.00	494/494	521/517	1730/1440	0.53/0.58	4.21/4.18	1.3/1.4
9.50	494/494	522/517	1715/1435	0.51/0.56	4.23/4.20	1.2/1.3

[a] $\lambda^{abs}_{max}$and λ^{fl}_{max} − absorbance and fluorescence wavelength maxima; $\Delta\nu^{fl}_{1/2h}$− half-width of the emission spectrum. [b] Φ_f − fluorescence quantum yield; τ_f − average fluorescence lifetime; $k_f = \Phi_f/\tau_f$ − emission rate constant. [c] Error limits are ±40 cm^{-1} (**7a**) and ±30 cm^{-1} (**7b**).

toluene in multigram quantities. Then, we examined the cyclohexylcarbonyl (Chc) as a protecting group for fluorescein. It was found that 3′,6′-*O*-diacylation of 5(6)-carboxyfluorescein with Chc_2O proceeds smoothly and gives similar yields (71% for two-step one-pot procedure **1** → **3** → (**4b** + **4d**) compared to 67% for **1** → **2** → (**4a** + **4c**)). However, the more pronounced difference in chromatographic mobility between **4b** and **4d** (ΔR_f 0.09) in toluene makes their separation easier than Piv derivatives **4a** and **4c** (ΔR_f 0.07). The small difference in mobility ($\Delta\Delta R_f$ 0.02) can be felt strongly while separating decigram quantities of these isomer derivatives.

The structures of compounds **4a**−**d** were assigned using 2D $^1H-^{13}C$ HMQC and HMBC NMR spectra.

trans-4-Aminocyclohexanol was reported as an achiral precursor for phosphoramidites which are more stable in acetonitrile solution than phosphoramidites derived from primary alcohols (*26*). The individual regioisomers of pentafluorophenyl esters **4a**−**d** were reacted with *trans*-4-aminocyclohexanol to give amides **5a**−**d** in high yields. The following phosphitylation with *N*,*N*-diisopropylamino-2-cyanoethoxychlorophosphine furnishes the desired phosphoramidites **6a**−**d** (Scheme 1).

Reagents **6a**−**d** were used in the last coupling step of machine-assisted solid-phase DNA synthesis for the preparation of 5′-labeled 23-mer 5′-**X**CACTTTGTATATTGGTTGAAACT-3′. The conjugates prepared were cleaved from their supports, deprotected by conc aq ammonia treatment at 55 °C overnight, analyzed by reversed-phase HPLC and MALDI-TOF MS, and purified by PAGE for fluorescence studies. Reagents **6a** and **6b** gave conjugate 5′-(5-FAM)-CACTTTGTATATTGGTTGAAACT-3′ (**7a**) (Chart 1); the products from two syntheses were identical by HPLC and MALDI-TOF MS and showed the same electrophoretic mobilities. There was no substantial difference in the amounts of isolated products. In a similar manner, the use of reagents **6c** or **6d** gave one labeled oligonucleotide−5′-(6-FAM)-CACTTTGTATATTGGTTGAAACT-3′ (**7b**). Conjugates **7a** and **7b** were characterized by UV−vis and fluorescence spectra (Figure 1, Table 1).

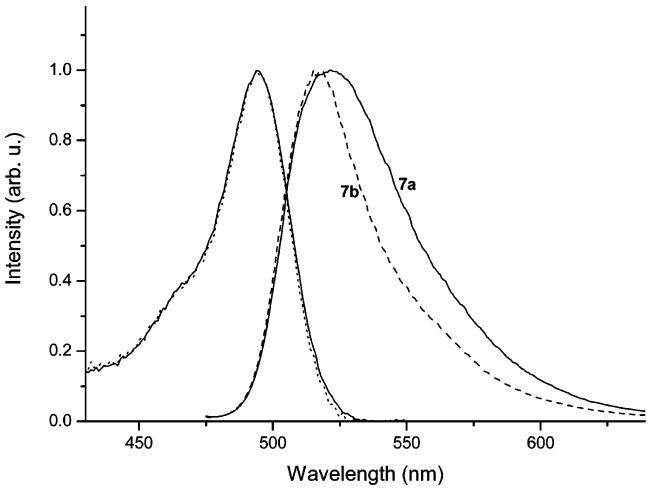


Figure 1. Normalized absorption (left) and fluorescence (right) spectra of labeled oligonucleotides **7a** (straight lines) and **7b** (doted and dashed lines) in sodium carbonate buffer, pH 8.35, excitation at λ = 470 nm.

There is no difference between UV−vis absorbances of 5- and 6-FAM attached to an oligonucleotide (Figure 1). The study of fluorescent properties of 5- and 6-FAM isomers in the usual pH range for fluorescein (8.35−9.5) showed that the differences in their photophysical characteristics depending on the pH are within error limits. In contrast, spectroscopic characteristics of 5- and 6-FAM are not essentially similar: 5-isomer displays a 3−5 nm red shift of the fluorescence maximum against 6-isomer (Figure 1, Table 1). The emission band in the spectrum of 5-FAM is substantially broader.

The obvious consequences from the presented data for 5- and 6-FAM are that these dyes in aqueous solutions within the pH range 8.35−9.5 have similar absorbance and fluorescence quantum yields, and, therefore, similar fluorescence brightness.

However, in the case of multiplex detection of several fluorochromes, the sharper emission band of 6-FAM could be an advantage.

To conclude, we have shown that 5- and 6-carboxyfluoresceins can be easily separated as Pfp esters of 3′,6′-*O*-diacyl derivatives, and Chc is the acyl protecting group of choice for this procedure; inexpensive phosphoramidite reagents can be easily prepared from the above esters in two steps; spectroscopic and photophysical data for 5- and 6-FAM on oligonucleotide were collected and compared.

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