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4′,5′-Dichloro-2′,7′-dimethoxy-5(6)-carboxyfluorescein (JOE): Synthesis and Spectral Properties of Oligonucleotide Conjugates

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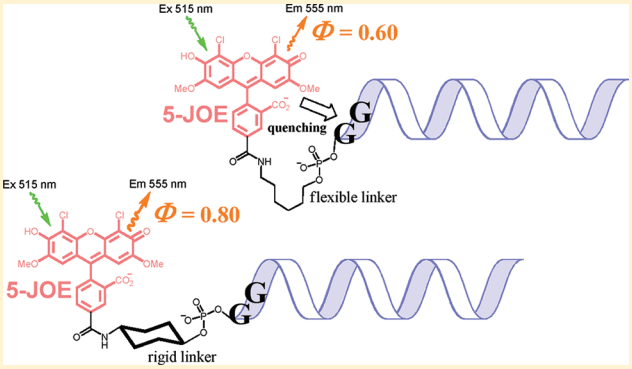
Supporting Information

ABSTRACT: A convenient procedure for the preparation of the fluorescent dye 4′,5′-dichloro-2′,7′-dimethoxy-5(6)-carboxyfluorescein (JOE) is reported; the overall yield achieved starting from isovanillin is 10 times higher (40% vs 4%) compared to the known procedure. Isomers (5- and 6-) are easily chromatographically separable as pentafluorophenyl esters of 3′,6′-*O*-bis(cyclohexylcarbonyl) derivatives. Four non-nucleoside JOE phosphoramidites based on 5- and 6-isomers and flexible 6-aminohexanol (AH) or rigid 4-*trans*-aminocyclohexanol (ACH) linkers have been prepared and used for oligonucleotide labeling. Spectral and photophysical properties of 5′-JOE-modified oligonucleotides have been studied. Fluorescence quantum yield of the dye correlates with the nature of the linker (rigid vs flexible) and with the presence of dG nucleosides in close proximity to a JOE residue.



INTRODUCTION

Fluorescent dyes play an exceptional role in nucleic acids technologies.[1−4] Xanthene fluorophore 4′,5′-dichloro-2′,7′-dimethoxy-5(6)-carboxyfluorescein (JOE), developed three decades ago,[5,6] possesses an absorbance (~525 nm) and emission (~550 nm) red-shifted compared to those of carboxyfluorescein (FAM). The substantial difference in emission wavelengths of FAM and JOE makes them spectrally resolvable and allows simultaneous use of both dyes in multiplex assays. In the late 1980s, JOE had been applied as a fluorescent label in DNA sequencing,[7,8] PCR,[9] and LCR[10] amplifications. Later on, the dye became one of the common fluorophores used in various formats of DNA sequencing based on the laser excitation of electrophoretically separated labeled DNA fragments, e.g., two dyes (FAM and JOE) labeled primers,[11,12] four dyes (FAM, JOE, TAMRA, ROX) labeled primers,[13−15] energy transfer primers,[16−19] and dye-labeled terminator[20] techniques. Oligonucleotides labeled with JOE found applications in an assay functionally equivalent to Southern blotting,[21] short tandem repeat (STR),[22−24] and single strand conformational polymorphism (SSCP)[25−27] techniques. Many real-time PCR platforms have spectral channels suitable for JOE,[28] and it is a common reporter dye in TaqMan probes,[29−36] PNA molecular beacons,[37] self-quenched probes LUX,[38−40] and HyBeacons.[41] Other applications worth mentioning of JOE in nucleic acid technology are fluorescent detection of mRNA,[42] DNA arrays[43] and solid phase minisequencing,[44] multiple SNP genotyping,[45] methylation assay,[46] detection of DNA-binding proteins[47] and ribosome−mRNA complexes,[48] and attaching of JOE-modified oligonucleotides to gold nanoparticles.[49] JOE applications nonrelated to nucleic acids are relatively rare, e.g., the labeling of cell surface proteins.[50]

The widespread use of JOE-labeled DNA fragments requires a scalable and reproducible method for the preparation of the carboxy dye derivatives as pure regioisomers. The existing approach to JOE synthesis[5,51] includes the preparation of 2-chloro-4-methoxyresorcinol followed by its condensation with trimellitic anhydride. The limitations of the synthesis reported are tedious chromatographic separations and poor yield. At present, JOE is available mainly as oxysuccinimide ester (6-JOE-SE) suitable for postsynthetic labeling of amino-modified oligonucleotides.[16,17,52] On the other hand, phosphoramidite reagents are compounds of choice to produce 5′-labeled oligonucleotides in automatic synthesizers. Although few JOE phosphoramidites are now commercially available, there are still no reports on their synthesis. Hence, the aims of the present work were the development of a simple and easily scalable procedure for the synthesis of 4′,5′-dichloro-2′,7′-dimethoxy-5(6)-carboxyfluorescein, the synthesis of phosphoramidite reagents derived from both isomers, and the study of spectral and photophysical properties of JOE-labeled oligonucleotides.

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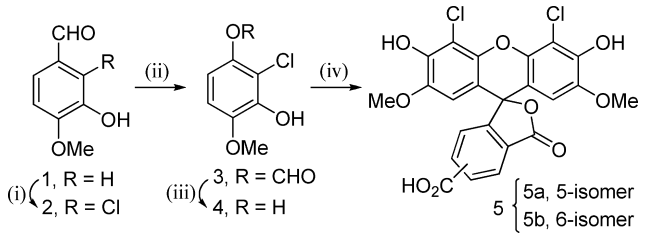
RESULTS AND DISCUSSION

The synthesis of 5(6)-carboxy-JOE was performed according to a previously described scheme,[5,51] with modifications that allowed an increase in the overall yield of the desired dye **5** by an order of magnitude (Scheme 1).

Scheme 1. Synthesis of JOE Chromophore[a]

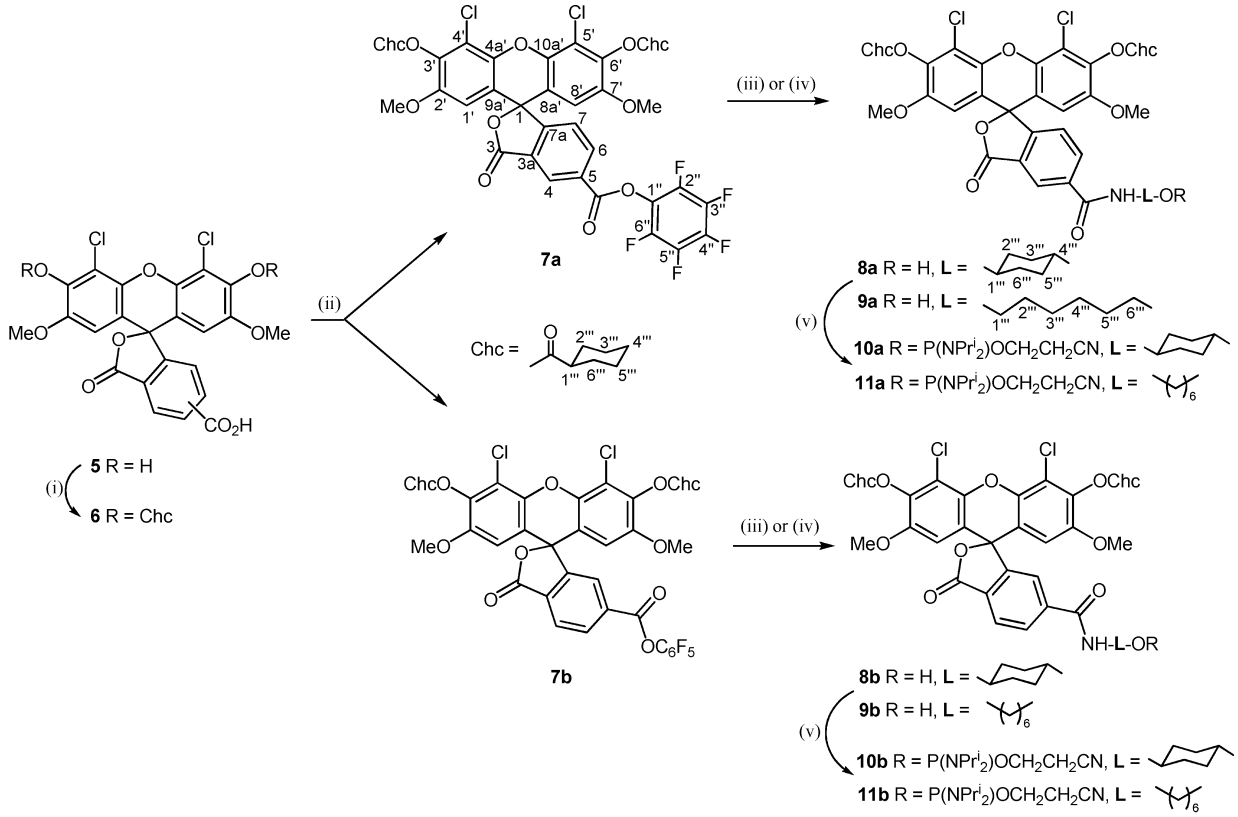


[a]Reaction conditions: (i) Cl_2, $CHCl_3$, 2.5 h, 78%; (ii) H_2O_2, SeO_2, CH_2Cl_2, 48 h; (iii) HCl, MeOH, 0.5 h, 84%; (iv) trimellitic anhydride, CH_3SO_3H, $SnCl_4$, 40 °C, 6 h, 61%.

Chlorination of the isovanillin was carried out as described[51,53] by bubbling chlorine gas through a solution of aldehyde **1** in chloroform. The precipitated 2-chloroisovanillin **2** was subjected to oxidation to yield the formate **3**, which was subsequently hydrolyzed to resorcinol **4**. Two last steps were combined in a one-pot procedure. Because the reaction of 2-chloroisovanillin **2** with MCPBA gives acidic byproducts interfering with isolation and thus decreasing the yield of resorcinol **4**,[51] hydrogen peroxide was tried for the oxidation step.[54] The reaction of 2-chloroisovanillin **2** with aq H_2O_2 in the presence of catalytic amounts of SeO_2 proceeded smoothly. The completion of oxidation step was determined easily: 2-cloroisovanillin is slightly soluble in dichloromethane; on the contrary, the formate **3** is well soluble. Thus, the initial suspension toward the end of oxidation became homogeneous. After $NaHSO_3$ workup, ester **3** was used without additional purification. Instead of the alkaline hydrolysis of formate ester,[5,51] we used acidic methanolysis to avoid unnecessary inorganic salt formation. The interesterification step proceeded smoothly and gave product that could be easily purified by crystallization from Et_2O–hexanes. These improvements increase the yield of resorcinol **4** up to four times as compared to the previously described method.[51]

The key step of the JOE synthesis is an assembly of xanthene dye **5** from resorcinol **4** and trimellitic anhydride. Condensation of these components in the presence of $ZnCl_2$[5] or heating in methanesulfonic acid at 160 °C[51] gave low yields of the desired dye. We observed that the condensation reaction indeed begins at ~160 °C; however, at this temperature large amounts of byproducts are formed. Surprisingly, we found that the use of a mixture of Lewis acid $SnCl_4$ with methanesulfonic acid as a dissolvent allows for dramatic reduction of the reaction temperature. The reaction proceeds smoothly at 40 °C with minimal byproduct formation to give the 61% isolated yield of the desired 5(6)-carboxy-JOE (**5**). Summarily, the overall yield 40% of dye **5** from isovanillin **1** was achieved in

Scheme 2. Synthesis of Four JOE Phosphoramidites[a]



[a]Reagents and conditions: (i) Chc_2O, Py, 70 °C, 1 h; (ii) C_6F_5OH, DCC, EtOAc, 2 h, 34% (**7a**), 22% (**7b**) from **5**; (iii) *trans*-4-aminocyclohexanol hydrochloride, DIEA, CH_2Cl_2, DMF, 2 h, 93% (**8a**), 97% (**8b**); (iv) 6-aminohexanol, DIEA, CH_2Cl_2, 2 h, 98% (**9a**), 96% (**9b**); (v) $Pr^i_2N(Cl)POCH_2CH_2CN$, DIEA, CH_2Cl_2, 1 h, 72% (**10a**), 70% (**10b**), 60% (**11a**), 64% (**11b**).

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Table 1. Spectroscopic (λ_{max}^{abs}, λ_{max}^{em})[a] and Photophysical (τ_{fl}, Φ_{fl})[b] Characteristics of Fluorescent Oligonucleotide Conjugates ON1−ON8 at Various pH (20°C)

#	modifying phosphoramidite	sequence, 5′ → 3′	pH	λ_{max}^{abs}, nm	λ_{max}^{em}, nm	τ_{fl},[c] ns	Φ_{fl}[d]
ON1	**10a**	**5-JOE^{ACH}**-agccacagtttacaacatttgtatct	8.35	522	555	4.54	0.81
			8.50	522	555	4.53	0.81
			9.00	522	555	4.56	0.87
ON2	**10b**	**6-JOE^{ACH}**-agccacagtttacaacatttgtatct	8.35	523	551	4.08	0.75
			8.50	524	551	4.10	0.74
			9.00	523	550	4.13	0.76
ON3	**11a**	**5-JOE^{AH}**-agccacagtttacaacatttgtatct	8.35	526	561	4.70	0.76
			8.50	525	560	4.70	0.77
			9.00	526	561	4.70	0.80
ON4	**11b**	**6-JOE^{AH}**-agccacagtttacaacatttgtatct	8.35	529	555	4.28	0.70
			8.50	529	557	4.19	0.69
			9.00	527	556	4.32	0.74
ON5	**10a**	**5-JOE^{ACH}**-ggagttgcagttgatgt	8.35	523	555	4.53	0.79
			8.50	523	555	4.51	0.79
			9.00	522	555	4.55	0.80
ON6	**10b**	**6-JOE^{ACH}**-ggagttgcagttgatgt	8.35	523	552	3.93	0.71
			8.50	523	550	3.92	0.71
			9.00	523	550	3.96	0.71
ON7	**11a**	**5-JOE^{AH}**-ggagttgcagttgatgt	8.35	525	559	3.98	0.58
			8.50	525	559	4.00	0.56
			9.00	526	558	4.29	0.63
ON8	**11b**	**6-JOE^{AH}**-ggagttgcagttgatgt	8.35	529	557	3.86	0.59
			8.50	529	557	3.76	0.59
			9.00	529	556	3.80	0.58

[a] λ_{max}^{abs} and λ_{max}^{em} = absorption and fluorescence wavelength maxima. [b] τ_{fl} = average fluorescence lifetime; Φ_{fl} = fluorescence quantum yield. [c] Error limit is ±0.05 ns. [d] Error limit is ±0.05.

contrast to the published 4% (8% yield of less than 50% pure compound).[51]

Pure isomers of 5- and 6-JOE can be obtained from the mixture **5**[51] and used for oxysuccinimide esters preparation. However, phosphoramidite reagents are preferred in the case of automatic solid-phase synthesis of 5′-modified oligonucleotides. Previously, we reported the convenient method for preparation of isomerically pure 5- and 6-FAM phosphoramidites and introduced the cyclohexanecarbonyl (Chc) protection group for 3′ and 6′ phenolic hydroxyls of fluorescein.[55] A similar strategy was applied for the synthesis of 5- and 6-JOE phosphoramidites (Scheme 2).

The first step for phosphoramidite synthesis is acyl protection of 3′- and 6′-OH groups of the dye. Acylation of **5** with either pivalic or cyclohexanecarboxylic anhydrides in DMF/DIEA[55] gave poor yields of 3′,6′-*O*-diacyl derivatives. Fortunately, we have found that 3′,6′-*O*-bis(cyclohexylcarbonyl) derivatives **6** form rapidly in pyridine as major products. In contrast, an unsatisfactory result was observed when pivalic anhydride was used in pyridine for acylation of phenolic groups, presumably because of the steric hindrance from chlorine and methoxy substituents in the xanthene ring. So, the cyclohexylcarbonyl group is protecting group of choice for JOE. Subsequent esterification of 3′,6′-*O*-bis(Chc) derivatives **6** with pentafluorophenol resulted in a mixture of isomeric products **7a** + **7b**, which is easily separable by column chromatography on silica gel. The chromatography uses basically toluene, well suitable solvent for recycling, as eluent, and thus is scaleable (dozens of grams in our hands).

trans-4-Aminocyclohexanol was used as an achiral linker arm for phosphoramidites[55−59] to make them more stable in acetonitrile solution compared to phosphoramidites derived from primary alcohols. Individual isomers **7a** (**7b**) reacted with *trans*-4-aminocyclohexanol to give amides **8a** (**8b**). The following phosphitylation with *N*,*N*-diisopropylamino-2-cyanoethoxychlorophosphine led to desired phosphoramidites **10a** (**10b**). Surprisingly, it was found that **10a** is prone to crystallization when dissolved in acetonitrile. The poor solubility in acetonitrile makes this reagent inconvenient for the standard, massive solid-phase DNA synthesis. So, we synthesized another two amides **9a** (**9b**) with more usual 6-aminohexanol linker arm. Phosphitylation of **9a** (**9b**) gives phosphoramidites **11a** (**11b**). Their solubility in MeCN was suitable for standard oligonucleotide synthesis.

Recently, we demonstrated that 5- and 6-regioisomers of xanthene dyes (FAM, TAMRA) attached to oligonucleotides show slightly different emission spectra.[55,59] In this paper, we continue our studies of spectroscopic and photophysical properties of xanthene dye-labeled oligonucleotides.

Phosphoramidites **10a**,**b** and **11a**,**b** were used as 0.1 M solutions in acetonitrile (except **10a**, which was dissolved in MeCN−CH_2Cl_2 1:1) in the last coupling step of machine-assisted solid-phase DNA synthesis for the preparation of 5′-labeled PCR primers: 5′-**JOE**-agccacagtttacaacatttgtatct-3′ (**ON1**−**ON4**) and 5′-**JOE**-ggagttgcagttgatgt-3′ (**ON5**−**ON8**). The second sequence containing 5′-ggag site was chosen for maximal dye quenching. Each conjugate was prepared using four phosphoramidites, containing 5- or 6-isomers of JOE, as well as *trans*-4-aminocyclohexanol (ACH) or 6-aminohexanol (AH) linker arms. The conjugates were characterized with UV−vis and fluorescence spectra at three different pH values (Table 1), usual conditions for fluorescein derivatives, e.g., for FAM fluorescence studies.[55]

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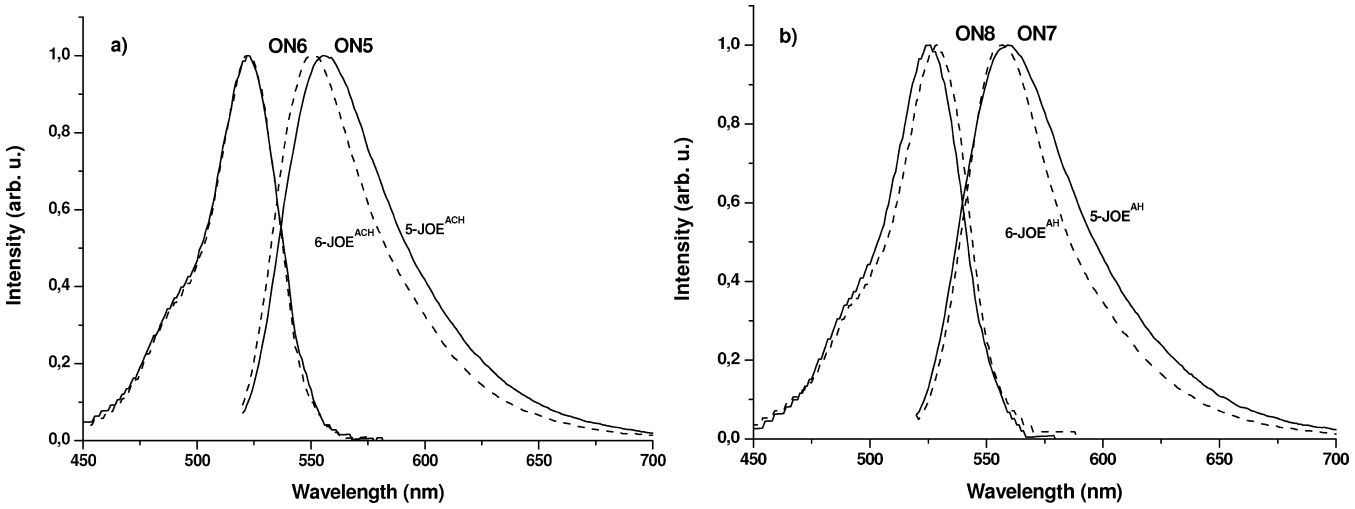

Figure 1. Normalized absorption (left) and fluorescence (right) spectra of JOE-labeled oligonucleotides (a) **ON5** (solid) and **ON6** (dashed); (b) **ON7** (solid) and **ON8** (dashed), in sodium bicarbonate buffer, pH 8.50, excitation at λ = 515 nm.

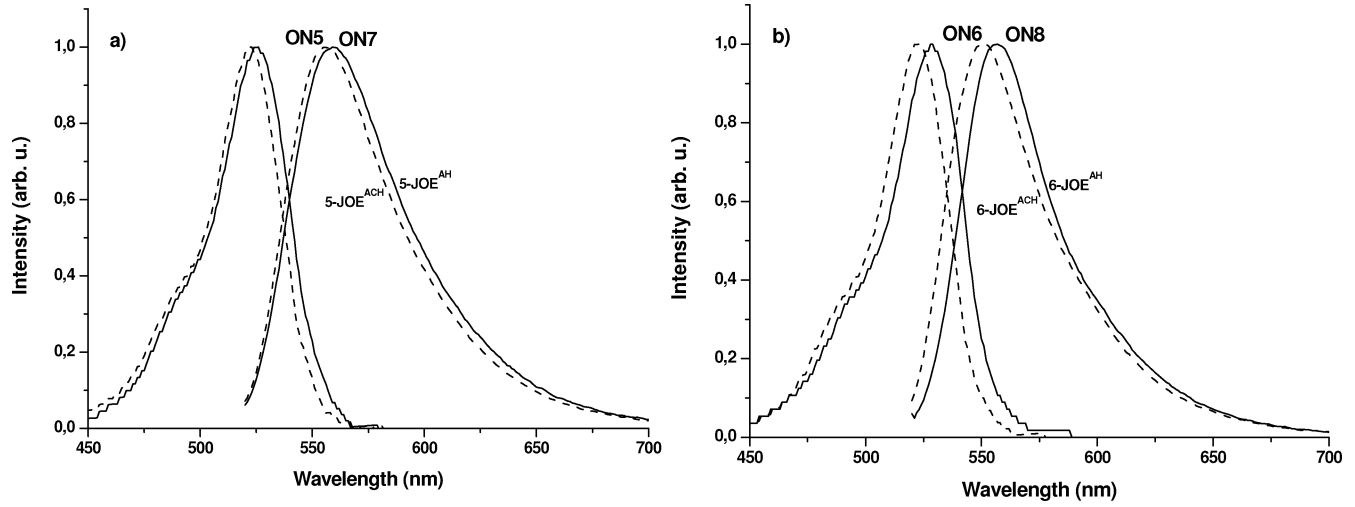

Figure 2. Normalized absorption (left) and fluorescence (right) spectra of JOE-labeled oligonucleotides (a) **ON5** (dashed) and **ON7** (solid); (b) **ON6** (dashed) and **ON8** (solid), in sodium bicarbonate buffer, pH 8.50, excitation at λ = 515 nm.

The data presented in Table 1 show that JOE-labeled oligonucleotides **ON1**−**ON8** have constant properties within pH range 8.35−9.00. Other factors, position of carboxamide group in regioisomers (5 vs 6), the nature of the linker arm (ACH vs AH), and the oligonucleotide sequence (the presence of 5′-dG nucleoside adjacent to JOE residue) show explicit influence on spectroscopic and photophysical characteristics of conjugates.

5-Isomers vs 6-Isomers (ON1 vs ON2, ON3 vs ON4, ON5 vs ON6, and ON7 vs ON8; Table 1, Figure 1). 6-Isomers show +1 to +4 nm in absorption maxima and −2 to −7 nm in emission maxima compared to 5-isomers (thus, Stokes shifts for 6-isomers are always 5−10 nm shorter); their average fluorescence lifetime is reduced by 0.3−0.6 ns (10−15%) and fluorescence quantum yield by approximately 0.05 (except for **ON6**/**ON7** pair). Absorption of 5- and 6-isomers is more (Figure 1a) or less (Figure 1b) similar; emission of 6-isomers is slightly blue-shifted and sharper (Figure 1a,b).

ACH vs AH Linkers (ON1 vs ON3, ON2 vs ON4, ON5 vs ON7, and ON6 vs ON8; Table 1, Figure 2). The more flexible AH linker always adds +3 to +6 nm to absorption and +3 to +7 nm to emission maxima, thus giving evidence of increased dye−oligonucleotide interaction (possibly, in the ground state). Indeed, the fluorescence quantum yield of ACH-linked dye is always higher. The effect is most pronounced in the case of **ON5**−**ON8**, containing two consecutive dG residues close to the dye; rigid ACH vs flexible AH linker adds 10% to quantum yield in the case of 6-JOE and a remarkable 20% for 5-JOE. Quenching of fluorescent dyes by G nucleobase is well-known.[60,61] Thus, the use of the rigid ACH linker and 5-JOE isomer (reagent **10a**) allows the complete neutralization of the negative effect of adjacent guanines. This is an important issue for the design of fluorescent DNA probes. Interestingly, cyclohexane derivatives were recently used to "insulate" perylene diimide residue and to prevent its fluorescence quenching with nucleobases.[62] Studies of the linker influence on the fluorescence of JOE-labeled DNA probes, including hybridization effects, are in progress and will be reported elsewhere.

To conclude, we have developed a simple procedure for the preparation of individual isomers of 4′,5′-dichloro-2′,7′-dimethoxy-5(6)-carboxyfluoresceins (JOE), described four JOE phosphoramidite reagents containing individual dye isomers, prepared a series of 5′-labeled oligonucleotides, and

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compared spectral and photophysical spectra of JOE–oligonucleotide conjugates. We also showed that the use of 5- or 6-isomer of JOE along with different type of linker arm allowed the adjustment of spectroscopic characteristics of labeled conjugates.

EXPERIMENTAL SECTION

General Methods. Reagents obtained from commercial suppliers were used without further purification. Pyridine and CH_2Cl_2 were used freshly distilled from CaH_2. DMF was distilled and stored over 4 Å molecular sieves under argon. Other solvents were used as received. *N*,*N*-Diisopropylamino-2-cyanoethoxychlorophosphine[63,64] was prepared as described. 600 MHz ^{1}H, 150 MHz ^{13}C, 564.4 MHz ^{19}F, and 243 MHz ^{31}P NMR spectra were referenced to $CDCl_3$ (7.26 ppm for ^{1}H and 77.16 ppm for ^{13}C), DMSO-d_6 (2.50 ppm for ^{1}H and 39.52 ppm for ^{13}C),[65] $PhCF_3$ (−63.72 ppm for ^{19}F), and 85% aq H_3PO_4, (0.00 ppm for ^{31}P). ^{1}H NMR coupling constants are reported in hertz (Hz) and refer to apparent multiplicities. The assignments of signals in ^{1}H and ^{13}C NMR spectra were done using 2D ^{1}H–^{13}C HSQC and HMBC NMR spectra. High resolution mass spectra (HRMS) were recorded in positive ion mode using electrospray ionization (ESI). Melting points were 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 by Primetech LLC (Minsk, Belarus) on a 200 nmol scale using standard protocols. Oligonucleotides were cleaved from solid supports, deprotected by concentrated ammonia treatment (60 °C, 6 h), purified using 20% denaturing PAGE (7 M urea) in Tris-borate buffer (pH 8.3), and desalted by gel filtration on Sephadex G-25 column in 1 × 10^{-5} M Tris-HCl buffer (pH 7.5, "saltless" buffer). UV absorption and fluorescence spectra were recorded in 0.1 M bicarbonate buffer (pH 8.35–9.00). Rhodamine 6G (R6G) in ethanol was used as a quantum yield standard. 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 (515 nm) did not exceed 0.05. The fluorescence quantum yields (Φ_{fl}) were calculated using the relative method according to the following equation:

$$\Phi_{fl} = \Phi_{fl(R6G)} \cdot \frac{I}{I_{R6G}} \cdot \frac{A_{R6G}}{A} \cdot \frac{n^2}{n_{R6G}^2}$$

where $\Phi_{fl\,(R6G)}$ is rhodamine 6G quantum yield in ethanol ($\Phi_{fl\,(R6G)}$ = 0.94[66]); I and I_{R6G} are integrated intensities of sample and R6G dye solutions, respectively; A and A_{R6G} are optical densities of sample and R6G dye solutions, respectively; and n and n_{R6G} are refractive indexes of ethanol and 0.1 M bicarbonate buffer, 1.3591 and 1.3340 at 20 °C, respectively. Fluorescence lifetimes were determined by the method of time-correlated single-photon counting using a nanosecond lifetime fluorometer. The parameters of the fluorescence decays were analyzed using T900 software. The samples used in quantum yield measurements were not degassed.

*2-Chloro-4-methoxyresorcinol (**4**).* To a suspension of 2-chloro-3-hydroxy-4-methoxybenzaldehyde **2**[51,53] (37.32 g, 0.20 mol) in CH_2Cl_2 (650 mL), SeO_2 (1.78 g, 0.016 mol) and 30% aq H_2O_2 (61 mL, 0.60 mol) were added. The mixture was vigorously stirred 48 h at ambient temperature. The organic layer was separated, washed with 10% $NaHSO_3$ (200 mL), dried over Na_2SO_4, and evaporated. The resulting oil was dissolved in methanolic HCl, obtained by addition of AcCl (20 mL) to MeOH (400 mL). The solution was stirred for 30 min at ambient temperature and evaporated, and the resulting lilac oil was crystallized from Et_2O–hexanes to give **4** as a crystalline solid (29.33 g, 84%, mp 76–77 °C (lit.[5] mp 69–70 °C, lit.[51] mp 74–76 °C)): R_f 0.36 (MeOH–$CHCl_3$ 1:19 v/v); ^{1}H NMR (DMSO-d_6) δ 9.44 (s, 1H, O*H*), 9.19 (s, 1H, O*H*), 6.72 (d, $J_{5,6}$ = 8.0 Hz, 1H, H-5), 6.35 (d, $J_{5,6}$ = 8.0 Hz, 1H, H-6), 3.71 (s, 6H, OCH_3); ^{13}C NMR (DMSO-d_6) δ 148.0 (C1), 143.9 (C3), 141.2 (C4), 111.0 (C5), 108.3 (C6), 104.8 (C2), 56.8 (OCH_3).

*4′,5′-Dichloro-2′,7′-dimethoxy-5(6)-carboxyfluorescein (**5**).* 2-Chloro-4-methoxyresorcinol **4** (17.45 g, 0.10 mol) and trimellitic anhydride (9.60 g, 0.05 mol) were dissolved in methanesulfonic acid (50 mL). After addition of $SnCl_4$ (5.85 mL, 0.05 mol), the mixture was stirred for 6 h at 40 °C and then cooled to room temperature. The mixture was poured into vigorously stirred water (300 mL), and the solid material was collected by filtration. It was flash chromatographed on neutral alumina, eluting with 30 → 50% ammonia (28% aq solution) in 2-propanol. The both isomers of **5** were collected as the second and the third colored bands. The fractions were evaporated and dissolved in water (250 mL), and the obtained solution was adjusted to pH 2 with concentrated HCl. The precipitate was collected and dried in a desiccator to give **5** as a red-brick solid (15.41 g, 61%) mixture of isomers: R_f 0.14 (5-isomer), 0.27 (6-isomer) (Et_3N–MeOH–$CHCl_3$ 1:5:14 v/v/v). To obtain the individual analytical samples of both isomers, 310 mg of the mixture was separated on a preparative column to give ammonium salts of **5a** (187 mg) and **5b** (138 mg).

*4′,5′-Dichloro-2′,7′-dimethoxy-5-carboxyfluorescein, Diammonium Salt (**5a·2NH$_3$**).* Spectral data: ^{1}H NMR (DMSO-d_6) δ 8.60 (d, 1H, $^4J_{4,6}$ = 1.4 Hz, H-4), 8.17 (dd, 1H, $J_{6,7}$ = 7.8 Hz, $^4J_{4,6}$ = 1.4 Hz, H-6), 7.40 (d, 1H, $J_{6,7}$ = 7.8 Hz, H-7), 6.01 (br. s, 2H, H-1′,8′), 3.43 (s, 6H, OCH_3).

*4′,5′-Dichloro-2′,7′-dimethoxy-6-carboxyfluorescein, Diammonium Salt (**5b·2NH$_3$**).* Spectral data: ^{1}H NMR (DMSO-d_6) δ 8.16 (dd, 1H, $J_{4,5}$ = 8.1 Hz, $^4J_{5,7}$ = 1.3 Hz, H-5), 8.12 (d, 1H, $J_{4,5}$ = 8.1 Hz, H-4), 7.81 (d, 1H, $J_{5,7}$ = 1.3 Hz, H-7), 6.01 (br. s, 2H, H-1′,8′), 3.44 (s, 6H, OCH_3).

3′,6′-*O*-Bis(cyclohexylcarbonyl)-4′,5′-dichloro-2′,7′-dimethoxy-5(6)-carboxyfluoresceins, Pentafluorophenyl Esters (7a,b); General Procedure. 4′,5′-Dichloro-2′,7′-dimethoxy-5(6)-carboxyfluorescein **5** (5.05 g, 10.0 mmol) was dissolved in pyridine (25 mL), and cyclohexanecarboxylic anhydride (7.14 g, 30.00 mmol) was added. The mixture was heated for 1 h at 70 °C, the solvent was evaporated, and the residue was dissolved in EtOAc (150 mL), washed with 0.5 M HCl (150 mL) and brine (150 mL), and dried over $MgSO_4$. After evaporation, the residue was dissolved in EtOAc (30 mL) and pentafluorophenol (2.02 g, 11.00 mmol) in EtOAc (7 mL), followed by the solution of DCC (2.47 g, 12.00 mmol) in EtOAc (7 mL). The mixture was stirred for 2 h at ambient temperature, the precipitate of dicyclohexylurea was filtered off, and the solution was concentrated to viscous oil. The residue was purified by column chromatography on silica, eluting with a step gradient of EtOAc in toluene (toluene → 1% EtOAc in toluene) to afford pure individual isomers **7a** (3.03 g, 34%) and **7b** (1.96 g, 22%).

*3′,6′-Bis(cyclohexylcarbonyloxy)-4′,5′-dichloro-2′,7′-dimethoxy-3-oxo-spiro[isobenzofuran-1(3H),9′-[9H]xanthene]-5-carboxylic acid, Pentafluorophenyl Ester (**7a**).* Spectral data: R_f 0.50 (EtOAc–toluene 1:19 v/v); ^{1}H NMR ($CDCl_3$) δ 8.86 (m, 1H, H-4), 8.48 (dd, 1H, $J_{6,7}$ = 8.0 Hz, $^4J_{4,6}$ = 1.0 Hz, H-6), 7.41 (d, 1H, $J_{6,7}$= 8.0 Hz, H-7), 6.24 (s, 2H, H-1′,8′), 3.63 (s, 6H, OCH_3), 2.69 (tt, 2H, $J_{a,a}$ = 11.0 Hz, $J_{a,e}$ = 3.7 Hz, H-1*a*‴), 2.12 (m, 4H, H-2*e*‴,6*e*‴), 1.83 (m, 4H, H-3*e*‴,5*e*‴), 1.72–1.61 (m, 6H, H-4*e*‴,2*a*‴,6*a*‴), 1.43–1.26 (m, 6H, H-3*a*‴,4*a*‴,5*a*‴); ^{13}C NMR ($CDCl_3$) δ 172.4 (2C, COCH), 167.5 (C3), 160.9 ($CO_2C_6F_5$), 158.1 (C7a), 149.1 (2C, C2′,7′), 141.3 (2C, C4a′,10a′), ~141 (m, 2C, $^1J_{CF}$ ~ 250 Hz, C2″,6″), ~140 (m, $^1J_{CF}$ ~ 250 Hz, C4″), 139.7 (2C, C3′,6′), ~138 (m, 2C, $^1J_{CF}$ ~ 250 Hz, C3″,5″), 137.4 (C6), 129.5 (C5), 128.3 (C4), 125.6 (C3a), 124.9 (m, 2C, C7,1″), 118.2 (2C, C4′,5′), 115.2 (2C, C8a′,9a′), 106.5 (2C, C1′,8′), 82.3 (C1), 56.6 (2C, OCH_3), 42.8 (2C, C1‴), 28.9 (4C, C2‴,6‴), 25.6 (2C, C4‴), 25.2 (4C, C3‴,5‴); ^{19}F NMR ($CDCl_3$), δ −152.20 (d, 2F, $J_{2'',3''}$ = $J_{5'',6''}$ = 19.8 Hz, F-2″,6″), −156.53 (t, 1F, $J_{3'',4''}$ = $J_{4'',5''}$ = 21.5 Hz, F-4″), −161.40 (m, 2F, F-3″,5″); HRMS (ESI+) *m*/*z* [M + H]$^+$ calcd for $C_{43}H_{34}{}^{35}Cl_2F_5O_{11}{}^+$ 891.1393, found 891.1355.

*3′,6′-Bis(cyclohexylcarbonyloxy)-4′,5′-dichloro-2′,7′-dimethoxy-3-oxo-spiro[isobenzofuran-1(3H),9′-[9H]xanthene]-6-carboxylic acid, Pentafluorophenyl Ester (**7b**).* Spectral data: R_f 0.31 (EtOAc–toluene 1:19 v/v); ^{1}H NMR ($CDCl_3$) δ 8.45 (dd, 1H, $J_{4,5}$ = 8.0 Hz, $^4J_{5,7}$ = 1.1 Hz, H-5), 8.21 (d, 1H, $J_{4,5}$ = 8.0 Hz, H-4), 7.97 (br. s, 1H, H-

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7), 6.23 (br. s, 2H, H-1′,8′), 3.63 (s, 6H, OCH_3), 2.69 (tt, 2H, $J_{a,a}$ = 11.0 Hz, $J_{a,e}$ = 3.7 Hz, H-1*a*‴), 2.11 (m, 4H, H-2*e*‴,6*e*‴), 1.83 (m, 4H, H-3*e*‴,5*e*‴), 1.71−1.60 (m, 6H, H-4*e*‴,2*a*‴,6*a*‴), 1.42−1.27 (m, 6H, H-3*a*‴,4*a*‴,5*a*‴); ^{13}C NMR ($CDCl_3$) δ 172.5 (2C, COCH), 167.7 (C3), 160.8 ($CO_2C_6F_5$), 153.6 (C7a), 149.0 (2C, C2′,7′), 141.4 (2C, C4a′,10a′), ∼141 (m, 2C, $^1J_{CF}$ ∼ 250 Hz, C2″,6″), ∼140 (m, $^1J_{CF}$ ∼ 250 Hz, C4″), 139.7 (2C, C3′,6′), ∼138 (m, 2C, $^1J_{CF}$ ∼ 250 Hz, C3″,5″), 132.5 (C5), 126.1 (2C, C4,7), 133.7 (C6), 129.4 (C3a), 124.6 (m, C1″), 118.2 (2C, C4′,5′), 115.2 (2C, C8a′,9a′), 106.6 (2C, C1′,8′), 82.4 (C1), 56.6 (2C, OCH_3), 42.8 (2C, C1‴), 28.9 (4C, C2‴,6‴), 25.7 (2C, C4‴), 25.2 (4C, C3‴,5‴); ^{19}F NMR ($CDCl_3$) δ −151.57 (m, 2F, F-2″,6″), −156.59 (t, 1F, $J_{3'',4''} = J_{4'',5''}$ = 21.1 Hz, F-4″), −161.56 (m, 2F, F-3″,5″); HRMS (ESI+) *m*/*z* [M + H]$^+$ calcd for $C_{43}H_{34}{}^{35}Cl_2F_5O_{11}{}^+$ 891.1393, found 891.1381.

Amides 8a,b; General Procedure. To a solution of activated ester **7a** (**7b**) (0.89 g, 1 mmol) in CH_2Cl_2 (10 mL), *trans*-4-aminocyclohexanol hydrochloride (0.15 g, 1 mmol) and DIEA (0.34 mL, 2 mmol) were added. The suspension was diluted with DMF (10 mL) and stirred for 2 h at ambient temperature to give a homogeneous solution. This was diluted with EtOAc (100 mL), washed with H_2O (100 mL), saturated $NaHCO_3$ (100 mL), and brine (100 mL), and dried over $MgSO_4$. After evaporation, the residue was chromatographed on silica, eluting with a step gradient 0 → 5% MeOH in toluene to give desired amides **8a** (**8b**).

*3′,6′-Bis(cyclohexylcarbonyloxy)-4′,5′-dichloro-2′,7′-dimethoxy-5-(4-hydroxy-trans-cyclohexylamino-carbonyl)-3-oxo-spiro-[isobenzofuran-1(3H),9′-[9H]xanthene] (**8a**).* Yield 0.76 g (92%): R_f 0.21 (acetone−toluene 3:7 v/v); ^{1}H NMR (DMSO-*d*$_6$) δ 8.70 (m, 1H, N*H*), 8.55 (m, 1H, H-4), 8.28 (dd, 1H, $J_{6,7}$ = 8.0 Hz, $^4J_{4,6}$ = 1.3 Hz, H-6), 7.60 (d, 1H, $J_{6,7}$ = 8.0 Hz, H-7), 6.42 (s, 2H, H-1′,8′), 4.56 (d, 1H, *J* = 4.3 Hz, O*H*), 3.67 (m, 1H, H-4″), 3.56 (s, 6H, OCH_3), 3.32 (m, 1H, H-1″), 2.74 (tt, 2H, $J_{a,e}$ = 3.6 Hz, $J_{a,a}$ = 10.8 Hz, H-1*a*‴), 1.99 (m, 4H, H-2*e*‴,6*e*‴), 1.73 (m, 8H, H-2*e*″,3*e*″,5*e*″,6*e*″,3*e*‴,5*e*‴), 1.62−1.50 (m, 6H), 1.40−1.09 (m, 10H) (H-2*a*″,3*a*″,5*a*″,6*a*″,2*a*‴,3*a*‴,4*a*‴,-4*e*‴,5*a*‴,6*a*‴); ^{13}C NMR (DMSO-*d*$_6$) δ 172.0 (2C, COCH), 167.7 (C3), 164.2 (CONH), 153.2 (C7a), 148.6 (2C, C2′,7′), 140.6 (2C, C4a′,10a′), 138.7 (2C, C3′,6′), 136.7 (C5), 134.9 (C6), 125.7 (C3a), 124.2 (2C, C4,7), 116.5 (2C, C4′,5′ or C8a′,9a′), 116.2 (2C, C4′,5′ or C8a′,9a′), 108.3, 108.2 (C1′,8′), 81.2 (C1), 68.1 (C1″), 56.7 (2C, OCH_3), 48.1 (C4″), 41.7 (2C, C1‴), 34.0 (2C, C2″,6″), 30.0 (2C, C3″,5″), 28.5 (4C, C2‴,6‴), 25.2 (2C, C4‴), 24.5 (4C, C3‴,5‴); HRMS (ESI+) *m*/*z* [M + Na]$^+$ calcd for $C_{43}H_{45}{}^{35}Cl_2NNaO_{11}{}^+$ 844.2262, found 844.2250.

*3′,6′-Bis(cyclohexylcarbonyloxy)-4′,5′-dichloro-2′,7′-dimethoxy-6-(4-hydroxy-trans-cyclohexylaminocarbonyl)-3-oxo-spiro-[isobenzofuran-1(3H),9′-[9H]xanthene] (**8b**).* Yield 0.80 g (97%): R_f 0.17 (acetone−toluene 3:7 v/v); ^{1}H NMR (DMSO-*d*$_6$) δ 8.37 (d, 1H, *J* = 5.5 Hz, N*H*), 8.22 (dd, 1H, $J_{4,5}$ = 8.0 Hz, $^4J_{5,7}$ = 0.8 Hz, H-5), 8.15 (d, 1H, $J_{4,5}$ = 8.0 Hz, H-4), 7.85 (m, 1H, H-7), 6.42 (s, 2H, H-1′,8′), 4.56 (d, 1H, *J* = 4.3 Hz, O*H*), 3.67 (m, 1H, H-4″), 3.57 (s, 6H, OCH_3), 3.32 (m, 1H, H-1″), 2.74 (tt, 2H, $J_{a,e}$ = 3.6 Hz, $J_{a,a}$ = 10.8 Hz, H-1*a*‴), 2.00 (m, 4H, H-2*e*‴,6*e*‴), 1.75 (m, 8H, H-2*e*″,3*e*″,5*e*″,6*e*″,3*e*‴,5*e*‴), 1.65−1.52 (m, 6H), 1.43−1.12 (m, 10H) (H-2*a*″,3*a*″,5*a*″,6*a*″,-2*a*‴,3*a*‴,4*a*‴,4*e*‴,5*a*‴,6*a*‴); ^{13}C NMR (DMSO-*d*$_6$) δ 172.0 (2C, COCH), 167.6 (C3), 163.6 (CONH), 151.6 (C7a), 148.7 (2C, C2′,7′), 141.1 (C6), 140.4 (2C, C4a′,10a′), 138.7 (2C, C3′,6′), 130.3 (C5), 127.2 (C3a), 125.6 (C4), 122.1 (C7), 116.3 (2C, C4′,5′ or C8a′,9a′), 116.2 (2C, C4′,5′ or C8a′,9a′), 108.4 (2C, C1′,8′), 81.2 (C1), 68.3 (C1″), 56.8 (2C, OCH_3), 48.3 (C4″), 41.7 (2C, C1‴), 34.2 (2C, C2″,6″), 30.2 (2C, C3″,5″), 28.5 (4C, C2‴,6‴), 25.2 (2C, C4‴), 24.5 (4C, C3‴,5‴); HRMS (ESI+) *m*/*z* [M + Na]$^+$ calcd for $C_{43}H_{45}{}^{35}Cl_2NNaO_{11}{}^+$ 844.2262, found 844.2272.

Amides 9a,b; General Procedure. To a solution of activated ester **7a** (**7b**) (0.89 g, 1 mmol) in CH_2Cl_2 (5 mL), DIEA (0.174 mL, 1 mmol) and 6-aminohexanol (0.117 g, 1 mmol) in CH_2Cl_2 (1 mL) were added. The solution was stirred for 2 h at ambient temperature. This was diluted with CH_2Cl_2 (50 mL), washed with H_2O (50 mL), saturated $NaHCO_3$ (50 mL), and brine (50 mL), and dried over $MgSO_4$. After evaporation, the residue was chromatographed on silica, eluting with a step gradient 0 → 20% acetone in toluene to give desired amides **9a** (**9b**).

*3′,6′-Bis(cyclohexylcarbonyloxy)-4′,5′-dichloro-2′,7′-dimethoxy-5-(6-hydroxyhexylaminocarbonyl)-3-oxo-spiro[isobenzofuran-1(3H),9′-[9H]xanthene] (**9a**).* Yield 0.81 g (98%): R_f 0.22 (acetone−toluene 3:7 v/v); ^{1}H NMR (DMSO-*d*$_6$) δ 8.85 (t, 1H, *J* = 5.5 Hz, N*H*), 8.55 (m, 1H, H-4), 8.28 (dd, 1H, $J_{6,7}$ = 8.0 Hz, $^4J_{4,6}$ = 1.3 Hz, H-6), 7.60 (d, 1H, $J_{6,7}$ = 8.0 Hz, H-7), 6.42 (s, 2H, H-1′,8′), 4.35 (t, 1H, *J* = 5.3 Hz, O*H*), 3.56 (s, 6H, OCH_3), 3.39 (m, 2H, H-6″), 3.30 (m, 2H, H-1″), 2.73 (tt, 2H, $J_{a,e}$ = 3.7 Hz, $J_{a,a}$ = 10.7 Hz, H-1*a*‴), 1.99 (m, 4H, H-2*e*‴,6*e*‴), 1.72 (m, 4H, H-3*e*‴,5*e*‴), 1.63−1.49 (m, 8H, H-2″,2*a*‴,4*e*‴,6*a*‴), 1.45−1.20 (m, 12H, H-3″,4″,5″,3*a*‴,4*a*‴,5*a*‴); ^{13}C NMR (DMSO-*d*$_6$) δ 172.0 (2C, COCH), 167.7 (C3), 164.2 (CONH), 153.2 (C7a), 148.6 (2C, C2′,7′), 140.6 (2C, C4a′,10a′), 138.7 (2C, C3′,6′), 136.7 (C5), 134.9 (C6), 125.7 (C3a), 124.2 (2C, C4,7), 116.5 (2C, C4′,5′ or C8a′,9a′), 116.2 (2C, C4′,5′ or C8a′,9a′), 108.3, 108.2 (C1′,8′), 81.2 (C1), 60.7 (C6″), 56.8 (2C, OCH_3), 41.7 (2C, C1‴), ∼40 (C1″), 32.5 (C5″), 29.1 (C2″), 28.5 (4C, C2‴,6‴), 26.5 (C3″), 25.3 (C4″), 25.2 (2C, C4‴), 24.5 (4C, C3‴,5‴); HRMS (ESI+) *m*/*z* [M + Na]$^+$ calcd for $C_{43}H_{47}{}^{35}Cl_2NNaO_{11}{}^+$ 846.2418, found 846.2436.

*3′,6′-Bis(cyclohexylcarbonyloxy)-4′,5′-dichloro-2′,7′-dimethoxy-6-(6-hydroxyhexylaminocarbonyl)-3-oxo-spiro[isobenzofuran-1(3H),9′-[9H]xanthene] (**9b**).* Yield 0.79 g (96%): R_f 0.17 (acetone−toluene 3:7 v/v); ^{1}H NMR (DMSO-*d*$_6$) δ 8.65 (t, 1H, *J* = 5.4 Hz, N*H*), 8.21 (dd, 1H, $J_{4,5}$ = 8.0 Hz, $^4J_{5,7}$ = 0.8 Hz, H-5), 8.15 (d, 1H, $J_{4,5}$ = 8.0 Hz, H-4), 7.83 (br.s, 1H, H-7), 6.44 (s, 2H, H-1′,8′), 4.32 (m, 1H, O*H*), 3.57 (s, 6H, OCH_3), ∼3.30 (m, 2H, H-6″), 3.19 (m, 2H, H-1″), 2.73 (tt, 2H, $J_{a,e}$ = 3.7 Hz, $J_{a,a}$ = 10.8 Hz, H-1*a*‴), 1.99 (m, 4H, H-2*e*‴,6*e*‴), 1.72 (m, 4H, H-3*e*‴,5*e*‴), 1.63−1.58 (m, 2H, H-4*e*‴), 1.57−1.50 (m, 4H, H-2*a*‴,6*a*‴), 1.46 (m, 2H, H-2″), 1.41−1.33 (m, 6H, H-3*a*‴,4*a*‴,5*a*‴), 1.30−1.20 (m, 6H, H-3″,4″,5″); ^{13}C NMR (DMSO-*d*$_6$) δ 172.0 (2C, COCH), 167.6 (C3), 164.2 (CONH), 151.6 (C7a), 148.7 (2C, C2′,7′), 141.2 (C6), 140.4 (2C, C4a′,10a′), 138.7 (2C, C3′,6′), 130.1 (C5), 127.1 (C3a), 125.7 (C4), 122.1 (C7), 116.4 (2C), 116.2 (2C) (C4′,5′,8a′,9a′), 108.4 (2C, C1′,8′), 81.1 (C1), 60.6 (C6″), 56.8 (2C, OCH_3), 41.7 (2C, C1‴), ∼40 (C1″), 32.4 (C5″), 29.0 (C2″), 28.5 (4C, C2‴,6‴), 26.5 (C3″), 25.2 (3C, C4″,4‴), 24.5 (4C, C3‴,5‴); HRMS (ESI+) *m*/*z* [M + Na]$^+$ calcd for $C_{43}H_{47}{}^{35}Cl_2NNaO_{11}{}^+$ 846.2418, found 846.2429.

Phosphoramidites 10−11a,b; General Procedure. Amide **8a,b** (**9a,b**) (0.41 g, 0.50 mmol) was evaporated with dry CH_2Cl_2 (2 × 10 mL), dissolved in dry CH_2Cl_2 (3 mL) and DIEA (0.104 mL, 0.60 mmol), followed by *N*,*N*-diisopropylamino-2-cyanoethoxychlorophosphine (0.142 g, 0.60 mmol). The mixture was stirred under argon for 1 h, diluted with CH_2Cl_2 (20 mL), washed with saturated $NaHCO_3$ (2 × 20 mL) and brine (20 mL), and dried for 1 h over Na_2SO_4. After evaporation, the residue was chromatographed on silica, eluting with 1% Py + 5% acetone in toluene to give desired phosphoramidites **10a,b** (**11a,b**).

*3′,6′-Bis(cyclohexylcarbonyloxy)-4′,5′-dichloro-2′,7′-dimethoxy-5-[4-(N,N-diisopropylamino-2-cyanoethoxyphosphinyloxy)-trans-cyclohexylaminocarbonyl]-3-oxo-spiro[isobenzofuran-1(3H),9′-[9H]xanthene] (**10a**).* Yield 0.37 g (72%): R_f 0.80 (Et_3N−acetone−toluene 1:4:15 v/v/v); ^{1}H NMR (DMSO-*d*$_6$) δ 8.62 (d, 1H, *J* = 7.8 Hz, N*H*), 8.58 (br.s, 1H, H-4), 8.29 (dd, 1H, $J_{4,5}$ = 8.1 Hz, $^4J_{5,7}$ = 1.2 Hz, H-6), 7.60 (d, 1H, $J_{4,5}$ = 8.1 Hz, H-7), 6.40 (m, 2H, H-1′,8′), 3.84 (m, 1H), 3.77−3.65 (m, 3H) (H-1″,4″, $POCH_2$), 3.60 (m, 2H, $CHCH_3$), 3.56 (s, 6H, OCH_3), 2.77 (t, 2H, *J* = 6.0 Hz, CH_2CN), 2.73 (tt, 2H, $J_{a,e}$ = 3.6 Hz, $J_{a,a}$ = 10.8 Hz, H-1*a*‴), 2.05−1.86 (m, 8H, H-2*e*″,3*e*″,5*e*″,6*e*″,2*e*‴,6*e*‴), 1.72 (m, 4H, H-3*e*‴,5*e*‴), 1.63−1.21 (m, 14H, H-2*a*″,3*a*″,5*a*″,6*a*″, H-2*a*‴,3*a*‴,5*a*‴,6*a*″,2*a*‴,3*a*‴,4*a*‴,5*a*‴,6*a*‴), 1.15 (d, 12H, *J* = 7.2 Hz, CCH_3); ^{13}C NMR (DMSO-*d*$_6$) δ 172.0 (2C, COCH), 167.7 (C3), 163.6 (CONH), 153.2 (C7a), 148.6 (2C, C2′,7′), 140.6 (2C, C4a′,10a′), 138.7 (2C, C3′,6′), 136.7 (C5), 135.1 (C6), 125.6 (C3a), 124.2 (2C, C4,7), 119.1 (CN), 116.4 (2C), 116.2 (2C) (C4′,5′,8a′,9a′), 108.3 (2C, C1′,8′), 81.3 (C1), 71.8 (d, $^2J_{P,C}$ = 18 Hz, C1″), 58.0 (d, $^2J_{P,C}$ = 18 Hz, $POCH_2$), 56.8 (2C, OCH_3), 47.8 (C4″), 42.4 (d, $^2J_{P,C}$ = 13.5 Hz, PNCH), 41.7 (2C, C1‴), 32.8, 32.7 (C2″,6″), 29.9 (2C, C3″,5″), 28.5 (4C, C2‴,6‴), 25.2 (2C, C4‴), 24.5 (4C, C3‴,5‴), 24.4 (d, 2C, $^3J_{P,C}$ = 7.5 Hz), 24.3 (d, 2C, $^3J_{P,C}$ = 7.5 Hz) ($CHCH_3$), 19.8 (d, $^3J_{P,C}$ = 7.5 Hz, CH_2CN); ^{31}P NMR (DMSO-*d*$_6$) δ

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144.85; HRMS (ESI+) m/z [M + Na]$^+$ calcd for $C_{52}H_{62}{}^{35}Cl_2N_3NaO_{12}P^+$ 1044.3340, found 1044.3349.

*3′,6′-Bis(cyclohexylcarbonyloxy)-4′,5′-dichloro-2′,7′-dimethoxy-6-[4-(N,N-diisopropylamino-2-cyanoethoxyphosphinyloxy)-trans-cyclohexylaminocarbonyl]-3-oxo-spiro[isobenzofuran-1(3H),9′-[9H]xanthene] (**10b**).* Yield 0.36 g (70%): R_f 0.65 (Et_3N–acetone–toluene 1:4:15 v/v/v); ^{1}H NMR (DMSO-d_6) δ 8.39 (d, 1H, J = 7.8 Hz, N*H*), 8.22 (m, 1H, H-5), 8.15 (d, 1H, $J_{4,5}$ = 8.1 Hz, H-4), 7.85 (br.s, 1H, H-7), 6.42 (s, 2H, H-1′,8′), 3.75–3.62 (m, 4H, H-1″,4″, POCH_2), 3.57 (s, 6H, OCH_3), 3.56–3.50 (m, 2H, C*H*CH$_3$), 2.74 (m, 2H, H-1*a*‴), 2.02–1.94 (m, 4H, H-2*e*‴,6*e*‴), 1.90–1.70 (m, 8H, H-2*e*″,3*e*″,5*e*″,6*e*″,3*e*‴,5*e*‴), 1.63–1.50 (m, 6H), 1.41–1.15 (m, 10H) (H-2*a*″,3*a*″,5*a*″,6*a*″,2*a*‴,3*a*‴,4*a*‴,4*e*‴,5*a*‴,6*a*‴), 1.12 (m, 12H, CHCH_3); ^{13}C NMR (DMSO-d_6) δ 172.0 (2C, *C*OCH), 167.6 (C3), 163.6 (CONH), 151.6 (C7a), 148.7 (2C, C2′,7′), 141.1 (C6), 140.4 (2C, C4a′,10a′), 138.7 (2C, C3′,6′), 128.9 (C5), 127.2 (C3a), 125.6 (C4), 122.1 (C7), 119.1 (*C*N), 116.4 (2C), 116.2 (2C) (C4′,5′,8a′,9a′), 108.4 (2C, C1′,8′), 81.2 (C1), 71.8 (m, C1″), 57.9 (d, $^2J_{P,C}$ = 19.5 Hz, POCH_2), 56.8 (2C, OCH_3), 47.9 (C4″), 42.4 (d, $^2J_{P,C}$ = 19.5 Hz, PNCH), 41.7 (2C, C1‴), 32.7 (m, 2C, C2″,6″), 29.9 (m, 2C, C3″,5″), 28.5 (4C, C2‴,6‴), 25.2 (2C, C4‴), 24.5 (4C, C3‴,5‴), 24.4 (d, 2C, $^3J_{P,C}$ = 10.5 Hz), 24.3 (d, 2C, $^3J_{P,C}$ = 10.5 Hz) (CH*C*H$_3$), 19.8 (d, $^3J_{P,C}$ = 10.5 Hz, *C*H$_2$CN); ^{31}P NMR (DMSO-d_6) δ 144.79; HRMS (ESI+) m/z [M + Na]$^+$ calcd for $C_{52}H_{62}{}^{35}Cl_2N_3NaO_{12}P^+$ 1044.3340, found 1044.3320.

*3′,6′-Bis(cyclohexylcarbonyloxy)-4′,5′-dichloro-2′,7′-dimethoxy-5-[6-(N,N-diisopropylamino-2-cyanoethoxyphosphinyloxy)-hexylaminocarbonyl]-3-oxo-spiro[isobenzofuran-1(3H),9′-[9H]-xanthene] (**11a**).* Yield 0.31 g (60%): R_f 0.82 (Et_3N–acetone–toluene 1:4:15 v/v/v); ^{1}H NMR (DMSO-d_6) δ 8.85 (m, 1H, N*H*), 8.55 (m, 1H, H-4), 8.28 (dd, 1H, $J_{6,7}$ = 8.0 Hz, $^4J_{4,6}$ = 1.2 Hz, H-6), 7.60 (d, 1H, $J_{6,7}$ = 8.0 Hz, H-7), 6.42 (s, 2H, H-1′,8′), 3.77–3.50 (m, 6H, H-6″, POCH_2, C*H*CH$_3$), 3.56 (s, 6H, OCH_3), 3.30 (m, 2H, H-1″), 2.77–2.68 (m, 4H, H-1*a*‴, CH_2CN), 2.00 (m, 4H, H-2*e*‴,6*e*‴), 1.72 (m, 4H, H-3*e*‴,5*e*‴), 1.65–1.47 (m, 8H, H-2″,2*a*‴,4*e*‴,6*a*‴), 1.43–1.20 (m, 12H, H-3″,4″,5″,3*a*‴,4*a*‴,5*a*‴), 1.12 (d, 6H, J = 6.8 Hz), 1.11 (d, 6H, J = 6.8 Hz) (CHCH_3); ^{13}C NMR (DMSO-d_6) δ 172.0 (2C, *C*OCH), 167.7 (C3), 164.2 (CONH), 153.2 (C7a), 148.6 (2C, C2′,7′), 140.6 (2C, C4a′,10a′), 138.7 (2C, C3′,6′), 136.7 (C5), 134.9 (C6), 125.7 (C3a), 124.2 (2C, C4,7), 119.1 (*C*N), 116.5 (2C), 116.2 (2C) (C4′,5′,8a′,9a′), 108.3 (2C, C1′,8′), 81.2 (C1), 62.9 (d, $^2J_{P,C}$ = 16.0 Hz, C6″), 58.1 (d, $^2J_{P,C}$ = 19.0 Hz, POCH_2), 56.8 (2C, OCH_3), 42.4 (d, 2C, $^2J_{P,C}$ = 12.0 Hz, PNCH), 41.7 (2C, C1‴), ~40 (C1″), 30.7 (d, $^3J_{P,C}$ = 7.0 Hz, C5″), 29.0 (C2″), 28.5 (4C, C2‴,6‴), 26.2 (C3″), 25.3 (C4″), 25.2 (2C, C4‴), 24.5 (4C, C3‴,5‴), 24.4 (m, 4C, CHCH_3), 19.8 (d, $^3J_{P,C}$ = 7.0 Hz, *C*H$_2$CN); ^{31}P NMR (DMSO-d_6) δ 146.23; HRMS (ESI+) m/z [M + Na]$^+$ calcd for $C_{52}H_{64}{}^{35}Cl_2N_3NaO_{12}P^+$ 1046.3497, found 1046.3505.

*3′,6′-Bis(cyclohexylcarbonyloxy)-4′,5′-dichloro-2′,7′-dimethoxy-6-[6-(N,N-diisopropylamino-2-cyanoethoxyphosphinyloxy)-hexylaminocarbonyl]-3-oxo-spiro[isobenzofuran-1(3H),9′-[9H]-xanthene] (**11b**).* Yield 0.33 g (64%): R_f 0.66 (Et_3N–acetone–toluene 1:4:15 v/v/v); ^{1}H NMR (DMSO-d_6) δ 8.66 (m, 1H, N*H*), 8.21 (m, 1H, H-5), 8.15 (m, 1H, H-4), 7.83 (br.s, 1H, H-7), 6.44 (s, 2H, H-1′,8′), 3.77–3.50 (m, 6H, H-6″, POCH_2, C*H*CH$_3$), 3.56 (s, 6H, OCH_3), 3.19 (m, 2H, H-1″), 2.77–2.68 (m, 4H, H-1*a*‴, CH_2CN), 2.00 (m, 4H, H-2*e*‴,6*e*‴), 1.72 (m, 4H, H-3*e*‴,5*e*‴), 1.65–1.47 (m, 8H, H-2″,2*a*‴,4*e*‴,6*a*‴), 1.43–1.20 (m, 12H, H-3″,4″,5″,3*a*‴,4*a*‴,5*a*‴), 1.11 (d, 6H, J = 6.8 Hz), 1.08 (d, 6H, J = 6.8 Hz) (CHCH_3); ^{13}C NMR (DMSO-d_6) δ 172.0 (2C, *C*OCH), 167.7 (C3), 164.2 (CONH), 151.7 (C7a), 148.7 (2C, C2′,7′), 141.2 (C6), 140.4 (2C, C4a′,10a′), 138.8 (2C, C3′,6′), 130.2 (C5), 127.1 (C3a), 125.7 (C4), 122.1 (C7), 119.1 (*C*N), 116.4 (2C), 116.2 (2C) (C4′,5′,8a′,9a′), 108.4 (2C, C1′,8′), 81.2 (C1), 62.9 (d, $^2J_{P,C}$ = 20.0 Hz, C6″), 58.1 (d, $^2J_{P,C}$ = 22.8 Hz, POCH_2), 56.8 (2C, OCH_3), 42.4 (d, 2C, $^2J_{P,C}$ = 15.0 Hz, PNCH), 41.8 (2C, C1‴), ~40 (C1″), 30.6 (m, $^3J_{P,C}$ = 6.8 Hz, C5″), 29.0 (C2″), 28.6 (4C, C2‴,6‴), 26.2 (C3″), 25.3 (C4″), 25.2 (2C, C4‴), 24.5 (4C, C3‴,5‴), 24.4 (m, 4C, CHCH_3), 19.8 (d, $^3J_{P,C}$ = 10.0 Hz, *C*H$_2$CN); ^{31}P NMR (DMSO-d_6) δ 146.22; HRMS (ESI+) m/z [M + Na]$^+$ calcd for $C_{52}H_{64}{}^{35}Cl_2N_3NaO_{12}P^+$ 1046.3497, found 1046.3496.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H and ^{13}C NMR spectra for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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