Method for Preparing New Flavin Derivatives: Synthesis of Flavin−Thymine Nucleotides and Flavin−Oligonucleotide Adducts

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In order to link to the 5′-end of oligonucleotides the flavin analogs **9a**,**b** possessing only one terminal hydroxy group on the side chain, the phosphoramidite and the H-phosphonate coupling methods were developed. Surprisingly, after reaction of compounds **9a,b** with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite, the flavin phosphoramidates **11a,b** were isolated instead of the expected phosphoramidite derivatives **10a,b**. A very efficient photooxidation process occurred probably during the isolation of the products. From the prepared flavin H-phosphonates **12a,b**, flavin−thymine nucleotides and flavin−oligonucleotide adducts were synthesized for the first time. The versatility of the method was demonstrated in the oxidation step with the synthesis of the flavin−thymine nucleotides **15**−**17** possessing a phosphodiester, a phosphorothioate, and a methyl phosphate linkage, respectively. This method is of general interest with regard to the extensive research developed for preparing flavin analogs and modified oligonucleotides possessing interesting biological or/and catalytic properties.

Introduction

Artificial modulation of gene expression can be achieved by synthetic oligonucleotides.[1] In the antisense strategy, the ability of oligonucleotides to base pair in the Watson−Crick mode is used to recognize a specific *m*-RNA sequence and inhibit translation.[1,2] Hoogsteen or reverse Hoogsteen hydrogen bondings are involved in the antigene strategy with triplex-forming oligonucleotides targeted to double-stranded DNA sequences in order to inhibit transcription.[1,3]

Modifications of the oligonucleotides have been achieved to increase their stability with regard to nuclease degradation, to improve their cellular uptake or their DNA binding capacities, and/or to incorporate a reactive group that provides the oligonucleotides with, for example, a nucleic acid-cleavage function.[4] Oligonucleotides have been linked to a large number of chemical groups such as intercalators, hydrophobic residues, and chemically reactive molecules.[5]

The development of conjugates with new properties should improve the efficiency of oligonucleotides in the control of gene expression and should give new tools for *in vitro* and *in vivo* studies of nucleic acids. Flavins are naturally occurring fluorescent compounds[6] that are involved as cofactor or/and substrate in many enzymatic redox processes.[7] Flavin nucleotides, FMN (2) or/and FAD (3) (Scheme 1), are prosthetic groups in the large family of proteins named flavoproteins including dehydrogenases, transferases, oxidases, monooxygenases, ..., which activate molecular oxygen.[7a] Riboflavin, FMN, and FAD are also substrates of enzymes named NAD(P)H: flavin oxidoreductases discovered recently and present in various human tissues.[7b] As a consequence of their remarkable photophysical, photochemical,[6] and biological properties, we found flavins, such as riboflavin (**1**) (Scheme 1), worth investigating as reactive moieties within oligonucleotide adducts. Light-activated riboflavin generates DNA modifications such as guanine degradations revealed by strand breaks induced by hot alkali treatment.[8] Furthermore, chemically or enzymatically reduced riboflavin also can generate oxygen radicals during electron transfer to molecular oxygen.[7c] It was thus reasonable to hope that oligonucleotide−flavin adducts might have efficient DNA-degrading properties.

Methods for preparing flavin adducts should be also of general interest with regard to the extensive chemistry developed for synthesizing biosensors[9a] and enzyme models[9b] in which flavins are involved in electron transfers. We report here the results obtained through the development of a coupling method for attaching the flavin analogs **9a,b** to oligonucleotides using the phosphoramidite and the H-phosphonate chemistry.

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(1) Zamecnik, P. C.; Stephenson, M. L. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 280−284. For a review see: Scanlon, K. J.; Ohta, Y.; Ishida, H.; Kijima, H.; Ohkawa, T.; Kaminski, A.; Tsai, J.; Horng, G.; Kashani-Sabet, M. *FASEB J.* **1995**, *9*, 1288−1296.

(2) For a review see: De Mesmaeker, A.; Häner, R.; Martin, P.; Moser, H. E. *Acc. Chem. Res.* **1995**, *28*, 366−374.

(3) Le Doan, T.; Perrouault, L.; Praseuth, D.; Habhoub, N.; Décout, J. L.; Thuong, N. T.; Lhomme, J.; Hélène, C. *Nucleic Acids Res.* **1987**, *15*, 7749−7760. Moser, H. E.; Dervan, P. B. *Science* **1987**, *238*, 645−650. For reviews see: Thuong, N. T.; Hélène, C. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 666−690. Hélène, C. *Anti-Cancer Drug Des.* **1991**, *6*, 569−584. Frank-Kamenetskii, M. D.; Mirkin, S. M. *Annu. Rev. Biochem.* **1995**, *64*, 65−95.

(4) For a review see: Varma, R. S. *Synlett* **1993**, 621−637.

(5) For a review see: Goodchild, J. *Bioconjugate Chem.* **1990**, *1*, 166−187.

(6) Heelis, P. F. In *Flavins and Flavoproteins*; Muller, F., Ed.; CRC Press: Boca Raton, 1991; pp 171−193.

(7) (a) For a minireview see: Massey, V. *J. Biol. Chem.* **1994**, *269*, 22459−22462. (b) Reichard, P.; Eliason, R.; Fontecave, M. *J. Biol. Chem.* **1987**, *262*, 12325−12331. Fieschi, F.; Nivière, V.; Frier, C.; Décout, J.-L.; Fontecave, M. *J. Biol. Chem.* **1995**, *270*, 30392−30400. (c) Gaudu, P.; Touati D.; Nivière, V.; Fontecave, M. *J. Biol. Chem.* **1994**, *269*, 8182−8188.

(8) Kasai, H.; Yamaizumi, Z.; Berger, M.; Cadet, J. *J. Am. Chem. Soc.* **1992**, *114*, 9692−9694. Ito, K.; Inoue, S.; Yamamoto, K.; Kawanishi, S. *J. Biol. Chem.* **1993**, *268*, 13221−13227.

(9) (a) Wingard, L. B.; Gurecka, J. L., Jr. *J. Mol. Catal.* **1980**, *9*, 209−217. Cosnier, S.; Innocent, C. *J. Electroanal. Chem.* **1992**, *338*, 339−345. (b) For a review see: Murakami, Y.; Kikuchi, J.-I.; Hisaeda, Y.; Hayashida, O. *Chem. Rev.* **1996**, *96*, 721−758. For examples see: Takeda, J.; Ohta, S.; Hirobe, M. *J. Am. Chem. Soc.* **1987**, *109*, 7677−7688. Carell, T.; Epple, R.; Gramlich, V. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 620−623.

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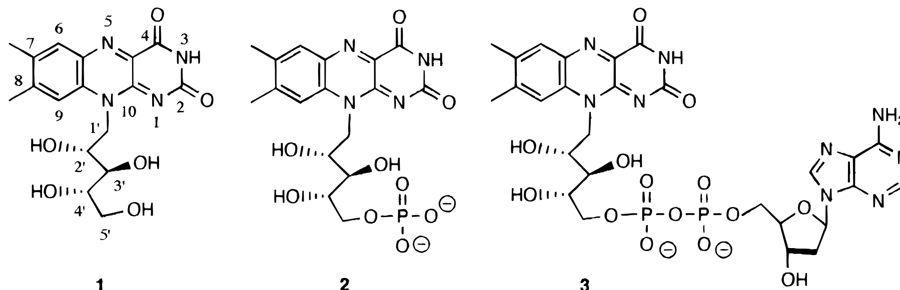
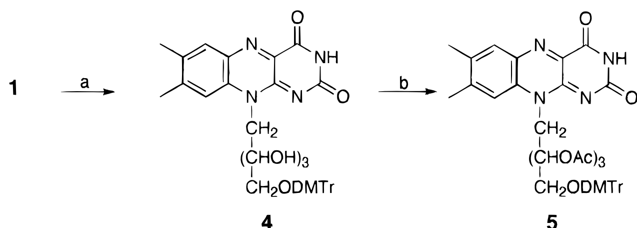
Scheme 1. Structures of Riboflavin (1), FMN (2), and FAD (3)

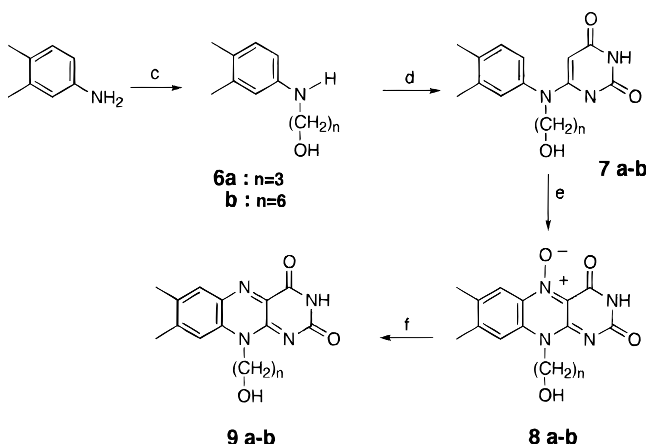


Scheme 2. Protection of the Hydroxy Functions in Riboflavin 1[a]



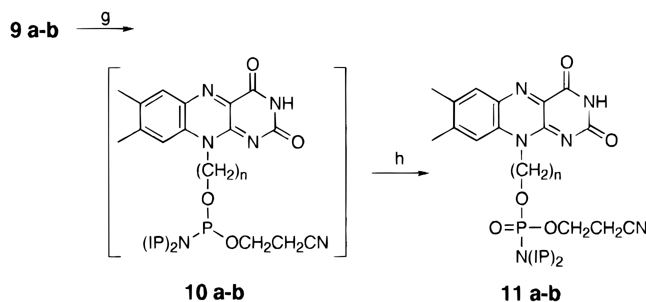
[a] Key: (a) DMTrCl/Py, 100 °C; (b) Ac_2O/Py, rt.

Scheme 3. Synthesis of Flavin Analogs 9a and 9b[a]



[a] Key: (c) $HO(CH_2)_nX/Et_3N$, reflux ($n = 3$, X = Br; $n = 6$, X = Cl); (d) 6-chlorouracil/H_2O/dioxane, reflux; (e) $NaNO_2$/AcOH, rt; (f) DTT or 2-mercaptoethanol/EtOH, reflux.

Scheme 4. Tentative Preparation of Flavin Phosphoramidites 10a,b, Obtention of Phosphoramidates 11a,b[a]



[a] Key: (g) 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite/*N,N*-diisopropylethylamine/Py; (h) isolation (O_2, $h\nu$).

Results

Preparation of the Riboflavin Analogs 9a,b. In order to preserve the physicochemical properties of the flavin isoalloxazine ring within the flavin–oligonucleotide adducts prepared, we chose to link the flavin at the extremity of its side chain. Riboflavin (**1**), also named vitamin B2, possesses four hydroxy groups on the ribityl side chain, and thus, its linking to oligonucleotides required a series of steps of protection and deprotection. The chemistry is further complicated by its poor solubility in organic solvents and by the relative position of the hydroxy functions on the side chain, which allows migration of acyl or silyl protective groups as reported for 1,2-diols.[10] For example, after a selective dimethoxytritylation at the 5′-position of riboflavin (Scheme 2, compound **4**) and then acetylation of the remaining hydroxy functions (compound **5**), we observed, by TLC and ^{1}H NMR spectrometry, a migration of the acetyl groups from the 4′- to the 5′-position (see numbering in Scheme 1) under the acid conditions of detritylation (2% dichloroacetic acid in CH_2Cl_2).

We thus decided to prepare the derivatives **9a,b** in which only one hydroxy function is present at the extremity of the side chain. These compounds were synthesized according to the method of preparation of 10-alkylisoalloxazines and riboflavin described by Yoneda *et al.*[11] The conditions for the preparation of the 6-(*N*-substituted anilino)uracile intermediates **7a,b** were modified, and the *N*-oxides **8a,b** were reduced into the corresponding flavins **9a,b** by *threo*-1,4-dimercapto-2,3-butanediol (dithiothreitol, DTT) or 2-mercaptoethanol instead of sodium dithionite (Scheme 3).

Attempts of Preparation of the Phosphoramidites 10a,b. Modifications of oligonucleotides at their extremity or at an internal position in their sequence have been achieved by incorporation of the corresponding modified phosphoramidites during the solid-phase synthesis.[12] Thus, we tried to prepare the phosphoramidites **10a,b** (Scheme 4) from the flavin analogs **9a,b**. Due to the poor solubility of these compounds in organic solvents, pyridine and *N,N*-diisopropylethylamine (DIPEA) in excess were necessary for complete dissolution. In each case, reaction with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (CP) under argon led essentially to one product. The ^{1}H NMR spectrum of the product obtained from **9a** or **9b** showed the concomitant presence of the cyanoethyl and diisopropylethylamino groups and of the flavin moiety. For each derivative, a unique ^{31}P

(10) Doerschuk, A. *J. Am. Chem. Soc.* **1952**, *74*, 4202–4203. Kupchan, S. M.; Slade, P.: Young, R. J.; Milne, G. W. A. *Tetrahedron* **1962**, *18*, 499–506. Reese, C. B.; Trentham, D. R. *Tetrahedron Lett.* **1965**, *29*, 2467–2472. Jones, S. S.; Reese, C. B. *J. Chem. Soc., Perkin Trans. 1* **1979**, *11*, 2762–2764. Mulzer, J.; Schöllhorn, B. *Angew. Chem., Int. Ed. Engl.* **1990**, *29*, 431–432.

(11) Yoneda, F.; Sakuma, Y.; Ichiba, M.; Shimnomura, K. *J. Am. Chem. Soc.* **1976**, *98*, 830–835.

(12) For reviews see: Beaucage, S. L.; Iyer, R. P. *Tetrahedron* **1993**, *46*, 10441–10488; **1993**, *49*, 1925–1963.

NMR signal was detected at 6.6 ppm that is quite different from the signal for the phosphorus(III) atom in *O*-alkylphosphoramidites, for example, in nucleoside 3′-*O*-phosphoramidites, expected in the 140−150 ppm range.[13a] This was indicative of an oxidation of the phosphoramidite functions leading to phosphoramidate groups.[13b] High-resolution mass spectrometry and elemental analysis (C, H, P) confirmed this assumption and allowed us to assign to these compounds the structures **11a,b** (Scheme 4). The same compounds were obtained when the reaction and the isolation were conducted in strongly attenuated light as far as possible.

In order to investigate the mechanism of this surprising formation of phosphoramidates, the reaction with compound **9b** was monitored by ^{31}P NMR spectrometry in argon-purged solution. A new peak at 144 ppm ascribable to the phosphoramidite intermediate was detected, but it disappeared slowly in the presence of dioxygen and daylight with the concomitant appearance of the peak corresponding to the phosphoramidate **11b**. The presence of *N,N*-diisopropylethylamine was required for phosphorylation.

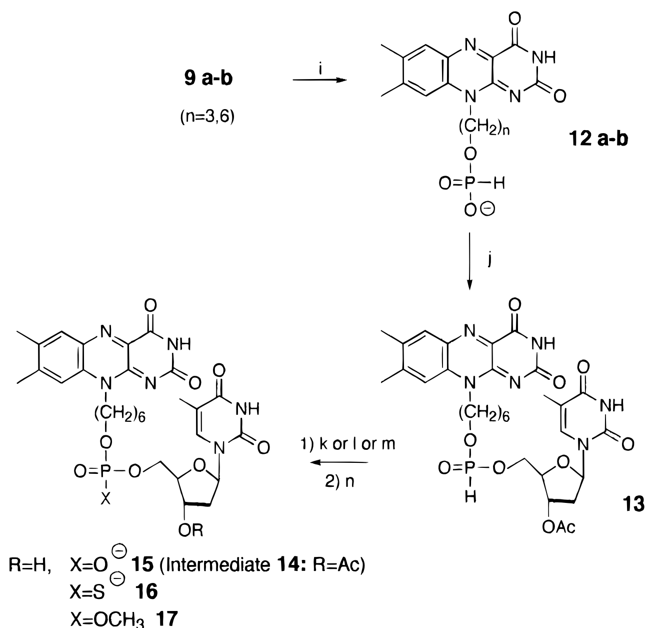
Thus, the obtention of the pure phosphoramidites and their use for preparing flavin adducts appeared very difficult.

Preparation of the Flavin H-Phosphonates 12a,b. Oligodeoxyribonucleotides and oligoribonucleotides can be synthesized on solid supports from nucleoside 3′-H-phosphonates.[14a] This chemistry has been extended to the synthesis of phosphate analogs of oligodeoxynucleosides such as phosphorothioates.[14a,b]

Preparation of **12a,b** from the flavin analogs **9a,b** was conducted with phosphorous acid in the presence of 2,4,6-triisopropylbenzenesulfonyl (TPS) chloride at room temperature (Scheme 5). The H-phosphonate **12b** was obtained in very good yield (90%) after C_{18} reversed-phase chromatography, whereas the H-phosphonate **12a** was isolated in a lower 62% yield. In this latter case, unconverted flavin was recovered in spite of further addition of sulfonyl chloride and phosphorous acid. Other phosphorylating agents such as 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one[15a] or phosphorus trichloride in the presence of 1,2,4-triazole[15b] gave lower yields. Finally, a better yield (84%) was obtained for **12a** using the less hindered coupling reagent *p*-toluenesulfonyl chloride. These H-phosphonates were characterized by ^{1}H, ^{13}C, and ^{31}P NMR (to compare to data for 3′-thymidine H-phosphonate[15b,16]), mass spectrometry, and elemental analysis.

Coupling of the Flavin H-Phosphonate 12b and 3′-*O*-Acetylthymidine: A Model Reaction for the Synthesis of Flavin−Oligonucleotide Adducts. For coupling with the 5′-hydroxy function of an oligonucleotide, the flavin H-phosphonates **12a,b** have to be activated as a mixed anhydride intermediate.[14a] In a model reaction of **12b** with 3′-*O*-acetylthymidine,[17] 1-adamantanecarboxylic acid chloride was used as a coupling reagent (Scheme 5). The major product of the reaction was isolated in 75% yield and characterized as the H-phosphonate diester **13**. The ^{31}P NMR spectrum revealed the presence of the two diastereoisomers characterized by doublets at 7.2 and 5.9 ppm, respectively (to compare to data for bis(thymidine) 3′,5′-H-phosphodiester[18]). In the 500 MHz ^{1}H NMR spectrum of this mixture, the characteristic signals of the flavin and nucleoside moieties were detected and assigned for each diastereoisomer by homonuclear shift correlation (COSY 45) and decoupling experiments. For each of them, two characteristic doublets were assigned to the hydrogen atom linked to the phosphorus (6.90 and 6.89 ppm, J_{H-P} = 708 and 704 Hz, respectively). Integration allowed us to estimate the relative proportion of the diastereoisomers to 70:30. Mass spectrometry and elemental analysis confirmed the structure **13**. The mixture of diastereoisomers was oxidized with iodine, and then the 3′-acetyl group was removed with aqueous ammonia to lead to the phosphodiester **15** in 75% yield after purification on reversed-phase chromatography (^{31}P NMR: to compare to data for bis(thymidine) 3′,5′-phosphodiester[16]). The acetylated phosphodiester intermediate **14** was also characterized.

Scheme 5. Synthesis of Flavin H-Phosphonates 12a,b and Flavin−Thymidine Nucleotide Analogs 13 and 15−17[a]



[a] Key: (i) H_3PO_3/$ArSO_2Cl$/Py (*n* = 3, Ar = Ts; *n* = 6, Ar = TPS); (j) adamantanecarboxylic acid chloride/3′-acetylthymidine/Py; (k) I_2/THF/Py; (l) S_8/CS_2/Py; (m) MeOH/*N*-methylimidazole/TEA/CCl_4; (n) NH_3,H_2O.

These results showed that the coupling and oxidation reactions appeared convenient for attaching the flavin analogs to oligonucleotides by a phosphodiester linkage.

Another advantage of the H-phosphonate chemistry developed here resides in the possibility of varying the nature of the link between the flavin and the oligonucle-

(13) (a) Sinha, N. D.; Biernat, J.; Mc Manus, J.; Köster, H. *Nucleic Acids Res.* **1984**, *12*, 4539−4557. (b) Peyrottes, S.; Vasseur, J. J.; Imbach, J.-L.; Rayner, B. *Nucleic Acids Res.* **1996**, *24*, 1841−1848.

(14) (a) Froehler, B. C. In *Methods in molecular biology, Vol. 20: Protocols for oligonucleotides and analogs;* Agrawal, S., Ed.; Humana Press: Totowa, NJ, 1993; pp 63−80. (b) Zon, G.; Stec, W. J. In *Oligonucleotides and analogues. A practical approach*; Eckstein, F., Ed.; IRL Press: Oxford, New York, Tokyo, 1991; pp 87−108. Eckstein, F. *Angew. Chem., Int. Ed. Engl.* **1983**, *22*, 423−439.

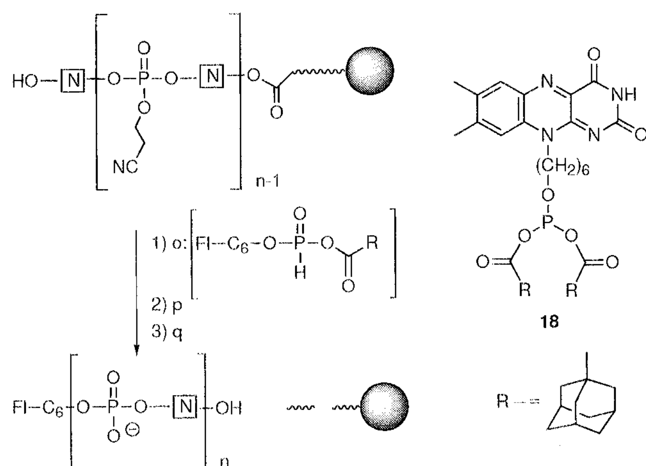
(15) (a) Marugg, J. E.; Tromp, M.; Kuyl-Yeheskiely, E.; van der Marel, G. A.; van Boom, J. H. *Tetrahedron Lett.* **1986**, *27*, 2661−2664. (b) Froehler, B. C.; Ng, P. G.; Matteucci, M. D. *Nucleic Acids Res.* **1986**, *14*, 5399−5407.

(16) Froehler, B. C.; Matteucci, M. D. *Tetrahedron Lett.* **1986**, *27*, 469−472.

(17) Décout, J.-L.; Lekchiri, Y.; Lhomme, J. *Photochem. Photobiol.* **1992**, *55*, 639−645.

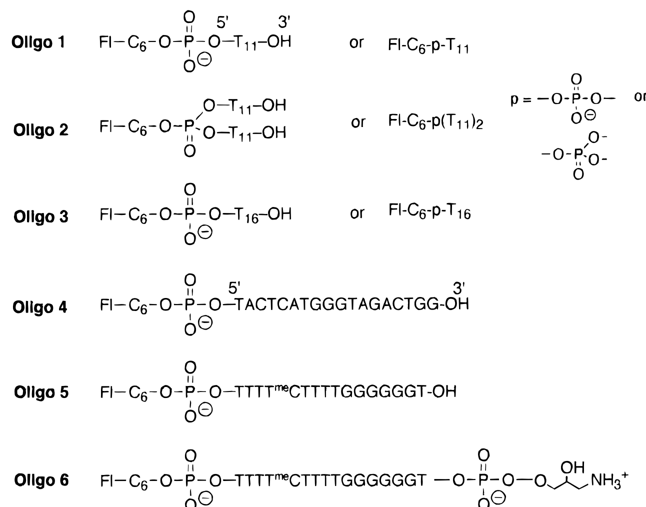
(18) Stawinski, J; Thelin, M. *J. Org. Chem.* **1994**, *59*, 130−136.

Scheme 6. Synthesis of Flavin−Oligonucleotide Adducts (Oligos 1−6, See Scheme 7)[a]



[a] Key: N = nucleoside; (o) **12b**/adamantanecarboxylic acid chloride or trimethylacetyl chloride/Py; (p) I_2/THF/Et_3N; (q) NH_3/H_2O or NaOH/H_2O/CH_3OH. Structure of intermediate **18** probably formed by overactivation of **12b** (o).

Scheme 7. Structures of the Flavin−Oligonucleotide Adducts Prepared[a]



[a] Key: Fl, isoalloxazine; C, cytosine nucleotide; ^{me}C, 5-methylcytosine nucleotide; T, thymine nucleotide; A, adenine nucleotide; G, guanine nucleotide.

otide during the oxidation step. Charged phosphodiesters and phosphorothioates and uncharged phosphate triesters, phosphoramidates, and alkyl phosphonates could be prepared from the H-phosphonate diester intermediate such as **13**. Such modifications should modify the properties of the flavin−oligonucleotide adducts in terms of their stability with regard to nuclease degradation and/or cellular uptake and affinity for the target nucleic acid. The phosphorothioate **16** and the methyl phosphate analogs **17** of the flavin−thymidine adducts **13** and **15** were prepared. Compound **16** was obtained in 78% yield after reaction of the H-phosphonate diester **13** with sulfur in carbon disulfide and then deprotection of the 3′-hydroxy group of the thymidine moiety with aqueous ammonia (^{31}P NMR: to compare to data for bis(thymidine) 3′,5′-phosphorothioate[19]). Reaction of **13** with methanol in the presence of triethylamine and carbon tetrachloride[20a] and then deacetylation afforded the methyl phosphotriester **17** in 42% yield (^{31}P NMR: to compare to data for bis(thymidine) 3′,5′-methylphosphotriester[20b]). A competitive hydrolysis of the phosphotriester function occurs under these conditions.

Synthesis of Flavin−Oligonucleotide Adducts. We chose to attach the flavin analog **9b** at the 5′-extremity of oligonucleotides synthesized on solid support by the phosphoramidite method. Flavins are insoluble compounds, and they tightly bind to glassware and plastic tubing. As a consequence, compound **9b** was not coupled at the end of the oligonucleotide synthesis into the automatized synthesizer but into classical glassware. After the solid-phase synthesis of the oligonucleotide, the last 4,4′-dimethoxytrityl group was removed, and then the insoluble solid support was treated with an excess of flavin−adamantanecarboxylic acid chloride mixture in pyridine at room temperature (Scheme 6). After this coupling step, the H-phosphodiester obtained was oxidized with iodine. Cleavage of the oligonucleotide from the solid support and deprotection were performed with an aqueous ammonia or a sodium hydroxide solution. The oligonucleotides were purified by HPLC on C_{18} reversed-phase and by anion-exchange chromatography (see the next part).

The first experiments were conducted with an undecathymidylate ($[Tp]_{10}T$) synthesized on a 1 μmol scale, using a large excess of a flavin−adamantanecarboxylic acid chloride mixture (60 and 120 equiv, respectively). Under these conditions, two flavin−oligonucleotide adducts were obtained and purified. They were identified by absorption spectrometry, ^{1}H NMR (see the next part), and mass spectrometry, respectively, as the expected flavin−T_{11} adduct (Fl−C_6−p−T_{11}: oligo 1, Scheme 7) and as a coupling product of a flavin and two T_{11} linked to one phosphorus atom Fl−C_6−p$(T_{11})_2$ (oligo 2, Scheme 7). These oligonucleotides were obtained in a 3:1 ratio. The side product is likely to result from overactivation of the flavin H-phosphonate **12b** with acyl chloride leading to the intermediate **18** (Scheme 6) as shown from model reactions with ethyl H-phosphonate, for example.[21] Here, the large proportion of overactivation product can be explained from steric considerations. The H-phosphonate function in **12b** is less hindered and thus more susceptible to overactivation than the same function at the 3′-position in nucleoside H-phosphonates used for preparing oligonucleotides. The isolated side oligonucleotide (oligo 2) possesses a new and very interesting structure that could find applications in the antisense strategy.

In order to avoid this side reaction, a 1:1.1 flavin−adamantanecarboxylic acid chloride mixture were used in the next experiment run with an oligothymidylate T_{16}. Under these conditions, the reaction appeared very selective and the Fl−C_6−p−T_{16} adduct (oligo 3) was obtained in 54% yield and characterized by ^{1}H NMR and mass spectrometry. Oligo 1 was also prepared in an excellent 62% yield using trimethylacetyl chloride as the activating reagent.

Three flavin−oligonucleotide adducts incorporating the four natural nucleosides and 5-methyl-2′-deoxycytidine

(19) Lesnikowski, Z. J.; Niewiarowski, W.; Zielinski, W. S.; Stec, W. J. *Tetrahedron* **1984**, *40*, 15−32.
(20) (a) Froehler, B. C. *Tetrahedron Lett.* **1986**, *27*, 5575−5578. (b) Kuijpers, W. H. A.; Huskens, J.; Koole, L. H.; van Boeckel, C. A. A. *Nucleic Acids Res.* **1990**, *18*, 5197−5205.
(21) Garegg, P. J.; Regberg, T.; Stawinski, J.; Strömberg, R. *Nucleosides Nucleotides* **1987**, *6*, 283−286. Froehler, B. C.; Matteucci, M. D. *Ibid.* 287−291. Garegg, P. J.; Regberg, T.; Stawinski, J.; Strömberg, R. *Ibid.* 655−662.

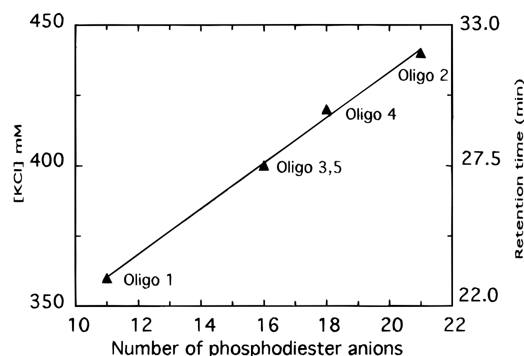


Figure 1. Identification of the flavin−oligonucleotide adducts prepared by anion-exchange chromatography eluting with a linear gradient of aqueous solutions A (KH_2PO_4/K_2HPO_4 buffer pH 6.0−acetonitrile 80:20) and B (1 M KCl in solution A): Variation of KCl concentration corresponding to adduct detection as a function of the number of anionic charges ($r = 0.998$).

(Scheme 7, oligos 4−6) were also synthesized and characterized (yields 30, 52, and 43%, respectively). Two of them (oligos 5 and 6) are triple-helix forming oligonucleotides directed against the polypurine tract (PPT) of HIV-1, a 16-nucleotide polypurine sequence present in the *nef* and *pol* genes.[22] Some side products were observed after deprotection of the amino functions of bases in ammonia at room temperature in the dark (removal of isobutyryl and benzoyl groups). The proportions of these side products were decreased using a 0.2 M sodium hydroxide solution in methanol−water 50:50 in place of ammonia. The yields might be improved with more labile protecting groups of bases such as phenoxyacetyl or dimethylformamidine groups.[23]

Identification and Characterization of the Flavin−Oligonucleotide Adducts. The flavin−oligonucleotide adducts were purified by HPLC on reversed-phase (C_{18}) and anion-exchange chromatography ($-CH_2N^+(CH_3)_3$ groups). During these purifications, the adducts were identified from their dual absorption at 254 and 450 nm and from the linear correlation between the number of their phosphate groups and their retention time (Figure 1, $r = 0.998$) observed by anion-exchange chromatography.

They were additionally characterized by absorption spectrophotometry, by ^{1}H NMR and mass spectrometry, and by acid hydrolysis and enzyme digestion.

In the ^{1}H NMR spectrum (500 MHz) of the conjugate $Fl-C_6-p-T_{11}$ (Figure 2), three signals could be assigned to the hydrogen atoms of the isoalloxazine ring: an isolated singlet at 7.94 ppm that could correspond to the C-6H or C-9H (F6-H or F9-H, see numbering in Scheme 1) and two singlets at 2.57 and 2.46 ppm corresponding to the methyl groups at the C-7 and C-8 positions (F7-CH_3 and F8-CH_3).

In order to confirm this assumption, a series of NOE experiments were performed (Figure 2). Irradiation corresponding to the singlet at 7.94 ppm resulted in the appearance of a signal at 2.46 ppm assigned to one of the flavin methyl groups. Irradiation at 7.7 ppm in the large multiplet corresponding to the C-6 protons of the thymine ring (T6-H) and probably to the other flavin proton F6-H or F9-H (as shown by integration) led to the appearance of the signal at 2.57 ppm assigned to the second flavinic methyl group. Irradiation of the signal at 4.70 ppm ascribable to the F1′-CH_2 allowed us to correlate the singlet at 7.7 ppm to the F9-H proton and as a consequence assign the singlet at 2.57 ppm to the F8-CH_3 (Figure 2). All the inverse correlations were also observed. It can be noted that the anomeric protons were detected as a broad multiplet between 6.37 and 6.22 ppm except for the anomeric proton of one 3′-thymidine, which appeared as a multiplet shielded at 6.08 ppm. A similar spectral profile was also observed for the other sugar protons.

In the ^{1}H NMR spectrum of the $Fl-C_6-p(T_{11})_2$ and $Fl-C_6-p-T_{16}$ adducts, a similar spectral profile was observed. The characteristic signals of the flavin moiety previously evidenced could be detected in the spectrum of each of the adducts prepared.

Acid hydrolysis and enzyme digestion of the oligonucleotides provided also a confirmation of the structures on the basis of the detection of the corresponding bases and nucleosides, respectively, and of the flavin moiety detected at 450 nm.

Discussion

The remarkable photochemical and enzymatic ability of flavins to activate molecular oxygen makes them very attractive to use as DNA-cleaving agents.[7,8] The isoalloxazine ring of flavins has been previously linked to netropsin and distamycin, which specifically recognize and bind A + T-rich nucleotide sequences in the minor groove of DNA.[24] The hybrid molecules obtained were able to cleave supercoiled DNA under visible light irradiation in the vicinity of A + T-rich sequences. Thus, we consider flavin−oligonucleotide adducts very promising molecules as a new class of specific and reactive antisense or antigene oligonucleotides. The presence of the fluorescent and photoreactive isoalloxazine ring on the oligonucleotides should confer to the adducts interesting properties.

The H-phosphonate chemistry allowed us to prepare, for the first time, flavin−thymine nucleotides and flavin−oligonucleotide adducts that could not be synthesized using the phosphoramidite method. This is also the first time that H-phosphonates are used for attaching a probe or/and a reactive group to oligonucleotides in such a way.

The H-phosphonate method offers some advantages for this type of oligonucleotide synthesis. Flavin H-phosphonates **12a,b** are very stable, and the unreacted large excess used for attachment to oligonucleotides is recovered after hydrolysis of the mixed anhydride intermediate. Moreover, various modified phosphorylated linkages can be generated as demonstrated with the synthesis of the flavin−thymine nucleotides **15**−**17**.

The flavin phosphoramidites **10a,b** could not be obtained in the reaction of the flavin analogs **9a,b** with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite in the presence of *N,N*-diisopropylethylamine. During their isolation, they were oxidized into the corresponding phosphoramidates **11a,b** in the presence of dioxygen and daylight as indicated by ^{31}P NMR experiments. The same reaction occurred even when light exposition was minimized. A very efficient photooxidation process occurs

(22) Wain-Hobson, S.; Sonigo, P.; Danos, O.; Cole, S.; Alizon, M. *Cell* **1985**, *40*, 9−17. Giovannangéli, C.; Rougée, M.; Garestier, T.; Thuong, N. T.; Hélène, C. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 8631−8635.

(23) Chaix, C.; Molko, D.; Téoule, R. *Tetrahedron Lett.* **1989**, *30*, 71−74. Mc Bride, L. J.; Kierzek, R.; Beaucage, S. L.; Caruthers, M. H. *J. Am. Chem. Soc.* **1986**, *108*, 2040−2048.

(24) Herfeld, P.; Helissey, P.; Giorgi-Renault, S.; Goulaouic, H.; Pager, J.; Auclair, C. *Bioconjugate Chem.* **1994**, *5*, 67−76.

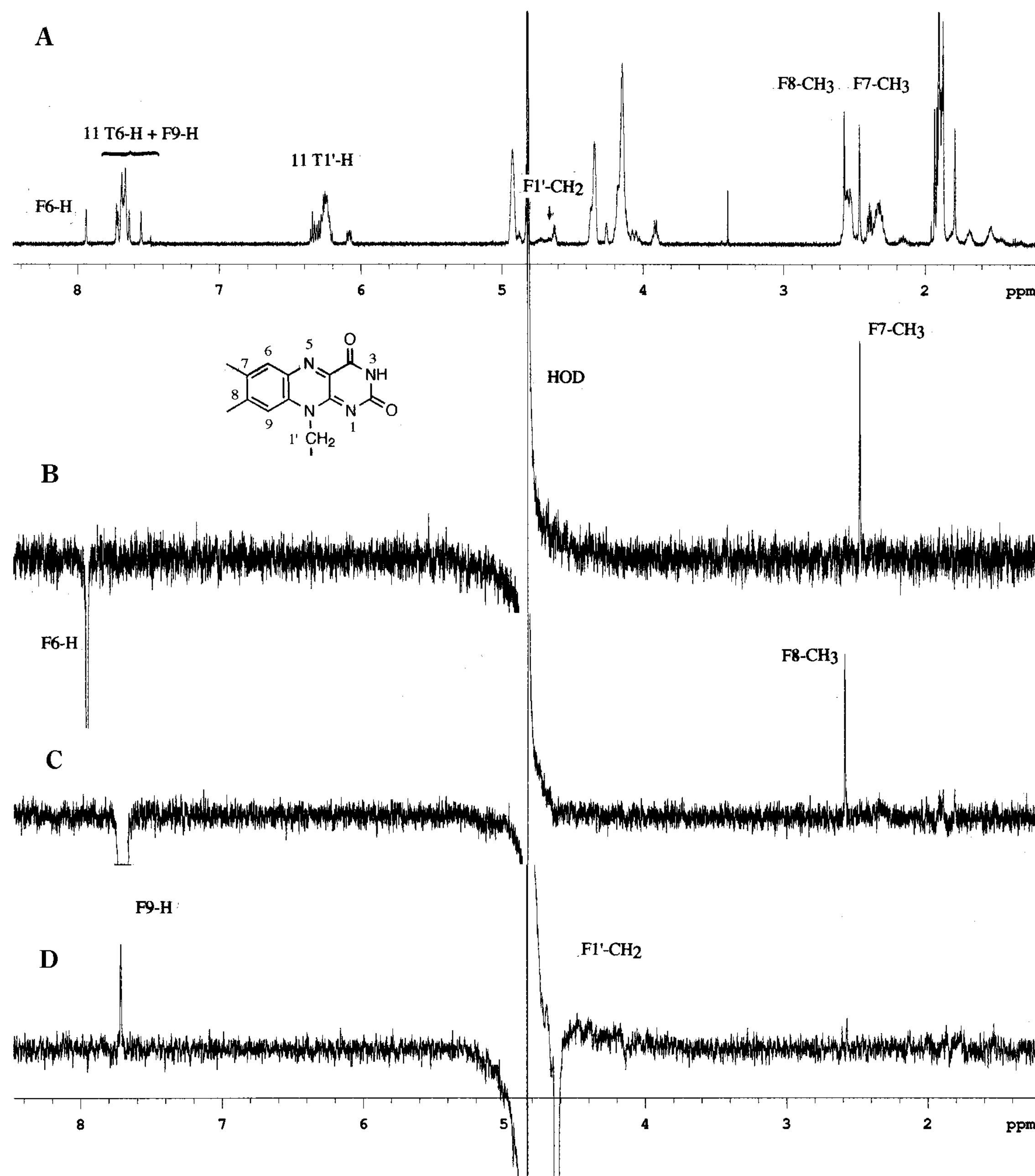


Figure 2. ^{1}H NMR spectrum (A) and 1D NOE ^{1}H spectra (B, C, D) of Fl−C_6−p−T_{11} (oligo 1, Scheme 7, D_2O, F: flavin moiety, T: thymidine). (B) irradiation corresponding to the singlet at 7.94 ppm (F6-H); (C) irradiation corresponding to the multiplet at 7.7 ppm (F9-H + 11 T6-H); (D) irradiation corresponding to the multiplet at 4.7 ppm (F1′-CH_2).

probably under the conditions of isolation. Photosensitized oxidations can occur by two processes named type-I and type-II photosensitizations.[25] Type-I involves the direct interaction of photosensitizer, usually in the triplet state, with the substrate through electron-transfer reactions. Type-II involves energy transfer from the triplet state of photosensitizer to molecular oxygen resulting in the formation of singlet oxygen. A very selective photooxidation of the guanine bases in DNA in the presence of riboflavin under UV irradiation has been reported via a type-I mechanism.[8] Flavins have been also used to photooxidize aromatic organic polluents in water.[26a]

Here, the situation is more complex due to the possible role of *N,N*-diisopropylethylamine. A single photoelectron transfer could occur between the tertiary aliphatic amine and flavin and then lead to activation of molecular oxygen. The photochemical oxidation of ethylenediamine tetracetic acid (EDTA) by riboflavin is known and has been used for a long time.[26b] Another example of such a transfer between cyclopropylamines and 3-methyllumiflavin has been used to induce photochemical reactions.[26c]

These results point to the very interesting ability of flavins to activate molecular oxygen. This property could

(25) Koizumi, M.; Kato, S.; Mataga, N.; Matsuura, T.; Usui, Y. In *Photosensitized reactions*; Kagakudajin Publishing Co.: Kyoto, 1978. Rodgers, M. A. J. In *Primary photo-processes in biology and medicine*; Bensasson, R. V., Jori, G., Land, E. J., Truscott, T. G., Eds.; Plenum Press: New York, 1985; pp 181−195.

(26) (a) Larson, R. A.; Stackhouse, P. L.; Crowley, T. O. *Environ. Sci. Technol.* 1992, **26**, 1792−1798. Tatsumi, K.; Ichikawa, H.; Wada, S. *J. Contam. Hydrol.* **1992**, *9*, 207−219. (b) Enns, K.; Burgess, W. H. *J. Am. Chem. Soc.* **1965**, *87*, 5766−5770. Heelis, P. F.; Hartman, R. F.; Rose, S. D. *Tetrahedron Lett.* **1994**, *35*, 6053−6056. (c) Kim, J.-M.; Bogdan, M. A.; Mariano, P. S. *J. Am. Chem. Soc.* **1991**, *113*, 9251−9257.

be used for the preparative oxidation of various organic compounds and should allow a very efficient photocleavage of the targeted nucleic acid with the oligonucleotide–flavin adducts prepared. Preliminary experiments with $Fl-C_6-p-T_{11}$ (oligo 1) have revealed a selective photocleavage of a 45-mer oligodeoxynucleotide containing a targeted undecadeoxyadenylate sequence (pA_{11}) at guanine residues in close proximity to the flavin in the duplex (unpublished results).

Experimental Section

General Methods. Melting points are reported uncorrected. Chemical shifts are in ppm relative to the residual signal of the solvent, except for the ^{31}P-NMR chemical shifts, which are relative to the signal of orthophosphoric acid as external reference. NMR peak assigments are included as Supporting Information.

Synthesis. 5′-*O*-(4,4′-Dimethoxytrityl)riboflavin 4. Riboflavin (**1**) (3 g, 7.97 mmol) was dissolved in dry pyridine (300 mL) at reflux under argon in the dark, and then 4,4′-dimethoxytrityl chloride (4 g, 11.9 mmol) was added. After 4 h at 100 °C, the solution was allowed to cool at room temperature. After addition of methanol (10 mL), the solvents were evaporated, and residual pyridine was coevaporated with toluene. Dichloromethane (200 mL) was added to the residue, and the insoluble material was removed by filtration. The filtrate was washed with water (50 mL), and the aqueous layer was extracted with dichloromethane (2 × 50 mL). The combined organic extracts were dried over $MgSO_4$ and evaporated. The residue was chromatographed on silica gel in dichloromethane–methanol–pyridine (97.5:2:0.5) to yield compound **4** (2.83 g, 3.98 mmol, 50%): mp 166–168 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.45 (1H, s), 8.03 (1H, s), 7.86 (1H, s), 7.25 (9H, m), 6.78 (4H, m), 5.04 (2H, m), 4.45 (1H, s), 4.17 (1H, m), 3.95 (1H, m), 3.75 (6H, s), 3.68 (2H, m), 3.45 (3H, m), 2.45 (6H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 159.8, 157.8, 155.2, 150.2, 145.8, 145.2, 136.7, 136.0, 135.9, 135.5, 133.9, 131.8, 130.8, 129.7, 127.8, 127.5, 126.3, 117.2, 112.9, 85.1, 73.8, 71.2, 68.7, 65.8, 54.9, 47.3, 20.8, 18.8; LRMS [FAB$^-$, glycerol] *m/z* 679 [M]$^-$, 377 [riboflavin]$^-$. Anal. Calcd for $C_{38}H_{38}N_4O_8{\cdot}CH_3OH$ (710.78): C, 65.90; H, 5.96; N, 7.88. Found: C, 65.61; H, 5.90; N, 8.25.

2′,3′,4′-Tri-*O*-acetyl-5′-(4,4′-dimethoxytrityl)riboflavin (5). To an ice-cold solution of 5′-*O*-(dimethoxytrityl)-riboflavin (**4**) (1 g, 1.40 mmol) in pyridine (200 mL) was added acetic anhydride (5 mL) under argon in the dark. The resulting solution was stirred at room temperature for 15 h, and then methanol (10 mL) was added at 0 °C. After evaporation and coevaporation with toluene, the residue was chromatographed on silica gel in dichloromethane–methanol–pyridine (97.5:2:0.5) to lead to compound **5** (0.86 g, 1.05 mmol, 75%): mp 171–173 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.00 (1H, s), 7.53 (1H, s), 7.35 (1H, s), 7.23 (9H, m), 6.75 (4H, d), 5.60 (2H, m), 5.32 (2H, m), 4.78 (1H, m), 3.75 (6H, s), 3.28 (2H, m), 2.50 (2H, s), 2.41 (2H, s), 2.32 (2H, s), 1.97 (3H, s), 1.65 (3H, s). Anal. Calcd for $C_{44}H_{44}N_4O_{11}{\cdot}0.5H_2O$ (813.86): C, 64.90; H, 5.55; N, 6.88. Found: C, 65.10; H, 5.66; N, 7.21.

Synthesis of Isoalloxazines 9a,b. *N*-(6′-Hydroxyhexyl)-3,4-dimethylaniline (6b). A mixture of 3,4-dimethylaniline (9.1 g, 75 mmol), triethylamine (15 mL), and 6-chlorohexanol (3.40 g, 24.88 mmol, 3.3 mL) was stirred at 110 °C for 5 h. After cooling and addition of dichloromethane (200 mL), the solution was washed with aqueous Na_2CO_3 (10%, 40 mL). The aqueous layer was extracted with dichloromethane (2 × 100 mL). The combined organic extracts were dried over $MgSO_4$ and evaporated. Compound **6b** was obtained after chromatography on silica gel in dichloromethane–methanol (98:2) and crystallization from hexane (5.40 g, 24.4 mmol, 98%): mp 42–43 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.90 (1H, d, $J = 8$ Hz), 6.40 (1H, d, $J < 2$ Hz), 6.34 (1H, dd, $J = 8$, $J < 2$ Hz), 3.62 (2H, t), 3.35 (2H, m), 3.05 (2H, t), 2.16 (3H, s), 2.12 (3H, s), 1.55 (4H, m), 1.38 (4H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 146.6, 137.2, 130.2, 125.1, 114.6, 110.2, 62.8, 44.2, 32.6, 29.6, 27.0, 25.5, 20.0, 18.6; LRMS [FAB$^+$, NBA] *m/z* 222 [M + H]$^+$. Anal. Calcd for $C_{14}H_{23}NO$ (221.34): C, 75.97; H, 10.47; N, 6.33. Found: C, 76.22; H, 10.61; N, 6.48.

6-[*N*-(6′-Hydroxyhexyl)-3,4-xylidino]uracil (7b). To a solution of compound **6b** (4.6 g, 20.8 mmol) in water–dioxane (1:1; 45 mL) refluxed under argon was added 6-chlorouracil (1 g, 6.82 mmol) with stirring. After 15 h reflux and then cooling, the pH was increased to 11 by addition of aqueous NaOH (10%). The resulting solution was extracted with dichloromethane (3 × 100 mL) to remove unreacted starting compound **6b**. Aqueous HCl was added to the aqueous layer to reach pH 3. The resulting precipitate was collected by filtration, washed with water, and then crystallized from water. A second fraction of **7b** was obtained after evaporation of the filtrates: the residue was stirred with methanol and filtered, methanol was evaporated, and the residual solid was crystallized from water (1.45 g, 4.32 mmol, 63%): mp 188–190 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 10.30 (1H, br s), 10.10 (1H, br s), 7.20 (1H, d, $J = 4$ Hz), 7.00 (1H, s), 6.95 (1H, d, $J = 4$ Hz), 4.30 (1H, t), 4.10 (1H, s), 3.55 (2H, t), 3.35 (2H, t), 2.20 (6H, s), 1.35 (4H, m), 1.25 (4H, m); ^{13}C NMR (75 MHz, DMSO-d_6) δ 164.3, 154.6, 151.3, 139.7, 138.5, 136.4, 131.1, 128.8, 125.2, 76.1, 60.8, 51.3, 32.5, 27.5, 25.9, 25.5, 19.7, 19.3; LRMS [FAB$^+$, NBA] *m/z* 333 [M + H]$^+$. Anal. Calcd for $C_{18}H_{25}N_3O_3{\cdot}0.25H_2O$ (335.92): C, 64.38; H, 7.60; N, 12.51. Found: C, 64.15; H, 7.58; N, 12.49.

Isoalloxazine 5-Oxide (8b). Sodium nitrite (1.7 g, 25 mmol) was added to a solution of compound **7b** (1.7 g, 5.06 mmol) in acetic acid (12 mL) in the dark. The mixture was stirred at room temperature for 3 h, and then water (6 mL) was added. The suspension was stirred again for 3 h, and the solvents were evaporated. After addition of water and filtration, the solid was washed with water and then crystallized from ethyl acetate–ethanol (50:50) for **8b** (1.70 g, 4.75 mmol, 94%): mp 222 °C dec; ^{1}H NMR (200 MHz, DMSO-d_6) δ 11.00 (1H, br s), 8.07 (1H, s), 7.76 (1H, s), 4.75 (1H, br s), 4.49 (2H, m), 3.38 (2H, m), 2.48 (3H, s), 2.37 (3H, s), 1.65 (2H, m), 1.40 (6H, m); LRMS [FAB$^+$, NBA] *m/z* 359 [M + H]$^+$. Anal. Calcd for $C_{18}H_{22}N_4O_4$ (358.16): C, 60.32; H, 6.19; N, 15.63. Found: C, 60.17; H, 6.34; N, 15.68.

***N*-(6′-hydroxyhexyl)isoalloxazine (9b).** An aqueous solution of dithiothreitol (1.4 g, 9.08 mmol, 20 mL) was added to a suspension of *N*-oxide **8b** (0.715 g, 1.99 mmol) in ethanol (500 mL). The mixture was refluxed with stirring for 20 min under argon. The solvent was evaporated, and the residue was crystallized from ethanol (0.63 g, 1.79 mmol, 90%): mp 270 °C dec; ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.24 (1H, br s), 7.90 (1H, s), 7.77 (1H, s), 4.56 (2H, t), 4.31 (1H, m), 3.39 (2H, m), 2.49 (3H, s), 2.39 (3H, s), 1.71 (2H, m), 1.42 (6H, m); ^{13}C NMR (75 MHz, DMSO-d_6) δ 159.9, 155.6, 150.0, 146.5, 139.9, 135.7, 133.7, 130.9, 130.7, 115.9, 60.5, 44.1, 32.3, 26.46, 26.0, 25.2, 20.5, 18.7; LRMS [FAB$^+$, NBA] *m/z* 343 [M + H]$^+$, 259 [M + H − $(CH_2)_6OH$]$^+$. Anal. Calcd for $C_{18}H_{22}N_4O_3{\cdot}0.5H_2O$ (351.4) C, 61.52; H, 6.60; N, 15.94. Found: C, 61.43; H, 6.69; N, 16.09.

Flavin Phosphoramidates 11a,b. Compound **9** (**a**, 50 mg; **b**, 57 mg, 0.160 mmol) was dissolved in dry pyridine (7 mL) and *N,N*-diisopropylethylamine (2 mL, 22.8 mmol) under argon in the dark. To the solution was added 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (260 μL, 1.15 mmol). The mixture was stirred for 24 h, and then aqueous sodium hydrogencarbonate (5%, 3 mL) was added. The resulting solution was extracted with dichloromethane (2 × 10 mL). The combined extracts were dried over $MgSO_4$ and then evaporated. The residue was purified by flash chromatography in dichloromethane–methanol–triethylamine (94:4:2) to yield compound **11** (**a**, 67 mg, 0.123 mmol, 77%; **b**, 72 mg, 0.126 mmol, 79%).

Compound **11a**: mp 188 °C dec; ^{31}P NMR (81 MHz, $CDCl_3$) δ 6.6; ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.40 (1H, br s), 8.00 (1H, s), 7.60 (1H, s), 4.90 (2H, m), 4.21 (4H, m), 3.42 (2H, m), 2.81 (2H, m), 2.54 (3H, s), 2.42 (3H, s), 1.61 (2H, m), 1.24 (12H, dd); LRMS [FAB$^+$, NBA] *m/z* 517 [M + H]$^+$; 462 [M + H − CH_2CH_2CN]$^+$; 447 [M + H − OCH_2CH_2CN]$^+$; HRMS exact mass calcd for $C_{24}H_{33}N_6O_5P$ 517.2328 [M + H]$^+$, found [FAB$^+$,

MCA] *m/z* 517.2343. Anal. Calcd for $C_{24}H_{33}N_6O_5P{\cdot}1.5\ H_2O$ (543.56): C, 52.56; H, 6.60; P, 6.40. Found: C, 52.78; H, 6.64; P, 6.41.

Compound **11b**: mp 192 °C; ^{31}P NMR (81 MHz, $CDCl_3$) δ 6.6; ^1H NMR (300 MHz, MeOD) δ 8.10 (1H, br s), 7.90 (1H, s), 4.95 (2H, m), 4.15 (4H, m), 3.30 (2H, m), 2.90 (2H, m), 2.60 (3H, s), 2.48 (3H, s), 2.10 (2H, m), 1.90 (2H, m), 1.80 (4H, m), 1.20 (12H, dd); LRMS [FAB$^+$, glycerol] *m/z* 559 [M + H]$^+$; 506 [M + H − CH_2CH_2CN]$^+$, 461 [M + H − N(iPr)$_2$]$^+$; HRMS exact mass calcd for $C_{27}H_{39}N_6O_5P$ 559.2797 [M + H]$^+$, found [FAB$^+$, MCA] *m/z* 559.2806. Anal. Calcd for $C_{27}H_{39}N_6O_5P{\cdot}1.5H_2O$ (567.62): C, 57.08; H, 6.87; P, 5.46. Found: C, 57.29; H, 7.02; P, 5.52.

Flavin H-Phosphonates 12a,b. Compound **9** (**a**, 50 mg; **b**, 57 mg, 0.160 mmol) was dissolved in dry pyridine (10 mL) at 100 °C under argon in the dark. To the solution cooled at 30 °C were added phosphorous acid (54 mg, 0.66 mmol) and then the arylsulfonyl chloride (0.160 mmol; **a**, *p*-toluenesulfonyl chloride, 30.5 mg; **b**, 2,4,6-triisopropylbenzenesulfonyl chloride, 48.5 mg). The mixture was stirred at room temperature for 15 h, and the solvent was evaporated. The residue was chromatographed on a short column of reversed phase (C_{18}-Sep-Pak Waters, 1g cartridge) in water to yield compound **12** (mixture of acid and its pyridinium salt 2:1; **a**, 54 mg, 0.130 mmol, 84%; **b**, 64 mg, 0.140 mmol, 90%).

Compound **12a**: mp 200 °C dec; ^{31}P NMR (81 MHz, DMSO-d_6) δ 7.2 (J_{PH} = 657 Hz); ^1H NMR (200 MHz, DMSO-d_6) δ 11.27 (1H, s), 8.58 (d), 7.88 (1H, s), 7.79 (1H, s), 7.77 (m), 7.39 (m), 6.76 (1H, d, J_{HP} = 657 Hz), 4.65 (2H, t), 4.05 (2H, m), 2.49 (3H, s), 2.39 (3H, s), 2.07 (2H, m); LRMS [FAB$^+$, NBA] *m/z* 365 [M + H]$^+$, 301 [M + H − PO_2H]; UV (H_2O, 50 mM, Tris HCl, pH 7.7) λ_{max} (ϵ) 444 (12 600). Anal. Calcd for $C_{15}H_{16}N_4O_5P{\cdot}1H_2O$ (381.30): C, 47.25; H, 4.76; N, 14.69; P, 8.12. Found: C, 47.07; H, 4.74; N, 14.66; P, 8.01.

Compound **12b**: mp 175 °C dec; ^{31}P NMR (81 MHz, DMSO-d_6) δ 7.4 (J_{PH} = 656 Hz); ^1H NMR (200 MHz, DMSO-d_6) δ 11.28 (1H, s), 8.56 (d), 7.88 (1H, s), 7.79 (1H, s), 7.41 (m), 7.38 (m), 6.69 (1H, d, J_{HP} = 656 Hz), 4.56 (2H, t), 3.90 (2H, m), 2.49 (3H, s), 2.39 (3H, s), 1.65 (2H, m), 1.60 (2H, m), 1.56 (4H, m); ^{13}C NMR (75 MHz, D_2O) δ 163.3, 160.3, 153.5, 151.5, 150.1, 144.1, 142.1, 136.3, 136.2, 133.3, 133.2, 130.3, 119.0, 67.2, 48.8, 32.7, 29.3, 28.2, 27.6, 23.6, 21.8; LRMS [FAB$^+$, glycerol] *m/z* 406 [M + H]$^+$; UV (H_2O, 50 mM, Tris HCl, pH 7.7) λ_{max} (ϵ) 444 (11 800). Anal. Calcd for $C_{18}H_{22}N_4O_5P{\cdot}1H_2O$ (423.38): C, 51.05; H, 5.72; N, 13.24. Found: C, 51.16; H, 5.75; N, 13.28.

Flavin−Thymidine H-Phosphodiester 13. To a solution of compound **12b** (0.80 g, 1.80 mmol) in dry pyridine (60 mL) were added dropwise, under argon in the dark, a solution of 1-adamantanecarboxylic acid chloride (0.6 g, 3.0 mmol) in pyridine (5 mL) and then a solution of 3′-acetylthymidine (0.73 g, 2.5 mmol) in pyridine (5 mL). The mixture was stirred for 24 h, and then water (30 mL) was added. The resulting solution was extracted with dichloromethane (2 × 150 mL). The combined organic extracts were dried over $MgSO_4$ and evaporated. Compound **13** was purified by chromatography on silica gel in dichloromethane−methanol (95:5) (0.95 g, 1.35 mmol, 75%): mp 147 °C dec; ^{31}P NMR (81 MHz, $CDCl_3$), two diastereoisomers A and B, δ 7.2 (d, J_{PH} = 704 Hz), 5.9 (d, J_{PH} = 708 Hz); ^1H NMR (500 MHz, $CDCl_3$), two diastereoisomers A and B, δ 8.67 (2H, s), 8.60 (2H, s), 8.05 (2H, s), 7.50 (1H, s), 7.44 (1H, s), 7.38 (2H, s), 6.90 (1H, d, J_{HP} = 708 Hz), 6.89 (1H, d, J_{HP} = 708 Hz), 6.35 (1H, dd, J = 9, 5.6 Hz), 6.32 (1H, dd, J = 9, 5.6 Hz), 5.25 (2H, m), 4.67 (4H, br m), 4.35 (4H, m), 4.16 (2H, m), 4.11 (4H, m), 2.55 (6H, s), 2.45 (6H, s), 2.43 (1H, m), 2.41 (1H, m), 2.21 (1H, m), 2.19 (1H, m), 2.08 (6H, s), 1.84 (4H, m), 1.72 (4H, m), 1.57 (6H, s), 1.54 (4H, m), 1.51 (4H, m); H-phosphonate doublet 200 MHz δ_A 7.61, 6.19 (J_{HP} = 708 Hz), δ_B 7.60, 6.19 (J_{HP} = 704 Hz); 300 MHz δ_A 8.08, 5.72 (J_{HP} = 708 Hz), δ_B 8.06, 5.72 (J_{HP} = 704 Hz); 500 MHz δ_A 8.67, 5.13 (J_{HP} = 708 Hz), δ_B 8.65, 5.13 (J_{HP} = 704 Hz); LRMS [FAB$^+$, NBA] *m/z* 673 [M + H]$^+$; HRMS exact mass calcd for $C_{30}H_{37}N_6O_{10}P$ 673.2387 [M + H]$^+$, found [FAB$^+$, MCA] *m/z* 673.2368. Anal. Calcd for $C_{30}H_{37}N_6O_{10}P{\cdot}CH_3OH$ (704.67): C, 52.82; H, 5.86; N, 11.93. Found: C, 52.73; H, 5.90; N, 11.89.

Flavin−Thymidine Phosphodiester 15. Compound **13** (100 mg, 0.142 mmol) was dissolved in 0.2 M iodine in pyridine−THF (1:1, 10 mL) under argon in the dark, and triethylamine (1 mL) was added. The mixture was stirred for 5 h, and then water was added. The resulting solution was extracted with dichloromethane to remove the traces of unreacted starting compound **13**. The aqueous layer was evaporated to dryness, and the residue was chromatographed on C_{18} reversed-phase (C_{18}-Sep-Pak Waters, 1 g cartridge) in water to yield the acetylated intermediate **14**. A solution of this compound in concentrated aqueous ammonia (15 mL) was stirred for 3 h. After evaporation, the residue was chromatographed on a short column of reversed phase (C_{18}-Sep-Pak Waters, 1 g cartridge) in water−methanol (90:10). Precipitation from diethyl ether yielded compound **15** (75 mg, 0.1 mmol, 75%).

Acetylated intermediate **14**: ^{31}P NMR (81 MHz, D_2O) δ 0.8; ^1H NMR (300 MHz, D_2O) δ 7.62 (1H, s), 7.58 (1H, s), 7.35 (1H, s), 5.82 (1H, m), 5.23 (1H, m), 4.45 (2H, br m), 4.00 (2H, m), 3.90 (1H, m), 3.86 (2H, m), 2.51 (3H, s), 2.39 (3H, s), 2.24 (1H, m), 2.12 (4H, m), 1.74 (4H, m), 1.66 (3H, s), 1.56 (4H, br m); LRMS [FAB$^+$, glycerol] *m/z* 690 [M + H]$^+$.

Compound **15**: mp 138 °C dec; ^{31}P NMR (81 MHz, D_2O) δ 0.9; ^1H NMR (500 MHz, D_2O) δ 7.74 (1H, s), 7.66 (1H, s), 7.33 (1H, d), 5.86 (1H, q), 4.45 (2H, br m), 4.03 (1H, m), 3.92 (1H, m), 3.90 (4H, m), 2.54 (3H, s), 2.43 (3H, s), 2.20 (1H, m), 2.05 (1H, m), 1.64 (3H, s), 1.80 (2H, m), 1.71 (2H, m), 1.58 (2H, m), 1.40 (2H, m); ^{13}C NMR-DEPT (75 MHz, D_2O) δ 139.5, 133.6, 119.0, 88.7, 87.6, 74.0, 69.0, 68.0, 48.8, 42.0, 32.1, 29.5, 28.0, 27.0, 23.6, 21.6, 14.6; LRMS [FAB$^+$, NBA] *m/z* 645 [M + H]$^+$. Anal. Calcd for $C_{28}H_{34}N_6O_{10}PNa{\cdot}2.5H_2O$ (713.61): C, 47.17; H, 5.47; N, 11.78. Found: C, 46.87; H, 5.40; N, 11.76.

Flavin−Thymidine Phosphorothioate 16. Compound **13** (100 mg, 0.142 mmol) was dissolved in a solution of 0.8 M sulfur S_8 in a pyridine−carbon disulfide mixture (1:1, 10 mL) under argon in the dark. The mixture was stirred for 24 h, and water was added. The resulting solution was extracted with dichloromethane to remove the unreacted starting compound **13**. The aqueous layer was evaporated to dryness. The residue was stirred with concentrated aqueous ammonia (15 mL) for 3 h. After evaporation, water was added, and the resulting solution was extracted with dichloromethane. The aqueous layer was evaporated, and the residue was chromatographed on a short column of reversed phase (C_{18}-Sep-Pak Waters, 1 g cartridge) in water−methanol (95:5). Precipitation from diethyl ether yielded pure compound **16** (68 mg, 0.094 mmol, 78%): mp 122 °C dec; ^{31}P NMR (81 MHz, D_2O), two diastereoisomers A and B, δ 56.2, 56.0; ^1H NMR (300 MHz, D_2O), two diastereomers A and B, δ 7.55 (4H, s), 7.36 (1H, s), 7.39 (1H, s), 5.95 (2H, m), 4.67 (2H, m), 4.47 (4H, br m), 4.10 (2H, m), 3.97 (4H, m), 3.93 (4H, m), 2.50 (6H, s), 2.38 (6H, s), 2.20 (2H, m), 2.10 (2H, m), 1.71 (3H, s), 1.69 (8H, m), 1.68 (3H, s), 1.56 (8H, m); ^{13}C NMR-DEPT (75 MHz, D_2O) δ 139.7, 133.4, 119.0, 88.7, 87.8, 74.3, 68.8, 67.7, 48.8, 42.0, 32.0, 29.5, 28.2, 27.4, 23.6, 21.6, 14.6; LRMS [FAB$^+$, NBA] *m/z* 661 [M + H]$^+$. Anal. Calcd for $C_{28}H_{34}N_6O_9SPNa{\cdot}2.5\ H_2O$ (729.67): C, 46.10; H, 5.35; N, 11.52; P, 4.25. Found: C, 45.99; H, 5.39; N, 11.48; P, 3.91.

Flavin−Thymidine Methylphosphotriester 17. Compound **13** (90 mg, 0.128 mmol) was dissolved in 10% methanol in a mixture of *N*-methylimidazole−TEA−CCl_4 (5:5:90, 15 mL) under argon in the dark. The mixture was stirred for 6 h, and dichloromethane was added. The resulting solution was washed with water, and the aqueous layer was extracted twice with dichloromethane. The combined organic extracts were dried over $MgSO_4$ and evaporated. The residue was stirred with concentrated aqueous ammonia (15 mL) for 1 h. After evaporation, dichloromethane was added, and the resulting solution was washed with water and dried over $MgSO_4$. The residue obtained after evaporation was chromatographed on silica gel in dichloromethane−methanol (95:5) to yield compound **17** (37 mg, 0.053 mmol, 42%): mp 157−158 °C dec; ^{31}P NMR (81 MHz, $CDCl_3$), two diastereoisomers A and B, δ −1.0, −1.6; ^1H NMR (300 MHz, $CDCl_3$), two diastereomers A and B, δ 8.60 (2H, br s), 8.05 (2H, s), 7.43 (1H, s), 7.39 (1H, s), 7.37 (2H, s), 6.32 (2H, m), 4.67 (4H, br m), 4.54 (2H, m), 4.30 (2H, m), 4.12 (4H, m), 3.80 (4H, m), 2.56 (6H, s), 2.44 (6H, s), 2.18 (4H, m), 1.93 (3H, s), 1.92 (3H, s), 1.85 (4H, m), 1.72 (4H,

m), 1.52 (8H, m); LRMS [FAB$^+$, NBA] m/z 661 [M + H]$^+$. Anal. Calcd for $C_{29}H_{37}N_6O_{10}P\cdot 1.5H_2O$ (687.64): C, 51.10; H, 5.87; N, 12.33. Found: C, 50.95; H, 5.68; N, 12.23.

Synthesis of the Flavin−Oligonucleotide Adducts. Columns containing the starting oligonucleotides were obtained from Eurogentec S.A. (phosphoramidite method, 1 μmol scale, DMTr protecting group removed). The solid support was dried by flushing argon through the column and was put in a test tube that was stoppered with a rubber septum. A mixture of the H-phosphonate **12b** (24 mg, 0.052 mmol; mixture of acid and its pyridinium salt 2:1 dried by coevaporation with toluene) and 1-adamantanecarboxylic acid chloride (10.4 mg, 0.058 mmol) or trimethylacetyl chloride (8 μL, 0.064 mmol) in dry pyridine was added to the solid support. The mixture was stirred at room temperature for 30 min. The solid support was isolated by filtration and washed with pyridine and then acetonitrile. After successive treatments with oxidizing reagents (I_2 (0.2 M) in THF for 5 min, I_2 (0.2 M) in *N*-methylmorpholine−water−THF (1:1:8) for 10 min and then I_2 (0.2 M) in triethylamine−water−THF (1:1:8) for 3 min), the support was washed with acetonitrile. The oligonucleotide was cleaved from the support with concentrated aqueous ammonia or with 0.2 M sodium hydroxide in methanol−water 50:50 with stirring at room temperature. The supernatant was evaporated (neutralization with aqueous HCl after deprotection with NaOH). The residue was chromatographed on a short column of reversed phase (C_{18}-Sep-Pak Waters, 1 g cartridge) in water−methanol.

The oligonucleotides were purified by two chromatographies (detection at 254 nm and 450 nm): first, on C_{18} reversed phase using a linear gradient of methanol (0% initially, then increasing 1.25%/min up to 35%) in 20 mM sodium phosphate buffer pH 6 at flow rate of 1mL/min; secondly, on polyanionic resin (MonoQ Pharmacia) using a linear gradient of potassium chloride (20% initially, then increasing 0.9%/min up to 60%) in a mixture of sodium dihydrogenphosphate 20 mM pH 6 acetonitrile (80:20) at flow rate 1.5 mL/min. Finally, desalting (C_{18}-Sep-Pak Waters, 1 g cartridge) yielded oligonucleotide (oligo 1, 62%; oligo 3, 54%; oligo 4, 30%; oligo 5, 52%; oligo 6, 43%).

Oligo 1 Fl−C_6−p−T_{11} (11 mer): ^{1}H NMR (500 MHz, D_2O) δ 7.94 (1H, s), 7.72 (1H, d), 7.71 (1H, s), 7.70−7.65 (8H, m), 7.63 (1H, s), 7.55 (1H, s), 6.37−6.22 (10H, m), 6.08 (1H, m), 4.92 (11H, m), 4.78−4.60 (2H, m), 4.35 (10H, m), 4.25 (1H, m), 4.20−4.00 (22H, m), 3.91 (2H, m), 2.57 (3H, s), 2.60−2.50 (11H, m), 2.46 (3H, s), 2.43−2.10 (11H, m), 1.93−1.86 (34H, m), 1.85−1.40 (4H, m), 1.79 (3H, s); MS (electrospray ES$^-$/MS, ammonium salt) exact mass calcd for $C_{128}H_{165}N_{26}O_{80}P_{11}$ 3688.5, found 3688.5.

Oligo 2 Fl−C_6−p(T_{11})$_2$ (22 mer): ^{1}H NMR (500 MHz, D_2O) δ 7.94 (1H, s), 7.72 (2H, s), 7.70−7.64 (19H, m), 7.39 (2H, s), 6.36−6.22 (20H, m), 6.18 (2H, m), 4.92 (22H, m), 4.70−4.60 (2H, m), 4.34 (22H, m), 4.20−4.10 (46H, m), 2.60−2.48 (25H, m), 2.45 (3H, s), 2.43−2.22 (22H, m), 1.96−1.85 (64H, m), 1.75−1.40 (4H, 3 m), 1.79 (3H, s), 1.76 (3H, s); MS (electrospray ES$^-$/MS, ammonium salt) exact mass calcd for $C_{238}H_{305}N_{48}O_{154}P_{21}$ 6952.7, found 6952.8.

Oligo 3 Fl−C_6−p−T_{16} (16 mer): ^{1}H NMR (500 MHz, D_2O) δ 8.02 (1H, s, F6-*H*), 7.82 (2H, s, 2 T6-*H*), 7.81 (1H, s, F9-*H*), 7.80−7.72 (12H, m, 12 T6-*H*), 7.63 (2H, s, 2 T6-*H*), 6.40−6.30 (15H, m, 15 T1′-*H*), 6.16 (1H, m), 5.02 (16H, m), 4.84−4.70 (2H, m), 4.44 (15H, m), 4.35 (1H, m), 4.32−4.10 (32H, m), 4.00 (2H, m), 2.66 (3H, s), 2.70−2.62 (16H, m), 2.55 (3H, s), 2.50−2.38 (16H, m), 2.20−1.74 (49H, m), 1.87 (3H, s), 1.82−1.60 (4H, 2 m); MS (electrospray ES$^-$/MS, ammonium salt) exact mass calcd for $C_{178}H_{230}N_{36}O_{115}P_{16}$ 5209.5, found 5209.0.

Oligo 5−6 (16 mers): ^{1}H NMR (500 MHz, D_2O) δ 7.96 (1H, s), 7.76 (1H, m), 4.88−4.60 (2H, m), 3.97 (2H, m), 2.61 (3H, s), 2.50 (3H, s), 2.10−1.82 (4H, m), 1.90−1.40 (4H, m).

Enzyme Digestion. A solution of nuclease P1 (0.3 U/μL) in 20 μL of buffer (30 mM sodium acetate, 1 mM $ZnSO_4\cdot 7H_2O$, pH 5.3) and a solution of phosphatase alkaline (1 U/μL) in 12 μL of buffer (50 mM Tris−HCl, 1 mM EDTA, pH 8) were prepared. Oligonucleotides were incubated with the solution of nuclease P1 (10 μL) at 37 °C for 2 h and after addition of the phosphatase alkaline solution (1 μL) for 1 h. The digests were analyzed by HPLC on C_{18} reversed phase under the conditions described for the purification of oligonucleotides.

Acid Hydrolysis. The oligomers were heated in aqueous hydrochloric acid (0.1 N, 250 μL) at 100 °C for 1 h 30 min, and then aqueous ammonia (2.5 M, 100 μL) was added. After evaporation, the residue was dissolved in water, and the resulting solution was analyzed by HPLC on C_{18} reversed phase under the conditions described for the purification of oligonucleotides.

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Supporting Information Available: ^{1}H NMR data for all compounds synthesized (6 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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