Linker-Free Fluorophore-Labeled Oligonucleotides: Synthesis of 3′-Fluoresceinylthymidine Building Blocks and their Coupling Reactions

Niels Griesang, Eric Kervio, Clemens Richert*

Institut für Organische Chemie, Universität Karlsruhe (TH), 76131 Karlsruhe, Germany
Fax +49(721)6084825; E-mail: cr@rrg.uka.de

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Abstract: Reported here are the syntheses of two thymidine derivatives with an amide-linked 6-carboxyfluorescein residue at their 3′-position that are suitable for the synthesis of fluorophore-labeled oligonucleotides. The first is a 5′-phosphoramidite with a pivaloyl-protected carboxyfluorescein residue in the lactone form. It was prepared from 3′-azido-3′-deoxythymidine (AZT) in three steps and 73% overall yield and coupled on solid support. The second is an imidazolide of thymidine 5′-monophosphate that was obtained from AZT in five steps and 48% overall yield. The imidazolide can be coupled to amino-terminated nucleic acids in aqueous solution.

Key words: DNA, nucleosides, oligomerization, oligonucleotides, solid-phase synthesis

Introduction

Fluorophore-labeled nucleic acids play important roles in biomedical applications. For example, DNA sequencing by the chain terminator method[1] is predominantly performed with fluorophore-labeled dideoxynucleotides.[2] Massively parallel detection of gene expression can be achieved with DNA chips that detect fluorophore-labeled DNA or RNA targets.[3] Direct, in situ detection of specific sequences, including those formed during polymerase chain reactions (PCR) can be achieved with molecular beacons,[4] which carry a combination of a fluorophore and a quencher.[5] Förster resonance energy transfer (FRET) can be employed to measure spatial closeness of two chromophores in a structural motif.

Common to the fluorophore labeling approaches currently favored is the use of a flexible linker between the nucleic acid and the luminescent chromophore. This results in limited precision in the location of the labeled compound, a loss of correlation between fluorophore and DNA in fluorescence anisotropy measurements, an increase in the molecular weight, and often an increased likelihood of adsorption on surfaces. We have recently shown that small appendages at the termini of oligonucleotides can be successfully engaged in complex formation with the termini of DNA duplexes.[6] A more precise location in folded structures of nucleic acids is also desirable for fluorophores. Developing a construct where the precise location of the fluorophore is achieved through a direct amidic link to a nucleotide is one first step towards achieving the precise location and a possible engagement in tightly folded complexes. Further, such rigidly linked constructs may facilitate the in situ detection of binding through fluorescence anisotropy measurements.

A report on linker-free deoxyadenosine-fluorescein hybrids incorporated in synthetic oligonucleotides has recently appeared.[7] In these hybrids, the covalent link between a carboxyfluorescein and the nucleotide involved the nucleobase. In order to allay concerns that this may affect the base pairing properties, we decided to pursue an alternative approach, where the direct link is between the deoxyribose and carboxyfluorescein. For azo dyes it is known that they can be directly attached to the 3′- or 5′-position of nucleic acids via ester bonds.[8] We were interested in producing building blocks that can be employed both in solid phase syntheses of oligonucleotides and in non-enzymatic primer extension reactions involving deprotected nucleic acids in aqueous solution. The former synthetic approach calls for phosphoramidites of protected nucleosides,[9] whereas the latter synthetic approach is commonly based on imidazolides of nucleotides.[10] We chose carboxyfluorescein as fluorophore, as this chromophore is among the best established in biomolecular applications. Carboxyfluorescein is commercially available and shows favorable absorption and emission properties, even when it is linked to DNA.[11] An amidic link was considered most desirable, as it is small, uncharged, rigid, and stable toward the deprotection conditions typically employed at the end of a solid phase synthesis of oligonucleotides. Of the four deoxynucleotides that make up DNA, thymidine was chosen for the current study, as it does not complicate syntheses with a need for protection of the nucleobase. This led to compounds **1** and **2** as our primary synthetic targets (Figure 1).

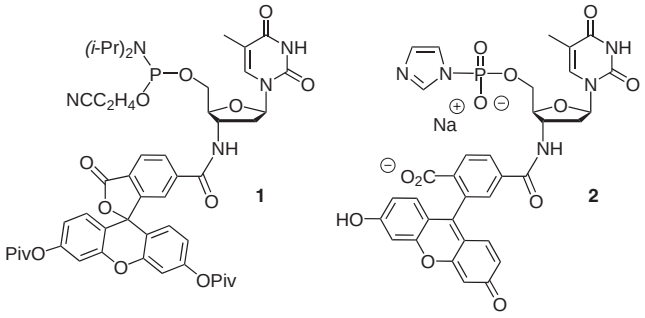

Figure 1 Building blocks for the synthesis of linker-free fluorescein-labeled oligonucleotides with 3′-terminal thymidine residues.

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Results and Discussion

The synthesis of **1** started from 3′-azido-3′-deoxythymidine (**3**) (Scheme 1). This antiviral compound is commercially available, and larger quantities could be prepared as described.[12] Hydrogenation gave amine **4**,[13] ready for coupling to the fluorophore. The *N*-hydroxysuccinimide (NHS) ester of 6-carboxyfluorescein dipivalate **5** was chosen as reaction partner. To generate it, the 6-isomer of carboxyfluorescein dipivalate was prepared from the commercial mixture of 5/6-carboxyfluorescein isomers following a literature protocol.[14] The NHS ester **5** was used, rather than an in situ activated acid, to avoid side reactions involving the lactone functionality. Such side reactions have been described for related xanthene dyes[15] and the 5-isomer of carboxyfluorescein[16] when coupling to a primary amine. For the conversion of carboxyfluorescein to the NHS ester, a protocol similar to that described by Laurent and collaborators for the mixture of fluorescein isomers[17] was employed. The use of EDC as coupling agent, in combination with an acidic aqueous work-up, gave sufficiently pure **5**, avoiding low-yielding chromatographic steps. The acylation occurred under conditions similar to those reported for other acylations of aminothymidine.[18] In this instance, amine **4** and NHS ester **5** were reacted in DMF without addition of a base to avoid the loss of pivaloyl groups. Thymidine-fluorescein hybrid **6** was thus obtained in 85% yield after chromatographic purification.

Compound **6** was 5′-phosphitylated to **1** (Scheme 1), which was purified by column chromatography after a basic aqueous work-up. Compound **1** was used in the last coupling step of DNA syntheses involving commercial 5′-phosphoramidites.[19] First, self-complementary hexamer **10** was prepared, starting from controlled pore glass (cpg) loaded with the 5′-terminal deoxyadenosine residue **7**. Automated synthesis gave pentamer **8**, followed by manual coupling of **1** to give the protected hexamer **9**. UV-melting curves for the duplex of deprotected **10** (Figure 2) showed a melting point (Tm) decrease of no more than 2.6 °C per modification when compared to that of unmodified control hexamer ACGCGT (**11**) (Table 1). Further, the duplex showed intense fluorescence (λ_{max} = 518 nm upon excitation at 495 nm). This was also true for the second oligonucleotide prepared (Scheme 2). Besides the 3′-fluoresceinyl residue, dodecamer **15** features a lysine residue at its 5′-terminus, linked to the DNA via a 2,2-dimethyl-3-hydroxypropionic acid residue.[20] This allows for immobilization of oligonucleotides on aldehyde-displaying glass slides in the form of microarrays.[21] Chain assembly started from functionalized support **12**[21] (Scheme 2) and proceeded to doubly modified **15**, which was obtained in mass spectrometrically pure form after RP-HPLC. The fluorophore-labeled oligonucleotide elutes four minutes later than the unlabeled control, and fractions containing this compound are easy to spot by their color and fluorescence. Again, the fluoresceinyl substituent had a minor effect on the melting point of the duplex with the target strand. When compared to the unmodified control duplex **16**:**17**, a ΔT_m of –2.0 °C was measured (Table 1). Lysine-terminated **15** is currently being used to optimize the immobilization procedure for oligonucleotides on aldehyde slides.

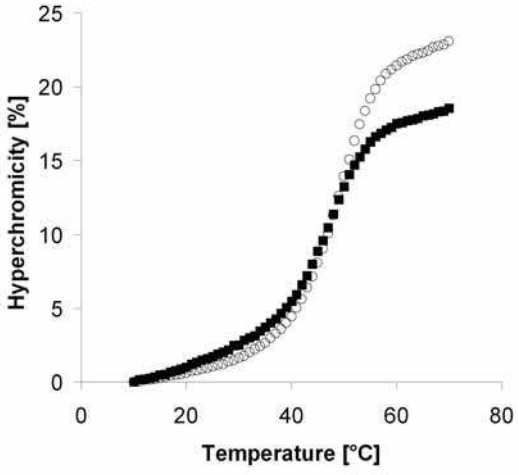
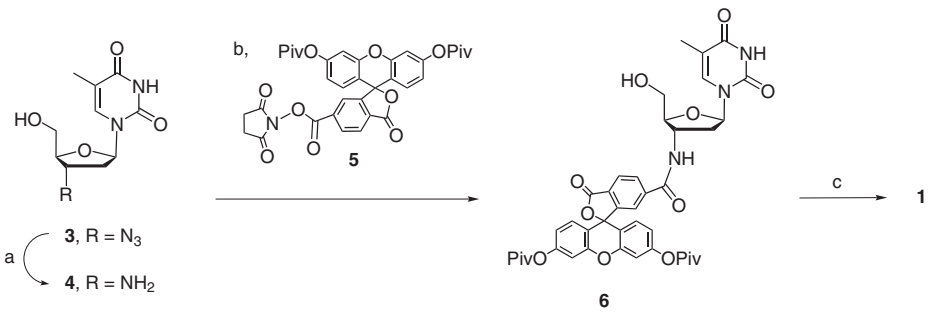


Figure 2 Representative UV-melting curves of the modified oligonucleotide **15** (filled squares) or unmodified 5′- TCATTC-TGTTCT-3′ (**16**) (open circles), each with their fully complementary strand **17** at 2.25 μM strand concentration, 1 M NaCl, 10 mM phosphate buffer, pH 7; monitored at 260 nm.

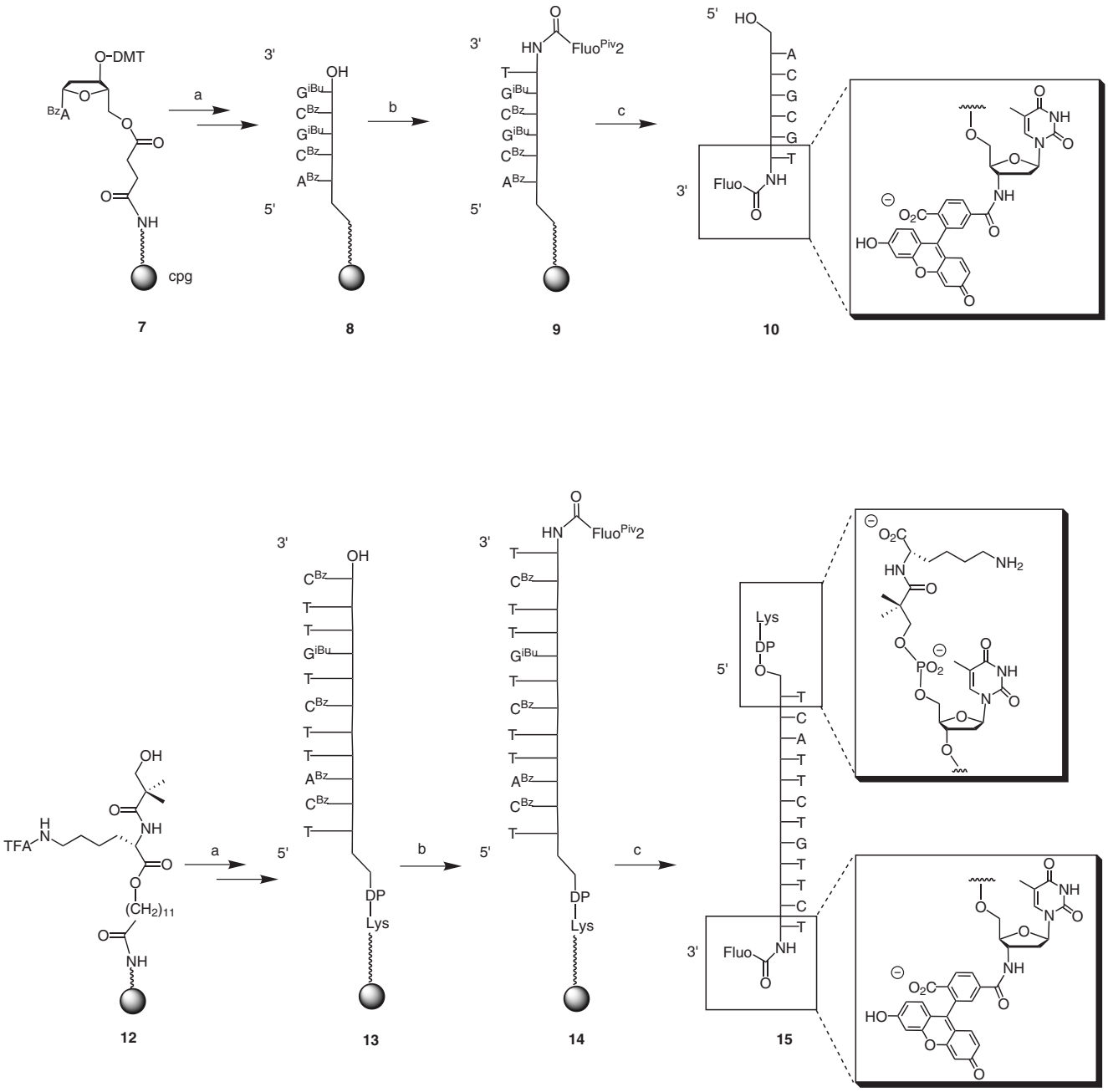


Scheme 1 *Reagents and conditions*: a) H_2, Pd/C, MeOH, 92%; b) **5**, DMF, 85%; (c) DIEA, chloro-*N*-(diisopropylamino)cyanoethoxyphosphoramidite, MeCN, 86%.

Since the linker-free labeling of the 3′-terminus of oligonucleotides seemed to be tolerated, we next turned to the synthesis of 5′-imidazolide **2**, which is envisioned to function in non-enzymatic, template-directed extension reactions.[23–26] Primer extension products from reactions involving **2**, when performed in microarray format, can be expected to be detectable by fluorescence scanning of chip surfaces. To evaluate routes without the complication of the hydrolytic lability of pivaloyl-protected fluoresceinyl residues, a model synthesis was performed with 3′-TBDMS-protected thymidine **18** (Scheme 3).

Imidazolides of nucleotides have been known for over 40 years,[27] and have been established as activated monomers in non-enzymatic replication.[28] Two possible routes to the imidazolide were considered: the oxidative generation of an imidazolide from a 5′-*H*-phosphonate under Atherton conditions,[29] and the coupling of imidazole to a 5′-phosphate. The phosphate could also have been generated directly from the 5′-alcohol,[30] or through oxidation of an *H*-phosphonate, which in turn should have been accessible from the alcohol using more reactive phosphorus(III) reagents.[31] Since the *H*-phosphonate promised the greatest flexibility, compound **19** was chosen as intermediate.

The synthesis of **18** followed the protocol of Lan and colleagues.[32] A more recent synthetic procedure[33] gave lower yields. Conversion to *H*-phosphonate **19** and the oxidative

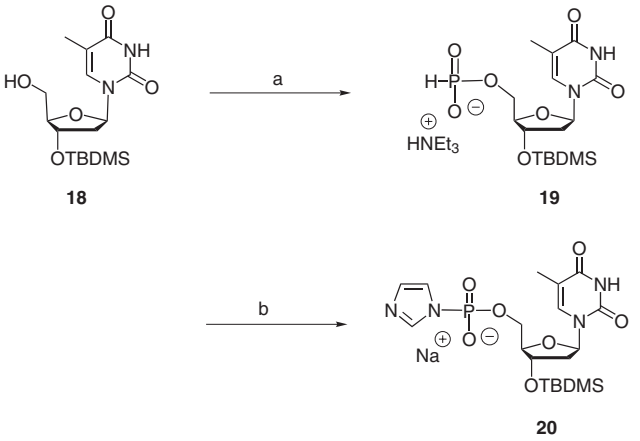


Scheme 2 *Reagents and conditions*: a) DNA synthesis with 5′-phosphoramidites; b) coupling cycle with **1**; c) NH_4OH. Fluo = fluorescein residue.

Table 1 UV Melting Points and Hyperchromicities of DNA Duplexes

Duplex	T_m (°C)[a]	ΔT_m (°C)[b]	Hyperchromicity (%)[b]
(11)$_2$ (control)	31.1 ± 0.3[22]		6.6 ± 1.4[22]
(10)$_2$	25.8 ± 2.3	–2.6	6.2 ± 2.3
16:**17** (control)	49.0 ± 0.3		23.1 ± 0.2
15:**17**	47.0 ± 0.6	–2.0	19.7 ± 1.0

[a] Average of four melting points ± one standard deviation; conditions: 1 M NaCl, 10 mM phosphate buffer, pH 7, strand concentration: 3.5 μM (entries 1, 2), 2.25 μM (entries 3, 4).
[b] Melting point difference to control strand (per fluoresceinyl group).



Scheme 3 *Reagents and conditions*: a) 1. diphenyl phosphite, pyridine, 2. H_2O, Et_3N, 50%; b) TMS-imidazole, CCl_4, Et_3N, MeCN, 36%.

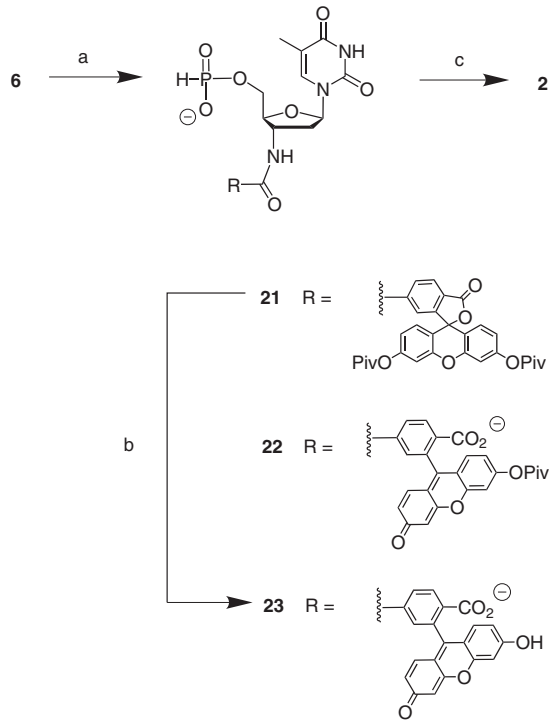
coupling to **20** were achieved in moderate yield, using an adaptation of the method of Ushioda and colleagues.[29]

The same route was employed for fluoresceinylthymidine **6** as starting material (Scheme 4). Phosphitylation with diphenyl phosphite generated a phenyl phosphite whose in situ hydrolysis with water–triethylamine gave *H*-phosphonate **21**. Initial attempts to purify **21** led to a mixture containing **22** and **23**, whose fluoresceinyl moieties lack pivaloyl groups. Hydrolysis was probably induced by residual triethylamine introduced during chromatography, though precipitation with $NaClO_4$ also gave partial loss of pivaloyl groups. Subsequently, the mixture was deliberately converted to **23** with sodium methoxide. Attempts to convert **23** to **2** analogously to the activation of **19** to **20** were unsuccessful, however, due to the low solubility of the deprotected intermediate. Ion exchange to the pyridinium form did not help. In a mixture of **21** and **23**, only the former was reactive toward the Atherton reagents. Separation of **2** and **23** by chromatography or precipitation failed.

Intermediate **21** could be isolated in satisfactory yields with 5% acetic acid in the eluent during chromatography.

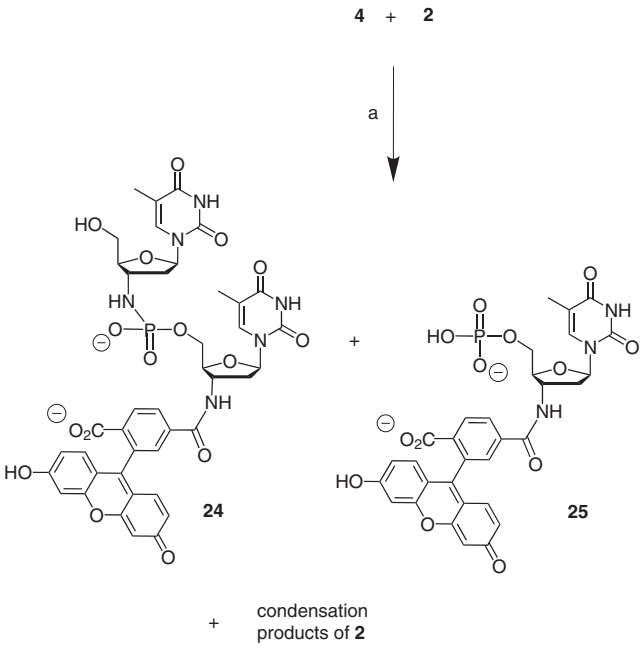
The free *H*-phosphonate of protected **21** was too insoluble in acetonitrile, however, for conversion to **2** under the conditions established for **19**. Both DMF and DMSO as solvents failed to give detectable conversion. Only DMF and Et_3N (1:1) gave conversion. In situ addition of water then led to the removal of the pivaloyl groups. Imidazolide **2** could thus be isolated as sodium salt after precipitation in the presence of sodium perchlorate.

An exploratory experiment with nucleosidic amine **4** as reaction partner in the absence of a template performed in aqueous buffer (Scheme 5) showed that **2** couples to amino-terminal nucleic acids, producing an (elongated) chain. The reaction was analyzed by MALDI MS under conditions that allow for quantitation.[34] After 40 hours, the imidazolide had disappeared from the spectrum and peaks for three products were detected at a ratio of approximately 9:2:3. These were for free phosphate **25**, the elongation product **24**, and a compound whose mass fits that of a dimerization product of **2** with hydrolyzed imidazolide group, respectively. Since small peaks compatible with condensation of **24** with an additional residue of **2** as well as a trimerization product of **2** were detected, it is fair to assume that the self-condensation of **2** observed in the absence of a template involves the phenolic hydroxyl groups of fluoresceinyl moieties and not the phosphate, which would have led to a pyrophosphate-linked dimer only. Oligomers of fluoresceinylnucleosides, such as those formed accidentally here, may be of interest as FRET fluorophore arrays with large photon capture cross sections.[35] Further, the detectable rate of coupling between **4** and **2**, even in the absence of a template, suggests



Scheme 4 *Reagents and conditions*: a) 1. diphenyl phosphite, pyridine, 2. H_2O, Et_3N, 85%; b) NaOMe, MeOH, 83%; c) 1. TMS-imidazole, CCl_4, Et_3N, DMF, 2. H_2O, 73%.

that a moderate template effect should suffice for elongation of a primer to dominate over hydrolysis of **2** and self-coupling reactions of the activated nucleotide. Imidazolide **2** may therefore become useful for screening catalysts for non-enzymatic primer extension reactions, including assays performed on microarrays analyzed by fluorescence microscopy. This use could complement the application of directly linked fluorescein-nucleoside hybrids as building blocks for oligomers with precisely positioned fluorophores, such as **10** and **15**.



Scheme 5 *Reagents and conditions*: a) 0.2 M $MgCl_2$, 1 M NaCl, 0.5 M aq 4-(2-hydroxyethyl)piperazin-1-ethansulfonic acid (HEPES) buffer, pH 7.9.

Anhyd solvents were purchased over molecular sieves and used without further purification. Reagents were the best available grade from Acros (Geel, Belgium) or Aldrich/Fluka/Sigma (Deisenhofen, Germany). Samples of 3′-azido-3′-deoxythymidine (AZT) were purchased from Toronto Research Chemicals Inc. (Ontario, Canada) or were prepared by the authors;[12] the isomeric mixture of 5(6)-carboxyfluorescein was from Fluka (Deisenhofen, Germany). Reversed or 5′-phosphoramidites were from Chemgenes (Ashland, MA, USA). All other reagents for DNA synthesis were from Proligo (Hamburg, Germany). Oligonucleotides were purified by reverse-phase HPLC, with a gradient of MeCN in 0.1 M Et_3NOAc (pH 7.0) and detection at 260 nm, using Nucleosil C4 columns for modified oligonucleotides and C18 columns for unmodified oligonucleotides (both 250 × 4.6 mm; Macherey-Nagel, Düren, Germany). Yields of oligonucleotides are based on the intensity of the product peaks in the integration of the HPLC traces of crude products. For modified oligonucleotides, the extinction coefficients were calculated as the sum of the extinction coefficient of the unmodified oligonucleotide and the extinction coefficient of any non-nucleosidic residue. MALDI-TOF spectra were acquired on a Bruker REFLEX IV spectrometer in negative, linear mode, using the software XACQ 4.0.4 and XTof 5.1. MALDI-TOF spectra were recorded in positive, linear mode in the case of small molecules, and in negative, linear mode in the case of oligonucleotides. MALDI matrixes were a mixture of 2,4,6-trihydroxyacetophenone (0.3 M in EtOH) and diammonium citrate (0.1 M in H_2O) (2:1) or diammonium tartrate (0.1 M in H_2O), abbreviated below as TC21. For small molecules, 2,5-dihydroxybenzoic acid (0.1 M in MeCN) or 6-aza-thio-2-thiothymidine (sat. solution in MeCN) was used as MALDI matrix. UV/Vis and Fluorescence spectra were recorded using a Perkin-Elmer spectrophotometer Lambda 10 and a Perkin-Elmer luminescence spectrometer LS50B respectively in 600 μL quartz cuvettes.

6-Carboxyfluorescein-3′,6′-dipivalate NHS-Ester (5)

The compound was synthesized as reported for the mixture of isomers,[17] starting from isomerically pure 6-carboxyfluorescein dipivalate; yield 92%; R_f 0.35 (CH_2Cl_2–MeOH, 98:2).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.40 (d, *J* = 7.9 Hz, 1 H), 8.18 (d, *J* = 7.9 Hz, 1 H), 7.95 (s, 1 H), 7.10 (d, *J* = 2.1 Hz, 2 H), 6.77–6.84 (m, 4 H), 2.90–2.89 (m, 4 H), 1.36 (br s, 18 H).

***N*-(3′,6′-Dipivaloylfluorescein-6-ylcarbonyl)-3′-amino-3′-deoxythymidine (6)**

To a stirred solution of amine **4** (230 mg, 0.954 mmol) in anhyd DMF (10 mL) was added the NHS ester **5** (916 mg, 1.19 mmol) under stirring. After 16 h at r.t., the mixture was diluted with CH_2Cl_2 and washed with 5% aq $NaHCO_3$ solution (2 ×) and brine (1 ×). The combined organic phases were dried (Na_2SO_4), and the solvent was removed by rotary evaporation. The residue was purified by column chromatography (silica gel) with a stepwise gradient of CH_2Cl_2–MeOH from 95:5 to 9:1 to give 620 mg of **6** (85%) as a yellow solid; R_f 0.35 (CH_2Cl_2–MeOH, 9:1).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 9.85 (br s, 1 H), 8.16 (d, *J* = 7.8 Hz, 1 H), 8.03 (d, *J* = 7.8 Hz, 1 H), 7.65 (2 s, each 1 H), 7.03–6.99 (m, 2 H), 6.77–6.67 (m, 4 H), 6.03 (t, *J* = 5.6 Hz, 1 H), 4.55 (m, 1 H), 3.83–3.70 (m, 3 H), 2.27–2.24 (m, 2 H), 1.79 (s, 3 H), 1.36 (s, 9 H), 1.35 (s, 9 H).

^{13}C NMR (126 MHz, $CDCl_3$). δ = 176.9, 176.8, 168.1, 166.2, 164.1, 153.2, 152.6, 152.5, 151.4, 151.3, 150.8, 139.8, 136.1, 129.8, 128.8, 128.7, 128.4, 125.5, 122.7, 117.9, 117.8, 115.5, 115.3, 110.9, 110.5, 110.4, 85.7, 84.5, 81.7, 62.0, 50.2, 37.0, 12.5.

MALDI-TOF MS (linear, positive, TC21): *m*/*z* calcd for $C_{41}H_{41}N_3O_{12}$ $[M + H]^+$: 768.8; found: 769.1.

***N*-(3′,6′-Dipivaloylfluorescein-6-ylcarbonyl)-3′-amino-3′-deoxythymidine-5′-*O*-yl-cyanoethyl-*N*,*N*-diisopropylphosphoramidite (1)**

To a stirred solution of **6** (75 mg, 100 μmol) and diisopropylethylamine (DIEA) (20 μL, 200 μmol) in anhyd MeCN (600 μL) was added 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (24 μL, 110 mol). After stirring for 45 min, the mixture was poured into aq sat. $NaHCO_3$ solution (20 mL) and extracted with CH_2Cl_2 (3 × 20 mL). The organic phase was dried (Na_2SO_4), and the solvent was removed by rotary evaporation to 0.3 mL. The residue was purified by column chromatography (silica gel previously neutralized with 0.5% Et_3N in elution mixture) using a mixture of cyclohexene–acetone (1:1). The title compound was obtained as pale yellow foam (83 mg, 86%); R_f 0.7 (hexane–acetone–Et_3N, 66:33:0.5).

^{31}P NMR (202 MHz, CD_3CN): δ = 150.1, 149.6.

MALDI-TOF MS (linear, positive, ATT): *m*/*z* calcd for $C_{50}H_{58}N_5O_{13}P$ ($[M + H]^+$): 968.9; found: 969.3.

Nucleoside 5′-*O*-Phosphonates 19 and 21; General Procedure

To a stirred solution of diphenyl phosphite (~10 equiv, 10 mmol, 1.93 mL) in anhyd pyridine (10 mL), was added dropwise a solution of the alcohol **18** or **6** (~1 mmol) in anhyd pyridine (2 mL). The mixture was stirred at r.t. under argon and the reaction progress was

monitored by TLC or MALDI-TOF MS until the phosphitylation was complete. After stirring for 1 h, the mixture was treated with H_2O–Et_3N (~500 μL). After stirring for 1 h, the solvents were evaporated in vacuo. The crude product was taken up in CH_2Cl_2 and washed with 1 M Et_3NHCO_3 buffer (2 ×) and brine (1 ×). The combined organic phases were dried (Na_2SO_4), and the solvent was removed by rotary evaporation. After purification by column chromatography (silica gel) the 5′-*O*-phosphonates **19** and **21** were obtained as yellow oils.

Triethylammonium 3′-*O*-(*tert*-Butyldimethylsilyl)thymidine-5′-*O*-phosphonate (19)

Prepared from **18** in 50% yield (261 mg) according to the general protocol. Column (silica gel): with a stepwise gradient of CH_2Cl_2–MeOH–Et_3N from 90:5:5 to 85:10:5; R_f 0.5 (CH_2Cl_2–MeOH–Et_3N, 90:5:5).

^{1}H NMR ($CDCl_3$, 250 MHz): δ = 7.69 (s, 1 H), 6.85 (d, *J* = 620 Hz, 1 H, HP), 6.30 (t, *J* = 6.4 Hz, 1 H), 4.41–4.39 (m, 1 H), 4.01–3.94 (m, 3 H), 3.04 [q, *J* = 7.3 Hz, 6 H, HN(CH_2CH_3)$_3$], 2.12–2.07 (m, 2 H), 1.87 (s, 3 H), 1.25 [t, *J* = 7.3 Hz, 9 H, HN(CH_2CH_3)$_3$], 0.86 (s, 9 H), 0.01 (s, 6 H).

^{31}P NMR (202 MHz, $CDCl_3$): δ = 4.52 (*J* = 620 Hz).

MALDI-TOF MS (linear, positive, DHB): *m*/*z* calcd for $C_{16}H_{28}N_2O_7PSi$ ([M + Na]$^+$): 443.4; found: 443.7.

Sodium 3′-*O*-(*tert*-Butyldimethylsilyl)thymidine-5′-*O*-phosphorimidazolide (20)

To a stirred solution of *H*-phosphonate **19** (57.1 mg, 0.109 mmol) in a mixture of MeCN–CCl_4 (2:1, 3 mL total volume) was added Et_3N (77 μL, 0.547 mmol). The mixture was then treated with trimethylsilylimidazole (80.8 μL, 0.547 mmol, 5 equiv) at r.t. After 2 h, the solution was then added in small portions to a solution of $NaClO_4$ (27 mg, 0.218 mmol) in anhyd acetone (18 mL) and anhyd Et_2O (9 mL). After stirring for 30 min, the white precipitate was isolated by filtration. The solid was washed three times with anhyd acetone (30 mL total) and twice with anhyd Et_2O (20 mL total) to give 20 mg (36%) of **20** as a white solid; R_f 0.45 (CH_2Cl_2–MeOH–Et_3N, 90:5:5).

^{1}H NMR (CD_3OD, 250 MHz): δ = 7.81 (s, 1 H), 7.63 (s, 1 H), 7.21 (s, 1 H), 6.96 (s, 1 H), 6.21 (t, *J* = 6.1 Hz, 1 H), 4.28–4.26 (m, 1 H), 3.94–3.80 (m, 3 H), 2.14–2.05 (m, 2 H), 1.86 (s, 3 H), 0.80 (s, 9 H), –0.03 (s, 6 H).

^{31}P NMR (202 MHz, CD_3OD): δ = –9.31.

MALDI-TOF MS (linear, positive, ATT): *m*/*z* calcd for $C_{19}H_{30}N_4O_7PSi$ ([M + Na]$^+$): 509.5; found: 509.5.

3′-Amino-3′-deoxy-*N*-(3′,6′-dipivaloylfluorescein-6-ylcarbonyl)thymidine-5′-*O*-phosphonate (21)

Prepared on a 0.086 mmol scale from **6** in 85% yield (61 mg) according to the general procedure. The reaction was monitored by MALDI-TOF MS. Column (silica gel) with the solvent mixture: CH_2Cl_2–MeOH–AcOH, 85:10:5); R_f 0.3 (CH_2Cl_2–MeOH–AcOH, 85:10:5).

^{1}H NMR (500 MHz, CD_3OD): δ = 8.22 (dd, *J* = 8.2, 0.9 Hz, 1 H), 8.15 (d, *J* = 8.2 Hz, 1 H), 7.82 (d, *J* = 0.9 Hz, 1 H), 7.76 (s, 1 H), 7.19–7.18 (m, 2 H), 6.93–6.87 (m, 4 H), 6.80 (d, *J* = 620 Hz, 1 H, HP), 6.28 (t, *J* = 6.5 Hz, 1 H), 4.67–4.65 (m, 1 H), 4.13–4.06 (m, 3 H), 2.44–2.32 (m, 2 H), 1.94 (s, 3 H), 1.37 (s, 18 H).

^{13}C NMR (126 MHz, CD_3OD): δ = 176.5, 168.5, 166.6, 165.0, 153.1, 153.0, 151.4, 150.9, 140.9, 136.5, 129.8, 128.9, 128.3, 124.9, 122.6, 117.9, 115.7, 110.6, 110.1, 84.6, 83.3, 83.2, 82.0, 63.3, 63.2, 50.8, 38.8, 36.8, 26.0, 11.1.

^{31}P NMR (202 MHz, CD_3OD): δ = 4.71 (*J* = 620 Hz).

MALDI-TOF MS (linear, positive, TT21): *m*/*z* calcd for $C_{41}H_{42}N_3O_{14}P$ ([M + H]$^+$): 832.7; found: 833.2.

Sodium 3′-Amino-3′-deoxy-*N*-(fluorescein-4-ylcarbonyl)thymidine-5′-*O*-phosphonate (23)

Crude **21** (166 mg, 0.2 mmol), prepared as described above, was dissolved in MeOH (3 mL) and treated with a solution of NaOMe in MeOH (1 M, 600 μL, 0.6 mmol, 3 equiv). After 2 h, the red solution was added to CH_2Cl_2 (40 mL). After stirring at 0 °C for 30 min, the red precipitate was isolated by filtration. The solid was washed twice with anhyd acetone (20 mL total) to give 113 mg (83%) of **23** as a red solid; R_f 0.8 (CH_2Cl_2–MeOH–Et_3N, 47:47:6).

^{1}H NMR (500 MHz, CD_3OD): δ = 8.08 (m, 2 H), 7.88 (m, 1 H), 7.74 (m, 1 H), 7.06–7.04 (m, 2 H), 6.56–6.52 (m, 4 H), 6.85 (d, *J* = 620 Hz, 1 H, HP), 6.37 (t, *J* = 6.5 Hz, 1 H), 4.76–4.74 (m, 1 H), 4.22–4.10 (m, 3 H), 2.48–2.44 (m, 2 H), 1.96 (s, 3 H).

^{13}C NMR (126 MHz, D_2O, NaOD): δ = 180.2, 179.9, 173.8, 169.2, 166.4, 158.7, 158.6, 157.6, 156.3, 151.6, 151.5, 142.6, 137.1, 133.9, 131.8, 131.2, 131.1, 128.7, 128.5, 128.4, 112.5, 111.6, 103.5, 84.7, 82.8, 63.2, 50.4, 36.2, 11.6.

^{31}P NMR (202 MHz, CD_3OD): δ = 4.78 (*J* = 620 Hz).

MALDI-TOF MS (linear, positive, TC21): *m*/*z* calcd for $C_{31}H_{25}N_3O_{12}P$ ([M + H]$^+$): 663.5; found: 664.3.

Sodium 3′-Amino-3′-deoxy-*N*-(fluorescein-4-ylcarbonyl)thymidine-5′-*O*-phosphorimidazolide (2)

To a stirred solution of the *H*-phosphonate **21** (61 mg, 0.073 mmol) in a mixture of DMF–CCl_4–Et_3N (1:1:1, 1.5 mL total) was added dropwise trimethylsilylimidazole (55 μL, 0.375 mmol, 5 equiv) at r.t., and the reaction was allowed to proceed for 5 h until MALDI-TOF MS showed full conversion. After stirring the yellow solution for 5 h, the mixture was treated with H_2O (13.5 μL, 0.75 mmol). The deprotection of the pivaloyl groups was monitored by MALDI-TOF MS. After 2 h, the dark red solution was then added in small portions to a solution of $NaClO_4$ (27 mg, 0.218 mmol) in anhyd acetone (27 mL). After stirring for 30 min, the red precipitate was isolated by filtration. The solid was washed three times with anhyd acetone (30 mL total) and twice with anhyd Et_2O (20 mL total), to give 40 mg (73%) of **2** as a red solid; R_f 0.8 (CH_2Cl_2–MeOH–Et_3N, 47:47:6).

^{1}H NMR (D_2O, 400 MHz, conc. and pH/pD dependent): δ = 7.83 (br s, 1 H), 7.78 (br s, 1 H), 7.75 (br s, 1 H), 7.42 (br s, 2 H), 7.10 (br s, 1 H), 7.05 (br s, 1 H), 6.92–6.78 (m, 2 H), 6.53–6.49 (m, 2 H), 6.45–6.40 (m, 2 H), 6.08 (t, *J* = 6 Hz, 1 H), 4.42 (q, *J* = 6 Hz, 1 H), 4.01–3.94 (m, 1 H), 3.93–3.91 (m, 1 H), 3.85–3.78 (m, 1 H), 2.33–2.25 (m, 1 H), 2.25–2.16 (m, 1 H), 1.79 (s, 3 H).

^{31}P NMR (202 MHz, D_2O): δ = –8.82.

MALDI-TOF MS: *m*/*z* calcd for $C_{34}H_{27}N_5O_{12}P$ ([M + H]$^+$): 730.8; found: 730.7.

Coupling of 4 with 2

Solutions of amine **4** (550 mM, 0.4 μL, 225 nmol) and imidazolide **2** (48.8 mM, 4.6 μL, 224 nmol) in HEPES buffer solution (500 mM; containing NaCl, 1 M and $MgCl_2$, 200 mM; pH 7.9) were combined to give a final concentration of 45 mM of either nucleoside/nucleotide. The mixture was kept at r.t., and the conversion was followed by MALDI-TOF mass spectrometry.

DNA Synthesis

Oligodeoxynucleotides were prepared on a 1 μmol scale, following the protocol recommended by the manufacturer of the DNA synthesizer (8909 Expedite, Perseptive Biosystems, system software

2.01). The couplings of the modified phosphoramidite **1** to solid-support-bound DNA **8** and **13** were carried out in a polypropylene vessel using a 0.1 M solution of the phosphoramidite in DCI activator solution (100 μL). The oligodeoxynucleotides were cleaved from the solid support and deprotected with 30% aq ammonia (400 μL) for 15 h at r.t. Excess ammonia was removed from the aqueous solutions with a gentle stream of compressed air. The solution was filtered (pore size 0.2 μm) and used directly for HPLC purification.

5′-ACGCGT-3′$^{NH\text{-}Fluo}$ (10)

HPLC: MeCN gradient 0% (in 0.1 M TEAA buffer) for 5 min to 15% in 55 min; t_R 36 min; yield: 61%.

MALDI-TOF MS: *m*/*z* calcd for $C_{79}H_{86}O_{39}N_{24}P_5$ $[M - H]^-$: 2147.5; found: 2147.9.

UV-Vis: λ (%) = 239.1 (100), 257.2 (94.7), 323.2 (15.2), 494.6 nm (59.4); ε_{260} (calcd): 77000 M^{-1} cm^{-1}.

5′-^{DPLys}TCATTCTGTTCT-3′$^{NH\text{-}Fluo}$ (15)

HPLC: MeCN gradient 0% (in 0.1 M TEAA buffer) for 5 min to 20% in 40 min; t_R 34 min; yield: 98%.

MALDI-TOF MS: *m*/*z* calcd for $C_{150}H_{157}O_{88}N_{36}P_{12}$ $[M - H]^-$: 4240.0; found: 4246.7.

UV: λ (%) = 261.5 (100), 331.4 (14.1), 494.2 nm (35.5); ε_{260} (calcd) = 122000 M^{-1} cm^{-1}.

UV-Melting Experiments

UV-melting data were acquired at 260 nm wavelength and 1 cm path length at heating and cooling rates of 1 °C/min, using solutions with equimolar concentrations of the DNA strands in 10 mM sodium phosphate buffer (NaH_2PO_4, Na_2HPO_4), pH 7, in previously deionized H_2O and the salt concentration given in Table 1. The melting temperatures and the thermodynamic data were determined with the program MeltWin 3.0.[36]

UV/Vis and Fluorescence Data

UV/Vis wavelengths scans were acquired between 200 and 600 nm at 20 °C of 1.97 μM solution of **10** and 2.43 μM of **15** in 10 mM sodium phosphate (NaH_2PO_4, Na_2HPO_4) buffer at pH 7.4. The fluorescence spectra were recorded between 510 and 650 nm for the same solutions at 20 °C using a 600 μL cuvette, an excitation wavelength of 495 nm, and emission and excitation slits of 3 nm.

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References

(1) Sanger, F.; Nicklen, S.; Coulson, A. R. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 5463.
(2) Lee, L. G.; Connell, C. R.; Woo, S. L.; Cheng, R. D.; McArdle, B. F.; Fuller, C. W.; Halloran, N. D.; Wilson, R. K. *Nucleic Acids Res.* **1992**, *20*, 2471.
(3) Pirrung, M. C. *Angew. Chem. Int. Ed.* **2002**, *41*, 1276; *Angew. Chem.* **2002**, *114*, 1326.
(4) Tyagi, S.; Kramer, F. R. *Nature Biotechnol.* **1996**, *14*, 303.
(5) Wittwer, C. T.; Herrmann, M. G.; Moss, A. A.; Rasmussen, R. P. *Biotechniques* **1997**, *22*, 130.
(6) (a) Bleczinski, C. F.; Richert, C. *J. Am. Chem. Soc.* **1999**, *121*, 10889. (b) Tuma, J.; Richert, C. *Biochemistry* **2003**, *42*, 8957. (c) Dogan, Z.; Paulini, R.; Rojas Stütz, J. A.; Narayanan, S.; Richert, C. *J. Am. Chem. Soc.* **2004**, *126*, 4762. (d) Tuma, J.; Paulini, R.; Rojas Stütz, J. A.; Richert, C. *Biochemistry* **2004**, *43*, 15680.
(7) Sigmund, H.; Pfleiderer, W. *Helv. Chim. Acta* **2003**, *86*, 2299.
(8) Gunnlaugsson, T.; Kellya, J. M.; Nieuwenhuyzenb, M.; O'Briena, A. M. K. *Tetrahedron Lett.* **2003**, *44*, 8571.
(9) (a) Letsinger, R. L.; Lunsford, W. B. *J. Am. Chem. Soc.* **1976**, *98*, 3655. (b) McBride, L. J.; Caruthers, M. H. *Tetrahedron Lett.* **1983**, *24*, 245. (c) Caruthers, M. H.; Barone, A. D.; Beaucage, S. L.; Dodds, D. R.; Fisher, E. F.; McBride, L. J.; Matteucci, M.; Stabinsky, Z.; Tang, J.-Y. *Methods Enzymol.* **1987**, *154*, 287.
(10) Lohrmann, R.; Orgel, L. E. *Tetrahedron Lett.* **1978**, *34*, 853.
(11) Jiao, G.-S.; Kim, T. G.; Topp, M. R.; Burgess, K. *Org. Lett.* **2004**, *6*, 1701.
(12) Czernecki, S.; Veréry, J. M. *Synthesis* **1991**, 239.
(13) Lin, T. S.; Prusoff, W. H. *J. Med. Chem.* **1979**, *21*, 109.
(14) Rossi, F. M.; Kao, J. P. Y. *Bioconjugate Chem.* **1997**, *8*, 495.
(15) Lyttle, M. H.; Carter, T. G.; Dick, D. J.; Cook, R. M. *J. Org. Chem.* **2000**, *65*, 9033.
(16) Theisen, P.; McCollum, C.; Upadhya, K.; Jacobson, K.; Vu, H.; Andrus, A. *Tetrahedron Lett.* **1992**, *33*, 5033.
(17) Laurent, A.; Debart, F.; Lamb, N.; Rayner, B. *Bioconjugate Chem.* **1997**, *8*, 856.
(18) Morr, M.; Waßmann, A.; Wray, V. *Tetrahedron* **1999**, *55*, 2985.
(19) (a) Uhlmann, E.; Engels, J. *Tetrahedron Lett.* **1986**, *27*, 1023. (b) v.d. Sande, J. H.; Ramsing, N. B.; Germann, M. W.; Elhorst, W.; Kalisch, B. W.; v. Kitzing, E.; Pon, R. T.; Clegg, R. C.; Jovin, T. M. *Science* **1988**, *241*, 551. (c) Koga, M.; Moore, M. F.; Beaucage, S. L. *J. Org. Chem.* **1991**, *56*, 3757. (d) Beier, M.; Stephan, A.; Hoheisel, J. D. *Helv. Chim. Acta* **2001**, *84*, 2089.
(20) Schwope, I.; Bleczinski, C. F.; Richert, C. *J. Org. Chem.* **1999**, *64*, 4749.
(21) Dombi, K. L.; Griesang, N.; Richert, C. *Synthesis* **2002**, 816.
(22) Connors, W. H.; Narayanan, S.; Kryatova, O. P.; Richert, C. *Org. Lett.* **2003**, *5*, 247.
(23) (a) Zielinski, W. S.; Orgel, L. E. *Nucleic Acids Res.* **1987**, *15*, 1699. (b) Orgel, L. E. *Nature* **1992**, *358*, 203. (c) Kozlov, I. A.; Pitsch, S.; Orgel, L. E. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 13448.
(24) (a) Kurz, M.; Göbel, K.; Hartel, C.; Göbel, M. W. *Helv. Chim. Acta* **1998**, *81*, 1156. (b) Hey, M.; Hartel, C.; Göbel, M. W. *Helv. Chim. Acta* **2003**, *86*, 844.
(25) Kanavarioti, A.; Stronach, M. W.; Ketner, R. J.; Hurley, T. B. *J. Org. Chem.* **1995**, *60*, 632.
(26) Luther, A.; Brandsch, R.; von Kiedrowski, G. *Nature* **1998**, *396*, 245.
(27) (a) Cramer, F.; Schaller, H.; Staab, H. A. *Chem. Ber.* **1961**, *94*, 1612. (b) Cramer, F.; Schaller, H.; Staab, H. A. *Chem. Ber.* **1961**, *94*, 1621.
(28) Lohrmann, R.; Orgel, L. E. *J. Mol. Biol.* **1980**, *142*, 555.
(29) (a) Atherton, F. R.; Openshaw, H. T.; Todd, A. R. *J. Chem. Soc.* **1945**, 660. (b) Ushioda, M.; Kadokura, M.; Moriguchi, T.; Kobori, A.; Aoyagi, M.; Seio, K.; Sekine, M. *Helv. Chim. Acta* **2002**, *85*, 2930.
(30) Bannwarth, W.; Trzeciak, A. *Helv. Chim. Acta* **1987**, *70*, 175.
(31) Wada, T.; Mochizuki, A.; Sato, Y.; Sekine, M. *Tetrahedron Lett.* **1998**, *39*, 7123.

(32) Lan, T.; McLaughlin, L. W. *J. Am. Chem. Soc.* **2000**, *122*, 6512.
(33) (a) Tallman, K. A.; Greenberg, M. M. *J. Am. Chem. Soc.* **2001**, *123*, 5181. (b) Tronchet, J. M. J.; Kovacs, I.; Dilda, P.; Seman, M.; Andrei, G.; Snoeck, R.; De Clercq, E.; Balzarini, J. *Nucleosides Nucleotides* **2001**, *20*, 1927.
(34) Sarracino, D.; Richert, C. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 2543.
(35) Lee, L. G.; Spurgeon, S. L.; Heiner, C. R.; Benson, S. C.; Rosenblum, B. B.; Menchen, S. M.; Graham, R. J.; Constantinescu, A.; Upadhya, K. G.; Cassel, J. M. *Nucleic Acids Res.* **1997**, *25*, 2816.
(36) McDowell, J. A.; Turner, D. H. *Biochemistry* **1996**, *35*, 14077.