DOI: 10.1002/ejoc.201000210

# Synthesis of 3′-BODIPY-Labeled Active Esters of Nucleotides and a Chemical Primer Extension Assay on Beads

**Kerstin Gießler,[a] Helmut Griesser,[b] Daniela Göhringer,[b] Thomas Sabirov,[b] and Clemens Richert*[a,b]**

**Keywords:** DNA / Oligonucleotides / Templated synthesis / Fluorescence

A solution-phase synthesis of active esters of 3′-fluorophore-labeled deoxynucleoside 5′-monophosphates was developed for thymine and cytosine as nucleobases by using two different BODIPY dyes. Starting from the respective 2′-amino-2′,3′-dideoxynucleoside-5′-monophosphate, the fluorescent oxyazabenzotriazolides can be prepared in one-pot procedures involving labeling and activation. Screening of a range of supports led to a chemical primer extension assay on beads with in situ detection of nucleobases in target DNA through optical read-out.

## Introduction

Labeled nucleotides are important tools in biomedicine and molecular biology. Radioactively labeled ATP is perhaps the most universal labeling reagent for DNA and RNA strands that are to be detected by gel electrophoresis and autoradiography.[1] Although radioactively labeled nucleic acids can be detected with exquisite sensitivity and accuracy, the radiation hazard complicates assays and adds a significant extra cost to experimental procedures. Sanger dideoxy sequencing with fluorophore-labeled terminator deoxynucleoside–triphosphates[2] (e.g. **1**, Figure 1) has been the backbone of genome sequencing methods that led to the elucidation of the human genome, as well as numerous other genomes from the different kingdoms of life. This method has been automated to an impressive degree, but it is probably too costly to compete with new generation sequencing methods in the long term.[3,4]

One approach to reducing the cost and effort of sequencing and genotyping is to avoid enzymes. Chemical primer extension (CPE) is a nascent technology that produces oligonucleotides containing nucleobases complementary to the sequence of a DNA template without enzymes.[5] It is an extension of methodologies developed in the context of studies of primitive self-replicating chemical systems.[6,7] It relies on the reaction of an active ester of a nucleoside monophosphate with a primer, as directed by the templating base of a DNA or RNA strand. Labeled monomers for CPE such as **2**[8] and **3**[9] (Figure 1) have been developed.

Chemical primer extension is sensitive[10] and rapid,[11] and it allows sequence information on DNA and RNA templates to be read,[12] but known versions have been limited to assays with a purification step before detection.[9] For example, in a published version of CPE with labeled monomers and read-out on DNA microarrays, labeled oligonucleotides had to be separated from excess monomers by spin columns.[9] The main cause for this undesirable feature of the assays was the background signal from unincorporated monomer on the surface of the microarrays, which was caused by unfavorable physical properties of the labeled monomers that we were unable to wash off from the surfaces of the microarray.

Further, the labeled monomers employed had to be prepared on a solid support[9] by using several costly phosphoramidites, limiting the scale on which the fluorescent building blocks could be prepared. Here we report fluorescent oxyazabenzotriazole esters of acylated 3′-amino-2′,3′-dideoxynucleoside-5′-monophosphates[14] with more favorable properties, together with primer extension reactions that can be performed directly on beads with subsequent washing and optical read-out.

For our current work, we chose BODIPY dyes as fluorophores for labeling. The fluorescent monomers were prepared by solution-phase syntheses and then employed in primer extension assays on beads, demonstrating that these monomers allow a nucleobase in a DNA template on the very support on which the extension assay is performed to be read-out, as required for efficient genotyping of single nucleotide polymorphisms (SNPs) and other genetic variations.

[a] Institut für Organische Chemie, Universität Karlsruhe (K.I.T.), 76131 Karlsruhe, Germany
Fax: +49-711-685-64321
E-mail: clemens.richert@oc.uni-stuttgart.de
[b] Institut für Organische Chemie, Universität Stuttgart, 70569 Stuttgart, Germany

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/ejoc.201000210.

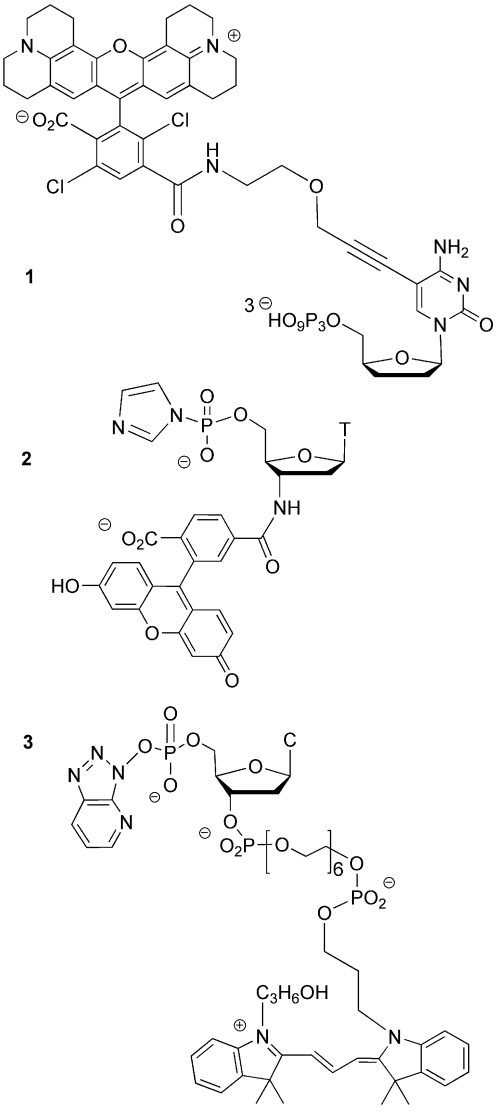


Figure 1. Fluorophore-labeled monomers that were developed for dideoxy sequencing (i.e., **1**)[2c] or chemical primer extension (i.e., **2**, **3**).[8,9]

## Results and Discussion

### Syntheses

The chemical primer extension system employed for the current study is shown in Scheme 1. It consists of primer **5** and a DNA 60mer template (**7a/g**) that is held on a support through base pairing to a capture oligonucleotide (**6**). Hybridization-based immobilization of templates ensures that assays can be performed in parallel fashion, with different templates hybridized to capture oligonucleotides on different spots of a microarray or different beads of a bead population. Bead-based sequencing of DNA in a massively parallel fashion has been described.[13]

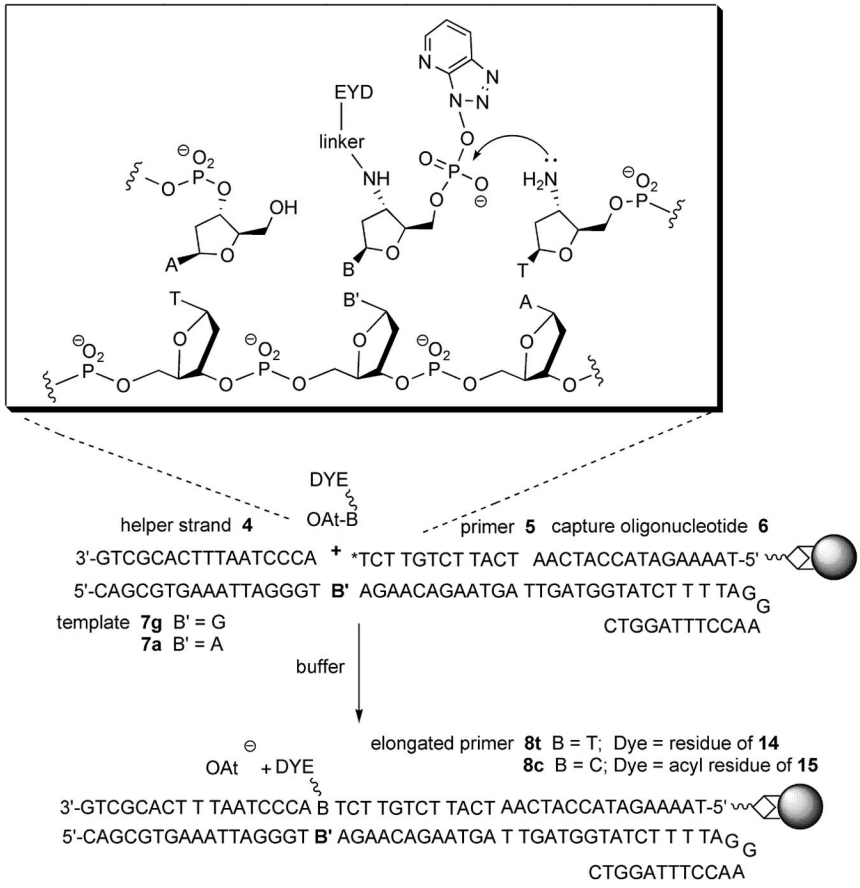
To determine the nucleobase of interest in the template strand, a labeled mononucleotide binds to the templating base (i.e., **B′**), followed by formation of a phosphoramidate linkage through attack of the amino-terminal primer.[14] Primer extension to **8c/t** can be accelerated by employing a third oligonucleotide (i.e., **4**) that binds immediately downstream from the templating base and that offers additional stacking interactions to the monomer. Such additional oligonucleotides are also dubbed "helper oligonucleotides".[10]

In our first assays, monomers with cyanine dyes as 3′-labels were employed, including compound **3** (Figure 1). With these, attempts to establish assay conditions for in situ detection of single bases in DNA through primer extension, followed by washing and optical detection on the same support, failed. Among the surface chemistries tested were glass slides decorated with an amino-terminal capture strand that had reacted with aldehyde groups,[15,16] NHS ester groups (CodeLink activated slides),[17] or isocyanate modified slides.[18] These monomer/surface combinations were also unsuccessful when combined with different passivation/blocking methods.[15] Treating the different surfaces with a 3.6 mM solution of **3** in the absence of any DNA confirmed that the overly strong background signal was due to unspecific adsorption of the fluorophore-labeled nucleotide. The problem with the background signal from the cyanine (Cy)-labeled nucleotides persisted, even when the unactivated monophosphate was used, the contact time was limited to 1 min, and when the washing buffer contained up to 0.2% SDS (sodium dodecyl sulfate).

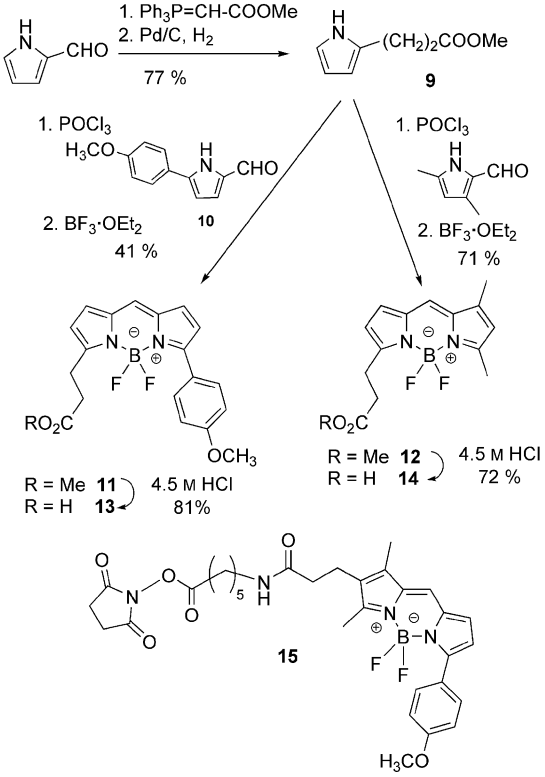
This prompted us to search for smaller dyes with more suitable properties. Among the fluorophores that fulfilled fundamental criteria, such as not being quenched by DNA, having a low tendency to intercalate, being stable under the coupling and assay conditions, and being accessible as a series of at least four different structures with sufficiently different emission spectra for each of the different nucleobases, the BODIPY dyes appeared most attractive. This class of dyes appeared readily accessible synthetically and has previously been proposed as fluorophores for automated DNA sequencing.[19] The NHS esters of the BODIPY chromophores with emission maxima similar to those of fluorescein and tetramethylrhodamine are commercially available under the trade names BODIPY-FL and BODIPY-TMR, albeit at very significant costs. We initially chose BODIPY-labeled nucleotides **21** and **22** as target molecules.

First, we synthesized **14** by following literature protocols[20,21] (see Supporting Information). This route produced large amounts of side products during an early condensation step and required a costly trichloroethyl ester. When ester cleavage was attempted under basic conditions, rather than the enzymatic protocol published, low yields were obtained. To avoid these problems, we employed different syntheses for both **14** and its redshifted analog **13** (Scheme 2). Both routes started from pyrrole-2-carbaldehyde and methyl (triphenylphosphoranylidene)acetate, which were reacted together to give **9** in two steps by a Horner–Wadsworth–Emmons reaction, followed by hydrogenation on Pd/C.[22] For the route to **14**, the required pyrrole aldehyde for the subsequent step was commercially available. For the route leading to **13**, aldehyde **10** was pre-




Scheme 1. Chemical primer extension reactions.




Scheme 2. Synthesis of BODIPY dyes **13** and **14** from pyrrol-2-carbaldehyde and structure of commercial labeling reagent **15**.

pared in three steps by pyrrole synthesis,[23] with *N*-allyl-4-methoxybenzamide,[24] followed by Vilsmayer Haack formylation in an overall yield of 70%. BODIPY dyes **11** and **12** were then prepared in moderate to good yield (41 and 71%, respectively) by using the method of Malan et al.[21] Liberation of the carboxylic acids from esters proved challenging at first. Even after careful optimization studies involving a series of bases (LiOH, KOH, $Li_2CO_3$, $Na_2CO_3$, $K_2CO_3$, $Cs_2CO_3$), solvents, and reaction temperatures, basic conditions gave no more than 63% yield of **14**.

In the case of BODIPY dye **13**, all attempts to hydrolyze the ester moiety by using alkaline hydroxides, carbonates,[25] or pig liver esterase (PLE)[21] again led to impure product in low yield. Synthetic procedures that involved a free acid as the starting material and that avoided the hydrolytic step[26] were considered, but reported yields are so low that they were not pursued. Hydrolysis under acidic conditions was studied instead. Sioni et al. describe the synthesis of dye **15** through ester hydrolysis with hydrochloric acid in THF under reflux conditions, resulting in 50% yield.[26] Another literature-known acidic hydrolysis,[27] performed with phosphoric acid, in our hands, gave the BODIPY acid in equal yield, but in lower purity. We found that hydrolysis of methyl esters **11** and **12** with more concentrated hydrochloric acid (4.5 M) in THF (1:1) at room temperature furnished free carboxylic acids **13** and **14** in analytically pure form in a yield of 81 and 72%, respectively.

With the two different fluorophores in hand, we then turned to the labeling reaction. Initially, a three-step procedure was employed, starting with separate activation of the BODIPY-FL propionic acid **14** to its OAt ester, followed by coupling to 3′-amino-3′-deoxythymidine-5′-monophosphate (**16**)[14] at pH 8.5. Amide **18** was then activated at its phosphate moiety by treatment with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 7-aza-1-hydroxybenzotriazole (HOAt) to give labeled active ester **21** in 67% yield. The absorption and fluorescence spectra of monomers **21** and **22** are shown in Figure 2.

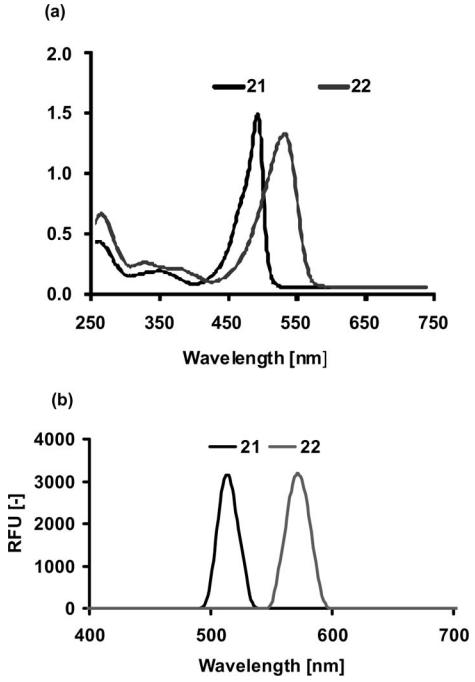
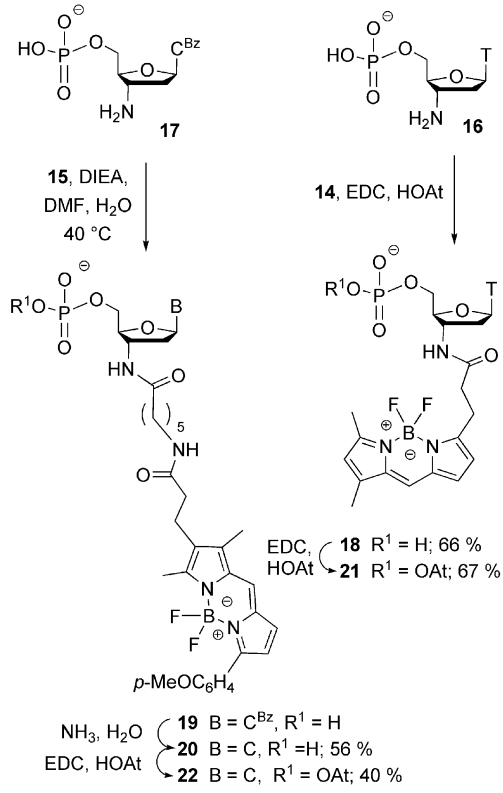



Figure 2. Optical properties of labeled nucleotides: (a) absorption spectra and (b) fluorescence spectra of **21** and **22**.

In our initial work, the second monomer to be employed in chemical primer extension assays (compound **22**) was synthesized starting from *N*-benzoyl-protected 3′-amino-2′3′-dideoxycytidine-5′-monophosphate[14] **17** (Scheme 3) and commercial BODIPY ester **15**. *N*-Hydroxysuccinimide ester **15** was coupled to 3′-amine **17** in aqueous DMF in the presence of *N*,*N*-diisopropylethylamine (DIEA), resulting in the protected, labeled cytidine monophosphate **19** that was deprotected with aqueous ammonia to give **20**. Reaction with EDC and HOAt at pH 5 gave monomer **22** for chemical primer extension assays.

To make the methodology more attractive for routine biomedical use, two steps were taken. The first was to develop a one-pot protocol for labeling and active ester formation. The second was to employ acid **13** (rather than the extremely costly **15**) for labeling 3′-amino-2′,3′-dideoxycytidine monophosphate, to access red monomer **24** at affordable cost. First, aminonucleotide **23** (Scheme 4) was generated through treatment of **17** with aqueous ammonia. For the one-pot conversion, BODIPY dyes **13** or **14** were then treated with EDC at 0 °C, followed by addition of the amino-

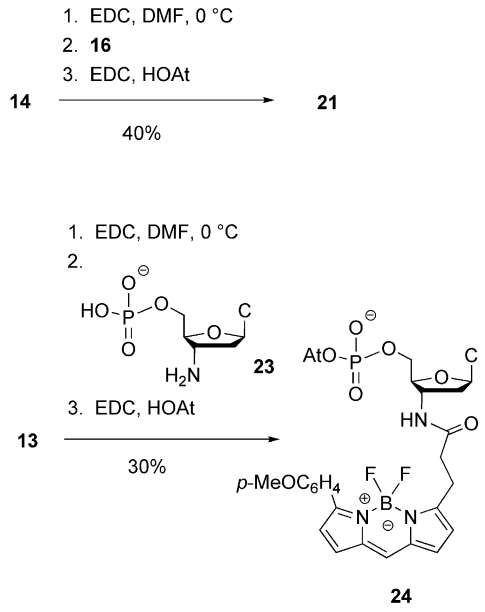



Scheme 3. Synthesis of activated BODIPY-labeled nucleotides **21** and **22**.

nonucleoside (**16** or **23**), and addition of more EDC and HOAt. The reaction solution was then directly applied to an RP-18 cartridge, from which **21** or **24** were eluted in a one-step purification process. The modest solubility of **24** in pure water also allows precipitation as a purification method, producing **24** in ca. 70% yield, contaminated with some **13**. Even with the cartridge step, the one-pot approach gave a very convenient protocol, producing labeled and activated monomers within a few hours without any HPLC or other costly or time-consuming steps.

Chemical primer extension assays were performed on different surfaces.[16,28] Initially, a capture oligonucleotide with a lysine residue at its 5′-terminus, identical in structure to that given in the literature[9] was immobilized on planar glass slides with epoxy groups on their surface through nucleophilic ring opening at pH 11. Templates **7a** and **7g** were then hybridized to different spots of the DNA-bearing surface, together with primer **5** and helper strand **4**. Finally, **22** was added to the entire slide surface in extension buffer containing 0.08 M $Mg_2Cl$, 0.4 M NaCl, and 0.2 M HEPBS [*N*-(2-hydroxyethyl)piperazine-*N*′-(4-butanesulfonic acid)], followed by primer extension and washing [1 × SSC (saline–sodium citrate) buffer containing 0.2% SDS and 1 × SSC buffer for 1 min at room temp.] and fluorescence imaging. The expected stronger fluorescence signal from spots with the templating base was not observed. Attempts to block unspecific adsorption and/or reactions on the surface by using different passivation agents did not solve this problem (Table 1).




Scheme 4. One-pot synthesis of activated BODIPY-labeled nucleotides **21** and **24**.

Table 1. Results of chemical primer extension assays with compound **22** on microarrays created from epoxy-derivatized glass surfaces.[a]

| Passivation reagent | Fluorescence intensity for spots with template **7g** | Fluorescence intensity for spots with template **7a** |
|---|---|---|
| None | 4500 | 4000 |
| Octylamine | 2600 | 3100 |
| Polyethylene glycolamine | 9900 | 8100 |

[a] Scans with neutral grey filter (1% transmission), exposure time 1 s, at 575 ± 15 nm emission wavelength, after assays with 3.6 mM solution of **22** in HEPBS buffer and a reaction time of 4 h.

To test whether the lack of selectivity was due to incorrect incorporation or unspecific adsorption, the same assay was performed on a planar gold surface generated with 5′-thiol-terminated capture oligonucleotides, as described in the literature.[29] Analysis by in situ MALDI-TOF mass spectrometry after primer extension showed incorporation of nucleotide **22** on spots with complementary templating base (template **7g**), but no detectable nucleotide incorporation on spots bearing the template with noncomplementary templating base (template **7a**). This suggested that unspecific adsorption was causing the incorrect fluorescence signal and thus the lack of selectivity.

Because exploratory assays with a number of different surface-activated slides suggested that the adsorption problem was widespread among planar surfaces, we decided to test sepharose beads instead, assuming that they would be less adsorptive. Bead-based sequencing methods are well established.[13,30] The sepharose particles allow fluorescence read-out and analysis by MALDI-TOF MS. Biotin-bearing capture oligonucleotide **6** was immobilized on streptavidin-coated beads, again followed by hybridization of templates **7a** or **7g**, primer **5**, and helper **4**. The beads were then incubated with labeled monomers **21** and **22**. Properly elongated primers **8c/t** were detected in the MALDI spectra, and fluorescence scans now showed the expected selectivity (Table 2).

Table 2. Results from chemical primer extension assays in HEPBS buffer (0.2 M HEPBS, 0.4 M NaCl, 0.08 M $MgCl_2$, pH 8.9) with compounds **21** and **22** on streptavidin-coated sepharose beads.

| Labeled monomer | Conc. monomer [mM] | Assay time [min] | Pyridine [mM] | S/N[b] |
|---|---|---|---|---|
| **21** | 3.6 | 360 | – | 1.9:1 |
| **22** | 3.6 | 360 | – | 2.3:1 |
| **21** | 2.0[a] | 20 | 100 | 4.2:1 |
| **21** | 2.0[a] | 10 | 100 | 4.0:1 |
| **21** | 2.0[a] | 5 | 100 | 4.7:1 |
| **21** | 2.0[a] | 10 | 100 | 6.0:1[c] |
| **21** | 2.0[a] | 5 | 100 | 11:1[c] |

[a] 18 mM unlabeled activated deoxynucleotides **33/34** (see Supporting Information) were included in the assay solution to achieve a modest level of labeling and thus an attenuated fluorescence signal. [b] Signal-to-noise ratios obtained by scanning at an exposure time of 0.01 s at 575 ± 15 nm emission wavelength for **22** and 530 ± 20 nm emission wavelength for **21**. The noise (N) or background signal is the fluorescence signal obtained on sepharose beads without oligonucleotides that were incubated with **21** or **22** in the same way. [c] Performed with purified EDC-free monomer.

Several assay parameters were then optimized. The monomer concentration was reduced to 2 mM, and the assay time was reduced from hours to minutes by using pyridine as catalyst.[11] Undiluted fluorophore-labeled monomers led to a signal that was so strong that it was difficult to avoid saturation of the detector of the microarray reader. To save precious monomers and to reduce the overly strong fluorescence signal, unlabeled monomers were included. Further, the excess amount of EDC was removed from the monomer solution and more extensive washing steps were introduced. Together, this led to a signal-to-noise ratio of 11:1 for incorporation of **21**. The noise or background signal is defined as the fluorescence signal obtained on sepharose beads without oligonucleotides. Those control beads were incubated with **21** or **22** in the same way as the beads displaying DNA to produce a control surface. The assay readily tolerated the addition of unlabeled monomer (Table 2, Entries 3–7) and showed a modest background signal.

We then proceeded to assays with mixtures of the two labeled monomers (**21** and **22**) as a first step toward genotyping, an application that requires determination of a single nucleobase rather than entire stretches of template with fluorophore-labeled nucleotides.[31] Primer extension assays were performed at a monomer concentration of 2 mM with 100 mM pyridine in the extension buffer at room temperature. Sometimes, mixture of different monomers give slower reactions than solutions containing the matched monomer alone,[10] so the reaction time was extended to 20 min, which may have reduced the selectivity. Figure 3a shows a MALDI-TOF spectrum of the partially elongated primer. This level of primer extension is more than sufficient for unambiguous fluorescence read-out of the templating base. The top row of Figure 3b shows the fluorescence images of

beads where the primer was extended in a template-directed fashion. The bottom row of images in Figure 3b shows scans of control beads lacking the templating base that were incubated with **22** and **21** under the same conditions. The fluorescence signal at 575 ± 15 nm for incorporation of **22** was found to be sevenfold higher than that for the fluorophore of **21** (530 ± 20 nm). Figure 3c shows that the mixture of labeled nucleotides **21** and **22** gave the desired sequence-specific incorporation of the BODIPY-labeled nucleotide complementary to the templating base. An exploratory experiment where the templating nucleobase was A (template **7a**) rather than G did show selective incorporation of **21** over **22**, albeit at a very modest level of selectivity (1.5:1), suggesting that there is further room for optimization of the experimental parameters. Given that the current concentration of labeled monomers is producing strong fluorescence readings, a further decrease in the concentration of **22**, which is less soluble than **21** and thus more prone to precipitate/adsorb upon evaporation of solvent from small samples, might lead to improved signal-to-noise ratios.

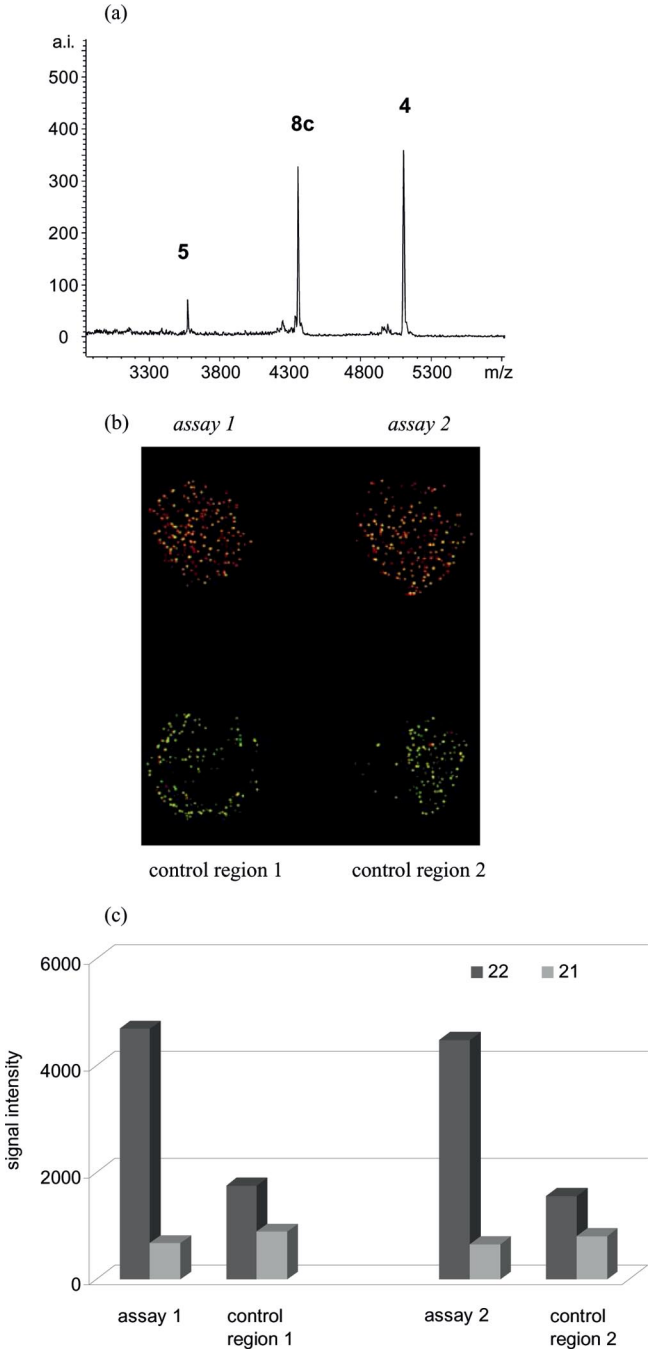



Figure 3. Results from chemical primer extension assay on sepharose beads with **22** and **21**. (a) MALDI spectra showing elongation of primer **5** to **8c** after 20 min at room temperature. (b) Fluorescence image of sepharose beads spread out on a microscope slide: (top row) beads with oligonucleotides and template **7g**, (bottom row) control beads. (c) Fluorescence intensities of sepharose beads detected at wavelength 530 ± 20 nm for **21** and 575 ± 15 nm for **22**.

Finally, to test whether slight structural differences in the linker and chromophore have a significant effect on the rate of incorporation, we performed an exploratory primer extension assay with an equimolar mixture of **22** and **24** in solution (Figure S24, Supporting Information). After activation of the mixture with EDC/HOAt, an aliquot of the resulting solution was directly added to a solution of primer and template in extension buffer. A MALDI-TOF mass spectrum acquired at the end of the assay (6 h) showed incorporation of either monomer with a slight bias towards extension by **22**, which reacted approximately three times more frequently than **24**.

## Conclusions

In conclusion, chemical primer extension with fluorescent monomers can be performed in a low-cost format, where multiple loci are undergoing enzyme-free nucleobase-selective reactions, followed by in situ read-out. Our results also highlight the importance of seemingly minor issues, such as background adsorption of the monomers. Low molecular weight fluorophores, such as BODIPY dyes, appear to be more favorable than larger dyes, which dominate the physicochemical properties of the resulting monomers to an even larger extent, for keeping adsorption at an acceptable level.

The monomers reported here are readily accessible by solution-phase syntheses, making them considerably less expensive than those requiring solid-phase methods.[9] The monomers described here are also less prone to oligomerize than their fluorescein-labeled counterparts,[8] and they show less propensity to adsorb onto surfaces, though a significant background signal remains. As we have previously shown that fluorophores may also be attached to 3′-amines of dideoxynucleotides through a photolabile linker,[9] it is interesting to ask whether the current results also help to pave the way for multiple, stepwise primer extension reactions with optical read-out. If so, an inexpensive alternative sequencing method may have moved one step closer to medical re-sequencing of short stretches of DNA. We are striving to adapt the solution-phase methodology reported here to the synthesis of such photolabile labeled monomers. However, even with the current methodology, there is room

for further improvement, as seen by the still very significant background signal observed in the fluorescence images (Figure 3).

## Experimental Section

**General Remarks:** Reagents were from Acros (Geel, Belgium) or Aldrich/Fluka/Sigma (Deisenhofen, Germany). HOAt was from MoBiTec (Göttingen, Germany). Dowex 50 WX8–200 cation exchange resin was from Acros (Geel, Belgium). Other reagents for DNA synthesis were from Proligo (Hamburg, Germany) or Chemgenes (Wilmington, USA, MA). BODIPY-TMR (NHS ester) **15** was from Molecular probes/Invitrogen (Karlsruhe, Germany). Sepharose beads were from GE Healthcare (Munich, Germany). Unmodified DNA oligomers and biotin-labeled DNA oligomers were purchased from Biomers (Ulm, Germany) or from Operon Biotechnologies GmbH (Cologne, Germany) in HPLC-purified form and were used without further purification. MALDI-TOF mass spectra were acquired with a Bruker BIFLEX IV spectrometer in negative, linear mode. MALDI matrices were a mixture of 2,4,6-trihydroxyacetophenone (0.3 M in EtOH) and diammonium citrate (0.1 M in $H_2O$) at a ratio of 2:1 (v/v) for oligonucleotides or ATT (6-aza-2-thiothymine, saturated solution in acetonitrile) for labeled nucleotides and activated monomers. UV/Vis spectra were recorded with a ND-1000 spectrophotometer (NanoDrop; peQlab, Erlangen, Germany). Labeled nucleotides were purified by reverse-phase HPLC on Nucleosil columns (C18; 250 mm × 4.6 mm by Macherey–Nagel, Düren, Germany). Unless otherwise noted, this involved a gradient of $CH_3CN$ (B) in 0.1 M triethylammonium acetate (pH 7.0) and detection at 260 nm. The read-out of the fluorescence signals from the spots of the slides or of beads was performed with an ArrayWoRxe Biochip Reader (Applied Precission, Issaquah, USA). The exposure time was set to 0.01 s, and a neutral gray filter (1% transmission, AHF Analysentechnik, Tübingen, Germany) was used. Fluorescence of **14** (BODIPY-FL) was detected by using the following filters: 480 ± 15 nm (excitation); 530 ± 20 nm (emission), and **15** (BODIPY-TMR) was detected with the following filter set: 546 ± 5 nm (excitation); 575 ± 15 nm (emission). The chromophores are commercially available from Invitrogen (Karlsruhe, Germany). Analysis of images was performed by using the ArrayWoRxe analysis software.

**Cyanine-Dye Labeled Nucleotide 3:** The synthesis of the Cy-labeled nucleotides was performed as published earlier.[9]

**Methyl 3-(1*H*-Pyrrole-2-yl)propionate (9):** Pyrrole methyl ester **9** was prepared by a method similar to that reported in the literature.[22] A detailed protocol and the analytical data are given in the Supporting Information.

**Methyl 3-[4,4-Difluoro-5-(4-methoxyphenyl)-4-bora-3a,4a-diaza-*s*-indacene-3-yl]propionate (11):** To a stirred solution of methyl 3-(1*H*-pyrrole-2-yl)propionate (**9**, 300 mg, 1.96 mmol) and 5-(4-methoxyphenyl)-1*H*-pyrrole-2-carbaldehyde (**10**, 433 mg, 2.15 mmol, 1.1 equiv.) in $CH_2Cl_2$ (30 mL) was added dropwise $POCl_3$ (335 mg, 2.19 mmol, 1.2 equiv.) in $CH_2Cl_2$ (4 mL) at –10 °C. Stirring was continued for 12 h at 0 °C and for 2 h at room temperature. The resulting black solution was cooled to –10 °C, and $BF_3{\cdot}OEt_2$ (1.04 mL, 8.3 mmol, 4.2 equiv.) and DIEA (1.45 mL, 8.3 mmol, 4.2 equiv.) were added. After stirring for another 12 h at 0 °C, the solution was washed with water (100 mL). The aqueous layer was extracted with $CH_2Cl_2$ (2 × 50 mL), and the combined organic layer was concentrated in vacuo. The crude product was purified by flash chromatography (50 g silica, petroleum ether, step gradient of 50–80% $CH_2Cl_2$) to give title compound **11** (307 mg, 0.80 mmol, 41%) as a greenish black solid. TLC (petroleum ether, $CH_2Cl_2$ 1:4). $R_f$ = 0.18. M.p. 105–107 °C. $^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 7.94 (d, $J$ = 8.8 Hz, 2 H, Ph), 7.14 (s, 1 H, H-8′), 7.06 (d, $J$ = 4.3 Hz, 1 H, H-7′), 7.00 (d, $J$ = 8.8 Hz, 2 H, Ph), 6.96 (d, $J$ = 4.2 Hz, 1 H, H-1′), 6.64 (d, $J$ = 4.4 Hz, 1 H, H-6′), 6.35 (d, $J$ = 4.2 Hz, 1 H, H-2′), 3.87 (s, 3 H, $OCH_3$), 3.68 (s, 3 H, $COOCH_3$), 3.31 (t, $J_{3,2}$ = 7.6 Hz, 2 H, H-3), 2.76 (t, $J_{2,3}$ = 7.6 Hz, 2 H, H-2) ppm. $^{13}$C NMR (125.8 MHz, $CDCl_3$): $\delta$ = 172.9 (CO), 161.1, 159.9, 159.2, 136.8, 134.5, 131.2, 131.1, 130.8, 129.6, 127.2, 124.7, 120.4, 118.5, 114.1, 114.0, 55.4 ($OCH_3$), 51.8 ($COOCH_3$), 33.1 (C-2), 24.2 (C-3) ppm. HRMS (ESI): calcd. for $C_{20}H_{19}BF_2N_2O_3$ [M + Na]$^+$ 407.1353; found 407.1359. UV/Vis ($CH_3CN/H_2O$, 1:1): $\lambda_{max}$ ($\varepsilon$, $M^{-1}cm^{-1}$) = 541 (59000) nm; $\lambda_{Em}$ = 565 nm.

**Methyl 3-[4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-yl]propionate (12):** The following protocol is similar to that reported for the corresponding trichloroethyl ester.[21] Briefly, to a stirred solution of **9** (1.066 g, 6.96 mmol) and 3,5-dimethyyl-1*H*-pyrrole-2-carbaldehyde (0.943 g, 7.66 mmol, 1.1 equiv.) in $CH_2Cl_2$ (55 mL) was added dropwise $POCl_3$ (1.175 g, 7.66 mmol, 1.1 equiv.) in $CH_2Cl_2$ (4 mL) at 0 °C. Stirring was continued for 0.5 h at 0 °C and for 6 h at room temperature. The resulting black solution was cooled to 0 °C, and $BF_3{\cdot}OEt_2$ (3.55 mL, 28 mmol, 4 equiv.) and DIEA (5.10 mL, 29.25 mmol, 4.2 equiv.) were added. After stirring for another 12 h at room temperature, water (100 mL) was added, and the resulting mixture was filtered through a bed of Celite. The precipitate was washed with $CH_2Cl_2$ (100 mL), and the aqueous layer of the combined filtrates was separated and extracted with $CH_2Cl_2$ (2 × 50 mL). The combined organic layer was concentrated in vacuo, and the crude product was purified by flash chromatography (100 g silica, $CH_2Cl_2$) to give title compound **12** in the form of red crystals in 71% yield. TLC (petroleum ether ether/ethyl acetate, 8:2): $R_f$ = 0.25. M.p. 76–78 °C. $^1$H NMR (500 MHz, $CDCl_3$): $\delta$ = 7.07 (s, 1 H, H-8′), 6.86 (d, $J$ = 4.0 Hz, 1 H, H-1′), 6.25 (d, $J$ = 4.0 Hz, 1 H, H-2′), 6.10 (s, 1 H, H-6′), 3.69 (s, 3 H, $OCH_3$), 3.29 (t, $J_{3,2}$ = 7.6 Hz, 2 H, H-3), 2.77 (t, $J_{2,3}$ = 7.6 Hz, 2 H, H-2), 2.56 (s, 3 H, $CH_3$), 2.23 (s, 3 H, $CH_3$) ppm. $^{13}$C NMR (125.8 MHz, $CDCl_3$): $\delta$ = 173.0 (CO), 160.4, 157.0, 143.9, 135.2, 133.3, 128.1, 123.9, 120.5, 116.7, 51.8 ($OCH_3$), 33.3 (C-2), 24.0 (C-3), 15.0 ($CH_3$), 11.3 ($CH_3$) ppm. HRMS (ESI): calcd. for $C_{15}H_{17}BF_2N_2O_2$ [M + Na]$^+$ 329.1246; found 329.1230. UV/Vis ($CH_3CN/H_2O$, 1:1): $\lambda_{max}$ ($\varepsilon$, $M^{-1}cm^{-1}$) = 503 (92000) nm; $\lambda_{Em}$ = 512 nm.

**3-[4,4-Difluoro-5-(4-methoxyphenyl)-4-bora-3a,4a-diaza-*s*-indacene-3-yl]propionic Acid (13):** To a stirred solution of methyl propionate (**11**, 38 mg, 0.1 mmol) in THF (4.9 mL) was added $H_2O$ (3.3 mL) and concentrated aqueous HCl (37%, 2.0 mL). After stirring for 36 h at room temperature, $CH_2Cl_2$ (30 mL) was added, the phases were separated, and the aqueous phase was washed with $CH_2Cl_2$ (2 × 20 mL). The combined organic phase was washed with brine and concentrated in vacuo. The crude was then purified by flash chromatography (20 g silica, $CH_2Cl_2$, step gradient of 0–2% $CH_3OH$) followed by precipitation in hexane to give **13** (30 mg, 0.081 mmol, 81%) as a greenish black solid. TLC ($CH_2Cl_2$/$CH_3OH$, 9:1): $R_f$ = 0.28. M.p. 197 °C. $^1$H NMR (500 MHz, $CD_3CN$): $\delta$ = 9.09 (br., 1 H, COOH), 7.93 (d, $J$ = 9.2 Hz, 2 H, Ph), 7.44 (s, 1 H, H-8′), 7.26 (d, $J$ = 4.3 Hz, 1 H, H-7′), 7.16 (d, $J$ = 4.2 Hz, 1 H, H-1′), 7.05 (d, $J$ = 9.1 Hz, 2 H, Ph), 6.76 (d, $J$ = 4.3 Hz, 1 H, H-6′), 6.45 (d, $J$ = 4.2 Hz, 1 H, H-2′), 3.87 (s, 3 H, $OCH_3$), 3.17 (t, $J_{3,2}$ = 7.6 Hz, 2 H, H-3), 2.71 (t, $J_{2,3}$ = 7.6 Hz, 2 H, H-2) ppm. $^{13}$C NMR (125.8 MHz, $CD_3CN$): $\delta$ = 173.7 (CO), 162.2, 161.2, 137.6, 135.5, 132.4, 132.1, 132.0, 131.4, 129.2, 125.5, 121.3, 119.4, 114.8, 56.1 ($OCH_3$), 32.6 (C-2), 24.7 (C-3) ppm. HRMS (ESI): calcd. for $C_{19}H_{17}BF_2N_2O_3$ [M + Na]$^+$ 393.1196;

found 393.1189. UV/Vis ($CH_3CN/H_2O$, 1:1): $\lambda_{max}$ ($\varepsilon$, $M^{-1}cm^{-1}$) = 541 (57000) nm; $\lambda_{Em}$ = 565 nm.

**3-[4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-yl]propionic Acid (14):** To a stirred solution of methyl ester **12** (80 mg, 0.26 mmol) in THF (12 mL) was added $H_2O$ (8 mL) and concentrated aqueous HCl (37%, 4.8 mL). After stirring for 36 h at room temperature, $CH_2Cl_2$ (50 mL) was added, the phases were separated, and the aqueous layer was washed with $CH_2Cl_2$ (2×40 mL). The combined organic layer was washed with brine and concentrated in vacuo. The crude was purified by flash chromatography (30 g silica, $CH_2Cl_2$, step gradient of 0–2% $CH_3OH$) followed by precipitation in hexane to yield **14** (55 mg, 0.188 mmol, 72%) as a red solid. TLC ($CH_2Cl_2/CH_3OH$, 9:1): $R_f$ = 0.33. M.p. 194–196 °C; $^1H$ NMR (500 MHz, $CD_3CN$): $\delta$ = 9.08 (br., 1 H, COOH), 7.38 (s, 1 H, H-8′), 7.00 (d, $J$ = 4.0 Hz, 1 H, H-1′), 6.33 (d, $J$ = 4.0 Hz, 1 H, H-2′), 6.23 (s, 1 H, H-6′), 3.15 (t, $J_{3,2}$ = 7.6 Hz, 2 H, H-3), 2.71 (t, $J_{2,3}$ = 7.6 Hz, 2 H, H-2), 2.51 (s, 3 H, $CH_3$), 2.26 (s, 3 H, $CH_3$) ppm. $^{13}C$ NMR (125.8 MHz, $CD_3CN$): $\delta$ = 173.9 (CO), 161.5, 157.7, 146.0, 136.1, 134.2, 129.3, 125.8, 121.5, 117.3, 32.9 (C-2), 24.5 (C-3), 15.0 ($CH_3$), 11.4 ($CH_3$) ppm. HRMS (ESI): calcd. for $C_{14}H_{15}BF_2N_2O_2$ $[M + Na]^+$ 315.1189; found 315.1094. UV/Vis ($CH_3CN/H_2O$, 1:1): $\lambda_{max}$ ($\varepsilon$, $M^{-1}cm^{-1}$) = 504 (88000) nm; $\lambda_{Em}$ = 513 nm.

**Stepwise Labeling and Activation**

**Compound 18:** To a stirred solution of EDC hydrochloride (13 mg, 66 μmol, 5 equiv.) and HOAt (5.6 mg, 41 μmol, 3 equiv.) in water (35 μL) at pH 5 was added compound **14** (4 mg, 13.7 μmol) dissolved in DMF (50 μL), and the mixture was stirred for 2 h at room temperature. Then, 3′-aminothymidine-5′-monophosphate (**16**, 3.9 mg, 12.2 μmol, 1.1 equiv.) dissolved in water (20 μL) at pH 8–9 was added, and the mixture was stirred for 12 h. The solution was lyophilized and resuspended in water, centrifuged to avoid filtration, and purified directly by HPLC. HPLC (RP, C-18 column; gradient of $CH_3CN$ in 0.1 M TEAA buffer 0% for 10 min, then to 30% in 30 min): $t_R$ = 24 min. Yield: 66%, as determined by integration. MS (MALDI-TOF): $m/z$ = 594.2 $[M - H]^-$.

**Compound 20:** To a stirred solution of 3′-amino-*N*4-benzoyl-2′,3′-dideoxycytidine-5′-monophosphate (**17**, 2 mg, 4.8 μmol, 1.5 equiv.) in water (50 μL) was added BODIPY NHS ester **15** (2 mg, 3.2 μmol, 1 equiv.) in DMF (75 μL), followed by the addition of DIEA (1.1 μL, 6.2 μmol, 1.9 equiv.). The solution was stirred at 40 °C for 12 h. Then, water (600 μL) was added, and the solution was extracted with ethyl acetate (3×2 mL). {An analytical sample gave MS (MALDI-TOF): $m/z$ = 903.0 $[M - H]^-$.} To the aqueous solution was added the same volume of 30% aqueous ammonia, and the resulting solution was heated to 45 °C for 30 min to remove the benzoyl group. The excess amount of ammonia was removed with a gentle stream of compressed air, and the resulting solution was used directly for HPLC purification to yield **20**. HPLC (RP, C-18 column; gradient of $CH_3CN$ in 0.1 M TEAA buffer 0% for 5 min, then to 90% in 40 min): $t_R$ 38 min. Yield: 56%. MS (MALDI-TOF): $m/z$ = 798.2 $[M - H]^-$.

**Compounds 21 and 22:** EDC hydrochloride (28.5 mg, 150 μmol) and HOAt (12.2 mg, 90 μmol) in a polypropylene vessel were suspended in water (200 μL). The pH was adjusted to 5.0 with NaOH solution (1.5 M; approx. 5 μL), resulting in a clear yellow solution. An appropriate volume of this stock solution (5 equiv. of EDC and 3 equiv. of HOAt) was added to the labeled nucleotide (**18** or **20**, 1 equiv.) in a polypropylene vessel. The dark solution was left to stand at room temperature for 2 h and then used directly for HPLC purification.

**Compound 21:** HPLC (RP, C-18 column; gradient of $CH_3CN$ in water 0% for 10 min, then to 20% in 50 min): $t_R$ 50–55 min. Yield: 67%. MS (MALDI-TOF): $m/z$ = 713.3 $[M - H]^-$.

**Compound 22:** HPLC (RP, C-18 column; gradient of $CH_3CN$ in water 0% for 10 min, then to 70% in 70 min): $t_R$ = 58 min. Yield: 40%. UV/Vis ($H_2O$): $\lambda_{max}$ = 542 nm; $\lambda_{Em}$ = 572 nm. MS (MALDI-TOF): $m/z$ =916.4 $[M - H]^-$.

**One-Pot Labeling and Activation**

**Compound 21:** To a solution of **14** (7.6 mg, 26 μmol) in DMF (120 μL) in a polypropylene vessel was added EDC hydrochloride (6.0 mg, 31.3 μmol, 1.2 equiv.) as a solid at 0 °C. The reaction mixture was shaken at this temperature for 5 min. After addition of 3′-amino-thymidine-5′-monophosphate (**16**, 9.0 mg, 26. μmol, 1 equiv.) in $H_2O$ (45 μL), the slurry was treated in a ultrasonic bath for 10 min, and the resulting homogeneous solution was shaken at room temperature for 30 min. After addition of EDC hydrochloride (15.0 mg, 78 μmol, 3 equiv.) and HOAt (17.7 mg, 130 μmol, 5 equiv.) as solids, shaking was continued at room temperature for 2 h. The reaction mixture was then diluted with a mixture of 0.1 M TEAB (triethylammonium bicarbonate) buffer/$CH_3CN$ (95:5), and title compound **21** was isolated by chromatography using a cartridge (Cromabond© C18; 0.1 M TEAB buffer/step gradient of 5–80% $CH_3CN$). Fractions (20–25% $CH_3CN$) containing desired product **21** were pooled, and the solvents were evaporated to dryness by lyophilization. Yield: 40% (determined by UV/Vis measurement, $\lambda$ = 505 nm). $^1H$ NMR (500 MHz, $D_2O$): $\delta$ = 8.52 (d, $J$ = 4.2 Hz, 1 H), 8.19 (d, $J$ = 8.5 Hz, 1 H), 7.32 (dd, $J$ = 4.4, 8.5 Hz, 1 H), 7.28 (s, 1 H), 6.89 (br., 1 H), 6.74 (br., 1 H), 6.12 (br., 1 H), 5.96 (t, $J$ = 5.9, 1 H, H-1′), 5.82 (br., 1 H), 4.40–4.28, 4.14, and 4.00 (3 m, 4 H, H-5′,4′,3′), 2.99 (m, 2 H, $CH_2$), 2.54 (m, 2 H, $CH_2$), 2.23–2.10 (m, 5 H, H-2′, $CH_3$), 1.85 (s, 3 H, $CH_3$), 1.47 (s, 3 H, $CH_3$) ppm. $^{31}P$ NMR (121.5 MHz, $D_2O$): $\delta$ = –1.31 ppm. HRMS (ESI): calcd. for $C_{29}H_{31}BF_2N_9O_8P$ $[M - H]^-$ 712.2016; found 712.2017. UV/Vis ($H_2O$): $\lambda_{max}$ = 505 nm; $\lambda_{Em}$ = 513 nm.

**Compound 24:** To a solution of **13** (10.6 mg, 28.6 μmol) in DMF (120 μL) in a polypropylene vessel was added EDC hydrochloride (6.6 mg, 34.4 μmol, 1.2 equiv.) as a solid at 0 °C. The reaction mixture was shaken at this temperature for 5 min. After addition of 3′-aminocytidine-5′-monophosphate **23** (9.4 mg, 26.6. μmol, 1 equiv.) in $H_2O$ (45 μL), the slurry was treated in an ultrasonic bath for 10 min, and the resulting homogeneous solution was shaken at room temperature for 30 min. After addition of EDC hydrochloride (16.4 mg, 85.8 μmol, 3 equiv.) and HOAt (19.4 mg, 143 μmol, 5 equiv.) as solids, shaking was continued at room temperature for 2 h. The reaction mixture was then diluted with a mixture of $H_2O$ (700 μL), and the precipitate was separated by using a centrifuge (6000 rpm, 5 min). The supernatant was removed, and the solid was washed twice with $H_2O$ (700 μL) and $H_2O/CH_3CN$ (500 μL, 95:5) followed by separation by using a centrifuge (6000 rpm, 5 min). The residue was dissolved in 0.1 M TEAB buffer/$CH_3CN$ (7:3, 1 mL, ultrasonic treatment) and title compound **24** was isolated by chromatography using a cartridge (Cromabond© C18; 0.1 M TEAB buffer/step gradient of 30–50% $CH_3CN$). Fractions (30–35% $CH_3CN$) containing desired product **24** were pooled, and the solvents were evaporated to dryness by lyophilization. Yield: 30% (determined by UV/Vis measurement, $\lambda$ = 544 nm). $^1H$ NMR (500 MHz, $D_2O/CD_3CN$): $\delta$ = 8.66 (br., $J$ = 4.2 Hz, 1 H), 8.36 (br. d, $J$ = 8.5 Hz, 1 H), 7.88–7.82 (m, 3 H), 7.43 (dd, $J$ = 4.5, 8.5 Hz, 1 H), 7.39 (br. s, 1 H), 7.22–7.12 (m, 2 H), 7.00 (m, 2 H), 6.68 (br., 1 H), 6.41 (br., 1 H), 6.06 (m, 1 H), 5.87 (t, $J$ = 6.7, 1 H, H-1′), 4.37, 4.22, and 4.04 (3 m, 4 H, H-5′,4′,3′), 3.81 (br. s, 3 H, $OCH_3$), 3.11 (t, $J$ = 7.1 Hz, 2 H, $CH_2$), 2.56 (t, $J$ = 7.1 Hz, 2 H, $CH_2$), 2.31–

2.21 (m, 2 H, H-2′) ppm. $^{31}$P NMR (202.5 MHz, $D_2O/CD_3CN$): $\delta$ = –3.56 ppm. HRMS (ESI): calcd. for $C_{33}H_{32}BF_2N_{10}O_8P$ [M – H]$^-$ 775.2125; found 775.2127. UV/Vis ($H_2O/CH_3CN$, 1:1): $\lambda_{max}$ = 542 nm; $\lambda_{Em}$ = 565 nm.

**Loading of Sepharose Beads:** First, the 5′-biotinylated capture strand **6** (Supporting Information) was immobilized through biotin-streptavidin linkages. For this, 100 μL of the bead suspension in 20% ethanol was washed three times with buffer (3 mL, 20 mM $NaH_2PO_4$, 80 mM $Na_2HPO_4$, 0.15 M NaCl, pH 7.5). To 100 μL of the resulting bead suspension (approx. 30 nmol loading) was added 360 μL (45 nmol, 1.5 equiv.) of a stock solution of capture strand **6**, followed by shaking overnight. The supernatant was removed, and the beads were washed twice with 200 μL of Buffer A containing 0.5 M HEPBS, 1 M NaCl, 0.2 M $MgCl_2$ at pH 8.9. Then, a solution of template **7a** or **7g**, primer **5**, and helper strand **4** (45 nmol each) in 125 μL Buffer A were added to the beads for 4 h at room temperature. After washing twice with Buffer A (1 mL each), the loaded beads were stored at 4 °C until usage.

**Chemical Primer Extension on Beads:** Chemical primer extension reactions were carried out with 3 μL of the sepharose bead suspension. The beads were centrifuged to the bottom of the polypropylene vessel, and the supernatant was removed. The total volume of the solution then added was 1 μL. It was made up from aliquots of stock solutions of Buffer A (HEPBS 0.5 M, NaCl 1 M, $MgCl_2$ 0.2 M, pH 8.9), pyridine in water (500 mM), and monomer **21** or **22** (10 mM) to give the following concentrations: 0.2 M HEPBS, 0.4 M NaCl, 0.08 M $MgCl_2$, 100 mM pyridine, 2 mM **21**, and 2 mM **22**. The assays were started through addition of **21** or **22** as the last stock solution and were carried out at 25 °C. Afterwards, 100 μL of Buffer A containing 0.2% SDS at 40 °C was added to the beads, followed by shaking for 2 min. Then, the beads were centrifuged down, and the supernatant was removed. This washing procedure was repeated a minimum of five times until the supernatant did not show fluorescence under a UV-lamp at 366 nm. The last washing step was performed in a sonication bath for 30 s. Then, the beads were suspended in aqueous $NH_4OAc$ (10 μL, 0.25 M), and an aliquot (3 μL) of the suspension was removed for MALDI-TOF analysis.

**MALDI-TOF Analysis:** The beads suspension (3 μL) was centrifuged, the supernatant was removed, and $H_2O$ (1 μL) was added. This mixture was heated to 70 °C for 15 min. The supernatant was quickly aspired, and the solution was treated with a few grains of Dowex cation exchange resin (ammonium form), followed by incubation for 5 min. The supernatant was spotted on a stainless steel MALDI target, allowed to dry, and the spot then treated with the matrix/comatrix mixture (0.3 M THAP in ethanol, 0.1 M diammonium citrate in water, 2:1; 1 μL).

**Fluorescence Read-Out:** The washed bead suspension was shaken, and 1 μL of the suspension was pipetted onto a glass microscope slide (PTFE coated microscope slide, Roth, Karlsruhe, Germany), and the beads were spread out on the slide surface, and were scanned in the microarray scanner, using a neutral grey filter (1% transmission) and an exposure time of 0.01 s at the following wavelengths 480 ± 15 nm (excitation) and 530 ± 20 nm (emission) for **14** and 546 ± 5 nm (excitation) and 575 ± 15 nm (emission) for **15**. The fluorescence images were integrated using ArrayWoRxe Software.

**Supporting Information** (see also the footnote on the first page of this article): Preparation of **9** and **14**; numbering scheme for the BODIPY fluorophore; structures of compounds **6**, **33**, and **34**; kinetic data for incorporation of **21**; MS (MALDI-TOF), NMR, absorption, and fluorescence spectra of **11**–**14**, **18**–**22**, and **24**.

## Acknowledgments

This work was supported by Deutsche Forschungsgemeinschaft [DFG, grant No. RI 1063/8-1 to C.R.]. The authors are grateful to A. Kaiser for performing exploratory experiments and to M. Röthlingshöfer, E. Kervio, A. Hochgesand, H. Vogel, and C. Deck for helpful discussions.

[1] J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning, A Laboratory Manual, Book 2*, 2nd ed., Cold Spring Habor Laboratory Press, New York, 1**989**, ch. 10, 10.51–10.67.
[2] a) L. G. Lee, C. R. Connell, S. L. Woo, R. D. Cheng, B. F. McArdle, C. W. Fuller, N. D. Halloran, R. K. Wilson, *Nucleic Acids Res.* **1992**, *20*, 2471–2483; b) L. G. Lee, S. L. Spurgeon, C. R. Heriner, S. C. Benson, B. B. Rosenblum, S. M. Menchen, R. J. Graham, A. Constantinescu, K. G. Upadhya, J. M. Cassel, *Nucleic Acids Res.* **1997**, *25*, 2816–2822; c) B. B. Rosenblum, L. G. Lee, S. H. Spurgeon, S. H. Khan, S. M. Menchen, C. R. Heiner, S. M. Chen, *Nucleic Acids Res.* **1997**, *25*, 4500–4504.
[3] R. F. Service, *Science* **2006**, *311*, 1544–1546.
[4] E. C. Hayden, *Nature* **2009**, *457*, 768–769.
[5] J. A. Rojas Stütz, E. Kervio, C. Deck, C. Richert, *Chem. Biodiversity* **2007**, *4*, 784–802.
[6] a) I. A. Kozlov, L. E. Orgel, *Mol. Biol.* **2000**, *34*, 781–789; b) L. E. Orgel, *Crit. Rev. Biochem. Mol. Biol.* **2004**, *39*, 99–123.
[7] Selected references: a) R. Lohrmann, L. E. Orgel, *Nature* **1976**, *261*, 342–344; b) D. Sievers, G. von Kiedrowski, *Nature* **1994**, *369*, 221–224; c) M. Kurz, K. Göbel, C. Hartel, M. W. Göbel, *Angew. Chem.* **1997**, *109*, 873–876; *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 842–845; d) A. Kanavarioti, C. F. Bernasconi, E. E. Baird, *J. Am. Chem. Soc.* **1998**, *120*, 8575–8581; e) M. Zielinski, I. A. Kozlov, L. E. Orgel, *Helv. Chim. Acta* **2000**, *83*, 1678–1684; f) J. C. Chaput, C. J. Switzer, *J. Am. Chem. Soc.* **2000**, *122*, 12866–12867; g) J. J. Chen, X. Cai, J. W. Szostak, *J. Am. Chem. Soc.* **2009**, *131*, 2119–2121.
[8] N. Griesang, E. Kervio, C. Richert, *Synthesis* **2005**, 2327–2334.
[9] N. Griesang, K. Giessler, T. Lommel, C. Richert, *Angew. Chem.* **2006**, *118*, 6290–6294; *Angew. Chem. Int. Ed.* **2006**, *45*, 6144–6148.
[10] P. Hagenbuch, E. Kervio, A. Hochgesand, U. Plutowski, C. Richert, *Angew. Chem.* **2005**, *117*, 6746–6750; *Angew. Chem. Int. Ed.* **2005**, *44*, 6588–6592.
[11] M. Röthlingshöfer, E. Kervio, T. Lommel, U. Plutowski, A. Hochgesand, C. Richert, *Angew. Chem.* **2008**, *120*, 6154–6157; *Angew. Chem. Int. Ed.* **2008**, *47*, 6065–6068.
[12] U. Plutowski, S. R. Vogel, M. Bauer, C. Deck, M. Pankratz, C. Richert, *Org. Lett.* **2007**, *9*, 2187–2190.
[13] See, for example: J. Shendure, G. J. Porreca, N. B. Reppas, X. Lin, J. P. McCutheon, A. M. Rosenbaum, M. D. Wang, K. Zhang, R. D. Mitra, G. M. Church, *Science* **2005**, *309*, 1728–1732.
[14] R. Eisenhuth, C. Richert, *J. Org. Chem.* **2009**, *74*, 26–37.
[15] K. L. Dombi, N. Griesang, C. Richert, *Synthesis* **2002**, *6*, 816–824.
[16] N. Zammatteo, L. Jeanmart, S. Hamels, S. Courtois, P. Louette, L. Hevesi, J. Remacle, *Anal. Biochemistry* **2000**, *280*, 143–150.
[17] CodeLink™-Activated microarray slides were from Amersham Biosciences, Piscataway, NJ, USA, following the recommended protocols of the manufacturer.
[18] T. Ameringer, M. Hinz, C. Mourran, H. Seliger, J. Groll, M. Moeller, *Biomacromolecules* **2005**, *6*, 1819–1823.
[19] M. L. Metzker, J. Lu, R. A. Gibbs, *Science* **1996**, *271*, 1420–1422.
[20] E. Cuevas-Yañez, J. M. Muchowski, R. Cruz-Almanza, *Tetrahedron* **2004**, *60*, 1505–1511.
[21] S. F. Malan, A. van Marle, W. B. Menge, V. Zuliana, M. Hoffman, H. Timmerman, R. Leurs, *Bioorg. Med. Chem.* **2004**, *12*, 6495–6503.

[22] a) R. Bihovsky, I. Pendrak, *Bioorg. Chem. Lett.* **1996**, *6*, 1541–1542; b) C. Peters, Dissertation, University of Dortmund, **2003**.
[23] A. Spaggiari, D. Vaccari, P. Davoli, F. Prati, *Synthesis* **2006**, 995–998.
[24] D. F. Harvey, D. M. Sigano, *J. Org. Chem.* **1996**, *61*, 2268–2272.
[25] M. L. Metzker, Substituted 4,4-Difluoro-4-bora-3a,4a-diaza-*s*-indacene compounds for 8-color DNA sequencing, WO Patent 20030668, **2003**.
[26] N. J. Meltola, R. Wahlroos, A. E. Soini, *J. Fluoresc.* **2004**, *14*, 635–647.
[27] H. C. Kang, R. P. Haugland, Long Wavelength Chemically Reactive Dipyrrometheneboron Difluoride Dyes and Conjugates, US Patent 5451663, **1995**.
[28] K. L. Dombi, N. Griesang, C. Richert, *Synthesis* **2002**, *6*, 816–824.
[29] a) U. Plutowski, C. Richert, *Angew. Chem.* **2005**, **117**, 627–631; *Angew. Chem. Int. Ed.* **2005**, *44*, 621–625; b) U. Plutowski, S. R. Vogel, M. Bauer, C. Deck, M. Pankratz, C. Richert, *Org. Lett.* **2007**, *9*, 2187–2190.
[30] M. Margulies, M. Egholm, W. E. Altman, S. Attiya, J. S. Bader, L. A. Bemben, J. Berka, M. S. Braverman, Y.-J. Chen, Z. Chen, S. B. Dewell, L. Du, J. M. Fierro, X. V. Gomes, B. C. Godwin, W. He, S. Helgesen, C. H. Ho, G. P. Irzyk, S. C. Jando, M. L. I. Alenquer, T. P. Jarvie, K. B. Jirage, J.-B. Kim, J. R. Knight, J. R. Lanza, J. H. Leamon, S. M. Lefkowitz, M. Lei, J. Li, K. L. Lohman, H. Lu, V. B. Makhijani, K. E. McDade, M. P. McKenna, E. W. Myers, E. Nickerson, J. R. Nobile, R. Plant, B. P. Puc, M. T. Ronan, G. T. Roth, G. J. Sarkis, J. F. Simons, J. W. Simpson, M. Srinivasan, K. R. Tartaro, A. Tomasz, K. A. Vogt, G. A. Volkmer, S. H. Wang, Y. Wang, M. P. Weiner, P. Yu, R. F. Begley, J. M. Rothberg, *Nature* **2005**, *437*, 376–380.
[31] J. Eid, A. Fehr, J. Gray, K. Luong, J. Lyle, G. Otto, P. Peluso, D. Rank, P. Baybayan, B. Bettman, A. Bibillo, K. Bjornson, B. Chaudhuri, F. Christians, R. Cicero, S. Clark, R. Dalal, A. de Winter, J. Dixon, M. Foquet, A. Gaertner, P. Hardenbol, C. Heiner, K. Hester, D. Holden, G. Kearns, X. Kong, R. Kuse, Y. Lacroix, S. Lin, P. Lundquist, C. Ma, P. Marks, M. Maxham, D. Murphy, I. Park, T. Pham, M. Phillips, J. Roy, R. Sebra, G. Shen, J. Sorenson, A. Tomaney, K. Travers, M. Trulson, J. Vieceli, J. Wegener, D. Wu, A. Yang, D. Zaccarin, P. Zhao, F. Zhong, J. Korlach, S. Turner, *Science* **2009**, *323*, 133–138.

Received: February 16, 2010
Published Online: May 21, 2010