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Synthesis of Oligonucleotides with 3′-Terminal 5-(3-Acylamidopropargyl)-3′-amino-2′,3′-dideoxyuridine Residues and Their Reactivity in Single-Nucleotide Steps of Chemical Replication

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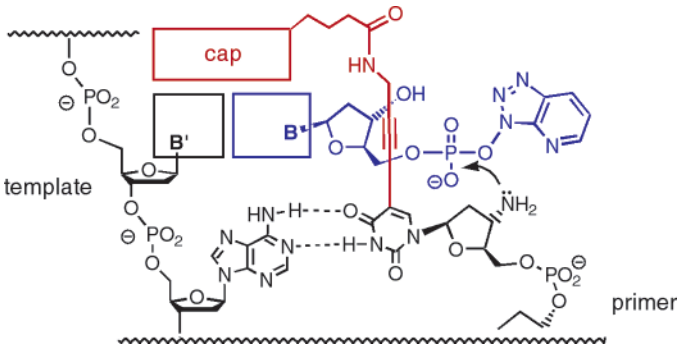

Oligonucleotides with a 3′-terminal 5-alkynyl-3′-amino-2′,3′-dideoxyuridine residue were prepared, starting from 2′-deoxyuridine. The optimized route employs a 2′,3′-dideoxy-3′-trifluoroacetamido-5-iodouridine 5′-phosphoramidite as building block for DNA synthesis and involves on-support Sonogashira coupling with *N*-tritylpropargylamine to generate oligonucleotides. The amino group of the propargylamine side chain was acylated to accelerate primer extension reactions involving the 3′-amino group. Three acyl groups were identified that decrease the half-life for DNA-templated extension steps with 7-azabenzotriazole esters of 2′-deoxynucleotides. The residue of 4-pyrenylbutyric acid was found to accelerate primer extension reactions and to render them more selective than those of the control primer. With this substituent, primer extension is also faster than previously measured for three-strand systems involving template, aminoprimer, and a downstream-binding helper oligonucleotide. Fast-reacting primers might become useful for genotyping single nucleotides.

Introduction

Replication is a pivotal reaction of life that precedes cell division. Bacterial cells achieve high-fidelity replications of entire genomes in under 20 min by enzymatically reacting nucleoside triphosphates with the terminal hydroxyl groups of growing DNA chains at a rate of approximately 1000 nucleotides per second.[1] Replication is based on template-directed oligomerization steps in which a primer is extended by one mononucleotide at a time. Primers are short strands of DNA or RNA that act as starting points for template-directed syntheses. Primer extension is also the basis of key biomedical applications, such as PCR and dideoxy DNA sequencing. Both in nature and in biomedical applications, primer extension requires polymerases as catalysts. Studying non-enzymatic alternatives to primer extension promises insights that might eventually lead to chemical versions of the polymerase chain reaction (PCR), the creation of self-replicating systems, and less expensive methods for determining DNA sequences.

Non-enzymatic replication has been studied for a number of years, and certain sequences of RNA have been shown to support the formation of complementary strands from activated mononucleotides.[2−4] Chemical primer extension has also been

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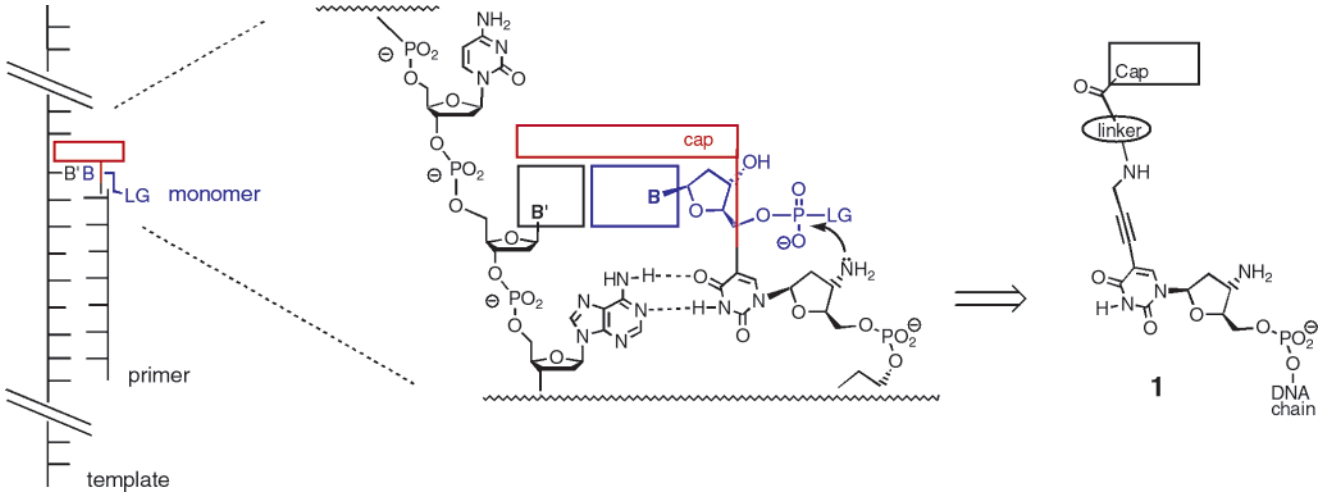

FIGURE 1. Chemical primer extension and primer with cap.

demonstrated for nucleic acids with modified backbones,[5−7] or nucleobases,[8] but neither has entered practical applications. Chemical ligation of short oligomers on a DNA or RNA template,[9−13] has reached a level of efficiency in generating a complementary strand that makes this reaction attractive for practical applications and for generating self-replicating systems.[14] To make non-enzymatic primer extension more suitable for diagnostic applications, however, an acceleration of the underlying reaction is needed.

To increase the reactivity toward activated mononucleotides, primers can be equipped with an amino group at their terminus. Amines are known to react rapidly with activated nucleotides in template-directed reactions in aqueous solutions.[15−17] Further, leaving groups other than the well-established 2-methylimidazolides[18] have recently been shown to lead to rate acceleration by up to 2 orders of magnitude.[19] A complementary approach to accelerate individual steps of chemical replication steps uses oligonucleotides whose termini are modified with substituents that assist in the capturing and/or conversion of monomers, mimicking, in a modest form, the effect of polymerases on primer extension in the cell. Such an effect has been observed for "capped" templates.[20] For practical applications, however, it is desirable to achieve this effect without the need to modify the template, so that DNA from natural sources can be employed. This calls for modified primers, e.g. in the form of oligonucleotides featuring both a terminal amino group and a cap that can assist in reactions with incoming nucleotides (Figure 1). Here, we report on the synthesis of such oligonucleotides and their increased reactivity in nonenzymatic primer extension reactions. Rapid single-nucleotide extension reactions allow for genotyping single-nucleotide polymorphisms with mass spectrometry as read-out technique.[21−23]

Results

Oligonucleotides with a 3′-terminal 3′-amino-2′,3′-dideoxyuridine residue featuring a substituent at position 5 (**1**, Figure 1) were the target molecules of our syntheses. Alkynyl groups at position 5 of pyrimidines are known to stabilize duplexes.[24,25] We chose propargylamine as the substituent at the 5 position of deoxyuridine, as the amino function allows for amide-forming reactions, so that the large pool of available carboxylic acids can be accessed in the search for caps that accelerate nonenzymatic primer extension reactions. Because amide-forming reactions involving oligonucleotides on controlled pore glass (cpg) are easiest to perform when the amino group is on the distal nucleotide of the immobilized chain,[26] we opted for DNA syntheses with 5′- or "reverse" phosphoramidites.[27,28]

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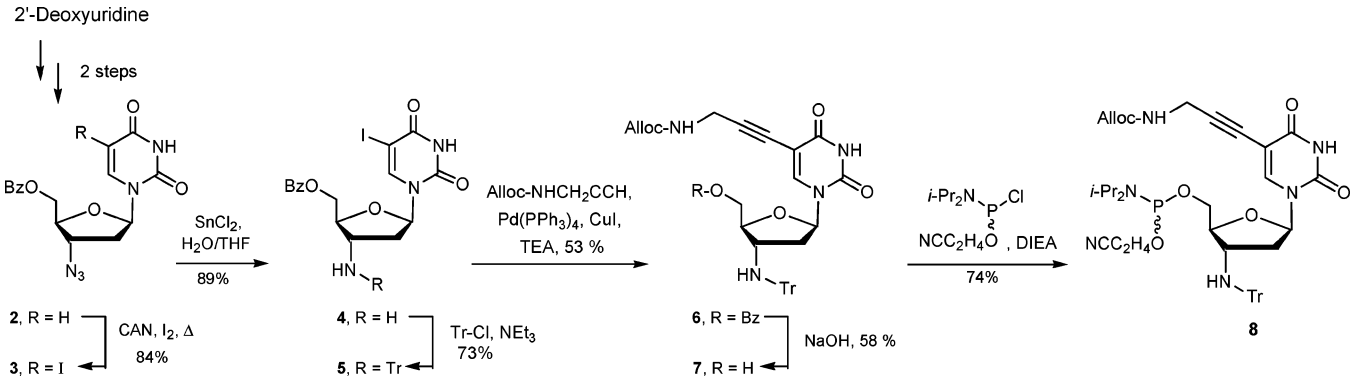
SCHEME 1



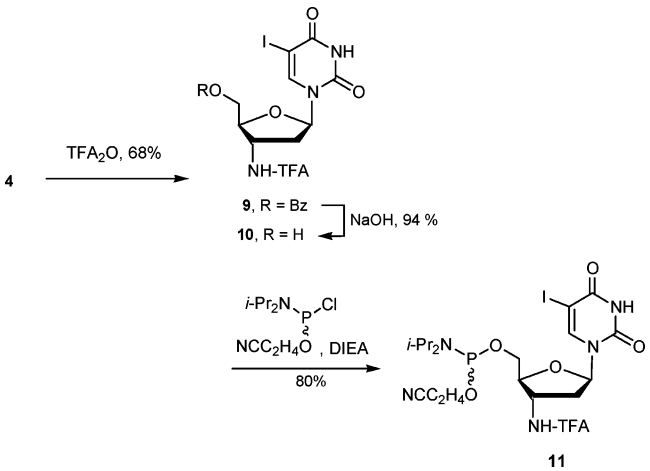
Several routes differing in the choice of reagents, protecting groups, and order of events were tested. The shortest route, involving a 3′-azide as the latent amine and no protecting groups at this position up to the oligonucleotide level, proved incompatible with phosphoramidite chemistry. After phosphitylation, decomposition, probably via intramolecular Staudinger reduction, set in before coupling to the remainder of the DNA chain could be achieved. Attempts to introduce the 3′-azido functionality via ring opening of 5-iodo-2,3′-anhydrouridine were also unsuccessful, probably because of the high reactivity of the iodo functionality. Schemes 1 and 2 show routes that led to full-length DNA chains on solid support. Either of them starts from 2′-deoxyuridine, which was converted to 2′-deoxy-2,3′-anhydrouridine in 85% yield, following literature protocols (Scheme 1).[29] Opening of the anhydrouridine with sodium azide in DMF gave **2**,[30] and subsequent iodination of position 5 with CAN and iodine[31] gave nucleoside **3** in 56% yield over two steps. Reduction of the azide proved more troublesome. Hydrogenation with palladium or platinum catalysts failed to reduce the azide. Instead, deiodination was observed without reduction of the azide (or the 5,6 double bond), even after long reaction times. Staudinger reduction yielded amine **4** in 67% yield, but scale-up and hydrolysis of the iminophosphorane proved difficult, and the purification remained cumbersome. Tin dichloride in water/THF[32] reacted smoothly to give amine **4** in 89% yield. This reaction was also suitable for scale-up to the gram scale.

The route then involved protection of the 3′-amino group with a trityl group to give **5**, followed by Sonogashira coupling with *N*-Alloc-protected propargylamine[33] to yield **6** (Scheme 1). The latter reaction gave variable yields, caused by incomplete conversion, making this route unsatisfactory. Subsequent debenzoylation led to **7** in moderate yield, but phosphitylation to **8** proceeded uneventfully. Although the coupling of **8** to the 3′-terminal hydroxyl group of DNA chains was high yielding, as evidenced by MALDI-TOF mass spectrometry of crude products obtained by deprotecting analytical samples with aqueous ammonia, the subsequent removal of the Alloc group again proceeded unsatisfactorily, with low conversion or decomposition when attempted under forcing conditions.

Scheme 2 shows the synthesis of a phosphoramidite that proved more reliable. The Sonogashira coupling was performed on cpg support,[34] and protecting groups were changed to shorten the route. A trifluoroacetyl group was used to protect 3′-amine **4**, giving **9** in reasonable yield. The TFA group later releases the free amine during the final deprotection step of the DNA. Debenzoylation of **9** to **10** proceeded without concomitant loss of the TFA group when 1.5 equiv of 1 M sodium hydroxide solution in MeOH was used for 1 h at ambient temperature. Conversion of **10** to phosphoramidite **11** using 2-cyanoethyl-*N*,*N*-diisopropyl-chlorophosphoramidite as the phosphitylating reagent gave the latter in 80% yield under unoptimized conditions.

A DNA hexamer of the sequence 5′-GCCACG-3′ was prepared, starting from cpg loaded with a deoxyguanosine residue (Scheme 3). After incorporation of the 5-iodonucleoside as the last monomer during automated DNA synthesis, giving **12**, on-support Sonogashira reaction[34] produced **13**. Trityl-protected propargylamine,[35] together with $Pd(PPh_3)_4$, CuI, and diisopropylethylamine (DIEA),[36] gave 80−96% conversion after 4 h at room temperature, as detected in MALDI-TOF mass

SCHEME 2



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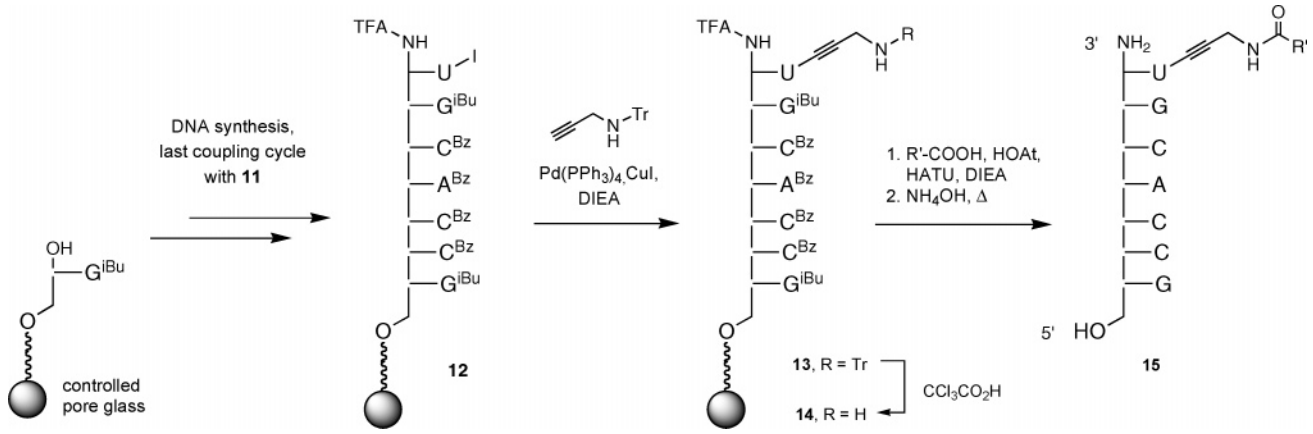
SCHEME 3



spectra of the crude products. The trityl group of **13** was then removed with the trichloroacetic acid solution in dichloromethane regularly employed for deblocking during DNA chain assembly. Amine (**14**) was then coupled to active esters of carboxylic acids, generating, after ammonia treatment, primers with an amidic cap (general structure **15**, Scheme 3).

Six different target oligonucleotides with caps were prepared to test the methodology. Two of these were generated through direct acylation of **14** with the respective carboxylic acids, two involved installation of a β-alanine linker, and two required a linker with a β-alanine and a glycine residue (Figure 2). The linkers were introduced via peptide coupling with Fmoc-protected amino acids, using HATU/HOAt, followed by Fmoc removal by treatment with piperidine/DMF (1:4) for 10 min and subsequent coupling, following a protocol similar to that reported for other acylated amino-terminal oligonucleotides.[37] For the direct acylation of **14**, 4-pyrenylbutyric acid and cholic acid were used to give **16** and **17**, respectively. Either of the acid residues is known to act as a cap for terminal base pairs,[38,39] and either contains an alkyl chain for bridging the distance between the attachment point to the primer and the stacking site above the incoming nucleotide (Figure 1). One-step HPLC purification gave **16** and **17** in yields between 30% and 35%. Modeling studies showed that an external spacer is necessary for acid residues lacking an internal alkyl spacer to reach above the base pair between templating base and incoming nucleotide. Trimethoxystilbene carboxylic acid was coupled to both the β-Ala and β-Ala-Gly-linked constructs, as the ability of this aromatic moiety to stack on terminal base pairs is well documented.[40] To add diversity, nalidixic acid was coupled to the β-Ala platform, and anthraquinone carboxylic acid was coupled to the β-Ala-Gly platform. Again, either of the acyl residues is known to stabilize duplexes when appended to the terminus of one strand.[41] In addition to cap-bearing primers **16**−**21**, which were obtained in overall yields between 15% and 41%, unmodified primer **22** was prepared as a control via standard DNA synthesis to a 3′-amino-3′-deoxyprimer.[19,20]

With oligonucleotides **16**−**22** in hand, exploratory primer extension reactions of the type shown in Figure 1 were performed. The assays employed azabenzotriazole esters of deoxynucleotides (**25a**−**t**) as activated monomers in aqueous buffer under conditions recently shown to give rapid reactions with 3′-amino-terminal primers.[19] Two different templates, one with weakly base-pairing adenine (**23**) and one with strongly base-pairing cytosine (**24**) as the templating base, were employed (Scheme 4). The A-templated reaction is the one giving the slowest reactions among the four different template bases (A, C, G, and T).[19] Exploratory assays showed that primers **17**−**19** do not undergo faster reactions with **25a**−**t** in the presence of **23** than control **22**. The remaining three primers (**16**, **20**, and **21**) exceeded **22** in reactivity and were selected for kinetic analysis.

Figure 3 shows representative spectra and kinetics for the fastest reaction found, and Table 1 lists half-lives of primer

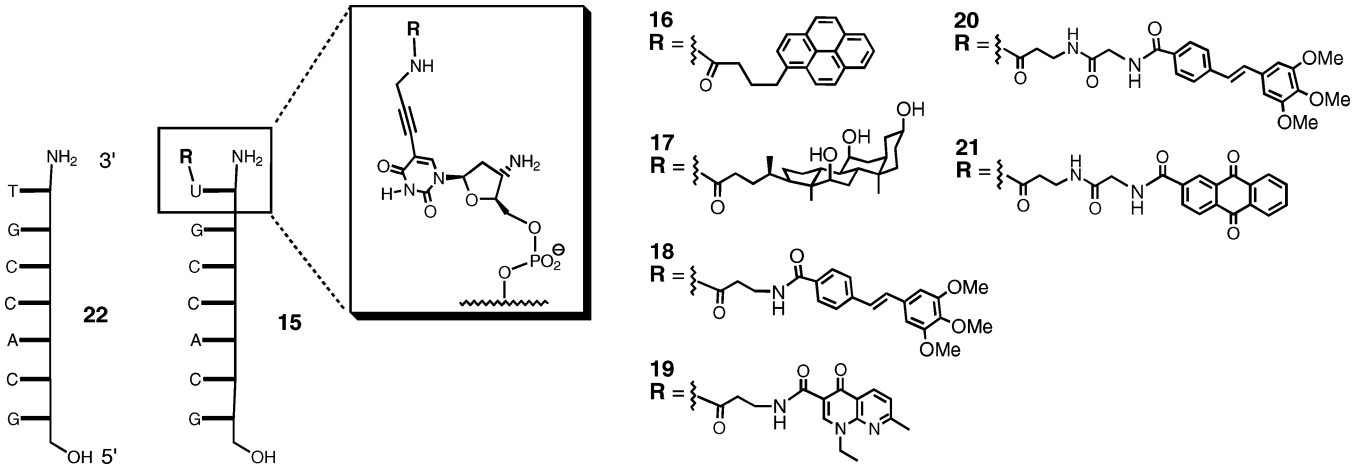


FIGURE 2. Structures of primers.

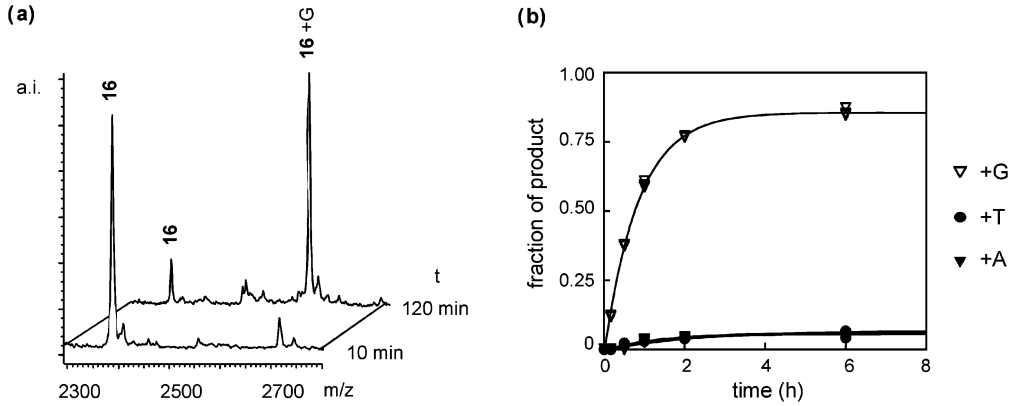


FIGURE 3. Competitive primer extension reactions with primer **16**, activated monomers **25a**−**t**, and template **24**: (a) MALDI-TOF mass spectra taken 10 and 120 min after initiation of the reaction. (b) Kinetics of formation of extension products. The legend shows the nucleotides by which **16** was extended; incorrect extension by deoxycytidine residues was below the detection limit.

SCHEME 4

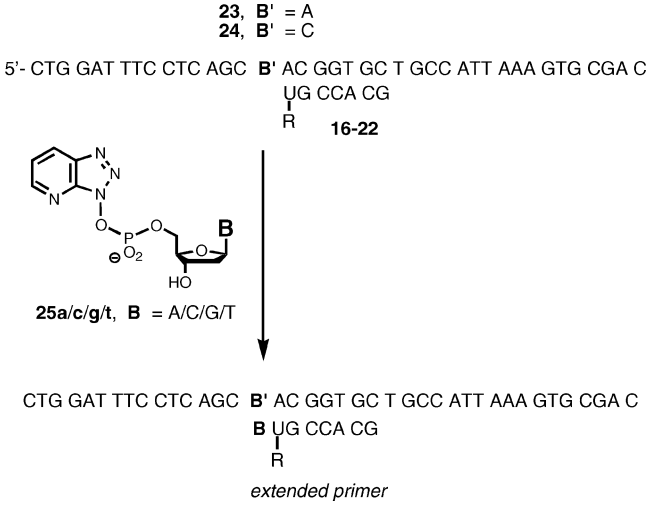


TABLE 1. Results of Competitive Template-Directed Primer Extension Reactions with Activated Monomers 25a−t[a]

template	primer	product ratio (primer + C/T/A/G)[b]	$t_{1/2}$ primer (h)
23	**22**	<1/**61**/12/26	2.58
23	**16**	<1/**72**/<1/26	0.67
23	**20**	7/**49**/17/27	1.58
23	**21**	<1/**55**/<1/43	1.15
24	**22**	<1/9/5/**85**	1.45
24	**16**	<1/6/6/**87**	0.61
24	**20**	6/13/10/**71**	1.03
24	**21**	<1/5/<1/**93**	0.62

[a] Conditions: 36 μM template and primer, 3.6 mM **25a**−**g**, 18 mM **25t,** 200 mM HEPES, pH 8.9, 400 mM NaCl, 80 mM $MgCl_2$. [b] Matched primer extension products highlighted in boldface.

conversion as well as product distributions for assays with either template. The "winner" compound, pyrenyl primer **16**, not only increased the rates of the primer extension up to 4-fold, but also improved its fidelity compared to control **22**, with less incorporation of mismatched nucleotides that do not fit the templating base of **23** or **24**. Compared to reactions with the same template performed in the presence of a "helper" oligonucleotide that binds immediately downstream of the templating nucleotide,[19] primer extension with **16** is faster for either template. Helper oligonucleotides create a binding pocket for incoming nucleotides by binding immediately downstream of the templating base. Perhaps most importantly for practical applications, such as non-enzymatic SNP genotyping,[19] the cap adjusts the rate of the extension reactions on the two different templates to very similar values, with half-lives of primer conversion of 0.67 and 0.61 h (Table 1). In the case of the three-strand system involving a helper oligonucleotide, the values are 0.8 and 1.3 h for the A and C templates, respectively, under the same conditions.[19] Anthraquinone-bearing primer **21** rivals **16** in reactivity and exceeds it in fidelity, when employed on template **24** but is inferior when reacting in the presence of **23**.

Discussion

These results establish a synthetic route to oligonucleotides with 3′-terminal diaminonucleotide residues. They also show that it is possible to generate primers for non-enzymatic single-nucleotide extension reactions with higher reactivity and better selectivity than control strands. This is encouraging, as it might lead to ways of more efficiently determining nucleotides in DNA strands without costly enzymes and their triphosphate substrates. Determining DNA sequences at individual positions known to harbor variances in the human genome is one focus of current genomic projects.[42,43] The step from caps on templates used in earlier work[20] to caps on primers is critically important for genotyping applications, as it ensures that unmodified DNA that comes from biological sources or a PCR reaction starting from genomic DNA can be probed.

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The present results are also interesting in the context of primer extension reactions themselves. They show that these reactions can occur more rapidly when the incoming nucleotide reacts in the presence of an aromatic substituent flexibly tethered to the primer. This is not trivial, as a substituent or cap might also *block* primer extension by stacking on the last base pair of the primer−template duplex, sealing the primer against extension. Because the caps tested do not feature cationic functional groups that can activate the monomers toward attack by the 3′-amino group of the primer, it is likely that the main effect is that of retaining the monomer at the reaction site. The accelerating effect of the current caps is similar to that of so-called helper oligonucleotides that bind immediately downstream of the templating nucleotide on the template,[19] even though the caps are not anchored in place through base pairing. It is possible that caps can be evolved that act *in the presence* of helper oligonucleotides, strengthening their effect. We have initiated a project that seeks to identify such caps.[44]

Even the rates achieved thus far are sufficient to determine nucleotides within 1 h, using suitable microarrays[45] and mass spectrometric read-out.[19] Rates for primer extension with **16** and both **23** and **24** are the highest on record for OAt esters **25a**−**t**, and up to a factor of 2 greater than those known for the three-strand system.[19] The latter system with its additional helper oligonucleotide has proven sufficiently reactive for on-chip genotyping in less than 3 h, starting from 500 fmol of template DNA.[19] The level of selectivity achievable with primers such as **16** is sufficient for making base calls in SNP genotyping assays, but probably too low for non-enzymatic replication of even a very small genome.

Only five different caps were tested in the current exploratory assays. All of them came from studies focused on sealing blunt ends of duplexes in the ground state.[37−41] Perhaps, optimized caps for primer extension reactions will look different. They might exploit interactions with both the substrates and the single-stranded overhang of the template through a combination of hemi-intercalation and stacking. To achieve this result, the cap should be too bulky for full intercalation to avoid sequestering it in the primer−template duplex upstream of the reaction site. Further, the linker should be rigid enough to disfavor blocking of the binding site for the incoming nucleotide. Finally, functional groups stabilizing the transition state directly or by attracting Mg^{2+} ions in a fashion similar to that of polymerases[46] could be beneficial.

With the route to 3′-amino primers with terminal 5-(acylamidopropargyl)deoxyuridine residues presented here, one can envision a search for caps via combinatorial libraries whose components compete in selection assays monitored by MALDI-TOF MS.[47] Peaks for primers with more efficient caps can be expected to appear first in the mass range of the extension products. Exploratory experiments leading to small libraries (chemsets), generated via mixed coupling with a reactivity-adjusted mixture,[48] suggest that spectrometrically monitored selection experiments (SMOSE) should be feasible. Last, but not least, it is worth mentioning that building block **11** might also become useful for the synthesis of oligonucleotides with phosphoramidate linkages[49] in the interior of the sequence. To this end, the protecting group scheme shown in Scheme 1 should be more suitable, as it ensures that the 3′-terminus can be deblocked selectively during chain assembly. The amino group at the propargyl side chain can be diversified in combinatorial searches for substituents that stabilize T−A base pairs via interactions in the major groove,[36,50] thus compensating for the weakness of U−A base pairs with just two hydrogen bonds.[51]

Experimental Section

3′-Azido-5′-*O*-benzoyl-2′,3′-dideoxyuridine (2). The following is a modification of the method of Czernecki and Valéry.[30] To a solution of 2,3′-anhydro-5′-*O*-benzoylthymidine[30] (0.79 g, 2.51 mmol) in dry DMF (5 mL) was added sodium azide (0.49 g, 7.54 mmol, 3 equiv), and the reaction mixture was heated to 120°C. After 18 h, the solution was treated with water (200 mL), and the pH was adjusted with 10% H_2SO_4 to pH 7. Three extractions with diethyl ether (200 mL), followed by drying of the combined organic phases over Na_2SO_4 and concentration in vacuo, gave a crude product that was chromatographed on silica (1:2 hexane/ethyl acetate, $R_F = 0.48$) to afford **2** as a pale yellow oil (0.51 g, 1.40 mmol, 56%). Spectroscopic data are in agreement with the literature.[30] HR-MS $C_{16}H_{16}N_5O_5$ calcd 358.1151, found 358.1135.

3′-Azido-5′-*O*-benzoyl-2′,3′-dideoxy-5-iodoridine (3). A solution of **2** (0.56 g, 1.59 mmol), iodine (0.24 g, 0.95 mmol, 0.6 equiv), and cerium ammonium nitrate (0.44 g, 0.8 mmol, 0.5 equiv) in acetonitrile (10 mL) was heated to 65°C. After 30 min, the solution was treated with saturated sodium hydrogen thiosulfate solution until the brown color of the solution disappeared and was then extracted with diethyl ether (2 × 150 mL). The combined organic phases were dried over Na_2SO_4 and concentrated in vacuo. Purification of the pale yellow oil on silica (1:1 hexane/ethyl acetate, $R_F = 0.60$) gave **3** as a pale yellow oil (0.65 g, 1.33 mmol, 84%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.41 (m, 1H), 2.61 (m, 1H), 4.27 (t, $J = 3.6$ Hz, 1H), 4.36 (m, 1H), 4.63 (d, $J = 3.7$ Hz, 2H), 6.12 (t, $J = 6.4$ Hz, 1H), 7.48 (t, $J = 8.0$ Hz, 2H), 7.61 (t, $J = 8.0$ Hz, 1H), 7.88 (s, 1H), 8.05 (d, $J = 8.3$ Hz, 2H), 9.66 (s, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 166.1, 160.1, 149.8, 144.0, 133.7, 129.8, 129.0, 128.9, 86.1, 82.5, 68.9, 63.7, 60.4, 38.3; MS (FAB, 3-NBA) *m*/*z* $C_{16}H_{15}IN_5O_5$ calcd 484.0, found 484.1; IR (drift) $\nu = 2106.9$ cm^{-1}.

5′-*O*-Benzoyl-2′,3′-dideoxy-3′-amino-5-ioduridine (4). To a solution of **3** (1 g, 2.07 mmol) in THF (10 mL) was added slowly a solution of tin dichloride (0.78 g, 4.14 mmol, 2 equiv) in water (5 mL). After 20 min, the reaction was stopped by adding 100 mL of saturated $NaHCO_3$ solution, and the pH was adjusted to 8 with NaOH solution (5 M). After triplicate extraction with 100 mL of ethyl acetate, the organic phase was dried over Na_2SO_4 and concentrated in vacuo. Amine **4** was obtained as a colorless oil (0.94 g, 2.06 mmol, 98%). ^{1}H NMR (CD_3OD, 400 MHz) δ 2.35 (m, 2H), 3.60 (m, 1H), 4.10 (m, 1H), 4.60 (dd, $J = 12.5$ Hz, 2.6 Hz, 2H), 6.14 (t, $J = 6.7$ Hz, 1H), 7.50 (m, 2H), 7.61 (t, $J = 7.4$ Hz, 1H), 8.01 (m, 3H); ^{13}C NMR (CD_3OD, 125.8 MHz) δ 166.1, 160.1, 149.8, 144.7, 133.1, 129.2, 128.4, 85.7, 84.7, 67.1, 63.5,

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50.8, 40.0; HR-MS (EI) $C_{16}H_{16}IN_3O_5$ calcd 457.0135, found 457.0132.

5′-*O*-Benzoyl-2′,3′-dideoxy-5-iodo-3′-(tritylamino)uridine (5). To a solution of **4** (0.2 g, 0.43 mmol) in dry CH_2Cl_2 (5 mL) was added NEt_3 (0.13 mL, 0.56 mmol, 1.3 equiv), followed by the dropwise addition of trityl chloride (0.16 g, 0.56 mmol, 1.3 equiv) in CH_2Cl_2 (2 mL). After 3 h, the reaction was quenched with saturated aqueous $NaHCO_3$ (100 mL) and extracted three times with 100 mL of CH_2Cl_2. The combined organic phases were dried over Na_2SO_4 and concentrated in vacuo. Purification on silica (20:1 CH_2Cl_2/MeOH, $R_F = 0.56$) afforded the title compound, which crystallized upon standing (0.22 g, 0.39 mmol, 73%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.41 (m, 1H), 2.61 (m, 1H), 4.27 (t, $J = 3.6$ Hz, 1H), 4.36 (m, 1H), 4.63 (d, $J = 3.7$ Hz, 2H), 6.12 (t, $J = 6.4$ Hz, 1H), 7.37 (m, 10H), 7.45 (m, 2H), 7.61 (m, 7H), 7.88 (s, 1H), 8.05 (d, $J = 8.3$ Hz, 3H), 9.2 (s, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 166.1, 160.1, 149.8, 144.0, 133.7, 129.8, 129.0, 128.9, 86.1, 82.5, 68.9, 63.7, 60.4, 38.3; FAB-MS (3-NBA) *m*/*z* 699.2.

5-[3-*N*-(Allyloxycarbonyl)aminopropyn-1-yl]-5′-*O*-benzoyl-2′,3′-dideoxy-3′-(tritylamino)uridine (6). A solution of **5** (150 mg, 0.21 mmol) in DMF (2 mL) was degassed under reduced pressure and placed under argon. Then, $Pd(PPh_3)_4$ (25.0 mg, 21 μmol, 0.1 equiv), CuI (8.0 mg, 42 μmol, 0.2 equiv), *N*-alloc-protected propargylamine (39.0 mg, 0.28 mmol, 1.3 equiv), and NEt_3 (0.09 mL, 0.63 mmol, 3 equiv) were added, and the solution was stirred for 4 h at room temperature. After addition of an aqueous solution of EDTA (0.14 M), the mixture was extracted with ethyl acetate (3 × 100 mL), and the combined organic phases were dried over Na_2SO_4, concentrated in vacuo, and subjected to chromatography (silica, 2:1 hexanes/ethyl acetate, $R_F = 0.5$), yielding a pale yellow oil (79 mg, 0.11 mmol, 53%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.27 (m, 1H), 1.75 (m, 1H), 1.90 (m, 2H), 3.40 (m, 1H), 3.90 (dq, $J =$ 17.6, 5.2 Hz, 2H), 4.03 (m, 1H), 4.39 (dd, $J = 12.5$, 4.0 Hz, 1H), 4.57 (m, 3H), 4.87 (br s, 1H), 5.29 (d, $J = 1.5$ Hz, 1H), 5.32 (d, $J = 1.5$ Hz, 1H), 5.91 (m, 1H), 5.99 (t, $J = 5.3$ Hz, 1H), 7.13−7.29 (m, 10 H), 7.45 (t, $J = 8.2$ Hz, 2H), 7.60−7.69 (m, 7H), 7.89 (dd, $J = 8.0$, 0.9 Hz, 2H), 8.84 (s br, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 166.2, 161.3, 155.8, 148.8, 145.8, 145.7, 142.1, 133.3, 132.6, 129.6, 129.3, 128.6, 128.4, 128.2, 126.9, 117.8, 99.3, 89.5, 85.7, 84.3, 74.1, 71.2, 65.8, 63.6, 53.6, 40.6, 31.5; FAB-MS (3-NBA) *m*/*z* 711.3.

5-[3-*N*-(Allyloxycarbonyl)aminopropyn-1-yl]-2′,3′-dideoxy-3′-(tritylamino) uridine (7). To a solution of **6** (73.0 mg, 0.1 mmol) in MeOH (3 mL) was added aqueous NaOH (0.3 mL, 1 M, 3 equiv) at room temperature. After 3 h, water (10 mL) was added, and the resulting mixture was extracted with ethyl acetate (3 × 100 mL). The combined organic phases were dried over Na_2SO_4 and concentrated in vacuo. Chromatographic purification on silica (1:2 hexanes/ethyl acetate, $R_F = 0.6$) gave a colorless solid (0.03 g, 0.06 mmol, 58%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.32 (m, 1H), 1.45 (m, 1H), 1.94 (s, 2H), 2.96 (s, 1H), 3.40 (m, 1H), 3.62 (m, 1H), 3.71 (m, 1H), 3.69 (m, 1H), 3.88 (m, 1H), 3.92 (m, 1H), 4.55 (d, $J = 5.3$ Hz, 1H), 5.20 (d, $J = 1.3$ Hz, 2H), 5.53 (d, $J = 4.9$ Hz, 1H), 5.94 (m, 1H), 5.99 (t, $J = 6.3$ Hz, 1H), 7.16 (t, $J = 7.1$ Hz, 3H), 7.20−7.30 (m, 6H), 7.53 (d, $J = 7.4$ Hz, 6H), 7.91 (s, 1H), 9.12 (s, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 171.2, 161.7, 156.1, 149.1, 146.1, 146.0, 143.8, 132.6, 132.1, 128.6, 128.1, 126.8, 117.9, 98.9, 89.6, 87.3, 85.5, 77.7, 77.2, 74.3, 71.2, 65.9, 61.7, 53.1, 40.4, 31.6; MALDI-TOF MS (linear, positive mode) *m*/*z* $C_{35}H_{34}N_4O_6$ calcd 606.2 [M + H], 629.1 [M + Na], found 606.8, 629.8.

5-[3-*N*-(Allyloxycarbonyl)aminopropyn-1-yl]-2′,3′-dideoxy-3′-(tritylamino) uridin-5′-*O*-yl-*β*-cyanoethyl-N,N-diisopropyl phosphoramidite (8). To a stirred solution of **7** (50.0 mg, 0.08 mmol) and diisopropylethylamine (42 μl, 0.24 mmol, 3 equiv) in anhydrous CH_3CN (2.0 mL) was added 2-cyanoethyl-*N*,*N*-diisopropyl chlorophosphoramidite (30 μL, 0.12 mmol, 1.5 equiv). The solution was stirred for 30 min at room temperature. After TLC indicated complete conversion, CH_2Cl_2 (20 mL) was added, and the solution was washed with saturated aqueous $NaHCO_3$ (2 × 30 mL) and with brine (30 mL). The organic phase was dried over Na_2SO_4, the volume was reduced in vacuo to 0.5 mL, and the solution was added dropwise to pentanes (20 mL). The supernatant was aspired, and the oily residue was dried in vacuo to give the title compound (mixture of diastereomers) as a colorless foam (49 mg, 0.06 mmol, 74%). ^{31}P NMR (CD_3CN-d_3, 300 MHz) δ 150.2, 150.5.

5′-*O*-Benzoyl-2′,3′-dideoxy-5-iodo-3′-(trifluoroacetylamido)-uridine (9). Amine **4** (0.92 g, 2.01 mmol) was dissolved in 25 mL of CH_2Cl_2, and trifluoracetic acid anhydride (640 mg, 3.02 mmol, 1.5 equiv) and pyridine (377 μL, 370 mg, 4.05 mmol, 2 equiv) were added under stirring. After 30 min, the solution was partioned between 100 mL of saturated aqueous $NaHCO_3$ and 100 mL of CH_2Cl_2. Concentration of the organic phase in vacuo and purification on silica [1:2 (v/v) hexanes/ethyl acetate, $R_F = 0.75$] gave a colorless solid. (0.76 g, 1.37 mmol, 68%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.31 (m, 1H), 2.71 (d, $J = 11.1$ Hz, 1H), 4.57−4.75 (m, 3H), 6.26 (s, 1H), 7.49 (t, $J = 7.5$ Hz, 2H), 7.62 (t, $J = 7.0$ Hz, 1H), 7.87 (s, 1H), 8.07 (d, $J = 7.8$ Hz, 2H), 8.2 (br s, 1H), 10.3 (br s, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 166.3, 144.9, 144.6, 133.4, 133.1, 129.3, 128.4, 128.4, 126.4, 90.6, 86.0, 84.9, 81.8, 63.6, 50.0, 36.5; HR-MS (EI) *m*/*z* for $C_{18}H_{15}N_3F_3IO_6$ calcd 552.9958, found 552.9957.

2′,3′-Dideoxy-5-iodo-3′-(trifluoroacetylamido)uridine (10). To a solution of **9** (100 mg, 0.18 mmol) in MeOH (4 mL) was added aqueous NaOH (0.72 mL, 1 M, 4 equiv). After TLC showed that the reaction was complete (typically 0.5−1 h), water (10 mL) was added, and the resulting mixture was extracted three times with 100 mL of ethyl acetate. The combined organic phases were dried over Na_2SO_4 and concentrated in vacuo. Purification on silica [1:2 (v/v) hexanes/ethyl acetate, $R_F = 0.5$] gave the title compound as a colorless solid (0.076 g, 0.17 mmol, 94%). ^{1}H NMR (MeOH-d_4, 500 MHz) δ 2.32 (t, $J = 6.6$ Hz, 2H), 3.59 (dd, $J = 12.1$, 3.3 Hz, 1H), 3.74 (dd, $J = 12.1$ Hz, 2.5 Hz, 1H), 3.88 (m, 1H), 4.50 (m, 1H), 6.08 (t, $J = 6.6$ Hz), 8.45 (s, 1H); ^{13}C NMR (MeOH-d_4, 125 MHz) δ 161.3, 157.6, 157.3, 150.4, 145.6, 114.4, 85.3, 84.7, 60.5, 49.1, 37.1; HR-MS (EI) *m*/*z* for $C_{11}H_{11}N_3F_3IO_5$ calcd 448.9695, found 448.9694.

2′,3′-Dideoxy-5-iodo-3′-(trifluoroacetylamido)uridin-5′-*O*-yl-*β*-cyanoethyl-*N*,*N*-diisopropyl-phosphoramidite (11). To a stirred solution of **10** (125 mg, 0.27 mmol) and diisopropylethylamine (141 μL, 0.81 mmol, 3 equiv) in anhydrous CH_3CN (2.0 mL) was added 2-cyanoethyl-*N*,*N*-diisopropyl chlorophosphoramidite (70 μL, 0.81 mmol, 1.1 equiv). The solution was stirred for 30 min. After TLC indicated complete conversion, CH_2Cl_2 (20 mL) was added, and the solution was washed with saturated $NaHCO_3$ solution (2 × 30 mL) and with brine (30 mL). The organic phase was dried over Na_2SO_4, and the solvent was evaporated to an approximate volume of 0.5 mL. This solution was added dropwise to pentanes (20 mL). The supernatant was aspired, and the oily residue was dried in vacuo, yielding the title compound (mixture of diastereomers) as a colorless foam (141 mg, 0.22 mmol, 80%). ^{31}P NMR (CD_3CN-d_3, 300 MHz) δ 149.9, 150.3.

DNA Synthesis. Oligodeoxynucleotides were prepared on 1 μmol scale on a model 8909 Expedite synthesizer, software 2.01 (Perseptive Biosystems) using the standard protocol, except that 5′-phosphoramidites (reverse phosphoramidites) were used. Coupling of **11** to the unmodified portion of the strand used a 0.4 M solution in CH_3CN that had been dried over 4-Å molecular sieves. All oligodeoxynucleotides were deprotected and cleaved from the solid support using concentrated aqueous ammonia for 5 h at 65 °C. After being cooled to room temperature., the supernatant was collected, and the residue was washed with deionized water. Excess ammonia was removed from the combined aqueous solutions with a gentle stream of compressed air, followed by lyophilization to dryness. The residue was taken up with deionized water, filtered, and subjected to HPLC purification.

Compound 22. The last extension cycle of the DNA synthesis used a 3′-MMT-protected phosphoramidite of 3′-amino-3′-deoxy-thymidine (ChemGenes). The MMT group was removed using the

deblocking solution for DNA synthesis [3:97 (v/v) trichloroacetic acid/CH_2Cl_2] for 10 min prior to cleavage from the solid support. HPLC 0% B for 5 min, to 30% B in 45 min, t_R = 21.2 min; yield 44%; MALDI-TOF MS calcd for $C_{67}H_{81}N_{27}O_{39}P_6$ ([M − H]$^-$) 2073, found 2071.

Support 13. A sample of controlled pore glass **12** (5.0 mg, 0.125 μmol loading) in a polypropylene vial was dried at 0.1 Torr for 1 h, and a slurry of $Pd(PPh_3)_4$ (8.0 mg, 6.25 μmol, 50 equiv) and CuI (2.0 mg, 10 μmol, 80 equiv) in dry DMF (200 μL) was added, followed by diisopropylethylamine (5 μL, 35 μmol, 280 equiv). After 2 min, *N*-tritylpropargylamine was added. After the mixture had been shaken for 5 h, the supernatant was aspired, and the support was washed once with CH_3CN (400 μL), twice with a solution of ethylenedithiocarbamic acid (sodium salt) in 400 μL of DMF [0.5% (v/v)], and thrice with CH_3CN (400 μL). The support was then dried at 0.1 Torr. Deprotection of an analytical sample showed >80% conversion by MALDI-TOF MS. MALDI-MS calcd for $C_{88}H_{97}N_{28}O_{39}P_6$ ([M − H]$^-$) 2355, found 2359.

Support 14. The trityl group on support **13** was removed as described for compound **22**, above. The deprotection was performed immediately prior to amide coupling. Deblocking solution (250 μL) was added to the solid support (10.0 mg, 0.25 μmol), and the mixture was shaken for 10 min. The supernatant was aspired, and the support was washed with CH_3CN (3 × 300 μL) and then dried at 0.1 Torr.

Oligonucleotides of General Structure 15. The carboxylic acid (100 μmol), HATU (15.0 mg, 90 μmol), and HOAt (2 mg, 90 μmol) were dissolved in DMF (300 μL), diisopropylethylamine (10 μL, 100 μmol, 1 equiv) was then added, and the resulting mixture was shaken for 5 min. The resulting solution was added to **14**, and the slurry was shaken for 3 h. The supernatant was aspired, and the support was washed with CH_3CN (3 × 300 μL) and then dried at 0.1 Torr. Afterward, the support was either subjected to subsequent Fmoc removal and coupling (compounds **18**−**22**) or deprotected with NH_4OH, as described for DNA Synthesis, above.

Compound 16. HPLC 0% B for 5 min, to 15% B in 55 min, t_R = 25.4 min; yield 30%, MALDI-MS calcd for $C_{89}H_{96}N_{28}O_{40}P_6$ ([M − H]$^-$) 2382, found 2388.

Compound 17. HPLC 0% B for 5 min, to 30% B in 45 min, t_R = 40.3 min; yield 35%, MALDI-MS calcd for $C_{93}H_{120}N_{28}O_{43}P_6$ ([M − H]$^-$) 2503, found 2509.

Compounds 18 and 19. The coupling of *N*-Fmoc-β-alanine (3.2 mg, 100 μmol) to support **14** (10.0 mg, 0.25 μmol loading) followed the protocol given for general structure **15** above. Then, a solution of piperidine in DMF [1:4 (v/v), 250 μL] was added to the support to remove the Fmoc groups, and the mixture was shaken for 10 min. The supernatant was aspired, and the support was washed with CH_3CN (3 × 300 μL) and then dried at 0.1 Torr. The support thus prepared was subjected to an amide-forming coupling reaction with trimethoxystilbene carboxylic acid[52] or nalidixic acid following the protocol given for **15**, above. Deprotection with NH_4OH again followed the general protocol given for DNA synthesis above.

Compound 18. HPLC 0% B for 5 min, to 30% B in 55 min, t_R = 30.2 min; yield 19%; MALDI-TOF MS calcd for $C_{90}H_{103}N_{29}O_{44}P_6$ ([M − H]$^-$) 2480, found 2486.

Compound 19. HPLC 0% B for 5 min, to 30% B in 55 min, t_R = 25.0 min; yield 15%; MALDI-TOF MS calcd for $C_{84}H_{97}N_{31}O_{42}P_6$ ([M − H]$^-$) 2398, found 2401.

Compounds 20 and 21. After coupling of Fmoc-β-alanine and removal of the Fmoc group, as described for **18**/**19**, *N*-Fmoc-glycine (3.0 mg, 100 μmol) was coupled with HATU, HOAt, and diisopropylethylamine following the protocol for **15**. Fmoc removal again followed the protocol for **18**/**19**. Coupling of trimethoxystilbene carboxylic acid or anthraquinone carboxylic acid was performed as described for **15**. Deprotection followed the protocol given under DNA Synthesis above.

Compound 20. HPLC 0% B for 5 min, to 30% B in 45 min, t_R = 35.9 min; yield 41%; MALDI-TOF MS calcd for $C_{92}H_{106}N_{30}O_{45}P_6$ ([M − H]$^-$) 2536, found 2532.

Compound 21. HPLC 0% B for 5 min, to 30% B in 45 min, t_R = 30.0 min; yield 30%; MALDI-TOF MS calcd for $C_{89}H_{96}N_{30}O_{44}P_6$ ([M − H]$^-$) 2475, found 2481.

Primer Extension. Template-directed extension reactions of oligonucleotides were carried out in 2.5 μL of an aqueous buffer solution at room temperature. The oligonucleotide template (**23** or **24**) plus one of the primers (**16**−**22**) were dissolved separately in water to give stock solutions (180 μM). An aliquot (0.5 μL) of each solution was added to 1 μL of a stock solution of the buffer containing HEPES (0.5 M), NaCl (1 M), and $MgCl_2$ (0.2 M) at pH 8.9 in a polypropylene reaction vessel. An aliquot (0.5 μL) of the stock solution of azabenzotriazole esters (**25a**−**t**) in water, with equal concentration for **25a**−**g** (18 mM) and 5-fold increased concentration for **25t** (90 mM), was added.[19] The final concentrations in the assay mixture were 3.6 mM for **25a**−**g**, 18 mM for **25t**, 200 mM for HEPES, 400 mM for NaCl, 80 mM for $MgCl_2$, 36 μM for the respective primer (**16**−**22**), and 36 μM for the template (**23** or **24**). Samples (0.4 μL) were drawn after 6, 30, 60, 120, and 360 min and 1 day and were diluted with water (15 μL). The resulting solution was kept over approximately 0.5 mg of Dowex cation-exchange resin (ammonium form) for 3 min. A sample (0.5 μL) of the supernatant was used for MALDI-TOF MS.

MALDI-TOF Analysis. Analysis was performed via MALDI-TOF mass spectrometry as previously described,[19] using conditions that allow for quantitative detection.[53] Briefly, aliquots of sample solution (0.5 μL) were spotted on a stainless steel MALDI target plate and treated with the matrix/comatrix mixture [0.4 μL, solution of 2,4,6-trihydroxyacetophenone (0.3 M in EtOH)] and diammonium citrate [0.1 M in H_2O, 2:1 (v/v)]. The resulting mixture was allowed to crystallize at room temperature and subjected to MALDI-TOF analysis. Three spectra were acquired per time point, using 300 shots per spot. Peak intensities were determined on the basis of peak heights. Fits to kinetic data were obtained as previously described.[19,20]

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Supporting Information Available: ^{1}H NMR spectra of stable new nucleosides, protocol for preparation of protected propargylamine, MALDI-TOF mass spectra of modified oligonucleotides, and additional kinetics for primer extension assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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