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A Versatile Modification of On-Column Oligodeoxynucleotides Using a Copper-Catalyzed Oxidative Acetylenic Coupling Reaction†

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Abstract: We report herein a versatile postsynthetic modification of on-column oligodeoxynucleotides (ODNs) using a copper-catalyzed oxidative acetylenic coupling reaction. Hexamers supported on resins via a methylamino-modified linker were prepared, and on-column modifications of ODNs were examined. ArgoPore resin proved to be the best choice for the modification, and introduction of functional molecules, such as anthraquinone, biotin, and fluorescein, resulted in good yields at not only the 5′-terminal but also the internal 3′-end of the ODNs. This method is applicable to the modification of 12mer ODN consisting of a random sequence. The resulting **ODN9** possessing fluorescein at its 5′-terminal acts as a non-RI primer for primer extension assays using the Klenow fragment.

Introduction

In recent years, synthetic oligodeoxynucleotides (ODNs) have become one of the most important molecular tools in scientific research. In addition to their role as antisense agents for therapeutic applications,[1] modified ODNs are now widely used in genomic studies as primers for DNA sequencing and polymerase chain reactions,[2,3] and in biotechnology as molecular beacons and probes for detection of gene expression in DNA microarrays.[4,5] However, to exploit these versatile techniques, site-specific modifications of ODNs with functional groups including fluorescent dyes and biotin are still needed.

The most straightforward method to introduce the functional groups into ODNs, the so-called presynthetic method, can be achieved by using an appropriate functionalized phosphoramidite through automated solid-phase synthesis.[6] It is reliable, and a variety of functionalized phosphoramidites are currently commercially available; however, these phosphoramidites are extremely expensive and sometimes difficult to prepare. In addition, the direct phosphoramidite approach cannot be applied if the functional group is unstable under the basic deprotection conditions in the solid-phase synthesis.

A convenient alternative, the so-called postsynthetic method, is currently being used.[7] With this method, once a convertible nucleoside unit is introduced into the ODN, the ODN can be modified with a variety of functional groups. Therefore, the postsynthetic method has been intensely investigated with the hope of developing a new strategy using various organic reactions. One typical approach is to react a primary amino group introduced into the ODN and a functional molecule possessing an isothiocyanate or an *N*-hydroxysuccinimidyl ester moiety.[8,9] However, because the coupling reaction is carried out with an unprotected ODN in aqueous solution, a large excess

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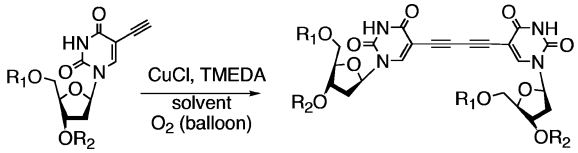
Table 1. The Copper-Catalyzed Oxidative Acetylenic Coupling Reaction of EdU Derivatives



1; R_1 = DMTr, R_2 = Lev
2; R_1, R_2 = H

3; R_1 = DMTr, R_2 = Lev
4; R_1, R_2 = H

entry	substrate	solvent	time (h)	product	yield (%)
1	**1**	DMF	24	**3**	78
2	**1**	CH_3CN	24	**3**	71
3	**2**	80% CH_3CN/H_2O	48	**4**	87
4	**2**	H_2O	72	**4**	68

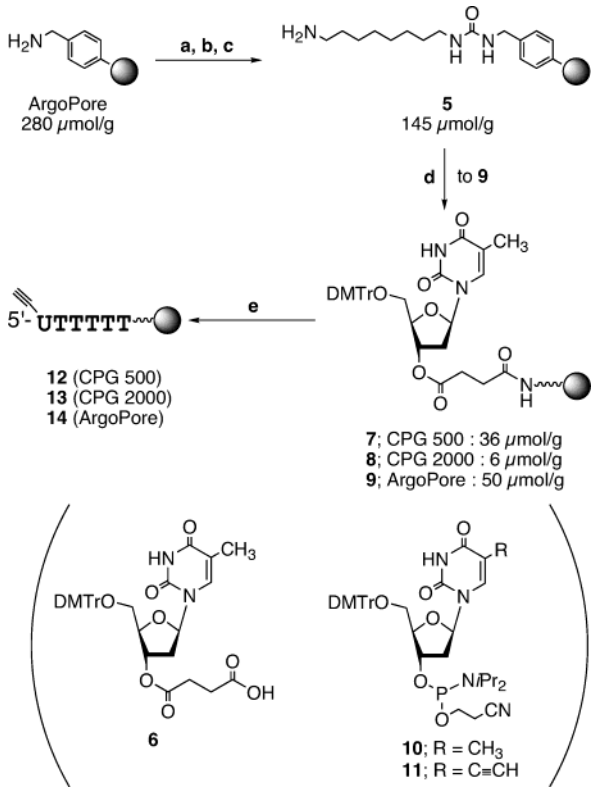
of the functional molecule is required and side reactions may take place with the exocyclic amino groups of the nucleobases. To overcome these problems, Greenberg et al. have developed a method for an efficient on-column modification using a photolabile phosphoramidite unit.[10] By employing a site-specific modification, their approach has the advantage of a facile purification of the resulting ODN; however, a photochemical apparatus is required. Grinstaff et al. reported a novel procedure that combines the advantages of on-column modification and Pd(0)-catalyzed cross-coupling reactions.[11] Although this method utilizes general Pd chemistry for an ODN modification, a rather unstable Pd(0) catalyst, $Pd(Ph_3P)_4$, is required. In addition, the coupling reaction proceeded well only when a convertible nucleoside (2′-deoxy-5-iodouridine, IdU) unit was located at the 5′-terminal of the on-column ODN.[11b] Consequently, development of a versatile and flexible procedure which is site-specific and high yielding to give modified ODNs would be highly desirable.[12]

Herein, we report a versatile modification of on-column ODNs using a copper-catalyzed oxidative acetylenic coupling reaction. To achieve the desired modification at various sites of the ODNs, we also examined resin-supported ODNs. As a result, we have found a suitable resin to introduce the functional molecules at not only the 5′-terminal but also at the internal 3′-end of on-column ODNs.

Results and Discussion

The copper-catalyzed oxidative acetylenic coupling reaction is a classical C−C bond forming reaction, one of the few which takes place under mild conditions in various solvents, including aqueous solution.[13] Therefore, the acetylenic coupling reaction was chosen for the site-specific modification of ODNs, polar macromolecules. Prior to using on-column ODNs, the reaction conditions were first examined on a nucleoside level (Table 1). When protected 5-ethynyl-2′-deoxyuridine (EdU) **1** was treated with 2 equiv of CuCl−TMEDA (*N*,*N*,*N*′,*N*′-tetramethylethylenediamine) complex in DMF under an O_2 atmosphere, the homo-coupling product **3** was obtained in 78% yield (entry 1). The reaction also proceeded in CH_3CN (entry 2). Although the reaction rate slowed, the acetylenic coupling reaction of EdU (**2**)[14] in aqueous solvent gave **4** in good yield (entries 3 and 4).

Scheme 1 [a]



[a] Reagents: (a) *p*-nitrophenylchloroformate, Et_3N, dioxane; (b) Ac_2O, pyridine; (c) 1,8-diaminooctane, dioxane; (d) **6**, EDC, DMF; (e) automated ODN synthesis.

Because the homo-coupling reaction proceeded well in the liquid phase, the reaction of the on-column ODN possessing an EdU unit with the EdU derivative to give the ODN with a diacetylene was examined. As mentioned in the Introduction, on-column modification by Pd(0)-catalyzed cross-coupling reactions proceeded well only when the IdU unit was located at the 5′-terminal of the ODN.[11b] This can probably be attributed to the physical properties of the resin-supported ODN, including its pore size. A 500 Å controlled pore glass (CPG500) resin is generally used for ODN synthesis, and the Pd(0)-catalyzed cross-coupling reaction was also carried out on this resin. Our thought was that this resin and its pore size are not suitable for on-column modification at the internal 3′-end of the on-column ODNs. On the basis of these considerations, we decided to employ 2000 Å controlled pore glass (CPG2000) and ArgoPore resins along with CPG500 for the on-column modification. Our proposed reaction was first examined with an on-column ODN with a convertible nucleoside, that is, the EdU unit, at the 5′-terminal. The synthesis of the resin-supported ODNs **12**−**14** is shown in Scheme 1. The reaction of 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-succinylthymidine (**6**) with commercially available CPG2000 resin (23 μmol/g) under standard conditions gave the solid support **8**. For the synthesis of support **9**, ArgoPore (280 μmol/

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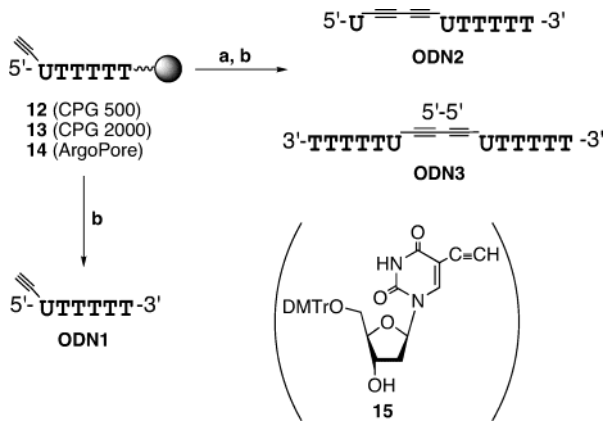
Scheme 2 [a]



[a] Reagents: (a) **15**, CuCl, TMEDA, DMF, O_2; (b) NH_4OH.

Table 2. The Copper-Catalyzed Oxidative Acetylenic Coupling Reaction of On-Column ODNs **12**−**14** with **15**

				yields (%)[a]		
entry	substrate	time (h)	temp	**ODN1**	**ODN2**	**ODN3**
1	**12**	24	room temp	60	38	2
2	**12**	48	room temp	51	43	6
3	**12**	24 × 2[b]	room temp	47	51	2
4	**13**	24 × 2[b]	room temp	28	72	trace
5	**14**	24 × 2[b]	room temp	35	58	6
6	**13**	24 × 2[b]	50 °C	2	90	8

[a] Yields were estimated from the ratio of peak areas. [b] The reaction was carried out for 24 h, and the resin was washed. Another cycle of the same coupling was then subjected for 24 h.

g) was first treated with *p*-nitrophenyl chloroformate in dioxane. After treatment with acetic anhydride to cap the remaining free amino groups, the resulting resin was reacted with 1,8-diaminooctane to give **5** (145 μmol/g), possessing an appropriate alkyl linker. Compound **6** was then coupled with **5** under the same conditions to give **9** (50 μmol/g). The resin-supported hexamers **12**−**14** containing the convertible unit at their 5′-termini were prepared on a DNA synthesizer using the phosphoramidites **10** and **11**.[15] The average coupling yield of the phosphoramidites was 99% using a 0.1 M solution of each phosphoramidite in CH_3CN and a coupling time of 300 s for **11** and 30 s for **10**, even when support **9** was used for the automated ODN synthesis.

With **12**−**14** in hand, on-column modification by the copper-catalyzed acetylenic coupling reaction was next examined. The reaction was conducted as follows: (1) 2 mg of the resin-supported hexamer was poured into a reactive tube for the solid-phase synthesis; (2) 6 mg of **15**[16] (large excess) and the CuCl−TMEDA complex in DMF (0.1 M, 500 μL) were added to the tube; (3) the mixture was shaken under an O_2 atmosphere; (4) the resin was washed with appropriate solvents; (5) the remaining resin was treated with NH_4OH to detach the ODNs from the resin; and (6) the resulting ODNs were analyzed by reversed-phase HPLC, and the yields were estimated from their peak areas (see Experimental Section). The results are summarized in Scheme 2, Table 2, and Figure 1. When **12** was treated with **15** for 24 h, the desired product **ODN2** was obtained in 38% yield along with 60% yield of **ODN1** (unreacted ODN)

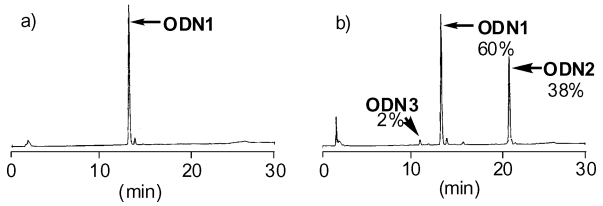


Figure 1. HPLC profiles of (a) **ODN1** (treatment of **12** with NH_4OH); (b) crude material after on-column reaction of **12** with **15** (entry 1).

and 2% yield of **ODN3**, a coupling product between on-column ODNs[17,18] (Table 2, entry 1 and Figure 1b). Although the chemical yield of the desired **ODN2** was slightly improved by increasing the reaction time (entry 2), **15** was nearly completely consumed after 48 h due to the formation of the homo-coupling product. Therefore, two cycles of the coupling reaction (procedures 2−4) were carried out, and these conditions gave **ODN2** in 51% yield (entry 3). With the reaction conditions fixed, the reaction of **13** and **14** was next examined. Both substrates gave **ODN2** in better yield than did **12** (entries 4 and 5), which suggests that the physical properties of the resins influence on-column modifications in copper-catalyzed acetylenic coupling reactions. Because **ODN1** was not completely consumed in every entry, the reaction was carried out at 50 °C. Consequently, the desired **ODN2** was obtained in 90% yield (entry 6), as estimated from peak areas in the HPLC analysis. However, the total peak areas of the HPLC under these conditions were approximately 40% less than those under conditions carried out at room temperature. Thus, cleavage of the succinyl linker supporting the ODN seemed to take place with heating. This is presumably due to a mechanism involving deprotonation of the amide nitrogen by TMEDA followed by intramolecular nucleophilic displacement at the ester carbonyl group.

In accordance with the results described above, a base-stable linker,[19] the methylamino-modified linker, was devised for the on-column modification. The requisite resins were prepared as shown in Scheme 3. According to the method reported by Brown et al.,[19a] the primary amino groups of CPGs were coupled with Boc-sarcosine,[20] followed by treatment with trifluoroacetic acid to give **16** and **17**, respectively. For the ArgoPore resin, **18** was prepared in a manner similar to that for **5**.[19b] The resulting resins were then coupled with **6** to give **19**−**21**, respectively. The resin-supported hexamers **24**−**26** were prepared on a DNA synthesizer in high average coupling yield. To examine the on-column modification at the internal 3′-end, the resin-supported hexamers **27** and **28** containing the convertible unit at their 3′-end were also prepared via the functionalized supports **22** and **23**, respectively. Prior to the reaction of the on-column ODN, the stability of the methylamino-modified linker under the reaction conditions was examined. When **19** was subjected to the acetylenic coupling conditions at 50 °C for 24 h in the absence of **15**, cleavage of the linker was not detected at all (data not shown).[21]

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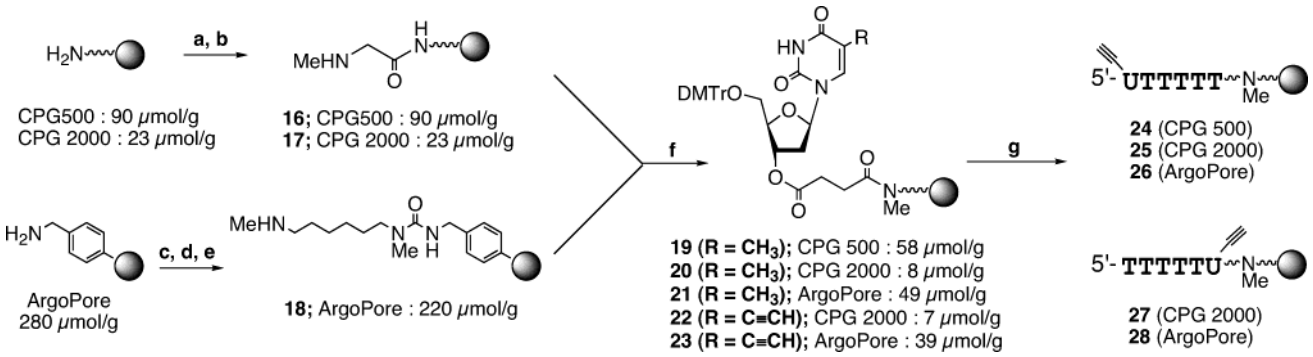
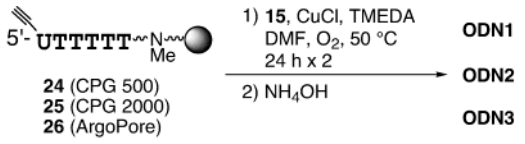
(16) Hurley, D. J.; Tor, Y. *J. Am. Chem. Soc.* **2002**, *124*, 3749−3762.
(17) When the reaction was carried out in the absence of **15**, **ODN3** was obtained in approximately 20% yield, which was analyzed by MALDI-TOF/MASS measurement.
(18) **ODN1**−**9** possess DMTr groups on their 5′-termini, except when the ODNs are used for MALDI-TOF/MASS measurements and primer extension assays.
(19) (a) Brown, T.; Pritchard, C. E.; Turner, G.; Salisbury, S. A. *J. Chem. Soc., Chem. Commun.* **1989**, 891−893. (b) Weiler, J.; Hoheisel, J. D. *Anal. Biochem.* **1990**, *189*, 218−227.
(20) In the original paper by Brown et al., FMOC-sarcosine was used.

Scheme 3 [a]



[a] Reagents: (a) Boc-sarcosine, EDC, CH_2Cl_2; (b) TFA, CH_2Cl_2; (c) *p*-nitrophenylchloroformate, Et_3N, dioxane; (d) Ac_2O, pyridine; (e) *N*,*N*′-dimethyl-1,6-diaminohexane; (f) **6** or 5-ethyl-5′-*O*-(4,4′-dimethoxytrytyl)-3′-*O*-succinyl-2′-deoxyuridine, EDC, DMF; (g) automated ODN synthesis.

Table 3. The Copper-Catalyzed Oxidative Acetylenic Coupling Reaction of On-Column ODNs **24**−**26** with **15**



		yields (%)[a]		
entry	substrate	**ODN1**	**ODN2**	**ODN3**
1	**24**	27	65	8
2	**25**	4	89	7
3	**26**	6	73	21

[a] Yields were estimated from the ratio of peak areas.

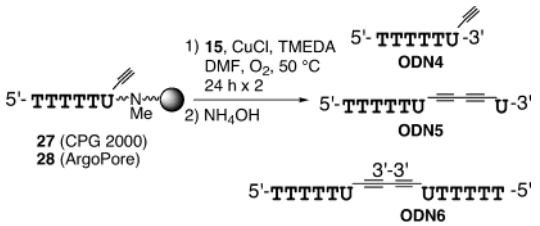
Because this linker tolerates the acetylenic coupling conditions, the reaction of on-column ODNs employing **24**−**26** was first examined (Table 3). When **24** was treated with **15** in the presence of the CuCl−TMEDA complex at 50 °C, the desired **ODN2** was obtained in 65% yield along with 27% of **ODN1** (entry 1). As we anticipated, when **25** was subjected to the coupling reaction under the same conditions, **ODN1** nearly completely disappeared, and the desired **ODN2** was obtained in much better yield (entry 2). Although a moderate yield of **ODN3** was obtained in the presence of a large excess of **15**, the reaction of **26** also resulted in a small amount of the remaining **ODN1** and a better yield of **ODN2** than that of **24** (entry 3).

The advantage of using CPG2000 and ArgoPore resins was clearly observed when the on-column acetylenic coupling reaction was examined at the internal 3′-end. The requisite reaction did not proceed when the hexamer on the CPG500 resin was subjected to the reaction,[22] and this result agreed well with that reported by Grinstaff et al.[11b] As can be seen in Table 4, when **27** and **28** were subjected to the reaction at 50 °C, **ODN4** (unreacted ODN) almost disappeared in both cases, and the desired **ODN5** was obtained in good yields (59% and 84%). Interestingly, contrary to the results using **25** and **26**, the reaction of **27** gave a moderate yield of **ODN6** (32%), a coupling product between two on-column ODNs, while that of **28** afforded **ODN5** in better yield (84%) with less **ODN6** being formed (16%).[18]

(21) When **7** was subjected to the acetylenic coupling reaction under the same conditions, the product of cleavage of the succinyl linker was detected in approximately 20% yield.

(22) Although we did not prepare a hexamer on CPG500 analogous to **27** (possessing a methylamino-modified linker), our preliminary attempt using a hexamer on CPG500 possessing a succinyl linker did not give the coupling product when the EdU unit was introduced at the internal 3′-end.

Table 4. The Copper-Catalyzed Oxidative Acetylenic Coupling Reaction of On-Column ODNs **27** and **28** with **15**



		yields (%)[a]		
entry	substrate	**ODN4**	**ODN5**	**ODN6**
1	**27**	9	59	32
2	**28**	trace	84	16

[a] Yields were estimated from the ratio of peak areas.

As mentioned above, we succeeded with the copper-catalyzed acetylenic coupling reaction of the on-column ODN at not only the 5′-terminal but also the internal 3′-end. This reaction was definitely affected by the kinds of resins supporting the ODN, which presumably depends on the physical properties of each resin.[23] A CPG resin is a silica-based support and is most common and suitable for efficient ODN synthesis. The typical property of this resin is nonswelling in organic solvents. Therefore, the accessibility of a reactant is not dependent on the swellability but probably on the pore size of the resin.[23] CPG500 is typically used and prefers a rather shorter ODN synthesis. The pore size of this resin tolerates the acetylenic coupling reaction when the reaction center is located at the 5′-terminal of the ODNs, whereas it is not suitable for the modification at the internal 3′-end located at the inner surface of the cavity because of poor accessibility of the reactant, such as **15**. This consideration is also supported by Grinstaff's results.[11b] In contrast, CPG2000 (a support preferring longer ODN synthesis) has a wider pore size, and thus the reactant can reach to the convertible unit even when it is located at the internal 3′-end. Unlike CPGs, ArgoPore is a highly cross-linked polystyrene resin, which was developed to facilitate the direct transfer of reaction conditions from known solution-phase methods to solid-phase methods. This resin offers the advantages of permitting rapid reaction kinetics and diffusion of reactants to the reaction center.[24] These properties would be convenient

(23) Bellon, L.; Wincott, F. In *Solid-Phase Synthesis. A Practical Guide*; Kates, S. A., Albericio, F., Eds.; Marcel Dekker Inc.: New York, 2000; pp 475−528 and references therein.

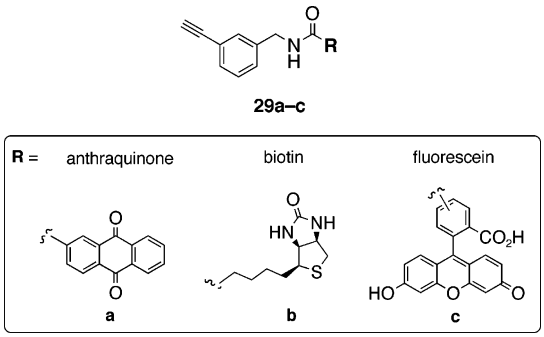
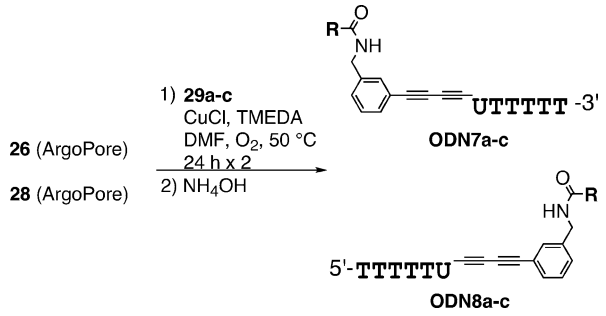


Figure 2. Structures of the functional molecules.

for modification of on-column ODNs, and the accessibility of the reactant should be the same either at the 5′-terminal or at the internal 3′-end of on-column ODNs. Although this consideration does not explain the product distribution of the reaction using **25** and **26** (the reaction at the 5′-terminal), and **27** and **28** (the reaction at the internal 3′-end), these physical and structural properties of the resins would partly explain the above-mentioned results. Thus far, although much effort has been dedicated to elucidating the effects of the resin on the synthesis of ODNs,[23] little is known about the effect of the resin on on-column modifications. To the best of our knowledge, only one example has been reported by De Napoli et al.,[25] in which an on-column synthesis of cyclic ODNs proceeded well when an ODN supported on Tentagel was used rather than the reaction of an ODN supported on CPG. Tentagel is a highly cross-linked polystyrene resin similar to ArgoPore, with a long poly(ethylene glycol) linker ($n = 70$).[23,26] Although this resin is known to be one of the most effective resins for ODN synthesis, our preliminary experiment using a Tentagel supported ODN resulted mainly in the formation of dimers such as **ODN3** (Scheme 2) and **ODN6** (Table 4) rather than the desired products **ODN2** and **ODN5** (data not shown). These results imply that the character of the linker and its length may also affect the chemical reactions of on-column ODNs, and ArgoPore possessing short alkyl linkers was better than Tentagel in our experiments.

To make this a versatile method, we next introduced functional molecules such as fluorescent dye into ODNs. Our results indicate that the CPG2000 resin also prefers ODN modification at the 5′-terminal end, while ArgoPore resin prefers the internal 3′-end. If the reaction between the interstrand ethynyl groups to form undesired **ODN3** could be avoided, ArgoPore would be better than CPG2000 for the ODN modification at either side because of its higher loadings. Therefore, we designed functional molecules possessing a more reactive ethynyl group, which would react readily with the convertible unit on the ODN and thus would diminish the formation of **ODN3**. To this end, the functional molecules **29a**−**c** were prepared (Figure 2).[27] When the on-column ODN **26** was subjected to the acetylenic coupling reaction in the presence of **29a** under optimized conditions, the **ODN7a** was obtained in 95% yield (Table 5, entry 1). As expected, formation of the undesired **ODN3** was negligible, and the HPLC trace after the reaction was quite simple without any purification (Figure 3a). Introduction of biotin and fluorescent dye using **29b** and **29c** also proceeded well to give **ODN7b** and **ODN7c** in good yields, respectively (entries 2 and 3). Reaction of **28** possessing the convertible unit at the internal 3′-end with **29a**−**c** gave **ODN8a**−**c** in good yields, respectively, equal to those of **26** (entries 4−6). As can be seen in Figure 3b, the HPLC trace was also unambiguous. Consequently, we succeeded in developing an efficient method to introduce functional molecules at arbitrary positions on on-column ODNs supported on ArgoPore.

Table 5. The Copper-Catalyzed Oxidative Acetylenic Coupling Reaction of On-Column ODNs **26** and **28** with Functional Molecules



entry	substrate	reactant	product	yields (%)[a]
1	**26**	**29a**	**ODN7a**	95
2	**26**	**29b**	**ODN7b**	91
3	**26**	**29c**	**ODN7c**	82
4	**28**	**29a**	**ODN8a**	91
5	**28**	**29b**	**ODN8b**	88
6	**28**	**29c**	**ODN8c**	76

[a] Yields were estimated from the ratio of peak areas.

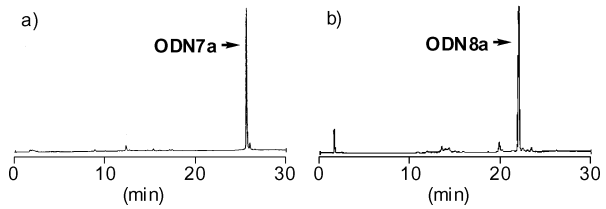
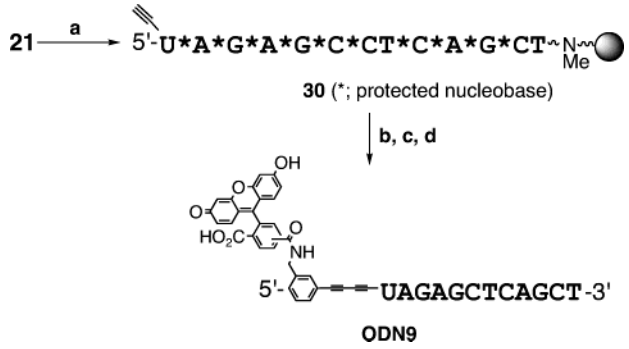


Figure 3. HPLC profiles of the crude material after on-column reaction of **26** (a) and **28** (b) with **29a**.

Our goal was to develop a versatile method for the introduction of functional molecules into ODNs which could be used as primers and probes. Although we were successful in introducing functional molecules such as fluorescent dyes into ODNs, our attempts resulted in only a simple and short sequence. In addition, the functional molecules are attached to ODNs with a rigid and somewhat shorter linker than usual, when the functional molecules are attached to ODNs via long alkyl chains. Therefore, we examined the modification of a 12mer ODN with a random sequence and evaluated the resulting ODN for a fluorescent dye labeled primer. Starting with **21**, a resin-supported 12mer **30** possessing the convertible unit on its 5′-terminal was prepared (Scheme 4). Treatment of **30** with **29c** in the presence of the CuCl−TMEDA complex in DMF for 48 h, followed by NH_4OH, gave **ODN9** possessing the fluorescent dye at its 5′-terminal in 83% yield regioselectively (estimated

(24) Resins and Reagent Catalog, Technical Handbook; Argonaut Technologies, Inc.; pp 144−148.
(25) (a) De Napoli, L.; Galeone, A.; Mayol, L.; Messere, A.; Piccialli, G.; Santacroce, C. *J. Chem. Soc., Perkin Trans. 1* **1993**, 747−749. (b) De Napoli, L.; Galeone, A.; Mayol, L.; Messere, A.; Montesarchio, D.; Piccialli, G. *Bioorg. Med. Chem.* **1995**, *3*, 1325−1329.
(26) Forns, P.; Fields, G. B. In *Solid-Phase Synthesis. A Practical Guide*; Kates, S. A., Albericio, F., Eds.; Marcel Dekker Inc.: New York, 2000; pp 1−77 and references therein.
(27) The synthesis of **29a**−**c** was presented in the Supporting Information.

Scheme 4 [a]



[a] Reagents: (a) automated ODN synthesis; (b) **29c**, CuCl, TMEDA, DMF, O_2, 50 °C; (c) NH_4OH, 55 °C; (d) 80% aq AcOH.

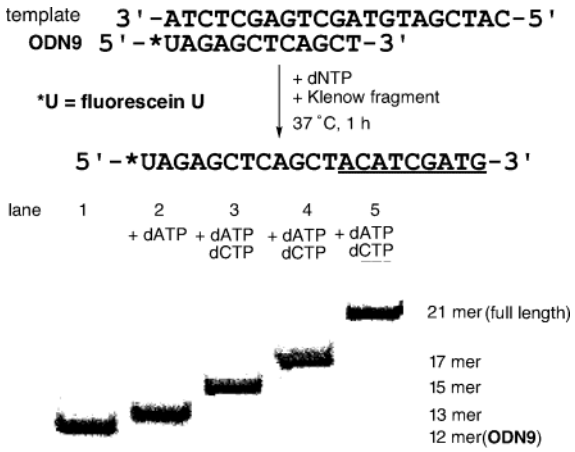


Figure 4. Primer extension assay using **ODN9**.

from HPLC analysis, Supporting Information). With **ODN9** in hand, a primer extension assay using the Klenow fragment (KF) was examined in the labeled **ODN9**−template duplex. The experiments were carried out at 37 °C for 1 h in the presence of varied mixtures of dNTPs. The primer extension reaction was analyzed by an Image Analyzer after denaturing polyacrylamide gel electrophoresis. As can be seen in Figure 4, the expected extension was observed in all conditions with 1.0 pmol of the primer **ODN9** (lanes 2−5). Consequently, it has been demonstrated that the fluorescent labeled **ODN9** acts as a non-RI primer.

In conclusion, we have demonstrated the on-column modifications of ODNs using a copper-catalyzed oxidative acetylenic coupling reaction. This reaction was obviously affected by the physical properties of the resin-supported ODN, and ArgoPore proved to be the best choice of the modification. The ODN, which includes the EdU unit, supported on ArgoPore reacts with a substrate possessing a terminal acetylene regioselectively at not only the 5′-terminal but also the internal 3′-end to give functionalized ODNs. Although the functional group is attached with an ODN through a rigid and somewhat short diacetylene linker, the fluorescent dye labeled ODN effectively acts as a non-RI primer. This method consists of (1) the readily available phosphoramidite derivative of EdU; (2) the usual automated ODN synthesis using ArgoPore resin; (3) preparation of functional molecules possessing a terminal acetylene; and (4) use of a stable CuCl complex. Our results have shown that the copper-catalyzed acetylenic coupling reaction is amenable to on-column modification of ODNs. This would be one of the most comprehensive modification methods of on-column ODNs because of its simple technique of organic and solid-phase synthesis. In addition, we would like to emphasize that our findings could be applicable to on-column modification of ODNs using other reaction systems.

Experimental Section

General Methods. Physical data were measured as follows. Melting points are uncorrected. ^1H and ^{13}C NMR spectra were recorded at 270 and 100 MHz instruments, respectively, in $CDCl_3$ or DMSO-d_6 as the solvent with tetramethylsilane as an internal standard. Chemical shifts are reported in parts per million (δ), and signals are expressed as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br (broad). Mass spectra were measured on a JEOL JMS-D300 spectrometer. TLC was done on Merck Kieselgel F254 precoated plates. Silica gel used for column chromatography was Merck silica gel 5715. CPG500 and CPG2000 resins were purchased from Glen Research. ArgoPore-NH_2 resin was purchased from Aldrich.

5-Ethynyl-5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-levulinyl-2′-deoxyuridine (1). To a solution of **15**[16] (1.70 g, 3.0 mmol) in CH_2Cl_2 (30 mL) were added levulinic acid (614 μL, 6.0 mmol), DMAP (730 mg, 6.0 mmol), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (1.4 g, 73 mmol), and the mixture was stirred for 1 h at room temperature. The reaction mixture was partitioned between AcOEt and H_2O, and the separated organic layer was washed with brine and dried (Na_2SO_4). The organic layer was concentrated in vacuo, and the residue was purified by a silica gel column, eluted with hexane/AcOEt (1:1−1:2) to give **1** (1.85 g, 94%) as a pale yellow foam: ^1H NMR (270 MHz, $CDCl_3$) δ 8.11 (br s, 1H), 7.42−7.21 (m, 10H), 6.86 and 6.83 (each s, each 2H), 6.30 (dd, 1H, J = 5.3 and 8.6 Hz), 5.02 (m, 1H), 4.17 (m, 1H), 3.79 (s, 6H), 3.49 (dd, 1H, J = 3.3 and 10.6 Hz), 3.33 (dd, 1H, J = 2.6 and 10.6 Hz), 2.86 (s, 1H), 2.77−2.56 (m, 5H), 2.44−2.34 (m, 1H), 2.20 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 205.95, 171.91, 160.72, 158.44, 148.74, 144.10, 143.30, 135.24, 134.95, 129.85, 127.95, 127.71, 126.89, 113.29, 113.26, 99.52, 87.31, 85.28, 84.53, 82.03, 77.20, 75.25, 73.77, 63.54, 55.29, 38.75, 37.88, 29.90, 28.04; FAB-LRMS (NaI) *m/z* 675 (MNa^+); FAB-HRMS (NaI) calcd for $C_{37}H_{36}N_2O_9Na$ (MNa^+) 675.2359; found 675.2339.

Bis[5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-levulinyl-2′-deoxyuridine-5-yl]diacetylene (3). A solution of CuCl (91 mg, 0.91 mmol) and TMEDA (140 μL, 0.91 mmol) in DMF (5 mL) was stirred for 30 min under O_2 atmosphere at room temperature. Compound **1** (300 mg, 0.46 mmol) was added to the solution, and the mixture was stirred for 24 h at room temperature. The reaction mixture was partitioned between AcOEt and H_2O, and the separated organic layer was washed with 5% EDTA aq, H_2O, and brine, and was dried (Na_2SO_4). The organic layer was concentrated in vacuo, and the residue was purified by a silica gel column, eluted with 0−5% MeOH in $CHCl_3$ to give **3** (230 mg, 78%) as a pale yellow foam: ^1H NMR (270 MHz, $CDCl_3$) δ 8.20 (br s, 2H), 8.00 (s, 2H), 7.38−7.15 (m, 18H), 6.86 and 6.83 (each s, each 4H), 6.22 (dd, 2H, J = 5.6 and 8.6 Hz), 5.46 (m, 2H), 4.20 (m, 2H), 3.74 and 3.73 (each s, each 6H), 3.53 (dd, 2H, J = 3.0 and 10.6 Hz), 3.27 (dd, 1H, J = 2.6 and 10.6 Hz), 2.79−2.56 (m, 10H), 2.40 (m, 1H), 2.20 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 206.16, 171.99, 160.53, 158.51, 148.95, 143.96, 135.20, 134.93, 129.88, 129.81, 128.02, 127.72, 127.07, 113.36, 99.27, 87.25, 86.03, 84.68, 78.28, 75.43, 73.13, 63.65, 55.25, 38.70, 37.82, 29.81, 27.97; FAB-LRMS *m/z* 1303 (MH^+); FAB-HRMS calcd for $C_{74}H_{71}N_4O_{18}$ (MH^+) 1303.4717; found 1303.4740.

Bis(2′-deoxyuridine-5-yl)diacetylene (4). A solution of CuCl (16 mg, 0.16 mmol) and TMEDA (24 μL, 0.16 mmol) in H_2O (1 mL) was stirred for 30 min under O_2 atmosphere at room temperature. Compound **2** (20 mg, 0.08 mmol) was added to the solution, and the mixture was further stirred for 72 h at room temperature. The reaction mixture was diluted with MeOH, and H_2S gas was bubbled through the mixture. The resulting precipitation was filtered, and the filtrate was concentrated in vacuo. The residue was purified by C_{18}-reversed phase column HPLC

and eluted with 5−50% MeOH in H_2O to give **4** (14 mg, 68%) as a pale yellow solid (crystallized from MeOH/H_2O): mp 242 °C (colored); ^{1}H NMR (270 MHz, DMSO-d_6) δ 11.72 (br s, 2H), 8.46 (s, 2H), 6.06 (dd, 1H, J = 6.6 and 5.9 Hz), 5.23 (d, 1H, J = 4.6 Hz), 5.17 (t, 1H, J = 4.6 Hz), 4.21 (m, 1H), 3.78 (m, 1H), 3.59 (m, 2H), 2.12 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.30, 148.97, 145.86, 96.54, 87.57, 85.09, 76.47, 75.39, 69.66, 60.64, 40.37; FAB-LRMS m/z 503 (MH^+). Anal. Calcd for $C_{22}H_{22}N_4O_{10}\cdot 0.5H_2O$: C, 51.67; H, 4.65; N, 10.77. Found: C, 51.42; H, 4.45; N, 10.65. UV (H_2O) λ_{max} 356.6 nm (ϵ 19 500), 333.6 nm (ϵ 28 900), 314.0 nm (ϵ 26 800), 238.8 nm (ϵ 25 900), 333.6 nm (ϵ 21 200), ϵ_{260} 17 900.

Preparation of ArgoPore Resin 5 Containing an Aminooctyl Linker. To a mixture of ArgoPore-NH_2 (1.00 g, 280 μmol/g), dioxane (10 mL), and Et_3N (1.0 mL) was added a solution of *p*-nitrophenylchloroformate (560 mg, 2.80 mmol) in dioxane (5 mL) at 0 °C, and the mixture was kept for 12 h at room temperature. The solid support was filtered and washed with CH_2Cl_2, and the remaining amino groups were capped by treatment with Ac_2O/pyridine (1:1, 10 mL) for 3 h. After the solid support was filtered and washed with CH_2Cl_2, a solution of 1,8-diaminooctane (288 mg, 2.0 mmol) in dioxane (10 mL) was added, and the mixture was kept for 48 h at room temperature. The resulting solid support **5** was filtered and washed with MeOH and CH_2Cl_2 and was dried under reduced pressure. The amount of the reactive amino group on the support was estimated by picrate assay[26,28] to be 145 μmol/g.

Preparation of Methylamino-Modified CPG 500 Resin 16. To a suspension of CPG500 (500 mg, 90.0 μmol/g) in CH_2Cl_2 (5 mL) were added Boc-sarcosine (34 mg, 0.18 mmol) and EDC (34 mg, 0.18 mmol), and the mixture was kept for 8 h at room temperature. The resin was filtered and washed with CH_2Cl_2, and the remaining amino groups were capped by treatment with Ac_2O/pyridine (1:1, 4 mL) for 3 h. After the resin was filtered and washed with CH_2Cl_2, the Boc group was removed by treatment with 50% TFA in CH_2Cl_2 for 1 h. The resulting resin **16** was filtered and washed with 5% ^{i}Pr$_2$NEt in CH_2Cl_2 (10 mL) and CH_2Cl_2 and was dried under reduced pressure. The amount of the methylamino group on the resin was estimated by picrate assay[26,28] to be 90.0 μmol/g.

Preparation of Methylamino-Modified CPG 2000 Resin 17. In the same manner as described for **16**, treatment of CPG 2000 (500 mg, 23 μmol/g) with Boc-sarcosine (9 mg, 46 μmol) gave **17** (23 μmol/g).

Preparation of Methylamino-Modified ArgoPore Resin 18. In the same manner as described for **5**, ArgoPore-NH_2 (1.00 g, 280 μmol/g) was treated with *p*-nitrophenylchloroformate (560 mg, 2.80 mmol), followed by *N*,*N*′-dimethyl-1,6-diaminohexane (500 μL, 2.8 mmol) to give **18** (220 μmol/g).

General Procedure of Attachment of Leader 3′-Nucleoside to Resins 8, 9, and 19−23. To a solution of **6** (34 mg, 53.5 μmol) in DMF (2 mL) were added EDC (10 mg, 53.5 μmol) and **16** (150 mg, 90 μmol/g), and the mixture was kept for 48 h at room temperature. The solid support was filtered and washed with pyridine. The remaining amino groups were capped by treatment with 0.1 M DMAP and 10% Ac_2O in pyridine. The resulting **19** was filtered and washed with EtOH and acetone and was dried under reduced pressure. The loading amount of the leader 3′-nucleoside unit was estimated by DMTr cation assay to be 58 μmol/g. In the same manner as described for **19**, resin-supported leader 3′-nucleoside units **8** (6 μmol/g), **9** (50 μmol/g), **20** (8 μmol/g), **21** (49 μmol/g), **22** (7 μmol/g), and **23** (39 μmol/g) were prepared.

Synthesis of ODNs.[18] Resin-supported ODNs **12**−**14**, **24**−**28**, and **30** were prepared on an Applied Biosystems 392 DNA/RNA synthesizer utilizing standard phosphoramidite chemistry at a 1.0 μmol scale. The phosphoramidite monomers were used at a concentration of 0.1 M in dry CH_3CN. The standard DNA synthesis cycle was used, except for the extended coupling time (300 s) of EdU phosphoramidite **11**. After completion of the synthesis of fully protected ODNs supported by resins, a small amount of the each resin (approximately 2 mg) was treated with concentrated NH_4OH (**12**−**14** and **24**−**28**, room temperature for 2 h; **30**, 55 °C for 12 h). After filtration of the resin, the filtrate was concentrated in vacuo to give **ODN1**, **ODN4**, and **ODN9** possessing a 5′-DMTr group, respectively. The resulting **ODN1**, **ODN4**, and **ODN9** were used as references for the subsequent on-column modification using the copper-catalyzed oxidative acetylenic coupling reaction. The remaining resin-supported ODNs were stored at −30 °C.

General Procedure of the Acetylenic Coupling Reaction Using Resin-Supported ODN. A solution of CuCl (5 mg, 50 μmol) and TMEDA (15 μL, 100 μmol) in DMF (500 μL) was stirred for 30 min under O_2 atmosphere at room temperature. To the resin-supported ODN (2 mg, containing 20−100 nmol of ODN) in a Valiant bond elute reservoir tube (5 mL, equipped with a polystyrene filter at the base) were added a reactant (**15**, **29a**, **29b**, or **29c**, 10 μmol) and the CuCl−TMEDA/DMF solution (500 μL), and the mixture was shaken under O_2 atmosphere at 50 °C for 24 h. The resin was washed with DMF (1 mL × 3), 5% EDTA aq (1 mL × 3), H_2O (1 mL × 3), and CH_3CN (1 mL × 3). The resulting resin was subjected to another cycle of the coupling reaction (24 h) and washed. The resin was then treated with concentrated NH_4OH (**12**−**14** and **24**−**28**, room temperature for 2 h; **30**, 55 °C for 12 h). After filtration of the resin, the filtrate was concentrated in vacuo, and the residue was dissolved in H_2O. This solution was analyzed by reversed-phase HPLC, using a J'sphere ODS-M80 column (4.6 × 150 mm, YMC) with a linear gradient of CH_3CN (from 23% to 41% over 20 min) in 0.1 M triethylammonium acetate (TEAA, pH 7.0), and the yields were estimated from peak areas of the HPLC chart.

Purification of ODNs for Measurement of MALDI-TOF/MASS and Primer Extension Assay.[18] The resulting ODNs after the acetylenic coupling reaction were purified by reversed-phase HPLC, using a J'sphere ODS-M80 column (10 × 150 mm, YMC) with a linear gradient of CH_3CN (from 23% to 41% over 20 min) in 0.1 M triethylammonium acetate (TEAA, pH 7.0). Fractions were concentrated, and the residue was treated with 80% AcOH aq for 20 min at room temperature. After successive coevaporation with H_2O, the residue was dissolved in H_2O and washed with AcOEt (three times). The water layer was concentrated in vacuo, and the residue was dissolved in H_2O and purified by reversed-phase HPLC using a J'sphere ODS-M80 column (10 × 150 mm, YMC) with a linear gradient of CH_3CN (**ODN1** and **ODN4**, from 23% to 41%; **ODN2**, **ODN3**, **ODN**5, and **ODN6**, from 7% to 20%; **ODN7a**−**c**, **ODN8a**−**c**, and **ODN9**, from 7% to 41% over 20 min) in 0.1 M triethylammonium acetate (TEAA, pH 7.0) to give the corresponding ODNs without 5′-DMTr groups.

MALDI-TOF/MASS Spectra of ODNs.[18] Spectra were obtained on a Voyager-DE pro (PerSeptive Biosystems), and the observed molecular weights supported their structure. **ODN2**: calculated mass, $C_{72}H_{86}N_{14}O_{45}P_5$ 2022.4 (M − H); observed mass, 2021.7. **ODN3**: calculated mass, $C_{122}H_{151}N_{24}O_{80}P_{10}$ 3543.4 (M − H); observed mass, 3544.4. **ODN5**: calculated mass, $C_{72}H_{86}N_{14}O_{45}P_5$ 2022.4 (M − H); observed mass, 2022.9. **ODN6**: calculated mass, $C_{122}H_{151}N_{24}O_{80}P_{10}$ 3543.4 (M − H); observed mass, 3542.6. **ODN7a**: calculated mass, $C_{85}H_{89}N_{13}O_{43}P_5$ 2135.6 (M − H); observed mass, 2136.8. **ODN7b**: calculated mass, $C_{80}H_{97}N_{15}O_{42}P_5S$ 2127.6 (M − H); observed mass, 2127.9. **ODN7c**: calculated mass, $C_{91}H_{93}N_{13}O_{46}P_5$ 2259.7 (M − H); observed mass, 2260.3. **ODN8a**: calculated mass, $C_{85}H_{89}N_{13}O_{43}P_5$ 2135.6 (M − H); observed mass, 2135.3. **ODN8b**: calculated mass, $C_{80}H_{97}N_{15}O_{42}P_5S$ 2127.6 (M − H); observed mass, 2127.6. **ODN8c**: calculated mass, $C_{91}H_{93}N_{13}O_{46}P_5$ 2259.7 (M − H); observed mass, 2261.8. **ODN9**: calculated mass, $C_{148}H_{162}N_{46}O_{76}P_{11}$ 4141.9 (M − H); observed mass, 4142.5.

Primer Extension Assay.[18] A solution of template ODN (2 pmol) and 5′-fluorescent primer **ODN9** (1 pmol) in H_2O (4 μL) was heated at 100 °C for 3 min and slowly cooled to room temperature. The primer

(28) Gisin, B. F. *Anal. Chim. Acta* **1972**, *58*, 248−249.

extension assay was carried out in a buffer containing 10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 7.5 mM dithiothreitol, and 1 unit of KF (Takara) in the presence of dNTP(s) (10 nmol each) and template ODN/**ODN9** complex at 37 °C for 1 h. The reaction was stopped by adding 40 μL of loading solution (90% formamide in tris-borate-EDTA), and the mixture was heated at 100 °C for 5 min. One-half of this mixture was analyzed by 20% PAGE including 7 M urea (16 × 20 × 0.1 cm, 500 V, 2 h), and the extension products were detected by a Fluorescent Image Analyzer FLA-2000 (FUJIFILM).

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Supporting Information Available: Synthetic procedures of **29a**−**c** and HPLC profiles of on-column modification of **30** with **29c** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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