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Site-Selective DNA Hydrolysis by Ce^{IV}–EDTA with the Use of One Oligonucleotide Additive Bearing Two Monophosphates

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Two deoxyuridine derivatives each bearing a monophosphate group at the 5-position with a C3 linker, were incorporated into an oligonucleotide. By using this modified oligonucleotide, a bulge was formed at a predetermined position in a DNA substrate, and two monophosphate groups were placed at both junctions of the bulge. Upon treatment of the mixture with Ce^{IV}–EDTA at pH 7.0, the phosphodiester linkages at the bulge site were selectively and efficiently hydrolyzed. The monophosphate groups introduced into the bulge site greatly accelerated site-selective DNA scission. Compared with the previously reported two-additive system, which combines two oligonucleotide additives each with a monophosphate at their termini, the present one-additive system is simpler and more convenient. Furthermore, site-selective DNA hydrolysis by using this one-additive system is successful even at high reaction temperatures (e.g., 55 °C). This reflects the thermodynamic stability of the duplexes formed between the substrate and the additive DNA.

Introduction

Artificial restriction nucleases are attracting interest mainly because their superior site-specificities could be beneficial for the manipulation of high-molecular-weight DNA molecules from animals and higher plants.[1–7] Much effort has been devoted to the development of catalysts that cleave DNA either through hydrolytic or oxidative pathways.[8,9] Among previously reported catalysts, the Ce^{IV} ion and its complexes are characterized by remarkable reactivity and their purely hydrolytic scission of DNA.[8] Site-selective DNA hydrolysis was achieved by attaching Ce^{IV} complexes to sequence-recognizing oligonucleotides.[10,11] For practical applications, however, the scission efficiency of this method was not satisfactorily high and its improvement has been desirable.

Recently, it was found that Ce^{IV}–EDTA (ethylenediamine-*N,N,N′,N′*-tetraacetate) complex hydrolyzes single-stranded DNA far more efficiently than double-stranded DNA.[12–16] Accordingly, this Ce^{IV} complex has been shown to preferentially hydrolyze gap-sites even when it is not covalently fixed to any sequence-recognizing moiety. By using two appropriate oligonucleotide additives, gap structures can be formed at any desired site in single-stranded DNA. These systems can therefore be used as potent new tools for future applications. Furthermore, this gap-selective hydrolysis was greatly accelerated by attaching monophosphate groups to the termini of oligonucleotide additives and then placing these groups at the gap site (the two-additive system shown in Figure 1 A).[17] EDTA groups can also be used instead of monophosphates.[18]

These results have indicated that still more useful tools for site-selective DNA scission could be obtained if oligonucleotides used in the two-additive system are covalently connected with each other (Figure 1 B). This paper reports the preparation of oligonucleotide additives that have two monophosphate groups in the middle and are well known for the one-additive strategy. By combining Ce^{IV}–EDTA with these oligonucleotide additives, DNA was selectively hydrolyzed at a predetermined site. Significantly, site-selective DNA scission with this one-additive system is successful even at high reaction temperatures. Under such conditions substrate–additive duplexes in the two-additive system mostly dissociate, and thus the site-selectivity of scission is poor. As expected, site-selective DNA scission with the one-additive system is successfully accomplished.

Results and Discussion

Incorporation of monophosphate groups into oligonucleotides for site-selective DNA scission with Ce^{IV}–EDTA

For site-selective DNA scission with the one-additive system, the target site in the substrate DNA must be single-stranded while the other parts should be kept double-stranded—the Ce^{IV}–EDTA complex preferentially hydrolyzes the single-stranded portion. Furthermore, two monophosphate groups should be placed near this site (Figure 1 B). In order to prepare oligonucleotide additives bearing two monophosphate groups at the desired sites, a new phosphoramidite monomer (**1**; Figure 1 C) was synthesized according to Scheme 1. The intermediate **3**, which was prepared from starting material **2** (5-

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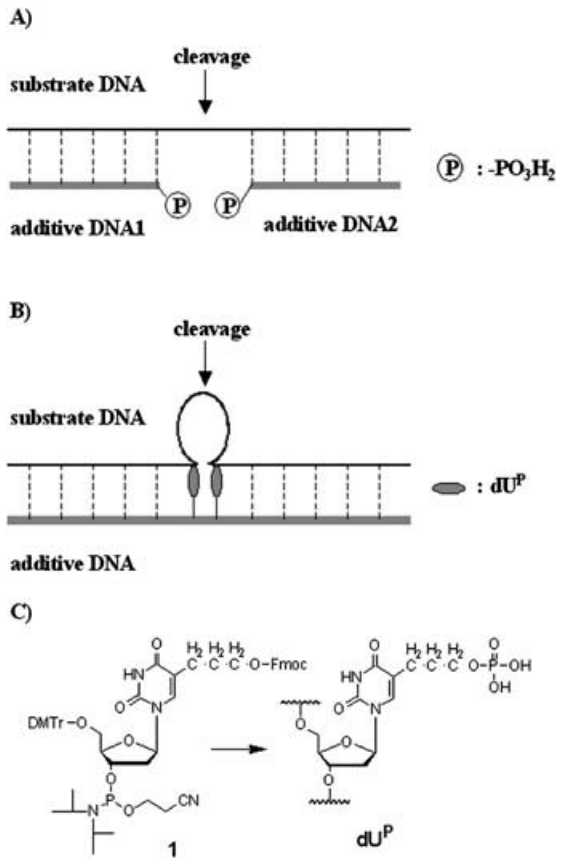


Figure 1. Schematic representation of A) two- and B) one-additive systems for site-selective DNA hydrolysis. C) Phosphoramidite monomer **1** and dU^P used in the one-additive system.

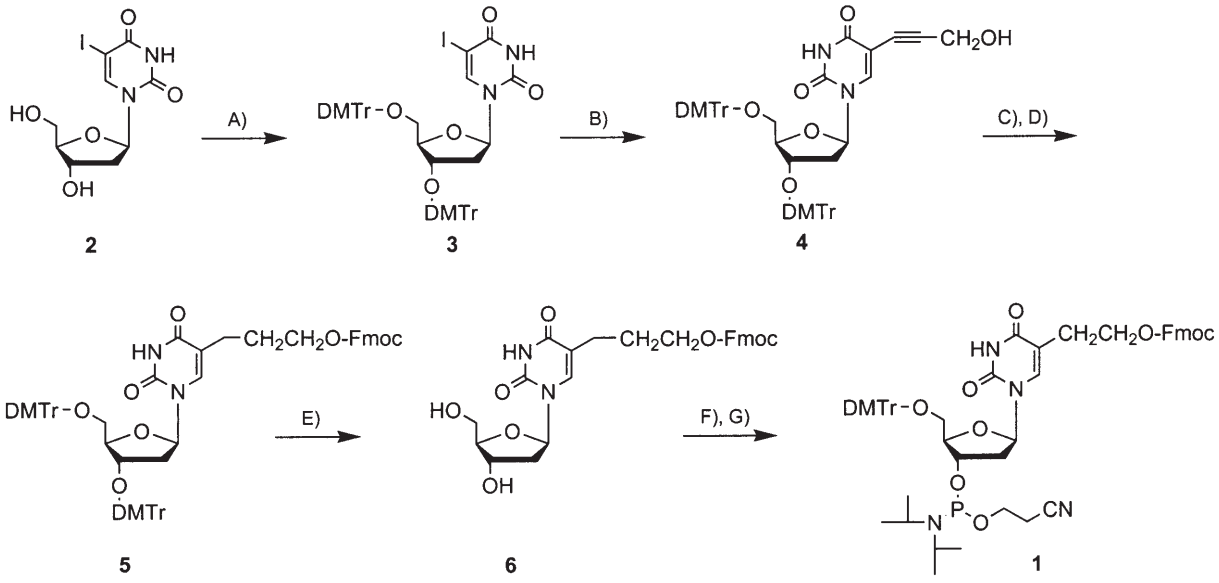
iodo-2′-deoxyuridine), was converted to 5-propynol derivative **4** by palladium-catalyzed coupling with prop-2-yn-1-ol.[19,20] Catalytic hydrogenation gave the 5-propanol derivative which was protected by a 9-fluorenylmethoxycarbonyl (Fmoc) group to afford compound **5**. The two dimethoxytrityl groups of **5** were removed with 2% trifluoroacetic acid (TFA) in dichloromethane. The 5′-hydroxy group of the resultant product **6** was selectively protected with a dimethoxytrityl group and then the free 3′-hydroxyl group was phosphitylated.

The new monomer **1** is a deoxyuridine derivative and has a hydroxypropyl residue at the C5 position (the hydroxyl group is protected by a Fmoc group). After DNA synthesis, the Fmoc group from **1** was deprotected and the resultant hydroxyl group was phosphorylated to give a dU^P residue at the desired site. The C3 linker that binds the monophosphate group to the C5 atom is flexible enough to allow this group to recruit Ce^{IV}–EDTA to the target phosphodiester linkages.[17]

Site-selective DNA hydrolysis with Ce^{IV}–EDTA in the presence of an oligonucleotide additive that bears two monophosphate groups (one-additive system)

The oligonucleotide additive, D40P2 (Figure 2), has dU^P residues at positions 20 and 21. When a 45-mer DNA substrate (S45) was combined with this additive, a five-base bulge was formed in the middle of S45 (T21–G25, underlined). Note that dU^P is a deoxyuridine derivative and forms a Watson–Crick base pair with A20 and A26 in S45. Thus, two monophosphate groups in these dU^P residues were placed at both junctions of the five-base bulge. The melting temperature (T_m) of the duplex between D40P2 and S45 is 64.8 °C, which is the same as the T_m of the duplex between unmodified oligonucleotide additive D40 and S45. Thus, the substitution of dT with dU^P did not interrupt duplex formation.

DNA hydrolysis was achieved simply by adding the Ce^{IV}–EDTA complex to aqueous solutions of DNA substrate S45 and oligonucleotide additive D40P2. In the left-hand side of the gel



Scheme 1. Synthetic route for phosphoramidite **1**. A) DMTr-Cl (3 equiv), pyridine, 24 h; B) prop-2-yn-1-ol, $(Ph_3P)_2PdCl_2$, CuI, Et_3N, DMF, 3 h; C) H_2, Pd/C, CH_3COOEt, overnight; D) Fmoc-Cl, pyridine, 3 h; E) 2% CF_3COOH in dichloromethane, 1 h; F) DMTr-Cl (1.2 equiv), pyridine, overnight; G) $(i\text{-}Pr_2N)_2POCH_2CH_2CN$, 1*H*-tetrazole, acetonitrile, 1 h.

substrate DNA

S40: 5′-CACTT AGAAT CAGGA ATGGA ACGCA GACTG TCGAC CTGAG-3′
S43: 5′-CACTT AGAAT CAGGA ATGGA **TTA** ACGCA GACTG TCGAC CTGAG-3′
S45: 5′-CACTT AGAAT CAGGA ATGGA **TTCAG** ACGCA GACTG TCGAC CTGAG-3′
S50: 5′-CACTT AGAAT CAGGA ATGGA **TTCAGCGTAC** ACGCA GACTG TCGAC CTGAG-3′
S45-N5: 5′-CAGTC AGGCA CCGTG TATGA **NNNNN** ACTCG AACGA TGCGC TCATC-3′

additive DNA

D40: 3′-GTGAA TCTTA GTCCT TACCT TGCGT CTGAC AGCTG GACTC-5′
D40P2: 3′-GTGAA TCTTA GTCCT TACC**dU^P dU^P**GCGT CTGAC AGCTG GACTC-5′
D40P2-B5: 3′-GTCAG TCCGT GGCAC ATAC**dU^P dU^P**GAGC TTGCT ACGCG AGTAG-5′
D40P1-R: 3′-GTGAA TCTTA GTCCT TACCT **dU^P**GCGT CTGAC AGCTG GACTC-5′
D40P1-L: 3′-GTGAA TCTTA GTCCT TACC**dU^P** TGCGT CTGAC AGCTG GACTC-5′
D20P-L: 3′-GTGAA TCTTA GTCCT TACC**dU^P**-5′
D20P-R: 3′-**dU^P**GCGT CTGAC AGCTG GACTC-5′

Figure 2. Sequences of substrate DNA molecules and oligonucleotide additives. The nucleotides that form bulge structures in substrate–additive duplexes are underlined. In S45-N5, N is C, T, A, or G.

in Figure 3A, the reaction temperature was at 55 °C. As shown in lane 4, substrate S45 was preferentially hydrolyzed in the bulge region (12% conversion). The scission process was analyzed in more detail with high-resolution gel electrophoresis (Figure 3B; the scission pattern obtained from this gel is presented in Figure 3C). The scission was strictly restricted to T21–G25, and most efficient in the middle of the bulge (3′-ends of T22 and C23). At 37 °C, scission with Ce^{IV}–EDTA in the presence of D40P2 was also sufficiently site-selective (Figure 3A, lane 11; 10% conversion).

For the purposes of comparison, S45 was treated with Ce^{IV}–EDTA in the presence of unmodified oligonucleotide additive D40 in which the two dU^P residues of D40P2 are replaced with conventional T. Here, a five-base bulge was also formed in the middle of S45 (T21–G25), but no monophosphate groups were present. DNA scission was found to be only marginal at both 55 °C (Figure 3A, lane 3) and 37 °C (lane 10). Notable promotion of site-selective DNA scission by the monophosphate groups of D40P2 is therefore evident. Additives bearing only one monophosphate group (D40P1-R and D40P1-L) were also more effective than D40, but they were less efficient than D40P2 (see Figure S1 in the Supporting Information). This is in accord with previous findings for site-selective DNA scission with the two-additive system.[17,18] As proposed in these latter studies, the monophosphate groups are thought to recruit the Ce^{IV} complex near the target site.

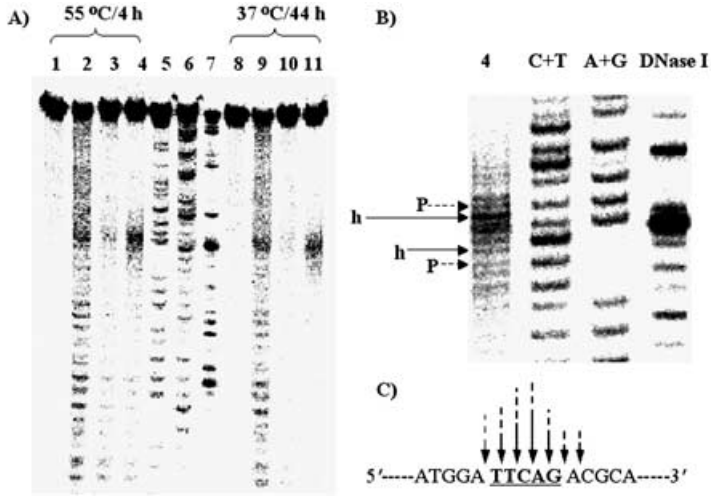


Figure 3. Gel-electrophoresis patterns for S45 hydrolysis (^{32}P-labeled at the 5′ end) with Ce^{IV}–EDTA in the presence of monophosphate-bearing oligonucleotide, D40P2. A) Lanes 1 and 8: controls without Ce^{IV}–EDTA; lanes 2 and 9: with Ce^{IV}–EDTA only; lanes 3 and 10: D40 (unmodified additive) with Ce^{IV}–EDTA; lanes 4 and 11: monophosphate-bearing D40P2 and Ce^{IV}–EDTA; lane 5: A+G Maxam–Gilbert sequencing; lane 6: C+T Maxam–Gilbert sequencing; lane 7: DNase I digestion. Reactions contained S45 (1.0 μM), D40P2 or D40 (2.0 μM), Ce^{IV}–EDTA (1 mM), and NaCl (200 mM) at pH 7.0 (7.5 mM HEPES buffer); 4 h at 55 °C for lanes 1–4; 44 h at 37 °C for lanes 8–11. B) High-resolution gel electrophoresis of the fragments from lane 4 in gel A) is presented parallel with Maxam–Gilbert sequencing lanes (3′-phosphate termini: P) and DNase I digests (3′-OH termini: h). C) Shows the scission efficiency. The solid parts of the arrows denote the formation of 3′-OH termini and the broken parts the formation of 3′-phosphate termini. 3′-OH termini were preferentially formed to 3′-phosphate termini.

In the presented site-selective DNA scission, two classes of fragments were formed. Fragments in one group co-migrated with the products of DNase I cleavage, whereas the second group co-migrated with the Maxam–Gilbert sequencing fragments (Figure 3B). Apparently, these two classes of scission products have 3′-OH termini (designated as h) and 3′-phosphate termini (designated as P), respectively; note that the DNA substrate was ^{32}P-labeled at the 5′ end. Relative frequencies of formation of these two types of termini for each of the scission fragments are represented in Figure 3C. For most fragments 3′-OH termini (shown by the solid parts of the arrows) were preferentially formed to 3′-phosphate termini (the broken parts). This manner of scission is consistent with previous results obtained with the two-additive system.[17] The completely hydrolytic nature of DNA scission by the Ce^{IV}–EDTA complex was substantiated by successful ligation of the products with DNA ligase.

Rapid site-selective DNA hydrolysis at high temperatures by using the one-additive system

One of the most significant advantages of the one-additive system presented here is that scission can be successfully achieved at a notable rate at high temperatures. The oligonucleotide additives used in this strategy are longer than those used in the two-additive system, and thus they form more stable duplexes with the substrate DNA molecules. Therefore, these duplexes should remain intact even at the high temperatures at which duplexes in the two-additive system would mostly dissociate.

As described above, the scission of S45 in the presence of D40P2 is satisfactorily selective even at

55 °C (see also Figure 4, lane 7). Furthermore, scission was strictly restricted to the bulge-site when three- (S43–D40P2) and ten-base bulges (S50–D40P2) were formed and the reaction was carried out at 55 °C (Figure 4, lanes 6 and 8). The melting temperatures of the duplexes in lanes 6, 7, and 8 were 65, 67, and 63 °C, respectively, and are sufficiently higher than the reaction temperature. Thus, these duplexes are stably formed in the reaction mixtures and the single-stranded portion is minimized. Otherwise, the single-stranded portion would have been promptly hydrolyzed by Ce^{IV}–EDTA without any site-selectivity. It is noteworthy that the site-selective DNA hydrolyses at 55 °C were more than tenfold faster than the corresponding reactions at 37 °C. Therefore, both high site-selectivity and rapid reactions could be satisfactorily accomplished with the presented one-additive system. This is an important feature for practical applications. When DNA scission was performed at 55 °C with the two-additive system, however, undesirable nonselective scission prevailed and site-selectivity was quite poor.

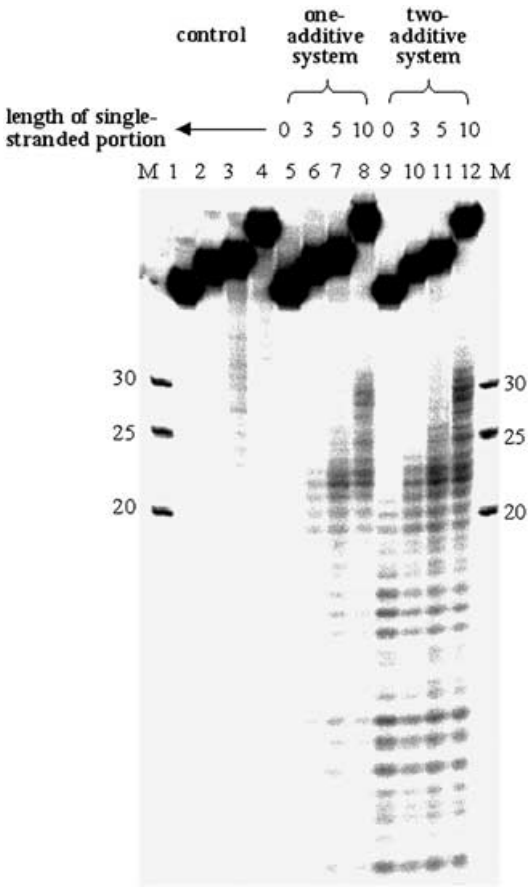


Figure 4. Site-selective DNA hydrolysis at 55 °C by using the one-additive system. Lanes 1–4: substrate DNA only; lane 5: fully matched S40–D40P2; lane 6: three-base bulge with S43–D40P2; lane 7: five-base bulge with S45–D40P2; lane 8: ten-base bulge with S50–D40P2. For the purposes of comparison results of scission with the two-additive system are also presented in lanes 9–12. Lane 9: nick obtained with S40–D20P-L/D20P-R; lane 10: three-base gap with S43–D20P-L/D20P-R; lane 11: five-base gap with S45–D20P-L/D20P-R; lane 12: ten-base gap with S50–D20P-L/D20P-R; M: 30-, 25-, and 20-mer markers. Reactions contained Ce^{IV}–EDTA (1 mM) and NaCl (100 mM) and were carried out at pH 7.0 and 55 °C for 4 h.

In lanes 10–12 in Figure 4, two 20-mer oligonucleotide additives (D20P-L and D20P-R) were combined with the corresponding DNA substrates, and gap structures were formed (three-, five-, and ten-base gaps, respectively). The monophosphates on the two oligonucleotide additives were placed near the single-stranded gaps (Figure 1 A). In addition to the bands resulting from scission in the gap site, others were observed especially at the bottom of the gel. Random scission was also evident in lane 9 where a nick structure was formed in the substrate DNA. As is apparent, the 5′ ends in the substrate DNA molecules which are complementary with D20P-L, were cleaved by Ce^{IV}–EDTA. Since the melting temperature of the duplex between D20P-L and the corresponding portion of the DNA substrates is only 54 °C, about half of the duplex is dissociated under the reaction conditions. These single-stranded portions are randomly hydrolyzed by Ce^{IV}–EDTA. Thus, only the 3′-side of the substrate DNA molecules is sufficiently protected from random scission (the T_m of the substrate duplex with D20P-R is 64 °C). Furthermore, the breathing motion of the substrate–additive duplexes is significant at higher temperatures. In order to minimize these factors and achieve DNA scission selectively, reactions with the two-additive system must be carried out at sufficiently low temperatures (e.g., 37 °C).[17] In this respect, the superiority of the one-additive system is evident.

Effects of size and base-sequence of the bulge portion on site-selective scission with the one-additive system

When the substrate DNA was completely complementary to the oligonucleotide additive (e.g., S40–D40P2), almost no scission occurred (Figure 4, lane 5). The single-stranded bulge region is crucial for the presented site-selective DNA hydrolysis. The ten- and five-base bulges were hydrolyzed 2.8- and 1.5-fold faster than the three-base bulge, respectively (lanes 6–8). Consistently, the efficiency of scission increased gradually with increasing bulge length from three to ten bases.

The DNA bases in the bulge sites were systematically varied by using a 45-mer substrate DNA (S45-N5, where N is C, T, A, or G) together with D40P2-B5 (Figure 2). Consequently, bulges involving five consecutive C, T, A, or G residues were formed, and two monophosphate groups were placed at these sites. All the bulges were efficiently hydrolyzed (Figure 5) and the relative scission efficiency was as follows: C5(2.7) > T5(1.3) > G5(1.2) > A5(1.0).

Conclusion

A deoxyuridine derivative with a monophosphate group that was connected to the 5-position with a C3 linker was prepared. By using its phosphoramidite monomer, two monophosphate groups were incorporated into desired positions in the middle of oligonucleotides. In duplexes between the oligonucleotide additive and substrate DNA a bulge was formed at specific positions in the substrate, and two monophosphate groups were placed at both junctions of the bulge. Upon treatment of this mixture with Ce^{IV}–EDTA at pH 7.0 and 55 °C, the substrate DNA

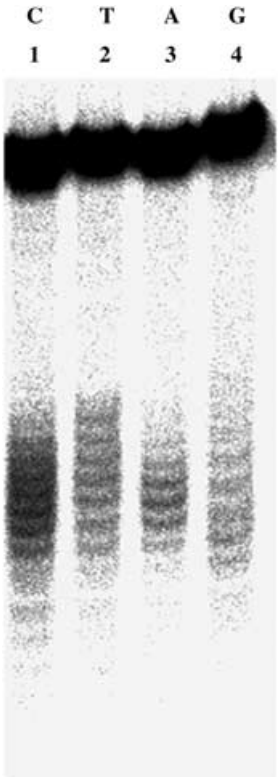


Figure 5. Site-selective hydrolyses with Ce^{IV}–EDTA at bulges formed by five consecutive C, T, A or G bases. Lane 1: C5 bulge with S45-C5–D40P2-B5; lane 2: T5 bulge with S45-T5–D40P2-B5; lane 3: A5 bulge with S45-A5–D40P2-B5; lane 4: G5 bulge with S45-G5–D40P2-B5. Reactions contained Ce^{IV}–EDTA (1 mM) and NaCl (200 mM) and were carried out at pH 7.0 and 55 °C for 4 h.

was selectively and efficiently hydrolyzed at the bulge sites. The main scission fragments had 3′-OH termini. Compared with the previously reported two-additive system, the present one-additive system is simpler and more convenient to use. Still more advantageously, DNA hydrolysis with this one-additive system was satisfactorily site-selective even at high reaction temperatures, mainly because of the large stability of the substrate–additive duplexes. Applications of these new tools to DNA manipulation are currently underway.

Experimental Section

Materials: The phosphoramidite monomer **1** was synthesized according to Scheme 1 and characterized as described in the Supporting Information. 5-Iodo-2′-deoxyuridine, prop-2-yn-1-ol, 4,4′-dimethoxytrityl chloride (DMTr-Cl), 9-fluorenylmethoxycarbonyl chloride (Fmoc-Cl), 1*H*-tetrazole, and 2-cyanoethyl *N,N,N′,N′*-tetraisopropylphosphordiamidite were obtained from Aldrich and used without further purification. Other phosphoramidite monomers were purchased form Glen Research Co.

Water was deionized with the MILLIPORE water purification system and sterilized in an autoclave immediately before use. The Ce^{IV}–EDTA complex was prepared by mixing equimolar amounts of $Ce(NH_4)_2(NO_3)_6$ (20 mM in water) and EDTA·4Na (20 mM in HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) buffer); the pH was adjusted to 7.0 with NaOH.

Synthesis of monophosphate-bearing oligonucleotides: All the oligonucleotides shown in Figure 2 were synthesized on an automated DNA synthesizer by using phosphoramidite **1** and conventional phosphoramidite monomers. First, the designed sequence was synthesized and the free 5′-OH group was capped with Ac_2O. Then the synthesis column was detached and the Fmoc group on **1** was removed by passing the deprotection solution (1 M 1,8-diazabicyclo[5.4.0]undec-7-ene in anhydrous acetonitrile) through the column over 5 min at RT. After extensive washing with acetonitrile (10 mL), the column was reinstalled onto the synthesizer and the hydroxypropyl group in the ologonucleotide was phosphorylated with chemical phosphorylation reagent II (CPRII, 0.2 M; Glen Research Co.). After the recommended workup, the products were purified by RP-HPLC.

The MALDI-TOF MS data of the modified oligonucleotides were in good agreement with the theoretical values. D40P2: 12471.1 (calcd = 12471.2); D40P1-R: 12344.3 (calcd = 12347.2); D40P1-L: 12344.3 (calcd = 12347.2); D20P-L: 6179.2 (calcd = 6179.1); D20P-R: 6229.3 (calcd = 6230.1); D40P2-B5: 12569.0 (calcd = 12570.3).

Site-selective DNA hydrolysis: The hydrolysis of DNA substrate (^{32}P-labeled at the 5′ end) was initiated by adding a solution of Ce^{IV}–EDTA complex to the reaction mixture. All reactions were carried out in HEPES buffer (7.5 mM, pH 7.0) at 55 °C, unless noted otherwise. The reaction mixtures consisted of DNA substrate (1.0 μM), each of the oligonucleotide additives (2.0 μM), and NaCl (200 mM). After a predetermined time, the reactions were stopped by adding EDTA disodium salt (10 mM) and inorganic phosphate (70 mM, $^1/_2$ of reaction mixture volume). The products were then analyzed with denaturing 20% polyacrylamide gel electrophoresis, and the scission fragments were quantified with a Fuji Film FLA-3000G imaging analyzer.

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