Prompt site-selective DNA hydrolysis by Ce(IV)–EDTA using oligonucleotide multiphosphonate conjugates

Tuomas Lönnberg,* Yuta Suzuki and Makoto Komiyama*

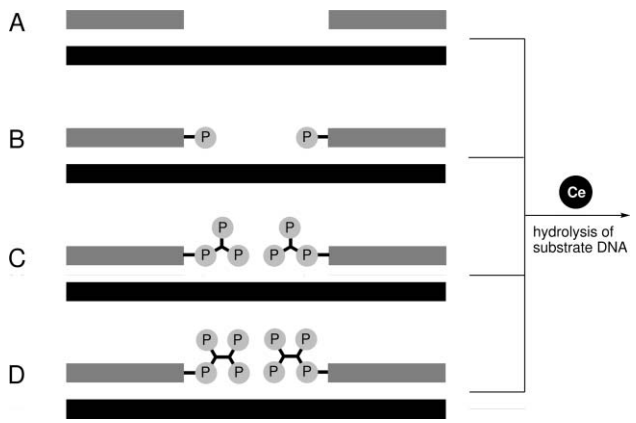
Received 7th May 2008, Accepted 17th June 2008
First published as an Advance Article on the web 16th July 2008
DOI: 10.1039/b807789d

Oligodeoxyribonucleotide multiphosphonate conjugates have been prepared by on-support oximation of aminooxy-functionalized oligonucleotides with 2-(4-formylphenoxy)ethyl esters of nitrilotris(methylenephosphonic acid) (NTP) and ethylenediaminetetrakis(methylenephosphonic acid) (EDTP). These conjugates, along with the corresponding oligonucleotides bearing hydroxy or monophosphate termini, were hybridized with a longer substrate DNA leaving a narrow single-stranded gap site in the substrate between the two additive oligonucleotides. Gap sites flanked by two of the multiphosphonate groups, in particular EDTP, were hydrolyzed by the Ce(IV)–EDTA complex significantly faster than the corresponding gap sites flanked by only hydroxy or monophosphate termini. Using the new oligonucleotide conjugates, efficient site-selective hydrolysis of the substrate DNA can be achieved at Ce(IV) concentrations where other single-stranded regions remain intact. At high Ce(IV) concentrations, the cleavage rate becomes independent on [Ce(IV)] and little improvement by the new multiphosphonate conjugates over oligonucleotides with monophosphate termini is observed, suggesting that the origin of the rate acceleration is the higher affinity of the NTP or EDTP ligands to Ce(IV) compared to hydroxy or monophosphate ligands.

Introduction

Natural restriction enzymes recognize a sequence of only 4 or 6 nucleotides, making them unsuitable for precise manipulation of large DNAs, *i.e.* longer than $4^6 = 4096$ base-pairs. For example, in human genome cleavage would occur at more than 700 000 sites. Artificial restriction enzymes capable of hydrolytic DNA scission with an enhanced site-selectivity are, hence, needed.[1] The most obvious strategy to achieve this is to covalently tether a catalytic group to an oligonucleotide which is complementary to the sequence next to the desired site of cleavage. Because of the great stability of DNA—the half-life for the hydrolysis of a DNA phosphodiester bond under neutral conditions at ambient temperature has been estimated to be 2×10^{14} years—this catalytic group has to be remarkably active.[2] Ce(IV) and its complexes have been shown to offer great catalytic potential, promoting DNA scission exclusively *via* a hydrolytic pathway.[3] Furthermore, the EDTA complex of Ce(IV) catalyzes the cleavage of single-stranded DNA markedly more than the cleavage of double-stranded DNA. This preference has been exploited to achieve site-selective DNA hydrolysis by using complementary additive oligonucleotides to introduce a single-stranded scissile gap site in the target DNA (Scheme 1A). Monophosphate termini flanking the gap site (Scheme 1B) have been reported to considerably enhance both the rate and the selectivity of the cleavage.[4]

Aminomethylphosphonate-type ligands, such as nitrilotris(methylenephosphonic acid) (NTP) or ethylenediaminetetrakis(methylenephosphonic acid) (EDTP), exhibit high affinity towards lanthanide ions,[5] so one might expect oligonucleotides bearing this kind of terminal groups to be particularly efficient in recruiting the catalytic Ce(IV) species to the gap site, resulting in more facile cleavage at lower Ce(IV) concentrations (Scheme 1C and D, respectively). Strong enough ligands should allow decrease of the Ce(IV) concentration employed down to a level where single-stranded DNA remains stable and only the gap site, where the local Ce(IV) concentration is high, would be cleaved.



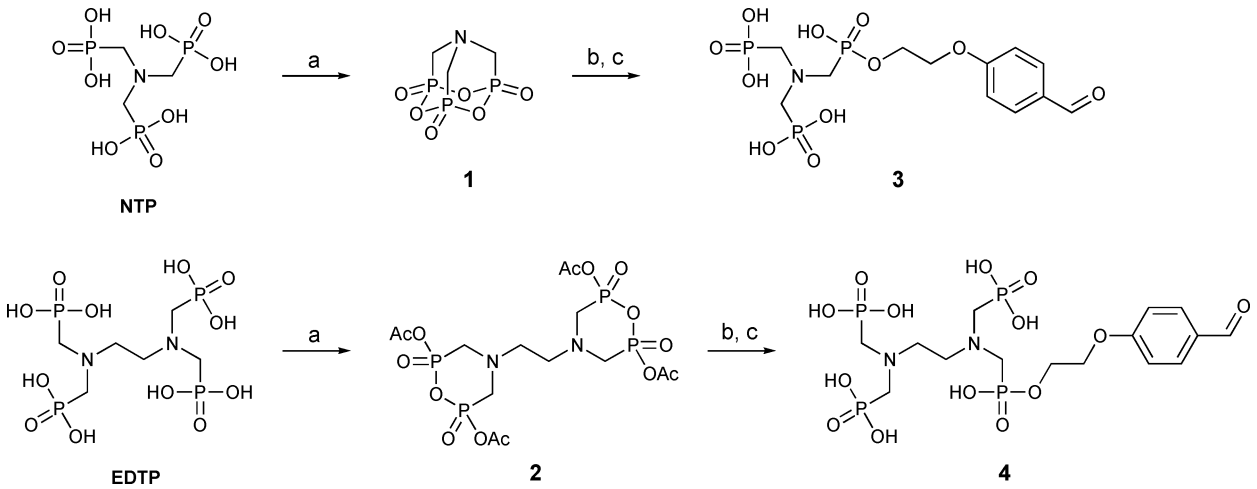
Scheme 1 Gap sites flanked by A) hydroxy termini, B) monophosphate termini, C) NTPs and D) EDTPs.

Results and discussion

Preparation of the multiphosphonate building blocks

For tethering to support bound oligonucleotides through an oxime bond, nitrilotris(methylenephosphonic acid) (NTP) and ethylenediaminetetrakis(methylenephosphonic acid) (EDTP) were converted to their 2-(4-formylphenoxy)ethyl esters (Scheme 2). First,

Research Center for Advanced Science and Technology, University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo, 153-8904, Japan. E-mail: komiyama@mkomi.rcast.u-tokyo.ac.jp; Fax: +81 3 5452 5209; Tel: +81 3 5452 5200



Scheme 2 Preparation of the multiphosphonate building blocks **3** and **4**. *Reagents and conditions*: a) acetic anhydride, DMF, b) 4-(2-hydroxyethoxy)-benzaldehyde, DMSO, c) water, DMSO.

NTP and EDTP were refluxed in a mixture of DMF and acetic anhydride to give the intramolecular phosphonic anhydride **1** and the mixed phosphonic–acetic anhydride **2**, respectively. Alcoholysis by 4-(2-hydroxyethoxy)benzaldehyde, followed by hydrolysis, then gave the desired building blocks **3** and **4**. The crude products were purified by semipreparative HPLC.

Preparation of the phthaloyl-protected aminooxy linkers

The phthaloyl-protected aminooxy group was introduced as *N*-hydroxyphthalimide as previously described, *i.e.* either by displacement of the bromides of 2-bromoethanol or 1,3-dibromopropan-2-ol to yield 2-phthalimidooxyethanol (**5**) or 1,3-diphthalimidooxypropan-2-ol (**6**), respectively, or under Mitsunobu conditions to dimethoxytrityl-protected tetra(ethylene glycol) (**7**) to yield *N*-[11-(4,4′-dimethoxytrityloxy)-3,6,9-trioxaundecanyloxy]phthalimide (**8**) (Scheme 3).[6] Conventional procedures were used for the dimethoxytrityl deprotection to yield 11-phthalimidooxy-3,6,9-trioxaundecanol (**9**) and the conversion of **5**, **6** and **9** to the phosphoramidite building blocks **10**, **11** and **12**.

Preparation of the oligonucleotide multiphosphonate conjugates

On-support oxime coupling has been previously described.[6] Compared to a primary amino group, the aminooxy group is more nucleophilic towards carbonyl compounds and the oxime product is much more stable than the imine.[7] Furthermore, oximation can be carried out in aqueous solution, making it a particularly attractive reaction for the coupling of oligonucleotides with the highly hygroscopic NTP and EDTP ligands that are essentially insoluble in organic solvents.

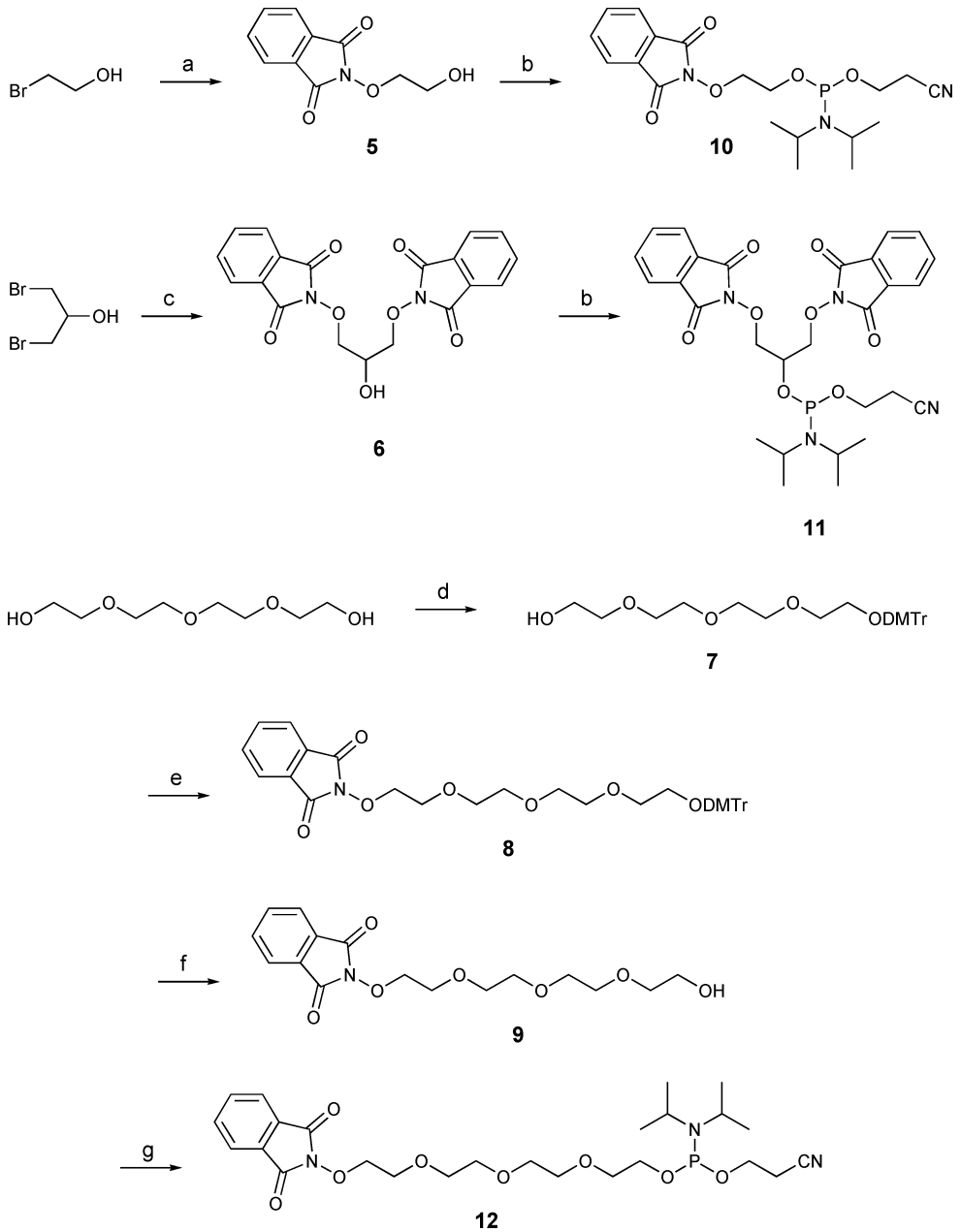
The oligodeoxyribonucleotides were assembled on a synthesizer by the conventional phosphoramidite method, except that a prolonged coupling time (600 s) was used for the protected aminooxy building blocks **10**, **11** and **12**. Selective removal of the phthaloyl protection was accomplished by treating the support-bound oligonucleotides with 0.5 M hydrazine acetate in pyridine, as previously described (Scheme 4).[6] Coupling with the phosphonate building blocks was carried out by shaking the supports in a 0.1 M aq. solution of **3** or **4** for 16 h at room temperature. The supports were then washed with water, after which conventional ammonolysis was carried out to release the oligonucleotide conjugates.

Cleavage of single-stranded DNA substrates

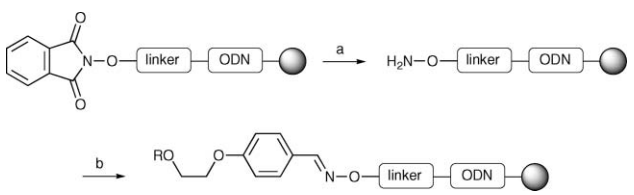
The substrate and additive oligonucleotides used in the study are summarized in Fig. 1. The NTP and EDTP building blocks **3** and **4** were attached to either the 3′- or the 5′-end of the additive oligonucleotides through either an ethylene glycol-, a tetra(ethylene glycol)- or a glycerol-type linker (building blocks **10**, **12** and **11**, respectively), whereas no linker was used between the monophosphate and the 3′- or 5′-terminal nucleotide. By using these additives, 5-nucleotide gaps flanked by various combinations of hydroxy, monophosphate, NTP and EDTP groups were formed in the 5′-FAM-labelled substrate DNAs.

Various assemblies of the 45-mer target **DNA45S** were incubated with 50 μM Ce(IV)–EDTA at pH 7.0 and 50 °C for 72 h (Fig. 2A). Under these conditions, no cleavage can be detected within the gaps flanked by hydroxy or monophosphate termini (lanes 1 and 2, respectively). Gaps flanked by only a single NTP or EDTP are hardly cleaved either (lanes 3, 4, 6 and 7), whereas efficient hydrolysis is observed in the gaps flanked by two of the new ligands (lanes 5 and 8). It should be pointed out that the gap flanked by two monophosphates (lane 2) represents the best system for cleavage of single-stranded DNA reported before this study.[4] Enlargement of the parts of lanes 8 (the EDTP–EDTP system) and M corresponding to the gap site is presented in Fig. 2B, together with a bar graphic showing the relative scission efficiencies at each individual phosphodiester linkage. As previously reported for the gaps flanked by monophosphates,[4] the most facile hydrolysis is observed in the middle of the gap.

The previously described system with two flanking monophosphates depends on hybridization of the whole target DNA, except for the gap site, for selectivity—otherwise, the remaining single-stranded portion is simultaneously hydrolyzed.[8] To test whether the present system could remove this limitation, *i.e.* whether gap-selectivity could be retained in the presence of other



Scheme 3 Preparation of the phthaloyl-protected aminooxy linkers. *Reagents and conditions*: a) *N*-hydroxyphthalimide, K_2CO_3, DMSO, b) 2-cyanoethyl diisopropylchlorophosphoramidite, DIPEA, MeCN, c) *N*-hydroxyphthalimide, sodium acetate, DMF, d) DMTrCl, 1,4-dioxane, e) *N*-hydroxyphthalimide, Ph_3P, DEAD, THF, f) acetic acid, water, g) 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite, 1*H*-tetrazole, MeCN.



Scheme 4 Coupling of the support-bound aminooxy-functionalized oligonucleotides with the phosphonate building blocks **3** and **4**. *Reagents and conditions*: a) hydrazine, acetic acid, pyridine, b) **3** or **4**, water.

single-stranded regions, a 5-base gap was formed in the middle of the 85mer-target **DNA85S** (Fig. 3). In addition to the 5-base gap site at T41–G45 and the 20-base double-stranded regions resulting from hybridization with the additive oligonucleotides, 20-base single-stranded overhangs remain in both ends of the target (G1–T20 and A66–A85). At pH 7.0 and 50 °C, phosphodiester bonds within these overhang regions are stable against hydrolysis by 50 μM Ce(IV)–EDTA (Fig. 3). In striking contrast, efficient and selective scission takes place in the gap sites flanked by two NTPs or EDTPs (lanes 5 and 8, 11% and 18% conversion, respectively). With two monophosphates, only modest scission is achieved (lane 2), whereas essentially no cleavage is observed with the other assemblies under the conditions used. Comparison with the markers (lane M) confirms that the scission is restricted

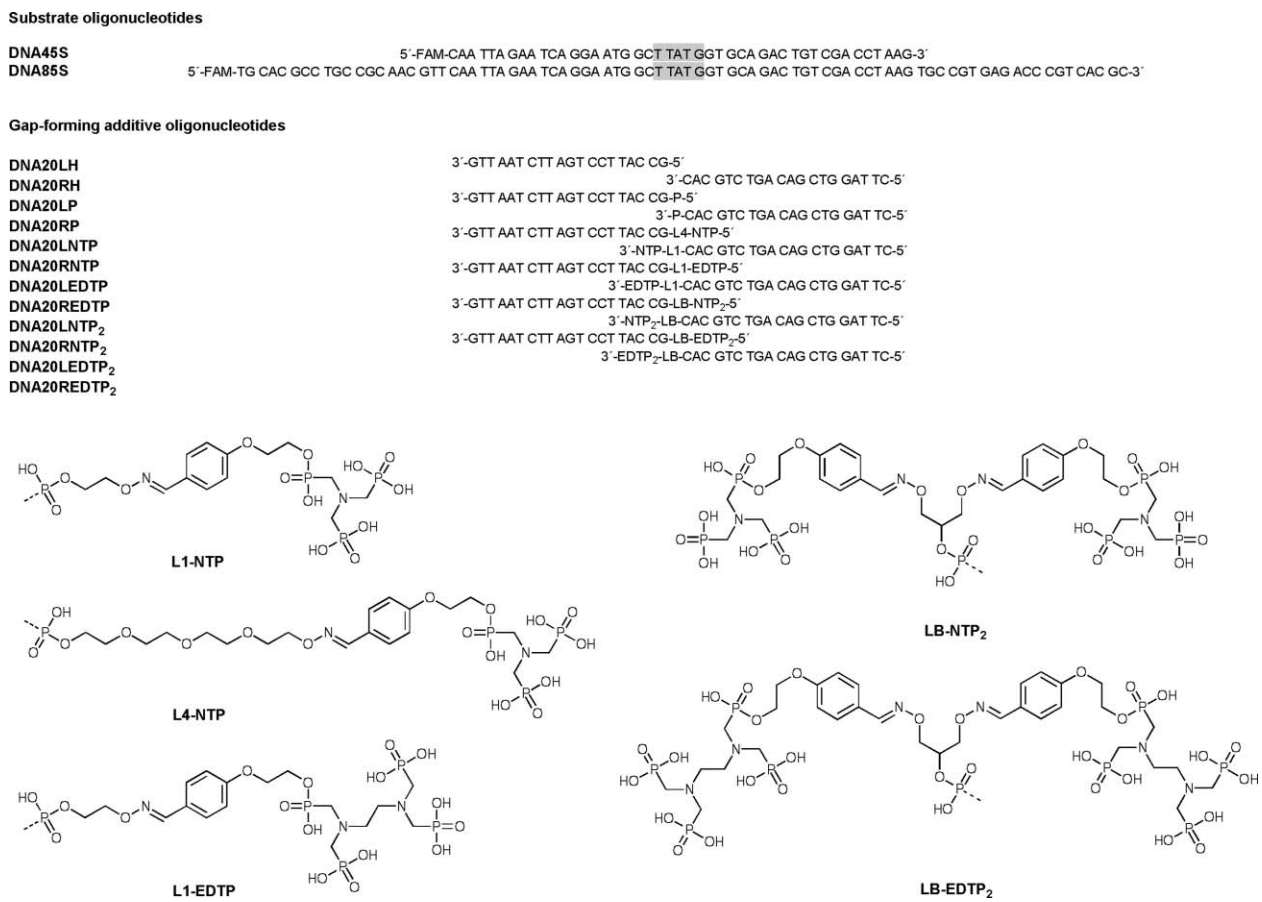


Fig. 1 The substrate and additive oligonucleotides used in the present study. In the substrate sequences, the nucleotides forming the gap site are shaded.

to the gap site. It should be pointed out that with a completely exposed substrate (lane P), no scission can be detected at the desired site. Furthermore, the non-specific cleavage observed with the exposed target is suppressed by the NTP- and EDTP-bearing oligonucleotides. Clearly, these new conjugates play an active role in the reaction, rather than merely preventing undesired cleavage by hybridizing with the target.

Comparison between lane 2 and 3, 4, 6 or 7 in Fig. 3 suggests that two ligands are needed for efficient scission—the gap flanked by two monophosphates is cleaved faster than the gaps flanked by only one of the superior NTP or EDTP ligands. To further elaborate on this point, oligonucleotides bearing two terminal NTPs or EDTPs were used to form gaps flanked from one or both sides by two of the new ligands and the rates of hydrolysis of these gaps and those flanked from both sides by only one ligand were compared (Fig. 4). In all cases, the scission is strictly restricted to the gap site. The gap flanked by four NTPs is cleaved significantly faster than the one flanked by two NTPs (lanes 4 and 1, 18% and 6% conversion, respectively), whereas no such acceleration can be observed with EDTP (lanes 8 and 5, 12% and 14% conversion, respectively). Furthermore, only modest cleavage is observed in the gaps flanked from one side by two NTPs or EDTPs (lanes 2, 3, 6 and 7), suggesting that for efficient hydrolysis, the two ligands have to be located on opposing sides of the gap.

Dependence of the extent of cleavage on Ce(IV) concentration

To shed light on the origin of the observed rate acceleration by the flanking NTP and EDTP groups, dependence of the extent of hydrolysis at the 5-base gap site in the 45mer target **DNA45S** on Ce(IV) concentration was studied over a range from 0 to 500 μM. Conversions of the target to the products of site-selective cleavage by flanking monophosphates, NTPs and EDTPs after 20 h are presented in Fig. 5. With the NTP and EDTP systems, saturation of the conversion at high Ce(IV) concentrations is observed. In all cases, dependence of the conversion on [Ce(IV)] may be expressed by eqn (1).

$$(c/100)\% = 1 - e^{-\frac{k_{max}[Ce]^n t}{K_d^n + [Ce]^n}} \quad (1)$$

K_d is the dissociation constant for the complex between the catalytic Ce(IV) species, the substrate DNA and the EDTP, NTP or monophosphate termini of the additive oligonucleotides. For this reactive complex, n is the number of Ce(IV) ions and k_{max} is the rate constant for the hydrolysis of the target DNA. The reaction time t = 72 000 s. The results obtained by fitting the experimental data to eqn (1) by a nonlinear least-squares method are presented in Table 1. For NTP and EDTP, fitting to eqn (1) suggests participation of two Ce(IV) ions in the catalysis. The need for two ligands to achieve efficient scission may also be interpreted

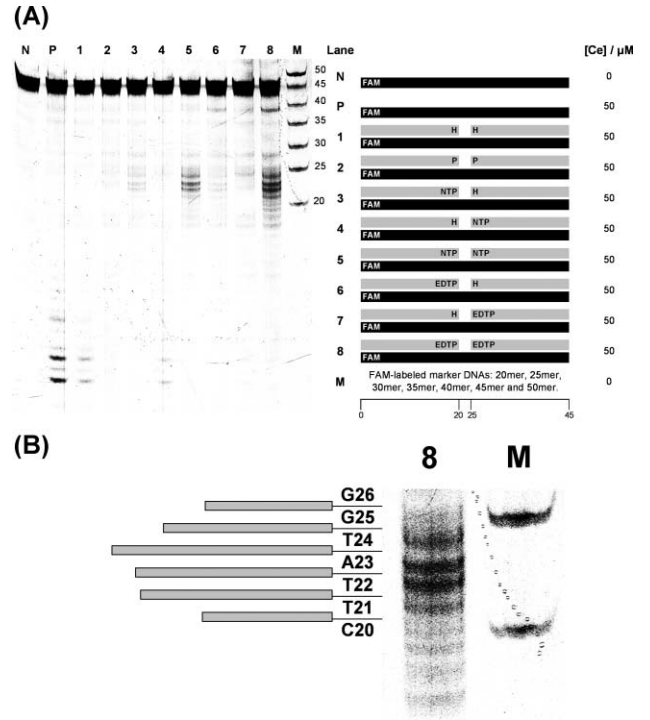


Fig. 2 (A) Polyacrylamide gel electrophoresis patterns for the hydrolysis of the 5′-FAM-labelled DNA target **DNA45S** at a 5-base gap by Ce^{IV}–EDTA complex. $t = 72$ h, pH = 7.0 (7.5 mM HEPES), $T = 50$ °C, [NaCl] = 100 mM, [target] = 1 μM, [additives] = 2 μM. (B) Enlargement of the parts of lanes 8 and M corresponding to the gap site. The bars represent the relative scission efficiencies at each individual phosphodiester linkage.

as evidence for a two metal mechanism, previously reported for various other metal ions.[9] At high Ce(IV) concentration, little difference in cleavage efficiency is observed between EDTP-, NTP- and monophosphate-flanked gaps. However, NTP and EDTP exhibit a greater affinity to Ce(IV) than monophosphate, making the NTP– and, especially, EDTP–oligonucleotide conjugates superior cleaving agents at low Ce(IV) concentration. Accordingly, site-selective scission is achievable by these conjugates even when other parts of the substrate remain single stranded.

Experimental

General

Nucleoside phosphoramidite monomers were purchased from Glen Research Co. The oligonucleotide multiphosphonate conjugates were prepared on an automated synthesizer, purified by the conventional methods, and characterized by MALDI-TOF MS. The substrate and marker oligonucleotides were commercial products of Sigma Genosys and were purified by the conventional methods before use. Water was deionized by a Millipore water purification system and sterilized by an autoclave immediately before use. Commercially obtainable $Ce(NH_4)_2(NO_3)_6$ (from NACALAI TESQUE) and EDTA4Na (from Tokyo Kasei Kogyo) were used without further purification. Homogeneous Ce(IV)–EDTA complex was prepared immediately before use by mixing equimolar amounts of $Ce(NH_4)_2(NO_3)_6$ and EDTA (4Na salt) in HEPES buffer, as previously described.[10]

The hydrolysis of the 5′-FAM-labeled DNA substrate was initiated by adding a solution of Ce(IV)–EDTA complex to the reaction mixtures and was carried out at pH 7.0 (5.0 mM Hepes buffer) and 50 °C unless noted otherwise; [target DNA] = 1.0 μM, [additive oligonucleotides] = 2.0 μM and [NaCl] = 100 mM. After a predetermined time, the reactions were stopped by adding EDTP to a final concentration of 1.5 mM. The reaction mixtures were then analyzed by denaturing 20% polyacrylamide gel electrophoresis, and the scission fragments were quantified with a Fuji Film FLA-3000G imaging analyzer.

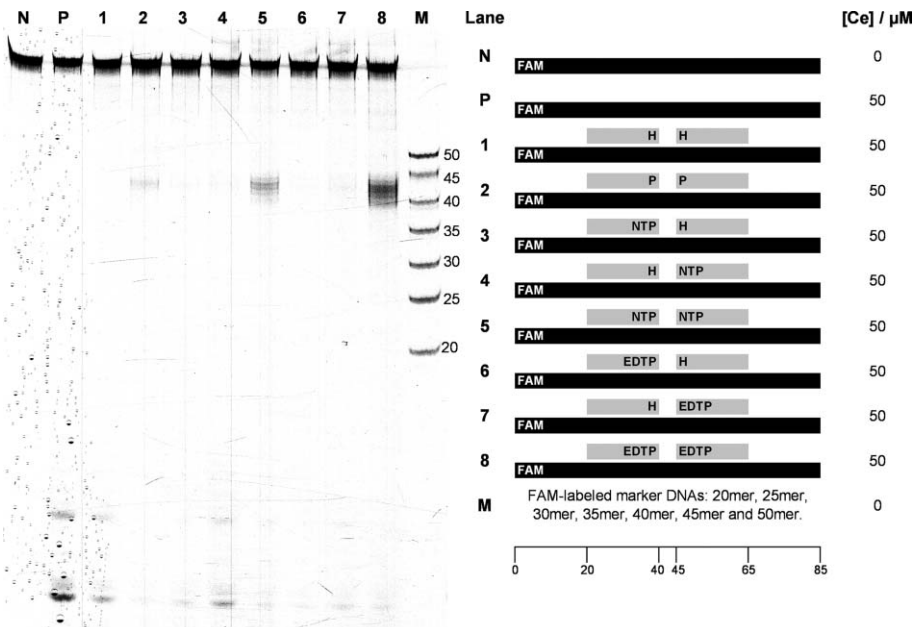


Fig. 3 Polyacrylamide gel electrophoresis patterns for the hydrolysis of the 5′-FAM-labelled DNA target **DNA85S** at a 5-base gap by Ce^{IV}–EDTA complex. $t = 114$ h, pH = 7.0 (7.5 mM HEPES), $T = 50$ °C, [NaCl] = 100 mM, [target] = 1 μM, [additives] = 2 μM.

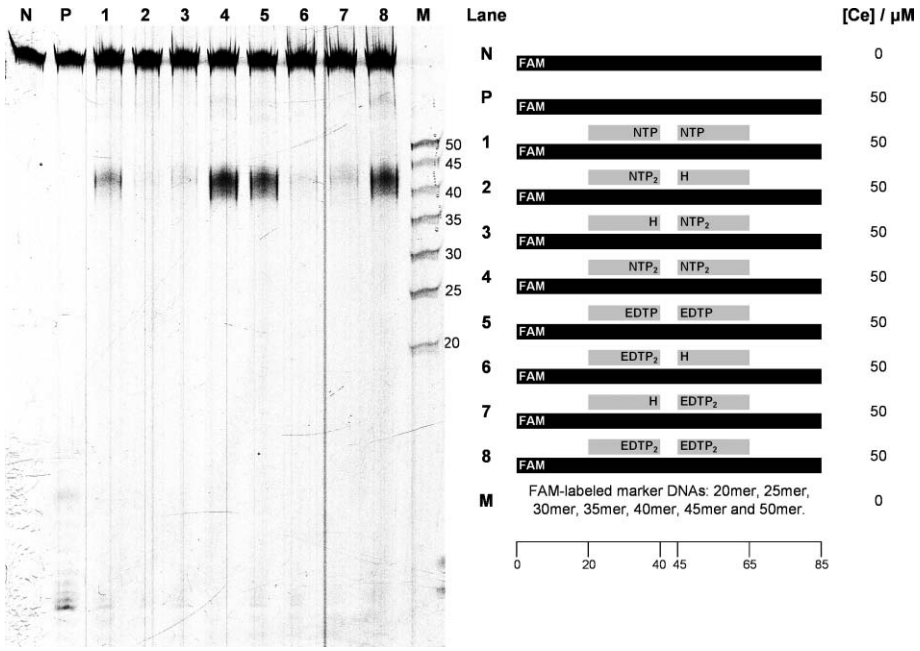


Fig. 4 Polyacrylamide gel electrophoresis patterns for the hydrolysis of the 5′-FAM-labelled DNA target **DNA85S** at a 5-base gap by Ce^{IV}–EDTA complex. $t = 72$ h, pH = 7.0 (7.5 mM HEPES), $T = 50$ °C, [NaCl] = 100 mM, [target] = 1 μM, [additives] = 2 μM.

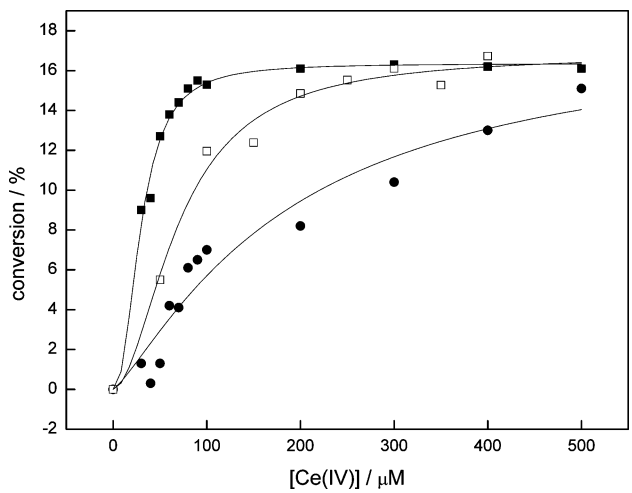


Fig. 5 Conversion of **DNA45S** to the products of hydrolysis at the 5-base gap site as a function of Ce(IV) concentration. **DNA20LP** : **DNA20RP** (●), **DNA20LNTP** : **DNA20RNTP** (▲) and **DNA20LEDTP** : **DNA20REDTP** (■), pH = 7.0 (7.5 mM HEPES), $T = 50$ °C, [NaCl] = 100 mM, [target] = 1 μM, [additives] = 2 μM.

Analytical TLC was conducted on silica gel 60 F254 plates (Merck) using the following eluents: A, ethyl acetate; B, hexane–dichloromethane–triethylamine (45 : 45 : 10, v/v).

2-Phthalimidooxyethanol (5)

The compound was prepared as previously described with the exception that purification was carried out by recycling GPC eluting with $CHCl_3$ (t_R = 59.5 min). ^{1}H NMR (δ_H)(500 MHz, $CDCl_3$) 3.50 (brs, 1H), 3.78–3.83 (m, 2H), 4.30 (d, 2H, $J = 4.4$ Hz), 1.45 (d, 6H, J = 7 Hz), 7.77–7.79 (m, 2H), 7.85–7.87 (m, 2H).

Table 1 Kinetic parameters for the hydrolysis at the 5-base gap site in DNA45S as a function of Ce(IV) concentration[a]

Ligands	Monophosphate	NTP	EDTP
$k_{max}/10^{-6}$ s^{-1}	3 ± 1	2.6 ± 0.1	2.48 ± 0.04
K_d/μM	200 ± 100	74 ± 7	31 ± 1
n	1.2 ± 0.4	1.9 ± 0.4	2.3 ± 0.3

[a] $T = 50$ °C, [NaCl] = 100 mM, [target] = 1 μM, [additives] = 2 μM.

^{13}C NMR (δ_C)(126 MHz, $CDCl_3$) 59.9, 80.3, 124.3 (2C), 129.2, 135.3 (2C), 164.9. ESI$^+$-MS: m/z 207.18 $[M + H]^+$. TLC: R_f(A) = 0.7.

1,3-Diphthalimidooxypropan-2-ol (6)

A two neck round bottom flask equipped with a magnetic stir bar and 3-way inlet cock was charged with *N*-hydroxyphthalimide (62.5 mmol, 10.1 g), sodium acetate (75 mmol, 6.15 g) and DMF (400 mL). The resulting mixture was stirred under nitrogen atmosphere at room temperature for 40 minutes. To this solution, 1,3-dibromopropan-2-ol (25 mmol, 2.56 mL) was added and the resulting mixture was stirred at 60 °C for 12 hours. After removal of solvents, the residue was dissolved in ethyl acetate (200 mL) and washed with aq. sodium bicarbonate until the red colour of the organic layer disappeared. The organic layer was dried with sodium sulfate and evaporated to dryness. To the residue, hot ethanol (250 mL) was slowly added to give a homogenous solution, from which recrystallization gave 2.05 g (22%) of **9** as a white powder. ^{1}H NMR (δ_H)(500 MHz, $CDCl_3$) 4.11 (d, 1H, J = 4 Hz), 4.28–4.31 (m, 1H), 4.35 (dd, 2H, $J_1 = 4$ Hz, $J_2 = 11$ Hz), 4.43 (dd, 2H, $J_1 = 4$ Hz, $J_2 = 11$ Hz), 7.75–7.78 (m, 4H), 7.81–7.84 (m, 4H); ^{13}C NMR (δ_C)(126 MHz, $CDCl_3$) 67.2, 79.3, 124.2, 129.2, 135.1, 164.2; ESI$^+$-MS: m/z 382.85 $[M + H]^+$, 421.05 $[M + Na]^+$.

O-2-(4-Formylphenoxy)ethylnitrilotris(methylenephosphonic acid) (3)

Nitrilotris(methylenephosphonic acid) (16.7 mmol, 5.0 g) was suspended in a mixture of acetic anhydride (20 mL) and DMF (20 mL). The resulting mixture was stirred under reflux for 3 h, after which it was evaporated to dryness. The residue (12.2 mmol, 3.0 g) and 4-(2-hydroxyethoxy)benzaldehyde (12.0 mmol, 2.0 g) were suspended in anhydrous DMSO (20 mL) and the resulting mixture was stirred under nitrogen atmosphere at 90 °C for 3 h. Water (10 mL) was added and the stirring at 90 °C continued for 1 h, after which the reaction mixture was evaporated to dryness. The residue was dissolved in water (100 mL) and washed with ethyl acetate (100 mL). The aqueous layer was evaporated to dryness and the residue purified by semipreparative HPLC. Overall yield starting from nitrilotris(methylenephosphonic acid) was 31%. ^{1}H NMR (δ_H)(500 MHz, D_2O) 3.68 (d, 4H, $J = 11$ Hz), 3.79 (d, 2H, $J = 11$ Hz), 4.33–4.36 (m, 4H), 7.18 (d, 2H, $J = 7$ Hz), 7.93 (d, 2H, $J = 7$ Hz), 9.77 (s, 1H). ^{31}P NMR (δ_P)(202 MHz, D_2O) 8.42 (2P), 10.68 (1P). ESI$^-$-MS: m/z 445.85 $[M - H]^-$.

O-2-(4-Formylphenoxy)ethylethylenediaminetetrakis(methylenephosphonic acid) (4)

Ethylenediaminetetrakis(methylenephosphonic acid) (11.5 mmol, 5.0 g) was suspended in a mixture of acetic anhydride (20 mL) and DMF (20 mL). The resulting mixture was stirred under reflux for 3 h, after which it was evaporated to dryness. The residue (8.8 mmol, 5.0 g), 4-(2-hydroxyethoxy)benzaldehyde (48.0 mmol, 8.0 g) and a catalytic amount of DMAP were suspended in anhydrous DMSO (15 mL) and the resulting mixture was stirred under nitrogen atmosphere at 100 °C for 2 h. Water (10 mL) was added and the stirring at 100 °C continued for 2 h, after which the reaction mixture was evaporated to dryness. The residue was dissolved in water (150 mL) and washed with ethyl acetate (2 × 100 mL). The aqueous layer was evaporated to dryness and the residue purified by semipreparative HPLC. Overall yield starting from ethylenediaminetetrakis(methylenephosphonic acid) was 26%. ^{1}H NMR (δ_H)(500 MHz, D_2O) 3.11 (d, 2H, $J = 10$ Hz), 3.25 (d, 2H, $J = 10$ Hz), 3.32 (m, 2H), 3.34 (d, 4H, $J = 10$ Hz), 3.50 (m, 2H), 4.29 (m, 2H), 4.35 (m, 2H), 7.19 (d, 2H, $J = 9$ Hz), 7.93 (d, 2H, $J = 9$ Hz), 9.77 (s, 1H). ^{31}P NMR (δ_P)(202 MHz, D_2O) 11.14 (2P), 17.07 (1P), 19.62 (1P). ESI$^-$-MS: m/z 583.05 $[M - H]^-$.

Preparation of the oligonucleotide multiphosphonate conjugates DNA20LNTP, DNA20RNTP, DNA20LEDTP, DNA20REDTP, DNA20LNTP$_2$, DNA20RNTP$_2$, DNA20LEDTP$_2$ and DNA20REDTP$_2$

The oligodeoxyribonucleotides were assembled on a synthesizer by the conventional phosphoramidite method, except that a prolonged coupling time (600 s) was used for the protected aminooxy building blocks **10**, **11** and **12**. The support-bound oligonucleotides were treated with a mixture of hydrazine, pyridine and acetic acid (1 : 32 : 8, *v*/*v*) for 30 minutes, after which they were washed with pyridine, methanol and acetonitrile and dried under vacuum. The supports were transferred to microcentrifuge tubes, a solution of either **3** or **4** (20 μmol) in water (200 μL) was added and the resulting heterogeneous mixture was shaken at room temperature for 12 h. The solution phases were then removed and the supports were washed with water and treated with a mixture of saturated aq. ammonia and ethanol (3 : 1, *v*/*v*) at 55 °C for 8 h, after which the solution phases were collected and evaporated to dryness. The residues were purified by denaturing polyacrylamide gel electrophoresis (20% acrylamide) and RP HPLC. MALDI-TOF-MS: m/z **DNA20LNTP**: 6753.3 (calcd 6754.5); **DNA20RNTP**: 6663.4 (calcd 6657.4); **DNA20LEDTP**: 6762.8 (calcd 6759.4); **DNA20REDTP**: 6798.5 (calcd 6794.5); **DNA20LNTP$_2$**: 7092.2 (calcd 7096.8); **DNA20RNTP$_2$**: 7124.0 (calcd 7131.8); **DNA20LEDTP$_2$**: 7367.9 (calcd 7370.2); **DNA20REDTP$_2$**: 7396.1 (calcd 7405.2).

Conclusions

Synthesis of oligonucleotide conjugates bearing one or two terminal nitrilotris(methylenephosphonic acid) (NTP) or ethylenediaminetetrakis(methylenephosphonic acid) (EDTP) ligands has been achieved using on-support oximation of aminooxy-functionalized oligonucleotides with 2-(4-formylphenoxy)ethyl esters of NTP and EDTP. Single-stranded gap sites formed by hybridizing a DNA substrate with two of these conjugates are efficiently hydrolyzed under conditions where other single-stranded regions of the substrate remain intact. In other words, the system described allows site-selective hydrolysis of single-stranded DNA targets of arbitrary length using short (*e.g.* 20mer) additive oligonucleotide conjugates.

Acknowledgements

This work was partially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, Culture, and Technology of Japan. Support by the Japan Society for the Promotion of Science and by the Global COE Program for Chemistry Innovation is also acknowledged.

References

1 M. Komiyama and J. Sumaoka, *Curr. Opin. Chem. Biol.*, 1998, **2**, 751; N. H. Williams, B. Takasaki, M. Wall and J. Chin, *Acc. Chem. Res.*, 1999, **32**, 485; A. Y. Chang and P. B. Dervan, *J. Am. Chem. Soc.*, 2000, **122**, 4856; A. Sreedhara and J. A. Cowan, *JBIC, J. Biol. Inorg. Chem.*, 2001, **6**, 337; K. D. Copeland, M. P. Fitzsimons, R. P. Houser and J. K. Barton, *Biochemistry*, 2002, **41**, 343.
2 N. H. Williams and P. Wyman, *Chem. Commun.*, 2001, 1268.
3 M. Komiyama, K. Matsumura, K. Yonezawa and Y. Matsumoto, *Chem. Express*, 1993, **8**, 85; M. Komiyama, T. Shiiba, T. Kodama, N. Takeda, J. Sumaoka and M. Yashiro, *Chem. Lett.*, 1994, **23**, 1025; B. K. Takasaki and J. Chin, *J. Am. Chem. Soc.*, 1994, **116**, 1121; M. Komiyama, N. Takeda, Y. Takahashi, H. Uchida, T. Shiiba, T. Kodama and M. Yashiro, *J. Chem. Soc., Perkin Trans. 2*, 1995, 269; M. E. Branum, A. K. Tipton, S. Zhu and L. Que Jr., *J. Am. Chem. Soc.*, 2001, **123**, 1898; M. Komiyama, N. Takeda and H. Shigekawa, *Chem. Commun.*, 1999, 1443; M. Komiyama, T. Shiiba, Y. Takahashi, N. Takeda, K. Matsumura and T. Kodama, *Supramol. Chem.*, 1994, **4**, 31; M. Komiyama, *J. Biochem.*, 1995, **118**, 665; R. T. Kovacic, J. T. Welch and S. J. Franklin, *J. Am. Chem. Soc.*, 2003, **125**, 6656; F. H. Zelder, A. A. Mokhir and R. Krämer, *Inorg. Chem.*, 2003, **42**, 8618.
4 W. Chen, Y. Kitamura, J.-M. Zhou, J. Sumaoka and M. Komiyama, *J. Am. Chem. Soc.*, 2004, **126**, 10285.
5 A. N. Pudovik, *Chemistry of Organophosphorus Compounds*, MIR, Moscow, 1983, p. 150.
6 H. Salo, P. Virta, H. Hakala, T. P. Prakash, A. M. Kawasaki, M. Manoharan and H. Lönnberg, *Bioconjugate Chem.*, 1999, **10**,

815; E. Trévisiol, A. Renard, E. Defrancq and J. Lhomme, *Tetrahedron Lett.*, 1997, **38**, 8687; E. Trévisiol, A. Renard, E. Defrancq and J. Lhomme, *Nucleosides, Nucleotides Nucleic Acids*, 2000, **19**, 1427; E. Trévisiol, E. Defrancq, J. Lhomme, A. Laayoun and P. Cros, *Eur. J. Org. Chem.*, 2000, 211; E. Trévisiol, E. Defrancq, J. Lhomme, A. Laayoun and P. Cros, *Tetrahedron*, 2000, **56**, 6501.

7 N. Isaacs, in *Physical Organic Chemistry*, Longman, Essex, 2nd edn, 1995, p. 276; W. P. Jencks, *Prog. Phys. Org. Chem.*, 1964, **2**, 63.

8 Y. Kitamura, S. Mori, W. Chen, J. Sumaoka and M. Komiyama, *JBIC, J. Biol. Inorg. Chem.*, 2006, **11**, 13.

9 T. C. Bruice, A. Tsubouchi, R. O. Dempcy and L. P. Olson, *J. Am. Chem. Soc.*, 1996, **118**, 9867; T. A. Steitz and J. A. Steitz, *Proc. Natl. Acad. Sci. U. S. A.*, 1993, **90**, 16498; B. K. Takasaki and J. Chin, *J. Am. Chem. Soc.*, 1994, **116**, 1121; N. H. Williams, B. Takasaki, M. Wall and J. Chin, *Acc. Chem. Res.*, 1999, **32**, 485.

10 M. Komiyama, Y. Aiba, Y. Yamamoto and J. Sumaoka, *Nature Protocols*, 2008, **3**, 655.