Chemical synthesis and thermodynamic characterization of oxanine-containing oligodeoxynucleotides

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ABSTRACT

Oxanine (Oxa, O), one of the major damaged bases from guanine generated by NO- or HNO2-induced nitrosative deamination, has been considered as a mutagen-potent lesion. For exploring more detailed properties of Oxa, large-scale preparation of Oxa-containing oligodeoxynucleotide (Oxa-ODN) with the desired base sequence is a prerequisite. In the present study, we have developed a chemical synthesis procedure of Oxa-ODNs and characterized thermodynamic properties of Oxa in DNA strands. First, 2'-deoxynucleoside of Oxa (dOxo) obtained from 2'-deoxyguanosine by HNO2-nitrosation was subjected to 5'-O-selective tritylation to give 5'-O-(4,4'-dimethoxytrityl)-dOxo (DMT-dOxo) with a maximum yield of 70%. Subsequently, DMT-dOxo was treated with conventional phosphoramidation, which resulted in DMT-dOxo-amidite monomer with a maximum yield of 72.5%. The amidite obtained was used for synthesizing Oxa-ODNs: the coupling yields for Oxa incorporation were over 93%. The prepared Oxa-ODNs were employed for analyzing the thermodynamic properties of DNA duplexes containing base-matches of O:N [N = C (cytosine), T (thymine), G (guanine) or A (adenine)]. Melting temperatures ($T_m$) and thermodynamic stability ($\Delta G^{\circ}_2$) were found to be lower by 6.83 to 13.41°C and 2.643 to 6.047 kcal mol$^{-1}$, respectively, compared with those of oligodeoxynucleotides, which had the same base sequence except that O:N was replaced by G:C (wild type). It has also been found that Oxa-pairing with cytosine shows relatively high stability in DNA duplex compared with other base combinations. The orders of $\Delta \Delta G^{\circ}_2$ were O:C > O:T > O:A > O:G. The chemical synthesis procedure and thermodynamic characteristics of Oxa-ODNs established here will be helpful for elucidating the biological significance of Oxa in relation to genotoxic and repair mechanisms.

INTRODUCTION

In 1996, we demonstrated for the first time that 2'-deoxyoxanosine (dOxo) forms as one of the main deaminated products from 2'-deoxyguanosine (dGuo) through nitric oxide (NO)- or nitrous acid (HNO2)-induced nitrosative oxidation (1). It was well known that DNA bases with exocyclic amino groups are converted to deaminated forms through nitrosative stress or high temperature (2–5), i.e. 2'-deoxyuridine (dUrd), 2'-deoxythymidine (dThd), 2'-deoxyinosine (dIno) and 2'-deoxyxanthosine (dXao) are resulted by the oxidative deamination from 2'-deoxycytidine (dCyd), 5-methyl-2'-deoxyuridine (d5meCyd), 2'-deoxyadenosine (dAdo) and dGuo, respectively (4). Compared with dXao and other deaminated nucleosides (dIno, dUrd and dThd), in which exocyclic nitrogen is oxidized, dOxo is a unique deaminated form in which an endocyclic nitrogen atom of guanine (Gua) is substituted by an oxygen atom (1,6,7).

Since then, theoretical and experimental efforts have been made to elucidate the chemical mechanism of dOxo formation, together with dXao, from dGuo by nitrosative deamination (7–11). Glaser and co-workers (7,10,11) presented that the ring-opened intermediate of Gua pyrimidine, after loss of dinitrogen in Gua diazonium ions, leads to the formation of xanthine (Xan, base moiety of dXao) as well as xanthine (Xan, base moiety of dXao). Furthermore, we have shown that the Oxa formation mechanism involves in such a ring-opened intermediate of Gua by isolating and characterizing the Gua-diazoo intermediate (10).
Since dOxo, which is a DNA lesion of dGuo, could be produced in the cellular system by NO, HNO2 or other nitrosating agent (1,6,12), its genotoxic properties including deglycosylation susceptibility, base-pairing stability and base-incorporation patterns have been analyzed (13,14). If Oxa is formed in DNA strands, Oxa can exist for a sufficient time since dOxo moiety is not easily hydrolyzed due to its stable N-glycosidic bond between base and sugar moieties. Also, it was suggested that Oxa could form two hydrogen bondings with either cytosine (Cyt) or thymine (Thy). Therefore, the Oxa generated in cellular genomes may induce the mis-incorporation of incorrect nucleotides causing G:C to A:T transition (13). In addition, our recent work has revealed that Oxa mediates a novel genotoxic mechanism related to the formation of DNA–protein cross-link (DPC) (15). Since Oxa has an O-acylisourea structure, it is likely that Oxa reacts with intracellular nucleophiles under physiological conditions and would lead to novel mutagenic or lethal events (15–17). In the case of the base-excision repair (BER) system, we observed that bacterial AlkA (3-methyladenine DNA glycosylase II) and endonuclease VIII possess repair activities on Oxa in oligodeoxynucleotides (ODNs) (18). Recently, Cao and co-workers (19,20) reported that bacterial endonuclease V and human AAG (alkyladenine glycosylase) shows BER activity on Oxa. However, another recent report by our group demonstrated that in the case of Oxa-related genotoxicity (Oxa-mediated DPC formation and its relevant events), the nucleotide excision and recombination repair (NER) system would play a more efficient role than BER system (21).

Oxa-containing oligodeoxynucleotide (Oxa-ODN) needs to be prepared to elucidate the biological significance of Oxa from biophysical properties to genotoxic threats in detail. Up to now, enzymatic methods using DNA polymerases, such as T4 polymerase (exo−) or Pol I klenow fragment, have been applied for the preparation of Oxa-ODNs [in this method, 2′-deoxyoxanosine triphosphate (dOTP) was used as a monomer and incorporated to pair with Cyt in DNA strands by DNA polymerase] (14,19–21). However, since the enzymatic method is not appropriate for the large-scale preparation and desired-sequence design, the chemical synthesis method has been required for extending biological and biotechnological research using Oxa-ODNs. Especially, to reveal the Oxa-induced cellular mechanisms, a large amount of Oxa-ODNs should be used as bait molecules for trapping Oxa-response enzymes or DPC-relevant proteins and as reporter molecules for identifying the Oxa-related genotoxic events in cellular system. Also, various kinds of Oxa-ODNs should be prepared for analyzing more detailed biophysical and biochemical properties of Oxa, such as thermodynamic characterization and structural analysis (NMR or X-ray crystallography). In addition, since Oxa has a unique property of reacting with several chemical functional groups such as amine or amine-containing molecules, Oxa-ODNs could be subjected to the recent hot topics of DNA-based nano-biotechnology.

In the present study, we report, for the first time, on the chemical synthesis procedures for preparing dOxo-amidite monomer and Oxa-ODNs. Also, we show the detailed characterization of the synthesized Oxa-ODNs in terms of thermodynamic stabilities of DNA duplexes containing Oxa base-pairings.

**Materials and Methods**

**Materials**

2-Cyanoethyl-N,N,N’,N’-tetraisopropylphosphoramidite was purchased from Aldrich Chemical Co. (Milwaukee, WI) and all other chemicals for chemical synthesis were obtained from Wako Pure Chemicals (Osaka, Japan). Solvents were acquired from Nacalai Tesques (Osaka, Japan) and anhydrous solvent was used for organic reactions. The reagents for oligonucleotide synthesis (including CPG column and appropriately protected normal nucleosides) were obtained from Glen Research Co. (Sterling, VA). The enzymes such as nuclease P1 and alkaline phosphatase required for ODN digestion were purchased from Roche Diagnostics (Mannheim, Germany).

**Instrument systems**

The reversed phase (RP-) HPLC system used for collection of dOxo was composed of a Tosho PX-8010 (controller), a CCPM (pump) and a UV-8010 (UV detector) (Tosho Co, Tokyo, Japan) with a preparative COSMOSIL C18-PAQ column [250 × 28 mm, 5 μm; Nacalai Tesques, (Osaka, Japan)]. For analysis and preparation of DMT-dOxo-amidite, the normal phase (NP-) HPLC system consisted of a Hitachi L-6200 (controller + pump) and an L4000 (UV detector) (Hitachi Co, Tokyo, Japan) with an Ultron VX-SIL column [150 × 4.6 mm (for analysis) or 250 × 20 mm (for preparation), 5 μm; Shinwa Co. (Kyoto, Japan)]. For purification of synthesized ODNs, an RP-HPLC system constructed with a Tosho PX-8020 (controller), a DP-8020 (pump), a CO-8020 (temperature controller) and a PD-8020 (diode detector) with an Ultrax VX-ODS column [150 × 4.6 mm (for analysis) or 250 × 10 mm (for purification), 5 μm; Shinwa Co. (Kyoto, Japan)] interfaced with a microcomputer and equipped with temperature controller. Melting temperature analysis was performed with Shimadzu TMSPC-8 Tm analysis system [Shimadzu Co (Kyoto, Japan)]. CD spectra of DNA complex were obtained on a Jasco spectropolarimeter J-720 [Japan Spectroscopic Co. (Tokyo, Japan)] interfaced with a microcomputer and equipped with temperature controller.

**Chemical synthesis of dimethoxytritylated dOxo-phosphoramidite**

(i) 2′-Deoxyoxanosine (dOxo):dGuo (4 mmol) was incubated with sodium nitrite (NaNO2; 100 mM) in 400 ml of sodium acetate buffer (3.0 M, pH 3.7) at 45°C for 4 h. After neutralization with sodium hydroxide (NaOH; 5 M), reaction mixtures were reduced to half of the volume by rotary evaporator. The solution was filtrated by Millex GS [0.22 μm; Millipore (Bradford, MA)] and applied to a preparative RP-HPLC system [isocratic elution, sodium phosphate buffer (400 μM, pH 7.4) containing 10% (v/v) acetonitrile (CH3CN); flow rate, 6 ml/min]. Further
purification and freeze drying of the appropriate fraction gave a white product of dOxo (0.6 mmol, 15% yield from dGuo) \(^{1}H\) NMR (600 MHz, D_{2}O at 30°C); \(\delta = 7.98\) (s, 1H, H-2), 6.26 (dd, 1H, H-1\'), 4.61 (dd, 1H, H-3\'), 4.12 (dd, 1H, H-4\'), 3.79 (m, 2H, H-5',5'\'), 2.77 (dd, 1H, H-2\'); UV: \(\lambda_{\text{max}} = 245, 286\) (pH 7); MS(FAB) \(m/z = 269\) (M+H\(^{+}\)).

(ii) \(S'\)-(4,4'-(dimethoxytrityl)-2'-deoxyoxanosine (DMT-dOxo);4,4'-(Dimethoxytritylchloride (DMT-Cl, 0.6–0.8 mmol) was added to a suspension of dOxo (0.4 mmol) in dry dimethylformamide (DMF; 3 ml) and then, imidazole (0.8 mmol) and diisopropylethylamine (DIEA-Mes, 0.8 mmol) were added to the solution. DIEA-Mes was prepared as reported by Kataoka and Hayakawa (22). The reaction mixture was allowed to stir at room temperature for 3 h. During this period, the mixture became homogeneous. The reaction mixture was poured into water (900 ml) and the resulting precipitate was collected by suction filtration and dried at room temperature for 24 h. The crude product was re-dissolved in dichloromethane (CH\(_2\)Cl\(_2\)) and dried using sodium sulfate (Na\(_2\)SO\(_4\)). The re-dissolved solution was concentrated by suction filtration and dried at room temperature for 3 h. The resulting solution was quenched by addition of methanol to a final concentration of 10% (v/v), filtered, and then added to a solution of Na\(_2\)SO\(_4\) to give a white solid. The solid was dried at room temperature for 24 h. After treatment with triethylamine-acetate buffer (TEAA; 2 M, pH 7.0), the mixture solution was subjected to a Poly-Pak cartridge [Glen Researches Co. (Sterling, VA)] to give a purified DMT-off ODN. Detritylated ODN was further purified by an RP-HPLC system using a gradient of CH\(_3\)CN using Eluent A [5% CH\(_3\)CN in 100 mM TEAA (pH 7.0)] and Eluent B [15% CH\(_3\)CN in 100 mM TEAA (pH 7.0)]; 26% (0 min)–90% (40 min) of Eluent B (flow rate : 2 ml/min). The appropriate sample was subjected to the final confirmation by an RP-HPLC system with the same gradient condition mentioned above (flow rate: 1 ml/min).

The purified ODNs were subjected to enzymatic digestion to analyze the component nucleosides. The ODN (10 \(\mu\)g) was dissolved in 50 \(\mu\)l of sodium acetate buffer (50 mM, pH 5.8) containing zinc chloride (5 mM). The DNA was digested to deoxyxynucleoside monophosphates by the addition of nuclease P1 (8 U) and incubation at 37°C for 3 h. Following addition of 50 \(\mu\)l of sodium acetate buffer (50 mM, pH 7.4), phosphatase groups were removed with alkaline phosphatase (15 U) and phosphodiesterase I (0.01 U; optional treatment) by incubation at 37°C for 6 h. The reaction mixture was directly analyzed by an RP-HPLC system with a linear gradient of 0% (0 min)–20% (20 min) of CH\(_3\)CN in 100 mM TEAA at a flow rate of 1 ml/min.

Biochemical characterization of oxanine-containing ODNs

For melting temperature analysis, all DNA solutions were prepared in a phosphate buffer composed of 1 M NaCl, 10 mM Na\(_2\)HPO\(_4\) and 1 mM Na\(_2\)EDTA adjusted to pH 7.0. ODN concentrations were determined on the absorbance values of nucleosides. Melting curves of DNA duplexes were obtained for the solutions containing a 1:1 strand ratio of ODNs with an increase in temperature from 20 to 90°C at a rate of 0.2°C/min. The melting temperatures were measured at various total concentrations (C\(_t\)) of ODN (between 1 and 50 \(\mu\)M). The thermodynamic parameters (enthalpy change, \(\Delta H;\) entropy change, \(\Delta S;\)) for each DNA duplex formation were estimated by the linear van’t Hoff equation, \(T_{m}^{-1} \text{versus ln}(C/C_{0});\) (23). For analysis of the whole DNA structure, CD spectra of DNA duplex were measured. All CD spectra were recorded from 350 to 210 nm at 25°C with a scan speed 100 nm/min in a jacketed cylindrical cuvette with a path length of 10 mm. The cuvette-holding chamber was flushed with a constant stream of dry N\(_2\) gas to avoid moisture condensation on the cuvette exterior. The total strand concentration of the samples was 16 \(\mu\)M in the same buffer solution as used for the melting temperature studies. All the CD data were accumulated 10 times and processed using a noise reduction program.

RESULTS AND DISCUSSION

Chemical preparation of DMT-dOxo-amidite

As summarized in Figure 1, a chemical preparation procedure of dimethoxytrityl-protected amidite of dOxo (DMT-dOxo-amidite) was developed in this study.
In the first step (preparation of dOxo), as reported previously (1), dGuo was incubated in a weakly acidic HNO₂ solution, which was mediated by NaNO₂ (100 mM) in a sodium acetate buffer (3.0 M, pH 3.7), at 45°C. Even though dGuo was not completely converted to deaminated products such as dXao and dOxo, the above nitrosative reaction was terminated in 4 h by neutralization of NaOH because the products including dOxo began to precipitate after 4 h incubation: It should be noted that the precipitates are difficult to re-dissolve in aqueous solution, making the recovering process of dOxo complex. HNO₂-treated dGuo solution was subjected to large-scale isocratic RP-HPLC separation to purify dOxo. The mobile phase employed in the preparative RP-HPLC was sodium phosphate buffer (400 mM, pH 7.4) containing 10% CH₃CN: low concentrated phosphate salt was found to be effective so far. The final dOxo yield from dGuo (the yield of dOxo from dIno is 26%) Moreover, the selectivity of tritylation was not higher than that achieved by the nirosation conversion from dGuo (the yield of dOxo from dIno is 26%). Moreover, for obtaining highly purified dOxo, RP-HPLC separation is also required. Therefore, our method described above is more effective so far.

In the second step (preparation of DMT-dOxo), a 5′-O selective dimethoxytritylation method was employed on the basis of another previous report (22). DIEA-Mes (2.0 equiv. to dOxo) was applied to acquire the 5′-O selectivity of tritylation (22). dOxo, DMT-Cl, DIEA-Mes and imidazole were incubated and their mole ratios were optimized. It was found that the amount of DMT-dOxo was dependent on the amount of DMT-Cl. However, when the ratio of DMT-Cl to dOxo is over 2:1, a large amount of by-product was observed to be formed (in the case that the ratio was over 3:1, the portion of by-product was ~45%). Ratios of DIEA-Mes and imidazole were not determinant factors to the yield of DMT-dOxo. The best ratio of dOxo, DMT-Cl, DIEA-Mes and imidazole was determined as 1:2:2:2. This 5′-O selective dimethoxytritylation method is efficient to yield 80% conversion of dOxo to DMT-dOxo. The final yield for the conversion of dOxo to DMT-dOxo was ~70%, which is comparable with that of DMT-dGuo synthesis (74%) obtained by a similar method (22).

In the third step (preparation of DMT-dOxo-amidite), DMT-dOxo was subjected to the normal phosphoramidation method, in which tetrazole and 2-cyanoethyl-\(N,N,N',N'\)-tetraisopropylphosphoramidite were used. To increase the purity and the yield of DMT-dOxo-amidite, the product was purified by preparative NP-HPLC. It should be noted that TEA, generally used as an additive modifier for increasing the resolution of NP-HPLC, was not appropriate for separation of DMT-dOxo-amidite. Instead, a small amount of MeOH (0.33%) was added to the NP-HPLC mobile phase composed of EtOAc and CH₂Cl₂ (65:35). As shown in Figure 2, two peaks of DMT-dOxo-amidite (diastereoisomer) were obtained. In this preparation step, DMT-dOxo was subjected to the phosphoramidite modification without protection of exo-NH₂. Therefore, this step using unprotected nucleoside substrate

![Figure 1](https://academic.oup.com/nar/article-abstract/33/18/5771/2401190)
Ultron VX-SIL (150 mm, 5 μm), temperature: ambient, detection: 11.86 and 12.79 min (diastereoisomer). Structural assignment performed using 1H, 13C and 31P NMR. DMT-dOxo-amidite is obtained from dGuo in only three steps. The large by-product would be practically sufficient since DMT-OxadOx treatment is not applicable for Oxa-ODN protection removal after the oligonucleotide synthesis. How- ever, conc. NH4OH-treatment is not applicable for Oxa-ODN protection removal after the oligonucleotide synthesis. How- ever, conc. NH4OH-treatment is not applicable for Oxa-ODN protection removal after the oligonucleotide synthesis.

Figure 2. NP-HPLC chromatogram of dimethoxytrityl-protected phosphoramidite of dOxo (DMT-dOxo-amidite). NP-HPLC condition: elution: isocratic with EtOAc:CH2Cl2:MeOH = 65:35:0.33, flow rate; 1 ml/min, column; Ultron VX-SIL (150 × 4.6 mm, 5 μm), temperature: ambient, detection; 11.86 and 12.79 min (diastereoisomer).

could be expected to generate a large quantity of by-product. However, the final yield from DMT-dOxo was as high as 72.5% (max.), which is comparable with the phosphoramidite yields obtained by the previous methods composed of several steps without base protection (25). Although the final yield from DMT-dOxo was as high as could be expected to generate a large quantity of by-product. However, the final yield from DMT-dOxo was as high as 72.5% (max.), which is comparable with the phosphoramidite yields obtained by the previous methods composed of several steps without base protection (25). Although the final yield from DMT-dOxo was as high as 72.5% (max.), which is comparable with the phosphoramidite yields obtained by the previous methods composed of several steps without base protection (25). Although the final yield from DMT-dOxo was as high as could be expected to generate a large quantity of by-product. However, the final yield from DMT-dOxo was as high as 72.5% (max.), which is comparable with the phosphoramidite yields obtained by the previous methods composed of several steps without base protection (25). Although the final yield from DMT-dOxo was as high as could be expected to generate a large quantity of by-product. However, the final yield from DMT-dOxo was as high as 72.5% (max.), which is comparable with the phosphoramidite yields obtained by the previous methods composed of several steps without base protection (25). Although the final yield from DMT-dOxo was as high as could be expected to generate a large quantity of by-product. However, the final yield from DMT-dOxo was as high as 72.5% (max.), which is comparable with the phosphoramidite yields obtained by the previous methods composed of several steps without base protection (25). Although the final yield from DMT-dOxo was as high as could be expected to generate a large quantity of by-product. However, the final yield from DMT-dOxo was as high as 72.5% (max.), which is comparable with the phosphoramidite yields obtained by the previous methods composed of several steps without base protection (25). Although the final yield from DMT-dOxo was as high as could be expected to generate a large quantity of by-product. However, the final yield from DMT-dOxo was as high as 72.5% (max.), which is comparable with the phosphoramidite yields obtained by the previous methods composed of several steps without base protection (25).

Chemical incorporation of Oxa base into ODNs

Using DMT-dOxo-amidite monomer prepared above, several kinds of oxanine-containing oligodeoxynucleotides (Oxa-ODNs) were synthesized as listed in Table 2. In the conventional method, ammonium hydroxide solution (28% NH4OH) is used for the CPG-cleavage and base-protection removal after the oligonucleotide synthesis. However, conc. NH4OH-treatment is not applicable for Oxa-ODN synthesis since Oxa base has high reactivity with primary amine as reported previously (16,17), and therefore, as suggested in Figure 3a, it reacts with NH3 to form irreversible ring-opened compound: after 1 h incubation of dOxo in conc. NH2OH-solution, irreversible compound was observed by RPHPLC and found to be a ring-opened form of dOxo-NH3 adduct (Structure II of Figure 3a) (HPLC data not shown). On the other hand, Oxa base was reported to exist in the ring opening/closure equilibrium in NaOH solution (13,26). As shown in Figure 3b, since Structure III, which is formed at high pH, is reversible to Structure I in neutral conditions, the Oxa base can be left intact. After 24 h incubation of dOxo in high pH, is reversible to Structure I in neutral conditions, the Oxa base can be left intact. After 24 h incubation of dOxo in high pH, is reversible to Structure I in neutral conditions, the Oxa base can be left intact. After 24 h incubation of dOxo in high pH, is reversible to Structure I in neutral conditions, the Oxa base can be left intact. After 24 h incubation of dOxo in high pH, is reversible to Structure I in neutral conditions, the Oxa base can be left intact. After 24 h incubation of dOxo in high pH, is reversible to Structure I in neutral conditions, the Oxa base can be left intact. After 24 h incubation of dOxo in high pH, is reversible to Structure I in neutral conditions, the Oxa base can be left intact. 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NaOH solution, no other compound derived from dOxo was observed in RP-HPLC data. Therefore, in the present study, conc. NaOH solution (0.5 M) was applied to CPG-cleavage and base-protection removal after the synthesis of Oxa-ODNs.

For efficient base-protection removal by NaOH, Ac-dC amidite (acetyl protected dC amidite), Pac-dC amidite (phe-noxyacetyl protected dA amidite) and iPr-Pac-dG amidite (4-isopropyl-pheoxyacetyl protected dG amidite) were used instead of benzoyl protected amidite (dC and dA) or isobutyl protected amidite (dG).

Next, we explored whether any change of Oxa takes place in ODN synthesis by analysis of OT-ODN (sequence 5'-OT-3'; O = Oxa and T = Thy). After DMT-dOxo-amidite was incorporated into the dT-CPG column (1 μmol scale), as depicted in Figure 4a, the DMT-off sample of OT-ODN was analyzed. When amine-unprotected phosphoramidite was employed in ODN synthesis, some by-products may be generated, i.e. free exo-NH₂ of amidite might react with another activated phosphoramidite monomer. If this is the case, two or several peaks would appear in ³¹P NMR spectra. Another ³¹P NMR analysis

**Figure 3.** Proposed mechanism of Oxa base response to NH₃OH (a) and NaOH (b), which shows ring-opened form of dOxo-NH₃⁺ adduct (a: Structure II is irreversible with Structure I) and ring opening/closure forms of dOxo in the presence of hydroxide (b: Structure III is reversible with Structure I).

**Figure 4.** ³¹P NMR analysis of the synthesized Oxa-ODN, which shows no change of Oxa base during the DNA synthesis using DMT-dOxo-amidite (a: schematic procedure of OT-ODN (5'-OT-3') preparation. b: ³¹P NMR (320 MHz) data of the synthesized OT-ODN in CD₃CN at 30°C).
reported that the amine-unprotected dAdo and dCyd induce some amount of by-product, whereas amine-free dGuo amidite present no by-product (25). In the case of OT-ODN sample, only one signal was observed at 0 p.p.m. in $^{31}$P NMR, as shown in Figure 4b, indicating that no by-product was produced in amine-free Oxal incorporation. Moreover, enzymatic digestion of the obtained OT-ODN sample with nuclease P1 and alkaline phosphatase showed only two peaks of dOxo and dThd (data not shown). These results demonstrate that the synthesized Oxal-ODN possesses intact form of Oxal.

Then, as summarized in Table 2, several kinds of Oxal-ODNs and their complementary ODNs were synthesized. In cases of synthesis of Oxal-ODNs, DMT cation released from detritylation of each step was monitored to evaluate the coupling efficiency before and after incorporation of DMT-dOxo-amidite into ODN. For each sequence, 1 μmol CPG-scale ODN synthesis was performed three times to get the average values of the coupling yield. As shown in Table 3, all the coupling yields of DMT-dOxo-amidite were observed to be over 93%. The coupling yields of Oxal after Gua (G), Ade (A), Cyt (C) and T were 93.39, 96.28, 98.06 and 99.09%, respectively. After Oxal incorporation, the coupling yield of the next amidite monomer was not influenced (c~d column in Table 3; the efficiencies of C, T, G and A were 97.82, 96.60, 97.28 and 97.60%, respectively). Moreover, before and after DMT-dOxo-amidite incorporation, the average of step-wise coupling yield was unchanged (a~b column versus d~e column in Table 3). In all these cases, the average values of step-wise coupling yield during the Oxal-ODN synthesis were around 97% (a~e column in Table 3). These results imply that the incorporated Oxal, amine-free form, does not mediate any severe influence to ODN chemical synthesis.

As given in Table 5, more detailed status of Oxal-ODN was presented by exemplifying 5mer Oxal-ODN (sequence 5'-GCOAT-3'). After conc. NaOH (0.5 M) was used for CPG-cleavage and deprotection of the synthesized ODN sample, alkali-tolerant Poly-Pak cartridge was employed for desalting NaOH and purifying Oxal-ODN. As shown in Figure 5a, the detritylated 5mer Oxal-ODN was obtained with over 90% purity. The appropriate fraction of Oxal-ODN was collected by further RP-HPLC purification and was subjected to enzymatic digestion with nuclease P1 and alkaline phosphatase. As presented in Figure 5b, the corresponding peaks of all the expected deoxynucleosides including dOxo were observed. Mass spectroscopy analysis (Supplementary Figure S-4) also supported that 5mer Oxal-ODN is the normal oligomers composed of dOxo and other four deoxynucleosides. All the data demonstrate that the Oxal incorporated into ODN can survive the various steps during and post DNA synthesis, i.e. the chemical preparation method established here produce Oxal-ODN successfully.

**Thermodynamic characterization of Oxal-ODNs**

To see whether the base-pairing between Oxal and normal bases affects DNA duplex conformation or not, CD analysis has been performed for all the DNA complementation types (Supplementary Figure S-5). Similarly to a previous report (13), all

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Position</th>
<th>Coupling yield</th>
<th>a~b (%)</th>
<th>b~c (%)</th>
<th>c~d (%)</th>
<th>d~e (%)</th>
<th>a~e (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COG-18</td>
<td>5'-TCT TCT GCO GCC TAC GAT-3'</td>
<td>96.96 ± 2.44%</td>
<td>93.39</td>
<td>97.82</td>
<td>96.92 ± 2.59%</td>
<td>96.70 ± 2.52%</td>
<td></td>
</tr>
<tr>
<td>TOA-18</td>
<td>5'-TCT TCT O ACC TAC GAT-3'</td>
<td>96.58 ± 2.23%</td>
<td>96.28</td>
<td>96.60</td>
<td>97.64 ± 1.19%</td>
<td>97.02 ± 1.79%</td>
<td></td>
</tr>
<tr>
<td>GOC-18</td>
<td>5'-TCT TCT GGO CCC TAC GAT-3'</td>
<td>97.09 ± 2.30%</td>
<td>98.06</td>
<td>97.28</td>
<td>96.59 ± 1.26%</td>
<td>97.16 ± 2.23%</td>
<td></td>
</tr>
<tr>
<td>AOT-18</td>
<td>5'-TCT TCT CAT C CO TAC GAT-3'</td>
<td>97.28 ± 1.86%</td>
<td>99.09</td>
<td>97.60</td>
<td>97.42 ± 1.32%</td>
<td>97.40 ± 1.86%</td>
<td></td>
</tr>
</tbody>
</table>

*aAverage step-wise coupling yield.

Figure 5. RP-HPLC separation of detritylated 5mer Oxal-ODN (5'-GCOAT-3') purified by Poly-Pak cartridge (a), and DNA monomer, dCyd, dGuo, dThd, dOxo and dAdo formed by the digestion of with nuclease P1 and alkaline phosphatase (b). HPLC condition: mobile phase; 100 mM TEAA solution (pH 7.0) with a gradient of CH$_3$CN [for (a), 7.6% (0 min)~14% (40 min) (please see Materials and Methods) and for (b), 0% (0 min)~20% (20 min)], with flow rate; 1 ml/min, column; Ultron VX-ODS columns [150 x 4.6 mm, 5 μm; Shinwa Co. (Kyoto, Japan)].
Table 4. Melting temperatures and thermodynamic parameters of DNA duplexes containing O:N and G:C base-pairing

<table>
<thead>
<tr>
<th>Type</th>
<th>Sequence</th>
<th>X</th>
<th>N</th>
<th>Tm  (ºC)</th>
<th>∆Tm (ºC)</th>
<th>−ΔHm (kcal mol⁻¹)</th>
<th>−ΔS°m (cal mol⁻¹ K⁻¹)</th>
<th>−ΔG°m (kcal mol⁻¹)</th>
<th>−ΔG°m (kcal mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-CGX-</td>
<td>5′-TCT CCT CCG</td>
<td>O</td>
<td>C</td>
<td>64.49</td>
<td>−6.83</td>
<td>130.396</td>
<td>371</td>
<td>15.293</td>
<td>2.643</td>
</tr>
<tr>
<td>-GGX-</td>
<td>3′-AGA GAA GGG</td>
<td>O</td>
<td>G</td>
<td>62.57</td>
<td>−8.75</td>
<td>105.994</td>
<td>303</td>
<td>11.889</td>
<td>6.047</td>
</tr>
<tr>
<td>-TXX-</td>
<td>5′-TCT CCT CTA</td>
<td>O</td>
<td>T</td>
<td>61.15</td>
<td>−10.17</td>
<td>126.664</td>
<td>365</td>
<td>13.494</td>
<td>4.442</td>
</tr>
<tr>
<td>-ANT-</td>
<td>3′-AGA GAA GAN</td>
<td>O</td>
<td>A</td>
<td>62.68</td>
<td>−8.64</td>
<td>113.296</td>
<td>323</td>
<td>13.073</td>
<td>4.863</td>
</tr>
<tr>
<td></td>
<td>GXX TCA GGA</td>
<td>G</td>
<td>C</td>
<td>71.32</td>
<td>0</td>
<td>135.595</td>
<td>379</td>
<td>17.936</td>
<td>0</td>
</tr>
</tbody>
</table>

aTm was measured in case that the total concentrations of ODNs is 4 µM.
bTm was calculated by Tm−Tm(G:C).
cΔG°m was calculated by ΔHm−(310.15 × ΔS°).
dΔG°m was calculated by ΔG°m(G:C)−ΔG°m(O:C).

CD data were almost identical to each other, indicating that such Oxa-pairing with normal bases does not evoke any severe influence on the whole conformation of DNA duplex. However, the local structure around Oxa in DNA duplex might be affected by the base-pair type correlated to thermodynamic stability of Oxa-containing DNA duplex (Oxa-DNA duplex).

For analyzing the thermodynamic stability of Oxa-DNA duplex, melting temperature and thermodynamic parameters were obtained using the prepared Oxa-ODNs as summarized in Table 4. In a previous report (13), we have already shown the melting temperatures (Tm) for Oxa-containing oligothymidinede, d(T2OT6), which was prepared by the HPLC purification of HNO2-treated d(T2GT6), the Tm for the duplexes of d(T2OT6)/d(A2NA6) (N = C, G, A and T) were 14.1~19.3°C, which were much lower than that of d(T2GT6)/d(A2CA6) (Tm = 32.8°C). Although these were the Tm values reported for Oxa-DNA duplex for the first time, the thermodynamic properties of Oxa-DNA duplex have not been elucidated in detail because of the low Tm values.

As represented in Table 4, various duplex formations between Oxa-ODNs and several complementary ODNs (the total concentrations of ODNs; 4 µM) were analyzed. In the cases of -CGG-/-GCC- type DNA duplexes, Tm of O:C, O:T, O:G and O:A were 64.49, 61.15, 62.57 and 62.68°C, respectively. Compared with a perfectly matched DNA duplex (Tm of -CGG-/-GCC--; 71.32°C), the differences of Tm [ΔTm = Tm (G:C) − Tm (O:N)] were 6.83°C (O:C), 10.17°C (O:T), 8.75°C (O:G) and 8.64°C (O:A). In the cases of -TOA-/-ANT- types, Tm values were 56.80°C (O:C), 55.37°C (O:T), 53.31°C (O:G) and 54.51°C (O:A). Compared with Tm of G:C (66.72°C), Tm values of O:C, O:T, O:G and O:A were lower by 9.92, 11.35, 13.41 and 12.21°C, respectively. These differences are similar to the common difference reported for one mismatch (i.e. the cases of G:T, G:G and G:A). Among them, O:C duplexes made relatively stable formations compared with other combinations of Oxa base-pairings.

After repeatedly measuring the Tm values in various total concentrations (Ct), the thermodynamic parameters were obtained for each duplex, as listed in Table 4. Then, ΔG°m (i.e. ΔHm−(310.15 × ΔS°)) were compared for analyzing the stability patterns of DNA duplex in 37°C according to Oxa base-pairings. In cases of -CGX-/-GNC- type, ΔG°m values were 15.293 (O:C), 13.494 (O:T), 15.980 (O:G) and 13.073 kcal mol⁻¹ (O:A). Their differences [ΔΔG°m = ΔG°m(G:C) − ΔG°m(C:G)] were 2.643 (O:C), 4.442 (O:T), 6.047 (O:G) and 4.863 (O:A) kcal mol⁻¹. The order of Oxa-pairing stabilities in -CGX-/-GNC- DNA duplex are O:C > O:T > O:A > O:G. The cases of -TOA-/-ANT- types showed the similar results to those of -CGX-/-GNC- ΔG°m values of O:C, O:T, O:G and O:A were 12.332, 11.118, 10.191 and 10.563 kcal mol⁻¹, respectively, and ΔΔG°m were 3.648, 4.862, 5.789 and 5.417 kcal mol⁻¹, respectively. Oxa-pairing stabilities in -TOA-/-ANT- type follows the same orders of -CGX-/-GNC- type as O:C > O:T > O:A > O:G.

Although all the base-matches of Oxa are less stable than a G:C perfect match in DNA duplex, Oxa forms relatively stable base-pairing with Cyt compared with other matches with Thy, Gua and Ade. As proposed in previous reports (13,14), Oxa with an acceptor–acceptor–donor configuration can form two hydrogen bondings with Cyt. This result suggested that Oxa would maintain relatively stable interaction with Cyt without severe influence on the local structure of DNA duplex even after Oxa is formed from Gua. It is probable that such a base-pairing stability of O:C would account for the recent results of repair enzyme responses, i.e. the general BER system is not effective for repairing Oxa in DNA strands (15,21). This is because BER enzyme might not easily recognize (and hydrolyze) Oxa due to the relative stable base-pairing with Cyt in DNA duplex (15,21).

In a previous paper, O:T pairs had been expected to show similar stability to that of O:C because Oxa might also have two possible hydrogen bondings with Thy. However, the expected hydrogen bonding between ring oxygen of Oxa and the imino proton of Thy would be weak or could not be formed because the ring oxygen atom of Oxa base has an sp² hybrid orbital and the lone pairs of electrons of oxygen exist out of plane. As represented in Table 4, O:T pairs in DNA duplex showed low thermodynamic stability compared to O:C pairs. In the cases of O:A and O:G base-pairings, low stabilities were also observed compared with O:C pairs indicating that Oxa has a very low chance to make hydrogen-bonding with Ade and Gua. However, O:A was observed to show better...
thermodynamic stability than O:G and similar stability to O:T.

In the previous reports involving base-incorporation and relevant mutagenesis, Oxa in DNA strand has been found to induce the misincorporation of Thy and Ade opposite Oxa, which may mediate the mutagenic conversion of G:C to A:T during the DNA polymerization (14,19,20). Thermodynamic stabilities of Oxa base-pairing, presented here, show a similar trend to the incorporation efficiency of four normal bases opposite Oxa in a DNA strand; O:C > O:T > O:A > O:G (14,19).

CONCLUSION

Since Oxa is one of the major damaged bases generated from guanine by NO- or HNO 2 -induced nitrosative deamination, Oxa has been analyzed in terms of its biochemical and biophysical properties (1,8,9,13–21,26). Although several papers have been reported previously, Oxa-related research still needs to be further investigated, especially, Oxa-related genotoxicity and repair mechanisms in cellular systems. To achieve these, there has been a need to establish a conventional method by which Oxa-ODN with desired base sequence can be prepared in large quantities. In this study, we have developed chemical synthesis procedures for Oxa-ODNs. For obtaining DMT-protected phosphoramidite of Oxa (DMT-dOxo-amidite), we have established the following three-steps procedures: (i) nitrosation of dGuo by acidic nitrite condition (dOxo; 15% yield), (ii) 5'-O-selective tritylation of dOxo on the basis of the method reported previously (22) (DMT-dOxo; 70% yield), (iii) phosphoramidation of DMT-dOxo (DMT-dOxo-amidite; 72.5% yield). Prepared DMT-dO-amidite was successfully used for synthesizing Oxa-ODN with a coupling efficiency of over 93%. Enzymatic digestion and spectroscopic data including NMR and mass spectroscopy supported that the incorporated Oxa is in an intact form in the ODN. Also, we have analyzed the thermodynamic properties of Oxa in DNA strands. Melting temperature (Tm) and thermodynamic parameters (ΔH°T, ΔS°T, and ΔG°T) were obtained to observe the thermodynamic stability of DNA duplexes containing Oxa-base pairings. All the duplexes containing O:N base pairs showed Tm values lower than that of a G:C perfect match by 6.83–10.17°C in 5'-COG-3' type duplexes and 9.92–13.41°C in 5'-TOA-3' types. Using ΔG°T, it was observed that all the DNA duplexes with O:N base pairs showed less thermodynamic stability than that of G:C by 2.643–6.047 kcal mol⁻¹. By comparing Tm and ΔG°T values, it has been found that O:N pairs have a relatively higher stability in DNA duplex than O:T, O:A and O:G.

Xan, another main damaged base of nitrosated Gua, has been investigated in terms of its biochemical role in DNA strands using the chemically synthesized Xan-containing ODN (27,28). However, in the case of Oxa, there has been no efficient method for the preparation of Oxa-ODN. To reveal the whole mechanism of NO-induced genotoxicity or cytotoxicity, it is also essential to study more detailed roles of both Oxa and Xan in cellular systems. Especially, a large amount of the designed Oxa-ODNs is required for analyzing Oxa-induced DPC formation and Oxa-response cellular events. Therefore, this paper introduces the total chemical synthesis procedure of Oxa-ODNs and thermodynamic characteristics of Oxa-ODNs for the first time. These results shown here will be helpful for elucidating the biological significance of Oxa related to genotoxic and repair mechanisms. Moreover, since Oxa possesses a kind of carboxylate function, Oxa-ODN can be used as one of the functional DNA oligomers, which can make covalent cross-linkage with amine or amine-containing biomolecules. Therefore, the results obtained here will be applied to extending the biotechnological application of Oxa-ODN, for example, novel biomolecule conjugations or nano-biostructure fabrication.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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