Synthesis and Evaluation of Artificial Nucleic Acid Bearing an Oxanorbornane Scaffold

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Abstract: Natural oligonucleotides have many rotatable single bonds, and thus their structures are inherently flexible. Structural flexibility leads to an entropic loss when unwound oligonucleotides form a duplex with single-stranded DNA or RNA. An effective approach to reduce such entropic loss in the duplex-formation is the conformational restriction of the flexible phosphodiester linkage and/or sugar moiety. We here report the synthesis and biophysical properties of a novel artificial nucleic acid bearing an oxanorbornane scaffold (OxNorNA), where the adamant oxanorbornane was expected to rigidify the structures of both the linkage and sugar parts of nucleic acid. OxNorNA phosphoramidite with a uracil (U) nucleobase was successfully synthesized over 15 steps from a known sugar-derived cyclopentene. Thereafter, the given phosphoramidite was incorporated into the designed oligonucleotides. Thermal denaturation experiments revealed that oligonucleotides modified with the conformationally restricted OxNorNA-U properly form a duplex with the complementally DNA or RNA strands, although the Tm values of OxNorNA-U-modified oligonucleotides were lower than those of the corresponding natural oligonucleotides. As we had designed, entropic loss during the duplex-formation was reduced by the OxNorNA modification. Moreover, the OxNorNA-U-modified oligonucleotide was confirmed to have extremely high stability against 3’-exonuclease activity, and its stability was even higher than those of the phosphorothioate-modified counterparts (Sp and Rp). With the overall biophysical properties of OxNorNA-U, we expect that OxNorNA could be used for specialized applications, such as conformational fixation and/or bio-stability enhancement of therapeutic oligonucleotides (e.g., aptamers).

Keywords: modified nucleic acid; modified oligonucleotide; oxanorbornane; conformational restriction; entropy; duplex-forming ability; mismatch discrimination; nuclease resistance

1. Introduction

The structural flexibility of natural oligonucleotides contributes to the formation of various higher-order structures. However, when unwound oligonucleotides form a specific structure (e.g., duplex), a large entropic loss generally arises. Thus, chemical modifications that restrict the structures of flexible phosphodiester linkages and/or sugar moieties to those seen in A-form DNA-RNA duplexes
have a useful role in producing high-affinity (i.e., potent) antisense oligonucleotides [1–3]. In our laboratory, a number of conformationally restricted artificial nucleic acids, for which a representative example is 2′,4′-bridged nucleic acid (2′,4′-BNA, commonly called locked nucleic acid (LNA)) (Figure 1), have been developed [4–8]. 2′,4′-BNA/LNA has an N-type sugar pucker commonly seen in the A-form duplex, and thus the 2′,4′-BNA/LNA-modified oligonucleotides exhibit a high duplex-forming ability toward single-stranded RNA (ssRNA). Leumann and co-workers have demonstrated that oligonucleotides modified with tricyclic nucleic acids, in which a fused ring system rigidifies the torsion angles γ and δ, exhibit increased ssRNA affinity relative to the natural oligonucleotides [9–12]. More recently, Heness and co-workers have successfully achieved restrictions of both the sugar ring and the torsion angle γ by designing α-L-triNA 1 [13]. Oligonucleotides containing α-L-triNA 1 are known to have a high binding affinity toward ssRNA.

Throughout our research on conformational restrictions of nucleic acids, we newly designed an artificial nucleic acid bearing an oxanorbornane scaffold (OxNorNA) (Figure 2). Oxanorbornane, 2-oxabicyclo[2,2,1]heptane, has a rigid structure, and thus the relative position of the phosphodiester linkage and nucleobase parts of OxNorNA can be strictly fixed. The linkage part of OxNorNA is considered a 4′–2′ system based on the position of nucleobase. This type of linkage system is rarely seen in the other artificial nucleic acids, although the effects of 5′–2′ and 3′–2′ linkage systems (isoDNA and TNA, respectively) on the duplex-forming ability and antisense potency have been intensely investigated [14–17]. Herein, we report a robust synthetic route for OxNorNA phosphoramidite with a uracil (U) nucleobase and the biophysical properties (duplex-forming ability, mismatch discrimination, and nuclease resistance) of OxNorNA-U-modified oligonucleotides.

![Figure 1. Conformational restrictions of natural oligonucleotides.](image)

![Figure 2. Structures of artificial nucleic acid bearing an oxanorbornane scaffold (OxNorNA), isoDNA, and TNA.](image)

2. Results and Discussion

2.1. Synthesis of OxNorNA-U Phosphoramidite

Synthesis of OxNorNA-U phosphoramidite was started from cyclopentene 1, derived over eight steps from commercially available α-ribose [18–20] (Scheme 1). At first, two hydroxyl groups of 1 were protected by a benzyl group, and the given compound 2 was subjected to a hydroboration reaction to produce the desired cyclopentanol 3 in a regio- and diastereoselective manner. Silylation was followed by hydrogenation using Pd/C afforded diol 5. The primary alcohol of compound 5 was then selectively protected, and the remaining secondary alcohol was converted into a leaving group (OTf). Subsequent treatment of sodium azide afforded compound 7 at a 71% yield over two steps. The reduction of the
azide group, using ammonium formate with Pd/C, underwent the removal of the TES group. A uracil ring was successively formed by a reaction with 3-methoxyacryloyl isocyanate, following the reported procedure [21–23]. The direct incorporation of the uracil nucleobase to the triflated compound of 6 was unsuccessful. The hydroxyl group of 9 was then mesylated, and the acetonide and TBS group were removed under mildly acidic conditions (CeCl3·7H2O, oxalic acid, MeCN, and rt) to afford triol 11. Intramolecular cyclization of 11 by aqueous (aq.) NaOH regio-selectively afforded the desired oxanorbornane 12 at a 72% yield. The regioselectivity is explained by the difference in the uracil nucleobase steric repulsion of the two possible products. That is, 12 obtained from the SN2 reaction of the 3'-hydroxyl group of 11 has the uracil nucleobase at a less hindered position than the other product that can be produced from the SN2 reaction of the 2'-hydroxyl group. The structure of 12 was confirmed by NMR (For 1H, 13C, 1H–1H COSY, DEPT, HMQC, and HMBC NMR spectra, see Supplementary Material) and X-ray crystal structure (Figure 3). The less hindered 2'-hydroxyl group of 12 was then protected by the dimethoxytrityl (DMTr) group, and the remaining alcohol was phosphorylated to afford 14, a suitable building block for the reverse (3'-to-5') oligonucleotide synthesis. We note that tritylation of 12 by using DMTrCl in pyridine resulted in no reaction, but DMTrOTf generated from a reaction of DMTrCl and AgOTf in CH2Cl2 successfully afforded compound 13 in 93% [16,24].

![Scheme 1. Synthesis of OxNorNA-uracil (U) phosphoramidite 14. Reagents and conditions: (i) NaH, BnBr, DMF, 0 °C, 92%; (ii) thexylborane, THF, 0 °C to rt; (iii) NaBO2·H2O, H2O, 0 °C to rt, 90% over 2 steps; (iv) 3-methoxyacryloyl isocyanate, THF, –40 °C to rt; (v) HCOONH4, Pd/C, THF, rt, 98%; (vi) 2-cyanoethyl N,N-diisopropylchlorophosphoramidite, N,N-diisopropylethylamine (DIPEA), 1-methylimidazole, MeCN, 0 °C to rt, 56%.](image-url)
2.2. Oligonucleotide Synthesis

Following the conventional phosphoramidite protocol, the given amidite \( 14 \) was successfully incorporated into the designed sequences (Table 1). The coupling time for the incorporation of the synthesized phosphoramidite \( 14 \) was prolonged to 12.5 min. In addition, the detritylation time of all reverse phosphoramidites was performed in 2 min. The above conditions afforded OxNorNA-U-modified oligonucleotides \( \text{ON1–ON3} \) in 22–47% yields (for high-performance liquid chromatography (HPLC) charts of purified oligonucleotides, see Supplementary Material).

![ORTEP drawing of the X-ray crystal structure of 12 (thermal ellipsoids at the 50% probability level).](image)

**Figure 3.** ORTEP drawing of the X-ray crystal structure of 12 (thermal ellipsoids at the 50% probability level).

| ID | Sequence *a* | Yield (%) | [M−H]− | [M−H]− |
|----|--------------|-----------|--------|--------|
| ON1 | 5'-d(GCG TTX TTT GCT)-3' | 22 | 3630.4 | 3630.3 |
| ON2 | 5'-d(GCG XTX TXT GCT)-3' | 31 | 3626.3 | 3626.3 |
| ON3 | 5'-d(TTT TTT TTT X)-3' | 47 | 2977.0 | 2976.6 |

*a* \( X = \text{OxNorNA-U}. \)

2.3. Duplex-forming Ability and Thermodynamic Parameters

The thermal stabilities of the duplexes formed by the OxNorNA-U-modified oligonucleotides (\( \text{ON1 and ON2} \)) with the complementary single-stranded DNA (ssDNA) or ssRNA were then evaluated by ultraviolet (UV) melting experiments. The given melting temperatures (\( T_m \) values) and thermodynamic parameters are summarized in Tables 2 and 3 (for van’t Hoff plots, see Figures S1 and S2 in Supplementary Material). As for the ssDNA complement (sequence: \( 5'-\text{d(AGCAAAAAACGC)}-3' \)) (Table 2), \( \text{ON1} \) with a single OxNorNA modification was found to properly form a duplex. However, the \( \text{ON1-ssDNA} \) duplex furnished a \( T_m \) value of 45.4 °C, which was 4.5 °C lower than that obtained for the corresponding \( \text{ON4-ssDNA} \) duplex (\( T_m = 49.9 \) °C). Further modification to \( \text{ON1} \) with OxNorNA resulted in a very low affinity for ssDNA (21.0 °C for \( \text{ON2} \)) as compared to the natural oligonucleotide (48.4 °C for \( \text{ON5} \)). Toward the ssRNA complement (sequence: \( 5'\text{-r(AGCAAAAAACGC)}-3' \)) (Table 3), OxNorNA-modified \( \text{ON1 and ON2} \) denoted similar trends, but they were found to prefer ssRNA rather than ssDNA (see their \( \Delta T_m/\text{mod. values} \)).

The low \( T_m \) values of the OxNorNA-modified oligonucleotides might be explained by an unusual 4'–2' linkage system that was changed from the general 5'–3' linkage system found in the natural oligonucleotides. The linkage shift may lead the nucleobase of OxNorNA to an unfavorable direction for the duplex formations (for the structure comparison between OxNorNA-U and \( 2',4'\text{-BNA/LNA-U nucleosides, see reference [25]} \)). Thermodynamic data obtained by van’t Hoff plots indicated that duplex formations by the OxNorNA-modified oligonucleotides were enthalpically unfavorably (e.g.,
base stacking), and entropy factors (e.g., structural restriction) had no compensatory effects on the duplex formations.

Table 2. \( T_m \) values and thermodynamic parameters of duplexes formed between oligonucleotides and the complementary single-stranded DNA (ssDNA) \(^a\).

| ID   | Sequence \(^b\)       | \( T_m(\Delta T_m/\text{mod.}) \) \(^c\) [°C] | \( \Delta H^\circ \) [kcal/mol] | \( \Delta S^\circ \) [kcal/mol] | \( \Delta G^\circ \) [kcal/mol] |
|------|------------------------|---------------------------------------------|---------------------------------|---------------------------------|---------------------------------|
| ON4  | 5\'d(GCG TTU TTG GCT)-3\' | 49.9                                        | -108.6                         | -310.1                          | -12.4                           |
| ON1  | 5\'d(GCG TTX TTG GCT)-3\' | 45.4 (-4.5)                                 | -76.4                          | -214.8                          | -9.8                            |
| ON5  | 5\'d(GCG UTU TUT GCT)-3\' | 48.4                                        | -81.4                          | -227.2                          | -11.0                           |
| ON2  | 5\'d(GCG TXT TXT GCT)-3\' | 21.0 (-9.1)                                 | -42.0                          | -117.2                          | -5.6                            |

\(^a\) Conditions: 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl and 4 \( \mu \)M of each oligonucleotide. \( T_m \) values are averages of at least three measurements. The sequence of ssDNA is 5\'d(AGCAAAAACGGC)-3\'. \( \Delta T_m/\text{mod.} \): the change in \( T_m \) value (\( \Delta T_m \)) per modification compared to the unmodified oligonucleotide. \( \Delta G^\circ \) values at 37 °C are shown. For thermodynamic parameters and van't Hoff plots, see Table S1 and Figure S1 in Supplementary Material.

\(^b\) \( X = \text{OxNorNA-U} \).

Table 3. \( T_m \) values and thermodynamic parameters of duplexes formed between oligonucleotides and the complementary single-stranded RNA (ssRNA) \(^a\).

| ID   | Sequence \(^b\)       | \( T_m(\Delta T_m/\text{mod.}) \) \(^c\) [°C] | \( \Delta H^\circ \) [kcal/mol] | \( \Delta S^\circ \) [kcal/mol] | \( \Delta G^\circ \) [kcal/mol] |
|------|------------------------|---------------------------------------------|---------------------------------|---------------------------------|---------------------------------|
| ON4  | 5\'d(GCG TTU TTG GCT)-3\' | 46.5                                        | -94.2                           | -268.3                          | -11.0                           |
| ON1  | 5\'d(GCG TTX TTG GCT)-3\' | 42.4 (-4.1)                                 | -92.0                           | -265.9                          | -9.5                            |
| ON5  | 5\'d(GCG UTU TUT GCT)-3\' | 44.7                                        | -86.8                           | -246.9                          | -10.2                           |
| ON2  | 5\'d(GCG TXT TXT GCT)-3\' | 24.7 (-6.7)                                 | -56.3                           | -163.6                          | -5.5                            |

\(^a\) Conditions: 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl and 4 \( \mu \)M of each oligonucleotide. \( T_m \) values are averages of at least three measurements. The sequence of ssRNA is 5\'r(AGCAAAAACGGC)-3\'. \( \Delta T_m/\text{mod.} \): the change in \( T_m \) value (\( \Delta T_m \)) per modification compared to the unmodified oligonucleotide. \( \Delta G^\circ \) values at 37 °C are shown. For thermodynamic parameters and van't Hoff plots, see Table S2 and Figure S2 in Supplementary Material.

\(^b\) \( X = \text{OxNorNA-U} \).

2.4. Mismatch Discrimination

We also evaluated mismatch discrimination of the OxNorNA-U-modified oligonucleotide. As shown in Tables 4 and 5, OxNorNA-U-modified ON1 exhibited much lower \( T_m \) values toward the one-base-mismatched sequences (\( N = G, C, T, \) and \( U \)) than toward the fully matched sequence (\( N = A \)). The base discrimination patterns of OxNorNA-U-modified ON1 were similar to the natural counterpart (ON4). This result indicated that a single modification with the adamant OxNorNA does not interfere with the common base-pairing rules, even the linkage moiety shifts from the natural nucleic acid.

Table 4. Mismatch discrimination of natural and OxNorNA-modified oligonucleotides toward ssDNA \(^a\).

| ID   | Sequence \(^b\)       | \( T_m(\Delta T_m = T_m[\text{mismatch}] - T_m[\text{match}]) \) [°C] |
|------|------------------------|--------------------------------------------------|
| ON4  | 5\'d(GCG TTU TTG GCT)-3\' | 49.9                                            |
|      |                         | 35.5 (-14.4)                                   |
|      |                         | 38.5 (-11.4)                                   |
|      |                         | 36.5 (-13.4)                                   |
| ON1  | 5\'d(GCG TTX TTG GCT)-3\' | 45.4                                            |
|      |                         | 33.2 (-12.2)                                   |
|      |                         | 36.0 (-9.4)                                    |
|      |                         | 32.5 (-12.9)                                   |

\(^a\) Conditions: 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl and 4 \( \mu \)M of each oligonucleotide. \( T_m \) values are averages of at least three measurements. The sequence of ssDNA is 5\'d(AGCAAAAACGGC)-3\'.

\(^b\) \( X = \text{OxNorNA-U} \).
Table 5. Mismatch discrimination of natural and OxNorNA-modified oligonucleotides toward ssRNA.

| ID  | Sequence b | T_m (ΔT_m = T_m [mismatch] − T_m [match]) [°C] |
|-----|------------|-----------------------------------------------|
|     |            | N = A  | N = G  | N = C  | N = U  |
| ON4 | 5′-d(GCG TTX TTT GCT)-3′ | 46.5   | 31.9 (−14.6) | 41.4 (−5.1) | 32.6 (−13.9) |
| ON1 | 5′-d(GCG TTX TTT GCT)-3′ | 42.4   | 27.8 (−14.6) | 35.1 (−7.3) | 27.6 (−14.8) |

a Conditions: 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl and 4 µM of each oligonucleotide. T_m values are averages of at least three measurements. The sequence of ssRNA is 5′-(AGCAAAAAACGC)-3′.

b X = OxNorNA-U.

2.5. Circular Dichroism Analysis

To analyze the structure of OxNorNA-U-modified oligonucleotide in a single-stranded or duplex form, circular dichroism (CD) spectra were recorded at 10 °C in the same buffer as that used for the UV melting experiments (Figure 4). As a result, ON1 with one OxNorNA modification showed similar CD spectra to the natural ON4 (Figure 4a,b); thus, ON1 and ON4 have similar secondary structures in the presence or absence of complementary strands (ssDNA and ssRNA). From the CD spectra in Figure 4b, ON1·ssRNA and ON4·ssRNA duplexes were found to form a typical A-form duplex (positive cotton band at 260 nm and negative cotton band at 210 nm). In contrast to the results in Figure 4a,b, ON2·ssDNA and ON2·ssRNA duplexes with multiple OxNorNA modifications displayed much weaker cotton effects than the corresponding ON5·ssDNA and ON5·ssRNA duplexes (Figure 4c,d). These results probably indicated disrupted base stacking in ON2·ssDNA and ON2·ssRNA duplexes and thus resulted in duplex destabilizations (low T_m values).

![Circular dichroism (CD) spectra of OxNorNA-modified oligonucleotides](image1)

Figure 4. Circular dichroism (CD) spectra of OxNorNA-modified oligonucleotides (ON1 and ON2) and their natural counterparts (ON4 and ON5) in the presence or absence of a complementary strand (ssDNA or ssRNA). (a) ON1 and ON4 in the presence or absence of ssDNA; (b) ON1 and ON4 in the presence or absence of ssRNA; (c) ON2 and ON5 in the presence or absence of ssDNA; (d) ON2 and ON5 in the presence or absence of ssRNA. Conditions: 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl and 4 µM of each oligonucleotide at 10 °C. The sequences of the target ssDNA and ssRNA are 5′-d(AGCAAAAAACGC)-3′ and 5′-(AGCAAAAAACGC)-3′, respectively.

2.6. Stability Against Nuclease Digestion

We next investigated the effect of OxNorNA modification on the stability of oligonucleotides against enzymatic degradation. Oligonucleotides with 3′-terminal modifications (sequence: 5′-d(TTT
TTT TTT X)-3’, X = OxNorNA-U (ON3); X = 5’-(R)-phosphorothioate (PS)-modified thymidine (ON6); X = 5’-(S)-PS-modified thymidine (ON7); X = locked nucleic acid (LNA) (ON8)) were prepared, incubated with 0.133 ug/mL 3’-exonuclease (snake venom phosphodiesterase, svPDE) at 37 °C, and the percentage of the remaining intact oligonucleotides was monitored by HPLC (Figure 5). Under the conditions we tested, the oligonucleotide modified with LNA (ON8) was digested within 20 min. Commonly used phosphorothioate (PS) modifications were found to be effective for improving the oligonucleotide stability against nuclease, but more than 30% of ON6 (Sp) and ON7 (Rp) were cleaved at 80 min. Conversely, over 80% of OxNorNA-modified ON3 was still intact after 80 min. It was expected that OxNorNA had successfully escaped from the nuclease recognition since it had an altered scaffold from the natural nucleoside and an unusual 4’–2’ linkage system.

Figure 5. Enzymatic stability of the OxNorNA-modified oligonucleotide. Conditions: 0.133 ug/mL snake venom phosphodiesterase (svPDE), 10 mM MgCl₂, 50 mM Tris-HCl (pH 8.0), and 2 µM each oligonucleotide at 37 °C. The sequence of the oligonucleotides used was 5’-d(TTT TTT TTT X)-3’. X = OxNorNA-U (red diamond, ON3), X = 5’-(S)-phosphorothioate (PS)-modified thymidine (orange square, ON6), X = 5’-(R)-PS-modified thymidine (gray triangle, ON7), and X = locked nucleic acid (LNA)-T (blue cross, ON8).

3. Materials and Methods

3.1. General Information

Reagents and solvents were purchased from commercial suppliers and used without purification unless otherwise specified. All experiments involving air and/or moisture-sensitive compounds were carried out under an Ar atmosphere. All reactions were monitored with analytical thin-layer chromatography (TLC; silica gel coated with fluorescent indicator F254; Merck, Darmstadt, Germany). Flash column chromatography was carried out using EPCLC–W–Prep 2XY (Yamazen, Osaka, Japan). NMR spectra were recorded on JNM-ECA-500, JNM-ECS-400, and JNM-ECS-300 spectrometers (JEOL, Akishima, Japan) using CDCl₃ or DMSO–d₆ as the solvent with tetramethylsilane (TMS) as an internal standard. For 31P NMR, 5% H₃PO₄ in D₂O (0.00 ppm) was used as an external standard. Infrared (IR) spectra we re recorded on an FT/IR–4200 spectrophotometer (JASCO, Hachioji, Japan). Optical rotations were recorded on a P–2200 instrument (JASCO). Mass spectra of all new compounds were measured on a SpiralTOF JMS-S3000 (for electrospray ionization, ESI) or a JMS-700 instrument (JEOL) (for fast atom bombardment, FAB). Solid-phase oligonucleotide synthesis was performed using an nS–8 Oligonucleotide Synthesizer (GeneDesign, Ibaraki, Japan). Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectra were recorded on an ultralifeXtreme or an Autoflex II mass spectrometer (Bruker Daltonics, Billerica, MA, USA). For HPLC, Shimadzu DGU-20A₃R, LC-20AD, CBM-20A, CTO-20AC, SPD-20A, and FRC-10A were utilized. For UV absorbance measurements, a UV-1800 spectrometer (Shimadzu, Kyoto, Japan) was utilized. UV melting experiments were performed using a UV–1650 or UV–1800 UV–Vis spectrophotometer equipped with a TMSPC–8 Tₘ analysis accessory (Shimadzu).
3.2. Synthesis of Compound 2

Compound 1 (3.0 g, 16.1 mmol) was dissolved in dry DMF (160 mL), and the solution was cooled in an ice bath. Sodium hydride (50% w/w in mineral oil, 2.30 g) was carefully added to the solution, and the resulting mixture was stirred for 30 min. Benzyl bromide (5.7 mL, 48.3 mmol) was then added dropwise, and the mixture was further stirred for 2 h at 0 °C. After the reaction was finished, the mixture was partitioned between CH₂Cl₂ and H₂O. The separated organic layer was washed with brine, dried over Na₂SO₄, and concentrated in vacuo. The residue was purified by silica gel column chromatography, eluted with hexane/AcOEt (7:1), to give compound 2 as a colorless oil (5.4 g, 92%). IR (KBr): νmax 3031, 2985, 2933, 2858, 1605, 1496, 1454 cm⁻¹; [α]D25 = −98.3 (c 1.00, MeOH); ¹H-NMR (500 MHz, CDCl₃): δ 7.40–7.25 (10H, m), 5.88–5.86 (1H, m), 4.94 (1H, dd, J = 5.7, 1.2 Hz), 4.85 (1H, d, J = 12.0 Hz), 4.77 (1H, dd, J = 5.7, 5.2 Hz), 4.59 (1H, d, J = 12.0 Hz), 4.54 (1H, d, J = 11.5 Hz), 4.48 (1H, d, J = 11.5 Hz), 4.33–4.35 (1H, m), 4.15 (2H, s), 1.46 (3H, s), 1.40 (3H, s); ¹³C-NMR (125 MHz, CDCl₃): δ 144.4, 138.4, 138.0, 128.0, 128.3, 128.2, 127.9, 127.8, 127.6, 127.5, 112.1, 82.3, 79.7, 77.6, 72.8, 71.9, 66.5, 27.7, 26.7; MS (FAB) m/z 389 [M + Na]⁺; HRMS (FAB) Calcd. for C₂₃H₂₉O₅Na [M + Na]⁺: 389.1729; found: 389.1731.

3.3. Synthesis of Compound 3

Compound 2 (6.9 g, 18.9 mmol) was dissolved in dry THF (189 mL), and the solution was cooled in an ice bath. The xylylborane (0.5 M in THF, 120 mL) was added dropwise to the solution, and the resulting mixture was stirred for 3 h at room temperature. After TLC indicated the consumption of the starting material, the mixture was cooled in an ice bath and was added to NaBO₂·4H₂O (14.6 g, 95 mmol) and H₂O (60 mL). The mixture was stirred for 1 h at room temperature, diluted with AcOEt, and washed with H₂O. The organic layer was further washed with brine, dried over Na₂SO₄, and concentrated in vacuo. The residue was purified by silica gel column chromatography, eluted with hexane/AcOEt (3:1), to give compound 3 as a colorless oil (6.5 g, 90%). IR (KBr): νmax 3499, 3063, 3031, 2984, 2928, 2917, 2867, 1497, 1454 cm⁻¹; [α]D23 = −65.5 (c 1.00, MeOH); ¹H-NMR (500 MHz, CDCl₃): δ 7.35–7.25 (10H, m), 4.78 (1H, d, J = 12.6 Hz), 4.64 (1H, dd, J = 7.5, 4.6 Hz), 4.46–4.53 (3H, m), 4.44 (1H, dd, J = 6.9, 2.3 Hz), 4.26–4.30 (1H, m), 3.98 (1H, dd, J = 5.2, 4.6 Hz), 3.73–3.76 (2H, m), 2.43 (1H, d, J = 2.3 Hz), 2.24–2.32 (1H, m), 1.43 (3H, s), 1.31 (3H, s); ¹³C-NMR (125 MHz, CDCl₃): δ 138.4, 138.0, 128.4, 128.2, 127.8, 127.7, 127.6, 127.5, 113.0, 86.1, 80.1, 78.0, 77.7, 73.4, 72.8, 68.4, 50.1, 25.9, 24.5; MS (FAB) m/z 385 [M + H]⁺; HRMS (FAB) Calcd. for C₂₃H₂₉O₅Na [M + Na]⁺: 385.2015; found: 385.2016.

3.4. Synthesis of Compound 4

Compound 3 (3.00 g, 7.81 mmol) was dissolved in dry DMF (80 mL), and imidazole (1.60 g, 23.4 mmol) and tert-butylidimethylchlorosilane (2.36 g, 15.6 mmol) were added. The resulting mixture was stirred for 1 h at room temperature. After the reaction was finished, the mixture was diluted with AcOEt and washed with saturated aq. NaHCO₃. The organic layer was washed with brine, dried over Na₂SO₄, and concentrated in vacuo. The residue was purified by silica gel column chromatography, eluted with hexane/AcOEt (8:1), to give compound 4 as a colorless oil (2.9 g, 74%). IR (KBr): νmax 3088, 3064, 3031, 2979, 2928, 2856, 1633, 1605, 1471, 1462, 1532 cm⁻¹; [α]D25 = −37.2 (c 1.00, MeOH); ¹H-NMR (500 MHz, CDCl₃): δ 7.23–7.36 (10H, m), 4.68–4.72 (1H, m), 4.51–4.64 (2H, m), 4.52 (1H, d, J = 12.1 Hz), 4.44 (1H, d, J = 12.1 Hz), 4.29–4.31 (1H, m), 4.11–4.13 (1H, m), 4.05–4.09 (1H, m), 3.77 (1H, dd, J = 9.2, 6.3 Hz), 3.62 (1H, dd, J = 9.2, 8.6 Hz), 2.50–2.38 (1H, m), 1.41 (3H, s), 1.28 (3H, s), 0.85 (9H, s), 0.08 (3H, s), 0.06 (3H, s); ¹³C-NMR (125 MHz, CDCl₃): δ 138.7, 138.4, 128.3, 127.9, 127.6, 127.6, 127.4, 112.0, 86.4, 79.5, 77.8, 76.9, 76.6, 73.0, 72.6, 67.2, 50.6, 25.8, 25.7, 25.6, 24.0, 17.9, −4.8, −5.0; MS (FAB) m/z 499 [M + H]⁺; HRMS (FAB) Calcd. for C₂₃H₂₉O₅Si [M + H]⁺: 499.2880; found: 499.2884.
3.5. Synthesis of Compound 5

Compound 4 (2.87 g, 5.75 mmol) was dissolved in EtOH (60 mL), and ammonium formate (1.60 g, 25.4 mmol) and 20% Pd(OH)2/C (500 mg) were added. The resulting suspension was refluxed for 3 h. The reaction mixture was cooled to room temperature and filtered through a Celite pad. The filtrate was concentrated in vacuo, and the resulting residue was purified by silica gel column chromatography, eluted with hexane/AcOEt (1:4), to give compound 5 as a colorless oil (1.7 g, 91%). IR (KBr): νmax 3427, 2954, 2932, 2858, 1633, 1472, 1463 cm⁻¹; [α]D 26°-44.0 (c 1.00, MeOH); 1H-NMR (500 MHz, CDCl₃): δ 4.56 (1H, dd, j = 7.5, 5.2 Hz), 4.41 (1H, dd, j = 7.5, 3.4 Hz), 4.28 (1H, dd, j = 9.2, 3.5 Hz), 4.18–4.23 (1H, m), 3.81–3.93 (2H, m), 2.71–2.75 (1H, m), 1.97–2.03 (1H, m), 1.51 (3H, s), 1.34 (3H, s), 0.89 (9H, t, J = 7.5, 3.4 Hz), 3.95 (1H, dd, J = 9.2, 4.0 Hz), 3.61–3.59 (2H, m), 1.87–1.81 (1H, m), 1.38 (3H, s), 1.21 (3H, s), 0.89 (9H, t, J = 8.0 Hz), 0.81 (9H, s), 0.53 (6H, q, J = 8.0 Hz), 0.03 (3H, s), 0.00 (3H, s); 13C-NMR (125 MHz, CDCl₃): δ 112.9, 86.9, 79.0, 69.8, 59.7, 54.2, 25.9, 25.7, 24.3, 17.9, 6.7, 4.3, −4.7, −5.1; MS (FAB) m/z 319 [M + H]+; HRMS (FAB) Calcd. for C₁₃H₃₁O₅Si [M + H]+: 319.1941; found: 319.1950.

3.6. Synthesis of Compound 6

Compound 5 (2.90 g, 9.09 mmol) was dissolved in dry CH₂Cl₂ (90 mL), and 2,6-lutidine (3.2 mL, 27.3 mmol) was added. The mixture was cooled to −78 °C, and then chlorotriethylsilane (1.68 g, 10.0 mmol) was added dropwise. The resulting mixture was stirred for 1 h at −78 °C. The reaction was quenched with an addition of MeOH and diluted with CH₂Cl₂. The mixture was partitioned between CH₂Cl₂ and saturated aq. NaHCO₃. The organic layer was concentrated in vacuo, and the resulting residue was purified by silica gel column chromatography, eluted with hexane/AcOEt (8:1), to give compound 6 as a colorless oil (3.39 g, 86%). IR (KBr): νmax 3530, 2954, 2877, 2856 cm⁻¹; [α]D 22°−32.6 (c 1.00, MeOH); 1H-NMR (500 MHz, CDCl₃): δ 4.57 (1H, dd, j = 7.5, 5.2 Hz), 4.36 (1H, dd, j = 6.9, 2.3 Hz), 4.22 (1H, dd, j = 8.6, 4.6 Hz), 3.92–4.00 (2H, m), 3.69 (1H, dd, j = 9.8, 5.7 Hz), 2.66–2.68 (1H, m), 2.08–2.16 (1H, m), 1.50 (3H, s), 1.32 (3H, s), 0.96 (9H, t, J = 8.0 Hz), 0.88 (9H, s), 0.60 (6H, q, J = 8.0 Hz), 0.08 (3H, s), 0.05 (3H, s); 13C-NMR (125 MHz, CDCl₃): δ 113.6, 87.1, 78.3, 71.4, 60.3, 53.0, 26.0, 25.7, 24.6, 17.9, −4.7, −5.2; MS (FAB) m/z 433 [M + H]+; HRMS (FAB) Calcd. for C₂₁H₄₄O₅Si₂ [M + H]+: 433.2806; found: 433.2804.

3.7. Synthesis of Compound 7

Compound 6 (3.20 g, 7.37 mmol) was dissolved in dry pyridine (74 mL), and the solution was cooled in an ice bath. Trifluoromethanesulfonic anhydride (1.5 mL) in dry CH₂Cl₂ (20 mL) was added dropwise, and the reaction mixture was stirred for 1 h at 0 °C. After the addition of water, the resulting mixture was extracted with CH₂Cl₂. The organic layer was washed with brine, dried over Na₂SO₄, and concentrated in vacuo. The crude product was co-evaporated with toluene three times and then used immediately for the next reaction without further purification. The triflate was dissolved in dry DMF (74 mL), and sodium azide (1.44 g, 22.1 mmol) was added. The resulting mixture was stirred for 8 h at room temperature. After the addition of water, the resulting mixture was extracted with AcOEt. The organic layer was washed with brine, dried over Na₂SO₄, and concentrated in vacuo. The resulting residue was purified by silica gel column chromatography, eluted with hexane/AcOEt (12:1), to give compound 7 as a colorless oil (2.40 g, 71% in 2 steps). IR (KBr): νmax 2879, 2103 cm⁻¹; [α]D 28°+27.1 (c 1.00, MeOH); 1H-NMR (500 MHz, CDCl₃): δ 4.38 (1H, dd, j = 8.0, 5.2 Hz), 4.27 (1H, dd, j = 7.5, 3.4 Hz), 3.95 (1H, dd, j = 9.2, 4.0 Hz), 3.63 (1H, dd, j = 5.2, 10.3 Hz), 3.61–3.59 (2H, m), 1.87–1.81 (1H, m), 1.38 (3H, s), 1.21 (3H, s), 0.89 (9H, t, J = 8.0 Hz), 0.81 (9H, s), 0.53 (6H, q, J = 8.0 Hz), 0.03 (3H, s), 0.00 (3H, s); 13C-NMR (125 MHz, CDCl₃): δ 112.9, 85.9, 83.1, 75.3, 64.6, 57.7, 53.8, 27.1, 25.7, 25.2, 17.9, 6.75, 4.33, −4.59, −5.19; MS (FAB) m/z 480 [M + Na]+; HRMS (FAB) Calcd. for C₂₁H₄₃N₃O₄Si₂Na [M + Na]+: 480.2690; found: 480.2701.
3.8. Synthesis of Compound 8

Compound 7 (10.1 g, 22.1 mmol) was dissolved in EtOH (220 mL), and ammonium formate (6.97 g, 111 mmol) and 20% Pd(OH)$_2$/C (2.00 g) were added. The suspension was vigorously stirred for 3 h at room temperature and then filtrated through a Celite pad. The filtrate was concentrated in vacuo, and the resulting residue was purified by silica gel column chromatography, eluted with CHCl$_3$/MeOH (8:2), to give compound 8 as a white solid (6.9 g, 98%). IR (KBr): $\gamma_{\text{max}}$ 3184, 2926, 2858, 1596 cm$^{-1}$; [$\alpha$]$_D^{23}$ = −13.4 (c 1.00, MeOH); $^1$H-NMR (500 MHz, DMSO-$d_6$): $\delta$ 8.33 (1H, s), 5.80 (2H, brs), 4.41 (1H, dd, $J = 4.6, 7.5$ Hz), 4.30 (1H, dd, $J = 4.6, 7.5$ Hz), 3.88 (1H, dd, $J = 5.2, 9.7$ Hz), 3.60 (1H, dd, $J = 3.5, 11.5$ Hz), 3.38 (1H, dd, $J = 4.0, 11.5$ Hz), 3.09 (1H, dd, $J = 4.6, 10.3$ Hz), 1.86−1.78 (1H, m), 1.38 (3H, s), 1.21 (3H, s); $^{13}$C-NMR (125 MHz, DMSO-$d_6$): $\delta$ 165.7, 112.0, 85.3, 82.7, 75.8, 56.1, 54.3, 53.4, 27.0, 25.7, 25.0, 17.7, −4.6, −5.0; MS (FAB) m/z 318 [M + H]$^+$; HRMS (FAB) Calcd. for C$_{19}$H$_{32}$N$_2$O$_8$Si [M + H]$^+$: 413.2101; found: 413.2104.

3.9. Synthesis of Compound 9

An amount of 3-methoxyacryloyl chloride (8.52 g, 70.8 mmol) was added to a suspension of silver cyanate (10.6 g, 70.8 mmol) in dry benzene (300 mL), and the mixture was refluxed for 30 min and cooled to room temperature. After concentrated in vacuo, the residue was purified by silica gel column chromatography, eluted with hexane/MeOH (8:2), to give a white solid. The given solid was dissolved in EtOH (60 mL) and treated with ammonium hydride (60 mL, 28% in water) in a sealed tube at 120 °C for 4 h. The resulting mixture was cooled at room temperature. After concentrated in vacuo, the residue was purified by silica gel column chromatography, eluted with hexane/AcOEt (1:2), to give compound 9 as a white solid (4.6 g, 48% in 2 steps). IR (KBr): $\gamma_{\text{max}}$ 3156, 2931, 2880, 1715, 1472 cm$^{-1}$; [$\alpha$]$_D^{23}$ = −16.2 (c 1.00, MeOH); $^1$H-NMR (500 MHz, CDCl$_3$): $\delta$ 9.60 (1H, brs), 7.32 (1H, d, $J = 8.0$ Hz), 5.72 (1H, d, $J = 8.0$ Hz), 4.82 (1H, dd, $J = 7.4, 5.2$ Hz), 4.53 (1H, dd, $J = 10.3, 4.6$ Hz), 4.47 (1H, dd, $J = 7.5, 4.1$ Hz), 4.05 (1H, dd, $J = 8.6, 4.0$ Hz), 3.57−3.72 (2H, m), 2.64 (1H, brs), 2.34−2.44 (1H, m), 1.51 (3H, s), 1.28 (3H, s), 0.89 (9H, s), 0.14 (3H, s), 0.10 (3H, s); $^{13}$C-NMR (125 MHz, CDCl$_3$): $\delta$ 163.4, 151.3, 142.9, 113.2, 113.2, 102.8, 85.6, 80.7, 62.9, 58.8, 52.6, 27.1, 25.7, 25.6, 25.0, 17.9, −4.6, −5.2; MS (FAB) m/z 413 [M + H]$^+$; HRMS (FAB) Calcd. for C$_{19}$H$_{33}$N$_2$O$_8$Si [M + H]$^+$: 413.2108; found: 413.2102.

3.10. Synthesis of Compound 10

Compound 9 (4.60 g, 11.2 mmol) was dissolved in dry CH$_2$Cl$_2$ (110 mL), and Et$_3$N (4.68 mL, 33.6 mmol) was added. The mixture was cooled to 0 °C, and methanesulfonyl chloride (952 µL, 12.3 mmol) was added dropwise. The resulting mixture was stirred for 1 h at 0 °C. The reaction was quenched with an addition of MeOH and diluted with CH$_2$Cl$_2$. The mixture was then partitioned between CH$_2$Cl$_2$ and saturated aq. NaHCO$_3$. The organic layer was concentrated in vacuo and the resulting residue was purified by silica gel column chromatography, eluted with hexane/AcOEt (1:2), to give compound 10 as a white solid (4.2 g, 77%). IR (KBr): $\gamma_{\text{max}}$ 3173, 2989, 2931, 2886, 2858, 1714, 1472 cm$^{-1}$; [$\alpha$]$_D^{23}$ = −24.1 (c 1.00, MeOH); $^1$H-NMR (500 MHz, DMSO-$d_6$): $\delta$ 11.34 (1H, brs), 7.81 (1H, d, $J = 8.0$ Hz), 5.61 (1H, dd, $J = 8.0, 2.3$ Hz), 4.75 (1H, dd, $J = 7.5, 5.2$ Hz), 4.49−4.57 (1H, m), 4.47 (1H, dd, $J = 8.1, 5.2$ Hz), 4.12−4.22 (2H, m), 3.88 (1H, dd, $J = 10.4, 5.8$ Hz), 3.12 (3H, s), 2.67−2.79 (1H, m), 1.43 (3H, s), 1.22 (3H, s), 0.87 (9H, s), 0.11 (3H, s), 0.08 (3H, s); $^{13}$C-NMR (125 MHz, DMSO-$d_6$): $\delta$ 163.2, 151.1, 112.7, 102.1, 84.1, 79.6, 75.6, 66.3, 48.1, 36.2, 27.1, 25.6, 25.1, 17.6, 17.6, −4.6, −5.2; MS (FAB) m/z 491 [M + H]$^+$; HRMS (FAB) Calcd. for C$_{20}$H$_{34}$N$_2$O$_8$Si [M + Na]$^+$: 491.1883; found: 491.1881.
3.11. Synthesis of Compound 11

Compound 10 (4.20 g, 8.57 mmol) was dissolved in MeCN (86 mL), and oxalic acid (77 mg, 0.86 mmol) and CeCl$_3$-7H$_2$O (9.58 g, 25.7 mmol) were added. The resulting mixture was stirred for 3 d at room temperature and then filtrated through a Celite pad. The filtrate was concentrated in vacuo, and the resulting residue was purified by silica gel column chromatography, eluted with CHCl$_3$/MeOH (7:3), to give compound 11 as a white solid (1.9 g, 66%). IR (KBr): $\nu_{\text{max}}$ 3343, 2931, 1690, 1349, 1173 cm$^{-1}$; $[\alpha]_D^{25}$ −75.5 (c 1.00, MeOH); $^1$H-NMR (500 MHz, DMSO-d$_6$): $\delta$ 11.21 (1H, s), 7.60 (1H, d, $J = 8.1$ Hz), 5.62 (1H, d, $J = 8.1$, 2.3 Hz), 5.34 (1H, brs), 4.93 (1H, brs), 4.47–4.42 (1H, m), 4.26–4.13 (3H, m), 3.72 (1H, dd, $J = 5.2$, 2.9 Hz), 3.58 (1H, dd, $J = 5.2$, 3.5 Hz), 3.12 (3H, s), 2.19–2.11 (1H, m); $^{13}$C-NMR (125 MHz, DMSO-d$_6$): $\delta$ 163.3, 151.4, 143.0, 136.4, 130.2, 128.3, 128.0, 127.2, 113.7, 101.1, 86.5, 81.8, 79.1, 78.2, 75.6, 71.4, 45.8; MS (FAB) $m/z$ 337 [M + H]$^+$; HRMS (FAB): Calcd. for C$_{11}$H$_{17}$N$_2$O$_8$BS [M + H]$^+$: 337.0706; found: 337.0700.

3.12. Synthesis of Compound 12

Compound 11 (1.90 g, 5.65 mmol) was dissolved in 1,4-dioxane (57 mL), and 2N aq. NaOH solution of compound 10 (21 mg, 0.0387 mmol) in dry MeCN (385 mL). After stirring for 2 h at room temperature, the reaction mixture was filtered through a Celite pad and the filtrate was concentrated in vacuo, and the resulting residue was purified by silica gel column chromatography, eluted with CHCl$_3$/MeOH (8:2), and by crystallization with AcOEt/MeOH (1:1) to give compound 12 as colorless crystals (980 mg, 72%). IR (KBr): $\nu_{\text{max}}$ 3387, 1685 cm$^{-1}$; $[\alpha]_D^{22}$ −170.2 (c 1.00, MeOH); $^1$H-NMR (500 MHz, DMSO-d$_6$): $\delta$ 11.18 (1H, s), 8.05 (1H, d, $J = 13.8$ Hz), 5.87 (1H, s), 5.54 (1H, d, $J = 13.0$ Hz), 5.02 (1H, d, $J = 10.7$ Hz), 4.54–4.43 (1H, m), 3.87 (1H, dd, $J = 3.8$, 12.2 Hz), 3.76 (1H, s), 3.64–3.53 (1H, m), 2.33 (1H, s); $^{13}$C-NMR (125 MHz, DMSO-d$_6$): $\delta$ 163.2, 151.9, 143.7, 100.0, 79.1, 78.2, 75.6, 71.4, 65.5, 45.8; MS (FAB) $m/z$ 241 [M + H]$^+$; HRMS (FAB): Calcd. for C$_{10}$H$_{13}$N$_2$O$_5$ [M + H]$^+$: 241.0824; found: 241.0826.

3.13. Synthesis of Compound 13

Silver triflate (241 mg, 0.938 mmol) was added portion wise to a solution of 4,4′-dimethoxytrityl chloride (337 mg, 0.995 mmol) in dry CH$_2$Cl$_2$ (1.5 mL). After stirring for 2 h at room temperature, the mixture left to stand for 1 h to precipitate AgCl. The supernatant (375 mL) containing DMTROTF was added to a solution of compound 12 (30.0 mg, 0.125 mmol) in dry pyridine (370 mL) and dry 2,6-lutidine (370 mL) at 0 °C, and the resulting mixture was stirred for 4 h at room temperature. The reaction was quenched with additions of saturated aq. NaHCO$_3$ and saturated aq. CuSO$_4$. The mixture was then filtrated through a Celite pad, and the filtrate was partitioned between AcOEt and saturated aq. CuSO$_4$. The resulting mixture was concentrated in vacuo, and the organic layer was then concentrated in vacuo, and the resulting residue was purified by silica gel column chromatography, eluted with CHCl$_3$/MeOH (9:1), to give compound 13 as a white solid (63 mg, 93%). IR (KBr): $\nu_{\text{max}}$ 3405, 3063, 2941, 2843, 1687, 1507, 1613, 1466 cm$^{-1}$; $[\alpha]_D^{28}$ −1.97 (c 1.00, MeOH); $^1$H-NMR (300 MHz, DMSO-d$_6$): $\delta$ 11.3 (1H, d, $J = 1.8$ Hz), 7.82 (1H, d, $J = 8.3$ Hz), 5.44–5.48 (2H, d, $J = 9.6$, 4.8 Hz), 7.26–7.34 (7H, m), 6.85–6.92 (4H, m), 5.96 (1H, d, $J = 3.2$ Hz), 5.46 (1H, d, $J = 3.2$, 1.6 Hz), 4.86 (1H, d, $J = 4.1$ Hz), 4.50 (1H,d, $J = 4.6$ Hz), 4.11 (1H, s), 3.89 (1H, d, $J = 4.6$, 2.3 Hz), 3.78 (6H, d, $J = 4.1$ Hz), 3.73–3.78 (1H, m), 2.69 (1H, s), 2.22 (1H, s); $^{13}$C-NMR (75 MHz, DMSO-d$_6$): $\delta$ 163.5, 168.6, 152.2, 145.9, 143.8, 136.4, 130.2, 128.0, 127.2, 113.7, 101.1, 86.5, 81.8, 78.1, 75.8, 72.5, 63.8, 55.5, 46.1, 31.4; MS (FAB) $m/z$ 543 [M + H]$^+$; HRMS (FAB): Calcd. for C$_{31}$H$_{31}$N$_2$O$_7$ [M + H]$^+$: 543.2131; found: 543.2129.

3.14. Synthesis of Compound 14

$N,N$-Disopropylethylamine (40 µL, 0.231 mmol), 1-methylimidazole (9.2 µL, 0.116 mmol), and 2-cyanoethyl $N,N$-disopropylchlorophosphoramidite (26 µL, 0.116 mmol) were added to a degassed solution of compound 13 (21 mg, 0.0387 mmol) in dry MeCN (385 µL) at 0 °C. The resulting mixture was stirred for 14 h at room temperature. The reaction was quenched with an addition of saturated aq. NaHCO$_3$. The mixture was concentrated in vacuo, and the resulting residue was purified by silica gel
column chromatography, eluted with hexane/AcOEt (7:1), and recrystallization to give compound 14 as a white solid (16 mg, 56%). IR (KBr): ν max 3469, 3064, 2964, 2925, 1690, 1507, 1615, 1460 cm−1; 1H-NMR (400 MHz, CDCl3): δ 8.53 (1H, s), 7.28–7.50 (8H, m), 6.76–6.82 (2H, m), 5.46 (1H, d, J = 8.2 Hz), 5.40 (1H, d, J = 8.2 Hz), 4.88 (1H, d, J = 3.7 Hz), 4.80 (1H, d, J = 3.7 Hz), 4.59 (1H, d, J = 4.1 Hz), 4.54 (1H, d, J = 4.1 Hz), 4.29 (1H, d, J = 8.7 Hz), 3.79–4.00 (1H, m), 2.97–2.57 (1H, m, 4.65 (1H, m), 1.28–1.29 (2H, m), 1.16 (3H, s), 1.14 (3H, s), 1.04 (3H, s), 1.03 (3H, d, J = 3.7 Hz); 13C-NMR (100 MHz, CDCl3): δ 158.6, 145.1, 143.2, 136.1, 136.0, 130.1, 130.0, 128.0, 127.9, 126.9, 117.1, 113.3 100.7, 87.0, 79.3, 72.0, 71.8, 64.9, 58.4, 58.2, 55.2, 46.0, 45.9, 43.3, 43.1, 43.0, 31.9, 31.6, 29.7, 29.6, 24.6, 24.5, 22.6, 20.0, 19.9, 14.1; 31P-NMR (162 MHz, CDCl3): δ 150.3, 149.2; MS (FAB) m/z 743 [M + H]+; HRMS (FAB) Calcd. for C40H48N4O8P [M + H]+: 743.3210; found: 743.3217.

3.15. Energy Minimized Structure (Spartan 16)

The molecular structure run out multi-step stabilized. At first, stable conformers were calculated by using conformer distribution (MMFF). The given conformers were further calculated using HF/3-21G (equilibrium geometry) and then ωB97X-D/6-31G* to eliminate high-energy and duplicate conformers. Finally, the most stable structures were searched by ωB97X-V/6-3111G,ω/6-31G density functional model.

3.16. X-ray Crystal Structure

A suitable crystal of compound 12 was carefully selected under an optical microscope and glued to thin glass fibers and mounted on the goniometer in a liquid nitrogen flow. X-ray diffraction data were collected on a Rigaku R-AXIS RAPID diffractometer employing graphite-monochromated CuKα radiation. The structure was solved by the direct method with the SIR-88 program [26] and refined with the SHELXL program [27]. The structural model was drawn with the ORTEP-3 program [28]. Further information on the crystal structure determinations has been deposited with the Cambridge Crystallographic Data Center (1989958). The data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html (or from the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK; Fax: +44 1223 336033; E-mail: deposit@ccdc.cam.ac.uk).

3.17. Oligonucleotide Synthesis

Oligonucleotides modified with OxNorNA-U (ON1–ON3) were synthesized by the reverse DNA synthesis using an nS–8 Oligonucleotide Synthesizer (GeneDesign) and a universal CPG solid support (0.2 μmol scale, Glen Research). The coupling time for OxNorNA phosphoramidite was extended to 12.5 min, and 5-[3,5-bis(trifluoromethyl)phenyl]-1H-tetrazole was used as an activator. The detritylation time of all reverse phosphoramidites was prolonged to 2 min (from 20 s), and 3 w/v% trichloroacetic acid/dichloromethane solution was used for the detritylation. The other synthetic procedures involved the standard phosphoramidite protocols. Cleavage from the solid support and removal of protecting groups was accomplished by mild conditions (tert-butylamine/water (1:3 v/v), 24 h at room temperature, and then 12 h at 60 °C). The resulting oligonucleotides were briefly purified using reverse-phase HPLC (Waters X Terra® MS C18 2.5 μm, 10 × 50 mm column, eluent A (0.1 M triethylammonium acetate (TEAA) in water), eluent B (MeCN), gradient: 5–9% or 6–15% of eluent B, flow rate = 5.0 mL/min). The resulting oligonucleotides were further purified with Sep-Pak® Plus C18 cartridges, where the DMTr group was removed by 2% TFA in water, and by reverse-phase HPLC (Waters X Terra® MS C18 2.5 μm, 10 × 50 mm column, eluent A (0.1 M triethylammonium acetate (TEAA) in water), eluent B (MeCN), gradient: 5–9% or 6–15% of eluent B, flow rate = 5.0 mL/min). The purified oligonucleotides were analyzed using reverse-phase HPLC (Waters X Terra® MS C18 2.5 μm, 4.6 × 50 mm column), and their compositions were confirmed by MALDI-TOF MS analysis (matrix: 3-hydroxypicorinic acid, additive: diammonium hydrogen citrate).
3.18. UV Melting Experiment

The UV melting experiments were performed on Shimadzu UV-1650B and UV-1800 spectrometers equipped with a $T_m$ analysis accessory. Samples containing oligonucleotide (4 $\mu$M), the target DNA or RNA (4 $\mu$M), and 100 mM NaCl in a 10 mM phosphate buffer (pH 7.2) were annealed at 100 °C and then cooled slowly to room temperature. The melting profile was recorded from 5 to 90 °C at a scan rate of 0.5 °C/min with detection at 260 nm. The $T_m$ value was obtained from the temperature for half-dissociation of the formed duplexes based on the first derivative of the melting curve. For van’t Hoff plots, $T_m$ values were determined at several oligonucleotide concentrations (0.90, 1.48, 2.44, 4.00, 6.52, and 13.6 $\mu$M) (see Figures S1 and S2 in Supplementary Material). The values of $\Delta H^\circ$, $\Delta S^\circ$, and $\Delta G^\circ$ were calculated according to the equations shown below (R is an ideal gas constant, and $C_t$ indicates oligonucleotide concentration):

$$1/T_m = (R/\Delta H^\circ) \ln(C_t/4) + \Delta S^\circ/\Delta H^\circ$$  \hspace{1cm} (1)

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$$  \hspace{1cm} (2)

3.19. CD Spectral Analysis

CD spectra were measured at 10 °C in a quartz cuvette with a 1-cm optical path length by using a J-720W spectrophotometer (JASCO). The sample solutions (360 $\mu$L) were prepared by dissolving oligonucleotide (4 $\mu$M) and NaCl (100 mM) in the phosphate buffer (10 mM, pH 7.2). The molar ellipticity was calculated from the equation $[\theta] = \theta/c/l$, where $\theta$ is the relative intensity, c is the sample concentration, and l is the cell path length in centimeters.

3.20. Enzymatic Stability Analysis

Samples (each sample volume: 130 $\mu$L) containing MgCl$_2$ (10 mM), oligonucleotide (2 $\mu$M), and snake venom phosphodiesterase (svPDE, 0.133 $\mu$g/mL) in the Tris-HCl buffer (50 mM, pH 8.0) were incubated at 37 °C. Portions of the samples (20 $\mu$L) were taken at respective time points, and svPDE was immediately deactivated by heating the sample at 90 °C for 2.5 min. The percentage of the remaining intact oligonucleotides was determined by reverse-phase HPLC (2.5 µm, 4.6 × 50 mm) and plotted against their reaction time.

4. Conclusions

We synthesized and evaluated structurally restricted OxNorNA-U-modified oligonucleotides. Although OxNorNA-U-modified oligonucleotides showed a lower duplex-forming ability as compared to the natural counterparts, the base discrimination ability of those was similar to that obtained for the natural oligonucleotides. Since OxNorNA has a rigid structure and exhibits extremely high enzymatic stability, we expect that OxNorNA could be useful for the point modifications of aptamers.

Supplementary Materials: The following are available online, Figure S1: van’t Hoff plots of the duplexes formed between oligonucleotides (ON1, ON2, ON4, and ON5) and complementary ssDNA, Figure S2: van’t Hoff plots of the duplexes formed between oligonucleotides (ON1, ON2, ON4, and ON5) and complementary ssRNA, Table S1: $T_m$ values of duplexes formed between oligonucleotides (ON1, ON2, ON4, and ON5) and complementary ssDNA, Table S2: $T_m$ values of duplexes formed between oligonucleotides (ON1, ON2, ON4, and ON5) and complementary ssRNA. Copies of the NMR spectra of all new compounds. Copies of the HPLC and MALDI-TOF MS charts of the synthesized oligonucleotides.

Author Contributions: K.M., Y.K., T.Y., and S.O. designed the experiments; H.K., S.M., K.M., K.I., and T.O. conducted the synthesis of OxNorNA-U phosphoramidite and OxNorNA-U-modified oligonucleotides, and the evaluation of the biophysical properties of OxNorNA-U-modified oligonucleotides; H.A. performed the X-ray crystal structure analysis; and T.Y. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Sample Availability: Not available.

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