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Synthesis and Properties of 2′-OMe-RNAs Modified with Cross-linkable 7-Deazaguanosine Derivatives

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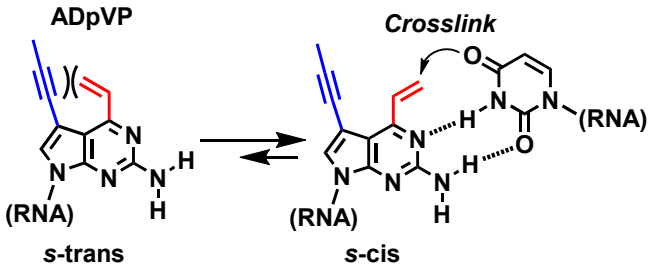


■ **Abstract**

Cross-linkable 7-deaza-6-vinylguanosine (ADVP) and 7-propynyl-7-deaza-6-vinylguanosine (ADpVP) derivatives were synthesized and successfully incorporated into 2′-OMe-RNA oligonucleotides by solid-phase oligonucleotide synthesis. Analysis of their cross-link properties revealed that the 7-propynyl substituent on ADpVP induces a significant enhancement of the cross-link kinetics of the proximal 6-vinyl group to the complementary uracil base in the target RNA compared to that of ADVP. In addition, the 2′-OMe-RNA oligonucleotide containing ADpVP exhibited a higher antisense effect on luciferase production in the cell lysate than that of ADVP. These results suggested that the 7-substituted 7-deaza-6-vinylguanosine (ADpVP) derivatives can be used as potent cross-linkers to target mRNA inside of cells.

1. Introduction

Synthetic oligonucleotides (ONs) are attractive biological tools with a high specificity to target nucleic acids and are used in many fields as diagnostics, therapeutics and many forms of nanotechnology. The chemical modification of the ONs can help expand their applicability. In particular, the modification of the sugar and phosphate moieties of the ONs enhances the binding affinity to targeting DNAs/RNAs and improves the metabolic stability for vivo use.[1] Less commonly, nucleobase modifications have been used as well, such as the tricyclic-type pyrimidine analogues (Phenoxazine, G-Clamp, Guanidino G-Clamp)[2-3], and 2,6-diaminopurine.[4] These modified bases within the ON increased the thermal stability of the duplex by an enhanced stacking effect or hydrogen bonding with the target RNA, improving the antisense silencing effect.[2, 5] Cross-link forming oligonucleotide (CFO) forms a covalent linkage to a target RNAs and several kinds of CFO activated by photo-irradiation have already been reported.[6-11] However, there are few reports about regulation of the gene expression in cells using CFO.[12] Here, we have developed several cross-linkable artificial nucleobases bearing the vinyl group as a reactive moiety, which exhibited a high selectivity and reactivity to the target base at the complementary site by the proximity effect.[13-18] In our previous report, fully 2′-O-methylated RNAs having the 2-amino-6-vinylpurine (AVP) efficiently suppressed translation *in vitro*, causing the production of the truncated protein.[19] In addition, this CFO containing the AVP inhibited the exogenous miRNA function by in-cell cross-linking to the target mRNA without activation of cross-linkers.[20] However, the inhibition of the endogenous miRNA function could not be achieved in cells, likely due to the

slow kinetics of the cross-link reaction. To achieve faster kinetics, we hypothesized that fixing the structural orientation of the 6-vinyl group to the target base in a duplex would enhance the reaction rate of the AVP without compromising the stability of cross-linker itself. With this in mind, the 7-substituted 7-deazapurine skeleton was designed, in which the substituent at the 7-position was expected to induce steric repulsion with the *s*-trans conformation of the 6-vinyl group to orient it into the *s*-cis conformation (**Figure 1**). To assess our hypothesis, we synthesized new cross-linkable AVP analogues, i.e., 2-amino-7-deaza-6-vinylpurine (ADVP) and 2-amino-7-deaza-7-propynyl-6-vinylpurine (ADpVP). The propynyl group was introduced as a substituent at the 7-position due to its synthetic easiness and stabilizing effect on the duplex DNA without perturbation of the duplex structure.[21-22] In an analogy to the previous result with AVP, we expected that *s*-cis-ADpVP would exhibit an efficient cross-linking with uracil (U) by forming two hydrogen bonds in a complementary duplex. In this paper, we report the synthesis and comparative evaluation of the cross-linking ability of AVP, ADVP and ADpVP to complementary RNA strands.

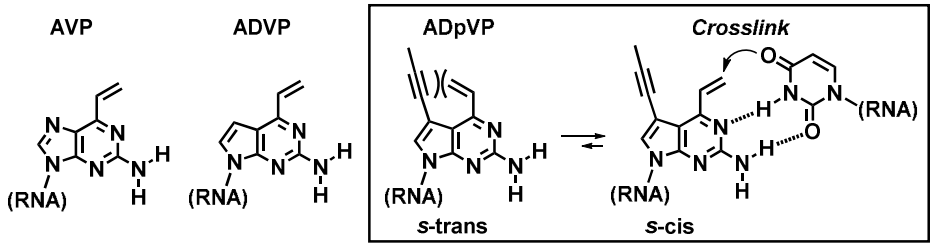
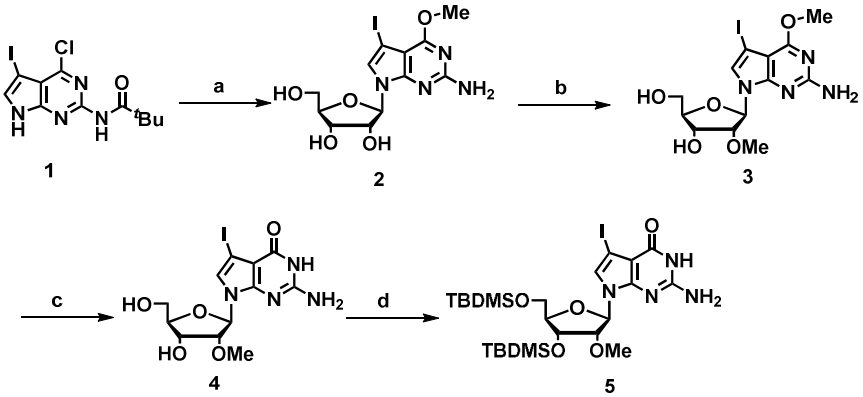


Figure 1. Chemical structures of 2-amino-6-vinylpurine (AVP), 2-amino-7-deaza-6-vinylpurine (ADVP), and 2-amino-7-deaza-7-propynyl-6-vinylpurine (ADpVP). Potential steric effect of 7-propynyl group on the orientation of 6-vinyl group.

2. Results and discussion

2.1 Synthesis of 2′-OMe-7-deaza-7-iodo-guanosine derivative (5) (Scheme 1).



Scheme 1. Synthesis of 7-deaza-7-iodo-guanosine derivative (**5**). (a)1) BSA, CH_3CN, rt, 20 min; 2) TMSOTf, 1-*O*-Acetyl-2,3,5-tri-*O*-benzoyl-β-L-ribofuranose, 50°C, 24 h, 66% (NMR yield); 3) 1.5M NaOMe-MeOH, reflux, 3 h, 63% (NMR yield); (b) NaH, DMF, 0 °C, 30 min, then CH_3I, rt, 16 h, 43%; (c) 2 M NaOH in H_2O-dioxane (6.6:1, v/v), reflux, 4 h, 97%; (d) TBDMSCl, imidazole, DMF, 0°C then rt, 2 h, 84%;

1-*O*-Acetyl-2,3,5-tri-*O*-benzoyl-*β*-(L)-ribofranose was added to the bis-halogenated 7-deazaguanine derivative (**1**),[23] transiently silylated by bis(trimethylsilyl)acetamide (BSA), in the presence of trimethylsilyl trifluoromethansulfonate (TMSOTf) to yield the nucleoside.[24] At this step, because of the difficult separation, a strong base treatment of this

nucleoside with 1.5 M NaOMe-MeOH was carried out without further purification to yield compound (**2**). This base treatment enabled both the deprotection of the hydroxyl groups of the sugar moiety and methoxylation at the 6-position of 7-deazaguanine by the aromatic nucleophilic substitution (SNAr) reaction. This proved advantageous as we utilized this O-6-methyl group as a protecting group for the amide of 7-deazaguanine during the following 2′-O-methylation. The N protection was necessary for the reaction as the pKa values of the hydroxyl group are generally higher than that of the amide NH.[25-27] The methylation of **2** mainly provided the mixture of the 2′- or 3′-OMe-nucleosides and they were separable by silica gel column chromatography. The 6-OMe group of the 2′-O-methylated compound (**3**) was hydrolyzed by a strong base treatment. After neutralizing the solution, the excess amount of salt proved to be an obstacle for purification, however, the hydrolyzed product (**4**) was efficiently precipitated in H_2O after evaporation of the excess 1,4-dioxane. The precipitation was repeatedly washed with water, which provided the desalted pure compound (**4**) in high yield (97%). Next, the hydroxyl groups of the sugar were protected with the *t*-butyldimethylsilyl (TBDMS) group to yield compound (**5**).

2.2 Synthesis of 2′-OMe-7-deaza-7-propynyl-guanosine derivative (6) by Sonogashira cross-coupling

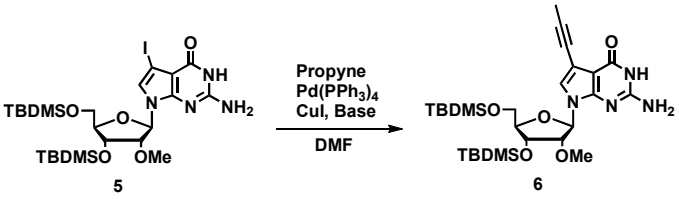


Table 1. Condition of Sonogashira cross-coupling

Entry	Base (eq)	Temp. (°C)	Time (h)	Yield (%)
1	TEA (2.8)	r.t.	48	63[b]
2	HMDS (12.0)	50	1	63
3	HMDS (8.0)	45	1	78
4	HMDS (6.0)	40	1	83

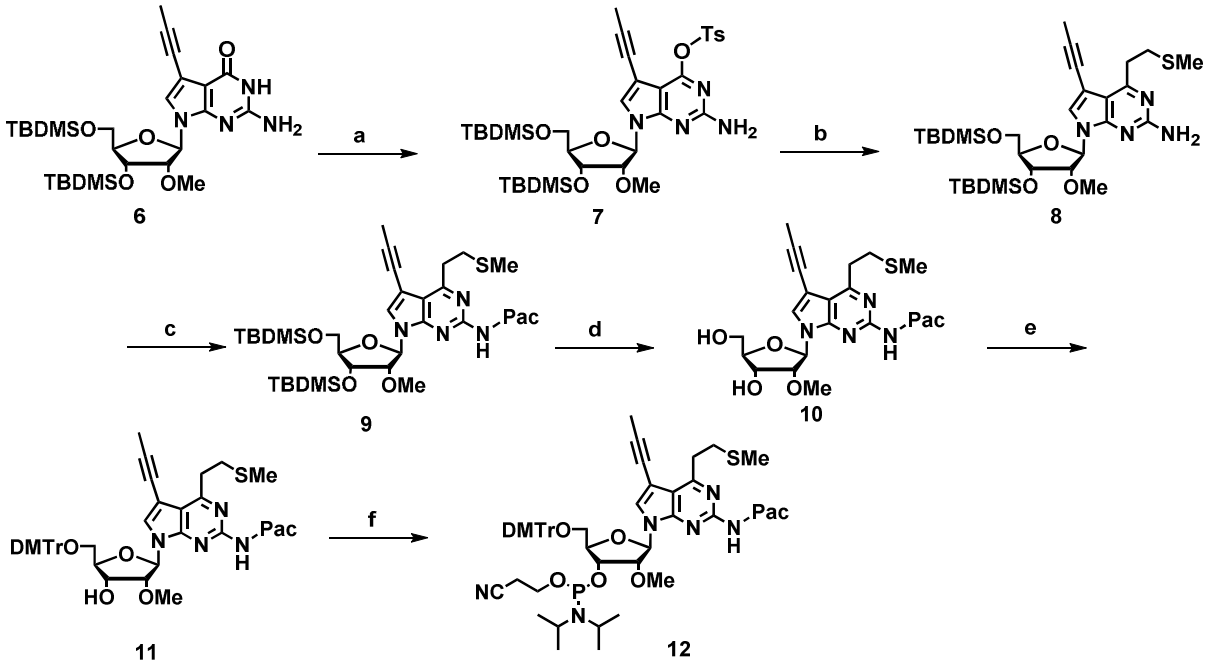
[a] 10 eq of Propyne, 0.1 eq of $Pd(PPh_3)_4$, 0.2 eq of CuI were used for all of the reaction conditions. [b]Yield of compound **8** with a mixture of palladium residues.

Initially, the Sonogashira cross-coupling[28-31], using triethylamine as a base was tested but we found it difficult to separate the palladium residue and side product by silica gel column chromatography. The mass of this side product corresponded to the mass of the compound with two propyne groups [**6** + one propyne group], suggesting that the propyne also reacted with the free amide[32] (**Figure S1**). We then focused on the effect of the free amide of the 7-deazaguanine on this reaction. As a test, the Sonogashira cross coupling efficiently proceeded using the he TBDMS-protected version of **2**, and the 7-propynylated product was purified as a pure material in 75% yield (data not shown). This implied that the masking of the amide of 7-deazaguanine with the proper protecting group could be important for this reaction to proced. Then alternative cross-coupling condition, which utilized hexamethyldisilazane (HMDS) as both a base and transient 6-carbonyl silylating reagent, was used.[33] In **Table 1**, entry 1-4, the equivalent of the base and reaction temperature were assessed, and the target product was obtained in 83% when the reaction was carried out at 40 °C in the presence of 6 equivalents of HMDS (entry 4).

2.3 Synthesis of 7-deaza-6-methylthioethyl-7-propynyl-6-vinylguanosine phosphoramidite derivative (12) (Scheme 2)

After the tosylation of **6**, the Suzuki-Miyaura cross-coupling[34] with **7** was carried out using $PdCl_2(PPh_3)_2$, K_2CO_3 and the

vinylboronic anhydride pyridine complex to produce 6-vinyl derivative. The 6-vinyl substituent was converted to a stable sulfide (**8**) by treatment with sodium thiomethoxide, because the vinyl group was anticipated to not tolerate future oligonucleotide synthesis conditions. After the phenoxyacetyl protection and deprotection of the TBDMS group, the 5′-hydroxyl group was protected with the dimethoxytrityl (DMTr) group to yield **11**. Phosphorylation at the 3′-hydroxyl group by the phosphorochloridate reagent produced the phosphoramidite (**12**).



Scheme 2. (a) TsCl, Et_3N, DMAP, CH_2Cl_2, 0 °C then rt, 3 h, 80%; (b) 1) vinylboronic anhydride pyridine complex, $PdCl_2(PPh_3)_2$, K_2CO_3, 1,4-dioxane-H_2O (3:1, v/v), 80 °C, 2 h, 2) MeSNa, rt, 1 h, 53% (2 steps); (c) Pac-Cl, pyridine, 0 °C then rt, 87%; (d) 0.1M TBAF-THF, rt, 1 h, 83%; (e) DMTr-Cl, pyridine, 0°C then rt, 3 h, 75%; (f) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite, DIPEA, CH_2Cl_2, 0 °C, 2 h, 73%.

2.4 Synthesis of oligonucleotides having vinyl-protected ADpVP (ON-1a), ADVP (ON-2a), and AVP (ON-3a)

The synthesis of oligonucleotides 5′-DMTr-2′-OMe-r[AGUAGCCC**X**GCGUGGUG]-3′ (**X** = $^{S\text{-}Me}$ADpVP for 5′-DMTr-ON-**1a**) were carried out using an automated RNA synthesizer except for the coupling of the phosphoramidite (**12)**, which were introduced into the 2′-OMe-RNA oligonucleotide by manual coupling to reduce the required amount of these phosphoramidites. The 2′-OMe-G (N-*i*Pr-Pac)-loaded solid support and phosphoramidites of 2′-OMe-U, 2′-OMe-G (N-Tac), 2′-OMe-A (N-Tac), and 2′-OMe-C (N-Tac) were used to carry out the *ultra-mild* oligonucleotide syntheses. For the deprotection and cleavage from the support, the oligonucleotide was treated with conc. NH_4OH for 6 h at 26 °C. This is slightly longer incubation time than that of the manufacturer's standard protocol because of slightly more stable bond of the 2N-Pac group on the 7-deazaguanosine derivatives compared to that of guanosine. After the DMTr-on purification by the C18-Seppak cartridge, further purification of the detritylated ON-**1a** was conducted by reverse-phase (RP) HPLC, and the separated major peak of ON-**1a** in the RP-HPLC profiles was characterized as the desired oligonucleotide by MALDI-TOF MS (**Figure S2**). To study the effect of the 7-substituent, 2′-OMe-[AGUAGCCC**X**GCGUGGUG]-3′ (**X** = $^{S\text{-}Oct}$ADVP, ON-**2a**), which contains the 7-deaza-6-(octylthio) ethyl-guanosine derivative ($^{S\text{-}Oct}$ADVP), was also prepared (**Scheme S1**). To comparatively study the effect of changing the canonical purine base to the 7-deazapurine base, the oligonucleotide having 2-amino-6-vinylpurine derivative ($^{S\text{-}Me}$AVP): 2′-OMe-r[AGUAGCCC**X**GCGUGGUG]-3′ (X = $^{S\text{-}Me}$AVP, ON-**3a**) was also prepared according to our previous report.[16]

2.5 Vinyl-conversion and purification/storage of activated ON-1a and ON-2a

To regenerate the C6-vinyl group of ADpVP of ON-**1a** and ADVP of ON-**2a**, the sulfide was oxidized to the sulfoxide by magnesium monoperphthalate (MMPP) treatment (**Figure S3, S5**). These oxidized ON-**1b** and ON-**2b** were subsequently treated with 0.4 M aq. NaOH to induce the E1cB-elimination of the sulfenic acid yielding vinyl substituents. The reaction was monitored by RP-HPLC and confirmed that the desired ON-**1c** and ON-**2c** were quantitatively synthesized (**Figure 2**). It should be noted that the relatively electron-rich properties of the 6-vinyl group of 7-deazapurine enabled HPLC purification and subsequent lyophilization without causing partial decomposition. These purified vinyl-active ON-**1c** and ON-**2c** were stably stored at -80 ^{o}C for several days before use.

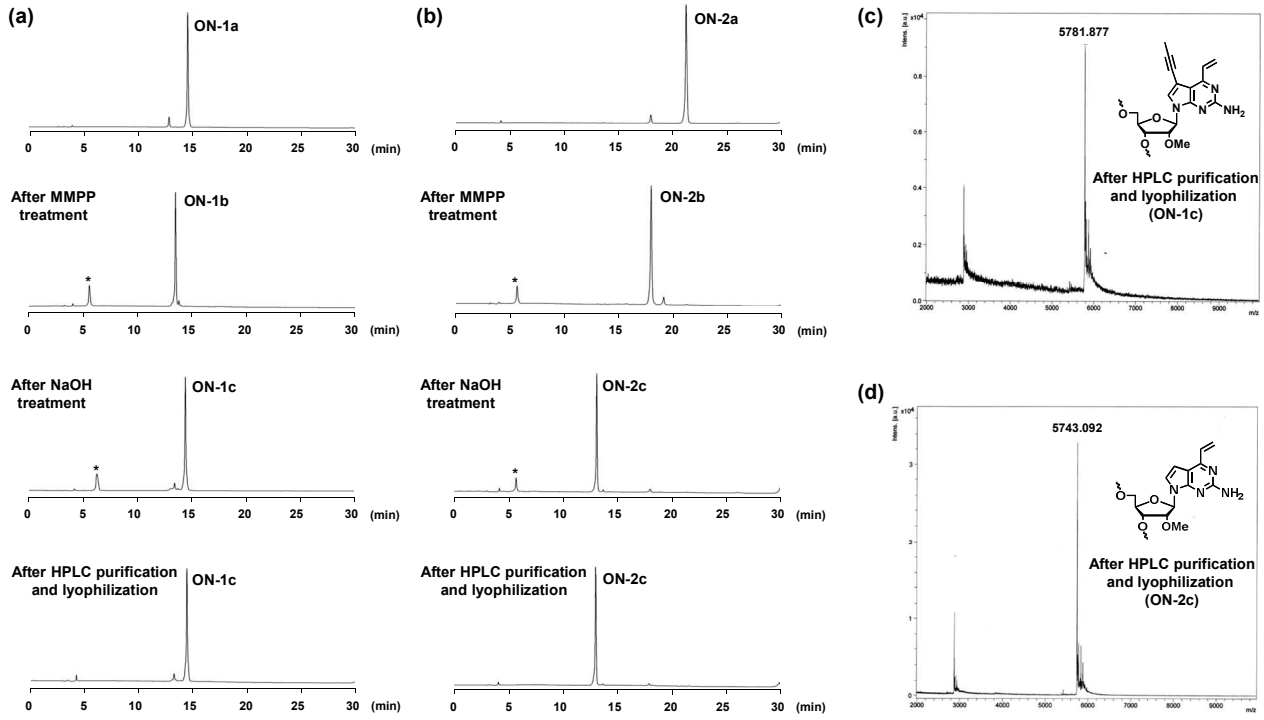


Figure 2. Reverse phase HPLC profiles after MMPP-mediated oxidation, NaOH treatment, and HPLC purification/lyophilization of (a) ON-**1a** and (b) ON-**2a.** HPLC conditions: COSMOSIL 5C18-MS-II (4.6 × 250 mm), solvent A: 0.1 M TEAA buffer (pH 7.0), B: CH_3CN, B: 5-25% in 30 min; (c) MALDI-TOF MS analysis of ON-**1c** after HPLC purification and lyophilization, mass calculated: 5781.9, mass observed 5781.9 $[M-H]^-$; (d) MALDI-TOF MS analysis of ON-**2c** after HPLC purification and lyophilization, mass calculated: 5743.8, found 5743.1 $[M-H]^-$.

2.6 Cross-link properties of ADpVP (ON-1c) and ADVP (ON-2c)

The cross-link reactions were conducted in 50 mM Mes buffer (pH 7) containing 100 mM NaCl at 37 ^{o}C using ON-**1c** (ADpVP), ON-**2c** (ADVP) and the complementary RNA (**Y** = A, G, C and U) labelled with fluorescein at the 5' end. The reactions were analyzed by 20% denaturing polyacrylamide gel electrophoresis. The cross-linked product was observed as the slower mobility band than the target RNA. The cross-link yields were calculated from the ratio of the cross-linked product to the remaining single-stranded cRNA and plotted versus the incubation time (**Figure 3**). ON-**1c** (ADpVP) exhibited a high cross-link reactivity and mostly selectivity to uracil (84% in 2 h, 89% in 8 h). On the other hand, ADVP showed slow but very selective reaction properties toward uracil (58% in 2 h, 91% in 24 h). The alkali-hydrolysis based foot printing of the cross-linked duplex between cRNA (**Y**=U) and ON-**1c** (ADpVP), ON-**2c** (ADVP) or ON-**3c** (AVP) was

carried out to identify the position of the cross-linking site of these cross-linkable bases to the complementary RNA strand.[18] These results suggested that the cross-linking reactions with three cross-linkable bases took place to uracil at the complementary site (**Figure S7**). We determined the cross-link adducts to RNA (**Y**=rU) with three cross-linkable bases by measuring MALDI-TOF MS (**Figure S8**). Based on the previous study[15], the cross-link with ADpVP might form with 4-oxygen of uracil. But we did not study the determination of the cross-linked structure and it is not clear which one is the correct structure of the adduct (A or B). The cross-linking reactions were shown not be reversible under the reaction conditions used after 48 h.

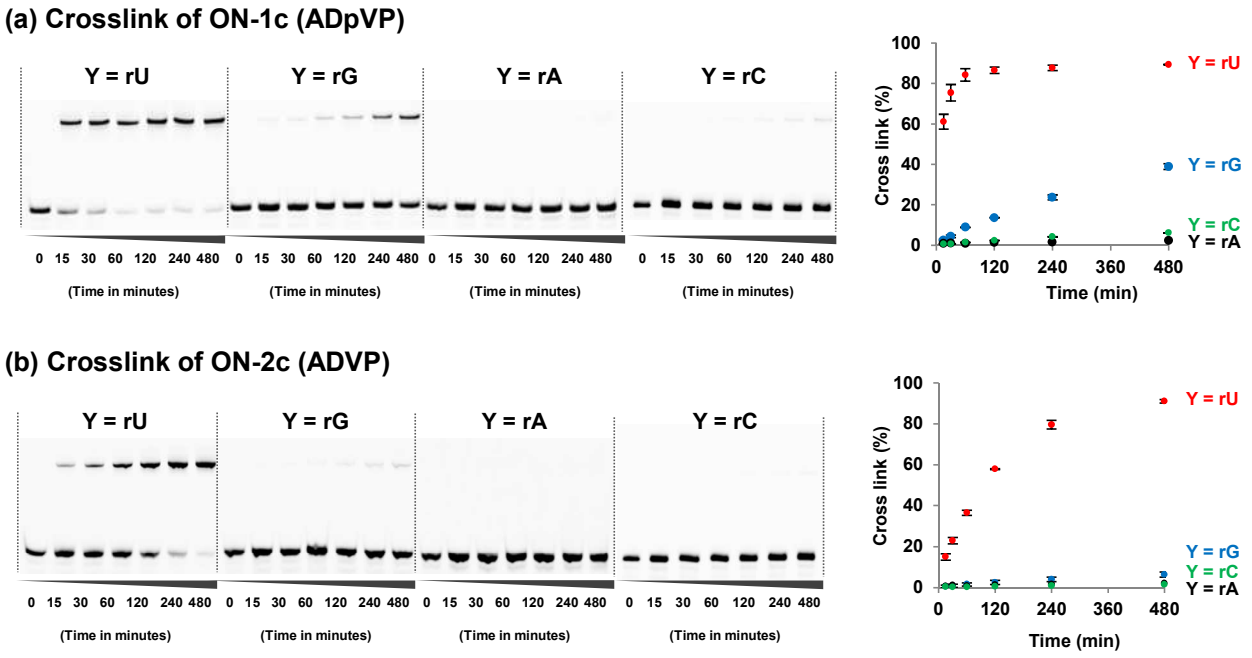


Figure 3. Complementary base dependency of cross-link reaction by (a) ON-**1c** and (b) ON-**2c**. The reaction was performed in 50 mM MES-buffer (pH 7.0) containing 100 mM NaCl, 4 μM ON-**1c** (or **2c**) and 2 μM cRNAs at 37 °C. Target RNAs used in this assay were 5′-FAM-r[CCACGCYGGGCUA]-3′ (**Y** = rU, G, A, C). (c) The speculative structures of the adducts for the cross-linked adducts to uracil with ADpVP .

2.7 Comparison of the cross-link reactivity of ADpVP (ON-1c), ADVP (ON-2c) and AVP (ON-3c)

The cross-link properties of ON-**1c** (ADpVP), ON-**2c** (ADVP), and ON-**3c** (AVP) to the complementary uracil base in RNA were evaluated. It was clearly demonstrated that the propynyl modification at the 7 position of 6-vinyl-7-deazaguanine significantly increased the reaction rate (**Figure 4a-d**). Each cross-link yield was analyzed based on the first-order kinetics, and the kinetics constants in the initial reaction time range (0-60 min) of ON-**1c**, ON-**2c**, and ON-**3c** were determined. The kinetic constant of ON-**1c** (ADpVP) was found to be one order higher than those of ON-**2c** and ON-**3c** [k (s^{-1}) = 7.66E-04 for ON-**1c**, 9.20E-05 for ON-**2c**, 9.24E-05 for ON-**3c**]. By comparison, the ADpVP (ON-**1c**) and ADVP (ON-**2c**) showed a

significant difference in reaction kinetics and it was obviously attributed to the effect of the 7-propynyl group. These results support our original hypothesis that the steric effect of the 7-substituents drives the proximal 6-vinyl group toward a favorable orientation and enabled the very efficient cross-link reaction, and thus enabled swift cross-link reaction with the target nucleotide.

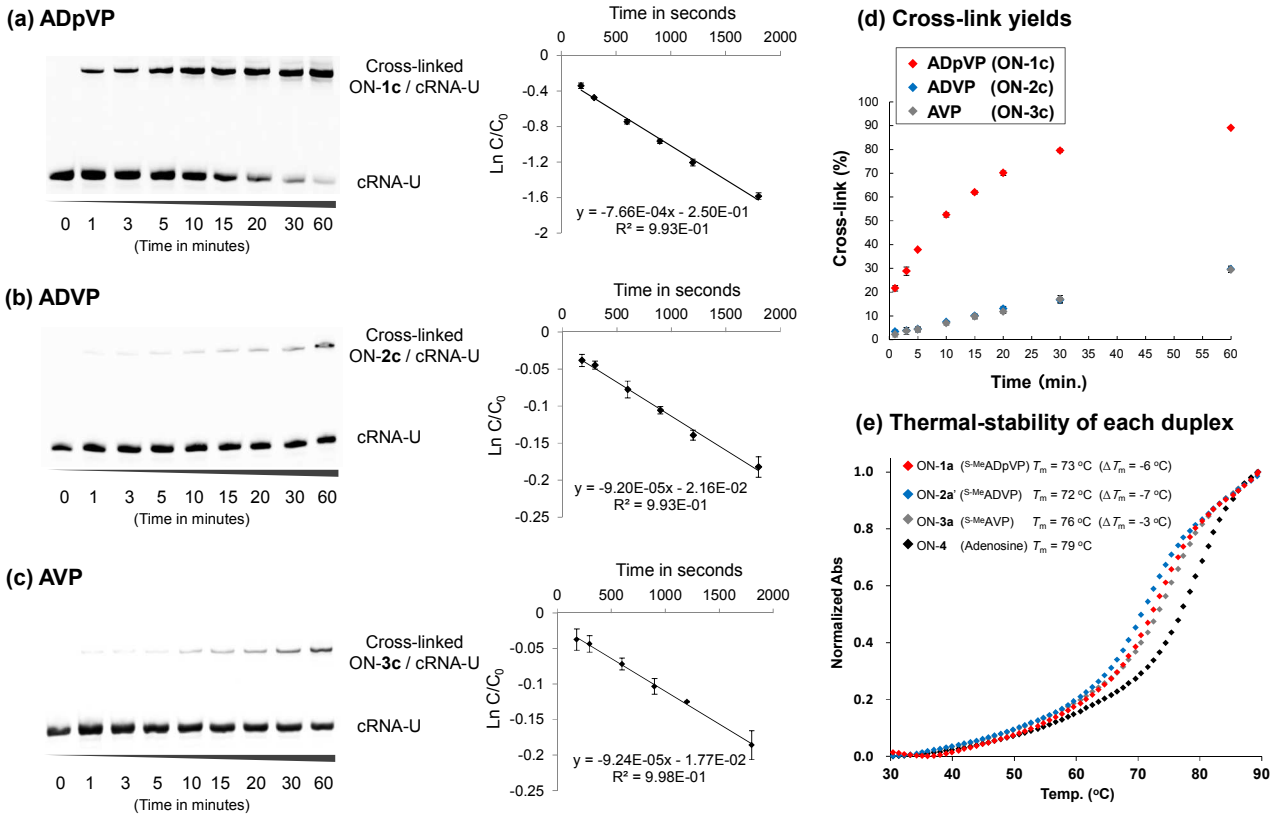


Figure 4. Kinetic analysis of cross-link reaction of (a) ON-**1c**, (b) ON-**2c**, (c) ON-**3c** with cRNA-U. 4 μM ON-**1c**, **2c** or **3c,** and 2 μM cRNA-U were incubated in 50 mM MES-buffer (pH 7.0) containing 100 mM NaCl at 37 °C. The kinetic constants were determined in triplicate experiments, C_0: Initial concentration of cRNA-U, C: concentration of remaining cRNA-U at each time point. (d) Quantification of cross-linked product on the gel at each time point. (e) Comparative T_m analysis of RNA duplex containing $^{S\text{-}Me}$ADpVP (ON-**1a**),$^{S\text{-}Me}$ADVP (ON-**2a'**), $^{S\text{-}Me}$AVP (ON-**3a**). T_m values were measured in 50 mM MES buffer (pH 7.0) containing 100 mM NaCl. ΔT_m = T_m (ON-**1a**, **2a'**,or **3a**) -T_m (ON-**4**). Sequence of 2′-OMe ONs: 5′-[AGUAGCCCXGCGUGGUG]-3′ (X = $^{S\text{-}Me}$ADpVP for ON-**1a**, $^{S\text{-}Me}$ADVP for ON-**2a'**, $^{S\text{-}Me}$AVP for ON-**3a**, Adenosine for ON-**4**, respectively). Sequence of cRNA-U: 5′-r[CCACGCUGGGCUA]-3′. T_m values were determined in triplicate experiments.

The thermal stability with the target RNA can potentially be determined to the cross-link efficiency. We then evaluated the thermostability of cRNA-U with the non-reactive 2′-OMe ONs containng $^{S\text{-}Me}$ADpVP (ON-**1a**), $^{S\text{-}Me}$ADVP (ON-**2a'**, see supplement), and $^{S\text{-}Me}$AVP (ON-**3a**), which are RNAs with vinyl masked non-reactive ADpVP, ADVP, and AVP derivatives, respectively (**Figure 4e**). The melting temperature (T_m) of the 2′-OMe ON (**1a~3a)** /RNA duplex was lower than that of the natural non-modified duplex. ON-**1a** and ON-**2a'** showed mostly equal T_m values, while ON-**3a** showed a slightly higher T_m than those of ON-**1a** and **2a**. These results suggested that the drastic change in the cross-link kinetics of ADpVP was not merely due to a difference in the duplex's thermostability, but attributed to the local structural impact of the 7-substituent onto the reactivity of proximal 6-vinyl group.

The kinetic behavior for the cross-linking reaction with ADpVP (ON-**1c**) and ADVP (ON-**2c**) was investigated at five different temperatures. The Arrhenius plot of the kinetic data for each reaction allowed the calculation of the activation energy (*Ea*), the enthalpy ($\Delta H^{\ddagger}$) and entropy ($\Delta S^{\ddagger}$) of activation (**Figure S9** and **Table S1**). The $\Delta G^{\ddagger}$ of ADpVP (ON-**1c**) is

4.5 kJ/mol lower than that of ADpVP (ON-**1c**), which has a slightly unfavorable contribution of the $\Delta H^{\ddagger}$ term and a favorable contribution of the $\Delta S^{\ddagger}$ term.

This result suggests that the vinyl group of ADpVP would require less conformational change than that of ADVP for the cross-link formation. The molecular orbital calculation (RB3LYP/6-31G*) revealed that the *s*-cis conformation of the vinyl group is more stable and that the energy difference between the *s*-cis and *s*-trans conformations is 3.92 kcal/mol for ADpVP and 1.56 kcal/mol for ADVP (**Figure 5**), further supporting the fact that the vinyl group of ADpVP is enforced in the *s*-cis conformation for the cross-linking reaction due to its 7-substituent.

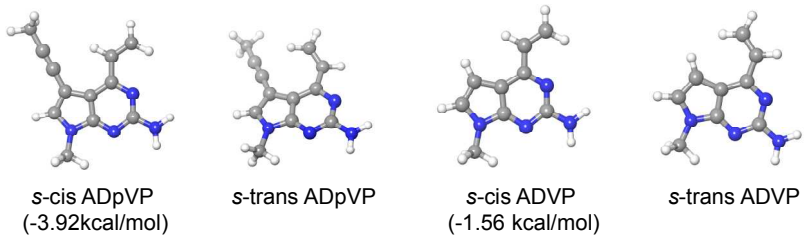


Figure 5. Conformations calculated by MO (RBLYP/6-31G*) for ADpVP and ADVP

2.8 Inhibition of Luciferase expression by cross-linking of ON-1c, ON2c, or ON-3c.

To demonstrate the beneficial aspect of the fast cross-linking rate by ADpVP, the effect of the cross-link with Luciferase mRNA on its in vitro translation was investigated. The mRNA was subjected to the cross-linking reaction with ON-**1c**, ON-**2c**, or ON-**3c** in a 50 mM MES buffer (pH 7.0) in the presence of 100 mM NaCl and 0.1 μM Luciferase mRNA for one hour. After the incubation, in vitro translation was conducted by the addition of rabbit reticulocyte, and the intensity of the Luciferase expression level was quantified (**Figure 6**).

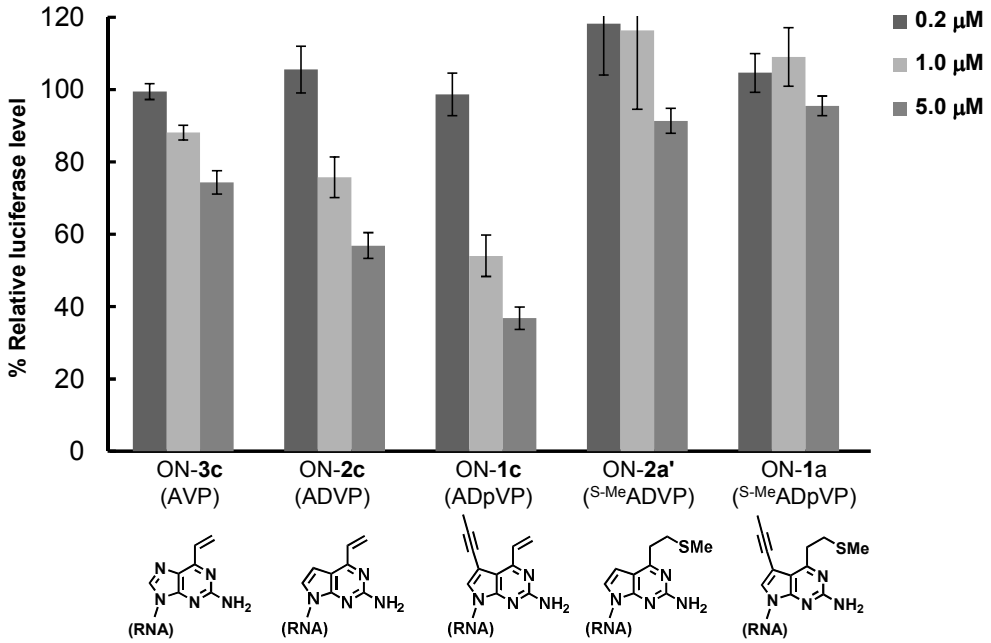


Figure 6. Relative luciferase levels after incubation in a buffer containing ON-**1c**, **2c**, **3c**, **1a**, or **2a'**, and luciferase mRNA.

The inhibition efficiency of the Luciferase translation is in the order of AVP (ON-**3c**) < ADVP (ON-**2c**) < ADpVP (ON-**1c**), which is in good agreement with that of their cross-linking reactivity to the short RNA target (**Figure 4d**). ON-**1a** and ON-**2a'** with the methylsulfide-masked vinyl group did not show any significant silencing in the Luciferase expression,

although they form stable duplexes with the target site on the mRNA (**Figure 4e**). These results support that the efficient cross-linking of the vinyl-ONs with the Luciferase mRNA is responsible for its silencing.

3. Conclusions

2′-OMe-RNAs having cross-linkable ADpVP and ADVP were successfully synthesized and evaluated. The steric effect of the 7-substituent on the reactivity of the 6-vinyl group to the complementary bases in the duplex form was found to ve productive as orginally hypothesized. Our data demonstrated several very important advantages of utilizing ADpVP in that (i) having a certain stability during the process of purification, lyophilization and storage, and (ii) very swift cross-linking occurs to uracil at the complementary site in the duplex. This advantage encourages us to further assess the *in-cell* stablity of the vinyl of ADpVP, and to investigate the efficient/selective covalant-binding toward various endogenous RNAs to induce a very solid/robust regulation of the gene expression. Further study of ADpVP and its derivatives is currently on-going in our laboratory.

4. Experimental section

4.1. General materials and methods

The NMR spectra were recorded using Brucker 400 or 600 MHz spectrometer. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded at 600 MHz (^{1}H-NMR, 600 MHz; ^{13}C-NMR, 150 MHz; ^{31}P-NMR, 162 MHz) or 400 MHz (^{1}H NMR, 400 MHz; ^{13}C NMR, 100 MHz). The chemical shifts were measured from tetramethylsilane (0 ppm), $CDCl_3$ (7.26 ppm) or DMSO-d_6(2.49 ppm) for ^{1}H NMR spectra, $CDCl_3$ (77.0 ppm) or DMSO-d_6 (39.7 ppm) for ^{13}C-NMR spectra, and 85% H_3PO_4 for ^{31}P-NMR spectra as external standards. The electrospray ionization (ESI) mass spectra were recorded on BioTOF II (Bruker Daltonics). The MALDI-TOF mass spectra were recorded on Autoflex speed mass spectrometer and the laser at 337 nm by negative or positive mode using 3-hydroxypicolinic acid as a matrix. Thin-layer chromatography (TLC) analysis was conducted using E. Merck Silica gel 60 F_{254} pre-coated glass plates. Column chromatography was performed with Kanto Chemical silica gel 60 N (spherical, neutral, 100-210 μm). Flash chromatography was performed with Kanto Chemical silica gel 60 N (spherical, neutral, 40-50 μm). The synthesis of modified oligonucleotides was performed using an automated DNA synthesizer 392 (Applied Biosystem). The unmodified oligonucleotides were purchased from JBioS, Co Ltd. HPLC was performed using Nacalai Tesque COSMOSIL 5C18-MS-II (4.6 or 10 × 250 mm) as a column. Anhydrous methanol, DMF, THF, CH_2Cl_2, 1,4-dioxane, pyridine, DMSO, toluene, and acetonitrile were purchased from Wako Pure Chemical Industries Ltd.

4.2. Synthesis of nucleoside derivatives

Synthesis of 2-Amino-5-iodo-4-methoxy-7-(β-D-ribofuranosyl)-*7H*-pyrrolo-[2,3-*d*]pyrimidine (**2**)

To a stirred suspension of **1** (2.6 g, 6.87 mmol) in anhydrous acetonitrile (46 mL) was added BSA (2.0 mL, 8.3 mmol) at room temperature. After stirring for 20 min, TMSOTf (1.6 mL, 8.93 mmol) was added and 1-O-acetyl-2,3,5-tri-O-benzoyl-D-ribofuranose (2.08 g, 4.12 mmol) was separately added three times at every 8h. The

reaction mixture was stirred at 50 °C for 24 h in total, cooled to room temperature, and diluted with CH_2Cl_2 (180 mL). The solution was washed with aqueous sat. $NaHCO_3$ (200 mL) and brine (200 mL), and after dried organic layer over Na_2SO_4, the solution was evaporated under reduced pressure. Obtained crude material was applied to silica gel column chromatography (eluent: Hexane / $CHCl_3$, 1:1) yielding the nucleoside as a yellow foam with slight amount of impurity (NMR yield 66 %). Without further purification, the nucleoside (731 mg; 0.89 mmol by NMR calculation) in anhydrous 0.5 M NaOMe-MeOH (48 mL) was heated under reflux for 3 h. The reaction mixture was neutralized with acetic acid, and then evaporated. The residue was taken up in EtOAc (400 mL) and washed with aqueous sat. $NaHCO_3$ (300 mL×2), and purified by a silica gel column chromatography (eluent: DCM / MeOH, from 40:1 to 8:1) yielding compound **2** (238 mg, 0.56 mmol, NMR yield 63%) was obtained as a white powder; ^{1}H-NMR (600 MHz, DMSO-d_6) δ 7.29 (s, 1H), 6.35 (br, 1H), 5.92-5.93 (d, 1H , *J* = 6.52 Hz), 5.22-5.24 (d, 1H, *J* = 6.16 Hz), 5.03-5.04 (d, 1H, *J* = 4.32 Hz), 4.96-4.99 (t, 1H, *J* = 5.44 Hz), 4.23-4.27 (m, 1H), 4.00-4.02 (m, 1H), 3.9 (s, 3H), 3.78-3.81 (m, 1H), 3.46-55 (m, 2H); ^{13}C-NMR (150 MHz, DMSO-*d6*): δ 164.3, 158.3, 151.8, 128.3, 102.8, 91.4, 87.2, 81.5, 70.8, 63.5, 58.8, 53.7, 50.2; HRMS (ESI) calcd for $C_{12}H_{16}IN_4O_5^+$ $[M+H]^+$ *m/z* 423.0160, found *m/z* 423.0158.

Synthesis of 2-Amino-5-iodo-4-methoxy-7-[(2-*O*-methyl)-β-D-ribofuranosyl)]-*7H*-pyrrolo-[2,3-*d*]pyrimidine (**3)**

A solution of the compound **2** (1.4 g, 3.32 mmol) in anhydrous DMF (140 mL) at 0 °C was added 60 % NaH (166 mg, 4.15 mmol) and stirred for 30 min. After adding CH_3I (247 μL, 3.98 mmol) dropwise to the solution, reaction mixture was warmed to an ambient temperature and stirred for 16 h. After the excess solvent was evaporated under reduced pressure, the residue was diluted with EtOAc (200 mL), washed with brine (150 mL×2, dried over Na_2SO_4, and evaporated. The crude was purified by silica gel column chromatography (eluent: 2% MeOH in DCM) yielding compound **3** (626 mg, 43%) ^{1}H-NMR (600 MHz, $CDCl_3$) δ 6.93 (br. s, 1H), 6.84 (s, 1H), 5.59 (d,1H, *J* = 7.8 Hz), 4.90 (br, 2H), 4.68 (m, 1H), 4.51 (m, 1H), 4.28 (br, 1H), 4.02 (s, 3H), 3.94 (m, 1H), 3.73 (m, 1H), 3.32 (s, 3H), 2.76 (br, 1H); ^{13}C-NMR($CDCl_3$, 150 MHz): δ 164.3, 158.3, 151.7, 128.3, 102.8, 91.4, 87.2, 81.5, 70.8, 63.5, 58.8, 53.7, 50.2; HRMS (ESI) m/z calcd for $C_{13}H_{18}IN_4O_5^+$ $[M+H]^+$ *m/z* 437.0316, found *m/z* 437.0324.

Synthesis of 2-Amino-5-iodo-7-[(2-*O*-methyl)-β-D-ribofuranosyl)]-3,7 dihydro-*4H*-pyrrolo-[2,3-*d*]pyrimidine-4-one (**4)**

Compound **3** (1.07 g, 2.45 mmol) was dissolved in 1,4-dioxane (8.2 mL) and added aqueous 2M NaOH (54 mL, 113 mmol). After the mixture was stirred under reflux for 4 h, the solution was neutralized to pH 8 with aqueous 2M HCl (46 mL), and then evaporated excess 1,4-dioxane under reduced pressure. During this process, the compound **4** was precipitated, which was collected by filtration and washed with iced water yielding compound **4** (1.0 g, 2.38 mmol, 97%); ^{1}H-NMR (600 MHz, DMSO-d_6); δ 10.53 (br, 1H), 7.15 (s. 1H), 6.35 (br, 1H), 5.92-5.93 (d, 1H, *J* = 7.2 Hz), 5.15 (d, 1H, *J* = 4.8 Hz), 5.03-5.05 (t, 1H, *J* = 5.4 Hz), 4.19-4.18 (dd, 1H, *J* = 4.7, *J* = 7.2 Hz), 3.97-3.99 (dd, 1H, *J* = 4.8 Hz, 6.6 Hz), 3.82-3.80 (dd, 1H, *J* = 4.2 Hz, 7.2 Hz), 3.53-3.49 (m, 2H), 3.52 (s, 3H); ^{13}C-NMR (150 MHz, DMSO-d_6) δ 158.6, 153.2, 151.6, 122.3, 100.4, 86.0, 84.2, 83.1, 69.2, 61.9, 57.8, 55.9; HRMS (ESI) m/z: calcd for $C_{12}H_{16}IN_4O_5^+$ $[M+H]^+$ m/z 423.0160, found m/z 423.0153.

Synthesis of 2-Amino-5-iodo-7-[(2-*O*-methyl-3,4-*O*-*t*-butyldimethylsilyl)-β-D-ribofuranosyl)]-3,7 dihydro-*4H*-pyrrolo-[2,3-*d*]pyrimidine-4-one (**5**)

To a suspension of compound **4** (1.0 g, 2.38 mmol) in anhydrous DMF (4.4 mL), imidazole (974 mg, 14.3 mmol) and TBDMSCl (1.07 g, 7.14 mmol) was added at 0 °C and stirred for 2 h at ambient temperature. The solution was diluted with

ethyl acetate and organic layer was washed with brine, dried over Na_2SO_4, filtered, and then evaporated. Obtained crude material was purified by silica gel column chromatography, which afforded compound **5** as a white form (1.30 g, 84%). ^{1}H-NMR(600 MHz, $CDCl_3$): δ 10.87 (br, 1HH), 7.10 (s. 1H), 6.14 (d, 1H, *J* = 4.2 Hz), 5.96 (br, 2H), 4.42 (t, 1H, *J* = 4.8 Hz), 4.02-4.00 (m, 1H, *J* = 2.4 Hz), 3.93-3.90 (dd, 1H, *J* = 2.4Hz, 11.4 Hz), 3.81-3.79 (t, 1H, *J* = 4.8 Hz), 3.77-3.74 (dd, 1H, *J* = 2.4 Hz, 11.4 Hz), 3.52 (s, 3H), 0.97 (s, 9H), 0.92 (s, 9H), 0.15 (s, 3H), 0.14 (s, 3H), 0.12 (s, 3H), 0.10 (s, 3H); ^{13}C-NMR (150 MHz, $CDCl_3$) δ 160.2 152.3, 151.5, 122.8, 100.9, 85.6, 84.6, 84.4, 70.0, 62.1, 58.4, 54.1, 26.2, 25.8, 18.6, 18.2, -4.6, -4.8, -5.2, -5.4; HRMS (ESI) m/z: calcd for $C_{24}H_{44}IN_4O_5Si_2^+$ $[M+H]^+$ m/z 651.1889, found m/z 651.1893.

Synthesis of 2-Amino-5-propynyl-7-[(2-*O*-methyl-3,5-*O*-*t*-butyldimethylsilyl)-β-D-ribofuranosyl)]-3,7 dihydro-*4H*-pyrrolo- [2,3-*d*]pyrimidine-4-one (**6**)

After compound **5** (700 mg, 1.08 mmol), $Pd(PPh_3)_4$ (125 mg, 0.11 mmol), and CuI (41 mg, 0.22 mmol) was left under vacuum in the flask for 10 min The flask was purged with argon, and anhydrous degassed DMF (3.3 mL) and degassed HMDS (1.2 mL) were added, and then stirred for 10 min at ambient temperature. To the solution, 1 M propyne in DMF solution (10.8 mL, 10.77 mmol) was added and stirred at 40 ^{o}C for 2 h. The solvent was then diluted with ethyl acetate, and organic layer was washed with brine, dried over Na_2SO_4, filtered, and evaporated. The crude product was purified by silica gel column chromatography (eluent: Et_2O / CH_3CN, from 60:1 to 4:1) to give compound **6** as a white form (503 mg, 83%). ^{1}H-NMR (600 MHz, $CDCl_3$): δ 11.04 (br, 1H), 7.15 (s, 1H), 6.12 (d, 1H, *J* = 4.2 Hz), 5.99 (br, 2H), 4.45-4.43 (t, 1H, *J* = 5.4 Hz), 4.01-3.99 (m, 1H), 3.92-3.89 (dd, 1H, *J* = 3.0 Hz, 11.4 Hz), 3.77-3.75 (m, 2H), 3.44 (s, 3H), 2.07 (s, 3H), 0.96 (s, 9H), 0.92 (s, 9H), 0.12 (s, 6H), 0.11 (s, 6H), 0.10 (s, 3H); ^{13}C-NMR (150 MHz, $CDCl_3$,): δ 160.0, 152.9, 150.8, 122.2, 100.4, 99.9, 86.1, 85.6, 84.4, 84.3, 73.1, 69.9, 62.0, 58.4, 26.1, 25.8, 18.5, 18.2, 4.8, -4.6, -4.8, -5.4, -5.5; HRMS (ESI) m/z: calcd for $C_{27}H_{47}N_4O_5Si_2^+$ $[M+H]^+$ 563.3080, found m/z 563.3080.

Synthesis of 2-Amino-5-propynyl-4-tosyloxy-7-[(2-*O*-methyl-3,5-*O*-*t*-butyldimethylsilyl)-β-D-ribofuranosyl)]-*7H*-pyrrolo-[2,3-*d*]pyrimidine (**7**)

The compound **6** (700 mg, 1.24 mmol) in anhydrous DCM (13 mL) was added anhydrous trimethylamine (698 μL, 4.97 mmol), *N*,*N*-dimethyl-4-aminopyridine (14.7 mg, 0.12 mmol), *p*-toluenesulfonyl chloride (472 mg, 2.48 mmol) at 0^{o}C, and the mixture was stirred for 3 h under argon at ambient temperature. After the solution was diluted with DCM, the organic layer was washed with brine, dried over Na_2SO_4, filtered, and then evaporated. Obtained crude material was purified by silica gel column choromatography (hexane / EtOAc, from 7:1 to 2:1) yielding compound **7** as a yellow form (708 mg, 80%). ^{1}H-NMR (600 MHz, $CDCl_3$); δ 8.13 (d, 2H, *J* = 8.4 Hz), 7.36-7.35 (m, 3H), 6.17-6.16 (d, 1H, *J* = 3.6 Hz), 4.88 (br, 2H), 4.41-4.43 (t, 1H, *J* = 5.4 Hz), 4.02-4.00 (m, 1H), 3.92-3.90 (dd, *J* = 3.0 Hz, 11.4 Hz), 3.76-3.74 (m, 2H), 3.43 (s, 3H), 2.46 (s, 3H), 1.90 (s, 3H), 0.95 (s, 9H), 0.92 (s, 9H), 0.12 (s, 3H), 0.11 (s, 3H), 0.10 (s, 3H), 0.09 (s, 3H); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 158.5, 157.0, 154.9, 145.1, 134.4, 129.5, 128.8, 125.7, 101.9, 97.3, 87.4, 85.7, 84.3, 84.2, 71.2, 69.7, 61.9, 58.4, 26.0, 25.8, 21.7, 18.5, 18.2, 4.3, -4.6, -4.8, -5.4, -5.5; HRMS (ESI) m/z: calcd for $C_{34}H_{53}N_4O_7SSi_2^+$ $[M+H]^+$ m/z 717.3168, found m/z 717.3176.

Synthesis of 2-Amino-5-propynyl-4-(2-methylthioethyl))-7-[(2-*O*-methyl-3,5-*O*-*t*-butyldimethylsilyl)-β-D-ribofuranosyl)]-*7H*-pyrrolo-[2,3-*d*]pyrimidine **(8)**

This compound **7** (220 mg, 307.1 μmol) in anhydrous degassed 1,4-dioxane (1.53 mL) was added degassed H_2O (505 μL), K_2CO_3 (29.7 mg, 215.0 μmol), vinylboronic anhydride pyridine complex (51.7 mg, 215.0 μmol), $PdCl_2(PPh_3)_2$ (21.5 mg, 30.7 μmol), and the mixture was stirred for 1 h under argon at 120 °C. After the reaction mixture was diluted with EtOAc, the organic layer was washed with brine, dried over Na_2SO_4, filtered and evaporated. Obtained crude material was purified by silica gel column chromatography (eluent: hexane/ EtOAc, from 7: 1 to 4:1) to give compound **8** as a yellow form with a slight amount of inpurity. HRMS (ESI) m/z: calcd. for $C_{29}H_{49}N_4O_4Si_2^+$ $[M+H]^+$ m/z 573.3287, m/z found 573.3302. Obtained material was subsequently used to the next reaction. The vinyl derivative was dissolved in anhydrous 1,4-dioxane (1.23 mL) and NaSMe (43.4 mg, 605.3 μmol) and H_2O (417 μL) was added. After stirring for 3 h at ambient temperature and the reaction mixture was diluted with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4, filtered and evaporated, which afforded compound **8** as an pure yellow form (142 mg, 95%). ^{1}H-NMR (600 MHz, $CDCl_3$): δ 7.40 (s, 1H, 8-H), 6.23 (d, 1H, *J* = 3.6 Hz), 4.82 (br, 2H), 4.46-4.45 (t, 1H, *J* = 5.4 Hz), 4.02-3.99 (m, 1H), 3.93-3.91 (dd, 1H, *J* = 3.0 Hz, 11.4 Hz), 3.80-3.78 (t, 1H, *J* = 4.2 Hz), 3.76-3.74 (dd, 1H, *J* = 2.4 Hz, 12Hz), 3.46 (s, 3H), 3.41-3.35 (m, 2H), 2.92-2.90 (m, 2H), 2.20 (s, 3H), 2.08 (s, 3H), 0.96 (s, 9H), 0.92 (s, 9H), 0.13 (s, 3H), 0.12 (s, 3H), 0.11 (s, 3H), 0.10 (s, 3H); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 163.2, 159.4, 152.2, 125.8, 111.0, 97.8, 87.4, 85.5, 84.2, 84.1, 73.0, 69.8, 61.9, 58.4, 34.5, 33.2, 26.1, 25.8, 18.5, 18.2, 15.5, 4.6, -4.6, -4.8, -5.4, -5.5; HRMS (ESI) m/z: calcd for $C_{30}H_{53}N_4O_4SSi_2^+$ $[M+H]^+$ m/z 621.3321, m/z found 621.3334.

Synthesis of 2-Phenoxyacetylamino-5-propynyl-4-(2-methylthioethyl)-7-[(2-*O*-methyl-3,5-*O*-*t*-butyldimethylsilyl) -β-D-ribofuranosyl)]-*7H*-pyrrolo-[2,3-*d*]pyrimidine (**9)**

To a solution of compound **8** (142 mg, 228.9 μmol) in anhydrous pyridine (1.8 mL) was added phenoxyacetyl chloride (47 μL, 343.4 μmol) at 0 °C, and stirred for 2h at ambient temperature, then diluted with ethyl acetate. The separated organic layer was washed with brine, dried over Na_2SO_4, filtered, and evaporated. Obtained crude material was purified by silica gel column chromatography to give compound **9** as a white form (151 mg, 87%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.80 (br, 1H), 7.79 (s, 1H), 7.38-7.34 (t, 2H, *J* = 7.6 Hz), 7.06-7.03 (m, 3H), 6.36 (d, 1H, *J* = 2.8 Hz), 4.82 (br, 2H), 4.50-4.48 (t, 1H, *J* = 4.8 Hz), 4.10-4.04 (m, 2H), 3.89-3.81 (m, 2H), 3.60 (s, 3H), 3.56-3.51 (m, 2H), 3.04-3.00 (t, 2H, *J* = 7.8 Hz), 2.22 (s, 3H), 2.10 (s, 3H), 1.00 (s, 9H), 0.93 (s, 9H), 0.18 (s, 3H), 0.17 (s, 3H), 0.12 (s, 3H), 0.11 (s, 3H); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 163.4, 157.3, 151.4, 105.2, 129.8, 129.6, 128.8, 122.1, 114.9, 114.7, 97.7, 88.1, 86.3, 84.4, 84.0, 72.4, 69.2, 68.0, 61.5, 58.4, 34.2, 33.0, 26.1, 25.8, 18.5, 18.2, 15.6, 4.5, -4.6, -4.8, -5.40, -5.43;HRMS (ESI) m/z: calcd for $C_{38}H_{59}N_4O_6SSi_2^+$ $[M+H]^+$ m/z 755.3688, found m/z 755.3697.

Synthesis of 2-Phenoxyacetylamino-5-propynyl-4-(2-methylthioethyl))-7-[(2-*O*-methyl-β-D-ribofuranosyl)]-*7H*-pyrrolo-[2,3-*d*]pyrimidine (**10)**

To a solution of **9** (150 mg, 198.8 μmol) in anhydrous THF solution (225 μL) was added 1.0 M tetrabutylammonium fluoride in THF solution (596.5 μmol, 597 μL) at 0 °C, and the mixture was stirred for 1 h at ambient temperature. The reaction mixture was concentrated under reduced pressure and obtained crude material was purified by silica gel column chromatography (eluent: EtOAc/hexane, from 4:1 to 100:0) to give the compound **10** (87 mg, 83%). ^{1}H-NMR (600 MHz, $CDCl_3$); δ 8.94 (br, 1H), 7.36-7.33 (m, 2H), 7.30 (s, 1H), 7.06-7.03 (m, 3H), 5.83 (d, 1H, *J* = 5.4 Hz), 4.72 (br, 2H), 4.67-466 (m, 2H), 4.24 (m, 1H) , 4.20 (m, 1H), 3.99-3.97 (m, 1H), 3.86-3.84 (m, 1H), 3.54-3.53 (m, 2H), 3.41 (s, 3H), 3.00-2.98 (t, 2H, *J* = 7.8 Hz), 2.66 (d, 1H, *J* = 3.0 Hz), 2.21 (s, 3H), 2.11 (s, 3H); ^{13}C-NMR (150 MHz, $CDCl_3$) δ 166.4, 164.5, 157.2, 151.1, 149.9, 131.2, 129.8, 122.3, 116.1, 115.0, 98.1, 90.1, 88.8, 86.2, 82.1, 71.8, 70.0, 67.8, 62.7, 58.8, 34.3,

33.0, 15.6, 4.6; HRMS (ESI) m/z calcd for $C_{26}H_{31}N_4O_6S^+$ $[M+H]^+$ m/z 527.1959, found m/z 527.1969.

Synthesis of 2-Phenoxyacetylamino-5-propynyl-4-(2-methylthioethyl))-7-[(2-O-methyl-5-O-(4,4'-O-dimethoxytrityl))-β-D-ribofuranosyl)]-*7H*-pyrrolo-[2,3-*d*]pyrimidine-4-one (**11**)

To a solution of **10** (87 mg, 165 μmol) in anhydrous pyridine (742 μL) was added 4,4'-dimethoxytrityl chloride (78 mg, 231 μmol) at 0 °C and stirred for 3 h at ambient temperature. The reaction mixture was diluted with ethyl acetate and organic layer was washed with brine, dried over Na_2SO_4, filtered, and evaporated. Obtained crude material was purified by silica gel column chromatography (eluent: EtOAc/hexane, from 2:3 to 2:1) yielding compound **11** (102 mg, 75%); ^{1}H-NMR (600 MHz, $CDCl_3$); δ 8.78 (br, 1 H), 7.55 (s, 1H), 7.46-6.82 (m, 18H), 6.34 (d, 1H, *J* = 2.4 Hz), 4.76 (br, 2H), 4.49-4.46 (dd, 1H, *J* = 7.2 Hz, 12.6 Hz), 4.24 (m., 2H), 3.79 (s, 6H), 3.64 (s, 3H), 3.52-48 (m, 3H), 3.46-3.45 (dd, 1H, *J* = 4.2 Hz, 10.8 Hz), 3.54-3.53 (m, 2H), 2.66 (d, 1H, *J* = 7.8 Hz), 2.21 (s, 3H), 2.06 (s, 3H); ^{13}C-NMR (150 MHz, $CDCl_3$); δ 163.7, 158.6, 157.3, 151.5, 150.3, 144.7, 135.9, 135.8, 130.2, 129.8, 128.4, 128.3, 127.9, 126.8, 122.2, 114.9, 113.3, 113.2, 98.3, 88.4, 88.6, 86.3, 83.8, 83.2, 72.1, 69.6, 68.1, 62.9, 58.8, 55.2, 34.2, 33.0, 15.6, 4.5; HRMS (ESI) m/z: calcd for $C_{47}H_{49}N_4O_8S^+$ $[M+H]^+$ m/z 829.3266, found m/z 829.3261.

Synthesis of 2-Phenoxyacetylamino-5-propynyl-4-(2-methylthioethyl)-7-[2-*O*-methyl-3-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite)-*5*-O-(4,4'-O-dimethoxytrityl)-β-D-ribofuranosyl]-*7H*-pyrrolo-[2,3-*d*]pyrimidine (**12**)

To a solution of compound **11** (50 mg, 60 μmol) in anhydrous CH_2Cl_2 (1.23 mL) was added *N,N'*-diisopropylethylamine (82 μL, 480 μmol) and 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (60 μL, 240 μmol) at 0 °C. After the reaction mixture was stirred for 2 h at 0 °C, the resulting mixture was diluted with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The obtained crude material was purified by silica gel column chromatography (eluent: hexane/EtOAc, from 5:2 to 3:2) to afford compound **12** as a colorless oil (45 mg, 73%); ^{1}H-NMR (600 MHz, $CDCl_3$); δ 8.72 (br, 1H), 7.59-7.25 (m, 11H), 7.06-7.03 (m, 3H), 6.87-6.84 (m, 4H), 39-6.37 (m, 1H), 4.84 (br, 2H), 4.61-4.20 (m, 3H), 3.97-3.88 (m, 2H), 3.82 (s, 6H), 3.64-3.60 (m, 2H), 3.54-3.41 (m, 7H), 3.02 (t, 2H, *J* = 7.8 Hz), 2.68-2.67 (m, 1H), 2.40-2.39 (1H, m), 2.24 (3H, s), 2.07-2.09 (3H, m), 1.20-1.06 (m, 12H); ^{31}P-NMR (152 MHz, $CDCl_3$) δ 150.91, 150.20; HRMS (ESI) m/z calcd for $C_{56}H_{66}N_6O_9PS^+$ $[M+H]^+$ m/z 1029.4344, found m/z 1029.4341

Synthesis of 4-Chloro-7-{5'-*O*-[(1,1-dimethylethyl)dimethylsilyl]-2',3'-*O*-(1-methylethylidene)-β-d-ribofuranosyl}-7*H*-pyrrolo[2,3-*d*]pyrimidine-2-amine **(14)**[36~38]

To a solution of 6-chloro-7-deazaguanine (676 mg, 4.0 mmol) in anhydrous CH_3CN (40 mL), 60% NaH in mineral oil (192 mg, 4.8 mmol) was added at 0 °C and compound **13** (625.5 mg, 2 mmol) in anhydrous THF (4 mL) was slowly added. The solution was stirred over night at ambient temperature, and the excess solvent was evaporated. The obtained crude material was diluted with excess ethyl acetate and the precipitate was filtered off. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and evaporated. Obtained crude material was purified by silica gel column chromatography (Hexane-EtOAc, 10/1, 8/1, 4/1) yielding compound **14** (602.7 mg, 33%). ^{1}H-NMR (600 MHz, $CDCl_3$) *δ* 7.13 (d, 1H, *J* = 3.6 Hz), 6.41 (d, 1H, *J* = 3.6 Hz), 6.24 (d, 1H, *J* = 3.0 Hz), 5.02 (br, 2H), 4.98 (dd, 1H, *J* = 3.0 Hz, 6.0 Hz), 4.92 (dd, 1H, *J* = 3.0 Hz, 6.0 Hz),4.27-4.25 (m, 1H), 3.84 (1H, dd, *J* = 3.6 Hz, 11.1 Hz), 3.79 (1H, dd, *J* = 4.2 Hz, 11.1 Hz), 1.63 (s, 3H), 1.38 (s, 3H), 0.91 (s, 9H), 0.07 (s. 6H); ^{13}C-NMR (150 MHz, $CDCl_3$) *δ* 158.6, 153.5, 152.6, 123.3, 114.1,111.1, 100.9, 89.3, 85.6, 84.6, 80.7, 63.3, 27.4, , 25.5, 18.4, -5.4, -5.5; HRMS (ESI) calcd. for $C_{20}H_{32}ClN_4O_4^+$ $[M + H]^+$ *m/z* 455.1876, found *m/z* 455.1879.

Synthesis of 4-Chloro-7-{(1,1-dimethylethyl)]-2‘,3‘-*O*-(1-methylethylidene)-β-d-ribofuranosyl}-7*H*-pyrrolo[2,3-*d*] pyrimidine-2-amine **(15)**

To a solution of compound **14** (555.1 mg, 1.22 mmol), 0.1 M TBAF-THF solution (61.2 mL, 6.12 mmol) was added and stirred at ambient temperature for 30 min. After evaporating excess THF, the excess EtOAc was added and washed with sat. NH_4Cl aq. and brine. The organic layer was dried over Na_2SO_4, filtered and evaporated. Obtained crude material was purified by silica gel column chromatography (CH_2Cl_2/MeOH, from 100:0 to 97:3) yielding compound **15** as a white foam (400.3 mg, 97%). ^{1}H-NMR (600 MHz, $CDCl_3$) δ 6.90 (d, 1H, J = 3.9 Hz), 6.38 (d, 1H, J = 3.9 Hz), 6.00 (d, 1H, J = 11.4), 5.70 (1H, d, J = 4.8), 5.20 (t, 1H, J = 5.4), 5.18 (br, 2H), 5.07 (dd, 1H, J = 1.8 Hz, 6.0 Hz) 4.47 (d, 1H, J = 1.8 Hz), 3.97 (d, 1H, J = 12.0), 3.76 (t, 1H, J = 1.8 Hz), 1.62 (s, 3H), 1.37 (s, 3H); ^{13}C-NMR (150 MHz, $CDCl_3$) δ 157.9, 153,8, 151.6, 126.1, 114.0, 112.4, 100.3, 95.5, 85.3, 82.6, 81.3, 63.3, 27.5, 25.2; HRMS (ESI) calcd. for $C_{14}H_{18}ClN_4O_4^+$ $[M + H]^+$ *m/z* 341.1011, found *m/z* 341.1012.

Synthesis of 2-Amino-4-chloro-7-(*β*-d-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (**16)**

Compound **15** (400.3 mg, 1.17 mmol) was dissolved in 80% aqueous AcOH and stirred for 1 h at ambient temperature. The reaction mixture was then further stirred for 3 h at 80 ^{o}C. After evaporation and co-evaporation with toluene, obtained crude material was purified by silica gel column chromatography (CH_2Cl_2/MeOH, from 98:2 to 90:10) yielding compound **16** as a white solid (297.4 mg, 78%). ^{1}H-NMR (600 MHz, DMSO-*d6*) δ 7.38 (d, 1H, J = 3.6 Hz), 6.68 (br, 2H), 6.36 (d, 1H, J = 4.2 Hz), 5.98 (d, 1H, J = 6.6 Hz), 5.28 (d, 1H, J = 6.0 Hz), 5.08 (d, 1H, J = 4.2 Hz), 4.97 (t, 1H, J = 5.4 Hz), 4.30 (dd, 1H, J = 6.0 Hz, 11.4 Hz), 4.05 (dd, 1H, J = 4.2 Hz, 7.8 Hz), 3.83 (dd, 1H, J = 3.6 Hz, 7.2 Hz), 3.59-3.56 (m, 1H), 3.52-3.49 (m, 1H); ^{13}C-NMR (150 MHz, DMSO-*d6*) δ 159.9, 154.9, 151.6, 123.8, 109.4, 100.3, 86.5, 85.3, 74.1, 71.1, 62.1; HRMS (ESI) calcd. for $C_{11}H_{14}ClN_4O_4^+$ $[M + H]^+$ *m/z* 301.0698, found *m/z* 301.0702.

Synthesis of 2-Amino-4-chloro-7-(2-*O*-methyl-*β*-d-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (**17**)

Compound **16** (90.2 mg, 0.30 mmol) was rendered anhydrous by repeated co-evaporation with anhydrous CH_3CN, and dissolved in anhydrous DMF (9.0 mL). To this solution, 60% NaH (13.2 mg, 0.33 mmol) and stirred for 30 min. Then CH_3I (18.7 μL, 0.30 mmol) was slowly added at 0 ^{o}C, and then stirred for 30 min at the same temperature. After diluting with excess EtOAc, the organic layer was washed with aqueous sat. NH_4Cl. The water layer was repeatedly extracted with EtOAc, and all organic layers were combined. This solution was dried over Na_2SO_4, filtered, and then evaporated. Obtained crude material was purified by silica gel column chromatography (CH_2Cl_2/MeOH, from 98:2 to 80:20) yielding pure 2′-O-methylated compound **17** as a white solid (26.1 mg, 28%). ^{1}H-NMR (600 MHz, CD_3CN) δ 7.22 (d, 1H, J = 3.9 Hz), 6.41 (d, 1H, J = 3.9 Hz), 5.94 (d, 1H, J = 6.6 Hz), 5.43 (br, 2H), 4.376-4.373 (m, 1H), 4.29 (dd, 1H, J = 5.4 Hz, 6.6 Hz), 4.28 (br, 1H), 3.33-3.32 (m, 4H); ^{13}C-NMR (150 MHz, CD_3CN) δ 159.9, 154.2, 153.4, 125.7, 111.9, 100.9, 88.2, 87.0, 83.6, 70.7, 63.1, 58.6; HRMS (ESI) calcd. for $C_{12}H_{16}ClN_4O_4^+$ $[M + H]^+$ *m/z* 315.0855, found *m/z* 315.0866.

Synthesis of 2-Phenoxyacetylamino-4-(2-methylthioethyl))-7-[(2-*O*-methyl-β-D-ribofuranosyl)]-*7H*-pyrrolo-[2,3-*d*] pyrimidine (**18)**

To a solution of compound **17** (19.7 mg, 62.5 μmol) in degassed 1,4-dioxane-H_2O (3:1, v/v) (2.4 mL) was added K_2CO_3 (9.5 mg, 68.8 μmol), vinylboronic anhydride pyridine complex (7.5 mg, 31.3 μmol), and $Pd(PPh_3)_4$ (7.2 mg, 6.25 μmol) at ambient temperature, and then stirred at 80 ^{o}C for 1.5 h. After cooling the reaction mixture to ambient temperature, 1-octanethiol (187.5 μL, 32.7 μmol) was added and stirred for 2.5 h. Further 1-octanethol (187.5 μL, 32.7 μmol) was added and stirred for 1 h, then diluted with excess CH_2Cl_2. After the precipitate was filtered off, the organic layer was washed with

brine, dried over Na_2SO_4, filtered, and evaporated. Obtained crude material was passed through silica gel column chromatography (CH_2Cl_2/MeOH, 97:3), and was used for the next reaction without further purification. A solution of the crude product (32.1 mg, 0.071 mmol) and imidazole (96.8 mg, 1.42 mmol) in DMF (140 µl) was added TBSCl (107 mg, 0.71 mmol) at room temperature. After being stirred for 12 h, the mixture was diluted with hexane/ethyl acetate = 3 and aq. $NaHCO_3$ was added. The mixture was extracted with hexane/ethyl acetate = 3. The organic layer was washed with brine and dried over Na_2SO_4, and evaporated. The mixture was passed through silica gel column chromatography (hexane/ethyl acetate = 10:3), and obtained TBS-protected compound containing a slight amount of unseparable impurity was used for the next reaction without further purification.

A solution of TBS-protected compound (68.2 mg, 0.100 mmol) in Pyridine (1 ml) was added PacCl (31.7 µl, 0.150 mmol) at 0 °C. After being stirred for 3 h, the mixture was diluted with hexane/ethyl acetate = 3 and aq. $NaHCO_3$ was added. The mixture was extracted with hexane/ethyl acetate = 3. The organic layer was washed with brine and dried over Na_2SO_4 and evaporated. The mixture was passed through silica gel column chromatography (hexane/ethyl acetate = 10:3). The mixture (40.8 mg, 0.0490 mmol) was dissolved in THF (1 ml) was added TBAF (1.0 M in THF) (0.107 ml, 0.107 mmol) at room temperature. The reaction mixture was evaporated to dryness. The residue was diluted with hexane/ethyl acetate = 1 and aq. $NaHCO_3$ was added. The mixture was extracted with hexane/ethyl acetate = 1. The organic layer was washed with brine and dried over Na_2SO_4. Concentration of the resulting solution gave **18** (17.9 mg, 0.031 mmol) in 44 % yield for 4 steps.

^{1}H-NMR (600 MHz, CD_3CN) δ9.02 (br, 1H), 7.34-7.23 (m, 3H), 7.04-7.02 (m, 3H), 6.52 (d, 1H, J = 3.6 Hz), 4.687-4.685 (m, 3H), 4.215-4.218 (1H, m), 3.98-3.96 (m, 1H), 3.85-3.82 (m, 1H), 3.55 (s, 3H), 3.23 (t, 2H, *J* = 7.7 Hz), 3.13 (br, 1H), 3.00 (t, 2H, *J* = 7.7 Hz), 2.57 (t, 2H, *J* = 7.2 Hz), 1.61-1.56 (m, 3H), 1.37-1.27 (m, 10H), 0.88-0.86 (m, 3H); ^{13}C-NMR (150 MHz, $CDCl_3$) δ 162.8, 157.1, 150.4, 129.6, 128.1, 122.1, 116.6, 114.8, 99.9, 89.8, 86.0, 82.0, 69.9, 67.7, 62.6, 58.5, 35.3, 32.30. 32.27, 31.6, 30.2, 29.5, 29.06, 29.05, 28.8, 22.5, 14.0; HRMS (ESI) calcd. for $C_{30}H_{43}N_4O_6S^+$ $[M + H]^+$ *m/z* 587.2898, found *m/z* 587.2912.

Synthesis of 2-Phenoxyacetylamino-4-(2-methylthioethyl)-7-[2-*O*-methyl-*5*-O-(4,4'-O-dimethoxytrityl)-β-D-ribofuranosyl]-*7H*-pyrrolo-[2,3-*d*]pyrimidine (**19**)

Compound **18** (127.9 mg, 0.21 mmol) was rendered anhydrous by repeated co-evaporation with anhydrous pyridine and dissolved in anhydrous pyridine (2.0 mL). To this solution, DMTr-Cl (88.6 mg, 0.26 mmol) was added and stirred for 2 h at ambient temperature. MeOH (2.0 mL) was then added and stirred for 10 min at ambient temperature. To this mixture, excess amount of ethyl acetate was added and repeatedly washed with sat. $NaHCO_3$aq. After drying organic layer over Na_2SO_4, filtered and evaporated, obtained crude material was purified by silica gel column chromatography (Hexane/CH_2Cl_2, 1:1, 1:2) yielding pure compound **19** (73.5 mg, yield not determined). ^{1}H-NMR (600 MHz, CD_3CN) δ 8.56 (br, 1H), 7.46-7.15 (m, 12H), 7.04-7.01 (m, 3H), 6.81-6.80 (d, 4H, *J* = 9.0 Hz), 6.43 (d, 1H, *J* = 3.6 Hz), 6.40 (d, 1H, *J* = 3.0 Hz), 4.79 (br, 2H), 4.55 (d, 1H, *J* = 6.6 Hz, 12.6 Hz), 4.17-4.12 (m, 2H), 3.77 (s, 6H), 3.62 (s, 3H), 3.55-3.52 (m, 1H), 3.43 (dd, 1H, *J* = 4.2 Hz, 10.8 Hz), 3.22 (t, 2H, *J* = 7.8 Hz), 3.01 (t, 2H, *J* = 7.8 Hz), 2.80 (d, 1H, *J* = 7.8 Hz), 2.57 (t, 2H, *J* = 7.5 Hz), 1.62-1.57 (m, 2H), 1.29-1.25 (m, 10H), 0.89 (t, 3H, *J* = 6.9 Hz); ^{13}C-NMR (150 MHz, CD_3CN) δ 162.2, 158.6, 157.4, 151.2, 151.1, 144.8, 135.84, 135.77, 130.3, 129.8, 128.4, 127.9, 127.0, 125.4, 122.2, 115.4, 115.0, 113.2, 100.3, 86.6, 86.2, 83.8, 83.3, 69.8, 68.1, 63.0, 58.8, 55.3, 35.6, 32.5, 31.9, 30.4, 29.7, 29.3, 29.0, 22.7, 14.2; HRMS (ESI) calcd. for $C_{51}H_{61}N_4O_8S^+$ $[M + H]^+$ *m/z* 889.4205, found *m/z* 889.4208, calcd. for $C_{51}H_{60}N_4O_8SNa^+$ $[M + Na]^+$ *m/z* 911.4024, found *m/z* 911.4027.

Synthesis of 2-Phenoxyacetylamino-4-(2-methylthioethyl)-7-[2-*O*-methyl-3-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl

phosphoramidite)-*5*-O-(4,4'-O-dimethoxytrityl)-β-D-ribofuranosyl]-*7H*-pyrrolo-[2,3-*d*]pyrimidine (**20**)

Compound **19** (73.5 mg, 82.7 μmol) was rendered anhydrous by repeated co-evaporation with anhydrous CH_3CN, and then dissolved into anhydrous CH_2Cl_2 (1.0 mL). To this solution N,N-diisopropylethylamine (43.2 μL, 248.1 μmol) and 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (27.6 μL, 124.1 μmol) were added at 0 ^oC. After stirring for 1 h at 0 ^oC, further N,N-diisopropylethylamine (43.2 μL, 248.1 μmol) and 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (27.6 μL, 124.1 μmol) were added and stirred for 1 h at 0^oC. The reaction mixture was diluted with excess CH2Cl2 and the organic layer was repeatedly washed with aqueous sat. $NaHCO_3$. After the organic layer was dried over Na_2SO_4, filtered and evaporated, obtained crude material was purified by silica gel column chromatofgraphy (Hexane/EtOAc, from 4:1 to 2:1) yielding compound **20** as a white foam (70.7 mg, 90%). ^{1}H-NMR (600 MHz, $CDCl_3$) δ 8.72 (br, 1H), 7.76-7.22 (m, 12H), 7.03-7.00 (m, 3H), 6.84-6.79 (m, 4H), 6.46-6.41 (m, 2H), 4.87 (br, 2H), 4.68-4.24 (m, 3H), 3.96-3.82 (m, 1H), 3.78 (s, 6H), 3.61-3.22 (m, 8H), 3.25-3.22 (m, 2H), 3.04-3.00 (m, 2H), 2.66-2.56 (m, 3H), 2.37-2.35 (m, 1H), 1.62-0.85 (m, 27H); ^{13}C-NMR (150 MHz, $CDCl_3$) δ 162.21, 162.20, 158.7, 151.9, 151.4, 144.7, 144.6, 135.8, 135.71, 135.68, 130.40, 130.38, 130.29, 129.83, 129.80, 128.5, 128.4, 127.9, 127.0, 127.1, 125.1, 122.1, 118.0, 117.5, 114.96, 114.94, 113.22, 113.20, 100.7, 86.8, 86.7, 85.8, 83.3, 83.1, 82.9, 82.8, 70.8, 70.6, 68.2, 63.4, 59.2, 59.1, 58.80, 58.78, 58.1, 55.33, 55.31, 43.5, 43.4, 43.3, 43.2, 35.6, 32.5, 31.9, 30.4, 29.7, 29.0, 24.70, 24.67, 24.66, 24.62, 24.61, 22.7, 20.44, 20.41, 20.22, 20.17, 14.2; ^{31}P-NMR (162 MHz, $CDCl_3$) δ 150.8, 150.0; HRMS (ESI) calcd. for $C_{60}H_{78}N_6O_9PS^+$ $[M + H]^+$ *m/z* 1089.5283, found *m/z* 1089.5299.

4.3. Oligonucleotide synthesis

*4.3.1 Synthesis of ON-**1a**, ON-**2a***

The synthesis of 2′-OMe-RNA oligonucleotides 5′-FAM-r[AGUAGCCC**X**GCGUGGUG]-3′ (**X** = ADpVP or ADVP) (ON-**1a** or ON-**2a**, respectively) was performed on ABI 392 automated RNA synthesizer. The 5′-DMTr-on synthesis was conducted by standard 1.0 μmol scale RNA phosphoramidite synthesis cycle, which consists of (i) detritylation, (ii) coupling, (iii) capping, and (iv) iodine oxidation. 5-ethylthiotetrazole was used for activating reagent, 3% dichloroacetic acid in DCM was used for detritylation. 5% $(Tac)_2O$ in THF and 16% *N*-methylimidazole in THF were used for capping reaction. 0.02 M I_2 in THF-pyridine-H_2O (7:2:1, v/v/v) was used for oxidation. All ADpVP and ADVP-phosphoramidite were prepared as 0.1 M CH_3CN solution and coupled for 600s. After the chemical chain elongation, deprotection and cleavage from the solid support was conducted by 45 mM K_2CO_3/MeOH solution containing methanthiol or 1-octanethiol at room temperature for 4 h. HPLC purification of 5′-DMTr-on oligonucleotide was conducted on Nacalai Tesque COSMOSIL 5C18-MS-II RP-HPLC column (10 × 250 mm) at a flow rate of 4 mL/min with a gradient mobile phase from 5% CH_3CN in 0.1 M TEAA buffer (pH 7.0) to 40 % CH_3CN for 20 min. HPLC profiles were obtained by monitoring UV absorption at 254 nm. Collected oligonucleotide was freeze-dried and treated with aqueous 10 % AcOH for 30 min at room temperature, and then centrifuged by centrifugation. Resulting oligonucleotide was further purified on Nacalai Tesque COSMOSIL 5C18-MS-II RP-HPLC column (10 × 250 mm) at a flow rate of 4 mL/min with a gradient mobile phase from 5% CH_3CN in 0.1 M TEAA buffer (pH 7.0) to 40 % CH_3CN for 25 min. Collected purified ON-**1a** and ON-**2a** were dried by freeze-dry and characterized by corresponding MALDI-TOF MS peak at 5831.5 $[M-H]^-$ (calcd m/z 5830.0) for ON-**1a**, and at 5906.3 $[M-H]^-$ (calcd m/z 5906.1) for ON-**2a**, respectively.

*4.2.2 Synthesis of ON-**1c** and ON-**2c** by oxidation and subsequent alkali treatment of ON-**1a** and ON-**2a***

The solution of ON-**1a** (or **2a)** (1 nmol) in distilled H_2O (23.3 μL) was added 5 mM magnesium monoperoxyphthalate

hexahydrate (MMPP-6H_2O) (5 nmol, 1 μL) and incubated at 26 °C for 1 h to synthesize sulfide-oxidized ON-**1b** (or **2b**). To this solution of ON-**1b** (or **2b**), aqueous 4 M NaOH (9.9 μmol, 2.47 μL) was added and incubated at 26 °C for 3 h to synthesize vinyl-converted ON-**1c** and the pH of reaction mixture was adjusted to 7.0-7.2 by aqueous 50% acetic acid. This solution was diluted with excess 0.1 M TEAA buffer (pH 7.0) and applied to RP-HPLC. RP-HPLC purification of ON-**1c** was conducted on Nacalai Tesque COSMOSIL 5C18-MS-II RP-HPLC column (4.6 × 250 mm) at a flow rate of 1 mL/min with a gradient mobile phase from 5% CH_3CN in 0.1 M TEAA buffer (pH 7.0) to 40 % CH_3CN for 25 min. Collected purified ON-**1c** was dried by freeze-dry and characterized by corresponding MALDI-TOF MS peak at 5781.9 $[M-H]^-$ (calcd m/z 5781.9). The in-situ generated sulfide-oxidized ON-**1b** was also characterized by using aliquot of separately conducted reaction sample and MALDI-TOF MS analysis observing a peak at 5846.8 (calcd m/z 5846.7). The conversion of ON-**2a** to ON-**2c** was carried out in a same protocol as that for the conversion of ON-**1a** to ON-**1c**. Oxidized ON-**2b** and vinyl converted ON-**2c** were characterized by corresponding MALDI-TOF MS peak at 5906.3 $[M-H]^-$ (calcd *m/z* 5906.1) for ON-**2b**, and at 5743.1 $[M-H]^-$ (calcd m/z 5743.8) for ON-**2c**, respectively.

*4.2.3 Synthesis of ON-**2a'**($^{S\text{-}Me}$ADVP)*

11.8 μM of ON-**2c** was incubated in a 50 mM MES-buffer (pH 7) containing 75 mM NaSMe at 26 °C for 6 h. The reaction mixture was diluted by excess 0.1 M TEAA buffer (pH 7) and purified by RP-HPLC in a same protocol as that for the purification of ON-**2c**. Purified ON-**2a'** was characterized as desired oligonucleotide by MALDI-TOF MS peak at 5791.6 (calcd *m/z* 5791.9).

4.3. Cross-link reaction and denature gel analysis of cross-linked products

In the cross-link assay, the active 4 μM ON-**1c**, **2c**, or **3c** were incubated with 2 μM FAM-labelled complementary RNAs 5′-FAM-r[CCACGC**Y**GGGXUA]-3′ (**Y** = U, G, A, or C) (cRNA-U, cRNA-G, cRNA-A, or cRNA-C, respectively) in 50 mM MES buffer (pH 7) containing 100 mM NaCl at 37 °C. Part of the reaction mixture were taken at each time points (0.25, 0.5, 1, 2, 4, 8 h) and quenched by adding 95% formamide containing 20 mM EDTA, subsequently frozen by liquid nitrogen, and then kept at -80 °C. Right before applying collected samples to denature gel, samples were heated at 95 °C for 1 min, iced, and then applied to the 20% formamide 20 % polyacrylamide gel containing 7 M urea. The cross-linked product was quantitated by using a fluorescent image analyzer (FLA-5100, FujiFilm). The yield of cross-link reaction was calculated from the ratio of the cross-linked product and remaining cRNAs.

4.4. Kinetics analysis of cross-link reaction

The cross-link reaction to cRNA-U by ON-**1c**, **2c**, or **3c** was carried out in 50 mM MES buffer (pH 7) containing 100 mM NaCl at 37 °C. Aliquots of reaction mixture at each time points (1, 3, 5, 10, 15, 20, 30, 60 min) were taken, quenched and cross-linked products were quantitated by the same protocol for the cross-link assay in longer time period. The kinetics for the inter-strand cross-link formation, which is the rate-limiting step, was approximated by ignoring the steps of very fast duplex formation, and the rate constant of disappearance of intact cRNA-U was calculated by fitting the data of the ratio of remaining cRNAs and cross-linked product to first-order reaction.

4.5. UV melting temperature analysis

1 μM cRNA-U and 1 μM ON-**1a**, **2a'**, **3a** or **4** were annealed in a MES buffer (pH 7) containing 100 mM NaCl by heating at 95 °C for 1 min and cooled down gradually to room temperature. T_m measurement was performed with temperature

controller. Both the heating and cooling curves were measured over a temperature range of 20 ºC to 95 ºC at 1.0 ºC/min in three times. The absorbance at 260 nm was recorded at every temperature points.

4.6. Luciferase mRNA inhibition assay

RNA transcription. To construct the luciferase reporter mRNA, the luciferase cDNA was first amplified using the ExTaq (Roche) using pmirGLO (Promega) as a template. The primers T7Luc-F: 5'-CGAAATTAATACGACTCAC TATAGGGTAAAGCCACCATGGAAGA-3' and LucR: 5'-AGCTTTTACACGGCGATCTT-3' were used for additio n of T7 promoter and amplification by PCR. The reaction product was purified by ethanol precipitation. G-capped RNAs were in vitro transcribed using the mMESSAGE mMACHINE kit (Ambion, Austin, TX) from the amplified DNA. The RNAs were polyadenylated with the Poly(A) Tailing kit (Ambion), purified with the MEGAclear kit (Ambion, Austin, TX).

In vitro translation. The firefly luciferase-coding mRNA (0.1 μM) was first incubated with 0.2, 1, or 5 μM of vinyl-active ON-**1c**, **2c** or **3c** were incubated in 50 mM MES buffer (pH 7.0) containing 100 mM NaCl at 37 ^{o}C for 1 h. This solution was then applied to in vitro translation using Rabbit reticulocyte lysate at 30°C for 90 min. Luciferase activities were determined with 2.5 μL of the sample using the Luciferase Assay System (Promega) and a GloMax 20/20 luminometer (Promega). The luciferase activity was normalized to control experiment without CFO. For comparative experiment, non-cross-linkable ON-**1a**, **2a'** was also used.

■ Supplementary Material

Copies of ^{1}H and ^{13}C NMR spectra for all new compounds and MALDI-TOF MS spectrum of oligonucleotides.

Kinetic analysis of the crosslink formation

Z-matrices and the number of imaginary frequencies for molecular orbital calculation results with ADVP and ADpVP.

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■ References

1. Khvorova, A.; Watts, J. K. The chemical evolution of oligonucleotide therapies of clinical utility, *Nat. Biotechnol*. **2017**, 35, 238-248.
2. Flanagan, W. M.; Wagner, R. W.; Grant, D.; Lin, K. Y.; Matteucci, M. D. Cellular penetration and antisense activity by a phenoxazine-substituted heptanucleotide, *Nat. Biotechnol*. **1999**, *17*, 48-52
3. Lin, K. Y.; Matteucci, M. D. A cytosine analogue capable of clamp-likebinding to a guanine in helical nucleic acids, *J. Am. Chem. Soc*., **1998**, *120*, 8531-8532.
4. Cheong, C.; Tinoco I. Jr.; Chollet, A. Thermodynamic studies of base pairing involving 2,6-diaminopurine, *Nucleic Acids Res*. **1988**, *16*, 5115-5122.
5. Kamiya, Y.; Donoshita, Y.; Kamimoto, H.; Murayama, K.; Ariyoshi, J.; Asanuma, H. Introduction of 2,6- diaminopurines into serinol

nucleic acid improves anti□miRNA performance, *Chembiochem* **2017**, *18*, 1917-1922.

6. Miller, P. S.; Bi, G.; Kipp, S. A.; Fok, V.; DeLong, R. K. Triplex formation by a psoralen-conjugated oligodeoxyribonucleotide containing the base analog 8-oxo-adenine, *Nucleic Acids Res.* **1996**, *24*, 730-736.
7. Yoshimura, Y.; Fujimoto, K. Ultrafast reversible photo-cross-linking reaction: toward in situ DNA manipulation, *Org. Lett.* **2008**, *10*, 3227-3230
8. Qiu, Z.; Lu, L.; Jian, X.; He, C. A diazirine-based nucleoside analogue for efficient DNA interstrand photocross-linking, *J. Am. Chem. Soc*. **2008**, *130*, 14398-14399.
9. Higuchi, M.; Kobori, A.; Yamayoshi, A.; Murakami, A. Synthesis of antisense oligonucleotides containing 2′-*O*-psoralenylmethoxyalkyl adenosine for photodynamic regulation of point mutations in RNA, *Bioorg. Med. Chem.* **2009**, *17*, 475-483
10. Op de Beeck, M.; Madder, A. Unprecedented C-Selective Interstrand cross-linking through *in situ* oxidation of furan-modified oligodeoxynucleotides, *J. Am. Chem. Soc*. **2011**, *133*, 796-807
11. Sloane, J. L.; Greenberg, M. M. Synthesis of cross-linked DNA containing oxidized abasic Site analogues, *J. Org. Chem*. **2014**, *79*, 9792-9798.
12. Sakamoto, T.; Shigeno, A.; Ohtaki, Y.; Fujimoto, K. Photo-regulation of constitutive gene expression in living cells by using ultrafast photo-cross-linking oligonucleotides, *Biomater. Sci.* **2014**, *2*, 1154-1157.
13. Nagatsugi, F.; Kawasaki, T.; Usui, D.; Maeda, M.; Sasaki, S. Highly efficient and selective cross-linking to cytidine based on a new strategy for auto-activation within a duplex, *J. Am. Chem. Soc*. **1999**, *121*, 6753-6754.
14. Hattori, K.; Hirohama, T.; Imoto, S.; Kusano, S.; Nagatsugi, F., Formation of highly selective and efficient interstrand cross-linking to thymine without photo-irradiation *Chem. Comm*. **2009**, *42*, 6463-6465.
15. Imoto, S.; Hori, T.; Hagihara, S.; Taniguchi, Y.; Sasaki, S.; Nagatsugi, F. Alteration of cross-linking selectivity with the 2'-OMe analogue of 2-amino-6-vinylpurine and evaluation of antisense effects, *Bioorg. Med. Chem. Lett*. **2010**, *20*, 6121-6124.
16. Nagatsugi, F.; Imoto, S. Induced cross-linking reactions to target genes using modified oligonucleotides, *Org. Biomol. Chem*. **2011**, *9*, 2579-2585.
17. Kusano, S.; Ishiyama, S.; Lam, S. L.; Mashima, T.; Katahira, M.; Miyamoto, K.; Aida, M.; Nagatsugi, F. Cross-linking reactions of 4-amino-6-oxo-2-vinylpyrimidine with guanine derivatives and structural analysis of the adducts, *Nucleic Acids Res.* **2015**, *43*, 7717-7730.
18. Yamada, K.; Ishiyama, S.; Onizuka, K.; Nagatsugi, F. Synthesis and properties of cross-linkable DNA duplex using 4-amino-2-oxo-6-vinyl-1,3,5-triazine, *Tetrahedron*, **2017**, *73*, 1424-1435.
19. Hagihara, S.; Kusano, S.; Lin, W. C.; Chao, X. G.; Hori, T.; Imoto, S.; Nagatsugi, F.Production of truncated protein by the cross-link formation of mRNA with 2′-OMe oligoribonucleotide containing 2-amino-6-vinylpurine, *Bioorg. Med. Chem.Lett.,* **2012**, *22*, 3870-3872.
20. Hagihara, S.; Lin, W. C.; Kusano, S.; Chao, X. G.; Hori, T.; Imoto, S.; Nagatsugi, F. The cross-link formation of 2′□OMe oligonucleotide containing 2□amino□6□vinylpurine protects mRNA from miRNA□mediated silencing , *ChemBioChem,* **2013**, *14*, 1427-1429.
21. Buhr, C.A.; Wagner, R.W.; Grant, D.; Froehler, B.C. Oligodeoxynucleotides containing C-7 propyne analogs of 7-deaza-2'-deoxyguanosine and 7-deaza-2'-deoxyadenosine., *Nucleic Acids Res.* **1996**, *24*, 2974-2980.
22. Seela, F.; Shaikh, K.I. Oligonucleotides containing 7-propynyl-7-deazaguanine: synthesis and base pair stability, *Tetrahedron*, **2005**, *61*, 2675-2681
23. Shi, J.; Van de Water, R.;, Hong, K.; Lamer, R. B.; Weichert, K. W.; Sandoval, C. M.; Kasibhatla, S. R.; Boehm, M. F.; Chao, J.; Lundgren, K.; Timple, N.; Lough, R.; Ibanez, G.; Boykin, C.; Burrows, F. J.; Kehry, M. R.; Yun, T. J.; Harning, E. K.; Ambrose, C.; Thompson, J.; Bixler, S. A.; Dunah, A.; Snodgrass-Belt, P.; Arndt, J.; Enyedy, I. J.; Li, P.; Hong, V. S.; McKenzie, A.; Biamonte, M. A. EC144 is a potent inhibitor of the heat shock protein 90, *J. Med. Chem*. **2012**, *55*, 7786–7795.
24. Seela, F.; Peng, X. 7-Functionalized 7-deazapurine ribonucleosides related to 2-aminoadenosine, guanosine, and xanthosine: glycosylation of pyrrolo[2,3-*d*]pyrimidines with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-d-ribofuranose, *J. Org. Chem*. **2006**, *71*, 81–90.

25. Prakash, T. P.; Prhavc, M.; Eldrup, A. B.; Dan Cook, P.; Carroll, S. S.; Olsen, D. B.; Stahlhut, M. W.; Tomassini, J. E.; MacCoss, M.; Galloway, S. M.; Hilliard, C.; Bhat, B. Synthesis and evaluation of *S*-acyl-2-thioethyl esters of modified nucleoside 5‘-monophosphates as inhibitors of hepatitis C virus RNA replication, *J. Med. Chem.* **2005**, *48*, 1199-1210.

26. Velikyan, I.; Acharya, S.; Trifonova, A.; Földesi, A.; Chattopadhyaya, J. The pK_a's of 2‘-hydroxyl group in nucleosides and nucleotides, *J. Am. Chem. Soc*. **2001**, *123*, 2893-2894.

27. Krishnamurthy, R. Role of pK_a of nucleobases in the origins of chemical evolution, *Acc. Chem. Res*. **2012**, *45*, 2035–2044.

28. Sonogashira, K.; Tohda, Y.; Hagihara, N. A convenient synthesis of acetylenes: catalytic substitutions of acetylenic hydrogen with bromoalkenes, iodoarenes and bromopyridines, *Tetrahedron Lett.* **1975**, *16*, 4467-4470.

29. Seela, F.; Becher, G.; Zulauf, M. 8-Aza-7-deazapurine DNA: synthesis and duplex stability of oligonucleotides containing 7-substituted bases, *Nucleosides Nucleotides*, **1999**, *18*, 1399-1400.

30. Hobbs, F. W. Jr. Palladium-catalyzed synthesis of alkynylamino nucleosides. A universal linker for nucleic acids, *J. Org. Chem*. **1989**, *54*, 3420-3422.

31. Robins, M. J.; Vinayak, R. S.; Wood, S. G. Solvent, not palladium oxidation state, is the primary determinant for successful coupling of terminal alkynes with iodo-nucleosides, *Tetrahedron Lett.***1990**, *31*, 3731-3734.

32. Clercq, E. D.; Descamps, J.; Balzarini, J.; Giziewicz, J.; Barr, P. J.; Robins, M. J. Nucleic acid related compounds. 40. Synthesis and biological activities of 5-alkynyluracil nucleosides, *J. Med. Chem.* **1983**, 26, 661-666.

33. Inouye, M.; Doi, Y.; Azuchi, J.; Shirato, W.; Chiba, J.; Abe, H., Hexamethyldisilazane-promoted sonogashira reaction of polyfunctionalized N-containing heterocycles, *Heterocycles*, **2010**, *82*, 1137-1141.

34. Nagatsugi, F.; Ogata, Y.; Imoto, S.; Sasaki, S. Efficient synthesis of 6-substituted purine derivatives using Pd-catalyzed cross-coupling reactions with 2’-deoxyguanosine O^6-tosylate, *Heterocycles* **2007**, *73*, 493-501.

35. Takano, S.; Tsuzuki, T.; Murayama, T.; Sakurai, T.; Fukuda, H.; Arisawa, M.; Shuto, S. Synthesis of 7-deaza-cyclic Adenosine-5′-diphosphate-carbocyclic-ribose and its 7-bromo derivative as intracellular Ca^{2+}-mobilizing agents, *J. Org. Chem*. **2015**, *80*, 6619-6627.

36. Wilcox, C. S.; Otoski, R. M. Stereoselective preparations of ribofuranosyl chlorides and ribofuranosyl acetates. Solvent effects and stereoselectivity in the reaction of ribofuranosyl acetates with trimethylallylsilane, *Tetrahedron Lett*. **1986**, *27*, 1011-1014.

37. Ramasamy, K.; Imamura, N.; Robins, R. K.; Revankar, G. R. A facile and improved synthesis of tubercidin and certain related pyrrolo[2,3□*d*]pyrimidine nucleosides by the stereospecific sodium salt glycosylation procedure, *J. Heterocyclic Chem*. **1988**, *25*, 1893-1898