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Structure–activity relationship of phosmidosine: importance of the 7,8-dihydro-8-oxoadenosine residue for antitumor activity

Mitsuo Sekine,[a,b,*] Kazuhisa Okada,[a] Kohji Seio,[b,c] Hideaki Kakeya,[d] Hiroyuki Osada[d] and Takuma Sasaki[e]

[a]*Department of Life Science, Tokyo Institute of Technology, Nagatsuda, Midoriku, Yokohama 226-8501, Japan*
[b]*Frontier Collaborative Research Institute, Tokyo Institute of Technology, Nagatsuda, Midoriku, Yokohama 226-8501, Japan*
[c]*CREST, JST (Japan Science and Technology Corporation), Nagatsuda, Midoriku, Yokohama 226-8501, Japan*
[d]*Antibiotics Laboratory, The Institute of Physical and Chemical Research (RIKEN), 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan*
[e]*Cancer Research Institute, Kanazawa University, Takara-machi, Kanazawa 920-8640, Japan*

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Abstract—To study the structure–activity relationship of phosmidosine, a variety of phosmidosine derivatives **9a**–**g** were synthesized by condensation of *N*-diisopropyl *N*′-(*N*-tritylprolyl)phosphorodiamidite **6** with appropriately protected nucleoside derivatives **7a**–**g**. As the result, replacement of the 7,8-dihydro-8-oxoadenine base by adenine and 6-*N*-acetyladenine did not affect the antitumor activity. However, phosmidosine derivatives containing uracil, cytosine, and guanine in place of the 7,8-dihydro-8-oxoadenine base did not show significant activity. A plausible explanation for the selective expression of phosmidosine compared with that of phosmidosine analogs having other amino acids in place of proline is also discussed. These results suggest that phosmidosine serves as an inhibitor of prolyl adenosine 5′-phosphate (prolyl-AMP) to inhibit the peptide synthesis in cancer-related cells.
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1. Introduction

Phosmidosine (**1**) was discovered as an antifungal antibiotic in 1991.[1] The structure of **1** was determined based on mass spectroscopy in 1993.[2] Later, this naturally occurring product has proved to have biological activity capable of morphological reversion of src^{ts}NKR cells.[3] As an intriguing characteristic of this molecule, it serves as a G1 arrest anticancer drug in a cell cycle.[3] Phosmidosine regulates hyperphosphorylation of RB proteins by inhibition of Cyclin D1 so that RB proteins remain in inactive forms keeping binding to EF2.[4] This inhibition takes place at the G1 phase. However, phosmidosine itself is somewhat unstable under physiological conditions.[2] Phosmidosine B (**2**) is one degradation product of phosmidosine.[1] This demethylated species has still 1/20 of the morphological reversion activity of phosmidosine.[1] In 2000, we first synthesized phosmidosine B and found that this compound has significant antitumor activities against various cancer-related cell lines.[5] Recently, we also succeeded in synthesizing phosmidosine.[6] However, it turned out that this *O*-methyl ester tends to decompose during the isolation procedure so that the isolation yield is not so high (27%). This is due to intermolecular and intramolecular transfer reactions of the methyl group on the phosphoramidate linkage.[1] Therefore, we have quite recently synthesized a variety of more stable derivatives of phosmidosine that can maintain the antitumor activity.[7] Among them, the *O*-ethyl ester derivative (**3**) proved to be sufficiently stable and exhibited sufficient antitumor activities against KB and L1210 cell lines.[7] Furthermore, it was found that both the prolyl and 7,8-dihydro-8-oxoadenosyl residues are important for the biological activity. The substitution of an acetyl group or other aminoacyl groups for the prolyl group resulted in considerable loss of the activity.

Keywords: Phosmidosine; Structure–activity relationship; 8-Oxoadenosine; Antitumor activity; Aminoacyl adenylate analog.
* Corresponding author. Tel.: +81-45-924-5706; fax: +81-45-924-5772; e-mail: msekine@bio.titech.ac.jp

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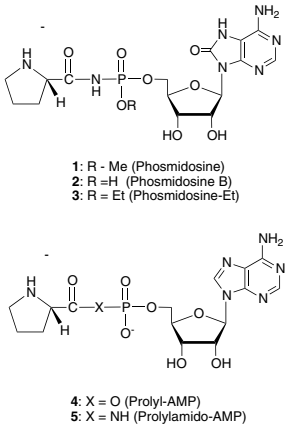


Figure 1. Various derivative of phosmidosine and prolyl-AMP.

The prolyl group is an essential element. The replacement of 7,8-dihydro-8-oxoadenosine with a simple ethyl group led to drastic loss of the activity. With an increase of the alkyl group in place of the methyl group on the phosphoramidate linkage, the activity decreased slightly.

In connection with our recent studies, we previously reported the synthesis[8,9] of several adenosine 5′-[*N*-(aminoacyl)phosphoramidate] derivatives [aminoacylamido-AMPs] containing an analog (**5**) of prolyl adenylate (**4**: prolyl-AMP) that would be useful as co-factors for X-ray analysis of aminoacyl-tRNA synthetase complexes (Fig. 1). It was also found that the synthetic aminoacyl-amido AMPs have weak antitumor activities against various cell lines.

Phosmidosine has structural elements close to those of prolyl-AMP (**4**), which serves as a carrier of a proline amino acid to the 3′-terminal site of $tRNA^{Pro}$ via a tri-particle complex with a prolyl-tRNA synthetase. Therefore, it is strongly suggested that phosmidosine might show significant antitumor activity in rapidly growing cancer cells as an inhibitor of the peptide synthesis.

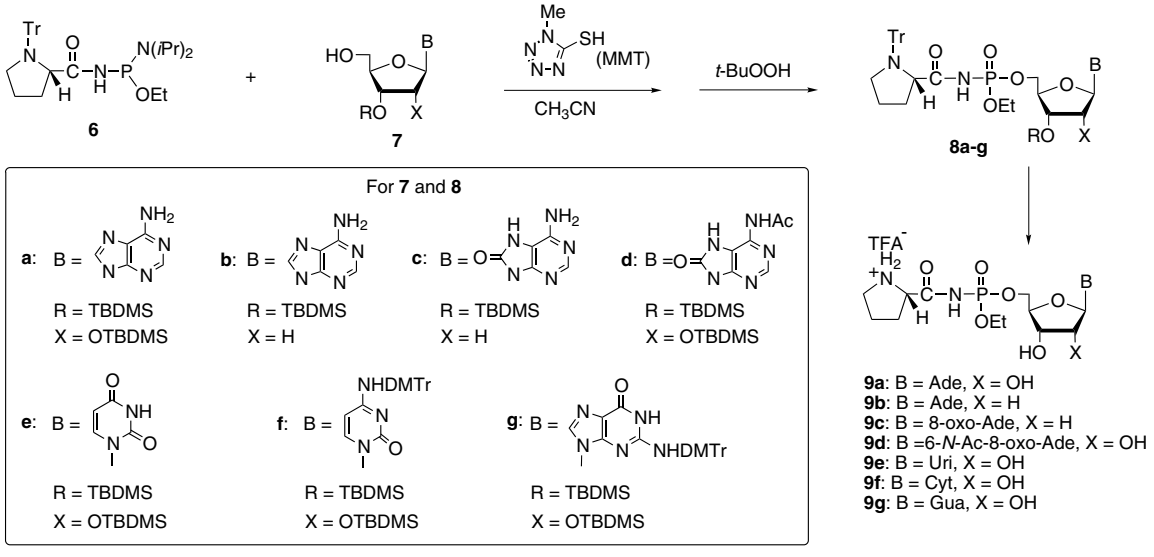
To clarify this possibility, the structure–activity relationship of phosmidosine is of great importance. Particularly, it should be clarified if the 7,8-dihydro-8-oxoadenosine component can be replaced by other elements such as the deoxy counterpart, the adenosine, and other ribonucleosides without loss of the activity.

In this paper, we report the synthesis of various derivatives of the phosmidosine *O*-ethyl ester by replacement of the adenosine moiety by other nucleoside derivatives and also their antitumor activities.

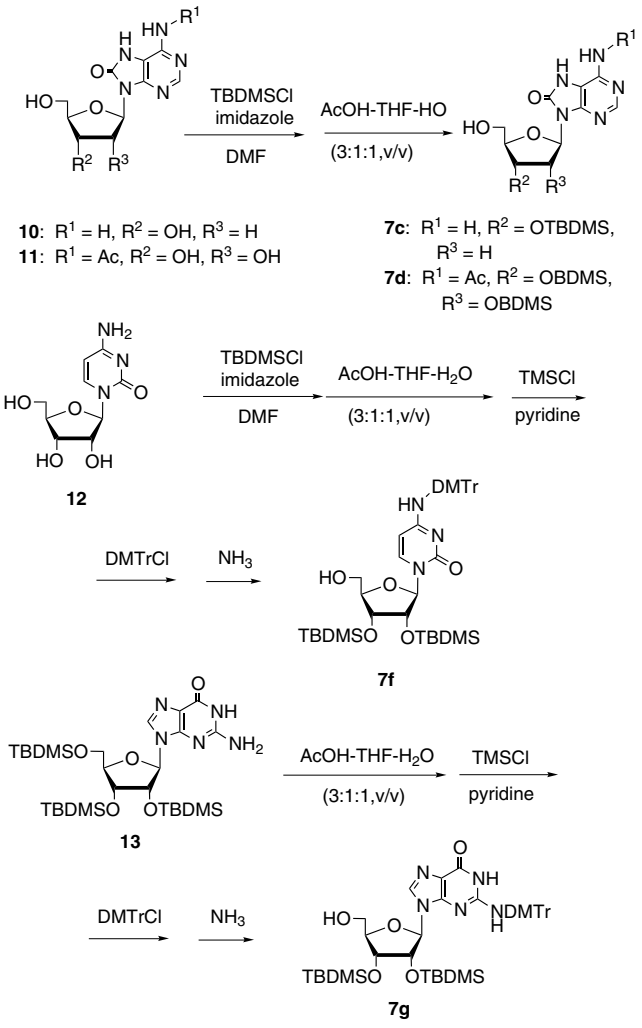
2. Results and discussion

To study the structure–activity relationship of phosmidosine, we synthesized a series of phosmidosine derivatives (**9a**–**g**). Phosmidosine has a unique structure of the 7,8-dihydro-8-oxoadenine base. Therefore, to see if the 7,8-dihydro-8-oxoadenine moiety is essential for the antitumor activity, we synthesized the *O*-ethyl ester derivative (**9a**) of prolylamido AMP replaced by adenosine in place of 7,8-dihydro-8-oxoadenosine. Moreover, to check the necessity of the ribose residue, the 2′-deoxyadenosine and 2′-deoxy-7,8-dihydro-8-oxoadenosine derivatives **9b** and **9c** were also prepared. In addition, we synthesized 6-*N*-acetylphosmidosine derivative **9d** to check if the 6-amino group of phosmidosine can be modified without loss of the activity. We also synthesized several compounds **9e**–**g** having other nucleobases in place of 7,8-dihydro-8-oxoadenine. The synthesis of these compounds is outlined in Scheme 1.

For the construction of the *N*-prolylphosphoramidate linkage, ethyl *N*-diisopropyl-*N*′-[*N*-tritylprolyl]phosphorodiamidite (**6**)[7] was activated by the action of 5-mercapto-1-methyl-1*H*-tetrazole (MMT)[10,11] to react with



Scheme 1. Synthesis of phosmidosine analogs by MMT-catalyzed phosphoramidite coupling reactions.



Scheme 2. Synthesis of key intermediates required for the synthesis of phosmidosine analogs.

appropriately protected nucleoside derivatives (**7a**–**g**). The oxidation of the resulting tervalent phosphorus intermediates was carried out by use of *t*-BuOOH.[12] The starting materials **7a**,[13] **7b**,[14] and **7e**[13] were obtained by the literature method. 3′-*O*-TBDMS-7,8-dihydro-8-oxodeoxyadenosine (**7c**) was synthesized from 8-bromodeoxyadenosine via 8-oxodeoxyadenosine (**10**), as shown in Scheme 2. Compound **7d** was also synthesized by a two-step reaction of 6-*N*-acetyl-8-oxodeoxyadenosine (**11**).

Table 1. Condensation of **6** with **7a**–**g** to give the *N*-prolylphosphoroamidate derivatives **8a**–**g**

Compd (**7**/**6**)	MMT/**6**	Product	Condensation time (min)	Oxidation time (min)	Yield (%)
7a 2.0	1.26	**8a**	60	10	60
7b 2.0	1.26	**8b**	10	10	58
7c 1.5	1.67	**8c**	60	10	43
7d 2.0	1.25	**8d**	10	10	55
7e 2.0	1.25	**8e**	10	10	89
7f 2.0	1.25	**8f**	10	10	91
7g 2.0	1.25	**8g**	10	10	72

For the synthesis of the cytidine and guanosine derivatives **9f** and **9g**, 4-*N*-DMTr-2′,3′-*O*-bis(*tert*-butyldimethylsilyl)cytidine **7f** and *N*-DMTr-2′,3′-*O*-bis(*tert*-butyldimethylsilyl)guanosine **7g** were synthesized, as shown in Scheme 2. Compound **7f** was synthesized in 43% yield from cytidine (**12**) via a five-step procedure without isolation of each intermediate. Compound **7g** was prepared in 63% yield from 2′,3′,5′-*O*-tris(*tert*-butyldimethylsilyl)guanosine (**13**).[13]

The results of the coupling reactions between **6** and **7a**–**g** are shown in Table 1. Desilylation followed by detritylation of **8a**–**g** gave the final products **9a**–**g** in satisfactory yields except for **9a** and **9b**. The details of the deprotection are summarized in Table 2. Compounds **9a** and **9f** were isolated as the trifluoroacetate salts but **9b**–**e** and **9g** were obtained as *N*-unprotonated species. The presence of excess TBAF, which was used for desilylation, might inhibit the salt formation of **9b**–**e** and **9g**.

The antitumor activities of these compounds obtained by the MTT assay[15] are shown in Table 3. For the assay, a set of diastereoisomers were used, since in the previous study each of the diastereoisomers of the *O*-alkylated phosmidosine derivative showed similar biological activities in anticancer tests and morphological reversion activity.[7]

The IC_{50} values of Table 3 show that the compounds tested exhibited similar antitumor activities against KB and L1210 cell lines except for the result of the deoxy counterpart **9b** in the L1210 cell line. Therefore, the presence of the 2′-hydroxyl group or 8-oxo function is not so important for the biological expression. It turned out that the *N*-acetyl derivative **9d** also showed similar antitumor activity to that of **9a**. This result implies that an *N*-acyl modification is useful for functionalization of phosmidosine to search for biomolecules, which interact

Table 2. Deprotection of fully protected phosmidosine derivatives **8a**–**g**

Compd	Desilylation		Detritylation		Product	Yield of **9**
	TBSF (equiv)	Time (h)	Conditions	Time		
8a			80% HCOOH	42 h	**9a**	19
8b	TBAF 3.9	6	1% TFA H_2O–CH_3CN (1:1, v/v)	15 min	**9b**	72
8c	TBAF 6.0	4	1% TFA H_2O–CH_3CN (1:1, v/v)	15 min	**9c**	58
8d	TBAF 10.0	4	1% TFA H_2O–CH_3CN (1:1, v/v)	15 min	**9d**	29
8e	TBAF 7.9	2	1% TFA H_2O–CH_3CN (1:1, v/v)	15 min	**9e**	91
8f	TBAF 8.1	1	4% TFA H_2O–CH_3CN (1:1, v/v)	3 + 12 h	**9f**	60
8g	TBAF 8.2	3	4% TFA H_2O–CH_3CN (1:1, v/v)	1 h	**9g**	69

Table 3. Antitumor activity of phosmidosine analogs

Compd	IC_{50} (μM)	
	KB	L1210
Phosmidosine-Et **3**	3.44	3.62
A-phosmidosine-Et **9a**	5.12	3.62
dA-phosmidosine-Et **9b**	3.85	21.6
8-Oxo-dA-phosmidosine-Et **9c**	3.23	5.68
N-Ac-8-oxo-A-phosmidosine-Et **9d**	2.89	4.10
U-phosmidosine-Et **9e**	>170	>170
C-phosmidosine-Et **9f**	>170	>170
G-phosmidosine-Et **9g**	>200	>200

with phosmidosine. The most plausible binding molecule might be a tRNA synthetase. From the results of the MTT assay obtained above, it is strongly suggested that prolyl-tRNA synthetase binds to phosmidosine like aminoacyl AMPs.

When the U-, G-, and C-phosmidosine-Et derivatives **7e**–**g** were tested, no significant antitumor activities were detected, as shown in Table 3. These results suggest that the replacement of the 7,8-dihydro-8-oxoadenine base with other bases such as uracil, guanine, and cytosine resulted in loss of the activity. This is reasonable if phosmidosine recognizes aminoacyl-tRNA synthetase, which would allow binding with the adenine base and its close analogs and thereby serves as an inhibitor.

3. Conclusion

In conclusion, it turned out that the 7,8-dihydro-8-oxoadenine is exchangeable to an adenine moiety and the deoxy counterpart did not affect the biological activity in KB cells but decreased it four times in the L1210 cell line. At any rate, the base part is essentially more important than the ribose moiety. Essentially, at least the adenine skeleton must be required. Since it is clear that the adenine or 7,8-dihydro-8-oxoadenine base is essential for antitumor activity, the possibility that phosmidosine actually interacts with an aminoacyl-tRNA synthetase increases. On the other hand, we have also reported that replacement of the prolyl group by other amino acid residues resulted in poorer antitumor activity.[7] If phosmidosine serves as an inhibitor in the peptide synthesis, these derivatives having other amino acids should express similar activities. Future studies are required to address this point. However, we also noticed the *O*-methyl phosmidosine derivatives having other amino acids tend to decompose even under neutral conditions on storage and were less stable than phosmidosine. This is because the other amino acids have primary amino groups so that intramolecular *N*–*N* rearrangement easily occurs compared with phosmidosine that has a secondary amino group on the five-membered ring not accessible to such cyclization.[7] Based on these discussions, it is likely that such modified phosmidosine derivatives cannot exist in cells for sufficient time to interact with the corresponding aminoacyl-tRNA synthetases so that only phosmidosine, which has a longer lifetime, survives in cells and shows antitumor and morphological reversion activities in cancer cells.

Apart from our studies, 5′-*O*-[*N*-aminoacylsulfamoyl]-adenosine and its analogs have been synthesized[16–18] in connection with the structure–activity relationship of ascamysine,[19] which has a 2-chloroadenosine moiety. These studies clearly indicate that the aminoacyl group recognizes the corresponding aminoacyl-tRNA synthetases. In these cases, such restricted recognition takes place since the sulfonamide ester linkage is chemically stable and no *N*–*N* rearrangement of the sulfonyl group occurs.

At the next stage of our study, extensive study should be done to isolate biomolecules, which interact with phosmidosine by use of suitably modified phosmidosine derivative having a biotin residue at the 6-*N*-acyl chain. Further study is now under way in this direction.

4. Experimental section

4.1. General remarks

^{1}H, ^{13}C, and ^{31}P NMR spectra were obtained at 270, 68, and 109 MHz, respectively. The chemical shifts were measured from tetramethylsilane (0 ppm) or DMSO-d_6 (2.49 ppm) for ^{1}H NMR, $CDCl_3$ (77.0 ppm), DMSO-d_6 (39.7 ppm) or DMF-d_7 (2.74 ppm) for ^{13}C NMR, and 85% phosphoric acid (0 ppm) for ^{31}P NMR. Column chromatography was performed with silica gel C-200. Reverse-phase column chromatography was performed by use of 37–55 μm C18 (125 Å) particles, which were set up in a glass column of a medium pressure preparative HPLC system. Elution was performed with the following solvent systems I–II for 500 min at a flow rate of 2.0 mL/min. Solvent system I: water–acetonitrile (100–0 to 70:70, v/v); solvent system II: water–MeOH–trifluoroacetic acid (93:7:0.1, v/v/v). Reverse-phase HPLC was performed using C18 columns (3.9×150 mm and 7.8×300 mm, respectively) with a linear gradient of 0–15% CH_3CN/H_2O containing 0.1 M NH_4OAc (pH 7.0) at 50 °C at a flow rate of 1.0 and 3.0 mL/min, respectively, for 30 min. Mass spectra were measured by use of an ESI-mass spectrophotometer and a MALDI-TOF mass spectrophotometer. UV spectra were measured by a U-2000 spectrophotometer. TLC was performed with silica gel 60 (F_{254}) plates. In vitro analysis of the antitumor activity in various cancer cell lines was carried out by the literature method reported by Carmichael et al.[15] and us.[7] The morphological reversion activity test was conducted according to the literature method.[3] Compounds **6** were synthesized according to the previous method reported.[7] Compounds **7a** and **7e**–**g** were synthesized according to the literature method.[14] Compound **7b** was synthesized by the Robins method.[14] Compound **11** was synthesized by our previous method.[6]

4.1.1. 3′-*O*-*tert*-Butyldimethylsilyl-7,8-dihydro-8-oxodeoxyadenosine (7c). Compound **10**[20] (802 mg, 3.0 mmol) was rendered anhydrous by coevaporation three times

with dry pyridine and finally dissolved in dry DMF (3 mL). To the solution were added *tert*-butyldimethylsilyl chloride (1.09 g, 7.2 mmol) and imidazole (980 mg, 14.4 mmol). After being stirred under argon atmosphere at room temperature for 3 h, the mixture was diluted with AcOEt. The solution was washed three times with 5% $NaHCO_3$, and the organic layer was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was dissolved in acetic acid–THF–water (3:1:1, v/v/v, 30 mL). After being stirred at 80 °C for 5 h, the mixture was diluted $CHCl_3$. The $CHCl_3$ solution was washed successively twice with water and with 5% $NaHCO_3$. The organic layer was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel with $CHCl_3$–MeOH (98:2, v/v) to give **7c** (360 mg, 31%): ^{1}H NMR (270 MHz, DMSO-d_6) δ 0.00 (6H, s), 0.79 (9H, s), 1.86–1.94 (1H, m, $J_{2'\text{-Ha},2'\text{-Hb}}$ = 6.3 Hz), 2.92–3.02 (1H, m), 3.32–3.38 (1H, m, $J_{5'\text{-Ha},5'\text{-Hb}}$ = 6.9 Hz), 3.48–3.53 (1H, m), 3.68–3.69 (1H, m), 4.50 (1H, m), 4.98–5.02 (1H, m, $J_{5'\text{-OH},5'\text{-Ha}} = J_{5'\text{-OH},5'\text{-Hb}}$ = 4.6 Hz), 6.01 (1H, t, $J_{1',2'\text{-Ha}}$ = 6.6 Hz, $J_{1',2'\text{-Hb}}$ = 7.3 Hz), 6.44 (2H, br s), 7.91 (1H, s), 10.24 (1H, br s); ^{13}C NMR (DMSO-d_6) δ −4.75, −4.71, 17.78, 25.75, 36.20, 61.94, 73.05, 81.16, 87.39, 103.37, 146.22, 146.96, 150.41, 151.03. ESI-mass *m/z* calcd for $C_{16}H_{28}N_5O_4Si$ 382.1911; observed [M + H] 382.1944.

4.1.2. 6-*N*-Acetyl-2′,3′-*O*-di-*tert*-butyldimethylsilyl-7,8-dihydro-8-oxoadenosine (7d). Compound **11**[6] (1.11 g, 3.42 mmol) was rendered anhydrous by coevaporation three times with dry pyridine and finally dissolved in dry pyridine (34 mL). To the solution was added 4,4′-dimethoxytrityl chloride (1.27 g, 3.76 mmol). After being stirred under argon atmosphere at room temperature for 3 h, the mixture was quenched by addition of MeOH (25 mL). The mixture was partitioned between $CHCl_3$ and 5% $NaHCO_3$. The organic layer was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was coevaporated three times with dry pyridine and finally dissolved in dry DMF (34 mL). To the mixture were added *tert*-butyldimethylsilyl chloride (1.13 g, 7.52 mmol) and imidazole (1.02 g, 15.0 mmol). After being stirred at room temperature for 12 h, the mixture was diluted $CHCl_3$. The $CHCl_3$ solution was washed three times with 5% $NaHCO_3$. The organic layer was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was dissolved in a 2% solution of trifluoroacetic acid in $CHCl_3$ (34 mL). After being stirred at room temperature for 30 min, the mixture was diluted $CHCl_3$. The $CHCl_3$ solution was washed three times with 5% $NaHCO_3$. The organic layer was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel with $CHCl_3$–MeOH (99:1, v/v) to give **7d** (1.10 g, 58%): ^{1}H NMR (270 MHz, DMSO-d_6) δ −0.33 (3H, s), −0.13 (3H, s, CH_3 of TBDMS), 0.06 (3H, s), 0.07 (3H, s), 0.69 (9H, s), 0.86 (9H, s), 2.08 (3H, s), 3.39–3.48 (1H, m, $J_{5'\text{-Ha},5'\text{-Hb}}$ = 6.6 Hz), 3.58–3.69 (1H, m), 3.84 (1H, m), 4.33–4.35 (1H, m), 4.89–4.93 (1H, m), 5.22–5.15 (1H, t, $J_{5'\text{-OH},5'\text{-Ha}}$ = 4.9 Hz), 5.75 (1H, d, $J_{1',2'}$ = 6.6 Hz), 8.38 (1H, s), 10.32 (1H, br s), 10.82 (1H, br s); ^{13}C NMR (DMSO-d_6) δ −5.3, −4.7, −4.63, −4.59, 17.6, 17.8, 23.2, 25.5, 25.8, 61.4, 70.5, 72.6, 84.9, 85.9, 110.9, 138.2, 149.5, 150.0, 150.9, 169.2. ESI-mass *m/z* calcd for $C_{24}H_{44}N_5O_6Si_2$ 554.2830; observed [M + H] 554.2742.

4.1.3. 2′,3′-*O*-Di-*tert*-butyldimethylsilyl-4-*N*-(4,4′-dimethoxytrityl)cytidine (7f). Cytidine (**12**) (973 mg, 4.0 mmol) was rendered anhydrous by coevaporation three times with dry pyridine and finally dissolved in dry DMF (8 mL). To the mixture were added *tert*-butyldimethylsilyl chloride (2.17 g, 14.4 mmol) and imidazole (1.96 g, 28.8 mmol). After being stirred at room temperature for 10 h, the mixture was diluted $CHCl_3$. The $CHCl_3$ solution was washed three times with 5% $NaHCO_3$. The organic layer was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was dissolved in acetic acid–THF–water (3:1:1, v/v/v) (40 mL). After being stirred at 80 °C for 14 h, the mixture was diluted $CHCl_3$. The $CHCl_3$ solution was washed twice with water and three times with 5% $NaHCO_3$. The organic layer was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was rendered anhydrous by coevaporation three times with dry pyridine and finally dissolved in dry pyridine (40 mL). To the solution was added trimethylsilyl chloride (1.01 mL, 8.0 mmol). After the mixture was stirred under argon atmosphere at room temperature for 30 min, 4,4′-dimethoxytrityl chloride (1.49 g, 4.4 mmol) was added. After being stirred at room temperature for 3 h, the mixture was quenched by addition of 28% aqueous ammonia (10 mL). The mixture was stirred at room temperature for an additional 30 min. The mixture was evaporated under reduced pressure and diluted $CHCl_3$. The $CHCl_3$ solution was washed three times with 5% $NaHCO_3$. The organic layer was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel with hexane–AcOEt–pyridine (70:30:1–60:40:1, v/v/v) to give **7f** (1.32 g, 43%): ^{1}H NMR (270 MHz, DMSO-d_6) δ −0.21 (3H, s), −0.11 (3H, s), 0.00 (3H, s), 0.01 (3H, s), 0.74 (9H, s), 0.81 (9H, s), 3.47–3.54 (2H, m, 5′-H), 3.66 (6H, s), 3.76–3.77 (1H, m), 3.99–4.02 (1H, m), 4.10–4.12 (1H, m), 5.11 (1H, m), 5.61 (1H, d, $J_{1',2'}$ = 5.6 Hz), 6.19 (1H, d, $J_{5,6}$ = 7.3 Hz), 6.75 (4H, d, $J_{meta,ortho}$ = 8.6 Hz), 7.06–7.17 (9H, m), 7.70 (1H, d, 6-H), 8.31 (1H, br s); ^{13}C NMR (DMSO-d_6) δ −5.0, −4.82, −4.77, −4.6, 17.7, 17.79, 17.84, 25.68, 25.74, 54.9, 60.6, 69.3, 72.1, 74.4, 79.1, 85.3, 88.1, 96.3, 112.5, 113.2, 123.7, 125.9, 127.2, 128.3, 129.7, 135.9, 136.7, 140.0, 144.8, 149.4, 154.0, 157.2, 163.0; ESI-mass *m/z* calcd for $C_{42}H_{60}N_3O_7Si_2$ 774.3970; observed [M + H] 774.3973.

4.1.4. 2′,3′-*O*-Di-*tert*-butyldimethylsilyl-2-*N*-(4,4′-dimethoxytrityl)guanosine (7g). Compound **13**[13] (939 mg, 1.5 mmol) was dissolved in acetic acid–THF–water (3:1:1, v/v/v) (15 mL). After being stirred at 80 °C for 10 h, the mixture was diluted $CHCl_3$. The $CHCl_3$ solution was washed twice with water and with 5% $NaHCO_3$. The organic layer was collected, dried over

Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was rendered anhydrous by coevaporation three times with dry pyridine and finally dissolved in dry pyridine (15 mL). To the solution was added trimethylsilyl chloride (123 μL, 2.0 mmol). After the mixture was stirred under argon atmosphere at room temperature for 1 h, 4,4′-dimethoxytrityl chloride (508 mg, 1.5 mmol) was added. After being stirred at room temperature for 4 h, the mixture was quenched by addition of 28% aqueous ammonia (6 mL). The mixture was stirred at room temperature for an additional 2 h. The mixture was evaporated under reduced pressure and diluted $CHCl_3$. The $CHCl_3$ solution was washed three times with 5% $NaHCO_3$. The organic layer was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel with hexane–AcOEt–pyridine (70:30:1–60:40:1, v/v/v) to give **7g** (767 mg, 63%): ^{1}H NMR (270 MHz, DMSO-d_6) δ −0.34 (3H, s, CH_3 of TBDMS), −0.16 (3H, s), 0.03 (3H, s, CH_3 of TBDMS), 0.08 (3H, s), 0.70 (9H, s), 0.85 (9H, s), 3.43 (2H, m), 3.68–3.73 (7H, m), 4.08–4.10 (1H, m), 4.42–4.45 (1H, m), 5.02 (1H, m), 5.26–5.29 (1H, d, $J_{1',2'}$ = 7.9 Hz), 6.78 (4H, d, $J_{ortho,meta}$ = 8.2 Hz), 7.16–7.25 (9H, m), 7.55 (1H, br s, 2-NH), 7.88 (1H, s), 10.59 (1H, br s); ^{13}C NMR (DMSO-d_6) δ −4.2, −3.9, −3.80, −3.76, 18.5, 18.6, 55.7, 55.7, 62.1, 70.3, 73.7, 75.5, 80.0, 84.5, 86.9, 113.5, 113.5, 117.4, 124.6, 127.1, 128.2, 129.1, 130.5, 136.8, 137.5, 137.8, 145.9, 150.3, 151.5, 151.6, 157,3, 158.2, 158.3. ESI-mass *m/z* calcd for $C_{43}H_{60}N_5O_7Si_2$ 814.4031; observed [M + H] 814.4508.

4.1.5. Typical procedure for the synthesis of nucleoside 5′-[ethyl *N*-(*N*-trityl-L-prolyl)phosphoroamidate] derivatives 8a–g.

4.1.5.1. 2′,3′-*O*-Di-*tert*-butyldimethylsilyladenosine 5′-[ethyl *N*-(*N*-trityl-L-prolyl)phosphoroamidate] (8a). A mixture of **6** (443 mg, 0.86 mmol) and **7a** (212 mg, 0.43 mmol) was coevaporated four times with dry acetonitrile and finally dissolved in dry acetonitrile (5 mL). To the mixture was added MMT (125 mg, 1.08 mmol), and the solution was stirred under argon atmosphere at room temperature for 1 h and then a 6 M solution of *tert*-butyl hydroperoxide in decane (717 μL, 4.3 mmol) was added. After being stirred at room temperature for an additional 30 min, the mixture was diluted with $CHCl_3$. The $CHCl_3$ solution was washed with 5% $NaHCO_3$, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel with hexane–AcOEt–pyridine, (100:0:1–99:1:1, v/v/v) to give a diastereomeric mixture of **8a** (244 mg, 60%): ^{1}H NMR (270 MHz, $CDCl_3$) δ −0.30 (3H, 2s, CH_3 of TBDMS), −0.13 (3H, 2s, CH_3 of TBDMS), −0.01 (6H, 2s), 0.68 (9H, 2s), 0.80 (9H, s), 0.81–1.51 (7H, m, $J_{POCH2CH3}$ = 7.9 Hz), 2.90 (1H, m), 3.20 (1H, m), 3.90 (1H, m), 4.18–4.53 (6H, m), 4.70 (1H, m), 5.93 (1H, 2d, $J_{1',2'}$ = 4.9 Hz), 6.70 (2H, br s), 6.99–7.08 (9H, m), 7.33–7.35 (6H, m), 8.13 (1H, 2s), 8.40 (1H, 2s); ^{13}C NMR ($CDCl_3$) δ −5.2, −5.1, −4.91, −4.86, −4.8, −4.6, −4.5, 16.0, 16.05, 16.10, 16.2, 17.65, 17.70, 17.8, 24.1, 25.5, 25.6, 25.7, 31.5, 50.4, 64.0, 64.1, 65.3, 65.4, 66.0, 66.1, 71.78, 71.80, 74.74, 74.75, 77.2, 77.9, 78.0, 82.81, 82.83, 83.0, 88.17, 88.18, 119.5, 119.6, 126.2, 126.3, 127.4, 127.6, 128.3, 128.8, 139.4, 143.75, 143.78, 149.3, 152.58, 152.60, 155.6, 177.28, 177.34; ^{31}P NMR ($CDCl_3$) δ −1.31, −1.46. ESI-mass *m/z* calcd for $C_{48}H_{69}N_7O_7PSi_2$ 942.4535; observed [M + H] 942.4823.

4.1.5.2. 3′-*O*-*tert*-Butyldimethylsilyldeoxyadenosine 5′-[ethyl *N*-(*N*-trityl-L-prolyl)phosphoroamidate] (8b). In a manner similar (see Table 1) to that described for the synthesis of **8a**, this compound was synthesized in 58% yield: ^{1}H NMR (270 MHz, $CDCl_3$) δ 0.08 (3H, s), 0.10 (3H, 2s), 0.64–0.90 (10H, m), 1.05–1.61 (6H, m, 3″-H, 4″-Hb, $J_{POCH2CH3}$ = 6.9 Hz), 2.11–2.44 (1H, m), 2.69–2.82 (1H, m), 2.92–3.10 (1H, m), 3.20–3.36 (1H, m), 3.92–3.99 (1H, m), 4.16–4.32 (4H, m, J_{POCH} = 10.6 Hz), 4.42–4.44 (1H, m), 4.66–4.67 (1H, m, $J_{3',2'\text{-Ha}}$ = 2.6 Hz), 6.44–6.56 (3H, m, $J_{1',2'\text{-Ha}}$ = 5.9 Hz, $J_{1',2'\text{-Hb}}$ = 7.9 Hz), 7.04–7.24 (9H, m), 7.41–7.44 (6H, m), 8.24 (1H, 2s), 8.28 (1H, 2s); ^{13}C NMR ($CDCl_3$) δ −4.80, −4.76, −4.72, −4.67, 16.1, 16.2, 16.3, 17.9, 24.3, 25.68, 25.71, 31.7, 40.5, 41.0, 50.6, 50.7, 64.15, 64.22, 64.3, 65.5, 65.6, 66.8, 72.2, 72.3, 77.2, 78.2, 83.9, 84.1, 85.5, 85.6, 119.6, 119.7, 126.4, 126.5, 127.6, 127.7, 128.5, 129.0, 139.0, 139.1, 143.7, 143.8, 149.3, 149.3, 152.8, 155.5, 155.6, 177.4, 177.5; ^{31}P NMR ($CDCl_3$) δ −1.61, −1.63. ESI-mass *m/z* calcd for $C_{42}H_{55}N_7O_6PSi$ 812.3721; observed [M + H] 812.22703.

4.1.5.3. 3′-*O*-*tert*-Butyldimethylsilyl-8-oxoadenosine 5′-[ethyl *N*-(*N*-trityl-L-prolyl)phosphoroamidate] (8c). In a manner similar (see Table 1) to that described for the synthesis of **8a**, this compound was synthesized in 43% yield: ^{1}H NMR (270 MHz, $CDCl_3$) δ 0.09 (3H, s), 0.10 (3H, s), 0.85–0.90 (10H, m), 1.06–1.58 (6H, m, $J_{POCH2CH3}$ = 7.3 Hz), 2.12–2.20 (1H, m, $J_{2'\text{-Ha},2'\text{-Hb}}$ = 5.6 Hz), 2.85–2.97 (1H, m), 3.06–3.41 (2H, m), 3.89–3.97 (1H, m), 4.06–4.38 (5H, m, J_{POCH} = 9.9 Hz), 5.70 (2H, 2br s), 6.34 (1H, 2t, $J_{1',2'\text{-Ha}}$ = 6.6 Hz, $J_{1',2'\text{-Hb}}$ = 6.9 Hz), 7.09–7.30 (9H, m), 7.40–7.46 (6H, m), 8.02 (1H, 2s); ^{13}C NMR ($CDCl_3$) δ −4.74, −4.72, −4.65, 16.1, 16.19, 16.22, 16.3, 18.0, 24.3, 24.4, 25.8, 25.9, 31.65, 31.68, 36.7, 36.8, 50.6, 64.6, 64.7, 65.3, 65.5, 67.25, 67.32, 72.4, 72.6, 77.2, 78.1, 81.5, 84.8, 85.0, 103.9, 126.4, 126.7, 127.6, 127.7, 128.5, 129.0, 143.7, 143.9, 144.0, 146.5, 146.6, 147.0, 147.1, 151.0, 152.1, 177.90, 177.94, 178.0, 178.1; ^{31}P NMR ($CDCl_3$) δ −1.41, −1.50. ESI-mass *m/z* calcd for $C_{42}H_{55}N_7O_7PSi$ 828.3670; observed [M + H] 828.3648.

4.1.5.4. 6-*N*-Acetyl-2′,3′-*O*-di-*tert*-butyldimethylsilyl-7,8-dihydro-8-oxoadenosine 5′-[ethyl *N*-(*N*-trityl-L-prolyl)phosphoroamidate] (8d). In a manner similar (see Table 1) to that described for the synthesis of **8a**, this compound was synthesized in 55% yield. Chromatography was performed by use of hexane–AcOEt (99.5:0.5–98.5:1.5, v/v): ^{1}H NMR (270 MHz, $CDCl_3$) δ −0.18 (3H, 2s), −0.03 (3H, 2s), 0.10–0.12 (6H, 4s), 0.78–0.91 (18H, m, 4″-Ha), 1.09–1.39 (5H, m, $J_{POCH2CH3}$ = 6.9 Hz), 1.61 (1H, m), 2.19 (3H, s), 2.90–3.10 (1H, m),

3.22–3.34 (1H, m), 3.88–3.92 (1H, m), 4.21–4.65 (6H, m, J_{POCH} = 0.2 Hz), 5.06–5.12 (1H, m, 2′-H), 6.00 (1H, 2d, $J_{1',2'}$ = 4.9 Hz), 7.08–7.27 (9H, m), 7.39–7.44 (6H, m), 8.19 (1H, 2s), 8.75 (1H, 2br s), 9.49 (1H, 2br s); ^{13}C NMR ($CDCl_3$) δ −4.81, −4.78, −4.7, −4.6, −4.5, −4.4, −4.3, 15.3, 16.18, 16.23, 16.29, 16.34, 17.92, 17.93, 18.07, 18.09, 23.0, 23.8, 24.25, 24.33, 25.71, 25.73, 25.9, 31.6, 31.7, 50.58, 50.60, 50.7, 52.0, 59.3, 64.35, 64.40, 64.43, 64.48, 65.46, 65.51, 65.6, 66.9, 66.95, 66.96, 67.00, 71.6, 71.9, 72.3, 72.4, 77.2, 78.1, 78.2, 81.9, 82.4, 82.5, 86.3, 86.4, 108.56, 108.62, 126.5, 126.3, 126.6, 126.7, 127.0, 127.5, 127.6, 127.70, 127.74, 127.8, 128.4, 128.5, 129.0, 137.7, 143.7, 144.0, 144.3, 146.7, 150.11, 150.13, 150.2, 150.7, 150.9, 151.0, 170.0, 170.1, 177.4, 177.5; ^{31}P NMR ($CDCl_3$) δ −1.79, −1.99; ESI-mass *m/z* calcd for $C_{50}H_{71}N_7O_9PSi_2$ 1000.4589; observed [M + H] 1000.4662.

4.1.5.5. 2′,3′-*O*-Di-*tert*-butyldimethylsilyluridine 5′-[ethyl *N*-(*N*-trityl-L-prolyl)phosphoroamidate] (8e). In a manner similar (see Table 1) to that described for the synthesis of **8a**, this compound was synthesized in 89% yield. Chromatography was performed by use of hexane–AcOEt (60:40, v/v): ^{1}H NMR (270 MHz, $CDCl_3$) δ 0.06–0.13 (12H, 6s), 0.87–0.92 (19H, m), 1.13–1.25 (1H, m), 1.36–1.45 (4H, m), 1.62 (1H, m, 3″-Hb), 2.96–3.09 (1H, m), 3.24–3.41 (1H, m), 3.88–3.97 (1H, m), 4.12–4.47 (7H, m), 5.74 (1H, 2d, 5-H, $J_{5,6}$ = 8.2 Hz), 5.93 (1H, d, $J_{1',2'}$ = 4.6 Hz), 6.75 (1H, br s), 7.12–7.27 (9H, m), 7.45–7.49 (6H, m), 7.86 (1H, 2d), 10.32 (1H, 2br s); ^{13}C NMR ($CDCl_3$) δ −4.9, −4.82, −4.77, −4.74, −4.66, −4.40, −4.35, 16.1, 16.20, 16.21, 16.3, 17.8, 17.87, 17.90, 17.93, 24.1, 24.2, 24.3, 25.6, 25.70, 25.73, 34.1, 50.3, 50.6, 63.9, 64.0, 64.06, 64.14, 64.9, 65.4, 65.45, 65.51, 65.57, 65.64, 65.7, 71.0, 74.9, 75.0, 77.2, 78.0, 78.11, 78.14, 82.4, 82.47, 82.51, 88.3, 88.4, 102.3, 102.4, 126.2, 126.4, 126.5, 127.5, 127.7, 128.9, 143.0, 143.8, 144.4, 150.4, 150.5, 163.5, 163.6, 177.16, 177.23, 177.25, 177.32; ^{31}P NMR ($CDCl_3$) δ −0.89, −1.14; ESI-mass *m/z* calcd for $C_{47}H_{68}N_4O_9PSi_2$ 919.4263; observed [M + H] 919.4391.

4.1.5.6. 2′,3′-*O*-Di-*tert*-butyldimethylsilyl-4-*N*-(4,4′-dimethoxytrityl)cytidine 5′-[ethyl *N*-(*N*-trityl-L-prolyl)phosphoroamidate] (8f). In a manner similar (see Table 1) to that described for the synthesis of **8a**, this compound was synthesized in 91% yield. Chromatography was performed by use of hexane–$CHCl_3$ (20:80–0:100, v/v): ^{1}H NMR (270 MHz, $CDCl_3$) δ 0.00–0.78 (12H, m), 0.84–1.54 (25H, m), 2.97–3.10 (1H, m, 5″-Ha), 3.19–3.29 (1H, m), 3.70 (6H, 2s), 3.74 (1H, m), 3.86–4.40 (7H, m), 5.09 (1H, 2d, 1′-H, $J_{1',2'}$ = 6.5 Hz), 5.87 (1H, d, 5-H, $J_{5,6}$ = 4.3 Hz), 6.74 (4H, d, $J_{ortho,metq}$ = 8.9 Hz), 7.07–7.24 (18H, m), 7.40–7.56 (7H, m); ^{13}C NMR ($CDCl_3$) δ −5.0, −4.91, −4.88, −4.7, −4.5, −4.4, −4.3, 15.3, 16.1, 16.2, 16.3, 17.9, 17.95, 17.98, 24.1, 24.20, 24.23, 25.7, 25.77, 25.80, 25.9, 31.5, 31.6, 34.2, 50.3, 50.6, 55.0, 55.1, 59.2, 63.96, 64.00, 64.0, 64.1, 64.9, 65.4, 65.5, 65.6, 66.1, 66.07, 66.09, 66.12, 66.2, 69.9, 70.8, 71.1, 75.0, 75.1, 77.2, 78.1, 78.2, 81.7, 89.67, 89.69, 94.7, 94.8, 113.3, 126.2, 126.5, 126.9, 127.1, 127.4, 127.6, 127.7, 128.0, 128.26, 128.33, 129.0, 129.6, 136.0, 136.1, 141.0, 141.3, 143.1, 143.8, 144.2, 144.4, 146.7, 158.25, 158.28, 164.95, 165.00, 176.75, 176.81, 176.95, 177.00; ^{31}P NMR ($CDCl_3$) δ −1.62, −1.93; ESI-mass *m/z* calcd for $C_{68}H_{87}N_5O_{10}PSi_2$ 1220.5729; observed [M + H] 1220.5531.

4.1.5.7. 2′,3′-*O*-Di-*tert*-butyldimethylsilyl-2-*N*-(4,4′-dimethoxytrityl)guanosine 5′-[ethyl *N*-(*N*-trityl-L-prolyl)phosphoroamidate] (8g). In a manner similar (see Table 1) to that described for the synthesis of **8a**, this compound was synthesized in 72% yield. Chromatography was performed by use of hexane–MeOH (99.5:0.5–99:1, v/v): ^{1}H NMR (270 MHz, $CDCl_3$) δ −0.27 (3H, 2s), −0.08 (3H, 2s), 0.08 (3H, 2s), 0.11 (3H, 2s), 0.75–0.80 (9H, m), 0.81–1.09 (10H, m), 1.20–1.38 (5H, m), 1.55–1.61 (1H, m), 2.94–3.04 (1H, m), 3.27–3.29 (1H, m), 3.68 (6H, s), 3.87–3.94 (1H, m), 4.11–4.26 (6H, m), 4.38–4.40 (1H, m), 4.56–4.60 (1H, m), 5.61 (1H, 2d, $J_{1',2'}$ = 6.5 Hz), 6.75 (4H, d, $J_{ortho,meta}$ = 7.9 Hz), 7.04–7.30 (18H, m), 7.40–7.44 (6H, m), 7.70 (1H, s), 8.66 (1H, s), 8.88 (1H, 2br s); ^{13}C NMR ($CDCl_3$) δ −4.5, −4.3, −4.0, −3.9, −3.8, −3.74, −3.73, 16.85, 16.92, 16.96, 17.02, 18.56, 18.61, 18.67, 18.68, 18.70, 18.74, 24.97, 25.00, 26.4, 26.45, 26.49, 32.2, 35.0, 51.3, 51.4, 55.8, 55.9, 64.8, 64.87, 64.92, 65.0, 66.1, 66.2, 66.25, 66.31, 67.47, 67.51, 67.56, 67.58, 70.8, 70.9, 73.1, 73.3, 75.3, 78.0, 78.85, 78.88, 84.09, 84.10, 84.2, 84.3, 84.4, 86.9, 114.1, 114.8, 118.35, 118.40, 127.1, 127.2, 127.4, 127.8, 128.19, 128.24, 128.3, 128.4, 128.47, 128.76, 128.84, 129.1, 129.2, 129.7, 130.3, 136.15, 136.22, 136.27, 136.32, 136.7, 136.9, 143.9, 144.4, 144.6, 144.8, 144.9, 151.5, 151.6, 151.9, 152.0, 157.3, 159.0, 159.6, 177.87, 177.93, 178.1, 178.2; ^{31}P NMR ($CDCl_3$) δ −1.61, −1.83; ESI-mass *m/z* calcd for $C_{69}H_{87}N_7O_{10}PSi_2$ 1260.5791; observed [M + H] 1260.5618.

4.1.6. Adenosine 5′-[ethyl *N*-(L-prolyl)phosphoroamidate] (A-phosmidosine) trifluoroacetic acid salt (9a). Compound **8a** (244 mg, 0.26 mmol) was dissolved in 80% formic acid (2.6 mL). After being stirred at room temperature for 42 h, the mixture was diluted with distilled water. The aqueous solution was washed three times with $CHCl_3$, evaporated under reduced pressure, and coevaporated with distilled water under reduced pressure. The residue was chromatographed on a column of C_{18} by using medium pressure chromatography with solvent system II. The fractions containing **9a** were collected and lyophilized. The residue was rechromatographed on a column of C18 with water–acetonitrile (95:5, v/v) followed by lyophilization from its aqueous solution to give **9a** as the TFA salt (29 mg, 19%): ^{1}H NMR (270 MHz, D_2O) δ 1.13 (3H, t, $J_{POCH2CH3}$ = 6.9 Hz), 1.81–1.91 (3H, m), 2.32 (1H, m), 3.24 (2H, m), 3.97–4.07 (2H, m), 4.29–4.35 (5H, m), 4.65 (1H, m), 6.01 (1H, d, $J_{1',2'}$ = 2.0 Hz), 8.28 (1H, s), 8.33 (1H, s); ^{13}C NMR (D_2O) δ 17.88, 17.90, 17.97, 17.99, 26.1, 31.98, 32.02, 49.1, 63.0, 63.2, 68.5, 68.56, 68.60, 68.65, 69.2, 69.3, 69.4, 72.2, 76.5, 85.0, 85.07, 85.14, 85.2, 91.1, 91.2, 112.4, 116.6, 120.9, 121.2, 121.3, 125.2, 144.87, 144.91, 147.0, 150.7, 152.3, 164.5, 165.0, 165.5, 166.0, 174.0, 174.1; ^{31}P NMR (D_2O) δ −1.15, −1.21. ESI-mass *m/z* calcd for $C_{17}H_{27}N_7O_7P$ 472.1710; observed [M + H] 472.1729.

4.1.7. Deoxyadenosine 5′-[ethyl *N*-(L-prolyl)phosphoroamidate] (dA-phosmidosine) (9b). Compound **8b** (118 mg, 0.15 mmol) was dissolved in THF (1.5 mL), and $Bu_4NF{\cdot}H_2O$ (152 mg, 0.58 mmol) was added. After being stirred at room temperature for 6 h, the mixture was diluted with $CHCl_3$. The $CHCl_3$ solution was washed three times with 5% $NaHCO_3$, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was dissolved in a 1% solution of TFA in water–acetonitrile (1:1, v/v, 1.5 mL). After being stirred at room temperature for 15 min, the mixture was diluted with distilled water. The aqueous solution was washed three times with $CHCl_3$, evaporated under reduced pressure, and coevaporated with distilled water under reduced pressure. The residue was chromatographed on a column of C_{18} by using medium pressure chromatography with solvent system I. The fractions containing **9b** were collected and lyophilized. The residue was rechromatographed on a column of C18 with water–acetonitrile (90:10, v/v) followed by lyophilization from its aqueous solution to give **9b** as the free form (48 mg, 72%): ^{1}H NMR (270 MHz, D_2O) δ 1.09 (3H, 2t, $J_{POCH2CH3}$ = 6.9 Hz), 1.81–2.02 (3H, m), 2.25–2.38 (1H, m), 2.57–2.66 (1H, m, $J_{2'\text{-Ha},2'\text{-Hb}}$ = 6.6 Hz), 2.77–2.88 (1H, m), 3.24–3.41 (2H, m), 3.73–3.84 (2H, m, J_{POCH} = 10.6 Hz), 4.07–4.16 (3H, m), 4.24–4.25 (1H, m), 4.69–4.74 (1H, m, $J_{3',2'\text{-Hb}}$ = 4.3 Hz), 6.37 (1H, dd, $J_{1',2'\text{-Ha}}$ = 6.3 Hz, $J_{1',2'\text{-Hb}}$ = 6.6 Hz), 8.09 (1H, s), 8.27 (1H, 2s); ^{13}C NMR (D_2O) δ 17.9, 18.0, 26.4, 32.4, 41.3, 41.4, 48.8, 64.7, 65.0, 65.4, 65.47, 65.50, 65.58, 67.56, 67.64, 67.7, 67.8, 73.4, 86.28, 86.34, 87.8, 87.88, 87.91, 120.9, 141.9, 150.79, 150.82, 154.9, 157.6, 178.5, 178.6; ^{31}P NMR (D_2O) δ 10.57; ESI-mass *m*/*z* calcd for $C_{17}H_{27}N_7O_6P$ 456.1761; observed [M + H] 456.1582.

4.1.8. 7,8-Dihydro-8-oxodeoxyadenosine 5′-[ethyl *N*-(L-prolyl)phosphoroamidate] (8-oxo-dA-phosmidosine) (9c). This compound was synthesized in 58% yield as the free form in a manner similar to that described for the synthesis of **9b**: ^{1}H NMR (270 MHz, D_2O) δ 1.10–1.16 (3H, 2t, $J_{POCH2CH3}$ = 6.9 Hz), 1.90–2.07 (3H, m), 2.30–2.40 (2H, m, 3″-Hb), 3.15–3.39 (3H, m), 3.81–3.95 (2H, m, J_{POCH} = 9.9 Hz), 4.11 (4H, m), 4.71 (1H, m, 3′-H), 6,21 (1H, t, $J_{1',2'\text{-Ha}} = J_{1',2'\text{-Hb}}$ = 6.6 Hz), 7.98 (1H, s); ^{13}C NMR ($CDCl_3$) δ 17.9, 18.0, 26.4, 32.4, 37.6, 48.8, 64.7, 65.1, 65.3, 65.4, 67.96, 67.98, 68.03, 68.1, 73.4, 73.5, 84.05, 84.12, 86.8, 87.0, 106.4, 148.8, 149.5, 153.3, 155.1, 178.7, 178.8; ^{31}P NMR ($CDCl_3$) δ 10.79, 10.87. ESI-mass *m*/*z* calcd for $C_{17}H_{27}N_7O_7P$ 472.1710; observed [M + H] 472.5253.

4.1.9. 6-*N*-Acetyl-7,8-dihydro-8-oxodeoxyadenosine 5′-[ethyl *N*-(L-prolyl)phosphoroamidate] (N^6-Ac-dA-phosmidosine) (9d). This compound was synthesized in 29% yield as the free form in a manner similar to that described for the synthesis of **9b**: ^{1}H NMR (270 MHz, D_2O) δ 1.13 (3H, t, $J_{POCH2CH3}$ = 6.9 Hz), 1.96–2.07 (3H, m), 2.27–2.32 (4H, m), 3.30–3.44 (2H, m), 3.83–3.93 (2H, m), 4.09–4.22 (4H, m), 4.65–4.69 (1H, m), 5.17–5.20 (1H, m), 5.95 (1H, d, $J_{1',2'}$ = 4.6 Hz), 8.40 (1H, s); ^{13}C NMR (D_2O) δ 17.9, 18.0, 32.4, 48.8, 64.7, 65.1, 65.4, 65.5, 67.6, 67.7, 72.3, 73.2, 73.3, 84.5, 84.7, 88.8, 113.5, 140.3, 152.8, 153.0, 154.8, 175.2, 178.6, 178.7. ^{31}P NMR (D_2O) δ 10.70; ESI-mass *m*/*z* calcd for $C_{19}H_{29}N_7O_9P$ 530.1764; observed [M + H] 530.1832.

4.1.10. Uridine 5′-[ethyl *N*-(L-prolyl)phosphoroamidate] (U-phosmidosine) (9e). This compound was synthesized in 91% yield as the free form in a manner similar to that described for the synthesis of **9b**: ^{1}H NMR (270 MHz, D_2O) δ 1.33–1.38 (3H, 2t, $J_{POCH2CH3}$ = 6.9 Hz), 2.01–2.18 (3H, m), 2.43–2.59 (1H, m), 3.34–3.51 (2H, m), 4.22–4.55 (8H, m, J_{POCH} = 11.2 Hz), 5.88–5.92 (2H, 2d, 1′-H, 5-H, $J_{1',2'}$ = 5.3 Hz), 7.74 (1H, 2d, $J_{6,5}$ = 8.2 Hz); ^{13}C NMR (D_2O) δ 18.1, 18.2, 26.4, 32.5, 48.9, 64.8, 65.2, 65.65, 65.68, 65.72, 67.17, 67.20, 67.22, 67.24, 67.27, 67.29, 67.3, 71.9, 70.0, 76.2, 76.3, 85.0, 85.1, 85.2, 85.3, 91.6, 104.8, 144.0, 144.1, 154.1, 168.6, 178.85, 178.88, 178.93, 179.0; ^{31}P NMR (D_2O) δ 11.07; ESI-mass *m*/*z* calcd for $C_{16}H_{26}N_4O_9P$ 449.1437; observed [M + H] 449.1453.

4.1.11. Cytidine 5′-[ethyl *N*-(L-prolyl)phosphoroamidate] (C-phosmidosine) TFA salt (9f). Compound **8f** (223 mg, 0.18 mmol) was dissolved in THF (1.8 mL), and $Bu_4NF{\cdot}H_2O$ (383 mg, 1.46 mmol) was added. After being stirred at room temperature for 1 h, the mixture was diluted with $CHCl_3$. The $CHCl_3$ solution was washed three times with 5% $NaHCO_3$, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was dissolved in a 4% solution of TFA in water–acetonitrile (1:1, v/v, 1.8 mL). After the mixture was stirred at room temperature for 3 h, trifluoroacetic acid (73 μL, 0.99 mmol) was added. After being stirred at room temperature for an additional 12 h, the mixture was diluted with distilled water. The aqueous solution was washed 3 times with AcOEt, evaporated under reduced pressure, and coevaporated with distilled water under reduced pressure. The residue wad chromatographed on a column of C_{18} by using medium pressure chromatography with solvent system I. The fractions containing **9e** were collected and lyophilized. The residue was rechromatographed on a column of C_{18} with water–acetonitrile (95:5, v/v) followed by lyophilization from its aqueous solution to give **9f** as the TFA form (62 mg, 60%): ^{1}H NMR (270 MHz, D_2O) δ 1.32 (3H, t, $J_{POCH2CH3}$ = 6.9 Hz), 1.96–2.13 (3H, m), 2.47–2.52 (1H, m), 3.39–3.41 (2H, m), 4.18–4.49 (8H, m), 5.84–5.85 (1H, m), 6.11–6.13 (1H, m), 7.79–7.83 (1H, m); ^{13}C NMR (D_2O) δ 17.9, 18.01, 18.03, 26.1, 31.96, 31.99, 49.0, 49.2, 63.0, 63.2, 68.5, 68.56, 68.6, 69.1, 69.16, 69.23, 71.26, 71.33, 76.2, 84.0, 84.1, 93.1, 98.1, 112.3, 116.6, 120.9, 125.19, 145.1, 155.5, 155.6, 164.5, 165.0, 165.2, 165.3, 165.5, 166.1, 174.09, 174.12; ^{31}P NMR (D_2O) δ −0.90, −0.95; ESI-mass *m*/*z* calcd for $C_{16}H_{27}N_5O_8P$ 448.1597; observed [M + H] 448.1583.

4.1.12. Guanosine 5′-[ethyl *N*-(L-prolyl)phosphoroamidate] (G-phosmidosine) (9g). This compound was synthesized in 69% yield as the free form in a manner similar to that described for the synthesis of **9b**: ^{1}H NMR (270 MHz, D_2O) δ 1.16 (3H, t, $J_{POCH2CH3}$ = 7.3 Hz), 2.85–2.06 (3H, m), 2.26–2.39 (1H, m), 3.24–3.43 (2H, m), 3.81–3.97 (2H, m, J_{POCH} = 11.5 Hz), 4.09–4.32 (4H, m), 4.45–4.50 (1H, m), 4.68 (1H, m, $J_{2',3'}$ = 4.9 Hz), 5.83 (1H, d, $J_{1',2'}$ = 4.6 Hz), 7.92 (1H, d); ^{13}C NMR

(D_2O) δ 17.9, 17.95, 18.03, 18.1, 26.4, 32.4, 48.8, 64.7, 65.1, 65.5, 65.6, 65.7, 67.1, 67.2, 67.3, 67.4, 72.55, 72.59, 76.25, 76.30, 85.4, 85.5, 89.75, 89.78, 118.40, 118.43, 139.4, 139.5, 153.77, 153.80, 156.07, 156.09, 160.9, 178.6, 178.67, 178.70, 178.8; ^{31}P NMR (D_2O) δ 10.63, 10.73. ESI-mass *m/z* calcd for $C_{17}H_{27}N_7O_8P$ 488.1659; observed [M + H] 488.1658.

4.1.13. 7,8-Dihydro-8-oxodeoxyadenosine. 8-Bromodeoxyadenosine[21] (6.6 g, 20 mmol) was dissolved in a mixture of acetic acid–acetic anhydride (1:1, v/v, 400 mL), and sodium acetate (30 g, 366 mmol) was added. After being stirred at 120 °C for 30 min, the mixture was diluted with $CHCl_3$. The $CHCl_3$ solution was washed five times with distilled water, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was dissolved in AcOEt. The AcOEt solution was washed 5% $NaHCO_3$, filtered, and evaporated under reduced pressure. The residue was dissolved in ethanol (86 mL), and NaOH (1.7 g, 43 mmol) was added. After being stirred at room temperature for 3 h, the mixture was stirred at 60 °C for an additional 1 h. The mixture was neutralized by addition of 4 M HCl (10 mL) and 5% $NaHCO_3$. The precipitates were removed by filtration and washed three times with distilled water. The filtrate and washings were collected and evaporated under reduced pressure. The residue was chromatographed on a column of C_{18} with water–acetonitrile (100:0–98:2, v/v) to give the title compound as ocherous solids (1.6 g, 28%): ^{1}H NMR (270 MHz, DMSO-d_6) δ 1.92–2.00 (1H, m, $J_{2'\text{-Ha},2'\text{-Hb}}$ = 4.6 Hz), 2.89–2.99 (1H, m), 3.41–3.47 (1H, m), 3.57–3.63 (1H, m), 3.79–3.80 (1H, m), 4.36–4.38 (1H, m, $J_{3',2'\text{-Hb}}$ = 5.3 Hz), 6.13 (1H, dd, $J_{1',2'\text{-Ha}}$ = 6.3 Hz, $J_{1',2'\text{-Hb}}$ = 8.2 Hz), 7.19 (2H, br s), 7.91 (1H, s); ^{13}C NMR (DMSO-d_6) δ 36.5, 62.5, 71.5, 81.6, 87.5, 104.7, 146.1, 147.6, 149.7, 152.1. ESI-mass *m/z* calcd for $C_{10}H_{14}N_5O_4$ 268.1046; observed [M + H] 268.1027.

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