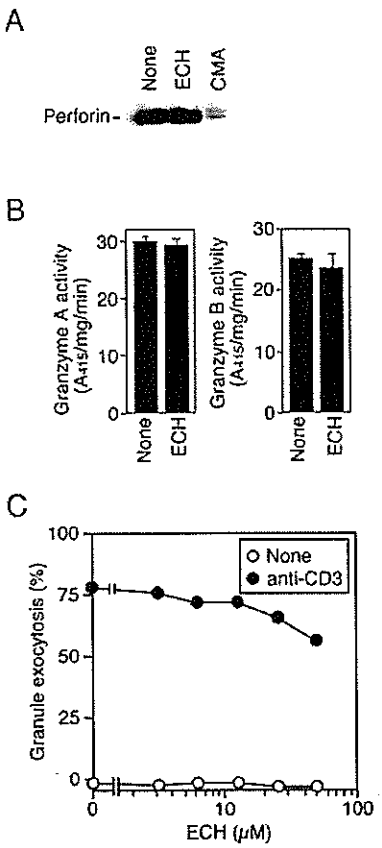

FIGURE 4. Effect of ECH on the components of lytic granules and granule exocytosis. *A*, OE4 cells were treated with 50 μM ECH or 100 nM CMA for 4 h or were left untreated. Postnuclear lysates were analyzed by Western blotting using anti-perforin Ab. *B*, OE4 cells were treated with or without 50 μM ECH for 4 h. Postnuclear lysates were measured for the enzyme activity of granzymes A and B. *C*, OE4 cells were incubated with (●) or without (○) immobilized anti-CD3 Ab in the presence of serial dilutions of ECH for 4 h. Culture supernatants were measured for the granzyme A activity. Data points represent the mean ± SD of triplicate cultures.

effector CTL were exposed to ECH at half the initial concentrations for pretreatment of target cells. To avoid any direct effects on lytic granules and the granule exocytosis pathway during the killing assay, ECH was used at concentrations <100 μM for pretreatment of target cells.

ECH does not inhibit perforin-dependent DNA fragmentation mediated by CD8$^+$ CTL

To determine whether ECH inhibits perforin-dependent DNA fragmentation, two different Fas-negative lymphomas were used as target cells against OE4 cells. As shown in Fig. 1, A20.FO and L5178Y cells were completely resistant to soluble FasL. Therefore, DNA fragmentation of these target cells induced by OE4 cells is solely dependent on the perforin/granzyme system. ECH did not affect DNA fragmentation of A20.FO cells (Fig. 5, *A* and *B*) and L5178Y cells (Fig. 5*C*) at concentrations up to 50 μM. Only slight reduction of DNA fragmentation was observed when A20.FO cells (Fig. 5*A*) and L5178Y cells (Fig. 5, *C* and *D*) were pretreated with 100 μM ECH.

ECH inhibits FasL-dependent DNA fragmentation mediated by CD8$^+$ CTL

Fas-positive target cells are killed by OE4 cells via the perforin-dependent pathway and the FasL-dependent pathway. To block the perforin-dependent killing pathway, OE4 cells were pretreated with 100 nM CMA for 2 h. ECH alone did not affect DNA fragmentation of Fas-positive A20 cells induced by OE4 cells (Fig. 6, *A* and *B*). However, FasL-dependent DNA fragmentation induced by CMA-treated OE4 cells was prevented by ECH in a dose-dependent manner, and ECH completely inhibited DNA fragmentation when A20 cells were pretreated with 50 μM (Fig. 6, *A* and *B*). These results demonstrate that ECH inhibits FasL-dependent DNA fragmentation mediated by CD8$^+$ CTL, but not perforin/granzyme-dependent DNA fragmentation. Likewise, in L5178Y-Fas cells, ECH dose-dependently inhibited DNA fragmentation induced by CMA-treated OE4 cells, and complete inhibition was observed when L5178Y-Fas cells were pretreated with 100 μM ECH (Fig. 6, *C* and *D*). The CMA-insensitive killing of OE4 cells corresponded to 40 and 70% in A20 cells (Fig. 6*B*) and L5178Y-Fas cells (Fig. 6*D*), respectively, suggesting that the FasL-dependent killing pathway plays a more dominant role in the induction of apoptosis in L5178Y-Fas cells than A20 cells. This difference might explain the observation that ECH alone exerts a stronger inhibitory activity toward DNA fragmentation of L5178Y-Fas cells.

ECH inhibits FasL-dependent DNA fragmentation, but not perforin-dependent DNA fragmentation, mediated by alloantigen-specific bulk CTL

To generalize the selective inhibitory effects of ECH on FasL-dependent DNA fragmentation, alloantigen-specific bulk CTL were induced by in vitro MLC for 4 days and used as effector CTL. In agreement with the observations using OE4 cells, ECH barely influenced DNA fragmentation of A20.FO cells induced by MLC cells (Fig. 7, *A* and *B*). In Fas-positive A20 cells, FasL-dependent DNA fragmentation induced by CMA-treated MLC cells was almost completely inhibited by ECH at 25 μM (Fig. 7*C*). By contrast, ECH alone did not significantly influence DNA fragmentation induced by MLC cells under these conditions (Fig. 7, *C* and *D*).

ECH inhibits FasL-dependent target cell lysis, but not perforin-dependent target cell lysis, in CTL-mediated cytotoxicity

The ^{51}Cr release assay has been widely used for the measurement of target cell lysis in CTL-mediated cytotoxicity. As observed with the DNA fragmentation assay (Fig. 1*C*), ECH inhibited cytolysis of A20 cells induced by soluble FasL in a dose-dependent manner (Fig. 8*A*). Consistent with this observation, ECH strongly prevented FasL-dependent target cell lysis mediated by BK-1 cells (Fig. 8*B*) as well as OE4 cells pretreated with CMA (Fig. 8*C*). By contrast, perforin-dependent target cell lysis mediated by OE4 cells was only marginally affected by ECH (Fig. 8*D*).

Discussion

Recently, we have shown that ECH inhibits Fas-mediated apoptosis by blocking activation of procaspase-8 in the DISC (24). In this work we have investigated whether ECH inhibits the perforin-dependent killing pathway and the FasL-dependent killing pathway in CTL-mediated cytotoxicity. In the short term killing assay based on two different murine Fas-positive/negative target cells vs the CD4$^+$ and CD8$^+$ CTL clones as well as alloantigen-specific bulk MLC cells, ECH profoundly blocked the FasL-dependent DNA fragmentation and cytolysis of target cells, but barely prevented the perforin/granzyme-dependent DNA fragmentation and cytolysis of target cells. Moreover, ECH did not influence the cellular levels of perforin and granzymes A/B and only marginally reduced the granule exocytosis pathway in response to CD3 stimulation.

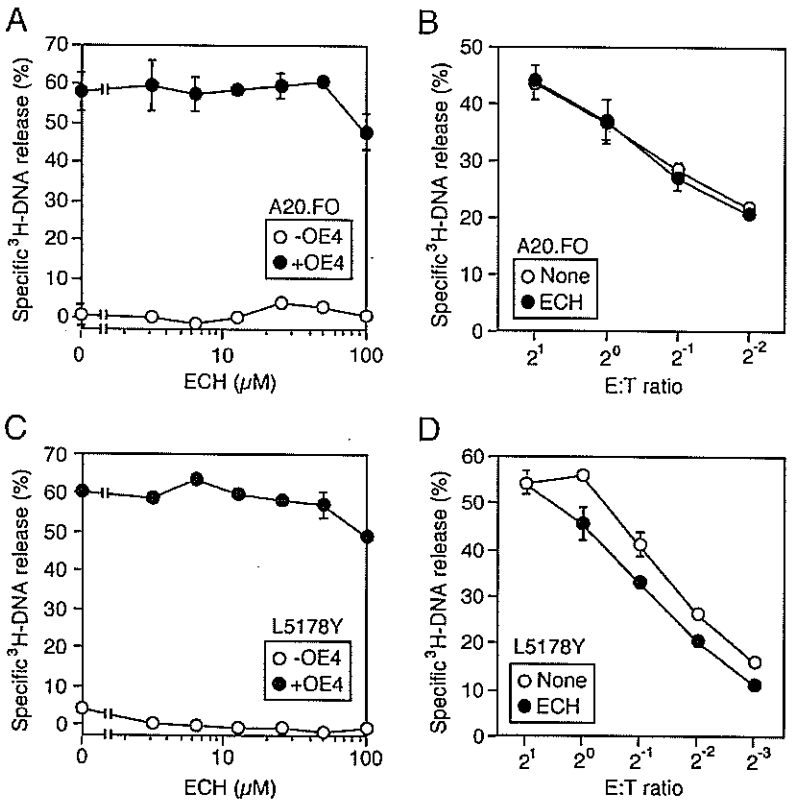

FIGURE 5. ECH does not affect perforin-based DNA fragmentation mediated by the CD8$^+$ CTL clone. *A*, [^{3}H]TdR-labeled A20.FO cells were pretreated with serial dilutions of ECH for 1 h. The target cells were mixed with (●) or without (O) OE4 cells (E:T cell ratio = 2), then incubated for 4 h. *B*, [^{3}H]TdR-labeled A20.FO cells were pretreated with (●) or without (O) 50 μM ECH for 1 h. The target cells were mixed with different numbers of OE4 cells, then incubated for 4 h. *C*, [^{3}H]TdR-labeled L5178Y cells were pretreated with serial dilutions of ECH for 2 h. The target cells were mixed with (●) or without (O) OE4 cells (E:T cell ratio = 2), then incubated for 4 h. *D*, [^{3}H]TdR-labeled L5178Y cells were pretreated with (●) or without (O) 100 μM ECH for 2 h. The target cells were mixed with different numbers of OE4 cells, then incubated for 4 h. The radioactivity of fragmented DNA was measured. Data points represent the mean ± SD of triplicate cultures.

Thus, our present results demonstrate that ECH is a highly selective inhibitor to block the FasL-dependent killing pathway in CTL-mediated cytotoxicity.

Death receptor-independent apoptosis induced by chemical compounds (i.e., staurosporine, MG-132, and ceramide) and UV irradiation was insensitive to ECH, whereas ECH markedly inhibited apoptosis induced by anti-Fas Ab, FasL, or TNF (24). These results suggest that ECH selectively blocks death receptor-mediated apoptosis that requires activation of procaspase-8. In the granule-dependent killing pathway, granzymes A and B have major roles in inducing target cell death upon translocation into the cytosol (1, 2). Granzyme A induces a caspase-independent cell death

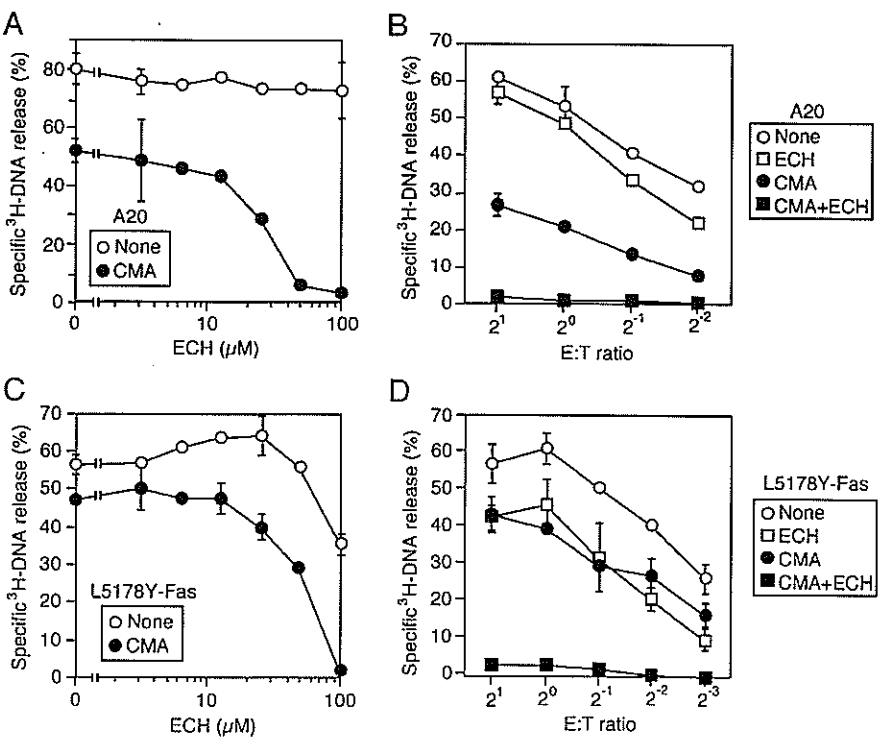

FIGURE 6. ECH selectively blocks FasL-based DNA fragmentation mediated by the CD8$^+$ CTL clone. *A*, OE4 cells were pretreated with (●) or without (O) 100 nM CMA for 2 h. [^{3}H]TdR-labeled A20 cells were pretreated with serial dilutions of ECH for 1 h. The target cells were mixed with OE4 cells (E:T cell ratio = 2), then incubated for 4 h. *B*, OE4 cells were pretreated with (● and ■) or without (O and □) 100 nM CMA for 2 h. [^{3}H]TdR-labeled A20 cells were pretreated with (□ and ■) or without (O and ●) 50 μM ECH for 1 h. The target cells were mixed with different numbers of OE4 cells, then incubated for 4 h. *C*, OE4 cells were pretreated with (●) or without (O) 100 nM CMA for 2 h. [^{3}H]TdR-labeled L5178Y-Fas cells were pretreated with serial dilutions of ECH for 2 h. The target cells were mixed with OE4 cells (E:T cell ratio = 2), then incubated for 4 h. *D*, OE4 cells were pretreated with (● and ■) or without (O and □) 100 nM CMA for 2 h. [^{3}H]TdR-labeled L5178Y-Fas cells were pretreated with (□ and ■) or without (O and ●) 100 μM ECH for 2 h. The target cells were mixed with different numbers of OE4 cells, then incubated for 4 h. The radioactivity of fragmented DNA was measured. Data points represent the mean ± SD of triplicate cultures.

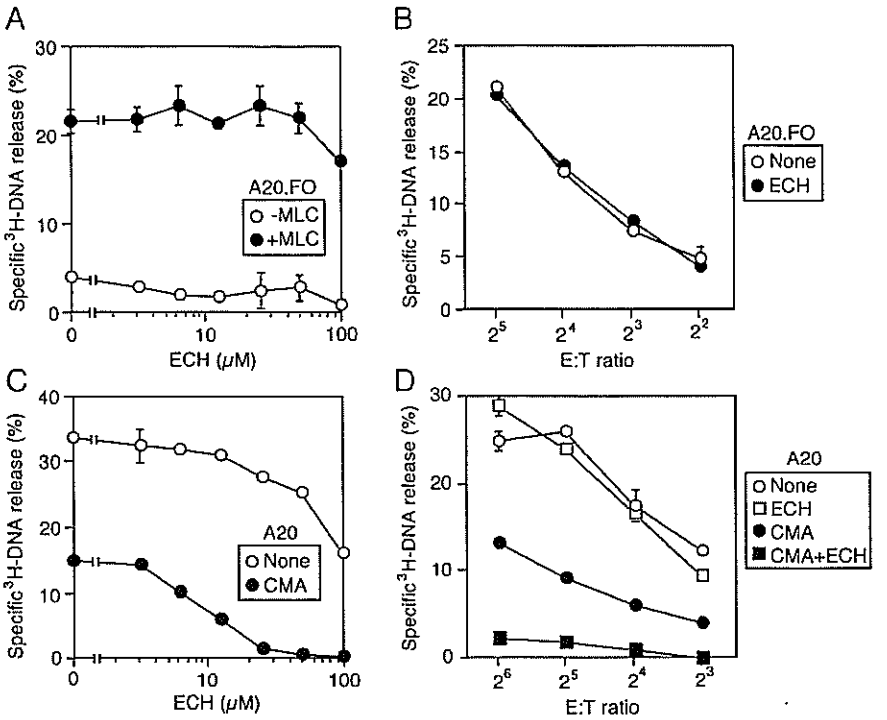

FIGURE 7. ECH selectively blocks FasL-based DNA fragmentation mediated by MLC cells. Responder spleen cells prepared from C57BL/6 mice were cultured with mitomycin C-treated stimulator spleen cells prepared from BALB/c mice for 4 days. *A*, [^{3}H]TdR-labeled A20.FO cells were pretreated with serial dilutions of ECH for 1 h. The target cells were mixed with (●) or without (O) MLC cells (E:T cell ratio = 32), then incubated for 4 h. *B*, [^{3}H]TdR-labeled A20.FO cells were pretreated with (●) or without (O) 50 μM ECH for 1 h. The target cells were mixed with different numbers of MLC cells, then incubated for 4 h. *C*, MLC cells were pretreated with (●) or without (O) 100 nM CMA for 2 h. [^{3}H]TdR-labeled A20 cells were pretreated with serial dilutions of ECH for 1 h. The target cells were mixed with MLC cells (E:T cell ratio = 16), then incubated for 4 h. *D*, MLC cells were pretreated with (● and ■) or without (O and ●) 100 nM CMA for 2 h. [^{3}H]TdR-labeled A20 cells were pretreated with (■ and □) or without (● and O) 50 μM ECH for 1 h. The target cells were mixed with different numbers of MLC cells, then incubated for 4 h. The radioactivity of fragmented DNA was measured. Data points represent the mean ± SD of triplicate cultures.

characterized by ssDNA nicks, which are mediated by granzyme A-activated DNase, NM23-H1 (34), whereas granzyme B initiates procaspase-3 processing, and the release of proapoptotic mitochondrial factors that facilitate the full activation of procaspase-3 (8–10). In the short term killing of target cells, granzyme B is critically involved in a rapid induction of DNA fragmentation (13). Consistent with this idea, the synthetic caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone completely prevented DNA fragmentation of A20 cells induced by the CD8$^+$ CTL clone in the short term assay (data not shown), confirming that granzyme B is a main factor that induces DNA fragmentation in the perforin-dependent killing pathway. ECH failed to prevent the perforin/granzyme B-dependent DNA fragmentation. These findings provide additional evidence that ECH specifically inhibits death receptor-mediated apoptosis, but does not affect death receptor-independent apoptosis.

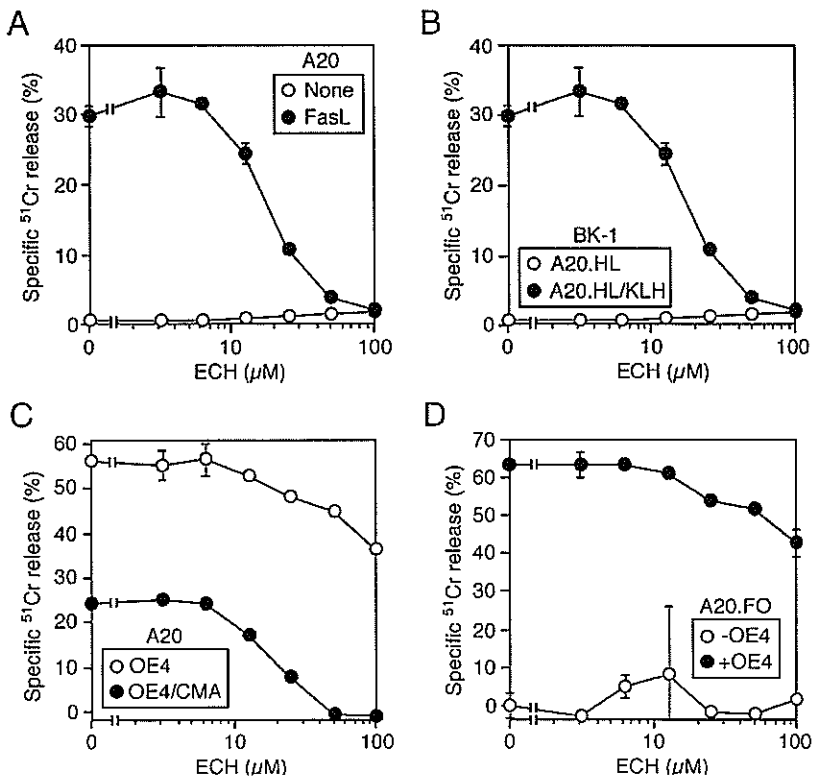

FIGURE 8. ECH inhibits FasL-dependent target cell lysis, but not perforin-dependent target cell lysis, in CTL-mediated cytotoxicity. *A*, ^{51}Cr-labeled A20 cells were preincubated with serial dilutions of ECH for 1 h, then incubated with (●) or without (O) cross-linked FasL (50 ng/ml) for 4 h. *B*, ^{51}Cr-labeled A20.HL cells were pulsed with or without KLH (300 μg/ml) before assay. KLH-pulsed A20.HL (●) and nonpulsed A20.HL (O) cells were preincubated with serial dilutions of ECH for 1 h. The target cells were mixed with BK-1 cells (E:T cell ratio = 8), then incubated for 4 h. *C*, OE4 cells were pretreated with (●) or without (O) 100 nM CMA for 2 h. ^{51}Cr-labeled A20 cells were pretreated with serial dilutions of ECH for 1 h. The target cells were mixed with OE4 cells (E:T cell ratio = 2), then incubated for 4 h. *D*, ^{51}Cr-labeled A20.FO cells were pretreated with serial dilutions of ECH for 1 h. The target cells were mixed with (●) or without (O) OE4 cells (E:T cell ratio = 2), then incubated for 4 h. The radioactivity of the supernatant was measured. Data points represent the mean ± SD of triplicate cultures.

The major molecular target of ECH in Fas-mediated apoptosis is procaspase-8, and ECH affected neither active caspase-8 nor activation of procaspase-3 and procaspase-9 at the cellular level (24). ECH has a molecular structure of α,β-unsaturated ketone and epoxide that might be reactive to thiol residues of proteins. In agreement with this hypothesis, glutathione or cysteine neutralized ECH binding to procaspase-8 (24). Recently, we have reported that the mycotoxin penicillic acid (PCA) inhibits Fas-mediated apoptosis by blocking activation of procaspase-8 (23). Although ECH and PCA are structurally unrelated, PCA exhibited the inhibitory activity similarly to ECH, in that PCA preferentially inhibited activation of procaspase-8, but did not affect active caspase-8 in living cells (23). Moreover, activation of procaspase-3 and procaspase-9 in the cell was only weakly inhibited by PCA (23). PCA contains α,β-unsaturated lactone able to bind to sulfhydryl groups. Analysis by mass spectrometry revealed that PCA binds to the cysteine residue of the active center in procaspase-8 (23). Although we have not yet determined the binding sites of ECH on procaspase-8, it seems likely that ECH binds to the active center cysteine and inactivates its intrinsic proteolytic activity.

DNA fragmentation was partially reversed when ECH was removed from A20 cells during the 4-h coculture with FasL. In our earlier paper (24), we showed that ECH affects neither cell surface Fas expression nor Fas-FasL interaction. Thus, it seems likely that a portion of procaspase-8 becomes functional by dissociation from ECH or is newly synthesized during the 4-h incubation, although we cannot rule out the possibility that ECH affects FasL expression on CTL.

In the short term culture, ECH alone did not induce apoptosis or necrosis. However, in the long term culture, ECH markedly reduced live cells without an induction of DNA fragmentation, whereas a portion of ECH-treated cells became positive for trypan blue staining. Thus, these results indicate that ECH inhibits proliferation, but can also induce necrotic cell death in the long term culture. Molecular mechanisms of ECH on inhibiting proliferation or inducing necrosis remain to be elucidated.

ECH inhibited Fas-mediated apoptosis in several human cell lines (24) (data not shown) and two murine lymphoma cell lines tested in this paper at the minimum inhibitory concentrations of ECH ranging from 10–100 μM. ECH inhibited Fas-mediated apoptosis at relatively lower concentrations in human cell lines (24), and 100 μM ECH was required for the complete inhibition of the Fas-mediated apoptosis in L5178Y-Fas cells. The culture medium for murine lymphoma cells contains 50 μM 2-ME, which might neutralize ECH due to the presence of thiol residues. However, the inhibitory doses of ECH unaltered in culture medium without 2-ME (data not shown). In addition, the FasL susceptibility is unlikely to involve the inhibitory doses of ECH, because L5178Y-Fas cells and A20 cells were equivalently susceptible to FasL, but Fas-mediated apoptosis in A20 cells was more strongly inhibited by ECH. A possible explanation might be that intracellular competitors such as glutathione and/or thiol-containing protein(s) antagonize ECH and determine the strength of its inhibitory activity on procaspase-8.

We previously showed that CMA is a specific inhibitor of the perforin-dependent killing pathway (18). CMA induces inactivation and subsequent proteolytic degradation of perforin in lytic granules upon neutralization of acidic pH (17, 19, 20). By contrast, the FasL-dependent killing pathway mediated by Ag-specific CTL was suppressed by an inhibitor of glycoprotein transport, brefeldin A (18), as FasL is newly synthesized upon TCR activation and transported to the cell surface. Ca^{2+} dependency was frequently used to distinguish between the perforin-based cytotoxicity and the FasL-based cytotoxicity. However, Ca^{2+} is not only required for the function of perforin, but also for TCR-mediated early signaling leading to FasL expression (35–37). Therefore, the Ca^{2+}-independent cytotoxicity previously observed is largely due to the preexisting FasL and is believed to be insensitive to brefeldin A. As ECH blocks activation of procaspase-8 in target cells, it is thought that ECH is a more general nonpeptide inhibitor of the FasL-mediated killing pathway exerted by CTL, NK cells, or other types of cells, even though they constitutively express cell surface FasL.

The Fas/FasL system plays an essential role in lymphocyte homeostasis, and mutations in this system lead to the accumulation of abnormal T cells in the peripheral lymphoid organs and the development of severe autoimmune diseases in humans and mice (3–5). In addition to death-inducing functions, our and other groups reported that Fas provides costimulatory signals for human T cells, and caspase inhibitors block T cell proliferation (38–40), suggesting that caspase activation is required for T cell proliferation. The FasL-dependent killing of target cells by CTL is involved in the pathogenesis of experimental autoimmune encephalomyelitis and fulminant hepatitis (41–43). In neuronal diseases such as Huntington diseases, procaspase-8 is activated independently of death receptors (44, 45). Taken together, caspase-8 inhibitors such as ECH might be therapeutic candidates to treat autoimmune diseases and neuronal diseases.

Mice deficient in perforin, granzymes, Fas, or FasL have been used to study the biological significance of the perforin/granzyme system and the Fas/FasL system in various in vivo models and in vitro killing assays. These knockout mice, however, are not always applicable to all experimental settings. In this report we have shown that ECH is a specific inhibitor of the FasL-dependent killing pathway, but does not affect the perforin/granzyme-dependent killing pathway. Thus, ECH is a highly useful tool to evaluate the FasL-dependent killing pathway in cell-mediated cytotoxicity and might be applicable for all CTL-target combinations.

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

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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.

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.
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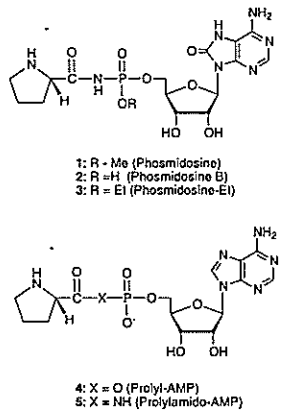


Figure 1. Various derivative of phosmidosine and prolyl-AMP.

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.

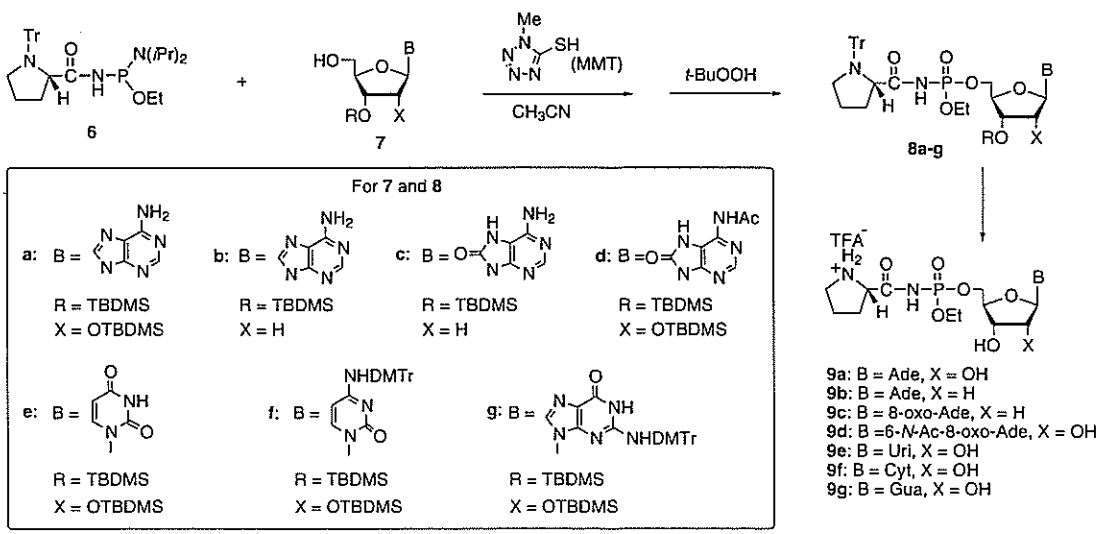
To clarify this possibility, the structure–activity relationship of phosmidosine is of great importance. Particularly, it should be clarified if the 7,8-dihydro-8-oxoadenosine component can be replaced by other elements such as the deoxy counterpart, the adenosine, and other ribonucleosides without loss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

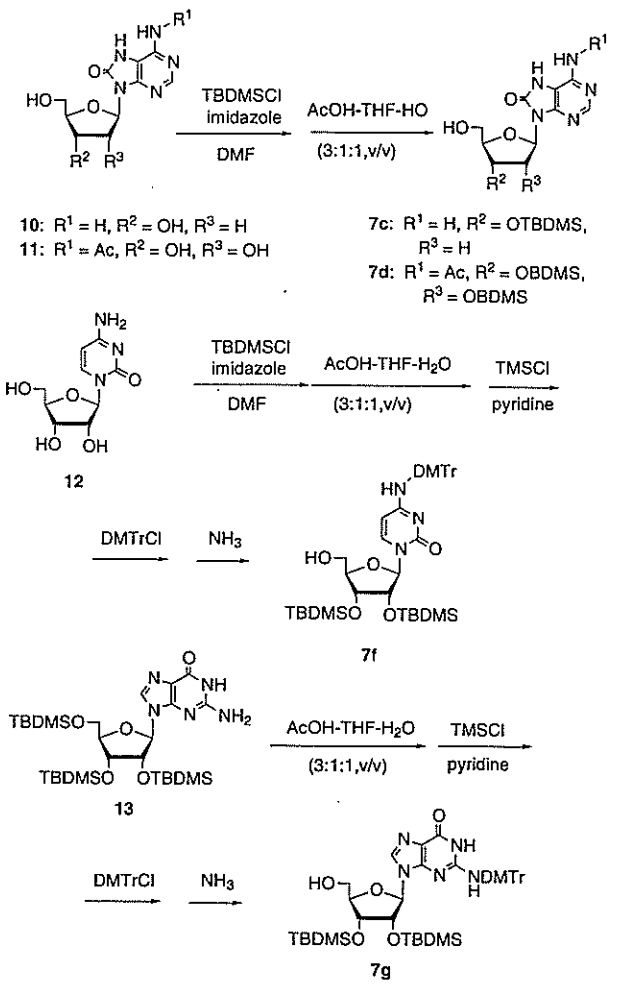
2. Results and discussion

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-oxo-adenosine 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, $J_{ortho,meta}$ = 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 4M 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%): ^1H 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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Synthesis of a biotin-conjugate of phosmidosine *O*-ethyl ester as a G1 arrest antitumor drug

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Abstract—This paper deals with the synthesis of a stable biotin–phosmidosine conjugate molecule **3** that is required for isolation of biomolecules that bind to phosmidosine (**1**). It was found that introduction of a biotin residue into the 6-N position of phosmidosine could be carried out by reaction of an N^7-Boc-7,8-dihydro-8-oxoadenosine derivative **13** with phenyl chloroformate followed by displacement with a diamine derivative **6** along with the simultaneous removal of the Boc group and one of the two phenoxycarbonyl groups and the successive condensation with an *N*-tritylated biotin derivative **5**. The condensation of an *N*-prolylphosphorodiamidite derivative **4** with an appropriately protected 7,8-dihydro-8-oxoadenosine derivative **17** having the biotin residue gave the coupling product **18**, which was deprotected to give the biotin–phosmidosine (*O*-ethyl ester) conjugate **3**.

1. Introduction

Recently, we have extensively studied the synthesis of a series of *N*-acylphosphoramidate derivatives involving phosmidosine (**1**) and related compounds (**2**) having *N*-amimoacylphosphoramidate linkages since these compounds have proved to have antitumor activity against various cancer-related cell lines.[1–6] In 1991, phosmidosine was first isolated as an antibiotic having morphological reversion activity of temperature-sensitive v-src^{ts}NKR cells.[7] Later, its structure was finally determined by use of mass spectrometry.[8] Osada and co-workers also reported that phosmidosine stops cell growth at the G_1 phase in the cell cycle.[9] This activity proved to be associated with inhibition of hyperphosphorylation of RB proteins by RB-kinases as a result of the inhibition of cyclin D1 expression.[10] We also reported the prolyl group and 7,8-dihydro-8-oxoadenine base are both responsible for expression of antitumor and morphological reversion activity by using a variety of phosmidosine analogs. These studies strongly suggest that phosmidosine serves as an antagonist of prolyl-AMP in tRNA aminoacylation mediated by prolyl-tRNA synthetase. However, this possibility has not been clarified to date (Fig. 1).

In this paper, we report the synthesis of a biotin–phosmidosine conjugate molecule **3**, which would be useful

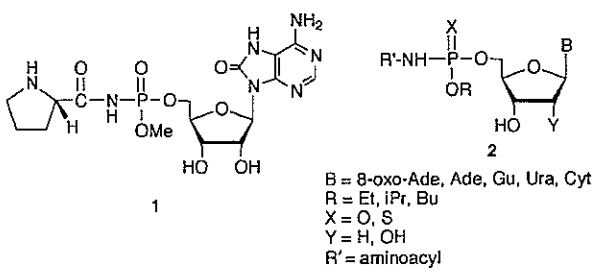

Figure 1. Phosmidosine (**1**) and its analog (**2**).

Keywords: Phosmidosine; Structure–activity relationship; 8-Oxoadenosine; Antitumor activity; Aminoacyl adenylate analog.
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for isolation of biomolecules that interact with phosmidosine.

2. Results and discussion

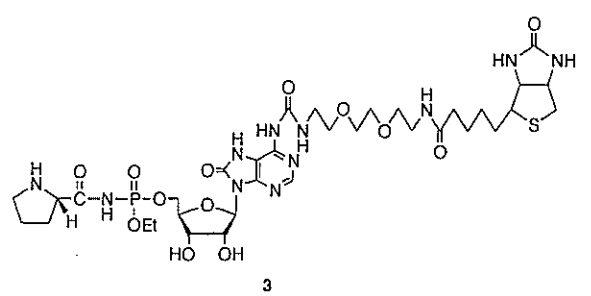
2.1. Determination of the site for introduction of a biotin residue into phosmidosine

In our previous studies, we have shown that the 8-oxoadenine moiety can be replaced by adenine and 6-*N*-acetyladenine bases without loss of the antitumor activity.[3] On the other hand, it was also reported that the ribose residue is exchangeable with the deoxy counterpart.[4] In consideration of the easiness of introduction of an acyl group into the exo-amino group, we decided to synthesize a 6-*N*-substituted phosmidosine derivative **3** where a biotin molecule is linked to the amino group via a linker. Phosmidosine has a methyl group in the *N*-prolylphosphoramidate linkage but the methyl group tends to be eliminated even under neutral conditions[8] and during the synthetic process, particularly when phosmidosine is concentrated to a-condensed solution.[3] Therefore, we introduced an ethyl group[3] in place of the methyl group in compound **3** to avoid self-decomposition due to the inherent instability of phosmidosine. This design was supported by the fact that *O*-ethyl ester analogs of phosmidosine did not affect the antitumor activity.[3] We also confirmed that a set of diastereoisomers generated by introduction of the ethyl group in a non-stereoselective manner are both active and there is no significant difference in antitumor activity between the two diastereoisomers[2,3] (Fig. 2).

Streptoavidine, which is well known to bind to four biotin molecules, is a relatively large protein so that there should be sufficient space between biotin and phosmidosine to keep the biological activity when phosmidosine binds to target biomolecules. Therefore, we used an 8-amino-3,6-dioxaoctanamine[11] as a linker, with solubility in aqueous solution in mind.

2.2. Synthesis of a biotin component

For construction of the *N*-prolylphosphoramidate linkage, we have recently employed a combination of *N*-diisopropyl-*N'*-[*N*-tritylprolyl]phosphorodiamidite **4** and 5'-unprotected 7,8-dihydro-8-oxoadenosine derivatives in the phosphoramidite coupling strategy.[3,4] Biotin has a hydrophilic character in the urea structure so that we used a 4,4'-dimethoxytrityl (DMTr) group as the protecting group of the urea function. Reaction of biotin with DMTrCl gave *N*-DMTr-product **5**[12] in 88% yield. This product was further condensed with a diamine **6** in the presence of DCC and HOBt to afford the amide **7** in 65% yield.



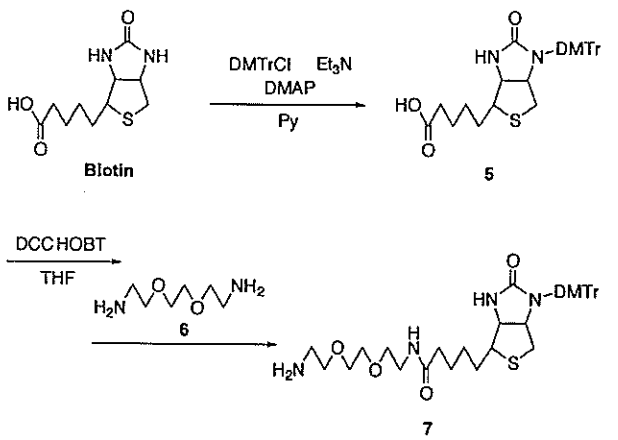
3

Figure 2. Biotin-containing phosmidosine derivative 3.

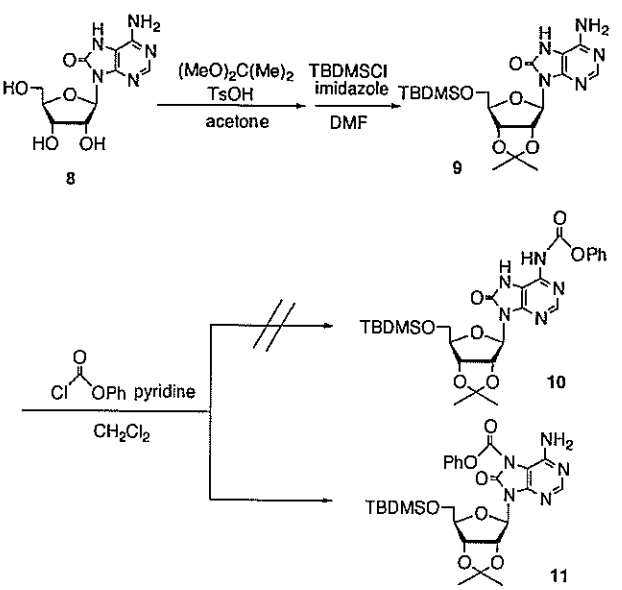
2.3. Synthesis of 7,8-dihydro-8-oxoadeonosine derivatives

First, we synthesized 2',3'-*O*-isopropylidene-5'-*O*-(*tert*-butyldimethylsilyl)-7,8-dihydro-8-oxoadenosine (**9**)[2] in 86% yield by use of a two-step reaction from 7,8-dihydro-8-oxoadenosine (**8**),[3] as shown in Scheme 1.

When compound **9** was allowed to react with phenyl chloroformate, the 6-*N*-phenoxycarbonyl-8-oxo-deoxyadenosine derivative **10** could not be obtained. Instead, the N^7-phenoxycarbonyl-8-oxo-deoxyadenosine derivative **11** was isolated in 92% yield (Scheme 2). The



Scheme 1. Synthesis of biotinamide derivative 7.

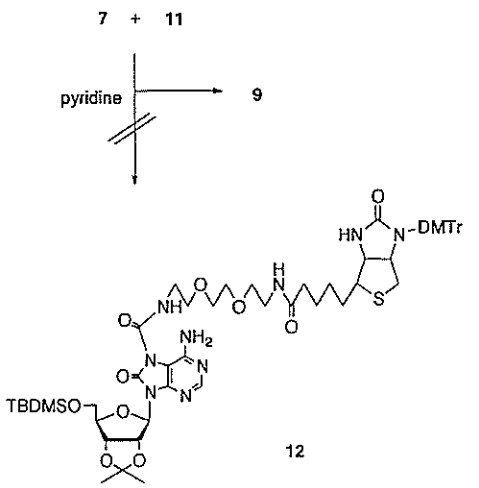


Scheme 2. Synthesis of N^7-phenoxycarbonyl-8-oxoadenosine derivative 11.

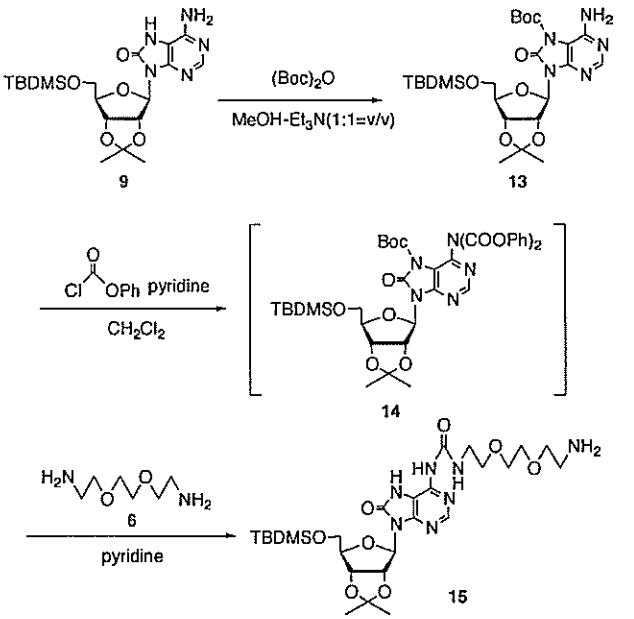
identification of this product was done by detailed analysis of ^{1}H NMR spectra. We previously encountered a similar result in a reaction of *tert*-butyl chloroformate with **9** giving rise to the N^7-Boc product in high yield. Previously, 6-*N*-acetyl-7,8-dihydro-8-oxoadenosine has been known as a 6-*N*-acylated derivative. This compound was obtained by peracetylation of 8-bromoadenosine followed by alkali hydrolysis. Therefore, no examples have been known of the synthesis of 6-*N*-acylated 7,8-dihydro-8-oxoadenosine derivative by direct acylation. With the previous result in mind, the 7-position of the 7,8-dihydro-8-oxoadenine moiety is considered more nucleophilic than the exo amino group. Although it is unknown if N^7-substituted derivatives of phosmidosine can maintain antitumor activity, we attempted to synthesize a biotin–phosmidosine conjugate **12** by condensation of **11** with **7** in pyridine (Scheme 3).

Surprisingly, however, we could not obtain a urea-type product **12**. The only product isolated was the deacylated species **9**. This result can be explained in terms of the potential leaving ability of the 7,8-dihydro-8-oxoadenine moiety. It is likely that the leaving ability of the phenoxy group is inferior to that of the base moiety.

Based on these unexpected results, we reconsidered use of an *N*-Boc protected species **13**, which we reported in our previous paper.[2] Reaction of **13** with 2.2 equiv of phenyl chloroformate gave an *N*,*N*-bis(phenoxycarbonyl) derivative **14**, which was further allowed to react with the diamine **6**. Although we expected it would be somewhat difficult to acylate the amino group because of the steric hindrance of the neighboring Boc group, compound **13** underwent rapid diacylation in 1 h. In contrast to this result, we have long experienced in the synthesis of phosmidosine derivatives that phosphitylation did not occur on this moiety. There is a sharp difference in reactivity between acylation and phosphitylation. In the second-step reaction, it was found that the Boc group and one of the phenoxycarbonyl groups were simultaneously removed by the action of the amine.



Scheme 3. Unexpected deacylation of **11** by the action of **7**.

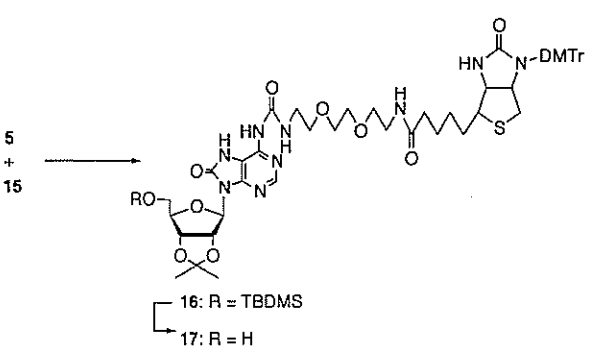


Scheme 4. Syntheis of 6-*N*-acylated 8-oxoadenosinde derivative **15**.

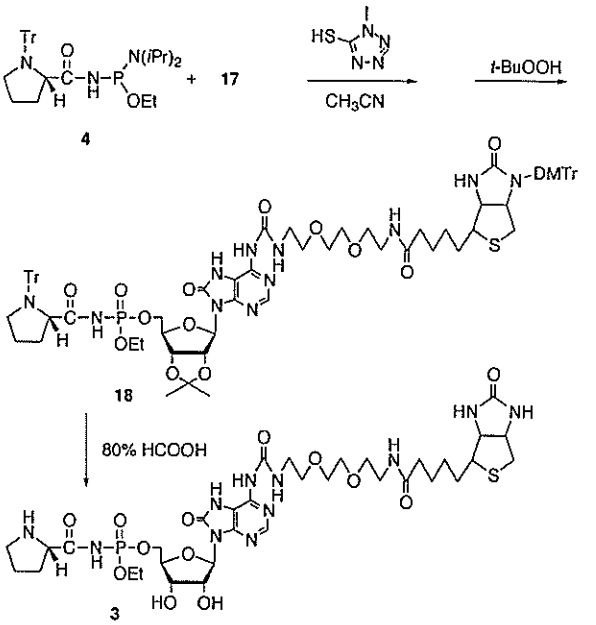
Consequently, the carbamoyl-type product **15** could be obtained in 70% yield. The elimination of the Boc group can be explained by the above same reason (Scheme 4).

Next, condensation of the *N*-carbamoyl product **15** with the *N*-DMTr biotin derivative **5** was carried out in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and HOBt. As the result, the coupling product **16** was obtained in 25% yield (Scheme 5).

Desilylation of **16** gave the 5′-OH component **17** in 79% yield. Finally, reaction of **17** with **4** in the presence of 5-mercapto-1-methyl-1*H*-tetrazole (MMT)[13,14] followed by oxidation with *t*BuOOH[15] gave the coupling product **18** in 69% yield. During this coupling reaction, there were observed no significant side reactions at the base part. Therefore, our strategy proved to be useful for modification on the 6-N position of phosmidosine derivatives. The usual deprotection of this protected species **18** with 80% formic acid afforded the fully deprotected target molecule **3** in 32% yield (Scheme 6).



Scheme 5. Synthesis of biotinylated 8-oxoadenosine derivative **17**.



Scheme 6. Synthesis of biotinylated phosmidosine analog.

Table 1. Antitumor activity of biotin–phosmidosine *O*-ethyl ester conjugate

Compd	IC_{50} (μM)	
	KB	L1210
Phosmidosine-Et	3.44	3.62
6-*N*-Acetylphosmidosine	2.89	4.10
Conjugate 3	54.9	>100

We also tested this final product to see if this molecule maintains antitumor activity. Consequently, it turned out that in the KB cell line the activity decreased 16 times more than that of the phosmidosine *O*-ethyl ester while in L1210 the activity dropped sharply. Although the antitumor activity of the final product considerably decreased, the figure observed in the KB cell line is at the level such that this molecule can be applied to affinity column chromatograpy to catch biomolecules that might bind to phosmidosine. Further study is under way. These results will be reported elsewhere in the near future (Table 1).

3. Conclusion

Here, we have succeeded in synthesizing a biotin–phosmidosine conjugate molecule. During the synthesis of this molecule, we found the inherent reactivity of the 7,8-dihydro-8-oxoadenine moiety toward acylating reagents. These results would provide new insight into the design of functionalized phosmidosine derivatives. Particularly, it is interesting that a naturally occurring base has not only a more nucleophilic character but also potential leaving ability. These features would be useful for the designing of artificial DNA or RNA molecules having enzyme activity by incorporation of new functional nucleotide building blocks.

4. Experimental section

4.1. General remarks

^1H, ^{13}C, and ^{31}P NMR spectra were obtained on a GX-270 apparatus at 270, 68, and 109 MHz, respectively. The chemical shifts were measured from tetramethylsilane (0 ppm) or DMSO-d_6 (2.49 ppm) for ^1H 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 Wako silica gel C-200. Reverse-phase column chromatography was performed by use of μBondasphere 37–55 mm C-18 (125A) particles, which was set up in a glass column of a medium pressure preparative HPLC system. Elution was performed with 0.1 M ammonium acetate (pH 7.0)–acetonitrile (100:0–50:50, v/v) for 50 min at a flow rate of 2.0 mL/min: Reverse-phase HPLC was performed using μBondasphere and μBondapak C-18 columns (Waters Co., Ltd, 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. ESI mass spectra were measured on Mariner™. MALDI-TOF mass spectra were measured on Voyager RP. TLC was performed with Merck silica gel 60 (F_{254}) plates. 8-Bromoadenosine was purchased from Sigma–Aldrich Co., Ltd. The morphological reversion activity test was conducted according to the literature method.[9] Compound **13** was prepared by the method reported by us.[2]

4.1.1. *N*-4,4′-Dimethoxytrityl-(+)-biotin (5).[12] (+)-Biotin (1.95 g, 8.0 mmol) was rendered anhydrous by coevaporation three times with dry pyridine and finally dissolved in dry pyridine (40 mL). To the solution were added 4,4′-dimethoxytrityl chloride (8.13 g, 24.0 mmol), triethylamine (1.11 mL, 8.0 mmol), and 4-(dimethylamino)pyridine (244 mg, 2.0 mmol). After being stirred at 70 °C for 4 h, the mixture was diluted with $CHCl_3$. The $CHCl_3$ solution was washed three times with 5% sodium citrate, and 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 (97:3, v/v) to give **5** as a white foam (3.86 g, 88%): ^1H NMR (270 MHz, DMSO-d_6): δ 1.33–1.64 (6H, m, CH_2), 2.17–2.20 (4H, m, SCH_2, $COCH_2$), 3.12 (1H, m, SCH), 3.90 (6H, s, CH_3 of PhOMe), 4.30–4.32 (2H, m, NCH), 6.76 (1H, s, NH), 6.84 (4H, d, *ortho* Ar–H of PhOMe, $J_{ortho,meta} = 8.9$ Hz), 7.02–7.29 (9H, m, Ar–H of Ph, *meta* Ar–H of PhOMe), 12.00 (1H, br s, COOH); ^{13}C NMR (DMSO-d_6): δ 24.53, 28.05, 28.44, 33.49, 54.24, 54.98, 59.25, 64.42, 71.66, 112.47, 126.32, 127.08, 129.23, 130.86, 135.74, 143.90, 157.55, 160.57, 174.19; ESI-mass *m/z* calcd for $C_{31}H_{35}N_2O_5S$ 547.2267; observed [M+H] 547.2113.

4.1.2. *N*-(3,6-Dioxa-8-aminooctyl)-*N*-(4,4′-dimethoxytrityl)-(+)-biotinylamide (7). To a solution of compound **5** (1.09 g, 2.0 mmol) in THF (20 mL) were added *N*,*N*′-dicyclohexyl carbodiimide (619 mg, 3.0 mmol) and 1-hydroxybenzotriazole (416 mg, 3.0 mmol). After the mixture had been stirred under argon atmosphere

at room temperature for 3 h, a solution of 8-amino-3,6-dioxaoctylamine **6** (1.46 mL, 16.3 mmol) in THF (20 mL) was dropwise added to the mixture over 15 min. Stirring was continued at room temperature for an additional 27 h. The mixture was diluted with $CHCl_3$, and 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 chromatographed on a column of silica gel with $CHCl_3$–MeOH (99:1–98:2, v/v) to give **7** as a white foam (882 mg, 65%): ^{1}H NMR (270 MHz, DMSO-d_6): δ 1.59–1.93 (6H, m, CH_2 of biotin), 2.34–2.39 (2H, m, SCH_2), 2.80 (2H, m, $COCH_2$), 2.93 (2H, t, NH_2CH_2, J = 5.6 Hz), 3.46–3.52 (3H, m, SCH, $NHCH_2$), 3.70 (4H, t, OCH_2CH_2O, J = 5.6 Hz), 3.89 (4H, m, OCH_2), 4.04 (6H, s, CH_3 of PhOMe), 7.07 (1H, s, NH of biotin), 7.15 (4H, d, *ortho* Ar–H of PhOMe), 7.32–7.38 (4H, m, *meta* Ar–H of PhOMe), 7.49–7.63 (5H, m, Ar–H of Ph), 8.19 (1H, br s, CONH); ^{13}C NMR (DMSO-d_6): δ 25.27, 28.15, 28.44, 35.11, 38.44, 40.70, 54.28, 54.98, 59.24, 64.43, 69.10, 69.47, 71.59, 71.68, 112.45, 126.31, 127.06, 129.23, 130.86, 135.72, 135.74, 143.90, 157.55, 160.58, 171.88; ESI-mass *m/z* calcd for $C_{37}H_{49}N_4O_6S$ 677.3373; observed [M+H] 677.3357.

4.1.3. 5′-*O*-*tert*-Butyldimethylsilyl-2′,3′-*O*-isopropylidene-7,8-dihydro-8-oxoadenosine (9).[2] To a suspension of 7,8-dihydro-8-oxoadenosine (2.83 g, 10 mmol) in acetone (100 mL) were added acetone dimethylacetal (24.6 mL, 200 mmol) and *p*-toluenesulfonic acid monohydrate (3.80 g, 20 mmol). The resulting mixture was stirred at room temperature for 20 min. The mixture was quenched by addition of satd $NaHCO_3$, and evaporated under reduced pressure. The residue was partitioned between $CHCl_3$–*i*PrOH (3:1, v/v) and 5% $NaHCO_3$. The organic layer was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was dissolved in dry DMF (20 mL), and *tert*-butyldimethylsilyl chloride (1.81 g, 12 mmol) and imidazole (1.63 g, 24 mmol) were added. After being stirred at room temperature for 1 h, the mixture was diluted with AcOEt. The AcOEt solution was washed three times 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 $CHCl_3$–MeOH (97:3, v/v) to give **9**[2] as a white foam (3.74 g, 86%). This compound was identified by comparison of it ^{1}H and ^{13}C NMR spectra with those of the authentic sample.[2]

4.1.4. 5′-*O*-*tert*-Butyldimethylsilyl-2′,3′-*O*-isopropylidene-N^7-phenoxycarbonyl-7,8-dihydro-8-oxoadenosine (11). To a mixture of **9** (1.31 g, 3.0 mmol) in pyridine (363 μL, 4.5 mmol) in CH_2Cl_2 (60 mL) was added phenyl chloroformate. After being stirred under argon atmosphere at room temperature for 30 min, the mixture was diluted with $CHCl_3$, and 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 chromatographed on a column of silica gel with hexane–AcOEt (50:50, v/v) to give **11** as a white foam (1.54 g, 92%): ^{1}H NMR (270 MHz, DMSO-d_6): δ 0.00 (6H, s, CH_3 of TBDMS), 0.84 (9H, s, $(CH_3)_3C$ of TBDMS), 1.33 (3H, s, CH_3 of isop), 1.53 (3H, s, CH_3 of isop), 3.68–3.83 (2H, m, 5′H), 4.08–4.15 (1H, m, 4′H), 4.92–4.96 (1H, m, 3′H), 5.44–5.46 (1H, m, 2′H), 6.02 (1H, d, 1′H, $J_{1',2'}$ = 6.6 Hz),7.11 (2H, br s, 6-NH_2), 7.31–7.54 (5H, m, Ar–H of Ph), 8.20 (1H, s, 2H); ^{13}C NMR (DMSO-d_6): δ −5.38, −5.28, 18.01, 25.23, 25.74, 26.98, 63.15, 81.48, 82.11, 86.41, 87.57, 100.84, 112.72, 121.20, 126.30, 129.52, 147.67, 148.69, 149.31, 149.84, 150.45, 153.49; ESI-mass *m/z* calcd for $C_{26}H_{36}N_5O_7Si$ 558.2384; observed [M+H] 558.2371.

4.1.5. Reaction of 7 with 11. A mixture of **7** (676.9 mg, 1.0 mmol) and **11** (557.7 mg, 1.0 mmol) was dissolved in dry pyridine (10 mL). After being stirred at room temperature for 25 h, the mixture was diluted with $CHCl_3$, and 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 chromatographed on a column of silica gel with hexane–AcOEt (50:50, v/v) to give **9** as a white foam (496 mg, 89%).

4.1.6. 5′-*O*-*tert*-Butyldimethylsilyl-6-*N*-[*N*-[3,6-dioxa-8-aminooctyl]carbamoyl]-2′,3′-*O*-isopropylidene-7,8-dihydro-8-oxoadenosine (15). Compound **13**[2] (537.7 mg, 1.0 mmol) was rendered anhydrous by coevaporation three times with dry pyridine and finally dissolved in dry CH_2Cl_2 (10 mL). To the mixture were added phenyl chloroformate (277.8 mL, 2.2 mmol) and dry pyridine (355.1 mL, 4.4 mmol). After being stirred under argon atmosphere at room temperature for 1 h, the mixture was diluted with $CHCl_3$, and 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 dry pyridine (10 mL), and 3,6-dioxa-8-aminooctylamine (2.96 g, 20 mmol) was added. After being stirred under argon atmosphere at room temperature for 40 min, the mixture was diluted with $CHCl_3$, and 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 chromatographed on a column of silica gel with $CHCl_3$–MeOH (98:2, v/v) to give **15** as a white foam (429 mg, 70%): ^{1}H NMR (270 MHz, $CDCl_3$): δ −0.04 (3H, s, CH_3 of TBDMS), −0.03 (3H, s, CH_3 of TBDMS), 0.82 (9H, s, $(CH_3)_3C$ of TBDMS), 1.34 (3H, s, CH_3 of isop), 1.54 (3H, s, CH_3 of isop), 2.86–2.90 (2H, m, NH_2CH_2), 3.49–3.79 (12H, m, OCH_2-CH_2O, $NHCH_2$, OCH_2, 5′H), 4.14–4.19 (1H, m, 4′H), 4.93–4.97 (1H, m, 3′H), 5.51 (1H, dd, 2′H, $J_{2',3'}$ = 6.3 Hz), 6.13 (1H, d, 1′H, $J_{1',2'}$ = 2.3 Hz), 8.25 (1H, s, 2H); ^{13}C NMR ($CDCl_3$): δ −5.26, −5.20, 18.41, 25.56, 25.93, 27.26, 40.15, 41.32, 63.42, 69.66, 70.15, 72.31, 77.21, 82.00, 82.22, 86.88, 87.22, 86.88, 87.22, 107.35, 113.43, 113.61, 115.37, 129.25, 140.14, 146.84, 146.94, 148.89, 149.24, 151.07, 151.89, 152.95, 155.85. ESI-mass *m/z* calcd for $C_{26}H_{46}N_7O_8Si$ 612.3177; observed [M+H] 612.3184.

4.1.7. 5′-*O*-*tert*-Butyldimethylsilyl-6-*N*-[*N*-[3,6-dioxa-8-[*N*′-(4,4′-dimethoxytrityl)-(+)-biotinylamido]octyl]carbamoyl]-2′,3′-*O*-isopropylidene-7,8-dihydro-8-oxoadenosine (16). To a solution of **5** (277.8 mg, 0.46 mmol) in dry

DMF (6 mL) was added 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (126.5 mg, 0.66 mmol). After the mixture was stirred under argon atmosphere at room temperature for 15 min, compound **15** (367.1 mg, 0.60 mmol) was added. After being stirred under argon atmosphere at room temperature for 30 min, the mixture was diluted with $CHCl_3$, and the $CHCl_3$ solution was washed three times with 5% sodium citrate, 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 **16** as a white foam (189.1 mg, 25%): ^{1}H NMR (270 MHz, $CDCl_3$): δ −0.03 (6H, s, CH_3 of TBDMS), 0.83 (9H, s, $(CH_3)_3C$ of TBDMS), 1.32 (3H, s, CH_3 of isop), 1.38–1.73 (9H, m, CH_2 of biotin, CH_3 of isop), 2.21–2.55 (4H, m, $COCH_2$, SCH_2), 3.10 (1H, m, SCH), 3.39–3.76 (20H, m, OCH_2CH_2O, OCH_2, NCH_2, 5′H, CH_3 of PhOMe), 4.08–4.14 (1H, m, 4′H), 4.36–4.46 (2H, m, NCH of biotin), 4.87–4.91 (1H, m, 3′H, $J_{3',4'}$ = 3.3 Hz), 5.38 (1H, dd, 2′H, $J_{2',3'}$ = 6.6 Hz), 5.97 (1H, d, 1′H, $J_{1',2'}$ = 2.3 Hz), 6.48 (1H, br s, NH of biotin), 6.72-6.78 (4H, m, *ortho* Ar–H of PhOMe), 7.14–7.32 (9H, m, *meta* Ar–H of PhOMe, Ar–H of Ph), 7.94 (1H, br s, 6-NH), 8.14 (1H, s, 2H), 8.45 (1H, br s, amide-NH of biotinylamide), 9.35 (1H, br s, NH of carbamoyl), 10.43 (1H, br s, 7-NH); ^{13}C NMR ($CDCl_3$): δ −5.31, −5.23, 18.33, 25.59, 25.88, 25.99, 27.19, 28.33, 28.47, 36.36, 38.83, 39.47, 54.81, 55.11, 59.63, 63.26, 65.68, 69.13, 69.58, 69.78, 70.29, 72.80, 77.21, 81.97, 82.04, 86.75, 86.90, 105.81, 112.76, 113.39, 126.86, 127.48, 129.48, 131.07, 134.96, 135.12, 140.12, 143.03, 147.86, 148.51, 149.93, 155.88, 158.22, 162.69, 172.70; ESI-mass *m/z* calcd for $C_{57}H_{78}N_9O_{12}SSi$ 1140.5260; observed [M+H] 1140.5325.

4.1.8. 6-*N*-[*N*-[3,6-Dioxa-8-[*N*′-(4,4′-dimethoxytrityl)-(+)-biotinylamido]octyl]carbamoyl]-2′,3′-*O*-isopropylidene-7,8-dihydro-8-oxoadenosine (17). To a solution of **16** (189.1 mg, 0.17 mmol) in THF (1.7 mL) was added TBAF–H_2O (130.2 mg, 0.50 mmol). After being stirred under argon atmosphere at room temperature for 4 h, the mixture was diluted with $CHCl_3$, and 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 chromatographed on a column of silica gel with $CHCl_3$–MeOH (99.4:0.6–99:1, v/v) to give **17** as a white foam (135.3 mg, 79%): ^{1}H NMR (270 MHz, $CDCl_3$): δ 1.30 (3H, s, CH_3 of isop), 1.38–1.72 (9H, m, CH_2 of biotin, CH_3 of isop), 2.20–2.51 (4H, m, $COCH_2$, SCH_2), 3.10 (1H, m, SCH), 3.38–3.84 (20H, m, OCH_2CH_2O, OCH_2, NCH_2, 5′H, CH_3 of PhOMe), 4.32 (1H, m, 4′H), 4.43 (2H, m, NCH of biotin), 4.93–4.98 (1H, m, 3′H, 5′-OH), 5.14 (1H, dd, 2′H, $J_{2',3'}$ = 5.9 Hz), 5.90 (1H, d, 1′H, $J_{1',2'}$ = 4.9 Hz), 6.49 (1H, br s, NH of biotin), 6.73–6.77 (4H, m, *ortho* Ar–H of PhOMe), 7.13–7.31 (9H, m, *meta* Ar–H of PhOMe, Ar–H of Ph), 7.93 (1H, br s, 6-NH), 8.14 (1H, s, 2H), 8.59 (1H, br s, amide-NH of biotinylamide), 9.29 (1H, br s, NH of carbamoyl), 10.54 (1H, br s, 7-NH); ^{13}C NMR ($CDCl_3$) 25.36, 25.98, 27.50, 28.28, 28.44, 36.33, 38.82, 39.36, 39.48, 54.84, 55.11, 59.56, 63.35, 65.66, 69.12, 69.51, 69.75, 70.28, 72.76, 77.21, 81.12, 81.41, 85.10, 88.31, 105.95, 112.77, 113.61, 126.88, 127.49, 129.48, 130.98, 131.07, 134.96, 135.05, 140.54, 142.97, 147.29, 148.35, 149.91, 155.70, 158.19, 158.21, 162.69, 172.70; ESI-mass *m/z* calcd for $C_{51}H_{64}N_9O_{12}S$ 1026.4395; observed [M+H] 1026.4407.

4.1.9. 6-*N*-[*N*-[3,6-Dioxa-8-[*N*′-(4,4′-dimethoxytrityl)-(+)-biotinylamido]octyl]carbamoyl]-2′,3′-*O*-isopropylidene-7,8-dihydro-8-oxoadenosine 5′-[ethyl *N*-(*N*-trityl-L-prolyl)phosphoramidate (18). A mixture of **17** (135.3 mg, 0.13 mmol) and **3** (140.3 mg, 0.26 mmol) was rendered anhydrous by coevaporation three times with dry pyridine and finally dissolved in dry acetonitrile (2 mL). To the solution was added MMT (38.3 mg, 0.33 mmol), and the mixture was stirred a under argon atmosphere at room temperature for 30 min. A 6 M solution of *tert*-butyl hydroperoxide in decane (220 μL, 1.32 mmol) was added. After being stirred under argon atmosphere at room temperature for 15 min, the mixture was diluted with $CHCl_3$, and 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 chromatographed on a column of silica gel with $CHCl_3$–MeOH (99.2:0.8, v/v) to give **18** as a white foam (134.9 mg, 69%): ^{1}H NMR (270 MHz, $CDCl_3$): δ 0.73–0.96 (1H, m, 4″Ha), 1.08–1.74 (18H, m, 3″H, 4″Hb, CH_2 of biotin, CH_3 of POEt, CH_3 of isop), 2.21–2.52 (4H, m, $COCH_2$, SCH_2), 2.89–3.01 (1H, m, 5″Ha), 3.11 (1H, m, SCH), 3.20–3.32 (1H, m, 5″Hb), 3.39–3.73 (18H, m, OCH_2CH_2O, OCH_2, NCH_2, CH_3 of PhOMe), 3.86–3.90 (1H, m, 2″H), 4.16–4.43 (7H, m, 4′H, 5′H, NCH of biotin, CH_2 of POEt), 5.00–5.07 (1H, m, 3′H), 5.32–5.34 (1H, m, 2′H), 6.04 (1H, 2d, 1′H, $J_{1',2'}$ = 6.3 Hz), 6.54 (1H, br s, NH of biotin), 6.75 (4H, 2d, *ortho* Ar–H of PhOMe, $J_{ortho,ortho}$ = 5.3 Hz), 7.06–7.43 (24H, m, *meta* Ar–H of PhOMe, Ar–H of Ph), 7.90 (1H, br s, 6-NH), 8.15 (1H, 2s, 2H), 8.53 (1H, br s, amide-NH of biotinylamide), 9.33 (1H, br s, NH of carbamoyl), 10.44 (1H, br s, 7-NH); ^{13}C NMR ($CDCl_3$): δ 16.07, 16.15, 16.17, 16.26, 24.17, 24.25, 25.45, 25.99, 27.09, 28.29, 28.44, 31.58, 36.34, 38.83, 39.38, 39.46, 50.55, 50.59, 54.83, 55.09, 55.11, 59.58, 64.16, 64.22, 64.25, 64.30, 65.45, 65.57, 65.65, 66.90, 66.94, 66.98, 67.01, 67.04, 67.09, 67.11, 69.15, 69.57, 69.74, 70.29, 72.75, 77.20, 78.12, 81.90, 82.10, 82.50, 82.75, 85.01, 85.13, 85.24, 86.67, 86.77, 105.82, 112.73, 113.69, 126.41, 126.65, 126.88, 127.49, 127.71, 128.46, 128.94, 128.98, 129.45, 131.05, 134.93, 134.95, 135.13, 140.20, 142.99, 143.82, 143.87, 147.53, 147.62, 148.65, 149.86, 149.88, 155.82, 158.17, 158.20, 162.69, 172.74, 177.19, 177.24. ^{31}P NMR ($CDCl_3$): 1.53, −2.10; ESI-mass *m/z* calcd for $C_{77}H_{91}N_{11}O_{15}PS$ 1472.6155; observed [M+H] 1472.6185.

4.1.10. 6-*N*-[*N*-[3,6-Dioxa-8-[(+)-biotinylamido]octyl]carbamoyl]-2′,3′-*O*-isopropylidene-7,8-dihydro-8-oxoadenosine 5′-[ethyl *N*-(L-prolyl)phosphoramidate (3). Compound **18** (100 mg, 0.12 mmol) was dissolved in 10% trifluoroacetic acid in water–THF (1:1, v/v, 1.2 mL). After being stirred at room temperature for 25 h, the mixture was diluted by addition of water. The aqueous solution was three times washed with