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Structure–activity relationships of epolactaene derivatives: structural requirements for inhibition of Hsp60 chaperone activity

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Abstract—Epolactaene is a microbial metabolite isolated from the fungal strain *Penicillium* sp. It arrests the cell cycle at the G_0/G_1 phase and induces the outgrowth of neurites in human neuroblastoma SH-SY5Y cells. In this communication, we report the structure–activity relationships (SARs) of new epolactaene derivatives, including those lacking the epoxylactam moiety and having various side chains. These derivatives were evaluated for their ability to inhibit the growth of human cancer cell lines. They were also analyzed for their ability to affect human heat shock protein 60 (Hsp60), which we have already identified as a protein that binds to epolactaene. We also identified the important structural framework of epolactaene/ETB (epolactaene tertiary butyl ester) for not only binding to Hsp60 but also inhibiting Hsp60 chaperone activity.

1. Introduction

Epolactaene is a microbial metabolite isolated by Kakeya et al. from the fungal strain *Penicillium* sp. BM 1689-P.[1] It was originally isolated for its effectiveness in promoting neurite outgrowth and arresting the cell cycle at the G_0/G_1 phase in a human neuroblastoma cell line.[2] Epolactaene contains characteristic structures that invoke certain biological activities, such as a highly oxidized γ-lactam and a conjugated (*E,E,E*)-triene in the side chain. It also has electrophilic characteristics in its α,β-unsaturated ketone, epoxide, and hemiaminal carbon, which are potentially reactive with biological nucleophiles, such as the sulfhydryls of cysteines. Because of epolactaene's interesting biological properties and its highly unusual structure, it has been an attractive target for organic chemists, and several groups, including our own, have undertaken its total synthesis.[3–8] In addition to the synthesis research, several reports have screened epolactaene analogues to investigate its biological activity and mode of action.[9,10] We also investigated the interaction between epolactaene and a proteasome complex, because lactacystin, a potent proteasome inhibitor, promotes neurite outgrowth and inhibits cell cycle progression in both the G_0/G_1 and G_2/M phases in mouse neuroblastoma Neuro 2A cells.[11,12] However, epolactaene did not inhibit the proteasome peptidase activities in vitro at a dose sufficient to inhibit growth in SH-SY5Y cells.[13]

In our recent report, we revealed that epolactaene binds to human Hsp60 and inhibits Hsp60 chaperone activity.[13] The Hsp60 family is known as a molecular chaperone assisting in protein folding.[14] In addition, mammalian Hsp60 has been reported to be involved in several biological processes, such as apoptosis,[15–18] immunoregulatory function,[19,20] and cell spreading.[21] Despite its importance, mammalian Hsp60 has been little studied compared with members of the prokaryotic Hsp60 family, such as GroEL.[14] In this report, we study the structure–activity relationships (SARs) of epolactaene derivatives to clarify the structural requirements for exhibiting biological activities. We prepared new epolactaene derivatives with various epoxylactam rings and side-chain moieties. We investigated these

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analogues for their ability to inhibit cell growth. Furthermore, we disclosed the key structural that enables epolactaene to inhibit human Hsp60 chaperone activity.

2. Chemistry

Epolactaene (Fig. 1, Epo) is a fungal metabolite that contains an epoxide inside a γ-lactam ring and an alkyl side chain bearing a triene moiety. Because each moiety could possibly induce biological activities, we are interested in clarifying the roles of these moieties in epolactaene's biological activity. For this purpose, we synthesized nine epolactaene derivatives with different epoxy lactam moieties and side-chain structures (Fig. 1).

Epolactaene tertiary butyl ester (ETB) was prepared so that we could observe the importance of the ester moiety. ETB was synthesized by the same procedures as those of Epo.[7] Bio-ETB as a probe was synthesized from ETB by the following reactions: (1) treatment of ETB with CF_3CO_2H afforded carboxylic acid, (2) coupling of the carboxylic acid with (11-hydroxyundecyl)carbamic acid *tert*-butyl ester by the use of EDC·HCl and DMAP gave amide, (3) treatment of the amide with CF_3CO_2H afforded amine, (4) coupling of the amine with (+)-biotin by the use of EDC·HCl and DMAP gave bio-ETB (74% yield in four steps). As to the importance of the lactam ring moiety, the complete loss or alteration of the lactam moiety gave rise to Epo-E, Epo-F, and Epo-G. They were prepared from tetrahydropyran-2-ol by highly stereoselective reactions, the details of which have already been reported.[7] For the side-chain derivation, compounds Epo-J, Epo-K, Epo-L, and Epo-M were synthesized. Epo-J, Epo-K, and Epo-L were prepared from the corresponding aldehydes by the diastereoselective reactions shown in Scheme 1. The aldehydes were treated with a newly developed Horner–Emmons reagent in the presence of *t*-BuOK in THF–HMPA to afford β-ketonitriles **2**. The Knoevenagel condensation between β-ketonitriles **2** and (*S*)-2-triethylsiloxypropanal prepared from (*S*)-methyl lactate proceeded in the presence of a catalytic amount of ethylenediammonium diacetate, affording the adducts **3** as single *E*-isomers, which were treated with TrOOLi at −78 °C to afford epoxides **4** with high diastereoselectivity. Both the bulky nucleophile (TrOOLi) and the TES protecting group are essential for the high selectivity. The deprotection of the TES group with AcOH and NH_4F in aq THF, hydrolysis of the nitrile by silica gel on TLC, and ammonolysis of the lactones formed **6** by NH_4OH in MeOH afforded the hydroxyamide **7**. The mild hydrolysis of the nitrile by silica gel should be ascribed to the intramolecular assistance of the hydroxy group. The final oxidation was achieved using SO_3·pyridine,[22] DMSO, and NEt_3 in CH_2Cl_2, affording Epo-J, Epo-K, and Epo-L. Finally, Epo-M was synthesized from Epo-L by hydrogenolysis catalyzed by Pd/C under a hydrogen atmosphere in an 84% yield.

3. Biological results

3.1. Effects of epolactaene derivatives on cell viability

We analyzed the SAR of the epolactaene derivatives for their inhibition of the growth of human neuroblastoma SH-SY5Y and human T-lymphoma Jurkat cells. The cell viability was assessed by MTT assay.[13] The 50% growth-inhibitory concentrations (IC_{50}s) are listed in Table 1. ETB was found to be as effective as epolactaene, whereas compound Epo-E, lacking the γ-lactam, resulted in the great loss of activity. The change to a lactone or reductive opening of the lactam ring also reduced activity greatly (compounds Epo-F and Epo-G). Furthermore, compounds Epo-J and Epo-L, each with a long hydrophobic alkyl chain lacking triene and ester moieties, were as active as epolactaene, aside from the less active Epo-K. Epo-M, with an α,β-saturated ketone in the side chain, retained a potent growth-inhibitory effect. From these results, we designed a biotin-conjugated epolactaene that retained its biological activity. Because the methyl ester moiety could be substituted by a bulky group such as tertiary butyl ester, we conjugated a biotin linker at the ester

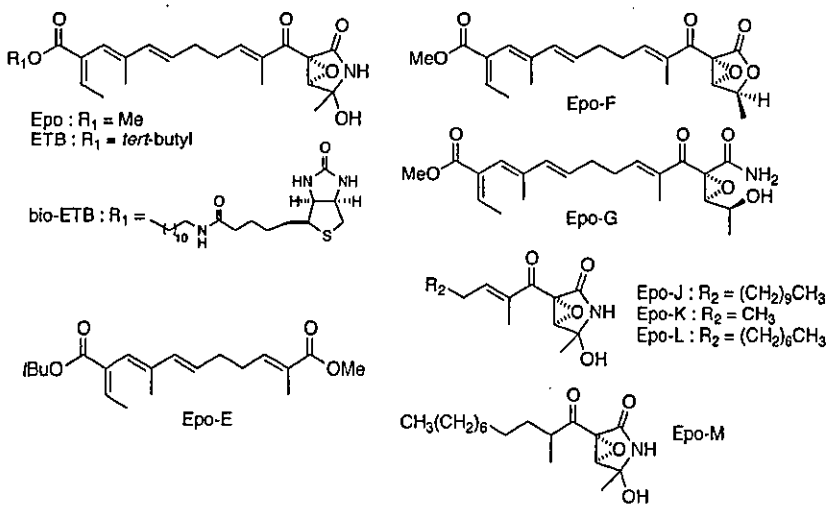
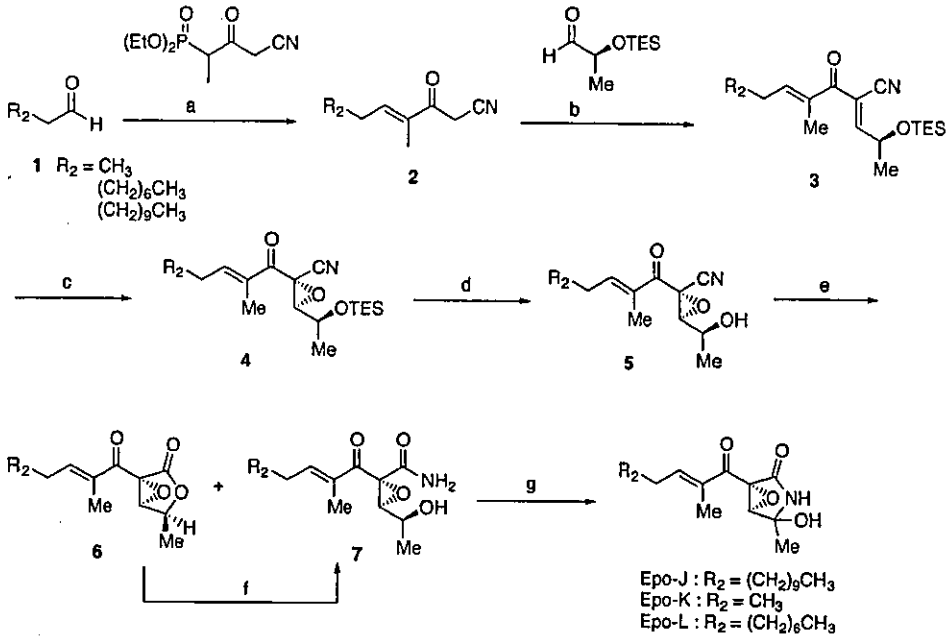


Figure 1. Structures of epolactaene and its derivatives used in this study.



Scheme 1. Synthesis of Epo-J, Epo-K, and Epo-L. Reagents and conditions: (a) *t*-BuOK, THF–HMPA (92–98%); (b) cat. ethylenediammonium diacetate, MeOH (83–89%); (c) TrOOH, *n*-BuLi, THF, −78 °C (78–84%); (d) AcOH, NH_4F, THF, H_2O; (e) TLC on silica gel; (f) NH_4OH, MeOH (71–77% from 4); (g) SO_3·Py, DMSO, NEt_3, CH_2Cl_2 (70–76%).

Table 1. IC_{50} acting against SH-SY5Y and Jurkat cell viability, competitive binding to Hsp60, and inhibition of Hsp60 chaperone activity on MDH reactivation by epolactaene derivatives

Compound	IC_{50} (μM)[a]		Binding competition[b]	In vitro MDH reactivation %[c]
	SH-SY5Y	Jurkat		
Epo	3.9	1.5	++	7.2±0.9
ETB	1.1	2.0	+++	1.9±6.8
Epo-E	>300.0	90.0	–	85.9±5.3
Epo-F	>130.0	20.0	+	53.1±11.8
Epo-G	38.0	20.0	–	62.7±8.1
Epo-J	5.7	1.2	+	31.2±6.2
Epo-K	18.0	8.0	–	35.1±2.4
Epo-L	13.0	1.5	+++	13.2±3.1
Epo-M	n.d.[d]	3.0	+++	70.0±8.9
Bio-ETB	6.5	3.0		n.d.

[a] IC_{50}: 50% inhibitory concentration.
[b] Binding competition percent of each derivative was generated relative to untreated sample as the control and shown as follows: +++, >90%; ++, 60–90%; +, 20–60%; –, <20%. Experimental conditions: see text.
[c] MDH reactivation % by BSA was subtracted as the baseline, and then the reactivation derived using untreated Hsp60 was taken as 100%. Experimental condition: see text.
[d] Not determined.

position (bio-ETB in Fig. 1). This biotin-labeled epolactaene retained its growth-inhibitory effect (Table 1).

3.2. Competitive effects by epolactaene derivatives on bio-ETB binding to human Hsp60

Epolactaene has electrophilic structures in its α,β-unsaturated ketone, epoxide, and hemiaminal carbon, which are potentially reactive with biological nucleophiles, such as the sulfhydryls of cysteines. Indeed, as we reported recently, human Hsp60 Cys-442 was the crucial amino acid residue for binding to epolactaene.[13]

To observe the structural requirement for interactions with Hsp60, we assessed the performance of epolactaene derivatives in a binding experiment. We utilized bio-ETB as an active molecular probe and analyzed the ability of each derivative to compete bio-ETB binding to recombinant Hsp60-His_6 protein. Human Hsp60-His_6 (14 μM in phosphate buffered saline (PBS)) pretreated with 1.3 equiv of each derivative at 4 °C. After 2 h, 2 equiv of bio-ETB was added to the mixture (the final concentration of Hsp60 was 2.8 μM) and incubated at 4 °C for another 0.5 h. SDS-PAGE loading buffer was added, and the resulting mixture was analyzed by

SDS-PAGE followed by blotting using HRP-conjugated streptavidin. The loss of labeling, that is, competition, showed the degree to which each derivative blocked the binding of bio-ETB with Hsp60. As summarized in Table 1, ETB almost completely blocked the binding of bio-ETB. Epo-L and Epo-M also competed remarkably, and there were no big differences in their inhibitory activities. Epo, Epo-F, and Epo-J also competed the binding, although they were less effective than ETB. Epo-E, Epo-G, and Epo-K, however, did not block the binding. We recently revealed that epolactaene/ETB selectively bind to Cys-442 of Hsp60, although human Hsp60 has two other cysteines, Cys-237 and Cys-447.[13] To confirm that the observed competitive binding of Epo-L and Epo-M was through Cys-442 of Hsp60, we also analyzed the competitive effect using the Ala mutant of both Cys-237 and Cys-447 of Hsp60. The competitive binding experiment by Epo-L and Epo-M using the double Ala mutant of Cys-237 and Cys-447 in Hsp60-His_6 gave the same results as those with the wild-type Hsp60-His_6 protein (data not shown).

3.3. Inhibitory effects of epolactaene derivatives on human Hsp60 chaperone activity

Next, we investigated the inhibitory effects of epolactaene derivatives against human Hsp60 chaperone activity. Hsp60 chaperone activity was measured by analyzing the chaperonin-assisted refolding of pig mitochondrial malate dehydrogenase (MDH).[13] Briefly, MDH was denatured in 10 mM HCl for 2 h at room temperature and diluted to a concentration of 100 nM in a buffer (0.1 M Tris(hydroxymethyl)aminomethane–HCl, pH 7.6, 7 mM KCl, 7 mM $MgCl_2$, 10 mM dithiothreitol) containing reconstituted chaperones (2.1 μM Hsp60 treated with 1.3 equiv epolactaene derivatives for 15 h at 4 °C, and 4.2 μM Hsp10). The refolding reaction was started by the addition of 2 mM ATP at room temperature. We tested the reactivation of MDH, which is dependent on the chaperone activity of Hsp60, treated with each derivative. The reactivation (%) of MDH by BSA was subtracted as the baseline, and then reactivation derived using Hsp60 treated without derivative was taken as 100%. The data are summarized in Table 1. Epolactaene, ETB, and Epo-L significantly inhibited reactivation. Epo-F, Epo-J, and Epo-K blocked reactivation slightly or moderately, though Epo-E did not effectively interfere with the chaperone activity. Epo-M showed weak inhibition relative to the extent of its binding competition compared with Epo-L. This loss in the inhibition of Epo-M invoked us the importance of α,β-unsaturated ketone that is the only difference between Epo-L and Epo-M. In the recent paper, we have already shown that epolactaene/ETB covalently binds with Hsp60 through Cys-442 and this binding was suggested to be responsible for the effective inhibition on the chaperone activity. There are several moieties in epolactaene that is potentially reactive with cysteine, that is, α,β-unsaturated ketone, epoxide, and hemiaminal carbon. To examine the possible reaction site on epolactaene, we analyzed the binding between bio-ETB and Hsp60. When we incubated Hsp60 already bound with bio-ETB in a buffer A (8 M guanidium–HCl, 0.5 M Tris(hydroxymethyl)aminomethane–HCl, pH 8.5, 1% dithiothreitol), the biotin-labeled amount of Hsp60 reduced (Fig. 2). It demonstrated that the binding between bio-ETB and Hsp60 could be reversible in buffer A, thus Michael addition to α,β-unsaturated ketone is reasonable.

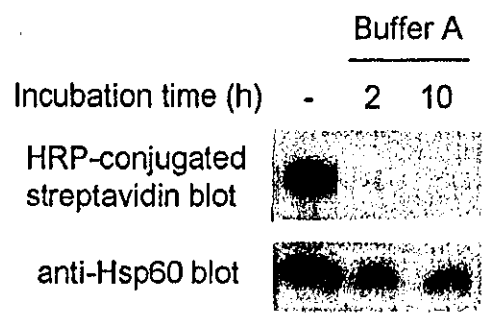


Figure 2. Reversible binding between bio-ETB and Hsp60 protein. Hsp60-His_6 protein (14 μM) in PBS was pretreated with 1.5 equiv bio-ETB. The mixture was then diluted 10-fold in buffer A (8 M guanidium–HCl, 0.5 M Tris(hydroxymethyl)aminomethane–HCl, pH 8.5, 1% dithiothreitol) and incubated for indicated time at 37 °C. The mixture was separated by SDS-PAGE and analyzed by Western blotting using HRP-conjugated streptavidin (*upper panel*) and anti-Hsp60 antibody (*lower panel*).

4. Discussion

In the present study, we described the SARs of new epolactaene derivatives. We evaluated the ability of each to inhibit the growth of human cancer cell lines, to compete the binding of biotin-conjugated epolactaene with human Hsp60, and to inhibit Hsp60's chaperone activity. The results for growth inhibition suggest that the γ-lactam moiety is important for the biological activity. Triene and ester moieties may not be always necessary, although the lack of a long side chain leads to a great loss of the growth-inhibitory effect.

The potency of epolactaene derivatives for competing bio-ETB binding to Hsp60 showed almost the same tendency observed for growth inhibition. Although the extent of competition depends on the experimental conditions, such as preincubation and labeling times, the comparison of relative ability is worthwhile. The results from the competitive binding experiment again revealed the importance of the lactam ring. The failure of Epo-E and Epo-G to compete the binding would be attributable to the absence of the lactam ring. Although Epo-F inhibits the labeling of bio-ETB to an extent, it is likely that the lactone moiety gave rise to another reactive site that does not exist in the original epolactaene. Epo-K, which has a shorter side chain, did not inhibit the binding. It is also noteworthy that Epo-M blocked the binding as effectively as Epo-L did.

The SAR study of the inhibition of Hsp60 chaperone activity is very significant. The results almost correspond to those of the growth inhibition and binding exper-

iments, except for the results with Epo-M. Epo-M inhibited growth and competed the binding as effectively as ETB and Epo-L. However, the inhibitory effect of Epo-M on Hsp60 chaperone activity was much weaker than that of Epo-L. The only difference between Epo-L and Epo-M is that the former contains α,β-unsaturated ketone. This moiety should have a fundamental role in inhibiting chaperone activity. We have already shown that epolactaene/ETB covalently binds Hsp60 through Cys-442 and suggested that this binding is responsible for the chaperone activity inhibition.[13] There are several moieties in epolactaene that is potentially reactive with cysteine, that is, α,β-unsaturated ketone, epoxide, and hemiaminal carbon. The reversibility of the binding in a buffer A (Fig. 2) demonstrates the Michael addition to the α,β-unsaturated ketone is reasonable. Because the chaperone cycle of Hsp60, which is a homologue of GroEL, probably depends on its conformational change,[23] covalent modification of Hsp60 may be important for the effective inhibition of chaperone activity. However, we cannot completely exclude the possibility that Epo-M lost inhibitory effect on chaperone activity because of flexibility in the side chain. This result, that Epo-M effectively inhibits growth but not Hsp60 chaperone activity, also suggests that epolactaene's growth inhibition effect is not always dependent solely on the inhibition of Hsp60 chaperone activity. Because the SAR results from the Hsp60 binding experiment almost correspond to the growth-inhibitory effect, binding to Hsp60 may result in the alteration of Hsp60-associated proteins to affect cell viability. It is also possible that epolactaene modifies other proteins too that more effectively inhibit growth.

In conclusion, we identified epolactaene's structural requirement for biological activities, as follows. We have proven that epolactaene's unique lactam moiety is critical to the growth inhibition of human cancer cells and to the modification of human Hsp60. The ester and triene moieties are likely not essential, although the length of the alkyl side chain is important to the effectiveness of the biological activities. The SAR studies here also revealed that α,β-unsaturated ketone is important for the effective inhibition of human Hsp60 chaperone activity. As a result, we identified potent derivatives, such as ETB, Epo-L, and the unique analogue Epo-M, which may serve as probes in further studies of epolactaene's biological activities and the biological analysis of human Hsp60 functions.

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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

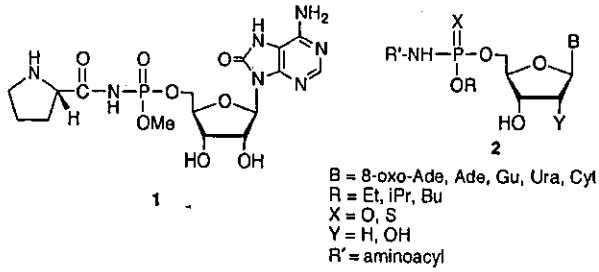

Figure 1. Phosmidosine (**1**) and its analog (**2**).

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

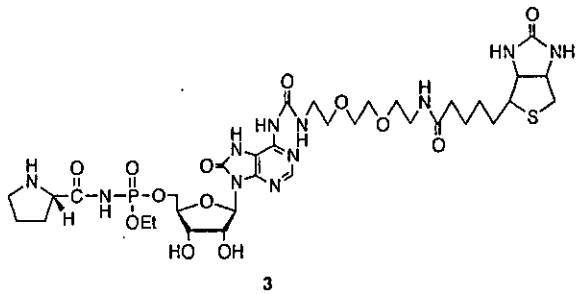
2.1. Determination of the site for introduction of a biotin residue into phosmidosine

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.



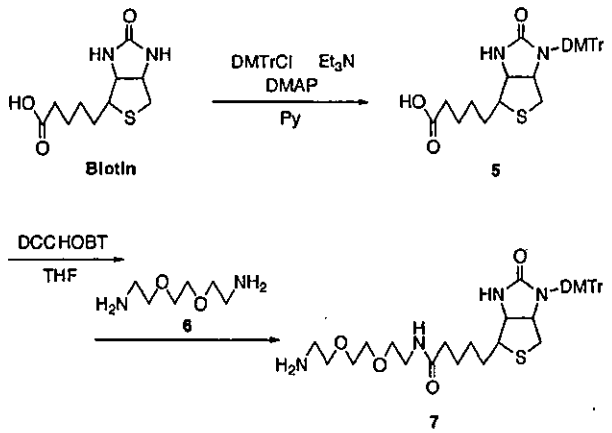
3

Figure 2. Biotin-containing phosmidosine derivative **3**.

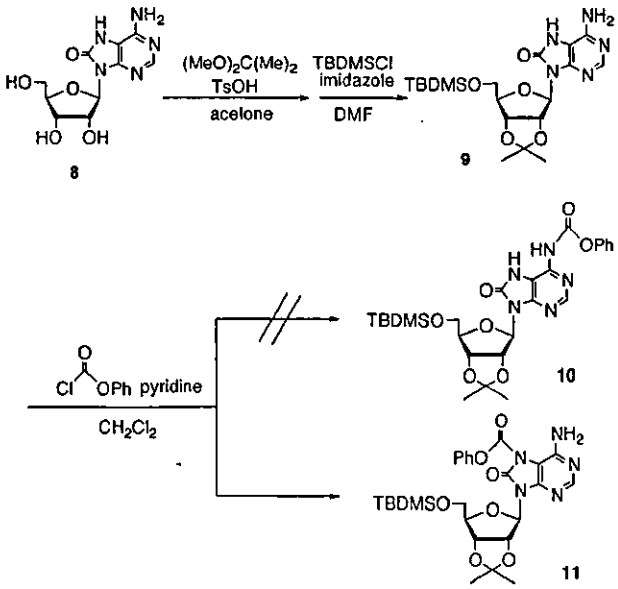
2.3. Synthesis of 7,8-dihydro-8-oxoadeonosine derivatives

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**.

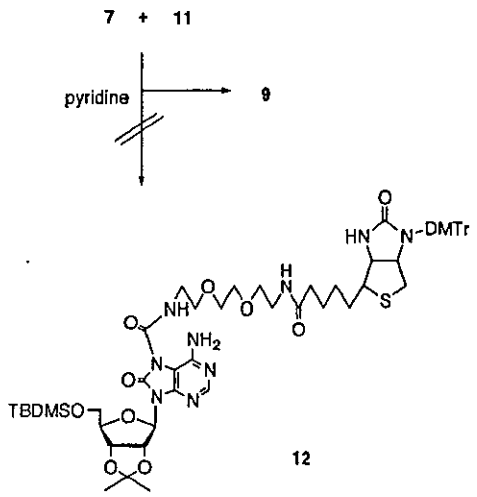


Scheme 2. Synthesis of N^7-phenoxycarbonyl-8-oxoadenosine derivative **11**.

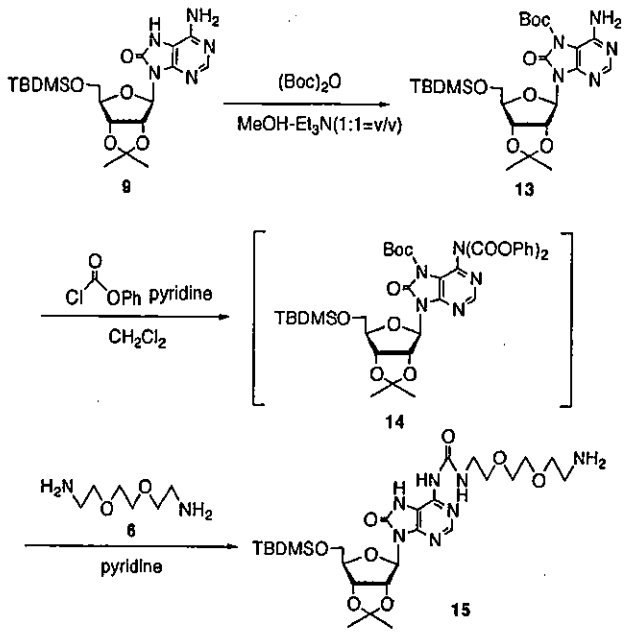
identification of this product was done by detailed analysis of ^{1}H NMR spectra. We previously encountered a similar result in a reaction of *tert*-butyl chloroformate with **9** giving rise to the N^7-Boc product in high yield. Previously, 6-*N*-acetyl-7,8-dihydro-8-oxoadenosine has been known as 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 2.2equiv 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**.

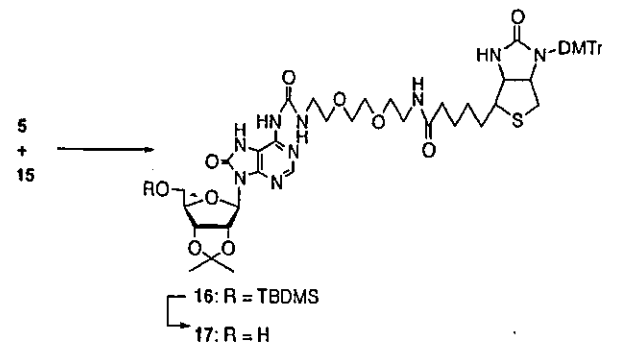


Scheme 4. Syntheis of 6-*N*-acylated 8-oxoadenosinde derivative **15**.

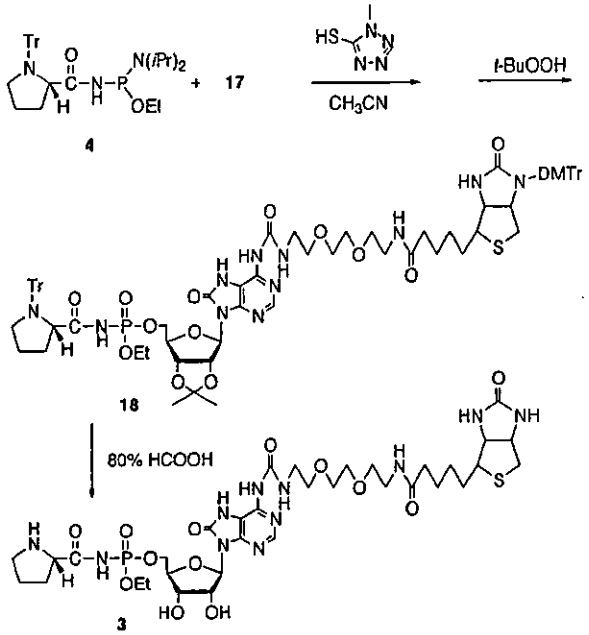
Consequently, the carbamoyl-type product **15** could be obtained in 70% yield. The elimination of the Boc group can be explained by the above same reason (Scheme 4).

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%): ^1H 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 ^1H 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%): ^1H 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%): ^1H 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

AcOEt and evaporated under reduced pressure. The residue was dissolved in 80% formic acid (1.2 mL). After being kept at room temperature for 5 h, the mixture was evaporated under reduced pressure. The residue was coevaporated three times with distilled water to remove the last traces of formic acid. The residue was chromatographed on a column of C-18 with solvent system III by using a medium pressure reverse-phase chromatography. The fractions containing **3** were collected and evaporated under reduced pressure. Rechromatography on a C-18 column with water–acetonitrile (90:10) followed by lyophilization from its aqueous solution to **3** (21.7 mg, 32%): ^{1}H NMR (270 MHz, D_2O): δ 1.10–1.53 (9H, m, CH_2 of biotin, CH_3 of POEt, $J_{POCH_2CH_3}$ = 6.9 Hz), 1.76–192 (3H, m, 3″Ha, 4″H), 2.01–2.05 (2H, m, $COCH_2$), 2.20 (1H, m, 3″H), 2.53–2.79 (2H, m, SCH_2), 2.99–3.06 (1H, m, SCH), 3.20–3.35 (6H, m, 5″H, NCH_2), 3.45–3.56 (8H, m, OCH_2-CH_2O, OCH_2), 3.73–3.83 (2H, m, NCH of biotin), 3.97–4.20 (5H, m, 5′H, 2″H, CH_2 of POEt), 4.37–4.42 (1H, m, 4′H), 4.47–4.50 (1H, m, 3′H), 4.96–5.02 (1H, m, 2′H, $J_{2',3'}$ = 5.6 Hz), 5.77 (1H, 2d, 1′H, $J_{1',2'}$ = 2.0 Hz), 8.17 (1H, s, 2H); ^{13}C NMR (D_2O): δ 17.96, 18.06, 26.43, 26.44, 27.78, 30.26, 30.52, 32.38, 38.02, 41.51, 42.07, 42.08, 42.29, 48.86, 57.86, 62.75, 64.55, 64.65, 64.74, 65.01, 65.56, 65.58, 65.64, 65.67, 67.81, 67.85, 67.88, 67.90, 67.92, 67.94, 71.40, 71.79, 72.08, 72.16, 72.29, 72.39, 73.26, 73.29, 84.42, 84.44, 84.47, 84.50, 84.55, 84.56, 84.60, 88.83, 88.86, 110.09, 110.11, 110.13, 142.81, 151.44, 152.33, 154.93, 154.99, 157.77, 167.54, 178.42, 178.48, 178.98. ^{31}P NMR (D_2O): 10.26, 10.28; ESI-mass *m/z* calcd for $C_{34}H_{55}N_{11}O_{13}PS$ 888.3439; observed [M+H] 888.3446.

4.1.11. Assay of in vitro antitumor activity. The tetrazolium-based semi-automated colorimetric assay (MTT assay) developed by Carmichael et al.[16] was modified and used to determine the in vitro antitumor activity of phosmidosine analogs. The activity was determined by using mouse leukemia L1210 cells and human epidermoid carcinoma KB cells. Roswell Park Memorial Institute Medium 1610 supplemented with 10% heat-inactivated fetal bovine serum and 50 μ/mL of kanamycine was used as the cell culture medium. Tumor cells (2×10^3 cells/well) plated into flat-bottomed 96-well plates (NUNC, Roskilde, Denmark) were incubated in a CO_2 gas incubator at 37 °C for 72 h in 200 μL of medium containing various concentrations of the test compounds. Cell growth was measured by using MTT reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma, St. Louise, Missouri, USA). After the addition of 25 μL of MTT solution (2 mg/mL), each well was incubated at 37 °C for an additional 4 h. Then the medium was removed and 200 μL of dimethyl sulfoxide (DMSO) were added. After mixing with a mechanical plate mixer for 5 min, absorbance at 540 nm was measured with Immuno Reader NJ-2000 (Nippon InterMed, Tokyo, Japan). The percentage of cell growth inhibition was calculated by the following formula: % inhibition = [1 − OD of sample wells/OD of control wells] × 100. The IC_{50} (μM) was given as the concentration at 50% inhibition of cell growth. Its value was determined graphically from the dose-response curve with at least three drug concentration points.

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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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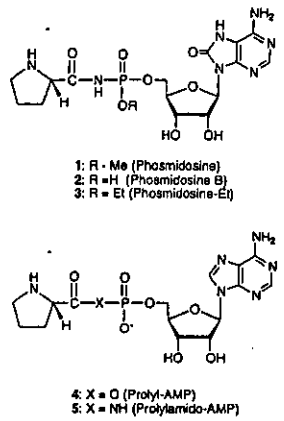


Figure 1. Various derivative of phosmidosine and prolyl-AMP.

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.

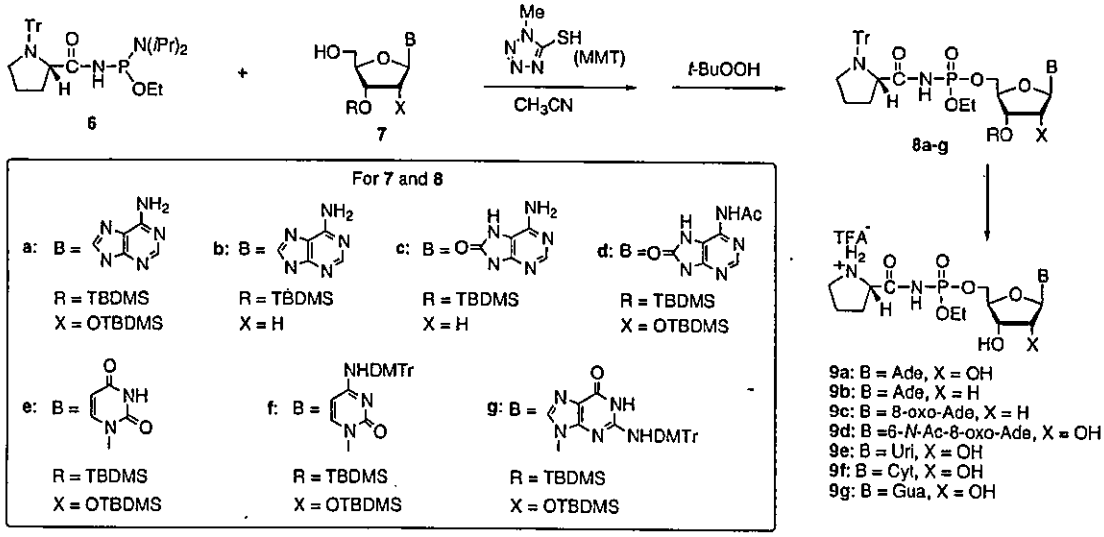
To clarify this possibility, the structure–activity relationship of phosmidosine is of great importance. Particularly, it should be clarified if the 7,8-dihydro-8-oxoadenosine component can be replaced by other elements such as the deoxy counterpart, the adenosine, and other ribonucleosides without loss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

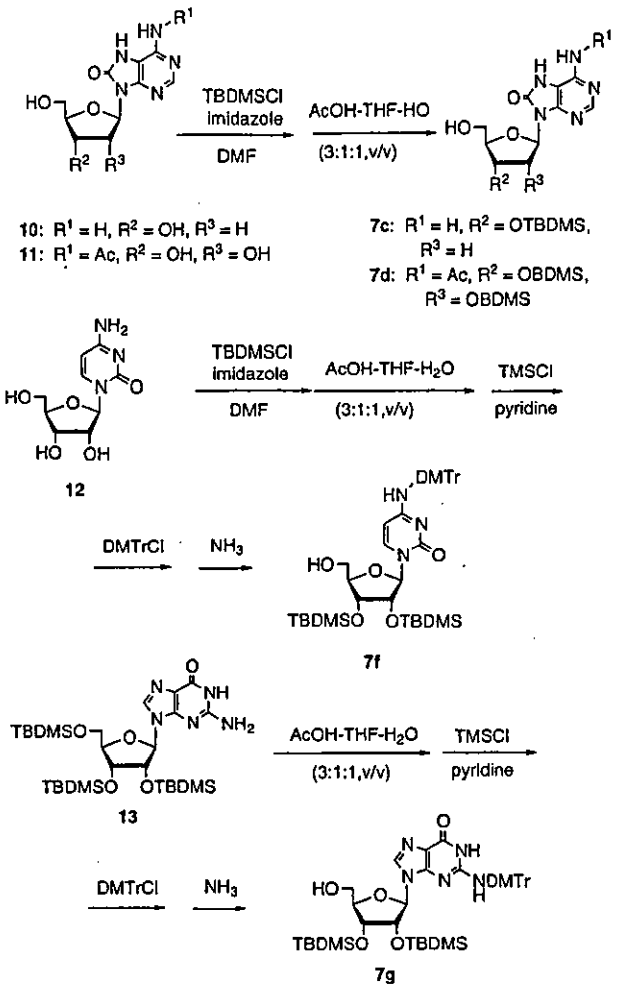
2. Results and discussion

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-oxoadenosine derivatives **9b** and **9c** were also prepared. In addition, we synthesized 6-*N*-acetylphosmidosine derivative **9d** to check if the 6-amino group of phosmidosine can be modified without loss of the activity. We also synthesized several compounds **9e–g** having other nucleobases in place of 7,8-dihydro-8-oxoadenine. The synthesis of these compounds is outlined in Scheme 1.

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



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



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

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

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

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

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

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

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

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

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

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