Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry 12 (2004) 6343–6349
Bioorganic & Medicinal Chemistry

Synthesis of a biotin-conjugate of phosmidosine *O*-ethyl ester as a G1 arrest antitumor drug

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

[a]*Department of Life Science, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8503, Japan*
[b]*CREST, JST (Japan Science and Technology Corporation), 4259 Nagatsuta, Midori-ku, Yokohama 226-8503, Japan*
[c]*Frontier Collaborative Research Center, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8503, Japan*
[d]*Cancer Research Institute, Kanazawa University, Takara-machi, Kanazawa 920-8640, Japan*
[e]*Antibiotics Laboratory, Discovery Research Institute, RIKEN, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan*

Received 7 September 2004; revised 22 September 2004; accepted 22 September 2004
Available online 12 October 2004

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

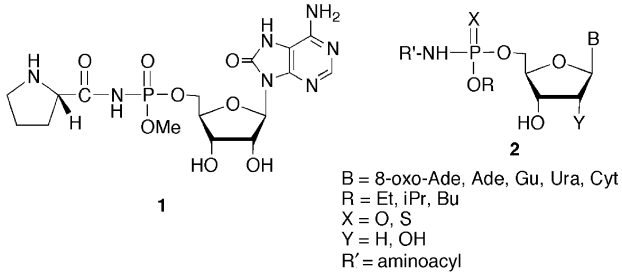


Figure 1. Phosmidosine (**1**) and its analog (**2**).

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

0968-0896/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2004.09.037

for isolation of biomolecules that interact with phosmidosine.

2. Results and discussion

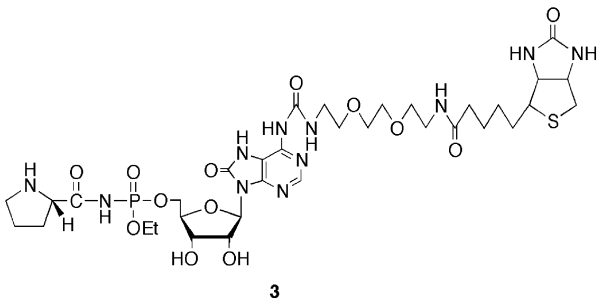
2.1. Determination of the site for introduction of a biotin residue into phosmidosine

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.



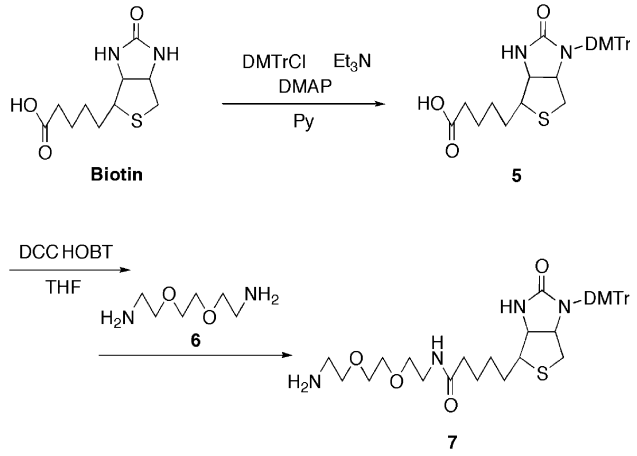
3

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

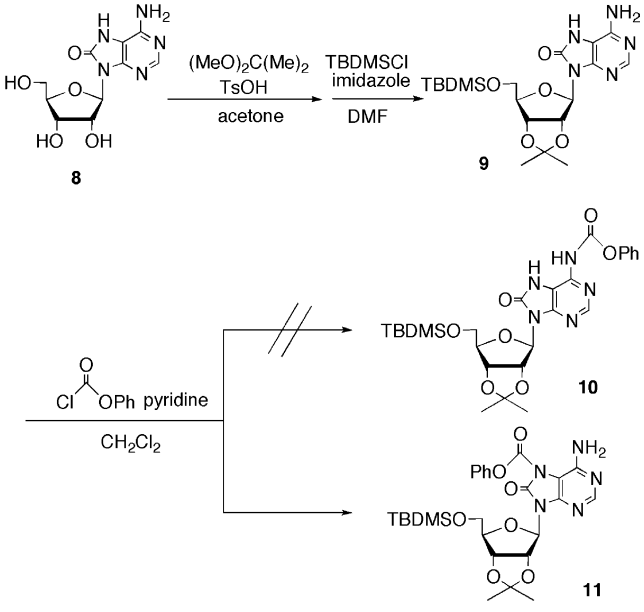
2.3. Synthesis of 7,8-dihydro-8-oxoadeonosine derivatives

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**.

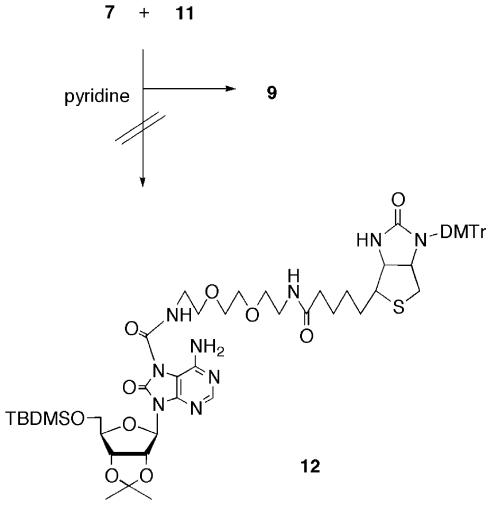


Scheme 2. Synthesis of N^7-phenoxycarbonyl-8-oxoadenosine derivative **11**.

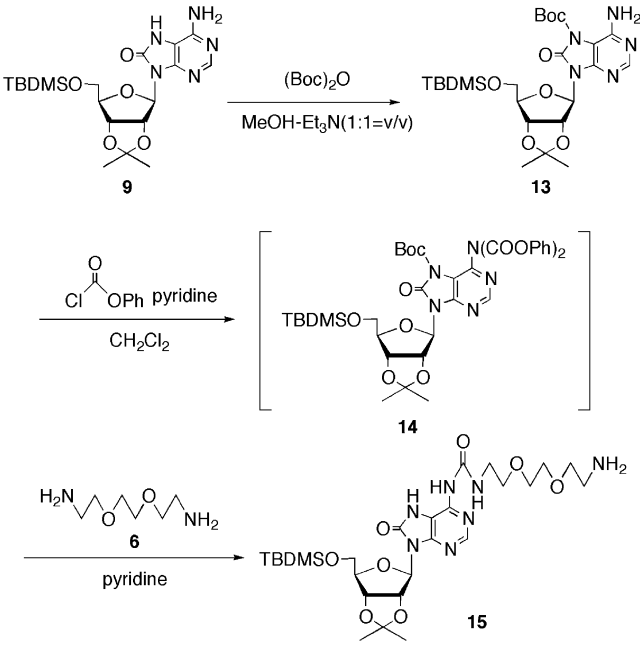
identification of this product was done by detailed analysis of ^{1}H NMR spectra. We previously encountered a similar result in a reaction of *tert*-butyl chloroformate with **9** giving rise to the N^7-Boc product in high yield. Previously, 6-*N*-acetyl-7,8-dihydro-8-oxoadenosine has been known as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

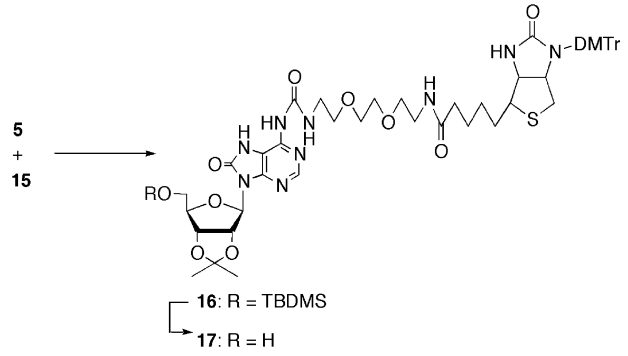


Scheme 4. Syntheis of 6-*N*-acylated 8-oxoadenosinde derivative **15**.

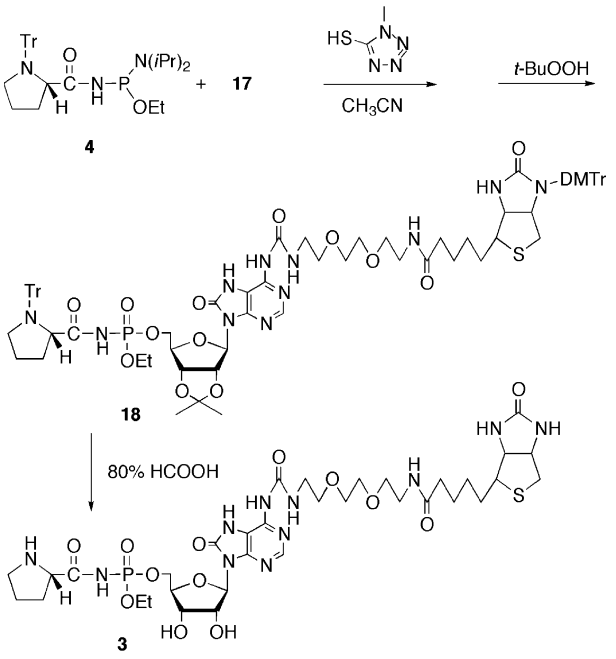
Consequently, the carbamoyl-type product **15** could be obtained in 70% yield. The elimination of the Boc group can be explained by the above same reason (Scheme 4).

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

^{1}H, ^{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 ^{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 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%): ^{1}H 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

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.

Acknowledgements

This work was supported by Grant-in-Aid for Scientific Research on Priority Areas (C) 'Genome Science' from the Ministry of Education, Culture, Sports, Science and Technology of Japan. This work was also supported by a Grant from CREST of JST (Japan Science and Technology Corporation).

References and notes

1. Moriguchi, T.; Asai, N.; Wada, T.; Seio, K.; Sasaki, T.; Sekine, M. *Tetrahedron Lett.* **2000**, *41*, 5881.
2. Moriguchi, T.; Asai, N.; Okada, K.; Seio, K.; Sasaki, T.; Sekine, M. *J. Org. Chem.* **2002**, *67*, 3290.
3. Sekine, M.; Okada, K.; Seio, K.; Kakeya, H.; Osada, H.; Obata, T.; Sasaki, T. *J. Org. Chem.* **2004**, *69*, 314.
4. Sekine, M.; Okada, K.; Seio, K.; Kakeya, H.; Osada, H.; Sasaki, T. *Bioorg. Med. Chem.* **2004**, *12*, 5193.
5. Moriguchi, T.; Yanagi, T.; Wada, T.; Sekine, M. *Tetrahedron Lett.* **1998**, *39*, 3725.
6. Moriguchi, T.; Yanagi, T.; Kunimori, M.; Wada, T.; Sekine, M. *J. Org. Chem.* **2000**, *24*, 8229.
7. Uramoto, M.; Kim, C. J.; Shin-ya, K.; Kusakabe, H.; Isono, K.; Phillips, D. R.; McCloskey, J. A. *J. Antibiot.* **1991**, *44*, 375.
8. Phillips, D. R.; Uramoto, M.; Isono, K.; McCloskey, J. A. *J. Org. Chem.* **1993**, *58*, 854.
9. Matsuura, N.; Onose, R.; Osada, H. *J. Antibiot.* **1996**, *49*, 361.
10. Kakeya, H.; Onose, R.; Liu Phillip, C.-C.; Onozawa, C.; Matsumura, F.; Osada, H. *Cancer Res.* **1998**, *58*, 704.
11. (a) Lamsa, M.; Raitamaa, K.; Pursiainen, J. J. *Phys. Org. Chem.* **1999**, *12*, 557; (b) Golisade, A.; Wiesner, J.; Herforth, C.; Jomaa, H.; Link, A. *Bioorg. Med. Chem.* **2002**, *10*, 769.
12. Kremsky, J. N.; Pluskal, M.; Casey, S.; Perry-O'Keefe, H.; Kates, S. A.; Sinha, N. D. *Tetrahedron Lett.* **1996**, *37*, 4313.
13. Filippov, D.; Timmers, C. M.; van der Marel, G. A.; van Boom, J. H. *Nucleos. Nucleot.* **1997**, *16*, 1403.
14. Filippov, D.; Timmers, C. M.; Roerdink, A. R.; van der Marel, G. A.; van Boom, J. H. *Tetrahedron Lett.* **1998**, *39*, 4891.
15. Jaeger, A.; Engels, J. *Tetrahedron Lett.* **1984**, *25*, 1437.
16. Carmichael, J.; DeGraff, W. G.; Gazar, A. F.; Minna, J. D.; Mitchell, J. B. *Cancer Res.* **1987**, *47*, 936.