Development of an Efficient Intermediate, α-[2-(Trimethylsilyl)ethoxy]-2-*N*-[2-(trimethylsilyl)ethoxycarbonyl]folic Acid, for the Synthesis of Folate (γ)-Conjugates, and Its Application to the Synthesis of Folate−Nucleoside Conjugates†

Makoto Nomura,‡,§ Satoshi Shuto,‡ and Akira Matsuda*,‡

Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812, Japan, and Hanno Research Center, Taiho Pharmaceutical Co. Ltd., 1-27 Misugidai, Hanno, Saitama 357-8527, Japan

Received February 2, 2000

Introduction

Folic acid (**1**, Figure 1) is transported into cells by a folate receptor via receptor mediated endocytosis, a process called potocytosis.[1,2] Consequently, the folate receptor is attractive as a potential drug delivery vehicle, since covalently bound folate drug conjugates are also transported into cells. Therefore, toxin,[3a,b] liposome containing DOX[3c,e] or antisense oligodeoxynucleotide (ODN),[3d] ODN,[3j,k] antibody,[3i] or [^{111}In]DTPA[3g,h] have all been conjugated with folate, and their biological effects have been studied. Antitumor agents are likely to be the most suitable for conjugating to folate, since the folate receptor is known to be overexpressed on epithelial malignancies such as ovarian, colorectal, and breast cancer, whereas in normal tissue it is expressed at very low levels.[2]

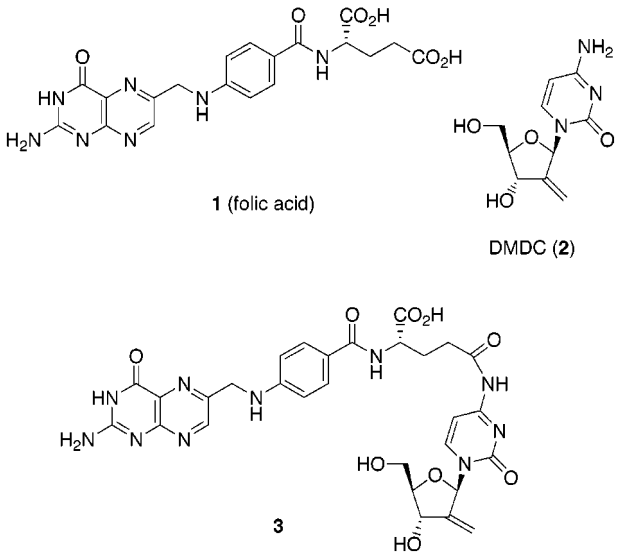
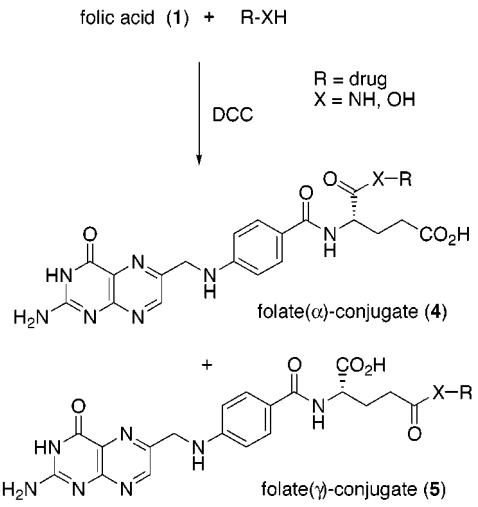


Figure 1.

Scheme 1



Although the biological importance of folic acid and its derivatives has been widely recognized, an efficient general method for preparing folate conjugates has not yet been developed. Until now, drugs have usually been conjugated to an unprotected folate using a condensing agent such as DCC to produce an often inseparable mixture of α-conjugate **4** and γ-conjugate **5** (Scheme 1).[3a−f,i−l,4] A practical method for preparing γ-conjugate **5** would therefore be extremely useful, since only γ-conjugate can be recognized by the folate receptor.[3f] Most recently, Fuchs et al. reported an effective method for preparing the γ-conjugate **5** via folate (γ)-methyl ester **6** (Figure 2), which was successfully utilized for the synthesis of DTPA−folate conjugates.[3g,h] Although their method is suitable for certain kinds of folate conjugates, further development of the method is needed for preparing the γ-conjugate since (1) the key intermediate, methyl ester **6**, is almost insoluble in organic solvents, which is

* To whom correspondence and reprint requests should be addressed. Phone: +81-11-706-3228. Fax: +81-11-706-4980. E-mail: matuda@pharm.hokudai.ac.jp.

† This paper constitutes part 196 of *Nucleosides and Nucleotides*; for part 195 in the series, see: Fukuoka, M.; Shuto, S.; Minakawa, N.; Ueno, Y, Matsuda, A. *J. Org. Chem.*, in press.

‡ Hokkaido University.

§ Taiho Pharmaceutical Co. Ltd.

(1) Rothberg, K. G.; Ying, Y.; Kolhouse, J. F.; Kamen, B. A.; Anderson, R. G. W. *J. Cell Biol.* **1990**, *110*, 637−649.

(2) (a) Campbell, I. G.; Jones, T. A.; Foulkes, W. D.; Trowsdale, J. *Cancer Res.* **1991**, *51*, 5329−5338. (b) Weitman, S. D.; Lark, R. H.; Coney, L. R.; Fort, D. W.; Frasca, V.; Zurawski, Jr., V. R.; Kamen, B. A. *Cancer Res.* **1992**, *52*, 3396−3401. (c) Ross, J. F.; Chaudhuri, P. K.; Ratnam, M. *Cancer* **1994**, *73*, 2432−2443. (d) Franklin, W. A.; Waintrub, M.; Edwards, D.; Christensen, K.; Prendegrast, P.; Woods, J.; Bunn, P. A.; Kolhouse, J. F. *Int. J. Cancer* **1994**, *Suppl. 8*, 89−95. (e) Mantovani, L. T.; Miotti, S.; Ménard, S.; Canevari, S.; Raspagliesi, F.; Bottini, C.; Bottero, F.; Colnaghi, M. I. *Eur. J. Cancer* **1994**, *30A*, 363−369.

(3) (a) Leamon, C. P.; Low, P. S. *J. Biol. Chem.* **1992**, *267*, 24966−24971. (b) Leamon, C. P.; Pastan, I.; Low, P. S. *J. Biol. Chem.* **1993**, *268*, 24847−24854. (c) Lee, R. J.; Low, P. S. *J. Biol. Chem.* **1994**, *269*, 3198−3204. (d)Wang, S.; Lee, R. J.; Cauchon, G.; Gorenstein, D. G.; Low, P. S. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 3318−3322. (e) Lee, R. J.; Low, P. S. *Biochim. Biophys. Acta* **1995**, *1233*, 134−144. (f) Wang, S.; Lee, R. J.; Mathias, C. J.; Green, M. A.; Low, P. S. *Bioconjugate Chem.* **1996**, *7*, 56−62. (g) Luo, J.; Smith, M. D.; Lantrip, D. A.; Wang, S.; Fuchs, P. L. *J. Am. Chem. Soc.* **1997**, *119*, 10004−10013. (h) Wang, S.; Luo, J.; Lantrip, D. A.; Waters, D. J.; Mathias, C. J.; Green, M. A.; Fuchs, P. L.; Low, P. S. *Bioconjugate Chem.* **1997**, *8*, 673−679. (i) Cho, B. K.; Roy, E. J.; Patrick, T. A.; Kranz, D. M. *Bioconjugate Chem.* **1997**, *8*, 338−346. (j) Li, S.; Deshmukh, H. M.; Huang, L. *Pharm. Res.* **1998**, *15*, 1540−1545. (k) Habus, I.; Xie, J.; Iyer, R. P.; Zhou, W.-Q.; Shen, L. X.; Agrawal, S. *Bioconjugate Chem.* **1998**, *9*, 283−291. (l) Gabizon, A. G.; Horowitz, A. T.; Goren, D.; Tzemach, D.; Mandelbaum-Shavit, F.; Qazen, M. M.; Zalipsky, S. *Bioconjugate Chem.* **1999**, *10*, 289−298.

(4) Nomura, Y.; Ueno, Y.; Matsuda, A. *Nucleic Acids Res.* **1997**, *25*, 2784−2791.

10.1021/jo000132a CCC: $19.00
Published on Web 07/14/2000

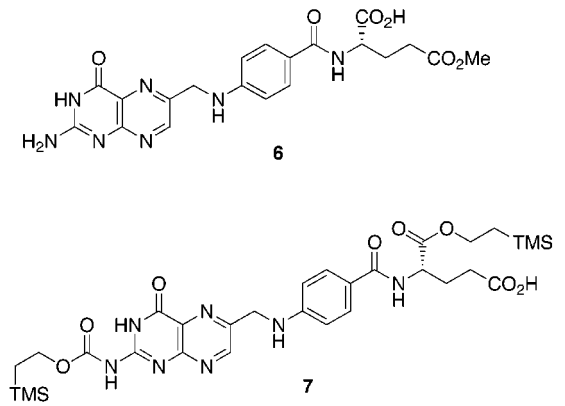


Figure 2.

often troublesome for routine organic reactions and purification, and (2) the reactivity of the (γ)-methyl ester may be insufficient for the reaction with relatively weak nucleophiles, such as aromatic amines including the 4-amino group of cytosine nucleosides. In this report, we describe a new and efficient method for preparing folate (γ)-drug conjugates using a novel protected folate unit **7** (Figure 2) and its application to the synthesis of folate (γ)-conjugate of an antitumor nucleoside **3**, 1-(2-deoxy-2-methylene-β-D-*erythro*-pentofuranosyl)cytosine (DMDC, **2**). The folate-conjugated nucleoside phosphoramidite unit **18** designed for ODN synthesis was also synthesized using this method.

Design and Synthesis of the Protected Folate Unit 7. We designed the γ-carboxylate-free folate derivative **7** as a novel unit for conjugating drugs to folate, in which the 2-amino group of the pteroic acid moiety and the α-carboxylate of the glutamic acid moiety were protected with a 2-(trimethylsilyl)ethoxycarbonyl (Teoc) group[5] and a 2-(trimethylsilyl)ethyl (TMSEt) group, respectively. We expected the two lipophilic silyl-protecting groups, which could be simultaneously deprotected with TBAF, to improve the solubility of the molecule in organic solvents. We also thought that the protection of the 2-amino group would effectively decrease the nucleophilicity of the nitrogen and thus avoid side reactions.

The protected folate unit **7** was prepared from the 2-*N*-Teoc-pteroic acid derivative and the α-carboxyl-protected glutamic acid, as shown in Scheme 2. Enzymatic hydrolysis of folic acid (**1**) with carboxypeptidase G gave pteroic acid (**8**),[3g] which, without purification, was successively treated with carbonyldiimidazole (CDI) and 2-trimethylsilylethanol in dimethyl sulfoxide (DMSO) to give 1-(2-*N*-Teoc-pteroyl)imidazole (**9**) in 50% yield from folic acid (**1**). This compound was soluble in organic solvents such as $CHCl_3$−MeOH and stable at room temperature and could be purified by normal silica gel column chromatography.

N-Boc-L-Glu (OBn)-OH (**10**) was also successively treated with CDI and 2-trimethylsilylethanol in CH_2Cl_2 to give the fully protected glutamate, which was hydrogenated with Pd−C to give in turn the γ-carboxyl-free glutamate **11** in 87% yield. The *N*-Boc group of **11** was deprotected with TsOH and afforded the crystalline α-(2-TMS-ethyl) glutamate **12** in 57% yield.

Condensation between 1-(2-*N*-Teoc-pteroyl)imidazole (**9**) and α-(2-TMSEt) glutamate (**12**) was next investigated with various bases, i.e., Et_3N, tetramethylguanidine,[3g] $^iPr_2NEt$, and *N*-methyl-1,5,9-triazabicyclo[4.4.0]-decene (MTBD).[3g] While the condensations with Et_3N or *i*-Pr_2NEt were unsuccessful, the desired product **9** was obtained in 47% yield using tetramethylguanidine as a base. When MTBD[3g] was used, the best result was observed; treatment of **9** with **12** (1.5 equiv) in the presence of MTBD (3 equiv) in DMSO at room temperature gave the protected folate unit **7** in 71% yield, which was stable enough to be purified by normal silica gel column chromatography. As expected, it was also soluble in organic solvents.

Synthesis of the Folate−Cytosine Nucleoside Conjugates. To confirm the usefulness of the folate unit **7**, the folate−cytidine and −deoxycytidine conjugates were first synthesized with the mixed-anhydride method. The protected folate **7** was therefore successively treated with $ClCO_2i$-Bu/Et_3N and cytidine or 2′-deoxycytidine in dimethylformamide (DMF) at room temperature. These reactions gave the desired condensation products **13** and **14** in 67 and 70% yield, respectively, in which the γ-carboxyl group was conjugated at the *N*-4-position of the cytosine moiety. The protected folate−cytosine nucleoside conjugates **13** and **14** were readily purified by silica gel column chromatography. The silyl protecting groups of these conjugates were simultaneously removed by treatment with TBAF in THF−DMSO. The reactions resulted in the target folate−cytosine nucleoside conjugates as the tetrabutylammonium salts, which were subsequently treated with NaOAc in a mixed solvent of MeOH/EtOH to give the sodium salts of the folate−nucleoside conjugates **15** and **16** as yellow precipitates. Using this method, we easily obtained the analytically pure conjugates **15** and **16** in excellent yields without further purification. We have thus developed an efficient method for preparing folate−cytosine nucleoside conjugates.

In our studies, DMDC **2**[6] proved to be a potent antitumor nucleoside, which significantly inhibited the growth of various human solid tumor cells both in vitro and in vivo. On the basis of these data, we designed a folate conjugate of DMDC (**3**) as a highly tumor selective antimetabolite. We expected the folate conjugate **3** to show an even more remarkable antitumor effect than the parent drug **2**, since **3** could be delivered selectively to tumor tissues due to its affinity for the folate receptor overexpressed on tumor cells. Therefore, the procedure used with folate unit **7** was successfully applied to the synthesis of the target folate−DMDC conjugate **3** via the protected intermediate **17**, as shown in Scheme 3.

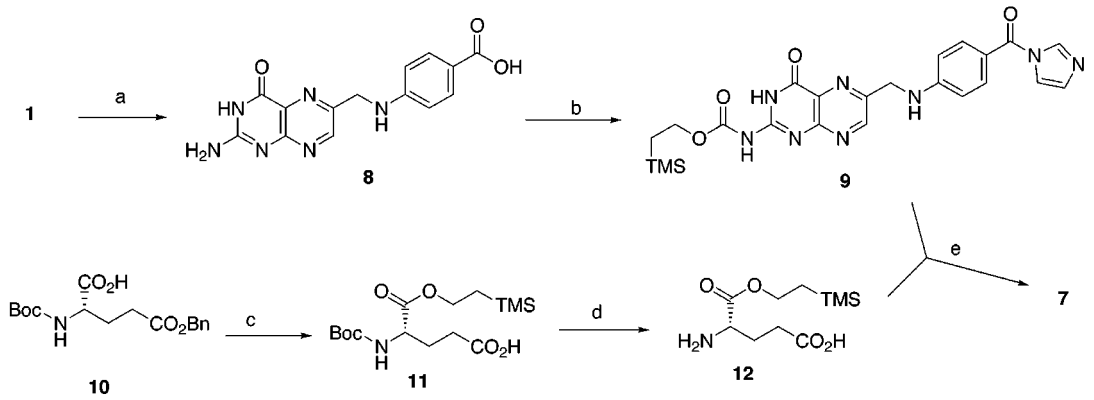
Synthesis of the Folate-Conjugated Nucleoside Phosphoramidite Unit for ODN Synthesis. Antisense ODNs have shown great efficacy in the selective inhibition of gene expression of tumor cells.[8] However, the therapeutic applications of such antisense ODN are often

(5) Carpino, L. A.; Tsao, J.-H. *J. Chem. Soc., Chem. Commun.* **1978**, 358−359.

(6) (a) Takenuki, K.; Matsuda, A.; Ueda, T.; Sasaki, T.; Fujii, A.; Yamagami, K. *J. Med. Chem.* **1988**, *31*, 1063−1064. (b) Matsuda, A.; Takenuki, K.; Tanaka, M.; Sasaki, T.; Ueda, T. *J. Med. Chem.* **1991**, *34*, 812−819. (c) Yamagami, K.; Fujii, A.; Arita, M.; Okumoto, T.; Sakata, S.; Matsuda, A.; Ueda, T.; Sasaki, T. *Cancer Res.* **1991**, *51*, 2319−2323. (d) Ono, T.; Fujii, A.; Hosoya, M.; Okumoto, T.; Sakata, S.; Matsuda, A.; Sasaki, T. *Biochem. Pharmacol.* **1996**, *52*, 1279−1285. (e) Miwa, M.; Eda, H.; Ura, M.; Ouchi, K. F.; Keith, D. D.; Foley, L. H.; Ishitsuka, H. *Clin. Cancer Res.* **1998**, *4*, 493−497. (f) Eda, H.; Ura, M.; Foley, L. H.; Ouchi, K. F.; Tanaka, Y.; Miwa, M.; Ishitsuka, H. *Cancer Res.* **1998**, *58*, 1165−1169.

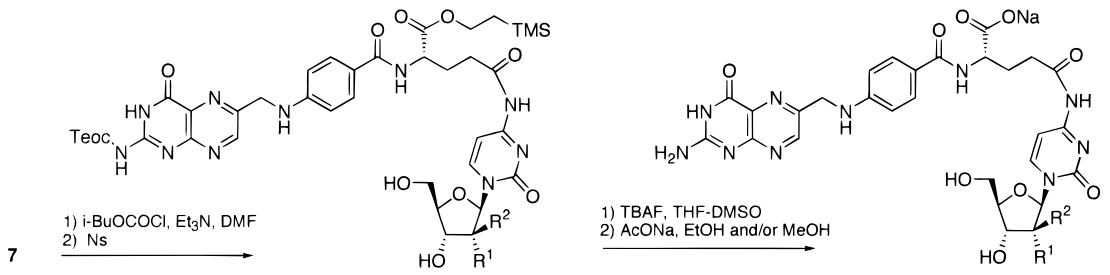
(7) Boral, A. L.; Dessain, S.; Chabner, B. A. *Cancer Chemother. Pharmacol.* **1998**, *42 (Suppl)*, S3−S21.

Scheme 2



Conditions: (a) carboxypeptidase G, TRIS-HCl buffer (pH 7); (b) 1) CDI, Et_3N, DMSO, 2) $TMSCH_2CH_2OH$; (c) 1) CDI, CH_2Cl_2, 2) $TMSCH_2CH_2OH$, 3) H_2, Pd/C, EtOH; (d) TsOH, H_2O-dioxane; (e) MTBD, DMSO.

Scheme 3



compound	R^1	R^2	yield (%)
13	-OH	-H	67
14	-H	-H	70
17	$=CH_2$		66

compound	R^1	R^2	yield (%)
15	-OH	-H	90
16	-H	-H	75
3	$=CH_2$		80

limited by their low cellular uptake.[3d,j,k,8] Conjugation of folic acid to antisense ODN at the γ-carboxyl group may enhance their intracellular delivery due to binding to the folate receptor overexpressed on tumor cells. Such folate-conjugated antisense ODNs may show significant antitumor effects; consequently, they have been extensively studied.[3j,k] A folate-conjugated nucleoside phosphoramidite unit, which is applicable to the usual DNA synthesizer, would be very efficient for these antisense studies. We therefore designed a folate-conjugated 2′-deoxynucleoside derivative **18** (Scheme 4) as an amidite unit for folate-conjugated antisense DNA synthesis and planned to investigate its synthesis using the silyl-protected folate unit **7** described above. The silyl protecting groups on the folate moiety of **18** were expected to be stable under the usual conditions for DNA synthesis by the phosphoramidite method. They would be removed postsynthetically.

The silyl-protected folate **7** was first converted to the activated ester **19** by treatment with *N*-hydroxysuccinimide and EDC in DMSO. The thymidine derivative **20**[4] bearing an aminohexyl tether at the 5-position was selected as a 2′-deoxynucleoside unit for conjugating to folate. The reaction of **19** with **20** (1.2 equiv) was performed in the presence of Et_3N in CH_2Cl_2 at room temperature to give the corresponding condensation product **21** in 83% yield, after purification by silica gel column chromatography. The phosphoramidyl group was introduced at the 3′-position of thymidine moiety by the usual procedure[8] to complete the desired folate-conjugated amidite unit **18**. The synthesis of folate-conjugated ODNs using the amidite unit **18** is now under investigation.

Conclusions

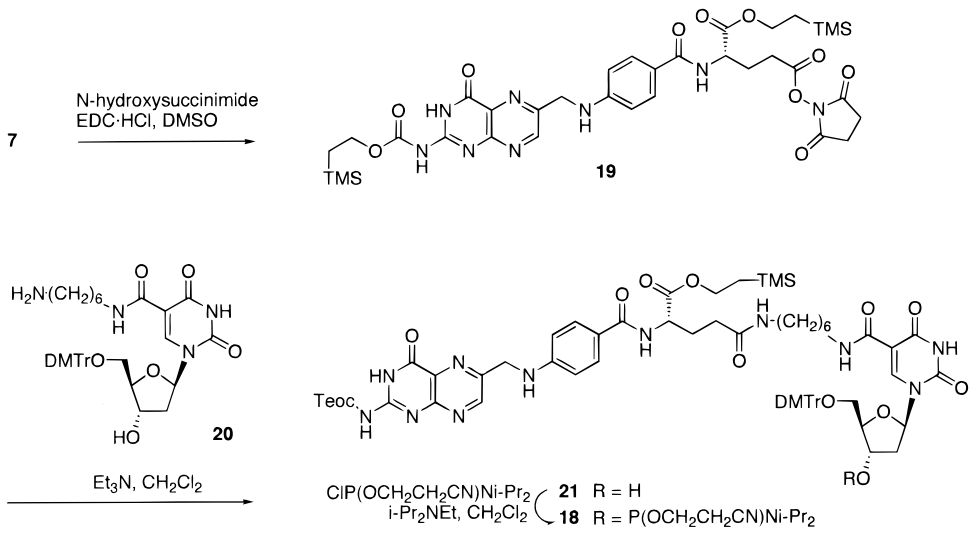
For synthesizing folate-drug conjugates, we have developed a novel protected folate unit, α-[2-(trimethylsilyl)-ethoxy]-2-*N*-[2-(trimethylsilyl)ethoxycarbonyl]folic acid (**7**), which is soluble in organic solvents and readily purified by silica gel chromatography. This unit **7** can be conjugated to various drugs having an amino or a hydroxyl group at the γ-carboxyl of folate. It was successfully applied to the synthesis of the antitumor cytosine nucleoside−folate conjugate **3** as well as to the folate-conjugated nucleoside phosphoramidite unit **18**. We have thus shown that this method can be applied to various types of folate−drug conjugate syntheses.

Experimental Section

Melting points were not corrected. ^1H and ^{13}C spectra were recorded at 400 or 270 MHz, and chemical shifts are reported in parts per million downfield from Me_4Si. All exchangeable

(8) Sinha, N. D.; Biernat, J.; Köster, H. *Tetrahedron Lett.* **1983**, *24*, 5843−5846.

Scheme 4



protons were detected by disappearance on the addition of D_2O. Mass spectra were obtained by fast atom bombardment (FAB) methods. Thin-layer chromatography was done on Merck coated plate $60F_{254}$. Silica gel or neutralized silica gel chromatography was done on Merck silica gel 5715 or ICN silica 60A, respectively. Reactions were carried out under an argon atmosphere.

1-[2-*N*-[2-(Trimethylsilyl)ethoxycarbonyl]pteroyl]-imidazole (9). A mixture of folic acid (**1**, 17.7 g, 40.0 mmol), $ZnCl_2$ (48 mg, 0.35 mmol), and carboxypeptidase G (Sigma, 7 mg, 20 units) dissolved in Tris-HCl buffer (pH 7.3, 0.1 M, 780 mL) was stirred at 30 °C for 2 weeks. The pH of the mixture was adjusted to 4.0 with 3 N HCl, and the resulting yellow precipitate was filtered and dried to give crude pteroic acid **8** (12.1 g). The suspension of the crude pteroic acid obtained (5.0 g), Et_3N (8.92 mL, 64.0 mmol), and CDI (10.4 g, 64.0 mmol) in DMSO (80 mL) was stirred at room temperature for 3.5 h. To the resulting solution was added 2-(trimethylsilyl)ethanol (18.3 mL, 128 mmol), and the whole was stirred at the same temperature for 5 h. The reaction mixture was poured into a mixture of water (550 mL), AcOH (16 mL), and Et_2O (320 mL). The resulting yellow precipitate was collected by filtration, dried, and purified on a silica gel column with 10% MeOH in $CHCl_3$ to give a yellow solid, which was washed with Et_2O to give **9** (4.13 g, 50%, as a yellow powder) in a pure form: mp > 300 °C; FAB-MS *m*/*z* 507 (MH^+); ^1H NMR (DMSO-d_6) 11.74 (br s, 2H), 8.87 (s, 1H), 8.15 (s, 1H), 7.68 (t, 1H, J = 6.1 Hz), 7.61 (d, 2H, J = 8.8 Hz), 7.60 (s, 1H), 7.09 (s, 1H), 6.77 (d, 2H, J = 8.8 Hz), 4.65 (d, 2H, J = 6.1 Hz), 4.26−4.30 (m, 2H), 1.01−1.07 (m, 2H), 0.05 (s, 9H); ^{13}C NMR (DMSO-d_6) 164.87, 159.39, 159.23, 154.90, 154.65, 153.05, 151.36, 149.18, 137.99, 132.72, 130.06, 129.67, 118.71, 117.61, 111.70, 64.62, 45.63, 17.00, −1.54. Anal. Calcd for $C_{23}H_{26}N_8O_4Si$: C, 54.53; H, 5.17; N, 22.12. Found: C, 54.43; H, 5.21; N, 21.92.

***N*-(*tert*-Butoxycarbonyl)-α-[2-(trimethylsilyl)ethyoxy]-L-glutamic Acid (11).** After a mixture of CDI (8.11 g, 50 mmol) and **10** (16.9 g, 50.0 mmol) in CH_2Cl_2 (230 mL) was stirred at room temperature for 1 h, 2-(trimethylsilyl)ethanol (7.17 mL, 50 mmol) was added, and the mixture was further stirred at room temperature for 18 h. Water (150 mL) was added, and the resulting mixture was partitioned. The organic layer was dried (Na_2SO_4), filtered, and evaporated. The residue was purified on a silica gel column with 25% EtOAc in hexane to give a colorless oil. The oil was dissolved in EtOH (230 mL) and 10% Pd/C (3.0 g) was added, and the whole was stirred at room temperature for 2 h under a hydrogen atmosphere. The mixture was filtered through a Celite pad, and the filtrate was evaporated. The residue was purified on a silica gel column with 10% MeOH in $CHCl_3$ to give **11** (15.2 g, 87%, as a colorless oil): FAB-MS *m*/*z* 348 (MH^+); ^1H NMR ($CDCl_3$) 5.17 (br d, 1H, J = 7.3 Hz), 4.31 (m, 1H), 4.20−4.25 (m, 2H), 2.38−2.53 (m, 2H), 2.16−2.20 (m, 1H); 1.88−1.97 (m, 1H), 1.44 (s, 9H), 0.98−1.05 (m, 2H), 0.05 (s, 9H); ^{13}C NMR ($CDCl_3$) 177.88, 172.15, 155.35, 80.08, 64.04, 52.85, 30.11, 28.30, 27.78, 17.41, −1.48. Anal. Calcd for $C_{15}H_{29}NO_6Si$: C, 51.84; H, 8.42; N, 4.03. Found: C, 51.50; H, 8.23; N, 3.99.

α-[2-(Trimethylsilyl)ethoxy]-L-glutamic Acid (12). A mixture of **11** (10.4 g, 30.0 mmol) and *p*-TsOH·H_2O (8.58 g, 45.0 mmol) in water (18 mL) and dioxane (54 mL) was stirred at 60 °C for 3 h. The reaction mixture was neutralized by Dowex 1 × 8 (HCO_3^- form, 18 g), which was filtered off, and the filtrate was evaporated and purified on a silica gel column with 20−33% MeOH in $CHCl_3$. Crystallization from MeOH afforded **12** in a pure form (4.20 g, 57%, as colorless needles): mp 122−123 °C; FAB-MS *m*/*z* 248 (MH^+); ^1H NMR (DMSO-d_6) 4.09−4.14 (m, 2H), 3.31 (dd, 1H, J = 4.9, 8.3 Hz), 2.22−2.31 (m, 2H), 1.77−1.85 (m, 1H), 1.55−1.64 (m, 1H), 0.92−0.96 (m, 2H), 0.02 (s, 9H); ^{13}C NMR (DMSO-d_6) 174.61, 174.06, 62.26, 53.15, 30.84, 29.13, 16.90, −1.43. Anal. Calcd for $C_{10}H_{21}NO_4Si\cdot{}^1/_4H_2O$: C, 47.40; H, 8.55; N, 5.53. Found: C, 47.57; H, 8.15; N, 5.29.

α-[2-(Trimethylsilyl)ethoxy]-2-*N*-[2-(trimethylsilyl)-ethoxycarbonyl]folic Acid (7). A mixture of **9** (1.52 g, 3.0 mmol), **12** (1.11 g, 4.5 mmol), and MTBD (1.29 mL, 9.0 mmol) in DMSO (15 mL) was stirred at room temperature for 21 h. The resulting mixture was poured into a mixture of aqueous AcOH (1 M, 600 mL), MeOH (250 mL), and $CHCl_3$ (600 mL), and the whole was partitioned. The organic layer was washed with aqueous AcOH (1 M)−MeOH (1:1, 400 mL) and then twice with H_2O−MeOH (2:1, 600 mL). The organic layer was dried (Na_2SO_4), filtered, and evaporated. The residue was purified on a silica gel column with $CHCl_3$−MeOH−AcOEt−AcOH (17:1:2:0.08) and then $CHCl_3$−MeOH−AcOH (9:1:0.025) to give a yellow solid, which was washed with $CHCl_3$−Et_2O to give **7** (1.47 g, 71% as a yellow powder): mp 137−138 °C; FAB-HRMS calcd for $C_{30}H_{44}N_7O_8Si_2$ *m*/*z* 686.2790, found *m*/*z* 686.2790 (MH^+); ^1H NMR (DMSO-d_6) 11.84 (br s, 2H), 8.81 (s, 1H), 8.29 (br d, 1H, J = 6.4 Hz), 7.63 (d, 2H, J = 8.8 Hz), 7.02 (t, 1H, J = 6.1 Hz), 6.64 (d, 2H, J = 8.8 Hz), 4.57 (d, 2H, J = 6.1 Hz), 4.31−4.35 (m, 1H), 4.24−4.29 (m, 2H), 4.08−4.12 (m, 2H), 2.30 (t, 2H, J = 7.2 Hz), 1.87−2.04 (m, 2H), 1.00−1.05 (m, 2H), 0.89−0.94 (m, 2H), 0.05 (s, 9H), 0.00 (s, 9H); ^{13}C NMR (DMSO-d_6) 173.65, 172.12, 166.23, 159.31, 154.80, 154.40, 151.92, 150.54, 149.05, 148.94, 129.85, 128.93, 121.15, 111.13, 64.59, 62.43, 51.96, 45.96, 30.24, 25.83, 17.06, 16.84, −1.43, −1.45. Anal. Calcd for $C_{30}H_{43}N_7O_8Si_2\cdot{}^1/_2H_2O$: C, 51.85; H, 6.38; N, 14.11. Found: C, 51.60; H, 6.26; N, 14.04.

Gereral Procedure for the Coupling 7 with Cytosine Nucleosides. After a mixture of Et_3N (46.0 μL, 0.33 mmol), $ClCO_2i$-Bu (42.8 μL, 0.33 mmol), and **7** (206 mg, 0.30 mmol) in DMF (2.0 mL) was stirred at room temperature for 12 min, a nucleoside (0.60 mmol) was added to the mixture, which was further stirred at room temperature for 2 h. The reaction mixture was poured into a mixture of aqueous AcOH (0.5 M, 60 mL),

MeOH (60 mL), and $CHCl_3$ (80 mL), and then the whole was partitioned. The organic layer was washed twice with a mixture of water (60 mL) and MeOH (50 mL), dried (Na_2SO_4), filtered, and evaporated. The residue was purified on a silica gel column with $CHCl_3$−MeOH−AcOEt−AcOH (9:1:1:0.04 to 6:1:1:0.03) to afford a yellow foam. The foam was dissolved in DMSO (5 mL) and poured into an aqueous AcOH (0.1 M, 150 mL), and the resulting yellow powder was collected by filtration to give the corresponding coupling product.

1-(β-D-Ribofuranosyl)-4-*N*-[γ-[α-[2-(trimethylsilyl)ethoxy]-2-*N*-[2-(trimethylsilyl)ethoxycarbonyl]folyl]]cytosine (13). Cytidine (146 mg, 0.6 mmol) was used as a nucleoside to give **13** (182 mg, 67% as a yellow powder): mp 214−217 °C (decomp.); FAB−HRMS calcd for $C_{39}H_{55}N_{10}O_{12}Si_2$ *m/z* 911.3540, found *m/z* 911.3536 (MH^+); ^{1}H NMR (DMSO-d_6) 11.75, 11.66 (each br s, each 1H), 10.84 (s, 1H), 8.83 (s, 1H), 8.40 (d, 1H, *J* = 7.6 Hz), 8.21 (d, 1H, *J* = 7.6 Hz), 7.64 (d, 2H, *J* = 8.8 Hz), 7.17 (d, 1H, *J* = 7.6 Hz), 7.01 (t, 1H, *J* = 6.1 Hz), 6.65 (d, 2H, *J* = 8.8 Hz), 5.76 (d, 1H, *J* = 2.7 Hz), 5.44 (d, 1H, *J* = 4.9 Hz), 5.13 (t, 1H, *J* = 4.9 Hz), 5.02 (d, 1H, *J* = 5.6 Hz), 4.58 (d, 2H, *J* = 6.1 Hz), 4.30−4.34 (m, 3H), 4.27−4.29 (m, 2H), 3.93−3.98 (m, 2H), 3.87−3.90 (m, 1H), 3.74 (ddd, 1H, *J* = 2.9, 4.9, 12.2 Hz), 3.59 (ddd, 1H, *J* = 3.2, 4.9, 12.2 Hz), 2.52 (t, 2H, *J* = 7.6 Hz), 2.04−2.14 (m, 1H), 1.90−1.98 (m, 1H), 1.02−1.06 (m, 2H), 0.90−0.94 (m, 2H), 0.05 (s, 9H), −0.01 (s, 9H); ^{13}C NMR (DMSO-d_6) 172.91, 172.06, 166.24, 162.05, 159.18, 154.58, 154.48, 151.92, 150.53, 149.09, 148.79, 145.23, 129.85, 128.96, 121.20, 111.12, 95.14, 90.09, 84.12, 74.45, 68.62, 64.60, 62.46, 59.89, 51.92, 45.96, 40.41, 32.94, 25.52, 17.05, 16.81, −1.47. Anal. Calcd for $C_{39}H_{54}N_{10}O_{12}Si_2 \cdot {}^1/_2H_2O$: C, 50.91; H, 6.02; N, 15.22. Found: C, 50.69; H, 5.91; N, 14.83.

1-(2-Deoxy-β-D-ribofuranosyl)-4-*N*-[γ-[α-[2-(trimethylsilyl)ethoxy]-2-*N*-[2-(trimethylsilyl)ethoxycarbonyl]folyl]]-cytosine (14). 2′-Deoxycytidine (136 mg, 0.6 mmol) was used as a nucleoside to give **14** (187 mg, 70% as a yellow powder): mp 217−219 °C (dec); FAB-HRMS calcd for $C_{39}H_{55}N_{10}O_{11}Si_2$ *m/z* 895.3591, found *m/z* 895.3581 (MH^+); ^{1}H NMR (DMSO-d_6) 11.75, 11.66 (each br s, each 1H), 10.83 (s, 1H), 8.83 (s, 1H), 8.31 (d, 1H, *J* = 7.6 Hz), 8.21 (d, 1H, *J* = 7.6 Hz), 7.64 (d, 2H, *J* = 8.8 Hz), 7.19 (d, 1H, *J* = 7.6 Hz), 7.02 (t, 1H, *J* = 5.9 Hz), 6.64 (d, 2H, *J* = 8.8 Hz), 6.08 (t, 1H, *J* = 6.3 Hz), 5.24 (d, 1H, *J* = 4.2 Hz), 5.03 (t, 1H, *J* = 5.1 Hz), 4.58 (d, 2H, *J* = 5.9 Hz), 4.27−4.34 (m, 3H), 4.18−4.22 (m, 1H), 4.07−4.11 (m, 2H), 3.85 (q, 1H, *J* = 3.7 Hz), 3.60 (ddd, 1H, *J* = 3.7, 5.1, 12.0 Hz), 3.56 (ddd, 1H, *J* = 3.7, 5.1, 12.0 Hz), 2.52 (t, 2H, *J* = 7.3 Hz), 2.23−2.30 (m, 1H), 2.06−2.14 (m, 1H), 1.89−2.03 (m, 2H), 1.02−1.06 (m, 2H), 0.90−0.94 (m, 2H), 0.05 (s, 9H), −0.01 (s, 9H); ^{13}C NMR (DMSO-d_6) 172.91, 172.11, 166.26, 162.02, 159.20, 154.67, 154.59, 154.28, 151.95, 150.55, 149.12, 148.83, 144.86, 129.89, 128.99, 121.19, 111.14, 95.20, 87.83, 86.08, 69.89, 64.64, 62.49, 60.91, 51.94, 45.97, 40.89, 32.94, 25.53, 17.07, 16.82, −1.44. Anal. Calcd for $C_{39}H_{54}N_{10}O_{11}Si_2 \cdot {}^1/_2H_2O$: C, 51.81; H, 6.13; N, 15.49. Found: C, 51.56; H, 6.11; N, 15.18.

1-(2-Deoxy-2-methylene-β-D-*erythro*-pentofuranosyl)-4-*N*-[γ-[α-[2-(trimethylsilyl)ethoxy]-2-*N*-[2-(trimethylsilyl)-ethoxycarbonyl]folyl]]cytosine (17). DMDC (**2**) (144 mg, 0.6 mmol) was used as a nucleoside to give **17** (180 mg, 66% as a yellow powder): mp 219−222 °C (dec); FAB-HRMS calcd for $C_{40}H_{55}N_{10}O_{11}Si_2$ *m/z* 907.3590, found *m/z* 907.3577 (MH^+); ^{1}H NMR (DMSO-d_6) 11.75, 11.66 (each br s, each 1H), 10.88 (s, 1H), 8.83 (s, 1H), 8.20 (d, 1H, *J* = 7.9 Hz), 8.09 (d, 1H, *J* = 7.6 Hz), 7.64 (d, 2H, *J* = 8.6 Hz), 7.18 (d, 1H, *J* = 7.6 Hz), 7.02 (t, 1H, *J* = 5.9 Hz), 6.64 (d, 2H, *J* = 8.6 Hz), 6.53 (s, 1H), 5.66 (d, 1H, *J* = 5.9 Hz), 5.33, 5.30 (each s, each 1H), 5.01 (t, 1H, *J* = 4.9 Hz), 4.58 (d, 2H, *J* = 5.9 Hz), 4.47−4.53 (br, 1H), 4.26−4.32 (m, 3H), 4.06−4.12 (m, 2H), 3.54−3.76 (m, 3H), 2.52 (t, 2H, *J* = 7.1 Hz), 1.86−2.13 (m, 2H), 1.01−1.07 (m, 2H), 0.89−0.95 (m, 2H), 0.05 (s, 9H), −0.01 (s, 9H); ^{13}C NMR (DMSO-d_6) 172.98, 172.10, 166.26, 162.22, 159.21, 154.60, 151.95, 150.55, 150.22, 149.12, 148.81, 145.94, 129.90, 128.98, 121.19, 111.55, 111.14, 95.85, 85.00, 84.87, 69.28, 64.64, 62.49, 60.21, 51.88, 45.96, 32.93, 25.48, 17.07, 16.81, −1.43. Anal. Calcd for $C_{40}H_{54}N_{10}O_{11}Si_2 \cdot {}^1/_2H_2O$: C, 52.44; H, 6.05; N, 15.29. Found: C, 52.04; H, 6.04; N, 14.92.

4-*N*-(γ-Folyl)cytosine Nucleoside Sodium Salt (General Procedure). A mixture of a protected folate−cytosine nucleoside conjugate (**13**, **14**, **17**) and TBAF (1 M in THF, 10 equiv) in DMSO (1 mL/0.1 mmol of the starting material) was stirred at room temperature for 10 h. After an additon of AcOH (1.25 mL/0.1 mmol of the starting material), the mixture was poured into a mixture of $CHCl_3$ and AcOEt (4:1, 25 mL/0.1 mmol of the starting material), and the precipitated yellow powder (a tetrabutylammonium salt) was collected by filtration. To a solution of the yellow powder obtained in EtOH−MeOH (1:1, 7 mL/0.1 mmol starting material) was added a solution of NaOAc in MeOH (13 mg/mL, 1.6 mL/0.1 mmol starting material), and the resulting mixture was centrifuged and decanted. MeOH and/or EtOH was added to the yellow precipitate, centrifuged, and decanted (×3) to afford the corresponding 4-*N*-(γ-folyl)cytosine nucleoside sodium salt (**15**, **16**, and **3**).

4-*N*-(γ-Folyl)-1-(β-D-ribofuranosyl)cytosine Sodium Salt (15). From **13** (128 mg, 0.14 mmol), the sodium salt **15** (98 mg, 90%, as a yellow powder) was obtained: mp 240−245 °C (dec); FAB-HRMS calcd for $C_{28}H_{30}N_{10}NaO_{10}$ *m/z* 689.2044, found *m/z* 689.2027 (MH^+); ^{1}H NMR (DMSO-d_6) 11.20 (s, 1H), 8.60 (s, 1H), 8.38 (d, 1H, *J* = 7.6 Hz), 7.57 (d, 1H, *J* = 6.1 Hz), 7.52 (d, 2H, *J* = 8.6 Hz), 7.28 (br s, 2H), 7.18 (d, 1H, *J* = 7.6 Hz), 6.90 (t, 1H, *J* = 5.9 Hz), 6.62 (d, 2H, *J* = 8.6 Hz), 5.75 (d, 1H, *J* = 2.0 Hz), 5.54 (br s, 1H), 5.18 (br s, 1H), 5.10 (br s, 1H), 4.45 (d, 2H, *J* = 5.9 Hz), 3.91−4.01 (m, 3H), 3.85−3.88 (m, 1H), 3.72 (br d, 1H, *J* = 12.2 Hz), 3.58 (br d, 1H, *J* = 12.2 Hz), 2.39 (t, 2H, *J* = 7.4 Hz), 1.86−2.04 (m, 2H);^{13}C NMR (DMSO-d_6) 174.44, 173.88, 165.12, 162.16, 154.97, 154.60, 150.40, 148.25, 147.93, 144.99, 128.31, 127.79, 122.09, 111.24, 95.29, 90.09, 84.13, 74.49, 68.57, 59.88, 53.20, 45.97, 33.49, 28.04. Anal. Calcd for $C_{28}H_{29}N_{10}NaO_{10} \cdot EtOH \cdot H_2O$: C, 47.87; H, 4.95; N, 18.61. Found: C, 47.76; H, 4.89; N, 18.23.

1-(2-Deoxy-β-D-ribofuranosyl)-4-*N*-(γ-folyl)cytosine Sodium Salt (16). From **14** (125 mg, 0.14 mmol), the sodium salt **16** (80 mg, 75%, as a yellow powder) was obtained: mp 230−235 °C (dec); FAB-HRMS calcd for $C_{28}H_{30}N_{10}NaO_9$ *m/z* 673.2095, found *m/z* 673.2081 (MH^+); ^{1}H NMR (DMSO-d_6) 11.21 (br s, 1H), 8.61 (s, 1H), 8.27 (d, 1H, *J* = 7.6 Hz), 7.58 (br s, 1H), 7.51 (d, 2H, *J* = 8.3 Hz), 7.22 (br s, 2H), 7.20 (d, 1H, *J* = 7.6 Hz), 6.90 (t, 1H, *J* = 5.6 Hz), 6.62 (d, 2H, *J* = 8.3 Hz), 6.08 (t, 1H, *J* = 6.3 Hz), 5.26 (d, 1H, *J* = 3.9 Hz), 5.06 (t, 1H, *J* = 5.1 Hz), 4.45 (d, 2H, *J* = 5.6 Hz), 4.17−4.21 (m, 1H), 3.94−3.99 (m, 1H), 3.81−3.84 (m, 1H), 3.51−3.62 (m, 2H), 2.39 (t, 2H, *J* = 7.6 Hz), 2.22−2.28 (m, 1H), 1.85−2.03 (m, 3H); ^{13}C NMR (DMSO-d_6) 174.29, 173.78, 165.02, 162.03, 161.89, 156.04, 154.89, 154.28, 150.33, 148.17, 147.83, 144.50, 128.22, 127.73, 122.06, 111.18, 95.29, 87.76, 85.95, 69.78, 60.86, 53.20, 45.95, 40.84, 33.49, 28.06. Anal. Calcd for $C_{28}H_{29}N_{10}NaO_9 \cdot EtOH \cdot H_2O$: C, 48.91; H, 5.06; N, 19.01. Found: C, 48.89; H, 5.05; N, 18.71.

1-(2-Deoxy-2-methylene-β-D-*erythro*-pentofuranosyl)-4-*N*-(γ-folyl)cytosine Sodium Salt (3). From **17** (91 mg, 0.10 mmol), the sodium salt **3** (60 mg, 80%, as a yellow powder) was obtained: mp 231−235 °C (dec); FAB-MS *m/z* 685 (MH^+); FAB-HRMS calcd for $C_{29}H_{30}N_{10}NaO_9$ *m/z* 685.2095, found *m/z* 685.2078; ^{1}H NMR (DMSO-d_6) 11.24 (br s, 1H, DMDC 4-NH), 8.61 (s, 1H), 8.04 (d, 1H, *J* = 7.6 Hz), 7.57 (br s, 1H), 7.52 (d, 2H, *J* = 8.0 Hz), 7.29 (br, 2H), 7.19 (d, 1H, *J* = 7.6 Hz), 6.90 (t, 1H, *J* = 5.6 Hz), 6.63 (d, 2H, *J* = 8.0 Hz), 6.53 (s, 1H), 5.72 (d, 1H, *J* = 5.6 Hz), 5.33, 5.30 (each s, each 1H), 5.07 (t, 1H, *J* = 5.1 Hz), 4.48−4.53 (m, 1H), 4.45 (d, 2H, *J* = 5.6 Hz), 3.96−4.00 (m, 1H), 3.58−3.74 (m, 3H), 2.39 (t, 2H, *J* = 7.5 Hz), 1.85−2.04 (m, 2H); ^{13}C NMR (DMSO-d_6) 174.21, 173.93, 165.10, 162.32, 161.86, 154.85, 154.70, 150.40, 150.22, 148.25, 147.96, 145.65, 128.28, 127.79, 122.10, 111.56, 111.24, 96.03, 84.98, 84.79, 69.28, 60.22, 53.17, 45.98, 33.50, 28.02; Anal. Calcd for $C_{29}H_{29}N_{10}NaO_9 \cdot EtOH \cdot H_2O$: C, 49.73; H, 4.98; N, 18.71. Found: C, 50.09; H, 4.98; N, 18.74.

α-[2-(Trimethylsilyl)ethoxy]-2-*N*-[2-(trimethylsilyl)-ethoxycarbonyl]folic Acid γ-(*N*-Hydroxysuccinimide) Ester (19). A mixture of **7** (617 mg, 0.90 mmol), *N*-hydroxysuccinimide (125 mg, 1.1 mmol), and EDC·HCl (173 mg, 0.90 mmol) in DMF (4.5 mL) was stirred at room temperature for 18 h. The reaction mixture was poured into water (300 mL), and the resulting yellow precipitate was collected by filtration to give **19** (647 mg, 87%, as a yellow powder): FAB-MS *m/z* 783 (MH^+); ^{1}H NMR (DMSO-d_6) 11.69 (br s, 2H), 8.83 (s, 1H), 8.31 (d, 1H, *J* = 7.1 Hz), 7.64 (d, 2H, *J* = 8.8 Hz), 7.05 (t, 1H, *J* = 6.1 Hz), 6.65 (d, 2H, *J* = 8.8 Hz), 4.58 (d, 2H, *J* = 6.1 Hz), 4.36−4.42 (m, 1H), 4.26−4.31 (m, 2H), 4.10−4.14 (m, 2H), 2.72−2.86 (m, 6H), 2.02−2.13 (m, 2H), 1.01−1.06 (m, 2H), 0.90−0.94 (m, 2H), 0.05

(s, 9H), −0.01 (s, 9H). Anal. Calcd for $C_{34}H_{46}N_8O_{10}Si_2 \cdot 3H_2O$: C, 57.70; H, 6.58; N, 11.05. Found: C, 57.99; H, 6.20; N, 10.84.

5-[6-[γ-[α-[2-(Trimethylsilyl)ethoxy]-2-*N*-[2-(trimethylsilyl)-ethoxycarbonyl]folyl]amino]hexylcarbamoyl]-1-(2-deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-ribofuranosyl)-uracil (21). A mixture of **19** (392 mg, 0.47 mmol), **20**[5] (387 mg, 0.575 mmol), and Et_3N (0.104 mL, 0.75 mmol) in CH_2Cl_2 (10 mL) was stirred at room temperature for 3.5 h. The reaction mixture was diluted with $CHCl_3$ (25 mL) and washed with water (25 mL × 2). The organic layer was dried (Na_2SO_4), evaporated, and purified on a neutralized silica gel column with $CHCl_3$−AcOEt−MeOH (25:3:1 to 17:2:1) and then 10% MeOH in $CHCl_3$ to give **21** (528 mg, 83%, as a yellow foam): FAB-MS *m*/*z* 1340 (MH^+); ^{1}H NMR (DMSO-d_6) 11.92, 11.74, 11.67 (each br s, each 1H), 8.82 (s, 1H), 8.65 (t, 1H, *J* = 5.4 Hz), 8.41 (s, 1H), 8.28 (d, 1H, *J* = 7.1 Hz), 7.77 (t, 1H, *J* = 5.1 Hz), 7.63 (d, 2H, *J* = 8.8 Hz), 7.15−7.25 (m, 9H), 7.01 (t, 1H, *J* = 6.3 Hz), 6.86 (d, 4H, *J* = 9.0 Hz), 6.64 (d, 2H, *J* = 8.8 Hz), 6.05 (t, 1H, *J* = 6.5 Hz), 5.32 (d, 1H, *J* = 4.4 Hz), 4.57 (d, 2H, *J* = 6.3 Hz), 4.23−4.30 (m, 3H), 4.06−4.10 (m, 3H), 3.91 (m, 1H), 3.71 (s, 6H), 3.15−3.23 (m, 4H), 2.95−3.00 (m, 2H), 2.10−2.29 (m, 4H), 1.97−2.05 (m, 1H), 1.83−1.93 (m, 1H), 1.38−1.45 (m, 2H), 1.29−1.35 (m, 2H), 1.15−1.28 (m, 4H), 1.01−1.06 (m, 2H), 0.89−0.93 (m, 2H), 0.04 (s, 9H), −0.02 (s, 9H). Anal. Calcd for $C_{67}H_{85}N_{11}O_{15}Si_2 \cdot {}^1/_2H_2O$: C, 51.47; H, 5.98; N, 14.08. Found: C, 51.56; H, 5.98; N, 14.15.

5-[6-[γ-[2-*N*-[α-[2-(Trimethylsilyl)ethoxy]-2-(trimethylsilyl)ethoxycarbonyl]folyl]amino]hexylcarbamoyl]-1-[3-*O*-[(2-cyanoethyl)(*N*,*N*-diisopropylamino)phosphinyl]-2-deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-ribofuranosyl]uracil (18). To a solution of **21** (380 mg, 0.282 mmol) in CH_2Cl_2 (4.2 mL) were added Et*N*i-Pr_2 (246 μL, 1.41 mmol) and 2-(cyanoethyl)-*N*,*N*-diisopropylchlorophosphoramidite (94.4 μL, 0.423 mmol), and the resulting mixture was stirred at room temperature for 5.5 h. The reaction mixture was poured into a mixture of $CHCl_3$ (100 mL) and saturated aqueous $NaHCO_3$ (100 mL), and the whole was partitioned. The organic layer was washed with saturated aqueous $NaHCO_3$, dried (Na_2SO_4), and evaporated. The residue was purified on a neutralized silica gel column with 67% CH_3-CN in $CHCl_3$ and then $CHCl_3$−MeOH−AcOEt (34:1:4 to 17:1:2) to give **18** (273 mg, 63% as a yellow foam); FAB-HRMS calcd for $C_{76}H_{103}N_{13}O_{16}PSi_2$ *m*/*z* 1540.6922, found *m*/*z* 1540.6920 (MH^+); ^{1}H NMR (DMSO-d_6) 11.94 (br s, 1H), 11.73 (br s, 2H), 8.83 (s, 1H), 8.67 (t, 1H, *J* = 5.6 Hz), 8.48 (s, 1/2H), 8.46 (s, 1/2H), 8.28 (d, 1H, *J* = 7.3 Hz), 7.78 (t, 1H, *J* = 5.6 Hz), 7.65 (d, 2H, *J* = 8.8 Hz), 7.18−7.37 (m, 9H), 7.01 (t, 1H, *J* = 6.1 Hz), 6.85−6.88 (m, 4H), 6.65 (d, 2H, *J* = 8.8 Hz), 6.09 (t, 1/2H, *J* = 6.1 Hz), 6.06 (t, 1/2H, *J* = 6.5 Hz), 4.59 (d, 2H, *J* = 6.1 Hz), 4.25−4.39 (m, 4H), 4.02−4.13 (m, 3H), 3.73 (s, 6H), 3.40−3.75 (m, 4H), 3.17−3.28 (m, 4H), 2.97−3.02 (m, 2H), 2.75 (t, 2/2H, *J* = 5.9 Hz), 2.63 (t, 2/2H, *J* = 5.9 Hz), 2.36−2.46 (m, 2H), 2.15−2.20 (m, 2H), 1.98−2.06 (m, 1H), 1.86−1.95 (m, 1H), 1.40−1.47 (m, 2H), 1.30−1.37 (m, 2H), 1.20−1.28 (m, 4H), 0.90−1.12 (m, 16H), 0.06 (s, 9H), 0.01 (s, 9H); ^{31}P NMR (acetone-d_6) 150.04, 149.86 (85% H_3PO_4 as an internal standard).

JO000132A