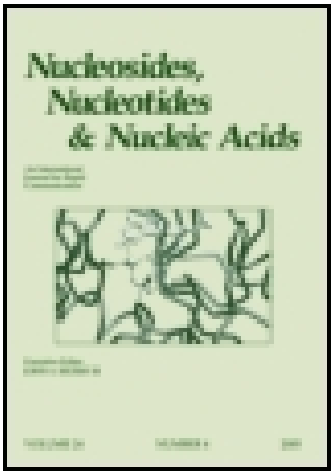

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Synthesis and Biological Evaluation of 1'-*C*-Cyano-Pyrimidine Nucleosides†

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ABSTRACTS: 2'-Deoxy-, 2'-bromo-, and arabino-1'-*C*-cyano-pyrimidine nucleosides were synthesized from O^2,2'-cyclouridine. Incorporation of cyano group at the anomeric position was achieved by treatment of 1',2'-unsaturated uridine with NBS in the presence of pivalic acid followed by TMS-cyanide and stannic chloride. Antineoplastic and antiviral activities of those compounds are also discussed.

INTRODUCTION

Several nucleoside antimetabolites have been used for clinical treatment of cancer and viral diseases. Among them, 1-β-D-arabinosylcytosine (araC)[1] for the treatment of acute myeloblastic leukemia and 3'-deoxy-3'-azidothymidine (AZT)[2] for the treatment of acquired immunodeficiency syndrome (AIDS) are well-known as important agents in the field of chemotherapy. The effectiveness of these compounds triggered to investigate the sugar modified nucleosides as antitumor and antiviral agents. After a lot of efforts have been made, some clinically effective compounds were found e.g., dideoxyinosine (DDI)[3], dideoxycytidine (DDC)[4], and dideoxydidehydrothymidine (D4T)[5] for AIDS treatment.

During the course of modification at the sugar portion, various new compounds substituted at 2'-, 3'-, and 4'-position have been reported to be synthesized. Among them, the nucleosides which had an electron-withdrawing 'cyano' group were particularly

† This paper is dedicated to Dr. Yoshihisa Mizuno on the occasion of his 75th birthday.

attractive. The analogue attaching cyano group at 2'-position with "up" configuration, 1-(2-*C*-cyano-2-deoxy-1-β-D-arabinosyl)cytosine (CNDAC), was synthesized. Its strong antineoplastic activities *in vitro* and *in vivo* were reported by Matsuda and his co-workers.[6] In addition, thymidine analogues, 3'-*C*-cyano-3'-deoxythymidine[7] and 4'-*C*-cyano-thymidine[8] having cyano group instead of 3'-hydroxyl group and 4'-hydrogen respectively, were synthesized. The latter showed a significant anti human immunodeficiency virus (HIV) activity and cytotoxicity. [8,9] (Chart 1)

Although numerous substituted nucleosides, such as the compounds described above, were synthesized, there were only limited examples concerned 1'-substituted analogues. The 1'-substituted derivatives were so far synthesized by the glycosylation using corresponding 1'-substituted riboses including psicofuranose,[10-12] or, started from an aminooxazoline derivative of fructose.[13] In these processes, the β-selectivity[10-12] and lengthy manipulation remained as problems.[10-13]

In order to overcome the problems, our colleagues synthesized 1'-substituted uridines started from a naturally occurring uridine in a stereoselective manner, as shown in Scheme 1.[14] The 1',2'-unsaturated uridine **6**, which was readily obtained by an elimination of O^2,2'-anhydrouridine, was succesfully converted to 1'-pivaloxy derivative **7** by β-face selective electrophilic addition. The Lewis acid mediated nucleophilic substitution of **7** have made possible to prepare 1'-carbon substituted nucleosides.
The stereoselectivity of these reactions were also noteworthy. In both reactions, the desired β-nucleosides were predominant, due to an assistance of 1',2'-epibromonium intermediate generated under the conditions. In this report, we describe the synthesis of 1'-*C*-cyano-pyrimidine nucleosides using above mentioned procedure, and discuss their biological activities, too.

RESULTS AND DISCUSSION

We envisaged to synthesize 2'-deoxy- (X = H), 2'-bromo- (X = Br) and 2'-arabino- (X = OH) 1'-*C*-cyano-pyrimidine nucleosides **A** (Chart 1), which could be obtained from **8**[14] (Scheme 1). The radical deoxygenation of **8** using tributyltin hydride gave 2'-deoxy derivative **9** in 77% yield, which was deprotected by tetrabutylammonium fluoride (TBAF), giving **10**. The conversion of **9** to cytosine counterpart, on the other hand, was performed by the triazole method[15] and the resulted **11** was desilylated by TBAF to afford 1'-*C*-cyano-2'-deoxycytidine **12** in 67 % yield. The same procedure could efficiently be applied to the synthesis of 2'-bromo derivative and compound **14** was obtained in moderate yield. However, the deprotection of **8** using TBAF caused an intramolecular cyclization and hydrolysis of cyano group (vide infra), thus, ammonium

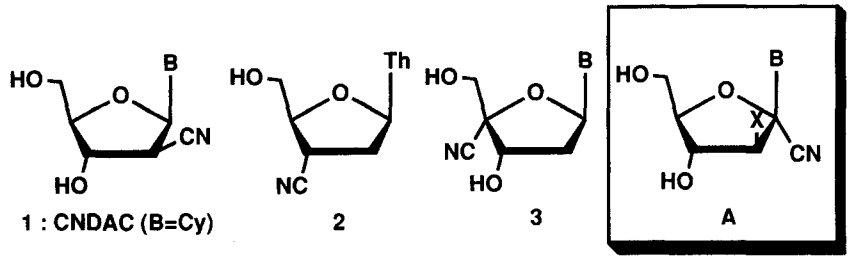
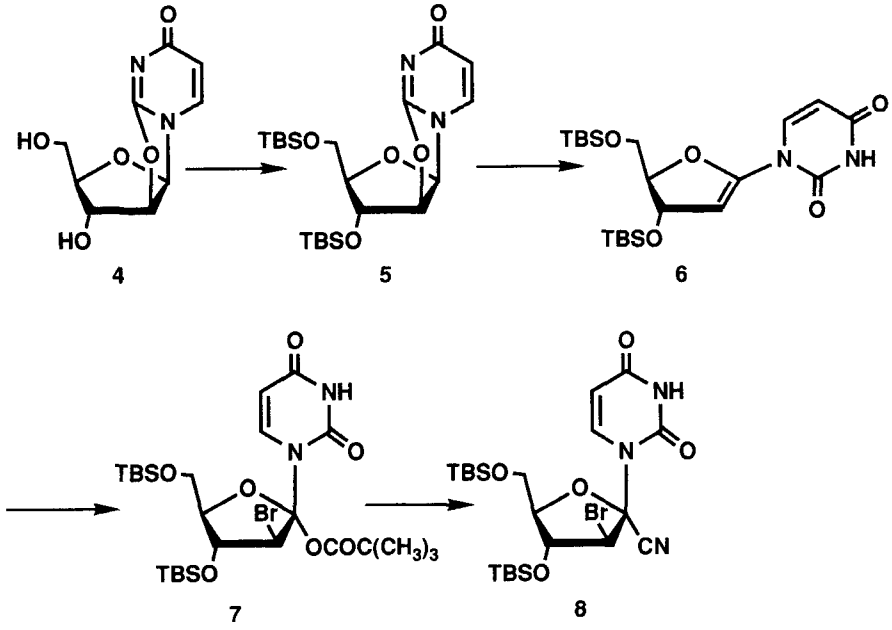


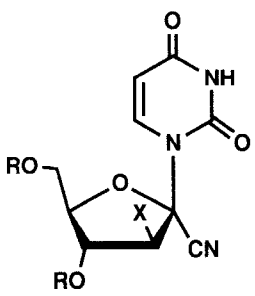
Chart 1



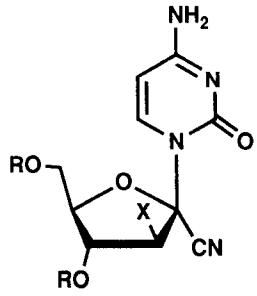
Scheme 1

hydrogen fluoride[16] in DMF, in place of TBAF, was employed to yield **15** in 33%. (Chart 2)

We further synthesized 5-substituted analogues, 5-iodouracil and thymine derivatives, from **8** or **9**. Iodination of **8** using iodine and ceric ammonium nitrate[17] afforded the 5-iodo derivative **16** and 5'-deprotected **17**. The ^{1}H NMR showed that the resonance of H-5' in compound **17** appeared around 4.0 ppm as multiplet, which was



9 : X=H, R=TBS
10 : X=R=H
15 : X=Br, R=H

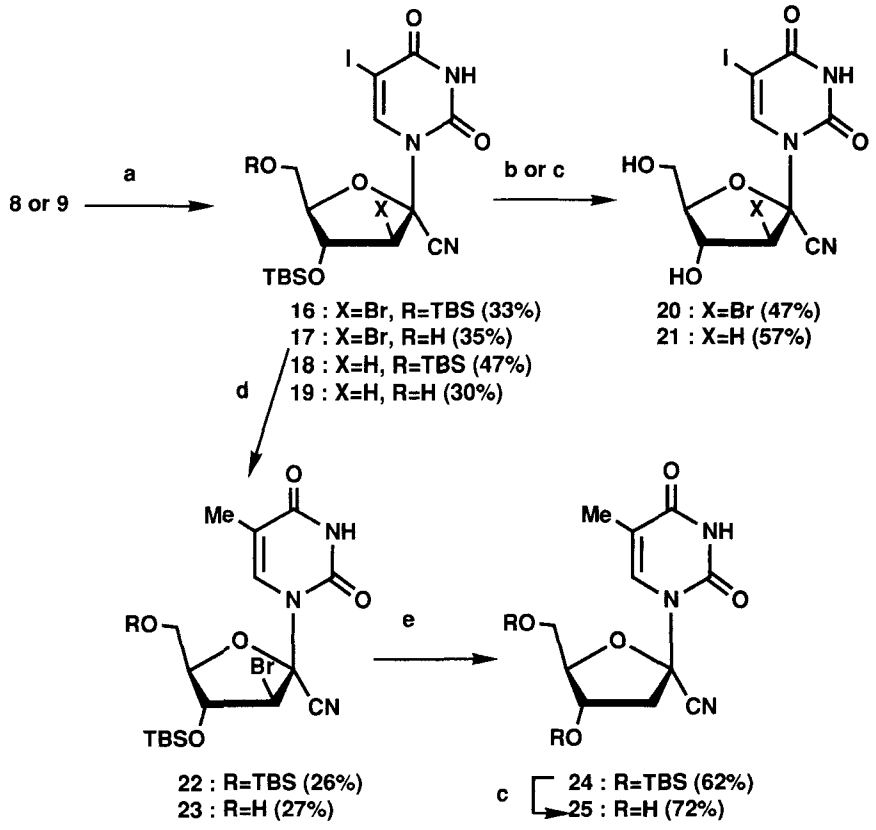


11 : X=H, R=TBS
12 : X=R=H
13 : X=Br, R=TBS
14 : X=Br, R=H

Chart 2

changed to doublet by D_2O treatment (data not shown). The compound **17** was readily reverted to **16** by the 5'-silylation and this also supported its structure. In a similar way, the reaction of **9** gave **18** and **19** in 47% and 30% yields, respectively. Deprotection of **16** and **18** furnished free nucleosides **20** and **21**, respectively. Since Hirota *et. al.* reported palladium assisted methylation of 5-iodouridine,[18] we intended to utilize the same reaction for the synthesis of thymidine analogues. The 5-iodo derivative **16** was treated with trimethylaluminum in the presence of a catalytic amount of bis(triphenylphosphine)palladium chloride, giving **22** and its 5'-desilylated compound **23** (the structure was confirmed by ^1H NMR, as described above) in 26% and 27% yields, respectively. Compound **22** was further treated with tributyltin hydride in the presence of AIBN to afford **24** in a good yield. Finally, 2'-deoxygenated **24** was deprotected by TBAF to afford 1'-*C*-cyano-thymidine **25**. (Scheme 2)

The synthesis of thymidine analogues required a long manipulation and the total yield was not satisfactory. Hence, we explored an alternative synthesis using a ribosylthymine. Cyclo-riboT **26** was silylated and the obtained **27** was further treated with potassium *t*-butoxide,[19] NBS, and pivalic acid followed by TMS-cyanide in the presence of stannic chloride, as in the case of cyclouridine, to afford 2'-bromo-1'-*C*-cyano-thymidine **22** through the 1',2'-unsaturated thymidine **28**. The obtained **22** was identical with that synthesized from cyclouridine described above. Thus, 2'-bromo-substituted thymidine derivative **30** was synthesized by deprotection of **22** in 17 % yield. (Chart 3)



Conditions: a) I_2, CAN, MeCN. b) NH_4F•HF, DMF. c) Bu_4NF, THF. d) Me_3Al, $(Ph_3P)_2PdCl_2$, THF. e) Bu_3SnH, AIBN, PhMe.

Scheme 2

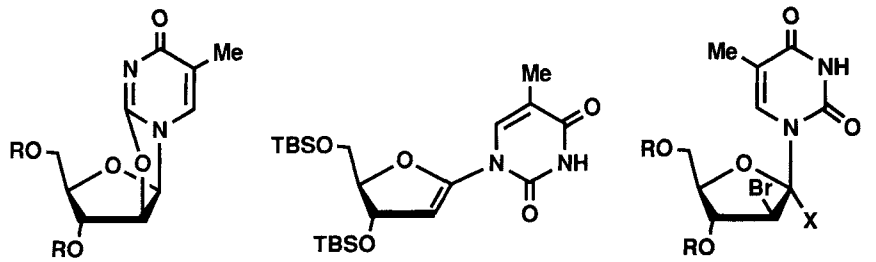
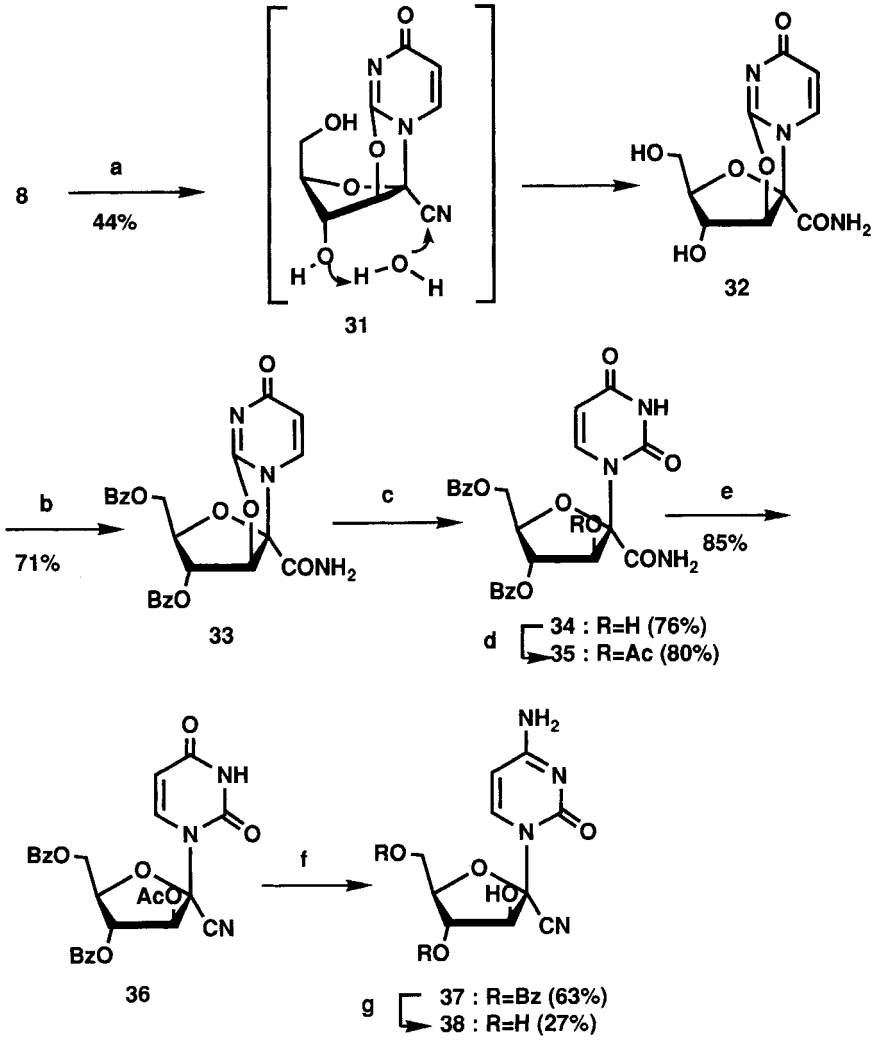


Chart 3

As a final target 1'-*C*-cyano-arabinosylcytosine was selected, because its potential antitumor activity as an analogue of araC was expected.[1] The deprotection of **8** using TBAF did not give any desired 1'-cyano-*O*-cyclo derivative **31**, but, an unexpected polar compound was obtained. The structure of the compound was determined as **32** in Scheme 3, from the following points : 1) ^{1}H NMR spectrum of **32** showed one set of broad signal at 8.11 and 8.16 ppm, which slowly disappeared by D_2O addition. 2) in FAB-mass spectrum of **32**, M^++H was observed at 270. Although hydrolysis of cyano group, in most cases, requires rather strong condition such as a basic hydrogen peroxide, the cyano-hydrolysis of **8** had occurred under mild basic condition. On the other hand, it is noteworthy that the deprotection of 2'-deoxygenated derivatives **9**, **18**, and **24** did not afford any carbamoyl products. These results suggest that the formation of *O*-cyclo-nucleoside is critical. Therefore, we postulated the reaction mechanism as follows: The treatment of **8** with TBAF caused a formation of 2',3'-epoxy derivative, which was spontaneously converted to O^2,2'-cyclo-nucleoside **31** under the condition.[14] The sugar puckering of *O*-cyclo-nucleoside **31** was fixed in C4'-endo-C3'-exo conformation,[20] where the 3'-hydroxyl group located closer to the 1'-cyano group. Thus, even under mild basic condition, the 3'-hydroxyl group could efficiently activate a water molecule and accelerate the hydrolysis.

We settled to utilize the carbamoyl nucleoside **32** for the further manipulation, since **32** was considered to have a masked and protected cyano group which could be recovered at the latter step. Compound **32** was benzoylated and *O*-cyclo moiety was hydrolyzed under acidic condition,[13b,c] then, acetylated to give **35** in a good yield. Among several attempts to recover a cyano group of **35**, the treatment with thionyl chloride in DMF allowed to convert the carbamoyl to the cyano group. The structure of **36** was confirmed by ^{1}H-NMR and FAB-mass spectrum. (See Experimental section) The uracil moiety of **36** was transformed to the cytosine by the triazole method[15] as described above, affording deacetylated cytidine derivative **37** in 65% yield. The deprotection of **37** was complicated. Treatment with sodium methoxide or methanolic ammonia did not give deprotected **38**, due to instability of the cyano group. Hence, the deprotection was achieved by the treatment with triethylamine in methanol to furnish 1'-*C*-cyano-arabinosylcytosine **38** .

The synthesized 1'-*C*-cyano-nucleosides were subjected to the evaluation of antitumor and antiviral activities. The antitumor activity was evaluated as their cytotoxity towards human T-cell line, CCRF-HSB-2, using MTT method[21] and KB cell using dye uptake method.[22] The antiviral activity against HSV-1 were assayed by a cytopathic-effect (CPE) inhibition.[23] The results were summarized in Table 1. The cytidine analogues **12**, **14**, and **38** exhibited moderate to potent antineoplastic activities.



Conditions: a) Bu_4NF, THF. b) BzCl, Pyr. c) 2N HCl, DMF. d) Ac_2O, DMAP, Pyr. e) $SOCl_2$, DMF. f) triazole, Et_3N, $POCl_3$, MeCN then aq. NH_4OH. g) Et_3N, MeOH

Scheme 3

Table 1: Antineoplastic and Antiviral Activity of 1'-*C*-Cyano Nucleosides

			Antineoplastic Activity (IC_{50} μg/ml)		Antiviral Activity (ED_{50} μg/ml)
comp	B	X	CCRF-HSB-2[a]	KB[b]	HSV-1[c]
10	Ura	H	>10	>10	>10
12	Cyt	H	1.0	1.3	3.2
14	Cyt	Br	0.25	>10	>10
15	Ura	Br	>10	>10	>10
20	5-I-Ura	Br	>10	>10	>10
21	5-I-Ura	H	>10	>10	>10
25	Thy	H	>10	>10	3.2
30	Thy	Br	>10	>10	>10
38	Cyt	OH	0.015	2.5	>10
araC	-	-	0.012	0.45	-

[a]MTT method, [b]Dye uptake method, [c]CPE inhibition

In contrast, none of the uracil nucleosides showed the activities. The 5-iodouracil derivatives **20** and **21** were also inactive, and, thymidine derivative **25** revealed a weak activity against HSV-1. The cytotoxity of cytidine series is noteworthy. Especially, 1'-cyano-araC **38** was equally cytotoxic, compared with araC, towards CCRF-HSB-2. The 2'-deoxy derivative **12** was a rather weak cytotoxic against T-cell line compared with **38**. However, it was as effective for KB cell as **38**. The results that only cytidine analogues showed potent activities suggest that all 1'-cyano-cytidines tested could be substrates for deoxycytidine kinase which converted araC and other cytidine derivatives to the active metabolites. On the other hand, the results also suggest that the 5'-phosphorylation by the virus coded thymidine kinase could hardly occur.

In conclusion, we have synthesized 1'-*C*-cyano-pyrimidine nucleosides including uridine, cytidine, thymidine, and 5-iodouridine, using a novel 1'-manipulation methodology. Among them, 1'-*C*-cyano-araC exhibited potent antineoplastic activity and 2'-deoxy-cytidine analogue also showed cytotoxity toward KB cell. Further evaluation of these compounds and their metabolic fate will be the problem in the future.

EXPERIMENTAL SECTION

General Methods. Physical data were measured as follows: Melting points were determined on a Yanagimoto MP-500D micro melting point apparatus and are uncorrected. ^{1}H-NMR spectra were recorded on a JEOL JNM-GSX-400 instrument in $CDCl_3$ or DMSO-d_6 as the solvent with tetramethylsilane as internal standard. UV-spectra were recorded with a Shimadzu UV-160A spectrophotometer. Low- and high-resolution mass spectra were taken on a JEOL JMS-AX500 spectrometer.

THF was freshly distilled under argon from sodium/benzophenone before use whereas dichloromethane was distilled from calcium hydride. All the reactions described below were performed under argon atmosphere and monitored by Thin-layer chromatography (TLC). TLC and preparative TLC were carried out on Merck pre-coated plates Kieselgel 60F254. Silica gel for chromatography was Merck Kieselgel 60.

1-(1-*C*-Cyano-2-deoxy-3,5-di-*O*-*t*-butyldimethylsilyl-β-D-*erythro*-pentofuranosyl)-uracil (9). To a toluene solution (20 mL) of **8** (224 mg, 0.40 mmol) were added tributyltin hydride (0.12 mL, 0.44 mmol) and AIBN (10 mg), and the mixture was kept at 100 °C for 2 h. After cooled to room temperature, the solvent was removed under reduced pressure and the residue was purified over silica gel column chromatography (17 % AcOEt in n-hexane), giving **9** (149 mg, 77%, crystallized from n-hexane/AcOEt): mp 194-195 °C; ^{1}H NMR ($CDCl_3$) δ 0.04, 0.05, 0.10, and 0.12 (12H, each as s), 0.85 and 0.92 (18H, each as s), 2.60-2.65 (1H, m), 3.14-3.18 (1H, m), 3.70-3.83 (2H, m), 4.37-4.40 (2H, m), 5.72 (1H, d, $J_{5,6}$ = 8.3 Hz), 7.80 (1H, d), 8.53 (1H, br); FAB-MS *m/z* 482 (M^++H), 455 (M^+-CN), 424. Anal. Calcd for $C_{22}H_{39}N_3O_5Si_2$ 0.25 H_2O : C, 54.34; H, 8.19; N, 8.64. Found: C, 54.49; H, 8.28; N, 8.57.

1-(1-*C*-Cyano-2-deoxy-β-D-*erythro*-pentofuranosyl)uracil (10). To a THF solution (8 mL) of **9** (120 mg, 0.25 mmol) was added TBAF (1 M solution of THF 0.55 mL, 0.55 mmol) and the mixture was stirred at room temperature for 2 h. After the solvent was removed under reduced pressure, the residue was purified over silica gel column chromatography (10 % MeOH in $CHCl_3$), giving **10** (31 mg, 49%, crystallized from n-hexane/AcOEt): mp 102-103 °C; UV (H_2O) λ_{max} = 257nm (ε 10200); ^{1}H NMR (DMSO-d_6) δ 2.65-2,70 (1H, m), 2.90-2.94 (1H, m), 3.46-3.57 (2H, m), 4.24-4.30 (2H, m), 5.07 (1H, br), 5.51 (1H, d, $J_{3',OH}$ = 2.9 Hz), 5.64 (1H, d, $J_{5,6}$ = 8.3 Hz), 7.87 (1H, d), 11.59 (1H, br); FAB-MS *m/z* 254 (M^++H). Anal. Calcd for $C_{10}H_{11}N_3O_5$.0.25AcOEt: C, 48.00; H, 4.76; N, 15.27. Found: C, 47.72; H, 4.83; N, 15.40.

1-(1-*C*-Cyano-2-deoxy-3,5-di-*O*-*t*-butyldimethylsilyl-β-D-*erythro*-pentofuranosyl)-cytosine (11). To an acetonitrile solution (5 mL) of triazole (237 mg, 3.43 mmol) were added triethylamine (0.48 mL, 3.43 mmol) and phosphorus oxychloride (0.10 mL, 1.03 mmol), and the mixture was stirred at room temperature for 1 h. To the resulting

products, a solution of **9** (150 mg, 0.31 mmol) in acetonitrile (2 mL) was added and the reaction mixture was stirred at room temperature for 17 h. 28% Ammonium hydroxide (3.1 mL), was added and the mixture was stirred at room temperature for 2 h. To the reaction mixture, $CHCl_3$ and water were added and the separated organic phase was dried (Na_2SO_4). The filtrate was concentrated and the residue was purified over silica gel column chromatography (3% MeOH in $CHCl_3$), giving **11** (100 mg, 67%) as a foam: UV (MeOH) λ_{max} = 241 and 264 nm; UV (pH 2) λ_{max} = 277 nm; ^{1}H NMR ($CDCl_3$) δ 0.05, 0.06, 0.07, and 0.09 (12H, each as s), 0.86 and 0.91 (18 H, each as s), 2.60-2.65 (1H, m), 3.17-3.21 (1H, m), 3.70-3.84 (2H, m), 4.30-4.36 (2H, m), 5.72 (1H, d, $J_{5,6}$ = 7.8 Hz), 7.85 (1H, d); FAB-MS *m/z* 481 (M^++H). Anal. Calcd for $C_{22}H_{40}N_4O_4Si_2$: C, 54.96; H, 8.39; N, 11.65. Found: C, 55.21; H, 8.52; N, 11.32.

1-(1-*C*-Cyano-2-deoxy-β-D-*erythro*-pentofuranosyl)cytosine (12). The compound **12** was obtained in 67% yield from **11** as described in the synthesis of **10**. Crystallization from n-hexane/AcOEt gave an analytically pure sample: mp 198-199 °C (dec.); UV (H_2O) λ_{max} = 232 (ε 8100) and 268 nm (ε 8800); UV (pH 2) λ_{max} = 275nm (ε 13100);^{1}H NMR (DMSO-d_6) δ 2.43-2,50 (1H, m), 2.95-2.99 (1H, m), 3.43-3.56 (2H, m), 4.19-4.21 (1H, m), 4.25-4.27 (1H, m), 5.03 (1H, br), 5.46 (1H, d, $J_{3',OH}$ = 3.4 Hz), 5.75 (1H, d, $J_{5,6}$ = 7.8 Hz), 7.36 (2H, br), 7.84 (1H, d, $J_{5,6}$ = 7.8 Hz); FAB-MS *m/z* 253 (M^++H). Anal. Calcd for $C_{10}H_{12}N_4O_4$.0.55AcOEt: C, 48.73; H, 5.50; N, 18.63. Found: C, 48.42; H, 5.70; N, 18.24.

1-(2-Bromo-1-*C*-cyano-2-deoxy-3,5-di-*O*-*t*-butyldimethylsilyl-β-D-*arabino*-pentofuranosyl)cytosine (13). The compound **13** was obtained in 65% yield as described in the synthesis of **11**. Crystallization from n-hexane/AcOEt gave an analytically pure sample: mp 197-198 °C; UV (MeOH) λ_{max} = 241 nm; UV (pH 2) λ_{max} = 272 nm; ^{1}H NMR ($CDCl_3$) δ 0.11, 0.13 and 0.17 (12H, each as s), 0.92 and 0.94 (18H, each as s), 3.88-3.93 (2H, m), 4.37-4.40 (1H, m), 4.69 (1H, d, $J_{3',4'}$ =2.9 Hz), 5.14 (1H, s), 5.81 (1H, d, $J_{5,6}$ =7.8 Hz), 7.59 (1H, d); FAB-MS *m/z* 559, 561 (M^++H). Anal. Calcd for $C_{22}H_{39}BrN_4O_4Si_2$.0.5 H_2O: C, 46.47; H, 7.09; N, 9.85. Found: C, 46.41; H, 7.09; N, 10.01.

1-(2-Bromo-1-*C*-cyano-2-deoxy-β-D-*arabino*-pentofuranosyl)cytosine (14). The compound **14** was obtained in 45% yield as described in the synthesis of **10**. Crystallization from Et_2O / EtOH gave an analytically pure sample: mp 205-206 °C (dec.); UV (H_2O) λ_{max} = 235 (ε 8500) and 267 nm (ε 9000); UV (pH 2) λ_{max} = 275 nm (ε 12600); ^{1}H NMR (DMSO-d_6) δ 3.69-3.72 (2H, m), 4.20-4.23 (1H, m), 4.47-4.48 (1H, m), 5.03 (1H, s), 5.22 (1H, br), 5.83 (1H, d, $J_{5,6}$ = 7.8 Hz), 6.42 (1H, br), 7.53 (2H, br), 7.76 (1H, d, $J_{5,6}$ = 7.8 Hz); FAB-MS *m/z* 331, 333 (M^++H). Anal. Calcd for $C_{10}H_{11}BrN_4O_4$.1.3H_2O: C, 33.88; H, 3.87; N, 15.80. Found: C, 34.07; H, 3.60; N, 15.38.

1-(2-Bromo-1-*C*-cyano-2-deoxy-β-D-*arabino*-pentofuranosyl)uracil (15). To a DMF solution (10 mL) of **8** (448 mg, 0.80 mmol) was added ammonium hydrogen fluoride (91 mg, 1.6 mmol) and the mixture was stirred at room temperature for 20 h. After the reaction was quenched with sat. $NaHCO_3$ solution, the solvent was removed under reduced pressure. The residue was purified over silica gel column chromatography (5% MeOH in chloroform), giving **15** (88 mg, 33%, crystallized from AcOEt): mp 128-129 °C; UV (H_2O) λ_{max} = 255 nm (ε 10500); ^1H NMR (DMSO-d_6) δ 3.67-3.77 (2H, m), 4.24-4.27 (1H, m), 4.50-4.51 (1H, m), 5.00 (1H, s), 5.30 (1H, dd, J = 5.9 and 6.3 Hz), 5.78 (1H, d, $J_{5,6}$ = 8.3 Hz), 6.48 (1H, d, $J_{3',OH}$ = 3.4 Hz), 7.85 (1H, d), 11.87 (1H, br); FAB-MS *m/z* 332,334 (M^++H). Anal. Calcd for $C_{10}H_{10}BrN_3O_5$.0.5AcOEt: C, 38.32; H, 3.75; N, 11.17. Found: C, 38.14; H, 3.67; N, 10.87.

1-(2-Bromo-1-*C*-cyano-2-deoxy-3,5-di-*O*-*t*-butyldimethylsilyl-β-D-*arabino*-pentofuranosyl)-5-iodouracil (16) and 1-(2-Bromo-1-*C*-cyano-2-deoxy-3-*O*-*t*-butyldimethylsilyl-β-D-*arabino*-pentofuranosyl)-5-iodouracil (17). To an acetonitrile solution (60 mL) of **8** (1.12 g, 2.0 mmol) were added iodine (600 mg, 2.4 mmol) and ceric ammonium nitrite (1.10 g, 2.0 mmol) and the mixture was kept reflux for 2h. After cooled to room temperature, the solvent was removed under reduced pressure. The residue was partitioned between AcOEt and 1% sodium thiosulfate solution. The separated organic phase was washed with brine, and dried (Na_2SO_4). The filtrate was concentrated and the residue was purified over silica gel column chromatography (12.5% AcOEt in n-hexane), giving **16** (447 mg, 33%, foam) and **17** (394 mg, 35%). **16**: UV (MeOH) λ_{max} = 277 nm;^1H NMR ($CDCl_3$) δ 0.14 and 0.18 (12H, each as s), 0.94 (18H, s), 3.94-3.95 (2H, m), 4.39-4.42 (1H, m), 4.70 (1H, d, $J_{3',4'}$ = 2.4 Hz), 4.96 (1H, s), 7.97 (1H, s), 8.37 (1H, br); FAB-MS *m/z* 688 (M^++H). Anal. Calcd for $C_{22}H_{37}BrIN_3O_5Si_2$: C, 38.49; H, 5.43; N, 6.12. Found: C, 38.49; H, 5.45; N, 6.04. **17**: ^1H NMR ($CDCl_3$) δ 0.15 and 0.19 (6H, each as s), 0.94 (9H, s), 1.86 (1H, dd, J =5.4 and 5.9 Hz), 4.00-4.03 (2H, m), 4.46-4.49 (1H, m), 4.68 (1H, d, $J_{3',4'}$ = 2.9 Hz), 5.00 (1H, d, J = 1.0 Hz), 7.98 (1H, s), 8.50 (1H, br).

1-(1-*C*-Cyano-2-deoxy-3,5-di-*O*-*t*-butyldimethylsilyl-β-D-*arabino*-pentofuranosyl)-5-iodouracil (18) and 1-(1-*C*-Cyano-2-deoxy-3-*O*-*t*-butyldimethylsilyl-β-D-*arabino*-pentofuranosyl)-5-iodouracil (19). The compounds were obtained in 47 and 30% yield, respectively, from **9** as described in the synthesis of **16** and **17**. **18**: UV (MeOH) λ_{max} = 214 and 279 nm; ^1H NMR ($CDCl_3$) δ 0.06, 0.07, 0.10, and 0.13 (12H, each as s), 0.84 and 0.93 (18H, each as s), 2.54-2.59 (1H, m), 3.18-3.22 (1H, m), 3.70-3.74 (1H, m), 3.81-3.85 (1H, m), 4.39-4.45 (2H, m), 8.12 (1H, s), 8.32 (1H, br); FAB-MS *m/z* 608 (M^++H), 581 (M^+-CN), 550 (M^+-*t*Bu). Anal. Calcd for $C_{22}H_{38}IN_3O_5Si_2$.0.25H_2O: C, 43.17; H, 6.34; N, 6.86. Found: C, 42.95; H, 6.37; N, 6.61. **19**: ^1H NMR ($CDCl_3$) δ 0.09

and 0.10 (6H, each as s), 0.91 (9H, s), 2.58-2.67 (1H, m), 3.07-3.12 (1H, m), 3.65-3.70 (1H, m), 3.78-3.83 (1H, m), 4.36-4.38 (1H, m), 4.45-4.47 (1H, m), 4.58(1H, dd, J = 4.4 and 4.9 Hz), 8.29 (1H, s), 11.45 (1H, br); FAB-MS m/z 494 (M^++H).

1-(2-Bromo-1-*C*-cyano-2-deoxy-β-D-*arabino*-pentofuranosyl)-5-iodouracil (20). The compound **20** was obtained in 47% yield from **16** as described in the synthesis of **15**. Crystallization from $CHCl_3$/AcOEt gave an analytically pure sample: mp 138-139 °C; UV (H_2O) λ_{max} = 284 nm (ε 7000); ^1H NMR (DMSO-d_6) δ 3.65-3.78 (2H, m), 4.20-4.24 (1H, m), 4.46-4.47 (1H, m), 4.96 (1H, s), 5.39 (1H, br), 6.46 (1H, d, $J_{3',OH}$ = 3.4 Hz), 8.18 (1H, s), 12.24 (1H, br); FAB-MS m/z 460 (M^++H). Anal. Calcd for $C_{10}H_9BrIN_3O_5.0.4AcOEt$: C, 28.25; H, 2.49; N, 8.52. Found: C, 27.86; H, 2.73; N, 8.27.

1-(1-*C*-Cyano-2-deoxy-β-D-*arabino*-pentofuranosyl)-5-iodouracil (21). The compound **21** was obtained in 57% yield from **18** as described in the synthesis of **10**. Crystallization from Et_2O/MeOH gave an analytically pure sample: mp 201-202 °C (dec.); UV (H_2O) λ_{max} = 217 (ε 9800) and 284 nm (ε 7500); ^1H NMR (DMSO-d_6) δ 2.70-2,75 (1H, m), 2.83-2.88 (1H, m), 3.41-3.49 (1H, m), 3.59-3.62 (1H, m), 4.22-4.23 (1H, m), 4.27-4.30 (1H, m), 5.18 (1H, br), 5.51 (1H, br), 8.25 (1H, s), 11.92 (1H, br); FAB-MS m/z 380 (M^++H). Anal. Calcd for $C_{10}H_{10}IN_3O_5.0.2H_2O$: C, 31.38; H, 2.74; N, 10.98. Found: C, 31.52; H, 2.66; N, 10.58.

1-(2-Bromo-1-*C*-cyano-2-deoxy-3,5-di-*O*-*t*-butyldimethylsilyl-β-D-*arabino*-pentofuranosyl)thymine (22) and 1-(2-Bromo-1-*C*-cyano-2-deoxy-3-*O*-*t*-butyldimethylsilyl-β-D-*arabino*-pentofuranosyl)thymine (23). To a THF solution (30 mL) of **16** (466 mg, 0.68 mmol) was added bis(triphenylphosphine)palladium chloride (24 mg) and the mixture was kept reflux for 5 min. Trimethylaluminum (1 M n-hexane solution 2.7 mL, 2.7 mmol) was added to the mixture and the whole was kept reflux for 2 h. After the reaction was quenched with water, the solvent was removed under reduced pressure. To the residue, AcOEt and water were added and undissolved materials were removed by filtration (celite). The separated organic phase was washed with 1% EDTA solution and brine, then dried (Na_2SO_4). The filtrate was concentrated and the residue was purified over silica gel column chromatography (8% AcOEt in n-hexane), giving **22** (103 mg, 26%, crystallized from n-hexane/AcOEt) and **23** (86 mg, 27%). **22** : mp 172-173 °C; UV (MeOH) λ_{max} = 259nm; ^1H NMR ($CDCl_3$) δ 0.12, 0.14, and 0.18 (12H, each as s), 0.93 and 0.94 (18H, each as s), 1.95 (3H, d, $J_{6,Me}$ = 1.5 Hz), 3.93-3.95 (2H, m), 4.38-4.39 (1H, m), 4.69 (1H, d, $J_{3',4'}$ =2.9 Hz), 5.00 (1H, s), 7.41 (1H, d), 8.31 (1H, br); FAB-MS m/z 574, 576 (M^++H). Anal. Calcd for $C_{23}H_{40}BrN_3O_5Si_2$: C, 48.07; H, 7.02; N, 7.31. Found: C, 47.73; H, 7.10; N, 7.03. **23** : ^1H NMR ($CDCl_3$) δ 0.14 and 0.17 (6H, each as s), 0.93 (9H, s), 1.93 (3H, d, $J_{5,Me}$ = 1.0 Hz), 3.87-3.88 (2H, m), 4.37-4.40 (1H, m), 4.41-4.42 (1H, m), 4.62 (1H, br), 5.00 (1H, s), 7.60 (1H, d), 11.08 (1H, br). The

compound **22** was obtained in 80% yield from **29** as follows: To a dichloromethane solution (40 mL) of **29** (0.60 g, 0.92 mmol) was added trimethylsilyl cyanide (0.62 mL, 4.6 mmol) and $SnCl_4$ (1M dichloromethane solution 1.2 ml, 1.2 mmol) at -40 °C. The mixture was allowed to warm to room temperature over 3h, quenched with sat. $NaHCO_3$.Undissolved materials were removed by filtration (celite), and the separated organic phase was washed with water and brine, then dried (Na_2SO_4). The filtrate was concentrated and the residue was purified over silica gel column chromatography.

1-(1-*C*-Cyano-2-deoxy-3,5-di-*O*-*t*-butyldimethylsilyl-β-D-*erythro*-pentofuranosyl)-thymine (24). The compound **24** was deoxygenated in 62% yield from **22** as described in the synthesis of **9**. Crystallization from n-hexane/AcOEt gave an analytically pure sample: mp 146-147 °C ;^{1}H NMR ($CDCl_3$) δ 0.02, 0.04, 0.10, and 0.12 (12H, each as s), 0.83 and 0.92 (18H, each as s), 1.93 (3H, d, $J_{6,Me}$ = 1.5 Hz), 2.55-2.60 (1H, m), 3.18-3.22 (1H, m), 3.69-3.82 (2H, m), 4.38-4.40 (2H, m), 7.56 (1H, d), 8.20 (1H, br). FAB-MS *m/z* 496 (M^++H).Anal. Calcd for $C_{23}H_{41}N_3O_5Si_2$: C, 55.72; H, 8.34; N, 8.48. Found: C, 55.53; H,8.20; N, 8.19.

1-(1-*C*-Cyano-2-deoxy-β-D-*erythro*-pentofuranosyl)thymine (25). The compound **25** was deblocked in 72% yield from **24** as described in **10**. Crystallization from n-hexane/AcOEt gave an analytically pure sample: mp 102-103 °C; UV (H_2O) λ_{max} = 264 nm (ε 9600); ^{1}H NMR (DMSO-d_6) δ 1.79 (3H, s), 2.63-2,68 (1H, m), 2.89-2.93 (1H, m), 3.46-3.60 (2H, m), 4.23-4.25 (1H, m), 4.27-4.28 (1H, m), 5.08 (1H, br), 5.50 (1H, br), 7.76 (1H, s), 11.59 (1H, br); FAB-MS *m/z* 268 (M^++H). Anal. Calcd for $C_{11}H_{13}N_3O_5.0.5H_2O$: C, 47.83; H, 5.11; N, 15.21. Found: C, 47.73; H, 5.46; N, 14.92.

3',5'-Di-*O*-*t*-butyldimethylsilyl-*O*^{2},2'-cyclothymidine (27). To a DMF solution (120 mL) of **26** (9.4 g, 39.2 mmol) were added imidazole (9.3 g, 137 mmol) and *t*-butyldimethylsilyl chloride (14.8 g, 97.9mmol) and the mixture was stirred at room temperature for 3h. After the reaction was quenched with water, the solvent was removed under reduced pressure. The residue was partitioned between AcOEt and water, and the separated organic phase was washed with brine and dried (Na_2SO_4). The filtrate was concentrated and the residue was crystallized from n-hexane/AcOEt, giving **27** (14.0 g, 76%): mp 124-125 °C; ^{1}H NMR ($CDCl_3$) δ 0.01, 0.15, and 0.17 (12H, each as s), 0.84 and 0.92 (18H, each as s), 1.99 (3H, d, $J_{6,Me}$ = 1.5 Hz), 3.34-3.38 (1H, m), 3.53-3.57 (1H, m), 4.13-4.15 (1H, m), 4.62 (1H, s), 5.06-5.08 (1H, m), 6.11-6.13 (1H, m), 7.20 (1H, d, $J_{6,Me}$ = 1.5 Hz); FAB-MS *m/z* 469 (M^++H). Anal. Calcd for $C_{22}H_{40}N_2O_5Si_2$: C, 56.37; H, 8.60; N, 5.98. Found: C, 56.05; H, 8.68; N, 6.29.

1-(3,5-Di-*O*-*t*-butyldimethylsilyl-2-deoxy-D-*erythro*-pent-1-enofuranosyl)thymine (28). To a DMF solution (20 mL) of t-BuOK (449 mg, 4 mmol) was added **27** (936 mg,

2.0 mmol) at 0°C and the mixture was stirred at room temperature for 1h. After neutralized by acetic acid, the solvent was removed under reduced pressure. The residue was partitioned between AcOEt and water, and the organic phase was washed with brine, and dried (Na_2SO_4). The filtrate was concentrated and the residue was purified over a silica gel column chromatography (16.7% AcOEt in n-hexane), giving **28** (211 mg, 23%, foam): ^{1}H NMR ($CDCl_3$) δ 0.17, 0.19, and 0.21 (12H, each as s), 1.00 (18H, s), 2.06 (3H, d, $J_{6,Me}$=1.5 Hz), 3.73-3.88 (2H, m), 4.50-4.53 (1H, m), 5.11-5.12 (1H, m), 5.63-5.64 (1H, m), 7.69 (1H, d, $J_{6,Me}$=1.5 Hz), 8.46 (1H, br); FAB-MS *m/z* 469 (M^++H). Anal. Calcd for $C_{22}H_{40}N_2O_5Si_2$: C, 56.37; H, 8.60; N, 5.98. Found: C, 56.24; H, 8.80; N, 5.73.

1-(2-Bromo-2-deoxy-3,5-di-*O*-*t*-butyldimethylsilyl-1-pivaloyloxy-β-D-*arabino*-pentofuranosyl)thymine (29). To an ether solution (100 mL) of **28** (1.31 g, 2.80 mmol) were added triethylamine (1.95 mL, 14.0 mmol), pivalic acid (1.43 g, 14.0 mmol) and NBS (0.60 g, 3.40 mmol), and the mixture was stirred at room temperature for 1 h. After the reaction was quenched by sat. $NaHCO_3$ solution, the separated organic phase was washed with water and brine, then dried (Na_2SO_4). The filtrate was concentrated and the residue was purified over silica gel column chromatography (14% AcOEt in n-hexane), giving **7** (0.79 g, 43%, crystallized from n-hexane): mp 144-145 °C ; ^{1}H NMR ($CDCl_3$) δ 0.09, 0.12, and 0.17 (12H, each as s), 0.91 (18H, s), 1.20 (9H, s), 1.93 (3H, s), 3.88-3.90 (2H, m), 4.09-4.11 (1H, m), 4.71-4.72 (1H, m), 4.91 (1H, s), 7.67 (1H, s), 8.15 (1H, br); FAB-MS *m/z* 649, 651 (M^++H). Anal. Calcd for $C_{27}H_{49}BrN_2O_7Si_2$: C, 49.91; H, 7.60; N, 4.31. Found: C, 49.77; H, 7.46; N, 4.03.

1-(2-Bromo-1-*C*-cyano-2-deoxy-β-D-*arabino*-pentofuranosyl)thymine (30). The compound **30** was deblocked in 17% yield from **22** as described in **13**. Crystallization from n-hexane/AcOEt gave an analytically pure sample: mp 208-209 °C (dec.); UV (H_2O) λ_{max} = 264 nm (ε 10400); ^{1}H NMR (DMSO-d_6) δ 1.83 (3H, s) 3.72-3.75 (2H, m), 4.22-4.26 (1H, m), 4.49-4.51 (1H, m), 4.99 (1H, s), 5.30 (1H, dd, *J* = 5.9 and 6.3 Hz), 6.46 (1H, d, $J_{3',OH}$ = 3.4 Hz), 7.72 (1H, s), 11.85 (1H, br); FAB-MS *m/z* 346, 348 (M^++H). Anal. Calcd for $C_{11}H_{12}BrN_3O_5$.0.5MeOH: C, 38.14; H, 3.90; N, 11.60. Found: C, 38.04; H, 3.57; N, 11.33.

1'-*C*-Carbamoyl-O^2,2'-cyclouridine (32). To a THF solution (60 mL) of **8** (1.92 g, 4.0 mmol) was added TBAF (5.7 mL , 1 M solution of THF, 5.7 mmol) and the mixture was stirred at room temperature for 12 h. The solvent was removed under reduced pressure and the residue was purified over silica gel column chromatography (17% MeOH in chloroform), giving **32** (477 mg, 44%, crystallized from EtOH): mp 262-263 °C (dec.); UV (MeOH) λ_{max} = 225 and 247 nm; ^{1}H NMR (DMSO-d_6) δ 3.32-3.40 (2H, m), 4.36-4.39 (1H, m), 4.45 (1H, s), 5.05 (1H, br), 5.13 (1H, s), 5.86 (1H, d, $J_{5,6}$ = 7.8 Hz), 6.04

(1H, br), 7.64 (1H, d, $J_{5,6}$ = 7.8 Hz), 8.11 (1H, br), 8.16 (1H, br); FAB-MS *m/z* 270 (M^++H). Anal. Calcd for $C_{10}H_{11}N_3O_6$.0.75MeOH : C, 44.14; H, 4.57; N, 14.37. Found: C, 43.90; H, 4.42; N, 14.30.

3',5'-Di-*O*-benzoyl-1'-*C*-carbamoyl-O^2,2'-cyclouridine (33). To a pyridine solution (5 mL) of **32** (80 mg, 0.32 mmol) was added benzoyl chloride (0.09 mL, 0.79 mmol) at 0 °C and the mixture was stirred at room temperature for 2 h. The solvent was removed under reduced pressure and the residue was partitioned between AcOEt and water. The separated organic phase was washed with brine and dried (Na_2SO_4). The filtrate was concentrated and the residue was purified over silica gel column chromatography (4% MeOH in chloroform), giving **33** (104 mg, 71%, crystallized from EtOH/Et_2O): mp 213-214 °C; ^{1}H NMR (DMSO-d_6) δ 4.43-4.55 (2H, m), 5.12-5.16 (1H, m), 5.65 (1H, s), 5.80-5.81 (1H, m), 5.99 (1H, d, $J_{5,6}$ = 7.3 Hz), 7.42-7.52 (5H, m), 7.58-7.68 (2H, m), 7.94-7.96 (2H, m), 8.04-8.06 (2H, m), 8.18 (1H, br), 8.26 (1H, br); FAB-MS *m/z* 478 (M^++H). Anal. Calcd for $C_{24}H_{19}N_3O_8$: C, 60.38; H, 4.01; N, 8.80. Found: C, 60.43; H, 4.06; N, 8.60.

1-(3,5-Di-*O*-benzoyl-1-*C*-carbamoyl-β-D-*arabino*-pentofuranosyl)uracil (34). To a DMF solution (27 mL) of **33** (497 mg, 1.04 mmol) was added 2 N HCl solution (11 mL) and the mixture was stirred at room temperature for 48 h. After neutralized by sodium bicarbonate, the solvent was removed under reduced pressure. The residue was partitioned between AcOEt and water and the organic phase was washed with brine, and dried (Na_2SO_4). The filtrate was concentrated and the residue was purified over silica gel column chromatography (75% AcOEt in n-hexane), giving **34** (390 mg, 76%, crystallized from n-hexane/AcOEt): mp 192-193 °C; UV (MeOH) λ_{max} = 230 and 262 nm; ^{1}H NMR (DMSO-d_6) δ 4.51-4.63 (3H, m), 5.21-5.23 (1H, m), 5.29-5.31 (1H, m), 5.49 (1H, d, $J_{5,6}$ =8.3 Hz), 6.29 (1H, d, $J_{2',OH}$ = 5.9 Hz), 7.20 (1H, s), 7.51-7.57 (5H, m), 7.66-7.71 (2H, m), 7.75 (1H, d), 8.00-8.05 (4H, m), 11.5 (1H, br); FAB-MS *m/z* 496 (M^++H). Anal. Calcd for $C_{24}H_{21}N_3O_9$ 0.5H_2O: C, 57.14; H, 4.40; N, 8.33. Found: C, 57.31; H, 4.20; N, 8.03.

1-(2-*O*-Acetyl-3,5-di-*O*-benzoyl-1-*C*-carbamoyl-β-D-*arabino*-pentofuranosyl)uracil (35). To a pyridine solution (30 mL) of **34** (1.80 g, 0.79 mmol) were added acetic anhydride (0.51 mL, 5.45 mmol) and DMAP (15 mg) and the mixture was stirred at room temperature for 2 h. After water was added, the mixture was stirred for 10 min. The solvent was removed under reduced pressure and the residue was partitioned between AcOEt and water. The separated organic phase was washed with brine, and dried (Na_2SO_4). The filtrate was concentrated and the residue was purified over silica gel column chromatography (2% MeOH in chloroform), giving **35** (1.56 g, 80%, crystallized from n-hexane/AcOEt): mp 130-131 °C; ^{1}H NMR ($CDCl_3$) δ 1.75 (3H, s), 4.62-4.72

(2H, m), 4.93-4.97 (1H, m), 5.44-5.46 (1H, m), 5.54 (1H, d, $J_{5,6}$ = 8.3 Hz), 6.51 (1H, d, $J_{2',3'}$ = 1.0 Hz), 7.39-7.62 (8H, m), 7.81 (1H, d), 8.03-8.14 (4H, m), 10.65 (1H, br); FAB-MS *m/z* 538 (M^++H). Anal. Calcd for $C_{26}H_{24}N_3O_{10}.0.5H_2O$: C, 57.04; H, 4.60; N, 7.67. Found: C, 57.11; H, 4.53; N, 7.37.

1-(3,5-Di-*O*-benzoyl-1-*C*-cyano-β-D-*arabino*-pentofuranosyl)uracil (36). To a DMF solution (80 mL) of **35** (1.56 g, 2.9 mmol) was added thionyl chloride (0.85 mL, 11.6 mmol) and the mixture was stirred at room temperature for 2 h. After neutralized by sat. $NaHCO_3$ solution, the solvent was removed under reduced pressure. The residue was partitioned between AcOEt and water and the separated organic phase was washed with brine, then, dried (Na_2SO_4). The filtrate was concentrated and the residue was purified over silica gel column chromatography (25% AcOEt in n-hexane), giving **36** (1.38 g, 85%, crystallized from n-hexane/AcOEt): mp 107-108 °C; ^{1}H NMR (DMSO-d_6) 1.70 (3H, s), 4.75-4.79 (1H, m), 4.84-4.87 (1H, m), 4.97-5.01 (1H, m), 5.49 (1H, d, $J_{3',4'}$ = 2.0 Hz), 5.82 (1H, dd, $J_{5,6}$ = 8.3 Hz, $J_{5,NH}$ = 2.0 Hz), 6.16 (1H, s), 7.42-7.69 (6H, m), 7.72 (1H, d), 8.05-8.15 (4H, m), 8.41 (1H, br); FAB-MS *m/z* 520 (M^++H), 493 (M^+-CN). Anal. Calcd for $C_{26}H_{22}N_3O_9$: C, 60.00; H, 4.26; N, 8.07. Found: C, 59.85; H, 4.35; N, 7.78.

1-(3,5-Di-*O*-benzoyl-1-*C*-cyano-β-D-*arabino*-pentofuranosyl)cytosine (37). The compound **37** was obtained in 63% yield from **36** as described in the synthesis of **11**. Crystallization from n-hexane/AcOEt gave an analytically pure sample: mp 187-188 °C (dec.) ; UV (MeOH) λ_{max} = 232 nm; UV (pH2) λ_{max} = 231 and 276 nm; ^{1}H NMR ($CDCl_3$) δ 4.62-4.69 (2H, m), 4.92-4.98 (2H, m), 5.42 (1H, m), 5.79 (1H, d, $J_{5,6}$ = 7.3 Hz), 6.98 (1H, d, $J_{2',OH}$ = 5.9 Hz), 7.44 (2H, br), 7.53-7.74 (7H, m), 8.05-8.10 (4H, m); FAB-MS *m/z* 477 (M^++H). Anal. Calcd for $C_{24}H_{20}N_4O_7.0.75H_2O$: C, 58.83; H, 4.42; N, 11.44. Found: C, 58.92; H, 4.19; N, 11.63.

1-(1-*C*-Cyano-β-D-*arabino*-pentofuranosyl)cytosine (38). To a MeOH solution (20 mL) of **37** (92.5 mg, 0.19 mmol) was added triethylamine (4 mL) and the mixture was kept at 50 °C for 8 h. The solvent was removed under reduced pressure and the residue was purified over silica gel column chromatography (20% MeOH in chloroform), giving **38** (14.4 mg, 27%, crystallized from H_2O): mp 117-118 °C (dec.) ; UV (H_2O) λ_{max} = 235 (ε 7400) and 267 nm (ε 7900); UV (pH2) λ_{max} = 275 nm (ε 11900); ^{1}H NMR (DMSO-d_6) δ 3.55-3.57 (2H, m), 3.93 (1H, s), 4.17-4.20 (1H, m), 4.57 (1H, d, $J_{2',OH}$ = 6.4 Hz), 5.17 (1H, dd, $J_{5',OH}$ = 5.4 and 5.9 Hz), 5.74 (1H, d, $J_{3',OH}$ = 2.9 Hz), 5.75 (1H, d, $J_{5,6}$ = 7.8 Hz), 6.10 (1H, d), 7.33 (2H, br), 7.71 (1H, d); FAB-MS *m/z* 269 (M^++H). Anal. Calcd for $C_{10}H_{12}N_4O_5.2H_2O$: C, 39.48; H, 5.30; N, 18.41. Found: C, 39.60; H, 5.47; N, 18.02.

Growth inhibition test for CCRF HSB-2 cell.[21] A cell suspension of human T-cell acute lymphoblastoid leukemia cells, CCRF-HSB-2, containing 1.1 x 10^5 cells per mL was prepared in RPMI 1640 medium supplemented with 10% fetal bovine serum. 90 μL of the cell suspension was seeded in a 96 multi-well plate, and 10 μL of test compound solution in serial 0.5 $\log_{10}$ dilution was added to each well. Cells were incubated in a CO_2-incubator at 37 °C. After 3 days, 10 μL of MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, purchased from Sigma Chemical Co., St. Louis, MO, USA), prepared in a concentration of 5 mg/mL in phosphate buffered saline (PBS), was added to each well. After another 4 h incubation at 37 °C, 100 μL of extraction buffer (0.02 N HCl solution of 50% DMF containing 20% of SDS) was added to each well to solubilize MTT-formazan. Absorbance at 570 nm (test wave length) and 690 nm (reference wave length) were measured using a microplate reader Tosoh MPR-A4. The percentage of cell growth inhibition was calculated by the following formula: Inhibition (%) = [1-(Tx - C0 / Cx - C0)] x 100: Tx: Absorbance at the end of incubation with test drug. Cx: Absorbance at the end of incubation without drug. C0: Absorbance at beginning incubation. The IC_{50} value of the test compound was determined graphically from dose-inhibition curve.

Growth inhibition test for KB cell.[22] Cell suspension containing 1.1 x 10^5 cells per mL of growth medium was prepared by trypsin digestion of parent culture, and 90 μL of the cell suspension was seeded in a 96 multi-well plate. After 4 h incubation, 10 μL of test compound solution in serial 0.5 $\log_{10}$ dilution was added to each well. The cultures were incubated in a CO_2-incubator at 37 °C for 3 days, and then incubated at 37 °C for 1h with 100 μL of medium containing 0.005% neutral red to stain them after removing culture fluid. The neutral red solution was discarded, and cells were washed with 100 μL of PBS. Dye in each well was extracted with 170 μL of 0.01 N HCl containing 50% ethanol, and absorbance at 550 nm was measured by a microplate reader. Cells stained at the time of replacement with drug containing medium was referred to the value of 0 time. The percentage of cell growth inhibition and the IC_{50} value were determined as described above.

Antiviral assay using HSV-1.[23] Antiviral activity against HSV-1 was examined by a CPE inhibition method as described earlier[22]: HEL cells growing in a 96 multi-well plate were infected with about 100 plaque forming units (p. f. u.) of HSV-1 (VR-3 strain). After 30 min of virus absorption, virus inoculum was discarded, and the infected cells were incubated with a test compound in serial 0.5 $\log_{10}$ dilution in a CO_2-incubator at 37 °C for 2 days. The CPE in each well was determined by microscopic examination. The antiviral activity was expressed as ED_{50} at which HSV-1 induced CPE were suppressed at least 50%.

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