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Synthesis of the Cyclic and Acyclic Acetal Derivatives of 1-(3-*C*-Ethynyl-β-D-*ribo*-pentofuranosyl)cytosine, a Potent Antitumor Nucleoside. Design of Prodrugs to be Selectively Activated in Tumor Tissues Via the Bio-Reduction–Hydrolysis Mechanism†

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Abstract—We have designed and synthesized the acetal derivatives of 1-(3-*C*-ethynyl-β-D-*ribo*-pentofuranosyl)cytosine (ECyd, **1**), the 2′,3′-*O*-nitrobenzylidene derivatives **2** and **3** and the 5′-*O*-(alkoxy)(nitrophenyl)methyl derivatives **6**–**10** as potential prodrugs of ECyd. These prodrugs can be selectively activated in tumor tissues via a bio-reduction–hydrolysis mechanism owing to the characteristic properties of tumor tissues, such as hypoxia and lower pH. Although the 2′,3′-*O*-(4-nitrobenzylidene) derivatives **2** and **3** were converted bio-reductively into the corresponding 4-aminobenzylidene derivatives by rat S-9 mix, the reduction products, that is, the corresponding amino congeners **4** and **5**, proved to be rather stable in an aqueous solution at pH 6.5 used as a pH model for acidic tumor tissues. In contrast, the 5′-*O*-(alkoxy)(4-nitropheny)methyl derivatives **6**–**8** were also reduced by rat S-9 mix to the corresponding amino congeners **11**–**13**, which were hydrolyzed to release ECyd more effectively at pH 6.5 than at pH 7.4. Accordingly, the acyclic acetals **6**–**8** may be efficient prodrugs of ECyd, that are effectively reduced under physiological conditions releasing ECyd in acidic tumor tissues.

Introduction

A number of antitumor agents are widely used today in cancer chemotherapy. However, serious problems limiting their antitumor effect still exist. If an antitumor agent could be selectively distributed to tumor tissues via its prodrug, a masked form of the drug, toxicities against normal tissues could then be decreased and many of these problems would possibly be eliminated, or at least minimized.

In recent years, we have synthesized a variety of sugar-modified nucleoside analogues and have found 1-(3-*C*-ethynyl-β-D-*ribo*-pentofuranosyl)cytosine (ECyd, **1**, Fig. 1)[1] to be a potent antitumor nucleoside that significantly inhibits the growth of various human solid tumor cells both in vitro and in vivo. It is now in clinical trials. Studies on the antitumor mechanism of action of ECyd showed that after being metabolized to its 5′-triphosphate (ECTP), it strongly inhibits RNA synthesis through inhibition of RNA polymerases.[2] This mechanism is different from that of other known antitumor antimetabolites, such as ara-C, 5-FU or gemcitabine. Although ECyd is expected to be an efficient antitumor drug because of its remarkable antitumor effects in experimental models, ECyd is however somewhat toxic to rapidly growing normal host cells. If ECyd distributes selectively to tumor tissues, the toxicity against the normal cells would be decreased. Consequently, a prodrug of ECyd, activated selectively in tumor tissues, is a very attractive alternative.

It is known that tumor tissues have properties different from normal tissues. One such characteristic observed in

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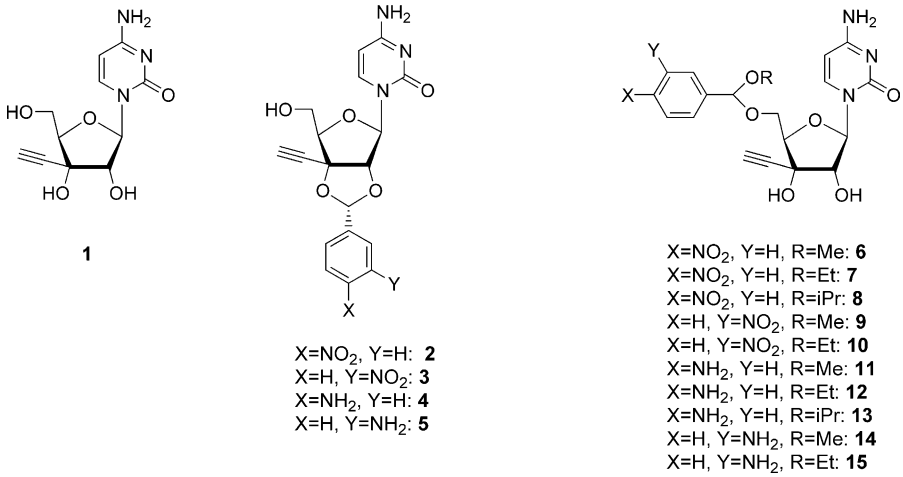


Fig 1. ECyd and its potential prodrugs.

tumor tissues, particularly in solid tumors, is hypoxia, which often decreases the response to chemotherapeutic agents and radioionizing therapy. Under hypoxic conditions, bio-reductive reactions would be promoted,[3] therefore much effort has been devoted to developing antitumor prodrugs which can be activated selectively in tumor tissues with bio-reductive potency. For example, nitrophenyl and quinone derivatives are known to be activated under bio-reductive conditions.[4,5]

Recently, Threadgill and co-workers proposed a potential bio-reductively activated prodrug system using the cyclic acetal of 5-nitrofuranylaldehyde for diol-containing drugs.[6] They hypothesized that the nitro group could be reduced to an amino group in vivo, and that the resulting 5-aminofuranyl compound would be readily hydrolyzed because of the electron-donating effect of the amino group, which produces the deprotected drug having a diol moiety. They confirmed that the 5-nitrofuran-2-ylmethylidene group was hydrolyzed via reduction by a chemical reductant system such as $NaBH_4$/Pd-C using 1,2-dihydroxyphenylethane as a diol model; however, its bio-reduction has not been examined. Such a cyclic acetal system may be suitable for developing effective prodrugs of ECyd, since it has a *cis*-diol moiety in the molecule. Therefore, we designed 2′,3′-*O*-(4-nitrobenzylidene)ECyd (**2**) and its regio-isomer 2′,3′-*O*-(3-nitrobenzylidene)ECyd (**3**) as potential prodrugs producing ECyd in vivo selectively in tumor tissues via the bio-reduction–hydrolysis mechanism. We also designed the acyclic acetal derivatives **6**–**10**, bearing an (alkoxy)(nitrophenyl)methyl group at the 5′-hydroxyl of ECyd (Fig. 1). These acyclic acetals were also expected to produce ECyd in vivo via the bio-reduction–hydrolysis mechanism. We speculated that the 4-nitrophenyl group would be more suitable than the 3-nitrophenyl group in the bio-reduction-hydrolysis strategy, since the bio-reduced 4-amino moiety is able to donate electrons by resonance thus promoting the hydrolysis as shown in Scheme 1.

In this report, we describe the synthesis of these cyclic and acyclic acetal derivatives of ECyd and also their hydrolysis to release ECyd via the bio-reduction–hydrolysis mechanism.

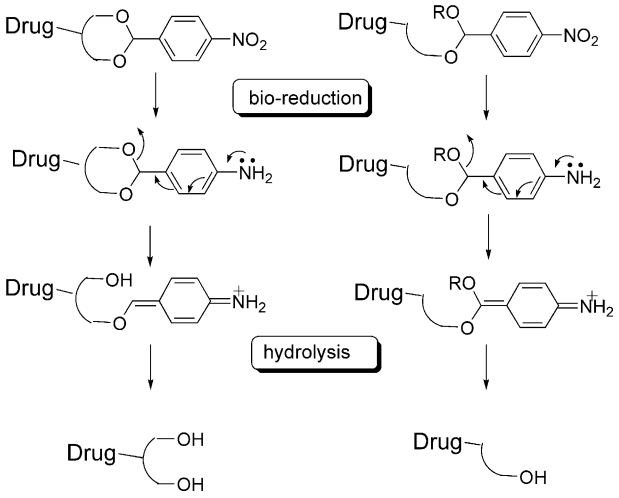
Results and Discussion

Synthesis of the benzylidene derivatives of ECyd

In addition to the target compounds **2**, **3** and **6**–**10** described above, we also planned to synthesize the corresponding amino congeners **4**, **5** and **11**–**15** (Fig. 1), the probable reduction products of **2**, **3** and **6**–**10**, to determine if they could be readily hydrolyzed.

The synthesis of the 2′,3′-*O*-benzylidene derivatives of ECyd **2**–**5** is shown in Scheme 2. Successive treatment of ECyd (**1**) with dimethoxypropane/$HClO_4$ in acetone and BzCl/DMAP/Et_3N in MeCN gave the fully protected ECyd derivative **16**. Removal of the isopropylidene group of **16** with aqueous 90% TFA gave the diol **17**, which was heated with 3- or 4-nitrobenzaldehyde dimethyl acetal in the presence of H_2SO_4 under reduced pressure to give the corresponding 2′,3′-*O*-nitrobenzylidene derivatives **18** and **19**, respectively. Reduction of the nitro groups of **18** and **19** was performed with $Na_2S_2O_4$ in the presence of the electron-phase transfer catalyst **22**[7] to give the aminobenzylidene derivatives **20** and **21**, respectively. Treatment of the benzylidene derivatives **18**, **19**, **20** and **21**, with NaOMe/MeOH afforded the corresponding target compounds, **2**, **3**, **4** and **5**, respectively. The stereochemistry of the benzylidene moiety was confirmed as *endo* by the nOe experiment of **2**, as shown in Figure 1.

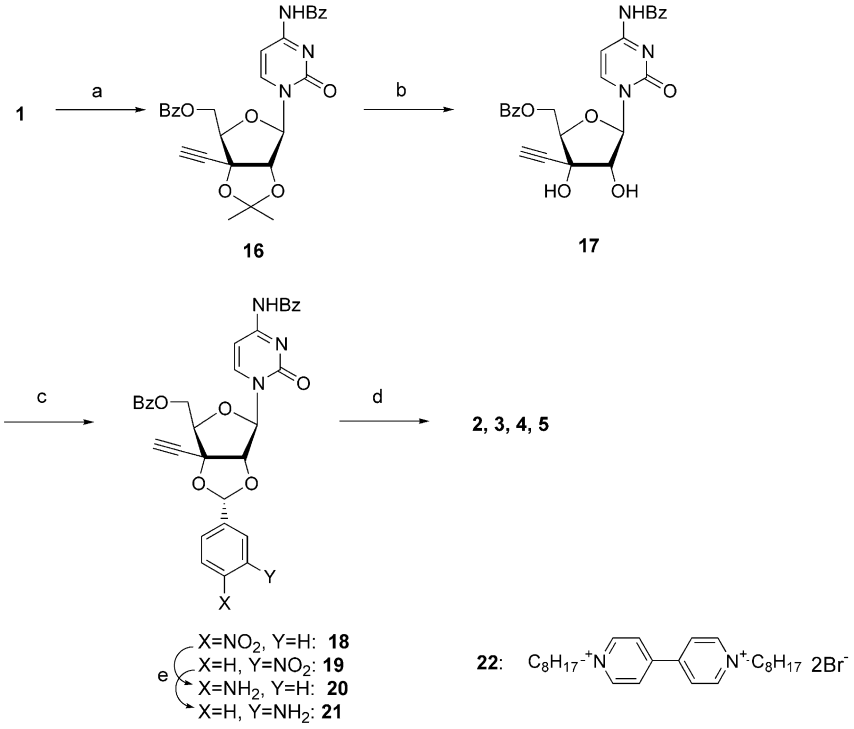
The synthesis of the acyclic acetal derivatives of ECyd **6**–**15** is shown in Scheme 3. ECyd was treated with 3- or 4-nitrobenzaldehyde dialkyl acetal and H_2SO_4 in DMSO under reduced pressure at room temperature to 50 °C to afford the corresponding acyclic acetal derivatives **6**–**10**. Reduction of the nitro group was performed using the same procedure as that for the synthesis of **4** and **5** to afford the 5′-*O*-(alkoxy)(aminophenyl)methyl derivatives **11**–**15**.



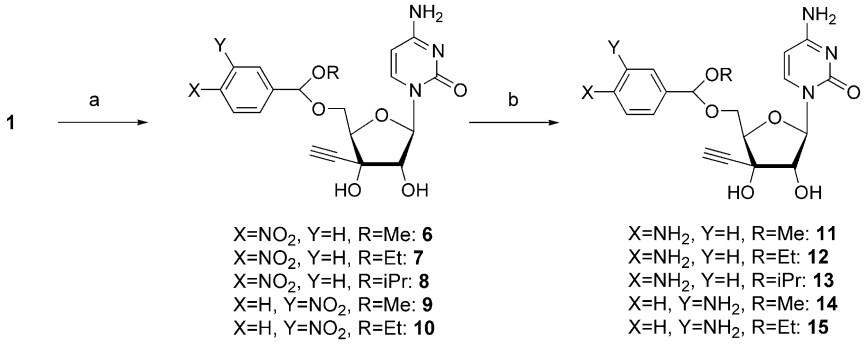
Scheme 1. The bio-reduction-hydrolysis mechanism.

Stability of the acetal derivatives of ECyd in aqueous solution

It is recognized that tumor tissues are often more acidic than normal tissues. The pH values of tumor tissues are usually between 6.5 and 7.0,[8] and sometimes lower (about 6.0).[8a,8c] Since acetals are unstable to hydrolysis under acidic conditions, the designed prodrugs may be hydrolyzed selectively in acidic tumor tissues to release ECyd. Therefore, it would be important to estimate the effect of the pH medium on the cleavage process of the potential prodrugs of ECyd. Two phosphate buffers, one at pH 6.5 as a model for acidic tumor tissues and one at the physiological pH 7.4, were used for the evaluations. The substrates were incubated at 37 °C in these buffers, and the time course of ECyd production was investigated by HPLC. The results are summarized in Table 1.



Scheme 2. Synthesis of the 2′,3′-*O*-benzylidene derivatives of ECyd. (a) (1) $(CH_3)_2C(OCH_3)_2$, acetone, $HClO_4$, (2) BzCl, Et_3N, DMAP, CH_3CN; (b) 90% TFA–H_2O, rt; (c) $RCH(OCH_3)_2$, cH_2SO_4, 90 °C, reduced pressure; (d) MeONa, MeOH; (e) **22**, $Na_2S_2O_4$, K_2CO_3, CH_2Cl_2–H_2O.



Scheme 3. Synthesis of the 5′-acetal derivatives of ECyd. (a) 3- or 4-NO_2-$PhCH(OR)_2$, cH_2SO_4, rt–50 °C, reduced pressure; (b) **22**, $Na_2S_2O_4$, K_2CO_3, CH_2Cl_2–H_2O.

Table 1. Stability of the acetal derivatives of ECyd in aqueous media[a]

Substrate	pH 6.5		pH 7.4	
	$t_{1/2}$ (min)	ECyd release at 2 h (%)	$t_{1/2}$ (min)	ECyd release at 2 h (%)
2	>1000	0	>1000	0
3	>1000	0	>1000	0
4	>1000	<1	>1000	0
5	>1000	0	>1000	0
6	>1000	0	>1000	0
7	>1000	0	>1000	0
8	>1000	0	>1000	0
9	>1000	0	>1000	0
10	>1000	0	>1000	0
11	14	100	74	72
12	8.4	100	32	87
13	4.8	100	20	100
14	>1000	3	>1000	<1
15	535	7	>1000	2

[a]The yields were determined by HPLC analysis.

All of the nitro compounds, that is, the 2′,3′-*O*-nitrobenzylidene derivatives **2** and **3** and 5′-(alkoxy)(nitrophenyl)methyl derivatives **6–10**, were completely resistant to hydrolysis under both of these conditions. The 2′,3′-*O*-(3-aminobenzylidene) derivative **5** was also stable at both pH 6.5 and pH 7.4. The corresponding 4-aminobenzylidene derivative **4** was very slowly hydrolyzed at pH 6.5 to form a trace of ECyd after 2 h, although it was stable at the physiological pH 7.4. We expected the electron-donating amino-substituent to promote the hydrolysis when it was attached at the resonantly effective *para*-position. However, the experiments showed that the rate of ECyd release from the 4-aminobenzylidene derivative **4** was very low.

In contrast, the acyclic acetal derivatives **11–15** were hydrolyzed more rapidly than the cyclic benzylidene derivatives. The 5′-*O*-(alkoxy)(4-aminophenyl)methyl derivatives **11–13** were hydrolyzed completely at pH 6.5 within 2 h, where the $t_{1/2}$ values were 14, 8.4, and 4.8 min, respectively. Although the acyclic acetals **11–13** were also hydrolyzed at the neutral pH 7.4, the rates were slower compared with those under the acidic conditions. Thus, the acyclic acetals **11–13** seemed to be hydrolyzed in acidic tumor tissues more effectively than in neutral normal tissues. On the other hand, the 5′-*O*-(alkoxy)(3-aminophenyl)methyl derivatives **14** and **15** were not readily hydrolyzed at pH 6.5 or pH 7.4.

These results suggest that the acyclic acetal derivatives of ECyd **11–13** having a 5′-*O*-(alkoxy)(4-aminophenyl)methyl group, may release ECyd selectively in acidic tumor tissues, if these are reductively produced from the corresponding nitro derivatives **6–8** in vivo.

Bio-reduction of the acetal derivatives of ECyd by S-9 mix

We next examined whether the cyclic 2′,3′-*O*-nitrobenzylidene and the acyclic 5′-*O*-(alkoxy)(4-nitrophenyl)methyl derivatives of ECyd could be biologically reduced using rat S-9 mix as the model bio-reductant, which is a 9000 g supernatant of rat liver homogenate containing P450 reductases and cofactors. The compounds were incubated with S-9 mix in a sodium phosphate buffer (pH 7.4) at 37 °C, and the resulting reduction products, that is, the corresponding amino congeners, were analyzed by HPLC.[9] The results are shown in Table 2. In the case of the acyclic acetals **6–8** having a 5′-*O*-(alkoxy)(4-nitrophenyl)methyl group, the corresponding reduction products were not detected, probably because they were rapidly hydrolyzed under the reaction conditions. However, one of the hydrolysis products, 4-aminobenzaldehyde, derived from the hydrolysis of the amino congeners **11–13**, was detected and its yield was measured by HPLC (Table 2).

The nitro group of the 2′,3′-*O*-(4-nitrobenzylidene) and -(3-nitrobenzylidene) derivatives **2** and **3** was reduced effectively by S-9 mix to form the aminobenzylidene congeners **4** and **5**, respectively; after 3 h, compounds **4** (41%) and **5** (29%) from **2** and **3**, respectively, were detected by HPLC. The reduction product of **2** was isolated by MCI gel column chromatography and identified as **4** by comparing the ^{1}H NMR and mass spectra and retention time on HPLC with those of a chemically synthesized authentic sample. The acyclic acetals **9** and **10** having a 5′-*O*-(alkoxy)(3-nitrophenyl)methyl group were reduced by S-9 mix as well, but the rates were somewhat lower than those of the 2′,3′-*O*-nitrobenzylidene derivatives; after 3 h, compounds **14** (21%) and **15** (25%) from **9** and **10**, respectively, were formed. Similarly, reduction of the acyclic acetals **6–8** having a 5′-*O*-(alkoxy)(4-nitrophenyl)methyl group to the corres-

Table 2. Bio-reduction of the acetal derivatives of ECyd[a]

Substrate	Product	Yield (%)	
		1 h	3 h
2	**4**	19	41
3	**5**	16	29
6	*p*-NH_2PhCHO	12	16
7	*p*-NH_2PhCHO	11	12
8	*p*-NH_2PhCHO	9	8
9	**14**	10	21
10	**15**	11	25

[a]The yields were determined by HPLC analysis.

ponding amino congeners **11–13** by S-9 mix was suggested, since formation of 4-aminobenzaldehyde was observed after a 3 h reaction in 16, 12 and 8% yields, respectively.

These results suggest that the cyclic and the acyclic acetal derivatives are substrates of P450 and can be converted into the corresponding amino congeners in vivo.

Conclusion

We identified the 5′-*O*-(alkoxy)(4-nitrophenyl)methyl derivatives **6–8** as candidate prodrugs of ECyd to be selectively activated in tumor tissues via the bio-reduction–hydrolysis mechanism owing to such characteristic properties as hypoxia and lower pH of these tissues. The compounds **6–8** were reduced by rat S-9 mix to the corresponding amino congeners **11–13**, which were hydrolyzed to release ECyd more effectively at pH 6.5 than at pH 7.4. This prodrug system with an acyclic acetal of 4-nitrobenzaldehyde would be applicable to other antitumor agents possessing a hydroxyl group. We are now planning to evaluate the antitumor effect of the cyclic and the acyclic acetal derivatives of ECyd.

Experimental

General methods

Melting points were measured on a Yanagimoto MP-3 micromelting point apparatus and are not corrected. The ^{1}H- and ^{13}C NMR spectra were recorded on a Jeol AL-400 (400 MHz) or Jeol JNM-EX 270 (270 MHz) spectrometer with tetramethylsilane (0.00 ppm) as an internal standard. Chemical shifts were reported in parts per million (δ), and signals were expressed as a s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br (broad). Coupling constants (*J*) were reported in Hz. All exchangeable protons were detected by disappearance on the addition of D_2O. Fast atom bombardment mass spectrometry (FAB-MS) was done on a Jeol JMS-HX110 instrument at an ionizing voltage of 70 eV. TLC was done on Merck Silica gel 60 F_{254} precoated plates. The silica gel used for column chromatography was Merck Silica gel 60 (70–230 mesh). The NH-silica gel used for column chromatography was Fuji Silysia DM1020 (100–200 mesh).

4-*N*-Benzoyl-1-(3-*C*-ethynyl-2,3-*O*-isopropylidene-5-*O*-benzoyl-β-D-*ribo*-pentofuranosyl)cytosine (16). To a suspension of ECyd[1b] (**1**, 5.34 g, 20.0 mmol) in a mixture of acetone (150 mL) and 2,2-dimethoxypropane (4.9 mL) was added 70% $HClO_4$ (4.3 mL, 50 mmol). The mixture was stirred at room temperature for 40 min. To the resulting clear solution were added 28% NH_4OH (6 mL) and evaporated. The residue was purified on a silica gel column (33–50% MeOH in $CHCl_3$) to give an $HClO_4$ salt of 1-(3-*C*-ethynyl-2,3-*O*-isopropylidene-β-D-*ribo*-pentofuranosyl)cytosine, a mixture of which, Et_3N (13.9 mL), DMAP (244 mg, 2.0 mmol) and BzCl (5.8 mL, 50 mmol) in CH_3CN (60 mL) was stirred at room temperature for 5 h. After addition of MeOH (6 mL), the reaction mixture was diluted with $CHCl_3$, washed with H_2O, dried (Na_2SO_4), and evaporated. The residue was purified on a silica gel column (20–50% EtOAc in $CHCl_3$) and then crystallized from Et_2O to give pure **16** (6.5 g, 63% as white crystals): mp 207–208 °C; found: C, 65.17; H, 4.88; N, 8.00. $C_{28}H_{25}N_3O_7$ requires C, 65.24; H, 4.89; N, 8.15%; ^{1}H NMR (DMSO-d_6) δ 11.25 (1H, br s, 4-NH), 8.16 (1H, d, J = 7.9 Hz, H-6), 7.97 (2H, d, J = 7.3 Hz, Bz), 7.86 (2H, d, J = 7.3 Hz, Bz), 7.45–7.65 (6H, m, Bz), 7.31 (1H, br d, J = 7.9 Hz, H-5), 5.98 (1H, d, J = 2.0 Hz, H-1′), 5.03 (1H, d, J = 2.0 Hz, H-2′), 4.57–4.69 (3H, m, H-4′, H-5′), 3.99 (1H, s, 3′-ethynyl), 1.48, 1.55 (each 3H, each s, 2′,3′-*O*-isopropylidene); ^{13}C NMR (DMSO-d_6) δ 167.08, 165.11, 163.32, 154.13, 144.98, 133.38, 132.90, 132.64, 129.02, 128.87, 128.60, 128.31, 115.28, 96.28, 91.30, 90.65, 84.77, 82.14, 80.69, 78.54, 63.80, 27.32, 25.93; FAB-MS m/z 516 (MH^+).

4-*N*-Benzoyl-1-[3-*C*-ethynyl-5-*O*-benzoyl-β-D-*ribo*-pentofuranosyl]cytosine (17). A solution of **6** (6.02 g, 11.7 mmol) in aqueous 90% TFA (250 mL) was stirred at room temperature for 1 h and poured into a mixture of CH_2Cl_2 (1 L) and H_2O (1.2 L). The organic layer separated was washed with saturated aqueous $NaHCO_3$, dried (Na_2SO_4), and evaporated. The residue was purified on a silica gel column (10% MeOH in $CHCl_3$) and then crystallized from $CHCl_3$–Et_2O to give pure **17** (4.72 g, 85% as white crystals): mp 208–209 °C; found: C, 62.01; H, 4.26; N, 8.59. $C_{25}H_{21}N_3O_7 \cdot 1/3H_2O$ requires C, 62.37; H, 4.54; N, 8.73%; ^{1}H NMR (DMSO-d_6) δ 11.27 (1H, br s, 4-NH), 8.24 (1H, d, J = 7.9 Hz, H-6), 7.47–8.00 (10H, m, Bz), 7.31 (1H, br d, J = 7.9 Hz, H-5), 6.25 (1H, s, 3′-OH), 6.17 (1H, d, J = 5.9 Hz, 2′-OH), 5.85 (1H, d, J = 4.6 Hz, H-1′), 4.65 (2H, d, J = 4.6 Hz, H-5′), 4.34 (1H, t, J = 4.6 Hz, H-4′), 4.24 (1H, dd, J = 4.6 Hz, 5.9 Hz, H-2′), 3.68 (1H, s, 3′-ethynyl); ^{13}C NMR (DMSO-d_6) δ: 167.19, 165.45, 163.15, 154.52, 145.57, 133.43, 132.94, 132.65, 129.29, 129.11, 128.73, 128.33, 95.98, 89.95, 82.86, 81.94, 78.79, 72.06, 64.66; FAB-MS m/z 476 (MH^+).

4-*N*-Benzoyl-1-[3-*C*-ethynyl-2,3-*O*-(4-nitrobenzylidene)-5-*O*-benzoyl-β-D-*ribo*-pentofuranosyl]cytosine (18). A mixture of **17** (147 mg, 0.30 mmol), 4-nitrobenzaldehyde dimethyl acetal (1.91 g, 9.7 mmol) and cH_2SO_4 (16 μL) in toluene (1 mL) was stirred at 90 °C under reduced pressure for 3.5 h. After addition of Et_3N (92 μL), the resulting mixture was partitioned between $CHCl_3$ and H_2O, and the organic layer was dried (Na_2SO_4) and evaporated. The residue was purified on a silica gel column (20–66% EtOAc in $CHCl_3$) and then crystallized from $CHCl_3$–Et_2O to give **18** (164 mg, 90% as a white powder): mp 201–202 °C; Found: C, 62.17; H, 4.10; N, 8.82. $C_{32}H_{24}N_4O_9 \cdot 1/2H_2O$ requires C, 62.24; H, 4.08; N, 9.07%; ^{1}H NMR (DMSO-d_6) δ 11.24 (1H, s, 4-NH), 8.33 (2H, d, J = 7.6 Hz, Ar), 8.24 (1H, d, J = 7.3 Hz, H-6), 7.93–7.95 (4H, m, Ar, Bz), 7.73 (2H, d, J = 7.6 Hz, Bz), 7.42–7.64 (6H, m, Bz), 7.25 (1H, d, J = 7.3 Hz, H-5), 6.38 (1H, s, Ar-CH), 5.99 (1H, s, H-1′), 5.21 (1H, s, H-2′), 5.04 (1H, dd, J = 2.9, 4.1 Hz, H-4′), 4.75 (1H, dd, J = 2.9, 12.2 Hz, H-5′a), 4.67 (1H, dd, J = 4.1, 12.2 Hz, H-5′b), 4.15 (1H, s, 3′-ethynyl); ^{13}C NMR (DMSO-d_6) δ

167.01, 165.00, 163.37, 154.28, 148.51, 145.41, 141.56, 133.37, 132.90, 132.63, 128.93, 128.69, 128.54, 128.32, 123.67, 104.52, 96.10, 93.29, 91.29, 84.48, 84.41, 81.76, 76.71, 64.19; FAB-MS *m*/*z* 609 (MH^+).

4-*N*-Benzoyl-1-[3-*C*-ethynyl-2,3-*O*-(3-nitrobenzylidene)-5-*O*-benzoyl-β-D-*ribo*-pentofuranosyl]cytosine (19). Compound **19** (165 mg, 90% as white crystals) was prepared from **17** (147 mg, 0.30 mmol) as described for preparing **18** using 3-nitrobenzaldehyde dimethyl acetal instead of 4-nitrobenzaldehyde dimethyl acetal, after purification on a silica gel column (20–66% EtOAc in $CHCl_3$) and subsequent crystallization from MeOH: mp 111–114 °C; found: C, 62.86; H, 4.09; N, 9.13. $C_{32}H_{24}N_4O_9$: C, 63.16; H, 3.98; N, 9.21%; ^{1}H NMR (DMSO-d_6) δ 11.23 (1H, s, 4-NH), 8.45 (1H, d, *J* = 1.0 Hz, Ar), 8.35 (1H, dd, *J* = 1.0, 8.3 Hz, Ar), 8.24 (1H, d, *J* = 7.8 Hz, H-6), 8.12 (1H, d, *J* = 7.8 Hz, Ar), 7.94 (2H, d, *J* = 8.1 Hz, Bz), 7.42–7.82 (9H, m, Bz, Ar), 7.25 (1H, d, *J* = 7.8 Hz, H-5), 6.40 (1H, s, Ar-CH), 6.00 (1H, d, *J* = 1.7 Hz, H-1′), 5.21 (1H, d, *J* = 1.7 Hz, H-2′), 5.05 (1H, dd, *J* = 3.2, 4.6 Hz, H-4′), 4.75 (1H, dd, *J* = 3.2, 12.0 Hz, H-5′a), 4.66 (1H, dd, *J* = 4.6, 12.0 Hz, H-5′b), 4.14 (1H, s, 3′-ethynyl); ^{13}C NMR (DMSO-d_6) δ 167.00, 165.00, 163.37, 154.30, 147.75, 145.44, 136.95, 133.73, 133.36, 132.92, 132.63, 130.34, 128.94, 128.68, 128.56, 128.32, 125.00, 121.85, 104.46, 96.07, 93.39, 91.29, 84.50, 84.40, 81.71, 76.77, 64.19; FAB-MS *m*/*z* 609 (MH^+).

4-*N*-Benzoyl-1-[3-*C*-ethynyl-2,3-*O*-(4-aminobenzylidene)-5-*O*-benzoyl-β-D-*ribo*-pentofuranosyl]cytosine (20). A solution of K_2CO_3 (319 mg, 2.31 mmol) and $Na_2S_2O_4$ (366 mg, 2.1 mmol) in H_2O (2.5 mL) was added to a mixture of **18** (305 mg, 0.50 mmol) and di(C_8H_7)-viologen **22**[7] (5 mg, 9.2 μmol) in a mixture of CH_2Cl_2 (5 mL) and H_2O (1 mL) under N_2 atmosphere. The resulting mixture was stirred at 40 °C for 24 h and then poured into $CHCl_3$ (50 mL). The organic layer separated was washed with H_2O, dried (Na_2SO_4), and evaporated. The residue was purified on a silica gel column (4% MeOH in $CHCl_3$) to give **20** (127 mg, 44% as a colorless amorphous): found: C, 62.68; H, 4.34; N, 8.95. $C_{32}H_{26}N_4O_7{\cdot}1/3CHCl_3$ requires C, 62.80; H, 4.29; N, 9.06%; ^{1}H NMR (DMSO-d_6) δ 11.22 (1H, br s, 4-NH), 8.22 (1H, d, *J* = 7.3 Hz, H-6), 7.95 (2H, d, *J* = 7.6 Hz, Bz), 7.76 (2H, d, *J* = 7.6 Hz, Bz), 7.41–7.65 (6H, m, Bz), 7.26 (1H, d, *J* = 7.3 Hz, H-5), 7.24 (2H, d, *J* = 8.6 Hz, Ar), 6.58 (2H, d, *J* = 8.6 Hz, Ar), 6.01 (1H, s, Ar-CH), 5.99 (1H, d, *J* = 2.3 Hz, H-1′), 5.37 (2H, s, Ar-NH_2), 5.04 (1H, d, *J* = 2.3 Hz, H-2′), 4.92 (1H, dd, *J* = 3.6, 5.3 Hz, H-4′), 4.72 (1H, dd, *J* = 3.6, 12.2 Hz, H-5′a), 4.64 (1H, dd, *J* = 5.3, 12.2 Hz, H-5′b), 4.03 (1H, s, 3′-ethynyl); ^{13}C NMR (DMSO-d_6) δ 166.95, 164.97, 163.21, 154.13, 150.35, 145.29, 133.26, 132.88, 132.53, 128.95, 128.69, 128.49, 128.28, 128.23, 120.97, 113.00, 107.22, 96.11, 93.00, 90.52, 84.37, 83.20, 81.02, 77.42, 64.14; FAB-MS *m*/*z* 579 (MH^+).

4-*N*-Benzoyl-1-[3-*C*-ethynyl-2,3-*O*-(3-aminobenzylidene)-5-*O*-benzoyl-β-D-*ribo*-pentofuranosyl]cytosine (21). Compound **21** (178 mg, 62% as a colorless amorphous solid) was prepared from **19** (305 mg, 0.50 mmol) as described for preparing **20**, after purification on a silica gel column (5% MeOH in $CHCl_3$): Found: C, 62.77; H, 4.31; N, 9.01. $C_{32}H_{26}N_4O_7{\cdot}1/3CHCl_3$ requires C, 62.80; H, 4.29; N, 9.06%; ^{1}H NMR (DMSO-d_6) δ 11.22 (1H, br s, 4-NH), 8.24 (1H, d, *J* = 7.6 Hz, H-6), 7.96 (2H, d, *J* = 7.0 Hz, Bz), 7.76 (2H, d, *J* = 7.3 Hz, Bz), 7.42–7.66 (6H, m, Bz), 7.26 (1H, d, *J* = 7.6 Hz, H-5), 7.09 (1H, dd, *J* = 7.6, 7.9 Hz, Ar), 6.81 (1H, t, *J* = 1.8 Hz, Ar), 6.73 (1H, d, *J* = 7.6, 1.8 Hz, Ar), 6.65 (1H, dd, *J* = 7.9, 1.8 Hz, Ar), 6.03 (1H, s, Ar-CH), 5.97 (1H, d, *J* = 2.1 Hz, H-1′), 5.23 (2H, s, Ar-NH_2), 5.11 (1H, d, *J* = 2.1 Hz, H-2′), 4.96 (1H, dd, *J* = 3.6, 5.1 Hz, H-4′), 4.74 (1H, dd, *J* = 3.6, 12.2 Hz, H-5′a), 4.67 (1H, dd, *J* = 5.1, 12.2 Hz, H-5′b), 4.07 (1H, s, 3′-ethynyl); ^{13}C NMR (DMSO-d_6) δ 167.18, 165.18, 163.48, 148.81, 145.39, 145.29, 135.43, 133.45, 133.08, 132.70, 129.07, 128.87, 128.81, 128.66, 128.40, 115.48, 114.46, 112.15, 106.68, 96.17, 93.26, 90.99, 84.63, 83.69, 81.31, 77.16, 64.21; FAB-MS *m*/*z* 579 (MH^+).

Removal of the benzoyl group (general procedure). A mixture of a substrate (**18**–**21**) (225 μmol) and NaOMe (1.25 M in MeOH, 0.36 mL) in MeOH (7 mL) was stirred at room temperature for 1 h, and the reaction was then quenched with AcOH (30 μL). The mixture was evaporated, and the residue was purified as described below.

1-[3-*C*-Ethynyl-2,3-*O*-(4-nitrobenzylidene)-β-D-*ribo*-pentofuranosyl]cytosine (2). After purification of the residue from the reaction of **18** on a silica gel column developed with 10–20% MeOH in $CHCl_3$, **2** (85 mg, 94% as a colorless solid) was obtained: mp 139–142 °C; Found: C, 50.07; H, 3.95; N, 12.28. $C_{18}H_{16}N_4O_7{\cdot}0.35CHCl_3$ requires C, 49.85; H, 3.73; N, 12.67%; ^{1}H NMR (DMSO-d_6) δ 8.30 (2H, d, *J* = 7.8 Hz, Ar), 7.89 (2H, d, *J* = 7.8 Hz, Ar), 7.66 (1H, d, *J* = 7.3 Hz, H-6), 7.34, 7.29 (each 1H, each br s, 4-NH_2), 6.34 (1H, s, Ar-CH), 6.01 (1H, d, *J* = 3.2 Hz, H-1′), 5.79 (1H, d, *J* = 7.3 Hz, H-5), 5.13 (1H, t, *J* = 5.0 Hz, 5′-OH), 4.97 (1H, d, *J* = 3.2 Hz, H-2′), 4.27 (1H, dd, *J* = 4.2, 5.6 Hz, H-4′), 4.08 (1H, s, 3′-ethynyl), 3.75 (1H, ddd, *J* = 4.2, 5.0, 12.0 Hz, H-5′a), 3.69 (1H, ddd, *J* = 5.0, 5.6, 12.0 Hz, H-5′b); NOE, irradiates the *ortho* proton of the nitrobenzylidene group, observe H-1′ (0.8%); ^{13}C NMR (DMSO-d_6) δ 165.59, 154.65, 148.42, 141.78, 141.19, 128.43, 123.64, 105.24, 94.63, 90.55, 89.25, 85.60, 83.67, 81.44, 77.59, 60.95; FAB-MS *m*/*z* 401 (MH^+).

1-[3-*C*-Ethynyl-2,3-*O*-(3-nitrobenzylidene)-β-D-*ribo*-pentofuranosyl]cytosine (3). After purification of the residue from the reaction of **19** on a silica gel column developed with 10–20% MeOH in $CHCl_3$, 3 (64 mg, 94% as a colorless solid) was obtained: mp 115–120 °C; found: C, 48.67; H, 3.71; N, 11.92. $C_{18}H_{16}N_4O_7{\cdot}1/2H_2O$ requires C, 48.30; H, 3.62; N, 12.18%; ^{1}H NMR (DMSO-d_6) δ 8.32–8.41 (2H, m, Ar), 8.08 (1H, d, *J* = 7.3 Hz, Ar), 7.78 (1H, t, *J* = 7.9 Hz, Ar), 7.66 (1H, d, *J* = 7.6 Hz, H-6), 7.31, 7.26 (each 1H, each br s, 4-NH_2), 6.36 (1H, s, Ar-CH), 6.01 (1H, d, *J* = 3.3 Hz, H-1′), 5.78 (1H, d, *J* = 7.6 Hz, H-5), 5.10 (1H, br s, 5′-OH), 4.96 (1H, d, *J* = 3.3 Hz, H-2′), 4.28 (1H, dd, *J* = 4.0, 6.6 Hz, H-4′), 4.06 (1H, s, 3′-ethynyl), 3.76 (1H, dd, *J* = 4.0, 11.9 Hz,

H-5′a), 3.69 (1H, dd, $J=6.6$, 11.9 Hz, H-5′b); ^{13}C NMR (DMSO-d_6) δ 165.60, 154.66, 147.72, 141.21, 137.11, 133.61, 130.32, 124.90, 121.76, 105.22, 94.60, 90.61, 89.35, 85.68, 83.61, 81.40, 77.65, 60.95; FAB-MS m/z 401 (MH^+).

1-[3-*C*-Ethynyl-2,3-*O*-(4-aminobenzylidene)-β-D-*ribo*-pentofuranosyl]cytosine (4). The residue from the reaction of **20** was partitioned between H_2O and $CHCl_3$, and the aqueous layer was evaporated and purified on an ODS column (0–15% MeCN in H_2O) to give **4** (68 mg, 81% as a colorless solid): Found: C, 55.95; H, 4.93; N, 14.44. $C_{18}H_{18}N_4O_5\cdot H_2O$ requires C, 55.67; H, 5.19; N, 14.43%; ^{1}H NMR (DMSO-d_6) δ 7.63 (1H, d, $J=7.4$ Hz, H-6), 7.32, 7.26 (each 1H, each br s, 4-NH_2), 7.20 (2H, d, $J=7.8$ Hz, Ar), 6.57 (2H, d, $J=7.8$ Hz, Ar), 6.02 (1H, d, $J=3.1$ Hz, H-1′), 5.98 (1H, s, Ar-C<u>H</u>), 5.79 (1H, d, $J=7.4$ Hz, H-5), 5.35 (2H, br s, Ar-N<u>H</u>$_2$), 5.07 (1H, br, 5′-OH), 4.77 (1H, d, $J=3.1$ Hz, H-2′), 4.18 (1H, dd, $J=4.0$, 6.8 Hz, H-4′), 3.96 (1H, s, 3′-ethynyl), 3.74 (1H, dd, $J=4.0$, 12.0 Hz, H-5′a), 3.69 (1H, dd, $J=6.8$, 12.0 Hz, H-5′b); ^{13}C NMR (DMSO-d_6) δ 165.67, 154.75, 150.46, 141.22, 128.35, 121.32, 113.12, 108.11, 94.71, 90.02, 88.99, 85.77, 82.41, 80.73, 78.38, 61.05; FAB-MS m/z 371 (MH^+).

1-[3-*C*-Ethynyl-2,3-*O*-(3-aminobenzylidene)-β-D-*ribo*-pentofuranosyl]cytosine (5). The residue from the reaction of **21** was partitioned between H_2O and $CHCl_3$, and the aqueous layer was evaporated and purified on an ODS column (0–15% MeCN in H_2O) to give **5** (74 mg, 89% as a colorless solid): found: C, 55.86; H, 4.93; N, 14.36. $C_{18}H_{18}N_4O_5\cdot H_2O$ requires C, 55.67; H, 5.19; N, 14.43%; ^{1}H NMR (DMSO-d_6) δ 7.56 (1H, d, $J=7.6$ Hz, H-6), 7.26, 7.22 (each 1H, each br s, 4-NH_2), 6.99 (1H, dd, $J=7.6$, 7.9 Hz, Ar), 6.69 (1H, br, Ar), 6.60 (1H, d, $J=7.6$ Hz, Ar), 6.56 (1H, d, $J=7.9$ Hz, Ar), 5.95 (1H, d, $J=3.0$ Hz, H-1′), 5.93 (1H, s, Ar-C<u>H</u>), 5.73 (1H, d, $J=7.6$ Hz, H-5), 5.14 (2H, s, Ar-N<u>H</u>$_2$), 5.04 (1H, br s, 5′-OH), 4.78 (1H, d, $J=3.0$ Hz, H-2′), 4.12 (1H, dd, $J=4.0$, 6.6 Hz, H-4′), 3.94 (1H, s, 3′-ethynyl), 3.68 (1H, dd, $J=4.0$, 11.9 Hz, H-5′a), 3.61 (1H, dd, $J=6.6$, 11.9 Hz, H-5′b); ^{13}C NMR (DMSO-d_6) δ 165.47, 154.52, 148.57, 140.98, 135.39, 128.66, 115.23, 114.23, 111.91, 107.42, 94.60, 90.37, 88.98, 85.85, 82.60, 80.90, 78.03, 60.95; FAB-MS m/z 371 (MH^+).

Synthesis of the acyclic acetal derivatives 6–10 (general procedure). A mixture of **1** (2.0 g, 7.5 mmol), the corresponding nitrobenzaldehyde dialkyl acetal (10 mmol), and cH_2SO_4 (600 μL, 11.3 mmol) in DMSO (9 mL) was stirred at ambient temperature under reduced pressure for 6 h, and then Et_3N (4.74 mL) was added. The mixture was poured into H_2O (20 mL), and the resulting mixture was extracted with EtOAc. The organic layer was washed with H_2O, dried (Na_2SO_4), and evaporated. The residue was purified on a silica gel column (10–20% MeOH in $CHCl_3$) to give the corresponding acyclic acetal.

1-[3-*C*-Ethynyl-5-*O*-[1-methoxy-1-(4-nitrophenyl)methyl]-β-D-*ribo*-pentofuranosyl]cytosine (6). Compound **6** (996 mg, 31%) was obtained as a colorless foam: found: C, 51.75; H, 4.52; N, 12.65. $C_{19}H_{21}N_4O_8\cdot 1/2H_2O$ requires C, 51.70; H, 4.80; N, 12.69%; ^{1}H NMR (DMSO-d_6) δ 8.23–8.28 (2H, m, Ar), 7.66–7.71 (2.6H, m, Ar, H-6), 7.55 (0.4H, $J=7.3$ Hz, H-6), 7.18 (2H, br s, 4-NH_2), 5.95 (1H, br s, 3′-OH), 5.81–5.88 (2H, m, H-1′, 2′-OH), 5.68–5.70 (1.6H, m, H-5, PhC*H*), 5.55 (0.4H, d, $J=7.3$ Hz, H-5), 4.00–4.10 (2H, m, H-2′, H-4′), 3.68–3.86 (2H, m, H-5′), 3.58 (0.4H, s, 3′-ethynyl), 3.57 (0.6H, s, 3′-ethynyl), 3.32 (3H, s, C*H*$_3$O); FAB-MS m/z 433 (MH^+).

1-[3-*C*-Ethynyl-5-*O*-[1-ethoxy-1-(4-nitrophenyl)methyl]-β-D-*ribo*-pentofuranosyl]cytosine (7). Compound **7** (755 mg, 23%) was obtained as a colorless foam: Found: C, 51.38; H, 5.12; N, 11.89. $C_{20}H_{22}N_4O_8\cdot H_2O$ requires C, 51.72; H, 5.21; N, 12.06%; ^{1}H NMR (DMSO-d_6) δ 8.23–8.27 (2H, m, Ar), 7.66–7.71 (2.7H, m, Ar, H-6), 7.58 (0.3H, $J=7.3$ Hz, H-6), 7.19 (2H, br s, 4-NH_2), 5.95 (1H, br s, 3′-OH), 5.80–5.88 (2H, m, H-1′, 2′-OH), 5.76 (0.3H, s, PhC*H*), 5.73 (0.7H, s, PhC*H*), 5.68 (0.3H, d, $J=7.3$ Hz, H-5), 5.66 (0.7H, d, $J=7.6$ Hz, H-5), 4.01–4.10 (2H, m, H-2′, H-4′), 3.78–3.82 (1.4H, m, H-5′), 3.59–3.72 (2.6H, m, H-5′, $CH_3C\mathit{H}_2O$), 3.57 (0.3H, s, 3′-ethynyl), 3.56 (0.7H, s, 3′-ethynyl), 1.13-1.20 (3H, m, $C\mathit{H}_3CH_2O$); FAB-MS m/z 447 (MH^+).

1-[3-*C*-Ethynyl-5-*O*-[1-isopropoxy-1-(4-nitrophenyl)methyl]-β-D-*ribo*-pentofuranosyl]cytosine (8). Compound **8** (755 mg, 22%) was obtained as a colorless foam: Found: C, 52.52; H, 5.22; N, 11.51. $C_{21}H_{24}N_4O_8\cdot H_2O$ requires C, 52.72; H, 5.48; N, 11.71%; ^{1}H NMR (DMSO-d_6) δ 8.23–8.27 (2H, m, Ar), 7.61–7.71 (2.7H, m, Ar, H-6), 7.58 (0.3H, $J=7.6$ Hz, H-6), 7.19 (2H, br s, 4-NH_2), 5.92 (1H, br s, 3′-OH), 5.78–5.89 (3H, m, H-1′, 2′-OH, PhC*H*), 5.67 (0.5H, d, $J=7.6$ Hz, H-5), 5.60 (0.5H, d, $J=7.6$ Hz, H-5), 3.94–4.10 (3H, m, H-2′, H-4′, $(CH_3)_2C\mathit{H}O$), 3.73–3.75 (1H, m, H-5′), 3.60–3.66 (1H, m, H-5′), 3.57 (0.5H, s, 3′-ethynyl), 3.56 (0.5H, s, 3′-ethynyl), 1.12–1.22 (6H, m, $(C\mathit{H}_3)_2CHO$); FAB-MS m/z 461 (MH^+).

1-[3-*C*-Ethynyl-5-*O*-[1-methoxy-1-(3-nitrophenyl)methyl]-β-D-*ribo*-pentofuranosyl]cytosine (9). Compound **9** (980 mg, 30%) was obtained as a colorless foam: Found: C, 50.04; H, 4.24; N, 12.05. $C_{19}H_{21}N_4O_8\cdot 1/4CHCl_3$ requires C, 50.02; H, 4.42; N, 12.12%; ^{1}H NMR (DMSO-d_6) δ 8.21–8.25 (2H, m, Ar), 7.85–7.89 (1H, m, Ar), 7.67–7.74 (1.7H, m, Ar, H-6), 7.58 (0.3H, $J=7.6$ Hz, H-6), 7.21, 7.17 (each 1H, br s, 4-NH_2), 5.95 (1H, s, 3′-OH), 5.83–5.95 (2H, m, H-1′, 2′-OH), 5.78 (0.3H, s, PhC*H*), 5.76 (0.7H, s, PhC*H*), 5.69 (0.7H, d, $J=7.6$ Hz, H-5), 5.57 (0.3H, d, $J=7.6$ Hz, H-5), 4.00–4.10 (2H, m, H-2′, H-4′), 3.57–3.80 (4H, m, H-5′, $CH_3C\mathit{H}_2O$), 3.54 (0.7H, s, 3′-ethynyl), 3.53 (0.3H, s, 3′-ethynyl), 1.14–1.21 (3H, m, $C\mathit{H}_3CH_2O$); FAB-MS m/z 433 (MH^+).

1-[3-*C*-Ethynyl-5-*O*-[1-ethoxy-1-(3-nitrophenyl)methyl]-β-D-*ribo*-pentofuranosyl]cytosine (10). Compound **10** (1.02 g, 31%) was obtained as a colorless foam: found: C, 52.76; H, 4.96; N, 12.26. $C_{20}H_{22}N_4O_8\cdot 1/2H_2O$ requires C, 52.75; H, 5.09; N, 12.30%; ^{1}H NMR (DMSO-d_6) δ 8.21–8.25 (2H, m, Ar), 7.84–7.89 (1H, m,

Ar), 7.66–7.74 (1.5H, m, Ar, H-6), 7.56 (0.5H, $J=7.3$ Hz, H-6), 7.19, 7.16 (each 1H, br s, 4-NH_2), 5.95 (1H, s, 3′-OH), 5.83–5.95 (2H, m, H-1′, 2′-OH), 5.68–5.71 (1.5H, m, PhC*H*, H-5), 5.56 (0.5H, d, $J=7.3$ Hz, H-5), 4.01–4.10 (2H, m, H-2′, H-4′), 3.67–3.86 (2H, m, H-5′), 3.55 (0.5H, s, 3′-ethynyl), 3.54 (0.5H, s, 3′-ethynyl), 3.33 (3H, m, CH_3O); FAB-MS m/z 447 (MH^+).

Reduction of the acyclic acetals 6–10 (general procedure). A solution of K_2CO_3 (120 mg, 0.87 mmol) and $Na_2S_2O_4$ (140 mg, 0.81 mmol) in H_2O (1 mL) was added to a mixture of a substrate (**6–10**, 0.1 mmol)) and di(C_8H_7)-viologen **22**[7] (2 mg, 3.8 μmol) in a mixture of CH_2Cl_2 (2 mL) and H_2O (0.1 mL) under N_2 atmosphere. The resulting mixture was stirred at room temperature for 2 h and then partitioned between CH_2Cl_2 and H_2O. After washing with CH_2Cl_2 (3×), the aqueous layer was saturated with NaCl and then extracted with THF (4×). The combined THF layer was dried (Na_2SO_4) and evaporated. The residue was purified on a NH-silica gel column (15% MeOH in $CHCl_3$) to give the corresponding reduction product.

1-[3-*C*-Ethynyl-5-*O*-[1-methoxy-1-(4-aminophenyl)methyl]-β-D-*ribo*-pentofuranosyl]cytosine (11). Compound **11** (3 mg, 7.6%) was obtained as a colorless amorphous: ^{1}H NMR (DMSO-d_6) δ 7.74 (0.6H, d, $J=7.3$ Hz, H-6), 7.60 (0.4H, $J=7.3$ Hz, H-6), 7.19 (2H, br s, 4-NH_2), 7.04 (0.8H,d, $J=8.3$ Hz, Ph), 7.03 (1.2H, d, $J=8.3$ Hz, Ph), 6.53 (0.8H, d, $J=8.3$ Hz, Ph), 6.52 (1.2H, d, $J=8.3$ Hz, Ph), 5.90 (0.4H, d, $J=6.3$ Hz, H-1′), 5.88 (0.6H, d, $J=6.3$ Hz, H-1′), 5.80 (2H, br s, 2′-OH, 3′-OH), 5.67 (0.6H, d, $J=7.3$ Hz, H-5), 5.54 (0.4H, d, $J=7.3$ Hz, H-5), 5.38 (0.6H, m, PhC*H*), 5.34 (0.4H, m, PhC*H*), 5.12 (2H, s, PhNH_2), 3.99–4.09 (2H, m, H-2′, H-4′), 3.57–3.74 (2H, m, H-5′), 3.54 (1H, s, 3′-ethynyl), 3.45–3.52 (2H, m, CH_3CH_2O), 1.11 (3H, q, $J=6.6$ Hz, CH_3CH_2O); FAB-MS (negative) m/z 401 [$(M-H)^-$]; FAB-HRMS (negative) 401.1481 [$(M-H)^-$, $C_{19}H_{21}N_4O_6$ requires m/z 401.1461].

1-[3-*C*-Ethynyl-5-*O*-[1-ethoxy-1-(4-aminophenyl)methyl]-β-D-*ribo*-pentofuranosyl]cytosine (12). Compound **12** (3.7 mg, 8.9%) was obtained as a colorless amorphous: ^{1}H NMR (DMSO-d_6) δ 7.74 (0.5H, d, $J=7.6$ Hz, H-6), 7.65 (0.5H, $J=7.6$ Hz, H-6), 7.20, 7.16 (each 1H, each br s, 4-NH_2), 7.04 (2H,d, $J=8.3$ Hz, Ph), 6.53 (1H, d, $J=8.3$ Hz, Ph), 6.52 (1H, d, $J=8.3$ Hz, Ph), 5.90 (0.5H, d, $J=6.6$ Hz, H-1′), 5.89 (0.5H, d, $J=6.6$ Hz, H-1′), 5.85 (2H, br s, 2′-OH, 3′-OH), 5.66 (0.5H, d, $J=7.3$ Hz, H-5), 5.51 (0.5H, d, $J=7.3$ Hz, H-5), 5.33 (0.5H, m, PhC*H*), 5.28 (0.5H, m, PhC*H*), 5.12 (2H, s, PhNH_2), 3.99–4.09 (2H, m, H-2′, H-4′), 3.62–3.77 (2H, m, H-5′), 3.54 (1H, s, 3′-ethynyl), 3.19 (3H, s, CH_3O); FAB-MS (negative) m/z 415 [$(M-H)^-$]; FAB-HRMS (negative) 415.1623 [$(M-H)^-$, $C_{20}H_{23}N_4O_6$ requires 415.1618].

1-[3-*C*-Ethynyl-5-*O*-[1-isopropoxy-1-(4-aminophenyl)methyl]-β-D-*ribo*-pentofuranosyl]cytosine (13). Compound **13** (1.7 mg, 4.0%) was obtained as a colorless amorphous: ^{1}H NMR (DMSO-d_6) δ 7.74 (0.5H, d, $J=7.3$ Hz, H-6), 7.72 (0.5H, $J=7.3$ Hz, H-6), 7.19, 7.15 (each 1H, each br s, 4-NH_2), 7.05 (1H,d, $J=8.3$ Hz, Ph), 7.04 (1H,d, $J=8.3$ Hz, Ph), 6.52 (1H, d, $J=8.3$ Hz, Ph), 6.51 (1H, d, $J=8.3$ Hz, Ph), 5.89 (0.5H, d, $J=6.6$ Hz, H-1′), 5.87 (0.5H, d, $J=6.6$ Hz, H-1′), 5.79 (2H, br, 2′-OH, 3′-OH), 5.66 (0.5H, d, $J=7.3$ Hz, H-5), 5.51 (0.5H, d, $J=7.3$ Hz, H-5), 5.43 (0.5H, m, PhC*H*), 5.37 (0.5H, m, PhC*H*), 5.09 (2H, s, PhNH_2), 3.56–4.08 (5H, m, H-2′, H-4′, H-5′, $(CH_3)_2CHO$), 3.54 (0.5H, s, 3′-ethynyl), 3.53 (0.5H, s, 3′-ethynyl),1.05–1.22 (6H, m, $(CH_3)_2CHO$); FAB-MS (negative) m/z 429 [$(M-H)^-$]; FAB-HRMS (negative) 429.1794 [$(M-H)^-$, $C_{21}H_{25}N_4O_6$ requires 429.1774].

1-[3-*C*-Ethynyl-5-*O*-[1-methoxy-1-(3-aminophenyl)methyl]-β-D-*ribo*-pentofuranosyl]cytosine (14). Compound **14** (2.9 mg, 7.2%) was obtained as a colorless amorphous: ^{1}H NMR (DMSO-d_6) δ 7.72 (0.5H, d, $J=7.6$ Hz, H-6), 7.59 (0.5H, $J=7.6$ Hz, H-6), 7.18 (2H, br s, 4-NH_2), 6.97–7.04 (1H, m, Ph), 6.61 (1H, s, Ph), 6.50–6.54 (2H, d, Ph), 5.93 (1H, s, 3′-OH), 5.89 (0.5H, d, $J=6.3$ Hz, H-1′), 5.87 (0.5H, d, $J=6.3$ Hz, H-1′), 5.81 (1H, br, 2′-OH), 5.68 (0.5H, d, $J=7.6$ Hz, H-5), 5.51 (0.5H, d, $J=7.6$ Hz, H-5), 5.35 (0.5H, m, PhC*H*), 5.31 (0.5H, m, PhC*H*), 5.07 (2H, s, PhNH_2), 3.99–4.09 (2H, m, H-2′, H-4′), 3.56–3.80 (2H, m, H-5′), 3.55 (1H, s, 3′-ethynyl), 3.22 (1.5H, s, CH_3O). 3.21 (1.5H, s, CH_3O); FAB-MS (negative) m/z 401 [$(M-H)^-$]; FAB-HRMS (negative) 401.1495 [$(M-H)^-$, $C_{19}H_{21}N_4O_6$ requires 401.1461].

1-[3-*C*-Ethynyl-5-*O*-[1-ethoxy-1-(3-aminophenyl)methyl]-β-D-*ribo*-pentofuranosyl]cytosine (15). Compound **15** (3.3 mg, 8.0%) was obtained as a colorless amorphous: ^{1}H NMR (DMSO-d_6) δ 7.72 (0.6H, d, $J=7.6$ Hz, H-6), 7.62 (0.4H, $J=7.6$ Hz, H-6), 7.17 (2H, br s, 4-NH_2), 6.96–7.03 (1H,m, Ph), 6.49–6.63 (3H, d, Ph), 5.91 (1H, s, 3′-OH), 5.88 (0.4H, d, $J=6.3$ Hz, H-1′), 5.87 (0.6H, d, $J=6.3$ Hz, H-1′), 5.80 (1H, br s, 2′-OH), 5.67 (0.6H, d, $J=7.6$ Hz, H-5), 5.55 (0.4H, d, $J=7.6$ Hz, H-5), 5.40 (0.6H, m, PhC*H*), 5.36 (0.4H, m, PhC*H*), 5.06 (2H, s, PhNH_2), 3.98–3.98 (2H, m, H-2′, H-4′), 3.57–3.74 (2H, m, H-5′), 3.56 (0.6H, s, 3′-ethynyl), 3.54 (0.4H, s, 3′-ethynyl), 3.45–3.54 (2H, m, CH_3CH_2O), 1.04–1.16 (3H, m, CH_3CH_2O); FAB-MS m/z 455 [$(M+K)^+$]; FAB-HRMS 455.1338 [$(M+K)^+$, $C_{20}H_{24}N_4O_6K$ requires 455.1333].

Stability of the acetal derivatives of ECyd in aqueous solution. An aqueous cytidine solution (0.50 mM, 50 μL) was added to a potassium phosphate buffer (200 mM, pH 6.0 or 7.4, 900 μL) as an internal standard and the solution was preincubated at 37 °C. To the solution was added a solution of a substrate (2.0 mM in DMSO, 50 μL), and the resulting mixture was kept at 37 °C and analyzed by HPLC. HPLC conditions for **2–5**: column, Inertsil ODS-2 5 μm 4.6×150 mm; eluent A, 2% CH_3CN in potassium phosphate buffer (50 mM, pH 7.0); eluent B, 30% CH_3CN in potassium phosphate buffer (50 mM, pH 7.0); gradient, 0–8 min, A 100%, 15–35 min, B 100%; flow rate, 1.0 mL/min; detection, UV (280 nm). Internal standard (cytidine) was eluted at 2.8 min, ECyd at 4.5 min, **2** at 26.0 min, **3** at 25.5 min, **4** at 21.2 min, and **5** at 21.7 min. HPLC conditions for **9–15**: column, Inertsil ODS-2 5 μm 4.6×150 mm; eluent A, 2% CH_3CN in potassium phosphate buffer (50 mM, pH 7.0); eluent B, 40% CH_3CN in potassium phosphate

buffer (50 mM, pH 7.0); gradient, 0–8 min, A 100%, 15–35 min, B 100%; flow rate, 1.0 mL/min; detection, UV (280 nm). Internal standard (cytidine) was eluted at 2.8 min, ECyd at 4.5 min, **6** at 20.0 min, **7** at 21.3 min, **8** at 22.8 and 22.9 min, **9** at 19.9 min, **10** at 21.1 min, **11** at 17.7 min, **12** at 18.6 min, **13** at 19.4 and 19.5 min, **14** at 18.0 min, and **15** at 18.9 min.

Reduction of the acetal derivatives of ECyd by S-9 mix. A mixture of a cofactor solution (0.9 mL) described below and an aqueous substrate solution (2.0 mM, 0.1 mL) was preincubated at 37 °C for 5 min. To the solution was added rat liver S-9 (WAKO, phenobarbital and 5,6-benzoflavone induced, 0.1 mL), and the mixture was kept at 37 °C. The reaction was quenched by an addition of CH_3CN (3 mL) at 0.5, 1 and 3 h. The resulting mixture was centrifuged (6400 rpm, 6 min), and the supernatant (1.2 mL) was dried under N_2 flow at 50 °C. To the residue was added sodium phosphate buffer (200 mM, pH, 6.0, 0.3 mL), and the solution was filtrated through Millipore Ultrafree C3LCC by centrifuging. The filtrate was analyzed by HPLC under the conditions described above. Concentration of the cofactor solution: $MgCl_2$, 8.9 mM; KCl, 37 mM; G-6-P, 5.6 mM; NADPH, 4.4 mM; NADH, 4.4 mM; sodium phosphate buffer (pH 7.4), 11 mM.

Isolation of the product 4 by the S-9 mix reduction of 2. A mixture of a cofactor solution (45 mL) described above, an aqueous solution of **2** (2.0 mM, 5 mL, 10 mmol) and rat liver S-9 (WAKO, phenobarbital and 5,6-benzoflavone induced, 5 mL) was kept at 37 °C for 5 h, and CH_3CN (3 mL) was added. The supernatant was evaporated, and the residue was diluted with H_2O (5 mL) and filtrated through PTFE membrane filter. The filtrate was purified on an MCI gel (CHP-20P) column (1.5 cm I. D. ×15 cm, 0–10% CH_3CN in H_2O) to give **4**, of which ^1H NMR and mass spectra and retention time on HPLC were identical to those of the authentic **4**.

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9. HPLC analysis of ECyd (**1**), which is possible to be produced in the bio-reduction system, was unsuccessful, because cofactors used for the bio-reduction could not be separated from **1** on HPLC.