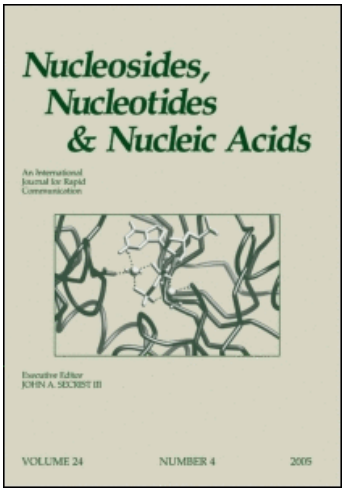

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Hiroshi Shiragami[a]; Takashi Ineyama[a]; Yumiko Uchida[a]; Kunisuke Izawa[a]
[a] Central Research Laboratories, Ajinomoto Co., Inc., Kawasaki, Japan

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SYNTHESIS OF 1-(2,3-DIDEOXY-β-D-*GLYCERO*-PENT-2-ENOFURANOSYL)THYMINE (d4T; STAVUDINE) FROM 5-METHYLURIDINE†

Hiroshi Shiragami, Takashi Ineyama, Yumiko Uchida and Kunisuke Izawa*
Central Research Laboratories, Ajinomoto Co., Inc.
1-1 Suzuki-cho, Kawasaki-ku, Kawasaki 210, Japan

Abstract: A practical synthetic method of d4T (**3**) from 5-methyluridine (**2a**) was developed. The Marumoto-Mansuri method was modified using 2',3'-*O*-methoxyethylidene-5-methyluridine (**10**) as an intermediate to afford 1-(3,5-di-*O*-acetyl-2-bromo-2-deoxy-β-D-ribofuranosyl)thymine (**6a**) in high yield with less formation of by-products. The reaction mechanism was also discussed.

Introduction

Nucleoside analogues, particularly those belonging to the 2',3'-dideoxynucleoside family including 3'-deoxy-3'-azidothymidine (AZT),[1] 2',3'-dideoxyinosine (ddI)[1,2] and 2',3'-dideoxycytidine (ddC),[3] are the only drugs approved for treatment of HIV (human immunodeficiency virus)[4] infection. 1-(2,3-Dideoxy-β-D-*glycero*-pent-2-enofuranosyl)-thymine (d4T; Stavudine)[5] which has an olefinic bond at the 2' and 3' positions of the sugar moiety was recently approved.

d4T is synthesized from thymidine by several methods. For example, processes utilizing ring opening of a 3',5'-anhydro intermediate,[6] β-elimination of a 3'-selenoxide,[7] reductive β-elimination of an O^2,3'-cyclic nucleoside[8] and base-promoted β-elimination of a 3'-mesylate[9] were reported. However, there are several disadvantages, such as its shortage and price considering thymidine as a raw material, because it is usually obtained from natural products such as salmon sperm.

† Dedicated to Dr. Yoshihisa Mizuno on the occasion of his 75th birthday.

Recent development[10] of enzymatic transribosylation of guanosine (**1**) to 5-methyluridine (**2a**) offered us an alternative starting material for thymine nucleosides. Here we wish to report a synthesis of d4T (**3**) from 5-methyluridine (**2a**), which provides a hybrid process[11] to d4T from easily available guanosine (FIG. 1).

Results and Discussion

For the synthesis of d4T from 5-methyluridine (**2a**) processes based on tin hydride reduction,[12a] Zn-Cu reduction[12b] and the Garegg-Samuelsson reaction[12c] were reported. From industrial standpoint, however, these methods have difficulties due to, for instance, special or expensive reagents, low yield and complicated purification.

In order to develop a convenient synthetic route to d4T 1-(3,5-di-*O*-acetyl-2-bromo-2-deoxy-β-D-ribofuranosyl)thymine (**6a**) was considered as a key intermediate. In 1974, Marumoto *et al.*[13] reported a direct synthesis of 1-(3,5-di-*O*-acetyl-2-bromo-2-deoxy-β-D-ribofuranosyl)uracil (**6b**) by reacting uridine (**2b**) with acetyl bromide, and later Mansuri *et al.*[12b] applied this method to 5-methyluridine (**2a**). Although we extensively studied the conditions of this reaction, **6a** could not be obtained in high yield (maximum 48%) and several by-products were formed. A 5'-brominated nucleoside (**7a**), 2',3',5'-tri-*O*-acetyl-5-methyluridine (**8a**) and thymine (**9a**) were found to be major by-products (FIG. 2).

Chu *et al.*[14] improved Marumoto's method by adding HBr/AcOH to the reaction and reported over 95% yield of **6b** from **2b**. When this method was applied to 5-methyluridine (**2a**) and the reaction conditions were optimized, yield of **6a** remained moderate (maximum 63%) and the by-products were still formed in a considerable amount (**7a** 6.3%, **8a** 3.3% and **9a** 8.4%). Trace experiments of Chu's report gave high yield (77.1-78.6%) of **6b** together with 2',3',5'-tri-*O*-acetyluridine (**8b**, 5.2-6.6%) and a 5'-bromide (**7b**, 3.1-3.2%). It is noteworthy that two isomers of **6b** (**X1** and **X2**, 12.9-13.3%) which could not be separated each other were found in the trace reaction. Structures of **X1** and **X2** were determined by mass and ^{1}H-NMR spectra. Mass spectra of **6b** and a mixture of **X1** and **X2** showed the same MH^+ (391 and 393, 1:1) and their fragment patterns were very similar to each other. ^{1}H-NMR spectra of **X1** and **X2** were compared with related compounds (TABLE 1). These spectral data suggest that **X1** and **X2** are isomers of **6b** and have 2'-acetoxy-3'-bromoxylofuranoside and 3'-acetoxy-2'-bromoarabinofuranoside configurations respectively, though the stereochemistry of **X2** is

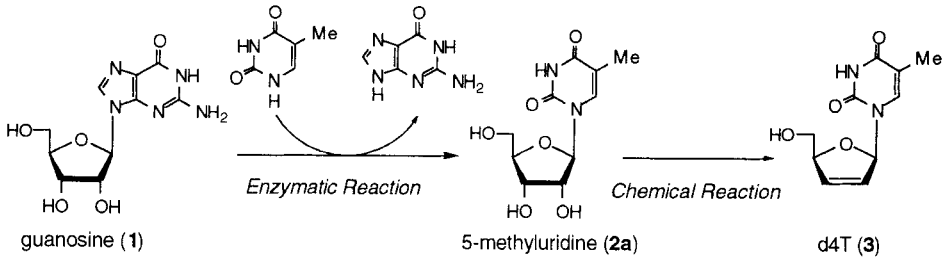


FIG. 1. Hybrid process from guanosine to d4T

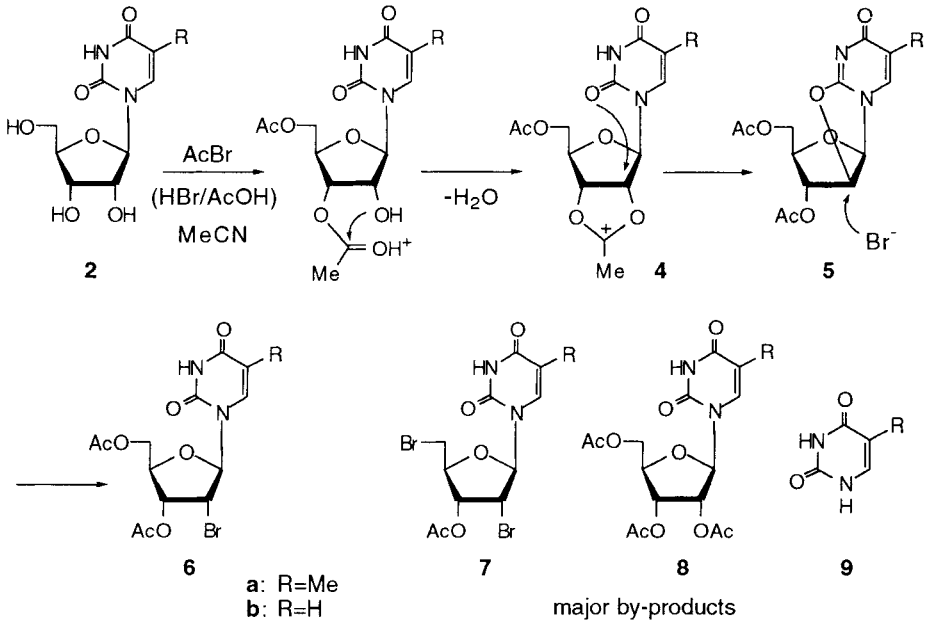


FIG. 2. Reaction of uridine derivatives with acetyl bromide

rather speculative. In the case of 5-methyluridine (**2a**), on the contrary, isomers like **X1** and **X2** were only detected by HPLC (1.6-2.6%). These results suggest that mode of reaction is somewhat different between uridine and 5-methyluridine. It is postulated that the 5-methyl group, electronically and/or sterically, affects the formation of an O^2,2'-anhydro intermediate (**5**) from a dioxolanium cation (**4**) and attack of bromide anion to **4**.

The 5'-brominated by-product (**7a**) was troublesome because it finally gave 5'-bromo-5'-deoxy-d4T which could not be removed effectively by industrially suitable

TABLE 1. ^{1}H-NMR[a] of **X1**, **X2** and related compounds[b]

	X1	**X2**	**6b**	**7b**	**8b**	**12**	**13**	**14**
1'	5.98	5.98	6.20	6.29	6.04	6.29	6.27	6.26
2'	5.43	4.72	4.58	4.51	5.33	5.42	5.41	5.74
3'	4.34	5.39	5.14	5.21	5.33	5.10	5.40	4.44
4'	ca. 4.42	4.24	ca. 4.42	4.40	4.35	4.21	4.52	ca. 4.50
5'a	ca. 4.42	4.51	ca. 4.40	3.77	4.35	4.43	4.34	ca. 4.47
5'b	ca. 4.42	4.51	ca. 4.40	3.75	4.35	4.39	4.03	ca. 4.47
5	5.82	5.78	5.80	5.85	5.79	5.75	6.09	-
6	7.71	7.59	7.44	7.58	7.39	7.51	7.35	-
Ac	2.166	2.161	2.191	2.199	2.150	2.154	2.170	2.186
Ac	2.130	2.140	2.143	-	2.135	2.135	2.017	2.132
Ac	-	-	-	-	2.108	2.049	-	-
*J*1'-2'	2.3	3.7	5.5	7.6	5.0	4.0	5.8	2.3
*J*2'-3'	1.1	1.0	5.9	6.2	nd	1.7	ca. 0	1.4
*J*3'-4'	3.3	2.8	4.9	3.2	nd	3.4	1.4	3.8
*J*4'-5'a	nd[d]	ca. 5	nd	ca. 3	nd	6.5	3.7	nd
*J*4'-5'b	nd	ca. 5	nd	ca. 3	nd	4.2	3.4	nd
*J*5'a-5'b	nd	nd	nd	ca. 12	nd	11.9	12.5	nd
*J*5-6	8.2	8.2	8.2	8.2	8.2	8.2	7.5	-
*J*5-3[c]	yes	yes	yes	yes	yes	yes	no	-

[a] 300MHz; $CDCl_3$; chemical shifts in δ ppm; coupling constants (*J*) in Hz.

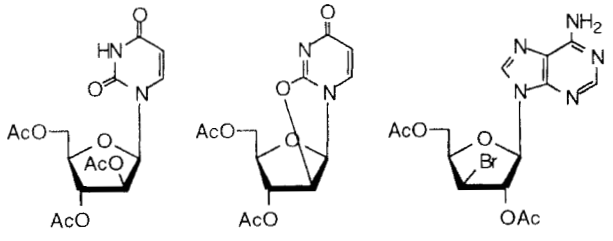
[b] **12** 1-(2, 3, 5-Tri-*O*-acetyl-β-D-arabinofuranosyl)uracil

13 3', 5'-Di-*O*-acetyl-O^2, 2'-cyclouridine

14 9-(2, 5-Di-*O*-acetyl-3-bromo-3-deoxy-β-D-xylofuranosyl)adenine[17]

[c] Coupling between 5-H and 3-NH.

[d] Could not be determined due to overlapping of signals.



12 **13** **14**

purification methods such as extraction and recrystallization. Conversion of 5'-bromo-5'-deoxy-d4T to d4T by hydrolysis of the 5'-bromo group under various conditions was not successful. Compound **7a** was found to have the same configuration as **6a** by ^{1}H-NMR analysis, which suggested that **7a** was formed in a similar fashion with **6a**. It was confirmed that **6a** was not converted into **7a** under the reaction conditions. Thus **7a** was supposed to be formed from a 5'-hydroxy intermediate either by direct bromination or by indirect one *via* an O^2,5'-anhydro compound. The reaction of 5-methyluridine with acetyl bromide produces water *in situ* as is apparent from the mechanism (FIG. 2), which may prevent acetylation of the 5'-position and consequently cause the formation of **7a**. Based on this hypothesis we investigated an alternative indirect route for the synthesis of **6a**, which consists of methoxyethylidenation of 5-methyluridine (**2a**), followed by acetoxy bromination (FIG. 3). Since formation of the intermediary dioxolanium cation (**4**) gives no water in this reaction, formation of **7a** should be diminished.

The results are summarized in TABLE 2. 5-Methyluridine (**2a**) was reacted with trimethyl orthoacetate in acetic acid at 50 °C for 1 hour. The methoxyethylidene derivative (**10**) was isolated from the reaction mixture, but it could be used for further reaction simply after concentrating the reaction mixture under reduced pressure. The residue was then incubated with acetyl bromide and/or HBr/AcOH at 50-60 °C for 4 hours. As a result **6a** was obtained in high yields (81-91%) and the formation of **7a** was successfully diminished (0-2.2%) as expected. Best results were obtained when a mixture of acetyl bromide and HBr/AcOH was used (entries 2, 3 and 4). The yield of **6a** was significantly decreased by addition of water (entry 8), but the yield of **7a** was not so affected as expected from the hypothesis. Addition of methanol showed no decrease of **6a** (entry 9). Therefore a considerable part of low yield by the Marumoto-Chu method can be attributed to the presence of water which may affect the formation and degradation of **4**. Another possibility that the cyclic intermediate (**10**) gives **4** more efficiently than the direct reaction should also be considered. The cause of the formation of **7a** is yet to be revealed.

Several methods for reductive β-elimination of acetoxy-bromo nucleosides were reported. For example Robins *et al.*[15] and Mansuri *et al.*[12b] used Zn-Cu couple and Amino *et al.*[16] used viologen. These methods, especially if scaled up, seem to have serious problems such as lability of the olefinic product under the reaction conditions and complicated processes for removal of waste metal complex. We examined a more convenient method using zinc powder as a reducing agent and ethylenediamine tetraacetic

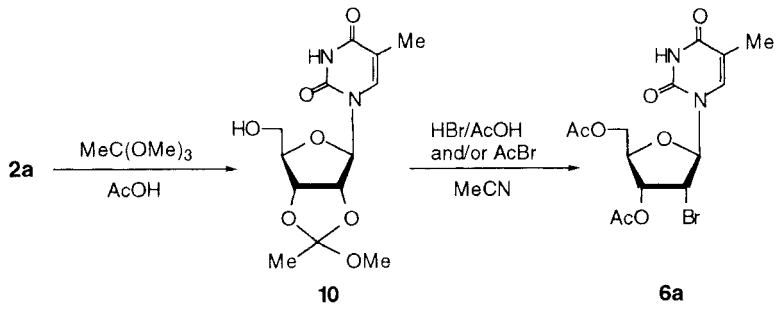


FIG. 3. Synthesis of **6a** *via* methoxyethylidenation

TABLE 2. Synthesis of **6a** from 5-methyluridine (**2a**) *via* **10**[a]

entry	reagent (equiv.)			yield (%)[b]			
	AcBr	HBr/AcOH	Ac_2O	**6a**	**7a**	**8a**	**9a**
1	3	0	0	81.0	0.1	3.6	0.3
2	3	1	0	90.5	0.0	2.6	6.0
3	2	2	0	90.0	1.3	1.8	4.7
4	1	3	0	91.0	1.9	1.5	4.5
5	0	4	0	81.2	2.2	1.6	4.3
6	0	4	1	81.7	1.5	2.2	3.9
7	0	3	1	88.3	1.3	2.0	4.8
8[c]	3	0	0	68.5	1.3	6.5	9.7
9[d]	3	0	0	87.5	0.5	2.1	7.4

[a] Concentrated crude **10** was used.
[b] Based upon **2a**.
[c] Water (1.0 equiv.) was added to **10**.
[d] Methanol (1.0 equiv.) was added to **10**.

acid (EDTA) as a metal-extracting agent. The β-elimination proceeded smoothly by adding zinc powder to **6a** in acetonitrile at room temperature and more than 90% yield of 5'-*O*-acetyl-d4T (**11**) was formed (FIG. 4). After completion of the reaction the mixture became homogeneous and waste zinc was effectively removed by washing the reaction mixture with aqueous EDTA sodium salt. d4T acetate (**11**) thus obtained was hydrolyzed with aqueous NaOH and finally purified with a synthetic adsorption resin to give d4T.

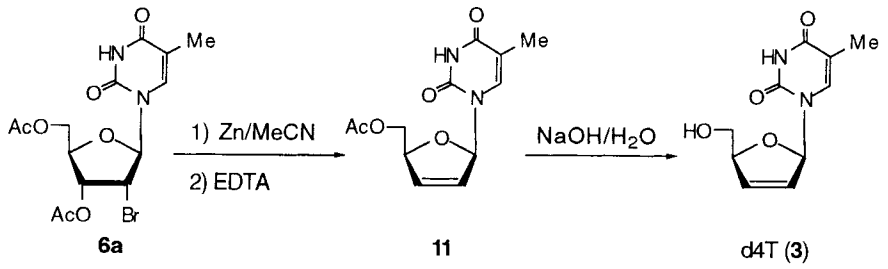


FIG. 4. Synthesis of d4T from **6a**

In conclusion, d4T was successfully obtained from 5-methyluridine in four steps. Combined with the enzymatic transribosylation this synthesis provided an economical route to d4T from guanosine which is industrially produced in a large scale fermentation.

The acetoxy-bromo compound (**6a**) can also be a useful intermediate for other thymine nucleosides such as thymidine and AZT. Synthesis of such nucleosides from 5-methyluridine is under investigation.

Experimental Section

Uridine was purchased from Yamasa Shoyu Co. and was used without further purification. Melting points were determined on a Yamato melting point apparatus and are not corrected. Ultraviolet absorption spectra were recorded on a Hitachi U-3200 spectrophotometer. ^{1}H-NMR spectra were obtained with a Varian Gemini 300 (300MHz), JEOL JNM-GX400 (400MHz) or Bruker AMX600 (600MHz) spectrometer using tetramethylsilane (in $CDCl_3$ and Me_2SO-d_6) or sodium 3-(trimethylsilyl)propanesulfonate (in D_2O) as an internal standard. Mass spectra (MS) were obtained with a JEOL JMS-DX300 spectrometer with fast atom bombardment (FAB) ionization.

5-Methyluridine (2a). 5-Methyluridine was prepared according to the literatures[10] and dried under reduced pressure for 2 h at 140 °C. mp. 183-184 °C (Lit.[18] 183-185 °C); $\lambda_{max}(H_2O)$ 267 nm (ε 1.00x10^5), $\lambda_{min}(H_2O)$ 234 nm (ε 2.46x10^3); ^{1}H-NMR (Me_2SO-d_6) δ 1.77 (s, 3H, CH_3), 3.54 (ddd, 1H, J =12.2, 4.9, 3.4 Hz, H-5'a), 3.63 (ddd, 1H, J =12.2, 5.4, 3.4 Hz, H-5'b), 3.82 (dt, 1H, J =3.9, 3.4 Hz, H-4'), 3.97 (dd, 1H, J =4.9, 3.9 Hz, H-3'), 4.03 (dt, 1H, J =5.9, 5.4 Hz, H-2'), 5.05 (d, 1H, J =4.9 Hz, 3'-OH), 5.09 (dd, 1H, J =5.4, 4.9 Hz, 5'-OH), 5.32 (d, 1H, J =5.9 Hz, 2'-

OH), 5.78 (d, 1H, J =5.4 Hz, H-1'), 7.73 (d, 1H, J =1.0 Hz, H-6), 11.29 (s, 1H, NH); MS m/z 259 (MH^+).

2',3'-*O*-Methoxyethylidene-5-methyluridine (10). To **2a** (2.58 g, 10.0 mmol) in acetic acid (5ml, 8.7 equiv.) was added trimethyl orthoacetate (1.73 ml, 1.4 equiv.), and the mixture was stirred at 50 °C for 1 h. The reaction mixture was concentrated under reduced pressure. Chloroform was added to the residue and the mixture was washed with brine. The organic layer was separated and concentrated. Purification by silica gel column chromatography (eluent $CHCl_3$/MeOH, 10/1) gave **10** (white crystals, 3.0 g, 95%) as a mixture of two diastereomers. mp. 114-120 °C; $\lambda_{max}(H_2O)$ 266 nm (ε 9.75x10^3), $\lambda_{min}(H_2O)$ 234 nm (ε 2.53x10^3); ^{1}H-NMR ($CDCl_3$) **A** δ 1.68(s, 3H, CH_3), 1.91 (br s, 3H, 5-CH_3), 3.27 (s, 3H, OCH_3), 3.82 (dd, 1H, J =12, 4 Hz, H-5'a), 3.92 (dd, 1H, J =12, 3 Hz, H-5'b), 4.24 (m, 1H, H-4'), 5.11 (dd, 1H, J =6.8, 3.7 Hz, H-3'), 5.18 (dd, 1H, J =6.8, 2.7 Hz, H-2'), 5.51 (d, 1H, J =2.7 Hz, H-1'), 7.16 (br s, 1H, H-6), 9.8 (br s, 1H, NH). **B** δ 1.61 (s, 3H, CH_3), 1.89 (br s, 3H, 5-CH_3), 3.38 (s, 3H, OCH_3), 3.82 (dd, 1H, J =12, 4 Hz, H-5'a), 3.93 (dd, 1H, J =12, 3 Hz, H-5'b), 4.37 (m, 1H, H-4'), 5.05 (dd, 1H, J =7.6, 4.1 Hz, H-3'), 5.15 (dd, 1H, J =7.6, 3.0 Hz, H-2'), 5.67 (d, 1H, J =3.0 Hz, H-1'), 7.18 (br s, 1H, H-6), 9.8 (br s, 1H, NH); MS m/z 315 (MH^+).

1-(3,5-Di-*O*-acetyl-2-bromo-2-deoxy-β-D-ribofuranosyl)thymine (6a). To a suspension of **2a** (5.0 g, 19.4 mmol) in acetic acid (50 ml) was added trimethyl orthoacetate (3.45 ml, 27.1 mmol) and the mixture was stirred at 50 °C for 1 h. The reaction mixture was concentrated to 8.5 g under reduced pressure. To the residual solution was added 40 ml of acetonitrile and the resulting solution was heated to 50 °C. A mixture of 30% HBr/AcOH (15.7 g, 3 equiv.) and AcBr (1.43 ml, 1 equiv.) was added dropwise at 50 °C during 1 h. The reaction temperature was raised to 60 °C and the mixture was stirred for another 4 h, then cooled to 10 °C and 10 ml of water was added. Neutralization with 25% aqueous NaOH followed by usual workup afforded **6a** (7.54 g 17.5 mmol, 90% from **2a**). mp. 128-131 °C (Lit.[12b] 55-57 °C); $\lambda_{max}(H_2O)$ 264 nm (ε 9.59x10^3), $\lambda_{min}(H_2O)$ 233 nm (ε 2.73x10^3); ^{1}H-NMR (600MHz, $CDCl_3$) δ 1.97 (s, 3H, 5-CH_3), 2.17 (s, 3H, Ac), 2.21 (s, 3H, Ac), 4.38 (m, 3H, H-4' and 5'ab), 4.54 (dd, 1H, J =6.1, 6.1 Hz, H-2'), 5.18 (dd, 1H, J =6.1, 3.7 Hz, H-3'), 6.22 (d, 1H, J =6.1 Hz, H-1'), 7.19 (s, 1H, H-6), 8.56 (br s, 1H, NH); MS m/z 405, 407 (1:1, MH^+).

1-(3-*O*-Acetyl-2,5-dibromo-2,5-dideoxy-β-D-ribofuranosyl)thymine (7a). This by-product was isolated by silica gel column chromatography from the

reaction mixture of **6a** which was obtained by Marumoto's procedure[15] from 5-methyluridine (**2a**). $\lambda_{max}(H_2O)$ 262 nm (ε 7.90x10^3), $\lambda_{min}(H_2O)$ 234 nm (ε 3.33x10^3); ^{1}H-NMR (600MHz, $CDCl_3$) δ 1.97 (s, 3H, 5-CH_3), 2.21 (s, 3H, Ac), 3.77 (m, 2H, H-5'ab), 4.38 (dt, 2H, J =2.9, 2.9 Hz, H-4'), 4.55 (dd, 1H, J =7.6, 6.6 Hz, H-2'), 5.24 (dd, 1H, J =6.6, 2.9 Hz, H-3'), 6.30 (d, 1H, J =7.6 Hz, H-1'), 7.39 (s, 1H, H-6), 9.61 (br s, 1H, NH); MS *m/z* 424, 426, 428 (1:2:1, MH$^+$).

1-(2,3-Dideoxy-β-D-*glycero*-pent-2-enofuranosyl)thymine (d4T; 3). To a solution of **6a** (6.21 g, 15.4 mmol) in acetonitrile was added 2.35 g (37.0 mmol, 2.4 equiv.) of zinc powder, and the mixture was stirred at room temperature for 2 h. Examination by HPLC showed the reaction completed. To the reaction mixture was added aqueous EDTA sodium salt separately prepared as follows: To EDTA•2Na•2H_2O (26.80 g, 74 mmol) in 100 ml of water was added 25% NaOH to make pH 7.9. The organic layer was separated and the aqueous layer was extracted twice with 50 ml of acetonitrile. The organic layers were combined and concentrated, followed by addition of aqueous 25% NaOH to make pH 12. The solution was stirred for 30 min and then 1M HCl was added to make pH 7.0. Examination by HPLC showed that 2.76 g (12.3 mmol) of d4T (**3**) had been produced in the solution (80% yield). The resulting solution was purified with Diaion SP-207 (Mitsubishi Chemical Co.) synthetic adsorption resin (resin volume 160 ml, eluted with water and aqueous 10-40% methanol) and finally crystallized from water to give **3** as white crystals (72%) which was identical in ^{1}H-NMR and HPLC analyses with an authentic sample prepared from thymidine.[6c] mp. 165-167 °C (Lit.[6c] 165-166 °C); $\lambda_{max}(H_2O)$ 266 nm (ε 9.79x10^3), $\lambda_{min}(H_2O)$ 235 nm (ε 2.55x10^3); ^{1}H-NMR (Me_2SO-d_6) δ 1.73 (d, 3H, J =1.0 Hz, CH_3), 3.60 (dd, 2H, J =4.9, 3.4 Hz, H-5'ab), 4.78 (br s, 1H, H-4'), 5.01 (dd, 1H, J =5.4, 4.9 Hz, OH), 5.91 (ddd, 1H, J =5.9, 2.0, 1.5 Hz, H-2'), 6.39 (dt, 1H, J =5.9, 1.5 Hz, H-3'), 6.82 (dt, J =3.4, 1.5Hz, 1H, H-1'), 7.64 (d, 1H, J =1.0 Hz, H-6), 11.29 (s, 1H, NH); MS *m/z* 225 (MH$^+$).

1-(5-*O*-Acetyl-2,3-dideoxy-β-D-*glycero*-pent-2-enofuranosyl)-thymine (5'-*O*-acetyl-d4T; 11). By the same procedure for **3** except hydrolysis by aqueous 25% NaOH, **11** was obtained. mp. 167-168 °C (Lit.[12b] 179-181 °C); $\lambda_{max}(H_2O)$ 266 nm (ε 9.27x10^3), $\lambda_{min}(H_2O)$ 235 nm (ε 2.47x10^3); ^{1}H-NMR (Me_2SO-d_6) δ 1.78 (d, 3H, J =1.5 Hz, CH_3), 2.02 (s, 3H, Ac), 4.18 (dd, 1H, J =12.3, 3.2 Hz, H-5'a), 4.22 (dd, 1H, J =12.3, 4.4 Hz, H-5'b), 4.97 (br s, 1H, H-4'), 6.01 (ddd, 1H, J =5.9, 2.4, 1.5 Hz, H-2'), 6.41 (ddd, 1H, J =5.9, 2.0, 1.5 Hz, H-3'), 6.81 (ddd, 1H,

J=3.4, 2.0, 1.5 Hz, H-1'), 7.26 (d, 1H, J=1.0 Hz, H-6), 11.38 (s, 1H, NH); MS *m/z* 267 (MH^+). *Anal.* Calcd for $C_{12}H_{14}N_2O_4$: C, 54.13; H, 5.30; N, 10.52. Found: C, 54.20; H, 5.19; N, 10.34.

Reaction of uridine (2b) with AcBr and HBr/AcOH. The same reaction conditions as reported by Chu *et al.*[14] were used. To a suspension of **2b** (4.89 g, 20.0 mmol) in dry acetonitrile (122 ml, dried over molecular sieves 4A) was added 30% HBr/AcOH (4.89 ml) and the mixture was heated to 55 °C. Acetyl bromide (4.85 ml) was added dropwise to the mixture at 55 °C during 2 h. The resultant solution was heated at 55-60 °C for 3 h, then cooled to room temperature. HPLC analysis showed formation of **6b** and by-products as follows: **6b** 79%, **7b** 3%, **8b** 5% and two isomers of **6b** (**X1** and **X2**) 13%. It should be noted that ratio of the by-products to **6b** was in a same range during the reaction (**7b**:**8b**:**X1**+**X2**:**6b**=3.6-4.3:6.9-6.5:16.6-16.3:100). The solution was added slowly to water (40 ml) with simultaneous neutralization by 25% aqueous NaOH (3-5 °C, pH 3.7-4.3), and pH was finally adjusted to 6.8. The organic layer was separated and concentrated *in vacuo* to give crude product. Analytical samples of **6b**, **7b**, **8b** and a 10:3 mixture of **X1** and **X2** were isolated from the crude product by preparative thin layer chromatography (E. Merck Kieselgel 60 F254 2mm thickness, developed with ethyl acetate). **X1** and **X2** could not be separated each other even by a reversed phase HPLC.

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