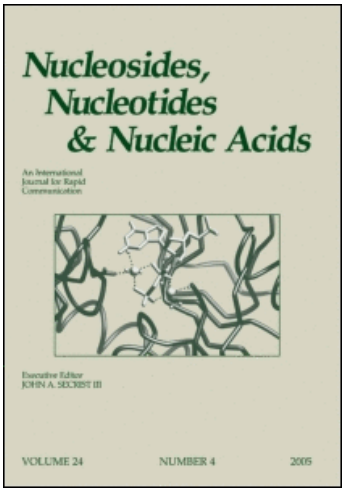

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SYNTHESIS OF A NEW 5'-*O*-TRIPHOSPHATE ANALOG OF 5-METHYL 2'-*O*-DEOXYCYTIDINE. PRELIMINARY IN VITRO LABELING FOR NON-RADIOACTIVE DETECTION OF DNA

Ch. Rodríguez-Tanty[a]; R. Pérez[a]; J. Miranda[b]; H. Vélez-Castro[a]; A. Rosado[a]; A. Macías[a]; V. Vidal-Alles[c]; M. J. Araña[c]; N. Pérez Souto[d]; D. Higginson-Clarke[b]; A. M. Riverón[b]
[a] Organic Chemistry Department, National Center for Scientific Research, Havana, Cuba [b] Molecular Biology Department, National Center for Scientific Research, Havana, Cuba [c] Biological Research Center, Havana, Cuba [d] Drug Research and Development Center, Havana, Cuba

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SYNTHESIS OF A NEW 5′-*O*-TRIPHOSPHATE ANALOG OF 5-METHYL 2′-*O*-DEOXYCYTIDINE. PRELIMINARY IN VITRO LABELING FOR NON-RADIOACTIVE DETECTION OF DNA

Ch. Rodríguez-Tanty,[1,*] R. Pérez,[1] J. Miranda,[2] H. Vélez-Castro,[1] A. Rosado,[1] A. Macías,[1] V. Vidal-Alles,[3] M. J. Araña,[3] N. Pérez Souto,[4] D. Higginson-Clarke,[2] and A. M. Riverón[2]

[1]Organic Chemistry Department
[2]Molecular Biology Department, National Center for Scientific Research, P.O. Box 6880, Havana, Cuba
[3]Biological Research Center, P.O. Box 6996 Havana, Cuba
[4]Drug Research and Development Center, P.O. Box 10600, Havana, Cuba

ABSTRACT

We report the synthesis of the triphosphate of 5-methyl 4-*N*-[6-(*p*-bromobenzamido)hex-1-yl]-2′-*O*-deoxycytidine 3**A**. We also analyzed the formation of intramolecular H-bonds of 5-methyl 4-*N*-{*n*-[6-(*p*-bromobenzamido) caproyl amino]alk-1-yl}-2′-deoxycytidine compounds, and confirmed their presence by ^{1}H-NMR studies. *In vitro* DNA labeling with modified nucleotides is preliminarily evaluated.

The labeling of nucleosides and nucleotides with haptenic groups creates new reagents for non-radioactive DNA detection based on immunochemical methods. Consequently, the efforts to efficiently synthesize nucleoside analogs and to improve the sensitivity of the methods for the immunological detection of labeled DNA molecules have considerably increased during the last decade.[1–4]

*Corresponding author.

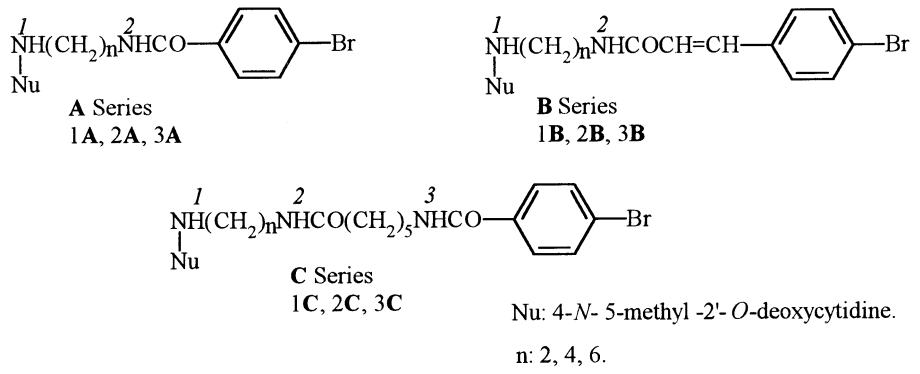


Figure 1. Structures of 5-methyl 4-*N*-[*n*-(*p*-bromobenzamido)alk-1-yl]-2′-*O*-deoxycytidine (**A** *series*); 5-methyl 4-*N*-[*n*-(*p*-bromocinnamoylamino)alk-1-yl]-2′-*O*-deoxycytidine (**B** series) and 5-methyl 4-*N*-{*n*-[-ε-(*p*-bromobenzamido) caproylamino]alk-1-yl}-2′-*O*-deoxycytidine (**C** series), *n*: 2, 4 or 6.

We previously reported the obtainment of the monoclonal antibody (MAb) 5H11E5 that recognized the hapten 6-(*p*-bromobenzamido) caproyl radical[5] (BLC). BLC was introduced at the 4-position of 5-methyl-2′-*O*-deoxycytidine through spacer arms of different sizes. We obtained three series of nucleosides containing the BLC radical (Fig. 1, **A**, **B** and **C**), defined their relative affinities for 5H11E5, and determined that in the antigen-antibody reaction it binds the *p*-bromobenzoyl fragment of BLC.

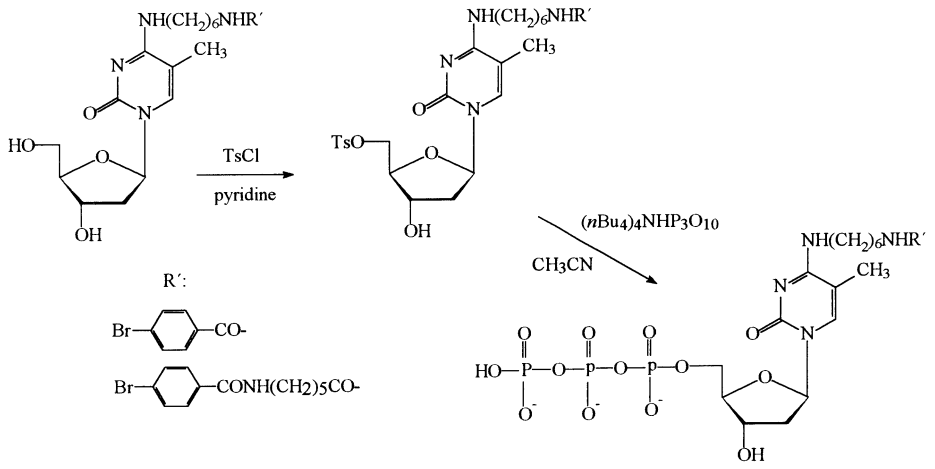
Here, we selected the 5-methyl 4-*N*-[6-(*p*-bromobenzamido)hex-1-yl]-2′-*O*-deoxycytidine (3**A**) and the 5-methyl 4-*N*-{6-[6-(*p*-bromobenzamido) caproylamino]hex-1-yl}-2′-*O*-deoxycytidine (3**C**)[6,7] for phosphorylation. Both analogs were well recognized by the MAb and their spacer arms were separated from the nucleoside by 7 and 14 atoms, lengths that have been considered optimal for sensitively detecting the label.[8,9] Additionally, we evaluated the *in vitro* DNA labeling using the triphosphate nucleotide.

RESULTS AND DISCUSSION

Chemical Discussion

The triphosphate synthesis of 3**A** and 3**C** were attempted according to literature procedures.[10–13] (*Scheme 1*).

We performed the synthesis of 5′-*O*-tosyl 5-methyl 4-*N*-[6-(*p*-bromobenzamido)hex-1-yl]-2′-*O*-deoxycytidine (**7**) as described.[14] Work up of the reaction mixture, followed by silica gel column chromatography yielded 32% of **7**. Its structure was determined by ^{1}H-NMR and FAB-MS ($m/z = 679$ $M + 1^+$) spectra. However, we could not obtain the tosyl derivative of (3**C**), despite temperature increasing during the reaction and the addition of catalytic amounts of 4-(dimethylamino)pyridine.



Scheme 1.

This result suggests that the BLC radical is occluding the 5′-hydroxylic group, inhibiting the tosylation by steric hindrance, similarly to the results obtained by Cosstick and Douglas,[15] who failed to introduce the dimethoxytritylate group in position 5 of 6-*N*-(2-aminoethyl)-2′-*O*-deoxyadenosine possibly, according to the authors, because the 2-phtalimidoethyl group partially occludes the 5′-hydroxy function. Probably in our case, the steric hindrance is due to the formation of H-bonds between the hydrogen atom of any of the NH groups and the oxygen atom of any of the carbonyl groups of the nucleoside.

To verify whether intramolecular hydrogen bonds are indeed formed in **3C**, we studied the ^{1}H-NMR spectra of the 3′- and 5′-diacetylated compounds of **C** series: 3′-, 5′-di-*O*-acetyl 5-methyl-4-N-{2-[-ε-(*p*-bromobenzamido) caproylamino]eth-1-yl}-2′-*O*-deoxycytidine (**4**), 3′-, 5′-di-*O*-acetyl 5-methyl-4-*N*-{4-[-ε-(*p*-bromobenzamido) caproylamino]but-1-yl}-2′-*O*-deoxycytidine (**5**), 3′, 5′-di-*O*-acetyl 5-methyl-4-*N*-{6-[-ε-(*p*-bromobenzamido) caproylamino] hex-1-yl}-2′-*O*-deoxycytidine (**6**). The spectra were recorded in $CDCl_3$ (in order to eliminate the solvation of the polar groups of the molecule with the solvent) and $CDCl_3$-D_2O at different temperatures.

The assignments of the protons of the different NH groups were carried out by the analysis of the H-H COSY spectra of all the compounds of series **A**, **B**, and **C**.

The protons of the 1-NH groups of **4**, **5** and **6** displayed similar chemical shifts when the spectra were recorded in $CDCl_3$ at 296 K (Table 1, rows 1, 2 and 3). Under the same experimental conditions, the protons of the 2-NH group also had similar chemical shift values (Table 1, rows 4, 5 and 6). However, the signal of the proton of the 3-NH group of **4** was shifted downfield compared to the signals of the protons of the remaining analogs

Table 1. Chemical Shift (δ, ppm) in ^{1}H-NMR Spectra of the H of NH Groups of the Compounds of 3′, 5′-Di-*O*-Acetyl 5-Methyl-4-*N*-{*n*-[-6-(*p*-bromobenzamido) Caproyl-amino]alk-1-yl}-2′-*O*-deoxycytidine, (C series), Recorded in $CDCl_3$ at 296 K and in $CDCl_3$-D_2O at 296, 305, 310 and 315 K

Compounds	NH groups	$CDCl_3$ 296 K	$CDCl_3$-D_2O 296 K	$CDCl_3$-D_2O 305 K	$CDCl_3$-D_2O 310 K	$CDCl_3$-D_2O 315 K
4	*1*	6,66	–	–	–	–
5		7,15	7,2	7,09	7,05	–
6		7,00	7,0	*	6,8	–
4	*2*	7,29	7,2	7,16	–	–
5		7,47	7,47	7,39	7,33	–
6		7,65	7,70	7,52	–	–
4	*3*	7,02	7,06	7,00	6,96	6,92
5		6,20	–	–	–	–
6		5,97	–	–	–	–

*The NMR spectrum of **6** was not recorded at 305 K. – proton signal disappeared.

(**5** and **6**) (Table 1, rows 7, 8 and 9). On the other hand, when the spectra were recorded in $CDCl_3$-D_2O at 296 K the protons of some NH groups were not exchanged with deuterium atoms. In particular, the protons of 1-NH and 2-NH groups of **5** and **6** (Table 1, rows 2, 3 and 5, 6) and the protons of 2-NH and 3-NH groups of **4** (Table 1, rows 4 and 7). Therefore, these protons are forming intramolecular H-bonds that avoid fast isotopic exchange. However, the isotopic exchanges occurred when we increased the temperature of the experiment, excluding the proton of the 3-NH group of compound **4**, (Table 1, columns 3, 4 and 5). This result indicated the high strength of the H-bond formed in **4**.

Although we did not use X ray diffraction techniques to determine the spatial conformation adopted by **4**, **5** and **6** molecules, the ^{1}H-NMR spectral data suggest the presence of the intramolecular H-bonds. These H-bonds in the molecules of **C** series hide the 5′-hydroxyl group and thus hinder the synthesis of the tosylate derivatives.

Synthesis of 5-methyl 4-N-[6-(p-bromobenzamido)hex-1-yl]-2′-O-deoxycytidine 5′-O-triphosphate (**8**): It was done as described[10]. At the beginning of the reaction two new compounds, having similar R_f. in TLC, were obtained. They were isolated according to literature procedure[10] and further purified through a Sephadex G10 column.

The structures of diphosphate and triphosphate of **3A** were determined by FAB mass-spectroscopy. See for instance, the FAB mass spectrum of **8** (Fig. 2). The structure of **8** was also corroborated by[31] P-NMR spectroscopy[10] and the compound was pure according HPLC-UV chromatography.

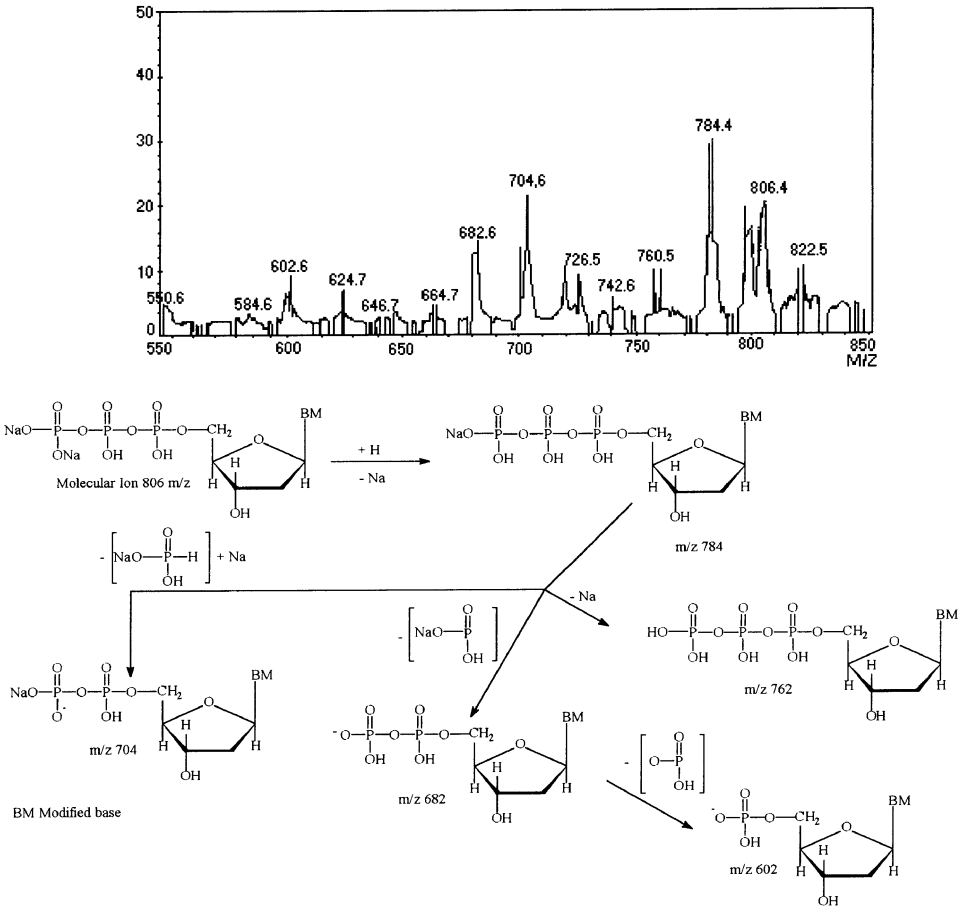


Figure 2. FAB mass spectrum (negative ions) and the most probably fragmentation scheme of 5-methyl-4-*N*-[-6-(*p*-bromobenzamido)hex-1-yl]-2′-*O*-deoxycytidine -5′-*O*-Triphosphate (**8**).

Biological Discussion

In vitro DNA incorporation of the modified nucleotide triphosphate (8): By comparing the thymidine, cytidine and modified nucleotide (**8**) structures, three important features can be noted: 1) as cytidine, the O^2, N^3 and N^4 atoms of **8** seem to behave in hydrogen bonding as proton-acceptor, proton-acceptor and proton-donor, respectively; 2) the BLC is attached to the N^4 atom of **8** and might influence its proton-donor capacity; and 3) as thymidine, **8** has a CH_3 group at the 5-position of the pyrimidine base. Therefore, compound **8** has a pyrimidine structure that resembles both the thymidine and cytidine nucleosides. Consequently, we performed random priming *in vitro* labeling experiments[15], in which we evaluated the incorporation of **8** to DNA as thymidine and cytidine analogs.

Incorporation of 8 to DNA when it replaced dCTP or dTTP in double labeling experiments, using [α-^{32}P]dATP as a tracer radionucleotide. To determine whether **8** acts as a thymidine or a cytidine analog, we designed a double labeling experiment which uses [α-^{32}P]dATP as a tracer radionucleotide and the Bam H1 restriction fragment of pBluescript plasmid as DNA template (Table 2, Experimental part). Assuming 100% labeling efficiency the incorporation of [α-^{32}P]dATP to the template in the random primer reactions with dNTPs; when **8** was used instead of dTTP, the mean labeling efficiency was nearly 42%, but it was slightly larger (49%) when **8** substituted dCTP (Fig. 3).

Detection level of DNA molecules in hybridization experiments using the probes simultaneously labeled with 8 and [α-^{32}P]dATP. We also made hybridization experiments using the single labeled (control +) and double labeled Bam H1 fragment of pBluescript plasmids (Lb1 and Lb2). In the experiments, a solution containing 5 ng of the unlabeled plasmid was two fold serially diluted and spotted onto nylon membrane (up to 2.5 pg, Fig. 4). After hybridization, the intensities of the radioactive signals in the autoradiographies decreased proportionally with the amount of DNA spotted onto the filters (Fig. 4). When the single labeled fragment (control +) was used in the hybridization solution, the radioactive signals appeared up to 2.5 pg of DNA (upper part of Fig. 4).

Similar results were obtained when the Bam H1 fragment was double labeled with a [α-^{32}P] dATP and the nucleotide **8** replacing dCTP (Lb2 Fig. 4); however, the radioactive signals were considerably more intense in the control +. When we used a [α-^{32}P]dATP and **8** instead of dTTP to double label the Bam H1 fragment, the smallest detectable amount of DNA in the autoradiography was 5 pg (Lb1 of Fig. 4). These results indicated that **8** behaves as both pyrimidine nucleotides, thymidine and cytidine, as previously discussed, though it replaces cytidine more efficiently in labeling reactions.

Table 2. Schematic Representation of the Nucleotides Used in the Reaction Mixtures for In Vitro Labeling of DNA by Random Primer Method[15]

	Nucleotides Used in the Random Priming Reaction				
Experiment	DGTP	dTTP	dCTP	[α-^{32}P]dATP	Nucleotide (**8**)
Control (+)	X	X	X	X	–
Double Lb_1	X	–	X	X	X
Double Lb_2	X	X	–	X	X

Lb: Labeling. Nucleotide (**8**): 5-methyl-4-*N*-[-6-(*p*-bromobenzamido)hex-1-yl]-2′-*O*-deoxycytidine 5′-*O*-Triphosphate (**8**).

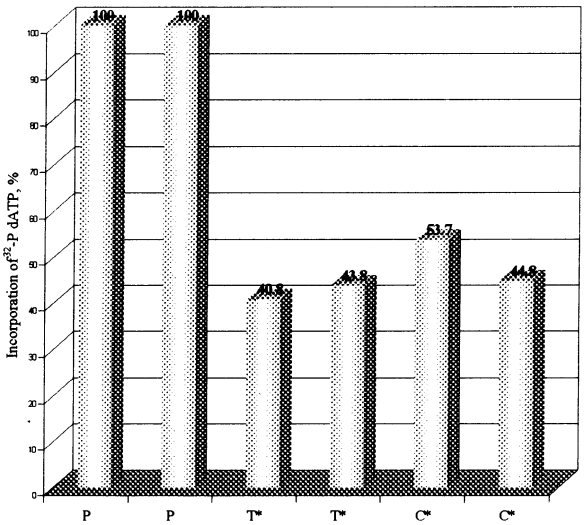


Figure 3. Labeling efficiency of [α-^{32}P]dATP to Bam H1 restriction fragment of pBluescript plasmid DNA in single (P) and double labeling (T* and C*) experiments (control +, Lb1 and Lb2, respectively in Table 2). In Lb1, 5-methyl-4-*N*-[-6-(*p*-bromobenzamido)hex-1-yl]-2′-*O*-deoxycytidine 5′-*O*-Triphosphate (**8**) was used as a thymide analog, in Lb2, **8** was used as cytidine analog. The percents were calculated as: for T*: [CPM(Lb1)/CPM(control +,)] × 100, for C*: [CPM(Lb2)/CPM(control +)] × 100 and for P: [CPM(control +)/CPM(control +)] × 100. CPM: counts per minute determined by Cerenkov's method [17] in the scintillation counter.

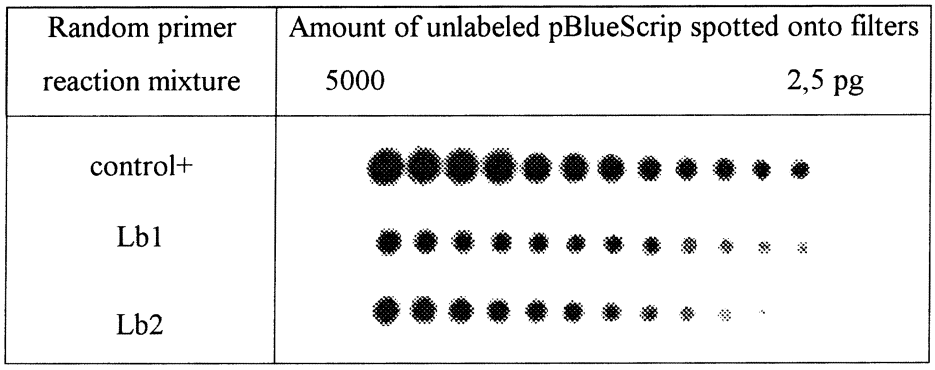


Figure 4. Results obtained in the autoradiographies after hybridizing pBlueScript plasmid DNA (the target) with its labeled Bam H1 restriction fragment (the probe). Control +: the probe was labeled with [α-^{32}P] dATP alone. Lb2 and Lb1: the probe was labeled with [α-^{32}P] dATP and with 5-methyl-4-*N*-[-6-(*p*-bromobenzamido)hex-1-yl]-2′-*O*-deoxycytidine 5′-*O*-Triphosphate (**8**). **8** was added to the reaction mixtures instead of dCTP or dTTP, respectively.

The ability of **8** to replace dCTP and dTTP in DNA synthesis could be advantageous to label DNA and to improve the sensitivity of non-radioactive DNA detection methods. Although the results of the hybridization experiments were satisfactory (Fig. 4) in our experiments, the mistakes introduced in the nucleotide sequences while the polymerase is copying the DNA template may cause incorrect base pairing between the complementary strands and could interfere with DNA renaturation. In addition, if **8** is used for Polymerase Chain Reaction (PCR), where DNA molecules need to be copied several times, the fidelity of the copies could be compromised. The results also suggest that **8** and its corresponding nucleoside analog might have mutagenic activity.

EXPERIMENTAL

Chemical Synthesis. General Procedures

The ^{1}H-NMR and ^{13}C-NMR spectra (δ, ppm) were obtained on a Bruker Model AC 250 F spectrometer with TMS as internal reference at 250 and 62.89 MHz, respectively. The ^{31}P-NMR spectra were obtained at 109,25 MHz on a Jeol Model GX-270 (85% H_3PO_4, as external reference). The assignment in ^{1}H-NMR spectra was mainly based on the off diagonal correlation peaks encountered in the H-H-COSY experiments. Used Keys: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet and AA′BB′, *p*-substituted aromatic system. In the ^{13}C-NMR spectra the following abbreviations were used to describe the different substitutions at C-atom, which were made with the aid of DEPT experiments: p, primary carbon; s, secondary carbon; t, tertiary carbon and c, quaternary carbon. NMR-Exchange experiments were made with heavy water 99.98% D at the indicated temperatures. Fast-Atom-Bombardment mass spectra (FAB-MS, positive and negative ions) were recorded on a JEOL spectrometer model JMS-HX-110 (glycerol as matrix, 10 KeV, Xe atoms). IR (cm^{-1}) spectra were recorded in KBr tablets on a Carl Zeiss SPECORD 71 IR spectrophotometer (Key: s, strong; m, medium and w, weak). Melting points (uncorrected) were determined on an Electrothermal apparatus. Pyridine and acetonitrile were dried and stored according to described procedures.[5,6] Paper chromatography to detect phosphorus compounds was carried out with Whatman N° 4 paper (solvent system i-PrOH: H_2O: 20% NH_3: Cl_3CCOOH 50-16.6-0.2-3.33), and the spot were visualized by means of sprinkling the paper with two solutions: 1)- 1% of ammonium molybdate in 3 M HCl and 2)- 1% of ascorbic acid in water. Thin layer chromatography (TLC) was performed on precoated aluminum sheets of silica gel 60 F_{254} (*E. Merck*, solvent system $CHCl_3$: MeOH 10:1). Dowex AG 50W-X8 cation exchange resin column was purchased from Bio-Rad Laboratories (100–200 mesh) and was used as H^+ and NH_4^+ forms. The desired triphosphate

compound was purified through a Sephadex G10 column that was equilibrated with distilled water. Its purity was assessed by reverse phase high-performance liquid chromatography with UV detection (HPLC-UV). The chromatographic system consisted of an isocratic pump (*Merck*, model L-7110), connected to an injector (*Rheodyne*, 77251) and a multiwavelength detector (*Knauer*, model 87.00). The obtained data was processed by means of an integrator (*Shimadzu*, model C-R6A). Two chromatographic Merck's columns were used (both 5 μm particle size, 250 by 4 mm i.d.): Superspher 60 RP-8 and Lichrospher 100 RP-18. Several solvent systems were studied and the effluent was monitored at the following wavelength: 210, 254 and 280 nm. The flow rate was usually set to 1 ml/min, and 20 μl were injected at concentrations of 10, 100, and 500 μg/ml of mobile phase. No impurities were detected in all experiments.

All reagents were obtained from commercial suppliers and were pure for synthesis. Sodium tripolyhosphate was recrystallized six times from water-ethanol and its purity was determined by paper chromatography.

Acetylation of 5-methyl 4-*N*-{*n*-[-ε-(*p*-bromobenzamido) caproylamino]-alk-1-yl}-2′-*O*-deoxycytidine. General Procedure.

The acetylation of the compounds of the **C** series[6] were done as reported[17].

3′-, 5′-di-*O*-Acetyl 5-methyl-4-*N*-{2-[-ε-(*p*-bromobenzamido)caproylamino] eth-1-yl}-2′-*O*-deoxycytidine (**4**).

IR (KBr, cm^{-1}): 3500 s, 3400 s, 2940 m, 2820 m, 1740 m, 1620 s, 1540 m, 1520 m. ^{1}H-NMR ($CDCl_3$/299 K): 7.72 (2H, AA′, *o*-CO-CH=); 7.50 (2H, BB′, *m*-CO-CH=); 7.29 (1H, m, 6-CH); 7.19 (1H, broad t, 2-NH); 6.88 (1H, broad t, 3-NH); 6.50 (1H, broad t, 1-NH); 6.37 (1H,d,d, $J_{H1'H2''} = 8.19$; $J_{H1'H2''} = 5.50$; 1-CH); 5.19 (1H, d,t, $J_{H3'H2'} = 6.13$; $J_{H3'H2''} = J_{H3'H4'} = 1.62$; 3′-CH); 4.35 (2H, d, $J_{H5'H4'} = 3.72$; 5′-CH_2); 4.22 (1H, m, 4′-CH); 3.58 (2H, m, CH_2NH-1); 3.40 (2H, t, CH_2NHCO); 2.53 (1H, d,d,d, $J_{H2'H2''} = 14.19$; 2′-H); 2.23 (2H, t, NHCOCH_2); 2.13 (3H, s, CH_3CO); 2.10 (3H, s, CH_3CO); 2.05 (1H, m, 2″-H); 1.95 (3H, broad s, CH_3); 1.65 (4H, m, 2 CH_2); and 1.4 (2H, m, CH_2). FAB-MS (m/z) = 665 $(M + 1)^+$.

3′-, 5′-di-*O*-Acetyl 5-methyl-4-*N*-{4-[-ε-(*p*-bromobenzamido) caproylamino] but-1-yl}-2′-*O*-deoxycytidine (**5**).

IR (KBr, cm^{-1}): 3400 s; 2950 m; 2850 m; 1740 s, 1640 s, 1540 s; 1520 s. ^{1}H-NMR ($CDCl_3$/299 K): 7.78 (2H, AA′, *o*-CO-CH=); 7.52 (2H, BB′,

m-CO-CH=); 7.28 (1H, m, 6-CH); 7.15 (1H, broad t, 2-NH); 6.65 (1H, broad t, 1-NH); 6.35 (1H, d,d, $J_{H1'H2'} = 8.21$; $J_{H1'H2''} = 5.52$; 1′-CH); 5.65 (1H, broad t, 3-NH); 5.2 (1H, d,t, $J_{H3'H2'} = 6.13$; $J_{H3'H2''} = J_{H3'H4'} = 1.62$; 3′-CH); 4.34 (2H, d, $J_{H5'H4'} = 3.74$; 5′-CH_2); 4.25 (1H, m, 4′-CH); 3.46 (4H, m, 2 CH_2NHCO); 3.28 (2H, t, CH_2NH-1); 2.55 (1H, d,d,d, $J_{H2'H2''} = 14.17$; 2′-H); 2.25 (2H, t, NHCOCH_2); 2.10 (3H, s, CH_3CO); 2.08 (3H, s, CH_3CO); 2.05 (1H, m, 2″-H); 1.95 (3H, broad s, CH_3); 1.70 and 1.35 (10H, m, 5 CH_2). FAB-MS $(m/z) = 693$ $(M+1)^+$.

3′-, 5′-di-*O*-Acetyl 5-methyl-4-*N*-{6-[-ε-(*p*-bromobenzamido) caproylamino] hex-1-yl}-2′-*O*-deoxycytidine (**6**).

IR (KBr, cm^{-1}): 3320 s, 3100 m, 2950 s, 2860 s, 1700 s, 1640 s, 1540 s, 1500 s. ^{1}H-NMR ($CDCl_3$/298 K): 7.75 (2H, AA′, *o*-CO-CH=); 7.50 (2H, BB′, *m*-CO-CH=); 7.42 (1H, m, 6-CH); 7.42 (1H, broad t, 2-NH); 6.65 (1H, broad t, 1-NH); 6.35 (1H, d,d, $J_{H1'H2'} = 8.17$; $J_{H1'H2''} = 5.52$;1′-CH); 5.62 (1H, broad t, 3-NH); 5.19 (1H, d,t, $J_{H3'H2'} = 6.15$; $J_{H3'H2''} = J_{H3'H4'} = 1.63$; 3′-CH); 4.38 (2H, d, $J_{H5'H4'} = 3.70$; 5′-CH_2); 4.25 (1H, m, 4′-CH); 3.45 (4H, m, 2 CH_2NHCO); 3.25 (2H, q, CH_2NH-1); 2.56 (1H, d,d,d, $J_{H2'H2''} = 14.2$; 2′-H); 2.25 (2H, t, NHCOCH_2); 2.15 (3H, s, CH_3CO); 2.12 (3H, s, CH_3CO); 2.05 (1H, m, 2″-H); 1.95 (3H, broad s, CH_3); 1.75 and 1.25 (14H, m, 7 CH_2). FAB-MS *(m/z) = 720 (M + 1)* $^+$.

5′-*O*-Tosyl 5-methyl 4-*N*-[-6-(bromobenzamido) hex-1-yl]-2′-*O*-deoxycytidine (**4**):

The reaction with **3A**[6] was done as reported[14]. The product was purified by silica gel column chromatography ($CHCl_3$:MeOH from 99:1 to 97:3). IR (KBr, cm^{-1}): 3500 s, 2980 m, 1640 s, 1560 m, 1520 m. ^{1}H-NMR (DMSO/298 K): 8.55 (1H, s, 2-NH); 7.8–7.5 (9H, m, Ts + *p*-BrBz), 7.5 (1H, s, 6-CH); 7.28 (1H, s, 1-NH); 6.20 (1H, m, 1′-CH); 5..41 (1H, d, 3′-OH); 4.21 (2H, m, 5′-CH_2); 3.86 (1H, m, 4′-CH); 3.28 (5H, m, 3′-CH + 2 CH_2NH); 2.51 (3H, s, *p*-CH_3Ph); 2.07 and 1.38 (2H, m, 2′-CH_2); 1.84 (3H, s, CH_3). FAB-MS $(m/z) = 679$ $(M+1)^+$.

Tetra (tetra-*n*-butylammonium) Hydrogen Triphosphate:

The synthesis was accomplished according to a described procedure[10]. The yield of the lyophilized product was 85%. It was stored over P_2O_5 at −20 °C until used. The purity of the compound was determined by paper chromatography. FAB-MS $(m/z) = 1224$ $(M+2)^+$. Anal. calcd for $C_{64}H_{145}N_4O_{10}P_3$: C: 62,90; H: 11.86; N: 4.58; O: 13.09; P: 7.60. Found: C: 61.90; H: 11.01; N: 4.67.

5-Methyl-4-*N*-[-6-(*p*-bromobenzamido)hex-1-yl]-2′-*O*-deoxycytidine 5′-*O*-Tri phosphate (**8**):

To a solution of **7** (0.536 mmol) in CH_3CN (0.6 mL) was added 3.338 mmol of tetra (tetra-*n*-butylammonium) hydrogen triphosphate, and the reaction mixture was stirred at room temperature for 67 h. CH_3CN was evaporated under reduced pressure, the remaining oily syrup was dissolved in 0.5 mL of water, and the solution was loaded onto the ion exchange column (NH_4^+ form). The product was eluted with 5 mL of water and the collected fractions were pooled and further lyophilized. The white solid obtained in the previous step was dissolved in distilled water, loaded onto a Sephadex G 10 column and eluted with water. The fractions of the first peak were pooled and lyophilized. The yield was 19%. The purity of the compound was determined by HPLC-UV chromatography. ^{31}P-NMR (D_2O): -10. 193 (α-P); -11.069 (γ-P); -22.462 (β-P). FAB-MS (m/z) = 806 $(M-1)^-$.

Biological Procedures

Bacterial Strains. An *Escherichia coli* XL1-Blue strain (New England Biolabs Inc.) containing an amp^r plasmid (pBluescript KS vector) was used.

Plasmid Extraction. The pBluescript plasmid was obtained by the alkaline lysis method,[19] and it was finally purified by gel filtration as previously described.[20]

Labeling Procedures. The Bam H1 restriction fragment of pBlueScript plasmid DNA (the probe) was *in vitro* labeled by means of the random primer method[15] (Multiprime DNA Labeling System from Amersham, Little Chalfont, United Kingdom). The labeled probes were further used in hybridization experiments. In all assays we used in the reaction mixtures [α-^{32}P]dATP, as a tracer radionucleotide. The unincorporated nucleotides were removed by gel filtration through Sephadex G-50. Incorporated radioactivity was measured in a Scintillation Counter (Rackbeta, LKB Wallace) and the specific activity of the different probes ranged from 5×10^8 to 1×10^9 cpm/μg.

Labeling Conditions to Evaluate if 8 acts as Thymidine or Cytidine Analog. We assayed one reaction mixture containing normal nucleosides (control + in Table 2) and two reaction mixtures, one containing **8** instead of thymidine (Double Lb1 in Table 2) and the other, containing **8** instead of cytidine (Double Lb2 in Table 2). In all assays, we counted the radioactive particles by Cêrenkov's method[18] (CPM or counts per minute). The CPM of control + were considered as the 100% labeling efficiency, whereas the CPM obtained in Lb1 and Lb2 expressed as percentage of the CPM of control +, is an indirect measure of the incorporation of **8**.

Dot Blot Hybridization Experiments. An initial solution (5 ng), containing unlabeled pBlueScript plasmid DNA as target molecules, was twofold serially diluted in water and NaOH was added to 0.1 M final concentration. Then, the solutions were heated at 65 °C for 10 min and quickly cooled at 4 °C. For neutralization, NaH_2PO_4 was used at 0.15 M final concentration.

Denatured plasmid DNA molecules (5 ng to 2.5 pg, Fig. 4) were spotted onto Zeta-Probe GT membranes (Biorad, Richmond, CA) using a vacuum filtration apparatus (Schleicher & Schuell, Dassel, Germany). The filters were further dried in vacuum and baked for 30 min at 80 °C.

The immobilized and denatured plasmid DNA molecules were then hybridized with single and double labeled Bam H1 fragments of pBlueScript plasmid DNA. The experimental conditions of hybridization were as described.[19] Briefly, the filters were prehybridized at 65 °C for 5 min in a solution of 0.25 M Na_2HPO_4 pH 7.2 and 7% SDS. The hybridization with labeled probes was done under the same experimental conditions, but using 18 h of incubation. After the hybridization, the filters were washed twice at 65 °C for 5 min. in a solution containing 0.25 M Na_2HPO_4, pH 7.2, 7% SDS. Afterwards, they were washed for 30–60 min in 20 mM Na_2HPO_4 pH 7.2, 5% SDS solution; and finally, in 20 mM Na_2HPO_4 pH 7.2, 1% SDS solution. The membranes were covered with Saran Wrap, and two X-ray films were exposed to the radioactivity emitted by the probes at −70 °C for 12 h. Dot-blot experiments were replicated twice.

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