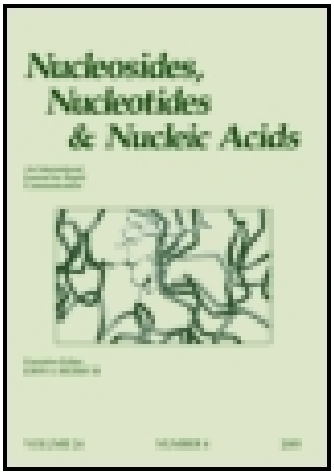

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INTRODUCTION OF AN IMMUNOCHEMICAL LABEL IN A CYTIDINE ANALOGUE

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Abstract: A new immunochemical label was synthesized and used as an artificial hapten to obtain a monoclonal antibody that specifically recognizes it. The new cytidine analogues, 5-methyl-4-N-{4-[6-(p-bromobenzoylamido)caproylamido]but-1-yl}-2'-deoxycytidine and 4-N-[ε-(p-bromobenzoylamido)caproylamido]cytidine, which could be useful for non-radioactive detection of DNA and RNA, were also synthesized.

Immunochemical methods are widely used for non radioactive detection of DNA. This is currently done by incorporating modified pyrimidine nucleosides and nucleotides into DNA, hybridization of a target-DNA with the modified probe, and detection of the DNA-DNA hybrids by a specific antibody to this chemical label[1-5]. These methods avoid the disadvantages associated with the use of radioactivity[6].

However, it has been considered[6] that the nonspecific recognition of DNA bases by the monoclonal antibody might give undesirable positive signals from unlabelled DNA. For this reason, the artificial hapten was synthesized first, and the corresponding monoclonal antibody against it was then obtained. The 5-methyl-2'-deoxycytidine (**7**) and modified cytidine (**8**) analogues were synthesized by linking the hapten to the nucleosides through a spacer arm.

RESULTS AND DISCUSSION

Chemical Discussion:

The immunochemical label, ε-(p-Bromobenzoylamido)caproic-N-hydroxysuccinimide ester (BLC-NHS or 3 in Scheme 1), was synthesized (Scheme 1) according to the methodology already described for the synthesis of peptides[7]. The overall yield was 64%. The BLC-NHS is a gentle acylating agent, which is stable in an aqueous medium.

The immunochemical label was linked to the molecule of 5-methyl-4-N-[4-(amino)but-1-yl]-2'-deoxycytidine (**6**) and to the molecule of cytidine. The synthesis of **7** was carried out according to Scheme 2.

In order to avoid steric hindrance in the recognition of 5-methyl-2'-deoxycytidine analogue labelled-DNA by the monoclonal antibody we introduced the label at the analogue through a spacer arm (5 atoms).

3',5'-Di-O-Acetylthymidine (**4**) was obtained according to the procedure previously reported[8].

The substitution of the triazole group was performed with 1,4-diaminobutane in pyridine at -20 °C. When the reaction is over, the temperature is kept at -20 °C and the amine **6** should be transformed to the corresponding oxalate salt to avoid the transacetylation reaction. The deprotection was carried out using NH_3 in methanol at room temperature. Under these conditions, acetylated amine as by-product was practically not observed. After the removal of salts, the amine was used without further purification.

The 5-methyl-2'-deoxycytidine labelling was carried out in DMF at 70 °C for two hours. An overall yield of 49% was obtained.

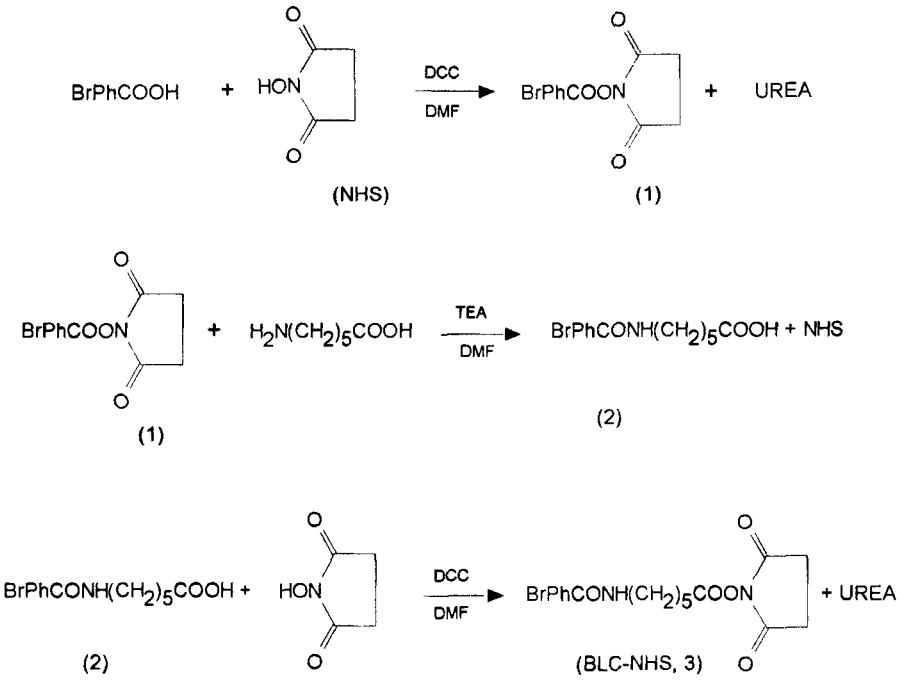
Compound **8** was synthetized in order to calculate the dissociation constant of the monoclonal antibody.

Biological Discussion:

Culture supernatant of the selected clone and the corresponding purified monoclonal antibody specifically bind with the artificial hapten as well as with the corresponding modified cytidine (**8**) (Kd = 1,53 x 10^{-9}mol/L), while it does not cross-react with natural pyrimidine bases of DNA or RNA (Figure 1).

The immunization protocol using the conjugated hapten-protein (BLC-keyhole limpets hemocyanin, BLC-KLH) instead of the conjugated protein-cytidine-analogue avoids immunological response to the chemical structure of natural nucleotides. Consequently, the recognition of cross targets, such as pyrimidine nucleotides, by the monoclonal antibody becomes practically impossible. These facts make the new 5-methyl-2'-deoxycytidine analogue (**7**) a possible alternative for non-radioactive detection of DNA.

The applications of compound 7 in the labelling of DNA will be described in a future report.



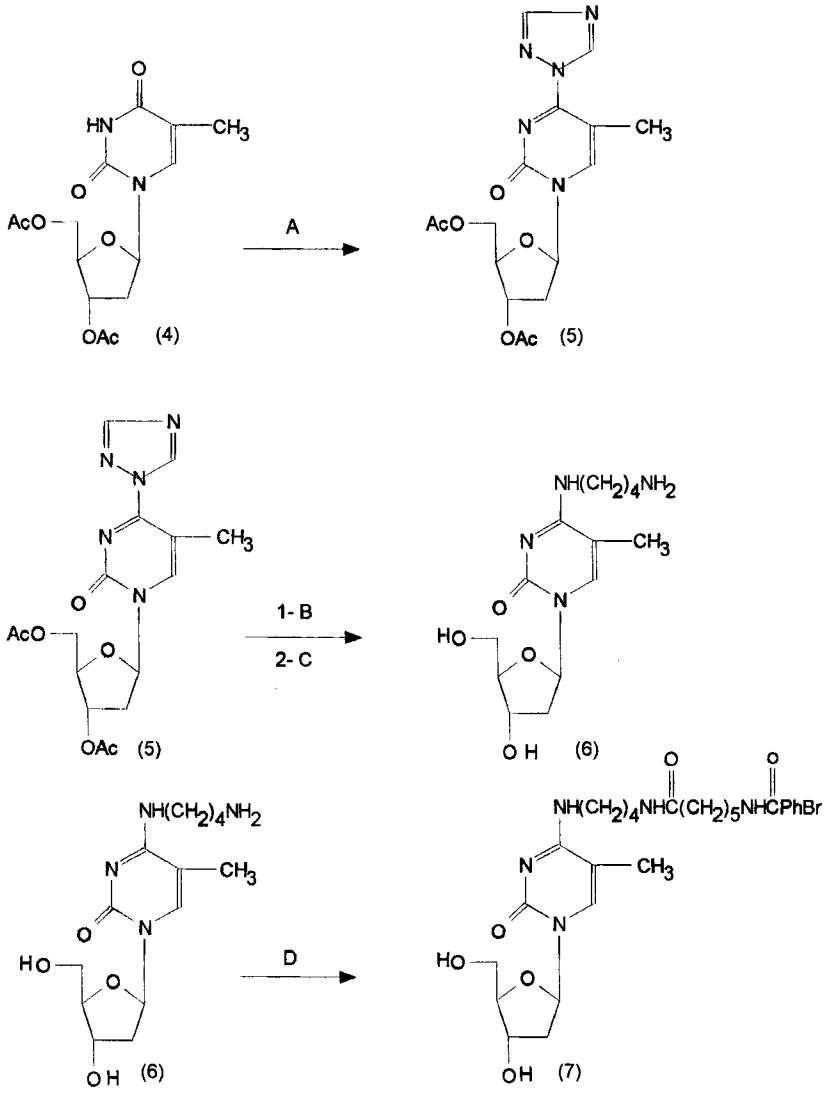
SCHEME 1

EXPERIMENTAL

Chemical Synthesis. General Procedures

Pyridine was dried over KOH, then distilled over P_2O_5 and stored over KOH. Dimethylformamide (DMF) was distilled and stored over molecular sieves (4Å). Acetonitrile was dried over P_2O_5, then distilled and stored over molecular sieves (4Å). All reagents were obtained from commercial suppliers and were pure for synthesis. Thin layer chromatography (TLC) was performed on precoated aluminum sheets of silica gel 60 F_{254} (*E. Merck*). Spots were visualized in a viewing chamber under 254 nm UV light. Column chromatography was performed on *Merck* silica gel 70-230 mesh.

Melting points (uncorrected) were taken on an Electrothermal apparatus. Elemental analysis were determined on a Carlo Erba Model EA-1108 analyzer. The ^1H NMR spectra (δ, ppm) were obtained at 250 MHz on a Brüker Model AC 250F spectrometer, TMS was used as internal reference and the assignment was based on COSY experiments. The multiplicities are recorded using the following abbreviations: s, singlet; d, doublet; t, triplet; q, quarted; m, multiplet and AA'BB', p-substituted aromatic system. ^{13}C NMR spectra



AC: CH_3

A: 1,2,4- Triazole + $POCl_3$ + Triethylamine/CH_3CN

B: $H_2N(CH_2)_4NH_2$/Pyridine, HOOCCOOH/H_2O; C: NH_3/CH_3OH (7N)

D: BLC-NHS/DMF

SCHEME 2

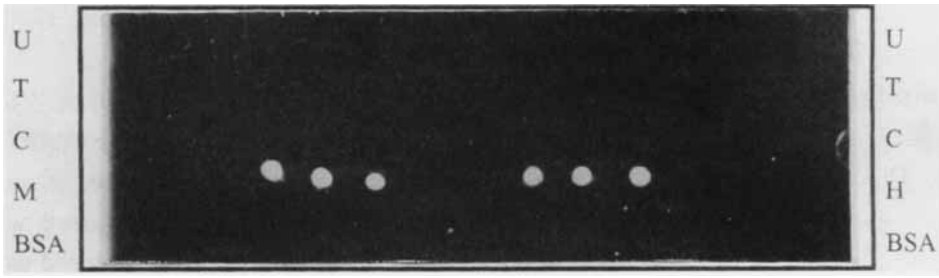


Figure 1. Chemiluminescent detection using the monoclonal antibody (Clone H11E5) against modified cytidine. U: uridine-BSA; T: tymidine-BSA; C: cytidine-BSA; M: *modified cytidine(8)-BSA* (left); H: *Hapten-BSA* (right) and BSA. Each pyrimidine nucleoside amount is ten fold greater than the one of cytidine *modified* or *Hapten*:0.2 nmol (1), 0.1 nmol (2) and 0.05 nmol (3).

were obtained at 62,8 MHz on a Brüker Model AC 250F. The following abbreviations are used to describe the kinds of carbon which were made with the aid of DEPT experiments: p, primary carbon; s, secundary carbon; t, terciary carbon and c, cuaternary carbon. Fast-atom-bombardment mass spectra (FAB-MS) were recorded in the positive-ion mode on a JEOL spectrometer model JMS-HX-110. For all experiments the matrix was glycerol. IR spectra were recorded in KBr tablets on a Carl Zeiss SPECORD 71 IR spectrophotometer, absorption bands in cm^{-1}. The following abbreviations are used to describe the intensity of the signal: s, strong; m, medium and w, weak.

P-Bromobenzoic acid N-Hydroxysuccinimide ester (1): p-Bromobenzoic acid (1g, 0,0049 moles) and N-hydroxysuccinimide (NHS, 0,57g, 0,0049 moles) were dissolved in DMF (16,5 mL). Dicyclohexylcarbodiimide (DCC) was added (1,02g, 0,0049 moles) and the reaction mixture was stirred at room temperature and left overnight. The precipitate obtained (dicyclohexylurea, DCU) was filtered and washed with ethyl acetate. The filtrate was concentrated under reduced pressure and the solid obtained was recrystallized from acetone to yield 95,6%, m.p.: 227-30 °C. **IR**(KBr): 3100m, 2980m, 1820s, 1730s, 1580s, 1480w, 1420w, 1400m, 1380m, 1300w, 1280w, 1260w, 1240s, 1220s, 1180m, 1080s, 1060s, 1040m, 1020m, 1000s, 840m, 820w, 740m, 700w, 680m, 640m, 600w.

ε(p-Bromobenzoylamido)caproic acid (2): 1 (1,47g, 0,0049 moles) and 6-aminocaproic acid were dissolved in DMF (16,3 mL). Triethylamine (TEA, 0,75 mL, 0,0054 moles) was added to the solution and the reaction mixture was stirred at 70 °C for 3 hours. The reaction mixture was poured into a cold solution of water:HCl (10:1) (100 mL) and the precipitate was filtered and washed with water until neutral pH. The product was recrystallized from ethanol to yield 89,0%, m.p.: 122-24 °C. **IR**(KBr): 3400s, 3040m,

2980s, 2900s, 1700s, 1640s, 1600m, 1540m, 1450m, 1460m, 1420m, 1360m, 1300m, 1280m, 1260s, 1120m, 1080m, 1020m, 960m, 880m, 840w, 640w.

ε(p-Bromobenzoylamido)caproic acid N-Hydroxysuccinimide ester (BLC-NHS, 3): 2 (3,26g, 0,0103 moles) and NHS (1,78g, 0,0154 moles) were dissolved in DMF (35 mL). DCC (3,09g, 0,015 moles) was added and the reaction mixture was stirred at room temperature and left overnight. The precipitate (DCU) was filtered and washed with acetone. The filtrate was evaporated to dryness using a vacuum pump. The obtained solid was recrystallized from ethyl acetate to yield 75,5%, m.p.: 121-23 °C., **IR**(KBr): 3360s, 2980m, 2860w, 1820m, 1780m, 1740s, 1620s, 1550m, 1480w, 1440w, 1380m, 1310w, 1260w, 1220s, 1160w, 1080s, 1050m, 1020w, 940w, 880w, 840m, 760w, 666m.

Anal. calcd. for $C_{17}H_{19}N_2O_5Br$: C: 44.63; H: 4.62; N: 4.36.

Found: C: 44.50; H: 4.78; N: 4,05.

3',5'-Di-O-Acetyl-4-(1,2,4-Triazol -1-yl)-2'-deoxythymidine (5): A solution of tris(1H-1,2,4-triazol -1-yl)phosphine oxide[10] (0,0038 moles) in acetonitrile (3 mL) was added dropwise to a solution of 3',5'-diacetylthymidine (1g, 0,0019 moles) in acetonitrile (6,5 mL). The reaction mixture was stirred at room temperature during 2 hours. The mixture was poured into a cold solution of $NaHCO_3$ (5%, 50 mL) and extracted with chloroform (3 x 10 mL). The organic phase was washed with water (3 x 10 mL) and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the obtained product was purified by column chromatography (chloroform:methanol, 99: 1) to yield 89,7% of a white solid. m.p.: 120-22 °C. **IR** (KBr): 3220m, 3000m, 1740s, 1680s, 1640m, 1540m, 1520s, 1440s, 1400s, 1340m, 1300s, 1280m, 1260s, 1240s, 1180m, 1140m, 1100m, 1080m, 1060m, 980m, 940w, 920m, 780w, 740w, 680m, 620m. **^{1}H NMR**($CDCl_3$): 9,29 (1H, s, *6-CH*); 8,53 and 8,08 (2H, 2 s, 2 *CH*-triazole); 6,29 (1H, t, *1'-CH*); 5,26 (1H, m, *4'-CH*); 4,44 (1H, s, *3'-CH*); 2,91 and 2,19 (2H, m, *2'-CH_2*); 2,49 (2H, s, *5'-CH_2*); 2,14 and 2,11 (9H, 3s, 2 CH_3CO + CH_3). **FAB-MS** (m/z): 378 (m+1).

5-Methyl-4-N-[4-(amino)but-1-yl]-2'-deoxycytidine (6): A solution of 4 (1g, 0,0025 moles) in pyridine (8 mL) was added dropwise to a cold solution (-20 °C) of 1,4-diaminobutane (1g, 0,0114 moles). The reaction mixture was stirred at -20 °C for 1 hour. Then, a solution of oxalic acid (3,15g, 0,025 moles) in water (30 mL) was added to the reaction mixture at -20 °C and it was stirred and left at room temperature for 1 hour. Then, the organic solvent was evaporated under reduced pressure and the traces of pyridine were coevaporated with toluene. The syrup obtained was dissolved in a solution of NH_3/CH_3OH (7N) and the mixture was stirred at room temperature for 3 hours. The precipitated salts were filtered and washed with CH_3OH and the solvent was evaporated under reduced pressure. The oily syrup obtained was used without further purification.

5-Methyl-4-N-{4-[ε-(p-bromobenzoylamido)-caproylamido]but-1-yl}2'-deoxy-cytidine (7): 3 (1,02g, 0,0025 moles) was added to a solution of 5 (0,0025 moles) in DMF (8,3 mL). The reaction mixture was stirred at 70 °C for 2 hours. Then, DMF was evaporated under reduced pressure and the remaining oily syrup was washed with ethylacetate (5 mL x 3). The ethyl acetate was removed to dryness using a vacuum pump and the obtained product was purified by column chromatography (chloroform:methanol, from 99:1 to 95:5). The overall yield for the two steps was 54%. m.p.: 130-32 °C. **IR**(KBr): 3350s, 3220m, 2950s, 2900s, 1640s, 1560s, 1510s, 1440s, 1360s, 1320s, 1100s, 1080s, 1060s, 1020s, 860m, 800m, 760m.**^{1}H NMR** (DMSO-d_6): 8,55 (1H, t, *3*-N*H*); 7,8 (1H, t, *2*-N*H*); 7,8-7,65 (4H, AA'BB', p-Br*Bz*); 7,59 (1H, s, *6*-C*H*); 7,15 (1H, t, 1-N*H*); 6,2 (1H, t, *1'*-C*H*); 5,2 (1H, d, *3'*-O*H*); 5,0 (1H, t, *5'*-O*H*); 4,2 (1H, s, *4'*-C*H*); 3,8 (1H, m, 3'-CH); 3,57 (2H, m, *5'*-CH_2); 3,45-3,20 (4H, m, 2 CH_2NHCO); 3,05 (2H, dd, CH_2NH); 2,15-1,95 (4H, m, CH_2CONH + *2'*-CH_2); 1,85 (3H, s, CH_3); 1,6 -1,25 (10H, m, 5 CH_2). **^{13}C NMR** (DMSO-d_6): 171,79 (c, NH*C*OCH$_2$); 165,08 (c, *4-C*); 162,64 (c, p-BrBz-*C*O); 154,97 (c, *2*-*C*O); 137,15 (t, *6*-*C*H); 133,81 (c, *C*arom-CO); 131,11 and 129,17 (2 t, *C*Harom); 124,50 (c, *C*arom-Br); 101,50 (c, *5*-*C*); 87,01 (t, *4'*-*C*H); 84,57 (t, *1'*-*C*H); 70,39 (t, *3'*-*C*H); 61,39 (s, *5'*-CH_2); 43,10 (2 s, CH_2NHCO + CH_2CONH); 42,10 (2 s, *2'*-CH_2 + CH_2NHCO); 39,29 (s, CH_2NH); 32,74; 30,60; 30,08 (2); 29,01 (5 s, 5 CH_2); 12,84 (p, CH_3). **FAB-MS**(m/z): 609 (m+1).

Anal. calcd. for $C_{27}H_{38}N_5O_6Br$: C: 53,31; H: 6,24; N: 11,51.

Found: C: 53,26; H: 6,15; N: 11,43.

4-N-[ε-(p-bromobenzoylamido)caproylamido]cytidine(8): 3 (1,68g, 0,0041 moles) was added to the solution of cytidine (1g, 0,0041 moles) in DMF (14 mL). The reaction mixture was stirred at 70 °C for 48 hours. Then, DMF was evaporated under reduced pressure and the remaining oily syrop was washed with ethylacetate (5 mL x 3) in order to remove the NHS. The solid obtained was recrystallized from ethanol to yield 61 % of **8**. m.p.: 202-04 °C. **UV** (CH_3OH) λmax = 250 nm (ε = 22537 L/mol). **IR** (KBr): 3500s, 3420s, 2950m, 1640s, 1570m, 1550m, 1500m, 1390m, 1320m, 1120m, 1080m, 850w, 800w, 760w, 720w, 640w. **^{1}H NMR** (DMSO-d_6): .10,8 (1H, s, 1-N*H*); 8,55 (1H, t, 2-N*H*); 8,45 (1H, d, *5*-C*H*); 7,8 and 7,65 (4H, AA'BB', p-Br*Bz*); 7,2 (1H, d, *6*-C*H*); 5,9 (1H, s, *1'*-C*H*); 4,45 (1H, d, *3'*-O*H*); 5,15 (1H, t, 5'-O*H*); 5,05 (1H, d, *2'*-O*H*); 4,05-3,9 (3H, m, *4'*-C*H* + *3'*-C*H* + 2'-C*H*); 3,8-3,55 (2H, m, *5'*-CH_2); 3,25 (2H, dd, CH_2NHCO); 2,4 (2H, t, CH_2CONH); 1,6-1,32 (6H, m, 3 CH_2). **^{13}C NMR** (DMSO-d_6): 173,78 (c, NH*C*OCH$_2$); 165,04 (c, *4-C*); 162,23 (c, p-BrBz-*C*O); 154,61 (c, *2*-*C*O); 145,25 (t, *6*-*C*H); 133,68(c, *C*arom-CO); 131,14 and 129,18 (2 t, *C*Harom); 124,62 (c, *C*arom-Br); 95,17 (t, 5-*C*H); 90,07 (t, *1'*-*C*H); 84,14 (t, *4'*-*C*H); 74,45 (t, *3'*-*C*H); 68,60 (t, *2'*-*C*H); 59,86 (s, *5'*-CH_2);

39.01(s, CH_2CONH); 36,24 (s, CH_2NHCO); 28,71; 25,88 and 24,11 (3 s, 3 CH_2). **FAB-MS (m/z): 540 (m+1).**

Anal. calcd. for $C_{22}H_{27}N_4O_7Br$: C: 49,01; H: 5,00; N: 10,39.

Found: C: 48,97; H: 4,95; N: 10,21.

Biological Procedures

Pyrimidine nucleosides, cytidine analogue (**8**) and hapten (BLC-NHS) conjugation to immunogenic proteins: The nucleosides were conjugated to bovine serum albumin (BSA), while the hapten was conjugated to keyhole limpets hemocyanin (KLH) and BSA as described[11-13]. The number of substitutions per mol was determined by measuring the absorbance of conjugates at 280 nm and at the λmax of the corresponding nucleoside or hapten.

Hybridoma production: The immunization protocol was as follows: Adult BALB/C mice were injected intraperitonealy with 40 μg of BLC-KLH in Freund's complete adjuvant on day 0.

Later, on days 7 and 14, they were subcutaneously injected with 40 μg of BLC-KLH in Freund's incomplete adjuvant. Mice were boosted intravenously with 10 μg of antigen in phosphate buffered saline (PBS) on day 21.

Mice showing higher antibody titre were sacrified 3 days after the last booster. Spleen cells were fused to the murine myeloma cell line P3.X63.Ag8.653 by means of polyethylene glycol-4000 (*Merck*) using a standard fusion protocol[14]. The cells were suspended in HAT (hypoxanthine/aminopterine/ thymidine) medium and distributed into 96-well culture plates. They were cultured for ten days and their supernatants were tested for antibody production by ELISA. Positive wells were subsequently cloned twice by limiting dilution at density of 0.5 cell/well.

Culture supernatants of selected hybridoma clones were assayed for their binding capacity to the artificial hapten by colorimetric ELISA and by a more sensitive chemiluminescent immunodot. One clone (5H11E5) was selected from thousands of clones and the corresponding monoclonal antibody was purified on Protein A Sepharose.

ELISA: The 96-wells microtiter PVC plate (Titertek) were coated at 4 °C with 0.2 nmol of hapten, pyrimidine nucleosides and cytidine analogue (**8**) conjugated to BSA, in 0.01 mol/L $NaHCO_3$ buffer, pH 9.55. They were further blocked with 5 % Skim milk in PBS. Then, hybridoma supernatants or purified antibodies were added to the coated microtitre wells and incubated at 37 °C for 1 hour. After three washes with 0.05 % Tween 20, the plates were incubated at 37 °C for 1 hour with Sheep anti-mouse IgG antibodies isolated from whole serum[15] and labelled with horseradish peroxidase as

described[16] elsewhere. The plates were washed and each well was incubated with 100 μL of substrate solution (0.04 mg of o-phenylenediamine in 3.5 mmol/L of H_2O_2) in buffer 0.1 mol/L of Na_2HPO_4, 0.2 mol/L of citric acid, pH 5.0. The reaction was stopped by the addition of 50 μl of 2.5 M H_2SO_4, and the absorbance was measured in a plate reader (*Multiskan, Flow Lab*) at 492 nm.

The affinity of the monoclonal antibody for cytidine analogue (**8**) was estimated by measuring its dissociation constant by a kinetic method[17].

Chemiluminescent Inmunodot: Spotted filters with 0,2 nmol of conjugated hapten-BSA, pyrimidine nucleosides-BSA and modified cytidine-BSA were treated with blocking solution [(0.3 % wt/vol Casein acc. to Hammarsten) in NT buffer (50 mmol/L Tris-HCL, pH 8.6, 150 mmol/L NaCl)]. They were incubated with diluted supernatant or purified monoclonal antibody in blocking solution plus 0.1% (wt/vol) Tween 20. After three washes with NT buffer plus 0.1% (wt/vol) Tween 20, the membranes were incubated with horseradish peroxidase-labelled sheep anti-mouse immunoglobulin. Finally, the filters were incubated for 1 min in chemiluminescence mix reagent (1.375 mmol/L Luminol, 1.8 mmol/L p-Iodophenol, 0.081 mmol/L H_2O_2 in 0.1 mol/L Tris-HCl, pH 9.5) and exposed to X-Ray film for 1 to 60 min.

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