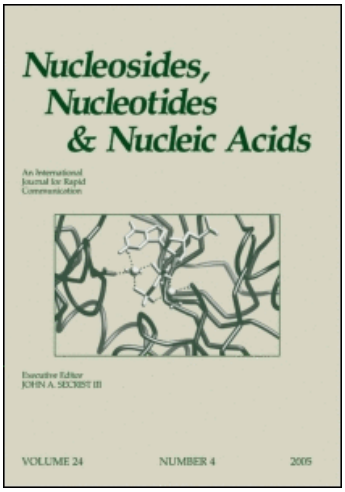

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SYNTHESIS OF A NEW ANALOG OF THYMIDINE FOR *IN VIVO* NON-RADIOACTIVE LABELING OF DNA

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ABSTRACT: The introduction of 6-(*p*-bromobenzoylamino)caproyl radical in the methyl group of 2′-*O*-deoxythymidine is described. *In vivo* incorporation of this nucleoside to DNA was determined using a monoclonal antibody that recognized the radical.

Inmunochemical labels have been successfully introduced at the base position of nucleosides [1-8]; however, they have never been introduced in the methyl group of thymidine. Here, we describe a procedure for synthesizing 5-{-*N*-[caproyl-6-(*p*-bromobenzamine)]aminomethyl}-2′-*O*-deoxyuridine (7), which contains 6-(*p*-bromobenzamine)caproyl (BLC)[7] in the methyl group of thymidine. Results of *in vivo* DNA labeling using this new nucleoside are also discussed.

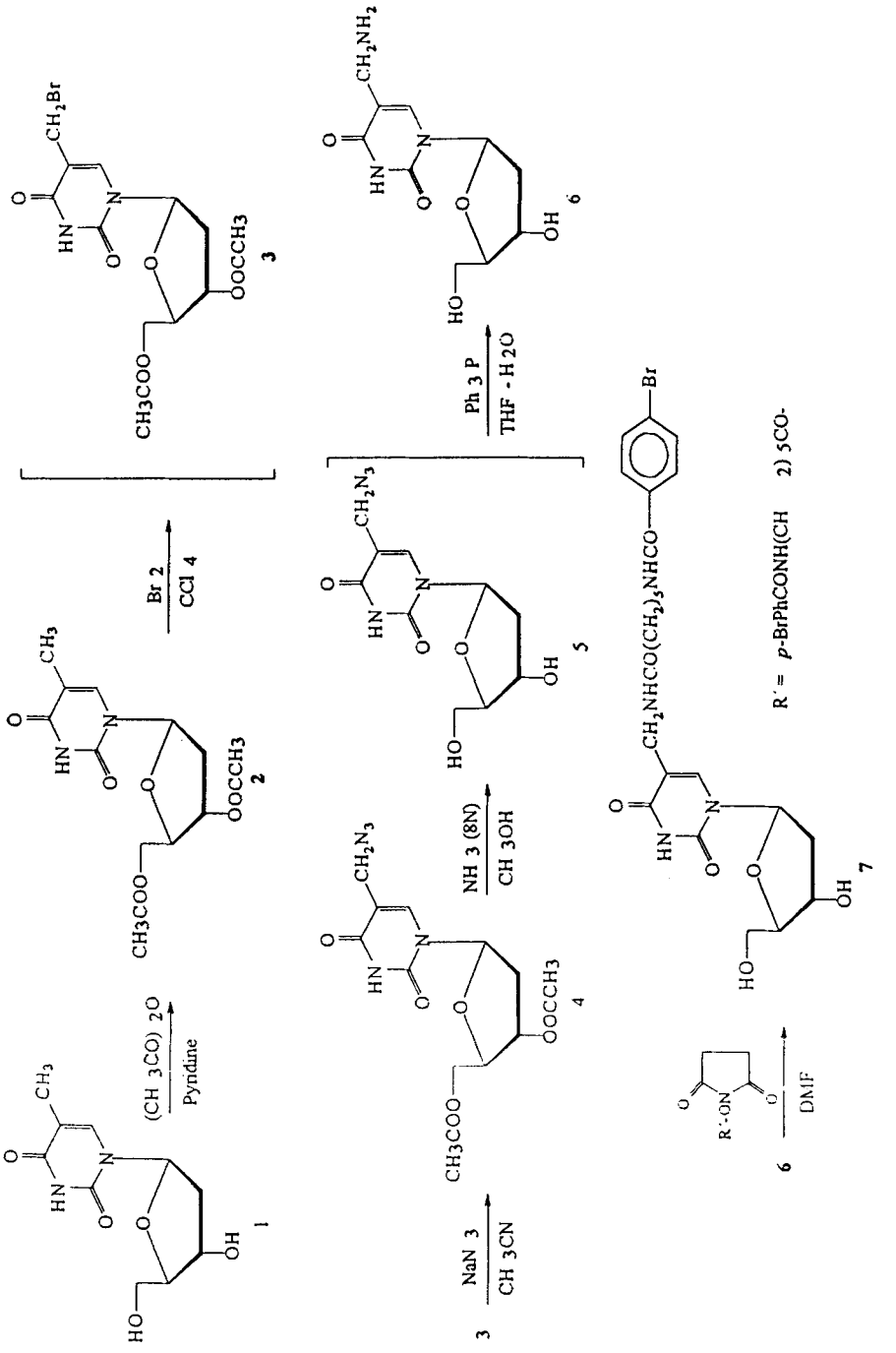
RESULTS AND DISCUSSION

Chemical Discussion: Compound 7 was synthesized in six steps *(scheme 1)* and three of them (bromination, azidation and deacylation) were done in one pot. The intermediate 3 was prepared from 2 (*scheme 1*) as reported[9], but modifying the purification step (see Experimental). Further, the azide anion displaced the bromine atom of 3, and finally, the acetyl groups were removed from 4. The overall yield of product 5, starting from 2 was 36 %. Azide group of 5 was reduced according to Knouzi[10]. The structure of compounds

5and 6 were determined using IR, ^{1}H-, ^{13}C-NMR and FAB-MS spectra. Finally, to obtain compound 7, the 6 was treated with 6-(*p*-bromobenzoylamino)caproic acid *N*-hydroxysuccinimide [7,8]. IR; NMR; FAB-MS and quantitative elemental analysis were used to characterize 7.

Biological Discussion. *In vivo* incorporation of modified thymidine to DNA can be done efficiently using Thy$^-$ mutants (cells lacking the ability of endogenous thymidine synthesis) and adding to the growth medium the nucleoside analog. A direct competitive ELISA determined *in vivo* incorporation of 7 to nuclear *Escherichia coli* (Thy$^-$) DNA. Previous to the ELISA, labeled DNA molecules were hydrolyzed by using DNAase, and further, free modified nucleotides and the immobilized hapten compete for capturing the MAb. To estimate the nucleotide concentrations, the recorded absorbances were replaced in a previously obtained calibration curve. The calibration curve, $Abs = b2 + (b1-b2) / [1 + (nucl.conc/b3)^{b4}]$ [11] (Eq. 1), had b1 = 9.05571 $x10^{-2}$, b2 = 2.32773, b3 = 9.48242 $x10^{-8}$ and b4 = 1.86328. In three independent labeling experiments, 1 μg of DNA was isolated, and their absorbances in the ELISAs were 0.262, 0.262 and 0.292, resulting in 2.49 $x10^{-8}$ M, 2.49 $x10^{-8}$ M and 2.74 $x10^{-8}$ M nucleotide concentration, respectively. These concentrations indicate that nearly 0.05 % of thymidine was replaced by the analog during DNA synthesis.

EXPERIMENTAL. Chemical Synthesis. All solvent were dried and stored as described [7,8]. Spectral measures were recorded as reported [7,8]. 5-Bromo 3′, 5′-di-*O*-acetyl-2′-*O*-deoxythymidine (3): was obtaining from 3′,5′-di-*O*-acetyl-2′-*O*-deoxythymidine[12], as reported[9]. The product was isolated by removing the solvent *in vacuum*, and it was used without further purification in the next step. 5-Azido-2′-*O*-deoxythymidine (5): Sodium azide (1,19 g, 0,018 mol) was added to a solution of 3 (0.0092 mol) in dry acetonitrile (120 mL). When the reaction was over (checked by TLC), the solvent was removed, and the syrup (4) was dissolved in a solution of NH_3/CH_3OH (8 N) for removing the acetyl groups. The product was purified by column chromatography (chloroform:methanol, from 90:1 to 90:3). Yield: 36%, m.p.: 139-41 °C (lit[13] m.p.: 130-32 °C). IR: 3400s, 3050s, 2950s, 2150s (νN_3), 1680s, 1460s, 1420s, 1280s, 1200m, 1100s, 1060s, 1000m, 960w, 880m, 760m, 660w. ^{1}H-NMR (DMSO-d_6): 11,55 (1H, s, N*H*); 7,6 (1H, m, 6-C*H*); 6,16 (1H, t, 1′-C*H*); 5,8 (1H, s, 3′-O*H*); 5,05 (1H, s, 5′-O*H*); 4,25 (1H, s, 3′-C*H*); 4,05 (2H, s, CH_2N_3); 3,8 (1H, s, 4′-C*H*); 3,55 (2H, s, 5′-CH_2); 2,1 (2H, s, 2′-CH_2). ^{13}C-NMR (DMSO-d_6): 162,76 (c, 4-C'O); 150,08 (c, 2-C'O); 139,77 (t, 6-CH); 108,14 (c, 5-C'); 87,36 (t, 4′-CH); 84,15 (t, 1′-CH); 70,12 (t, 3′-CH); 61,11 (s, 5′-CH_2); 46,78 (s, CH_2N_3); 39,53 (s, 2′-CH_2). FAB-MS (m/z) = 284 $(M+H)^+$. 5-Amino-2′-O-deoxythymidine (6): The synthesis was carried out according to the procedure for reducing azide group to amine group[10]. Yield: 74%, m.p.: 172-74 °C. IR: 3400s, 3050m, 2890m, 1660s, 1520s,



SCHEME 1

1480s, 1400m, 1360m, 1320m, 1280m, 1200w, 1100m, 1060m, 800w, 730w, 700w. ^{1}H-NMR (DMSO-d_6): 7,75(1H, s, N*H*); 7,6 (1H, m, *6*-C*H*); 6,2 (1H, t, *1'*-C*H*); 5,5-4,5 (4H, m, 5'-OH + 3'-OH + NH_2); 4,25 (1H, s, *3'*-C*H*); 3,68 (1H, a, *4'*-C*H*); 3,6-3,5 (4H, *5'*-CH_2 + CH_2NH); 2,14 (2H, s, *2'*-CH_2). ^{13}C-NMR (DMSO-d_6): 163,19 (c, *4*-*C*O); 150,31 (c, *2*-*C*O); 135,89 (t, *6*-*C*H); 115,51 (c, *5*-*C*); 87,23 (t, *4'*-*C*H); 83,79 (t, *1'*-*C*H); 70,36 (t, *3'*-*C*H); 61,29 (s, *5'*-*C*H_2); 39,29 (s, 2'-*C*H_2); 38,37 (s, *C*H_2NH$_2$). FAB-MS (m/z) = 258 $(M+H)^+$. 5-{*-N*-[caproyl-6-(*p*-bromobenzamine)] aminomethyl}-2'-*O*-deoxyuridine (7): The compound was synthesized as described[7,8]. Yield: 41%, m.p.: 200-02 °C. IR: 3420s, 2940w, 2860w, 1720m, 1660s, 1640s, 1540w,1480w, 1400w, 1280m, 1100w, 1060w, 800w, 760w. ^{1}H-NMR (DMSO-d_6): 11,38 (1H, s, N*H*); 8,51 (1H, s, 3-N*H*); 8,1 (1H, s, 2-N*H*); 7,8-7,65 (5H, m, *p*-BrBz + 6-C*H*); 6,2 (1H, t, 1'-C*H*); 5,26 (1H, s, 3'-O*H*); 4,95 (1H, s, 5'-O*H*); 4,25 (1H, s, 3'-CH); 3,94-3,73 (3H, m, 4'-C*H* + 5'-C*H*$_2$); 3,51 (2H, s, C*H*$_2$NHCO); 3,29-3,2 (2H, q, C*H*$_2$NHBz-*p*-Br); 2,18-1,99 (4H, m, 2'-C*H*$_2$ + NHCOC*H*$_2$); 1,6-1,42 and 1,38-1,16 (6H, m, 3 C*H*$_2$). ^{13}C-NMR (DMSO-d_6): 172,17 (c, NHCOCH$_2$); 165,03 (c, *4*-*C*O); 162,69 (c, *p*-BrBz-*C*O); 150,14 (c, *2*-*C*O); 137,37 (t, *6*-*C*H); 133,67 (c, *C*arom-CO); 131,14 and 129,18 (2 t, CHarom.); 124,61 (c, *C*arom-Br); 110,87 (c, *5*-*C*); 87,35 (t, *4'*-*C*H); 83,98 (t, *1'*-*C*H); 70,46 (t, *3'*-*C*H); 61,41 (s, *5'*-*C*H_2); 39,41 (s, 2'-*C*H_2); 39,07(2); 38,74 (3s, 2 *C*H_2NHCO + NHCO*C*H_2); 28,75; 26,11 and 24,84 (3 s, 3 *C*H_2). FAB-MS m/z = 554 $(M+1)^+$. Anal. Cald. for $C_{23}H_{29}N_4O_7Br$: C, 49,93; H, 5,24; N, 10,12; Br, 14,44. Found: C: 49,34 , H: 5,13, N: 9,98.

Biological Procedures. Bacterial Strains: *Escherichia coli* cells (DH5α) Thy$^-$ mutants were obtained as described[14]. ***In vivo* DNA Labelling**: Thy$^-$ cells were grown in LB medium alone, and in LB supplemented with 200 μg/mL of 7, or thymidine. The cultures were shaken overnight at 37 °C. Phenylmethylsulfonylfluoride was added to cell lysate for protecting the BLC peptic bond from protease attack. Nuclear DNA molecules were obtained as reported[15]. **ELISA**. To measure the incorporation of the modified nucleotide **8** to DNA we designed a direct competitive ELISA[8]. The MAb and gelatin-hapten conjugate[8] optimum amounts, for coating the wells, were determined. To obtain a calibration curve, the concentration of free nucleoside was varied to attain the function relating absorbance with nucleotide concentration. The absorbance obtained in the competitive ELISA of hydrolyzed labeled DNA was used to estimate in the calibration curve the incorporation of thymidine analog to DNA. The experimental details in the direct competitive ELISA were described in a previous paper[8]. **Statistical method to estimate the incorporation of the modified nucleotide to DNA**. The function describing the relationship between the absorbances and known free nucleoside concentrations was fitted to a sigmoidal curve according to described[11]. Solving this equation for the absorbances obtained in the ELISA, we estimated the thymidine analog concentration. The equation was solved using an iterative method (ref).

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