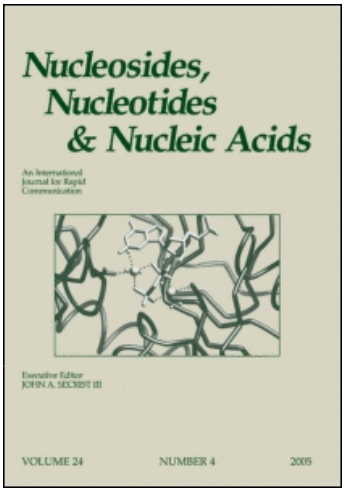

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SYNTHESIS OF 3'-O-PROPARGYLTHYMIDINE AS A CANDIDATE ANTIRETROVIRAL AGENT

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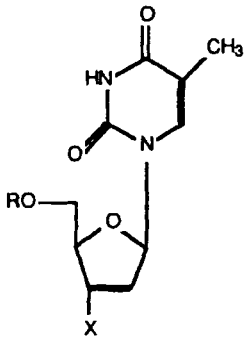
SUMMARY

3'-O-Propargylthymidine, which may be viewed as a structural analogue of the potent antiretroviral agent 3'-azido-3'-deoxythymidine (AZT), was synthesized from 5'-O-(4,4'-dimethoxytritylthymidine by reaction with propargyl bromide followed by gentle acidolysis. The 3'-O-propargyl derivative was tested for antiretroviral activity in SC-1 mouse fibroblasts infected with Rauscher murine leukemia virus (MuLV). No inhibition of MuLV proliferation was observed at concentrations of 3'-O-propargylthymidine from 0.001 to 100 μM, whereas the IC_{50} against the host cells was 30 μM. By comparison, AZT had an IC_{50} for MuLV growth of 0.01 μM and an IC_{50} for cell growth of >100 μM. Thus, replacement of the 3'-N-N≡N group in AZT by a 3'-$OCH_2C{\equiv}CH$ group increased cytotoxicity but decreased antiretroviral activity relative to AZT.

The 5'-triphosphate ester of 3'-O-methylthymidine (**1**) has been reported to be a chain-terminating inhibitor of DNA synthesis with a high degree of specificity toward reverse transcriptase (RT) from Rous sarcoma virus (RSV) and avian myeloblastosis virus (AMV).[1,2] Inhibition of calf thymus DNA polymerase α was negligible, suggesting that if **1** were converted to a 5'-triphosphate by cellular kinases, it might show antiretroviral activity with minimal toxicity

toward mammalian host cells. The 5'-triphosphates of other 3'-modified thymidine analogues such as 3'-amino-3'-deoxythymidine and 3'-fluoro-3'-deoxythymidine were also active in these assays, though none of them was as effective as 2',3'-dideoxythymidine 5'-triphosphate (ddTTP) in blocking reverse transcription. Other mammalian DNA polymerases such as rat liver DNA polymerase β and calf thymus terminal deoxynucleotidyl transferase, on the other hand, remained susceptible to inhibition by ddTTP. As part of a broader study of sugar-modified pyrimidine nucleosides as potential antiretroviral agents, we desired to prepare an analogue of **1** in which the O-alkyl group contained a *carbon-carbon triple bond.* An attractive feature of such a compound was that it might more closely resemble the antiretroviral agent 3'-azido-3'-deoxythymidine (AZT, **2**),[3,4] which is currently in clinical use for the treatment of human acquired deficiency syndrome (AIDS)[5] and similarly acts by chain termination of reverse transcriptase mediated viral DNA synthesis after being converted to a 5'-triphosphate.[6,7] This Note reports the synthesis and proof of structure of the heretofore undescribed AZT analogue 3'-O-propargylthymidine (**3**).



1 : X = OMe, R = H

2 : X = N-N≡N, R = H

3 : X = $OCH_2C{\equiv}CH$, R = H

4 : X = $OCH_2C{\equiv}CH$, R = $(C_6H_5)(4\text{-}MeOC_6H_4)_2C$

5 : X = $OCH_2C{\equiv}CH$, R = CH_3CO

Alkylation of 5'-O-tritylthymidine has been reported to occur on either N^3 or the 3'-OH group depending on the reagent used, the mole ratio of reactants used, and the solvent. Baker and coworkers[8] observed that alkylation of 5'-unprotected thymidine with an equimolar amount of 1-bromopentane, potassium carbonate, and sodium iodide at 80-90°C in dimethylsulfoxide afforded N^3-alkylation. In contrast, Griffin and Todd[9] found that alkylation of 5'-O-tritylthymidine with a 6-fold excess of benzyl chloride in a mixture of benzene and dioxane at 70°C led predominantly to

3'-O-benzylation. Hampton and coworkers,[10] on the other hand, found that alkylation of 5'O-tritylthymidine with either a 6-fold or 2-fold excess of ethyl iodide under these conditions yielded a mixture of 3'-O-ethylthymidine and 3-ethyl-3'-O-ethylthymidine. In preliminary experiments using 5'-O-dimethoxytritylthymidine[11] and propargyl bromide, we likewise observed mono- and di-alkylation, with 3'-O-alkylation apparently occurring first. However, in contrast to previous workers, we found it expedient to terminate the reaction as soon as the first trace of dialkylated byproduct began to form, even though this meant that substantial amounts of starting material remained unchanged. This was because the 3'-O-propargyl and 3-propargyl-3'-O-propargyl derivatives proved much more difficult to separate from each other than from starting material. The monoalkylated product, **4**, was readily separated by silica gel chromatography and was subsequently detritylated to **3** in 63% yield by treatment with 1% trifluoroacetic acid in chloroform at 0°C for 1 h. That alkylation had occurred on the 3'-OH group rather than on N^3 was established unequivocally by 5'-O-acetylation of the detritylated nucleoside with acetic anhydride in pyridine. The resultant product, **5**, analyzed correctly for a *monoacetyl derivative* and yielded an NMR spectrum with singlets of equal area at δ 1.93 (5-CH_3)and δ 2.13 (3'-O-acetyl). The presence of the 3'-O-propargyl group was clearly indicated in the spectra of **3** and its 5'-O-acetyl derivative **5** by a triplet at δ 2.7 for the $CH_2C{\equiv}C\mathit{H}$ proton and a doublet at δ 4.2 for the $C\mathit{H}_2C{\equiv}CH$ protons.

Compound **3** was tested *in vitro* for cytotoxicity to SC-1 mouse fibroblasts and for antiretroviral activity against Rauscher murine leukemia virus (MuLV) in the same cells.[13] Some inhibition of cell growth was already evident at 0.01 μM and reached 53% at 10 μM. Thus, in order to be selective, **3** would have to inhibit viral proliferation at concentrations substantially lower than 10 μM. However, virus proliferation was not observed at drug concentrations from 0.001 to 10 μM, whereas AZT under the same assay conditions had an IC_{50} for viral growth of 0.01 μM and an IC_{50} for cell growth of >100 μM. According to a recent report,[14] the IC_{50} of 3'-O-methylthymidine (**1**) against cultured human T-lymphocytes is ca. 90 μM while its antiretroviral activity at this concentration is very low, giving it a therapeutic index of <1. The 3'-O-ethyl analogue was similarly unpromising.[14] Our results show that replacement of the 3'-N-N≡N group in AZT by 3'-$OCH_2C{\equiv}CH$ as opposed to 3'-OCH_3 or 3'-OC_2H_5 increases cytotoxicity but not antiviral activity. The fact that **3** was cytotoxic suggested that the 5'-triphosphate of **3** probably formed in the cells, and that lack of therapeutic selectivity was most likely due to the inability of this 5'-triphosphate to selectively inhibit viral versus cellular to DNA polymerase activity.

Experimental

^1H magnetic resonance spectra were obtained with a Varian T60 spectrometer using Me_4Si as the reference. TLC was carried out on Whatman MK6F and Baker 250F silica gel plates containing a fluorescent indicator, with spots being visualized under 254 nm ultraviolet light or with the aid of I_2. Column chromatography was performed on Baker 3405 (60-200 mesh) or Baker "Flash" (40 μm) silica gel. Melting points were measured on a Fisher-Johns hot stage apparatus and are not corrected. Reported yields are non-optimized. Reagent grade solvents were redistilled, and were routinely stored over 4A molecular sieves. 5'-O-(4,4'-Dimethoxytrityl)-thymidine was obtained in 81% yield as described previously;[11] mp 129-130°C (lit.[11] 123-124°C). Other chemicals were from Aldrich (Milwaukee, WI).

5'-O-(4,4'-Dimethoxytrityl)-3'-O-propargylthymidine (4). A solution of 5'-O-(4,4'-dimethoxytrityl)thymidine (3.27 g, 6 mmol) in a mixture of benzene (36 mL) and dioxan (12 mL) was treated with propargyl bromide (1.43 g, 12 mmol) and powdered KOH (0.673 g, 12 mmol). The mixture was heated at 70°C for 3 h,at which time TLC analysis (silica gel, 95:5 $CHCl_3$-MeOH indicated a single product (R_f 0.73) in addition to a substantial amount of unchanged starting material (R_f 0.35). The mixture was evaporated to dryness under reduced pressure, the residue was taken up in MeOH (3 mL), and the solution poured into H_2O (60 mL). Extraction with $CHCl_3$ (3 x 150 mL), drying (Na_2SO_4), and evaporation left a foam, which was chromatographed on a silica gel column (30 x 5 mL) using $CHCl_3$ followed successively by 1% and 2.5% MeOH in $CHCl_3$ as the eluents. A colored impurity eluted quickly with $CHCl_3$ and was discarded. The $CHCl_3$-MeOH eluates were monitored by TLC, and homogeneous fractions were pooled and evaporated to obtain **4** as a foam (0.608 g, 17% yield) followed by starting material (2.68 g, 83% recovery). The yield of **4** based on consumed starting material was 96%; NMR ($CDCl_3$) δ 1.5 (s 3H, C_5-CH_3), 2.2-2.5 (m, 3H, $C_{2'}$-H and $CH_2C{\equiv}CH$), 3.4 (s, 2H, $C_{5'}$-H), 3.8 (s, 9H, OCH_3), 4.1 (d, 1H, $CH_2C{\equiv}CH$), 4.4-4.7 (m, 2H, $C_{3'}$-H and $C_{4'}$-H), 6.3 (m, 1H, $C_{1'}$-H), 5.7-7.4 (m, 13H, aromatic), 7.5 (s, 1H, C_6-H). Attempts to increase the yield of **4** by extending the time of reaction were unsuccessful due to apparent formation of the N^3,$O^{3'}$-dipropargyl derivative, which could be detected by TLC as a fast moving spot with R_f 0.8. Compound **4** could not be crystallized, but was analytically pure and used in the next reaction without additional purification. Anal. ($C_{34}H_{34}N_2O_7.0.5H_2O$) Calcd.: C, 68.96; H, 5.95; N, 4.73. Found: C, 68.99; H, 6.03; N, 4.58.

3'-O-Propargylthymidine (3). To a stirred solution of **4** (600 mg, 1.03 mmol) in $CHCl_3$ (25 mL) was added 1% trifluoroacetic acid in $CHCl_3$ (25 mL) at 0°C. After 1 h, pyridine

(2.5 mL) and H_2O (2.5 mL) were added, and the mixture was extracted with H_2O (3 x 20 mL). The aqueous layer was evaporated under reduced pressure, and the residue was chromatographed on a silica gel column (25 x 2 cm), with 2.5% MeOH in $CHCl_3$ as the eluent. TLC-homogeneous fractions (R_f 0.18; silica gel, 95:5 $CHCl_3$-MeOH) were pooled and evaporated to dryness to obtain a white solid (180 mg, 62.5% yield): mp 137-140°C; NMR (CD_3OD + $CDCl_3$) δ 1.9 (s, 3H, C_5-CH_3), 2.3 (m, 2H, $C_{2'}$-H), 2.7 (t, 2H, $CH_2C{\equiv}CH$), 3.8 (d, 2H, $C_{5'}$-H), 4.0 (m, 1H, $C_{4'}$-H), 4.2 (d, 1H, $CH_2C{\equiv}CH$), 4.4 (m, 1H, $C_{3'}$-H), 6.2 (m, 1H, $C_{1'}$-H), 7.7 (s, 1H, C_6-H). Anal. ($C_{13}H_{16}N_2O_5$) Calcd.: C, 55.69; H, 5.75; N, 9.99. Found: C, 55.68; H, 5.75; N, 9.76.

5'-O-Acetyl-3'-O-propargylthymidine (5). Acetic anhydride (5 mL) was added to a solution of **3** (50 mg, 0.18 mmol) in dry pyridine (5 mL), and the mixture was stirred at ambient temperature for 16 h. The reaction mixture was poured into ice water (20 mL), the product was extracted into $CHCl_3$ (50 mL), and the organic layer was washed with 5% $NaHCO_3$ and H_2O, dried (Na_2SO_4), and evaporated to dryness. The residue was chromatographed on a silica gel column (20 x 0.5 cm) with 2.5% MeOH in $CHCl_3$ as the eluent. TLC-homogeneous fractions (R_f 0.32; silica gel, 95:5 $CHCl_3$-MeOH) were combined and evaporated to obtain pure **5** as a clear oil (51 mg, 89% yield); NMR ($CDCl_3$) δ 1.93 (s, 3H, C_5-CH_3), 2.13 (s, 3H, CH_3CO), 2.2-2.6 (m, 3H, $C_{2'}$-H and $CH_2C{\equiv}CH$), 4.2-4.4 (m, 5H, $C_{2'}$-H, $C_{3'}$-H, $C_{4'}$-H, and $CH_2C{\equiv}CH$), 6.2 (t, 1H, $C_{1'}$-H), 7.2 (s, C_6-H). The presence of a *three-proton* singlet at δ 2.13 , corresponding to a single O-acetyl group, indicated that propargylation had occured on $O^{3'}$ rather than N^3. Anal. ($C_{15}H_{18}N_2O_6$) Calcd.: C, 55.89; H, 5.63; N, 8.69. Found: C, 55.77; H, 5.97; N, 8.47.

Bioassay. Stocks of a Rauscher murine leukemia virus (MuLV)[15] strain were prepared by injecting freshly thawed virus intravenously into 6-week old BALB/c mice, which were sacrificed on day 20. Single cell suspensions of splenocytes were prepared in Dulbecco's minimal essential medium (DME) containing 20% fetal bovine serum (FBS). The cells were spun down, and the virus-containing supernatant was passed through a sterile 0.45 μm Nalgene filter. The filtrate typically had a viral titer of $5x10^6$ plaque forming units/mL as determined by a standard XC plaque assay described in the literature.[16] An adpatation of the same assay was also used to determine antiviral activity. Briefly, SC-1 mouse fibroblasts were plated onto 60-mm plastic dishes containing DME supplemented with 10% heat-inactivated FBS and antibiotics. The initial cell density was 10^5/dish. The cultures were treated with various concentrations of drug from 10^{-10} to 10^{-5} M, and after 24 h were inoculated with a range of dilutions of virus in the presence of Polybrene (8 μg/mL) to promote infection. After another 24 h of incubation, the cells were

washed, suspended in fresh DME, and allowed to grow to confluence. The confluent cells were then lethally irradiated with UV light and overlayered with XC rat sarcoma indicator cells at a density of 10^6 cells/dish. Three days later the medium was removed, and the cells were washed, and stained with methylene blue and carbofuchsein in methanol. Plaques comprising non-stained areas surrounded by syncytia were counted and compared with controls. For the determination of cytotoxicity, uninfected SC-1 cells were incubated with various concentrations of drug until control cultures reached confluence. Untreated and drug-treated cells were then trypsinized, stained with trypan blue, and counted. Assays were performed in triplicates and results were averaged.

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