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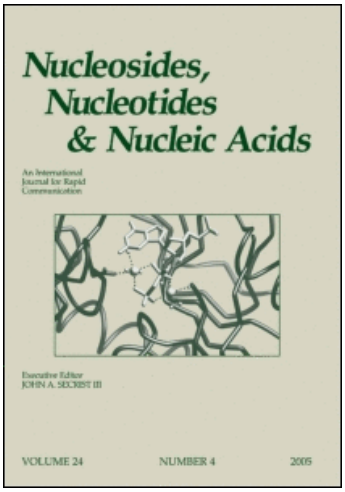

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
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Shin HongKang[a]; A. K. Sinhababu[b]; Moo J. Cho[a]
[a] School of Pharmacy, University of North Carolina, Chapel Hill, NC, USA [b] Research Triangle Park, Glaxo Wellcome Research Institute, NC, USA

To cite this Article HongKang, Shin , Sinhababu, A. K. and Cho, Moo J.(1998) 'Synthesis and Biological Activity of BIS(Pivaloyloxymethyl) Ester of 2'-Azido-2'-Deoxyuridine 5'-Monophosphate', Nucleosides, Nucleotides and Nucleic Acids, 17: 6, 1089 — 1098
To link to this Article: DOI: 10.1080/07328319808004222
URL: http://dx.doi.org/10.1080/07328319808004222

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NUCLEOSIDES & NUCLEOTIDES, 17(6), 1089-1098 (1998)

SYNTHESIS AND BIOLOGICAL ACTIVITY OF BIS(PIVALOYLOXYMETHYL) ESTER OF 2'-AZIDO-2'-DEOXYURIDINE 5'-MONOPHOSPHATE

Shin Hong Kang[a], A. K. Sinhababu[b], and Moo J. Cho[a*].

[a]School of Pharmacy, University of North Carolina, Chapel Hill, NC 27514 and
[b]GlaxoWellcome Research Institute, Research Triangle Park, NC 27709 USA

Abstract: Bis(pivaloyloxymethyl) ester of 2'-azido-2'-deoxyuridine 5'-monophosphate was prepared as a prodrug to generate 2'-azido-2'-deoxyuridine 5'-diphosphate inside the cell. A synthetic route utilizing stannyl phosphate was adopted in the preparation. The prodrug was evaluated for cell growth inhibition against a variety of tumor cell lines along with 2'-azido-2'-deoxyuridine and 2'-azido-2'-deoxycytidine.

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INTRODUCTION

A substrate-like potent inhibitor of ribonucleotide reductase (EC 1.17.4.1), 2'-azido-2'-deoxyuridine 5'-diphosphate[1] (N_3dUDP), is of little therapeutic potential because of its poor membrane permeability caused by charges on the pyrophosphate group. It is conceivable that cellular delivery of N_3dUDP could be achieved by masking all negative charges on the hydroxyl groups of the 5'-pyrophosphate with a membrane-permeable pro-moiety. However, this particular strategy has several problems. First, the synthesis of a desired derivative of a pyrophosphate can not be easily achieved mainly due to the instability of asymmetric tetra-ester of pyrophosphate.[2] Secondly, during the generation of the parent diphosphate, the intermediate pyrophosphate with intact pro-moieties can be broken down directly to the monophosphates. Although several pyrophosphate diesters have been reported as nucleoside phospholipid prodrugs,[3] they are not prodrugs of a diphosphate. These pyrophosphate diesters were first degraded into monophosphate

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derivatives by cellular pyrophophatase. These were then phosphorylated all the way to the corresponding triphosphates to exert biological activity.[4]

In a permeabilized cell system where even charged molecular species can access to cellular kinases as well as the reductase, 2′-azido-2′- deoxyuridine 5′-monophosphate (N_3dUMP), but not 2′-azido-2′- deoxyuridine (N_3dUrd), showed an inhibitory activity against the reductase similar to that of N_3dUDP.[5] A further study on N_3dUMP suggested that it could be readily phosphorylated by cellular kinases into N_3dUDP which is a true inhibitor of the reductase.[5] Albeit it is membrane-permeable, N_3dUrd can not be a viable precursor of its diphosphate because of its inefficient phosphorylation.[5] Based on this observation and the consideration discussed in the preceding paragraph, a prodrug approach was tested with the azidonucleoside monophosphate to generate the azido-nucleoside diphosphate inside the cell.

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As a pro-moiety, pivaloyloxymethyl group had been introduced to improve the absorption of ampicillin and α-methyldopa from the gastrointestinal tract.[6,7] In these early studies, pivaloyloxymethyl group was found to be the best among many acyloxyalkyl groups which were designed to be hydrolyzed *in vivo*. In the 1980s, the moiety was incorporated to a prodrug of nucleoside monophosphate by Farquhar and his colleagues.[8] Since then, the bis(pivaloyloxymethyl) ester prodrugs have been well characterized and successfully used to achieve cellular delivery of dideoxyuridine 5′-monophosphate,[9] 9-(2-phosphonylmethoxyethyl)-adenine,[10] and 2′-deoxy-5-fluorouridine 5′-monophosphate.[11] These nucleoside monophosphate prodrugs were found to revert back to the parent compounds efficiently without toxic by-products.[11-13] Based on these successful applications, it was decided to pursue a prodrug approach using pivaloyloxymethyl moiety for the cellular delivery of N_3dUMP.

In this paper, we describe a new synthetic route to prepare acyloxyalkyl ester of phosphate in high yield. We also report that the charged nucleotide, N_3dUMP, can be delivered inside the cells via its neutral prodrug to inhibit the growth of tumor cell lines in the standard National Cancer Institute (NCI) test panel.[14]

SYNTHESIS

In several instances the synthesis of acyloxyalkyl ester of a simple phosphate or a

phosphonate has been accomplished by direct alkylation of the corresponding silver or tetraalkylammonium salt with acyloxyalkyl halides.[8,13] In general, this direct method is not applicable to the synthesis of acyloxyalkyl esters of nucleotides, in part, due to poor solubility of the nucleotide salts in a desired reaction solvent. Indeed, efficient general methods for the synthesis of the nucleotide acyloxyalkyl esters are lacking. The approaches that have been described in the literature include (a) alkylation of nucleoside 5'-deoxy-5'-halides with silver salts of preformed acyloxyalkyl phosphates,[8] (b) condensation of cyclic nucleotides containing highly hydrophobic moieties and acyclic nucleoside phosphonates with acyloxyalkyl halides in the presence of hindered bases,[13] and (c) condensation of nucleosides with bis(pivaloyloxymethyl) phosphate in the presence of Mitsunobu reagent.[11]

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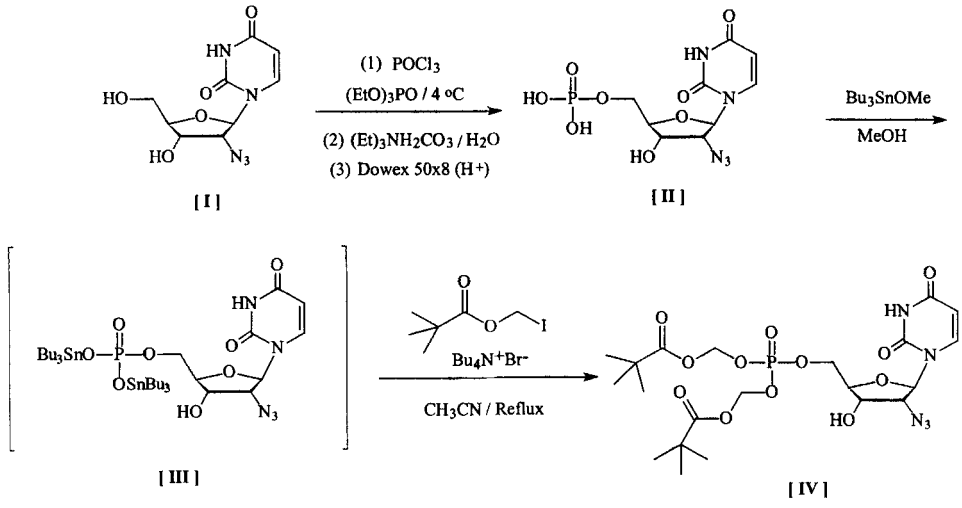
Recently, Ohuchi *et al.*[15] described an efficient method for the *O*-alkylation of dialkylphosphates, via the corresponding stannyl intermediates, with simple alkyl bromides in the presence of tetraalkylammonium bromide. We have successfully adopted this method for the preparation of bis(acyloxyalkyl)esters of pyrimidine nucleoside 5'-monophosphates (**Scheme 1**). Thus, the required tributylstannyl phosphate **III** was prepared by simply mixing N_3dUMP (free acid **II**) with Bu_3SnOMe in methanol at room temperature. The formation of **II** was conformed by ^{1}H-decoupled ^{31}P NMR which displayed a singlet at -2.7 ppm compared to a singlet at 5.8 ppm for **II**. Treatment of a solution of **III** in CH_3CN with iodomethylpivalate in the presence of $Bu_4N^+Br^-$ resulted in quantitative conversion of **II** to **IV**. The final product **IV** was isolated in 75% yield after chromatographic purification. Application of this method to 5'-monophosphates of uridine gave the corresponding bis(acyloxyalkyl) derivatives in greater than 80 % yield.

The prodrug **IV** was identified by spectral data (^{1}H NMR, ^{31}P NMR, MS). A noteworthy feature of the mass spectrum of **IV** was two sequential losses of elements of CH_2O (30 amu) from the molecular ion at *m/z* 578. Acylium migration can account for this mass spectral observation,[16] and may serve as a diagnostic feature in the characterization of these acyloxyalkyl esters of nucleotides.

BIOLOGICAL ACTIVITY

When Chinese hamster ovary (CHO) cells were exposed to the nucleoside analogue



Scheme 1. Preparation of bis(pivaloyloxymethyl) ester of 2′-azido-2′-deoxyuridine 5′-monophosphate (**IV**) from N_3dUrd (**I**).

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(N_3dUrd), its 5′-monophosphate (N_3dUMP), or 5′-diphosphate (N_3dUDP), up to a concentration of 100 μM, the cells were unaffected; however, the bis(pivaloyloxymethyl) ester of N_3dUMP (**IV**) inhibited the cell growth with IC_{50} of 3.0 μM (**Fig. 1**). This result, combined with the observation that N_3dUrd did not inhibit ribonucleotide reductase in permeabilized cells,[5] clearly suggests that the nucleoside analogue requires phosphorylation for biological activity. Considering both N_3dUMP and N_3dUMP blocked the reductase activity in permeabilized cells,[5] the lack of cell growth inhibition by N_3dUMP and N_3dUDP appears to be due to the inability of these charged molecules to enter the cells. On the contrary, the bis(pivaloyloxymethyl) ester of N_3dUMP (**IV**) can penetrate the cellular membrane, most likely via passive diffusion, and revert to N_3dUMP inside the cells. The resulting N_3dUMP can be phosphorylated by cellular kinases to N_3dUDP. The latter inhibits cell growth through interfering with the reduction of nucleoside dipohosphates by ribonucleotide reductase.

The prodrug (**IV**), N_3dCyd, and N_3dUrd were evaluated for inhibition of cell growth with 60 tumor cell lines at the National Cancer Institute. These cell lines were derived from nine different types of cancer; i.e. leukemia, non-small cell lung cancer, colon

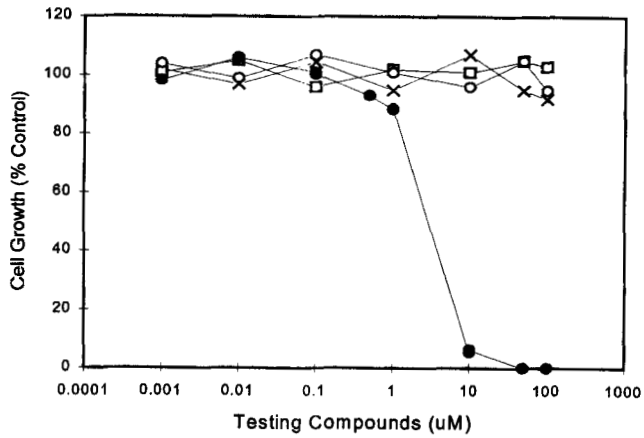

Figure 1. Inhibition of CHO cell growth by N_3dUrd (×),N_3dUMP (□),N_3dUDP (○), and the prodrug **IV** (●) after 48-hr incubation. Each point is the average of triplicate determinations. The ranges in the data points were less than 10 %.

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cancer, CNS cancer, melanoma, ovarian cancer, renal cancer, prostate cancer, and breast cancer. Results from the nine cell lines are summarized in **Table 1**. The inhibitory activity of the tumor cell growth was shown in terms of GI_{50} representing primary growth inhibition.[16] The prodrug **IV** inhibited the tumor cell growth from each of the nine panels with a GI_{50} value in low μM range. N_3dCyd showed an activity slightly better than or comparable to **IV** against several cell lines (CCRF-CEM, SNB-19, PC-3, DU-145), however, in the other cell lines **IV** was more active than N_3dCyd. Interestingly, while **IV** has comparable activity to most tumor cell lines tested with moderate differences, N_3dCyd demonstrated a high cell-line specificity: i.e. CCRF-CEM is 1000-fold sensitive to N_3dCyd than HOP-92. This might be due to difference in deoxycytidine kinase level as observed with 3T6 and CHO cells.[5]

N_3dUrd it did not show any growth inhibitory activity in the range of concentration used (up to mM). It is likely due to its poor conversion to N_3dUMP, which is a prerequisite for its activation to biologically active species, N_3dUDP. When the rate-limiting phosphorylation is overcome by the prodrug **IV**, N_3dUDP could be effectively generated resulting inhibition of tumor cell growth.

Table 1. Effect of N_3dCyd, N_3dUrd, and Bis(pivaloyloxymethyl) Ester of 2′-Azido-2′-deoxyuridine 5′-Monophosphate (**IV**) on Growth of Various Tumor Cell Lines. The inhibitory activity is represented by IG_{50} in μM.

Panel	Cell Line	N_3dCyd	N_3dUrd	IV
Leukemia	CCRF-CEM	0.53	> 875	2.53
	RRMI-8226	244	> 875	1.62
Non-small Cell	HOP-92	483	> 875	1.82
Lung Cancer	NCI-H23	28.4	> 875	4.44
Colon Cancer	HCC-2998	75.8	> 875	2.75
	HCT-15	38.0	> 875	3.07
CNS Cancer	SF-268	198	> 875	0.75
	SNB-19	6.68	> 875	12.9
Melanoma	MALME-3M	103	> 875	0.26
	M14	24.8	> 875	3.00
Ovarian Cancer	OVCAR-3	109	> 875	0.40
	OVCAR-8	56.8	> 875	3.06
Renal Cancer	ACHN	31.1	> 875	4.19
	UO-31	238	> 875	1.62
Prostate Cancer	PC-3	2.09	> 875	3.81
	DU-145	2.99	> 875	4.94
Breast Cancer	MCF7	16.3	> 875	3.81
	HS 578T	6.18	> 875	0.80

The test was performed by NCI, Drug synthesis & Chemistry Branch. The results with 18 cell lines out of 60 were summarized above (two cell lines for each panel): the one most sensitive to N_3dCyd, the other to **IV**.

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EXPERIMENTAL

Nuclear magnetic resonance spectra, ^{1}H NMR and ^{31}P NMR, were recorded on a Varian VXR-300S NMR spectrometer. ^{1}H and ^{31}P chemical shifts are reported in ppm relative to TMS (δ) and to 85 % H_3PO_4 (δ), respectively. Mass spectra were obtained on a Finnigan TSQ-700 triple quadrupole mass spectrometer, using atmospheric pressure chemical ionization (APCI). Analytical high-pressure liquid chromatography (HPLC) was

performed on a Hewlett Packard 1090 Chromatograph on a reversed phase column (BDS-Hypersil®-C8, 250 x 4.6 mm; Keystone Scientific Inc.). Preparative HPLC was performed on a Rainin HPXL Chromatograph using a reversed phase column (Zorbax®-C18, 250 x 21.2 mm, DuPont). UV spectra were recorded on a Hewlett Packard 300 Diode Array Spectrophotometer. 2'-Azido-2'-deoxyuridine was purchased from Sigma Chemical Co. (St. Louis, MO). All the other chemicals and solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI). Chinese hamster ovary (CHO) cells were obtained from American Type Culture Collection (Rockville, MD).

2'-Azido-2'-deoxyuridine 5'-Monophosphates (II): A mixture of 2'-azido-2'-deoxyuridine (200 mg, 0.74 mmol) and $POCl_3$ (300 μL, 3.2 mmol) in anhydrous $(EtO)_3PO$ (2 mL) was stirred overnight at 4 °C. Triethylammonium bicarbonate (1 M, pH 8.5, 6.5 mL) was added and the resulting mixture was extracted with Et_2O (3 × 10 mL). The product **II** in the aqueous layer was collected and purified on a 2.5 x 30 cm column of Sephadex A-25 with 0-0.5 M linear gradient (2 L) of $Et_3NH^+HCO_3^-$ (pH 7.5). The appropriate fractions were identified by UV absorbance, pooled, and evaporated in vacuo. To remove the inorganic phosphate still remaining, the residue was dissolved in H_2O (10 mL) and $Ba(OH)_2$ (185 mM) was then added until the pH of the solution was 12. Carbon dioxide was then bubbled in to adjust the pH to 7. The mixture was centrifuged to remove precipitated $Ba_3(PO_4)_2$ and the barium salt of **II** (^{1}H-decoupled ^{31}P NMR (D_2O): δ 4.22, singlet) was obtained from the supernatant. The barium salt of **II** in H_2O was passed through a column of Dowex-50 x 8 (H^+ form) ion exchange resin. Elution with H_2O followed by lyophilization to give 170 mg of **II** (free acid, 0.49 mmol) in 65 % yield. UV: λ_{max} (H_2O) 262 nm (ε 9.6 × 10^3); ^{1}H NMR (D_2O): δ 7.77 (d, 1H, J = 8.1 Hz, H-6), 5.84 (d, 1H, J = 4.9 Hz, H-1'), 5.78 (d, 1H, J = 8.1 Hz, H-5), 4.40 (t, 1H, H-3'), 4.19 (t, 1H, H-2'), 3.97-4.11 (m, 3H, H-5' and H-4'); ^{1}H-decoupled ^{31}P NMR (D_2O): δ -0.06 (s); MS: *m/z* 348 ([M - H]$^-$).

Bis[(pivaloyloxy)methyl] 2'-Azido-2'-deoxyuridine 5'-Monophosphates (IV): A mixture of **II** (35 mg, 0.10 mmol) and tributylstannyl methoxide (65 mg, 0.20 mmol) in CH_3OH (3 mL) was stirred at 25 °C for 30 min. Methanol was removed by evaporation. To remove the residual CH_3OH, dissolution of the residue in CH_3CN (3 mL) followed by

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evaporation of solvent was repeated 3 times. To the residue in CH_3CN (3 mL) were added $Bu_4N^+Br^-$ (65 mg, 0.20 mmol) and iodomethyl pivalate (324 mg, 1.0 mmol) prepared by reacting chloromethyl pivalate with NaI in CH_3CN. The mixture was refluxed for 1 h and then cooled to 25 °C. The mixture was concentrated to a small volume (~ 0.3 mL) under reduced pressure and then applied on a silica gel column. The column was eluted with a mixture of CH_2Cl_2 and CH_3OH (95:5, v/v). The appropriate fractions were identified by UV absorbance, pooled and evaporated in vacuo. The crude **IV** was purified by HPLC on Zorbax C18 column to give purified **IV** (93 mg, 0.16 mmol) in 80 % yield. ^{1}H NMR(CH_3OH-*d4*): δ 7.60 (d, 1H, *J* = 8.1 Hz, H-6), 5.78 (d, 1H, *J* = 4.4 Hz, H-1′), 5.68 (d, 1H, *J* = 8.1 Hz, H-5), 5.59-5.64 (m, 4H, OCH_2O), 4.31-4.38 (m, 2H, H-2′ and H-3′), 4.29-4.21 (m, 1H, H-4′), 4.03-4.09 (m, 2H, H-5′), 1.16 (s, 18H, $C(CH_3)_3$); ^{1}H-decoupled ^{31}P NMR(CH_3OH-*d4*): δ -3.71 (s); MS: *m/z* 578 ($[M + H]^+$).

CHO Cell Culture: CHO cells were maintained as monolayer in α-minimal essential medium (Sigma) supplemented with antibiotics and 10% heat-inactivated fetal calf serum (GIBCO, Grand Island, NY) at 37 °C in a humidified atmosphere containing 5% CO_2. All experiments were performed with cells in exponentially growing phase. Cell number was determined on a hemocytometer with an inverted microscope (Nikon, Model TMS).

CHO Cell Growth Study: The culture medium for an exponentially growing CHO cell monolayer was replaced with that containing various concentrations of **IV**. Due to the poor solubility of **IV** in H_2O, a stock solution of **IV** in EtOH was added to the cell in monolayer (the final concentration of EtOH was 1%). Up to 1 mM, **IV** did not precipitate out in 1% EtOH. The cells were incubated under the same condition as described before. After incubated for 48 h, the cells were harvested and viable cells were counted using 0.4% Trypan Blue solution on a hemocytometer on an inverted microscope.

Antitumor Screening against 60 Tumor Cell Lines: These *in vitro* tests were performed by the National Cancer Institute. Experimental details can be found elsewhere.[14] Briefly, each cell line was seeded onto 96-well microtiter plates and then preincubated for 24-28 h in the absence of drug. Subsequently, **IV** or **I** was added in five 10-fold dilutions and the culture was incubated for 48 h. The end-point cell viability or cell growth was determined by *in situ* fixation of cells, followed by staining with a protein-binding dye,

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sulforhodamine B. The bound stain was solubilized and measured spectrophotometrically to determine relative cell growth or viability in treated and untreated cells.

ACKNOWLEDGMENTS

The authors are grateful for the kind arrangement made by Ms Jill Johnson of the NCI Drug Synthesis and Chemistry Branch for testing the compounds disclosed in this report in their screening program.

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