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Research Paper

Bis(carbamoyloxymethyl) Esters of 2′,3′-Dideoxyuridine 5′-monophosphate (ddUMP) as Potential ddUMP Prodrugs

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Purpose. We previously reported the synthesis of bis(pivaloyloxymethyl) 2′,3′-dideoxyuridine 5′-monophosphate (POM_2-ddUMP) (**1a**) as a membrane-transport prodrug formulation of the free parent nucleotide, ddUMP. Although successful at delivering ddUMP into cells in culture, POM_2-ddUMP was rapidly degraded by plasma carboxylate esterases after intravenous administration to experimental animals, and therefore has limited therapeutic potential as a systemically administered prodrug. We now report the synthesis of bis(*N,N*′-dimethylcarbamoyloxymethyl)- and bis(*N*-piperidinocarbamoyloxymethyl) 2′,3′-dideoxyuridine 5′-monophosphate [DM_2-ddUMP (**1b**) and DP_2-ddUMP (**1c**), respectively], analogues of POM_2-ddUMP that were designed to be more resistant to degradation by plasma esterases.

Methods. After entering cell by passive diffusion, it was anticipated that loss of one of the carbamoyloxymethyl groups of **1b** and **1c** would occur by spontaneous chemical hydrolysis to give the intermediate phosphodiesters, **2b** and **2c.** Cleavage of the remaining carbamoyloxymethyl groups by cellular phosphodiesterase I would generate ddUMP. **1b** and **1c** were prepared by condensation of 2′,3′-dideoxyuridine (ddU) with the appropriate bis(*N*-alkylcarbamoyloxymethyl) phosphate in DMA in the presence of triphenylphosphine and diethyl azodicarboxylate (the Mitsunobo reagent).

Results. The half-lives of **1b** and **1c** when incubated at a concentration of 10^{-4} M in human plasma at 37°C were 3.5 h and 3.7 h, respectively, similar to the half-lives observed under the same temperature conditions in 0.05 M aqueous phosphate buffer, pH 7.4. By contrast, the half-life of the POM_2 prodrug, **1a,** in plasma was only 5 min. The initial products of degradation of **1b** and **1c** were the phosphodiesters **2b** and **2c.** The latter compounds gave rise to ddUMP when incubated with snake venom phosphodiesterase I.

Conclusion. These findings support the premise inherent in the design of **1b** and **1c,** namely that the carbamate prodrugs are far more resistant to hydrolysis by plasma carboxylate esterases than their POM counterparts and can revert to the free parent 5′-mononucletides by successive chemical and enzymatic hydrolysis. Further studies of **1b** and **1c** as membrane-permeable prodrugs of ddUMP are in progress.

KEY WORDS: anticancer; 2′,3′-dideoxyuridine 5′-monophosphate; membrane-permeable prodrugs.

INTRODUCTION

5′-Mononucleotides are obligatory intermediates in the activation of most anticancer and antiviral nucleoside analogues (1–4). However, their utility as chemotherapeutic agents is severely compromised because they are charged at physiologic pH and penetrate poorly into cells (5–6). 5′-Mononucleotides are also susceptible to rapid degradation to the corresponding nucleosides in tissues by 5′-nucleotidases and other nonspecific phosphohydrolases (7–8). To overcome these limitations, we reported a series of neutral bis(pivaloyloxymethyl) [POM_2] 5′-nucleotide esters (e.g., **1a** (12)) as potential membrane-permeable prodrugs of the parent 5′-mononucleotides (7–10). We showed that these compounds penetrated facilely into cells and reverted to the parent 5′-mononucleotides (11), (e.g., **3**) after successive cleavage of the POM groups by carboxylate esterases and phosphodiesterases, respectively (Scheme 1). However, a significant shortcoming of this approach *in vivo* is that the POM_2 prodrugs are rapidly degraded in human plasma by carboxylate esterases (S. Khan and D. Farquhar, unpublished results) before they have an opportunity to reach their target therapeutic sites. To address this problem, we have investigated the potential of bis(carbamoyloxymethyl) esters of 5′-mononucleotides as potential esterase-resistant prodrugs of the free parent nucleotides. Carbamate groups should be less susceptible than ester groups to esterase-mediated hydrolysis and, therefore, bis(carbamoyloxymethyl) 5′-nucleotide prodrugs should have superior systemic bioavailability. Once administered *in vivo*, cleavage of the first carbamoyloxymethyl group is anticipated to occur by spontaneous chemical hydrolysis and, by analogy with mono-POM 5′-nucleotides (10), cleavage of the second should occur by nonspecific phosphodiesterases. The objective of this study was to prepare model bis(carbamoyloxy-

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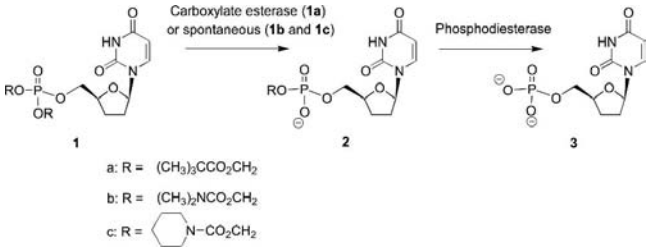


Sch. 1. Mechanism of reversion of the prodrugs to the patient ionic compounds.

methyl) 5′-nucleotide prodrugs and to study their stability and chemical fate in aqueous buffers and in human plasma. Bis(*N,N′*-dimethylcarbamoyloxymethyl) and *bis*(N-piperidinocarbamoyloxymethyl) diesters of 2′,3′-dideoxyuridine 5′-monophosphate (**1b** and **1c,** respectively) were selected for this purpose. A brief preliminary account of part of this work has previously appeared (12).

MATERIALS AND METHODS

2′,3′-Dideoxyuridine (ddU) and phosphodiesterase I (E.C 3.1.4.1) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). ddUMP was prepared from ddU by the general method of Yoshikawa *et al.* (13) All reactions were carried out in dry glassware and were protected from atmospheric moisture. Solvents were dried over freshly activated (300°C/4 h) molecular sieves (type 4A). Reactions with silver salts were conducted in dry glassware in the dark and were protected from atmospheric moisture. The silver salts were dried *in vacuo* over P_2O_5 before use. The homogeneity of the products was determined by ascending TLC on silica-coated glass plates (silica gel 60 F 254, Merck, Darmstadt, Germany) using mixtures of $CHCl_3$-MeOH (typically 1% to 10% MeOH) as the eluting solvent. Chromatograms were visualized under a UV lamp (254 nm) or by placing the air-dried plates in a tank of I_2 vapor. Compounds containing pivaloyloxymethyl or carbamoyloxymethyl groups were identified by spraying the plates with a 0.25% solution of 4-amino-3-hydrazino-5-mercapto-1,2,4-tetrazole (Purpald) in 0.5 N NaOH solution and heating them in an oven at 85°C for 5 min. The liberated formaldehyde reacted with the Purpald reagent to form purple spots against a white background. Preparative separations were performed by flash chromatography on silica (Merck, 230-400 mesh) using mixtures of EtOAc/hexane or $CHCl_3$/MeOH as eluent. Chemical reagents were purchased from Aldrich Chemicals Co. (Milwaukee, WI, USA) or from Sigma Chemical Co. (St. Louis, MO, USA). Nuclear magnetic resonance spectra (^{1}H NMR, ^{13}C NMR) were recorded at ambient temperature on an IBM-Bruker Model NR/200 AF spectrometer (Boston, MA, USA) in the Fourier transform mode, in $CDCl_3$, using tetramethylsilane as an internal standard. Mass spectra were obtained on a Finnegan Model 3300 quadruple spectrometer (Platte City, MO, USA) in the electron impact mode, or the chemical ionization mode using methane as the reagent gas. Elemental analyses were performed by Galbraith Laboratories, Inc. (Knoxville, TN, USA) and, where indicated only by the symbols of elements, the results were within ±0.4% of the theoretical values. Melting points were determined on a Hoover capillary apparatus (Pittsburgh, PA, USA) and are uncorrected.

Chemical Synthesis

*Disilver Benzyl Phosphate (**7**)*

Phosphorus acid (dried over P_2O_5) (25.0 g, 0.30 mol) was dissolved in a solution of benzyl alcohol (500 ml) and Et_3N (150 ml, 33.75 mmol). Solid I_2 (116 g, 0.45 mol) was added in 10 g portions with cooling in an ice bath at 5°C. The mixture was stirred for 30 min. It was then poured into acetone (2000 ml) and an excess of cyclohexylamine (100 ml) was added. The precipitate which formed was collected by filtration, washed with acetone, and recrystallized from aqueous EtOH (H_2O, 200 ml; EtOH, 1100 ml) containing 1% (v/v) of cyclohexylamine (14). The yield was 69.0 g (78%). The mother liquor was evaporated to dryness and the residual solid was recrystallized to give a further 17 g of product. The total yield of cyclohexylammonium benzyl phosphate was 86.0 g (97%); mp 229–233°C (lit. mp 233–234°C). A solution of this product (50.0 g, 0.13 mol) in distilled H_2O (500 ml) was passed through a column (5 × 50 cm) of Bio-Rad AG (Richmond, CA, USA) 50W × 12 cation-exchange resin (200-400 mesh, 2.3 mEq per ml of resin bed) in the Na^+ form. The resin was washed with 3 column volumes of distilled H_2O. The combined effluents were concentrated to ca. 300 ml on a rotary evaporator under reduced pressure at <40°C and then added, dropwise, to a rapidly stirred solution of $AgNO_3$ (48.3 g, 0.29 mol) in H_2O (300 ml). The copious white precipitate that formed was filtered and washed sequentially with distilled H_2O, EtOH, and Et_2O, and air-dried. The product was stored *in vacuo* over P_2O_5. The yield was 48.9 g, 94%. Anal. ($C_7H_7O_4PAg_2$) C, H, Ag.

*(N,N′-Dimethyl)carbamoyloxymethyl Chloride (**6b**)*

A solution of dimethylamine **4b** (20 g, 0.44 mol) in toluene (250 ml) was added dropwise over 30 min with vigorous stirring at 0°C to chloromethyl chloroformate (28.7 g, 20 ml, 0.22 mol) contained in a 1-L flask. The mixture was stirred overnight at room temperature and then filtered to remove precipitated salts. The filtrate was concentrated under reduced pressure, and the residue was taken up in toluene (100 ml), and filtered to remove additional precipitated salts. The filtrate was washed successively with 5% $NaHCO_3$ solution (50 ml) and H_2O (3 × 100 ml), and dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure (30 mmHg) at <30°C to give (*N,N′*-dimethyl)carbamoyloxymethyl chloride, **6b,** as a light yellow viscous liquid in 83% yield. ^{1}H NMR ($CDCl_3$): δ 5.78 (s, 2 H, CH_2), 2.97 (s, 6 H, $N(CH_3)_2$. MS: m/z 138 (MH^+). Anal. ($C_4H_8ClNO_2$) C, H, N.

*N-Piperidinocarbamoyloxymethyl Chloride (**6c**)*

A solution of piperidine **4c** (9.9 g, 11.5 ml, 0.11 mol) in toluene (60 ml) was added dropwise over 30 min with vigorous stirring at 0°C to chloromethyl chloroformate, **5** (7.5 g, 6.0 ml, 0.06 mol). The mixture was stirred overnight at room temperature and then filtered to remove precipitated salts. The filtrate was concentrated under reduced pressure, and the residue was taken up in toluene (50 ml), and again filtered to remove additional precipitated salts. The filtrate was washed successively with 5% $NaHCO_3$ solution (50 ml) and H_2O (3 × 100 ml), and dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure (30 mmHg) at <30°C to give *N*-piperidinocarbamoyloxymethyl chloride,

6c, as a light yellow viscous liquid in 85% yield. ^{1}H NMR (IBM-Bruker Model NR/200; $CDCl_3$): δ 5.79 (s, 2 H, OCH_2), 2.97 (m, 4 H, $N(CH_2)_2$; piperidino α protons), 2.97 (m, 6 H, $(CH_2)_3$; piperidino β and γ protons). MS: m/z 178 (MH^+). Anal. ($C_7H_{12}ClNO_2$) C, H, N.

*Bis[(N,N′-Dimethyl)carbamoyloxymethyl] Benzyl Phosphate (**8b**)*

A solution of **6b** (11.5 g, 0.067 mol) in dry toluene (100 ml), was added, dropwise, over 30 min to a suspension of finely divided disilver benzyl phosphate, **7** (3,6) (13.0 g, 0.033 mol) in toluene (50 ml). An exothermic reaction ensued. The reaction mixture was stirred vigorously at 80°C for 24 h. The precipitated salts were filtered and the filtrate was washed successively with 5% $NaHCO_3$ solution (1 × 100 ml) and H_2O (3 × 50 ml), then dried over anhydrous Na_2SO_4. Evaporation of the solvent yielded bis[(*N*,*N*′-dimethyl)carbamoyloxymethyl] benzyl phosphate, **8b,** as a colorless, viscous oil (13.7 g, 89%). ^{1}H NMR ($CDCl_3$): δ 7.23 (s, 5 H, C_6H_5), 5.63 (d, 4 H, OCH_2O, J = 14 Hz), 5.10 (d, 2 H, $C_6H_5CH_2$, J = 8 Hz), 2.93 (s, 12 H, $N(CH_3)_2$). MS: m/z 391 (MH^+). Anal. ($C_{15}H_{23}N_2O_8P$) C, H, N.

*Bis[(N-piperidino)carbamoyloxymethyl] Benzyl Phosphate (**8c**)*

A solution of **6c** (3.5 g, 0.02 mol) in dry toluene (20 ml), was added, dropwise, over 30 min to a suspension of finely divided disilver benzyl phosphate, **7** (3.8 g, 0.09 mol) in toluene (20 ml). The reaction mixture was maintained with stirring at 80°C for 24 h. The precipitated salts were filtered, and the filtrate was washed successively with 5% $NaHCO_3$ solution (1 × 100 ml) and H_2O (3 × 50 ml), then dried over anhydrous Na_2SO_4. Evaporation of the solvent yielded bis[(*N*-piperidinocarbamoyloxymethyl] benzyl phosphate, **8c,** as a colorless, viscous oil (4.2 g, 89%). ^{1}H NMR ($CDCl_3$): δ 7.28 (s, 5 H, C_6H_5), 5.80 (d, 4 H, OCH_2O, J = 14 Hz), 5.20 (d, 2 H, $C_6H_5CH_2$, J = 8 Hz), 3.43 (m, 8 H, $N(CH_2)_2$; piperidino α protons), 1.56 (m, 12 H, $(CH_2)_3$; piperidino β and γ protons). MS: m/z 471 (MH^+). Anal. ($C_{21}H_{31}N_2O_8P$) C, H, N.

*Bis[(N,N′-dimethyl)carbamoyloxymethyl] Hydrogen Phosphate (**9b**)*

A solution of **8b** (10.6 g, 0.03 mol) in cyclohexane (200 ml) was hydrogenated over 5% Pd/C (500 mg) at a pressure of 30 psi. After 1 h, an additional 500 mg of catalyst was added and hydrogenolysis was continued for a further 1 h. The catalyst was filtered and the solution was evaporated under reduced pressure to give bis[(*N*, *N*′-dimethyl)carbamoyloxymethyl] hydrogen phosphate, **9b,** as a colorless viscous oil. The yield was 7.3 g (68%). ^{1}H NMR ($CDCl_3$): δ 5.64 (d, 4 H, OCH_2O, J = 14 Hz), 2.93 (s, 12 H, $N(CH_3)_2$). MS: m/z 301 (MH^+). Anal. ($C_8H_{17}N_2O_8P$) C, H, N.

*Bis[(N-piperidino)carbamoyloxymethyl] Hydrogen Phosphate (**9c**)*

A solution of **8c** (3.9 g, 0.08 mol) in cyclohexane (200 ml) was hydrogenated over 5% Pd/C (100 mg) at a pressure of 30 psi. After 1 h, an additional 100 mg of catalyst was added and hydrogenolysis was continued for a further 1 h. The catalyst was filtered and the solution was evaporated under reduced pressure to give bis(*N*-piperidinocarbamoyloxymethyl) hydrogen phosphate, **9c,** as colorless viscous oil. The yield was 8 g (89%). ^{1}H NMR ($CDCl_3$): δ 5.52 (d, 4 H, OCH_2O, J = 14 Hz), 3.19 (m, 8 H, $N(CH_2)_2$; piperidino α protons), 1.49 (m, 12 H, $N(CH_2)_3$; piperidino β and γ protons). MS: m/z 381 (MH^+). Anal. ($C_{14}H_{25}N_2O_8P$) C, H, N.

*Bis[(N,N′-dimethyl)carbamoyloxymethyl] 2′,3′-Dideoxyuridine 5′-monophosphate (**1b**)*

Compound **9c** (297 mg, 0.99 mmol), 2′,3′-dideoxyuridine (140 mg, 0.66 mmol), and triphenylphosphine (136 mg, 0.99 mmol) were dissolved in DMA (3.0 ml) and the solution was stirred magnetically for 10 min. A solution of diethylazodicarboxylate (0.16 ml, 0.99 mmol) in DMA (0.5 ml) was added with stirring and the reaction mixture was maintained at 60°C for 5 days under a N_2 atmosphere. The DMA was evaporated under reduced pressure. The remaining oil was triturated several times with 10 ml portions of *n*-hexane/EtOAc (3:1) and the supernatants were decanted. The contents of the flask were maintained under high vacuum for 24 h to remove residual DMA, and the remaining product was taken up in the minimum of $CHCl_3$ and chromatographed on a column of silica (70 g) using $CHCl_3$-MeOH (95:5) as eluent. Bis[(*N*,*N*′-dimethyl)carbamoyloxymethyl] 2′,3′-dideoxyuridine 5′-monophosphate **1b** (14) was isolated as a viscous oil. Yield, 144 mg (45%). ^{1}H NMR ($CDCl_3$): δ 7.65 (d, 1 H, H-6, J = 8 Hz), 6.0 (dd, 1 H, H-1′), 5.77 (d, 1 H, H-5, J = 8 Hz), 5.70 (d, 4 H, $P(O)OCH_2O$, J_{PH} = 12 Hz), 4.28 (m, 2 H, H-5′), 2.97 (s, 12 H, $N(CH_3)_2$), 2.22 (m, 2 H, H-3′), 2.15 (m, 1 H, H-4′), 1.71 (m, 2 H, H-2′). MS: m/z 495 (MH^+). Anal. ($C_{17}H_{27}N_4O_{11}P$): C, H, N.

*Bis[(N-piperidino)carbamoyloxymethyl] 2′,3′-Dideoxyuridine 5′-monophosphate (**1c**)*

The product was prepared from **9c** (565 mg, 1.5 mmol), 2′,3′-dideoxyuridine (210 mg, 0.99 mmol), triphenylphosphine (390 mg, 1.5 mmol) and diethylazodicarboxylate (0.24 ml, 1.5 mmol) as described for **1b.** The crude product was purified on a column of silica (100 g) using $CHCl_3$/MeOH (9:1) as eluent to afford **1c** (15) as a viscous oil. Yield, 111 mg (35%). ^{1}H NMR ($CDCl_3$): δ 7.73 (d, 1 H, H-6, J = 8 Hz), 6.20 (dd, 1 H, H-1′), 5.78 (d, 1 H, H-5, J = 8 Hz), 5.71 (d, 4 H, $P(O)OCH_2O$, J_{PH} = 12 Hz), 4.40 (m, 2 H, H-5′), 3.45 (m, 8 H, $N(CH_2)_2$; piperidino α protons), 1.51 (m, 12 H, $N(CH_2)_3$; piperidino β and γ protons), 2.22 (m, 2 H, H-3′), 2.15 (m, 1 H, H-4′), 1.71 (m, 2 H, H-2′). MS: m/z 575 (MH^+). Anal. ($C_{23}H_{35}N_4O_{11}P$): C, H, N.

*Mono[(N,N′-Dimethyl)carbamoyloxymethyl] 2′, 3′-Dideoxyuridine 5′-phosphate (**2b**)*

NH_4HCO_3 buffer (0.05 M), pH 8 (5 ml), was added to **1b** (15 mg, 0.03 mmol) in a round-bottomed flask, and the mixture was stirred at room temperature for 30 min. The contents of the flask were transferred to a 20 ml vial that was maintained at 37°C for 4 h. The progress of the formation of the monocarbamoylmethyl derivative was monitored by high-pressure liquid chromatography (HPLC) on a μBondapak C-18 reversed-phase column (150 × 3.90 mm, i.d.; Phenomenex, Torrance, CA, USA) using a stepwise gradient of MeOH:H_2O (1:1) and 0.05 M NH4OAc, pH 4.4, as described

under aqueous stability studies, above. After completion of the reaction, the solution was lyophilized. H_2O (10 ml) was added and the solution was again lyophilized; this procedure was twice repeated. Yield: 11 mg (93%). The identity and purity of the product was confirmed by HPLC and NMR analysis. ^{1}H NMR (MeOD): δ 7.65 (d, 1 H, H-6, J = 8 Hz), 6.0 (dd, 1 H, H-1′), 5.77 (d, 1 H, H-5, J = 8 Hz), 5.66 (d, 2 H, $P(O)OCH_2O$, J_{PH} = 12 Hz), 4.25 (m, 2 H, H-5′), 2.92 (s, 6 H, $N(CH_3)_2$), 2.25 (m, 2 H, H-3′), 2.12 (m, 1 H, H-4′), 1.74 (m, 2 H, H-2′). MS: m/z 378 (MH^+). Anal. ($C_{13}H_{20}N_3O_8P$): C, H, N.

*Mono[(N-piperidino)carbamoyloxymethyl] 2′,3′-Dideoxyuridine 5′-phosphate (**2c**)*

This compound was prepared from **1c** (30 mg, 0.06 mmol) as described for the dimethyl analogue, **2b,** above. Yield: 21 mg (80%). ^{1}H NMR (MeOD): δ 7.73 (d, 1 H, H-6, J = 8 Hz), 6.20 (dd, 1 H, H-1′), 5.78 (d, 1 H, H-5, J = 8 Hz), 5.71 (d, 2 H, $P(O)OCH_2O$, J_{PH} = 12 Hz), 4.4 (m, 2 H, H-5′), 3.45 (m, 4 H, $N(CH_2)_2$; piperidino α protons), 1.49 (m, 6 H, $(CH_2)_3$; piperidino β and γ protons), 2.22 (m, 2 H, H-3′), 2.15 (m, 1 H, H-4′), 1.71 (m, 2 H, H-2′). MH^+ = 418.21. Anal. ($C_{13}H_{20}N_3O_8P$): C, H, N. 3.19

Aqueous Solubility of ddUMP Prodrugs

Potassium phosphate buffer (0.05 M), pH 7.4 (3 ml), was added to 10 ml round-bottomed flasks containing 15.2 mg each of **1a, 1b,** and **1c.** The mixtures were stirred at room temperature for 2 h, then the contents of each flask were transferred to 10 ml centrifuge tubes and centrifuged at 2000 rpm for 10 min. The supernatants were decanted and passed through a 0.22 pm Millipore filter to remove any remaining particulate matter. The concentrations of **1a, 1b,** and **1c** in solution were determined by UV adsorption using the measured value of 7838 for the molar extinction coefficient (ε) of these compounds at wavelength of 268 nm.

Partition Coefficient of ddUMP Prodrugs

1-Octanol (3 ml; previously equilibrated with 0.05 M potassium phosphate buffer, pH 7.4) was added to solutions of **1a, 1b,** and **1c** (ca. 6 mg each) in 0.05 M phosphate buffer, pH 7.4 (3 ml; previously equilibrated with 1-octanol) contained in 15 ml capped centrifuge tubes. The mixtures were agitated for 5 min. The octanol and aqueous layers were separated, and the concentration of **1a, 1b,** and **1c** in each phase was determined by UV absorption using a value of 7838 for the molar extinction coefficient (ε) of each compound a wavelength of 268 nm.

Stability Studies of ddUMP Prodrugs in Aqueous Buffers

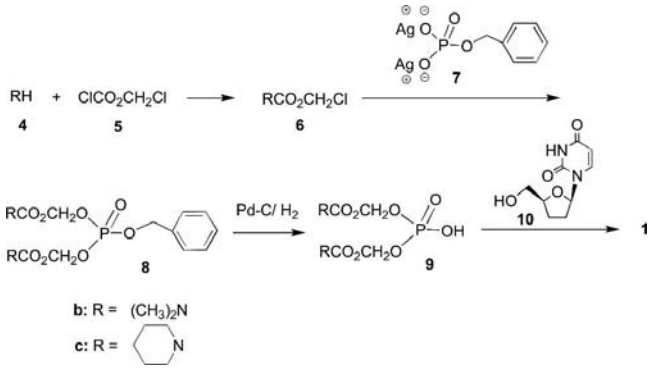
Aliquots of a stock solution of POM_2-ddUMP (**1a**), DM_2-ddUMP (**1b**), and DP_2-ddUMP (**1c**) in H_2O (10^{-3} M) were diluted with various buffers to a final concentration of 10^{-4} M. These solutions were stirred at room temperature. At selected time intervals (typically, 2, 4, 8, 12, 24, 30, 50, and 100 h) aliquots (50 μl) were removed and analyzed immediately for parent drug by HPLC on a μBondapak C-18 reversed phase column (150 × 3.90 mm, i.d.; Phenomenex) protected with a Waters Guard-Pak precolumn filter (0.22 μm). The mobile phase was a stepwise gradient of MeOH:H_2O (1:1) (solution A) and 0.05 M ammonium acetate, pH 4.4 (solution B) at a flow rate of 1 ml per min delivered according to the following program: 10 min of A; 10 min of A:B, 80:20 (v/v); 10 min of A:B, 60:40 (v/v); and 10 min of B. Eluted compounds were monitored with a variable-wavelength UV detector set at 268 and 0.005 absorbance unit full scale (AUFS) sensitivity. The retention times for POM_2-ddUMP, POM_1-ddUMP, ddU, and ddUMP were 28.79, 25.10, 14.72, and 8.48 min, respectively. For DM_2-ddUMP, DM_1-ddUMP, ddU, and ddUMP the retention times were 28.81, 25.31, 13.88, and 8.46 min, respectively, and for DP_2-ddUMP, DP_1-ddUMP, ddU, and ddUMP the retention times were 28.92, 25.33, 13.78, and 8.16 min, respectively.

Stability Studies of ddUMP Prodrugs in Human Plasma

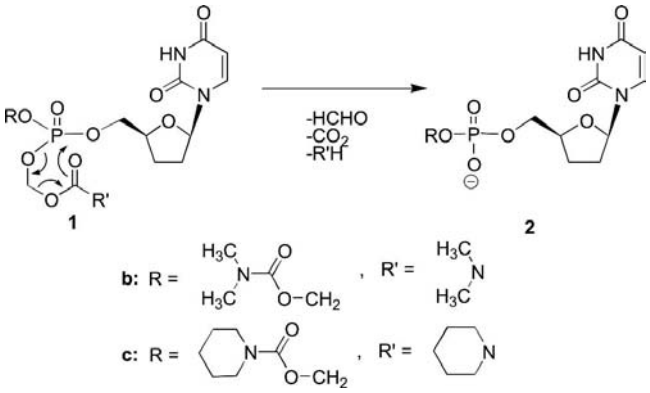
Human plasma was obtained from the Blood Bank (M. D. Anderson Cancer Center, Houston, TX). The reaction was started by adding 100 μl of stock solutions (1×10^{-2} M) of POM_2-ddUMP (**1a**), DM_2-ddUMP (**1b**), and DP_2-ddUMP (**1c**) in EtOH to plasma (1900 μl) contained in separate 3 ml vials such that the final concentrations of prodrug were 1×10^{-4} M. The samples were agitated for 20 s on a Vortex shaker to ensure thorough mixing and then incubated at 37°C. Samples (100 μl) from each vial were withdrawn at 0, 1, 5, 15, 30, 60, 120, 180, 360, and 1440 min and diluted with 4 volumes of ice-cold MeOH. The mixtures were agitated on a Vortex shaker for 1 min and then centrifuged at 10,000 rpm for 10 min to sediment precipitated protein. The supernatants were analyzed by HPLC for parent compounds and degradation products as described above for aqueous stability studies.

Stability of POM_1-ddUMP and DP_1-ddUMP in the Presence of Snake Venom (*Bothrops atrox*) Phosphodiesterase I

Snake venom (*Bothrops atrox*) phosphodiesterase I was obtained from Sigma Chemical Co. and used as received. The specific activity of the preparation was 0.029 U/mg of protein where 1 U is defined as the amount that will hydrolyze 1.0 μmol of bis(*p*-nitrophenyl) phosphate per minute at pH 8.8 and 37°C. DP_1-ddUMP was dissolved in 0.05 M phosphate buffer, pH 7.4, at a concentration of 10^{-4} M. One-milliliter aliquots of this solution contained in 5.0 ml screw-capped glass vials were incubated at 37°C in the absence or the presence of the enzyme. For the enzyme studies, 1 and 2 U of phosphodiesterase I per μmol of substrate were used. A control reaction was run using 2 U of enzyme that had previously been boiled for 10 min. To begin reaction the enzyme was added to 0.2 ml of the drug solution contained in 1.5 ml micro centrifuge tubes that were preequilibrated at 37°C. At intervals of 0, 0.17, 0.34, 0.67, 1, 2, 3, 5, 8, 25 h thereafter, aliquots (0.1 ml) of the reaction mixture were withdrawn and added to 0.1 ml of ice cold MeOH were added to deactivate the enzyme. The mixtures were agitated on a Vortex mixer (Queens Village, NY, USA) for 20 s and then centrifuged at 2000 rpm for 5 min. The supernatants were analyzed by HPLC on a μBondapak C-18 reversed phase column (150 × 3.90 mm, i.d.; Phenomenex) protected with a Waters Guard-Pak precolumn filter (Milford, MA, USA) (0.22 μm). The mobile phase was a stepwise gradient of MeOH:H_2O (1:1) (solution A) and 0.05 M ammonium acetate, pH 4.4 (solution B) at a flow rate of



Sch. 2. Synthesis of prodrugs **1b** and **1c.**



Sch. 3. Mechanism of release of **2a** and **2b.**

1 ml per min as described above under aqueous stability studies. The retention times for POM_1-ddUMP and ddUMP were 25.10 and 8.48 min, respectively. For DP_1-ddUMP and ddUMP, the retention times were 25.33 and 8.16 min, respectively. The substrate concentrations were determined spectrophotometrically by UV absorption using the measured value of 7838 for the molar extinction coefficient (ε) at a wavelength of 268 nm. The half-lives were determined by linear least-square regression analysis of the pseudo-first-order reactions.

RESULTS AND DISCUSSION

Preparation of Bis(carbamoyloxymethyl) Esters of ddUMP

1b and **1c** were prepared as shown in Scheme 2. Reaction of the secondary amine, **4,** with chloromethyl chloroformate, **5,** yielded the chloromethyl carbamate, **6,** which was reacted with disilver benzyl phosphate, **7,** to generate the corresponding phosphotriester, **8.** Catalytic hydrogenation of **8** yielded the free acid **9** which was condensed with 2′,3′-dideoxyuridine in the presence of triphenylphosphine and diethyl azodicarboxylate (DEAD) (the Mitsunobo reagent) to give the target phosphotriesters, **1b** and **1c** (16).

Preparation of Monocarbamoyloxymethyl Esters of ddUMP

Mono(*N,N*′-dimethylcarbamoyloxy-methyl)- and mono(*N*-peridinocarbamoyloxymethyl) 2′,3′-dideoxyuridine 5′-monophosphate (**2b** and **2c,** respectively) were prepared by selective hydrolysis of the parent diesters, **1b** and **1c,** with 0.05 M NH_4HCO_3 buffer at pH 7.0.

Table I. Half-lives[a] of **1a, 1b,** and **1c** in Aqueous Buffers

Buffer	Half-life (h)		
	1a	**1b**	**1c**
0.05 M potassium chloride, pH 1.0	88	2.7	2.9
0.05 M sodium acetate, pH 4.0	176	4.2	3.3
0.05 M potassium phosphate, pH 7.4	157	5.0	3.4
0.05 M Tris-HCl, pH 9.0	25	0.7	3.6

[a] Half-lives represent the average of triplicate determinations.

Aqueous Solubility

The maximum solubility of **1a, lb,** and **1c** in 0.05 M phosphate buffer, pH 7.4, determined by stirring the compounds with the buffer for 2 h at ambient temperature, were 3.6 mg/ml for **1a,** 8.65 mg/ml for **1b,** and 1.9 mg/ml for **1c.** The 4-fold greater aqueous solubility of **1b** over **1c** is obviously due to the reduced lipophilicity of the dimethylamino groups compared to the piperidino groups.

Partition Coefficient

The partition coefficients *(P)* of **1a, 1b,** and **1c** between l-octanol and 0.05 M potassium buffer, pH 7.4, were 15.1 (log $P = 1.18$), 0.016 (log $P = -1.78$), and 5.54 (log $P = 0.72$), respectively. Compounds with positive log *P* values (such as **1a** and **1c**) generally have move favorable membrane-permeability characteristics than compounds with negative *P* values (such as **1b**).

Stability in Aqueous Buffers

When stirred at a concentration of 10^{-4} M in 0.05 M aqueous buffers over the pH range 1 to 9, **1b** was slowly degraded with half-lives ranging from 0.7 h to 5.0 h. The piperidino analogue, **1c,** showed similar degradation kinetics but was significantly more stable than **1b** at alkaline pH. The only products observed were the monocarbamoyloxymethyl compounds **2b** and **2c.** By comparison, the POM_2 analogue **1a** was markedly more stable under all pH conditions with half-lives ranging from 1 day to 7 days (Table I).

The greater lability of **1b** and **1c** over **1a** is most likely due to the greater nucleophilicity of the carbamoyl group compared to the acyl group. It is likely that DM_2-ddUMP and DP_2-ddUMP rearrange by attack of the carbamoyl oxygen atom on the phosphoryl group with cleavage of the P-OCH_2

Table II. Half-lives[a] of **1a, 1b,** and **1c** in Human Plasma[b]

Compound	Half-life (h)
POM_2-ddUMP, **1a**	0.08
DM_2-ddUMP, **1b**	3.7
DP_2-ddUMP, **1c**	3.5

[a] Half-lives represent the average of triplicate determinations.
[b] Initial drug concentration: 10^{-4} M.

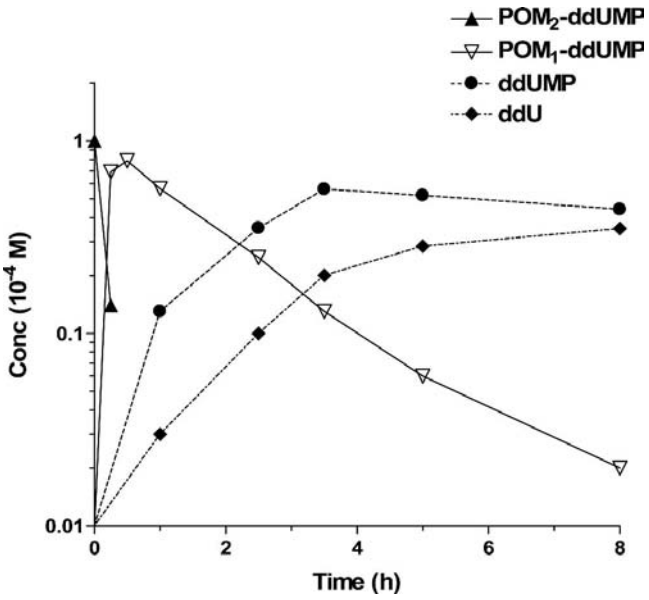


Fig. 1. Stability of POM_2-ddUMP, **1a,** in human plasma at 37°C.

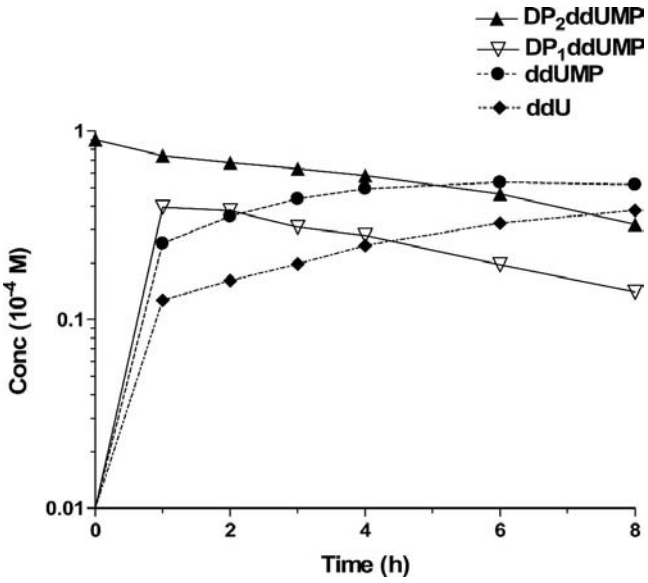


Fig. 3. Stability of DP_2-ddUMP, **1c,** in human plasma at 37°C.

linkage and release of formaldehyde as shown in Scheme 3. The intermediate mixed anhydride is then hydrolyzed to give the phosphodiester **2** and a carbamic acid, the latter spontaneously breaking down to give carbon dioxide, and the corresponding secondary amine. This type of reaction is likely to be less facile with POM groups because the electron density at the carbonyl oxygen atom in carboxylate esters is lower than that of the corresponding carbamates.

Stability in Human Plasma

When incubated at a concentration of 10^{-4} M in human plasma, **1a** was degraded rapidly. Fifteen minutes after the start of the incubation, none of the parent compound was detectable (Fig. 1). By comparison, the half-life of **1a** in phosphate buffer, pH 7.4, was 157 min (Table II). The degradation of **1a** was accompanied by the formation of a product that reached a peak concentration at 45 min and then gradually disappeared to give rise to two other products. The initial product was identified as POM_1-ddUMP, **2a,** and the two derived products as ddUMP and ddU. The comparatively slow rate of hydrolysis of the second ester group compared to the first suggests the involvement of an enzyme other than carboxylate esterase. The most likely candidate is 5′-nucleotide phosphodiesterase I (EC 3.1.4.1), an enzyme abundant in mammalian tissues (17,18) and known to catalyze the hydrolysis of a wide range of naturally occurring and synthetic phosphodiesters (16). Although the mammalian enzyme was not available to test this hypothesis, **2a** was rapidly hydrolyzed to ddUMP when incubated with phosphodiesterase I derived from snake venom (*Bothrops atrox*), demonstrating that it is an excellent substrate for this enzyme (Fig. 4). Conversion of the derived ddUMP to ddU most likely occurs by nonspecific phosphatases present in human plasma.

The stabilities of the two bis(carbamoyloxymethyl) compounds, **1b** and **1c,** in human plasma were markedly different from that of **1a** (Figs. 2 and 3). Thus, the half-lives of **1b** and **1c** were 3.7 h and 3.5 h, respectively, similar to that observed in 0.05 M phosphate buffer, pH 7.4. These findings demonstrate that, unlike the POM_2 compound, the two bis(carbamoyloxy-

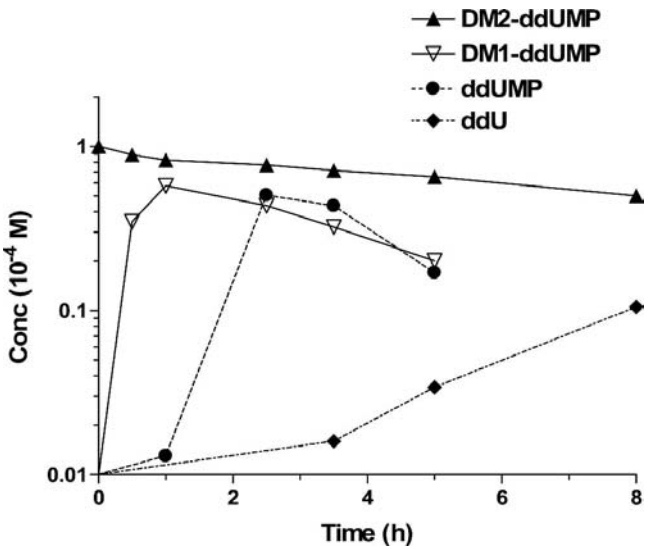


Fig. 2. Stability of DM_2-ddUMP, **1b,** in human plasma at 37°C.

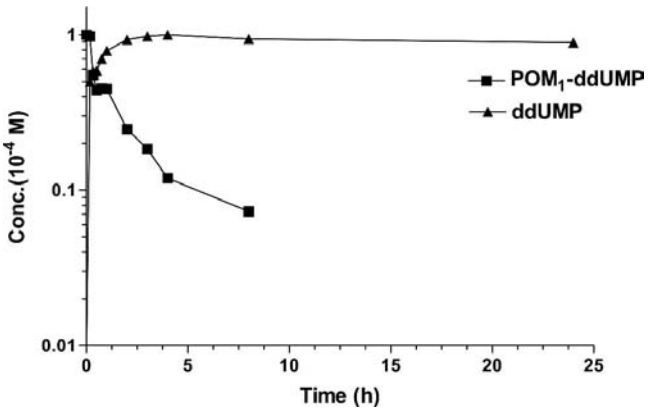


Fig. 4. Stability of POM_1-ddUMP, **2a,** in the presence of phosphodiesterase I (0.001 U enzyme/mmol of **2a**).

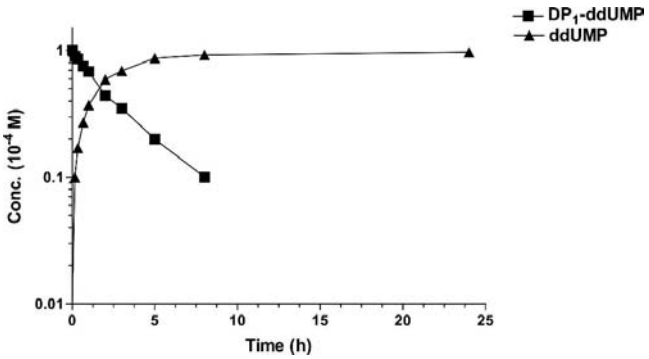


Fig. 5. Stability of DP_1-ddUMP, **2c,** in the presence of phosphodiesterase I (0.001 U enzyme/mmol of **2c**).

methyl) prodrugs are essentially unaffected by carboxylate esterases and other hydrolytic enzymes present in human plasma. As with **1a,** the phosphophodiester intermediates **2b** and **2c** were formed initially and then gradually disappeared to give rise to ddUMP and ddU. When incubated with snake venom phosphodiesterase I (19–21), **2c** was rapidly hydrolyzed to ddUMP demonstrating that, like **2a,** it is an excellent substrate for this enzyme (Fig. 5).

CONCLUSIONS

DP_2-ddUMP and DM_2-ddUMP were synthesized as potential membrane permeable prodrugs of ddUMP, and their stabilities were evaluated in human plasma and aqueous buffers. The findings clearly demonstrate that these compounds are far more resistant to degradation by plasma enzymes than the POM_2 analogue reported earlier. However, we have not yet ascertained that these bis(carbamoyloxymethyl) prodrugs give rise to ddUMP within cells. This requires the availability of radiolabeled formulations of DM_2-ddUMP and DP_2-ddUMP, the synthesis of which are in progress. Success in this prodrug approach requires that the diester generated within cells by spontaneous hydrolysis of the first carbamoyloxymethyl group is further hydrolyzed by cellular phosphodiesterases to ddUMP. Although this biotransformation cannot be assured, the excellent facility with which hydrolysis occurs in the presence of snake venom phosphodiesterase I lends confidence that it will also occur within cells. Further studies of bis(carbamoyloxymethyl) 5′-nucleotides as potential prodrugs of the free parent nucleotides are underway and will be the subject of a future communication.

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