Simplified Synthesis of Mono(4-aminophenyl)-3′-Thymidilic Acid Ester Used as Thymidine Kinase Specific Ligand for Affinity Chromatography

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INTRODUCTION

Herpes simplex viruses (HSV) need virally induced thymidine kinase (TK) for their DNA replication, whereas this enzyme is not important for mammalian metabolism. Its inhibition offers the possibility for specific antiviral therapy (1). In order to predict cytotoxic and antiviral effects of new substances, enzyme-assays are used to measure the phosphorylation of a substance by viral TK.

Affinity chromatography serves to isolate enzymes without denaturation. The affinity matrix, using mono(4-aminophenyl)-3′-thymidilic acid ester (5) as specific ligand, developed by Kowal and Markus (2), is most often used for the isolation of animal (2), human (3), and viral TK (4,5).

In this paper, we describe a simplified, highly efficient synthesis of mono(4-aminophenyl)-3′-thymidilic acid ester (5) (Scheme I). Also, we optimized the synthesis of mono(4-nitrophenyl)-3′-thymidilic acid ester (4), since the ammonium salt of this compound is no longer commercially available (6).

Although Stuart *et al.* (7) found moderate antiviral and cytotoxic activity of 4 and 5 in a rabbit–kidney 13-cell test, we tested the compounds again in a general antiviral screening program.

MATERIALS AND METHODS

Synthesis

Melting points were determined on a Büchi melting-point apparatus 510 and are uncorrected. The IR spectra (KBr) were obtained on a Perkin–Elmer FT-IR 1750 spectrometer. The NMR spectra were recorded with a Bruker AC-250 spectrometer (^{1}H-NMR, 250 MHz; ^{13}C-NMR, 62.2 MHz). For thin-layer chromatography (TLC) Merck silica gel 60F-254 aluminia plates and for column chromatography Merck 50- to 200-mesh silica gel were used. As chromatographic solvents we used dichlormethane/ethanol (1 + 1; eluant A), chloroform/methanol/concentrated ammonium hydroxide (65 + 35 + 6; eluant B), and ethyl acetate (eluant C). All chemicals except thymidine were purchased from E. Merck, Darmstadt, FRG.

5′-Tritylthymidine (2) (9). Thymidine (4.3 g, 18 mmol) and triphenylmethyl chloride (7.2 g, 26 mmol) were dissolved in 100 mL of dry pyridine and stirred for 30 min at 100°C. The solution was cooled and added dropwise to 1.5 L of vigorously stirred ice water. The product was collected by filtration, washed thoroughly with water followed by methanol, and dried under reduced pressure. The off-white solids crystallized from acetone–toluene 1/2 (7.92 (89% yield). mp 128°C (lit. 128–130°C), R_f 0.62 (C); $C_{29}H_{28}N_2O_5$ (484.59). ^{1}H-NMR (d_6-DMSO), δ (ppm): 11.16 (s, 1H, NH); 7.36 (m, 16 H, C-6, C-5′ Ph_3C); 6.30 (t, 1H, C-1′); 5.2 (d, 1H, OH); 4.36 (t, 1H, C-3′); 3.96 (q, 1H, C-4′); 3.30 (d, 2H, C-5′); 2.20 (m, 2H, C-2′); 1.52 (s, 3H, CH_3).

Mono(4-nitrophenyl)-5′-trityl-3′-thymidylic Acid Ester (3). A solution of *p*-nitrophenyl phosphorodichloridate (2.0 g, 7.8 mmol) in 10 mL of dry dioxane and 1.2 mL of dry pyridine was added over 20 min to 2 dissolved in 10 mL of dry dioxane and pyridinium hydrochloride precipitates. The mixture was stirred under an argon atmosphere for 3 hr at room temperature. Five milliliters of water and 1 mL of pyridine were added to dissolve the precipitation. After removal of the solvents under reduced pressure a clear, sticky oil was obtained which was chromatographed on a silica gel column (2.5 × 50 cm) using eluant A. The fractions were concentrated, and the precipitated product was collected by filtration, washed with methanol and dried. Fine white crystals were obtained (2.1 g, 66% yield). mp 218°C (dec). R_f 0.67 (A). $C_{35}H_{32}N_3O_{10}P$ (685.67). ^{1}H-NMR (d_6-DMSO), δ (ppm): 11.17 (s, 1H, NH); 8.15 (d, 2H, ArH-3,5); 7.60 (s, 1H, C-6); 7.20 (m, 17H, C-5′ Ph_3C, ArH-2,6); 6.10 (t, 1H, C-1′); 4.86 (t, 1H, C-3′); 4.12 (m, 1H, C-4′); 3.18 (m, 2H, C-5′); 2.29 (m, 2H, C-2′), 1.40 (c, 3H, CH_3).

Mono(4-nitrophenyl)-3′-thymidylic Acid Ester (4). Substance 3 (2.0 g, 3 mmol) was heated in acetic acid (80%) at 100°C for 20 min. The substance was completely dissolved. After cooling the solvent was distilled off. To remove the residual acetic acid, it was codistilled with toluene and ethanol. The solid residue was further purified by trituration with diethyl ether. The resulting off-white crystals could be purified on a silica gel column using eluant B (1.0 g, 73% yield). mp 225°C (dec), R_f 0.57 (A), 0.27 (B). $C_{16}H_{18}N_3O_{10}P$ (443.34). IR (KBr): ν = 3435 (NH, OH); 1717 (C=O); 1593, 1493 (Ar); 1518, 1348 (NO_2); 1253 (P=O); 1111 (R-O-R) cm^{-1}. ^{1}H-NMR (d_6-DMSO), δ (ppm): 8.35 (d, 2H, ArH-3,5); 7.87 (s, 1H, C-6); 7.57 (d, 2H, ArH-2,6); 6.29 (t, 1H, C-1′); 5.20 (s, 1H, OH); 4.93 (t, 1H, C-3′); 4.12 (d, 1H, C-4′); 3.70 (m, 2H, C-5′; 2.37 (m, 2H, C-2′); 1.95 (s, 3H, CH_3). ^{13}C-NMR (d_6-DMSO), δ (ppm): 163.63 (C-4); 158.00 (ArC-4); 150.42 (C-2); 142.06 (ArC-1); 135.77 (C-6); 125.18 (ArC-3,5); 120.24 (ArC-2,6); 109.55 (C-5); 85.30 (C-4′); 83.74 (C-1′); 76.3 (C-3′); 61.39 (C-5′); 12.22 (CH_3). MS (FAB, 14.49 eV), *m/z* (%) = 444 (86, $M^+ + 1$); 466 (100, $M^+ + Na^+$); 225 (90, 3′-deoxythymidine);

Mono(4-aminophenyl)-3′-thymidylic Acid Ester (5). Compound 4 (0.5 g, 1.1 mmol) was dissolved in a mixture of

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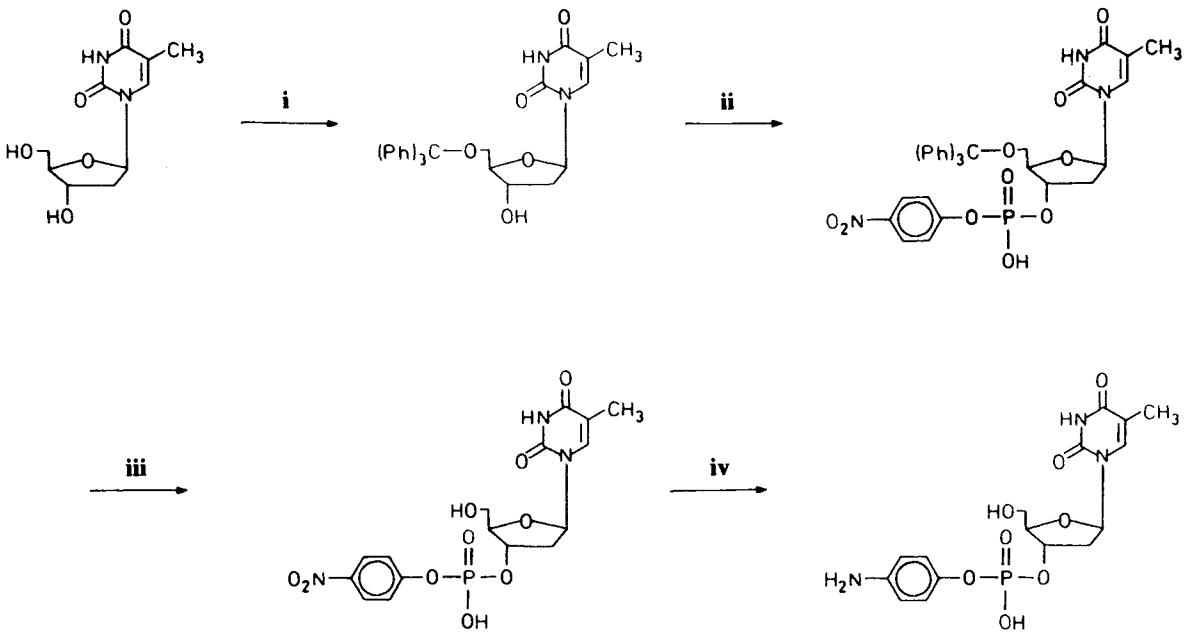

Scheme I. Reagents: i, $(Ph)_3CCl$; ii, p-NO_2-C_6H_4-PO_2Cl_2; iii, HOAc; iv, H_2.

30 mL of water and 60 mL of methanol. The catalyst, 10% palladium on carbon, was added and stirred under a hydrogen atmosphere at 1000 mbar/RT for 1 hr. The catalyst was collected by filtration and the solvent was evaporated. The brownish crude product was chromatographed on a silica gel column (0.25 × 50 cm) using eluant B. Compound 5 was isolated as off-white, hygroscopic crystalls (0.43 g, 94%). mp 140°C. R_f 0.35 (B). $C_{16}H_{20}N_3O_8P$ (413.36). IR (KBr): ν = 3425 (OH, NH); 1690 (C=O); 1510, 1476, 1385 (Ar); 1220 (P=O); 1103 (R-O-R) cm^{-1}. ^{1}H-NMR (d_6-DMSO), δ (ppm): 7.87 (s, 1H, C-6); 7.00 (d, 2H, ArH-2,6); 6.64 (d, 2H, ArH-3,5); 6.30 (t, 1H, C-1′); 4.86 (t, 1H, C-3′); 4.10 (q, 1H, C-4′); 3.70 (t, 2H, C-5′); 2.37 (m, 2H, C-2′); 2.00 (s, 3H, CH_3). ^{13}C-NMR (d_6-DMSO), δ (ppm): 163.73 (C-4); 150.44 (C-2); 144.14 (ArC-1); 143.55 (ArC-4); 136.02 (C-6); 120.45 (C-4′); 120.39 (ArC-5); 114.26 (ArC-2,6); 109.49 (C-5); 86.06 (C-4′); 83.72 (C-1′); 75.24 (C-3′); 61.48 (C-5′); 40.54 (C-2′); 12.25 (CH_3) MS (FAB, 7.71 eV), m/z (%) = 414 (75, M^+); 436 (68, $M^+ + Na^+$); 225 (100, 3′-deoxythymidine).

Elementary analysis data gave no satisfactory results because of the hygroscopicitiy of 5.

Biological Methods

The substances were tested at concentrations of 0.05, 0.5, 5, 50, and 500 μg/mL using mice embryo cell cultures that were incubated for 3 days. Afterward the cell plaques were counted. Growth inhibition or destruction of noninfected cells was a measure of cytotoxicity. As a parameter of antiviral effects, the reduction or inhibition of cell destruction of HSV 1 and HSV 2 incubated cell cultures was used.

RESULTS AND DISCUSSION

The main disadvantage of the published synthesis of 5 (2,7,10) is the high-pressure reduction of 4, which requires sizable equipment and provokes side reactions, resulting in difficult purification and moderate yields (66%). Because of the poor solubility of 4 in the reaction medium, only small quantities of 5 can be synthesized. Important physicochemical data, such as melting points and complete ^{1}H- and ^{13}C-NMR-data are not given. Because of the hygroscopicity of the substance, only the more stable Na, Ba, and Li salts are described.

We performed catalytic hydrogenation with 10% palladium on carbon at atmospheric pressure in a water–methanol mixture (1:2) with high yields (90%) in a reaction time not longer than 30 min. The crude product 5 is purified on a silica gel column. The pure product can be stored in an argon atmosphere at room temperature protected from light for months. It was shown to serve as an efficient TK-specific ligand for affinity chromatography which is now available in large amounts and sufficient purity.

Further, we investigated the cytotoxic and antiviral activity of 4 and 5. Using a plaque reduction test system with mice embryo cells, we did not observe any antiviral effect against HSV 1 and 2 up to a maximum concentration of 500 μg/mL for 5. At a concentration of 500 μg/mL, compound 4 showed a moderate cytotoxic effect. However, Stuart *et al.* found antiviral and cytotoxic effects of 4 and 5 employing a test system with rabbit kidney cells (7).

We used mice embryo cell cultures instead of rabbit kidney cells, since it is our experience that they form better virally induced plaques than rabbit kidney cells or Vero cells (8). The reference substance 5-ethyldeoxyuridine (EDU) showed 100% inhibition at a concentration of 5 μg/mL in our test system.

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