AN ALTERNATIVE SYNTHESIS OF HPMPC AND HPMPA DIPHOSPHORYL DERIVATIVES

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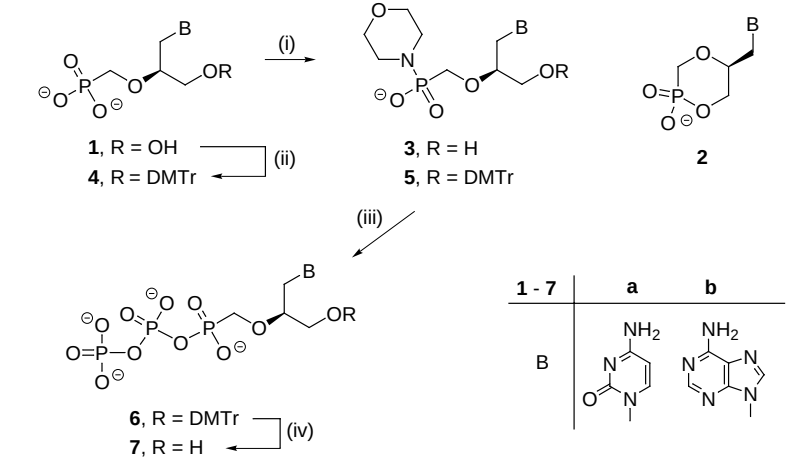
In (*S*)-1-[3-hydroxy-2-(phosphonomethoxy)propyl]cytosine (HPMPC) and (*S*)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine (HPMPA) with 3-hydroxy functions protected with 4,4′-dimethoxytrityl (DMTr) groups, phosphonate groups were transformed to the morpholides and treated with bis(tributylammonium) diphosphate. Selective cleavage of the DMTr group in the presence of the labile diphosphate residue was achieved in water at pH 2.5. Purification by charcoal adsorption followed by anion exchange chromatography afforded phosphonate-diphosphate compounds (HPMPCpp, HPMPApp).

Keywords: Acyclic nucleotide analogs; Nucleotides; HPMPCpp; HPMPApp; Triphosphates; Diphosphates; Phosphonates; Phosphorylation.

HPMPC (Cidofovir, Vistide®; **1a**), an acyclic nucleoside phosphonate active against a broad spectrum of DNA viruses, is now marketed for treatment of CMV retinitis[1,2]. Many studies were devoted to its mechanism of action[2–8]. In cells, nucleotide phosphonates are usually phosphorylated to form a parallel to natural nucleoside triphosphates. In that form (bearing the diphosphate residue linked to the phosphonate function), they interact with enzymes whose inhibition is considered responsible for their biological action (*e.g.*, DNA polymerases)[9,10]. Synthetic diphosphorylated nucleoside phosphonates are required for the kinetic studies on these enzymes.

The standard methodology for the synthesis of nucleoside triphosphates from nucleotides consists usually of a transformation of the phosphate into its activated intermediate (morpholide[11], imidazolide[12] or a mixed anhydride with diphenyl hydrogenphosphate[13]) followed by treatment with inorganic diphosphate[14]. This activation principle was easily applicable to phosphonate analogues of nucleotides; for example, 2-(phosphonomethoxy)ethyl derivatives of nucleobases were routinely transformed to the di- and triphosphate analogues by morpholide procedure[15]. However, this

approach repeatedly failed in one special case. It was in 3-hydroxy-2-(phosphonomethoxy)propyl derivatives of cytosine and adenine **1** (HPMP-type; Scheme 1), wherein the phosphonomethoxy group neighbours on a primary hydroxy group. In this case, a sterically favoured six-membered cyclic ester (*e.g.*, compound **2**) was formed during the preparation of an activated intermediate (*e.g.*, morpholide **3**). Even use of an excess of the activating agent did not avoid formation of the cyclic ester **2** as the main product[15]. It is apparent that, for overcoming this obstacle, the primary hydroxy group must be protected. The corresponding 3-benzyloxy derivatives, whose using for this purpose was recently described[5,6], are not accessible from 3-hydroxy-2-(phosphonomethoxy)propyl derivatives **1**.



(i) 1,3-Dicyclohexylcarbodiimide, morpholine, 2-methylpropan-2-ol, reflux;
(ii) 4,4'-dimethoxytrityl chloride, tributylamine, DMSO; (iii) $(Bu_3NH)_2H_2P_2O_7$, DMSO; (iv) pH 2.5

SCHEME 1

Hence, this approach requires a total synthesis of 3-benzyloxy derivatives (i) by alkylation of (protected) heterocyclic bases with an appropriate phosphonate-bearing synthon or (ii) by an introduction of phosphonomethoxy grouping onto the 3-benzyloxy-2-hydroxypropyl function of the acyclic nucleotide intermediate. Because of the strong liability of cytosine ring to reduction under formation of the 5,6-dihydro derivative, the final removal of the benzyl group cannot be done by hydrogenolysis, but it was accomplished by a hydrogen-transfer reaction with ammonium formate and Pd/C (refs[5,6]). Nevertheless, there is still a risk of contamination of the final product with 5,6-dihydro derivative of HPMPCpp, which is difficult to

detect and which could substantially interfere in enzyme assays. Therefore, we sought another protecting group which could be directly introduced at the primary hydroxy group of the HPMP derivatives **1** and which could be easily removed leaving intact both the cytosine ring and the labile diphosphate function. The use of acyl protecting groups is contraindicated because of the alkaline conditions required for the deprotection. We selected the 4,4′-dimethoxytrityl group (DMTr) as the most convenient candidate for the purpose.

RESULTS AND DISCUSSION

HPMPC (**1a**) was transformed to its tributylammonium salt in methanol. Selective tritylation of the primary hydroxy group was achieved in DMSO with a three-fold excess of DMTr-Cl and 20 equivalents of tributylamine. The desired *O*-dimethoxytrityl derivative **4a** was obtained in almost quantitative yield. Using a larger excess of DMTr-Cl leads to a formation of compound with M = 883, which we consider to be the N^4,*O*-bis(dimethoxytrityl) derivative of HPMPC. During attempts of its isolation by crystallization from ethyl acetate it was very liable to partial deprotection, which gives predominantly a compound with a slightly lower R_F than **4a**, which we consider to be N^4-dimethoxytrityl derivative. Crystallization from ethyl acetate was found to be a convenient method for isolation and purification of the product **4a**. Surprisingly, it was obtained as a free acid, even though the reaction mixture had contained the excess of tributylamine. The crystalline free acid **4a** can be stored in freezer for several months without significant decomposition; in protic solvents it is deprotected by its own acidity after several hours. The phosphonate **4a** was transformed into the morpholidate **5a** which, on treatment with bis(tributylammonium) diphosphate, gave the protected phosphonate-diphosphate compound **6a**. Selective removal of DMTr group in the presence of the labile diphosphate function was achieved in water at pH 2.5. Removal of the residual diphosphate anion was accomplished by adsorption of the crude product **7a** on activated charcoal at pH 2.5, wash of unadsorbed inorganic salts with water, and liberation of the partially purified product with 10% ammonia. Final isolation was accomplished *via* chromatography on a DEAE Sephadex anion-exchanger column by applying a linear gradient of 0–0.5 M triethylammonium hydrogencarbonate (TEAB). Identity and purity of the diphosphoryl derivative **7a** was proved by NMR, MS and by comparison with the authentic product, obtained by enzymatic phosphorylation[3], on HPLC with diode-array detector. The adenine derivative, HPMPApp (**7b**),

was obtained in the same manner. The presented protocol for chemical phosphorylation of HPMP derivatives avoids the disadvantages connected with the use of the described[5,6] benzyl protection for the primary hydroxy group. Moreover, our method is significantly more simple and less laborious.

EXPERIMENTAL

HPLC was performed on a Waters HPLC system (996 PDA detector, PDA software Millenium[32], version 3.05, 616 pump with 600 S controller and Waters fraction collector II) equipped with 15 cm × 4 mm Supelcosil™ LC 18T 3 μm reverse-phase column. The linear gradient (curve No. 6) of counter-ion solvent system at a flow rate 0.75 ml/min was used: 0 (100% A)–100% B, 30 min (solvent A: 50 mM potassium dihydrogenphosphate, 3 mM tetrabutylammonium hydrogensulfate, pH 3.19; solvent B: 50 mM potassium dihydrogenphosphate, 3 mM tetrabutylammonium hydrogensulfate, 30% acetonitrile, pH 3.19). Anion-exchanger column chromatography was done on DEAE Sephadex purchased from Sigma. TLC was carried out on silica gel pre-coated aluminium plates with fluorescent indicator (Merck 5554, 60 F_{254}) in the system propan-1-ol–concentrated aqueous ammonia–water (11 : 7 : 2) and propan-2-ol–concentrared aqueous ammonia–water (7 : 1 : 2), and on PEI-cellulose pre-coated plastic sheets Polygram® Cel 300 PEI/UV_{254} (Macherey–Nagel, Germany) in the system 4 M LiCl – 1 M AcOH (1 : 4). Paper electrophoresis (20 V/cm) was performed on a Whatman No. 3MM paper in 1 M acetic acid. Acid-washed activated charcoal was purchased from Sigma. FAB mass spectra were recorded on a ZAB-EQ (VG Analytical) spectrometer, using ionization by Xe, accelerating voltage 8 kV, and thioglycerol–glycerol 3 : 1 mixture as a matrix. ^{1}H NMR spectra were recorded at 500 MHz on a Varian UNITY 500 instrument in DMSO-d_6 or in deuterium oxide with the solvent signal (δ 2.50) or sodium 3-(trimethylsilyl)propane-1-sulfonate (DSS) as an internal standard. ^{13}C NMR APT spectra were recorded at 127.5 MHz on a Varian UNITY 500 instrument in DMSO-d_6 with the solvent signal as an internal reference (δ 39.7) or in deuterium oxide with dioxane as an external standard (δ 66.86). ^{31}P NMR spectra were recorded at 80.98 MHz on a Varian UNITY 200 instrument with the use of phosphoric acid as an external standard. Chemical shifts are given in ppm (δ-scale), coupling constants (*J*) in Hz. HPMPC was obtained from Gilead Sciences (U.S.A.), HPMPA was synthesized according to ref.[16]. 4,4′-Dimethoxytrityl chloride was purchased from Sigma.

(*S*)-1-{3-[4,4′-Dimethoxytrityl)oxy]-2-(phosphonomethoxy)propyl}cytosine (Free Acid) (**4a**)

To a suspension of (*S*)-1-[3-hydroxy-2-(phosphonomethoxy)propyl]cytosine dihydrate (free acid, **1a**, 158 mg, 0.5 mmol) in methanol (15 ml), tributylamine (1.6 g, 10 mmol) was added; the suspension of the free acid easily dissolved to a clear solution of tributylammonium salt. Methanol was evaporated; the residue, co-evaporated with acetonitrile, was dissolved in DMSO, 4,4′-dimethoxytrityl chloride (510 mg, 1.5 mmol) was added and the reaction mixture was stirred for 3 h. Procceding of the reaction was monitored by TLC in propan–1-ol-ammonia–water (11 : 7 : 2). If the reaction was not completed, an additional portion of 4,4′-dimethoxytrityl chloride was added (200 mg, 0.6 mmol) and the reaction time prolonged overnight. DMSO and tributylamine were extracted down with ether; prod-

uct **4a** was collected by filtration, recrystallized from ethyl acetate (tributylammonium chloride is soluble in ethyl acetate), and dried *in vacuo*. Yield 275 mg (95%), white crystals, m.p. 140–142 °C. MS (FAB⁻): *m/z* 580 (100%, M – H). HRMS (FAB⁻): for $C_{29}H_{31}N_3O_8P$ (M – H) calculated 580.1849, found 580.1793. ^{1}H NMR (500 MHz, DMSO-d_6): 8.70 br, 1 H (P-OH); 8.45 br, 1 H (P-OH); 7.80 d, 2 H (DMTr); 7.64 d, 1 H, *J* = 7.1 (6-H); 7.60 br, 1 H (Ha-NH); 7.55 m, 5 H (DMTr); 7.19 t, 2 H (DMTr); 6.88 d, 4 H (DMTr); 6.70 br, 1 H (Hb-NH); 5.77 d, 1 H, *J* = 7.1 (5-H); 4.00 dd, 1 H, *J* = 12.0, 3.0 (1′a-H); 3.83 m, 1 H (2′-H); 3.71 s, 6 H (DMTr); 3.64 dd, 1 H, *J* = 12.4, 10.0 (Ha-CP); 3.63 dd, 1 H, *J* = 12.0, 7.5 (1′b-H); 3.40 dd, 1 H, *J* = 12.4, 9.3 (Hb-CP); 3.10 dd, 1 H, *J* = 10.5, 3.0 (3′a-H); 2.95 dd, 1 H, *J* = 10.5, 4.0 (3′b-H). ^{13}C NMR (127.5 MHz, DMSO-d_6): 163.30 (4-C); 158.25, 2 C (DMTr); 152.48 (2-C); 148.50 (6-C); 144.94 (DMTr); 135.69 (DMTr); 135.62 (DMTr); 129.87, 4 C (DMTr); 128.04, 2 C (DMTr); 127.88, 2 C (DMTr); 126.81 (DMTr); 113.395, 4 C (DMTr); 93.34 (5-C); 85.68 (DMTr); 78.22 d, *J* = 7.8 (2′-C); 67.02 d, *J* = 161.1 (C-P); 62.80 (3′-C); 55.185, 2 C (DMTr); 50.15 (1′-C).

(*S*)-9-[3-(4,4′-Dimethoxytrityl)oxy-2-(phosphonomethoxy)propyl]adenine (Free Acid) (**4b**)

This compound was prepared from (*S*)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine (**1b**, 150 mg, 0.5 mmol) as described above for **4a**. Yield 225 mg (74%), white crystals, m.p. 129–131 °C. MS (FAB⁻): 604 (100%, M – H). HRMS (FAB⁻): for $C_{30}H_{31}N_5O_7P$ (M – H) calculated 604.1961, found 604.1934. ^{1}H NMR (500 MHz, DMSO-d_6): 8.105 s, 1 H (2-H or 8-H); 8.06 s, 1 H (2-H or 8-H); 7.35 br, 2 H (NH_2); 7.35 d, 2 H (DMT); 7.27 t, 2 H (DMTr); 7.19 m, 5 H (DMTr); 6.83 d, 2 H (DMTr); 6.82 d, 2 H (DMTr); 4.38 dd, 1 H, *J* = 14.2, 4.5 (1′a-H); 4.32 dd, 1 H, *J* = 14.4, 5.7 (1′b-H); 3.95 br quint, 1 H, Σ*J* = 19.9 (2′-H); 3.72 s, 6 H (DMTr); 3.70 dd, 1 H, *J* = 12.9, 10.3 (Ha-CP); 3.57 dd, 1 H, *J* = 12.9, 10.0 (Hb-CP); 3.005 dd, 1 H, *J* = 10.0, 4.9 (3′a-H); 2.91 dd, 1 H, *J* = 10.0, 5.0 (3′b-H). ^{13}C NMR (127.5 MHz, DMSO-d_6): 158.20 (DMTr); 155.60 (6-C); 151.80 (2-C); 149.63 (4-C); 144.85 (DMTr); 141.62 (8-C); 135.52, 2 C (DMTr); 129.81 (DMTr); 127.97 (DMTr); 127.83, 2 C (DMTr); 126.79 (DMTr); 118.40 (5-C); 113.30, 4 C (DMTr); 85.75 (DMTr); 78.94 d, *J* = 11.7 (2′-C); 66.23 d, *J* = 161.2 (C-P); 62.83 (3′-C); 55.17, 2 C (DMTr); 43.76 (1′-C).

HPMPCpp Sodium Salt (**7a**)

Compound **4a** (290 mg, 0.5 mmol) was dissolved in methanol (15 ml) containing morpholine (130 mg, 1.5 mmol). Methanol was evaporated, 2-methylpropan-2-ol (10 ml) and 1,3-dicyclohexylcarbodiimide (DCC, 60 mg, 0.3 mmol) was added, and the reaction mixture was heated to reflux. In 1 h intervals, further four portions of DCC (60 mg, 0.3 mmol) in 2-methylpropan-2-ol (1 ml) were added; then reflux was prolonged for additional 2 h. The solvent was evaporated and DCC was extracted with ether. The precipitate was collected by filtration, dissolved in a solution of bis(tributylammonium) diphosphate (820 mg, 1.5 mmol) in DMSO (5 ml), and left at room temperature overnight. Reaction mixture was diluted with water (40 ml) and pH was adjusted to 2.5 with diluted hydrochloric acid. Proceeding of the removal of DMTr group was monitored by TLC in propan-1-ol–concentrated ammonia–water (11 : 7 : 2). After 1 h, activated charcoal (10 g) was added and the reaction mixture was stirred for 30 min. Charcoal, containing adsorbed product **7a**, was filtered over celite and washed with water (100 ml). The crude product was liberated by wash with 10% aqueous ammonia (100 ml). The ammonia solution was evaporated to dryness; the residue was dissolved in water and passed through a Dowex 50 (Na^+) column (20 ml). The eluate

was concentrated to 40 ml volume, pH adjusted to 2.5, and the adsorption-desorption process mentioned above was repeated. (*N*,*N*′-Dicyclohexyl(morpholine-4-carboxamidinium) salts of diphosphosphoric acid can be adsorbed on charcoal and contaminate the product.) The evaporated crude product was purified on a DEAE Sephadex column (60 ml) applying a linear gradient of triethylammonium hydrogencarbonate (0–0.5 mol/l). Appropriate fractions, identified by TLC or by UV detector, were collected, evaporated to dryness, and co-evaporated with water. The residue was dissolved in water and passed through a Dowex 50 (Na^+) column (20 ml). The eluent was evaporated to dryness and co-evaporated with ethanol; the product **7a** was precipitated from a methanol–ether (1 : 4) mixture. Yield 55 mg (22%), counted for trisodium salt, a white powder. MS (FAB^+): 527 (M – 4 H + 4 Na), 505 (M – 3 H + 3 Na), 483 (M – 2 H + 2 Na). HRMS (FAB^+): for $C_8H_{14}N_3Na_3O_{12}P_3$ (M – 3 H + 3 Na) calculated 505.9483, found 505.9431; for $C_8H_{15}N_3Na_2O_{12}P_3$ (M – 2 H + 2 Na) calculated 483.9664, found 483.9600. ^{1}H NMR (500 MHz, D_2O): 7.81 d, 1 H, *J* = 7.6 (6-H); 6.13 d, 1 H, *J* = 7.6 (5-H); 4.13 dd, 1 H, *J* = 14.3, 3.5 (1′a-H); 3.91 dd, 1 H, *J* = 13.1, 9.6 (Ha-CP); 3.89 dd, 1 H, *J* = 14.3, 7.0 (1′b-H); 3.83 dd, 1 H, *J* = 12.4, 3.7 (3′a-H); 3.79 m, 1 H (2′-H); 3.78 dd, 1 H, *J* = 13.1, 9.8 (Hb-CP); 3.60 dd, 1 H, *J* = 12.4, 4.3 (3′b-H). ^{13}C NMR (127.5 MHz, D_2O): 161.08 (4-C); 152.04 (2-C); 147.18 (6-C); 92.995 (5-C); 77.82 d, *J* = 11.7 (2′-C); 63.70 d, *J* = 163.1 (C-P); 58.14 (3′-C); 47.71 (1′-C). ^{31}P NMR (80.98 MHz, D_2O): 9.21 d, *J* = 24.4 (α-P); –9.67 d, *J* = 19.5 (γ-P); –22.04 dd, *J* = 26.4, 19.5 (β-P).

HPMPApp Sodium Salt (**7b**)

Compound **4b** (300 mg, 0.5 mmol) provided, using the above protocol, product **7b** (60 mg, 23%, counted for trisodium salt, a white powder). MS (FAB^+): 508 (M – H + 2 Na), 530 (M – 2 H + 3 Na), 552 (M – 3 H + 4 Na). ^{1}H NMR (500 MHz, D_2O): 8.305 s, 1 H (2-H or 8-H); 8.26 s, 1 H (2-H or 8-H); 4.51 dd, 1 H, *J* = 15.0, 4.0 (1′a-H); 4.43 dd, 1 H, *J* = 15.0, 6.3 (1′b-H); 3.94 m, 1 H (2′-H); 3.90 dd, 1 H, *J* = 12.8, 10.4 (Ha-CP); 3.795 dd, 1 H, *J* = 12.8, 9.3 (Hb-CP); 3.79 dd, 1 H, *J* = 12.6, 3.9 (3′a-H); 3.53 dd, 1 H, *J* = 12.6, 4.9 (3′b-H). ^{13}C NMR (127.5 MHz, D_2O): 155.09 (6-C); 151.91 (2-C); 147.55 (4-C); 143.27 (8-C); 117.90 (5-C); 79.82 d, *J* = 11.7 (2′-C); 63.75 d, *J* = 166.0 (C-P); 59.95 (3′-C); 43.58 (1′-C). ^{31}P NMR (80.98 MHz, D_2O): 8.99 d, *J* = 26.4 (α-P); –9.32 d, *J* = 19.5 (γ-P); –22.03 dd, *J* = 26.4, 19.5 (β-P).

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