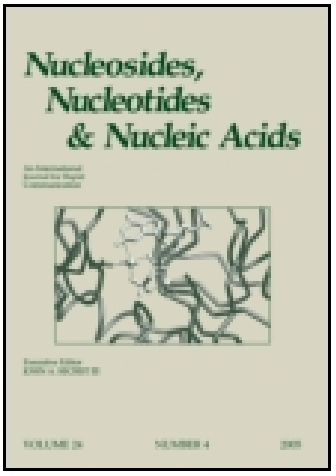

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Nucleotides Part LXXX: Synthesis of 3′-O Fluorescence Labeled Thymidine Derivatives and Their 5′-O-Triphosphates

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NUCLEOTIDES PART LXXX: SYNTHESIS OF 3′-O FLUORESCENCE LABELED THYMIDINE DERIVATIVES AND THEIR 5′-O-TRIPHOSPHATES

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□ *A new labeling technique attaching a fluorescent pteridine derivative (**3**, **5**) via a linker onto the 3′-OH group of 5′-O-dimethoxytritylthymidine (**7**) was developed to lead to the conjugates **8** and **11**. After detritylation to give **9** and **12**, the final conversion into the corresponding 5′-triphosphates (**13**, **14**), which were isolated as sodium salts, was performed by known methods.*

Keywords Isoxanthopterin derivatives; fluorescent marker; thymidine conjugates; nucleoside 5′-triphosphates

INTRODUCTION

Nucleoside 5′-triphosphates with fluorescent labels can be efficient tools for structural and functional investigations of oligonucleotides and nucleic acids.[1] Over the last few decades, some approaches to syntheses of 2′-deoxynucleoside 5′-triphosphates bearing at the 3′-position acyloxy groups with fluorescent markers have been described.[2–5] A large variety of labels have been introduced in various positions of the nucleoside[6–8] for specific reasons. We concentrated our interest on pteridine derivatives, which show strong fluorescence, high quantum yields, and high extinctions in the region of 360 nm. The aim of these investigations was the synthesis of modified thymidine 5′-triphosphates bearing at the 3′-OH group via a C_6- and a triethoxy-carbonyl linker the 8-(3,6-dimethylisoxanthopterinyl) residue as the fluorescing marker.

SYNTHESIS

Starting from 2-amino-6-chloro-3-methyl-5-nitro-4(3H)pyrimidone,[9] reaction with 6-aminohexanoic acid and 2-[2-(2-aminoethoxy)ethoxy]ethanol,

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respectively, led in good yields to 6-(2-amino-3-methyl-5-nitro-4-oxo-3,4-dihydropyrimidin-6-ylamino)hexanoic acid (**1**) and 2-amino-6-{2-[2-(2-hydroxyethoxy)ethoxy]ethylamino}-3-methyl-5-nitropyrimidin-4(3*H*)-one (**2**). Catalytic reduction of **1** with Pd/C under hydrogen atmosphere to the corresponding 5-amino derivative led on condensation with ethyl pyruvate to a mixture of 6-(2-amino-3,6-dimethyl-4,7-dioxo-3,4-dihydropteridin-8(7*H*)-yl)hexanoic acid (**3**) and 6-(2-amino-1,8-dimethyl-6-oxo-1,6-dihydropurin-9-yl)hexanoic acid (**4**), which were separated by column chromatography to give yields of 50% and 23%, respectively. Similar hydrogenation of **2** and subsequent reaction with ethyl pyruvate gave 2-amino-8-{2-[2-(3-hydroxyethoxy)ethoxy]ethyl}-3,6-dimethyl-pteridin-4,7-(3*H*,8*H*)-dione (**5**) in 44% yield. Coupling of **3** with 5′-dimethoxytritylthymidine (**7**)[10] afforded activation of the carboxylgroup by ethyl choroformate to the mixed anhydride leading to the conjugate **8** at 0°C in 50% yield. As a side-product ethyl 6-(2-amino-3,6-dimethyl-4,7-dioxo-3,4-dihydropteridin-8(7*H*)-yl)hexanoate (**6**) was isolated in 20% yield. The analogous conjugate **11** resulted from the reaction between **5** and 5′-O-dimethoxytrityl-3′-O-(4-nitrophenyl-oxycarbonyl)thymidine (**10**) in pyridine in the presence of DMAP at room temperature in 73% yield. Treatment of **8** and **11**, respectively, in 80% acetic acid led under detritylation in 81% to **9** and **12**. In the final step, the 5′-triphosphates **13** and **14** were prepared by a slightly modified Ludwig's one-pot procedure[11] working in pyridine at −10°C with two equivalents of $POCl_3$. The intermediary dichlorophosphate was then treated with bis-(tri-n-butylammonium) pyrophosphate and after quenching with triethylammonium bi-arbonate and DEAE-cellulose chromatography the tetra(triethylammonium) salts were isolated and then converted into the tetrasodium salts[12] **13** and **14**, respectively (Figure 1).

EXPERIMENTAL

General

Products were dried under high vacuum. All solvents used were of anh. grade. TLC: precoated silica gel (SiO_2) thin-layer sheets 60 *F254* (Merck, Germany) and cellulose thin-layer sheets F 1440 LS254 (Schleicher & Schüll, Germany). m.p.: Büchi-B-545 melting-point apparatus; uncorrected. UV/VIS: Perkin-Elmer Lambda 5; λ_{max} (log ε) in nm. ^{1}H-NMR: Bruker AC 250; ^{31}P-NMR: Jeol JMN-GX 400; δ in ppm rel. to Me_4Si or $CDCl_3$ as internal standard, J in Hz. Elemental analyses were performed by the Analytical Laboratory of the Department of Chemistry, Konstanz University.

6-(2-Amino-3-methyl-5-nitro-4-oxo-3,4-dihydropyrimidin-6-ylamino)hexanoic Acid (1)

A suspension of 2-amino-6-chloro-3-methyl-5-nitropyrimidin-4-one[9] (5.75 g, 28 mmol) and 6-amino hexanoic acid (8.11 g, 62 mmol) in EtOH

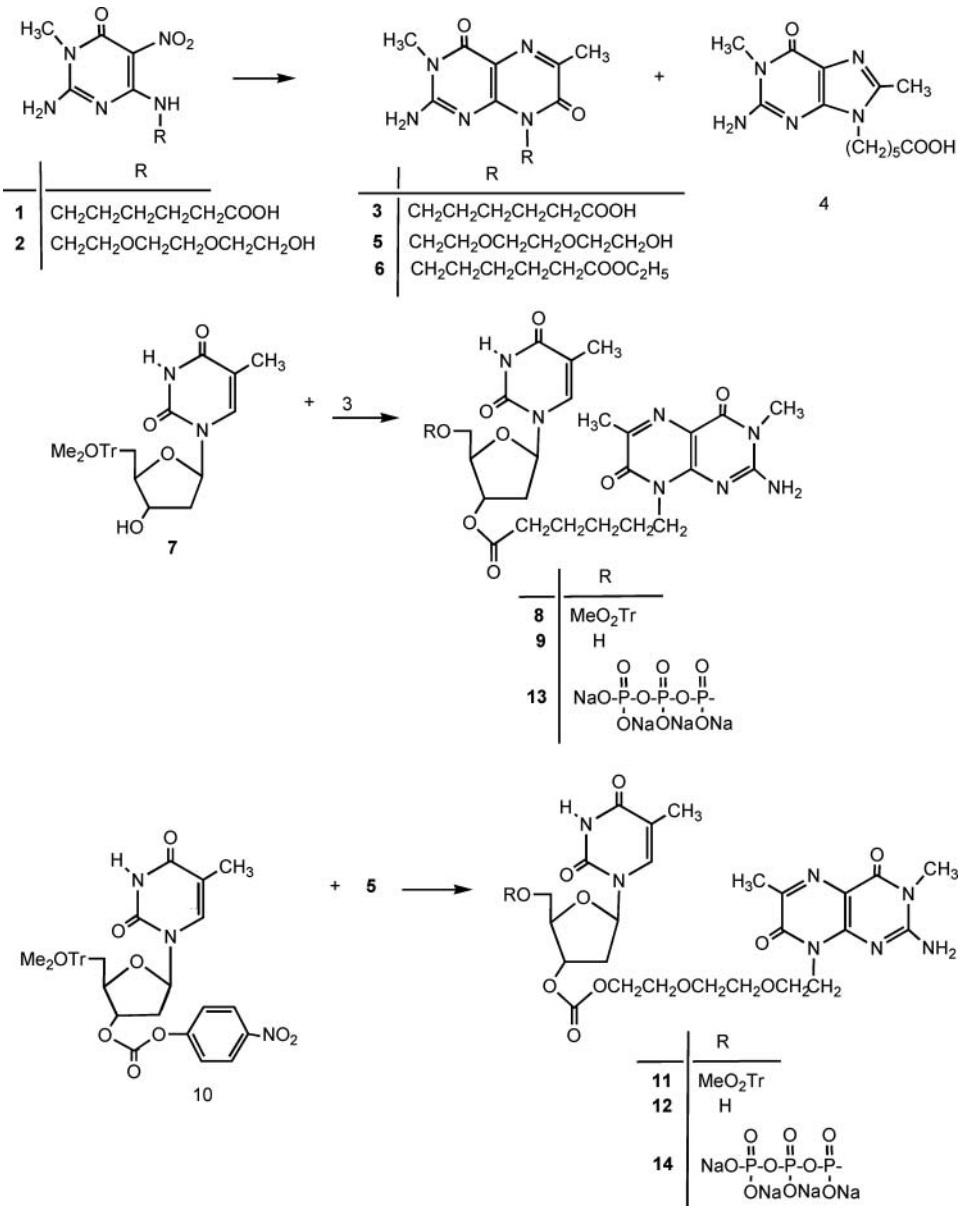

FIGURE 1 Reaction scheme.

(200 ml) was refluxed for 5 hours. After cooling the solid was collected and dried at 80°C to give 7.56 g (90%) of **1** as colorless crystals. f.p. 229°C. UV (MeOH): 216 (4.29), 230 (sh 4.13), 298 (sh 3.68), 331 (4.03). ^{1}H-NMR (DMSO-d_6): 11.87 (bs, COOH); 9.33 (t, *HN*-CH_2); 7.76 (bs, NH_2); 3.47 (m, HN-C*H*$_2$); 3.18 (s, N-CH_3), 3.50 (m, CH_2); 1.55 (m, 4H, 2 x CH_2); 1.33 (m, CH_2). Anal. Calc. for $C_{11}H_{17}N_5O_5$ (299.3): C 44.15, H 5.73, N 23.40; Found: C 44.27, H 5.78, N 23.21.

2-Amino-6-{2-[2-(2-hydroxyethoxy)ethoxy]ethylamino}-3-methyl-5-nitropyrimidin-4(3*H*)-one (2)

A mixture of 2-amino-6-chloro-3-methyl-5-nitropyrimidin-4-one[9] (10.2 g, 0.05 mol) 8-aminoethoxyethoxyethanol (7.5 g, 0.05 mol) and triethylamine (10 ml) in EtOH (150 ml) was refluxed for 30 minutes. The hot solution was filtered from little precepitate and the filtrate stored overnight in the icebox. The solid was collected and dried at 60°C to give 13.1 g (83%) of **2** as colorless crystals. f.p.125–127°C. UV (MeOH): 215 (4.30), 236 (sh 4.11), 284 (sh 3.58), 330 (4.07). ^{1}H-NMR (DMSO-d_6): 9.38 (t, *HN*-CH_2); 7.88 (bs, NH_2), 4.52 (t, OH); 3.65 (m, N-CH_2); 3.60 (m, CH_2), 3.55 (m, 4H, 2 x CH_2); 3.50 (m, CH_2); 3.42 (m, CH_2); 3.18 (s, N-CH_3).

Anal. Calc. for $C_{11}H_{19}N_5O_6$ (317.3): C 41.64, H 6.04, N 22.07; Found: C 41.56, H 5.99, N 21.95.

6-(2-Amino-3,6-dimethyl-4,7-dioxo-3,4-dihydropteridin-8(7*H*)-yl)hexanoic Acid (3) and 6-(2-Amino-1,8-dimethyl-6-oxo-1,6-dihydropurin-9-yl)hexanoic Acid (4)

A suspension of compound **1** (0.9 g. 3 mmol) and Pd/C (90 mg, 10%) in H_2O/MeOH (100 ml, 1:1) was shaken for 36 hours under H_2-atmosphere. The catalyst was filtered off, washed with hot H_2O (100 ml) and hot MeOH (100 ml). The combined filtrates were concentrated to 100 ml and after addition of ethyl pyruvate (0.6 g, 5 mmol) and AcOH (2 ml) refluxed for 5 hours. It was evaporated, coevaporated with MeOH (5 × 10 ml). The residue was treated by flash column chromatography on silica-gel (25 g, with CH_2Cl_2/MeOH (100:5, 400 ml), (100:7.5, 400 ml) and (100:10, 400 ml). The first product fractions yielded after evaporation 0.48 g (50%) of **3** as yellow foam. Recrystallization from MeOH gave brownish crystals. f.p.. 232°C. UV (MeOH): 217 (4.56), 293 (4.01), 340 (4.15). ^{1}H-NMR (DMSO-d_6): 12.01 (bs, COOH); 7.63 (bs, 2H, NH_2); 4.07 (t, N-CH_2); 3.29 (s, N-CH_3); 2.24 (s, C-CH_3); 2.20 (t, CH_2); 1.53 (m, 4H, 2 x CH_2); 1.29 (m, CH_2).

Anal. calc. for $C_{14}H_{19}N_5O_4$ (321.3): C 52.33, H 5.96, N 21.79; Found: C 51.66, H 6.02, N 21.64.

The second product fraction gave after evaporation and treatment with MeOH 0.2 g (23%) of **4** as colorless foam. UV (MeOH): 205 (4.26), 255 (4.08), 264 (sh 4.00). ^{1}H-NMR (DMSO-d_6): 12.05 (bs, COOH); 6.91 (bs, 2H, NH_2); 3.85 (t, N-CH_2); 3.28 (s, N-CH_3); 2.32 (s, C-CH_3); 2.19 (t, CH_2); 1.70–1.40 (m, 4H, 2 x CH_2); 1.24 (m, CH_2).

Anal. calc. for $C_{13}H_{19}N_5O_3$ x H_2O (311.3): C 50.15, H 6.80, N 22.49; Found: C 50.86, H 6.34, N 22.67.

2-Amino-8-{2-[2-(3-hydroxyethoxy)ethoxy]ethyl}-3,6-dimethylpteridin-4,7-(3*H*,8*H*)-dione (5)

A suspension of **2** (3.17 g, 0.01 mol) and Pd/C (0.3 g, 10%) in MeOH (100 ml) was shaken under H_2-atmosphere for 20 hours. MeOH/HCl (5 ml, 10%) was added, the catalyst filtered off and the filtrate concentrated to 50 ml. After addition of ethyl pyruvate (2 ml) the mixture was stirred at room temperature for 18 hours. The solution was treated with charcoal, filtered and evaporated. The residue was recrystallized from EtOH to give 1.5 g (44%) of **5** as yellow crystals. f.p.. 170°C.

UV (MeOH): 216 (4.55), 293 (3.99), 340 (4.11). ^{1}H-NMR (DMSO-d_6): 7.67 (bs, NH_2), 4.53 (t, OH); 4.31 (t, N-CH_2); 3.61 (t, CH_2), 3.55 (t] CH_2); 3.48 (m, 4H, 2 x CH_2); 3.38 (t, CH_2); 3.31 (s, N-CH_3), 2.26 (s, C-CH_3).

Anal. Calc. for $C_{14}H_{21}N_5O_5$ x H_2O (357.4): C 47.05, H 6.48, N 19.59; Found: C 46.87, H 6.15, N 19.43.

5′-O-Dimethoxytrityl-3′-O-[6-(2-amino-3,6-dimethyl-4,7-dioxo-3,4,7,8-tetrahydropteridin-8-yl)hexanoyl]thymidine (8) and Ethyl 6-(2-amino-3,6-dimethyl-4,7-dioxo-3,4,7,8-tetrahydro-pteridin-8-yl)hexanoate (6)

To a cooled (ice bath) stirred suspension of hexanoic acid **3** (0.321 g, 1 mmol) in dry CH_2Cl_2 (5 ml) triethylamine (0.101 mg, 1 mmol) and ethyl chloroformate (0.108 mg, 1 mmol) were added. The mixture was stirred for 20 minutes, then 5′-O-dimethoxytritylthymidine (**7**)[10] (0.817 g, 1.5 mmol) and after 5 minutes DMAP (24 mg, 0.02 mmol) were added. After stirring at 0°C for 1 hour the mixture was diluted with CH_2Cl_2 (30 ml), washed with aqueous $NaHCO_3$, the organic layer separated, dried over Na_2SO_4, and evaporated. The residue was purified by flash column chromatography on silica-gel (30 g) with CH_2Cl_2 (100 ml), CH_2Cl_2/MeOH 100:1 (100 ml), CH_2Cl_2/MeOH 100:2 (200 ml), CH_2Cl_2/MeOH 100:3 (300 ml), CH_2Cl_2/MeOH 100:4 (400 ml) and CH_2Cl_2/MeOH 100:5 (500 ml). The product fractions were collected to give starting material 7 (0.4 g), compound 8 (0.425 g, 50%) and ethyl 6-(2-amino-3,6-dimethyl-4,7-dioxo-3,4,7,8-tetrahydropteridin-8-yl)hexanoate (**6**) 70 mg (20%).

Compound **8**: UV (MeOH): 204 (4.86), 211 (sh 4.79), 229 (sh 4.53), 251 (sh 4.16), 289 (sh 4.03), 340 (4.13). ^{1}H-NMR (DMSO-d_6): 11.38 (s, NH); 7.61 (bs, NH_2); 7.52 (s, H-6); 7.40–7.20 (m, 9 H, arom. H); 6.88 (d, 4H, *o* to OCH_3); 6.20 (t, H-C(1′)); 5.29 (m, H-C(3′)); 4.06 (m, 3H, H-C(4′), N-CH_2); 3.72 (2, 6H, 2 x OCH_3); 3.32 (m, 3H, H-C(5′), N-CH_3); 3.21 (m, H-C(5″)); 2.45 (m, H-C(2′)); 2.31 (m, 3H, H-C(2″), CH_2); 2.23 (s, C-CH_3); 1.55 (m, 4H, 2 x CH_2); 1.41 (s, C-CH_3); 1.12 (m, H-C(2′)).

Anal. Calc. for $C_{45}H_{49}N_7O_{10} \times 1.5\ H_2O$ (874.9): C 61.78, H 6.00, N 11.21; Found: C 61.57, H 5.87, N 11.30.

Compound **6**: UV (MeOH): 217 (4.55), 293 (3.9), 341 (4.13). ^{1}H-NMR ($CDCl_3$): 5.77 (s, 2H, NH_2); 4.15 (m, 4H, N-CH_2, O$CH_2$$CH_3$); 3.51 (s, N-$CH_3$); 2.47 (s, C-$CH_3$); 2.32 (t, CH_2); 1.73 (m, 4H, 2 x CH_2); 1.42 (m, CH_2) 1.26 (t, OCH_2CH_3).

Anal. calc. for $C_{16}H_{23}N_5O_4$ (349.4): C 55.00, H 6.64, N 20.04; Found: C 54.78, H 7.19, N 19.81.

3′-O-[6-(2-Amino-3,6-dimethyl-4,7-dioxo-3,4,7,8-tetrahydropteridin-8-yl)]hexanoyl-thymidine (9)

A solution of compound **8** (0.425 g, 0.5 mmol) in 80% AcOH (5 ml) was stirred at room temperature for 1 hour. Evaporation and coevaporation with MeOH (4 × 10 ml) and flash chromatography (FC) (SiO_2) with CH_2Cl_2/MeOH (100:2, 100 ml), (100:4, 100 ml), (100:6, 100 ml), (100:7, 100 ml), (100:8, 100 ml) gave in the product fraction 0.22 g (81%) of **9** as a colorless foam. UV (MeOH): 216 (4.58), 258 (4.08), 290 (3.99), 340 (4.11). ^{1}H-NMR (DMSO-d_6): 11.35 (s, NH); 7.72 (s, H-6); 7.63 (bs, NH_2); 6.20 (dd, H-C(1′)); 5.21 (m, 2H, H-C(3′), 5′-OH); 4.11 (m, N-CH_2); 3.93 (m, H-C(4′)); 3.60 (m, 3H, H-C(5′,5″)); 3.29 (s, N-CH_3); 2.35 (t, CH_2); 2.23 (m, 5H, H-C(2′, 2″), C-CH_3); 1.77 (s, CH_3 Thy); 1.59 (m, 4H, 2 x CH_2); 1.30 (m, CH_2).

Anal. Calc. for $C_{24}H_{31}N_7O_8 \times 1.5\ H_2O$ (572.6): C 50.34, H 5.98, N 17.12; Found: C 50.50, H 5.86, N 17.03.

5′-O-Dimethoxytrityl-3′-O-(4-nitrophenoxycarbonyl)thymidine (10)

The nucleoside **7** (1.09 g, 2 mmol) was coevaporated with dry pyridine (3 × 5 ml) and then dissolved in dry pyridine (5.0 ml) and dry CH_2Cl_2 (5 ml). After addition of 4-nitrophenyl chloroformate (0.605 g, 3 mmol) the mixture was stirred at room temperature for 24 hours. The solvent were evaporated, the residue dissolved in CH_2Cl_2 (30 ml), washed with aqueous $NaHCO_3$ solution, the organic layer separated, dried over Na_2SO_4, evaporated, and coevaporated with toluene (3 × 5 ml). The residue was purified by FC (40 g SiO_2) with n-hexane/EtOAc 4:1 (200 ml); 2:1 (300 ml), 1:1 (300 ml) and EtOAc (300 ml). Evaporation of the product fraction 1.0 g (70%) of **10** as colorless foam. UV (MeOH): 203 (4.88), 234 (4.43), 266 (4,32). ^{1}H-NMR ($CDCl_3$): 8.54 (s, HN); 8.29 (m, 2H, *o* to NO_2); 7.62 (s, H-C(6)); 7.40–7.20 (m, 11H, arom. H); 6.84 (d, 4H, *o* to OCH_3); 6.51 (dd, H-C(1′)); 5.44 (d, H-C(3′)); 4.31 (t, H-C(4′)); 3.79 (s, 6H, 2 x OMe); 3.59 (dd, H-C5′)); 3.47 (dd, H-C(5″)); 2.60 (m, H-C(2′)); 2.51 (m, H-C(2″)); 1.41 (s, C-CH_3).

5′-O-Dimethoxytrityl-3′-O-[9-(2-amino-3,6-dimethyl-4.7-dioxo-3,4,7,8-tetrahydropteridin-8-yl) ethoxyethoxyethoxycarbonyl]thymidine (11)

The nucleoside **10** (0.74 g, 1.04 mmol) was coevaporated with dry pyridine (3 × 5 ml), then dissolved in dry pyridine (10 ml) and after addition of

compound **5** (0.27 g, 0.8 mmol) and DMAP (0.122 g, 1 mmol) the mixture was stirred at room temperature for 24 hours. The work-up is analogous to **8** to give on evaporation of the product fraction 0.53 g (73%) of **11** as a yellowish foam. UV (MeOH): 203 (4.90), 229 (sh 4.55), 252 (sh 4.19), 275 (sh 4.16), 340 (4.12). ^{1}H-NMR (DMSO-d_6): 11.40 (s, NH); 7.68 (bs, NH_2); 7.51 (s, H-6); 7.40–7.20 (m, 9 H, arom. DMTr); 6.88 (d, 4H, *o* to OCH_3); 6.19 (t, H-C(1′)); 5.22 (m, H-C(3′)); 4.28 (t, N-CH_2); 4.13 (m, 3H, H-C(4′), O-CH_2); 3.72 (2, 6H, 2 x OCH_3); 3.55 (m, 8H, 4 x CH_2); 3.29 (m, 4H, H-C(5′), N-CH_3); 3.20 (m, H-C(5″)); 2.43 (m, H-C(2′,2″)); 2.23 (s, C(6)-CH_3); 1.41 (s, C(5)-CH_3).

Anal. Calc. for $C_{46}H_{51}N_7O_{13}$ x H_2O (927.9): C 59.54, H 5.76, N 10.56; Found: C 59.68, H 5.72, N 10.54.

3′-O-[9-(2-Amino-3,6-dimethyl-4.7-dioxo-3,4,7,8-tetrahydropteridin-8-yl)ethoxyethoxy-ethoxycarbonyl]thymidine (12)

A solution of **11** (0.5 g, 0.55 mmol) in 80% AcOH (5 ml) was stirred at room temperature for 1 hour. The work up was performed analogous to **9** by FC to give 0.27 g (81%) of **12** as a yellowish powder. UV (MeOH): 216 (4.61), 259 (4.09), 341 (4.11). ^{1}H-NMR (DMSO-d_6): 11.34 (s, NH); 6.71 (s, H-C(6)); 6.68 (bs, NH_2); 6.14 (t, H-C(1′)); 5.23 (t, 5′-OH); 5.13 (m, H-C(3′)); 4.28 (t, N-CH_2); 4.17 (t, O-CH_2); 4.03 (m, H-C(4′)), 3.55 (m, 10H, 4 x CH_2, H-C(5′,5″)); 3.29 (s, N-CH_3); 2.30 (m, H-C(2′,2″)); 2.24 (s, C(6)-CH_3); 1.76 (s, C(5)-CH_3).

Anal. Calc. for $C_{24}H_{31}N_7O_8 \times 0.5\ H_2O$ (616.6): C 48.70, H 5.56, N 15.89; Found: C 48.41, H 5.84, N 15.37.

3′-O-[6-(2-Amino-3,6-dimethyl-4,7-dioxo-3,4,7,8-tetrahydropteridin-8-yl)]hexanoyl-thymidine 5′-triphosphate Tetrasodium Salt (13)

The conjugate **9** (57 mg, 0.1 mmol) was coevaporated with dry pyridine (3 × 1 ml), then dissolved in dry pyridine (1 ml) and after cooling to −10°C under Ar-atmosphere $POCl_3$ (20 μl, 0.2 mol) added. The mixture was stirred for 10 minutes, warmed to 0°C and under vigorous stirring tributylamine (130 μl) and 0.5 M bis-(tri-n-butylammonium pyrophosphate in anhydrous DMF (1 ml) added. After stirring for 30 minutes was 0.2 M TEAB buffer (12.5 ml) slowly added and stirring continued for another 45 minutes. The resulting solution was applied on a DEAE cellulose column in the bicarbonate form. The column was washed with H_2O and then with a linear gradient of aqueous tributylammonium bicarbonate (0.01–0.5 M). The pooled triphosphate peak was evaporated and the residue coevaporated with MeOH (5 × 5 ml). The final residue was dissolved in MeOH (0.5 ml) and then under stirring a solution of 1 M NaI in acetone (0.5 ml) followed

by acetone (2 ml) added. The precipitate was collected, washed with acetone (4 × 1 ml), and dried overnight in vacuum over P_4O_{10} to give 0.18 g (20%) of **13** as a colorless powder. UV (MeOH): 216 (4.50), 258 (4.01), 340 (3.97). ^{1}H-NMR (D_2O): 7.62 (s, H-6); 5.97 (t, H-C(1′)); 5.30 (m, H-C(3′)); 4.20–3.95 (m, 5H, N-CH_2, H-C(4′), H-C(5′,5′)); 3.28 (s, N-CH_3); 2.33 (t, CH_2); 2.22 (m, 5H, C-CH_3, H-C(2′, 2″)); 1.82 (s, CH_3 Thy); 1.57 (m, 4H, 2 x CH_2); 1.27 (m, CH_2). ^{31}P-NMR (D_2O): -8.22 (d, γP), -10.47 (d, αP), -21.48 (t, βP).

3′-O-[9-(2-Amino-3,6-dimethyl-4,7-dioxo-3,4,7,8-tetrahydropteridin-8-yl)ethoxyethoxy-ethoxycarbonyl]thymidine 5′-triphosphate Tetrasodium Salt (14)

Analogous to **13** with **12** (62 mg, 0.1 mmol) to give 0.38 g (40%) of **14** as a colorless powder. UV (MeOH): 215 (4.49), 260 (4.02), 341 (4.00). ^{1}H-NMR (D_2O): 7.62 (s, H-6); 6.04 (t, H-C(1′)); 5.27 (m, H-C(3′)); 4.35–4.05 (m, 7H, N-CH_2, O-CH_2, H-C(4′), H-C(5′,5′)); 3.75–3.50 (m, 8H, 4 x CH_2); 3.30 (s, N-CH_3); 2.52 (m, H-C(2′, 2″)); 2.24 (s, C-CH_3(6)); 1.81 (s, CH_3 Thy). ^{31}P-NMR (D_2O): -9.69 (d, γP), -10.54 (d, αP), -21.82 (t, βP).

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