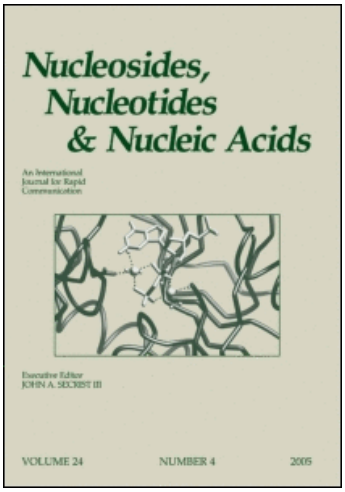

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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Online publication date: 11 June 2010

To cite this Article Miazga, A. , Ziemkowski, P. , Siwecka, M. A. , Lipniacki, A. , Piasek, A. and Kulikowski, T.(2010) 'Synthesis, Biological Properties and Anti-HIV-1 Activity of New Pyrimidine P_1,P_2-Dinucleotides', Nucleosides, Nucleotides and Nucleic Acids, 29: 4, 438 — 444
To link to this Article: DOI: 10.1080/15257771003738642
URL: http://dx.doi.org/10.1080/15257771003738642

Nucleosides, Nucleotides and Nucleic Acids, 29:438–444, 2010

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257771003738642

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SYNTHESIS, BIOLOGICAL PROPERTIES AND ANTI-HIV-1 ACTIVITY OF NEW PYRIMIDINE P_1,P_2-DINUCLEOTIDES

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□ *New homo- and hetero-P_1,P_2-dinucleotides were prepared with the use of multistep procedures starting from the monophosphates of 3′-fluoro-2-thiothymidine, 3′-fluoro-4-thiothymidine, AZT and 1-[(2-hydroxyethoxy)-methyl-5-propyl-6-phenylselenenyl]uracil. Anti-HIV properties of the synthesized P_1,P_2-dinucleotides were evaluated against laboratory syncytia inducing strain HIV-1 in CEM-T4 cells. Anti-HIV activities were in the range of 5–45 nM, and therapeutic indexes were higher than 4666–14000. Interactions of the above mentioned compounds with recombinant HIV-1 reverse transcriptase were also investigated. The obtained results point to reverse transcriptase inhibition, with somewhat lower inhibitory activity than that of their parental nucleoside-5′-triphosphates. Compound **6** may be regarded as a potent anti-HIV/AIDS drug.*

KEYWORDS P_1,P_2-dinucleotides; anti-HIV agents; reverse transcriptase inhibitors

INTRODUCTION

It is known that 2′,3′-dideoxynucleoside analogues commonly used in HIV/AIDS therapy are poorly phosphorylated to their active forms–5′-triphosphates- in monocyte derived macrophages (MDM) and, therefore, their therapeutic effects are limited. Direct introduction of nucleotides into animal cells essentially does not occur. To overcome these limitations, some 5′,5′-P_1,P_2 dinucleotides were previously synthesized; it was shown that after encapsulation in erythrocytes (RBC) they protected in vitro human macrophages from HIV-1 infection.[1] Such compounds are more soluble in aqueous media than the parent nucleosides, and show better, as compared with nucleosides, penetration into the cell in undegraded form. 5′,5′-P_1,P_2-dinucleotides undergo intracellular hydrolysis by dinucleotide

This work was supported by KBN 3PO5F 00825 grant.
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pyrophosphatase to nucleoside 5′-monophosphates, which may be further phosphorylated by kinases to 5′-triphosphates.[2]

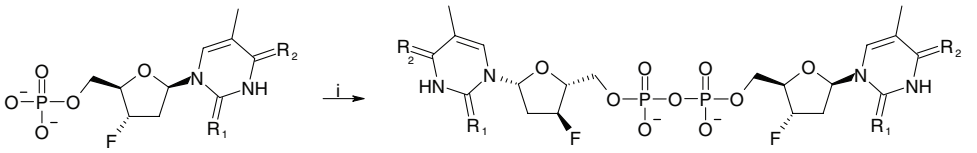
Analogues of 3′-fluorothymidine thiated at the heterocyclic moiety are selective inhibitors of HIV-1.[3] To enhance their solubility in water and penetration into T- lymphocytes, we decided to synthesize thiated homo- and hetero- 5′,5′-P_1,P_2-dinucleotides and investigate, without encapsulation in RBC, their inhibitory activity toward HIV-1 in CEM-T4 cells.

RESULTS AND DISCUSSION

Chemistry

5′-Mononucleotides of 3′-azidothymidine and thiated analogues of 3′-fluorothymidine were prepared from appropriate nucleosides in reaction with $POCl_3$, 1,2,4-triazole and triethylamine. 1-[(2-Hydroxyethoxy)methyl-5-propyl-6- phenylselenenyl]uracil phosphate was synthesized with the use of Yoshikawa procedure, employing direct phosphorylation with $POCl_3$ in trimethylphosphate.

P_1,P_2-homodinucleotides **3** and **4** were synthesized by condensation of 5′-mononucleotides in the presence of tosyl chloride (Scheme 1). P_1-3′-fluoro-2-thiothymidine-P_2-3′-azidothymidine pyrophosphate (**6**) was prepared with the use of a multistep procedure, involving the conversion of 2′,3′-dideoxy-3′-fluoro-2-thiothymidine 5′-mononucleotide to P_1-3′-fluoro-2-thiothymidine-5′-P_2-diphenylpyrophosphate followed by condensation with AZTMP (Scheme 2). 5′-Mononucleotide of 2′,3′-dideoxy-3′-fluoro-2-thiothymidine was converted into morpholine derivative in reaction with morpholine and 1,3-dicyclohexylcarbodiimide in t-BuOH/H_2O. Coupling of 3′-fluoro-2-thiothymidine 5′-monophosphate morpholidate with tri-n-butylammonium salt of 1-[(2-hydroxyethoxy)methyl-5-propyl-6-phenylselenenyl]uracil phosphate led to heterodinucleotide **8** (Scheme 3).

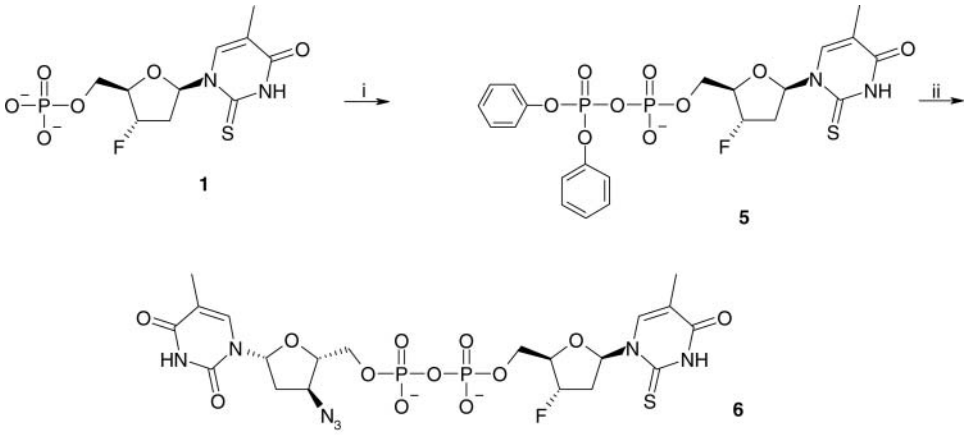


1: R_1 = S, R_2 = O
2: R_1 = O, R_2 = S

3: R_1 = S; R_2 = O
4: R_1 = O; R_2 = S

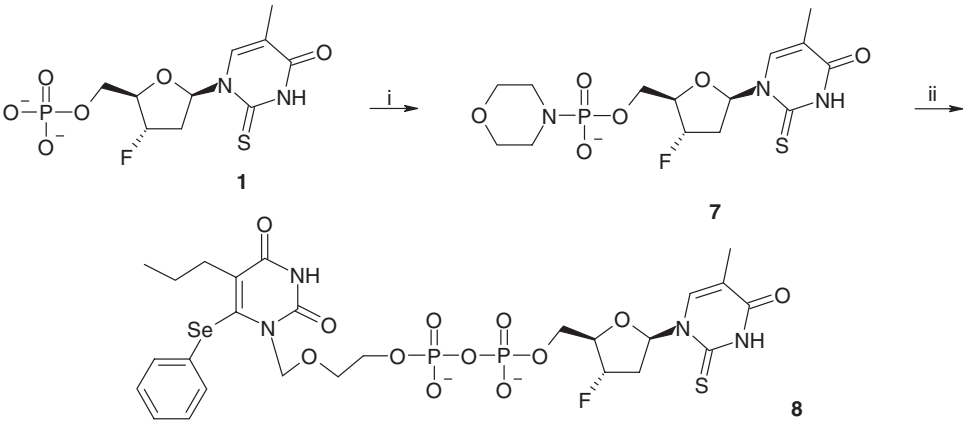
i: TsCl, DMAP, pyridine, RT, 24 h

SCHEME 1 Synthesis of homodinucleotides (**3**, **4**).



i: diphenyl chlorophosphate, tri-n-butylamine, 1,4-dioxane/DMF, RT, 3 h
ii: AZTMP, pyridine, RT, 18 h

SCHEME 2 Synthesis of P_1-3′-fluoro-2-thiothymidine-P_2-3′-azidothymidine pyrophosphate (**6**).



i: morpholine, DCC, t-BuOH, 90°C, 3 h
ii: 1-[(2-hydroxyethoxy)methyl-5-propyl-6-phenylselenenyl]uracil phosphate, pyridine, RT, 18 h

SCHEME 3 Synthesis of P_1-3′-fluoro-2-thiothymidine-P_2-1-[(2-hydroxyethoxy)methyl-5-propyl-6-phenylselenenyl]uracil pyrophosphate (**8**).

Biology

New P_1,P_2 dinucleotides **3, 4,** and **6** cells exhibited potent antiviral activity in CEM-T4 (ED_{50} 15, 45, and 5 nM, respectively). Lower activity of **8** (ED_{50} 200 nM) is most likely caused by larger nonpolar aryloseleno- substituent at position 2 of uracil ring, while very high activity of compound **6** is probably due to the presence of AZT as a part of the dinucleotide molecule.

No cytotoxicity was observed in CEM-T4 cells at the highest tested concentration (100 μM), so that the therapeutic indexes were higher than 4,666–14,000.

To explain the mode of the antiviral action of the above mentioned thiated P_1,P_2-pyrimidine analogues, corresponding nucleoside 5′-triphosphates were synthesized, and their interactions with HIV-1 recombinant reverse transcriptase (HIV-1 RT) were investigated and compared with those of the P_1,P_2-dinucleotide analogues. The most active inhibitors were 5′-triphosphates of 2′,3′-dideoxy-3′-fluoro-2-thiothymidine and 2′,3′-dideoxy-3′-fluoro-4-thiothymidine, with inhibitory activity (IC_{50}) 0.17 μM and 0.2 μM, respectively, while the corresponding P_1,P_2-dinucleotides **3** and **4** exhibited somewhat lower inhibitory activities (IC_{50} 0.2 and 0.9 μM, respectively). The obtained results show that antiviral activities of thiated pyrimidine P_1,P_2-dinucleotides are only to some extent correlated with HIV RT inhibition, and rather the 5′-triphosphates, resulting from the metabolism of 5′-monophosphates, are responsible for the antiviral activity.

EXPERIMENTAL

General Procedures

High resolution ^{1}H NMR spectra were recorded on a Varian 500 MHz in D_2O, with DSS as internal standard. ^{31}P NMR spectra were recorded on a Varian 200 MHz in D_2O, with 85% H_3PO_4 as external standard. Thin-layer chromatography (TLC) was run on Merck silica gel F_{254} plates. The solvent used for TLC was i-PrOH - conc. aq. NH_3 -H_2O 11:7:2. DEAE-Sephadex A-25 was purchased from Pharmacia. All other reagents, materials and solvents were from Sigma-Aldrich (St. Louis, MO, USA).

Bis(2′,3′-dideoxy-3′-fluoro-2-thiothymidine)-5′,5′-P^1,P^2-pyrophosphate (3). 5′-Monophosphate of 2′,3′-dideoxy-3′-fluoro-2-thiothymidine (**1**) (51 mg, 0.14 mmol) was transformed into tri-n-butylammonium salt and dissolved in 5 mL of dry DMF. To this solution was added 0.5 mL of dry pyridine, catalytic amount of 4-dimethylaminopyridine and tosyl chloride (78 mg, 0.4 mmol). The solution was stirred at room temperature for 24 hours and 2 mL of water was added. After evaporation of the solvents the product was purified by ion-exchange column chromatography with a DEAE-Sephadex A-25 resign with a linear gradient (0–0.4 M) of aqueous triethylammonium bicarbonate buffer as the mobile phase. The homogenous fractions containing pure pyrophosphate were pooled and concentrated under reduced pressure. The product was dissolved in a small amount of methanol and solution of 100 mg NaI in 5 mL of acetone was added. The precipitated sodium salt was filtered off, washed several times with acetone, and dried over P_2O_5. Yield: 34.6 mg (70%); TLC R_f 0.72; ^{1}H NMR (D_2O) δ 7.87 (1H, d, H6), 7.06 (1H, dd, H1′) 5.52–5.4 (1H, dd, H3′, $J_{3'\text{-F}} = 53.06$ Hz), 4.55–4.49

(1H, m, H4′, $J_{4'\text{-F}} = 27.03$ Hz), 4.22 (2H, d, H5′, H5″), 2.84–2.75 (1H, m, H2″), 2.33–2.19 (1H, m, H2′), 1.97 (3H, s, CH_3); ^{31}P NMR (D_2O) δ -11.18; MS *m/z* 661 $(M\text{-}H)^-$.

Bis(2′,3′-dideoxy-3′-fluoro-4-thiothymidine)-5′,5′-P^1,P^2-pyrophosphate (4). Compound received in the same manner as compound **3**. Yield: 33 mg (67%); TLC R_f 0.70; ^{1}H NMR (D_2O) δ 7.77 (1H, d, H6), 6.37 (1H, dd, H1′), 5.51–5.39 (1H, dd, H3′, $J_{3'\text{-F}} = 52.56$ Hz), 4.52–4.47 (1H, m, H4′, $J_{4'\text{-F}} = 27.53$ Hz), 4.18 (2H, d, H5′, H5″), 2.67–2.59 (1H, m, H2″), 2.45–2.32 (1H, m, H2′), 2.05 (3H, s, CH_3); ^{31}P NMR (D_2O) δ −11.15; MS *m/z* 661 $(M\text{-}H)^-$.

P^1-(2′,3′-dideoxy-3′-azidothymidine)-P^2-(2′,3′-dideoxy-3′-fluoro-2-thiothymidine) pyrophosphate (6). Compound **1** (68 mg, 0.2 mmol) was converted into tri-n-butylammonium salt (free acid was dissolved in 3 mL of methanol with 0.4 mL tri-n-butylamine and stirred for 30 minutes) and dissolved in the mixture of dry 1,4-dioxane (1 mL) and DMF (0.25 mL). Diphenyl chlorophosphate (42 μL, 0.2 mmol) and tri-n-butylamine (55 μL, 0.23 mmol) was added and stirred in room temperature for 3 hours. After evaporation of the solvents diethyl ether (10 mL) was added, and the reaction mixture was left standing for 1 hour in refrigerator. Diethyl ether was decanted, and the traces of ether were removed by evaporation with dioxane. Resulting P^1-nucleoside-5′-P^2-diphenylpyrophosphate **5** was dissolved in 1 mL of dry pyridine and triethylammonium salt of 3′-azido-2′,3′-dideoxytymidine 5′-monophosphate (69.4 mg, 0.2 mmol) was added and stirred in room temperature for 18 hours. Reaction mixture was evaporated to dryness under reduced pressure and extracted two times with diethyl ether. The residue was purified by ion-exchange column chromatography with a DEAE-Sephadex A-25 resign with a linear gradient (0–0.4 M) of aqueous triethylammonium bicarbonate buffer as the mobile phase. The fractions containing pure pyrophosphate were pooled and concentrated under reduced pressure and converted to sodium salt. Yield: 17.3 mg (12%); TLC R_f 0.67; ^{1}H NMR (D_2O) δ 7.88 (1H, d, H6), 7.64 (1H, s, $H6_{AZT}$), 7.05 (1H, dd, H1′) 6.18 (1H, t, $H1'_{AZT}$), 5.50–5.39 (1H, dd, H3′, $J_{3'\text{-F}} = 52.73$ Hz), 4.54–4.47 (2H, m, H4′, $H3'_{AZT}$), 4.22–4.10 (5H, m, H5′, H5″, $H4'_{AZT}$), 2.82–2.73 (1H, m, H2″), 2.44 (2H, m, $H2'_{AZT}$, $H2''_{AZT}$), 2.32–2.17 (1H, m, H2′), 1.95 i 1.89 (6H, 2 s, 2 CH_3); ^{31}P NMR (D_2O) δ −11.21, −11.07; MS *m/z* 668 $(M\text{-}H)^-$.

P^1-(1-[(2-hydroxyethoxy)methyl]-6-phenylselenenyl-5-propyluracil)-P^2-(2′,3′-dideoxy-3′-fluoro-2-thiothymidine) pyrophosphate (8). To the solution of **1** (44.2 mg, 0.13 mmol) in 2 mL of water *tert*-butanol (2 mL) and morpholine (45 μL, 0.52 mmol) were added, and reaction mixture was heated at 90°C. 1,3-Dicyclohexylcarbodiimide (107.5 mg, 0.52 mmol) in 3 mL of *tert*-butanol was added and the reaction mixture was stirred and heated at 90°C for 3 hours. After this time 2 mL of water was added and the mixture was extracted with diethyl ether (3 × 10 mL). The combined organic fractions were dried over Na_2SO_4 and evaporated under reduced pressure

to syrup. Received morpholine derivative of o-P^1-nucleosidephosphate **7** was dissolved in 1 mL of anhydrous pyridine, and tri-n-butylammonium salt of 1-[(2-hydroxyethoxy)methyl]-6-phenylselenenyl-5-propyluracil 5′-monophosphate (50 mg, 0.11 mmol) was added. The mixture was stirred at room temperature for 18 hours. After evaporation of the solvents the residue was extracted two times with diethyl ether. The residue was purified by ion-exchange column chromatography with a DEAE-Sephadex A-25 resign with a linear gradient (0–0.4 M) of aqueous triethylammonium bicarbonate buffer as the mobile phase. The fractions containing pure pyrophosphate were pooled and concentrated under reduced pressure and converted to sodium salt. Yield: 13.5 mg (15%); TLC R_f 0.7; ^{1}H NMR (D_2O) δ 7.90 (1H, d, H6), 7.45–7.33 (5H, m, Ph), 7.04 (1H, dd, H1′), 5.60 (2H, s, OCH_2N), 5.52–5.41 (1H, dd, H3′), 4.56–4.51 (1H, m, H4′), 4.27–4.2 (2H, m, H5′, H5″), 3.96–3.74 (4H, m, CH_2CH_2), 2.83–2.74 (1H, m, H2″), 2.52 (2H, m, $\underline{CH_2}CH_2CH_3$), 2.29–2.15 (1H, m, H2′), 1.95 (3H, s, CH_3), 1.18 (2H, m, $CH_2\underline{CH_2}CH_3$)0.79 (3H, t, $CH_2CH_2\underline{CH_3}$); ^{31}P NMR (D_2O) δ −11.27, −10.57; MS *m/z* 784 $(M\text{-}H)^-$.

Inhibition of HIV-1 Virus Replication

Antiviral activity was determined as follows: CEM-T4 cells (kindly provided by NIH AIDS Research and Reference Program) inoculated with HIV-1 (syncytia inducing phenotype) were cultured for up to 10 days in 96 well culture plate with various concentrations of the tested compounds in triplicate. The media over the cell cultures were collected at various time intervals, and the amounts of p24 protein were determined. The efficacy of inhibition of HIV-1 replication by the tested compounds was compared and related to the control culture grown in standard medium, as well as to cells cultured in media enriched with known amounts of AZT.

The cytotoxicity of the investigated compounds was tested in CEM-T4 cells. After 10 days of culture at various concentrations of the tested compounds, the cell viability was determined by the standard MTT method.

HIV-1 Reverse Transcriptase Assay

The activity of HIV-1 RT was determined using a colorimetric method involving incorporation of digoxigenin- and biotin-labeled dUTP into DNA[4]. Standard reaction mixture contained 46 mM Tris-HCl (pH 7.8), 9.2 mM DTT, 27.5 mM $MgCl_2$, 266 mM KCl, 0.05% Triton X-100, 1.25 mg/m BSA, 0.075 mM EDTA, 0.1 mM poly(A)oligo$(dT)_{15}$, digoxigenin- and biotin-labeled nucleotides dUTP/dTTP (10μM), 3 μL RT (30 mU/μL), and 20 μL (0.01–5 μM) of inhibitor solution. The mixture was incubated at 37°C for 1 hour, and unreacted substrates were washed out. Antibodies against digoxigenin (200 μL) conjugated with peroxidase (200 mU/mL) were added and

incubated at 37°C for 1 hour. Antibodies were washed out, and 200 μL of 2,2′-azino-di(3-ethylbenzothiazoline) was added, left for 30 minutes at room temperature, and absorbance at 405 nm was measured with the use of ELISA microtiter plate reader.

CONCLUSION

Among the above mentioned P_1,P_2-dinucleotides, compound **6** is the most potent and selective anti HIV-1 agent, in which the influence of the 3′-azido-substituent is clearly visible. It may be regarded as a potential anti-HIV/AIDS drug.

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