Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry Letters 18 (2008) 1364–1367
Bioorganic & Medicinal Chemistry Letters

Syntheses of N^3-substituted thymine acyclic nucleoside phosphonates and a comparison of their inhibitory effect towards thymidine phosphorylase

Karel Pomeisl,* Antonín Holý, Ivan Votruba and Radek Pohl

Gilead Sciences & IOCB Research Centre, Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, v.v.i, Centre for New Antivirals and Antineoplastics (IOCB), Flemingovo nám. 2, 16610 Prague, Czech Republic

Received 22 November 2007; revised 2 January 2008; accepted 3 January 2008
Available online 8 January 2008

Abstract—A series of N^3-substituted thymine acyclic nucleoside phosphonates bearing a number of (phosphonomethoxy)alkyl groups were synthesized and investigated for their ability to inhibit the human thymidine phosphorylase expressed in V79 Chinese hamster cells, as well as thymidine phosphorylase from SD-lymphoma, *Escherichia coli* and human placenta. In comparison to N^1- substituted analogues which possess a considerable inhibitory activity towards thymidine phosphorylase from SD-lymphoma, the results showed a marginal inhibitory effect of these compounds. None of the presented N^3-substituted derivatives possess a significant cytostatic activity.

Pyrimidine acyclic nucleoside phosphonates (ANPs) are compounds which possess significant antiviral and cytostatic activity.[1] The scale of biological effects for pyrimidine ANP derivatives could be also extended in connection with their potential inhibitory potency towards thymidine phosphorylase (TP).[2] This enzyme, which is identical to platelet-derived endotherial cell growth factor (PD-ECGF),[3] catalyses the reversible phosphorolysis of thymidine to thymine and 2-deoxy-D-ribose 1-phosphate.[3a] The dephosphorylated product of the latter, 2-deoxy-D-ribose, plays an important role in tumour angiogenesis.[4] Therefore, inhibitors of TP may find utility as suppressors of tumour growth.[4c]

The aim of our work has been the development of new ANP multisubstrate inhibitors of this enzyme bearing pyrimidine base and various phosphonoalkyl groups to interfere at thymine and phosphate-binding sites.[2,5] The determination of inhibitory activity of our previously reported compounds[6–8] was performed with TP expressed in V79 Chinese hamster cells, human placenta, *Escherichia coli* and newly used SD-lymphoma. The results show a considerable selective multisubstrate effect of a number of side-chain modified pyrimidine ANPs towards TP isolated from SD-lymphoma[6–8] whereas the marginal inhibitory activity on the other enzymes has been observed. In contrast, various kinds of known 5-halogeno 6-amino substituted[4c,9] uracils possess a significant effect on human TP. However, the low values of their inhibitory activity on the enzyme purified from spontaneous T-cell lymphoma of an inbred Sprague–Dawley rat strain were found.[9b] Therefore, we guess the structures of mentioned enzymes could probably be different in recognition of active sites as well as the interactions of known substituted uracils,[10] thymine[2], and/or 7-deazaxantyl[5] ANPs predicted on *E. coli* and human TP.

Based on this assumption, we systematically deal with modifications of structure in catabolically stable pyrimidine ANPs to influence their interaction with human TP. In this report, we described in particular one of the possible modifications of structures in known thymine (phosphonomethoxy)alkyl derivatives[6] such as 1-[2-(phosphonomethoxy)ethyl]thymine (PMET), 1-[3-hydroxy-2-(phosphonomethoxy)propyl]thymine (HPMPT) and 1-[3-fluoro-2-(phosphonomethoxy)-propyl]thymine (FPMPT) which were found as efficient inhibitors of TP isolated from SD-lymphoma (Fig. 1). That means we synthesized their new N^3-substituted analogues **6**, **7**, and **10**.

Keywords: Acyclic nucleoside phosphonates; Thymidine phosphorylase; Fluorination; Pyrimidine; Alkylation.
* Corresponding author. Tel.: +420 220183475; fax: +420 220183560; e-mail: pomeisl@uochb.cas.cz

0960-894X/$ - see front matter © 2008 Published by Elsevier Ltd.
doi:10.1016/j.bmcl.2008.01.006

In this case, we investigated N^3-selective and effective alkylation of the thymine moiety with a number of (phosphonomethoxy)alkyl groups which have previously never been tested with ANPs and therefore still remains a synthetic and biochemical challenge. For biological studies we prepared both the optical isomers to compare their activity in N^3-substituted HPMPT and FPMPT derivatives. In conclusion, the inhibitory activity of obtained compounds **6**, **7**, and **10** was compared with those of our reported N^1-substituted pyrimidine ANPs.

It is well known that the N^3-position of pyrimidines plays an important role in the forming of hydrogen interactions in various biological systems such as the base pairing, e.g., in DNA.[11] It seems that the hydrogen at pyrimidine N^1 and/or N^3-position also interacts with TP as recently reported by the proposal of inhibitor binding in *E. coli* and human TP crystal structures.[2,10] Our effort was to evaluate the substitution of N^3-position with (phosphonomethoxy)alkyl groups while the hydrogen at N^1-position of base would be available for potential interaction with the enzyme.[10] We expected it could influence the inhibitory effect of our synthesized ANPs towards TP in different directions. Therefore, the results of biochemical screening could be helpful in better understanding of the enzyme function in the thymine active site and to design more efficient ANP inhibitors in future.

As demonstrated in the described novel syntheses of phosphonates **6a**, **6b**, and **10**, for the preparation of required compounds we used the selective N^3-alkylation of 1-(tetrahydro-2*H*-pyran-2-yl)thymine (**1**)[12] with chiral oxiranes **2a**, **2b** and halogenoalkylphosphonate **8** as a key step followed by deprotection (Scheme 1). Alkylation of the protected base proceeded in good preparative 57–70% yields of corresponding intermediates **3** and **9** in the presence of sodium hydride in dimethylformamide.[13] Compounds **3a** and **3b** were further converted to **4a** and **4b** by their reaction with 2-[(diisopropoxyphosphoryl)methyl tosylate followed by mild hydrogenation over 10% palladium on charcoal in the presence of glacial acetic acid. For the replacement of hydroxy group with fluorine in the intermediates **4a** and **4b** we applied our simple improved method,[7] developed for the preparation of N^1-substituted FPMPT from easily accessible HPMPT intermediates using commercial and weakly corrosive perfluorobutane-1-sulfonyl fluoride as a fluorination agent. All the compounds

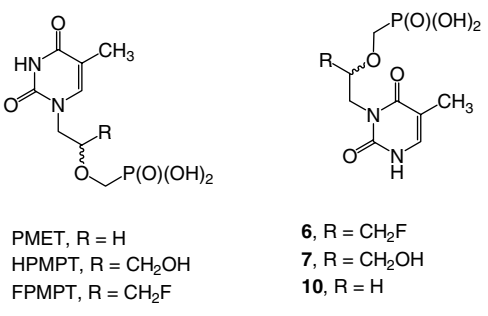
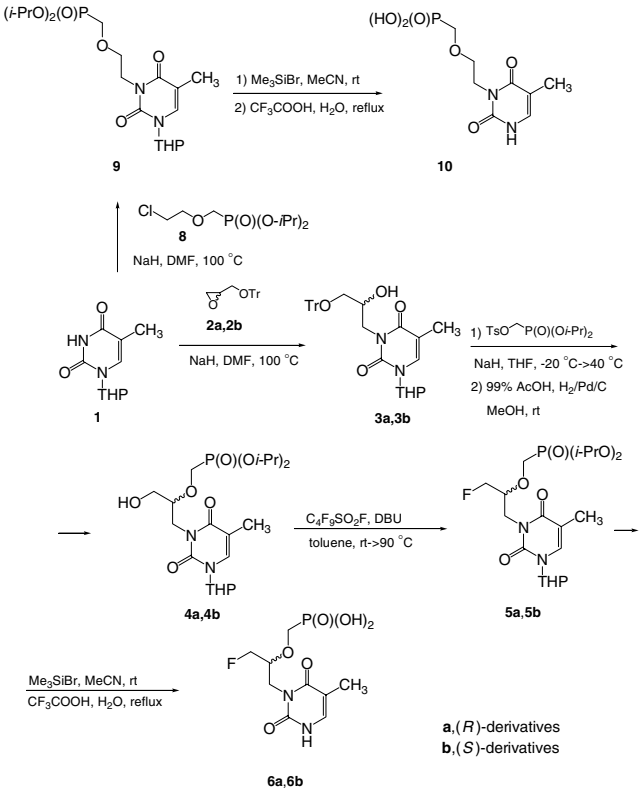


Figure 1. N^1- and N^3-substituted pyrimidine ANPs under study.



Scheme 1. Syntheses of N^3-substituted thymine ANPs.

obtained (e.g., **5** and **9**, see Scheme 1) were deprotected by their reaction with bromotrimethylsilane followed by hydrolysis in the presence of trifluoroacetic acid.[14]

The inhibitory activity of compounds **6a**, **6b**, **7a**, **7b**, and **10** was compared with those of N^1-phosphonoalkyl thymines. Data listed in Table 1 show that none of the synthesized phosphonates possesses an inhibitory effect on TP isolated from *E. coli*, human placenta and TP expressed in V79 Chinese hamster cells. In contrast to N^1-substituted phosphonoalkyl thymines,[6] alkylation of N^3-position even results in the significant decrease of the inhibitory activity on TP from rat spontaneous T-cell lymphoma. Furthermore, the influence of chirality and substitution on the side chain of the phosphonoalkyl group to inhibitory effect is marginal towards TP from SD-lymphoma as shown for described N^3-substituted analogues **6a**, **6b**, and **10** of some efficient ANP inhibitors[6] (see Table 1).

None of the presented compounds **6**, **7**, and **10** possess, at a concentration of 10 μmol L^{-1}, a significant cytostatic activity in tissue cultures estimated in mouse lymphocytic leukemia L1210 cells (ATCC CCL 219), CCRF-CEM T lymphoblastoid cells (human acute lymphoblastic leukemia, ATCC CCL 119), human promyelocytic leukemia HL-60 cells (ATCC CCL 240) and human cervix carcinoma HeLa S3 cells (ATCC CCL 2.2).[16,17]

Based on these biochemical results, we have proved that the inhibitory activity of pyrimidine ANPs towards TP from SD-lymphoma is strongly dependent on their thymine substitution with phosphonoalkyl groups. The de-

Table 1. Inhibition of thymidine phosphorylases by ANPs

Compound	Inhibition of thymidine phosphorylase[a], V_i/V_0			
	E. coli	Human, V79 expressed	SD-Lymphoma	Human placenta
PMET[b]	1.00	1.02	0.27	0.85
(*R*)-HPMPT[b]	0.98	0.84	0.31	0.84
(*R*)-FPMPT[b]	0.93	0.82	0.11	0.56
6a	0.98	1.01	0.75	n.d.
6b	0.93	1.05	n.d.	n.d.
7a	1.01	0.79	0.78	0.91
7b	0.94	0.95	n.d.	1.02
10	1.07	1.12	1.44	0.82

[a] 100 μM [^{3}H]-2′-deoxythymidine, 250 μM P_i (pH 6,7), tested compound 10 μmol·l^{-1}, an appropriate amount of enzyme, 10 min incubation at 37 °C Ref. 6,9b,15. The inhibitory efficacy is expressed by V_i/V_0 (V_i. . .rate of phosphorolysis in the presence of inhibitors, V_0. . . rate of phosphorolysis in the absence of inhibitors).

[b] The structures of compared N^1-substituted thymine ANPs are shown in Figure 1.

crease of the inhibitory effect could be induced by low affinity of N^3-substituted ANPs to enzyme in contrast to N^1-substituted derivatives. This probably results from the differences in recognition of the thymine active site by a variable formation of potential interactions between heteroatoms of N^1- and N^3-substituted thymine moiety and SD-lymphoma TP or by missing of the thymine essential carbonyl group in correct direction which may exclude an interaction with those of TP. However, the marginal values of inhibition on human TP could also indicate the significant diversity in the phosphate binding site in both enzymes. Therefore, we have assumed that newly used TP from SD-lymphoma is not an appropriate model enzyme to human TP probably due to a supposable short length between the thymine and phosphate binding sites and this is a subject of current research. On the other hand, data obtained from SD-lymphoma TP afford a valuable information and a more comprehensive view on problems of pyrimidine multisubstrate inhibitors and their potential utilization on model and commercial enzymes.

Acknowledgments

The study has been performed as a part of research project #Z 40550506 of the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic and the Centre of New Antivirals and Antineoplastics 1M0508 supported by the Ministry of Education of the Czech Republic. The financial support of the Program of target projects of Academy of Sciences of the Czech Republic (Grant #1QS400550501) and of Gilead Sciences (Foster City, CA, USA) is gratefully acknowledged.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.01.006.

References and notes

1. (a) Holý, A. *Curr. Pharm. Design* **2003**, *9*, 2567; (b) Holý, A. In *Recent Advances in Nucleosides: Chemistry and Chemotherapy*; Chu, C. K., Ed.; Elsevier: Amsterdam, 2002; p 167.
2. Esteban-Gamboa, A.; Balzarini, J.; Esnouf, R.; De Clercq, E.; Camarasa, M.-J.; Pérez-Pérez, M.-J. *J. Med. Chem.* **2000**, *43*, 971.
3. (a) Friedkin, M.; Roberts, D. W. *J. Biol. Chem.* **1954**, *207*, 245; (b) Miyazono, K.; Okabe, T.; Urabe, A.; Takaku, F.; Heldin, Ch. *J. Biol. Chem.* **1987**, *262*, 4098; (c) Usuki, K.; Sarasi, J.; Waltenberger, J.; Miyazono, K.; Pierce, G.; Thomason, A.; Heldin, Ch. *Biochem. Biophys. Res. Commun.* **1992**, *184*, 1311.
4. (a) Furakawa, T.; Yoshimura, A.; Sumizawa, T.; Haraguchi, M.; Akiyama, S.; Fukui, K.; Ishizawa, M.; Yamada, Y. *Nature* **1992**, *356*, 668; (b) Haraguchi, M.; Miyadera, K.; Uemura, K.; Sumizawa, T.; Furukawa, T.; Yamada, K.; Akiyama, S.; Yamada, Y. *Nature* **1994**, *368*, 198; (c) Matsushita, S.; Nitanda, T.; Furukawa, T.; Sumizawa, T.; Tani, A.; Nishimoto, K.; Akiba, S.; Miyadera, K.; Fukushima, M.; Yamada, Y.; Yoshida, H.; Kanzaki, T.; Akiyama, S. *Cancer Res.* **1999**, *59*, 1911.
5. Balzarini, J.; Degréve, B.; Esteban-Gamboa, A.; Esnouf, R.; De Clercq, E.; Engelborghs, Y.; Camarasa, M.-J.; Pérez-Pérez, M.-J. *FEBS Lett.* **2000**, *483*, 181.
6. Votruba, I.; Pomeisl, K.; Tloušťová, E.; Holý, A.; Otová, B. *Biochem. Pharmacol.* **2005**, *69*, 1517.
7. Pomeisl, K.; Pohl, R.; Holý, A.; Votruba, I. *Collect. Czech. Chem. Commun.* **2005**, *70*, 1465.
8. Pomeisl, K.; Votruba, I.; Holý, A.; Pohl, R. *Collect. Czech. Chem. Commun.* **2006**, *71*, 595.
9. (a) Klein, R. S.; Lenzi, M.; Lim, T. H.; Hotchkiss, K. H.; Wilson, P.; Schwartz, E. L. *Biochem. Pharmacol.* **2001**, *62*, 1257; (b) Nencka, R.; Votruba, I.; Hřebabecký, H.; Tloušťová, E.; Horská, K.; Masojídková, M.; Holý, A. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 1335.
10. Norman, R. A.; Barry, S. T.; Bate, M.; Breed, J.; Colls, J. G.; Ernill, R. J.; Luke, R. W. A.; Minshull, C. A.; McAlister, M. S. B.; McCall, E. J.; McMiken, H. H. J.; Paterson, D. S.; Timms, D.; Tucker, J. A.; Pauptit, R. A. *Structure* **2004**, *12*, 75.
11. Voet, D.; Voet, J. G. In *Biochemistry*; John Wiley & sons Inc, 1990; p 795, 809.
12. Noell, C. W.; Cheng, C. C. *J. Heterocycl. Chem.* **1966**, *3*, 5.
13. A typical N^3-alkylation procedure in the synthesis of **9**: a mixture of compound **1** (505 mg, 2.4 mmol) and 60% sodium hydride dispersion (144 mg, 3.6 mmol) in dimethylformamide (30 mL) was stirred at room temperature. After 1 h of stirring, the mixture was allowed to warm to 60 °C and compound **7** (620 mg, 2.4 mmol) in dimethylformamide (20 ml) was added. The resulting mixture was heated at 100 °C for 9 h until the conversion of **1** finished. The mixture was concentrated in vacuo to a minimum volume. The residue was codistilled with toluene (3× 20 mL) and diluted with chloroform (30 mL). The mixture was filtered through a Celite pad and the filtrate was concentrated to a minimum volume. The residue was chromatographed on neutral aluminium oxide (ethyl acetate/chloroform/methanol = 26:25:1). The crude product was purified by preparative TLC (ethyl acetate/chloroform/methanol = 26:25:1). The relevant fractions were combined and evaporated in vacuo. Yield 592 mg (57%) of a colourless liquid: IR ν_{max} (CCl_4) 2980, 1707, 1672, 1651, 1462, 1451, 1386, 1375, 1260, 1250, 1181, 1142, 1107, 1089, 1063, 1046, 1010, 991, 889, 556, 503 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.322, 1.324, 1.346 and 1.35 (4× d, 4× 3H, J_{vic} = 6.2, $(CH_3)_2CH$), 1.57–1.78 (m, 4H, CH_2–THP), 1.87

(m, 1H, CH_aH_b–THP), 1.93 (d, 3H, $J_{CH3,6}$ = 1.2, CH_3), 1.99 (m, 1H, CH_aH_b–THP), 3.77–3.84 (m, 5H, H-2′, H-4′, CH_aH_bO–THP), 4.12 (ddt, 1H, J = 11.7, 4.2, 2.3, CH_aH_bO–THP), 4.19 (t, 2H, $J_{1',2'}$ = 6.0, H-1′), 4.78–4.80 (m, 2H, $CH(CH_3)_2$), 5.60 (dd, 1H, J = 10.8, 2.4, CHO–THP), 7.24 (q, 1H, $J_{6,CH3}$ = 1.2, H-6); ^{13}C NMR ($CDCl_3$) δ 13.21 (CH_3); 22.74 (CH_2–THP), 23.94 and 24.07 (d, $J_{C,P}$ = 4, $(CH_3)_2CH$), 24.90 and 30.96 (CH_2–THP), 39.70 (CH_2-1′), 65.41 (d, $J_{C,P}$ = 167, CH_2-4′), 69.04 (CH_2O–THP), 69.51 (d, $J_{C,P}$ = 12, CH_2-2′), 71.02 (d, $J_{C,P}$ = 6, $CH(CH_3)_2$), 82.79 (CH–OTHP), 109.95 (C-5), 133.79 (CH-6), 150.56 (C-2), 163.34 (C-4); FAB-MS *m/z* 433 $[MH]^+$ (77); FABHRMS calcd for $C_{19}H_{34}N_2O_7P$ 433.2103: Found 433.2099.

14. A typical deprotection procedure in the synthesis of **10**: a mixture of compound **9** (562 mg, 1.3 mmol), acetonitrile (20 mL) and bromotrimethylsilane (1.9 g, 12.3 mmol) was stirred overnight at room temperature. The mixture was concentrated in vacuo and then codistilled with water (2× 2 mL). The residue was further refluxed in water (3 mL) and trifluoroacetic acid (10 mL) for 1.5 h. The mixture was cooled to room temperature and neutralized with dilute triethylammonium hydrogencarbonate. The mixture was extracted with chloroform. The water layer was separated and concentrated in vacuo. The residue was purified on 40 mL of DEAE-Sephadex (Cl^- form, 0–0.4 M triethylammonium hydrogencarbonate) with subsequent deionization on activated charcoal. The product was eluted with 12% aqueous ammonia/methanol = 1:1. The relevant fractions were combined, evaporated in vacuo and codistilled with water (3× 5 mL). The residue was dissolved in water (3 mL), applied onto a column of Dowex 50 × 8 (Li^+ form, 30 mL) and then the column was washed with water. The appropriate UV absorbing fraction containing product **10** was evaporated to dryness in vacuo. The residue was dissolved in water and lyophilized. The following compounds were obtained as dilithium salts. Yield 110 mg (32%) of a white solid, mp > 300 °C: IR ν_{max} (KBr) 3259, 3186, 3123, 1715, 1648, 1489, 1448, 1387, 1330, 1213, 1136, 1112, 1001, 913, 805, 771, 723, 581, 564, 480 cm^{-1}; ^{1}H NMR (D_2O) δ 1.88 (d, 3H, $J_{CH3,6}$ = 1.2, CH_3), 3.52 (d, 2H, $J_{H,P}$ = 8.5, H-4′), 3.77 4.19 (t, 2H, $J_{2',1'}$ = 5.8, H-2′), 4.14 (t, 2H, $J_{1',2'}$ = 5.8, H-1′), 7.33 (q, 1H, $J_{6,CH3}$ = 1.2, H-6); ^{13}C NMR (D_2O) δ 14.72 (CH_3), 42.87 (CH_2-1′), 71.09 (d, $J_{C,P}$ = 152, CH_2-4′), 71.90 (d, $J_{C,P}$ = 10, CH_2-2′), 112.40 (C-5), 139.84 (CH-6), 155.94 (C-2), 169.51 (C-4); ^{31}P NMR (202.3 MHz, D_2O) δ 14.41; FAB-MS m/z 277 [MH]+ (67); FABHRMS calcd for $C_8H_{12}Li_2N_2O_6P$ 277.0753: Found 277.0762.

15. Enzyme assay. The standard reaction mixture (50 μl) contained 20 μM bisTris–HCl, pH 6.4, 1 mM EDTA and 2 mM DTT, 100 μM [3H-methyl]thymidine, 250 μM potassium phosphate, pH 6,7, and 25.5 pU of enzyme according to [Ref.6] The reaction was carried out at 37 °C for 10 min and stopped by spotting a 2 μl aliquot onto Silica gel 60 F254 plate that had been prespotted with 0.01 μmol of each thymine and thymidine. The plate was developed in the non-aquaeous phase of the solvent system ethyl acetate/water/formic acid (60:35:5). The spots were visualised under UV light (254 nm) and cut out for radioactivity determination in the toluene-based scintillation cocktail.

16. Cytostatic activity assays. *L1210 cells*, *CCRF-CEM cells*, and *HL-60* cells were cultivated in RPMI 1640 medium supplemented with calf foetal serum using 24-well tissue culture plates. The endpoint of the cell growth was 72 h following the drug addition. Cells were then counted in Celtac MEK 5208 (NIHON KOHDEN) haematological analyzer. *HeLa S3 cells* were seeded to 24-well dishes in RPMI 1640 HEPES modification with foetal calf serum. 48 h following the drug addition the cultivation was stopped and the cell growth was evaluated after methylene blue addition.

17. Hocek, M.; Holý, A.; Votruba, I.; Dvořáková, H. *J. Med. Chem.* **2000**, *43*, 1817.