Anti-Hepatitis B Virus Activity of New Substituted Pyrimidine Acyclic Nucleoside Analogues

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Z. Naturforsch. **64c**, 767–772 (2009); received May 29/July 8, 2009

A number of *N*-substituted pyrimidine acyclic nucleosides were synthesized by coupling reaction of 2-(2-chloroethoxy)ethyl acetate or (2,2-dimethyl-1,3-dioxolan-4-yl)methyl 4-methylbenzenesulfonate with the corresponding base followed by deprotection. The synthesized compounds were tested for their antiviral activity against hepatitis B virus (HBV). The plaque reduction infectivity assay was used to determine virus count reduction as a result of treatment with the synthesized compounds which showed moderate to high antiviral activities.

Key words: Pyrimidines, Acyclic Nucleosides, Anti-Hepatitis B Virus

Introduction

Pyrimidines are an important class of compounds which have widespread applications from pharmaceuticals to materials (Brown, 1996). They have been recognized as important heterocyclic compounds due to their diverse biological activities, such as Tie-2 kinase inhibitory (Malibu and Kappe, 2007), HIV-1 inhibitory (Gadhachanda *et al.*, 2007), antimalarial (Agarwal *et al.*, 2005), secretive adenosine A1 receptor antagonistic (Chang *et al.*, 2004), anticancer (Capdeville *et al.*, 2002), analgesic (Zaki *et al.*, 2006), cardiovascular (Atwal, 1987), and antiallergic (Ozeki *et al.*, 1989) activities. Furthermore, it has been found that 3,4-dihydropyrimidin-2(1*H*)-ones (DHPMs) have an interesting multifaceted pharmacological profile as hepatitis B virus (HBV) replication inhibitors (Kappe, 2000; Deres *et al.,* 2003; Lengar and Kappe, 2004). A number of synthetic pharmacophores with antibacterial (Hegab *et al.*, 2007), antifungal (Atul *et al.*, 2008), and antimycotic activities (Keutzberger and Gillessen, 1985) are based on the pyrimidyl moiety. Also, pyrimidines are present in numerous natural products and, significantly, in the pyrimidine and purine bases ribo- and deoxyribonucleosides (Lister, 1971; Hoffmann, 1996; Hurst, 1980; Brown, 1984). On the other hand, nucleoside analogues are structurally, metabolically, and pharmacodynamically related agents that have diverse biological actions and therapeutic effects including antiviral (Remy and Secrist, 1985; Abdel-Aal *et al.,* 2006; Rashad *et al.,* 2008) and antitumour (Chu *et al.,* 1984; Park *et al.,* 1986; Lin and Liu, 1985) activities. Furthermore, the acyclic nucleoside (Mereyala and Gurijala, 1993; El Ashry *et al.*, 2005, 2006; El Ashry and El Kilany, 1996, 1997) analogues including modifications of both the acyclic glycon and aglycon parts are biological inhibitors and have stimulated extensive research as (El Ashry *et al.*, 2000, 2007). The importance of the substituted hydroxyalkyl chain conformation in the interaction of acyclic nucleosides with enzymes has been demonstrated (Alvarez *et al.*, 1994). Moreover, the 5-substituted derivatives of pyrimidine acyclonucleoside analogues have exhibited pronounced inhibitory properties with respect to uridine phosphorylase and have enhanced antitumour action (Park *et al.,* 1986; Lin and Liu, 1985). Owing to the above significance and our interest in the synthesis of new and potent antiviral nucleoside analogues (El-Sayed *et al.,* 2008; El-Zayate *et al.*, 2009; Ali *et al.*, 2007), herein we report the synthesis and anti-HBV activity of new substituted pyrimidine acyclic nucleosides.

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Results and Discussion

Chemistry

The coupling reaction at N-1 of the substituted pyrimidine derivatives with acyclic and cyclic oxygenated halides is the most efficient route to our target acyclic nucleosides. Thus, the reaction of the substituted pyrimidin-2,4-dione derivatives **1a–c** with 2-(2-chloroethoxy)ethyl acetate (**2**) in *N,N*-dimethylformamide (DMF) and in the presence of sodium hydride at room temperature (r.t.) afforded the corresponding substituted 2-{2-[2,4-dioxo-3-phenyl-3,4-dihydropyrimidin-(2*H*)-1-yl]ethoxy}ethyl acetate derivatives **3a–c** in

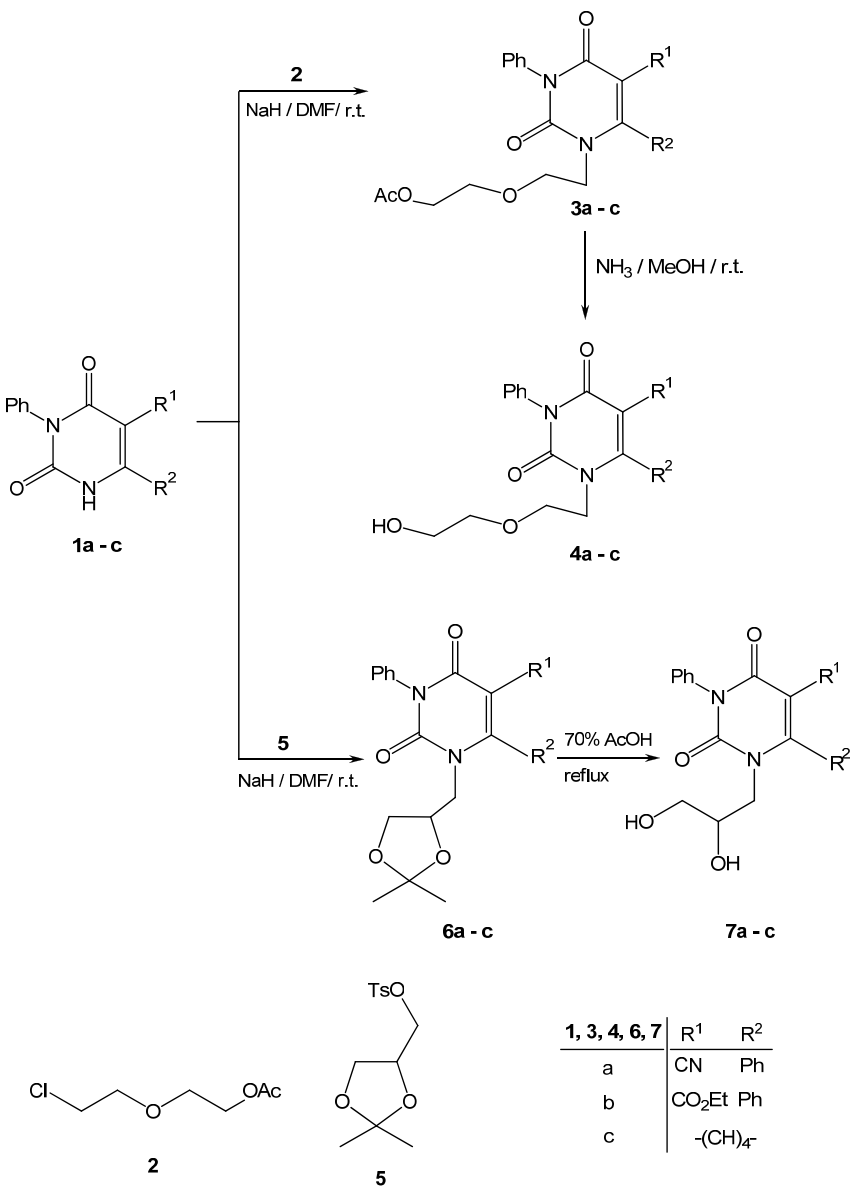

Fig. 1. Synthesis of new pyrimidine acyclic nucleosides.

75–80% yields (Fig. 1). The chemical structures of **3a–c** were confirmed by means of their spectral and analytical data. Thus, their IR spectra showed characteristic absorption bands for the C=O groups at 1665–1785 and 1735–1740 cm^{-1} in addition to an absorption band at 2205 cm^{-1} for the CN group of compound **3a**. The ^1H NMR spectra of **3a–c** showed four CH_2 groups, each as a triplet at δ 3.82–4.92 ppm, the methyl group at δ 2.38–2.44 ppm in addition to the signals of aromatic protons at δ 6.78–7.92 ppm. When the (2,4-dioxo-3,4-dihydropyrimidin-(2*H*)-1-yl)ethoxyethyl) acetate derivatives **3a–c** were treated with methanolic ammonia solution at room temperature, the corresponding substituted 1-[2-(hydroxymethoxy)ethyl]-2,4-dioxo-1,2,3,4-tetrahydropyrimidine derivatives **4a–c** were produced in 70–72% yields. The structures of the deprotected acyclic nucleoside analogues **4a–c** were established on the basis of their IR, ^1H NMR, and elemental analyses data which all agreed with the assigned structures. The IR spectra showed an absorption band at 3420–3440 cm^{-1} for the OH group and revealed the absence of the carbonyl ester group. Furthermore, the ^1H NMR spectra revealed the disappearance of the peak corresponding to the methyl group, and instead a signal corresponding to the OH group appeared at δ 4.35 ppm.

The reaction of the substituted pyrimidin-2,4-dione derivatives **1a–c** with (2,2-dimethyl-1,3-dioxolan-4-yl)methyl 4-methylbenzenesulfonate (**5**) in DMF and in the presence of sodium hydride at room temperature afforded the corresponding substituted ethyl 1-[(2,2-dimethyl-1,3-dioxolan-4-yl)methyl]-2,4-dioxo-1,2,3,4-tetrahydropyrimidines **6a–c** in 80–82% yields. The ^1H NMR spectrum of **6a** as a representative example showed δ 1.88, 1.92 (s, 6H, $2CH_3$), 4.58 (d, 2H, CH_2), 4.96 (d, 2H, CH_2), 5.08 (m, 1H, CH), 6.88 (m, 4H, Ar–H), 7.40 (m, 3H, Ar–H), 7.85 (m, 3H, Ar–3H). Treatment of **6a–c** with 70% acetic acid at reflux temperature afforded the corresponding deprotected acyclic nucleoside analogues, 1-(2,3-dihydroxypropyl)-2,4-dioxo-3,6-diphenyl-1,2,3,4-tetrahydropyrimidine derivatives **7a–c**, in 75–80% yields. Their chemical structures were proved by means of IR, ^1H NMR and elemental analyses data which were in full agreement with the assigned structures. The ^1H NMR spectra revealed the absence of the signals corresponding to the two methyl groups and instead the signals corresponding to the two hydroxy groups appeared at δ 4.15–4.65 ppm.

Antiviral activity

The synthesized compounds were tested for their antiviral activity against HBV using the HepG2.2–2.15 cell line, a human hepatoplastoma cell line producing hepatitis B viral particles (Korba and Gerin, 1992). The results of our preliminary screening indicated that compounds **4a–c** and **7a** showed the highest effect at 10 $\mu g/10^5$ cells, while compounds **7b** and **7c** showed moderate activity (Tables I and II).

In conclusion, new acyclic nucleosides were synthesized in order to increase the number of compounds screened for antiviral activity. Some of them displayed promising activities.

Experimental

General

Melting points were determined using a Büchi apparatus. IR spectra (KBr) were recorded with a Bruker-Vector22 instrument (Bruker, Bremen,

Table I. Inhibitory concentration (IC_{50}) and selectivity index (SI) of the synthesized compounds. The HepG2.2–2.15 CC_{50} value for all tested compounds is 100 μM.

Compound	IC_{50} [μM]	SI
Lamivudine	0.1	1000
4a	0.2	500.0
4b	0.3	333.3
4c	0.5	200.0
7a	0.6	166.6
7b	0.7	142.8
7c	0.7	142.8

Table II. Inhibition of HBV replication by the synthesized compounds.

Compound	Concentration [μM]	HBV DNA in supernatant	HepG2 viable cells
Lamivudine	0.1	0.25	1.00
4a	0.1	0.83	0.90
4b	0.1	0.21	0.63
4c	0.1	0.13	0.19
7a	0.1	0.17	0.56
7b	0.1	0.28	0.35
7c	0.1	0.28	0.35

Germany). ^{1}H NMR spectra were recorded with a Varian Gemini spectrometer at 300 MHz and 200 MHz with TMS as internal standard. Chemical shifts are reported in δ scale (ppm) relative to TMS as a standard, and the coupling constants (J values) are given in Hz. The progress of the reactions was monitored by TLC using aluminum silica gel plates 60 F_{245}. EI-mass spectra were recorded with a HP D5988 A 1000 MHz instrument (Hewlett Packard, Palo Alto, CA, USA). Antiviral activity against HBV was tested at the Liver Institute, Menoufia University, Shebin El-Koam, Egypt.

Antiviral activity

The synthesized compounds were tested for their antiviral activity against hepatitis B virus (HBV) using the HepG2.2–2.15 cell line (Sells *et al.*, 1987), a human hepatoplastoma cell line producing HBV particles (Korba and Gerin, 1992).

The cell line was maintained in RPMI-1640 (Glutamax) (Gibco BRL Life Technologies, Paisly, Scotland) culture medium containing 100 IU/mL nystatin (Gibco BRL Life Technologies), 380 μg/mL G418 (genetecin) (Gibco BRL Life Technologies), and 10% fetal calf serum (FCS) (Gibco BRL Life Technologies). The transferred HepG2.2–2.15 cells were kept in a tissue culture flask at 37 ºC and 5% CO_2. Subcultures were set up after one week by trypsination [10% versin/trypsin (Biochrome KG, Berlin, Germany)] and transferred to a 96-well tissue culture plate. Serial dilutions of the test compounds were added to the cell suspension and incubated for 6 d at 37 ºC and 5% CO_2. The antiviral activity and cytotoxic effect of the test compounds was estimated by comparing the DNA content in the culture supernatant and the viability of the cells containing the test compounds with those of HepG2.2–2.15 cells containing no compounds added to their supernatant (blank cells). The drug lamivudine (4-amino-1-[(2*R*,5*S*)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one, GlaxoSmithKline, Uxbridge, UK) which is a potent selective inhibitor of HBV replication has been used as a standard positive control. Each compound was tested in triplicate.

DNA extraction

DNA extraction was done by incubating 10 μL of diluted supernatant with 10 μL of 0.2 M NaOH at 37 ºC for 1 h, then carefully adding 9.6 μL of 0.2 M HCl, followed by addition of 90 μL of Tris-EDTA [2-amino-2-(hydroxymethyl)-1,3-propanediol-EDTA (TE) (Gibco BRL Life Technologies)] buffer.

PCR-ELISA detection of HBV DNA

The DNA content in the cell culture supernatant was determined by PCR amplification of the HBV DNA using 1 µmol/L of each of the following primers: HCID-1 (5′-GGAAAG AAGTCAGAAGGCA-3′) and HCID-2 (5′-TT-GGGGGAGGAGATTAGGTT-3′), in a reaction mixture containing 14 μL extracted supernatant, 4 mmol/L $MgCl_2$, 10 μmol/L DIG-11-dUTP (Roche, Munich, Germany), 190 μmol/L dTTP, 200 μmol/L dATP, dGTP, dCTP (Roche), 1.5 U Taq polymerase (Roche), in a total volume of 50 μL. PCR reaction conditions were: 32 cycles of 10 min at 94 ºC, 30 s at 58 ºC, and 30 s at 72 ºC, with a 3-s increment for each cycle, in a Perkin Elmer 480 thermal cycler (Perkin Elmer, Waltham, MA, USA). The PCR product was detected by the DIG-ELISA assay (Roche). The optical density (OD) of DNA of the test compounds was compared to that of the blank culture.

Cytotoxicity assay

3-(3,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA) is a colourless substrate that is transferred to a coloured product by any living cell but not by dead cells. The assay utilizes MTT to test the viability of the cells in the presence of the test compound compared to the viability of the blank cells (Fouad *et al.*, 1998).

Calculation of IC_{50}, CC_{50} and SI values

The 50% inhibitory concentration (IC_{50}) of an antiviral drug was determined by plotting the DNA content of the serial dilutions of the tested compound versus the concentration of this compound. The 50% cytotoxic effect (CC_{50}) was calculated from the average viability of the cells in proportion to the concentration of the drug. The selective index (SI) was calculated as CC_{50}/IC_{50}.

Abdel-Aal M. T., El-Sayed W. A., and El-Ashry E. S. H. (2006), Synthesis and antiviral evaluation of some sugar arylglycinoylhydrazones and their oxadiazoline derivatives. Arch. Pharm. Chem. Life Sci. **339**, 356–663.

Agarwal A., Srivastava K., Purib S. K., and Chauhana P. M. S. (2005), Synthesis of 2,4,6-trisubstituted pyrimidines as antimalarial agents. Bioorg. Med. Chem. **13**, 4645–4650.

Ali O. M., Amer H. H., and Abdel-Rahman A. A.-H. (2007), Synthesis of N^4-β-D-glycoside cytosines and sugar N^4-acetylcytosin-1-ylmethylhydrazones as antiviral agents. J. Chem. Res., 281–283.

Alvarez R., Velazquez S., San-Flex A., Aquaro S., de Clercq E., Perno C.-F., Karlsson A., Balzarini J., and Camarasa J. (1994), 1,2,3-Triazole-[2,5-bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3'-spiro-5''-(4''-amino-1'',2''-oxathiole-2'',2''-dioxide) (TSAO) analogs: Synthesis and anti-HIV-1 activity. J. Med. Chem. **37**, 4185–4194.

Atul R. G., Kiran S. T., Fazal S., Mukund V. D., and Kumar V. S. (2008), Efficient synthesis of antifungal pyrimidines via palladium catalyzed Suzuki/Sonogashira cross-coupling reaction from Biginelli 3,4-dihydropyrimidin-2(1*H*)-ones. Tetrahedron **64**, 10214–10223.

Atwal K. (1987), Preparation of 2-amino dihydropyrimidin-5-carboxylates as cardiovascular agents. US Patent 4,769,371, 1988.

Brown D. J. (1984), In: Comprehensive Heterocyclic Chemistry (Katritzky A. R. and Rees C. W., eds.). Pergamon Press, Oxford, Vol. 3, Chapter 2.13.

Brown D. J. (1996), In: Comprehensive Heterocyclic Chemistry II (Katritzky A. R. and Rees C. W., eds.). Pergamon Press, Oxford, Vol. 3, Chapter 2.13.

Capdeville R., Buchdunger E., Zimmermann J., Matter A., and Glivec S. T. (2002), Rationally developed targeted anticancer drug. Nat. Rev. Drug Disc. **1**, 493–502.

Chang L. C. W., Spanjersberg R. F., Jvon F. D. K., Mulder-Krieger T., van DenHout G., Beukers M. W., Brussee J., and Jzerman A. P. (2004), 2,4,6-Trisubstituted pyrimidines as a new class of selective adenosine a1 receptor antagonists. J. Med. Chem. **47**, 6529–6540.

Chu M. Y. W., Naguib F. N. M., Iltzsch M. H., El Kouni M. H., Chu S. H., Cha S., and Calabresi P. (1984), Potentiation of 5-fluoro-2'-deoxyuridine antineoplastic activity by the uridine phosphorylase inhibitors benzylacyclouridine and benzyloxybenzylacyclouridine. Cancer Res. **44**, 1852–1856.

Deres K., Schroder C. H., Paessens A., Goldmann S., Hacker H. J., Weber O., Kraemer T., Niewoehner U., Pleiss U., Stoltefuss J., Graef E., Koletzki D., Masantschek R. N. A., Reimann A., Jaeger R., Grob R., Beckermann B., Schlemmer K.-H., Haebich D., and Rubsamen-Waigmann H. (2003), Inhibition of hepatitis B virus replication by drug-induced depletion of nucleocapsids. Science **299**, 893–896.

El Ashry E. S. H. and El Kilany Y. (1996), Acyclonucleosides: Part 1. *seco*-Nucleosides. Adv. Heterocyclic Chem. **67**, 391–438.

El Ashry E. S. H. and El Kilany Y. (1997), Acyclonucleosides: Part 2. *diseco*-Nucleosides. Adv. Heterocyclic Chem. **68**, 1–88.

El Ashry E. S. H., Rashed N., and Shobier A. H. (2000), Glycosidase inhibitors and their chemotherapeutic value. Part 1. Pharmazie **55**, 251–262.

El Ashry E. S. H., Awad L. F., Abdel-Hamid H., and Atta I. A. (2005), Synthesis of interglycosidically *S*-linked 1-thio-oligosaccharides under microwave irradiation. J. Carbohydr. Chem. **24**, 745–753.

El Ashry E. S. H., Awad L. F., and Atta I. A. (2006), Synthesis and role of glycosylthio heterocycles in carbohydrate chemistry. Tetrahedron **62**, 2943–2998.

El Ashry E. S. H., Rashed N., Awad L. F., Ramadan E., Abdel-Mageed S. M., and Rezki N. (2007), Novel regioselective hydroxyl-alkylation of 4,5-diphenylimidazole-2-thione and a competitive intramolecular ring closure of the *S*-hydroxyalkyl-imidazoles to imidazo[2,1-*b*]thiazines and thiazoles. Role of catalyst, microwave irradiation, and solid support. Nucleosides, Nucleotides, Nucleic Acids **26**, 423–435.

El-Sayed W. A., Fathi N. M., Gad W. A., and El-Ashry E. S. H. (2008), Synthesis and antiviral evaluation of some 5-*N*-arylaminomethyl-2-glycosylsulphanyl-1,3,4-oxadiazoles and their analogues against hepatitis A and Herpes Simplex viruses. J. Carbohydr. Chem. **27**, 357–372.

El-Zayate W. A., El-Sayed W. A., and Abdel-Rahman A. A.-H. (2009), Anti-hepatitis B virus activity of new pyrimidine and adenine peptide nucleic acid (PNA) analogues. Z. Naturforsch. **64c**, 6–10.

Fouad T., Nielsen C., Brunn L., and Pederson E. B. (1998), Use of standardization cell culture assay to assess activities of some potent anti-HIV nucleoside analogues against hepatitis B virus replication. Sc. J. Az. Med. Fac. (Girls) **19**, 1173–1187.

Gadhachanda V. R., Wu B., Wang Z., Kuhen K. L., Caldwell J., Zondler H., Walter H., Havenhand M., and He Y. (2007), 4-Aminopyrimidines as novel HIV-1 inhibitors. Bioorg. Med. Chem. Lett. **17**, 260–265.

Hegab M. I., Hassan N. A., Rashad A. E., Fahmy A. A., and Abdel-Megeid F. M. E. (2007), Synthesis, reactions, and antimicrobial activity of some fused thieno[2,3-*d*]pyrimidine derivatives. Phosphorus, Sulfur, Silicon, Related Elements **182**, 1535–1556.

Hoffmann M. G. (1996), In: Houben-Weyl, Methoden der Organischen Chemie, Vol. E9 (Schaumann E., ed.). Georg Thieme Verlag, Stuttgart.

Hurst D. T. (1980), In: An Introduction to the Chemistry and Biochemistry of Pyrimidines, Purines and Pteridines. Wiley, Chichester.

Kappe C. O. (2000), Biologically active dihydropyrimidones of the Biginelli-type – a literature survey. Eur. J. Med. Chem. **35**, 1043–1052.

Keutzberger A. and Gillessen X. (1985), Antimykotische Wirkstoffe, 18. Mitt. Aromatisch substituierte 2-(4-Toluidino)pyrimidine. Arch. Pharm. **318**, 370–374.

Korba B. E. and Gerin J. L. (1992), Use of a standardized cell culture assay to assess activities of nucleoside analogs against hepatitis B virus replication. Antiviral Res. **19**, 55–70.

Lengar A. and Kappe C. O. (2004), Tunable carbon-carbon and carbon-sulfur cross-coupling of boronic acids with 3,4-dihydropyrimidine-2-thiones. Org. Lett. **6**, 771–774.

Lin T.-S. and Liu M.-C. (1985), Synthesis of 1-[[2-hydroxy-1-(hydroxymethyl)ethoxy]methyl]-5-benzyluracil and its amino analogue, new potent uridine phosphorylase inhibitors with high water solubility. J. Med. Chem. **28**, 971–973.

Lister J. H. (1971), In: The Chemistry of Heterocyclic Compounds, Vol. 24, Fused Pyrimidines, Part II, the Purines (Weissberger A. and Taylor E. C., eds.). Wiley-Interscience, New York.

Malibu M. and Kappe C. O. (2007), Microwave-assisted solution- and solid-phase synthesis of 2-amino-4-arylpyrimidine derivatives. J. Comb. Chem. **9**, 275–284.

Mereyala H. B. and Gurijala V. R. (1993), Use of 2-pyridyl-2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-1-thio-*β*-D-gluco-pyranoside as a glycosyl donor and methyl iodide as an activator for the synthesis of 1,2-*trans*-linked saccharides. Carbohydr. Res. **242**, 277–280.

Ozeki K., Ichikawa T., Hiroyuki T., Tanimury K., Sato M., and Yaginuna H. (1989), Studies on antiallergy agents. III. Synthesis of 2-anilino-1,6-dihydro-6-oxo-5-pyrimidinecarboxylic acids and related compounds. Chem. Pharm. Bull. **37**, 1780–1787.

Park K. S., El Kouni M. H., Krenitsky T. A., Chu S. H., and Cha S. (1986), Inhibition of uridine phosphorylase from *Escherichia coli* by benzylacyclouridines. Biochem. Pharmacol. **35**, 3853–3855.

Rashad A. E., Hegab M. I., Abdel-Megeid R. E., Micky J. A., and Abdel-Megeid F. M. E. (2008), Synthesis and antiviral evaluation of some new pyrazole and fused pyrazolopyrimidine derivatives. Bioorg. Med. Chem. **16**, 7102–7106.

Remy R. J. and Secrist J. A. (1985), Acyclic nucleosides other than acyclovir as potential antiviral agents. Nucleosides, Nucleotides **44**, 411–427.

Sells M. A., Chen M. L., and Acs G. (1987), Production of hepatitis B virus particles in HepG2 cells transfected with cloned hepatitis B virus DNA. Proc. Natl. Acad. Sci. USA **84**, 1005–1009.

Zaki M. E. A., Soliman H. A., Hiekal O. A., and Rashad A. E. (2006), Pyrazolopyranopyrimidines as a class of anti-inflammatory agents. Z. Naturforsch. **61c**, 1–5.