Full Paper

Novel Acyclic Phosphonylated 1,2,3-Triazolonucleosides with an Acetamidomethyl Linker: Synthesis and Biological Activity

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A new series of 4-substituted [(1,2,3-triazol-1-yl)acetamido]methylphosphonates as acyclic nucleotide analogs were synthesized from diethyl (2-chloroacetamido)methylphosphonate via azidation followed by 1,3-dipolar cycloaddition with selected alkynes derived from natural nucleobases or their mimetics. All compounds were tested for their antiviral activities against DNA and RNA viruses as well as for cytostatic activity or cytotoxicity. Among all tested compounds, [(1,2,3-triazol-1-yl)acetamido]-methylphosphonate **6e** substituted with the N^3-Bz-benzuracil moiety showed activity against the vesicular stomatitis virus ($EC_{50} = 45\ \mu M$) in HeLa cell cultures.

Keywords: Antiviral / Cycloaddition / Synthesis / Triazoles

Received: December 17, 2013; Revised: January 28, 2014; Accepted: January 31, 2014

DOI 10.1002/ardp.201300468

Introduction

Effective treatment of viral infections as well as cancer is still an unsolved problem of contemporary medicine, which has stimulated an intensive search for new drugs in the last decades.

Among available therapeutics, nucleosides and nucleotides belong to the most important classes of antiviral and cytostatic compounds. However, their clinical applications are hampered by toxic side effects [1] and drug resistance [2]. For this reason a search for new nucleoside mimetics with improved activity and lower toxicity is conducted by research groups around the world. So far, various structural analogs have been obtained including modification of both the nucleobase and sugar moieties. Among them, numerous acyclic nucleoside phosphonates (ANPs) with promising activity have been synthesized by connecting a phosphonate group with functionalized purine or pyrimidine bases via an acyclic linker.

(*S*)-9-(3-Hydroxy-2-phosphonylmethoxypropyl)adenine [(*S*)-HPMPA], which shows a broad spectrum of antiviral (anti-DNA and RNA viruses) activity, was obtained as early as in 1986 and is considered as the first biologically active ANP [3–5]. Small modifications within a carbon linker led to the discovery of other analogs including adefovir [PMEA] {9-[2-(phosphonomethoxy)ethyl]adenine}, which is active against herpesviruses, retroviruses, and HBV [6, 7], as well as tenofovir {(*R*)-9-[2-(phosphonomethoxy)propyl]adenine} [(*R*)-PMPA], which is used as an anti-HIV and anti-HBV agent [8]. On the other hand, cidofovir {(*S*)-9-[3-hydroxy-2-(phosphonomethoxy)-propyl]cytosine} [(*S*)-HPMPC] shows activity against herpesviruses (such as cytomegalovirus) and adenoviruses [9] (Fig. 1).

A significant body of information on the structure–activity relationship of ANPs has been collected in the literature [10–14]. However, there is still a great interest in search for new modified aliphatic spacers to achieve a correct balance between potency and selectivity, which resulted in elaboration of various ANP derivatives with modified carbon linkers (Fig. 2) [15–18].

Moreover, several analogs of ANPs containing an 1,2,3-triazole ring between the phosphonoalkyl and the nucleobase moieties have been reported and some of them showed interesting activity (Fig. 3) [19–21].

On the other hand, some known modified nucleosides are equipped with nucleobase moieties functionalized with, e.g., carbamoyl, carbamate, or ureidyl groups or they may serve as linkers as well (Fig. 4) [22–31]. However, the primary reason to incorporate the acetamido group next to the phosphate or diphosphate is a replacement of the ionic phosphate by the

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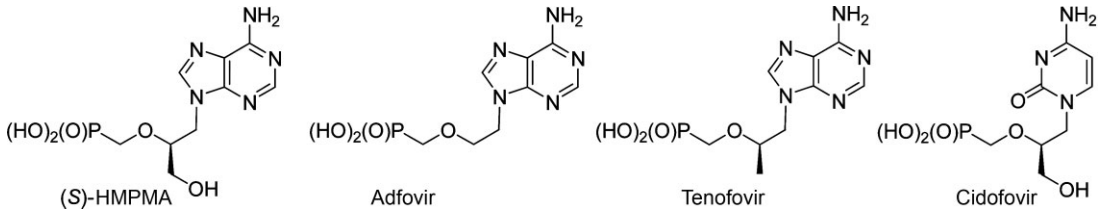


Figure 1. Acyclic nucleoside phosphonates (ANPs) active against viral infections.

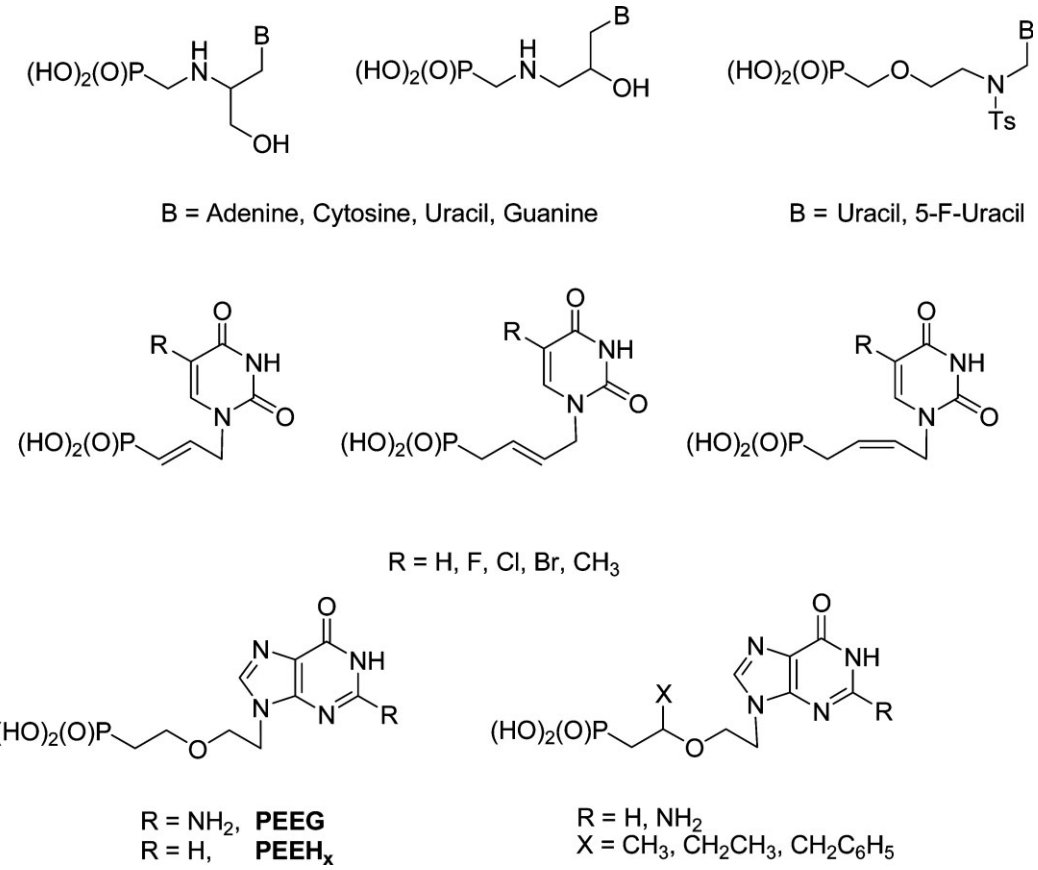


Figure 2. Acyclic nucleoside phosphonates (ANPs) with modified carbon linkers.

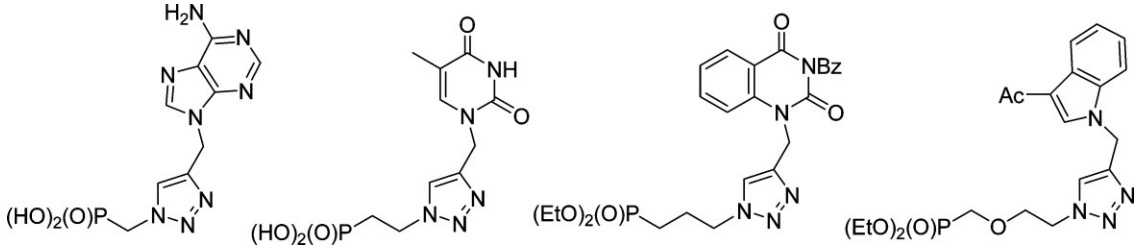


Figure 3. Acyclic nucleoside phosphonates (ANPs) having natural nucleobases (or their analogs) connected to the 1,2,3-triazole ring by a methylene linker.

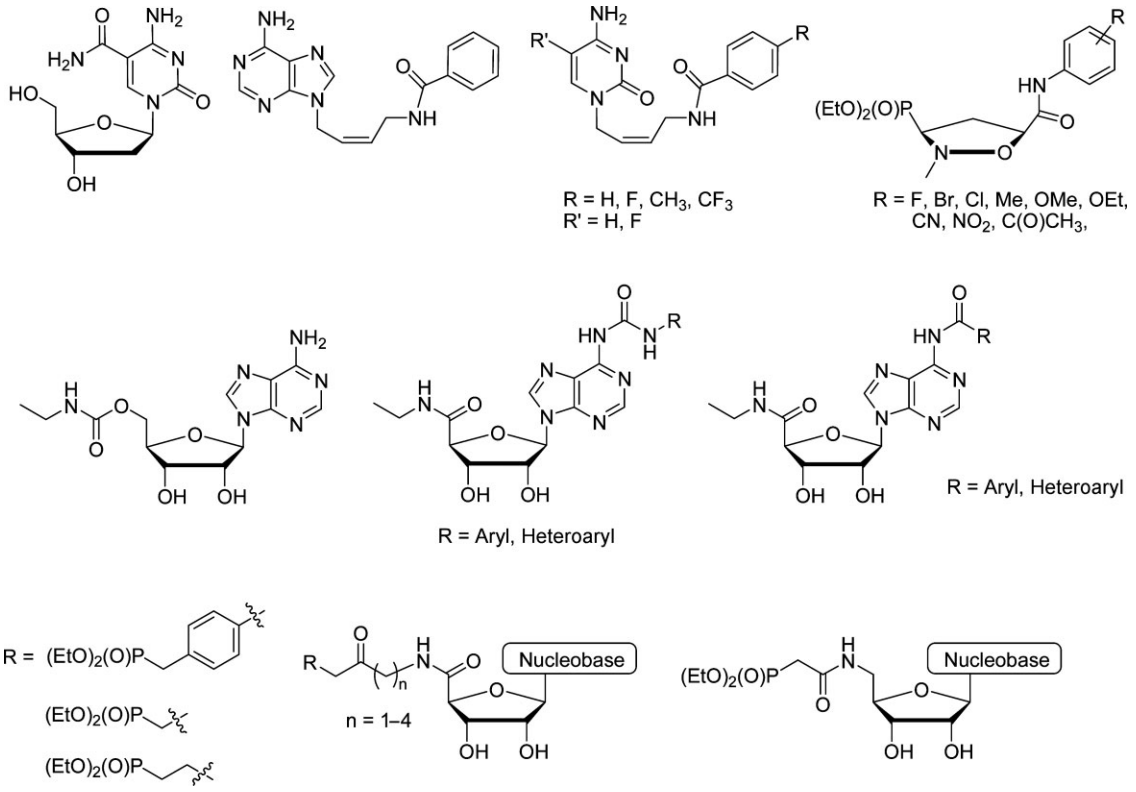


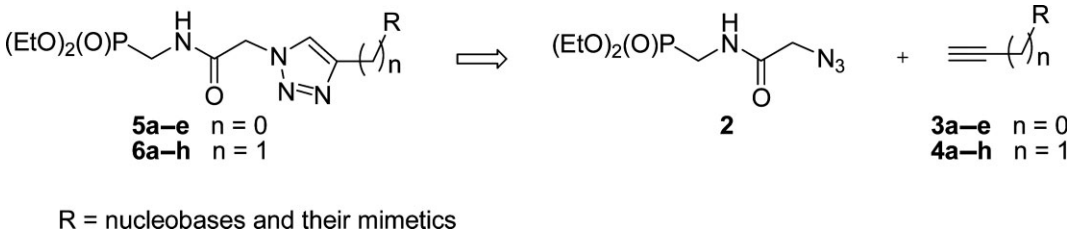
Figure 4. Modified nucleosides containing carbamoyl, carbamate, or ureidyl linker.

neutral amide bond, which may be considered as a non-charged phosphate bioisostere [32, 33]. Furthermore, the replacement of the phosphate internucleotide bonds with an acetamido linkage has also been investigated [34]. Although the phosphate and carbamoyl groups differ in length, flexibility, polarity, lipophilicity, and hydrogen bonding capabilities, increased stability toward enzymatic hydrolysis in comparison to phosphates makes this replacement valuable and thus it deserves further studies.

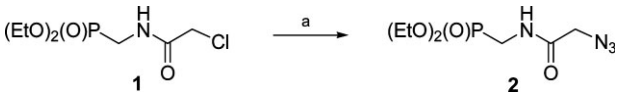
In continuation of our involvement in the search for new biologically active nucleotide analogs [20, 21, 35, 36], the synthesis and biological evaluation of a new series of acyclic phosphonylated 1,2,3-triazolonucleosides **5a–e** and **6a–h** containing an acetamidomethyl linker located between a phosphorus atom and a 1,2,3-triazole ring substituted at C4 with selected nucleobases are presented (Scheme 1). In addition to the canonical nucleobases (in **4a–4d**) and their close mimetics (in **4e** and **4f**), several nucleobase analogs containing substituted heterocyclic (in **3e**, **4g**, and **4h**) and benzene (in **3a–3d**) rings [37, 38] were used.

Results and discussion

The starting diethyl (2-chloroacetamido)methylphosphonate **1** was obtained from diethyl aminomethylphosphonate and chloroacetyl chloride following the literature procedure described for the preparation of analogous diethyl (2-bromoacetamido)methylphosphonate [39].



Scheme 1. Retrosynthesis of phosphonylated 1,2,3-triazoloacyclonucleosides.



Scheme 2. Reagents and conditions: (a) NaN_3, Bu_4NBr, toluene, 90°C, 4 h.

The conversion of phosphonate **1** into diethyl (2-azidoacetamido)methylphosphonate **2** was achieved with tetrabutylammonium azide, which was generated *in situ* from sodium azide and tetrabutylammonium bromide in toluene at 90°C (Scheme 2). This procedure is simple, safe, and efficient leading to the formation of **2** in 82% yield.

The 1,2,3-triazoloacyclonucleotides **5** and **6** were synthesized by the Hüisgen 1,3-dipolar cycloaddition of the diethyl (2-azidoacetamido)methylphosphonate **2** and the selected terminal alkynes **3** (phenylacetylene **3a**, 1-ethynyl-2-fluorobenzene **3b**, 1-ethynyl-3-fluorobenzene **3c**, 1-ethynyl-2,4-difluorobenzene **3d**, 5-ethynyl-1-methyl-1*H*-imidazole **3e**), natural propargylated nucleobases (N^9-propargyladenine **4a** [40], N^1-propargylthymine **4b** [40], N^1-propargyluracil **4c** [40, 41], and N^4-acetyl-N^1-propargylcytosine **4d** [41]) and several modified nucleobases (N^3-benzoyl-N^1-propargyluracil **4e** [21, 42], N^3-benzoyl-N^1-propargylquinazoline-2,4-dione **4f** [21], 5,6-dimethyl-N^1-propargylbenzimidazole **4g** [43], and 3-acetyl-*N*-propargylindole **4h** [44]) (Scheme 3).

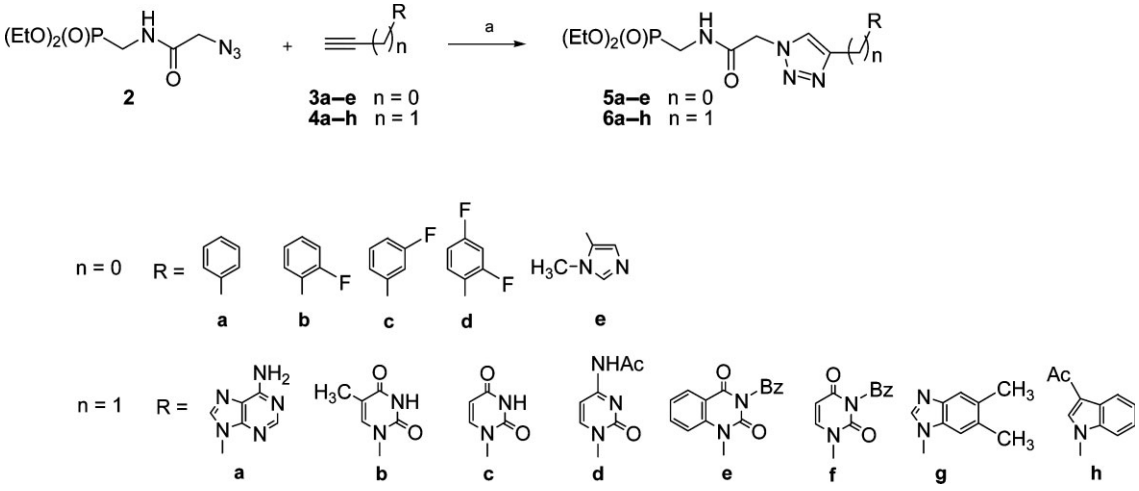
According to our previous experience, cycloadditions were carried out in a microwave oven at 40–45°C [20]. A complete conversion of azidophosphonate **2** into the respective 1,2,3-triazoloacyclonucleotides **5** or **6** was achieved in less than 10 min.

Structure and purity of all 1,2,3-triazoloacyclonucleotides were established by ^1H, ^{31}P, and ^{13}C NMR and IR techniques as well as by elemental analysis.

Antiviral activity and cytotoxicity evaluation

All the 1,2,3-triazoloacyclonucleotides **5** and **6** were evaluated for their antiviral activities against a wide variety of DNA and RNA viruses, using the following cell-based assays: (a) human embryonic lung (HEL) cells: herpes simplex virus-1 (KOS), herpes simplex virus-2 (G), herpes simplex virus-1 (TK^- ACV^r KOS), vaccinia virus, and vesicular stomatitis virus; (b) CEM cell cultures: human immunodeficiency virus [HIV-1 and HIV-2]; (c) Vero cell cultures: para-influenza-3 virus, reovirus-1, Sindbis virus, Coxsackie virus B4, and Punta Toro virus; (d) HeLa cell cultures: vesicular stomatitis virus, Coxsackie virus B4, and respiratory syncytial virus; (e) Crandell-Rees Feline Kidney (CRFK) cell cultures: feline corona virus (FIPV) and feline herpes virus (FHV); (f) Madin Darby Canine Kidney (MDCK) cell cultures: influenza A virus H1N1 subtype (A/PR/8), influenza A virus H3N2 subtype (A/HK/7/87), and influenza B virus (B/HK/5/72). Ganciclovir, cidofovir, acyclovir, brivudin, (*S*)-9-(2,3-dihydroxypropyl)adenine [(*S*)-DHPA], *Hippeastrum* hybrid agglutinin (HHA), *Urtica dioica* agglutinin (UDA), dextran sulfate (molecular weight 10,000 (DS-10000)), ribavirin, zanamivir, amantadine, and rimantadine were used as the reference compounds. The antiviral activity was expressed as the EC_{50}: the compound concentration required to reduce virus-induced cytopathogenicity by 50% (other viruses). In these series of tested compounds, only **6e** (R = N^3-Bz-benzuracil) was moderately active against vesicular stomatitis virus (EC_{50} = 45 μM) in HeLa, but not HEL cells.

The cytotoxicity of the tested compounds toward the uninfected host cells was defined as the minimum cytotoxic concentration (MCC) that causes a microscopically detectable alteration of normal cell morphology. The 50% cytotoxic concentration (CC_{50}), causing a 50% decrease in cell viability, was determined using a colorimetric 3-(4,5-dimethylthiazol-2-



Scheme 3. Reagents and conditions: (a) $CuSO_4 \cdot 5H_2O$ (0.05 equiv.), sodium ascorbate (0.1 equiv.), H_2O–EtOH (1:1), MW, 40–45°C, 10 min.

yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) assay system. None of the tested compounds appeared cytotoxic toward screened cell lines at 250 μM.

Evaluation of cytostatic activity

The cytostatic activity of the tested compounds was defined as the 50% inhibitory concentration (IC_{50}), causing a 50% decrease in cell proliferation. It was determined against murine leukemia L1210, human lymphocyte CEM, and human cervix carcinoma HeLa cells. None of the compounds were inhibitory (cytostatic) at 250 μM.

Conclusion

Transformation of diethyl (2-chloroacetamido)methylphosphonate **1** into the unknown diethyl (2-azidoacetamido)-methylphosphonate **2** was efficiently accomplished employing *in situ* generated tetrabutylammonium azide.

The 1,2,3-triazoloacyclonucleotides **5a–e** and **6a–h** were obtained in good yields from phosphonate **2** by the copper(I)-catalyzed 1,3-dipolar cycloaddition under microwave irradiation with several aromatic and heteroaromatic alkynes **3a–e** and propargylated natural nucleobases as well as their selected mimetics **4a–h**.

All synthesized compounds were examined for their antiviral activities against a variety of DNA and RNA viruses, and for their cytostatic activity or cytotoxicity.

Compound **6e** ($R = N^3$-Bz-benzuracil) showed activity against vesicular stomatitis virus ($EC_{50} = 45$ μM) in HeLa cell cultures.

The lack of any biological activity might be due to the poor uptake by the (virus-infected) cells and/or by a lack of efficient interaction with any potential target protein important for the integrity of the tumor cells or replication of the viruses.

Experimental

^{1}H NMR spectra were taken in $CDCl_3$ or CD_3OD on the following spectrometers: Varian Mercury-300 and Bruker Avance III (600 MHz) with TMS as an internal standard, chemical shifts δ in ppm with respect to TMS, coupling constants J in Hz. ^{13}C NMR spectra were recorded for $CDCl_3$, CD_3OD, or DMSO-d_6 solutions on a Varian Mercury-300 and Bruker Avance III (600 MHz) spectrometer at 75.5 and 151 MHz, respectively. ^{31}P NMR spectra were taken in $CDCl_3$ or CD_3OD on Varian Mercury-300 at 121.5 MHz.

IR spectral data were measured on an Infinity MI-60 FT-IR spectrometer. Melting points were determined on a Boetius apparatus and are uncorrected. Elemental analyses were performed by the microanalytical laboratory of this faculty on a Perkin Elmer PE 2400 CHNS analyzer.

The following adsorbents were used: column chromatography, Merck silica gel 60 (70–230 mesh), analytical TLC, Merck TLC plastic sheets silica gel 60 F_{254}. TLC plates were developed in chloroform–methanol solvent systems. Visualization of spots was effected with iodine vapors. All solvents were purified by methods described earlier in the literature.

All microwave irradiation experiments were carried out in a microwave reactor Plazmatronika RM 800. The reaction was carried out in 50 mL glass vials.

*Synthesis of diethyl (2-chloroacetamido)-methylphosphonate **1***

To a solution of chloroacetyl chloride (0.524 mL, 6.58 mmol) in toluene (10 mL) cooled to 0°C containing anhydrous potassium carbonate (1.161 g, 8.40 mmol), a solution of diethyl aminomethylphosphonate (1.100 g, 6.58 mmol) in toluene (2 mL) was added dropwise and the reaction mixture was stirred at 0°C for 40 min. Then, it was allowed to warm up to room temperature and it was further stirred for 20 h. The suspension was diluted in chloroform (15 mL) and filtered through a layer of Celite. The solution was concentrated *in vacuo*, and the crude product was purified on a silica gel column with a chloroform–methanol mixture (50:1 or 20:1 v/v) to give diethyl (2-chloroacetamido)-methylphosphonate **1** (1.283 g, 80%) as a white solid. m.p. = 62–63°C; IR (KBr): $\nu = 3263$, 3078, 2984, 2929, 1680, 1235, 1023, 969 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 6.83$ (brs, 1H, NH), 4.21–4.12 (m, 4H, 2 × POC*H*$_2$CH$_3$), 4.09 (d, $J = 1.1$ Hz, 2H, CH_2Cl), 3.74 (dd, $J = 12.4$ Hz, $J = 5.9$ Hz, 2H, PCH_2), 1.35 (2t, $J = 7.1$ Hz, 6H, 2 × POCH$_2$C*H*$_3$); ^{13}C NMR (75.5 MHz, $CDCl_3$): $\delta = 166.2$ (d, $J = 5.1$ Hz, C=O), 62.4 (d, $J = 6.6$ Hz, POC), 42.0 (s, CH_2Cl), 34.8 (d, $J = 157.5$ Hz, PC), 16.1 (d, $J = 5.7$ Hz, POC*C*); ^{31}P NMR (121.5 MHz, $CDCl_3$): $\delta = 22.65$ ppm. Anal. calcd. for $C_7H_{15}ClN_4O_4P$: C, 34.51; H, 6.21; N, 5.75. Found: C, 34.28; H, 6.40; N, 5.93.

*Synthesis of diethyl (2-azidoacetamido)-methylphosphonate **2***

A suspension of tetrabutylammonium bromide (0.096 g, 0.297 mmol) and sodium azide (0.245 g, 3.77 mmol) in toluene (8 mL) containing diethyl (2-chloroacetamido)methylphosphonate **1** (0.483 g, 1.98 mmol) was heated to 90°C for 4 h. After cooling, the suspension was filtered through a layer of Celite. The solution was evaporated *in vacuo*, and the crude product was purified on a silica gel column with a chloroform–methanol mixture (100:1 v/v) to give diethyl (2-azidoacetamido)methylphosphonate **2** (0.382 g, 77%) as a colorless oil; IR (film): $\nu = 3240$, 3066, 2985, 2931, 2107, 1688, 1220, 1052, 1028 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 6.78$ (brs, 1H, NH), 4.21–4.11 (m, 4H, 2 × POC*H*$_2$CH$_3$), 4.03 (d, $J = 1.1$ Hz, 2H, CH_2N_3), 3.73 (dd, $J = 12.4$ Hz, $J = 6.0$ Hz, 2H, PCH_2), 1.35 (2t, $J = 7.1$ Hz, 6H, 2 × POCH$_2$C*H*$_3$); ^{13}C NMR (75.5 MHz, $CDCl_3$): $\delta = 167.0$ (d, $J = 5.4$ Hz, C=O), 62.9 (d, $J = 6.6$ Hz, POC), 52.3 (s, CH_2N_3), 34.8 (d, $J = 157.3$ Hz, PC), 16.5 (d, $J = 5.9$ Hz, POC*C*); ^{31}P NMR (121.5 MHz, $CDCl_3$): $\delta = 22.89$ ppm. Anal. calcd. for $C_7H_{15}N_4O_4P$: C, 33.60; H, 6.04; N, 22.39. Found: C, 36.78; H, 6.12; N, 22.60.

General procedure for the preparation of 1,2,3-triazoles

To a solution of azidoalkylphosphonates **2** (1.00 mmol) in EtOH (1 mL) and H_2O (1 mL), $CuSO_4 \cdot 5H_2O$ (0.05 mmol), sodium ascorbate (0.10 mmol), and alkynes (1.00 mmol) were added sequentially. The suspension was irradiated in the microwave reactor at 40–45°C for 10 min. After cooling, the solvent was removed by vacuum evaporation. The residue was suspended in chloroform (5 mL) and filtered through a layer of Celite. The solution was concentrated *in vacuo*, and the crude product was

purified on a silica gel column with a chloroform–methanol mixture (50:1, 20:1, or 10:1 v/v) to give the appropriate 1,2,3-triazoles **5a–e** and **6a–h**.

*Diethyl [2-(4-phenyl-1,2,3-triazol-1-yl)acetamido]-methylphosphonate **5a***

Yield: 90%; after chromatography on a silica gel column with chloroform–methanol (100:1 v/v) appropriate fractions were crystallized from an ethyl acetate–hexane mixture to give white needles. m.p.: 131–132°C; IR (KBr): $\nu = 3244$, 3066, 2985, 2930, 1687, 1221, 1046, 1025 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): $\delta = 8.04$ (s, 1H, *H*C5′), 7.85 (brs, 1H, NH), 7.82–7.80 (m, 2H, $H_{aromat.}$), 7.45–7.31 (m, 3H, $H_{aromat.}$), 5.21 (s, 2H, CH_2N), 4.16–4.07 (m, 4H, $2 \times POCH_2CH_3$), 3.76 (dd, $J = 12.3$ Hz, $J = 6.0$ Hz, 2H, PCH_2), 1.27 (2t, $J = 7.1$ Hz, 6H, $2 \times POCH_2CH_3$); ^{13}C NMR (151 MHz, $CDCl_3$): $\delta = 165.5$ (d, $J = 5.4$ Hz, C=O), 148.2, 130.4, 128.9, 128.3, 125.8, 121.3, 62.9 (d, $J = 6.6$ Hz, POC), 52.6 (s, CH_2N_3), 35.0 (d, $J = 157.6$ Hz, PC), 16.3 (d, $J = 5.6$ Hz, POC*C*); ^{31}P NMR (121.5 MHz, $CDCl_3$): $\delta = 21.96$ ppm. Anal. calcd. for $C_{15}H_{21}N_4O_4P$: C, 51.13; H, 6.01; N, 15.90. Found: C, 51.44; H, 5.99; N, 15.66.

*Diethyl {2-[4-(2-fluorophenyl)-1,2,3-triazol-1-yl]acetamido}-methylphosphonate **5b***

Yield: 86%; after chromatography on a silica gel column with chloroform–methanol (100:1 v/v) appropriate fractions were crystallized from an ethyl acetate–hexane mixture to give white needles. m.p.: 127–129°C; IR (KBr): $\nu = 3215$, 3149, 3058, 2995, 2938, 1680, 1204, 1023, 755 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$): $\delta = 8.30$ (brt, $J = 4.9$ Hz, 1H), 8.24 (dt, $J = 7.6$ Hz, $J = 1.8$ Hz, 1H, $H_{aromat.}$), 8.18 (d, $J = 3.5$ Hz, 1H, *H*C5′), 7.30–7.26 (m, 1H, $H_{aromat.}$), 7.22 (dt, $J = 7.6$ Hz, $J = 1.1$ Hz, 1H, $H_{aromat.}$), 7.11 (ddd, $J = 10.9$ Hz, $J = 8.2$ Hz, $J = 1.1$ Hz, 1H, $H_{aromat.}$), 5.25 (s, 2H, CH_2N), 4.15–4.06 (m, 4H, $2 \times POCH_2CH_3$), 3.77 (dd, $J = 12.2$ Hz, $J = 5.9$ Hz, 2H, PCH_2), 1.27 (2t, $J = 7.1$ Hz, 6H, $2 \times POCH_2CH_3$); ^{13}C NMR (151 MHz, $CDCl_3$): $\delta = 165.9$ (d, $J = 5.5$ Hz, C=O), 159.2 (d, $J = 249.2$ Hz, CF), 141.4 (d, $J = 2.5$ Hz, C_{aromat}), 129.3 (d, $J = 8.5$ Hz, C_{aromat}), 127.8 (d, $J = 3.5$ Hz, C_{aromat}), 124.5 (d, $J = 3.1$ Hz, C_{aromat}), 124.4 (d, $J = 12.8$ Hz, C_{aromat}), 122.0, 115.6 (d, $J = 21.5$ Hz, C_{aromat}), 63.0 (d, $J = 6.7$ Hz, POC), 52.4 (s, CH_2N), 34.9 (d, $J = 157.6$ Hz, PC), 16.2 (d, $J = 6.0$ Hz, POC*C*); ^{31}P NMR (243 MHz, $CDCl_3$): $\delta = 21.67$ ppm. Anal. calcd. for $C_{15}H_{20}FN_4O_4P$: C, 48.65; H, 5.44; N, 15.13. Found: C, 48.51; H, 5.24; N, 15.19.

*Diethyl {2-[4-(3-fluorophenyl)-1,2,3-triazol-1-yl]acetamido}-methylphosphonate **5c***

Yield: 76%; after chromatography on a silica gel column with chloroform–methanol (100:1, 50:1 v/v) appropriate fractions were crystallized from an ethyl acetate–hexane mixture to give white needles. m.p.: 101–102°C; IR (KBr): $\nu = 3242$, 3058, 2989, 2935, 1688, 1231, 1049, 1028, 756 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$): $\delta = 8.31$ (brt, $J = 5.1$ Hz, 1H), 8.11 (s, 1H, *H*C5′), 7.59 (dt, $J = 7.6$ Hz, $J = 1.4$ Hz, 1H, $H_{aromat.}$), 7.56 (ddd, $J = 9.9$ Hz, $J = 2.6$ Hz, $J = 1.4$ Hz, 1H, $H_{aromat.}$), 7.37 (dt, $J = 8.4$ Hz, $J = 5.9$ Hz, 1H, $H_{aromat.}$), 7.02 (ddt, $J = 8.4$ Hz, $J = 2.4$ Hz, $J = 1.4$ Hz, 1H, $H_{aromat.}$), 5.23 (s, 2H, CH_2N), 4.18–4.09 (m, 4H, $2 \times POCH_2CH_3$), 3.78 (dd, $J = 12.2$ Hz, $J = 5.9$ Hz, 2H, PCH_2), 1.30 (2t, $J = 7.1$ Hz, 6H, $2 \times POCH_2CH_3$); ^{13}C NMR (151 MHz, $CDCl_3$): $\delta = 165.6$ (d, $J = 5.4$ Hz, C=O), 163.2 (d, $J = 245.5$ Hz, CF), 146.9 (d, $J = 3.0$ Hz, C_{aromat}), 132.6 (d, $J = 8.6$ Hz, C_{aromat}), 130.4 (d, $J = 8.6$ Hz, C_{aromat}), 121.9, 121.3 (d, $J = 2.8$ Hz, C_{aromat}), 115.0 (d, $J = 21.4$ Hz, C_{aromat}), 112.6 (d, $J = 23.1$ Hz, C_{aromat}), 63.1 (d, $J = 6.9$ Hz, POC), 52.4 (s, CH_2N), 35.0 (d, $J = 157.7$ Hz, PC), 16.3 (d, $J = 5.7$ Hz, POC*C*); ^{31}P NMR (243 MHz, $CDCl_3$): $\delta = 21.73$ ppm. Anal. calcd. for $C_{15}H_{20}FN_4O_4P$: C, 48.65; H, 5.44; N, 15.13. Found: C, 48.51; H, 5.24; N, 14.95.

*Diethyl {2-[4-(2,4-difluorophenyl)-1,2,3-triazol-1-yl]-acetamido}methylphosphonate **5d***

Yield: 82%; after chromatography on a silica gel column with chloroform–methanol (100:1, 50:1 v/v) appropriate fractions were crystallized from an ethyl acetate–hexane mixture to give white needles. m.p.: 105–106°C; IR (KBr): $\nu = 3242$, 3073, 2989, 2935, 1688, 1231, 1049, 1028, 756 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$): $\delta = 8.29$ (dt, $J = 8.6$ Hz, $J = 6.5$ Hz, 1H$_{aromat.}$), 8.12 (d, $J = 3.5$ Hz, 1H, *H*C5′), 7.59 (ddt, $J = 8.6$ Hz, $J = 2.6$ Hz, $J = 0.8$ Hz, 1H, $H_{aromat.}$), 7.33 (brs, 1H, NH), 6.94 (ddd, $J = 11.0$ Hz, $J = 8.6$ Hz, $J = 2.6$ Hz, 1H, $H_{aromat.}$), 5.22 (s, 2H, CH_2N), 4.17–4.12 (m, 4H, $2 \times POCH_2CH_3$), 3.77 (dd, $J = 12.4$ Hz, $J = 5.9$ Hz, 2H, PCH_2), 1.34 (t, $J = 7.1$ Hz, 3H, $POCH_2CH_3$), 1.32 (t, $J = 7.1$ Hz, 3H, $POCH_2CH_3$); ^{13}C NMR (151 MHz, $CDCl_3$): $\delta = 165.4$ (d, $J = 5.5$ Hz, C=O), 162.6 (dd, $J = 251.0$ Hz, $J = 12.0$ Hz, CF), 159.3 (dd, $J = 251.0$ Hz, $J = 12.0$ Hz, CF), 141.0 (d, $J = 1.7$ Hz), 128.8 (dd, $J = 9.7$ Hz, $J = 5.2$ Hz, C_{aromat}), 123.9 (d, $J = 12.2$ Hz), 114.8 (dd, $J = 13.3$ Hz, $J = 3.8$ Hz, C_{aromat}), 112.0 (dd, $J = 21.2$ Hz, $J = 3.2$ Hz, C_{aromat}), 104.1 (d, $J = 26.0$ Hz, C_{aromat}), 63.0 (d, $J = 6.6$ Hz, POC), 52.6 (s, CH_2N), 35.0 (d, $J = 157.8$ Hz, PC), 16.3 (d, $J = 6.0$ Hz, POC*C*); ^{31}P NMR (243 MHz, $CDCl_3$): $\delta = 21.29$ ppm. Anal. calcd. for $C_{15}H_{19}F_2N_4O_4P$: C, 46.40; H, 4.93; N, 14.43. Found: C, 46.48; H, 4.90; N, 14.38.

*Diethyl {2-[4-(1-methylimidazolo-5-yl)-1,2,3-triazol-1-yl]-acetamido}methylphosphonate **5e***

Yield: 76%; after chromatography on a silica gel column with chloroform–methanol (100:1, 50:1 v/v) appropriate fractions solidified as a white powder. m.p.: 111–112°C; IR (KBr): $\nu = 3564$, 3279, 3136, 2932, 1661, 1219, 1052, 1021 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): $\delta = 7.96$ (s, 1H, *H*C5′), 7.70 (brt, $J = 5.8$ Hz, 1H), 7.59 (s, 1H), 7.28 (s, 1H), 5.20 (s, 2H, CH_2N), 4.18–4.09 (m, 4H, $2 \times POCH_2CH_3$), 3.92 (s, 3H, CH_3), 3.75 (dd, $J = 12.2$ Hz, $J = 5.8$ Hz, 2H, PCH_2), 1.31 (2t, $J = 7.0$ Hz, 6H, $2 \times POCH_2CH_3$); ^{13}C NMR (151 MHz, $CDCl_3$): $\delta = 166.3$ (d, $J = 4.2$ Hz, C=O), 139.7, 137.4, 127.2, 123.8, 123.8, 62.8 (d, $J = 6.6$ Hz, POC), 51.6 (s, CH_2N), 34.4 (d, $J = 158.4$ Hz, PC), 32.8, 15.3 (d, $J = 5.7$ Hz, POC*C*); ^{31}P NMR (121 MHz, $CDCl_3$): $\delta = 22.10$ ppm. Anal. calcd. for $C_{13}H_{21}N_6O_4P$: C, 43.82; H, 5.94; N, 23.59. Found: C, 43.61; H, 5.74; N, 23.40.

*Diethyl {2-[4-((6-aminopurin-9-yl)methyl)-1,2,3-triazol-1-yl]-acetamido}methylphosphonate **6a***

Yield: 81%; after chromatography on a silica gel column with chloroform–methanol (100:1, 50:1 v/v) appropriate fractions were crystallized from a methanol–diethyl ether mixture to give white needles. m.p. = 176–178°C; IR (KBr): $\nu = 3310$, 3232, 3072, 2996, 2919, 1692, 1661, 1596, 1572, 1229, 1048, 1021 cm^{-1}; solubility of **6a** in methanol, water, and DMSO was insufficient to measure the ^{13}C NMR spectrum; ^1H NMR (600 MHz, $CDCl_3$): $\delta = 8.41$ (s, 1H), 8.02 (s, 1H), 7.87 (s, 1H), 6.43 (brs, 1H), 5.57 (brs, 2H), 5.54 (s, 2H, CH_2N), 5.07 (s, 2H), 4.15–4.09 (m, 4H, $2 \times POCH_2CH_3$), 3.70 (dd, $J = 12.2$ Hz, $J = 5.8$ Hz, 2H, PCH_2), 1.32 (t, $J = 7.0$ Hz, 6H, $2 \times POCH_2CH_3$); ^{31}P NMR (243 MHz, $CDCl_3$): $\delta = 21.21$ ppm. Anal. calcd. for $C_{15}H_{22}N_9O_4P \cdot 2H_2O$: C, 39.22; H, 5.70; N, 27.44. Found: C, 39.00; H, 5.97; N, 27.67.

Diethyl {2-[4-((5-methyl-2,4-dioxopyrimidin-1-yl)methyl)-1,2,3-triazol-1-yl]acetamido}methylphosphonate **6b**

Yield: 86%; after chromatography on a silica gel column with chloroform–methanol (50:1, 20:1 v/v) appropriate fractions were crystallized from a methanol–diethyl ether mixture to give white needles. m.p. = 175–176°C; IR (KBr): ν = 3305, 3147, 3085, 2990, 2934, 2882, 1677, 1048, 1026 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 9.74 (s, 1H, NH), 7.94 (s, 1H, *H*C5′), 7.83 (brt, J = 5.8 Hz, 1H, PCH_2NH), 7.35 (br q, J = 1.1 Hz, 1H, *H*C=CCH_3), 5.06 (s, 2H), 4.99 (s, 2H), 4.18–4.13 (m, 4H, 2 × $POCH_2CH_3$), 3.73 (dd, J = 12.5 Hz, J = 5.8 Hz, 2H, PCH_2), 1.92 (d, J = 1.1 Hz, 3H, HC=C*CH*$_3$), 1.33 (t, J = 7.1 Hz, 6H, 2 × $POCH_2CH_3$); ^{13}C NMR (75.5 MHz, CD_3OD): δ = 166.9 (d, J = 4.0 Hz, C=O), 166.2, 152.2, 143.3, 142.0, 126.5, 111.4, 63.9 (d, J = 6.6 Hz, POC), 52.7, 43.5, 35.6 (d, J = 158.0 Hz, PC), 16.8 (d, J = 6.0 Hz, POC*C*),12.5 (s, CH_3); ^{31}P NMR (243 MHz, $CDCl_3$): δ = 21.98 ppm. Anal. calcd. for $C_{15}H_{23}N_6O_6P \times H_2O$: C, 41.67; H, 5.83, N, 19.44. Found: C, 41.66; H, 5.56; N, 19.70.

Diethyl {2-[4-((2,4-dioxopyrimidin-1-yl)methyl)-1,2,3-triazol-1-yl]acetamido}methylphosphonate **6c**

Yield: 75%; after chromatography on a silica gel column with chloroform–methanol (50:1, 20:1 v/v) appropriate fractions solidified as a white powder. m.p. = 156–157°C; IR (KBr): ν = 3305, 3147, 3085, 2990, 2934, 2882, 1677, 1048, 1026 cm^{-1}; ^{1}H NMR (300 MHz, CD_3OD): δ = 8.03 (s, 1H, *H*C5′), 7.70 (d, J = 7.9 Hz, 1H, *H*C=CH), 5.66 (d, J = 7.9 Hz, 1H, *H*C=CH), 5.20 (d, J = 1.1 Hz, 2H), 5.03 (s, 2H), 4.15–4.10 (m, 4H, 2 × $POCH_2CH_3$), 3.75 (d, J = 11.9 Hz, 2H, PCH_2), 1.31 (t, J = 7.1 Hz, 6H, 2 × $POCH_2CH_3$); ^{13}C NMR (75.5 MHz, CD_3OD): δ = 166.9 (d, J = 5.4 Hz, C=O), 166.1, 152.1, 146.2, 143.1, 126.6, 102.7, 64.0 (d, J = 6.6 Hz, POC), 52.8, 43.8, 35.6 (d, J = 158.0 Hz, PC), 16.8 (d, J = 5.9 Hz, POC*C*); ^{31}P NMR (121.5 MHz, CD_3OD): δ = 22.22 ppm. Anal. calcd. for $C_{14}H_{21}N_6O_6P$: C, 42.00; H, 5.29; N, 20.99. Found: C, 42.24; H, 5.14; N, 21.20.

Diethyl {2-[4-((N^4-acetylamino-2-oxopyrimidin-1-yl)-methyl)-1,2,3-triazol-1-yl]acetamido}methylphosphonate **6d**

Yield: 83%; after chromatography on a silica gel column with chloroform–methanol (10:1, 5:1 v/v) appropriate fractions solidified as a white powder. m.p. = 175–177°C; IR (KBr): ν = 3426, 3281, 3145, 3042, 3009, 2934, 1710, 1666, 1050, 1025, 975 cm^{-1}; ^{1}H NMR (300 MHz, CD_3OD): δ = 8.03 (s, 1H, *H*C5′), 7.97 (d, J = 7.3 Hz, 1H, HC=C*H*),7.41 (d, J = 7.3 Hz, 1H, *H*C=CH), 5.13 (s, 2H), 5.11 (s, 2H), 4.17–4.07 (m, 4H, 2 × $POCH_2CH_3$), 3.69 (d, J = 12.1 Hz, 2H, PCH_2), 2.20 (s, 3H, C(O)CH_3), 1.31 (t, J = 6.9 Hz, 6H, 2 × $POCH_2CH_3$); ^{13}C NMR (75.5 MHz, CD_3OD): δ = 171.2, 165.4 (d, J = 4.5 Hz, C=O), 166.7, 156.5, 148.8, 141.4, 125.9, 97.4, 63.0 (d, J = 6.6 Hz, POC), 51.9, 45.1, 34.7 (d, J = 158.0 Hz, PC), 24.4, 16.2 (d, J = 5.7 Hz, POC*C*); ^{31}P NMR (121.5 MHz, CD_3OD): δ = 23.23 ppm. Anal. calcd. for $C_{16}H_{24}N_7O_6P \cdot H_2O$: C, 41.83; H, 5.70; N, 21.34. Found: C, 41.80; H, 5.78; N, 21.52.

Diethyl {2-[4-((3-benzoyl-2,4-dioxoquinazolin-1-yl)methyl)-1,2,3-triazol-1-yl]acetamido}methylphosphonate **6e**

Yield: 84%; after chromatography on a silica gel column with chloroform–methanol (20:1, 10:1 v/v) appropriate fractions solidified as a white powder. m.p. = 87–88°C; IR (KBr): ν = 3446, 3067, 2985, 1748, 1699, 1663, 1233, 1023, 811, 754, 679 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.17 (dd, J = 7.9 Hz, J = 1.4 Hz, 1H), 8.07 (brt, J = 5.9 Hz, 1H, PCH_2NH), 7.98–7.94 (m, 2H, $H_{aromat.}$), 7.92 (s, 1H, *H*C5′), 7.87 (d, J = 8.3 Hz, 1H, $H_{aromat.}$), 7.72 (ddd, J = 8.3 Hz, J = 7.4 Hz, J = 1.4 Hz, 1H, $H_{aromat.}$), 7.67–7.62 (m, 1H, $H_{aromat.}$), 7.52–7.46 (m, 2H, $H_{aromat.}$), 7.28 (dt, J = 7.4 Hz, J = 0.9 Hz, 1H, $H_{aromat.}$), 5.40 (s, 2H), 5.10 (s, 2H), 4.12–4.02 (m, 4H, 2 × $POCH_2CH_3$), 3.70 (dd, J = 12.2 Hz, J = 5.9 Hz, 2H, PCH_2), 1.27 (2t, J = 7.1 Hz, 6H, 2 × $POCH_2CH_3$); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 168.6, 165.3 (d, J = 5.4 Hz, C=O), 161.0, 149.4, 142.3, 140.2, 136.2, 135.1, 131.5, 130.4, 129.2, 129.2, 128.8, 125.5, 123.7, 115.4, 115.3, 63.1 (d, J = 4.9 Hz, POC), 52.3, 38.9, 34.9 (d, J = 157.5 Hz, PC), 16.5 (d, J = 5.8 Hz, POC*C*), 16.4 (d, J = 5.8 Hz, POC*C*); ^{31}P NMR (121.5 MHz, $CDCl_3$): δ = 22.72 ppm. Anal. calcd. for $C_{25}H_{27}N_6O_7P$: C, 54.15; H, 4.91; N, 15.16. Found: C, 54.40; H, 4.71; N, 15.36.

Diethyl {2-[4-((3-benzoyl-2,4-dioxopyrimidin-1-yl)methyl)-1,2,3-triazol-1-yl]acetamido}methylphosphonate **6f**

Yield: 93%; after chromatography on a silica gel column with chloroform–methanol (50:1, 20:1 v/v) appropriate fractions were collected as a colorless oil. IR (film): ν = 3246, 3079, 2989, 1747, 1698, 1667, 1237, 1050, 734, 699 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.20 (brt, J = 5.9 Hz, 1H, PCH_2NH), 7.95 (s, 1H, *H*C5′), 7.93–7.89 (m, 2H, $H_{aromat.}$), 7.67 (d, J = 8.0 Hz, 1H, *H*C=CH), 7.67–7.62 (m, 1H, $H_{aromat.}$), 7.51–7.46 (m, 2H, $H_{aromat.}$), 5.78 (d, J = 8.0 Hz, 1H, HC=C*H*), 5.13 (s, 2H), 5.01 (s, 2H), 4.15–4.06 (m, 4H, 2 × $POCH_2CH_3$), 3.72 (dd, J = 12.1 Hz, J = 5.9 Hz, 2H, PCH_2), 1.27 (t, J = 7.1 Hz, 6H, 2 × $POCH_2CH_3$); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 168.9, 165.5 (d, J = 5.4 Hz, C=O), 166.5, 162.5, 149.9, 144.6, 135.4, 131.3, 130.6, 129.3, 126.0, 102.5, 63.3 (d, J = 6.5 Hz, POC), 52.4, 43.6, 35.6 (d, J = 157.2 Hz, PC), 16.6 (d, J = 4.1 Hz, POC*C*); ^{31}P NMR (121.5 MHz, $CDCl_3$): δ = 22.94 ppm. Anal. calcd. for $C_{21}H_{25}N_6O_7P$: C, 50.00; H, 5.00; N, 16.66. Found: C, 50.22; H, 4.88; N, 16.40.

Diethyl {2-[4-((5,6-dimethylbenzimidazol-1-yl)methyl)-1,2,3-triazol-1-yl]acetamido}-methylphosphonate **6g**

Yield: 74%; after chromatography on a silica gel column with chloroform–methanol (50:1, 20:1 v/v) appropriate fractions solidified as a white powder. m.p. = 172–174°C; IR (KBr): ν = 3416, 3217, 3142, 3058, 2974, 2942, 1693, 1216, 1044, 979 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.18 (brt, J = 5.9 Hz, 1H, PCH_2NH), 7.95 (s, 1H), 7.63 (s, 1H), 7.53 (s, 1H), 7.21 (s, 1H), 5.42 (s, 2H), 5.05 (s, 2H), 4.13–4.02 (m, 4H, 2 × $POCH_2CH_3$), 3.70 (dd, J = 12.3 Hz, J = 5.9 Hz, 2H, PCH_2), 2.35 (s, 3H, CH_3), 2.34 (s, 3H, CH_3), 1.25 (2 × t, J = 7.1 Hz, 6H, 2 × $POCH_2CH_3$); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 165.2 (d, J = 5.0 Hz, C=O), 142.7, 141.9, 141.3, 132.6, 131.6, 124.2, 120.5, 119.5, 110.1, 62.9 (d, J = 6.6 Hz, POC), 52.0, 40.2, 34.8 (d, J = 157.5 Hz, PC), 20.6, 20.2, 16.3 (d, J = 5.4 Hz, POC*C*), 16.2 (d, J = 5.4 Hz, POC*C*); ^{31}P NMR (121.5 MHz, $CDCl_3$): δ = 22.73 ppm. Anal. calcd. for $C_{19}H_{27}N_6O_4P$: C, 52.53; H, 6.26; N, 19.34. Found: C, 52.31; H, 6.11; N, 19.67.

Diethyl {2-[4-((3-acetylindol-1-yl)methyl)-1,2,3-triazol-1-yl]-acetamido}methylphosphonate **6h**

Yield: 95%; after chromatography on a silica gel column with chloroform–methanol (50:1, 20:1 v/v) appropriate fractions were crystallized from a methanol–diethyl ether mixture to give a white solid. m.p. = 128–130°C; IR (KBr): ν = 3395, 3235, 3134, 3067, 2986, 2930, 1692, 1639, 1026, 754 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.37–8.33 (m, 1H), 7.87 (s, 1H), 7.70 (brt, J = 5.8 Hz, 1H, PCH_2NH), 7.62 (s, 1H), 7.43–7.40 (m, 1H), 7.31–7.25 (m, 2H), 5.46 (s, 2H), 5.06 (s, 2H), 4.12–4.02 (m, 4H, 2 × $POCH_2CH_3$), 3.68 (dd,

$J = 12.3$ Hz, $J = 5.8$ Hz, 2H, PCH_2), 2.51 (s, 3H, C(O)CH_3), 1.25 (t, $J = 6.8$ Hz, 6H, $2 \times POCH_2CH_3$); ^{13}C NMR (75.5 MHz, $CDCl_3$): $\delta = 193.3$ (s, C=O), 165.4 (d, $J = 5.2$ Hz, C=O), 142.8, 136.4, 135.3, 126.2, 124.2, 123.4, 122.6, 122.4, 117.2, 110.0, 63.1 (d, $J = 6.6$ Hz, POC), 52.1, 42.1, 34.9 (d, $J = 157.7$ Hz, PC), 27.6, 16.4 (d, $J = 5.7$ Hz, POC*C*); ^{31}P NMR (121.5 MHz, $CDCl_3$): $\delta = 22.62$ ppm. Anal. calcd. for $C_{20}H_{26}N_5O_5P \cdot H_2O$: C, 51.61; H, 6.06; N, 15.05. Found: C, 51.51; H, 5.76; N, 15.15.

Antiviral activity assays

The antiviral assays were based on inhibition of virus-induced cytopathicity in HEL [herpes simplex virus type 1 (HSV-1), HSV-2 (G), vaccinia virus and vesicular stomatitis virus], Vero (para-influenza-3, reovirus-1, Sindbis, Coxsackie B4, and Punta Toro virus), HeLa (vesicular stomatitis virus, Coxsackie virus B4, and respiratory syncytial virus), MDCK (influenza A (H1N1 and H3N1) and influenza B virus), or CRFK (feline herpes virus; feline corona virus (FIPV)) cell cultures. Confluent cell cultures in microtiter 96-well plates were inoculated with 100-cell culture inhibitory dose-50 ($CCID_{50}$) of virus (1 $CCID_{50}$ being the virus dose to infect 50% of the cell cultures) in the presence of varying concentrations (250, 50, 10, ... μM) of the test compounds. Viral cytopathicity was recorded as soon as it reached completion in the control virus-infected cell cultures that were not treated with the test compounds. The antiviral concentration was expressed as the EC_{50} or 50%-effective compound concentration required to inhibit virus-induced cytopathicity by 50%.

Cytotoxicity and cytostatic assay

The cytotoxicity of the test compounds was monitored as a microscopically visible alteration of cell morphology, and expressed as the MCC or compound concentration required to afford a microscopically detectable alteration of cell culture morphology.

The cytostatic activity of the test compounds was determined as the 50% cytostatic concentration (IC_{50}) or compound concentration required to inhibit cell proliferation by 50%. For this purpose, cells were seeded in 200-μL wells of 96-well microtiter plates and allowed to proliferate for 2 (L1210) to 3 (CEM) or 4 (HeLa) days in the absence or presence of different serial concentrations of the test compounds. At the end of the exponential proliferation phase, the cells were counted by an automated Coulter ZI particle counter (Analis, Ghent, Belgium).

The authors wish to express their gratitude to Mrs. Małgorzata Pluskota, Mrs. Leentje Persoons, Mrs. Frieda De Meyer, and Mrs. Lizette van Berckelaer for excellent technical assistance. The synthetic part of this work was supported by the Medical University of Łódź internal funds (503-3014-01). The virological/cytostatic part of this work was supported by the KU Leuven (GOA No. 10/014).

The authors have declared no conflict of interest.

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