Design and synthesis of phosphonoacetic acid (PPA) ester and amide bioisosters of ribofuranosylnucleoside diphosphates as potential ribonucleotide reductase inhibitors and evaluation of their enzyme inhibitory, cytostatic and antiviral activity

Stefano Manfredini[1]*, Nicola Solaroli[1,4], Angela Angusti[1], Federico Nalin[1], Elisa Durini[1], Silvia Vertuani[1], Sabrina Pricl[2], Marco Ferrone[2], Silvio Spadari[3], Federico Focher[3], Annalisa Verri[3], Erik De Clercq[5] and Jan Balzarini[5]

[1]Department of Pharmaceutical Sciences, University of Ferrara, Ferrara, Italy
[2]Computer-aided Systems Laboratory, Department of Chemical Engineering, University of Trieste, Trieste, Italy
[3]Istituto di Genetica Molecolare, CNR, Pavia, Italy
[4]Division of Clinical Virology, Huddinge University Hospital, Huddinge/Stockholm, Sweden
[5]Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, Belgium

*Corresponding author: E-mail: s.manfredini@unife.it

Continuing our investigations on inhibitors of ribonucleotide reductase (RNR), the crucial enzyme that catalyses the reduction of ribonucleotides to deoxyribonucleotides, we have now prepared and evaluated 5′-phosphono acetic acid, amide and ester analogues of adenosine, uridine and cytidine with the aim to verify both substrate specificity and contribution to biological activity of diphosphate mimic moieties. A molecular modeling study has been conducted on the RNR R1 subunit, in order to verify the possible interaction of the proposed bioisosteric moieties. The study compounds were finally tested on the recombinant murine RNR showing a degree of inhibition that ranged from 350 µM for the UDP analogue 5′-deoxy-5′-N-(phosphon-acetyl)uridine sodium salt (amide) to 600 µM for the CDP analogue 5′-O-[(diethyl-phosphon)acetyl]cytidine (ester). None of the tested compounds displayed noteworthy cytostatic activity at 100–500 µM concentrations, whereas ADP analogue 5′-N-[(diethyl-phosphon)acetyl]adenosine (amide) and 5′-deoxy-5′-N-(phosphon-acetyl)adenosine sodium salt (amide) showed a moderate inhibitory activity (EC$_{50}$: 48 µM) against HSV-2 and a modest inhibitory activity (EC$_{50}$: 110 µM) against HIV-1, respectively.

Keywords: synthesis, phoshonoacetic ester and amide, diphosphate isosters, RNR inhibitors, cytostatic and antiviral activity, molecular modelling.

Introduction

The ribonucleotide reductase (RNR) is a crucial enzyme in the *de novo* synthesis of DNA; it converts all ribonucleoside diphosphates (NDP) into the corresponding 2′-deoxyribonucleotides (dNDP) in prokaryotic and eukaryotic cells. Viral RNRs are also known: herpes simplex virus (HSV)- and varicella zoster virus (VZV)-infected cells express a viral ribonucleotide reductase, distinct from that present in uninfected cells, which is endowed with a proper enzymatic activity that ensures sufficient 2′-deoxynucleotide supply and DNA synthesis (Boehmer *et al.*, 1997; Heineman *et al.*, 1994). Over the last decade, this enzyme has attracted increasing interest as an important target in the control of the replication of neoplastic cells as well as of pathogenic viral agents. The ribonucleotide diphosphate reductase

(RDR) of the *E. coli*, mammalian and HSV, is structurally formed by two homodimeric proteins, namely R1 and R2, each of them is composed of two polypeptidic chains characterized by an α2β2 structure. The R1 subunit contains the binding sites for the ribonucleoside diphosphates, which are the specific substrates, and for the allosteric effectors, which control the speed and the specificity of dNDP formation. In previous studies, aimed at discovering new mechanism-based inhibitors of RNR (Manfredini *et al.*, 1999), we pointed out the significant role of the diphosphate moiety of the nucleotide during the interaction with RNR. In fact, molecular modelling studies clearly showed that both natural and nucleoside diphosphate analogues accomplish their strong hydrogen bonds with Glu623,

Thr624, Ser625 and Thr209 of the RNR R1 subunit, through the diphosphate group and particularly with the beta-phosphate. Eukaryotic class I RNRs, recognize natural ribonucleoside diphosphates (NDP) as well as mechanism-based inhibitors with a nucleoside structure (for example, gemcitabine), after their conversion to the corresponding 5′-diphosphate form, by action of specific kinases (Plunkett *et al*., 1996). However, as widely reported, the nucleoside kinases recognition can be lost when considerable structural modifications, aimed to gain enzyme inhibitors, are accomplished on the natural substrates (Johansson & Eriksson, 1996; Kukhanova *et al*., 2000). Moreover, it is known that the clinical use of nucleotides is limited by their low stability to non-specific phosphohydrolases (Wagner *et al*., 2000).

To overcome these drawbacks, different approaches have been reported in the literature (Bazzanini *et al*., 1999; Manfredini *et al*., 1999). Among these, interesting results were obtained by SAR studies conducted to discover new bio-analogues of the nucleotide phosphate group with increased stability. An effective application of this approach on the diphosphate nucleotides, was reported by Macchia *et al*. (1994) in the nineties; the authors studied a series of bioisosters of the diphosphate group in anti-HSV agents such as 5-iododeoxyuridine. A few years before Lambert WR and co-workers (1989) reported the antiviral activity of phosphonoacetic (PAA) and phosphonoformic acid (PFA) esters of 5-bromo-2′-deoxyuridine; these compounds were found to be relatively stable in contact with alkaline and intestinal phosphatase. Charvet *et al*. (1994) also modified the 2′,3′-dideoxy-3′-thiacytidine (3TC, lamivudine) by introduction of PFA and PAA moieties and found these derivatives relatively stable to plasma esterase with a half-life of up to 120 min. Moreover, a well-established approach in medicinal chemistry involves the replacement of ester functions with heterocycles, such as isoxazoles, oxadiazoles, furans. (Manfredini *et al*., 2000; Manfredini *et al*., 1996; Manfredini *et al*., 1996). Design, synthesis, modelling studies and biological evaluation of the synthesized compounds, will be herein described.

Materials and methods

Chemistry

Reaction courses were routinely monitored by thin-layer chromatography (TLC) on silica gel precoated Macherey-Nagel durasil-25, with detection under a 254-nm UV lamp and/or by spraying the plates with 10% H_2SO_4/CH_3OH and heating and/or by spraying the plates with ammonium molybdate reagent. Column chromatography was performed with Macherey-Nagel 0.063–0.2 mm/70–230 mesh silica gel. MALDI-MS (matrix-assisted laser desorp-

tion ionization time-of-flight) spectra were obtained on a Hewlett-Packard HPG2025A mass spectrometer operative on a positive linear mode. Nuclear magnetic resonance spectra were determined in *d6*-DMSO, D_2O and $CDCl_3$ solution with a Brucker AC-200 spectrometer and chemical shifts are presented in ppm from internal tetramethylsilane as a standard; ^{31}P NMR spectra were determined in *d6*-DMSO, D_2O and $CDCl_3$ with a Brucker AM-200 spectrometer and chemical shifts presented in ppm from internal 85% H_3PO_4/H_2O solution as a standard. Ultraviolet spectra were recorded on a Kontron UVIKON 922 spectrometer. Melting points were determined by Kofler melting point apparatus (Thermovar, C Reichert AG, Vienna) and are uncorrected. All drying operations were performed over anhydrous sodium sulphate or magnesium sulphate; room temperature varied between 22° and 25°C.

Biology

Enzyme

Murine recombinant RNR proteins R1 and R2 were expressed and purified as previously reported (Mann *et al*., 1991; Thelander *et al*., 1994). Ribonucleotide reductase activity of recombinant murine protein was measured using the ^{3}H-CDP reduction assay described earlier (Engström *et al*., 1979) with some modifications. The enzyme (10 and 0.7 micrograms of recombinant R2 and R1 respectively) was incubated at 37°C in 15 μl of a mixture containing 40 mM Hepes-K^{+}, pH 7,6, 10 mM ATP, 6.4 mM MgCl$_2$, 10 μM DDT, 100 mM KCl, 20 μM FeCl$_3$ and 30 μM 3H-CDP (450 cpm/pmole). The reaction was stopped by the addition of 18 μl of 2 M perchloric acid and precipitated proteins were removed by centrifugation. 0.25 μmoles of dCMP and CMP were added as carrier to the supernatant. After hydrolysis for 10 min at 100°C, the deoxycytidine monophosphate formed was isolated by thin layer chromatography with the following solvent: 2% boric acid-2 M LiCl (2:1, v/v). The marker spots were located under UV light, cut out and placed in vials with 4 ml of scintillation fluid. The radioactivity was counted in a Beckman liquid scintillation counter and all values were corrected by subtracting the blank values.

When ADP was used as substrate, the reaction mixture (30 μl) was the same as described above except for the substrate and the positive effectors (ADP: 40 μM; ATP: 3 mM and dGTP: 500 μM). After 30 min at 37°C the reaction was stopped by boiling for 1 min. Precipitated proteins were removed by centrifugation. ADP reduction was determined after separation of the product from the substrate by HPLC chromatography using the BioRad (Hercules, CA) 100 MAPS preparative system. A 4.6×125 mm Partisphere SAX column (Wathman) was used at room temperature

under the following conditions: injection volume, 25 μl; detection, UV 260 nm; eluent, buffer A (20 mM ammonium phosphate, pH 3.7) and buffer B (1 M ammonium phosphate, pH 3.7). Gradient conditions were 40 min linear gradient from buffer A to buffer B. The flow rate was 1 ml/min.

Cytostatic activity of test compounds

All assays were performed in 96-well microtitre plates. To each well were added $5–7.5×10^4$ cells and a given amount of the test compound. The cells were allowed to proliferate for 48 h (murine leukemia L1210, murine mammary carcinoma FM3A) or 72 h (human lymphocyte CEM and Molt) at 37°C in a humidified CO_2-controlled atmosphere. At the end of the incubation period, the cells were counted in a Coulter counter. The IC_{50} (50% inhibitory concentration) was defined as the concentration of the compound that reduced the number of viable cells by 50%.

Antiviral activity of the test compounds

The compounds were evaluated against a variety of viruses in cell culture. The antiviral assays, other than HIV-1, were based on inhibition of virus-induced cytopathic effect in either E_6SM (HSV-1, HSV-2, VV, VSV), HEL (VZV, CMV) or Vero (vesicular stomatitis virus, respiratory syncytial virus, Coxsackie virus B4, parainfluenza-3 virus, Sindbis virus, Punta Toro virus, reovirus-1) cell cultures. Briefly, confluent cell cultures in 96-well microtitre plates were inoculated with 100 $CCID_{50}$ of virus, 1 $CCID_{50}$ being the virus dose required to infect 50% of the cell cultures. After a 1 h virus adsorption period, residual virus was removed and the cell cultures were incubated in the presence of varying concentrations (400, 200 and 100 μg/ml) of the test compounds. Viral cytopathic effect was recorded as soon as it reached completion in the control virus-infected cell cultures that were not treated with the test compounds.

The assay to examine inhibition of HIV-1-induced cytopathic effect in CEM cells was as follows: human CEM (~$3×10^5$ cells/ml) cells were infected with 100 $CCID_{50}$ of HIV-1(IIIB)/ml and seeded in 200 μl wells of a microtitre plate, containing appropriate dilutions of the test compounds. After 4 days of incubation at 37°C, virus-induced CEM giant cell formation was examined microscopically.

Molecular modelling

Computational studies were conducted on a Silicon Graphics Origin 200, using the molecular modelling packages Cerius2 (v. 4.2) Accerlys Inc., San Diego (Calif., USA), and AMBER (v. 6.0) (Case *et al.*, 1999; Pearlman *et al.*, 1995). The starting 3-D model of the R1 subunit of ribonucleotide reductase (RNR) was based on its X-ray crystallographic structure downloaded from the Protein Data Bank (PBD entry code 4R1R (Eriksson *et al.*, 1997), data 4, C292A mutant with bound GDP and dTTP, and reduced active site. Water molecules in the coordinate file were removed and hydrogen was added to the protein backbone and side chains with the PARSE module of AMBER. All ionizable residues were considered in the standard ionization state at neutral pH. The all-atom force field (FF) parameters by Cornell *et al.*(Cornell *et al.*, 1995) (in parm94.dat file of the AMBER 6.0 code) was applied for protein relaxation. The primary cut-off distance for non-bonded interaction was set to 12 Å and the cut-off tapers for the Coulomb and van der Waals interactions were 1.2 and 2, respectively. The GB/SA continuum solvation model (Jayaram *et al.*, 1998; Weiser *et al.*, 1999) was used to mimic a water environment. Geometry refinement was carried out using the SANDER module via a combined steepest descent-conjugate gradient algorithm, using as a convergence criterion for the energy gradient the root-mean-square of the Cartesian elements of the gradient equal to 0.01 kcal/(mol Å). As expected, no relevant structural changes were observed between the R1 subunit relaxed active site and the original 3-D structure. To prevent global conformational changes of the enzyme, the backbone of the protein binding site were constrained by a harmonic force constant of 100 kcal/mol, whereas the amino acid side chains and the ligands were allowed moving without any constraint. Each nucleotide analogue/ enzyme complex resulting from the procedure described above was further refined in the AMBER suite using the quenched molecular dynamics (QMD) method. In this case, 100 ps MD simulation at 298 K were employed to sample the conformational space of the ligand-enzyme complex. The integration step was equal to 1 fs. After each ps, the system was cooled to 0 K, the structure was extensively minimized and stored. The lowest energy structure for each ligand/receptor complex was selected for further analysis.

2′,3′-O-isopropyliden-5′-O-[(diethyl-phosphon)acetyl]-adenosine (3a)

Compound **1a** (Hampton, 1961) (200 mg, 0.651 mmol), DCC (322.4 mg, 1.562 mmol) and 4-DMAP (7.95 mg, 0.0651 mmol) were dissolved in DMF (8 ml) and, to the stirred solution, diethyl phosphonacetic acid (210 ml, 1.302 mmol) was slowly added under argon atmosphere. After 20 h at room temperature (TLC: CH_2Cl_2/MeOH, 9:1), the reaction was filtered. The solvent was evaporated and co-evaporated with EtOH (3×10 ml). The residue was dissolved in CH_2Cl_2 (15 ml) and the organic phase was washed with H_2O (20 ml), dried, filtered and evaporated to dryness. The resulting oil was purified by silica gel column chromatography (CH_2Cl_2/MeOH 8:2 with 5 drops/l of TEA) to give 220 mg of compound **3a**: yield 70%, yellow

oil. ^{1}H NMR (DMSO-*d6*): δ 1.14–1.26 (m, 6H, 2×CH$_3$); 1.32 (s, 3H, CH$_3$-C); 1.54 (s, 3H, CH$_3$-C); 3.11 (2H, J$_{PH}$=24 Hz, CH$_2$-P); 3.91-4.07 (m, 5H, 2×CH$_2$ and H5″); 4.22–4.36 (m, 2H, H4′ and H5′); 5.02–5.06 (m, 1H, H3′); 5.45–5.50 (m, 1H, H2′); 6.19 (d, 1H, J=2.63 Hz, H1′); 7.37 (sbr, 2H, NH$_2$); 8.16 (s, 1H, H2); 8.35 (s, 1H, H8); 31P NMR, (DMSO-*d6*): δ −20.75; MALDI-TOF MS: *m/z* 485 Da (M)+; 508 Da (M+Na)$^+$ and 524 Da (M+K)$^+$. C$_{19}$H$_{28}$N$_5$O$_8$P requires 485.484. Anal. (C$_{19}$H$_{28}$N$_5$O$_8$P) C,H,N.

2′,3′-O-isopropyliden-5′-O-[(diethyl-phosphon)acetyl]-uridine (4a)

Compound 2a (Hampton, 1961) (200 mg, 0.704 mmol) was reacted with diethyl phosphonacetic acid (114 ml, 0.704 mmol) as described for compound **3a**. After the usual work-up, the residue was purified by silica gel column chromatography (CH$_2$Cl$_2$/MeOH 9:1) to give 235 mg of compound **4a**, yield 72%; yellow oil. ^{1}H NMR (CDCl$_3$): δ 1.29–1.36 (m, 9H, 2×CH$_3$ and CH$_3$-C); 1.55 (s, 3H, CH$_3$-C); 3.0 (2H, J$_{PH}$ =21.17 Hz, CH$_2$-P); 4.08–4.23 (m, 4 H, 2×CH$_2$); 4.30–4.40 (m, 3H, H4′, H5′ and H5″); 4.83–4.88 (m, 1H, H3′); 4.99–5.02 (m, 1H, H2′); 5.73–5.77 (m, 2H, H1′ and H5); 7.43 (d, 1H, J=8.05 Hz, H6); 8.0 (sbr, 1H, NH); 31P NMR, (CDCl$_3$): δ −19.91; MALDI-TOF MS: *m/z* 462 Da (M)$^+$; 485 Da (M+Na)$^+$ and 501 Da (M+K)$^+$. C$_{18}$H$_{27}$N$_2$O$_{10}$P requires 462.388. Anal. (C$_{18}$H$_{27}$N$_2$O$_{10}$P) C,H,N: H calcd. 5.89; found 5.86.

5′-O-[(diethyl-phosphono)acetyl]adenosine (5a)

The compound **3a** (50 mg, 0.103 mmol) was dissolved in CF$_3$COOH 50% (3 ml) and stirred for 2 h at room temperature (TLC: CH$_2$Cl$_2$/MeOH, 9:1). The solution was co-evaporated with EtOH (3×10 ml) and the resulting oil was purified by silica gel column chromatography (CH$_2$Cl$_2$/MeOH, linear gradient from 9:1 to 7:3) to give 32 mg of compound **5a**, 72% yield; colourless foam. ^{1}H NMR (DMSO-*d6*): δ 1.13–1.20 (m, 6H, 2×CH$_3$); 3.07 (d, 2H, J$_{PH}$=19.05 Hz, CH$_2$-P); 3.96-4.04 (m, 6H, 2×CH$_2$, H5′ and H5″); 4.20–4.42 (m, 3H, H4′, H3′ and H2′); 4.65–4.75 (m, 1H, OH3′); 5.75–5.76 (m, 1H, OH2′); 5.91 (d, 1H, J=5.5 Hz, H1′); 7.48 (sbr, 2H, NH$_2$); 8.17 (s, 1H, H2); 8.42 (s, 1H, H8); 31P NMR, (DMSO-*d6*): δ −21; MALDI-TOF MS: *m/z* 469 Da (M+Na)$^+$ and 484 Da (M+K)$^+$. C$_{16}$H$_{24}$N$_5$O$_8$P requires 445.5. Anal (C$_{16}$H$_{24}$N$_5$O$_8$P) C,H,N.

5′-O-[(diethyl-phosphon)acetyl]uridine (6a)

Compound **6a** was prepared as described for **5a** starting from compound **4a** (50 mg, 0.108 mmol). After usual work-up, the residue was purified by silica gel column chromatography (linear gradient from CH$_2$Cl$_2$/MeOH 9:1 to 7:3) to give 26 mg of compound **6a**, 57% yield; colourless foam. ^{1}H NMR (DMSO-*d6*): δ 1.19–1.26 (m, 6H, 2×

CH$_3$); 3.2 (d, 2H, J$_{PH}$=21.4 Hz, CH$_2$-P); 3.97–4.11 (m, 7H, 2×CH$_2$, H5′, H5″ and H4′); 4.23–4.25 (m, 2H, H3′, H2′); 5.30–5.33 (m, 1H, OH3′); 5.46–5.49 (m, 1H, OH2′); 5.65 (d, 1H, J=8.1 Hz, H5); 5.77 (d, 1H, J=5.35 Hz, H1′); 7.70 (d, 1H, J=8.1 Hz, H6); 11.38 (sbr, 1H, NH); 31P NMR, (DMSO-*d6*): δ −21.09; MALDI-TOF MS: *m/z* 445 Da (M+Na)$^+$, 461 Da (M+K)$^+$ and 485 Da (M+Na+K)$^+$. C$_{15}$H$_{23}$N$_2$O$_{10}$P requires 422.324. Anal (C$_{15}$H$_{23}$N$_2$O$_{10}$P) C,H,N.

5′-O-(phosphon-acetyl)adenosine sodium salt (7a)

To a solution of compound **3a** (188.8 mg, 0.389 mmol) in CH$_2$Cl$_2$ (8 ml), TMSBr (1 ml, 7.78 mmol) was added and the solution was stirred under argon atmosphere and at room temperature for 9 h (TLC: iPrOH/H$_2$O/NH$_4$OH 6:3:1). The solvent was evaporated and the residue was purified by column chromatography on DEAE Sephadex A-25 (2×30 cm column), using as eluent TEAB (linear gradient from 0.01 to 1 M), at pH 7.5 and with a flow rate of 50 ml/h. The collected fractions, containing the expected product, were evaporated and the residue dissolved in water. Tetra-*n*-butyl ammonium cation was exchanged for sodium by passing the solution through a Dowex AG 50W-X2 column (50–100 mesh, Na$^+$ form). The solution was freeze dried to give 45 mg of **7a**, yield 30%; white solid, mp198°–201°C. ^{1}H NMR (D$_2$O): δ 2.81 (d, 2H, J$_{PH}$= 20.47 Hz, CH$_2$-P); 3.12–3.64 (m, 3H, H5′, H5″ and H4′); 4.41–4.50 (m, 2H, H3′, H2′); 6.07 (d, 1H, J=4.85 Hz, H1′); 8.19 (s, 1H, H2,); 8.36 (s, 1H, H8); 31P NMR, (DMSO-*d6*): δ 12.76; MALDI-TOF MS: m/z 390.3 Da (M+H)$^+$; 412.3 Da (M+Na)$^+$; C$_{12}$H$_{14}$N$_5$Na$_2$O$_8$P requires 433.038; C$_{12}$H$_{16}$N$_5$O$_8$P requires 389.26. Anal. (C$_{12}$H$_{16}$N$_5$O$_8$P) C,H,N: H calcd. 4.14; found 4.12.

5′-O-(phosphon-acetyl)uridine sodium salt (8a)

The compound **8a** was prepared as described for **7a** starting from **4a** (104.5 mg, 0.226 mmol). The residue, after work-up, was purified by DEAE Sephadex A-25 (2×30 cm) column using TEAB as eluent to give 28 mg of compound **8a**, yield 30%; yellow solid, mp >300°C. ^{1}H NMR (D$_2$O): δ 2.77 (d, 2H, J$_{PH}$=19.6 Hz, CH$_2$-P); 4.33–4.41 (m, 5H, H5′, H5″, H4′, H3′ and H2′); 5.91 (d, 1H, J=5.02 Hz, H1′); 5.95 (d, 1H, J=8.1 Hz, H5); 7.80 (d, 1H, J=8 Hz, H6); 31P NMR, (DMSO-*d6*) δ 12.75; MALDI-TOF MS: *m/z* 389 Da (M+Na)$^+$. C$_{11}$H$_{13}$N$_2$Na$_2$O$_{10}$P requires 410.010; C$_{11}$H$_{15}$N$_2$O$_{10}$P requires 366.218. Anal. (C$_{11}$H$_{15}$N$_2$O$_{10}$P) C,H,N: N calcd. 7.65; found 7.69.

2′-3′-O-isopropyliden-5′-N-[(diethyl-phosphon)acetyl]-adenosine (3b)

Compound **3b** was prepared as described for the compound **3a** starting from **1b** (128 mg, 0.418 mmol) (MacCoss *et al.*, 1980). After work-up, the residue was purified by silica gel

column chromatography (CH_2Cl_2/MeOH 9:1) to give 56 mg of compound **3b**, yield 28%; yellow foam. ^{1}H NMR ($CDCl_3$): δ 1.27–1.34 (m, 6H, 2×CH_3); 1.36 (s, 3H, CH_3-C); 1.61 (s, 3H, CH_3-C); 2.99 (m, 2H, J_{PH}=22.6 Hz, CH_2-P); 3.30-3.45 (m, 2H, H5′, H5″); 4.07–4.24 (m, 4H, 2×CH_2); 4.48–4.49 (m, 1H, H4′); 4.85–4.89 (m, 1H, H3′); 5.31–5.41 (m, 1H, H2′); 5.89 (d, 1H, J=4.42 Hz, H1′); 6.31 (sbr, 2H, NH_2); 7.95 (s, 1H, H2); 8.36 (s, 1H, H8); 8.52–8.54 (m, 1H, NH); ^{31}P NMR, ($CDCl_3$) δ 22.7. MALDI-TOF MS: *m/z* 485.5 Da (M+H)[+]; 507 Da (M+Na)[+]. $C_{19}H_{29}N_6O_7P$ requires 484.443. Anal. ($C_{19}H_{29}N_6O_7P$) C,H,N: H calcd. 6.03; found 6.00.

2′-3′-O-isopropyliden-5′-N-[(diethyl-phosphon)acetyl]-uridine (4b)

Compound **4b** was prepared as described for **3a**, starting from **2b** (236 mg, 0.833 mmol) (MacCoss *et al.*, 1980). After work-up, the residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH 9:1) to give 320 mg of compound **4b**, yield 83%; yellow oil.

^{1}H NMR ($CDCl_3$): δ 1.22–1.33 (m, 9H, CH_3-C, 2× CH_3); 1.49 (s, 3H, CH_3-C); 2.9 (d, 2H, J_{PH}=21.43 Hz, CH_2-P); 3.4-3.8 (m, 2H, H5′, H5″); 4.08–4.19 (m, 5H, 2×CH_2 and H4′); 4.73–4.75 (m, 1H, H3′); 4.97–5.00 (m, 1 H, H2′); 5.58 (d, 1H, J=2.55 Hz, H1′); 5.69 (d, 1H, J=8.0 Hz, H5); 7.41 (d, 1H, J=8.0 Hz, H6); 13 (sbr, 1H, NH); ^{31}P NMR, ($CDCl_3$) δ 23.22. MALDI-TOF MS: *m/z* 462.5 Da (M+H)[+]; 485 Da (M+Na)[+]. $C_{18}H_{28}N_3O_9P$ requires 461.403. Anal. ($C_{18}H_{28}N_3O_9P$) C,H,N: N calcd. 9.11; found 9.16.

5′-N-[(diethyl-phosphon)acetyl]adenosine (5b)

Compound **5b** was prepared as described for **5a**, starting from **3b** (57 mg, 0.118 mmol). After work-up, the residue was purified by silica gel column chromatography (linear gradient from CH_2Cl_2/MeOH 9:1 to 7:3) to give 34 mg of compound **5b**, yield 65%; yellow foam. ^{1}H NMR (DMSO-d6): δ 1.14–1.23 (m, 6H, 2×CH_3); 2.91 (d, 2H, J_{PH}=21.25 Hz, CH_2-P); 3.93–4.09 (m, 7H, H5′, H5″, H4′, 2×CH_2, H3′); 4.63–4.72 (m, 1H, H2′); 5.35–5.37 (m, 1H, OH3′); 5.49–5.52 (m, 1H, OH2′); 5.85 (d, 1H, J=6.3 Hz, H1′); 7.36 (sbr, 2H, NH_2); 8.17 (s, 1 H, H2); 8.39 (s, 1 H, H8); 8.43 (sbr, $NHCH_2$); ^{31}P NMR, (DMSO-*d6*) δ 22.89; MALDI-TOF MS: *m/z* 445.4 Da (M+H)[+]; 467.5 Da (M+Na)[+] $C_{16}H_{25}N_6O_7P$ requires 444.385. Anal. ($C_{16}H_{25}N_6O_7P$) C,H,N.

5′-deoxy-5′-N-[(diethyl-phosphon)acetyl]uridine (6b)

Compound **6b** was prepared as for **5a**, starting from **4b** (50 mg, 0.108 mmol). After work-up, the residue was purified by silica gel column chromatography (linear gradient from CH_2Cl_2/MeOH 9:1 to 7:3) to give 30 mg of compound **6b**, yield 66%; brown foam. ^{1}H NMR (DMSO-d6): δ 1.18-

1.25 (m, 6H, 2×CH_3); 2.9 (d, 2H, J_{PH}=21.36 Hz, CH_2-P); 3.78–3.85 (m, 2H, H5′, H5″); 3.97–4.05 (m, 7H, H4′, 2× CH_2, H3′, H2′); 5.19–5.21 (m, 1H, OH3′); 5.39–5.41 (m, 1H, OH2′); 5.63 (d, 1H, J=8 Hz, H5); 5.74 (d, 1H, J=6.7 Hz, H1′); 7.71 (d, 1H, J=8.0 Hz, H6); 8.16 (sbr, 1H, NHCH_2); 11.4 (sbr, 1H, NH); ^{31}P NMR, (DMSO-*d6*) δ 23.87; MALDI-TOF MS: *m/z* 444.6 Da (M+Na)[+]; 460.5 Da (M+K)[+]; $C_{15}H_{24}N_3O_9P$ requires 421.34. Anal. ($C_{15}H_{24}N_3O_9P$) C,H,N: H calcd. 9.97; found 9.93.

5′-deoxy-5′-N-(phosphon-acetyl)adenosine sodium salt (7b)

The compound **7b** was prepared as for **7a**, starting from **3b** (90.3 mg, 0.187 mmol). After work-up the residue was purified by column chromatography on DEAE Sephadex A-25 (2×30 cm) column with TEAB as eluent, to give 16 mg of compound **7b**, yield 22%; white solid; m.p. >300°C. ^{1}H NMR (D_2O): δ 2.71 (d, 2H, J_{PH}=20 Hz, CH_2-P); 3.16–3.69 (m, 3H, H5′, H5″ and H4′); 4.29–4.36 (m, 2H, H3′, H2′); 6.02 (d, 1H, J=5.6 Hz, H1′); 8.22 (s, 1H, H2); 8.32 (s, 1H, H8); ^{31}P NMR, (DMSO-*d6*) δ 18.18; MALDI-TOF MS: *m/z* 389.7 Da (M+H)[+]; 411.7 Da (M+Na)[+]; $C_{12}H_{15}N_6Na_2O_7P$ requires 432.054; $C_{12}H_{17}N_6O_7P$ requires 388.09. Anal. ($C_{12}H_{17}N_6O_7P$) C,H,N: H calcd. 4.41; found 3.38.

5′-deoxy-5′-N-(phosphon-acetyl)uridine sodium salt (8b)

The compound **8b**, was prepared as for **7a**, starting from **4b** (121 mg, 0.262 mmol). After work-up, the residue was purified by a DEAE Sephadex A-25 (2×30 cm) column with TEAB to give 45 mg of compound **8b**, yield 47%; yellow solid; m.p. >300°C. ^{1}H NMR (DMSO-d6): δ 2.75 (d, 2H, J_{PH}=20.3 Hz, CH_2-P); 3.31–3.36 (m, 2H, H5′, H5″); 3.77–4.05 (m, 2H, H4′, H3′ and H2′); 4.8–5.3 (m, 2H, OH3′, OH2′); 5.62 (d, 1H, J=8.1 Hz, H5); 5.73 (d, 1H, J=5.82 Hz, H1′); 7.74 (d, 1H, J=8.1 Hz, H6); 7.95 (sbr, 1H, NH CH_2); 11.4 (sbr, 1H, NH); ^{31}P NMR, (DMSO-d6) δ 18.46; MALDI-TOF MS: *m/z* 365 Da (M)[+]; 388 Da (M+Na)[+]; 405 Da (M+K)[+]. $C_{11}H_{14}N_3Na_2O_9P$ requires 432.24; $C_{11}H_{16}N_3O_9P$ requires 365.223. Anal. ($C_{11}H_{16}N_3O_9P$) C,H,N.

5′-O-terbutyl-dimethyl-silyl-2′,3′-O-isopropyliden-4-N-(benzyl-oxy-carbonyl)cytidine (10a)

Compound **9** (602 mg, 1.25 mmol) was dissolved in pyridine (20 ml) and benzyl chloroformate (429 μl, 3.043 mmol) was added drop-wise at 0°C. The mixture was stirred at room temperature under positive argon pressure, and the reaction was monitored by TLC (CH_2Cl_2/MeOH 9:1). After 15 h the mixture was evaporated and co-evaporated with EtOH (3×10 ml) and the residue was dissolved in CH_2Cl_2 (15 m). The organic phase was then washed

with H_2O (2×20 ml), dried, filtered and evaporated to dryness. The resulting oil was purified by silica gel column chromatography (CH_2Cl_2/MeOH 9:1) to give 679 mg of compound **10a**, yield 78%; yellow oil. ^{1}H NMR (CDCl$_3$): δ 0.07 (s, 6H, (CH$_3$)$_2$-Si); 0.87 (s, 9H, tBut-Si); 1.35 (s, 3H, CH$_3$-C); 1.58 (s, 3H, CH$_3$-C); 3.81–3.93 (m, 2H, H5′, H5″); 4.40–4.41 (m, 1H, H4′); 4.72–4.80 (m, 2H, H3′, H2′); 5.15–5.25 (m, 2H, CH$_2$-Ph); 5.95 (s, 1H, H1′); 7.21 (d, 1H, J=7.54 Hz, H5); 7.30–7.45 (m, 5H, Ph) 8.09 (d, 1H, J=7.54 Hz, H6); 8.6 (sbr, 1H, NH); MALDI-TOF MS: *m/z* 532 Da (M+H)$^+$; 554 Da (M+Na)$^+$; 570 Da (M+K)$^+$. C$_{26}$H$_{37}$N$_3$O$_7$Si requires 531.673. Anal. (C$_{26}$H$_{37}$N$_3$O$_7$Si) C,H,N.

5′-O-terbutyl-dimethyl-silyl-2′,3′-O-isopropyliden-4-N-(t-butyl-oxy-carbonyl)cytidine (10b)

Compound **9** (200 mg, 0.5 mmol) was dissolved in THF (5 ml) and di-tBut-dicarbonate (200 mg, 0.93 mmol) was added. The stirred mixture was then heated under reflux conditions and under positive argon pressure for 5 h (TLC: CH_2C_{12}/MeOH, 9:1). After evaporation, the resulting residue was purified by silica gel column chromatography (linear gradient from CH_2Cl_2/MeOH 9.9:0.1 to 9.8:0.2) to give 231 mg of compound **10b**, yield 93%; white foam. ^{1}H NMR (CDCl$_3$): δ 0.06 (s, 6H, (CH$_3$)$_2$-Si); 0.89 (s, 9H, tBut-Si); 1.36 (s, 3H, CH$_3$-C); 1.51 (s, 9H, tBut-O); 1.59 (s, 3H, CH$_3$-C); 3.76-3.40 (m, 2H, H5′, H5″); 3.76–3.40 (m, 2H, H5′, H 5″); 4.38–4.40 (m, 1H, H4′); 4.75–4-83 (m, 2H, H3′, H2′); 5.96 (s, 1H, H1′); 7.14 (d, 1H, J=7.54 Hz, H5); 7.49 (sbr, 1H, NH); 8.05 (d, 1H, J=7.54 Hz, H6). MALDI-TOF MS: *m/z* 498.6 Da (M+H)$^+$; 421 Da (M+Na)$^+$; 537 Da (M+K)$^+$. C$_{26}$H$_{37}$N$_3$O7Si requires 497.657. Anal. (C$_{23}$H$_{39}$N$_3$O$_7$Si) C,H,N: N calcd. 8.44; found 8.40.

2′,3′-O-isopropyliden-4-N-(benzyl-oxy-carbonyl)-cytidine (11a)

Compound **10a** (700 mg, 1.68 mmol) was dissolved in THF (35 ml) and TEA×HF (480 ml, 2.94 mmol) was added. The mixture was stirred at room temperature and under positive argon pressure. After 24 h (TLC: CH_2Cl_2/MeOH 9:1) the mixture was evaporated and the residue purified by silica gel column chromatography (CH_2Cl_2/MeOH 9:1) to give 717 mg of compound **11a**, yield 77%; yellow foam. ^{1}H NMR (CDCl$_3$): δ 1.35 (s, 3H, CH$_3$-C); 1.57 (s, 3H, CH$_3$-C); 3.6–4.0 (m, 2H, H5′ and H5″); 4.32–4.42 (m, 1H, H4′); 5.10–5.25 (m, 3H, H3′, H2′, OH5′); 5.15–5.25 (m, 2H, CH$_2$-Ph); 5.51 (s, 1H, H1′); 7.3–7.4 (m, 6H, Ph and H5); 8.0 (d, 1H, J = 7.5 Hz, H6); 8.6 (sbr, 1H, NH); MALDI-TOF MS: *m/z* 419 Da (M+H)$^+$; 440 Da (M+Na)$^+$; 456 Da (M+K)$^+$. C$_{20}$H$_{23}$N$_3$O$_7$ requires 417.42. Anal. (C$_{20}$H$_{23}$N$_3$O$_7$) C,H,N: H calcd. 5.55; found 5.52.

2′,3′-O-isopropyliden-4-N-(t-butyl-oxy-carbonyl)-cytidine (11b)

Compound **10b** (380 mg, 0.76 mmol) was deprotected as described for compound **11a**. After work-up the residue was purified by silica gel column chromatography (linear gradient from CH_2Cl_2/MeOH 9.9:0.1 to 9.8:0.2) to give 247 mg of compound **11b**, yield 85%; white foam. ^{1}H NMR (DMSO-*d6*): δ 1.28 (s, 3H, CH$_3$-C); 1.45 (s, 9H, tBut-O); 1.48 (s, 3H, CH$_3$-C); 3.57–3.61 (m, 2H, H5′ and H5″); 4.16–4.18 (m, 1H, H4′); 4.75–4.77 (m, 1H, H3′); 4.84–4.85 (m, 1H, H2′); 5.05–5.10 (m, 1H, OH5′); 5.80 (d, 1H, J=1.9 Hz, H1′); 7.0 (d, 1H, J=7.5 Hz, H5); 8.15 (d, 1H, J=7.5 Hz, H6); 10.4 (sbr, 1H, NH); MALDI-TOF MS: *m/z* 385 Da (M+H)$^+$; 406 Da (M+Na)$^+$; 422 Da (M+K)$^+$. C$_{17}$H$_{25}$N$_3$O$_7$ requires 383.40. Anal. (C$_{17}$H$_{25}$N$_3$O$_7$) C,H,N.

2′,3′-O-isopropyliden-5′-O-[(diethyl-phosphon)acetyl]4-N-(benzyl-oxy-carbonyl)cytidine (12a)

Compound **12a** was prepared as for **3a**, starting from **11a** (293.3 mg, 0.703 mmol). After work-up, the residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH 9:1) to give 405 mg of compound **12a**, yield 97%; white solid; mp: 153–157°C. ^{1}H NMR (CDCl$_3$): δ 1.24–1.37 (m, 9H, 2×CH$_3$, CH$_3$-C); 1.55 (s, 3H, CH$_3$-C); 2.91 (d, 2H, J$_{PH}$=15.05 Hz, CH$_2$-P); 3.75–3.95 (m, 2H, H5′ and H5″); 4.07–4.18 (m, 4H, 2× CH$_2$); 4.38–4.41 (m, 3H, H4′); 4.85–4.95 (m, 1H, H3′); 5.04–5.07 (m, 1H, H2′); 5.20 (s, 2H, CH$_2$-Ph); 5.72 (s, 1H, H1′); 7.26 (d, 1H, J=7.50 Hz, H5); 7.34–7.45 (m, 5H, Ph); 7.78 (d, 1H, J=7.50 Hz, H6); 7.9 (sbr, 1H, NH); 31P NMR, (CDCl$_3$): δ 19.87; MALDI-TOF MS: *m/z* 596 Da (M+H)$^+$; 618 Da (M+Na)$^+$; 634 Da (M+K)$^+$. C$_{26}$H$_{34}$N$_3$O$_{11}$P requires 595.535. Anal. (C$_{26}$H$_{34}$N$_3$O$_{11}$P) C,H,N.

2′,3′-O-isopropyliden-5′-N-[(diethyl-phosphon)acetyl]4-N-(t-butyl-oxy-carbonyl)cytidine (12b)

Compound **12b** was prepared as described for compound **3a** starting from **11b** (580 mg, 1.52 mmol). After work-up, the residue was purified by silica gel column chromatography (linear gradient from Hexane/Acetone 1:1 to Acetone 100% with 5 drops/l of TEA) to give 300 mg of compound **12b**, yield 35%; yellow oil. ^{1}H NMR (DMSO-*d6*): δ 1.16-1.27 (m, 9H, 2×CH$_3$, CH$_3$-C); 1.38–1.47 (m, 12H, CH$_3$-C, tBut-O); 2.05 (d, 2H, J$_{PH}$=33.3 Hz, CH$_2$-P); 3.36–3.82 (m, 2H, H5′ and H5″); 4.04–4.28 (m, 5H, 2×CH$_2$, H4′); 4.85–4.90 (m, 1H, H3′); 5.20–5.23 (m, 1H, H2′); 5.41 (s, 1H, H1′); 7.20 (d, 1H, J=7.4, H5); 7.60 (d, 1H, J=7.4, H6); 8.04 (sbr, 1H, NHCH$_2$); 8.79 (sbr, 1H, NH); MALDI-TOF MS: *m/z* 583 Da (M+Na)$^+$; 599 Da (M+K)$^+$.

$C_{23}H_{37}N_4O_{10}P$ requires 560.534. Anal. $(C_{23}H_{37}N_4O_{10}P)$ C,H,N: N calcd. 10.00; found 10.05.

2′,3′-O-isopropyliden-5′-O-[(diethyl phosphon)acetyl]-cytidine (13a)

Compound **12a** (450 mg, 0.576 mmol), was suspended in cyclohexene (6.51 ml, 0.064 mmol) and 10% Pd/C (146 mg) in EtOH (13 ml) was then added. The mixture was heated at reflux conditions for 3 h (TLC: CH_2Cl_2/MeOH 9:1). The suspension was filtered on a celite pad and the solution evaporated to dryness. The residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH 9:1) to give 120 mg of compound **13a**: yield 34%, colourless oil. ^{1}H NMR (CDCl$_3$): δ 1.27–1.39 (m, 9H, 2×CH$_3$, CH$_3$-C);1.52 (s, 3H, CH$_3$-C); 2.99 (d, 2H, J_{PH}=29.1 Hz, CH$_2$-P); 4.06–4.20 (m, 4H, 2×CH$_2$); 4.31–4.43 (m, 3H, H4′, H5′ and H5″); 4.91–4.92 (m, 1H, H3′); 5.12–5.15 (m, 1H, H2′); 5.48 (s, 1H, H1′); 5.91 (d, 1H, J=7.3 Hz, H5); 7.31 (d, 1H, J=7.3 Hz, H6); ^{31}P NMR, (CDCl$_3$): δ 20.05; MALDI-TOF MS: m/z 462.5 Da (M+H)$^+$; 484.5 Da (M+Na)$^+$; 500 Da (M+K)$^+$. $C_{18}H_{28}N_3O_9P$ requires 461.403. Anal. $(C_{18}H_{28}N_3O_9P)$ C,H,N: H calcd. 6.12; found 6.09.

5′-O-[(diethyl-phosphon)acetyl]cytidine (14a)

Compound **14a** was prepared as described for compound **5a** starting from **13** (64 mg, 0.139 mmol). After work-up, the residue was purified by silica gel column chromatography (linear gradient from CH_2Cl_2/MeOH 9:1 to 7:3) to give 48 mg of compound **14a**: yield 82%, yellow foam. ^{1}H NMR (DMSO-$d6$): δ 1.14–1.26 (m, 6H, 2×CH$_3$); 2.61 (d, 2H, J_{PH}=45.13 Hz, CH$_2$-P); (m, 7H, 2×CH$_2$, H4′, H5′ and H5″); 4.25–4.35 (m, 2H, H2′, H3′); 5.35 (sbr, 1H, OH3′); 5.55 (sbr, 1H, OH2′); 5.76 (d, 1H, J=3.17 Hz, H1′); 6.03 (d, 1H, J=7.65 Hz, H5); 7.86 (d, 1H, J=7.65 Hz, H6); 8.75 (sbr, 1 H, NH$_2$); ^{31}P NMR, (DMSO-$d6$): δ 22.95; MALDI-TOF MS: m/z 422.5 Da (M+H)$^+$, 444 Da (M+Na)$^+$, 460 Da (M+K)+. $C_{15}H_{24}N_3O_9P$ requires 421.34. Anal. $(C_{15}H_{24}N_3O_9P)$ C,H,N.

5′-N-[(diethyl-phosphon)acetyl]cytidine (14b)

Compound **12b** (95 mg, 0.17 mmol), was dissolved in 50% CF$_3$COOH (8 ml) and stirred for 2 h at room temperature. After 15 h (TLC: CH_2Cl_2/MeOH, 8:2), the solution was evaporated and co-evaporated with EtOH (3×10 ml) and the resulting oil was purified by silica gel column chromatography (linear gradient from CH_2Cl_2/MeOH 9.5:0.5 to 8:2) to give 26 mg of compound **14b**, yield 36%; colourless foam. ^{1}H NMR (DMSO-$d6$): δ 1.14–1.25 (m, 6H, 2× CH$_3$); 2.89 (d, 2H, J_{PH}=21.34 Hz, CH$_2$-P); 3.74–3.92 (m, 3H, H4′, H5′ and H5″); 3.93–4.09 (m, 6H, 2×CH$_2$, H2′, H3′); 5.12 (sbr, 1H, OH3′); 5.30 (sbr, 1H, OH2′); 5.72–5.82 (m, 2H, H1′, H5); 7.12–7.47 (m, 2H, NH$_2$); 7.63 (d, 1H, J=7.37 Hz, H6); 8.19 (sbr, 1H, NHCH$_2$); ^{31}P

NMR, (DMSO-$d6$): δ 23, 87; MALDI-TOF MS: m/z 421.4 Da (M+H)$^+$; 443.7 Da (M+Na)$^+$; 459.6 Da (M+K)$^+$. $C_{15}H_{25}N_4O_8P$ requires 420.354. Anal. $(C_{15}H_{25}N_4O_8P)$ C, H, N: H calcd. 5.99; found 5.96.

5′-O-(phosphon-acetyl)cytidine sodium salt (15a)

The compound **15a** was prepared as described for compound **7a** starting from **13** (268 mg, 0.581 mmol). After work-up, the residue was purified by a DEAE Sephadex A-25 (2×30 cm) column with TEAB to give 45 mg of compound **15a**: yield 30%, white solid, mp >300°C. ^{1}H NMR (D$_2$O): δ 2.90 (d, 2H, J_{PH}=20.48 Hz, CH$_2$-P); 4.30–4.40 (m, 3H, H5′, H5″, H4′); 4.42–4.52 (m, 2H, H3′, H2′); 5.91(d, 1H, J=3.13 Hz, H1′); 6.11 (d, 1H, J= 7.54, Hz, H5); 7.82 (d, 1H, J=7.54 Hz, H6); ^{31}P NMR, (DMSO-$d6$): δ 12.76; MALDI-TOF MS: m/z 404 Da (M+K)$^+$. $C_{11}H_{14}N_3Na_2O_9P$ requires 409.20; $C_{11}H_{16}N_3O_9P$ requires 365.223. Anal. $(C_{11}H_{16}N_3O_9P)$ C,H,N: H calcd. 4.42; found 4.40.

5′-N-(phosphon-acetyl)cytidine sodium salt (15b)

The compound **15b** was prepared as described for compound **7a** starting from **14b** (240 mg, 0.57 mmol). After work-up, the residue was purified by a DEAE Sephadex A-25 (2×30 cm) column with TEAB, to give 160 mg of compound **15b**: yield 70%; white solid; mp >300°C. ^{1}H NMR (D$_2$O): δ 2.79 (d, 2H, J_{PH}=20.34 Hz, CH$_2$-P); 3.15–3.45 (m, 2H, H5′, H5″); 3.68–3.71 (m, 1H, H4′); 4.07–4.32 (m, 2H, H3′, H2′); 5.87 (d, 1H, J=4.06 Hz, H1′); 6.22 (d, 1H, J=7.72 Hz, H5); 7.88 (d, 1H, J=7.72 Hz, H6); ^{31}P NMR, (DMSO-$d6$): δ 18.54; MALDI-TOF MS: m/z 365.4 Da (M+H)$^+$; 3.87 Da (M+Na)$^+$; 403.5 Da (M +K)$^+$. $C_{11}H_{15}N_4Na_2O_8P$ requires 408.20; $C_{11}H_{17}N_4O_8P$ requires 364.248. Anal. $(C_{11}H_{17}N_4O_8P)$ C,H,N: N calcd. 13.72; found 13.66.

Results

Chemistry

In order to obtain nucleotide the 5′phosphonoacetic acid derivatives, uridine and adenosine were protected at the 2′ and 3′ positions (**1a**, **2a**), as described by Hampton (1961). To prepare the corresponding 5′-amino-5′-deoxy nucleotide derivatives (**1b**, **2b**), we adapted the synthetic strategy described by MacCoss *et al*. (1980) starting from **1a** and **2a**. The protected compounds **1a**, **2a** and **1b**, **2b** were then reacted with phosphonoacetic acid (PAA) in the presence of dicyclohexylcarbodiimide (DCCI) and DMF (Figure 1).

The resulting ester (**3a**, **4a**) and amide (**3b**, **4b**) derivatives were finally deprotected by using two different deprotection methods. Compounds **5a, b** and **6a, b** were obtained by treatment with TFA, whereas compounds **7a, b** and **8a,**

Table 1. Inhibitory activity (IC$_{50}$) and binding energies (ΔG) of the study compounds

Compounds	IC$_{50}$ µM	ΔG Kcal/mol
8a	655	—5.35
8b	350	—7.74
5a	500	—6.29
5b	N.I.	—5.15
6a	760	—5.08
6b	N.I.	—4.94
7a	880	—5.20
7b	N.I.	—4.70
14	600	—6.14
14b	N.I.	—5.04
15	773	—5.06
15b	N.I.	—4.92

N.I., not inhibitory.

b, were obtained by reaction with trimethylsilyl bromide (TMSBr) followed by treatment with Dowex Na$^+$ Form, (50×2–100).

In the case of cytidine, previous protection of the N4 position of the pyrimidine ring was required to avoid competition during the coupling step. Indeed, the related 2′,3′,5′-protected compound **9** (Griffey & Poulter 1983), was treated with benzyloxychloroformiate in pyridine or with di-tBut-dicarbonate (BOC) in THF/dioxane to give the compounds **10a** and **10b**, respectively (Figure 2). The corresponding phosphonic acid derivatives (**12a, b**) were obtained, as described above for uridine and adenosine (Figure 1), after the removal of the t-butyl-dimethylsilyl (TBDMS) protecting group at the 5′-position by treatment with TEA×3HF (**11a, b**). The final synthetic step involved the deprotection of the N^4 position of the pyrimidine ring

and the 2′, 3′ positions of the sugar moiety. Hydrogenation of **12a** over Pd/C gave the partially deprotected compound **13a**, which was subsequently treated with 50% TFA to give **14a**. Finally, direct treatment of the BOC-protected derivative **12b** with TFA 50% led to compound **14b** (Figure 2). The disodium salt derivatives **15a, b** were obtained by reaction with TMSBr followed by treatment with Dowex Na$^+$ (50×2–100) starting from compounds **13a** and **14b**.

Biology

RNR inhibition

The synthesized compounds were evaluated for their *in vitro* inhibitory activity on murine recombinant RNR proteins R1 and R2, showing lower inhibitory activity than expected: IC$_{50}$ values ranged from 350 µM for the UDP analogue **8b** (amide) to 600 µM for the CDP analogue **14a** (ester). The IC$_{50}$ value is defined as the concentration of the compound that inhibits the reduction of the substrate by 50% in the assay conditions (Table 1).

Cytostatic and antiviral activity

The cytostatic activity of the prepared nucleosides was evaluated against murine leukemia L1210, mammalian carcinoma FM3A and human T4-lymphocyte Molt and CEM cells. None of the studied compounds were cytostatic at 100–500 µM (Table 2), whereas ADP analogues showed moderate activity (EC$_{50}$: 48 µM, **5b**, amide) against herpes simplex virus type 2 (HSV-2) in E$_6$SM cell cultures and modest activity (EC$_{50}$: 110 µM, **7b**, amide) against HIV-1 in CEM cell cultures (Tables 2 and 3).

The virtual lack of biological activity, in absence of any other different mechanism, at the concentration range used for the tested compounds, is in agreement with the observed poor inhibition of the isolated enzyme.

Figure 1. Synthesis of 5′–N or 5′–O–diethyl phophonoacetyl derivatives of adenosine and uridine and of corresponding sodium salts
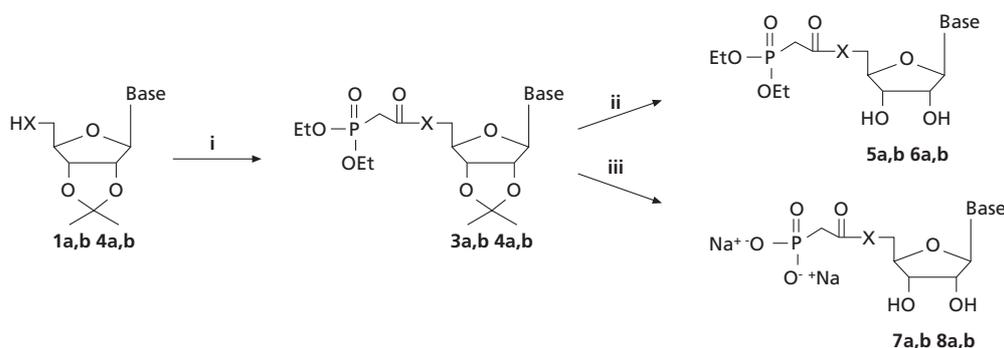
1,3,5,7: Base=adenine; 2,4,6,8: base=uracil
a: X=O; b: X=NH
i: DCCI; PPA-diethyl ester, DMF; ii: TFA 50%; iii: TMSBr, CH2Cl$_2$, Dowex 50W × 2 –100, Na$^+$ form.

Figure 2. Synthesis of 5′–N– or 5′–O-diethyl phophonoacetyl derivatives of cytidine
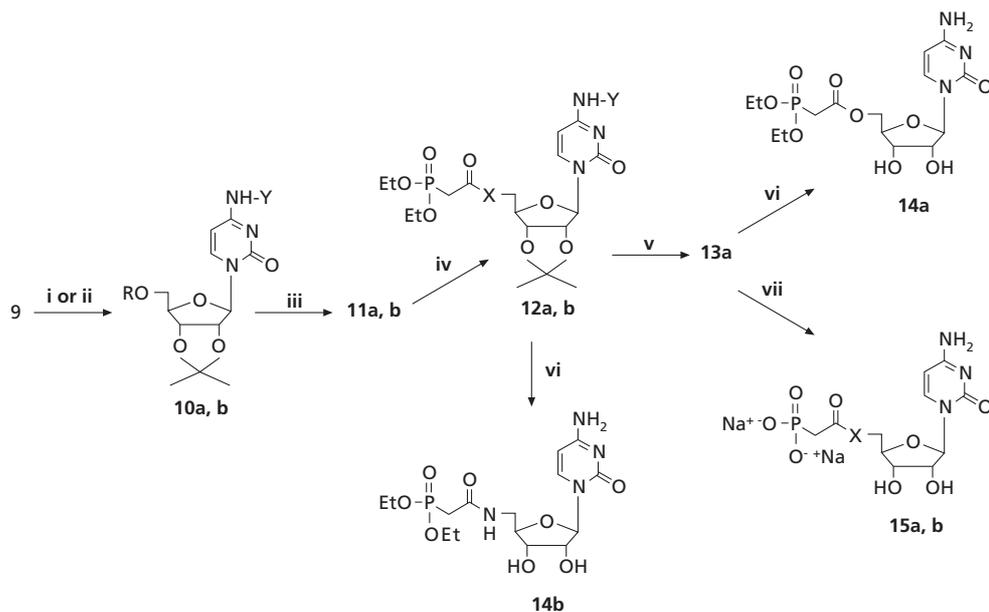
9,10: R=TBDMS; **11**: R=H; **a**: Y=BnOCO; **b**: Y=BOC; **12a**: X=O, Y= BnOCO; **13a**: X=O, Y=H; **15a**: X=O; **15b**: X=NH
i: Benzyl-chloroformate, pyridine; ii: di-tBut-dicarbonate, THF, dioxane; iii: TEAX3HF; iv: DCCI, PPA-diethyl ester, DMF; v: cyclohexene, Pd/C 10%, H2; vi: TFA 50%; vii: TMSBr, CH2Cl₂, Dowex 50W×2–100 Na⁺ form 100

Molecular modelling

The enzyme was studied with different isosteric nucleotide analogues (heterocycles, phosphonic acid, esters and amides) of the four natural substrates (ADP, GDP, CDP and UDP). These were docked into the binding site of the R1 subunit by modifying the 5′-position of the GDP contained in the crystallographic structure, by substitution of the diphosphate group with the appropriate bioisosteric residue. Figures **3a** and **b** report, as a graphic example, the comparison between the structures of the **5a**/R1 and the crystallographic GDP/R1 complexes, respectively. The resulting structures of the enzyme complexed with the considered nucleotide analogues, even though not superimposed to the corresponding reference X-ray structure of the GDP/R1 assembly, presented interesting data on the energy's point of view. In fact, the relevant calculations indicated high stability of the substrate-enzyme complexes in the case of the amide and ester derivatives; in particular, a detailed analysis of these structures revealed an increased number of hydrogen bonds as compared to the natural substrate suggesting a potential inhibition activity. In the case of the heterocyclic isosters, the steric hindrance limited the docking of the analogues into the active site. Therefore, the phosphonic acid ester and amide bioisosters were selected for the synthetic studies.

Discussion

Nucleoside diphosphates represent the only substrate forms recognized by RNR. Notwithstanding that, the potential therapeutic use of nucleotide analogues, as widely demonstrated (Plunkett *et al.*, 1996), is prevented by their instability in biological fluids and, when administered in a non-phosphorylated form, strongly conditioned by metabolic activation by specific kinases (Balzarini, 1993; Meier, 1998). Our previous studies (Manfredini *et al.*, 1999) pointed out that the principal interactions between the

Table 2. Cytostatic and anti-HIV-1 activity of test compounds in cell culture

Comp.	CC₅₀ (μM)				EC₅₀ (μM)
	L1210	FM3A	Molt4/C8	CEM	HIV-1(IIIB)
8a	>500	>500	> 500	>500	250
8b	>100	>100	>100	>100	
5a	>100	>100	>100	>100	
5b	>100	>100	>100	>100	
6a	>100	>100	>100	>100	
6b	>100	>100	>100	>100	
7a	480	>500	>500	>500	>250
7b	413	>500	>500	>500	110
14a	>500	>500	>500	>500	>250
14b	>500	>500	>500	>500	>250
15a	>500	>500	>500	>500	>250
15b	>500	>500	>500	>500	>250
PMEA[a]				69	7.0

CC₅₀, 50% cytostatic concentration, or compound concentration, required to inhibit tumour cell proliferation by 50%; EC₅₀, 50% effective concentration, or compound concentration, required to inhibit virus-induced syncytium formation by 50%.

Table 3. Antiviral activity of test compounds in cell culture

Comp.	EC$_{50}$ (µM)											
	E$_6$SM cell cultures				HEL cell cultures		HeLa cells	Vero cell cultures				
HSV-1	HSV-2	VV	VSV	CMV[a]	VZV[a]	RSV	CS B4	PI 3	Sindbis	PTV	RV-1	
8a	>40	>40	120	>40	>50	>50	>200	>40	>40	>40	>40	>40
8b	>80	>80	>80	>80	>200	>200	>400	>80	>80	>80	>80	>80
5a	240	>400	>400	>400	>200	>200	>400	>80	>80	>80	>80	>80
5b	240	48	240	240	>200	>200	>16	>80	>80	>80	>80	>80
6a	>400	>400	>400	>400	>200	>200	>400	>80	>80	>80	>80	>80
6b	240	240	>400	>400	>200	>200	>400	>80	>80	>80	>80	>80
7a					>200	>200						
7b					>200	>200						
14a	>80	>80	240	>80	>50	>200	>80	>80	>80	>80	>80	>80
14b					>200	>200	>80	>80	>80	>80	>80	>80
15a	>80	>80	240	>80	>50	>200						
15b					>200	>200						
BVDV	0.07	>80	3.2	>80	>80	0.007	>80	>80	>80	>80	>80	>80
GCV	0.010	0.019	>100	>100	2.0	1.0	>80	>80	>80	>80	>80	>80

[a]Data are for CMV strain AD-169 and Davis, and for VZV strains YS, OKA, 07/1 (TK-deficient) and YS/R (TK-deficient).
CMV, cytomegalovirus; CS B4, coxsackie B4; EC$_{50}$ 50% effective concentration, or compound concentration required to inhibit virus-induced cytopathicity by 50%; HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type and type 2; PI 3, para-influenza 3; PTV, punta toro virus; RV-1, reovirus-1; RSV, respiratory syncytial virus; VSV; vesicular stomatitis virus; VV, vaccinia virus; VZV, varicella-zoster virus

Figure 3. The active-binding site cleft of the RNR R1 subunit with the docked nucleotide analogue **5a** (A) and with the natural substrate molecule GDP (B)*

A

B
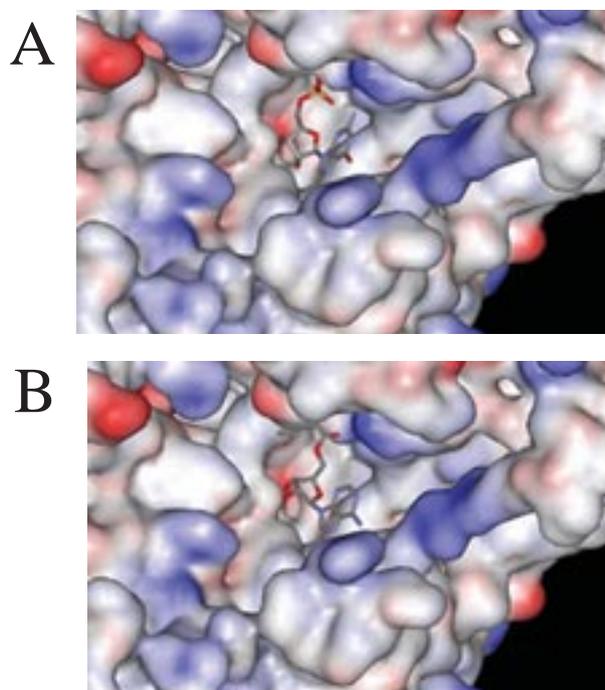
*The docked molecules are shown in stick representation.

active site and the natural and nucleoside analogue diphosphates concern the diphosphate group (Eriksson *et al.* 1997).

Taking all this into account, our investigation started with a molecular modelling study on the RNR R1 subunit, which aimed to explore the possibility of substituting the diphosphate group of natural substrates by bioisosteric modifications. This approach is well-known from the literature: the pyrophosphate (PP) analogue foscarnet (PFA) that inhibits viral DNA polymerase activity, interferes with cytomegalovirus, herpes virus and HIV replication (Kudlacek *et al.*, 2001). In this work we considered heterocycles, PAA esters and amides. As described in the modelling section, calculations indicated the PAA amide and ester as the best candidates for bioisosteric replacement of the diphosphate. Thus, PAA-amides and esters of cytidine, uridine and adenosine were prepared and investigated for their inhibitory activity on RNR and for their cytostatic and antiviral activities. Among these compounds, only the UDP analogue **8b** (amide), ADP analogue **5a** (ester) and CDP analogue **14a** (ester) were endowed with a moderate inhibitory activity against the isolated enzyme (IC$_{50}$ 350, 500 and 600 µM, respectively). None of the compounds showed any cytostatic activity at 100–500 µM, whereas compound **5b** (ADP analogue, amide) showed a moderate activity (EC$_{50}$ 48 µM) against HSV-2 and compound **7b**

(ADP analogue, amide) a modest but interesting activity (EC$_{50}$ 110 µM) against HIV-1. As discussed above a significant degree of interaction with the enzyme's active site, comprising two orders of DG values magnitude, was predicted by the modelling study for the designed molecules (Table 1). Thus, although modelling studies predicted improvements in the binding capabilities by replacement of the diphosphate moiety for a corresponding PAA amide and ester bioisosters, only a fair agreement exists for the most potent inhibitors **8b, 5a** and **14a,** and all the other compounds were inactive. However, some final conclusions can be drawn. It is worth noting that the compounds **5a** and **14a**, notwithstanding the lack of acid phosphate moiety characteristic of natural substrates, were also endowed with a inhibitory activity on RNR, although modest. Moreover, the only compounds provided with a limited activity (antiviral) in tumour cell lines, **5b** and **7b**, both PAA-amides and ADP analogues, were found not inhibitory against the isolated enzyme. Thus, it may be assumed that the observed activity should be ascribed to a mechanism different than RNR inhibition or to interaction with RNRs different than the human one (Boehmer *et al.*, 1997; Heineman *et al.*, 1994). Finally, as both **5b** and **7b** are PAA-amides, the reversible nature of the ester linkage may also play a role in determining the inactivity of the corresponding PAA-ester ADP analogues in cell cultures, possibly due to degradation by cellular enzymes.

Acknowledgements

References

Ballatore C, McGuigan C, De Clercq E & Balzarini J (2001) Synthesis and evaluation of novel amidate prodrugs of PMEA and PMPA. *Bioorganic and Medicinal Chemistry Letters* **11**:1053–1056.

Balzarini J (1993) Metabolism and mechanism of antiretroviral action of purine and pyrimidine derivatives. *Pharmacy World Science* **16**:113–126.

Bazzanini R, Manfredini S, Durini E, Groschel B, Cinatl J, Balzarini J, De Clercq E, Imbach JL, Perigaud C & Gosselin G (1999) Synthesis, cytostatic activity and inhibition of ribonucleotide reductase by 5′-phosphoramidates and 5′- diphosphate, of 2′-O-allylarabinofuranosyl nucleosides. *Nucleosides Nucleotides* **18(4–5)**:971–972.

Boehmer PE, Lehman IR (1997) Herpes simplex virus DNA replication. *Annual Review of Biochemistry* **66**:347–84.

Case DA, Pearlman DA, Caldwell JW, Cheatham III TE, Ross WS, Simmerling CL, Darden TA, Merz KM, Stanton RV, Cheng AL, Vincent JJ, Crowley M, Tsui V, Radmer RJ. Duan Y, Pitera J, Massova I, Seibel GL, Singh UC, Weiner PK, Kollman PA (1999) *AMBER 6*. University of California: San Francisco, USA.

Charvet AS, Camplo M, Faury P, Graciet JC, Mourier N, Chermann JC & Kraus JL (1994) Inhibition of human immunodeficiency virus type 1 replication by phosphonoformate- and phosphonoacetate-2′,3′-dideoxy-3′-thiacytidine conjugates. *Journal of Medicinal Chemistry* **37**:2216–2223.

Charvet, AS, Turin F, Faury P, Hantz O; Camplo M, Mourier N, Berthillon P, Graciet JC. Chermann JC, Trepo C & Kraus J-L (1994) Synthesis and antiviral activity of new carbonylphosphonate 2′,3′-dideoxy-3′-thiacytidine conjugates. *Antiviral Research* **25**:161–168.

Cornell WD, Cieplak P, Bayly CI, Gould IR, Merz KM Jr., Ferguson DM, Spellmeyer DC, Fox T, Caldwell JW & Kollman PA (1995) A second generation force field for the simulation of proteins, nucleic acids, and organic molecules. *Journal of American Chemical Society* **117**:5179–5197.

Engström Y, Eriksson S, Thelander L & Åkerman M (1979) Ribonucleotide reductase from calf thymus. Purification and properties. *Biochemistry* **14**:2941–2948.

Eriksson M, Uhlin U, Ramaswamy S, Ekberg M, Regnström K, Sjöberg BM & Eklund H (1997) Binding of allosteric effectors to ribonucleotide reductase protein R1: reduction of active-site cysteines promotes substrate binding. *Structure* **5**:1077–1092.

Griffey RH & Poulter CD (1983) Detection of the imino hydrogen bond in G-C pairs by H and N nuclear magnetic resonance spectroscopy. *Tetrahedron Letters* **24**:4067–4070.

Hampton A (1961) A new procedure for the conversion of ribonucleosides to 2′,3′-O-isopropilidene derivates. *Journal of the American Chemical Society* **83**:3640–3645.

Heineman TC, Cohen JI (1994) Deletion of the varicella-zoster virus large subunit of ribonucleotide reductase impairs growth of virus *in vitro*. *Journal of Virology* **68**:3317–3323.

Jayaram B, Sprous D, & Beveridge DL (1998) Solvation free energy of biomacromolecules: parameters for a modified generalized Born model consistent with the AMBER force field. *Journal of Physical Chemistry B* **102**:9571–9576.

Johansson NG & Eriksson S (1996) Structure-activity relationships for phosphorylation of nucleoside analogs to monophosphates by nucleoside kinases. Review. *Acta Biochimica Polonica* **43**: 143–160.

Kudlacek O, Mitterauer T, Nanoff C, Hohenegger M, Tang WJ, Freissmuth M & Kleuss C (2001) Inhibition of adenylyl and guanylyl cyclase isoforms by the antiviral drug foscarnet. *Journal of Biological Chemistry* **276**:3010–3016.

Kukhanova M, Krayevsky A, Prrusoff W & Cheng YC (2000) Design of anti-HIV compounds: from nucleoside to nucleoside 5′-triphosphate analogs. Problems and perspectives. Review. *Current Pharmaceutical Design* **6**: 585–598.

Lambert RW, Martin JA, Thomas GJ, Duncan IB, Hall MJ & Heimer EP (1989) Synthesis and antiviral activity of phosphonoacetic and phosphonoformic acid esters of 5-bromo-2′-deoxyuri-

dine and related pyrimidine nucleosides and acyclonucleosides. *Journal of Medicinal Chemistry* **32**:367–374.

Macchia M, Martinelli A, Parkin A & Rossello A (1994) Bioisosters of the diphosphate group in activated forms of antiherpes virus agents. A theoretical study. *Farmaco* **49**: 325–332.

MacCoss M, Ryu E K, White S R & Last L R (1980) A new synthetic use of nuleoside N1-oxides. *Journal of Organic Chemistry* **45**:788-794.

Manfredini S, Baraldi PG, Bazzanini R, Simoni D, Vertuani S, Pani A, Scintu F, Pinna E, Lichino D & La Colla P (1996) Pyrazole related nucleosides. Design, synthesis and antiproliferative activity of methyl-4-iodo-1-β-D-ribofuranosyl-pyrazole-3-carboxylate (IPCAR) and derivatives against human leukemias, lymphomas and solid tumors cell *in vitro. Medicinal Chemistry Research* **6**:93–311.

Manfredini S, Bazzanini R, Marangoni M, Baraldi PG, Simoni D, Vertuani S, Pani A, Scintu F, Pinna E, Lichino D & La Colla P (1996) Design, synthesis and antiproliferative activity of methyl-4-iodo-1-β-D-ribofuranosyl-pyrazole-3-carboxylate and related compounds. *Bioorganic & Medicinal Chemistry Letters* **11**:1279–1284.

Manfredini S, Baraldi PG, Durini E, Balzarini J, De Clercq E, Karlsson A, Buzzoni V & Thelander L (1999) Synthesis, cytostatic activity and inhibition of ribonucleotide reductase by 5′-phosphoramidates and 5′- diphosphate, of 2′-O-allylarabinofuranosyl nucleosides. *Nucleosides Nucleotides* **18(4–5)**:1007–1008.

Manfredini S, Baraldi PG, Durini E, Vertuani S, Balzarini J, De Clercq E, Karlsson A, Buzzoni V & Thelander L (1999) 5′-Phosphoramidates and 5′-Diphosphates of 2′-O-Allyl-D-arabino-furanosyl- uracil, -cytosine, and -adenine: inhibition of ribonucleotide reductase. *Journal of Medicinal Chemistry* **42**:3243–3250

Manfredini S, Lampronti I, Vertuani S, Solaroli N, Recanatini M, Bryan D & McKinney M (2000) Design, synthesis and binding at

cloned muscarinic receptors of N-[5-(1′-substituted-ace-toxymethyl)-3-oxadiazolyl] and N-[4-(1′-substituted-ace-toxymethyl)-2-dioxolanyl] dialkyl amines. *Bioorganic and Medicinal Chemistry* **8**:1559–1566.

Mann GJ, Gräslund A, Ochiai EI, Ingemarson R & Thelander L (1991) Purification and characterization of recombinant mouse and Herpes Simplex Virus ribonucleotide reductase R2 subunit. *Biochemistry* **30**:1939–1947.

Meier C. (1998) Pro-nucleotides. Recent advances in the design of efficient tools for the delivery of biologically active nucleoside monophosphates. *Synthesis Letters* 233–242.

Pearlman DA, Case DA, Caldwell JW, Ross WS, Cheatham III TE, DeBolt S, Ferguson D, Seibel GL & Kollman PA (1995) AMBER, a package of computer programs for applying molecular mechanics, normal mode analysis, molecular dynamics and free energy calculations to simulate the structural and energetic properties of molecules. *Computer Physics Communications* **91**:1–41.

Plunkett W, Huang P, Searcy CE & Gandhi V (1996) Gemcitabine: preclinical pharmacology and mechanisms of action. Review. *Seminars in Oncology* **5**: 3–15.

Thelander M, Mann GJ, Behravan G, Soucy F, Beaulieu P, Lavallée P, Gräslund A & Thelander L (1994) Purification, characterization and localization of subunit interaction area of recombinant mouse ribonucleotide reductase R1 subunit. *Journal of Biological Chemistry* **269**:23171–23176.

Wagner CR, Iyer VV & Mc Intez EJ (2000) Pronucleotides: toward the *in vivo* delivery of antiviral and anti-cancer nucleotides. *Medicinal Research Reviews* **20**: 417–451.

Weiser J, Shenkin PS, Still WC (1999) Approximate atomic surfaces from linear combinations of pairwise overalps (LCPO). *Journal of Comparative Chemistry* **20**:217–230.