5′-Phosphoramidates and 5′-Diphosphates of 2′-*O*-Allyl-β-D-arabinofuranosyl-uracil, -cytosine, and -adenine: Inhibition of Ribonucleotide Reductase

Stefano Manfredini,*[,†] Pier Giovanni Baraldi,[†] Elisa Durini,[†] Silvia Vertuani,[†] Jan Balzarini,[‡] Erik De Clercq,[‡] Anna Karlsson,[§] Valentina Buzzoni,[†,⊥] and Lars Thelander[⊥]

Department of Pharmaceutical Sciences, Ferrara University, Italy, Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, Belgium, Karolinska Institute, Huddinge University Hospital, Stockholm, Sweden, and Department of Medical Biochemistry and Biophysics, Umeå University, Sweden

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Continuing our studies on ribonucleotide reductase (RNR) mechanism-based inhibitors, we have now prepared the diphosphates (DP) of 2′-*O*-allyl-1-β-D-arabinofuranosyl-uracil and -cytosine and 2′-*O*-allyl-9-β-D-arabinofuranosyl-adenine and evaluated their inhibitory activity against recombinant murine RNR. 2′-*O*-Allyl-araUDP proved to be inhibitory to RNR at an IC_{50} of 100 μM, whereas 2′-*O*-allyl-araCDP was only marginally active (IC_{50} 1 mM) and 2′-*O*-allyl-araADP was completely inactive. The susceptibility of the parent nucleosides to phosphorylation by thymidine kinase and 2′-deoxycytidine kinase was also investigated, and all nucleosides proved to be poor substrates for the above-cited kinases. Moreover, prodrugs of 2′-*O*-allyl-araU and -araC monophosphates, namely 2′-*O*-allyl-5′-(phenylethoxy-L-alanyl phosphate)-araU and -araC, were prepared and tested against tumor cell proliferation but proved to be inactive. A molecular modeling study has been conducted in order to explain our results. The data confirm that for both the natural and analogue nucleoside diphosphates, the principal determinant interaction with the active site of RNR is with the diphosphate group, which forms strong hydrogen bonds with Glu623, Thr624, Ser625, and Thr209. Our findings indicate that the poor phosphorylation may represent an explanation for the lack of marked in vitro cytostatic activity of the test compounds.

Introduction

Ribonucleotide reductase (RNR) is an essential enzyme in DNA synthesis: it is responsible for the de novo synthesis of all deoxyribonucleoside diphosphates (dNDP) in prokaryotic (*E. coli*) and eukaryotic (mammalian) cells.[1] The enzyme comprises two homodimers termed R1 and R2: the site of reduction is contained in the R1 subunit (i.e. Cys462 and Cys225 in *E. coli*), whereas the radical is on the R2 subunit (i.e. diferric-tyrosyl cofactor, Tyr122, in *E. coli*) maintained by a cofactor. The enzymatic activity requires subunit association and transfer of the free radical from R2 onto R1 subunit. Because RNR is the rate-limiting enzyme in dNDP synthesis in eukaryotic cells and because RNR gene overexpression has been associated with malignant transformation and metastatic potential,[2] RNR has been considered as an attractive target for antitumor/antiviral chemotherapy, and a number of potent inhibitors have been discovered and proposed as potential antitumor/antiviral drugs. As a result of these studies three main classes of inhibitors have emerged: (a) compounds that scavenge the tyrosyl radical on the R2 subunit and/or inhibit its transfer to the R1 subunit (i.e. hydroxyurea, HU); (b) compounds that prevent R1 and R2 association (i.e. HSV R2−C-terminal amino acid peptidomimetics); (c) mechanism-based inhibitors, mainly modified nucleosides that must enter the active site at the R1 subunit to block the enzyme (i.e. gemcitabine, 2′,2′-difluoro-2′-deoxycytidine, dFdC).[3] HU has been used for a long time as a cancer chemotherapeutic agent, and dFdC has been recently approved by the FDA for the treatment of pancreatic cancer. We have recently reported the results of our studies directed to the design of new nucleoside analogues potentially active as RNR mechanism-based inhibitors.[4,5]

Continuing our efforts, we have now prepared the diphosphates of 2′-*O*-allyl-1-β-D-arabinofuranosyl derivatives[5] and tested them for their inhibitory activity against the purified recombinant murine RNR. The susceptibility of the parent nucleosides to phosphorylation by thymidine and 2′-deoxycytidine kinases has been investigated, and a molecular modeling study has been conducted. The pronucleotide approach has also been evaluated by preparing the prodrug of 2′-*O*-allyl-araU and -araC monophosphate, namely 2′-*O*-allyl-5′-(phenylethoxy-L-alanyl phosphate)-araU and -araC. In this paper, we also describe the experimental procedures for the synthesis of the parent 2′-*O*-allyl-araU, -araC, and -araA compounds.

Chemistry

Although the 2′-*O*-allylation of ribonucleosides has been reported without protecting the 3′- and 5′-hydroxy groups, in the case of our arabinofuranosides we proceeded with protected precursors, the reason being that a mixture of 2′- and 3′-*O*-allyl derivatives is usually obtained with unprotected nucleosides.[6,7] Thus, 2,2′-anhydro-1-β-D-arabinofuranosyl-uracil (**1**)[8] was chosen as a common starting material for the preparation of **4** and **7**. This intermediate is particularly useful for our

† Ferrara University.
‡ Katholieke Universiteit Leuven.
§ Karolinska Institute.
⊥ Umeå University.

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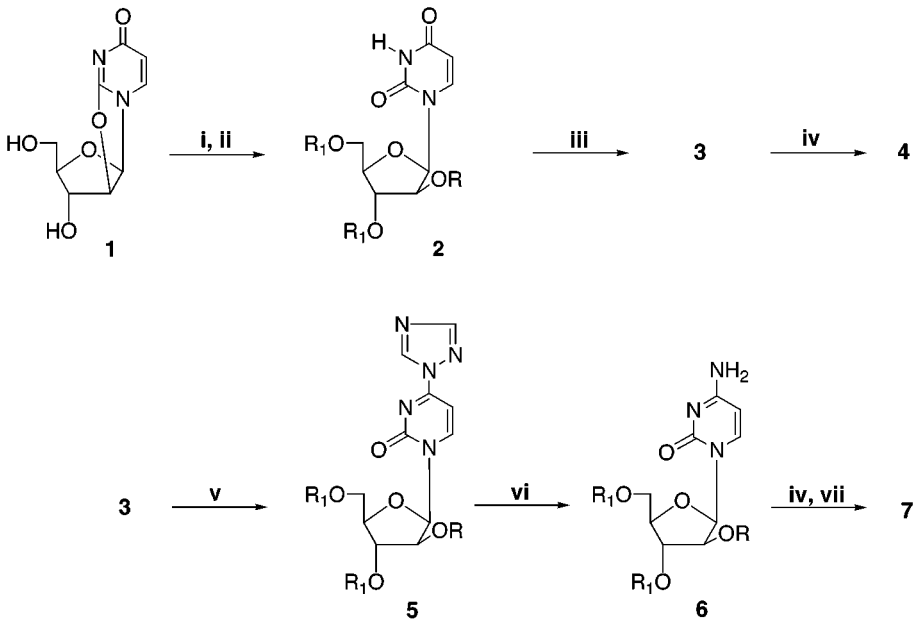
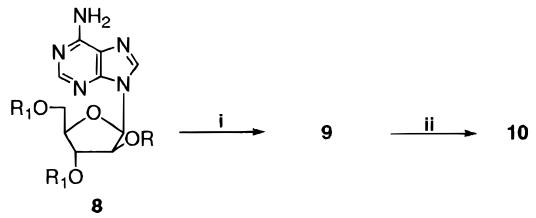
Scheme 1[a]



2: R = H, R_1 = THP; **3:** R = allyl, R_1 = THP; **4:** R = allyl, R_1 = H; **5:** R = allyl, R_1 = THP; **6:** R = allyl, R_1 = THP; **7:** R = allyl, R_1 = H

[a] (i) DHP, TsOH·H_2O, CH_3CN, (ii) KOH, EtOH; (iii) allyl bromide, NaH (60%), THF; (iv) TsOH·H_2O, MeOH; (v) 1,2,4-triazole, $POCl_3$, Et_3N, CH_3CN; (vi) 30% NH_4OH/H_2O, dioxane; (vii) Dowex 1X2 resin (OH^- form), H_2O.

Scheme 2[a]



8: R = H, R_1 = TBDMS; **9:** R = allyl, R_1 = TBDMS; **10:** R = allyl, R_1 = H

[a] (i) Allyl bromide, NaH (50%), THF; (ii) NH_4F/MeOH.

purpose because it can be easily protected in a regiospecific manner at the 5′- and 3′-hydroxy groups and it can also be converted to the corresponding cytosine derivative by known procedures.[9] Briefly, 2,2′-anhydro-1-β-D-arabinofuranosyl-uracil (**1**) was protected as the 3′,5′-*O*-THP derivative, and the crude residue was treated with KOH in ethanol to give 3′,5′-*O*-THP-araU (**2**) (Scheme 1) which was finally allylated at the 2′-position with allyl bromide and sodium hydride, as the base, in THF to give compound **3** (58% overall yield, as compared to **1**). This latter was deprotected to give the expected 2′-*O*-allyl-araU (**4**) by treatment with toluenesulfonic acid (TsOH) in 63% yield or converted into the corresponding 3′,5′-*O*-THP-2′-*O*-allyl-araC (**6**) in two steps. Next treatment with TsOH gave the deprotected **7** in 27% yield.

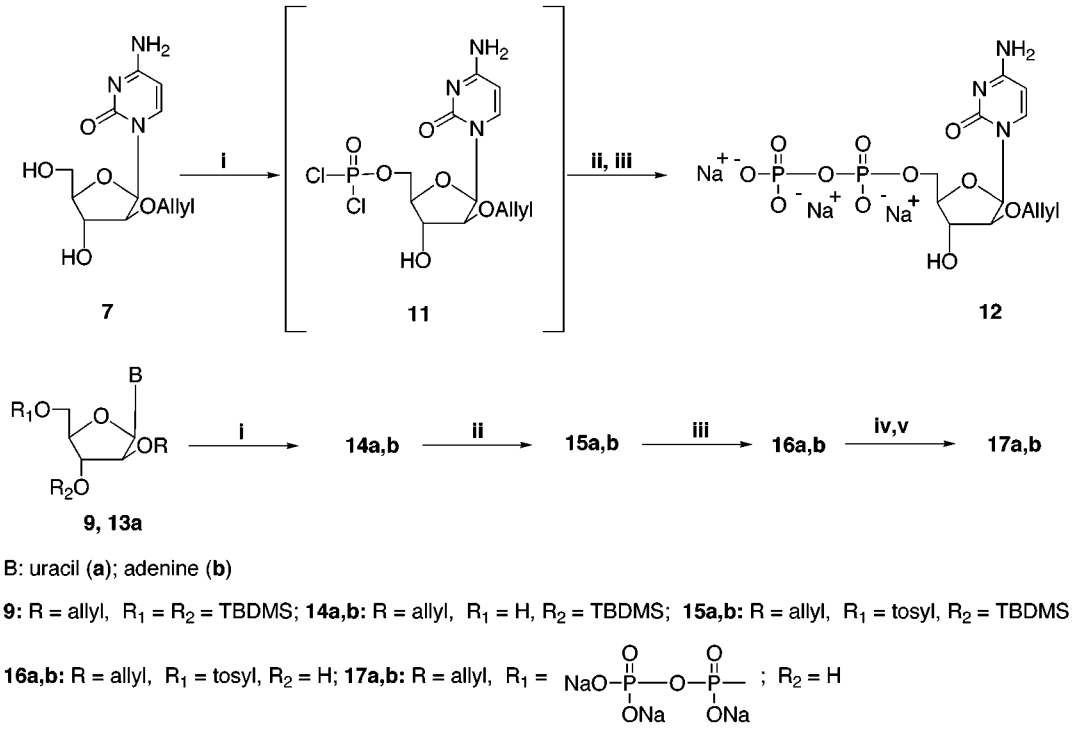
In the case of araA, better results were achieved if the 3′- and 5′-hydroxyl moieties were protected with *tert*-butyldimethylsilyl groups (TBDMS). Allylation of **8**[10] with allyl bromide and sodium hydride gave the expected **9** in 32% yield, with concomitant formation of mixtures of byproducts alkylated at the N^6- and/or 2′-*O*-positions (Scheme 2). The TBDMS groups were then removed by treatment with ammonium fluoride/methanol (NH_4F/MeOH) to give the final **10** in 82% yield.[11]

Synthesis of Nucleoside Diphosphates. Method A: Taking advantage of the procedure described by Kovács and Ötvös[12] for the synthesis of dNTPs, the starting nucleoside **7** (Scheme 3) was treated with $POCl_3$/trimethyl phosphate solution to give the monophosphate dichloride **11**, which was in turn reacted with tri-*n*-butylammonium orthophosphate (0.5 M solution) to give, after purification and displacement of the butylammonium ion, 2′-*O*-allyl-1-β-D-arabinofuranosyl-cytosine 5′-diphosphate (**12**) as the sodium salt in 13% yield.

Method B: We adapted the procedure described by Poulter et al.;[13] 2′-*O*-allyl-5′-*O*-tosyl-1-β-D-arabinofuranosyl-uracil (**16a**) and -adenine (**16b**) were reacted with the tris(tetra-*n*-butylammonium)hydrogen pyrophosphate to displace the tosyl group and to obtain, after purification and displacement of the butylammonium ion, the expected diphosphates **17a** and **17b** (19% and 38% yield, respectively). Both 5′-tosyl nucleosides **16a** and **16b** were prepared by conventional procedures from the corresponding starting nucleosides in four steps. Thus, 2′-*O*-allyl-3′,5′-*O*-TBDMS-araU (**13a**), obtained from compound **4** by reaction with *tert*-butyldimethylsilyl chloride, and 2′-*O*-allyl-3′,5′-*O*-TBDMS-araA (**9**) were deprotected at the 5′-position by treatment with 80% acetic acid to give the corresponding 2′-*O*-allyl-3′-*O*-TBDMS nucleosides **14a** and **14b** in 54% and 67% yield, respectively. Next, reaction of these intermediates with tosyl chloride in the presence of 4-dimethylaminopyridine (DMAP) gave the 2′-*O*-allyl-3′-*O*-TBDMS-5′-*O*-tosyl derivatives **15a** and **15b** in 84% and 80% yield, respectively. Removal of the silyl protecting group with 2% HF gave 2′-*O*-allyl-5′-*O*-tosyl-araU (**16a**) and 2′-*O*-allyl-5′-*O*-tosyl-araA (**16b**) in 89% and 59% yield, respectively.

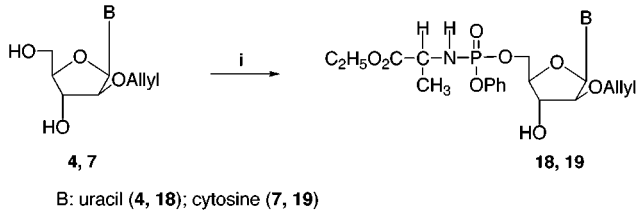
Synthesis of Nucleoside Phosphoramidates. Pronucleotides of 2′-*O*-allyl-1-β-D-arabinofuranosyl-uracil and -cytosine, namely compounds **18** and **19**, were

Scheme 3[a]



[a] For **7**, **11**, **12**: (i) $(Me_3O)_3PO$, Proton Sponge, $POCl_3$, 0 °C; (ii) *n*-$Bu_3NH^+H_2PO_4^-$, *n*-Bu_3N, TEAB (0.2 M); (iii) Dowex AG 50 W-X2 (Na^+ form). For **9**, **13a**, **14a**,**b**−**17a**,**b**: (i) AcOH (80%), 60 °C; (ii) tosyl chloride, 4-DMAP, 0 °C; (iii) 2% HF/H_2O, CH_3CN; (iv) $[(n\text{-But})_4N]_3HP_2O_7$, CH_3CN; (v) Dowex AG 50W-X2 (Na^+ form).

Scheme 4[a]



[a] (i) Phenylethoxy-L-alanyl phosphorochloridate, 1-methylimidazole, THF.

prepared by reaction of nucleosides **4** and **7** with phenylethoxy-L-alanyl phosphorochloridate, adapting a procedure described by McGuigan et al.[14] (Scheme 4). The compounds were obtained in 20% and 18% yield, respectively. All the compounds were characterized by ^1H and ^{31}P NMR, mass spectroscopy, and HPLC.

Modeling Parameters

The enzyme was studied with the three natural substrates (CDP, ADP, and UDP) docked in the binding site of the R1 subunit, by modifying the structure of the natural substrate GDP contained in the crystallographic structure of the enzyme.[15] After placement of the substrate in the binding site, the substrate, along with the amino acid residues of the binding site in the range of 8 Å from the substrate itself, was optimized for 500 steps of steepest descent (SD) and 2000 steps of polar Ribiere conjugate gradient (PRCG). A distance-dependent dielectric constant was used in conjunction with a 12 Å cutoff in all calculations. The substrate was then subjected to 25 ps of molecular dynamics (MD) at 300 K and reoptimized using the conjugate gradient algorithm until the gradient norm fell below 0.01 kcal/mol/Å. The resulting conformation was further optimized for 1500 steps of SD and 2000 steps of PRCG, and the structure of the appropriate substrate was modified at the 2′-position, on the glycosylic moiety, by substitution of the β-hydrogen with the β-*O*-allyl group and of the α-hydroxy group with an α-hydrogen. The structure was finally optimized until convergence. These calculations indicated high stability of the substrate−enzyme complexes and close similarity between the structures of the enzyme complexed with the studied synthetic molecules and the X-ray structure. This confirms that the calculations utilized for the structural analysis are able to reproduce the real situation very closely. For the operations above-described, MacroModel (5.5) software and the Amber force field were used.

Biology

RNR Inhibition. Murine recombinant RNR proteins R1 and R2 were obtained and purified as previously reported.[16,17] RNR activity of recombinant murine R1 and R2 protein was measured using the [^{3}H]CDP reaction assay as described earlier.[18] ADP reduction was determined after separation of the product from the substrate by boronate affinity chromatography as described by Shewach.[19] The degree of inhibition of RNR is shown in Figure 1 with the best IC_{50} (100 μM) for 2′-*O*-allyl-araUDP. No clear evidence for a time-dependent inhibition was obtained, and therefore, the simplest explanation is that the inhibition is competitive.

Phosphorylation of Nucleosides by Kinases. The susceptibility of the parent nucleosides to phosphorylation by cellular and viral-encoded kinases has been investigated against purified cytosolic thymidine kinase (TK-1) derived from human T-lymphocyte CEM cells and cytosolic deoxycytidine kinase (dCyd) and mito-

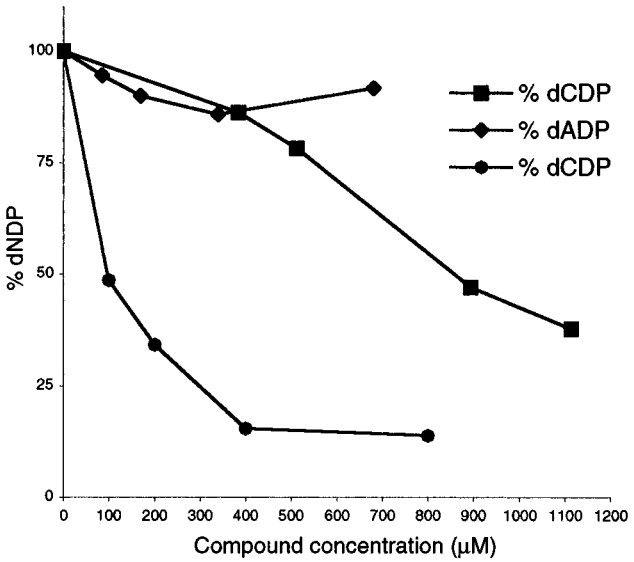


Figure 1. Inhibition of RNR by 2′-*O*-allyl-araCDP (■), 2′-*O*-allyl-araADP (◆), or 2′-*O*-allyl-araUDP (●). In the experiments with araCDP- and araUDP-*O*-allyl derivatives the concentration of [^{3}H]CDP was 0.5 mM and dGTP was used as a positive effector. With the araADP-*O*-allyl derivative the concentration of [^{14}C]ADP was 0.02 mM formed after 30-min incubation at 37 °C, where 100% is the activity in the absence of drug.

Table 1. Cytostatic Activity of Compounds **4**, **7**, **10**, **18**, and **19**

	IC$_{50}$ (μM)[a]			
compd	L1210	Molt4/C8	CEM	FM3A
4	>500	>500	>500	>500
7	38 ± 8.4	>500	61 ± 6.0	325 ± 32
10	181 ± 20	285 ± 30	79 ± 1.7	316 ± 28
18	>200	>200	>200	>200
19	>200	>200	>200	>200

[a] 50% inhibitory concentration, or compound concentration required to inhibit tumor cell proliferation by 50%.

chondrial TK-2 thymidine kinase, both derived from human liver source. None of the compounds served as substrates for the three nucleoside kinases (IC$_{50}$ > 1000 μM).

Cytostatic Activity. The cytostatic activity of the nucleosides was evaluated against murine leukemia L1210 and mammary FM3A cells and human T4-lymphocyte Molt and CEM cells. The uracil derivative **4** was not cytostatic at 500 μM, the cytosine derivative **7** was cytostatic between 37 and 325 μM, and the adenine derivative **10** was cytostatic between 80 and 316 μM (Table 1). The pronucleotides 2′-*O*-allyl-5′-(phenylethoxy-L-alanyl phosphate)-1-β-D-arabinofuranosyl-uracil (**18**) and -cytosine (**19**) were virtually inactive (IC$_{50}$ > 200 μM).

Discussion

We started the present study in order to evaluate whether the activity observed in preliminary in vitro evaluation conducted on some 2′-*O*-allyl-β-D-arabinofuranosyl derivatives,[5] previously designed by us as potential RNR mechanism-based inhibitors, was directly correlated to an interaction with the enzyme. The 2′-*O*-allyl-araC, -araU, and -araA diphosphates were prepared and tested for their inhibitory effect on recombinant murine RNR, R1 and R2 subunits. Under the conditions tested, 2′-*O*-allyl-araUDP was endowed with an IC$_{50}$ of 100 μM, whereas 2′-*O*-allyl-araCDP was only marginally active (IC$_{50}$ ~ 1 mM) and 2′-*O*-allyl-araADP was completely inactive. The susceptibility of the parent 2′-*O*-allyl nucleosides to phosphorylation by thymidine and 2′-deoxycytidine kinases was investigated, and they were shown to be very poor substrates for the above-cited kinases. Since for many nucleoside analogues the first phosphorylation step is, in general, the rate-limiting step in the activation of the drug, we have therefore prepared the 2′-*O*-allyl-araU and -araC monophosphate prodrugs, namely 2′-*O*-allyl-5′-(phenylethoxy-alanyl phosphate)-araU (**18**) and -araC (**19**). In particular, 2′-*O*-allyl-araU was chosen because it was inactive in cell culture but the most inhibitory to RNR. However, when the pronucleotides were evaluated for their effects on cell proliferation, they also proved inactive. Although the phosphoramidate pronucleotide approach does not necessarily guarantee a release of the monophosphate, absence of biological activity can also be explained by the lack of further phosphorylation of the drug to its 5′-di(and 5′-tri)phosphate.

Finally, the first crystallographic structure of the R1 subunit complexed to the natural substrate GDP has been recently obtained by Eklund et al.[15] Starting from these data the three synthetic nucleotides 2′-*O*-allyl-araUDP (**17a**), -araCDP (**12**), and -araADP (**17b**) have been placed into the active site and their interactions have been studied through molecular mechanics calculations. Briefly, the order of the observed inhibitory activity (2′-*O*-allyl-araUDP > -araCDP ≫ -araADP) could be explained by the more hindered conformation adopted by 2′-*O*-allyl-araADP into the active site. Another important observation emerging from this study is that for both natural and analogue diphosphates the principal interaction with the active site concerns the diphosphate group, which forms strong hydrogen bonds with Glu623, Thr624, Ser625, and Thr209 (Figure 2). In our opinion, these data are of relevance in the design of new RNR mechanism-based inhibitors.

Experimental Section

Chemistry. Melting points were determined in open capillary tubes and are uncorrected. Reaction courses were routinely monitored by thin-layer chromatography (TLC) on silica gel precoated F254 Merck plates with detection under a 254-nm UV lamp and/or by spraying the plates with 10% H_2SO_4/MeOH and heating and/or by spraying the plates with ammonium molybdate reagent. Nuclear magnetic resonance spectra were determined in DMSO-d_6, $CDCl_3$, CD_3OD, or D_2O solution with a Bruker AC-200 spectrometer, and chemical shifts are presented in ppm from internal tetramethylsilane as a standard; ^{31}P NMR spectra were determined in DMSO-d_6, $CDCl_3$, or D_2O solution with a Bruker AM-200 spectrometer, and chemical shifts are 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. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) spectra were obtained on a Hewlett-Packard HPG2025A mass spectrometer operating in a positive linear mode. HPLC (analytical and preparative) separations were performed on a Waters 600E chromatographic system; reverse-phase Waters C18 columns (150 × 4.6 mm, 150 Å) were used. Column chromatography was performed with Merck 60−200 mesh silica gel. Room temperature varied between 22 and 25 °C; all drying operations were performed over anhydrous magnesium sulfate. Microanalyses, unless stated, were in agreement with calculated values ±0.4%.

3′,5′-Bis-*O*-tetrahydropyranyl-araU (2). 2,2′-Anhydro-araU[8] (**1**) (500 mg, 2.21 mmol) was dissolved in CH_3CN (30

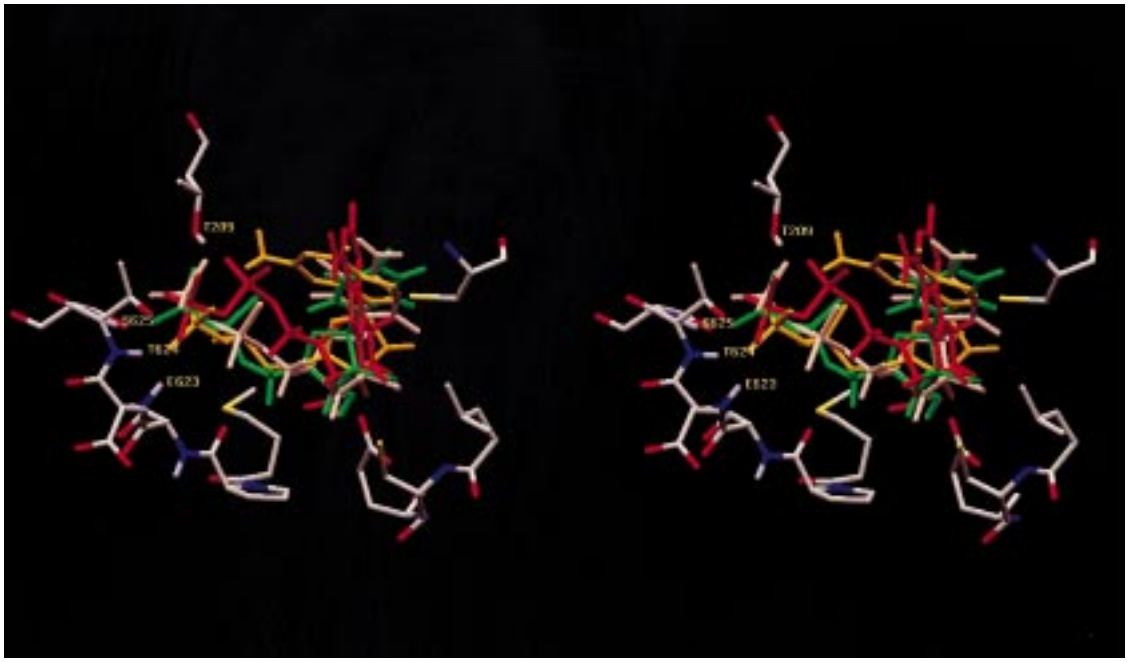


Figure 2. Substrate-binding cleft (stereoview) of the R1 subunit with the natural substrate GDP (orange) and the three synthetic diphosphates 2′-*O*-allyl-araCDP (**12**, pink), 2′-*O*-allyl-araADP (**17a**, red), and 2′-*O*-allyl-araUDP (**17b**, green). The β-phosphate groups strongly bind to the hydrogens of residues Glu623, Thr624, Ser625, and Thr209.

mL), and dihydropyrane (DHP; 2 mL, 221 mmol) and *p*-toluenesulfonic acid monohydrate (TsOH; 42 mg, 0.22 mmol) were added. After 3 h at room temperature, TLC (CH_2Cl_2/MeOH, 9:1) indicated complete reaction, the solvent was evaporated, and the residue was dissolved in CH_2Cl_2 (10 mL) and washed with saturated $NaHCO_3$ (1 × 10 mL). The organic layer was dried and evaporated to give a solid, which was dissolved in 0.1 M KOH/EtOH (15 mL). The mixture was heated at reflux condictions for 4 h (TLC: EtOAc/hexane, 8:2). The solvent was then evaporated, and the resulting residue was purified by silica gel column chromathography (EtOAc/hexane, 7:3) to give 662 mg of **2** as a yellow oil: yield 76%; ^{1}H NMR ($CDCl_3$) δ 8.60−8.46 (m, 1H, NH); 7.88 (m, 1H, H6); 6.27 (m, 1H, H1′); 6.09 (m, 1H, OH2′); 5.67 (m, 1H, H5); 4.70−3.50 (m, 11H, H2′, H3′, H4′, H5′, H5”, CHO- and CH_2O-THP); 1.80−1.55 (m, 12H, CH_2 × 6, THP).

3′,5′-Bis-*O*-tetrahydropyranyl-2′-*O*-allyl-araU (3). Compound **2** (614 mg, 1.49 mmol) was dissolved in THF (10 mL), and 60% NaH (149 mg, 3.72 mmol) was added under vigorous stirring under positive argon pressure; after 10 min, allyl bromide (315 μL, 3.72 mmol) was added and the mixture was stirred at room temperature for 17 h (TLC: EtOAc/hexane, 7:3). The solvent was then evaporated, and the residue was dissolved in CH_2Cl_2 (20 mL), washed with aqueous saturated NH_4Cl solution (1 × 10 mL) and H_2O (1 × 10 mL), dried, and evaporated. The resulting residue was purified by column chromatography (Et_2O/hexane, 8:2) to give 270 mg of **3** as a white foam: yield 40%; ^{1}H NMR ($CDCl_3$) δ 8.65−8.50 (m, 1H, NH); 7.77 (m, 1H, H6); 6.25 (m, 1H, H1′); 5.75−5.65 (m, 2H, H5 and Hb-allyl); 5.25−5.10 (m, 2H, Hc-allyl); 4.80−4.65 (m, 2H, Ha-allyl); 4.25−3.50 (m, 11H, H2′, H3′, H4′, H5′, H5”, CHO- and CH_2O-THP); 1.80−1.55 (m, 12H, CH_2 × 6, THP).

2′-*O*-Allyl-araU (4). Compound **3** (276 mg, 0.6 mmol) was dissolved in MeOH (5 mL), and TsOH monohydrate (221 mg, 1.16 mmol) was added. The mixture was vigorously stirred at room temperature until complete conversion to the deprotected compound (TLC: CH_2Cl_2/MeOH, 9:1). After evaporation, the crude residue was purified by column chromatography (CH_2Cl_2/MeOH, linear gradient from 9.5:0.5−9:1) and preparative HPLC using CH_3CN/H_2O (linear gradient from 1:9 to 6:4). This gave, on freeze-drying of the appropriate fractions, 110 mg of compound **4** as a white solid: yield 63%; mp 168−170 °C ($MeOH/Et_2O$); UV (MeOH) λ_{max} 264 nm (ϵ 9700), λ_{min} 232 nm (ϵ 2100); ^{1}H NMR (DMSO-d_6) δ 11.33 (sbr, 1H, NH); 7.67 (d, J = 7.9 Hz, 1H, H6); 6.13 (d, J = 5.0 Hz, 1H, H1′); 5.75−5.50 (m, 3H, Hb-allyl, H5 and OH); 5.15−5.00 (m, 3H, Hc-allyl and OH); 4.05−3.55 (m, 7H, H2′, H3′, H4′, H5′, H5” and Ha-allyl); MALDI MS $(M + H)^+$ 285.3 Da. Anal. ($C_{12}H_{16}N_2O_6$) C, H, N.

***N*^{4}-Triazolyl-3′,5′-bis-*O*-tetrahydropyranyl-2′-*O*-allyl-araU (5).** 1,2,4-Triazole (539 mg, 7.8 mmol) and $POCl_3$ (152 μL, 1.67 mmol) were dissolved in CH_3CN (6 mL), and Et_3N (1 mL, 7.47 mmol) was added dropwise at 0 °C. Compound **3** (265 mg, 0.58 mmol) dissolved in CH_3CN (4 mL) was added to the above prepared mixture, and the suspension obtained was stirred at room temperature for 5 h (TLC: EtOAc/hexane, 6:4). The mixture was then treated with Et_3N (0.72 mL, 5.16 mmol) and H_2O (0.25 mL, 13.9 mmol), and after 10 min the solvent was evaporated and the residue was dissolved in CH_2Cl_2 (10 mL) and washed with aqueous saturated $NaHCO_3$ solution (1 × 5 mL). The aqueous layer was further extracted with CH_2Cl_2 (2 × 10 mL). The combined organic layers were dried and evaporated, and the crude residue was purified by silica gel column chromatography (EtOAc/hexane, linear gradient from 1:1 to 7:3) to give 163 mg of compound **5** as a pale-yellow oil: yield 56%; ^{1}H NMR ($CDCl_3$) δ 9.29 (s, 1H, H-triazole); 8.50−8.30 (m, 1H, H6); 8.13 (s, 1H, H-triazole); 7.05−6.95 (m, 1H, H5); 6.36 (m, 1H, H1′); 5.70−5.60 (m, 1H, Hb-allyl); 5.20−5.05 (m, 2H, Hc-allyl); 4.80−4.70 (m, 2H, Ha-allyl); 4.30−3.50 (m, 11H, H2′, H3′, H4′, H5′, H5”, Ha-THP, CH_2O-THP); 1.80−1.55 (m, 12H, CH_2 × 6, THP).

2′-*O*-Allyl-araC (7). Compound **5** (150 mg, 0.3 mmol) was dissolved in dioxane (1 mL), and 30% NH_4OH (1 mL) was added; the mixture was then stirred at room temperature (TLC: CH_2Cl_2/MeOH, 9:1). After 20 h, the solvent was evaporated and the crude residue (compound **6**, 100 mg, about 0.22 mmol) was dissolved in MeOH (5 mL). TsOH monohydrate (80 mg, 0.42 mmol) was added to the solution, which was then vigorously stirred at room temperature for 3 h (TLC: CH_2Cl_2/MeOH, 9:1). After this time, the reaction was complete, the solvent was evaporated and the residue was purified by column chromatography (CH_2Cl_2/MeOH, 9:1) and preparative HPLC with CH_3CN/H_2O (linear gradient from 1:9 to 6:4). This gave, on freeze-drying of the appropriate fractions, compound **7** toluenesulfonic acid salt as a white solid. Further purification on Dowex 1-X2 resin (200−400 mesh, OH^- form) gave, after freeze-drying of appropriate fractions, 23 mg of

compound **7** (free base) as a gray solid: yield 27%; mp 87−93 °C (MeOH/Et_2O); UV (MeOH) λ_{max} 273 nm (ϵ 7300), λ_{min} 253 nm (ϵ 5500); ^{1}H NMR (DMSO-d_6) δ 7.58 (d, J = 7.4 Hz, 1H, H6); 7.15 (m, 2H, NH_2); 6.14 (d, J = 5.0 Hz, 1H, H1′); 5.75−5.50 (m, 3H, Hb-allyl, H5 and OH); 5.15−4.95 (m, 3H, Hc-allyl and OH); 4.15−3.50 (m, 7H, H2′, H3′, H4′, H5′, H5" and Ha-allyl); MALDI MS $(M)^+$ 283.8 Da. Anal. ($C_{12}H_{17}N_3O_5$) C, H, N.

3′,5′-Bis-*O*-*tert*-butyl-dimethylsilyl-araA (8). Compound **8** was prepared following and adapting the procedure reported by Baker et al.[10] Ara-A (250 mg, 0.94 mmol) was dissolved in anhydrous DMF (5 mL) under positive argon pressure, and freshly distilled Et_3N (0.65 mL, 4.7 mmol) and TBDMS-Cl (531 mg, 3.52 mmol) were then added. After 72 h at 60 °C (TLC: CH_2Cl_2/MeOH, 9:1) the mixture was diluted with EtOAc (10 mL) and washed with H_2O (10 mL). The organic layer was dried and evaporated, and the crude residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH, 9.5:0.5) to give 348 mg of **8:** yield 74%; mp 175−178.[10]

3′,5′-Bis-*O*-*tert*-butyldimethylsilyl-2′-*O*-allyl-araA (9). Compound **8** (174 mg, 0.35 mmol) was dissolved in THF (6 mL), and NaH 60% (35 mg, 0.88 mmol) was added under vigorous stirring; after 15 min allyl bromide (75 μL, 0.88 mmol) was added and the mixture was stirred under positive argon pressure at room temperature for 16 h (TLC: EtOAc/hexane, 8:2). When the reaction was complete, the solvent was evaporated and the residue was dissolved in CH_2Cl_2 (10 mL), washed with aqueous saturated NH_4Cl solution (2 × 10 mL), dried, and evaporated. The resulting solid was purified by silica gel column chromatography (EtOAc/hexane, 6:4) to give 61 mg of **9**: yield 32%; white foam; ^{1}H NMR ($CDCl_3$) δ 8.34 (s, 1H, H2); 8.20 (s, 1H, H8); 6.52 (d, J = 5.1 Hz, 1H, H1′); 6.00 (sbr, 2H, NH_2); 5.62−5.45 (m, 1H, Hb-allyl); 5.38−4.92 (m, 2H, Hc-allyl); 4.52−4.49 (m, 1H, H3′); 4.05−3.65 (m, 6H, H2′, H4′, H5′, H5" and Ha-allyl); 0.91 and 0.93 (s, 18H, 2 × tBut-Si); 0.14, 0.09, 0.07 and 0.02 (s, 12H, 2 × Me_2−Si).

2′-*O*-Allyl-araA (10). The protected compound **9** (57 mg, 0.11 mmol) was dissolved under positive argon pressure in dry MeOH (2 mL), and NH_4F (47 mg, 1.27 mmol) was added. The mixture was heated at reflux conditions until complete conversion to deprotected compound **10** (TLC: CH_2Cl_2/MeOH, 9:1). After evaporation, the crude residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH, 9.5:0.5) to provide 29 mg of **10**: yield 82%; white solid; mp 219−224 °C (MeOH/Et_2O); UV (MeOH) λ_{max} 260 nm (ϵ 12500); ^{1}H NMR (DMSO-d_6) δ 8.22 (s, 1H, H2); 8.13 (s, 1H, H8); 7.27 (sbr, 2H, NH_2); 6.39 (d, J = 5.7 Hz, 1H, H1′); 5.70−5.45 (m, 2H, Hb-allyl and OH); 5.15−4.70 (m, 3H, Hc-allyl and OH); 4.5−4.3 (m, 1H, H3′); 4.30−3.55 (m, 6H, H2′, H4′, H5′, H5" and Ha-allyl); MALDI MS $(M)^+$ 307.6 Da. Anal. ($C_{13}H_{17}N_5O_4$) C, H, N.

2′-*O*-Allyl-araC 5′-Diphosphate (12). Compound **7** (80 mg, 0.28 mmol) and Proton Sponge (150 mg, 0.7 mmol) were dissolved in anhydrous trimethyl phosphate (1.4 mL), and freshly distilled $POCl_3$ (52 μL, 0.56 mmol) was added dropwise under positive argon pressure at 0 °C. After 4 h (TLC: *i*-PrOH/NH_4OH/H_2O, 11:7:2) a solution of 0.5 M tri-*n*-butylammonium orthophosphate (*n*-$But_3NH^+H_2PO_4^-$) (2.8 mL, 1.4 mmol) and tributylamine (0.28 mL) was added to the stirred suspension followed by a 0.2 M solution of triethylammonium bicarbonate (TEAB) (pH 7.5, 15 mL). The solvent was then evaporated, maintaining bath temperature at about 40−45 °C, and the residue was purified by a DEAE Sephadex A-25 (2 × 30 cm) column with TEAB (linear gradient from 0.01 to 1 M), pH 7.5, at a flow rate of 33 mL/h over 10 h. Tetra-*n*-butylammonium cation was exchanged for sodium by passing the solution through a Dowex AG 50W-X2 column (50−100 mesh, Na^+ form) and eluting with 10 column volumes of deionized water. After freeze-drying of appropriate fractions, the solid mass obtained was further purified by HPLC with CH_3CN/H_2O (linear gradient from 1:9 to 6:4) to give, on freeze-drying, 19 mg of compound **12**: retention time = 5.90; yield 13%; white solid; mp > 300 °C (MeOH/Et_2O); UV (H_2O) λ_{max} 278 nm (ϵ 10200) and 242 nm (ϵ 9500); ^{1}H NMR (CD_3OD) δ 7.95 (d, J = 7.6 Hz, 1H, H6); 6.27 (d, J = 4.6 Hz, 1H, H1′); 5.90−5.65 (m, 1H, Hb-allyl); 5.20−4.80 (m, 3H, Hc-allyl and H5); 4.24−4.10 (m, 1H, H3′); 4.08−3.90 (m, 6H, Ha-allyl, H2′, H4′, H5′, H5"); ^{31}P NMR (DMSO-d_6) δ −7.87 (d, Jp,p = 20.33 Hz, 1P, Pα); −15.36 (d, Jp,p = 20.2 Hz, 1P, Pβ); MALDI MS $(M + Na)^+$ 466.2 Da.

2′-*O*-Allyl-3′,5′-bis-*O*-*tert*-butyldimethylsilyl-araU (13a). Compound **4** (200 mg, 0.7 mmol) was dissolved in anhydrous DMF (5 mL); freshly distilled Et_3N (0.66 mL, 5.5 mmol) and TBDMS-Cl (351 mg, 3.85 mmol) were then added, and the mixture was heated at 60 °C under vigorous stirring and under positive argon pressure for 20 h (TLC: CH_2Cl_2/MeOH, 9.5:0.5). The solvent was evaporated; the residue was dissolved in CH_2Cl_2 (10 mL), washed with saturated $NaHCO_3$ (1 × 10 mL) and brine (1 × 10 mL), dried, filtered, and evaporated. The resulting solid was purified by silica gel column chromatography (EtOAc/hexane, 3:7) to give 183 mg of compound **13a**: yield 51%; white foam; ^{1}H NMR ($CDCl_3$) δ 8.50 (sbr, 1H, NH); 7.80 (d, J = 8 Hz, 1H, H6); 6.37 (d, J = 4.6 Hz, 1H, H1′); 5.94−5.60 (m, 2H, Hb-allyl and H5); 5.40 (dd, J = 6.6 Hz and J = 1 Hz, 2H, Hc-allyl); 4.38−4.00 (m, 3H, H2′, H3′ and H4′); 3.95−3.60 (m, 4H, Ha-allyl, H5′ and H5"); 0.93 and 0.91 (s, 18H, 2 × tBut-Si); 0.12 and 0.1 and 0.08 and 0.03 (s, 12H, 2 × Me_2−Si); MALDI MS $(M + Na)^+$ 535.9 Da; $(M + K)^+$ 552.0 Da.

2′-*O*-Allyl-3′-*O*-*tert*-butyldimethylsilyl-araU (14a). Compound **13a** (676 mg, 1.32 mmol) was dissolved in 80% acetic acid (10 mL), and the solution was stirred at 60 °C for 6 h (TLC: EtOAc/hexane, 1:1). The mixture was then coevaporated with EtOH (4 × 10 mL), and the residue was dissolved in (CH_2Cl_2), washed with aqueous saturated $NaHCO_3$ solution (1 × 10 mL) and brine (1 × 10 mL), dried, and evaporated. The resulting crude solid was purified by silica gel column chromatography (EtOAc/hexane, linear gradient from 3:7 to 8:2) to give 283 mg of **14a**: yield 54%; white foam; ^{1}H NMR ($CDCl_3$) δ 8.25 (sbr, 1H, NH); 7.70 (d, J = 6 Hz, 1H, H6); 6.10 (d, J = 4 Hz, 1H, H1′); 5.80−5.50 (m, 2H, H5 and Hb-allyl); 5.10 (dd, J = 1 Hz and J = 6.6 Hz, 2H, Hc-allyl); 4.90 (sbr, 1H, OH); 4.30−4.10 (m, 3H, H2′, H3′ and H4′); 3.85−3.75 (m, 2H, H5′ and H5"); 3.78−3.45 (m, 2H, Ha-allyl); 0.80 (s, 9H, tBut-Si); 0.03 and 0.018 (s, 6H, Me_2−Si); MALDI MS $(M + Na)^+$ 422.1 Da; $(M + K)^+$ 438.6 Da.

2′-*O*-Allyl-3′-*O*-*tert*-butyldimethylsilyl-5′-*O*-tosyl-araU (15a). Compound **14a** (283 mg, 0.71 mmol) was dissolved in anhydrous CH_2Cl_2 (6 mL), and 4-dimethylaminopyridine (DMAP) (174 mg, 1.42 mmol) was added under positive argon pressure. Tosyl chloride (203 mg, 1 mmol) was dissolved in freshly distilled CH_2Cl_2 (2 mL), and the solution obtained was added dropwise at the reaction mixture under vigorous stirring at 0 °C. After 20 h at 4 °C and 30 min at room temperature, TLC (EtOAc/hexane, 4:6) indicated complete reaction; the solvent was evaporated, and the residue was purified by silica gel column chromatography (EtOAc/hexane, 3:7); 330 mg of compound **15a** was obtained: yield 84%; white foam; ^{1}H NMR ($CDCl_3$) δ 8.84 (sbr, 1H, NH); 7.75 (d, J = 8.4 Hz, 2H, Ph); 7.30 (d, J = 8.4 Hz, 2H, Ph); 7.25 (d, J = 6 Hz, 1H, H6); 6.15 (d, J = 3.8 Hz, 1H, H1′); 5.58−5.50 (m, 2H, Hb-allyl and H5); 5.06 (dd, J = 1 Hz and J = 6.6 Hz, 2H, Hc-allyl); 4.40−4.10 (m, 3H, H2′, H3′ and H4′); 3.94−3.88 (m, 2H, H5′ and H5"); 3.84−3.77 (m, 2H, Ha-allyl); 2.39 (s, 3H, Me-Ph); 0.80 (s, 9H, tBut-Si); 0.03 and 0.01 (s, 6H, Me_2-Si); MALDI MS $(M + Na)^+$ 576.2 Da; $(M + K)^+$ 592.5 Da.

2′-*O*-Allyl-5′-*O*-tosyl-araU (16a). Compound **15a** (322 mg, 0.58 mmol) was dissolved in CH_3CN (15 mL), and 2% HF/H_2O (3 × 15 mL) was added at room temperature every 12 h (TLC: CH_2Cl_2/MeOH, 9.5:0.5). The mixture was neutralized with saturated NH_4HCO_3, and the solvent was evaporated to give, on freeze-drying, a crude residue, which was dissolved in a CH_2Cl_2/MeOH (9:1) solution, filtered, and purified by column chromatography (CH_2Cl_2/MeOH, linear gradient from 9.8:0.2 to 9.5:0.5) to give 226 mg of **16a**: yield 89%; white foam; ^{1}H NMR ($CDCl_3$) δ 8.80 (sbr, 1H, NH); 7.73 (d, J = 8.4 Hz, 2H, OTs); 7.32 (d, J = 8.4 Hz, 2H, OTs); 7.20 (d, J = 6 Hz, 1H, H6); 6.10 (d, J = 3.8 Hz, 1H, H1′); 5.55−5.50 (m, 3H, Hb-allyl, H5 and OH); 5.05 (dd, J = 1 Hz and J = 6.6 Hz, 2H, Hc-allyl);

4.43−4.13 (m, 3H, H2′, H3′ and H4′) 3.95−3.88 (m, 2H, H5′ and H5"); 3.84−3.77 (m, 2H, Ha-allyl); 2.40 (s, 3H, Me-Ph); MALDI MS $(M + H)^+$ 439.8 Da; $(M + Na)^+$ 462.0 Da; $(M + K)^+$ 478.0 Da.

2′-*O*-Allyl-araU 5′-diphosphate (17a). Compound **16a** (226 mg, 0.51 mmol) was dissolved in dry CH_3CN (300 μL), and tris(tetra-*n*-butylammonium)hydrogen pyrophosphate[7] (698 mg, 0.77 mmol) was added (TLC: CH_2Cl_2/MeOH, 9.8:0.2). After 48 h at room temperature, the solvent was evaporated and the crude residue was purified by DEAE Sephadex A-25 (2 × 30 cm) column with TEAB (linear gradient 0.01−1 M), pH 7.5, at a flow rate of 50 mL/h over 10 h. On freeze-drying of the appropriate fraction a white mass was obtained. The tetra-*n*-butylammonium cation was then exchanged for sodium by Dowex AG 50W-X2 column chromatography (50−100 mesh, Na^+ form) and eluting with 2 column volumes of deionized water. The eluent was freeze-dried, and the solid obtained was further purified by HPLC with CH_3CN/H_2O (linear gradient from 1:9 to 6:4) to give, on freeze-drying, 50 mg of compound **17a**: retention time = 3.08; yield 19%; white solid; mp 120−123 °C (MeOH/Et_2O); UV (H_2O) λ_{max} 263 nm (ϵ 11700), λ_{min} 225 nm (ϵ 3500); ^{1}H NMR (D_2O) δ 7.87 (d, J = 8 Hz, 1H, H6); 6.37 (d, J = 5.6 Hz, 1H, H1′); 5.94 (d, J = 8 Hz, 1H, H5); 5.90−5.75 (m, 1H, Hb-allyl); 5.29−5.16 (m, 2H, Hc-allyl); 4.50−4.40 (m, 2H, H2′ and H3′); 4.30−4.10 (m, 3H, H4′, H5′ and H5"); 4.10−3.90 (m, 2H, Ha-allyl); ^{31}P NMR (D_2O) δ −9.4 (d, J p,p = 19.6 Hz, 1P, Pα); −10.3 (d, J p,p = 19.18 Hz, 1P, Pβ); MALDI MS $(M + Na)^+$ 468.4 Da.

2′-*O*-Allyl-3′-*O*-*tert*-butyldimethylsilyl-araA (14b). Compound **9** (129 mg 0.24 mmol) was deprotected with 80% acetic acid (2.5 mL) as described for compound **13a**. The resulting oil was purified by silica gel column chromatography (EtOAc/hexane, 8:2) to give 68 mg of **14b**: yield 67%; colorless syrup; ^{1}H NMR ($CDCl_3$) δ 8.28 (s, 1H, H2); 8.04 (s, 1H, H8); 6.43 (d, J = 5.6 Hz, 1H, H1′); 5.86 (sbr, 2H, NH_2); 5.62−5.40 (m, 1H, Hb-allyl); 5.05−4.95 (m, 3H, Hc-allyl and OH); 4.70−4.50 (m, 1H, H3′); 4.19−3.64 (m, 6H, Ha-allyl, H2′, H4′, H5′, H5"); 0.90 (s, 9H, 1 × tBut-Si); 0.16 (s, 6H, 2 × Me_2-Si); MALDI MS $(M)^+$ 422.9 Da.

2′-*O*-Allyl-3′-*O*-*tert*-butyldimethylsilyl-5′-*O*-tosyl-araA (15b). Compound **14b** (250 mg, 0.59 mmol) was reacted with tosyl chloride (124 mg, 0.65 mmol) as described for compound **14a**. The residue was purified by silica gel column chromatography (EtOAc/hexane, 1:1); 272 mg of **15b** was obtained: yield 80%; white foam; ^{1}H NMR ($CDCl_3$) δ 8.31 (s, 1H, H2); 7.96 (s, 1H, H8); 8.27 (d, J = 10.8 Hz, 2H, Ph); 7.29 (d, J = 10.8. Hz, 2H, Ph); 6.46 (d, J = 4.4 Hz, 1H, H1′); 5.75 (sbr, 2H, NH_2); 5.52−5.44 (m, 1H, Hb-allyl); 5.04−4.95 (m, 2H, Hc-allyl); 4.40−3.98 (m, 1H, H3′); 4.30−4.20 (m, 2H, H2′ and H4′); 4.15−3.90 (m, 2H, H5′ and H5"); 3.85−3.60 (m, 2H, Ha-allyl); 2.45 (s, 3H, Me-Ph); 0.89 (s, 9H, 1 × tBut-Si); 0.12 (s, 6H, 2 × Me_2-Si).

2′-*O*-Allyl-5′-*O*-tosyl-araA (16b). Compound **15b** (120 mg, 0.21 mmol) was dissolved in CH_3CN (5 mL) and deprotected with 2% HF/H_2O (3 × 5 mL) as described for compound **15a**. The residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH, linear gradient from 9.5:0.5 to 9:1) to give 57 mg of compound **16b**: yield 59%; white foam; ^{1}H NMR ($CDCl_3$) δ 8.30 (s, 1H, H2); 8.06 (s, 1H, H8); 7.72 (d, J = 8.2 Hz, 2H, OTs); 7.25 (d, J = 8.2 Hz, 2H, OTs); 6.52 (d, J = 5 Hz, 1H, H1′); 6.15 (sbr, 2H, NH_2); 5.50−5.41 (m, 2H, Hb-allyl and OH); 5.01−4.91 (m, 2H, Hc-allyl); 4.70−4.57 (m, 1H, H3′); 4.40−4.10 (m, 4H, H2′, H4′, H5′ and H5"); 4.00−3.60 (m, 2H, Ha-allyl); 2.39 (s, 3H, Me-Ph).

2′-*O*-Allyl-araA 5′-diphosphate (17b). Compound **16b** (155 mg, 0.33 mmol) was dissolved in anhydrous CH_3CN (200 μL) and converted into the corresponding diphosphate with tris(tetra-*n*-butylammonium)hydrogen pyrophosphate[7] (451 mg, 0.38 mmol) as described for compound **16a**. The appropriate fractions, deriving from the Dowex AG 50W-X2 (50−100 mesh, Na^+ form) column chromatography, were freeze-dried, and the solid obtained was further purified by HPLC (linear gradient from H_2O to CH_3CN/H_2O, 6:4) to give, on freeze-drying, 69 mg of compound **17b**: retention time = 3.58; yield 38%; white solid; mp 101−103 °C (MeOH/Et_2O); UV (H_2O) λ_{max} 259 nm (ϵ 13600), λ_{min} 226 nm (ϵ 3900); ^{1}H NMR (D_2O) δ 8.52 (s, 1H, H2); 8.29 (s, 1H, H8); 6.59 (d, J = 6 Hz, 1H, H1′); 5.51−5.43 (m, 1H, Hb-allyl); 5.01−4.91 (m, 2H, Hc-allyl); 4.68−4.57 (m, 1H, H3′); 4.35−4.20 (m, 4H, H2′, H4′, H5′ and H5"); 4.18−3.70 (m, 2H, Ha-allyl); ^{31}P NMR (D_2O) δ −6.96 (d, Jp,p = 14.8 Hz, 1P, Pα); −10.25 (d, Jp,p = 24 Hz, 1P, Pβ); MALDI MS $(M + H)^+$ 468.4 Da; $(M + Na)^+$ 490.3 Da; $(M + K)^+$ 506.1 Da.

2′-*O*-Allyl-5′-(phenylethoxy-L-alanyl phosphate)-araU (18). Compound **4** (155 mg, 0.4 mmol) was dissolved in dry THF (10 mL). Ethoxy-L-alanyl monochlorophosphate (1.2 mL, 1.2 mmol), prepared according to McGuigan et al.,[14] and 1-methylimidazole (192 μL, 2.4 mmol) were added dropwise to the solution at room temperature under vigorous stirring and positive argon pressure; a green gum precipitated from the pale-yellow solution. After 5 h, ethoxy-L-alanyl monochlorophosphate (0.5 mL, 0.5 mmol) and 1-methylimidazole (65 μL, 0.8 mmol) were further added; after 72 h, the solvent was evaporated and the residue was dissolved in CH_2Cl_2 (15 mL) and washed with 1 M HCl (1 × 10 mL), aqueous saturated $NaHCO_3$ solution (1 × 10 mL), and brine (1 × 10 mL). The organic layer was dried and evaporated, and the crude solid was purified by silica gel column chromatography (CH_2Cl_2/MeOH, linear gradient from 9.8:0.2 to 9:1) and also by preparative HPLC (linear gradient from H_2O to CH_3CN) to give, on freeze-drying, 43 mg of compound **18**: yield 20%; white foam; ^{1}H NMR ($CDCl_3$) δ 11.0−10.50 (br, 1H, NH); 8.70−8.55 (br, 1H, NH); 7.40−7.10 (m, 6H, H−Ar and H6); 6.21 and 6.25 (d × 2, J = 5.4 Hz, 1H, H1′); 5.75−5.45 (d × 2, J = 8 Hz and m, 2H, H5 and Hb-allyl); 5.45−5.30 (br, 1H, OH); 5.30−5.00 (m, 2H, Hc-allyl); 4.29−3.62 (m, 10H, Ha-allyl, H2′, H3′, H4′, H5′, H5", C*H*NH, C*H*$_2$OC); 1.22−1.13 (m, 6H, *Me*CHNH, *Me*CH_2OC); ^{31}P NMR ($CDCl_3$) δ 3.97, 3.65 (1:1); MALDI MS $(M + Na)^+$ 562.8 Da. Anal. ($C_{23}H_{30}N_3O_{10}P$) C, H, N.

2′-*O*-Allyl-5′-(phenylethoxy-L-alanyl phosphate)-araC (19). Compound **7** (88 mg, 0.31 mmol) was dissolved in dry THF (10 mL) and reacted with ethoxy-L-alanyl monochlorophosphate (0.9 mL, 0.9 mmol)[14] and 1-methylimidazole (150 μL, 1.86 mmol) as described for compound **4**. The crude residue was purified by silica gel column chromatography (linear gradient from CH_2Cl_2 to CH_2Cl_2/MeOH 9:1) and preparative HPLC with CH_3CN/H_2O (linear gradient from 1:9 to 6:4) to give, on freeze-drying, 30 mg of compound **19**: yield 18%; pale-yellow foam; ^{1}H NMR ($CDCl_3$) δ 10.0−9.85 (sbr, 1H, NH); 7.41 and 7.38 (d × 2, J = 8.2 Hz, 1H, H6); 7.30−7.16 (m, 5H, H−Ar); 7.15 (m, 2H, NH_2); 6.20 and 6.23 (d × 2, J = 5.4 Hz, 1H, H1′); 5.75−5.49 (m, 2H, Hb-allyl and OH); 5.45−5.30 (d × 2, J = 8 Hz, 1H, H5); 5.13−5.03 (m, 2H, Hc-allyl); 4.29−3.60 (m, 10H, Ha-allyl, H2′, H3′, H4′, H5′, H5", CHNH, CH_2OC); 1.22−1.13 (m, 6H, *Me*CH, *Me*CH_2OC); ^{31}P NMR ($CDCl_3$) δ 5.26, 5.04 (1:1); MALDI MS $(M + Na)^+$ 561.2 Da. Anal. ($C_{23}H_{31}N_4O_9P$) C, H, N.

Biology. Materials and methods: Enzymatic assays on RNR (R1 and R2 subunits) were performed according to the procedures described by Engström[18] and Shewach.[19]

Cytostatic activity of test compounds: All assays were performed in 96-well microtiter 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 and 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% effective inhibitory concentration) was defined as the concentration of compound that reduced the number of viable cells by 50%.

Nucleoside kinase assay: Purified cytosolic human thymidine kinase and human deoxycytidine kinase activity was assayed as follows: the reaction mixture contained 50 mM Tris HCl, pH 8.0, 2.5 mM $MgCl_2$, 10 mM dithiothreitol, 2.5 mM ATP, 1 mg/mL bovine serum albumin, 10 mM NaF, and [*methyl*-^{3}H]thymidine or [5-^{3}H]deoxycytidine at 1 μM (≈0.1−0.4 μCi/assay) in a total volume of 50 μL. Assays were perfomed at 37 °C during a 30-min incubation period. Aliquots of 45 μL

of the reaction mixtures were spotted onto Whatman DE-81 filter paper disks. The filters were subsequently washed three times for 5 min in 1 mM ammonium formate, one time for 1 min in H_2O, and one time for 5 min in ethanol. The radioactivity was determined by scintillation counting.

Molecular modeling: Computational studies were conducted on a Silicon Graphics Indigo 2 and O2 workstations using MacroModel[20] (5.5) software and the Amber[21] force field.

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