Eur. J. Med. Chem. 34 (1999) 423–431

Short communication

Study of human deoxycytidine kinase binding properties using intrinsic fluorescence or new fluorescent ligands

Manijeh Shafiee[a], Gilles Gosselin[a], Jean-Louis Imbach[a], Gilles Divita[b], Staffan Eriksson[c], Georges Maury[a]*

[a]*Laboratoire de Chimie Bioorganique, UMR 5625 du CNRS, Département de chimie organique fine, Case courrier 006, Université Montpellier II des Sciences et Techniques du Languedoc,Place Bataillon, 34095 Montpellier Cedex 5, France*
[b]*Centre de Recherche de Biochimie Macromoléculaire, UPR-1086 du CNRS, BP 5051, 34293 Montpellier Cedex 5, France*
[c]*Department of Veterinary Medical Chemistry, The Swedish University of Agricultural Sciences, The Biomedical Centre, Box 575, SE-751 23 Uppsala, Sweden*

(Received 15 July 1998; accepted 3 December 1998)

Abstract – A series of D- and L-enantiomers of cytidine or adenosine analogues and fluorescent N-methylanthraniloyl (MeNHBz) cytidine derivatives regiospecifically synthesized from cytidine or deoxycytidine were used to quantify the enantioselectivity of human deoxycytidine kinase (dCK) and to characterize its binding states. Both D- and L-enantiomers of these compounds induced significant bimodal quenchings of the intrinsic fluorescence of the enzyme. The ratios of dissociation constants Kd_D/Kd_L for the high affinity binding of non fluorescent cytidine derivatives were remarkably similar. β-D- and β-L-ATP gave monophasic titration curves and the enzyme displayed a preference for the natural enantiomer. This demonstrates the lack of enantioselectivity of dCK in the substrate binding steps of its mechanism. The results of other fluorescence experiments with MeNHBz-cytidine derivatives were consistent with an enzyme mechanism in which nucleotide binding precedes nucleoside binding. © Elsevier, Paris

fluorescent cytidine derivatives / deoxycytidine kinase / enantioselectivity

1. Introduction

Deoxycytidine kinase (EC 2.7.1.74) is an important enzyme in the salvage reactions of nucleoside synthesis. It has a broad substrate specificity and catalyzes the phosphorylation of all three natural deoxyribonucleosides, β-D-dC, β-D-dA and β-D-dG by transferring the γ-phosphoryl group of β-D-ATP or β-D-UTP [1]. As a result, it may activate a large number of nucleoside analogues and is therefore important in antiviral or anticancer chemotherapies. An ordered Bi-Bi mechanism has been postulated with either ATP [2] or the deoxynucleoside [3] as the first substrate to bind to the enzyme. However, the occurrence of bimodal kinetic curves, negative cooperativity [4] and the apparent existence of two binding sites or states [5] suggest that the enzymatic mechanism is more complex than previously believed [1]. Furthermore, deoxycytidine kinase has been reported to have a markedly relaxed enantioselectivity being able to catalyze the phosphorylation of both enantiomers of a series of cytidine analogues [6]. In this context, human deoxycytidine kinase (dCK) appears as the key enzyme in the activation of a number of unnatural L-nucleoside analogues into their 5′-triphosphate derivatives since the 5′-monophosphorylation of nucleoside analogues is generally considered as the most critical step [7].

We are currently studying the enantioselectivity of recombinant dCK and we have previously shown that the enzyme catalyzes the phosphorylation of, not only a broad series of L-cytidine analogues [8], but also L-adenosine analogues [9]. In the present work, we have determined the affinity of dCK with respect to a series of newly prepared fluorescent cytidine derivatives as well as non fluorescent enantiomeric nucleoside or nucleotide

*Correspondence and reprints

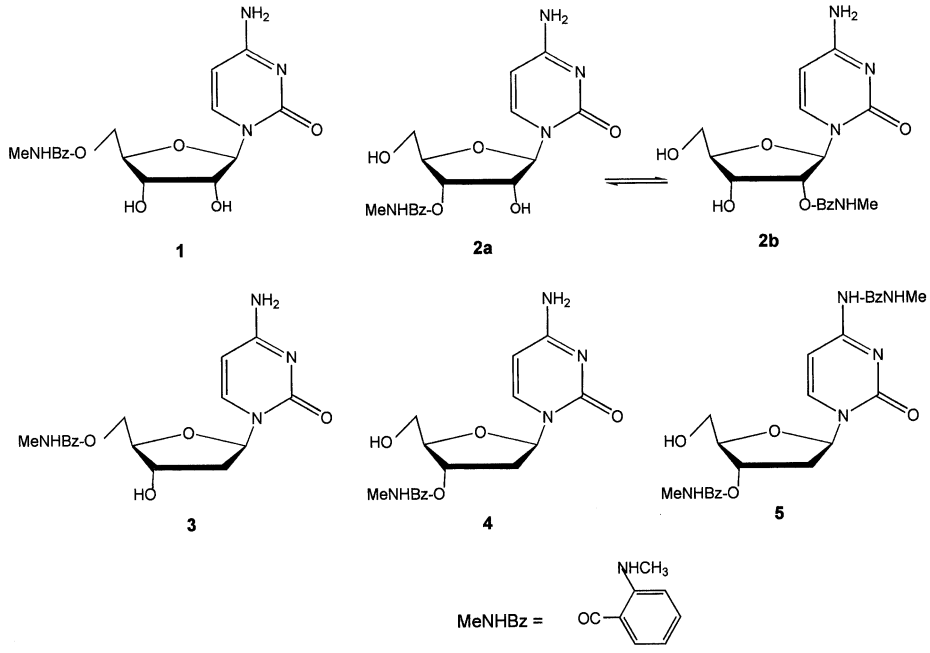

Figure 1. N-Methyl anthraniloyl cytidine derivatives synthesized.

analogues using fluorescence spectroscopy, in order to study the binding sites and the enantioselectivity of the enzyme.

2. Chemistry

The N-methylanthraniloyl (MeNHBz) fluorophore has often been used for the characterization of various nucleoside or nucleotide binding proteins [10–12]. The efficiency of this marker is probably due to its high quantum yield and its modest bulk resulting in relatively slight modifications of the binding efficiency to the enzyme compared to the parent compounds [13]. The introduction of the N-methylanthraniloyl group into a nucleoside or a nucleotide molecule has been achieved in basic aqueous medium through nucleophilic attack of N-methyl isatoic anhydride by an alcoholate anion of the substrate [10]. However, this reaction is restricted to purine nucleoside or nucleotide labelling and yields only mixtures of 2′- or 3′-*O*-N-methylanthraniloyl derivatives in rapid equilibrium [12]. To characterize the binding sites and study the enantioselectivities of several enzymes of cytidine or deoxycytidine metabolism [9], we needed cytidine analogues regiospecifically substituted at various positions on the molecule. The compounds **1–5** *(figure 1)* were prepared through selective protection of the adequate hydroxyl and amino groups of cytidine or deoxycytidine with monomethoxytrityl chloride or *t*-butyldimethylsilyl chloride, subsequent reaction with N-methyl isatoic anhydride in the presence of sodium hydride, and acidic deprotection. Under these conditions, good yields of mono- or, in one instance, bis-MeNHBz derivatives were obtained. The rapidly interconverting **2a** and **2b**, although isolated by preparative HPLC, were not stable enough to be used separately in binding experiments and were used as an approximately 65:35 mixture. All compounds **1–5** fluoresce strongly in water with a maximum of emission at 440 nm when excited in the 320–350 nm range. These properties compare to that of 2′(3′)-MeNHBz-adenosine which presents a maximum of emission at 428 nm in water upon excitation at 330–350 nm [10].

3. Biological results and discussion

Prior to studying the binding of compounds **1–5** to human deoxycytidine kinase, we determined the kinetic properties of **2**, **4** and **5** having a free 5′-OH group and thus being potential substrates towards dCK. Under our conditions, only **2** and **4** were substrates of the enzyme with Km constants comparable to that of β-D-dC and β-D-riboC, respectively (*figure 2, table I*) [8]. In contrast, Vm constants were two orders of magnitude lower than that of the reference compounds. The bis-MeNHBz

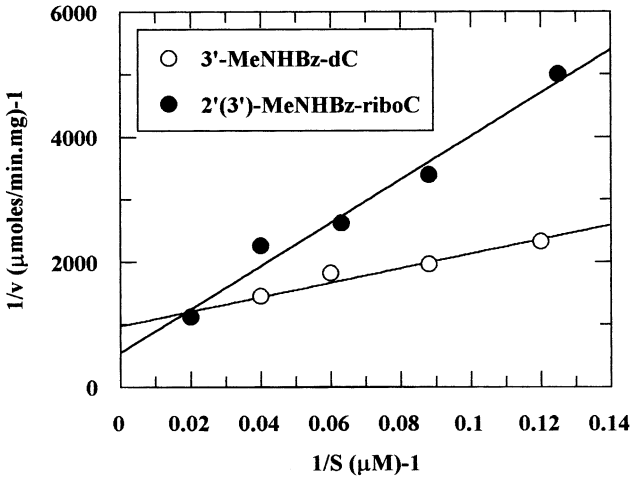

Figure 2. Substrate properties of 3´-MeNHBz-riboC (●) and 2´(3´)-MeNHBz-dC (○) with respect to dCK. The medium contained 50 mM Tris HCl pH 7.5, 5 mM ATP, 5 mM $MgCl_2$, 1 mM dithiothreitol, 45 µg.ml^{-1} dCK.

derivative **5** was not a substrate. Thus, the affinity of the MeNHBz-derivatives **1**, **3** and **5** for dCK can be directly assessed by steady state fluorescence studies of the binding to the enzyme in the presence of $MgCl_2$ and ATP.

The intrinsic fluorescence of human dCK is mostly due to the presence of seven tryptophan residues per monomer in the dimeric molecule. It has been previously used to study the binding properties of β-D-dC, β-D-dA or β-D-dU assuming the exclusive occurrence of static quenching of fluorescence in the presence of these compounds [5]. The enzyme fluorescence quenching using β-D-dC or β-D-dA was bimodal implying the existence of at least two binding sites or two conformational states of the enzyme. We used this method to determine the enantioselectivity of the ligand binding step in the dCK mechanism with respect to a series of β-D- or β-L-cytidine analogues. Assuming that there is a linear relation between the fluorescence of free enzyme or enzyme-ligand complex and enzyme or enzyme-ligand concentration, respectively, dCK was titrated with the nucleoside or nucleotide analogues in the presence of $MgCl_2$ *(figure 3)*. We observed a decrease of intrinsic fluorescence in all titrations without any shift of emission wavelength maximum. The results were fitted to the equations (1) or (2) (see experimental) and the probability that the two fits were equally appropriate was determined *(table II)*. We found that the quenching of all cytidine ligands **6–13** was biphasic irrespective of stereochemistry and that the constants Kd_1 and Kd_2 corresponding to enzyme-ligand complex dissociation were practically independent of the sugar structure. In all cases, the enantioselectivity of dCK based on the ratio of Kd constants favoured only slightly the D-enantiomer in both phases of the titration (Kd_D/Kd_L ratios were in the range of 0.5–0.6 and 0.3–0.5 for Kd_1 and Kd_2, respectively). The situation was less straightforward for D- and L-dA but, whatever the binding mode, dCK showed again a relaxed enantioselectivity *(table II)*. The enzyme was also non enantioselective in the binding to β-D- or β-L-ATP. In contrast with nucleoside analogues, in the case of β-ATP, the results were best interpreted assuming that only one binding site or enzymatic state was involved with a preference for the natural enantiomer (Kd_D/Kd_L: 0.4) (*figure 3*, *table II*).

The prepared N-methylanthraniloyl cytidine derivatives **1–4** all bound to dCK regardless of the fluorescent label position in the molecule. The quenching of intrinsic dCK fluorescence was most probably unimodal for **1** and **2** and bimodal for **3** and **4** *(table II)*. Because of the substitution at position 5´, steady state titration experiments were possible with **3** in the presence of saturating concentrations of ATP and $MgCl_2$. The presence of ATP induced a reversal to unimodal quenching with a distinct increase in the stability of the enzyme-ligand complex

Table I. Substrate properties of MeNHBz-cytidine derivatives with respect to dCK and as compared to unlabelled compounds. The reaction medium contained 5 mM ATP, 5 mM $MgCl_2$, the substrate (10 to 50 µM) and the enzyme (45 µg/mL for the MeNHBz compounds and 135 ng/mL for the unlabelled compounds).

Compound	Km (µM)	Vm (µmol/min.mg)	Vm/Km
ß-D-dC	9	0.24	1
ß-D-riboC	60	0.21	0.13
ß-D-2´(3´)-MeNHBz-riboC, **2**	63	0.002	0.001
ß-D-3´-MeNHBz-dC, **4**	12	0.001	0.003
ß-D-3´,N^4-BisMeNHBz-dC, **5**	a	a	–

[a]No reaction observed.

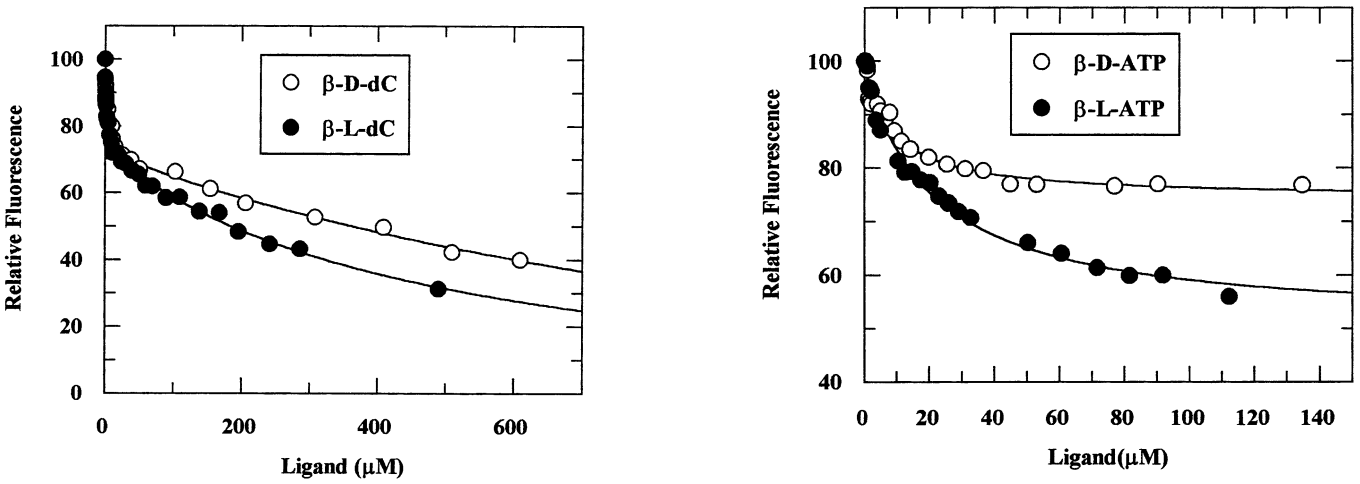

Figure 3. Titration of 0.5 μM dCK with the enantiomers of **A**. β-dC or **B**. β-ATP using the intrinsic tryptophan fluorescence of the enzyme.

(table II). This suggests that the ATP binding site of the enzyme is indirectly involved in the binding of **3** to dCK in the absence of ATP, so that the preliminary binding of ATP-$MgCl_2$ to the enzyme favours the subsequent binding of **3**. This may be consistent with the enzyme mechanism in which nucleotide binding precedes nucleoside binding [2].

In a different approach to the determination of the affinities of **1–5** for dCK, the fluorescence of the N-methylanthraniloyl group was used to monitor the titration of the ligand with the enzyme. Depending on the ligand, a 30–100% increase in fluorescence intensity was observed without any shift of the emission wavelength maximum. Displacement experiments of **3** from its preformed complex with dCK were performed by adding saturating concentrations of a high affinity substrate of the enzyme. Introducing 15 μM to 150 μM β-D-dC in a first phase of the titration (concentration of added enzyme below 0.2 μM) gave no change in fluorescence intensity, whereas the addition of 150 μM β-D-dC in a second

Table II. Affinities of β-nucleoside and nucleotide derivatives to dCK: binding modes and values of dissociation constants (μM) determined with the Grafit program. The medium contained 50 mM Tris HCl pH 7.5, 100 mM KCl, 1 mM dithiothreitol, 5 mM $MgCl_2$, and 10 % glycerol.

Ligand	Biphasic binding (I)		Monophasic binding (II)	Probability that II was
	Kd_1	Kd_2	Kd	more appropriate than I
D-dC, **6**	0.6 ± 0.2	170 ± 30	–	0.002
L-dC, **7**	1.3 ± 0.3	300 ± 90	–	0.001
D-ddC, **8**	1.5 ± 0.4	75 ± 20	–	0.003
L-ddC, **9**	2.4 ± 0.9	270 ± 100	–	0.008
D-araC, **10**	1.2 ± 0.7	45 ± 10	–	0.06
L-araC, **11**	2.4 ± 0.6	140 ± 50	–	0.001
D-riboC, **12**	1.5 ± 0.8	75 ± 40	–	0.09
L-riboC, **13**	2.7 ± 0.4	220 ± 140	–	0.001
D-dA, **14**	9.3 ± 3	230 ± 40	25 ± 4	0.12
L-dA, **15**	2 ± 1.7	95 ± 20	65 ± 8	0.12
D-ATP, **16**	–	–	8.4 ± 1	0.93
L-ATP, **17**	–	–	21 ± 2	0.22
5'-MeNHBz-riboC, **1**	–	–	23 ± 4	0.16
2'(3')-MeNHBz-riboC, **2**	–	–	24 ± 3	0.50
5'-MeNHBz-dC, **3**	4.3 ± 1	220 ± 150	–	0.005
Idem + 5mM ATP	–	–	1.7 ± 0.1	0.38
3'-MeNHBz-dC, **4**	4.3±0.7	160±80	–	0.08

phase of the titration (concentration of added enzyme above 1 μM) resulted in only a 30–40% decrease in fluorescence intensity. The titration of **3** by dCK in the presence of 5 mM ATP increased about 2-fold the fluorescence intensity compared to the titration in the absence of ATP. This effect, already observed in the intrinsic fluorescence data, is probably due to an improved binding of the ligand to the protein in the presence of ATP as well as a possible change of the binding site polarity. The addition of β-D-dC in the final stage of the titration decreased the fluorescence intensity to the same level as in the absence of ATP. Fitting the experiment data to equation (2) yielded two largely different Kd constants: 0.01 μM and 1.8 μM *(figure 4)*. The value of the second dissociation constant is comparable to Kd_1 obtained for the same compound using the intrinsic fluorescence of the enzyme *(table II)*. These results may be explained by the occurrence of a strong binding of **3** at a non specific site of dCK as well as a weaker binding at the substrate site. Such a non specific binding could not be detected in intrinsic fluorescence experiments with **1–5** because ligand concentrations were much larger than enzyme concentrations. Similar examples have been previously reported in the literature in which the label on a fluorescent probe induces binding to an enzyme at a different site than expected from its structure [14, 15]. Comparatively low fluorescence variations were observed in the binding of **1** to dCK, and displacement experiments (as in the case of **3**) failed to give any significant fluorescence change. In this case, the experimental data were best correlated with equation (1), thus suggesting that the binding was mainly non specific (Kd: 0.01 μM) *(figure 4)*.

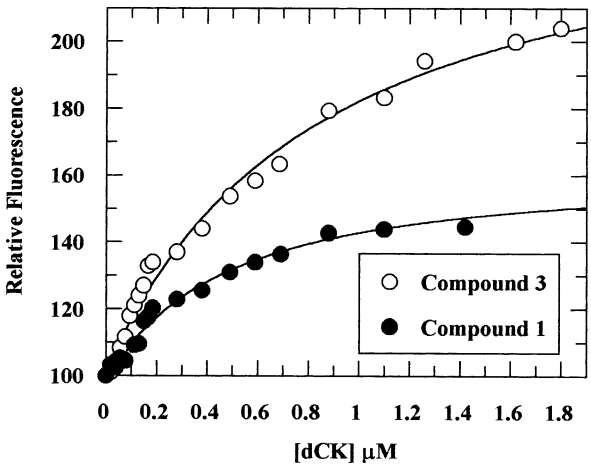

Figure 4. Titration of N-methyl anthraniloyl cytidine derivatives **1** or **3** (0.1 μM) with human deoxycytidine kinase using the fluorescence of the enzyme ligands.

Finally, the results of our study of dCK affinity for various ligands reflect the complexity of this enzyme, already evident in kinetic studies of its mechanism [1]. Multiphasic variations of intrinsic or extrinsic fluorescence intensities in titrations are probably related to the existence of several permanent or transient conformational states or binding sites of the enzyme depending on substrate concentration. The justification for these multiple specific bindings of ligands as well as the non specific binding in the case of **1** and **3** must await the disclosure of the 3D-structure of the enzyme. Our results further demonstrate that the lack of enantioselectivity in the mechanism of human dCK primarily concerns the binding steps of the substrates to the enzyme. They also suggest that the binding of β-D-ATP to dCK precedes the binding of nucleosidic substrates in the enzymatic mechanism.

4. Experimental protocols

4.1. Chemistry

UV spectra were recorded on an Urikson 810 (KONTRON) spectrophotometer. ^{1}H NMR spectra were obtained with a Bruker AC 250 spectrometer. Deuterium exchange and decoupling experiments were performed to confirm proton assignments. FAB mass spectra were recorded in the positive-ion or negative-ion mode on a JEOL DX 300 mass spectrometer: the matrix was a mixture (50/50, v/v) of glycerol and thioglycerol (G/T). Elemental analyses were carried out by the Service Central de Microanalyse du CNRS and were within ± 0.4% of the calculated values, unless otherwise stated. Chromatograms were obtained with a Waters System Prep 4 000 equipped with an automatic injector WISP 715 ULTRA and a diode array detector 990. An SSQ 7 000 Finnegan MAT mass spectrometer was directly connected to the chromatograph, and analyses were performed either in the positive or in the negative mode of electron spray ionization (ESI).

β-D-dC and β-D-ribo C were obtained from Sigma and β-D-araC from Upjohn. β-D-ddC was a gift from J. Balzarini (University of Leuven, Belgium). β-L-dC [16], β-L-ddC [17], and β-L-dA [18] were synthesized as previously described. β-L-riboC, β-L-araC and β-L-ATP were stereospecifically synthesized by multi-step reaction sequences starting from commercially available L-sugars, and the details of their synthesis will be reported else-

where. N-Methyl isatoic anhydride has been purchased from Aldrich.

4.1.1. β-D-5′-O-(N-Methylanthraniloyl)cytidine **1**

To a stirred solution of the hydrochloride of β-D-2′,3′-*O*-(isopropylidene)cytidine (450 mg, 1.4 mmol, 1eq) in anhydrous DMF (6 mL), sodium hydride (169 mg, 4.2 mmol) was added at room temperature under argon. The mixture was heated at 70 °C and N-methylisatoic anhydride was added in small portions. After 40 h, 20 mL of water were added and the product extracted with dichloromethane and ethyl acetate. The residual oil was chromatographed on silica gel (eluent: gradient of 0–5% ethyl acetate in chloroform) to give β-D-2′,3′-*O*-(isopropylidene)-5-*O*-(N-methylanthraniloyl)cytidine (308 mg, 0.74 mmol, 53%) as an oil. ^{1}H NMR (DMSO-d_6, TMS) δ 7.70 (1H, m, H-6a), 7.70 (1H, m, H-6), 7.68 (1H, m, H-3a), 7.45 (1H, m, N*H*CH_3), 7.42 (2H, s, NH_2), 6.65 (1H, dd, $J_{3a,\ 4a}$ = J_{4a-5a} = 8.5Hz, H-4a), 6.5 (1H, t, *J* = 8.2 Hz, H-5a), 5.7, (1H, d, *J* = 3.4 Hz, H-1′), 5.62 (1H, d, *J* = 7.4Hz, H-5), 5.0 (1H, m, H-2′), 4.85 (1H, m, H-3′), 4.4 (2H, m, H-5′ and H-5″), 4.3 (1H, m, H-4′), 2.8 (1H, d, *J* = 5Hz, NH-*CH*$_3$), 1.4 (3H, s, CH_3), 1.25 (3H, s, CH_3). A solution of β-D-2′,3′-*O*-(isopropylidene)-5′-*O*-(N-methylanthraniloyl)cytidine (270 mg, 0.65 mmol) in 50% aqueous formic acid (9 mL) was stirred for 48 h at room temperature. After evaporation of the solvent, addition of ethanol and coevaporation, the residual product was chromatographed on silica gel (eluent: gradient of 0–10% ethyl acetate in dichloromethane) then purified by preparative HPLC (eluent: gradient of 0–50% acetonitrile in water in 40 min, 1mL/min), retention time: 20 min. **1** (113 mg, 0.3 mmol, 46%) was obtained as a white solid: m.p. 133–136 °C. MS (FAB > 0, G/T): 377 $[M + H]^+$, 753 $[2M + H]^+$. MS (FAB < 0, G/T): 375 $[M - H]^-$. UV (EtOH-95): λmax 254 (ε 14 800), 274 (ε 8 100). ^{1}H NMR (DMSO-d_6) δ 7.7 (1H, dd, $J_{5a,\ 6a}$ = 8Hz, $J_{4a,\ 6a}$ = 1.6 Hz, H-6a), 7.5 (1H, d, *J* = 7.4Hz, H-6), 7.45 (1H, m, N*H*CH_3), 7.38 (1H, m, H-3a), 7.1 (2H, d, NH_2), 6.65 (1H, dd, $J_{4a,\ 5a}$ = $J_{3a,\ 4a}$ = 8.5 Hz, H-4a), 6.5 (1H, t, *J* = 8.3Hz, H-5a), 5.7 (1H, d, *J* = 3.4Hz, H-1′), 5.59 (1H, d, *J* = 7.4Hz, H-5), 5.45 (1H, s, OH-2′), 5.2 (1H, s, OH-3′), 4.5 and 4.3 (2H, 2m, H-5′ and H-5″), 4.1 (1H, m, H-4′), 3.97 (1H, m, H-3′), 3.94 (1H, m, H-3′), 3.95 (1H, m, H-2′), 2.7 (3H, d, *J* = 5Hz, NH*CH*$_3$). Anal. $C_{17}H_{20}N_4O_6$, H_2O (C, H, N).

4.1.2. β-D-2′(3′)-O-(N-Methylanthraniloyl)cytidine **2**

β-D-5-*O*,N^4-Bis(monomethoxytrityl)cytidine was prepared from cytidine using the procedure previously described for adenosine derivatives [19], after heating the reaction mixture 12 h at 100 °C. The product was purified by chromatography on silica gel (eluent: 0–5% methanol in dichloromethane) as a yellow solid (74%). MS (FAB > 0, G/T): 788 $[M + H]^+$. MS (FAB < 0, G/T):786 $[M - H]^-$, 514 $[M - mMTr]^-$. ^{1}H NMR (DMSO-d_6) δ 7.65 (1H, d, *J* = 7.6Hz, H-6), 7.2 (24H, m, H-arom.rings), 7.0 (1H, m, NH*CH*$_3$), 6.78 (4H, m, H-arom.rings), 6.2 (1H, d, *J* = 7.6Hz, H-5), 5.8 (1H, d, *J* = 5.3Hz, H-1′), 5.7 (1H, s, OH-2′), 4.3 (1H, s, OH-3′), 4.1 (1H, m, H-3′), 3.75 (1H, m, H-2′), 3.7 (6H, s, O*CH*$_3$), 3.65 (1H, m, H-4′), 2.8 (2H, m, H-5′ and H-5″). To a solution of β-D-5′-*O*, *N*^{4}-bis(monomethoxytrityl)cytidine (5 g, 6.3 mmol, 1 eq) in 50 mL of DMF, sodium hydride (305 mg, 7.6 mmol) was added. The solution was then heated to 70 °C and N-methylisatoic anhydride (909 mg, 7 mmol, 1.1 eq) was added under argon. After 4 h, 50 mL of cold water were added and the product extracted with chloroform. After washing and drying, the residual oil was chromatographed on silica gel (eluent: 0.1% methanol in chloroform) to give β-D-5′-*O*, *N*^{4}-bis(monomethoxytrityl)-2′(3′)-*O*-(N-methylanthraniloyl)cytidine (4 g, 4.4 mmol, 70%). MS(FAB > 0, G/T): 921 $[M + H]^+$, 833 $[2M + H]^+$. MS (FAB < 0, G/T): 919 $[M - H]^-$.

A solution of β-D-5′-*O*, *N*^{4}-bis(monomethoxytrityl)-2′(3′)-*O*-(N-methylanthraniloyl)cytidine (3 g, 3.2 mmol) in 80% aqueous acetic acid (50 mL) was stirred for 3 d at room temperature. After evaporation and coevaporation with ethanol, the residue was chromatographed on silica gel (eluent: gradient of 0–10% methanol in dichloromethane). The residual solid was further purified by preparative HPLC (eluent: gradient of 0–50% acetonitrile in water, in 30 min) to give **2** as a solid (50 mg, 0.32 mmol, 62%) directly characterized by MS (retention times: 18.3 and 20.4 min for the 2′-and the 3′-isomer, respectively). M.p. 154–160 °C. UV (EtOH): λmax 254 (ε 15 100), 274 (ε 8 300). MS (FAB > 0, G/T): 377 $[M + H]^+$, 753 $[2M + H]^+$. MS (FAB < 0, G/T): 375 $[M - H]^-$, 751 $[2M - H]^-$. ^{1}H NMR (DMSO-d_6): **2a** (65%): δ 7.9 (1H, dd, $J_{5a,\ 6a}$ = 8Hz, $J_{4a,\ 6a}$ = 1.6 Hz, H-6a), 7.7 (1H, d, *J* = 7.4 Hz, H-6), 7.4 (1H, m, CH_3*NH*), 7.4 (1H, m, H-3a), 7.2 (2H, d, NH_2), 6.7 (1H, dd, $J_{4a,\ 5a}$ ≈ $J_{3a,\ 4a}$ = 8.5 Hz, H-4a), 6.6 (1H, m, H-5a), 5.9 (1H, d, *J* = 5.9 Hz, H-1′), 5.75 (1H, d, *J* = 7.6Hz, H-5), 5.73 (1H, m, OH-2′), 5.18 (1H, m, H-3′), 4.35 (1H, m, H-2′), 4.15 (1H, m, H-4′), 3.65 (2H, m, H-5′ and H-5′′), 2.8 (3H, d, *J* = 3.8Hz, NH*CH*$_3$). **2b** (35%): δ 7.7 (2H, H-6 and H-6a), 7.4 (2H, m, CH_3*NH* and H-3a), 7.2 (2H, d, NH_2), 6.7(1H, dd, $J_{4a,\ 5a}$ ≈ $J_{3a,\ 4a}$ = 8.5 Hz, H-4a), 6.6 (1H, m, H-5a), 6.5 (1H, d, *J* = 5.1Hz, H-1′), 5.75 (1H, d *J* = 7.7Hz, H-5), 5.18 (1H, m, H-3′), 5.1 (1H, m, OH-3′), 4.35 (1H, m, H-2′), 3.9 (1H, m, H-4′), 3.1 (3H, s, NH*CH*$_3$). Anal. $C_{17}H_{20}N_4O_6$,1/2 H_2O (C, H, N).

4.1.3. β-D-5′-O-(N-Methylanthraniloyl)-2′-deoxycytidine **3**

β-D-5′-*O*-*t*-Butyldimethylsilyl-2′-deoxycytidine was prepared in 95% yield from 2′-deoxycytidine using a published procedure [20]. From this compound (1.6 g, 4.7 mmol), β-D-5′-*O*-*t*-butyldimethylsilyl-N^4, 3′-bis-(monomethoxytrityl)-2′-deoxycytidine was prepared in 61% yield using conditions already published [19]. MS (FAB > 0, G/T): 886 $[M + H]^+$, 614 $[M – mMTr + H]^+$, 273$[mMTr]^+$. MS (FAB < 0, GT): 884$[M – H]^–$, 612$[M – mMTr – H]^–$. ^{1}H NMR (DMSO-d_6): δ 7.68 (1H, d, *J* = 7.26 Hz, H-6), 7.5 (24H, m, H-arom.rings), 7.0 (1H, s, CH_3*NH*), 6.91 and 6.8 (2[2H], 2d, *J* = 8.9 and 6.3 Hz, H-arom.rings), 6.3 (1H, d, *J* = 7.1 Hz, H-5), 6.2(1H, s, H-1′), 4.1 (1H, s, H-3′), 3.9 (1H, s, H-4′), 3.8 (6H, 2s, 2 [OCH_3]), 3.49 and 3.2 (2H, 2m, H-5′ and H-5″), 1.6 and 1.38 (2H, 2m, H-2′ and H-2″), 0.8 (9H, s, C-*t*Bu), 0.0(6H, s, Si-*t*Bu). A solution of dry β-D-5′-*O*-*t*-butyldimethylsilyl-N^4, 3′-bis(monomethoxytrityl)-2′-deoxycytidine (2.3 g, 2.6 mmol) in 15 mL of THF was treated with tetrabutylammonium fluoride (2 mL, 1 eq) and stirred for 2 h at room temperature. Addition of 30 mL of cold water, extraction with chloroform, washing with water, drying and evaporation gave a solid which was chromatographed on silica gel (eluent: 0.2% ethyl acetate, 0.2% triethylamine in chloroform) and purified as a white solid (1.76 g, 2.3 mmol, 88%). MS (FAB > 0, G/T): 772 $[M + H]^+$, 544 $[M – mMTr + 2Na^+]^+$, 273$[mMTr]^+$. MS (FAB < 0, G/T): 770 $[M – H]^–$, 534 $[M – mMTr + Cl]^–$, 498 $[M – mMTr]^–$. β-D-N^4,3′-Bismonomethoxytrityl-2′-deoxycytidine (1.71 g, 2.2 mmol) thus obtained was dissolved in 20 mL of dry DMF and treated with sodium hydride (133 mg, 3.3 mmol). A solution of N-methylisatoic anhydride (742 mg, 5.7 mmol) in 5 mL of DMF was added and the mixture was stirred for 10 h at 60 °C. Upon addition of 30 mL of cold water, β-D-5′-*O*-(N-methylanthraniloyl)-N^4,3′-bismonomethoxytrityl-2′-deoxycytidine precipitated as a white solid which was dried (1.4 g, 1. 55 mmol, 90%). MS (FAB > 0, G/T): 905 $[M + H]^+$, 632 $[M – mMTr + H]^+$, 273$[mMTr]^+$. MS (FAB < 0, G/T): 903 $[M – H]^–$, 633 $[M – mMTr – H]^–$. ^{1}H NMR (DMSO-d_6): δ 7.7 (1H, dd, $J_{5a,\ 6a}$ = 8Hz, $J_{6a,\ 4a}$ = 1.55 Hz, H-6a), 7.5 (27H, m, H-6, NH, H-arom.ring and H-5a), 7.1 and 6.85 (2[2H], 2d, *J* = 8.9 and 6.3 Hz, H-arom.rings), 6.7(1H, d, $J_{5a,\ 4a}$ = $J_{3a,\ 4a}$ = 8.4 Hz, H-4a), 6.55 (1H, t, *J* = 8.3 Hz, H-3a), 6.15 (1H, d, *J* = 7.4 Hz, H-5), 5.9 (1H, t, *J* = 6.9 Hz, H-1′), 4.45 and 4.35 (2H, 2m, H-5′ and H-5″), 4.25 (1H, s, H-3′), 3.97 (1H, s, H-4′), 3.6 (2[3H], 2s, 2[OCH_3]), 2.8 (3H, d, *J* = 4.9 Hz, NHCH_3), 1.45 (2H, m, H-2′ and H-2″). A solution of β-D-5′-*O*-(N-methylanthraniloyl)-N^4,3′-bismonomethoxytrityl-2′-deoxycytidine (1.3 g, 1.44 mmol) in 30 mL of 80% aqueous acetic acid was stirred for 48 h at 50 °C. After evaporation and coevaporation of the reaction medium with ethanol, the residue was chromatographed on silica gel (eluent: gradient of 0–12% methanol in chloroform) yielding 400 mg of **3** as a solid which was further purified by HPLC (eluent: gradient of 0–50% acetonitrile in water, in 40 min) as a white solid (350 mg, 1 mmol, 75%). M.p. 215–219 °C. MS (FAB > 0, GT): 361 $[M + H]^+$, 721$[2M + H]^+$, 383$[M + Na^+]^+$, 249[M-MeNHBz + $H]^+$, 134$[MeNHBz]^+$. MS (FAB < 0, G/T): 359$[M – H]^–$, 719$[2M – H]^–$. UV (EtOH 95): λmax 254 (ε14 600), λmax 274 (ε 8 100). ^{1}H NMR (DMSO-d_6) δ 7.75 (1H, dd, $J_{5a,\ 6a}$ = 8Hz, $J_{6a,\ 4a}$ = 1.6 Hz, H-2a), 7.55 (1H, d, *J* = 7.4 Hz, H-6), 7.45 (1H, d, *J* = 5 Hz, CH_3*NH*), 6.35 (1H, t, $J_{3a,\ 5a}$ = 1.5 Hz, $J_{3a,\ 4a}$ = 8.5 Hz, H-5a), 7.2 (2H, d, NH2), 6.75 (1H, d, $J_{5a,\ 4a}$ = $J_{3a,\ 4a}$ = 8.5 Hz, H-4a), 6.6 (1H, t, H-3a), 6.15 (1H, t, *J* = 7.5 Hz, H-1′), 5.6 (1H, d, *J* = 7.4 Hz, H-5), 5.4 (1H, s, OH-3′), 4.4 (1H, m, H-5′), 4.35 (1H, m, H-5″), 4.3 (1H, m, H-3′), 4.05 (1H, m, H-4′), 2.8 (3H, d, *J* = 5 Hz, NHCH_3), 2.2 (1H, m, H-2′), 2.05 (1H, m, H-2″). Anal. $C_{17}H_{20}N_4O_5$ (C, H, N).

4.1.4. β-D-3′-O-(N-Methylanthraniloyl)-2′-deoxycytidine **4**

β-D-5′-*O*-Monomethoxytrityl-2′-deoxycytidine was prepared from 2′-deoxycytidine using a previously reported procedure for adenosine derivatives [19], after heating the reaction mixture for 48 h at 40 °C. The product was chromatographed on silica gel (eluent: gradient of 0–12% methanol in chloroform) and obtained as a solid (49%). MS (FAB > 0, G/T): 500 $[M + H]^+$, 999 $[2M + H]^+$, 1 498 $[3M + H]^+$. MS (FAB < 0 G/T): 498 $[M – H]^–$, 534 $[M + Cl]^–$, 997 $[2M – H]^–$, 1 033 $[2M + Cl]^–$. ^{1}H NMR (DMSO-d_6) δ 7.65 (1H, d, *J* = 7.6 Hz, H-6), 7.1 (14H, m, H-arom.rings), 7.1 (2H, m, NH_2), 6.75 (2H, d, *J* = 8.8 Hz, H-arom.rings), 5.98 (1H, t, *J* = 6.3, H-1′), 5.55 (1H, d, *J* = 7.6 Hz, H-5), 5.22 (1H, d, *J* = 3.9 Hz, OH-3′), 4.1 (1H, m, H-3′), 3.75 (1H, m, H-4′), 3.6 (3H, s,-O-CH_3), 3.1 (2H, m, H-5′ and H-5″), 2.1 and 1.95 (2H, 2m, H-2′ and H-2″). To a solution of β-D-5′-*O*-monomethoxytrityl-2′-deoxycytidine (1.9 g, 3.8 mmol, 1 eq) in 20 mL of DMF, sodium hydride (167 mg, 4.2 mmol) then N-methylisatoic anhydride (742 mg, 5.7 mmol, 1.5 eq) in 5 mL of DMF were added, and the mixture was stirred for 36 h at room temperature. After addition of 20 mL of cold water, extraction with 100 mL of chloroform then with 50 mL of ethylacetate, the product was chromatographed on silica gel (eluent: gradient of 0–5% methanol in chloroform) to yield 1 g of a mixture of two compounds. MS (FAB > 0, G/T): 634 $[M + 2H]^+$, 833 $[2M + H]^+$, 273$[mMTr]^+$. MS (FAB < 0,

G/T): 631 $[M - H]^-$, 1 263 $[2M - H]^-$, 667 $[M + Cl]^-$, 764$[M - 2H]^{2-}$.

The mixture was dissolved in 30 mL of 80% aqueous acetic acid and the solution was stirred for 3 d at room temperature. After evaporation, then coevaporation with ethanol, the residue was chromatographed on silica gel (eluent: gradient of 0–12% methanol in chloroform) then purified by HPLC (eluent: gradient of 0–50% acetonitrile in water, for 40 min) which yielded 2 products: (i) **4** (300 mg, 0.83 mmol, 22%) as a white solid (m.p. 223–227 °C). HPLC/UV/MS: Retention time 26.2 min, MS ESI > 0: 361 $[M + H]^+$, 462 $[M + NEt_3 + H]^+$, 721 $[2M + H]^+$. MS (FAB > 0, GT): 361 $[M + H]^+$, 721 $[2M + H]^+$, 743 $[2M + Na^+]^+$. MS (FAB < 0, G/T): 359 $[M - H]^-$. UV (EtOH 95: λmax 254 (ε 15 600), λmax 274 (ε 8 600). ^{1}H NMR (DMSO-d_6) δ 7.85 (2H, m, H-6 and H-6a), 7.58 (1H, m, CH_3*NH*), 7.4 (1H, t, $J_{3a,\ 5a}$ = 1.5Hz, $J_{3a,\ 4a}$ = 8.5 Hz, H-3a), 7.19 (2H d, NH_2), 6.7 (1H, d, $J_{5a,\ 4a}$ = 8.5 Hz, H-4a), 6.6 (1H, t, $J_{5a,\ 4a}$ = 8.5 Hz, $J_{5a,\ 6a}$ = 8Hz, H-5a), 6.25 (1H, dd, *J* = 5.63 and 8.6Hz, H-1′), 5.8 (1H, d, *J* = 7.4 Hz, H-5), 5.35 (1H, d, *J* = 5.9 Hz, H-3′), 5.21 (1H, s, OH-5′), 4.1 (1H, d, *J* = 1.9Hz, H-4′), 3.7 (2H, s, H-5 and H-5″), 2.8 (3H, d, *J* = 5Hz, NH*CH*$_3$), 2.4 and 2.3 (2H, 2m, H-2′ and H-2″). Anal. $C_{17}H_{20}N_4O_5$: C, 56.66; H, 5.59; N, 15.55. Found: C, 56.26; H, 5.55; N, 14.99. (ii) **5** (130 mg, 0.26 mmol, 7%) as a white solid (m.p. 226–229 °C). HPLC/UV/MS: Retention time 29.6 min. MS ESI > 0: 494 $[M + H]^+$, 595 $[M + NEt_3 + H]^+$, 988 $[2M + 2H]^{2+}$. MS (FAB > 0, GT): 494 $[M + H]^+$, 228$[M - 2MeNHBz + H]^+$, 134$[MeNHBz]^+$. MS (FAB < 0, GT): 492 $[M - H]^-$, 985 $[2M - H]^-$. UV (EtOH 95): λmax 257 (ε 19 300), λmax 283 (ε 15 200). ^{1}H NMR (DMSO-d_6) δ 7.9 (1H, dd, *J* = 7.7 and 2.8 Hz, H-6, 7.78 (2H, m, 2 H-6a), 7.48 (7H, m, 2NH, H-3a and H arom.ring), 6.7 (1H, d, $J_{3a,\ 4a} = J_{5a,\ 4a}$ = 8.5 Hz, H-4a), 6.6 (1H, t, $J_{5a,\ 6a}$ = 7.5 Hz, H-3a), 6.28 (1H, t, *J* = 6.9 Hz, H-1′), 5.4 (1H, t, *J* = 6.7 and 7.8 Hz, H-3′), 5.23 (1H, dd, *J* = 7.7 and 2.8 Hz, H-5), 5.21 (1H, s, OH-5′, 4.1 (1H, s, H-4′), 3.65 (2H, s, H-5 and H-5″), 3.3 (3H, s, NH*CH*$_3$), 2.8 (3H, d, *J* = 5 Hz, NH*CH*$_3$), 2.4 and 2.2 (2H, 2m, H-2′ and H-2″). Anal. $C_{25}H_{27}N_5O_6$ (C, H, N).

4.2. Kinetic experiments

Recombinant human deoxycytidine kinase has been prepared as previously published [21, 22] with a histidine tag sequence. It was more than 90% pure and had very similar properties as compared to the enzyme without a tag sequence. The enzyme specific activity was 240 U/mg protein, 1 unit of enzymatic activity being defined as the amount of enzyme that catalyzes the phosphorylation of 1 nmol of β-D-2′-deoxycytidine per min at 37 °C.

The enzyme activity was measured by HPLC analysis of the reaction medium which contained 50 mM Tris HCl pH 7.5, 5 mM ATP, 1 mM dithiothreitol, 5 mM $MgCl_2$, and the enzyme (45 mg/mL). After 10 min of preincubation at 37 °C, the substrate (10–50 μM) was added. Analyses of the reaction mixture were performed by HPLC on a Hypersil ODS 3μ column with the following eluent (A: Triethylammonium acetate 20 mM pH 6.6, B: Triethylammonium acetate and 50% of acetonitrile): 10 min of 90% A + 10% B, 15 min from 90% A + 10% B to 100% B, 10 min of 100% B (1 mL /min). The retention times in min of each substrate and its 5′-monophosphate (when observed) were, respectively: **2** (23.4 and 25.3, 21.2 broad), **4** (27.4, 23.2), **5** (29.6). The products were characterized by UV and by their mass spectrum which is directly determined by connecting the HPLC chromatograph to the MS spectrometer (mode ESI < 0). The method did not allow reliable determination of kinetic parameters at low substrate concentration. For this reason, initial concentrations of substrates were kept above 10 μM. In each case, the reaction was run until about 20% of the substrate had been transformed. For each concentration of substrate, the kinetic curves were determined by at least three measurements of substrate transformation as a function of time. The kinetic parameters were determined from initial rates with the Lineweaver-Burk method and the Grafit program (Erithacus Software, 1992). The compounds **1–5** were weak or very weak inhibitors of dCK since a large excess (100 μM) was needed in all cases to detect the inhibition with respect to 20 μM β-D-2′-deoxycytidine as substrate: 30% for **1** and **2**, 55% for **3**, 50% for **4** and 0% for **5**, under the same conditions as in substrate studies.

4.3. Fluorescence measurements

The experiments were performed on a SLM-Smart 8 000 spectrofluorometer equipped with a 450 W lamp. The tryptophan intrinsic fluorescence was excited at 290 nm to minimize the substrate inner-filter effect, and the emission was measured at 332 nm with a spectral band pass of 2 nm or 4 nm for excitation or emission, respectively. Experiments were carried out at 25 °C in 50 mM Tris HCl pH 7.5, 100 mM KCl, 1 mM dithiothreitol, 5 mM $MgCl_2$ and 10% glycerol. In measurements of intrinsic tryptophan fluorescence variations, the enzyme (0.5 μM) was equilibrated for 2 h in the fluorescence buffer then titrated with each ligand (0–600 μM). The results were corrected for dilution and for internal quenching as previously described [23]. Curve fitting was performed with the program Grafit using the equations

(1) or (2) which assume the existence of one or two binding sites or enzyme states, respectively:

(1) $F = Fmin–\{(Eo + Lo + Kd) – [(Eo + Lo + Kd)^2 – 4EoLo]^{0.5}\}(\Delta F)/2Eo$

where F is the observed relative fluorescence intensity, Fmin is the fluorescence intensity at the beginning of the titration, ΔF is the variation of fluorescence intensity between the initial value and at saturating concentration of substrate, Eo is the total enzyme concentration, Lo is the total ligand concentration and Kd the dissociation constant of the enzyme-ligand complex;

(2) $F = Fmin – (\{(Eo + Lo + Kd_1) – [(Eo + Lo + Kd_1)^2 – 4EoLo]^{0.5}\}(F_1)/2Eo + [F_2Lo/(Kd_2 + Lo)])$

where F_1 and F_2 are the fluorescence quenching values induced by the first and the second binding respectively, Kd_1 and Kd_2 are the corresponding dissociation constants. The Grafit program was used to determine the standard errors and to compare the curve fits corresponding to the equations (1) or (2). All calculations were performed assuming that the stoichiometry of binding is one ligand per dimer of protein [5].

In experiments using the fluorescence of MeNHBz-derivatives, a solution of a fixed concentration of fluorescent ligand (0.1 μM) in the fluorescence buffer (700 mL) was excited at 350 nm and the emission observed at 440 nm with a spectral band pass of 4 or 8 nm for excitation or emission, respectively. The MeNHBz-nucleoside was equilibrated at 25 °C then titrated with the enzyme up to a concentration of 1.5 μM or 5μM depending on the concentration of the ligand, and the increase of fluorescence intensity corrected for dilution. In attempting to determine the dissociation constants by this method, the equations (1) and (2) were used with the following modifications: the minus sign after Fmin was replaced by a plus sign in both equations, and F_1 and F_2 are the fluorescence increases induced by the first and the second binding, respectively.

References

[1] Arner E.S.J., Eriksson S., Pharmacol. Ther. 67 (1995) 155–186.

[2] Kim M.Y., Ives D.H., Biochemistry 28 (1989) 9043–9047.

[3] Datta N.S., Shewach D.S., Mitchell B.S., Fox I.H., J. Biol. Chem. 264 (1989) 9359–9364.

[4] Sarup J.C., Fridland A., Biochemistry 26 (1987) 590–597.

[5] Kierdaszuk B., Rigler R., Eriksson S., Biochemistry 32 (1993) 699–707.

[6] Furman P.A., Wilson J.E., Reardon J.E., Painter G.R., Antivir. Chem. Chemother. 6 (1995) 345–355.

[7] Nair V., Jahnke T.S., Antimicrob. Agents Chemother. 39 (1995) 1017–1029.

[8] Shafiee M., Griffon J.F., Gosselin G., Cambi A., Vincenzetti S., Vita A., Eriksson S., Imbach J.-L., Maury G., Biochem. Pharmacol. 56 (1998) 1237–1242.

[9] Pelicano H., Pierra C., Eriksson S., Gosselin G., Imbach J.-L., Maury G., J. Med. Chem. 40 (1997) 3969–3973.

[10] Hiratsuka T., Biochim. Biophys. Acta 742 (1983) 496–508.

[11] Woodward S.K.A., Eccleston J.F., Geeves M.A., Biochemistry 30 (1991) 422–430.

[12] Rensland H., Lautwein A., Wittinghofer A., Goody R.S., Biochemistry 30 (1991) 11181–11185.

[13] Pelicano H., Maury G., Elalaoui A., Shafiee M., Imbach J.-L., Goody R.S., Divita G., Eur. J. Biochem. 248 (1997) 930–937.

[14] Delahunty M.D., Wilson S.H., Karpel R.L., J. Mol. Biol. 236 (1994) 469–479.

[15] Rao R., Al-Shawi M.K., Senior A.E., J. Biol. Chem. 263 (1988) 5569–5573.

[16] Holy A., Collect. Czech. Chem. Commun. 37 (1972) 4072–4087.

[17] Gosselin G., Mathe C., Bergogne M.C., Aubertin A.-M., Sommadossi J.-P., Schinazi R., Imbach J.-L., Nucleosides Nucleotides 14 (1995) 611–617.

[18] Urata H., Ogura E., Shinohara K., Ueda Y., Akagi M., Nucleic Acids Res. 20 (1992) 3325–3332.

[19] Herdewijn P., Pauwels R., Baba M., Balzarini J., De Clercq E., J. Med. Chem. 30 (1987) 2131–2137.

[20] Ogilvie K.K., Can. J. Chem. 51 (1973) 3799–3807.

[21] Chottinger E.G., Shewach D.S., Data N.S., Ashcraft E., Gribbin D., Ginsburg D., Fox I.H., Mitchell B.S., Proc. Natl. Acad. Sci. USA 88 (1991) 1531–1535.

[22] Usova E., Eriksson S., Eur. J. Biochem. 248 (1997) 762–766.

[23] Elalaoui A., Divita G., Maury G., Imbach J.-L., Goody R.S., Eur. J. Biochem. 221 (1994) 839–846.