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ARTICLE

Synthesis and study of (*R*)- and (*S*)-β-hydroxyphosphonate acyclonucleosides as structural analogues of (*S*)-HPMPC (Cidofovir)

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A synthetic pathway to new acyclonucleoside phosphonates, designed as analogues of Cidofovir, is described. The reduction of a β-ketophosphonate intermediate, readily available from the nucleobase and benzylacrylate, afforded an enantiomeric mixture of (*R*)- and (*S*)-β-hydroxyphosphonate derivatives which was resolved. The assignment of the absolute configuration was proposed on the basis of NMR studies. The influence of this modification, presence of the hydroxyl group and chirality on the β-position related to the phosphorus atom, on antiviral activity against a broad variety of DNA and RNA viruses and also on the capacity to be recognized as substrates by human NMP kinases was investigated.

1. Introduction

Current search for novel antiviral chemotherapy focuses primarily on the outburst of (re-)-emerging viral diseases, such as new strains of herpes viruses, hepatitis viruses and H1N1, HHV-6, SRAS coronavirus, Influenza, Ebola virus, West Nile virus, as well as a number of exotic viruses, which although still located in small areas of the world, have the potential for pandemic outbreak.[1] Notably, considerable progress remains to be made in these last areas. The clinical fruit of all of these research advances has been an armamentarium of drugs that can be used to successfully treat a variety of viral illnesses. Among the various classes of antiviral drugs, nucleos(t)ide analogues[2, 3] and especially acyclic nucleoside phosphonates (ANPs)[4] have received considerable attention, due to their ability to inhibit viral DNA polymerases and reverse transcriptase, which play key roles in the viral cycles. The synthesis and study of novel series of ANPs are still one of the promising areas for the development of new compounds that may interfere selectively with viral proliferation. ANPs belong to the family of modified nucleotide analogues, in which the sugar moiety has been replaced by a functionalized acyclic chain linking the nucleobase at one end and the phosphonic acid group at the other. Therefore, ANPs are metabolically stable due to the presence of a phosphonate linkage (P–C) instead of the labile phosphoester (P–O), making these compounds resistant towards enzymatic hydrolysis. Furthermore, they do not require the first intracellular phosphorylation step which is often considered as a limiting-step within the metabolic pathway leading to the biologically relevant derivative (i.e. the 5'-triphosphate nucleoside analogue). Indeed, ANPs share their mechanism of action with other nucleoside analogues. Briefly, after two subsequent phosphorylation steps the ANP is converted to its corresponding triphosphate derivative, which can then interfere with nucleic acid biosynthesis.[4]

In 1996, (*S*)-HPMPC namely Cidofovir (a cytosine containing ANP, Fig. 1, compound **1**) became the first approved drug for the treatment of CMV retinitis in AIDS patients.[5] In the literature, several series of ANPs have been reported and modifications are mainly involving two structural changes: 1) keeping C-O-C functionality and varying the nature of the side chain[6] and/or the nucleobase,[7] 2) bio-isosteric replacement of the C-O-C functionality with other functional group.[8, 9] These literature data prompted us to design new ANPs as potential anti-infectious agents, and as a part of our ongoing research program we reported herein the synthesis and the study of novel derivatives structurally related to (*S*)-HPMPC. These last incorporate cytosine as nucleobase and possess a carbinol (CH-OH) moiety in place of the oxygen atom of the phosphonomethoxyethyl (C-O-C) group of the acyclic chain (Fig. 1). We assumed that the resulting β-hydroxyphosphonate derivatives will present similar structural features such as distance in between the nucleobase and the phosphonate moiety, the flexibility which enables the compounds to adopt a conformation suitable for interaction with the active site of the phosphorylating enzymes (i.e. kinases) as well as their targets (i.e. viral polymerases). We also postulated that the secondary β-hydroxyl group may allow the formation of supplementary hydrogen bonds and it might also mimic one of the hydroxyl functions of the natural sugar counterpart (either ribo- or deoxyribofuranose), which is important for the interaction with metabolic enzymes and / or with polymerases.

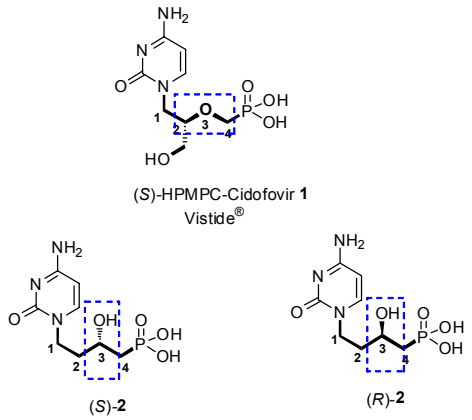


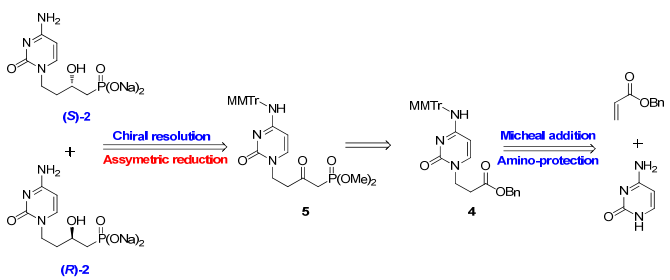
Fig. 1 Structures of a cytosine containing marketed ANP (Vistide®) and of the target analogues.

Synthesis of enantiomerically pure β-hydroxyphosphonate derivatives received significant attention due to their potential biological importance.[10] Thus, there are numbers of methods for their asymmetric synthesis reported in the literature[10-12] such as stereoselective reduction[10, 11] or chemoenzymatic[12] reduction of the corresponding β-ketophosphonate intermediate or kinetic resolution,[13] as well as the use of chiral derivatizing agents[10, 14, 15] from racemic mixture of β-hydroxyphosphonates. Herein, we report the synthesis and resolution of enantiomerically pure (*R*)- and (*S*)-β-hydroxyphosphonate derivatives (compound 2, Fig. 1) and the use of (*S*)- methoxyphenyl acetic acid [(*S*)-MPA] as chiral derivatizing agent. Then compounds were evaluated in vitro for antiviral activity and their potential phosphorylation by human NMP kinases was also studied.

2. Results and discussion

2.1. Chemistry

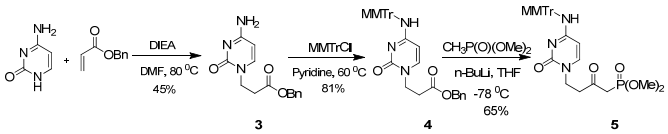
The retro-synthesis proposed for the preparation of the target β-hydroxyphosphonate derivatives (*R*)-**2** and (*S*)-**2** is shown in Scheme 1. Both isomers may be obtained via reduction of the corresponding β-ketophosphonate intermediate **5** via asymmetric reduction using chiral reducing agents or resolution of the diastereomeric pair with a suitable chiral derivatizing agent. The assignment of the absolute configuration would then be addressed using NMR spectroscopy. The key intermediate **5** could be prepared through treatment of a suitable ester (compound 4, scheme 1) with the lithium salt of dimethylmethylphosphonate. Compound **4** would be available through the Michael addition of cytosine to benzyl acrylate as the Michael acceptor.



Scheme 1 Retrosynthesis for derivatives (*R*)-**2** and (*S*)-**2**

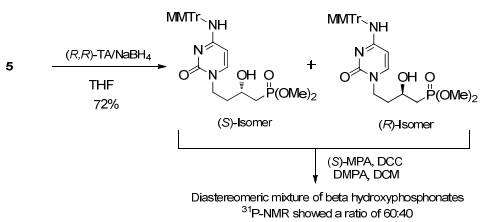
In the literature, such Michael additions suffer from low yields and lack of regioselectivity. We recently reported such kind of regioselective Michael addition reaction with adenine as nucleobase using diisopropylethylamine (DIEA) as a suitable base,[16] allowing the exclusive formation of the *N*-9 alkylated product in good yield. Similar conditions were applied to cytosine and exclusive *N*-1 alkylated derivative was obtained in 45% yield (compound 3, scheme 2). After removal of the solvent, the addition of ethyl acetate to the crude afforded pure **3** as a precipitate without further purification. The exclusive formation of the *N*-1 alkylated product was confirmed by NMR and UV absorption studies.[17]

The exocyclic amine function of **3** was then protected with a monomethoxytrityl (MMTr) group to give rise to **4** in 88% yield (Scheme 2). The MMTr moiety was chosen as nucleobase protecting group to enhance the solubility of such intermediate in less polar solvents, commonly used in the next step of the synthesis (such tetrahydrofuran or dichloromethane). Compound **4** was treated with an excess of the lithium salt of dialkylmethylphosphonate at low temperature and afforded the desired β-ketophosphonate **5** in 65% yields.



Scheme 2 Synthesis of β -ketophosphonate intermediate **5**

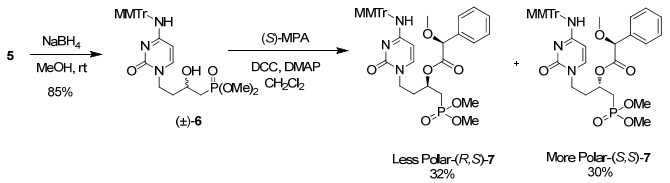
With the key intermediate **5** in hand, we initially envisaged to apply an asymmetric reduction methodology. Indeed, Nesterov et al[18] reported an efficient and stereoselective synthesis of α- and β-hydroxyphosphonates from the corresponding ketophosphonates via asymmetric reduction using a chiral reactant derived from sodium borohydride and natural (*R,R*)- or unnatural (*S,S*)-tartaric acid. Thus, we tested this straight ford method and treatment of **5** with $NaBH_4$ in presence of (*R, R*)-tartaric acid gave a mixture of the two β-hydroxyphosphonates in 72% yield (Scheme 3). The enantioselectivity of this step was evaluated afterward by derivatization of the crude mixture with (*S*)-MPA in the presence of 1,3-dicyclohexylcarbodiimide (DCC) and 4-*N*,*N*-(dimethylamino)pyridine (DMAP) in dichloromethane. ^{31}P-NMR spectra showed two clearly distinct signals in the ratio of 60/40 that corresponds to an estimated 20% diastereomeric excess.



Scheme 3 Asymmetric reduction of **5** using $NaBH_4$/(*R, R*)-tartaric acid (TA) as chiral reactant.

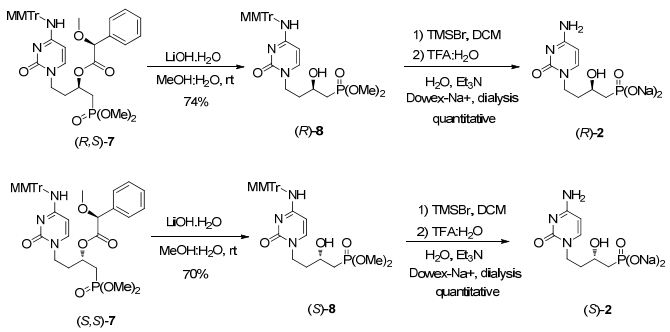
Due to the poor stereoselectivity of this reaction when applied to our substrate, we then decided to perform the standard reduction of the β-ketophosphonate followed by a chiral resolution step using (*S*)-methoxyphenylacetic acid [(*S*)- MPA], a well-known chiral derivatizing agent (scheme 4). Thus, reduction of compound **5** was carried out with $NaBH_4$ in methanol at room temperature and

afforded a racemic mixture of corresponding β-hydroxyphosphonates (±)-**6** in 85% yield. Then, coupling of (±)-**6** with (*S*)-MPA in the presence of DCC and a catalytic amount of DMAP provided an equimolar mixture of the two mandelates which were separated by silica gel column chromatography (Scheme 4). The less polar (*R*,*S*) and more polar (*S*,*S*) diastereomers **7** were isolated in 32% and 30% yields, respectively.



Scheme 4 Synthesis and chiral resolution of the diastereomeric pair: (*R*,*S*)-**7** and (*S*,*S*)-**7.**

Once the absolute configuration was determined by NMR spectroscopy, the final steps of the synthesis were separately carried out for both derivatives (Scheme 5). Treatment of the mandelates (*R*,*S*)-**7** or (*S*,*S*)-**7** with LiOH.H_2O, in a mixture of MeOH/H_2O (8:2) at room temperature, led to enantiomerically pure (*R*)-**8** or (*S*)-**8** after flash column purification in 74% and 70% yields, respectively. Finally, these (*R*)- and (*S*)- isomers were treated with an excess of trimethylsilylbromide (TMSBr) followed by treatment with TFA:H_2O mixture at room temperature to remove both the dimethylphosphonate and MMTr protecting groups in two successive steps, and afford the desired derivatives (*R*)-**2** and (*S*)-**2** in quantitative yields after passing through a Dowex Na^+ ion exchange resin and dialysis.



Scheme 5 Final steps of the synthesis of ANPs (*R*)-**2** and (*S*)-**2**.

2.2. Assignment of the absolute configuration by NMR studies

After separation, the absolute configuration of each diastereoisomers was proposed on the basis of the study of chemical shift differences in NMR spectra.[19] According to the Newman projections (Fig. 2) the substituent under the shielding cone of the phenyl ring from (*S*)-MPA moved up field.[20] When phenyl group is eclipsed with the cytosinyl-methylene moiety (Cy-CH_2-CH_2) in the less polar diastereoisomer the two methylene groups are shielded and should appear upfield than the more polar one. Indeed, we observed that the Cy-CH_2-*CH_2* signal resonates at 1.96 ppm in the less polar diastereoisomer whereas it resonates at 2.08 ppm in the more polar one (Δδ 0.12 ppm). Similarly the Cy-*CH_2*-CH_2 resonates at 3.16 ppm in the less polar diastereoisomer, whereas it resonates at 3.33 ppm in the more polar one (Δδ 0.17ppm). On the other hand, when phenyl group is eclipsed with the $(CH_3O)_2P$ and the $CH_2P(O)$ groups, as in more polar diastereoisomer, both methyl and methylene protons are shielded and should appear upfield with respect to the less polar one. Indeed, the two methyl protons of the $(CH_3O)_2P$ group resonate at 3.53 and 3.5 ppm in the more polar diastereoisomer (Fig. 2), whereas in the less polar one they resonate at 3.67 and 3.65 ppm (Δδ 0.14 and Δδ 0.15 ppm) respectively. Similarly the protons of the *CH_2*P(O) group resonate at 1.86 and 1.92 ppm in the more polar diastereoisomer whereas in less polar one they appeared at 2.03 and 2.08 ppm (Δδ 0.17 and Δδ 0.16 ppm) respectively. In addition, chemical shift differences were also observed in the ^{13}C and ^{31}PNMR spectra of the two diastereoisomers and are summarized in Table 1.

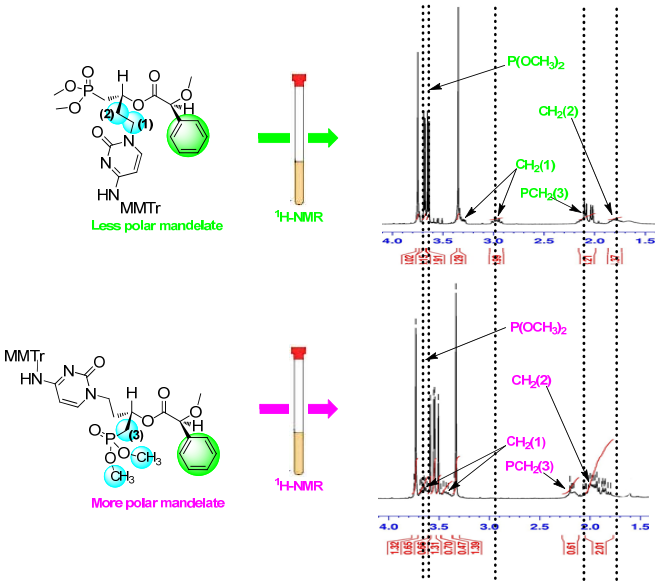


Fig. 2 Assignment of the absolute configuration of the diastereomeric pair based on the NMR chemical shift differences due to the shielding effect exerted by the anisotropy cone of the phenyl ring from (*S*)-MPA.

Table 1 Chemical shift data of the diastereomeric pair of β-hydroxyphosphonates (*R*,*S*)-**7** and (*S*,*S*)-**7**

Entry	Group	Less polar-**7**	More polar-**7**	Δδ
	^{1}H-NMR (300 MHz)			
1	$CH_2P(O)$	2.03	1.86	0.17
		2.08	1.92	0.16
2	$(CH_3O)_2P$	3.65	3.50	0.15
		3.67	3.53	0.14
3	Cy-CH_2CH_2	1.96	2.08	0.12
4	Cy-CH_2CH_2	3.16	3.33	0.17
5	H-5 (Cytosinyl)	5.97	6.59	0.62
	^{31}P-NMR (121 MHz)			
6	$(CH_3O)_2P$	28.03	27.83	0.2
	^{13}C-NMR (75 MHz)			
7	$CH_2P(O)$	29.8	29.5	0.3
8	Cy-CH_2CH_2	33.8	33.6	0.2
9	Cy-CH_2CH_2	46.4	46.33	0.1

* Spectra were recorded in $CDCl_3$

Furthermore, the ^{31}P NMR chemical shift for more polar diastereomer was 27.83 ppm while for less polar diastereomer was 28.03 ppm, giving a difference of 0.2 ppm. Similarly, π-stacking

effect was also observed in cytosine derivatives. When phenyl group was eclipsed with the nucleobase, as in the less polar diastereoisomer, the H-5 proton of the nucleobase was shielded and resonates at 5.97 ppm whereas in more polar one it appears at 6.59 ppm (Δδ 0.62 ppm). Consequently, the careful study of the ^{1}H-, ^{13}C- and ^{31}P-NMR spectra of both isolated diastereoisomers strongly suggest that the absolute configuration of the less polar compound is (*R*,*S*)-**7** while the more polar compound is (*S*,*S*)-**7**.

2.3. Biological evaluation and structure-based molecular docking

Compounds (S)-**2** and (R)-**2** were evaluated for antiviral activity in cell culture experiments (including HEL, CRFK, Vero, MDCK and HeLa cells) toward the following viruses: varicella zoster virus (VZV), cytomegalovirus (CMV), herpes simplex virus type 1 (HSV-1,KOSand TK- KOS), HSV-2 (G), vaccinia virus and vesicular stomatitis viruses, feline herpes and corona viruses, parainfluenza- 3, reovirus-1, Sindbis, Coxsackie B4 and Punta Toro virus, influenza A (H1N1 and H3N2) and influenza B viruses and respiratory syncytial virus. However, none of them revealed a significant effect up to 100 µM. Cytotoxicity was also evaluated on HEL, Vero, HeLa and MDCK cell cultures and the studied compounds were found to be non-toxic (up to 100 µM).

The biological effect of ANPs is highly dependent of their sequential phosphorylation by cellular kinases to their corresponding mono- and diphosphate derivatives, which may act as potential inhibitors of targeted polymerases. The first step in this metabolization process being mediated by nucleoside monophosphate kinases (NMP), we investigated the capacity of our compounds to act as substrates for related proteins (hTMPK and hUMP-CMPK) in comparison to natural nucleotides and cidofovir. The presence of the β-hydroxyphosphonate moiety prevents any reaction of the two novel derivatives with the tested enzymes namely human and vaccinia virus TMP kinase as well as human UMP-CMP kinase.

As UMP-CMP kinase is responsible for the first stage of cidofovir activation,[21] we thus compared the positioning of our analogues to this of cidofovir by means of structure-based docking calculations. The best poses were analyzed. The analysis revealed a greater flexibility of both enantiomers of compound **2** that may be associated to the presence of the carbinol moiety within the acyclic chain compared to cidofovir (Figure 3A-D). Thus, the phosphonomethoxyethyl linkage in cidofovir allows a positioning of the phosphonate group favourable to the phosphotransfer through a stabilizing hydrogen bond network and a proper orientation towards the ATP site (Figure 3B-D). Nevertheless, one should note that as initially proposed the β-hydroxyl function on the acyclic chain of our derivatives promotes the formation of an additional hydrogen bond with the guanidine group of Arg 151.

3. Conclusion

In conclusion, we have synthesized, separated and characterized a new set of enantiomerically pure (*R*)- and (*S*)-β-hydroxyphosphonic acids belonging to the cytosine family. These derivatives were designed as structural analogues of (*S*)-HPMPC (Cidofovir), a well-known antiviral ANP drug.

These novel ANPs were assayed in various cell lines for antiviral activity against a wide variety of DNA and RNA viruses. No activity was found nor was any cytotoxicity to the host cells.

Further studies were undertaken to tentatively explain the lack of biological activity and we observed that none of these derivatives were recognized and phosphorylated by the appropriate human NMP kinases. Structure-based molecular docking allowed addressing structural determinant of this lack of reactivity towards human UMP-CMP kinase in particular.

These data highlighted that the margins of structural alteration within the acyclic nucleotides' skeleton are really narrow and contribute to the global SAR study aiming to identify new ANPs as potential antiviral agents.

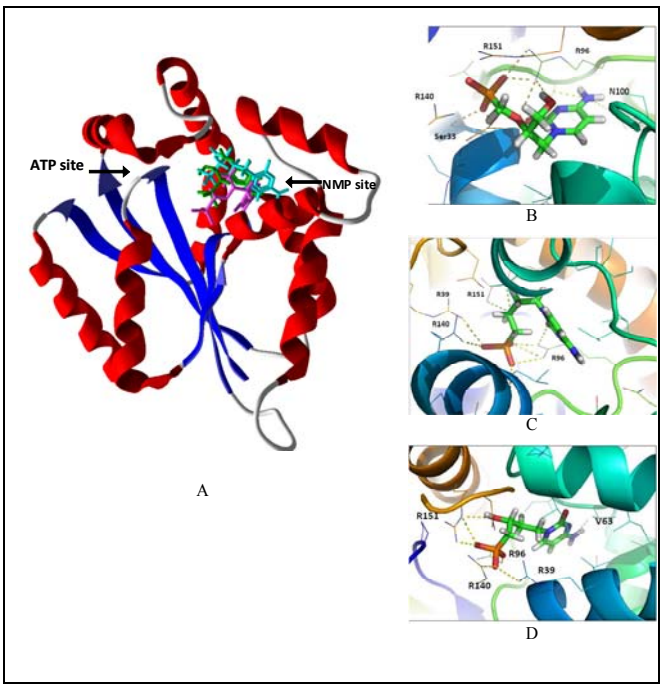


Fig. 3 **Putative positioning of our novel ANPs compared to cidofovir within hUCK NMP site**. A. Overall view of the superimposition of the three molecules within hUCK NMP site (Cidofovir in magenta, (*S*)-**2** in green and (*R*)-**2** in cyan). Positioning of cidofovir (B), Positioning of (*S*)-**2** (C), and (*R*)-**2** (D). Structure of hUCK was obtained from this of *Dictyostelium discoideum* (pdb code : 2UKD), ANP analogues are shown as sticks using CPK colours, key residues are indicated and hydrogen bonds are highlighted by yellow dotted lines.

4. Experimental section

4.1. General conditions

Tetrahydrofuran (THF) was distilled from sodium/benzophenone immediately prior to use, DMF and methanol from CaH_2, dichloromethane from P_2O_5, pyridine and Et_3N from KOH. Solids were dried over P_2O_5 under reduced pressure at rt. Moisture sensitive reactions were performed under argon atmosphere using oven-dried glassware. ^{1}H-NMR spectra were recorded at 300 MHz and ^{13}C-NMR spectra at 75 MHz with proton decoupling at 25°C using a Bruker 300 Advance. Chemical shifts are given in δ values referenced to the residual solvent peak ($CHCl_3$ at 7.26 and 77 ppm or DMSO-d_5 at 2.49 and 39.5 ppm) relative to TMS. COSY experiments were performed in order to confirm proton assignments. Coupling constants, *J*, are reported in Hertz (Hz). 2D ^{1}H–^{3}C heteronuclear COSY spectra were recorded for the attribution of ^{13}C signals. ^{31}P NMR spectra were recorded at ambient temperature at 121 MHz with proton decoupling. Chemical shifts are reported

relative to external H_3PO_4. Specific rotations were measured with a Perkin–Elmer Model 341 spectropolarimeter (path length 1 cm) and are given in units of 10^{-1} deg.cm^2.g^{-1}. TLC was performed on pre-coated aluminium sheets of silica gel 60 F_{254} (Merck, Art. 9385), visualization of products being accomplished by UV absorbance followed by charring with 5% ethanolic sulphuric acid and then heating.

4.2. *N*-9-(2-benzyloxycarbonylethyl)cytosine 3

To a suspension of cytosine (2.5 g, 22.5mmol) in dry DMF (25mL) diisopropylethylamine (9.82mL, 56.25mmol) was added and the mixture was heated at 80°C for 1h under argon. Then, the reaction mixture was cooled to room temperature and benzylacrylate (9.12g, 56.25mmol) added. The mixture was heated at 80°C for 3 days. The solvent was evaporated under high pressure and the crude was suspended in ethyl acetate and filtered, the resulting solid was washed with ethyl acetate to give 3 as a fine powder (2.9 g, 45% yield). λ_{max} (EtOH 95)/nm 273 (ε/dm^3 mol^{-1} cm^{-1} 7600). δ_H (300 MHz; DMSO-d_6) 2.74 (2H, t, *J* = 6.6Hz, Cy-CH_2-C*H*$_2$), 3.85 (2H, t, *J* = 6.6Hz, Cy-C*H*$_2$-CH_2), 5.09 (2H, s, CH_2-Ph), 5.58 (1H, d, *J* = 6Hz, 6-H), 7.01 (2H, br d, *J* = 12.3Hz, NH_2) 7.36 (5H, m, H_{arom}), 7.51 (1H, d, *J* = 7Hz, 5-H). δ_C (75 MHz, DMSO-d_6) 32.8 (Cy-CH_2-*C*H$_2$), 45.3 (Cy-*CH*$_2$-CH_2), 65.6 (*CH*$_2$-Ph), 93.0 (C-5), 127.9, 127.9, 128.4, 135.9, 146.4 (6-C), 155.5 (2-C), 166 (4-C), 170.9 (C=O). ESI-QTof > 0: *m/z* 274.1 (M+H)$^+$, HRMS Found: 274.1192. Calc. for $C_{14}H_{16}N_3O_3$: 274.1201.

4.3. [*N*-6-Monomethoxytrityl-*N*-9-(2-benzyloxycarbonyl-ethyl)]cytosine 4

To a solution of **3** (1.5 g, 5.48 mmol) in dry pyridine (32 mL) 4-methoxytrityl chloride (3.38 g, 10.94 mmol) was added portion wise and the reaction mixture was heated at 60 °C for 24h. The solvent was evaporated, residue was co-evaporated with toluene: methanol (1:1). The crude was dissolved in ethyl acetate and washed with saturated aqueous solution of $NaHCO_3$, water, brine, and dried over $MgSO_4$, concentrated in vacuum. The crude product was purified by column chromatography (5% acetone in dichloromethane) and the resulting solid was passed through neutral alumina to remove unwanted colouring material before to afford **4** (2.42 g, 81%) as colourless solid. R_f (5% acetone in dichloromethane) 0.32. λ_{max} (EtOH 95)/nm 283 nm (ε/dm^3 mol^{-1} cm^{-1} 7400). δ_H (300 MHz, $CDCl_3$) 2.89 (2H, t, *J* = 6Hz, Cy-CH_2-*CH*$_2$), 3.81 (3H, s, OCH_3), 3.96 (2H, t, *J* = 6Hz, Cy-*CH*$_2$-CH_2), 4.95 (1H, d, *J* = 7.5Hz, 5-H), 5.08 (2H, s, CH_2Ph) 6.83 (2H, d, *J* = 12Hz, H_{arom}), 7.12 (1H, br s, NH), 7.08 (1H, d, *J* = 7.5Hz, 6-H), 7.34 (17H, m, H_{arom}). δ_C (75 MHz, $CDCl_3$) 32.9 (Cy-CH_2-*CH*$_2$), 4.4 (Cy-*CH*$_2$-CH_2), 55.2 (OCH_3), 66.6 (*C*H$_2$-Ph), 70.6 (C-Ph$_3$), 94.0 (5-C), 113.6 (C_{arom}), 127.5, 128.2, 128.3, 128.4, 128.6, 129.9, 135.4, 135.8, 135.4, 135.8, 144.1 (C_{arom}), 146.4 (6-C), 155.3, 158.7 (2-C), 165.5 (4-C), 171.5 (C=O). ESI-QTof > 0: *m/z* 546.3 (M+H)$^+$, HRMS Found: 546.2393. Calc. for $C_{34}H_{32}N_3O_4$: 546.2400.

4.4. [*N*-6-Monomethoxytrityl-*N*-9-(3-oxo-4-dimethyl-phosphonobutyl)]cytosine 5

A solution of dimethylmethylphosphonate (1.98 g, 16.03 mmol) in dry THF (27.5 mL) was cooled to -78°C and n-BuLi (40.25 mL, 16.03 mmol) was added dropwise. The resulting mixture was stirred at -78°C for 1h and a solution of **4** (3.5 g, 6.414 mmol) in THF (18 mL) was added drop wise. The reaction mixture was stirred at -78°C for 2h, and then the reaction mixture was added to a cooled saturated NH_4Cl aqueous solution. The resulting organic layer was separated and the aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with water, brine, and dried over $MgSO_4$, concentrated under reduced pressure. The crude product was purified by flash column chromatography (4% MeOH in DCM) to afford **5** (2.35 g, 65%) as colourless solid. R_f (4% MeOH in dichloromethane) 0.39. λ_{max} (EtOH 95)/nm 283 nm (ε/dm^3 mol^{-1} cm^{-1} 8700). δ_H (300 MHz, $CDCl_3$) 3.0 (2H, d, *J* = 22.5 Hz, CH_2P), 3.08 (2H, t, *J* = 5.7Hz, Cy-CH_2-*CH*$_2$), 3.61, 3.63 (6H, 2s, $(CH_3O)_2$P), 3.73 (3H, s, OCH_3), 3.83 (2H, t, *J* = 5.7Hz, Cy-*CH*$_2$-CH_2),4.9 (1H, d, *J* = 7.2Hz, 5-H) 6.75 (2H, d, *J* = 9Hz, H_{arom}), 7.08 (1H, d, *J* = 7.5, 6-H) 7.21 (12H, m, H_{arom}). δ_C (75 MHz, $CDCl_3$) 41.4 (d, *J* = 127.12Hz, CH_2P), 42.1 (Cy-CH_2-*CH*$_2$), 45.4 (Cy-*CH*$_2$-CH_2), 52.7, 52.9 (2s, $(CH_3O)_2$P), 55.2 (OCH_3), 70.7 (C-Ph$_3$), 93.9 (5-C), 113.6, 127.5, 128.3, 128.6, 129.9, 135.7, 144.0 (C_{arom}), 146.9 (6-C), 155.1 (2-C), 158.7 (C_{arom}), 165.3 (4-C), 200.4 (d, *J* = 6.6Hz, C=O). δ_P (121 MHz, $CDCl_3$) 21.9. ESI-QTof > 0: *m/z* 562.2 (M+H)$^+$, HRMS Found: 562.2106. Calc. for $C_{30}H_{33}N_3O_6P$: 562.2107.

4.5. (±)-[*N*-6-Monomethoxytrityl-*N*-9-(3-hydroxy-4-dimethylphosphonobutyl)]cytosine 6

To a solution of **5** (2.0 g, 3.65 mmol) in methanol (16 mL) was added sodium borohydride (0.83 g, 21.9 mmol) and the resulting mixture was stirred for 1h. Then, the reaction mixture was cooled to 0°C and quenched with saturated NH_4Cl aqueous solution. The solvent was removed under reduced pressure, and the crude was dissolved in water, extracted with ethyl acetate. The resulting organic layer was washed with brine, dried over $MgSO_4$, concentrated under reduced pressure. The crude product was purified by flash column chromatography (4% MeOH in dichloromethane) to afford (±)-**6** (1.7 g, 85%) as colourless solid. R_f (4% MeOH in dichloromethane) 0.3. λ_{max} (EtOH 95)/nm 283 nm (ε/dm^3 mol^{-1} cm^{-1} 11000). δ_H (300 MHz, $CDCl_3$) 1.76 (2H, 2m, Cy-CH_2-*CH*$_2$), 1.86 (1H, d, *J* = 5.4Hz, CHP), 1.91 (1H, d, *J* = 4.5Hz, CHP), 3.6 (1H, m, C*H*(OH)), 3.65 (3H, d, *J* = 6.6Hz, (CH_3O)P), 3.68 (3H, s, *J* = 6.6Hz, (CH_3O)P), 3.73 (3H, s, OCH_3), 3.9 (2H, 2m, Cy-*CH*$_2$-CH_2), 4.45 (1H, br s, CH(O*H*)), 4.96 (1H, d, *J* = 7.2Hz, 5-H), 6.76 (2H, d, *J* = 7.5Hz, H_{arom}), 6.95 (1H, d, *J* = 7.5Hz, 6-H), 7.22 (12H, m, H_{arom}). δ_C (75 MHz, $CDCl_3$) 32.4 (d, *J* = 138Hz, CH_2P), 37.6 (d, *J* = 15.3Hz, Cy-CH_2-*CH*$_2$), 46.7 (Cy-*CH*$_2$-CH_2), 52.4 (d, *J* = 17.9Hz, (CH_3O)P), 52.5 (d, *J* = 17.6Hz, (CH_3O)P), 55.2 (OCH_3), 62.7 (d, *J* = 3.9Hz, CH(OH)), 70.6 (C-Ph$_3$), 94.8 (5-C), 113.6, 127.5, 128.3, 128.6, 129.9, 135.8, 144.1, (C_{arom}), 145.5 (6-C), 156.6 (2-C), 158.7, (C_{arom}) 165.5 (4-C). δ_P (121 MHz, $CDCl_3$) 32.1. ESI-QTof > 0: *m/z* 564 (M+H)$^+$, HRMS Found: 564.2264. Calc. for $C_{30}H_{35}N_3O_6P$: 564.2263.

4.6. Derivatization of (±)-6 with (*S*)-MPA

To a solution of (±)-**6** (1.2 g, 3.09 mmol) and (*S*)-methoxyphenyl acetic acid (0.72 g, 4.32 mmol) in dichloromethane (27 mL) were added 1,3-dicyclohexylcarbodiimide (890 mg) and a catalytic amount of dimethylaminopyridine at room temperature. The resulting mixture was stirred during 1hr, then filtered and the solvent was evaporated under reduced pressure. The crude product was purified by column chromatography (5% ethanol in diethyl ether) to afford less polar compound (*R,S*)-**7** (492 mg, 32%) and more polar compound (*S,S*)-**7** (472 mg, 30%).

4.6.1. Less Polar Diastereoisomer (R,S)-7.

R_f (5% ethanol in diethyl ether) 0.2. $[\alpha]_D^{20}$ +34.6 (*c* 0.74 in MeOH). λ_{max} (EtOH 95)/nm 283 nm (ε/dm^3 mol^{-1} cm^{-1} 10400). δ_H (300 MHz, $CDCl_3$) 1.96 (2H, d, *J* = 128Hz, Cy-CH_2-*CH*$_2$), 2.03 (1H, d, *J* = 6Hz, CHP), 2.08 (1H, d, *J* = 6.2Hz, CHP), 3.16 (2H, 2m, Cy-*CH*$_2$-CH_2), 3.37 (3H, s, OCH_3), 3.65 (3H, d, *J* = 11Hz, (CH_3O)P), 3.67 (3H, d, *J* = 11Hz, (CH_3O)P), 3.75 (3H, s, OCH_3), 4.68 (1H, s, CH), 5.97 (1H, d, *J* = 7.4Hz, 5-H), 6.9 (2H, d, *J* = 6.7Hz, H_{arom}), 6.95 (1H, d, *J* = 7.5Hz, 6-H), 7.16 (12H, m, H_{arom}). δ_C (75 MHz, $CDCl_3$) 29.8 (d, *J* = 140Hz, CH_2P), 33.8 (d, *J* = 8Hz, Cy-CH_2-*CH*$_2$), 46.4 (Cy-*CH*$_2$-CH_2),

52.6 (d, J = 24Hz, $(CH_3O)P$), 52.6 (d, J = 24Hz, $(CH_3O)P$), 55.3 (OCH_3), 57.4 (OCH_3), 67.2 (d, J = 3Hz, CH(OH)), 70.5 (C-Ph_3), 82.1 ($C(OCH_3)$, 94.2 (5-C), 113.6, 127.2, 127.5, 128.3, 128.6, 128.8, 128.9, 130.0, 135.9, 136.4, 144.1, (C_{arom}), 145.2 (6-C), 155.5 (2-C), 158.8,(C_{arom}) 165.4 (4-C), 170.0 (C=O). δ_P (121 MHz, $CDCl_3$) 28.0. ESI-QTof > 0: *m/z* 712 $(M+H)^+$, HRMS Found: 712.2786. Calc. for $C_{39}H_{43}N_3O_8P$: 712.2788.

*4.6.2. More Polar Diastereoisomer (S,S)-***7.**

R_f (5% ethanol in diethyl ether) 0.18. $[\alpha]_D^{20}$ + 38.6 (*c* 0.89 in MeOH). λ_{max} (EtOH 95)/nm 283 nm (ε/dm^3 mol^{-1} cm^{-1} 10400). δ_H (300 MHz, $CDCl_3$) 1.86 (1H, ddd, J = 7.4Hz, CHP), 1.92 (1H, ddd, J = 5.5Hz, 5.4Hz, 5.5Hz, CHP), 2.08 (2H, 2m, Cy-CH_2-CH_2), 3.33 (3H, s, OCH_3), 3.5 (2H, 2m, Cy-CH_2-CH_2), 3.53 (3H, d, J = 18.2Hz, $(CH_3O)P$), 3.56 (3H, d, J = 18.2Hz, $(CH_3O)P$), 3.73 (3H, s, OCH_3), 4.69 (1H, s, CH), 4.84 (1H, d, J = 7.3Hz, 6-H). 5.0 (1H, m, CH), 6.59 (1H, d, J = 7.4Hz, 5-H), 6.76 (2H, d, J = 4.75Hz, H_{arom}), 6.95 (1H, d, J = 7.5Hz, 6-H), 7.16 (12H, m, H_{arom}). δ_C (75 MHz, $CDCl_3$) 29.5 (d, J = 139Hz, CH_2P), 33.6 (d, J = 6Hz, Cy-CH_2-CH_2), 46.3 (Cy-CH_2-CH_2), 52.5 (d, J = 6Hz, $(CH_3O)_2P$), 55.3 (OCH_3), 57.5 (OCH_3), 68.0, 70.5 (C-Ph_3), 82.4 ($C(OCH_3)$, 94.4 (5-C), 113.6, 127.1, 127.5, 128.3, 128.6, 128.7, 128.8, 130.0, 135.9, 136.9, 144.2, (C_{arom}), 145.2 (6-C), 155.5 (2-C), 158.7, (C_{arom})165.5 (4-C), 170.2 (C=O). δ_P (121 MHz, $CDCl_3$) 27.8. ESI-QTof > 0: *m/z* 712 $(M+H)^+$, HRMS Found: 712.2786. Calc. for $C_{39}H_{43}N_3O_8P$: 712.2788.

4.7. (*R*)-[*N*-6-Monomethoxytrityl-*N*-9-(3-hydroxy-4-dimethyl phosphonobutyl)]cytosine 8

To a solution of (*R,S*)-**7** (260 mg, 0.36 mmol) in methanol/water (8:2, v/v, 10 mL) was added LiOH.H_2O (30.36 mg, 0.72 mmol), and the resulting mixture was stirred at room temperature until completion of the reaction was indicated by TLC. Then, the solvents were evaporated under reduced pressure and the crude was dissolved in water and extracted with ethyl acetate. The organic layer was washed with brine, dried over $MgSO_4$, concentrated under reduced pressure. The crude product was purified by flash column chromatography (4% MeOH in dichloromethane) to afford (*R*)-**8** (182 mg, 88.4%). R_f (4% MeOH in dichloromethane) 0.3. $[\alpha]_D^{20}$ + 22.28 (c, 0.5 in MeOH). λ_{max} (EtOH 95)/nm 284 (ε/dm^3 mol^{-1} cm^{-1} 11000). The NMR chemical shifts are identical to (±)-**6**. HRMS Found 564.2264. Calc. for $C_{30}H_{35}N_3O_6P$: 564.2263..

4.8. (*S*)-[*N*-6-Monomethoxytrityl-*N*-9-(3-hydroxy-4-dimethyl phosphonobutyl)]cytosine 8

The above procedure was applied to compound (*S,S*)-**7** (270 mg, 0.38 mmol) to afford (*S*)-8 (196 mg, 91.7%). R_f (4% MeOH in dichloromethane) 0.3. $[\alpha]_D^{20}$ - 28.36 (c, 0.48 in MeOH). λ_{max} (EtOH 95)/nm 283 nm (ε/dm^3 mol^{-1} cm^{-1} 11000). The NMR chemical shifts are identical to (±)-**6**. HRMS Found: 564.2264. Calc. for $C_{30}H_{35}N_3O_6P$: 564.2263.

4.9. (*R*)-*N*-9-(3-hydroxy-4-phosphonobutyl)cytosine 2

To a cooled solution of (*R*)-**8** (180 mg, 0.319 mmol) in dry dichloromethane (7 mL) was added trimethylsilylbromide (247.67 µl, 1.91 mmol), and the reaction mixture was stirred at room temperature until the completion of the reaction was indicated by TLC (isopropanol/water/ammonia 7:2:1). Then, water was added and the reaction mixture was stirred at room temperature for 1h. The aqueous layer was separated, washed with diethyl ether and concentrated under high vacuum. After freeze-drying, the crude product was treated with triethylamine until pH 7 and then passed through a Dowex Na^+ ion exchange column, the desired fractions were collected and lyophilized to give (*R*)-**2** quantitatively as a hygroscopic salt . R_f (iPrOH/H_2O/NH_4OH 30%, 7:2:1, v/v/v) 0.16. $[\alpha]_D^{20}$ +8.6 (*c* 0.47 in H_2O). λ_{max} (H_2O)/nm 274 (ε/dm^3 mol^{-1} cm^{-1} 8300). δ_H (300 MHz, D_2O) 1.73 (2H, m, CH_2P), 1.82 (2H, 2m, Cy-CH_2-CH_2), 3.76 (1H, m, CH(OH)), 3.82 (2H, m, Cy-CH_2-CH_2), 5.87 (1H, d, J = 7.2Hz, 5-H), 7.52 (1H, d, J = 7.2Hz, 6-H). δ_C (75 MHz, $CDCl_3$) 35.7 (d, J = 91.7Hz, CH_2P), 36.5 (d, J = 46.6Hz, Cy-CH_2-CH_2), 47.1 (Cy-CH_2-CH_2), 65.7 (d, J = 2.6Hz, CH(OH)), 95.5 (5-C), 147.2 (6-C), 158.4 (2-C), 166.4 (4-C). δ_P (121 MHz, $CDCl_3$) 20.0. ESI-QTof > 0: *m/z* 308 $(M+H)^+$, HRMS Found: 308.0388. Calc. for $C_8H_{13}N_3O_5Na_2P$: 308.038.

4.10. (*S*)-*N*-9-(3-hydroxy-4-phosphonobutyl)cytosine 2

The procedure described above was applied to compound (*S*)-**8** (160 mg, 0.284 mmol) to afford (*S*)-**2** quantitatively as hygroscopic salt. R_f (iPrOH/H_2O/NH_4OH 30%, 7:2:1, v/v/v) 0.16. $[\alpha]_D^{20}$ -8.6 (*c* 0.47 in H_2O). λ_{max} (H_2O)/nm 274 (ε/dm^3 mol^{-1} cm^{-1} 8100). The NMR chemical shifts are identical to (*R*)-**2**. ESI-QTof > 0: *m/z* 308 $(M+H)^+$, HRMS Found: 308.0388. Calc. for $C_8H_{13}N_3O_5Na_2P$: 308.0388.

4.11. NMP kinase enzymatic assays

Protein expression purification and enzymatic activity measurements

Human his-tagged UMP-CMP and TMP kinases were expressed and purified to homogeneity as described previously.[22, 23] The kinase enzymatic activities were followed using the coupled spectrophotometric assay as in ref.[24]

Structure based molecular docking

Nucleotide analogues were drawn using Chemsketch software (http://www.nmrsoftware.com/products/chem_dsn_lab/chemsketch/) to produce 2D molecules subsequently translates to pdb files. The structure file of cidofovir was retrieved from PubChem database (https://**pubchem**.ncbi.nlm.nih.gov/)

The docking studies for both enantiomers of compound **2**, and in comparison to cidofovir, were carried out using the docking engine Molegro virtual docker version 5.5.0. The human UMP-CMP kinase structure is an homology model obtained from 2UKD pdb file from*Dictyostelium discoideum* [23, 25] Heteroatoms and ligands were removed from the crystal structures and hydrogen atoms were added. The scoring function Moldock Score and the Moldock SE search algorithm were used, the resolution of the grid for estimation of the computed binding affinity was 0.3 Å with a radius of 15 Å around the key NMP site residues. Fifteen runs were launched and up to 50 (maximum) poses subsequently analyzed. Positioning through NMP site pockets and hydrogen bond network for each nucleotide analogue/NMP kinase model were examined. Figures were prepared with Pymol (DeLano Scientific LLC, San Carlos, CA, USA).

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Notes and references

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† Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.
Electronic Supplementary Information (ESI) available: [spectral data for all compounds]. See DOI: 10.1039/b000000x/

References

1. E. De Clercq, in *Annual Review of Pharmacology and Toxicology*, ed. A. K. Cho, 2011, vol. 51, pp. 1-24.
2. E. De Clercq, *Biochem. Pharmacol.*, 2007, **73**, 911-922.
3. E. De Clercq, *Biochem. Pharmacol.*, 2011, **82**, 99-109.
4. E. De Clercq and A. Holy, *Nat. Rev. Drug Discovery*, 2005, **4**, 928-940.
5. X. F. Xiong, J. L. Smith and M. S. Chen, *Antimicrob. Agents Chemother.*, 1997, **41**, 594-599.
6. O. Baszczynski, P. Jansa, M. Dracinsky, B. Klepetarova, A. Holy, I. Votruba, E. de Clercq, J. Balzarini and Z. Janeba, *Bioorg. Med. Chem.*, 2011, **19**, 2114-2124.
7. M. Krecmerova, M. Masojidkova and A. Holy, *Bioorg. Med. Chem.*, 2010, **18**, 387-395.
8. M. R. Harnden, A. Parkin, M. J. Parratt and R. M. Perkins, *J. Med. Chem.*, 1993, **36**, 1343-1355.
9. D. Topalis, U. Pradere, V. Roy, C. Caillat, A. Azzouzi, J. Broggi, R. Snoeck, G. Andrei, J. Lin, S. Eriksson, J. A. C. Alexandre, C. El-Amri, D. Deville-Bonne, P. Meyer, J. Balzarini and L. A. Agrofoglio, *J. Med. Chem.*, 2011, **54**, 222-232.
10. O. I. Kolodiazhnyi, *Tetrahedron: Asymmetry*, 2005, **16**, 3295-3340.
11. M. Kitamura, M. Tokunaga and R. Noyori, *J. Am. Chem. Soc.*, 1995, **117**, 2931-2932.
12. A. Woschek, W. Lindner and F. Hammerschmidt, *Adv. Synth. Catal.*, 2003, **345**, 1287-1298.
13. O. Pamies and J. E. Backvall, *J. Org. Chem.*, 2003, **68**, 4815-4818.
14. T. Gajda, *Tetrahedron: Asymmetry*, 1994, **5**, 1965-1972.
15. M. Ordonez, A. Gonzalez-Morales, C. Ruiz, R. De la Cruz-Cordero and M. Fernandez-Zertuche, *Tetrahedron: Asymmetry*, 2003, **14**, 1775-1779.
16. M. Kasthuri, L. Chaloin, C. Périgaud and S. Peyrottes, *Tetrahedron: Asymmetry*, 2011, **22**, 1505-1511.
17. C. Hildebrand and G. E. Wright, *J. Org. Chem.*, 1992, **57**, 1808-1813.
18. V. V. Nesterov and O. I. Kolodiazhnyi, *Tetrahedron*, 2007, **63**, 6720-6731.
19. B. M. Trost, J. L. Belletire, S. Godleski, P. G. McDougal, J. M. Balkovec, J. J. Baldwin, M. E. Christy, G. S. Ponticello, S. L. Varga and J. P. Springer, *J. Org. Chem.*, 1986, **51**, 2370-2374.
20. K. M. Blazewska and T. Gajda, *Tetrahedron: Asymmetry*, 2009, **20**, 1337-1361.
21. T. Cihlar and M. S. Chen, *Mol. Pharmacol.*, 1996, **50**, 1502-1510.
22. C. Pasti, S. Gallois-Montbrun, H. Munier-Lehmann, M. Veron, A. M. Gilles and D. Deville-Bonne, *Eur. J. Biochem.*, 2003, **270**, 1784-1790.
23. D. Topalis, B. Collinet, C. Gasse, L. Dugue, J. Balzarini, S. Pochet and D. Deville-Bonne, *FEBS J.*, 2005, **272**, 6254-6265.
24. C. Blondin, L. Serina, L. Wiesmuller, A. M. Gilles and O. Barzu, *Anal.l Biochem.*, 1994, **220**, 219-221.
25. D. Topalis, H. Kumamoto, M.-F. A. Velasco, L. Dugue, A. Haouz, J. A. C. Alexandre, S. Gallois-Montbrun, P. M. Alzari, S. Pochet, L. A. Agrofoglio and D. Deville-Bonne, *FEBS J.*, 2007, **274**, 3704-3714.