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Synthesis of substituted 5'-aminoadenosine derivatives and evaluation of their inhibitory potential towards CD73

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Abstract: Derivatives of 5'-amino-adenosine containing methyl carboxylate, methyl phosphonate, gem-bisphosphonate, bis(methylphosphonate), and α -carboxymethylphosphonate or phosphonoacetic moieties were synthesized from a key intermediate 5'-aminonucleoside. These nucleos(t)ide analogues were envisaged as 5'-mono or diphosphate nucleoside mimics. All compounds were evaluated for CD73 inhibition in a cell-based assay (MDA-MB-231) and towards the purified recombinant protein. Most of them failed to reach significant inhibition of AMP hydrolysis by CD73 at 100 μ M. Among the new compounds, the most interesting ones were derivatives **5** and **7**, inhibiting recombinant CD73 by 36 and 47% and cellular CD73 by 61 and 45% at 100 μ M, respectively. Molecular modeling allowed to explain partially this lack of activity as predicted docking score calculated beforehand were initially encouraging, especially for compound **7**.

Introduction

Ecto-5'-nucleotidase CD73 is a glycoposphatidyl-inositol-anchored di-Zn²⁺ metallo-phosphatase. It is an important enzyme involved in the extracellular catabolism of nucleosides and nucleotides working together with the membrane-bound CD39. The latter catalyzes the dephosphorylation of ATP into AMP, whereas CD73 dephosphorylates extracellular AMP into adenosine and inorganic phosphate. Thus, CD73, as a modulator of adenosine production, has been identified as an active player and a validated drug target in several diseases, such as cancer, autoimmunity, allergy and ischemia-reperfusion injury.^[1] Numerous studies have shown that the inhibition of CD73 activity, both in mice models and in cell cultures, by a substrate analogue mimicking ADP (APCP, 5'-methylene-bisphosphonate adenosine, Figure 1), a monoclonal antibody or siRNA, is associated with decreased tumor growth and metastasis development, decreased tumor angiogenesis and increased activity of immune-modulating therapeutic antibodies.^[2] In the last few years, exciting progress in the

development of immunotherapy for the treatment of cancer patients have been achieved. Thus, modulation of the immune system through a small-molecule approach offers several unique advantages (lower cost, facilitated administration...) being complementary to and potentially synergistic with immunoncology approaches using monoclonal antibodies.^[3] This is exemplified by the association of two mAbs, one targeting CD73 (MEDI-9447^[4]) and one neutralizing the immune check point PD-1. However, approaches currently using small molecules to inhibit CD73 activity are not yet suitable for clinical use. Indeed, one of the first and well-known small molecule inhibitor (APCP) has low bioavailability, poor metabolic stability and off-target related effects presumably associated with chemical instability and adenosine release.^[5] To our knowledge, the most potent inhibitors of CD73 were described by the team of Prof. Müller and are structurally related to the APCP skeleton (Figure 1).^[6] In addition, nucleoside analogues incorporating two carboxylate groups have been patented.^[7] However, no data are available on the *in vivo* activity of these two series of compounds. Therefore, it is still of great interest to design new small-molecule CD73 inhibitors.

Study of APCP in the active site of CD73 (Figure 2) showed key structural interactions with the terminal domains in the substrate binding site.^[8] The adenine-base formed hydrophobic π -stacking interaction between two phenylalanine residues (F417 and F500), and the diol group of the ribose interacts mainly with D506. The α -phosphonate group binds with R354 and N245 residues, whereas the β -phosphonate group binds to R395 of C-terminal domain as well as to N85 and H243 of the N-terminal domain. In addition, α,β -phosphonate groups chelate the two Zn²⁺ ions through the formation of monodentate and bidentate interactions. Based on these observations and as illustrated in Figure 1, two types of nucleos(t)ide analogues were designed using adenosine as scaffold. In particular, 5'-amino-adenosine was selected as the nitrogen atom allows di-substitution or formation of additional H-bond interaction. Thus, compounds **1**, **4** and **5** incorporate either a carboxylate or a phosphonate group that may be viewed as equivalent of the phosphate of AMP (the

natural CD73 substrate). In order to mimic the interaction of the α,β -methylene diphosphate group of APCP with R354 and N245, a carboxyl oxygen or a phosphoryl oxygen was present in derivatives **2**, **3**, and **6-9**, whereas the two oxygen atoms of the phosphonate group of bis(methylphosphonate), α -carboxyl-methylphosphonate or phosphonoacetic moieties may mimic the chelation of the Zn^{2+} ions by the β -phosphonate of APCP.

We report herein the synthesis of novel series of N-substituted, 5'-amino-adenosine derivatives (compounds **1-9**, Figure 1) as potential AMP or ADP mimics and evaluate their potential as CD73 inhibitors *in vitro*.

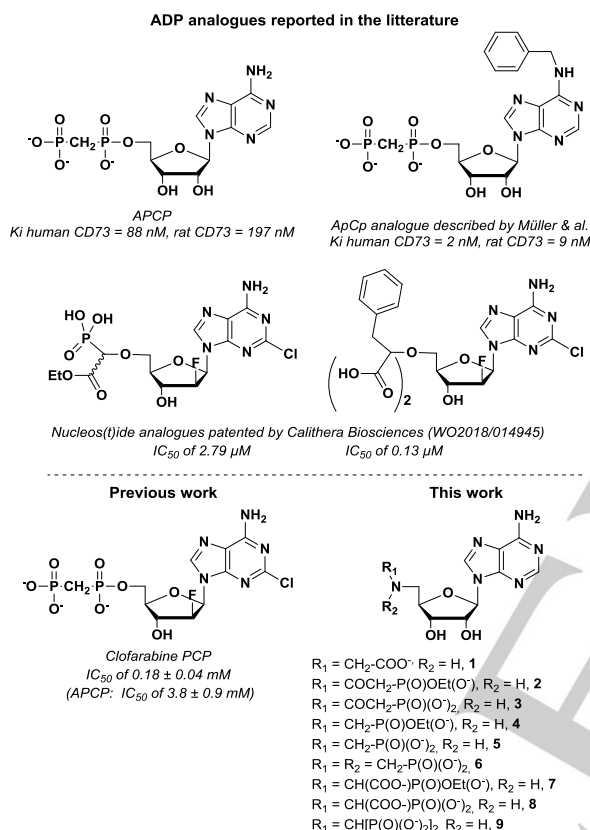


Figure 1. Structures of ADP analogues as potent CD73 inhibitors and studied compounds **1-9**.

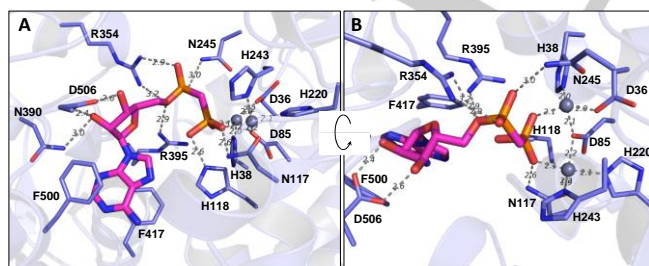


Figure 2. APCP binding mode within the active site of CD73 (crystal structure of the closed form solved by Knapp et al.^[8]). A) and B) correspond to two orientations. APCP is depicted as pink sticks while connecting residues are shown in blue thin sticks and zinc ions as grey spheres.

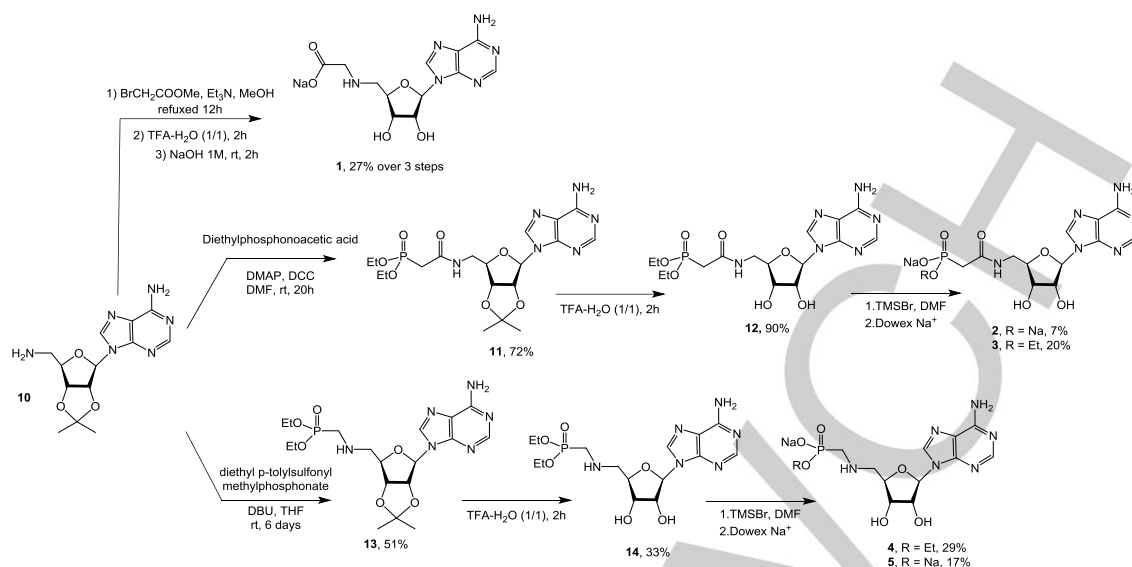
Results and Discussion

Synthesis

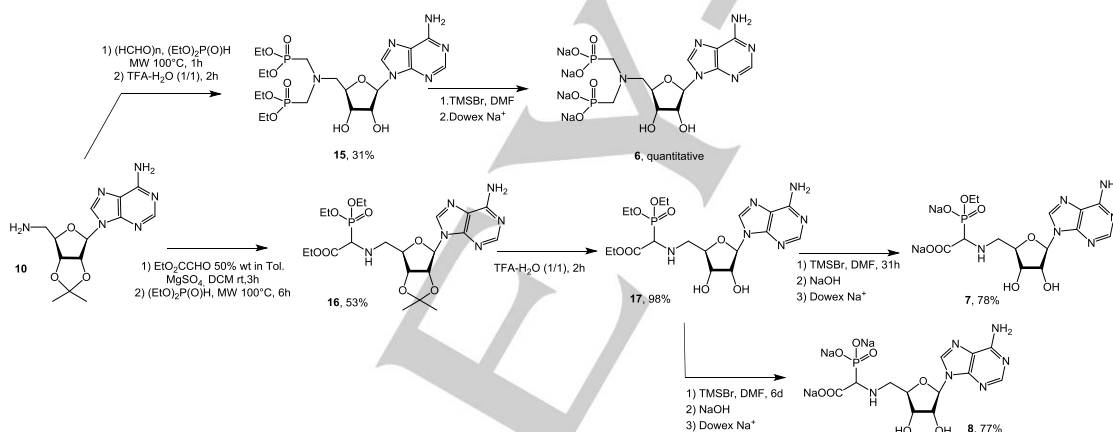
The synthesis of adenosine analogues **1-8** began with 5'-amino-2',3'-O-isopropylidene-adenosine **10** (Schemes 1 & 2), which can be easily accessed in three steps from commercially available adenosine with overall yield about 71%.^[9] Synthesis of derivatives **1** to **5** (Scheme 1) was envisaged through nucleophilic displacement by using methyl bromoacetate^[10] or diethyl *p*-toluene sulfonyloxymethylphosphonate (obtained before^[11]) in presence of a base (either NEt_3 or DBU), or coupling of diethylphosphonoacetic acid^[12] using previously described or adapted conditions. Attempts to obtain di-substituted derivatives, by increasing the number of equivalents and/or reaction time, and temperature were unsuccessful. Concerning the synthesis of compound **1**, the 5'-deoxy-2,3-O-(isopropylidene)-5'-N-methyl-acetate-5'-aminoadenosine was contaminated with triethylammonium salts and directly engaged in the next step leading to N-alkylated derivative in 45% yield over the two steps. The latter was treated with an equimolar amount of 1M NaOH to afford derivative **1**. Intermediates **11** and **13** were isolated in good yields, and removal of the sugar-protecting group in acidic conditions afforded derivatives **12** and **14** in 90 and 33% yields, respectively. Deprotection of the diethylphosphonoester group for compounds **12** and **14** was performed with TMSBr in DMF (Scheme 1). Partially deprotected derivatives were eventually isolated such as compounds **3** and **4**, as well as the desired phosphonic acids **2** and **5**, which were obtained as sodium salts through ion-exchange on Dowex (Na^+ form).

Synthesis of derivative **6** incorporating two phosphonomethyl groups and derivatives **7** and **8** incorporating a carboxylate and a phosphonate groups was also carried out starting from 5'-amino-5'-deoxy-2',3'-O-isopropylidene-adenosine **10** (Scheme 2). The double Kabachnik-Fields reaction of **10**, paraformaldehyde, and diethyl phosphite was performed under microwave activation and solvent-free conditions.^[13] The resulting derivative was directly treated in acidic conditions to afford compound **15** in 31% yield over the two steps. Then, removal of the phosphonic protecting groups was carried out as previously described and the desired 5'-N-[bisphosphonomethyl] adenosine **6** was obtained quantitatively.

The α -carboxyphosphonate derivative **16** was obtained by using the Aza-Pudovick reaction, consisting in the addition of diethylphosphite to the *in situ* generated imine from **10** and ethylglyoxylate. This step was also performed under microwave-assisted and solvent-free conditions as described in the literature.^[14] Removal of the various protecting groups was carried out successively under acidic conditions, affording compound **17**, and then in presence of TMSBr. During this last step, the α -carboxy(monoethyl)phosphonate ester **7** was isolated as major derivative after 31 hours, whereas formation of the fully deprotected derivative **8** required 6 days of reaction. In both case, α -carboxyphosphonate derivatives **7** and **8** were isolated as equimolar mixture of the two diastereoisomers.



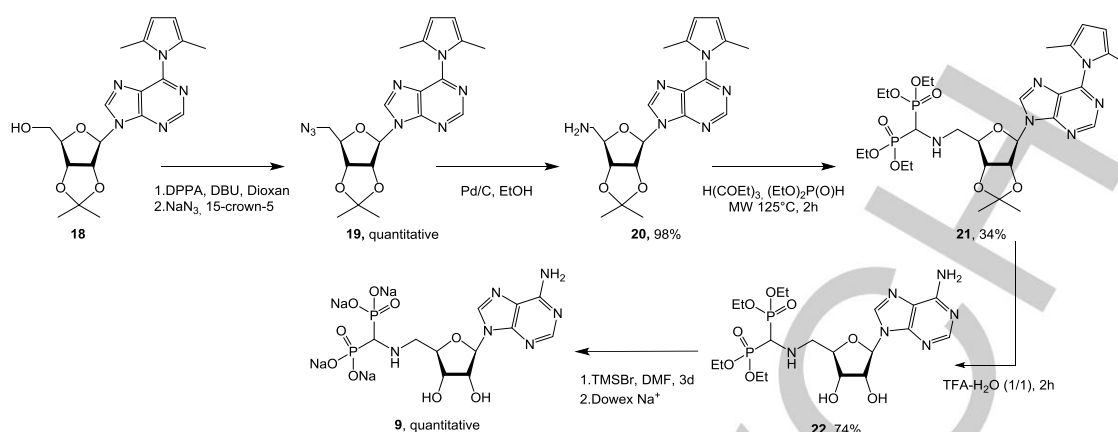
Scheme 1. Synthetic pathways for derivatives 1-5.



Scheme 2. Synthetic pathways for derivatives 6-8.

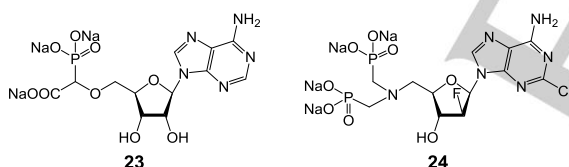
Preparation of (aminomethylene)bisphosphonate derivative **9** (Scheme 3) was envisaged through a three-component condensation involving **10**, triethyl orthoformate and diethyl phosphite.^[15] Despite several attempts, only small amounts of the desired compound were obtained and formation of the bis-adduct corresponding to the reaction of the exocyclic amino function of the purine ring was also observed. Thus, the use of a fully protected precursor appeared necessary. Protection of the amino group in position 6 of the 2',3'-O-isopropylidene-adenosine by the 2,5-dimethylpyrrole was selected as it can be hydrolyzed in the same conditions as of the ribose protecting group. Thus, compound **18** was obtained in 36% yield from

commercially available adenosine, according to previously published procedure.^[16] Then azidation in position 5' was performed and compound **19** was subjected to hydrogenation to afford the 9-(5-amino-5-deoxy-2,3-O-isopropylidene-β-D-ribofuranosyl)-6-(2,5-dimethylpyrrol-1-yl)purine **20**. With this fully protected intermediate in hand, the method elaborated by E. Balint et al.^[15] was successfully applied to a nucleosidic substrate, affording the gem(bisphosphonate) derivative **21** in 34% yield. Final steps consisted in removal of the various protecting groups as previously reported and lead to the target compound **9**.



Scheme 3. Synthesis of derivative 9.

In addition to compounds **1-9**, we synthesized two analogues, such as the α -carboxyphosphonate derivative of adenosine as a reference (Figure 3, compound **23**), to study the impact of the nitrogen atom in position 5' (in comparison to compound **8**), and the analogue of derivative **6** incorporating Clofarabine (Figure 3, compound **24**) instead of adenosine to study the influence of the nucleosidic residue. Indeed, literature data^[7a, 17] tend to show that the use of Clofarabine as nucleoside scaffold led to increased potency for 5'-ectonucleotidase inhibition (cf. Clofarabine PCP, Figure 1). Synthetic pathways of these two derivatives are detailed in the SI as compound **23** has already been reported in the literature^[18] and compound **24** was obtained by adapting procedure described in Scheme 2.

Figure 3. Structures of compounds **23** and **24**.

Inhibitory activity of synthesized compounds towards CD73

The enzymatic activity of CD73 was assessed in presence of all adenosine-based derivatives to determine their potential inhibitory effect on CD73. Inhibition levels promoted by the compounds are shown in Table 1 at 10 or 100 μ M with recombinant protein, and at 100 μ M in the cell based assay. Compounds **1-4**, **6** and **8** inhibited CD73 activity only weakly at high concentration (between 10-44% of inhibition) in both conditions. Compounds **5**, **7**, **9** and **24** exhibited a moderate enzymatic inhibition with inhibition values ranging from 45 to 61% at 100 μ M in at least one of the two assays. In contrast, derivative **23** was found to promote a strong inhibition on both recombinant ($76 \pm 3\%$) and cellular ($82 \pm 4\%$) CD73 at high concentrations, and still significantly ($35 \pm 4\%$) at 10 μ M (Figure 4A). As expected, APCP, as well as our previously developed inhibitor Clofarabine PCP,^[17] used here as positive controls,

were able to inhibit CD73 activity by more than 90% at high concentrations. MTT assays were performed with MDA-MB-231 cells and studied compounds to estimate cytotoxicity, and showed marginal (76% survival for compound **3**) or no such effect (data not shown).

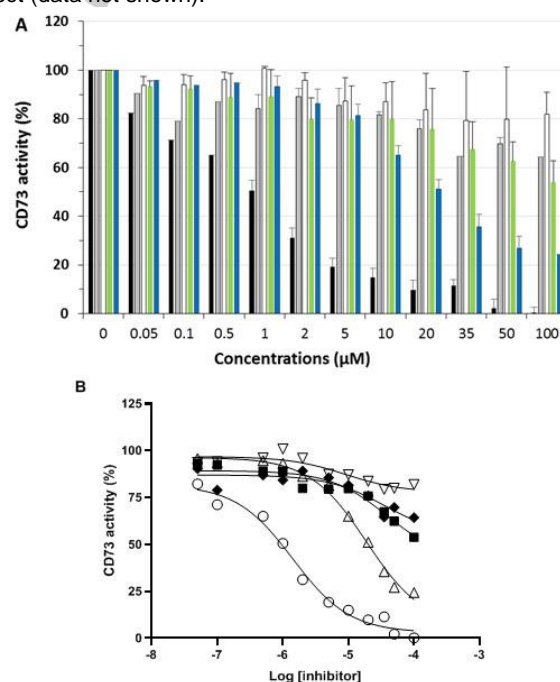


Figure 4. A) *In vitro* enzymatic activity measured in the presence of representative compounds: APCP (black bars), derivative **5** (grey), **6** (white), **7** (green) and **23** (blue). B) IC₅₀ determination for weak compounds **5** (♦), **6** (▽), **7** (■), modest **23** (Δ) compared to strong inhibitor, APCP (○).

For the most interesting derivative (**23**), a detailed inhibition curve was obtained in order to determine its IC₅₀ values and compared to the ones of weak (**5**, **6** and **7**) or strong (APCP) inhibitors (Figure 4B). For all compounds, dose response curves followed a regular sigmoidal shape even for derivatives **5-7**. IC₅₀ was determined at 35 ± 6 μ M for compound **5**, around 35 μ M and 92 μ M for compounds **6** and **7**, respectively (with important uncertainty for compounds **5-7** in this concentration range). In contrast, derivative **23** gave a value of 19 ± 6 μ M and APCP at 1.4 ± 1.0 μ M.

Table 1. Summary of biochemical properties of compounds **1–9** and **24**, and reference compounds (APCP, **23** and Clofarabine PCP).

Compound #	Structure	Docking Score ^[a]	% of inhibition @ 100 μ M (% \pm SEM) toward purified recombinant-CD73	% of inhibition @ 10 μ M (% \pm SEM) toward purified recombinant-CD73	% of inhibition @ 100 μ M (% \pm SEM) in cell based assay
APCP		147.2	99.5 \pm 0.5	81.2 \pm 6.7	91 \pm 3
1		106.4	39.4 \pm 5	13.7 \pm 3	12 \pm 1
2		108.8	32.3 \pm 2.8	11.9 \pm 7.4	27 \pm 9
3		114.3	19.9 \pm 3	18.9 \pm 3.5	44 \pm 5
4		110.7	36 \pm 5	10 \pm 8.5	29 \pm 9
5		118.4	36 \pm 6	18.5 \pm 2	61 \pm 8
6		132	18 \pm 9	13 \pm 8	n.d.a. ^[c]
7		143.7	46 \pm 9	20.2 \pm 3	45 \pm 6
8		135.3	36.2 \pm 5	5.7 \pm 4	23 \pm 8
9		136.6	51.9 \pm 2	10.5 \pm 3	31 \pm 7
23		134.8	76 \pm 2.5	35 \pm 4	82 \pm 4
24		132.5	n.a. ^[b]	n.a. ^[b]	45 \pm 9
Clofarabine PCP		142.8	98.5 \pm 1.5	96 \pm 2.5	92 \pm 4

[a] Docking score for AMP is 108.5. [b] n.a.: not active. [c] n.d.a.: no data available. Data represents Mean \pm SEM of 3 independent experiments.

Molecular modeling

To understand the relatively weak and disappointing inhibition observed with most of the 5'-amino-adenosine derivatives, a molecular docking study was carried out by using the crystal structure of CD73 (4H2I). Best-ranked docking poses (according to their docking scores given by the GoldScore fitness function which takes into account H-bonding energy, van der Waals energy, metal interaction and ligand torsion strain) were analyzed for defining the various interactions with CD73 residues arising upon binding of the compound (Figure 5). Binding orientation differed significantly for derivatives **1–4** as compared to that of APCP (Figure 5A). Indeed, two major features distinguish their binding, first the positioning of the nucleobase that is shifted and made weaker π - π interactions with the two phenyl residues (F417 and F500) from CD73, and consequently the displacement of the ribose weakening its interaction with D506 (two H-bonds). Overall, the binding of such compounds altered electrostatic interactions between R354 (and R395) and 5'-oxygen and those from the α -phosphonate for APCP, as it is replaced by a single phosphonate and an amino group (preferentially H-bond donor than H-bond acceptor). For compound **5**, the binding is strongly different to that of APCP as seen for compounds **1–4** and again interactions are lost

between D506 and ribose hydroxyls but also with R354 and N245 due to the missing α -phosphonate and between H118 or R395 with the missing oxygen of β -phosphonate (Figure 5B). This may be explained by the length of the amino-phosphonate chain that is shorter for **5** compared to the one of APCP and since the electrostatic component is the main driving force to connect oxygen near the magnesium ion; this induces a translation of the rest of the molecule. The difference in inhibition between APCP and the potent CD73 inhibitor (**23**) may be explained by a weakening of electrostatic interactions between R354 and oxygen atoms (5'-oxygen, as seen by the larger distance of 5.4 Å compared to 3 Å with APCP) and similarly with R395 (distance increases from 2.9 to 3.3 Å). In addition to this, a slight shift of the ribose moiety is observed inducing an increase of the H-bond distance between 2'-oxygen and D506 (from 2.4 to 2.9 Å) (Figure 5C). A lower activity was observed for compound **9** compared to **23** although these two molecules differ only by one atom at 5' position (nitrogen versus oxygen) and presented similar DS values. Firstly, docking analysis failed to identify important modifications between binding modes for these two compounds as they almost superimposed (Figure 6A).

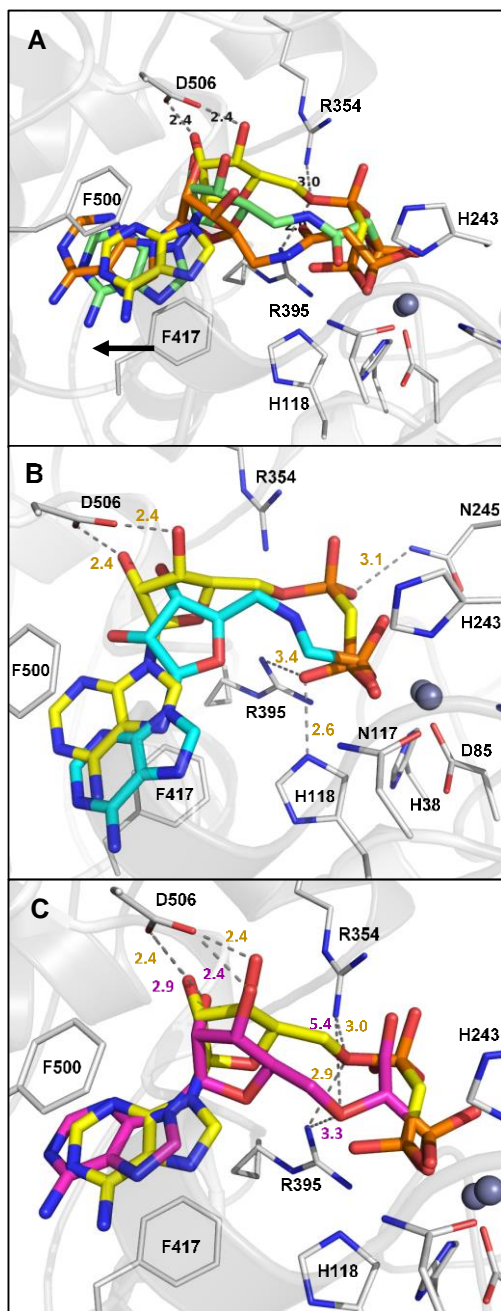


Figure 5. Binding prediction by docking for various compounds and comparison of interactions mediated with CD73 amino acids. A) Docking poses for weak inhibitors such as **2** (light green) and **3** (orange) and showing a displacement (black arrow) compared to that of APCP (depicted as yellow sticks). B) Large difference observed in binding orientation of compounds **5** (cyan) and APCP (yellow). C) Comparison between APCP (yellow) and **23** (pink sticks) highlighting changes of interaction distances between 5'-oxygen and R354/R395, and for ribose hydroxyl groups and D506.

However, a thorough analysis showed that subtle changes occurred such as the orientation of the nucleobase between both phenyl residues (possibly lowering π - π interactions) and an increase of the H-bond formed by the 2'-hydroxyl group and D506 (2.9 Å for **23** and 3.2 Å for **9**). Most importantly, this single atom substitution may reflect either a weakening of the interaction with R395 (stronger with an oxygen than a nitrogen because of the electronegativity) or a change / break in the H-bond network formed between water molecules, amino acids and compound. Although five water molecules were included

during the docking, no changes was observed at the vicinity of the substituted atom. This loss of activity may also be due to a change in the protonation state of H118 during the enzymatic reaction (achieved at pH 6.5) leading to a decrease in binding affinity of **9** (because of the supplementary hydrogen). Comparison of binding poses of compounds **6** and **24** (Figure 6B), and compound **24** and Clofarabine PCP (Figure 6C), highlighted the impact of both the nature of the nucleoside (adenosine or Clofarabine) and the bis(methylphosphonate) chain on the global positioning of these derivatives. These modifications have a strong impact on the orientation of the ribose moiety and consequently on the nucleobase positioning for compounds **6** and **24**.

Whereas, for methylene bisphosphonate derivatives (APCP and Clofarabine PCP) modification of the nucleoside moiety did not change the final orientation of the nucleobase, as previously described^[17]. In Figure 6E, overlay of binding poses of compounds **7** and **9** show the differences of interactions between the gem-bisphosphonate (**7**) and α -carboxylmethylphosphonate (**9**) with a slight move of the ribose weakening the interaction with D506 and R354. More notably, this binding pose suggested the loss of a major interaction between N245 and the third phosphate oxygen.

Discussion

We designed novel AMP or ADP analogues incorporating methyl carboxylate, methyl phosphonate, bis(methylphosphonate), gem-bisphosphonate, α -carboxylmethylphosphonate or -phosphonoacetic moieties as potential CD73 inhibitors. The replacement of the oxygen atom in the 5'-position of endogenous adenosine could allow obtaining di-substituted derivatives and may lead to additional interaction(s) in the catalytic site of the protein. First of all, the docking scores (DS) of the targeted compounds (**1-9** and **24**) were evaluated and compared to the ones of AMP (the natural substrate), APCP, Clofarabine PCP and compound **23** as reference compounds (Table 1). Most of the derivatives showed a DS higher than AMP (108.5), except the methyl carboxylate derivative (**1**). Based on these data, bis(methylphosphonate) (**6**), gem-bisphosphonate (**7**), and α -carboxylmethylphosphonate (**8** and **9**) analogues attracted our attention as they showed DS values in the same range as APCP (147.2) and Clofarabine PCP (142.8). Compound **7**, including the gem-bisphosphonate moiety, seemed to be the most promising one with a DS value of 143.7. Thus, this novel series of 5'-aminoadenoside analogues was successfully obtained in moderate to good yields and we next investigated their ability to interfere with CD73. In all cases, replacement of the methylene bisphosphonate group of APCP lead to a decreased ability to inhibit CD73. Whereas substitution of the oxygen in 5'-position by a nitrogen atom did not seem to affect the ligand potency (DS values of 136.6 for compound **9** and 134.8 for compound **23**), it significantly affects the ability to inhibit the protein. When, the adenosine moiety was replaced by Clofarabine (compounds **6** and **24**), it slightly increased the potential of the inhibitors but this effect was not able to counterbalance the replacement of the nature of the hetero-atom in position 5'. Despite our effort in designing original nucleotide analogues, the replacement of the P-CH₂-P-O5' pattern, and related interaction within the catalytic site of CD73, appears not suitable following the here proposed modifications.

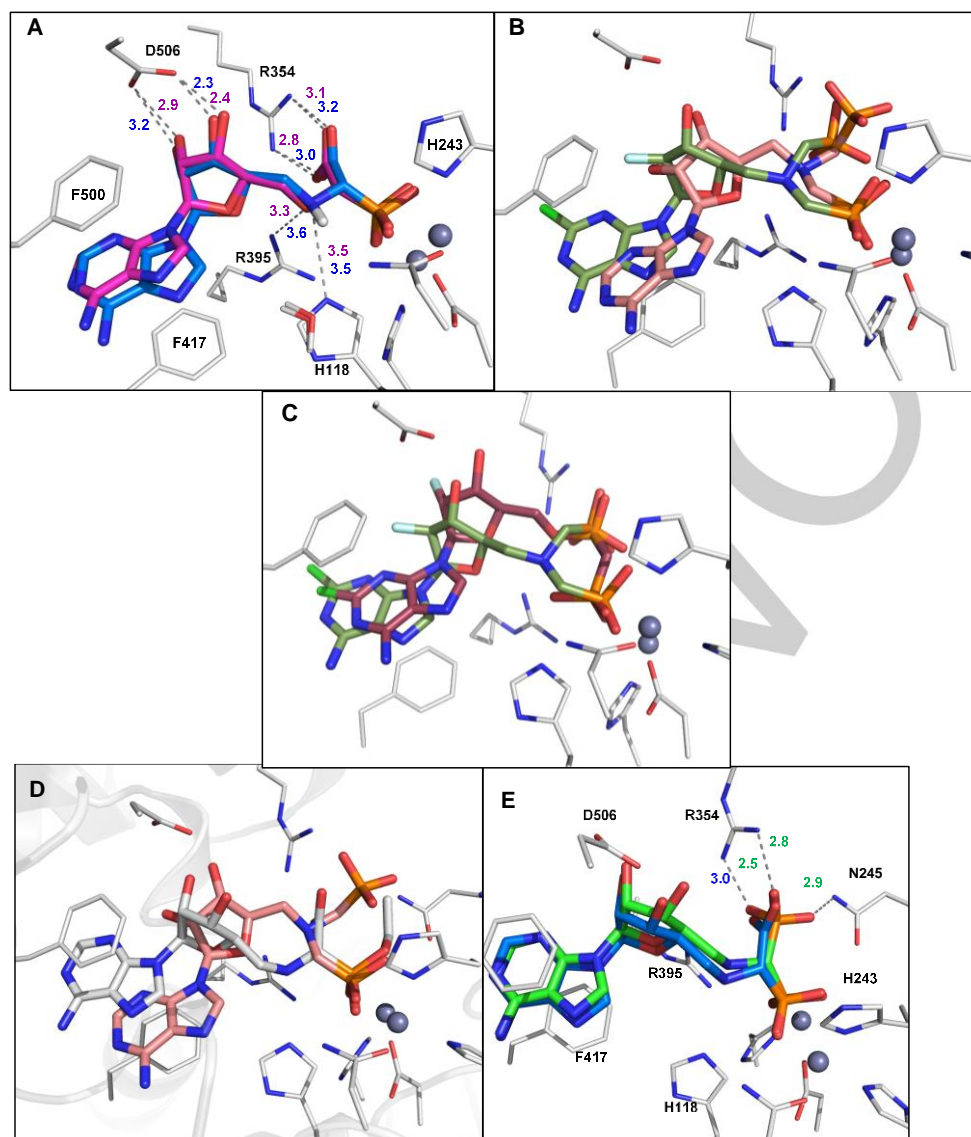


Figure 6. A) Deciphering the differential activity of compound **9** (blue) and **23** (pink sticks) by analyzing their binding mode. B) Comparison of binding mode of **6** (salmon sticks) versus **24** (dark green). C) Binding modes of **24** (dark green) and Clofarabine PCP (brown). D) Overlay of binding poses of derivatives **6** (salmon) and **8** (white). E) Binding comparison of between compounds **7** (green) and **9** (blue sticks).

According to the important contribution of the negatively charged groups over the final orientation of the inhibitor, it would be interesting to increase the distance in between the carboxylate and the phosphonate groups, and flexibility in this region may be beneficial for zinc ions chelating. In addition, as the location of carboxylate and/or phosphonate groups seems to govern the correct positioning of the nucleoside moiety, increasing this distance may lead to stronger interactions (reduction of the bonding length with D506 and hydroxyl groups and improving the π -stacking between the nucleobase and aromatic residues).

Conclusions

Novel series of mono- or di-substituted 5'-aminonucleotide analogues including carboxylated or phosphonylated substituents, or both, were successfully obtained. All synthesized compounds were evaluated for their ability to inhibit 5'-ectonucleotidase CD73. Among them, phosphonic acids **5** and **24** showed marginal inhibition of CD73 in the cell based

assay (45-61% inhibition at 100 μ M), as well as derivatives **7** and **9** with the purified recombinant protein (46-52% inhibition at 100 μ M). We assume that these differences may arise from the use of the soluble form of the protein, whereas in the cell-based assay the protein is anchored to the membrane. In these two cases, our derivatives may access and/or accommodate in a different fashion the catalytic site of the protein. In addition, molecular modeling showed that the modifications envisaged deeply impact the binding modes of the new derivatives in comparison to APCP and/or Clofarabine APCP.

Considering the literature data related to the design of nucleos(t)ide analogues as CD73 inhibitors, these results will certainly contribute to the establishment of a SAR study and further development of novel derivatives.

Experimental Section

^1H NMR, ^{13}C NMR and ^{31}P spectra were recorded with proton decoupling at ambient temperature on the following spectrometers: Bruker Avance III

(600, 500 or 400 MHz). Chemical shifts (δ) are quoted in parts per million (ppm) referenced to the residual solvent peak chloroform (CDCl_3) at 7.26 ppm and 77.16 ppm, dimethyl sulfoxide ($\text{DMSO}-d_6$) at 2.50 ppm and 39.52 ppm, methanol (CD_3OD) at 3.41 ppm and 49.00 ppm, and deuterium oxide (D_2O) at 4.63 ppm relative to tetramethylsilane (TMS). For ^{31}P NMR spectra, chemical shifts are reported relative to external phosphoric acid (H_3PO_4). COSY experiments were performed in order to confirm proton assignments as well as 2D ^1H - ^{13}C heteronuclear COSY for the attribution of ^{13}C signals. Coupling constants, J , are given in Hertz. Mass spectra were recorded on a Micromass Q-ToF mass spectrometer using electrospray ionization. The high-resolution mass spectra (HRMS) were obtained with a Waters Synapt G2S spectrometer equipped with positive electrospray source ionization (ESI), using Leu-enkephalin as an internal standard. The capillary voltage was set to 1.2 kV and the sampling cone voltage was set to 30 V. Thin layer chromatography was performed on pre-coated aluminum sheets of Silica Gel 60 F254 (Merck, Art. 5554), visualization of products was accomplished by UV absorbance followed by spraying with Hanes molybdate reagent. Column chromatography was carried out on Silica Gel 60 (Merck, Art. 9385). All moisture sensitive reactions were carried out in anhydrous conditions under argon atmosphere using oven-dried glassware. Solvents were dried and distilled prior to use and solids were dried over P_2O_5 under reduced pressure at room temperature.

General Procedure (A) for removal of acid labile sugar protecting groups. The protected derivative was dissolved in $\text{TFA}-\text{H}_2\text{O}$ (ratio 1/1, 30 mL/mmol) at room temperature and stirred until TLC indicated completion of the reaction. Then, the solvents were evaporated and the crude was co-evaporated with EtOH under reduced pressure. The crude material was purified by flash chromatography on silica gel to afford the desired product.

General Procedure (B) for diethyl phosphonate removal. The protected phosphonate (1 eq.) was dissolved in anhydrous DMF (20 mL/mmol) and trimethylsilyl bromide (15 eq.) was added dropwise at 0°C . The reaction mixture was stirred at room temperature until completion of the reaction was indicated by TLC ($\text{PrOH}/\text{NH}_4\text{OH}/\text{H}_2\text{O}$, 7/2/1, v/v/v). Then, the reaction was stopped by adding triethylammonium bicarbonate buffer (TEAB 1 M, pH 7) and concentrated to dryness under high vacuum. Column chromatography of the crude materials on reverse phase (gradient: H_2O to MeOH 100%) gave the expected phosphonic acid (as triethylammonium salt), which was passed through a Dowex Na^+ ion exchange column, the desired fractions were collected and freeze dried leading to the title compound as sodium salt.

5'-Amino-5'-deoxy-2',3'-O-(isopropylidene)adenosine (10) was obtained from commercially available adenosine in 3 steps with 71% overall yield according to previously published procedures. [9] Characterizations (^1H , ^{13}C and MS) were in agreement with the literature. R_f ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 9/1) 0.04. ^1H NMR (300 MHz, CDCl_3): δ 8.31 (s, 1H, H-2), 7.91 (s, 1H, H-8), 6.11 (s, 2H, NH_2), 6.02 (d, $J = 3.0$ Hz, 1H, H-1'), 5.46 (dd, $J = 6.5, 3.0$ Hz, 1H, H-2'), 5.01 (dd, $J = 6.5, 3.5$ Hz, 1H, H-3'), 4.24 (dd, $J = 9.3, 4.5$ Hz, 1H, H-4'), 2.99 (qd, $J = 13.4, 5.2$ Hz, 2H, H-5'), 1.72 (s, 2H, $\text{CH}_2\text{-NH}_2$), 1.60 (s, 3H, CH_3), 1.37 (s, 3H, CH_3). ^{13}C NMR (75 MHz, CDCl_3): δ 155.9 (C-6), 153.3 (C-2), 149.5 (C-4), 140.0 (C-8), 120.5 (C-5), 114.7 ($\text{C}(\text{CH}_3)_2$), 90.8 (C-1'), 87.7 (C-4'), 83.8 (C-2'), 82.0 (C-3'), 44.0 (C-5'), 27.4 (CH_3), 25.5 (CH_3). Q-TOF MS E^+ : m/z 307.15 ($\text{M}+\text{H}^+$). HRMS Q-TOF MS E^+ : Calculated for $\text{C}_{13}\text{H}_{19}\text{N}_6\text{O}_3$ [$\text{M}+\text{H}^+$] 307.1519; found 307.1521.

5'-N-acetate-5'-deoxyadenosine (1) To a suspension of compound **10** (535 mg, 1.61 mmol) in anhydrous MeOH (32 mL) and under argon atmosphere was added freshly distilled triethylamine (566 μL , 2.5 eq.). Methyl bromoacetate (312 μL , 2.05 eq.) was then dropwise added over a period of 2 h and the reaction mixture was refluxed overnight before concentration under reduced pressure. Purification by flash chromatography on silica gel ($\text{CH}_2\text{Cl}_2/\text{MeOH}$, 19/1 to 9/1) afforded the alkylated intermediate as white solid (705 mg containing 20% of

triethylammonium salts). This last was directly used in the next step without further purification. R_f ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 9/1) 0.41. Q-TOF MS E^+ : m/z 379.17 ($\text{M}+\text{H}^+$). HRMS Q-TOF MS E^+ : Calculated for $\text{C}_{16}\text{H}_{23}\text{N}_6\text{O}_5$ [$\text{M}+\text{H}^+$] 379.1730; found: 379.1728. This derivative (200 mg, 0.44 mmol) was treated following general procedure A. Purification was achieved by flash chromatography on silica gel (EtOAc/MeOH , 100/0 to 50/50) to give the deprotected intermediate (as a white solid, 246 mg, 45% for two steps) which was engaged in the saponification step. R_f (EtOAc/MeOH 1/4) 0.40. Q-TOF MS E^+ : m/z 339.14 ($\text{M}+\text{H}^+$). Q-TOF MS E^- : m/z 337.10 ($\text{M}-\text{H}^-$). HRMS Q-TOF MS E^+ : Calculated for $\text{C}_{13}\text{H}_{19}\text{N}_6\text{O}_5$ [$\text{M}+\text{H}^+$] 339.1417; found: 339.1418. UV (EtOH abs) $\lambda_{\text{max}} = 259$ nm ($\epsilon_{\text{max}} = 9710$). Thus, methyl ester derivative (194 mg, 0.58 mmol) was dissolved in NaOH 1M solution and stirred at room temperature for 2 h. The volatiles were removed under reduced pressure and the crude was purified by flash chromatography on silica gel (EtOAc/MeOH , 1/2 to 0/100) to afford compound **1** as a white solid (118 mg, 60%). R_f (EtOAc/MeOH 1/9) 0.05. ^1H NMR (600 MHz, D_2O): δ 8.31 (s, 1H, H-2), 8.20 (s, 1H, H-8), 6.08 (d, $J = 5.5$ Hz, 1H, H-1'), H-2' (signal in D_2O peak), 4.39 – 4.35 (m, 2H, H-3', H-4'), 3.45 – 3.38 (m, 2H, $\text{CH}_2\text{-CO}$), 3.17 (d, $J = 5.6$ Hz, 2H, H-5'). ^{13}C NMR (151 MHz, D_2O): δ 176.5 (C=O), 155.5 (C-6), 152.8 (C-2), 148.7 (C-4), 140.3 (C-8), 118.9 (C-5), 88.1 (C-1'), 82.2 (C-4'), 73.5 (C-2'), 71.5 (C-3'), 51.3 ($\text{CH}_2\text{-CO}$), 49.9 (C-5'). Q-TOF MS E^+ : m/z 347.11 ($\text{M}+\text{H}^+$). HRMS Q-TOF MS E^+ : Calculated for $\text{C}_{12}\text{H}_{17}\text{N}_6\text{O}_5$ [$\text{M}-\text{Na}+2\text{H}^+$] 325.1260; found: 325.1261.

5'-Deoxy-5'-N-[(diethyl-phosphono)acetyl]-2',3'-O-isopropylidene-adenosine (11) Diethyl phosphonoacetic acid (321 μL , 2 eq.) was slowly added, at room temperature and under an argon atmosphere, to a solution of compound **10** (306 mg, 1 mmol), DCC (495 mg, 2.4 eq.) and 4-DMAP (12 mg, 0.1 eq.) in anhydrous DMF (12 mL). After 20 h, the reaction mixture was filtered and the filtrate was evaporated under reduced pressure. The resulting oil was then co-evaporated 3 times with EtOH (10 mL) before dissolution with CH_2Cl_2 . The organic layer was washed with H_2O (twice), dried (MgSO_4), filtered and concentrated to dryness. The resulting oil was purified by silica gel column chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}$, 9/1) to give compound **11** as a yellow foam (233 mg, 72%). R_f ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 9/1) 0.33. ^1H NMR (300 MHz, CDCl_3): δ 8.39 (brs, 1H, NH), 8.37 (s, 1H, H-2), 7.93 (s, 1H, H-8), 6.00 (brs, 2H, NH_2), 5.87 (d, $J = 4.5$ Hz, 1H, H-1'), 5.37 (dd, $J = 6.2, 4.5$ Hz, 1H, H-2'), 4.86 (dd, $J = 6.3, 2.3$ Hz, 1H, H-3'), 4.46 (dd, $J = 5.9, 3.3$ Hz, 1H, H-4'), 4.20 – 4.01 (m, 5H, O- CH_2 and H-5'), 3.38 – 3.33 (m, 1H, H-5'), 2.97 (dd, $J = 21.4, 4.7$ Hz, 2H, $\text{CH}_2\text{-P}$), 1.60 (s, 3H, CH_3), 1.35 (s, 3H, CH_3), 1.33 – 1.27 (m, 6H, O- $\text{CH}_2\text{-CH}_3$). ^{13}C NMR (75 MHz, CDCl_3): δ 165.4 (d, $J = 4.9$ Hz, C=O), 155.8 (C-6), 152.8 (C-2), 148.7 (C-4), 140.3 (C-8), 119.8 (C-5), 114.9 ($\text{C}(\text{CH}_3)_2$), 91.1 (C-1'), 83.8 (C-4'), 82.9 (C-2'), 81.5 (C-3'), 63.1 (d, $J = 11.2$ Hz, O- $\text{CH}_2\text{-CH}_3$), 41.2 (C-5'), 35.2 (d, $J = 134.1$ Hz, $\text{CH}_2\text{-P}$), 27.0 ($\text{C}(\text{CH}_3)_2$), 25.0 ($\text{C}(\text{CH}_3)_2$), 15.98 (d, $J = 6.1$ Hz, O- $\text{CH}_2\text{-CH}_3$). ^{31}P NMR (121 MHz, CDCl_3): δ 22.1. Q-TOF MS E^+ : m/z 485.19 ($\text{M}+\text{H}^+$). HRMS Q-TOF MS E^+ : Calculated for $\text{C}_{19}\text{H}_{29}\text{N}_6\text{O}_7\text{P}$ [$\text{M}+\text{H}^+$] 485.1914; found: 485.1923.

5'-Deoxy-5'-N-[(diethyl-phosphono)acetyl]adenosine (12) The title compound was obtained as a white solid (350 mg, 90%) from compound **11** (424 mg, 0.876 mmol) using general procedure A. Purification was achieved by flash chromatography on silica gel ($\text{CH}_2\text{Cl}_2/\text{MeOH}$, 9/1). R_f ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 9/1) 0.08. ^1H NMR (300 MHz, $\text{DMSO}-d_6$): δ 8.39 (s, 1H, H-2), 8.19 (s, 1H, H-8), 7.40 (brs, 2H, NH_2), 5.86 (d, $J = 6.3$ Hz, 1H, H-1'), 5.43 (brs, 1H, OH), 5.28 (brs, 1H, OH), 4.68 (t, $J = 5.7$ Hz, 1H, H-2'), 4.07 – 3.93 (m, 6H, H-3', H-4' and O- $\text{CH}_2\text{-CH}_3$), H-5' (signal in H_2O peak.), 2.91 (d, $J = 21.4$ Hz, 2H, $\text{CH}_2\text{-P}$), 1.21 (t, $J = 7.1$ Hz, 6H, CH_3). ^{13}C NMR (75 MHz, $\text{DMSO}-d_6$): δ 164.3 (C=O), 156.0 (C-6), 152.3 (C-2), 149.3 (C-4), 140.4 (C-8), 119.4 (C-5), 87.6 (C-1'), 83.5 (C-4'), 72.7 (C-2'), 71.1 (C-3'), 61.7 (d, $J = 6.1$ Hz, O- $\text{CH}_2\text{-CH}_3$), 41.3 (C-5'), 34.6 (d, $J = 132.3$ Hz, $\text{CH}_2\text{-P}$), 16.2 (d, $J = 5.8$ Hz, CH_3). ^{31}P NMR (121 MHz, $\text{DMSO}-d_6$): δ 22.8. Q-TOF MS E^+ : m/z 445.16 ($\text{M}+\text{H}^+$).

5'-Deoxy-5'-N-(phosphono-acetyl)adenosine (disodium salts) (2) The title compound was obtained as a white solid (17 mg, 7%) from

compound **12** (287 mg, 0.646 mmol) using general procedure B. Purification was achieved by flash chromatography on reverse phase (H₂O/MeOH, 100/0 to 50/50). *R_f* (iPrOH/H₂O/NH₄OH, 6/3/1) 0.39. ¹H NMR (400 MHz, D₂O): δ 8.31 (s, 1H, H-2), 8.19 (s, 1H, H-8), 6.01 (d, *J* = 5.6 Hz, 1H, H-1'), H-2' signal in D₂O peak, 4.35 (t, *J* = 4.8 Hz, 1H, H-2'), 4.28 (q, *J* = 4.8 Hz, 1H, H-4'), 3.65 and 3.56 (ABX, *J* = 14.5, 5.3, 4.8 Hz, 2H, H-5'), 2.62 (d, *J* = 19.2 Hz, 2H, CH₂-P). ¹³C NMR (101 MHz, D₂O): δ 172.4 (d, *J* = 4.4 Hz, C=O), 155.4 (C-6), 152.7 (C-2), 148.7 (C-4), 140.2 (C-8), 118.8 (C-5), 87.4 (C-1'), 83.2 (C-4'), 73.2 (C-2'), 70.9 (C-3'), 40.9 (C-5'), 38.4 (d, *J* = 115.1 Hz, CH₂-P). ³¹P NMR (162 MHz, D₂O): δ 12.45. Q-TOF MS E⁺: *m/z* 389.10 (M+H)⁺. HRMS Q-TOF MS E⁺: for Calculated C₁₂H₁₈N₆O₆P [M+H]⁺ 389.0975; found: 389.0975. UV (EtOH abs) λ_{max} = 261 nm (ε_{max} = 12300).

5'-Deoxy-5'-N-(ethyl-phosphono-acetyl)adenosine (monosodium salt) (3) Compound **3** was obtained as a white solid (60 mg, 20%) from compound **12** (350 mg, 0.790 mmol) using general procedure B. Purification was achieved by flash chromatography on reverse phase (H₂O/MeOH, 100/0 to 50/50). *R_f* (iPrOH/H₂O/NH₄OH, 6/3/1) 0.59. ¹H NMR (400 MHz, D₂O): δ 8.31 (s, 1H, H-2), 8.25 (s, 1H, H-8), 6.01 (d, *J* = 5.6 Hz, 1H, H-1'), 4.84 – 4.81 (m, 1H, H-2'), 4.35 – 4.26 (m, 2H, H-3' and H-4'), 3.92 – 3.83 (m, 2H, O-CH₂-CH₃), 3.70 and 3.51 (ABX, *J* = 15.5, 5.2, 3.7 Hz, 2H, H-5'), 2.72 (d, *J* = 20.3 Hz, 2H, CH₂-P), 1.15 (t, *J* = 7.1 Hz, 3H, CH₃). ¹³C NMR (101 MHz, D₂O): δ 170.3 (d, *J* = 5.7 Hz, C=O), 155.6 (C-6), 152.9 (C-2), 148.8 (C-4), 140.5 (C-8), 119.0 (C-5), 87.7 (C-1'), 83.3 (C-4'), 73.1 (C-2'), 70.9 (C-3'), 61.3 (d, *J* = 5.9 Hz, O-CH₂-CH₃), 40.9 (C-5'), 36.4 (d, *J* = 122.0 Hz, CH₂-P), 15.83 (d, *J* = 6.2 Hz, CH₃). ³¹P NMR (162 MHz, D₂O): δ 16.0. Q-TOF MS E⁺: *m/z* 439.11 (M+H)⁺. Q-TOF MS E⁺: *m/z* 415.11 (M-Na)⁺. HRMS Q-TOF MS E⁺: Calculated for C₁₄H₂₁N₆O₆NaP [M+H]⁺ 439.1107; found: 439.1108. UV (EtOH abs) λ_{max} = 259 nm (ε_{max} = 13900).

5'-Deoxy-5'-N-[(diethyl-phosphono)methyl]-2',3'-O-isopropylidene-adenosine (13) A solution of compound **10** (408 mg, 1.2 mmol) in anhydrous THF (4 mL) was added, at room temperature, to a mixture of (diethoxyphosphoryl)methyl 4-methylbenzenesulfonate (322 mg, 1.0 mmol) and DBU (152 μL, 1.0 mmol) under an argon atmosphere. The reaction mixture was stirred at 50°C for 4 days before concentration under reduced pressure. The crude was purified by flash chromatography on silica gel (CH₂Cl₂/MeOH, 9/1) to give **13** as a white solid (232 mg, 51%). *R_f* (CH₂Cl₂/MeOH 9/1) 0.40. ¹H NMR (400 MHz, CDCl₃): δ 8.35 (s, 1H, H-2), 7.92 (s, 1H, H-8), 6.00 (d, *J* = 3.0 Hz, 1H, H-1'), 5.77 (s, 2H, NH₂), 5.42 (dd, *J* = 6.3, 3.1 Hz, 1H, H-2'), 5.02 (dd, *J* = 6.3, 3.3 Hz, 1H, H-3'), 4.35 – 4.33 (m, 1H, H-4'), 4.16 – 4.07 (m, 4H, O-CH₂-CH₃), 3.07 – 2.93 (m, 4H, H-5' + CH₂-P), 1.60 (s, 3H, CH₃), 1.37 (s, 3H, CH₃), 1.35 – 1.27 (m, 6H, O-CH₂-CH₃). ¹³C NMR (101 MHz, CDCl₃): δ 155.7 (C-6), 153.4 (C-2), 149.6 (C-4), 140.0 (C-8), 120.5 (C-5), 114.7 (C(CH₃)₂), 90.9 (C-1'), 85.6 (C-4'), 83.6 (C-2'), 82.3 (C-3'), 62.2 (d, *J* = 6.6 Hz, O-CH₂-CH₃), 52.5 (d, *J* = 13.0 Hz, C-5'), 45.3 (d, *J* = 154.5 Hz, CH₂-P), 27.4 (CH₃), 25.5 (CH₃), 16.6 (d, *J* = 5.7 Hz, O-CH₂-CH₃). ³¹P NMR (162 MHz, CDCl₃): δ 25.7. Q-TOF MS E⁺: *m/z* 457.20 (M+H)⁺. HRMS Q-TOF MS E⁺: Calculated for C₁₈H₃₀N₆O₆P [M+H]⁺ 457.1964; found: 457.1965.

5'-Deoxy-5'-N-[(diethyl-phosphono)methyl]adenosine (14) The title compound was obtained as a white solid (60 mg, 33%) from compound **13** (200 mg, 0.438 mmol) using general procedure A. Purification was achieved by flash chromatography on reverse phase (H₂O/MeOH, 100/0 to 50/50). *R_f* (CH₂Cl₂/MeOH 9/1) 0.06. ¹H NMR (300 MHz, D₂O): δ 8.27 (s, 1H, H-2), 8.25 (s, 1H, H-8), 6.07 (d, *J* = 5.4 Hz, 1H, H-1'), 4.86 – 4.82 (m, 1H, H-2'), 4.47 – 4.41 (m, 2H, H-3' and H-4'), 4.19 – 4.06 (m, 4H, O-CH₂-CH₃), 3.55 (d, *J* = 13.5 Hz, 2H, H-5'), 3.49 (d, *J* = 5.8 Hz, 2H, CH₂-P), 1.23 (t, *J* = 6.9 Hz, 3H, CH₃), 1.19 (t, *J* = 6.8 Hz, 3H, CH₃). ¹³C NMR (75 MHz, D₂O): δ 155.3 (C-6), 152.3 (C-2), 148.4 (C-4), 141.0 (C-8), 119.3 (C-5), 89.2 (C-1'), 80.1 (C-4'), 73.0 (C-2'), 71.6 (C-3'), 64.6 (d, *J* = 6.5 Hz, O-CH₂-CH₃), 50.3 (d, *J* = 9.6 Hz, C-5'), 41.2 (d, *J* = 153.1 Hz, CH₂-P), 15.5 – 15.3 (m, CH₃). ³¹P NMR (121 MHz, D₂O): δ 20.6. Q-TOF MS E⁺:

m/z 417.17 (M+H)⁺. HRMS Q-TOF MS E⁺: Calculated for C₁₅H₂₆N₆O₆P [M+H]⁺ 417.1651; found: 417.1651.

5'-Deoxy-5'-N-[(ethyl-phosphono)methyl]adenosine (monosodium salt) (4) The title compound was obtained as a white solid (40 mg, 29%) from compound **14** (140 mg, 0.336 mmol) using general procedure B. Purification was achieved by flash chromatography on reverse phase (H₂O/MeOH, 100/0 to 50/50). *R_f* (iPrOH/NH₄OH/H₂O, 7/2/1) 0.43. ¹H NMR (400 MHz, D₂O): δ 8.30 (s, 1H, H-2), 8.24 (s, 1H, H-8), 6.07 (d, *J* = 5.6 Hz, 1H, H-1'), 4.85 – 4.82 (m, 1H, H-2'), 4.39 – 4.35 (m, 2H, H-3', H-4'), 3.89 – 3.84 (m, 2H, O-CH₂-CH₃), 3.26 – 3.24 (m, 2H, H-5'), 3.05 – 2.93 (m, 2H, CH₂-P), 1.13 (t, *J* = 7.1 Hz, 3H, CH₃). ¹³C NMR (101 MHz, D₂O): δ 155.6 (C-6), 152.9 (C-2), 148.7 (C-4), 140.4 (C-8), 119.0 (C-5), 88.2 (C-1'), 81.9 (C-4'), 73.2 (C-2'), 71.6 (C-3'), 60.9 (d, *J* = 5.8 Hz, O-CH₂-CH₃), 51.3 (d, *J* = 10.2 Hz, C-5'), 44.0 (d, *J* = 142.9 Hz, CH₂-P), 15.8 (d, *J* = 5.9 Hz, CH₃). ³¹P NMR (162 MHz, D₂O): δ 17.7. Q-TOF MS E⁺: *m/z* 411.12 (M+H)⁺. Q-TOF MS E⁺: *m/z* 387.12 (M-Na)⁺. HRMS Q-TOF MS E⁺: Calculated for C₁₃H₂₁N₆O₆NaP [M+H]⁺ 411.1158; found: 411.1161. UV (EtOH abs) λ_{max} = 259 nm (ε_{max} = 15800).

5'-Deoxy-5'-N-phosphonomethyladenosine (disodium salt) (5) The title compound was obtained as a white solid (29 mg, 17% over the 2 steps) from compound **13** (182 mg, 0.4 mmol) using general procedure B. The residue was purified on reverse phase (H₂O/MeOH: 100/0) to give the intermediate deprotected phosphonate (37 mg). This last was subsequently treated following procedure A. Purification on reverse phase (H₂O/MeOH: 100/0) followed by an ion exchange on DOWEX 50WX2 (Na⁺ form). ¹H NMR (500 MHz, D₂O): δ 8.27 (s, 1H, H-8), 8.23 (s, 1H, H-2), 6.07 (d, *J* = 5.4 Hz, 1H, H-1'), H-2' signal in D₂O peak, 4.48 – 4.45 (m, 1H, H-4'), 4.42 (t, *J* = 4.8 Hz, 1H, H-3'), 3.60 – 3.52 (m, 2H, H-5'), 3.00 (d, *J* = 11.6 Hz, 2H, CH₂-P). ¹³C NMR (126 MHz, D₂O): δ 155.5 (C-6), 152.9 (C-2), 148.6 (C-4), 140.5 (C-8), 119.0 (C-5), 88.6 (C-1'), 80.0 (C-4'), 73.2 (C-2'), 71.4 (C-3'), 50.8 (C-5'), 46.6 (d, *J* = 128.9 Hz, CH₂-P). ³¹P NMR (162 MHz, D₂O): δ 7.34. Q-TOF MS E⁺: *m/z* 405.07 (M+H)⁺. HRMS Q-TOF MS E⁺: calculated for C₁₁H₁₆N₆O₆PNa₂ [M+H]⁺ + 405.0664 found; 405.0663.

5'-Deoxy-5'-N-[(tetraethyl-bisphosphono)methyl]adenosine (15) Compound **10** (302.4 mg, 0.98 mmol), paraformaldehyde (62.9 mg, 2 eq.) and diethylphosphite (0.26 mL, 2 eq.) were sealed in a microwave reactor. Under stirring, microwave irradiations were applied for 1h at 100°C with a power of 850 Watts. The crude mixture was purified on silica gel flash chromatography (CH₂Cl₂/MeOH: 90/10) to afford protected intermediate (300 mg, 0.5 mmol). This latter was treated following procedure A. The residue was co-evaporated with EtOH and gave, after purification on reverse phase (H₂O/MeOH: 100/0 to 0/100) and freeze-drying, the desired compound **15** as a white solid (172.3 mg, 31%). *R_f* (iPrOH/NH₄OH/H₂O, 7/1/2) 0.81. ¹H NMR (500 MHz, CDCl₃): δ 8.25 (s, 1H, H-2), 8.11 (s, 1H, H-8), 6.27 (br s, 2H, NH₂), 6.01 (d, *J* = 3.9 Hz, 1H, H-1'), 4.65 – 4.63 (m, 1H, H-2'), 4.55 – 4.53 (m, 1H, H-3'), 4.21 – 4.18 (m, 1H, H-4'), 4.12 – 4.08 (m, 8H, O-CH₂-CH₃), 3.26 – 3.11 (m, 6H, CH₂-P + H-5'), 1.30 – 1.27 (m, 12H, O-CH₂-CH₃). ¹³C NMR (126 MHz, CDCl₃): δ 155.6 (C-6), 152.9 (C-2), 149.6 (C-4), 139.6 (C-8), 119.8 (C-5), 89.2 (C-1'), 83.3 (C-4'), 74.5 (C-2'), 71.2 (C-3'), 62.5 (O-CH₂-CH₃), 57.8 (C-5'), 52.4 (d, *J* = 7.5 Hz, CH₂-P), 51.1 (d, *J* = 7.5 Hz, CH₂-P), 16.6 (O-CH₂-CH₃). ³¹P NMR (202 MHz, CDCl₃): δ 24.6. Q-TOF MS E⁺: *m/z* 567.21 (M+H)⁺. HRMS Q-TOF MS E⁺: calculated for C₂₀H₃₇N₆O₉P₂ [M+H]⁺ 567.2097; found 567.2103.

5'-Deoxy-5'-N-[(bisphosphono)methyl]adenosine (tetra sodium salt) (6) The title compound was obtained as a white solid (158 mg, quantitative yield) from compound **15** (165 mg, 0.3 mmol) using general procedure B. After stirred at room temperature for 24 h, trimethylsilyl bromide (200 μL, 5 eq.) was added and the mixture stirred at room temperature for 5 h. Purification by reverse-phase column chromatography with H₂O followed by ion exchange on DOWEX 50WX2 (Na⁺ form) provided compound **6** as a white solid (158 mg, quantitative yield). ¹H NMR (500 MHz, D₂O): δ 8.38 (s, 1H, H-2), 8.30 (s, 1H, H-8),

6.14 (d, $J = 5.3$ Hz, 1H, H-1'), H-2' signal in D₂O peak, 4.67 – 4.63 (m, 1H, H-4'), 4.43 – 4.41 (m, 1H, H-3'), 4.06 – 3.84 (m, 2H, H-5'), 3.61 – 3.47 (m, 4H, CH₂-P). ¹³C NMR (126 MHz, D₂O): δ 155.4 (C-6), 152.6 (C-2), 148.7 (C-4), 140.7 (C-8), 119.2 (C-5), 88.7 (C-1'), 78.7 (C-4'), 73.1 (C-2'), 71.6 (C-3'), 58.1 (C-5'), 52.6 (CH₂P), 51.6 (CH₂P). ³¹P NMR (121 MHz, D₂O): δ 7.9. Q-TOF MS E⁺: m/z 475.05 (M-H)⁺. HRMS Q-TOF MS E⁺: calculated for C₁₂H₁₈N₆O₉NaP₂ [M-H]⁺: 475.0508; found 475.0511.

5'-Deoxy-5'-N-[(diethoxyphosphorylethylacetate)]-2',3'-O-isopropylidene-adenosine (16) To a stirred solution of **10** (419.4 mg, 1.37 mmol) in freshly distilled dichloromethane (7 mL) was added MgSO₄ (300 mg, 5 eq.) and ethylglyoxylate (50% solution (wt.) in toluene, 272 μ L, 1.2 eq.) under an argon atmosphere. After stirring at room temperature for 3 h, the mixture was filtered and concentrated in vacuum to afford the intermediate imine. It was transferred in a microwave reactor and diethyl phosphite (700 μ L, 4 eq.) was added, then the mixture was irradiated by microwave for 6 h at 100°C with a power of 850 Watts. Purification by flash chromatography on silica gel (CH₂Cl₂/MeOH: 90/10) afforded 1/1 mixture of diastereomers **16** as a white solid (375 mg, 53%). R_f (CH₂Cl₂/MeOH, 9/1) 0.48. ¹H NMR (500 MHz, CDCl₃): δ 8.39 (s, 1H, H-2, dia1 or dia2), 8.36 (s, 1H, H-2, dia1 or dia2), 8.06 (s, 1H, H-8, dia1 or dia2), 7.95 (s, 1H, H-8, dia1 or dia2), 6.06 (d, $J = 2.9$ Hz, 1H, H-1', dia1), 6.03 (d, $J = 3.2$ Hz, 1H, H-1', dia2), 5.74 (br s, 4H, NH₂, dia1 + dia2), 5.42 (dd, $J = 6.5$, 3.0 Hz, 1H, H-2', dia1), 5.39 (dd, $J = 6.4$, 3.2 Hz, 1H, H-2', dia2), 5.07 (dd, $J = 6.4$, 3.2 Hz, 1H, H-3', dia2), 5.02 (dd, $J = 6.5$, 3.4 Hz, 1H, H-3', dia1), 4.37 – 4.32 (m, 2H, H-4', dia1 + dia2), 4.24 – 4.11 (m, 12H, O-CH₂-CH₃, dia1 + dia2), 3.77 (s, 1H, CHP, dia1 or dia2), 3.72 (s, 1H, CHP, dia1 or dia2), 3.10 (dd, $J = 4.0$, 12.7 Hz, 1H, H-5', dia2), 2.98 – 2.86 (m, 2H, H-5', dia1 + dia2), 2.79 (dd, $J = 3.8$, 12.7 Hz, 1H, H-5' dia2), 1.61 (s, 6H, CH₃ dia1 + dia2), 1.38 (s, 6H, CH₃, dia1 + dia2), 1.33 – 1.29 (m, 12H, O-CH₂-CH₃, dia1 + dia2), 1.24 – 1.21 (m, 6H, O-CH₂-CH₃, dia1 + dia2). ¹³C NMR (126 MHz, CDCl₃): δ 169.2 (CO, dia1 or dia2), 169.1 (CO, dia1 or dia2), 155.6 (C-6, dia1 + dia2), 153.5 (C-2, dia1 or dia2), 153.3 (C-2, dia1 or dia2), 149.6 (C-4, dia1 + dia2), 140.1 (C-8, dia1), 139.9 (C-8, dia2), 120.5 (C-5, dia1 or dia2), 120.4 (C-5, dia1 or dia2), 114.8 (C(CH₃)₂, dia1), 114.7 (C(CH₃)₂, dia2), 91.0 (C-1', dia2), 90.4 (C-1', dia1), 86.3 (C-4', dia1), 85.9 (C-4', dia2), 83.9 (C-2', dia1), 83.7 (C-2', dia2), 82.3 (C-3', dia1 + dia2), 63.6 (O-CH₂-CH₃, dia1 + dia2), 63.5 – 63.4 (O-CH₂-CH₃, dia1 + dia2), 61.9 (O-CH₂-CH₃, dia1 + dia2), 61.4 – 61.3 (CHP, dia1 or dia2), 60.2 (CHP, dia1 or dia2), 50.8 – 50.6 (C-5', dia1 + dia2), 27.5 (CH₃, dia1 + dia2), 27.4 (CH₃, dia1 + dia2), 16.6 – 16.5 (P-O-CH₂-CH₃, dia1 + dia2), 14.2 (C-O-CH₂-CH₃, dia1 + dia2). ³¹P NMR (202 MHz, CDCl₃): δ 17.6 (dia1 or dia2), 17.5 (dia1 or dia2). Q-TOF MS E⁺: m/z 529.22 (M+H)⁺. HRMS Q-TOF MS E⁺: calculated for C₂₁H₃₄N₆O₈P [M+H]⁺ 529.2176; found 529.2177.

5'-Deoxy-5'-N-[(diethoxyphosphorylethylacetate)]adenosine (17) The title compound was obtained as a white solid (330.2 mg, 98%) from compound **16** (365.2 mg, 0.69 mmol) using general procedure A. Purification was carried by flash chromatography on silica gel (CH₂Cl₂/MeOH, 9/1). R_f (CH₂Cl₂/MeOH, 9/1) 0.32. ¹H NMR (300 MHz, CD₃OD): δ 8.36 – 8.26 (m, 4H, H-2 & H-8, dia1 + dia2), 5.98 (d, $J = 5.5$ Hz, 1H, H-1', dia1), 5.94 (d, $J = 5.9$ Hz, 1H, H-1', dia2), H-2' dia2 signal in H₂O peak, 4.80 – 4.77 (m, 1H, H-2', dia1), 4.38 (dd, $J = 5.3$, 3.5 Hz, 1H, H-3', dia2), 4.26 – 4.10 (m, 15H, O-CH₂-CH₃, H-3' dia1 and H-4' dia1 + dia2), 3.15 (dd, $J = 12.8$, 3.1 Hz, 1H, H-5' dia2), 3.03 – 3.00 (m, 2H, H-5', dia1 + dia2), 2.83 (dd, $J = 12.8$, 4.9 Hz, 1H, H-5', dia2), 1.34 – 1.20 (m, 18H, O-CH₂-CH₃, dia1 + dia2). ¹³C NMR (126 MHz, CD₃OD): δ 170.4 (CO, dia1 + dia2), 156.6 (C-6, dia1 or dia2), 156.4 (C-6, dia1 or dia2), 152.8 (C-2, dia1), 152.7 (C-2, dia2), 150.6 (C-4, dia1), 150.5 (C-4, dia2), 142.5 (C-8, dia2), 142.1 (C-8, dia1), 120.8 (C-5, dia2), 120.7 (C-5, dia1), 90.8 (C-1', dia2), 90.4 (C-1', dia1), 86.3 (C-4', dia2), 85.9 (C-4', dia1), 75.0 (C-2', dia1), 74.6 (C-2', dia2), 73.0 (C-3', dia1), 72.9 (C-3', dia2), 65.3 – 64.8 (m, O-CH₂-CH₃, dia1 + dia2), 63.0 (CHP, dia1 + dia2), 51.9 – 51.4 (C-5', dia1 + dia2), 16.8 – 14.4 (m, C(O)CH₂-CH₃ and P(O)-CH₂-CH₃, dia1 + dia2). ³¹P NMR (202 MHz, CD₃OD): δ 18.4 (dia1 or dia2), 18.3 (dia1 or dia2). Q-TOF MS E⁺: m/z 489.19 (M+H)⁺. HRMS Q-TOF MS E⁺: calculated for C₁₈H₃₀N₆O₈P [M+H]⁺ 489.1863; found 489.1866.

5'-Deoxy-5'-N-[(ethoxyphosphorylacetate)]adenosine (disodium salt) (7) The title compound was obtained as a white solid (71.6 mg, 78%) from compound **17** (94.5 mg, 0.19 mmol) using general procedure B. Purification by reverse-phase column chromatography with H₂O/MeOH (100/0 to 0/100) afforded the desired intermediate as a triethylammonium salt. This last was treated with NaOH solution (1M in H₂O, 300 μ L, 1.5 eq.) and the mixture was stirred at room temperature overnight. The mixture was neutralized with a saturated aqueous solution of NH₄Cl. After freeze-drying, purification by reverse phase column chromatography with H₂O/MeOH (100/0 to 0/100) and ion exchange on DOWEX 50WX2 (Na⁺ form) afforded the desired mixture of diastereomers **7**. R_f (iPrOH/NH₄OH/H₂O, 7/1/2) 0.62. ¹H NMR (500 MHz, D₂O): δ 8.33 – 8.29 (m, 4H, H-2 and H-8, dia1 + dia2), 6.11 – 6.09 (m, 2H, H-1', dia1 + dia2), H-2' dia1 + dia2 signal in D₂O peak, 4.55 – 4.48 (m, 4H, H-4' and H-3', dia1 + dia2), 4.01–3.86 (m, 6H, CHP and O-CH₂-CH₃, dia1 + dia2), 3.75 – 3.60 (m, 4H, H-5', dia1 + dia2), 1.17 – 1.09 (m, 6H, CH₃, dia1 + dia2). ¹³C NMR (126 MHz, D₂O): δ 168.9 (CO, dia1 + dia2), 155.2 (C-6, dia1 + dia2), 152.4 (C-2, dia1 or dia2), 152.3 (C-2, dia1 or dia2), 148.5 (C-4, dia2), 148.4 (C-4, dia1), 141.2 (C-8, dia1 + dia2), 119.4 (C-5, dia1 + dia2), 89.4 (C-1', dia1 + dia2), 79.9 (C-4', dia2), 79.2 (C-4', dia1), 73.3 (C-2', dia1), 73.0 (C-2', dia2), 71.5 (C-3', dia1), 71.4 (C-3', dia2), 62.6 – 59.9 (m, CHP and O-CH₂-CH₃, dia1 + dia2), 49.3 – 48.8 (m, C-5', dia1 + dia2), 15.9 – 15.8 (m, CH₃, dia1 + dia2). ³¹P NMR (202 MHz, D₂O): δ 7.9 (dia1 or dia2), 7.8 (dia1 or dia2). Q-TOF MS E⁺: m/z 477.09 (M+H)⁺. HRMS Q-TOF MS E⁺: calculated for C₁₄H₂₀N₆O₈PNa₂ [M+H]⁺ 477.0876; found 477.0876.

5'-Deoxy-5'-N-[(phosphorylacetate)]adenosine (tri sodium salt) (8) The title compound was obtained as a white solid (78.9 mg, 77%) from compound **17** (219.2 mg, 0.45 mmol) using general procedure B. Purification was carried out by reverse phase column chromatography with H₂O/MeOH (100/0 to 0/100) to afford desired compound as a triethylammonium salt. This last was then dissolved in a NaOH solution (1M in H₂O, 2.1 mL, 10 eq.) and stirred at room temperature for a week the solution was adjusted to pH = 5 with an ion exchange resin DOWEX 50WX2 (H⁺ form). Freeze drying, followed by reverse phase column chromatography with H₂O/MeOH (0 to 100) and ion exchange on DOWEX 50WX2 (Na⁺ form) provided the desired mixture of diastereomers **8**. R_f (iPrOH/NH₄OH/H₂O, 5/1/4) 0.22. ¹H NMR (500 MHz, D₂O): δ 8.28 (d, 2H, H-8, dia1 + dia2), 8.22 (d, 2H, H-2, dia1 + dia2), 6.09 – 6.06 (m, 2H, H-1', dia1 + dia2), H-2' dia1 + dia2 signal in D₂O peak, 4.53 – 4.48 (m, 2H, H-4', dia1 + dia2), 4.46 – 4.44 (m, 1H, H-3', dia1), 4.41 – 4.39 (m, 1H, H-3', dia2), 3.76 (t, $J = 14.0$ Hz, 2H, CHP, dia1 + dia2), 3.69 – 3.52 (m, 4H, H-5', dia1 + dia2). ¹³C NMR (126 MHz, D₂O): δ 171.7 – 171.6 (m, CO, dia1 + dia2), 155.4 (C-6, dia1 + dia2), 153.0 (C-2, dia1), 152.8 (C-2, dia2), 148.5 (C-4, dia1 + dia2), 140.5 (C-8, dia1 or dia2), 140.4 (C-8, dia1 or dia2), 119.0 (C-5, dia1 + dia2), 88.8 (C-1', dia1), 88.6 (C-1', dia2), 79.8 (C-4', dia1), 79.5 (C-4', dia2), 73.4 (C-2', dia2), 73.2 (C-2', dia1), 71.4 (C-3', dia2), 71.2 (C-3', dia1), 64.2 – 63.1 (m, CHP, dia1 + dia2), 50.1 – 49.9 (m, C-5', dia1 + dia2). ³¹P NMR (202 MHz, D₂O): δ 5.8 (dia1 + dia2). Q-TOF MS E⁺: m/z 405.09 (M+H)⁺. HRMS Q-TOF MS E⁺: calculated for C₁₂H₁₈N₆O₈P [M+H]⁺ 405.0924; found 405.0921.

9-(2,3-O-isopropylidene- β -D-ribofuranosyl)-6-(2,5-dimethylpyrrol-1-yl)purine (18)^[16] Compound **18** was obtained from commercially available adenosine with 36% yield according previously published procedure.^[16] Characterization (¹H) was in agreement with the literature. R_f (Petroleum ether / EtOAc, 7/3) 0.23. ¹H NMR (400 MHz, CDCl₃): δ 8.92 (s, 1H, H-2), 8.17 (s, 1H, H-8), 6.00 (s, 2H, CH), 5.98 (d, $J = 5.0$ Hz, 1H, H-1'), 5.32 – 5.29 (m, 1H, H-2'), 5.16 (dd, $J = 5.9$, 1.2 Hz, 1H, H-3'), 4.59 (s, 1H, H-4'), 4.02 and 3.85 (AB, $J = 12.8$, 1.8 Hz, 2H, H-5'), 2.21 (s, 6H, CH₃), 1.67 (s, 3H, CH₃), 1.40 (s, 3H, CH₃).

9-(5-azido-5-deoxy-2,3-O-isopropylidene- β -D-ribofuranosyl)-6-(2,5-dimethylpyrrol-1-yl)purine (19) To a stirred solution of compound **18** (300 mg, 0.78 mmol) in anhydrous 1,4-dioxane (3 mL) was added diphenyl phosphoryl azide (336 μ L, 2 eq.) and 1,8-

diazabicyclo[5.4.0]undec-7-ene (350 μ L, 3 eq.) under an argon atmosphere. The mixture was stirred overnight at room temperature before the addition of sodium azide (253 mg, 5 eq.) and a catalytic amount of crown ether (15-crown-5, 1.7 mg, 0.01 eq.). This mixture was heated at reflux for 4 h, before filtration and concentration to dryness under reduced pressure. Purification by column chromatography with Petroleum ether/EtOAc (80/20) afforded compound **19** as a white solid (319 mg, quantitative yield). R_f (Petroleum ether/EtOAc, 7/3) 0.73. ^1H NMR (600 MHz, CDCl_3): δ 8.95 (s, 1H, H-2), 8.24 (s, 1H, H-8), 6.23 (d, J = 2.5 Hz, 1H, H-1'), 5.99 (s, 2H, 2xCH), 5.48 – 5.47 (m, 1H, H-2'), 5.08 – 5.07 (m, 1H, H-3'), 4.44 – 4.42 (m, 1H, H-4'), 3.69 – 3.62 (m, 2H, H-5'), 2.20 (s, 6H, CH_3 pyrrole), 1.65 (s, 3H, CH_3), 1.41 (s, 3H, CH_3). ^{13}C NMR (151 MHz, CDCl_3): δ 152.9 (C-4), 152.7 (C-2), 150.8 (Cq), 143.9 (C-8), 130.0 (C-5), 129.4 (C-6), 115.4 (Cq), 109.3 (CH), 90.7 (C-1'), 85.2 (C-4'), 84.1 (C-2'), 81.8 (C-3'), 52.4 (C-5'), 27.4 (CH_3), 25.5 (CH_3), 13.7 (CH_3). Q-TOF MS E^+ : m/z 411.19 ($\text{M}+\text{H}^+$). HRMS Q-TOF MS E^+ : calculated for $\text{C}_{19}\text{H}_{23}\text{N}_6\text{O}_3$ [$\text{M}+\text{H}^+$] 411.1893; found 411.1891.

9-(5-amino-5-deoxy-2,3-O-isopropylidene- β -D-ribofuranosyl)-6-(2,5-dimethylpyrrol-1-yl)purine (20) Compound **19** (587.5 mg, 1.4 mmol) was dissolved in absolute ethanol (7 mL) then Palladium on carbon 10% (44 mg, 0.03 eq.) was added and the heterogeneous mixture was stirred overnight under a hydrogen atmosphere. After filtration through a pad of Celite, the residual mixture was concentrated in vacuum. Purification by column chromatography with $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (90/10) afforded compound **20** as a white solid (539 mg, 98%). R_f ($\text{CH}_2\text{Cl}_2/\text{MeOH}$, 9/1) 0.58. ^1H NMR (600 MHz, CDCl_3): δ 8.93 (s, 1H, H-2), 8.28 (s, 1H, H-8), 6.15 (d, J = 3.3 Hz, 1H, H-1'), 5.98 (s, 2H, 2xCH), 5.48 – 5.46 (m, 1H, H-2'), 5.07 – 5.06 (m, 1H, H-3'), 4.33 – 4.31 (m, 1H, H-4'), 3.13 – 3.02 (m, 2H, H-5'), 2.20 (s, 6H, CH_3), 1.65 (s, 3H, CH_3), 1.40 (s, 3H, CH_3). ^{13}C NMR (151 MHz, CDCl_3): δ 153.0 (C-4), 152.6 (C-2), 150.7 (Cq), 144.2 (C-8), 129.9 (C-5), 129.5 (C-6), 115.2 (C), 109.3 (CH), 90.9 (C-1'), 87.1 (C-4'), 83.8 (C-2'), 81.8 (C-3'), 43.7 (C-5'), 27.4 (CH_3), 25.5 (CH_3), 13.7 (CH_3). Q-TOF MS E^+ : m/z 385.20 ($\text{M}+\text{H}^+$). HRMS Q-TOF MS E^+ : calculated for $\text{C}_{19}\text{H}_{25}\text{N}_6\text{O}_3$ [$\text{M}+\text{H}^+$] 385.1988; found 385.1986.

9-(5-deoxy-2,3-O-isopropylidene- β -D-ribofuranosyl-5-N[(tetraethyl(methylene bisphosphonate))]-6-(2,5-dimethylpyrrol-1-yl)- purine (21) Compound **20** (260mg, 0.68 mmol), triethyl orthoformate (135 μ L, 1.2 eq.) and diethylphosphite (306 μ L, 3.5 eq.) were sealed in a microwave reactor under stirring. Microwave irradiations were applied for 2 h at 125°C with a power of 850 Watts. The resulting brown oil was purified by column chromatography $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (95/5) to afford the (aminomethylene) bisphosphonate **21** as a white solid (157 mg, 34%). R_f ($\text{CH}_2\text{Cl}_2/\text{MeOH}$, 9/1) 0.72. ^1H NMR (500 MHz, CDCl_3): δ 9.01 (s, 1H, H-2), 8.33 (s, 1H, H-8), 6.12 (d, J = 3.8 Hz, 1H, H-1'), 5.98 (s, 2H, 2xCH), 5.43 (dd, J = 6.5, 3.8 Hz, 1H, H-2'), 5.13 (dd, J = 6.5, 3.2 Hz, 1H, H-3'), 4.40 – 4.38 (m, 1H, H-4'), 4.23 – 4.15 (m, 8H, O- CH_2), 3.41 – 3.32 (m, 2H, H-5' and CHP), 3.11 – 3.08 (m, 1H, H-5'), 2.20 (s, 6H, CH_3), 1.64 (s, 3H, CH_3), 1.39 (s, 3H, CH_3), 1.32 – 1.27 (m, 12H, O- CH_2 - CH_3). ^{13}C NMR (101 MHz, CDCl_3): δ 153.2 (C-4), 152.9 (C-2), 150.7 (Cq), 144.0 (C-8), 130.0 (C-5), 129.4 (C-6), 115.1 (Cq), 109.2 (CH), 90.8 (C-1'), 85.7 (C-4'), 83.5 (C-2'), 81.9 (C-3'), 63.3 – 63.0 (m, O- CH_2), 56.3 – 53.4 (m, CHP), 51.5 (C-5'), 27.5 (CH_3), 25.6 (CH_3), 16.6 (O- CH_2 - CH_3), 13.7 (CH_3). ^{31}P NMR (202 MHz, CDCl_3): δ 19.5 – 19.0 (m, 2P). Q-TOF MS E^+ : m/z 671.27 ($\text{M}+\text{H}^+$). HRMS Q-TOF MS E^+ : calculated for $\text{C}_{28}\text{H}_{45}\text{N}_6\text{O}_9\text{P}_2$ [$\text{M}+\text{H}^+$] 671.2723; found 671.2720.

5'-Deoxy-5'-N[(tetraethyl(methylene bisphosphonate))]adenosine (22) The title compound was obtained as a white solid (89 mg, 74%) from compound **21** (146.2 mg, 0.2 mmol) using general procedure A (with a ratio of TFA/ H_2O , 9/1). Purification was achieved by column chromatography with $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (90/10). R_f ($\text{CH}_2\text{Cl}_2/\text{MeOH}$, 9/1) 0.22. ^1H NMR (500 MHz, CD_3OD): δ 8.32 (s, 1H, H-2), 8.29 (s, 1H, H-8), 5.94 – 5.93 (m, 1H, H-1'), H-2' signal in H_2O peak, 4.39 – 4.38 (m, 1H, H-3'), 4.24 – 4.14 (m, 9H, O- CH_2 - CH_3 and H-4'), 3.38 – 3.34 (m, 1H, H-5'), 3.06 – 3.03 (m, 1H, H-5'), 1.33 – 1.27 (m, 12H, CH_3). ^{13}C NMR (126 MHz, CD_3OD): δ 157.0 (C-6), 153.6 (C-2), 150.6 (C-4), 142.2 (C-8), 120.9 (C-

5), 90.8 (C-1'), 86.4 (C-4'), 74.6 (C-2'), 72.9 (C-3'), 64.9 – 64.5 (m, O- CH_2 - CH_3), 52.6 – 52.5 (m, CHP and C-5'), 16.8 – 16.7 (m, CH_3). ^{31}P NMR (202 MHz, CD_3OD): δ 20.3 – 19.2 (m, 2P). Q-TOF MS E^+ : m/z 553.19 ($\text{M}+\text{H}^+$). HRMS Q-TOF MS E^+ : calculated for $\text{C}_{19}\text{H}_{35}\text{N}_6\text{O}_9\text{P}_2$ [$\text{M}+\text{H}^+$] 553.1941; found, 553.1938.

5'-Deoxy-5'-N(methylene bisphosphonate)adenosine (Tetra sodium salts) (9) The title compound was obtained as a white solid (79 mg, quantitative yield) from compound **22** (82 mg, 0.15 mmol) using general procedure B (but with 20 eq. trimethylsilyl bromide). Purification was achieved by reverse-phase column chromatography with H_2O followed by ion exchange on DOWEX 50WX2 (Na^+ form). R_f ($i\text{PrOH}/\text{NH}_4\text{OH}/\text{H}_2\text{O}$, 6/1/3) 0.36. ^1H NMR (400 MHz, D_2O): δ 8.38 (s, 1H, H-8), 8.36 (s, 1H, H-2), 6.13 (d, J = 5.7 Hz, 1H, H-1'), H-2' signal in D_2O peak, 4.62 – 4.58 (m, 1H, H-4'), 4.52 – 4.50 (m, 1H, H-3'), 3.99 – 3.84 (m, 2H, H-5'), 3.62 (t, J = 17.7 Hz, 1H, CHP). ^{13}C NMR (151 MHz, D_2O): δ 154.2 (C-6), 151.6 (C-2), 147.2 (C-4), 139.22 (C-8), 117.7 (C-5), 87.3 (C-1'), 78.4 (C-4'), 72.1 (C-2'), 69.9 (C-3'), 53.4 (CHP), 48.3 (C-5'). ^{31}P NMR (162 MHz, D_2O): δ 7.28 (P), 7.07 (P). Q-TOF MS E^- : m/z 439.05 (M-H). HRMS Q-TOF MS E^- : calculated for $\text{C}_{11}\text{H}_{17}\text{N}_6\text{O}_9\text{P}_2$ [M-H] $^-$ 439.0532 found; 439.0536.

5'-O-[(phosphorylacetate)]adenosine (trisodium salt) (23) This compound was obtained according to the procedure describe by Debarge et al.^[18] Characterizations (^1H and MS) were in agreement with the literature. ^1H NMR (400 MHz, D_2O): δ 8.62 (s, 1H, H-8, dia1 or dia2), 8.60 (s, H-8, 1H, dia1 or dia2), 8.18 (s, 2H, H-2, dia1 + dia2), 6.07 (s, 1H, H-1', dia1 or dia2), 6.06 (s, 1H, H-1', dia1 or dia2), H-2' signal in D_2O peak, 4.53 – 4.45 (m, 2H, H-4', dia1 or dia2), 4.34 – 4.30 (m, 2H, H-3', dia1 or dia2), 3.99 – 3.94 (m, 2H, CHP, dia1 or dia2), 3.90 – 3.61 (m, 4H, H-5', dia1 + dia2). HRMS Q-TOF MS E^+ calculated for $\text{C}_{12}\text{H}_{17}\text{N}_5\text{O}_9\text{P}$ [$\text{M}+\text{H}^+$] 406.0764 found; 406.0763.

2'-Arabino-fluoro-5'-N-[(bisphosphono)methyl]-2-chloro-2',5'-dideoxyadenosine (24) The title compound was synthesized from commercially available Clofarabine according to procedure detailed in the SI. R_f ($i\text{PrOH}/\text{NH}_4\text{OH}/\text{H}_2\text{O}$, 7/1/2) 0.04. ^1H NMR (600 MHz, D_2O): δ 8.53 (s, 1H, H-8), 6.46 (dd, J = 16.1, 4.1 Hz, 1H, H-1'), 5.36 – 5.26 (m, 1H, H-2'), 4.72 – 4.66 (m, 1H, H-3'), 4.41 – 4.38 (m, 1H, H-4'), 3.67 – 3.51 (m, 2H, H-5'), 3.05 (d, J = 10.8 Hz, 4H, N- CH_2). ^{13}C NMR (151 MHz, D_2O) δ 156.4 (C-2), 153.8 (C-6), 149.9 (C-4), 142.0 (C-8), 117.1 (C-5), 94.5 (d, J = 191.3 Hz, C-2'), 82.2 (d, J = 17.1 Hz, C-1'), 80.1 (C-4'), 75.6 (d, J = 25.4 Hz, C-3'), 57.4 (C-5'), 55.4 (d, J = 137.1 Hz, $\text{H}_2\text{C-N}$). ^{31}P NMR (400 MHz, MeOD) δ 13.2. ^{19}F NMR (400 MHz, MeOD) δ -198.4. Q-TOF MS E^- : m/z 489.03 (M-H). HRMS Q-TOF MS E^- : Calculated for $\text{C}_{12}\text{H}_{17}\text{N}_6\text{O}_8\text{FP}_2\text{Cl}$ [M-H] $^-$ 489.0256; found: 489.0256

Molecular modelling and docking. All compounds were modelled using the VegaZZ molecular modelling suite and subjected to energy minimization of the potential energy by 500 steps of steepest-descent gradient followed by 5, 000 steps of conjugate gradient algorithm (Tripos force field and Gasteiger-Marsili partial charges) until convergence with a gradient tolerance of 0.001 kcal/mol. Å).^[19] Molecular docking was achieved with Gold v5.6 (Genetic Optimization for Ligand Docking) from the Cambridge Crystallographic Data Centre (CCDC, Software Limited) on the crystal structure of CD73 (PDB 4H2I) solved at 2 Å of resolution in a closed conformation (crystal form III from Knapp K. et al.^[8]). Target binding site was defined by a spherical area around the CZ atom of Arg395 (as this residue is located in the center of the substrate binding site and in contact with phosphonate oxygen's of APCP) with a radius 10 Å and scrutiny of cavity. Water molecules from the crystal were preserved and allowed for translation / rotation freedoms within a 2 Å window. The search of best docking poses was performed by executing 50 runs of genetic algorithms (search-based optimization technique based on Genetics and natural selection) and the ranking of docking solutions was computed by the GoldScore fitness function by using the complete linkage clustering method from the RMSD matrix of generated solutions.^[20] The GoldScore scoring function was selected as it has been optimized for the prediction of ligand binding positions and takes into

account factors such as H-bonding energy, van der Waals energy, metal interaction and ligand torsion strain (thus, the score reflects the theoretical affinity and is expressed as arbitrary units in GOLD). The highest-ranked poses were selected for structural analysis and interaction measurements with CD73 amino acids using the PyMol Molecular Graphics System (v1.8, Schrödinger, LLC).

Enzymatic inhibition assays. CD73 activity was evaluated using the purified recombinant enzyme as previously described^[17] by quantifying the inorganic phosphate release upon AMP hydrolysis. Briefly, the amount of inorganic phosphate produced was determined by using the Green Malachite Phosphate assay kit (Gentaur) and a standard phosphate concentration range (0-50 μ M) for normalizing raw data. To determine the enzymatic inhibition, recombinant CD73 (2 nM, final concentration) was incubated in a reaction buffer (Tris 50 mM, pH 7.5, NaCl 100 mM, MgCl₂ 1 mM, CaCl₂ 1 mM and ZnCl₂ 5 μ M) in the absence or in the presence of the indicated compounds at various concentrations for 1 minute at room temperature. The reaction was started by addition of the substrate, AMP (100 μ M) and incubated for 2 minutes at 37°C under gentle shaking. Then, reaction was stopped by addition of the Green Malachite reagent (containing a strong acid) and inorganic phosphate produced was quantified by reading the optical density (OD) at 630 nm on a plate reader (Tecan Sunrise). The percentage of enzymatic inhibition was calculated by using the following formula: $1 - [(OD_{AMP+inhibitor} - OD_{min}) / (OD_{max} - OD_{min})]$. OD_{min} refers to the absorbance without enzymatic reaction (background signal) and OD_{max}, the full reaction in absence of inhibitor. To determine IC₅₀ values of the inhibitory compounds, GraphPad Prism was used.

Cell-based assays. Inhibition of CD73 activity on cells were assessed using CD73-positive human breast cancer MDA-MB-231 cells. Cells (25,000 per well) were seeded in 96-well plates in 100 μ L complete media and left to adhere overnight. After 4 washes with 100 μ L buffer (MgCl₂ 2 mM, NaCl 120 mM, KCl 5 mM, glucose 10 mM, Hepes 20 mM pH 7.4), cells were incubated with 100 μ L containing (or not) 200 μ M AMP and 100 μ M inhibitors for 10 minutes. Then, the reaction was stopped by incubating the plate on ice, and inorganic phosphate was quantified with the Green Malachite Phosphate Assay kit (Gentaur). The inhibition activity was calculated as $1 - (OD_{AMP+inhibitor} - OD_{inhibitor}) / (OD_{AMP} - OD_{only\ cells})$. Every condition was performed in triplicate and at least three times.

Potential toxicity of studied compounds to human cells was estimated after exposing the same cells (3000 cells in 96 well plates) to 100 μ M of compounds for 72 h. Then, the relative number of cells was determined using the MTT assay.

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Conflict of Interest

The authors declare no conflict of interest

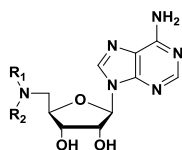
Keywords: 5'-ectonucleotidase • enzyme inhibitors • nucleos(t)ide analogue • immunotherapy • cancer

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Entry for the Table of Contents



$R_1 = H$, $R_2 = CH_2-COO^-$, or $COCH_2-P(O)(O^-)OEt(O^-)$, or $COCH_2-P(O)(O^-)_2$, or $CH_2-P(O)(O^-)OEt(O^-)$, or $CH_2-P(O)(O^-)_2$, or $CH(COO^-)P(O)(O^-)OEt(O^-)$, or $CH(COO^-)P(O)(O^-)_2$, or $CH[P(O)(O^-)_2]_2$

$R_1 = R_2 = CH_2-P(O)(O^-)_2$

Low to modest inhibitors of CD73

Novel nucleos(t)ide analogues based on a 5'-aminoadenosine scaffold were synthesized and their ability to inhibit 5'-ectonucleotidase CD73 was assessed.