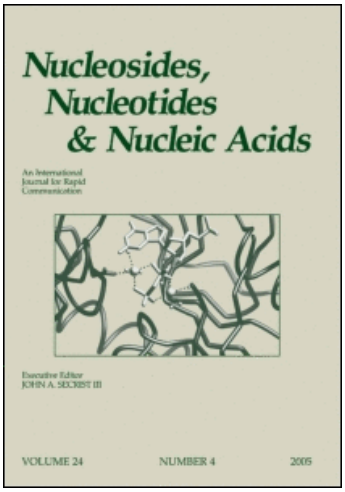

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MOLECULAR RECOGNITION OF ADENOSINE DEAMINASE: ^{15}N NMR STUDIES

Avital Laxer, Hugo E. Gottlieb, and Bilha Fischer □ *Department of Chemistry, Gonda-Goldschmied Medical Research Center, Bar-Ilan University, Ramat-Gan, Israel*

□ *The elucidation of the molecular recognition of adenosine deaminase (ADA), the interpretation of the catalytic mechanism, and the design of novel inhibitors are based mostly on data obtained for the crystalline state of the enzyme. To obtain evidence for molecular recognition of the physiologically relevant soluble enzyme, we studied its interactions with the in situ formed inhibitor, 6-OH-purine riboside (HDPR), by 1D-^{15}N- and 2D-(^{1}H-^{15}N)- NMR using the labeled primary inhibitor [$^{15}N_4$]-PR. We synthesized both [$^{15}N_4$]-PR and an [$^{15}N_4$]-HDPR model, from relatively inexpensive ^{15}N sources. The [$^{15}N_4$]-HDPR model was used to simulate H-bonding and possible Zn^{2+}-coordination of HDPR with ADA. We also explored possible ionic interactions between PR and ADA by ^{15}N-NMR monitored pH-titrations of [$^{15}N_4$]-PR. Finally, we investigated the [$^{15}N_4$]-PR-ADA 1:1 complex by 2D-(^{1}H-^{15}N) NMR. We found that HDPR recognition determinants in ADA do not include any ionic-interactions. HDPR N1 H is an H-bond acceptor, and not an H-bond donor. Despite the proximity of N7 to the Zn^{2+}-ion, no coordination occurs; instead, N7 is an H-bond acceptor. We found an overall agreement between the crystallographic data for the crystallized ADA:HDPR complex and the ^{15}N-NMR signals for the corresponding soluble complex. This finding justifies the use of ADA's crystallographic data for the design of novel inhibitors.*

Keywords Adenosine deaminase; nucleosides; ^{15}N NMR; Purine riboside

INTRODUCTION

Adenosine deaminase (ADA [Adenosine Aminohydrolase E.C.3.5.4.4]) is a relatively small monomeric enzyme (33 KDa) that catalyzes irreversibly the hydrolytic deamination of adenosine and 2′-deoxyadenosine to inosine and 2′-deoxy-inosine, respectively.[1,2] Hydrolysis of adenosine and ammonia liberation occurs via a tetrahedral intermediate bearing the amine and hydroxyl groups at C6.[3] The enzymatic deamination reaction is accelerated by about 2×10^{12}-times, as compared to deamination in aqueous solution.[4]

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In mammalians, ADA is found in all cells,[5] and a decrease[6] or increase[7] in ADA levels triggers various pathological conditions. Therefore, the addition of an efficient and specific inhibitor to ADA is the key to controlling the activity of endogenous adenosine, involved in various physiological and pathological conditions. Specifically, by elevating adenosine levels,[8] ADA inhibitors were proposed for the treatment of cerebral and myocardial ischemia.[9,10]

In addition, inhibition of ADA prevents rapid de-activation of adenosine-based drugs. For instance, ring-expanded purine nucleosides, including coformycin and pentostatin, proved synergistic antitumor and/or antiviral antibiotics which potentiate the effects of other antitumor or antiviral compounds, through the inhibition of adenosine deaminase.[11] Other potent ADA inhibitors include deaza-analogues of adenosine and erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) analogues.[12–14]

The development of specific and efficient inhibitors requires a thorough understanding of the molecular recognition determinants of ADA.

The molecular recognition of ADA was investigated by x-ray crystallography of ADA-inhibitor complexes.[15,16] For instance, the crystal structure of the ADA-purine riboside, PR, complex revealed that PR, **1**, undergoes hydration in the catalytic site to form 6-hydroxy-purine-riboside, HDPR, **2a**. In this way, the crystallographic data provided significant information on the transition state of the catalytic reaction, and binding interactions. HDPR is stabilized in the catalytic site by a network of H-bonds involving the ribose hydroxyls, and the purine's N1H, N3, and N7 nitrogen atoms. For instance, N1H H-bonds to active-site carboxyl (Glu217), while the 6-OH group interacts with the catalytic site via both H-bonding (Asp295, His238) and coordination with Zn^{2+} ion.[16,17]

The crystallographic data provided the basis for the mechanism of catalysis. [16,18] So far, the elucidation of the molecular recognition of ADA, the interpretation of the catalytic mechanism, and the design of novel inhibitors, were based on evidence obtained for the crystalline state of the enzyme. However, to obtain evidence for molecular recognition of the soluble enzyme under physiologically relevant conditions, spectroscopic methods are more suitable than x-ray crystallography.

The application of various spectroscopic techniques is complicated nevertheless by the multitude data resulting from both the enzyme and inhibitor partners. Considering both this severe limitation and binding-modes involving PR nitrogen atoms, we chose ^{15}N NMR for studying the molecular recognition of PR by soluble ADA.

Specifically, interactions of **1** with the enzyme due to coordination with metal-ions, protonation, and H-bonds, may be explored by 1D-^{15}N- and 2D-(^{1}H-^{15}N)- NMR using ^{15}N labeled PR.

Here, we report on the synthesis of [$^{15}N_4$]-PR, **1**, from the relatively inexpensive ^{15}N sources: $^{15}NH_4Cl$, $^{15}NH_4OH$, and $Na^{15}NO_2$. Furthermore,

we report on the synthesis of [$^{15}N_4$]-HDPR model, **2b**. This model was used to simulate H-bonding and possible Zn^{2+}-coordination of HDPR **2a** in the catalytic site, as monitored by 1D-^{15}N-NMR. Likewise, we explored possible ionic interactions between PR and ADA by ^{15}N-NMR monitored pH-titrations of [$^{15}N_4$]-PR. Finally, we describe the investigation of [$^{15}N_4$]-PR-ADA 1:1 complex by 2D-(^{1}H-^{15}N) NMR to elucidate interactions of HDPR with the catalytic site. This NMR experiment with physiologically relevant, soluble ADA, complements the molecular recognition picture obtained by crystallography and can confirm or reject hypotheses on binding interactions deduced for the ADA-HDPR crystalline state.

RESULTS AND DISCUSSION

[$^{15}N_4$]-PR, **1**, was the inhibitor of choice for studying the molecular recognition of ADA by ^{15}N NMR as the affinity of PR to the enzyme is extremely high (K_i of HDPR is ca. 10^{-13} M),[19] and it exchanges slowly (k_{on} is ca. 10^8 sec^{-1} mol^{-1}).[20] Such a slow exchange makes it possible to monitor only the enzyme-bound inhibitor without detecting any free inhibitor in solution.

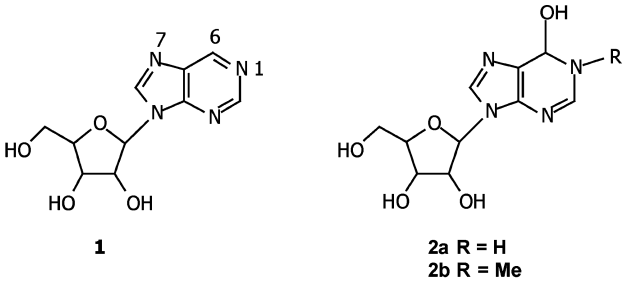
Synthesis of [$^{15}N_4$]-PR

Previously, we reported the synthesis of [$^{15}N_5$]-2-thiohexyl-adenine,[21] **9**, in five steps from [$^{15}N_2$]-malononitrile and [$^{15}N_2$]-thiourea (Scheme 1). Each of the starting materials was prepared in two steps utilizing relatively inexpensive ^{15}N sources: $^{15}NH_4Cl$, $^{15}NH_4OH$, and $Na^{15}NO_2$.[21,22]

Here, we utilized labeled adenine analogue **9** as the starting material for the preparation of [$^{15}N_4$]-PR, **1**. The synthetic scheme involved desulfurization of **9** to obtain [$^{15}N_5$]-adenine followed by coupling to 1-O-acetyl-2,3,5-tri-O-benzoyl-β-D-ribofuranose, **11**, to produce protected and labeled adenosine analogue, **12** (Scheme 2). Finally, deamination and deprotection were expected to provide [$^{15}N_4$]-PR, **1**. However, desulfurization product, **10**, was obtained only in a 25% yield due to its low solubility in organic solvents and difficulties in removing the product from Ra-Ni.

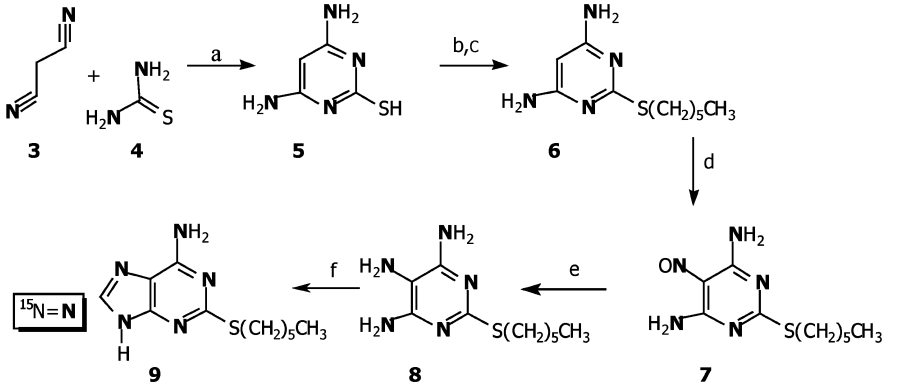
Alternatively, we coupled [$^{15}N_4$]-2-thiohexyladenine **9** with **11** to produce nucleoside **13** in 69% yield (Scheme 3). The coupling reaction was regioselective, occurring at N9 rather than N7. In addition, this reaction was highly stereoselective, producing the β-isomer as the major product.[23] The product yield depended on the molar ratio of the base and ribose. With a 1:1 ratio, the yield was 54%, while with a 1.2:1 base:ribose ratio, the yield increased to 69%. The excess labeled base can be isolated by chromatography and recycled.

Desulfurization of **13** was achieved by Ra-Ni in EtOH. Although TLC indicated the full conversion of the starting material to a single product,



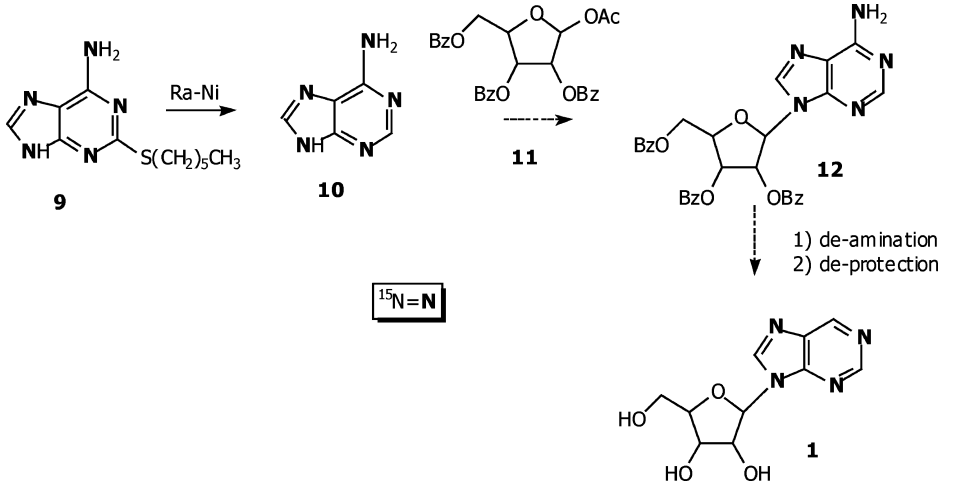
1

2a R = H
2b R = Me



Key: (a) EtONa/EtOH, reflux, 3 h, 71% (b) 0.25 M NaOH/MeOH, rt; (c) $Br(CH_2)_5CH_3$/DMF, rt, 89%; (d) $NaNO_2$/HOAc, rt, 5 min, 62%;(e) H_2(1 atm)/PtO_2/EtOH, rt, 3 h, 100%; (f) $HCONH_2$, reflux, 1 h, 80%.

SCHEME 1



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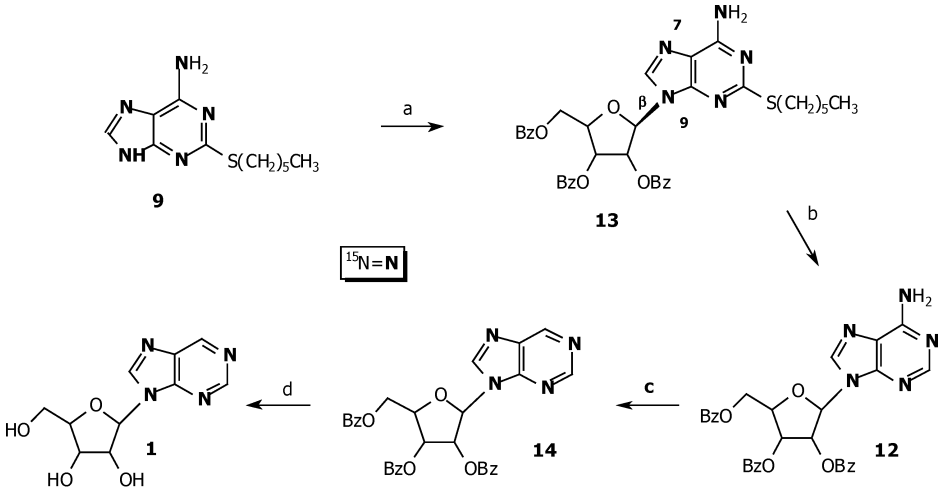


Key: (a) HMDS, $(NH_4)_2SO_4$, evaporation; **11**, 1,2-DCE, TMSOTf, 69% (b) Ra-Ni, DMF, 100°C, 1.5 h, 60%; (c) iso-amylnitrite, THF, reflux, 3 days, 93%; (d) NH_4OH, MeOH, dioxane, 80°C, 24 h, 90%.

SCHEME 3

12, the yield was only 35%, due to the adsorption of the product on the Ra-Ni, and the limited solubility of **12** in EtOH. Therefore, the reaction was repeated in DMF at 100°C. Upon the completion of the reaction, Ra-Ni, was filtered and washed with warm DMF. In this way, the yield exceeded 60%. Deamination of **12** was achieved by diazotation using isoamylnitrite.[24,25] THF in this reaction served as both the solvent and the source of H atoms reacting with the purine radical formed in the reaction. The protected PR **14** was obtained in 93% yield. Finally, deprotection of **14** in a mixture of dioxane-NH_4OH-MeOH at 80°C for 24 hours provided **1** in 90% yield. This method was found significantly superior to the common deprotection method in 2 M ethanolic ammonia at 100°C/24 hours.

Comparison of ^{15}N-chemical shifts of **13** to those of **12** and **14,** reveals a clear downfield shift for all nitrogen atoms in **14** (Table 1). This shift was most pronounced for N1 and N3. Thus, N1 shifts downfield by 39 ppm due to removal of the N^6-amine and the 2-thioether groups. The lack of these exocyclic groups, which increase electron density at N1 and N3, results in deshielding of these nitrogen atoms.

We also compared ^{15}N NMR spectrum of labeled PR in D_2O and DMSO. The large effect of the protic solvent on the nitrogen atoms' chemical shifts was clearly observed. N1, N3, and N7 accept H-bonds from water and their signals shift upfield, compared to their chemical shifts in aprotic solvent (DMSO), due to reduction of π-bond order on H-bond acceptors.[26] This effect was not observed for N9 whose lone pair is engaged in the aromatic system (Table 1).

TABLE 1 ^{15}N NMR data for labeled PR and synthetic intermediates[a]

Comp no.	Derivative	NH_2	N9	N7	N3	N1
13	2-Shex-Ad (Bz)	−306.0	−215.7	−142.4	−164.5	−157.5
12	Ad (Bz)	−305.5	−215.1	−139.3	−154.7	−143.5
14	PR (Bz)	—	−214.9	−136.3	−129.4	−118.2
1 (D_2O)	PR	—	−212.7	−147.0	−135.5	−112.0
1 (DMSO)	PR[i]	—	−211.1	−138.1	−129.9	−101.0

[a]Chemical shifts of non-labeled PR.

Upon labeling the PR nitrogen atoms with ^{15}N isotopes, additional $^2J_{\text{N-H}}$ couplings appear in the ^{1}H NMR (Figure 1). Specifically, H6, H2, and H8 appear as follows: d, $J = 10$; t, $J = 14$; dd, $J = 8$, 11 Hz, respectively.

DOES MOLECULAR RECOGNITION OF PR BY ADA INVOLVE IONIC INTERACTIONS?

Possible protonation of PR by acidic amino-acids in the catalytic site might affect the binding mode of PR and the catalytic mechanism. Protonation of PR, if any, will result in ionic interactions between the enzyme and inhibitor partners.

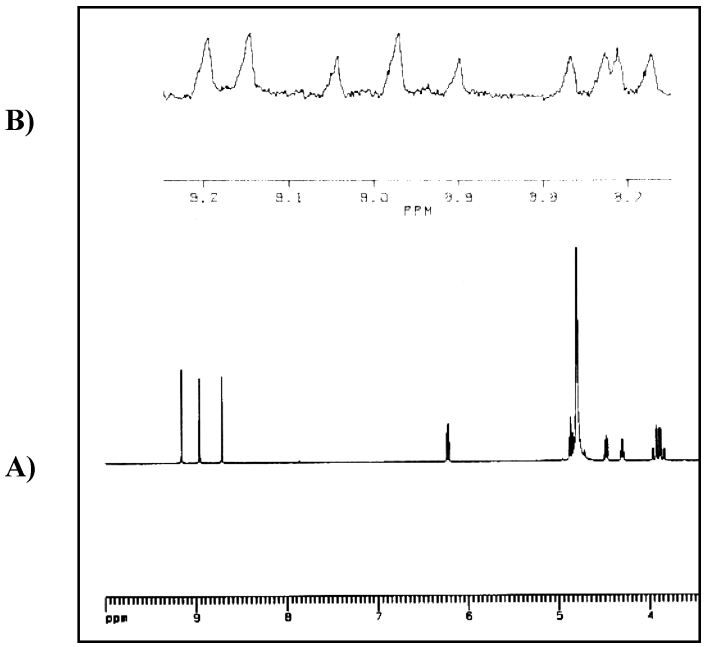


FIGURE 1 ^{1}H NMR spectrum of [$^{15}N_4$]-PR, **1**: A) full spectrum. b) $^2J_{(N,H)}$ couplings of H-2, H-6, and H-8.

TABLE 2 pH-dependence of PR nitrogens ^{15}N NMR chemical shifts

pH	N1	N3	N7	N9
5.63	−112.1	−135.5	−147.0	−212.7
4.61	−112.3	−135.5	−147.2	−212.8
3.65	−114.4	−135.4	−147.0	−212.5
3.04	−115.8	−135.3	−147.1	−212.3
2.36	−129.8	−134.5	−146.3	−212.0
1.90	−139.9	−133.2	−145.3	−209.1
1.39	−171.1	−131.8	−144.0	−206.7
0.97	−185.4	−131.1	−143.2	−205.1

To establish if any ionic interactions are involved in the primary molecular recognition of PR, we determined protonation site and pK_a value of PR by ^{15}N NMR.

In general, ionization constants of nucleos(t)ides are measured by pH-titration monitored by a variety of spectroscopic methods.[17,27,28] However, most of these methods do not provide any information on the protonation site in the nucleobase (e.g., N1 vs. N7). An alternative spectroscopic method is ^{15}N NMR, which offers unambiguous determination of the protonation site.[29] Due to the limited solubility of nucleosides, previous ^{15}N NMR protonation studies of nucleosides have been performed by trifluoroacetic acid-titrations in DMSO, resulting in no pK_a determination.[29,30] Recently, we demonstrated the use of ^{15}N NMR for the determination of pK_a values of substituted adenine nucleotides.[31]

Here, we first studied the pH-dependent δ of PR nitrogen atoms (Table 2). Upon acidification of [$^{15}N_4$]-PR solution from pH 5.63 to 0.97, all nitrogen atoms shifted upfield, however, the most pronounced shift was observed for N1 with $\Delta\delta$ of 73 ppm. The pK_a of PR was determined by the second derivative of the fitted sigmoidal graph of the pH-dependent N1 chemical shift (Figure 2). The PR pK_a value was 1.58 (±0.02). This value

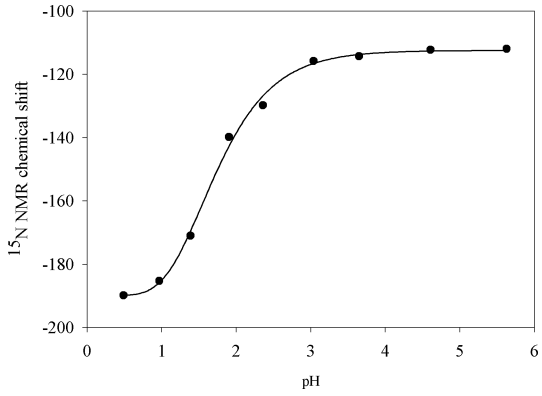


FIGURE 2 pH-titration of [$^{15}N_4$]-PR, **1**, monitored by ^{15}N NMR. The sigmoidal graph depicts $\Delta\delta$ values of the [$^{15}N_4$]-PR N1 atom.

is lower as compared to values found in the literature (2.05–2.07).[3,32] Differences in pK_a values may be due to the different determination methods (UV and ^{13}C NMR vs. ^{15}N NMR), solvent (^{13}C NMR-based pK_a was determined in phosphate buffer), and sample concentrations.

The x-ray crystal structure of the ADA-HDPR complex indicated the presence of Asp296 in the vicinity of PR's N7.[15] To test if N7 undergoes any protonation in the catalytic site, we titrated PR with a strong acid and monitored it by ^{15}N NMR. To a DMSO-d_6 solution of PR we added first 1 eq. of H_2SO_4 until full protonation of N1 was attained. Then we added HBr ($pK_a = -9$), but noticed no change in the N7 (or any other nitrogen) chemical shift. Namely, PR N7 is not basic, unlike the measurable basicity of adenosine N7 ($pK_a = -1.6$).[33] As this experiment was performed in DMSO, where ionization of acids differs from that in water, we cannot obtain any quantitative information on PR N7 pK_a. However, as N7 is not protonated by an extremely strong acid as HBr, the basicity of N7 is negligible.

It has been proposed that PR undergoes instantaneous N1-protonation by Glu217 in the catalytic site.[34,35] However, the low pK_a value we determined for PR N1 implies that the catalytic site must be extremely acidic for PR protonation to occur. A negative pK_a value for Glu is unlikely. Therefore, we conclude that PR is not initially recognized by ADA via any ionic-interactions. Furthermore, regarding the C6-hydration mechanism of PR at the catalytic site, we conclude that N1 is H-bonded to Glu217, rather than protonated, thus, making C6 more susceptible to hydrolysis.

[$^{15}N_4$]-HDPR MODEL IS REQUIRED FOR ^{15}N NMR STUDIES OF HDPR:ADA COMPLEX

When PR binds to ADA's catalytic site a rapid hydration (in less than 4 msec)[20] of the C6-N1 double bond occurs to provide 6-hydroxy-1,6-dihydropurine riboside, HDPR, **2a**. As the addition of labeled PR to ADA will form labeled HDPR, we had to obtain first ^{15}N NMR data for **2a**, for the assignment of the ADA-bound HDPR ^{15}N chemical shifts in the next experimental step.

However, HDPR, **2a**, is impossible to obtain. Although in the enzyme, the hydration of PR is quantitative, only traces of HDPR are present in an aqueous solution, as the equilibrium constant for the hydration is $1.1 \cdot 10^{-7}$.[36] Generally, there is no evidence for hydration of purines and pyrimidines in an aqueous solution,[37,38] due to the loss of aromaticity upon hydration. Apparently, HDPR is most stable in ADA's catalytic site, due to its resemblance to the substrate's transition state, and the multitude of binding interactions with the catalytic site.

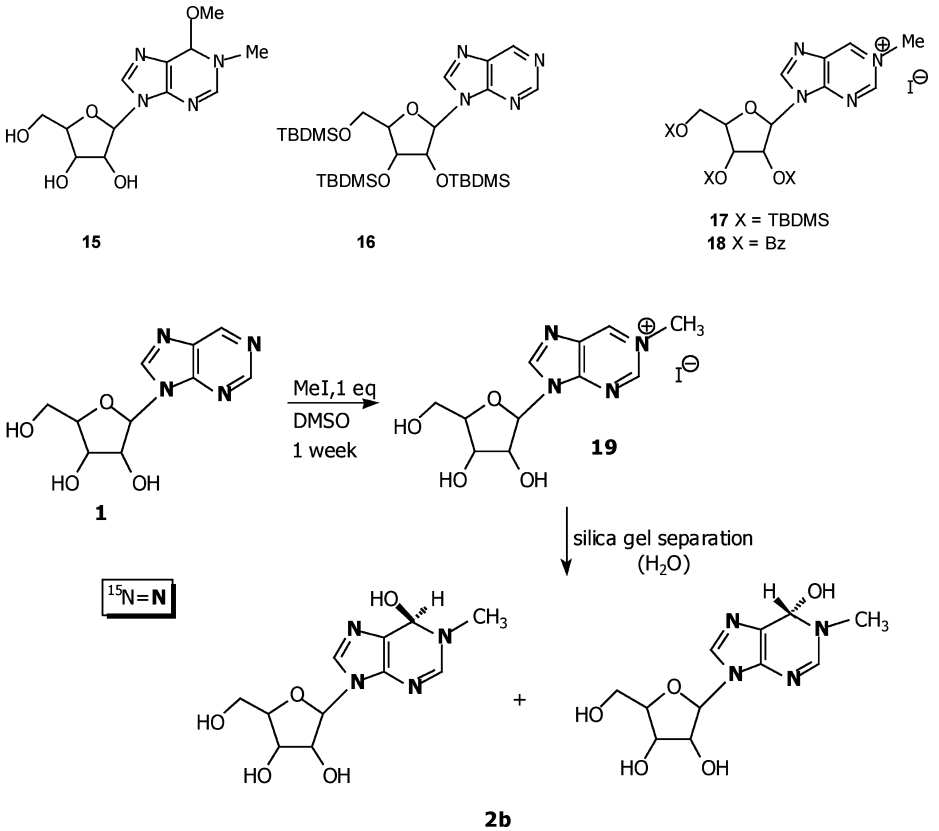
SYNTHESIS OF [$^{15}N_4$]-HDPR MODEL

As HDPR is impossible to obtain, we aimed to synthesize a stable model of HDPR, for example, **15**. This model may be obtained from

PR by alkylation of N1, followed by the addition of a methoxide ion to C6.

For this purpose, we protected the PR ribose hydroxyls by either benzoyl or TBDMS groups (**14**, **16**) and have then added MeI. Next, we added either MeOH/NaOH or MeOH/K_2CO_3 as methoxide sources. However, no nucleophilic addition occurred at C6-position in the N1-methylated PR analogue, **17**, under various reaction conditions. Apparently, the silyl groups reduce the electrophilic nature of **17**. Alternatively, we added MeOH/K_2CO_3 to N1-methyl per-benzoate PR, **18**, in acetonitrile at room temperature overnight. The addition of MeO^- to **18** resulted in the cleavage of the glycosidic bond and the production of the unstable 1-methyl-6-hydroxy-dihydropurine, which was detected by MS.

Finally, the desired HDPR model, **2b**, was obtained rather unexpectedly, in a short and facile way, with no need for protection of the ribose hydroxyls (Scheme 4). Specifically, PR was selectively N1-methylated under mild conditions by the addition of only 1 eq. MeI in DMSO at room temperature for 1 week.[36] Separation of the methylated product, **19**, on a silica-gel column resulted in the formation of the desired HDPR model **2b**. Although



SCHEME 4

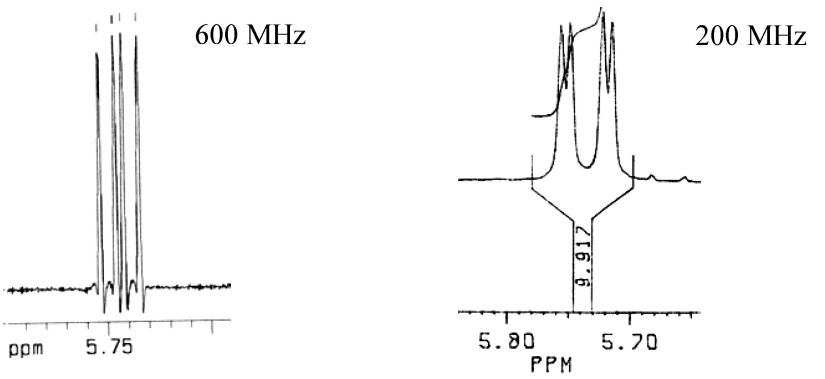


FIGURE 3 Pattern of HDPR model **2b** H-1′ signal evaluated at different fields (200 and 600 MHz).

19 had been synthesized before, product **2b** was not identified, as **19** was isolated on cellulose rather than silica gel.[36]

Product **2b** was obtained as a mixture of two diastereoisomers, indicated by the presence of two typical H1′-signals (two doublets at 5.73 and 5.74 ppm, J = 7 Hz). To ensure that these signals are indeed two doublets and not a dd signal, we measured the ^{1}H NMR spectrum at different fields (200 and 600 MHz) (Figure 3). The different patterns prove that the ^{1}H signals result from two diastereoisomeric products.

HDPR model, **2b**, obtained easily in one step and in high yield, was relatively stable in anhydrous solution. Thus, **2b** survived in MeOH solution at −18°C for several weeks. However, traces of water triggered the decomposition of **2b**. In an aqueous solution, **2b** decomposed within minutes.

Assignment of ^{15}N NMR Chemical Shifts of HDPR Model

To assign the chemical shifts of ADA-bound HDPR, we measured the ^{15}N NMR spectrum for **2b** in a protic medium to mimic H-bond network in the enzyme. Due to the instability of **2b** in water, the spectrum was measured in CD_3OD (Figure 4). The spectrum of **2b** was significantly different as compared to the PR spectrum. Specifically, N1 shifted upfield to ca. 270 ppm due to its sp^3 hybridization. Likewise, N3 shifted upfield by ca. 60 ppm due to the loss of aromaticity.

DOES MOLECULAR RECOGNITION OF HDPR INVOLVE N7-COORDINATION WITH Zn^{2+}?

As crystallographic data for the HDPR-ADA complex indicate a relatively short distance between Zn^{2+} ion and N7 (3.75 Å), HDPR N7 may coordinate with Zn^{2+} in soluble ADA. To test this hypothesis, we simulated a possible

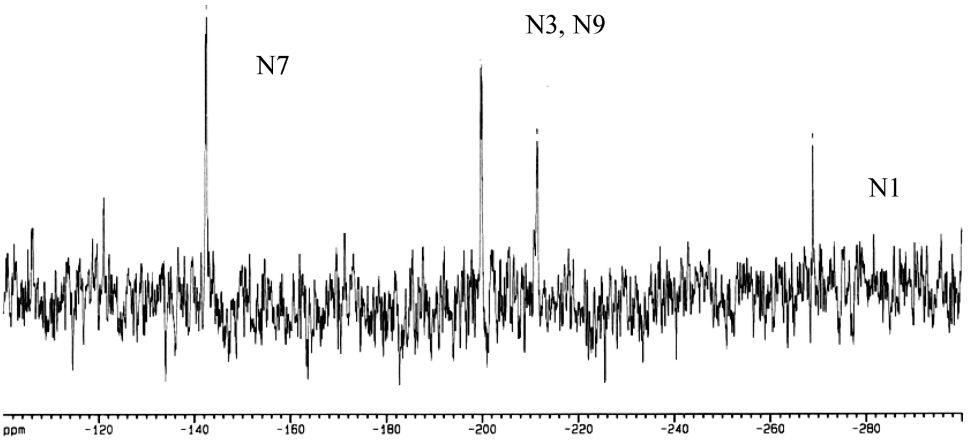


FIGURE 4 ^{15}N NMR spectrum of [$^{15}N_4$]-**2b** in CD_3OD at 60.8 MHz.

coordination by the addition of 1 eq. of $ZnCl_2$ to a solution of **2b** in CD_3OD and measurement of the resulting ^{15}N NMR chemical shifts. We observed a significant change of N7 chemical shift by more than 30 ppm, indicating the coordination of N7 with Zn^{2+}. This coordination reduces the aromatic character of N7, which turns to be a "pyrrole"-like, trivalent nitrogen. This observation of upfield shift is consistent with a previous report on an imidazole-Zn^{2+} complex.[30,39]

EXPLORING [$^{15}N_4$]-PR RECOGNITION BY ADA BY 2D(^{1}H-^{15}N) NMR

The recognition of PR by ADA may be explored by a high quality 1D-^{15}N NMR spectrum of a 1:1 PR:ADA complex (exchange is slow). However, for this purpose, 7–10 mM solutions are required. These high concentrations result in problems such as low solubility of the protein and high viscosity of the sample.

To overcome these problems, we chose the 2D-(^{1}H-^{15}N)-INEPT experiment[40] to study interactions between ADA and PR. In this NMR experiment the detection of the less sensitive nucleous (^{15}N) is achieved via the more sensitive nucleus (^1H). In this way, we could use less concentrated samples and reduce measurement time. The INEPT experiment increases sensitivity 30-fold, and, therefore, a 1–2 mM ADA:PR solution was sufficient for measurements. A disadvantage of this method is its applicability only to nitrogens that are coupled to hydrogens. However, polarization transfer is not limited only to $^1J_{N\text{-}H}$, the value of which is 90 Hz, and information may be obtained also from long range N-H couplings, when $18 > {}^3J_{N\text{-}H}, {}^2J_{N\text{-}H} > 5$.[41]

In purines $^2J_{N\text{-}H}$ are 7–17 Hz, while in pyrimidines they are ca. 17 Hz, and in imidazole J values are close to 7 Hz.[42]

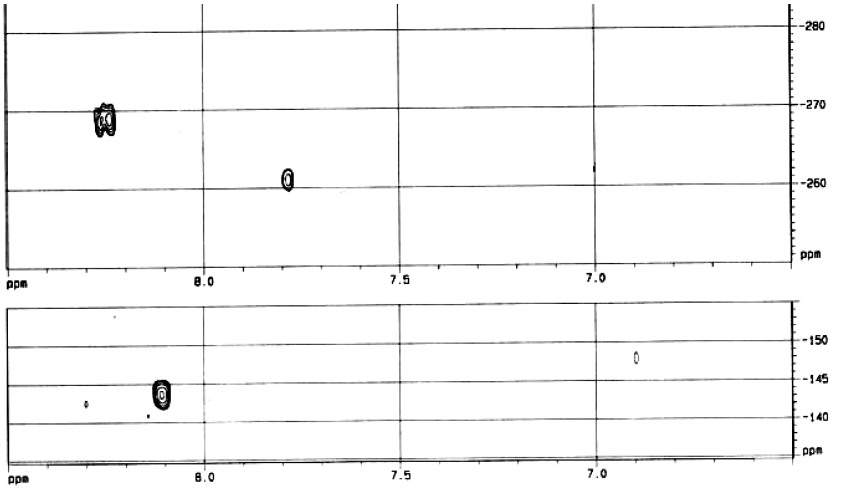


FIGURE 5 2D-(^{1}H-^{15}N)-HMBC experiment at 1.8 mM [$^{15}N_4$]-PR:ADA complex in 50 mM phosphate buffer in D_2O (pD 7).

To eliminate the large signal of water in the spectrum, we used D_2O for the preparation of phosphate buffer. Although we gain a clearer spectrum, we lose one bond coupling information resulting from the N1-H labile proton. Therefore, our experiment of choice was HMBC (suitable for ^{1}H-^{15}N detection through 2–3 bonds) rather than HMQC (suitable for detection through one bond), as nitrogen atoms in PR are coupled with the purine hydrogen atoms through two bonds.

The 2D-(^{1}H-^{15}N)-HMBC experiment of ADA:PR 1:1 complex was performed at 1.8 mM concentration in 50 mM phosphate buffer in D_2O (pD 7). The HMBC spectrum showed a cross peak of a nitrogen atom at −268.9 ppm coupled with a proton at 8.22 ppm (Figure 5). This chemical shift of N fits perfectly the chemical shift of N1 in the HDPR model, **2b** (Figure 4). The proton is H2 and $^3J_{N\text{-}H}$ is 12 Hz. Another cross-peak is observed for a nitrogen atom that appears at −144.0 ppm coupled with a proton at 8.08 ppm. Here, we also noticed a high correlation with the chemical shift of N7 and H8 in the HDPR model, **2b** in CD_3OD. We could not assign the small cross peak at -260.9 ppm. Cross-peaks corresponding to N3 or N9 were not observed in the HMBC spectrum. In the HMBC experiment, no information will be obtained if the peak's width is larger than the ^2J or ^3J coupling constant.

CONCLUSIONS

By labeling the inhibitor, rather than the enzyme partner, we were able to obtain information on the binding interactions of soluble ADA:PR complex through the 2D-(^{1}H-^{15}N)-HMBC experiment in D_2O.

The HMBC spectrum obtained proved helpful for the understanding of the molecular recognition of ADA.

A single set of peaks was observed in the HMBC spectrum, thus, confirming the high affinity of PR to ADA. However, the relatively narrow width of labeled HDPR N1 and N7 signals in the spectrum, ca. 10 Hz, was rather unexpected as HDPR is bound to a 33 KDa protein.

The HMBC spectrum provides an additional evidence for the hydration of PR in the enzyme's catalytic site by the chemical shift of N1 in the ADA-bound inhibitor which fits perfectly to the chemical shift of the N1 in HDPR model, **2b**.

Both the H-bonding ADA binding-site and the H-bonding solvent, MeOH, affect similarly the chemical shift of N1 in [$^{15}N_4$]-HDPR and [$^{15}N_4$]-HDPR model **2b**, respectively. MeOH donates H-bond to N1 in **2b**, which appears at –269 ppm. The chemical shift of ADA-bound HDPR N1H is identical to this shift. Therefore, we conclude that in ADA's catalytic site, HDPR N1 probably experiences an environment similar to that of a methanolic solution. Furthermore, as in model **2b**, there is no hydrogen atom on N1, but a methyl group, N1 interacts with MeOH only as an H-bond acceptor. Namely, HDPR N1H in the enzyme catalytic site is also an H-bond acceptor, and not an H-bond donor. Indications for strong H-bonds of the HDPR N1H were provided before by Raman[19] and time-dependent saturation transfer NOE studies.[43] Yet, these studies could not establish the donor or acceptor role of HDPR N1H.

The chemical shift of N7 in labeled HDPR in the enzyme fits perfectly with the chemical shift of N7 (an H-bond acceptor) in model **2b** in methanolic solution. This is consistent with the crystallographic evidence for the interaction of HDPR N7 with Asp296 in ADA.[15]

Based on the null basicity of the PR N7, the low basicity of N1, and comparison to **2b** chemical shifts found here, we conclude that HDPR recognition determinants in ADA do not include any ionic-interactions with the neighboring carboxylic acids Asp296 and Glu217, respectively. Likewise, we did not observe any Zn^{2+}-ion coordination with HDPR N7, unlike N7 in the free **2b**. Although Zn^{2+} ion is within coordination distance,[34] no significant upfield shift (of ca. 30 ppm) was observed for ADA:HDPR N7 nitrogen. Therefore, we conclude that despite the proximity of N7 to the Zn^{2+}-ion, no coordination occurs with the metal-ion in the soluble inhibitor-enzyme complex.

In summary, we conclude that physiologically relevant, soluble, ADA recognizes HDPR by donating H-bonds to both N1 and N7. We found an overall agreement between crystallographic data for the ADA:HDPR complex and the 2D-(^{1}H-^{15}N)HMBC experiment for soluble ADA. This finding justifies the design of novel ADA inhibitors based on ADA's crystallographic structure.

EXPERIMENTAL

General

NMR spectra were recorded on a Bruker DPX-300 instrument (300.1, 75.5, and 30.4 MHz for ^{1}H, ^{13}C, ^{15}N, respectively) or on a Bruker DMX-600 instrument (600.1, 150.9, and 60.8 MHz for ^{1}H, ^{13}C, ^{15}N, respectively). The chemical shifts are reported in ppm relative to tetramethylsilane (TMS) as an internal standard. ^{15}N NMR spectra were recorded with nitromethane ($\delta = 0$ ppm) as an external standard. Negative chemical shifts are upfield from nitromethane. Difficulties in obtaining ^{15}N spectra with good signal to noise ratios are quite common due to the negative Nuclear Overhauser Effect that results from the negative magnetogyric ratio of the ^{15}N nucleus, and also the relative long T_1 relaxation times. In order to obtain the necessary data, spectra were recorded in more than one of the following techniques: proton coupling, proton decoupling, and inverse gated. Mass spectra were recorded on AutoSpec-E fision VG high-resolution mass spectrometer. Nucleotides were characterized by FAB (fast atom bombardment) and high-resolution FAB using glycerol matrix under FAB negative conditions on AutoSpec-E fision VG high-resolution mass spectrometer (Micromass, Hertsfordshire, UK). $^{15}NH_4Cl$ (99% atom ^{15}N) and $Na^{15}NO_2$ (min 99% atom ^{15}N) were purchased from Isotec Inc. (Miamisburg, OH, USA). ($^{15}N_2$)-Thiourea (96% atom ^{15}N) and $^{15}NH_4OH$ (98% atom ^{15}N) were purchased from Eurisotop (Saint Aubin, France), and from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA) respectively.

[$^{15}N_4$]-Purine Riboside, PR (1): [$^{15}N_4$]-2′,3′,5′-Tri-O-benzoyl-purine riboside **14** (45 mg) was dissolved in dioxane (1.5 mL) and methanol (1 mL) and added to 28% ammonium hydroxide solution (2 mL) in a sealed pressure flask equipped with a magnetic stir-bar. The reaction mixture was sealed and heated at 70–80°C for 24 hours. The reaction was shown to be complete by TLC (MeOH:$CHCl_3$; 20:80). The reaction mixture was concentrated under reduced pressure and the residue was applied to a column of silica gel and eluted with a gradient of 5–10% MeOH in $CHCl_3$. The product was obtained in quantitative yield as a white solid. ^{1}H NMR (D_2O, 300 MHz) δ: 9.16 (d, $^{2}J_{(N,H)} = 10$ Hz, 1H, H-6), 8.96 (t, $^{2}J_{(N,H)} = 14$ Hz, 1H, H-2), 8.72 (dd, $^{2}J_{(N,H)} = 11$, 8 Hz, 1H, H-8), 6.23 (d, $^{2}J_{(H,H)} = 5.7$ Hz, 1H, H-1′), 4.87 (t, $^{2}J_{(H,H)} = 5.3$ Hz, 1H, H-2′), 4.48 (t, $^{2}J_{(H,H)} = 4$ Hz, 1H, H-3′), 4.30 (q, $^{2}J_{(H,H)} = 4$ Hz, 1H, H-4′), 3.94 (dd, $^{2}J_{(H,H)} = 12.8$, 3 Hz, 1-H, H-5′), 3.86 (dd, $^{2}J_{(H,H)} = 12.8$, 4 Hz, 1-H, H-5′). ^{13}C NMR (D_2O, 150 MHz) δ: 152.8 (t, $^{1}J_{(C,N)} = 2.8$ Hz, C-2), 151.6 (C-4), 149.3 (C-6), 146.6 (d, $^{1}J_{(C,N)} = 11.8$ Hz, C-8), 134.8 (C-5), 89.2 (d, $^{1}J_{(C,N)} = 10.8$ Hz, C-1′), 86.4 (C-4′), 74.6 (C-2′), 71.2 (C-3′), 62.1 (C-5′). ^{15}N NMR (D_2O, 61 MHz) δ: −112.7 (t, $^{2}J_{(N,H)} = 11.6$, N-1), −136.3 (dd, $^{2}J_{(H,H)} = 14.6$, 3 Hz, N-3), −147.6 (N-7), −213.4 (d, $^{2}J_{(N,H)} = 8$, N-9). HRMS (DCI, CH_4): calcd for $C_{10}H_{12}15N_4O_4$ (M^+) 256.0739, found 256.0749.

1,6-Dihydro-6-hydroxy-1-methyl-9-β-D-ribofuranosyl-9H-purine (2b): A solution of [$^{15}N_4$]-purine riboside, **1** (42 mg, 0.166 mmol) and methyl iodide (0.01 mL, 0.166 mmol) in DMSO (0.5 mL) was stirred for one week at room temperature. The reaction mixture was separated by column chromatography on silica gel and eluted with $CHCl_3$:MeOH 30:70 to give the hydration product **2b** as an oil (two diastereomers) in a quantitative yield. ^{1}H NMR (CD_3OD, 600 MHz) δ: 7.77 (s, 1 H, H-8), 7.76 (s, 1H, H-8), 7.41 (s, 1H, H-2), 6.24 (s, 1H, H-6), 5.74 (d, $^2J_{(H,H)}$ = 7 Hz, 1H, H-1′), 5.73 (d, $^2J_{(H,H)}$ = 7 Hz, 1H, H-1′), 4.62 (dd, $^2J_{(H,H)}$ = 7, 5 Hz, 1H, H-2′), 4.57 (dd, $^2J_{(H,H)}$ = 7, 5 Hz, 1H, H-2′), 4.27–4.25 (m, 1H, H-3′), 4.13 (q, $^2J_{(H,H)}$ = 2.3 Hz, 1H, H-4′), 4.12 (q, $^2J_{(H,H)}$ = 2.3 Hz, 1H, H-4′), 3.85–3.80 (m, 1H, H-5′), 3.68 (dt, $^2J_{(H,H)}$ = 12.5, 3 Hz, 1H, H-5′), 3.23 (s, 3H, N-CH_3), 3.22 (s, 3H, N-CH_3). ^{13}C NMR (CD_3OD, 150 MHz) δ: 149.81 (C-2), 149.78 (C-2), 137.7 (C-4), 137.6 (C-4), 136.7 (C-8), 136.6 (C-8), 118.4 (C-5), 118.3 (C-5), 91.2 (C-1′), 91.1 (C-1′), 88.2 (C-4′), 88.1 (C-4′), 86.1 (C-6), 86.0 (C-6), 75.73 (C-2′), 75.68 (C-2′), 72.8 (C-3′), 72.7 (C-4′), 63.64 (C-5′), 63.59 (C-5′), 38.0 (N-CH_3), 37.95 (N-CH_3). ^{15}N NMR (CD_3OD, 60 MHz) δ: −142.4 (d, $^2J_{(N,H)}$ = 13.4 Hz, N-7), −199.8 (d, $^2J_{(N,H)}$ = 12 Hz, N-3), −211.5 (t, $^2J_{(N,H)}$ = 7.5 Hz, N-9), −268.9 (t, $^2J_{(N,H)}$ = 7.5 Hz, N-1). MS: not indicative due to the deglycosylation of **2b** in the mass-spectrometer.

[$^{15}N_5$]-2′,3′,5′-Tri-O-benzoyladenosine (12): A suspension of Raney nickel and water (50% w/w, about . . . 1 teaspoon) was added to a solution of [$^{15}N_5$]-2-hexylthio-2′,3′,5′-tri-O-benzoyladenosine **13** (230 mg, 0.328 mmol) in 30 mL DMF. The heterogeneous solution was heated (100°C) for 1.5 hours and then filtered and washed with warm DMF. The solvent was evaporated and co-evaporated with ethanol. The oily residue was purified by column chromatography and eluted with a gradient of 1–5% MeOH in $CHCl_3$ to give the product in 60% yield (112 mg). ^{1}H NMR (Acetone-d_6, 600 MHz) δ: 8.30 (dd, $^2J_{(N,H)}$ = 11.8, 7.2 Hz, 1H, H-8), 8.20 (t, $^2J_{(N,H)}$ = 15.6 Hz, 1H, H-2), 8.13–7.95(m, 6H, Bz), 7.67–7.58 (m, 3H, Bz), 7.52–7.39 (m, 6H, Bz), 6.89 (dd, $^1J_{(N,H)}$ = 90.5, $^2J_{(N,N)}$ = 3 Hz, 2H, NH_2), 6.69–6.63 (m, 2H, H-1′, H-2′), 6.46 (t, $^2J_{(N,H)}$ = 5.4 Hz, 1H, H-3′), 4.98–4.94 (m, 1H, H-4′), 4.92 (dd, $^2J_{(H,H)}$ = 12, 3.5, 1H, H-5′), 4.78 (dd, $^2J_{(H,H)}$ = 12, 3.5, 1H, H-5′). ^{13}C NMR (Acetone-d_6, 75 MHz) δ: 166.5 (CO), 166.8 (CO), 165.7 (CO), 157.4 (dm, $^1J_{C\text{-}N}$ = 20.2 Hz, C-6), 154.0 (t, $^1J_{(C,N)}$ = 2 Hz, C-2), 150.5 (dm, $^1J_{(C,N)}$ = 19.5 Hz, C-4), 141.1 (d, $^1J_{(C,N)}$ = 10.5 Hz, C-8), 134.5, 134.4, 134.1, 130.7, 130.4, 130.1, 129.9, 129.44, 129.38, 88.1 (d, $^1J_{(C,N)}$ = 12 Hz, C-1′), 80.9 (C-4′), 74.5 (C-2′), 72.2 (C-3′), 64.3 (C-5′). ^{15}N NMR (Acetone-d_6, 61 MHz) δ: −139.4 (d, $^2J_{(N,H)}$ = 14.6 Hz, N-7), −143.6 (N-1), −154.7 (d, $^2J_{(N,H)}$ = 17.7 Hz, N-3), −215.1 (d, $^2J_{(N,H)}$ = 5.5 Hz, N-9), −305.4 (td, $^1J_{(N,H)}$ = 91.5, $^2J_{(N,N)}$ = 4.3 Hz, NH_2). HRMS (DCI, CH_4): calcd for $C_{31}H_{26}15N_5O_7$ (MH^+) 585.1683, found 585.1698.

[$^{15}N_5$]-2-Hexylthio-2′,3′,5′-tri-O-benzoyladenosine (13): A solution of [$^{15}N_5$]-2-hexylthioadenine **9** (142 mg, 0.554 mmol) and $(NH_4)_2SO_4$ (catalytic amount) in HMDS (2 mL) was stirred under reflux for 30 minutes. The solution was cooled to room temperature and evaporated under high vacuum. A solution of 1-O-acetyl-2,3,5-tri-O-benzoyl-β-D-ribofuranose **11** (155.4 mg, 0.31 mmol) in 1,2-DCE (2 mL) was added to the oily residue of the silylated base followed by a solution of trimethylsilyl triflate (0.15 mL, 0.83 mmol) in 1,2-DCE (1.85 mL). The mixture was then heated under reflux for 1 hour, at which time TLC on a silica gel plate ($CHCl_3$:MeOH 9:1) indicated a complete consumption of the sugar and formation of the product ($R_f = 0.69$). At this point, the reaction mixture was diluted with methylene chloride and washed with saturated $NaHCO_3$ solution and brine. The organic layer was dried with $MgSO_4$, filtered, concentrated, and flash chromatographed on silica gel by using 3% MeOH in $CHCl_3$ to give the desired nucleoside **13** in 69% yield (257 mg). ^{1}H NMR ($CDCl_3$, 600 MHz) δ: 8.01–7.19 (m, 6H, Bz), 8.86 (dd, $^2J_{(N,H)} = 11.5$, 8.5 Hz, 1H, H-8), 7.58–7.53 (m, 3H, Bz), 7.43–7.36 (m, 6H, Bz), 6.40 (ddd, $J_{(H,H)} = 5.7$, 4, 1.5, 1H, H-1′), 6.33 (d, $J_{(H,H)} = 4$ Hz, 1H, H-2′), 6.20 (t, $J_{(H,H)} = 6.0$ Hz, 1H, H-3′), 6.17 (d, $^1J_{(N,H)} = 90.5$ Hz, 2H, NH_2), 4.85 (dd, $J_{(H,H)} = 12$, 3.6, 1H, H-5′), 4.82–4.79 (m, 1H, H-4′), 4.68 (dd, $J_{(H,H)} = 12$, 4.5 Hz, 1H, H-5′), 3.26–3.11 (m, 2H, SCH_2), 1.74–1.63 (m, 2H, SCH_2CH_2), 1.40–1.16 (m, 6H), 0.80 (t, $J_{(H,H)} = 7$ Hz, 3H, CH_3). ^{13}C NMR ($CDCl_3$, 150 MHz) δ: 166.10 (CO), 165.93 (CO), 165.06 (d, $^1J_{(C,N)} = 4.4$ Hz, C-2), 154.46 (dt, $^1J_{(C,N)} = 20.8$ Hz, C-6), 150.10 (dm, $^1J_{(C,N)} = 21.4$ Hz, C-4), 138.15 (d, $^1J_{(C,N)} = 10.9$ Hz, C-8), 133.75, 133.64, 133.39, 129.81, 129.76, 129.59, 129.17, 128.64, 128.56, 128.52, 128.48, 128.46, 117.32 (d, $^1J_{(C,N)} = 9.4$ Hz, C-5), 87.38 (d, $^1J_{(C,N)} = 12.2$ Hz, C-1′), 80.10 (C-4′), 74.13 (C-2′), 71.18 (C-3′), 63.44 (C-5′), 31.33, 31.29, 28.77, 28.52, 22.44, 13.95 (CH_3). ^{15}N NMR (CD_3OD, 61 MHz) δ: −142.7 (N-7), −157.7 (N-1), −164.5 (d, $^2J_{(N,N)} = 3.6$ Hz, N-3), −215.7 (d, $^2J_{(N,H)} = 4.9$ Hz, N-9), −306.0 (td, $^1J_{(N,H)} = 91.5$, $^2J_{(N,N)} = 5.5$ Hz, NH_2). HRMS (DCI, CH_4): calcd for $C_{37}H_{37}15N_5O_7S$ (M^+) 700.2265, found 700.2222.

[$^{15}N_4$] -2′,3′,5′-Tri-O-benzoylpurine Riboside (14): A solution of [$^{15}N_5$] -2′,3′,5′-tri-O-benzoyladenosine **12** (78 mg, 0.133 mmol) in dry THF (3.5 mL) was treated with dry *iso*-amyl nitrite (133 μL, 0.98 mmol) in dry THF (1 mL). The mixture is immediately purged with nitrogen and then heated under reflux for 24 hours. An additional aliquot of *iso*-pentyl nitrite (133 μL) in dry THF (1 mL) was added each day for 2 more days. After 72 hours, the yellow-orange mixture was evaporated and purified by silica gel column (eluted with 1% MeOH in $CHCl_3$). The product was obtained as a yellow oil in 93% yield (73 mg). ^{1}H NMR ($CDCl_3$, 600 MHz) δ: 9.20 (bs, 1H, H-6), 8.91 (t, $^2J_{(N,H)} = 14$ Hz, H-2), 8.40 (t, $^2J_{(N,H)} = 10$ Hz, H-8), 8.10–7.90 (m, 6 H, Bz), 7.62–7.54 (m, 3 H, Bz), 7.47–7.34 (m, 6 H, Bz), 6.53 (d, $^2J_{(H,H)} = 4.8$ Hz, 1H, H-1′), 6.41 (t, $^2J_{(H,H)} = 4.7$ Hz, 1H, H-2′), 6.26 (t, $^2J_{(H,H)} = 5.5$ Hz, 1H,

H-3′), 4.94 (dd, $^2J_{(H,H)} = 12.2$, 3.2, 1H, H-5′), 4.87 (q, $^2J_{(H,H)} = 4.2$ Hz, 1H, H-4′), 4.73 (dd, $^2J_{(H,H)} = 12.2$, 4.2, 1H, H-5′). ^{13}C NMR ($CDCl_3$, 150 MHz) δ: 166.0 (CO), 165.3 (CO), 165.1 (CO), 151.7 (C-4), 151.4 (C-2), 147.2 (C-6), 145.1 (C-8), 134.4 (C-5), 133.9, 133.8, 133.5, 129.8, 129.6, 129.5, 129.2, 128.60, 128.56, 128.5, 128.1, 87.2 (d, $^1J_{(C,N)} = 11.4$ Hz, C-1′), 81.0 (C-4′), 73.9 (C-2′), 71.3 (C-3′), 63.3 (C-5′). ^{15}N NMR ($CDCl_3$, 61 MHz) δ: −118.2 (N-1), −129.4 (d, $^2J_{(N,H)} = 14$ Hz, N-3), −136.3 (N-7), −214.9 (N-9). HRMS (DCI, CH_4): calcd for $C_{31}H_{25}15N_4O_7$ (MH^+) 569.1604, found 569.1646.

1-Methyl-9-(2′,3′,5′-tri-O-*tert*-butyldimethylsilanyl-β-D-ribofuranosyl)-9H-Purinium Iodide (17): A solution of 2′,3′,5′-tri-O-(tert-butyldimethylsilyl)purine riboside **15** (80 mg, 0.134 mmol) and CH_3I (0.13 mL, 2.08 mmol) in CH_3CN (7 mL) was heated under reflux for 6 hours. After stirring for an additional 15 hours at room temperature, the solvent was evaporated under reduced pressure to give the crude ammonium iodide salt, which was used without further purification in the next synthetic step. ^{1}H NMR (CD_3OD, 200 MHz) δ: 9.73 (d, $^2J_{(H,H)} = 1.5$ Hz, 1H, H-6), 9.48 (d, $^2J_{(H,H)} = 1.5$ Hz, 1H, H-2), 9.44 (s, 1H, H-8), 6.26 (d, $^2J_{(H,H)} = 4$ Hz, 1H, H-1′), 4.78 (t, $^2J_{(H,H)} = 4$ Hz, 1H, H-2′), 4.52–4.45 (m, 4H, H-3′, N-CH_3), 4.24–4.16 (m, 1H, H-4′), 4.00 (dd, $^2J_{(H,H)} = 12.5$, 3 Hz, 1H, H-5′), 3.81 (dd, $^2J_{(H,H)} = 12.5$, 2.8 Hz, 1H, H-5′), 0.95 (s, 9H, t-Bu), 0.89 (s, 9H, t-Bu), 0.87 (s, 9H, t-Bu), 0.15 (s, 3H, $SiCH_3$), 0.10 (s, 3H, $SiCH_3$), 0.50 (s, 3H, $SiCH_3$), 0.02 (s, 3H, $SiCH_3$), 0.0 (s, 3H, $SiCH_3$).

Preparation of Adenosine Deaminase Solution for an Enzymatic Assay and NMR Experiment

A 3.2 M suspension of adenosine deaminase (ADA) from calf intestine (Roche Diagnostics Corporation, Indianapolis, IN, USA) in $(NH_4)_2SO_4$ solution was exchanged for 50 mM D_2O potassium phosphate buffer, pD 7, by repeatedly concentrating the sample to less than 0.5 mL and rediluting to 2 mL with fresh buffer in a distilled-water-rinsed Amicon Centricon-10 centrifuge concentrator. Six exchange cycles were repeated with the phosphate buffer in D_2O.

ADA reactivity was assayed as follows:[44] 0.1 mL of 1.4 mM adenosine solution in 0.05 M phosphate buffer, pH 7.4, was added to ice cold 2.88 mL solution of ca. 0.8 units/mL of ADA in 0.05 M phosphate buffer, pH 7.4 (prepared as described above). The decrease in the UV absorbance of adenosine (at 265 nm) due to its deamination, was measured as a function of time and the enzyme activity was found to be 146 units/mg.

^{1}H-^{15}N HMBC Experiment of ADA:[$^{15}N_4$]-PR Complex

The NMR sample was prepared as follows: 1.8 mM solution of ADA (0.5 mL, prepared as described above) and [$^{15}N_4$]-PR (0.9 μmol) in 50 mM D_2O

potassium phosphate buffer, pD 7 (7.7 μL). 2D-(^{1}H-^{15}N) HMBC spectrum was recorded on a Bruker DMX-600 instrument (Rheinstetten, Germany). (600.1 and 60.8 MHz for ^{1}H and ^{15}N, respectively). The reference for ^{1}H was the residual HDO peak, corrected for the temperature.[45]

The HMBC spectrum was collected using a conventional HMBC pulse sequence from the Bruker pulse program library. The experiment was run with a delay time, D6, of 0.04 second, which is optimized for the detection of 12.5 Hz (N-H) coupling constants. The ^{1}H aquisition time was 142.6 msec. The relaxation delay after each acquisition was set at 2 seconds. The total experiment time was 16.2 hours. The 2D experiment consisted of 64 time increments of 1024 data points each. After zero filling in both dimensions and forward linear prediction in F1 (^{15}N), the final data matrix consisted of 256 × 2048 data points. This resulted in digital resolutions of 1.8 and 47.5 Hz/point for ^{1}H and ^{15}N, respectively.

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