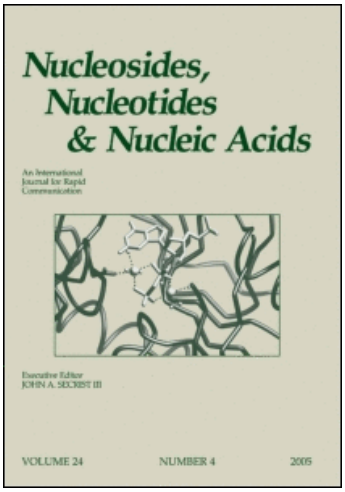

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Preparation of Imino and Amino N-15 Enriched 2-Aminopurine Deoxynucleoside

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PREPARATION OF IMINO AND AMINO N-15 ENRICHED 2-AMINOPURINE DEOXYNUCLEOSIDE

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Abstract

We report the synthesis of N-15 enriched 2-aminopurine-2'-deoxynucleoside (APdR). Both ring and 2-amino nitrogens were labelled via a Dimroth rearrangement of the free base. The corresponding deoxynucleoside was prepared enzymatically. Results of both proton and N-15 NMR studies show that the predominant tautomeric form of APdR is amino and the N1 position is shown to be the site of protonation.

INTRODUCTION

2-Aminopurine is a potent mutagen in procaryotic and eucaryotic systems, and it has been extensively used in mutagenesis studies in E. coli and bacteriophage T4 infected E. coli (see ref. 1 for review). Transition mutations induced by 2-aminopurine result from the formation of 2-aminopurine-cytosine base pairs during DNA replication (2-4). Suggested base pairing schemes between AP and C involve rare tautomer formation, as originally proposed by Freese (2), formation of a protonated base pair in Watson-Crick geometry (5,6) and wobble base pair formation (6,7).

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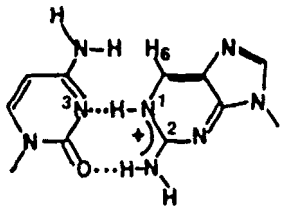
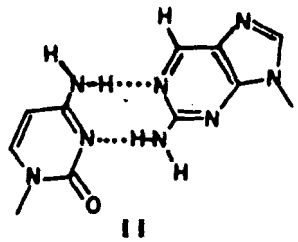
Recent NMR studies on an AP·C mismatch in a duplex oligonucleotide suggested that the most probable structure was the protonated base pair, structure I (6). Base mispairs involving either AP or C as disfavored tautomers were ruled out. However, not all the exchangeable hydrogen-bonding protons were observed so that an AP·C wobble base mispair (structure II) or an equilibrium between structures I and II could not be completely excluded. A more direct and sensitive probe to distinguish between the different hydrogen bonding possibilities would be to study an N-15 labelled AP·C mismatch.



I



II

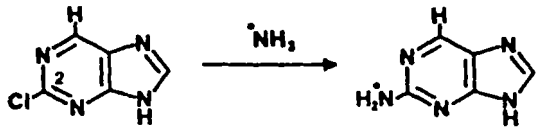
As a first step toward the N-15 NMR studies, we have synthesized and characterized N-15 enriched APdR. Very little has been published on the isotopic enrichment of purines and only recently have the preparation of the normal N-15 enriched deoxynucleosides been reported (8,9). Two characteristics of 2-aminopurine introduce special problems for synthesis of the N-15 enriched derivative. First, it is well known that substitution at the 2-position of purines is more difficult than substitution at the 6-position (10). Second, the glycosidic linkage of APdR is much more labile than that of the other deoxynucleosides which precludes most substitution reactions on the intact deoxynucleoside (11).

We first prepared the N-15 enriched free base, AP, which was then converted to the deoxynucleoside utilizing *trans*-N-deoxyribosylase (9,12). Enzymes have not been generally appreciated in organic synthesis because they can be difficult to prepare and the reaction scale may be severely limited. In the preparation of isotopically labelled molecules, however, enzymes have proven to be very useful. We demonstrate here that the deoxynucleosides may be conveniently prepared on the hundred milligram scale with *trans*-N-deoxyribosylase, and perhaps more importantly, reactions proceed with near quantitative yield under extremely mild conditions.

RESULTS AND DISCUSSION

A. Preparation of N-15 labelled APdR

Leonard and Henderson (13) previously demonstrated that adenine, N-15 enriched in the amino group, could be obtained by reacting isotopically enriched ammonia in n-butanol with 6-chloropurine in a sealed bomb at 150°C. The same strategy was used here utilizing methanol and 2-chloropurine (III) to prepare amino-enriched 2-aminopurine (IV). Because substitution of the 2-position of purine is more difficult than the 6-position (10) the displacement was conducted at 160°C rather than 150°C. The product, N-15 enriched 2-aminopurine was obtained in 40% yield and was chromatographically and spectrally indistinguishable from an authentic sample.



III IV

We had expected to obtain 2-aminopurine exclusively labelled in the amino group (IV), in analogy with the previously reported adenine reaction, however, the ^{15}N-^{1}H NMR coupling pattern was found to be more complicated that anticipated (see below). 2-Aminopurine free base was enzymatically converted to the deoxyribonucleoside in near quantitative yield. The N-15 enriched derivative displayed the same chromatographic properties and pH dependent UV spectra as an authentic sample. The spectrophotometrically determined pK value for APdR (25°C, μ = 0.1 M) was found to be 3.80 (FIG. 1) NMR spectra, as discussed below, are consistent with the proposed structure.

B. NMR spectra of enriched APdR: Identification of the sites of enrichment

The proton NMR spectrum of unenriched APdR in DMSO is shown in FIG. 2A and the proton spectrum of the N-15 enriched derivative in FIG. 2B. The 6-proton resonance of AP and APdR is to low field of the 8-resonance (14). The observed spectrum in FIG. 2B corresponds neither to that of the unenriched APdR nor to that expected for N-15 labelling at the 2-position of APdR. We find that the 6-proton resonance now gives a pseudo triplet with an 11.6 Hz splitting between outer resonances (FIG. 2B). The chemical shift of the central resonance, which corresponds to roughly 60% of the total integrated area, is coincident with that observed in the unenriched sample, FIG. 2A. The amino resonance gives a similar pattern. We observe a doublet which corresponds to roughly 40% of the

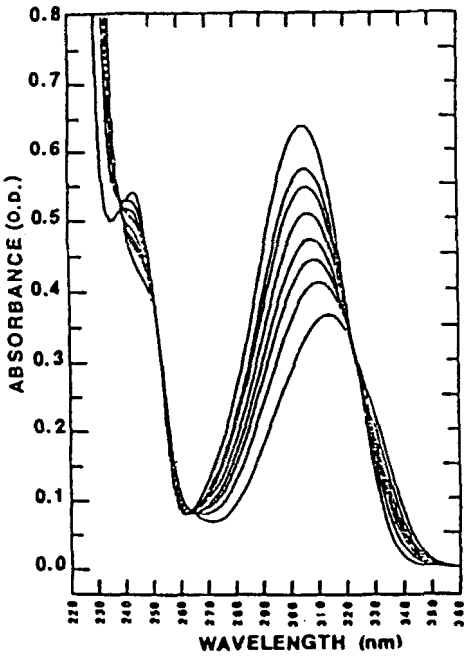


FIG. 1. Ultraviolet spectrum of APdR as a function of pH (25°C) Values of pH with decreasing absorption at 300nm are 7.33, 4.22, 4.07, 3.80, 3.58, 3.38, 3.05 and 1.52.

integrated pseudo triplet intensity. In addition the central amino resonance shows long distance coupling. These patterns are identical to those observed for the enriched base (not shown).

Clearly, both the N1 and 2-amino groups have undergone enrichment. From the integrated intensities of the proton resonances (FIG. 2B) we estimate that the N1 ring nitrogen is 40% enriched and the amino group is 60% enriched. The ^{1}J coupling of the amino protons and ^{2}J coupling on H6 are both slightly smaller than the corresponding coupling constants observed for enriched adenine (15). In addition, the amino protons of APdR show small three bond coupling to N1.

The N-15 NMR spectrum of enriched APdR is water revealed two N-15 resonances (FIG.3). The proton coupled N-15 NMR spectrum of APdR at pH 7 is shown in FIG. 3A. The lower field resonance in the aromatic region (N1 ring nitrogen) has a chemical shift of 174 ppm downfield from external N-15 aniline. The amino resonance of enriched APdR is at 21 ppm. The amino resonance in the

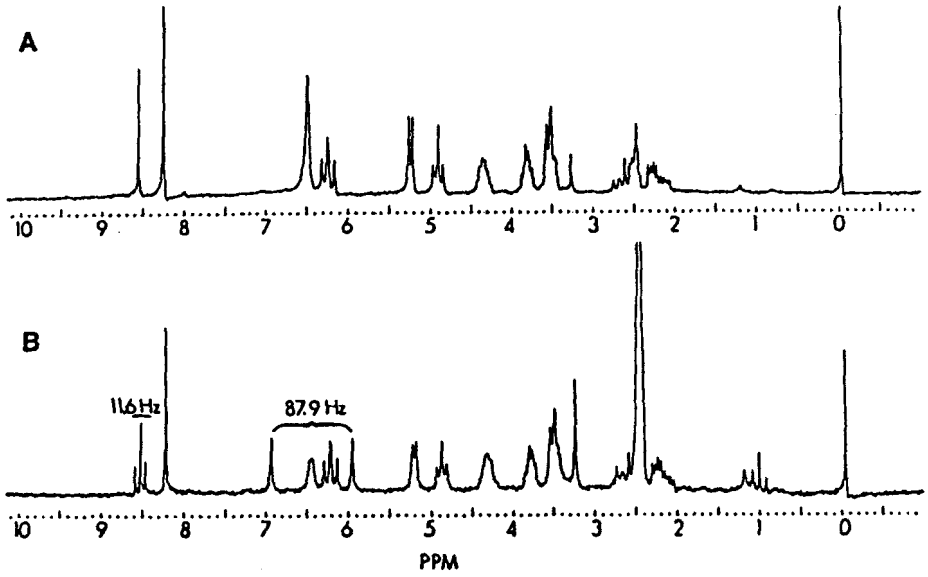


FIG. 2. Proton NMR spectrum of (A) unenriched APdR and (B) APdR partially N-15 enriched in both the N1 and 2-amino positions. Spectra were recorded at 90MHZ in deuterated DMSO and chemical shifts are reported relative to internal TMS.

proton coupled spectrum (FIG. 3A) is split into a broad triplet (^{1}J N-H; 89 Hz) and the N1 resonance is split into a doublet (^{2}J N-H; 9.8 Hz). The N1 resonance for APdR is 153 ppm to low field of the 2-amino resonance, which corresponds well with the difference in chemical shifts between the N1 and 6-amino resonances in dA (150 ppm, ref 8).

In the proton decoupled spectrum at pH 7 (FIG. 3B), the N1 resonance disappears because of a small, negative NOE, and the 2-amino resonance becomes a singlet. The large negative NOE observed for the amino resonance is consistent with the amino conformation and has been observed previously for amino resonances of both adenine and cytosine derivatives (15,16). The influence of protonation on the N-15 NMR spectrum (FIG. 3C) is discussed below.

The results of the proton and N-15 NMR studies show that both the 2-amino and N1 positions have undergone partial enrichment. We propose that enrichment at both the amino and exocyclic positions proceeds via a Dimroth rearrangement (13,17) of the free base which is shown schematically for AP in FIG.4. Reaction of the 98% enriched ammonia would first occur exclusively at the 2-

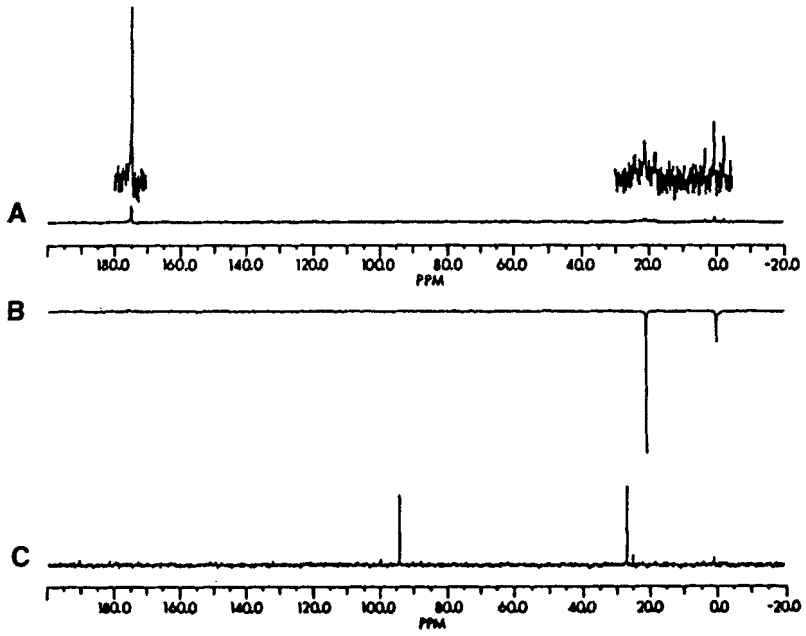


FIG. 3. N-15 NMR spectra of N-15 enriched APdR in water. (A) Proton coupled spectrum pH 7; (B) Proton decoupled spectrum, pH 7; and (C) Proton coupled spectrum pH 1.5.

position. In the basic environment at high temperature, catalytic quantities of water would attack the 6-position (IV --> V, FIG. 4) and result in ring opening between the 1 and 6-positions (VI). The amino groups attached to the 2-carbon are then chemically identical. Rotation around the 2-3 bond (VI --> VIa) followed by ring closure (IVa --> Va) would then result in exchange of label between the N1 and exocyclic amino positions.

Interestingly, under similar conditions, at somewhat lower temperature, amination of 6-chloropurine does not result in Dimroth rearrangement and simultaneous labelling of the ring nitrogen (13). The observed rearrangement of AP was not anticipated, however, it provides an extremely convenient method for labelling the ring nitrogen position. The Dimroth rearrangement has not been previously reported for the 2-position of purines.

C. APdR is predominantly in the amino tautomeric form in solution

The results of this study show that the preferred tautomeric form of APdR, both in DMSO and H_2O, is amino (structure VII), not imino (structure VIII).

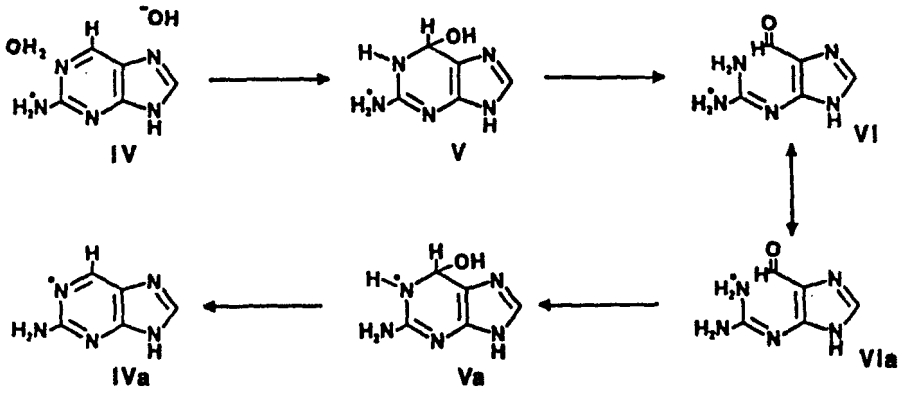
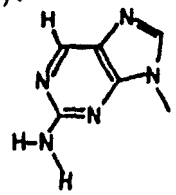
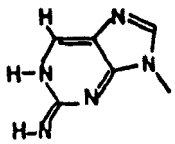


FIG. 4 Schematic representation of the proposed Dimroth rearrangement.

The proton spectrum of unenriched APdR in water is shown in FIG. 5. At lowest field are the 6 and 8 nonexchangeable proton resonances (8.6 and 8.25 ppm, respectively). The anomeric proton is at 6.4 ppm and the amino resonance at 6.01 ppm integrates for two protons when taking into account the nonlinear excitation pulse form. As the amino protons give rise to a single resonance, the amino group of APdR must rotate rapidly in water, as has been demonstrated for dA (18,19).


VII


VIII

The 8-proton is only half the size of the 6-proton (FIG. 5) due to selective deuteration (20). Selective deuteration was utilized to confirm the relative assignment of the 8 and 6-protons and the assignment presented here for APdR is the same as that given for the free base by Coburn et. al (14). In DMSO, the amino protons also give rise to a single resonance (FIG. 2A). If the amino tautomer predominated, two resonances would be expected. As only one resonance is observed for the amino protons, the amino group must be rotating rapidly.

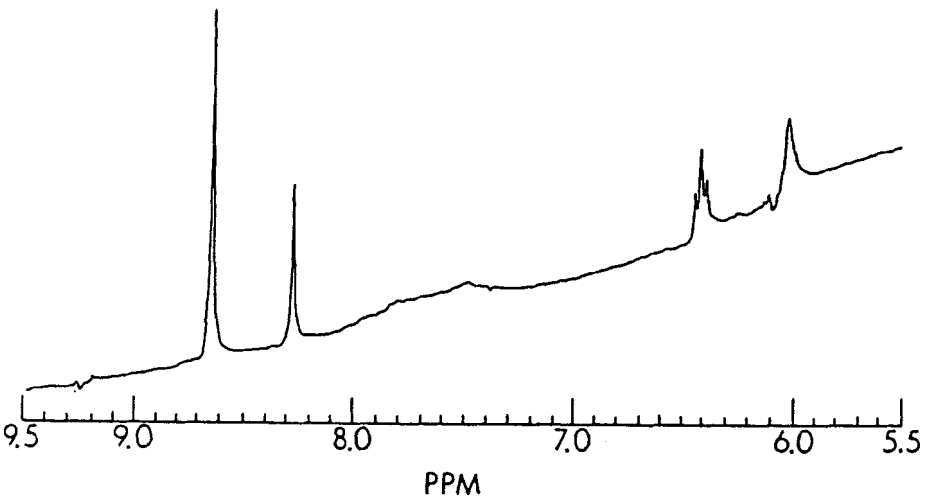


FIG. 5 Proton NMR spectrum of APdR in 90%H_2O/10%D_2O, 10mM nucleoside concentration, 0.1 M phosphate buffer, pH 7.

Rapid rotation of the amino group indicates that the C2-amino nitrogen bond has very little double bond character. If the imino conformation was populated for any significant length of time, the increased double bond character would slow the rate of rotation and result in line broadening or the appearance of two separate resonances.

The chemical shifts of the N-15 resonances in APdR are consistent with the amino tautomeric form and are inconsistent with the imino tautomer. An imino-type ring nitrogen would have resonated greater than 60 ppm to higher field (21,22). In the proton-coupled N-15 spectrum, the amino nitrogen appeared as a triplet and in the decoupled spectrum, gave a large negative NOE, consistent with the amino conformation (15,16) and the only splitting of the N1 resonance arose from two-bond coupling to H6. If predominantly in the imino tautomeric form, the N1 resonance would have been split into a doublet with a coupling constant on the order of 90Hz, as with guanosine (16), and the exocyclic nitrogen would also have appeared as a doublet.

D. Influence of protonation on the N-15 NMR spectrum of APdR

A previous proton NMR study (6) indicated that the most probable structure for the AP·C mispair in DNA was a protonated base pair in Watson-Crick geometry (structure I). We therefore investigated the influence of protonation on the

N-15 NMR spectra of enriched APdR. Proton coupled N-15 spectra were recorded at pH 1.5 as shown in FIG. 3C. Lowering the pH to 1.5 (pK APdR; 3.8) results in a large upfield shift (81 ppm) of the N1 resonance and a small downfield shift of the amino resonance (5 ppm). The large upfield shift of the N1 and small downfield shift of the amino resonance are consistent with protonation-induced shifts observed for other nucleosides (14,15,20) and confirms that N1 of APdR is the site of protonation (7,23). The magnitude of the upfield shift of the N1 resonance resulting from protonation of APdR is larger than that observed for the N1 of adenosine (72 ppm) and the downfield shift of the amino resonance (5 ppm) is smaller than that reported for adenosine (9 ppm, ref. 16).

Protonation results in a significant increase in the rate of amino proton exchange (24) which would be expected to significantly alter proton-nitrogen coupling (16). As can be seen by comparing the proton coupled spectra of APdR at pH 7 and 1.5 (Figs. 3A and 3C), the amino resonance at low pH is no longer split into a triplet. At low pH, the N1-H6 coupling constant has been reduced to 3 Hz.

E. N-15 NMR and elucidation of base pairing interactions in DNA

The mutagenicity of 2-aminopurine historically has been attributed to an enhanced tendency to assume the imino tautomeric form (2). Theoretical studies, however, indicate that AP is less likely to assume the rare imino tautomeric form than is adenine (25). While the present study indicates that the predominant form of APdR in solution is amino, these results can not be used to quantitate the tendency of AP to assume the imino tautomeric form, relative to A.

In order to clarify the mechanism of mispair formation by AP, however, it will be necessary to directly investigate an AP·C base pair in DNA. Freese has suggested that formation of second hydrogen bond between AP and C in DNA could result from a "trigger mechanism" (2). Formation of a first hydrogen bond would then stimulate the a tautomeric shift of AP generating a second hydrogen bond. Similarly, formation of an AP·C base pair with one hydrogen bond could stimulate acquisition of a proton from solvent water generating a protonated base pair with two hydrogen bonds (5,6).

N-15 NMR data may be used to clarify and confirm the nature of the hydrogen bonding between AP and C in oligonucleotides containing N-15 enriched C and AP residues. Data presented here indicates that neutral amino, imino and protonated forms of AP can be distinguished unambiguously by N-15 NMR

spectroscopy. Examination of site-specifically enriched oligonucleotides will allow unequivocal resolution of the mechanism of AP mutagenesis.

EXPERIMENTAL SECTION

Materials: 2-chloro-4,5-diaminopyrimidine was obtained from Sigma Chemical Company, St. Louis MO. Tri-ethyl-orthoformate was obtained from Aldrich Chemical Co., Milwaukee, WI. Isotopically enriched N-15 ammonia was purchased from Monsanto Research Corp., Miamisburg, Ohio. 2-chloropurine was prepared from 2-chloro-4,5-diaminopyrimidine and triethyl-ortho-formate according to Montgomery (26). Lactobacillus helviticus culture was obtained from American Type Culture Collection, Rockville, MD.

UV spectra were obtained with Perkin Elmer Lambda 3B UV/Visible or Cary 219 spectrophotometers.

Methanolic ammonia was prepared by introducing 15g of 98+% N-15 enriched ammonia into an evacuated flask cooled in a dry ice acetone bath. 100ml cold methanol was then slowly added to the condensed ammonia. This solution was maintained in a stoppered flask under refrigeration.

Enriched 2-aminopurine was obtained by treating 1.5 g 2-chloropurine in 15 ml methanolic ammonia in a sealed bomb at 160°C for 10 hours. The reaction mixture was concentrated by evaporation of methanolic ammonia under reduced pressure and then fractionated by silica gel chromatography eluting with 10% methanol in chloroform. Chromatographically pure enriched 2-aminopurine (0.52 g) was obtained in 40% yield. Enriched 2-aminopurine obtained by this method had UV spectral properties in neutral and acidic solution identical those of commercially available 2-aminopurine.

Trans-N-deoxyribosylase was isolated from Lactobacillus helviticus as described previously (12). Lactobacillus helviticus cultures (4 x 2.5l) were grown overnight at 37°C without agitation. Approximately 40g of cells were isolated from the culture media by continuous flow centrifugation. Cells were washed in 0.15M NaCl and then sonicated for 10 min. Cell debris was removed by centrifugation and the supernatant was dialyzed against 0.05 M phosphate buffer, pH 5.1 overnight. After dialysis, the suspension was heated at 55°C for 10 min, cooled on ice and again centrifuged. Streptomycin sulfate was added to the supernatant and the suspension was cooled on ice for 10 min. The precipitate which formed was removed by centrifugation. The supernatant, containing the *trans*-N-deoxyribosylase activity was kept at 0°C. This procedure yielded roughly 100 ml of a suspension of *trans*-N-deoxyribosyase with an activity of 0.097 units/ml. One unit catalyzes the production of 1μmol of deoxynucleoside per minute.

APdR was obtained from AP using *trans*-N deoxyribosylase. 100mg enriched AP was dissolved in 85 ml water with 1.8 g 2'deoxycytidine as deoxyribose donor, 5 ml of the enzyme preparation and 10 ml 0.2M tris maleate buffer, pH 6. The reaction was maintained at 37°C overnight. Thin layer silica gel chromatography indicated the complete conversion of AP to APdR. The reaction mixture was concentrated under reduced pressure and deoxynucleosides were extracted with 10% methanol in chloroform (100ml). The organic solvent was removed under reduced pressure and APdR was isolated by silica gel chromatography (10% methanol in chloroform) and recrystallized from methanol.

0.95g of chromatographicallly pure APdR (98% conversion efficiency) was obtained.

Proton NMR spectra in deuterated DMSO were obtained with a JEOL FX-90 NMR spectrometer operating at 89.87 MHz with TMS as internal standard. Proton spectra in water were obtained in 90% H_2O/10%D_2O, 10mM nucleoside concentration, 0.1 M phosphate buffer, pH 7.2, using a Bruker 270SY NMR spectrometer operating at 270 MHz. A 1-τ-1 hard pulse sequence was used for supression of the solvent peak (27). Chemical shifts of protons in water are reported relative to tetramethylammonium chloride (3.188 ppm).

N-15 spectra were obtained in water with a Bruker 270 MHz NMR spectrometer operating at 27.384 MHz. N-15 chemical shifts are reported in ppm downfield from external N-15 aniline in deuterated acetonitrile. A time delay of 5 sec was used in the accumulation of N-15 spectra and 400 scans were accumulated for each spectrum. APdR was 0.073 M in 0.1 M phosphate buffer pH 7 or 1.5.

ACKNOWLEDGEMENTS

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