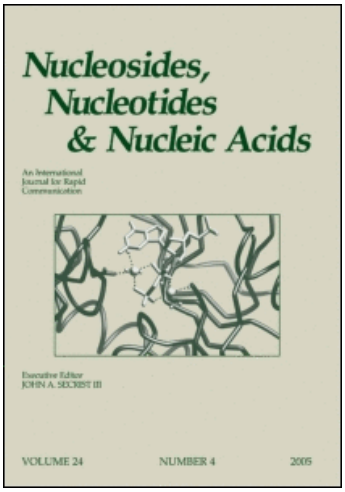

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Synthesis and Utilization of $^{13}C(8)$-Enriched Purines

Christopher J. Lafrancois[a]; June Fujimoto[a]; Lawrence C. Sowers[a]
[a] Division of Pediatrics, City of Hope National Medical Center Duarte, CA

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SYNTHESIS AND UTILIZATION OF ^{13}C(8)-ENRICHED PURINES

Christopher J. LaFrancois, June Fujimoto, and Lawrence C. Sowers*

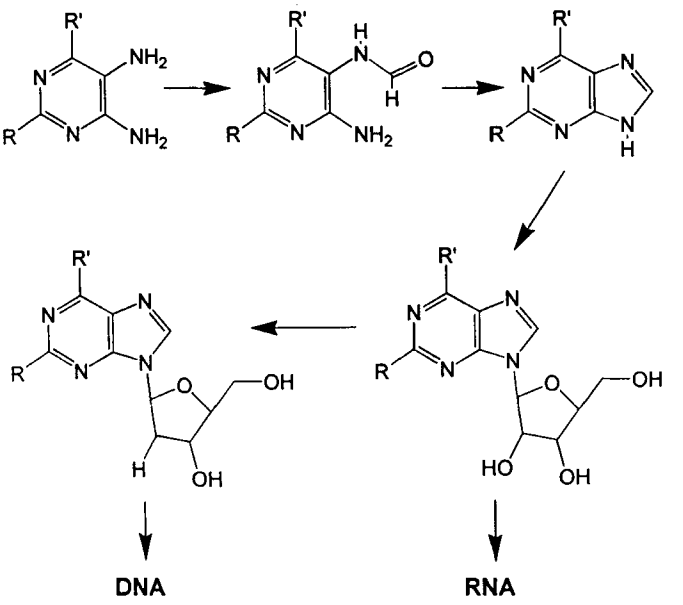
Division of Pediatrics
City of Hope National Medical Center
Duarte, CA 91010

ABSTRACT A new and more efficient method is presented for the synthesis of ^{13}C(8)-enriched adenine. In addition, we present for the first time the synthesis of ^{13}C(8)-enriched 2-aminopurine and purine. All three analogues have been converted to the corresponding ribonucleosides. The adenine analogue has been further converted to the 2'-deoxy-nucleoside and incorporated into a synthetic oligonucleotide. Data is presented demonstrating the utility of ^{13}C-enrichment in heteronuclear isotope-edited NMR spectra.

INTRODUCTION

Interest in nucleoside analogues, selectively enriched with stable isotopes, has been growing due to their utility in heteronuclear NMR studies[1-15] as well as mass-labeled standards for analytical studies utilizing mass spectrometry methods.[15-20] The rate-limiting event in the utilization of such derivatives is the synthesis. Due to the limited scope to date of synthetic efforts, this powerful strategy remains underutilized.

Adenine was first prepared synthetically, via the 5-formamido intermediate, by boiling 4,5,6-triaminopyrimidine in formic acid.[21] Enrichment with ^{13}C in the 8-position has been accomplished with ^{13}C-enriched formic acid.[22-24] The critical step in the synthesis is ring closure of the 5-formamidopyrimidine, which was accomplished by heating. However, yields for the ring closure reaction were modest, prompting the use of alternative methods. Such methods, however, resulted in substantial carbon exchange which decreased their utility for the synthesis of ^{13}C-enriched adenine.



SCHEME 1: Synthetic route for preparation of ^{13}C-enriched purine derivatives. adenine; R = H, R' = NH_2 / 2-aminopurine; R = NH_2, R' = H / purine; R = H, R' = H

We report here that the 4,5,6-triaminopyrimidine can be converted quantitatively to the corresponding formamido intermediate (**Scheme 1**). Previously we converted ^{15}N-enriched 2,4,5-aminopyrimidine to 2-aminopurine by heating in acetic anhydride with ethylorthoformate as the carbon donor[25] as suggested by Montgomery.[26] Ring closure was accomplished by heating in acetic anhydride. Here, we have used enriched formic acid to prepare the intermediate formamidopyrimidine, and then acetic anhydride to induce ring closure which allows recovery of enriched adenine in 98% yield.

Enriched adenine has been converted to the ribonucleoside by the method of Vorbruggen,[27] to the deoxynucleoside by the method of Robins[28] and incorporated into an oligonucleotide. Isotope-edited NMR experiments demonstrate that the H8 proton of the selectively enriched A residue can be identified even in the single strand, which contains five other adenine residues.

Previous studies have demonstrated that the mutagenically important adenine-cytosine mispair in DNA exists as an equilibrium between protonated wobble and neutral

reverse wobble configurations when the base pair is embedded within a duplex DNA domain.[29,30] The chemical shift of the H8 proton proves to be an important monitor of the state of protonation. The equilibrium of this mispair at a putative DNA replication fork may undergo different dynamic changes. In a dynamic and thermodynamically less stable region, assignment of proton resonances by standard methods may not be possible. Therefore, the labeled derivative reported here, in conjunction with heteronuclear NMR editing methods, may prove uniquely valuable in examining the dynamics of such structures in solution.

Interest in 2-aminopurine (2AP) also derives from its mutagenic base pair with cytosine.[14,31,32] A recent study has similarly demonstrated a pH-dependent equilibrium between protonated and neutral structures.[14] To our knowledge, ^{13}C-enriched 2AP has not been previously prepared, and such synthesis is described here.

An ^{15}N-enriched 2AP has been previously reported and converted to the corresponding deoxynucleoside in low yield enzymatically.[25] To our knowledge, conversion to the nucleoside has not yet been accomplished by chemical synthetic methods, and such synthesis is reported here. Recently, we reported on the conversion of 2AP-riboside to the corresponding 2'-deoxynucleoside and its incorporation into oligodeoxynucleotides,[33] providing a complete pathway for the incorporation of ^{13}C-enriched 2AP into synthetic oligonucleotides.

Similarly, unsubstituted purine has not been previously prepared with ^{13}C, and this synthesis is reported here. Purine ribonucleoside, or nebularine, has been used as an antibiotic.[34] (ref 34 and references cited therein) Our interest in purine ribonucleoside derives from the observations that it undergoes ring opening in moderately alkaline solution, generating the corresponding formamidopyrimidine (FAPY) derivative.[35,36] FAPY derivatives of the normal DNA bases, A and G, are considered important DNA damage products.[37,38] Purine can be incorporated into oligos and has been studied by NMR.[39-41] We are interested in placing the enriched purine derivative into oligos and opening the ring in alkali generating FAPY, which could be monitored by NMR editing. To date, the synthesis of oligos with FAPY A or G has not been successful, as the alkali needed for opening would deformylate and destroy the DNA. Nebularine may therefore provide an important and accessible model for FAPY derivatives in DNA.

EXPERIMENTAL

Instrumentation. NMR spectra were obtained with Varian Unity 300 and 500 Plus NMR spectrometers. NMR spectra were recorded in deuterated DMSO. Proton chemical shifts are reported downfield from TMS, and ^{13}C-chemical shifts (75.429 MHz) are reported relative to DMSO at 39.5 ppm. The GC/MS analysis was performed with a Hewlett Packard 5890 GC interfaced to a 5970 mass selective detector. UV spectra were recorded with a Perkin Elmer Lambda IIIB double beam spectrophotometer. HPLC separations were performed with a Perkin Elmer series IV pump, Vydac C-18 semi-preparative column and LKB-Pharmacia diode array detector. Oligonucleotides were synthesized with a Pharmacia gene assembler.

Materials. The ^{13}C-enriched formic acid was obtained from Cambridge Isotopes (Andover, MA). The starting materials, 4,5,6-triaminopyrimidine sulfate, 4,5-diaminopyrimidine and 2,4-diamino-5-nitropyrimidine were obtained from Sigma Chemical Co. (St. Louis, MO). All other solvents and materials, including ß-D-ribofuranose 1-acetate 2,3,5-tribenzoate and trimethylsilyltriflate are commercially available and were of the highest quality available.

SYNTHESIS CONDITIONS AND RESULTS

Adenine (A). A quantity of 4,5,6-triaminopyrimidine sulfate (2.0g, 9.0 mmol) was heated in 50 mL 0.1 N HCl at 90°C and 0.7 mL (20 mmol) of enriched formic acid was added. In approximately 30 min all of the starting material had dissolved. Examination of an aliquot by UV indicated conversion of the triamino- to the FAPY-derivative (**TABLE 1**). The UV spectrum shifts from 279 nm[42] to 259 nm[43] upon formylation.

A 10 µl aliquot was dried and then silylated for analysis by GC/MS. The derivatized product was injected on the GC/MS. One predominant peak was observed as shown in the chromatogram of **FIG. 1A**. The parent ion was observed at 370 *m/z* (**FIG. 1B**). The fragmentation pattern and relative abundance of ions in the mass spectrum are identical with that reported previously for FAPY-A.[44] A sample was also taken for analysis by NMR (**TABLE 3**).

The solvent was evaporated and the material was used for the ring closure without further purification. Ring closure of the formamidopyrimidine to adenine was

TABLE 1: UV characteristics of purine precursors and derivatives.

Compound	pH	λ max (nm)	ε x 10^3 ($M^{-1}cm^{-1}$)	ref.
4,5,6-triaminopyrimidine	6.5	279	8.7	42
4,6-diamino-5-formamidopyrimidine	6.5	259	4.7	43
adenine	6.5	261	12.5	42
adenosine	7.0	259	15.4	48
2-aminopurine	7.0	305	6.0	56
2AP riboside	6.8	304	6.8	57
purine	5.7	263	7.9	56
purine riboside	water	262	7.0	59

accomplished by heating in acetic anhydride. Acetic anhydride (50 mL) was added to the flask which was fitted with a reflux condenser. The temperature was rapidly increased by placing the flask into a hot heating mantle. After approximately five min, the solvent began to reflux and all of the starting material had dissolved. When the flask had cooled to room temperature, the residual acetic anhydride was removed under reduced pressure.

Aqueous ammonia was then added to the residue and the solution was heated at 60°C for 1 h to hydrolyze any acetylated adenine to adenine. The solvent was removed under reduced pressure and the residue taken up into water. The pH was brought to neutral with sodium hydroxide and the solution was concentrated to a small volume. Adenine began to precipitate and the precipitation was allowed to proceed overnight in the refrigerator. The adenine was washed with cold water and analyzed. The UV (**TABLE 1**), mass spectral (**TABLE 2**), and NMR (**TABLE 3**) characteristics were consistent with ^{13}C(8)-enriched adenine. The chemical shifts of the H6 and H8 protons, relative assignments,[8] ^{13}C chemical shift and ^{13}C-^{1}H coupling constant[12,45,46] are consistent with data previously obtained at natural abundance.

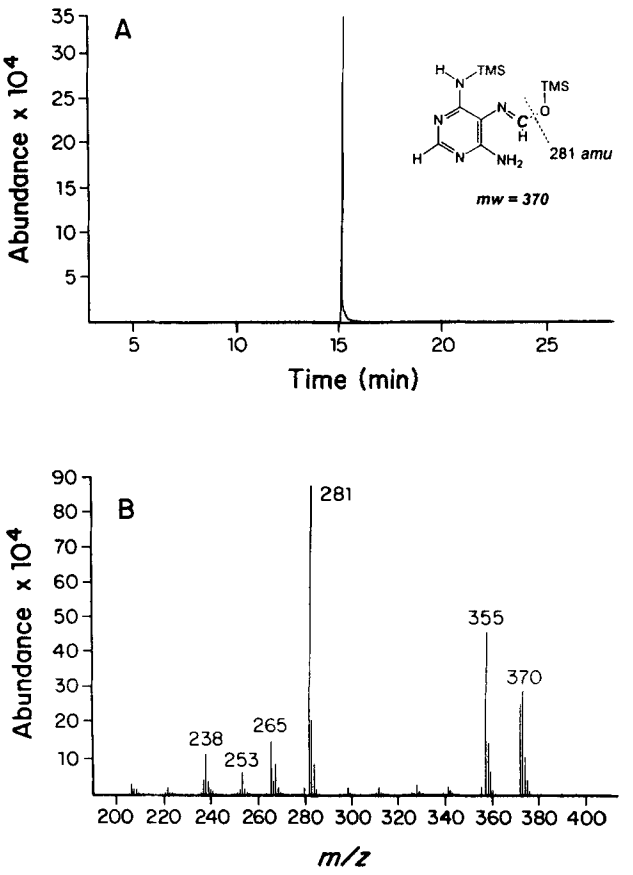


FIG. 1: A) Gas chromatogram of the ^{13}C-enriched 4,6-diamino-5-formamidopyrimidine. B) Mass spectrum of the peak.

The material was pure by GC/MS analysis and NMR and was used without further purification. The amount of material obtained was 1.2 g, for a yield of 98 % relative to the staring 4,5,6-triaminopyrimidine or 60 % relative to the labeled formic acid. Enriched adenine (2.0 g, 15 mmol) was then benzylated according to a published procedure[47] and used without further purification. The benzylated adenine derivative was converted to the ribonucleoside by coupling with a sugar derivative according to Vorbruggen.[27] Following deprotection in sodium methoxide, ^{13}C(8)-enriched adenosine was purified by silica gel chromatography with 10% methanol in dichloromethane. The UV spectra of the enriched adenosine[48] was indistinguishable from an authentic sample.

TABLE 2: Calculated and observed exact mass for protonated ^{13}C-enriched purines and nucleosides, ionized via CI and ESI+ respectively. The mass spectra were obtained in the positive ion mode.

Compound	Formula	Calc.	Expt.
adenine	$C_4H_6N_5(^{13}C)$	137.0657	137.0659
adenosine	$C_9H_{14}N_5O_4(^{13}C)$	269.1079	269.1068
2-aminopurine	$C_4H_6N_5(^{13}C)$	137.0657	137.0656
2AP riboside	$C_9H_{14}N_5O_4(^{13}C)$	269.1079	269.1076
purine	$C_4H_5N_4(^{13}C)$	122.0548	122.0548
purine riboside	$C_9H_{13}N_4O_4(^{13}C)$	254.0970	254.0962

TABLE 3: ^{1}H chemical shifts, ^{13}C chemical shifts (ppm) and ^{1}H-^{13}C coupling constants (Hz).

Compound	H2	H6	H8	C8	$^{1}J_{C8-H8}$	$^{3}J_{C8-H1'}$
4,6-diamino-5-formamidopyrimidine	7.74	--	8.08	160.77	194.9	--
adenine	8.13	--	8.11	138.89	209.4	--
adenosine	8.14	--	8.35	139.91	213.1	4.0
2-aminopurine	--	8.56	8.04	140.97	209.5	--
2AP riboside	--	8.61	8.32	140.88	213.5	4.0
purine	8.93	9.14	8.63	146.10	210.4	--
purine riboside	8.97	9.22	8.86	145.50	214.5	4.0

The identity and purity of the product was further confirmed by mass spec and NMR. The assignment of H2 and H8, and ^{13}C-H8 coupling constant are consistent with literature values.[12]

Labeled adenosine was converted to 2'-deoxyadenosine by the method of Robins et al.[28] The amino group was benzylated, the 5'-hydroxyl was converted to the 5'-dimethoxytrityl derivative,[49] and the 3'-hydroxyl converted to the cyanoethyldiisopropyl-phosphoramidite by standard procedures.[50]

The labeled adenine phosphoramidite was then used for solid phase oligo-nucleotide synthesis using standard methods. The sequence of the oligonucleotide prepared is:

5' d(C T G **A** G A A A A C C C)

where **A** is the enriched adenine derivative. This sequence corresponds to the sequence examined by Eckert and Kunkel for the pH-dependence of formation of the A-C mispair by DNA polymerase.[51,52] In general, approximately 100 mg of the free base generates 100 mg of the deoxynucleoside, which in turn generates approximately 100 mg of the protected phosphoramidite. That quantity of the phosphoramidite is sufficient to generate NMR quantities of the labeled oligonucleotide.

Following synthesis, the oligo was deblocked and cleaved from the solid support with ammonium hydroxide at 60°C overnight. The DMT-containing product oligonucleotide was purified by HPLC using a PRP polystyrene column, detritylated with 80% acetic acid at room temperature for 30 min, and again purified by HPLC using a C-18 reverse phase column.

A sample was prepared for NMR analysis of the oligonucleotide containing a unique ^{13}C-enriched adenine residue. The sample was prepared in 150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.5, and the strand concentration was 44 mM. The proton NMR spectrum, obtained with ^{13}C-decoupling is shown in **FIG. 2A**. A multitude of the expected aromatic resonances are seen in the spectrum between 10 and 5 ppm. A ^{13}C-edited spectrum[53,54] was then obtained and is shown in **FIG. 2B**. The edited spectrum was obtained, based upon a carbon-proton one-bond coupling constant of 212 Hz.[1,12] Only protons with strong coupling are obtained in the edited spectrum. As can be seen, this method allows immediate and unequivocal assignment to the H8 proton resonance of the adenine residue at position 4 of this sequence in solution.

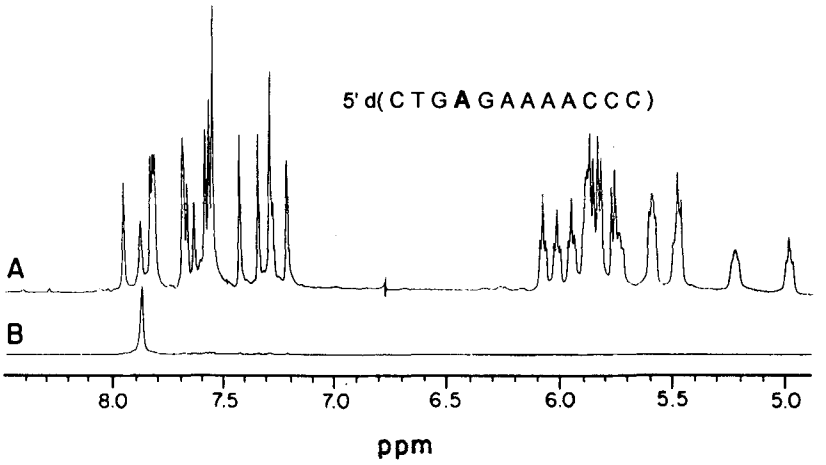


FIG. 2: A) Proton NMR spectrum of the oligonucleotide duplex containing a unique enriched adenine residue at position 4, obtained with ^{13}C-decoupling. B) Edited spectrum of the same duplex, obtained with ^{13}C-decoupling during the acquisition.

2-Aminopurine (2AP). Commercially available 2,4-diamino-5-nitropyrimidine was converted to 2,4,5-triaminopyrimidine by reduction with sodium dithionite according to Albert et al.[55] Attempts were made to convert this product to 2AP by the same methods described above for adenine. In aqueous solution, formation of the intermediate formamidopyrimidine proceeded with very low yields.

The reaction was then carried out in dimethylacetamide. The 2,4,5-triaminopyrimidine (1.0 g, 8.0 mmol) was placed in 10 mL of dimethylacetamide and 0.7 mL (20 mmol, 2 eq) of enriched formic acid was added. The vial was sealed and heated at 120°C overnight. The solvent was removed under reduced pressure and the crude product analyzed. The UV spectrum[56] and GC/MS analysis of the product indicated a high yield for the conversion to the ring closed purine. Thus, under these conditions, both formylation and ring closure occur. The product was isolated by silica gel chromatography and 351 mg of a white solid was obtained (32 % yield). Standard analytical criteria of UV, GC/MS, high resolution mass spectrometry and NMR spectroscopy were consistent with the assigned structure. The chemical shifts of the H2 and H8 protons,[25,46] ^{13}C chemical shift and ^{13}C-^{1}H coupling constant[45] are consistent with data previously obtained at natural abundance.

The 2AP free base (1.0 g, 7.4 mmol) was benzylated with benzoyl chloride in pyridine at 60° for 1 h. Thin layer analysis revealed that the reaction had gone to completion. The benzylated derivative was then used for sugar coupling by the method of Vorbruggen.[27] Following deprotection in sodium methoxide, the 2AP-riboside was purified by silica gel chromatography. A white material was obtained (1.03 g) in 52 % yield, chromatographically indistinguishable from material made by the method of Fox.[57] Conversion of unenriched 2AP riboside to the 2'-deoxynucleoside and incorporation into oligonucleotide was recently reported by our group.[33]

Purine (P). As with 2AP above, we were unable to convert 4,5-diaminopyrimidine to purine in two steps as with adenine. Alternatively we used the conditions which generated 2AP in a one step procedure. 4,5-Diaminopyrimidine (1.0 g, 9.0 mmol) was placed in a vial with 10 mL dimethylacetamide and 0.7 mL (2 eq) of ^{13}C-enriched formic acid. The vial was sealed and heated at 120°C overnight. The solvent was removed under reduced pressure and the crude product purified by silica gel chromatography. An off-white material was obtained which was analyzed by standard chemical methods including UV,[56] GC/MS, NMR and high resolution mass spec. Enriched purine (900 mg) was obtained in 83 % yield based upon 4,5-diaminopyrimidine. The chemical shifts of the H2, H6 and H8 protons, relative assignments,[58] ^{13}C chemical shift and ^{13}C-^{1}H coupling constant[45] are consistent with data previously obtained at natural abundance.

Purine (1.0 g) was converted directly to purine riboside by the method of Vorbruggen et al.[27] The product (2.0 g) was purified in 96 % yield. The product was chromatographically indistinguishable from authentic standard. The UV spectrum was consistent with the reported values[59] as was the ^{13}C spectrum.[60]

In **FIG. 3A** we present the proton NMR spectrum of commercially available unenriched nebularine. In **FIG. 3B**, we show the edited spectrum of the enriched purine riboside obtained with ^{13}C-decoupling and a delay set to observe 216 Hz coupling. In **FIG 3C**, we show the edited spectrum of the enriched purine riboside obtained with ^{13}C-decoupling and a delay set to observe 4 Hz coupling. It is shown that the edited experiments allow immediate and unambiguous assignment and identification of the purine ribonucleoside H8 and H1' protons.

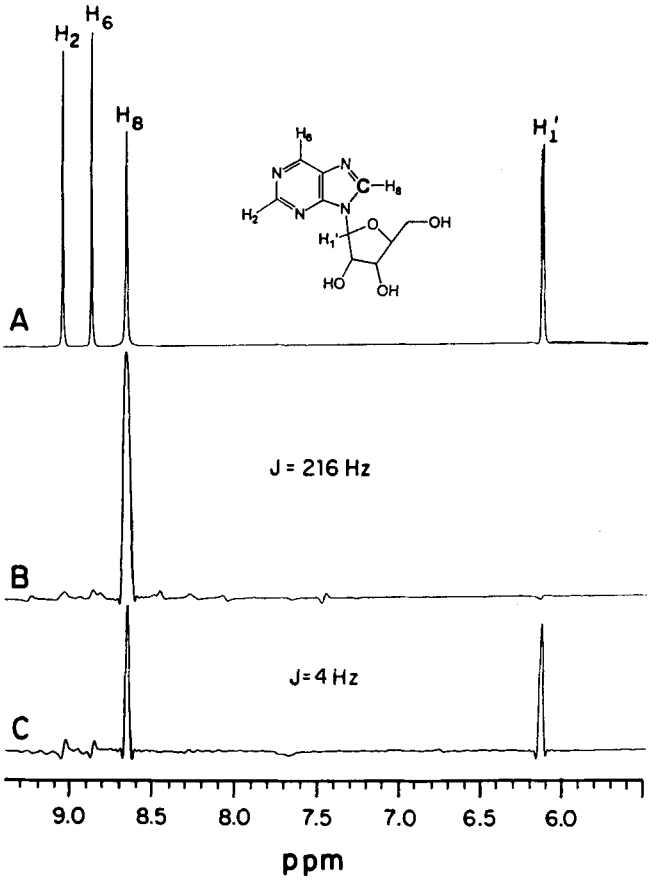


FIG. 3: A) Proton NMR spectrum of commercially available nebularine in D_2O. B) Edited spectrum of the enriched purine riboside obtained with ^{13}C-decoupling and a delay set to observe 216 Hz coupling. C) Edited spectrum of the enriched purine riboside obtained with ^{13}C-decoupling and a delay set to observe 4 Hz coupling.

CONCLUSIONS

We present here a facile synthesis of three ^{13}C-enriched purine derivatives, adenine, 2AP and purine. These derivatives have further been converted to the corresponding nucleosides. The identity of the products has been established by UV, NMR and mass spectral methods.

Heteronuclear edited NMR spectra are further presented which demonstrate that such methods allow immediate and unequivocal identification and assignment of the H8

resonance. The H8 resonance chemical shift has been valuable in other systems for identifying equilibria between structures in DNA.[29] Most aberrant base pairs studied to date by NMR methods appear as a set of structures in equilibrium with one another.[61] The labeling methods reported here should prove invaluable in investigating structures which are not easily solved by standard proton NMR methods.

ACKNOWLEDGMENTS

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