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SYNTHESIS OF [1, 3, 6, 7 - ^{15}N, 8 - ^{13}C] ADENINE

Maria CHIRIAC[*], *Damian AXENTE*[*], *Nicolae PALIBRODA*[*], *and*

Constantin T. CRAESCU[‡]

[*]INSTITUTE OF ISOTOPIC AND MOLECULAR TECHNOLOGY, 3400 CLUJ-NAPOCA,

P. O. BOX 700, ROMANIA;

[‡]INSTITUT CURIE, SECTION BIOLOGIE INSERM U-350, 91405 ORSAY, FRANCE

SUMMARY

The synthesis of [1,3,6,7-^{15}N, 8-^{13}C] adenine, using [1,3,7-^{15}N, 8-^{13}C] xanthine as starting labelled material, is presented.

The experimental procedure is an adaptation of the synthesis methods for the corresponding unlabelled compounds.

Key Words: [^{15}N, ^{13}C] adenine, synthesis, [^{15}N, ^{13}C] xanthine, $^{15}NH_3$, chlorination, ^{15}N-amination, catalytic hydrogenation.

INTRODUCTION

The heteronuclear NMR spectroscopy in the structural analysis of proteins and nucleic acids requires labelling with ^{15}N or ^{13}C. These NMR studies using oligonucleotides, specifically labelled with ^{15}N and ^{13}C, may provide valuable information regarding nucleic acid structure, drug binding, and nucleic acid-protein interaction (1,2,3).

In the last years, several groups have reported a number of syntheses of ^{15}N-labelled purine nucleosides (4).

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For the purpose of preparation of labelled nucleotides by enzymatic methods, we intend to synthesise purines labelled with ^{15}N and ^{13}C in different positions.

The first labelled purine prepared by us was [1, 3, 7 - ^{15}N, 8 - ^{13}C] xanthine, its synthesis being presented elsewhere (5).

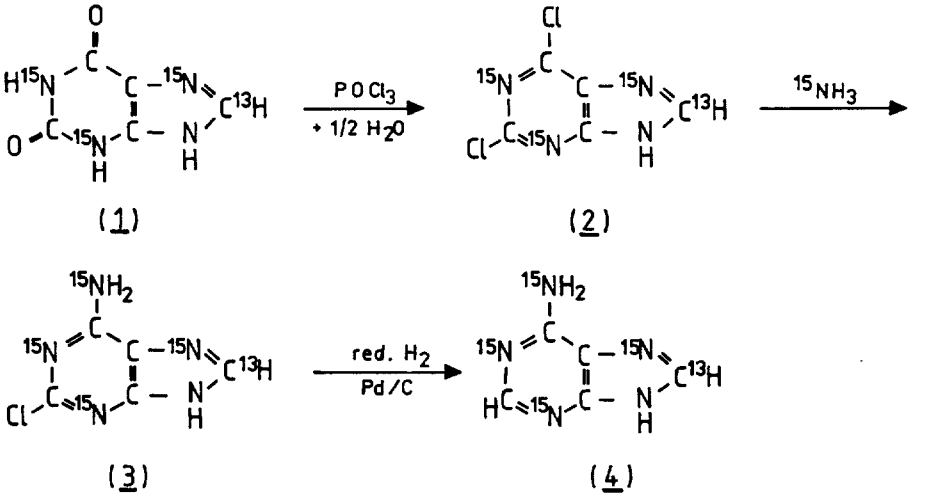
Because of the possibility to transform purines one into another (6), we prepared [1, 3, 6, 7 - ^{15}N, 8 - ^{13}C] adenine, using [1, 3, 7 - ^{15}N, 8 - ^{13}C] xanthine as starting material.

The ^{15}N-labelled compounds, $(^{15}NH_2)_2CO$, $Na^{15}NO_2$ and $^{15}NH_3$, used as starting material for the synthesis of labelled xanthine and adenine, were obtained from $H^{15}NO_3$ 99 at. % ^{15}N, produced at the Institute of Isotopic and Molecular Technology, Cluj-Napoca, Romania. For the ^{13}C labelling of xanthine, formic-^{13}C acid 99 atom % ^{13}C from Sigma, was used.

The structure of adenine was confirmed using NMR spectroscopy and mass spectrometry and the isotopic label was determined by MS on the molecular compound.

EXPERIMENTAL

[1,3,6,7-^{15}N, 8-^{13}C] adenine synthesis is presented in Scheme 1.



Scheme 1; Synthesis of [1,3,6,7-^{15}N, 8-^{13}C] adenine (4).

[1,3,7-^{15}N, 8-^{13}C] - 2,6 - Dichloropurine (2)

It is known that the chlorination of xanthine with phosphoryl chloride and trimethylamine led only to 2,6-bis-(dimethylamino)-purine (7).

The addition of dimethylaniline to phosphoryl chloride facilitated the chlorination of hydroxypyrimidines (8), uric acid (9) and hypoxanthine (10) (under reflux conditions), but this compound does not improve the chlorination of xanthine.

The addition of water to phosphoryl chloride, in an amount of a half molar equivalent, favours the formation of the reagent "pyrophosphoryl chloride", used in chlorination reactions (11). We adapted this method for the chlorination of labelled xanthine (1).

Xanthine was chlorinated at a temperature around 165^{0}C to 2,6-dichloropurine. At lower temperatures a considerable amount of xanthine was not chlorinated, while at higher temperatures a dark brown material appeared, which contained organically bound chloride, was insoluble in pyrophosphoryl chloride and seemed to be some polymer of 2,6-dichloropurine.

Preparation of "pyrophosphoryl chloride": 2.5 ml of water were added drop wise to 25 ml of phosphoryl chloride and the mixture was boiled for 1.5 hours to dispel the hydrogen chloride. Then, after cooling, the top layer was decanted from the thick syrup at the bottom and used later for the chlorination of xanthine (1).

A mixture of 160 mg xanthine [1,3,7 - ^{15}N, 8 - ^{13}C] (1) and 1.5 ml of "pyrophosphoryl chloride" was heated in a sealed glass tube at 165^{0}C for 19 hours. After cooling, the brown solution was decanted from the solid residue and the volatile material was removed under reduced pressure.

The syrupy residue was poured onto 10 g of crushed ice. A small amount of tan precipitate which appeared, was removed, and the filtrate extracted six times with ~10 ml portions of diethyl ether. After removing the solvent by distillation in vacuo, 78.5 mg of product (2) were obtained (yield = 39%).

[1,3,6,7-^{15}N, 8-^{13}C] - 2 Chloro - 6 - aminopurine (3)

We tried the ^{15}N-amination of compound (2) by J.Leonard's method (12) for the ^{15}N-amination of 6-chloropurine, but the yield was not satisfactory. Tests performed on unlabelled compounds showed that the amination produced about 50% of 2,6-diaminopurine.

By an adaptation of the classical Fischer method (13) for amination of chloropurines with ammonia in water (or alcoholic solution) we obtained good results.

The amination of (2) was made by heating 78.5 mg compound with 0.26 l of $^{15}NH_3$ in 5 ml H_2O, in a sealed glass tube at 100°C for 6 hours. Then, the reaction mixture was concentrated under reduced pressure. The residue was dissolved by heating with 5 ml of water and, after standing overnight in the refrigerator, compound (3) was filtered. We obtained 70 mg [1,3,6,7-^{15}N, 8-^{13}C]-2 chloro - 6 - aminopurine (yield = 95 %).

The unreacted $^{15}NH_3$ was recovered as $^{15}NH_4Cl$.

[1,3,6,7-^{15}N, 8-^{13}C] Adenine (4)

The dechlorination of compound (3) was performed by catalytic hydrogenation, by an adaptation of the hydrogenation method of chloropurines, described by Brederek (14).
We used a 7% palladium-charcoal catalyst prepared as follows (15): 0.5 g of charcoal were refluxed in 7 ml 10% nitric acid solution. After filtration, washing with water at neutral pH and drying at 105°C, the charcoal was added to a mixture of 2.1 g sodium acetate in 7 ml water, and 37 mg $PdCl_2$ in 3.5ml 0.1N HCl.

The hydrogenation of the palladium catalyst was made at atmospheric pressure and room temperature, the solution being magnetically stirred. The catalyst was washed with distilled water, taking precautions to keep the catalyst all the time under water, and finally added to compound (3) dissolved in a sodium hydroxide solution (160 mg NaOH in 30 ml H_2O). The hydrogenation of compound (3) was made at atmospheric pressure and 30°C, under magnetical stirring. The filtrate obtained by removing the catalyst, was neutralised with 2N HCl. After cooling, compound (4) precipitated.

We obtained 50 mg of [1,3,6,7-^{15}N, 8-^{13}C] adenine (yield = 89 %).

The structure of adenine -^{15}N, ^{13}C synthesised by us, was confirmed by mass spectrometry and by NMR analysis.

The electron impact mass spectra were recorded using a double focusing mass spectrometer type MAT-311 (Varian MAT, Germany). The samples, representing raw or purified products of synthesis steps, performed on labelled or unlabelled material, were introduced using the direct inlet probe. Some 50 spectra were recorded with temperature programming from room temperature to 260°C for each run, using repetitive scans under computer control over the mass range 25-350, to identify compounds (1) - (4) as well as minor impurities. The mass spectra of the labelled compounds were compared with the mass spectra of the corresponding unlabelled compounds, for the intermediates and the final product along all synthesis steps. All mass spectra showed intense molecular ions, e.g. at m/z=135 for natural adenine and m/z=140 for the labelled adenine.

The isotope shift observed in the molecular ions of adenine is well correlated to the ^{15}N and ^{13}C label expected from synthesis. Mass shifts were also observed in some fragment ions, containing the label atoms.

Figure 1 shows the mass spectra of a standard adenine sample with the natural isotope composition (a) and the synthesised adenine containing $^{15}N_4{}^{13}C$ (b). The molecular ions M=135 and M=140 confirm by the shift of five mass units the $^{15}N_4$ and ^{13}C label.

All fragment ions of unlabelled adenine are found in the mass spectrum of adenine - ^{15}N, ^{13}C (b), shifted by a number of mass units equal to the number of ^{15}N and ^{13}C atoms of each fragment ion. The main fragment ion for instance, M-HCN, is M-27 in the unlabelled and M-28 in the labelled compound.

The final evidence of the nature of the reaction product was obtained by NMR analysis. The NMR spectra were obtained on a Varian Unity 500 spectrometer equipped with a tri-nuclear 5 mm probe, at 25°C. The sample (1mg/ml) was prepared in ^2H_2O (99.97%) or ^1H_2O containing 5% ^2H_2O. For the 2D (^1H-^{13}C) HMQC experiment we used the basic sequence proposed by Bax et al. (17).

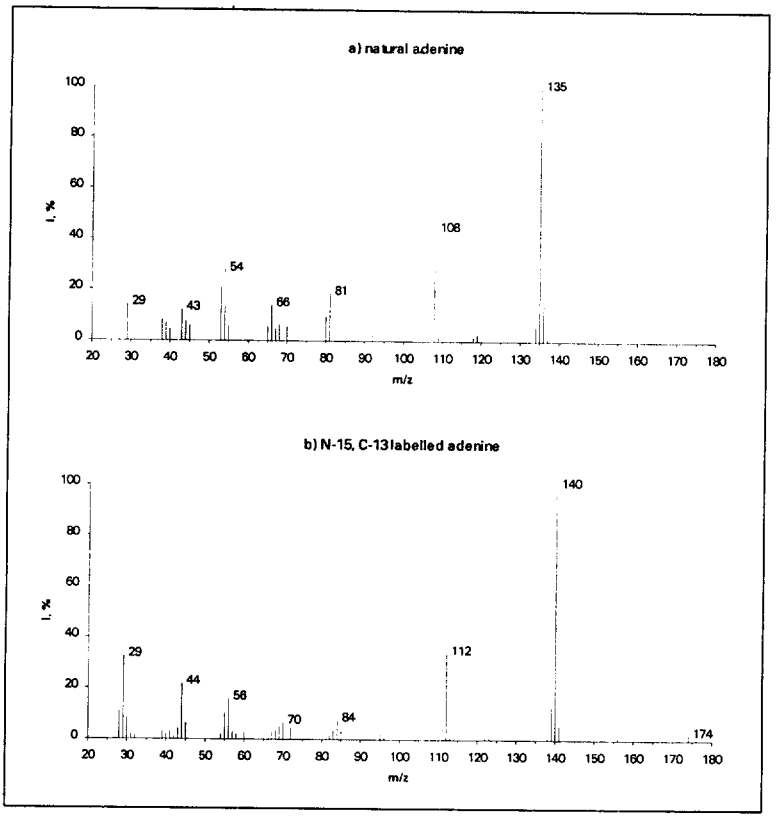


Fig. 1 EI mass spectra of (a) unlabelled adenine and (b) $^{15}N_4{}^{13}C$ labelled adenine.

Figure 2 shows a series of 1D proton NMR spectra and a portion of the (^{1}H-^{13}C) HMQC 2D spectrum of the labelled adenine in ^2H_2O. Comparison of non-decoupled 1D spectrum with those obtained under ^{13}C or ^{15}N decoupling enabled us to identify the carbon-bound protons and to verify the isotope labelling. Thus, the ^{13}C8-H proton (8.21 ppm) is readily identified from the 2D HMQC spectrum and from its large coupling ($^1J_{H\text{-}C}$ = 212.6 Hz) with ^{13}C, which could be removed by broadband decoupling. Its resonance is additionally splitted by the two-bond spin-spin coupling with ^{15}N7 ($^2J_{H\text{-}N}$ = 10.6 Hz). The ^{12}C2-bound proton, resonating at a slightly lower field (8.26 ppm), is a triplet generated by the spin-spin magnetic interaction with the two neighboring ^{15}N nuclei, as indicated by ^{15}N -decoupled experiment.

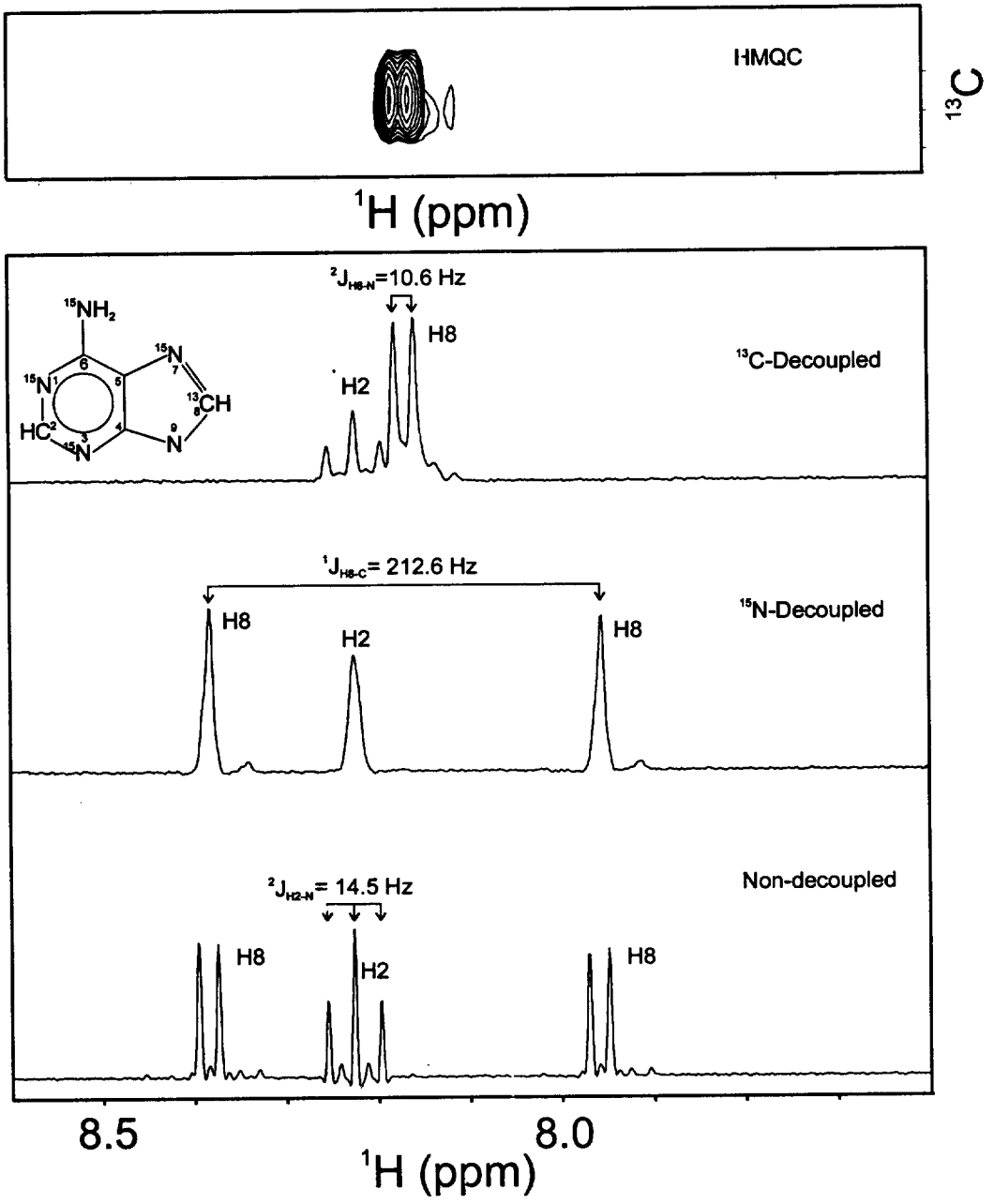


Fig.2 Proton 1D NMR spectra in absence or presence of heteronuclear decoupling (^{15}N or ^{13}C) of the labelled adenine (1 mg/ml) in $^{2}H_2O$, pH 6.7 at 25 °C. The top of the figure shows a portion of the (^{13}C - ^{1}H)-HMQC spectrum containing the ^{13}C8 - ^{1}H cross-peak. Spin-spin heteronuclear coupling constant values are also indicated in the figure.

Taken together, the present mass spectroscopy and NMR results provide an unambiguous structure confirmation and characterization of the enrichment of the adenine molecule.

The total isotope label was evaluated based on the molecular ions m/z 140, containing all five labelled atoms, and m/z 139 containing only four labelled atoms in the five label positions. The intensity ratio 0.11 of the ions m/z 139 and m/z 140, obtained in the labelled compound, showed an overall label of about 98 labelled atoms in 100 atoms of the label positions, which correlates well with the 99 at. % ^{15}N of the starting material and with the 99 at. % ^{13}C of the formic acid used in the synthesis of xanthine (5).

The amount of volatile organic impurities was estimated using the intensity areas of the molecular ions during sublimation in the high vacuum. The precursor 2-chloro-adenine-($^{15}N_4{}^{13}C$), M=174, and the byproduct 2-amino-adenine-($^{15}N_5{}^{13}C$), M=156, were found to be present in amounts of 5.2 and respectively 1.2 % of intensity area.

Further purification of the product by ion exchange, as recommended by R.Kunin (16), was not considered necessary, due to the high selectivity of the enzymatic syntheses to follow, using the labelled adenine.

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