Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 6, No. 10, pp. 1179-1182, 1996

0960-894X/96 $15.00 + 0.00

PII: S0960-894X(96)00194-1

Site-specific ^{15}N-labelling of Adenine in DNA for NMR Studies

Yao-Zhong Xu§*, Vasudevan Ramesh+ and Peter F. Swann§

§CRC Nitrosamine-induced Cancer Research Group, Department of Biochemistry and Molecular Biology University College London, London, UK and
+Department of Biochemistry and Biological NMR Centre, University of Leicester, Leicester, UK
** fax nr : + 44-171-380-7193 E-mail nr : UCLCYZX@UCL.AC.UK*

Abstract: a simple and efficient protocol is described for site-specific ^{15}N-labelling of adenine in synthetic oligodeoxynucleotides by post-synthetic substitution with ^{15}N-labelled ammonia of the oxidized methylthio group on purine. A labelled DNA of 20-mer was characterized by NMR spectroscopy.

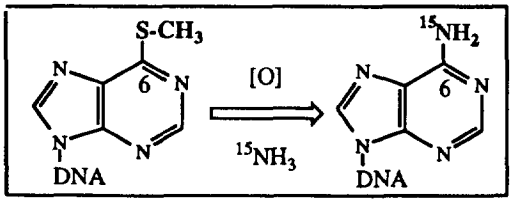
In recent years, the labelling of nucleic acids with stable isotopes (^{13}C, ^{15}N) has become an important and rapidly developing methodology to characterize the structure and interactions of biologically significant structural motifs of DNA and more recently of RNA. Combined with multidimensional NMR techniques[1] isotopic labelling offers a powerful approach to elucidate the three dimensional structure and dynamics of nucleic acids[2]. In addition to the direct effect of achieving spectral simplification, incorporation of isotopic labels permits the measurement of heteronuclear scalar coupling constants[3], which can be used as additional torsional angle geometrical constraints in the structure computing algorithms.

At the present, isotopic (^{13}C,^{15}N) labelling for NMR of nucleic acids is generally accomplished in two ways i.e. enzymatic and chemical synthesis. In enzymatic synthesis DNA templates and the enzyme T7 RNA polymerase have been used successfully to make labelled RNA from pre-labelled nucleoside triphosphates[4]. Very recently, using an analogous approach, uniform labelling (^{13}C,^{15}N) of DNA has been demonstrated[5]. The main drawback of the enzymatic approach is that incorporation of the isotopic label is done in an uniform and non-specific manner, covering the whole molecule and hence site-specific structural information cannot be achieved. In chemical synthesis labelled monomers are used as *precursors* in DNA synthesis by automated synthesizers (reviewed in ref. 6). This method provides an opportunity to probe the specificity of interaction of the labelled DNA with a target molecule, such as a protein or drug, by varying the site of labelling. Currently, a great deal of effort has been devoted to develop synthetic methods for preparation of ^{15}N-labelled bases or nucleosides (nucleotides) for their subsequent incorporation into nucleic acids[6]. However, this approach is inefficient in terms of utilisation of isotopically labelled monomers, prepared by multi-step synthesis, although attempts to recover the monomers have been made[7].

An alternative and a more efficient approach is to first synthesize chemically the DNA of the desired sequence and then, in the next step, *selectively* convert the chosen base into a labelled one. The significance of this *post-synthetic* approach is that the use of the isotopic agent is only made at the very last step. Recently, we have demonstrated this approach by incorporating a ^{15}N-label on the exocyclic 4-amino group of a cytosine in DNA and have observed the interaction of the site-specifically labelled *trp* operator with *E.coli trp* repressor protein by 2D-NMR spectroscopy[8]. As part of our ongoing effort to develop methods for site-specific labelling of exocyclic amino group of bases in synthetic DNA and RNA, we want to develop an efficient route to synthesis of DNA containing ^{15}N-labelled adenine and its characterization by NMR.

The major challenge in the post-synthetic, site-specific labelling of bases, including adenine, lies not only in the incorporation of suitable groups at a pre-determined site in DNA assembly and their stability to nucleophilic deprotection, but also in their replacement under mild conditions. Previously, 6-halopurines[9] and 6-phenoxypurine[10] have been employed as the precursor to 6-substituted adenines. However, it has been concluded in a recent paper[11] that "6-chloro and 6-fluoropurine derivatives were not efficient for producing oligonucleotides with 6-^{15}N-adenine probably due to depurination during the removal of the DMT". On the other hand, the reported 6-pentafluorophenoxypurine[11] is an improved variety of 6-phenoxypurine, but, its substitution even at the nucleoside level still needs severe treatment (16 hrs at 60°C with 3.3 M aqueous ammonia). We have previously reported that 6-dinitrophenylthiopurine[12] in synthetic DNA could be converted into a variety of 6-substituted adenine. This provides a possibility for the preparation of DNA containing 6-^{15}N-labelled adenine when ^{15}N-NH_3 is used as the substituting and deprotecting agent. However, the formation of 6-substituted purine analogues by this approach is sometimes accompanied by a small amount of unwanted 6-thiopurine analogues[12]. Recently, we have reported the synthesis of phosphoramidite of 6-methylthiopurine-2'-deoxynucleoside and its incorporation into DNA[13]. The methylthio group on the purine is stable towards concentrated ammonia at room temperature[13], but could be oxidized to become a good leaving group[14].

In this letter we present our work on site-specific ^{15}N-labelling of adenine in DNA for NMR studies. 6-methylthiopurine was used as the precursor to ^{15}N-labelled adenine as shown in **Scheme 1.** As a trial, a pentamer of the sequence, CGXAT (X: 6-methylthiopurine), was chemically synthesized. The modified pentamer was treated with very diluted m-chloroperoxybenzoic acid (MCPBA) in CH_3CN for a short time to selectively oxidize the methylthio group, then treated with concentrated ammonia at RT to substitute for the oxidized methylthio (i.e methylsulphonyl) group, to cleave the pentamer from the CPG support and to remove all base-labile protecting groups[15]. Nucleoside composition analysis (**Figure 1**) of the main product confirmed that 6-methylthiopurine in the pentamer was successfully transformed into adenine and other bases were not affected. We also observed that the oxidized methylthio group on purine in the pentamer could be quantitatively replaced with methylamine to form N^6-methyladenine. It has been noted that the oxidized methylthio group, on the 6-position of 9-β-D-ribofuranosylpurine was extremely labile, which when treated with ammonia in water gave only hypoxanthine, the hydrolysed product[14a]. However, in our experiments, the oxidized methylthio on the purine in deoxyoligonucleotides could be readily converted into the desired adenine with very little formation of hypoxanthine using concentrated aqueous ammonia.



Scheme 1 (above): Post-synthetic transformation of methylthiopurine into 6-^{15}N-adenine in DNA.

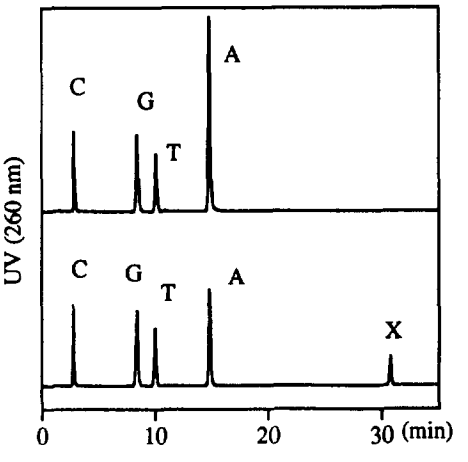


Figure 1 (right): HPLC profiles of nucleoside composition analysis of the pentamer containing 6-methylthiopurine (bottom part) and of the transformed pentamer with MCPBA-oxidation and NH_3-substitution (top part).

The obvious explanation for the formation of the pentamer containing hypoxanthine is the presence of water (see note 16). Further experiments[16] indicated that the substitution at the 6-position of the modified purine in the pentamer is quicker than the deprotection of the oligomer. Therefore, to reduce the formation of the hydrolysed product and to make full use of the isotopic reagent, $^{15}NH_3$, the substitution and deprotection steps should be done separately. One possible protocol is to use $^{15}NH_3$ in an aprotic solvent (such as CH_3CN or THF) as the substituting agent, followed by unlabelled concentrated aqueous ammonia as the deprotecting agent.

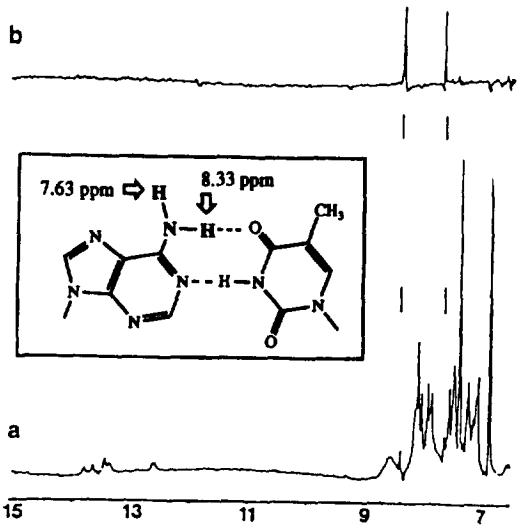


Figure 2: 600 MHz 1-D ^{1}H-NMR spectra of a) unlabelled 20-mer synthetic *trp* operator oligonucleotide b) ^{15}N-edited ^{1}H-NMR spectrum of site-specifically ^{15}N-labelled adenine residue of the same oligonucleotide.

As the byproduct, the oligomer containing hypoxanthine is only a very low percentage of the total and also is not ^{15}N-labelled, its presence is likely to be well within the tolerable limits for ^{15}N-NMR measurement of the ^{15}N-labelled adenine oligomer. Therefore, we have made a self complementary oligomer (belonging to the *trp* operator sequence[8]), d 5'-CGAΔCTAGTTAACTAGTTCG-3' (the underlined A is 6-^{15}N-adenine), by the post-synthetic transformation (see the experimental part). The successful transformation of the 20 mer has also be confirmed by nucleoside composition analysis. The duplex formed by annealing this labelled DNA has been characterized by NMR spectroscopy. **Figure 2** shows a comparison of the spectra of (a) unlabelled synthetic oligomer (20-mer) with (b) the site-specifically labelled ^{15}N-adenine oligomer of the same sequence. The spectral simplification achieved is clearly evident in spectrum b. The two well resolved resonances appearing at 8.33 ppm and 7.63 ppm in the ^{15}N-edited spectrum (b) correspond to the two protons attached to the ^{15}N-label of the 6-amino group of the labelled adenine in the DNA. The chemical shifts of these two resonances are characteristic of the amino group protons of the adenine residues in B-DNA type conformation and thus facilitate their assignment in an unambiguous manner. It can be observed that the low-field resonance at 8.33 ppm has a greater linewidth due to its involvement in Watson-Crick hydrogen bonding.

In conclusion, the work presented here illustrates a simple and efficient preparation of DNA containing site-specifically ^{15}N-labelled adenine for NMR studies. The same approach may possibly also be used for site-specific labelling of guanine at its 2-position. Furthermore, as 6-methylthio group on purine in DNA is stable towards conc. ammonia at RT[13] and its oxidized form(s) easily replaceable, these combined properties present a promising route to prepare DNA containing special groups (eg. radioactive atoms, carcinogenic adducts) by oxidizing and substituting fully deprotected and purified DNA bearing 6-methylthiopurine residues with a minimum amount of required hazardous agents at the very last step.

EXPERIMENTAL

General Methods: Syntheses of oligomers were carried out by ABI 391 DNA synthesizer (Applied Biosystems), using Expedite monomers and supports, of which the amino groups on the bases are protected with t-butylphenoxyacetyl group (Millipore). General methods such as purification with Nensorb Prep cartridges

(Du Pont) and nucleoside composition analysis by reversed phase HPLC were carried out as described before[12]. **Sample preparation**: A typical preparation of 6-^{15}N-labelled adenine oligomer is as follows: the CPG-support bearing oligomer containing 6-methylthiopurine with its 5'-end protected by the DMT group was prepared as described before[13]. Then the synthetic oligomer was treated with 10 ml of 1% MCPBA (Sigma) in CH_3CN for 5 minutes at room temperature, followed by thoroughly washing with CH_3CN. The resultant oligomer was converted into ^{15}N-labelled adenine oligomer and deprotected by 2 ml of ammonium hydroxide [6.4 N aqueous solution, 99.5 atom % ^{15}N (Isotech, USA)] at room temperature for 3 days. The oligomer was then purified with Nensorb cartridge and freeze-dried. Prior to NMR measurements the labelled oligomer was heated to 85°C for about 5 minutes and then very slowly cooled down to about 5°C (overnight) to ensure duplex formation. **NMR spectroscopy**: NMR spectra were recorded using a Bruker AMX 600 spectrometer operating at a ^{1}H-frequency of 600 MHz. Spectra were measured at 300K with a spectral width of 13.2 kHz and with the carrier frequency positioned on the water resonance. The intense signal arising from the water protons was minimised by low power gated irradiation during relaxation delay (1. 25 s) between successive transients. One dimensional ^{15}N-edited ^{1}H-NMR spectrum was measured with a double quantum delay set to 1/2J = 5.6 ms, $^1J_{N\text{-}H}$ = 94 Hz. The GARP composite pulse sequence was employed for ^{15}N-decoupling before data acquisition.

ACKNOWLEDGEMENT: This work is supported by the Cancer Research Campaign. We thank Prof. G.C. K. Roberts for providing access to the 600 MHz NMR spectrometer and Dr. Q. Zheng for discussion.

REFERENCES AND NOTES

1. Farmer, B.T.; Mueller, L.; Nikonowicz, E. and Pardi, A. *J. Biomol. NMR.* **1994** *4*, 129 - 133.
2. Moore, P.B. (1995) *Acc. Chem. Res.* 28, 251 - 256.
3. Eberstadt, M.; Gemmecker, G.; Mierke, D.F. and Kessler, H. *Angew. Chem.* **1995** *34*, 1671 - 1695.
4. a) Milligan, J.F.; Groebe, D.R.; Wetherell, G.W. and Uhlenbeck, O.C. *Nucl. Acids. Res.* **198715**, 8783 - 8798; b) Batey, R.T.; Inada, M.; Kujawinski, E.; Puglisis, J.D. and Williamson, J.R. *Nucl. Acids. Res.* **1992** *20*, 4515 - 4523; c) Nikonowicz, E.; Sirr, A.; Legault, P.; Jucker, F.M.; Baer, L.M. and Pardi, A. *Nucl. Acids. Res.* **1992** *20*, 4507 - 4713.
5. Zimmer, D.P. and Crothers, D.M. *Proc. Natl. Acad. Sci. (USA)* **1995** *92*, 3091 -3095.
6. Jones, R.A. in *Protocols for oligonucleotide conjugates - synthesis and analytical techniques* Ed. by Agrawal, S. **1994** 207 - 231 (and references therein) Humana Press, Totowa, New Jersey.
7. Gao, X.; Gaffney, B.L. and Jones, R.A. *Tetrahedron Lett.* **1991** *32*, 5477 - 5480.
8. Ramesh, V.; Xu, Y.-Z. and Roberts, G.C.K. (1995) *FEBS Lett.* **1995** *363*, 61 - 64.
9. a) Harris, C.M.; Zhou, L.; Strand, E.A.and Harris, T.M. *J. Am. Chem. Soc.* **1991** *113*, 4328-4329; b) Kim, S.J.; Stone, M.P; Harris, C.M. and Harris, T.M.*J. Am. Chem. Soc.* **1992** *114*, 5480-5481; c) Kim, S.J.; Jajoo, H.K.; Kim, H.Y.; Zhou, L.; Horton, P.; Harris, C.M. and Harris,T.M. *Bioorganic & Medical Chemistry* **1995** *3* 881-822.
10. a) Ferentz, A.E. and Verdine, G.L. *J. Am. Chem. Soc.* **1991** *113*, 4000 - 4002; b) Ferentz, A.E. and Verdine, G.L. *Nucleosides and Nucleotides* **1992***1*, 1749 - 1763.
11. Acedo, M.; Fabrega, C.; Avino, A.; Goodman, M.; Fagan, P.; Wemmer, D. and Eritja, R. *Nucl. Acids. Res.* **1994** *22*, 2982 - 2989.
12. a) Xu, Y.-Z.; Zheng, Q. and Swann, P.F.*Tetrahedron* **1992** *48*, 1729 - 1740; b) Xu, Y.-Z.; Zheng, Q. and Swann, P.F.*Tetrahedron Lett.* **1992** *33* , 5837 - 5840.
13. The work was presented as a poster at 11th International Round Table Nucleosides, Nucleotides and Their Biological Applications in Leuven Belgium in Sept. 1994 (see, Xu, Y.-Z.; Zheng, Q. and Swann, P.F. *Nucleosides and Nucleotides* **1995** *14*, 929 - 932).
14. Early work on utilization of oxidized methylthiopurine in nucleosides: a) Wetzel, R. and Eckstein, F. *J. Org. Chem.* **1975** *40*, 658 - 660; b) Seela, F.; Herdering, W. and Kehne, A. *Helv. Chim. Acta* **1987** *70* 1649-1660. On the oligonucleotide level, the oxidized methylthiopurine was used as a precursor to N6,N6-ethynoadenine in a polythymine [c) Matteucci, M.D. and Webb, T.R. *Tetrahedron Lett.* **1987** *28* 2469-2472] and to 6-pyridium-purine [d) Burdzy, A.; Skalsi, B.; Biala, E.; Kowalewski, A.; Paszyc, S. and Adamiak *Nucleosides and nucleotides* **1995** *14* 979-982.
15. The pentamer containing 6-methylthiopurine was treated with 5 ml of 1% MCPBA for 5 min and then with conc. aqueous ammonia at RT for 3 days. The transformed pentamer (containing adenine) was obtained in high yield, virtually the same as that of normal pentamer, and confirmed by nucleoside composition analysis.
16. The substitution of the oxidized pentamer with 2 M ammonia in methanol at RT overnight gave oligomer containing 6-methoxypurine as the major product with no (or little) amount of hydrolysed product when conc. aqueous ammonia (or 0.5 M NaOH) was employed at RT for 2 days for deprotection. However, the treatment with 0.5 M NaOH at RT for 3 days for the substitution and deprotection gave the hydrolysed product only. These experiments suggested the substitution is faster than the deprotection.

(*Received in Belgium* 22 *January* 1996; *accepted* 22 *April* 1996)