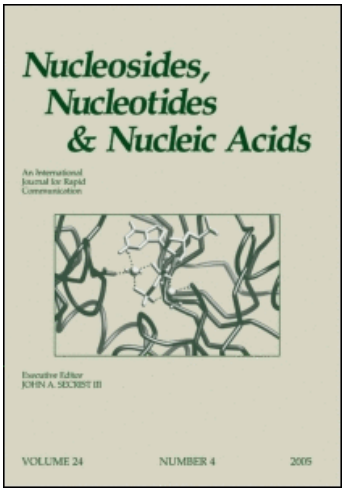


**Nucleosides, Nucleotides and Nucleic Acids**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

# Use of Both Direct and Indirect $^{13}C$ Tags for Probing Nitrogen Interactions in Hairpin Ribozyme Models by $^{15}N$ NMR

Anthony J. Shallop[a]; Barbara L. Gaffney[a]; Roger A. Jones[a]
[a] Department of Chemistry and Chemical Biology, Rutgers, The State University of New Jersey, Piscataway, New Jersey, USA

Online publication date: 02 October 2004

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS
Vol. 23, Nos. 1 & 2, pp. 273–280, 2004

# Use of Both Direct and Indirect $^{13}C$ Tags for Probing Nitrogen Interactions in Hairpin Ribozyme Models by $^{15}N$ NMR†

**Anthony J. Shallop, Barbara L. Gaffney, and Roger A. Jones***

Department of Chemistry and Chemical Biology, Rutgers, The State University of New Jersey, Piscataway, New Jersey, USA



## ABSTRACT

We have used the synthesis and $^{15}N$ NMR study of separate loop A and loop B domains of the hairpin ribozyme to demonstrate that multiple $^{15}N$ atoms can be incorporated into an RNA strand and be unambiguously distinguished through a combination of direct and indirect tagging by $^{13}C$ atoms. Absence of $^{15}N$ chemical shift changes shows that the G8N1 in loop A does not become deprotonated up to pH 8, and that the G21N7 of loop B does not bind to $Mg^{2+}$.

*Key Words:* Hairpin ribozyme; $^{15}N$ NMR; $^{13}C$ tag; Specific labeling.

## INTRODUCTION

$^{15}N$ NMR has proven to be a useful, non-perturbing tool for probing effects at nitrogen atoms in DNA and RNA. Changes in $^{15}N$ chemical shifts can report on several kinds of interactions, such as hydrogen bonding, protonation, metal binding and stacking, without introducing any modifications that could alter the structure.[1–10]

†In honor and celebration of the 70th birthday of Professor Leroy B. Townsend.

*Correspondence: Roger A. Jones, Department of Chemistry and Chemical Biology, Rutgers, The State University of New Jersey, Piscataway, NJ 08854, USA; E-mail: jones@rutchem.rutgers.edu.



DOI: 10.1081/NCN-120027834 1525-7770 (Print); 1532-2335 (Online)

Although the $^{15}N$ nucleus has a spin of 1/2 and gives straightforward NMR spectra,[11] its low natural abundance and low sensitivity require significant enrichment to be of practical use. Since uniform labeling usually leads to assignment difficulties, chemical labeling at specific sites in the nucleosides is desirable for this application. In recent years, we have reported high-yield methods to specifically incorporate $^{15}N$ into several sites at the same time, as well as $^{13}C$ to differentiate similar $^{15}N$ resonances.[12–16] A $^{13}C$ incorporated at the purine C2 acts as a direct tag for adjacent nitrogens, because of the substantial couplings (6–23 Hz).[12,16] In contrast, the very small C8–N7 coupling (<1 Hz) makes $^{13}C$ at the purine C8 more convenient as an indirect tag by taking advantage of the large C8–H8 coupling (213 Hz).[15] To demonstrate the incorporation and study of six different $^{15}N$ atoms in two nucleosides of one RNA strand, using both direct and indirect tagging by $^{13}C$, we report here our results for a model of the loop A domain of the hairpin ribozyme.



## RESULTS AND DISCUSSION

The hairpin ribozyme is found in the satellite RNA of tobacco ringspot virus.[17–19] This ribozyme encompasses a four-way helical junction, and it is the close interaction of non-Watson–Crick paired regions in two of the arms (A and B) that is primarily responsible for catalysis.[20] Loop A contains the cleavage site, and loop B is the catalytic unit. Metal ions are required for activity, but help to form and maintain the correct tertiary structure rather than participate in the catalytic step.[21,22] NMR structures of the separate loop A and B domains have been reported,[23,24] as well as X-ray crystal structures of the full hairpin ribozyme.[25,26] Comparison of these structures shows that significant conformational changes take place upon docking of the A and B domains.

We have synthesized two 14mer RNA strands that form the symmetrical loop A domain, with [2-$^{13}$C-1,7,NH$_2$-$^{15}N_3$]-guanosine at G8 and [8-$^{13}$C-1,7,NH$_2$-$^{15}N_3$]-adenosine at A9 (Figure 1). Resonances for the two amino groups can be distinguished, because that for A9 is a singlet, while that for G8 is split to a doublet (J = 24 Hz) by the $^{13}C2$ tag. The two N1 resonances can be distinguished by their markedly different chemical shifts (AN1 $\sim$ 223 ppm, GN1 $\sim$ 145 ppm). Although the $^{13}C8$-$^{15}N7$ coupling in A9 is too small to observe directly in 1 D $^{15}N$ NMR spectra (J < 1 Hz), the $^{13}C$ still serves as an indirect tag. In a $^{1}H$-$^{15}N$ HSQC NMR spectrum of the labeled strand (Figure 2), the H8,N7 crosspeak of G8 is unsplit, while the H8,N7 crosspeak of A9 is split by 213 Hz along the $^{1}H$ dimension, due to the C8–H8 coupling.

The unlabeled strand of the loop A domain was titrated into the labeled strand in thirds, and $^{15}N$ spectra at each step clearly show the progression from single to double strand, since chemical shifts for A9N7, G8N7, and A9N1 differ in the two forms

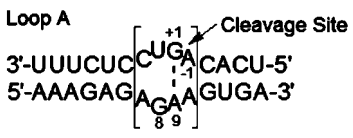


***Figure 1.*** Loop A domain synthesized with [2-$^{13}$C-1,7,NH$_2$-$^{15}N_3$]-guanosine at G8 and [8-$^{13}$C-1,7,NH$_2$-$^{15}N_3$]-adenosine at A9.

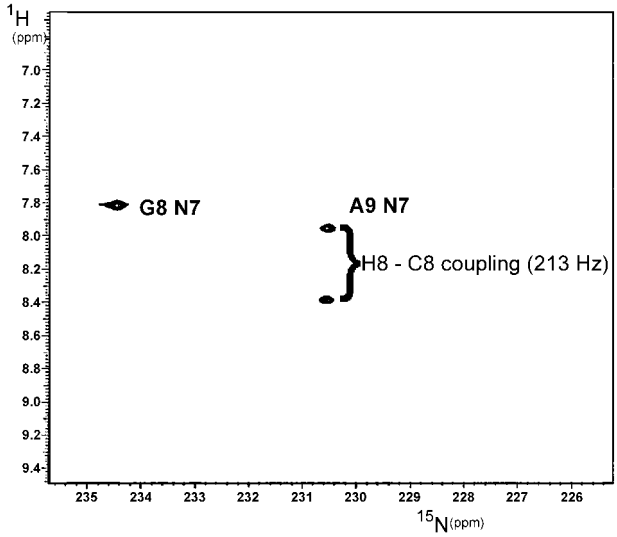


***Figure 2.*** $^{1}H$-$^{15}N$ HSQC NMR spectrum displaying the H8,N7 crosspeak of the loop A domain A9 that is split by 213 Hz along the $^{1}H$ dimension due to H8–C8 coupling, and that for G8 that is unsplit.

by 1–2 ppm (data not shown). This result indicates that the single strand and duplex are in slow exchange at 30.4 MHz. Figure 3 shows all six $^{15}N$ resonances for both forms.

G8 is in a non-Watson–Crick paired region of the loop A domain. Its N1H is close to the active site and is thought to participate in the cleavage step.[26,27] Since guanosine N1 normally has a pKa of 9.4,[28] the G8N1 would have to have a lowered pKa if it were to serve as an effective general acid/base catalyst at physiological conditions. Examples of such altered pKa's of nucleosides in very specific environments in RNA have been increasingly documented.[29–33] If the G8N1 in fact gives up its proton to any significant extent, its $^{15}N$ chemical shift should move downfield accordingly.[34] Although this process no doubt requires the docked complex of A and B, we wished to define the $^{15}N$ chemical shift behavior of the N1 of G8 in the isolated loop A domain. Figure 4 shows the $^{15}N$ NMR chemical shift of the G8N1 in the loop A duplex (closed squares) as a function of pH from 6 to 8, along with corresponding data for the G8N1 of the labeled loop A single strand (open squares) from pH 5.5 to 8. We did not go to a higher pH to avoid degradation. The superposition of the data shows that the duplex and the single strand have the same pH dependence in this range, indicating that the structure present in the isolated loop A domain does not lower the pKa of the G8N1.

In addition to the loop A domain, we also synthesized a model of the loop B domain, containing [8-$^{13}$C-1,7,$NH_2$-$^{15}N_3$]-guanosine at G21 (Figure 5), since it is a suspected metal binding site. Titration of the unlabeled strand into the labeled one again showed the progression from single strand to duplex (data not shown). In this case, the duplex signal is distinctly broader than that of the single strand, consistent with Feigon's observation that the isolated loop B domain is unusually dynamic and flexible.[35] Addition of 4 and 8 equivalents of $Mg^{2+}$ progressively increased the





MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

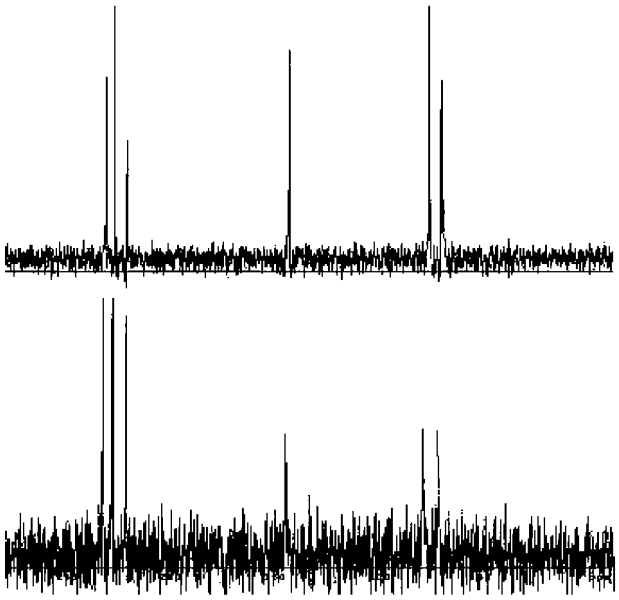

***Figure 3.*** NMR spectra of the labeled loop A domain single strand (top) and duplex (bottom) at 30°C. (*View this art in color at www.dekker.com.*)

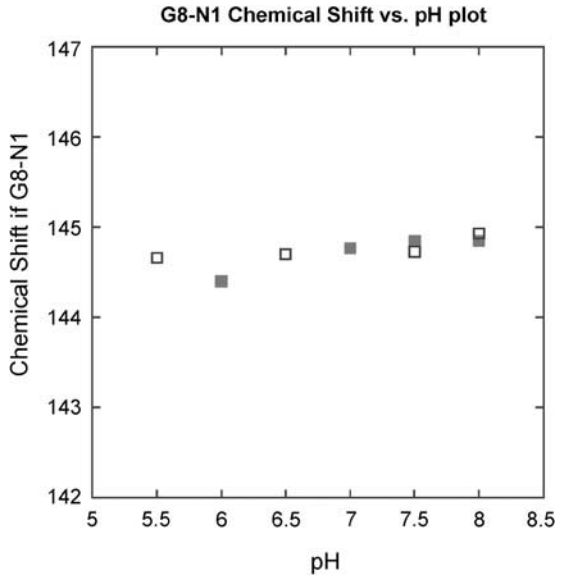


***Figure 4.*** pH titration showing $^{15}N$ NMR chemical shifts for the G8N1 in the loop A domain duplex (closed squares) and in the loop A domain labeled single strand (open squares) at 30°C. (*View this art in color at www.dekker.com.*)





Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

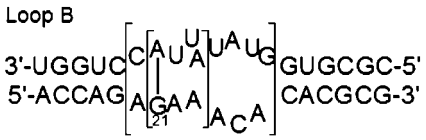


***Figure 5.*** Loop B domain synthesized with [8-$^{13}$C-1,7,$NH_2$-$^{15}N_3$]-guanosine at G21.

broadness of the signal (data not shown), but did not cause any upfield change, which would be expected for metal binding to the N7.[36] This finding shows that $Mg^{2+}$ has no significant binding to the G21N7, presumably because it is a hard metal and preferentially binds to oxygen.

## CONCLUSION

The results reported here demonstrate that at least six $^{15}N$ atoms can be incorporated into an RNA strand and be unambiguously distinguished through the use of both direct and indirect tagging by $^{13}C$ atoms. The behavior of their chemical shifts can be used to probe the presence or absence of local interactions like deprotonation and metal binding, without introducing a perturbing change. In the work described here, G8N1 in the loop A domain did not display any signs of deprotonation up to pH 8, and G21N7 in the loop B domain did not show any binding to $Mg^{2+}$.

## EXPERIMENTAL

The $^{13}C$,$^{15}N$ labeled nucleosides were synthesized[15] and protected[37] as described previously, and their phosphoramidites were made by standard methods using 2-cyanoethyl tetraisopropylphosphorodiamidite[38] and pyridinium trifluoroacetate.[39] The oligonucleotides were synthesized on controlled pore glass supports using a Pharmacia OligoPilot II synthesizer at scales of 20–60 μmol using standard phosphoramidite chemistry with either 0.5 M tetrazole or 1.0 M dicyanoimidazole[40] as an activator. The RNA was partially deprotected and removed from the support with aqueous methylamine at 65° for 10 minutes and then desilylated with $Et_3N \cdot HF$/NMP/$Et_3N$ at 65° for 2 hours.[41] Excess fluoride was scavenged with trimethylsilyl isopropyl ether.[42] The crude RNA was dissolved in 0.1 M TEAA and purified by reversed phase chromatography, first with the 5′-dimethoxytrityl group on, and again after detritylation. The pure RNA was desalted by reversed phase chromatography using 0.1 M $NH_4HCO_3$, and then converted to the sodium form by cation exchange. The identities of the oligonucleotides were confirmed using ESI-MS.

NMR samples were 300 μL and contained 150 mM NaCl and 20 mM 4-(2-hydroxyethyl)-1 piperazineethanesulfonic acid (HEPES) in 90% $H_2O$/10%$D_2O$. They were placed in Shegemi tubes after the pH was adjusted to 6.5–6.8, unless otherwise noted. The loop A sample was 6.2 mM total strand concentration and the loop B sample was 5.4 mM. Proton-decoupled 1D $^{15}N$ NMR spectra were acquired on a Varian

Oxford-300 NMR spectrometer at 30.4 MHz for about 14 hours and are reported relative to $NH_3$ using external 1 M [$^{15}$N]-urea in DMSO at 25° at 77.0 ppm as a reference.[43] Small volumes of $MgCl_2$ were titrated into the loop B sample as equivalents relative to the labeled strand. The $^{1}$H-$^{15}$N HSQC spectrum was acquired on a 600 MHz Varian Inova.

## ACKNOWLEDGMENTS

We thank Seho Kim for assistance with the 2D NMR. This work was supported by grants from the National Institutes of Health (GM48802 and GM60485).

## REFERENCES

1. Gaffney, B.L.; Wang, C.; Jones, R.A. Nitrogen-15-labeled oligodeoxynucleotides. 4. Tetraplex formation of d[G($^{15}$N$^{7}$)GTTTTTGG] and d[T($^{15}$N$^{7}$GGGT] monitored by $^{1}$H detected $^{15}$N NMR. J. Am. Chem. Soc. **1992**, *114*, 4047–4050.
2. Gaffney, B.L.; Goswami, B.; Jones, R.A. Nitrogen-15-labeled oligodeoxynucleotides. 7. Use of $^{15}$N NMR to probe H-bonding in an O$^{6}$MeG • C base pair. J. Am. Chem. Soc. **1993**, *115*, 12607–12608.
3. Gaffney, B.L.; Kung, P.-P.; Wang, C.; Jones, R.A. Nitrogen-15-labeled oligodeoxynucleotides. 8. Use of $^{15}$N NMR to probe Hoogsteen hydrogen bonding at guanine and adenine N-7 atoms of a DNA triplex. J. Am. Chem. Soc. **1995**, *117*, 12281–12283.
4. Gao, X.; Jones, R.A. Nitrogen-15-labeled oligodeoxynucleotides. Characterization by $^{15}$N NMR of d[CGTACG] containing $^{15}$N1- or $^{15}$N6-labeled deoxyadenosine. J. Am. Chem. Soc. **1987**, *109*, 3169–3171.
5. Goswami, B.; Gaffney, B.L.; Jones, R.A. Nitrogen-15-labeled oligodeoxynucleotides. 5. Use of $^{15}$N NMR to probe H-bonding in an O$^{6}$MeG · T base pair. J. Am. Chem. Soc. **1993**, *115*, 3832–3833.
6. Tanaka, Y.; Kojima, C.; Morita, E.H.; Kasai, Y.; Yamasaki, K.; Ono, A.; Kainosho, M.; Taira, K. Identification of the metal ion binding site on an RNA motif from hammerhead ribozymes using $^{15}$N NMR spectroscopy. J. Am. Chem. Soc. **2002**, *124*, 4595–4601.
7. Wang, C.; Gao, X.; Jones, R.A. Nitrogen-15-labeled oligodeoxynucleotides. 2. Solvent isotope effects on the chemical shift of the adenine N1 in an A • T base pair. J. Am. Chem. Soc. **1991**, *113*, 1448–1450.
8. Zhang, X.; Gaffney, B.L.; Jones, R.A. $^{15}$N NMR of a specifically labeled RNA fragment containing intrahelical GU wobble pairs. J. Am. Chem. Soc. **1997**, *119*, 6432–6433.
9. Zhang, X.; Gaffney, B.L.; Jones, R.A. $^{15}$N NMR of RNA fragments containing specifically labeled GU and GC pairs. J. Am. Chem. Soc. **1998**, *120*, 615–618.
10. Zhang, X.; Gaffney, B.L.; Jones, R.A. $^{15}$N NMR of RNA fragments containing specifically labeled tandem GA pairs. J. Am. Chem. Soc. **1998**, *120*, 6625–6626.

11. Levy, G.C. *Nitrogen-15 Nuclear Magnetic Resonance Spectroscopy*; John Wiley & Sons: New York, 1979.
12. Abad, J.L.; Gaffney, B.L.; Jones, R.A. $^{15}N$-Multilabeled adenine and guanine nucleosides. Syntheses of [1,3,$NH_2$-$^{15}N_3$]- and [2-$^{13}C$-1,3,$NH_2$-$^{15}N_3$]-labeled adenosine, guanosine, 2′-deoxyadenosine, and 2′-deoxyguanosine. J. Org. Chem. **1999**, *64*, 6575–6582.
13. Pagano, A.R.; Lajewski, W.M.; Jones, R.A. Syntheses of [6,7-$^{15}N$]-adenosine, [6,7-$^{15}N$]-2′-deoxyadenosine, and [7-$^{15}N$]-hypoxanthine. J. Am. Chem. Soc. **1995**, *117*, 11669–11672.
14. Pagano, A.R.; Zhao, H.; Shallop, A.; Jones, R.A. Syntheses of [1,7-$^{15}N_2$]- and [1,7,$NH_2$-$^{15}N_3$]-adenosine and 2′-deoxyadenosine via an N1-alkoxy mediated Dimroth rearrangement. J. Org. Chem. **1998**, *63*, 3213–3217.
15. Shallop, A.J.; Gaffney, B.L.; Jones, R.A. Use of $^{13}C$ as an indirect tag in $^{15}N$ specifically labeled nucleosides. Syntheses of [8-$^{13}C$-1,7,$NH_2$-$^{15}N_3$]-adenosine, -guanosine, and their deoxy analogs. J. Org. Chem. **2003**, *68*, 8657–8661.
16. Zhao, H.; Pagano, A.R.; Wang, W.; Shallop, A.; Gaffney, B.L.; Jones, R.A. Use of a $^{13}C$ atom to differentiate two $^{15}N$-labeled nucleosides. Syntheses of [6-$^{15}N$]-adenosine; [1,2-$^{15}N$]- and [1,2-$^{15}N$-2-$^{13}C$]-guanosines; [1,2,7-$^{15}N$]- and [1,2,7-$^{15}N$-2-$^{13}C$]-2′deoxyguanosine. J. Org. Chem. **1997**, *62*, 7832–7835.
17. Hampel, A.; Tritz, R.; Hicks, M.; Cruz, P. Hairpin catalytic RNA model, evidence for helices and sequence requirement for substrate RNA. Nucleic Acids Res. **1990**, *18*, 299–304.
18. Long, D.M.; Uhlenbeck, O.C. Self-cleaving catalytic RNA. FASEB J. **1993**, *7*, 25–30.
19. Symons, R.H. Small catalytic RNAs. Ann. Rev. Biochem. **1992**, *61*, 641–671.
20. Walter, N.G.; Burke, J.M. The hairpin ribozyme: structure, assembly and catalysis. Curr. Opin. Struct. Biol. **1998**, *2*, 24–30.
21. Nesbitt, S.; Hegg, L.A.; Fedor, M.J. An unusual pH-independent and metal-ion-independent mechanism for hairpin ribozyme catalysis. Chem. Biol. **1997**, *4*, 619–630.
22. Young, K.J.; Gill, F.; Grasby, J.A. Metal ions play a passive role in the hairpin ribozyme catalysed reaction. Nucleic Acids Res. **1997**, *25*, 3760–3766.
23. Butcher, S.E.; Allain, F.H.-T.; Feigon, J. Solution structure of the loop B domain from the hairpin ribozyme. Nat. Struct. Biol. **1999**, *6*, 212–216.
24. Cai, Z.; Tinoco, I., Jr. Solution structure of Loop A from the hairpin ribozyme from tobacco ringspot virus satellite. Biochemistry **1996**, *35*, 6026–6036.
25. Rupert, P.B.; Ferre-D'Amare, A.R. Crystal structure of a hairpin ribozyme-inhibitor complex with implications for catalysis. Nature (Lond.) **2001**, *410*, 780–786.
26. Rupert, P.B.; Massey, A.P.; Sigurdsson, S.T.; Ferre-D'Amare, A.R. Transition state stabilization by a catalytic RNA. Science **2002**, *298*, 1421–1424.
27. Pinard, R.; Hampel, K.J.; Heckmean, J.E.; Lambert, D.; Chan, P.A.; Major, F.; Burke, J.M. Functional involvement of G8 in the hairpin ribozyme cleavege mechanism. EMBO J. **2001**, *20*, 6434–6442.
28. Saenger, W. *Principles of Nucleic Acid Structure*; Springer-Verlag: New York, 1984.
29. Connell, G.J.; Yarus, M. RNAs with dual specificity and dual RNAs with similar specificity. Science **1994**, *264*, 1137–1141.






MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

30. Legault, P.; Pardi, A. Unusual dynamics and pKa shift at the active site of a lead-dependent ribozyme. J. Am. Chem. Soc. **1997**, *119*, 6621–6628.
31. Muth, G.W.; Ortoleva-Donnelly, L.; Strobel, S.A. A single adenosine with a neutral pKa in the ribosomal peptidyl transferase center. Science **2000**, *289*, 947–950.
32. Nakano, S.-i.; Chadalavada, D.M.; Bevilacqua, P.C. General acid-base catalysis in the mechanism of a hepatitis delta virus ribozyme. Science **2000**, *287*, 1493–1497.
33. Ravindranathan, S.; Butcher, S.E.; Feigon, J. Adenine protonation in domain B of the hairpin ribozyme. Biochemistry **2000**, *39*, 16026–16032.
34. Büchner, P.; Maurer, W.; Rüterjans, H. Nitrogen-15 nuclear magnetic resonance spectroscopy of $^{15}$N-labeled nucleotides. J. Magn. Reson. **1978**, *29*, 45–63.
35. Butcher, S.E.; Allain, F.H.-T.; Feigon, J. Determination of metal binding sites within the hairpin ribozyme domains by NMR. Biochemistry **2000**, *39*, 2174–2182.
36. Buchanan, G.W. Applications of $^{15}$N NMR spectroscopy to the study of molecular structure, stereochemistry and binding phenemena. Tetrahedron **1989**, *45*, 581–604.
37. Song, Q.; Wang, W.; Fischer, A.; Zhang, X.; Gaffney, B.L.; Jones, R.A. High yield protection of purine ribonucleosides for phosphoramidite RNA synthesis. Tetrahedron Lett. **1999**, *40*, 4153–4156.
38. Nielsen, J.; Taagaard, M.; Marugg, J.E.; Boom, J.H.v.; Dahl, O. Application of 2-cyanoethyl N,N,N′,N′-tetraisopropylphosphorodiamidite for in situ preparation of deoxyribonucleoside phosphoramidites and their use in polymer-supported synthesis of oligodeoxyribonucleotides. Nucleic Acids Res. **1986**, *14*, 7391–7403.
39. Sanghvi, Y.S.; Guo, Z.; Pfundheller, H.M.; Converso, A. Improved process for the preparation of nucleosidic phosphoramidites using a safer and cheaper activator. Org. Process Res. Dev. **2000**, *4*, 175–181.
40. Vargeese, C.; Carter, J.; Yegge, J.; Krivjansky, S.; Settle, A.; Kropp, E.; Peterson, K.; Pieken, W. Efficient activation of nucleoside phosphoramidites with 4,5-dicyanoimidazole during oligonucleotide synthesis. Nucleic Acids Res. **1998**, *26*, 1046–1050.
41. Wincott, F.; DeRenzo, A.; Shaffer, C.; Grimm, S.; Tracz, D.; Workman, C.; Sweedler, D.; Gonzalez, C.; Scaringe, S.; Usman, N. Synthesis, deprotection, analysis and purification of RNA and ribozymes. Nucleic Acids Res. **1995**, *23*, 2677–2684.
42. Song, Q.; Jones, R.A. Use of silyl ethers as fluoride scavengers in RNA synthesis. Tetrahedron Lett. **1999**, *40*, 4653–4654.
43. Wishart, D.S.; Bigam, C.G.; Yao, J.; Abildgaard, F.; Dyson, H.J.; Oldfield, E.; Markley, J.L.; Sykes, B.D. $^{1}$H, $^{13}$C and $^{15}$N chemical shift referencing in biomolecular NMR. J. Biomol. NMR **1995**, *6*, 135–140.

Received July 25, 2003
Accepted October 14, 2003

# Request Permission or Order Reprints Instantly!