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The synthesis of 5-iodocytidine phosphoramidite for heavy atom derivatization of RNA

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Abstract

The synthesis of an RNA phosphoramidite of 5-iodocytidine is reported. This heavy atom cytidine derivative was incorporated into four RNAs. Oligoribonucleotides with 5-iodocytidine will be useful for solving the X-ray crystallographic phase problem and for photochemical cross-linking studies. © 1999 Elsevier Science Ltd. All rights reserved.

The heavy atom method to solve the phase problem in X-ray crystallography has been widely used for DNA and proteins.[1] RNA structure determination has remained a challenge because there are few suitable solutions to the phase problem in oligoribonucleotides.[2] To overcome this hurdle we designed and synthesized the 5-iodocytidine phosphoramidite **6** (Scheme 1).

The incorporation of iodine at position 5 in cytidine is suitable for structural studies of short RNAs for two reasons. First, derivatization of the 5 position of cytidine does not disrupt Watson–Crick hydrogen bonding. Second, the iodine at the 5 position of cytidine protrudes into the major grove and therefore causes minimal perturbation to A-form duplex structure.

For the synthesis of the 5-iodocytidine phosphoramidite the protecting groups used were the same as those used in commercially available phosphoramidites with 2′ TBDMS protection.[3] The use of similar protecting groups allows easy incorporation of this heavy atom derivative into oligoribonucleotides via an automated chemical synthesizer.

As shown in Scheme 1, compound **2**[4] was prepared from cytidine by iodination with iodic acid and iodine.[5] Pure white crystals of 5-iodocytidine were obtained after recrystallization from methanol in 53% yield. Inspection of the proton NMR of **2** shows the disappearance of the H5 proton of cytidine and concomitant collapse of the H6 proton to a singlet. This assignment verifies the incorporation of iodine at the desired site. Compounds containing iodine are known to be light sensitive, therefore all the subsequent reactions were performed in the dark. Also, the subsequent reactions involved the use of water sensitive reagents and therefore particular care was taken to assure use of dry glassware and the reactions were conducted under argon.

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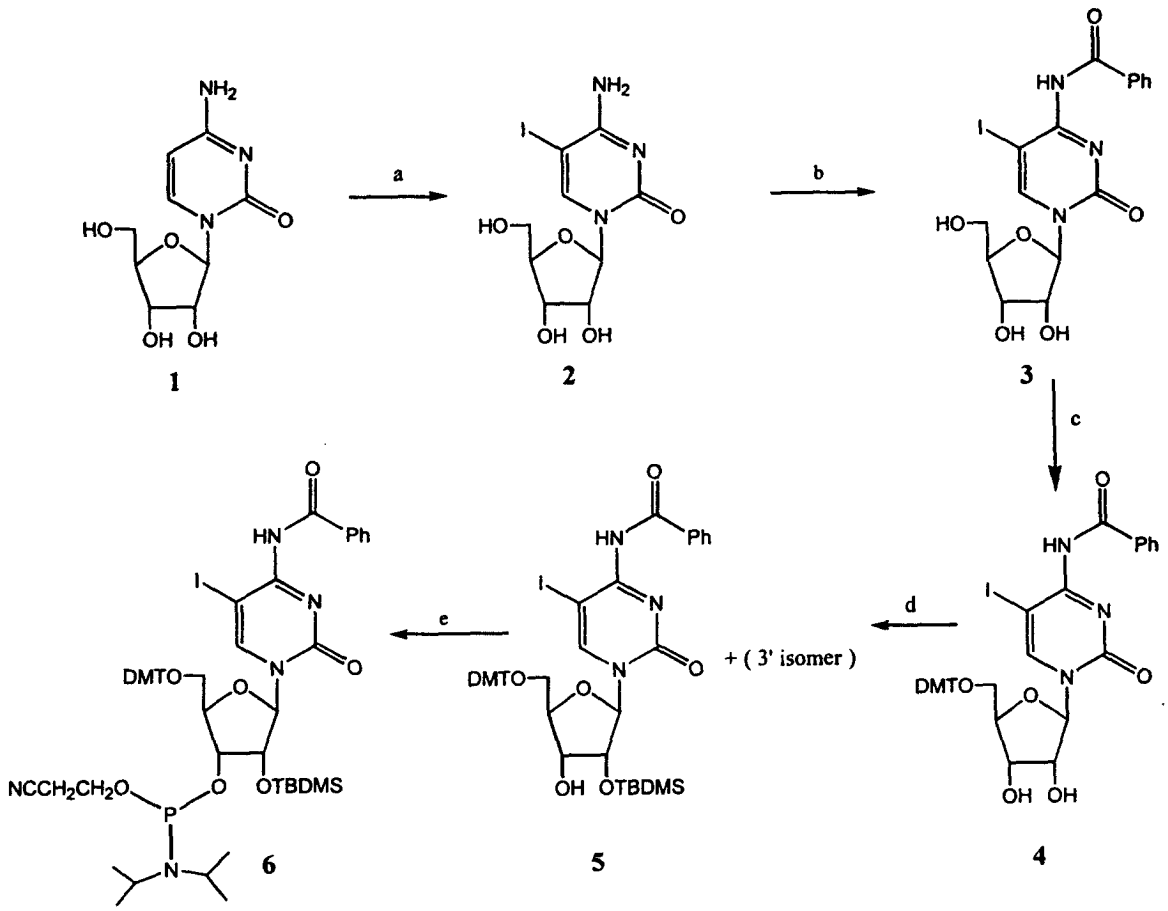

Scheme 1. Scheme for 5-iodocytidine phosphoramidite synthesis. Reagents and conditions: (a) iodine (1.0 equiv.), iodic acid (1.0 equiv.), acetic acid, H_2O, and CCl_4 (8:3:2), respectively, overnight; (b) TMSCl (3.5 equiv.)/pyridine, 0°C, 30 min, followed by benzoyl chloride (1.1 equiv.), 2 h; (c) DMTCl (1.1 equiv.)/pyridine, 25°C, 4 h; (d) TBDMSCl (1.5 equiv.)/pyridine, imidazole (2.5 equiv.), 6 h; (e) β-cyanoethyl *N,N*-diisopropylphosphoramidite chloride (1.1 equiv.)/THF, *N,N*-diisopropylethylamine (4 equiv.), and DMAP (0.2 equiv.), overnight, 25°C

Protection of the exocyclic amine was achieved via transient protection of the sugar alcohols as trimethylsilyl (TMS) ethers and subsequent reaction of the amine with benzoyl chloride.[6] After completion of the reaction, the TMS groups were removed by addition of water. Compound **3** was obtained in 66% yield.

Pyridine was added to compound **3**[7] and evaporated several times to remove any water from the sample. The tritylation was carried out at room temperature within 3–4 h to yield **4**.[8] Silica gel chromatography in the presence of triethylamine yielded **4** in 50% yield. The low yield of tritylation is probably due to steric hindrance from the iodine at C5. The yield may be improved by recovering unreacted **3**.

Compound **4** was reacted with *tert*-butyldimethyl chlorosilane in pyridine[9] to produce a mixture of **5**[10] and its 3′ isomer. This mixture was purified by column chromatography with toluene:ethyl acetate (3:2) and gave compound **5** in 45% yield. More of compound **5** (12%) was generated by isomerization of its 3′ isomer with 3% triethylamine/methanol. Silylation of **4** carried out in DMF[11] gave lower yields of **5** and higher yields of the undesired 3′ isomer. Phosphitylation of **5** was performed with

β-cyanoethyl *N,N*-diisopropylphosphoramidite chloride in tetrahydrofuran, *N,N*-diisopropylethylamine and 4-dimethylaminopyridine.[12] The 5-iodocytidine phosphoramidite **6**[13] was obtained in 53% yield. ^{31}P NMR of compound **6** demonstrated diastereotopic phosphorous resonances at 148.4 and 149.4 ppm, thereby confirming the formation of the phosphoramidite.[13]

The 5-iodocytidine amidite was successfully incorporated into four oligoribonucleotides {RRE strand I[14] r(5′-G^{I}CUGGGCGCAGG-3′), RRE strand II[14] r(5′-C^{I}CUGACGGUACAGC-3′), TAR loop[15] r(5′-GAGCCCUGGGAGG^{I}CUC-3′), and a tandem GA mismatch duplex[16] r(5′-GGCGAG^{I}CC-3′)}. The coupling efficiency for the new amidite is 50%, but this could probably be improved by removal of water from compound **6** and optimization of the coupling procedure. The resulting RNAs were deprotected with a standard protocol,[17] HPLC purified, and inspected by 1D and 2D NMR to verify the incorporation of 5-iodocytidine. Enzymatic digestion with ribonuclease P_1 and calf intestinal alkaline phosphatase was performed on the sequences, followed by reversed-phase HPLC analysis[18] to verify the incorporation of 5-iodocytidine (Fig. 1). The putative 5-iodocytidine HPLC peak was collected; the UV spectrum (absorbance maximum at 292 nm)[5] and 1D NMR of the compound confirmed the structure as 5-iodocytidine. X-Ray crystallographic studies of these oligoribonucleotides are in progress (Holbrook, S.; Irani, R. J.; SantaLucia Jr., J., unpublished).

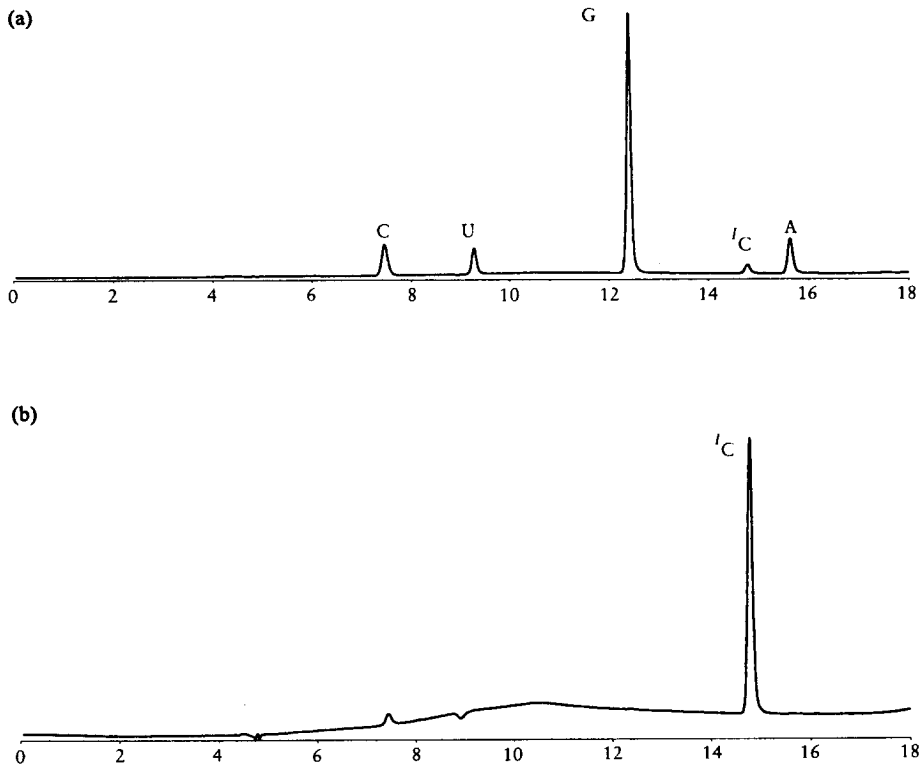

Figure 1. HPLC profile of: (a) enzymatic digestion mixture of r(5′-G^{I}CUGGGCGCAGG-3′); and (b) 5-iodocytidine standard. The enzymatic digestion was performed with nuclease P_1 and calf intestine alkaline phosphatase. The HPLC column is Hypersil 5 μ C18 (4.6×250 mm). The mixture was eluted with triethylamine (100 mM, pH=7) and 50% methanol/100 mM triethylamine, with a linear gradient from 0 to 27.5% methanol (18 min) at a flow rate of 1 ml/min. The absorbance was detected at 254 nm. The extinction coefficients at 254 nm for C, U, G, ^{I}C and A were 7.0×10^3, 9.3×10^3, 13.5×10^3, 4.2×10^3, 13.4×10^3, respectively. The retention times of C, U, G, ^{I}C and A were 7.49, 9.33, 12.49, 14.89 and 15.76 min, respectively. The ratio of peak areas measured after correction for extinction coefficients at 254 nm of C, U, G, ^{I}C and A are 2.18:1.00:6.70:0.77:1.06, respectively, with standard errors of ±10–20%

In conclusion, we have synthesized the 5-iodocytidine phosphoramidite and incorporated 5-iodocytidine site-specifically into small RNAs. The 5-iodocytidine phosphoramidite reported here will likely be useful for crystallographic studies of many RNAs. Site-specific incorporation of 5-iodocytidine will also allow the study of RNA–RNA and RNA–protein interactions via photochemical cross-linking.[19,20]

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4. ^{1}H NMR (DMSO-d_6) δ (ppm): 8.41 (1H, s, H6), 7.81 (1H, br s, NH), 6.62 (1H, br s, NH), 5.70 (1H, d, H1′, *J*=3.5 Hz), 5.37 (1H, d, 3′-OH, *J*=5.5 Hz), 5.22 (1H, t, 5′-OH, *J*=5 Hz), 4.99 (1H, d, 2′-OH, *J*=6 Hz), 3.94 (2H, m, H2′, H3′), 3.83 (1H, m, H4′), 3.63 (2H, m, H5′, H5″). MS (+FAB)=370 $[M+H]^+$.
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7. ^{1}H NMR (acetone-d_6) δ (ppm): 8.97 (1H, s, H6), 8.38 (2H, d, benzoyl, *J*=8 Hz), 7.59 (1H, t, benzoyl), 7.49 (2H, t, benzoyl), 5.91 (1H, d, H1′, *J*=3 Hz), 4.36 (2H, m, H2′, H3′), 4.12 (1H, m, H4′), 3.93 (2H, m, H5′, H5″). MS (+FAB)=474 $[M+H]^+$.
8. ^{1}H NMR (acetone-d_6) δ (ppm): 8.35 (1H, s, H6), 8.38 (2H, d, benzoyl, *J*=7 Hz), 7.60–7.24 (12H, m, DMT-Ar, benzoyl), 6.92 (4H, d, DMT-Ar, H3, H3′, H5, H5′ *J*=9 Hz), 5.95 (1H, d, H1′, *J*=4 Hz), 4.51 (1H, m, H2′), 4.46 (1H, m, H3′), 4.22 (1H, m, H4′), 3.78 (6H, s, DMT-OCH_3), 3.44 (2H, m, H5′, H5″). MS (+FAB)=798 $[M+Na]^+$.
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10. ^{1}H NMR (acetone-d_6) δ (ppm): 8.38 (1H, s, H6), 8.36 (2H, d, benzoyl, *J*=8 Hz) 7.61–7.26 (12H, m, DMT-Ar, benzoyl), 6.92 (4H, d, DMT-Ar, H3, H3′, H5, H5′ *J*=9.5 Hz), 5.94 (1H, d, H1′, *J*=4 Hz), 4.60 (1H, m, H2′), 4.34 (1H, m, H3′), 4.24 (1H, m, H4′), 3.80 (6H, s, DMT-OCH_3), 3.46 (2H, m, H5′, H5″), 0.94 (9H, s, $SiC(CH_3)_3$), 0.19 (3H, s, $SiCH_3$), 0.18 (3H, s, $SiCH_3$). MS (+FAB)=890 $[M+H]^+$.
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13. ^{1}H NMR (acetone-d_6) δ (ppm): 8.39 (1H, s, H6), 8.38 (2H, d, *J*=8 Hz) 7.61–7.34 (12H, m, DMT-Ar, benzoyl), 7.05 (1H, d, $NCCH_2CH_2OP$, *J*=9 Hz), 6.93 (2H, d, benzoyl, *J*=8 Hz), 6.74 (1H, d, $NCCH_2CH_2OP$, *J*=9 Hz), 5.94 (1H, d, H1′, *J*=4 Hz), 4.61 (1H, m, H2′), 4.40 (1H, m, H3′), 4.24 (1H, m, H4′), 3.80 (6H, DMT-OCH_3), 3.47 (2H, m, H5′, H5″), 2.78–2.92 (2H, m, $2CH(CH_3)_3$), 2.55 (2H, m, $NCCH_2CH_2OP$), 1.24 and 1.20 (12H, two d, $2CH(CH_3)_2$, *J*=7 Hz), 0.94 (9H, s, $SiC(CH_3)_3$), 0.19 (3H, s, $SiCH_3$), 0.18 (3H, s, $SiCH_3$); ^{31}P NMR D_2O (std. ref. TMP) δ (ppm): 148.4 and 149.4. MS (+FAB)=1090 $[M+H]^+$.
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