Isoguanosine Substitution of Conserved Adenosines in the Hammerhead Ribozyme†

Mabel M. P. Ng, Fritz Benseler, Thomas Tuschl, and Fritz Eckstein*

Max-Planck-Institut für experimentelle Medizin, Hermann-Rein-Strasse 3, D-37075 Göttingen, Germany

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ABSTRACT: Isoguanosine has been incorporated into a 34-mer hammerhead ribozyme by the solid-phase phosphoramidite method, using an acetamidine base protecting group. The activity of the hammerhead ribozyme when singly mutated to isoguanosine at the adenosine positions 6, 9, and 13 was 1–2-fold less than the wild-type activity. Mutations to 2-aminopurine ribonucleoside at positions 9 and 13 were 5-fold reduced in activity, but that at position 6 was approximately 30-fold reduced. These results support the view that the 6-amino functions of A6, A9, and A13 are not very important for catalysis. The 2-position of A6 tolerates a carbonyl function but not an amino group, whereas A9 and A13 tolerate both functional groups. The tolerance of a 2-amino group at A9 and A13 makes G(anti)/A(anti) Watson–Crick type base mispairing for G12/A9 and A13/G8 unlikely.

Isoguanosine is a naturally occuring substance first isolated from croton beans (Cherbuliez & Bernhard, 1932). There has been interest in expanding the genetic code using isoguanosine (Switzer et al., 1989, 1993; Bain et al., 1992), as well as exploring the biological properties of this adenosine analogue (Hagen, 1973; Leonard et al., 1962). Enzymatic incorporation of deoxyisoguanosine into oligodeoxynucleotides has been reported by Switzer et al. (1993), Golas et al. (1976), and Tor and Dervan (1993), and chemical incorporation using both the phosphonate and phosphoramidite methods has been reported by Switzer et al. (1993) and Seela et al. (1992). Isoguanosine incorporation into oligoribonucleotides has been achieved by the phosphonate method (Seela & Fröhlich, 1993, 1994).

We are interested in obtaining information on the possible interactions of the invariant nucleosides in the central core of the hammerhead ribozyme (Figure 1). To this end we have previously replaced the guanosines in this region by a number of unnatural nucleosides including isoguanosine (Tuschl et al., 1993). In these experiments the formamidine-base-protected isoguanosine phosphoramidite was used in the chemical synthesis of the oligoribonucleotide. It could only be incorporated as a terminal nucleotide, and chain extension beyond it was not possible. The hammerhead ribozymes containing isoguanosine at positions G5, G8, and G12 had to be constructed by ligation with T4 DNA ligase. Although this method gives the desired products, it is not amenable for routine synthesis, and alternatives are desirable. In an extension of this work we report here the synthesis and incorporation of the isoguanosine phosphoramidite into non-terminal positions of a 34-mer hammerhead ribozyme using solid-phase chemical synthesis, and subsequent activity of the hammerhead ribozyme with mutation of specific adenosines within the central core region to isoguanosine, 2-aminopurine ribonucleoside, and guanosine. It proved crucial to the synthesis and subsequent incorporation of the phosphoramidite of isoG to use amidine rather than acyl protecting groups. This method of synthesis represents an improvement over the H-phosphonate method in that it permits the synthesis of longer oligonucleotides (Regberg et al., 1988), as well as specific nucleotide oxidation to phosphorothioates (Zon & Stec, 1991).

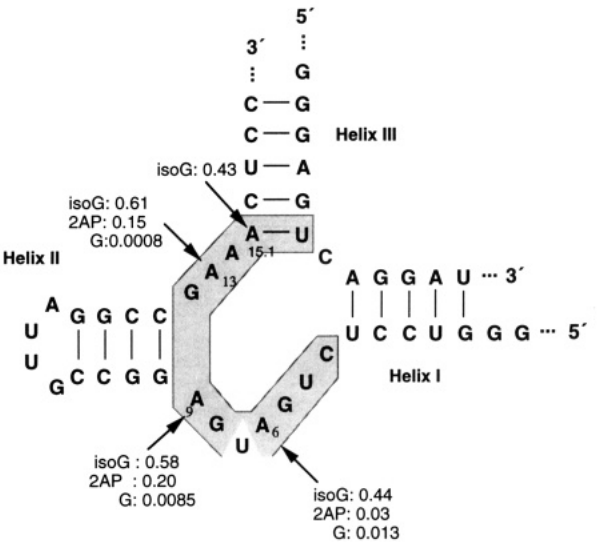

FIGURE 1: Hammerhead ribozyme with positions indicated for single mutations described in this work. Shaded area, invariant core with numbering of positions of nucleotides according to Hertel et al. (1992). Relative rates, taken from Table 1, are given for substitutions at the four positions of interest.

MATERIALS AND METHODS

HPLC grade methanol and acetonitrile were purchased from Baker (Deventer, Holland). Solvents acetone, dichloromethane, chloroform, ethyl acetate, *n*-hexane, toluene, silver nitrate, isobutyryl chloride, isobutyric anhydride, benzoyl chloride, phenoxyacetyl chloride, anhydrous sodium sulfate, sodium hydrogen carbonate, urea, and hydrofluoric acid (40%) were purchased from Merck (Darmstadt, Germany), and triethylamine and *N,N*-dimethylformamide were from Fluka AG (Neu-Ulm, Germany). Dry pyridine, 1,4-dioxane, and THF (all containing less than 0.01% H_2O) from Merck were stored over 4-Å molecular sieves (Merck). Dichloromethane used in the phosphitylation reaction was stored over sodium–lead alloy (10% Na, Merck) and passed down a column of basic alumina (Alumina B-Super I, ICN Biomedicals, Es-

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* Author to whom correspondence should be addressed. Tel. (49) 551-3899 274. Fax (49) 551-3899 388.

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chwege, Germany) immediately prior to use. TBDMSCl,[1] DMTCl, TMSCl, *N*-ethyldiisopropylamine, *N*-methylimidazole, 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite, 1.1 M TBAF in THF, and *N,N*-dimethylacetamide dimethyl acetal were purchased from Aldrich (Steinheim, Germany). Alkaline phosphatase (molecular biology grade from calf intestine, 22 units/μL) and snake venom phosphodiesterase (*Crotalus durissus*, 2 mg/mL) were obtained from Boehringer (Mannheim, Germany). T4 polynucleotide kinase was obtained from United States Biochemical Corp. [γ-^{32}P]ATP (10 μCi/μL) was obtained from Amersham. X-ray film (X-OMAT XAR-5) was purchased from Kodak.

Isoguanosine was prepared according to Chern et al. (1991), with the exception that the oily residue obtained after reaction with dicyclohexyldiimide was extracted with boiling toluene instead of partitioning between a mixture of water and chloroform. Removal of trimethylsilyl protecting groups following acylation was effected using either ammonia (Ti et al., 1982) or hydrofluoric acid (Gao et al., 1986) as detailed below. 1-Hydroxybenzotriazol was used in the synthesis of N^6-(phenoxyacetyl)isoguanosine (Benseler & McLaughlin, 1986).

^{1}H NMR spectra were recorded at 360.13 MHz on a Bruker WH 360 spectrometer with tetramethylsilane as the internal standard. CD_3OD was added to ^{1}H NMR samples for the identification of exchangeable protons. ^{31}P NMR spectra were recorded on the same instrument at 145.79 MHz with ^{1}H decoupling and 85% H_3PO_4 as the external standard.

Silica gel 60 F_{254} TLC plates (Merck) were developed in various solvent (v/v) systems: S1, CH_2Cl_2/MeOH, 8:2; S2, CH_2Cl_2/MeOH, 9:1; S3, CH_2Cl_2/MeOH, 6:4; S4, EtOAc/MeOH, 6:4. The compounds were visualized first by the use of UV light, followed by spraying with 5% aqueous sulfuric acid and heating. Flash column chromatography was performed on silica gel 60 (Merck) with particle size less than 0.063 mm.

Analytical HPLC was performed on columns (4.6 × 25 mm) containing ODS-Hypersil (5 μm, Shandon, Runcorn, England) using a DuPont 8800 instrument coupled to a DuPont 8800 UV detector.

Preparation of Oligoribonucleotides. Automated oligoribonucleotide syntheses were carried out on a 1-μmol scale, on an Applied Biosystems 380B DNA/RNA synthesizer, using monomeric ribonucleotide phosphoramidites coupled to control pore glass columns, as supplied by Milligen/Biosearch. The 2-aminopurine ribonucleoside phosphoramidite was prepared as described (Tuschl et al., 1993). The 34-mer oligoribonucleotides and the 12-mer substrate were synthesized and isolated according to the protocol described previously (Tuschl et al., 1993). Electroelution was performed with Schleicher & Schuell BIOTRAP elution chambers using membranes BT1 and BT2.

The homogeneity of the ribozymes was analyzed by analytical reverse-phase HPLC using a linear gradient of acetonitrile (1.4–14% in 15 min) in 50 mM aqueous triethylammonium acetate buffer, with a flow rate of 1.5 mL/min, as well as by electrophoresis on a denaturing 20% polyacrylamide gel of the 5′-^{32}P-labeled ribozymes. The labeling reaction was carried out as described (Tuschl et al., 1993).

Nucleoside composition and incorporation of the isoguanosine-containing ribozymes were determined after digestion of 0.4 A_{260} unit of the purified 34-mer with snake venom phosphodiesterase (2 μL) for 16 h in 50 μL of buffer solution (50 mM Tris-HCl and 10 mM $MgCl_2$, pH 8). Alkaline phosphatase (2 μL) was added 30 min prior to analysis of the sample by reverse-phase HPLC. This avoided deamination of adenosine to inosine (Usman et al., 1987). The resulting nucleoside mixture was analyzed by HPLC using a linear gradient of acetonitrile (1–17% in 25 min) in 50 mM aqueous triethylammonium acetate buffer, with a flow rate of 1.5 mL/min.

Kinetic Analysis. Cleavage rates of the 5′-^{32}P-labeled substrate by the ribozymes under saturating substrate conditions were determined according to the general protocol given previously (Tuschl et al., 1993). First-order rate constants were determined at both 500 and 800 nM substrate concentrations and at ribozyme concentrations from 5.0 to 50 nM in 10 mM $MgCl_2$ and 50 mM Tris-HCl (pH 7.5). Aliquots were taken at appropriate time intervals between 1 and 240 min of initiation of the reaction for analysis by gel electrophoresis. Radioanalytic scanning of gels was performed with a Fuji BAS2000 Bio-Imaging Analyzer.

N^6-Isobutyrylisoguanosine (**2a**). To an ice-cooled suspension of isoguanosine (150 mg, 0.54 mmol) in dry pyridine (10 mL) was added TMSCl (0.56 mL, 8.2 equiv), and the reaction mixture was stirred for 30 min. The reaction was followed by TLC (S1: R_f product, 0.76; R_f educt, 0.04) and was complete after 30 min, whereupon isobutyryl chloride (0.17 mL, 3 equiv) was added. The reaction solution was stirred at 25 °C for a further 2 h, after which TLC (S1) indicated completion of the reaction. Water (0.5 mL) was then added at 0 °C, followed by aqueous ammonia (0.21 mL of a 25% solution to give a 1 M solution), producing a white precipitate, which was removed immediately by centrifugation. The supernatant was evaporated to near dryness, the residue was redissolved in water, and the solution was extracted twice with dichloromethane. The aqueous layer was concentrated, and the residue was purified by chromatography on a column with ODS-Hypersil with a 10% to 20% ethanol:water gradient. Yield: 89 mg (48%). UV λ_{max} (ethanol) 305, 248, 213 nm. ^{1}H NMR (DMSO-d_6) δ = 11.92 (broad, s, 1H, NH), 11.58 (broad, s, 1H, NH), 8.32 (s, 1H, H8), 5.72 (d, J = 5.9 Hz, 1H, H$_1$′), 5.47 (d, J = 6.1 Hz, 1H, OH), 5.25 (m, 1H, OH), 5.18 (d, J = 4.8 Hz, 1H, OH), 4.48 (dd, J = 11.2, 5.8 Hz, 1H, H$_2$′), 4.10 (dd, J = 8.2, 4.7 Hz, 1H, H$_3$′), 3.92 (dd, J = 6.7, 3.4 Hz, 1H, H$_4$′), 3.65, 3.55 (m, 2H, C$_5$′, C$_5$″), 3.00 (sept, 1H, COC*H*Me$_2$), 1.12 (d, J = 6.8 Hz, 6H, COCH*Me*$_2$).

N^6-Benzoylisoguanosine (**2b**). To a suspension of isoguanosine (0.30 g, 1.06 mmol) in pyridine (20 mL) was added TMSCl (1.12 mL, 8.2 equiv), and the reaction mixture was stirred at room temperature for 1 h. Benzoyl chloride (0.30 mL, 2.4 equiv) was then added, and the solution was stirred for a further 12 h, after which the reaction was complete as indicated by TLC (S1: R_f product, 0.98; R_f educt, 0.04). HF (2 mL of a 40% aqueous solution) and ethanol (2 mL) were then added at 0 °C, and the reaction mixture was stirred at 0 °C for 30 min (TLC, S1: R_f product, 0.45). Water (20 mL) was then added, and the product was extracted with ethyl acetate (4 × 40 mL). The organic layer was dried over sodium sulfate and filtered; the filtrate was concentrated, and the residue was purified by flash column silica gel column chromatography, using a 0–20% ethanol gradient in dichloromethane as the eluant. The product eluted in the presence of 20% ethanol. Yield: 0.11 g (27%). UV λ_{max} (ethanol)

[1] Abbreviations: isoG, isoguanosine; 2AP, 2-aminopurine ribonucleoside; TMSCl, trimethylsilyl chloride; TBAF, tetrabutylammonium fluoride, DMTCl, 4,4′-dimethoxytrityl chloride; TBDMSCl, *tert*-butyldimethylsilyl chloride; PAC, phenoxyacetyl; Bz, benzoyl.

333, 303, 221 nm. ^{1}H NMR (DMSO-d_6) δ = 8.42 (s, 1H, H8), 8.10 (d, 2H, *ortho*-C_6H_5), 7.83 (s, broad, 1H, N*H*-COPh), 7.77 (dd, J = 7.5Hz, 1H, *para*-C_6H_5), 7.61 (dd, J = 7.7 Hz, 2H, *meta*-C_6H_5), 5.82 (d, 1H, J = 6.1 Hz), 5.50 (d, 1H, J = 6.2 Hz, OH), 5.19 (d, 1H, J = 4.8 Hz, OH), 5.14 (dd, 1H, J = 5.1 Hz, exchangeable with CD_3OD), 4.53 (m, 1H, H_1'), 4.10 (m, 1H), 3.92 (m, 1H), 3.61, 3.54 (m, 2H, H_5', H_5'').

N^6-(Phenoxyacetyl)isoguanosine (**2c**). To an ice-cooled suspension of isoguanosine (1 g, 3.57 mmol) in dry pyridine (100 mL) was added trimethylsilyl chloride (3.40 mL, 26.78 mmol), and the mixture was stirred at 0 °C for 1 h. In another flask, 1-hydroxybenzotriazole (0.5 g, 1.05 equiv) was suspended in acetonitrile (15 mL), and phenoxyacetyl chloride (0.74 mL, 1.5 equiv) was added. This suspension was immediately transferred to the above reaction mixture at 0 °C, and the reaction mixture was stirred at room temperature for 20 h (TLC, S1: R_f product, 0.82; R_f educt, 0.04). The mixture was cooled to 0 °C, and water (12 mL) was added followed by aqueous HF (9 mL of a 40% solution to give a 2 M solution). The reaction solution was then stirred at 0 °C for 6 h. The reaction volume was then concentrated to about 20 mL and partitioned between water and diethyl ether. The organic phase was reextracted with H_2O. The aqueous phase was concentrated to give an off-white product. Yield: 140 mg (10%). UV λ_{max} (ethanol) 345, 303, 244 nm. ^{1}H NMR (DMSO-d_6) δ = 11.51 (broad, s, 1H, NH), 8.38 (s, 1H, H2 or H_8), 8.01 (s, 1H, H_2 or H_8), 7.33–7.24, 6.97–6.95 (m, 5H, $CH_2OC_6H_5$), 6.89 (m, 2H, $CH_2OC_6H_5$), 5.78 (d, J = 5.7 Hz, 1H, H_1'), 5.50 (s, 1H, OH), 5.20 (s, 1H, OH), 4.82 (m, 1H, H_2'), 4.46 (m, 1H, H_3'), 4.12 (m, 1H, H_4'), 3.64, 3.63 (m, 2H, H_5', H_5'').

5'-O-(4,4'-Dimethoxytrityl)-N^6-isobutyrylisoguanosine (**3a**). To a solution of N^6-isobutyrylisoguanosine (**2a**) (0.78 g, 2.2 mmol) in pyridine (40 mL) was added DMTCl portionwise over a 7-h period (0.85 g, 1.1 equiv). After a further 2 h, when TLC (S2: R_f product, 0.46; R_f educt, 0.11) indicated completion, methanol (2 mL) was added. The volume was concentrated to about 10 mL, and the mixture was redissolved in dichloromethane and twice washed with aqueous sodium bicarbonate (5% solution), followed by washing with water (2×). The organic layer was dried over sodium sulfate, the filtrate was evaporated to dryness, and the residue was coevaporated three times with toluene, to give a yellow foam, and purified by flash column silica gel column chromatography. The column was packed in *n*-hexane containing 3% triethylamine and washed with dichloromethane (filtered through basic alumina) prior to loading of the column with the sample dissolved in dichloromethane. The product was eluted using a 1–2% gradient of ethanol in dichloromethane, containing 0.5% triethylamine. The required fractions were pooled and coevaporated in the presence of toluene to give a white product. Yield: 0.49 g (34%). ^{1}H NMR (DMSO-d_6) δ = 11.56 (broad, s, 1H, NH), 8.21 (s, 1H, H_8), 7.37–7.16, 6.86–6.82 (m, 13H, 4-MeO-C_6H_5), 5.77 (m, 1H, H1'), 5.60 (d, J = 5.6 Hz, 1H, OH), 5.22 (d, J = 6.1 Hz, OH), 4.53 (m, 1H), 4.25 (m, 1H), 4.02 (m, 1H), 3.72 (s, 6H, 4-*Me*O-phenyl), 3.22, 3.16 (m, 2H, C5', C5''), 2.98 (m, 1H, $CH(CH_2Me_2)$), 1.11 (d, J = 6.8 Hz, 6H, $CHMe_2$).

5'-O-(4,4'-Dimethoxytrityl)-N^6-[1-(dimethylamino)-ethylidene]isoguanosine (**6b**). To a stirred suspension of isoguanosine (1.8 g, 6.35 mmol) in DMF (100 mL) was added *N,N*-dimethylacetamide dimethyl acetal (2.58 mL, 15.87 mmol). After 10 min, the suspension cleared, and stirring was continued for a further 15 min (TLC, S3: R_f product, 0.50; R_f educt, 0.05). The reaction volume was then reduced to about 30 mL and concentrated twice more from DMF (2 × 60 mL) to a final volume of 30 mL, to which pyridine was added (25 mL). DMTCl (2.59 g, 9.17 mmol) was then added portionwise over a 6-h period, and the mixture was stirred at room temperature. When no more educt was apparent (TLC, S1: R_f product, 0.65; R_f educt, 0.19), ethanol (5 mL) was added. The reaction solution was then diluted with dichloromethane and washed with saturated aqueous sodium bicarbonate solution (3 × 150 mL). The combined bicarbonate fractions were back-extracted with dichloromethane, and the combined organic phases were dried over sodium sulfate. The filtrate was evaporated to approximately 50 mL, toluene (50 mL) was added, and the solution was evaporated to near dryness. Evaporation with dichloromethane/toluene was repeated twice to give a yellow foam.

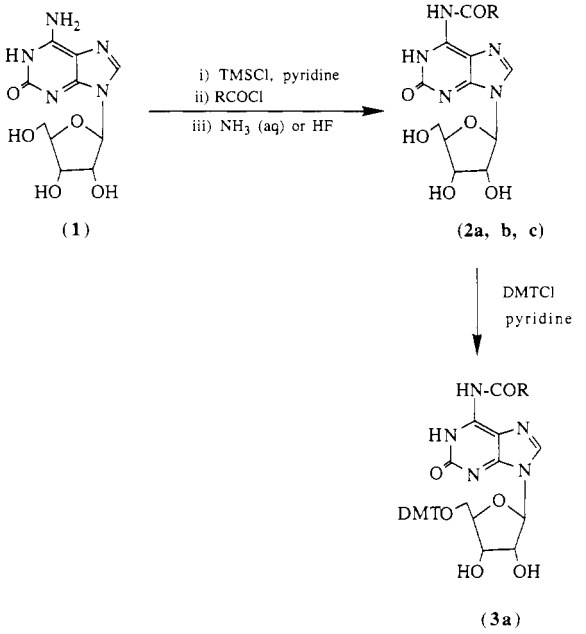
The crude product was purified by flash column silica gel chromatography using a 0–15% gradient of methanol in ethyl acetate, containing 0.5% triethylamine. The product-containing fractions, eluting between 15 and 17% methanol, were pooled and evaporated to dryness in the presence of toluene. The residue was redissolved in dichloromethane and precipitated into cold *n*-hexane. The precipitate was dried *in vacuo* at 37 °C overnight. Yield: 2.46 g (overall 71%). ^{1}H NMR (DMSO-d_6), δ = 10.95 (s, broad, 1H, N^1H), 7.90 (s, 1H, H_8), 7.37–7.18, 6.86–6.83 (m, 13H, 4-MeO-C_6H_5), 5.70 (d, 1H, J = 4.36 Hz, H_1'), 5.54 (d, J = 5.6 Hz, 1H, OH), 5.15 (d, J = 6.1 Hz, 1H, OH), 4.48 (dd, 1H, H_2'), 4.19 (dd, 1H, H_3'), 3.99 (m, 1H, H_4'), 3.72 (s, 6H, 4-*Me*O-C_6H_5), 3.48, 3.41 (m, 2H, H_5', H_5''), 3.15 (s, 3H, NCMeN*Me*$_2$), 3.08 (s, 3H, NCMeN*Me*$_2$) , 2.09 (s, 3H, NC*Me*NMe$_2$).

5'-O-(4,4'-Dimethoxytrityl)-2'-O-(tert-butyldimethylsilyl)-N^6-[1-(dimethylamino)ethylidene]isoguanosine (**7b**). To a solution of **6b** (0.90 g, 1.37 mmol) in pyridine (10 mL) was added silver nitrate (0.35 g, 1.50 equiv) and a solution of TBDMSCl (0.23 g, 1.10 equiv) in THF (15 mL). The reaction mixture was then stirred at room temperature in the dark for 1.5 h, after which time a further 1 equiv of TBDMSCl (0.21 g) together with 1.2 equiv of silver nitrate (0.28 g) was added over 4 h (TLC, S4: R_f product, 0.58; R_f educt, 0.37). The reaction mixture was then filtered into brine solution, which was extracted with dichloromethane (3 × 10 mL). The organic phase was dried over sodium sulfate and filtered, and the filtrate was evaporated to dryness in the presence of toluene.

The crude product was redissolved in dichloromethane and purified by flash column silica gel chromatography; it eluted between 5 and 10% methanol in dichloromethane. The product-containing fractions were pooled and coevaporated to dryness in the presence of toluene. The residue was redissolved in ethyl acetate and precipitated into cold *n*-hexane, and the white product was collected and dried at 40 °C overnight. Yield: 0.57 g (54%). ^{1}H NMR (DMSO-d_6) δ = 10.94 (s, broad, 1H, N^1H), 7.90 (s, 1H, H_8), 7.39–7.21, 6.88–6.85 (m, 13H, 4-MeO-C_6H_5), 5.72 (d, J = 5.0 Hz, 1H, H_1'), 5.06 (d, J = 6.2 Hz, 1H, 3'-OH), 4.59 (dd, J = 6.4 Hz, 1H, H_3'), 4.01 (m, 1H, H_4'), 3.73 (s, 6H, 4-*Me*O-C_6H_5), 3.48 (m, 1H, H_5'), 3.41 (M, 1H, H_5''), 3.21 (d, J = 3.6 Hz, 1H, H_2'), 3.14 (s, 3H, CMeN*Me*$_2$), 3.08 (s, 3H, CMeN*Me*$_2$), 2.08 (s, 3H, NC*Me*NMe$_2$), 0.78 (s, 9H, SiC*Me*$_3$), 0.01 (s, 3H, Si*Me*), –0.05 (s, 3H, Si*Me*).

5'-O-(4,4'-Dimethoxytrityl)-2'-O-(tert-butyldimethylsilyl)-N^6-[1-(dimethylamino)ethylidene)isoguanosine 3'-[O-(2-cyanoethyl) N,N'-diisopropylphosphoramidite] (**8b**). Compound **7b** (250 mg, 0.325 mmol) was dissolved in dry dichloromethane, and *N,N*-diisopropylethylamine (0.22 mL,

Scheme 1



4 equiv), *N*-methylimidazole (26 μL, 1 equiv), and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.14 mL, 2 equiv) were added under argon. After the solution was stirred under argon at room temperature for 2 h, a further 1.5 equiv of the phosphoramidite (0.11 mL) together with 2 equiv of diisopropylethylamine (0.11 mL) was added over 3 h (TLC, S2: R_f product, 0.62; R_f educt, 0.60). Methanol (1 mL) was then added, followed by dichloromethane (10 mL). The solution was washed with 1 M triethylammonium bicarbonate (pH 7.5) (2 × 5 mL). The combined organic phases were dried over sodium sulfate and filtered, and the filtrate was concentrated in the presence of toluene to afford an oil, which was purified by flash column silica gel chromatography. The product was eluted using dichloromethane/ethylacetate/triethylamine (v/v, 10:10:1). The required fractions were pooled and coevaporated in the presence of toluene. The oil obtained was dissolved in dry dichloromethane and precipitated into *n*-hexane cooled with dry ice/acetone. The white product was collected by centrifugation at 4 °C and dried *in vacuo* at room temperature overnight. Yield: 255 mg (81%). ^{31}P NMR (CD_3CN) 151.40 (s, 41%), 150.05 (s, 56%).

RESULTS

Isoguanosine was reacted with the acylating agents isobutyryl chloride, benzoyl chloride, and phenoxyacetyl chloride to yield the corresponding N^6-acylisoG derivatives (Scheme 1). While the N^6-isobutyrylisoG was isolable and stable, care had to be taken to separate the product from NH_4Cl, which precipitated out upon addition of ammonia required for removal of the trimethylsilyl transient protecting groups. Incomplete separation of the acylated isoguanosine from this side product invariably resulted in the loss of the acyl protecting group. Use of aqueous hydrofluoric acid for removal of the trimethylsilyl groups of N^6-acylisoG gave unsatisfactory yields.

When the removal of the different acyl protecting groups was examined in the presence of aqueous ammonia, it was observed that the benzoyl protecting group was more labile than the isobutyryl group, which in turn was more readily cleaved than the phenoxyacetyl group. The times for complete removal of the base-protecting group at 55 °C, in 32% aqueous ammonia/ethanol (3:1, v/v), as followed by TLC at various time points, were 16 min, 40 min, and 12 h for the benzoyl, isobutyryl, and phenoxyacetyl groups, respectively. This represents a surprising inverse in trend in base lability when compared to the removal of these groups from either adenosine or guanosine (Lehmann et al., 1989; Westman et al., 1993; Seela & Wenzel, 1992).

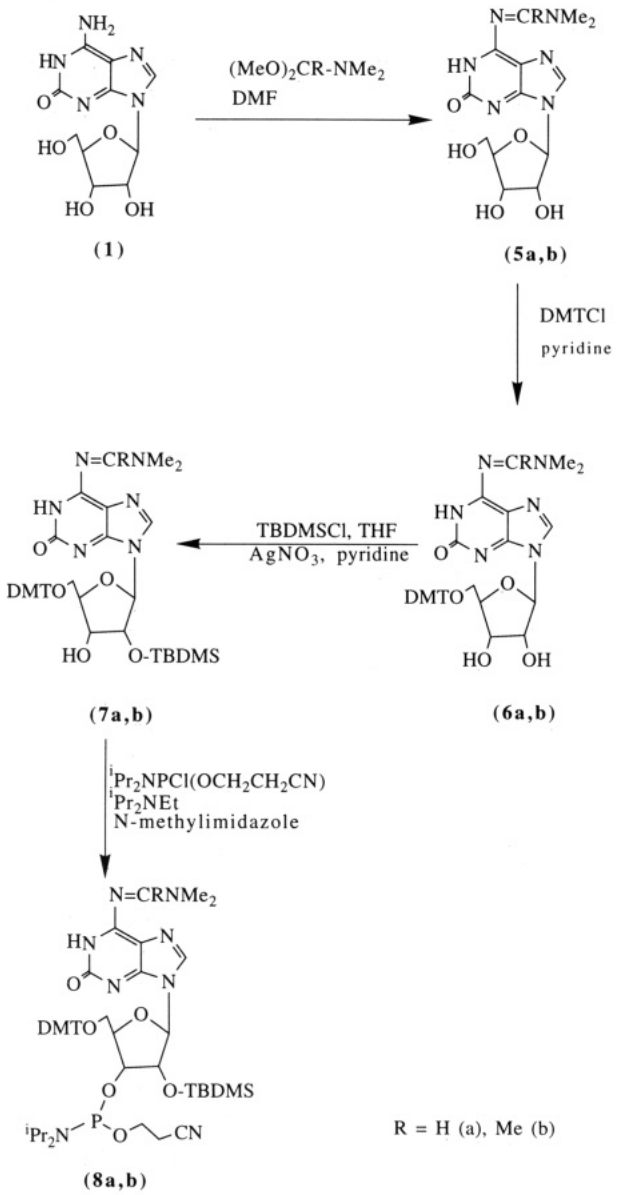
N^6-IsobutyrylisoG (**2a**) could be further reacted to 5′-DMT-N^6-isobutyrylisoG (**3a**). However, attempts to purify the product by silica gel column chromatography, either in the absence or in the presence of varying amounts of Et_3N, invariably led to significant amounts of degradation on the column. Only yields of 34% for 5′-DMT-N^6-isobutyrylisoG (**3a**) at best could be obtained after the single step of column purification. Reaction of **3a** with TBDMSCl according to the general protocol described previously (Tuschl et al., 1993) gave only a trace of a compound which had the expected TLC mobility. Loss of the DMT group as well as of the isobutyryl protecting group was also indicated by TLC. For these reasons, and as the yields of the other acyl-protected isoG derivatives were low, it was decided not to pursue further the acyl groups for the protection of isoG for oligoribonucleotide synthesis.

Instead, reaction of isoguanosine with *N,N*-dimethylacetamide dimethyl acetal and subsequent dimethoxytritylation gave the corresponding 5′-*O*-(4,4′-dimethoxytrityl)-N^6-[1-(dimethylamino)ethylidene]isoG nucleoside (Scheme 2, **6b**) in good yields after silica gel purification. It was further converted into the 2′-*O*-TBDMS 3′-*O*-phosphoramidite derivative **8b**, which could be purified by silica gel column chromatography.

When N^6-[1-(dimethylamino)ethylidene]isoG phosphoramidite **8b** was employed in the solid-phase synthesis, successful coupling as well as chain elongation was observed. Isoguanosine was incorporated at three positions in the hammerhead ribozyme (Figure 1). The integrity of isoguanosine incorporation into a 34-mer ribozyme was confirmed by nuclease and phosphatase digestion. Figure 2a shows the HPLC analysis at 260 nm of the digest of the ribozyme containing isoG at the A6 position. The peak eluting at 4.82 min was identified as isoG, since it coelutes with an authentic sample. In addition, in neutral solutions the absorbance of isoG has a maximum at 292 nm (Sepiol et al., 1976) and is virtually minimal at 260 nm, in contrast to the standard bases, which have virtually no absorption at 292 nm. Therefore, analysis of the digest at 292 nm strongly indicates the presence of isoG eluting at 4.73 min, significantly increased in comparison to the diminished peaks for uridine and adenosine (Figure 2b). This confirms that isoG was successfully incorporated into the oligoribonucleotide. Moreover, gel electrophoresis of the 5′-labeled ribozymes containing isoG clearly confirms that the synthesis yielded the required full-length product (Figure 3).

The activity of the hammerhead ribozyme with each mutation was investigated under substrate saturation conditions at both 500 and 800 nM and at 25 °C and pH 7.5. The rates of cleavage were measured and compared relative to the wild type, and the results are summarized in Table 1. The cleavage rates were measured at 500 nM substrate, at appropriate time intervals, and similar rates were also obtained at 800 nM substrate. Single mutations of the adenosines at positions 6 and 9 to guanosine resulted in significant decreases of ribozyme activity of 77- and 120-fold, respectively, whereas no cleavage activity could be detected for the G13 ribozyme (J. Thomson and F. Eckstein, unpublished). Mutations to

Scheme 2



2-aminopurine nucleoside at these positions decreased activities 34-, 5-, and 7-fold, respectively. With mutations at the same positions to isoguanosine, the activity was restored close to that of the wild type, with only a 2-fold decrease in activity observed. Mutation of A at position 15.1 to isoguanosine decreased activity to approximately half.

DISCUSSION

We are interested in an understanding of the structure–function relationship in the hammerhead ribozyme (Figure 1). One approach for addressing this problem is the incorporation of unnatural nucleosides in the central core of the ribozyme (Fu & McLaughlin, 1992a,b; Fu et al., 1993; Odai et al., 1990; Slim & Gait, 1992; Seela et al., 1993; Tuschl et al., 1993). Recently we have shown that the formamidine-base-protected isoguanosine phosphoramidite (**8a**) could only be incorporated as a terminal nucleotide in the solid-phase synthesis of oligoribonucleotides (Tuschl et al., 1993). Although this method gives the desired products, it is not amenable to routine synthesis. Isoguanosine-containing oligoribonucleotides of 10–12 nucleotides in length have been chemically synthesized using the formamidine isoguanosine

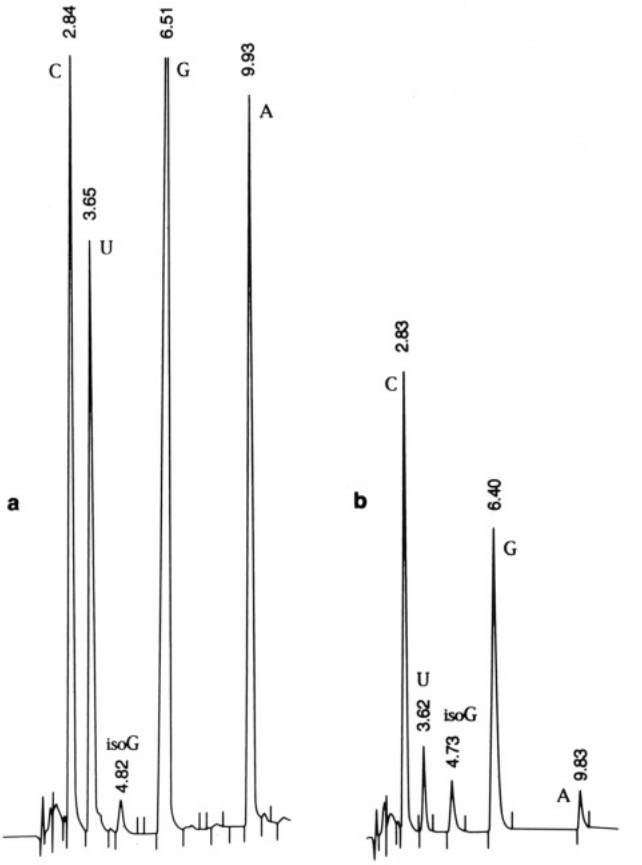


FIGURE 2: HPLC analyses of nucleoside composition of the oligoribonucleotide containing isoG at the A6 position after enzymatic digestion. Details are described in Materials and Methods. a, digest measured at 260 nm; b, digest measured at 292 nm.

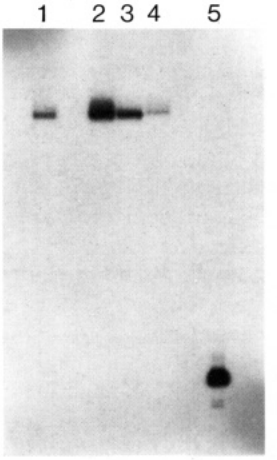


FIGURE 3: Gel electrophoretic analysis of 5′-^{32}P-labeled ribozymes containing isoguanosine. Lane 1, unmodified 34-mer ribozyme; lanes 2, 3, and 4, ribozymes containing isoG substitutions, at the A6, A9, and A13 positions, respectively; lane 5, 12-mer oligoribonucleotide substrate.

H-phosphonate as a building block (Seela & Fröhlich, 1994). The authors have noted that the coupling yields were diminished when an isoG was already incorporated at the 3′-end of the growing oligonucleotide chain, and they acknowledged problems in the synthesis of longer oligonucleotides (Seela & Fröhlich, 1994). Neither of these methods is well suited for the routine synthesis of isoguanosine-containing hammerhead ribozymes, 34 nucleotides in length. A more efficient synthesis, preferably using the phosphoramidite method, was therefore considered desirable.

Table 1: Comparison of Rates of Cleavage of Modified Ribozymes[a]

ribozyme	k_{obs} (min^{-1})	k_{rel}
unmodified	2.46	1
isoG/A6	1.08	0.44
isoG/A9	1.43	0.58
isoG/A13	1.51	0.61
isoG/A15.1	1.06	0.43
2AP/A6	0.073	0.030
2AP/A9	0.49	0.20
2AP/A13	0.38	0.15
G/A6	0.032	0.013
G/A9	0.021	0.0085
G/A13	<0.002	<0.0008[b]
P/A6		0.79[c]
P/A9		0.90[c]
		0.30[d]
P/A13		0.14[e]
		0.30[d]

[a] k_{obs} was determined as described in Materials and Methods. P, purine ribonucleoside; 2AP, 2-aminopurine ribonucleoside. [b] J. Thomson and F. Eckstein, unpublished. [c] Values taken from Fu and McLaughlin (1992a). [d] Values taken from Slim and Gait (1992). [e] Value taken from Fu et al. (1993).

Chemical Synthesis. Attempts toward the chemical synthesis of isoguanosine-containing ribozymes using acyl protecting groups for the 6-amino function of isoguanosine were unsuccessful. Although isoguanosine could be acylated to give the N^6-acylisoguanosine derivative, these protecting groups were not of sufficient stability for further reactions. Protection using the amidine group was therefore explored (Zemlicka et al., 1966; Froehler et al., 1983; McBride et al., 1986; Vu et al., 1990; Vinayak et al., 1992; Theisen et al., 1993, and references therein; Switzer et al., 1993). We had shown earlier that isoguanosine could be incorporated only into terminal positions of oligoribonucleotides using the formamidine protecting group (Tuschl et al., 1993). We show here that, in contrast, the acetamidine phosphoramidite of isoguanosine is suitable for the synthesis of ribozymes containing isoguanosine in internal positions. The failure to obtain such ribozymes with the formamidine derivative is explainable by loss of the protecting group under oligonucleotide synthesis conditions as has been noted for deoxyadenosine (McBride et al., 1986; McBride & Caruthers, 1983; Theisen et al., 1993, and references therein; Sonveaux, 1986). This loss facilitates subsequent phosphitylation of the exocyclic amine, which results in higher molecular weight "branched" oligodeoxynucleotide impurities (Gryaznov & Letsinger, 1991, 1992). The marked change in stability with use of the acetamidine-protected isoG in oligoribonucleotide synthesis makes this base-protecting group superior to formamidine, and the protecting group of choice in this case.

Kinetic Analysis. The incorporation of additional exocyclic functions at a nucleobase in the ribozyme central core might result in steric repulsion. Such modifications, in contrast to deletion modification of a particular exocyclic function, should display a much more pronounced effect on the destabilization of the transition state because of steric interference with the transition-state geometry. We substituted the 2-hydrogen atom of adenosines by an amino or a carbonyl function by using isoG and 2AP to monitor the influence of the 2-position of conserved adenosines on the chemical cleavage rate of the hammerhead ribozyme. Kinetic analysis of the activity of the hammerhead ribozymes containing isoG instead of adenosine at positions 6, 9, and 13 were undertaken (Table 1). It can be seen that isoguanosine can replace adenosine at each of these positions with not more than a 2-fold reduction in activity. It is thus a better substitute than the 2-aminopurine nucleoside, which shows a reduction in rate of 5- to 30-fold. This in turn is more efficient in supporting catalysis than guanosine at these positions. The value for the ribozyme with G at position 9 is in qualitative agreement with that reported by Ruffner et al. (1990) who observed a reduction in rate by a factor of 300 with guanosine at this position. However, there are discrepancies for the ribozyme with guanosine at position 6, where these authors could not detect any activity, and for that with G at position 13, which we found to be inactive. These discrepancies might be the result of the different ribozyme construct and the different method for the determination of the rate chosen by these authors. The effects of isoG and 2AP have been examined previously at positions 5, 8, and 12 where a guanosine is normally located (Tuschl et al., 1993). The observation made there was that activities of the ribozymes with these substitutions at all three positions were much more reduced than reported here for positions 6, 9, and 13. We have also investigated the kinetic consequences of substitution of A at position 15.1 by isoG. This substitution reduced activity to approximately half, indicating that the isoG/U base pair can be formed as would be expected (Tuschl et al., 1993) and that the 2-keto group does not interfere with transition-state formation.

Deletion-modified ribozymes that carried the purine ribonucleoside nebularine instead of adenosine at position 9 or 13 have been synthesized by Slim and Gait (1992). They found a reduction in cleavage rate of a factor of 3 for each of these modifications, although these values were not the result of a detailed kinetic analysis. Analogous nebularine substitutions at positions 9 and 13 by Fu et al. (1993) displayed a rate reduction by a factor of 1.1 and 7, respectively. The substitution of A6 by nebularine resulted in a 1.3-fold decreased cleavage activity (Fu & McLaughlin, 1992a). Thus, the presence of the A6, A9, or A13 6-amino group is not important for transition-state stabilization of the cleavage reaction.

Adenosine 6 is distinct from A9 or A13 as it does not tolerate a 2-amino group substitution. Although the isoG and nebularine modifications are active, the activity of the ribozyme with 2AP at position 6 is decreased 30-fold. Thus, the 2-position of A6 tolerates a carbonyl function but not an amino group. The guanosine modification when compared to 2AP decreased the activity further only by a factor of 3, which suggests that the 6-position of A6 can carry either a hydrogen, an oxygen, or an amino group. Furthermore, the fact that A6 is the only purine of the central core that does not tolerate 7-deaza substitution (Fu & McLaughlin, 1992b) indicates that its structural role in the transition state is distinct from that of A9 or A13.

It has been suggested that there are G12/A9 and A13/G8 mismatched base pairs in the hammerhead ribozyme (Li et al., 1991; Slim & Gait, 1992). So far, there are no structural studies of the hammerhead central core domain available such as NMR spectroscopy (Heus & Pardi, 1991) or X-ray crystallographic data (Pley et al., 1993), and thus the presence or absence of G/A pairs has to be discussed solely on the basis of functional group modification experiments. On the basis of the reactivity of functional group modified ribozymes, there seems to be no evidence that GA/AG "double mismatched" or "tandem" base mispairs (SantaLucia & Turner, 1993) (Figure 4a) are present but that these nucleotides must be involved in different tertiary interactions (Tuschl et al., 1993; Seela et al.,1993; Fu et al., 1993). The results reported here support this view. The high reactivity of ribozymes with changes of A9 and A13 to isoG or 2AP suggest that the 2-position of these adenosines can be either a keto or an amino

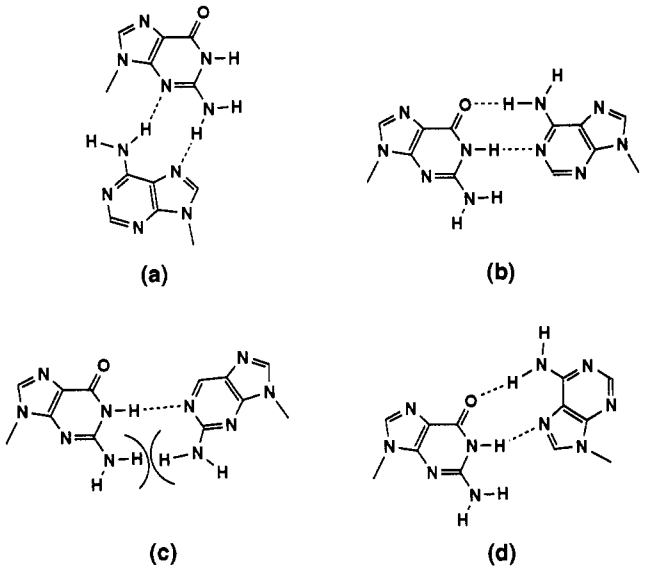


FIGURE 4: Possible G/A mismatches. a, reversed Hoogsteen G(anti)/A(anti) or "tandem" mismatched structure; b, Watson–Crick type G(anti)/A(anti); c, steric repulsion in a Watson–Crick type G(anti)/2AP(anti) base pair; d, Hoogsteen G(anti)/A(syn).

group without interference with transition-state geometry. We can therefore exclude G12/A9 and A13/G8 pairing of the G(anti)/A(anti) Watson–Crick type (Figure 4b) because the 2-amino groups of a G(anti)/2AP(anti) base pair should show strong repulsion (Figure 4c) and therefore severely reduced ribozyme cleavage activity. The existence of the related Hoogsteen G(anti)/A(syn) pairing (Figure 4d) or the guanosine 2-amino group involving G(anti)/A(anti) mispaired structure cannot be excluded, however, by these results. That the presence of G in positions 9 and 13 leads to such strong inhibition of activity might be a consequence of alternative tertiary structure by different hydrogen-bonding or stacking interactions. Even though there is no evidence for G/A double mismatch base pairs, due to the structural diversity of G/A mismatches one might still consider the presence of two structurally distinct G12/A9 and A13/G8 base mispairs in the ribozyme, as these G/A pairs display different effects on ribozyme cleavage if mutated in the same way. For example, A9 to P9 substitution reduces cleavage activity only 1–3-fold (Fu & McLaughlin, 1992a; Slim & Gait, 1992); A13 to P13 substitution reduces it by a factor of 3–7 (Slim & Gait, 1992; Fu et al., 1993). Similarly, G12 to I12 substitution results in a 380-fold rate reduction, whereas G8 to I8 substitution decreases the rate only 3-fold (Tuschl et al., 1993). These observations suggest that both G/A pairs, if they exist, are structurally distinct species.

The individual energetic contributions of exocyclic base functional groups to transition-state stabilization must not necessarily reflect the thermodynamically most stable orientations of G/A base pairs, as they were found in duplex structures (SantaLucia & Turner, 1993; Greene et al., 1994). In fact, the unsymmetrical environment of the central core suggests that, similar to ribosomal RNA (Traub & Sussman, 1982), a single G/A mismatch is sufficient to introduce a break in the double-helical structure of stem II to produce a nonlinear tertiary structure. The geometry of this helix-breaking G12/A9 base pair at the end of helix II might still be related to known G/A pairings, whereas G8 and A13 could be involved in other tertiary interactions.

In conclusion, we have shown that the phosphoramidite of isoG can be synthesized and chemically incorporated into a 34-mer hammerhead ribozyme by using (dimethylamino)-acetamidine as the base-protecting group. Mutations of the adenosines at positions 6, 9, and 13 to isoguanosine and of those at positions 9 and 13 to 2-aminopurine ribonucleoside affect activity only slightly, whereas that to 2-aminopurine ribonucleoside at position 6 reduces activity 30-fold. The results make the existence of G(anti)/A(anti) mismatched base pairs of the Watson–Crick type in the central core very unlikely.

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