Nucleic Acids Research, Vol. 19, No. 15 4161–4166

Synthesis of RNA containing inosine: analysis of the sequence requirements for the 5′ splice site of the *Tetrahymena* group I intron

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Received April 22, 1991; Revised and Accepted July 9, 1991

ABSTRACT

Two protected derivatives of the ribonucleoside inosine have been prepared to serve as building blocks for phosphoramidite-based synthesis of RNA. Two different synthetic routes address the unusual solubility characteristics of inosine and its derivatives. The final products of the different synthetic pathways, 5′-*O*-(dimethoxytrityl)-2′-*O*-(*t*-butyldimethylsilyl) inosine 3′-*O*-(β-cyanoethyldiisopropylamino) phosphoramidite 5a, and O^6-*p*-nitrophenylethyl-5′-*O*-(dimethoxytrityl)-2′-*O*-(*t*-butyldimethylsilyl) inosine 3′-*O*-(methyldiisopropylamino) phosphoramidite 5b, were chemically incorporated into short oligoribonucleotides which also contained the four standard ribonucleoside bases. The oligomers were chosen to study base-specific interactions between an RNA substrate and an RNA enzyme derived from the Group I *Tetrahymena* self-splicing intron. The oligomers were shown to be biochemically competent using a *trans* cleavage assay with the modified *Tetrahymena* intron. The results confirm the dependence of the catalytic activity on a wobble base pair, rather than a Watson-Crick base pair, in the helix at the 5′-splice site. Furthermore, comparison of guanosine and inosine in a wobble base pair allows one to assess the importance of the guanine 2-amino group for biological activity. The preparation of the inosine phosphoramidites adds to the repertoire of base analogues available for the study of RNA catalysis and RNA-protein interactions.

INTRODUCTION

Inosine is a purine analogue of guanosine which lacks the 2-amino group of the guanine base. It can form both a base pair with cytidine with a standard Watson-Crick geometry and a base pair with uridine in a wobble geometry (Figure 1). Its ability to form wobble base pairs allows it to play an important biological role as a component of the anticodon loop of tRNA molecules (1). In the area of chemical synthesis of nucleic acids some progress has been made in exploiting the wobble base-pairing properties of the hypoxanthine base. In particular, 2′-*deoxy*inosine has been incorporated into probes to serve as a 'universal' base that discriminates minimally between matched and mismatched base pairs (2,3). This work has involved exclusively *deoxy*oligonucleotides, however, and a phosphoramidite synthon for the incorporation of inosine into synthetic RNA would be useful.

A method for incorporating inosine into RNA polymers will facilitate studies of the role of inosine in RNA-RNA and protein-RNA interactions. Such a synthon will gain still greater value as methods for the non-enzymatic synthesis of RNA improve. Alkylsilyl protecting groups for the 2′-hydroxyl position in conjunction with the phosphoramidite method (4) have now permitted the synthesis of RNA molecules up to 77 nucleotides in length (5) that have modest biological activity and oligomers up to 36 nucleotides in length that have full biological activity (6). An inosine synthon having the 2′-hydroxyl protected as a tetrahydropyranyl derivative has previously been reported (7).

This report describes methods for protecting inosine and for its incorporation into oligoribonucleotides using RNA phosphoramidite chemistry. The 5′-hydroxyl group is protected with the dimethoxytrityl (DMT) group and the 2′-hydroxyl with the *t*-butyldimethylsilyl (TBDMS) group. The 3′-hydroxyl is derivatized as either a methyl or cyanoethyl diisopropylamino phosphoramidite. To address the unusual solubility characteristics of inosine, two different synthetic routes were followed, one of which involved protection of the *O*6 base position with the *p*-nitrophenylethyl (NPE) group (8) to increase the solubility of the intermediates.

The specific motivation for preparing inosine containing RNA in this work was to study the effect of different base pairing interactions at the cleavage site in the self-splicing reaction of the *Tetrahymena* intron. The U:G base pair (Figure 1 and 2) at the splice junction (Figure 2) is completely conserved among Group I introns known to self-splice *in vitro* (9). Mutagenesis of the *Tetrahymena* intron suggested a requirement for a wobble base pair at the 5′ exon-intron junction where the first cleavage occurs (10). In a *trans* cleavage assay (Figure 2), of all possible base pair combinations at the splice site (using the four standard

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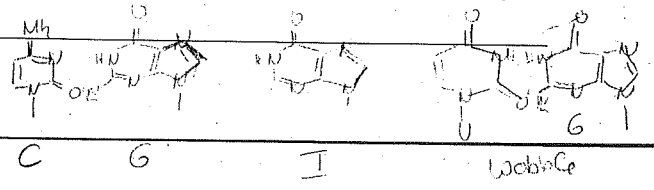


bases), only a C:A wobble base pair substitution retains a level of cleavage activity comparable (50%) to the wild type U:G. These active base pairs project different functional groups into the major and minor grooves, suggesting that the conformation of the wobble base pair in the helix, rather than the particular bases themselves, is recognized by the catalytic core. We chose to extend these studies to determine whether a U:I wobble base pair would similarly substitute for the standard U:G wobble base pair.

RESULTS AND DISCUSSION

Synthesis of Protected Inosine Phosphoramidites

The synthetic routes for the preparation of **5a** and **5b** are shown in Scheme 1. The first step in both pathways is the protection of the 5′-ribose hydroxyl group with the dimethoxytrityl protecting group. Standard conditions for this reaction in THF, CH_2Cl_2 and pyridine proved unsuccessful due to the insolubility of inosine in these solvents. As in the case of the protection of dI with the DMT group (3), very polar solvents such as DMF and DMSO (50/50 with pyridine) were required to achieve efficient tritylation. Addition of the DMT group increased the chromatographic mobility of the product, allowing for simple purification of **2** by silica gel chromatography.

The 2′-hydroxyl group of **2** was protected by reaction with *t*-butyldimethylsilyl chloride using pyridine as the solvent and mild base and $AgNO_3$ as a catalyst. This salt has been reported to increase the silylation selectivity toward the 2′- rather than the 3′-hydroxyl group (11). Care was taken to avoid generating the 2′,3′-bis-silyl product, which is difficult to separate from the 2′-*O*-silyl isomer on silica gel columns. The reaction was complete in several hours to yield **4a**. The desired 2′-*O*-silyl isomer was easily separated from the 3′-*O*-silyl isomer by silica gel chromatography. Interestingly, not only does the 2′-*O*-silyl isomer elute first (as it does for all four standard RNA bases—A, C, G, U) but the 3′-*O*-silyl isomer did not elute from the column under the solvent conditions used (solvent C in experimental). Purified **4a** is a dry, white, flaky solid that is insoluble in the solvents used in standard phosphitylation reactions (THF and CH_2Cl_2). The phosphitylation reaction was ultimately successful using THF/collidine (3/1) as a solvent system in which **4a** is marginally soluble. The reaction was complete in 1–2 hours and the compound **5a**, though difficult to resolve from starting material, can be purified from remaining phosphine reagent, *N*-methylimidazole and collidine on a silica column with solvent D, although considerable trailing was seen. Indeed, no TLC conditions are completely satisfactory for separating **4a** from **5a** due to the large amount of smearing seen in all solvent combinations tried.

The quantity of contaminating starting material is easily measured by ^1H NMR and was less than 1% in our preparation. The final product **5a** is a white foam which is readily soluble in standard solvents including acetonitrile, the solvent used to dissolve the phosphoramidite for the coupling reaction.

Deoxyinosine phosphoramidite has been synthesized without protection at the *O6* position and both ribo- and deoxy- guanosine are routinely used as protected phosphoramidites without *O6* protection. We therefore assumed initially that protection of the *O6* keto position would not be necessary. However, protecting the polar keto group (pathway **B**) was an obvious approach for alleviating the solubility problems encountered in pathway **A**. Thus, in pathway **B**, the *O6* position of the hypoxanthine base is protected as the *p*-nitrophenylethyl ether. We found the 'transient protection' Mitsunobu reaction (12) to be the simplest and the most effective method for this transformation. While pure **3** could not be isolated with one column chromatographic step, the material contaminated with triphenylphosphine oxide was sufficiently pure to permit direct silylation. As predicted, following the introduction of the *O6* protecting group, the subsequent intermediates were more soluble (**4b** *vs* **4a**) and more easily resolved on silica columns (**5b** *vs* **5a**) than their *O6* unprotected counterparts. In contrast to the results reported for deoxyguanosine by Gao *et al.* (12), column resolution following the Mitsunobu reaction was better when inosine had already been protected with the DMT group.

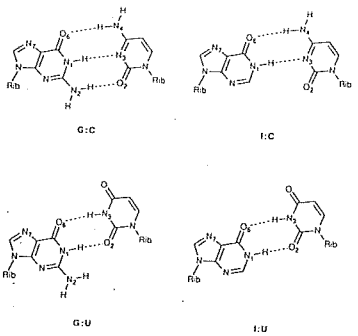
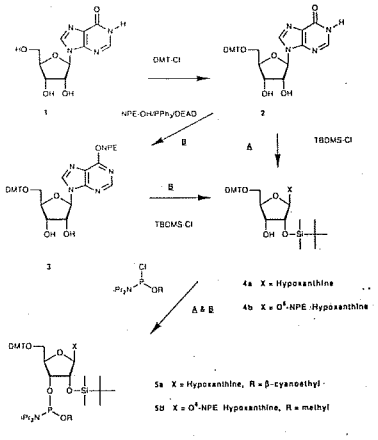


Figure 1. Watson-Crick base pairing between C:G and C:I. Wobble base pairing between U:G and U:I.



Scheme 1: Synthetic scheme for the preparation of 5a & b.

The silylation of **3** proceeded without difficulty in DMF containing imidazole; both the 2′-*O*-silyl and 3′-*O*-silyl isomer eluted from the column in the expected order (2′-*O*-silyl isomer first). The number of equivalents of TBDMS-Cl added was again minimized to avoid generating the 2′,3′-bis-silyl product. Compound **4b**, quite soluble in standard solvents (in contrast to 4a), was phosphitylated in CH_2Cl_2 using bis(diisopropylamino) methoxy phosphine (13,14). The final product **5b** (R_f = 0.6) was easily separated from starting material **4b** (R_f = 0.2) by TLC and by silica gel chromatography. It was therefore simple both to monitor the progress of the reaction and to separate the desired product from unreacted starting material.

It appears that either pathway **A** or **B** is suitable for the preparation of an inosine phosphoramidite which may be incorporated into an RNA molecule. While pathway **A** has the advantage of requiring one less step in the synthetic pathway, pathway **B** has the advantage of having intermediates which are easier to handle since they are soluble in most organic solvents. In addition, the protecting group at *O*6 reduces the possibility of adduct formation at *O*6 during oligonucleotide synthesis (15). Since the NPE group is removed during the treatment with tetra-*n*-butylammonium fluoride (TBAF) (16) that is required to remove the silyl protecting groups, the use of the NPE protecting group in RNA synthesis imposes no additional deprotection step. From the biochemical results (*vide infra*) there is no difference in the quality of the short oligoribonucleotides synthesized with or without O^6-NPE protection. For general use, **5a** is recommended, as its synthesis requires fewer steps. However, for large scale preparation of phosphoramidite, **5b** may be beneficial due to its increased solubility and simpler chromatographic behaviour.

Finally, we have fully characterized the protected ribonucleoside compounds **2**, **3**, **4a**, **4b**, **5a**, **5b** by ^{1}H NMR, the results of which are shown in Tables 1 and 2. ^{31}P NMR data for **5a** and **5b** appear in the experimental section.

Solid Phase Synthesis

The sequences shown in Table 3 were synthesized on a Milligen/Biosearch Cyclone automated nucleic acid synthesizer as described previously (6). Syntheses were conducted on a 1 μmol scale using derivatized 1000 Å CPG solid supports. Other reagents and details are described in the experimental section.

The first three sequences in Table 3 represent the splicing templates (IGS, internal guide sequence) for the *trans* cleavage assay described below. The sequence **r9G** is the wild type template. The sequence **r9I**, the template containing inosine in place of guanosine at the splicing recognition site, was synthesized using ribonucleoside phosphoramidite 5a. The sequence **r9I′** is identical to **r9I** except that it was prepared with the O^6-NPE-protected phosphoramidite, **5b**.

The last two sequences correspond to complementary strands used for the aminoacylation of simple RNA helices as recently described by Musier-Forsyth *et al.* (17). The **I3:U70** sequence introduces an inosine substitution at the G3:U70 recognition site of *E. coli* alanine aminoacyl synthetase. The **I4:C69** sequence retains the wildtype G3:U70 recognition site but introduces an inosine base pair at a site just below the determinant base pair. The biochemical results of this study will be described elsewhere (18).

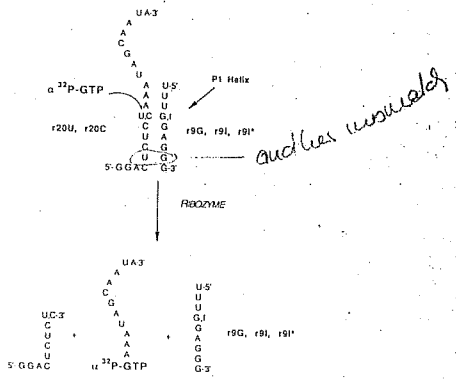


Figure 2. Cleavage assay used to study the splice site requirements of the *Tetrahymena* intron oligonucleotide substrates, **r20U** or **r20C**, anneal to **r9G**, **r9I** or **r9I′** to form the substrate with U:G, U:I, C:G or C:I base pairs at the splice junction. Ribozyme and α^{32}P-GTP carry out cleavage of the substrate resulting in the production of a labeled 12-mer.

Table 1 ^{1}H Chemical Shifts (ppm, $CDCl_3$)

	H8	H2	H1′	H2′	H3′	H4′	H5′	H5″	O-CH_3 (DMT)	P-O-CH_2-CH_2-CN or P-O-CH_3 [&/or NPE CH_2's]	tBu-Si	Me-Si	Me-Si
2†	8.26 s	8.06 s	5.97 d	4.65 t	4.30 t	4.12 dd	3.25 d	3.25 d	3.78 s				
3	8.00 s	7.53 s	5.95 d	4.46 dd	4.33 dd	4.23 m	3.41 ABq	3.37 ABq	3.75 s	[4.74 t - 3.16 t NPE (J_{HH} = 5.4 Hz)]			
4a	8.03 s	8.03 s	6.02 d	4.90 t	4.34 m	4.26 m	3.52 ABq	3.39 ABq	3.78 s		0.86 s	-0.01 s	-0.12 s
4b	8.00 s	7.44 s	5.91 d	4.31 m	4.28 m	4.24 m	3.46 ABq	3.34 ABq	3.78 s	[4.69 t - 3.23 t NPE (J_{HH} = 5.3 Hz)]	0.83 s	-0.02 s	-0.19 s
5a‡	8.02 s	7.92 s	5.93 d	4.87 t	4.40 m	4.31 m	~3.5 m	~3.4 m	3.75 s	3.95 m - 2.27 t (J_{HH} = 6.1 Hz)	0.74 s	-0.05 s	-0.22 s
	7.99 s	7.93 s	5.98 d	4.86 t	4.39 m	4.30 m	~3.5 m	~3.4 m	3.75 s	3.93 m - 2.26 t (J_{HH} = 6.1 Hz)	0.73 s	-0.06 s	-0.22 s
5b‡	8.02 s	7.47 s	5.96 d	4.38 m	4.32 m	4.25 m	~3.5 m	~3.4 m	3.78 s	3.42 d (J_{P-H} = 13 Hz) [4.88 t - 3.28 NPE (J_{HH} = 5.3 Hz)]	0.78 s	-0.00 s	-0.03 s
	7.99 s	7.44 s	5.87 d	4.38 m	4.32 m	4.25 m	~3.5 m	~3.4 m	3.78 s	3.19 d (J_{P-H} = 13 Hz) [4.82 t - 3.22 NPE (J_{HH} = 5.3 Hz)]	0.76 s	-0.21 s	-0.23 s

ABq = AB quartet, d = doublet, dd = doublet of doublets, m = multiplet, s = singlet, t = triplet, † = DMSO-d_6, ‡ = Two diastereomers.

Trans Cleavage Assay

The *Tetrahymena* self-splicing RNA, the most extensively studied Group I intron, shows a preference for wobble pairs (U:G and C:A) over Watson-Crick base pairs at the 5′ splice site of the intron (10). A *trans* cleavage assay (depicted in Figure 2) measured the cleavage activity of a *Tetrahymena* ribozyme derivative with the P1 helix oligonucleotide combinations, U:G, C:G, U:I and C:I, which form either a wobble or a Watson-Crick base pair at the splice site. In this assay, the catalytic core of the intron is dissociated from its substrate, the 5′ splice site, allowing the use of small synthetic substrates. The stem structure representing the 5′ splice site and internal guide sequence (P1 helix) is provided in *trans* as two separate oligonucleotides which anneal to form a cleavage substrate.

The three short oligoribonucleotides (9-mers **r9G**, **r9I** and **r9I***, Table 3) represent the IGS of the P1 helix with either G or I incorporated opposite the cleavage site. The complemetary strands (20-mers **r20C** or **r20U**, Table 3) containing either U or C at the cleavage site were prepared enzymatically by T7 transcription (19). In pairwise combinations, these oligonucleotides anneal to form either a wobble base pair (U:G or U:I) or a Watson-Crick base pair (C:G or C:I).

Sub-saturating levels of the different substrate combinations were incubated with enzyme and a trace amount of α-^{32}P-GTP. The product of the cleavage reaction was a labeled 12-mer (Figure 2), which was separated from unincorporated α-^{32}P-GTP on a denaturing gel. The results shown in Figure 3 demonstrate the preference of the ribozyme for wobble base pairs over Watson-Crick base pairs at the cleavage site. No detectable activity was seen for the C:G or C:I combination or for the 5′ splice sites alone (**r20U** or **r20C**, data not shown). The U:I pairing, while clearly more efficiently recognized by the ribozyme than the Watson-Crick pairing, is recognized 2.6-fold less well than the wild type combination U:G. The activities of the oligonucleotides containing **5a** and **5b** are indistinguishable, indicating that, at the level of sensitivity of the assay, the NPE deprotection was complete.

The 2.6 fold difference in activity observed between U:G and U:I could arise *via* any of a number of subtle influences that inosine may have on the structure of the splice site. The decrease in activity correlates roughly to the difference expected from the loss of a single weak hydrogen bonding interaction. Although the absence of the 2-amino group should not disturb the wobble base pair configuration within the helix (Figure 1), it could effectively disrupt the ability of the enzyme to recognize its splice site, *i.e. via* hydrogen bonding. While specific features of the bases clearly play some role in splice site recognition, the major component is probably recognition of the internal wobble base pair induced distortion of the phosphate backbone (20,21,22).

Strict requirement for a wobble base pair has been observed in the interaction between *E. coli* alanine tRNA synthetase in its cognate RNA acceptor stem helix (23,24). In that case a wobble base pair is necessary, but not sufficient, for recognition; there is an additional requirement for the unpaired guanine 2-amino group which facilitates the correct protein-RNA contact (18). This is in contrast to the ribozyme activity described above, It appears that there are a number of factors which may affect nucleic acid molecular recognition and these factors may be explored using nucleotide analogues.

The work described here demonstrates that the chemical synthesis of RNA in general, and of RNA containing unusual base components in particular, is a viable method for the study of RNA-RNA interactions. Another example is the recent synthesis of 2-aminopurine ribonucleoside phosphoramidite (25) and its incorporation in a ribozyme substrate. The preparation of the inosine phosphoramidites described in this work adds another base component to the repertoire of analogues available for the study of RNA.

Table 2 Coupling Constants (Hz)

	$J_{1'-2'}$	$J_{2'-3'}$	$J_{3'-4'}$	$J_{4'-5'(5'')}$	$J_{5'-5''}$
2	4.6	4.8	4.9	4.2	0
3	5.6	1.9	3.2	3.9	11
4a	5.9	5.2	1.4	~3	11
4b	6.0	nr	nr	2.8	10.6
5a	6.6	nr	nr	nr	nr
5b	6.4	nr	nr	nr	nr

nr = not resolved

Table 3 RNA Sequences

Name	Sequence 5′-3′	Comments
r9G	UUU GGA GGG	Wildtype template
r9I	UUU IGA GGG	Inosine template made with 5a
r9I*	UUU IGA GGG	Inosine template made with 5b
r20U	GGA CUC UCU AAA UAG CAA UA	Primer containing U at the splice site, T7 transcript
r20C	GGA CUC UCC AAA UAG CAA UA	Primer containing C at the splice site, T7 transcript
13:U76	GGI GCU AAG	Complementary strand for aminoacylation
14:C69	GGG ICU AAG	Complementary strand for aminoacylation

EXPERIMENTAL

General Materials and Methods

The synthesis of the ribonucleoside intermediates and the phosphoramidites, the automated synthesis of the oligoribonucleotides, and the deprotection of the synthetic RNA were performed according to the recently published methods of Scaringe *et al.* (6). Enzymes were obtained from Perkin-Elmer Cetus and New England Biolabs. Ultraviolet (UV) spectra and colorimetric trityl determinations were obtained on a Hewlett Packard 8451A spectrophotometer. Radioactive gels were quantitated on a Betascope two-dimensional array detector (Betagen, Waltham, MA).

NMR Analysis

^{1}H and ^{31}P nuclear magnetic resonance (NMR) spectra were obtained on a Varian XL-300 spectrometer equipped with a Varian 5 mm broad band probe. ^{1}H NMR spectra were

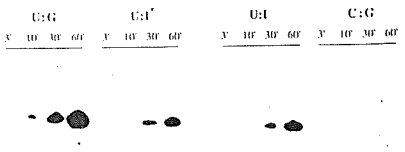


Figure 3. Autoradiogram of 20% polyacrylamide/7 M urea gel showing time courses of cleavage assay with U:G, U:I*, U:I and C:G at the splice site. Reactions were incubated at 45°C for the times indicated to produce the ^{32}P labeled 12-mer.

referenced to the internal $CHCl_3$ signal in the samples, δ=7.24 ppm. ^{31}P NMR chemical shifts were referenced to an external 85% H_3PO_4 standard. $CDCl_3$ (Cambridge Isotopes) was used as lock.

Chromatographic Solvent Systems:

A	95:5	/ CH_2Cl_2:MeOH
B	77:20:3	/ CH_2Cl_2:Acetone:MeOH
C	35:25:40	/ Hexanes:CH_2Cl_2:Acetone
D	25:35:38:2	/ Hexanes:CH_2Cl_2:Acetone:Triethylamine
E	20:30:40:10	/ Hexanes:CH_2Cl_2:Acetone:Triethylamine
F	97:3	/ $CHCl_3$:MeOH
G	87:10:3	/ CH_2Cl_2:Et_2O:MeOH
H	90:8:2	/ CH_2Cl_2:THF:MeOH
I	92:6:2	/ CH_2Cl_2:THF:MeOH
J	50:50	/ CH_2Cl_2:Et_2O
K	58:40:2	/ Hexanes:CH_2Cl_2:Triethylamine

Synthesis of 5′-*O*-(Dimethoxytrityl) inosine (2)

Inosine **1** (20 mmol, 1 eq) was coevaporated twice with pyridine and was then dissolved in 200 ml DMF and 40 ml pyridine. Dimethoxytrityl chloride (24 mmol, 1.2 eq) was dissolved in 30 ml pyridine and added slowly over 2 hr (5 ml per addition). The reaction was monitored by TLC (Solvent A; R_f=0.65) and was complete in 5 hr. Imidazole (40 mmol, 2 eq) was added and the solvent was removed *in vacuo*. The resulting residue was coevaporated twice with toluene (150 ml) and was resuspended in CH_2Cl_2 (200 ml) which was extracted with 5% $NaHCO_3$ (200 ml) and with saturated NaCl (200 ml). The organic fraction was dried over Na_2SO_4. The product was purified by silica gel chromatography (5×50 cm) using solvent B with an isolated yield of 75%.

Synthesis of 5′-*O*-(Dimethoxytrityl)-2′-*O*-(*t*-butyldimethylsilyl) inosine (4a)

2 (3 mmol, 1 eq) was dissolved in pyridine (10 ml) and $AgNO_3$ (7.2 mmol, 2.4 eq) was added. *t*-Butyldimethylsilyl chloride (3.6 mmol, 1.2 eq) was dissolved in pyridine (3 ml) and the solution was added dropwise over 30 min. TLC (Solvent C; R_f=0.53) indicated that the reaction was nearly complete in 4 hr, with minimal formation of 2′,3′-bis-silyl product. Imidazole (6 mmol, 2 eq) was added and the solvent was removed *in vacuo*. The residue was coevaporated twice with toluene (150 ml), resuspended in ethyl acetate (150 ml), and extracted with 5% $NaHCO_3$ (150 ml) and saturated NaCl (150 ml). The organic fraction was dried over Na_2SO_4. The desired product **4a** was obtained in 40% yield following silica gel chromatography (5×50 cm) using solvent C. The purified product is a white solid which is sparingly soluble in most common organic solvents (*i.e.* acetone, CH_2Cl_2, Et_2O, EtOAc, THF, *etc.*).

Synthesis of 5′-*O*-(Dimethoxytrityl)-2′-*O*-(*t*-butyldimethylsilyl) inosine 3′-*O*-(β-cyanoethyldiisopropylamino) phosphoramidite (5a)

Dry **4a** (0.5 mmol, 1 eq) was suspended in THF (10 ml, freshly distilled). Collidine was added until the compound dissolved (*ca* 3 ml). *N*-methyl imidazole (0.25 mmol, 0.5 eq) was added followed by (*N*,*N*-diisopropylamino)(β-cyanoethyl) phosphonamidic chloride (1.25 mmol, 2.5 eq). A pale white precipitate (collidine-HCl) appeared within seconds; the mixture was stirred for 2 hr to ensure that the reaction had gone to completion. TLC in solvent D (R_f=0.53) showed the formation of a new faster moving product, although streaking made it difficult to confirm that starting material had reacted to completion. The reaction mixture was placed in an ice bath and diluted with ethyl acetate (100 ml). 5% $NaHCO_3$ (150 ml) was added slowly (over 15 min, to avoid heat generation) to destroy excess phosphitylating reagent. The mixture was transferred to a separatory funnel and extracted with 5% $NaHCO_3$ (150 ml) followed by saturated NaCl (150 ml). The aqueous phases were extracted with EtOAc (150 ml). The organic layers were combined, dried over Na_2SO_4, and coevaporated with toluene (6×100 ml). Purification on a small silica gel column (3 cm×50 cm) with solvent E yielded product **5a** as a broad peak in 80% yield. The purity of the final product and the absence of starting material were confirmed by ^{31}P and ^1H NMR respectively. ^{31}P NMR: δ = 153.35 and 151.18 ppm.

Synthesis of O^6-*p*-Nitrophenylethyl-5′-*O*-(dimethoxytrityl) inosine (3)

To **2** (4 mmol, 1 eq) was added dioxane (20 ml) followed by *N*-(trimethylsilyl) imidazole (5.2 mmol, 1.3 eq). This mixture was stirred at room temperature for 15 min. Triphenylphosphine (6 mmol, 1.5 eq), 2-(*p*-nitrophenyl)ethanol (6 mmol, 1.5 eq), and diethylazodicarboxylate (6 mmol, 1.5 eq) were then added. TLC (solvent F; R_f=0.4) indicated that the reaction was complete in 20 min. To the reaction was added 25 ml HF/pyridine (12) for 15 min to remove the transient silyl protecting groups. The reaction mixture was poured into 5% $NaHCO_3$ (150 ml) and the mixture was extracted 3 times with CH_2Cl_2 (150 ml each); the organic layer was then dried over Na_2SO_4. Several coevaporations with toluene (100 ml) removed residual pyridine. Two successive silica gel columns, run in solvent G, yielded pure **3** in 48% yield.

Synthesis of O^6-*p*-Nitrophenylethyl-5′-*O*-(dimethoxytrityl)-2′-*O*-(*t*-butyldimethylsilyl) inosine (4b)

Dry **3** (1.8 mmol, 1 eq) was dissolved in dry DMF (5 ml). Imidazole (5.4 mmol, 3 eq) and *t*-butyldimethylsilyl chloride (1.8 mmol, 1 eq) were then added and the reaction mixture was stirred for 3 hr. TLC (solvent H; product R_f=0.6) indicated that some starting material remained but that the 2′,3′-bis-silyl product had not yet formed. The reaction was stopped by adding CH_2Cl_2 (50 ml). The organic phase was extracted with 5% $NaHCO_3$ (50 ml) and saturated NaCl (50 ml). The aqueous fractions were extracted with CH_2Cl_2 (100 ml) and the combined organic fractions were dried over Na_2SO_4. The desired 2′-*O*-silyl isomer **4b** was obtained in 40% yield following chromatography in solvent I.

Synthesis of O^6-*p*-Nitrophenylethyl-5′-*O*-(dimethoxytrityl)-2′-*O*-(*t*-butyldimethylsilyl) inosine 3′-*O*-(methyldiisopropylamino) phosphoramidite (5b)

Dry **4b** (0.8 mmol, 1 eq) was dissolved in dry CH_2Cl_2 (3 ml) and diisopropylethylammonium tetrazolide salt (0.4 mmol, 0.5 eq) was added. To the reaction mixture was added dropwise bis(diisopropylamino)methoxy phosphine (1.6 mmol, 2 eq)(13,14). The reaction mixture was stirred overnight, after which TLC (solvent J; R_f=0.40, 0.45) showed the reaction to be more than 95% complete. The two diastereomers were quite well resolved in this system and the starting material remained near the origin. The reaction was stopped by the addition of CH_2Cl_2 (10 ml) followed by extraction (3×) with saturated NaCl (20 ml). The aqueous phases were extracted with CH_2Cl_2 (40 ml) the combined organic fractions were dried over Na_2SO_4.

The product was purified on a silica gel column (1×20 cm) using solvent K to yield a white foam in 70% yield. ^{31}P NMR: δ = 152.12 and 150.00 ppm.

Automated Synthesis, Deprotection, and Purification of Inosine Containing Oligoribonucleotides

The oligoribonucleotides shown in Table 3 were synthesized using a Milligen/Biosearch Cyclone DNA synthesizer (6) and the standard ribonucleoside phosphoramidites (A, C, G, and U) were either purchased from Milligen/Biosearch or prepared according to Scaringe *et al.* (6). The protected inosine phosphoramidite was coupled manually by interrupting the synthesis during the coupling step (12 min) and flushing the column with a solution (200 μl) 0.1 M in ribonucleoside phosphoramidite and 0.5 M in tetrazole. The standard 1 μmol coupling program was modified to match the conditions described previously (4–6). Coupling efficiency, assayed by measuring the trityl cation released (A_{504}), was >98%. All of the oligomers, except **r9I'**, were deprotected and purified as described previously (6). Oligonucleotide **r9I'** was treated first with thiophenol reagent to remove the methyl phosphate protecting group (4). Following this step, **r9I'** was deprotected as above except that the characteristic dark brown colour of *p*-nitrophenylethylene, the elimination product of NPE deprotection, was observed during the TBAF step as expected.

The final purification of the 9-mers was performed by HPLC. A Dionex Nucleopak NA100 anion exchange column used with denaturing buffers (A: 0.2 M NaCl/25 mM NaOH and B: 2.0 M NaCl/25 mM NaOH) gave good resolution of the desired major peak (at 25% B). The products were kinased and checked on a 20% PAGE and all appeared as single bands.

Preparation of T7 Transcribed Splice Junction Oligoribonucleotides and the RNA Enzyme

The RNA oligomers representing the 5′ exon-intron junction containing either U, **r20U**, or C, **r20C**, were purified on a 15% acrylamide–7M urea gel (26) after transcription from a synthetic DNA oligomer containing the T7 promoter sequence (19). The enzyme LG12 was transcribed from a PCR DNA template (27) and was isolated on a 4% acrylamide–7M urea gel.

Trans Cleavage Reactions

The assay uses a ribozyme (derived from JD1100 described in Doudna and Szostak (28)) whose sequence includes the secondary structural elements beginning at the base of helix P2 and continuing through P9.2 (all but the first 29 and last 4 nucleotides of the intron). Enzyme at 0.12 μM was pre-incubated at 60°C for 2 min, followed by 5 min at 25°C, in 10 mM NH_4Cl, 30 mM Tris-HCl buffer, pH 7.4, and 20 mM $MgCl_2$ with each pairwise combination of oligoribonucleotides (**r9G**, **r9I**, and **r9I'** with **r20U** and **r20C**) at 12 μM. GTP (α-^{32}P-labelled) was added in trace amounts to start the reactions, which were incubated at 45°C. Time points were taken by adding aliquots (5 μl) of the reaction mixture directly to loading dye (0.2% bromphenol blue in 90% formamide, 25 mM EDTA) at 3 min, 10 min, 30 min and 60 min. Products were analyzed on a 20% acrylamide-7M urea sequencing gel (0.7 mm thick) and the amount of labelled GTP incorporated into cleaved 20-mer was quantitated on the Betascope from which relative reaction rates were determined.

ACKNOWLEDGEMENTS

We thank Milligen/Biosearch for providing the Cyclone DNA synthesizer, ancillary reagents and RNA phosphoramidites, Mr Adam Foley for obtaining NMR spectra, Mr Steven Scaringe for technical assistance, and Drs Loren Williams, David Bartel and Jennifer Doudna for critical reading of this manuscript. This research was supported by funds from the National Institutes of Health, The National Science Foundation, the Office of Naval Research, the National Aeronautics & Space Administration, the Swiss National Science Foundation, and Hoechst A.G. NU was the recipient of an NIH Fogarty International Research Fellowship 1988–89.

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