**Published online April 2, 2004**

*Nucleic Acids Research, 2004, Vol. 32, No. 6* ***2017–2022***
*DOI: 10.1093/nar/gkh517*

# Chemical syntheses of inhibitory substrates of the RNA–RNA ligation reaction catalyzed by the hairpin ribozyme

**Archna P. Massey and Snorri Th. Sigurdsson***

Department of Chemistry, University of Washington, Seattle, WA 98195-1700, USA

Received January 19, 2004; Revised and Accepted March 9, 2004

## ABSTRACT

**The chemical syntheses of RNA oligomers containing modifications on the 5′-carbon of the 5′-terminal nucleoside for crystallographic and mechanistic studies of the hairpin ribozyme are reported. Phosphoramidites 4 and 8 were prepared and used in solid phase syntheses of RNA oligomers containing the sequence 5′-N′UCCUCUCC, where N′ indicates either 5′-chloro-5′-deoxyguanosine or 5′-amino-5′-deoxyguanosine, respectively. A ribozyme ligation assay with the 5′-chloro- and 5′-amino-modified RNA oligomers demonstrated their inhibition of the hairpin-catalyzed RNA–RNA ligation reaction.**

## INTRODUCTION

Since the discovery of RNA catalytic activity over 20 years ago, significant research effort has been directed towards determining how RNA, a biopolymer with a limited number of functional groups, can catalyze reactions. In the past few years, several crystal structures of ribozymes have been determined, but these structures have given limited insights into the catalytic mechanisms of RNA (1). Although X-ray crystallography is the most powerful method for obtaining high resolution structures of RNA, this method has two shortcomings for studying RNA catalytic mechanisms. First, the structures present in the crystal may need to undergo a considerable conformational change to reach the transition state of the reaction. Thus, it may not be obvious from these ground state structures how specific functional groups facilitate lowering of the transition state energy of the reaction during catalysis. Secondly, due to the challenge in obtaining crystals of complex RNA molecules suitable for X-ray analysis, it is usually only possible to obtain a structure of one state on the reaction coordinate. For example, the initial structures of the hammerhead ribozyme (2,3) contained inhibitory substrates whereas the structure of the hepatitis delta virus ribozyme was obtained after substrate cleavage (4). However, conformational changes in the hammerhead ribozyme have been observed by crystallography, although this approach may not be generally applicable (5,6).

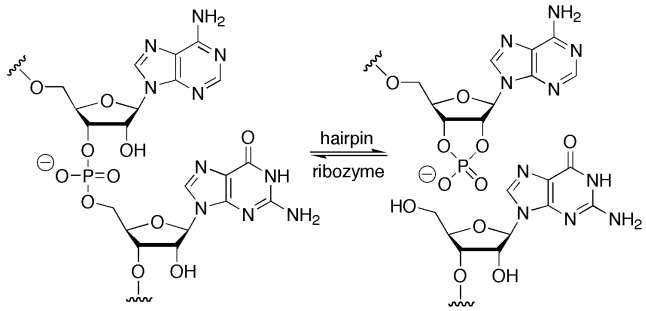


**Figure 1.** The phosphodiester transesterification reaction catalyzed by the hairpin ribozyme.

A crystal structure of the hairpin ribozyme, a catalytic RNA requiring ~50 nt for activity, was recently obtained bound to an inhibitory substrate containing a 2′-methoxy group at the cleavage site (7). This ribozyme catalyzes cleavage of an RNA substrate and can also be used for RNA–RNA ligations since the equilibrium constant for the cleavage and ligation is close to one (Fig. 1). Consistent with this, crystallization of the hairpin ribozyme in the presence of a cleavable substrate yielded crystals containing a mixture of the uncleaved (ligated) strand and the cleaved product. The presence of the cleaved product in the crystal indicated that crystal structure of the ribozyme–product complex could be obtained if the ligation reaction was inhibited. One strategy for inhibition of the ligation reaction is modification of the product's 5′ group at the cleavage site. In this paper, we describe the chemical syntheses of such inhibitory substrates of the RNA–RNA ligation reaction catalyzed by the hairpin ribozyme. The utility of one of these substrates has been demonstrated in crystallographic studies of the hairpin ribozyme (8).

## MATERIALS AND METHODS

### General experimental methods

All reagents and anhydrous *N*,*N*-dimethylacetamide and DMF were obtained from Aldrich and used without further purification. Anhydrous $CH_2Cl_2$ and pyridine were freshly distilled from calcium hydride. All reactions were carried out in oven-dried glassware under an inert atmosphere of argon. Thin-layer chromatography was performed on precoated plates

*To whom correspondence should be addressed. Tel: +1 206 616 8276; Fax: +1 206 685 8665; Email: snorrisi@hi.is

 

(silica gel 60 F254 from E-Merck) and visualized with UV light and a *p*-anisaldehyde dip [prepared by mixing *p*-anisaldehyde (6 ml), $H_2SO_4$ (8.0 ml) and $CH_3COOH$ (2.4 ml) in $C_2H_5OH$ (218 ml)] followed by charring on a hot plate. Flash column chromatography was carried out with EM type 60 (230–400 mesh) silica gel. $^1$H-, $^{13}$C- and $^{31}$P-NMR spectra were recorded on a Bruker 500 MHz DRX Avance FT-NMR spectrometer at frequencies 499.85, 125.70 and 202.34 MHz, respectively. Chemical shifts are reported in p.p.m. relative to DMSO-$d_6$ (2.50 p.p.m. for $^1$H and 39.51 p.p.m. for $^{13}$C), $CDCl_3$ (7.27 p.p.m. for $^1$H and 77.23 p.p.m. for $^{13}$C), $(CD_3)_2CO$ (2.05 p.p.m. for $^1$H and 29.92, 206.68 p.p.m. for $^{13}$C) or $CD_3OD$ (3.31, 4.87 p.p.m. for $^1$H and 49.15 p.p.m. for $^{13}$C). Chemical shifts for $^{31}$P-NMR are reported in p.p.m. relative to 85% phosphoric acid (0 p.p.m.) as an external standard. High-resolution mass spectra (HRMS-FAB) were recorded on JEOL HX-110 mass spectrometer. All new compounds were characterized by $^1$H-, $^{13}$C-NMR, and HRMS-FAB.

### Chemical synthesis of RNA

The chemical syntheses of the RNAs containing 5′ modifications were carried out by Dharmacon Research, using phosphoramidites **4** and **8**. After RNA synthesis using standard solid-phase chemistry (9), the RNA was cleaved off the solid support and the base-protecting groups and acetates on the 2′-ACE protecting groups were removed by 40% methylamine (aq.) at room temperature for 2.5 h (5′-chloro oligomer) and 1 h at 60°C (5′-amino oligomer). After deprotection, the methylamine solution was removed *in vacuo* and the crude oligomer treated with a solution of triethylamine-hydrofluoric acid (1 ml) at 25°C for 12 h to remove the TBDMS group. This solution was subsequently diluted to 50 ml with water, passed over a strong anion exchange column (Dionex DNAPac-PA-100), washed with 20 mM triethylammoniumbicarbonate (TEAB) (10 ml) and eluted off of the column using 2 M TEAB (3 × 1.5 ml). Most of the oligomer eluted in the first two fractions. Prior to purification of the oligomers by denaturing polyacrylamide gel electrophoresis, the ACE-protecting groups were removed using standard protocols (9).

### RNA–RNA ligation assays

Solutions of the 12mer substrate 5′-GGCCACCUGACA (65 μM), which contained a 2′,3′ cyclic phosphate (7), and a 92mer hairpin ribozyme (7) (65 μM) were independently incubated with each of the 9mer substrates 5′-N′UCCUCUCC (260 μM), where N′ indicates either guanosine, 5′-chloro-5′-deoxyguanosine, or 5′-amino-5′-deoxyguanosine at 37°C in a 50 mM Tris buffer, pH 7.0 containing 50 mM $MgCl_2$. After 1 h, the reaction mixtures were subjected to 20% denaturing polyacrylamide gel electrophoresis and the gel subsequently stained with toluidine blue.

### *N*2-isobutyryl-5′-chloro-5′-deoxyguanosine (2)

A solution of *N*2-isobutyrylguanosine (**1**) (10) (5 g, 14.0 mmol), $Ph_3P$ (7.32 g, 28.0 mmol) and $CCl_4$ (10.74 g, 70.0 mmol) in *N*,*N*-dimethylacetamide (40 ml) was stirred at 40°C. After 1 h, the reaction mixture was allowed to cool to 25°C, followed by addition of water (400 ml) and extraction with ethyl acetate. The organic layer was dried over $Na_2SO_4$ and concentrated under reduced pressure. The residue was subjected to column chromatography (10% MeOH/$CHCl_3$) to yield 5.99 g of white foam containing **2** and a phosphorus-containing impurity. An analytically pure sample of **2** was obtained by persilylation with trimethylsilylchloride (TMSCl), followed by chromatographic purification and removal of the silyl groups. A solution of the mixture containing **2** (0.3 g) in pyridine (3 ml) was cooled to –40°C, treated with TMSCl (0.292 g, 2.6 mmol) and stirred for 1 h. The temperature was allowed to rise to –10°C during the reaction time, after which the reaction mixture was diluted with $CH_2Cl_2$ (6 ml) and washed with water and brine. The organic layer was dried over $Na_2SO_4$, the solvent removed *in vacuo* and the residue purified by column chromatography (8% MeOH/$CHCl_3$) to give the bis(trimethylsilyl) derivative as a white solid (0.208 g). To a stirred suspension of the TMS derivative (0.050 g, 0.097 mmol) in pyridine (0.5 ml) at –40°C was added aq. ammonium hydroxide (29%) (0.6 ml). After stirring the reaction mixture at 25°C for 1 h, water was added (3 ml) and the aqueous solution washed with $CHCl_3$. The aqueous layer was evaporated *in vacuo* to give the 5′-chloro nucleoside **2** (0.036 g, 100%) as a white solid. ($R_f$ = 0.41 in 10% MeOH/$CHCl_3$); $^1$H-NMR (DMSO-$d_6$) δ 1.10 (br s, 6H), 2.76 (br s, 1H), 3.81(br s, 1H), 3.92 (br s, 1H), 4.08 (br s, 1H), 4.17 (br s, 1H), 4.63 (br s, 1H), 5.74 (s, 1H), 5.85 (br s, 1H), 6.57 (br s, 1H), 8.23 (s, 1H), 11.28 (br s, 1H); $^{13}$C-NMR (DMSO-$d_6$) δ 19.7, 35.6, 45.6, 72.0, 73.6, 84.5, 87.6, 121.1, 138.8, 149.1, 149.9, 155.8, 180.9; HRMS *m*/*z* calc. for $C_{14}H_{19}O_5N_5Cl$ (M+H$^+$) 372.1074, found 372.1072.

### Silylation of *N*2-isobutyryl-5′-chloro-5′-deoxyguanosine (2): preparation of compounds 3a–c

To a stirred solution of *N*2-isobutyryl-5′-chloro-5′-deoxyguanosine (**2**) (0.791 g), including the phosphorus-containing impurity, in DMF (4.7 ml) were added TBDMSCl (0.7 g, 4.68 mmol) and imidazole (0.635 g, 9.3 mmol) at 25°C. The reaction mixture was stirred for 1 h, after which water (15 ml) was added, followed by extraction with $CHCl_3$. The $CHCl_3$ layer was washed with water and brine, dried over $Na_2SO_4$ and concentrated *in vacuo*. The residue obtained was subjected to silica gel chromatography using 25% $Me_2CO/CH_2Cl_2$ to give the disilylated compound (**3c**) (0.088 g, 7.8%, two steps), the 2′-silylated compound **3a** (0.250 g, 27.5%, two steps) and the 3′-silyl compound **3b** (0.093 g, 10.2%, two steps). The $R_f$ values on silica gel TLC plates using 30% $Me_2CO/CH_2Cl_2$ as an eluent for **3a**, **3b** and **3c** were 0.5, 0.4 and 0.7, respectively. *N*2-isobutyryl-2′-*O*-(*tert*-butyldimethylsilyl)-5′-chloro-5′-deoxyguanosine (**3a**): $^1$H-NMR (DMSO-$d_6$) δ –0.16, –0.03 (2 × s, 6H), 0.72 (s, 9H), 1.12 (d, *J* = 4.99 Hz, 6H), 2.77 (m, 1H), 3.86 (m, 1H), 3.97 (m, 1H), 4.12 (m, 2H), 4.73 (br s, 1H), 5.35 (br s, 1H), 5.88 (d, *J* = 4.99 Hz, 1H), 8.25 (s, 1H), 11.62 (br s, 1H), 12.10 (br s, 1H); $^{13}$C-NMR (DMSO-$d_6$) δ –4.58, –4.09, 18.6, 19.7, 26.3, 35.6, 45.4, 71.9, 75.7, 85.2, 87.3, 121.1, 138.7, 149.1, 149.8, 155.6, 181.0; HRMS *m*/*z* calc. for $C_{20}H_{33}O_5N_5ClSi$ (M+H$^+$) 486.1939, found 486.1933.

*N2-isobutyryl-3′-O-(tert-butyldimethylsilyl)-5′-chloro-5′-deoxyguanosine (**3b**).* $^1$H-NMR (DMSO-$d_6$) δ 0.14 (s, 6H), 0.92 (s, 9H), 1.13 (br s, 6H), 2.77 (m, 1H), 3.78 (m, 1H), 3.94 (br s, 1H), 4.07 (br s, 1H), 4.25 (br s, 1H), 4.81 (br s, 1H), 5.63 (br s, 1H), 5.82 (br s, 1H), 8.29 (s, 1H), 11.62 (br s, 1H), 12.11 (br s,

1H); $^{13}$C-NMR (DMSO-$d_6$) δ –4.2, –3.55, 18.9, 19.7, 26.6, 35.6, 44.7, 72.6, 74.3, 85.8, 86.8, 121.2, 138.8, 149.1, 150.1, 155.7, 180.9**;** HRMS *m/z* calcd for $C_{20}H_{33}O_5N_5ClSi$ (M+H$^+$) 486.1938, found 486.1944.

*N2-isobutyryl-2′,3′-di-O-(tert-butyldimethyl-silyl)-5′-chloro-5′-deoxyguanosine (**3c**).* $^1$H-NMR (DMSO-$d_6$) δ –0.32, –0.058, 0.132, 0.160 (4 × s, 12H), 0.70, 0.83 (2 × s, 18H), 1.12 (d, *J* = 4.99 Hz, 6H), 2.79 (m, 1H), 3.83 (br s, 1H), 4.09 (br s, 2H), 4.28 (br s, 1H), 4.96 (br s, 1H), 5.85 (d, *J* = 6.98 Hz, 1H), 8.31 (s, 1H), 11.53 (br s, 1H), 12.14 (br s, 1H); $^{13}$C-NMR (DMSO-$d_6$) δ –3.8, 18.3, 18.6, 19.7, 26.3, 26.6, 35.6, 44.5, 74.2, 74.6, 86.3, 87.0, 121.3, 139.2, 149.0, 149.9, 155.6, 180.9; HRMS *m/z* calc. for $C_{26}H_{47}O_5N_5ClSi_2$ (M+H$^+$) 600.2804, found 600.2798.

**Phosphoramidite 4**

Diisopropylethylamine (0.056 ml, 0.321 mmol) was added to a stirred solution of 2′-silylated compound **3a** (0.094 g, 0.193 mmol) in $CH_2Cl_2$ (0.56 ml), which was subsequently treated with *N,N*-diisopropyl methylphosphonamidic chloride (0.045 g, 0.227 mmol) at 25°C. After 15 min, the reaction mixture was diluted with $CH_2Cl_2$ (2 ml) and the organic layer washed with saturated solution of $NaHCO_3$, followed by water and brine. The organic layer was dried ($Na_2SO_4$) and concentrated *in vacuo*. The organic residue was purified by silica gel flash column chromatography using 50% EtOAc/hexane as the eluent to furnish the phosphitylated product **4** (0.080 g, 64%). ($R_f$ = 0.6 in 60% EtOAc/hexane). $^{31}$P-NMR ($CDCl_3$) δ (a mixture of diasteromers) 151.54, 152.69; HRMS *m/z* calc. for $C_{27}H_{49}O_6N_6ClSiP$ (M+H$^+$) 647.2909, found 647.2902.

***N*2-isobutyryl-5′-azido-5′-deoxyguanosine (5)**

A solution of *N*2-isobutyryl-5′-chloro-5′-deoxyguanosine (**2**) (5.99 g, 16.0 mmol) and $NaN_3$ (15.48 g, 238 mmol) in DMF (46 ml) was stirred between 95 and 100°C. After 8 h, the reaction was determined by MS to be complete. The mixture was cooled to 25°C and the solvent removed *in vacuo*. The residue was subjected to column chromatography using 15% MeOH/$CHCl_3$ to yield **5**, along with the impurity from the starting material (1.96 g), as a white foam. An analytically pure sample of compound **5** was obtained by persilylation with TMSCl, followed by chromatographic purification and removal of the silyl groups. A solution of compound **5** and the impurity (0.1 g) in pyridine (1 ml) was treated with TMSCl (0.094 g, 0.874 mmol) at –40°C. After stirring for 1 h, during which time the temperature of the reaction mixture rose to –10°C, the reaction mixture was diluted with $CH_2Cl_2$ (2 ml) and washed with water and brine. The organic layer was dried over $Na_2SO_4$ and the solvent removed under reduced pressure. The residue was purified by column chromatography using 10% MeOH/$CHCl_3$ to give the bis-trimethylsilyl derivative of **5** (0.071 g) as a white solid. To a stirred suspension of the TMS derivative (0.071 g, 0.136 mmol) in pyridine (0.7 ml) at –40°C was added aq. ammonium hydroxide (29%) (1 ml). After stirring the reaction mixture at 25°C for 1 h, water was added (4 ml) and the aqueous solution washed with $CHCl_3$. The aqueous layer was evaporated *in vacuo* to give the 5′-azido nucleoside **5** (0.051 g, 100%) as a white solid. ($R_f$ = 0.416 in 10% MeOH/$CHCl_3$); $^1$H-NMR (DMSO-$d_6$) δ 1.13 (d, *J* = 4.9 Hz, 6H), 2.77 (m, 1H), 3.56 (dd, *J* = 4.9, 13.4 Hz, 1H), 3.66 (dd, *J* = 4.9, 13.4 Hz, 1H), 4.02 (br s, 1H), 4.11 (br s, 1H), 4.60 (d, *J* = 4.9 Hz, 1H), 5.38 (br s, 1H), 5.64 (br s, 1H), 5.84 (d, *J* = 4.9 Hz, 1H), 8.25 (s, 1H), 11.64 (br s, 1H), 12.09 (br s, 1H); $^{13}$C-NMR (DMSO-$d_6$) δ 19.7, 35.6, 52.7, 71.8, 73.8, 83.8, 87.9, 121.2, 138.9, 149.0, 149.7, 155.7, 180.9; HRMS *m/z* calc. for $C_{14}H_{19}O_5N_8$ (M+H$^+$) 379.1478, found 379.1479.

***N*2-isobutyryl-5′-deoxy-5′-trifluoroacetamidoguanosine (6)**

A suspension of *N*2-isobutyryl-5′-azido-5′-deoxyguanosine (**5**) (1.6 g, 4.24 mmol) in 50% AcOH/MeOH (70 ml) containing 10% Pd/C (1.6 g) was hydrogenated at 60 p.s.i. for 5 days. The reaction mixture was filtered through celite and solvent was evaporated on rotary evaporator to give the 5′-amino nucleoside as white solid. To a stirred suspension of the amine in MeOH (31 ml) at 25°C was added *N*-trifluoroacetyl imidazole (0.820 g, 5 mmol). After 6 h, the solvent was removed *in vacuo* and the residue purified by column chromatography using 14% MeOH/$CHCl_3$ to provide compound **6** (1.17 g, 22%, four steps). ($R_f$ = 0.7 in 14% MeOH/$CHCl_3$); $^1$H-NMR (MeOH-$d_4$) δ 1.24 (d, *J* = 4.9 Hz, 6H), 2.77 (m, 1H), 3.66 (m, 1H), 3.74 (m, 1H), 4.16 (m, 1H), 4.34 (m, 1H), 4.72 (m, 1H), 5.92 (d, *J* = 4.9 Hz, 1H), 8.08 (s, 1H); $^{13}$C-NMR (MeOH-$d_4$) δ 18.4, 35.9, 41.9, 72.0, 74.1, 82.6, 89.5, 115.4, 117.7, 120.7, 139.3, 148.6, 158.2, 158.5, 180.8; $^{19}$F-NMR (MeOH-$d_4$) δ –77.27, –77.49; HRMS *m/z* calc. for $C_{16}H_{20}O_6N_6F_3$ (M+H$^+$) 449.1396, found 449.1386.

**Silylation of *N*2-isobutyryl-5′-deoxy-5′-trifluoroacetamidoguanosine (6): preparation of compounds 7a–c**

To a stirred solution of *N*2-isobutyryl-5′-deoxy-5′-trifluoroacetamidoguanosine (**6**) (0.677 g, 1.5 mmol) in DMF (7 ml) was added TBDMSCl (0.459 g, 3.0 mmol) and imidazole (0.408 g, 6.0 mmol) at 25°C. The reaction mixture was stirred for 2 h, after which water (10 ml) was added, followed by extraction with $CHCl_3$. The organic layer was washed with water and brine, dried over $Na_2SO_4$ and concentrated *in vacuo*. The residue obtained was purified by silica gel chromatography using 25% $Me_2CO/CH_2Cl_2$ to give the disilylated compound (**7c**) (0.1 g, 10.0%), the 2′-silylated compound **7a** (0.225g, 26%) and the 3′-silyl compound **7b** (0.065 g, 7.6%). The $R_f$ values for **7a, 7b** and **7c** on silica gel TLC plates using 30% $Me_2CO/CH_2Cl_2$ as an eluent were 0.4, 0.3 and 0.5, respectively.

*N2-isobutyryl-2′-O-(tert-butyldimethylsilyl)-5′-deoxy-5′-trifluoroacetamidoguanosine (**7a**).* $^1$H-NMR [$(CD_3)_2CO$] δ –0.10 (s, 3H), 0.04 (s, 3H), 0.76 (s, 9H), 1.22 (d, *J* = 4.99 Hz, 6H), 2.91 (m, 1H), 3.72 (m, 1H), 3.88 (m, 1H), 3.91 (m, 1H), 4.18 (m, 1H), 4.48 (m, 1H), 4.86 (m, 1H), 5.91 (d, *J* = 4.99 Hz, 1H), 8.15 (s, 1H), 8.98 (br s, 1H), 10.75 (br s, 1H), 12.20 (br s, 1H); $^{13}$C-NMR [$(CD_3)_2CO$] δ –5.5, –5.2, 18.1, 18.8, 18.8, 25.6, 36.1, 42.1, 72.3, 75.9, 82.8, 89.2, 115.5, 117.8, 121.6, 138.8, 148.8, 149.0, 155.5, 157.5, 157.8, 180.6; $^{19}$F-NMR [$(CD_3)_2CO$] δ –75.19; ($CD_3OD$) δ –9.70, –77.48; HRMS *m/z* calc. for $C_{22}H_{34}O_6N_6F_3Si$ (M+H$^+$) 563.2261, found 563.2253.

*N2-isobutyryl-3′-O-(tert-butyldimethylsilyl)-5′-deoxy-5′-trifluoroacetamidoguanosine (**7b**).* $^1$H-NMR [$(CD_3)_2CO$] δ

0.196 (s, 6H), 0.92 (s, 9H), 1.25 (d, $J$ = 4.9 Hz, 6H), 2.06 (br s, 1H), 2.93 (m, 1H), 3.73 (m, 1H), 3.87 (m, 1H), 4.22 (br s, 1H), 4.42 (br s, 1H), 5.03 (m, 1H), 5.85 (d, $J$ = 6.4 Hz, 1H), 8.07 (s, 1H), 9.13 (s, 1H), 10.70 (s, 1H), 12.18 (br s, 1H); $^{13}$C-NMR [$(CD_3)_2CO$] δ –5.2, –4.7, 18.4, 18.9, 19.5, 25.8, 36.1, 42.4, 73.2, 74.4, 84.3, 88.7, 115.5, 117.8, 121.3, 139.1, 148.6, 149.2, 155.5, 157.6, 157.8, 180.5; $^{19}$F-NMR [$(CD_3)_2CO$] δ –75.15; ($CDCl_3$) δ –75.10, –79.65; HRMS *m/z* calc. for $C_{22}H_{34}O_6N_6F_3Si$ ($M+H^+$) 563.2266, found 563.2277.

*N2-isobutyryl-2′,3′-di-O-(tert-butyldimethylsilyl)-5′-deoxy-5′-trifluoroacetamidoguanosine (**7c**).* $^1$H-NMR [$(CD_3)_2CO$] δ –0.32, –0.03, 0.18 (4 × s, 12H), 0.77, 1.01 (2 × s, 18H), 1.23 (d, $J$ = 6.48 Hz, 6H), 2.91 (m, 1H), 3.82 (m, 1H), 3.93 (m, 1H), 4.24 (d, $J$ = 4.99 Hz, 1H), 4.41 (m, 1H), 5.07 (m, 1H), 5.93 (d, $J$ = 4.99 Hz, 1H), 8.20 (s, 1H), 9.20 (br s, 1H), 10.64 (br s, 1H), 12.30 (br s, 1H); $^{13}$C-NMR [$(CD_3)_2CO$] δ –5.8, –4.96, –4.8, –4.7, 17.9, 18.2, 18.8, 18.9, 25.6, 25.8, 36.1, 42.5, 74.4, 74.8, 84.8, 87.9, 115.5, 117.8, 121.8, 139.2, 148.8, 149.4, 155.6, 157.6, 180.6; HRMS *m/z* calc. for $C_{28}H_{48}O_6N_6F_3Si_2$ ($M+H^+$) 677.3126, found 677.3120.

### Phosphoramidite 8

*N*,*N*-Diisopropylethylamine (0.030 ml, 0.172 mmol) was added to a stirred solution of the 2′-silylated compound **7a** (0.060 g, 0.106 mmol) in $CH_2Cl_2$ (0.56 ml). This solution was treated with *N*,*N*-diisopropylmethylphosphonamidic chloride at 25°C (0.025 g, 0.126 mmol). After 20 min, the reaction mixture was concentrated *in vacuo* and the resulting residue purified by column chromatography using 50% EtOAc/hexane as the eluent to give the phosphitylated product **8** (0.077 g, 100%) ($R_f$ = 0.6 in 60% EtOAc/hexane); $^{31}$P-NMR ($CDCl_3$) δ (a mixture of diastereomers and rotamers) 151.05, 151.75, 153.41, 155.26; $^{19}$F-NMR ($CDCl_3$) δ –74.91, –75.09, –79.56, –79.75; HRMS *m/z* calc. for $C_{29}H_{50}O_7N_7F_3SiP$ ($M+H^+$) 724.323, found 724.3212.

## RESULTS AND DISCUSSION

The most important criterion that guided the design of the inhibitor substrates was that the functional group on the 5′-end at the cleavage site did not react with the 2′,3′-cyclic phosphate (Fig. 1). It is also desirable that the group should be able to participate in hydrogen bonding, like the 5′-hydroxyl group it replaces, and that the Van der Waals radius of the 5′-group be similar to an hydroxyl group. Based on these criteria, we chose to prepare two different substrates, one containing a 5′-halogen and another containing a 5′-amino group. Neither of these groups is nucleophilic; the amino group is expected to be protonated at pH 7. The halogen atom is a hydrogen bond acceptor and the protonated amino group a hydrogen bond donor.

### 5′-Chloro-modified phosphoramidite

Initially, we considered incorporation of a bromine atom because it can be easily located by crystallography due to its anomalous X-ray scattering. However, preliminary studies with 5′-bromo-5′-deoxythymidine revealed that a substantial amount of the bromo group was displaced by methylamine under the conditions used for the deprotection of RNA (data not shown). Therefore, we focused our efforts on incorporation of a chlorine atom, which is stable under the deprotection conditions. The synthetic route for phosphoramidite **4**, which can be used directly in chemical synthesis of RNA containing the 5′-chloro modification is shown in Figure 2.

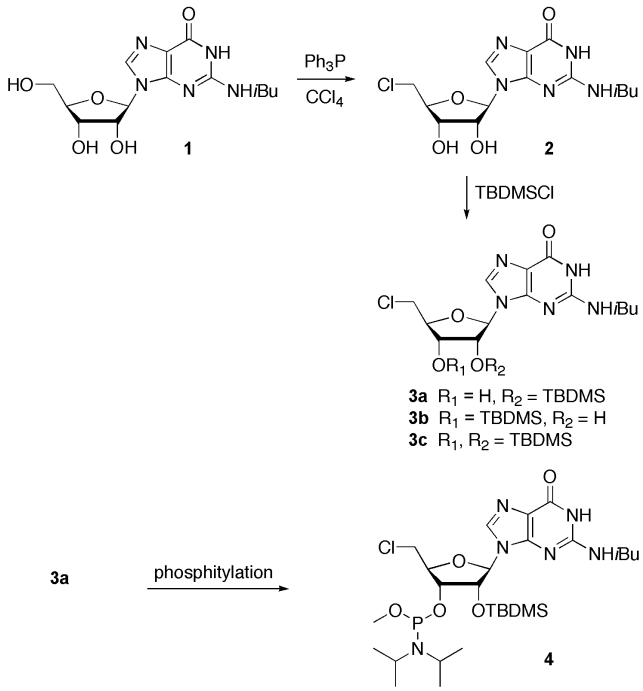


**Figure 2.** Synthesis of phosphoramidite **4**.

*N*2-isobutyrylguanosine (**1**) (10), was treated with triphenylphosphine and carbon tetrachloride to yield the chlorinated nucleoside **2**. After chromatographic purification, the $^1$H-NMR and the $^{31}$P-NMR spectra showed the presence of a non-nucleoside, phosphorus-containing impurity that had the same $R_f$ value as the desired product on silica gel in all the solvent systems that were tested. Therefore, this mixture was not further purified and used directly in the next step. An analytically pure sample of **2** was obtained by reaction with TMSCl, followed by chromatographic purification and removal of the silyl groups.

The 5′-chloro nucleoside **2** was silylated using TBDMSCl and imidazole in DMF to give the disilylated nucleoside **3c**, along with the 2′- and 3′-monosilylated compounds **3a** and **3b**, respectively (11). The two monosilylated guanosine derivatives had very similar chromatographic properties and silyl migration between the 2′- and 3′-hydroxyl groups was observed when protic solvents were used for their chromatographic purification. However, compounds **3a** and **3b** were successfully purified by silica gel flash column chromatography using a $CH_2Cl_2$/acetone mixture as the eluent. The position of silylation for each isomer was determined from $^{13}$C-NMR spectra as silylation of a sugar hydroxyl leads to a downfield shift of the carbon to which the silylated hydroxyl group is attached (11). The 2′-monosilylated compound **3a** was subsequently phosphitylated with *N*,*N*-diisopropyl methylphosphonamidic chloride to give the desired phosphoramidite **4** in 65% yield.

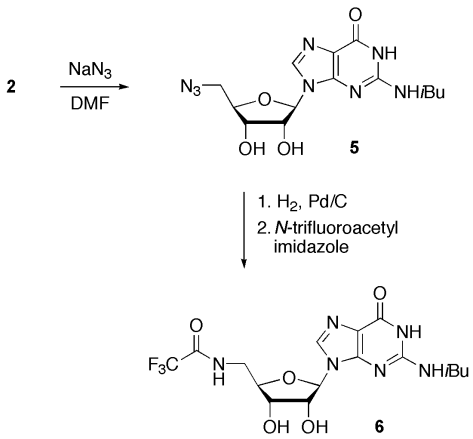


**Figure 3.** Synthesis of compound **6**.

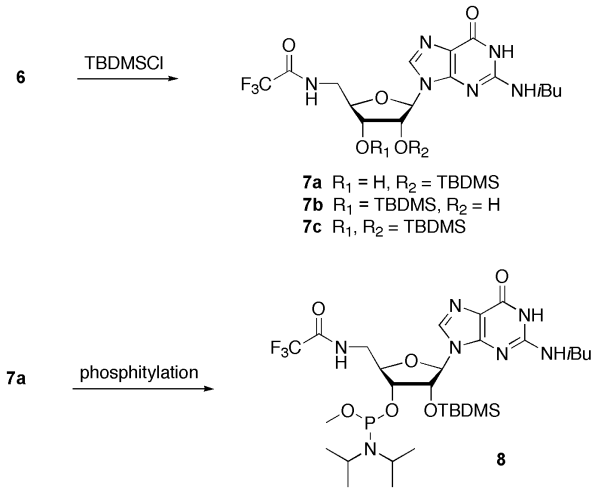


**Figure 4.** Synthesis of phosphoramidite **8**.

## 5′-Amino-modified phosphoramidite

The *N2*-isobutyryl-5′-chloro-5′deoxy-guanosine (**2**) containing the aforementioned impurity was reacted with sodium azide in DMF at 95–100°C to afford azide **5** (Fig. 3). This reaction was monitored by mass spectrometric analysis, due to the identical $R_f$ values of **2** and azide **5** by silica gel TLC in several different solvents. Chromatographic purification of the reaction mixture gave azide **5**, along with the phosphorus-containing impurity present in the starting material. An analytically pure sample of azide **5**, was obtained by disilylation with TMSCl, followed by chromatography and removal of the silyl groups.

When the crude azide **5** was reduced to the corresponding amine using standard reduction conditions (hydrogen at 55 p.s.i. over 10% Pd/C in methanol) (Fig. 3), the isobutyryl group was unexpectedly removed. This was probably due to excess sodium azide, which could, after reduction, facilitate the removal of the isobutyryl group. Indeed, when the crude azide was chromatographically purified, the isobutyryl group was not affected upon reduction. However, reduction of the azide was slow (15% in 3 days), presumably due to catalyst poisoning by the amine, but proceeded at an acceptable rate (completed after 5 days) when carried out in 50% acetic acid in methanol. Because of the high polarity of the resulting amine, it was used in the next step without chromatographic purification.

The trifluoroacetamide-protecting group for amines was determined to be readily removed under the deprotection conditions used for RNA synthesis [40% $MeNH_2$ (aq.), 55°C, 10 min]. Reaction of the 5′-amino nucleoside with *N*-trifluoroacetyl imidazole gave the 5′-trifluoroacetamide derivative **6** (22% yield from *N2*-isobutyrylguanosine, four steps). Compound **6** was reacted with TBDMSCl to give the 2′,3′-disilylated compound **7c**, along with the 2′ and 3′ silyl isomers **7a** and **7b**, which were separated by flash column chromatography (Fig. 4).

The final step in this synthesis was the phosphitylation of the 2′-silyl isomer **7a** with *N*,*N*-diisopropyl methylphosphonamidic chloride, which gave phosphoramidite **8** in quantitative yield after chromatographic purification (Fig. 4). Analysis of this product by $^{31}$P-NMR showed four peaks in the chemical shift range expected for a phosphoramidite. However, only two peaks are normally observed for the two diastereomeric phosphoramidite products resulting from different stereochemical configurations of the phosphorus atom. The $^{1}$H-NMR of the product contained multiple peaks for the individual proton resonances but the mass spectrum showed the expected mass for **8**. Taken together, these data indicated that there were four different isomers present in this sample.

The most obvious explanation for observation of the four isomers is that migration of the TBDMS group from the 2′-hydroxyl to the 3′-hydroxyl had occurred during the phosphitylation reaction. The phosphitylation of 2′-silyl isomer was carried out using the weaker bases, 2,4,6-collidine and *N*-methyl imidazole, but with the same result. To further investigate the possibility of a silyl migration during the phosphitylation reaction, a mixture of the monosilyl compounds **7a** and **7b** was phosphitylated. This resulted in a phosphoramidite that showed eight peaks in the $^{31}$P-NMR, not the expected four peaks if silyl migration was the reason for observation of the different isomers. Therefore, the isomers observed after phosphitylation did not originate from phosphitylation of different silyl isomers.

The observation of four isomers in phosphoramidite **8** by $^{31}$P-NMR could be due to different trifluoroacetamide rotamers, because amides are known to have limited rotation around the amide bond (12). Indeed, diol **6** showed two peaks in the $^{19}$F-NMR and phosphoramidite **8** showed four peaks, which could be evidence for the presence of rotamers. On the other hand, each of the 2′ and 3′ silyl isomers **7a** and **7b** exhibited only one peak in the $^{19}$F-NMR spectra, indicating the absence of rotamers that were stable on the NMR time scale. However, detection of amide rotamers by NMR has been shown to be solvent dependent (13–15). In fact, when the $^{19}$F-NMR spectra of compounds **7a** and **7b**, each of which exhibited one peak in a solution of acetone-$d_6$, were recorded in MeOH-$d_4$, both compounds showed two peaks as would be expected for the two rotamers. Since all the trifluoroacetamide compounds prepared in this study showed double the number of expected resonances when their $^{19}$F-NMR spectra were recorded in MeOH-$d_4$ or $CDCl_3$, we concluded that the doubling arose from trifluoroacetamide rotamers.

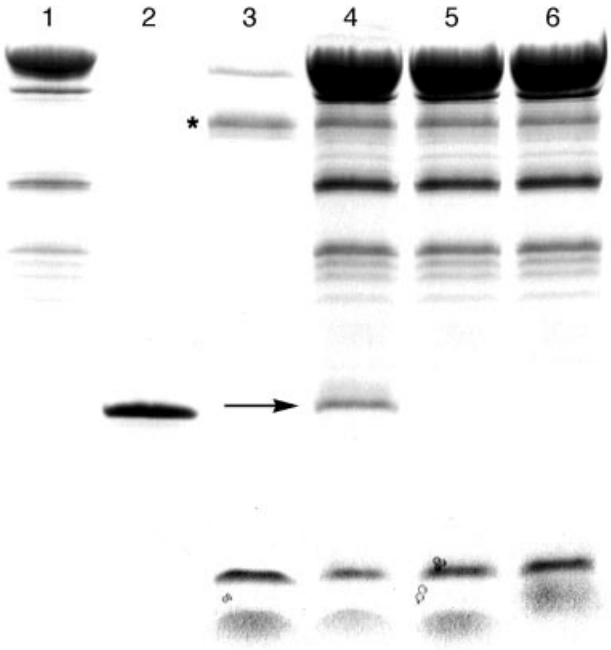


**Figure 5.** Analysis of RNA–RNA ligations catalyzed by the hairpin ribozyme using the substrates 5′-N′UCCUCUCC, where N′ indicates either guanosine (lane 4), 5′-chloro-5′-deoxyguanosine (lane 5) or 5′-amino-5′-deoxyguanosine (lane 6) using denaturing polyacrylamide gel electrophoresis. Lane 1, the hairpin ribozyme; lane 2, an authentic sample of the product resulting from a ligation reaction catalyzed by the hairpin ribozyme; lane 3, both of the substrates for the hairpin-catalyzed RNA–RNA ligation reaction, 5′-GUCCUCUCC and 5′-GGCCACCUGACA, the latter containing a 2′,3′-cyclic phosphate. The asterisk indicates an impurity in the latter oligomer. The arrow indicates a product of the hairpin-catalyzed reaction using the non-modified substrate.

### Chemical syntheses and characterization of RNAs containing 5′ modifications

The RNA oligomers 5′-N′UCCUCUCC, where N′ indicates either the 5′-chloro- or the 5′-amino-modified nucleoside described above, were synthesized using standard phosphoramidite chemistry. To verify that these RNA oligomers were indeed inhibitors of the RNA–RNA ligation reaction catalyzed by the hairpin ribozyme, a ligation assay was performed (Fig. 5). When the 92mer hairpin ribozyme (7) was incubated with two substrates, 5′-GUCCUCUCC and 5′-GGCCACCUGACA, the latter containing a 2′,3′-cyclic phosphate, a ligation product was formed (lane 4). When the substrate 5′-GUCCUCUCC was replaced with the substrates 5′-N′UCCUCUCC, where N′ is either 5′-chloro-5′-deoxyguanosine (lane five) or 5′-amino-5′-deoxyguanosine (lane six) no ligation product could be detected, confirming that these substrates inhibit the RNA–RNA ligation reaction catalyzed by the hairpin ribozyme. It is noteworthy that the short oligomer containing the 5′-amino-modified guanosine nucleoside has reduced mobility in the gel (lowest band, lane 6), relative to the other oligomers of the same length. This is not unexpected, because protonation of the 5′-amino group decreases the overall negative charge of the oligomer, causing it to run slower on the gel.

In conclusion, two hairpin ribozyme substrates have been prepared, in which the 5′-hydroxyl group of the 5′-terminal guanosine nucleoside has been replaced with either a 5′-chloro- or a 5′-amino group. These substrates have been shown to inhibit the RNA–RNA ligation reaction catalyzed by the hairpin ribozyme.

## ACKNOWLEDGEMENTS

We thank Dr A. R. Ferré-D'Amaré for helpful discussions and for performing the ligation assay, the Sigurdsson group for critical review of the manuscript and the National Institutes of Health (GM56947) for financial support.

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