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

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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′UCCUCUC, 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).

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 CH₂Cl₂ 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.

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(silica gel 60 F254 from E-Merck) and visualized with UV light and a p-anisaldehyde dip [prepared by mixing p-anisaldehyde (6 ml), H2SO4 (8.0 ml) and CH2COOH (2.4 ml) in C2H5OH (218 ml)] followed by charring on a hot plate. Flash column chromatography was carried out with EM type 60 (230–400 mesh) silica gel. 1H-, 13C- and 31P-NMR spectra were recorded on a Bruker 500 MHz DRX Avance FT-NMR spectrometer at frequencies 499.85, 125.70 and 40 MHz, respectively. Chemical shifts are reported in p.p.m. relative to DMSO-d6 (2.50 p.p.m. for 1H and 39.51 p.p.m. for 13C), CDCl3 (7.27 p.p.m. for 1H and 77.23 p.p.m. for 13C), (CD3)2CO (2.05 p.p.m. for 1H and 29.92, 206.68 p.p.m. for 13C) or CD2OD (3.31, 4.87 p.p.m. for 1H and 49.15 p.p.m. for 13C). Chemical shifts for 31P-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 1H-, 13C-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% methylene (aq.) at room temperature for 2.5 h (5′-chboro oligomer) and 1 h at 60°C (5′-amino oligomer). After deprotection, the methylene 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 DNAac-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′-GGCCACCUCAGA (65 μM), which contained a 2′,3′ cyclic phosphate (7), and a 92mer hairpin riboyme (7) (65 μM) were independently incubated with each of the 9mer substrates 5′-N′UCCUCUCU (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 MgCl2. After 1 h, the reaction mixtures were subjected to 20% denaturing polyacrylamide gel electrophoresis and the gel subsequently stained with toluidine blue.

N2-isobutyryl-5′-chloro-5′-deoxyguanosine (2)

A solution of N2-isobutyrylguanosine (I) (10) (5 g, 14.0 mmol), Ph3P (7.32 g, 28.0 mmol) and CCl4 (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 Na2SO4 and concentrated under reduced pressure. The residue was subjected to column chromatography (10% MeOH/CHCl3) 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 CH2Cl2 (6 ml) and washed with water and brine. The organic layer was dried over Na2SO4, the solvent removed in vacuo and the residue purified by column chromatography (8% MeOH/CHCl3) 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 CHCl3. The aqueous layer was evaporated in vacuo to give the 5′-chloro nucleoside 2 (0.036 g, 100%) as a white solid. (Rf = 0.41 in 10% MeOH/CHCl3); 1H-NMR (DMSO-d6) 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); 13C-NMR (DMSO-d6) 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 C51H49O17N16Cl2 (M+H+) 732.1074, found 732.1072.

Silylation of N2-isobutyryl-5′-chloro-5′-deoxyguanosine (2): preparation of compounds 3a–c

To a stirred solution of N2-isobutyryl-5′-chloro-5′-deoxyguanosine (2) (0.791 g), including the phosphorus-containing impurity, in DMF (4.7 ml) were added TBDMSI (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 CHCl3. The CHCl3 layer was washed with water and brine, dried over Na2SO4 and concentrated in vacuo. The residue obtained was subjected to silica gel chromatography using 25% Me2CO/CH2Cl2 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 Rf values on silica gel TLC plates using 30% Me2CO/CH2Cl2 as an eluent for 3a, 3b and 3c were 0.5, 0.4 and 0.7, respectively. N2-isobutyryl-2′-O-(tert-butylidemethylsilyl)-5′-chloro-5′-deoxyguanosine (3a): 1H-NMR (DMSO-d6) δ −0.16, −0.03 (2 × 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); 13C-NMR (DMSO-d6) 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 C52H52O19N15Si2 (M+H+) 846.1939, found 846.1933.

N2-isobutyryl-3′-O-(tert-butylidemethylsilyl)-5′-chloro-5′-deoxyguanosine (3b): 1H-NMR (DMSO-d6) 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), 8.31 (s, 1H), 11.53 (br s, 1H), 12.14 (br s, 1H); 13C-NMR found 486.1938, found 486.1944.

Phosphoramidite 4

Dissopropylethylamine (0.056 ml, 0.32 mmol) was added to a stirred solution of 2'-silylated compound 3a (0.094 g, 0.193 mmol) in CH2Cl2 (0.56 ml), which was subsequently treated with N,N-disopropyl methylphosphonamidic chloride (0.045 g, 0.227 mmol) at 25°C. After 15 min, the reaction mixture was diluted with CH2Cl2 (2 ml) and the organic layer was concentrated in vacuo over Na2SO4 and the solvent removed under reduced pressure.

Silylation of N2-isobutyryl-5'-deoxy-5'-trifluoroacetamidoguanosine (6): preparation of compounds 7a–c

To a stirred solution of N2-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 CHCl3. The organic layer was washed with water and brine, dried over Na2SO4 and concentrated in vacuo. The residue obtained was purified by silica gel flash column chromatography using 50% EtOAc/hexane as the eluent to furnish the phosphitylated product (4.080 g, 64%). 

HRMS m/z calc. for C20H33O5N5ClSi (M+H+): 379.1479, found 379.1479.
153.41, 155.26; 19F-NMR (CDCl3) (a mixture of diastereomers and rotamers) 151.05, 151.75, (data not shown). Therefore, we focused our efforts on the conditions used for the deprotection of RNA because it can be easily located by crystallography due to its anomalous X-ray scattering. However, preliminary studies with 5’-bromo-5’-deoxymethylidine revealed that a substantial amount of the bromo group was displaced by methylene 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.

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’-phosphate (Fig. 1). It is also desirable that the group should 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’-deoxymethylidine revealed that a substantial amount of the bromo group was displaced by methylene under the conditions used for the deprotection of RNA (data not shown). Therefore, we focused our efforts on

Figure 2. Synthesis of phosphoramidite 4.

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 CH2Cl2 (0.56 ml). This solution was treated with N,N-diisopropylmethyllphosphonamidic 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%) (Rf = 0.6 in 60% EtOAc/hexane); 31P-NMR (CDCl3) δ (a mixture of diastereomers and rotamers) 151.05, 151.75, 153.41, 155.26; 19F-NMR (CDCl3) δ −74.91, −75.09, −79.56, −79.75; HRMS m/z calc. for C28H48O6N6F3Si (M+H+) 724.323, found 724.3212.
5′-Amino-modified phosphoramidite

The 5′N2-isobutyl-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 Rf 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 isobutyl group was unexpectedly removed. This was probably due to excess sodium azide, which could, after reduction, facilitate the removal of the isobutyl group. Indeed, when the crude azide was chromatographically purified, the isobutyl 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% MeNH2 (aq.). 55°C, 10 min]. Reaction of the 5′-amino nucleoside with N-trifluoroacetyl imidazole gave the 5′-trifluoroacetamidc derivative 6 (22% yield from 5′N2-isobutylrylguanosine, four steps). Compound 6 was reacted with TBDMSCI 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 31P-NMR showed four peaks in the chemical shift range expected for a phosphoramidite. However, only two peaks are normally observed for the two ster conmutable. The 31P-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 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 31P-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 31P-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 19F-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 19F-NMR spectra, indicating the absence of rotamers that were stable on the NMR time scale. However, detection of amide rotamers by NMR has 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 19F-NMR spectra of compounds 7a and 7b, each of which exhibited one peak in a solution of acetone-d6, were recorded in MeOH-d4, 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 19F-NMR spectra were recorded in MeOH-d4 or CDCl3, we concluded that the doubling arose from trifluoroacetamide rotamers.
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 phosphoramide 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’-GGCCA-CCUGACAC, 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.

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