Replacement of the Cleavable Phosphodiester Bond of a Hammerhead Ribozyme Substrate by an Amide Linkage

Fabienne Burlina,[a] Alain Favre,[a] Jean-Louis Fourrey,*[b] and Martial Thomas[b]

Keywords: Amide linkage / Phosphodiester / Hammerhead ribozyme / Oligonucleotides

A nucleoside dimer in which the natural phosphodiester bond is replaced by an isosteric amide linkage has been prepared. This dimer analogue was subsequently incorporated chemically at the cleavage position of a hammerhead ribozyme substrate. Although the resulting substrate analogue exhibited a high affinity for the ribozyme as shown by gel retardation assays, the amide bond proved to be fully resistant to cleavage under standard conditions of ribozyme cleavage activity.

Introduction

The first ribozymes to be described were natural RNA molecules which were found to manifest an RNase activity.[1] Since this seminal discovery, an increasing number of RNA sequences endowed with the ability to catalyse a great number of chemical reactions have been proposed in the literature. In one series, artificial RNA constructs (derived from the natural ribozymes) efficiently catalyse phosphodiester bond cleavage within an RNA substrate with outstanding selectivity.[2] In this respect, the case of the most studied hammerhead ribozyme, which is comprised of a relatively short catalytic sequence, is very illustrative. On the other hand a number of RNA enzymes have been developed by in vitro selection and thus have greatly broadened the repertoire of catalytic activities exhibited by oligonucleotides.[3] In yet another remarkable step DNA enzymes with RNase activity have been proposed[4] and therefore by-pass the potential role of RNA 2′-hydroxyl groups for activity.

Hopefully, these nucleic acid derived tools will play an ever increasing role in gene inhibition strategies in the near future. As a prerequisite to achieve this goal it has become extremely important to investigate new methods for the stabilization of oligonucleotides in biological media.[5] For example, amide bonds have been proposed as surrogates of phosphodiester bonds. Thus, the replacement of phosphodiester backbone by amide bonds such as the „amide-3" linkages [6] has been shown to yield oligonucleotides mimics which exhibit a remarkable affinity for their complementary RNA and stability towards nucleases. Accordingly, we proposed to make use of these observations in the hammerhead ribozyme field by incorporating a few phosphodiester isoster amide linkages in place of the natural phosphodiester bonding in such systems. Moreover, we noticed that within the repertoire of ribozyme catalysed reactions, cases which involved the formation of the biologically important amide bond and cleavage reactions were relatively rare.[7] Accordingly, as a first step toward the construction of nuclease resistant hammerhead ribozymes incorporating amide bonds at crucial positions, we envisaged beginning our investigation by the synthesis of a hammerhead ribozyme substrate analogue containing an amide linkage in place of the normal scissile phophodiester bond in order to examine both its affinity for the ribozyme and, eventually, amide cleavage susceptibility.

For this investigation we considered as very appropriate the well-studied ribozyme system shown in Figure 1[8] whose 14-mer substrate **S** exhibits a CpU dimer at the cleavage site. We decided to replace this unit by the amide analogue **1** containing at the 3′-end a thymidine instead of uridine. The synthesis of the phosphoramidite **12** to be used for assembling the substrate analogue **S(am)** containing the amide dimer **1** in place of CpU is outlined in Scheme 1.2

Results and Discussion

Ketone **2**[9] was converted into the olefinic nucleoside **3** by a Wittig reaction and stereoselective hydrogenation of the latter in the presence of Pd/C catalyst by a well established procedure[10] gave the ester **4**. For transformation of **4** into the corresponding N^4-benzoylcytosine nucleoside **6**, we first of all prepared the 4-triazolyl derivative **5** by reaction of **4** with triazole in the presence of phosphorus oxychloride. Displacement of the triazolyl group of **5** was readily accomplished by treatment of the latter with the benzamide ion according to the method of Sanghvi[11] and gave the expected cytosine derivative **6** in an overall yield of 42% based on the starting material **2**. It is of importance to note here that it was our initial intention to use this scheme with the ketone derivative obtained by oxidation of *N4*-benzoyl-2′,5′-bis(*tert*-butyldimethylsilyl)cytidine, unfortunately the hydrogenation step after the Wittig reaction did not proceed satisfactorily with the cytidine analogue of **3**. Consequently, the scheme was modified as described above. Conversion of **6** to the expected phosphoramidite **12** was straightforward. Thus compound **6** was regioselectively desilylated at the 5′-

[a] Institut Jacques Monod, CNRS-Université Paris VII, 2 Place Jussieu, F-75251 Paris cedex 05, France
[b] Institut de Chimie des Substances Naturelles CNRS, F-91190 Gif-sur-Yvette, France

 © WILEY-VCH Verlag GmbH, D-69451 Weinheim, 2000 1434−193X/00/0204−0633 $ 17.50+.50/0

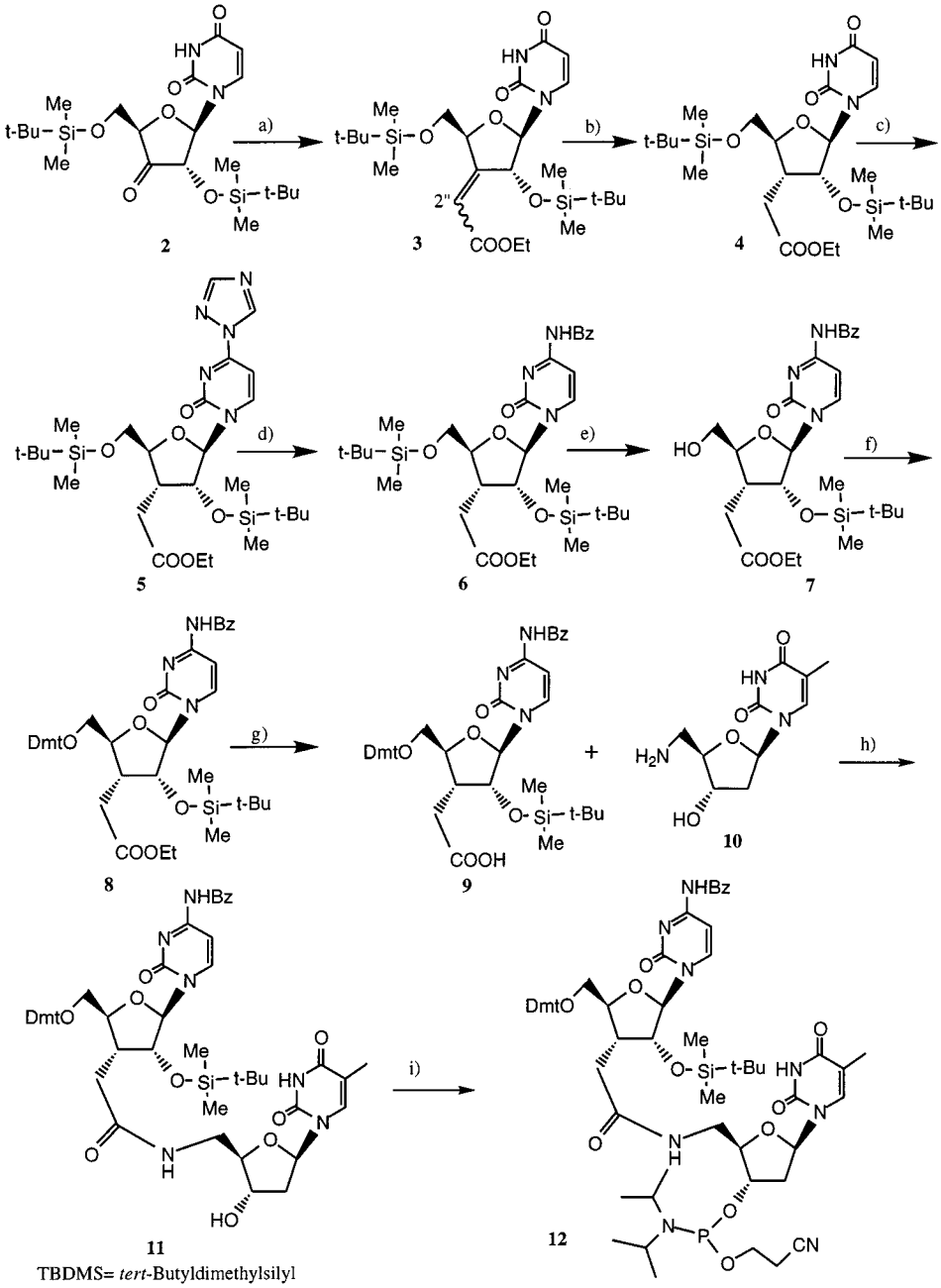
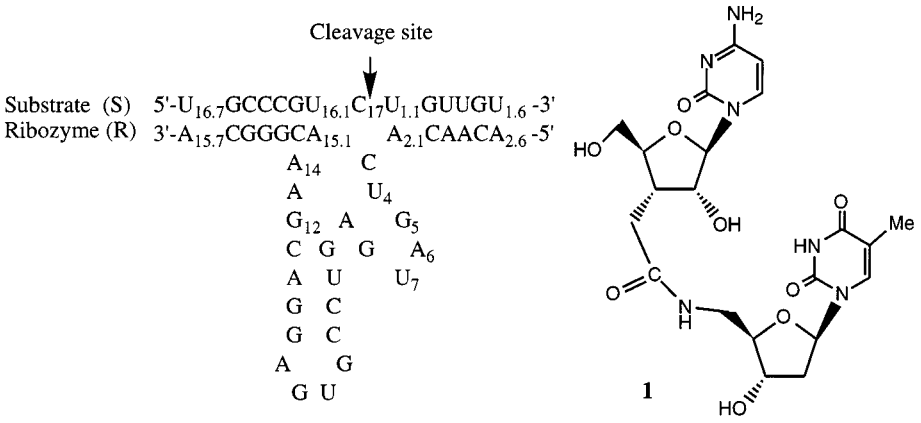


Figure 1. Ribozyme (**R**) and its substrate (**S**), arrow indicates the CpU cleavage site; **1** represents the amide analogue of CpU to be incorporated in **S(am)** at the cleavage site. For numbering see *Nucleic Acids Res.* **1992**, *20*, 3252.

O position by careful treatment with camphorsulfonic acid in methanol to yield compound **7** in moderate yield (52%) and dimethoxytritylation of **7** under standard conditions provided the 5′-protected derivative **8**. Some experimental work proved necessary to find reproducible conditions for the saponification of this compound. The best method for the synthesis of the acid **9** appeared to be treatment with sodium hydroxide in 95% ethanol followed by careful neutralisation. Coupling of **9** with 5′-amino-5′-deoxythymidine **10** was performed by HBTU in the presence of 1-hydroxybenzotriazole and gave **11** in a moderate yield (57%). Finally, the amide **11** was phosphitylated by treatment with cyanoethyl diisopropylchlorophosphoramidite to give **12** in 69% yield. This phosphoramidite **12** served for the chemical synthesis of the substrate analogue **S(am)** which contains the amide bond at the cleavage site (Figure 1).

S(am) and the nonmodified substrate **S** were both obtained by chemical RNA synthesis by standard protocols.[12] The synthesis was completed by treatment with ethanolic ammonium hydroxide, which caused cleavage of the oligoribonucleotide from the support and removal of both the cyanoethyl phosphate protecting groups and the base protecting groups. The remaining 2′-*O*-silyl groups were removed in the presence of triethylamine trishydrofluoride (TEA,3HF).[13] The oligoribonucleotides were finally purified by electrophoresis on 15% polyacrylamide gel in the



Scheme 1. a) $P(Ph)_3$ = CHCOOCEt, THF; b) H_2, Pd/C, EtOH; c) $POCl_3$, $(Et)_3N$, triazole, MeCN; d) benzamide, NaH, dioxane; e) camphorsulfonic acid, MeOH; f) dimethoxytrityl chloride, pyridine; g) NaOH, 95% EtOH; h) HBTU, HOBt, Et_3N, MeCN/THF (1:1); i) 2-cyanoethyl diisopropylchlorophosphoramite, *N,N'*-diisopropylethylamine, CH_2Cl_2.

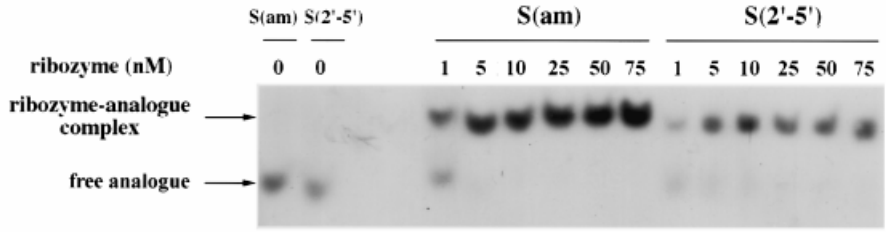


Figure 2. Autoradiogram obtained after native gel electrophoresis of the [5′-^{32}P]-labelled substrate analogues (0.2 nM) in the absence or the presence of increasing concentrations of ribozyme.

presence of 8 M urea. The substrate analogue **S(am)** was tested for cleavage using a transcribed 35-mer ribozyme **R**. Cleavage reactions were carried out under single turnover conditions for both **S(am)** and its reference substrate **S**. Thus, a trace of [5′-^{32}P]-labelled **S** or **S(am)** was incubated in the presence of a large excess of ribozyme (5 μM) either in 50 mM Tris-HCl buffer (pH 8), 20 mM $MgCl_2$ at 37 °C or 5 mM Hepes buffer (pH 7), 10 mM $MgCl_2$ at 10 °C. Under both sets of conditions, **S** is known to be efficiently cleaved with k_{cat} greater than 15 and 2 min^{-1} respectively.[14] In agreement with these experiments, full cleavage i.e. 98% at pH 8 and 80% at pH 7 was attained after 5 min of incubation. In contrast, no cleavage could be detected for **S(am)** under any of the above conditions even after an incubation period of 3 hours. We therefore investigated (by electrophoresis shift mobility assays in the presence of $MgCl_2$) whether the introduction of an amide linkage at the cleavage site affects the affinity for the ribozyme (Figure 2).

We used, as reference in these binding experiments, the noncleavable substrate analogue **S(2′-5′)** which had the same **S(am)** sequence but contained a single 2′,5′-phosphodiester bond at the cleavage site. This analogue was previously shown to be resistant to the ribozyme mediated cleavage and to behave as a competitive inhibitor of **S** in the ribozyme **R** mediated reaction.[15] In our first experiment the [5′-^{32}P]-labelled substrate analogues were incubated at 25 °C pH 7.5, 10 mM $MgCl_2$ in the presence of increasing concentrations of **R** (1 to 75 nM). As shown in Figure 2, even at the lowest ribozyme concentration, the analogues migrated on a nondenaturing polyacrylamide gel mostly as a retarded band due to formation of a ribozyme-substrate analogue complex with an increased friction coefficient. With both analogues a single retarded band can been seen independent of ribozyme concentration in agreement with the formation of a 1: 1 ribozyme-substrate analogue complex. In order to evaluate the apparent dissociation constants, another experiment was performed with lower **R** concentrations (0.2 to 5 nM). As shown in Figure 3, the two analogues behave quite similarly. The experimental data yielded an excellent fit with the curves (isotherms) derived3

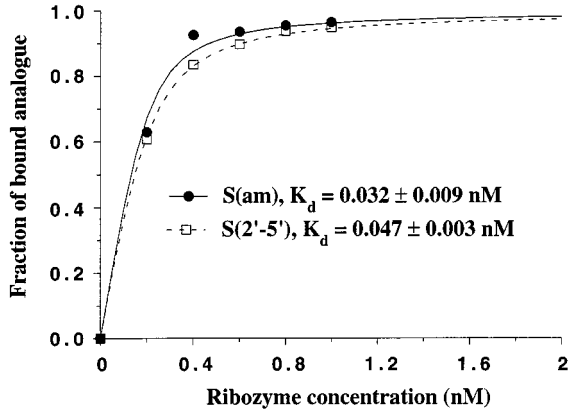


Figure 3. Determination of the equilibrium dissociation constants K_d. The fraction of bound substrate analogue was plotted as a function of the total ribozyme concentration. The lines represent the calculated best fits to the experimental data.

from a 1:1 equilibrium model and gave apparent K_d values close to 0.04 nM. Compared to **S(2′-5′), S(am)** appears to exhibit a slightly higher affinity which may be due in part to the fact that it lacks a negative charge at the 2′,5′-phosphate linkage in **S(2′-5′)** and thus decreases electrostatic repulsion within the core of the complex.

In conclusion, although **S(am)** binds to the hammerhead ribozyme **R** with high affinity, it is definitively not a substrate in the cleavage reaction. Our next objective will be the introduction of amide linkages within the nonconserved regions of ribozyme **R** as well as at sites of the catalytic core which are known to be highly RNase sensitive.[5]

Experimental Section

General: Solvents were dried by standard methods. – Standard reagents were of commercial quality (Acros, Aldrich or Lancaster) and were used without purification, 2-(*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was from France Biochem and γ-[^{32}P]-ATP (3000 Ci/mmol) was from Amersham (UK). – Microanalyses were performed by the "Service de Microanalyses de l'ICSN-CNRS". – ^{1}H, ^{13}C and ^{31}P NMR: Bruker AC200, AC250, AM300 and AM400 instruments. – NMR chemical shifts (δ) are given in ppm relative to TMS (^{1}H) or external PO_4H_3 (^{31}P). – For convenience standard numbering of nucleosides is used to describe the ^{1}H NMR spectra. – Mass spectrometry: AEI MS50 (EI); AEI MS9 (CI). – Flash chromatography: 32–63 mm silica-gel (SDS). – Thin layer chromatography: Silica gel 60 F_{254} coated sheets (SDS). – Standard RNA phosphoramidites were from PerSeptive Biosystems (France).

1-[2,5-Bis-*O*-(*tert*-butyldimethylsilyl)-3-deoxy-3-(ethoxycarbonyl)-methylene-β-D-ribofuranosyl]uracil (3): A solution of ketone **2** (3.7 g, 7.9 mmol) and (ethoxycarbonylmethylene)triphenylphosphorane (2.8 g, 8.37 mmol) in THF (60 mL) was stirred at room temp. for 18 h. The solvent was removed in vacuo and the residue was purified by flash chromatography on silica gel (heptane/EtOAc, 3:1) to give the pure title compound **3** (3.72 g, 65%). – ^{1}H NMR ($CDCl_3$): δ = 9.19 (s, 1 H, NH), 8.02 (d, 1 H, *J* = 8.1 Hz, H-6), 5.99 (d, 1 H, *J* = 7.6 Hz, H-1′), 5.90 (t, 1 H, *J* = 2.3 Hz, H2"), 5.79 (d, 1 H, *J* = 8.1 Hz, H-5), 5.36 (s, 1 H, H-4′), 4.69 (m, 1 H, H-2′), 4.26–3.91 (m, 4 H, 2 × CH_2), 1.31 (t, 3 H, *J* = 7.4 Hz, Me), 0.85 (s, 18 H, 6 × Me), 0.07 (s, 3 H, Me), 0.04 (s, 3 H, Me), 0.03 (s, 3 H, Me), –0.07 (s, 3 H, Me). – ^{13}C NMR ($CDCl_3$): δ = 165.3, 163.0, 159.4, 150.6, 140.0, 114.0, 103.4, 85.8, 80.1, 77.1, 64.7, 60.7, 25.6, 18.4, 17.9, –4.74, –4.94, –5.5. – CI-MS (*i*BuH); *m*/*z*: 541 [M + H]$^+$.

1-[2,5-Bis-*O*-(*tert*-butyldimethylsilyl)-3-deoxy-3-α-(ethoxycarbonyl)-methyl-β-D-ribofuranosyl]uracil (4): A solution of compound **3** (203 mg, 0.37 mmol) in ethanol (20 mL) containing 10% Pd/C (20 mg) was stirred for 16 h under a H_2 atmosphere. The solution was then filtered through Celite and the solid washed with ethanol. The combined filtrates were evaporated to give **4** (188 mg, 92%), mp: 134–135 °C. – ^{1}H NMR ($CDCl_3$): δ = 10.14 (s, 1 H, NH), 8.17 (d, 1 H, *J* = 7.2 Hz, H-6), 5.67–5.63 (m, 2 H, H-5, H-1′), 4.38 (s, 1 H, H2′), 4.07–3.69 (m, 5 H, H-4′, 2 × CH_2), 2.54–2.15 (m, 3 H, H-3′,CH_2), 1.21 (t, 3 H, *J* = 7.4 Hz, Me), 0.87 (s, 9 H, 3 × Me), 0.86 (s, 9 H, 3 × Me), 0.07 (s, 6 H, 2 × Me), 0.04 (s, 3 H, Me), 0.02 (s, 3 H, Me). – ^{13}C NMR ($CDCl_3$): δ = 171.6, 164.3, 150.5, 140.8, 140.5, 101.2, 91.3, 84.4, 77.6, 61.2, 60.4, 37.0, 29.1, 25.8, 18.4, 18.0, 14.2, –3.6, –4.4, –5.6, –5.7. – CI-MS (*i*BuH): *m*/*z*: 543 [M + H]$^+$. – $C_{25}H_{46}N_2O_7Si_2$ (542.81): calcd. C 55.32, H 8.54, N 5.16; found C 55.57, H 8.53, N 5.06.

1-[2,5-Bis-*O*-(*tert*-butyldimethylsilyl)-3-deoxy-3-α-(ethoxycarbonyl)-methyl-β-D-ribofuranosyl]-1,2-dihydro-4-(1-triazolyl)pyrimidin-2-one (5): Under an inert atmosphere, $POCl_3$, (0.5 mL, 5.46 mmol), followed by Et_3N (3 mL, 21.5 mmol) were added to a solution of 1,2,4-triazole (1.9 g, 27.5 mmol) in anhydrous acetonitrile (19 mL) at 0 °C. The solution was stirred for 30 min and then a solution of **4** (590 mg, 1.09 mmol) in anhydrous acetonitrile (9 mL) was added dropwise. After the addition, the reaction mixture was allowed to warm to room temp. and stirred for 18 h. The reaction was quenched with aqueous Et_3N (9.5 mL, 1:1, Et_3N/H_2O) and extracted with CH_2Cl_2 (25 mL). The solvent was evaporated in vacuo and the residue purified by flash chromatography (heptane/AcOEt, 3:1) on silica gel to give **5** (495 mg, 76.5%) as a powder. – ^{1}H NMR ($CDCl_3$): δ = 9.24 (s, 1 H, H-Tr), 8.89 (d, 1 H, *J* = 7.2 Hz, H-6), 8.09 (s, 1 H, H-Tr), 6.91 (d, 1 H, *J* = 7.2 Hz, H-5), 5.77 (s, 1 H, H-1′), 4.50 (d, 1 H, *J* = 3.6 Hz, H-2′), 4.23–3.67 (m, 5 H, H-4′, 2 × CH_2), 2.68–2.13 (m, 3 H, CH_2, H-3′) 1.21 (t, 3 H, *J* = 7.1 Hz, Me), 0.92 (s, 9 H, 3 × Me), 0.90 (s, 9 H, 3 × Me), 0.33 (s, 3 H, Me), 0.13 (s, 3 H, Me), 0.11 (s, 6 H, 2 × Me). – CI-MS (*i*BuH); *m*/*z*: 594 [M + H]$^+$. – $C_{27}H_{47}N_5O_6Si_2$ (593.86): calcd. C 54.60; H 7.98, N 11.56; found C 54.37, H 8.16, N 11.79.

***N*^{4}-Benzoyl-1-[2,5-bis-*O*-(*tert*-butyldimethylsilyl)-3-deoxy-3-α-(ethoxycarbonyl)methyl-β-D-ribofuranosyl]cytosine (6):** Sodium hydride (60% w/w, 330 mg, 8.25 mmol) was added to a solution of benzamide (1 g, 9 mmol) in anhydrous dioxane (25 mL). After 30 min, a solution of **5** (1.2 g, 2.02 mmol) in dioxane (24 mL) was added and the mixture stirred for 90 min. The reaction was quenched with acetic acid (2 mL, pH 6), the mixture diluted with CH_2Cl_2 (320 mL) and the organic phase washed twice with water (120 mL). The solvent was removed in vacuo and the reaction product purified by flash chromatography on silica gel (heptane/AcOEt, 7:3) to give **6** (1.187 g, 91%) as a powder. – ^{1}H NMR ($CDCl_3$): δ = 8.65 (d, 1 H, *J* = 6 Hz, H-6), 7.94–7.45 (m, 6 H, H-5, *N4*-BzC), 5.79 (s, 1 H, H-1′), 4.47 (d, 1 H, *J* = 3.2 Hz, H-2′), 4.22–3.69 (m, 5 H, H-4′, 2 × CH_2), 2.68–2.16 (m, 3 H, H-3′, CH_2), 1.16 (t, 3 H, *J* = 7.1 Hz, Me), 0.99 (s, 9 H, 3 × Me), 0.96 (s, 9 H, 3 × Me), 0.33 (s, 3 H, Me), 0.11 (s, 3 H, Me), 0.09 (s, 3 H, Me), 0.08 (s, 3 H, Me). – ^{13}C NMR ($CDCl_3$): δ = 171.6, 145.5, 133.1, 129.0, 127.9, 96.0, 92.6, 84.7, 77.6, 61.1, 60.1, 38.4, 36.6, 29.0, 26.0, 18.6, 18.2, 14.2, –4.1, –5.4, –5.8. – CI-MS (*i*BuH); *m*/*z*: 646 [M + H]$^+$. – $C_{32}H_{51}N_3O_7Si_2$ (645.93): calcd. C 59.50; H 7.96, N 6.50; found C 59.69; H 7.75; N 6.45.

***N*^{4}-Benzoyl-1-[2-*O*-(*tert*-butyldimethylsilyl)-3-deoxy-3-α-(ethoxycarbonyl)methyl-β-D-ribofuranosyl]cytosine (7):** A solution of **6** (1.017 g, 1.57 mmol) and camphorsulfonic acid (365 mg, 1.57 mmol) in methanol (10 mL) was stirred at room temp overnight. The reaction was quenched by the addition of Et_3N (0.22 mL, 1.57 mmol). The solvents were removed in vacuo and the residue purified by flash chromatography on silica gel. Elution with heptane/EtOAc (3:7) gave **7** (435 mg, 52%) as a white solid. – ^{1}H NMR ($CDCl_3$): δ = 8.73 (d, 1 H, *J* = 6.5 Hz, H-6), 7.93–7.47 (m, 6 H, H-5, *N*^{4}-BzC), 5.74 (s, 1 H, H-1′), 4.45 (d, 1 H, *J* = 3.4 Hz, H-2′), 4.17–3.67 (m, 5 H, H-4′, 2 × CH_2), 2.62–2.38 (m, 3 H, H-3′, CH_2), 1.22 (t, 3 H, *J* = 7.2 Hz, Me), 0.92 (s, 9 H, 3 × Me), 0.33 (s, 3 H, Me), 0.15 (s, 3 H, Me). – ^{13}C NMR ($CDCl_3$): δ = 174.0, 165.0, 145.8, 133.1, 129.0, 127.8, 96.2, 92.6, 85.7, 78.6, 61.3, 60.7, 36.1, 30.0, 26.0, 18.2, 14.2, –4.6, –5.5. – CI-MS (*i*BuH); *m*/*z*: 532 [M + H]$^+$.

***N*^{4}-Benzoyl-1-[2-*O*-(*tert*-butyldimethylsilyl)-3-deoxy-3-α-(ethoxycarbonyl)methyl-5-*O*-(dimethoxytrityl)-β-D-**

ribofuranosyl]cytosine (8): A solution of **7** (430 mg, 0.81 mmol) and dimethoxytrityl chloride (430 mg, 1.27 mmol) in pyridine (21 mL) was stirred overnight at room temp. The reaction was quenched by addition of methanol (2.1 mL). The mixture was diluted with EtOAc, the organic phase washed with water and concentrated in vacuo to give a residue which was purified by flash chromatography on silica gel (heptane/AcOEt, 3:2) to give **8** (521 mg, 77%) as a powder. – ^{1}H NMR ($CDCl_3$): δ = 8.72 (d, 1 H, *J* = 7.5 Hz, H-6), 7.93–6.83 (m, 19 H, H-5, Ar), 5.80 (s, 1 H, H-1′), 4.50 (d, 1 H, *J* = 3.4 Hz, H-2′), 4.14–3.27 (m, 5 H, H-4′, 2 × CH_2), 3.84 (s, 6 H, 2 × OMe), 2.57–1.86 (m, 3 H, H-3′, CH_2), 1.23 (t, 3 H, *J* = 4.8 Hz, Me), 0.89 (s, 9 H, 3 × Me), 0.35 (s, 3 H, Me), 0.11 (s, 3 H, Me). – $C_{47}H_{55}N_3O_9Si$ (834.04): calcd. C 67.69; H 6.65, N 5.04; found: C 67.51; H 6.75; N 5.26.

***N*^{4}-Benzoyl-1-[2-*O*-(*tert*-butyldimethylsilyl)-3-α-(carboxy)methyl-3-deoxy-5-*O*-(dimethoxytrityl)-β-D-ribofuranosyl]cytosine (9):** A 10% aqueous NaOH solution (0.5 mL) was added to a solution of **8** (245 mg, 0.29 mmol) in 95% ethanol (4.5 mL) and the resulting mixture stirred for 3 h at room temp. The reaction was quenched by the careful addition of a 10% aqueous HCl solution until the pH 6.5 . The solution was diluted with CH_2Cl_2, the organic phase washed with water and concentrated in vacuo. The residue was purified by flash chromatography on silica gel. Elution with heptane/EtOAc (6:4) gave unchanged **8,** which was used directly in the next step (53 mg, 21%), whilst elution with EtOAc/methanol (8:2) gave **9** (87 mg, 37%) as a foam. – ^{1}H NMR ($CDCl_3$): δ = 8.75 (d, 1 H, *J* = 7.5 Hz, H-6), 7.88–6.82 (m, 19 H, H-5, Ar), 5.77 (s, 1 H, H-1′), 4.48 (s, 1 H, H-2′), 4.06 (m, 1 H, H-4′), 3.77 (s, 6 H, 2 × OMe), 3.74 (d, 1 H, *J* = 9.8 Hz, H-5′), 3.26 (d, 1 H, *J* = 9.8 Hz, H-5′), 2.50 (m, 3 H, CH_2, H-3′), 0.84 (s, 9 H, 3 × Me), 0.27 (s, 3 H, Me), 0.05 (s, 3 H, Me).

***N*^{4}-Benzoyl-1-{2-*O*-(*tert*-butyldimethylsilyl)-3-deoxy-5-*O*-(dimethoxytrityl)-3-α-[*N*-(5′-deoxythymidin-5′-yl)carboxamido]-methyl-β-D-ribofuranosyl}cytosine (11):** A solution of **9** (150 mg, 0.19 mmol) in anhydrous acetonitrile/THF (1:1) (6 mL) maintained at room temp. and under an inert atmosphere, was treated with HBTU (90 mg, 0.282 mmol), followed by 1-hydroxybenzotriazole (20 mg, 0.148 mmol) and Et_3N (40 μL, 0.287 mmol). The resulting mixture was stirred for 2 h at room temp and then treated with 5′-amino-5′-deoxythymidine **10** (70 mg, 0.29 mmol) and Et_3N (60 μL, 0.43 mmol). The solution was stirred overnight, the solvents removed in vacuo and the residue purified by flash chromatography on silica gel. Elution with EtOAc/Methanol/Et_3N (97.8:2:0.2) gave **11** (145 mg, 74%). – Selected ^{1}H NMR data ($CDCl_3$): δ = 8.00–6.80 (m, 21 H, 2H-6, Ar, H-1′T), 5.90 (s, 1 H, H-1′C), 4.72–3.50 (m, 8 H, H-2′C, H-3′T, 2 H-4′, 4 H-5′), 3.60 (s, 6 H, 2 × OMe), 2.39 (m, 3 H, H-3′C, CH_2), 1.79 (s, 3 H, Me), 0.77 (s, 9 H, 3 × Me), 0.31 (s, 3 H, Me), 0.11 (s, 3 H, Me). – FAB-MS (LiCl); *m*/*z*: 1035 $[M + Li]^+$.

***N*^{4}-Benzoyl-1-{2-*O*-(*tert*-butyldimethylsilyl)-3-deoxy-5-*O*-(dimethoxytrityl)-3-α-[3′-*O*-(2-cyanoethyl-*N,N*-diisopropylphosphoramidite)-*N*-(5′-deoxythymidin-5′-yl)carboxamido]methyl-β-D-ribofuranosyl}cytosine (12):** A solution of compound **11** (145 mg, 0.14 mmol) and *N,N*′-diisopropylethylamine (100 μL, 0.57 mmol) in 1.5 mL of anhydrous dichloromethane under an inert atmosphere was treated with 2-cyanoethyl diisopropylchlorophosphoramidite (50 μL, 0.22 mmol). After 1 h, the reaction mixture was diluted with dichloromethane (10 mL), the solution extracted with 5% aqueous sodium bicarbonate solution and then with saturated aqueous NaCl. The organic layer was separated, dried over anhydrous sodium sulfate and the solvent removed in vacuo. The residue was purified by flash chromatography on silica gel. Elution with EtOAc/Et_3N (99.8:0.2) gave compound **12** as a white powder (104 mg, 69%). – ^{31}P NMR ($CDCl_3$): δ = 149.47–149.28.

Oligoribonucleotide Synthesis and Purification: The synthesis of the 14-mer Substrate **S** and the **S(am)** analogue were performed on an Applied Biosystems 392 DNA/RNA Synthesizer on a 1 μmol scale with commercial phosphoramidites and the standard RNA synthesis protocol.[12] Average stepwise coupling yields were in the 91.9–98.2% range for standard phosphoramidites and 78.9% for phosphoramidite **12**. After removal of the terminal 5′-*O*-dimethoxytrityl group, oligonucleotides were cleaved from the support by treatment (1 h) with a freshly prepared ethanolic 28% ammonia (3:1). The solution was maintained for 48 h at room temperature to remove the base labile protecting groups. Solvents were evaporated and the residue coevaporated with methanol in vacuo. The residue was taken up in doubly distilled water, transferred to an Eppendorf tube and lyophilized. The 2′-*O*-silyl groups were removed by treatment with 1 mL of triethylamine trihydrofluoride (98%)/DMF (4:1) for 1.5 h at 55 °C. The oligoribonucleotides were precipitated in butan-1-ol at –20 °C overnight and purified by preparative 15% polyacrylamide gel electrophoresis in the presence of 8M urea. Oligoribonucleotides were recovered from the gel by overnight elution with a 0.15 M NaCl at 4 °C. The eluted oligonucleotides were ethanol-precipitated at –20 °C, washed with cold 70% ethanol, dried under vacuum and redissolved in water. They were stored at –20 °C until used. A second run of purification on 15% polyacrylamide gel was performed after labelling the 5′-terminal nucleotide with [^{32}P] using T4-Polynucleotide kinase and [γ-^{32}P]-ATP. The radioactive band corresponding to the full-length oligoribonucleotide was recovered from the gel as described above and used for all subsequent experiments. The 35-mer ribozyme **R** was obtained by transcription using T7 RNA polymerase by standard protocol.[16]

Cleavage Experiments: Cleavage tests were performed in a 50 mM Tris-HCl buffer (pH 8), 20 mM $MgCl_2$ at 37 °C or in 5 mM Hepes buffer (pH 7), 10 mM $MgCl_2$ at 10 °C. To avoid adsorption of the oligoribonucleotides on the Eppendorf tubes, the reactions were carried out in tubes pretreated with a solution of dichlorodimethylsilane. A 15 μL volume solution containing a trace of [5′-^{32}P]-labelled substrate in the cleavage buffer was heated at 65 °C for 2 minutes and then incubated at the reaction temperature. The reaction was initiated by the addition of 10 μL of this solution to a 10 μL solution of ribozyme (final concentration 5μM). For each kinetic measurement, ten aliquots of 1.5 μL were removed at various times (up to 3 h) and the reaction was stopped in a 3 μL solution of 5 mM EDTA, 0.025% xylene cyanol, 80% formamide. Substrate and product were separated on a 20% polyacrylamide gel, 8 M urea. The product fraction was quantified using a Molecular Dynamics PhosphorImager and ImageQuant software.

Measurement of the Equilibrium Dissociation Constants: The K_d were measured using shift electrophoresis assays as described by Fedor *et al.*[17] [5′-^{32}P]-Labelled substrate analogues and ribozyme were heated separately for 2 min at 65 °C in 50 mM Tris-HCl buffer (pH 7.5) containing 5% sucrose, 0.02% bromophenol blue, 0.02% xylene cyanol, 10 mM $MgCl_2$ and then incubated at 25 °C for 10 min. Analogues (at final concentration 0.2 nM) were incubated in the presence of increasing concentrations of ribozyme (0.2 to 75 nM) for 15 min at 25 °C. Free substrate analogue and analogue-ribozyme complex were separated on a nondenaturing 15% polyacrylamide gel in 50 mM Tris-acetate buffer (pH 7.5), 10 mM $MgCl_2$. After 2 hours of pre-run at 25 °C of the gel, the samples were run for 4 h at 15 W. The amounts of free analogue and analogue-

ribozyme complex were determined using a Molecular Dynamics PhosphorImager and ImageQuant software. In order to properly evaluate the apparent dissociation constant K_d, the fraction f of substrate analogue, which migrated as a retarded band, was plotted as a function of the total concentration of ribozyme added R_T (0.2 to 5 nM). Assuming formation of a 1:1 complex, this curve was fitted with Kaleidagraph by means of the Equation

$$f^2 - f\left(1 + \frac{K_d + R_T}{S_T}\right) + \frac{R_T}{S_T} = 0$$

where $S_T = 0.2$ nM is the total substrate concentration and K_d the constant to be determined.

Acknowledgments

This work was supported by CNRS (ACC 5) and the "Ligue Nationale de Recherche sur le Cancer". We thank Sidaction for a postdoctoral fellowship (F. B.) and V. Lefort for her efficient assistance.

[1] [1a] K. Kruger, P. J. Grabowski, A. J. Zaug, J. Sands, D. E. Gottschling, T. R. Cech, *Cell* **1983**, *31*, 147–157. – [1b] C. Guerrier-Takada, K. Gardiner, T. Marsh, N. Pace, S. Altman, *Cell* **1983**, *35,* 849–857.

[2] [2a] N. K. Vaish, A. R. Kore, F. Eckstein, *Nucleic Acids Res.* **1998**, *26*, 5237–5242. – [2b] F. Eckstein, D. M. J. Lilley, *Catalytic RNA*, Springer Verlag, Berlin, Germany,**1997**. – [2c] L. Jaeger, *Curr. Opin. Chem. Biol*. **1997**, *7*, 324–335.

[3] [3a] J. R. Lorsch, J. W. Szostak, *Acc. Chem. Res.* **1996**, *29*, 103–110. – [3b] A. A. Beaudry, G. F. Joyce, *Science* **1992**, *257*, 635–641.

[4] G. F. Joyce, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 5845–5647.

[5] F. Burlina, A. Favre, J.-L. Fourrey, *Bioorg. Med. Chem*. **1997**, *5*, 1999–2010.

[6] [6a] G. Stork, C. Zhang, S. Gryaznov, R. Schultz, *Tetrahedron Lett*. **1995**, *36*, 6387–6390. – [6b] J. Lebreton, A. Walner, C. Lesueur, A. De Mesmaeker, *Synlett* **1994**, 137–140. – [6c] I. Idziak, G. Just, M. J. Damha, P. A. Giannaris, *Tetrahedron Lett*. **1993**, *34*, 5417–5420.

[7] [7a] B. Zhang, T. R. Cech, *Chem. Biol.* **1998**, *5*, 539–553. – [7b] A. Jenne, M. Famulok, *Chem. Biol.* **1998**, *5*, 23–34. – [7c] P. A. Lohse, J. W. Szostak, *Nature* **1996**, *381*, 442–444. – [7d] X. Dai, A. De Mesmaeker, G. F. Joyce, *Science* **1995**, *297*, 237–240.

[8] A. Woisard, J.-L. Fourrey, A. Favre, *J. Mol. Biol.* **1994**, *239*, 366–370.

[9] F. Hanske, D. Mades, M. J. Robins, *Tetrahedron* **1984**, *40*, 125–135.

[10] K. Lee, D. F. Wiemer, *J. Org. Chem.* **1993**, *58*, 7808–7812.

[11] M. Perbost, T. Hoshiko, F. Morvan, E. Swayze, R. H. Griffey, Y. Sanghvi, *J. Org. Chem*. **1995**, *60*, 5150–5156.

[12] [12a] M. J. Gait, C. Pritchard, G. Slim, *Oligonucleotides and analogues: A practical approach*, (Ed.: F. Eckstein), IRL Press, Oxford, UK, **1991**, pp. 25–48. – [12b] M. J. Damha, K. K. Ogilvie, *Methods Mol. Biol.* **1993**, *20*, 81–114.

[13] D. Gasparutto, T. Livache, H. Bazin, A. M. Duplaa, A. Guy, A. Khorlin, D. Molko, A. Roget, R. Teoule, *Nucleic Acids Res.* **1992**, *20*, 5159–5166.

[14] F. Burlina, A. Favre, J.-L. Fourrey, M. Thomas, *J. Chem. Soc.; Chem. Commun.* **1996**, 1623–1624.

[15] F. Burlina, J.-L. Fourrey, V. Lefort, A. Favre, *Tetrahedron Lett*. **1999**, *40*, 4559–4562.

[16] J. F. Milligan, O. C. Uhlenbeck, *Methods Enzymol.* **1989**, *180*, 51–62.

[17] M. J. Fedor, O. C. Uhlenbeck, *Biochemistry* **1992**, *31*, 12042–12054.

Received July 9, 1999
[O99431]