Cyclic Oligoribonucleotides (RNA) by Solid-Phase Synthesis

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Abstract: A novel solid-phase synthesis of small- to medium-sized cyclic RNA oligonucleotides is presented. A major advantage of the approach is the lack of restrictions on the sequence variety with respect to the four standard bases adenine, cytosine, guanine, and uracil. This has been demonstrated for cycles containing 2 to 21 nucleotide units. The approach allows fully automated assembly, and is related to a procedure known for the preparation of cyclic oligonucleotides in the DNA series (E. Alazzouzi, N. Escaja, A. Grandas, E. Pedroso, *Angew. Chem.* **1997**, *109*, 1564–1567; *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 1506–1508). It combines standard phosphoramidite chemistry for chain elongation and standard phosphotriester chemistry for ring closure. A key aspect of the method is use of the novel 2′-*O*-triisopropylsilyloxymethyl (TOM) protected RNA phosphoramidites (X. Wu, S. Pitsch, *Nucleic Acids Res.* **1998**, *26*, 4315–4323) instead of the classic *tert*-butyldimethyl silyl (TBDMS) protected amidites. Furthermore, the design of the final cleavage step is selective only for correctly cyclized oligoribonucleotides. This results, after deprotection, in HPLC profiles in which the crude oligonucleotide is represented by the major peak with typically more than 80% of the integrated area. The ring closure itself proceeds with an average yield of 15%.

Keywords: circular RNA · macrocycles · oligoribonucleotides · solid-phase synthesis

Introduction

Circular RNA is frequently found in nature. This is reflected in the large number of circular viroids[1] and in RNA cyclase ribozyme activity.[2] The biological significance of circularity often remains unclear and is a matter of intense current research.[3]

Circular DNA has received attention lately, as it can be used as efficient templates for DNA and T7-RNA polymerases, a process termed as rolling circle DNA/RNA synthesis.[4] Furthermore, cyclic oligodeoxynucleotides have been investigated in the context of antisense and antigene therapeutics because of their intrinsic resistance towards degradation by exonucleases.[5] In numerous structural studies it has been shown that conformational constraints by circularity cause previously unnoticed structural motifs in nucleic acids.[6]

So far, most techniques for the preparation of cyclic nucleic acids involve template-directed ligation of a linear precursor oligonucleotide. This approach is probably the most reasonable for large cycles (>30–35 nucleotides). Several variations are found in the literature. The template can be provided as a separate sequence[7] as well as an internal secondary structure.[8] The ligation itself proceeds either enzymatically (T4 DNA/RNA ligase)[7a, 8a] or is chemically controlled (BrCN/imidazole or *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide).[7b, 8b–c] Generation of circular RNA by autocatalytic splicing has yet to be shown to be an effective synthetic procedure.

Recently, an efficient solid-phase synthetic approach for small- to medium-sized cyclic DNA oligonucleotides has been described;[9] however, efficient procedures for the preparation of cyclic RNA oligonucleotides with comparable ring sizes have not yet been reported.[10] This objective is addressed and successfully achieved with the method described herein. As a major advantage, the method has no restriction on the sequence choice despite the fact that it is an operationally simple process. Therefore, this protocol provides a powerful tool to study structural and physicochemical properties of small- to medium-sized cyclic RNA oligonucleotides.

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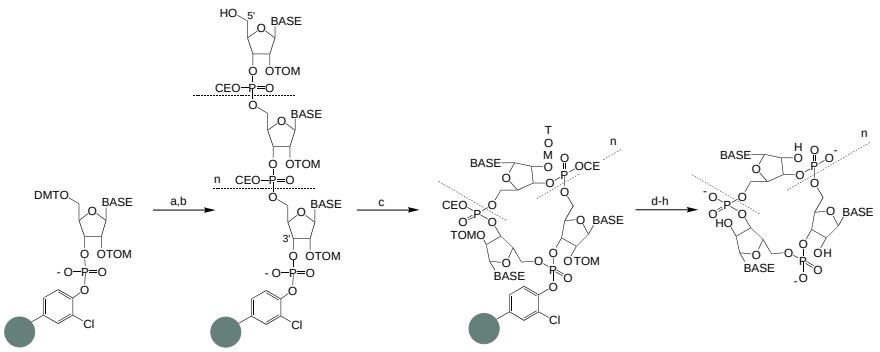
Results and Discussion

The novel approach for the preparation of cyclic oligoribonucleotides combines standard RNA phosphoramidite chemistry for chain elongation and standard phosphotriester chemistry for ring closure. Deprotection of the synthesized

  0947-6539/99/0507-2077 $ 17.50+.50/0

cyclic RNAs can also be achieved by typical standard procedures established in RNA automated synthesis, although the ribose 2′-OHs are not protected with the commonly used *tert*-butyl dimethylsilyl group (TBDMS) but with the recently introduced triisopropylsilyloxymethyl (TOM) protecting group.[11]



Scheme 1. Synthesis of cyclic oligoribonucleotides. a) Chain assembly; $n+1$ repeats of: detritylation ($CHCl_2COOH$), coupling (5′-*O*-DMT-2′-*O*-TOM-nucleoside-3′-*O*-P(OCE)N*i*Pr_2/benzylthiotetrazole), capping (Ac_2O), oxidation (I_2); b) detritylation ($CHCl_2COOH$); c) cyclization (MSNT); d) CE-deprotection (TEA/pyridine); e) cleavage from the solid support (tetramethylguanidinium pyridine-2-aldoximate); f) deprotection of the bases (NH_3 and/or $MeNH_2$); g) TOM-deprotection (TBAF, desalting - Sephadex G 10); h) purification (ion-exchange-MONO Q).

Figure 1 shows the modified solid-phase supports required. Their synthesis is straightforward: (3-chloro-4-hydroxyphenyl)acetic acid 2,4,5-trichlorophenyl ester[9] was treated with a 2′-*O*-TOM-nucleoside cyanoethylphosphoramidite in the presence of benzylthiotetrazole followed by oxidation with *tert*-butyl hydroperoxide to yield the corresponding nucleotide-linker. A polyethyleneglycol – polystyrene copolymer, commercially available as TentaGel S NH_2, served as the matrix. After its modification with 6-aminohexanoic acid,[12] coupling of the nucleotide-linker in the presence of *N*,*N*′-dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) to the amino-derivatized support followed by removal of the cyanoethyl (CE) group, afforded the anionic nucleotide support **1** with an average loading of 90 μmol g^{-1}.

Assembly of the oligonucleotide chain using 2′-*O*-TOM RNA CE-phosphoramidites proceeded with stepwise average coupling yields of more than 98 %. Final detritylation provided the 5′-terminal hydroxy group for cyclization with

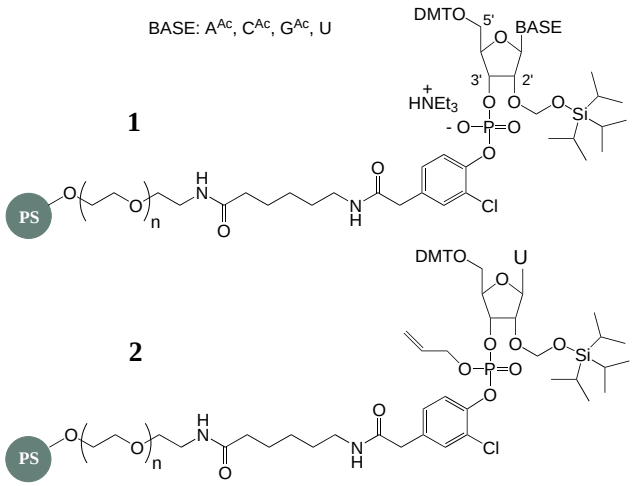


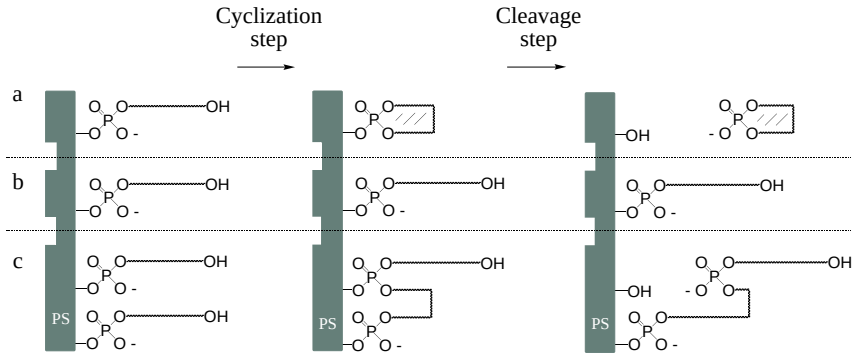
Figure 1. Modified solid supports, **1** and **2**, for the synthesis of cyclic oligoribonucleotides based on a TentaGel S NH_2 matrix; polystyrene (PS).

Abstract in German: *Die vorliegende Arbeit stellt eine neuartige Festphasensynthese für kleine bis mittelgrosse cyclische RNA-Oligonukleotide vor. Ein Hauptvorteil der Methode, nämlich ihre absolute Sequenzunahängigkeit im Bezug auf die vier Standardbasen Adenin, Cytosin, Guanin, und Uracil, wird mit der Darstellung und Charakterisierung von mehr als 20 Cyclen mit Ringgrössen von 2 bis 21 Nukleotideinheiten belegt. Die Methode erlaubt den automatisierten Strangaufbau und orientiert sich an der Darstellung cyclischer DNA (E. Alazzouzi, N. Escaja, A. Grandas, E. Pedroso, Angew. Chem.* ***1997****, 109, 1564 – 1567; Angew. Chem. Int. Ed. Engl.* ***1997****, 36, 1506 – 1508). Sie kombiniert Standardphosphoramiditchemie für die Strangverlängerung mit Standardphosphotriesterchemie für den Ringschluss. Ein entscheidender Aspekt der Methode ist die Verwendung der erst kürzlich eingeführten 2′-O-Triisopropylsilyloxymethyl (TOM) geschützten RNA-Phosphoramidite (X. Wu, S. Pitsch, Nucleic Acids Res.* **1998**, *26, 4315 – 4323) anstelle der klassischen tert-Butyl dimethylsilyl (TBDMS) geschützten Amidite. Weiters ist der Abspaltungsschritt vom Träger selektiv nur für die korrekt cyclisierten Oligoribonucleotide. Der Reinheitsgrad der Rohprodukte beträgt mehr als 80 %, der Ringschluss selbst erfolgt mit einer durchschnittlichen Ausbeute von 15 %.*

the 3′-terminal phosphodiester group (Scheme 1). Thereby, the coupling reagent of choice was 1-mesitylenesulfonyl-3-nitro-1,2,4-triazole (MSNT). Subsequent removal of the CE-phosphate protecting groups was performed in order to apply a selective cleavage step for the correctly cyclized oligoribonucleotides which were then bound exclusively through phosphotriesters to the resin. These *o*-chlorophenylphosphotriesters were readily cleaved by tetramethylguanidinium pyridine-2-aldoximate, while all noncyclized or crosslinked oligoribonucleotides remained attached to the solid support through phosphodiester linkages (Scheme 2).

Deprotection of the nucleobases was performed with saturated methylamine in ethanol/water at room temperature for cyclic oligonucleotides with less than 10 nucleotides. For larger cycles, previous treatment with concentrated ammonia in water/ethanol at 42 °C resulted in more satisfactory HPLC profiles of the crude product. TOM-deprotection of the ribose 2′-OHs was carried out with tetrabutylammonium fluoride (TBAF) followed by desalting over a Sephadex G 10 column. As a characteristic feature, the HPLC profile of a crude cyclic oligonucleotide (up to a decamer) showed a major peak of more than 85 % of the integrated area, and for a larger cycle it

0947-6539/99/0507-2078 $ 17.50+.50/0 *Chem. Eur. J.* **1999**, *5*, No. 7



Scheme 2. Depending on the length of the oligoribonucleotide about 5 to 40% are correctly cyclized. However, deprotection of the cyanoethyl groups from the phosphate backbone allows subsequent cleavage with tetramethylguanidinium pyridine-2-aldoximate which is selective for phosphotriesters. Therefore, the correctly cyclized oligonucleotides (a) are released whereas noncyclized (b) or cross-linked oligonucleotides (c) remain attached to the solid support.

was still more than 60% (Figure 2). Purification of all sequences was performed on an ion-exchange column. Purity was checked by capillary electrophoresis or reversed-phase HPLC and usually exceeded 95%. Absolute yields of the purified products were 15% for the smaller cycles and

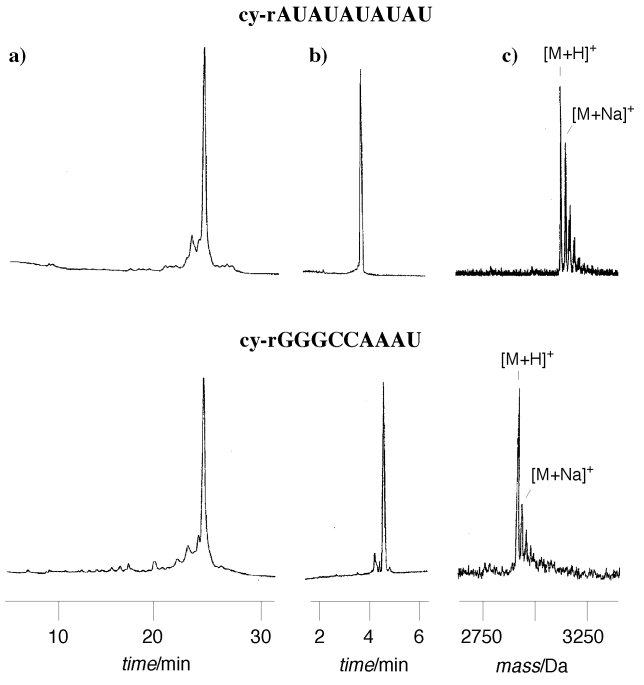


Figure 2. Documentation of the preparation of two cyclic oligonucleotides, cy-rAUAUAUAUAU and cy-rGGGCCAAAU: a) Anion-exchange HPLC chromatograms of the crude products after deprotection, gradient *I*. b) Open tube CE electropherograms after their purification. c) MALDI-TOF mass spectra (compare with Table 1; No. 16, 17). For details see the Experimental Section.

decreased for the larger ones. Table 1 lists some of the cyclic oligoribonucleotides prepared and their corresponding linear references together with characterization data obtained by MALDI-TOF mass spectrometry. Preparation of cyclic heneicosamer (No. 24) reflected the limitations of the method with respect to ring size and facile purification. So far, all sequences were synthesized on a 1 μmol scale, however, the method is amenable to scale-up.

All sequences in Table 1 were prepared with the anionic support **1**. Because of the unprotected phosphodiester group of **1**, chain elongation can be considered to be negatively affected through chain branching. In order to obtain experimental evidence concerning this question, the rU-support **2** with an allyl-protected phosphate group was prepared. Allyl protection is expected to be completely orthogonal to the other semipermanent phosphate protecting groups used (CE and *o*-chlorophenyl). For comparison, three sequences, namely cy-rCAU, cy-rACGU, and cy-rUUUUUU were synthesized on support **2**. The only difference in the preparation of cyclic oligonucleotides with this support was the allyl deprotection of the 3′-terminal phosphate involving tetrakis(triphenylphosphane)palladium before cyclization with MSNT. The subsequent steps remained unchanged. HPLC profiles of the crude cyclic oligonucleotides obtained were of comparable quality but yields were 5 to 10% less. Therefore, the allyl-protected support **2** is not superior to **1**.

Resistance to degradation of the cyclic oligoribonucleotides by calf-spleen phosphodiesterase was tested on cy-rACGU, cy-rGAGC$_4$U and cy-rU$_2$GA$_4$GAGA$_2$C$_3$U. Compared with their corresponding linear counterparts, resistance of the cyclic oligonucleotides was highly increased for the two shorter sequences whereas resistance was about the same for the hexadecamers (Figure 3). With these experiments, the expectation was confirmed that migration of the cyclic compounds is faster than migration of the corresponding linear reference sequences in capillary electrophoresis and reversed-phase HPLC.

A major reason for the success of the approach is founded on the use of RNA phosphoramidites that are TOM-protected at the ribose 2′-OH and *N*-acetylated at the bases. To the author's knowledge, only two cyclic oligoribonucleotides, namely cy-rC$_8$ and cy-rC$_{14}$, have previously been prepared with 2′-OH-TBDMS RNA phosphoramidites, by means of a different solid-phase approach.[10a] In general, TOM/acetyl-protected RNA phosphoramidites seem to be advantageous compared with the classic TBDMS/benzoyl-protected RNA phosphoramidites.[11] The combination of these protecting groups guarantees smooth and complete deprotection after synthesis. With respect to the solid support **1**, with ribose 2′-OH TBDMS protection, silyl migration to the anionic phosphodiester group which results in cleavage of the oligonucleotide from the solid support would have to be considered during chain assembly.

The sequences listed in Table 1 were chosen, on the one hand to show that the procedure has absolutely no restriction on the sequence variety, and on the other hand for their

Table 1. Synthesis and characterization of cyclic and linear oligoribonucleotides.

No.	Sequence[a]	Synthesis scale [μmol]	Yield[b] [OD]	[%]	$[M+H]^+$ calc.	obs.	Δ[%]
1	cy-rAU	1	4.2	15	652	651	−1.5
2	cy-rGU	1	2.7	14	638	636	−3.1
3	cy-rUU	1	3.1	15	613	613	0
4	cy-rAA	1	4.3	13	663	659	−6.1
5	cy-rCAU	1	1.9	6	942	941	−1.1
6	cy-rGAU	1	4.3	11	982	981	−1.0
7	cy-rACGU	1	2.2	5	1287	1286	−0.8
	rACGU-3′-p	2	22.4	25	1305	1304	−0.8
8	cy-rAGACAA	1	7.5	10	1968	1966	−1.0
9	cy-rAAAAAA	1	4.6	5	1976	1976	0
10	cy-rUUUUUU	1	1.8	3	1838	1838	0
11	cy-rCCCCCC	1	2.7	5	1832	1832	0
12	cy-rGGGGGG	1	2.0	3	2072	2072	0
13	cy-rGAGCCCCU	1	0.9	1	2548	2545	−1.2
	rGAGCCCCU-3′-OH	1	6.1	8	2486	2483	−1.2
14	cy-rAUUCAUUC	1	4.6	6	2496	2496	0
15	cy-rCUCGCUUG	1	2.1	3	2525	2526	+0.4
16	cy-rGGGCCAAAU	1	4.1	4	2941	2938	−1.0
17	cy-rAUAUAUAUAU	1	2.7	2	3178	3180	+0.6
18	cy-rGCGCGCGCGC	1	1.8	2	3253	3252	−0.3
19	cy-rGCGAAACGCUUU	1	1.8	1	3858	3859	+0.3
20	cy-rAAAGAGACACUC	1	1.7	1	3888	3889	+0.3
21	cy-rAUCAUUUUUCUGAU	1	1.3	1	4394	4388	−1.4
22	cy-rGAUGAGGCGhegACGACU[c]	1	0.7	1	5261	5257	−0.8
23	cy-rUUGAAAAGAGAACCCU	1	0.8	1	5175	5171	−0.8
	rUUGAAAAGAGAACCCU-3′-p	2	17.2	9	5193	5194	+0.2
24	cy-rGGAGAUCUGAGCGCUCUCUCC	1	0.6	1	6727	[d]	

[a] Cyclic rApAp (cy-rAA). [b] Yields were determined after purification. One optical density (1 OD) is equivalent to the amount of oligonucleotide dissolved in 1 mL water showing an extinction of 1 at a wavelength of 260 nm when measured in a cell with pathlength of 1 cm; yields were calculated by using the values for extinction coefficients given in the Experimental Section. [c] Hexaethylene glycol (heg). [d] The $[M+H]^+$ peak was not detected due to higher salt adducts.

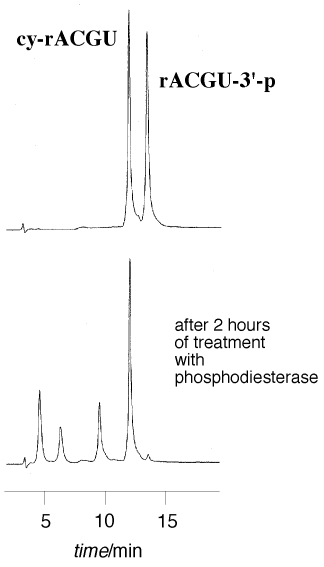


Figure 3. Enzymatic resistance and digestion, respectively, of cy-rACGU and rACGU-3′-p towards phosphodiesterase from calf spleen followed by reversed-phase HPLC, gradient *III*; start (above) and after 2 hours (bottom); for detailed reaction conditions see the Experimental Section.

possible structural or biological significance. For instance, the DNA analogues of cy-rAUUCAUUC (No. 14) and cy-rCUCGCUUG (No. 15) form a previously unrecognized general four-stranded DNA motif, the so-called bi-loop.[6] Cyclic diribonucleotides (No. 4) are known for their activity as RNA polymerase inhibitors,[10c] and in case of cy-rGG, as an activator for cellulose synthase.[12] The sequence cy-rGAUGAGGCGhegACGACU (No. 22) represents an artificial circular hammerhead ribozyme. Its biological activity is retained and its synthesis has also been achieved by a template-mediated ligation with T4 RNA ligase.[8a] Furthermore, cy-rGGAGAUCUGAGCGCUCUCUCC (No. 24) is a cyclic HIV-1 TAR RNA analogue; a whole family of these analogues show high *Tat*-binding activity.[13]

Conclusions

For the first time a solid-phase synthesis for small- to medium-sized cyclic oligoribonucleotides with no restrictions on the sequence variety is presented. The approach allows fully automated assembly with commercially available 2′-*O*-TOM RNA phosphoramidites. Ring closure is achieved by standard phosphotriester RNA chemistry. Selective cleavage from the solid support provides the cyclic oligoribonucleotide in high purity after deprotection.

In particular, because of their higher resistance to exonuclease cleavage, cyclic RNA molecules are potential targets in therapeutics and diagnostics. Provided that a specific research topic allows their use, cyclic RNAs are advantageous over linear sequences whenever applied in the presence of biological materials.[19]

0947-6539/99/0507-2080 $ 17.50+.50/0 *Chem. Eur. J.* **1999**, *5*, No. 7

Experimental Section

General: All chemicals (reagent grade) and solvents (puriss.) were from Aldrich or Fluka (exceptions mentioned). Tentagel S NH_2 (Advanced ChemTech; Louisville, Kentucky 40228, www.peptide.com), 2′-*O*-TOM-RNA phosphoramidites and benzylthiotetrazole (Xeragon AG; 8005 Zurich, Switzerland, rna@xeragon.com), dicyanoimidazole (GlenResearch; Sterling, Virginia 20166, support@glenres.com). Molecular sieves (4 Å) were activated at 250 °C under vacuum. UV spectroscopy was performed on a Cary 1 Bio spectrophotometer (Varian). For determination of oligonucleotide concentrations, the following extinction coefficients (260 nm) [$L\,mol^{-1}cm^{-1}$] were used: adenosine 15300, guanosine 11700, cytosine 7400, uridine 9900. All matrix-assisted laser desorption/ionization (MALDI) analyses were performed by the Mass Spectrometry Facility of The Scripps Research Institute on a PerSeptive Biosystems Voyager-Elite mass spectrometer with delayed extraction; matrix: 2,4,6-trihydroxyacetophenone; ions were detected in the positive mode. Abbreviations: acetyl (Ac), cyanoethyl (CE), *N*,*N*′-dicyclohexylcarbodiimide (DCC), 4,5-dicyanoimidazole (DCI), *N*,*N*-dimethylformamide (DMF), 4,4′-dimethoxytrityl (DMT), ethyl (Et), hexaethylene glycol (heg), 1-hydroxybenzotriazole (HOBt), 1-mesitylenesulfonyl-3-nitro-1,2,4-triazole (MSNT), polystyrene (PS), tetrabutylammonium fluoride (TBAF), triethylamine (TEA), triisopropylsilyloxymethyl (TOM), tris(hydroxymethyl)aminomethane (Tris).

Preparation of solid support 1: A solution (0.35 mL) of benzylthiotetrazole (0.12 mmol) in CH_3CN was added to a solution (0.8 mL) of 5′-*O*-DMT-2′-*O*-TOM-nucleoside CE-phosphoramidite (0.12 mmol) and (3-chloro-4-hydroxyphenyl)acetic acid 2,4,5-trichlorophenyl ester[9] (0.11 mmol) in CH_2Cl_2. The reaction mixture was shaken for 1 h under an argon atmosphere. *tert*-Butyl hydroperoxide (60 μL, 5.5 M in decane) was added, and 10 min later the reaction mixture was diluted with dry DMF/CH_2Cl_2 (1:4, 2 mL). Tentagel S NH_2 resin (300 to 350 mg) derivatized with 6-aminohexanoic acid[9, 14] (60 to 70 μmol NH_2) together with DCC (23 mg, 0.11 mmol), and HOBt (15 mg, 0.11 mmol) were added, and the suspension shaken for 2 h. Then, the resin was washed with DMF, CH_2Cl_2, CH_3CN, and again CH_2Cl_2. Capping of the remaining amino groups and additional oxidation with standard GeneSynthesizer reagents (PerSeptive) were followed by treatment with TEA/pyridine (1:1, 4 mL) for 3 h. After washing with pyridine, MeOH, CH_3CN, and CH_2Cl_2, the resin was dried under vacuum (2 d) to obtain a dry solid powder **1**. Degree of cleavage ranged from 80 to 110 μmol DMT g^{-1} resin.

Preparation of solid support 2: Preparation of **2** was performed analogously to the procedure described for **1** with 5′-*O*-DMT-2′-*O*-TOM-uridine allylphosphoramidite. No final treatment of the resin with TEA/pyridine was required. The degree of cleavage was 95 μmol DMT g^{-1} resin.

Oligoribonucleotide synthesis: All RNA syntheses were carried out on an Expedite 8909 Nucleic Acid Synthesis System (PerSeptive Biosystems). The standard 1 μmol DNA synthesis cycle was modified and included the following steps: detritylation with dichloroacetic acid in $ClCH_2CH_2Cl$ (5% *v*/*v*) for 315 s/150 pulses (1 pulse is equivalent to 16–18 μL); coupling with solutions of 5′-*O*-DMT-2′-*O*-TOM-nucleoside CE-phosphoramidites in CH_3CN (10% *w*/*v*) for 540 s/7 pulses (12 mg amidite/coupling) activated by benzylthiotetrazole (0.35 M) or DCI[15] (1 M); capping with PerSeptive capping solutions for 30 s/30 pulses; oxidation with iodine in CH_3CN/*sym*-collidine/water (100:9:45, 0.01 M) for 60 s/60 pulses. Amidite solutions, tetrazole solution, and CH_3CN (Wsh) were dried over molecular sieves overnight. The *O*-DMT-hexaethylene glycol CE-phosphoramidite used for the synthesis of sequence No. 22 was prepared following ref. [16]. The linear reference sequences rACGU-3′-p and $rU_2GA_4GAGA_2C_3U$-3′-p were synthesized on a solid support with a starter group of the type described in ref. [17]. All sequences were synthesized trityl-off.

Cyclization, CE-deprotection, and cleavage: The oligoribonucleotides synthesized on support **1** (trityl-off) were treated with 1-mesitylenesulfonyl-3-nitro-1,2,4-triazole (MSNT) solution in anhydrous pyridine (0.1 M, 0.45 mL). The cyclization time was 7 to 18 h depending on the length of the oligonucleotide. After the product had been washed with pyridine, the cyclization step with MSNT (0.1 M, 0.3 mL) was repeated for 3 to 7 h. The support was washed with pyridine, and treated with TEA/pyridine (1:1) for 3 to 5 h followed by washing with pyridine, CH_2Cl_2, and MeOH. Cleavage was performed with a solution of pyridine aldoxime/tetramethylguanidine (0.1 M) in dioxane/water (2:1, 0.5 mL) for 12 to 16 h. The treatment was repeated. Solutions containing the oligonucleotides and washings with dioxane/water were combined and evaporated to dryness.

Removal of the allyl protection group from the 3′-terminal phosphate of the oligoribonucleotides synthesized on support **2** (trityl-off) was achieved by suspension of the support in a mixture of $Pd[(C_6H_5)P_3]_4$ (30 mg), $(C_6H_5)_3P$ (30 mg), and $Et_2NH_2HCO_3$ (30 mg) in CH_2Cl_2 (2 mL) at room temperature.[18] The suspension was vigorously shaken for 7 to 9 h, and washed with CH_2Cl_2, acetone, and water, suspended in an aqueous sodium diethyldithiocarbamate solution (0.1 M, 2 mL) for 30 min, and washed with water, acetone, and CH_2Cl_2. Cyclization was then carried out as described above.

Oligoribonucleotide deprotection: Sequences with less than 10 nucleotides were treated with $MeNH_2$ in EtOH (8 M, 0.75 mL) and $MeNH_2$ in water (40%, 0.75 mL) for 2 to 5 h at room temperature; the solution was then evaporated to dryness. Removal of the 2′-*O*-silyl acetals was afforded by treatment of the oligonucleotide with tetrabutylammonium fluoride trihydrate (TBAF · $3H_2O$) in THF (1 M, 0.9 mL) for at least 12 h at room temperature. The reaction was quenched by the addition of Tris · HCl (1 M, pH 7.4, 0.9 mL). The volume of the solution was reduced to 1 mL and directly applied on a Sephadex G 10 column (30 × 1.5 cm) controlled by UV-detection at 260 nm and simultaneous detection of conductivity. The product was eluted with water and evaporated to dryness.
Sequences with more than 10 nucleotides were treated with NH_3 in water (25%, 1.0 mL) and EtOH (0.2 mL) at 42 °C for 3 h, the solution was then evaporated to dryness. Deprotection was continued with $MeNH_2$ as described above.

Oligoribonucleotide purification: All oligoribonucleotides were purified by ion-exchange chromatography on a Pharmacia ÄKTA purifier with a MONO Q HR 5/5 column (Figure 2). Flow rate: 1 mL min^{-1}; eluent A: 10 mM Na_2HPO_4 in H_2O (pH 10.5); eluent B: 10 mM Na_2HPO_4/1 M NaCl in H_2O (pH 10.5); detection at 260 nm; gradient *I* (for a purity check of the crude products after deprotection): 30 min 0–100% B in A; gradient *II* (for purification): Δ30% B in A within 30 min. Fractions with the purified oligonucleotide were desalted by loading onto a C18 SepPak cartridge (Waters/Millipore), followed by elution with $(Et_3NH)HCO_3$ (0.15 M), water, and then H_2O/CH_3CN (6:4). Combined fractions with the oligonucleotide were lyophilized to dryness.

The purity of the oligonucleotides was checked by capillary electrophoresis or by reversed-phase HPLC. Capillary electrophoresis was performed on a Beckman P/ACE System 5010; open tube fused silica capillary column, 50 μ id, length: 30 cm; Tris · borate buffer (0.1 M), pH 8.4, 30 °C, 20 kV, detection at 254 nm. Reversed-phase HPLC was performed on a Beckman System Gold equipped with the 126 solvent module and the 168 detector; Brownelee Aquapore RP-300 column (C8, 7 μM, 22 × 0.4 cm). Flow rate: 1 mL min^{-1}; eluent C: $(Et_3NH)OAc$ (0.1 M) in H_2O (pH 6.4); eluent D: $(Et_3NH)OAc$ (0.1 M) in H_2O/CH_3CN (1:4); gradient *III*: 30 min 0–40% D in C; elution at 35 °C; detection at 260 nm.

Enzymatic digestion: To an aqueous solution (100 μL) of cyclic oligoribonucleotide (cy-rACGU or cy-rGAGC$_4$U or cy-$rU_2GA_4GAGA_2C_3U$, 2.5×10^{-6} μM) and its corresponding linear reference sequence (rACGU-3′-p or rGAGC$_4$U-3′-OH or $rU_2GA_4GAGA_2C_3U$-3′-p, 2.5×10^{-6} μM), phosphodiesterase from calf spleen (1.5 μL, ≈0.01 U, Boehringer-Mannheim Cat. No. 108251, suspension in $(NH_4)_2SO_4$ solution (3.2 M), pH 6.0) was added. The mixture was kept at room temperature and analyzed at fixed times by reversed-phase HPLC (Figure 3).

Acknowledgments

An APART fellowship (Austrian Programme For Advanced Research and Technology) from the Austrian Academy of Science and funding from the Austrian Science Fund (FWF-project P-13216-CHE) is gratefully acknowledged. I thank Prof. Dr. A. Eschenmoser for generously supporting this research; the experimental work was performed as an academic guest in his laboratory at The Scripps Research Institute (La Jolla, CA). I thank Dr. S. Pitsch (ETH Zuerich, Switzerland) and Dr. R. Krishnamurthy (Scripps, La Jolla, CA) for helpful discussions. I also wish to express thanks to Dipl.-Chem. P. Weiss (Xeragon AG, Switzerland), Dr. R. Knochenmuss (ETH Zuerich, Switzerland), Prof. Dr. K. Grubmayr (University of Linz, Austria), and Prof. Dr. H. Falk (University of Linz, Austria).

[1] a) M. Puttaraju, A. T. Perrotta, M. D. Been, *Nucleic Acids Res.* **1993**, *21*, 4253 – 4258; b) J. M. Buzayan, H. van Tol, P. Zalloua, G. Bruening, *Virology* **1995**, *208*, 832 – 837.
[2] a) E. Ford, M. Ares Jr., *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 3117 – 3121; b) M. Puttaraju, M. D. Been, *Nucleic Acids Res.* **1992**, *20*, 5357 – 5364.
[3] a) C. Chen, P. Sarnow, *Science* **1995**, *268*, 415 – 417; b) A. D. Branch, J. A. Polaskova, D. R. Schreiber, *Nucleic Acids Res.* **1995**, 23, 4391 – 4399.
[4] a) A. M. Diegelman, E. T. Kool, *Nucleic Acids Res.* **1998**, *26*, 3235 – 3241; b) S. L. Daubendiek, E. T. Kool, *Nat. Biotechnol.* **1997**, *15*, 273 – 277.
[5] a) E. T. Kool, *Annu. Rev. Biophys. Biomol. Struct.* **1996**, *25*, 1 – 28; b) E. T. Kool, *Acc. Chem. Res.* **1998**, *31*, 502 – 510.
[6] a) S. A. Salisbury, S. E. Wilson, H. R. Powell, O. Kennard, P. Lubini, G. M. Sheldrick, N. Escaja, E. Alazzouzi, A. Grandas, E. Pedroso, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 5515 – 5518; b) C. González, N. Escaja, M. Rico, E. Pedroso, *J. Am. Chem. Soc.* **1998**, *120*, 2176 – 2177.
[7] a) L. Wang, D. E. Ruffner, *Nucleic Acids Res.* **1998**, *26*, 2502 – 2504; b) S. Wang, E. T. Kool, *Nucleic Acids Res.* **1994**, *22*, 2326 – 2333.
[8] a) D. Beaudry, J.-P. Perreaut, *Nucleic Acids Res.* **1995**, *23*, 3064 – 3066; b) G. W. Ashley, D. M. Kushlan, *Biochemistry* **1991**, *30*, 2927 – 2933; c) C. S. Lim, C. A. Hunt, *Nucleosides & Nucleotides* **1997**, *16*, 41 – 51.
[9] E. Alazzouzi, N. Escaja, A. Grandas, E. Pedroso, *Angew. Chem.* **1997**, *109*, 1564 – 1567; *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 1506 – 1508.
[10] a) L. DeNapoli, A. Galeone, L. Mayol, A. Messere, D. Montesarchio, G. Piccialli, *Bioorg. Med. Chem.* **1995**, *3*, 1325 – 1329; b) E. de Vroom, H. J. G. Broxterman, L. A. J. M. Sliedregt, G. A. van der Marel, J. H. van Boom, *Nucleic Acids Res.* **1988**, *16*, 4607 – 4620; c) C. J. Hsu, D. Dennis, R. Jones, *Nucleosides & Nucleotides* **1985**, *4*, 377 – 389.
[11] a) X. Wu, S. Pitsch, *Nucleic Acids Res.* **1998**, *26*, 4315 – 4323; b) S. Pitsch, X. Wu, P. Weiss, L. Jenny in *RNA '98: The Third Annual Meeting of the RNA Society*, *Program & Abstracts* (Eds.: D. Brown, R. Gesteland, F. Kramer, A. Pyle), University of Wisconsin, p. 554; see also c) S. Pitsch, *Helv. Chim. Acta* **1997**, *80*, 2286 – 2314.
[12] P. Ross, H. Weinhouse, Y. Aloni, D. Michaeli, P. Weinberger-Ohana, R. Mayer, S. Braun, E. de Vroom, G. A. van der Marel, J. H. van-Boom, M. Benziman, *Nature* **1987**, *325*, 279 – 281.
[13] M. Y.-X. Ma, K. McCallum, S. C. Climie, R. Kuperman, W. C. Lin, M. Sumner-Smith, R. W. Barnett, *Nucleic Acids Res.* **1993**, *21*, 2585 – 2589.
[14] D. W. Brown, M. M. Campell, R. G. Kinsman, P. D. White, C. A. Moss, D. J. Osguthorpe, P. K. C. Paul, B. I Baker, *Biopolymers* **1990**, *29*, 609 – 622.
[15] C. Vargeese, J. Carter, J. Yegge, S. Kriviansky, A. Settle, E. Kropp, K. Peterson, W. Pieken, *Nucleic Acid Res.* **1998**, *26*, 1046 – 1050.
[16] M. Durand, K. Cheyrie, M. Chassignol, N. Thuong, J. Maurizot, *Nucleic Acid Res.* **1990**, *18*, 6353 – 6359.
[17] a) V. A. Efimov, A. A. Buryakova, S. V. Reverdatto, O. G. Chkah-makhcheva, Y. A. Ovchinnikov, *Nucleic Acid Res.* **1983**, *11*, 8369 – 8387; b) M. Bolli, R. Micura, A. Eschenmoser, *Helv. Chim. Acta* **1997**, *80*, 1901 – 1951.
[18] Y. Hayakawa, M. Hirose, R. Noyori, *J. Org. Chem.* **1993**, *58*, 5551 – 5555.
[19] L. Wang, D. E. Ruffner, *Biochem. Biophys. Res. Commun.* **1998**, *250*, 711 – 719.

Received: October 14, 1998
Revised version January 25, 1999 [F1395]

  0947-6539/99/0507-2082 $ 17.50+.50/0