Making cyclic RNAs easily available

Miriam Frieden, Anna Grandas and Enrique Pedroso*

Departament de Química Orgànica, Facultat de Química, Universitat de Barcelona, Martí i Franquès 1-11, 08028 Barcelona, Spain. E-mail: pedroso@admin.qo.ub.es

Received (in Cambridge, UK) 17th June 1999, Accepted 13th July 1999

A simple solid-phase procedure allows cyclic oligoribonucleotides to be obtained as long as the linear precursor attached to the support has a 2′-deoxyribonucleoside or a 2′-*O*-methylribonucleoside at the 3′-end.

Some of the smallest oligoribonucleotides with known biological activity are cyclic molecules. For instance, c(GG) is an activator of cellulose synthase in *Acetobacter xilinum*,[1] and c(UU) and c(AU) are inhibitors of the DNA dependent RNA polymerase of *E. coli*.[2] At the other end of the scale, large cyclic RNAs are formed in the splicing processes of ribonucleic acids in certain organisms[3] and viroids have circular single stranded RNA as their genomic material.

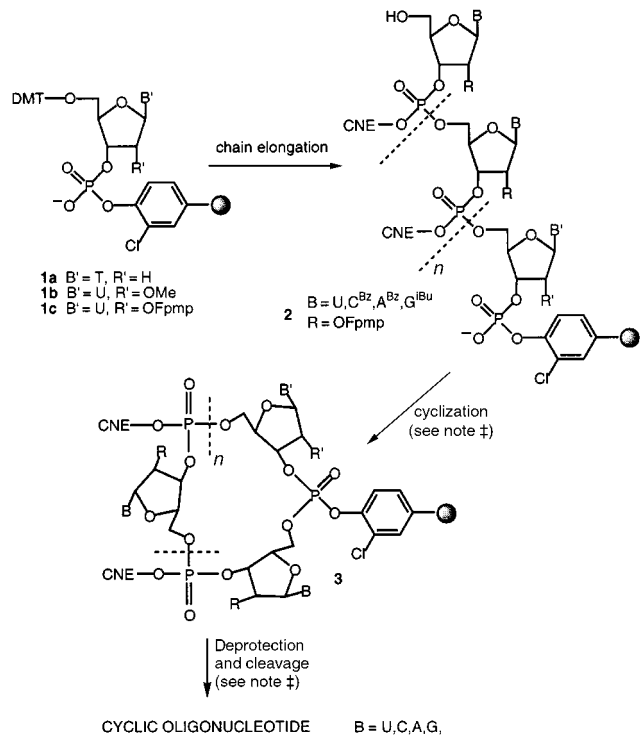
The structure of cyclic RNAs has been determined either by X-ray diffraction[4] or by NMR[5] only for very small molecules. Cyclic RNAs may be useful models for the study of a variety of RNA structural motifs,[6] such as hairpin loops, which could bring new insights into the structure–function relationship of ribonucleic acids.

In spite of their wide potential applications, few efforts have been dedicated so far to the development of chemical methods for the preparation of cyclic oligoribonucleotides. The synthesis of cyclic dinucleotides[7] and tetranucleotides[8,9] has been reported using the phosphotriester[7,8] or the H-phosphonate[9] methods in solution, whereas a solid-phase method has been employed for the preparation of cytidine homooligomers.[10] Larger cyclic RNAs have been obtained either by template-directed chemical[11] or enzymatic[12] ligation, and by rolling circle transcription and self-processing of circular DNA oligonucleotides encoding hairpin ribozymes.[13]

We have recently described a straightforward solid-phase procedure for obtaining small to medium-sized cyclic oligodeoxyribonucleotides.[14] The main advantage of the method is that, after the cyclization and cleavage reactions, non-cyclized chains, polymers and other by-products remain attached to the solid matrix. Therefore, fairly pure crude products are obtained, regardless of the size of the circles and the sometimes low cyclization yields. Scheme 1 shows the key steps in the extension of this methodology to the preparation of cyclic RNA. Synthesis of the ribonucleotide-resin **1** and chain elongation by the phosphite-triester approach using 2-cyanoethyl (CNE) phosphoramidites yields the oligonucleotide-resin **2** which, upon cyclization with 1-mesitylenesulfonyl-3-nitro-1,2,4-triazole (MSNT), affords **3**. After oximate cleavage of **3** and deprotection the cyclic RNA is obtained.

The choice of a suitable 2′-OH protecting group is the most crucial decision in RNA synthesis. It is well documented that the last step of the synthesis must be the deprotection of the 2′-hydroxy functions to avoid strand cleavage or 3′–5′ to 2′–5′ migration of the phosphodiester linkages.[15] The commonly used TBDMS group was discounted because of its lack of stability towards the oximate cleavage treatment.[16] Early elimination of the TBDMS group of the ribonucleoside directly attached to the support may result in the above mentioned undesirable side reactions.

For this reason, we turned our attention to the acid-labile 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl group (Fpmp),[17] whose stability to oximate was confirmed using 2′-*O*-Fpmp-uridine (data not shown). However, to our surprise, the first attempts to prepare small cyclic RNAs (2- to 6-mer) employing the Fpmp group were completely unsuccessful: very low yields and impure crude products were obtained. We reasoned that the key difference with respect to cyclic DNA synthesis was the presence of the bulky 2′-*O*-Fpmp group at the 3′-end of **2** that may be hindering the cyclization reaction. In order to test the validity of this assumption, three nucleotide resins **1** having differently hindered phosphate groups were prepared as previously described[14] on an NH_2-TentaGel™ resin, **1a** (B′ = T, R′ = H), **1b** (B′ = U, R′ = OMe) and **1c** (B′ = U, R′ - OFpmp), and their homogeneity was assessed by gel-phase ^{31}P NMR.† On these nucleotide-resins a series of dinucleotides were assembled, cyclized, cleaved and deprotected.‡ Nucleoside sequences, yields and mass spectrometric data are indicated in Table 1 (entries 1 to 5). These results clearly indicate that the lowest yields were obtained when the nucleotide-resin **1c** was employed (entries 4 and 5). In fact, c(UU) could not be isolated from the complex crude mixture, whilst the rest of the cyclic dinucleotides were easily purified by C18-HPLC chromatography and unequivocally characterized by mass spectrometry and enzymatic digestion.[14] Particularly striking is the difference in yield between the two syntheses of c(UT) [= c(TU)] (entries 3 and 4): much lower yield was obtained from the most hindered resin **1c** than from **1a**, thus demonstrating that steric hindrance in the vicinity of the 3′ terminus phosphate of linear precursor **2** plays a key role. Nevertheless, comparing the results of entries 4 and 5 we can assert that the rest of the chain has a non-negligible effect. Most probably, it hinders the dinucleotide in attaining a reactive conformation.



Scheme 1

Table 1 Synthesized cyclic oligoribonucleotides

Entry	Oligonucleotide-resin	Cyclic oligonucleotide	Crude yield (%)	Purity (%) (HPLC)	Mass spectrometry	
					calc.	found
1	T-T-**R**	c(TT)	50	>90	608.09	608.29[b]
2	T-U_{OMe}-**R**	c(TU_{OMe})	43	>90	624.08	624.20[b]
3	U_{OFpmp}-T-**R**	c(UT)	48	>90	610.07	610.39[b]
4	T-U_{OFpmp}-**R**	c(TU)	14	80	610.07	610.39[b]
5	U_{OFpmp}-U_{OFpmp}-**R**	c(UU)	3	—	612.09	—
6	UUU-T-**R**[a]	c(UUUT)	19	75	1222.12	1222.14[c]
7	CUC-T-**R**[a]	c(CUCT)	33	69	1220.15	1222.20[c]
8	UUUUU-T-**R**[a]	c(UUUUUT)	7	57	1834.17	1834.76[c]
9	UGCUUGC-T-**R**[a]	c(UGCUUGCT)	20	70	2523.49	2521.39[c]
10	UAUAG-U_{OMe}-**R**[a]	c(UAUAGU_{OMe})	5	80[d]	1935.57	1933.56[c]
11	UAGCA-U_{OMe}-**R**[a]	c(UAGCAU_{OMe})	4	80[d]	1934.26	1932.84[c]

[a] Ribonucleosides were 2′-*O*-Fpmp protected. [b] Electrospray MS. [c] MALDI-TOF MS. [d] Estimated by PAGE.

The synthesis of some larger circles was undertaken to further evaluate the possibilities of the method. Several cyclic oligoribonucleotides were obtained at the 1–2 μmol scale from resins **1a** and **1b**. Results are shown in Table 1 (entries 6 to 11). The cyclic products were submitted to Sephadex G-10 gel filtration and HPLC or PAGE purification, and were characterized as above.

Yields of crude products are generally lower than those typically obtained in the synthesis of cyclic DNA. The low yield obtained for c(U_5T) (entry 8) and the difference in yield between the two cyclic tetramers (entries 6 and 7) reflect the difficulty in getting high and reproducible yields in the cyclization reaction with MSNT. A 2′-*O*-methylribonucleotide at the 3′ end of the linear precursor seems to have a negative effect on the cyclization yield of the hexamers (entries 10, 11), which was not observed for a dinucleotide (entry 2). The homogeneity of the crude products detached from the resin is also slightly lower than in cyclic DNA synthesis. However, a major HPLC peak or PAGE band is always obtained, thus allowing easy purification of the circle and showing that the key advantage of the method is preserved.

In conclusion, cyclic RNA can be obtained provided that the linear precursor attached to the support has a 2′-deoxyribonucleoside or a 2′-*O*-methylribonucleoside at the 3′ end. Such single modification in the sequence of the cyclic RNAs should have little relevance for many purposes. For instance, enzymatically circularized hammerhead ribozymes containing non-nucleoside linkers have been shown to display increased biological activity and reduced divalent metal ion requirement.[18]

Work is in progress to prepare larger, 'all-ribonucleoside' cyclic RNAs by circumventing the problem of the steric hindrance at the 3′ end phosphate.

This work was supported by the Ministerio de Educación (DGES, grant PB97-941) and the Generalitat de Catalunya (Centre de Referència de Biotecnologia and SGR98-1).

Notes and references

† *Selected* ^{31}P *NMR data* (121.4 MHz, $CDCl_3$) for **1a**: δ_P −5.12. For **1b**: δ_P −6.99. For **1c**: δ_P −6.90.

‡ Oligonucleotide chains were assembled using 5′-*O*-DMT-nucleoside 3′-cyanoethylphosphoramidites (2′-*O*-Fpmp-protected, when required) and tetrazole for the coupling step, and Bu^tOOH for the oxidation. Conditions for the cyclization reaction are: 0.15 M MSNT in pyridine, three treatments (4 h + 4 h + overnight) with 20 equiv. MSNT each. Conditions for the deprotection and cleavage reactions are: (i) Et_3N–pyridine (1:1), 3 × 1 h; (ii) 0.2 M tetramethylguanidinium *syn*-pyridine-2-aldoximate in dioxane–water (1:1), 4 h + 4 h + overnight with 50 equiv. oximate; (iii) conc. aqueous NH_3, 55 °C, 12 h; (iv) 0.5 M AcONa, pH 4, 12 h, and then neutralization with 3 M Tris·HCl, pH 8.

1 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 and M. Benziman, *Nature*, 1987, **325**, 279.
2 C.-Y. L. Hsu and D. Dennis, *Nucleic Acids Res.*, 1982, **10**, 5637.
3 A. J. Zaug, P. J. Grabowski and T. R. Cech, *Nature*, 1983, **301**, 578; T. R. Cech, *Annu. Rev. Biochem.*, 1990, **59**, 543.
4 M. Egli, R. V. Gessner, L. D. Williams, G. J. Quigley, G. A. van der Marel, J. H. van Boom, A. Rich and C. A. Frederick, *Proc. Natl. Acad. Sci. U.S.A.*, 1990, **87**, 3235.
5 M. M. W. Mooren, S. S. Wijmenga, G. A. van der Marel, J. H. van Boom and C. W. Hilbers, *Nucleic Acids Res.*, 1994, **22**, 2658.
6 G. L. Conn and D. E. Draper, *Curr. Opin. Struct. Biol.*, 1998, **8**, 278.
7 C.-Y. L. Hsu, D. Dennis and R. A. Jones, *Nucleosides Nucleotides*, 1985, **4**, 377.
8 E. de Vroom, H. J. G. Broxterman, L. A. J. M. Sliedregt, G. A. van der Marel and J. H. van Boom, *Nucleic Acids Res.*, 1988, **16**, 4607; C. Sund, P. Agback and J. Chattopadhyaya, *Tetrahedron*, 1991, **47**, 9659.
9 C. B. Reese and Q. Song, *Nucleic Acids Res.*,1999, **27**, 963.
10 L. De Napoli, A. Galeone, L. Mayol, A. Messere, G. Piccialli and C. Santacroce, *J. Chem. Soc., Perkin Trans. 1*, 1993, 747; L. De Napoli, A. Galeone, L. Mayol, A. Messere, D. Montesarchio and G. Piccialli, *Bioorg. Med. Chem.*, 1995, **3**, 1325.
11 S. Wang and E. T. Kool, *Nucleic Acids Res.*, 1994, **22**, 2326.
12 T. Pan, R. R. Gutell and O. C. Uhlenbeck, *Science*, 1991, **254**, 1361; C. Y. Chen and P. Sarnow, *Science*, 1995, **268**, 415.
13 A. M. Diegelman and E. T. Kool, *Nucleic Acids Res.*, 1998, **26**, 3235.
14 E. Alazzouzi, N. Escaja, A. Grandas and E. Pedroso, *Angew. Chem., Int. Ed. Engl.*, 1997, **36**, 1506.
15 M. A. Morgan, S. A. Kazakov and S. M. Hecht, *Nucleic Acids Res.*, 1995, **23**, 3949.
16 K. K. Ogilvie, N. Y. Theriault, J.-M. Seifert, R. T. Pon and M. J. Nemer, *Can. J. Chem.*, 1980, **58**, 2686.
17 M. V. Rao, C. B. Reese, V. Schehlmann and P. S. Yu, *J. Chem. Soc., Perkin Trans. 1*, 1993, 43; D. C. Capaldi and C. B. Reese, *Nucleic Acids Res.*, 1994, **22**, 2209.
18 L. Wang and D. E. Ruffner, *Nucleic Acids Res.*, 1998, **26**, 2502; *J. Am. Chem. Soc.*, 1998, **120**, 7684.

Communication 9/04851K