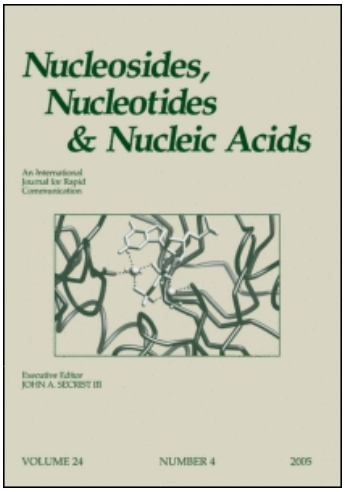

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IMPROVEMENTS TO THE CHEMICAL SYNTHESIS OF BIOLOGICALLY ACTIVE RNA USING 2'-*O*-Fpmp CHEMISTRY

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Abstract: The synthetic cycle protocol for the solid phase synthesis of RNA using 5'-*O*-(DMTr)-2'-*O*-(Fpmp)-ribonucleoside phosphoramidites is optimised. A simple and reliable two step deprotection procedure is developed to isolate biologically active RNA. It is demonstrated that fully deprotected RNA is completely stable under the deprotection conditions and that it does not undergo internucleotide cleavage and/or migration. Ribozymes and substrate RNAs synthesized using this chemistry were found to be catalytically active.

Introduction

There is an increasing interest in the chemical synthesis of biologically active RNA for biomedical, biochemical and physical studies. The use of chemically synthesized RNA in designing anti-sense oligonucleotides and ribozymes for potential therapeutic applications has further boosted the need for developing a reliable method for the synthesis of RNA and its modified analogues. Taking into account all of the associated problems for different 2'-*O*-protecting groups used in the chemical synthesis of RNA, we have developed and improved the chemistry involving 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl (Fpmp) [1-5] acetal protecting group especially for use in the solid phase synthesis of biologically active RNA.

The prime objectives of this study were: (i) to improve the solid phase synthesis of RNA using isomerically pure 2'-*O*-Fpmp ribonucleoside phosphoramidites, (ii) to develop simple and reliable deprotection procedures in order to isolate biologically active RNA and (iii) to prove that RNA is stable and does not undergo internucleotide cleavage and/or migration under the deprotection conditions.

Results and Discussion

Cruachem 5'-*O*-(DMTr)-2'-*O*-(Fpmp) ribonucleoside phosphoramidites and the 2'/3'-*O*-benzoyl ribonucleoside-loaded solid supports were used in this study. Solid phase oligoribonucleotide synthesis was carried out on 1.0 μM scale using the Cruachem PS250 DNA/RNA synthesizer.

TABLE: Improvements to the RNA synthetic cycle protocol.

Synthetic Cycle Steps	Reagent and/or Solvent	Duration (t/sec) in the Previous Versions	Duration in the Current Version
Coupling reaction	m-Nitrophenyltetrazole in CH_3CN	0.1M - 180	-
	0.5M Tetrazole in CH_3CN	-	10 min
Capping	Ac_2O, THF, Lutidine, NMI in THF	Flow time: 1 x 5 + 2 x 3	2 x 5
		Wait time: 3 x 60	30
Oxidation	0.1M I_2 in THF, Pyridine, Water	Flow time: 10	12
		Wait time: 30	-
Capping	Ac_2O, THF, Lutidine, NMI in THF	Flow time: -	2 x 5
		Wait time: -	30
Deblocking	2% TCA in Dichloromethane	50	60

In order to improve the solid phase chemical synthesis of RNA, we have optimised each and every step of the synthetic cycle protocol (see Table) to such an extent that the synthesis of RNA up to 50-mers has become routine.

The synthetic RNA sequences were released from the solid support and partially unblocked by treating either with aqueous ammonia (d = 0.88) at 55° for 8-10 hours or with triethylamine in aqueous ammonia (5:95, v/v) at 80°C for 2 hours. The 2'-*O*-Fpmp protecting groups are completely stable under these conditions of deprotection step 1. This is advantageous as it allows the 2'-protected RNA to be purified without any risk of being digested by contaminating traces of endonucleases.

Although a pH 2.0-2.5 acidic solution (0.01M hydrochloric acid or dilute acetic acid) was used to remove all the 2'-*O*-Fpmp protecting groups in order to isolate the fully unblocked RNA, ours as well as recent observations[5] clearly indicate that certain RNA sequences can undergo internucleotide cleavage and migration under these conditions. It has been realised[6] that the internucleotide linkages of RNA can both migrate and undergo cleavage under acidic conditions. It also has been realised[5] that the extent of acid catalysed internucleotide cleavage of RNA is about twice as much as the acid catalysed internucleotide migration. A systematic study of acid catalysed internucleotide cleavage of RNA indicated that it depends on the base sequence, pH as well as the nature of the acidic solution and temperature.

We have found that a solution of sodium acetate (pH 3.25) is quite suitable for removing all the 2'-*O*-Fpmp protecting groups from the 2'-protected RNA. Thus all the 2'-*O*-Fpmp protected RNA

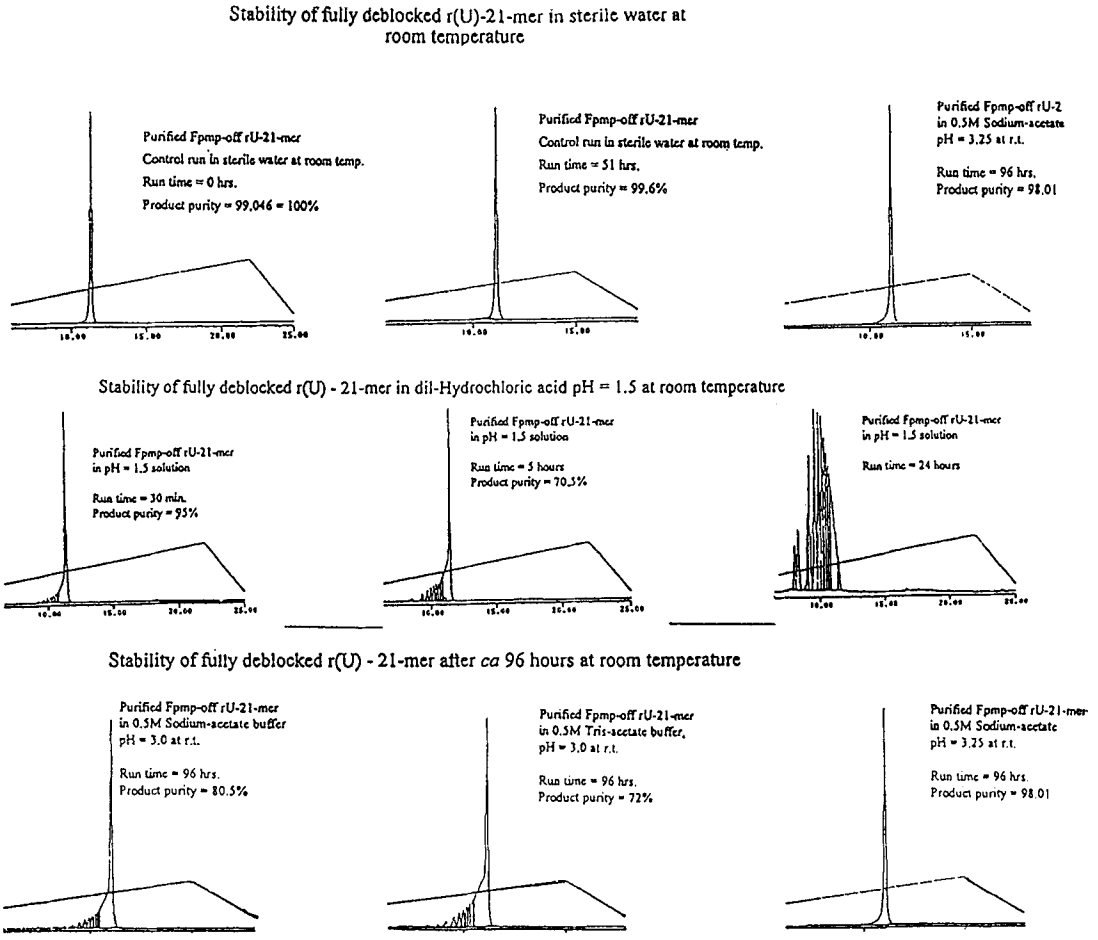
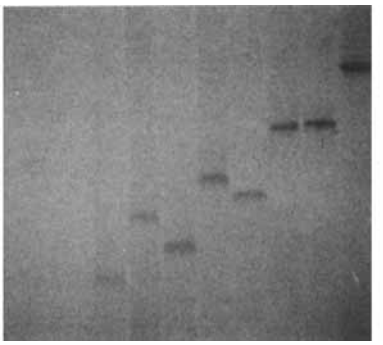


FIGURE 1: HPLC-profiles showing stability of oligo uridylic acids.

sequences were fully unblocked by treating with 0.5M sodium acetate buffer (pH 3.25, 500 µL for 1 µM scale synthesis) at 30°C for 36 hours or longer. After the appropriate time the acidic solution was neutralised with a solution of 3.0M tris-base (100 µL for 500 µL of acidic buffer) and the fully unblocked RNA was readily generated by ethanol precipitation without vacuum centrifugation.

Recent observations indicate that homo-oligomers, polyuridylic acids are particularly sensitive to acid catalysed internucleotide migration[7]. We have therefore carried out stability studies on purified, fully deblocked oligouridylic acids (U 21-mer) under different acidolysis conditions and have found that a solution of 0.5M sodium acetate (pH 3.25) at 30°C does not cause any internucleotide cleavage and/or migration, (Figure 1).



Lane L to R
1. Fully deblocked 21-mer
2. 5'-*O*-DMT - 2'-*O*-Fpmp 21-mer
3. Fully deblocked 30-mer
4. 5'-*O*-DMT - 2'-*O*-Fpmp 30-mer
5. Fully deblocked 40-mer
6. 5'-*O*-DMT - 2-*O*-Fpmp 40-mer
7. Fully deblocked 50-mer
8. 5'-*O*-DMT - 2'-*O*-Fpmp 50-mer

FIGURE 2: Page analysis of unpurified 2'-Fpmp protected and fully unblocked RNA mixed base sequences.

Furthermore, the fully protected U 21-mer, using the two step deprotecting procedures, was digested with ribonuclease A and bacterial alkaline phosphatase (pH 8.0) and the HPLC analysis of the hydrolysate indicated no detectable peak corresponding to the uridylyl (2'-5')-uridine[7].

Several synthetic RNA sequences were deprotected using the two step deprotection procedures and isolated the fully unblocked RNA in good yields. Figure 2 shows the PAGE analysis of the unpurified 2'-Fpmp protected and fully unblocked RNA mixed base sequences to indicate their mobility differences.

Ribozymes and substrate RNAs synthesized using this chemistry were found to be catalytically (biologically) active (data not shown).[8]

It is demonstrated that Fpmp chemistry, with the improved synthetic cycle protocol and with simplified post-synthesis handling procedures to isolate fully unblocked biologically active RNA, is the method of choice for the chemical synthesis of RNA.

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