Volume 17 Number 18 1989 Nucleic Acids Research

Solid phase synthesis of the 5'-half of the initiator t-RNA from *B.subtilis*

C.Chaix, A.M.Duplaa, D.Molko* and R.Téoule

Départment de Recherche Fondamentale, Laboratoires de Chimie, Centre d'Etudes Nucléaires de Grenoble, 85X 38041 Grenoble Cédex, France

Received March 30, 1989; Revised and Accepted August 11, 1989

ABSTRACT

Using cyanoethyldiisopropylamino phosphoramidite chemistry, four oligonucleotides constituting a part of the sequence of the initiator t-RNA from *B. subtilis* were synthesized. For the protection of the exocyclic amino functions of bases, phenoxyacetyl group was used for adenine and guanine, and acetyl group was preferred for cytosine. With these labile groups, final deprotection of the oligonucleotides can be performed in milder conditions, allowing the incorporation of 5,6-dihydrouridine in a 35-mer constituting the 5'-end of the t-RNA.

INTRODUCTION

The growing task by molecular biologists and biochemists for synthetic nucleic acid fragments has focussed the attention of most laboratories and firms involved in nucleic acid chemistry, to develop more reliable synthetic procedures. Most efforts have been devoted to the deoxy series. Especially, the technology of assembling monomeric units on a solid support has been considerably simplified (1−4). Oligoribonucleotide synthesisis not as significantly advanced. This is mainly due to the presence, in the 2' position of the sugar moiety of nucleosides, of an additional hydroxy function. The protection of this position is a difficult step which makes the preparation of protected nucleosides time-consuming. In addition, the protecting group used for the 2'-hydroxy function, induces a steric hindrance which deactivates the condensation of the monomeric units on the growing chains. There are other reasons why oligoribonucleotide synthesis is always lagging behind its deoxy homologue. The choice of appropriate protection for the exocyclic amino functions of nucleic bases is another key step in oligonucleotide synthesis. Oligoribonucleotides are sensitive to alkaline hydrolysis and thus there is a real need for milder deprotection conditions. Owing to the presence of fragile modified nucleosides in their primary structure, t-RNAs are particularly unstable and their chemical synthesis is dependent on the choice of appropriate protecting groups for the other nucleosides. We have proposed a new set of easily removable amino protecting groups useful for both deoxy oligonucleotide synthesis (5) and RNA synthesis (6). The aim of this article is to describe the preparation of oligoribonucleotides constituting a part of the sequence of *B.subtilis* formylmethionine t-RNA with a set of labile amino protecting groups. One of the sequences—a 35-mer—incorporates 5,6-dihydrouridine, a modified nucleoside present in most t-RNAs.

Stability of 5,6-dihydrouridine in basic conditions

To evaluate the stability of 5,6-dihydrouridine in alkaline conditions and subsequently to choose the optimum deprotection system for oligonucleotides bearing this nucleoside, 5,6-dihydrouridine was treated in an ethanol/concentrated aqueous ammonia (1/1) solution

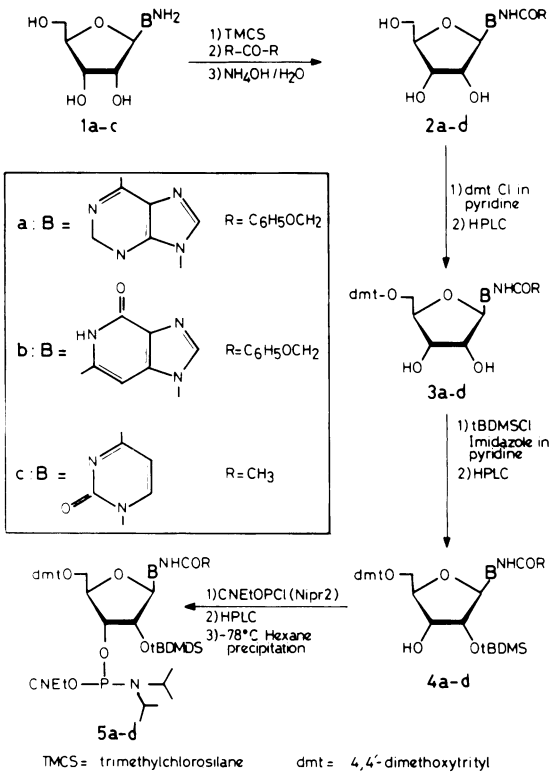

Figure 1. Reaction scheme used for the preparation of mononucleotides. d: B=5,6-dihydrouracil-1-yl.

at room temperature. Aliquots were taken, neutralised in acetic acid and analysed by silica gel TLC. 10% degradation appeared only after 5 hours, showing that a deprotection time of 4 hours could be acceptable in these alkaline conditions, without causing important damage to the synthetic molecule.

Protection of the exocyclic amino function of nucleosides

Various amino-protected ribonucleosides were synthesized according to the scheme depicted in Figure 1. Their synthesis is based on the transient protection method described by Ti *et al.* (7) in the deoxy series. To avoid the formation of coloured side products in the case of guanosine and adenosine, a mixture of phenoxyacetyl chloride and hydroxybenzotriazole (8) was used as the acylating agent. The yields were in the satisfactory range 60−70%.

To evaluate the deprotection kinetics of these nucleosides, HPLC on a Reversed-phase column was used. The nucleosides were treated in an ethanol/concentrated aqueous ammonia 1/1 solution at room temperature. Aliquots were taken, neutralized with 5% acetic acid and directly injected onto the column. The concentration of each nucleoside was plotted versus time and the half-life deprotection time was graphically determined for each nucleoside. The results listed in Table 1 showed that phenoxyacetyl group is suitable for both guanine and adenine. Isobutyryl is too stable in basic conditions and the acetyl group,

Table 1. Half-life deprotection time of various N-acyl nucleosides. Conditions: A NH_4OH (29%)/Ethanol (1/1), B NH_4OH (29%)/EtOH (3/1) (v/v).

Compound	t½	Conditions
N2-ibu Guo	> 24 hrs	A
N2-pac Guo	20 min	A
N6-pac Ado	4 min	A
N4-ace Ctd	10 min	A
N4-ace Ctd	5 min	B
N4-ibu Ctd	30 min	B

having a half-life of 10 minutes, was preferred for the protection of the amino function of cytosine.

Protection of the 5'-hydroxy function of nucleosides

In oligoribonucleotide synthesis, this protection is usually performed with the monomethoxytrityl group. This choice is perfectly reasonable since this group is more stable than its dimethoxy analog and as long as the N-glycosidic bond is more stable in acidic conditions than in the deoxy series. Nevertheless, our goal was to introduce in synthetic oligonucleotides, more acid-labile natural nucleosides such as those which are found in transfer RNAs. So we chose the dimethoxytrityl group which gives excellent results in the deoxy series and necessitates milder acidic conditions for its deblocking in each condensation cycle.

The introduction of this group on the base-protected nucleosides was made according to the general methodology described in the second step of Figure 1. In the ribo series, the selective tritylation of the 5' position is more difficult to realise than in the deoxy-series. Indeed, two secondary hydroxy functions (in the 2' and 3' positions) compete with the 5' hydroxyl group. This results in a complex mixture of mono and bis dimethoxytritylated compounds in which the appropriate product is the major one. It was purified by preparative HPLC on silica gel. Our attempts to increase selectivity for the 5' position failed. The best results were obtained when dimethoxytrityl chloride was added gradually to the sugar-free nucleoside.

Protection of the 2'-hydroxy functions

Three different protecting groups have been successfully used in the most recent approaches to solid phase oligoribonucleotide synthesis. These groups are: the nitro-phenyl group proposed by Ikehara *et al.* (9), the Ctmp group (10), developed by Reese *et al.* and the t-butyldimethylsilyl group (tBDMS) used by Ogilvie *et al.* (11) for the synthesis of a 77-nucleotide long RNA sequence. According to Ogilvie *et al.* (11) and to Stawinski *et al.* (12), this latter group proved to be stable both in phosphoramidite and H-phosphonate methods and was reported to give exclusively 3'−5' RNA sequences when pure 2' protected monomers were used. Consequently, we chose it for the protection of nucleosides.

Its introduction on the protected nucleosides led to a mixture of 2' and 3' isomers in approximately equal proportions and a small amount (10−15 %) of bis-silylated compound. In each case, this mixture was resolved both by TLC and preparative HPLC on silica gel. The 3' isomer collected during this separation was isomerised in a mixture of 2' and 3' isomers which yielded a second crop of the appropriate product, after another HPLC separation.

Table 2. Chemical shifts in ppm of fully protected nucleosides in deuterated acetone. * Mixture of diastereoisomers

Compound	1'	2'	3'	4'	5'	5''	2	8	5	6	^{31}P NMR
2a	6.21	4.98	4.55	4.30	3.92	3.92	8.70	8.76			
2b	6.01	4.78	4.54	4.22	3.91	3.91		8.27			
2c	5.87	4.10	4.10	4.05	3.86	3.86			7.42	8.57	
2d	5.94	4.39	4.24	4.12	3.84	3.84			2.65	3.70	
3a	6.27	5.12	4.76	4.39	3.54	3.54	8.70	8.61			
3b	6.05	4.87	4.59	4.33	3.51	3.51		8.12			
3c	5.99	4.38	4.58	4.32	3.61	3.61			7.30	8.54	
3d	6.00	4.30	4.30	4.08	3.40	3.40			2.65	3.65	
4a	6.27	5.24	4.64	4.40	3.58	3.57	8.71	8.62			
4b	6.06	4.93	4.52	4.34	3.57	3.52		8.18			
4c	5.95	4.49	4.58	4.32	3.64	3.34			7.27	8.61	
4d	6.03	4.48	4.26	4.08	3.41	3.48			2.65	3.70	
5a *	6.30	5.47	4.79	4.56	3.68	3.68	8.71	8.64			150.6 & 152.0
5b *	6.07 6.10	5.05			3.59	3.59		8.18 8.16			150.8 & 152.0
5c *	6.06 5.95								7.23 7.16	8.63 8.51	150.2
5d *	6.07	4.56			3.44	3.44			2.64		150.4 & 151.5

The identity and purity of the final products must be carefully checked. Traces of 3′ tBDMS in the 2′ isomer are unacceptable. They lead to unwanted 2′−5′internucleotidic linkages in the final oligonucleotide.

Proton NMR at 200 MHz was used to identify 2′ and 3′ isomers. The observation of the coupling between the residual sugar hydroxy proton and the 2′ (or 3′) proton in anhydrous deuterated acetone clearly indicates the position of the t-butyldimethylsilyl group. The chemical shifts of the protected nucleosides 4a−d are listed in Table 2.

The purity of compounds 4a−d was checked by reversed phase liquid chromatography. Each pair of isomers is well resolved on a Merck Li-Chrospher RP18e column and the absence of any contamination by the unwanted 3′-isomer was evidenced (data available on request).

Preparation of monomers for the assembling

Since the beginings of phosphoroamidite chemistry, two protecting groups have been developed for the internucleotide phosphate. Originally, the methyl group was introduced

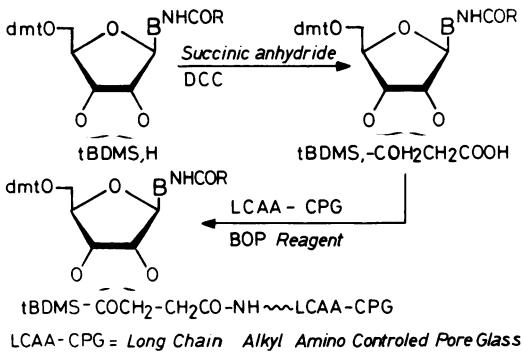

Figure 2. Reaction scheme used for the preparation of mononucleosides covalently linked to Controled Pore Glass.

by Caruthers *et al.* (1). Its deprotection necessitates a special step consisting of a treatment with the thiophenolate ion. Work-up of the reaction has been simplified with the introduction of cyanoethyl group (2). It is eliminated from the molecule simultaneously with the cleavage of the oligonucleotide from the support. The deprotection of labile groups on the exocyclic amino functions should be concomitant with that step .

Our first attempts to synthesize the mononucleotides (5a−d) as described in Figure 1 with the convenient reagent bis(diisopropylamino)cyanoethyl phosphoroamidite failed. The presence of the bulky tBDMS group on the 2′ position of the sugar moiety considerably slows down the condensation rate. The reaction took place with the more reactive cyanoethyldiisopropylamino chlorophosphine. The monitoring of the reaction by TLC was tricky. The use of silica gel plates is inefficient because the starting nucleoside and one of the diastereoisomers of the phosphoroamidite formed are not resolved. So, estimating the completion of the condensation is not easy. Reverse-phase plates gave better results but require longer development times. The problems encountered at the analytical level were also found for the preparative purification of the amidites. Using a shallow gradient of ethyl acetate in dichloromethane in the presence of 1% triethylamine, the mononucleotides 5a, 5c and 5d derived from adenine, cytosine and dihydrouridine were purified using silica gel preparative HPLC. The separation of the monomer 5b was obtained in two steps. The first was a reversed phase chromatographic step on a Li-Chroprep RP 18 column eluted with a gradient of acetonitrile in water. The second step was a normal phase silica gel purification. The monomers were then precipitated in hexane at −78°C. The absence of 2′-phosphoramidite compounds was checked by ^{1}H and ^{31}NMR and RPLC which confirms the stability of the tBDMS group in the phosphitylation conditions (11, 12). The physico-chemical data of the mono ribonucleotides are summarized in Table 2.

Preparation of 5,6-dihydrouridine derivative

5,6-dihydrouridine (DHU) was prepared from uridine by catalytic hydrogenation on Rhodium/alumina (13). It was protected in the 5′ and 2′ positions by dimethoxytrityl and tBDMS groups respectively, and phosphitylated according to the same procedures as described for the normal nucleosides. Compounds 3d, 4d and 5d were fully characterised by FAB mass spectrometry, ^{1}H and ^{31}P NMR spectroscopy.

Preparation of the functionalized support

The preparation of the solid support constituting the 3′-end of oligoribonucleotide sequences was made according to the scheme depicted in Figure 2. As the starting material we used a mixture of 2′ and 3′ tBDMS nucleosides which was not resolved during the

Table 3. Schedule for the solid phase assembling of oligoribonucleotides on a 0.2 μmol scale.

Step	Reagent	Time
Detritylation	2 % Trichloracetic acid in CH_2Cl_2	1.5min.
Condensation	0.1 M 5a-d + 0.5 M Tetrazole in CH_3CN 50 μl	16 min.
Capping	0.3 M Phenoxyacetic anhydride in Lutidine/THF 1/8 6.5 % Dimethylaminopyridine in THF	1 min.
Oxidation	0.1 M Iodine in Lutidine/THF/Water 10/40/1	1 min.

chromatographic separation after the silylation step. The fixing of the succinate 'arm' was made by reaction of succinic anhydride with the mixture of nucleosides, activated by dimethylaminopyridine. The condensation of this crude material on the amino-function of the support (Long Chain Alkyl Amino Controlled Pore Glass) was activated by benzotriazol-1-yl-Oxy-tris(dimethylamino) phosphonium hexafluorophosphate (BOP reagent) (14). The loading of the nucleoside was 32 μmol/g.

Assembling of the oligoribonucleotides

The assembling cycle used for the synthesis of oligoribonucleotides is depicted in Table 3. This cycle is identical to the classical assembling methodology used for DNA synthesis, except in two respects. Owing to the lower reactivity of phosphoroamidites in the ribo series, the condensation time was increased to 16 minutes. The capping step was also modified. To avoid a transamidation reaction (15) between the acetyl group used for the capping of unreacted hydroxy-functions and the phenoxyacetyl moiety of protected guanines, phenoxyacetic anhydride was used as the capping reagent (15). The coupling yield was monitored by colorimetric measurements of the trityl cation released after each condensation cycle. The average coupling yield was more than 95%.

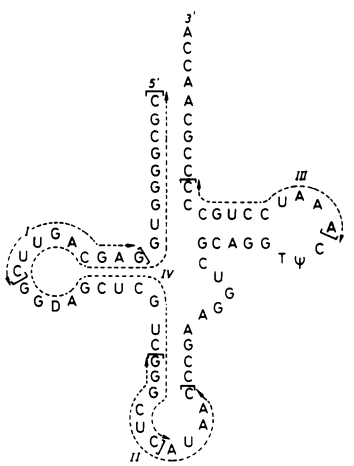

Figure 3: Nucleotide sequence of *B.subtilis* f-methionine t-RNA. The oligonucleotides noted in brackets are those described in this paper. They are noted I to IV in the text.

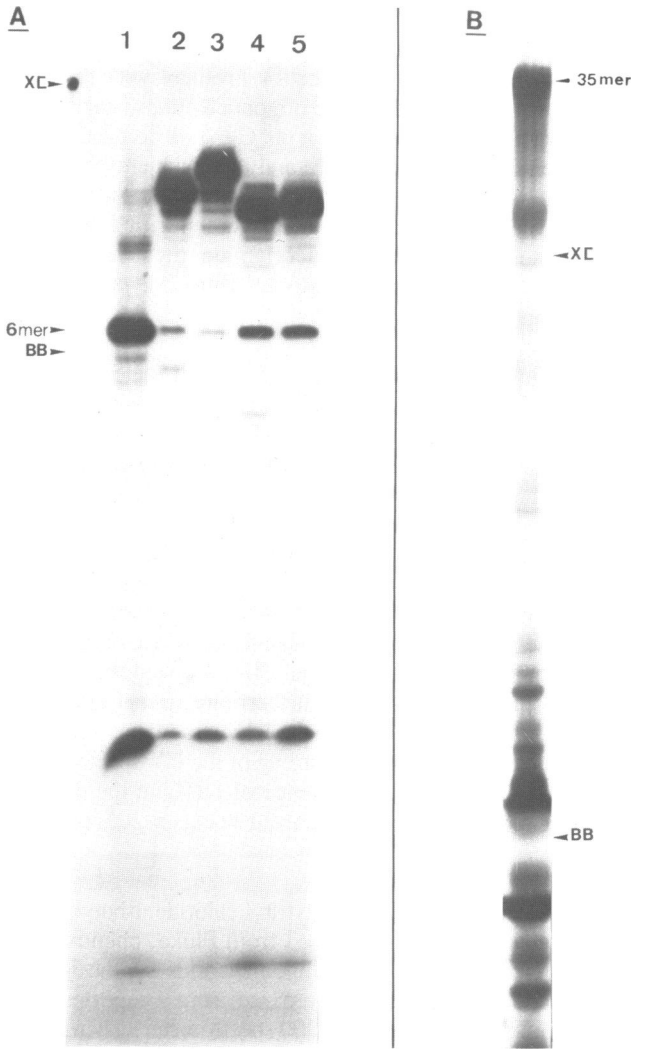

Figure 4: Autoradiographies of the oligoribonucleotide crude deprotection mixtures after migration on a 20% urea PAGE. Part A : 150 min. migration at 2 kV. Lane 1 6-mer deoxy; Lane 2 10-mer seq. CpUpUpGpApCpGpApGpU; Lane 3 11-mer seq. II; Lane 4 9-mer seq.I ; Lane 5 10-mer seq. III Part B : 240 min. migration at 2 kV. Crude 35-mer seq.IV.

The sequences prepared using the labile amino protecting groups belong to *B. subtilis* f-methionine tRNA. They are numbered I to IV in the sequence depicted in Figure 3.

Purification and analysis of the oligonucleotides

After the assembling cycles, the solid phases loaded with the oligonucleotides were treated in concentrated aqueous ammonia / ethanol (1/1) for 4 hours at room temperature. Then the tBDMS groups were removed according to Ogilvie *et al* (11). The crude deprotection mixtures were ^{32}P-labelled using polynucleotide kinase and γ^{32}P-ATP, after which they

were analysed by polyacrylamide gel electrophoresis. The autoradiography of the gels showed, for each oligonucleotide a major band with the correct mobility (Figure 4). To compare the efficiency of the 'short' deprotection protocol with the classical treatment, an aliquot of the deprotection mixtures of the oligonucleotides bearing only normal bases was taken and treated for 17 additional hours at 60°C. No difference was detected between the mixtures deprotected at room temperature and those which were treated for a longer time at 60°C.

On a preparative scale, the oligonucleotides were purified by polyacrylamide gel electrophoresis. After their elution from the acrylamide, they were desalted on Sephadex G25 columns. Typically 1−2 A_{254} were collected from 0.2 μmol cartridges.

The synthetic RNA fragments were characterised in several ways, using 5′ and 3′ labelling methods.

The identity of the nucleotide constituting the 5′ end of the 35-mer was evidenced by two-dimensional chromatography on PEI Cellulose plates. The crude digestion mixture of the 5′ ^{32}P labelled 35-mer with nuclease P1 was co-migrated with unlabelled mononucleotides. This showed a single radioactive spot corresponding to the appropriate nucleotide. The 3′-labelled oligonucleotides were treated with RNase T2 and chromatographed in the same system.

Further analysis were made using Nuclease P1 which interacts selectively with natural 3′-5′ internucleotidic bonds (16). 0.04 A_{254} of oligonucleotides III and IV (35 mer) were incubated with this enzyme and the subsequent nucleotide mixture was dephosphorylated with alkaline phosphatase. HPLC analysis revealed the presence of the expected nucleosides and the absence of partially undigested material. This showed the homogeneity of 3′−5′ linkages along the oligonucleotidic chains. Furthermore, integration of the peaks was in agreement with the nucleoside composition of the RNA fragments. The use of a photodiode array detector allowed both detection and recording of the UV spectrum of each component in the final enzymatic hydrolysate. The presence of DHU in the 35-mer was evidenced by its retention time and UV spectrum registered between 205 and 320nm.

MATERIAL AND METHODS

General conditions: Ribonucleosides, dimethoxytrityl chloride, t-butyl dimethylsilyl chloride and 1-H Hydroxybenzotriazole were purchased from Fluka, phenoxyacetyl chloride was from Janssen. All reagents for solid phase oligonucleotide synthesis were from Applied Biosystems. Phenoxyacetic anhydride was prepared from a reported procedure (15). ^1H NMR spectra were recorded on a Brüker AC 200 spectrometer. Chemical shifts are relative to tetramethylsilane as an internal standard in deuterated acetone or heavy water. ^{31}P NMR spectra were recorded with a Brüker WM 250 spectrometer. Chemical shifts are based upon 85% H_3PO_4 as an external standard. High resolution fast atom bombardment (FAB) mass spectra were made on a KRATOS MS 50 mass spectrometer working in positive or negative mode.

Thin layer chromatographic separations were performed on Merck 60F 254 silica gel plates. They were developed in various solvant systems:

—CH_2Cl_2/CH_3OH 9/1 v/v (System A)

—CH_2Cl_2/CH_3OH 8/2 v/v (System B)

—CH_2Cl_2/ AcOEt 1/1 v/v (System C).

Reversed phase TLC were performed on Macherey Nagel SIL RP18W/UV_{254} plates developed in CH_3CN/H_2O 70/30 v/v.

Preparative HPLC were performed on an axial compression system (Jobin-Yvon) equipped with two columns (40 and 80 mm internal diameter) and a LKB gradient former. Analytical HPLC were made on a Merck Li-Chrospher RP-18e column (125×4 mm) using a Waters 990 diode array detector.

N_6-Phenoxyacetyl-adenosine (2a): Adenosine (2.67 g—10 mM) was dried twice by evaporation from dry pyridine and suspended in 50 ml of dry pyridine. Trimethylchlorosilane (9.5 ml—75 mM) was added dropwise and the solution was mixed at room temperature for 25 minutes. During this period of time, 1-hydroxybenzotriazole (2.13 g—15.75 mM) was dried three times by evaporation of dry acetonitrile and subsequently suspended in 5 ml acetonitrile and 5 ml of pyridine. In this second flask was added the phenoxyacetyl chloride (2.1 ml—15 mM). After 5 minutes, both flasks are cooled with ice and the flask containing guanosine was added dropwise to the acylating agent through a rubber septum. The mixture was stirred overnight at room temperature. TLC in system A proved that acylation was complete. The mixture was cooled at 5°C and water (10ml) was added followed by concentrated aqueous ammonia (5 ml). After 15 minutes, solvents were removed by rotary evaporation and the subsequent gum was diluted in 300 ml water. This solution was washed by 2×150 ml chloroform. After evaporation of the solvent, the desired compound is crystalised in Ethanol−Water 9/1 v/v to yield 2.6g N_2-phenoxyacetyl-adenine (65%). Rf = 0.6 in system B.

N_2-Phenoxyacetyl-guanosine (2b): Guanosine (2.83 g—10 mM) was dried twice by evaporation from dry pyridine and suspended in 50 ml of dry pyridine. Trimethylchlorosilane (9.5 ml—75 mM) was added dropwise and the solution was mixed at room temperature for 25 minutes. During this period of time, 1-hydroxybenzotriazole (2.13 g—15.75 mM) was dried three times by evaporation of dry acetonitrile and subsequently suspended in 5 ml acetonitrile and 5 ml of pyridine. In this second flask was added the phenoxyacetyl chloride (2.1 ml−15 mM). After 5 minutes, both flasks were ice cooled and the flask containing guanosine was added dropwise to the acylating agent through a rubber septum. The mixture was stirred overnight at room temperature. TLC in system A proved that acylation was complete. The mixture was cooled at 5°C and water (10ml) was added followed by concentrated aqueous ammonia (5 ml). After 15 minutes, the solvents were removed by rotary evaporation and the subsequent gum was diluted in 300 ml water. This solution was washed by 2×150 ml chloroform. After evaporation of the solvent, the desired compound is crystallised in Ethanol/ Water 9/1 v/v to yield 2.5g N_2-phenoxyacetyl-guanosine (60%). Rf = 0.32 in system B.

N_4-Acetyl-Cytidine (2c): Cytidine (2.43 g—10 mM) was dried twice by evaporation from dry pyridine and suspended in 50 ml of dry pyridine. The solution was cooled to 5°C, trimethylchlorosilane (9.5 ml—75 mM) was added dropwise and the solution was mixed at room temperature for 25 minutes. After cooling at 5°C, acetyl chloride (1.4 ml—20 mM) was added dropwise throught a rubber septum and the solution was stirred overnight at 5°C. Water (10ml) was added followed by concentrated aqueous ammonia (5 ml). After 15 minutes, the solvents were removed by rotary evaporation and the subsequent gum was diluted in 300 ml water. This solution was washed by 2×150 ml chloroform. N_4-acetyl-cytidine (2.42 g—50%) was obtained after chromatography on a Merck Kieselgel silanized (70−230 mesh) column (5×20 cm), eluted by aceton−water 2/8. Rf = 0.55 in system B.

5,6-dihydrouridine: Uridine (4.88 g—20mM) was dissolved in water (250 ml) and Rhodium/Alumina (500 mg) was added. Hydrogen was applied under a pressure of 60

bars and the reaction was allowed to proceed for 4.5 hrs at room temperature. A TLC in system A showed the complete disappearence of UV absorbance at 254 nm, indicating that the reaction was complete. The reaction mixture was filtered on Celite, water was evaporated and DHU was applied to a preparative column of reversed phase silica gel (Li-Chrosorb RP 18) eluted with water. 4g (90%) were collected and submitted to tritylation reaction without further purification.

Half-life deprotection times: Approx. 10 mg nucleoside 2a−c were dissolved in 10 ml deprotection mixture (NH_4OH / EtOH 3/1 or 1/1). Aliquots (100 μl) were taken at 1 min., 2 min., 4 min. ... and immediatly neutralized in 1 ml 10% aqueous acetic acid. 1/10 of these solutions was then injected onto a Merck Li-Chrospher RP 18e column (5 μm—125×4 mm) eluted with a linear gradient of acetonitrile in 50 mM ammonium acetate (2 to 15% in 15 min.) and peaks were integrated at 260 nm with a Waters 990 UV monitor. Plotting the concentration of protected nucleosides versus time gave graphically the t ½ values.

Stability of DHU in deprotection conditions: 5,6-dihydrouridine (60 mg) was dissolved in an Ethanol/Conc. aqueous ammonia (1/1) solution (5 ml). Aliquots were taken every 30 min., neutralised in 10% aqueous acetic acid and analysed by silica gel TLC developed in system A. Spots were visualised by carbonization of the plate after spraying a 10% sulphuric acid in ethanol solution. The degradation was evidenced by the presence of spots having a Rf value lower than DHU. Less than 10% degradation appeared only after 5 hours.

5'-O-dimethoxytritylation of nucleosides: The base-protected nucleoside (10 mM) was dried by evaporating 2×10 ml of dry pyridine. It was taken up in 60 ml pyridine and dimethoxytrityl chloride in solution in 40 ml pyridine was added dropwise at 5°C over a 2 hour period. The solution was left overnight at 5°C and then checked for completion by TLC. Water (5 ml) was added and after 5 minutes, solvents were evapored to near dryness. The desired compound was then purified on silica gel HPLC.

Adenosine derivative (3a): Yield 55%. Rf = 0.82 in system A.

Guanosine derivative (3b): Yield 50%. Rf = 0.55 in system A.

Cytidine derivative (3c): Yield 45%. Rf = 0.59 in system A.

5,6-dihydrouridine derivative (3d): Yield 90%.

General method for the protection of 2' hydroxy function: The protected nucleoside (3a−d, 4 mM) was dried by evaporating 2×10 ml of dry pyridine. It was taken up in 40 ml pyridine and imidazole (0.79 g—11.6 mM) was added followed by t-butyldimethylsilyl chloride (0.35 g—2.4 mM). The reaction mixture was kept under magnetic stirring for one day and another crop of tBDMS chloride was added. This process was repeated another time. When TLC analysis showed that no starting material was present in the solution, water (5 ml) was added at 0°C and the solvents were evaporated to dryness. The yellow gum was taken up in chloroform (300 ml), washed with saturated aqueous sodium bicarbonate (2×150 ml) and water (150 ml). The compounds were separated from their 3' and bis 2',3'silylated isomers by silica gel HPLC. NMR data of compound 4a−d are listed in table 2.

Adenosine derivative (4a): Yield 35%. Rf in system C 0.82. High resolution mass spectrometry in positive mode: $C_{45}H_{52}N_5O_8Si$ Calc. Mass 818.3585 . Found 818.358 ± 0.004 for $(M + H)^+$.

Guanosine derivative (4b): Yield 32%. Rf in system C 0.45. High resolution mass spectrometry in positive mode: $C_{45}H_{52}N_5O_9Si$ Calc. Mass 834.3534 . Found 834.351 ± 0.004 for $(M + H)^+$.

Cytidine derivative (4c): Yield 43%. Rf in system C 0.57. High resolution mass spectrometry in positive mode: $C_{38}H_{48}N_3O_8Si$ Calc. Mass 702.3210 . Found 702.325 ± 0.004 for $(M + H)^+$.

5,6-dihydrouridine derivative (4d): Yield 25%. Rf in system C 0.74. High resolution mass spectrometry in positive mode: $C_{36}H_{47}N_2O_8Si$ Calc. Mass 662.3101. Found 663.308 ± 0.004 for $(M + H)^+$.

General method for the phosphitylation of nucleosides: 4 mM nucleosides 4a−c (1 mM—4d) were dried by 2 evaporations of pyridine and 1 evaporation of THF. They were dissolved in THF (10 ml) and kept under an argon atmosphere. Dimethylaminopyridine (49 mg—0.4 mM) and N,N,N-ethyldiisopropylamine (1.4 ml—8 mM) were added. Cyanoethyl diisopropylamino chlorophosphophine (1.1 ml—4.4 mM) was added dropwise through a rubber septum over a 30 min. period and the reaction was stirred at room temperature for 4 additional hrs. The mixture was then filtered to eliminate the precipitated ammonium chloride and the excess phophitylating agent was hydrolysed by 0.5 ml water. After evaporation of the solvents, the residual gum was dissolved in ethyl acetate (150 ml), washed with saturated aqueous sodium bicarbonate (150 ml) and water (2×150 ml). After the organic phase was dried over sodium sulfate and evaporated to dryness, the desired product was obtained by purification on silica gel (A 5a, DHU 5d and C 5c: yield 40%) or alternatively the guanosine derivative was first purified on a Merck Li-Chroprep RP 18 column (100 g), eluted with a gradient from 50 to 100% acetonitrile in 5% triethylamine−water. It was then purified again by silica gel chromatography to yield 0.8 g (38%) compound (5b). The mononucleotides were dissolved in 10 ml dichloromethane (Purine nucleosides) or alternatively 10 ml toluene (pyrimidine nucleosides) and precipitated in hexane at −78°C. They were stored as white powders under argon atmosphere.

Adenosine derivative 5a: High resolution mass spectrometry in negative mode: $C_{54}H_{67}N_7O_9PSi$ Calc. Mass 1016.4507 . Found 1016.453 ± 0.004 for $(M-H)^-$.

Guanosine derivative 5b: High resolution mass spectrometry in negative mode: $C_{54}H_{67}N_7O_{10}PSi$ Calc. Mass 1032.4456 . Found 1032.447 ± 0.004 for $(M-H)^-$.

Cytidine derivative 5c: High resolution mass spectrometry in negative mode: $C_{47}H_{63}N_5O_9PSi$ Calc. Mass 900.4133 . Found 900.412 ± 0.004 for $(M-H)^-$.

Dihydrouridine derivative 5d: High resolution mass spectrometry in negative mode: $C_{45}H_{62}N_4O_9PSi$ Calc. Mass 861.4024 . Found 861.405 ± 0.004 for $(M-H)^-$.

Preparation of the Cytidine solid support: A mixture of 2′ and 3′ tBDMS cytosine nucleosides (4c—0.9 g—1.3 mM) was dried by evaporation of 5 ml pyridine (3 times). It was dissolved in 20 ml dry pyridine, dimethylaminopyridine (80 mg—0.65 mM), succinic anhydride (0.4 g—4 mM) were added and the reaction mixture was allowed to stand under stirring for 70 hrs at room temperature. The solution was poured in water (50 ml) and the desired mixture of compounds were extracted by chloroform (3×50 ml). The organic phase was washed with aqueous sodium bicarbonate (2×50 ml), water (50 ml) and the solvent was evaporated under reduced pressure to give a white foam which was used without further purification.

Long Chain Alkyl Amino (LCAA) Controled Pore Glass (CPG) from Electronucleonics Inc. having a pore diameter of 500 A and a particle size of 80−120 μm (2 g—100 μM) was dried by evaporating 3 times in a mixture of pyridine (5 ml) and triethylamine (100 μl). The half-part of the previous mixture was added, followed by BOP reagent (600 g—1.5 mM) and the reaction was kept under mechanical stiring for 24 hours. The support was

poured in a sintered glass funnel and washed successively with pyridine (3×10 ml), acetic anhydride / lutidine / pyridine 1/1/1 (2×5 ml—5 min.), pyridine (2×10 ml), water (10 ml),acetone (5×10 ml) and ethylic ether (5×10 ml). After drying under vacuum, the loading of the support was measured as 32 μM/g by detritylating part of an aliquot and estimating spectroscopically at 500 nm the amount of trityl cation released.

Solid phase syntheses were performed on an Applied Biosystems 381 A DNA synthesizer working on a 0.2 μmol scale. Modifications of the condensation cycle from the standard one were as follows:

The condensation time was increased to 16 minutes by adding a fourth sequence of pumping tetrazole and monomers to column and adding a waiting step of 4 min. after each reagent was delivered to the column.

The capping reagent was 0.3 M phenoxyacetic anhydride in lutidine THF 1/8, 6.5% Dimethylaminopyridine in THF.

Deprotection of the oligonucleotides: The cartridges containing the support loaded with the oligonucleotide chains were treated with a 28−30% ammonia / ethanol solution 1/1 (4×500 μl) for 4 hrs. at room temperature. An aliquot of the deprotection mixtures of oligonucleotides I to III was taken and treated at 50°C for 17 additional hours. The solvent was evaporated under reduced pressure and the RNA fragments were further treated with tetrabutylammonium fluoride as described by Ogilvie *et al.*(11).

Analysis of the oligonucleotides: 5′-end labelling was made with γ^{32}P ATP and polynucleotide kinase; 3′-end labelling was made with T_4 RNA ligase and ^{32}P C P, on a 0.01 μg scale. The radioactive oligonucleotides were then purified by 8 M urea 20% polyacrylamide gel electrophoresis. The autoradiography showed major bands with the expected mobility. They were electroeluted in 5 ml phosphate buffer (20 mM, pH 7.8) and dialysed against 2 mM ammonium bicarbonate. No significant difference was observed between the compounds deprotected at room temperature and those which were treated at 50°C.

The 5′-labelled oligoribonucleotides were treated with Nuclease P1 (0.15 unit—Boehringer) for 2 hrs. at 37°C and analysed by two dimensional chromatography on PEI cellulose plates (Schleicher & Schüll) eluted by respectively isobutyric acid / 0.5 M ammonia 5/3 (v/v) and isopropanol / concentrated hydrochloric acid / water 70/15/15 (v/v/v). The migration of the radioactive spots was compared with that of natural witnesses.

The 3′ ends were determined by the same chromatographic procedure after digestion of the 3′ end labelled compounds by T_2 Nuclease (5 units—BRL) in 15 min. at 37°C.

The nucleoside composition of sequences I and IV was determined as follows. The oligonucleotide (0.04 A_{254}) was reacted with Nuclease P1 (1 Unit—Boehringer) for 2 hrs. at 37°C. Alkaline phosphatase (1 Unit—Genofit) was added and the reaction mixture was allowed to stand for 1 hr at 37°C. Water (500 μl) was added and the reaction mixture was centrifuged for 10 min. at 14000 rpm. 400 μl of the supernatant were directly injected onto a Li-Chrospher RP 18 e (5 μm—125×4 mm) column eluted by 10 mM triethylammonium acetate (TEAA) pH 6.0 for 2 min. and a linear gradient of Acetonitrile to 15% in 15 min. The peaks were recorded and integrated at 260 nm with a Waters 990 diode array UV detector.

Sequence 1 (10-mer): Calc. C (50%); U (20%); G (10%); A (20%). Found C (51.05%); U (18.92%); G (10.41%); A (19.62%).

Sequence 5 (35-mer): Calc. C (26.47%); U (17.65%); G (47.05%); G (8.82%). Found C (27.21%); U (16.82%); G (46.88%); A (9.09%).

*To whom correspondence should be addressed

REFERENCES

1. Barone, A.D., Tang, J.Y. and Caruthers, M.H. (1984) Nucleic Acids Res., **12**,4051.
2. Sinha, N.D., Biernat, J., Mc Namus, J. and Köster, H. (1984) Nucleic Acids Res., **12**,4539.
3. Froehler, B.C., Ng, P.G. and Matteucci, M.D. (1986) Nucleic Acids Res., **14**,5399.
4. Garegg, P.J., Lindh, I., Regberg, T., Stawinski, J. and Strömberg, R. (1985) Chem. Scripta, **25**,280.
5. Schulhof, J.C., Molko, D. and Téoule, R. (1987) Nucleic Acids Res., **15**,397.
6. Molko, D., Schulhof, J.C. and Téoule, R. Patent n° : E.N. 86 04 990 in France, Applications n° : 031781 in U.S.A., 87 400739 6 in Europe, 085 615in Japan
7. Ti, G.S., Gaffney, B.L. and Jones, R.A. (1982) J. Am. Chem. Soc., **104**,1316.
8. Benseler, F. and McLaughlin, L.W. (1986) Synthesis 45.
9. Tanaka, T., Tamatsukuri, S. and Ikehara, M. (1987) Nucleic Acids Res., **15**,7235.
10. 1-((2-chloro-4-methyl)phenyl)-4-methoxypiperidin-4-yl group.Rao, T.S., Reese, C.B., Serafinowska, H.T., Takaku, H. and Zappia,G. (1987) Tet. Lett., **28**,4897.
11. Ogilvie, K.K., Usman, N., Nicogoshian, K. and Cedergren, R.J. (1988) Proc. Natl. Acad. Sci. USA., **85**,5764.
12. Stawinski, J., Strömberg, R., Thelin, M. and Westman, E. (1988) Nucleic Acids Res., **16**,9285.
13. Schulhof, J.C., Molko, D. and Téoule, R. (1988) Nucleic Acids Res., **16**,319.
14. Castro, B., Dormoy, J.R., Evin ,G. and Selve, C. (1975) Tet. Lett., 1219.
15. Chaix, C., Molko, D. and Téoule, R. (1989) Tet. Lett., **30**,71.
16. Tanimura, H., Sekine, M. and Hata, T. (1987) Chem. Lett., 1057.

This article, submitted on disc, has been automatically converted into this typeset format by the publisher.