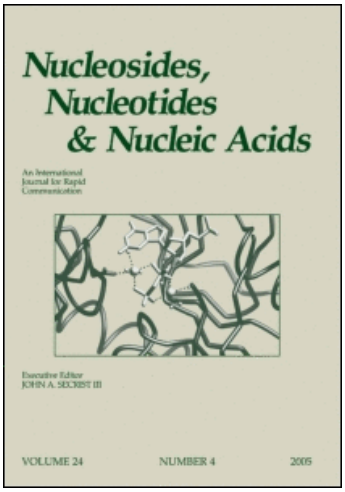

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Synthesis of Oligoribonucleotides by the H-Phosphonate Approach Using Base Labile 2'-O-Protecting Groups. V. Recent Progress in Development of the Method

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SYNTHESIS OF OLIGORIBONUCLEOTIDES BY THE H-PHOSPHONATE APPROACH USING BASE LABILE 2'-O-PROTECTING GROUPS. V. RECENT PROGRESS IN DEVELOPMENT OF THE METHOD

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Abstract: A simple and inexpensive method for the preparation of oligoribonucleotides using N-isopropoxyacetyl(phenoxyacetyl)-2'-O-(2-chlorobenzoyl)-5'-O-dimethoxytritylnucleoside H-phosphonate building blocks has been developed.

In the last years there has been a growing interest on antisense oligonucleotides which could constitute a new therapeutic principle[1]. After Zamecnik and Stephenson[2,3] had shown the possibility to inhibit the growth of Rous sarcoma virus by a short oligodeoxynucleotide, there were a number of reports on the use of the antisense DNA and RNA fragments against many viruses including HIV-I and HTLV-III[4-9].

On the other hand remarkable progress has been achieved in the chemical synthesis of oligoribonucleotides. Nucleases resistant, modified oligoribonucleotides also seem attractive for antisense approach because ribonucleosides are much less costly as starting materials than their deoxy analogues. Thus, investigations with the aim of developing more effective and simple methods for oligoribonucleotide synthesis are continued by

various groups. The most serious problem is the choice of the 2'-OH protecting group. Trialkylsilyl[10,11], acetal[12-16], o-nitrobenzyl[17,18] and p-nitrophenylethylsulphonyl[19] groups were used for this purpose.

However, most of the widely used protecting group techniques are too elaborate and costly for synthesizing antisense oligonucleotides on a large scale. Therefore, our attention was attracted to the selective benzoylation of N-protected 5'-O-dimethoxytritylated ribonucleosides described by Kempe and co-workers[20]. This method offered several advantages: simple and fast synthesis of protected ribonucleosides, stability of benzoyl groups in acidic media during detritylation and easy removal under mild basic conditions. Moreover, it seems to be a really inexpensive pathway to synthons for oligoribonucleotide synthesis.

RESULTS AND DISCUSSION

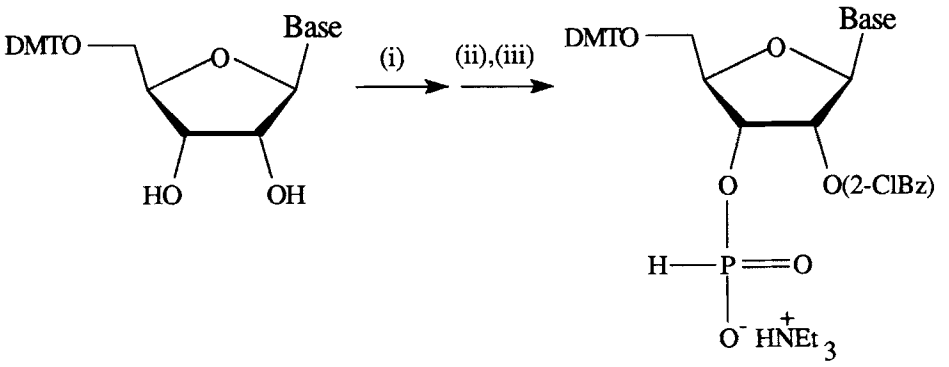
Our initial attempts[21] to synthesize native oligoribonucleotides using 2'-O-benzoyl protection[20] failed completely. Mixtures of modified oligomers have been obtained with various condensing agents and coupling conditions.

Hence, suspecting that n-butylamine, used by Kempe instead of ammonia solution to prevent a rapid breakdown of oligoribonucleotide during final deprotection step, was responsible for observed side-reactions we searched for another way to suppress both modification and degradation.

Recently, we reported[22] the preliminary experiments concerning this subject. In this manuscript synthesis of oligoribonucleotides using N-isopropoxyacetyl[23] (ipa) or N-phenoxyacetyl[24-27] (pac) and 2'-O-(2-chlorobenzoyl) (2-ClBz) protecting groups is described. The main reasons for choosing of the 2-chlorobenzoyl group are: *(i)* deprotection conditions are sufficiently mild and comparable with these for used N-protecting groups, all protecting groups can be completely removed in concentrate aqueous ammonia under conditions mild enough to avoid degradation of the oligoribonucleotide (25% NH_3/H_2O, 5h, R.T.). *(ii)* the corresponding benzoyl chloride is commercially available.

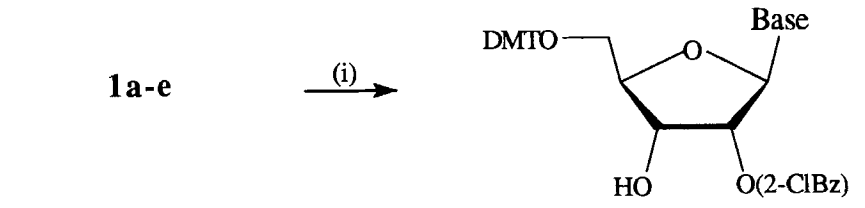
We also developed an improved synthesis of the protected ribonucleoside H-phosphonates **3a-e** (Scheme 1) which were obtained in a one pot procedure via the reaction of the protected ribonucleosides **1a-e** with 2-chlorobenzoyl chloride in methylenechloride/pyridine (9:1, v/v) at -

Scheme 1



1a-e **3a-e**

Scheme 2



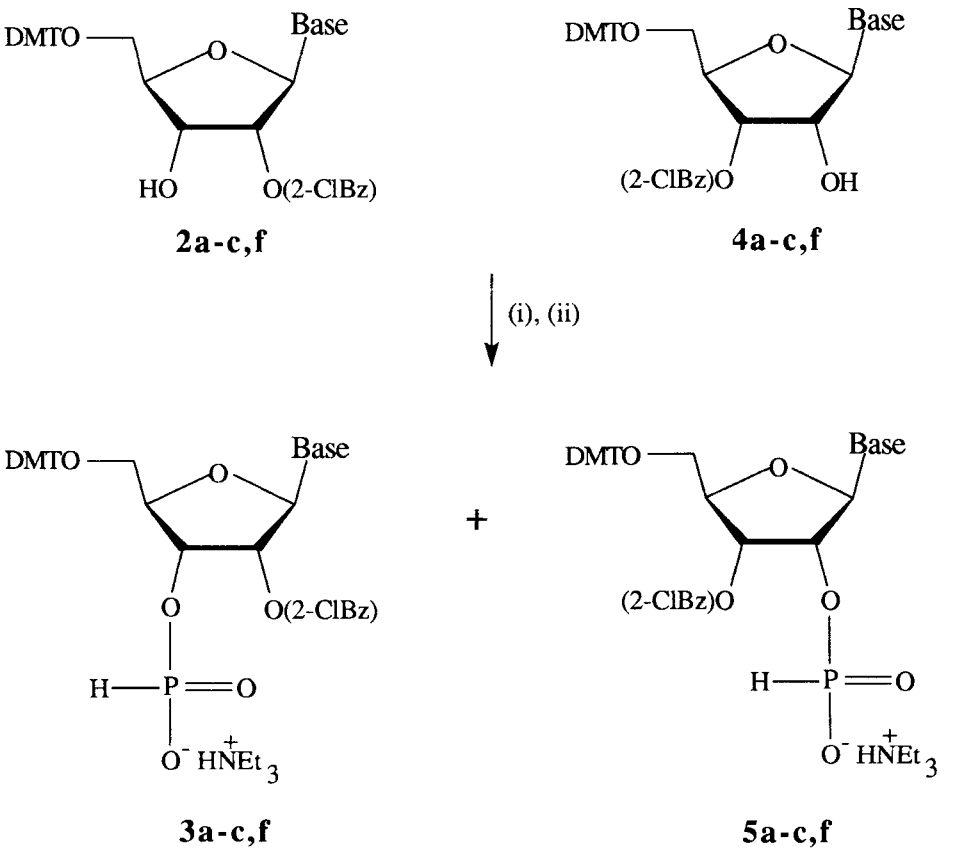
2a-e

Base = ipa^{6}Ade (a), Ura (b), ipa^{2}Gua (c), pac^{2}Gua (d), ipa^{4}Cyt (e); (i)- 2-chlorobenzoylchloride (1.1 eq) in CH_2Cl_2/Py, 9:1, at -78°C; (ii)- salicylchlorophosphite (1.5 eq); (iii)- 1.0 M triethylammonium bicarbonate (aq.).

78°C. After completion of the reaction (TLC, solvent A) salicylchlorophosphite was added, the reaction mixture was allowed to warm up to -30°C and then quenched with 1.0 M TEAB. Finally, the products **3a-e** were purified on silica gel column chromatography using a linear gradient (0-10%) of methanol in chloroform containing 1% of triethylamine.

This method in comparison with traditional way[20] involving isolation of 2'-O-benzoylated product **2a-e** (Scheme 2) provides indeed fast,

Scheme 3



Base = ipa^{6}Ade (a), Ura (b), ipa^{2}Gua (c), bz^{4}Cyt (f); (i)- PCl_3 (5 eq), imidazole, N-methylmorpholine in CH_2Cl_2 at -60°C; (ii)- 1.0 M triethylammonium bicarbonate (aq.).

simple and inexpensive route for synthesis of ribonucleoside H-phosphonates, synthons for oligoribonucleotide synthesis. Moreover, the problem of 2'-3' migration of aroyl groups is practically solved because the work up of **2a-e** which can lead to acyl migration is eliminated.

The major requirement to be met by 2'-O-acyl building blocks is their isomeric purity. We investigated the possibility to separate the nucleosides 3' and 2'-H-phosphonates having 2-chlorobenzoyl group in the ribose moiety by a simple silica gel column chromatography.

The 2'-O-(2-chlorobenzoyl)ribonucleosides **2a-c,f** (Scheme 3) were prepared by adding 2-chlorobenzoylchloride to a solution of 5'-O-tritylated ribonucleosides in methylenechloride containing 5% of pyridine (Scheme 2) and subsequently kept in ethanol/water (4:1) solution for a couple of hours (TLC, solvents B, C) to obtain mixtures of **2a-c,f** and **4a-c,f** in a ratio of approximately 1:1. The N-benzoyl[27] protection was used for cytidine instead of the N-isopropoxyacetyl group since this was not completely stable in ethanol/water. The obtained mixtures were precipitated from hexane and converted into the corresponding H-phosphonates without altering the customary reaction conditions. TLC analysis of products after precipitation from diethyl ether (Table 1) showed two main compounds in a ratio of approximately 1:1 (**3a-c,f** and **5a-c,f**).

The separation of isomeric H-phosphonates was performed by routine silica gel column chromatography using a linear gradient of methanol (0-10%) in chloroform containing 1% of triethylamine. All 2'-O-benzoylated H-phosphonates **3a-c,f** had higher R_f values than the corresponding 3'-O-benzoylated isomers (Table 1) and were eluted in the earlier fractions. The later fractions containing **5a-c,f** were slightly contaminated with **3a-c,f** (TLC analysis, solvent A) and were purified once more under the same conditions. Structures of separated **3a-c,f** and **5a-c,f** were confirmed by ^{1}H NMR (Table 1, 2).

The efficiency of separation was determined by HPLC (Table 3, Fig. 1). The isomeric 2' and 3'-H-phosphonates have different retention times allowing easy detection of even small amounts of the other isomer in separated fractions. The two isomers can be also distinguished by their phosphorus chemical shifts (Table 1).

Examination of the data in Table 3 suggests that the desired 2'-O-(2-chlorobenzoyl)-3'-O-hydrogenphosphonates **3a-c,f** can be efficiently separated from the 3'-O-(2-chlorobenzoyl) isomers by silica gel column

Table 1. R_f[a], ^{31}P and ^1H NMR[b] chemical shifts (δ, ppm) of the isomeric H-phosphonates **3a-c,f** and **5a-c,f**

									CH_3CH_2N						$(CH_3)_2CHO$				
compd	R_f	^{31}P	H1'	H2'	H3'	H4'	H5',H5''	H-P	CH_3	CH_2	OCH_3	H2(5)	H8(6)	arom.	CH_3	CH	OCH_2	NH
3a	0.29	2.2	6.53	6.24	5.35	4.59	3.51 3.57	6.89	1.33	3.05	3.77	8.19	8.70	6.81, 7.99 7.19-7.44	1.33	3.79	4.20	9.6 12.2
5a	0.25	2.6	6.40	5.79	5.89	4.54	3.49 3.55	6.85	1.21	2.95	3.75	8.36	8.70	6.74, 7.96 7.18-7.43	1.31	3.78	4.21	9.6 12.4
3b	0.23	2.1	6.36	5.78	5.21	4.48	3.53 3.60	6.89	1.20	2.96	3.78	5.25	7.80	6.85, 7.98 7.20-7.44	-	-	-	9.1 12.2
5b	0.20	2.3	6.22	5.20	5.64	4.41	3.50 3.55	6.89	1.21	2.97	3.76	5.39	7.78	6.82, 7.94 7.20-7.44	-	-	-	9.2 12.3
3c	0.21	1.5	6.32	6.13	5.50	4.52	3.45 3.50	6.88	1.23	3.01	3.76	-	7.82	6.78, 7.96 7.18-7.41	1.25	3.74	4.09	9.4, 10.4, 11.7
5c	0.19	2.1	6.19	5.63	5.85	4.47	3.45 3.48	6.87	1.17	2.94	3.76	-	7.98	6.78, 7.96 7.15-7.48	1.24	3.76	4.09	9.18, 11.8, 12.0
3f	0.22	1.5	6.27	5.82	5.22	4.46	3.60 3.68	6.94	1.34	3.07	3.77	7.91	8.38	6.84, 7.94 7.20-7.58	-	-	-	9.0, 10.5
5f	0.19	2.0	6.24	5.21	5.62	4.54	3.54 3.66	6.98	1.21	2.94	3.77	7.91	8.43	6.84, 7.92 7.20-7.56	-	-	-	9.8, 10.1

[a]TLC solvent A. [b]^{31}P NMR spectra were recorded on a JEOL GSX-270 spectrometer in pyridine. Phosphorus signals were referenced to external 2% phosphoric acid in D_2O. ^1H NMR spectra were recorded on a Bruker WM-360 spectrometer in $CDCl_3$, signals were referenced to internal TMS.

Table 2. Spin-spin coupling constants (Hz) in **3a-c,f** and **5a-c,f**

compd	H1',H2'	H2',H3'	H3',H4'	H2',P	H3',P	H4',H5"	H4',H5'	H5',H5"	HP	H5,H6
3a	5.9	5.3	3.7	-	10.2	2.9	4.0	10.5	629	-
5a	5.6	5.4	4.1	10.5	-	3.7	4.2	10.8	636	-
3b	6.0	5.1	3.7	-	10.8	2.2	2.6	10.6	629	8.0
5b	4.9	5.2	5.1	10.2	-	2.5	2.5	10.8	630	8.0
3c	5.3	5.3	6.0	-	10.5	2.8	3.6	10.2	631	-
5c	6.1	5.8	3.6	10.6	-	3.9	3.3	10.6	632	-
3f	3.0	4.8	6.4	-	11.5	2.3	2.8	11.1	634	8.0
5f	3.1	4.8	7.0	10.9	-	2.5	2.8	11.4	639	7.5

In all cases $^{3}J_{HH}(CH_3CH_2N)$=7.3 Hz and $^{3}J_{HH}((CH_3)_2CHO)$=6.2 Hz.

Table 3. HPLC[a] analysis of separated **3a-c,f** and **5a-c,f**.

Separated mixture[b]	Retention times (min)		Ratio **3a-c,f** / **5a-c,f**	
	3a-c,f	**5a-c,f**	First fraction	Second fraction
3a; 5a	8.47	12.80	99.98 : 0.02	1.35 : 98.65
3b; 5b	3.94	7.04	99.96 : 0.04	0.18 : 99.82
3c; 5c	6.04	11.05	99.87 : 0.13	0.56 : 99.44
3f; 5f	13.91	23.04	99.70 : 0.30	0.52 : 99.48

[a]HPLC was performed on a Silasorb SPH C-18 column 4x150 mm using acetonitrile/0.1 M triethylammonium acetate (pH 6.5), 45:55 (isocratic mode), flow rate 1.7 ml/min., λ=254 nm.
[b]The mixtures of 3 and 5 (approx. 1:1) were separated as described in Experimental Section.

chromatography. The separation of **5a-c,f**, however, is less efficiency and requires additional silica gel purification steps.

It is also clear that **3a-e** can be easily purified from the **5a-e** contamination (usually 1-2 %) arising during selective benzoylation. For the purpose of the present work we considered the small 3'-O-acyl contamination in **3a-e** (HPLC analysis, approx. 0.1%) to be sufficiently low to enable synthesis of short oligoribonucleotides. However, the data reported above suggest that the isomeric purity can be significantly improved to allow synthesis of longer RNA fragments.

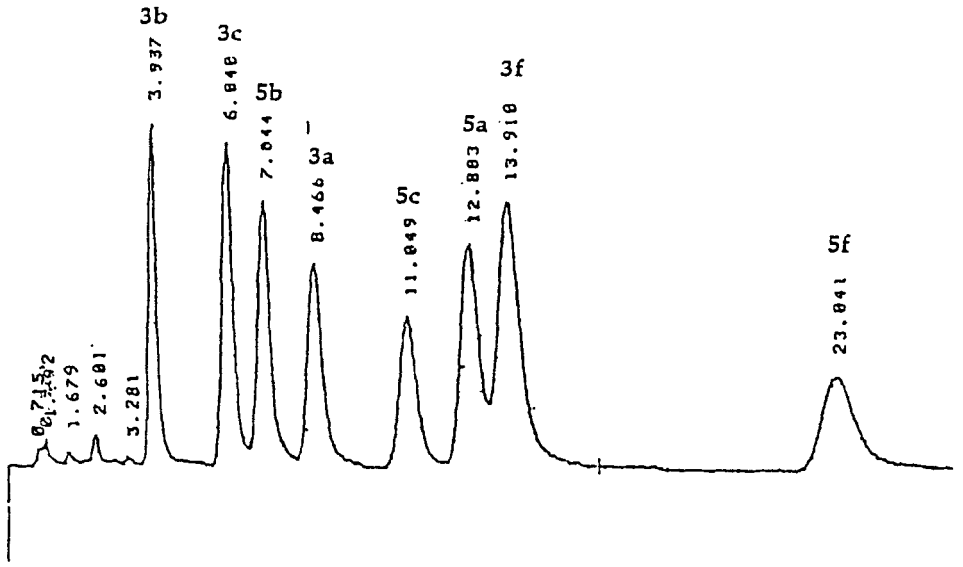


Fig. 1. Analytical HPLC separation of artificial mixture of **3a-c,f** and **5a-c,f**.

Table 4. Chemical steps for one synthetic cycle.

Step	Reagent or solvent	Volume	Time(s)
1.Wash	CH_2Cl_2	3x1ml	45
2.Detritylation	3% DCA/CH_2Cl_2	3x1ml	60
3.Wash	CH_2Cl_2	2x1ml	90
	CH_3CN	4x1ml	
4.Condensation	0.05 M **3a-e** in pyridine	1ml	150
	0.25 M Adamantoyl chloride in CH_3CN	1ml	
5.Wash	CH_3CN	1x1ml	15
6.Capping	0.05 M iPr H-phosphonate in pyridine	0.5ml	60
	0.25 M Adamantoyl chloride in CH_3CN	0.5ml	
7.Wash	CH_3CN	4x1ml	60

Total time 10 minutes

Table 5. Yields of oligoribonucleotides.

Oligoribonucleotide	Chain length	Yield per step	Overall yield
6. U_6	6	98	50
7. A_6	6	93	14
8. C_6	6	96	22
9. AUGAGG	6	94	11
10. AUGAGGAGG	9	94	4
11. UCUACCCC	8	98	22
12. GGAGUAGC	8	97	15
13. AUGAGGAGGC	10	97	7
14. UAAUGAGGAGGC	12	98	11
15. AGGAUUAUUUAUGC	14	96	5
16. UUACCCAUGUCCUCC	15	97	6

Syntheses of oligoribonucleotides (Table 5) were performed by a solid phase method in a syringe on the sample of controlled pore glass containing 2.5 μmol of the immobilized nucleoside. Oxidation of polymer bonded oligonucleoside H-phosphonates was carried out according to the described method[28] using 0.05 M I_2 in THF/Py/MeIm/H_2O 90:5:1:5 (2.5 min) and 0.05 M I_2 in THF/NEt_3/H_2O, 90:5:5 (2.5 min).

Cleavage of the oligoribonucleotides from the polymer support and removal of the N- and 2'-O-protecting groups were accomplished with concentrated aqueous ammonia solution within 5 hours at room temperature. It should be noted that under these conditions no significant degradation of oligoribonucleotides was observed. The prolonged time (10 hours) of deprotection caused no remarkable increase of degradation.

The oligoribonucleotides (Table 5) **6-10** were separated by ion exchange chromatography on DEAE-Sephadex A-25 as described previously[22]. The partially deprotected oligomers **11-16** bearing 5'-O-dimethoxytrityl groups were isolated by RP FPLC (Fig. 2a), compound corresponding to the main peak was detritylated with 80% aqueous acetic acid and purified by RP FPLC (Fig. 2b). The correct nucleotide ratio and native 3'-5' phosphodiester linkages were confirmed by enzymatic degradation (Ribonuclease A) followed by RP FPLC analysis.

Oligoribonucleotides synthesized using n-butylamine[21] and aqueous ammonia solutions as deprotection agents have been analyzed by 24%

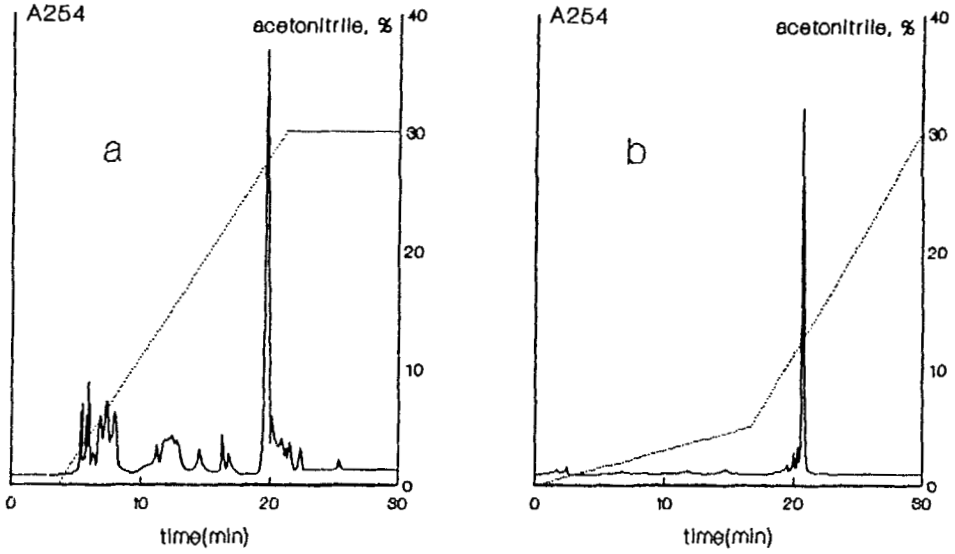


Fig. 2. Reversed-phase FPLC analysis of synthesized oligoribonucleotides: crude DMT(GGAGUAGC) **12** (a) and separated GGAGUAGC **12** (b).

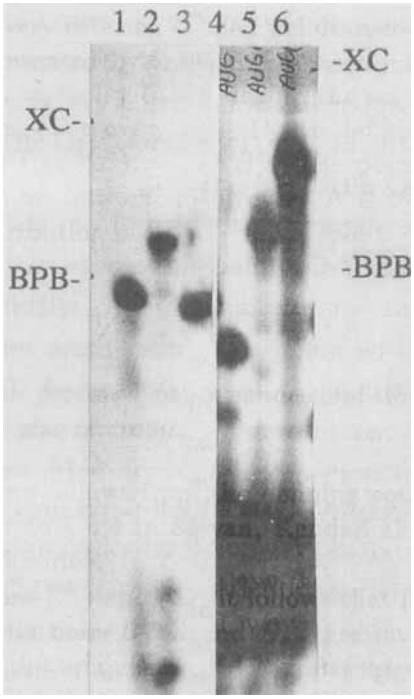


Fig. 3. Autoradiogram of 24% polyacrylamide/7M urea gel electrophoresis. Comparison of the 5'-end-^{32}P-labeled oligoribonucleotides synthesized using the new methodology: Lane 1 AUGAGG, Lane 2 AUGAGGAGG and synthesized using n-butylamine: Lane 5 AUGAGG, Lane 6 AUGAGGAGG. Lanes 3,4 independently synthesized markers AUGAGG and AUG.

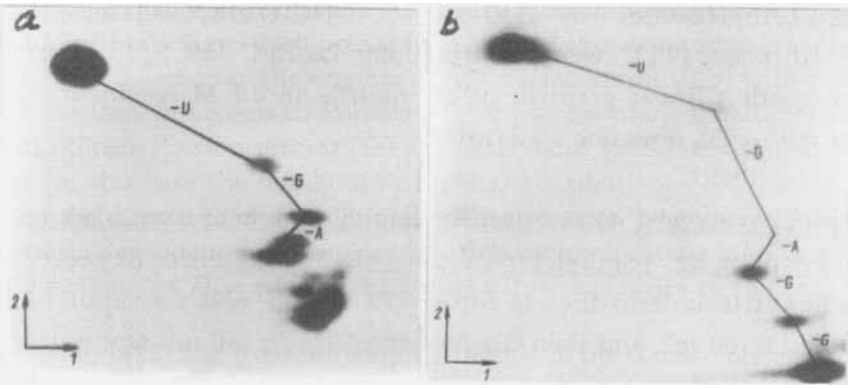


Fig. 4. Sengerprint sequencing of AUGAGG synthesized using n-butylamine (a) and the new methodology (b).

polyacrylamide/7M urea gel electrophoresis (Fig. 3), Sengerprint sequencing (Fig. 4) and RP FPLC (data not shown). It is clear that n-butylamine treatment leads to modified oligomers. These have a slower mobility on gel electrophoresis and seem more hydrophobic on RP FPLC. Our attempts to isolate individual compounds from modified oligomers were unsuccessful.

On the other hand the problem can be eliminated by using mild ammonia treatment made possible by the easily removable N-isopropoxy or phenoxyacetyl and 2'-O-(2-chlorobenzoyl) groups.

Thus, this new methodology allows a fast, simple and exceptionally inexpensive preparation of ribonucleoside H-phosphonates that makes it quite suitable for a large scale synthesis of native and modified oligoribonucleotides. Further investigations of various aspects of the use of 2'-O-aroyl groups in combination with H-phosphonate chemistry are under way and will be reported in forthcoming papers.

EXPERIMENTAL SECTION

Long chain alkylamino controlled pore glass (LCAA CPG, Pierce) with immobilized first nucleoside and 2'-O-(2-chlorobenzoyl) ribonucleosides **2a-f** were prepared as described previously[22]. TLC was performed on Silufol UV-254 plates (Lachema) using solvents A ($CHCl_3$/MeOH, 9:1, v/v), B

(toluene/ethylacetate, 1:1, v/v) and C ($CHCl_3$/ethylacetate, 3:7, v/v). Reversed-phase FPLC was performed on ProRPC HR 5/10 (Pharmacia) column with a linear gradient of acetonitrile in 0.1 M triethylammonium acetate (pH 6.95), flow rate 1 ml/min.

N-Isopropoxyacetyl-2'-O-(2-chlorobenzoyl)-5'-O-dimethoxytrityladenosine 3'-H-phosphonate, triethylammonium salt **3a**. N-Isopropoxyacetyl-5'-O-dimethoxytrityladenosine **1a** (4.69 g, 7 mmol) was coevaporated with pyridine (3x100 ml) and then dissolved in CH_2Cl_2 (90 ml) and pyridine (10 ml). The reaction mixture was cooled to -78°C (acetone-dry ice). A solution of 2-chlorobenzoyl chloride (1.26 ml, 7.7 mmol) in CH_2Cl_2 (10 ml) was then added during 15 min. under stirring and cooling. After stirring for 1 h at -78°C (TLC, solvent A) the solution of salicylchlorophosphite (1.35 ml, 10 mmol) in CH_2Cl_2 (10 ml) was added. During stirring for 1 h the mixture was allowed to warm up to -30°C. After quenching with 1.0 M TEAB, pH 8.5 (200 ml) the organic layer was separated and dried over $MgSO_4$. After evaporation the residue was chromatographed on a silica gel (Woelm, 200 μm) column (40x150 mm) using a linear gradient (0-10%) of methanol in chloroform containing 1% of NEt_3. The main fraction was collected, evaporated, coevaporated with dry acetonitrile (3x50 ml) and dried in vacuo to give **3a** as a white powder. Yield 3.28 g (48%).

2'-O-(2-chlorobenzoyl)-5'-O-dimethoxytrityluridine 3'-H-phosphonate, triethylammonium salt **3b**. Compound **1b** (3.82 g, 7 mmol) was treated analogously to **1a** and gave **3b**; yield 3.22 g (54%).

N-Isopropoxyacetyl-2'-O-(2-chlorobenzoyl)-5'-O-dimethoxytritylguanosine 3'-H-phosphonate, triethylammonium salt **3c**. Compound **1c** (4.77 g, 7 mmol) was treated analogously to **1a** and gave **3c**; yield 3.16 g (46%).

N-phenoxyacetyl-2'-O-(2-chlorobenzoyl)-5'-O-dimethoxytritylguanosine 3'-H-phosphonate, triethylammonium salt **3d**. Compound **1d** (5.03 g, 7 mmol) was treated analogously to **1a** and gave **3d**; yield 3.01 g (42%).

N-isopropoxyacetyl-2'-O-(2-chlorobenzoyl)-5'-O-dimethoxytritylcytidine 3'-H-phosphonate, triethylammonium salt **3e**. Compound **1e** (4.52 g, 7 mmol) was treated analogously to **1a** and gave **3e**; yield 3.53 g (53%).

Preparation of isomeric mixtures of **2a-c,f** and **4a-c,f**. Compounds **2a-c,f** were dissolved in ethanol/water, 4:1 and kept at room temperature for a couple of hours, whereupon TLC analysis (solvents B,C) showed formation of **2a-c,f** and **4a-c,f** in ratio approximately 1:1. After evaporation the residue was partitioned between chloroform and water. The organic layer was separated, dried over $MgSO_4$, evaporated and precipitated in hexane. R_f= **2a** 0.40, **4a** 0.26 (B); **2b** 0.52, **4b** 0.41 (B); **2c** 0.60, **4c** 0.45 (C); **2f** 0.35, **4f** 0.24 (B).

Synthesis and separation of **3a** and **5a**. Imidazole (5.58 g, 82 mmol) was suspended in CH_2Cl_2 (200 ml) and N-methylmorpholine (28 ml, 248 mmol) was added. The mixture was cooled to 0°C and PCl_3 (2.2 ml, 24.8 mmol) was added. The mixture was stirred under argon for 30 min. at room temperature, then cooled to -60°C (acetone-dry ice) and the mixture of **2a** and **4a** (3.35 g, 5 mmol) in CH_2Cl_2 (100 ml) was added for 10 min. The mixture was stirred for 30 min. at -60°C, quenched with 1.0 M TEAB, pH 8.5 (400 ml), the organic layer was separated and dried over $MgSO_4$. After evaporation the residue was chromatographed on silica gel (Silasorb 600, 30 μm) column (35x140 mm) using a linear gradient (0-10%) of methanol in chloroform containing 1% of NEt_3. The fractions were collected, evaporated and dried in vacuo. The fraction containing **5a** was purified once more under the same conditions. Yields: **3a** 1.4 g (29%) and **5a** 1.3 g (27%).

Synthesis and separation of **3b** and **5b**. The mixture of **2b** and **4b** (2.73 g, 5 mmol) was treated analogously to **2a** and **4a** and gave **3b**; yield 1.2 g (33%) and **5b**; yield 0.9 g (21%).

Synthesis and separation of **3c** and **5c**. The mixture of **2c** and **4c** (3.41 g, 5 mmol) was treated analogously to **2a** and **4a** and gave **3c**; yield 1.3 g (26%) and **5c**; yield 0.8 g (16%).

Synthesis and separation of **3f** and **5f**. The mixture of **2f** and **4f** (3.27 g, 5 mmol) was treated analogously to **2a** and **4a** and gave **3f**; yield 0.8 g (17%) and **5f**; yield 1.4 g (30%).

Preparation of oligoribonucleotides **6-16**.

Oligomers **6-10** were prepared and purified according to the published procedures[22]. Oligoribonucleotides **11-16** were prepared according to the

protocol (Table 4) as described above. After oxidation the oligoribonucleotide, attached to the solid support, was washed with acetonitrile and acetone. The support was removed from the syringe, moistured with ethanol and kept with conc. aqueous ammonia (1 ml) for 5 hours at room temperature (18-20°C). Solution was decanted, solid phase washed with water/ethanol, 1:1 (1 ml) and ethanol (1 ml) and the solution was evaporated near to dryness. The residue was dissolved in the chromatographic eluent and 5'-O-DMT-oligoribonucleotides were separated by RP FPLC (Fig. 2a). The main fraction was collected, evaporated and the trityl group was removed by 80% acetic acid (200 µl) at room temperature for 20 min. Water (1 ml) and ethanol (1 ml) were then added and the solution was evaporated to dryness. The residue was dissolved in the chromatographic eluent and purified by RP FPLC (Fig. 2b).

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