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Selective 2'-benzoylation at the *cis* 2',3'-diols of protected ribonucleosides. New solid phase synthesis of RNA and DNA-RNA mixtures

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ABSTRACT

5'-O-(Dimethoxytrityl)-2'-O-(benzoyl or 3,4,5-trimethoxybenzoyl)-base protected ribonucleosides have been prepared by selective benzoylation of the 2'-hydroxyl group. The isomerization of the 2'-benzoates to the 3'-benzoates was studied. The protected ribonucleosides have been converted to either methylphosphochloridites or methylphosphoamidites and used to synthesize oligoribonucleotides on silica gel solid support. The synthetic RNA were deprotected and isolated using conditions that minimize internucleotide cleavage. The use of 2'-benzoates as protecting groups for ribonucleosides has made it possible to easily prepare and isolate mixtures of DNA and RNA.

INTRODUCTION

The problem to chemically differentiate between the two hydroxyl groups of the cis 2',3'-diols of ribonucleosides has been studied by many groups. Relative reactivity between the two hydroxyl groups has been investigated and the 2'-hydroxyl group was demonstrated in certain systems to have an enhanced reactivity over the neighboring 3'-hydroxyl group. Lohrmann and Orgel[1] prepared dimers with 2'— 5' and 3'— 5' linkages in a study which showed a 6 to 9-fold increased reactivity for the 2'-hydroxyl group over the 3'-hydroxyl group. Complete to nearly complete selectivity was reported in benzyl ether formation with benzyl-, 2-nitrobenzyl- and 4-methoxybenzyl bromides and pyridine oxide analogues.[2-10] A slightly lower selectivity occurred in silylation[11-15] and carbamoylation[16] reactions. Specific protection of the 2'-hydroxyl groups has been obtained through intermediates where the 5'- and 3'-hydroxyl groups were protected[17-22]; e.g., diesters and 5',3'-cyclic phosphates[22] were prepared which left only the 2'-hydroxyl group available for reaction. Other examples were the use of cyclic 5',3'-disiloxanes[23] as intermediates for the specific phosphorylation of the

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2'-hydroxyl group[24] and specific removal of the hydroxyl group to give 2'-deoxynucleosides.[25,26].

The purpose of this study was to find a base-labile protecting group that could be introduced at the 2'-hydroxyl group of a ribonucleoside with high selectivity and that could be removed together with the base protecting groups. These protected ribonucleosides are potential starting materials for an RNA synthesis that follows the same synthetic scheme presently employed in DNA chemistry, such as synthesis by the phosphite methods.[27-29] Early work in RNA chemistry focused on the acyl and benzoyl protecting groups.[4,30-37] These ester groups offered two advantages: (1) they were readily formed and (2) they were easily removed under mildly basic conditions. However, it was discovered that the 2'-isomer easily converted to the 3'-isomer which appeared to be more thermodynamically stable.[37] Attempts to separate such compounds, for example, the 5'-O-(dimethoxytrityl)-N^6-benzoyl-2'-O-(benzoyl)cytidine and the corresponding adenosine from the 3'-O-benzoyl isomer, were reported not to be possible.[31,32]

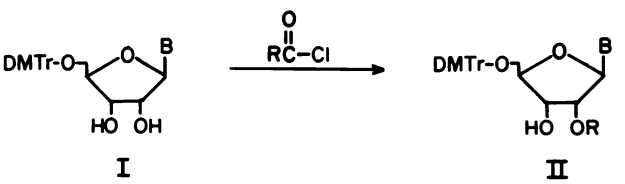
This paper shows that benzoylation of 5'-O-tritylated, base-protected ribonucleosides is very selective for the 2'-hydroxyl group even at room temperature and that the products are stable under proper conditions. Reaction conditions and work up procedures that prevent isomerization are described. The desired 2'-benzoates have been obtained in nearly quantitative yields; less than 1-4% of the 3'-isomers have been detected. These compounds have been fully explored as starting materials for oligoribonucleotide synthesis. In addition to a desired high degree of selectivity in a protection reaction, the 2'-benzoate was stable towards isomerization and hydrolysis during the phosphorylation of the 3'-hydroxyl group and towards the chemicals used in the solid phase synthesis.[27-29,38] The ester group was readily removed together with the base protecting groups giving a dimethoxytritylated oligomer which could be isolated and detritylated to give the fully deprotected RNA by HPLC purification; thus, the same purification steps used presently in DNA chemistry were followed. Moreover, any mixture of DNA and RNA could be made and purified under the same conditions. These mixed DNA-RNA molecules are used in new molecular biology applications, such as linkers, primers, probes, in site specific mutagenesis, and as a tool in RNA-cloning; the applications are presently under investigation and will be published elsewhere.

RESULTS AND DISCUSSION

Studies in the Benzoylation of the 2'-Hydroxyl Group of 5'-O-(Dimethoxytrityl)-uridine (DMTr-U), 5'-O-(Dimethoxytrityl)-N^6-benzoyladenosine (DMTr-A^{Bz}), 5'-O-(Dimethoxytrityl)-N^4-benzoylcytidine (DMTr-C^{Bz}) and 5'-O-(Dimethoxytrityl)-N^2-benzoylguanosine (DMTr-G^{Bz}, or the N^2-isobutyryl-guanosine, DMTr-G^{iBu}) (fig. 1)

Assignment of structure. In the preliminary experiments, DMTr-U was taken as the model compound for the study of benzoylation of ribonucleosides. Treatment of DMTr-U in pyridine with one equivalent of benzoyl chloride at room temperature gave a reaction mixture of three compounds, in addition to traces of unreacted starting material. TLC analysis on silica gel (toluene/ethyl acetate, 1:1, v/v) showed a major component (R_f = 0.38) and two minor components (R_f = 0.22, 0.50). When 2.1 equivalents of benzoyl chloride were added, one single compound (R_f = 0.50) was formed; it was assigned to be the 2',3'-dibenzoylated compound. Each reaction product was converted back to starting material when treated with aqueous ammonia in acetonitrile. The assignment of the major and minor components as benzoylated isomers of the 2'- and 3'-hydroxyl groups were shown by using them to synthesize dimers of 2'— 5' and 3'— 5' linkages.

The product mixture from reaction of 25 mMol of DMTr-U with 1 equivalent of benzoyl chloride was separated on preparative HPLC (Waters LC 500A) using 20% ethyl acetate/dichloromethane as eluent. A yield of 6.8 g of the major component and 0.9 g of the minor component (R_f = 0.22) in near purity was obtained. The products were precipitated from hexane and converted to methylphosphochloridites according to the method described for deoxynucleosides. These chlorophosphites were coupled to silica functionalized with rA^{Bz} on the automated gene machine NUCSYN (Analysteknik AB, Sweden). The



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B = U, A^{Bz}, G^{Bz}, G^{iBu}, C^{Bz}

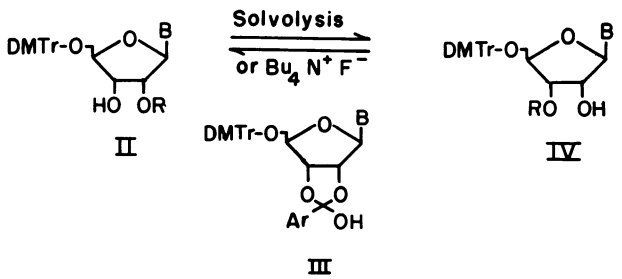
DMTr = 4,4- dimethoxytrityl

R = benzoyl or 3,4,5 trimethoxybenzoyl

Fig. 1.

unit file in Table 4 was used with the steps 3 and 5-11 edited out (rA-silica, 0.005 mMol/100 mg, 200 mg; chlorophosphite concentration: 0.2 mMol/mL in THF, 1 mL used, recycled 5 min). The products were deprotected and isolated according to the general scheme described in the experimental section. The fully deprotected dimers were compared on C-18 HPLC with authentic samples of rUpA (2'— 5') and rUpA (3'— 5') purchased from Sigma as reference compounds. The major component (R_f = 0.38) obtained in the benzoylation of DMTr-U produced the dimer corresponding to rUpA (3'— 5') and the minor component (R_f = 0.22), to the dimer rUpA (2'— 5'). The structural assignment of the product mixture was verified by these experiments; the major product in benzoylation of DMTr-U was the 2'-benzoate (DMTr-U-2'-Bz) and it could be purified to a high degree, contaminated by only traces of the 3'-benzoate (DMTr-U-3'-Bz). Moreover, both benzoylated isomers were compatible with the reactions conditions in solid phase synthesis.

Selectivity in benzoylation and isomerization of 2'- and 3'-benzoates (fig. 1 and 2). Benzoylation of DMTr-U in pyridine at room temperature with one equivalent of benzoyl chloride gave 2'/3' benzoates in the ratio of 95:5 as determined by HPLC analysis. When the reaction was done at -45°C in pyridine and at -78°C in dichloromethane/pyridine, an enhancement in selectivity was observed; a 99:1 ratio of 2'/3' benzoates was obtained. The remaining ribonucleosides (DMTr-A^{Bz}, DMTr-G^{Bz} and DMTr-C^{Bz}) were benzoylated



B = U, A^{Bz}, G^{Bz}, G^{iBu}, C^{Bz}
DMTr = 4,4 - dimethoxytrityl
R = benzoyl or 3,4,5 - trimethoxybenzoyl
Ar = Ph or 3,4,5 – trimethoxyphenyl

Fig. 2.

at the lower temperatures. Treatment of the tritylated nucleosides with the less reactive 3,4,5-trimethoxybenzoyl chloride produced a product mixture similar to that from the parent compound. It may be of interest to note that the reaction of DMTr-U with 4-nitrobenzoyl chloride and isobutyryl chloride in pyridine at -45°C gave a mixture of 2'- and 3'-isomers with a much lower selectivity for the 2'-isomer. Purification on silica gel of the 2'-benzoates and the 2'-trimethoxybenzoates from the unreacted starting material and the 3'-isomers resulted in nearly pure 2'-isomers; it appeared that a conversion to the 3'-isomers was occurring during the chromatography. Washing the silica gel with 10% pyridine/dichloromethane, followed by a wash of dichlromethane before separation did not preclude the isomerization. A loss of product from isomerization was also reflected in the low recovery. A higher yield was obtained form the 2'-trimethoxybenzoates, presumably due to less isomerization. Yields and isomeric purity are given in Table 1.

Under the same reaction conditions but with 1.1 equivalents of benzoyl chloride, the 2'-isomeric purity was 99% for DMTr-U and greater than 96% for the other ribonucleosides. The 10% excess reagent served to drive the reaction to completion; unreacted starting material was not observed. The product profile was essentially clean (traces of the 2',3'-dibenzoates) after work up of the crude benzoylation mixture. The increased yield of the 2'-benzoate relative to the 3'-benzoate in excess benzoyl chloride indicated that the 3'-benzoate was more readily converted to the 2',3'-dibenzoate. There appeared, however, a clear difference in reactivities towards dibenzoylation for the 3,4,5-trimethoxybenzoyl chloride. Formation of the dibenzoate from the 2'-monobenzoate was very slow, presumably due to bulkiness and lower reactivity of the protecting group. Therefore, treatment with 10% excess benzoyl chloride appeared to be the method of choice for selective 2'-benzoylation (Table 2 for 2'/3' isomeric ratio). All of the 2'-benzoylated (or 2'-trimethoxybenzoylated) ribonucleosides used in syntheses were hence not subjected to silica gel chromatography; minimal amounts of the 2',3'-dibenzoate were carried over into oligonucleotide syntheses and did not appear to interfere with any of the subsequent reactions.

The isomerization of the 2'-benzoates which had occurred during purification by column chromatography and preparative HPLC showed that careful work up and handling of these compounds was a necessity. The degree to which DMTr-U-2'-Bz isomerized was studied in various solvents at room temperature and at -20°C for a period of 24 hours. The resultant mixtures

Table 1. Separation of DMTr-ribonucleoside, 2'- and 3'- benzoates and 2'- and 3'-trimethoxybenzoates, on silica and reversed phase HPLC.

Ribonucleoside[a]	Yields[b] (isomeric ratio of 2'/3' isomers)	$R_{t(2')}$, $R_{t(3')}$ (min); solvent system[c,d]	$R_{f(2')}$, $R_{f(3')}$; solvent system[e]
DMTr-U-2'-Bz	46% (96:4)	5.0, 6.5; B	0.38, 0.22; G
DMTr-U-2'-Ar	44% (97:3)	10.5, 13.8; A	0.24, 0.12; G
DMTr-A^{Bz}-2'-Bz	55% (98:2)	8.5, 11.0; B	0.25, 0.12; G
DMTr-A^{Bz}-2'-Ar	60% (95:5)	13.5, 17.8; C	0.11, 0.04; G
DMTr-G^{Bz}-2'-Bz	24% (96:4)	10.0, 13.0; A	0.50, 0.27; F
DMTr-G^{Bz}-2'-Ar	49% (96:4)	12.3, 17.5; C	0.38, 0.17; F
DMTr-C^{Bz}-2'-Bz	29% (97:3)	13.8, 18.5; B	0.28, 0.10; G
DMTr-C^{Bz}-2'-Ar	47% (98:2)	15.3, 19.3; D	0.14, 0.06; G

a. Bz = benzoate, reaction conditions: ribonucleoside (20 mMol) in pyridine (100mL) treated with benzoyl chloride (20 mMol) at -40°C; Ar = 3,4,5-trimethoxybenzoate, reaction conditions: ribonucleoside (10 mMol) in dichloromethane (100 mL) and pyridine (7 mL) treated with 3,4,5-trimethoxybenzoyl chloride (10 mMol) at -78°C.

b. The mixtures were separated on preparative HPLC (Waters) in 20:80 EtOAc/CH_2Cl_2, v/v.

c. Analysis on analytical HPLC, Excalibar Spherisorb ODS, 5μ at a flow of 1 mL/min in either solvent A: 80:20 MeOH/H_2O or solvent B: 81:19 MeOH/H_2O.

d. Analysis on analytical HPLC, Altex Ultrasphere ODS, 5μ at a flow of 1 mL/min in either solvent A, solvent C: 82:18 MeOH/H_2O or solvent D: 85:15 MeOH/H_2O.

e. The samples and fractions were analyzed by TLC (silica gel plates, Merck) in either solvent F: 70:30 EtOAc/CH_2Cl_2 or solvent G: 50:50 toluene/EtOAc.

Table 2. Conversion of DMTr-ribonucleoside-2'-Bz compounds to amidites and reaction with functionalized silica to give DMTr-(3'-5') dimers and fully deprotected (3'-5') dimers.

Silica-rN	Ribonucleosides (isomeric ratio of 2'/3')	Dimers (isomeric ratio of 3'-5'/2'-5')[a]	Retention times in minutes DMTr-dimer[b]	fully deprotected dimer[c] (3'-5')	(2'-5')
rA	DMTr- U-2'-Bz (99:1)	rUpA (99:1)	32	24[d]	18[d]
rA	DMTr- U-3'-Bz (4:96)	rUpA (5:95)	41	24[d]	18[d]
rU	DMTr- A^{Bz}-2'-Bz (98:2)	rApU (98:2)	28	18	12
rC	DMTr- G^{Bz}-2'-Bz (96:4)	rGpC (97:3)	29	16	10
rC	DMTr- G^{iBu}-2'-Bz (97:3)	rGpC (98:2)	29	16	10
rG	DMTr- C^{Bz}-2'-Bz (99:1)	rCpG (99:1)	27	6	3

a. Ratio obtained after full deprotection of crude reaction mixture.

b. Analysis on Altex Ultrasphere ODS, 5μ semiprep column with a gradient of 25-45% acetonitrile/0.1 M triethylammonium acetate over 60 min.

c. Analysis on Excalibar Spherisorb ODS, 5μ analytical column with a gradient of 3-5% acetonitrile/0.1 M triethylammonium acetate over 30 min.

d. A gradient of 4-6% acetonitrile/0.1 M triethylammonium acetate over 30 min.

of DMTr-U-2'-Bz and DMTr-U-3'-Bz were analyzed on C-18 HPLC using 81% methanol/water as eluent. A summary of the results is given in Table 3. At room temperature the 2'-benzoate isomerized readily to the 3'-isomer in most solvents except for dichloromethane. At the lower temperature, significant isomerization was observed only in methanol. The corresponding 2'-trimethoxybenzoate was stable towards isomerization in dichloromethane and tetrahydrofuran at room temperature after 24 hours. However, a 26:74 ratio of 2'/3' trimethoxybenzoates was found in methanol, a result which was similar to that observed for the corresponding 2'/3' benzoates. A comparable mixture was also obtained upon treatment of the 2'-benzoate with tetrabutylammonium fluoride in acetonitrile. DMTr-U-2'-Bz (0.1 mMol) in 2 mL of acetonitrile isomerized rapidly to a 29:71 ratio of 2'/3' isomers when treated with 0.5 or 1 equivalent of tetrabutylammonium fluoride at room temperature; the mixture was obtained within 10 minutes and remained constant during a 1-hour period. Treatment of DMTr-U-2'-Bz with triethylamine in dichloromethane resulted in a new peak (R_t = 8.5 min) in HPLC analysis (C-18, 80% methanol/water). The starting material DMTr-U-2'-Bz (R_t = 7 min) and the 3'-benzoylated isomer (R_t = 10 min) were not present. The new peak (R_t = 8.5 min) was assumed to be the intermediate III (fig 2)[39] in the isomerization of 2'- and 3'-benzoates and that idea is presently under study. The isomerization of 2'-derivatives to the 3'-derivatives has been reported by other groups. Reese *et al.*[37] observed isomerization of the 2'-acetate to the 3'-acetate in methanol and Ogilvie *et al.*[40] reported that silyl ethers showed the same tendency.

Synthesis of Ribonucleotide Dimers (3'→5')

The ribonucleosides (DMTr-U-2'-Bz, DMTr-A^{Bz}-2'-Bz, DMTr-C^{Bz}-2'-Bz, DMTr-G^{iBu}-2'-Bz and DMTr-G^{Bz}-2'-Bz) which contained not more than 1-4% 3'-benzoyl isomers were phosphorylated with methoxydichlorophosphine (fig. 3) and coupled to U-, rA^{Bz}-, rC^{Bz}- and rG^{Bz}-silica; results are shown in Table 2. The dimers formed were deprotected at the phosphotriester with thiophenoxide[41]

Table 3. Isomerization of DMTr-U-2'-(benzoate or 3,4,5-trimethoxybenzoate) at room temperature and at -20°C for 24 hours.

Solvent	Ratio of 2'/3' benzoates 20°C	-20°C	Ratio of 2'/3' trimethoxybenzoates at 20°C
methanol	30:70	56:44	26:74
acetonitrile	69:31	96:4	94:6
tetrahydrofuran	73:27	94:6	98:2
dichloromethane	98:2	98:2	98:2

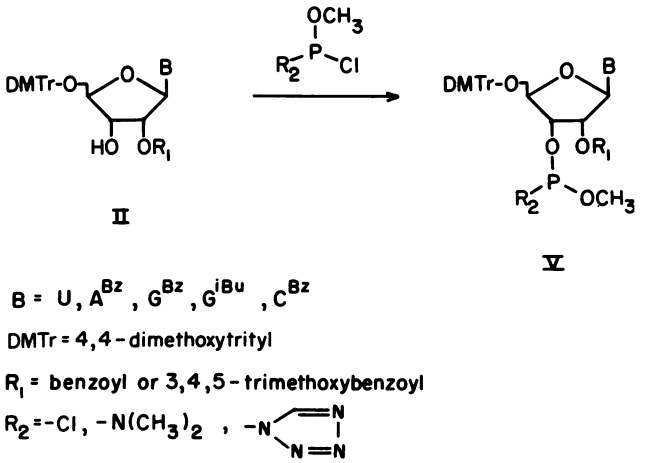


Fig. 3.

for 1.5 hours and then removed from the support by treatment with concentrated ammonia for 1 hour at room temperature. The ammonia solution was then heated at 50°C for 4 hours to complete the deprotection of the nucleotide bases. To determine whether the dimers were isomerically stable under detritylation and isolation procedures, the ammonia solution from each reaction was divided into two samples to be treated differently. One sample was analyzed as the dimethoxytritylated product on C-18 HPLC. One major peak was observed; isolation and detritylation of that peak gave the fully deprotected dimer which was again analyzed by HPLC[42]. It corresponded to the 3'— 5' dimer and not the 2'— 5' dimer, authentic samples of which were obtained from Sigma. The chromatographic systems and retention times are given in Table 2. All dimers made on the solid support and isolated in the 2-step HPLC procedure were isomerically pure 3'— 5' dimers which showed no detectable amounts of the wrong isomers. The other half of the samples were directly treated with 80% acetic acid-water without isolation at the tritylated stage. Analyses of these samples showed essentially pure 3'— 5' dimers; traces of the 2'— 5' dimers which corresponded to the 3'-benzoate contaminants were detected. The 3'-benzoate of DMTr-U was similarly treated and pure rUpA (2'— 5') was produced. Isomerically pure dimers were also obtained by use of the phosphoamidites of the benzoylated ribonucleosides.

The results from the above experiments showed that pure 2'-O-benzoyl isomers were isomerically stable under the reaction conditions of phosphor-

ylation with either methoxydichlorophosphine or chloro-N,N-dimethylamino-methoxyphosphine, the coupling by solid phase, and the procedures for deprotection and isolation. However, isomerization of the 3'— 5' dimers to the 2'— 5' dimers was observed when deprotection of the methyl phosphotriester with thiophenoxide was incomplete. Exclusion of thiophenoxide treatment and only ammonolysis gave 12% of the 2'— 5' isomer of rApU which under proper deprotection yielded exclusively the 3'— 5' isomer. The isomerization probably proceeded through a cyclic phosphate, which upon hydrolysis or ammonolysis could decompose to compounds of either 2'— 5' or 3'— 5' linkage.[43,44]

Synthesis of Oligoribonucleotides on Silica as Solid Support

Oligoribonucleotides (2'-benzoylated) were synthesized by solid phase chemistry in the same manner as the syntheses of the dimers; the phosphoamidites V (fig. 3) were used (see unit file, table 4). Synthesis of oligoribonucleotides on silica solid support have also been reported by Ogilvie et al.[45] using methylchlorophosphite reagents[46] of the 2'-silylated ribonucleosides.[11-15]

The three RNA which were prepared are listed in Table 6. Each nucleotide coupling proceeded in 85-95% yield as measured by the dimethoxytrityl cation absorption at 470nm in a flow-thru visible UV-cell. After the solid phase synthesis was complete the oligoribonucleotide attached to the support was treated with thiophenoxide for 1.5 hours and concentrated ammonia for 1 hour as previously described for deprotection and recovery of the dimers. The aqueous ammonia solution was separated from the support by filtration and the sample was evaporated to dryness.

Since prolonged treatment of RNA with ammonia resulted in internucleotide cleavage, reaction conditions were sought in which decomposition was minimized under conditions for removal of benzoyl and isobutyryl protecting groups from the purine and pyrimidine bases. The compound for the study was rA_{10} which was commercially available (Boerhinger-Mannheim). As seen in Table 5, ammonolysis completely cleaved the model compound rA_{10} within a few hours at 50°C. A drastically reduced internucleotide cleavage was obtained in a mixture of n-butylamine-methanol-dioxane (1:1:2, v/v) which removed N-benzoyl groups within 3-7 hours at 40°C. Those RNA which were synthesized and separated from the silica support were thus treated with the milder butylamine reagent for deprotection of the bases. Oligomers containing G^{Bz} were treated with the butylamine reagent for 7 hours and the ones with G^{iBu} for 5 hours. After the reaction was completed, the mixture

Table 4. A unit file, steps in automated synthesis on 200 mg of functionalized silica.

Steps	Minutes	Valves (Reagents, 2 mL/min)
1	10	DT[a]
2	5	THF
3	.2	MIX[b]
4	.2	U[c]
5	.1	MIX
6	.1	U
7	.1	MIX
8	.1	U
9	.1	MIX
10	.2	U
11	.2	MIX
12	5	RE[d]
13	4	THF
14	.2	RE
15	3	OX[e]
16	3	THF
17	2	CAP[f]
18	3.5	RE
19	3.5	THF
20	.2	RE

a. detritylating reagent: 70 g $ZnBr_2$ in 500 mL CH_3NO_2 and 1% H_2O.

b. activation reagent for phosphoamidite: 2.1 g 1H-tetrazole in 120 mL CH_3CN.

c. nucleotide: 1 mMol in 20 mL CH_3CN

d. recycling valve: is closed when extensive reaction time is required and when void volume has to be washed out.

e. oxidizing reagent: 1 g I_2 in 250 mL THF, 120 mL H_2O and 25 mL 2,6-lutidine

f. capping reagent: Solution A:B, 1:1, v/v; solution A: 100 mL sym-collidine, 80 mL Ac_2O and 400 mL THF; solution B: 25 g N-dimethylaminopyridine in 500 mL THF.

was evaporated and the residue taken up in water. The resultant dimethoxytritylated oligoribonucleotides were purified on HPLC with acetonitrile in 0.1 M triethylammonium acetate. The isolated, tritylated compounds were treated with 80% acetic acid-water for 10 minutes, the acid removed

Table 5. Studies on the deprotection of purines and internucleotide cleavage.

Base protected ribonucleoside	Treatment with conc. NH_3 at 50°C		Treatment with $nBuNH_2$-MeOH-dioxane (1:1:2, v/v) at 40°C	
	reaction time[a]	decomposition[b] of rA_{10}	reaction time[a,c]	decomposition[b,d] of rA_{10}
rA^{Bz}	2 h	93%	3 h	7%
rG^{iBu}	4 h	98%	5 h	12%
rG^{Bz}	5 h	100%	7 h	17%

a. >95% deprotection; extent of deprotection monitored by analytical TLC, silica gel, 20% MeOH/CH_2Cl_2

b. Decomposition from internucleotide cleavage determined by analysis on reversed phase HPLC.

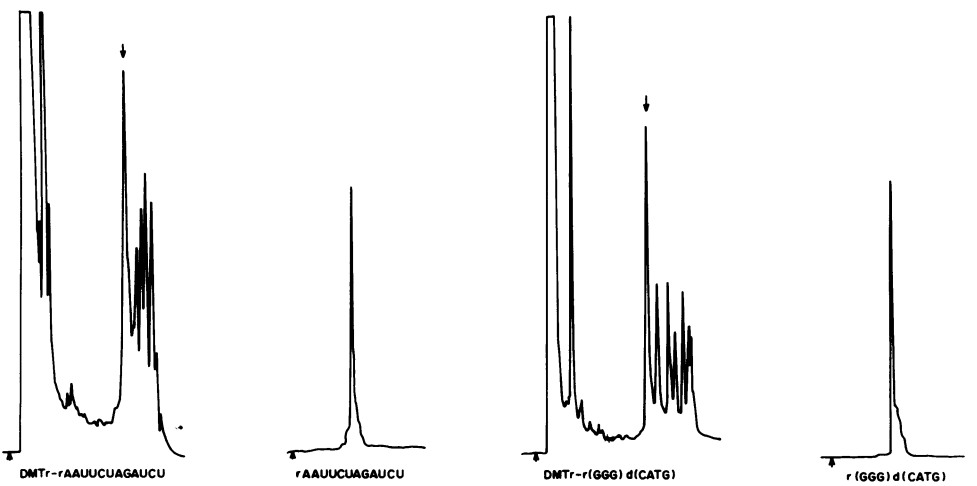
c. The same result was obtained in a study of the DMTr-ribonucleosides (analysis on TLC, silica gel, 10% MeOH/CH_2Cl_2).

d. The amount of decomposition corresponding to the length of reaction time was obtained from a linear plot of residual rA_{10} vs. time.

by evaporation under reduced pressure and the residue taken up in water for purification by HPLC as the fully deprotected oligoribonucleotide (see Table 6 and fig. 4). The synthetic RNA were converted to the 5'-[^{32}P]-labelled compounds and treated with snake venom phosphodiesterase. The partially digested mixtures were characterized by the methods of 2-dimensional electrophoresis and homochromatography.[47-49] Two of the oligoribonucleotides that were prepared, rU_6 and rA_{10}, could be compared with authentic samples (Boerhinger-Mannheim); they proved to be identical under analyses by HPLC and homochromatography.

Mixed DNA-RNA Synthesis

Oligomers containing both DNA and RNA have been previously synthesized by Gilham et al.[50,51] using solution chemistry with phosphotriester methods. The same group has also recently published a solid phase method on glass support, functionalized with a uridine derivative, giving a DNA molecule with a 3'-ribonucleoside.[52] The introduction of the 2'-benzoate as a protecting group for ribonucleotides in solid phase synthesis allowed the synthesis of any combination of DNA-RNA possible. The mixed DNA-RNA were synthesized using the unit file in Table 4. The 5'-O-(dimethoxytrityl)-deoxynucleoside-3'-(N,N-dimethylaminomethoxy)phosphine were prepared in the same manner as



HPLC profiles of an RNA and a mixed DNA-RNA

Fig. 4.

Table 6. HPLC separation and yields of oligonucleotides of RNA and mixed DNA-RNA.

Oligonucleotides	Gradient over 30 min of CH_3CN (%)/0.1 M $Et_3NH^+OAc^-$	R_t (min)	O.D.$_{260}$ (% yield)[c]
DMTr-rU_6	25-35%, a	21	70 (6.3%)
rU_6	10-20%, b	7	45 (4.0%)
DMTr-rA_{10}	20-30%, a	27	55 (3.7%)
rA_{10}	8-18%, b	25	36 (2.4%)
DMTr-r(AAUUCUAGAUCU)	20-30%, b	17	65 (2.0%)
r(AAUUCUAGAUCU)	10-20%, b	12	34 (1.0%)
DMTr-d(GAT)r(CCC)	25-35%, a	15	88 (5.9%)
d(GAT)r(CCC)	8-18%, a	13	50 (3.4%)
DMTr-r(GGG)d(CATG)	20-30%, a	24	71 (5.4%)
r(GGG)d(CATG)	10-20%, b	16	45 (3.4%)
DMTr-d(CCGAATTC)rA	20-30%, b	22	120 (12.6%)
d(CCGAATTC)rA	10-20%, b	18	88 (9.2%)
DMTr-r(UUU)d(CCGAATTC)rA	20-30%, b	18	115 (9.7%)
r(UUU)d(CCGAATTC)rA	10-20%, b	15	85 (7.2%)
DMTr-d(GAAA)r(UU)dGr(UU)dArUd(CC)	20-30%, a	23	75 (3.6%)
d(GAAA)r(UU)dGr(UU)dArUd(CC)	10-20%, a	22	51 (2.4%)

a. Altex Ultrasphere ODS, 5μ semiprep column, flow = 3 mL/min.

b. Altex Ultrasphere ODS, 5μ analytical column, flow = 1 mL/min.

c. Isolated from 200 mg of functionalized silica.

the amidites of ribonucleosides. A list of the mixed DNA-RNA prepared in this manner is given in Table 6 along with the HPLC isolation conditions. The compounds d(GAT)r(CCC) and r(GGG)d(CATG) were also prepared from the 2'-trimethoxybenzoylated ribonucleosides (fig. 3). Analysis by 2-dimensional electrophoresis and homochromatography[47,53,54] was also performed.

The use of these mixed DNA-RNA in molecular biology is currently under investigation and will be published at a later date. The DNA-RNA serve as useful probes in hybridization and priming reactions where the stabilization may be enhanced or the destabilization be reduced by the use of DNA-RNA hybrids, e.g., in site specific mutagenesis. The mixed DNA-RNA with a 3'-terminal ribonucleoside can also be a useful intermediate in building single-stranded DNA-RNA molecules using RNA ligase[55-57] which does not require a template for ligation. Studies in collaboration with R. I. Gumport (University of Illinois) to use these substrates to prepare long single-stranded oligomers are currently in progress.

EXPERIMENTAL SECTION

Materials

All chemicals were commercially available and used without further purification, except for the following: tetrahydrofuran, distilled under N_2 from sodium/benzophenone; acetonitrile, distilled under N_2 from CaH_2; toluene (ACS grade), pyridine (ACS grade), sym-collidine and 2,6-lutidine, dried over 4A molecular sieves (Union Carbide). Nucleosides were purchased from the following sources: uridine, guanosine, adenosine and cytidine (Sigma); deoxycytidine and deoxyguanosine (P-L Biochemicals), deoxyadenosine and thymidine (Calbiochem). Porasil C silica (80-100μ, Waters) was used as the isoluble solid support.

Materials for purification and analysis: pre-coated analytical TLC plates, silica gel 60 F-254 (Merck); 7.5:1 cellulose-DEAE plates (Analtech); column chromatography on silica gel 60, 230-400 mesh ASTM (Merck); preparative LC on PrePak-500/silica (Waters); reversed phase chromatography on Altex Ultrasphere ODS, 5μ semiprep and analytical column (Beckman) and Excalibar Spherisorb ODS, 5μ analytical column (Applied Science Division).

General Procedure for the Selective Benzoylation of the 2'-Hydroxyl Group of DMTr-ribonucleosides

The 5'-O-(dimethoxytrityl) base-protected ribonucleoside (U, rA^{Bz}, rG^{Bz}, rG^{iBu} or rC^{Bz}, 10 mMol) was coevaporated with dry pyridine (3 x 100 mL) in a 250-mL round bottom flask. In the third coevaporation, the volume was

reduced to 50 mL. The round bottom flask was equipped with a rubber septum and magnetic stir bar and cooled to -45°C with a dry ice-acetone-water bath. A solution of benzoyl chloride (11 mMol, 1.3 mL) in dichloromethane (4 mL) was slowly added to the stirred mixture. A 20% excess of benzoyl chloride (12 mMol, 1.4 mL) was used in the benzoylation of DMTr-rG^{Bz} and DMTr-rG^{iBu}. After 30 min the reaction mixture was warmed to -20°C and allowed to react at that temperature for 2 h. The mixture was analyzed on TLC using 10% MeOH/CH_2Cl_2; the TLC was usually eluted first with Et_2O to remove pyridine. In general, the starting material was consumed after this reaction period. The mixture was quenched with water (0.5 mL) and then concentrated at reduced pressure at 30°C to a gum. The gum was taken up in dichloromethane (250 mL) and washed with water (2 x 100 mL). The organic phase was dried over sodium sulfate and concentrated. The residue was dissolved in dichloromethane (30 mL) and the product was obtained by precipitation from hexane (1 L). The precipitate was filtered and dried in a dessicator over phosphorous pentoxide. This work up procedure minimized isomerization of the 2'-benzoate. Traces of the 3'-benzoate were detected by analysis on analytical C-18 HPLC (see Table 2).

The corresponding 2'-trimethoxybenzoate was obtained following this procedure using 1.1 eq. of 3,4,5-trimethoxybenzoyl chloride in dichloromethane with 5 eq. of pyridine at -78°C. The reaction was quenched with methanol at -78°C.

General Procedure for the Functionalization of Silica

Porasil C (88 g) and triethoxysilylpropylamine (65 mL) in toluene (300 mL) were refluxed for 7 h in a 1-L round bottom flask. The silica was then filtered and washed with pyridine (3 x 50 mL) and transferred to a 500-mL round bottom flask containing pyridine (100 mL). Trimethylsilylchloride (60 mL) was added and the mixture was shaken at RT for 1 h. The silica was filtered and washed with the following: pyridine (2 x 100 mL), water (100 mL), pyridine (2 x 100 mL), pyridine-triethylamine (9:1, 50 mL), pyridine (2 x 100 mL) and ether (3 x 100 mL). The silica was dried in a dessicator at reduced pressure. The aminated silica (10 g) was functionalized with either succinylated deoxyribonucleosides[29] or succinylated 2'-benzoylated ribonucleosides using the following procedure. The succinylated nucleoside (4 mMol) was dissolved in DMF (10 mL) with N-hydroxybenztriazole (10 mMol, 1.4 g) and treated with dicyclohexylcarbodiimide (20 mMol, 4.1 g) in DMF (10 mL). (DMF was distilled under N_2 from CaH_2 prior to use.) The dicyclohexylurea formed during the active ester formation precipitated

out of solution. The product mixture was centrifuged in a sealed serum bottle. The supernatant was removed and was added with a syringe to the silica (10 g) in pyridine (14 mL). The mixture was shaken at RT for 3 h. The silica was filtered, washed with pyridine (3 x 50 mL) and then treated with the capping solution used in the solid phase synthesis (see Table 4). A mixture of solutions A and B, 1:1, v/v (20 mL) was added to the silica in a sintered glass funnel and was allowed to react for 10 min at RT with gentle agitation. The reagent was filtered off and the silica was washed with pyridine (3 x 50 mL) and ether (3 x 50 mL). After drying in a dessicator under reduced pressure, the silica was ready for use in solid phase synthesis (see unit file, Table 4). The functionalization was in the range of 0.005-0.008 mMol/100 mg silica.

General Procedure for the Preparation of 5'-O-(Dimethoxytrityl)-2'-O-(benzoyl or 3,4,5-trimethoxybenzoyl)-(U, rA^{Bz}, rG^{Bz}, rG^{iBu} or rC^{Bz})-3'-(N,N-dimethylaminomethoxy)phosphine

An oven-dried 50-mL flask (e.g., a serum bottle, Wheaton) containing a magnetic stir bar was flushed with dry argon and sealed with a teflon/silicon septum. Dry THF (20 mL) was added through a syringe and the solution was cooled to -78°C with a dry ice-acetone bath. To the stirred solution was added chloro-N,N-dimethylaminomethoxyphosphine (3 mMol, 0.35 mL) and triethylamine (3.6 mMol, 0.48 mL). A solution of the protected ribonucleoside (3 mMol) dissolved in THF (10 mL) was added to the cooled mixture of phosphorylating agent. Triethylammonium hydrochloride immediately formed and precipitated out of solution. After 30 min the reaction mixture was removed from the cold-temperature bath and the hydrochloride was sedimented by centrifugation. The supernatant was transferred to a rubber septum-equipped round bottom flask containing dry toluene (100 mL). The product mixture was concentrated at reduced pressure at 30°C. To the dry residue (nucleoside phosphoamidite) was added another portion of toluene (3 x 100 mL) and the evaporation was repeated to remove excess triethylamine. The residue was dissolved in dichloromethane (6 mL) and precipitated from hexane (80 mL) cooled to -20°C in a sealed serum bottle. The precipitate was sedimented by centrifugation and the solvent removed. The precipitate was dried at reduced pressure in a dessicator over phosphorous pentoxide. The amidite was dissolved in acetonitrile at a concentration of 1 mMol/20 mL.

Isolation of RNA and Mixed DNA-RNA from the Solid Silica Support

The final step in the solid phase synthesis was a THF wash of the silica support in the reaction column (HPLC precolumn with 1/16-inch end fittings,

I.D. 3.2 mm, O.D. 1/4-inch, length 50 mm). The silica was emptied from the column into a 7-mL vial and dried in a dessicator at reduced pressure. The support containing the synthetic oligonucleotide was treated with thiophenol-triethylamine-dioxane (1:2:2, v/v, 1 mL) for 1.5 h at RT. The reagent was removed from the support by filtration through a pasteur pipet with a silylated glass wool plug. The support was washed with dioxane (4 x 2 mL) and water (2 x 2 mL). After the washes, the support was transferred to a second vial and treated with concentrated aqueous ammonia (3 mL) for 1 h at RT to cleave the oligonucleotide from the support. The aqueous phase was transferred to a polypropylene tube and concentrated at reduced pressure in a Speed-Vac concentrator (Savant). (When pure DNA was deprotected, the ammonia solution was transferred to another vial and heated for 5 h at 50°C.) To the dried sample was added $BuNH_2$-MeOH-dioxane (1:1:2, v/v, 1 mL) and the sealed tube was heated at 40°C for 7 h. The deprotecting reagent was removed by concentration at reduced pressure. The DMTr-oligomer thus obtained was dissolved in water (0.5 mL) and separated by analytical or semipreparative C-18 HPLC (see Tables 6 and 7). The isolated sample was concentrated and detritylated with 80% acetic acid-water (0.5 mL) for 10 min at RT. The solution was concentrated at reduced pressure and the residue taken up in water (0.3 mL). The fully deprotected oligomer was purified by C-18 HPLC. The purified sample was evaporated to dryness and stored at -20°C.

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