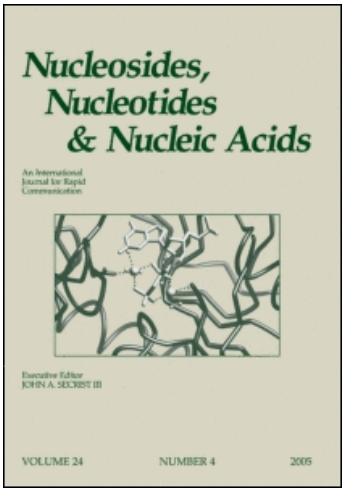

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THE CHEMICAL SYNTHESIS OF BIOCHEMICALLY ACTIVE OLIGORIBONUCLEOTIDES USING DIMETHYLAMINOMETHYLENE PROTECTED PURINE H-PHOSPHONATES

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Abstract: Dimethylaminomethylene was applied as the protecting group for the exocyclic amino groups of adenosine and guanosine in the automated chemical synthesis of oligoribonucleotides on a polymer bound support. The dimethylaminomethylene protecting group can be removed at room temperature under conditions where the concomitant loss of the 2'-protection group can be excluded. The transformation of 2'-O-(t-butyldimethylsilyl)-5'-O-(4,4'-dimethoxytrityl) protected nucleosides to 3'-H-phosphonates yields synthons, well suited for the automated chemical synthesis of oligoribonucleotides. Using these H-phosphonate monomers, a coupling time of two minutes is sufficient to obtain average coupling yields of more than 98 %. Synthesized RNA is recognized as a substrate in an enzymatic reaction, forms the expected secondary structures and is suitable for NMR structural investigations.

Introduction

Chemically synthesized oligoribonucleotides are required for the solution of many problems in RNA biochemistry and in RNA structure analysis. Synthetic constructs of RNA may be used to produce milligram quantities of proteins *in vitro*

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without the presence of microorganisms[1]. Symons and coworkers[2] have discovered short RNA sequences with self-cleaving abilities. These "hammerheads" act as sequence specific nucleases and could be handled as enzymes for the cleavage of specific RNA sequences. Another field where chemically synthesized oligoribonucleotides attract attention is the structural study of RNA by NMR[3,4] or X-ray[5] analysis. An alternative approach for the synthesis of RNAs of defined length and sequence is the use of T7 RNA polymerase and synthetic DNA templates[6]; however, to obtain the high quantities required for structural studies, or to insert modified nucleotides into the polymer chain, the chemical synthesis on a polymer bound support is the method of choice.

The automated synthesis of oligoribonucleotides was successfully developed on the basis of phosphoramidite chemistry by Ogilvie and coworkers[7,8,9], where average coupling efficiencies from 96 to 98 % were achieved with coupling times from five to fifteen minutes. Alternative to the use of the 4,4'-dimethoxytrityl (DMT) and the tert.-butyldimethylsilyl (tBDMS) protecting groups for the 5'- and 2'-position of the ribose, a set of different protecting groups was developed[10,11,12]. In an extensive study about the choice of protecting groups and deprotection conditions in automated RNA synthesis, Stawinski et al.[13] reported that the most critical step during the deprotection procedure is the use of elevated temperature (55°C) in combination with an ammonia/ethanol solution, which is necessary to remove the N^2-isobutyryl residue from the guanine base. Under these conditions 4 - 10 % of the tert.-butyldimethylsilyl group[14,8] are lost, leading to the cleavage of the RNA. This problem can be avoided by introduction of the phenoxyacetyl[15] or dimethylaminomethylene[16,17] protecting groups for adenine and guanine. In comparison to the overall yields for the preparation of the phenoxyacetyl derivatives, shown by Wu et al.[18], N^2-dimethylaminomethylene-2'-deoxyguanosine and N^6-dimethylaminomethylene-2'-deoxyadenosine were prepared in almost quantitative yields[19]. In addition we found that the presence of the dimethylaminomethylene group facilitates the difficult 2'-O-silylation of the N-acyl protected guanosine.

It is difficult to recover and reuse the synthons in the phosphoramidite approach, a fact that might be a great disadvantage using modified or isotopically labeled nucleotides. For this reason we decided to apply the H-phosphonate chemistry[13,20,21] in combination with the dimethylaminomethylene base protection of guanine and adenine for the synthesis of oligoribonucleotides, and we report here about the synthesis of RNA molecules with an average coupling efficiency of 98 %.

Materials and Methods

Nucleosides were purchased from Fluka, dichloromethane (absolute), pyridine (absolute), acetonitrile (HPLC grade), N,N-dimethylformamide diethylacetal, 4,4'-dimethoxytritylchloride, t-butyldimethylsilylchloride, triethylamine (puriss.) and imidazole (puriss.) came from Fluka and were used without further purification. Phosphorus trichloride (2 M solution in dichloromethane) was from Aldrich. CPG-supports with spacer were prepared as described[22] from 5'-O-(4,4'-dimethoxytrityl)-N-substituted ribonucleosides (25 - 40 μmoles/g). The 2'-O-(t-butyldimethylsilyl)-5'-O-(4,4'-dimethoxytrityl)-uridine-3'-H-phosphonate and the 2'-O-(t-butyldimethylsilyl)-5'-O-(4,4'-dimethoxytrityl)-N^4-benzoylcytidine-3'-H-phosphonate were synthesized according to known procedures[13]. Column chromatography was performed on Kieselgel 60 (Fluka). Thin-layer chromatography (TLC) was carried out on DC-Alufolien Kieselgel (Fluka). The 4,4'-dimethoxytrityl containing compounds were identified by exposure of the thin-layer plates to HCl vapors. The UV spectra were recorded on a Shimadzu UV-160 UV/VIS spectrometer. Scanning of the gels was done with an Ultroscan XL laser densitometer from LKB. Nuclease P1 was from Boehringer, alkaline phosphatase from *E.coli* came from Sigma, HPLC analysis was performed on a Beckman System Gold 126 solvent module using the model 167 multiwavelength UV-detector. ^{1}H-nuclear magnetic resonance spectra were recorded on a Bruker AM 500 spectrometer; the signals were referenced to an internal $CDCl_3$ signal (7.24 ppm) in the spectra of the synthons and to the methyl resonance of internal 3-(trimethylsilyl)-1-propanesulfonic acid in the case of the synthesized oligoribonucleotides. All NMR measurements were done at 277 K and the concentration of the duplexes ranged from 0.4 to 1.4 mM.

Preparation of the H-phosphonates (Ib, IIb)

The compounds were prepared by a slightly modified method of Froehler et al.[23]. A solution of imidazole (30 mmoles) in dichloromethane (75 ml) was cooled under stirring on ice and a 2 M solution of phosphorus trichloride in dichloromethane (4.32 ml) was added with a syringe, followed by the addition of triethylamine (4.2 ml). The mixture was stirred 15 minutes, then the solution of the protected ribonucleoside (2 mmoles, evaporated previously with 10 ml acetonitrile), which was prepared according to Arnold et al.[22] in dichloromethane (25

ml), was added dropwise during 20 minutes. After 1 hr, TLC in toluene / ethyl acetate (2/1) showed the complete disappearance of the starting substance. Water (20 ml) was then added, the mixture stirred for 5 minutes, the organic layer separated, the water layer extracted with chloroform (40 ml) and the combined extracts diluted with toluene and evaporated. The residue was dissolved in chloroform/triethylamine (99/1; 8 ml) and applied on a silica gel column (100 ml), equilibrated in the same solvent. Elution was performed with 100 ml portions of the starting solvent, containing successively 5, 10, 15 and 25 % of methanol. The appropriate fractions were evaporated, the residue was evaporated twice with chloroform and dried under oil pump vacuum, yielding glassy foams of triethylammonium salts of H-phosphonates Ib (1.46 g; 79 %; R_F in toluene / ethyl acetate (2/1): 0.55) and IIb (1.55 g; 86 %; R_F in toluene / ethyl acetate (2/1): 0.68).

Automated RNA synthesis

The automated RNA synthesis was performed on a Gene Assembler Plus DNA-synthesizer from Pharmacia. 1-Adamantanecarbonyl chloride and tetrabutylammonium fluoride were purchased from Fluka. Dichloroacetic acid in dichloroethane came from Pharmacia, low water containing pyridine and acetonitrile were from Baker.

The H-phosphonates were dissolved in pyridine/acetonitrile (1/1) to a concentration of 0.12 M each. 1-Adamantanecarbonyl chloride was dissolved in the same solution to reach a concentration of 0.48 M. The automated oligoribonucleotide synthesis was performed according to a modified synthesizer program, developed for the synthesis of DNA. The coupling time for each cycle was elevated from 1 min for DNA synthesis to 2, 5 or 20 min for RNA synthesis. Coupling was achieved by 10 alternating pulses (1.2 seconds each) of 0.12 M H-phosphonate and 0.48 M 1-adamantanecarbonyl chloride solution to the CPG-support column (flow rate 2.5 ml/min), coated with 1.5 μmol starting nucleoside. Then the activated H-phosphonates were recycled through the column for 2, 5 or 20 min. The following scheme shows the conditions for the automated oligoribonucleotide synthesis.

elongation cycle	reagents and solvents	time (s)
1. detritylation	3 % dichloroacetic acid in dichloroethane	60
2. wash	dichloroethane	45
3. wash	acetonitrile/pyridine (1/1)	45
4. condensation I	0.12 M H-phosphonate	1.2
	0.48 M 1-adamantanecarbonyl chloride	1.2

5. condensation II	repeat step 4. four times	
6. condensation III	recycling of the activated H-phosphonates through column	
7. wash	acetonitrile	30
8. wash	dichloroethane	60

The flow rate in each step was 2.5 ml/min.

At the end of the synthesis the oxidation of the polymer bound oligomeric H-phosphonates was achieved by a two step procedure according to Froehler et al.[23]. Cleavage of the crude oligoribonucleotide from the CPG-support and deprotection of the bases was performed in a one step procedure by treatment of the CPG-support with ethanol/25 % ammonia (1/3; v/v) for 24 - 48 hrs (depending on the sequence and on the secondary structure of the RNA) at room temperature. After filtration, the resulting solution was evaporated to dryness. The remaining 2'-blocked oligoribonucleotide was dissolved in 0.9 ml 1 M tetrabutylammonium fluoride in DMF and incubated at room temperature. After 3, 6, 9, 12 and 24 hrs 10 µl aliquots were removed from the reaction mixture, applied on a Nucleosil C-4 HPLC column (Macherey und Nagel, 4.6 mm x 250 mm) and eluted with a 70 min gradient from 100 % 50 mM triethylammonium acetate pH 7.3 to 100 % acetonitrile. 2'-Deprotection is complete after 12 - 24 hrs (depending on the sequence and on the secondary structure of the RNA), as demonstrated by a single peak fraction in the HPLC profile. The resulting crude RNA was desalted and analysed by 20 % PAGE in the presence of 7 M urea. For further purification the RNA was extracted from the gel and the solution subsequently was batched with Sepharose A25 in a buffer containing 200 mM sodium chloride, 20 mM sodium acetate pH 5.2 and 10 mM magnesium chloride. Elution of the bound RNA was achieved by washing the Sepharose A25 with a buffer containing 1 M sodium chloride, 20 mM sodium acetate pH 5.2 and 10 mM magnesium chloride. The resulting solution was desalted on Biogel-P6.

For analysis of the nucleoside composition the oligoribonucleotides were cleaved with nuclease P1 and treated with alkaline phosphatase from *E.coli* according to Gehrke et al.[24]. The resulting nucleosides were applied on a Supelcosil LC-18-S column (Supelco, 4.6 mm x 250 mm), equilibrated in 2.5 % methanol in 50 mM KH_2PO_4 pH 4.4 (v/v) and eluted with the following gradient at 35°C. 0 - 18 min: 2.5 % methanol in 50 mM KH_2PO_4 pH 4.4; 18 - 28 min: 8 % methanol in 50 mM KH_2PO_4 pH 4.4; 28 - 40 min: 8 - 20 % methanol in 50 mM KH_2PO_4 pH 4.4. The flow rate was 1 ml/min.

The purification of the oligoribonucleotides for NMR investigations was achieved with a Vydac C4 column (Promochem, 10 mm x 250 mm) equilibrated in

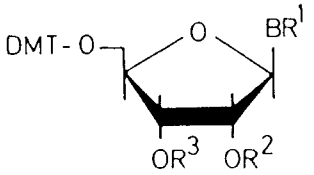

FIG. 1: DMT: -4,4'-dimethoxytrityl

Ia: B= guanine; R^1= dmm; R^2= tBDMS; R^3= H
Ib: B= guanine; R^1= dmm; R^2= tBDMS; R^3= H-P
IIa: B= adenine; R^1= dmm; R^2= tBDMS; R^3= H
IIb: B= adenine; R^1= dmm; R^2= tBDMS; R^3= H-P

H-P: $-PH(O)O^-NEt_3H^+$
dmm: $=CHN(CH_3)_2$; tBDMS: $-Si(CH_3)_2C(CH_3)_3$

100 mM triethylammonium acetate pH 6.0 and a linear gradient of acetonitrile in equilibration buffer. The collected RNA was finally desalted on Biogel-P6, lyophilized to dryness and dissolved in 10 mM sodium phosphate pH 6.5, 100 mM sodium chloride and 10 % (v/v) D_2O.

Results

Preparation of the H-phosphonates

The silylation of 5'-O-(4,4'-dimethoxytrityl)-N^2-dimethylaminomethylene-guanosine afforded predominantly (68 %) the desired 2'-O-silyl derivative (Fig. 1, Ia), which could be easily separated from the corresponding 3'-isomer on a silica gel column with chloroform/2-propanol as eluant. As monitored by spectroscopy at 300 nm of the compound Ia, the dimethylaminomethylene protecting group can be removed by treatment with an aqueous ammonia/ethanol solution at room temperature within 24 hrs. During this reaction the other protecting groups of Ia were stable and no other reaction product was detectable by TLC or HPLC. In the case of 5'-O-(4,4'-dimethoxytrityl)-N^6-dimethylaminomethyleneadenosine the same protecting strategy was used and the yield of the 2'-O-silylated compound (Fig. 1, IIa) was 62.5 %.

For the automated chemical synthesis of oligoribonucleotides, compounds Ia, IIa and the corresponding 2'-O-silylated derivatives of uridine and N^4-benzoyl-

TABLE 1: Some ^{1}H-NMR chemical shifts (in ppm) for the compounds in Fig. 1

Compound	H1'(d),$J_{1',2'}$,[a]	H2'(m)	H3'(m)	H8(s)	tBu(s)	MeSi(s)	SiMe(s)
Ia	5.98,(5.8)	4.68	4.30	7.80	0.82	0.00	-0.85
Ib	6.05,(5.8)	4.68	4.80	7.75	0.75	0.02	-0.84
IIa	6.07,(7.0)	5.00	4.30	8.45	0.68	-0.97	-0.80
IIb	6.17,(7.0)	4.98	4.80	8.40	0.74	0.03	-0.79

[a] J values in brackets are quoted in Hz
^{1}H-NMR spectra are referenced to an internal $CDCl_3$ standard (7.24 ppm downfield from TMS)

cytidine were converted to their H-phosphonates. All compounds were characterized by TLC, UV-spectroscopy and ^{1}H-NMR. The most important NMR data of the educts (Ia, IIa) and of the products (Ib, IIb) are summarized in table 1. For the analysis of purity with respect to positional isomers of protected nucleosides and nucleotides the H2' and H3' signals are of important diagnostic value.

Synthesis of oligoribonucleotides

The rate of N^4-benzoylcytidine deprotection is similar to the rate of hydrolysis of the dimethylaminomethylene group from purines. Therefore we used the classical 2'-O-(t-butyldimethylsilyl)-5'-O-(4,4'-dimethoxytrityl)-N^4-benzoylcytidine for the synthesis of RNA. The assembly of oligoribonucleotides from H-phosphonates was performed with a fourfold molar excess of 1-adamantanecarbonyl chloride as the condensing agent over the H-phosphonates, which were used in a 20 fold molar excess over the column bound nucleoside. The decrease of the coupling time from 20 min, described by Arnold et al.[22], to 2 min had no influence on the average coupling efficiency of 97 - 99 % and on the quality and quantity of the product (Fig. 2 and Tab. 2).

The syntheses of these oligouridines were started with each 1.5 μmol of support-bound uridine and the amount of the products (Tab. 2) was not reduced by shortening the coupling time. The use of a coupling time of 2 minutes decreased the time for an elongation cycle from initial 25 to 7 minutes.

In figure 3 the crude product of a 23mer (5'-UCU UCA UCU CCU CCU UUA UCA GG-3') synthesized with the coupling time of 2 minutes is shown. The synthesis amount was 144 A_{260} (48 % yield, based on 1.5 μmol starting nucleoside). Figure 3A and 3B and the high yield of the crude product clearly

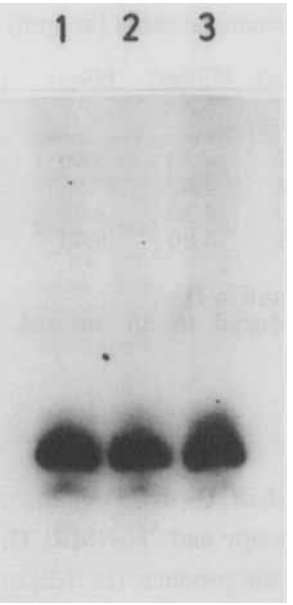

FIG. 2: 20 % PAGE (7M urea) of the crude products (after oxidation, deprotection and desalting) of each 0.1 A_{260} oligo U (15mer); lane 1) 2 min coupling, lane 2) 5 min coupling, lane 3) 20 min coupling.

TABLE 2: Yields of the oligouridine 15mers, synthesized from each 1.5 μmol starting nucleoside, using different coupling times.

coupling time (min)	amount of crude product (A_{260})	amount of pure product (% of crude product)
20	99.5	34.4
5	119.9	32.8
2	112.5	33.4

indicate that a coupling time of 2 minutes is also sufficient for the synthesis of oligoribonucleotides containing the four different bases.

Average coupling yields are summarized in table 3, indicating that the four-fold molar excess of 1-adamantanecarbonyl chloride over the H-phosphonates and the coupling time of two minutes are sufficient to obtain coupling efficiencies between 97 and 99 %.

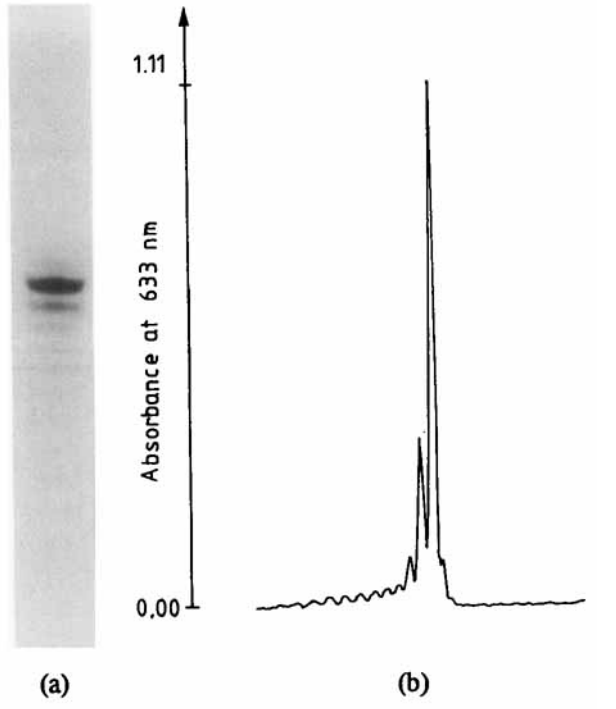

FIG. 3: A) 20 % PAGE (7M urea) of a 23mer (0.05 A_{260}, crude product) synthesized with 2 min coupling time. B) Scan of the bands in the gel at 633 nm; the integration of the peaks shows that the amount of the main product is about 55 %.

TABLE 3: Average coupling yields of various syntheses

No.	sequence	yield (%)*
1	AUGCAUGCAUGCAUAA	99.4
2	ACUGAAACCCUUUGGGG	98.9
3	CGCUUCAGGCCUGGAGCG	99.0
4	AUCAGACUUUUAAUCUGAG	98.2
5	GGUUCCGGGGAAACCUGGAG	97.6
6	CCUGAUCUCGUCUGAUCUCAGG	98.6
7	UCUUCAUCUCCUCCUUUAUCAGG	97.8
8	CCUGAUAAAGGAGGAGAUGAAGA	97.6
9	GCGCCGAAACACCGUGUCUCGAGC	98.0
10	AAAAAAAAAUAAUUUUAAAUAUUU	99.4
11	AAAUAUUUAAAAUUAUUUUUUUUUU	99.2
12	GGGGCUAAGCGGUUCGAUCCCGCUUAGCUCCACCA	98.2
13	GACCUCGGCGUGUUCGAAUCACGUCGGGGUCACCA	98.7

* The average coupling yields were determined with the UV detector and the software of the Gene Assembler.

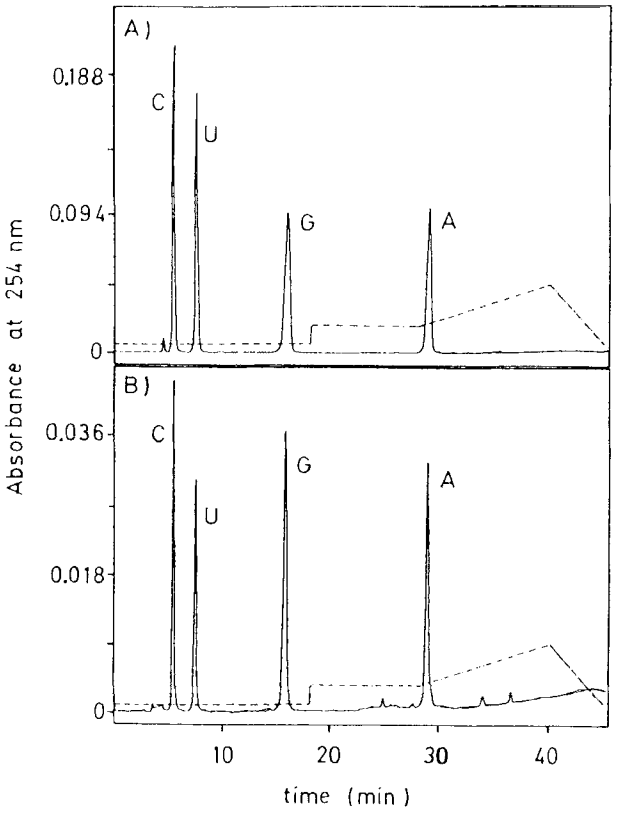

FIG. 4: HPLC analysis of A) the four standard nucleosides A, C, G and U and B) the nucleosides of a 35 nucleotides long Minihelix^{Ala} (0.15 A_{260}). The flow rate was 1 ml/min at 35^{o}C. The detection was done at 254 nm, the broken lines indicate the gradient of methanol in 50 mM KH_2PO_4 pH 4.4.

Deprotection of the crude DMT/off oligoribonucleotides was achieved by an aqueous ammonia/ethanol mixture within 24 - 48 hrs. Incomplete deprotection leads to the appearance of a smear during polyacrylamide gel electrophoresis. The rate of deprotection depends significantly on the sequence and secondary structure of the synthetic oligoribonucleotide. In order to ensure that no base modification had occured during the chemical synthesis and during the deprotection procedures and that deprotection is complete, a chemically synthesized Minihelix^{Ala} (5'-GGG GCU AAG CGG UUC GAU CCC GCU UAG CUC CAC CA-3')[25], purified by elution from a 20 % PAGE (7M urea), was cleaved with nuclease P1 and subsequently treated with alkaline phosphatase from *E.coli*. The resulting nucleosides

were analysed by a reversed phase HPLC according to Gehrke et al.[24]. The comparison of the retention times of the corresponding nucleosides (Fig. 4B) with the retention times of the standard nucleosides (Fig. 4A) shows that the nucleosides are completely (>98 %) deprotected by treatment with the ethanolic ammonia and 1M tetrabutylammonium fluoride.

Using the molar extinction coefficients of each nucleoside[26], which were corrected for the pH of the elution buffer, and the area of each major peak in figure 4B we found a ratio of 17.6/34.5/26.7/20.7 for A/C/G/U which agrees well with the ratio of 17.1/34.3/28.6/20.0, calculated from the sequence. In addition this Minihelix^{Ala}, synthesized with the H-phosphonate method, is a substrate for alanyl-tRNA synthetase from *E.coli*. The kinetic parameters of aminoacylation, K_M, v_{max} and the extent of aminoacylation, are similar to those reported by Francklyn and Schimmel[25] for the same RNA sequence prepared by T7 RNA polymerase, indicating a full functional competence of the chemically synthesized RNA in the enzymatic reaction.

A further method to study the biochemical activity of the synthesized oligoribonucleotides is given by NMR spectroscopy, where the resonances in the imino proton region of the spectrum (below 10 ppm) indicate whether two complementary strands are forming basepairs or not. For these studies the aminoacyl-stem and the aminoacyl-arm of tRNA^{Ala} from *E.coli* were synthesized and purified as shown in Materials and Methods. To suppress the strong water signal the 1-3-3-1 binomial pulse sequence after Hore[27] was chosen. The assignment of the signals in the following figures was achieved by NOE experiments and replacement of the G-U basepair by I-U and I-C basepairs. The U imino proton resonance of the A-U basepair 7 remains invisible (even at the low temperature of 4°C) due to the high opening rate of this basepair at the end of the helix ("fraying") and the resulting increased proton exchange, but all other imino proton resonances clearly could be identified.

In comparison to the aminoacyl-stem the presence of the 3'-terminal ACCA has a stabilizing influence on the RNA duplex. The imino proton resonances of the first and the second basepair of the duplex (Fig. 5A) are sharpened and shifted to higher fields by the addition of the 3'-terminal ACCA (Fig. 5B). This indicates a higher stability of the first two basepairs of the duplex (for details see Limmer et al.[28]) and is in good agreement with the rules for duplex stability according to Freier et al.[29].

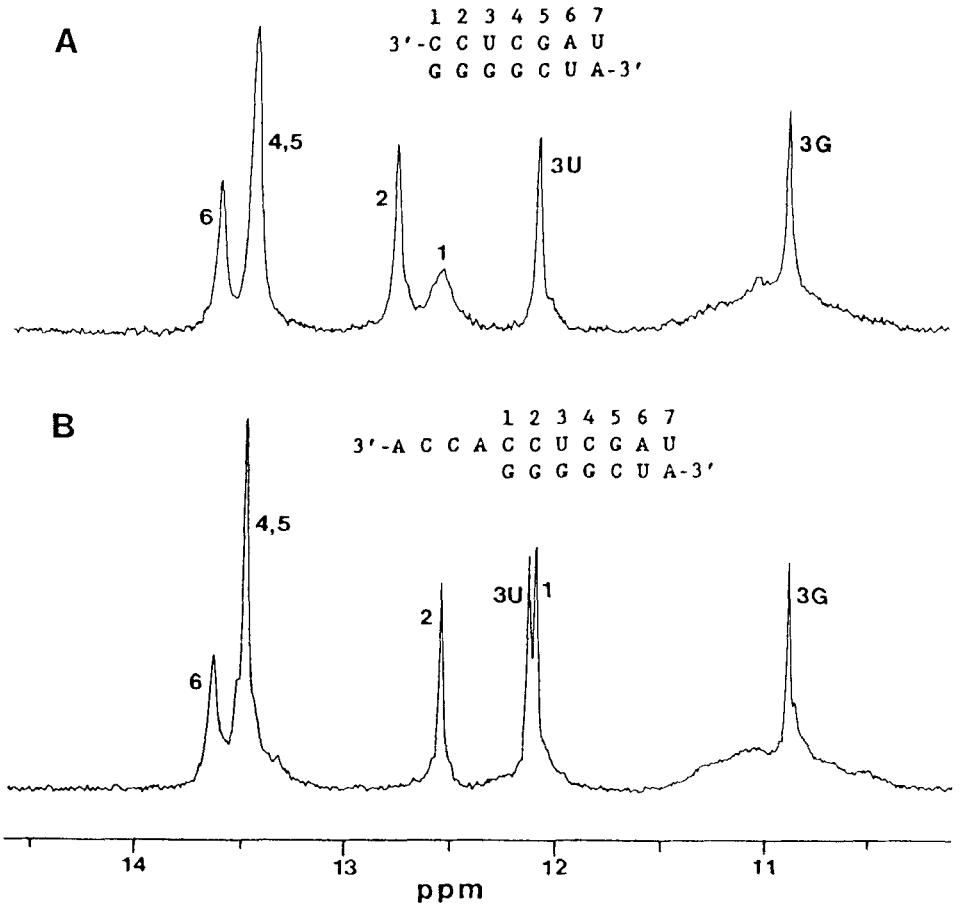

FIG. 5: Imino proton region in the ^{1}H-NMR spectrum of the synthesized aminoacyl-stem (A) and of the aminoacyl-arm (B) of $tRNA^{Ala}$ from *E.coli*. The assignment of the lines to the basepairs in the sequence is indicated.

The effect of the replacement of the G-U basepair by an I-U basepair is shown in figure 6. Both the G-U and the I-U basepairs have two imino protons which can be detected by ^{1}H-NMR. In comparison with the imino proton resonances of the G-U pair the resonances of the I-U basepair exhibit a reduced separation (Fig. 6A). The large downfield shift of the one imino proton of the third basepair in the duplex with the replaced G-U basepair is in good agreement with the formation of an I-U basepair and demonstrates the successful incorporation of a modified base into a RNA oligonucleotide by the H-phosphonate approach. In addition the presence of the I-U basepair influences the chemical shift of the imino

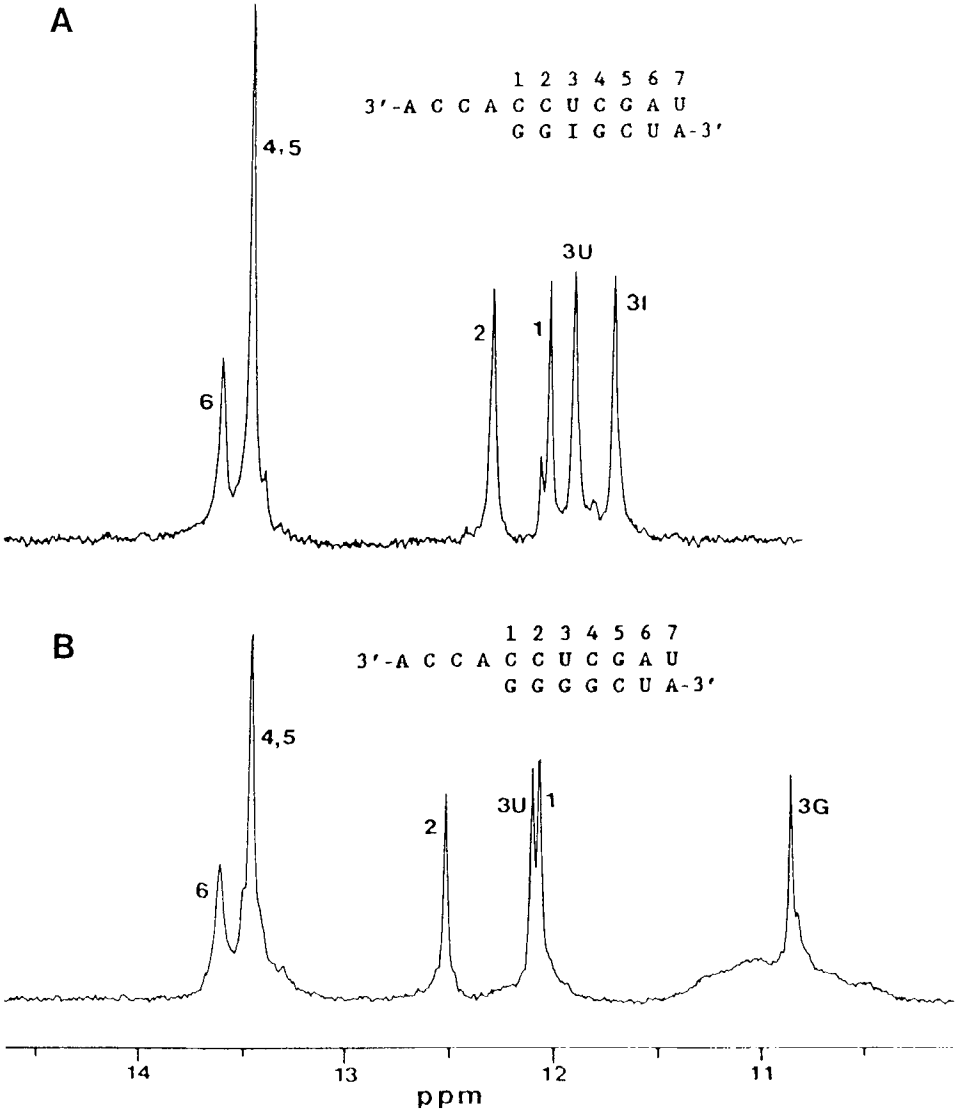

FIG. 6: Imino proton region in the ^{1}H-NMR spectrum of the synthesized aminoacyl-arm of $tRNA^{Ala}$ from *E.coli* with an I-U basepair in position 3 (A) and of the synthesized "wildtype" aminoacyl-arm (B).

proton resonance of the second basepair which appears at higher field as compared to the same basepair in the G-U containing duplex (Fig. 6B).

Discussion

There are two protecting groups for the exocyclic amines of adenine and guanine which can be removed under mild conditions at room temperature; the phenoxyacetyl- and the dimethylaminomethylene group. We decided to apply the dimethylaminomethylene group because i) its introduction proceeds in almost quantitative yield[19], ii) the depurination rate of adenosine under the acidic conditions used for detritylation should be much slower than in the case of the phenoxyacetyl group[17] and iii) the dimethylaminomethylene protection directs the silylation of both nucleosides predominantly to the 2'-position of the ribose[22]. In addition the column chromatographic separation of the 2'- and 3'-silylated isomers is easier when the dimethylaminomethylene protection groups are used for adenosine and guanosine as compared to acyl protection groups. This increases the yield of the desired protected nucleoside and simplifies the synthesis of the ribonucleoside H-phosphonates. As could be shown the dimethylaminomethylene group is stable during the synthesis of oligoribonucleotides and its removal under mild conditions prevents the loss of the tert.-butyldimethylsilyl group from the 2'-position of the ribose and the subsequent cleavage of the RNA. The protection of purine nucleosides by dimethylaminomethylene in combination with the H-phosphonate chemistry for RNA synthesis was first used by Arnold et al.[22]. Later Vinayak et al.[30] applied the same strategy to the phosphoramidite based RNA synthesis.

A comparison of the overall coupling efficiency (97 - 99 %) of the H-phosphonate ribonucleosides with the coupling efficiencies of the ribonucleoside phosphoramidites with various protecting groups either on the bases or at the 5'- and 2'-position of the ribose demonstrates that there is practically no difference in coupling efficiency between H-phosphonate and phosphoramidite chemistry, however the dimethylaminomethylene protected H-phosphonates still have certain advantages over the phosphoramidites, i) no phosphorus protecting group must be removed after the synthesis and ii) the oxidation of the H-phosphonate diesters occurs at the end of the whole synthesis. Furthermore it is possible to recover the unused excess of the activated H-phosphonates and transform them to synthons which can be used again. This is of great importance for the synthesis of RNA oligonucleotides with modified or isotopically labeled (^{15}N or ^{13}C) nucleotides.

After oxidation, cleavage from the support, deprotection and purification, the oligoribonucleotides synthesized with the described method are biochemically active. This was shown by aminoacylation of a RNA fragment of $tRNA^{Ala}$ from *E.coli* containing 35 nucleotides[25]. Using NMR spectroscopy the ability of the synthesized oligoribonucleotides to form double helices was demonstrated. NOE experiments, replacement of the G-U basepair by an I-U or I-C basepair and an assignment according to Hilbers[31] confirm the base-pairing pattern derived from the sequence of the oligoribonucleotides.

Up to now we are able to synthesize oligoribonucleotides with 30 nucleotides in length with excellent yields and in milligram amounts. To synthesize longer RNAs the use of a capping method for the unreacted 5'-hydroxyls via the H-phosphonate approach[32] is indispensable.

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