The effective synthesis of uridylyl/3'-5'/5-methylcytidylyl/3'-5'/guanosine

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ABSTRACT

The triester method was adapted to the synthesis of uridylyl/3'-5'/5-methylcytidylyl/3'-5'/guanosine. As the protecting groups 4-methoxy-5,6-dihydro-2H-pyran for 2'-OH and 5'-OH groups of uridine and 2'-OH group of 5-methylcytidine, methoxymethylidene for 2',3'-cis-diol system of guanosine, and benzoyl for the amino groups of 5-methylcytidine and guanosine were used. The obtained product was characterised by UV, electrophoresis, chromatography, an enzymatic digestion and alkaline hydrolysis.

INTRODUCTION

5-Methylcytidine has been found in RNAs from different sources [1-5]. Although the position of 5-methylcytidine in RNA strand has been established in several species [6-9] the effect of this nucleoside on chemical and biochemical properties of RNA still demands elucidation. In this communication the effective synthesis of modified triribonucleoside diphosphate, uridylyl/3'-5'/5-methylcytidylyl/3'-5'/guanosine /Upm⁵CpG/ is described.

RESULTS

As the substrates for the total synthesis of Upm⁵CpG trinucleotide the following compounds were used: 2',5'-di-O-methoxy-tetrahydropyranylluridine [10,11] /I/, N⁴-benzoyl-2'-O-methoxy-tetrahydropyranyl-5-methylcytidine /II/, and N²-benzoyl-2',3'-O-methoxymethylideneguanosine [12] /III/. Component /II/ was obtained by the series of reactions presented in the fig. 1.

Phosphorylation of /I/ was accomplished with the pyridinium salt of 2,2,2-trichloroethylphosphate and 2,4,6-triisopropylbenzenesulphonyl chloride /TPS/ in pyridine. The obtained nucleotide /Ia/ was condensed with N^4 -benzoyl-2'-O-methoxytetrahydropyran-5-methylcytidine /II/ in pyridine in the presence of TPS. The protected uridylyl/3'-5'/5-methylcytidine /IV/ was obtained after column chromatography on silica gel in 53% yield. One part of protected diribonucleoside monophosphate /IV/ was fully deblocked. The phosphotriester protecting group was removed with Zn-Cu in DMF [13], the alkali labile protecting group benzoyl was removed with half saturated methanolic ammonia [13] and the

methoxytetrahydropyranyl group was removed with 0.01N hydrochloric acid followed by ammonia [11,15]. The product was obtained in high, 87% yield after a purification by paper chromatography, and was characterised by UV, electrophoresis and paper chromatography in several systems. Enzymatic digestion of the synthetic modified diribonucleoside monophosphate with pancreatic ribonuclease and alkaline hydrolysis showed the presence of uridine 3'-phosphate and 5-methylcytidine in approximately equal amounts as was proved by chromatography, electrophoresis and UV analysis.

Second part of protected diribonucleoside monophosphate /IV/ was used for the synthesis of protected triribonucleoside diphosphate /VI/ which was obtained in 51% yield. The phosphorylating and condensing agents as well as the reaction conditions were similar as in the reaction $I + II \rightarrow IV$ /via Ia, fig. 2/.

The blocking groups in VI were removed similarly as in IV yielding 80% of uridylyl/3'-5'/5-methylcytidylyl/3'-5'/guanosine /VII/. Enzymatic digestion of the synthetic modified triribonucleoside diphosphate with pancreatic ribonuclease and alkaline hydrolysis showed the presence of uridine 3'-phosphate, 5-methylcytidine 3'-phosphate and guanosine in approximately equal amounts, which was proved by chromatography, electrophoresis and UV analysis.

The obtained results, especially the yield over 50% in every step of the reaction are in favour of the chosen protecting groups as well as of the chosen reaction conditions.

EXPERIMENTAL

UV spectra were recorded on a Unicam SP 700A and Specord UV-Vis Carl Zeiss Jena spectrophotometers. IR spectra were measured on a Unicam SP 200G spectrophotometer. NMR spectra 80 MHz were determined on a Tesla BS 487A and Varian 60 MHz EM-360 spectrometers using HMDSO, TMS and DSS as internal references.

Fig 2.

The melting points are uncorrected.

Pyridine was dried over CaH₂ and freshly distilled before every reaction. TPS /2,4,6-triisopropylbenzenesulphonyl chloride/ was obtained according to [14] and recrystallized before every condensation from n-pentane. The condensation reactions with TPS were carried out in darkness over argon. 4-Methoxy-5,6-2H-pyran was obtained according to [15] immediately before every blocking reaction from tetrahydropyran-4-on [16].

Koch-Light and Mecherey Nagel + Co silica gel 100-200 mesh and Mallinckrodt analytical grade silicic acid 100 mesh according to Ramsey and Patterson were used for column chromatography. The

conditions for column chromatography and silica gel activity were determined according to [17]. The results of paper and thin-layer chromatography are shown in Table 1. Electrophoresis on Whatman 3MM filter paper was carried out in 0.03M phosphate buffer /pH=7.1/ /E₁/ and 0.05M formate buffer /pH=2.7/ /E₂/ under 30V/cm migration rate related to uridine 3'-phosphate /1.00/. Pancreatic ribonuclease /preparation of Ciech, Poland, after five recrystallizations/ was used.

T₁, CHCl₃/MeOH, 85:15; T₂, CHCl₃/MeOH, 9:1; T₃, CHCl₃/MeOH, 98:2
T₄, CH₂Cl₂/MeOH, 9:1.

Table 1

Compound	R _f values in systems								
	Paper chromatography					Thin-layer chromatography			
	A	B	C	D	E	T ₁	T ₂	T ₃	T ₄
m ⁵ Cyt	0.32		0.57			0.14			
m ⁵ C	0.21		0.58			0.08			
I							0.43	0.33	
II							0.63		0.67
III		0.78	0.61						
IV							0.61	0.19	
Upm ⁵ C /V/			0.21	0.33					
VI							0.53	0.10	
Upm ⁵ CpG /VII/				0.56	0.60				

Paper and thin-layer chromatograms were run in a descending manner on Whatman 1 paper and plates coated with Merck Kieselgel HP₂₅₄ respectively in following solvent systems /measured by volume/:

A, n-BuOH saturated with water; B, n-BuOH/AcOH/H₂O, 5:2:3;
C, i-PrOH/NH₃aq/H₂O, 7:1:2; D, EtOH/1M AcONH₄ /pH=7.5/, 7:3;
E, isobutyric acid/NH₃aq/H₂O /pH=3.7/, 66:1:33;

5-Methylcytosine

5-Methylcytosine was obtained according to modified method [18]. Yield 84%, m.p. 268-70°C /with decompn./. UV /0.1N HCl/: $\lambda_{\max}$ 283 nm / $\epsilon_{\max}$ 9700/, $\lambda_{\min}$ 242 nm / $\epsilon_{\min}$ 960/; /0.1N NaOH/: $\lambda_{\max}$ 273 nm / $\epsilon_{\max}$ 6150/, $\lambda_{\min}$ 251 nm / $\epsilon_{\min}$ 3480/; NMR /CF₃COOH/: δ ppm 1.86/s,3,C⁵-CH₃/, 7.30/s,2,H-4/, 7.95/s,1,H-6/.

2',3',5'-Tri-O-benzoyl-5-methylcytidine

2',3',5'-Tri-O-benzoyl-5-methylcytidine was obtained according to general method [19]. Yield 54% /after column chromatography and recrystallization from ethanol/, m.p. 223-4°C. UV /0.1N HCl/: $\lambda_{\max}$ 234 nm and 285 nm / $\epsilon_{\max}$ 13210 and 11230/, $\lambda_{\min}$ 265 nm / $\epsilon_{\min}$ 1810/; /MeOH/: $\lambda_{\max}$ 277 nm / $\epsilon_{\max}$ 9140/, $\lambda_{\min}$ 257 nm / $\epsilon_{\min}$ 5870/; NMR /CDCl₃/: δ ppm 1.98/s,3,C⁵-CH₃/, 5.97/d,1, J_{1,2} = 3.5 Hz, H-1'/, 7.84/s,1,H-6/. Elemental analysis for C₃₁H₂₇N₃O₈, m.w. 569.55. Calc. 65.36%C, 4.77%H, 7.37%N; found 65.48%C, 4.73%H, 7.37%N.

5-Methylcytidine

2',3',5'-Tri-O-benzoyl-5-methylcytidine /1 g, 1.75 mM/ was dissolved in 60ml of anhydrous methanol and anhydrous methanol saturated with NH₃ at 0°C /5 ml/ was added. After 24 hr the solvent was evaporated, the residue dissolved in water and extracted with CHCl₃. Then the water layer was evaporated to dryness and the residue was dried by coevaporation with anhydrous ethanol and benzene. The dry powder was recrystallized from anhydrous ethanol yielding 95% of 5-methylcytidine, m.p. 210-11°C. UV /0.1N HCl/: $\lambda_{\max}$ 289 nm / $\epsilon_{\max}$ 11360/, $\lambda_{\min}$ 246 nm / $\epsilon_{\min}$ 690/; /0.1N NaOH/: $\lambda_{\max}$ 278 nm / $\epsilon_{\max}$ 8140/, $\lambda_{\min}$ 256 nm / $\epsilon_{\min}$ 5940/; NMR /D₂O/: δ ppm 1.99/s,3,C⁵-CH₃/, 6.01/d,1, J_{1,2} = 4 Hz, H-1'/, 8.46/s,1,H-6/. Elemental analysis for

$C_{10}H_{15}N_3O_5$, m.w. 257.24. Calc. 46.68%C, 5.87%H, 16.33%N; found 46.53%C, 5.87%H, 16.35%N.

3'-O-Acetyl-N⁴,5'-O-dibenzoyl-5-methylcytidine

5-Methylcytidine was transformed into 2',3'-O-methoxyethylidene-5-methylcytidine similarly as cytidine [20] with yield 86% and this compound gave 88% of N⁴,5'-O-dibenzoyl-2',3'-O-methoxyethylidene-5-methylcytidine according to general method [21].

N⁴,5'-O-Dibenzoyl-2',3'-O-methoxyethylidene-5-methylcytidine /0.595 g, 1.14 mM/ was dissolved in 5% acetic acid /7 ml/ and the solution was stirred for 45 min at ambient temperature. Then the mixture was extracted with $CHCl_3$ /5x4 ml/. The chloroform extract was dried over anhydrous Na_2SO_4 and the solvent was evaporated. The residue was separated by column chromatography on silica gel /20 g/. As the eluents CH_2Cl_2 , CH_2Cl_2 /MeOH /98:2, by vol/, and CH_2Cl_2 /MeOH /97.5:2.5, by vol/ were used. Pure 3'-O-acetyl-N⁴,5'-O-dibenzoyl-5-methylcytidine was obtained after two recrystallizations from anhydrous ethanol /0.277 g/. Yield 48% m.p. 196-8°C. UV /0.1N HCl/: λ_{max} 232 nm and 288 nm / ϵ_{max} 16700 and 8950/, λ_{min} 256 nm / ϵ_{min} 6150/; /MeOH/:

λ_{max} 230 nm and 276 nm / ϵ_{max} 18740 and 11100/, λ_{min} 264 nm / ϵ_{min} 9050/; NMR / d_6 -DMSO- D_2O /: δ ppm 1.85/s,1, C^5 - CH_3 /, 2.11 /s,3,3'- $OCOCH_3$ /, 5.96/d,1, $J_{1,2}$, = 3 Hz, H-1'/, 7.48-8.24/m,10, 4- $NCOC_6H_5$ and 5'- $OCOC_6H_5$ /, 8.51/s,1,H-6/. Elemental analysis for $C_{26}H_{25}N_3O_8$, m.w. 507.48. Calc. 61.53%C, 4.96%H, 8.28%N; found 61.37%C, 4.95%H, 8.21%N.

N⁴-Benzoyl-2'-O-methoxytetrahydropyranyl-5-methylcytidine /II/

3'-O-Acetyl-N⁴,5'-O-dibenzoyl-2'-O-methoxytetrahydropyranyl-5-methylcytidine /0.225 g, 0.36 mM/ obtained from 3'-O-acetyl-N⁴,5'-O-dibenzoyl-5-methylcytidine and 4-methoxy-5,6-dihydro-2H-pyran [15] according to method used for cytidine [10] was

dissolved without additional purification in 2.5 ml of anhydrous methanol and 2.5 ml anhydrous dioxane. To this solution 0.8 ml of 1N MeONa in MeOH was added and the solution was stirred at ambient temperature for 15 min. The Dowex 50W-X8 /pyridinium form/ was added and the mixture was left for 15 min. The resin was filtered off, washed with CHCl_3 /MeOH /1:1, by vol/. The filtrate was evaporated on a rotovap to dryness, purified by column chromatography on silicic acid with CHCl_3 /MeOH /9:1, by vol/ and recrystallized from CHCl_3 /n-pentane /1:3, by vol/. Yield of II 71% /0.122 g/, m.p. 183-4°C. UV /MeOH/: λ_{max} 289 nm / ϵ_{max} 16450/, λ_{min} 242 nm / ϵ_{min} 7360/; NMR / d_6 -DMSO- D_2O /: δ ppm 1.64/t,4,- CH_2 -, 1.88/s,3, C^5 - CH_3 /, 3.21/s,3,- OCH_3 /, 3.68/t,4,- OCH_2 -, 5.93/d,1, $\text{J}_{1,2}$ = 3.5 Hz, H-1'/, 7.35-8.15/m,5, 4- NCOC_6H_5 /, 8.48/s,1,H-6/. Elemental analysis for $\text{C}_{23}\text{H}_{29}\text{N}_3\text{O}_8$, m.w. 475.48. Calc. 58.09%C, 5.72%H, 8.83%N; found 58.26%C, 5.68%H, 8.90%N.

2',5'-Di-O-methoxytetrahydropyranylluridylyl [3'-/2,2,2-trichloroethyl/ 5'] N^4 -benzoyl-2'-O-methoxytetrahydropyranyl-5-methylcytidine /IV/

2,2,2-Trichloroethylphosphate dicyclohexylammonium salt [22] /54 mg, 0.1 mM/ was dissolved in pyridine /3.2 ml/ and converted into a pyridinium salt by coevaporation with pyridine /5x2.5 ml/, dissolved in pyridine 2.5 ml, and TPS /68mg, 0.2 mM/ was added. I /23 mg, 0.05 mM/ was added after 0.5 hr. TLC showed that I was completely converted into Ia after 2 days. TPS was destroyed by the addition of ice /2 g/ followed by water /5 ml/ after 0.5 hr. The reaction mixture was extracted with CH_2Cl_2 /5x3 ml/ and the organic layer was evaporated to dryness and dried by coevaporation with pyridine /4x3 ml/. To the residue TPS /34mg, 0.1 mM/ and pyridine /2.5 ml/ were added and after 0.5 hr followed by the addition of II /47 mg, 0.1 mM/

in pyridine /1 ml/. TLC showed a complete conversion of Ia and II into IV after 2 days. TPS was destroyed with ice and water and the product was extracted with CH_2Cl_2 /5x3 ml/. After evaporation of the solvent the residue was purified by column chromatography on silica gel /15 g/ in CH_2Cl_2 /MeOH /98.5:1.5, by vol/ and CH_2Cl_2 /MeOH /97.5:2.5, by vol/, yielding 29 mg /53%/ of IV. UV [CHCl_3 /MeOH /1:1, by vol/]: λ_{max} 256 nm and 291 nm / ϵ_{max} 47600 and 17900/, λ_{min} 238 nm and 273 nm / ϵ_{min} 20700 and 16400/.

Uridyl/3'-5'/5-methylcytidine /V/

IV /7 mg, 0.006 mM/ was dissolved in DMF /1 ml/ and stirred at 50°C with Zn-Cu couple. TLC showed complete transformation of IV into a product of $R_f=0$ [system CHCl_3 /MeOH /9:1, by vol/] after 1 day. Then half saturated at 0°C methanolic ammonia /1 ml/ was added and the reaction mixture was left at ambient temperature for 2 days. The unreacted Zn-Cu couple was filtered and washed with methanolic ammonia. The solvents were evaporated, the residue was dissolved in methanolic ammonia /pH=10/ and Dowex 50W-X8 NH_4^+ /0.4 g/ was added. The resin was filtered and washed with water after 15 min. The solution was concentrated to 10 ml on a rotavap and its pH brought up to 2 with 0.01N HCl.

After 1 day the pH was brought up to 9 with NH_3 aq. and neutralized after 0.5 hr. The preparative paper chromatography /system C/ of the reaction mixture gave Upm^5C /V/, 76 OD /pH 7 $\frac{\text{H}_2\text{O}}{260\text{nm}}$ / 87% yield.

UV /0.1N HCl/: λ_{max} 278 nm, λ_{min} 240 nm; $\epsilon_{\text{max}}/\epsilon_{\text{min}}$ 3.58; E_{250}/E_{260} 0.56; E_{280}/E_{260} 1.53; /0.1N NaOH/: λ_{max} 270 nm λ_{min} 237 nm; / H_2O / λ_{max} 271 nm, λ_{min} 237 nm. Paper electrophoresis: 0.52 /system E_1 /.

The enzymatic digestion and alkaline hydrolysis of Upm⁵C

The mixture of Upm⁵C /10 OD, pH $\overset{\text{H}_2\text{O}}{7.260}$ / in 50 ml of 0.05 M Tris-HCl buffer solution /pH=8/ and 15 ml of a pancreatic ribonuclease solution in the same buffer /content: 30µg of enzyme/ was incubated for 3 hr at 37°C. The paper electrophoresis of incubation mixture showed the presence of Up and m⁵C in approximately equal amounts as was shown by UV and paper chromatographic analysis of electrophoretic spots. TLC analysis of the digestion mixture /system C and D/ showed the presence of Up and m⁵C.

The same result was obtained after alkaline hydrolysis of Upm⁵C /5 OD, pH $\overset{\text{H}_2\text{O}}{7.260}$ / in 30 ml of 0.3N potassium hydroxide for 24 hr at 37°C.

2',5'-Di-O-methoxytetrahydropyranylluridylyl [3'-/2,2,2-trichloroethyl/ 5'] N⁴-benzoyl-2'-O-methoxytetrahydropyranyl-5-methylcytidylyl [3'-/2,2,2-trichloroethyl/ 5'] N²-benzoyl-2',3'-O-ethoxymethylideneguanosine /VI/

Condensation of IV /12 mg, 0.035 mM/ with III /24 mg, 0.0525 mM/ which was obtained from N²-benzoylguanosine [23] and triethyl orthoformate according to method used for guanosine [12] was accomplished via IVa similarly as Ia + II → IV. Yield of VI was 51%. UV [CHCl₃/MeOH /1:1, by vol/]: λ_{max} 252 nm and 286 nm /ε_{max} 41800 and 72300/, λ_{min} 231 and 267 nm /ε_{min} 19700 and 31400/.

Uridylyl/3'- 5'/5-methylcytidyl/3'- 5'/guanosine /VII/

The blocking groups in VI /10 mg, 0.0055 mM/ were removed similarly as in IV. Yield of Upm⁵CpG /VII/ 80%, 104 OD /pH $\overset{\text{H}_2\text{O}}{7.261}$ nm/. UV /0.1N HCl/: λ_{max} 268 nm, λ_{min} 230 nm ε_{max}/ε_{min} 2.43; E₂₅₀/E₂₆₀ 0.66; E₂₈₀/E₂₆₀ 0.91; /0.1N NaOH/: λ_{max} 261 nm, λ_{min} 237 nm, /H₂O/: λ_{max} 260 nm, λ_{min} 232 nm. Paper electrophoresis: 0.60 /system E₁/ and 0.44 /system E₂/.

The enzymatic digestion and alkaline hydrolysis of Upm⁵CpG

The enzymatic digestion and alkaline hydrolysis of Upm⁵CpG was done similarly as with Upm⁵C. The paper electrophoresis of incubation mixture showed the presence of Up, m⁵Cp and G in approximately equal amounts as was shown by UV and paper chromatographic analysis of electrophoretic spots. TLC analysis of the digestion mixture /system C and D/ showed presence of Up, m⁵Cp and G.

The same result was obtained after alkaline hydrolysis.

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