The modified nucleosides of tRNAs. II. Synthesis of 2'-O-methylcytidylyl (3'-5') cytidine

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Received 28 April 1975

ABSTRACT

The synthesis of 2'-O-methylcytidylyl (3'-5')cytidine by the triester method using as protecting groups, 2,2,2-trichloroethyl for phosphate hydroxyl group, p-chlorophenoxyacetyl for 5-hydroxyl group, methoxymethylidene for 2',3'-cis-diol system, and benzoyl for the exo-amino group of cytidine is presented. The obtained product was characterised by UV, electrophoresis, chromatography and an enzymatic digestion.

INTRODUCTION

2'-O-Methylnucleosides have been found in RNAs from different sources, as well as in rRNAs[1] and tRNAs[1, 2]. The knowledge of the chemical behaviour, structural and biological significance of 2'-O-methylnucleoside residues is still fragmentary. 2'-O-Methylated oligonucleotides are more stable under alkaline conditions than other oligoribonucleotides [3]. The synthesis of an alkali-stable diribonucleoside monophosphate, 2'-O-methylcytidylyl (3'-5')cytidine (fig. 1, CmpC) is presented in this communication*.

The synthesis of 2'-O-methylcytidylyl (3'-5')cytidine (CmpC)

* Since we completed the synthesis described in this communication, the chemical synthesis of another oligoribonucleotide containing a 2'-O-methyl-nucleoside has been published by T. Neilson et al. [4].
CmpC (fig. 1) was synthesised by the triester method (fig. 2) using as protecting groups.
groups, 2,2,2-trichloroethyl for phosphate hydroxyl group [5], p-chlorophenoxyacetyl for the 5'-hydroxyl group [6], methoxymethylidene for the 2',3'-cis-diol system [7], and benzoyl for the exo-amino group of cytidine.

Cytidine (fig.2.1) was methylated using diazomethane in the presence of SnCl2.2H2O [8] giving 2'-O-methylcytidine (II) in 67% yield.

We should like to point out that in our preparation 70% of the yield was obtained by crystallisation directly from the reaction mixture without laborious chromatography*. 3'-O-Methylcytidine (IIa) was obtained also after Dowex 1 x 2 (OH-) chromatography of mother liquors in 11% yield. N4-Benzoyl-2'-O-methylcytidine (III) was obtained in 75% yield by selective N-benzylation of 2'-O-methylcytidine with benzoyl anhydride as described for cytidine [10]. N4-Benzoyl-2'-O-methylcytidine was treated with p-chlorophenoxyacetyl chloride in CH3CN and 2,6-lutidine.

N4-Benzoyl-5'-O-p-chlorophenoxyacetyl-2'-O-methylcytidine (IV) was obtained after column chromatography as a crystalline product in 40% yield. The 3'-ester (V) and 3',5'-diester (VI) were observed also. N4-Benzoyl-3',5'-di-O-p-chlorophenoxyacetyl 2'-O-methylcytidine (VI) was obtained as a crystalline product in 14% yield.

Studies on the improvement of yield of IV are in progress. N4-Benzoyl-2',3'-O-methoxymethylidene cytidine (VIII) was synthesised from cytidine by a two step method in 49% overall yield, whereas the synthesis described in the literature [7] follows a three-step procedure resulting in a 48% overall yield. Our procedure distinctly simplifies and shortens the synthesis. N4-Benzoylcytidine (VII), synthesised according to the literature [10], was treated with trimethylorthoformate leading to two main products in approximately equal amounts, the mono-orthoester (VIII) and the bis-orthoester (IX). The bis-orthoester was quantitatively transformed into N4-benzoyl-2',3'-O-methoxymethylidene cytidine under mildly acidic conditions, i.e. in chloroform solution in the presence of silicic acid, or methylene chloride-methanol solution in the presence of catalytic amounts of acid.

* A similar result was obtained for N4-2'-O-dimethylcytidine [9].
N*-Benzoyl-5'-O-p-chlorophenoxyacet-2'-O-methylcytidine (IV) was phosphorylated with the pyridinium salt of 2,2,2-trichloroethylphosphate and 2,4,6-trisopropylbenzenesulphonyl chloride (TPS) in pyridine [5]. The obtained nucleotide (X) was condensed with N*-benzoyl-2',3'-O-methoxymethylidenecytidine (VIII) in pyridine in the presence of TPS. The protected 2'-O-methylcytidylyl (3'-S')cytidine (XI) was obtained after short column chromatography in 72% yield. The 5'-protecting group of (XI) may be selectively eliminated [11] and the obtained partially blocked dinucleosidemonophosphate was used for the synthesis of longer oligonucleotides. The diribonucleoside monophosphate (XI) was then fully deblocked. The phosphotriester protecting group was removed with a Zn-Cu couple in DMF [5], the alkali labile protecting groups benzoyl and p-chlorophenoxacyethyl were removed with half-saturated methanolic ammonia [5, 6], and the methoxymethylidene group was removed using 0.01N hydrochloric acid followed by ammonia [7]. The product was obtained in 25% yield after a purification by paper-chromatography, and was characterised by UV, electrophoresis, paper- and thin-layer-chromatography in several systems. The chromatographic, electrophoretic and UV analysis of the enzymatic digestions of synthetic dinucleoside monophosphate with a snake venom phosphodiesterase showed the presence of 2'-O-methylcytidine and cytidine 5'-phosphate in approximately equal amounts and proved it to be 2'-O-methylcytidylyl (3'-S')cytidine.

On the basis of above results, it can be stated that the chosen protecting groups had accomplished their task well.

EXPERIMENTAL

Pyridine was dried over CaH2 and freshly distilled. TPS (2,4,6-trisopropylbenzenesulphonyl chloride) was obtained according to [12]. The condensation reactions with TPS were carried out in darkness. Melting points are uncorrected. UV spectra were recorded with a Unicam SP 700 A spectrophotometer. IR spectra were measured with a Unicam SP 200 G spectrophotometer. NMR spectra 80 MHz were determined on a Tesla BS 487 A spectrometer using HMDSO and t-butanol as internal references for non-aqueous and deuterium oxide solutions respectively. The results of UV data and an elemental analysis of obtained compounds are listed in Table 1. The results of paper- and thin-layer-chromatography are shown in Table 2.
Table 1

<table>
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<tr>
<th>Compound</th>
<th>Solvent*</th>
<th>λ max (nm)</th>
<th>ε max x 10^3</th>
<th>λ min (nm)</th>
<th>ε min (nm)</th>
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<th>calculated for C, H, N</th>
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<td></td>
<td>pH12</td>
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<td>254</td>
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<tr>
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<tr>
<td></td>
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* Two diastereomers in approximately equal amounts.

UV spectra at pH1 and 12 were performed in water.

Table 2

<table>
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<tr>
<th>Compound</th>
<th>R f x 100 values in systems</th>
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<tr>
<td>VIII</td>
<td>46</td>
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<tr>
<td>X</td>
<td>15</td>
</tr>
<tr>
<td>XI</td>
<td>73</td>
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</table>

Paper and thin layer chromatograms were run in a descending manner on Whatman 1 paper and plates coated with Merck Kieselgel GF 254 respectively in following solvent systems (measured by volume):

A: i-PrOH/NH₃aq/ H₂O, 7:1:2; B, n-BuOH sat. with water; C, EtOH/1M AcO Na + 3.3 mM EDTA, 7:3; D, n-BuOH/ AcOH/H₂O, 5:2:3; E₁, CHCl₃/MeOH, 75:25; E₂, CHCl₃/MeOH, 85:15; E₃, CHCl₃/MeOH, 90:10; E₄, CHCl₃/MeOH, 92.5:7.5; F, i-PrOH/0.5M NH₄HCO₃aq, 8.2.
Koch-Light and Schuchardt silica gel 100-200 mesh was used for column chromatography. The conditions for column chromatography and silica gel activity were determined according to [13].

Electrophoresis on Whatman 1 filter paper was carried out in 0.05M borate buffer (pH 9.2) under 10 V/cm, migration rate related to uridine 3'-phosphate (1.00).

Phosphodiesterase from Crotales adamanteus snake venom (Sigma) was used.

2'-O-Methylcytidine (II) and 3'-O-methylcytidine (IIa)
To the stirred solution of 3.000 g (12.33 mM) anhydrous cytidine 90 mg SnCl₂·2H₂O in 720 ml anhydrous methanol, CH₂N₂ solution in 1,2-dimethoxyethane (100 ml) [14] was added. The chromatography has shown that the reaction was completed after a few minutes. Solution was concentrated in vacuo, resulting oil dissolved in MeOH, and solution once more concentrated. This procedure was repeated several times. The crystallizing oil was dissolved in warm methanol 40 ml, which gave after crystallization 1.805 g II. 57% yield, m.p. 242-254°C, NMR D₂O: 6 ppm 7.90 (d,1, J₁,6 = 8Hz, H-6), 6.06 (d,1, J₅,6 = 8Hz, H-5), 5.98 (d,1, J₁₂ = 4Hz, H-1'), 4.30 (m,1, H-3'), 4.10 (m,1, H-4'), 4.00 (m,1, H-2'), 3.88 (m,2, H-5'), 3.53 (s,3, 3'-OCH₃).

Mother liquors containing II and IIa, and traces of other compounds (tlc, El system, Rf 0.58, 0.37, 0.29) after purification with column chromatography on silica gel, were separated on Dowex 1 x 2 OH⁻ column according to [15]. Further amounts of II (0.324 g, total yield 67%) and crystalline IIa, 0.360 g, 11.5% yield, m.p. 194-195°C, NMR D₂O: 6 ppm 7.85 (d,1, J₅,6 = 7.5 Hz), 6.04 (d,1, J₅,6 = 7.5 Hz, H-5), 5.88 (d,1, J₁₂ = 3.5 Hz, H-1'), 4.45 (m,1, H-2'), 4.17 (m,1, H-4'), 3.89 (m,1, H-3'), 3.86 (m,2, H-5'), 3.46 (s,3, 3'-OCH₃) were obtained.

N*-Benzoyl-2'-O-methylcytidine (III)
The solution of II (1.000 g, 3.89 mM) and benzoyl anhydride (1.00 g) in 100 ml of anhydrous methanol was refluxed. The further 1.00 g portions of benzoyl anhydride was added after 1, 2, 3 and 4 hrs of the refluxing. The solution was concentrated under diminished pressure after 10 hrs, and a residue
extracted with 50 ml of diethyl ether. The undissolved residue containing III and unreacted II, was chromatographed on a silica gel column (30 g) in CHCl3/MeOH (90:10, by vol) and gave III, 0.802 g, 57% yield, m.p. 181-182°C, NMR d6-DMSO: δ ppm 8.54 (d,1, J5, 6 = 7.7 Hz, H-6), 7.31 (d,1, J5, 6 = 7.7 Hz, H-5), 7.42-8.11 (m,5,4-NCOC6H5), 5.86 (d,1, J1, 2 = 2.5 Hz, H-1'), 4.09 (m,1, H-3'), 3.91 (m,1, H-4'), 3.71 (m,3, H-2' and H-5'), 3.44 (s,3, 2'-OCH3). Also 0.216 g of unreacted II was recovered. The further amount of III (0.260 g, total yield 75.5%) was obtained after silica gel chromatography of the ether extract.

N^4-Benzoyl-5'-O-p-chlorophenoxyacetyl-2'-O-methylcytidine (IV) and N^4-benzoyl-3',5'-di-O-p-chlorophenoxyacetyl-2'-O-methylcytidine (VI)

p-Chlorophenoxyacetyl chloride (174 mg, 0.85 mM) was added to the solution of III (200 mg, 0.555 mM) in anhydrous acetonitrile (80 ml) and 2,6-lutidine (0.52 ml). The reaction was quenched after 5 days with water (2 ml) and the solution was concentrated under reduced pressure. The residue was chromatographed on a silica gel column (60 g) in CH2Cl2/MeOH (97:3, by vol). The obtained VI was crystallised (petroleum ether-benzene), 55 mg, 14% yield, m.p. 138-142°C, NMR (CDCl3): δ ppm 7.92 (d,1, J5, 6 = 8 Hz, H-6), 6.76 (d, overlapped with p-chlorophenoxyacetyl, H-5), 5.88 (d,1,J1, 2' = 2.5 Hz, H-1'), 4.74 (m,1, H-3'), 4.62 and 4.60 (2 x s, 2 x 2, 3'- and 5'-O-COCH2C6H4Cl), 4.44 (m,2, and 5'-O-COCH2C6H4Cl), 4.08 (m,1, H-2'), 3.88 (s,3, 2'-OCH3). IR (KBr): cm^-1 1781 νc = 0 5'-ester, 1759 νc = 0 3'-ester. The separated IV was crystallised (MeOH):114 mg, 39% yield, m.p. 174-175°C, NMR (d6 = DMSO): δ ppm 11.2 (s,1, -NH), 8.09 (d,1, J5, 6 = 7 Hz, H-6), 6.90 (d,1, overlapped with p-chlorophenoxyacetyl, H-5), 5.85 (d,1,J1, 2' = 2.5 Hz, H-1'), 4.84 (s,2, 5'-O-COCH2C6H4Cl), 4.38 (m,2, H-5'), 4.07 (m,2, H-3' and H-4'), 3.77 (m,1, H-2'), 3.41 (s,3, 2'-OCH3). IR (KBr):cm^-1 3360 νO-H 3-hydroxyl, 1790 νc = 0 5'-ester.

N^4-Benzoylcytidine VII was obtained according to Watanabe and Fox [10].

N^4-Benzoyl-2',3'-O-methoxymethylidenecytidine VIII

VII (1.00 g, 2.88 mM), p-toluenesulphonic acid monohydrate (0.137 g, 0.72 mM, 0-25 equal), trimethyl orthoformate (5 ml) and DMF (7.5 ml) were stirred together at room temperature with exclusion of moisture. After ca 30 minutes

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VII was dissolved. TLC showed that the reaction had gone to completion after ca 50 minutes, leading to two main products VIII and IX in approximately equal amounts, and to minor products (TLC, system E4, Rf 0.15 and 0.10). The reaction mixture was neutralised pH 7 with methanolic (N-M) sodium methylate, and VIII crystallised from reaction mixture, 0.350 g, 31% yield, m.p. (after recrystallisation from EtOH) 202-206°C. TLC (table 2, system E 3) as well as NMR analysis showed that obtained VIII was a mixture of two diastereoisomers in approximately equal amounts. NMR (d6-DMSO): δ ppm 11.2 (s, 1, 4-NH), 8.26 (d, 1, J5, 6 = 7.5 Hz, H-6), 7.31 (d, 1, J5, 6 = 7.5 Hz, H-5), 6.07 and 5.81 (2 x s, 1, CH of dioxolane systems of two diastereoisomers), 5.95 (d, 1, J1, 2 = 2 Hz, H-1'), 4.90 (m, 2, H-2' and H-3'), 4.23 (m, 1, H-4').

The mother liquors were concentrated, extracted with CHCl3 (200 ml). The residue after an evaporation of CHCl3 was dissolved in CH2Cl2/MeOH (20 ml 90:2, by vol) containing traces of acid. TLC after 24 hrs showed a complete transformation of IX into VIII. The solution was concentrated and the residue was crystallised from EtOH giving an additional amount of VIII, 0.335 g, total yield 61% m.p. 188-198°C.

N4-Benzoyl-5'-O-p-chlorophenoxyacetyl-2'-O-methylcytidyl 3'-2,2,2-trichloroethyl-5' N4-benzoyl-2',3'-O-methoxymethylidene-cytidine (XI)

2,2,2-Trichloroethylphosphate dicyclohexylammonium salt (135 mg 0.305 mM) was dissolved in pyridine (10 ml), and converted into a pyridinium salt by coevaporation with pyridine (4 x 10 ml), dissolved in pyridine (10 ml), and followed by addition of TPS (183 mg, 0.605 mM). IV (79 mg, 0.149 mM) was added after 1.5 hrs. TLC showed that IV was completely converted into X after 1 day. TPS was destroyed by the addition of ice (4 g), followed by water (50 ml) after 0.5 hr. The reaction mixture was extracted with CH2Cl2 (4 x 10 ml) and the organic layer was concentrated and dried by coevaporation with pyridine (10 ml), TPS (183 mg, 0.605 mM) was added and after 0.5 hr was followed by the addition of VIII (79 mg, 0.204 mM). TLC showed a complete conversion of X into XI after 2 days. TPS was destroyed by the addition of ice (5 g), followed by water (50 ml) after 0.5 hr. The mixture was extracted with CH2Cl2 (4 x 10 ml), the organic layers were washed with water, dried (MgSO4), concentrated and chromatographed on a silica gel column (40 g) in
CHCl₃/MeOH (99:1, by vol) and gave XI as an insoluble in MeOH solid 120 mg, 72% yield.

2′-O-Methylcytidylyl(3′-5′)cytidine (CmpC)

(i) The deblocking procedure of XI into CmpC

XI (11 mg, 10 nM) was dissolved in DMF (1.0 ml) and stirred at 50°C with freshly prepared Zn-Cu couple. TLC showed complete transformation of XI into a product of Rf = 0 (system E4) after 1 day. Half-saturated at 0°C methanolic ammonia (1.0 ml) was added and the reaction mixture was left at room temperature. The unreacted Zn-Cu couple was filtered and washed with diluted methanolic ammonia after 2 days. Solvents were evaporated, the residue was dissolved in methanolic ammonia (pH 8-10) and Dowex 50 W x 8 NH₄⁺ (1 g) was added. The resin was filtered and washed with water after 15 minutes. The solution was concentrated to 10 ml and its pH brought down to 2 with ca 0.5N HCl aq. The pH was brought up to 9 with NH₃ aq. after 2 days, and neutralised after 0.5 hr. The preparative paper chromatography (system A) of the reaction mixture gave CmpC, 40 OD (pH7 H₂O), 25% yield. UV (0.1N HCl): max 282 nm, min 246 nm, ε max/ε min 2.72; E₂₅₀/E₂₆₀ 0.66; E₂₈₀/E₂₆₀ 1.71. Paper electrophoresis: 0.69.

(ii) The enzymatic analysis of CmpC with a snake venom phosphodiesterase

The mixture of CmpC (2 OD, pH H₂O 7.270 nm) in water (30 µl), 0.3% solution of a snake venom phosphodiesterase in water (40 µl) and 1M ammonium formate buffer (pH 9.2, 30 µl) was incubated at 37°C during 24 hrs. The paper electrophoresis of incubation mixture showed the presence of cytidine 5′-phosphate (pC) and 2′-O-methylcytidine (II) in approximately equal amounts as it was shown by UV and paper chromatographic analysis of electrophoresis spots.

TLC analysis of the digestion mixture (systems A and F) showed the presence of pC and II also.

This paper was supported by the Polish Academy of Sciences Project 09.3.1.
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