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Transesterifications in the Synthesis of Dinucleoside Phosphates Containing Dihydropyrimidine Nucleosides Residues

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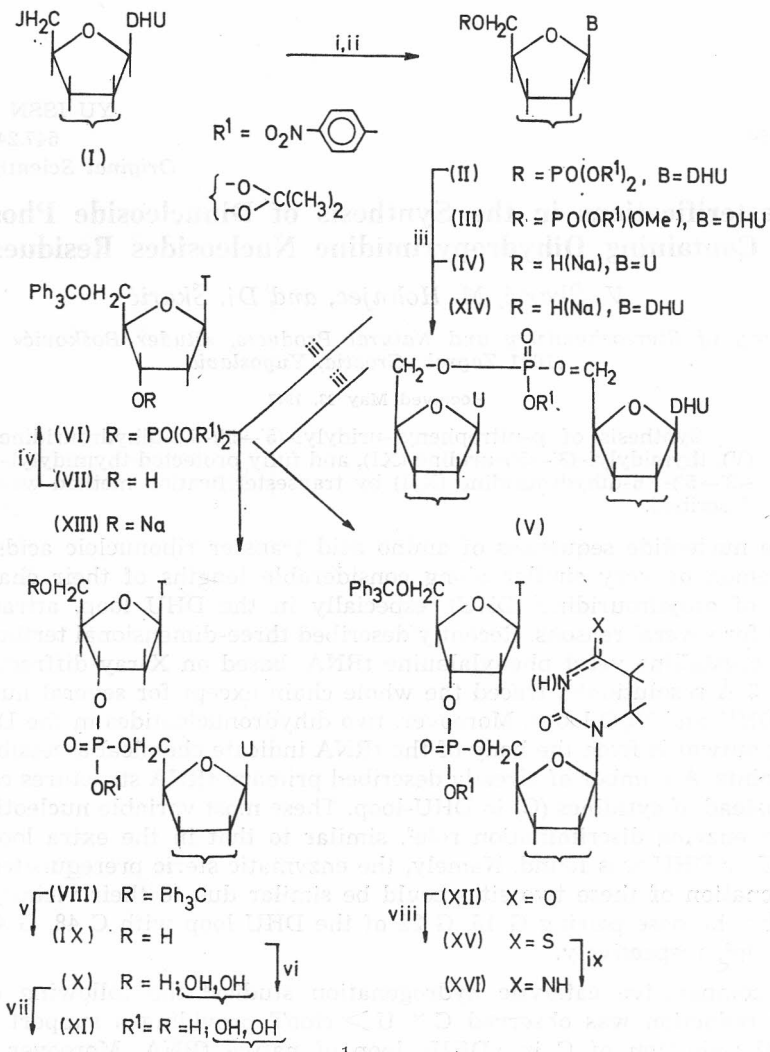
Synthesis of *p*-nitrophenyl-uridylyl-(5'→5')-5,6-dihydrouridine (V), thymidylyl-(3'→5')-uridine (XI), and fully protected thymidylyl-(3'→5')-5,6-dihydrouridine (XII) by transesterification method was described.

The nucleotide sequences of amino acid transfer ribonucleic acids (*t*RNA) are common or very similar along considerable lengths of their chains. The regions of dihydrouridine (DHU), especially in the DHU loop, attracted our interest for several reasons. Recently described three-dimensional tertiary structure of crystalline yeast phenylalanine *t*RNA, based on X-ray diffraction analysis at 3 Å resolution[1,2], traced the whole chain except for several nucleotides in the DHU and TψC loops. Moreover, two dihydronucleotides in the DHU loop arching outwards from the body of the *t*RNA indicate chemical accessibilities at these points. A number of already described primary *t*RNA structures contained DHU instead of cytidines (C) in DHU-loop. These most variable nucleotides may have an enzyme discrimination role[2], similar to that in the extra loop where either U or DHU was found. Namely, the enzymatic steric prerequisites for the hydrogenation of these two sites could be similar due to their vicinity, established by the base pairing G 15, G 22 of the DHU loop with C 48, G 46 of the extra loop[2], respectively.

In comparative catalytic hydrogenation studies[3] the following order of ease of reduction was observed C ≫ U ≻ riboT providing a support for preferential reduction of C in »DHU« loop of native *t*RNA. Moreover Zachau[4] indicated the AGC sequence conversion into AGDHU in several E. coli *t*RNA's. Our hypothesis for the biosynthetic relationship between C and U and possible alteration of some C to DHU[5] was based on the smooth displacement of the amino-group of DHC by water as nucleophile.

Furthermore, the incorporation of 5-fluorouracil into nascent *t*RNA's[6] affected U and its derivatives (ψ and riboT), but not »DHU« in DHU loop suggesting the absence of U derivative in this region of native *t*RNA's.

In order to clarify the above mentioned modifications of cytidine-containing oligonucleotides we investigated the synthesis of dinucleoside phosphates containing the chemically sensitive 5,6-dihydrocytidine residue. The selective monothiation of 5,6-dihydrouridine into 4-thio analogue[7] and its amination into



Reagents: i, $PhCH_3$-$AgOPO(OR^1)_2$; ii, $Na^+ {}^-OMe$-MeOH-CH_2Cl_2; iii, Na-$O(CH_2CH_2)_2O$; iv, $(R^1O)_2OPCl$-py; v, 80% HOAc (5 min); vi, 40% HOAc (90 min); vii, snake venom phosphodiesterase-0.1 M Tris buffer; viii, P_2S_5-py; ix, NH_3-$O(CH_2CH_2)_2O$.

5,6-dihydrocytidine[5] in the preformed dinucleoside phosphate offered an approach mild enough to preserve dihydrocytidylic moiety.

The fact that the cyclic structure of 1-*N*-substituted 5,6-dihydrouracil was preserved in the presence of sodium methoxide[8] encouraged us to attempt the dinucleoside phosphate synthesis by transesterification method. Thus 2,3'-*O*--isopropylidene-5,6-dihydrouridine-5'-di-*p*-nitrophenyl phosphate (II) was successfully transesterified when treated with sodium methoxide in methanol or with a dioxan solution of in situ obtained 5'-*O*-sodium-2',3'-*O*-isopropylidene-

-uridine (IV, R=Na) yielding 2,3'-*O*-isopropylidene-5,6-dihydrouridine-5'-*p*-nitrophenyl-methyl phosphate (III) and *p*-nitrophenyl-2',3'-*O*-isopropylidene-uridylyl-(5'→5')-2',3'-*O*-isopropylidene-5,6-dihydrouridine (V), respectively.

The 5'-*O*-triphenylmethyl-thymidine-3'-*O*-di-*p*-nitrophenyl phosphate (VI) was then examined in transesterification reaction with 5'-*O*-sodium-2',3'-*O*-isopropylidene uridine (IV, R=Na). Thus obtained *p*-nitrophenyl-5'-*O*-triphenylmethyl-thymidylyl-(3'→5')-2',3'-*O*-isopropylidene-uridine (VIII) was deprotected by 80% acetic acid (5 min) and then by 40% (90 min) affording *p*-nitrophenyl--thymidylyl-(3'→5')-2',3'-*O*-isopropylidene uridine (IX) and then *p*-nitrophenyl thymidylyl-(3'→5')-uridine (X).

The dinucleoside phosphates having masked the anionic site by *p*-nitrophenyl group were soluble in organic solvents and amenable for conventional separation and characterization techniques. In addition *p*-nitrophenyl group in phosphotriester X was susceptible to selective cleavage in 0.1 mol/dm^3. Tris buffer solution and in the presence of 0.3 mol/dm^3 $Mg(OAC)_2$ and moderate amount of snake venom phosphodiesterase. The formation of thymidylyl-(3'→5')--uridine (XI) was easily followed by the precipitation of the yellow coloured *p*-nitrophenolate. The contribution of phosphodiesterase in this specific hydrolysis remained to be examined.

Since the transesterification method provided a convenient route to dinucleoside phosphate containing dihydrouridylic acid, phosphotriester VI, in reaction with in situ obtained 5'-*O*-sodium-2',3'-*O*-isopropylidene-5,6-dihydrouridine (XIV, R=Na), was successfully transformed into the *p*-nitrophenyl-methyl-thymidylyl-(3'→5')-2',3'-*O*-isopropylidene-5,6-dihydrouridine (XII). It is worth noting that the synthesis of this dinucleoside phosphate from phosphotriester II showed much lower reactivity toward a freshly prepared dioxan solution of 5'-*O*-triphenylmethyl-3'-*O*-sodium thymidine (XIII).

In a preliminary small-scale reaction to test the effectiveness of the thiation of dinucleoside phosphate XII it was found that the reaction with phosphorous pentasulphide was selective enough to give *p*-nitrophenyl-5'-*O*-triphenylmethyl--thymidylyl-(3'→5')-2',3'-*O*-isopropylidene-5,6-dihydro-4-thiouridine (XV). The product XV isolated from this reaction was then aminated by anhydrous ammonia in dioxan into the corresponding thymidylyl-(3'→5')-5,6-dihydrocytidine derivative XVI. The UV absorption maxima of dinucleoside phosphates XV and XVI at λ_{max} 283 nm and 260 nm, respectively, are consistent with indicated structural transformations.

EXPERIMENTAL

The same techniques and apparatus were used as described previously[5].

2',3'-O-*Isopropylidene-uridine-5'-di*-p-*nitrophenyl Phosphate*

To a solution of 5'-deoxy-5'-iodo-2',3'-*O*-isopropylideneuridine[9] (394 mg, 1 mmol) in anhydrous toluene (100 ml) silver di-*p*-nitrophenyl phosphate (894 mg, 2 mmol) was added and the suspension refluxed for 20 h. The precipitate was filtered off, and the filtrate evaporated to dryness. The product crystallized from ethanol in quantitative yield, m. p. 118—120 °C (Lit.[10] 118—120 °C).

2',3'-O-*Isopropylidene-5,6-dihydrouridine-5'-di*-p-*nitrophenyl Phosphate* (II)

From 5'-iodo-5,6-dihydrouridine (I) (396 mg, 1 mmol) in toluene (100 ml) and silver di-*p*-nitrophenyl phosphate (670 mg, 1.5 mmol) following above described procedure a quantitative yield of the product was obtained, m. p. 196—198 °C (from ethanol).

Anal. $C_{24}H_{25}N_4O_{13}P$ (608.44) calc'd.: C 47.37; H 4.15; N 9.21; P 5.07%
found: C 47.70; H 4.02; N 9.57; P 5.27%

2',3'-O-Isopropylidene-5,6-dihydrouridine-5'-p-nitrophenyl, Methyl Phosphate (III)

2',3'-O-Isopropylidene-5,6-dihydrouridine-5'-di-*p*-nitrophenyl phosphate (II) (61 mg, 0.1 mmol) in methanol-methylene chloride (4 ml, 1:1) was treated with 0.1 mol/dm³ sodium methoxide in methanol (1 ml) and set aside for 50 min at room temperature. The solution was concentrated to 1 ml and the foamy product (44 mg, 88%) separated by preparative TLC [ether-methylene chloride (3:2), ether as eluant], R_f ca. 0.2.

Anal. $C_{19}H_{24}N_3O_{11}P$ (501.37) calc'd.: C 45.51; H 4.83; N 8.38; P 6.18%
found: C 45.85; H 5.05; N 8.57; P 6.29%

p-*Nitrophenyl 2',3'-O-isopropylidene-uridylyl-(5'→3')-2',3'-O-isopropylidene-5,6-dihydrouridine* (V)

To a solution of 2',3'-isopropylidene-uridine (IV, R=H) (142 mg, 0.5 mmol) in anhydrous dioxan (5 ml) sodium (9 mg, 0.375 mmol) was added and refluxed for 15 h. Thus obtained solution was treated with 2',3'-O-isopropylidene-5,6-dihydrouridine-5'-di-*p*-nitrophenyl phosphate (II) (152 mg, 0.25 mmol) and set aside at room temperature for 20 h. A yellow precipitate (135 mg) separated by filtration and the filtrate passed through a silica gel (3 g) column. The eluate was then evaporated to dryness and the preparative TLC [ether-acetone-methylene chloride (2:1:1)] separated: compounds (II), R_f ca. 0.9 (37 mg) and (IV), R_f ca. 0.4 (52 mg), as starting materials, and the product, R_f ca. 0.35 (70 mg). An additional amount of the product (28 mg) was obtained from organic layer when the afore-mentioned yellow precipitate had been partitioned between water and methylene chloride [overall yield 98 mg, 68.5% based on phosphate (II)].

Anal. $C_{30}H_{38}N_5O_{16}P$ (755.61) calc'd.: C 47.68; H 5.07; N 9.27; P 4.09%
found: C 48.01; H 5.35; N 9.55; P 3.95%

5'-O-Triphenylmethyl-thymidine-3'-O-di-p-nitrophenyl Phosphate (VI)

To a solution of di-*p*-nitrophenyl phosphorochloridate[11] (540 mg, 1.5 mmol) in pyridine (20 ml) 5'-O-triphenylmethylthymidine (VII) (500 mg, 1.035 mmol) was added, stirred at room temperature for 1 h and then evaporated to dryness. From the silica gel (40 g) column methylene chloride (100 ml) eluted and unidentified mixture. Methylene chloride-ether (1:1, 200 ml) eluted a foamy product (633 mg, 76%).

Anal. $C_{41}H_{35}N_4O_{12}P$ (806.69) calc'd.: C 61.04; H 4.37; N 6.95; P 3.84%
found: C 61.19; H 4.32; N 7.19; P 3.78%

UV spectrum: λ_{max} 223 and 270 br nm (log ε 4.05 and 4.25), λ_{min} 242 nm (log ε 3.78).

p-*Nitrophenyl 5'-O-Triphenylmethyl-thymidylyl-(3'→5')-2',3'-O-isopropylidene-uridine* (VIII)

To a solution of 2',3'-O-isopropylidene-uridine (IV, R=H) (142 mg, 0.5 mmol) in anhydrous dioxan (5 ml) sodium (9 mg, 0.375 mmol), was added and refluxed for 15 h. Thus obtained solution was treated with 5'-O-triphenylmethyl-thymidine-3'-di-*p*-nitrophenyl phosphate (VI) (202 mg, 0.25 mmol) at room temperature for 24 h and then evaporated to dryness. The residue was partitioned between water and methylene chloride and the organic layer chromatographed on a silica gel (10 g) column. Ether-methylene chloride (2:1, 250 mg) eluted the product (145 mg, 59,3%), R_f ca. 0.3 [TLC in ether-methylene chloride (2:1)].

Anal. $C_{47}H_{46}N_5O_{15}P$ (951.85) calc'd.: C 59.30; H 4.87; N 7.36%
found: C 59.24; H 4.97; N 7.79%

p-*Nitrophenyl Thymidylyl-(3'→5')-2',3'-O-isopropylidene-uridine* (IX)

A solution of *p*-nitrophenyl 5'-*O*-triphenylmethyl-thymidylyl-(3'→5')-2',3'-*O*-isopropylidene-uridine phosphate (VIII) (240 mg, 0.252 mmol) in 80% acetic acid (10 ml) was refluxed for 5 min and then evaporated to dryness. The foamy product (109 mg, 61%) separated by preparative TLC [ether-acetone (2:1), acetone as eluant], R_f ca. 0.2.

Anal. $C_{28}H_{32}N_5O_{15}P$ (709.55) calc'd.: C 47.39; H 4.55; N 9.87; P 4.36%
found: C 47.32; H 4.30; N 9.58; P 4.11%

p-*Nitrophenyl Thymidylyl-(3'→5')-uridine* (X)

To a solution of *p*-nitrophenyl thymidylyl-(3'→5')-2',3'-*O*-isopropylidene-uridine (IX) (50 mg, 0.07 mmol) in 40% acetic acid was refluxed for 90 min and then evaporated to dryness. The residue was purified by TLC [methylene chloride-acetone (1 : 1), acetone as eluant], R_f ca. 0.2, to a foamy product (31 mg, 66%).

Anal. $C_{25}H_{28}N_5O_{15}P$ (669.48) calc'd.: C 44.85; H 4.21; N 10.46; P 4.63%
found: C 44.70; H 4.43; N 10.22; P 4.30%.

Mass spectrum calc'd.: (M^+) T 258, U 244; found: T 257, U 243.

Thymidylyl-(3'→5')-uridine (XI)

To *p*-nitrophenyl thymidiylyl-(3'→5')-uridine (X) (18 mg, 0.027 mmol) in 0.1 mol/dm³. Tris buffer solution (1 ml) 0.3 mol/dm³ $Mg(OAc)_2$ (0.5 ml) was added and then treated at 37 °C with 30 units of phosphodiesterase (CalBiochem), from Russell's viper venom (Crotalus adamanteus). The electrophoresis of the product was performed on thyn layer cellulose in 0.05 mol/dm³ Na_2HPO_4 buffer solution at 400 V cm^{-1}, 5 mA, and 45 min. duration. After 10 h the completion of digestion was proved by the disappearance of electrophoretic mobilities of starting material at 0.15 (mmV^{-1} h^{-1} cm) and the appearance of the product at 0.57 (mmV^{-1}, h^{-1} cm) relative to uridylic acid. *p*-Nitrophenol appeared at 1.0 (mmV^{-1}, h^{-1} cm).

p-*Nitrophenyl 5'-O-triphenylmethyl-(3'→5')-2',3'-O-isopropylidene-5,6-dihydrouridine* (XII)

(a) A solution of 5'-*O*-triphenylmethyl thymidine (VII) (242 mg, 0.5 mmol) in anhydrous dioxan (5 ml) was treated with sodium (9 mg, 0.375 mmol) and refluxed for 20 h. To this solution of 3'-*O*-sodium-thymidine (XIII) 2',3'-*O*-isopropylidene-5,6-dihydrouridine-5'-di-*p*-nitrophenyl phosphate (II) (152 mg, 0.25 mmol) was added and the mixture stirred at room temperature for 28 h. The yellow crystalline product, as sodium *p*-nitrophenolate (28 mg), was filtered off and the filtrate evaporated to dryness. The residue was partitioned between methylene chloride-water. From the organic layer preparative TLC [ether-methylene chloride (2 : 1), acetone as eluant] separated a fraction (213 mg), R_f ca. 0.6, as 5'-*O*-triphenylmethyl-thymidine (VII) and the product, R_f 0.1 (34 mg, 14%).

Anal. $C_{47}H_{48}N_5O_{15}P$ (953.86) calc'd.: C 59.18; H 5.07; N 7.34%
found: C 58.91; H 5.27; N 7.06%.

UV spectrum: λ_{max} 268 br nm (log ε 3.91) λ_{min} 234 nm (log ε 3.65).

(b) To a solution of 2',3'-isopropylidene-5,6-dihydrouridine (XIV, R=H) (500 mg, 1.75 mmol) in anhydrous dioxan (15 ml) sodium (30 mg, 1.3 mmol) was added and refluxed for 15 h. This solution of 5'-*O*-sodium-5,6-dihydrouridine (XIV, R = Na) was treated with 5'-*O*-triphenylmethyl-thymidine-3'-di-*p*-nitrophenyl phosphate (VI) (271 mg, 0.34 mmol) and set aside at 40 °C for 18 h. The precipitate was filtered off, and the filtrate evaporated to dryness. The residue was chromatographed in methylene chloride on a silica gel (15 g) column. Ether-methylene chloride (1 : 1) eluted the product (152 mg, 47.5%), R_f ca. 0.1 [ether-methylene chloride (2 : 1)].

p-*Nitrophenyl 5'-O-triphenylmethyl-thymidylyl-(3'→5')-2',3'-O-isopropylidene-5,6-dihydro-4-thiouridine* (XV)

Thymidylyl-(3'→5')-5'6-dihydrouridine (XII) (30 mg, 0.032 mmol) in freshly distilled pyridine (2 ml) was treated with phosphorous pentasulphide (15 mg, 0.067 mmol) and

refluxed for 30 min. The solvent was removed by evaporation, the residue extracted with methylene chloride, and then purified by preparative TLC [methylene chloride--ether (1 : 1)]. The yellow coloured fraction, R_f ca. 0.4, was eluted with ether. Yield 14 mg (46%), λ_{max} 283 br nm (log ε 4.11), λ_{min} 238 nm (log ε 3.62). 2',3',5'-Tri-*O*-acetyl--4-thio-5,6-dihydrouridine[5] showed λ_{max} at 279 nm (log ε 4.22).

p-Nitrophenyl 5'-O-triphenylmethyl-thymidylyl-(3'→5')-2',3'-O-isopropylidene--5,6-dihydrocytidine (XVI)

Into a solution of thymidylyl-(3'→5')-5,6-dihydro-4-thiouridine (XV) (10 mg) in anhydrous dioxan (1 ml) anhydrous ammonia was bubbled for 30 min, and then evaporated to dryness. The residue was chromatographed on preparative TLC [methylene chloride-methanol (6 : 1)]. Methanol eluted the product (3 mg, 29%), R_f ca. 0.2, λ_{max} 260 br nm (log ε 3.98).

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SAŽETAK

Transesterifikacije u sintezi dinukleozid-fosfata s dihidropirimidin-nukleozidima kao komponentama

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Metodom transesterifikacije sintetizirani su *p*-nitrofenil-uridilil-5'→5')-5,6-dihidrouridin (V), timidilil-(3'→5')uridin (XI) i potpuno zaštićeni timidilil-(3' 5')-5,6-dihidrouridin.

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