Hydrolysis of 2'- and 3'-*C*-Methyluridine 2',3'-Cyclic Monophosphates and Interconversion and Dephosphorylation of the Resulting 2'- and 3'-Monophosphates: Comparison with the Reactions of Uridine Monophosphates

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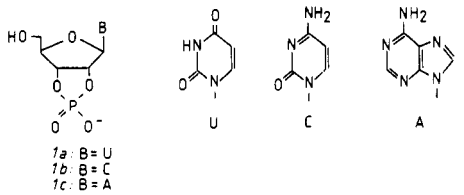
2',3'-Cyclic monophosphates of 2'- and 3'-*C*-methyluridines have been prepared and shown to hydrolyze to a mixture of the corresponding 2'- and 3'-monophosphates. The predominant product isomer is the one having the tertiary hydroxyl group phosphorylated, but on longer treatment a phosphate migration from the tertiary to secondary hydroxyl function takes place. Hydrolytic dephosphorylation competes with the phosphate migration, the tertiary hydroxyl group being dephosphorylated 1 order of magnitude faster than the secondary one. Kinetics of the partial reactions have been described and compared to the data obtained with uridine 2',3'-cyclic monophosphate. The tertiary monophosphate has been shown to be exceptionally susceptible to nucleophilic attack of the neighboring hydroxyl group.

Introduction

Five-membered cyclic phosphodiesters appear as intermediates in the enzymic hydrolysis of ribonucleic acids (RNA). Ribonucleases, i.e., the enzymes catalyzing the cleavage of the internucleosidic phosphodiester bonds, exert their catalytic action in two steps: nucleophilic attack of the neighboring 2'-hydroxyl group on tetracoordinated phosphorus with concomitant loss of the 5'-linked nucleoside gives a nucleoside 2',3'-cyclic monophosphate that is subsequently hydrolyzed to nucleoside 3'-monophosphate.[1] Hydrolysis of monomeric nucleoside 2',3'-cyclic monophosphates (2',3'-cNMP, **1**) has been the subject of considerable interest as a model reaction of the latter step of ribonuclease action. This reaction is known to be strongly exothermic, the $\Delta H°$ values ranging from −33 kJ mol^{-1} (2',3'-cUMP, **1a**) to −40 kJ mol^{-1} (2',3'-cGMP).[2] The acid-catalyzed reaction is of second order in hydronium ion concentration under conditions where the substrate monoanion is the major form, indicating that the reaction takes place via the monocationic phosphodiester.[3-5] The hydroxide-ion-catalyzed hydrolysis is, in turn, a first-order reaction.[3,4] Moreover, a kinetically significant uncatalyzed hydrolysis occurs.[4,5] All reactions give a mixture of nucleoside monophosphates, consisting of 40% 2'-isomer (2'-NMP) and 60% 3'-isomer (3'-NMP).[5] Neither the product composition nor the hydrolysis rate is markedly influenced by the structure of the base moiety.[3]



1a: B = U
1b: B = C
1c: B = A

Besides hydronium and hydroxide ions, undissociated acids and bases may catalyze the hydrolysis of 2',3'-cNMP. Eftink and Biltonen[4] established that both imidazole and imidazolium ion may act as a catalyst and proposed a concerted mechanism on the basis of the observed second-order dependence of rate on imidazole concentration. Yoshinari and Komiyama,[6] in turn, reported on significant catalysis by monocations of alkylene diamines and suggested a bifunctional mechanism involving a general acid catalysis by the protonated amino group (protonation of the phosphate group) and a general base catalysis by the neutral amino function (deprotonation of the attacking water molecule).

It is also known that the hydrolysis products, 2'- and 3'-NMP, undergo interconversion under neutral and acidic conditions.[5,7] This mutual isomerization, however, leads to the same equilibrium composition of 2'- and 3'-NMP as the much faster hydrolysis of 2',3'-cNMP, and hence the product distribution of the latter reaction remains unchanged as a function of time.

In 2',3'-cNMPs both esterified hydroxyl functions are secondary hydroxyl groups. Accordingly, it is not surprising that both P-O2' and P-O3' bonds are cleaved at comparable rates, and that migration of the phosphate group between O2' and O3' atoms yields an equilibrium mixture consisting of comparable amounts of 2'- and 3'-NMP. The present study is aimed at clarifying how the situation is changed if one of the esterified hydroxyl groups is a tertiary one. For this purpose, hydrolysis of 2',3'-cyclic monophosphates of 2'-*C*-methyl- (**2**) and 3'-*C*-methyluridine (**3**) and interconversion of the resulting 2'- and 3'-monophosphates (**4a,b**, **5a,b**) have been studied. These nucleotides still contain all functionalities of the naturally occurring nucleotides, i.e., all the possible binding sites for enzymes. It is known that (i) the 5'-triphosphate of **4c** is a substrate of DNA-dependent RNA polymerase from *E. coli* and may substitute 5'-UTP in enzymic RNA synthesis,[8,9] (ii) the 5'-triphosphate of **5c** is a terminating substrate of the same enzyme,[8,10] and (iii) **3** (but not **2**) is

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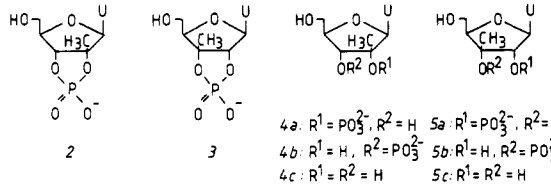
Table I. First-Order Rate Constants and Product Distributions for Hydrolysis of 2',3'-Cyclic Monophosphates of Uridine (1a), 2'-C-Methyluridine (2), and 3'-C-Methyluridine (3) in Aqueous Alkali at 323.2 K[a]

compd	$[OH^-]$/mol/L	$k_{obs}/10^{-4}$ s^{-1}	$\chi(3')$[b]
1a	0.05	8.6 ± 0.4	0.59
	0.01	1.37 ± 0.03	0.58
2	0.05	5.29 ± 0.01	0.31
	0.01	0.843 ± 0.005	0.33
3	0.05	5.84 ± 0.09	0.70
	0.01	0.844 ± 0.008	0.72

[a] The ionic strength adjusted to 0.1 mol/L with sodium chloride. [b] Mole fraction of the 3'-monophosphate in the mixture of 2'- and 3'-monophosphates.

slowly hydrolyzed by pancreatic RNAse A.[11,12] Understanding of the solvolytic reactions may, in part, help to elucidate the enzymic reactions.



Results and Discussion

2'-C-Methyl- (2) and 3'-C-methyl-2',3'-cyclic monophosphates (3) were obtained by phosphorylating the 2',3'-O-dibutylstannylidene derivatives[13] of 4c[14] and 5c[11,15] with diphenyl chlorophosphate and cleaving the phenyl blocking groups by successive hydrolysis in water and aqueous alkali. The resulting isomeric mixtures of 2'- and 3'-monophosphates (4a/4b; 5a/5b) were cyclized to 2 and 3 in the presence of N,N'-dicyclohexylcarbodiimide.

Hydrolysis of 2'-C-methyl- (2) and 3'-C-methyluridine 2',3'-monophosphates (3) was followed by HPLC, and the products were identified by ^{1}H NMR spectroscopy. Table I records the pseudo-first-order rate constants and product compositions observed in aqueous alkali for 2 and 3 and their unmodified analogue, 2',3'-cUMP (1a). With all these compounds the reaction is first-order in hydroxide ion concentration and yields a mixture of 2'- and 3'-monophosphates. The C-methyluridine derivatives 2 and 3 are hydrolyzed from 30 to 40% less readily than 2',3'-cUMP. The predominant isomer among the products is in both cases the one having the tertiary hydroxyl group phosphorylated. Neither dephosphorylation nor migration of the phosphate group from the tertiary to secondary hydroxyl function could be observed.

The observed predominance of the tertiary monophosphates (4a, 5b) among the products is consistent with the guidelines presented by Westheimer[16] for hydrolysis of phosphoesters. According to this concept, a nucleophilic attack at tetracoordinated phosphorus results in a trigonal bipyramidal intermediate (or transition state). The entering group is assumed to adopt an apical position, having

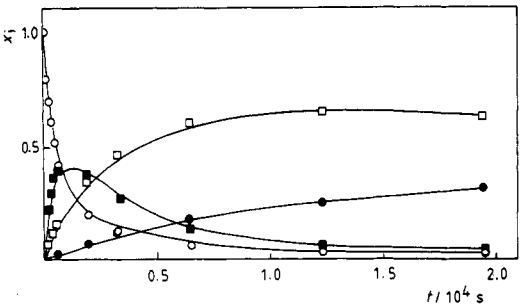
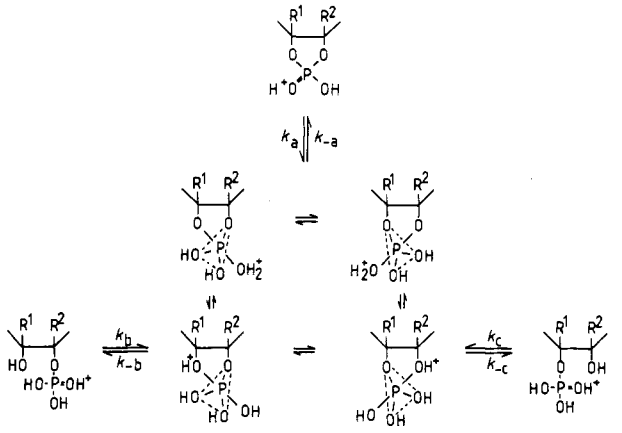


Figure 1. Time-dependent product distribution for the hydrolysis of 3'-C-methyluridine 2',3'-cyclic monophosphate (3; ○) in aqueous hydrogen chloride (0.010 mol/L; I = 0.10 mol/L with NaCl) at 363.2 K. Notation: 3'-C-methyluridine 2'-monophosphate (5a; □), 3'-C-methyluridine 3'-monophosphate (5b; ■), and 3'-C-methyluridine (5c; ●). The lines drawn are based on the Runge–Kutta simulation.

Scheme I



a longer bond length to phosphorus than the three equatorial ligands, and departure of the leaving group is possible from apical position only. Electronegative ligands prefer an apical position. Since the secondary hydroxyl group, i.e., 3'-OH in 4a and 2'-OH in 5b, is undoubtedly more acidic than the tertiary one (2'-OH in 4b and 3'-OH in 5a), it is expected that this group preferentially takes an apical position and hence leaves more rapidly than the tertiary one.

Under acidic conditions the reactions of 2 and 3 are more complex. Figure 1 shows the time-dependent product distribution for the hydrolysis of 3 in aqueous hydrogen chloride (0.01 mol dm^{-3}). Disappearance of the starting material is accompanied with formation of a mixture of 2'- (5a) and 3'-monophosphates (5b) of 3'-C-methyluridine. The composition of this mixture (23% 5a; 77% 5b) is similar to that obtained by alkaline hydrolysis, and it remains practically unchanged during the first half-life of the hydrolysis reaction. However, after this initial period the 2'-monophosphate (5a) becomes gradually the predominant isomer ($\chi_{5a} > 0.9$). Simultaneously, 3'-C-methyluridine (5c) begins to accumulate, most likely via hydrolytic dephosphorylation of 5a and 5b. The results obtained with the 2'-C-methylated cyclic monophosphate (2) were analogous: a mixture of 2'- (4a) and 3'-monophosphates (4b), consisting of 65% 4a, is initially formed, and subsequently a phosphate migration from the tertiary 2'-OH to secondary 3'-OH takes place. Accordingly, the reactions taking place under acidic conditions may be depicted by Scheme I.

When 3'-C-methyluridine 3'-monophosphate (5b) was used as a starting material instead of the 2',3'-cyclic monophosphate 3, the time-dependent product distribu-

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Table II. First-Order Rate Constants for Hydrolysis of the 2′,3′-Cyclic Monophosphates of Uridine (1a), 2′-*C*-Methyluridine (2), and 3′-*C*-Methyluridine (3) and Interconversion and Dephosphorylation of the Resulting 2′- and 3′-Monophosphates under Acidic Conditions at 363.2 K[a]

compd	−log ([H⁺]/ mol/L)	$k_i/10^{-4}$ s^{-1}							
		k_1	k_{-1}	k_2	k_{-2}	k_3	k_{-3}	k_4	k_5
1a	2.0	7.3	0.02[b]	13.0	0.02[b]	0.25[c]	0.17[c]	0.11[c]	0.11[c]
	5.0[d]	0.0042		0.010		0.050[c]	0.033[c]	0.12[c]	0.12[c]
2	2.0	10.0	3.1	5.0	0.11	1.7	0.11	e	e
3	2.0	3.6	0.085	12.2	4.4	0.085	1.3	0.081	0.71
	5.0[d]	0.0027	0.000 10	0.011	0.0043	0.029	0.44	0.100	1.04

[a] For the rate constants see Scheme I. The ionic strength adjusted to 0.1 mol/L with sodium chloride. [b] Estimated on the basis of the previous observation,[10] according to which incorporation of ^{18}O from solvent water to 2′/3′-NMP is 1 order of magnitude slower than the phosphate migration. [c] From ref 7. [d] Adjusted with an acetic acid/sodium (0.02/0.04 mol/L) acetate buffer.

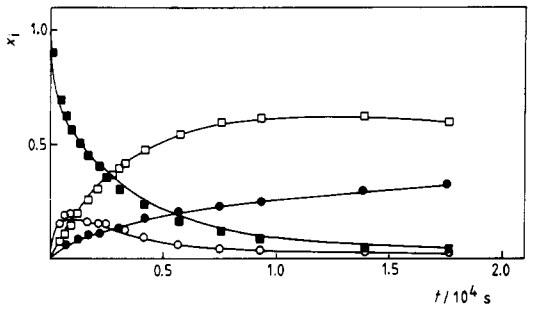
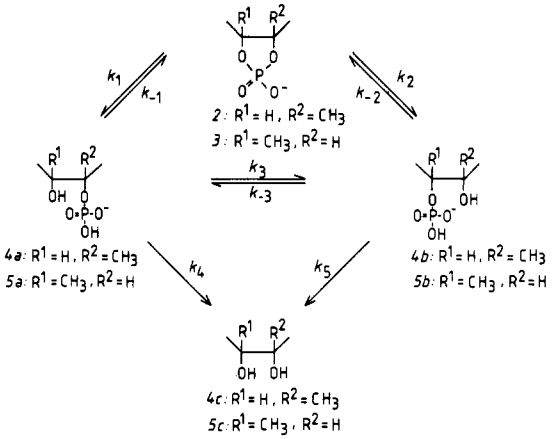


Figure 2. Time-dependent product distribution for the isomerization of 3′-*C*-methyluridine 3′-monophosphate (**5b**; ■) to the corresponding 2′-monophosphate (**5a**; □) in aqueous hydrogen chloride (0.010 mol/L; I = 0.10 mol/L with NaCl) at 363.2 K. Notation: 3′-*C*-methyluridine 2′,3′-cyclic monophosphate (**3**; ○) and 3′-*C*-methyluridine (**5c**; ●). The lines drawn are based on the Runge–Kutta simulation.

Scheme II



tion depicted in Figure 2 was obtained. Consistent with Scheme I, the isomerization of **5b** to **5a** is accompanied by formation of the cyclic phosphate **3**. The mole fraction of this species is, however, markedly reduced when **5a** becomes the predominant isomer. In other words, the equilibrium constant for the cyclization reaction is considerably greater with the tertiary 3′-monophosphate than with the secondary 2′-monophosphate. As noted above, dephosphorylation of **5a** and **5b** competes with their interconversion.

Table II records the values obtained by Runge–Kutta simulation for the rate constants indicated in Scheme I. These data allow the following conclusions to be drawn. (i) The hydrolysis rates of **2** and **3** ($k_1 + k_2$) are almost equal, and about 80% of that of 2′,3′-cUMP. (ii) As in aqueous alkali, the predominant hydrolysis product is the tertiary monophosphate, **4a** or **5b** (k_2/k_1 is 0.5 and 3.4 with **2** and **3**, respectively). (iii) The tertiary monophosphates (**4a** and **5b**) are cyclized to 2′,3′-cyclic monophosphates (**2** and **3**) 50 times as rapidly as the secondary monophosphates (k_{-2}/k_{-1} is 0.035 and 50 with **4a,b** and **5a,b**, respectively). The cyclization rates of the secondary monophosphates are comparable to that of 2′- and 3′-NMP,[5] and hence cyclization of the tertiary monophosphates is unexpectedly rapid. (iv) The phosphate migration from tertiary to secondary hydroxyl group is 1 order of magnitude faster than the reverse reaction (k_{-3}/k_3 is 0.065 and 0.15 with **4a,b** and **5a,b**, respectively) and also faster than migration between secondary 2′- and 3′-OH groups of NMPs.[5,7] (v) The tertiary monophosphates are dephosphorylated 1 order of magnitude faster than the secondary ones (k_5/k_4 is 10 with **5a,b**). (vi) About 40% of the migration takes place via intermediary formation of the cyclic monophosphate. With 2′,3′-cAMP (**1c**) this route has been estimated to be less important.[5]

The acid-catalyzed hydrolysis of 2′,3′-cNMPs and interconversion of the resulting 2′- and 3′-NMP has been suggested[5] to take place via a common pentacoordinated intermediate, which is formed either by an attack of a water molecule on the monocationic cyclic phosphodiester or by an intramolecular attack of the neighboring hydroxyl group on monocationic phosphate group of 2′- or 3′-NMP (Scheme II). According to the pseudorotation concept of Westheimer,[16] the attacking nucleophile initially adopts an apical position, but the various phosphorane structures obtained may equilibrate via pseudorotation and protolytic rearrangement. Comparison of Scheme II to the minimal reaction scheme (Scheme I) enables estimation of the ratios of the partial rate constants, k_{-b}/k_{-c} and k_b/k_c, indicated in the former scheme. When hydrolysis of **3** is used as an example, the following equations may be written: $k_{-b}/k_{-c} = k_1/k_2 = 0.3$ and $(k_c k_{-b})/(k_{-c} k_b) = k_3/k_{-3}$. Substitution of the values of k_{-b}/k_{-c} and k_3/k_{-3} in the latter equation gives $k_b/k_c = 0.02$. Accordingly (i) the secondary oxygen ligand (O2′) departs from the phosphorane intermediate three times as readily as the tertiary one (O3′), consistent with its greater acidity or apicophilicity, and (ii) the secondary hydroxyl group (2′-OH) attacks the neighboring phosphate group 50 times as readily as the tertiary one (3′-OH). For the following reason we believe that the exceptionally high susceptibility of tertiary monophosphates **4a**, **5b** to nucleophilic attack of the neighboring hydroxyl group is of electronic origin and does not result from the effect of *C*-methyl substituents on sugar ring puckering. The previous data[11,14] together with the values of the $^1H,^1H$-coupling constants (Table III) indicate that **2** and its 2′-monophosphate (**4a**) adopt an N-type conformation, while **3** and its 3′-monophosphate (**5b**) prefer S puckering. However, with both **4a** and **5b** the attack on the phosphorus atom linked to a tertiary hydroxyl group is exceptionally rapid, i.e., considerably faster than the corresponding reaction with 2′,3′-cUMP.

The dephosphorylation of 2′- and 3′-NMP has been suggested[5,7] to proceed by the dissociative mechanism

Table III. LC Retention Times (t_R) and ^1H NMR Spectroscopic Data for the Compounds Studied

		^1H chemical shifts/$^1H,^1H$ and $^1H,P$ coupling constants								
		chemical shifts[b]								
compd	t_R/min[a]	H6	H5	H1′	H2′	H3′	H4′	H5′	H5″	CH_3
2	7.1	7.76	5.89	6.08		4.47	4.25	3.96	3.86	1.35
3	6.0	7.74	5.87	5.84	4.69		4.23	3.90	3.79	1.51
4a	4.4	7.89	5.87	6.35		3.90	4.10	4.00	3.85	1.47
4b	6.9	7.90	5.89	5.97		4.31	4.14	4.02	3.90	1.24
4c	10.3	7.87	5.87	5.98		3.86	4.01	4.01	3.82	1.18
5a	7.6	7.92	5.89	6.07	4.48		4.06	3.79	3.75	1.39
5b	3.8	7.90	5.89	6.00	4.11		4.58	3.81	3.72	1.57
5c	8.7	7.90	5.90	5.95	4.14		4.07	3.78	3.72	1.32

	coupling constants[c]						
	$J_{H5,H6}$	$J_{H1',H2'}$	$J_{H3',H4}$	$J_{H4',H5'}$	$J_{4',5''}$	$J_{P,H2'}$	$J_{P,H3'}$
2	8.1		6.2	3.3	5.2		13.7
3	8.0	3.8		3.9	7.3	9.7	
4a	8.1		9.2	2.5	4.4		
4b	8.1		9.2	2.4	4.1		9.4
4c	8.2		9.3	2.3	4.2		
5a	8.1	8.0		3.2	4.0	9.8	
5b	8.1	7.9		3.4	5.0	2.6	
5c	8.1	7.7		3.5	5.0		

[a] On a Hypersil ODS column (4 × 250 mm, 5 μm), flow rate 1 mL min^{-1}, eluent acetic acid buffer (pH 4.2) containing 0.1 mol/L ammonium chloride. [b] Given as ppm from external TMS. The spectra recorded on a Jeol GX-400 spectrometer in ^2H_2O at 309 K. [c] Given in Hz.

established[17] for the hydrolysis of simple monoalkyl phosphates. Accordingly, the reactive species is the monoanionic phosphoester, and the reaction involves a preequilibrium proton transfer from the hydroxyl ligand to the esterified oxygen and a subsequent rate-limiting heterolysis to free alcohol and a metaphosphate ion. The latter species is, however, too short-lived to be regarded as a real intermediate in aqueous solution. Most likely the metaphosphate ion is released, not as a free species, but preassociated with a water molecule.[17,18] The data in Table II reveal that the phosphate group is hydrolyzed from the secondary hydroxyl group (3′-OH of **4b** or 2′-OH of **5a**) approximately as rapidly as from the secondary hydroxyl groups of 2′,3′-cNMPs,[5,7] whereas the dephosphorylation of the tertiary hydroxyl group (2′-OH of **4a** or 3′-OH of **5b**) occurs 1 order of magnitude faster. Since it is known that 2′- and 3′-UMPs are dephosphorylated almost 10 times as fast as 5′-UMP,[7] the hydrolysis rate of phosphomonoesters derived from tertiary, secondary, and primary hydroxyl groups appears to decrease in this order.

In summary, the results of the present investigation show that insertion of a methyl group on C2′ or C3′ of a nucleoside 2′,3′-cyclic monophosphate has only a modest effect on the hydrolytic stability of the five-membered cyclic phosphodiester but affects markedly the product distribution. The phosphate migration from tertiary to secondary hydroxyl group and the cyclization and dephosphorylation of the tertiary monophosphates all proceed considerably faster than the corresponding reactions of 2′- and 3′-NMPs. Consistent with the facile phosphate migration from tertiary to secondary hydroxyl function, it has been noted that acetylation of 5′-O-benzoyl-3′-C-methyluridine with excess of acetic anhydride or tosyl chloride yielded only the 2′-O-acetylated product.[19] Analogously, 2′-O-benzoyl-3′-C-methyluridine did not show any sign of isomerization during half a year storage in pyridine at 293 K, whereas 2′- and 3′-O-benzoyluridines are known to undergo mutual isomerization in the same solvent.[20]

Experimental Section

Materials. Preparation of 2′-C-methyluridine (**4c**),[14] 3′-C-methyluridine (**5c**),[11] and 3′-C-methyluridine 3′-monophosphate (**5b**)[11] has been described earlier. Uridine 2′,3′-cyclic monophosphate was a commercial product of Sigma, and it was used as received.

2′-C-Methyluridine 2′,3′-Cyclic Monophosphate (2). A mixture of 2′-C-methyluridine (**4c**; 0.5 mmol) and dibutylstannoxane (0.55 mmol) was heated in dry methanol (30 mL) until the dissolution was complete (1 h) and cooled to 20 °C, after which tributylamine (5 mmol) and diphenyl chlorophosphate (5 mmol) were added. The mixture was stored for 1 h at 20 °C and concentrated to dryness in vacuo. The residue was suspended in water (30 mL), mixed for 30 min, and extracted with chloroform (5 × 30 mL). pH of the aqueous layer was adjusted to 13 with lithium hydroxide and the solution stored at 20 °C for 16 h and neutralized with Dowex 50 (H^+-form). The solution and combined washings of the resin were applied on a column of DEAE-Toyopearl 650 M (30 mL, HCO_3^--form), washed with water (200 mL) and dilute aqueous ammonium bicarbonate (0.05 mol/L, 200 mL), and eluted with 0.125 mol dm^{-3} aqueous ammonium bicarbonate. Pooled fractions were coevaporated with water (5 × 10 mL), dissolved in water (10 mL), and desalted with Dowex 50 (H^+-form). The residue evaporated to dryness was dried by repeated coevaporations with methanol and dissolved in dry methanol (5 mL). N,N′-Dicyclohexylcarbodiimide (3 mmol) was added, and the mixture was stirred for 16 h at 20 °C. The mixture was evaporated to dryness, and the residue was distributed between water and chloroform (30 mL). The aqueous layer was extracted with chloroform (5 × 30 mL) and applied on a column of DEAE-Toyopearl 650 M (30 mL). The column was washed with water (200 mL), and the product was eluted with aqueous ammonium bicarbonate (0.05 mol/L). The pooled fractions were desalted and lyophilized. The overall yield was 45%. Table III records the ^1H NMR shifts and $^1H,^1H$-couplings for the product obtained. The ^{31}P NMR spectrum (^2H_2O, pH 6) showed only one signal at 19.87 ppm (from external phosphoric acid; $J_{P,H3'}$ = 13.7 Hz).

3′-C-Methyluridine 2′,3′-Cyclic Monophosphate (3). **3** was prepared from 3′-C-methyluridine (**5c**) as described above for **2**. The product was identical with that synthesized previously.[11]

Identification of Reaction Products. All reaction products were separated by HPLC on a reversed-phase column and characterized by ^1H NMR spectroscopy. Table III summarizes the retention times and NMR spectroscopic data.

Kinetic Measurements. The reactions were followed by the HPLC technique described previously.[21] The initial substrate

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concentration was 5×10^{-4} mol/L, and the temperature was maintained within 0.1 K. Chromatographic separations were carried out on a Hypersil ODS column (4 × 250 mm, 5 μm), using an acetic acid buffer (pH 4.2) containing 0.1 mol/L of ammonium chloride as eluant. The concentrations were assumed to be proportional to the integrated areas of the UV signals, since the structure of the base moiety was not changed by the reactions followed.

Calculation of Rate Constants. The first-order rate constants indicated in Scheme I were obtained by simulating the time-dependent product distributions with the aid of a numerical intergration method based on the Runge-Kutta algorithm.[22] Equations 1-6 were used to describe the reaction system. Here A stands for **2** (or **3**), B for **4a** (or **5a**), C for **4b** (or **5b**), and D for **4c** (or **5c**). $(\chi_B/\chi_C)_{init}$ denotes the ratio of the mole fractions of B and C during the early stage of the hydrolysis of A, and $(\chi_B/\chi_C)_{eq}$ denotes the same ratio after equilibration of B and C. The method of least-squares was applied to fit the experimental data.

$$d\chi_A/dt = -(k_1 + k_2)\chi_A + k_{-1}\chi_B + k_{-2}\chi_C \quad (1)$$

$$d\chi_B/dt = k_1\chi_A - (k_{-1} + k_3 + k_4)\chi_B + k_{-3}\chi_C \quad (2)$$

$$d\chi_C/dt = k_2\chi_A + k_3\chi_B - (k_{-2} + k_{-3} + k_5)\chi_C \quad (3)$$

$$d\chi_D/dt = k_4\chi_B + k_5\chi_C \quad (4)$$

$$k_1/k_2 = (\chi_B/\chi_C)_{init} \quad (5)$$

$$k_{-3}/k_3 = (\chi_B/\chi_C)_{eq} \quad (6)$$

Registry No. **1a**, 606-02-0; **2**, 141635-70-3; **3**, 87215-04-1; **4a**, 141635-71-4; **4b**, 141635-72-5; **4c**, 31448-54-1; **5a**, 87215-02-9; **5b**, 87215-03-0; **5c**, 80541-15-7.

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Enzyme-Catalyzed Glycosylation of Peptides Using a Synthetic Lipid Disaccharide Substrate

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A lipid disaccharide, consisting of chitobiose linked to dolichol via an α-1-pyrophosphate, has been synthesized for use as a substrate in the enzyme-catalyzed glycosylation of peptides. For the purpose of confirming the structure of the reaction product, the expected glycopeptide was synthesized via an unambiguous, convergent method. Chromatographic and spectral comparison of the synthetic vs biosynthetic glycopeptides showed that they were identical. Thus, glycosylation of synthetic peptides by a synthetically accessible lipid disaccharide can be effected using oligosaccharyltransferase isolated from yeast.

Introduction

A key reaction in the biosynthesis of N-linked glycoproteins involves the coupling of a growing peptide to a lipid-linked oligosaccharide.[1] This is a cotranslational process, catalyzed by the enzyme, dolichyl-diphosphooligosaccharide-protein glycotransferase (EC 2.4.1.119), commonly referred to as oligosaccharyltransferase (OST).[2] As shown in Figure 1, biosynthesis of the lipid-linked oligosaccharide substrate involves a series of glycosyl transfer reactions in which the sugar donor is either a nucleotide sugar or a dolicyl sugar.[3] The entire process occurs in the rough endoplasmic reticulum and thus involves a series of reactions which are catalyzed by membrane-bound enzymes.[4] The intact lipidoligosaccharide (LOS, **1a**) containing the so-called "core" oligosaccharide is shown in Figure 2. The standard method used to isolate lipid-linked substrates for studying the OST-catalyzed reaction (eq 1) involves microsomal preparations in which a specific radioactive nucleotide sugar is added in order to obtain radioactive lipidoligosaccharides labeled at a specific sugar residue. Using full-length biosynthetic **1a**, we have previously shown that only isomer **3** is obtained following enzyme-catalyzed hydrolysis of the GlcNAc-GlcNAc bond of the primary glycopeptide product **2a**, isolated from the yeast OST-catalyzed reaction (eq 1).[5] However, preparation of LOS is a cumbersome process, the yields are low, and the isolated biosynthetic LOS is unstable over periods of several months, even at -80 °C (J. Lee, R. S. Clark, and J. K. Coward, unpublished results).

For the purposes of carrying out mechanistic studies on the reaction catalyzed by OST, we required a lipid-linked oligosaccharide which would be amenable to total chemical synthesis by which we could ultimately introduce selected isotopic probes. Previous work with truncated biosynthetic lipid-linked oligosaccharides[6] or with yeast mutants unable to carry out specific steps in the biosynthetic pathway[7] have revealed that a variety of lipid-linked oligosaccharides are able to act as substrates for the OST-catalyzed reaction. Although these studies provide good precedent for the use of shorter lipid oligosaccharides as glycosyl donors in the OST-catalyzed reaction, the quantities of materials

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