The reaction mechanism of *N*-benzoylimidazole with ribonucleotides

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ABSTRACT

The reaction of uridine 3'-phosphate with benzoylimidazole in the absence and presence of a strong base was followed up by ^{31}P and ^1H nmr as well as paper electrophoresis. Possible reaction courses were proposed, the reaction rate constants were calculated and the reaction mechanism was discussed. It is possible to selectively acylate ribonucleotides with benzoylimidazole by appropriate choice of the base used.

INTRODUCTION

N-Acylimidazole was first employed as acylating agent to acylate alcohols, sugars and amines by Staab¹. In nucleic acid chemistry there have been only a few reports on the use of acylimidazole^{2,3}. In our previous papers⁴⁻⁶ we had briefly reported that the reaction course as well as the reaction products from ribonucleotides and *N*-acylimidazole varied markedly with conditions: eg. whether a strong organic base was present or not. For instance, in the absence of a strong organic base, the reaction led predominately to ribonucleoside 2',3'-cyclophosphate. When a strong base such as morpholino-*N,N'*-dicyclohexylcarboxamide (MDCAI) or tetraethylammonium hydroxide (TEAH) was present in the reaction system, not only the formation of ribonucleoside 2',3'-cyclophosphate was satisfactorily suppressed, but also the acylation of both hydroxyl groups of the ribose moiety and the amino group of the heterocyclic ring was greatly accelerated.

In the present paper the details of kinetic study on the reaction mechanism of ribonucleotide with acylimidazole followed up by ^{31}P and ^1H nmr⁷ spectroscopy and chemical synthesis are reported.

EXPERIMENTAL

Materials

Uridine 2',3'-cyclophosphate (Up), 2'-O-benzoyluridine 3'-phosphate (Ubzp), 2',5'-di-O-benzoyluridine 3'-phosphate [(Bz)Ubzp], their mixed anhydrides with benzoic acid (Ubzpbz and (Bz)Ubzpbz) and uridine pyrophosphate [(Up)₂O] were synthesized as reference compounds for ³¹P and ¹H nmr spectroscopy, chromatography and paper electrophoresis⁸.

The pyridinium salt of UMP(Up.py) was obtained by exchanging the sodium salt of UMP (product of Shanghai Reagent Factory No.2) on cation exchanger column with pyridine. MDCAI⁹ and N-benzoylimidazole(BzIm)¹⁰⁻¹² were prepared according to known methods. DMF was dried over potassium hydroxide and distilled before use. TEAH was obtained from TEAH aqueous solution (10 or 25%, A.R., Shanghai Reagent Factory) by repeated vacuum evaporation with added pyridine. All melting points were uncorrected.

General procedures for kinetic determination

A Varian XL-200 nmr spectrometer was employed for recording spectral changes of ³¹P and ¹H nmr during the reaction course of uridine 3'-phosphate with acylimidazole under different conditions. ³¹P-¹H heteronuclear decoupling technique was also employed. For ³¹P nmr, a 10 mm sample tube was used with DMF as solvent, 85% H₃PO₄ as external standard, D₂O as frequency lock at a frequency of 80.9 MHz. The number of accumulations varied from 30 to 50. The sum of width and interval of pulse was 1 second. For ¹H nmr, a 5 mm sample tube was employed with TMS as internal standard and DMF-d₇ or DMSO-d₆ as solvent. The concentrations of the reacting substances and products were calculated from the areas of the corresponding nmr peaks, assuming that UMP and its derivatives have approximately the same peak area per unit concentration.

For checking the products formed during the reaction of Up.py with benzoylimidazole, paper electrophoresis was employed. Several drops of the reaction mixture were taken out at definite time intervals and added with stirring to an excess of dried benzene or ether containing a drop of acetic acid-pyridine to stop the reaction. The precipitated product was collected by centrifugation, dissolved in 50% aqueous pyridine and examined

by paper electrophoresis. Paper electrophoresis was performed on Xinhua No.3 filter paper [(5-10) x 60 cm], wetted with 0.05 M triethylammonium bicarbonate buffer(pH 7.5), 4000-5000 V, 20-30 mA, and 30-45 min. Relative mobilities (R_m) of various compounds to that of uridine 3'-phosphate were determined. The relative amounts of starting materials, intermediates and final products were determined by Shimatsu Dual-wavelength TLC Scanner CS-910 at 260 nm.

Sample preparation:

Triethylammonium salts of $U\gg p^{13}$ and (Bz)Ubzp⁴ were prepared respectively according to known methods and purified through a column of DEAE-Cel $\cdot$ AcO⁻.

Triethylammonium salt of (Bz)U>p was synthesized through the reaction of triethylammonium salt of $U\gg p$ with BzIm (in 1:7 molar ratio) in DMF at 17°C for 20 h. The product was purified on a column of DEAE-Cel $\cdot$ AcO⁻.

Triethylammonium salt of (Bz)Up¹⁴ was prepared from the hydrolyzation of (Bz)U>p by ribonuclease A and purified by chromatography on DEAE-Cel $\cdot$ AcO⁻ column.

Triethylammonium salt of (Bz)Ubzpbz: In the presence of TEAH, Up.py was reacted with BzIm at 20°C in 1:10:3 molar ratios of Up.py:BzIm:TEAH for 3 h. The product was purified by chromatography on DEAE-Cel $\cdot$ AcO⁻ column and crystallized from dichloromethane/ether, m.p. 151-153°C. ¹H nmr: 8.1-9.1(16H,m), Anal. C₃₆H₄₀N₃O₁₂P Calcd.: C 58.61, H 5.46, N 5.69, P 4.10. Found: C 58.50, H 5.42, N 5.73, P 3.80.

Triethylammonium salt of Ubzpbz: In the presence of MDCAI, MMT-Up.py¹⁴ was reacted with BzIm (in 1:5:8 molar ratios of MMT-Up.py:MDCAI: BzIm) in DMF at 20°C for 24 h. The reaction mixture was added dropwise with stirring into an excess of dried ether and the precipitate formed was collected by centrifugation and dried in a vacuum desiccator. The residue was dissolved in 80% acetic acid, kept for 30 min at room temperature and evaporated under reduced pressure. The residue was taken up in dried pyridine and dropped into a great volume of anhydrous ether. The precipitate was collected by centrifugation and purified by DEAE-Cel $\cdot$ AcO⁻ column chromatography. R_m of the product is same as that of Ubzpbz prepared according to Avison's method¹⁵.

Table 1. ^{31}P NMR chemical shifts of the triethylammonium salts of UMP and its derivatives in DMF

Compound	(ppm)	Compound	(ppm)
UMP	2.56	U>p	18.1
Ubzp	1.3	(Bz)U>p	17.9
(Bz)Up	2.2	Ubzpbz	-7.9
(Bz)Ubzp	1.1	(Bz)Ubzpbz	-8.1

Triethylammonium salt of Ubzp: On decomposing the mixed anhydride (Ubzpbz) by the usual method¹⁵ yielded triethylammonium salt of Ubzp which was purified by chromatography on DEAE-Cel $\cdot$ AcO $^-$ column.

Triethylammonium bis(2',5'-di-O-benzoyluridine) pyrophosphate: Khorana's general method¹⁶ was adopted. To a solution of 310 mg (0.5 mmol) of triethylammonium salt of (Bz)Ubzp in 3 ml of dried pyridine was added a solution of 1.39 g (7.5 mmol) of triethylamine and 3.1 g (15 mmol) of DCC in 10 ml dried pyridine. After 24 h, a solution of 450 mg (7.5 mmol) of glacial acetic acid in 10 ml of water was added, and the reaction mixture filtered. The filtrate was extracted twice with ether and the aqueous layer was concentrated under reduced pressure by repeated evaporations with pyridine. The residue was dissolved in anhydrous pyridine and dropped into an excess of anhydrous ether. The precipitate was collected by centrifugation and dried in a desiccator. The crude product was purified through chromatography on DEAE-Cel $\cdot$ AcO $^-$ column. Anal. $\text{C}_{58}\text{H}_{70}\text{N}_6\text{O}_{21}\text{P}$ Calcd.: C 53.38, H 5.48, N 6.73, P 4.96. Found: C 53.34, H 5.71, N 6.57, P 4.61. ^{31}P nmr: -10.6 ppm.

RESULTS AND DISCUSSION

Table 1 shows the ^{31}P nmr chemical shifts of triethylammonium salts of UMP and its derivatives in DMF. The formation of mixed anhydride between Up and benzoic acid causes up-field shift by about 10 ppm; the formation of U>p results in down-field shift by about 15 ppm; the conversions of UMP into Ubzp and (Bz)Up give up-field shifts of about 1 and 0.5 ppm respectively.

By means of ^{31}P and ^1H nmr in combination with paper

Fig. 1 ^{31}P nmr spectra of the reaction of Up.py (0.074 mol) with BzIm (0.451 mol) in 0.25 ml DMF at 20°C .

chromatography and paper electrophoresis as well as actual isolation of the intermediate products formed during the reactions, we were able to trace the paths of the acylation of Up.py with acylimidazole in the presence of different bases. A series of reactions of uridine 3'-phosphate with acylimidazole under different conditions were followed up. With BzIm as a model acylating reagent in DMF in the absence of a strong organic base, the ^{31}P nmr spectral changes recorded during the reaction are shown in Fig.1. The Up.py (A, 1.6 ppm) first reacted with BzIm to form an active intermediate (B, -6.7 ppm) which has been ascertained from the chemical shift to be the mixed anhydride, uridine 3'-phosphoric benzoic anhydride (Upbz). As the reaction was going on, there appeared a new signal (C, 18.0 ppm) which

Table 2. ^1H NMR chemical shifts of the triethylammonium salts of UMP and its derivatives

Compound	¹ H NMR (ppm)					
	1'	5	2'	3'	4'	5'
UMP [*]	6.02 (d)	5.71 (d)	4.43 (m)	4.79 (m)	4.28 (m)	3.80 (d)
(Bz)Ubzp [*]	6.22 (d)	5.65 (d)	5.80 (m)	5.24 (m)	4.82 (m)	4.69 (d)
Ubzpbz [*]	6.37 (d)	5.69---5.78		5.40 (m)	4.44 (m)	3.90 (m)
(Bz)Ubzpbz [*]	6.31 (d)	5.68 (d)	5.91 (m)	5.61 (m)	4.86 (m)	4.74 (m)
U>p [*]	6.10 (d)	5.69 (d)	4.95 (m)	4.83 (m)	4.23 (m)	3.77 (m)
Ubzp ^{**}	6.05 (d)	5.76 (d)	5.54 (m)	4.86 (m)	overlapped	
(Bz)Up ^{**}	5.82 (d)	5.56 (d)	4.54 (m)			4.30 (m)
(Bz)Ubzp ^{**}	6.09 (d)	5.58 (d)	5.74 (m)	5.10 (m)	overlapped	
(Bz)Ubzpbz ^{**}	6.12 (d)	5.67 (d)	5.76 (m)	5.30 (m)	4.73 (m)	4.58 (m)
(BzUbzp) ₂ O ^{**}	5.98 (d)	5.58 (d)	5.63 (m)	5.12 (m)	overlapped	
U>p ^{**}	5.85 (d)	5.60 (d)	4.70 (m)	4.50 (m)	4.00 (m)	3.60 (m)
(Bz)U>p ^{**}	5.86 (d)	5.60 (d)	4.85 (m)	4.74 (m)	4.55 (m)	4.40 (m)

* In DMSO-d₆, ** In DMF-d₇

has been identified by comparison with synthetic uridine 2',3'-cyclophosphate. Todd had reported 15 to 18 ppm¹⁷ for the chemical shift of the cyclophosphate. So the anhydride was turned into U>p which was further changed partly into (Bz)U>p as indicated by ^1H nmr spectroscopy (Table 2). The total yield of both products was about 85% as estimated from ^{31}P nmr. The above mentioned reaction path was the main one. There was a side path in which Ubzp (D, 1.3 ppm) was first formed and then changed into Ubzpbz (E, -8.0 ppm) and partly into (Bz)Ubzpbz (in about 15% yield for the sum of both). The latter two compounds had been identified by comparison of their ^{31}P nmr spectra with those of the synthetic ones (-7.9 and -8.1 ppm, Table 1).

As there is no significant difference between ^{31}P chemical shifts of UMP (or U>p or Ubzp) and the respective 5'-O-benzoyl-derivatives, ^1H nmr was used to follow the above reaction. The

Fig. 2 ^1H nmr spectra of the reaction of Up.py (0.094 mol) with BzIm (0.677 mol) in 2.5 ml DMF-d_7 at 20°C .

result was shown in Fig.2. No significant change of 5'-H peak could be observed within 1 h while under the same condition almost all of the starting material Up.py had been exhausted in the same interval as estimated from ^{31}P nmr peak A (Fig.1). It seems therefore that in the absence of a strong base the benzylation at 5'-OH is very slow.

Based on the sequence of the appearance of particular signals and the relative intensities of the particular peaks of ^{31}P

Scheme 1

Reaction courses of Up.py with BzIm in DMF

nmr at different times, Scheme 1 was proposed for the possible reaction courses of the above reaction.

For the first-approximation, the reaction rates can be represented by the following differential equations:

$$d[A]/dt = -k_p[A][BzIm] - k_{2'}[A][BzIm] \quad (1)$$

$$d[B]/dt = k_p[A][BzIm] - k_c[B] - k_{p2'}[B][BzIm] \quad (2)$$

$$d[C]/dt = k_c[B] \quad (3)$$

$$d[D]/dt = k_{2'}[A][BzIm] - k_{p'}[D][BzIm] \quad (4)$$

$$d[E]/dt = k_{p'}[D][BzIm] + k_{p2'}[B][BzIm] \quad (5)$$

where k_p , k_c , $k_{p2'}$, $k_{2'}$ and $k_{p'}$ represent specific rate constants (Scheme 1); [A] stands for [Up], [B] for [Upbz], [C] for the sum of [U>p] and [(Bz)U>p], [D] for [Ubzp] and [E] for the sum of [Ubzpbz] and [(Bz)Ubzpbz]. A "PAREST" (Parameter Estimation) computer program¹⁸ has been developed for solving the nonlinear differential equations. Besides the cyclization which was treated as first order reaction, the other reactions were treated as second order reactions. The experimentally determined kinetic curves of the reaction of Up.py with BzIm were shown in Fig.3 and the rate constants were calculated and listed

Fig. 3. The kinetic curves of the reaction of Up.py (0.065 mol) with BzIm (0.26 mol) in 2.5 ml DMF at 20°C. Experimental: Up—○, Upbz—x, U>p—△, Ubzp—■, Ubzpbz—●. Computer-simulated: -----

in Table 3. The computer-simulated kinetic curves (Fig.3) agree quite well with the experimental ones except the curve of Upbz which indicated some deviation.

An addition of triethylamine to the above reaction system caused some retardation of the formation of the mixed anhydride. Therefore, the product of cyclization was decreased. At the end, the yield of uridine 2',3'-cyclophosphate was only about 15%. The order of the appearance and disappearance of the peaks in the reaction is very similar to that in the pyridinium salt system. The rate constants of k_p , k_c , $k_{p2'}$, k_2 , and k_p' were also calculated and listed in Table 3.

In the presence of an excess of MDCAI ([Up]:[MDCAI] = 1:3), the ^{31}P nmr spectral changes are shown in Fig.4. The MDCAI

Table 3. Rate constants of Up.py(I) with BzIm(II) in the absence and presence of Et₃N(III) in DMF at 20°C

[I]:[II]:[III]	k _p (a)	k _c (b)	k _{p2} (a)	k ₂ (a)	k _{p'} (a)
1 : 6 : 0	13	2.8	0.76	2.7	6.2
1 : 7 : 3.5	0.83	0.83	0.0	5.0	0.5

(a): $\times 10^4 \times \text{mol}^{-1} \text{sec}^{-1}$ (b): $\times 10^4 \text{sec}^{-1}$

salt of UMP (A, 3.9 ppm) reacted with BzIm first to form Ubzp (D, 2.5 ppm), then (Bz)Ubzp (F, 1.8 ppm) and finally (Bz)Ubzpbz (G, -8.8 ppm) (Scheme 2). The calculated rate constants and thermodynamic data are listed in Table 4. The main products isolated were identical with (Bz)Ubzp and (Bz)Ubzpbz. In the

Fig. 4 ³¹P nmr spectra of the reaction of Up.py (0.077 mol) with BzIm in the presence of MDCAI at 30°C in 2.5 ml DMF ([Up.py] : [MDCAI] : [BzIm] = 1:3:7).

Scheme 2

Reaction courses of Up.py with BzIm in DMF in the presence of MDCAI or TEAH

presence of equivalent MDCAI, the peak of Ubzp (2.89 ppm) appeared first, and then the peak of U_p (18.0 ppm) appeared. Ubzp was isolated as the main product. When an insufficient amount of MDCAI ([Up]:[MDCAI] = 1:0.67) was used, the salt of UMP (2.64 ppm) reacted with BzIm to a greater extent to form Upbz (-6.8 ppm) which was later cyclized into U_p (17.9 ppm), and to a smaller extent, to form Ubzp, which was then changed into (Bz)Ubzp (0.83 ppm) and finally into (Bz)Ubzpbz (-8.1 ppm).

In the presence of an excess of TEAH ([Up]:[TEAH] = 1:3) (Fig.5), the TEAH salt of UMP (A, 6.3 ppm) reacted with BzIm to form first (Bz)Up (H, 5.9 ppm) (peak H was confirmed to be (Bz)Up, not starting material, by electrophoresis and isolation of product) and later (Bz)Ubzp (F, 4.2 ppm), which was further very slowly converted into (Bz)Ubzpbz (G, -7.6 ppm) (Scheme 2). Because the initial reaction of UMP with BzIm in the presence of TEAH was too rapid to be followed up with ³¹P nmr, the rate constants of reaction were not quantitatively determined, but from the spectral data (Fig.5) it is apparent that $k_5 > k_2 > k'_p$.

From the above results it is clear that, Upbz is the active intermediate in these reactions. Although a mixed anhydride as a possible active intermediate in the 2',3'-cyclophosphate formation had been suggested by earlier workers^{19,20}, it has never been actually detected. Now the appearance and disappearance of the active intermediate cyclophosphate were spectrometrically determined for the first time.

Table 4. Rate constants and thermodynamic parameters of Up.py with BzIm in DMF in the presence of MDCAI*

Functional group	k x 10 ³			E _a	ΔS	ΔH	ΔG
	20°C	30°C	40°C				
2'-OH	10	12	15	1.7	-52	1.2	17
5'-OH	5	7.5	10	3.3	-32	2.7	12
3'-P-OH	0.25	0.40	0.67	4.6	-27	4.6	12

* Unit of k, ΔS and E_a (ΔH, ΔG) is 1/mol.sec, cal/deg.mol and kcal/mol respectively.

From the rate constants in Tables 3 and 4, it is obvious that in the presence of a strong base the formation rate of the mixed anhydride is depressed, and that the reaction rates depend upon the basicity of the base present in the reaction system. These results could be interpreted in terms of: (1) the degrees of ionization (pK_a's) and oxygen nucleophilicities of the 2'-and 5'-hydroxyl and 3'-phosphate groups of the nucleotide in the system at a given pH; (2) the ease of the departure of the

Fig. 5 ³¹P nmr spectra of the reaction of Up.py (0.083 mol) with BzIm in the presence of TEAH at 20°C in 2.5 ml DMF ([Up.py] : [TEAH] : [BzIm] = 1:3:5.2)

leaving group (Im or Im⁻) which occurs during the attack of the nucleophile group (2'-OH, 5'-OH, -O-PO₃H⁻ or -OPO₃⁼) of the nucleotide molecule upon BzIm²⁵; and (3) the steric hindrance of the reacting group (2'-OH or 5'-OH).

In aqueous solution, the pK_a of P-OH group of UPO₃H⁻ is 5.88²¹. The pK_a of the 2'-OH of ribo-nucleosides or -nucleotides may be estimated to be about 12.5^{22,23} and that of primary 5'-OH, which is similar to ethyl alcohol, assumed to be about 15-16²⁴. Although the change of the medium may cause the change in pK_a value, the order of pK_a's for these -OH groups is mostly not to be altered, namely P-OH < 2'-OH < 5'-OH. In the following discussion we used the data of pK_a's of aqueous solutions for reactions taking place in DMF. The order of the nucleophilicity of these groups is most possibly 5'-O⁻ > 2'-O⁻ > 3'-O-PO₃⁼.

In the presence of a weak base like pyridine (pK_a 5.26) or triethylamine (pK_a 11.01), the 3'-OPO₃H⁻ group of the ribo-nucleotide forms much more favorably with BzIm the conjugate acid BzImH⁺²⁵ than either of the two hydroxyl groups. The nucleophilic attack of 3'-OPO₃⁼ on BzImH⁺ should give rise to an easily leaving group (Im) so that the formation of the mixed anhydride is facilitated. The 2',3'-cyclophosphate is predominantly formed and only a little amount of 2'-O-benzoyl-uridine phosphate derivative was produced.

In the presence of a strong base like MDCAI of which the conjugate acid has pK_a of ca. 13²⁶, being of the same order as pK_a of 2'-OH of UMP, but is lower than pK_a of 5'-OH of UMP, so that BzIm may form conjugate acid BzImH⁺ with 2'-OH but not so easily with 5'-OH. The attack of 2'-O⁻ on BzImH⁺ yields 2'-O-benzoyl-derivative. As to the 3'-phosphate group which should exist mainly as -O-PO₃⁼ species could no more form BzImH⁺ with BzIm and its nucleophilic attack on BzIm would only lead to the formation of Im⁻ which is difficult to leave²⁵; and furthermore, ROPO₃⁼ is a weak nucleophile and it is difficult to form the mixed anhydride. The result is that MDCAI depresses k_p and increases greatly k₂, and Ubzp was obtained as the main product. Therefore, one may predict that the 2'-OH group of the ribonucleotide can be selectively acylated by acylimidazole

under suitable conditions. The prediction has been experimentally realized²⁷.

In the presence of TEAH, because its conjugate acid has pK_a over 15²⁸, being of the same order as that of 5'-OH of UMP. The 3'-phosphate group is expected to be nearly all in the form of $3'\text{-OPO}_3^-$ which could hardly react with BzIm and both 2'-OH and 5'-OH are highly ionized. Due to stronger nucleophilicity and less steric hindrance of $5'\text{-RO}^-$ than that of $2'\text{-RO}^-$, the formation of 5'-O-acylated derivative is much preferred than that of 2'-O- derivative in the presence of excess of TEAH. The selective acylation of 5'-OH with acylimidazole under suitable condition has also been achieved experimentally²⁷. Although in the presence of an excess of MDCAI or TEAH, 3-NH (its pK_a 9.4²⁹) of uracil moiety of Up or U>p is also ionized, the imido-nitrogen, however, is less nucleophilic than $2'\text{-RO}^-$ and $5'\text{-RO}^-$ of the nucleotide, so no benzylation on the imino-nitrogen is found under the experimental condition³⁰.

It should be pointed out that some deviation between calculated concentrations and experimental ones of mixed anhydride was observed at the late stage of the reaction in the absence of a strong base. The main cause of the deviation may be attributed to the imidazole acting as a base liberated and accumulated during the reaction of Up.py with BzIm, resulting in the retardation of the formation of U>p²⁷.

Similar reaction courses and mechanisms have been found when AcIm or other acylimidazoles were employed instead of BzIm to acylate ribonucleotides^{4,27} in the presence or absence of a strong base.

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