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Kinetic Isotope Effects and Stereochemical Studies on a Ribonuclease Model: Hydrolysis Reactions of Uridine 3′-Nitrophenyl Phosphate

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The reactions of a ribonuclease model substrate, the compound uridine-3′-*p*-nitrophenyl phosphate, have been examined using heavy-atom isotope effects and stereochemical analysis. The cyclization of this compound is subject to catalysis by general base (by imidazole buffer), specific base (by carbonate buffer), and by acid. All three reactions proceed by the same mechanistic sequence, via cyclization to cUMP, which is stable under basic conditions but which is rapidly hydrolyzed to a mixture of 2′- and 3′-UMP under acid conditions. The isotope effects indicate that the specific base-catalyzed reaction exhibits an earlier transition state with respect to bond cleavage to the leaving group compared to the general base-catalyzed reaction. Stereochemical analysis indicates that both of the base-catalyzed reactions proceed with the same stereochemical outcome. It is concluded that the difference in the nucleophile in the two base-catalyzed reactions results in a difference in the transition state structure but both reactions are most likely concerted, with no phosphorane intermediate. The ^{15}N isotope effects were also measured for the reaction of the substrate with ribonuclease A. The results indicate that considerably less negative charge develops on the leaving group in the transition state than for the general base-catalyzed reaction in solution.

INTRODUCTION

Ribonucleases catalyze the cleavage of the (5′–O–P bond of RNA. Because of the biological importance of this reaction its mechanistic details have been the object of numerous studies, involving both the enzyme itself and model systems (*1*). Williams and co-workers have conducted linear free energy studies on a ribonuclease model, uridine 3′-aryl phosphates (*2*). These compounds undergo the first step of the ribonuclease reaction, namely cleavage of the P–O bond, with formation of a 2′,3′ cyclic phosphate. In that work, the Brønsted parameters were measured for the general base reaction catalyzed by imidazole buffer and the specific base reaction in carbonate

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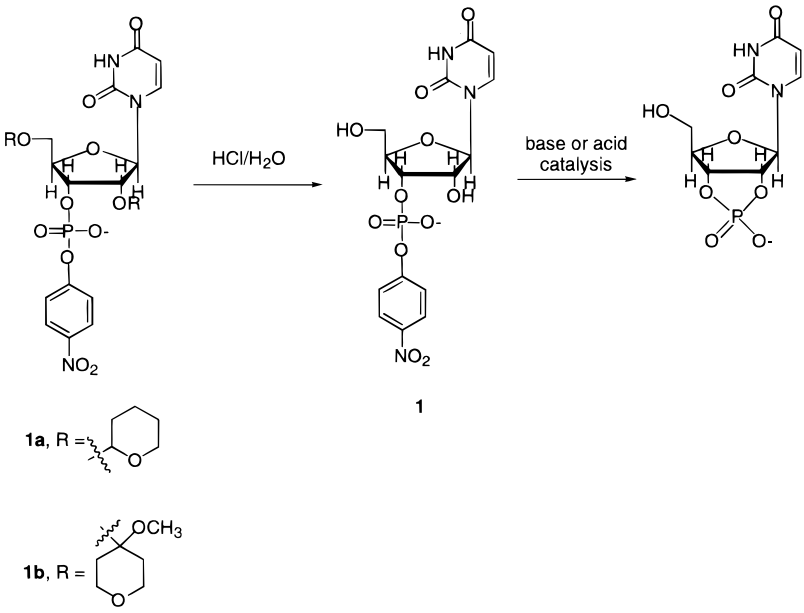
buffer. It was concluded that both the general and the specific base-catalyzed reactions were concerted, with bond cleavage to the leaving group and bond formation to the nucleophilic 2′ hydroxyl group each occurring in the transition state (*2*). In this paper, we describe the results of heavy-atom isotope effects and of stereochemical studies with uridine 3′-*p*-nitrophenyl phosphate (Scheme 1) under conditions of general base and specific base catalysis. In addition, the isotope effects were measured for the acid-catalyzed hydrolysis reaction, and the ^{15}N isotope effects were measured for the ribonuclease-A-catalyzed reaction of the substrate at pH 6.2 and 7.6.

MATERIALS AND METHODS

Pyridine was distilled from calcium hydride under nitrogen before use. DMSO was stored over KOH and 3-Å molecular sieves. Chloroform and hexane were dried in evacuated ampoules over P_2O_5 and lithium aluminum hydride, respectively, and were distilled directly into reaction vessels under vacuum. Other reagents and solvents were purchased from commercial sources and used as received unless otherwise specified. Ribonuclease A was purchased from Sigma.

The product identification NMR experiments were performed on a Bruker 500-MHz instrument, operating at 202.34 MHz for ^{31}P experiments. These ^{31}P spectra were referenced to 80% phosphoric acid in D_2O (external reference). Thin layer chromatography was performed on precoated silica gel plates (silica gel 60, F_{254}, Merck) using phosphomolybdic acid/ethanol reagent for visualization. Column chromatography was performed on silica gel 60 (40–63 mm, Merck).

[^{14}N]-*p*-Nitrophenol (*3*) and [^{15}N, phenolic-^{18}O]-*p*-nitrophenol (*4*) were prepared



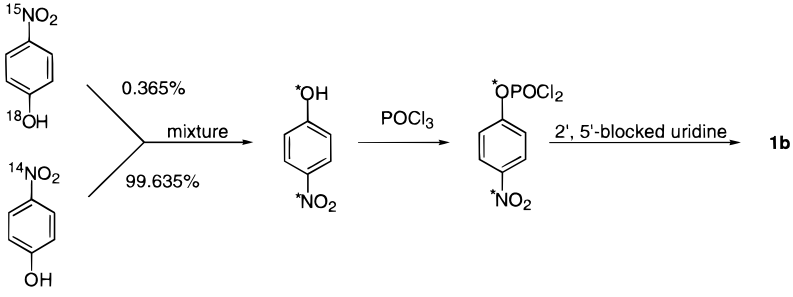
SCHEME 1

as previously described. 2′,5′-bis(*tert*-Butyldiphenylsilyl)uridine (**2**) was prepared according to Ogilvie *et al.* (*5*). [^{18}O]-Phosphorus oxychloride was prepared by hydrolysis of phosphorus pentachloride with 48% [^{18}O]-H_2O. Phosphoryl-[^{18}O]-*p*-nitrophenyl phosphorodichloridate was prepared from this material by reaction of an excess of phosphorus oxychloride with *p*-nitrophenol as described (*6*).

*Preparation of natural abundance 2′,5′-bis(tetrahydropyranyl)-uridyl-3′-p-nitrophenyl phosphate (**1a**).* This compound was prepared by the general procedure of Williams *et al.* (*2*). Uridine was benzoylated at the 3′ position using dibutyltin oxide and benzoyl chloride (*7*), followed by protection of the 2′- and 5′-hydroxy groups as their tetrahydropyranyl ethers using 5,6-dihydro-2*H*-pyran, with subsequent removal of the benzoyl group with ammonia. The resulting 2′,5′-bis(tetrahydropyranyl) uridine (900 mg) was dissolved in 4.5 mL of dry dioxane. This solution was added to a solution of *p*-nitrophenyl phosphorodichloridate (725 mg) and pyridine (450 μL) in 3 mL of dry dioxane, and the mixture was stirred under nitrogen for 6 h. Then a mixture of water (4 mL) and pyridine (1 mL) was added, and the reaction mixture was stirred for 15 min. After concentration *in vacuo*, the crude product was extracted from water with methylene chloride, purified by reverse phase chromatography eluting with methanol/water, and finally converted into the sodium salt by ion exchange. The compound was stored as the 2′,5′-bis(tetrahydropyranyl) ether and was deblocked in dilute HCl solution just before use.

Preparation of remote labeled p-nitrophenyl phosphorodichloridate (Scheme 2). Quantities of [^{14}N]-*p*-nitrophenol and [^{15}N, phenolic-^{18}O]-*p*-nitrophenol were mixed in proportion to closely duplicate the 0.365% natural abundance of ^{15}N. The isotopic ratio was confirmed by isotope ratio mass spectrometry. This mixture was then used to prepare remote labeled *p*-nitrophenyl phosphorodichloridate by the method previously described (*3*).

*Preparation of remote labeled 2′,5′-bis(4-methoxytetrahydropyran-4-yl)-uridyl-3′-p-nitrophenyl phosphate (**1b**).* Uridine was protected at the 2′- and 5′-positions as the bis (4-methoxytetrahydropyran-4-yl) ether by the same reaction sequence described above for the preparation of 2′,5′-bis(tetrahydropyranyl) uridine, except that 4-methoxy-5,6-dihydro-2*H*-pyran was used in place of 5,6-dihydro-2*H*-pyran (*8*). This compound was reacted with remote labeled *p*-nitrophenyl phosphorodichloridate and purified as described above for the natural abundance compound. The compound was



SCHEME 2

stored as the 2′,5′-bis(4-methoxytetrahydropyran-4-yl) ether and deblocked in dilute HCl solution just before use in measurements of bridge-^{18}O isotope effects. The achiral 4-methoxytetrahydropyran-4-yl ether protecting group was used to avoid differential rates in the acid-catalyzed removal of the protecting groups which could occur with the chiral tetrahydropyranyl ether.

*Preparation of 3′-O-[2′,5′-bis(tert-butyldiphenylsilyl)uridyl]-O-methyl-O-p-nitrophenyl ^{18}O-phosphate (**3**) (Fig. 1).* Protected uridine **2** (948 mg, 2 mmol) and pyridine (230 μL) were dissolved in hexane/chloroform (5:1 v/v, 5 mL) and the resulting solution was treated with a solution of *p*-nitrophenyl-[^{18}O]-phosphorodichloridate (517 mg, 2 mmol) in 1 mL of the same solvent at 0°C. A 0.5-mL aliquot of the reaction mixture was transferred into an NMR tube and the progress of the reaction was monitored by ^{31}P NMR. The formation of two diastereomers of the product giving rise to a doublet at -3 and -4 ppm was observed, along with unidentified by-products. The reaction was continued to reach the optimum yield of the product. The reaction mixture was then mixed with 230 μL of pyridine and 120 μL of methanol. TLC (hexane/acetone, 3:1) of the reaction mixture showed formation of a distinct product spot along with some unreacted protected uridine. The mixture was concentrated and purified by chromatography eluting with hexane/acetone (5:1) to give a 2:1 mixture of the diastereomers of **3**, along with some *p*-nitrophenyl dimethyl phosphate. This mixture was inseparable by TLC and was subjected to demethylation without further purification. ^{31}P NMR (C_6D_6) diastereomer a: -8.13 [^{16}O], -8.17 [^{18}O]; diastereomer b: -8.18 [^{16}O], -8.22 [^{18}O]; *p*-nitrophenyl dimethyl phosphate: -6.57 [^{16}O], -6.71 [^{18}O].

*Preparation of 3′-O-[2′,5′-bis(tert-butyldiphenylsilyl)uridyl] O-p-nitrophenyl phosphate (**4**).* The triesters **3a,b** (141 mg, 0.205 mmol) and tetra-*n*-butylammonium iodide (150 mg, two-fold excess) were dissolved in dry acetonitrile (0.5 mL), and the solution was stored at room temperature. After 12 h TLC could detect no remaining substrate. The mixture was chromatographed on silica gel, eluting first with chloroform/methanol (20:1) to elute excess tetrabutylammonium iodide and some *p*-nitrophenol and then with chloroform/methanol (7:1) to give pure product (178 mg). ^{31}P NMR (CD_3OD) δ -7.70, -7.73 ppm.

*Desilylation of **4** to diester **5** and subsequent cyclization to cyclic 2′,3′-uridine phosphate **6*** (Fig. 1). Cleavage of the TBDMS groups in **4** with tetra-*n*-butylammonium fluoride in THF or with HCl in aqueous methanol were found to give unsatisfactory results due to the rapid formation of 2′,3′-cyclic UMP following deprotection of the 2′-hydroxyl group. The tetra-*n*-butylammonium salt of diester **4** (120 mg) from the previous step was converted to the potassium salt using the potassium form of Dowex 50-X8 cation exchange resin. This salt was dissolved in methanol/water (5:3) and the solution was treated with Dowex 50-X8 resin in the proton form. ^{31}P and ^{1}H NMR were used to monitor the progress of deprotection of the 5′-hydroxyl group, and the reaction was judged complete within approximately 0.5 h as revealed by an upfield shift of the ^{31}P chemical shift by 0.25 ppm. Deprotection of the 2′-hydroxyl was slower and required several hours and resulted in a further upfield shift of 0.18 ppm. In parallel with deprotection, cyclization and hydrolysis of 2′,3′-cyclic UMP proceeded which manifested itself by the appearance of two new signals at 2.9 and 2.7 ppm, which were ascribed to 2′- and 3′-UMP. After 3 h the concentration of

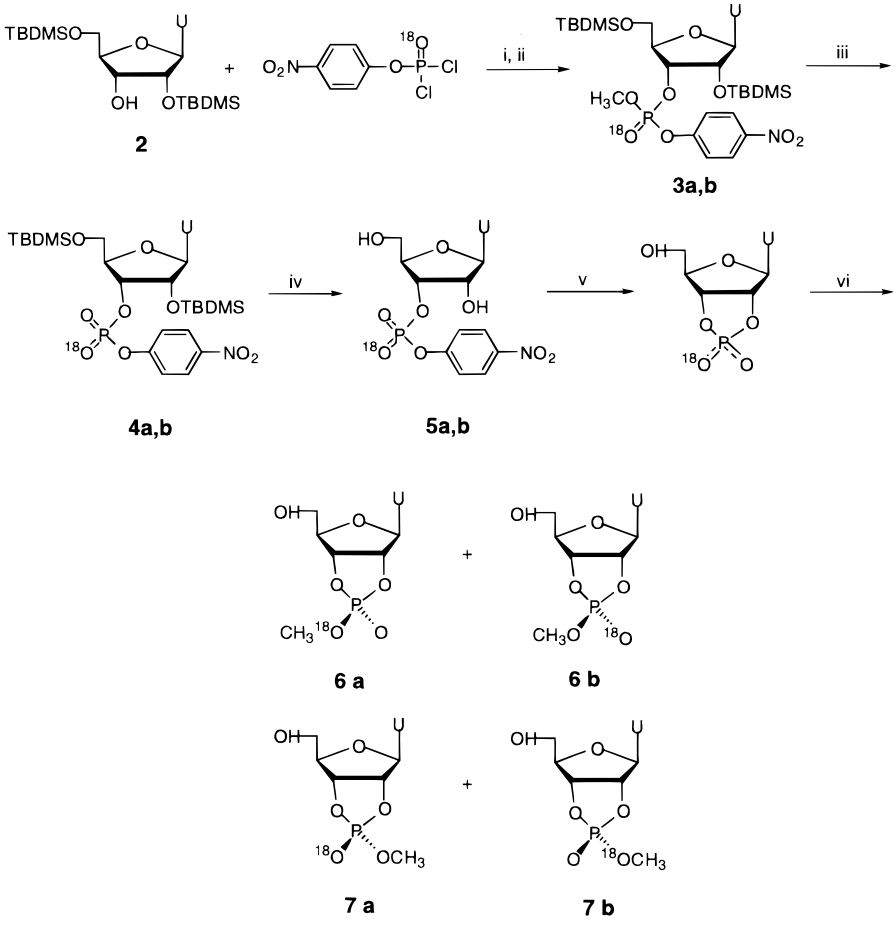


FIG. 1. Outline of the sequence used to produce the diastereomeric mixture of **5**, its subsequent cyclization to cUMP with imidazole or carbonate buffer, and subsequent methylation for ^{31}P NMR analysis. i: Pyridine; ii: methanol, pyridine; iii: $Bu_4N^+I^-$; iv: H^+-Dowex 50-X8; v: K_2CO_3 (pH 9.6) or imidazole (pH 6.7) buffers; vi: CH_3I, dibenzo-18-crown-6.

diester **5** reached a maximum and began to decline. At this point the mixture was split into two equal portions. The first was added to imidazole–HCl buffer (20 mL, 0.2 M, pH 6.7), and the second was added to potassium carbonate buffer (20 mL, 0.2 M, pH 9.6). The pH of the solutions was checked immediately and found to be within 0.1 unit of the pH of the original buffer. ^{31}P NMR spectra recorded shortly after mixing showed formation of the cyclic UMP at 19.2 ppm. The sample in carbonate buffer was passed through a Dowex 50-X8 column in the Et_3NH^+ form, the eluate concentrated under vacuum, and the residue was chromatographed on DEAE–Sephadex eluting with triethylammonium bicarbonate buffer. The pure cUMP obtained was freed of the residual buffer salt by absorbption on charcoal, washes with excess water, and elution with ethanol/aqueous ammonia (100:3). The eluate

was concentrated, passed through a Dowex 50-X8 column in the potassium form, and concentrated to give cUMP as its potassium salt. ^{31}P NMR δ 19.10 [^{16}O], 19.07 [^{18}O]. The sample treated with imidazole buffer was worked up in a similar manner except that it was treated sequentially with charcoal, DEAE–Sephadex chromatography, and cation exchange with K^+-Dowex.

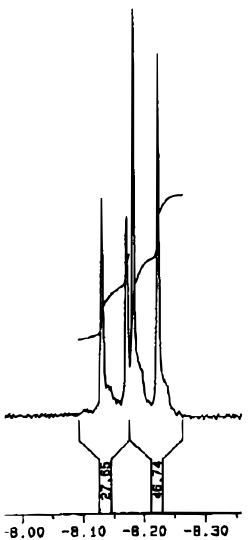
Configurational analysis of [^{16}O, ^{18}O] cUMP. An aqueous mixture of the foregoing potassium salts of [^{16}O, ^{18}O] cUMP (half of the total quantity) was transferred to a 5-mm NMR tube and freeze-dried. The solid residue was made anhydrous by repeated dispersion in warm, dry dioxane and freeze-drying. The anhydrous solid was dissolved in dry DMSO (0.4 mL), dibenzo-18-crown-6 (15 mg) was added, and the homogeneous sample was treated with methyl iodide (100 μL) at room temperature. The progress of the reaction to form **6** and **7** was followed by ^{31}P NMR. The reaction was complete within 3 h, as evidenced by the appearance of two groups of signals at 19.5 and 18.8 ppm. In addition to the two groups of signals arising from exo- and endomethyl esters of [^{16}O, ^{18}O] cUMP, the formation of a minor quantity of upfield (carbonate sample) and down field (imidazole sample) was observed (Fig. 2). These signals displayed a similar intensity pattern as the major peak clusters and are probably the product of further alkylations occurring on the uridine moiety. **7**: ^{31}P NMR (DMSO) δ 19.476 (^{16}O–P=^{16}O), 19.459 (^{18}O–P=^{16}O), 19.434 (^{16}O–P=^{18}O), 18.796 (^{16}O–P=^{16}O), 18.778 (^{18}O–P=^{16}O), 18.753 (^{16}O–P=^{18}O).

General procedures for isotope effect determinations. Removal of the protecting groups on the 2′- and 5′-hydroxyl groups was accomplished by dissolving in 30 mL of dilute HCl (pH 1.5) for 45 min at room temperature in the case of 2′,5′-tetrahydropyranyl groups and by identical conditions for 30 min for labeled 2′,5′-(4-methoxytetrahydropyran-4-yl) groups. NMR experiments monitoring the removal of the tetrahydropyranyl groups versus hydrolysis showed that under these conditions deprotection was complete while hydrolysis of the deprotected diester was undetectable by ^{13}P NMR. For ^{15}N isotope effects, the natural abundance 2′,5′-bis(tetrahydropyranyl)uridine-3′-p-nitrophenyl phosphate (**1a**) was used, and for the ^{18}O isotope effect experiments, remote labeled 2′,5′-bis(4-methoxytetrahydropyran-4-yl) uridine-3′-p-nitrophenyl phosphate (**1b**) was used. It was noted that the four diastereomers of **1a** resulting from the chiral tetrahydropyranyl groups exhibited slightly different rates of deprotection. There was a possibility that if the diastereomers differed slightly in their isotopic composition, then in the isotopic mixture used for the bridge ^{18}O isotope effects differential deprotection rates might result in slightly different isotopic mixtures from experiment to experiment. For this reason the achiral protecting groups used in **1b** were used for the remote label experiments.

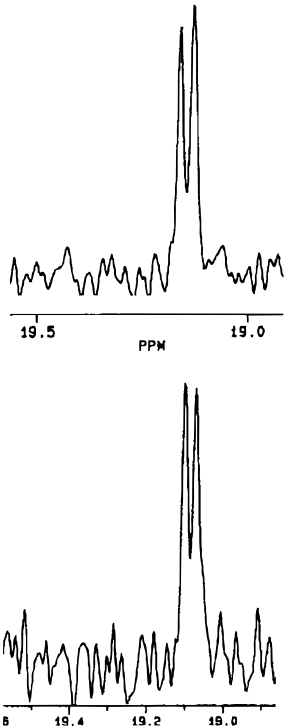
Deblocking of **1a** and **1b** was monitored by adding 10-μL samples to a cuvette containing 0.4 N NaOH and reading the absorbance at 400 nm. The deblocked substrate undergoes nearly instantaneous release of *p*-nitrophenolate under these conditions. After deblocking was complete, the solution was divided into three equal portions treated under the specific reaction conditions described below.

The isotope effects were calculated using the isotopic ratios of the nitrogen atom in the nitro group in the product nitrophenol. This ratio was measured for the product isolated after partial reaction and that in the unreacted substrate following its complete hydrolysis. The isotope ratio in the product and that in the residual substrate, compared

Mix of diastereomers of the triester 3



cUMP products from imidazole (top) and carbonate (bottom) reactions



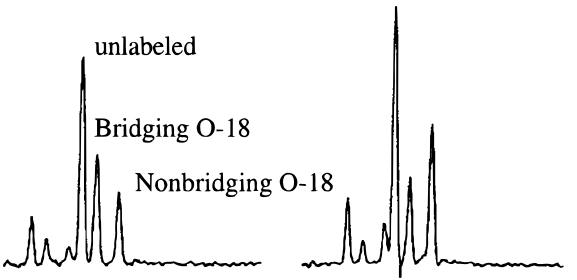
cUMP products after methylation

Imidazole buffer products

unlabeled

Bridging O-18

Nonbridging O-18



Carbonate buffer products