ISOTOPE EXCHANGE REACTIONS OF THE HYDROGEN H-5 OF SELECTED PYRIMIDINE DERIVATIVES AND THE PREPARATION OF TRITIUM-LABELED PYRIMIDINES

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Dedicated to the 75th anniversary of Professor Antonín Holý's birthday and the 25th anniversary of the discovery of antiviral nucleoside phosphonates.

The hydrogen-to-deuterium isotope exchange reaction of hydrogen in position 5 of pyrimidine derivatives was studied using NMR techniques. The dependence of the reaction rate on the pH and on the solvent composition was explored. In tracer experiments using tritiated water, the application of this exchange reaction was tested for the preparation of pyrimidine derivatives labeled by tritium.

Keywords: Isotopic labeling; NMR spectroscopy; Nucleobases; Pyrimidines.

The pyrimidine moiety is an essential structural motif that appears in a large variety of natural products of biological importance[1–3] and in many synthetic drugs[4,5]. There is a large class of pharmacologically important pyrimidine derivatives which act as dihydrofolate reductase (DHFR) inhibitors[6], compounds with anti-HIV [7,8], anti-adenoviruses[9] and anti-HBV activities[10], inhibitors of tetrahydrobiopterin synthesis[11], regulators of pain sensitivity and persistence[12], antidepressants[13] and inhibitors of cyclin-dependent kinase[14,15] or inhibitors of human thymidine phosphorylase[16,17] as a potential drug candidate for cancer therapy.

Two decades ago, a novel class of biologically active compounds with an interesting antiviral activity, acyclic nucleoside phosphonates (ANP), was discovered by Holý[18]. These compounds (e.g. tenofovir, Viread®) are characterized by the presence of a phosphonomethyl ether function at the β-position of a short alkyl chain linked specifically to the N9-position of purine (adenine, guanine or 2,6-diaminopurine) base and show a high and

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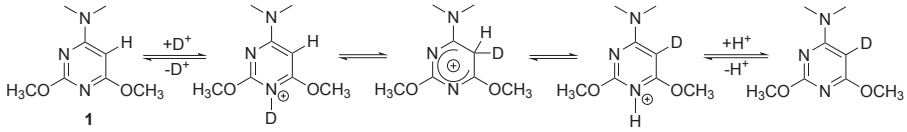
specific activity against DNA-viruses (e.g. herpes-, pox- or adenoviruses), retroviruses (e.g. HIV-1 and 2) and hepatitis B virus[7,8].

In the so-called open-ring ANP, the imidazole ring of the purine heterocyclic system is replaced by the phosphonomethoxyalkoxy substituent of the 2,4-disubstituted (amino, hydroxy) pyrimidine at position O-6 [19,20]. These compounds have essentially the same specific properties in terms of their qualitative antiviral activity when compared to their purine counterparts. For the animal experiments as well as for the metabolic studies, both of which are unavoidable parts of the preclinical phase of any potential novel drug development, it would have been very useful to use a material labeled by (a) radioisotope(s).

A common method for labeling pyrimidines exploits the 5-bromo derivatives easily accessible by direct bromination with elemental bromine or *N*-bromoacetamide[20]. The catalytic tritiodehalogenation on palladium with tritium gas proceeds smoothly and yields the expected radioisotope-containing product[21].

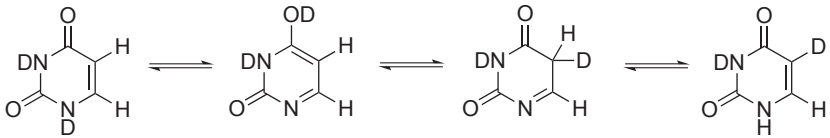
Recently, we have studied the hydrogen-to-deuterium isotope exchange of the hydrogen in position 5 of various pyrimidine derivatives[22,23]. According to our observations, there are two possible mechanisms of the isotope exchange reaction of the hydrogen in position 5 of pyrimidine derivatives. We have shown that C-protonated pyrimidines are the key intermediates in the exchange reaction in acidic media. It has been theoretically and experimentally confirmed that pyrimidine derivatives with electron-donating substituents at the 2-, 4- and 6-position can be protonated at the C-5 position[22,24,25], leading to an equilibrium between the C-5 and N-1/N-3 protonated forms. The positive charge is stabilized by the resonance effect of the substituents. The mechanism of the isotope exchange reaction of H-5 in 4-(*N*,*N*-dimethylamino)-2,6-dimethoxypyrimidine (**1**) in acidic media is depicted in Scheme 1.



SCHEME 1
The mechanism of the isotope exchange reaction of compound **1** in acidic media

We have also studied the mechanism of the isotopic exchange reaction of hydrogen H-5 of uracil and its methyl-derivatives in water and in organic

solvents at neutral pH. The key intermediate of the reaction is the C-5 tautomer of uracil, where the carbon atom at position 5 has two hydrogen atoms, its hybridization is changed from sp^2 to sp^3, and the aromaticity of the pyrimidine ring is lost[23]. Here, C-protonation is not probable, because the pK_as of uracil are –2.4 and 9.4 [26], indicating that the concentration of ionic forms in a neutral solution is very low. The reaction rates depended strongly on the solvent used. The highest reaction rate was observed in 4-methoxypyridine, where the reaction was three orders of magnitude faster than in acetone and two orders of magnitude faster than in water. In organic solvents, we proposed a mechanism with the participation of a solvent molecule. We suggested the following mechanism of isotopic exchange (Scheme 2): The exchange reaction involves a very rare uracil tautomer, which has an sp^3 hybridized carbon atom in position 5. First, the canonical diketo form of uracil is changed to a ketoenol form (3,4-U), which is a fast stepwise process[27–29]. The 3,4-U form is then transformed to the rare 3,5-U tautomer. The organic solvents could work as catalysts in the rare tautomer formation. In the transition state, a solvent molecule could form a complex with the uracil molecule, and the free electron pair on the solvent heteroatoms lowers the barrier of the proton shift from oxygen to carbon C-5. The influence of the electronic structure of the solvent molecules was demonstrated on the series of pyridine derivatives. The electron-donating substituents ($–CH_3$, $–OCH_3$) in position 4 of pyridine increased (with respect to pyridine) the reaction rates. On the other hand, the electron-withdrawing carbonitrile substituent decreased the exchange reaction rate.



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SCHEME 2
The mechanism of the isotope exchange reaction of uracil (**2**)

Here, we report our observations of the pH and solvent dependence of the hydrogen-to-deuterium exchange reaction of simple pyrimidine derivatives and the possible exploitability of this surprising phenomenon for the preparation of [5-^{3}H]uracil derivatives.

RESULTS AND DISCUSSION

We studied the kinetics of the H-5 isotope exchange reaction of pyrimidine derivatives with various buffers and mixtures of solvents. The intensity of the H-5 signal in ^{1}H NMR spectra of compound **1** dissolved in a buffer or a mixture of solvents was continuously decreasing because of the exchange of the proton with deuterium as confirmed by APT ^{13}C NMR spectra, where the signal of C-5 appeared at δ ~ 100 as a singlet pointing down (a CH group). Within a few hours after the sample preparation, the intensity of this signal decreased, and a new signal appeared in the same position. This new signal pointed up (a carbon without any attached proton) and was split into a triplet with equal line intensity because of a coupling with deuterium. This deuteration indicated by NMR was also confirmed by a MS experiment. The ability of a particular pyrimidine derivative to undergo the exchange reaction and even the reaction rate can be roughly estimated from the value of the C-5 chemical shifts. The dependence of the logarithm of the reaction rates on the C-5 chemical shifts for a previously studied[22] series of pyrimidine derivatives with various substituents in positions 2, 4 and 6 is depicted in Fig. 1. Usually, the exchange reaction of the proton in position 5 is very fast for compounds with a $\delta_{C\text{-}5}$ lower than 75 ppm, and the non-deuterated form cannot be observed in acidic D_2O solutions at all. On the other hand, compounds with a $\delta_{C\text{-}5}$ higher than 105 ppm can be

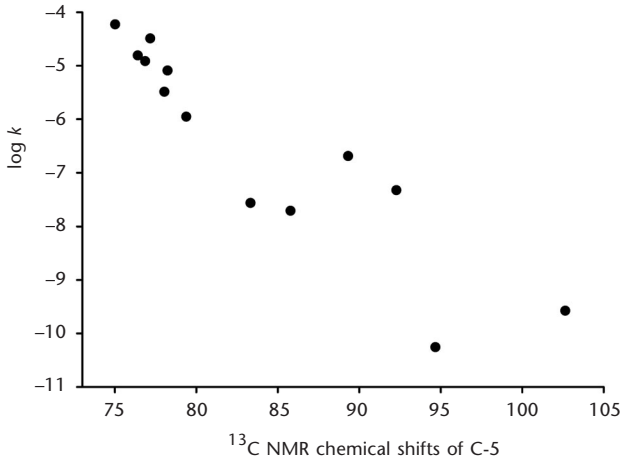


FIG. 1
The dependence of the logarithm of the isotope exchange reaction rate constants on the C-5 chemical shifts for 13 pyrimidine derivatives with various substituents in positions 2, 4 and 6

stored for months in D_2O solutions without any isotope exchange being detected. The ^1H NMR spectra of the samples in a D_2O buffer or in a mixture of solvents were measured repeatedly after specified intervals of time and the intensity of the signal of the H-5 was compared with other signals in the spectra. The H-5 exchange reaction was apparently a pseudo-first-order reaction, and the rate constants were obtained from plots of the logarithm of the starting compound concentrations (with ^1H in position 5) with the points graphed as a function of time. The data fitted first-order kinetics very well; the correlation coefficient was usually higher than 0.99.

We measured the dependence of the exchange rates of the H-5 in compound **1** on the pH of the buffer at room temperature (Table I). The reaction is very sensitive to pH with the reaction rate being almost 20,000 times slower in the pH 10 buffer than in the pH 1.3 buffer. This finding supports the proposed mechanism, where the protonated/deuterated species is the key intermediate of the reaction.

TABLE I
The pH dependence of the isotope exchange reaction of the H-5 of compound **1**

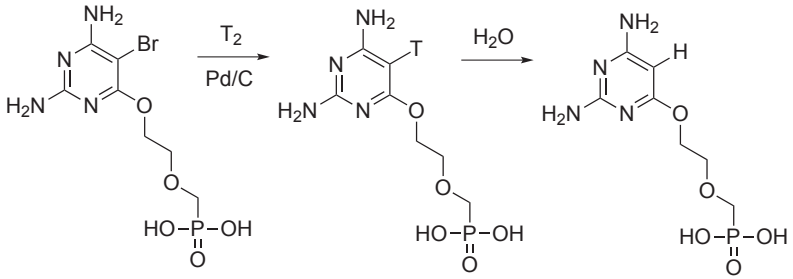
pH	1.3	2.9	4.1	6.0	7.9	8.8	10.0
k, 10^{-4} s^{-1}	8.08	9.22	0.27	0.21	0.058	0.012	0.00042

We measured the isotope exchange rates of 2-amino-4,6-dimethoxypyrimidine in a phosphate buffer with pH 1.3 and in mixtures of the buffer with DMSO in ratios 1:1, 1:5 and 1:30. In the 1:1 mixture, the exchange reaction was only slightly slower than in the pure buffer (2.8×10^{-4} s^{-1} vs 3.3×10^{-4} s^{-1}), but in the 1:5 and 1:30 mixtures, the reaction was 580 and 38,000 times slower, respectively. There are probably two main reasons for the reaction being so much slower in the organic solvents. First, the concentration of D_2O (which acts as a source of the deuterium cation for the C-5 deuteration) is lower and, second, the organic solvents cannot stabilize the intermediate C-protonated form as effectively as water and therefore the $\Delta G^{\#}$ barrier is higher. We observed a similar decrease of the reaction rates also for the isotope exchange reactions of 2-amino-4,6-dimethoxypyrimidine and compound **1** in the 1:30 mixture of the phosphate buffer and tetrahydrofuran.

It follows from our observations that H-5 labeled pyrimidine derivatives with electron-donating substituents should not be used in acidic water solutions, because the label can easily be exchanged with the hydrogen from

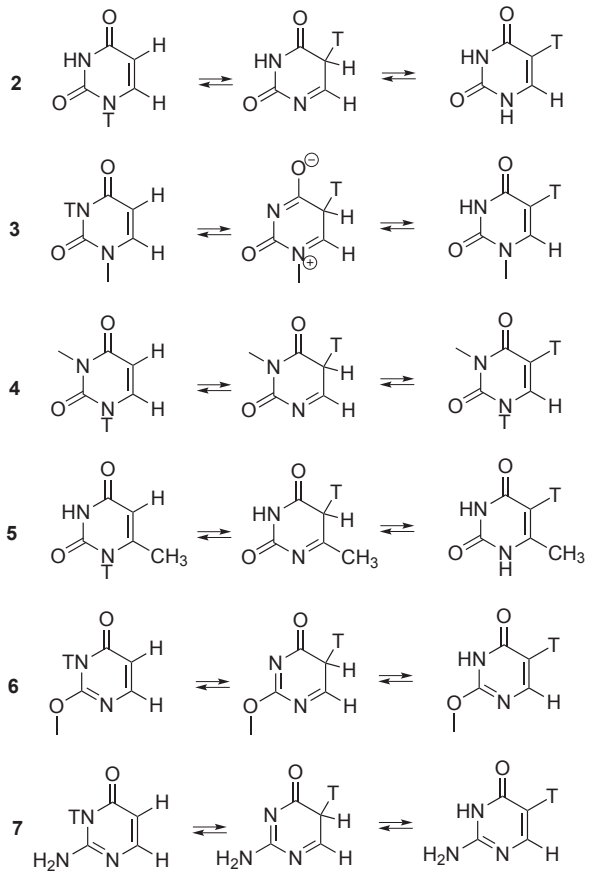
the environment. In alkaline solutions and in organic solvents, the exchange reaction is suppressed. The isotope exchange reaction in acidic water media is not suitable for the preparation of H-5 labeled pyrimidine derivatives since a great excess of the labeled water is necessary (the reaction is very slow in organic solvents with a lower concentration of labeled water) and the acid has to be neutralized and the final product has to be subsequently desalinated.

An example of the undesirable isotope exchange of H-5 may be the reaction of ((2-((2,6-diaminopyrimidin-4-yl)oxy)ethoxy)methyl)phosphonic acid, the so-called open-ring ANP [19,20] (Scheme 3), where the tritium-labeled compound was prepared by the palladium-catalyzed reaction of the 5-bromo derivative[20] with tritium gas. However, on passing the product through the cation exchange resin in an acidic form, all the radioactivity had completely disappeared. Therefore, this purification step had to be avoided. This instability of the 5-isotopic label was also supported by the finding that it had also disappeared after the treatment of the product with the cell-free extract from the appropriate host cells.



SCHEME 3
The isotope exchange of the H-5 of ((2-((2,6-diaminopyrimidin-4-yl)oxy)ethoxy)methyl)-phosphonic acid

In this work, we verified in tracer experiments the suitability of the isotope exchange reaction for the preparation of tritium-labeled uracil derivatives (Scheme 4). The pyridine solutions of compounds **2**–**7** and HTO with a specific activity of 1.3 mCi/mmol in a molar ratio of 1:100, respectively, were heated in sealed ampoules at 100 °C for 3 days. After workup, the reaction mixtures were analyzed by radio-HPLC. Derivatives **3** and **4** turned out to be too volatile and their recovery was too low for proper characterization. The reaction mixture with derivative **6** turned dark brown and only [^{3}H]uracil (**2**) was recovered in a sufficient amount for characterization. The results for **2**, **5** and **7** are summarized in Table II. The position of the radio-



SCHEME 4
The mechanism of the tritium labeling of compounds **2**–**7**

isotope label of compounds **2**, **5** and **7** was verified by ^{3}H NMR spectroscopy. In the spectra, we observed only signals with the chemical shifts corresponding to tritium atom in the position 5. The ^{3}H NMR spectrum of compound **5** is depicted in Fig. 2.

The advantages of this isotope exchange method for pyrimidine-derivative labeling are that: *1*) No chemical synthesis is necessary. *2*) Neither catalysts nor special reagents are necessary. Only dry pyridine and an HTO solution are added to the reaction mixture. *3*) No laborious isolation of the labeled product is necessary. The reaction mixtures can simply be evaporated several times to dryness from H_2O to exchange the labile tritons

TABLE II
The results of the exchange reactions of uracil derivatives with HTO in pyridine

	uracil (**2**)	6-methyluracil (**5**)	isocytosine (**7**)
Recovery of mass [%]	81.5	76.5	79.9
Recovery of radioactivity [μCi]	10.5	10.7	13.5
Specific activity [mCi/mmol]	0.61	1.01	0.67
% of theoretical specific activity[a]	46.8	77.6	58.4
Radiochemical purity [%]	88.6	91.5	94.3

[a] Under equilibrium conditions, the specific activities of isolated pyrimidine derivatives **2**, **5** and **7** at a given molar ratio of 1:100 in favor of HTO should be 98% of the specific activity of HTO, i.e. 1.3 mCi/mmol.

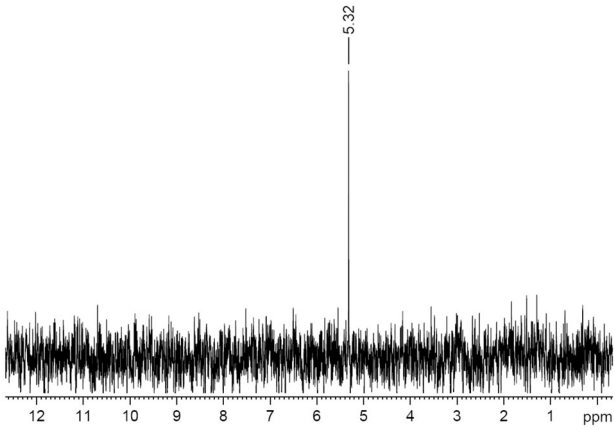


FIG. 2
^{3}H NMR spectrum of [5-^{3}H]-6-methyluracil (**5**)

(OT and NT groups) back to protons. *4*) No radiolysis of the products was observed because of the low concentration of the radiolabel. The biggest drawback of the reaction is that only low specific activity of the products can be obtained with commercially available HTO solutions.

EXPERIMENTAL

Starting Material

Compounds **2**–**5** and **7** were purchased from Sigma–Aldrich. Compounds **1** [22] and **6** [30] were prepared according to the literature. The pyridine for the exchange experiments was

redistilled from P_2O_5 and stored over a molecular sieve. [^{3}H]H_2O (HTO) was purchased from ARC (USA).

^{3}H NMR spectra of the labeled compounds were measured on a Bruker Avance II 300 MHz spectrometer at 320 MHz in DMSO-d_6, with the signal of water at 4.7 ppm taken as an external standard. Broad band ^{1}H decoupling was applied during accumulation of spectra.

The radio-HPLC was performed on a system consisting of a WATERS Delta 600 Pump and Controller, a WATERS 2487 UV detector and a RAMONA radio chromatographic detector from Raytest (Germany). The column effluent was mixed with a Zinsser Quickszint Flow 302 cocktail at a 1:3 ratio. The data were collected and processed using Empower 2.0 software. The radioactivities were measured on a Perkin–Elmer Tri-Carb 2900TR liquid scintillation counter in a Quicksafe A cocktail from Zinsser (Germany). The evaporations were done using a CentriVap Concentrator from Labconco (USA).

Exchange-Rate Determination

Various 50 mM phosphate buffers of the required pH values were prepared (using H_2O), lyophilized and dissolved in D_2O. The pyrimidine derivatives (usually 2 mg) were dissolved in 600 µl of a buffer. The ^{1}H NMR spectra of the samples stored at room temperature were acquired periodically (with a suitable delay between them). The NMR spectra were measured with a Bruker Avance II 500 instrument (499.84 MHz for ^{1}H). At least ten spectra were measured for each sample. The amount of ^{1}H in position 5 was obtained by the integration of the H-5 signal with respect to an integral intensity of another ^{1}H signal of the molecule. Derivatives without any suitable ^{1}H signal in the molecule were measured with one drop of dioxane as the internal integration standard. The rate constants were obtained from the plots of the logarithm of the ^{1}H-5 concentrations versus time. The experimental error of the reaction-rate determination was estimated to be less than 2%.

Exchange Reactions of Compounds **2–7** with HTO

The uracil derivatives were dried over phosphorus pentoxide under vacuum overnight. The NMR standard 5 mm NMR tubes were dried just before weighing by heat gun. The corresponding uracil derivative was weighed (2 mg) to the NMR tube and 500 µl of pyridine and 30 µl of HTO (specific activity 1.3 mCi/mmol) were added. The NMR tube was flame-sealed and heated in a silicone oil bath at a temperature of 100 °C for 72 h. After cooling, the ampoule was opened, the reaction mixture was transferred by Pasteur pipette to a glass centrifugation tube (the ampoule was washed with one 250 µl portion of pyridine) and evaporated to dryness. The residue was dissolved in 1 ml of HPLC grade water and evaporated to dryness. This step was repeated once more to complete the labile activity elimination. The solid colorless residue was then dissolved in 600 µl of water and the radioactivity was assayed. The composition of the reaction mixture was assayed by radio-HPLC. The specific activity of the tritium labeled uracil derivative was calculated using an UV-based mass assay by HPLC and LSC radioactivity measurement (corrected by radiochemical purity).

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