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Kinetic and mechanistic studies on the dephosphoryl reaction catalyzed in nucleoside 5′-amino acid phosphoramidates

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Abstract

Dephosphorylation catalyzed by a base occurs in nucleoside 5′-phosphoramidates (**1a**–**3b**) and nucleoside 5′-thiophosphoramidates (**4a**–**5b**). The reaction rates for both classes of compounds increase with the basicity of the catalysts in the solution. The kinetics and mechanisms of the reaction were investigated by NMR spectroscopy and a semi-empirical quantum mechanics method. Our experimental results showed that the reaction was pseudo first order. The rate constants of the dephosphorylation for compounds **1a**–**3b** were faster than those for compounds **4a**–**5b**. The effects of the structures of compounds on the reaction were discussed.

Keywords: Kinetics; Dephosphorylation; Phosphoramidates; Thiophosphoramida

1. Introduction

It is well known that phosphorylation and dephosphorylation of proteins play a very important role in regulating complicated biochemical processes in all living organisms [1]. Most of the enzymes catalyzing protein phosphorylation and dephosphorylation reactions employ the hydroxyl group on the serine or threonine residue of their active site [2,3]. In addition to their roles in the normal metabolism process, reversible phosphorylation reactions also contribute to abnormal cancer formation pathways [4–6]. However, the chemistry of the phosphorylation/dephosphorylation mechanism in biological systems is still not very clear. It is even worthwhile to note that the development of nucleoside produgs capable of undergoing intracellular activation to the corresponding nucleotide has become an area of intense interest [7–9]. Several nucleoside analogs are important weapons in the anticancer and antiviral chemotherapeutic arsenal. The biological activity of most of these analogues requires intracellular metabolism to 5′-mononucleotides by kinase-mediated phosphorylation. Some nucleotide-5′-phosphoramidates are very efficient HIV inhibitors and have attracted attention as antiviral nucleoside prodrugs [7,8].

Recently, the first synthesis of a series of nucleoside 5′-phosphoramidates (**1a**–**3b**) and nucleoside 5′-thiophosphoramidates (**4a**–**5b**) have been achieved and characterized by mass spectroscopy (MS), nuclear magnetic resonance (NMR) and elemental analysis. These compounds were designed to act as membrane-soluble prodrugs of bioactive free nucleotides and some have shown selective anti-HIV activity in MT-4 cells [9–11]. It is interesting to find that dephosphoryl reactions catalyzed by a general base can take place for both compounds. Here we present the kinetic and mechanistic studies on the dephosphoryl reactions and the factors that affect the reactions.

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2. Experimental

2.1. Materials

Nucleoside 5′-phosphoramidates (**1a**–**3b**) were prepared according to published procedures [9], and nucleoside 5′-thiophosphoramidates (**4a**–**5b**) were synthesized according to the method reported in [10,11]. Their structures were characterized by ESI-MS, ^{1}H-NMR, ^{13}C-NMR, ^{31}P-NMR, 2D-NMR and elemental analysis.

2.2. Methods

All NMR experiments were carried out on a Bruker AC-200P or a Bruker AM-500 FT NMR spectrometer. The ^{1}H- and ^{13}C-NMR chemical shifts are referred to the TMS and $CDCl_3$, respectively. ^{31}P-NMR spectra were obtained using 85% phosphoric acid as an external reference. Kinetic studies were performed by time evolution of the proton-decoupled ^{31}P-NMR spectra of the mixture. All the measurements were performed at room temperature.

3. Results and discussion

3.1. Kinetic study

The chemical structures of *O*-[2′,3′-isopropylidene](*O*-[2′,3′-diacetyl])uridine-5′-yl *O*-isopropyl *N*-phosphoryl serine (threonine) methyl esters **1a**–**2b**, *O*-[2′,3′-diacetyl] thymidine-5′-yl *O*-isopropyl *N*-phosphoryl serine (threonine) methyl esters **3a**–**3b**, *O*-[2′,3′-isopropylidene]uridine-5′-yl *O*-isopropyl *N*-thiophosphoryl serine (threonine) methyl esters **4a**–**4b**, and *O*-[2′,3′-isopropylidene]uridine-5′-yl methoxyserinyl(threonenyl) thiophosphate **5a**–**5b** are illustrated in Scheme 1.

^{31}P-NMR is an important method to monitor the process of reaction and to get information about the reaction of phosphorous compounds, especially when the intermediates and products are unstable. The ^{31}P-NMR spectra showed that the dephosphorylation reaction of compounds **1a**–**5b** can take place in CH_3CN when they were treated with a little NEt_3. For example, when the base was added, after 20 min the ^{31}P-NMR signal of compound **3a** (**A** at δ_P: 7.80 ppm) was transformed into an intermediate (**B** at δ_P: 21.5 ppm), while the signal of the product (**C** at δ_P: 5.38 ppm) emerged (Fig. 1). The final **C** reached its maximum after 12 h. Serine methyl ester and 2′,3′-*O*-isopropylidene serine were obtained by TLC (coated with silica gel) in almost quantitative yields after the reaction was complete. It is interesting to note that the intermediate was a penta-coordinate, due to giving a signal at 21.5 ppm. Similar observations were observed for other compounds.

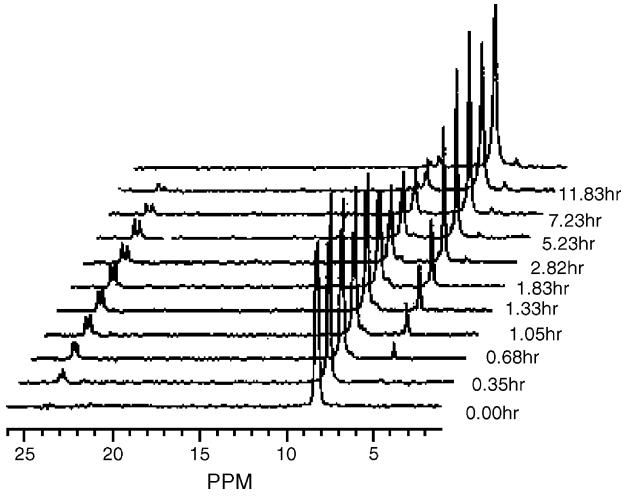
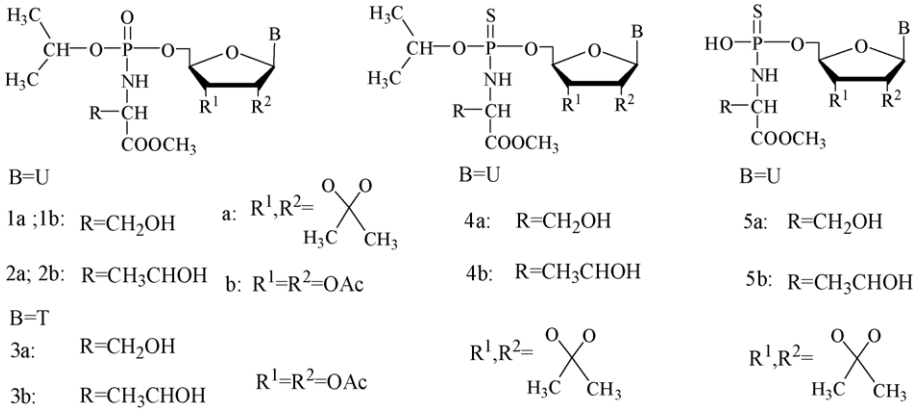


Fig. 1. ^{31}P-NMR stack spectra for dephosphoryl reaction of compound **3a**.

The kinetic data were determined by the integral area of the quantitative ^{31}P-NMR peaks, and the rate of reactants disappearance was considered as the rate of the reaction. Integrating the appropriate peaks gave the ln *C*–*t* curves (*C*: concentration, *t*: time), which were found to be a straight line. These results indicated that the dephosphorylation reaction of **1a**–**5b** was a first order kinetic reaction. The rate constants



Scheme 1. Structures of compounds **1a**–**5b**.

Table 1
The k (s^{-1}) and $t_{1/2}$ (h) of the reaction (T, °C)

	Compound					
	1a (40 °C)	**2a** (40 °C)	**3a** (22 °C)	**3b** (22 °C)	**4b** (60 °C)	**5b** (60 °C)
k	3×10^{-5}	1×10^{-4}	9×10^{-5}	2×10^{-4}	1.8×10^{-6}	2.0×10^{-6}
$t_{1/2}$	6.42	1.92	2.14	0.96	10.38	9.42

(k) and half-life time ($t_{1/2}$) were calculated according to Eqs. (1) and (2) and are summarized in Table 1.

$$\ln \frac{C_0}{C} = kt \quad (1)$$

$$t_{1/2} = \frac{\ln 2}{k} \quad (2)$$

3.2. Effect of basicity of catalysts on the reaction

Experimental results indicated that the reaction was catalyzed by a general base. The rate constants k were affected by the relative basicity of the catalysts and the concentrations of the reactants. In order to study the effect of the basicity of catalysts on the reactions, the reaction rates of compound **1a** were determined with five catalysts with different basicity, respectively. The data demonstrated that the dephosphoryl reaction in the presence of catalysts with different basicity was also first order kinetic reaction and was accelerated when the basicity of the catalysts was increased, with the exception of imidazole (Table 2).

It is worthwhile to mention that imidazole is an interesting catalyst in biological systems. It contains two nitrogen atoms and has a unique resonance structure. It can catalyze many biological and chemical reactions, including cleavage and isomerization of a dinucleotide [12], and enantioselective hydrolysis of esters of amino acids [13]. It has previously been shown that the formation of penta-coordinate intermediates is promoted by imidazole [14], which means the reaction is catalyzed more efficiently by imidazole than by some catalysts that have higher basicity. This is also in agreement with the catalytic mechanism of the present paper.

However, there are several types of catalysts in Table 2. Triethylamine is an aliphatic amine, and trimethylpyridene and pyridine are aromatic amines. Comparing their basicity and structure versus reaction rates in Table 2, it seems that aspects of the structure also play an important role in catalyzing reactions, and the details of the mechanism of catalysis is under further investigation.

Table 2
Comparison of the kinetic data of compound **1a** with different catalysts

Base	T (°C)	pK_b	k (s^{-1})	$t_{1/2}$ (h)
Hexahydropyridene	22	2.88	7×10^{-4}	0.28
Triethylamine	22	3.28	3×10^{-5}	6.42
Imidazole	40	7.05	1×10^{-5}	19.25
Trimethylpyridene	40	6.57	7×10^{-6}	27.51
Pyridine	40	8.83	6×10^{-6}	32.09

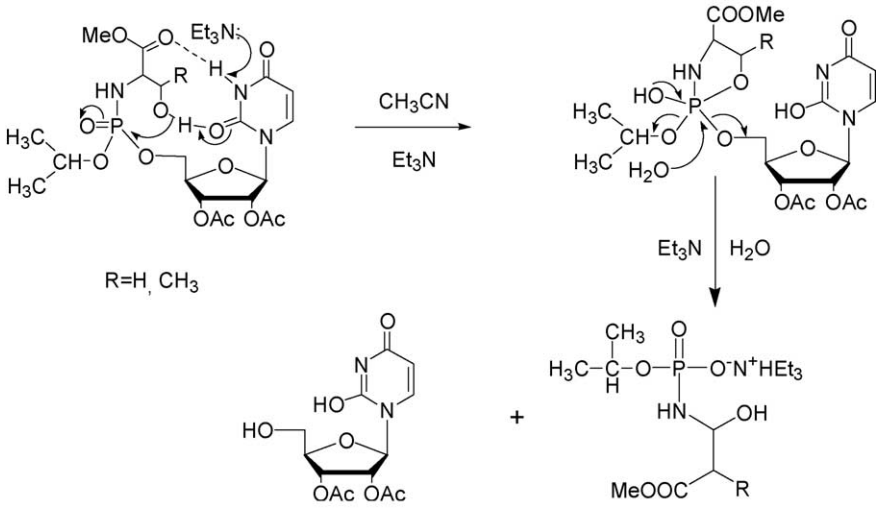
3.3. Structural effects on the reaction

There are significant differences in the reaction rates of 5′-phosphoramidates (**1a**–**3b**) and nucleoside 5′-thiophosphoramidates (**4a**–**5b**). The results of our study showed that compounds **1a**–**3b** dephosphorylated more easily than compounds **4a**–**5b** under the same conditions. The reaction for compounds **1a**–**3b** finished in less than 12–20 h at room temperature, but it took about 24–36 h for compounds **4a**–**5b** to react completely at 60 °C. It seems that substrate structures play an important role in the reactions. The compounds differ only in the electro negativity of the atom related to reactivity. The electro negativity of the oxygen atom in compounds **1a**–**3b** is higher than that of the sulfur atom in compounds **4a**–**5b**, resulting in higher nucleophilicity of the P atom in the former than in the latter.

In addition, we found that the dephosphorylation reaction of 5′-phosphoramidates bearing threonine residue was faster than those for compounds with serine residue under the same conditions. This suggested that the structure had an effect on the reactions also. The difference between serine and threonine residues is the $-CH_3$ group that acts as an electron donor. This leads to a higher nucleophilicity of the −OH group in threonine than that in serine.

3.4. Mechanism studies

In order to study the reaction mechanism, it is necessary to note the conformation of compounds. Pyrimidine nucleoside is generally believed to favor anti conformation in solution. However, the results from our NOE (NMR) experiments indicated the glycosyl bonds in nucleoside 5′-phosphoramidates (**1**–**3**) and nucleoside 5′-thiophosphoramidates (**4**–**5**) favor syn orientation. There were significant NOE cross-peaks between H-1′and H-2′ of the ribose ring and H-6 of the pyrimidine of compounds in the spectra, namely, the 2-keto group of the pyrimidine base over the ribose ring. Because this conformation was formed, we assume that there are probably two pairs of hydrogen bonds between carbonyl, hydroxyl groups of serine (threonine) and the pyrimidine base (Scheme 2). The results from the semi-empirical quantum mechanics computation support this assumption to some degree. The model established by full geometric structure optimization (AM1 method) revealed two pairs of



Scheme 2. The proposed catalytic route for the dephosphorylation.

intramolecular hydrogen bonds. For instance, the distances between the (U) C (2)=O to HO-β (Ser) and (U) N (3)H to O=C (Ser) for compound **1b** were calculated to be 2.126 and 2.227 Å, respectively. This suggested that there was a mechanism of intramolecular catalyzed dephosphorylation. The hydroxyl group on the serine (threonine) side chain is activated by this intramolecular hydrogen bonding and could attack the P atoms nucleophilicily. The participation of intramolecular hydrogen bonds increased the nucleophilicity of the reactants. The reaction proceeded via a penta-coordinate intermediate, which gave a signal at 21.5 ppm (δ_P). This peak disappeared eventually and led to the self-cleavage of the phosphodiester bond. The proposed mechanism for the intramolecular catalytic dephosphorylation reactions of compounds **1**–**5** is illustrated in Scheme 2.

According to the mechanism suggested, it provided the example that the hydrogen bond between the amino acid side chain of the protein and the base group of nucleic acid is the limiting factor for intramolecular catalytic dephosphoryl reactions, which might be related to the enzymatic nucleotide transfer reaction, nucleic acid cleavage or other biochemical reactions.

4. Conclusion

The dephosphorylation of phosphoramidates and thiophosphoramidates was catalyzed by a base and proceeded via a penta-coordinate intermediate. The rate constants of the reaction were affected by the basicity of the catalysts and the structures of the reactants.

Acknowledgments

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