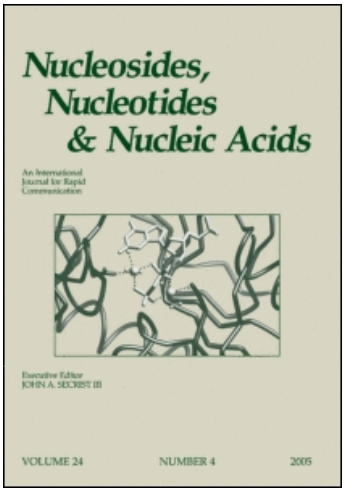

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SUBSTRATE PROPERTIES OF ADENOSINE- AND URIDINE- 3'-PHENYLPHOSPHONATES FOR 3'-NUCLEOTIDASE/NUCLEASES[1)]

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ABSTRACT: Adenosine- and uridine- 3'-phenylphosphonates have been synthesized and evaluated as substrates of 3'-nucleotidase/nucleases. No other nucleases hydrolyzed these compounds. Since V_{max} values for the adenosine derivative were comparable to those for 3'-AMP and the apparent K_m were 1.4-2.6 mM, it may be a useful substrate.

INTRODUCTION

A class of endonucleases, liberating 5'-mono- and oligonucleotides from single-stranded RNA and DNA without significant base specificity, has been purified from various plants and fungi.[1] The enzymes recognize nucleoside 3'-phosphate as a substrate and attack 3'-O-P bond, thus always show 3'-nucleotidase activity.[2-8] Their application to nucleic acid structure studies and gene manipulations are well known. However, there is very little information on the biological role of 3'-nucleotidase/nuclease in plants and fungi.

An enzyme with the similar specificity has been purified from the membrane fraction of the protozoan family Trypanosomatidae.[9,10] The investigations of the level of the activity at the various concentration of purine in the culture medium suggest that it functions in the acquisition of the required purines from exogenous sources which are not capable of being transported across the surface membrane.[11]

It is interesting to investigate the change of the activity of 3'-nucleotidase/nuclease in plants and fungi during growth and its distribution using specific substrates, because these results may serve to give an information in understanding their biological role. To the purpose, nucleoside 3'-phosphonate esters appear suitable compounds, because the derivatives would be hydrolyzed by the enzymes, but be not by 5'-O-P bond-hydrolyzing nucleases and phosphatase. In this paper, we prepared 3'-phenylphosphonate esters of adenosine and uridine as substrates of 3'-nucleotidase/nuclease and examined their substrate properties. A part of preliminary results were reported.[12]

[1)] This article is dedicated to Dr. Yoshihisa Mizuno for celebration of his 75th birthday.

MATERIALS AND METHODS

Materials.

5'-*O*-Trityl derivatives of adenosine or uridine, phenylphosphonic dichloride, snake venom phosphodiesterase, spleen phosphodiesterase, micrococcal nuclease, and RNase T_2 were obtained from Sigma Chemical Co. (St. Louis, MO). Adenosine, uridine, 3'-AMP, 3'-UMP and P1 nuclease were products of Seikagaku Kogyo Co. Ltd. (Tokyo, Japan). Mung bean nuclease and S1 nuclease were from Takara Shuzo Co. Ltd. (Kyoto, Japan). Dowex AG-1 x 8 was from Bio-Rad Laboratories (Richmond, CA) and Cosmosil 75C18-OPN was from Nacalai Tesque Inc. (Kyoto, Japan). Good's buffers were from Dojindo Laboratories (Kumamoto, Japan). All other chemicals were of analytical grade.

Analyses.

HPLC was carried out with a Cosmosil packed 5C18-MS column (4.6 x 250 mm, Nacalai Tesque Inc., Kyoto) in a Shimadzu LC-9A apparatus (Kyoto, Japan) at 50°C. The solvent was composed of 5 mM NaH_2PO_4 and acetonitril (10:1). The flow rate was 1.0 ml/min and elution was monitored at 260 nm. Integration was done with a Shimadzu C-R6A Chromatopac (Kyoto, Japan).

UV absorption spectra were recorded on a UV-visible recording spectrophotometer model UV-160, Shimadzu, Kyoto, Japan.

Enzymatic interconversions of nucleotide analogs and nucleosides were carried out with P1 nuclease in 0.1 M Tris-HCl buffer, pH 7.5, at 37°C for 2 h, and analyses were done by HPLC.

^{1}H-NMR spectra were run on a JEOL JNM-GX 200 Fourier transform NMR spectrometer (270 MHz, Tokyo, Japan) with residual HDO as an internal standard (δppm 4.8). Signal assignments of nucleotide analogs were done by comparison of those of their respective 3'-ribonucleotide.[13]

Preparation of adenosine 3'-phenylphosphonate (3'-APP) and uridine 3'-phenylphosphonate (3'-UPP).

To a solution of 5'-*O*-trityladenosine (1.63 g, 3.2 mmol) in 50 ml of pyridine, phenylphosphonic dichloride (0.93 ml, 1.28 g, 6.4 mmol) was added. The mixture was stirred in an ice bath for 1 h and at room temperature for additional 2.5 h, and evaporated to dryness below 40°C. The residue was dissolved in 20 ml of 80% acetic acid, heated for 15 min at 100°C, and neutralized with 1N NaOH. After being filtered, the filtrate was diluted 50-fold with 0.01 M formate buffer, pH 3.0, and put on a Dowex AG-1 x 8 column (2.6 x 50 cm) equilibrated with 0.01 M formate buffer, pH 3.0. The column was washed with the same buffer, and eluted with a 2000 ml linear gradient from 0.01 to 0.4 M formate buffer, pH 3.0. The eluate containing the compound converted to adenosine on treatment with P1 nuclease were pooled, concentrated by evaporation below 40°C, and put on a Cosmosil 75C18-OPN column (2.6 x 25 cm) equilibrated with distilled water. After the column was washed with distilled water, the product was eluted with 20% acetonitrile and lyophilized (0.63 g, 1.8 mmol, 57%).

This preparation showed a single peak by HPLC analysis. The UV spectra at pH 1, 7, and 10 of this preparation were essentially identical with those of 3'-AMP. The preparation was dissolved in 99.8% D_2O and lyophilized twice, and the final lyophilized sample was dissolved in 99.8% D_2O. The chemical shifts of ^{1}H-NMR spectrum were

8.13 (1H, s, H-8), 8.01 (1H, s, H-2), 7.45-7.66 (5H, m, phenyl), 6.04 (1H, d, H-1'), 4.55 (1H, m, H-3'), 4.11 (1H, m, H-4'), and 3.25-3.57 (2H, m, H-5', 5''). The H-2' signal overlapped with a residual HDO peak.

Uridine 3'-phenylphosphonate (0.38 g. 1.2 mmol, 29%) was prepared from 5'-*O*-trityl-uridine (2.0 g, 4.1 mmol) by the reaction with phenylphosphonic dichloride according to the method described above. The preparation showed a single peak by HPLC analysis. The UV spectra at pH 1, 7, and 10 of this preparation were essentially identical with those of 3'-UMP. The chemical shifts of ^{1}H-NMR spectrum were 7.55-7.85 (6H, m, H-6 and phenyl), 5.89 (1H, d, H-5), 5.97 (1H, d, H-1'), 4. 53 (1H, m, H-3'), 4. 31 (1H, m, H-2'), 4.19 (1H, m, H-4'), and 3.53-3.76 (2H, m, H-5', 5'').

Enzymatic activity.

Mung bean nuclease (1.0 U), S1 nuclease (1.0 U), P1 nuclease (0.01 U), phosphatase alkaline (2.0 U), snake venom phosphodiesterase (2.0 U), spleen phosphodiesterase (2.0 U), micrococcal nuclease (2.0 U), and RNase T_2 (2.0 U) were assayed for their activities for 3'-APP and 3'-UPP, where the activities (U) were expressed according to the supplier's definitions. A reaction mixture containing 1.0 mM the compound, 20 mM acetate buffer, pH 5.0, or Tris-HCl buffer, pH 8.0, and enzyme in a total volume of 0.2 ml was incubated at 37°C overnight. The nucleosides produced were analyzed by HPLC.

Mung bean nuclease (24 U), S1 nuclease (40 U), and P1 nuclease (0.02 U) were assayed at various pH for their activities for 3'-APP, 3'-UPP, 3'-AMP and 3'-UMP. A reaction mixture containing 20 mM buffer, the enzyme, and 5.0 mM substrate in a total volume of 1.0 ml was incubated at 37°C for 10 min. The buffer used were; pH 4 - 5.5, acetate buffer; pH 5 - 7, MES buffer; pH 7 - 8, HEPES buffer; pH 8 - 9, CHES buffer. As the substrate was 3'-APP or 3'-UPP, the amount of the nucleoside produced was measured by HPLC. When 3'-AMP or 3'-UMP was used as a substrate, the release of Pi from the 3'-nucleotides was determined according to the method descibed before.[8]

RESULTS AND DISCUSSION

Mung bean nuclease, S1 nuclease, and P1 nuclease hydrolyzed 3'-APP and 3'-UPP to adenosine and uridine, respectively. Assays for activities of the 3'-nucleotidase/nucleases in the crude preparation using 3'-ribonucleotides as a substrate are interfered by phosphatase, and those using RNA are by the other nucleases. Phosphatase and the other nucleases tested did not hydrolyze 3'-APP and 3'-UPP with the amount indicated. At the amount, the enzymes completely hydrolyzed 5 mM 3'-ribonucleotides or 10 mg/ml of yeast RNA overnight. The present results suggested that the assays with 3'-APP and 3'-UPP were not interfered with the other nucleases.

Mung bean nuclease, S1 nuclease, and P1 nuclease had maximum activities at pH 4.5 - 6.0 for 3'-APP and 3'-UPP, and at pH 7.0 - 8.0 for 3'-AMP and 3'-UMP (Fig. 1). The optimum pH of the enzymes for the nucleoside 3'-phenylphosphonates were similar to those for RNA and single-stranded DNA.[5,14,15] The enzymes might recognize the phosphonate group of the nucleoside 3'-phenylphosphonates as analog of the phosphodiester group of RNA and single-stranded DNA.

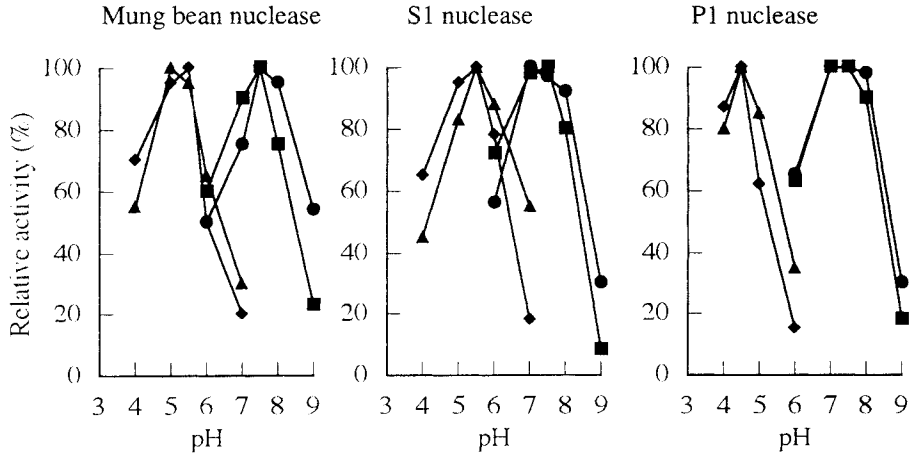

Fig. 1. Optimum pH for 3'-APP, 3'-UPP, 3'-AMP, and 3'-UMP of 3'-Nucleotidase/nucleases. At optimum pH, the hydrolysis rates for 3'-APP (▲), 3'-UPP (◆), 3'-AMP (●), and 3'-UMP (■) of mung bean nuclease were 0.0040, 0.0003, 0.0031, and 0.0012 μmol/min U, those of S1 nuclease were 0.00112, 0.00011, 0.00038, and 0.00037 μmol/min U, those of P1 nuclease were 4.2, 6.9, 4.6, 0.5 μmol/min U, respectively.

Table 1. Apparent K_m and V_{max} for 3'-APP and 3'-UPP of 3'-nucleotidase/nucleases.

	Mung bean nuclease		S1 nuclease		P1 nuclease	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
Substrate	(mM)	(μmole/min U)	(mM)	(μmole/min U)	(mM)	(μmole/min U)
3'-APP[a)]	2.2	0.0052	1.4	0.00105	2.6	4.7
3'-AMP[b)]	0.6	0.0016	0.4	0.00036	0.3	4.5
3'-UPP[a)]	22.0	0.0024	23.7	0.00124	28.6	7.4
3'-UMP[b)]	4.8	0.0019	1.1	0.00038	1.2	7.2

a) Values were obtained in 20 mM acetate buffer, pH 5.0.
b) Values were obtained in 20 mM Tris-HCl buffer, pH 7.0.

At optimum pH, the V_{max} values of the 3'-nucleotidase/nucleases for 3'-APP and 3'-UPP were comparable to those for 3'-AMP and 3'-UMP, and the apparent K_m values for 3'-APP and 3'-UPP were approximately one order of magnitude higher than those for 3'-AMP and 3'-UMP, respectively (Table 1). Judging from the apparent K_m (1.4 - 2.6 mM) and V_{max} for 3'-APP, it is a useful substrate of the enzymes.

Adenosine (phosphate) deaminases have been purified from the various microorganisms.[16-22] We reported that an adenosine (phosphate) deaminase from the squid liver can deaminate 3'-APP to inosine 3'-phenylphosphonate.[23] Investigation using 3'-APP of 3'-nucleotidase/nuclease activity in preparation containing the deaminase may be limited. But this problem can be overcome by using 3'-UPP, even though it is a rather poor substrate. Alternatively, it will be resolved by the use of 3'-APP together with an adenosine deaminase inhibitor, for example coformycine. Now investigation using 3'-APP and 3'-UPP of the change of the nuclease activities in plants and fungi during growth and their distribution is in progress. An enzyme, identified as a 3'-nucleotidase/nuclease, has been shown to localize in the surface membrane of several members of the protozoan family Trypanosomatides.[9-11] An enzyme with similar specificity has not been identified in mammals. The compounds may be used for diagnosis of the diseases caused by Leishmania and African trypansomes.

REFERENCES

1. Kowalski, D.; Laskowski, M. Sr. in "Handbook of Biochemistry and Molecular Biology", 3th edn., ed. by G. D. Fasman, Chemical Rubber Co., Cleveland, Ohio, 1976, Vol. 2, pp. 491-531.
2. Suno, A.; Nomura, A.; Mizuno, Y. *J. Biochem.* 1973, 73, 1291.
3. Sung, S. C.; Laskowski, M .Sr. *J. Biol .Chem.* 1962, 237, 506.
4. Nomura, A.; Suno, M.; Mizuno, Y. *J. Biochem.* 1971, 70, 993.
5. Fujimoto, S.; Kuninaka, A.; Yoshino, H. *Agric. Biol. Chem.* 1974, 38, 777.
6. Kroeker, W. D.; Hanson, D. M.; Fairley J. L. *J. Biol. Chem.* 1975, 250, 3767.
7. Shishido, K. ; Habuka, N. *Biochim. Biophys. Acta* 1986, 884, 215.
8. Uchida, H.; Wu, Y. -D.; Takadera, M.; Miyashita, S.; Nomura, A. *Biosci. Biothech. Biochem.* 1993, 57, 2139.
9. Neubert, T. A.; Gottlieb, M. *J. Biol. Chem.* 1990, 265, 7236.
10. Gbenle, G. O.; Dwyer, D. M. *Biochem. J.* 1992, 285, 41.
11. Gottlieb, M. *Science* 1985, 227, 72.
12. Nomura, A.; Tamura, M. Nucleic Acids Research 1978, Special Publication No. 5, 415.
13. Davis, D. B.; Danyluk, S. S. *Biochemistry* 1975, 14, 543.
14. Kroeker,W. D.; Kowalski, D.; Laskowski, M. Sr. Biochemistry 1976, 15, 463.
15. Ando, T. *Biochim. Biophys. Acta* 1966, 114, 158.
16. Minato, S.; Tagawa, T.; Nakanishi, K. *J. Biochem.* 1965, 58, 519.
17. R. Wolfenden, R.; Sharpless, T. K.; Allan, R. J. Biol. Chem. 1967, 5, 977.
18. Su, J. -C.; Li, C. -C.; Ting, C. C. *Biochemistry* 1966, 5, 536.
19. Chung, S. -T.; Aida, K. *J. Biochem.* 1967, 61, 1.
20. Yates, M. G. *Biochim. Biophys. Acta* 1969, 171, 299.

21. Nishizawa, K.; Okada, Y.; Kubo, K.; Anzai, H. *Jap. J. Phycol.* 1980, 28, 205.
22. Jun, H .-K.; Kim, T. -S.; Sakai, T *J. Ferment. Bioeng.* 1991, 71, 6.
23. Uchida, H.; Narita, Y.; Masuda, A.; Matsui, Y.; Chen, Y. -X.; Takahashi, I.; Maeda, S.; Nomura, A. *Biosci. Biotech. Biochem.* in press.