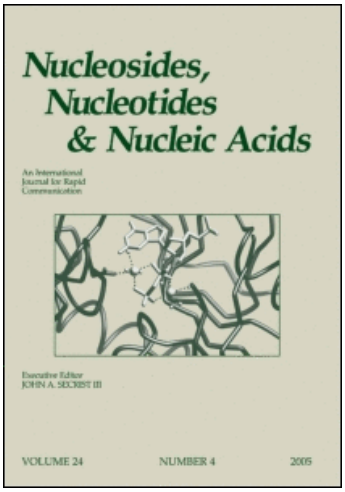

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SUBSTRATE PROPERTIES OF ADENOSINE- AND URIDINE- 3', 5'-BISPHENYLPHOSPHONATES FOR 3'-NUCLEOTIDASE/NUCLEASES

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ABSTRACT: 3', 5'-Bisphenylphosphonate and 5'-phenylphosphonate esters of adenosine and uridine were synthesized to investigate the substrate properties of the 3', 5'-bisphenylphosphonates for 3'-nucleotidase/nucleases. The V_{max}/apparent K_m values of the enzymes for them were found to be 9 to 21-fold higher than those for the corresponding nucleoside 3'-phenylphosphonates.

INTRODUCTION

A class of endonucleases, recognizing nucleoside 3'-phosphate as a substrate and attacking 3'-O-P bond, has been purified from various plants and fungi, and protozoan family Trypanosomatidae.[1-11] The enzymes, 3'-nucleotidase/nucleases, liberate 5'-mono- and oligonucleotides from single-stranded RNA and DNA without significant base specificity and show 3'-nucleotidase activity.

In our previous report, we have shown that 3'-phenylphosphonate esters of adenosine and uridine are hydrolyzed by mung bean nuclease, nuclease S1, and nuclease P1 but not by phosphatase and the other nucleases tested.[12] Assays for activities of 3'-nucleotidase/nucleases in crude preparations using the compounds as substrates can be done without interference by phosphatase and the other nucleases. The apparent K_m values of the 3'-nucleotidase/nucleases for the compounds were approximately one order of magnitude higher than those for the corresponding nucleoside 3'-monophosphates. The optimum pH of the enzymes for them were similar to those for RNA and single-stranded DNA. The enzymes might recognized the phosphonate group with no secondary ionizable hydroxyl group as an analog of the phosphodiester group of the polynucleotides.

The existence and nature of subsite interaction have been elucidated in studies on various nucleases.[13-16] Our previous inhibition studies using nucleosides and 5'-nucleotides have shown the possibility that a 3'-nucleotidase/nuclease from potato tubers has not only a phosphate-binding site for 3'-phosphate but also one for 5'-phosphate in an active site.[4] Thus, a nucleoside with 3'- and 5'-phosphonate groups might be better substrates of 3'-nucleotidase/nucleases than that with a 3'-phosphonate group. Comparison of the kinetic parameters for them might lead us to get the information about subsite interactions of polynucleotides with the enzymes.

In this paper, we described preparations of 3', 5'-bisphenylphosphonate and 5'-phenylphosphonate esters of adenosine and uridine and the substrate properties of the 3', 5'-bisphenylphosphonates for plant and fungal 3'-nucleotidase/nucleases. They were better substrates than the corresponding nucleoside 3'-phenylphosphonates. The existence of the additional 5'-phosphonate group affected substrate binding and reaction rate.

MATERIALS AND METHODS

Materials. Adenosine, uridine, 2', 3'-*O*-isopropylidene derivatives of adenosine or uridine, phenylphosphonic dichloride, Mung bean nuclease, nuclease S1, nuclease P1, RNase T2, RNase A, spleen phosphodiesterase, and phosphatase alkaline were obtained from Sigma Chemical Co. (St. Louis, MO). Adenosine- and uridine 3'-phenylphosphonates were synthesized as described before.[12] DEAE-Sephadex A-25 and Cosmosil 75C_{18}-OPN were purchased from Pharmacia (Uppsala, Sweden) and Nacalai Tesque Inc. (Kyoto, Japan), respectively. 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 5C_{18}-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 acetonitrile (8:1). 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 conversions of nucleoside di- to monophosphonates were carried out with nuclease P1 in 0.1 M acetate buffer, pH 5.5, at 37°C for 2 h, and analyses were done by HPLC. Phosphorus content was measured by the method as described by Allen.[17] The ratio of phosphorus to base was estimated from the content of phosphorus and absorbance at 260 nm. ^{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 (δ_{ppm} 4.8). Signal assignments of nucleotide analogs were done by comparison of those of their respective 3'-ribonucleotide.[18] Sample was dissolved in 99.8% D_2O and lyophilized twice, and the final lyophilized sample was dissolved in 99.8% D_2O and analyzed.

Preparation of 3', 5'-bisphenylphosphonate esters of adenosine and uridine. To a solution of adenosine (1.08 g, 4.0 mmol) in 50 ml of pyridine, phenylphosphonic dichloride (1.70

ml, 2.34 g, 12.0 mmol) was added. The mixture was stirred in an ice bath for 1 h and at room temperature for additional 3 h, and evaporated to dryness below 40°C. The residue was dissolved in 20 ml of distilled water and neutralized with 1N NaOH. After being filtered, the filtrate was diluted 50-fold with 0.02 M formate buffer, pH 3.0, and put on a DEAE-Sephadex A-25 column (2.6 x 40 cm) equilibrated with 0.02 M formate buffer, pH 3.0. The column was washed with the same buffer, and eluted with a 2000 ml linear gradient from 0.02 to 1.0 M formate buffer, pH 3.0. The eluate containing the compound converted to adenosine 5'-phenylphosphonate prepared as described below on treatment with nuclease P1 were pooled. The pooled fractions were concentrated by evaporation below 40°C, and put on a Cosmosil 75C_{18}-OPN column (2.6 x 25 cm) equilibrated with distilled water. After the column was washed with distilled water, the product was eluted with 10% acetonitrile and lyophilized (0.18 g, 0.33 mmol, 8.3 %). The UV spectra at pH 1, 7, and 13 of this preparation were essentially identical with those of 5'-AMP and 3'-AMP. The ratio of phosphorus to base was 1.92. ^{1}H-NMR (D_2O) δ 8.13 (1H, s, H-8), 8.01 (1H, s, H-2), 7.45-7.66 (10H, m, phenyl), 6.04 (1H, d, H-1'), 4.55 (1H, m, H-3'), 4.11 (1H, m, H-4'), and 3.77 (2H, m, H-5', 5"). The H-2' signal overlapped with a residual HDO peak.

Uridine 3', 5'-bisphenylphosphonate (0.15 g. 0.29 mmol, 7.3 %) was prepared from uridine (0.98 g, 4.0 mmol) by the reaction with phenylphosphonic dichloride according to the method described above. The UV spectra at pH 1, 7, and 13 of this preparation were essentially identical with those of 5'-UMP and 3'-UMP. The ratio of phosphorus to base was 2.05. ^{1}H-NMR (D_2O) δ 7.75 (1H, d, H-6), 7.40-7.65 (10H, m, phenyl), 5.78 (1H, d, H-5), 5.96 (1H, d, H-1'), 4. 33 (1H, m, H-3'), 4. 24 (1H, m, H-2'), 4.12 (1H, m, H-4'), and 3.60 (2H, m, H-5', 5").

Preparation of 5'-phenylphosphonate esters of adenosine and uridine. To a solution of 2', 3'-*O*-isopropylidene adenosine (1.53 g, 5.0 mmol) in 50 ml of pyridine, phenylphosphonic dichloride (1.41 ml, 1.95 g, 10.0 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, neutralized with 1N NaOH, and filtered. From the filtrate, adenosine 5'-phenylphosphonate (0.82 g, 2.0 mmol, 40%) was purified by the method applied to purification of adenosine 3'-phenylphosphonate.[12] The UV spectra at pH 1, 7, and 13 of this preparation were essentially identical with those of 5'-AMP. ^{1}H-NMR (D_2O) δ 8.22 (1H, s, H-8), 8.18 (1H, s, H-2), 7.25-7.65 (5H, m, phenyl), 6.05 (1H, d, H-1'), 4.47 (1H, m, H-3'), 4.32 (1H, m, H-4'), and 4.04 (2H, m, H-5', 5"). The H-2' signal overlapped with a residual HDO peak.

Uridine 5'-phenylphosphonate (0.77 g. 2.0 mmol, 40%) was prepared from 2', 3'-*O*-isopropylidene uridine (1.42 g, 5.0 mmol) by the reaction with phenylphosphonic dichloride according to the method described above. The UV spectra at pH 1, 7, and 13 of this preparation were essentially identical with those of 5'-UMP. ^{1}H-NMR (D_2O) δ 7.50-7.88 (6H, m, H-6 and phenyl), 5.78 (1H, d, H-5), 5.92 (1H, d, H-1'), 4. 24-4.38 (3H, m, H-2', 3', and 4'), and 4.00 (2H, m, H-5', 5").

Enzymatic activity. Mung bean nuclease (1.0 U), nuclease S1 (1.0 U), nuclease P1 (0.0005 U), phosphatase alkaline (1.0 U), spleen phosphodiesterase (1.0 U), RNase T2 (1.0 U), and RNase A (1.0 U) were assayed for their activities for adenosine- and uridine 3', 5'-bisphenylphosphates, 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 products of hydrolysis were identified as the nucleoside 5'-phenylphosphonates by comparison of retention times in HPLC of the products with those of synthesized materials.

Mung bean nuclease (4.0 U), S1 nuclease (4.0 U), and P1 nuclease (0.002 U) were assayed at various pH for their activities for adenosine- and uridine 3', 5'-bisphenylphosphates. A reaction mixture contained 20 mM buffer, the enzyme, and 5.0 mM substrate in a total volume of 0.2 ml. The buffer used were; pH 2.5 - 3.5, glycine-HCl buffer; pH 3.6 - 5.5, acetate buffer; pH 5.5 - 7, MES buffer. After incubation at 37°C for 10 min, the amount of the nucleoside 5'-phenylphosphonate produced was measured by HPLC.

RESULTS AND DISCUSSION

Mung bean nuclease, nuclease S1, and nuclease P1 hydrolyzed adenosine 3', 5'-bisphenylphosphonate (3', 5'-ABPP) and uridine 3', 5'-bisphenylphosphonate (3', 5'-UBPP) to adenosine 5'-phenylphosphonate and uridine 5'-phenylphosphonate, respectively. But phosphatase and the other nucleases tested did not.

The 3'-nucleotidase/nucleases had maximum activities at pH 3.6 - 6.0 for 3', 5'-ABPP and 3', 5'-UBPP (FIG. 1). The optimum pH of the enzymes for the compounds were similar to those for adenosine 3'-phenylphosphonate (3'-APP), uridine 3'-phenylphosphonate (3'-UPP), RNA, and single-stranded DNA.[5,12,19,20]

The apparent K_m and V_{max} values at pH 5.5 of the 3'-nucleotidase/nucleases for 3', 5'-ABPP and 3', 5'-UBPP were measured and compared with those for 3'-APP and 3'-UPP. The V_{max} values for 3', 5'-ABPP and 3', 5'-UBPP were higher than those for the corresponding nucleoside 3'-phenylphosphonates and the apparent K_m values for the former compounds were lower than those for the latter compounds (TABLE 1). Judging from the V_{max}/apparent K_m values, 3', 5'-ABPP and 3', 5'-UBPP are 9 to 21-fold better substrates of the enzymes than the corresponding nucleoside 3'-phenylphosphonates. These results also indicate that the 5'-phosphonate group of the substrates plays important roles in the reactions catalyzed by the enzymes; the enzymes have subsites which interact with the 5'-phosphonate group.

The hydrolytic reactions catalyzed by nuclease P1 and S1 have been shown to proceed without involving the participation of covalent enzyme intermediates.[21, 22] The mechanism of the reaction catalyzed by mung bean nuclease may be the same as that by nuclease S1 and P1 since many similarities exist among the enzymes. The differences in the V_{max} and apparent K_m values of the enzymes for 3', 5'-ABPP, 3', 5'-UBPP, 3'-APP, and 3'-UPP suggest that the interactions between their subsites and the additional 5'-phosphonate groups accelerate their catalytic rates as

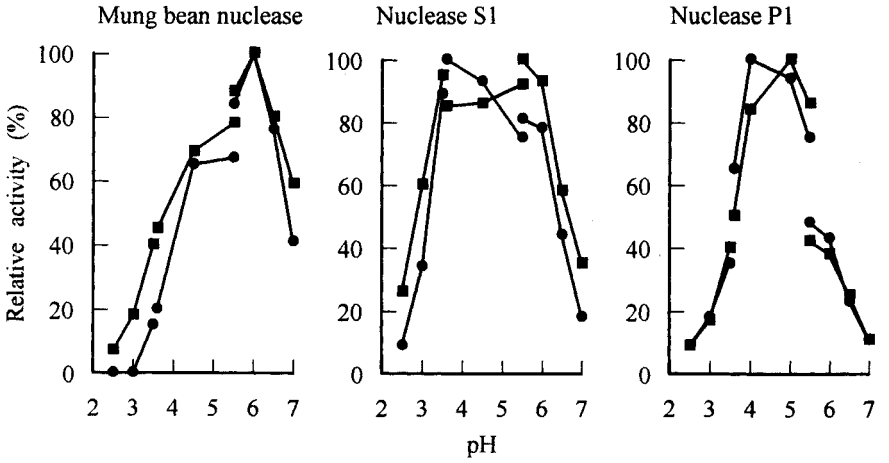

FIG. 1. Optimum pH for 3', 5'-ABPP and 3', 5'-UBPP of 3'-Nucleotidase/nucleases.

At optimum pH, the hydrolysis rates for 3', 5'-ABPP (■) and 3', 5'-UBPP (●) of mung bean nuclease were 0.0061 and 0.0059 μmol/min U, those of nuclease S1 were 0.0050 and 0.0031 μmol/min U, and those of nuclease P1 were 14 and 6.2 μmol/min U, respectively.

TABLE 1. Apparent K_m and V_{max} of 3'-nucleotidase/nucleases for 3', 5'-ABPP and 3', 5'-UDPP.

After incubation at 37°C for 10 min of a reaction mixture containing 20 mM acetate buffer, pH 5.5, the enzyme, and a various concentration of substrate in a total volume of 0.2 ml, the amount of product was measured by HPLC. The enzymes used were; 1.0 and 3.0 U mung bean nuclease, 1.0 and 5.0 U nuclease S1, and 0.0004 and 0.002 U nuclease P1 for the nucleoside 3', 5'-bisphenylphosphonates and the nucleoside 3'-phenylphosphonates, respectively.

Enzyme	Substrate	apparent K_m (mM)	V_{max} (μmole/min U)	V_{max}/apparent K_m	Relative V_{max}/apparent K_m [a)]
Mung bean	3', 5'-ABPP	0.28	0.0063	0.023	11
nuclease	3'-APP	1.5	0.0032	0.0021	1
	3', 5'-UBPP	3.1	0.0076	0.0025	19
	3'-UPP	18	0.0024	0.00013	1
Nuclease S1	3', 5'-ABPP	0.34	0.0051	0.015	16
	3'-APP	1.2	0.0011	0.00092	1
	3', 5'-UBPP	1.9	0.0033	0.0017	21
	3'-UPP	17	0.0014	0.00082	1
Nuclease P1	3', 5'-ABPP	0.33	14	42	14
	3'-APP	1.2	3.5	2.9	1
	3', 5'-UBPP	6.4	16	2.5	9
	3'-UPP	24	6.5	0.27	1

a) The ratio of V_{max} / apparent K_m for nucleoside 3',5'-diphenylphophonate to that for the corresponding nucleoside 3'-phenylphophonate.

well as substrate bindings. Phosphonate group appears to be an analog of phosphodiester group. In hydrolysis of polynucleotide by the enzymes, 5'-phosphodiester group might interact subsite and exhibit same effects. Further characterization of the subsite is in progress.

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