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A route to 2′,5′-oligoadenylates with increased stability towards phosphodiesterases

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The rates of enzymatic hydrolysis of 2′,5′-oligoadenylates and their synthetic analogs have been measured. These compounds were treated with either NIH 3T3 cell lysates, mouse liver homogenates or snake venom phosphodiesterase. All analogs with 3′-terminal acyclic nucleoside residues demonstrated greater stability compared with the natural compound adenylyl(2′-5′)adenylyl(2′-5′)adenosine.

2′,5′-Oligoadenylate analog; Phosphodiesterase; Enzyme stability

1. INTRODUCTION

2′,5′-Oligoadenylates (2-5As) are believed to be mediators of the action of interferon [1]. This suggests the use of 2-5As or their derivatives as potential antiviral or antitumor agents. The following difficulties, however, have to be overcome: 2-5As are not readily taken up by the intact eukaryotic cell and are rapidly hydrolysed by phosphodiesterases present in cell extracts. Chemical modification of the structures of 2-5As might provide solutions to these problems ([2,3] and references therein).

Unfortunately, at present, we cannot rationalize our knowledge of the process of oligonucleotide penetration through cell membranes for the preparation of active compounds in this series. This is also true, but to a lesser extent, of the mechanism of action and specificity of 2′,5′-phosphodiesterase which is responsible for degradation of 2-5A to 5′-AMP and ATP [4]. It may be assumed that the mechanisms of oligonucleotide hydrolysis and specificity of this enzyme are similar to those of snake venom phosphodiesterase which hydrolyses oligonucleotides to yield the corresponding 5′-nucleotides. The substrate specificity of this enzyme has been well documented in the literature [5]. Both enzymes can hydrolyse 2′,5′- and 3′,5′-internucleotide diester bonds [4,5].

Analogs stable towards snake venon phosphodiesterase would also be inhibitors of 2′,5′-phosphodiesterase and other similar enzymes, which could cause elevation of endogenous 2-5A levels followed by certain physiological effects.

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2. MATERIALS AND METHODS

The following oligonucleotides have been synthesized previously: 2′,5′-ApApA (I) [6], the corresponding 3′-deoxy analog, II [7], compound VII [8], acyclic analogs X and XI [9] and analogs III–VI, VIII and IX [10] (scheme 1). Snake venom phosphodiesterase was obtained from the Experimental Plant of the Institute of Chemistry (Estonian SSR Academy of Sciences, Tallin).

2.1. *Hydrolysis of 2-5A analogs with venom phosphodiesterase*

2-5A and its analogs were treated with phosphodiesterase in a solution containing 0.1 M Tris-HCl and 6 mM $MgCl_2$ (pH 8.0). Kinetic measurements were monitored using spectral methods on a Specord M 40 spectrometer (Carl Zeiss, GDR). The pH was determined with a GK 2401 C combined electrode

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(Radiometer, Denmark) using a pH-340 pH-meter (USSR). In order to assay 2-5A concentrations and to calculate the rates of 2-5A hydrolysis, the molar extinction coefficients and the changes in their values during the course of hydrolysis were determined as follows. A specified amount of nucleotides was completely hydrolysed by the phosphodiesterase. Using a value of ϵ = 15400 for 5′-AMP, we determined the concentration of products in the hydrolysate and calculated the initial substrate concentration and $\Delta\epsilon$. A value of $\epsilon = 15400 \times 3 - \Delta\epsilon$ was taken as the molar extinction of the substrate. The ϵ values thus calculated for the compounds studied varied from 38700 to 41200 at 262 nm and pH 8.0. The values of $\Delta\epsilon$ varied from 4800 (compound III) to 11300 $M^{-1} \cdot cm^{-1}$ (compound II); ϵ was taken as 39200 $M^{-1} \cdot cm^{-1}$ for 2-5A analogs which were hydrolysed by the enzyme either very slowly (compounds VII–IX) or not at all (compounds X, XI). $\Delta\epsilon$ for compounds VII–IX was taken to be 7000 $M^{-1} \cdot cm^{-1}$. The enzyme concentration was assayed using ϵ = 148000 $M^{-1} \cdot cm^{-1}$ at pH 8.0 [11].

The values of k_{cat} and K_m were calculated from the dependences of the initial reaction rates on substrate concentration.

Snake venom phosphodiesterase hydrolyses oligonucleotides via an exonucleolytic mechanism [5]. Therefore, all measured kinetic parameters for the hydrolysis reaction refer to the 2′–5′-phosphodiester bond adjacent to the modified nucleoside residue.

2.2. *Determining the stability of 2-5A analogs in the presence of an NIH 3T3 cell lysate*

An NIH 3T3 cell lysate was prepared as in [12]. The lysate contained cell protein at a concentration of ~2 mg/ml. A 10 mM solution of analog in water was added to the lysate (1:2, v/v) and incubation performed for 48 h at 37°C. Samples of the incubation mixture were then applied to Silufol UV-254 plates and chromatograms developed using a mixture of isopropanol/25% ammonia/water (7:1:2, by vol.). Spots corresponding to substrate and reaction products were cut off, eluted with water and the concentrations determined spectrophotometrically.

2.3. *Determination of the stability of 2-5A analogs in the presence of mouse liver homogenate*

Mouse liver was homogenised in a glass homogeniser with 3 vols of a solution containing 30 mM KCl, 5 mM $MgCl_2$, 1 mM mercaptoethanol and 50 mM Tris-HCl (pH 7.9) and then centrifuged at 5000 × g for 10 min. The resultant homogenate contained protein at about 10 mg/ml. The subsequent procedure was the same as that described above, except that incubation lasted up to 6 h.

3. RESULTS AND DISCUSSION

Modification of a carbohydrate residue in the 3′-terminal nucleoside, for example, incorporation of an acyclic nucleoside analog, has been shown to decelerate the rate of hydrolysis of oligonucleotides by snake venom phosphodiesterase [13]. The aim of this work was to create 2-5A analogs with increased stability towards phosphodiesterases and, at the same time, retaining their affinity to these enzymes. To this end, we prepared 2-5A analogs with a 3′-terminal acyclic nucleoside residue (III–IX) [8,10] as well as 2′,5′-ApApA and 3′,5′-ApApA acyclic analogs X and XI [9], and studied their stability in the

Table 1

Hydrolysis of compounds I–XI

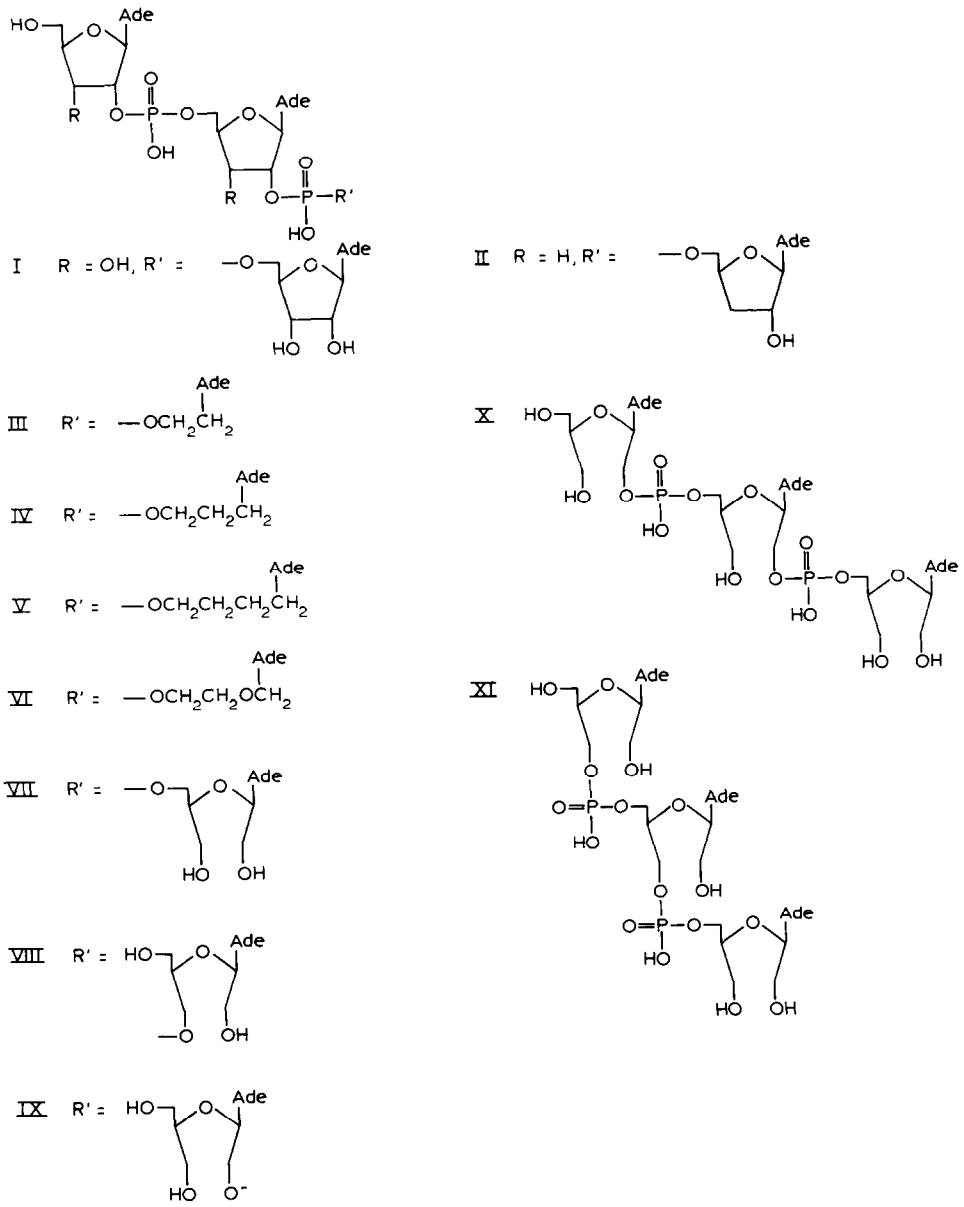
Compound	Hydrolysis by snake venom phosphodiesterase			Hydrolysis (%) in the presence of	
	K_m ($\times 10^5$) (M)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	NIH 3T3 cell lysate[a]	Mouse liver homogenate[b]
I	0.8	4.4	5.7	15	60
II	1.8	1.9	1.1	6	20
III	1.7	0.06	0.035	1	1
IV	1.9	0.2	0.105	1	5
V	1.5	0.8	0.53	1	10
VI	1.4	0.44	0.31	1	50
VII	2.2	0.04	0.018	1	1
VIII	1.9	0.1	0.053	1	1
IX	1.4	0.05	0.036	1	n.d.
X	n.h.	(K_i 10^{-4} M)[c]		1	1
XI	n.h.	(K_i 3 × 10^{-4} M)[c]		1	n.d.

[a] Incubation time 24 h

[b] Incubation time 3 h

[c] K_i values were determined from the hydrolysis of the *p*-nitrophenyl ester of 5′-thymidylic acid

n.h., not hydrolysed; n.d., not determined



Scheme 1. Structures of compounds I–XI.

presence of snake venom phosphodiesterase and cell lysates.

The kinetic parameters for hydrolysis of these compounds by snake venom phosphodiesterase are listed in table 1. One can see that all analogs tested that possess a 3′-terminal acyclic nucleoside residue (compounds III–IX) have identical affinity for the enzyme (similar K_m values), virtually coinciding with the corresponding values for compounds I and II. The rates of hydrolysis vary for compounds III–IX due to the differences in their molecular kinetic constants k_{cat}. This variation exceeds two orders of magnitude compared with natural 2-5A; the analog based on 2′,3′-seco-

adenosine (VII) is characterised by the lowest value.

Analogs X and XI, containing three acyclic carbohydrate fragments of nucleotides, are virtually not hydrolysed by snake venom phosphodiesterase.

When 2-5A analogs were incubated with the NIH 3T3 cell lysate, the linear stage of hydrolysis (i.e. the stage during which the extent of hydrolysis depended on time in a linear manner) lasted 24 h for native 'core' 2-5A. Since the extent of hydrolysis of this compound was the highest (table 1), the results on hydrolysis of 2-5A analogs in the presence of cell lysates are presented in this table for the 24 h incubation.

As demonstrated by these data, a high degree of hydrolysis comparable with that for natural core 2-5A is observed only for the 3′-deoxy analog (II). All other 2-5A analogs were hydrolysed to a minor extent.

The situation was essentially the same in the presence of mouse liver homogenate (the linear step lasting up to 3 h) (table 1). Compound VI was an exception, since the extent of its hydrolysis was close to that of 2-5A.

Therefore, the synthesized compounds are sufficiently stable under the given conditions. One might expect the same results in the case of phosphodiesterases other than those used in this work. However, for such compounds to exert their physiological activity, they must not only be stable but also actively bind to phosphodiesterases which hydrolyse 2-5A. The content of endogenous 2-5A in the cell will then rise owing to inhibition of these enzymes and give rise to the hypothetical effect (this mechanism has been proposed by Kimchi et al. [14]). The compounds studied here exhibit strong affinity for snake venom phosphodiesterase (table 1) and, quite possibly, for other phosphodiesterases as well. Hence, the proposed modification of 2-5A, namely the incorporation of an acyclic adenosine analog, yields compounds with greater stability against the action of phosphodiesterases and with high-affinity binding to these enzymes.

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