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Di(1,N⁶-ethenoadenosine)5′, 5‴-P¹,P⁴-tetraphosphate, a fluorescent enzymatically active derivative of Ap_4A

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Di(1,N⁶-ethenoadenosine)5′, 5‴-P¹,P⁴-tetraphosphate, ε-(Ap_4A), a fluorescent analog of Ap_4A has been synthesized by reaction of 2-chloroacetaldehyde with Ap_4A. At neutral pH this Ap_4A analog presents characteristic maxima at 265 and 274 nm, shoulders at ca 260 and 310 nm and moderate fluorescence (λ_{exc} 307 nm, λ_{em} 410 nm). Enzymatic hydrolysis of the phosphate backbone produced a slight hyperchromic effect but a notorious increase of the fluorescence emission. Cytosolic extracts from adrenochromaffin tissue as well as cultured chromaffin cells were able to split ε(Ap_4A) and catabolize the resulting ε-nucleotide moieties up to ε-Ado.

Ap_4A; Ap_4A fluorescent derivative; Ap_4A splitting enzyme; Ectoenzyme; Chromaffin cell

1. INTRODUCTION

The biochemistry of diadenosine polyphosphates, mainly Ap_4A, has received increasing interest throughout recent years [1,2], however, their precise biological functions remain to be clearly understood. Roles as alarmone [3,4] or as inductor of DNA synthesis [1] have been proposed for Ap_4A. Moreover the presence of Ap_4A and Ap_3A in human platelets [5–7] and of Ap5-A and Ap_4A in the secretory granules of chromaffin cells [8] suggest a potential implication of these dinucleotides in platelet and neurosecretory functions. On the other hand the strong inhibition of adenosine kinase by Ap_4A [9] and its inhibitory effect on evoked catecholamine secretion [10] has been reported.

Specific enzymes that split Ap_4A, and can consequently modulate the cellular levels of this dinucleotide, have been isolated and studied from many biological sources [11–13]. The most sensitive methods to assay such enzymes involve the use of luminometric [14] and radiometric techniques [15].

The acquisition of fluorescent and enzymatically active derivatives of Ap_4A would be of great interest in order to open the study of Ap_4A biochemistry through a new fluorimetric approach. The fluorescent etheno derivatives of common adenosine containing nucleotides (e.g. ε-AMP, ε-ADP, ε-ATP or ε-NAD^+), obtained by reaction of 2-chloroacetaldehyde with adenine rings, have been useful fluorescent probes to study kinetic and binding properties of some kinases and dehydrogenases [16–18]. In this article we report the synthesis and some properties of a fluorescent Ap_4A derivative and show that this analog is efficiently catabolized by intact chromaffin cells and extracts from adrenomedullary tissue.

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Abbreviations: Ap_4A, Diadenosine 5′,5‴-P¹, P⁴-tetraphosphate; ε-(Ap_4A), Di (1, N⁶-ethenoadenosine)5′,5‴-P¹,P⁴-tetraphosphate; ε-ATP, εADP, ε-AMP and ε-Ado refer to the 1,N⁶ etheno derivatives of ATP, ADP, AMP and adenosine respectively; the symbol 'ε' stands for etheno, [16]; HPLC, high-performance liquid chromatography

2. MATERIALS AND METHODS

2.1. *Chemical*

ε-ATP, ε-ADP, ε-AMP, ε-Ado and Ap_4A were from Sigma (St. Louis, MO, USA), 2-chloroacetaldehyde was from Merck (Darmstadt, Germany) and *C. durissus* phosphodiesterase was from Boehringer (Mannheim, Germany). Methanol and salts used for HPLC were of HPLC grade from Merck. All other products were reagent grade.

2.2. *HPLC*

The chromatographic system was made up of a M-510 pump, a U6K injector, a 440 UV dectector at a fixed wavelength of 254 nm, a 420 AC fluorescent detector at λ_{exc} 300 nm and λ_{em} 400 nm, a μBondapack C-18 column (300 × 4 mm, 10 μ particle size) and a Guard Pak module packed with C-18 material to protect the analytical columns, all from Waters Assoc. (Milford, MA, USA). Chromatograms were obtained by an HP 3390 A integrator (Hewlett-Packard, Avondale, CA, USA) and by a Pharmacia LKB recorder (Uppsala, Sweden). A UV and fluorescence detector were connected in this order. Occasionally a Lichrosorb RP 18 column (250 × 4 mm, 10 μ particle size) purchased from Kontron (Zurich, Switzerland) was used. The mobile phase was composed of 0.1 M KH_2PO_4, 10% methanol, pH 6.0 and was pumped at a flow rate of 1.5 ml/min

through the μBondapack C-18 column or at 1.0 ml/min through the Lichrosorb RP 18 column. Preparation of the mobile phase and standards was as described previously [19]. The elution order of etheno derivatives was ε-ATP, ε-ADP, ε-AMP, ε-(Ap_4A) and ε-Ado. Sep-pak Accell QMA cartridges (anion exchange) used in the preparative separation of ε-(Ap_4A) were from Waters.

2.3. *Spectroscopy*

Ultraviolet and uncorrected fluorescence spectra were obtained by means of a DU-40 spectrophotometer (Beckman, S. Ramón, CA, USA) and a F-2000 spectrofluorimeter (Hitachi, Tokyo, Japan). Spectra of ε-(Ap_4A) were recorded in solutions of 20 mM potassium phosphate buffer, 4 mM $MgCl_2$, pH 7.0 or 50 mM Tris-HCl, 4 mM $MgCl_2$, pH 8.0 (1.0 ml) in quartz cuvettes (Sigma). Then, 1 μl containing 0.6 mU of *C. Durissus* phosphodiesterase was added and spectra were again recorded after the completion of enzymatic hydrolysis.

2.4. *Biological extracts, cell cultures and enzyme assays*

Metabolite-free cytosolic extracts from bovine adrenal medulla, were prepared as described [8,19]. Reaction mixtures to monitor enzymatic degradation of ε-(Ap_4A) contained 50 mM Tris-HCl, 4 mM $MgCl_2$ pH 8.0, 10 μM ε-(Ap_4A) and 50 μl of extract in a final volume of 1.0 ml and were incubated at 37°C. At the required times 125 μl aliquots were taken up, heated for 2 min in a boiling water bath to stop enzyme action and centrifuged to eliminate precipitated protein. 10–50 μl aliquots of the clear supernatant were processed by reversed-phase HPLC according to section 2.2.

Chromaffin cell cultures were performed as previously described [20,21]. To monitor the ectoenzymatic degradation of ε-(Ap_4A), 3×10^6 cells/well were incubated at 37°C in 0.5 ml of Locke's solution containing 2–10 μM ε-(Ap_4A). At the required times 50 μl aliquots were taken up and processed by reversed-phase HPLC according to section 2.2.

3. RESULTS AND DISCUSSION

3.1. *Synthesis and chromatographic purification of ε-(Ap_4A)*

The reaction conditions were similar to those described for the synthesis of etheno derivatives of adenosine and related common nucleotides [16]. A solution of 1 ml containing 5 mM Ap_4A, 1.0 M 2-chloroacetaldehyde and 50 mM ammonium acetate buffer, pH 4.5 was incubated at 35°C. Formation of etheno derivatives was followed by measuring the decrease of the absorbance quotient A_{265}/A_{275}; after about 70 h the quotient remained practically stable around 1.30. Analytical reversed-phase HPLC of the reaction mixture at different incubation times revealed the progressive diminution of the Ap_4A peak (under UV detection) and the simultaneous appearance of a major and highly retained peak under both UV and fluorescence detection; other minor peaks were also evident (Fig. 1). The last peak of the chromatograms was tentatively identified as an etheno derivative of Ap_4A. This presumed etheno derivative was isolated from the reaction mixture as follows: 200 μl aliquots of the mixture were diluted with water to 1 ml and applied to a Sep pak Accell QMA cartridge; the cartridge was washed with 4 ml of water and the nucleotides eluted with 2 ml of a solution containing 0.1 N HCl and 0.2 N KCl. The eluate was rapidly neutralized to pH 6 with 10 N KOH and injected into the chromatograph to collect the fluorescent derivative.

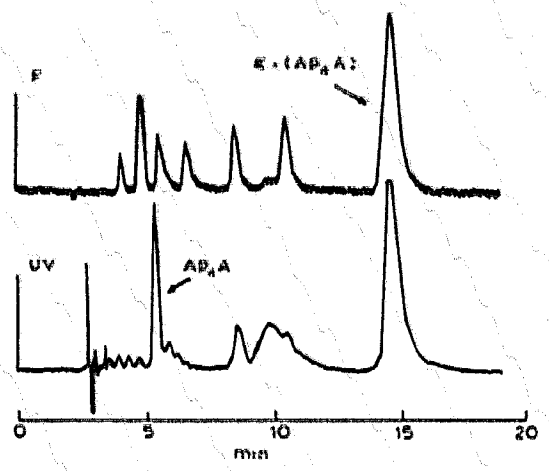


Fig. 1. Chromatographic profiles (F, fluorimetric; UV, ultraviolet) of the reaction mixture of ε-(Ap_4A) synthesis after 70 h reaction. About 35% of Ap_4A was still present. Column: Lichrosorb C18, chromatographic conditions according to section 2.2.

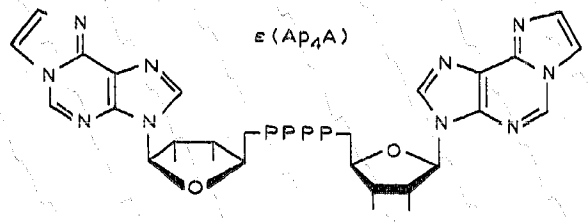
Using a μBondapak C-18 Column, quantities of 0.4–1.0 mg could be isolated in one chromatographic run.

Aliquots of the HPLC-isolated fraction were subjected to complete enzymatic hydrolysis by *C. durissus* phosphodiesterase and then chromatographed; only one peak that co-eluted with authentic ε-AMP under both UV and fluorescence detection was obtained, this being consistent with an Ap_4A derivative containing both adenine rings modified by a $1,N^6$-etheno bridge; ε-(Ap_4A) is the abbreviation proposed for such compound; (Scheme 1).

3.2. *Properties of ε-(Ap_4A)*

UV absorption spectra at neutral pH present two characteristic maxima at 265 and 274 nm and shoulders at ca 260 and 310 nm (Fig. 2). No changes were noticed in the pH range 6.0–8.0 when using phosphate or Tris buffers. Hydrolysis by *C. durissus* phosphodiesterase produced a slight hyperchromic effect (Fig. 2). By measuring the absorbance of ε-(Ap_4A) at 265 nm before and after enzymatic hydrolysis and from the knowledge of the molar absorption coefficient for ε-AMP ($6.0 \times 10^3\ M^{-1} \cdot cm^{-1}$ [16] a value of $8.7 \times 10^3\ M^{-1} \cdot cm^{-1}$ was calculated for the molar absorption coefficient of ε-(Ap_4A) at 265 nm.

Upon excitation at 307 nm and neutral pH, ε-(Ap_4A) fluoresces with a maximum at 410 nm (Fig. 3), being the



Scheme 1

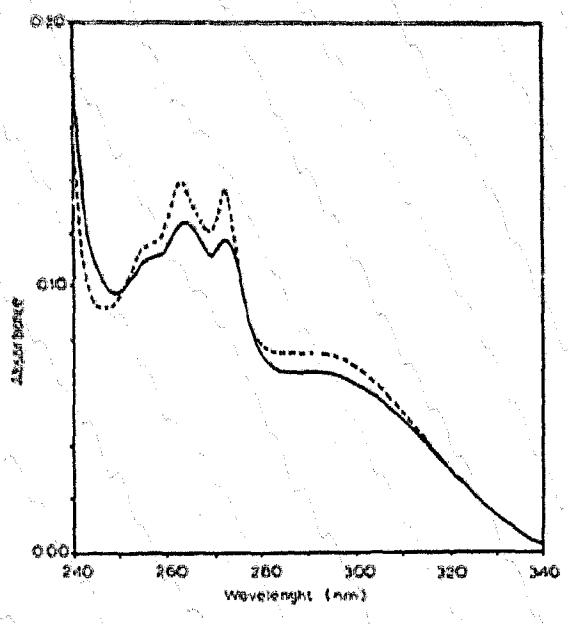


Fig. 2. Ultraviolet absorption spectra of ε-(Ap_4A) in 50 mM Tris-HCl, 4 mM $MgCl_2$, pH 8.0 before (—) and after (----) hydrolysis by *C. durissus* phosphodiesterase.

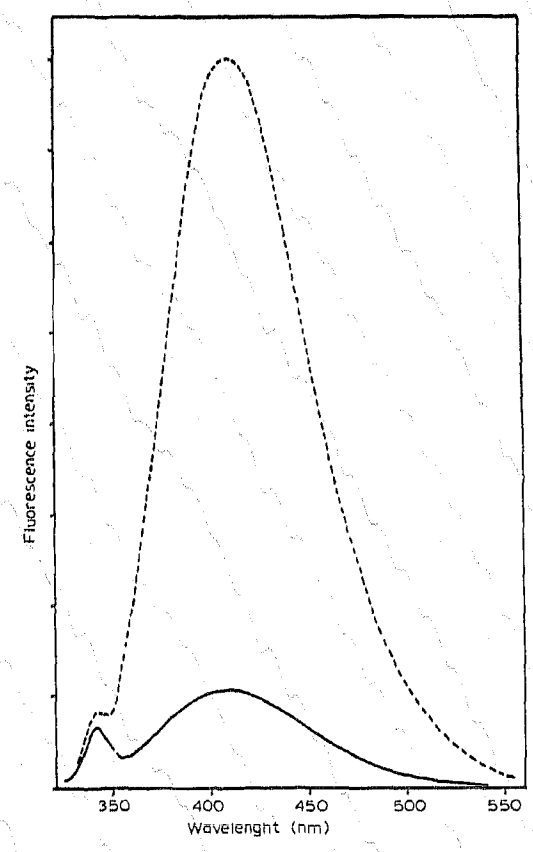


Fig. 3. Fluorescence emission spectra of ε-(Ap_4A) in 50 mM Tris-HCl, 4 mM $MgCl_2$ pH 8.0 before (—) and after (----) hydrolysis by *C. durissus* phosphodiesterase.

fluorescent emission notoriously less than that of ε-AMP, this in spite of containing two ε-AMP moieties. Hydrolysis of ε-(Ap_4A) by *C. durissus* phosphodiesterase produced a large increase of fluorescence emission. The maximum wavelength of emission was unchanged but an intensity increase of 7.5 was measured (Fig. 3). So, enzyme splitting of ε-(Ap_4A) can be measured in a very sensible way by following the increase in fluorescence intensity with time at 410 nm. These fluorescence properties of ε-(Ap_4A) appear to be similar to those reported for the etheno derivatives of NaC^+ and are probably due to an intramolecular stacking of the heterocyclic rings that produce an effective quenching of fluorescence [17,18]. No noticeable changes in fluorescence emission or excitation were observed performing hydrolysis in phosphate or Tris buffers.

3.3. ε-(Ap_4A) as a substrate of Ap_4A cleaving enzymes

Cleaving activities on Ap_4A and other diadenosine polyphosphates have been reported in cytosolic extracts from adreno-chromaffin tissue [8,19] and in the external surface of cultured chromaffin cells [22]. Conse-

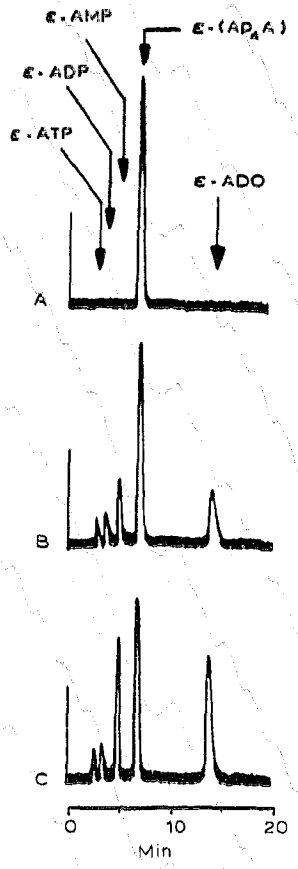


Fig. 4. Fluorescence chromatographic profiles illustrating the ectoenzymatic degradation of ε-(Ap_4A) by cultured chromaffin cells. 3×10^6 cells/well were incubated at 37°C in 2.0 ml of Locke's solution containing 5 μM ε-(Ap_4A); at the indicated times (A, 0 min; B, 60 min; C, 120 min) 50 μl were taken up and injected into the chromatograph. Column, μBondapak C18; chromatographic conditions are according to section 2.2.

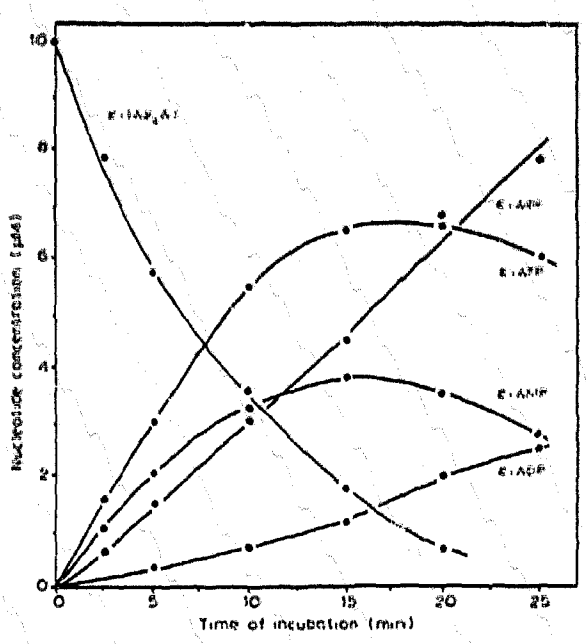


Fig. 5. Enzymatic degradation of ε-(Ap_4A) by a cytosolic extract from bovine adrenal medulla as a function of time, 1-ml reaction mixtures were incubated and processed according to section 2.4. 50 μl aliquots were injected on to a μBondapak C-18 column according to section 2.2.

quently these biological sources were chosen to investigate the usefulness of ε-(Ap_4A) as a fluorogenic substrate for Ap_4A cleaving enzymes.

As shown in Figs 4 and 5 both intact cultured chromaffin cells and their cytosolic extracts were able to split ε-(Ap_4A) and then continue the degradation of the ε-nucleotide produced moieties up to ε-Ado; the nucleoside accumulating in the medium. This additionally indicates that ε-ATP, ε-ADP and ε-AMP are substrates of both ecto and cytosolic nucleotides. The presence and kinetic characterization of ectonucleotidases hydrolysing extracellular adenine nucleotides in chromaffin cells has recently been published [23]. The pattern of ε-nucleotide release from ε-(Ap_4A) indicates an asymmetrical cleavage, similar to that of the unmodified nucleotide Ap_4A [8,22].

K_m values for Ap_4A cleaving enzymes from bovine adrenal chromaffin and aortic endothelial cells lie in the low micromolar range [8,22,24]. The results reported here demonstrate that ε-(Ap_4A) is biologically active at similar concentrations as is Ap_4A, and open up the possibility to assay Ap_4A cleaving enzymes by direct fluorescence measurements as a complementary or even alternative approach to the use of radiometric or luminometric techniques. It is noteworthy that ε-Ado derived nucleotides can be fluorimetrically detected at concentrations as low as 10^{-8} M. Another interesting property of ε-(Ap_4A) is that ε-Ado produced from it is not further catabolized via adenosine deaminase because of the inability of this enzyme to recognize ε-Ado as a substrate [25]; this allows, if desired, to follow specifically the degradation of adenosine nucleotides.

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