Synthesis and biological activity of a fluorescent analogue of 2–5A

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A fluorescent analogue of 2–5A, ppp5′ϵA2′p5′ϵA2′p5′ϵA, was obtained by reaction of 2′,5′$(pA)_3$ with chloroacetaldehyde followed by conversion to the 5-triphosphate through reaction of the corresponding phosphoroimidazolide with pyrophosphate anion. The 5′-monophosphate, p5′ϵA2′p5′ϵA2′p5′ϵA, was not an antagonist of 2–5A action in extracts of mouse L cells. Neither did the 5′-triphosphate, ppp5′ϵA2′p5′ϵA2′p5′ϵA, inhibit translation. Moreover, the 5′-triphosphate was bound to the 2–5A-dependent endonuclease 5000-times less effectively than 2–5A itself.

Interferon *2–5A* *2–5A-Activated endonuclease* *Ethenoadenosine* *Fluorescence* *Oligonucleotide synthesis*

1. INTRODUCTION

When certain vertebrate cell lines are treated with interferon, several new proteins are synthesized and a cellular antiviral state develops [1–3]. One newly induced protein is referred to as 2–5A synthetase which synthesizes, after activation by dsRNA, a unique 2′,5′-linked oligoadenylate, 2–5A [4]. This oligonucleotide may then activate a latent cellular endoribonuclease which can degrade mRNA, thereby preventing translation [1–3]. This 2–5A system, i.e., the 2–5A synthetase, 2–5A itself, and the 2–5A-activated ribonuclease, may be the basis of interferon's antiviral action, at least in the instance of several virus infections [5,6].

Fluorescent 1,N^6-etheno analogues of adenine nucleotides and polynucleotides have been synthesized and found to be substrates in many enzymatic reactions [7 and references cited therein]. In the present context, a fluorescent analogue of 2–5A could be of value in studying the conformation and/or oligonucleotide–enzyme interactions of the 2–5A-activated endonuclease and perhaps also the 2–5A synthetase and 2–5A phosphodiesterase [1–3]. An earlier report [8] had suggested that ϵATP was a substrate for 2–5A synthetase and that the resulting 2,5′pp(pϵA)$_3$ (2–5ϵA) was an efficient activator of the endonuclease. In order to confirm and extend these findings, and because of our interest in delineating the structural parameters involved in binding of 2–5A to the endonuclease [9], we have chemically synthesized 2′,5′$(p\epsilon A)_3$ and 2′,5′pp$(p\epsilon A)_3$ and evaluated their interaction with the 2–5A-dependent endonuclease (also referred to as RNase L or F [1–3]).

Abbreviations: ϵA,1,N^6-ethenoadenosine i.e., (3-β-D-ribofuranosylimidazo[2.1-i]purine); 2′,5′$(p\epsilon A)_3$, p5′ϵA2′p5′ϵA2′p5′ϵA; 2′,5′pp$(p\epsilon A)_3$, ppp5′ϵA2′p5′ϵA2′p5′ϵA; 2–5A, ppp5′A2′p5′$(A2'p5')_n$A, where $n \geqslant 1 - \sim 10$

2. MATERIALS AND METHODS

2.1. *Spectroscopy*

^{1}H-NMR spectra were recorded with a Varian HR220 instrument operating at 220 MHz. The solvent was D_2O with dioxane ($\delta = 3.75$ ppm from TMS) as an internal standard. Chemical shifts are reported as δ in ppm. Multiplicity is abbreviated as

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s (singlet), d (doublet) or m (multiplet). ^{31}P-NMR spectra were obtained with a Varian instrument at 109 MHz with D_2O as solvent and 0.15% H_3PO_4 as external reference.

The concentrations and quantities of ethenoadenosine in the products were determined with a Hewlett–Packard 8450 A UV/VIS spectrophotometer using the following values: $\epsilon^{258} = 11.5 \cdot 10^{-3}$ for 2′,5′(pA)$_3$, $\epsilon^{275} = 4.5 \cdot 10^{-3}$ and $\epsilon^{258} = 3.75 \cdot 10^{-3}$ for 2′,5′(pϵA)$_3$. Calculations of the degree of modification with ϵA residues were based on a previous observation [10] that the UV spectra of poly(ϵA,A) copolymers (3′,5′-linked) with varying ratios of the bases have two isosbestic points at 243.5 nm and at 280 nm. Thus, the degree of modification (or the % of 1,N^6-ethenoadenine residues) can be represented by the equation [10]:

$$\alpha = \frac{A_{294}\sigma}{(A_{258} - A_{294} \cdot \sigma)\frac{\epsilon^{258}_{(p\epsilon A)_3}}{\epsilon^{258}_{(pA)_3}} + A_{294}\sigma}$$

where:

A = absorption at given wavelength

$$\sigma = \frac{\epsilon^{258}_{(p\epsilon A)_3}}{\epsilon^{294}_{(p\epsilon A)_3}}.$$

The fluorescence emission spectra were recorded with a Perkin–Elmer MPF-44A Fluorescence spectrophotometer. All measurements were done at room temperature in 0.1 M phosphate buffer (pH 7.2) and with the excitation wavelength 305 nm. The apparent spectra were corrected and relative quantum efficiencies were determined according to the literature [11]. For relative quantum efficiency determination, ethenoadenosine 5′-monophosphate (P-L Biochemicals) was used as a reference compound, quantum efficiency 0.59 [12].

2.2. *Thin-layer chromatography*

Thin-layer chromatography was performed on E. Merck precoated PEI-cellulose F plates with solvent systems: (A) 0.1 M NH_4HCO_3; (B) 0.25 M NH_4HCO_3; or (C) 1 M LiCl; or on cellulose F plates with system D, 1 M ammonium acetate (pH 4.9) CH_3OH (1:2).

2.3. *1,N^6-ethenoadenylyl(2′−>5′)-1,N^6-ethenoadenylyl(2′−>5′)-1,N^6-ethenoadenosine 5′-monophosphate[2′,5′(pA)$_3$]*

The oligomer, 2′,5′(pA)$_3$ (2850 A_{260} units, 0.083 mmol, prepared as in [13]) was dissolved in 10% chloroacetaldehyde solution (3 ml, 3.8 mmol, η_D^{20} 1.3375, prepared from dimethylacetal as in [12]) and the solution's pH was adjusted to 4.9 with 1 M sodium acetate (pH 4.3). After 48 h incubation at 37°C, 90% of the adenine residues had reacted according to calculations made from the UV spectrum of the crude mixture (vide ante). The reaction mixture then was applied to a DEAE Sephadex A-25 (HCO_3^-) column (1.6 × 35 cm), and elution was performed with a linear gradient of 0.05–0.5 M triethylammonium bicarbonate buffer (pH 7.5). Two partly overlapping fractions were collected. The first (450 A_{275} units, 40%) contained oligoadenylate with 96% of the adenosine residues converted to ethenoadenosine, and the second fraction contained oligomer with 89% of the adenosine converted to ethenoadenosine as determined by UV spectroscopy (vide ante). This first fraction therefore is referred to as 2′,5′(pϵA)$_3$. On TLC, 2′,5′(pϵA)$_3$ ran as a single spot with R_f's of 0.38 (system A), 0.67 (system C) and 0.13 (system D).

^{31}P-NMR: δ 1.62 ppm, s, 1P; δ −1.11 ppm, s, 2P.

^{1}H-NMR: δ 5.72 ppm (d, $J < 2$ Hz, 1H); δ 5.93 ppm (d, $J < 2$ Hz, 1H); δ 6.05 ppm (d, $J < 2$ Hz, 1H); δ 6.85 (s, 1H); δ 7.13 (s, 1H); δ 7.30 (s, 1H); δ 7.38 (s, 1H); δ 8.53 (s, 1H); δ 8.60 (s, 1H).

Relative fluorescence quantum efficiency: 0.051.

2.4. *1,N^6-ethenoadenylyl(2′−>5′)-1,N^6-ethenoadenylyl(2′−>5′)-1,N^6-ethenoadenosine 5′-triphosphate [2′,5′pp(pϵA)$_3$]*

An aqueous solution of 2′,5′(pϵA)$_3$ (240 A_{275} units, 0.018 mmol) was evaporated to dryness and was then dried by two consecutive additions and evaporations of dry DMF (2 × 2 ml). Gentle warming was necessary to dissolve the residue. DMF (1 ml) and carbonyl diimidazole (30 mg, 0.19 mmol) were then added, and the mixture was stirred at ambient temperature for 1 h. The reaction mixture was poured into a solution of sodium iodide in acetone (20 ml of 1% solution), and the precipitate of sodium salt of the 5′-phosphoroimidazolide of 2′,5′(pϵA)$_3$ that formed, was centrifuged down,

washed several times with acetone and then dried in vacuo.

Di-(tri-*n*-butylammonium)-pyrophosphate (250 μl of 0.5 M in DMF) was added to the dried phosphoroimidazolide and the mixture was kept at room temperature for 24 h after which it was diluted with cold water (1 ml) and applied to a column of DEAE-Sephadex (A-25, HCO_3^-, 1 × 10 cm). Elution was with a linear gradient of triethylammonium bicarbonate (0.3–0.6 M, 400 ml total). The yield was 78 OD_{275} units (6 μmol, 33%). The product, pp(pϵA)$_3$, was homogeneous by TLC in solvent systems B ($R_f = 0.42$) and C ($R_f = 0.14$).

^{31}P NMR: δ −1.04 (s, 1P) and −1.14 (s, 1P); δ −7.07 (d, $J = 20.0$ Hz, P_γ); δ −11.55 (d, $J = 19.3$ Hz, P_α); δ −22.70 (m, $J = 20.0$ Hz, P_β).

^{1}H-NMR: δ 8.78 (s, 1H); δ 8.73 (s, 1H); δ 8.22 (s, 1H); δ 8.15 (s, 1H); δ 7.99 (s, 1H); δ 7.88 (s, 1H); δ 7.55 (s, 2H); δ 7.27 (s, 1H); δ 7.13 (s, 1H); δ 6.95 (s, 1H); δ 6.15 (d, $J < 2$ Hz, 1H); δ 6.09 (d, $J < 2$ Hz, 1H); δ 5.89 (d, $J < 2$ Hz, 1H).

Relative fluorescence quantum efficiency: 0.063.

2.5. *Cell-free protein synthesis and radiobinding assays*

These were performed as in [14–15]. The radioactive probe ppp5′A2′p5′A2′p5′A3′[^{32}P]p5′C3′p, was obtained from Amersham and had a specific activity of 3000 Ci/mmol.

3. RESULTS AND DISCUSSION

The 5′-monophosphorylated analogue, 2′,5′(pA)$_3$ was prepared in 40% yield by the reaction of 2′,5′(pA)$_3$ with aqueous chloroacetaldehyde solution under conditions similar to those in [12,17]. A product was obtained which, by UV spectroscopic estimates, consisted of at least 96% ethenoadenosine residues with no more than 4% adenosine residues. This estimate was confirmed by ^{1}H-NMR which revealed, in addition to the 3 anomeric protons, a total of 12 aromatic protons corresponding to 6 from the purine ring system and 6 from the etheno substitution. Integration of the PMR spectrum indicated complete replacement of the adenosine residues with ethenoadenosine. ^{31}P-NMR

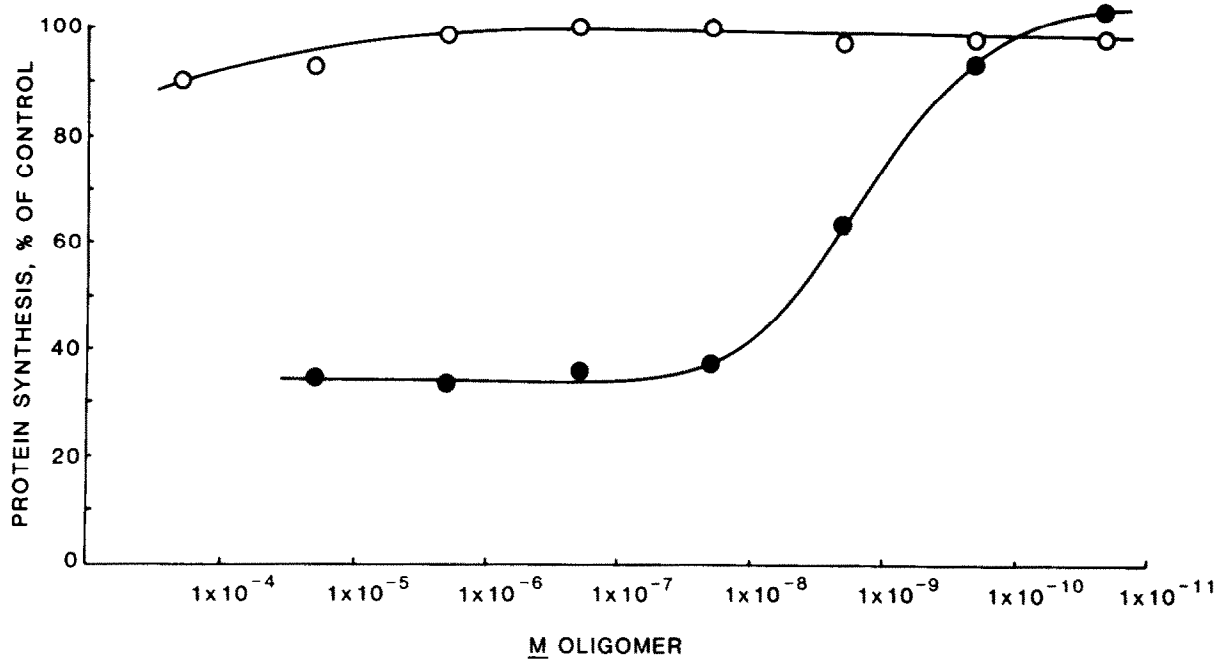

Fig.1a. Ability of 2′,5′(pϵA)$_3$ (○) or 2′,5′(pA)$_3$ (●) to prevent the protein synthesis inhibitory action of 2–5A. In these experiments, the concentration of 2–5A was constant at $2 \cdot 10^{-8}$ M, and the concentration of the oligonucleotide monophosphate varied. 100% corresponds to the level of protein synthesis obtained in the absence of any additions. The appropriate controls also included the effect of addition of 2′,5′(pϵA)$_3$ (△) alone and the effect of addition of 2′,5′(pA)$_3$ alone (▲).

revealed the obligatory internucleotide and 5′-monophosphate phosphorus resonances. This monophosphate was transformed, using standard procedures, to the corresponding 5′-triphosphate, 2′,5′pp(pεA)$_3$, the structure of which was confirmed by ^{31}P and ^{1}H-NMR.

It has been demonstrated [15] that the 5′-monophosphate, 2′,5′(pA)$_3$, is an antagonist of 2–5A action since it can bind to the 2–5A-dependent nuclease and displace 2–5A itself. This phenomenon has been used to ascertain the ability of various 5′-monophosphates to interact with the endonuclease [9]. Under conditions where 10^{-6} M 2′,5′(pA)$_3$ was able to prevent 50% of the protein synthesis, inhibitory action of 2–5A, 2′,5′(pεA)$_3$ did not reveal any significant antagonistic effect even at 10^{-4} M (fig.1a), suggesting that its affinity for the endonuclease must be at least 60-times less than that of 2–5A. In agreement with this finding, when 2′,5′pp(pεA)$_3$ was evaluated as an inhibitor of translation in encephalomyocarditis virus RNA-programmed extracts of mouse L cells, it was found to be devoid of activity; i.e., it did not cause significant inhibition of translation at 10^{-4} M, whereas 2–5A itself gave 50% of maximum protein synthesis inhibition at 10^{-9} M (fig.1b). Finally, pp(pεA)$_3$ was checked for its ability to bind to the 2–5A-dependent endonuclease using the radioactive assay in [16]. The fluorescent analogue, pp(pεA)$_3$, was bound at least 5000-times less effectively than 2–5A as judged by the ability of such ligands to displace the probe ppp5′A2′p5′A2′p5′A2′p5′A3′[^{32}P]p5′C3′p from the endonuclease (fig.1c).

In contrast to an earlier report [8] which provided no verification of the structure of the purported 2′,5′pp(pεA)$_n$ product, we have found that 2′,5′pp(pεA)$_3$ binds very poorly to the 2–5A-dependent endoribonuclease and cannot give rise to inhibition of protein synthesis even at concentrations of 10^5 M that are necessary to inhibit translation in the case of 2–5A action. These results indicate that the etheno modification, altering as it does the N^1 and N^6 sites of the purine ring, is not compatible with binding to the 2–5A-dependent endonuclease. Consistent with this, other

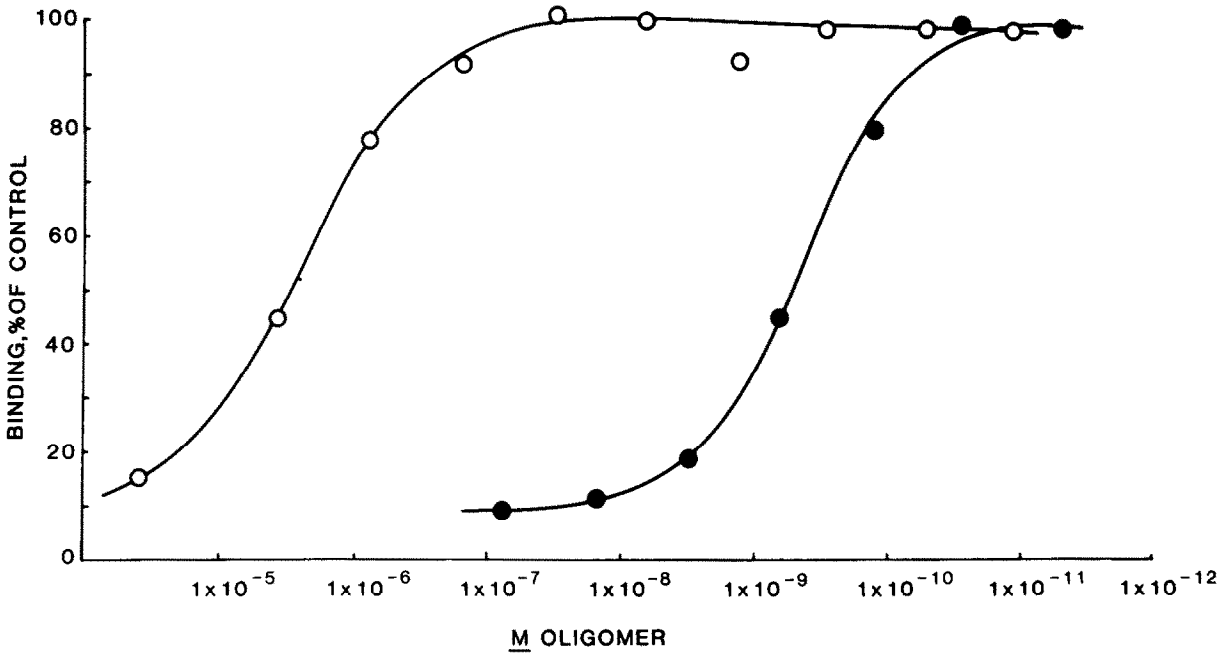

Fig.1b. Ability of the 5′-triphosphate pp(pεA)$_3$ to inhibit protein synthesis in extracts of mouse L cells programmed by encephalomyocarditis virus RNA. 100% protein synthesis represents the level of translation in the absence of any additions. The effect of addition of varying quantities of 2′,5′pp(pεA)$_3$ (○) is compared to the effect of addition of varying amounts of 2′,5′pp(pA)$_3$ (●).

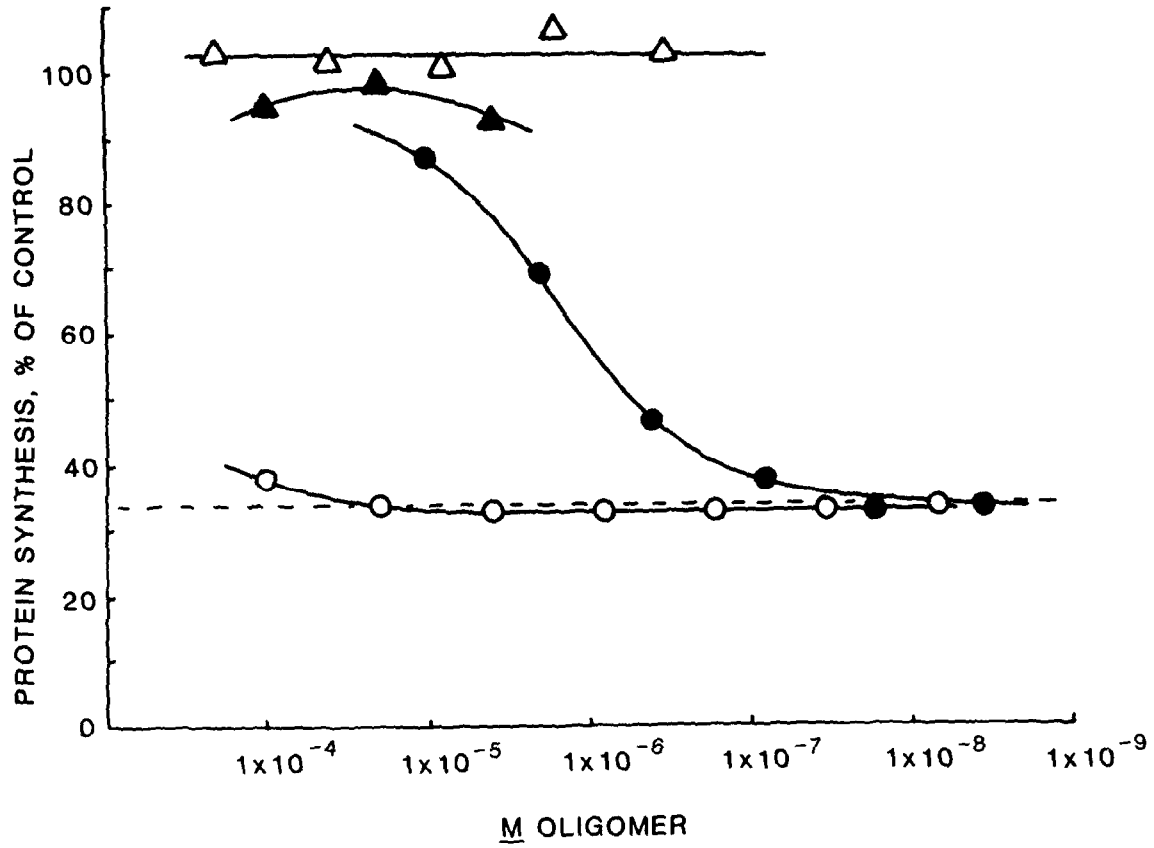

Fig.1c. Ability of 2',5'pp(pεA)$_3$ (○) and 2',5'(pA)$_3$ (●) to bind to the 2–5A-activated endonuclease of L cells as ascertained by the radiobinding assay in [16]. Increasing amounts of 2',5'pp(pεA)$_3$ or 2',5'pp(pA)$_3$ were added to a constant concentration of the probe ppp5'A2'p5'A2'p5'A2'p5'A3'-[^{32}P]p5'C3'p in a buffer containing extracts of mouse L cells [16].

studies in this laboratory [9 and, Lesiak, Sawai and Torrence, unpublished observations], have shown that replacement of the adenine ring with uracil, cytosine, or inosine gives analogues with greatly reduced affinity for the endonuclease. It appears, therefore, that determinants of the adenine ring are vital in the interaction of 2–5A with the 2–5A-dependent endonuclease.

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