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# SYNTHESIS AND PROPERTIES OF POLY(5-ETHYNYLURIDYLIC ACID)

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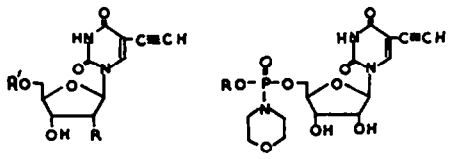
**Abstract**—5-Ethynyluridine 5'-diphosphate has been synthesised from 5-ethynyluridine under carefully controlled conditions to prevent any reaction with the ethynyl group. The compound is a substrate for polynucleotide phosphorylase from *Micrococcus luteus* and has been polymerised to give poly(5-ethynyluridylic acid). The polymer has a stable secondary structure with a $T_m$ of 76° accompanied by a 16% hyperchromicity in solutions of high ionic strength and forms a 1:1 complex with poly(A) with a $T_m$ of 78°. Thus the presence of the ethynyl group in the pyrimidine ring causes the polymer to have and to form much more stable secondary structures than the corresponding unsubstituted polynucleotide poly(U).

Many 5-substituted uracils have recently been synthesised, the deoxynucleosides of which have been shown to have antiviral or antitumour properties.[1-3] Many homopolyribonucleotides have also been synthesised by the action of polynucleotide phosphorylase on the corresponding 5-substituted uridine 5'-diphosphate and these have often been shown to interact with polyadenylic acid to give double or triple stranded structures possessing a wide range of stabilities depending upon the nature of the 5-substituent. In particular poly(5-methyluridylic acid) and poly(5-ethyluridylic acid) form more stable complexes with polyadenylic acid than does polyuridylic acid itself.[4] We have previously reported attempts to synthesise poly(5-acetyluridylic acid) but the nucleoside 5'-diphosphate was not a substrate for the polymerizing enzyme.[5] Double-stranded homopolynucleotide complexes are known to induce interferon and it appears that there is a minimum stability required for the secondary structure, below which no interferon iis produced[6] and as several 5-alkylated uracil-containing polynucleotides form very stable complexes with polyadenylic acid, it was decided to attempt the synthesis of poly(5-ethynyluridylic acid).

The UV absorption spectrum of 5-ethynyluridine (2) $\lambda_{max}$ 290 nm, $\varepsilon$ 10,700 at pH 7) is very different from that of 5-methyluridine (ribothymidine) or 5-ethyluridine which indicates that there must be considerable delocalization of the $\pi$-electrons from the triple bond into the $\pi$-system of the aromatic ring, making the properties of such a polymer likely to be of particular interest. X-ray analysis of the crystal structure of the deoxynucleoside also yields evidence for conjugation between the C(5)-C(6) double bond and the ethynyl group.[9]

The deoxynucleoside of 5-ethynyluracil (1) has been shown to possess activity against leukaemia cells[7] and also to show activity against herpes virus[8] and although attempts to get the base to replace



1 R=H; R'=H
2 R=OH; R'=H
3 R=$Cl_3CCH_2$
4 R=H
5 R=OH; R'=$(HO)_2OPO(OH)OP$—

thymine residues in DNA have so far failed, X-ray studies of the crystalline $\beta$-deoxynucleoside have shown that the overall shape of the molecule is very similar to that of thymine and is likely to be able to be accommodated in a polynucleotide structure.[9]

Homopolynucleotides have also been shown to possess antiviral activity against RNA-containing viruses (encephalomyocarditis [EMC] virus) under conditions in which no interferon production could be expected or was detected. It is thought that the homopolynucleotides mimic similar tracts which occur in the virion RNA or its complement in the minus strand and interfere with the process of virus replication.[10]

Due to the ease with which the ethynyl group is hydrated under mild acidic conditions,[11] it was necessary to avoid such conditions during the preparation of 5-ethynyluridine 5'-diphosphate. The synthesis of the ribonucleoside (2) has already been reported.[12]

Some preliminary experiments were performed to investigate the possibility of using a wheat shoot phosphotransferase[13] for the preparation of the 5'-monophosphate in order to avoid the rather drastic conditions that are usually present at some stage during most chemical phosphorylation procedures. However, in our hands, 5-ethynyluridine was a poor substrate for the enzyme and the maximum phosphorylation achieved as estimated by tlc was only 16%. Thus it was decided to use the phosphorylating agent 2,2,2-trichloroethylphosphoromorpholino chloridate, described by Owen *et al.*[14]

This was selective for the 5'-position and gave compound **3** in an isolated yield of 52%. The trichloroethyl group could be removed under mild, non-acidic conditions[15] to give the morpholidate (**4**) which is the derivative required for the preparation of the 5'-diphosphate.

Phosphorylation of this phosphomorpholidate was achieved using mono-(tri-n-butylammonium) phosphate and orthophosphoric acid as described by Van Boom *et al.*,[16] to give the diphosphate (**5**) which was purified by chromatography on DEAE-Cellulose to give a product (26% yield) which was shown to be homogeneous upon tlc on polyethyleneimine (PEI)-cellulose and silica, and had NMR and UV spectra consistent with the required nucleoside 5'-diphosphate. The low yield of this preparation can in part be explained by the presence of other nucleoside 5'-diphosphates in the final mixture. These were separated in the ion-exchange chromatographic step and in particular, one of these products which was present in considerable quantities but which was not identified, had a UV spectrum consistent with the base moiety being a uracil derivative bearing a saturated grouping at the 5-position.

5-Ethynyluridine 5'-diphosphate (**5**) was shown to act as a substrate for polynucleotide phosphorylase from *Micrococcus luteus* to give a yield of polymer of 56% in 5 h. Analysis on the ultracentrifuge showed that the polymer was rather heterogeneous in size with an $S_{20}$ value in 0.05 M potassium phosphate buffer pH 7.0 containing 0.1 M NaCl of 5.2. Enzymatic digestion of the polymer with snake venom phosphodiesterase and with ribonuclease A confirmed the expected structure for the polynucleotide with the sole product in each case being 5-ethynyluridine 5'-phosphate and 5-ethynyluridine 2'-(3)-phosphate respectively although the reaction with the former enzyme was very slow.

The UV absorption spectrum of poly(5-ethynyluridylic acid). $\lambda_{max}$ 288 nm, ($\varepsilon_p$ 10,000), $\lambda_{min}$ 252 nm in water at pH 7.0 was similar to that of the nucleoside 5-ethynyluridine,[12] $\lambda_{max}$ 290 nm ($\varepsilon$ 10,700), $\lambda_{min}$ 253 nm at pH 7.0. The hyperchromicity obtained by enzymatic digestion of the polynucleotide depended markedly on the ionic strength of the solution. At low ionic strength (0.02 M Tris-HCl pH 8.0) the hyperchromicity was 3.4% and at high ionic strength (0.15 M NaCl, 0.015 M sodium citrate), the hyperchromicity was 17%. These figures agree well with those obtained by thermal denaturation (Fig. 1) which gave the corresponding figures of 3.5% (low ionic strength, $T_m$ 34°) and 16% (high ionic strengh, $T_m$ 76°). The corresponding figures for polyuridylic acid [poly(U)] are not measurable at low ionic strength ($T_m \sim 0°$)and 18.5% (high ionic strength, $T_m$ 8.5°)[17] Poly(5-methyluridylic acid)[18] shows 50% hyperchromicity ($T_m$ 33°) and poly(5-ethyluridylic acid) shows 30% hyperchromicity[4] ($T_m \sim -2°$) in the presence of 10 mM $Mg^{2+}$.

It is clear that unlike poly(U), poly(5-methyluridylic acid) and poly(5-ethyluridylic acid), poly(5-ethynyluridylic acid) possesses an ordered structure even at low temperatures at low ionic strength or in the absence of magnesium ions and forms a structure with considerable stability at high ionic strength. This is presumably due to the increased delocalization of electrons in the pyrimidine ring caused by the presence of the unsaturated ethynyl grouping thus enabling the base residues to stack better in an ordered tertiary structure.

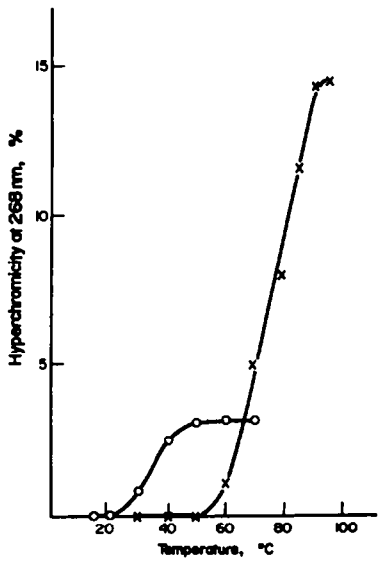



Fig. 1. Determination of $T_m$ of poly(5-ethynyluridylic acid) in (a) 0.02 M Tris-HCl (pH 8.0) ⊙—⊙. (b) in 0.15 M NaCl, 0.015 M sodium citrate pH 6.7 ×——×.

The continuous variation method[19] was used to investigate the complexes formed between poly(5-ethynyluridylic acid) and poly(adenylic acid).

The results (Fig. 2) show that poly(5-ethynyluridylic acid) forms a helical complex with poly(A). Unlike the complex formed between poly(U) and poly(A), no evidence for anything

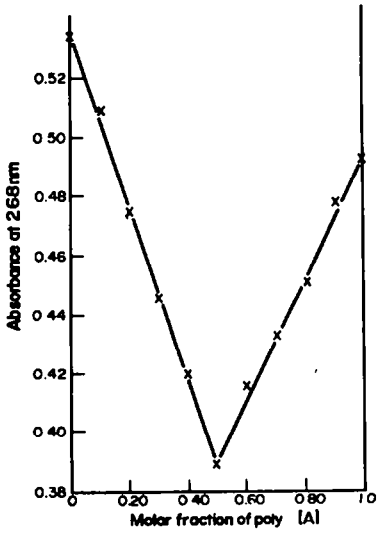



Fig. 2. Absorbance of mixtures of poly(A) and poly(5-ethynyluridylic acid) after 18 h at 4° in 0.15 M NaCl, 0.015 M sodium citrate pH 6.7.

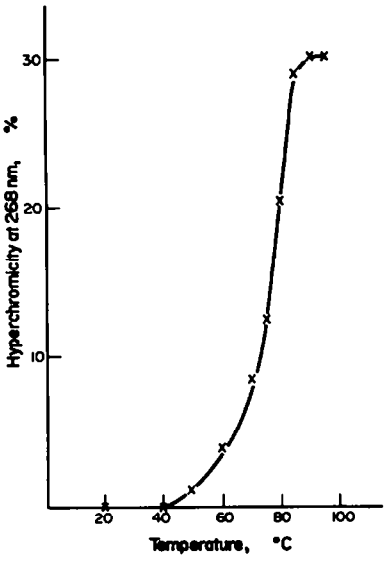



Fig. 3. Determination of the $T_m$ of poly(A). poly(5-ethynyluridylic acid) hybrid in 0.15 M NaCl, 0.015 M sodium citrate pH 6.7.

other than a double-stranded structure could be found although the stability of this structure varied over a wide range depending upon the exact ionic conditions used.[20,21] Neutral or slightly alkaline conditions have to be used, particularly if the solution is to be heated for $T_m$ determination as the ethynyl group is particularly easily hydrolysed to an acetyl group which results in a change in the UV absorption spectrum of the polynucleotide and at certain wavelengths could be interpreted as a hyperchromic effect.

The $T_m$ of the poly(A):poly(5-ethynyluridylic acid) hybrid in 0.15 M NaCl, 0.015 M sodium citrate solution is 78° (Fig. 3) and demonstrates a sharp cooperative melting profile under conditions which in an adjacent cuvette, poly(A):poly(U) (1:1) gave a $T_m$ of 50°. It appears to be necessary to have this control experiment performed under identical conditions of ionic strength and temperature as the $T_m$ (and the stoichiometry) of poly(A):poly(U) hybrids is very dependent upon ionic strength and many different values have been quoted although apparently some of the differences can be explained by assuming that the reaction mixtures had not reached equilibrium.[21] Under the conditions described here, no significant change in hybrid formation was found over a period of 48 h.

Thus the poly(A): poly(5-ethynyluridylic acid) hybrid is stable and thus potentially capable of inducing interferon production. It has been also found that poly(5-ethynyluridylic acid) itself is an inhibitor of influenza virus transcriptase and these biological results will be reported elsewhere.

## EXPERIMENTAL

NMR spectra were recorded at 100 MHz in $(CD_3)_2SO$ unless otherwise stated. Tlc was carried out on silica gel (MN Kieselgel G/$UV_{254}$) or cellulose (MN 300 G/$UV_{254}$) or polyethyleneimine (PEI/MN 300 G/$UV_{254}$) (Machery, Nagel & Co., W. Germany). Column chromatography was carried out on Kieselgel 60 (70–120 mesh ASTM) (type 7734) (E. Merck A. G., W. Germany). The following chromatography solvents were used: (1) chloroform-methanol (8:2); (2) propan-2-ol-ammonia (Sp. g. 0.88)-water (7:1:2); (3) 1 M LiCl; (4) 2-methylpropanoic acid-ammonia (Sp. g. 0.88)-water (66:1:33).

*Enzymes*

*Micrococcus luteus* polynucleotide phosphorylase (nucleoside diphosphate, polynucleotide nucleotidyltransferase, E.C. 2.7.7.8) was obtained from P-L Biochemicals Inc., Wisconsin, U.S.A. Venom phosphodiesterase (E.C. 3.1.4.1.) and pancreatic ribonuclease A (E.C. 2.7.7.16) were obtained from the Worthington Biochemical Corporation, New Jersey, U.S.A.

*β,β,β-Trichoroethylester of 5-ethynyluridinephosphoromorpholidate* (3). 5-Ethynyluridine 2 (439 mg, 1.64 mmol) was dissolved in pyridine (10 ml) and evaporated to dryness (×2) to remove traces of water. Then to a soln of the nucleoside in pyridine, β,β,β-trichloroethylphosphoromorpholinochloridate (676 mg, 2.13 mmol) was added and the soln left at room temp for 24 h. Then a further 250 mg (0.82 mmol) of phosphorylating agent was added and the reaction allowed to continue for a further 2 days. Phosphate buffer (0.05 M, pH 7.0, 15 ml) was added and after 30 min the mixture was evaporated to dryness. The resulting oil was dissolved in chloroform (150 ml), washed with water, dried and then added dropwise to an excess of petroleum ether (60–80°) to give a ppt of a crude product (829 mg) as an off-white solid. The product was further purified by chromatography on silica gel using MeOH-$CHCl_3$ (6:94) as eluant. Fractions containing the pure compound were combined and the pure product isolated as before by precipitation from chloroform with petroleum ether to yield a chromatographically homogeneous product (silica, solvent 1, $R_f$ 0.46) (468 mg, 52%) (Found: C, 38.1; H, 4.0; N, 8.2. $C_{16}H_{21}N_3O_7Cl_3P$ requires: C, 38.08; H, 4.19; N, 8.32%); $\lambda_{max}$ 228 nm (ε, 7700) and 288 nm (ε, 9200), $\lambda_{min}$ 250 nm (ε, 1900) in MeOH; δ 3.35 (10 H, m, morpholino H's and H-5'), 4.1 (1H, s, acetylenic H), 4.60 (1H, d, $—CH_2CCl_3$, J = 1 Hz), 4.66 (1H, d, $—CH_2CCl_3$, J = 1 Hz), 5.76 (1H, d, H-1', $J_{1'2'}$ = 5 Hz), 7.90 and 7.94 ppm (1H, 2s, H-6 of diasteriomers).

*5-Ethynyluridinephosphoromorpholidate* (4). Compound 3 (313 mg, 0.56 mmol) was dissolved in pyridine (8.4 ml), Zn (399 mg, 6.13 mmol) and acetylacetone (4.0 ml, 3.9 mM) added and the mixture stirred vigorously for 15 min.[15] The reaction was monitored by tlc on silica in solvent 1 which showed that the reaction was by this time complete. The Zn was removed by filtration, washed with pyridine and the soln taken to dryness. The resulting oil was dissolved in pyridine and the product precipitated by the addition of an excess of diethyl ether, collected by centrifugation, washed with ether and dried to give a white powder from which traces of Zn ions were removed by passing a soln of the product down a Chelex 100 ($Na^+$) resin (Bio-Rad Laboratories). The product (300 mg) was chromatographically homogeneous (silica, solvent 2, $R_f$ 0.33) but was contaminated with traces of inorganic material and was used for the next step without further purification. δ 3.29 (8H, m, morpholino Hs'), 4.04 (1H, s, acetylenic H), 5.76 (1H, d, H-1', $J_{1'2'}$ = 6 Hz), 8.58 ppm (1H, s, H-6).

*5-Ethynyluridine 5'-diphosphate* (5). Compound 4 (700 mg, 1.21 mmol) was suspended in anhyd pyridine and mixed with a soln of mono (tri-*n*-butylammonium) phosphate[16] (0.86 ml, 3.63 mmol) and 88% orthophosphoric acid (0.225 ml, 3.63 mmol) in anhyd pyridine. The mixture was evaporated to dryness (×3) under reduced pressure to remove any traces of water and then pyridine (12 ml) added and the mixture shaken for 1 h. The flask and contents were then transferred to a dry atmosphere

and after 48 h tlc on silica in solvent 2 of on PEI-cellulose in solvent 3, showed that the reaction was complete. The mixture was then evaporated to dryness, all traces of pyridine removed and the resulting oil dissolved in water (12 ml) containing 495 mg (4.86 mmol) of lithium acetate. The solution was extracted with ether, the aqueous layer adjusted to pH 11.0 with LiOH and the solution left at 0° for 1 h. The lithium phosphate formed was removed by centrifugation, the soln neutralised with Dowex 50 W $H^+$), reduced in volume to 5 ml and applied to a column of DEAE-52 Cellulose (30 cm × 3 cm) which had been pre-equilibrated with 0.02 M triethylammonium bicarbonate soln pH 8.0. A linear gradient of 0.02 M→0.3 M triethylammonium bicarbonate pH 8.0 in 4 l total volume was used. Fractions containing 5-ethynyluridine 5'-diphosphate were pooled, evaporated to dryness and repeatedly evaporated with MeOH to remove traces of triethylammonium bicarbonate. A soln of the pyrophosphate was then passed down a column of Dowex 50 W ($Na^+$) and the eluate lyophilised to give the product (156 mg, 26%). The compound was chromatographically homogeneous (PEI-cellulose, solvent 3, $R_f$ 0.13; cellulose, solvent 4, $R_f$ 0.38). $\lambda_{max}$ 288 nm (ε, 9600), $\lambda_{min}$ 258 nm (ε, 2800) at pH 7.0; $\lambda_{max}$ 286 nm (ε, 7600), $\lambda_{min}$ 258 nm (ε, 2200) at pH 12; δ ($D_2O$) 3.63 (1H, s, acetylenic H), 4.30 (5H, m, H-5', H-4', H-3', H-2'), 5.90 (1H, d, H-1', $J_{1',2'}$ = 5 Hz), 8.15 ppm (1H, s, H-6).

*Poly(5-ethynyluridylic acid)*. Compound **5** (29 mg, 0.059 mmol, 535 $A_{288}$ units) were dissolved in water (4 ml) containing $MgCl_2$ (10 mM), EDTA (5 mM) Tris-HCl (pH 9.0, 66 mM) and *M. luteus* polynucleotide phosphorylase (20 units) and incubated at 37° for 6 h. The release of inorganic phosphate was monitored[22] and was found to reach a plateau at around 40% in 5 h. The reaction was stopped by the addition of 90% phenol soln (4 ml) and the soln deproteinised in the usual way.[23] The combined aqueous layers were dialysed exhaustively against 0.02 M Tris-HCl pH 8.0 buffer at 5° to yield a solution of polymer containing 300 $A_{288}$ units (56% yield, ignoring hypochromicity). The polynucleotide was eluted in the void volume of a Sephadex G-50 column and was stored frozen at −40°. When lyophilised, the resulting white powder was not soluble in aqueous solutions or in dimethylformamide.

*Enzymatic digestion of poly(4-ethynyluridylic acid)*. (1) Poly(5-ethynyluridylic acid) (0.5 $A_{288}$ units) was incubated with snake venom phosphodiesterase (5 μl of 1 mg/ml solution) in Tris-HCl buffer (pH 8.0, 0.02 M, 50 μl) at 37° for 21 h. Electrophoresis and chromatography on silica in solvent 4 ($R_f$ 0.43) and on PEI-cellulose in solvent 3 ($R_f$ 0.32) confirmed the sole product to be 5-ethynyluridine 5'-phosphate. The hyperchromicity shown was 3.4%. The enzymatic hydrolysis is very slow; under similar conditions, the hydrolysis of poly(U) was complete in 30 min.

(2) Poly(5-ethynyluridylic acid) (0.5 $A_{288}$ units) was incubated with ribonuclease A (2 μl of 1 mg/ml solution) in 0.15 M NaCl-0.015 M sodium citrate buffer (pH 7.0, 50 μl) at 37° for 21 h. Chromatography as above confirmed that the sole product of digestion was 5-ethynyl uridine 2'(3')-phosphate. The hyperchromicity shown was 22% after 21 h with a 20% increase being evident after 12 mins.

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