SYNTHESIS OF THE URACIL ANALOG OF THURINGIENSIN AND ITS INHIBITORY EFFECT ON DNA-DEPENDENT RNA POLYMERASE OF *Escherichia coli*

Ladislav KALVODA, Květa HORSKÁ and Karel ŠEBESTA

Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6

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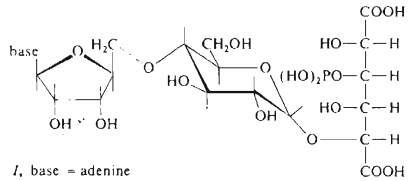
An analog of thuringiensin (*II*), containing uracil instead of adenine, was synthetized by the method employed for thuringiensin. Similarly to the parent substance this analog inhibits the DNA-dependent RNA polymerase of *Escherichia coli*, simultaneously competing with the natural substrate, *i.e.* UTP. The inhibition constant ($K_i = 30$ μM) shows that the uracil analog of thuringiensin is an inhibitor of RNA polymerase roughly as efficient as thuringiensin.

It has been shown in the preceding studies that the phosphorylated derivative of adenosine, thuringiensin (*I*), is a natural inhibitor of both prokaryotic and eukaryotic DNA-dependent RNA polymerases[1-6]. The inhibitory effect of this compound, containing in addition to adenosine glucose and phosphorylated allaric acid[7], consists in the competition with ATP for the nucleotide binding site on RNA polymerase[1]. Studies carried out with the analogs of this natural inhibitor have demonstrated the relative role of the individual parts of the molecule for the inhibitory effect. The transformation of the sugar moiety of the molecule brings about a decrease of the inhibitory effect[8]. A change of the purine base shows that the binding to the active site and thus also the inhibition depend on the base pairing according to the Watson–Crick rule[1,2]. The dephosphorylation of thuringiensin leads to a complete loss of enzymatic activity[9].

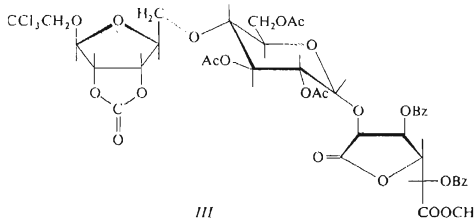
The ribonucleoside triphosphates derived from the purine bases, adenine and guanine, are involved in the RNA polymerase catalyzed reaction both during the inhibition and the elongation phase. It was therefore of interest to examine the inhibitory properties of the above analog containing a pyrimidine base, *i.e.* uracil instead of adenine. The present paper deals with the synthesis of a pyrimidine analog of thuringiensin and with the determination of its inhibitory effect.

The synthesis of the uracil analog of thuringiensin (*II*) was carried out by a method analogous to that used for the synthesis of thuringiensin[10]. The original synthesis was modified only slightly: The transformation of the triacetate (*III*) to the pentaacetate (*IV*) was simplified and the time of the conversion of the hexaacetate (*V*) into the anomerous mixture of halogenoses (*VI*) was prolonged. To ensure the pre

ferential formation of the β-anomer the nucleosidation was carried out in acetonitrile in the presence of mercuric bromide[11]. The cleavage of the lactone ring and the phosphorylation were carried out as described in the original paper[10].



I, base = adenine
II, base = uracil



III

The comparison of the inhibitory effect of thuringiensin (*I*) with the effect of its uracil analog *II* (Fig. 1) shows that the inhibition curve characterizing both com-

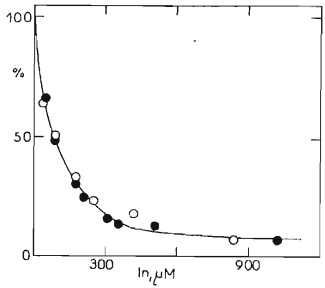
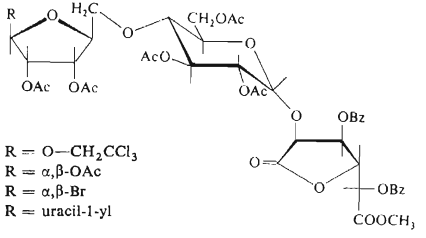


FIG. 1

Inhibition of RNA Polymerase by Thuringiensin (*I*) and the Uracil-Analog of Thuringiensin (*II*)

The reaction mixture contained in a final volume of 0·125 ml: salts, DNA, nucleotide triphosphates and the enzyme as given in Experimental. Varying amounts of the inhibitor were added to the reaction mixture before the enzyme. The reaction mixture was incubated for 10 min at 37°C. Values for thuringiensin ○ and the uracil-analog of thuringiensin ●. In denotes inhibitor.

pounds has the same course. The inhibitory effect can be reversed by the corresponding nucleoside triphosphate only, *i.e.* by UTP (Table I), similarly to thuringiensin (*I*) which is antagonized by ATP only. The competition between the uracil analog (*II*) and UTP follows from the Dixon plot (Fig. 2). The plot permits the graphical determination of the K_i-value showing that the uracil analog of thuringiensin (K_i = = 20 μM) has approximately the same inhibitory effect as thuringiensin itself (K_i = = 20 μM). The uracil analog of thuringiensin therefore acts analogically to the parent substance. It can therefore be used to adavantage as a specific inhibitor of RNA polymerase wherever the presence of the adenine moiety is not desirable.



IV, R = O—CH_2CCl_3
V, R = α,β-OAc
VI, R = α,β-Br
VII, R = uracil-1-yl

TABLE I

Relation between the Inhibitor of RNA Polymerase and Varying Concentrations of Nucleoside Triphosphates

The reaction mixture contained in a final volume of 0·125 ml: salts, DNA and enzyme as given in Experimental; labeled ATP (25 nC) and labeled UTP (25 nC), respectively; nucleoside triphosphates and uracil-thuringiensin analog (*II*) as shown in the Table. The reaction mixture was incubated 10 min at 37°C. The labeled triphosphate is marked by an asterisk.

Concentration of inhibitor *II* μM	Concentration, μM				Enzyme activity %
	ATP	CTP	GTP	UTP	
—	80	80	80	20*	100·0
73	80	80	80	20*	42·3
73	240	80	80	20*	37·9
73	80	240	80	20*	35·4
73	80	80	240	20*	41·3
73	20*	80	80	80*	46·6
73	20*	80	80	160	58·6
73	20*	80	80	240	68·9

EXPERIMENTAL

Thin-layer chromatography was carried out on Merck Kieselgel GF_{254} (Type 60). The solutions were dried by anhydrous magnesium sulfate and concentrated at a bath temperature of 40°C. The samples for analysis were dried 10 h at 60°C and 13 Pa. The UV-spectra were measured in a Unicam SP-700 spectrophotometer.

Methyl 3,5-Di-O-benzoyl-2-O-[2,3,6-tri-O-acetyl-4-O-2,2,2-trichloroethyl(-2,3-di-O-acetyl--β-D-ribofuranosid-5-yl)-α-D-glucopyranosyl]-(2*R*)-allaro-1,4-lactone-6-ate (*IV*)

Pyridine (30 ml) and water (30 ml) were added to 2·46 g (2 mmol) of *III* and the mixture was heated at 100°C with stirring. The mixture was then cooled down, concentrated, and the residue was codistilled with pyridine (2 . 10 ml). Subsequently, pyridine (20 ml) and acetic anhydride (20 ml) were added to the dry residue and the mixture was set aside for 1 h at room temperature; it was concentrated afterwards and the residue codistilled with xylene (2 . 20 ml). The obtained syrup (*IV*) was used in the subsequent step without any purification. The sample for analyses was purified by chromatography on silica gel in the system benzene–ethyl acetate 8 : 2 (R_F 0·33 on TLC in the same system). For $C_{34}H_{47}Cl_3O_{23}$ (1050) calculated: 50·23% C, 4·51% H, 10·13% Cl; found: 50·46% C, 4·41% H, 10·32% Cl.

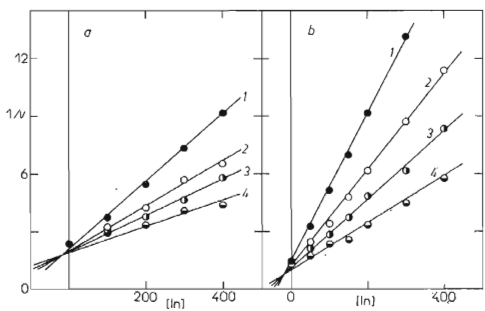


FIG. 2

Double Reciprocal Plot of RNA Polymerase Activity at Different Ratios of UTP/Uracil Analog of Thuringiensin (*II*) (*a*) and ATP/Thuringiensin (*I*) (*b*) *1* 80, *2* 120, *3* 160, *4* 240 μM UTP (*a*) or ATP (*b*) in the reaction mixture. [In] denotes the micromolar concentration of thuringiensin and the UTP-analog of thuringiensin, respectively, *v* is the reaction rate of the reaction with the inhibitor expressed in counts/min. For details of the reaction mixture see Experimental.

Methyl 3,4-Di-O-benzoyl-2-O-(2,3,6-tri-O-acetyl-4-O-(2′,3′-di-O-acetyl-uridin-5-yl)-α-D-glucopyranosyl)-(2*R*)-allaro-1,4-lactone-6-ate (*VII*)

Product *IV* obtained from 2·42 g (2 mmol) of *III* was dissolved in methanol (25 ml), zinc powder (4·0 g) was added to the solution which was subsequently treated dropwise with hydrochloric acid (1·0 ml). The mixture was filtered 15 min later, the filter was washed with methanol, and the filtrate was concentrated. After the addition of water (20 ml) and chloroform (20 ml) the emulsion formed was broken up by the addition of a few drops of acetic acid; the chloroform layer was dried and concentrated. The dry residue was mixed with pyridine (5 ml) and acetic anhydride and the mixture was allowed to stand 1 h at room temperature; it was concentrated afterwards and the residue was codistilled with xylene (3 . 10 ml). The dry residue of the anomerous mixture of acetates *V* was dissolved in the mixture chloroform (10 ml)–xylene (10 ml) and hydrogen bromide gas bubbled through the solution 45 min at room temperature; the solution was concentrated afterwards. To the dry residue of the anomerous mixture of halogenoses *VI* were added mercuric bromide (50 mg) and a solution of 2,4-bis(trimethylsiloxy)pyrimidine[3] (780 mg; 3·0 mmol) in acetonitrile (30 ml); the mixture was allowed to stand 48 h at room temperature. Pyridine (2 ml) and acetic acid (2 ml) were subsequently added, the mixture was concentrated and the residue codistilled with toluene (2 × 10 ml). Product *VII* was isolated by chromatography on silica gel (4 × 25 cm column) in the system benzene–acetone 7 : 3 (TLC: R_F of *VII* 0·42 in the same system). Yield 975 mg (48% in terms of *III*) $[\alpha]_D^{25}$ +50·9° (*c* 0·5 in chloroform). For $C_{46}H_{48}O_{24}N_2$ (1013) calculated: 54·54% C, 4·77% H, 2·76% N; found: 54·61% C, 5·12% H, 2·45% N.

2(*R*)-O-(4-O-Uridine-5′-yl-α-D-glucopyranosyl)-4-O-phosphorylallaric Acid (*II*)

Pyridine (1·0 ml) was added to the solution of nucleoside *VII* (250 mg; 0·25 mmol) in methanol (10 ml) and the mixture was boiled for 45 min. The mixture was then acidified by hydrochloric acid, diluted with water (50 ml), and extracted with ethyl acetate (2 . 30 ml); the extract was concentrated and the residue was codistilled with ethyl acetate (3 . 20 ml). The dry residue was treated with ethyl acetate (20 ml). then stepwise with a 1M solution of phosphorus oxychloride (4 ml) and with 1M pyridine solution (4 ml) (both solutions in ethyl acetate); the mixture was stirred 2 h at room temperature afterwards. Water was added and the mixture was stirred for another 15 min period. The aqueous layer was separated, extracted with ethyl acetate (15 ml), both extracts were pooled, dried, and concentrated. The dry residue was dissolved in pyridine (10 ml) and 1M sodium hydroxide (20 ml) was added to the solution. The mixture was allowed to stand overnight at room temperature and filtered through Dowex 50 in NH_4^+-form; the filtrate was concentrated and the dry residue was codistilled with water (2 . 20 ml). The product was isolated from the residue by ion exchange chromatography on DEAE-cellulose (25 × 300 mm column). The column was eluted by a gradient of triethylamine carbonate (pH 8, 0·1M—0·5M, constant volume 1 000 ml). The UV-absorbing fraction emerging between 500 and 1 100 ml was collected. The fraction was evaporated to dryness and the residue was codistilled with water (2 . 50 ml). The dry residue was dissolved in water (5 ml) and the solution was filtered through Dowex 50 in H^+-form (10 × 20 mm column), the filtrate was made alkaline by ammonia, concentrated and lyophilized. Yield 35 mg (21%) of 88% concentrate (determined spectroscopically), uracil: P 1 : 1·16, λ_{min} 231 nm, λ_{max} 262 nm in 0·1M-HCl; CD spectrum (H_2O): $[\Theta]_{275nm}$ 25 260°, $[\Theta]_{262nm}$ 0°, $[\Theta]_{244nm}$ —46 190°, $[\Theta]_{225nm}$ (min) —18 880°, $[\Theta]_{215nm}$ —36 180°.

Biochemical Preparations

The isolation of thuringiensin has been described elsewhere[12,13]. The DNA-dependent RNA polymerase was prepared from *E. coli* K12 by the modified method of Burgess[14], including

the chromatography on Heparin-Sepharose according to Sternbach[15]. The solution of the enzyme in 50% glycerol containing 10 mg of protein per ml was stored at —20°C. The DNA from *Bacillus subtilis* SB 19 was prepared by the method of Marmur[16]. Unlabeled nucleotide triphosphates were purchased from the California Corporation for Biochemical Research (U.S.A.). [^{14}C]-UTP and [^{14}C]-ATP were from the Institute for Research Production and Application of Radioisotopes, Prague. Dithiothreitol was purchased from Koch-Light Laboratories (England). Instafluor and Soluene 300 were from Packard (The Netherlands). Whatman GF/C glass fibre filters (diameter 24 mm) were used.

Enzyme assay. The reaction mixture contained in a final volume of 0·125 ml: 50 mM Tris-HCl (pH 7·5), 0·8 mM-Mg^{2+}, 1 mM-Mn^{2+}, 4 μM dithiothreitol, 80 μM-UTP, CTP and GTP, respectively, 20 μM-ATP and 0·8 μM [^{14}C]-ATP (25 nCi) (in experiments with labeled UTP the concentration of ATP was 80 μM, of UTP 20 μM and of [^{14}C]-UTP 0·8 μM, 25 nCi); the reaction mixture contained further 12 μg of DNA and 10 μg of enzyme protein. After incubation at 37°C for 10 min, 0·01 ml of bovine serum albumin (7·5 mg/ml) was added as a carrier followed by 1·0 ml of 5% (w/v) trichloroacetic acid. After standing for 30 min at 0°C the acid-precipitable material was collected on Whatman GF/C glass fibre filters and washed with 5 ml of 5% and 0·5% trichloroacetic acid and with 5 ml of an ether–ethanol mixture (1 : 1). After drying (10 min, 80°C) the filter was suspended in 0·2 ml of Soluene 300 for 30 min. The radioactivity was measured 60 min after addition of 5 ml of a scintillation cocktail (2 ml of Instafluor and 3 ml of toluene scintillator) in an Isocarb liquid scintillation counter.

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REFERENCES

1. Šebesta K., Horská K., Biochim. Biophys. Acta *209*, 357 (1970).
2. Šebesta K., Sternbach H.: FEBS (Fed. Eur. Biochem. Soc.) Lett. *8*, 233 (1970).
3. Klier M. A., Lecadet M.-M., deBarjac H.: C. R. Acad. Sci., Ser. D *277*, 2805 (1973).
4. Beebee T., Korner A., Bond R. P. M.: Biochem. J. *127*, 619 (1972).
5. Smuckler E. A., Hadjiolov A. A.: Biochem. J. *129*, 153 (1972).
6. Horská K., Šebesta K.: This Journal *46*, 673 (1981).
7. Farkaš J., Šebesta K., Horská K., Samek Z., Dolejš L., Šorm F.: This Journal *42*, 909 (1977).
8. Horská K., Kalvoda L., Šebesta K.: This Journal *41*, 3837 (1976).
9. Šebesta K., Horská K., Vaňková J.: This Journal *34*, 1786 (1969).
10. Kalvoda L., Prystaš M., Šorm F.: This Journal *41*, 800 (1976).
11. Píškala A., Šorm F.: Czech. 165 899 (1975).
12. Nishimura T., Shimizu B.: Chem. Pharm. Bull. *12*, 352 (1964).
13. Šebesta K., Horská K., Vaňková J.: This Journal *34*, 891 (1969).
14. Burgess R. R.: J. Biol. Chem *244*, 6160 (1969).
15. Sternbach H., Engelhardt R., Lezius A. G.: Eur. J. Biochem. *60*, 51 (1975).
16. Marmur J.: J. Mol. Biol. *3*, 208 (1961).

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