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Erythromycin Series. X. Inhibitory Activity of Several New Erythromycin Derivatives in Cell-Free Amino Acid Polymerization Systems*

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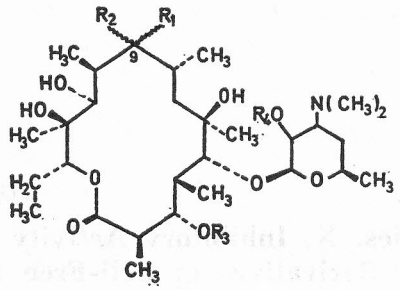
Erythromycin A (I), erythromycin A 9-oxime (II), 9(S)-erythromycylamine (V), and several new derivatives of these compounds, were assayed for their ability to inhibit the poly(A)-directed synthesis of polylysine and the poly(C)-directed synthesis of polyproline in cell-free systems from *Escherichia coli*. The rate of polypeptide synthesis was inhibited 50% by concentrations between 0.5 and 1.5 $\mu mol \cdot dm^{-3}$ of the eight examined compounds, in the following decreasing order of activity: methylsuccinate of V (VI), I, V, II, methylsuccinate of II (III), *p*-toluenesulfonyl-V (VII), *p*-acetylamino-benzenesulfonyl-V (VIII), and ethylsuccinate of I (IV). The derivative of VII lacking cladinose (IX) showed lower but still significant activity. Hence, none of the substitutions in the position 9 of the macrolide ring, present in these compounds, impairs the ability of I to bind the prokaryotic ribosome and inhibit its function, which is the basis for antibacterial activity of erythromycines.

Erythromycin belongs to the macrolide group of antibiotics. Its antibacterial activity is due to the specific inhibition of protein synthesis in prokaryotes (for reviews see Pestka[1,2]). The sensitivity to erythromycin is associated with the large ribosomal subunit[3,4]. Prokaryotic 70 S, but not eukaryotic 80 S ribosomes can strongly and specifically bind one molecule of the drug[5]. The specific binding site was traced to the isolated protein L 15 of the large ribosomal subunit, though the absolute requirement for an additional protein, L 16, was demonstrated in the reconstituted 50 S subunit[6]. Using radioactive erythromycin, the association constant of $7.2 \cdot 10^7$ $dm^3 \cdot mol^{-1}$ was determined for erythromycin : 70 S ribosome complex[1]. Pestka and his co-workers have subsequently assayed an impressive number of erythromycin derivatives (over fifty) for their ability to inhibit the binding of radioactive erythromycin to ribosomes[1,7], providing thus very accurate data about their activity. A good correlation was found between ribosomal binding of a derivative and its antibacterial activity[8].

* Taken in part from the M. Sc. theses submitted by P. M. and N. F. to Zagreb University



I $R_1, R_2 = =O$, R_3 = cladinosyl, $R_4 = -H$

II $R_1, R_2 = =N-OH$, R_3 = cladinosyl, $R_4 = -H$

III $R_1, R_2 = =N-O-CO-(CH_2)_2-COOCH_3$, R_3 = cladinosyl, $R_4 = -H$

IV $R_1, R_2 = =O$, R_3 = cladinosyl, $R_4 = -CO-(CH_2)_2COOC_2H_5$

V $R_1 = -NH_2$, $R_2 = -H$, R_3 = cladinosyl, $R_4 = -H$

VI $R_1 = -NH-CO-(CH_2)_2-COOCH_3$, $R_2 = -H$, R_3 = cladinosyl, $R_4 = -H$

VII $R_1 = -NH-SO_2-C_6H_4-CH_3$, $R_2 = -H$, R_3 = cladinosyl, $R_4 = -H$

VIII $R_1 = -NH-SO_2-C_6H_4-NH-CO-CH_3$, $R_2 = -H$, R_3 = cladinosyl, $R_4 = -H$

IX $R_1 = -NH-SO_2-C_6H_4-CH_3$, $R_2 = -H$, $R_3 = -H$, $R_4 = -H$

The availability of several new compounds of the erythromycin series (Chart 1), including *N*-(substituted-benzenesulfonyl) erythromycylamines[9], all displaying high antibacterial activity, prompted our investigation of their activity at the very site of the erythromycin action, i. e. on the prokaryotic ribosome. Information about this activity seemed particularly important for erythromycin derivatives which could be considered, at the same time, as derivatives of benzenesulfonylamine, and potentially capable of an additional or different biological activity. However, lacking radioactive erythromycin, we had to perform measurements of the inhibition of polymerization of amino acids in the *Escherichia coli* cell-free system. A kinetic approach to such a study has been recently developed[10].

MATERIALS AND METHODS

Escherichia coli MRE600 was grown to late exponential phase as described earlier[11]. The cells were broken, and pre-incubated 30 000 g supernatant prepared by the usual procedures[10,11]. All detailes of the cell-free polypeptide synthesis were recently described[10], except for the synthesis of polyproline. Here, the concentration of poly(C) was 0.16 mg · cm^{-3}, and [^{14}C]-L-proline (specific radioactivity 120 Ci · mol^{-1}) was used at 20 μmol · dm^{-3}; Mg^{2+} concentration was raised to 14 mmol · dm^{-3}, and the reaction was terminated by the addition of 20% trichloroacetic acid. All reaction times were 10 min. Though the rate of polymerization was not strictly linear after

the first five minutes, higher ammounts of the polypeptide product, accumulated in longer reactions, enabled more accurate measurements.

The synthesis of new erythromycin derivatives has been described previously[9].

RESULTS

We have studied the inihibitory effect of several derivatives of I in two cell-free systems: synthesis of polylysine, directed by poly(A), and synthesis of polyproline, directed by poly(C). The reaction conditions were chosen as to measure reaction rates, rather than final levels of polymerazition[10]. Under such conditions, I was found, at least formally, to be a competitive inhibitor with respect to the synthetic polynucleotide, which, in turn, stimulated the polypeptide synthesis in a non-linear, pseudo-cooperative manner[10]. Since we were unable to reconcile the first effect with the known mechanism of the action of I, and could not find a plausible explanation for the cooperative effect, we did not attempt to run detailed kinetic experiments with all derivatives we examined in the present research. Instead, the rate of polymerization of amino acids was measured as a function of the inhibitor concentration. Determining the 50%-inhibitory concentrations was sufficient for comparison of the activity of individual derivatives.

Dependence of the rate of polylysine synthesis on the inhibitor concentration, i, is shown in Figure 1. Rates of synthesis in the presence of inhibitor, v_i, are expressed as fractions of the rate in the un-inhibited control, v, which varied in individual experiments with various samples of cell-free extracts, from 0.15 to 0.22 nmol · cm^{-3} · min^{-1}. Corresponding »blank« values, obtained in samples with no poly(A) added, were subtracted from each point; they amounted to less than 10% of total incorporation. In this way, only the effect of inhibitors on the synthes s directed by poly(A) was measured.

Similar experiments were performed in the cell-free system synthesizing polyproline under the direction of poly(C). The rate of polymerization without inhibitor was about 40 pmol · cm^{-3} · min^{-1}. Typical inhibition curves are

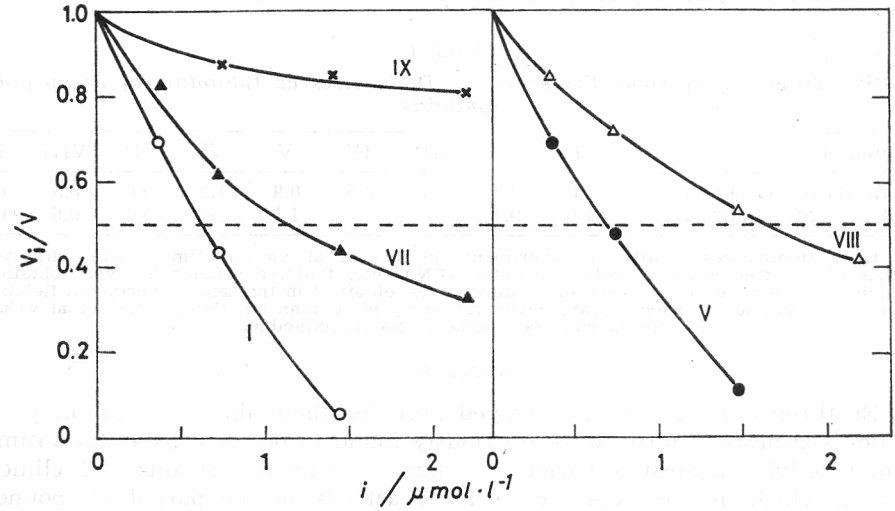


Figure 1. Inhibition of polylysine synthesis by several derivatives of erythromycin. i, concentration of inhibitor; v, reaction rate in un-inhibited control; v_i, reaction rate in the presence of inhibitor. Structures I to IX are shown in Chart 1.

shown in Figure 2. Though the synthesis of polyproline is somewhat more resistant to I and its derivatives than the synthesis of polylysine, relative efficiencies of individual derivatives agree very well in the two systems. We have also assayed our compounds for the inhibition of polyphenylalanine synthesis directed by poly(U). Poor inhibition was obtained in all cases, in agreement with earlier findings for the parent compound (I)[12]; hence, no meaningful comparison of derivatives could be performed in this system.

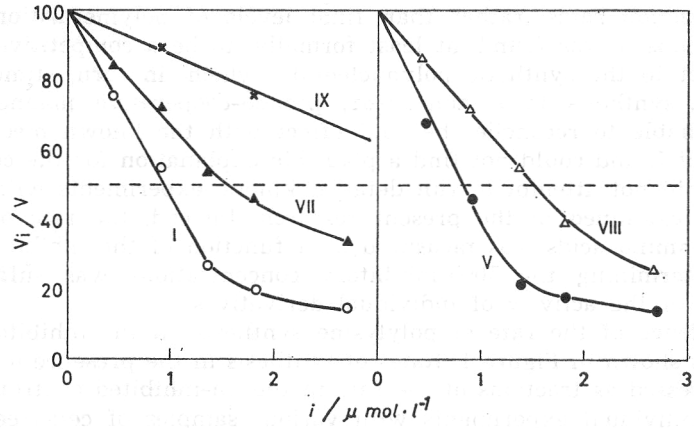


Figure 2. Inhibition of polyproline synthesis by several derivatives of erythromycin. For symbols see Figure 1.

Numerous experiments with each derivative in the other two systems, i. e. poly(A) and poly(C)-directed polymerizations, are summarized in Table I. Among the compounds examined, one (VI) was found more efficient than I, several were 50 to 90% as active as I (II, III, V, VII and VIII), while only the derivative lacking cladinose (IX) was considerably less active.

TABLE I

Relative Efficiency of Some Erythromycin Derivatives as Inhibitors of Polypeptide Synthesis

Compound	I	II	III	IV	V	VI	VII	VIII	IX
Synthesis of polylysine	1.0	0.9	0.7	0.5	0.9	1.3	0.6	0.5	0.1
Synthesis of polyproline	1.0	0.7	—	—	1.1	—	0.6	0.6	0.2

The table summarizes results of experiments performed at various times, using different batches of cell-free extracts, polynucleotides, tRNA, etc. Relative efficiencies were obtained by dividing i_{50} for I by i_{50} for each of its derivatives, obtained in the same experiment. Relative efficiencies, obtained in such a way, never varied by more than 0.1, though the actual values for i_{50} were occasionally less reproducible.

DISCUSSION

Erythromycin derivatives, assayed here for their ability to inhibit polypeptide synthesis *in vitro*, were previously found to posses substantial antimicrobial activity against a variety of standard bacterial strains and clinical isolates, which in some cases exceed the activity of the parent compound[9]. In spite of their importance for possible application, these data do not allow direct conclusions about structural requirements for the activity of I and

its derivatives on the bacterial target organelle, i. e. the ribosome. Permeability of bacterial membranes for individual compounds, as well as possible metabolic degradation or modification, can in fact determine the apparent activity. This is undoubtely true for I itself, which is inactive against intact Gram-negative bacteria, but active in cell-free extracts derived from them. Such a drawback does not exist when the interaction of radioactive I with purified ribosomes is studied *in vitro*. Hence, the competition of erythromycin derivatives with radioactive I was used to asses the importance of functional groups of I for its activity[1,7].

We have good reason to believe that our approach, in which we measure the inhibition of amino acid polymerization, gives equally meaningfull results. In a cell-free system, the permeability barrier does not exist, and the incubation time of several minutes is too short to allow appreciable metabolic modification. Nevertheless, there is an apparent discrepancy between our results and those obtained by the ribosome-binding method, when compounds tested by both methods (I, II and V) are compared. However, using reported association constants for these derivatives[7,13], one can calculate that, at the ribosome concentration we used (ca. 10^{-6} mol · dm^{-3}), the fraction of ribosomes not in complex with the inhibitors at their 50%-inhibitory concentrations, is close to one-half. Hence, the two methods are in good agreement, though the binding experiments are more sensitive.

Our data for new derivatives of I demonstrate that new substitutions in position 9 of the macrolide ring, both in oxime and amine, do not impair the activity of the drug. The substituents can vary from simple alkyles or aryles (cf. ref. 1) to methylsuccionyl (III, VI) and *p*-substituted benzenesulfonyl (VII, VIII). On the other hand, the same substitutions improve some other properties of the drug, e. g. acid stability, and more significantly, high level in serum for many hours after oral administration[9]. Esterification of the —OH group in the desosamine part of the molecule with ethylsuccinic acid leads to the very active compound IV; previous substitutions in the same position[1] resulted in somewhat decreased activity. The derivative of VII lacking cladinose (IX) shows lower, but significant activity. Some activity of I lacking cladinose has been previously reported[1], and it seems that substitutions in position 9, like in IX, at least cause no further inactivation, and possibly compensate for some loss of activity.

In conclusion, our experiments add several new compounds to the list of erythromycin derivatives with strong inhibitory activity on bacterial ribosome. Though the substitutions in compounds II to VIII do not improve the activity to any significant degree, they add some other favourable properties to the drug[9]. Therefore, these compounds may be of considerable practical interest.

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SAŽETAK

Eritromicinska serija. X. Inhibitorska aktivnost nekoliko novih derivata eritromicina u polimerizacijskim sistemima bez stanica

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Ispitana je sposobnost eritromicina A (I), eritromicin-A-9-oksima (II), 9(S)-eritromicilamina (V), te nekih derivata tih spojeva da inhibiraju sintezu polilizina upravljanu poliadenilnom kiselinom i sintezu poliprolina upravljanu policitidilnom kiselinom, u sistemu bez stanica iz bakterije *Escherichia coli*. Osam ispitanih spojeva inhibiralo je brzinu sinteze polipeptida 50% kod koncentracija između 0,5 i 1,5 $\mu mol \cdot dm^{-3}$, dajući ovaj redoslijed aktivnosti: metilsukcinat-V (VI), I, V, II, metilsukcinat-II (III), *p*-toluensulfonil-V (VII), *p*-acetilamino-benzensulfonil-V (VIII), etilsukcinat-I (IV). Derivat spoja VII bez kladinoze (IX) pokazao je manju, ali ipak značajnu aktivnost. Prema tome, nijedna supstitucija u položaju 9 makrolidnog prstena u proučenim spojevima ne poništava sposobnost spoja I da se veže na prokariotski ribosom i inhibira njegovu funkciju, što čini osnovu antimikrobne aktivnosti eritromicina.

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