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INFLUENCE OF FERROCENE AND ITS DERIVATIVES ON GROWTH OF *Escherichia coli* (ATTC 25922)*

This study is a continued investigation of the influence of ferrocene and its derivatives on trypsin activity. The goal was to examine the effect in vivo, by monitoring the growth of the bacteria Escherichia coli. The growth of the bacteria with the addition of ferrocene and derivatives of various concentrations was followed up spectrophotometrically, measuring changes in OD, correlating OD with the number of formed bacterial colonies and comparing the results as the mean generation time. The obtained results in relation to control experiments indicate a very strong inhibitory action of ferrocene and (dimethylaminomethyl)ferrocene, a medium or modest inhibitory effect of methyl 1'-acetamidoferrocene-1-carboxylate and benzyl 1'-methoxycarbonyl-1-ferrocenecarbamate; influence of benzyl 1'-carboxy-1-ferrocenecarbamate is negligible, while 1'-acetamidoferrocene-1-carboxylic acid causes the increase in the growth of Escherichia coli.

Key words: ferrocene; E. coli; growth inhibition.

Ferrocene is an organometallic compound which is the subject of many studies and its applications in chemistry are indeed vast: it is being used in electrochemical enzyme biosensors [1]; in catalysis of chiral compounds [2]; ferrocene based polymers have been shown to exhibit interesting electrochemical, optical, thermal, morphological, pharmacological and magnetic properties [3]; ferrocifens (ferrocene-substituted tamoxifen analogs) are proven to be not only more effective against breast cancer than tamoxifen, but also with less acute toxicity [4]; ferroquine (chloroquine analog, a drug used for malaria treatment) is much more potent and safer drug than chloroquine [4]; metallocenes of transition metals exhibit antitumor activity against sarcoma, melanoma, colon and lung carcinoma [5,6].

Papers describing experiments with *Plasmodium falciparum* (responsible for malaria) and drugs with incorporated ferrocene unit are suggesting that redox properties of ferrocene are crucial for antiplasmodial activity because they directly participate in processes that involve the quenching and generation of free radicals [7]. An alternative approach to the development of clinically useful protease inhibitors utilizes coordination chemistry of transition metal ions and their complexes [8]. It was found that five-coordinated Ti-sulfate completely and irreversibly blocks trypsin [9].

Since ferrocene, with its cyclopentadienyl rings, meets the necessary five-coordinate geometry, the subject of the investigation was whether ferrocene could also inhibit trypsin and if the introduction of the desirable functional groups in ferrocene derivatives could improve the interaction with the enzyme's active site (especially with the negatively charged carboxylate of the trypsin Asp 189 residue) [10,11]. It was found that ferrocene and investigated derivatives at greater concentrations inhibit trypsin, and the inhibition was, according to Dixon [12,13], uncompetitive. In order to examine the influence of ferrocene and its derivatives on trypsin *in vivo*, the growth of the bacteria *Escherichia coli* was studied, with the addition of different concentrations of these compounds, and the obtained results are presented here.

EXPERIMENTAL

Materials: ferrocene (Aldrich, 98%); (dimethylaminomethyl)ferrocene (Aldrich, 96%); ferrocene deri-

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vatives: methyl 1'-acetamidoferrocene-1-carboxylate, 1'-acetamidoferrocene-1-carboxylic acid, benzyl 1'--methoxycarbonyl-1-ferrocenecarbamate, benzyl 1'--carboxy-1-ferrocenecarbamate were obtained from the Faculty of Food Technology and Biotechnology, University of Zagreb [14] (numbered structures shown in Figure 1; the term „inhibitor #" is being used hereafter instead of proper chemical nomenclature in order to avoid the repetition of longer terms); Tris buffer (Lachema); nutrient broth powder (Institute for Immunology and Virology – Torlak, Belgrade); commercial culture *Escherichia coli* ATTC 25922 (Veterinary Institute "Vaso Butozan", Banja Luka).

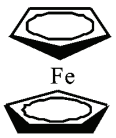
The growth media was prepared by dissolving 23 g of nutrient broth powder in 1 L of 0.1 M Tris-HCl buffer, pH 8.2. It was autoclaved at 120 °C; pH was re-checked and, if necessary, corrected. Buffered nutrient broth (10 ml) was inoculated from a slope of *E. coli* pure culture. After 24 h of incubation at 37 °C, 1 ml of the obtained bacterial suspension was transferred into 10 ml of nutrient broth. The previous step was repeated in the same way and thus obtained *E. coli* starting inoculum was used in further experiments.

The bacteria growth was monitored at three different concentrations of the inhibitors (concentrations given in Table 1). The exceptions were ferrocene (inhibitor 1) and inhibitor 5 (only one concentration used) because of the turbidity occurring when an alcoholic inhibitor solution at greater concentrations is mixed with the aqueous nutrient broth. The maximum working concentration for these two inhibitors was found to be 0.14 mM in the reaction mixture.

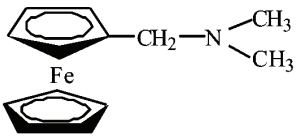
A series of probes was prepared. Probes were incubated at 37 °C and OD at λ = 600 nm was measured spectrophotometrically (UV-Vis Jenway 6305) every 60 min in total duration of 6 h. In order to avoid contamination during measurement, a separate probe was prepared for each measuring time (7 probes, from t_0 to t_6) and discarded after OD determination.

Series 1 consisted of 7 probes containing nutrient broth (5.0 ml), starting bacterial suspension (0.5 ml) and sterile distilled water (0.1 ml). These probes were used to monitor the bacterial growth without ethanol or an inhibitor added. Instead of sterile distilled water, the probes from the second series contained 96% ethanol in order to monitor the bacterial growth with the addition of ethanol (ferrocene and its derivatives were dissolved in 96% ethanol, being water insoluble). Series 3, 4 and 5 contained different concentrations of the inhibitors instead of distilled water or ethanol.

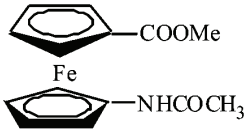
The volume ratio (nutrient media: bacterial suspension/ethanol) was obtained empirically, aiming to: select optimal A_{600} working range values; determine optimal alcohol volume so it does not prevent bacterial growth; account for solubility limits (some inhibitors are only slightly soluble in ethanol) and finally, to avoid turbidity due to mixing the inhibitor solution at greater concentrations with the aqueous media. The blank OD values (blank probe contained 5.5 ml nutrient broth and 0.1 ml of the inhibitor solution of



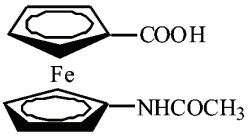
ferrocene 1



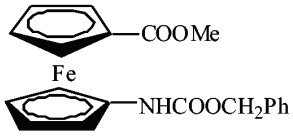
(dimethylaminomethyl)ferrocene 2



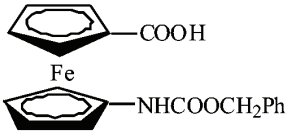
methyl 1'-acetamidoferrocene-1-carboxylate 3



1'-acetamidoferrocene-1-carboxylic acid 4



benzyl 1'-methoxycarbonyl-1-ferrocenecarbamate 5



benzyl 1'-carboxy-1-ferrocenecarbamate 6

Figure 1. Ferrocene and its derivatives.

various concentrations) were subtracted from the OD values obtained in the experiments with bacteria. The wavelength λ = 600 nm was selected because the nutrient broth has a very low OD value at this wavelength, and literary [15], the bacterial growth monitoring is usually performed at 550-600 nm working range. The bacterial growth inhibition experiments were performed using much greater inhibitor concentrations: 2, 5 and 10 times those used in the purified enzyme experiments [10,11], given the chemical and physical differences of the test systems (trypsin encapsulated in the bacteria versus a low concentration of free trypsin in the solution).

It should be noted that these experiments were monitoring *E. coli* growth trend in the presence of certain compound relative to growth trend in the absence of that compound because freshly prepared starting inoculum always has slightly different OD; the adjustment period and growth dynamics are not always the same, thus reflecting on the inhibition start. Statistically relevant number of tests can be performed using a multiple well UV microplate reader.

OD and cfu correlation: *E. coli* starting inoculum (0.5 ml) was transferred to 5.0 ml of nutrient broth (7 probes for t_0 to t_6, and 7 parallel probes); OD (A_0) was immediately determined for t_0 probe and at the same time by a standard dilution method inoculated media for cell count (three plates for each dilution) in order to correlate the initial cfu N_0 and OD. The same procedure was repeated in 60 min for t_1, and continued in the same way to t_6 (to cover the experiment duration of 6 h). Cfu were counted after 48 h of incubation at 37 °C and correlated to OD, as presented in Figure 2.

RESULTS AND DISCUSSION

Figure 3 refers to (dimethylaminomethyl) ferrocene 2 as an example of the bacterial growth inhibition. The logarithm of the cell count values resulted from the averaged parallel experiments, obtained at the same time, with the same initial culture.

The influence of ferrocene and its derivatives on *E. coli* growth can be expressed mathematically using mean generation time, T, or specific growth rate, k [16]. When bacterial growth is followed experimentally the results are usually expressed by a graph in which log N is plotted against time, as in Figure 3. For the exponential phase of the growth this results in a linear plot from which, based on similar triangles, the following equation is derived:

$$\frac{\log N - \log N_0}{t} = \frac{\log 2}{T} \quad (1)$$

where T is "the mean generation time" (the average generation time for all cells in the culture), and t is the time taken for the population to increase exponentially from N_0 to N.

The mean generation time values upon addition of the inhibitor (of different concentrations) and ethanol were determined (Table 1).

Figure 4 illustrates the influence of investigated inhibitors (including benzamidine, a standard trypsin inhi-

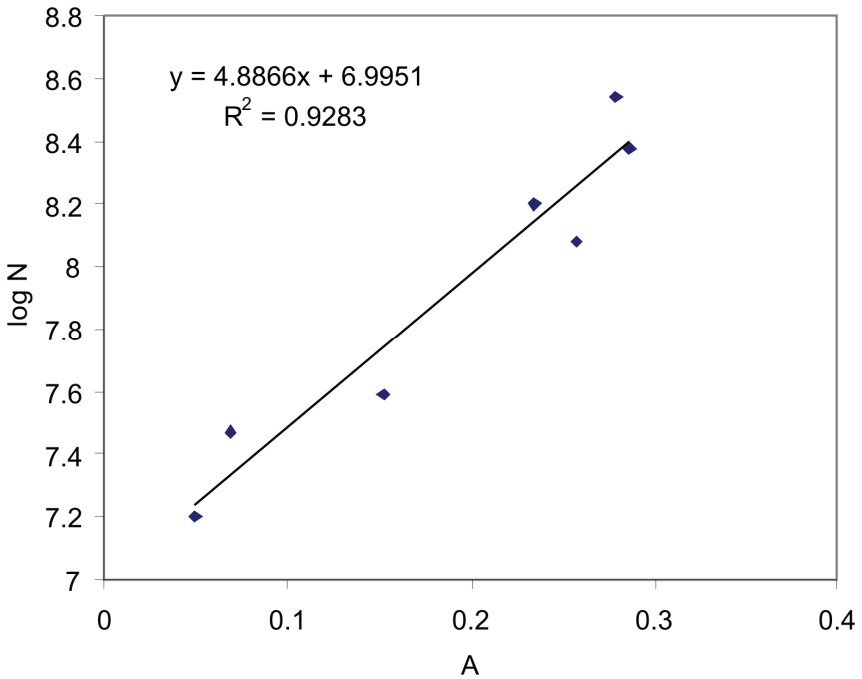


Figure 2. OD and log N correlation.

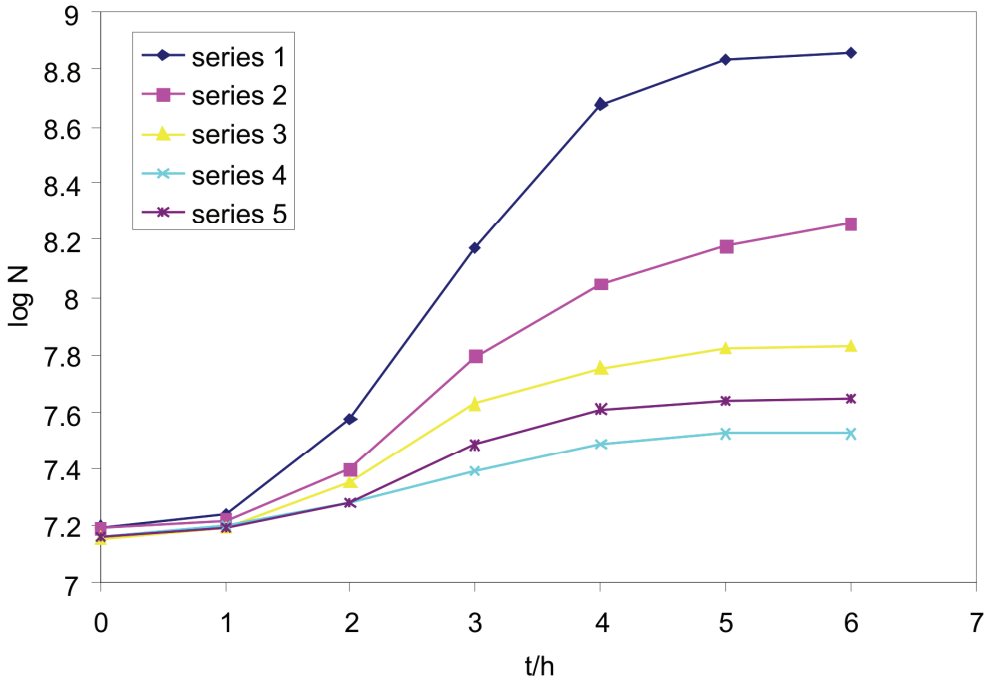


Figure 3. (Dimethylaminomethyl)ferrocene, logarithmic plot of bacterial cell number against time.

bitor), expressed as a percentage of growth reduction or increase relative to the control growth of *E. coli* with ethanol.

The obtained results indicate that (dimethylaminomethyl) ferrocene exhibits the most prominent bacterial growth reduction, especially at 0.3548 mM concentration (215.53% growth reduction, relative to ethanol). Ferrocene has a strong inhibitory effect too (106.52% growth reduction relative to ethanol) at its maximum working concentration of 0.1401 mM. Inhibitor 4 is the only inhibitor exhibiting the bacterial growth increase at all three concentrations (ranging from 9.58 to 14.52% compared to the ethanol control), and it can be regarded as a growth activator for *E. coli*. Benzamidine has a modest inhibitory effect (growth reduction ranges from 4.76 to 8.65% relative to the control), as well as the inhibitor 5 (growth reduction by 6.96% compared to the control). It seems that the inhibitor 6 has a negligible influence since percentages of growth reduction or increase ranged from 0.89 to 4.05%. The inhibitor 3 inhibits the bacterial growth at its highest concentration (32.04% relative to EtOH), while at lower concentrations its effect on *E. coli* growth can be regarded as negligible.

If these results are compared with the results obtained from the experiments with purified trypsin [10], it is obvious that (dimethylaminomethyl) ferrocene 2 in both cases exhibits the strongest inhibitory effect (K_i = 0.048 mM with purified enzyme). Structurally, this inhibitor is the only monosubstituted ferrocene derivative and the only tertiary amine. Standard trypsin inhibitors are mostly amines, the positively charged nitrogen atom of which fits into the bottom of the pocket that determines the specificity of trypsin for positively charged amino acid residues (Lys and Arg) and this is why ferrocene derivatives with amino functional group were selected.

The most common trypsin inhibition pathways are:

- *via* the interaction of the inhibitor's amino group and the free carboxyl group of Asp-189 at the bottom of the substrate binding pocket, as well as through many other hydrogen bonds between the main chain of trypsin and the inhibitor (not the case with the in-

Table 1. Mean generation time (min) for various inhibitors

c / 10^{-3} mol dm^{-3}	Inhibitor						Benzamid.
	1	2	3	4	5	6	
0.141	168.00	90.07	80.45	44.81	76.96	53.59	86.00
0.355	-	175.34	86.83	47.09	-	56.26	87.88
0.709	-	110.12	107.18	47.40	-	55.17	89.19
Ethanol	81.35	55.57	81.17	52.42	71.95	54.07	82.09

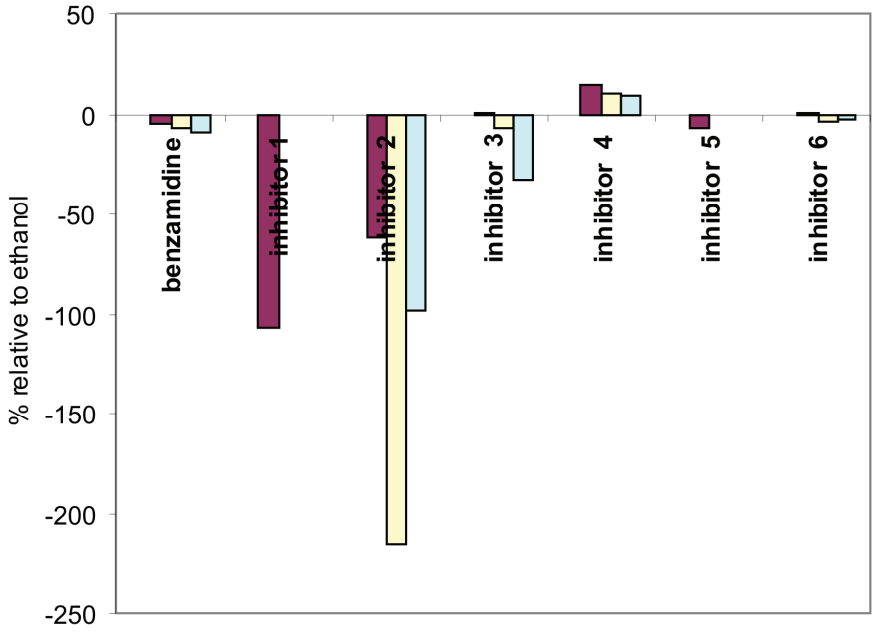


Figure 4. Influence of inhibitors on E. coli growth.

hibitor 2; as a tertiary amine, it is unable to engage in hydrogen bonding),

- if carbonyl group and surrounding atoms of the inhibitor fit snuggly in the oxyanion hole of the enzyme (steric factors may play a role with the inhibitor 2); maybe the fact that this is the only monosubstituted ferrocene derivative allows better fitting in the trypsin`s active site) and

- irreversible trypsin inhibition when the inhibitor reacts with the serine residue (certainly not the case here).

Benzamidine (standard trypsin inhibitor, K_i = 18 μM), inhibits the growth of *E. coli* very modestly, suggesting that good inhibition of purified enzyme does not imply good inhibition *in vivo*. In order to examine whether ferrocene and its derivatives specifically inhibit trypsin activity in the bacteria, it would be useful to experiment with the addition of these compounds to the *E. coli* lysate.

CONCLUSION

The investigation of the influence of ferrocene and its derivatives on trypsin activity *in vivo*, by *Escherichia coli* growth studies indicated that (dimethylaminomethyl)ferrocene had the strongest inhibitory effect on the bacterial growth. The most prominent inhibition *in vivo*, as well as with purified trypsin could be due to steric factors, since this compound is the only monosubstituted ferrocene derivative examined. In the future work, it would be interesting to investigate the effect ferrocene and its derivatives have on trypsin released in *E. coli* lysate.

Nomenclature

OD Optical density;
cfu Colony forming units;
A_{600} Absorbance measured at 600 nm.

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