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ENZYMATIC OXIDATION OF THIAZOLIDINES IN THE PRESENCE OF CYCLODEXTRINS: EVALUATION OF ANTIMICROBIAL ACTIVITY OF THE REACTANTS AND PRODUCTS

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Abstract: The antimicrobial activity of native and H_2O_2-oxidized thiazolidine derivatives was investigated using horseradish peroxidase (EC 1.11.1.7) in a 33% aqueous solution of dimethylsulfoxide (DMSO). To increase the solubility of thiazolidines, 4% solutions of α- and β-cyclodextrins were used. The presence of α-CD increased the solubility of the studied compounds by 1.25-2 times, and β-CD by 1.66-2.87 times. Antimicrobial activity was assessed using the MTT test on cultures of *Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa*. The native and oxidized forms of the compounds used in the work differed in their effect on microorganisms. It was concluded that the use of α- and β-cyclodextrins

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is promising in increasing the number of synthesized compounds, which indicates significant potential of cyclodextrins for the enzymatic synthesis of water - and DMSO-insoluble organic compounds.

Keywords: thiazolidine, horseradish peroxidase, cyclodextrins, enzymatic synthesis, antimicrobial activity.

Introduction

Cyclodextrins (CD) are cyclic oligosaccharides that have a shape similar to a torus, with an outer hydrophilic surface and an inner hydrophobic cavity, which gives them a unique structure for the formation of inclusion complexes. In these complexes, lipophilic (hydrophobic) molecules can enter the inner cavity of the CD without forming covalent bonds.[1] CDs are commonly used in the pharmaceutical industry for various purposes: they can increase the solubility of medicinal compounds in water,[2] improve the chemical and physical stability of compounds,[3] reduce local toxicity (with oral or topical application),[4] enhance delivery to and through biological membranes,[5] mask the unpleasant taste and odor of compounds,[6] and convert liquid compounds into amorphous powders.[7] The three main types of CDs are α-, β-, and γ-CD, which contain 6, 7, or 8 α-D-glucopyranoside residues, respectively, which are bound in positions 1,4.[8] The solubility of CD in water is lower than that of glucose and its di- and trisaccharides due to strong interactions between CD molecules within the crystal lattice. For instance, α-CD can dissolve in up to 14.5 g per 100 g of water, and β-CD can dissolve in up to 1.85 g per 100 g of water at a temperature of +20°C. However, as the temperature increases, the solubility of CD also increases. The low water solubility of CD results from the formation of hydrogen bonds between hydroxyl groups, and any substitution of these groups (even with hydrophobic fragments) leads to a significant increase in water solubility.[9] Unlike D-glucose and its linear oligosaccharides, CDs exhibit good solubility in both water and dimethyl

sulfoxide (DMSO), making them highly versatile for use in many applications. In fact, DMSO is often the preferred solvent for many organic compounds, and CDs' ability to increase the solubility of hydrophobic compounds in water can be extremely beneficial in synthetic works, particularly for fermentative reactions that require an aqueous medium and a neutral pH. It should be noted that some heterocyclic compounds, for example, 4-aminoantipyrine (4-amino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one) upon oxidation with hydrogen peroxide in the presence of peroxidase give a red-colored quinoneimine complex that is used to determine various biologically active compounds (such as uric and lactic acid, cholesterol, triglycerides, and glucose) in biological liquids.[10] This has led to speculation that the enzymatic reaction of hydrogen peroxide oxidation in the presence of horseradish peroxidase could also be used to oxidize a number of derivative thiazolidines. Our research has shown that this reaction can occur in water-DMSO solutions with DMSO concentrations up to 50%. Herein, we report the investigation of the effect of CD on the enzymatic oxidation reaction of thiazolidines with the participation of horseradish peroxidase in water-DMSO solutions. Additionally, we compare the antimicrobial properties of the original compounds with the products of their oxidation. These findings could have significant implications for the development of new methods for synthesizing and analyzing various organic compounds, including those with potential antimicrobial properties.

Results and Discussion

Carrying out the reaction in water-DMSO media

As thiazolidine and its derivatives are significantly more soluble in DMSO than in water, we aimed to investigate the reaction mechanisms of peroxidase in aqueous DMSO media at the outset of our research.

We discovered that peroxidase is only slightly soluble in pure DMSO, with a solubility of less than 0.1 mg/mL. However, when dissolved in 50% DMSO, its solubility increases to over 2 mg/mL, which is sufficient for the enzymatic reaction. Guaiacol was used as a donor of hydrogen ions, which is often used as a test compound in the study of peroxidase activity. To enable this reaction, we introduced an organic solvent and conducted the oxidation reaction of guaiacol by peroxidase both in an aqueous medium and in a DMSO medium, following the procedure outlined in:[11]

a) *In aqueous media*. To 0.5 mL of a 0.7% solution of guaiacol, 1.9 mL of dist. water, 0.1 mL of 0.02% peroxidase solution and 0.05 mL of 1.5% hydrogen peroxide solution were added. We observed the appearance of a brown color, which quickly turned into dark brown, and upon prolonged standing (30 min or more), the formation of a dark precipitate.

b) *In DMSO*. To 0.5 mL of a 0.7% solution of guaiacol in DMSO, 1.9 mL of DMSO, 0.1 mL of a 0.02% aqueous peroxidase solution, and 0.05 mL of a 1.5% hydrogen peroxide solution (the final concentration of DMSO in the reaction mixture was about 91%) were added. A brown coloration was observed, but it was much less intense than in previous experiment. Analyzing the results, we decided to investigate the rate of the enzymatic reaction in an aqueous medium and in media containing an organic solvent. For this purpose, a standard oxidation reaction of guaiacol with peroxidase was used. But the reaction was carried out in an aqueous medium (control) and water-DMSO solutions, with the content of 20% and 50% DMSO, respectively (Figure 1).

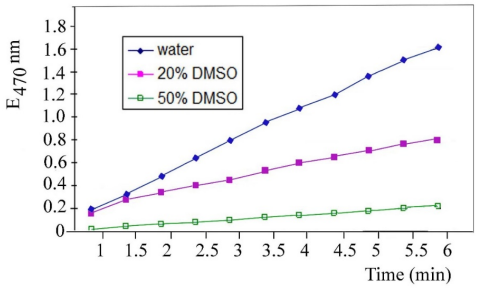

Figure 1. The rate of guaiacol oxidation by peroxidase in water-DMSO media.

Methodology for studying the reaction rate. Peroxidase solution was added to the reaction mixture containing solutions of guaiacol and hydrogen peroxide, and the extinction value was measured at 470 nm on a spectrophotometer. The optical density was monitored in the course of 6 min (Figure 1).

Calculations: the speed of the reaction in water was considered as 1 (or 100%).

The speed of reaction in 20% DMSO:

$$V=\frac{E_{470\,DMSO\,20\%}}{E_{470\,H_2O}}$$

where: $E_{470DMSO20\%}$ - extinction value of the reaction mixture in 20% DMSO at 470 nm;

E_{470H2O} - extinction value of the reaction mixture in water at 470 nm;

V = 0.46/0.8 = 0.58 (value at 3 min exposure).

That is, the reaction rate in 20% DMSO is 58% of the rate in water. Similarly, in 50% DMSO the speed of reaction would be 0.08/0.8 = 0.1 (or 10% of the rate in water). Thus, the reaction of oxidation of organic compounds by peroxidase is linear, directly proportional to the concentration of peroxidase, organic substrate, and the time of its implementation (at a constant concentration of hydrogen peroxide). The reaction proceeds most rapidly in an aqueous medium and slower in water-DMSO media. If the rate of the enzymatic reaction in an aqueous medium is taken as 1, then in 50% DMSO the reaction rate is approximately 10 times lower: and in 20% DMSO it is 1.7 times lower compared to the reaction in an aqueous medium. Although the reaction is slowed down in organic solvents, it is possible to achieve complete oxidation of the organic substrate by increasing the reaction time or increasing the concentration of peroxidase.

Study of the thiazolidine derivatives solubility in aqueous-DMSO media

The thiazolidines used in the work (Figure 2) were mostly yellow or orange compounds, soluble in 100% DMSO at a concentration of 0.3% to 10%.

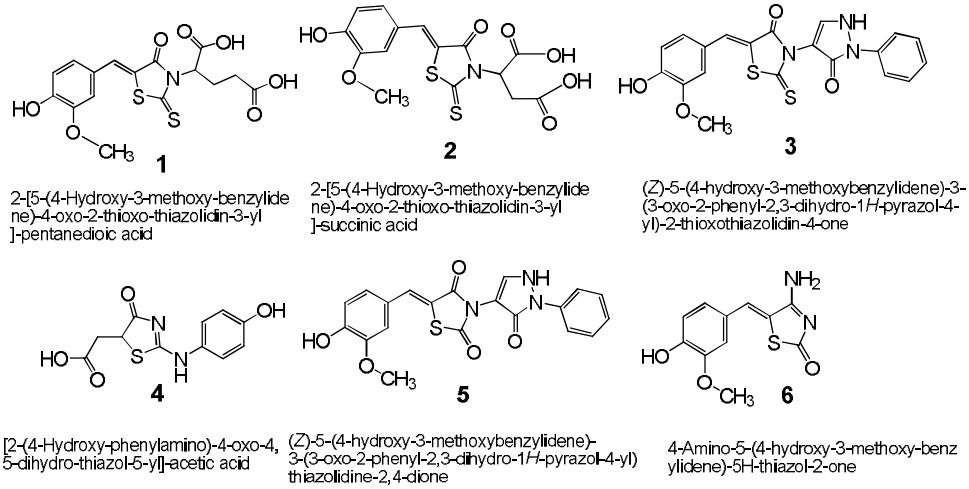

Figure 2. Structures of thiazolidine derivatives **1-6**.

In water-DMSO media, the solubility sharply decreases with increasing of water concentration (Table 1).

Table 1. Solubility of thiazolidines in aqueous-DMSO solutions.

Compound	Solubility (mg/mL), pH 7.0			
	100% DMSO	75% DMSO	50% DMSO	25% DMSO
1	220	120	12.5	1.0
2	135	22.5	12.5	2.5
3	122	12.2	8.1	4.0
4	160	65	27.5	20
5	115	10	1.3	0.9
6	120	20	3.2	0.16

For the investigated compounds, the increase in solubility is different, which most likely depends on its structure. Interestingly, in the presence of 4% α-CD and β-CD, the solubility of thiazolidines is considerably increased (Table 2). We used 33% DMSO for the reasons that such a concentration of DMSO in most cases does not have high antimicrobial activity and will allow a more objective assessment of the antimicrobial effect of thiazolidines solutions. This is also confirmed by a number of other researchers.[12]

Table 2. Effect of the presence of 4% α-CD and β-CD on the solubility of thiazolidines in 33% DMSO.

Compound	**Solubility (**mg/mL**), pH 7.0**				
	33% DMSO	33% DMSO + 4% α-CD	The multiplicity of increased solubility	33% DMSO + 4% β-CD	The multiplicity of increased solubility
1	2.2	4.4	2.0	5.8	2.63
2	9.6	12.0	1.25	13.6	1.42
3	3.5	5.0	1.42	7.0	2.0
4	20.4	26.7	1.35	34.0	1.66
5	0.8	1.2	1.50	2.3	2.87
6	1.86	3.8	2.02	4.3	2.31

Thus, the presence of 4% α-CD increases the solubility of thiazolidines by 1.25–2 times, and the presence of β-CD by 1.66–2.87 times. This means that the solubility of thiazolidines in the presence of cyclodextrins increases quite significantly, which can be important in the enzymatic oxidation of a large amount of thiazolidines, because it is possible to introduce into the reaction 1.25–2.87 times more substance and, accordingly, obtain several times more reaction product. The presence of

4% α-CD and β-CD does not affect the oxidation of thiazolidines with hydrogen peroxide in the presence of peroxidase. If it is necessary to remove α-CD and β-CD from the reaction mixture after the enzymatic reaction, then as our studies have shown, in many cases they can be precipitated with acetone. Cyclodextrins precipitate when two volumes of acetone are added, while thiazolidine and their oxidation products remain in solution under these conditions. If it is not possible to separate thiazolidine and cyclodextrins in this way, gel chromatography on a Sephadex G-10 column in an aqueous DMSO medium can be used.

Performing the oxidation reaction of thiazolidines with hydrogen peroxide in the presence of peroxidase in a water-DMSO medium

The oxidation reaction of thiazolidines with hydrogen peroxide in the presence of horseradish peroxidase was carried out similarly to the oxidation reaction of guaiacol, taking into account that thiazolidines are practically insoluble in water, but soluble in water-DMSO mixtures. The following technique was used: 0.4 mL of a solution of thiazolidine in 33% DMSO and 0.05 mL of a 3% solution of hydrogen peroxide in 33% DMSO and 0.05 mL of a solution of horseradish root peroxidase (2 mg/mL) were mixed. The control sample was prepared by adding 0.1 mL of distilled water to 0.4 mL of a solution of thiazolidine in 33% DMSO. After 30 minutes of incubation of the reaction mixture, the UV absorption spectrum of the starting material and the UV absorption spectrum of the reaction product were measured (Fig. 3). For example, Figure 3 shows the UV-visible absorption spectra of compound **1** before and after the reaction.

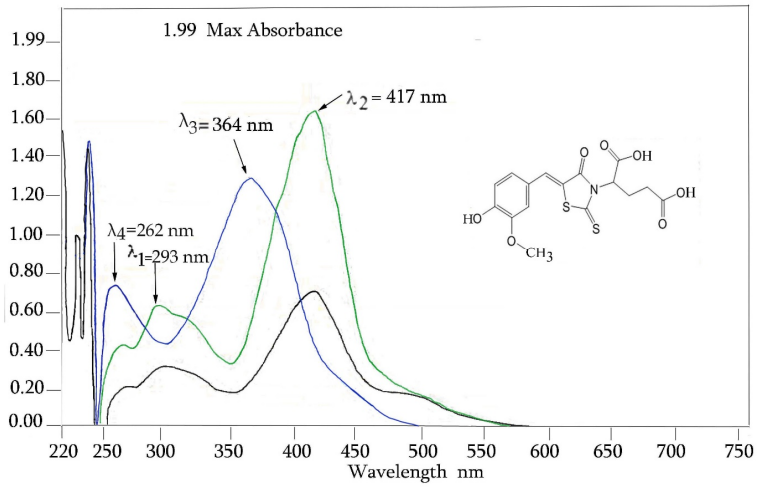

Figure 3. Examples of UV-Vis spectra of a saturated solution of compound **1** in 33% DMSO (black line); the UV-spectrum of compound **1** in the presence of 4% α-CD (green line); and the UV spectrum in the presence of 4% β-CD after reaction with horseradish peroxidase (blue line).

The increase in the extinction value in the presence of 4% β-CD compared with 4% α-CD is explained by the greater solubility of this compound in the presence of β-CD. In this particular case, after carrying out the enzymatic reaction, the UV-spectrum of the obtained compound is shifted to the UV zone (blue line). The adsorption maxima of the oxidized compound are observed at 364 nm and 262 nm against 417 nm and 293 nm in the unoxidized compound. Similarly, for compound **2**, (Figure 4) differing from the previous one in the absence of CH_2-CH_2 groups in the side chain, an increase in solubility in the presence of 4% α-CD and β-CD leads to an increase in the extinction of the original compound at 412 nm. After the enzymatic oxidation of this substance, the UV- spectrum of the obtained substance shifted to the UV zone with a maximum at 402 nm (purple line).

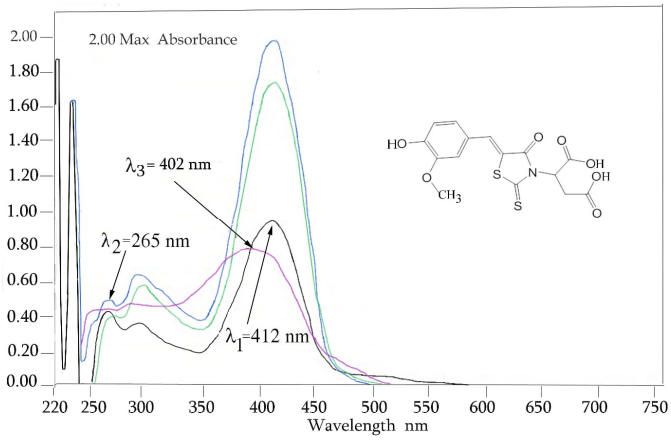

Figure 4. UV-Vis spectra of a saturated solution of compound **2** in 33% DMSO in the presence of cyclodextrins before and after oxidation (black line - 0.96 mg/mL compound **2**, control; green line - 1.2 mg/mL compound **2** in the presence of 4% α-CD; blue line - 1.3 mg/mL compound **2** in the presence of 4% β-CD; purple line -2 mg/mL of compound **2** in the presence of 4% α-CD after the oxidation reaction).

For compound **3**, an increase in solubility was also observed in the presence of 4% α-CD and 4% β-CD, which was accompanied by a corresponding increase in the extinction value at 509 nm. After oxidation of the compound, one of the maxima is shifted from 509 nm to 372 nm, while the second at 269 nm strongly increases (Figure 5).

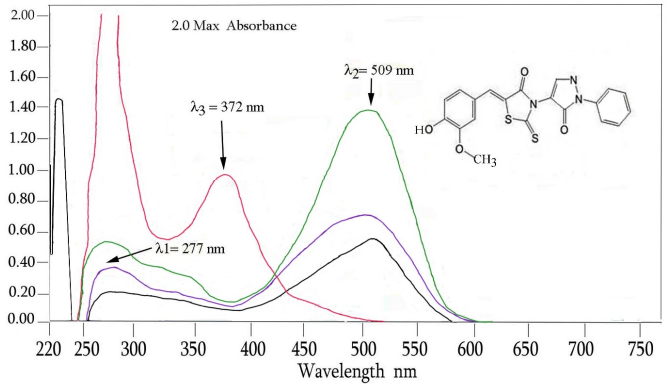

Figure 5. UV absorption spectra of a saturated solution of compound **3** in 33% DMSO in the presence of cyclodextrin before and after oxidation (black line – 3.5 mg/mL compound **3**, control; purple line – 5 mg/mL compound **3** in the presence of 4% α-CD; green line – 7.0 mg/mL compound **3** in the presence of 4% β-CD; red line –3.5 mg/mL of compound **3** in the presence of 4% α-CD after the oxidation reaction).

Unfortunately, in case of compounds **4-6** the formation of a precipitate during the reaction was observed and the corresponding reaction mixtures were not further investigated.

In case of compounds **1** and **2**, the reaction products were identified by the value of molecular ions in the chromato-mass spectra for the oxidation products of compounds **1** and **2** (m/z = 380.0, $[M-H]^+$ and 366.0, $[M-H]^+$, respectively). According to the spectral data, it was found that oxidation with hydrogen peroxide in the presence of the peroxidase enzyme results in the transformation of 2-thioxo-4-thiazolidinine derivatives into the corresponding 2,4-thiazolidinedione derivatives (Figure 6).

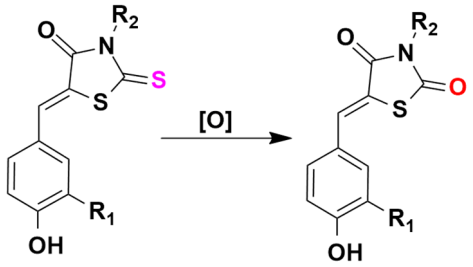

Figure 6. Possible oxidation of compound **1-3.**

Study of the antimicrobial action of thiazolidines and their oxidation products

The next step in our study was to study the antimicrobial activity of thiazolidines and their oxidation products. The study was carried out directly with the reaction mixture containing the corresponding thiazolidine or its oxidation product in the presence or absence of 4% α-CD or β-CD. To inactivate horseradish peroxidase, the reaction mixture was kept in a boiling water bath for 10 minutes before the experiments. Antimicrobial activity testing was performed on *Staphylococcus aureus (S. aureus)*, *Escherichia coli (E. coli)* and *Pseudomonas aeruginosa (P. aeruginosa)*.

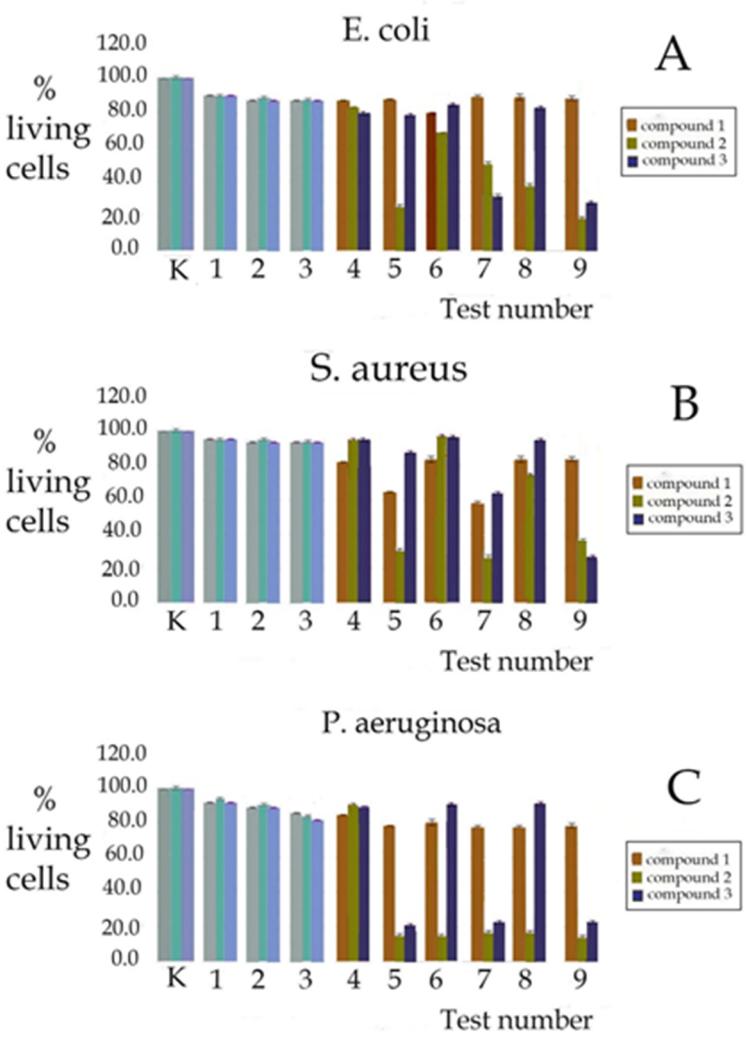

Figure 7. Effect of native and oxidized compounds on the growth of *E. coli* (A), *S. aureus* (B) and *P. aeruginosa* (C) in the presence and absence of α-CD or β-CD. **Notes:** K – cultural environment; 1 – 33% DMSO solution; 2 – 4% α-CD solution in 33% DMSO; 3 – 4% β-CD solution in 33% DMSO; 4 – a solution of compounds **1-3** in 33% DMSO (control 1); 5 – a solution of compounds **1-3** in 33% DMSO after oxidation (experiment 1); 6 – a solution of compounds **1-3** in 33% DMSO with 4% α-CD (control 2); 7 – solution of compounds **1-3** in 33% DMSO with 4% α-CD after oxidation (experiment 2); 8 – solution of compounds **1-3** in 33% DMSO with 4% β-CD (control 3); 9 – a solution of compounds **1-3** in 33% DMSO with 4% β-CD after oxidation (experiment 3).

As it can be observed from Figure 7, the growth of all studied microorganisms in the presence of 33% DMSO was slightly inhibited, by approximately 5-10% (Fig. 7 A, B, C, column 1). The addition of 4% α- and β-CD to 33% DMSO did not increase the growth inhibition of *S. aureus* and *E. coli* but increased the growth inhibition of *P. aeruginosa* by ≈10% (Figure 7 A, B, C, columns 2 and 3). It is possible to note a slight difference in the effect on microorganisms of saturated solutions of non-oxidized compound **2** and **3** in 33% DMSO (Figure 7, column 4) in comparison with the effect of 33% DMSO in the control (Figure 7, column 1). Only compound **1** was 15% stronger action on *S. aureus*. Also, no significant effect of unoxidized compound **1** on the growth of *E. coli, S. aureus* and *P. aeruginosa* in concentrations from 0.22% to 0.58% (Figure 7 columns 4, 6 and 8) and its oxidized product (Figure 7 columns 5, 7 and 9). Unoxidized compound **2** at a concentration of 0.96% inhibited the growth of *E. coli* by approximately 18% (Figure 7, column 4). Increasing their concentrations due to the presence of α- and β-CD to 1.20% and 1.30%, respectively, led to a greater inhibition of *E. Coli* growth by 12% and 44%, respectively (Figure 7, columns 6 and 8). After oxidation of compound **2** in the presence of peroxidase, the growth of *E. coli* was inhibited by approximately 50%. Increasing the concentration of oxidized product to 0.50% and 0.70% using 4% α- and β-CD in 33% DMSO resulted in inhibition of *E. coli* growth by 62% and 82%, respectively (Figure 7, columns 5, 7 and 9). The inhibitory effect of the unoxidized and oxidized form of compound **2** on *P. aeruginosa* is well expressed and approximately the same (at the level of 85-80%).

An increase in the concentration of unoxidized compound **3** from 0.35% to 0.50% and 0.70%, respectively, in the presence of α- and β-CD did not reveal antimicrobial activity of this compound (Figure 7, columns 4, 6 and 8). At the same time, after its oxidation, an increase in the antimicrobial activity against all tested microorganisms was noted. A

particularly strong increase in antimicrobial activity was observed for *P. aeruginosa* (Figure 7, columns 5, 7 and 9). Thus, compound **2** from the three studied derivatives showed the greatest antimicrobial activity. This effect was enhanced after enzymatic oxidation of the compound, especially for *E. coli* and *S. aureus*.

Experimental

Materials

The chemicals such as α- and β-CD, DMSO were purchased from Aldrich (Germany). All the purchased reagents were used without further purification. Distilled water was utilized in all performed experiments and stock solutions preparation. Saburo's medium for the cultivation of microorganisms was purchased from "Sistema Optimum" (Lviv, Ukraine). Peroxidase of horseradish roots (*Armoracia rusticana*) (EC 1.11.1.7) of medium degree of purification (RZ = 1.5) was obtained from SPK Lektinotest (Lviv, Ukraine). Thiazolidine derivatives were synthesized in accordance with previous reports (Fig. 1).[13-15]

Solubility investigations of thiazolidines

To study the solubility of thiazolidines in DMSO and water-DMSO mixtures, the following procedure was used: to an exact amount of thiazolidine, 100% DMSO in portions was added while observing the dissolution of the compound at room temperature. If the compound did not dissolve completely, the mixture was heated in a water bath to +70°C, then cooled to room temperature and observed for 10 minutes. If the compound did not precipitate during this time, its solubility in DMSO was recorded. Next, distilled water in portions was added to achieve concentrations of 75%, 50%, and lower, and monitored if the solutions become cloudy. Then, the solutions were heated to +70°C and observed the dissolution of the precipitate. If necessary, an aqueous solution of DMSO was added in

appropriate concentrations in portions until complete dissolution was achieved. After cooling, the concentration was adjusted by adding the required amount of solvent. We repeated these steps to determine the solubility of thiazolidines when dissolved in a mixture containing 4% α- or β-CD.

Oxidation of thiazolidines in the presence of peroxidase

To a test tube, 0.4 mL of thiazolidine solution, 0.05 mL of 3% hydrogen peroxide solution, and 0.05 mL of 0.2% horseradish root peroxidase solution were added. All compounds were prepared in water-DMSO basis and incubated at pH 5.5-7.5 with or without 4% α-CD or β-CD at +37°C in a water bath. After incubating the reaction mixture for 15 minutes, the optical density was measured using a NanoDrop 1000 spectrophotometer 3.8.1 (USA) in the range of 220–750 nm. In parallel, the optical density of the thiazolidine solution was determined in the absence of peroxidase and hydrogen peroxide solutions, and the volume of the corresponding water-DMSO mixture was added instead.

Determination of the viability of microorganisms using the MTT assay

To assess the antimicrobial activity, we employed the MTT assay, which involves the reduction of yellow tetrazolium salt (MTT) to purple formazan. The number of viable cells was determined by measuring the absorbance at 570 nm. The method of determination was described earlier.[16]

Determination of value of molecular ions

Determination of value of molecular ions carried out using the device Agilent 1260 Infinity II with single-quadrupole mass-selective detector Agilent 6125 (Agilent Technologies, Santa Clara, CA, USA.

Conclusion

Cyclodextrins possess unique properties that make them ideal for use in enzymatic oxidation reactions of organic compounds with hydrogen peroxide. One of their most notable properties is their ability to increase the solubility of organic compounds in water. Additionally, cyclodextrins are highly soluble in both water and dimethyl sulfoxide, which is particularly useful in studying the biological effects of many organic compounds, for which DMSO is often the only solvent available. In our research, we observed that the presence of 4% α-CD in solutions increased the solubility of thiazolidines by 1.25-2 times, while β-CD increased the solubility by 1.66-2.87 times. This allowed us to examine the antimicrobial activity of native and oxidized compounds across a wider range of concentrations. Since the solubility of these compounds in aqueous DMSO solutions is generally low, achieving complete inhibition of microbial growth has been difficult. However, using α- and β-CD, we were able to increase the solubility of the studied compounds and identify potential antimicrobial agents. Overall, the unique properties of cyclodextrins make them valuable in enzymatic oxidation reactions and in the study of organic compounds biological effects. Hence, when using α- and β-CD, it was possible to increase the solubility of the studied compounds and to identify compounds that could potentially be of interest as antimicrobial compounds.

Our research using peroxidase as a catalyst actually falls within the framework of a new integrative scientific direction - "green" or "ecologically rational chemistry".[17]

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