Study on Antibacterial Activity of 2-Methyl-3-(methylthio) pyrazine Against Three Strains of Spoilage Bacteria

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Abstract Previous studies have shown that glycine and proline are pharmacophores that display antibacterial activity. In the present study, glycine and proline were derivated to diketopiperazine compounds by chemical synthesis method, and their antibacterial activities were evaluated by three strains of spoilage bacteria, *Escherichia coli*, *Pseudomonas aeruginosa* and *Shewanella putrefaciens*, and the relationship of their antibacterial activities and structures was also investigated. Uv-vis spectrophotometry was used to determine the growth curves of three kinds of active indicator bacteria. Minimum inhibitory concentration (MIC) was determined by micro broth dilution method. The results showed that 2-methyl-3-(methylthio) pyrazine had significant antibacterial activity against three strains of the bacteria, and its MIC was 1.25%. It indicates 2-methyl-3- (methylthio) pyrazine has the potential to be developed as a kind of preservative in future.

Key words 2-Methyl-3-(methylthio) pyrazine; Growth curve; Minimum inhibitory concentration

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Since the 19th century, the advantages of microbial-derived preservatives, such as safety, broad spectrum, high efficiency, no toxic side effects, and strong antibacterial properties, have aroused widespread interest among scientific researchers[1]. Lactic acid bacteria have unique advantage and are defined as food-grade microorganisms, so they are considered to be the most valuable in developing potential preservatives[2]. At present, although there are a large number of studies on the classification and purification of antibacterial products against lactic acid bacteria, only one active molecule, nisin, is widely used commercially, while its antibacterial function on Gram-negative bacteria is extremely poor, which greatly limits its application in the field of antisepsis and preservation[3].

In this study, a small molecule cyclodipeptide compound with antibacterial activity obtained from the fermentation broth of *Bacillus coagulans* was structurally modified to study the antibacterial activity of diketopiperazine compounds. Chemical synthesis is a good way to modify the structures of small molecules for further activity screening. In the present study, glycine and proline were derivated to diketopiperazine compounds by chemical synthesis method, and their antibacterial activities were evaluated by three strains of spoilage bacteria, *Escherichia coli*, *Pseudomonas aeruginosa* and *Shewanella putrefaciens*, and the relationship of their antibacterial activities and structures was also investigated.

Materials and Methods

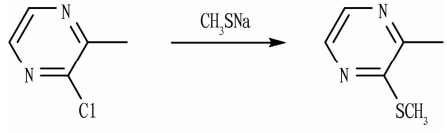
Experimental reagents

2-Methylpyrazine, cuprous bromide, sodium methyl mercaptide were purchased from Shanghai Bide Pharmaceutical Tech Co., Ltd. (Shanghai, China). Broth medium was purchased from Haibo Biotech Co., Ltd. (Qingdao, China). All other reagents were analytically pure.

Organic synthesis

First, 9.41 g (0.1 mol) of 2-methylpyrazine was dissolved in 182.30 g of 30.0% hydrochloric acid (containing 1.5 mol of hydrogen chloride). With stirring, the temperature was controlled to 0 - 15 ℃, and 25.0% hydrogen peroxide solution (16.32 g) was added dropwise. After the dropwise addition was completed, the reaction temperature was controlled to 0 - 15 ℃ to allow the reaction system to undergo a chlorination reaction. During the reaction process, samples were taken regularly and detected with a high-performance liquid chromatograph. When 2-methyl-3-chloride in the reaction system was detected, and when the mass of pyrazine accounted for 73.5% of the total organic matter mass in the reaction system, 17.21 g (0.12 mol) of copper bromide was added to terminate the reaction, and a liquid containing 2-methyl-3-chloropyrazine was obtained[4].

Subsequently, 63.78 g of methylene chloride was added to the material liquid. After 2 h of extraction with stirring and 1 h of standing, the organic phase was taken and cooled to the range from -15 to -5 ℃, so that the solid matter was fully separated from the organic phase, and 8.36 g of the fixed substance obtained was 2-methyl-3-chloropyrazine. After calculation, the yield was 65.5%. The compound structure was analyzed by 1H NMR. After determination, the content was 97.8%.



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Sodium methyl mercaptide was added to the reaction kettle dropwise, and the temperature was controlled to 80 – 95 ℃ to complete the reflux reaction for 2-methyl-3-chloropyrazine. After the reaction was completed, the reaction system was cooled down, the methyl tert-butyl ether extract was kept while discarding the aqueous phase. Next, the organic phase was returned to the kettle, and hydrochloric acid solution was added dropwise into the kettle to form a salt. After standing for separation, the lower aqueous phase was neutralized with alkali solution to pH = 8, and distillation was performed to extract with DCM and concentrate it[5]. The target product 2-methyl-3-(methylthio)pyrazine was obtained with a yield of 92%.

Bacteria culture and activation

E. coli., *P. aeruginosa*, and *S. putrefaciens* strains were provided by Biological Research Center, Huanghe Science and Technology University, and cultured on solid AGAR medium. The strains were activated by inoculating the colonies to slope AGAR culture-medium twice, and then incubated at 37 ℃ and subcultured for 24 h.

Determination of growth curve for strains

Broth medium was used to detect the growth curve for the three strains. The bacteria on the inclined medium were collected with normal saline and prepared into a bacterial suspension with OD_{600} of 0.5. Next, 0.5 ml of bacterial suspension was diluted with broth medium and cultured in an oscillating incubator. The bacteria on the slope medium were collected with normal saline and prepared into a bacterial suspension with OD_{600} of 0.5. Next, 0.5 ml of bacterial suspension was diluted with broth medium and cultured in an oscillating incubator at 37 ℃ and in rotation speed of 180 rpm. OD_{600} absorbance of each bacterial suspension was determined at 5, 6, 7, 8, 9, 10 and 20 h, respectively. The growth curve was drawn with time as the horizontal coordinate and OD_{600} absorbance as the vertical coordinate[6].

Determination of antibacterial activity

Screening of solvents The bacterial colonies (*E. coli*, *P. aeruginosa*, *S. putrefaciens*) on the inclined surface were washed with sterilized normal saline to prepare the bacterial suspension. The absorbance of the bacterial suspension in 600 nm was diluted by normal saline to 0.5.

2-Methyl-3-(methylthio) pyrazine was a liquid drug. Its dissolution effect was investigated with DMSO and tween 80, and its bacteriostatic effect on three indicator bacteria was investigated through bacteriostatic zone experiment.

The prepared bacterial suspension was diluted by 10 times, and 100 μl of bacterial solution was pipetted for coating. Next, 7 μl of tween 80 with different concentration gradients were pipetted, and then dripped onto a piece of aseptic filter paper 6 mm in diameter and cultured in a constant temperature incubator at 37 ℃ for 24 h, respectively. According to above steps, three parallel experiments were done to observe whether a bacteriostatic zone was generated.

Drug sensitivity test Drops of 2-methyl-3-(methylthioyl) pyrazine diluted by 7 μl of tween 80 were added to a sterile filter paper with a diameter of 6 mm. Tween 80 of different concentrations was used as blank control and norfloxacin of saturated concentration was used as positive control. And the bacteria were cultured at 37 ℃ for 24 h. Three experiments were done in parallel to measure the mean diameter of antibacterial zone[7-8].

Determination of minimum inhibitory concentration

The mother liquor of 2-methyl-3-(methylthiophenyl) pyrazine was prepared with the concentration of 25% with Tween 80, and the compounds were diluted with broth medium to 12.5%, 6.25%, 3.125%, 1.562 5%, 0.781 25%, 0.390 625%, 0.195%, respectively.

Sterilized conical bottles with a volume of 25 ml were used for the determination of MIC concentration. The system consisted of broth medium 9.4 ml, bacterial solution 100 μl and compound solution 500 μl, and the blank group (sterile solution) and control group (no compound) were set up at 37℃, 180 r/min. The culture of *E. coli* and *P. aeruginosa* was 4 h, and the culture of *S. putrefaciens* was 5 h. The absorbance of OD_{600} was determined by an ultraviolet spectrophotometer[9].

Results and Discussion

Structural characterization

The structure of organic synthesis product was analyzed by NMR and the result is shown in Fig. 1 and it was determined as 2-methyl-3-(methylthioyl) pyrazine(Fig. 2).

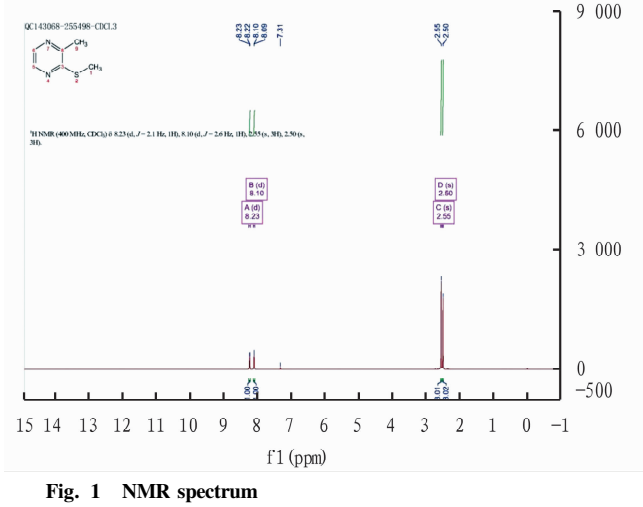


Fig. 1 NMR spectrum

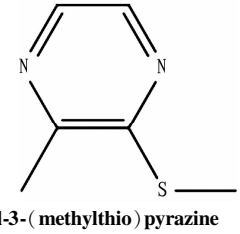


Fig. 2 2-Methyl-3-(methylthio)pyrazine

Drawing of growth curve

The results of the growth curves of the three strains are shown in Fig. 3 – Fig. 5. As can be seen from the figures, the logarithmic growth period of *E. coli* and *P. aeruginosa* was 2 – 6 h, and

that of *S. putrefaciens* was 4 – 6 h. The growth curve initially rose linearly and gradually flattened after 6 h. Bacteria in the logarithmic growth phase are more sensitive to changes in external environmental factors, so it is best to choose bacteria in the logarithmic growth phase when studying the inhibitory effect of drugs on bacteria.

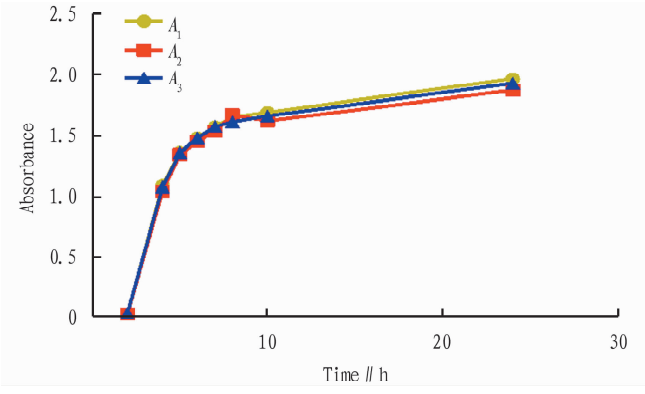


Fig. 3 Growth curve of *E. coli*

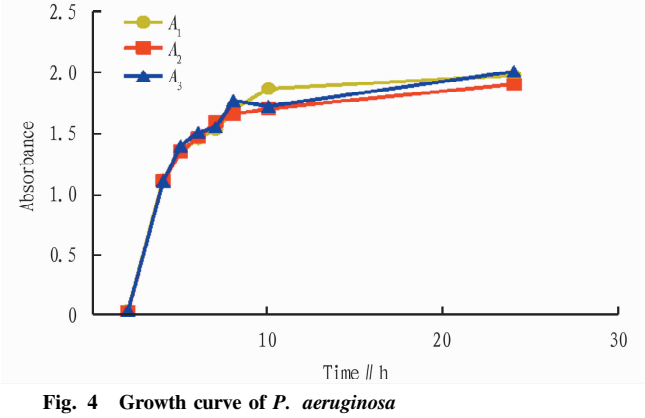


Fig. 4 Growth curve of *P. aeruginosa*

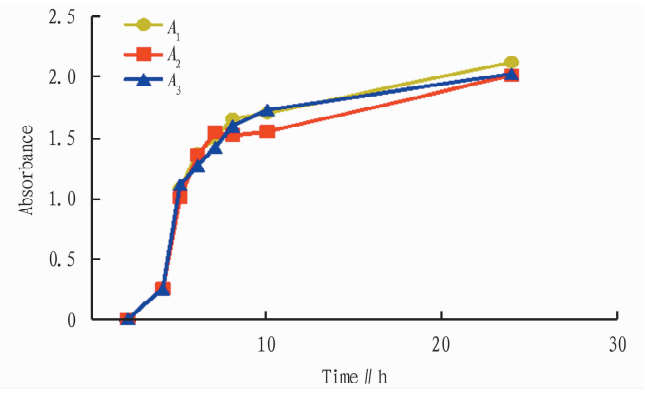


Fig. 5 Growth curve of *S. putrefaciens*

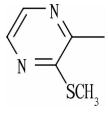
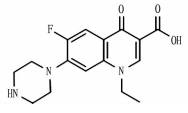
Screening of solvents

The dissolving effect of 2-methyl-3-(methylthiophenyl) pyrazine was very poor when DMSO was used as solvent, and the dissolving effect of tween 80 was better. The inhibition zone test showed that tween 80 had no inhibitory effect on the three indicator bacteria. As can be seen from Table 1, 2-methyl-3-(methylthio) pyrazine had obvious antibacterial effects on *E. coli*, *P. aeruginosa* and *S. putrefaciens*.

Determination of minimum inhibitory concentration

The minimum inhibitory concentration of 2-methyl-3-(methylthioyl) pyrazine against three putrefaction strains was analyzed by microbroth dilution method[10], and the results are shown in Fig. 6 – Fig. 8. It can be seen from the figures that with the increase of drug concentration, the antibacterial effect on the three putrefaction strains was gradually enhanced, and the bacterial growth in the test tube of the control group tended to be normal, and the minimum antibacterial concentration of 2-methyl-3-(methylthioyl) pyazine on *E. coli*, *P. aeruginosa* and *S. putrefaciens* was 1.25%.

Table 1 Antibacterial activity of drugs indicated by diameter of the antibacterial zone

Compound	Structure	Concentration	Anti-bacteria activity	Diameter // mm		
				E. coli	*P. aeruginosa*	*S. putrefaciens*
2-Methyl-3-(methylthio) pyrazine		100.0 ml/ml	+	15.1 ± 0.8	13.3 ± 1.1	12.2 ± 0.9
Norfloxacin		0.7 mg/ml	+	24.0 ± 1.0	23.7 ± 2.1	20.8 ± 1.9

In this study, a small molecule cyclodipeptide compound with antibacterial activity obtained from the fermentation broth of *Bacillus coagulans* was structurally modified to study the antibacterial activity of diketopiperazine compounds[11]. The aim was to further investigate the antibacterial activity of 2-methyl-3-(methylthio) pyrazine against putrefaction bacteria, in order to provide research basis for the development of the subsequent compound 2-methyl-3-(methylthio) pyrazine as an antiseptic preservative and produce social and economic benefits. *Bacillus coagulans* has been widely used as a bacteriostatic agent, which has the advantages of high temperature resistance and easy storage, *etc.* 2-Methyl-3 -(methylthioyl) pyrazine is obtained by structural modification from the compound isolated from its metabolites, and few reports have been reported[11-12]. Tween 80 is a non-ionic surfactant, which can be used as emulsifier, dispersant, solutizer or stabilizer, and is widely used in medicine and food, *etc.* Its content ranges from less than 1% to 12%[13], and the minimum antibacterial concentration in this study was only 3.75%, indicating that it could be used

safely. The results showed that the derivatives of cyclic dipeptide compound were obtained by chemical synthesis with amino acid as the core skeleton, and showed good antibacterial activity with MIC value of 1.25%.

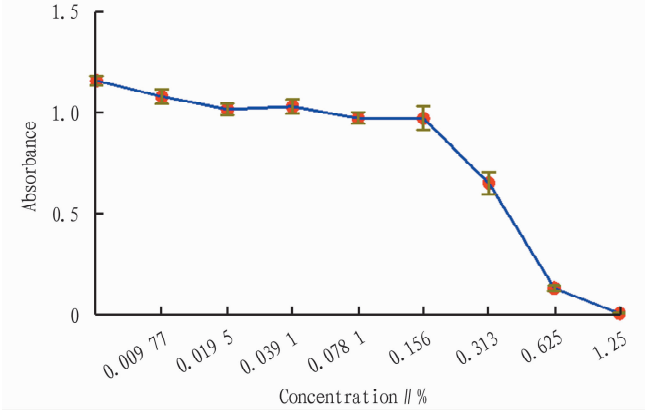


Fig. 6 OD_{600} value of *E. coli* at 4 h

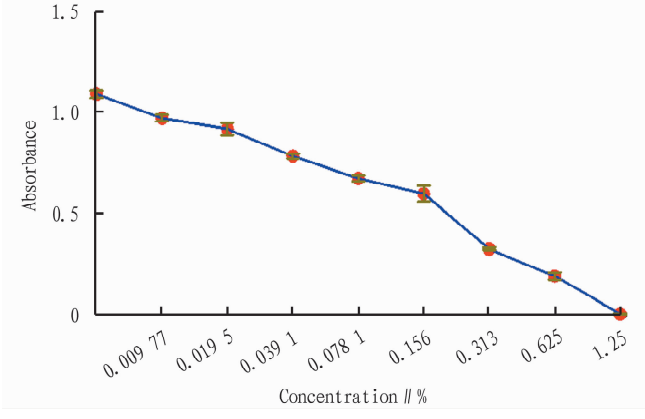


Fig. 7 OD_{600} value of *P. aeruginosa* at 4 h

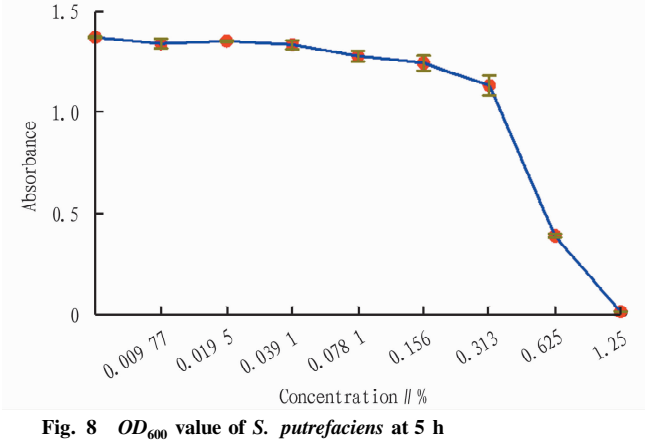


Fig. 8 OD_{600} value of *S. putrefaciens* at 5 h

Conclusions

The bacteriostatic activity of 2-methyl-3-(methylthioyl) pyrazine, a derivative of *B. coagulans* metabolite, was investigated in this study. The logarithmic growth period of *E. coli*, *P. aeruginosa* and *S. putrefaciens* was first determined to be 4 – 5 h, and tween 80 was selected as the best solvent. Broth dilution method was used to prepare a series of 2-methyl-3-(methylthioyl) pyrazine solution, and its inhibitory effect on bacterial growth was investigated. The lowest inhibitory concentration of the compounds that could inhibit the normal growth and reproduction of *E. coli*, *P. aeruginosa* and *S. putrefaciens* was 1.25%. The results indicated that 2-methyl-3-(methylthio) pyrazine showed significant antibacterial activity against the three putrefaction bacteria, which provides a theoretical basis for the development of this compound as an antiseptic preservative.

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