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# IN VITRO STUDY OF THE ANTIMICROBIAL EFFECT OF SYNTHESIZED COPOLYMER COMPOUNDS AGAINST *STAPHYLOCOCCUS AUREUS* AND *ESCHERICHIA COLI* STRAINS

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**ABSTRACT**

The newly synthesized antimicrobial copolymers i.e. [poly N-vinylpyrrolidone -block- poly ε-caprolactone -ω-hydroxyethylmethacrylate] (KFB), [(poly N-vinylpyrrolidone -block- poly ε-caprolactone -ω-hydroxyethylmethacrylate) -graft- poly-4-Vinylpyridine] (KFC) and [(poly N-vinylpyrrolidone -block- poly ε-caprolactone -ω-hydroxyethylmethacrylate) -graft- poly-4-Vinylpyridine octyl bromide] (KFCQ), were tested *in vitro*, using diffusion disc method (MDD) against two microbial strains: gram-positive bacteria *Staphylococcus aureus* ATCC 25923 (*S. aureus*) and gram-negative bacteria *Escherichia coli* ATCC 25922 (*E. coli*). Good antibacterial activities were observed for all tested copolymers. It was demonstrated that gram-positive bacteria were more sensitive than gram-negatives, and KFCQ agent was more active than KFB and KFC.



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## Introduction

Bacteria appeared in the earth 3.8 billion years ago [1] and they are always present around us. They exist in all planetary ecosystems, whether fresh or salt water, soil or air. The human body contains bacterial cells ten times more than human cells [2]. They are present on our skin, mucous membranes, and intestine, although the vast majority of these bacteria are beneficial to us, there are also some pathogenic bacteria responsible for infectious diseases. In recent decades, microbial resistance has evolved to the point where it presents a major problem in the field of public health worldwide, including the resistance of *Staphylococcus aureus*, *Pseudomonas aerugienosa* and *Escherichia coli* for a wide range of antibiotics [3].

Microbial resistance can be intrinsic or acquired. Microbes can acquire a new resistance when a spontaneous mutation occurs in their genes or when there is a transfer of new genes from another species. Genetic resistance information on chromosomes or plasmids is transferred from one species to another as extrachromosomal DNA molecules, which contain genes that are not necessary for the replication or survival for the host cell [4]. As the number of multidrug-resistant microorganisms increases, there is a big concern about the inefficiency of many antibiotics [5, 6].

Products, commonly commercialized as antimicrobials are efficient as they are impregnated with elements such as triclosan or silver ion. These elements are limited by the short effective life [7, 8]. Due to bacterial resistance [9, 10], Materials that kill bacteria or inhibit their growth because of their structure have the potential to be commonly used in infections as useful biomaterials. Cationic polymers such as poly (4-vinyl-N- pyridinium bromide), have shown their ability in killing bacteria because of their structures [8, 11-13]. The electrostatic attraction of the cation on the negatively charged bacterial membrane allows the rupture of the membrane by the quaternizing alkyl tail and leads to cell death [14].

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The application of polyvinyl pyridine (PVP) metal complex as an antibacterial agent was carried out by Kantouch et al. [15]. The authors showed that Cu / oxidized PVP and Ag / oxidized PVP significantly retards the growth of bacteria, and Ag / oxidized PVP has a better biocidal effect.

Ping Li et al. [16], prepared water with highly dispersible silver sulfadiazine (SSD) by the liquid phase method along with polyvinylpyrrolidone (PVP) as a surface modifying agent. Therefore, the resultant SSD particle decorated with PVP (P-SSD) was promising to exhibit excellent antibacterial effects against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, respectively.

In this context, it seems interesting to evaluate the antimicrobial activity of synthesized copolymers namely [poly N-vinylpyrrolidone -block- poly ε-caprolactone -ω-hydroxyethylmethacrylate] (KFB), [(poly N-vinylpyrrolidone -block- poly ε-caprolactone -ω-hydroxyethylmethacrylate) -graft- poly-4-Vinylpyridine] (KFC) and [(poly N-vinylpyrrolidone -block- poly ε-caprolactone -ω-hydroxyethylmethacrylate) -graft- poly-4-Vinylpyridine octyl bromide] (KFCQ), against two bacterial strains, namely, *Staphylococcus aureus* and *Escherichia Coli* using the Disc Diffusion Method (MDD).

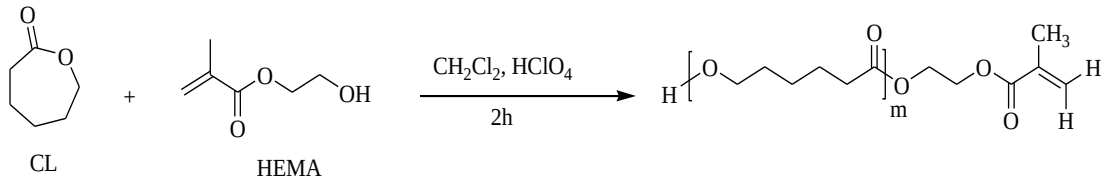
## Materials and Methods

### Chemical materials and synthesized copolymers

Hydroxyethylmethacrylate (HEMA), N-vinylpyrrolidone (NVP), 4-Vinylpyridine (VP) and ε-caprolactone (CL) were purchased from Sigma–Aldrich USA. Initiators Azobisisobutyronitrile (AIBN) and perchloric acid ($HClO_4$) were obtained from Sigma–Aldrich USA. Solvent tetrahydrofuran (THF), dichloromethane (DCM) and methanol ($CH_3OH$) were purchased from Shanghai Chemical Group, China.

- **Synthesis of the [Poly ε-caprolactone (PCL)-ω-hydroxyethylmethacrylate (HEMA)]**

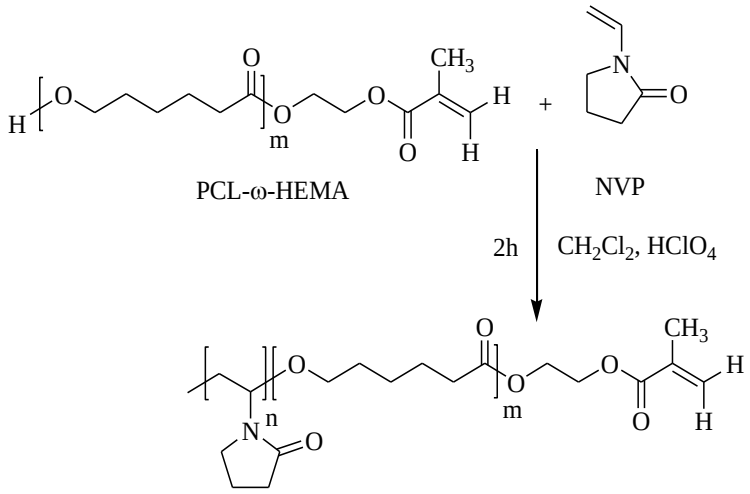
PCL-ω-HEMA macromonomer was prepared under nitrogen atmosphere, by cationic polymerization in the presence of monomer ε-CL and perchloric acid ($HClO_4$) as an initiator in dichloromethane ($CH_2Cl_2$). After homogenization of the mixed reaction, the hydroxyethylmethacrylate (HEMA) was added. The reaction system was kept at room temperature for 2h; the macromonomer was obtained and then precipitated in methanol ($CH_3OH$), washed in dichloromethane (DCM) and dried (See Scheme 1) [17].




**Scheme 1.** Synthesis of the (PCL-ω-HEMA)

- **Synthesis of the [(poly N-vinylpyrrolidone (PNVP)-block-poly ε-caprolactone (PCL)-ω-hydroxyethylmethacrylate (HEMA)]**.

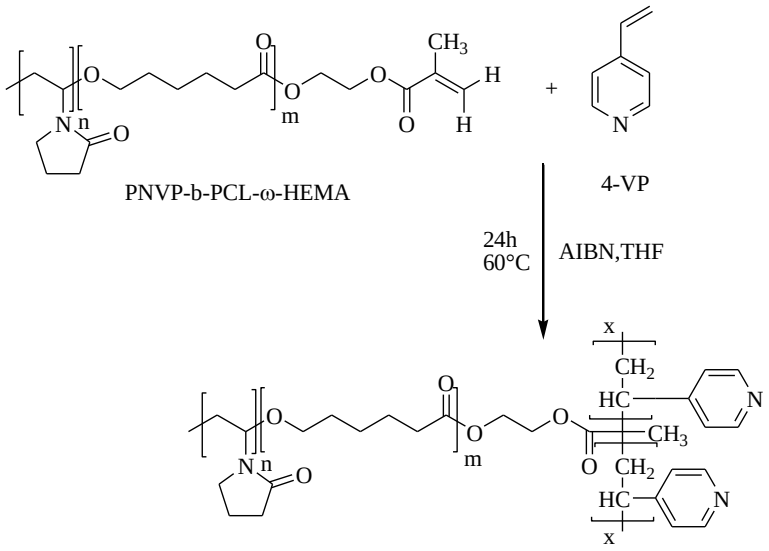
NVP was polymerized in dichloromethane (DCM) under nitrogen atmosphere, using (PCL-ω-HEMA) macro-initiator in the presence of perchloric acid ($HClO_4$). The reaction was carried at room temperature for 2h. The copolymer (PNVP-b-PCL-ω-HEMA) (KFB) was obtained and then precipitated in methanol ($CH_3OH$), washed in dichloromethane (DCM) and dried (See scheme 2) [17].




**Scheme 2.** Synthesis of the (PNVP-b-PCL-ω-HEMA) (KFB)

- **Synthesis of the [(poly N-vinylpyrrolidone (PNVP)-block- poly ε-caprolactone (PCL)-ω-hydroxyethylmethacrylate(HEMA)-graft-poly-4-Vinylpyridine (PVP)]**

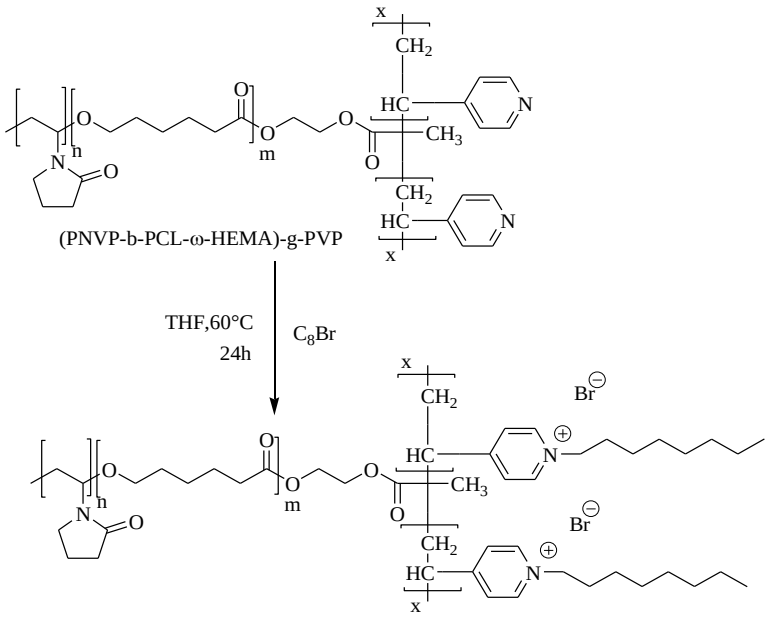
Poly-4-Vinylpyridine (PVP) was prepared by radical polymerization of 4-vinyl pyridine (VP) in tetrahydrofuran (THF), under vacuum, with 2, 2-azobisisobutyronitrile (AIBN) as initiator. The PNVP-b-PCL was added to the reaction mixture. The flask was thermostated at 60 °C for 24h of stirring. The product [(PNVP-b-PCL-ω-HEMA)-g-PVP] (KFC) was precipitated in methanol ($CH_3OH$), washed in chloroform ($CHCl_3$) and dried (See Scheme 3) [17].




**Scheme 3.** Synthesis of the [(PNVP-b-PCL-ω-HEMA)-g-PVP] (KFC)

- **Synthesis of the [(poly N-vinylpyrrolidone (PNVP)-block- poly ε-caprolactone (PCL)-ω-hydroxyethyl methacrylate(HEMA)-graft poly-4-Vinylpyridine (PVP) octyl bromide ($C_8Br$)]**

The [(PNVP-b-PCL-ω-HEMA)-g-PVP$C_8$Br] was obtained by mixing [(PNVP-b-PCL-ω-HEMA) -g-PVP] to Bromo octyl ($C_8Br$) in tetrahydrofuran (THF). The mixture was stirred at 60°C for 24h, the product named [(PNVP-b-PCL-ω-HEMA)-g-PVP$C_8$Br] (KFCQ) was obtained then precipitated in methanol ($CH_3OH$), washed in chloroform ($CH_3Cl$) and dried (See Scheme 4) [17].




**Scheme 4.** the synthesis of [(PNVP-b-PCL-ω-HEMA)-g-PVP$C_8$Br] (KFCQ)

**Biological materials and microorganism**

To test the antimicrobial effect of the synthesized chemical molecules we used two indicator strains, *Staphylococcus aureus* ATCC 25923 (Gram+) and *Escherichia coli* ATCC 25922 (Gram-).

These strains were from the University Hospital Establishment (EHU) of Oran November 1st, microbiology department.

- **Culture centre**

  - Mueller-Hinton agar

Composition of Mueller-Hinton agar (MH)

Casein peptone ……..17.5g
Beef extract ……2g
Corn starch ……...5g
Agar-agar……20g
Distilled water ……..1L
pH……6.8

Solutions at the concentrations of 1000 µg/ml, 500 µg/ml and 250 µg/ml KFB, KFC and KFCQ respectively were prepared in the tetrahydrofuran (THF) as a solvent.

To test the activity of polymers on both bacterial strains (*Staphylococcus aureus)* ATCC 25923 and *Escherichia Coli* ATCC 25922*)*, we chose the method of diffusion discs of polymer solutions on agar medium in Petri dishes. This method makes it possible to estimate the effectiveness of each concentration of the chemical solution on the growth of the tested microorganisms.

The principle of this method is analogous to that of an antibiogram [18-20].

The different bacterial strains were subcultured by the Mueller Hinton streak method and incubated in an oven at 37 ° C for 18 to 24 hours. From these young cultures, pure colonies were isolated to prepare bacterial inoculum. Each colony was suspended in 2.5 ml of sterile physiological saline.

The turbidity of the suspension was measured using a densitometer and adjusted at 0.5 Mc Farland ($10^8$ UFC/mL). The inoculation was done by making tight streaks on the agar plate by the swab. This operation was repeated 3 times by turning the plate 60 degrees.

Sterile discs of blotting paper with 6 mm in diameter (Rundfilter 25 Mn 86/90 type) were impregnated with the polymer solution. Four discs which were impregnated by different concentrations of KFB, KFC, and KFCQ as well as THF solvent as a control, were deposited on the seeded medium. After incubation at 37 °C for 24 hours, the diameters of the clear zones of inhibition around the discs were measured in mm according to Biyiti et al. [21].

Before incubating at 37 °C, the dishes were left at room temperature for 15 minutes, for good diffusion of the contents of the disc.

Three repetitions are performed for each strain as well as for the controls.

**Results and Discussion**

**Antimicrobial activity**

We tested *in vitro* the antibacterial activity of the KFB, KFC and KFCQ copolymers, on two bacterial strains (*Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922).

The antimicrobial activity was estimated by the presence or absence of inhibition zones. The results of the various tests are grouped in Table 1 and illustrated by Figures 1 and 2.

**Table 1:** The inhibition area diameters (mm) of the growth of two strains

| Bacteria | *Staphylococcus aureus* ATCC 25923 (Gram+) | | | | *Escherichia coli* ATCC 25922 (Gram-) | | | |
|---|---|---|---|---|---|---|---|---|
| Compounds | 0 µg/ml | 250 µg/ml | 500 µg/ml | 1000 µg/ml | 0 µg/ml | 250 µg/ml | 500 µg/ml | 1000 µg/ml |
| | Diameters of the zones of inhibition (mm) | | | | | | | |
| KFB | 0 | 11 | 13 | 15 | 0 | 9 | 13 | 11 |
| KFC | 0 | 14 | 16 | 20 | 0 | 13 | 15 | 18 |
| KFCQ | 0 | 16 | 17 | 23 | 0 | 15 | 18 | 21 |

0: corresponds to the control disc impregnated by THF

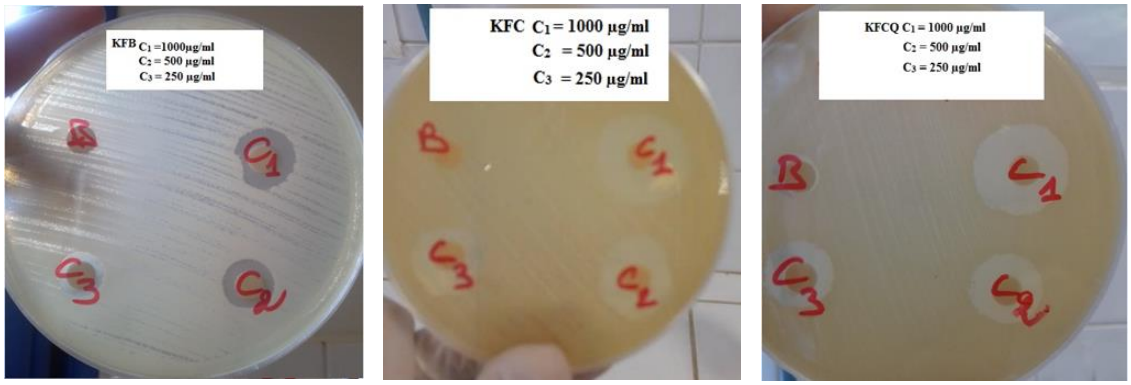



**Figure 1:** Effect of KFB, KFC and KFCQ copolymers on the growth of *Staphylococcus aureus* (ATCC 25923).

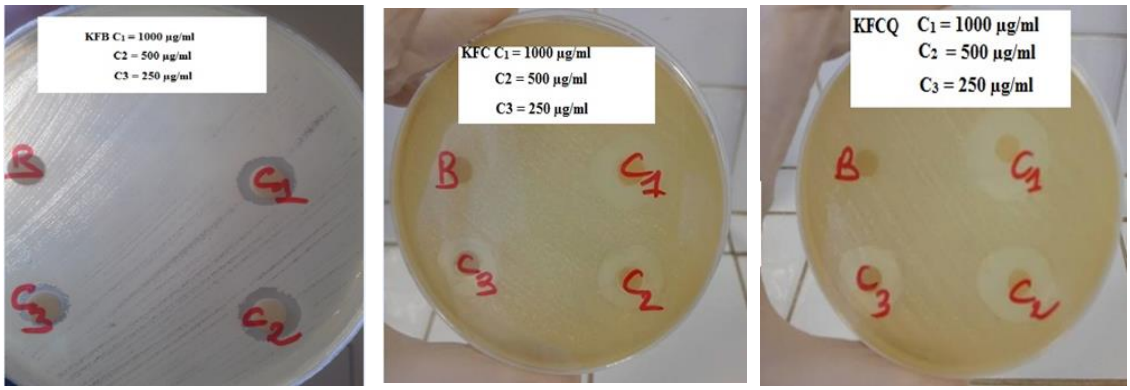



**Figure 2:** Effect of KFB, KFC and KFCQ copolymers on the growth of *Escherichia coli* (ATCC 25922).

The majority of our tested products exhibited antibacterial activities based on the diameters of the inhibition zones according to Table 1 and Figures 1 and 2.

All products, tested at three concentrations were active on Gram-positive and Gram-Negative bacteria, *Staphylococcus aureus* (ATCC 25923) and *Escherichia Coli* (ATCC 25922), respectively.

The KFB, KFC, and KFCQ had high antimicrobial activities against the tested microorganisms and had a high inhibition against both microbial strains.

The results showed that the Gram-positive bacteria, *Staphylococcus aureus* were more sensitive than *Escherichia Coli* with the inhibition zones of 15 mm, 20 mm, and 23 mm for 1000 µg/ml of KFB, KFC, and KFCQ respectively.

In addition, *Escherichia coli* had lower inhibition with the smaller zone of inhibition of 11 mm, 18 mm, and 21 mm for at 1000 µg/ml of KFB, KFC KFCQ) respectively.

Some authors have reported that macromolecules can interact more effectively with Gram-positive bacteria cells because their external layer of polyglycan is packed enough to allow deep penetration of the polymer chain into the cell to interact with the cytoplasmic membrane. On the other hand, a Gram-negative bacteria cell has an additional membrane with a phospholipid bilayer structure that further protects the internal cytoplasmic membrane against the harmful action of the biocidal polymers [22].

Many authors showed that polyvinyl pyridine (PVP) has pyridine fragments that can lead to pyridinium like antimicrobial polymers [22, 23].

Bradley C. Allison et al. [24] revealed that the quaternized polyvinylpyridine has intrinsic antimicrobial properties which are efficient against Gram-positive bacteria, Gram-negative bacteria, viruses, and yeasts.

It is well known that most bacterial cell walls have a negative charge and phosphatidylethanolamine (70%) as the main component; so most of the antimicrobial polymers are positively charged. Therefore, polymers with quaternary ammonium groups are probably the most studied type of biocidal polymers. It is generally accepted that the mechanism of the bactericidal action of polycationic biocides involves a destructive interaction with the cell wall and/or cytoplasmic membranes [25]. In addition, some authors have also revealed that poly(ε-caprolactone) also has antibacterial properties [26, 27].

In order to visualize the action of the copolymers on the microbial strains tested, it seemed more appropriate to represent the results as a histogram as shown in figure 3.

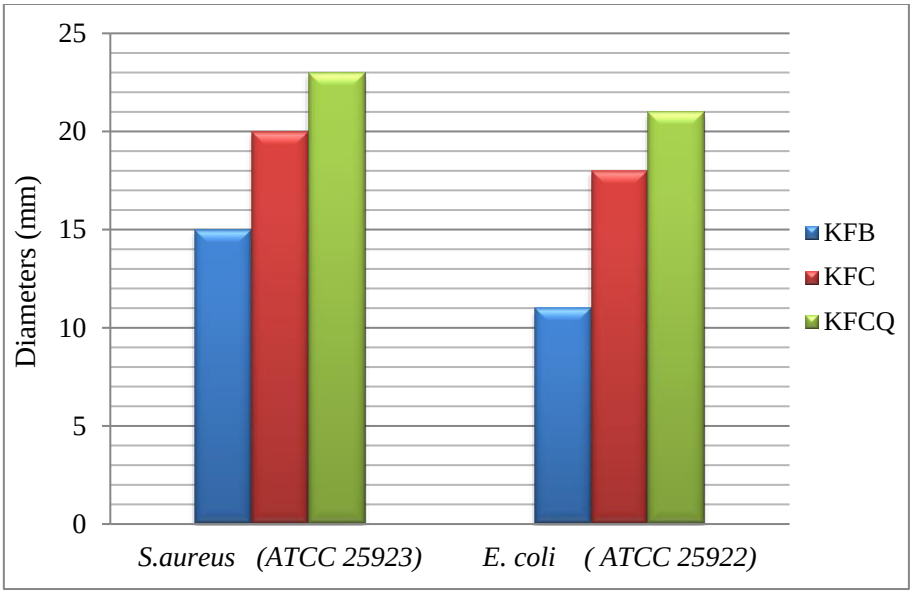



**Figure 3:** Diameters of the inhibition zones of the microbial strains tested with regard to the copolymers KFB, KFC, and KFCQ

**Conclusion**

The antimicrobial activity of the copolymers KFB, KFC and KFCQ were carried out *in vitro* by disc diffusion method (MDD).

The antimicrobial tests performed by this method clearly showed that the copolymers namely KFB, KFC, and KFCQ**,** have significant activity against all tested microbial strains. Gram-positive bacteria, *Staphylococcus aureus* was more sensitive microorganism than *Escherichia coli*.

Moreover, the [poly N-vinylpyrrolidone -block- poly ε-caprolactone -ω-hydroxyethylmethacrylate) -graft- poly-4-Vinylpyridine octyl bromide] was relatively more active than the [(poly N-vinylpyrrolidone -block- poly ε-caprolactone -ω-hydroxyethylmethacrylate] and [(poly N-vinylpyrrolidone -block- poly ε-caprolactone -ω-hydroxyethylmethacrylate) -graft-poly-4-Vinylpyridine].

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