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RESEARCH ARTICLE

# Cu(II)-catalyzed degradation of ampicillin: effect of pH and dissolved oxygen

Yiming Guo[1,2] · Daniel C. W. Tsang[3] · Xinran Zhang[1,2] · Xin Yang[1,2]

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**Abstract** Cu(II)-catalyzed hydrolysis of β-lactam antibiotics has been well-identified and recognized as the key mechanism of antibiotic degradation. However, the overlooked Cu(II) oxidation susceptibly also plays an important role comparably with hydrolysis. This study evaluated the roles of hydrolysis and oxidation in Cu(II)-catalyzed degraded ampicillin (AMP), as a typical β-lactam antibiotic, under relevant environmental conditions (pH 5.0, 7.0, and 9.0; oxygen 0.2 and 6.2 mg/L). Under AMP and Cu(II) molar ratio of 1:1, AMP degradation was the fastest at pH 9.0, followed by pH 5.0 and pH 7.0. The facilitation of oxygen on AMP degradation was notable at pH 5.0 and 7.0 rather than pH 9.0. AMP degradation rate increased from 21.8% in 0.2 mg/L $O_2$ solution to 85.9% in 6.2 mg/L $O_2$ solution at pH 7.0 after 4-h reaction. AMP oxidation was attributed to both oxygen-derived Cu(I)/Cu(II) cycle and intermediate reactive oxygen species ($HO^{\cdot}$ and $O_2^{\cdot-}$). Several intermediate and final products in AMP degradation were firstly identified by LC-quadrupole time-of-flight-MS analysis. Phenylglycine primary amine on the AMP structure was the essential complexation site to proceed with the oxidation reaction. The oxidation of AMP preferentially occurred on the β-lactam structure. The inherent mechanisms related to pH and oxygen conditions were firstly investigated, which could enhance the understanding of both oxidation and hydrolysis mechanisms in AMP degradation. This study not only has an important implication in predicting β-lactam antibiotic transformation and fate in natural environment but also benefits the developing of strategies of antibiotic control to reduce the environmental risk.

**Keywords** β-Lactam antibiotics · Ampicillin · Copper redox · Complexation · Oxidation · Hydrolysis

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✉ Xinran Zhang
zhangxinr@mail.sysu.edu.cn

✉ Xin Yang
yangx36@mail.sysu.edu.cn

[1] School of Environmental Science and Engineering, Sun Yat-sen University, Guangzhou 510275, China

[2] Guangdong Provincial Key Laboratory of Environmental Pollution Control and Remediation Technology, Guangzhou 510275, China

[3] Department of Civil and Environmental Engineering, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong, China

## Introduction

Typical β-lactam antibiotics, including penicillins and cephalosporins, are among the most frequently utilized antibiotics (Sharma et al. 2016). They are among the largest category of antibiotic consumption for human use in the USA, UK, France, India, and China (Van Boeckel et al. 2014). A large portion of the antibiotics were excreted unchanged, and they still remained bioactive in water and soil (Subbiah et al. 2011). Taking ampicillin (AMP) as an example, approximately 30% would be excreted for oral intake while up to 75% would be excreted for intravenous intake (Clarke et al. 1986; Clarke et al. 1986). The unchanged portion is released to urban sewage system or directly to the surrounding environment (Watkinson et al. 2009). In conventional wastewater treatment plants, biodegradation processes hardly removed ampicillin (Li and Zhang 2010). Other antibiotic removal methods have also been investigated, including absorption (Carabineiro

et al. 2011; Carabineiro et al. 2012), chlorination (Dodd et al. 2005), ozonation, and advanced oxidation processes (Huber et al. 2003). However, these methods present limited efficiency due to the interference of water matrix. Consequently, they are frequently detected in surface water, groundwater, and wastewater effluent (Tim et al. 2016; Zhang et al. 2015; Van Boeckel et al. 2014). Their persistence in wastewater treatment plant effluent and aqueous environment may promote antibiotic-resistant bacteria as well as antibiotic-resistant gene (Rodriguez-Mozaz et al. 2015).

When released to the aqueous environment, hydrolysis is an important pathway for β-lactam degradation. Penicillins, one group of β-lactam antibiotics, have a unique molecule consisting of a fused β-lactam–thiazolidine ring system, wherein the β-lactam ring is susceptible to cleavage by a variety of reagents, such as enzyme, acid base reagents, and metal ions (Hou and Poole 1971; Gensmantel et al. 1980). Penicillins could be hydrolyzed by β-lactamase enzyme and transformed to acid derivatives. In addition, acid and alkali promote penicillin degradation into different products. In acid solution, the β-lactam ring cleaves and gives an array of complex products, including penilloic acid, penicillamine, and penilloaldehyde (Blaha et al. 1976). In contrast, in alkaline solutions (pH 7.5–9.0), penicillins degrade rapidly and generate penicilloic acid alone (Robinson-Fuentes et al. 1997). Metal ions, such as Hg(II), Zn(II), Cu(II), and Co(II), could promote the degradation of penicillins. Penicillin–metal complex forms firstly, followed by hydrolyzing β-lactam ring. Among these metal ions, Cu(II) appears to present a strong catalytic ability on penicillin degradation, which was at least four orders of magnitude higher than other ions such as Hg(II), Zn(II), and Co(II) (Gensmantel et al. 1980).

Previous studies attributed the Cu(II) catalytic degradation of penicillins to Cu(II)-catalyzed hydrolysis reaction (Fernandez-Gonzalez et al. 2005; Lapshin and Alekseev 2009). It has been proposed that Cu(II) and penicillins undergo rapid complexation and then rate-limiting hydrolysis of the complex, yielding the corresponding penicilloic acid–Cu(II) complex (Cressman et al. 1969). Several other products such as penicillamine and penicillenic acids are also reported, which are generated via penicilloic acid intermediate (Gunther 1950). Apparently, the complexation between penicillins and Cu(II) is an important step for penicillin degradation. The complexation sites between penicillins and Cu(II) have been proposed as either (i) the β-lactam nitrogen and the carboxylate group or (ii) the β-lactam carbonyl group and the side-chain amide nitrogen, with benzylpenicillin as an example (Alekseev 2012). Another research suggests the phenylglycine primary amine group might also participate in the complexation (Chen et al. 2016). As to AMP, owning to the presence of phenyglycine primary amine group in the side chain and the thiazolidine ring fused to the β-lactam ring moiety, the complexation of AMP should be much more complicated than cefalexin and benzylpenicilin.

A recent study found that Cu(II) plays dual roles in benzylpenicillin degradation—not only as a hydrolysis catalyst but also as an oxidant (Chen et al. 2015). The benzylpenicillin degradation products were proposed to bebenzylpenicilloic acid as the hydrolysis product and phenylacetamide as the oxidized products. Moreover, a subsequent study also identified that Cu(II) plays dual roles in cefalexin degradation (Chen et al. 2016). In view of the fact that AMP has the same structure of phenylglycine primary amine group with cefalexin, the oxidation reaction between Cu(II) and AMP should be taken into consideration. However, our understanding of the synergistic effects of hydrolysis and oxidation on Cu(II)-promoted degradation of AMP, including the identification of degradation products and the relevant reaction mechanisms, is still very limited.

In addition, solution pH has been reported to present great impact on Cu(II)-catalyzed degradation of β-lactam antibiotics (Chen et al. 2016). Previous study on Cu(II)-catalyzed benzylpenicillin G degradation suggests that the fastest degradation rate is at pH 7.0 followed by pH 9.0 and 5.0 (Chen et al. 2015). However, cefalexin, with a phenylglycine amino moiety, degraded much faster at pH 9.0 than pH 7.0 and 5.0 (Chen et al. 2016). Solution pH could affect the structure of the complex through influencing the extent of protonation/deprotonation of function groups. Furthermore, solution pH may affect the redox cycle of Cu(I)–Cu(II), which is critical in oxidation reaction (Yuan et al. 2012). Oxygen should be a critical impact factor in the oxidation reaction between penicillins and Cu(II), which may also change the AMP degradation intermediates and products (Chen and Huang 2009). The effects of pH together with oxygen on the degradation kinetics and pathways for Cu(II)-catalyzed degradation of other β-lactam antibiotics deserve further investigation.

The objective of this study was to deepen the understanding of the Cu(II)-catalyzed degradation mechanisms of β-lactam antibiotic from two perspectives: hydrolysis and oxidation. AMP was chosen as the model compound because it has more complicated structure compared with previously reported β-lactam antibiotic (cefalexin and benzylpenicillin). To our best knowledge, although Cu(II)-catalyzed hydrolysis of AMP has been well defined, the systematic study of Cu(II)-induced oxidation of AMP, including the degradation mechanisms and transformation products, has not been reported yet. In this study, the roles of hydrolysis and oxidation in Cu(II)-catalyzed degradation of AMP were investigated under relevant environmental pH and oxygen conditions. The mechanisms of AMP degradation and the related degradation products were identified by MS analysis. Understanding of the dependence of pH and oxygen in AMP degradation is not only important for predicting β-lactam antibiotics transformation and fate in natural environment, but is also beneficial for developing

strategies of antibiotic control to reduce the environmental risk.

## Material and methods

### Chemical reagents

Ampicillin trihydrate was obtained from J&K (China). Copper(II) sulfate pentahydrate (> 99.999% purity) and copper(I) chloride beads (> 99.99% purity) were obtained from Sigma-Aldrich (USA). Other chemicals were of the highest purity available purchased from Sigma-Aldrich (USA). All experimental solutions were prepared with ultrapure water.

### Experimental methods

Batch experiments of AMP degradation were conducted in a 100-mL glass flask wrapped with aluminum foil to prevent photocatalytic degradation of AMP. The initial concentration of AMP solution was 100 μM in order to ensure sufficient transformation product could be generated and identified. The experimental solution pH was firstly adjusted at pH 5.0, 7.0, and 9.0, in order to cover the typical environmental pH condition. Solution pH was maintained with 5 mM different buffer reagents, in terms of 2-(*N*-morpholino) ethanesulfonic acid (MES) for pH 5.0, 4-morpholinepropanesulfonic acid (MOPS) for pH 7.0 and pH 8.0, and 2-(cyclohexylamino) ethanesulfonic acid (CHES) for pH 9.0. These buffers were chosen because they have negligible complex with copper ion (Chen and Huang 2009). The reaction between AMP and Cu(II) was initiated by adding desirable amount of Cu(II) to buffered AMP solution, with the initial Cu(II) concentrations of 25, 50, 100, 200, 300, and 400 μM. The solution was constantly stirred at room temperature (25 ± 1 °C). In oxygen-rich experiments, experimental solutions were exposed to the ambient air and the concentration of dissolved oxygen in the solution was around 6.2 mg/L. As to oxygen-limited condition, experimental solutions were purged by nitrogen gas and the dissolved oxygen concentration was kept below 0.2 mg/L. Then, samples were collected at predetermined time intervals. Samples for AMP and its product analysis were immediately quenched by residual Cu(II) and Cu(I) by an excess amount of ethylenediamine tetraacetic acid (EDTA, 0.5 mM) and analyzed within 24 h. Samples for Cu(I) analysis and complexation were conducted immediately without the addition of EDTA. All the experiments were conducted in duplicate or more.

### Analytical methods

The AMP-Cu(II) complex was investigated using UV–vis spectrophotometry using Shimadzu UV-2700 UV–VIS spectrophotometer. The differential UV absorbance (Δabs) was calculated by the absorbance of complex subtracted by the absorbance of both Cu(II) alone and AMP alone, as shown in Eq. 1 (Dimitrovska et al. 1996):

$$\Delta abs = \mathrm{abs}^{(\mathrm{Cu(II)+AMP})} - \mathrm{abs}^{(\mathrm{Cu(II)\ only})} - \mathrm{abs}^{(\mathrm{AMP\ only})} \quad (1)$$

The concentration of Cu(I) was measured by the spectrophotometric absorbance of the complex of Cu(I) and bathocuproine (Yuan et al. 2012). Before the addition of bathocuproine, 20 mM EDTA was firstly added into samples to inhibit the interference from Cu(II) with bathcuproine. The reaction of Cu(I) with bathocuproine generated a stable orange complex, which could be measured by a UV/vis spectrophotometer at 484 nm. During the experiment, $N_2$ was purged to exclude oxygen throughout the entire time.

Ampicillin was analyzed by a Thermo Ultimate 3000 high-performance liquid chromatography (HPLC) equipped with a diode array detector. The sample was separated by a Syncronis C18 column (250 mm × 4.6 mm, 5 μm) with an injection volume of 20 μL. The mobile phase gradient elution was with 0.1% formic acid in water (A) and pure methanol (B) at 50:50 *v*/*v* at a flow rate of 0.8 mL/min. The detection wavelength was 220 nm.

The minimum reporting limit (MRL) of AMP concentration was defined as the lowest concentration that gives a signal-to-noise ratio higher than 3, and the corresponding MRL value of AMP was 1 μM. Since the initial concentration of AMP solution was obviously higher than the MRL, solid-phase extraction (SPE) method was not employed in this study. The samples were also not spiked with internal standards. The Pearson correlation coefficient ($R^2$) for the linear regression of the calibration curve was 0.9939, suggesting this detection method is suitable for AMP analysis. The relative standard deviation (RSD) of five replicates was required to be within 5% to meet the quality assurance-/quality control (QA/QC)-reporting requirements for AMP concentration determination.

Transformation products were analyzed by ultra-high-performance liquid chromatography coupled with a quadrupole time-of-flight mass spectrometer (UHPLC-QTOF-MS). Samples were separated by Waters Acquity BEH C18 analytical column (2.1 × 100 mm, 1.8 μm) at a flow rate of 0.4 mL/min. The sample injection volume was 20 μL, and the gradients of mobile phases were (A) water containing 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid. The products were analyzed by positive electrospray ionization mode (ESI+) at 70–220 eV of fragmentation voltage and scan range of 50–1000. Other typical parameters were set as

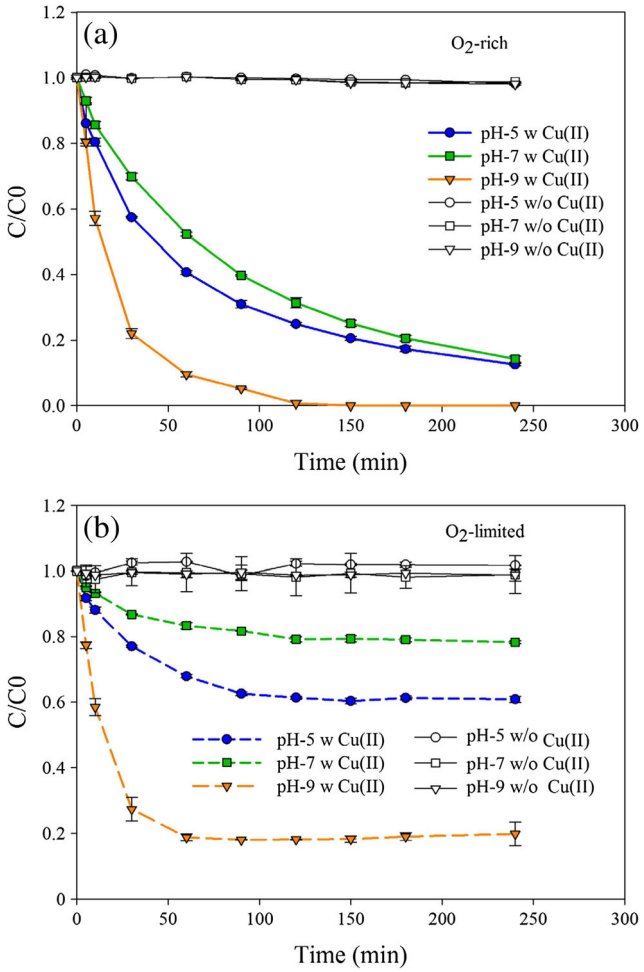



**Fig. 1** The degradation of AMP in the absence (w/o Cu(II)) and presence of Cu(II) (w Cu(II)) at different pH in **a** oxygen-rich condition (open to air) and **b** oxygen-limited condition (closed to air and purged by nitrogen). Conditions: $[Cu(II)]_0 = [AMP]_0 = 100\ \mu M$

follows: nebulizer gas 6.0 bar, capillary voltage 2000 V, and fragmentor 40 V.

## Result and discussion

### Accelerated AMP degradation by Cu(II): effect of pH and oxygen

The effects of pH and oxygen on Cu(II)-promoted AMP degradation were investigated under three pH values (5.0, 7.0, and 9.0) and two oxygen concentrations ($O_2$-rich and $O_2$-limited conditions). As shown in Fig. 1, in the absence of Cu(II), the loss of AMP was negligible at pH 5.0 and less than 1% at pH 9.0 within 4 h under both $O_2$-rich and $O_2$-limited conditions. However, the addition of Cu(II) in the solution significantly enhanced the AMP degradation in all the pH and oxygen conditions in this study. It can be seen that the effect of pH on Cu(II)-catalyzed degradation of AMP was obvious in both $O_2$-rich and $O_2$-limited conditions. In both oxygen levels, the degradation rate of AMP was the fastest at pH 9.0, followed by pH 5.0 and pH 7.0.

By comparing Fig. 1a, b, the degradation trends of AMP were different under the two oxygen concentrations. In the $O_2$-limited condition, Cu(II)-catalyzed AMP degradation was fast initially, slowed down, and then ceased after 120 min. Meanwhile, the increase of $O_2$ concentration significantly enhanced the degradation of AMP under all of the three pH conditions. At pH 7.0, the AMP degradation rate increased from 21.8% in $O_2$-limited solution to 85.9% in $O_2$-rich solution after 4-h reaction. As for the other two pH conditions, the increase of AMP degradation with oxygen concentration was also observed, which was from 39.2 to 87.5% at pH 5.0 and from 80.2 to 100% at pH 9.0. These results suggested that both pH condition and oxygen concentration imposed significant effects on Cu(II)-promoted degradation of AMP.

The Cu(II)-catalyzed degradation of AMP increased monotonically with the increase of Cu(II) doses. As shown in Fig. 2, under $O_2$-rich and pH 7.0 conditions, the degradation of AMP after 4 h reached 14.6, 33.0, 66.7, 85.6, and 85.9% with the addition of Cu(II) at 20, 40, 60, 80, and 100 μM, respectively. Figure S1-1 suggested that the Cu(II)-promoted degradation of AMP followed first-order kinetics. The degradation constants of AMP were $7 \times 10^{-4}$/min, $1.7 \times 10^{-3}$/min, $8.2 \times 10^{-3}$/min, and $2.0 \times 10^{-2}$/min for Cu(II)/AMP molar ratios of 0.2:1, 0.4:1, 1:1, and 2:1, respectively. AMP degradation represented a Cu(II) dosage-dependent tendency at pH 7.0, which was similar with that at pH 5.0 and 9.0 (data not shown). Another experiment was conducted involving the provision of additional 100 μM Cu(II) into the reactor when AMP degradation was slow after 150 min under the oxygen-limited condition. The degradation of the remaining AMP continued to proceed and slowed down in a similar trend to that during the first 150 min (Fig. SI-2). These results were consistent with

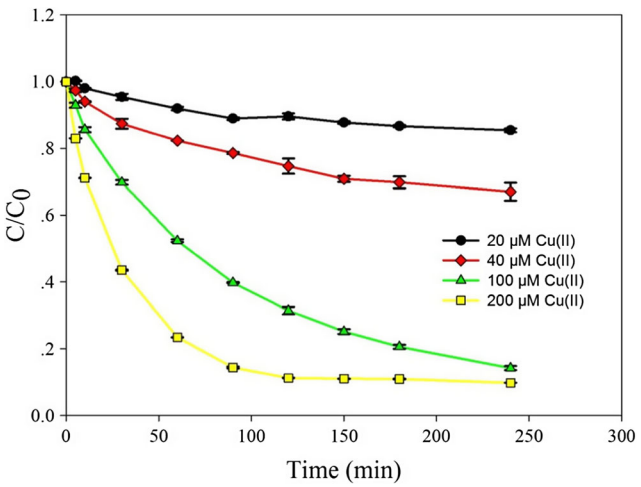



**Fig. 2** Effect of initial Cu(II) concentration on AMP degradation. Condition: $[AMP]_0 = 100\ \mu M$, pH = 7.0 (buffered by 10 mM MOPS), open to air

previous studies, suggesting that the interaction between Cu(II) and AMP played an important role in AMP degradation.

### Effect of pH on the complexation between AMP and Cu(II)

Complexation of Cu(II) with β-lactam function group plays a crucial role in AMP hydrolysis and further promotes AMP degradation. As shown in Fig. SI-3, the addition of excess competing ligands (EDTA) almost completely inhibited AMP degradation, implying that the complexation between AMP and Cu(II) was a prerequisite for Cu(II)-catalyzed degradation of AMP. Complexation of AMP with Cu(II) was investigated at Cu(II)/AMP ratios from 0.25:1 to 4:1 under three pH conditions. The determination was conducted immediately after Cu(II) and AMP mixed together to minimize the oxidation between AMP and Cu(II). The absorbance characteristics of Cu(II)-AMP complex were expressed by the difference in UV absorbance (Δabs).

As shown in Fig. 3, two absorbance bands were observed after complexation at pH 7.0. One is in the range of 280–400 nm with a peak at 315 nm, and another one is in the range of 230–270 nm with a peak at 243 nm. A previous study reported that the peak of 315 nm only occurred in the complexation of Cu(II) with penicillins containing phenylglycine amino group (e.g., amoxicillin, cefalexin, cefadroxil), but this peak was absent from Cu(II) complex with non-phenylglycine-type penicillins (e.g., cefapirin, penicillin G) (Chen et al. 2015). In addition, the peak at 243 nm could be observed at all of the investigated penicillins with carboxylate group (Dimitrovska et al. 1996). Another study using nuclear magnetic resonance spectroscopy confirmed that carboxylate group was a complex site of penicillin for metal ions and the β-lactam nitrogen was suggested at the other complex site (Gensmantel et al. 1980). Therefore, the peak at 315 nm seems to indicate the complex of Cu(II) involving the side-chain primary amine moiety of AMP, whereas the peak at 243 nm likely refers to the Cu(II) complexation with carboxylate group of AMP.

The complexation of Cu(II) and AMP at pH 5.0 and 9.0 was also investigated, and the results are shown in Fig. SI-4. At pH 9.0, the two absorbance peaks at 243 and 310 nm were also obvious. At pH 5.0, the value of Δabs became smaller, indicating weaker complexation. Meanwhile, the peak shifted from 315 to 325 nm. A previous study also reported the absorption maximum at 325 nm at pH 3.5 and 5.4 from the UV–Vis spectra of ampicillin in the presence of Cu(II) ions (Fernandez-Gonzalez et al. 2005). At pH 5.0, Cu(II) complexation may also involve the carboxyl group at the thiazolidine ring and the tertiary nitrogen on the β-lactam ring. It is noted that AMP has two p*K*a values at 2.5 and 7.3, which correspond to deprotonation of the carboxyl group on the

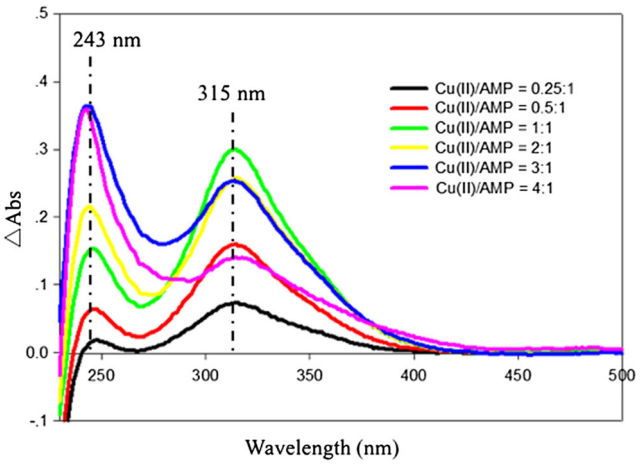



**Fig. 3** The changes of UV absorbance under different Cu(II)/AMP molar ratios. Condition: $[AMP(II)]_0$ = 100 μM, pH = 7.0 (buffered by 10 mM MOPS)

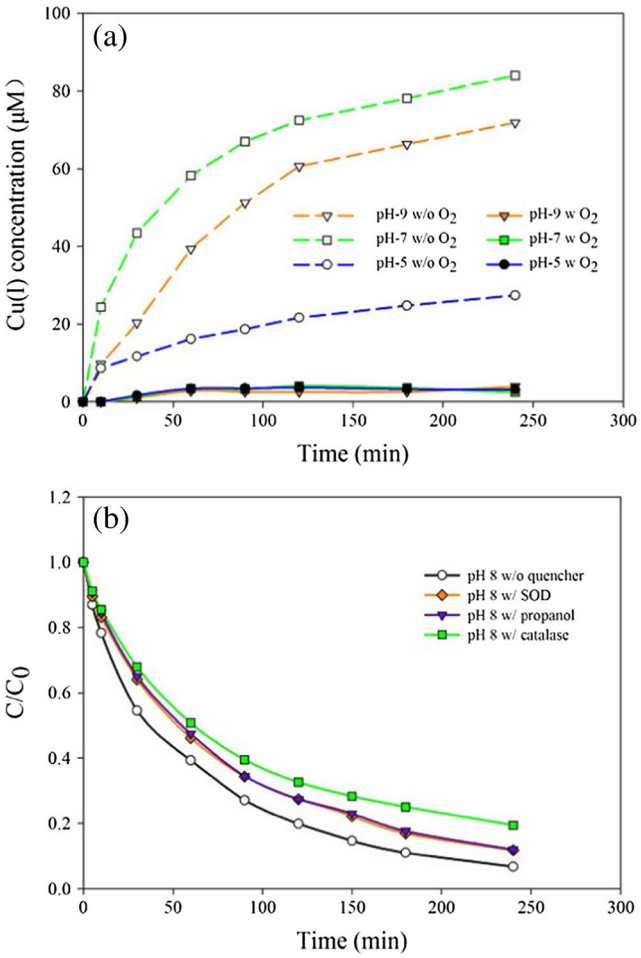



**Fig. 4** **a** Accumulation of Cu(I) during AMP degradation at different pH under oxygen-limited condition. **b** Effects of reactive oxygen species (ROS) on AMP degradation. Conditions: $[Cu(II)]_0$ = $[AMP]_0$ = 100 μM, **b** pH buffered by 10 mM MOPS, with air purging; [2-pronpanol] = 26 mM, [catalase] = [SOD] = 400 units/mL

dihydrothiazine ring and protonation of the primary amine group on the phenylglycine side chain, respectively. In the pH range of 5.0–9.0 used in this study, the carboxyl group was deprotonated. The side-chain primary amine was nearly 100% protonated at pH 5.0, 49.9% protonated at pH 7.0, and almost 100% deprotonated at pH 9.0. The deprotonated amine was favorable for complexation with Cu(II). Although the complex at pH 5.0 was weak, the high degradation rate reported above indicated that acid hydrolysis in the presence of Cu(II) was important at low pH condition.

### Oxidative species involved in AMP reaction

The oxidative role of Cu(II) in the degradation of benzylpenicillin has been proposed in the previous study (Chen et al. 2015). The oxidation reaction in Cu(II)-promoted AMP degradation was evaluated in this study. As shown in Fig. 4a, the accumulation of Cu(I) was observed in the oxygen-limited condition, but not in the oxygen-rich condition. This result clearly evidenced the occurrence of oxidation reaction between AMP and Cu(II). The results represented in Fig. 4a are comparable with the AMP degradation shown in Fig. 1, implying that the Cu(I) was converted back to Cu(II) via the reaction with oxygen and continued degradation of AMP in the oxygen-rich solution. Meanwhile, the accumulation of Cu(I) was the highest at pH 7.0, followed by pH 9.0 and pH 5.0. The relatively less AMP degradation at pH 7.0 (shown in Fig. 1) was possibly due to the lower Cu(II) concentrations than that of pH 9.0 and pH 5.0 in the oxygen-limited condition.

In addition, the Cu(I)-Cu(II) redox cycle may involve the generation of reactive oxygen species (ROS), which can enhance the transformation of AMP. Three kinds of ROS scavengers, i.e., 2-propanol, SOD, and catalase, were added into the solution to quench hydroxyl radical (·OH), superoxide radical ($\cdot O_2^-$), and hydrogen peroxide ($H_2O_2$), respectively. The experiment was conducted at pH 8.0 because the pH of SOD was in a range of 7.6–10.5. As shown in Fig. 4b, the degradation of AMP was slowed down by 5.3, 5.1, and 12.8% with the addition of 2-propanol, SOD, and catalase, respectively. Catalase mainly scavenged $H_2O_2$ thereby reducing the generation of ·OH, while it could also quench superoxide radical. Thus, the quenching with catalase had the highest inhibition of AMP degradation. This result indicated that both $\cdot O_2^-$ and ·OH generated in oxidation process play a role in the Cu(II)-catalyzed AMP degradation.

### Identification of Cu(II)-promoted AMP degradation products

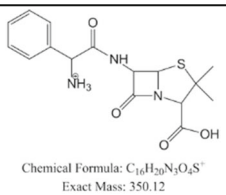
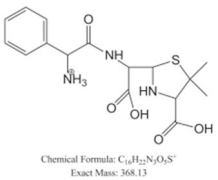
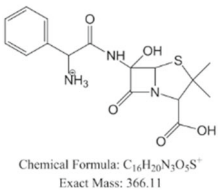
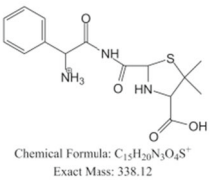
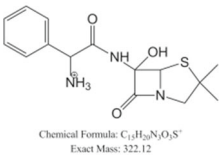
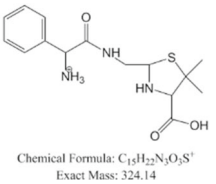
In order to figure out the mechanisms of Cu(II)-catalyzed degradation of AMP, transformation products were identified by LC-QTOF. In this study, ESI-MS/MS determination was conducted at positive mode, in which $[M + H]^+$ was the predominant ion. Negative mode was also used, but no transformation product was observed. The chemical formula of products was proposed based on the chemical formula of the parent AMP, the observed mass spectra, and their secondary fragments obtained by from MS/MS analysis.

As shown in Fig. SI-5, chromatographic peak eluted at 3.44 min with the cluster *m/z* of 350.1178 was identified as AMP, which matched well with the theoretical isotopic mass by Isotope Pattern Calculator v4.0. The stable AMP degradation products were separated and subjected to MS/MS analysis. Five AMP degradation products were identified: 368.1275, 324.1379, 338.1177, 366.1126, and 322.1128, corresponding to $P_{368}$, $P_{324}$, $P_{338}$, $P_{366}$, and $P_{322}$, respectively. All the clusters of *m/z* signals ranged from 297 to 370, which was close to that of parent AMP (*m/z* at 350.12). The products $P_{368}$ and $P_{324}$ detected in this study were consistent with a recent report (Chen et al. 2016), whereas $P_{338}$, $P_{366}$, and $P_{322}$ were firstly found in this study. The proposed products and their exact *m/z* and chemical formulas were summarized in Table 1. Furthermore, the intensity variation of these products with a function of AMP degradation time at the three pH values was shown in Fig. SI-6.

The product $P_{368}$ with the characteristic *m/z* of 368.1275 was the prominent intermediate product in all the three pH and with/without oxygen conditions, as illustrated in Figs. 5 and SI-6. The observed mass of $P_{368}$ corresponded to the chemical formula of $C_{16}H_{22}N_3O_5S^+$, which could undergo epimerization and generate an array of isomers $P_{368\ a–d}$ with the different retention times but the same fragmentation pattern. The two $[M + H]^+$ ions generated fragments at *m/z* of 307.1104, 147.0925, and 106.0656, which were relative to $C_{15}H_{19}N_2O_3S^+$, $C_6H_{15}N_2S^+$, and $C_7H_8N^+$, respectively. The corresponding $MS^2$ results and the proposed structures of fragments were illustrated in Fig. SI-7. Based on these results, $P_{368}$ could be identified to be ampicilloic acid (AMPA), as the β-lactam hydration product (368 = 350 + 18). In addition, $P_{324}$ was related to the cleavage of carboxylic acid moiety from $P_{368}$ (324 = 368–45 + 1), which was observed at pH 9.0 in oxygen-rich condition.

Interestingly, $P_{338}$ (*m/z* ratio of 338.1177) was observed during the Cu(II)-promoted AMP degradation at pH 7.0 and 9.0 in oxygen-rich solution, which has not been reported previously. This compound was not present either at pH 5.0 or in oxygen-limited condition. Figure SI-6 indicated the abundance of $P_{338}$ slightly increased at the beginning of reaction (30 min), but significantly increased accompanied with the decrease of $P_{368}$ in the presence of oxygen. These results imply that $P_{338}$ may be an oxidized product of AMP, and the intermediate product AMPA seems to be the major precursor. According to the mass spectra, $P_{338}$ could be fixed at a formula of $C_{15}H_{20}N_3O_4S^+$. According to the fragments (Fig. SI-8),

**Table 1** Characteristics of AMP and its stable intermediate products generated in Cu(II)-promoted degradation of AMP

| Compound | RT (min) | Experiment Mass [a] | Theoretical Mass [b] | **Mass error** | Proposed formula and structure |
|---|---|---|---|---|---|
| Ampicillin (AMP) | 3.44 | 350.1177 | 350.1175 | 0.6 |  Chemical Formula: $C_{16}H_{20}N_3O_4S^+$ Exact Mass: 350.12 |
| P368 | 2.60<br>2.71<br>2.98<br>3.10 | 368.1286 | 368.1280 | 1.6 |  Chemical Formula: $C_{16}H_{22}N_3O_5S^+$ Exact Mass: 368.13 |
| P366 | 4.12 | 366.1126 | 366.1124 | 0.5 |  Chemical Formula: $C_{16}H_{20}N_3O_5S^+$ Exact Mass: 366.11 |
| P338 | 4.34 | 338.1174 | 338.1175 | -0.3 |  Chemical Formula: $C_{15}H_{20}N_3O_4S^+$ Exact Mass: 338.12 |
| P322 | 4.70 | 322.1227 | 322.1225 | 0.6 |  Chemical Formula: $C_{15}H_{20}N_3O_3S^+$ Exact Mass: 322.12 |
| P324 | 3.23 | 324.1378 | 324.1382 | -1.2 |  Chemical Formula: $C_{15}H_{22}N_3O_3S^+$ Exact Mass: 324.14 |

[a] Obtained from the UHPLC-QTOF mass spectrometer

[b] Calculated using data from Isotope Pattern Calculator, v4.0

$P_{338}$ retained the structures of phenylmethanamine (*m/z* of 106.0657) and thiazolidine ring (*m/z* of 160.0432). Therefore, $P_{338}$ had a similar structure with AMP and AMPA, suggesting that both of these two moieties might not involve the oxidation reaction. Based on the structural characteristics and $MS^2$ spectral information, $P_{338}$ seems to be generated through the oxidation of hydrolyzed β-lactam ring moiety, and the proposed structure is presented in Table 1 and Fig. SI-8.

The generation of $P_{338}$ suggested a different Cu(II)-promoted degradation pathway of AMP from that of previously investigated β-lactam antibiotics. In the Cu(II)-catalyzed hydrolysis of benzylpenicillin, phenylacetamide was observed as the oxidation product at pH higher than 7 from the cleavage of the amide side chain. But AMP did not generate the 2-amino-2-phenylacetamide product (*m/z* around 150) to a comparable extent. Meanwhile, during Cu(II) oxidation of cefalexin, an oxidized product, benzamide (*m/z* around 121) was observed, which was proposed to be generated from the Cu(II) oxidation of the phenylglycine side chain. However, this product was also not found in AMP oxidation. Notably, this result provided a strong evidence for the proposed oxidative role of Cu(II) on AMP degradation, and furthermore, the

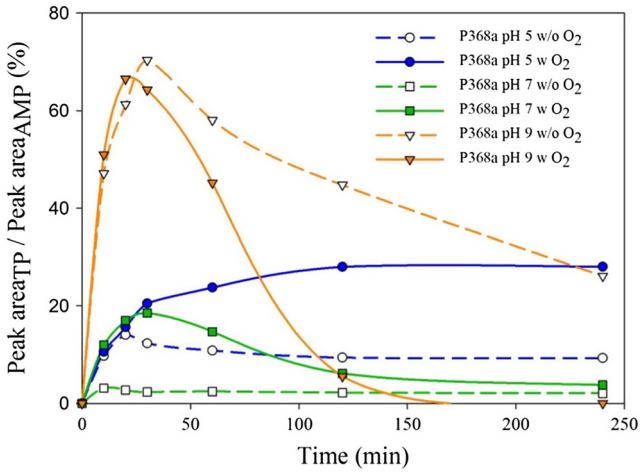



**Fig. 5** The evolution of $P_{368}$ in Cu(II)-promoted degradation of AMP at different pH and oxygen conditions. Conditions: $[Cu(II)]_0 = [AMP]_0 = 100$ μM

oxidation site was the β-lactam ring rather than the phenylglycine side chain.

Besides, $P_{366}$ (*m/z* of 366.1126) was detected at all the three pH values and its formation was more obvious at oxygen-limited conditions. The chemical formula of $P_{366}$ corresponds to $C_{16}H_{20}N_3O_5S^+$ as shown in Table 1 and Fig. SI-9. The corresponding $MS^2$ results illustrate that $P_{366}$ still contained phenylglycine side chain and thiazolidine ring moiety. The formation of $P_{366}$ might involve the addition of a hydroxyl moiety on the β-lactam structure of AMP molecule (366 = 350 + 17–1). Furthermore, $P_{322}$ (*m/z* of 322.1128) was observed at pH 9.0 in oxygen-limited condition. Based on the chemical formula of $C_{15}H_{20}N_3O_3S^+$ and $MS^2$ fragment structures, $P_{322}$ seemed to be the secondary product of $P_{366}$ generated from the cleavage of carboxylic acid moiety from $P_{366}$ (322 = 366–45 + 1).

## Impact of oxygen and pH on the proposed reaction mechanism

Figure 5 illustrates the evolution of $P_{368}$, as the main intermediate product, under the three pH values with/without oxygen in the solution. The concentration of $P_{368}$ showed an increase followed by a decreasing trend with increasing reaction time. At pH 9.0, the abundance of $P_{368}$ showed the fastest increase during the first 20 min followed by the fastest decrease with prolonging reaction time compared to pH 5.0 and 7.0. The accumulation of $P_{368}$ was also faster at pH 5.0 than at pH 7.0. Meanwhile, pH 9.0 led to the maximum $P_{368}$ generation amount, followed by pH 5.0 and pH 7.0, which was consistent with the parent AMP degradation at the three pH values (Fig. 1). Furthermore, the presence of oxygen enhanced the yields of $P_{368}$ at pH 5.0–9.0. The degradation of $P_{368}$ was significantly accelerated at alkaline conditions in oxygen-rich condition. The evolution of other intermediates at different pH values and oxygen concentrations isalso presented in Fig. SI-6. All of these results indicated that the pH value and oxygen concentration impose a substantial effect on Cu(II)-catalyzed degradation of AMP.

In general, the pH value affects AMP degradation in the following aspects: (1) Solution pH determines the dissociation of AMP, thus the protonation of the primary amine group on the phenylglycine side chain and the deprotonation of

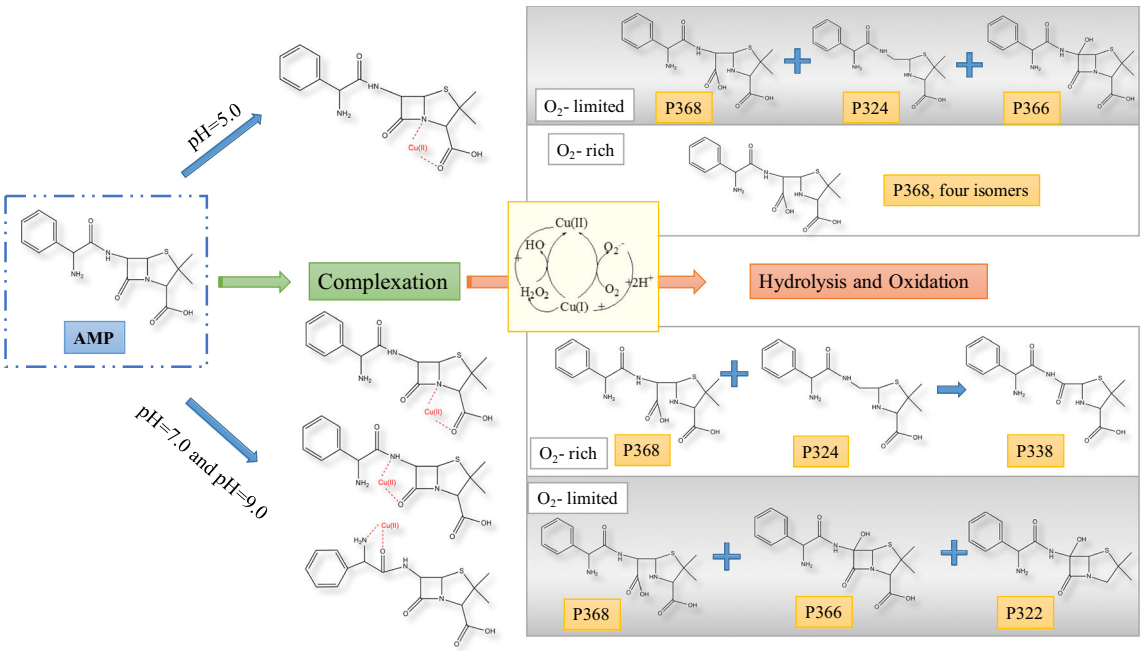



**Fig. 6** Proposed pathway of Cu(II)-catalyzed degradation of AMP

carboxyl group in the dihydrothiazine ring, which will affect the complexation with Cu(II). At pH 5.0, Cu(II) complexation may only involve the carboxyl group at the thiazolidine ring and the tertiary nitrogen on the β-lactam ring. At pH 7.0 and 9.0 where the primary amine group is partially or completely deprotonated, Cu(II) complexation involves the primary amine group and the C=O group close to it. (2) Solution pH influences the redox cycle of Cu(I)–Cu(II) and the generation of ROS species in the presence of oxygen. The overall oxidation rate of Cu(I) increases with the increasing pH. At acidic pH, Cu(I) reacts with superoxide radical to generate Cu(II) and $H_2O_2$. The reaction of Cu(I) and $H_2O_2$ also happens in acidic condition and generated hydroxyl radical. As a result, the hydrolysis of the Cu(II)-AMP complex led to the formation of AMPA, a β-lactam ring open product, which was faster at pH 9.0 followed by pH 5.0 and 7.0. The reason for the difference in hydrolysis rate may be attributed to the difference in the complex site at different pH values. At the same time, the different complex sites resulted in the different oxidation products. For example, in oxygen-rich condition, $P_{338}$ only existed at pH 7.0 and 9.0, but not at pH 5.0.

Oxygen concentration affects the AMP degradation in the following aspects: (1) The presence of oxygen in abundance can re-oxidize Cu(I) back to Cu(II). The AMP degradation can be consequently enhanced by increasing concentration of Cu(II). Thus, the Cu(II) from Cu(I) oxidation by oxygen sustained the fast degradation of AMP. (2) The involvement of oxygen produces reactive oxygen species during the copper redox reaction. The reactive oxygen species, including $HO^{\cdot}$ and $O_2^{\cdot-}$, can oxidize AMP and its hydrolysis products, which promote the AMP degradation in comparison to the oxygen-limited conditions. For instance, $P_{366}$ was detectable in oxygen-limited condition rather than oxygen-rich condition. The difference in reaction products in the presence and absence of oxygen support the different reaction schemes.

Based on the results of degradation kinetics and the identified products, a reaction scheme demonstrating the Cu(II)-catalyzed AMP degradation is proposed and shown in Fig. 6. The presence of Cu(II) catalyzed the hydrolysis of AMP to form AMPA as the major product. In oxygen-limited condition, the further degradation of AMPA generated $P_{366}$ and $P_{322}$. In the oxygen-rich condition, AMPA could undergo oxidation and yield $P_{338}$ at pH ≥ 7, whereas it proceeded with merely isomerization at pH 5.0. Therefore, Cu(II) performed two roles in AMP degradation. On one hand, the complex with AMP enhanced the hydrolysis of AMP to generate AMPA. On the other hand, Cu(II) oxidized AMP and AMPA to generate oxidized products. The Cu(II)-catalyzed hydrolysis of AMP was similar to benzylpenicillin with the β-lactam ring opening. However, the oxidation of AMP involved the β-lactam rather than the thiazolidine ring and phenylglycine side chain, which was different from either benzylpenicillin or cefalexin reported previously.

## Conclusions

The Cu(II)-catalyzed degradation of AMP was pH- and dissolved oxygen-dependent. The AMP degradation was the highest at pH 9.0, followed by pH 5.0 and 7.0. The alkaline condition promoted the deprotonation of the amine moiety on AMP, which was favorable for complexation with Cu(II). Although the complex at pH 5.0 was weak, acid-catalyzed hydrolysis was dominant. The complexation of Cu(II) with phenylglycine primary amine of AMP was essential to proceed with the oxidation reaction, which only occurred at pH ≥ 7.0. Dissolved oxygen accelerated AMP degradation, via both Cu(I)/Cu(II) redox cycle and the generation of reactive oxygen species. LC-QTOF-MS analysis indicated the hydrolysis and oxidation prefer to involve β-lactam ring rather than thiazolidine ring or phenylglycine primary amine group of AMP. These results were different from other reported β-lactam antibiotics, such as cefalexin and penicillin G. Therefore, pH and oxygen affect the hydrolysis and oxidation process on Cu(II)-catalyzed degradation of AMP, which should be considered in the prediction of AMP environmental fate and transformation products.

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