ISOLATION OF A CEPHALOSPORINASE INHIBITOR DERIVED FROM CLOXACILLIN SULFONE IN THE PRESENCE OF PHOSPHATE ANIONS

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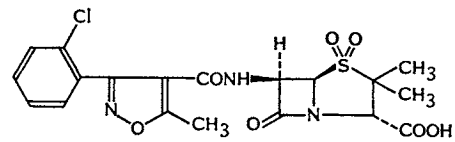
In a previous study[1], we demonstrated that cloxacillin sulfone (**1A**) is a powerful mechanism-based inactivator for TEM-type penicillinases, but not for *Citrobacter freundii* cephalosporinase. On the other hand, we found the interesting phenomenon that **1A** was converted into a specific inhibitor for the cephalosporinase during its incubation with phosphate anions. This finding was originally referred to as a delayed inactivation phenomenon because the inhibitory effect of the specific inhibitor appeared just after completion of **1A** degradation[2]. We called the specific inhibitor the "secondary inhibitor". The phenomenon was assumed to be due to the compound(s) derived from **1A** through a phosphate-mediated reaction. The reasons for this assumption were as follows: 1) The inhibitor was not produced in the absence of phosphate anions. 2) When **1A** was preincubated with phosphate anions, the secondary inhibitor activity was detected even after the free anions had been removed from the reaction mixture. 3) The level of the inhibitory activity increased with increasing phosphate anion concentration and increasing preincubation period.

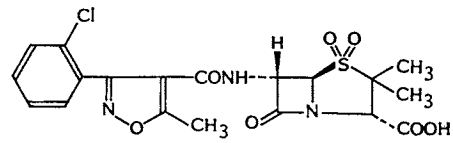
In this study, we tried to isolate the secondary inhibitor after prolonged incubation of **1A** with phosphate anions.

Cephalosporinase was purified from cells of *C. freundii* GN346 as previously described[3]. **1A** (Fig. 1) was synthesized from cloxacillin *via* cloxacillin sulfoxide and then purified by HPLC, as reported previously[1]. The 6-epimer of **1A** (**1B**) (Fig. 1) was synthesized from cloxacillin sulfoxide as follows: Cloxacillin sulfoxide was epimerized in the presence of 1,5-diazabicyclo-[4.3.0]non-5-ene (DBN) by a modification of

Fig. 1. Chemical structures of cloxacillin sulfone (**1A**) and its 6-epimer (**1B**).



1A



1B

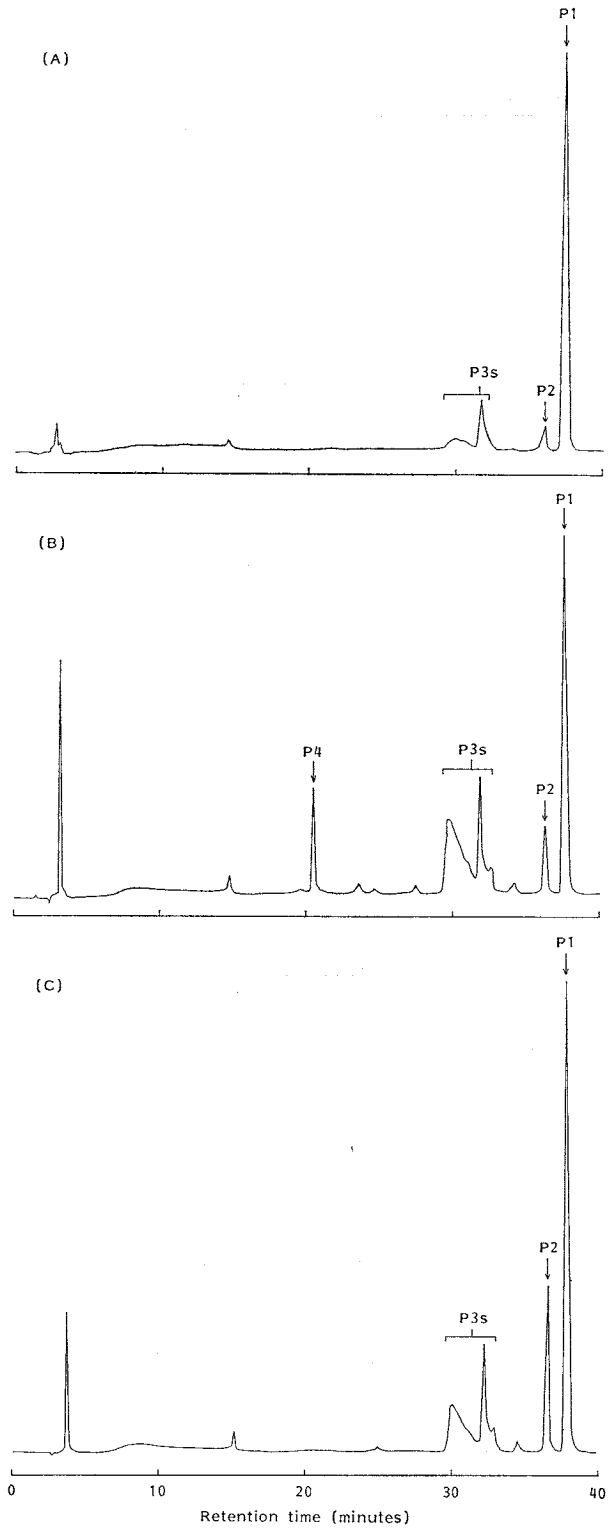
the method of PANT and STOODLEY[4]. The resulting mixture was oxidized with $KMnO_4$. **1B** was separated from **1A** by HPLC.

As shown in Fig. 2A, freshly solubilized **1A** gave one major HPLC peak, P1 (at 37 minutes) accompanied by several minor peaks, P2 (at 36 minutes) and P3s (ranging from 29 to 33 minutes). The P2 and P3s compounds were identified as **1B** and hydrolysis products, respectively, on the basis of the HPLC pattern for an authentic sample of **1B** and alkaline hydrolyzates of **1A** (data not shown).

When **1A** was incubated in 50 mM phosphate buffer (pH 7.0) at 30°C for 15 hours, the decrease in peak P1 was followed not only by concomitant increases in peaks P2 and P3s, but also by the appearance of a new peak at 21 minutes (P4) (Fig. 2B). Peak P4 did not appear when **1A** was incubated in distilled water (Fig. 2C), whereas the decrease in peak P1 followed by increases in peaks P2 and P3s was observed even in distilled water. It should be noted that **1A** preincubated in distilled water did not exhibit the secondary inhibitor activity[2]. It could therefore be presumed that the P4 compound is the secondary inhibitor. It was also confirmed that the amount of the P4 compound was largely dependent upon the concentration of phosphate anions and the length of the incubation period (data not shown).

The peak P1, P2, P3s and P4 fractions were pooled separately, then they were examined as to the secondary inhibitor activity in the absence of phosphate anions (Fig. 3). Only the P4 fraction showed strong irreversible inactivation

Fig. 2. HPLC profiles of cloxacillin sulfone (**1A**) preparations.

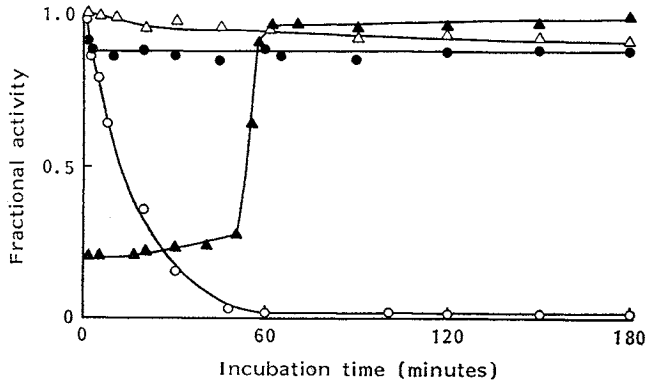


The 20-μl aliquots of **1A** solutions (5 mM) were applied to a reverse-phase C8 column (Senshupak C-8, 4.6 mm×15 cm) equilibrated with 50 mM triethylamine-AcOH, pH 6.0, followed by elution with a linear gradient of 0~40% CH_3CN for 40 minutes at a flow rate of 1.0 ml/minute. Peaks were detected by measuring the absorbance at 230 nm.

(A) Freshly dissolved **1A** in 50 mM sodium phosphate buffer, pH 7.0. (B) The **1A** dissolved as in (A) was incubated at 30°C for 15 hours. (C) The **1A** solubilized in distilled water was incubated at 30°C for 15 hours.

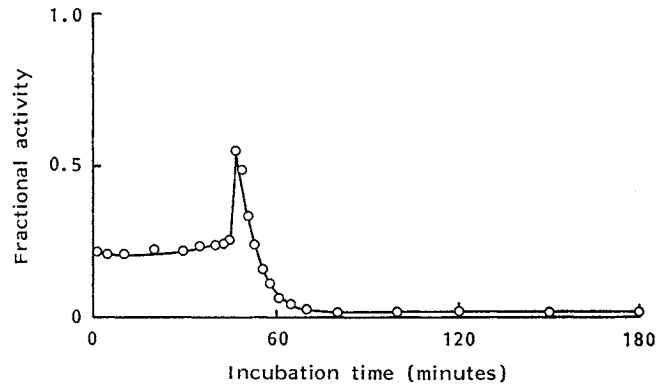
Fig. 3. Time course of *Citrobacter freundii* cephalosporinase activity when the enzyme was incubated with the peak fractions.

▲ P1, △ P2, ● P3s, ○ P4.



The 5-μM of the enzyme was incubated with 100 μl of each pooled peak fraction in 200 μl of 50 mM HEPES - NaOH buffer, pH 7.0, at 30°C. The residual enzyme activity was measured by the dilution method, as described previously[2], using 100 mM cephalothin as substrate.

Fig. 4. Time course of *Citrobacter freundii* cephalosporinase activity when the enzyme was incubated a mixture of the P1 and P4 fractions.



The assay conditions were the same as in Fig. 3 except that 100 μl of the P4 and P1 fractions, respectively, was added.

of the cephalosporinase.

When the P4 fraction was mixed with the P1 fraction (**1A**) in the ratio of 1 : 1, and then the cephalosporinase was added to the mixture in the absence of phosphate anions, the delayed inactivation phenomenon was seen (Fig. 4). This result also indicated that the P4 compound is really the secondary inhibitor. Phosphate anions may be required in the medium only for the formation of the secondary inhibitor, and are not necessary for the inhibitor to inactivate the enzyme. The lag in the appearance of the secondary inhibition in the presence of **1A** may be due to competition of the inhibitor with **1A** at the active site on the enzyme.

It was suggested by the results of preliminary experiments that the secondary inhibitor molecule contains a covalently-bound phosphate. The only group of β-lactamase inhibitors containing covalently-bound phosphate so far reported are the monophosphams[5], which are derivatives of monocyclic β-lactams. However, our IR analysis results indicated that the secondary inhibitor has no β-lactam ring, suggesting that it belongs to a new group of β-lactamase inhibitors.

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