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Synthesis of a novel β-lactamase hydrolysis resistant penicillin analog

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من المعتقد أن المتبقي Lys^{234} residue يساهم في تحفيز خميرة بيتالاكتاميز فهو يؤدي دور المرتكز الإلكتروستاتي لمجموعة الكربوكسيلات المرتبطة بذرة الكربون الثالثة في جزئ البنيسيللين. وتهدف هذه الدراسة إلى اختبار دور مجموعة الكربوكسيلات الموجودة على ذرة الكربون الثالثة في الارتباط بالإنزيم. ولهذا الغرض فقد تم تحضير مشتق بنسلين جديد هو 3-أمينوميثيل-6-فينيل أسيتاميدو بنسيلينات حيث تم فيه استبدال مجموعة الكربوكسيل الموجودة على ذرة الكربون الثالثة بمجموعة أمينية. وقد تحقق ذلك باختزال أنهيدريد مختلط لدواء بنيسيللين G للحصول على 6-فينيل أسيتاميدوبنيسيلليناييل الكحول. وقد تم بحث سلوك هذه المجموعة الكحولية في التفاعل مع المكونات الحمضية حسب تفاعل ميتسيونوبو، وتم تحضير 3- ثنائي ثلاثي بيوتوكسيكاربونيل أمينوميثيل -6- فينايل اسيتاميدوبنيسيللينات كمركب خام. وبعد التنقية باستخدام كروماتوغرافيا العمود، فإن المركب الخام قد نزعت منه المجموعة الواقية لمجموعة الأمين ليعطي المركب المطلوب وهو 3- أمينوميثايل -6- فينايل اسيتاميدوبنيسيللينات. وقد تم تقدير ودراسة حلمأة هذا المركب بواسطة انزيم بيتالاكتاميز وانزيم بيتالاكتاميز المعدل. كما أنه قد تم التعديل في انزيم بيتالاكتاميز بتغيير المتبقي Lys^{234} residue إلى حمض الجلوتاميك المتبقي باستخدام التبدل الخلقي النوعي.

It has been suggested that Lys^{234} residue participates in β-lactamase catalysis by acting as an electrostatic anchor for the C-3 carboxylate of penicillin. The aim of the present work is to test the role of the carboxylate group at C-3 in binding with the enzyme. A novel penicillin derivative, 3-aminomethyl-6-phenylacetamidopenicillanate, was prepared in which the carboxylic acid group at C-3 was replaced by an amino group. This was achieved by the reduction of a mixed anhydride of penicillin G to obtain 6-phenylacetamidopenicillanyl alcohol. The behavior of the alcoholic function in reacting with acidic components, following Mitsunobu reaction, was investigated, and 3-di-*tert*-butoxycarbonylaminomethyl-6-phenylacetamidopenicillanate was prepared as a crude product. After purification using column chromatography, the crude product undergoes deprotection of the amino group to produce the desired compound 3-aminomethyl-6-phenylacetamidopenicillanate. The hydrolysis of this compound by β-lactamase and the altered β-lactamase was determined and studied. The alteration in β-lactamase was done by changing the lys^{234} residue to glutamic acid residue using site specific mutagenesis.

Key words: 6-Phenylacetamidopenicillanyl alcohol, 3-aminomethyl-6-phenylacetamidopenicillanate, site specific mutagenesis, Mitsunobu reaction, B-lactamase-resistance.

Introduction

The ideal antibiotic would be the one that is selectively toxic towards a parasite without affecting the host. In the case of β-lactam antibiotics, the cell wall appears to be the target that permits selective inhibition of the parasite. Bacteria have a thick cell wall, in contrary to mammalian cells, so the β-lactam antibiotic is very close to be the ideal. The bacterial cell wall consists of a rigid structure that, among other things, protects the fragile cytoplasmic membrane from the high osmotic pressure within the cell. If this cell wall has been damaged for any reason, the cystoplasmic membrane may be damaged beyond repair and, unless the cell is in a high osmotic environment, it will undergo lysis. There is evidence that β-lactam antibiotics generally exert their antibacterial activity by interfering with the synthesis of the cell wall in susceptible organisms. Because of this, β-lactam antibiotics rapidly kill dividing cells but not resting cells (1).

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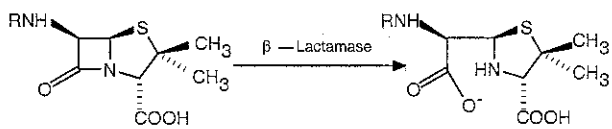
Gram-positive bacteria generally have a thick peptidoglycan (murein) structure (50-100 molecular layers) surrounding the cell membrane, whilst gram-negative bacteria have a peptidoglycan structure of only one or two molecular layers, surrounded by an outer membrane. This outer membrane constitutes a permeability barrier to the β-lactam antibiotics.

Penicillins kill gram-positive bacteria more effectively than gram-negative bacteria (1).

The highly reactive amide bond of the β-lactam ring of penicillin resembles the geometry of acylated D-alanyl-D-alanine, so the enzyme mistakenly accepts it as its normal substrate. When fixed to the transpeptidase, a very facile acylation of the transfer site would occur with opening of the β-lactam ring A penicilloyl enzyme would be formed thus inactivating the transpeptidase. Penicillin is, in fact, irreversibly bound to the "penicillin-binding component" of the bacterial cell, which is probably the protein component of a lipoprotein complex located on the outside of the cell (2,3), presumably the transpeptidase (4- 6).

Bacterial resistance to penicillin can be due to the production of enzymes known as β-lactamases, the most common being pencillinase (EC 3.5.2.6). These hydrolyze the antibiotic as shown in Scheme 1, rendering it an inactive compound.



Scheme 1. Hydrolysis of β-Lactams by β-Lactamases.

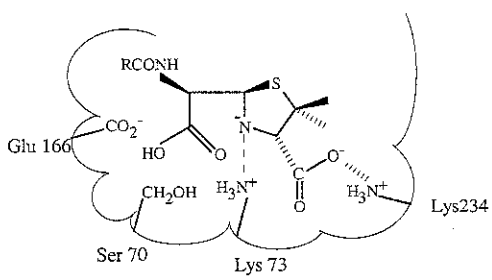


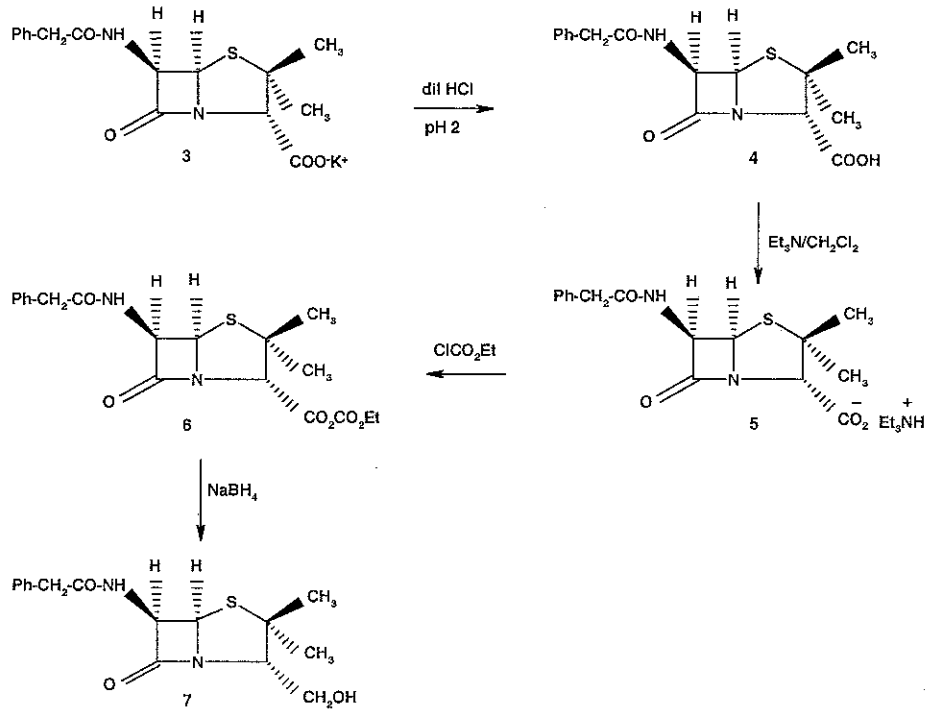
Figure 1. Mode of binding between β-Lactamases and β-Lactams.

β-Lactams have been in clinical use for about 50 years. During that period the ability to produce β-lactamases has become widespread amongst pathogenic bacteria through the mechanism of plasmid exchange (7-9). As a result, the usefulness of the first generation of β-lactam antibiotics has been considerably reduced. A detailed knowledge of β-lactamase structure will be very important in improving the design of β-lactam antibiotics which will bypass this defense mechanism.

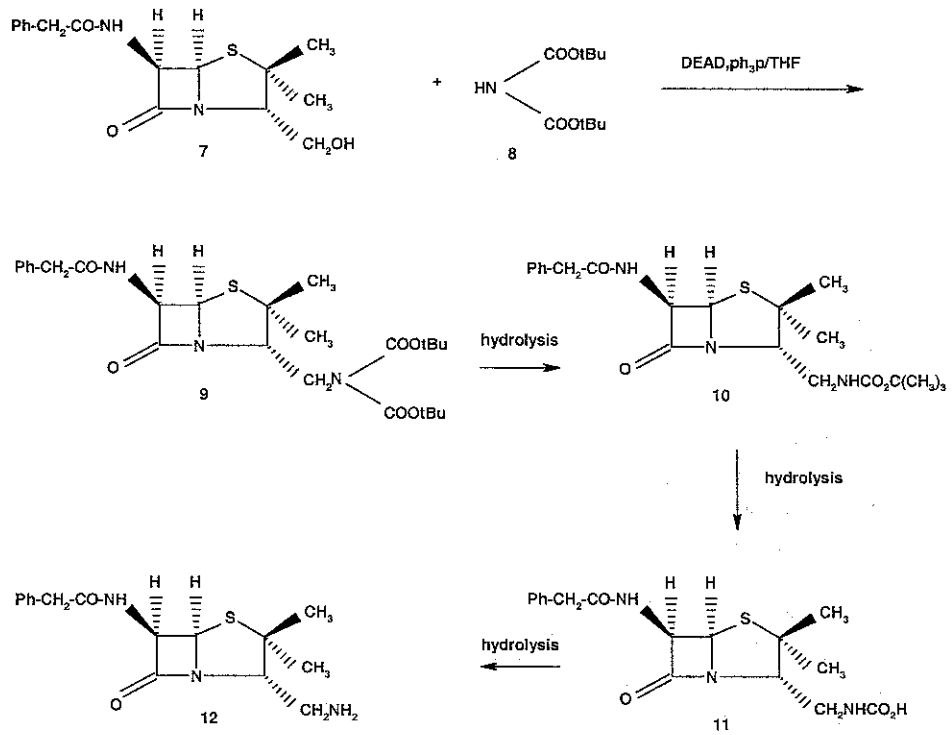
The size of the depression in the enzyme surface, the position of the catalytically important group and the other side chains, and the relatively rigid nature of β-lactam molecules have suggested a mode of binding for these substrates. The following points summarize the bonding between the enzyme and the substrate as shown in Figure 1. (10-13).

1) The carbonyl-carbon atom of the β-lactam ring must approach O_γ of Ser^{70} in order to form the acyl enzyme compound. 2) The carboxyl group on the thiazolidine ring, must make an appropriate electrostatic interaction with the enzyme. The only candidate for this interaction is the side chain ammonium group of Lys^{234}. 3) Points 1 and 2 determine the approximate position of the β-lactam nucleus with respect to the enzyme surface. Two different orientations of the fairly rigid β-lactam ring system are possible. One of these places the carbonyl oxygen of the ring over the side chain ammonium group of Lys^{73}, the other places it over the two exposed main chain amides of Ser^{70} and Gln^{237}. After the second orientation, Lys^{73} makes no direct electrostatic interactions with the substrate but, because of its salt bridge with Glu^{166}, it has an acceptable environment, and the amino moieties of Ser^{70} and Gln^{237} interact in a satisfactory manner with the β-lactam ring carbonyl oxygen. 4) The stereochemically unhindered hydrophobic end groups on the side chain of the antibiotic interact with the hydrophobic side chains of Val^{103} and Ile^{239} (14, 15).

In this work a novel compound, 3-aminomethyl-6-phenylacetamidopenicillanate (**12**), was prepared starting from commercially available penicillin G potassium salt (**3**). The ability of β-lactamase to bind to **12** was studied.



Scheme 2. The synthesis of 6-phenylacetamidopenicillanyl alcohol (**7**).



Scheme 3. The synthesis of 3-aminomethyl-6-phenylacetamidopenicillanate (**12**).

Materials and Methods

Melting points were determined on a Kofler hot-stage apparatus. ^{1}H-nmr spectra were recorded on a Bruker WM360 (360 MHz) FT instrument. NMR data are reported by parts per million (δ) and are referenced to TMS as internal standard. The splitting pattern abbreviations are as follows: s, singlet; m, multiplet; d, doublet; br, broad; t, triplet; q, quartet; dd, doublet of doublet. ^{13}C-nmr spectra were recorded on the Bruker WM360 (90.6 MHz). Infra-red spectra were recorded on a Perkin Elmer 1720 FT instrument. Mass spectra were recorded on Kratos MS80 and MS25. UV spectra were recorded on a Philips PU8720 spectrophotometer. Optical rotation were measured on a Perkin Elmer PE241 polarimeter using a 1 dm path length micro cell. Thin layer chromatography was carried out on Merck Kieselgel 60 F_{254} pre-coated silica gel plates of thickness 0.2 mm. Column chromatography was performed using Merck Kieselgel 60 (230-400 mesh). Diethyl ether, and tetrahydrofuran were dried over lithium aluminum hydride. Dichloromethane, triethylamine were dried over calcium hydride. Petroleum ether (60-80°C) was dried over calcium chloride. All other reagents were obtained from commercial suppliers, and were used without further purification.

Synthesis of 6-Phenylacetamidopenicillanylalcohol(7):

Triethylamine (12.1 g, 16.7 ml, 120 mmol) was added to a solution of 6-phenylacetamidopenicillanic acid (**4**, 33.4 g, 100 mmol) in dichloromethane (100 ml). The resulting solution was cooled to -20 °C and a solution of ethyl chloroformate (13.0 g, 9.4 ml, 120 mmol) in dichloromethane (20 ml) was added dropwise with stirring The reaction mixture was stirred for one and half hours at -10 to -20 °C, washed with water (50 ml), brine (50 ml), and dried (Na_2SO_4). The solvent was removed *in vacuo* to give the crude mixed anhydride as a foam in 95% yield. The mixed anhydride showed the presence of the carbonyl group of the β-lactam at 1790 cm^{-1} and the mixed anhydride at 1810 cm^{-1}. The mixed anhydride was dissolved in dioxane (150 ml) and *pH* 7 phosphate buffer (3 ml) was added. The mixture cooled in an ice-bath (CARE NOT TO FREEZE) and sodium borohydride (7.56 g, 200 mmol) was added in small portions during the next 30 min. The reaction mixture was allowed to warm up slowly to room temperature and stirred at this temperature for *ca* one hour. Brine was added to the reaction mixture, which was extracted with ethyl acetate (2× 100 ml) and dried ($MgSO_4$). The solvent was removed *in vacuo* to give a foam, which was purified by column chromatography on silica gel eluting with (1:1) petroleum ether (60-80) and ethyl acetate to give 6-phenylacetamidopenicillanyl alcohol (**7**) as brittle foam. **M. p.** 170-175 °C;$[\alpha]_D^{25}$ = +186° (c=0.046, $CHCl_3$); **m.s. [+ve FAB (EtOH/ Glycerol)] m/e:** 641 $[2M+H]^+$, 466 $[2M-C_{10}H_9NO_2+H]^+$, 321 $[MH]^+$, 188 $[M-C_6H_5CH_2CONH+2H]^+$, 146 $[M-C_6H_5CH_2CONHCHCO+H]^+$; **^{1}H-nmr ($CDCl_3$) δ**: 7.33 (m, 5H, $C_6\underline{H}_5$), 6.08 (d, 1H, J=8.8 Hz, -N<u>H</u>), 5.58 (dd, 1H, J=4.2, 8.8 Hz, C-6 <u>H</u>), 5.29 (d, 1H, J=4.2 Hz, C-5 <u>H</u>), 3.85 (dd, 1H, J=4.8, 8.5 Hz, C-3 <u>H</u>), 3.70 (dd, 1H, J=4.8, 11.3 Hz, -C<u>H</u>OH), 3.63 (2H, AB_2, $PhC\underline{H}_2CO$-), 3.58 (dd, 1H, J=8.5, 11.3 Hz, -C<u>H</u>OH), 2.50 (br s, 1H, $CH_2O\underline{H}$), 1.47 (s, 3H, $C\underline{H}_3$), 1.34 (s, 3H, $C\underline{H}_3$); **^{13}C-NMR ($CDCl_3$) δ**:174.99 (-<u>C</u>ON), 170.74 (-<u>C</u>ONH), 130 ($\underline{C}_6H_5$-), 70.20 (-<u>C</u>-6), 65.84(-<u>C</u>-5), 62.83 (-<u>C</u>-2), 59.75 (-$\underline{C}H_2$OH), 58.63 (-<u>C</u>-3), 43.20 (Ph$\underline{C}H_2$-), 32.75 (-$\underline{C}H_3$), 24.90 (-$\underline{C}H_3$); **I.R. ($CHCl_3$)ν_{max}/cm^{-1}**: 3280 (NH, OH), 1770 (C=O, β-lactam ring), 1660, 1530 (C=O, amide); **$C_{16}H_{20}N_2O_3S$ (320.40)** Requires: 59.98% C, 6.29% H, 8.75% N, Found: 59.88% C, 6.11% H, 8.69% N.

Synthesis of 3-aminomethyl-6-phenylacetamido-penicillanate (12):

To a stirred solution of triphenylphosphine (0.73 g, 2.78 mmol), Diethyl azodicarboxylate (0.5 g, 0.43 ml, 2.77 mmol) and di-*tert*-butyliminodicarboxylate (**8**), (0.35 g, 2.77 mmol) in dry tetrahydrofuran (30 ml), 6-phenylacetamidopenicillanyl alcohol (**7**), (0.8 g, 2.52 mmol) was added at -20 °C. The reaction mixture was stirred at this temperature for four hours and allowed to warm up to room temperature and stirred for 48 hours. Diethyl ether (10 ml) was added and diethyl hydrazinedicarboxylate was precipitated. The solvent was removed *in vacuo* to give 3-di-*tert* butoxycarbonylaminomethyl -6- phenylacetoamido-penicillanate (**9**) as orange oil. The crude product (**9**) was purified by column chromatography three times on silica gel eluting with dichloromethane to give 3-aminomethyl-6-phenylacetamido-penicillanate (**12**) as a colorless oil. **m.s.[+ve FAB (EtOH/Glycerol)]**

m/e: 339 $[M+H_2O+2H]^+$, 321 $[MH]^{+2}$; **^{1}H-nmr ($CDCl_3$)** δ: 7.27 (m, 5H, -$C_6\underline{H}_5$), 6.02 (d, 1H, J=8.5 Hz, -N$\underline{H}$), 5.50 (dd, 1H, J=4.1, 8.5 Hz, C-6 $\underline{H}$), 5.22 (d, 1H, J=4.1 Hz, C-5 $\underline{H}$), 3.91 (dd, 1H, J=5.4, 8.23 Hz, C-3 $\underline{H}$), 3.70 (dd, 1H, J=5.4, 11.5 Hz, C$\underline{H}$NH_2), 3.55 (AB_2, 2H, PhC$\underline{H}_2$CO-), 3.51 (dd, 1H, J=8.23, 11.5Hz, C$\underline{H}$NH_2), 1.41 (s, 3H, C$\underline{H}_3$), 1.26 (s, 3H, C$\underline{H}_3$); **^{13}C-nmr ($CDCl_3$)** δ: 175.50 (-$\underline{C}$ON), 170.00 (-$\underline{C}$ONH), 130 ($\underline{C}_6H_5$-), 70.00 (-$\underline{C}$-6), 66,00(-$\underline{C}$-5), 62.70 (-$\underline{C}$-2), 60.00 (-$\underline{C}$-3), 43.20 (CH_2NH_2), 41.50 (Ph$\underline{C}H_2$-), 32.50 (-$\underline{C}H_3$), 25.00 (-$\underline{C}H_3$); **I.R. ($CHCl_3$)** ν_{max}/cm^{-1}: 3340 (NH, NH_2), 1779 (C=O, β-Lactam ring), 1660 (C=O, amide).

Compound **(10)** was isolated as orange oil when 3-di-*tert* butoxycarbonylaminomethyl -6- phenylacetoamidopenicillanate **(9)** was purified by column chromatography (one time) on silica gel eluting with dichloromethane. **m.s.[+ve FAB (EtOH/Glycerol)] m/e**: 419 $[M]^+$, 362 $[M-C(CH_3)_3]^+$, 119 $[C_6H_5CH_2CO]^+$; **^{1}H-nmr ($CDCl_3$)** δ: 7.27 (m, 5H, -$C_6\underline{H}_5$), 6.02 (d, 1H, J=8.5 Hz, N$\underline{H}$), 5.50 (dd, 1H, J=4.1, 8.5 Hz, C-6 $\underline{H}$), 5.22 (d, 1H, J=4.1 Hz, C-5 $\underline{H}$), 3.91 (dd, 1H, J=5.4, 8.23 Hz, C-3 $\underline{H}$), 3.70 (dd, 1H, J=5.4, 11.5 Hz, C$\underline{H}$NH_2), 3.55 (AB_2, 2H, PhC$\underline{H}_2$CO-), 3.51 (dd, 1H, J=8.23, 11.5Hz, C$\underline{H}$NH_2), 1.44-1.20 (overlap beaks, 15H, C$\underline{H}_3$).

Compound **(11)** was also isolated as orange oil when 3-di-*tert*-butoxycarbonylaminomethyl-6-phenyl acetamidopenicillanate **(9)** was purified by column chromatography (two times) on silica gel eluting with dichloromethane **m.s. [+ve FAB (EtOH/ Glycerol)] m/e**: 362 $[MH]^+$, 232 $[M-C_6H_5CH_2CONH+H]^+$, 189 $[M-C_6H_5CH_2CONHCHCO+H]^+$; **I.R. ($CHCl_3$)** ν_{max}/cm^{-1}: 3200 (NH), 3100 (COOH, acid), 1780 (C=O, β-Lactam ring), 1660 (C=O, amide).

Visualization of Free and Complexed Enzymes by infra red Spectrometry:

Analyses were performed using an infra red spectrometer. Inhibition reactions were carried out at 37 °C by mixing 3 μl of the 3-aminomethy-6-phenylacetamidopenicillinyl **(12)** solution (18 mM in water) with: a) 10 μl of the β-lactamase solution (1 mg/ml in 10 mM ammonium bicarbonate, *pH* 7). b) 10 μl of the altered β-lactamase solution (1mg/ml in 10 mM ammonium bicarbonate, *pH* 7). Similarly, 3 μl of penicillin G was mixed with 10 μl of the altered β-lactamase solution (1mg/ml in 10 mM ammonium bicarbonate, *pH* 7). After 3 hours of incubation, the reactions were monitored by infrared spectrophotometry.

Results and Discussion

Reaction of the alcohol with acidic components (pKa ≤ 11) in the presence of triphenylphosphine and diethyl azodicarboxylate, was first reported by Mitsunobu (16). This convenient stereospecific method was followed to prepare 3-aminomethyl-6-phenylacetamidopenicillinate **(12)** by reacting 6-Phenylacetamidopenicillanyl alcohol **(7)** with di-*tert*-butyliminodicarboxylate **(8)**. The crude ^{1}H-nmr spectrum showed a mixture of unreacted alcohol **(7)**, 3-di-*tert* butoxycarbonylaminomethyl-6-phenyl-acetamidopenicillanate **(9)**, and diethyl hydrazine-dicarboxylate which was precipitated upon addition of diethyl ether to the reaction mixture.

Attempts were made to isolate 3-di-*tert*-butoxycarbonylaminomethyl-6-phenyl - acetamida-penicillanate **(9)** from the crude reaction mixture using column chromatography, three times eluting with dichloromethane. This resulted in the appearance of 3-aminomethyl-6-phenylacetamido-penicillinate **(12)** rather than 3-di-*tert*-butoxy-carbonylaminomethyl-6-phenylacetamidopenicillan-ate **(9)**, which was confirmed by ^{1}H-nmr spectrum. Therefore, it was concluded that purification of compound **(9)** using column chroma-tography three times caused hydrolysis and cleavage of di-*tert*-butoxy carbonyl group, due to the acidic properties of silica gel, and the formation of compounds **(10)**, **(11)**, **(12)** after one, two and three times respectively, of eluting compound **(9)** on silica gel.

Binding and hydrolysis of 3-aminomethyl-6-phenylacetamidopenicillinate (12) by β-lactamase:

As mentioned before, the carboxyl group at position-3 on a β-lactam antibiotic makes an appropriate electrostatic interaction with side chain ammonium group of Lys^{234} at the β-lactamase. Using site-specific mutagenesis (17,18), the Lys^{234} residue was changed to a glutamic acid residue to investigate whether changing the carboxylic acid group at position-3 to an amino group in β-lactam would still make electrostatic interaction.

The ability of β-lactamase to hydrolyze β-lactam depends on the ability of the enzyme to make an electrostatic interaction with β-lactam. Therefore, β-lactamase could not open the β-lactam ring of 3-aminomethyl-6-phenylacetamidopenicillanate **(12)** and ultimately does not cause hydrolysis due to the absence of this electrostatic interaction between β-

lactamase and compound **(12)**. This was confirmed by infra red spectrum, which showed a strong peak at 1779 cm^{-1} corresponding to the carbonyl group of the intact β-lactam ring.

Further evidence on the significance of electrostatic interaction came from the next experiment, when the altered β-lactamase, changing the Lys^{234} moiety to glutamic acid, could not hydrolyze the β-lactam ring in 6-phenylacetamidopenicillanic acid **(4)** due to the absence of electrostatic interaction between the carboxylic acid group of glutamic acid moiety on the enzyme and the carboxylic acid group at C-3 on 6-phenylacetamidopenicillanic acid **(4)**. Whereas, the altered β-lactamase hydrolyzes the β-lactam ring of 3-aminomethyl-6-phenylacetamidopenicillanate **(12)** and an acyl–enzyme complex was formed. This hydrolysis was confirmed by infra red spectrum which showed a peak at 1680 cm^{-1} corresponding to carbonyl group of the opened β-lactam ring.

When the Lys^{234} moiety of β-lactamase was changed to glutamic acid, it makes an appropriate electrostatic interaction with the amino moiety at position C-3 of 3-aminomethyl-6-phenylacetamido-penicillanate **(12)**. This electrostatic interaction allows the ser^{70} of altered β-lactamase to approach the carbonyl-carbon atom of the β-lactam ring.

This interaction and hydrolysis confirm the necessity to have an amino group on one side and a carboxylic group on the other side of the interacting β-lactamase and β-lactam to allow the hydrolysis of β-lactam ring and the formation of the acyl-enzyme complex.

Further future microbiological and pharmacological studies will be conducted to examine whether the 3-aminomethyl-6-phenyl-acetamidopenicillanate **(12)** and other related analogs have similar antimicrobial effect compared to the known β-lacatm antibiotics.

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