IN VITRO MICROBIOLOGICAL EVALUATION OF TEI-1194 AND TEI-2012, NOVEL ANTIPSEUDOMONAL SEMISYNTHETIC PENICILLINS

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TEI-1194, sodium 6-[D-(−)-α-(coumarin-3-carboxamide)-phenylacetamide] penicillanate and TEI-2012, sodium 6-[D-(−)-α-(8-hydroxy-coumarin-3-carboxamide)-phenylacetamide] penicillanate are new semisynthetic penicillin derivatives both possessing a broad spectrum of *in vitro* antibacterial activities. Minimal inhibitory concentrations of both agents were compared with carbenicillin. TEI-1194 and TEI-2012 were clearly found to have more potent activities especially against *Pseudomonas aeruginosa* than carbenicillin. At a concentration at 6.25 μg/ml, 85~90% of a total of 50 strains of clinically isolated *P. aeruginosa* were inhibited by TEI-1194 and TEI-2012, whereas carbenicillin had no effect. Evaluation of the antibacterial activity against a series of mutants producing different levels of β-lactamases and test of the susceptibilities to some β-lactamases demonstrated that TEI-1194 and TEI-2012 had low susceptibility to various cephalosporinases. However, both compounds were susceptible to penicillinase from *Klebsiella pneumoniae* H-2 at a rate of about 15% of penicillin-G taking its absolute rate as 100.

Recently, the opportunistic infectious diseases have progressively increased and become a serious problem in chemotherapy. These have been mainly brought about by various Gram-negative bacteria such as *Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli, Enterobacter cloacae, Proteus* spp. and *Serratia marcescens, etc.* Carbenicillin (CBPC), sulbenicillin (SBPC) and recently ticarcillin were developed for the purpose of effective treatment of *P. aeruginosa*-infections. The unfortunate emergence of bacteria possessing high resistance to CBPC[1,2] has stimulated the search for newer antibiotics.

It has been known that some attempts of modification of an amino group of ampicillin (ABPC) have led to an improval of the antibacterial activity, particularly, to *Pseudomonas* spp[3,4,5]. In our laboratory, the synthesis of new α-carboxamide benzyl penicillin derivatives have been studied and the biological activities have been investigated. Among these derivatives, two new compounds, TEI-1194 and TEI-2012, having a coumarin carboxamide group at the α position of benzylpenicillin, have been demonstrated as possessing marked antipseudomonal activity compared with CBPC. In this study, the *in vitro* activities of TEI-1194 and TEI-2012 were tested against standard bacteria and also against 50 strains of clinically isolated *P. aeruginosa*. Furthermore, the levels of resistance to various β-lactamases were evaluated, thereby making use of mutants producing different levels of β-lactamase as reported in a previous paper[6].

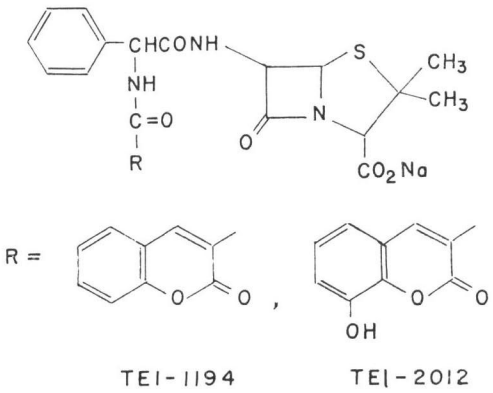
Materials and Methods

Antibiotics

TEI-1194, sodium 6-[D-(−)-α-(coumarin-3-carboxamide)-phenylacetamide] penicillanate, and TEI-2012, sodium 6-[D-(−)-α-(8-hydroxy-coumarin-3-carboxamide)-phenylacetamide] penicillanate,

shown in Fig. 1, were synthesized by chemists of the Teijin Institute for Biomedical Research[11]. Other antibiotics used for comparison were commercially obtained.

Fig. 1. Chemical structure of TEI-1194 and TEI-2012.



Media

Media employed in our study were as follows: Tryptosoya broth (TSB) was purchased from Nissui Pharmaceutical Co., Ltd., Japan. Heart infusion broth (HIB) and heart infusion agar (HIA) were products of Eiken Chemical Co., Ltd.

Strains

Strains stocked in our laboratory were used as standards except for *E. coli* K-12 ML1410 carrying RGN238 or RGN823 that were kindly supplied by T. SAWAI, Chiba University, Chiba, Japan. The clinical isolates of *P. aeruginosa* were gifts from the Kitasato Institute. Isolation and some properties of β-lactamase-mutants used in this study have been reported in the preceding paper[6].

Determination of MICs

Minimal inhibitory concentrations (MICs) were determined by an agar dilution technique. Serial two-fold dilutions of freshly prepared antibiotic solutions were mixed with melted HIA and the mixture was poured into Petri dishes.

Plates were inoculated with one loopful of 10^{-2} -fold diluted overnight culture of organisms in TSB unless specially described. The MIC values (μg/ml) were determined after 18-hour incubation at 37°C.

Determination of MBCs

Minimal bactericidal concentrations (MBCs) were determined by a standard two-fold serial dilution method with HIB as follows: An overnight culture of each strain in HIB was diluted to a final concentration of 10^4 cells/ml or 10^6 cells/ml with HIB containing serial two-fold dilutions of antibiotics. MICs were determined after incubation at 37°C for 18 hours. One loopful of each culture tube in the MIC test series was inoculated onto antibiotic-free HIA plates, and, after incubation at 37°C for 18 hours, the minimal bactericidal concentrations (MBCs) were recorded as the minimal concentration of drug destroying the ability of treated bacteria to grow on HIA plates.

Stability to β-Lactamase

The enzyme preparations were prepared as follows: Seven ml of a culture in TSB of each strain was diluted 10-fold with the same broth and incubated at 37°C. Penicillin G was added to the culture of each strain in a final concentration of 2 mg/ml for the induction of β-lactamase formation. After incubation for 3.0 hours, the grown cells were harvested and washed once with 0.1 M Na-phosphate buffer (pH 7.0). The cells were disrupted by ultrasonic oscillation. After centrifugation, the supernatant was used as crude enzyme. β-Lactamase activity was determined by a modification of SARGENT's method[7,8] unless specially described.

Specific activity was expressed as units per mg of protein per hour. Cephalothin (CET) was used a substrate for β-lactamase (cephalosporinase) from *P. aeruginosa*, *E. cloacae* or *Serratia* spp. For β-lactamase (penicillinase) from *K. pneumoniae*, penicillin G (PCG) was used as a standard substrate. Susceptibility to β-lactamase was expressed as a relative rate of the six β-lactam substrates, taking the absolute rate of CET or PCG as 100.

Inhibition of β-Lactamase

The β-lactamase assay by modified SARGENT's method[8] was carried out in the presence of 10, 20, 50, 100 and 200 μM of TEI-1194 or TEI-2012 or ABPC or CBPC as inhibitor. The activity was ex-

pressed as a relative rate of the hydrolysis of CET or PCG, taking the absolute rate in inhibitor-free assay as 100.

Results and Discussion

Antibacterial Spectrum

The spectrum of antibacterial activities of TEI-1194 and TEI-2012 against standard Gram-positive and Gram-negative organisms are shown in Table 1. As compared with CBPC, TEI-1194 and TEI-2012 had characteristically improved antibacterial activities particularly against *P. aeruginosa* including CBPC-resistant strains. Both compounds were moderately active against *Staphylococcus epidermidis* 1194, *E. coli* ML1410/RGN238 and *E. cloacae* 147 which are highly resistant to CBPC. Nevertheless, against *K. pneumoniae* H-2 and *E. coli* K-12 ML1410/RGN823 which are known to produce high level of type Ib penicillinase[9], TEI-1194 and TEI-2012 were not effective and the MICs were not less than 200 μg/ml.

In Vitro Activity against Clinical Isolates of *P. aeruginosa*

Against fifty of clinically isolated *P. aeruginosa*, the MICs of TEI-1194 and TEI-2012 were compared with those of gentamicin Cx (GM), CBPC and ABPC. The results are presented in Fig. 2. The median MICs of TEI-1194, TEI-2012 and CBPC were located at concentrations of 6.25, 1.6 and 50 μg/ml respectively (Fig. 2a). At a concentration of 6.25 μg/ml, a total of 85~90% of strains were inhibited by TEI-1194 and TEI-2012, but CBPC and ABPC did not inhibit the growth of any strain (Fig. 2b).

The antibacterial activity of GM was the highest in all the tested compounds, followed by TEI-2012, TEI-1194 and CBPC in that order. Although the activities of TEI-2012 and TEI-1194 were highly effective agaist almost all the strains at a concentration of 200 μg/ml or less, 2~3% of strains of *P. aeruginosa* remained highly resistant to these agents (MIC, higher than 400 μg/ml).

Bactericidal Activity

The *in vitro* minimal bactericidal concentration (MBC) of TEI-2012 was compared with that of CBPC and ABPC. As shown in Table 2, against *P. aeruginosa* IFO 3080, TEI-2012 seemed to possess more potent bactericidal activity than CBPC and ABPC. Similar results were

Table 1. Antibacterial spectrum of TEI-1194 and TEI-2012 against standard strains of bacteria.

Test organism	MIC (μg/ml)[a]		
	TEI-1194	TEI-2012	CBPC
Staphylococcus aureus FDA 209P	≤0.4	≤0.4	≤0.4
S. aureus D-1	50	50	50
Staphylococcus epidermidis 1194	25	50	>200
Bacillus subtilis ATCC 6633	≤0.4	≤0.4	≤0.4
Escherichia coli K-12 NIHJ	≤0.4	≤0.4	≤0.4
E. coli K-12 ML 1410/RGN 238	50	6.25	>200
E. coli K-12 ML 1410/RGN 823	200	200	>200
E. coli LA 290/R 55	3.13	12.5	>200
Klebsiella pneumoniae H-2	>200	>200	>200
Enterobacter cloacae 147	1.56	3.13	>200
E. cloacae IFO 12937	25	50	6.2
Proteus morganii G-2	3.13	1.56	≤0.4
Proteus rettgeri Tid-21	1.56	1.56	25
Proteus inconstans 164	1.56	3.13	≤0.4
Serratia G-18	12.5	12.5	100
Pseudomonas aeruginosa IFO 3080	0.8	0.4	12.5
P. aeruginosa O-37	50	25	>200
P. aeruginosa Tid-53	12.5	6.25	>200
P. aeruginosa GN 315	3.13	1.56	100
P. aeruginosa 130	1.56	1.56	25
P. aeruginosa C-23	12.5	12.5	>200

a) These results were obtained by means of the standard two-fold serial dilution method using agar based upon the standard method of the Japan Society of Chemotherapy.

Table 2. Minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) of TEI-2012 compared with ABPC and CBPC.

Strains tested	Cells/ml	TEI-2012		CBPC		ABPC	
		MIC[a] (μg/ml)	MBC[a] (μg/ml)	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)
P. aeruginosa IFO 3080	10^4	0.2	0.8	25	25	50	100
〃	10^6	0.4	1.6	50	50	100	100
E. coli NIHJ	10^4	0.2	0.2	0.8	0.8	1.6	3.2
〃	10^6	0.4	0.4	1.6	1.6	1.6	3.2

[a] These tests were carried out by the method described in the text.

Fig. 2. Antibacterial activity of TEI-1194 and TEI-2012 against 50 strains of *Pseudomonas aeruginosa* clinically isolated.
(a) Isolation frequency of strains inhibited.
(b) Cumulative percentage of strains inhibited.

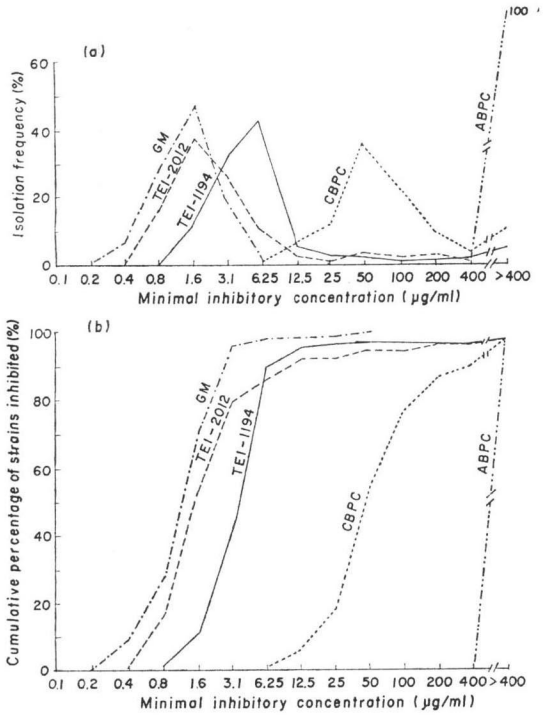
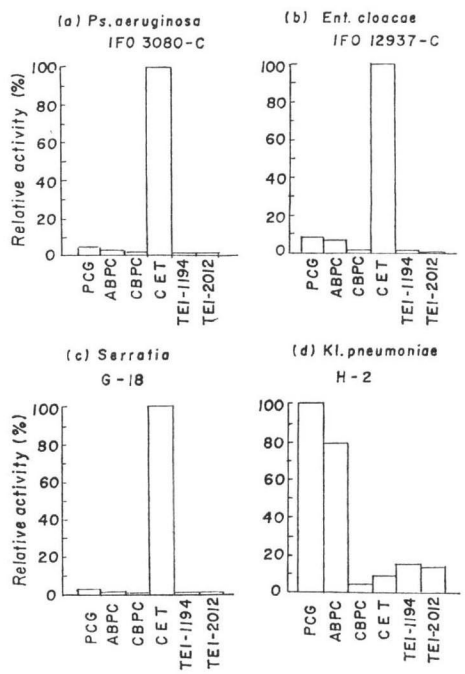


Fig. 3. Susceptibility of TEI-1194 and TEI-2012 to β-lactamases from some Gram-negative bacteria compared with PCG, ABPC, CBPC and CET.
The experimental method was described in the text.



obtained in experiments with *E. coli* K-12 NIHJ.

Resistance to β-Lactamase

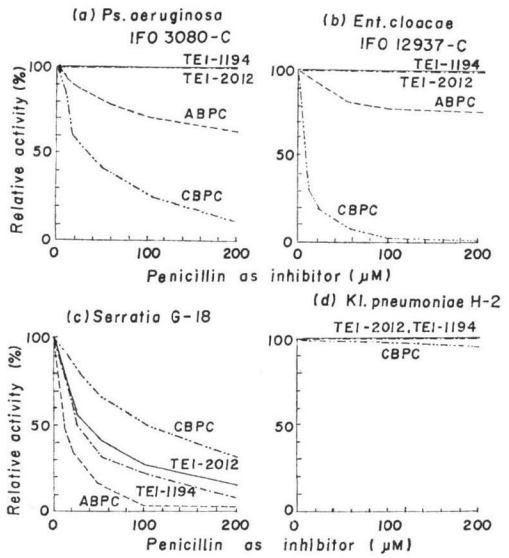
The relative rates of hydrolysis of TEI-1194 and TEI-2012 by cephalosporinases from *P. aeruginosa* IFO 3080-C, *E. cloacae* IFO 12937-C and *Serratia* G-18 were compared with those of CBPC and other agents (Figs. 3a, 3b and 3c). According to the microiodometrical method of NOVICK[10], TEI-1194 and TEI-2012 were slightly hydrolyzed by cephalosporinase from *E. cloacae* IFO 12937 C at a rate ranging from 0.1 to 0.25% of CET taking the absolute rate of the latter as 100. The levels of resistance

of TEI-1194 and TEI-2012 are almost the same as that of CBPC. On the other hand, penicillinase from *K. pneumoniae* could hydrolyze TEI-1194 and TEI-2012 at a rate of about 15% taking the absolute rate of hydrolysis of PCG as 100 (Fig. 3d). Susceptibility of various kinds of penicillinase mediated by R-plasmids remain to be elucidated and are now undertaken.

Inhibitory Effects of TEI-1194, TEI-2012 and Other Penicillins against Some β-Lactamases

For the purpose of preliminary evaluation of the affinity of TEI-1194 and TEI-2012 to the β-lactamase molecule, the inhibitory effects of both compounds were also examined. Various concentrations of TEI-1194 or TEI-2012 or ABPC or CBPC as inhibitory agent were added to the β-lactamase assay system and the relative rate of hydrolysis of PCG as substrate was determined (Fig. 4). CBPC and ABPC had inhibitory effects against the cephalosporinase produced from *P. aeruginosa* IFO 3080-C and *E. cloacae* IFO 12937-C (Figs. 4a, 4b). CBPC and ABPC gave about 60% and 20% inhibition respectively at 50 μM against cephalosporinase from *P. aeruginosa* IFO 3080-C (Fig. 4a). As presented in Fig. 4b, about 90% and 20% inhibition were obtained at 50 μM CBPC and ABPC respectively against the enzyme from *E. cloacae* IFO 12937-C. On the contrary, TEI-1194 and TEI-2012 had no inhibitory effects toward both enzymes even at 200 μM (Figs. 4a, 4b). However, TEI-1194 and TEI-2012 had more potent inhibitory-effects against cephalosporinase from *Serratia* G-18 than CBPC but their effects were poorer than that of ABPC (Fig. 4c). These tendencies are strikingly different from the results presented in Fig. 4a or 4b although the mode of substrate-specificities of the enzymes from *P. aeruginosa* IFO 3080-C and *E. cloacae* IFO 12937-C are known to be similar to that of the enzyme from *Serratia* G-18 as previously presented (Figs. 3a, 3b and 3c). Finally, TEI-1194 and TEI-2012, like CBPC were rather weak inhibitors against the enzyme from *Klebsiella pneumoniae* H-2 (Fig. 4d). The results of these studies suggest that TEI-1194 and TEI-2012 have relatively low affinities to the molecules of cephalosporinases from *P. aeruginosa* IFO 3080-C and *E. cloacae* IFO 12937-C as well as to the penicillinase from *K. pneumoniae* H-2. On the other hand, TEI-1194 and TEI-2012 might have significant affinities to cephalosporinase from *Serratia* G-18 but the precise mode of their bindings to the enzyme and the mechanism of inhibition remains to be explained.

Fig. 4. Inhibition of β-lactamases by TEI-1194, TEI-2012 and some other penicillins.
a), b) and c); CET was used as substrate. d); PCG was used as substrate.



Antibacterial Activities of TEI-1194 and TEI-2012 against Mutants Producing Different Levels of β-Lactamase

In 1977, we isolated β-lactamase-deficient mutants (L-mutants) from some clinically important Gram-negative bacteria and described about their properties[6]. These sets of the mutants containing β-lactamase-constitutive derivatives proved to be very useful tools for the direct elucidation of suscep-

Table 3. Antibacterial activities of TEI-1194 and TEI-2012 compared with ABPC, CBPC, CER and CET against the mutants producing various levels of β-lactamase.

Microorganism	Specific[a),c)] activity		MIC (μg/ml)					
	−I	+I[b)]	TEI-1194	TEI-2012	ABPC	CBPC	CER	CET
Pseudomonas aeruginosa IFO 3080	<1.8	288	1.6	0.2	100	25	>3,200	>3,200
P. aeruginosa IFO 3080-C	384	396	3.2	0.8	3,200	100	>3,200	>3,200
P. aeruginosa IFO 3080-L	<1.8	<1.8	0.8	0.2	12.5	25	50	200
P. aeruginosa Tid-53	<1.8	156	1.6	6.25	>3,200	400	>3,200	>3,200
P. aeruginosa Tid-53-C	216	294	25	50	>3,200	400	>3,200	>3,200
P. aeruginosa Tid-53-L	<1.8	<1.8	1.8	3.1	50	200	200	3,200
Enterobacter cloacae IFO 12937	1.8	372	25	50	200	6.25	200	400
E. cloacae IFO 12937-C	2,658	3,012	100	400	3,200	800	800	>3,200
E. cloacae IFO 12937-L	<1.8	<1.8	6.25	6.25	6.25	6.25	3.1	50
Serratia G-18	12	294	25	25	100	100	3,200	>3,200
Serratia G-18-L	<1.8	<1.8	1.6	1.6	12.5	12.5	25	800
Klebsiella pneumoniae H-2	90	96	>400	>400	>3,200	>3,200	25	50
K. pneumoniae H-2-L	<1.8	<1.8	12.5	25	25	25	3.1	3.1

a) β-Lactamase activity was expressed as μmoles of PCG or CET hydrolyzed per hour per mg of protein.
b) The induction was carried out with 2 mg/ml of PCG at 37°C for 3 hours.
c) PCG was used as the substrate for the assay of β-lactamase activity from *Klebsiella pneumoniae*. In other cases, CET was employed.

tibility-levels of newly synthesized and developed β-lactam antibiotics to various β-lactamases in cell systems and they made possible to evaluate the role of β-lactamase in the net resistance-level. MIC values of TEI-1194 and TEI-2012 against the series of mutants are summarized in Table 3.

In experiments with *P. aeruginosa* IFO 3080, 3080-C and 3080-L, the MICs of TEI-1194 and TEI-2012 were rather independent from the producibility of β-lactamase, whereas those of ABPC, CBPC, CER and CET were considerably affected by the levels of β-lactamase. In contrast to these results, the MICs of TEI-1194 and TEI-2012 were significantly changed in the cases of strains Tid-53, -53-C and -53-L. Interestingly, that of CBPC was not affected by β-lactamase producibility compared with TEI-1194, TEI-2012, ABPC, CER and CET. A clarification of the factor harbouring the residual level of CBPC-resistance (MIC, 200 μg/ml) in Tid-53-L was tried by an R-transfer-experiment. However transconjugation of this level of resistance to *P. aeruginosa* IFO 3080-L Rif^r *met* (MIC of CBPC, 50 μg/ml) as a recipient of an R-plasmid did not result in the isolation of a conjugate able to grow at 50 μg/ml of CBPC.

E. cloacae IFO 12937-C produces a high level of cephalosporinase even without induction and its specific activity is much higher than that of the *P. aeruginosa* IFO 3080-C and *P. aeruginosa* Tid-53-C enzymes. Working with strains *E. cloacae* IFO 12937, IFO 12937-C and -L, the MICs of TEI-1194 and TEI-2012 seem to be controlled by the producibility of cephalosporinase. This is apparently in contrast to earlier results which had demonstrated a relatively low susceptibility of both agents to this cephalosporinase as well as to the enzyme from *P. aeruginosa* IFO 1080-C (Figs. 3a and 3b). The contradiction might be explained by the difference of specific activities of the enzymes. Actual-

ly, the enzyme preparation (0.25 mg protein equivalent) from *E. cloacae* IFO 12937-C was able to degrade 1 mM TEI-2012 after incubation for 18 hours at 37°C, whereas the enzyme (0.25 mg protein equivalent) from *P. aeruginosa* IFO 3080-C could hydrolyze only 10% of 1 mM TEI-2012 under the same condition.

According to the microiodometric method, CBPC is about ten-thousands-fold more resistant to the cephalosporinase from *E. cloacae* IFO 12937-C than CET. Nevertheless, the MIC value of CBPC remains moderately high (MIC; 800 μg/ml). On this type of CBPC-resistance mechanism we would report in our next paper[11].

Against *Serratia* G-18 and its β-lactamase-deficient mutant (G-18-L), TEI-1194 and TEI-2012 were more effective than ABPC, CBPC, CER and CET. This would suggest that TEI-1194 and TEI-2012 seem to have not only resistance to cephalosporinase from *Serratia* G-18 but also improved penetration to the cells of this *Serratia* strain.

Compared with cephaloridine (CER) or CET, TEI-1194 and TEI-2012 were seem to have "latent" antibacterial activity against *K. pneumoniae* H-2 because the MICs of TEI-1194 and TEI-2012 against this strain are much higher than those of CER or CET, whereas against the β-lactamase-deficient mutant (H-2-L), the levels of MICs are not very different. Therefore, high resistance of *K. pneumoniae* to TEI-1194 and TEI-2012 is supposed to be predominantly brought about by β-lactamase produced by this strain.

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