ANTIBACTERIAL AND PHARMACOKINETIC PROPERTIES OF M14659, A NEW INJECTABLE SEMISYNTHETIC CEPHALOSPORIN

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In vitro and *in vivo* antibacterial activities and pharmacokinetics of M14659 were investigated. *In vitro* activity of M14659 was superior to that of ceftazidime against *Staphylococcus aureus*. Against Gram-negative bacteria except *Pseudomonas aeruginosa*, its activity was almost equal to that of ceftazidime. M14659 was more active against *P. aeruginosa* including multi-drug resistant strains than cefsulodin, cefoperazone or ceftazidime. Affinities of M14659 for penicillin-binding proteins (PBPs) of *Escherichia coli* and *P. aeruginosa* were 2 to 14 times higher for PBP-1A and PBP-1B than found for ceftazidime, and almost the same for PBP-3. *In vivo* activity of M14659 against *S. aureus* was superior to that of cefamandole, cefotaxime or ceftazidime. Against Gram-negative bacteria including *P. aeruginosa*, M14659 was 2 to 220 times more active than cefotaxime or ceftazidime. Plasma half-life of M14659 in mice was about 3 times longer than that of ceftazidime. M14659 administered intravenously to mice was mainly excreted in urine without metabolism, and its recovery rate was almost equal to that of ceftazidime.

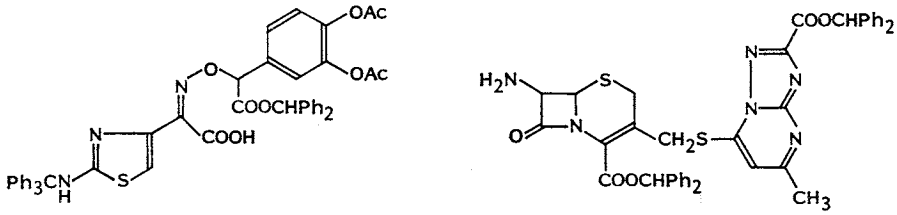
In recent years a number of cephalosporins with enhanced activity against Gram-negative bacteria has been developed[1]. Among them, ceftazidime is the most potent agent against *Pseudomonas aeruginosa*[1,2], and is often used alone in pseudomonal infections as an alternative to aminoglycosides[2]. However, ceftazidime has a notable weakness against *Staphylococcus aureus*[1], and ceftazidime-resistant strains of *P. aeruginosa* have already emerged[3,4]. Thus, there is continued interest in the development of new cephalosporins which have a balanced spectrum of activity and more potent anti-pseudomonal activity than ceftazidime. M14659 is a new injectable semisynthetic cephalosporin antibiotic which has a (2-carboxy-5-methyl-s-triazolo[1,5-a]pyrimidine-7-yl)thiomethyl group at the 3 position of the cephalosporin nucleus and a 2-(2-amino-4-thiazolyl)-2-[*Z*-[(*S*)-carboxy(3,4-dihydroxyphenyl)methyl]oxyimino]acetamido group at its 7 position (compound **3** in Scheme 1). In preliminary comparisons, M14659 was very active against Gram-positive and Gram-negative bacteria, and showed more potent antibacterial activities than ceftazidime against *S. aureus* and *P. aeruginosa* including ceftazidime-resistant strains[5]. In the present report, we further investigated *in vitro* properties, *in vivo* antibacterial activities and pharmacokinetics in mice of M14659 in comparison with those of ceftazidime.

Materials and Methods

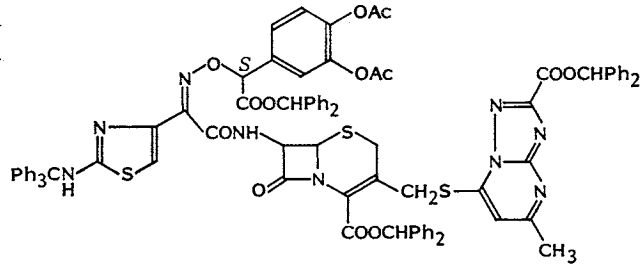
Antibiotics

M14659 was synthesized as described in Experimental. Nitrocefin was synthesized at Fuji Central Research Laboratory, Mochida Pharmaceutical Co., Ltd., Shizuoka, Japan. The other antibiotics

Scheme 1.



Compound **1**



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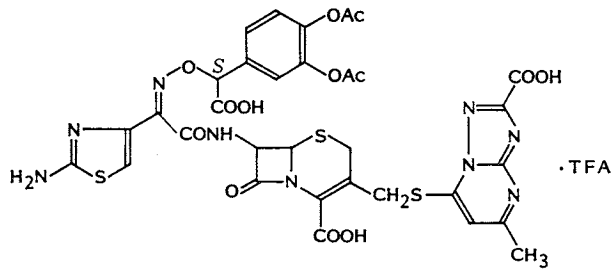

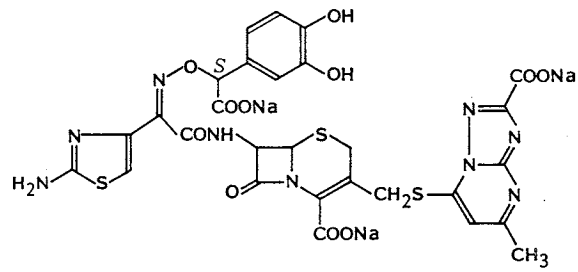

M14659 (compound **3**)

were commercially obtained from the manufactures as follows: Cefazolin, Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan; cefmetazole, Sankyo Co., Ltd., Tokyo, Japan; cephaloridine, cefamandole, Shionogi & Co., Ltd., Osaka, Japan; cefsulodin, Takeda Chemical Industries, Ltd., Osaka, Japan; cefotaxime, Chugai Pharmaceutical Co., Ltd., Tokyo, Japan; cefoperazone, Toyama Chemical Co., Ltd., Toyama, Japan; ceftazidime, Glaxo Inc., London, England.

Bacterial Strains

Standard strains of bacteria were obtained from Institute for Fermentation, Osaka, Japan. A total of 279 bacterial isolates including β-lactamase producing resistant strains of *P. aeruginosa* were recent clinical isolates collected from several hospitals in Japan.

Determination of MIC

MIC values were determined by the 2-fold agar dilution method with Mueller-Hinton medium (Difco Laboratories, Detroit, U.S.A.). Bacteria were cultured overnight at 37°C, and diluted to a final inoculum of 10^6 cfu/ml with Mueller-Hinton broth (MHB, Difco Laboratories, Detroit, U.S.A.). 5 μl of the diluted broth culture was inoculated onto the agar plates containing antibiotics with a Microplanter (Sakuma Seisakusho, Ltd., Tokyo, Japan) to give a final inoculum of 5×10^3 cfu and bacterial growth was observed 18 hours after the inoculation at 37°C. The MIC was defined as the lowest antibiotic concentration which prevented visible bacterial growth.

Determination of MBC

MBC values were determined with 18-hour broth cultures of bacteria in MHB. Bacteria were cultured overnight at 37°C, and diluted to 2×10^4 cfu/ml with MHB. Test tubes containing 1 ml of the culture dilutions and 1 ml of serial 2-fold dilutions of antibiotics were incubated at 37°C for 18 hours. After incubation, the broth dilution MIC was determined as the lowest antibiotic concentration inhibiting visible bacterial growth. 5 μl from each tube inhibiting visible bacterial growth was inoculated on agar plates with a Microplanter. After 18 hours of incubation at 37°C, MBC was determined as the lowest antibiotic concentration which prevented bacterial growth on the agar plate.

β-Lactamases

β-Lactamases obtained from *P. aeruginosa* 3R, 12R and 16R were prepared according to the method of MINAMI *et al.*[6]. Bacteria were cultured at a concentration of 5×10^8 cfu/ml in MHB at 37°C for 4 hours, and collected by centrifugation at $7,500\times g$ for 10 minutes. Then, the bacteria were sonicated and centrifuged at $13,000\times g$ for 30 minutes. The resulting supernatant was used as a crude enzyme solution. β-Lactamase obtained from *Enterobacter cloacae* was purchased from Sigma Chemical Company, St. Louis, U.S.A. β-Lactamases obtained from *Citrobacter freundii* GN 7391 and *Proteus vulgaris* 1427 were kindly provided from Dr. M. TAJIMA.

Assay of β-Lactamase

β-Lactamase assay was carried out by the spectrophotometric method of Ross and O'CALLAGHAN[7]. The decrease in absorbance of the substrate at an appropriate wavelength was measured in a temperature-controlled spectrophotometer (Hitachi Model 220A, Tokyo, Japan) at 30°C. The absorption maxima of the cephalosporins as follows: Cephaloridine, 260 nm; nitrocefin, 510 nm; cefotaxime, 264 nm; ceftazidime, 257 nm; M14659, 298 nm. The Vmax and *Km* were calculated using the Michaelis-Menten equation. For the determination of *Ki* by Dixon plots, rates of hydrolysis of 20 and 40 μM nitrocefin were determined in the presence of various concentrations of the inhibitor with an autoanalyzer (COBAS FARA, F. Hoffmann-La Roche & Co., Basel, Switzerland). In these experiments enzyme was preincubated with the inhibitor for 5 minutes before the addition of nitrocefin.

Affinity for Penicillin-binding Proteins (PBPs)

Membrane fractions were prepared from *E. coli* NIHJ JC-2 and *P. aeruginosa* IFO 3445 exponentially grown in Antibiotic Medium No. 3 (Difco Laboratories, Detroit, U.S.A.) according to the method of SPRATT[8], and suspended at 15 mg/ml in 0.05 M sodium phosphate buffer. 30 μl of the membrane preparation was preincubated with 3 μl of either 0.05 M phosphate buffer or dilutions of nonradioactive antibiotics for 10 minutes at 30°C. 3 μl of [^{14}C]benzylpenicillin (162 μCi/ml, specific activity;

54 mCi/mmol, Amersham International plc, England) was added and incubated for further 10 minutes at 30°C. The binding was terminated by the addition of 2 μl of 15% (w/v) Sarkosyl solution containing 45 mg unlabeled benzylpenicillin per ml (Banyu Pharmaceutical Co., Ltd., Tokyo, Japan). After the suspension was allowed to stand for 20 minutes at 20°C, the inner membrane fraction (Sarkosyl-soluble fraction) was obtained by centrifugation at 14,000$\times g$ for 30 minutes at 20°C. The fraction was mixed with 15 μl of 0.2 M Tris-HCl buffer (pH 6.8) containing sodium dodecyl sulfate 3% (w/v), glycerol 30% (w/v), bromophenol blue 0.002% (w/v), and 4.5 μl of 2-mercaptoethanol, and heated in a boiling water bath for 2 minutes. 10 μl of the solution was then subjected to slab gel electrophoresis according to the method of NOGUCHI *et al.*[9]. After electrophoresis, the gel was fixed in 300 ml of 30% methanol - 10% acetic acid for 15 hours at room temp, and washed 5 times for 0.5~1 hour each with 300 ml of the same solution. The gel was soaked in 300 ml of Enlightning (New England Nuclear, U.S.A.) with gentle shaking for 45 minutes, and dried *in vacuo* on Whatman No. 3 MM paper. A fluorogram was prepared by exposing the gel to X-ray film (Kodak X-Omat AR film) at −80°C for 3~4 weeks. The concentrations of antibiotics required for 50% binding inhibition of ^{14}C-labeled benzylpenicillin to PBPs (IC_{50}s) were determined with a Densitometer (Model CS-900; Shimadzu Corp., Kyoto, Japan) from the fluorograms.

Determination of *In Vivo* Activity

In vivo antibacterial activities of antibiotics were studied in 4-week-old male ICR strain mice (Shizuoka Laboratory Animal Center, Shizuoka, Japan). Ten mice per group were intraperitoneally infected with 2 to 8 times the 50% lethal doses of the bacteria suspended in 0.2 ml of sterile saline containing 5% gastric mucin (Nakarai Chemicals, Ltd., Tokyo, Japan). Challenge doses ranged from 7×10^4 to 3×10^8 cfu per mouse. Appropriate doses of antibiotics were administered intravenously 1 hour after infection. The 50% effective dose (ED_{50}) was calculated by the Litchfield-Wilcoxon method[10] from the number of mice surviving 7 days after infection.

Serum Protein Binding

One volume of an antibiotic solution (1,000 μg/ml) in 1/15 M phosphate buffer solution (pH 7.0) was added to 9 volumes of fresh sera of mice, rats and humans. The reaction mixture was incubated at 37°C for 1 hour with gentle shaking and then subjected to centrifugal ultrafiltration with a MPS-3 device (Amicon Corp., Denvers, U.S.A.). The protein-free ultrafiltrate, containing the unbound antibiotic, was microbiologically assayed by using *P. vulgaris* GN 5298 as a test organism.

Plasma Concentrations of Antibiotics

Antibiotics dissolved in sterile physiological saline just before use, were administered intravenously to ICR strain mice at a dose of 20 mg/kg. Blood samples of mice in groups of five were withdrawn from the heart with heparinized syringes at 1, 2.5, 5, 10, 20, 30, 40, 60, 120 and 240 minutes after administration, respectively. Plasma was separated from these heparinized blood samples by centrifuging at 1,400$\times g$ at 4°C, and 50 μl of 0.14 N hydrochloric acid was added to a portion of 100 μl of each plasma sample of M14659 for HPLC analysis. Plasma samples were stored at −80°C until analyzed. Antibiotic concentration of the plasma was determined by bioassay using *P. vulgaris* GN 5298 as a test organism. Plasma concentrations of M14659 were also determined by HPLC.

Urinary Concentrations of Antibiotics

Antibiotics dissolved in sterile physiological saline just before use, were administered intravenously to two ICR strain mice per metabolic cage at a dose of 20 mg/kg. Urine samples from five cages were collected at 0 to 3, 3 to 6, 6 to 9, and 9 to 24 hours after administration, and 400 μl of 0.5 M acetate buffer (pH 4.0) was added to a portion of 100 μl of each urine sample of M14659 for HPLC analysis. Urine samples were stored at −80°C until analyzed. Antibiotic concentration of the urine was determined as described above.

HPLC Analysis

50 μl of distilled water was added to 150 μl of the plasma sample containing 0.14 N hydrochloric acid. After centrifugation, supernatant was filtered with Millipore Column Guard (0.45 μm, Nihon

Millipore Kogyo K.K.), and the filtrate was analyzed by HPLC. Urine samples were also analyzed by HPLC after centrifugation and filtration with the membrane. A standard curve was determined by using standard antibiotics dissolved in normal mouse plasma or urine (concentrations and % recovery of standards: 0.5~200 μg/ml and 97% for plasma, 20~1,000 μg/ml and 96% for urine, respectively) and it was confirmed that there were no peaks having the same retention time as M14659 in normal mouse plasma or urine at the same time. The correlation coefficient of the standard curve was >0.999. The lower limits of HPLC sensitivity were 0.5 μg/ml for plasma, and 20 μg/ml for urine. In this HPLC analysis internal or external standards were not used. The equipments and conditions for HPLC were as follows:

Pump: TRI ROTOR-VI (Japan Spectroscopic Co.), sample injector: Model 7125 (Reodine Co.), UV detector: UVIDEC-100-VI (Japan Spectroscopic Co.), recorder: Chromatograph processor Model 8000A (System Instruments Corp.), column: TSK-GEL ODS-120A, 4×250 mm (Toyo So Co.), mobile phase for plasma: Ammonium phosphate buffer (100 mM, pH 7.0) - acetonitrile (91.5 : 8.5), mobile phase for urine: Ammonium phosphate buffer (10 mM, pH 7.0) - acetonitrile (94.0 : 6.0), flow rate: 1.0 ml/minute, wave length: 300 nm, injection volume: 20 μl (plasma), 40 μl (urine).

Pharmacokinetic Analysis

The two-compartment model was used for pharmacokinetic analysis of M14659, and the one-compartment model for ceftazidime. The plasma half-lives ($T_{1/2}(\alpha)$ and $T_{1/2}(\beta)$), the total body clearance (CL_{tot}), and the area under the plasma concentration versus time curve extrapolated to time infinity (AUC) were calculated by means of residual method and the following equations:

$$T_{1/2}(\alpha)=0.693/k\alpha\,, \quad T_{1/2}(\beta)=0.693/k\beta\,, \quad CL_{tot}=D/AUC\,, \quad AUC=C_0/k\beta\,,$$

where D represents the dose, C_0 is the extrapolated initial plasma concentration, and $k\beta$ is the elimination rate constant.

Results

Antibacterial Spectrum and *In Vitro* Activity of M14659

M14659 is a potent, broad-spectrum cephalosporin, equally active on a weight basis against *S. aureus* and *P. aeruginosa* at concentrations in the range 0.20 to 3.13 μg/ml. Enterobacteriaceae were susceptible to very low concentrations of M14659; MIC_{90} values were in the range 0.10 to 1.56 μg/ml for selected and clinical isolates (Tables 1 and 2).

M14659 has excellent anti-pseudomonal activity (MIC_{90} 0.20 μg/ml). The range of MICs was narrow for clinical isolates and suggests that the cephalosporin has considerable potential for use in pseudomonal infections. In comparative studies, M14659 was shown to be considerably more active than cefsulodin or cefoperazone in terms of MIC_{90}. Ceftazidime was active at comparable concentrations against some strains but M14659 had greater activity against some ceftazidime-resistant isolates. This property was further demonstrated for three β-lactamase producing *P. aeruginosa* strains (Table 3). M14659 retained its activity, whereas cefsulodin, cefoperazone, cefotaxime and ceftazidime were inactive against these isolates.

M14659 has good activity against *S. aureus* and *Staphylococcus epidermidis*; MIC_{90} 6.25 μg/ml for clinical isolates. This is less active on a weight basis than cefamandole but M14659 is more active *in vitro* than ceftazidime. All of the cephalosporins were inactive against methicillin-resistant strains.

The *in vitro* activity of M14659 against Enterobacteriaceae is typical of aminothiazolyl cephalosporins and in comparative studies, M14659 was shown to be as effective as cefotaxime or ceftazidime. Against *Enterobacter* strains, M14659 was effective against 46 isolates over a narrow concentration range, 0.02 to 1.56 μg/ml. Both cefotaxime and ceftazidime were inactive to some of these isolates

Table 1. Antibacterial spectra of M14659 and other cephalosporins against standard strains.

Organism	MIC (μg/ml)					
	M14659	Cefazolin	Cefmetazole	Cefamandole	Cefotaxime	Ceftazidime
Micrococcus luteus IFO 12708	0.78	0.78	0.20	0.05	<0.05	0.39
Staphylococcus aureus 209 P	3.13	0.39	0.78	0.39	3.13	12.5
S. aureus NIHJ IID	0.78	0.10	0.39	0.10	0.78	3.13
S. aureus Smith	0.78	0.10	0.39	0.20	1.56	6.25
S. epidermidis IFO 3762	1.56	0.20	0.78	0.20	0.78	6.25
S. epidermidis IFO 12993	3.13	0.39	1.56	0.39	0.78	6.25
S. epidermidis IFO 13889	12.5	0.39	1.56	0.39	3.13	12.5
Enterococcus faecalis IFO 12964	50	25	>100	50	>100	>100
E. faecalis IFO 12966	>100	50	>100	50	>100	>100
E. faecalis IFO 12969	100	50	>100	50	>100	>100
E. faecium IFO 12367	>100	>100	>100	>100	>100	>100
Bacillus subtilis ATCC 6633	12.5	0.10	0.39	0.05	0.78	6.25
B. subtilis NIHJ PCI 219	6.25	0.20	0.78	3.13	1.56	6.25
Escherichia coli NIHJ JC-2	0.20	1.56	0.78	1.56	0.10	0.39
E. coli IFO 13168	0.05	1.56	0.78	0.20	<0.05	0.05
Salmonella enteritidis IFO 3313	<0.05	3.13	0.39	0.39	0.10	0.10
Citrobacter freundii IFO 12681	0.78	25	25	6.25	0.78	0.78
Klebsiella pneumoniae IFO 3317	<0.05	0.78	0.39	0.10	<0.05	0.05
K. pneumoniae FDA PCI 602	<0.05	0.78	0.39	0.20	<0.05	0.10
Enterobacter aerogenes IFO 13534	<0.05	25	>100	1.56	0.10	0.39
Serratia marcescens IFO 3736	0.05	>100	1.56	1.56	0.10	<0.05
S. marcescens IFO 3759	0.05	>100	6.25	25	0.20	0.05
S. marcescens IFO 12648	0.78	>100	6.25	50	0.20	0.10
Morganella morganii IFO 3848	0.39	0.78	0.78	0.78	0.05	0.39
Proteus mirabilis IFO 3849	<0.05	6.25	3.13	1.56	0.05	0.05
P. vulgaris IID HX-19	0.05	12.5	1.56	0.39	<0.05	<0.05
P. vulgaris GN 5298	0.20	100	1.56	25	<0.05	0.05
Vibrio parahaemolyticus IFO 12711	100	25	6.25	25	50	100
Acinetobacter calcoaceticus IFO 12552	6.25	>100	100	100	12.5	6.25
Pseudomonas aeruginosa IFO 3445	1.56	>100	>100	>100	12.5	1.56
P. aeruginosa No. 13	1.56	>100	>100	>100	12.5	1.56
P. aeruginosa No. 19	0.20	>100	>100	>100	25	3.13
Flavobacterium meningosepticum IFO 12535	>100	>100	12.5	>100	25	>100

Inoculum size: 5×10^3 cfu.

Table 2. *In vitro* antibacterial activities of M14659 and other cephalosporins against clinical isolates.

Organism (No.)	Antibiotic	MIC (μg/ml)[a]		
		50%	90%	Range
Staphylococcus aureus (14)	M14659	1.56	6.25	1.56 ~50
	Cefamandole	0.78	0.78	0.39 ~50
	Ceftazidime	6.25	12.5	6.25 ~>100
MRSA[b] (15)	M14659	25	>100	6.25 ~>100
	Cefamandole	12.5	25	3.13 ~50
	Ceftazidime	100	>100	12.5 ~>100
Escherichia coli (63)	M14659	0.10	0.39	0.012~6.25
	Cefotaxime	0.05	0.10	0.012~0.39
	Ceftazidime	0.10	0.20	0.024~0.78
Klebsiella sp. (50)	M14659	0.05	0.39	0.012~0.78
	Cefotaxime	0.024	0.05	0.012~0.20
	Ceftazidime	0.10	0.20	0.024~0.39
Serratia sp. (10)	M14659	0.78	0.78	0.39 ~3.13
	Cefotaxime	0.78	0.78	0.20 ~1.56
	Ceftazidime	0.10	0.20	0.10 ~0.78
Enterobacter sp. (46)	M14659	0.39	1.56	0.024~1.56
	Cefotaxime	0.10	3.13	≤0.006~100
	Ceftazidime	0.20	0.39	0.024~50
Citrobacter sp. (10)	M14659	0.20	1.56	0.024~1.56
	Cefotaxime	0.10	0.39	0.05 ~6.25
	Ceftazidime	0.20	0.20	0.05 ~12.5
Indole-positive *Proteus* sp. (6)	M14659	0.10	0.20	0.024~0.20
	Cefotaxime	0.05	0.20	≤0.006~0.20
	Ceftazidime	0.05	0.20	0.05 ~0.20
Indole-negative *Proteus* sp. (15)	M14659	0.012	0.10	≤0.006~0.10
	Cefotaxime	0.024	0.024	≤0.006~0.024
	Ceftazidime	0.05	0.05	≤0.006~0.05
Pseudomonas aeruginosa (50)	M14659	0.10	0.20	≤0.05 ~3.13
	Cefsulodin	3.13	50	0.78 ~>100
	Cefoperazone	6.25	100	0.39 ~>100
	Ceftazidime	1.56	6.25	0.39 ~50

[a] MIC of antibiotic that inhibited 50 and 90%, respectively, of the isolates.
[b] Methicillin-resistant *Staphylococcus aureus*.
Inoculum size: 5×10^3 cfu.

Table 3. Antibacterial activities of M14659 and other cephalosporins against β-lactamase producing resistant strains of *Pseudomonas aeruginosa*.

Organism	MIC (μg/ml)				
	M14659	CTX	CFS	CPZ	CAZ
Pseudomonas aeruginosa 3R	0.20	>100	>100	>100	50
P. aeruginosa 12R	1.56	>100	100	>100	50
P. aeruginosa 16R	0.39	>100	12.5	100	25

Abbreviations: CTX; cefotaxime, CFS; cefsulodin, CPZ; cefoperazone, CAZ; ceftazidime.
Inoculum size: 5×10^8 cfu.

at concentrations up to 100 μg/ml. This finding suggests that M14659 may have potential for use against *Enterobacter* strains which are currently resistant to other cephalosporins.

M14659 is bactericidal at concentrations close to the MIC (Table 4). MBCs of both ceftazidime and M14659 were similar for three selected isolates. These preliminary findings indicate that M14659

Table 4. MBCs and MICs of M14659 and ceftazidime.

Organism	M14659			Ceftazidime		
	MIC (μg/ml)	MBC (μg/ml)	MBC/MIC	MIC (μg/ml)	MBC (μg/ml)	MBC/MIC
Staphylococcus aureus 209 P	1.56	6.25	4	6.25	12.5	2
Escherichia coli NIHJ JC-2	0.20	0.20	1	0.20	0.20	1
Pseudomonas aeruginosa IFO 3445	0.78	1.56	2	0.39	0.78	2

MIC was determined by broth dilution method.
Inoculum size: 10^4 cfu/ml.

Table 5. Kinetics of hydrolysis of M14659 and other cephalosporins by β-lactamases obtained from various Gram-negative bacteria.

Organism	Vmax[a]					*Km* (μM)			*Ki* (μM)[b]		
	CER	NC	M14659	CTX	CAZ	CER	NC	CTX	M14659	CTX	CAZ
P. v. 1427	100	72	<0.1	23	<0.1	246	53	203	95	NT	4,010
E. c.	100	240	<0.1	<0.1	<0.1	482	220	NT	6.8	0.03	5.6
C. f. GN 7391	100	149	<0.1	<0.1	<0.1	165	66	NT	0.24	0.0004	0.37
P. a. 3R	100	1,157	<0.1	<0.1	<0.1	307	273	NT	62	0.28	16
P. a. 12R	100	2,050	<0.1	<0.1	<0.1	76	216	NT	29	0.05	10
P. a. 16R	100	2,524	<0.1	<0.1	<0.1	77	299	NT	59	0.30	16

Abbreviations: CER; Cephaloridine, NC; nitrocefin, CTX; cefotaxime, CAZ; ceftazidime, *E.c.*; *Enterobacter cloacae*, *C.f.*; *Citrobacter freundii*, *P.v.*; *Proteus vulgaris*, *P.a.*; *Pseudomonas aeruginosa*.
[a] Rates of hydrolysis of antibiotics are expressed in percent hydrolysis of cephaloridine.
[b] *Ki* values were determined by using nitrocefin as a substrate.
NT: Not tested.

should be effective as a bactericidal agent.

Kinetics of Hydrolysis

M14659 was very stable to β-lactamases from *P. vulgaris*, *E. cloacae*, *C. freundii* and *P. aeruginosa* (Table 5); ceftazidime showed similar stability in terms of relative hydrolysis rates (Vmax) compared with the labile compounds cephaloridine and nitrocefin. M14659 was more stable than cefotaxime in the presence of β-lactamase from *P. vulgaris*. M14659 showed similar affinity to ceftazidime for enzymes from *E. cloacae* and *C. freundii*. *Ki* values 6.8 μM and 0.24 μM respectively compared with 5.6 μM and 0.37 μM for ceftazidime. In the presence of *Pseudomonas* β-lactamases, M14659 showed 2 to 4-fold lower affinity compared with ceftazidime and at least 100 times lower affinity than cefotaxime.

Inhibition of PBPs

Affinities of M14659 and ceftazidime for PBPs are shown in Table 6. M14659 like ceftazidime inhibits PBP-3 preferentially at a similar IC_{50}, approximately 0.2 μg/ml, in *E. coli*. M14659 inhibited PBPs 1A and 1B of *E. coli* at lower concentrations than ceftazidime, 0.3 and 0.7 μg/ml, respectively. Additionally M14659 acts at 12 μg/ml against PBP-2. This property may be of advantage as this PBP is an essential protein like PBP-3 in *E. coli*. Similarly in *P. aeruginosa* M14659 inhibited PBPs 1A and 1B at lower IC_{50}s than ceftazidime, 0.08 and 6.0 μg/ml, respectively, and PBPs 3 and 4 at similar IC_{50}s to ceftazidime, 0.08 and 1.0 μg/ml, respectively.

In Vivo Activity of M14659

M14659 was effective in treating experimental septicaemia in mice following challenge with a range of Gram-positive and Gram-negative organisms (Table 7). *S. aureus* infections responded well, ED_{50} values for M14659 were comparable with those for cefazolin, and lower than those of cefotaxime

Table 6. Affinities of M14659 and ceftazidime for penicillin-binding proteins of *Escherichia coli* NIHJ JC-2 and *Pseudomonas aeruginosa* IFO 3445.

Organism	Antibiotic	IC_{50} (μg/ml)					
		Penicillin-binding proteins					
		1A	1B	2	3	4	5/6
E. coli NIHJ JC-2	M14659	0.254 (0.0011)	0.673 (0.0030)	12.2 (0.0545)	0.272 (0.0012)	>224 (>1.0)	>224 (>1.0)
	Ceftazidime	1.48 (0.0085)	9.46 (0.0545)	71.2 (0.410)	0.210 (0.0012)	>174 (>1.0)	>174 >(1.0)
P. aeruginosa IFO 3445	M14659	0.079 (0.0011)	5.99 (0.0800)	>74.8 (>1.0)	0.082 (0.0011)	0.973 (0.0130)	>74.8 (>1.0)
	Ceftazidime	0.132 (0.0023)	13.9 (0.240)	>57.9 (>1.0)	0.061 (0.0011)	0.966 (0.0167)	>57.9 (>1.0)

The number in parentheses is a molar ratio to ^{14}C-labeled benzylpenicillin required for 50% inhibition binding of labeled benzylpenicillin.

Table 7. *In vivo* antibacterial activities of M14659 and other cephalosporins against experimental systemic infections in mice.

Test organism (inoculum size, cfu/mouse)[a]	Antibiotic	MIC[b] (μg/ml)	ED_{50} (mg/kg)
Staphylococcus aureus Smith (3.0×10^7)	M14659	0.78	0.20
	Cefazolin	0.10	0.22
	Cefotaxime	1.56	1.18
S. aureus No. 128 (1.5×10^8)	M14659	1.56	1.59
	Cefazolin	0.39	1.35
	Cefotaxime	1.56	5.48
	Ceftazidime	6.25	20.6
MRSA 4[c] (1.0×10^8)	M14659	12.5	1.44
	Cefazolin	50	42.1
	Cefmetazole	6.25	24.6
	Cefamandole	6.25	11.8
	Cefotaxime	25	18.4
MRSA 4-2[c] (1.0×10^8)	M14659	50	2.98
	Cefazolin	100	75.8
	Cefmetazole	25	29.8
	Cefamandole	12.5	5.05
	Cefotaxime	50	20.8
	Ceftazidime	>100	>100
MRSA 58[c] (1.0×10^7)	M14659	12.5	9.79
	Cefazolin	>100	>100
	Cefmetazole	25	54.8
	Cefamandole	12.5	14.6
MRSA 242[c] (1.5×10^8)	M14659	100	2.04
	Cefazolin	100	>100
	Cefamandole	25	4.23
	Cefotaxime	50	>100
	Ceftazidime	100	>100

Table 7. (Continued)

Test organism (inoculum size, cfu/mouse)[a]	Antibiotic	MIC[b] (μg/ml)	ED_{50} (mg/kg)
Escherichia coli No. 67	M14659	0.10	0.89
(5.0×10^7)	Cefotaxime	<0.05	2.83
	Ceftazidime	0.10	4.63
E. coli No. 111	M14659	0.10	1.73
(1.0×10^5)	Cefotaxime	<0.05	4.60
	Ceftazidime	0.05	3.91
Klebsiella pneumoniae IFO 3317	M14659	<0.05	0.14
(3.0×10^8)	Cefotaxime	<0.05	2.35
	Ceftazidime	<0.05	1.59
Proteus mirabilis IFO 3849	M14659	<0.05	9.65
(3.0×10^8)	Cefotaxime	<0.05	>100
	Ceftazidime	0.10	18.8
P. vulgaris GN 5298	M14659	0.20	0.16
(7.0×10^4)	Cefotaxime	0.05	6.00
	Ceftazidime	0.10	0.97
Enterobacter sp. No. 5	M14659	0.78	0.10
(1.0×10^7)	Cefotaxime	0.20	1.77
	Ceftazidime	0.10	1.04
Enterobacter sp. No. 8	M14659	<0.05	1.04
(8.4×10^7)	Cefotaxime	<0.05	>30
	Ceftazidime	0.05	8.94
Citrobacter sp. No. 6	M14659	0.10	0.09
(1.0×10^7)	Cefotaxime	0.10	2.26
	Ceftazidime	0.20	1.73
Citrobacter sp. No. 11	M14659	1.56	0.13
(3.0×10^7)	Cefotaxime	0.10	0.91
	Ceftazidime	0.20	0.83
Serratia marcescens IFO 3736	M14659	0.05	0.19
(4.0×10^7)	Cefotaxime	0.10	42.4
	Ceftazidime	0.10	1.73
S. marcescens IFO 3759	M14659	0.05	3.68
(4.0×10^7)	Cefotaxime	0.10	71.8
	Ceftazidime	0.05	26.6
S. marcescens IFO 12648	M14659	0.78	2.88
(4.0×10^7)	Cefotaxime	0.39	78.4
	Ceftazidime	0.20	42.3
Pseudomonas aeruginosa No. 3	M14659	0.20	69.5
(3.0×10^7)	Ceftazidime	25	240
P. aeruginosa No. 13	M14659	1.56	48.9
(1.0×10^7)	Ceftazidime	1.56	218
P. aeruginosa No. 19	M14659	0.20	45.0
(3.0×10^7)	Ceftazidime	1.56	238
P. aeruginosa No. 22	M14659	0.39	51.1
(4.0×10^7)	Ceftazidime	6.25	178
P. aeruginosa No. 34	M14659	0.39	32.0
(1.0×10^8)	Ceftazidime	1.56	170
P. aeruginosa No. 35	M14659	0.20	64.8
(1.0×10^7)	Ceftazidime	3.13	244

[a] Mice were infected intraperitoneally with 0.2 ml of a bacterial suspension in saline containing 5% gastric mucin.

[b] Inoculum size: 5×10^5 cfu.

[c] Methicillin-resistant *Staphylococcus aureus*.

Table 8. Binding of M14659 and ceftazidime to serum proteins of different animal species.

Species of animal	Antibiotic concentrations (μg/ml)	Serum binding (%)	
		M14659	CAZ
Human	100	90.8±0.5	25.5±3.7
Mouse	100	63.0±0.9	4.2±1.4
Rat	100	95.4±0.1	12.0±1.3

CAZ: Ceftazidime.
Results are given as means±SE of 3 tests.

Table 9. Plasma concentrations and half-lives of M14659 and ceftazidime after single 20-mg/kg iv injections in mice.

Parameter	Time (minutes)	Bioassay		HPLC
		M14659	Ceftazidime	M14659
Plasma concentration (μg/ml)	1	113.2±23.8	47.1±6.0	101.1±10.6
	2.5	43.8±7.3	52.3±7.1	58.8±7.1
	5	28.2±2.7	26.6±4.8	48.4±1.0
	10	23.0±1.2	17.3±0.7	33.4±2.3
	20	19.2±1.0	8.7±1.8	21.1±1.3
	30	15.3±3.8	4.1±0.3	17.3±1.3
	40	12.7±2.1	2.4±0.1	11.7±0.3
	60	7.6±1.0	1.2±0.1	8.6±0.7
	120	1.3±0.1	ND	2.0±0.8
	240	ND	ND	0.4±0.3
$T_{1/2}(\alpha)$[a] (minutes)		0.6	ND	3.0
$T_{1/2}(\beta)$[b] (minutes)		26.3	10.6	29.6
AUC (μg·minutes/ml)		1,386	569	1,725
CL_{tot} (ml/minute/kg)		14.4	35.1	11.6

Results are given as means±SE for 5 mice.
ND: Not detected.
[a] Plasma half-life at α phase.
[b] Plasma half-life at β phase.

and ceftazidime. Some response to M14659 was also seen in infections due to methicillin-resistant Staphylococci (MRSA), though its MICs were relatively high; ED_{50} values of M14659 ranged from 1.44 to 9.79 mg/kg, and were lower than those of cefazolin, cefmetazole, cefamandole, cefotaxime or ceftazidime. Even in the infection due to MRSA 4-2, which has been isolated from MRSA 4 as a single colony by using an agar plate containing 25 μg of cefazolin per ml after the culture at 44°C for 8 hours to eliminate penicillinase-encoding plasmids and induces PBP-2′, M14659 was more active than the comparative compounds. Therefore, the activity of M14659 to the MRSA infections seemed to be attributable not to the heterogenicity of the isolates but to its activity *in vivo*.

Pseudomonas infections responded at ED_{50}s in the range 32 to 69.5 mg/kg M14659; ceftazidime was 3 to 5-fold less active than M14659.

M14659 was as effective as ceftazidime in protection tests using *E. coli* and *Proteus* species, and more active than ceftazidime against *Klebsiella pneumoniae* and *Serratia marcescens*. Two *Enterobacter* infections were more susceptible to M14659 than to cefotaxime or ceftazidime. This finding correlates with the low affinity of M14659 for *Enterobacter* β-lactamase and the narrow MIC range observed for clinical isolates. *Citrobacter* strains which produce a similar chromosomal β-lactamase were also effectively eradicated in experimental infections.

Table 10. Urinary excretion of M14659 and ceftazidime after single 20-mg/kg iv injections in mice.

Time (hours)	Recovery rate (%)		
	Bioassay		HPLC
	M14659	Ceftazidime	M14659
0~3	42.0±8.8	49.6±9.4	47.5±6.2
3~6	23.3±8.7	20.7±7.1	16.9±3.8
6~9	1.6±0.7	3.2±2.0	2.6±2.1
9~24	1.0±0.4	0.6±0.1	ND
0~24	67.8±3.2	74.2±5.0	67.0±2.0

Results are given as means±SE for 10 mice.
ND: Not detected.

Serum Protein Binding of M14659

The percentages of binding of M14659 to human, mouse and rat serum were 90.8, 63.0 and 95.4%, respectively. Corresponding values for ceftazidime to the respective sera were 25.5, 4.2 and 12.0%, respectively. Thus, M14659 was highly bound to the serum proteins of humans, mice and rats as compared with ceftazidime (Table 8).

Plasma Concentrations of M14659

After intravenous administration plasma concentrations of M14659 in mice were markedly higher than those of ceftazidime (Table 9), and M14659 was still detected at a concentration of 1.3 μg/ml by bioassay and 2.0 μg/ml by HPLC in mouse plasma 120 minutes after administration, when ceftazidime was not detected. Further, the plasma half-lives of M14659 were 0.6 minute at α phase and 26.3 minutes at β phase by bioassay, and 3.0 and 29.6 minutes by HPLC, respectively. AUC and CL_{tot} of M14659 were 1,386 μg·minutes/ml and 14.4 ml/minute/kg by bioassay, and 1,725 μg·minutes/ml and 11.6 ml/minute/kg by HPLC, respectively. An α phase was not observed with ceftazidime, and the plasma half-life at β phase was 10.6 minutes by bioassay. AUC and CL_{tot} of ceftazidime were 569 μg·minutes/ml and 35.1 ml/minute/kg by bioassay (Table 9).

Urinary Concentrations of M14659

Recovery of M14659 in mouse urine collected at 0 to 24 hours after administration was 67.8% by bioassay, and 67.0% by HPLC. The recovery of ceftazidime was 74.2% by bioassay (Table 10).

Discussion

A number of anti-pseudomonal cephalosporins like ceftazidime has been developed as alternative treatments for serious pseudomonal infections which have been traditionally treated with aminoglycosides alone or in combination with β-lactam antibiotics. Although ceftazidime is reported to be the most potent anti-pseudomonal agent among currently clinically used cephalosporins[1], it is also true that its anti-staphylococcal activity is poor[1]. Further, some clinical isolates of *P. aeruginosa* resistant to ceftazidime have already been reported[3,4]. Our search for a new cephalosporin with a balanced spectrum of activity and more potent anti-pseudomonal activity than ceftazidime has resulted in the development of M14659.

M14659 has a broad antibacterial spectrum covering Gram-positive and Gram-negative bacteria, similar to that exhibited by cefotaxime and ceftazidime. M14659 was more active *in vitro* than ceftazidime against clinical isolates of *S. aureus* including methicillin-resistant strains, and equipotent to ceftazidime against Gram-negative isolates excepting *P. aeruginosa*. Against clinical isolates of *P. aeruginosa*, M14659 was superior to anti-pseudomonal cephalosporins such as cefsulodin, cefoper-

azone and ceftazidime. Generally, *Pseudomonas* species are known to be resistant to β-lactam antibiotics *via* three possible mechanisms[4]: (1) Production of chromosomal or plasmid mediated β-lactamases, (2) altered permeability to β-lactam antibiotics, or (3) altered penicillin-binding proteins. Mechanism (3), however, is thought to be infrequent[4]. Our study using β-lactamase producing strains of *P. aeruginosa*, which are thought to be resistant to cefsulodin, cefoperazone and ceftazidime mainly *via* mechanism (1) or both (1) and (2), indicated that M14659 was also active against resistant strains of *P. aeruginosa*, and M14659 was very stable to the β-lactamases obtained from the strains. Further, the affinities of M14659 for the β-lactamases, which were expressed as *Ki* values, were lower than those of cefotaxime and ceftazidime. Recently, it has been proposed that the tight binding of non-hydrolyzable cephems by periplasmic β-lactamase molecules, *i.e.*, trapping, could prevent them from binding to target PBPs and create the resistance seen in certain strains of bacteria such as *Enterobacter*, *Serratia*, and *Pseudomonas*[11,12]. If this theory is true, the excellent activity of M14659 against the β-lactamase producing resistant strains of *P. aeruginosa* may be attributable to its low affinity for their chromosomal β-lactamase. Furthermore, M14659 showed greater affinity than ceftazidime for PBP-1A and PBP-1B of *E. coli* and *P. aeruginosa*. Both these factors may be important in the bactericidal activity of M14659 and its activity against *P. aeruginosa* which are resistant to other so-called third-generation cephalosporins.

In vivo activity of M14659 against experimental systemic infections due to *S. aureus* including methicillin-resistant strains was superior to that of cefotaxime or ceftazidime. Against infections due to Gram-negative bacteria except *P. aeruginosa*, M14659 was 3 to 220 times more active than cefotaxime and 2 to 20 times than ceftazidime on the basis of ED_{50} values, though MICs of M14659 against the challenge strains were similar to or higher than those of cefotaxime and ceftazidime. Furthermore, its *in vivo* activity against *P. aeruginosa* was marginally superior to that of ceftazidime, probably mainly reflecting the higher *in vitro* activity of the compound. Against the other test organisms M14659 showed a more potent therapeutic effect than might be expected in comparison of its MICs with those of ceftazidime. This might be explained by more advantageous pharmacokinetic properties of the former compound. M14659 was found more highly bound to human, mouse and rat serum proteins than ceftazidime. Plasma half-life of M14659 in mice was, however, much longer than that of ceftazidime, and M14659 at a concentration thought to inhibit many Gram-negative bacteria was still detected in mouse plasma 120 minutes after an intravenous dose of 20 mg/kg of the compound, similar to what has been reported for ceftriaxone[13]. However, the urinary excretion of M14659 in mice was as high as that of ceftazidime. These results suggest that M14659 is a long-acting compound similar to ceftriaxone, and these characteristics may contribute to the excellent therapeutic properties of M14659. However, since several β-lactam antibiotics have been reported to show strong *in vivo* activities because of their (i) synergism with serum factors[14], (ii) facilitation of phagocytosis of neutrophils through antibiotic action on bacteria[15,16], or (iii) activation of phagocyte functions[17], further studies on participation of these factors may be required to explain the good therapeutic effect of M14659 more fully.

In conclusion, these findings indicate that M14659 is a novel anti-pseudomonal cephalosporin which has a balanced spectrum of activity and promising therapeutic activity in experimental infections in mice. If preliminary findings are borne out in man, M14659 has considerable potential for the treatment of a wide range of infections and may be a valuable alternative to currently available anti-pseudomonal cephalosporins.

Experimental

NMR spectra were determined with a Jeol FX-90Q spectrometer. IR spectra were recorded on a Nicolet 5DX spectrometer. Optical rotation was measured with a Jasco DIP-181 polarimeter.

2-(2-Triphenylmethylamino-4-thiazolyl)-2-[*Z*-[diphenylmethyloxycarbonyl(3,4-diacetoxyphenyl)-methyl]oxyimino]acetic Acid (Compound 1)

Thionyl chloride (60 ml) was added to a suspension of (3,4-diacetoxyphenyl)acetic acid (51.1 g) in 105 ml of CCl_4, and the mixture was heated at 70°C for 1 hour. After cooling to room temp, *N*-

bromosuccinimide (42.3 g), CCl_4 (105 ml) and a little amount of HBr were added, and the mixture was heated for an additional 1 hour. The resulting mixture was concentrated under reduced pressure, and the residue was redissolved in CCl_4. After filtering off insoluble material, the filtrate was dissolved in 400 ml of Me_2CO and the pH of the soln was adjusted to 4.0 with satd $NaHCO_3$ aq soln under ice-cooling. The resulting mixture was extracted with $CHCl_3$. The $CHCl_3$ layer was washed with satd NaCl aq soln and dried over anhydrous Na_2SO_4. The dried soln was concentrated under reduced pressure to give 61.4 g of 2-bromo-2-(3,4-diacetoxyphenyl)acetic acid (**I**). Then, diphenyldiazomethane (39.6 g) was added to a soln of the product **I** (61.4 g) in 500 ml of Me_2CO, and the soln was stirred at room temp for 1 hour. The resulting soln was concentrated under reduced pressure and the residue was purified by silica gel column chromatography to give 48.4 g of diphenylmethyl 2-bromo-2-(3,4-diacetoxyphenyl)acetate (**II**). The product **II** (48.4 g) in 200 ml of MeCN and Et_3N (13.6 ml) were added to an ice-cooled suspension of *N*-hydroxyphthalimide (15.9 g) in 300 ml of MeCN. The mixture was stirred under ice-cooling for 1.5 hours. The resulting soln was concentrated under reduced pressure and redissolved in EtOAc. The soln was washed with H_2O, 1 N citric acid soln and with satd NaCl aq soln in that order. The washed soln was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure, and the residue was purified by silica gel column chromatography to give 15.3 g of diphenylmethyl 2-phthalimidooxy-2-(3,4-diacetoxyphenyl)acetate (**III**). To a soln of the product **III** (15.3 g) in 200 ml of CH_2Cl_2, methylhydrazine (1.34 ml) was slowly added at $-60°C$, and the mixture was allowed to stand until room temp was reached. After stirring for 2 hours, methylhydrazine (0.07 ml) was added to the mixture, followed by stirring for an additional 30 minutes. The insoluble material was filtered off, the filtrate was concentrated under reduced pressure, and the residue was purified by silica gel column chromatography to give 8.7 g of diphenylmethyl 2-aminooxy-(3,4-diacetoxyphenyl)acetate (**IV**). A soln of the product **IV** (8.7 g) in 150 ml of MeOH was added dropwise to a soln of (2-triphenylmethylamino-4-thiazolyl)glyoxylic acid (7.62 g) in 400 ml of MeOH. The mixture was stirred at room temp for 1.5 hours, and concentrated under reduced pressure to give 16.0 g of the objective compound **1**: IR (KBr) cm^{-1} 1772, 1256, 1209, 1180, 754, 701; ^1H NMR ($CDCl_3$) δ 8.9 (1H, s), 7.8~7.2 (28H, m), 6.9 (1H, s), 6.8 (1H, s), 5.9 (1H, s), 2.3 (6H, s).

Diphenylmethyl (6*R*,7*R*)-7-[2-(2-Triphenylmethylamino-4-thiazolyl)-2-[*Z*-[(*S*)-diphenylmethyloxycarbonyl(3,4-diacetoxyphenyl)methyl]oxyimino]acetamido]-3-[(2-diphenylmethyloxycarbonyl-5-methyl-s-triazolo[1,5-a]pyrimidin-7-yl)thiomethyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate (Compound **2**)

To an ice-cooled soln of the compound **1** (5.6 g) and diphenylmethyl (6*R*,7*R*)-7-amino-3-[(2 diphenylmethyloxycarbonyl-5-methyl-s-triazolo[1,5-a]pyrimidin-7-yl)thiomethyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate[18] (5.0 g) in 170 ml of CH_2Cl_2, 1.4 g of dicyclohexylcarbodiimide was added, and the mixture was stirred at room temp for 5 hours. After filtering off the insoluble material, the filtrate was concentrated under reduced pressure. The residue was dissolved in EtOAc and the material was filtered off. The filtrate was washed with satd NaCl aq soln and dried over anhydrous Na_2SO_4. The dried soln was concentrated under reduced pressure, and the residue was purified by silica gel column chromatography to give 0.73 g of the objective compound **2**: IR (KBr) cm^{-1} 1780, 1742, 1737, 1507, 1249, 1205, 1182, 700; ^1H NMR (DMSO-d_6) δ 9.7 (1H, d, J=8 Hz), 8.9 (1H, br s), 7.5~7.1 (50H, m), 6.9 (1H, s), 6.82 (1H, s), 6.78 (1H, s), 5.9 (1H, s), 5.8 (1H, dd, J=4 and 8 Hz), 5.2 (1H, d, J=4 Hz), 4.3 (2H, br s), 3.6 (2H, ABq), 2.6 (3H, s), 2.2 (6H, s).

(6*R*,7*R*)-7-[2-(2-Amino-4-thiazolyl)-2-[*Z*-[(*S*)-carboxy(3,4-dihydroxyphenyl)methyl]oxyimino]-acetamino]-3-[(2-carboxy-5-methyl-s-triazolo[1,5-a]pyrimidin-7-yl)thiomethyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic Acid Sodium Salt (M14659, Compound **3**)

To a soln of the compound **2** (0.73 g) in 3 ml of $ClCH_2CH_2Cl$, anisole (0.4 ml) and CF_3COOH (0.8 ml) were added under ice-cooling, and the resulting soln was stirred at room temp for 3 hours. Additional CF_3COOH (0.6 ml) was added and the mixture was stirred for another 30 minutes. After removing the solvent by decantation, the residue was washed with $ClCH_2CH_2Cl$ and crystallized with Et_2O to give 0.3 g of the product **V**. The product **V** (0.27 g) was suspended in 11 ml of H_2O, and the

pH of the mixture was adjusted to 8.0 with $NaHCO_3$. After stirring at room temp for 6 hours, the resulting soln was applied to a Diaion HP-10 column. The appropriate fractions eluted with H_2O were collected and lyophilized to give 0.14 g of M14659: IR (KBr) cm^{-1} 1763, 1599, 1514, 1474, 1404, 1360, 1314; ^1H NMR (D_2O) δ 7.2 (1H, s), 7.0~6.8 (4H, m), 5.7 (1H, d, J=5 Hz), 5.4 (1H, s), 5.0 (1H, d, J=5 Hz), 4.3 (2H, ABq), 3.4 (2H, ABq), 2.6 (3H, s); $[\alpha]_D^{25}$ +27.4° (c 1.0, H_2O).

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