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Studies on Anti-MRSA Parenteral Cephalosporins

IV. A Novel Water-soluble *N*-Phosphono Type Prodrug for Parenteral Administration

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A systematic approach for improving the water-solubility of anti-MRSA (methicillin-resistant *Staphylococcus aureus*) cephalosporin derivatives is described. We first tried to improve the water-solubility of 7β-[2-(5-amino-1,2,4-thiadiazol-3-yl)-2(*Z*)-fluoromethoxyimino-acetamido]-3-[(*E*)-2-(1-methylimidazo[1,2-*b*]pyridazinium-6-yl)thiovinyl]-3-cephem-4-carboxylate (**1a**) by substitution of the C-3′ pharmacophore. Replacement of the C-3′ pharmacophore with a 1-methyl-4-pyridinio group improved the water-solubility without decreasing the anti-MRSA activity. Furthermore, we applied the *N*-modified prodrug strategy to the C-7 acyl group in order to enhance the water-solubility drastically. Among the compounds prepared, the *N*-phosphono type prodrugs **2a** (1-methylimidazo[1,2-*b*]pyridazinium derivative) and **2b** (1-methyl-4-pyridinio derivative) showed water-solubility appropriate for a product intended for intravenous injection and *in vivo* anti-MRSA activity comparable to that of vancomycin.

As we reported previously[1], 7β-[2-(5-amino-1,2,4-thiadiazol-3-yl)-2(*Z*)-fluoromethoxyiminoacetamido]-3-[(*E*)-2-(1-methylimidazo[1,2-*b*]pyridazinium-6-yl)thiovinyl]-3-cephem-4-carboxylate (**1a**, Fig. 1) exhibits potent antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA), and its activity was found to be comparable to that of vancomycin (VCM) both *in vitro* and *in vivo*. In general, the recently reported anti-MRSA β-lactam derivatives, represented by **1a**, are characterized by a zwitterionic structure, which is essential for broad antibacterial activity against Gram-positive and Gram-negative bacteria and/or good *in vivo* efficacy. However, insufficient water-solubility that is ascribed to this structural feature has restricted their further development toward clinical studies[2,3].

In fact, lyophilized **1a** has low water-solubility (1.0 mg/ml), and the crystalline form of **1a** shows poor water-solubility (less than 0.05 mg/ml). Assuming that the extreme water-insolubility of **1a** was due to the nature of the C-3′ pharmacophore, we continued to search for cephalosporins with suitable cationic C-3′ pharmacophores and whose anti-MRSA activity is at least as potent as that of **1a**.

On the other hand, with the aim of finding a compound with substantially improved water-solubility, a prodrug strategy was designed. In order to avoid the restriction of administration options due to insufficient water-solubility, which is ascribed to physicochemical properties of the compound, the prodrug strategy has been applied widely in pharmaceutical development[4,5]. In cephalosporin derivatives containing a 2-aminothiazolyl or 5-amino-1,2,4-thiadiazolyl group in the C-7 acyl moiety, the amino group has been the main target for chemical modification. *N*-Carboxylation[6,7] and *N*-phosphorylation[8] of 5-amino-1,2,4-thiadiazole derivatives have been reported to be useful methods for obtaining water-soluble prodrugs. In addition, *N*-alanylation of the 2-aminothiazole moiety in ceftizoxime played an important role in the development of

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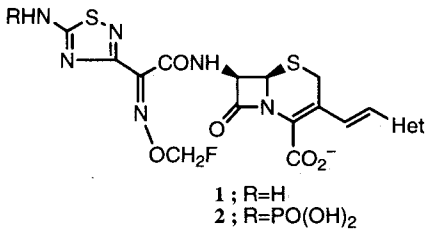
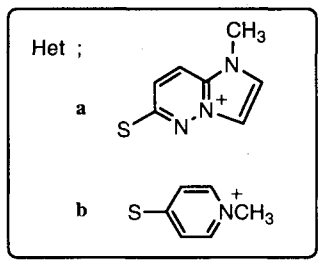
Fig. 1.





the orally active prodrug AS-924[9]. Accordingly, we focused on *N*-phosphono and *N*-sulfo derivatives, because these functions are expected to produce excellent hydrophilicity as well as physiologically acceptable properties.

In this paper, our approach for improvement of the water-solubility of the zwitterionic cephalosporins is discussed. Especially, the synthesis, antibacterial activity and pharmacokinetic profile of the novel *N*-phosphono prodrug derivatives **2a** and **2b** (Fig. 1) together with the parent compounds **1a** and **1b** are presented.

Chemistry

Preparation of 3-[(*E*)-2-(1-Methylpyridinio)thiovinyl]-7β-[2-(5-phosphonoamino-1,2,4-thiadiazol-3-yl)-2(*Z*)-fluoromethoxyiminoacetamido]-3-cephem-4-carboxylates (**1b**~**1d**) with Improved Water-Solubility

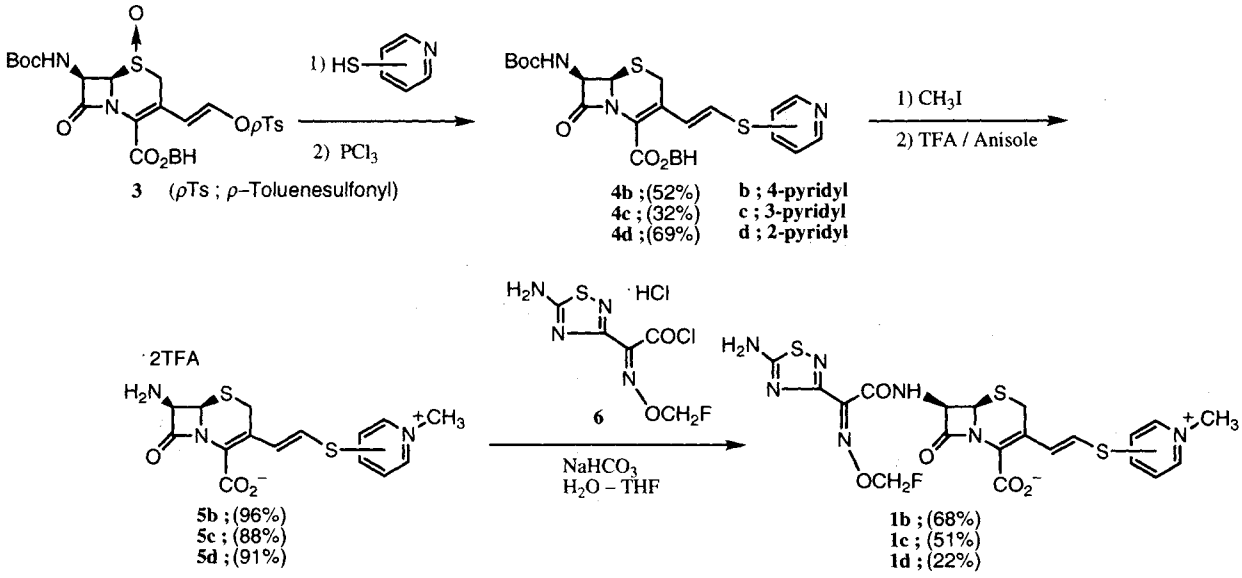
Synthesis of the 3-[(*E*)-thiovinyl] cephalosporin derivatives having various quaternary heteroaromatic rings as the C-3′ pharmacophore was accomplished by the previously reported procedure[1]. Among the derivatives prepared, only a series of 1-methylpyridinio derivatives showed improved water-solubility compared with that of **1a**, while still maintaining excellent anti-MRSA activity. Apart from our study, several literature articles[10,11] have emphasized the excellent antibacterial activity of a series of 1-methylpyridinio derivatives, but none have mentioned the physicochemical properties of the compounds. As illustrated in Scheme 1, reaction of the (*E*)-2-vinyltosylate[12] **3** with 2-mercapto-, 3-mercapto- and 4-mercaptopyridine followed by treatment with phosphorus trichloride afforded the corresponding (*E*)-2-(pyridylthio)vinyl cephem derivatives **4b**~**4d**, respectively, with complete retention of the (*E*)-configuration[13] in 32~69% yields. After methylation of **4b**~**4d** with iodomethane, removal of *t*-butoxycarbonyl (Boc) and benzhydryl (BH) groups was achieved by TFA/anisole treatment to give the corresponding 7β-amino derivatives **5b**~**5d** in good yields. Condensation of **5b**~**5d** with 2-(5-amino-1,2,4-thiadiazol-3-yl)-2(*Z*)-fluoromethoxyiminoacetyl chloride hydrochloride[14] (**6**) under aqueous conditions gave the desired 7β-[2-(5-amino-1,2,4-thiadiazol-3-yl)-2(*Z*)-fluoromethoxyiminoacetamido]-3-[(*E*)-2-(1-methylpyridinio)thiovinyl]-3-cephem-4-carboxylates (**1b**~**1d**) in 22~68% yields.

We measured the water-solubility of lyophilized **1b**~**1d** (2~5 mg/ml) and the crystalline form (1.1 mg/ml) of **1b**. Water-solubility of these analogs was improved as compared with that of **1a** but did not reach the target threshold of 10 mg/ml to satisfy the administration protocol.

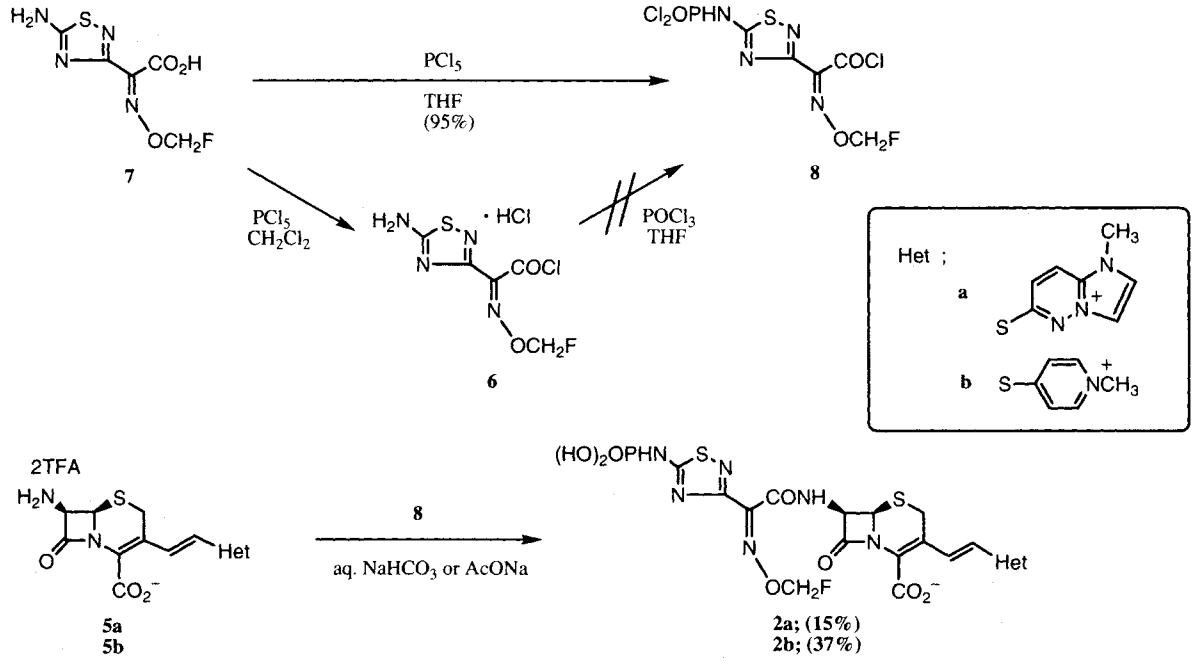
Preparation of the *N*-Modified Prodrugs of **1a** and **1b**

The novel *N*-phosphono derivatives bearing a 1-methylimidazo[1,2-*b*]pyridazinium-6-yl (**2a**) or 1-methyl-4-pyridinio (**2b**) group as the C-3′ pharmacophore were synthesized as shown in Scheme 2. The patent which covers *N*-phosphono prodrug cephalosporin derivatives having a 5-amino-1,2,4-thiadiazole moiety was issued to a group of researchers at Fujisawa in 1984[8]. According to the patent specifications and examples, the scope and limitation of the strategy are somewhat obscure for practical use. In addition, concerning the preparation of the acylating agent bearing a dichlorophosphorylamino group in the 1,2,4-thiadiazole moiety, another literature article[15] suggested suitable reaction conditions. When 2-(5-amino-1,2,4-thiadiazol)-2(*Z*)-fluoromethoxyiminoacetic acid[16] (**7**) was converted into the corresponding acid chloride **6** by treatment

Scheme 1.

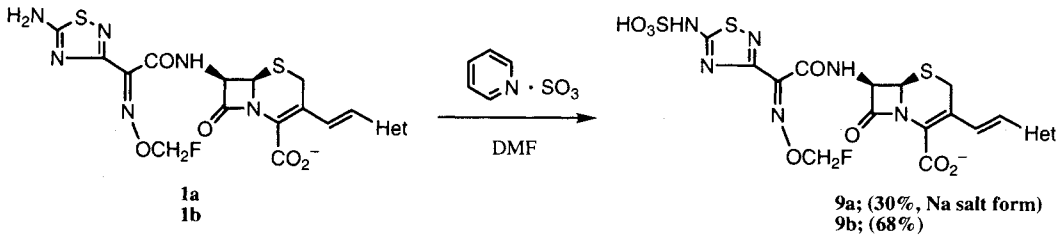


Scheme 2.



with phosphorus pentachloride in dichloromethane, substitution of the solvent with *THF* or *EtOAc* could alter the reaction mode to give 2-(5-dichlorophosphorylamino-1,2,4-thiadiazol)-2(*Z*)-fluoromethoxyiminoacetyl chloride (**8**) as the major product. Under conditions similar to those described in the literature (see experimental section), **7** was smoothly converted into **8** in a 95% yield. Although the reaction mechanism was not clear, the plausible

Scheme 3.



1a
1b

9a; (30%, Na salt form)
9b; (68%)

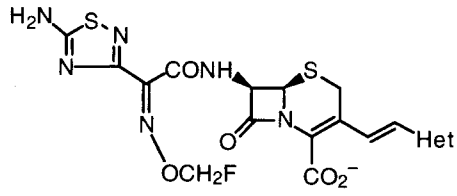
mechanism, dichlorophosphorylation of the amino group by phosphorus oxychloride generated *in situ*, was ruled out because of little conversion of the isolated acid chloride **6** into **8** upon treatment with phosphorus oxychloride in THF. The obtained dichlorophosphorylated acid chloride **8** was condensed with the 7β-amino derivatives **5a**[1] and **5b** in the presence of aqueous sodium bicarbonate or sodium acetate. During the acylation, the dichlorophosphoryl group was concomitantly hydrolyzed to a phosphono group. Column chromatographic purification of the crude product afforded the desired *N*-phosphono derivatives **2a** and **2b** in 15% and 37% yields, respectively. However, under acidic conditions, the P–N bond was too labile to get highly pure prodrugs. As a result, the lyophilized *N*-phosphono prodrugs **2a** and **2b** were inevitably contaminated (less than 3%) with the parent compounds **1a** and **1b**, respectively. Preparation of the *N*-sulfo derivatives **9a** and **9b** was achieved by direct sulfonation of the parent compounds **1a** and **1b** with pyridine sulfur trioxide in 30% and 68% yields, respectively (Scheme 3). The imidazo[1,2-*b*]pyridazinium derivative **9a** was isolated as the sulfonic acid sodium salt. In contrast to the phosphono derivatives, the *N*-sulfo derivatives **9a** and **9b** could be obtained with high purity as cleavage of the S–N bond under work-up conditions was negligible.

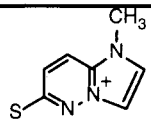
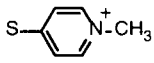
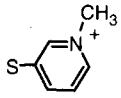
We measured the water-solubility of the prodrug-type compounds **2a**, **2b** and **9b** in their lyophilized forms, and it was found that in all cases the water-solubility was not high enough (2~5 mg/ml) and the pH of the resulting solution was too low (pH 1~2) for intravenous administration. However, when the pH of the solution was adjusted to a physiologically acceptable value by the addition of sodium bicarbonate, the solubility of the prodrug-type compounds **2a**, **2b** and **9b** was dramatically increased. Consequently, the sodium salt forms of the prodrug-type compounds **2a**, **2b**, **9b** and **9a** were found to have sufficient water-solubility (over 100 mg/ml).

Biological Results and Discussion

Table 1 shows the MICs of the 2-thiovinyl derivatives bearing a 1-methyl-4-pyridinio (**1b**), 1-methyl-3-pyridinio (**1c**) and 1-methyl-2-pyridinio (**1d**) group as the C-3′ pharmacophore. All of the derivatives **1b**~**1d** exhibited potent anti-MRSA activity. Among them, the 1-methyl-4-pyridinio derivative **1b** showed the highest anti-MRSA activity, and it was slightly superior to that of the 1-methylimidazo[1,2-*b*]pyridazinium derivative **1a**. In addition, **1b** was more active than **1a** against Gram-negative bacteria, though it was less active than cefozopran (CZOP) against *Pseudomonas aeruginosa*. Table 2 shows the MIC_{90} values of **1b** and reference compounds against 84 clinical isolates of MRSA and their affinity for penicillin binding protein 2′ (PBP2′). The MIC_{90} value of **1b** (1 μg/ml) was lower than that of **1a** (2 μg/ml) and the same as that of VCM. In addition, **1b** showed high affinity for PBP2′ with IC_{50} value of 1.1 μg/ml, which is nearly eighty times as high as that of CZOP. It was considered that this high affinity of **1b** for PBP2′ was reflected in its potent anti-MRSA activity. The prodrug-type derivatives showed less than one tenth the *in vitro* antibacterial activity of their parent compounds against all strains tested (data not shown). As described in the chemistry section, the prodrug derivatives were slightly contaminated with the parent compounds. In addition, it was likely that the prodrugs were converted into the parent compounds during the procedure used for MIC determination. Therefore, we speculated that the antibacterial activity of the prodrug derivatives was due to contamination with the parent compounds and that the prodrugs themselves possessed hardly any *in vitro* antibacterial activity.

Table 1. Antibacterial activity (MIC, μg/ml) of 7β-[2-(5-amino-1,2,4-thiadiazol-3-yl)-2(*Z*)-fluoromethoxyiminoacetamido]-3-[2(*E*)-(1-methylpyridinio)thiovinyl]-3-cephem-4-carboxylates (**1b**~**1d**), **1a** and CZOP.



Compd.	Het	*S. a.*	MRSA1	MRSA2	MRSA3	*E.c.*	*E.cl.*	*S.m.*	*P.v.*	*P.a.*1	*P.a.*2
1a		0.2	0.78	1.56	3.13	0.013	0.1	0.2	0.05	6.25	100
1b		0.2	0.78	0.78	1.56	0.013	0.025	0.05	0.025	6.25	50
1c		0.2	0.78	1.56	6.25	0.025	0.1	0.05	0.05	6.25	50
1d		0.2	0.78	1.56	6.25	0.013	0.05	0.025	0.025	25	50
CZOP		0.78	12.5	25	100	0.05	0.1	0.1	0.2	1.56	6.25

S. a., *Staphylococcus aureus* 308A-1; MRSA1, *S. aureus* N295 ; MRSA2, *S. aureus* N133 ; MRSA3, *S. aureus* OFU4 ; *E.c.*, *Escherichia coli* NIHJ JC-2 ; *E. cl.*, *Enterobacter cloacae* GN5788 ; *S. m.*, *Serratia marcescens* IFO 12648 ; *P.v.*, *Proteus vulgaris* IFO 3988 ; *P.a.*1, *Pseudomonas aeruginosa* P9 ; *P.a.*2, *P. aeruginosa* U31.

Table 2. Comparative MIC_{90} against clinical isolates of MRSA and PBP2′ affinity of **1a**, **1b** and reference compounds.

Compd.	MIC_{90}[#] (μg/ml)	IC_{50}[$] ($\mu$g/ml)
1a	2	2.70
1b	1	1.10
CZOP	64	78.0
VCM	1	NT

NT; not tested

[#] Value against 84 clinical isolates of MRSA.

[$] Affinity for PBP2' of *S. aureus* N200P.

In terms of *in vivo* efficacy, rapid conversion of the prodrug into the parent compound is a key issue. We examined the *in vivo* anti-MRSA activity of the prodrugs against experimental systemic infection in mice (Table 3).

Table 3. Protective effect of the selected compounds and reference compounds against experimental systemic infection caused by *S. aureus* N133 in mice.

Compd.	ED_{50}[#] (mg/kg)	MIC (μg/ml)
1a	4.82	1.56
2a	5.57	ND
9a	>12.5	ND
1b	1.70	0.78
2b	1.20	ND
9b	25	ND
VCM	2.21~5.05	1.56
CZOP	22.6	25

ND; Not determined.

[#] Compounds were administered subcutaneously immediately after the bacterial challenge.

Although the *N*-sulfo derivatives **9a** and **9b** showed little *in vivo* protective effect, the *N*-phosphono derivatives **2a** and **2b** showed protective effect comparable to that of their parent compounds **1a** and **1b**. Especially, the 1-methyl-4-pyridinio derivative **2b** exhibited more potent *in vivo* anti-MRSA activity than VCM. Consistent with the *in vivo* efficacy of **2b**, the pharmacokinetic study demonstrated that **2b** was converted into **1b** within 5 minutes after intravenous administration to mice (Fig. 2). These results suggested that the *N*-phosphono prodrug strategy for *in vivo* use of cephalosporin derivatives would be useful, irrespective of the C-3′ pharmacophore structure.

In conclusion, in order to improve the water-solubility of anti-MRSA cephalosporins, we pursued the *N*-modified prodrug strategy as well as substitution of the C-3′ pharmacophore. Ultimately, we discovered 3-[(*E*)-2-(1-methylimidazo[1,2-*b*]pyridazinium-6-yl)thiovinyl]-7β-[2-(5-phosphonoamino-1,2,4-thiadiazol-3-yl)-2(*Z*)-fluoro-methoxyiminoacetamido]-3-cephem-4-carboxylate (**2a**) and its 1-methyl-4-pyridinio derivative **2b**, which were found to have water-solubility sufficient for intravenous injection and excellent anti-MRSA activity *in vivo*. Further biological evaluation of **2a** and **2b** is now in progress.

Experimental

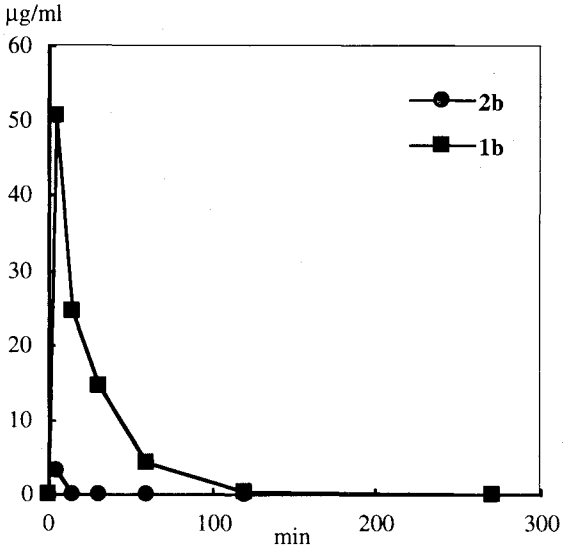
MPs were determined with a Yanagimoto micro melting point apparatus and are uncorrected. IR spectra were taken on a Hitachi 215 or Horiba FT-200 spectrophotometer. ^{1}H-NMR spectra were recorded on a Varian gemini 200 (200 MHz) spectrometer using TMS as the internal standard. Py. is used as an abbreviation for pyridyl group. Column chromatography was carried out on Merck Kieselgel 60 (Art No. 7734), YMC ODS-AM, and Mitsubishi Chemical MCI gel CHP-20P, HP-20 and SP-207.

Determination of *In Vitro* Antibacterial Activity

The MICs against selected strains of Gram-positive and Gram-negative bacteria were determined by the standard serial 2-fold agar dilution method with Mueller-Hinton agar as the test medium. The agar plates were inoculated with about 10^4 CFU of microorganisms per spot and were incubated overnight at 37°C.

In some experiments, clinical isolates of MRSA, which were kindly supplied by three clinical laboratories in Japan in 1993 (54 strains) and 1995 (30 strains), were used for the determination of anti-MRSA activity of test compounds. The MIC_{90} was defined as the concentration of a compound required to inhibit the growth of 90% of the strains.

Fig. 2. Plasma concentrations of **1b** and **2b** after a single intravenous administration of **2b** at a dose of 20 mg/kg as **1b** to mice.



Determination of *In Vivo* Antibacterial Activity

S. aureus strain N133 was cultured overnight at 37°C in brain heart infusion broth, suspended in 5% mucin and inoculated intraperitoneally into ICR male mice. Compounds were dissolved in a 10% aq DMSO solution (**1a** and **1b**) or saline containing two equivalents of $NaHCO_3$ to the compound (**2a** and **2b**) and administered subcutaneously immediately after the bacterial challenge. The 50% effective dose (ED_{50}) was calculated from the survival rate recorded on day 5 after infection.

Determination of Affinity for Penicillin Binding Protein 2′

Membrane was prepared from *S. aureus* N200P cells grown to the late exponential phase in trypticase soy broth and incubated with [^{14}C]benzylpenicillin. Binding affinity of antibiotic for PBP2′ was assessed by a competition assay, in which the membrane was incubated with dilutions of the antibiotic at 30° for 10 minutes and then labeled with [^{14}C]benzylpenicillin for 10 minutes. PBPs were separated by SDS-polyacrylamide gel electrophoresis and detected by fluorography. Binding affinity was expressed in terms of the concentration required to prevent [^{14}C]benzylpenicillin binding by 50% (IC_{50}).

Determination of the Pharmacokinetic Profile

After intravenous administration (5, 15 and 30 minutes and 1, 2 and 4.5 hours) of **2b** to ICR male mice at a dose of

20 mg/kg as **1b**, the mice were sacrificed at each time point, and blood samples were obtained. To a 100 μl portion of the plasma sample, a 900 μl portion of 0.03 M phosphate buffer (pH 7) was added on an ice-bath. The mixture obtained was then ultrafiltered using a Centrifree apparatus (Amicon, Inc., U.S.A.), and **1b** and **2b** in the filtrate were analyzed by HPLC systems under the following conditions. The column was a YMC-ODS AM-302 (4.6×150 mm, 5 mm particle size; YMC Co. Ltd. Japan). The mobile phase was a mixture of 0.03 M phosphate buffer (pH 7) and acetonitrile. Gradient elution was carried out, in which the acetonitrile concentration was increased from 5 to 13% in 17 minutes. The flow rate was 1 ml/minute. The injection volume was 50 μl, and each compound was detected at 254 nm.

Benzhydryl 7β-*t*-Butoxycarbonylamino-3-[(*E*)-2-(4-pyridyl)-thiovinyl]-3-cephem-4-carboxylate (**4b**)

4-Mercaptopyridine (49.2 g, 443 mmol) was added to a solution of benzhydryl 7β-*t*-butoxycarbonylamino-3-[(*E*)-2-tosyloxyvinyl]-1-oxide-3-cephem-4-carboxylate[12] (**3**, 100 g, 147 mmol) in DMSO (1.0 liter), and the mixture was stirred at 50°C for 6 hours. After cooling at room temperature, the reaction mixture was poured into a mixture of EtOAc (3.8 liter), cooled water (3.0 liter) and brine (1.0 liter), and the mixture was stirred for 10 minutes. The separated organic layer was washed with water (3.5 liter) and brine (500 ml) successively and dried over $MgSO_4$. After filtration, the filtrate was evaporated under reduced pressure. The residue was dissolved in a mixture of THF (500 ml) and *N*,*N*-dimethylacetamide (49 ml). Under cooling at −10°C, phosphorus trichloride (25.9 ml, 300 mmol) was added to the solution dropwise, and the mixture was stirred at the same temperature for 35 minutes. The reaction mixture was diluted with a mixture of EtOAc (1.5 liter) and saturated aq $NaHCO_3$ (3.0 liter). The mixture was stirred at room temperature for 30 minutes. The separated organic layer was washed with brine (1.5 liter), dried over $MgSO_4$ and filtered. The filtrate was evaporated under reduced pressure. The residue was purified by silica gel column chromatography (1.0 kg : eluents=*n*-hexane - EtOAc=3 : 2~*n*-hexane - EtOAc - MeOH=20 : 10 : 1). The fractions eluted with *n*-hexane - EtOAc - MeOH=20 : 10 : 1 were concentrated under reduced pressure to give **4b** (45.7 g, 52%): ^{1}H NMR ($CDCl_3$) δ 1.47 (9H, s, Boc), 3.59, 3.71 (2H, ABq, J=18 Hz, C_2-H), 5.02 (1H, d, J=5 Hz, C_6-H), 5.38 (1H, d, J=8 Hz, C_7-NH), 5.66 (1H, dd, J=5 and 8 Hz, C_7-H), 6.67 (1H, d, J=16 Hz, vinyl), 6.98 (1H, s, CH), 7.1~7.5 (13H, m, Ph, Py. and vinyl), 8.47 (2H, dd, J=1 and 4 Hz, Py.).

Benzhydryl 7β-*t*-Butoxycarbonylamino-3-[(*E*)-2-(3-pyridyl)thiovinyl]-3-cephem-4-carboxylate (**4c**)

3-Mercaptopyridine (291 mg, 2.65 mmol) and *N*,*N*-diisopropylethylamine (1.15 ml, 6.61 mmol) were successively added to a solution of **3** (900 mg, 1.33 mmol) in DMF (7 ml), and the mixture was stirred at room temperature for 5 hours. The reaction mixture was diluted with EtOAc (150 ml), and the solution was successively washed three times with water (each 100 ml) and with brine (40 ml). The separated organic layer was dried over $MgSO_4$ and filtered. The filtrate was evaporated under reduced pressure. The residue was purified by silica gel column chromatography (25 g: eluents=*n*-hexane~*n*-hexane - EtOAc=2 : 3). The fractions eluted with *n*-hexane - EtOAc=2 : 3 were concentrated under reduced pressure. The residue was dissolved in a mixture of dichloromethane (56 ml) and *N*,*N*-dimethylacetamide (0.43 ml). Under cooling at −12°C, phosphorus trichloride (0.12 ml, 1.41 mmol) was added to the solution, and the mixture was stirred at the same temperature for 2 hours. The reaction mixture was concentrated under reduced pressure. The concentrate was portioned in a mixture of EtOAc (150 ml) and aq $NaHCO_3$ (100 ml). The separated organic layer was washed with brine (100 ml), dried over $MgSO_4$ and filtered. The filtrate was evaporated under reduced pressure. The residue was purified by silica gel column chromatography (20 g: eluents=*n*-hexane~*n*-hexane - EtOAc=1 : 2). The fractions eluted with *n*-hexane - EtOAc=1 : 2 were concentrated under reduced pressure to give **4c** (450 mg, 32%): ^{1}H NMR ($CDCl_3$) δ 1.46 (9H, s, Boc), 3.50, 3.61 (2H, ABq, J=16 Hz, C_2-H), 5.00 (1H, d, J=5 Hz, C_6-H), 5.42 (1H, d, J=10 Hz, C_7-NH), 5.63 (1H, dd, J=5 and 10 Hz, C_7-H), 6.61 (1H, d, J=16 Hz, vinyl), 6.96 (1H, s, CH), 7.1~7.5 (12H, m, Ph, vinyl and Py.), 7.70 (1H, m, Py.), 8.53 (1H, dd, J=2.4 and 4.8 Hz, Py.), 8.61 (1H, d, J=2.4 Hz, Py.).

The 2-pyridyl derivative **4d** was prepared by a method similar to that used for **4c**. The yield was 69%. The analytical data were as follows: ^{1}H NMR ($CDCl_3$) δ 1.47 (9H, s, Boc), 3.68, 3.82 (2H, ABq, J=18 Hz, C_2-H), 5.02 (1H, d, J=5 Hz, C_6-H), 5.33 (1H, d, J=9.6 Hz, C_7-NH), 5.63 (1H, dd, J=5 and 9.6 Hz, C_7-H), 6.98 (1H, s, CH), 7.0~7.6 (14H, m, Ph, vinyl and Py.), 7.67 (1H, d, J=16 Hz, vinyl), 8.48 (1H, m, Py.).

7β-Amino-3-[(*E*)-2-(1-methyl-4-pyridinio)thiovinyl]-3-cephem-4-carboxylate Bistrifluoroacetic Acid (**5b**)

Iodomethane (14.5 ml, 228 mmol) was added to a solution of **4b** (45.7 g, 75.9 mmol) in DMF (143 ml), and the mixture was stirred at room temperature for 4 hours. The reaction mixture was diluted with a mixture of *n*-

hexane (2.0 liter) and diethyl ether (2.0 liter). After being stirred at room temperature for 30 minutes, the mixture was allowed to stand. The resulting upper layer was removed by decantation. The residual oil was dissolved in a mixture of dichloromethane (430 ml) and anisole (85 ml). TFA (215 ml) was added to the mixture dropwise, and the mixture was stirred at room temperature for 2 hours. The reaction mixture was diluted with diethyl ether (2.8 liter). The resulting precipitate was collected by filtration, washed with diethyl ether (1.0 liter) and dried under a vacuum to give **5b** (42.0 g, 96%): ^{1}H NMR (DMSO-d_6) δ 3.77, 3.88 (2H, ABq, J=16 Hz, C_2-H), 4.23 (3H, s, CH_3), 5.15 (1H, d, J=5 Hz, C_6-H), 5.24 (1H, d, J=5 Hz, C_7-H), 7.30, 7.42 (2H, d, J=15 Hz, vinyl), 8.12, 8.76 (each 2H, d, J=7 Hz, Py.).

The derivatives **5c** and **5d** were prepared by a method similar to that used for **5b**. The yields and analytical data were as follows:

5c (88%): ^{1}H NMR (DMSO-d_6) δ 3.82, 4.10 (2H, ABq, J=17 Hz, C_2-H), 4.34 (3H, s, CH_3), 5.19 (1H, d, J=5 Hz, C_6-H), 5.27 (1H, d, J=5 Hz, C_7-H), 7.24, 7.33 (2H, d, J=15 Hz, vinyl), 8.10 (1H, dd, J=8 and 6 Hz, Py.), 8.63 (1H, d, J=8 Hz, Py.), 8.88 (1H, d, J=6 Hz, Py.), 9.12 (1H, s, Py.).

5d (91%): ^{1}H NMR (DMSO-d_6) δ 3.88, 4.23 (2H, ABq, J=18 Hz, C_2-H), 4.22 (3H, s, CH_3), 5.28 (1H, d, J=5 Hz, C_6-H), 5.33 (1H, d, J=5 Hz, C_7-H), 7.16, 7.49 (2H, d, J=15 Hz, vinyl), 7.87 (1H, m, Py.), 8.17 (1H, d, J=9 Hz, Py.), 8.43 (1H, m, Py.), 9.02 (1H, d, J=6 Hz, Py.).

7β-[2-(5-Amino-1,2,4-thiadiazol-3-yl)-2(*Z*)-fluoromethoxyiminoacetamido]-3-[(*E*)-2-(1-methyl-4-pyridinio)-thiovinyl]-3-cephem-4-carboxylate (**1b**)

Under ice-cooling, 2-(5-amino-1,2,4-thiadiazol-3-yl)-2(*Z*)-fluoromethoxyiminoacetyl chloride hydrochloride[14] (**6**, 40.3 g, 146 mmol) was added portionwise to a solution of **5b** (65.0 g, 113 mmol) in a mixture of THF (1.5 liter), H_2O (1.5 liter) and 0.6 M $NaHCO_3$ (865 ml), and the mixture was stirred at 5°C for 30 minutes. The reaction mixture was concentrated under reduced pressure. The concentrate was diluted with H_2O (36.0 liter) and purified by MCI gel HP-20 column chromatography (6.0 liter: eluents=H_2O~15% aq EtOH). The fractions eluted with 15% aq EtOH were concentrated under reduced pressure, and the concentrate was lyophilized to give **1b** (42.4 g, 68%). The analytical results are shown in Table 4 and Table 5.

The derivatives **1c** and **1d** were prepared by a method similar to that used for **1b**. The yields are shown in Scheme 1. The analytical results are shown in Table 4 and Table 5.

Crystallization of **1b**

The lyophilized **1b** (42.4 g, 76.9 mmol) was suspended in a mixture of EtOH (551 ml) and H_2O (382 ml). To the suspension was added conc HCl (12.8 ml), and the mixture became a clear solution. After sonication, the solution was allowed to stand at room temperature for 1.5 hours. The resulting hydrochloride crystals of **1b** were collected by filtration and washed with EtOH (100 ml) and H_2O (30 ml) successively. The crystals (MP 230~250°C (dec.); *Anal* Calcd for $C_{20}H_{19}N_7O_5S_3ClF\cdot 1.0H_2O$: C 39.64, H 3.49, N 16.18, Cl 5.85. Found: C 39.82, H 3.67, N 15.86, Cl 5.87.) were suspended in a mixture of acetonitrile (135 ml) and H_2O (400 ml). To the suspension was added $NaHCO_3$ (5.4 g, 59.5 mmol), and the mixture became a clear solution. The solution was stirred at room temperature for 3 hours. The resulting crystals were collected by filtration, washed with a cooled mixture of acetonitrile (18 ml) and H_2O (54 ml) and air-dried to give **1b** as crystals (26.4 g, 62%): MP 195~210°C (dec.)

2-(5-Dichlorophosphorylamino-1,2,4-thiadiazol)-2(*Z*)-fluoromethoxyiminoacetyl Chloride (**8**)

Under ice-cooling, phosphorus pentachloride (35.9 g, 172 mmol) was added portionwise to a suspension of 2-(5-amino-1,2,4-thiadiazol-3-yl)-2(*Z*)-fluoromethoxyiminoacetic acid[16] (**7**, 25.3 g, 115 mmol) in THF (150 ml), and the mixture was stirred at 5°C for 1 hour to afford a clear solution. The reaction solution was concentrated under reduced pressure. The concentrate was diluted with *n*-hexane (300 ml). The resulting upper layer was removed by decantation. The separated residual oil was washed twice with *n*-hexane (each 300 ml) by decantation and suspended in toluene (150 ml). Under stirring, *n*-hexane (450 ml) was added to the suspension dropwise. The mixture was stirred at room temperature for 30 minutes and allowed to stand at 5°C for 1 hour. The resulting crystals were collected by filtration, washed with a mixture of *n*-hexane (45 ml) and toluene (15 ml) and dried under a vacuum to give **8** (38.7 g, 95%): MP 55~60°C; ^{1}H NMR ($CDCl_3$) δ 5.80 (2H, d, J=53 Hz, CH_2F).; IR (KBr) cm^{-1} 2986, 1790, 1574, 1539, 1224, 1076, 1014.

3-[(*E*)-2-(1-Methylimidazo[1,2-*b*]pyridazinium-6-yl)-thiovinyl]-7β-[2-(5-phosphonoamino-1,2,4-thiadiazol-3-yl)-2(*Z*)-fluoromethoxyiminoacetamido]-3-cephem-4-carboxylate (**2a**)

Under ice-cooling, the pH of a solution of 7β-amino-3-[(*E*)-2-(1-methylimidazo[1,2-*b*]pyridazinium-6-yl)thiovinyl]-3-cephem-4-carboxylate bistrifluoroacetic acid[1] (**5a**, 6.2 g, 10.0 mmol) in a mixture of H_2O (150 ml) and THF (150 ml)

Table 4. IR and analytical data for the prepared cephem derivatives.

Compd No.	Formula	*Anal* Calcd (%) C	H	N	Found (%) C	H	N	IR (KBr, cm^{-1})		
1b	$C_{20}H_{18}N_7O_5S_3F \cdot 4.5H_2O$	37.97	4.30	15.50	37.93	4.19	15.39	1765	1670	1635
1c	$C_{20}H_{18}N_7O_5S_3F \cdot 3.5H_2O$	39.08	4.10	15.95	38.85	4.16	15.65	1770	1670	1600
1d	$C_{20}H_{18}N_7O_5S_3F \cdot 4.0H_2O$	38.52	4.20	15.72	38.44	4.12	15.51	1760	1670	1605
2a	$C_{21}H_{19}N_9O_8S_3FP \cdot 4.0H_2O$	33.92	3.66	16.95	34.00	3.43	17.19	1765	1675	
2b	$C_{20}H_{19}N_7O_8S_3FP \cdot 2.0H_2O$	35.98	3.47	14.69	36.09	3.55	14.78	1770	1675	1630
9a	$C_{21}H_{17}N_9O_8S_4FNa \cdot 4.5H_2O$	32.56	3.38	16.27	32.70	3.30	16.37	1760	1670	1600
9b	$C_{20}H_{18}N_7O_8S_4F \cdot 1.0H_2O$	36.97	3.10	15.09	37.08	3.34	14.71	1770	1680	1630

Table 5. ^1H NMR spectral data for the prepared cephem derivatives.

Compd No.	Chemical shift (*J*=Hz) (DMSO-d_6, δ) Cephem nuclei C_2-H ABq(17)	C_6-H d(5)	C_7-H dd(5&8)	C_7-NH d(8)	Thiadiazole NHR br s	OCH_2F d (55)	CH=CH d (16)	CH_3 s	Heteroazolium
1b	3.56 3.79	5.10	5.67	9.78	8.25	5.80	6.50 7.52	4.19	7.97 (2H,d,*J*=6.6Hz), 8.66 (2H,d,*J*=6.6Hz).
1c	3.54 3.74	5.09	5.66	9.76	8.23	5.79	6.46 7.50	4.34	8.00 (1H,dd,*J*=8&6Hz), 8.47 (1H,d,*J*=8Hz), 8.78 (1H,d,*J*=6Hz), 8.98 (1H,s).
1d	3.58 3.79	5.12	5.70	9.78	8.24	5.80	6.42 7.67	4.19	7.77 (1H,dd,*J*=7&6Hz), 8.04 (1H,d,*J*=8Hz), 8.35 (1H,dd,*J*=8&7Hz),8.94 (1H,d,*J*=6Hz).
2a	3.69 3.98	5.24	5.86	9.82	9.20	5.80	7.18 7.30	4.10	7.99, 8.68 (each1H,d,*J*=10Hz), 8.40, 8.70 (each1H,s).
2b	3.67 4.13	5.24	5.87	9.85	9.27	5.81	7.08 7.32	4.21	8.06 (2H,d,*J*=7Hz), 8.72 (2H,d,*J*=7Hz).
9a	3.56 3.70	5.13	5.70	9.79	11.02	5.81	6.68 7.50	4.08	7.96, 8.64 (each1H,d,*J*=10Hz), 8.37, 8.71 (each1H,d,*J*=2Hz).
9b	3.69 4.16	5.26	5.89	9.86	11.02	5.81	7.14 7.34	4.21	8.08 (2H,d,*J*=6Hz), 8.71 (2H,d,*J*=6Hz).

was adjusted to 6.9 with aq sodium acetate. To the solution was added portionwise **8** (7.1 g, 20.0 mmol), and the mixture was stirred at 5°C for 30 minutes. The reaction mixture was concentrated under reduced pressure. The concentrate was purified by MCI gel SP-207 column chromatography (1.0 liter: eluents=H_2O~10% aq EtOH). The fractions eluted with 10% aq EtOH were concentrated under reduced pressure, and the concentrate was lyophilized. The obtained crude **2a** was dissolved in H_2O (30 ml) containing $NaHCO_3$ (534 mg, 6.35 mmol) and subjected to ODS-AM column chromatography (450 ml). After elution with 1 N HCl (10 ml) and H_2O (1.0 liter) successively, the fractions eluted with H_2O-acetonitrile= 7.5:1 were concentrated under reduced pressure. The concentrate was lyophilized to give **2a** (1.0 g, 15%). The content of **1a** in **2a** was determined to be 2.1% by HPLC analysis.

The analytical results are shown in Table 4 and Table 5.

3-[(*E*)-2-(1-Methyl-4-pyridinio)thiovinyl]-7β-[2-(5-phosphonoamino-1,2,4-thiadiazol-3-yl)-2(*Z*)-fluoromethoxyiminoacetamido]-3-cephem-4-carboxylate (**2b**)

Under ice-cooling, **8** (10.5 g, 29.5 mmol) was added portionwise to a solution of **5b** (8.5 g, 14.8 mmol) in a mixture of H_2O (338 ml) and 0.6 M aq $NaHCO_3$ (110 ml), and the mixture was stirred at 5°C for 1 hour. The reaction mixture was diluted with H_2O (10 liter) and purified by MCI gel SP-207 column chromatography (1.0 liter:

eluents=H_2O~15% aq EtOH). The fractions eluted with 15% aq EtOH were concentrated under reduced pressure, and the concentrate was lyophilized. The obtained crude **2b** was dissolved in H_2O (14 ml) containing $NaHCO_3$ (1.34 g, 16 mmol), and subjected to ODS-AM column chromatography (1.0 liter). After elution with 1 N HCl (60 ml) and H_2O (1.6 liter) successively, the fractions eluted with H_2O-acetonitrile=10:1 were concentrated under reduced pressure. The concentrate was lyophilized to give **2b** (3.41 g, 37%). The content of **1b** in **2b** was determined to be 2.4% by HPLC analysis.

The analytical results are shown in Table 4 and Table 5.

3-[(*E*)-2-(1-Methylimidazo[1,2-*b*]pyridazinium-6-yl)-thiovinyl]-7β-[2-(5-sulfoamino-1,2,4-thiadiazol-3-yl)-2(*Z*)-fluoromethoxyiminoacetamido]-3-cephem-4-carboxylate Sodium Salt (**9a**)

Pyridine sulfur trioxide (239 mg, 1.50 mmol) was added to a suspension of 7β-[2-(5-amino-1,2,4-thiadiazol-3-yl)-3-[(*E*)-2-(1-methylimidazo[1,2-*b*]pyridazinium-6-yl)thiovinyl]-2(*Z*)-fluoromethoxyiminoacetamido]-3-cephem-4-carboxylate[1] (**1a**, 296 mg, 0.50 mmol) in DMF (3 ml), and the mixture was stirred at room temperature. After 5 hours, additional pyridine sulfur trioxide (239 mg, 1.50 mmol) was added to the reaction mixture, and the mixture was stirred at room temperature for an additional 16 hours. The reaction mixture was diluted with EtOAc (80 ml), and the mixture was stirred at room temperature for 10 minutes. The resulting upper layer was removed by decantation. The residue was dissolved in aq sodium acetate (80 ml) and washed with EtOAc (50 ml). The separated aqueous layer was concentrated under reduced pressure. The concentrate was purified by MCI gel CHP-20P column chromatography (150 ml: eluents=H_2O~5% aq EtOH). The fractions eluted with 5% aq EtOH were concentrated under reduced pressure, and the concentrate was lyophilized to give **9a** (105 mg, 30%). The analytical results are shown in Table 4 and Table 5.

3-[(*E*)-2-(1-Methyl-4-pyridinio)thiovinyl]-7β-[2-(5-sulfoamino-1,2,4-thiadiazol-3-yl)-2(*Z*)-fluoromethoxyiminoacetamido]-3-cephem-4-carboxylate (**9b**)

Pyridine sulfur trioxide (239 mg, 1.50 mmol) was added to a suspension of **1b** (276 mg, 0.50 mmol) in DMF (3 ml), and the mixture was stirred at room temperature. After 5, 20 and 44 hours, additional pyridine sulfur trioxide (239 mg, 1.50 mmol) was added to the reaction mixture, and the mixture was stirred at room temperature for an additional 16 hours. The reaction mixture was diluted with EtOAc (80 ml), and the mixture was stirred at room temperature for 10 minutes. The resulting upper layer was removed by decantation. The residue was dissolved in aq sodium acetate (50 ml) and washed with EtOAc (50 ml). The separated aqueous layer was concentrated under reduced pressure. The concentrate was purified by MCI gel SP-207 column chromatography (100 ml: eluents=H_2O~10% aq EtOH). The fractions eluted with 10% aq EtOH were concentrated under reduced pressure, and the concentrate was lyophilized to give **9b** (215 mg, 68%). The analytical results are shown in Table 4 and Table 5.

References

1) ISHIKAWA, T.; K. KAMIYAMA, Y. NAKAYAMA, Y. IIZAWA, K. OKONOGI & A. MIYAKE: Studies on anti-MRSA parenteral cephalosporins. III. Synthesis and antibacterial activity of 7β-[2-(5-amino-1,2,4-thiadiazol-3-yl)-2(*Z*)-alkoxyiminoacetamido]-3-[(*E*)-2-(1-alkylimidazo[1,2-*b*]pyridazinium-6-yl)thiovinyl]-3-cephem-4-carboxylates and related compounds. J. Antibiotics 54: 257~277, 2001
2) GREENLEE, M. L.; J. B. LAUB, G. P. ROUEN, F. DININNO, M. L. HAMMOND, J. L. HUBER, J. G. SUNDELOF & G. G. NAMMOND: Dicationic 2-fluorenonylcarbapenems: potent anti-MRS agents with improved solubility and pharmacokinetic properties. Bioorg. Med. Chem. Lett. 9: 3225~3230, 1999
3) HECKER, S. J.; I.-S. CHO, T. W. GLINKA, Z. J. ZHANG, M. E. PRICE, V. J. LEE, B. G. CHRISTENSEN, A. BOGGS, S. CHAMBERLAND, F. MALOUIN, T. R. PARR, T. ANNAMALAI, J. BLAIS, E. L. BOND, L. CASE, C. CHAN, J. CRASE, R. FRITH, D. GRIFFITH, L. HARFORD, N. LIU, M. LUDWIKOW, K. MATHIAS, D. REA & R. WILLIAMS: Discovery of MC-02,331, a new cephalosporin exhibiting potent activity against methicillin-resistant *Staphylococcus aureus*. J. Antibiotics 51: 722~734, 1998
4) WERMUTH, C. G.: Preparation of water-soluble compounds by covalent attachment of solubilizing moieties. *In* The Practice of Medicinal Chemistry. *Ed.*, C. G. WERMUTH, pp. 755~776, Academic Press Ltd., London, 1996
5) FREEMAN, S. & K. C. ROSS: Prodrug design for phosphates and phosphonates. Prog. Med. Chem. 34: 111~147, 1997
6) SAKANE, K.; N. YASUDA & S. NISHIMURA (Fujisawa Pharmaceutical Co., Ltd.): Water-soluble compositions containing heteronioalkylcephem derivatives, their preparation, and formulations containing them. EP 303,223, Feb 15, 1989 [Chem. Abstr. 111: 23293, 1989]
7) MIYAKE, A.; H. SHIMIZU & K. NAITO (Takeda Chemical Industries, Ltd.): 7β-[(*Z*)-2-(5-Carboxylatoamino-1,2,4-thiadiazol-3-yl)-2-methoxyiminoacetamido]-3-[(1-imidazo[1,2-*b*]pyridazinium)methyl]-3-cephem-4-carboxylate salts and their preparation. JP 03 14,586, Jan 23, 1991 [Chem. Abstr. 114: 228632, 1991]
8) TERAJI, T.; K. SAKANE & J. GOTO (Fujisawa Pharmaceutical Co., Ltd.): Cephem compounds. EP 99, 553, Feb 01, 1984 [Chem. Abstr. 100: 209522, 1984]
9) KASAI, M.; S. HATANO, M. KITAGAWA, A. YOSHIMI, K.

NISHIMURA, N. MORI, A. SAKAI & T. SUGIHARA: AS-924, a novel orally active bifunctional prodrug of ceftizoxime. Synthesis and relationship between physicochemical properties and oral absorption. Chem. Pharm. Bull. 47: 1081~1088, 1999

10) HANAKI, H.; H. AKAGI, S. NOMURA, N. UNEMI & K. HIRAMATSU: Structure-activity relationships of cephalosporin derivatives against methicillin-resistant *Staphylococcus aureus* and *Enterococcus faecalis*. J. Antibiotics 49: 402~404, 1996

11) TSUBOUCHI, H.; K. TSUJI, K. YASUMURA, M. MATSUMOTO, T. SHITSUTA & H. ISHIKAWA: Synthesis and biological evaluation of a series of new parenteral optically active 3-[[(alkylpyridinium-4′-yl)thio]methyl]-2-oxaisocephems. J. Med. Chem. 38: 2152~2157, 1995

12) FARGE, D.; P. L. ROY, C. MOUTONNIER & J. F. PEYRONEL (Rhone-Poulenc Industries S. A.): 3-Vinyl-cephalosporins. DE 3,019,445, Dec 04, 1980 [Chem. Abstr. 95: 7307, 1981]

13) PEYRONEL, J. F.; C. MOUTONNIER & B. PLAU: Synthesis of 3-substituted cephalosporins from penicillins. *In* Recent Advances in The Chemistry of β-Lactam Antibiotics. *Ed.*, A. G. BROWN *et al.*, pp. 336~342, Third International Symposium, 1984

14) KANAI, T.; A. IMAI, K. SATO, H. SHINOHARA & K. OISHI (Eisai Chemical Co., Ltd.): Preparation of aminothiadiazolyl(alkoxyimino)acetyl halides. EP 590,681, Apr 06, 1994 [Chem. Abstr. 120: 323580, 1994]

15) KANAI, T.; Y. MORITA, H. SHINOHARA, Y. KAI & K. OGURA: Reaction of insoluble 2-(5-amino-1,2,4-thiadiazol-3-yl)-2-[(fluoromethoxy)imino]acetic acid with insoluble PCl_5 in CH_2Cl_2. Chem. Express 7: 805~808, 1992

16) KANAI, T.; Y. KAI, N. SATO, T. NAITO, T. KAMIYA, T. NAKAMUTA & K. OGURA: Efficient preparation of (*Z*)-2-(5-amino-1,2,4-thiadiazol-3-yl)-2-[(fluoromethoxy)-imino]acetic acid. Bull. Chem. Soc. Jpn. 66: 2335~2338, 1993