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Structural requirements for the stability of novel cephalosporins to AmpC β-lactamase based on 3D-structure

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Abstract—AmpC β-lactamase is one of the leading causes of *Pseudomonas aeruginosa* (*P. aeruginosa*) resistance to cephalosporins. FR259647 is a cephalosporin having a novel pyrazolium substituent at the 3-position and exhibits excellent activity (MIC = 1 μg/mL) against the AmpC β-lactamase overproducing *P. aeruginosa* FP1380 strain in comparison with the third-generation cephalosporins FK518 [*Abstracts of Papers*, 30th Interscience Conference on Antimicrobial Agents and Chemotherapy, Atlanta, GA, October 21–24, 1990, Abs. 454; *Abstracts of Papers*, 30th Interscience Conference on Antimicrobial Agents and Chemotherapy, Atlanta, GA, October 21–24, 1990, Abs. 455; *Abstracts of Papers*, 30th Interscience Conference on Antimicrobial Agents and Chemotherapy, Atlanta, GA, October 21–24, 1990, Abs. 456; *Abstracts of Papers*, 30th Interscience Conference on Antimicrobial Agents and Chemotherapy, Atlanta, GA, October 21–24, 1990, Abs. 457] (MIC = 16 μg/mL) and ceftazidime (CAZ) (MIC = 128 μg/mL). The stability of FR259647 and FK518 to AmpC β-lactamase was evaluated using MIC assays against both the *P. aeruginosa* PAO1 strain and a PAO1 mutant strain overproducing AmpC β-lactamase as a differential assay, which indicates that the main difference derives from their stability to AmpC β-lactamase. A structural analysis using computer simulations indicated that the difference in stability may be due to steric hindrance of the 3-position substituents causing differential affinity. This steric hindrance may disturb entry of the cephalosporins into the binding pocket. We predicted the possibility of inhibition of entry as a potential means of enhancing stability by conformational analysis. In order to validate this speculation, novel FR259647 derivatives **4**–**9** were designed, calculated, synthesized, and evaluated. As a result, we demonstrated that their probability of entry correlated with the MIC ratio of the mutant strain to the parent strain and supports the validity of our model.

1. Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a ubiquitous versatile environmental bacterium that is a leading cause of opportunistic human infections.[1] Infection of immune-suppressed patients, such as elderly or hospitalized patients, with this pathogen is now an important clinical issue because it often leads to high mortality.[2] *P. aeruginosa* has an intrinsic resistance to many antimicrobial agents because of its low outer membrane permeability, multiple efflux pumps, and chromosomal AmpC β-lactamase.[3] Only a few antimicrobial agents, such as amikacin, imipenem, ceftazidime (CAZ) (**1**), and ciprofloxacin, are used for treatment of *P. aeruginosa* infections, however, these antimicrobials often have low activity because *P. aeruginosa* can acquire various resistant mechanisms.[4,5] Recently, the emergence of resistant strains against these antimicrobials is a growing threat to human health. Accordingly, it is an urgent issue to generate effective antibacterial drugs against the resistant strains.

Keywords: AmpC β-lactamase; Conformational studies; Cephalosporin; *Pseudomonas aeruginosa*.
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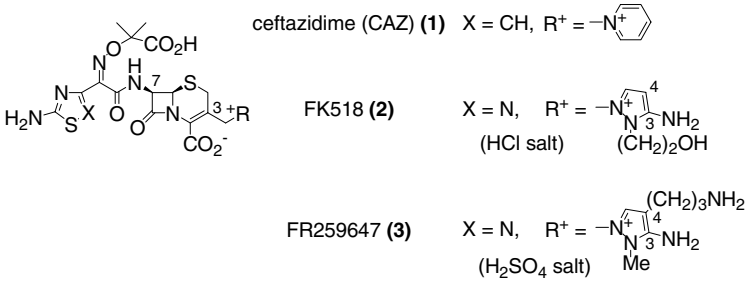
Pseudomonas aeruginosa is naturally susceptible to the third-generation cephalosporin CAZ.[6] However, it is known that CAZ has low activity against *P. aeruginosa* that overproduces AmpC β-lactamase, which is the primary mechanism of *P. aeruginosa* resistance to cephalosporins.[2] *P. aeruginosa* produces an inducible, chromosomally encoded AmpC β-lactamase and this enzyme is usually expressed at very low levels.[7] CAZ has effective antibacterial activity against *P. aeruginosa* producing the enzyme at this level because it works as a suicide inhibitor against the enzyme.[8] However, CAZ has decreased antibacterial activity against *P. aeruginosa* overproducing the enzyme, because it is labile to the overproduced enzyme.[9]

In order to generate promising anti-*P. aeruginosa* agents, research in our group has been directed toward the development of novel cephalosporins. Our group reported that the cephalosporins FK518 (**2**)[10a–d] and FR259647 (**3**)[11] had 16- and 128-fold improved antibacterial activity (MIC) against AmpC β-lactamase overproducing clinical isolates of *P. aeruginosa* FP1380 strains in comparison with CAZ, respectively (Table 1). Furthermore, the stability of these cephalosporins to AmpC β-lactamase was evaluated using MIC assays against both the *P. aeruginosa* PAO1 strain and a PAO1 *ampD*-defective mutant (Δ*ampD*/PAO1) strain overproducing AmpC β-lactamase as a differential assay and was indicated as the Δ*ampD*/PAO1:PAO1 strain MIC ratio.[7,12,13] The Δ*ampD*/PAO1 strain was constructed by the disruption of the *ampD* gene, which suggested that the total amount of penicillin-binding protein (PBP) and the affinity of the cephalosporins to PBPs in the mutant strain were the same as those of the parent strain.[7,12] Their MIC ratios indicated that FR259647 was more stable to AmpC β-lactamase than FK518 and CAZ (Table 1). These data suggested that improvement of the antipseudomonal activity of FR259647 may be due to increased stability to AmpC β-lactamase, which could be due to the slight structural difference between FR259647 and FK518. This difference gave us a clue to analyze the structural requirements for the stability of FR259647 derivatives to AmpC β-lactamase.

AmpC β-lactamase has strong activity for the hydrolysis of cephalosporins, deactivating them.[14] AmpC β-lactamase is a serine-dependent enzyme and its catalytic reaction mechanism consists of two steps, acylation of the active site and hydrolytic deacylation.[14,15] Recently, two X-ray crystal complex structures of AmpC β-lactamase from *Escherichia coli* (*E. coli*) with cephalosporins, CAZ and cephalothin, have been reported and revealed snapshots of the 3D-structure, such as the complex of AmpC β-lactamase with intact cephalothin[15,16] and the acyl-enzyme intermediate,[8,15] in its hydrolysis mechanism.

So far, the reported structure-based drug design research using the 3D-structure of AmpC β-lactamase has been mainly directed toward the design of inhibitors of the enzyme, not cephalosporins which are stable to the enzyme.[17a–f] In this paper, we attempted to analyze the structural requirements for the stability to AmpC β-lactamase based on the structural difference in the 3-side chain of FR259647 and FK518, considering the 3D-structure of the 3-side chain binding pocket of AmpC β-lactamase. The results led to the speculation that steric hindrance of the 3-side chain of cephalosporin nucleus against AmpC β-lactamase can disturb the entry of cephalosporins into the 3-side chain binding pocket. We predicted the possibility of entry by conformational analysis and considered the relationship between the probabilities of entry and the MIC ratios of the mutant strain to the parent strain. From this consideration, we proposed structural requirements for the stability of cephalosporins having a pyrazolium ring at the 3-position to AmpC β-lactamase, and verified the validity of

Table 1. MICs (μg/mL) of FR259647, FK518, and CAZ against *P. aeruginosa* FP1380, PAO1, and Δ*ampD*/PAO1 strains and probability of entry through the gate of the 3-side chain binding pocket by conformational analysis



Compounds	MIC (μg/mL)			Calculation	
	FP1380	PAO1	Δ*ampD*/PAO1	MIC ratio[a]	Probability of entry[b] (%)
FR259647 (**3**)	1	0.5	0.5	1	0.10
FK518 (**2**)	16	0.5	4	8	100
CAZ (**1**)	128	2	64	32	N.D.[c]

[a] MIC ratio of Δ*ampD*/PAO1 to PAO1 strain.
[b] Vide Section 2.2.
[c] No data.

this proposal by design and synthesis of novel FR259647 derivatives using the probability of entry as an index.

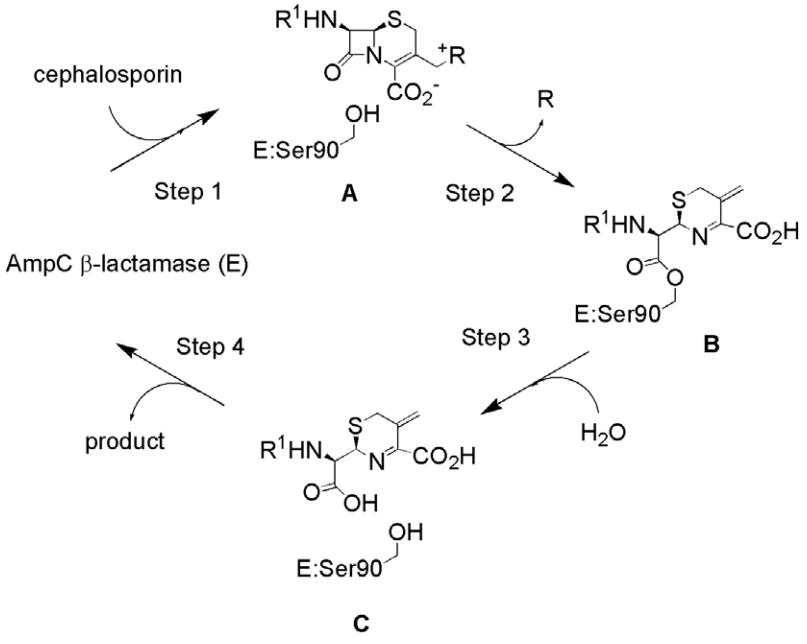
2. Results and discussion

2.1. Consideration of the stability of FR259647 and FK518 to AmpC β-lactamase

In the reaction pathway for AmpC β-lactamase, as shown in Scheme 1, a cephalosporin first binds to the enzyme to form a precovalent first encounter complex (complex A). Following nucleophillic attack by the catalytic serine, the enzyme and β-lactam are covalently bound in an acyl-enzyme complex (intermediate B). A water molecule then attacks the carboxyl carbon of the intermediate B, hydrolyzing the covalent enzyme–ligand bond and generating a hydrolyzed product and the intact enzyme (complex C). Finally, the product leaves, and the active enzyme is regenerated. Therefore, the stability of cephalosporin to AmpC β-lactamase is determined by the following two factors; formation ability of the complex A, and the thermodynamic stability of intermediate B. The first factor indicates that compounds which are poorly recognized by the enzyme should be stable to the enzyme, and the second factor indicates that compounds converted to a stable intermediate B inhibit the enzyme because they can work as a suicide inhibitor, like CAZ.[8] The only structural difference between FR259647 and FK518 is the substituent on the 3-pyrazolium ring moiety, as shown in Table 1, which induces the difference in stability to AmpC β-lactamase. Because FR259647 and FK518 have the same 7-side chain, the intermediate B derived from them by reaction with Ser90 in the active center of AmpC β-lactamase should be exactly the same structure, as shown in Scheme 1. Therefore, this indicates that the difference in the stability between them is due to FR259647 being only poorly recognized by the enzyme in comparison with FK518. So we decided to focus only on Step 1 as the key factor for understanding the stability (Scheme 1).

2.2. Construction of model of the structural requirements for stability to AmpC β-lactamase

In order to investigate the difference in recognition ability between FR259647 and FK518 by AmpC β-lactamase, the 3D-structure of AmpC β-lactamase from *P. aeruginosa* was constructed from the X-ray structure of AmpC β-lactamase from *E. coli* (PDB entry 1KVL)[15] by the homology modeling method using the FAMS (Full Automatic Modeling System) program.[18] The modeling structure showed that cephalosporin binding pocket has a cleft structure and a tunnel structure bridged by Ala312-Pro322 over the 3-side chain binding site in the cleft (Fig. 1a). Outside of each end of the tunnel, the L side locating at the 7-side chain binding site (B site) and the catalytic center (A site), and the R side located at the outside of the 3-side chain binding site (C site), was exposed to solvent as shown in Figure 1a and b. It was speculated that FR259647 and FK518 approached the binding pocket from the direction of the L side, because the size of their 7-side chain was larger than the space of the tunnel and their 7-side chain cannot pass through the tunnel from the R side. A gate on the L side of the tunnel located in the white rectangle was surrounded by the following four residues, Ala319, Tyr177, Thr343, and Asn373, as shown in Figure 1c. The gate was almost the same size as the tunnel, so we speculated that the 3-side chain that has passed through the gate could be accommodated in the 3-side



Scheme 1. Hydrolysis pathway of cephalosporins by AmpC β-lactamase (E); (A) a precovalent first encounter complex of the enzyme and cephalosporin (R^1, 7-side chain; R, 3-side chain); (B) an acyl-enzyme complex; (C) a complex of a hydrolyzed product and the intact enzyme.

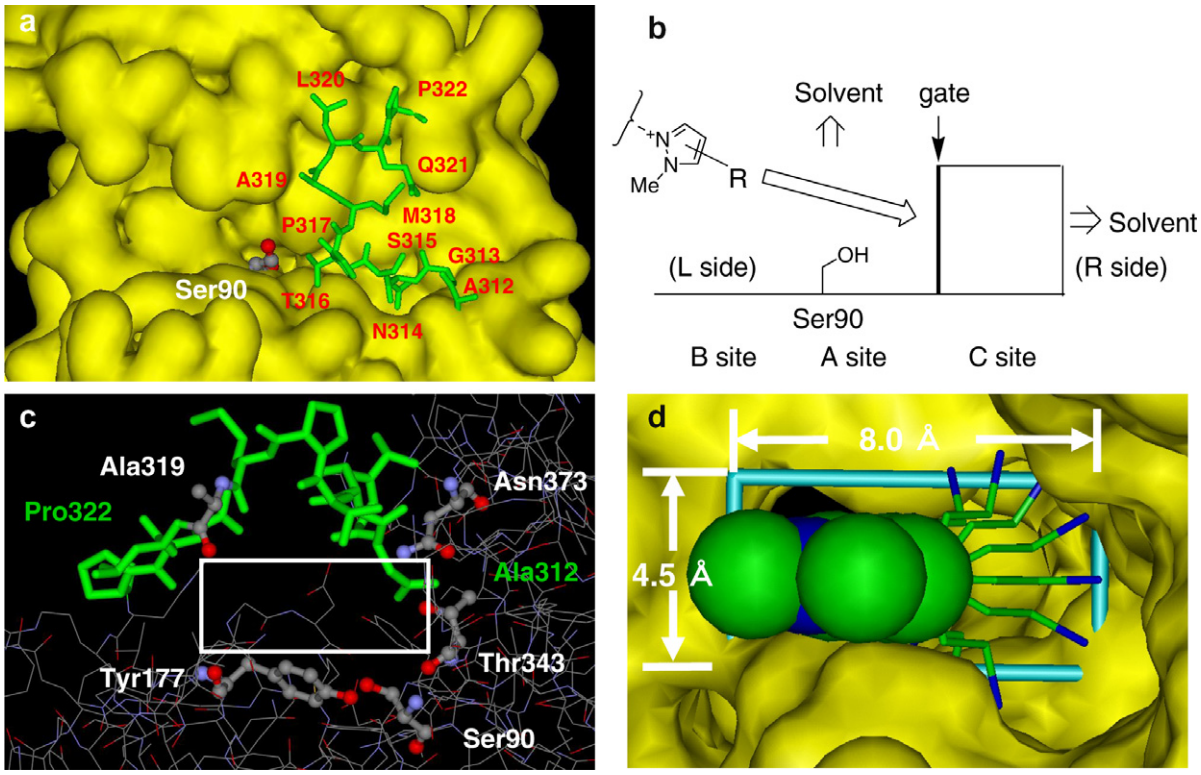


Figure 1. (a) Cephalosporin binding pocket of AmpC β-lactamase. Ser90 (ball and stick display) is a catalytic center. The binding pocket forms a cleft structure and the Connolly surface is displayed with a yellow color. The loop Ala312-Pro322 (green stick display) forms a bridged structure over the cleft, which shows a tunnel structure. (b) Diagrammatic illustration of cephalosporin binding pocket: A site (Ser90) shows active center; B site shows 7-side chain binding site; C site shows 3-side chain binding site; solid line shows the gate of the 3-side chain binding pocket; rectangle shows tunnel structure part; outline arrow indicated direction of approach of FR259647 derivatives to binding pocket. (c) The 3-side chain binding site viewed from L side (see b). The entrance of the 3-side chain binding site displays white rectangle surrounded by the following four residues, Ala319, Tyr177, Thr343, and Asn373 (ball and stick display). Green stick display shows the loop Ala312-Pro322 except for Ala319. (d) The gate of the 3-side chain binding pocket of AmpC β-lactamase and 3-side chain of FR259647 approaching the gate, rotating aminopropyl group on pyrazolium ring; CPK display shows pyrazolium ring colored by atom type. Stick display shows rotating aminopropyl moiety colored by atom type. Yellow solid display shows active pocket Connolly surface. The blue part shows the gate of the 3-side chain binding pocket. Pictures (a) and (c) were drawn by Viewer Lite 5.0. Picture (d) was drawn by InsightII 2000.

chain binding pocket. Therefore, among various possible causes for the stability, we speculated that steric hindrance between a cephalosporin and the gate in its entry into the 3-side chain binding pocket prevented and inhibited formation of complex A, and this inhibition resulted in the stability against AmpC β-lactamase. In order to validate this speculation, the Connolly surface of the binding pocket was displayed and the 3D-structure of the gate was investigated. The shape of the gate was approximated to a rectangle, and the gate size was measured by creating manually the rectangle fitted to the surface in the proximity of the white rectangle shown in Figure 1c using the Discover/Insight II program.[19] The result showed that the gate was a rectangle of 4.5 Å × 8.0 Å displayed as blue as shown in Figure 1d. Considering the structure of both FR259647 and the gate, we reasoned that FR259647 would approach the gate from the L side, rotating the 4-aminopropyl moiety on the pyrazolium ring, putting the 3- or 4-substituent on the ring at the head (Fig. 1d). If FR259647 is bound to AmpC β-lactamase, the 3-side chain of FR259647 has to pass through the gate of the 3-side chain binding pocket. Therefore, we predicted the possibility of entry into the binding pocket as the probability of entry through the gate by conformational analysis and attempted to evaluate the relationship between the probability of entry and the stability to AmpC β-lactamase. Conformational analysis of the structure of the 3-side chain in the case of FR259647 was executed for the rotatable bond $\tau_1 - \tau_3$ of the aminopropyl moiety as a variable using the systematic search module in SYBYL 6.9 (Fig. 2a).[20] According to the conditions described above, we defined that a conformer can pass through the gate of the 3-side chain binding pocket in the case that the maximum value (D_{max}) of the distance D between the π-plane of the pyrazolium ring and each atom of 3- or 4-substituent on the pyrazolium ring including van der Waals radius is less than the height H (4.5 Å) of the gate as shown in Figure 2b. The probability $P(i)$ of conformer i amongst all possible conformers of FR259647 was calculated from total energy of each conformer using the Maxwell–Boltzmann distribution rule (Eq. 1), and the sum of the probability $P(i)$ of the conformers which can pass through the gate is defined as the probability of entry of FR259647. As shown in Figure 2b, in the case that D_{max} is less than H, the conformers could pass through the gate to be bound to the active center. In the case that D_{max} is more or equal to H, the

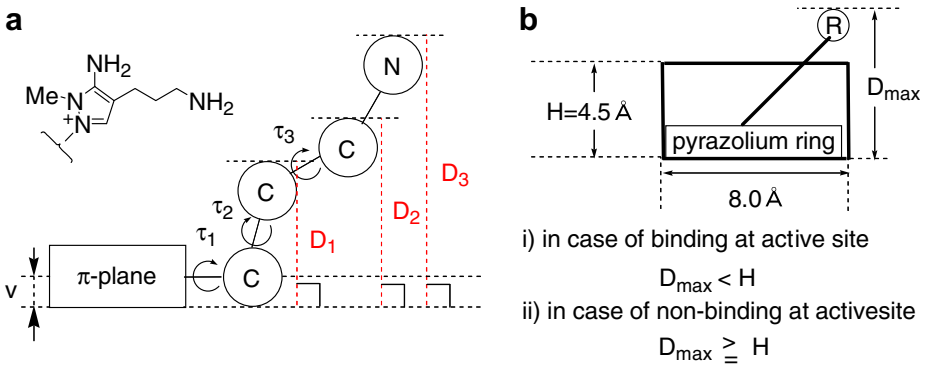


Figure 2. (a) The 3-side chain of FR259647; π-plane shows the π-plane of pyrazolium ring; $\tau_1 - \tau_3$ shows free rotatable bond. *v* shows van der Waals radius. Circles surrounded around atomic symbol show van der Waals surface. Red dashed lines show distance $D_1 - D_3$ between π-plane of pyrazolium ring and each atom of the aminopropyl moiety. (b) Condition whereby FR259647 derivatives can pass through the gate from the L side (see Fig. 1b and d) for binding to active center (Ser90) of AmpC β-lactamase: solid rectangle shows the gate of the 3-side chain binding pocket; *H* shows height of the gate; D_{max} shows maximum value of distance of each atom of 3- or 4-substituent R on pyrazolium ring from π-plane of pyrazolium ring including van der Waals radius.

conformers may not pass through the gate. We reasoned that compounds with a higher probability of the latter type of conformer amongst all conformers should be more stable to the enzyme

$$P(i) = \exp(-Ei/RT) / \sum_{i=1}^{N} \exp(-Ei/RT) \qquad (1)$$

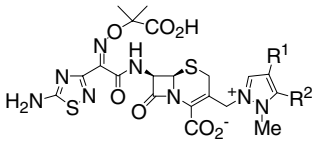
$P(i)$: probability of conformer i amongst all possible conformers of a compound,
N: the total number of conformers,
i: conformer number,
Ei: total energy of conformer i,
R = 0.00199 kcal/mol/K,
T = 298.15 K.

As shown in Table 1, the probabilities of entry of FR259647, bearing a 4-(3-aminopropyl) group, and FK518 bearing an 3-amino group were 0.10% and 100%, respectively. From these calculation results, we proposed FR259647 derivatives with lower probability of entry would be more stable to AmpC β-lactamase.

2.3. Model validation of structural requirements for the stability to AmpC β-lactamase using designed compounds

We next attempted to verify the validity of the structural requirements using designed compounds. Considering the conformational space of compounds, we speculated that compounds with a short methylene chain at the 3- or 4-position on the pyrazolium ring would have a higher probability of entry and could be labile to AmpC β-lactamase, and that compounds with a longer methylene chain on the same position would have a lower probability of entry and could be stable because of steric hindrance against the gate. A part of the gate of the 3-side chain binding pocket of AmpC β-lactamase comprises of loop (Ala312-Pro322). PBP, which is a target enzyme for cephalosporins, and AmpC β-lactamase are closely related and have a similar 3D-structure, but such a loop does not exist in PBP.[21] The loop of AmpC β-lactamase has been inserted in the location of the binding for the second strand in PBP.[21] Accordingly, we considered that modification of the substituents at the 3- or 4-position of the pyrazolium ring of FR259647, aiming at steric hindrance of the gate, may generate cephalosporins which are stable to AmpC β-lactamase, keeping their affinity for PBP. Compounds **4**–**9** with various lengths of methylene chain at the 3- or 4-position on the pyrazolium ring were designed, calculated, synthesized, and evaluated. Conformational analysis of the structure of the 3-side chain of each compound was executed for the rotatable bonds of the amino, aminoalkyl or alkyl moiety at the 3- or 4-position on the pyrazolium ring as a variable, and the probability of entry of each compound was calculated using a procedure similar to that employed for the calculation of FR259647. As shown in Table 2, the probabilities of entry of compound **4**, bearing an 4-amino group on the pyrazolium ring, and compound **5** bearing a 4-aminomethyl group were 100% and 85%, respectively. These results predicted that compounds **4** and **5** would be labile to AmpC β-lactamase. The Δ*ampD*/PAO1:PAO1 strain MIC ratios of compounds **4** and **5** were 8, and indicated that these compounds were labile to AmpC β-lactamase. On the other hand, the probabilities of entry of compound **6** bearing a 4-(4-aminobutyl) group on the pyrazolium ring, compound **7** bearing a 4-(5-aminopentyl) group, and compound **8** bearing a 3-(2-aminoethyl) group were 0.71%, 0.43%, and 5.9%, respectively. These results predicted that compounds **6**–**8** would be stable to AmpC β-lactamase. The Δ*ampD*/PAO1:PAO1 MIC ratios of **6**–**8** were all 2, indicating that these compounds were stable to AmpC β-lactamase. However, these designed compounds all have an amino group at the edge of the 3- or 4-substituent on the pyrazole ring, it was also considered that FR259647 might be stable because of electrostatic repulsion between the amino moiety of the aminoalkyl group on the pyrazolium ring of FR259647 and the basic group of AmpC β-lactamase. In order to evaluate the possibility of repulsion, compound **9**, without the amino group of 4-(3-aminopropyl)

Table 2. MICs (μg/mL) of compounds **4**–**9** against *P. aeruginosa* PAO1 and Δ*ampD*/PAO1 strain, and probability of entry through the gate of the 3-side chain binding pocket by conformational analysis



No.	Compounds		Δ*ampD*/PAO1[a]	MIC ratio[b]	Calculation probability of entry (%)
	R^1=	R^2=	PAO1[a]		
4	$-NH_2$	$-NH_2$	4/0.5	8	100
5	$-CH_2NH_2$	$-NH_2$	4/0.5	8	85
6	$-(CH_2)_4NH_2$	$-NH_2$	1/0.5	2	0.71
7	$-(CH_2)_5NH_2$	$-NH_2$	2/1	2	0.43
8	–H	$-(CH_2)_2NH_2$ (H_2SO_4 salt)	1/0.5	2	5.9
9	$-(CH_2)_2CH_3$	$-NH_2$	2/1	2	2.7

[a] MIC (μg/mL).
[b] MIC ratio of Δ*ampD*/PAO1 to PAO1 strain.

group on the pyrazolium ring from FR259647, was synthesized and evaluated. The probability of entry of compound **9** was 2.7% and this result predicted that compound **9** would be stable to AmpC β-lactamase. If compound **9** is labile to AmpC β-lactamase, our model is not supported. The Δ*ampD*/PAO1:PAO1 MIC ratio of **9** was 2 and indicated that this compound was stable to AmpC β-lactamase. Because both FR259647 and **9** were stable to AmpC β-lactamase, this revealed that electrostatic repulsion of the amino moiety of the aminoalkyl group on the pyrazolium ring of FR259647 did not induce the stability. These results supported the validity of our proposal and revealed the structural requirements for stability and that FR259647 derivatives with lower probability of entry were more stable to AmpC β-lactamase.

Taken together, these studies indicated that the probability of entry of FR259647 derivatives through the gate of the 3-side chain binding pocket of AmpC β-lactamase correlated with the MIC ratio of the mutant strain Δ*ampD*/PAO1 to the parent strain PAO1, and that FR259647 derivatives with lower probability of entry are more stable to AmpC β-lactamase (Fig. 3).

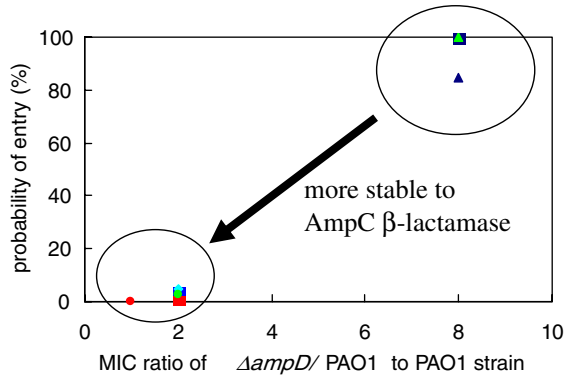
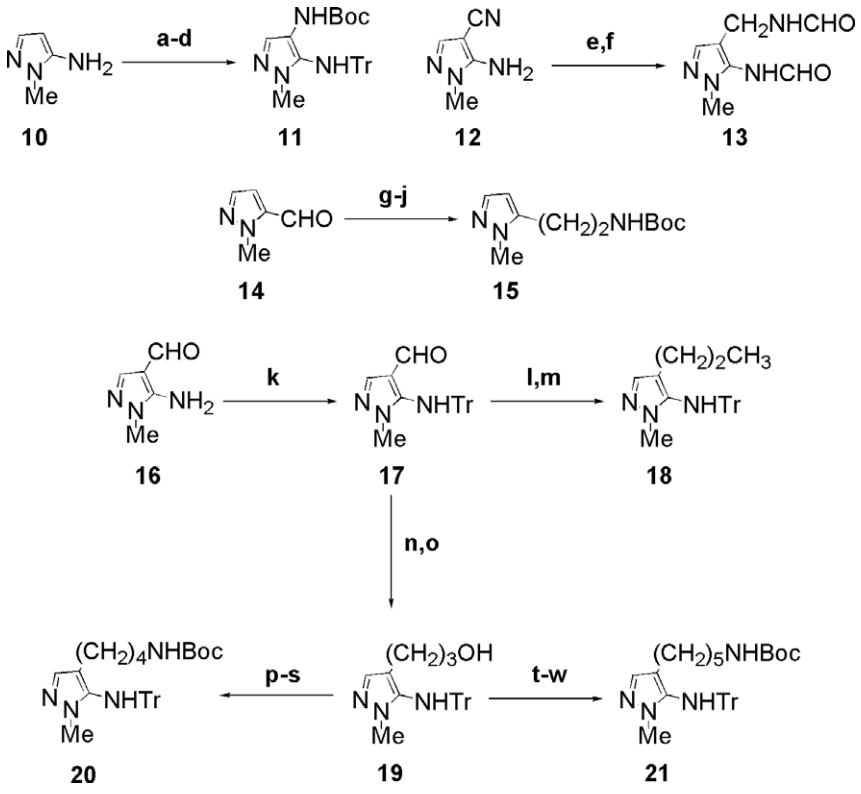


Figure 3. Correlation between MIC ratio of Δ*ampD*/PAO1 to PAO1 strain and probability of entry. FR259647 derivatives with lower probability of entry are more stable to AmpC β-lactamase.

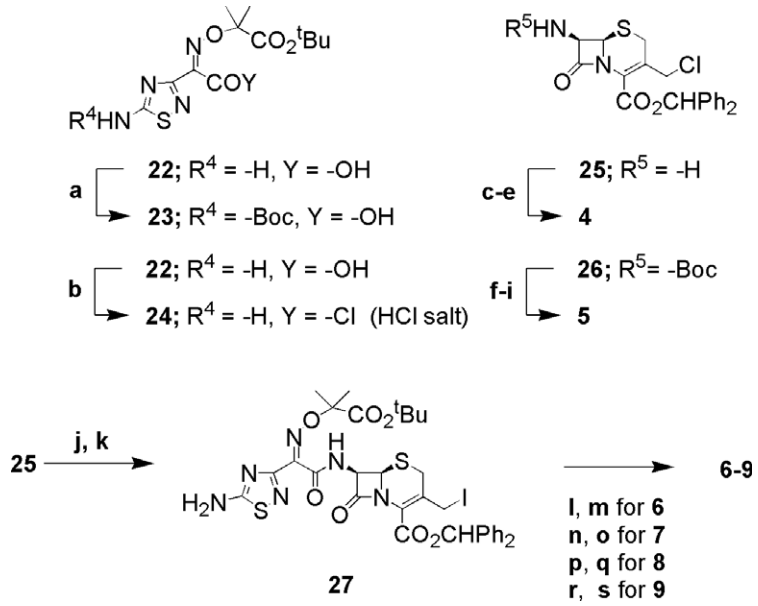
3. Chemistry

The preparation of the 3-side chain pyrazoles was performed according to the procedures shown in Scheme 2. 4-*tert*-Butoxycarbonylamino-1-methyl-5-tritylaminopyrazole (**11**) was prepared by nitrosation of 5-amino-1-methylpyrazole (**10**), followed by hydrogenation, *N*-*tert*-butoxycarbonylation, and tritylation. 5-Formylamino-4-formylaminomethyl-1-methylpyrazole (**13**) was prepared by reduction of 5-amino-4-cyano-1-methylpyrazole (**12**) with $LiAlH_4$, followed by formylation. *tert*-Butyl 2-(1-methylpyrazol-5-yl)-ethylcarbamate (**15**) was prepared by Horner–Emmons reaction of 1-methylpyrazole-5-carbaldehyde (**14**), followed by hydrogenation, hydrolysis, and Curtius rearrangement. l-Methyl-5-tritylaminopyrazole-4-carbaldehyde (**17**) was prepared by tritylation of 5-amino-1-methylpyrazole-4-carbaldehyde (**16**). 4-(1-Propyl)-1-methyl-5-tritylaminopyrazole (**18**) was prepared by Wittig reaction of **17**, followed by hydrogenation. 4-(3-Hydroxypropyl)-5-tritylamino-1-methylpyrazole (**19**) was prepared by Horner–Emmons reaction of **17**, followed by reduction with $LiAlH_4$. 4-(4-*tert*-Butoxycarbonylaminobutyl)-5-tritylamino-1-methylpyrazole (**20**) was prepared by iodination of **19** and treatment with NaCN, $LiAlH_4$, and Boc_2O. 4-(5-*tert*-Butoxycarbonylaminopentyl)-5-tritylamino-1-methylpyrazole (**21**) was prepared by Swern oxidation of **19**, followed by Horner–Emmons reaction, reduction with $LiAlH_4$, and treatment with Boc_2O.

Cephalosporins **4**–**9** were obtained by coupling of protected cephalosporin derivatives and the pyrazoles according to the procedures shown in Scheme 3. Cephalosporin **4** was prepared by Vilsmeier acylation of 7-ACA derivative **25**[22] and acetic acid derivative **23**, which was obtained by *N*-*tert*-butoxycarbonylation of the known carboxylic acid **22**,[23] followed by coupling with pyrazole **11**, and subsequent removal of the protecting groups. Cephalosporin **5** was prepared by coupling of pyrazole **13** and 7-ACA derivative **26**,[24] followed by removal of the protecting groups, and acylation with acyl chloride **24**, which was prepared by treatment of **22** with PCl_5, and subsequent removal of the protecting groups.



Scheme 2. Reagents and conditions: (a) $NaNO_2$, concd HCl, H_2O, 5 °C; (b) H_2, 10% Pd–C, H_2SO_4, H_2O, rt, 73% in 2 steps; (c) Boc_2O, *i*-Pr_2EtN, CH_2Cl_2, rt; (d) TrCl, Et_3N, CH_2Cl_2, rt, 23% in 2 steps; (e) $LiAlH_4$, THF, rt; (f) HCO_2H, Ac_2O, MeOH, rt, 43% in 2 steps; (g) $(EtO)_2POCH_2CO_2Et$, NaH, THF, 0 °C to rt, 62%; (h) H_2, 10% Pd–C, EtOH, rt, 98%; (i) 1 N NaOH, EtOH, 70 °C, 71%; (j) $(PhO)_2P(O)N_3$, Et_3N, *t*-BuOH, reflux, 86%; (k) TrCl, Et_3N, CH_2Cl_2, rt, 92%; (l) Ph_3PEtBr, NaH, DMSO, 70 °C–rt, 36%; (m) H_2, 10% Pd–C, EtOAc–THF, rt, 91%; (n) $(EtO)_2POCH_2CO_2Et$, NaH, THF, 0 °C–rt, 76%; (o) $LiAlH_4$, THF, rt, 74%; (p) I_2, imidazole, Ph_3P, THF, rt, 34%; (q) NaCN, NH_4Cl, DMF, 70 °C, 83%; (r) $LiAlH_4$, THF, reflux, 90%; (s) Boc_2O, CH_2Cl_2, rt, 51%; (t) $(COCl)_2$, DMSO, *i*-Pr_2EtN, CH_2Cl_2, −78 to 0 °C, 25%; (u) $(EtO)_2POCH_2CN$, NaH, THF, rt, 74%; (v) $LiAlH_4$, THF, rt, 99%; (w) Boc_2O, CH_2Cl_2, rt, 28%.



Scheme 3. Reagents and conditions: (a) Boc_2O, sodium bis(trimethylsilyl)amide, THF–DMF, 0 °C, 47%; (b) PCl_5, CH_2Cl_2, −20 to −10 °C, 76%; (c) *N*-(trimethylsilyl)acetamide (MSA), THF, then **23**, $POCl_3$, DMF, AcOEt–THF, −10 to 0 °C, 87%; (d) **11**, NaI, DMF–CH_2Cl_2, rt; (e) TFA, anisole, CH_2Cl_2, rt, 3.4% in 2 steps; (f) **13**, NaI, DMF–CH_2Cl_2, rt; (g) TFA, anisole, CH_2Cl_2, rt; (h) **24**, MSA, THF–DMF, 0 °C; (i) concd HCl, MeOH, rt, 2.2% in 4 steps; (j) **24**, MSA,THF, 0 °C, 87%; (k) NaI, Aliquat 336, toluene-phosphate buffer, rt, 88%; (l) **20**, MSA, DMF, 37 °C; (m) TFA, anisole, CH_2Cl_2, rt, 16% in 2 steps; (n) **21**, MSA, DMF, 37 °C; (o) TFA, anisole, CH_2Cl_2, rt, 21% in 2 steps; (p) **15**, MSA, DMF, 37 °C; (q) TFA, anisole, CH_2Cl_2, rt, 3.8% in 2 steps; (r) **18**, MSA, DMF, 37 °C; (s) TFA, anisole, CH_2Cl_2, rt, 18% in 2 steps.

Key intermediate iodomethyl cephalosporin derivative **27** was prepared by acylation of **25** with acyl chloride **24**, followed by iodination. Cephalosporins **6**–**9** were prepared by coupling of the pyrazoles **20**, **21**, **15**, and **18** with **27**, respectively, and subsequent removal of the protecting groups. The cephalosporin compounds were then purified by column chromatography by HPLC and HP-20 to evaluate biological activities.

4. Conclusions

We have investigated the structural requirements for the stability of FR259647 derivatives to AmpC β-lactamase based on 3D-structure. Comparison of the structural difference between FR259647 and FK518, considering the 3D-structure of the active pocket of AmpC β-lactamase, led to structural requirements for stability to the enzyme that steric hindrance of the 3- or 4-substituent on the pyrazole ring at the 3-position of cephalosporin nucleus against the gate of the 3-side chain binding pocket interfered with the entry of FR259647 into the 3-side chain binding pocket. We predicted the possibility of the entry into the binding pocket as the probability of entry by conformational analysis. In order to validate our speculation, novel FR259647 derivatives **4**–**9** were designed, calculated, synthesized, and evaluated. As a result, we demonstrated that their probability of entry correlated with their MIC ratio of Δ*ampD*/PAO1 strain to PAO1 strain as an index of stability and validity of our model. In conclusion, we propose novel structural requirements for the stability of FR259647 derivatives to AmpC β-lactamase that the derivatives with the lower probability of entry through the gate of the 3-side chain binding pocket of AmpC β-lactamase are more stable. Our continuing efforts to generate more potent cephalosporins, which are more stable to AmpC β-lactamase than FR259647, against *P. aeruginosa* overproducing AmpC β-lactamase based on these structural requirements will be reported in the future.

5. Experimental

5.1. Homology modeling

Homology modeling of AmpC β-lactamase from *P. aeruginosa* was performed with the FAMS (Full Automatic Modeling System) program[18] using the crystal structure of AmpC β-lactamase from *E. coli* as a template. The amino acid sequences of *P. aeruginosa* and *E. coli* AmpC β-lactamase have 43% identical residues, two insertion residues, and 10 deletion residues. A modeled structure from *P. aeruginosa* was obtained using the crystal structure of complex of mutant AmpC β-lactamase S64G from *E. coli* with cephalothin (PDB entry 1KVL) as a template.[15,16] We used the sequence from Lys35 to Leu384 as sequence information for *P. aeruginosa* AmpC β-lactamase in accordance with amino acid sequence of template crystal structures that are visible in the electron density. In order to compare their structures, the modeling structure of *P. aeruginosa* was superimposed on the complex crystal structure of *E. coli* AmpC β-lactamase (1KVL) by overlapping corresponding Cα atoms using the Discover/Insight II program[19] on a Silicon Graphics Workstation Octane2 with R12000 CPU, respectively. Superimposition of the modeling structure on the template X-ray structure indicated that root mean square distance between Cα atoms was 0.50 Å. The amino acid residues of the active pocket of AmpC β-lactamase less than 6 Å from the bound cephalothin have 60% identical residues, no insertions, and no deletions. These results show the validity of the modeled structure.

5.2. Conformational analysis and calculation method of probability of entry

Conformational studies of the 3-side chain structures of FR259647, FK518, and compounds **4**–**9** were performed with the systematic search module in SYBYL 6.9[20] on a Silicon Graphics Workstation Octane2 with R12000 CPU. Each torsion angle τ was rotated in 30° increments. Other parameters used default values. Total energy of each conformer was calculated by systematic search module, and distance D between the π-plane of the pyrazolium ring and each atom of the 3- or 4-substituent on the pyrazolium ring of each conformer was calculated using PLANE in the SYBYL function on a spread sheet and was added to the van der waals radius of each atom.[20] The probability $P(i)$ of conformer i amongst all possible conformers of each compound was calculated from the total energy of each conformer using the Maxwell–Boltzmann distribution rule (Eq. 1). The probability of entry of each compound was calculated as a sum of the probability $P(i)$ of the conformers which satisfied the following conditions: maximum value (D_{max}) of the distance D is less than the height H (4.5 Å) of the gate. The size of the gate was measured by creating manually a rectangle fitting to the surface of the 3D-structure of AmpC β-lactamase using the Discover/Insight II program.[19]

5.3. Antibacterial assay against Δ*ampD*/PAO1 and PAO1 strains

It is known that *P. aeruginosa* PAO1 *AmpD*-defective mutant (Δ*ampD*/PAO1) leads to the overproduction of AmpC β-lactamase.[7,12] Construction of Δ*ampD*/PAO1 mutant was performed by disrupting *ampD* of *P. aeruginosa* PAO1 using a streptomycin-resistant gene cassette according to the literature method.[7,13] The antibacterial activity (MIC) ratio of Δ*ampD*/PAO1 to PAO1 was used as an index of stability to AmpC β-lactamase. MICs were determined by an agar dilution method using Mueller-Hinton agar (Becton–Dickinson, Tokyo, Japan) and an inoculum of 10^4 CFU per spot. Results of susceptibility testing were recorded according to Clinical and Laboratory Standards Institute reference method M7-A5, after inoculation at 35 °C for 18 h.[25]

5.4. Chemistry

5.4.1. General methods. Proton NMR spectra were recorded on a Brucker BIOSPIN AVANCE400 or

DPX200. δ Values in ppm relative to tetramethylsilane are given. IR spectra were recorded with the compound (neat) on a sodium chloride disk or as KBr pellets or Nujol suspension using HORIBA FT-710. Mass spectra were recorded with Hewlett Packard 1100LC/MSD. High resolution mass spectra were recorded by micromass LCT. Elemental analysis was obtained with Perkin-Elmer 2400II.

5.4.2. Starting materials. (*Z*)-2-(5-Amino-1,2,4-thiadiazol-3-yl)-[2-(1-*tert*-butoxycarbonyl-1-methylethoxy)-imino]acetic acid (**22**), benzhydryl 7β-amino-3-chloromethyl-3-cephem-4-carboxylate (**25**), and benzhydryl 7β-*tert*-butoxycarbonylamino-3-chloromethyl-3-cephem-4-carboxylate (**26**) were prepared according to the procedure described in the literature.[22–24]

5.4.3. Synthesis of cephalosporin 4.

5.4.3.1. 4-*tert*-Butoxycarbonylamino-1-methyl-5-tritylaminopyrazole (11). To a solution of 5-amino-1-methylpyrazole (**10**) (100 g, 1.03 mol) in water (700 mL) were added concd HCl (86 mL) and sodium nitrite (63.9 g, 927 mmol) in water (200 mL) below 10 °C. The reaction mixture was stirred for 30 min at 5 °C. The precipitated solid was filtered off and dried to give 5-amino-1-methyl-4-nitrosopyrazole (117 g). To a suspension of this product (117 g, 928 mmol) in water (819 mL) were added concd H_2SO_4 (91 g, 928 mmol) and 10% Pd–C (58 g) at room temperature. The resulting mixture was hydrogenated under balloon pressure for 10 h at room temperature. The reaction mixture was filtered and the resulting filtrate was concentrated in vacuo. To the resulting suspension was added isopropanol (IPA; 2.3 L) and the mixture was stirred for 1 h. The precipitated solid was filtered and dried to give 4,5-diamino-1-methylpyrazole sulfate (158 g, 73%): ^{1}H NMR (D_2O) δ 3.74 (3H, s), 7.80 (1H, s).

To an ice-cooled solution of 4,5-diamino-1-methylpyrazole sulfate (30 g, 143 mmol) in CH_2Cl_2 (300 mL) were added di-*tert*-butyl dicarbonate (34.3 g, 157 mmol) and diisopropylethylamine (49.7 mL, 285 mmol). The solution was stirred for 4 h at room temperature. To the resulting mixture was added water and extracted with EtOAc. The organic layer was washed with water twice and brine. The extract was dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The residue was triturated with diisopropyl ether (IPE), filtered, and dried in vacuo to give 4-*tert*-butoxycarbonylamino-1-methyl-5-aminopyrazole (10.8 g). To a solution of this product (10.5 g, 49.5 mmol) and triethylamine (34.5 mL, 247 mmol) in CH_2Cl_2 (150 mL) was added trityl chloride (16.5 g, 59.4 mmol) at room temperature and was stirred at room temperature overnight. The resulting mixture was concentrated in vacuo and the organic layer was washed with water three times and brine. The extract was dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The residue was triturated with IPE, filtered, and dried in vacuo to give **11** (14.2 g, 23%): ^{1}H NMR (DMSO-d_6) δ 1.35 (9H, s), 2.70 (3H, s), 5.66 (1H, s), 7.00–7.40 (17H, m); MS (APCI) *m*/*z* 455 $(M+H)^+$.

5.4.3.2. (*Z*)-2-{5-[(*tert*-Butoxycarbonyl)amino]-1,2,4-thiadiazol-3-yl}-2-[(1-*tert*-butoxycarbonyl-1-methylethoxy)imino]acetic acid (23). To a solution of **22**[23] (5.0 g, 15.1 mmol) in a mixed solvent of THF (80 mL) and DMF (20 mL) was added a solution of sodium bis(trimethylsilyl)amide (8.33 g, 45.4 mmol) in THF (12 mL), and the mixture was stirred for 15 min. To the reaction mixture was added a solution of di-*tert*-butyl dicarbonate (3.3 g, 15.1 mmol) in THF (20 mL) under ice-cooling, and the mixture was stirred under ice-cooling for 3 h. To the reaction mixture was added EtOAc, and the mixture was washed with 10% aqueous $KHSO_4$ solution and then washed with a phosphate buffer (pH 6.86). The organic layer was separated, dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The residue was triturated with IPE, filtered, and dried in vacuo to give **23** (3.10 g, 47%): ^{1}H NMR (DMSO-d_6) δ 1.37 (9H, s), 1.45 (6H, s), 1.50 (9H, s), 12.7 (1H, s); IR(KBr) ν_{max} 3192, 2981, 1714, 1551, 1153, 1001 cm^{-1}; MS (ESI): *m*/*z* 429 $(M-H)^+$.

5.4.3.3. 7β-[(*Z*)-2-(5-Amino-1,2,4-thiadiazol-3-yl)-2-(1-carboxy-1-methylethoxyimino)acetamido]-3-[3,4-diamino2-methyl-1-pyrazolio]methyl-3-cephem-4-carboxylate (4). A mixture of DMF (0.648 mL, 8.37 mmol) and $POCl_3$ (0.781 mL, 8.37 mmol) was stirred at room temperature for 30 min. To the mixture was added **23** (3 g, 6.98 mmol) in THF (4 mL) at 4 °C, and the reaction mixture was stirred at room temperature for 1 h. Meanwhile, a mixture of **25**[22] (3 g, 6.65 mmol) and *N*-(trimethylsilyl)acetamide (8.72 g, 66.4 mmol) in THF (15 mL) was warmed to make a clear solution. The solution was then cooled to −20 °C and added to the activated acid solution obtained above. The reaction mixture was stirred at −10 °C to 0 °C for 1 h and poured into mixture of EtOAc and water. The aqueous layer was separated, and the organic layer was washed with brine, dried over anhydrous $MgSO_4$, and filtered. The filtrate was concentrated in vacuo and purified by column chromatography on silica gel eluting with hexane/EtOAc (3:2) to give benzhydryl 7β-[(*Z*)-2-(5-*tert*-butoxycarbonylamino-1,2,4-thiadiazol-3-yl)-2-(1-*tert*-butoxycarbonyl-1-methylethoxyimino)acetamido]-3-chloromethyl-3-cephem-4-carboxylate (4.79 g, 87%): ^{1}H NMR (DMSO-d_6) δ 1.39 (9H, s), 1.48 (6H, s), 1.50 (9H, s), 3.58 (1H, d, J = 18.3 Hz), 3.76 (1H, d, J = 18.3 Hz), 4.44 (2H, s), 5.29 (1H, d, J = 5.0 Hz), 6.01 (1H, dd, J = 8.6, 5.0 Hz), 6.97 (1H, s), 7.2–7.6 (10H, m), 9.65 (1H, d, J = 5.0 Hz), 12.7 (1H, s); IR(KBr) ν_{max} 2981, 1794, 1720, 1525, 1371, 1247, 1151 cm^{-1}; MS (ESI): *m*/*z* 849 $(M+Na)^+$.

To a solution of benzhydryl 7β-[(*Z*)-2-(5-*tert*-butoxycarbonylamino-1,2,4-thiadiazol-3-yl)-2-(1-*tert*-butoxycarbonyl-1-methylethoxyimino)acetamido]-3-chloromethyl-3-cephem-4-carboxylate (0.65 g, 0.786 mmol) in a mixed solvent of DMF (2 mL) and CH_2Cl_2 (2 mL) was added sodium iodide (130 mg, 0.864 mmol), and the mixture was stirred for 30 min at room temperature. To the reaction mixture was added **11** (714 mg, 1.57 mmol). The whole mixture was stirred for 36 h at room temperature and poured into a mixture of EtOAc and water, and extracted with EtOAc. The organic layer was washed with water, a mixture of aqueous 10% sodium thiosulfate and

brine, brine, and aqueous 10% trifluoroacetic acid sodium salt solution, and dried over $MgSO_4$, filtered, and evaporated in vacuo. The residue was dissolved in a small amount of EtOAc and poured into IPE. The resulting precipitates were filtered. The filter cake was washed with IPE and dried in vacuo. To a solution of the resulting solid in CH_2Cl_2 (1.5 mL) were added anisole (0.5 mL) and trifluoroacetic acid (1.0 mL). The resulting solution was stirred for 2 h at room temperature and poured into IPE. The resulting precipitate was collected by filtration and dried in vacuo. The crude product was purified by preparative HPLC utilizing ODS. The first eluate containing the desired product was concentrated to about 30 mL in vacuo. The concentrate was adjusted to about pH 3 by addition of concd HCl and purified by column chromatography on HP-20 eluting with 20% aqueous IPA. The eluate was concentrated to about 30 mL in vacuo and lyophilized to give **4** (15.5 mg, 3.4%): ^{1}H NMR (D_2O) δ 1.53 (6H, s), 3.13 and 3.30 (2H, ABq, J = 17.7 Hz), 3.65 (3H, s), 4.50–4.95 (2H, m), 5.20 (1H, d, J = 4.7 Hz), 5.84 (1H, d, J = 4.7 Hz), 7.57 (1H, s); IR(KBr) ν_{max} 1772 cm^{-1}; HRMS (ESI) m/z calcd for $C_{20}H_{25}N_{10}O_7S_2$ $[M+H]^+$ 581.1349. Found: 581.1350. Anal. Calcd for $C_{20}H_{24}N_{10}O_7S_2$ 3.89H_2O: C, 36.92; H, 4.92; N, 21.53. Found: C, 37.32; H, 5.42; N, 21.03.

5.4.4. Synthesis of cephalosporin 5

5.4.4.1. 5-Formylamino-4-formylaminomethyl-1-methylpyrazole (13). To ice-cooled THF (300 mL) was added $LiAlH_4$ (6.21 g, 164 mmol) under nitrogen gas. To the ice-cooled mixture was added 5-amino-4-cyano-1-methylpyrazole (**12**) (10.0 g, 81.9 mmol) and stirred for 2 h at room temperature under nitrogen gas. To the ice-cooled mixture were successively added sodium fluoride (30.0 g) and water (12 mL). The resulting mixture was filtered and concentrated in vacuo. The residue was dissolved in toluene, and the solution was concentrated in vacuo again to give 5-amino-4-aminomethyl-1-methylpyrazole (6.34 g). To formic acid (11.5 mL) was added acetic anhydride (14.2 mL), and the mixture was stirred at room temperature for 30 min. The resulting mixture was added to 5-amino-4-aminomethyl-1-methylpyrazole (6.34 g, 50.2 mmol) in MeOH (19.4 mL) and the mixture was stirred for 2 h at room temperature. After concentration in vacuo, to the residue was added water. The mixture was adjusted to pH 9.5 with DIAION® SA10A (OH^-). The mixture was filtered and concentrated in vacuo. The residue was purified by column chromatography on silica gel eluting with CH_2Cl_2/MeOH (3:1) to give **13** (3.97 g, 43%): ^{1}H NMR (DMSO-d_6) δ 3.59 (3H, s), 3.98 (2H, d, J = 5.8 Hz), 7.30 (1H, s), 8.01 (1H, t, J = 5.8 Hz), 8.15 (1H, s), 8.39 (1H, s), 9.95 (1H, s) MS (ESI): m/z 205 $(M+H)^+$.

5.4.4.2. (*Z*)-2-(5-Amino-1,2,4-thiadiazol-3-yl)-2-(1-*tert*-butoxycarbonyl-1-methylethoxyimino)acetyl chloride hydrochloride (24). To a solution of PCl_5 (11.1 g, 53.4 mmol) in CH_2Cl_2 (168 mL) was added **22** (16.78 g, 50.8 mmol) at −20 °C. The resulting mixture was stirred at −20 to −10 °C for 1.5 h, and to the mixture was added dropwise IPE (671.2 mL) at −20 to −10 °C. The mixture was stirred under ice-cooling for 1 h and the resulting precipitate was collected by filtration to give **24** (14.8 g, 76%): IR (Nujol) ν_{max} 3430, 3270, 3130, 1815, 1750, 1725, 1640 cm^{-1}.

5.4.4.3. 7β-[(*Z*)-2-(5-Amino-1,2,4-thiadiazol-3-yl)-2-(1-carboxy-1-methylethoxyimino)acetamido]-3-[3-amino-4-aminomethyl-2-methyl-1-pyrazolio]methyl-3-cephem-4-carboxylate (5). To a solution of **26**[24] (3.50 g, 6.80 mmol) in DMF (4.5 mL) was added sodium iodide (1.02 g, 6.80 mmol), and the mixture was stirred for 30 min at room temperature. To the reaction mixture was added **13** (3.71 g, 20.4 mmol). The whole mixture was stirred for 29 h at room temperature and poured into a mixture of EtOAc and water. The aqueous layer was separated, and the organic layer was washed with brine, dried over anhydrous $MgSO_4$, and filtered. The filtrate was concentrated to about 20 mL in vacuo. The concentrate was poured into IPE (300 mL), and the resulting precipitate was collected by filtration and dried in vacuo. To a solution of the resulting solid in CH_2Cl_2 (10.5 mL) were added anisole (3.5 mL) and trifluoroacetic acid (7 mL). The resulting solution was stirred for 3 h at room temperature and poured into IPE. The resulting precipitate was collected by filtration and dried in vacuo to give crude 7β-amino-3-[3-formamido-4-formamidomethyl-2-methyl-1-pyrazolio]methyl-3-cephem-4-carboxylate bistrifluoroacetate (3.07 g). To a solution of the crude product (3.07 g, 4.93 mmol) and *N*-(trimethylsilyl)acetamide (6.47 g, 49.3 mmol) in a mixed solvent of DMF (15 mL) and THF (15 mL) was added **24** (1.9 g, 4.93 mmol) under ice-cooling. The solution was stirred for 2 h at the same temperature. The reaction mixture was poured into EtOAc (300 mL), and the mixture was stirred for 30 min. The resulting precipitate was collected by filtration, successively washed with EtOAc and IPE, and dried in vacuo to give a solid (3.28 g). To a suspension of the resulting solid in CH_2Cl_2 (9 mL) were added anisole (3 mL) and trifluoroacetic acid (6 mL) under ice-cooling. The resulting solution was stirred for 3 h at room temperature and poured into IPE. The resulting precipitate was collected by filtration and dried in vacuo to give crude 7β-[(*Z*)-2-(5-amino-1,2,4-thiadiazol-3-yl)-2-(1-carboxy-1-methylethoxyimino)acetamido]-3-[3-formamido-4-formamidomethyl-2-methyl-1-pyrazolio]methyl-3-cephem-4-carboxylate (3.44 g). To a solution of the crude product (2.5 g) in MeOH (25 mL) was added concd HCl solution (2.5 mL) at room temperature. The mixture was stirred for 17 h at room temperature. The reaction mixture was adjusted to about pH 7 by addition of saturated aqueous Na_2CO_3 solution and concentrated in vacuo to remove MeOH. The resulting residue was purified by preparative HPLC utilizing ODS. The eluate containing the desired product was concentrated to about 30 mL in vacuo. The concentrate was adjusted to about pH 1 by addition of concd HCl and purified by column chromatography on HP-20 eluting with 20% aqueous IPA. The eluate was concentrated in vacuo and lyophilized to give **5** (50.0 mg, 2.2%) as an amorphous solid: ^{1}H NMR (D_2O) δ 1.53 (3H, s), 1.54 (3H, s), 3.20 and 3.48 (2H, ABq, J = 17.8 Hz), 3.73 (3H, s), 4.08 (2H, s), 4.99 and 5.19 (2H, ABq, J = 15.4 Hz), 5.26 (1H, d, J = 4.9 Hz), 5.86

(1H, d, $J = 4.9$ Hz), 8.04 (1H, s); IR(KBr) ν_{max} 3346, 3184, 1770, 1595, 1398 cm^{-1}; HRMS (ESI) *m/z* calcd for $C_{21}H_{27}N_{10}O_7S_2$ $[M+H]^+$ 595.1506. Found: 595.1505. Anal. Calcd for $C_{21}H_{26}N_{10}O_7S_2$ $6H_2O$: C, 35.89; H, 5.45; N, 19.93. Found: C, 36.03; H, 5.85; N, 19.89.

5.4.5. Synthesis of cephalosporin 6

5.4.5.1. 1-Methyl-5-(tritylamino)-pyrazole-4-carbaldehyde (17). To a suspension of 5-amino-1-methylpyrazole-4-carbaldehyde (**16**) (25 g, 200 mmol) and Et_3N (22.2 g, 220 mmol) in CH_2Cl_2 (500 mL) was added trityl chloride (61.3 g, 220 mmol) at room temperature. The mixture was stirred at room temperature for 70 h. The reaction mixture was washed with 10% aqueous citric acid solution, 10% aqueous $NaHCO_3$ solution, and brine. The extract was dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The residue was triturated with EtOAc, filtered, washed with EtOAc, and dried in vacuo to give **17** (67.6 g, 92%) as a colorless solid: ^1H NMR ($CDCl_3$) δ 2.84 (3H, s), 7.26–7.34 (15H, m), 7.60 (1H, s), 8.95 (1H, br s), 9.58 (1H, s); MS (APCI) *m/z* 366 $(M-H)^+$.

5.4.5.2. 3-(1-Methyl-5-triphenylmethylaminopyrazol-4-yl) propanol (19). To a suspension of sodium hydride (60% dispersion in mineral oil, 4.80 g, 120 mmol) in THF (200 mL) was added dropwise triethyl phosphonoacetate (4.80 g, 120 mmol) under ice-cooling. The mixture was stirred under ice-cooling for 1 h. To the reaction mixture was added **17** (36.7 g, 100 mmol) at room temperature, and the mixture was stirred at room temperature for 17 h. After concentration in vacuo, the residue was dissolved in $CHCl_3$. The solution was washed with 10% aqueous citric acid solution, 10% aqueous $NaCO_3$ solution, and brine. The extract was dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The residue was triturated with EtOAc, filtered, and dried in vacuo to give ethyl (*E*)-3-(1-methyl-5-triphenylmethylaminopyrazol-4-yl)acrylate (33.0 g, 76%) as a colorless solid: ^1H NMR (DMSO-d_6) δ 1.14 (3H, t, $J = 7.0$ Hz), 3.04 (3H, s), 3.99 (2H, q, $J = 7.0$ Hz), 5.79 (1H, d, $J = 16.0$ Hz), 6.26 (1H, s), 7.07 (1H, d, $J = 16.0$ Hz), 7.15–7.35 (15H, m), 7.62 (1H, s); MS (APCI) *m/z* 460 $(M+Na)^+$.

To a suspension of $LiAlH_4$ (5.7 g, 150 mmol) in THF (200 mL) was added ethyl (*E*)-3-(1-methyl-5-triphenylmethylaminopyrazol-4-yl)acrylate (21.9 g, 50 mmol) at room temperature under nitrogen gas. The mixture was stirred at room temperature for 1 h. After cooling on an ice bath, potassium fluoride (34 g) and water (10 mL) were added to the reaction mixture. The insoluble materials were removed by filtration. The filtrate was concentrated in vacuo, and the residue was dissolved in $CHCl_3$. The solution was washed with 10% aqueous citric acid solution. The organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The oily residue was purified by column chromatography on silica gel eluting with $CHCl_3$ to give **19** (14.8 g, 74%) as a solid: ^1H NMR (DMSO-d_6) δ 1.10–1.30 (2H, m), 1.83 (2H, d, $J = 7.7$ Hz), 2.76 (3H, s), 3.05–3.20 (2H, m), 4.20–4.30 (1H, m), 5.67 (1H, s), 6.98 (1H, s), 7.10–7.30 (15H, m); MS (APCI) *m/z* 396 $(M-H)^+$.

5.4.5.3. 4-(4-*tert*-Butoxycarbonylaminobutyl)-5-tritylamino-1-methylpyrazole (20). To a solution of **19** (3.0 g, 7.55 mmol), imidazole (1.03 g, 15.1 mmol), and triphenylphosphine (3.96 g, 15.1 mmol) in THF (35 mL) was added iodine (3.83 g, 15.1 mmol) at room temperature. To the solution was further added iodine until the color of the solution changed to pale yellow. To the solution was added saturated aqueous $NaHCO_3$ solution and the mixture was extracted with $CHCl_3$. The organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by column chromatography on silica gel eluting with a mixed solvent of EtOAc and hexane to give 4-(3-iodopropyl)-5-tritylamino-1-methylpyrazole (1.31 g, 34%): ^1H NMR (DMSO-d_6) δ 1.35–1.55 (2H, m), 1.85–2.00 (2H, m), 2.76 (3H, s), 2.93 (2H, t, $J = 7.1$ Hz), 5.74 (1H, s), 7.02 (1H, s), 7.10–7.35 (15H, m); MS (APCI) *m/z* 508 $(M+H)^+$, 530 $(M+Na)^+$.

A solution of 4-(3-iodopropyl)-5-tritylamino-1-methylpyrazole (1.00 g, 1.97 mmol), sodium cyanide (97.6 mg, 1.99 mmol), and ammonium chloride (21.1 mg, 0.394 mmol) in DMF (5 mL) was stirred at 70 °C for 5 h. After concentration in vacuo, the residue was extracted with EtOAc. The organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo to give 4-(3-cyanopropyl)-5-tritylamino-1-methylpyrazole (664 mg, 83%): ^1H NMR ($CDCl_3$) δ 1.30–1.50 (2H, m), 1.85–2.00 (2H, m), 2.06 (2H, t, $J = 7.2$ Hz), 2.86 (3H, s), 4.13 (1H, s), 7.10–7.35 (16H, m); MS (APCI) *m/z* 429 $(M+Na)^+$.

To ice-cooled THF (13 mL) was added $LiAlH_4$ (185 mg, 4.87 mmol) under nitrogen gas. To the ice-cooled suspension was added 4-(3-cyanopropyl)-5-tritylamino-1-methylpyrazole (660 mg, 1.62 mmol) and then refluxed for 5 h. After cooling to room temperature, to the ice-cooled mixture were successively added sodium fluoride (1.02 g) and water (350 μL). After filtration, the solution was concentrated in vacuo and to the residue was added toluene and the solution was concentrated in vacuo again to give 4-(4-aminobutyl)-5-tritylamino-1-methylpyrazole (602 mg, 90%): ^1H NMR (DMSO-d_6) δ 0.90–1.20 (4H, m), 1.70–1.85 (2H, m), 2.36 (2H, t, $J = 6.5$ Hz), 2.75 (3H, s), 5.66 (1H, s), 6.98 (1H, s), 7.10–7.35 (15H, m); MS (APCI) *m/z* 409 $(M-H)^+$.

To an ice-cooled solution of 4-(4-aminobutyl)-5-tritylamino-1-methylpyrazole (600 mg, 1.46 mmol) in CH_2Cl_2 (5 mL) was added dropwise a solution of di-*tert*-butyl dicarbonate (335 mg, 1.53 mmol) in CH_2Cl_2 (1 mL). After stirring for 3 h at room temperature and concentrating in vacuo, the residue was extracted with EtOAc. The organic layer was successively washed with water and brine and dried over $MgSO_4$. After filtration and concentration, the residue was purified by column chromatography on silica gel eluting successively with CH_2Cl_2 and a mixed solvent (CH_2Cl_2/EtOAc = 3:1) to give **20** (383 mg, 51%): ^1H NMR (DMSO-d_6) δ 0.90–1.20 (4H, m), 1.37 (9H, s), 1.70–1.85 (2H, m), 2.70–2.80 (2H, m), 2.74 (3H, s), 5.66 (1H, s), 6.60–6.75 (1H, m), 6.98 (1H, s), 7.10–7.35 (15H, m); MS (APCI) *m/z* 511 $(M+H)^+$, 533 $(M+Na)^+$.

5.4.5.4. Benzhydryl 7β-[(*Z*)-2-(5-amino-1,2,4-thiadiazol-3-yl)-2-(1-*tert*-butoxycarboxy-1-methylethoxyimino)acetamido]-3-iodomethyl-3-cephem-4-carboxylate (27). To a solution of **25**[24] (140 g, 310 mmol) in THF (1.4 L) was added *N*-(trimethylsilyl)acetamide (142 g, 1.09 mol) and the mixture was stirred at about 35 °C for 40 min. To the mixture was added **24** (143 g, 372 mmol) under ice-cooling and the reaction mixture was stirred at the same temperature for 1 h. The resulting reaction mixture was added to mixture of EtOAc and ice-water. The mixture was adjusted to pH 1.5 with aqueous $NaHCO_3$ solution, and then the organic layer was separated. To the organic layer was added aqueous $NaHCO_3$ solution to adjust to pH 7.5, and then the organic layer was separated. The aqueous layer was extracted again with EtOAc and the combined organic layers were washed successively with water and brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated to about 0.8 kg in vacuo. The concentrate was slowly poured into IPE (7 L), and the resulting precipitate was collected by filtration, washed with IPE, and dried in vacuo to give benzhydryl 7β-[(*Z*)-2-(5-amino-1,2,4-thiadiazol-3-yl)-2-(1-*tert*-butoxycarboxy-1-methylethoxyimino)acetamido]-3-chloromethyl-3-cephem-4-carboxylate (220 g, 98%). This product was identified by deprotection to the final compound. To a mixture of the product (2.95 g, 4.06 mmol) in a mixed solvent of CH_2Cl_2 (6 mL) and anisole (1.5 mL) was added trifluoroacetic acid (2.95 mL) at 0–5 °C. After stirring at 0 °C for 2 h, the resulting mixture was poured into a mixed solvent of IPE (60 mL) and hexane (120 mL) under ice-cooling. The resulting precipitate was filtered, washed with hexane, and dried over P_2O_5 to give 7β-[(*Z*)-2-(5-amino-1,2,4-thiadiazol-3-yl)-2-(1-*tert*-butoxycarboxy-1-methylethoxyimino)acetamido]-3-chloromethyl-3-cephem-4-carboxylic acid trifluoroacetic acid salt (2.41 g, 88%): ^{1}H NMR (DMSO-d_6) δ 1.39 (9H, s), 1.40 (6H, s), 3.47, 3.75 (2H, ABq, J = 18 Hz), 4.55 (2H, s), 5.20 (1H, d, J = 5 Hz), 5.85 (1H, dd, J = 5 Hz, 8 Hz), 9.48 (1H, d, J = 8 Hz).

To a solution of benzhydryl 7β-[(*Z*)-2-(5-amino-1,2,4-thiadiazol-3-yl)-2-(1-*tert*-butoxycarboxy-1-methylethoxyimino)acetamido]-3-chloromethyl-3-cephem-4-carboxylate (60 g, 82.5 mmol) in toluene (600 mL) were added a solution of sodium iodide (61.8 g, 412 mmol) in 0.05 mol phosphate buffer (pH 7, 500 mL) and tricaprylylmethylammonium chloride (Aliquat 336) (6.67 g, 16.5 mmol). The mixture was stirred at room temperature for 15 h. The reaction mixture was added to a mixture of EtOAc and water. The organic layer was washed with water and brine, and then dried over $MgSO_4$. The $MgSO_4$ was filtered off, and the filtrate was evaporated to 255 g under reduced pressure. The concentrate was poured into IPE (2 L). The resulting precipitate was collected by filtration and dried to give **27** (59.4 g, 88%): ^{1}H NMR (DMSO-d_6) δ 1.39 (9H, s), 1.46 (6H, s), 3.57 and 3.87 (2H, ABq, J = 18.0 Hz), 4.30 (2H, br s), 5.25 (1H, d, J = 4.9 Hz), 5.94 (1H, dd, J = 4.8, 8.7 Hz), 6.95 (1H, br s), 7.15–7.60 (10H, m), 8.17 (2H, br s), 9.53 (1H, d, J = 8.7 Hz).

5.4.5.5. 7β-[(*Z*)-2-(5-Amino-1,2,4-thiadiazol-3-yl)-2-(1-carboxy-1-methylethoxyimino)acetamido]-3-[3-amino-4-(4-aminobutyl)-2-methyl-1-pyrazolio]methyl-3-cephem-4-carboxylate (6). To a solution of **27** (550 g, 0.672 mmol) in DMF (1.7 mL) was added *N*-(trimethylsilyl)acetamide (441 mg, 3.36 mmol), and the mixture was stirred at room temperature for 1.5 h. To the reaction mixture was added **20** (377 mg, 0.739 mmol) and the mixture was stirred at 37 °C overnight. After cooling to room temperature, to the resulting reaction mixture was added water and the solution extracted with EtOAc. The organic layer was washed with water, a mixture of aqueous 10% sodium thiosulfate solution and brine, brine, and aqueous 10% sodium trifluoroacetate solution, dried over $MgSO_4$, and filtered. The filtrate was concentrated to about 3 mL in vacuo. The concentrate was poured into IPE, and the resulting precipitate was collected by filtration and dried in vacuo. To a solution of the resulting solid in CH_2Cl_2 (1.8 mL) were added anisole (0.6 mL) and trifluoroacetic acid (1.8 mL), and the mixture was stirred at room temperature for 2 h. The reaction mixture was poured into IPE, and the resulting precipitate was collected by filtration and dried in vacuo to give a crude product, which was purified by preparative HPLC utilizing ODS. The eluate containing desired product was concentrated to about 30 mL in vacuo. The concentrate was adjusted to about pH 3 with concd HCl and purified by column chromatography on HP-20 eluting with 30% aqueous IPA. The eluate was concentrated to about 30 mL in vacuo and lyophilized to give **6** (69 mg, 16%): ^{1}H NMR (D_2O) δ 1.53 (6H, s), 1.40–1.80 (4H, m), 2.30–2.50 (2H, m), 2.90–3.10 (2H, m), 3.16 and 3.37 (2H, ABq, J = 17.7 Hz), 3.68 (3H, s), 4.91 and 5.18 (2H, ABq, J = 15.8 Hz), 5.23 (1H, d, J = 4.7 Hz), 5.84 (1H, d, J = 4.7 Hz), 7.74 (1H, s); IR(KBr) ν_{max} 1772 cm^{-1}; HRMS (ESI) *m/z* calcd for $C_{24}H_{33}N_{10}O_7S_2$ $[M+H]^+$ 637.1975. Found: 637.1976. Anal. Calcd for $C_{24}H_{32}N_{10}O_7S_2$ $5.8H_2O$: C, 38.89; H, 5.93; N, 18.90. Found: C, 38.93; H, 6.04; N, 18.76.

5.4.6. Synthesis of cephalosporin 7

5.4.6.1. 4-(5-*tert*-Butoxycarbonylaminopentyl)-5-tritylamino-1-methylpyrazole (21). To a solution of oxalyl chloride (857 μL, 9.82 mmol) in CH_2Cl_2 (15 mL) was added DMSO (2.14 mL, 30.2 mmol) at −78°C under a nitrogen gas flow and the solution was stirred at the same temperature for 40 min. To the solution was added **19** (3.00 g, 7.55 mmol) in CH_2Cl_2 (5 mL) and stirred for 1 h. To the solution was added diisopropylethylamine (6.58 mL, 37.8 mmol) and gradually increased the temperature to 0 °C with stirring for 1 h. To the mixture was added water and the solution extracted with EtOAc. The organic layer was successively washed with water and brine. The extract was dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography eluting successively with hexane and a mixed solvent (hexane/EtOAc = 1:1) to give 4-(2-formylethyl)-5-tritylamino-1-methylpyrazole (754 mg, 25%): ^{1}H NMR (DMSO-d_6) δ 1.95–2.20 (4H, m), 2.73 (3H, s), 5.77 (1H, s), 7.00 (1H, s), 7.10–7.30 (15H, m), 9.39 (1H, s); MS (APCI) *m/z* 394 $(M-H)^+$.

To an ice-cooled solution of diethyl cyanomethylphosphonate (806 mg, 4.55 mmol) in THF (15 mL) was added so-

dium hydride (60% dispersion in mineral oil, 182 mg, 4.55 mmol) under flowing nitrogen gas and then stirred for 1 h at room temperature. To the ice-cooled reaction mixture was added 4-(2-formylethyl)-5-tritylamino-1-methylpyrazole (1.50 g, 3.79 mmol) and then stirred at room temperature overnight. To the mixture was added water and the solution extracted with EtOAc. The organic layer was successively washed with water and brine. The extract was dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo to give 4-(4-cyanobut-3-enyl)-5-tritylamino-1-methylpyrazole (1.18 g, 74%): ^{1}H NMR (DMSO-d_6) δ 1.90–2.10 (4H, m), 2.74 and 2.76 (3H, s), 5.43 and 5.56 (1H, d, J = 16.6 Hz and 11.0 Hz) 5.76 and 5.77 (1H, s), 6.28 and 6.46 (1H, d, J = 11.0 Hz and 16.6 Hz) 7.02 and 7.04 (1H, s), 7.10–7.35 (15H, m); MS (APCI) m/z 417 $(M-H)^+$.

To ice-cooled THF (23 mL) was added $LiAlH_4$ (313 mg, 8.24 mmol) under flowing nitrogen gas. To the ice-cooled suspension was added 4-(4-cyanobut-3-enyl)-5-tritylamino-1-methylpyrazole (1.15 g, 2.75 mmol) and the temperature gradually increased to room temperature with stirring. The mixture was stirred for 2 h under the same conditions. To the ice-cooled resulting mixture were successively added sodium fluoride (1.73 g) and water (594 μL). After filtration, the solution was evaporated in vacuo to give 4-(5-aminopentyl)-5-tritylamino-1-methylpyrazole (1.15 g, 99%): ^{1}H NMR (DMSO-d_6) δ 0.80–1.30 (6H, m), 1.70–1.90 (2H, m), 2.43 (2H, t, J = 6.9 Hz), 2.75 (3H, s), 5.67 (1H, s), 6.98 (1H, s), 7.05–7.40 (15H, m).

To a solution of 4-(5-aminopentyl)-5-tritylamino-1-methylpyrazole (1.10 g, 2.59 mmol) in CH_2Cl_2 (11 mL) was added di-*tert*-butyl dicarbonate (622 mg, 2.85 mmol) at room temperature and stirred at the same temperature overnight. To the reaction mixture was added saturated aqueous $NaHCO_3$ solution and extracted with EtOAc. The organic layer was successively washed with water and brine. The extract was dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo to give **21** (383 mg, 28%): ^{1}H NMR (DMSO-d_6) δ 0.85–1.05 (4H, m), 1.10–1.30 (2H, m), 1.38 (9H, s), 1.70–1.85 (2H, m), 2.74 (3H, s), 2.75–2.90 (2H, m), 5.66 (1H, s), 6.65–6.80 (1H, m), 6.98 (1H, s), 7.10–7.35 (15H, m); MS (APCI) m/z 525 $(M+H)^+$.

5.4.6.2. 7β-[(*Z*)-2-(5-Amino-1,2,4-thiadiazol-3-yl)-2-(1-carboxy-1-methylethoxyimino)acetamido]-3-[3-amino-4-(5-aminopentyl)-2-methyl-1-pyrazolio]methyl-3-cephem-4-carboxylate (7). This compound was prepared from **21** and **27** using a procedure similar to that employed for the preparation of **6** (90 mg, 21%): ^{1}H NMR (D_2O) δ 1.25–1.80 (6H, m), 1.53 (3H, s), 1.54 (3H, s), 2.40 (2H, t, J = 7.2 Hz), 2.98 (2H, t, J = 7.5 Hz), 3.14 and 3.34 (2H, ABq, J = 17.6 Hz), 3.67 (3H, s), 4.80–5.00 (2H, m), 5.23 (1H, d, J = 4.9 Hz), 5.84 (1H, d, J = 4.9 Hz), 7.73 (1H, s); IR(KBr) ν_{max} 1772 cm^{-1}; HRMS (ESI) m/z calcd for $C_{25}H_{35}N_{10}O_7S_2$ $[M+H]^+$ 651.2132. Found: 651.2126. Anal. Calcd for $C_{25}H_{34}N_{10}O_7S_2$ $5.5H_2O$: C, 40.05; H, 6.05; N, 18.68. Found: C, 40.41; H, 6.00; N, 18.20.

5.4.7. Synthesis of cephalosporin 8

5.4.7.1. *tert*-Butyl 2-(1-methyl-pyrazol-5-yl)ethylcarbamate (15). To a solution of triethyl phosphonoacetate (26.9 g, 120 mmol) in THF (200 mL) was added sodium hydride (60% dispersion in mineral oil, 4.80 g, 120 mmol) portionwise under ice-cooling. The mixture was stirred for 1 h at the same temperature. To the reaction mixture was added 1-methyl-1*H*-pyrazole-5-carbaldehyde (**14**) (33.0 g, 300 mmol) in THF (165 mL) at room temperature and the mixture was stirred for 1.5 h at the same temperature. To the resulting solution was added 10% aqueous $KHSO_4$ solution. It was then extracted with EtOAc twice. The combined organic layers were successively washed with saturated aqueous $NaHCO_3$ solution and brine. The extract was dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography eluting with a mixed solvent (hexane/EtOAc = 95:5 to 2:1) to give ethyl (2*E*)-3-(1-methyl-pyrazol-5-yl)-2-propenoate (33.4 g, 62%) as oil: ^{1}H NMR (DMSO-d_6) δ 1.26 (3H, t, J = 7.1 Hz), 3.88 (3H, s), 4.20 (2H, q, J = 7.1 Hz), 6.54 (1H, d, J = 15.8 Hz), 6.88 (1H, d, J = 1.8 Hz), 7.45 (1H, d, J = 1.8 Hz), 7.60 (1H, d, J = 15.8 Hz); IR(neat) ν_{max} 2981, 2943, 1713, 1703, 1180, 781 cm^{-1}; MS (APCI) m/z 181 $(M+H)^+$.

A solution of ethyl (2*E*)-3-(1-methyl-pyrazol-5-yl)-2-propenoate (33.4 g, 185 mmol) in EtOH (500 mL) was treated with 10% Pd–C (6.60 g) under a hydrogen atmosphere for 2 h at room temperature. After the catalyst was filtered off, the filtrate was concentrated in vacuo to give ethyl 3-(1-methyl-pyrazol-5-yl)propanoate (33.0 g, 98%) as a colorless oil: ^{1}H NMR (DMSO-d_6) δ 1.17 (3H, t, J = 7.1 Hz), 2.64 (2H, t, J = 7.3 Hz), 2.86 (2H, t, J = 7.3 Hz), 3.33 (3H, s), 4.07 (2H, q, J = 7.1 Hz), 6.01 (1H, d, J = 1.7 Hz), 7.26 (1H, d, J = 1.7 Hz); IR(neat) ν_{max} 2981, 2941, 1733, 1541, 1398, 1182 cm^{-1}; MS (APCI) m/z 183 $(M+H)^+$.

To a solution of ethyl 3-(1-methyl-pyrazol-5-yl)propanoate (33.0 g, 181 mmol) in EtOH (330 mL) was added 1 N aqueous NaOH solution (362 mL), and the mixture was stirred at 70 °C for 1 h. After cooling to room temperature, EtOH was evaporated off. The solution was extracted with Et_2O. The aqueous layer was acidified to pH 3 using aqueous 1 N HCl solution and the mixture was extracted with EtOAc four times. The combined organic layers were dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The residue was triturated with IPE, filtered, and dried in vacuo to give 3-(1-methyl-pyrazol-5-yl)propanoic acid (20.1 g, 71%) as a colorless solid: ^{1}H NMR (DMSO-d_6) δ 2.56 (2H, t, J = 7.7 Hz), 2.82 (2H, t, J = 7.7 Hz), 3.73 (3H, s), 6.01 (1H, d, J = 1.7 Hz), 7.26 (1H, d, J = 1.7 Hz), 11.90–12.70 (1H, br s); IR(KBr) ν_{max} 1718, 1302, 1190, 1009, 945, 802, 633 cm^{-1}; MS (APCI) m/z 155 $(M+H)^+$.

To a solution of 3-(1-methyl-pyrazol-5-yl)propanoic acid (10.0 g, 64.9 mmol) in *t*-BuOH (200 mL) were added diphenylphosphoryl azide (16.8 mL, 77.8 mmol) and triethylamine (10.8 mL, 77.8 mmol). The mixture was refluxed for 7 h. After cooling to room temperature, the reaction mixture was diluted with EtOAc, and the solution was successively washed with saturated aqueous $NaHCO_3$ solution, water, and brine. The extract was dried over anhydrous $MgSO_4$, filtered, and concen-

trated in vacuo. The residue was purified by silica gel column chromatography eluting with a mixed solvent (CH_2Cl_2/EtOAc = 9:1 to 1:1) to give **15** (12.6 g, 86%) as an oil: ^{1}H NMR (DMSO-d_6) δ 1.37 (9H, s), 2.72 (2H, t, J = 7.0 Hz), 3.15 (2H, dt, J = 5.5, 7.0 Hz), 3.71 (3H, s), 6.04 (1H, d, J = 1.7 Hz), 6.96 (1H, t, J = 5.5 Hz), 7.27 (1H, d, J = 1.7 Hz); IR(KBr) ν_{max} 1701, 1541, 1275, 1250, 777 cm^{-1}; MS (ESI) m/z 226.4 $(M+H)^+$.

5.4.7.2. 7β-[(*Z*)-2-(5-Amino-1,2,4-thiadiazol-3-yl)-2-(1-carboxy-1-methylethoxyimino)acetamido]-3-[3-(2-aminoethyl)-2-methyl-1-pyrazolio]methyl-3-cephem-4-carboxylate hydrogen sulfate (8). To a solution of **27** (2.5 g, 3.44 mmol) in DMF (5.0 L) was added *N*-(trimethylsilyl)acetamide (2.26 g, 17.2 mmol). After stirring at room temperature for 0.5 h, **15** (968 mg, 4.3 mmol) was added. Stirring was continued for 24 h at 37 °C. The resulting mixture was poured into water and extracted with EtOAc. The organic layer was successively washed with water, aqueous 10% sodium thiosulfate, brine, and aqueous 10% trifluoroacetic acid sodium salt, and dried over $MgSO_4$, filtered, and evaporated in vacuo. The residue was dissolved in a small amount of EtOAc and added to IPE dropwise. The precipitates were filtered, and the filter cake washed with IPE and dried under vacuum. To a solution of the resulting solid in CH_2Cl_2 (3.6 mL) were added anisole (1.8 mL) and trifluoroacetic acid (5.4 mL). The resulting solution was stirred for 4 h at room temperature and poured into IPE. The resulting precipitate was collected by filtration and dried in vacuo. The crude product was purified by preparative HPLC utilizing ODS. The first eluate containing the desired product was concentrated to about 30 mL in vacuo. The concentrate was adjusted to about pH 3 by addition of concd HCl and purified by column chromatography on HP-20 eluting with 30% aqueous IPA. The eluate was concentrated to about 30 mL in vacuo, 2 M aqueous H_2SO_4 (80 μL, 0.16 mmol) was added, and the solution lyophilized to give **8** (90 mg, 3.8%): ^{1}H NMR (D_2O) δ 1.61 (6H, s), 3.26 and 3.56 (2H, ABq, J = 17.9 Hz), 3.27 (2H, t, J = 7.4 Hz), 3.43 (2H, t, J = 7.4 Hz), 4.01 (3H, s), 5.29 (1H, d, J = 4.8 Hz), 5.33 and 5.46 (2H, ABq, J = 15.5 Hz), 5.90 (1H, d, J = 4.8 Hz), 6.79 (1H, d, J = 3.1 Hz), 8.21 (1H, d, J = 3.1 Hz); IR(KBr) ν_{max} 1781, 1716, 1676, 1633, 1153 cm^{-1}; HRMS (ESI) m/z calcd for $C_{22}H_{28}N_9O_7S_2$ $[M+H]^+$ 594.1553. Found: 594.1554. Anal. Calcd for $C_{22}H_{27}N_9O_7S_2{\cdot}H_2O_4S$ $5.5H_2O$: C, 33.42; H, 5.10; N, 15.94. Found: C, 33.32; H, 4.71; N, 15.61.

5.4.8. Synthesis of cephalosporin 9

5.4.8.1. 4-(2-Propyl)-1-methyl-5-tritylaminopyrazole (18). A suspension of sodium hydride (60% dispersion in mineral oil, 392 mg, 16.3 mmol) in DMSO (15 mL) was stirred at 70 °C for 1 h. After cooling to room temperature, to the suspension was added ethyltriphenylphosphonium bromide (6.37 g, 17.1 mmol) and stirred at room temperature for 1 h. To the mixture was added **17** (3.00 g, 8.16 mmol) in THF (10 mL) and stirred at room temperature for 2 h. To the resulting mixture was added water and the mixture was extracted with EtOAc. The organic layer was successively washed with water and brine. The extract was dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo to give 4-(1-propenyl)-1-methyl-5-tritylaminopyrazole as mixture of Z: E = ca. 6:1 (1.12 g, 36%): the ratio of E/Z was determined by the ratio of the integral of the methyl group in the NMR spectrum. Major product; ^{1}H NMR (DMSO-d_6) δ 1.60 (3H, dd, J = 7.0, 1.5 Hz), 2.91 (3H, s), 5.0 (1H, dd, J = 11.4, 7.0 Hz), 5.67 (1H, dd, J = 11.4, 1.5 Hz), 5.83 (1H, s), 6.44 (1H, s), 7.15–7.35 (15H, m); Minor product; ^{1}H NMR (DMSO-d_6) δ1.44 (3H, d, J = 5.2 Hz), 2.81 (3H, s), 4.9–5.1 (1H, m), 5.5–5.7 (1H, m), 5.83 (1H, s), 6.44 (1H, s), 7.15–7.35 (15H, m); mixture; MS (APCI) m/z 378 $(M-H)^+$.

A mixture of 4-(1-propenyl)-1-methyl-5-tritylaminopyrazole (1.00 g, 2.64 mmol) and 10% Pd–C (500 mg) in a mixed solvent of EtOAc (5 mL) and THF (1 mL) was stirred under a H_2 atmosphere for 10 min at room temperature. After the catalyst was filtered off, filtrate was concentrated in vacuo to give **18** (911 mg, 91%): ^{1}H NMR (DMSO-d_6) δ 0.62 (3H, t, J = 7.2 Hz), 0.9–1.1 (2H, m), 1.79 (2H, t, J = 7.8 Hz), 2.75 (3H, s), 5.67 (1H, s), 6.44 (1H, s), 7.10–7.35 (15H, m); MS (APCI) m/z 380 $(M-H)^+$.

5.4.8.2. 7β-[(*Z*)-2-(5-Amino-1,2,4-thiadiazol-3-yl)-2-(1-carboxy-1-methylethoxyimino)acetamido]-3-[3-amino-2-methyl-4-(1-propyl)-1-pyrazolio]methyl-3-cephem-4-carboxylate (9). This compound was prepared from **18** and **27** using a procedure similar to that employed for the preparation of **6** (203 mg, 18%): ^{1}H NMR (D_2O) δ 0.89 (3H, t, J = 7.2 Hz), 1.56 (6H, s), 1.45–1.65 (2H, m), 2.34 (2H, t, J = 7.3 Hz), 3.12 and 3.27 (2H, ABq, J = 17.6 Hz), 3.64 (3H, s), 4.6–4.8 and 4.92 (2H, ABq, J = 16.0 Hz), 5.20 (1H, d, J = 4.8 Hz), 5.84 (1H, d, J = 4.8 Hz), 7.74 (1H, s); IR (KBr) ν_{max} 1780, 1774 cm^{-1}; HRMS (ESI) m/z calcd for $C_{23}H_{30}N_9O_7S_2$ $[M+H]^+$ 608.1710. Found: 608.1703. Anal. Calcd for $C_{23}H_{29}N_9O_7S_2$ $3.7H_2O$: C, 40.97; H, 5.44; N, 18.69. Found: C, 40.95; H, 5.26; N, 18.37.

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