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Novel broad-spectrum and long-acting parenteral cephalosporins having an acyl cyanamide moiety at the C-3 terminal: Synthesis and structure-activity relationships

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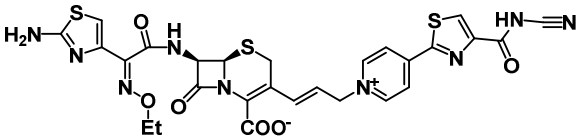

PRSP	0.25 μg/mL
BLNAR	0.063 μg/mL
Mouse AUC	20.9 μg·hr/mL

Title

Novel broad-spectrum and long-acting parenteral cephalosporins having an acyl cyanamide moiety at the C-3 terminal: Synthesis and structure-activity relationships

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Abstract

A series of novel 7β-[2-(2-aminothiazole-4-yl)-2-(Z)-(alkoxyimino)acetamido]-cephalosporins having pyridinium-linked acyl cyanamide at the C-3 position were prepared and their antibacterial activities and pharmacokinetics profiles were evaluated. Most of the compounds exhibited potent antibacterial activities against penicillin-resistant *Streptococcus pneumoniae* (PRSP) and β-lactamase non-producing penicillin-resistant *Haemophilus influenzae* (BLNAR). Introduction of a propenyl group between the cephalospoin core and the side chains at the C-3 position improved the pharmacokinetics profile. Among these compounds, 7β-[2-(2-aminothiazole-4-yl)-2-(Z)-(alkoxyimino)acetamido]-3-(pyridin-1-ium-1-yl)prop-1-en-1-yl)cephalosporins (**32j**) showed well-balanced antibacterial activity against *S. pneumoniae* and *H. influenzae* which included resistant strains and also other Gram-positive or Gram-negative pathogens. Furthermore, **32j** showed a long half-life comparable to that of Ceftriaxone in mice and monkeys.

Keywords

Cephalosporin

PRSP

BLNAR

acyl cyanamide

long acting

Highlights

- New cephalosporin compounds having acyl cyanamide group were synthesized.
- Most of them showed good antibacterial activity against PRSP and BLNAR.
- In particular, the compounds introduced propenyl group as a linker showed long acting feature.

1. Introduction

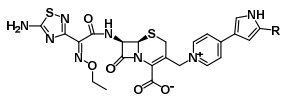
Cephalosporin antibiotics have been the focus of extensive research around the world, and a number of compounds have been developed as anti-infective agents playing clinically important roles. Above all, Ceftriaxone (CTRX) [1], displaying strong activity against Gram-negative bacteria [2] as a third-generation cephalosporin, has been contributing to the improvement of patient QOL (quality of life). It has also aided in suppressing medical expenses by making possible outpatient treatment as a once-daily administration cephalosporin drug due to its extremely long half life [3].

Although antibacterial agents including cephalosporins offer protection from the threat of infectious diseases, their excessive use has given rise to the emergence of resistant pathogens [4], including penicillin-resistant *Streptococcus pneumoniae* (PRSP) [4-6] and beta-lactamase non-producing ampicillin-resistant *Haemophilus influenzae* (BLNAR) [7, 8] as pathogenic bacteria of community-acquired pneumonia. The increase of such resistant pathogens has become a clinical problem. There is a limited number of safe drugs that are effective against such resistant bacteria, and new drugs need to be developed.

Under these circumstances, we started research to find antimicrobial agents possessing not only strong antibacterial activity against such resistant bacteria but also against other important Gram-positive and Gram-negative bacteria involved in community acquired bacterial infection, such as *Staphylococcus aureus*, *Escherichia coli* and *Moraxella catarrhalis* [4]. In addition, we thought that the candidate should have a long-acting pharmacokinetic profile similar to that of ceftriaxone.

Our previous studies [9] identified Compound **A** as having broad and potent antibacterial activity against Gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) and Gram-negative bacteria including *Pseudomonas aeruginosa*. We studied this compound as a next-generation antimicrobial agent, but found it to not be suitable for parenteral dosing because of its very poor solubility. Compound B with a carboxylic acid group at the 2-position of pyrrole had greatly improved solubility in water by forming a sodium salt, but unfortunately showed decreased antimicrobial activity. We next tried to identify a bioisostere instead the carboxylic acid without loss of antimicrobial activity. This led us to compound **C** with an acyl cyanamide group, which showed moderate antibacterial activity and good solubility. Interestingly, compound C had very long blood

durability in monkeys, comparable to that of ceftriaxone. This motivated us to conduct lead optimization around this compound.



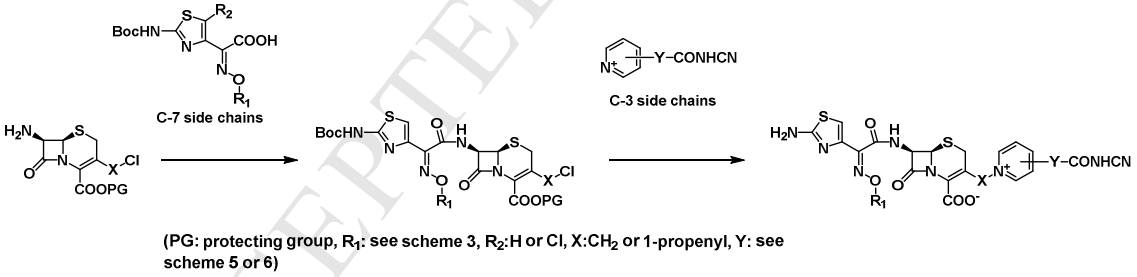
		MIC						PK Mouse			PK Monkey		
	R	*S.aureus* SMITH	*S.aureus* SR2030(MRSA)	*S.aureus* SR3626(MRSA)	*E.coli* NIHJ JC-2	*P.aeruginosa* PAO1	*P.aeruginosa* SA24	AUC (ug·hr/mL)	$T_{1/2}$ (hr.)	CLtot (mL/min/kg)	AUC (ug·hr/mL)	T 1/2 (hr.)	CLtot (mL/min/kg)
A	—H	0.4	1.6	6.3	0.02	0.8	0.8						
B	—COOH	3.1	12.5	25	0.05	0.8	0.8						
C	—CONHCN	1.6	12,5	12.5	0.1	0.8	0.4	27.3	0.4		22	2	
Cefozopuran		0.8	12.5	50	0.05	0.8	0.4	30.3	0.4		15	1.2	
CTRX								60.8	0.64	5.49	21.9	1.95	0.77

Table 1. SAR table of previous work

Here we describe the detailed synthesis of novel cephalosporins, and the effects of functional group transformations on antibacterial activity and pharmacokinetics.

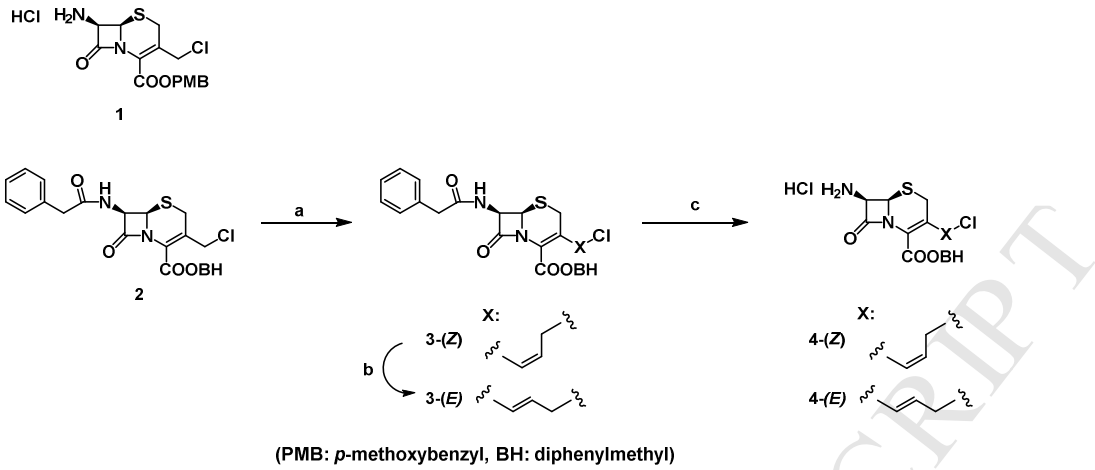
2. Chemistry

The outline of the synthetic route for novel cephalosporin derivatives is shown in Scheme 1. First, the carboxylic acid having an aminothiazole and an oxime moiety was made to react with the amino group at C-7 of the cephalosporin nucleus to introduce the C-7 side chain. Next, the pyridine derivative synthesized separately was introduced at the C-3 position, followed by deprotection to afford a variety of compounds.



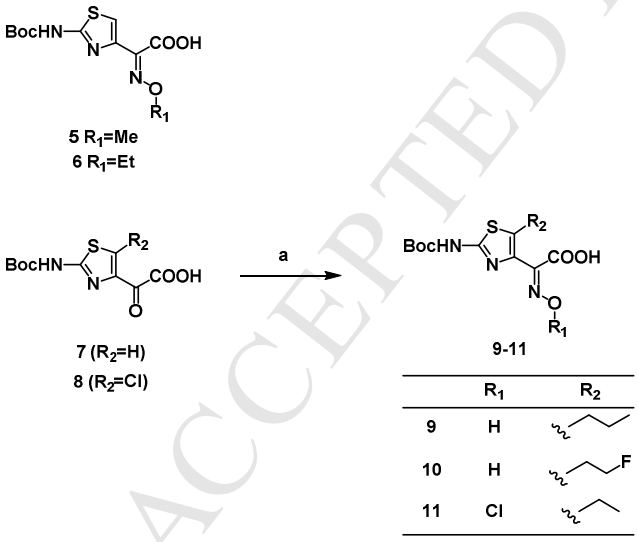
Scheme 1. Outline of the synthesis for novel cephalosporins

The cephalosporin nucleus having a chloromethyl group at the C-3 position was obtained from commercially available 7-amino-3-chloromethyl-3-cephem-4-carboxylic acid *p*-methoxybenzyl ester hydrochloride (ACLE· HCl) **1** [10] as shown in Scheme 2. The cephalosporin nucleus with a chloropropenyl group introduced at the C-3 position was prepared by removing a phenylacetyl group of compound **2-(*Z*)** or **2-(*E*)**, which were derived from commercially available 7-Phenylacetamido-3-chloromethyl-3-cephem-4-carboxylic acid diphenylmethyl ester (GCLH) [10] in accordance with a known route. [11]



Scheme 2. Reagents and conditions: (a) (1) NaI, THF, rt; (2) PPh_3, EtOAc, rt; (3) 2N NaOH aq., CH_2Cl_2, rt; (4) Chloroacetaldehyde, N,O-Bis(trimethylsilyl)acetamide, THF, DMSO, rt; (b) 2,2'-Azobis(2,4-dimethylvaleronitrile), 4-Chlorothiophenol, CH_2Cl_2, reflux; (c) (1) PCl_5, Pyridine, rt; (2) MeOH, 0°C

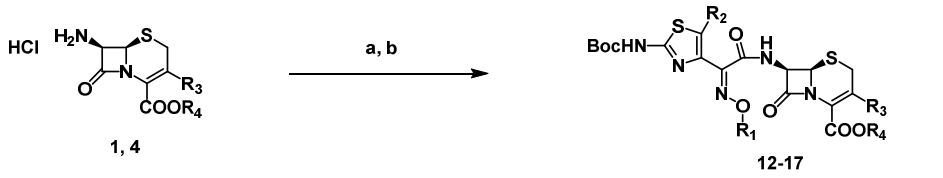
In terms of the C-7 side chains, **5** and **6** were prepared by a known procedure [12] and the other side chains **9**, **10** and **11** were obtained by oximation of ketoacid **7** and **8** with the corresponding alkoxylamine, respectively.

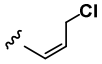
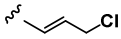


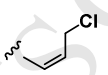
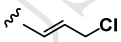
	R_1	R_2
9	H	
10	H	F
11	Cl	

Scheme 3. Reagents and conditions: (a) $R_2O\text{-}NH_2$, Et_3N, MeOH, rt

The cephalosporin intermediates **12** to **17** were prepared by condensation reaction of carboxylic acid and cephalosporin nucleus **1** or **4**, using phenyl phosphorodichloridate or phosphorus chloride in the presence of *N*-methylmorpholine.

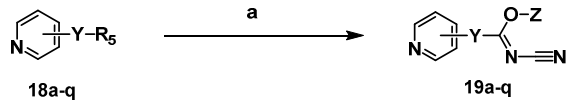


	R3	R4
1		PMB
4-(*Z*)		BH
4-(*E*)		BH

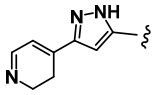
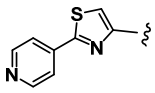
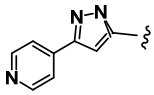
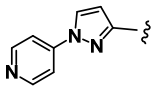
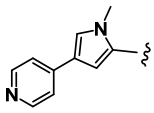
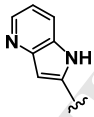
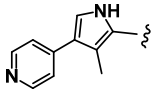
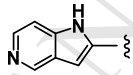
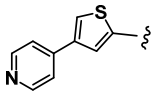
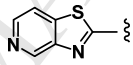
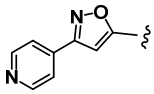
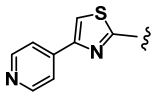
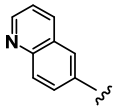
	R1	R2	R3	R4
12	Me	H		PMB
13	Et	H		PMB
14		H		PMB
15		H		PMB
16	Et	Cl		PMB
17-(*Z*)	Et	H		BH
17-(*E*)	Et	H		BH

Scheme 4. Reagents and conditions: (a) Phenylphosphoryl dichloride or $POCl_3$, N-Methylmorpholine, EtOAc, -20°C; (b)NaI, THF, 15°C, for **12-16**

The synthesis of C-3 side chains is shown in **Scheme 5**. First, the corresponding intermediates having carboxylic acids or esters were prepared. Using carboxylic acid intermediates, the carboxylic acid part was converted to the active acylimidazolide group by reaction with 1,1'-Carbonyldiimidazole (CDI) followed by introducing sodium cyanamide to afford the C-3 side chain having an acylcyanamide group (Method A). When the ester intermediates were used as materials, they were made to directly react with sodium cyanamide in an alcohol solvent to afford the desired compounds (Method B). The obtained C-3 side chains were further transformed to the sodium salts using MeONa, except for **19c** which was used for the next step as a free base.

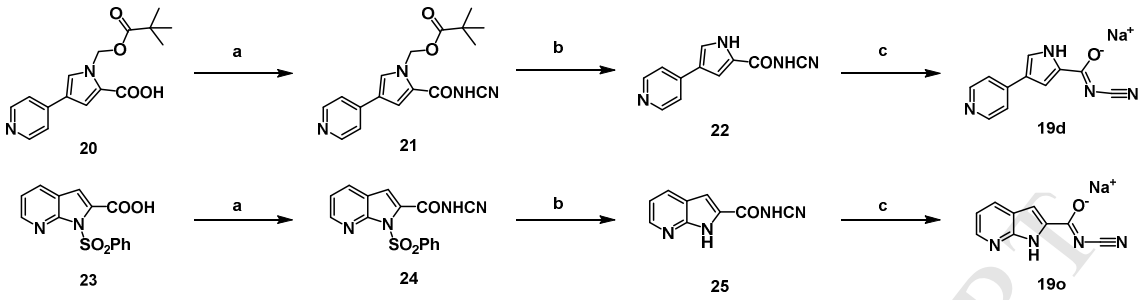


18a-q 19a-q

		R_5	Z			R_5	Z
a		COOH	Na	j		COOEt	Na
b		COOEt	H	k		COOEt	Na
c		COOEt	H	l		COOEt	Na
e		COOH	Na	m		COOH	H
f		COOH	Na	n		COOH	H
g		COOMe	Na	p		COOEt	Na
h		COOEt	Na	q		COOMe	Na
i		COOEt	Na	r		COOH	Na

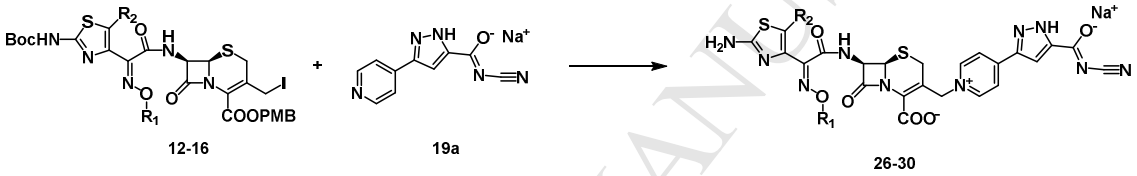
Scheme 5. Reagents and conditions: (a) Method A: (1) 1,1'-Carbonyldiimidazole, DMF, rt; (2) Sodium cyanamide, DMF, rt; Method B: Sodium cyanamide, MeOH, rt

Other synthetic methods for some C-3 side chains are shown in **Scheme 6**. Compound **20** or **23** with a protected nitrogen on the pyrrole ring had cyanamide groups introduced via acyl imidazole, using CDI in the same manner as Method A. After removing the respective protecting groups, the obtained free acylcyanamide group was neutralized with sodium methoxide to obtain **19d** or **19o**.



Scheme 6. Reagents and conditions: (a) (1) 1,1'-Carbonyldiimidazole, DMF, rt; (2) Sodium cyanamide, DMF, rt; (b) 2N NaOH aq., MeOH or EtOH, rt; (3) MeONa, MeOH, rt

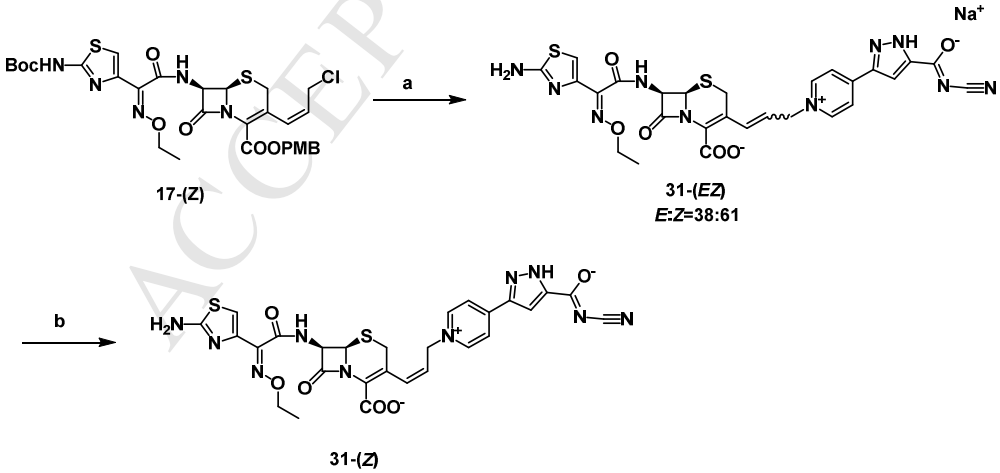
The coupling reaction of the mother nucleus with the C-3 side chains followed by a deprotection reaction is shown in **Scheme 7** to **9**.



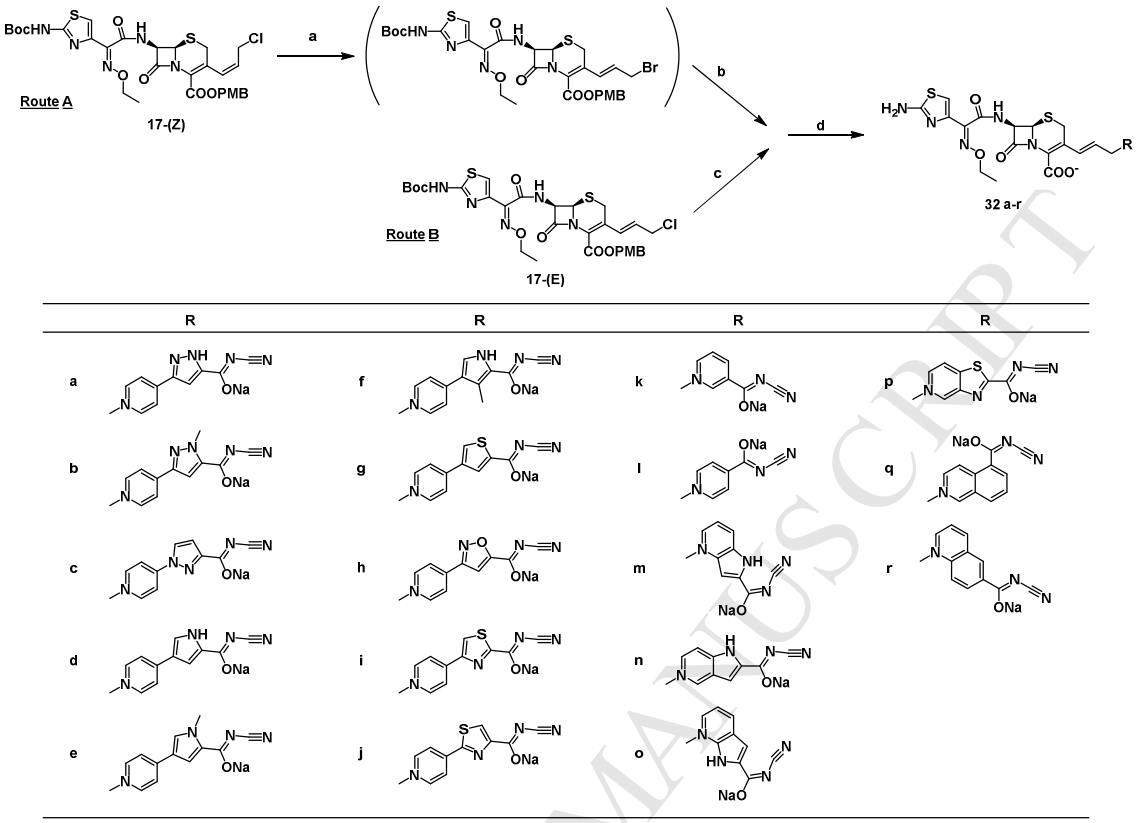
	R1	R2
12 or 26	Me	H
13 or 27	Et	H
14 or 28		H
15 or 29		H
16 or 30	Et	Cl

(PMB: p-methoxybenzyl)

Scheme 7. Reagents and conditions: (a) DMSO, rt; (b) 1M/L $AlCl_3$ in CH_3NO_2, CH_2Cl_2, -20°C



Scheme 8. Reagents and conditions: (a) (1)**19a**, NaBr, DMA, rt; (2) 1M/L $AlCl_3$ in CH_3NO_2, CH_2Cl_2, -20°C; (c) Separation by HP20SS chlomatography



Scheme 9. Reagents and conditions: (a) NaBr, DMF, rt, 5hours; (b) **19a**, **b**, **h**-**j**, **m** or **p**, DMF, rt; (c) **19c**-**g**, **k**, **l**, **n**, **o**, **q** or **r**, NaBr, DMF, rt; (d) 1M/L $AlCl_3$ in CH_3NO_2, CH_2Cl_2, -20°C

After coupling of 3-iodomethyl cephalosporins **12** to **16** with **19a** in DMSO, the resulting compound was deprotected with aluminum chloride in the presence of anisole, purified by HP20SS column chromatography, and lyophilized to give **26** to **30**.

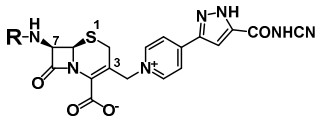
Synthesis of **31** having *Z*-propenyl and a mixture of *E*/*Z*-propenyl linker at the C-3 position was carried out as follows. First, **17-(Z)** was coupled with the C-3 side chain **19a** in the presence of sodium bromide in DMA. The resulting compound was deprotected similar to the procedure for synthesizing methylene type compounds to obtain a cis-trans mixture **31-(*EZ*)**. Chromatographic separation afforded **31-(*Z*)** in only the cis form.

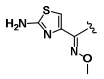
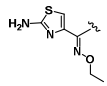
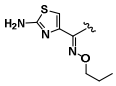
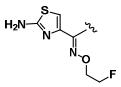
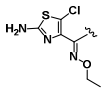
Synthesis of the trans isomer of **32** having an *E*-propenyl group was carried out using cis chloride **17-(Z)** or trans chloride **17-(E)** as the material. The cis chloride **17-(Z)** was made to react with sodium bromide in DMF for 5 hours at room temperature with monitoring for completion of isomerization to the trans form by NMR analysis. Subsequent coupling with the sodium salt of the C-3 chain gave the intermediate before deprotection (Route A). The trans chloride **17-(E)** was directly coupled with the sodium salt of the C-3 side chain in the presence of sodium bromide in DMF to give the same intermediate (Route B). Each intermediate obtained was deprotected to give

trans compounds **32**, except for **32c**. To obtain **32c**, **19c** was coupled with **17-(E)** in the presence of sodium bromide and N,O-bis(trimethylsilyl) acetamide in DMF and then deprotected.

3. Results and discussion

Initially, we investigated the effect of C-7 side chains on compounds having a pyrazole ring at the C-3 position of cephalosporins. The pyrazole ring was selected as a C-3 side chain because many analogues are more easily prepared than with the pyrrole ring. Compound **26** with an aminothiazole and a methoxyimino group at the C-7 position of cephalosporins showed potent and broad antibacterial activity against the target bacteria, especially PRSP and BLNAR. Also, compound **26** showed longer $T_{1/2}$ in mouse than compound **A**. Compound **27** and **28,** which have extended alkyl groups of the oxime moiety, maintained good antibacterial activity and exhibited improved activity against *M. catarrhalis*, one of the important pathogens in community-acquired pneumonia [13]. On the other hand, a slight decrease of activity was observed against *E. coli*, representative of Gram-negative bacteria. Compound **29** with a fluoroethyl group introduced to the oxime moiety showed comparable activity with compound **27**. The alkyl chain extension of the oxime part at C-7 position (Compound **27** - **29**) resulted in a decrease of AUC in mouse. Compound **30** with a chlorine substituent at the aminothiazole ring showed decreased activity against BLNAR compared to compound **27**. Compound **30** showed the highest protein binding rate (PB) and thereby had better AUC in mice than other compounds. As a result, we decided to continue SAR study by modifying compound **27** having an ethoxyimino group because this compound showed relatively well-balanced antibacterial activity among these five compounds.



	R	MIC							Mouse PK			
		S.a. [a]	PRSP 1[b]	PRSP 1[c]	BLNAR 1[d]	BLNAR 2[e]	*M.c.* [f]	*E.c.* [g]	AUC (ug·hr/mL)	$T_{1/2}$ (hr.)	CLtot (mL/min/kg)	PB (%)
26		1	0.25	0.5	0.063	0.25	2	0.25	18.1	0.91	18.4	15
27		1	0.25	0.5	0.063	0.25	0.5	0.5	10.8	1.14	30.8	23
28		0.5	0.25	0.5	0.063	1	1	1	8.9	0.98	37.4	37
29		1	0.25	0.5	0.063	0.125	1	0.25	12.7	1.06	26.2	24
30		1	0.125	0.25	0.25	2	0.125	2	41.5	0.59	8	57
	CTRX	2	1	2	0.125	0.25	1	0.125	60.8	0.64	5.49	94

[a]*Staphyloccocus aureus* SMITH

[b]PRSP 1, *Streptococcus pneumoniae* SR16675

[c]PRSP 2, *Streptococcus pneumoniae* SR16750

[d]BLNAR 1, *Haemophilus influenzae* SR11435

[e]BLNAR 2, *Haemophilus influenzae* SR24106

[f]*M.c.*, *Moraxella catarrahalis* SR24290

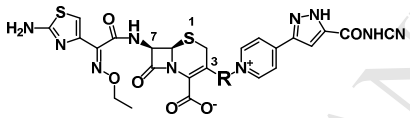
[g]*E.c.*, *Escherichia coli* NIHJ JC-2

Table 2. In vitro antibacterial activities (MIC, μg/mL) and pharmacokinetic profiles of compound **26**-**30**

Athough good antibacterial activity against pathogenic bacteria for respiratory infections was obtained through modification of the C-7 side chain, there was still room for improvement of activity against *Staphylococcus aureus*. We next focused on modification of the linker between the core and the pyridinium moiety on the C-3 side chain. Cepharosporins having a propenyl linker at the C-3 position have been known as examples of Cefluprenam, and this propenyl linker resulted in enhancement of antibacterial activity against *Staphylococcus aureus* [14]. In addition, we hypothesized that introduction of a lipophilic vinyl group would give a long-acting pharmacokinetic profile caused by increase of the PB.

We prepared **31-(EZ)** as an *E*/*Z* = 36/64 mixture to compare its antibacterial activity with the methylene type analogue **27**. Compound **31-(EZ)** showed 4-fold improvement of activity against *S.*

aureus and *E. coli*, as well as maintaining activity against PRSP and BLNAR, but had slightly reduced activity against *M. catarrahalis*. Evaluation of the pharmacokinetic profiles in mice given intravenous injections showed that the AUC of **31-(EZ)** was better than that of compound **27** in spite of a comparable PB value. This result encouraged us to separate each geometric isomer of the vinyl group at the C-3 position and compare the biological characteristics of the isomers. The antibacterial activity against PRSP and BLNAR of the *E* isomer **32a** was 4-fold superior to that of the *Z* isomer **31-(*Z*)**, and each isomer showed comparable activity against other pathogens. Although the CLtot parameter of **32a** in mice was quite similar to that of **31-(EZ)**, the $T_{1/2}$ of **32a** was slightly better. Therefore, we attempted to modify the hetero ring moiety in place of pyrazole in the most favorable cephalosporin skeleton having an (*E*)-propenyl linker at the C-3 position. Moreover, we considered the possibility of prediction of the pharmacokinetics profiles based on the lipophilic parameters of these compounds.



	R	MIC							Mouse PK			
		S.a.	PRSP 1	PRSP 1	BLNAR 1	BLNAR 2	*M.c.*	*E.c.*	AUC (ug·hr/mL)	$T_{1/2}$ (hr.)	CLtot (mL/min/kg)	PB (%)
27		1	0.25	0.5	0.063	0.25	0.5	0.5	10.8	1.14	30.8	23
31-(*EZ*)	 *E*:*Z*=36:64	0.25	0.25	0.5	0.125	0.25	1	0.125	17.4	0.85	19.1	24
31-(*Z*)		0.25	0.5	1	0.25	0.5	1	0.125				
32a		0.25	0.125	0.25	0.063	0.125	2	0.125	17.4	1.03	19.2	25
	CTRX	2	1	2	0.125	0.25	1	0.125	60.8	0.64	5.49	94

*Abbreviations: see footnote in Table 2

Table 3. In vitro antibacterial activities (MIC, μg/mL) and pharmacokinetic profiles of compound **27**, **31-(*EZ*)**, **31-(*Z*)**, **32a** and CTRX

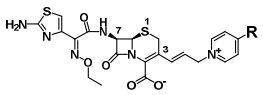
Compound **32b** with a methyl group introduced at the pyrazole nitrogen of compound **32a** showed decreased activity against *M. catarrhalis*. Compound **32c** with the nitrogen of the pyrazole ring directly bonded to a pyridinium ring had comparable activity with compound **32a**.

Next, we attempted to replace the pyrazole ring with another heterocyclic ring in order to further improve the activities as well as the pharmacokinetic profiles. Compound **32d**, **32e** and **32f** having a

pyrrole ring or methylated pyrrole with an acylcyanamide did not show better activities or pharmacokinetic profiles than those of compound **32a**.

Compound **32g** with a thiophene ring showed comparable activity to compound **32a**, and showed low CLtot in mice. Furthermore, compound **32h** with an isoxazole ring had improved antibacterial activity against *M. catarrhalis*, and showed a long-acting characteristic on $T_{1/2}$ in monkey. We also prepared compound **32i** and **32j** having a thiazole ring. These two compounds showed generally good antibacterial activity including activity against PRSP and BLNAR. Compound **32j** showed the most potent activity against BLNAR.

We investigated the relationship between pharmacokinetic parameters and lipophilicity and found that ClogP [15] could predict the AUC values in mouse with good correlation ($R^2 = 0.44$) as shown in **Figure 1**.



		MIC							Mouse PK				Monkey PK				
	R	*S.a.*	PRSP 1	PRSP 1	BLNAR 1	BLNAR 2	*M.c.*	*E.c.*	AUC (ug·hr/mL)	$T_{1/2}$ (hr.)	CLtot (mL/min/kg)	PB (%)	AUC (ug·hr/mL)	$T_{1/2}$ (hr.)	CLtot (mL/min/kg)	PB (%)	CLogP
32a		0.25	0.125	0.25	0.063	0.125	2	0.125	17.4	1.03	19.2	25	35.4	4.43	0.48	73	-7.35
32b		0.5	0.125	0.5	0.063	0.25	8	0.25	21.3	0.82	13.2	41	21.8	2.96	0.81	59	-7.59
32c		0.5	0.25	0.5	0.063	0.125	2	0.125	16.8	0.87	19.8	14	32.1	2.80	0.54	86	-7.45
32d		0.25	0.125	0.25	0.063	0.125	8	0.125	17.6	0.93	18.9	25	20.2	4.25	0.84	70	-7.05
32e		0.5	0.25	0.5	0.063	0.125	8	0.25	26.0	0.87	12.8	33	32.1	4.08	0.66	77	-6.89
32f		0.25	0.125	0.125	0.063	0.125	4	0.125	22.0	0.86	15.2	46	42.2	5.19	0.40	84	-6.86
32g		0.25	0.125	0.25	0.063	0.125	2	0.125	30.4	0.75	11.0	37	25.9	4.67	0.66	64	-6.33
32h		0.5	0.25	0.25	0.063	0.125	0.5	0.125	14.8	0.85	22.6	22	59.8	5.14	0.29	94	-7.62
32i		0.5	0.125	0.25	0.063	0.25	2	0.125	27.4	0.74	12.2	31	48.0	5.16	0.41	77	-7.40
32j		0.5	0.25	0.25	0.031	0.063	1	0.063	20.9	0.67	16.0	19	37.5	3.88	0.49	73	-7.40
	CTRX	2	1	2	0.125	0.25	1	0.125	60.8	0.64	5.49	94	21.9	1.95	0.77	95	

*Abbreviations: see footnote in Table 2

Table 4. In vitro antibacterial activities (MIC, μg/mL) and pharmacokinetic profiles of compound 3**2a-32j** and CTRX

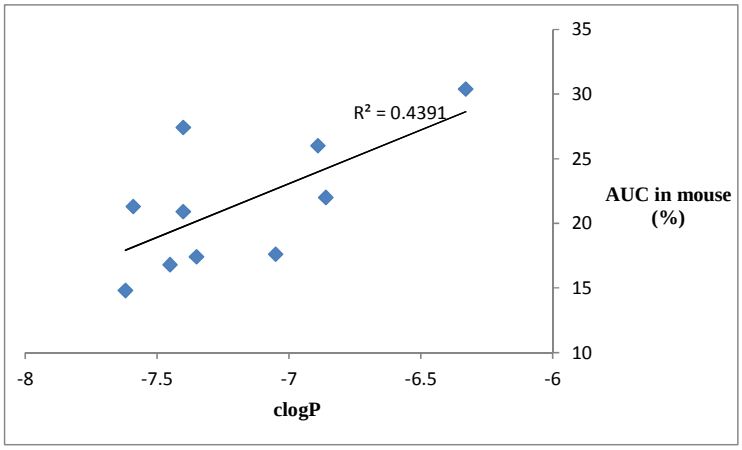
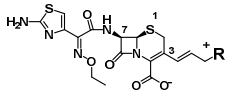


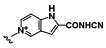
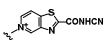
Figure 1. Correlation between clogP and AUC in mouse for **32a** to **32j**

Next, we prepared two compounds with a cyanamide group directly introduced to the pyridine ring without a hetero ring linker, and evaluated their biological profiles. These two compounds **32k** and **32l,** which have different substitution patterns, had good antibacterial activity similar to those of other compounds having a hetero ring linker. The overall pharmacokinetics profiles of these two compounds were not good, probably due to low lipophilicity, according to our hypothesis. We considered that the second ring between pyridinium and cyanamide was necessary to increase the lipophilicity and thereby improve the pharmacokinetic features. From that point of view, we next tested some compounds having lipophilic fused-ring sidechains at the C-3 position by using CLogP as an index for lipohilicity .

Three derivatives **32m**, **32n** and **21o** with fused a pyrrole with pyridinim at the C-3 position were prepared. These three compounds had approximately one log higher CLogP values than those of **32k** and **32l,** and showed enhanced AUC in the mouse as expected. These compounds also maintained good antibacterial activity although the activity of compound **32n** against *M. catarrhalis* was decreased. Next, we prepared compound **32p** with thiazole as an example of one having a low CLogP value. Unexpectedly, the AUC in mouse was relatively good. Also, compounds **32q** and **32r** with a quinoline or isoquinoline group at the C-3 side chain did not have high AUCs in mice though their CLogP values are the highest among those of the fused-pyridinium ring series. Most of the fused-pyridinium ring compounds did not show good pharmacokinetics profiles in monkeys.

As shown above, the relationship between the pharmacokinetic parameters in mouse and CLogP among compound **32k** to **32r** did not show any obvious correlation. This indicates that the pharmacokinetic features might be affected by not only lipophilicity but also other factors such as acidity of the acyl cyanamide group or the distance between the acyl cyanamide group and the cephalosporin nucleus.



		MIC							Mouse PK				Monkey PK				
	R	*S.a.*	PRSP 1	PRSP 1	BLNAR 1	BLNAR 2	*M.c.*	*E.c.*	AUC (ug·hr/mL)	$T_{1/2}$ (hr.)	CLtot (mL/min/kg)	PB (%)	AUC (ug·hr/mL)	$T_{1/2}$ (hr.)	CLtot (mL/min/kg)	PB (%)	CLogP
32k		0.5	0.25	0.5	0.063	0.125	2	0.125	14.1	0.55	23.7	6	9.5	1.33	1.79	33	-7.94
32l		0.5	0.25	0.5	0.063	0.125	1	0.25	14.6	0.72	22.9	8	9.7	1.22	1.84	41	-7.94
32m		0.5	0.25	0.5	0.031	0.125	1	0.125	23.6	0.73	14.1	36	9.8	2.03	1.74	60	-6.85
32n		0.5	0.25	0.5	0.063	0.125	4	0.25	30.4	0.63	10.9	27	20.9	3.35	0.80	67	-7.06
32o		0.5	0.25	0.5	0.063	0.25	0.5	0.125	22.2	0.64	15.0	33	29.2	4.35	0.57	94	-7.06
32p		0.5	0.125	0.25	0.063	0.063	0.5	0.25	23.7	0.70	14.0	41	24.5	2.86	0.68	84	-7.77
32q		0.5	0.25	0.5	0.063	0.125	2	0.125	14.2	0.73	23.5	3	10.6	1.52	1.59	58	-6.52
32r		0.25	0.25	0.5	0.031	0.125	0.5	0.125	12.9	0.95	25.7	10	8.2	1.74	2.08	37	-6.31
	CTRX	2	1	2	0.125	0.25	1	0.125	60.8	0.64	5.5	94	21.9	1.95	0.77	95	

*Abbreviations: see footnote in Table 2

Table 5. In vitro antibacterial activities (MIC, μg/mL) and pharmacokinetic profiles of compound **32k-32r** and CTRX

The results showed that all of the compounds having both an aminothiazole alkoxyimino group at the C-7 position and a cyanamide group at the pyridinium ring via the propenyl group at the 3-position of cephalosporins displayed potent antibacterial activity against BLNAR, PRSP, and *S. aureus*. Also, the activity against *M. catarrhalis* was affected by the functional group and the substitution pattern of the pyridinium group at the C-3 position. Introduction of a heteroaromatic ring between the pyridinium ring and the cyanamide group led to enhancement of PB and thereby resulted in good pharmacokinetic features compared with a direct connection.

After consideration of antibacterial activity and the pharmacokinetics profile in mice and monkeys, compound **32j** was selected for further detailed evaluation. This compound had water solubility good enough to use as an injection drug, showing more than 10% solubility in saline.

The results of the in vivo efficacy of **32j** and CTRX against *P. pneumoniae* in the mouse systemic infection model are shown in Table 6. The strains used for the infection model were penicillin-susceptible *P. pneumoniae* SR16605 and penicillin-resistant *P. pneumoniae* SR16754. The efficacy of each compound was expressed as 50% effective dose (ED_{50}), which was calculated from the number of mice surviving at 7 days after injection. Compound **32j** was 2 or 3 times more

effective, respectively, than CTRX against PSSP or PRSP when the MIC values were aligned.

Compound	*S.peumoniae* SR16605(PSSP)		*S.peumoniae* SR16754(PRSP)	
	MIC (ug/ml)	ED50 (mg/kg)	MIC (ug/ml)	ED50 (mg/kg)
32j	0.008	0.017	0.25	0.71
CTRX	0.016	0.11	1	5.65

Table 6. In vivo efficacy of **32j** in the mouse systemic infection model

4. Conclusion

Chemical modification of the side chains was conducted at the C-3 or C-7 position of cephalosporins having an acyl cyanamide group, and the biological properties of the resulting compounds were evaluated. Many compounds exhibited good antibacterial activity against Gram-positive and Gram-negative bacteria including PRSP and BLNAR. Introducing a propenyl group between the cephalosporin core and the 3-side chain improved the pharmacokinetics profiles. The relationship between lipophilicity and pharmacokinetic profiles was also investigated. We could not identify a clear correlation for all compounds prepared, but higher CLogP values as a parameter of lipophilicity led to improved AUCs in mice in the hetero-ring linker series.

Among the compounds prepared, compound **32j** showed potent and well-balanced antibacterial activity including against PRSP and BLNAR, and showed a better pharmacokinetics profile in monkeys compared to CTRX. Furthermore, *in vivo* evaluation of mice with this compound against penicillin-susceptible and -resistant *S. pneumoniae* resulted in excellent therapeutic efficacy that reflected the in vitro activity and pharmacokinetics profiles.

5. Experimental

^{1}H NMR (300MHz) was recorded on a Varian Gemini 300 spectrometer. Chemical shifts are reported in ppm using tetramethylsilane (TMS) as an internal standard. HR-FAB/MS were recorded on a JEOL LMS-SX/SX102A. Column chromatography was performed with Merck silica gel 60 (230-400 mesh).

5.1. Synthesis of C-7 side chain

Compound **5** and **6** were prepared by a known procedure [12].

5.1.1. (Z)-2-(2-((tert-Butoxycarbonyl)amino)thiazol-4-yl)-2-(propoxyimino)acetic acid (9). To a solution of **7** (4.08 g, 15.0 mmol) in MeOH (30 ml) was added triethylamine (2.29 ml, 16.5 mmol)

and O-propylhydroxylamine hydrochloride (1.84 g, 16.5 mmol) and then the mixture was stirred at room temperature for 1 h 40 min. The mixture was concentrated and the residue obtained was poured into dilute hydrochloric acid. The mixture was extracted with ethyl acetate and the combined organic layer was washed with H_2O and brine, dried over $MgSO_4$, filtered, and evaporated. 2-Propanol was added to the residual solid, which was collected by filtration and dried to give **9** (3.95 g, 80%) as a crystalline solid. ^{1}H-NMR ($CDCl_3$) δ: 0.95 (3H, t, *J* = 7.4 Hz), 1.59 (9H, s), 1.77 (2H, dt, *J* = 14.2, 7.1 Hz), 4.24 (2H, t, *J* = 6.7 Hz), 7.36 (1H, s).

Compound **10** and **11** were prepared by the procedure used for compound **9**.

5.1.2. (Z)-2-(2-((tert-Butoxycarbonyl)amino)thiazol-4-yl)-2-(fluoroethoxyimino)acetic acid (10). Crystalline solid, 76% yield. ^{1}H-NMR ($CDCl_3$) δ: 1.56 (9H, d, *J* = 8.6 Hz), 4.49 (2H, dt, *J* = 28.3, 3.9 Hz), 4.69 (2H, dt, *J* = 47.6, 4.1 Hz), 7.33 (1H, s).

5.1.3. (Z)-2-(2-((tert-Butoxycarbonyl)amino)-5-chlorothiazol-4-yl)-2-(ethoxyimino)acetic acid (11). Crystalline solid, 78% yield. ^{1}H-NMR ($CDCl_3$) δ: 1.36 (3H, t, *J* = 7.0 Hz), 1.57 (8H, s), 4.30 (2H, q, *J* = 7.0 Hz).

5.2. Introduction of C-7 side chain

Compound **12** was prepared by a known procedure [16].

5.2.1. Compound 14. (Step 1) To a suspension of **9** (1.65 g, 5.0 mmol), **1** (2.03 g, 5 mmol) and phenyl phosphorodichloridate (0.891 ml, 6 mmol) in EtOAc (25 ml) was added dropwise N-methylmorpholine (2.20 ml, 20.0 mmol) for 5 min at
-20°C and then the mixture was stirred at -20°C for 1 h 35 min. To the mixture was added dilute hydrochloric acid and additional EtOAc. The organic layer was separated and washed with H_2O and brine, dried over $MgSO_4$, filtered, and evaporated. The obtained residue was purified by column chromatography on silica gel to obtain an intermediate having a chloromethyl group at the C-3 position of cephalosporin. (3.00 g, 88%) as a foam. ^{1}H-NMR ($CDCl_3$) δ: 0.99 (3H, t, *J* = 7.5 Hz), 1.58 (9H, s), 1.78-1.83 (2H, m), 3.54, 3.72 (2H, ABq, *J* = 24.4 Hz), 3.86 (3H, s), 4.29 (2H, t, *J* = 6.8 Hz), 4.48, 4.61 (2H, ABq, *J* = 15.8 Hz), 5.10 (1H, d, *J* = 5.0 Hz), 5.25, 5.30 (2H, dd, *J* = 15.6 Hz), 6.04 (1H, dd, *J* = 9.0, 4.9 Hz), 6.95 (2H, d, *J* = 8.5 Hz), 7.20-7.41 (6H, m).

(Step 2) To a solution of above obtained intermediate (2.97 g, 14.4 mmol) in THF (30 ml) was added sodium iodide (1,97 g, 13.1 mmol) at 15°C and then the mixture was stirred at 15°C for 30 min. To

the mixture was added aqueous NaS_2O_3 solution and the mixture was extracted with ethyl acetate. The organic layer was separated and washed successively with H_2O and brine, dried over $MgSO_4$, filtered, and evaporated to give **14** as a foam. ^{1}H-NMR ($CDCl_3$) δ: 1.00 (3H, t, *J* = 7.5 Hz), 1.58 (9H, s), 1.75-1.87 (2H, m), 3.57, 3.83 (2H, ABq, *J* = 24.4 Hz), 3.86 (3H, d, *J* = 1.7 Hz), 4.30 (2H, t, *J* = 6.9 Hz), 4.41, 4.45 (2H, ABq, *J* = 12.2 Hz), 5.09 (1H, d, *J* = 4.9 Hz), 5.27, 5.32 (2H, ABq, *J* = 15.8 Hz), 5.99 (1H, dd, *J* = 8.9, 4.9 Hz), 6.96 (2H, d, *J* = 8.4 Hz), 7.30-7.38 (4H, m), 8.41 (1H, s).

Compound **13**, **15** and **16** were prepared by the procedure used for compound **14**.

5.2.2. Compound 13. Foam, 89% yield in two steps. ^{1}H-NMR ($CDCl_3$) δ: 1.35 (3H, t, *J* = 7.1 Hz), 1.54 (9H, s), 3.53, 3.79 (2H, ABq, *J* = 18.6 Hz), 3.82 (3H, s), 4.35 (2H, q, t = 6.8 Hz), 4.36, 4.41 (2H, ABq, J = 9.40 Hz), 5.05 (1H, t, *J* = 4.9 Hz), 5.22, 5.27 (2H, ABq, *J* = 11.8 Hz), 5.95 (1H, dd, *J* = 9.0, 4.9 Hz), 6.91 (2H, d, *J* = 8.4 Hz), 7.22 (1H, d, *J* = 8.8 Hz), 7.31 (1H, s), 7.37 (2H, d, *J* = 8.4 Hz), 8.39 (1H, s).

5.2.3. Compound 15. Foam, 97% yield in two steps. ^{1}H-NMR ($CDCl_3$) δ: 1.58 (9H, s), 3.56, 3.82 (2H, ABq. *J* = 24.0 Hz), 3.86 (3H, s), 4.43 (2H, s), 4.57 (2H, dt, *J* = 27.9, 4.1 Hz), 4.74 (2H, dt, *J* = 47.3, 4.2 Hz), 5.10 (1H, d, *J* = 5.0 Hz), 5.26, 5.32 (2H, ABq, *J* = 15.8 Hz), 5.99 (1H, dd, *J* = 8.9, 4.9 Hz), 6.95 (2H, d, *J* = 8.7 Hz), 7.27-7.31 (1H, m), 7.34 (1H, s), 7.41 (2H, d, *J* = 8.5 Hz).

5.2.4. Compound 16. Foam, 93% yield in two steps. ^{1}H-NMR ($CDCl_3$) δ: 1.41 (3H, t, *J* = 7.0 Hz), 1.57 (9H, s), 3.83, 3.37 (2H, ABq, *J* =20.0 Hz), 3.86 (3H, s), 4.36-4.47 (4H, m), 5.07 (1H, d, *J* = 5.0 Hz), 5.25, 5.34 (2H, ABq, *J* =13.6 Hz), 5.99 (1H, dd, *J* = 8.9, 5.0 Hz), 6.95 (2H, d, *J* = 7.9 Hz), 7.41 (2H, d, *J* = 8.2 Hz), 7.50 (1H, d, *J* = 9.0 Hz), 8.45 (1H, s).

Compound **17-(*Z*)** and **17-(*E*)** were prepared by the procedure in Step 1 for compound **14**.

5.2.5. Compound 17-(*Z*). Foam, 86% yield. This compound contained approximately 0.15 equivalent of **17-(*E*)**. ^{1}H-NMR ($CDCl_3$) δ: 1.37(3H, t, J = 7.1Hz), 1.54(9H, s), 3.30-3.90(4H, m), 4.38(2H, q, J = 7.1Hz), 5.12(0.16H, d, J = 4.9 Hz), 5.16 (0.84H, d, J = 4.9Hz), 5.64(1H, m), 6.06(1H, m), 6.23(0.84H, d, J = 11.4Hz), 6.93-6.99 (m, s), 7.00(0.16H, d, J = 15.0Hz), 7.20-7.50(11H, m)

5.2.6. Compound 17-(*E*). Foam, quantitative yield. ^{1}H-NMR ($CDCl_3$) δ: 1.41 (3H, t, *J* = 7.0 Hz), 1.57 (9H, s), 3.83, 3.37 (2H, ABq, *J* =20.0 Hz), 3.86 (3H, s), 4.36-4.47 (4H, m), 5.07 (1H, d, *J* = 5.0 Hz), 5.25, 5.34 (2H, ABq, *J* =13.6 Hz), 5.99 (1H, dd, *J* = 8.9, 5.0 Hz), 6.95 (2H, d, *J* = 7.9 Hz), 7.41 (2H, d, *J* = 8.2 Hz), 7.50 (1H, d, *J* = 9.0 Hz), 8.45 (1H, s).

5.3. Synthesis of C-3 side chain

5.3.1. Compound 19f. (Method A) To a suspension of **18f** (1.16 g, 5.74 mmol) in DMF (10 ml) was added CDI (973 mg, 6 mmol) and then the mixture was stirred at room temperature for 40 min. To the mixture was added sodium cyanamide (845 mg, 13.1 mmol), followed by stirring at room temperature for 1 h. The mixture was then diluted with H_2O, and the pH was adjusted to 6.1 with 2 M/L hydrochloric acid, and the precipitated solid was filtered. The solid obtained was suspended in MeOH and the pH was adjusted to 6.1 with 1 M/L MeONa/MeOH, and then evaporated to give compound **19f** (1.33 g, 93%) as a solid. ^{1}H-NMR (DMSO-D_6) δ: 2.51 (3H, s), 7.06 (1H, s), 7.42 (2H, d, *J* = 4.6 Hz), 8.47 (2H, d, *J* = 4.6 Hz).

Compound **19a**, **19e** and **19r** were prepared by the procedure used for compound **19f**, and compound **19c**, **19m** and **19n** by that used for compound **19f**, except for the transformation process of free cyanamide to sodium salt.

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5.3.2. Compound 19a. Solid, 69% yield. ^{1}H-NMR (d_6-DMSO) δ: 7.08(1H, s), 7.79(2H, d, J = 6.0 Hz), 8.56(1H, d, J = 6.3Hz).

5.3.3. Compound 19c. Solid, 98% yield. ^{1}H-NMR (d_6-DMSO) δ: 7.17 (1H, d, J = 2.7 Hz), 8.05 (2H, dd, J = 1.5, 4.8 Hz), 8.78 (2H, dd, J = 1.8, 4.8 Hz), 8.90 (1H, d, J = 2.7 Hz).

5.3.4. Compound 19e. Solid, 68% yield. ^{1}H-NMR (d_6-DMSO) δ: 4.88(1H, s), 6.99(1H, d, J = 2.1Hz), 7.46(3H, m), 8.40(2H, d, J = 6.0Hz).

5.3.5. Compound 19m. A pale pink solid, 58% yield. ^{1}H-NMR (d_6-DMSO) δ: 7.01 (1H, s), 7.63 (1H, dd, J = 5.7, 8.1 Hz), 8.40 (1H, d, J = 8.1 Hz), 8.65 (1H, d, J = 5.7 Hz), 12.8 (1H, brs).

5.3.6. Compound 19n. An ocherous solid, 87% yield. ^{1}H-NMR (d_6-DMSO) δ: 7.24 (1H, s), 7.79 (1H, d, J = 6.9 Hz), 8.37 (1H, d, J = 6.9 Hz), 9.25 (1H, s), 12.9 (1H, brs).

5.3.7. Compound 19r. Solid, 72% yield. ^{1}H-NMR (DMSO-D_6) δ: 7.55 (1H, dd, *J* = 8.2, 4.1 Hz), 7.95 (1H, d, *J* = 8.9 Hz), 8.31 (1H, dd, *J* = 8.7, 1.8 Hz), 8.48 (1H, d, *J* = 8.2 Hz), 8.57 (1H, d, *J* = 1.7 Hz), 8.92 (1H, dd, *J* = 4.2, 1.8 Hz).

5.3.8. Compound 19h. (Method B) To a solution of **18h** (27.96 g, 128 mmol) in MeOH (300 ml)

was added sodium cyanamide (9.8 g, 148 mmol), followed by stirring at room temperature for 1.5 h. The mixture was concentrated to 50 ml, and then i-PrOH was added. The resulting precipitate was filtered and dried to give **19h** (31.5 g, 82%) as a solid. ^{1}H-NMR (d_6-DMSO) δ: 7.43(1H, s), 7.89(2H, d, J = 6.0 Hz), 8.73(2H, d, J = 6.0Hz).

Compound **19g**, **19i**, **19j**, **19k**, **19l**, **19p** and **19q** were prepared by the procedure used for compound **19h**, and compound **19b** was prepared by that used for compound **19h,** except for the transformation process of free cyanamide into sodium salt.

5.3.9. Compound 19b. Solid, 74% yield. ^{1} H-NMR (d_6-DMSO) δ: 4.20 (3H, s), 7.50 (1H, s), 8.29 (2H, brs), 8.81 (2H, brs).

5.3.10. Compound 19g. A pale brown solid, 55% yield. ^{1} H-NMR (d_6-DMSO) δ: 7.71(2H, d, J = 6.3Hz), 7.90(1H, d, J = 1.5 Hz), 8.12(1H, d, J = 1.8Hz), 8.55(2H, d, J = 6.0Hz).

5.3.11. Compound 19i. A white solid, 51% yield. ^{1} -NMR (d_6-DMSO) δ: 7.91 (2H, dd, J = 1.5, 4.5 Hz), 8.43 (1H, s), 8.63 (2H, dd, J = 1.5, 4.5 Hz .

5.3.12. Compound 19j. An ocherous solid, 86% yield. ^{1}H-NMR (d_6-DMSO) δ: 7.89 (2H, dd, J = 1.8, 4.5 Hz), 8.18 (1H, s), 8.70 (2H, dd, J = 1.8, 4.5 Hz).

5.3.13. Compound 19k. Solid, 61% yield. ^{1}H-NMR (d_6-DMSO) δ: 7.30 – 7.34 (1H, m), 8.14 – 8.18 (1H, m), 8.52 - 54 (1H, m), 9.00 (1H, m).

5.3.14. Compound 19l. Solid, 56% yield. ^{1}H-NMR (d_6-DMSO) δ: 7.74 (2H, dd, J = 1.5, 4.5 Hz), 8.55 (2H, dd, J = 1.5, 4.5 Hz).

5.3.15. Compound 19p. Solid, 49% yield. ^{1} H-NMR (d_6-DMSO) δ: 8.13 (1H, dd, J = 0.9, 5.4 Hz), 8.52 (1H, d, J = 5.4 Hz), 9.29 (1H, d, J = 0.9 Hz).

5.3.16. Compound 19q. Solid, 75% yield. This compound was a 1:1 mixture of the corresponding compound having carboxylic acid instead of acyl cyanamide. ^{1}H-NMR (DMSO-D_6) δ: 7.58-7.62 (1H, m), 7.97-8.21 (2H, m), 8.41-8.47 (1H, m), 8.84-8.99 (1H, m), 9.22-9.27 (1H, m).

5.3.17. Compound 21 This compound was prepared by the procedure used for compound **19f,** except for the transformation process of free cyanamide to sodium salt. A yellow green powder, 99%

yield. ^{1}H-NMR (d_6-DMSO) δ: 1.11 (9H, s), 6.32 (2H, s), 7.43 (1H, d, J = 1.8 Hz) , 7.96 (2H, d, J = 6.6 Hz), 8.09 (1H, d, J = 1.8 Hz), 8.64 (2H, d, J = 6.6 Hz).

5.3.18. Compound 19d. To a suspension of **21** (50.2 g, 154 mmol) in MeOH (1000 ml) was added dropwise 2 N NaOH (385 ml, 770 mmol), and then the mixture was stirred at room temperature for 1 h. The pH of the mixture was adjusted to 7.0 with 2 N HCl (385 ml, 770 mmol). The mixture was concentrated to remove MeOH, and the solid that precipitated was obtained by filtration. The precipitate was washed successively with H_2O, 2-propanol, and Et_2O. Finally, the precipitate was dried to give **22** (32 g, 98%) as a white solid. ^{1}H-NMR (d_6-DMSO) δ: 7.29 (1H, s), 7.88 (1H, m), 7.96 (2H, d, J = 6.8 Hz), 8.58 (2H, d, J = 6.2 Hz), 12.2 (1H, m).

To a suspension of **22** (32 g, 152 mmol) in MeOH (60 ml) was added dropwise 1.14 M/L MeONa/MeOH (135 ml, 154 mmol) at 0°C and then the mixture was stirred at 0°C for 10 min. To the mixture was added 2-propanol (350 ml), followed by concentration of the mixture to remove MeOH and then filtration to obtain the precipitated solid. The solid obtained was washed successively with 2-propanol, and Et_2O. The precipitate was dried to give **19d** (35.6 g, 100%) as a pale yellow solid. ^{1}H-NMR (d_6-DMSO) δ: 6.91 (1H, m), 7.39 (1H, m), 7.53 (2H, d, J = 6.0 Hz), 8.39 (2H, d, J = 5.8 Hz), 11.44 (1H, m).

5.3.19. Compound 24 This compound was prepared by the procedure used for compound **19f,** except for the transform process of free cyanamide to sodium salt, 88% yield. ^{1}H-NMR ($CDCl_3$) δ: 7.08 (1H, s), 7.21 (1H, dd, J = 4.2, 7.8 Hz), 7.45 – 7.57 (3H, m), 7.85 (1H, d, J = 7.8 Hz), 8.24 (2H, d, J = 7.5 Hz), 8.52 (1H, d, J = 4.2 Hz .

5.3.20. Compound 19o. To a solution of **24** (1.41 g, 4.32 mmol) in EtOH (14 ml) was added 2 N NaOH (10.8 ml, 21.6 mmol) and then the mixture was stirred at room temperature for 4 h. To the mixture was added 2 M/L HCl (14 ml, 28 mmol) at 0°C. The resulting precipitate was filtered and dried under reduced pressure to give **25** (567 mg, 70%). ^{1}H-NMR (d_6-DMSO) δ: 7.20 (1H, dd, J = 4.5, 7.8 Hz), 7.33 (1H, s) , 8.20 (1H, dd, J = 1.8, 7.8 Hz), 8.44 (1H, brd, J = 4.5 Hz).

To a suspension of **25** (448 mg, 2.42 mmol) in MeOH (9 ml) was added 1 M/L MeONa/MeOH (2.4 ml, 2.4 mmol) followed by evaporation to give **19o** (552 mg, 110%). ^{1}H-NMR (d_6-DMSO) δ: 6.76 (1H, s), 7.03 (1H, dd, J = 4.5, 7.8 Hz), 7.94 (1H, d, J = 7.8 Hz), 8.22 (1H, d, J = 4.5 Hz), 11.6 (1H, br).

5.4. Introduction of C-3 side chain and deprotection

5.4.1. Compound 28. To a solution of **14** (772 mg, 1.0 mmol) in DMSO (2 ml) was added **19a** (235

mg, 1.0 mmol) and then the mixture was stirred at room temperature for 1 h. The mixture was poured into 5% NaCl in H_2O at 0°C. The resulting precipitate was filtered, H_2O was added, and lyophilization was done to obtain a coupled intermediate (843 mg, 98%).

To a solution of the above intermediate (833 mg, 0,97 mmol) and anisole (0.97 ml, 8.9 mmol) in CH_2Cl_2 (10 ml) was added 1 M/L $AlCl_3/CH_3NO_2$ (5.82 ml, 5.82 mmol) at -20°C and then the mixture was stirred at -20°C for 30 min. The mixture was poured into 0.3 M/L aqueous HCl (20 ml) and MeCN (20 ml) at 0°C, and then this was added to i-Pr_2O (40 ml). The resulting oily residue and supernatant liquid were separated by decantation. The supernatant liquid was separated to obtain the aqueous phase. The oily residue was dissolved using dilute aqueous HCl and MeCN, and combined with the above aqueous phase. To the aqueous crude solution was added HP20SS and the mixture was concentrated, charged onto an HP20SS column, and chromatographed with aqueous MeCN. The fractions containing the desired product were collected, concentrated and lyophilized to give **28** (338 mg, 55%) as a powder. ^{1}H-NMR (DMSO-D_6) δ: 0.90 (3H, t, *J* = 7.4 Hz), 1.60 (2H, td, *J* = 14.2, 7.2 Hz), 3.44, 3.55 (2H, ABq, *J* = 18.3 Hz), 3.97 (2H, t, *J* = 6.6 Hz), 5.20 (1H, d, *J* = 5.0 Hz), 5.39, 5.51 (2H, ABq, *J* = 14.9 Hz), 5.89 (1H, dd, *J* = 8.1, 4.9 Hz), 6.68 (1H, s), 7.21 (2H, s), 7.47 (1H, s), 8.51 (2H, d, *J* = 7.0 Hz), 8.91 (2H, d, *J* = 6.7 Hz), 9.61 (1H, d, *J* = 8.1 Hz).

Compound **26**, **27**, **29** and **30** were prepared by the procedure used for compound **28**.

5.4.2. Compound 26. A pale yellow powder, 30% yield. ^{1}H-NMR (D_2O) δ: 3.22, 3.71 (2H, ABq, *J* = 18.0 Hz), 3.87 (3H, s), 5.28 (1H, d, *J* = 4.8 Hz), 5.22, 5.55 (2H, ABq, *J* = 14.4 Hz), 5.77 (1H, d, *J* = 4.7 Hz), 6.76 (1H, s), 7.16 (1H, s), 8.22 (2H, d, *J* = 6.0 Hz), 8.90 (2H, d, *J* = 6.3 Hz).

5.4.3. Compound 27. A pale yellow powder, 52% yield. ^{11}H-NMR (D_2O) δ: 1.22 (3H, t, *J* = 7.2 Hz), 3.24, 3.69 (2H, ABq, *J* = 18.0 Hz), 4.15 (2H, q, *J* = 6.6 Hz), 5.28 (1H, d, *J* = 4.8 Hz), 5.26, 5.54 (2H, ABq, *J* = 14.6 Hz), 5.80 (1H, d, *J* = 4.5 Hz), 6.81 (1H, s), 7.23 (1H, s), 8.26 (2H, d, *J* = 7.0 Hz), 8.90 (2H, d, *J* = 6.7 Hz).

5.4.4. Compound 29. Powder, 57% yield. ^{1}H-NMR (DMSO-D_6) δ: 3.43, 3.57 (2H, ABq, *J* = 18.3 Hz), 4.26 (2H, dt, *J* = 29.7, 3.9 Hz), 4.62 (2H, dt, *J* = 47.8, 4.0 Hz), 5.22 (1H, d, *J* = 4.9 Hz), 5.39, 5.52 (2H, ABq, *J* = 14.6 Hz), 5.90 (1H, dd, *J* = 8.1, 4.9 Hz), 6.74 (1H, s), 7.23 (2H, s), 7.47 (1H, s), 8.51 (2H, d, *J* = 6.9 Hz), 8.92 (2H, d, *J* = 6.9 Hz), 9.67 (1H, d, *J* = 8.1 Hz).

5.4.5. Compound 30. Powder, 52% yield. ^{1}H-NMR (DMSO-D_6) δ: 1.21 (3H, t, *J* = 7.0 Hz), 3.43, 3.54 (2H, ABq, *J* = 18.2 Hz), 4.08 (2H, q, *J* = 7.0 Hz), 5.18 (1H, d, *J* = 5.0 Hz), 5.48, 5.51 (2H, ABq, *J* = 12.5 Hz), 5.87 (1H, dd, *J* = 8.5, 4.9 Hz), 7.37 (2H, s), 7.47 (1H, s), 8.51 (2H, d, *J* = 6.9 Hz), 8.92

(2H, d, *J* = 6.7 Hz), 9.58 (1H, d, *J* = 8.5 Hz).

5.4.6. Compound 31-(*EZ*). To a solution of **17-(*Z*)** (700 mg, 0.95 mmol) and **19a** (245 mg, 1.0 mmol) in DMA (2.6 ml) was added NaBr (245 mg, 1.0 mmol) at 0°C and then stirred at room temperature for 5 h. The mixture was poured into H_2O (20 ml). The resulting precipitate was filtered, washed with Et_2O, and dried under reduced pressure to give the coupled intermediate (630 mg, 73%).

To a solution of the above intermediate (630 mg, 0.69 mmol) and anisole (0.47 ml, 4.1 mmol) in CH_2Cl_2 (8 ml) was added 2 M/L $AlCl_3/CH_3NO_2$ (2.07 ml, 4.13 mmol) at -20°C and then the mixture was stirred at -20°C for 40 min. The mixture was poured into 0.3 N aqueous HCl (25 ml) and MeCN (25 ml) at 0°C and then this was added to Et_2O (25 ml). The mixture was separated to obtain the aqueous phase. To the aqueous crude solution was added HP20SS, and the mixture was concentrated, charged onto HP20SS column, and chromatographed with aqueous MeCN. The fractions containing the desired product were collected, concentrated and lyophilized to give **31-(*EZ*)** (225 mg, 50%) as an ocherous powder. The geometric isomer ratio of this compound was *E*:*Z* = 36:64. ^{1}H- NMR (D_2O) δ: 1.18 (3H, m), 3.28-3.61(2H, m), 4.14 (2H, m), 4.96-5.19 (3H, m), 5.68 and 5.72 (1H, d, J =4 .5Hz) , 5.79 and 6.04 (1H, m), 6.41 (0.64H, d, J = 10.8Hz), 6.82 and 6.83 (1H, s), 6.92 (0.36H, d, J = 17.7Hz), 7.07 and 7.10 (1H, s), 8.13 (2H, m), 8.57 and 8.63 (1H, d, J = 6.6Hz). IR (KBr) cm^{-1}:3418, 2979, 2164, 1763, 1637, 1603, 1532, 1473, 1439, 1396, 1348, 1207. MS(FAB): 649^+ $(M\text{-}Na+2H)^+$. Anal. Calcd for $C_{26}H_{23}N_{10}O_6S_2Na\cdot 3.9H_2O$: C, 43.77; H, 4.19; N, 18.90; S, 8.66; Na, 3.10 (%). Found: C, 43.73; H, 3.95; N, 29.18; S, 8.43; Na, 3.36 (%).

5.4.7. Compound 31-(*Z*). The **31-(*EZ*)** (*E*:*Z* = 25:75) was prepared separately by the procedure described above. The mixture was dissolved in aqueous $NaHCO_3$ solution and chromatographed with aqueous MeCN. The fractions containing the desired product were collected, concentrated and lyophilized to give **31-(*Z*)** (39 mg, 6%) as a white powder. ^{1}H H-NMR (D_2O) δ: 1.30 (3H, t, J = 7.2Hz), 3.43, 3.67 (2H, ABq, J = 17.7 Hz), 4.26 (2H, q, J = 7.5Hz), 5.06-5.29 (2H, m), 5.30 (1H, d, J = 2.7Hz) , 5.83 (1H, m), 5.90 (1H, m), 5.51 (1H, d, J = 11.1Hz), 6.98 (1H, s), 7.32 (1H, s), 8.29 (2H, m), 8.69 (2H, d, J = 5.7Hz).

5.4.8. Compound 32a. To a solution of **17-(*Z*)** (3.69 g, 5 mmol) in DMF (5 ml) was added NaBr (2.57 g, 25 mmol) and then the mixture was stirred at room temperature for 5 h. Isomerization to the trans form of geometry at the C3-position propenyl group was confirmed by NMR analysis. To the mixture was added **19a** (1.18 g, 5 mmol) at 0°C and the mixture was then stirred at 0°C for 18 h. The mixture was poured into 5% NaCl in H_2O (200 ml) at 0°C. The resulting precipitate was filtered, and dried to give the coupled intermediate. (4.60 g, 100%).

To a solution of the above intermediate (4.60 g, 5 mmol) and anisole (5.0 ml, 46 mmol) in CH_2Cl_2 (30 ml) was added 2 M/L $TiCl_4/CH_2Cl_2$ 15 ml, 30 mmol) at -20°C and then the solution was stirred at -20°C for 30 min. The mixture was poured into 0.3 N aqueous HCl (100 ml) and i-Pr2O (100 ml) at 0°C, and the resulting precipitate was filtered. The obtained precipitate was dissolved in dilute aqueous HCl and MeCN. To the crude solution was added HP20SS and the mixture was concentrated, charged onto HP20SS column, and chromatographed with aqueous MeCN. The fractions containing the desired product were collected, concentrated and lyophilized to give a solid (0.59 g, 0.91 mmol). The obtained solid was dissolved in $NaHCO_3$ (76 mg, 0.91 mmol) solution in H_2O (20 ml), and lyophilized to give **32a** (614 mg, 18%). ^{1}H-NMR (D_2O) δ: 1.28 (3H, t, *J* = 7.1 Hz), 3.66, 3.70 (2H, ABq, *J* = 17.9 Hz), 4.22 (2H, q, *J* = 7.0 Hz), 5.25-5.27 (3H, m), 5.80 (1H, d, *J* = 4.7 Hz), 6.08-6.18 (1H, m), 6.93 (1H, s), 6.98 (2H, d, *J* = 15.6 Hz), 7.23 (1H, s), 8.25 (2H, d, *J* = 6.4 Hz), 8.76 (2H, d, *J* = 6.6 Hz). IR (KBr) cm^{-1}:3419, 2967, 1762, 1634, 1527, 1474, 1431, 1359, 1249. MS(ESI): 649^+ $(M\text{-}Na+2H)^+$. Anal. Calcd for $C_{27}H_{23}N_6O_7S_2Na\cdot 3.6\ H_2O\cdot 0.7NaHCO_3$: C, 44.11; H, 4.13; N, 11.14; S, 8.50; Na, 5.18 (%). Found: C, 43.81; H, 4.01; N, 11.47; S, 8.41; Na, 5.24 (%).

Compound **32h**, **32i**, **32j** and **32p** were prepared by the procedure used for compound **32a**.

5.4.9. Compound 32h. Powder, 14% yield. ^{1}H-NMR (D_2O) δ: 1.27 (3H, t, J = 7.1), 3.67 (2H, s), 4.22 (2H,q, J = 7.1), 5.26 (1H, d, J = 4.5 Hz), 5.37 (1H, d, J = 6.9 Hz), 5.80 (1H, d, J = 4.5 Hz), 6.12 (1H, dt, J = 6.9, 15.6 Hz), 6.92 (1H, s), 7.02 (1H, d, J = 15.6 Hz), 7.47 (1H, s), 8.47 (2H, d, J = 6.6 Hz), 8.99 (2H, d, J = 6.6). IR (KBr) cm^{-1}: 3411, 2982, 2171, 1763, 1607, 1561, 1532, 1475, 1439, 1389, 1357, 1299, 1201, 1150, 1091, 1034, 1000. MS(ESI): 650^+ $(M–Na+2H)^+$. Anal. Calcd for $C_{27}H_{22}N_9NaO_7S_2\cdot 4.9\ H_2O$: C, 42.67; H, 4.22; N, 16.59; S, 8.44; Na, 3.03 (%). Found: C, 42.60; H,4.03; N, 16.41; S, 9.04; Na, 3.46 (%).

5.4.10. Compound 32i. Powder, 4% yield. ^{1}H-NMR (D_2O) δ: 1.27 (3H, t, J = 6.9 Hz), 3.65 (1H, d, J = 17.4 Hz), 3.72 (1H, d, J = 17.4 Hz), 4.22 (2H,q, J = 6.9 Hz), 5.25 – 5.30 (2H, m), 5.80 (1H, d, J = 4.8 Hz), 6.14 (1H, dt, J = 7.2, 15.3 Hz), 6.92 (1H, s), 7.00 (1H, d, J = 15.3 Hz), 8.39 (1H, d, J = 6.9 Hz), 8.56 (1H, s), 8.81 (2H, d, J = 6.9 Hz). IR (KBr) cm^{-1}: 3419, 2983, 2183, 2154, 1763, 1636, 1602, 1532, 1491, 1461, 1364, 1291, 1203, 1155, 1090, 1036, 1001. MS(ESI): 688^+ $(M+H)^+$. Anal. Calcd for $C_{27}H_{22}N_9NaO_6S_3\cdot 4.9\ H_2O\cdot 0.1\ NaHCO_3$: C, 41.50; H, 4.10; N, 16.07; S, 12.26; Na, 3.22 (%). Found: C, 41.40; H, 3.96; N, 16.22; S, 12.31; Na, 3.42 (%).

5.4.11. Compound 32j. Powder, 7% yield. ^{1}H-NMR (D_2O) δ: 1.28 (3H, t, J = 7.2 Hz), 3.65 (1H, d, J = 17.4 Hz), 3.71 (1H, d, J = 17.4 Hz), 4.22 (2H,q, J = 7.2 Hz), 5.26 (1H, d, J = 4.5 Hz), 5.33 (1H, d,

J = 6.9 Hz), 5.80 (1H, d, J = 4.5 Hz), 6.14 (1H, dt, J = 6.9, 15.3 Hz), 6.93 (1H, s), 7.01 (1H, d, J = 15.3 Hz), 8.40 (1H, s), 8.47 (2H, d, J = 6.9 Hz), 8.91 (2H, d, J = 6.9 Hz). IR (KBr) cm^{-1}: 3846, 3418, 3055, 2984, 2162, 1762, 1635, 1598, 1532, 1469, 1440, 1357, 1300, 1203, 1156, 1124, 1091, 1037, 1002. MS(ESI): 688^{+} $(M+H)^{+}$. Anal. Calcd for $C_{27}H_{22}N_9NaO_6S_3 \cdot 5.3\ H_2O$: C, 41.41; H, 4.20; N, 16.10; S, 12.28; Na, 2.94 (%). Found: C, 41.31; H, 4.20; N, 16.08; S, 12.36; Na, 3.24 (%).

5.4.12. Compound 32p. Powder, 7% yield. ^{1}H-NMR (D_2O) δ: 1.28 (3H, t, J = 7.2 Hz), 3.67 (2H, s), 4.22 (2H,q, J = 7.2 Hz), 5.26 (1H, d, J = 4.5 Hz), 5.47 (1H, d, J = 6.6 Hz), 5.80 (1H, d, J = 4.8 Hz), 6.17 (1H, dt, J = 6.6, 15.6 Hz), 6.93 (1H, s), 7.03 (1H, d, J = 15.6 Hz), 8.67 (1H, d, J = 6.6 Hz), 8.74 (1H, d, J = 6.6 Hz), 9.72 (1H, s). IR (KBr) cm^{-1}: 3425, 2180, 1762, 1606, 1532, 1363, 1304, 1200, 1166, 1123, 1084, 1036. MS(ESI): 662^{+} $(M+H)^{+}$. Anal. Calcd for $C_{25}H_{20}N_9NaO_6S_3 \cdot 3.9\ H_2O$: C, 41.02; H, 3.83; N, 17.22; S, 13.14; Na, 3.14 (%). Found: C, 40.98; H, 3.72; N, 17.21; S, 13.11; Na, 3.47 (%).

5.4.13. Compound 32b. To a suspension of **19b** (369 mg, 1.62 mmol) in MeOH (3 ml) was added 1 M/L MeONa/MeOH (1.62 ml, 1.62 mmol) and then the mixture was evaporated to give the sodium salt of **19b** (455 mg, 119%). The title compound was prepared by the procedure used for compound **32a** with the sodium salt of **19b**. Powder, 17% yield. ^{1}H-NMR (D_2O) δ: 1.28 (3H, t, J = 7.2 Hz), 3.64 (1H, d, J = 17.7 Hz), 3.71 (1H, d, J = 17.7 Hz), 4.11 (3H, s), 4.22 (2H, q, J = 7.2 Hz), 5.25 – 5.26 (2H, m), 5.80 (1H, d, J = 4.5 Hz), 6.12 (1H, dt, J = 6.9, 15.6 Hz), 6.91 (1H, s), 6.99 (1H, d, J = 15.6 Hz), 7.23 (1H, s), 8.22 (2H, d, J = 7.2 Hz), 8.97 (2H, d, J = 7.2 Hz). IR (KBr) cm^{-1}: 3419, 2980, 2186, 2151, 1764, 1636, 1604, 1522, 1475, 1437, 1388, 1347, 1301, 1201, 1155, 1035, 1001. MS(ESI): 663^{+} $(M–Na+2H)^{+}$. Anal. Calcd for $C_{28}H_{25}N_{10}NaO_6S_2 \cdot 4.6\ H_2O$: C, 43.81; H, 4.49; N, 18.25; S, 8.36; Na, 3.00 (%). Found: C,4 3.81; H, 4.28; N, 18.15; S, 8.24; Na, 3.26 (%).

5.4.14. Compound 32m. This compound was prepared by the procedure used for compound **32b**. Powder, 12% yield. ^{1}H-NMR (D_2O) δ: 1.27 (3H, t, J = 6.9 Hz), 3.62 (2H, s), 4.22 (2H,q, J = 6.9 Hz), 5.22 (1H, d, J = 4.8 Hz), 5.43 (2H, d, J = 6.3 Hz), 5.79 (1H, d, J = 4.8 Hz), 6.15 (1H, dt, J = 6.3, 15.9 Hz), 6.87 (1H, d, J = 15.9 Hz), 6.93 (1H, s), 7.23 (1H, s), 7.69 (1H, dd, J = 6.0, 8.5 Hz), 8.48 (1H, d, J = 8.5 Hz), 8.59 (1H, d, J = 6.0 Hz). IR (KBr) cm^{-1}: 3409, 2984, 2164, 1762, 1664, 1592, 1532, 1460, 1335, 1201, 1160, 1122, 1091, 1036. MS(ESI): 644^{+} $(M+H)^{+}$. Anal. Calcd for $C_{26}H_{22}N_9NaO_6S_2 \cdot 4.3\ H_2O \cdot 0.2\ NaHCO_3$: C, 42.65; H, 4.21; N, 17.08; S, 8.69; Na, 3.74 (%). Found: C, 42.66 ; H, 4.13; N, 16.87; S,9.18; Na, 3.77 (%).

5.4.15. Compound 32g. To a solution of **17-(*E*)** (738 mg, 1 mmol) and **19g** (251 mg, 1 mmol) in DMF (2 ml) was added NaBr (307 mg, 3 mmol) and then the mixture was stirred at room

temperature for 4 h 20 min. The mixture was poured into 5% NaCl in H_2O (30 ml) at 0°C. The resulting precipitate was filtered, H_2O was added, and lyophilization gave the coupled intermediate. (851 mg, 102%).

To a solution of the above intermediate (845 mg, 1 mmol) and anisole (1 ml, 7.77 mmol) in CH_2Cl_2 (5 ml) was added 2 M/L $TiCl_4/CH_2Cl_2$ 3 ml, 6 mmol) at -30°C and then the mixture was stirred at -30°C for 1 h 30 min. The mixture was poured into 0.3 N aqueous HCl (30 ml) and i-Pr_2O (30 ml) at 0°C, and the resulting precipitate was filtered. The obtained precipitate was dissolved in dilute aqueous HCl and MeCN. To the crude solution was added HP20SS, and the mixture was concentrated, charged onto HP20SS column, and chromatographed with aqueous MeCN. The fractions containing the desired product were collected, concentrated and lyophilized to give solid (266 mg, 0.41 mmol). The obtained solid was dissolved in NaHCO3 (34 mg, 0.40 mmol) solution in H2O (7 ml), and lyophilized to give **32g** (272 mg, 35%). ^{1}H-NMR (d_6-DMSO) δ: 1.22(3H, t, J = 7.2Hz), 3.43, 3.49(2H, ABq, J = 17.1 Hz), 4.09(2H, q, J = 7.2Hz), 5.05(1H, d, J = 4.8Hz), 5.21(2H, d, J = 7.2Hz) , 5.60(1H, dd. J = 4.8, 8.1Hz), 5.87(1H, m), 6.72(1H, s), 7.22(2H, s), 7.29(1H, d, J = 15.9Hz), 8.17(1H, d, J = 1.8Hz), 8.48(2H, d, J = 6.9Hz), 8.65(1H, d, J = 1.5Hz), 8.93(2H, d, J = 7.2Hz), 9.54(1H, d, J = 8.1Hz). IR (KBr) cm^{-1}:3417, 2982, 2177, 1762, 1634, 1602, 1538, 1470, 1434, 1382, 1353, 1251, 1204. MS(ESI): 709$^+$ $(M+H)^+$. Anal. Calcd for $C_{30}H_{25}N_8O_6S_2Na\cdot 4.5\ H_2O\cdot 0.2NaHCO_3$: C, 46.59; H, 4.43; N, 14.39; S, 8.24; Na, 3.54 (%). Found: C, 46.60; H ,4.43; N, 14.63; S, 7.45; Na, 3.48 (%).

Compound **32d**, **32e**, **32f**, **32k**, **32l**, **32o**, **32q** and **32r** were prepared by the procedure used for compound **32g**.

5.4.16. Compound 32d. Powder, 27% yield. ^{1}H-NMR (D_2O) δ: 1.29 (3H, t, J = 6.9 Hz), 3.67 (2H, s), 4.23 (2H, q, J = 6.9 Hz), 5.26 – 5.28 (3H, m), 5.81 (1H, d, J = 4.5 Hz), 6.07 – 6.17 (1H, m), 6.95 – 6.99 (3H, m), 8.31 (2H, d, J = 7.2 Hz), 8.49 (1H, d, J = 2.7 Hz), 8.85 (2H, d, J = 7.2 Hz). IR (KBr) cm^{-1}: 3420, 2981, 2162, 1764, 1638, 1599, 1536, 1475, 1446, 1384, 1332, 1203, 1158, 1036. MS(ESI): 649$^+$ $(M–Na+2H)^+$. Anal. Calcd for $C_{27}H_{23}N_{10}NaO_6S\cdot 6.5\ H_2O$: C, 41.17; H, 4.61; N, 17.78; S ,8.14; Na, 2.92 (%). Found: C, 41.04; H, 4.09; N, 17.77; S, 8.31; Na, 3.06 (%).

5.4.17. Compound 32e. Powder, 3% yield. ^{1}H-NMR (d_6-DMSO) δ: 1.22(3H, t, J = 6.3Hz), 3.35-3.48(2H, m), 3.92(3H, s), 4.09(2H, q, J = 6.3Hz), 5.03(1H, d, J = 4.8Hz), 5.07(2H, m) , 5.58(1H, m), 5.81(1H, m), 6.72(1H, s), 7.21-7.28(4H, m), 7.92(1H, m), 8.11(1H, d, J = 6.6Hz), 8.66(1H, d, J = 7.5Hz), 9.53(1H, d, J = 8.4Hz). IR (KBr) cm^{-1}:3420, 2983, 2181, 2146, 1763, 1634, 1604, 1561, 1496, 1469, 1433, 1402, 1334, 1220. MS(FAB): 662$^+$ $(M-Na+2H)^+$. Anal. Calcd for $C_{29}H_{26}N_9O_6S_2Na\cdot 5.0\ H_2O\cdot 0.1NaHCO_3$: C, 44.68; H, 4.65; N, 16.12; S, 8.20; Na, 3.23 (%). Found: C,

44.74; H, 4.67; N, 15.85; S, 8.15; Na, 3.50 (%).

5.4.18. Compound 32f. Powder, 33% yield. ^{1}H-NMR (d_6-DMSO) δ: 1.22(3H, t, J = 7.2Hz), 2.62(3H, s), 3.42, 3.49(2H, ABq, J = 17.1 Hz), 4.09(2H, q, J = 6.9Hz), 5.04(1H, d, J = 5.1Hz), 5.12(2H, d, J = 7.2Hz) , 5.60(1H, dd, J = 4.5, 8.1Hz), 5.83(1H, m), 6.72(1H, s), 7.23-7.28(3H, m), 7.65(1H, s), 8.08(2H, d, J = 7.2Hz), 8.66(2H, d, J = 7.2Hz), 9.54(1H, d, J = 8.1Hz), 11.76(1H, s). IR (KBr) cm^{-1}:3398, 2154, 1762, 1633, 1604, 1565, 1537, 1487, 1471, 1442, 1392, 1329, 1220. MS(FAB): 684$^+$ $(M+H)^+$. Anal. Calcd for $C_{29}H_{26}N_9O_6S_2Na\cdot 6.0\ H_2O$: C, 43.99; H, 4.84; N, 15.92; S, 8.10; Na, 2.90 (%). Found: C, 44.12; H, 4.42; N, 15.85; S, 8.07; Na, 2.82 (%).

5.4.19. Compound 32k. Powder, 18% yield. ^{1}H-NMR (D_2O) δ: 1.30 (3H, t, J = 7.2 Hz), 3.65 (2H, s), 4.25 (2H,q, J = 7.2 Hz), 5.26 (1H, d, J = 4.8 Hz), 5.36 (2H, d, J = 6.9 Hz), 5.82 (1H, d, J = 4.8 Hz), 6.11 (1H, dt, J = 6.9, 13.5 Hz), 6.96 (1H, d, J = 13.5 Hz), 6.99 (1H, s), 8.10 (1H, t, J = 7.2 Hz), 8.51 – 8.94 (2H, m), 9.29 (1H, s). IR (KBr) cm^{-1}: 3418, 2984, 2168, 1763, 1636, 1601, 1533, 1380, 1304, 1203, 1166, 1136, 1037, 1002. MS(ESI): 605$^+$ $(M+H)^+$. Anal. Calcd for $C_{24}H_{21}N_8NaO_6S_2\cdot 3.7\ H_2O$: C, 42.94; H, 4.26; N, 16.69; S, 9.55; Na, 3.42 (%). Found: C, 42.99; H, 4.14; N, 16.61; S, 9.36; Na, 3.49 (%).

5.4.20. Compound 32l. Powder, 19% yield. ^{1}H-NMR (D_2O) δ: 1.30 (3H, t, J = 7.2 Hz), 3.64 (2H, s), 4.25 (2H,q, J = 7.2 Hz), 5.26 (1H, d, J = 4.8 Hz), 5.34 (2H, d, J = 7.2 Hz), 5.82 (1H, d, J = 4.8 Hz), 6.09 (1H, dt, J = 7.2, 15.6 Hz), 6.97 (1H, d, J = 15.6 Hz), 6.98 (1H, s), 8.37 (2H, d, J = 6.9 Hz), 8.91 (2H, d, J = 6.9 Hz). IR (KBr) cm^{-1}: 3420, 3055, 2982, 2160, 1762, 1605, 1561, 1533, 1457, 1367, 1301, 1200, 1150, 1122, 1036, 1001. MS(ESI): 605$^+$ $(M+H)^+$. Anal. Calcd for $C_{24}H_{21}N_8NaO_6S_2\cdot 3.6\ H_2O$: C, 43.06; H, 4.25; N, 16.74; S, 9.58; Na, 3.43 (%). Found: C, 43.09; H, 4.22; N, 16.73; S, 9.48; Na, 3.64 (%).

5.4.21. Compound 32o. Powder, 7% yield. ^{1}H-NMR (d_6-DMSO) δ: 1.21 (3H, t, J = 6.9 Hz), 4.08 (2H, q, J = 6.9 Hz), 5.01 (1H, d, J = 5.1 Hz), 5.24 (1H, dd, J = 6.3, 14.4 Hz), 5.52 – 5.62 (2H, m), 5.90 (1H, m), 6.71 (1H, s), 7.02 – 7.07 (2H, m), 7.21 (2H, brs), 7.31 (1H, d, J = 15.6 Hz), 8.18 (1H, d, J = 6.3 Hz), 8.28 (1H, d, J = 7.8 Hz), 9.53 (1H, d, J = 7.8 Hz). IR (KBr) cm^{-1}: 3409, 2981, 2286, 2153, 1762, 1611, 1533, 1475, 1456, 1394, 1365, 1301, 1259, 1200, 1163, 1122, 1089, 1037. MS(ESI): 622$^+$ $(M–2Na+3H)^+$. Anal. Calcd for $C_{26}H_{21}N_9Na_2O_6S_2\cdot 4.7\ H_2O$: C,41.62 ; H,4.08 ; N,16.08 ; S,8.55 ; Na,6.13 (%). Found: C,41.47 ; H,3.75 ; N,16.86 ; S,8.52 ; Na,6.18 (%).

5.4.22. Compound 32q. Powder, 6% yield. ^{1}H-NMR (D_2O) δ: 1.29 (3H, t, J = 6.9 Hz), 3.67 (2H, s), 4.24 (2H, q, J = 6.9 Hz), 5.27 (1H, d, J = 4.8 Hz), 5.44 (2H, d, J = 6.9 Hz), 5.82 (1H, d, J = 4.8 Hz),

6.17 (1H, dt, J = 6.9, 15.6 Hz), 6.96 (1H, s), 7.04 (1H, d, J = 15.6 Hz), 8.01 (1H, t, J = 7.8 Hz), 8.45 - 8.48 (2H, m), 8.54 (1H, d, J = 7.2 Hz), 8.95 (1H, d, J = 7.2 Hz), 9.73 (1H, s). IR (KBr) cm^{-1}: 3418, 2984, 2162, 1763, 1607, 1535, 1474, 1456, 1390, 1365, 1293, 1243, 1202, 1168, 1133, 1090, 1037, 1001. MS(ESI): 655^{+} $(M+H)^{+}$. Anal. Calcd for $C_{28}H_{23}N_8NaO_6S_2 \cdot 2.8\ H_2O \cdot 0.6\ NaHCO_3$: C, 45.47; H, 3.90; N, 14.83; S, 8.49; Na, 4.87 (%). Found: C, 45.36; H, 4.11; N, 15.01; S, 8.91; Na, 4.73 (%).

5.4.23. Compound 32r. Powder, 14% yield. ^{1}H-NMR (DMSO-D_6) δ: 1.21 (3H, t, *J* = 7.0 Hz), 3.38, 3.48 (2H, ABq, *J* = 22.4 Hz), 4.07 (3H, q, *J* = 7.0 Hz), 5.02 (1H, d, *J* = 4.9 Hz), 5.59 (1H, dd, *J* = 8.2, 4.7 Hz), 5.74 (2H, br s), 5.90-5.98 (1H, m), 6.70 (1H, s), 7.21 (2H, s), 7.33 (1H, d, *J* = 15.9 Hz), 8.16 (1H, dd, *J* = 8.3, 5.9 Hz), 8.53 (1H, d, *J* = 9.2 Hz), 8.68 (1H, d, *J* = 9.0 Hz), 8.96 (1H, s), 9.38 (1H, d, *J* = 8.4 Hz), 9.50-9.55 (2H, m). IR (KBr) cm^{-1}: 3418, 2983, 2155, 1763, 1629, 1600, 1531, 1471, 1374, 1231, 1200, 1166, 1121, 1090, 1036, 1002. Anal. Calcd for $C_{28}H_{23}N_8NaO_6S_2 \cdot 5.7\ H_2O \cdot 0.15\ NaHCO_3$: C, 43.92; H, 4.52; N, 14.55; S, 8.33; Na, 3.43 (%). Found: C, 43.89; H, 4.31; N, 14.71; S, 9.11; Na, 3.42 (%).

5.4.24. Compound 32n. To a suspension of **19n** (410 mg, 2.20 mmol) in MeOH (5 ml) was added 1 M/L MeONa/MeOH (2.0 ml, 2.0 mmol). The pH of the mixture was adjusted to 7.3 with aqueous HBr, and then the mixture was evaporated to give a sodium salt of **19n**. The title compound was prepared by the procedure used for compound **32g** giving the sodium salt of **19n** as a powder, 9% yield. ^{1}H-NMR (D_2O) δ: 1.28 (3H, t, J = 6.9 Hz), 3.65 (2H, ABq, J = 17.8 Hz), 4.22 (2H,q, J = 6.9 Hz), 5.24 (3H, d, J = 4.8 Hz), 5.79 (1H, d, J = 6.6 Hz), 6.13 (1H, dt, J = 6.9, 15.9 Hz), 6.89 – 6.94 (2H, m), 7.27 (1H, s), 7.82 (1H, d, J = 7.2 Hz), 8.29 (1H, d, J = 7.2 Hz). IR (KBr) cm^{-1}: 3425, 2160, 1762, 1598, 1535, 1372, 1333, 1202, 1165, 1120, 1092, 1036, 1003. MS(ESI): 644^{+} $(M+H)^{+}$. Anal. Calcd for $C_{26}H_{22}N_9NaO_6S_2 \cdot 5.3\ H_2O \cdot 0.2\ NaHCO_3$: C, 41.63; H, 4.37; N, 16.68; S, 8.48; Na, 3.65 (%). Found: C, 41.76; H, 4.33; N, 16.16; S, 8.88; Na, 3.85 (%).

5.4.25. Compound 32c. To a suspension of **19c** (213 mg, 1.0 mmol) in DMF (3 ml) was added N,O-bis trimethylsilyl acetamide (494 µl, 2.0 mmol) and mixture was sonicated to a clear solution. To the obtained solution was added to **17-(E)** (738 mg, 1 mmol) and NaBr (309 mg, 3.0 mmol), and then the mixture was stirred at room temperature for 7.5 h. The mixture was poured into 5% NaCl in H_2O (80 ml) at 0°C. The resulting precipitate was filtered and dried under reduced pressure to give coupled intermediate (934 mg, 94%).

To a solution of the above intermediate (924 mg, 0.93 mmol) and anisole (652 µl, 6.0 mmol) in CH_2Cl_2 (15 ml) was added 2 M/L $TiCl_4/CH_2Cl_2$ 3 ml, 6 mmol) at -40°C and then the mixture was stirred at -30°C for 50 min. The mixture was poured into 0.3 N aqueous HCl (40 ml) and to the

mixture was added i-Pr_2O (30 ml) at 0°C and resulting precipitate was obtained by filtration then dissolved in aqueous $NaHCO_3$ solution. The crude solution was chromatographed on HP20SS column with aqueous MeCN. The fractions containing the desired product were collected, concentrated and rechromatographed on ODS column with aqueous MeCN. The fractions containing the desired product were collected, concentrated and lyophilized to give **32c** (215 mg, 27%) as a pale brown powder. ^{1}H-NMR (D_2O) δ: 1.29 (3H, t, J = 6.9 Hz), 3.67 (2H, s), 4.23 (2H, q, J = 6.9 Hz), 5.26 – 5.28 (3H, m), 5.81 (1H, d, J = 4.5 Hz), 6.07 – 6.17 (1H, m), 6.95 – 6.99 (3H, m), 8.31 (2H, d, J = 7.2 Hz), 8.49 (1H, d, J = 2.7 Hz), 8.85 (2H, d, J = 7.2 Hz). IR (KBr) cm^{-1}: 3420, 2981, 2162, 1764, 1638, 1599, 1536, 1475, 1446, 1384, 1332, 1203, 1158, 1036. MS(ESI): 649^+ $(M–Na+2H)^+$. Anal. Calcd for $C_{27}H_{23}N_{10}NaO_6S_3 \cdot 6.5\ H_2O$: C, 41.17; H, 4.61; N, 17.78; S, 8.14; Na, 2.92 (%). Found: C, 41.04; H, 4.09; N, 17.77; S, 8.31; Na, 3.06 (%).

6. Pharmacokinetics evaluation.

6.1. Test compound administration. Three to five mice per group received an intravenous bolus dose of the test compounds at 10 mL/kg and 20 mg/kg for animal weights and dosing volumes. Blood samples were obtained at 0.083, 0.25, 0.5, 1, 2 and 3 h after administration by cardiac puncture following anesthesia. Plasma samples were separated by centrifugation at 3,000 x g for 10 minutes at 4 °C.

Three monkeys per group were dosed with an intravenous bolus injection of the test compounds at 1 mL/kg, 20 mg/kg for animal weight and dosing volume. Blood samples were obtained at 0.083, 0.25, 0.5, 1, 2, 3, 4, 6 and 8 h post-dose from the femoral vein. Plasma samples were separated by centrifugation at 3,000 x g for 10 minutes at 4°C. All the samples were stored at −80°C until assay.

6.2. Analysis. For the evaluation of pharmacokinetic (PK) profile in mice, the concentration of the compounds in plasma was assayed by the band-culture method. *Escherichia coli* 7437 was used as the test organism in Mueller-Hinton medium (Difco, New Jersey, USA). The compound concentrations in plasma were calculated from calibration curves for the compounds dissolved in normal plasma. $AUC_{0-\infty}$, and $T_{1/2}$ were calculated with the WinNonlin program (Pharsight, NJ, USA) based on a 1-compartment model. For the PK evaluation in monkeys, the plasma concentration was calculated by high-performance liquid chromatography (HPLC) analysis (Shimadzu, Japan). $AUC_{0-\infty}$, CLtot and $T_{1/2}$ were calculated using the WinNonlin program, and the concentrations of the test compounds were fitted to a 2-compartment model.

7. *In vitro* evaluation

MICs were determined with an agar dilution method using Sensitivity Disk Agar-N (Nissui Pharmaceutical Tokyo, Japan). The overnight cultures of antibacterial strains in Mueller Hinton broth (Becton Dickinson) were diluted to about 10^6 CFU/mL. Bacterial suspensions of 1 μL were spotted onto agar plates containing the antibiotic of 2-fold serial dilution concentrations and incubated for 20 h at 37 °C before the MICs were scored.

8. *In vivo* evaluation

Systemic infection models: The *in vivo* potency of **32j** was determined using a mouse model of septicemia. The mice were injected intraperitoneally with 0.5 mL suspension of *Streptococcus pneumoniae* SR16605 (penicillin-susceptible strain) and SR16754 (penicillin-resistant strain) were injected as a suspension with hog mucin (ICN, Cleveland, Ohio). **32j** and CTRX were administered subcutaneously 1 and 5 h after infection. Mortality was recorded over 7 days to estimate the 50% effective dose (ED_{50}) and 90% confidence limits, which were determined by the logit method.

9. Animals

Six-week-old, male Jcl: ICR mice (body weight 25 to 33 g for PK evaluation and 19 to 25 g for in vivo evaluation) obtained from CLEA Japan, Inc. and female cynomolgus monkeys obtained from Hamri Co., Ltd. were used. The animals were maintained in accordance with the criteria of the Institutional Animal Care and Use Committee of Shionogi. All studies with animals were approved by the Institutional Animal Care and Use Committee of Shionogi & Co., Ltd.

10. Measurement of protein binding rate

The serum protein binding of the compounds was determined by ultrafiltration with a micropartition system. Just before use, the pH of the plasma was adjusted to 7 using carbonic acid gas, and 0.01 ml of compound solution was added to 0.99 ml of plasma from mice and monkeys (final concentration, 10 μg/ml). The mixture was allowed to react at 37°C for 30 minutes. The sample was placed in an ultrafiltration device (ULTRACENT-30, Tosho Corp., Japan) and centrifuged at 3200 rpm for 30 minutes at 25°C. The concentration of compound in the filtrate was determined by bioassay to calculate the degree of protein binding.

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