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MexAB-OprM specific efflux pump inhibitors in *Pseudomonas aeruginosa*. Part 7: Highly soluble and in vivo active quaternary ammonium analogue D13-9001, a potential preclinical candidate

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Abstract—A series of 4-oxo-4*H*-pyrido[1,2-*a*]pyrimidine derivatives, substituted at the 2-position with piperidines bearing quaternary ammonium salt side chains, were synthesized and evaluated for their ability to potentiate the activity of the fluoroquinolone levofloxacin (LVFX) and the β-lactam aztreonam (AZT) in *Pseudomonas aeruginosa*. Attachment of the charged entity using an *N*-ethylcarbamoyloxy linker led to the discovery of the highly soluble compound **22** (**D13-9001**), which maintained good potency in vitro and displayed excellent activity in vivo in a rat pneumonia model of *P. aeruginosa*.

1. Introduction

The emergence of drug resistance in many organisms is a serious public health issue and, therefore, has attracted much attention in recent years. In particular, reports of multidrug resistance in Gram-negative organisms such as *Pseudomonas aeruginosa* have increased significantly.[1] A major component of this problem, and of intrinsic resistance, is attributable to the phenomenon of efflux, whereby a differentially low concentration of the antibiotic is maintained inside the bacterium. At least seven RND (resistance-nodulation-cell division) superfamily efflux pumps are known to exist in *P. aeruginosa*.[2–5] Among these, the MexAB-OprM system is constitutive and commonly overexpressed in clinical isolates.[5] Therefore, we targeted a MexAB-OprM specific efflux pump inhibitor (EPI) for use as a potentiator of existing antibacterial agents through combinational use. This kind of agent would be useful in the treatment of the increasingly common clinically refractory infections caused by *P. aeruginosa*.

In previous papers, we reported two types of pyridopyrimidine-based EPI: (i) vinyl tetrazoles with piperidine 2-substituents such as **1** and (ii) acrylic acid analogues such as **2** (Fig. 1) bearing aryl residues in place of the piperidine, but each class bore liabilities as clinical candidates.[6–11] Compound **1** showed excellent potency in vitro, but the solubility was very poor for intravenous use. On the other hand, the more soluble compound **2** possessed a reasonable safety profile in an acute toxic assay, but unfortunately showed only moderate potentiation activity in vivo. Thus we set out to improve the

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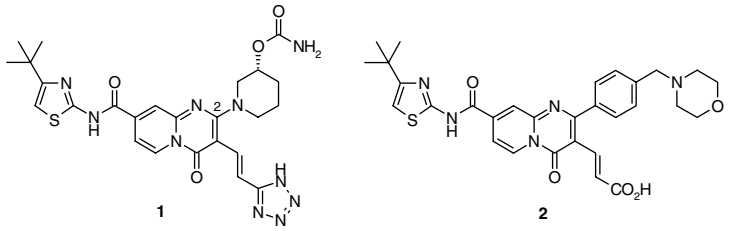


Figure 1. Structure of the pyridopyrimidine-based MexAB-OprM specific efflux pump inhibitors.

solubility of the strong potentiator **1** by incorporating highly water-soluble moieties such as quaternary ammonium salts, directed by our cumulative knowledge of the SAR in both series.

Herein, we report the design, the synthesis, and evaluation of analogues resulting from this effort.

2. Chemistry

We planned to synthesize the quaternary ammonium salts from the appropriate intermediate (**11**) by attachment of the linker moiety, quaternization with a suitable alkyl halide, and finally by deprotection of *p*-methoxybenzyl group, as depicted in Scheme 2. A series of substituted piperidines was introduced via nucleophilic displacement of a diphenyl phosphonate at the C-2 position. The primary carbamate **1** was synthesized via an *N*-trichloroacetyl protected intermediate as displayed in Scheme 1. The stereochemistry of the 3-hydroxyl group on the piperidine was fixed as (*R*) since the (*S*)-isomer exhibited less potency.[9] The appropriate alcohol (**11**) was reacted with 1,1′-carbonyldiimidazole and then with ω-dimethylaminoalkylamine to provide a series of *N*,*N*-dimethylamino intermediates (**13**, **14**), which were useful for the preparation of various quaternary ammonium salts. Treatment with alkyl halides, followed by deprotection of tetrazole moiety (and ester hydrolysis, where necessary), gave the desired derivatives (**20**–**23**).

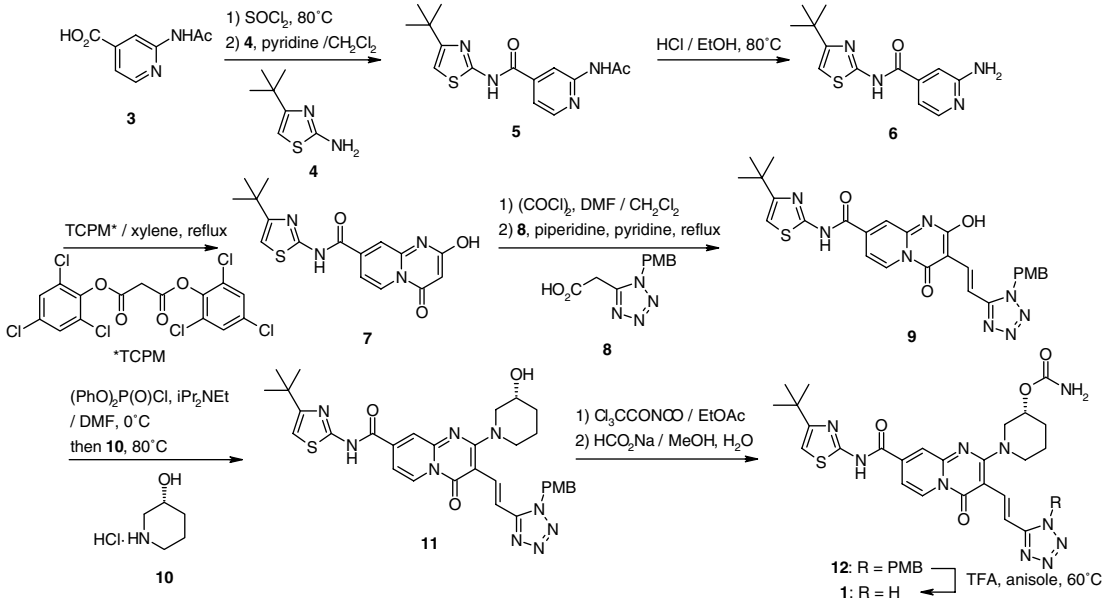
A compound in which the carbamate linker was replaced by amide was synthesized as shown in Scheme 3. After attachment of 3-piperidineacetic acid ester (**24**) to the pyridopyrimidine nucleus, ester hydrolysis, EDC-mediated amide formation with *N*,*N*-dimethylethylenediamine, quaternization, and treatment with TFA provided the piperidine acetic acid derivative (**26**).

In an attempt to remove the chiral center, two avenues were explored. The asymmetric carbonate linker was replaced with an acrylamide as illustrated in Scheme 4. The acrylic acid moiety was generated by Horner–Wadsworth–Emmons reaction on the 3-oxo-piperidine (**27**), and subsequent steps analogous to earlier analogues afforded the desired compound (**31**).

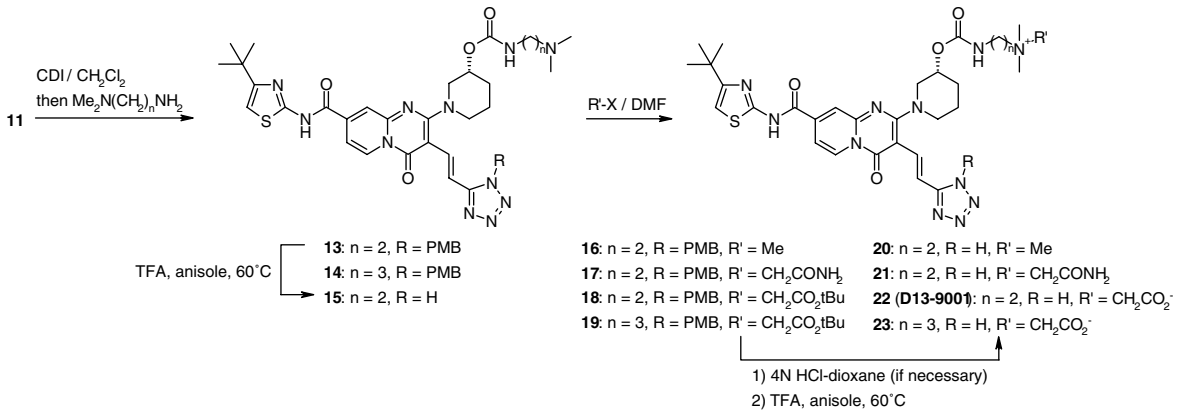
Finally, an achiral 4-piperidinyl variant (**34**) was synthesized using the same route as that described for **22** (Scheme 5).

3. Results and discussion

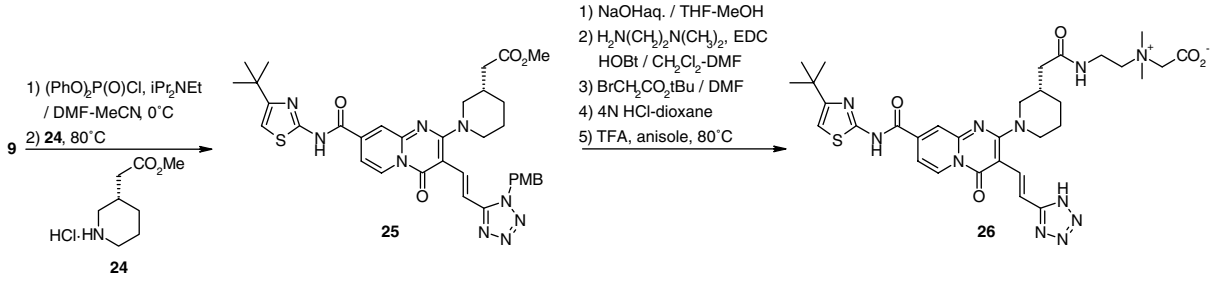
The compounds prepared in this study were tested for in vitro potentiation of the activity of levofloxacin



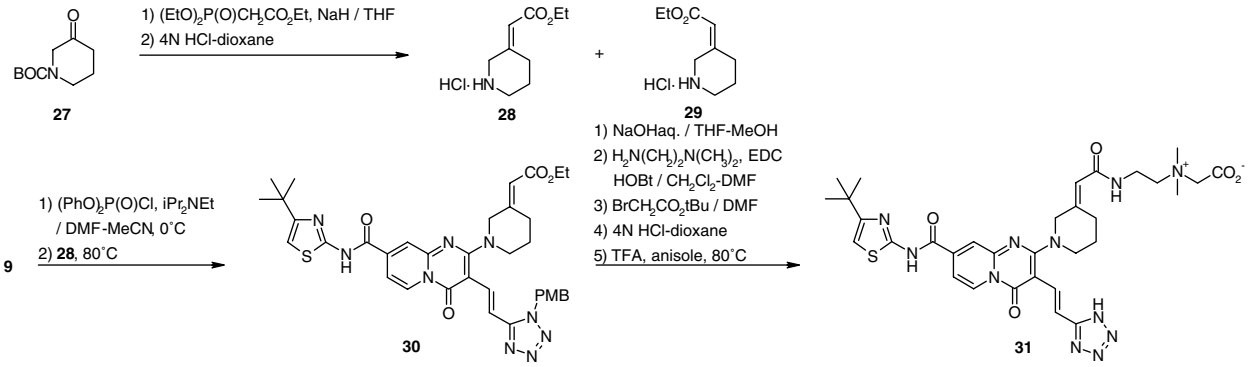
Scheme 1. Synthesis of 2-piperidine attached vinyl tetrazole analogue **1**.



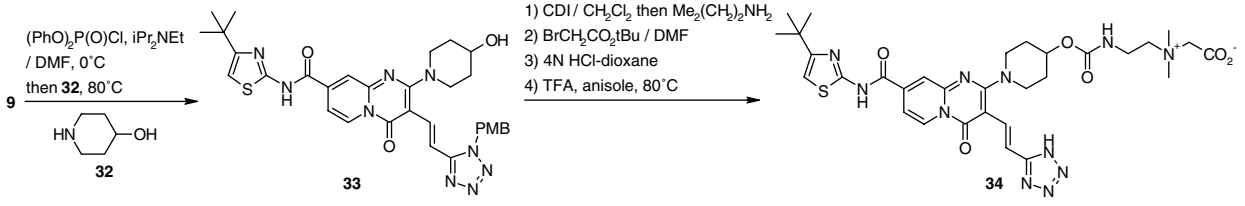
Scheme 2. Synthesis of quaternary ammonium salt derivatives.



Scheme 3. Synthesis of piperidineacetic acid linker analogue **26**.



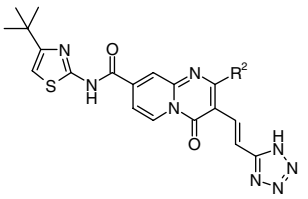
Scheme 4. Synthesis of acrylamide linker analogue **31**.

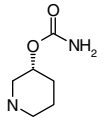
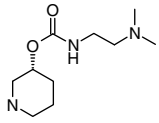
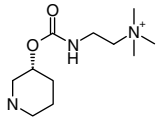
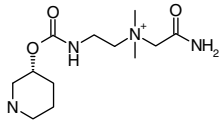
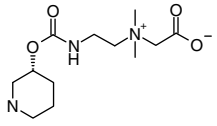
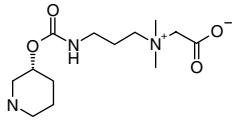
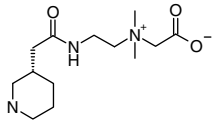
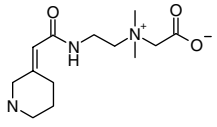
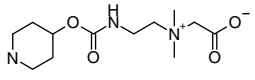


Scheme 5. Synthesis of 4-hydroxypiperidine linker analogue **34**.

(LVFX) and aztreonam (AZT) against PAM1723, a laboratory strain of *P. aeruginosa* in which the MexAB-OprM pump is over-expressed and the MexCD-OprJ and MexEF-OprN pumps are disrupted.[12] The results, expressed as the minimum concentration of inhibitor required to decrease (potentiate) the minimum inhibitory concentration (MIC) of the combination agent 8-fold (MPC_8, μg/mL), together with aqueous solubilities (in pH 6.8 buffer solution, μg/mL), are presented in Table 1. Antimicrobial assays were

Table 1. Chemical structures and potentiation activity of quaternary ammonium analogues[a]



Compound	R^2	MPC_8 (LVFX) (μg/mL)		MPC_8 (AZT) (μg/mL)	Sol (pH 6.8) (μg/mL)
		Without HSA	With 0.125% HSA		
1		0.5	2	⩽0.25	9.2
15		2	4	1	—[c]
20		2	4	2	—[c] (547)[d]
21		4	4	4	95
22 (D13-9001)		2	4	2	747
23		4	4	2	610
26		4	8	4	860
31		16	>16[b]	8	insoluble[e]
34		8	16	4	170

[a] All compounds lacked intrinsic antibacterial activity.
[b] The apparent lack of activity is attributable to precipitation of the compound.
[c] Not tested.
[d] The solubility of its enantiomer.
[e] Precipitation was observed and the solubility was not quantitated.

conducted in the presence and absence of 0.125% human serum albumin (HSA) in order to evaluate the effects of protein binding. The previously reported 3-(*R*)-carbamoyloxy piperidine derivative (**1**) showed excellent potency. However, this compound did not possess adequate solubility for intravenous use (empirically, ca. >100 μg/mL). In order to solve this problem, the attachment of a quaternary ammonium

moiety through a variety of tethers was explored. Although the introduction of a dimethylaminoethyl substituent onto the carbamate slightly reduced the potency, we chose the ethylene linker for this study because the compound **15** still displayed good activity as potentiator of AZT. Moreover, the influence of the addition of HSA was lower than **1**.

The trimethylammonium analogue (**20**) showed almost the same activity as **15**. This compound, when dosed intravenously, proved lethal to 0/3 and 2/3 mice at 50 and 100 mg/kg, respectively. More hydrophilic quaternary compounds were also tested. The acetamide analogue (**21**) displayed an improved safety profile (no deaths at 100 mg/kg), but was less potent than **20**, and its rather low solubility was disappointing. On the other hand, the acetic acid analogue **22** (**D13-9001**) retained good activity in vitro and was not lethal to mice at 100 mg/kg; moreover, its solubility profile was excellent.

The length of the linker was extended in **23**. The potency and solubility were slightly reduced.

Varying the nature and position of the carbamate also changed the biological profile of the analogues. The carba analogues **26** and **31** displayed rather reduced potency, and the introduction of the double bond in **31** reduced the solubility dramatically.

The migration of the carbamate from the 3-position to the 4-position of the piperidine, generating an achiral compound, reduced the activity by 2- to 4-fold. Interestingly, this transformation also led to significant lower solubility (**22** vs **34**).

Because of its high solubility and improved safety characteristics, we chose compound **22** (**D13-9001**) as a prototypical EPI to perform in vivo profiling in combination with AZT, evaluating its potentiation activity in a lethal pneumonia model in rats using the *P. aeruginosa* strain PAM1020.[13] As shown in Figure 2, the combination of 1.25, 5, and 20 mg/kg of **22** (**D13-9001**) with 1000 mg/kg of AZT gave improved survival rates at the end of day seven, whereas no obvious effect was observed on treatment with AZT alone.

Figure 3 displays the serum concentration-time curve observed in rats after the administration of **22** (**D13-9001**) with 1000 mg/kg of AZT by intravenous infusion over 2 h (dose levels 1.25, 5, and 20 mg/kg). Pharmacokinetic parameters are shown in Table 2. The compound exhibits reasonably linear PK characteristics in the dose range from 5 to 20 mg/kg and therapeutic efficacy in vivo was clearly demonstrated at these exposures. The serum levels of AZT were the same with and without co-administration of the EPI (data not shown). In addition, the PK parameters of **22** (**D13-9001**) in monkeys are presented in Table 3. Here again reasonable dose linearity was observed, with serum levels at 5 mg/kg exceeding those in the rat at the same dose. Hence we expect that in vivo efficacy would be obtainable at a lower doses in mammals.

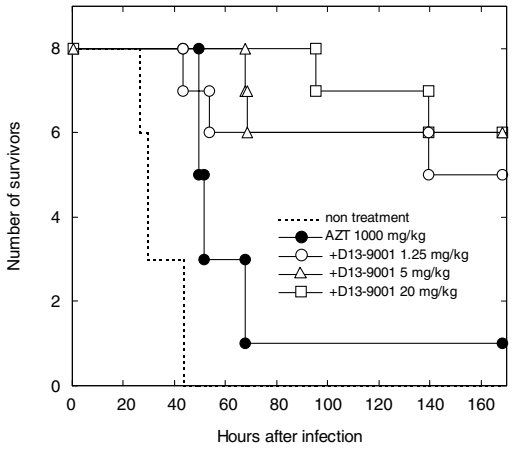


Figure 2. Efficacy of AZT combined with **22** (**D13-9001**) by intravenous infusion in a model of *P. aeruginosa* pneumonia in rats.

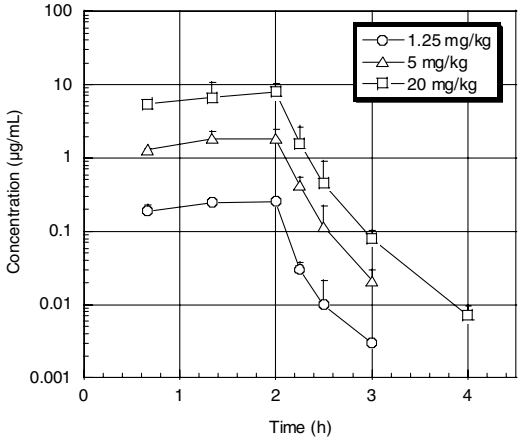


Figure 3. Serum concentration of **22** (**D13-9001**) after intravenous infusion to rats.

4. Conclusions

In summary, we have discovered a series of pyridopyrimidine derivatives, substituted at the 2-position with piperidines bearing quaternary ammonium salt side chains, that are highly water soluble. Of these, ammonium acetic acid analogue **22** (**D13-9001**) exhibited potent efficacy in vivo, high solubility, and a good safety profile in an acute toxicity assay. Additional research is in progress in order to extend to a clinically effective efflux pump inhibitor versus *Pseudomonas aeruginosa*.

5. Experimental

5.1. General

Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. [1-(4-Methoxybenzyl)-1*H*-tetrazol-5-yl]acetic acid (**8**) was prepared according to the literature procedures.[14] Melting points were taken on a Yanako MP-500D melting point apparatus and are uncorrected.

Table 2. PK parameters of **22** (**D13-9001**) after intravenous infusion to rats

Dose (mg/kg)	C_{2h} (μg/mL)	AUC_{∞} (μg·h/mL)	MRT_{∞} (h)	CL_{∞} (mL/min/kg)	$V_{dss\infty}$ (L/kg)	$t_{1/2}$ (t = 2.25−3.0) (h)
1.25	0.262	0.486	1.186	42.84	3.049	0.23
5	1.836	3.529	1.209	23.61	1.712	0.18
20	8.040	14.213	1.215	23.45	1.710	0.25

Linear extrapolation to time 0. Approximated concentration was used in extrapolation to time ∞.

Table 3. PK parameters of **22** (**D13-9001**) after intravenous infusion to monkeys

Dose (mg/kg)	C_{1h} (μg/mL)	AUC_{∞} (μg·h/mL)	MRT_{∞} (h)	CL_{∞} (mL/min/kg)	$V_{dss\infty}$ (L/kg)	$t_{1/2}$ (h)
1	1.224	1.491	0.614	11.23	0.414	0.28 (t = 1.25–2.0)
5	7.635	7.891	0.625	10.70	0.400	0.41 (t = 1.5–3.0)

Linear extrapolation to time 0. Approximated concentration was used in extrapolation to time ∞.

Optical rotations were measured in a 0.5-dm cell at ca. 25 °C at 589 nm with a HORIBA SEPA-300 polarimeter. ^{1}H NMR spectra were determined on a JEOL JNM-EX400 spectrometer. Chemical shifts are reported in parts per million relative to tetramethylsilane as internal standard. Significant ^{1}H NMR data are tabulated in the following order: number of protons, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad), coupling constant(s) in hertz. Infrared (IR) spectra were obtained on a HORIBA FT-720 spectrometer. High-resolution mass spectra (HRMS) were obtained on a JEOL JMS-T100LP mass spectrometer under electron spray ionization conditions (ESI). Elementary analyses were conducted at Research Technology Center, Daiichi Pharmaceutical Co., Ltd. Column chromatography refers to flash column chromatography conducted on Merck silica gel 60, 230–400 mesh ASTM. Thin-layer chromatography (TLC) was performed with Merck silica gel 60 F_{254} TLC plates, and compound visualization was effected with a 5% solution of phosphomolybdic acid in ethanol, UV lamp, or Wako ninhydrin spray. Unless otherwise specified, reactions were carried out at ambient temperature. Combined organic extracts were dried over anhydrous Na_2SO_4. Reagents and solvents were removed from reaction mixture or combined organic extracts by concentration in vacuo using a rotary evaporator with bath at 35–45 °C.

5.2. Synthesis

5.2.1. 2-(Acetylamino)-*N*-(4-*tert*-butyl-1,3-thiazol-2-yl)isonicotinamide (5). To **3** (500 mg, 2.79 mmol) was added dropwise thionyl chloride (5 mL, 68.5 mmol), and the mixture was stirred at 80 °C for 30 min. The mixture was concentrated, and the residue was added to a solution of 4-*tert*-butyl-1,3-thiazol-2-amine (525 mg, 3.34 mmol) in pyridine (0.25 mL)-CH_2Cl_2 (5 mL) at 0 °C. The mixture was stirred at 0 °C for 30 min and further at ambient temperature for 2.5 h. The mixture was diluted with water and extracted with $CHCl_3$. The organic layer was dried and concentrated. The residue was purified by silica gel column chromatography ($CHCl_3 \rightarrow CHCl_3$/MeOH = 80:1 → 50:1) to afford **5** (546 mg) as a white solid. ^{1}H NMR ($CDCl_3$) δ: 1.31 (9H, s), 2.26 (3H, s), 6.61 (1H, s), 7.63 (1H, dd, J = 5.1, 0.5 Hz), 8.44 (1H, d, J = 5.1 Hz), 8.51 (1H, br), 8.72 (1H, s). HRMS (ESI) Calcd for $C_{15}H_{19}N_4O_2S$ ($[M + H]^+$): 319.1229. Found: 319.1191.

5.2.2. 2-Amino-*N*-(4-*tert*-butyl-1,3-thiazol-2-yl)isonicotinamide (6). To a solution of **5** (546 mg, 1.71 mmol) in EtOH (12 mL) was added dropwise conc. HCl (1.2 mL), and the mixture was stirred at 80–90 °C for 1 h. The mixture was concentrated, neutralized with 1 N NaOH aq and extracted with $CHCl_3$. The organic layer was dried and concentrated. The residue was purified by silica gel column chromatography ($CHCl_3 \rightarrow CHCl_3$/MeOH = 60:1 → 20:1) to afford **6** (247 mg) as a white solid. ^{1}H NMR (CD_3OD) δ: 1.33 (9H, s), 6.63 (1H, d, J = 0.7 Hz), 7.05 (1H, t, J = 0.7 Hz), 7.09 (1H, ddd, J = 5.4, 1.5, 0.7 Hz), 8.13 (1H, dd, J = 5.4, 0.7 Hz).

5.2.3. *N*-(4-*tert*-Butyl-1,3-thiazol-2-yl)-2-hydroxy-4-oxo-4*H*-pyrido[1,2-*a*]pyrimidine-8-carboxamide (7). To a suspension of **6** (200 mg, 0.724 mmol) in xylene (20 mL) was added TCPM (370 mg, 0.799 mmol), and the mixture was stirred at 130 °C for 1 h. The mixture was cooled and concentrated. The residue was diluted with $CHCl_3$, and the precipitated solid was collected by filtration, and washed with $CHCl_3$ to afford **7** (209 mg) as an orange solid. ^{1}H NMR (DMSO-d_6) δ: 1.31 (9H, s), 3.45 (2H, m), 6.88 (1H, s), 7.77 (1H, d, J = 7.1 Hz), 8.02 (1H, s), 8.99 (1H, d, J = 7.1 Hz). HRMS (ESI) Calcd for $C_{16}H_{17}N_4O_3S$ ($[M + H]^+$): 345.1021. Found: 345.1013.

5.2.4. *N*-(4-*tert*-Butyl-1,3-thiazol-2-yl)-2-hydroxy-3-{(*E*)-2-[1-(4-methoxybenzyl)-1*H*-tetrazol-5-yl]vinyl}-4-oxo-4*H*-pyrido[1,2-*a*]pyrimidine-8-carboxamide (9). (i) To a solution of DMF (16.9 mL, 218 mmol) in CH_2Cl_2 (500 mL) was added dropwise oxalyl chloride (18.6 mL, 218 mmol) at 0 °C. After the mixture was stirred at 0 °C for 15 min, **7** (25.0 g, 72.6 mmol) was added at 0 °C and stirring was continued for 2 h. The mixture was diluted with satd $NaHCO_3$ aq at 0 °C and acidified to pH 3 with 1 N HCl aq at 0 °C. The precipitated solid was collected by filtration and washed with water to afford *N*-(4-*tert*-butyl-1,3-thiazol-2-yl)-3-formyl-2-hydroxy-4-oxo-4*H*-pyrido[1,2-*a*]pyrimidine-8-carboxamide (15.5 g) as a yellow solid. ^{1}H NMR (DMSO-d_6) δ: 1.31 (9H, s), 6.88 (1H, br s), 7.88–7.92 (2H, m), 9.07 (1H, d, J = 7.8 Hz), 10.06 (1H, s). HRMS (ESI) Calcd for $C_{17}H_{17}N_4O_4S$ ($[M + H]^+$): 373.0971. Found: 373.0998.

ii) *N*-(4-*tert*-Butyl-1,3-thiazol-2-yl)-3-formyl-2-hydroxy-4-oxo-4*H*-pyrido[1,2-*a*]pyrimidine-8-carboxamide (11.6 g, 28.4 mmol) and **8** (10.0 g, 40.3 mmol) were dissolved in pyridine (200 mL)-piperidine (40 mL), and the mixture was refluxed for 3 h. The mixture was cooled and concentrated, and the residue was diluted with 1 N HCl aq and EtOAc. The precipitated solid was collected by filtration, and washed with water and EtOAc to afford **9** (11.3 g) as a red solid. ^{1}H NMR (DMSO-d_6) δ: 1.30 (9H, s), 3.71 (3H, s), 5.57 (2H, s), 6.86 (1H, d, J = 12.0 Hz), 6.92 (1H, d, J = 8.6 Hz), 7.22 (1H, d, J = 8.6 Hz), 7.70 (1H, d, J = 15.9 Hz), 7.93 (1H, d, J = 7.3 Hz), 7.95 (1H, s), 8.05 (1H, d, J = 15.9 Hz), 9.14 (1H, d, J = 7.3 Hz). HRMS (ESI) Calcd for $C_{27}H_{27}N_8O_4S$ ([M + H]$^+$): 559.1876. Found: 559.1928.

5.2.5. *N*-(4-*tert*-Butyl-1,3-thiazol-2-yl)-2-[(3*R*)-3-hydroxypiperidin-1-yl]-3-{(*E*)-2-[1-(4-methoxybenzyl)-1*H*-tetrazol-5-yl]vinyl}-4-oxo-4*H*-pyrido[1,2-a]pyrimidine-8-carboxamide (11). To a suspension of **9** (10.8 g, 19.3 mmol) in MeCN (130 mL)-DMF (65 mL) were added diisopropylethylamine (10.0 mL, 57.4 mmol) and diphenyl chlorophosphate (4.20 mL, 20.3 mmol) at 0 °C. After stirring at 0 °C for 50 min under Ar, **10** (2.00 g, 19.8 mmol) was added at 0 °C, and the mixture was stirred at 80 °C for 1 h. After concentration, the residue was diluted with EtOAc and washed with 1 N HCl aq and satd $NaHCO_3$ aq. The organic layer was dried and concentrated. The residue was purified by silica gel column chromatography (CH_2Cl_2 → $CHCl_3$/EtOAc = 2:1 → 1:1) to afford **11** (10.8 g) as a red amorphous material. ^{1}H NMR ($CDCl_3$) δ: 1.15–1.60 (2H, m), 1.39 (9H, s), 1.67–2.06 (3H, m), 3.36–4.15 (5H, m), 3.77 (3H, s), 5.50 (2H, s), 6.47 (1H, s), 6.90 (2H, d, J = 8.8 Hz), 7.35 (2H, d, J = 8.8 Hz), 7.39–7.45 (1H, m), 7.77 (2H, s), 7.99 (1H, br), 8.80 (1H, d, J = 7.3 Hz). $[\alpha]_D^{24.7}$ −12.4° (c 1.019, $CHCl_3$). HRMS (ESI) Calcd for $C_{32}H_{36}N_9O_4S$ ([M + H]$^+$): 642.2611. Found: 642.2569.

5.2.6. (3*R*)-1-(8-{[(4-*tert*-Butyl-1,3-thiazol-2-yl)amino]carbonyl}-3-{(*E*)-2-[1-(4-methoxybenzyl)-1*H*-tetrazol-5-yl]vinyl}-4-oxo-4*H*-pyrido[1,2-*a*]pyrimidin-2-yl)piperidin-3-yl carbamate (12). To a solution of **11** (100 mg, 0.156 mmol) in EtOAc (3 mL) was added trichloroacetyl isocyanate (74.3 μL, 0.312 mmol) at 0 °C, and the mixture was stirred for 45 min. After concentration, the residue was dissolved in MeOH (2 mL)-H_2O (1 mL) and sodium formate (100 mg, 1.47 mmol) was added. After stirring for 2 h, more sodium formate (100 mg, 1.47 mmol) was added and stirring was continued for 18.5 h. After the mixture was concentrated, the residue was diluted with water and extracted with $CHCl_3$ (3×). The organic layer was dried and concentrated. The residue was purified by PTLC ($CHCl_3$/EtOAc = 1:1) to afford **12** (76.7 mg) as a red solid. ^{1}H NMR ($CDCl_3$) δ: 1.32 (9H, s), 1.64–1.91 (3H, m), 2.04–2.18 (3H, m), 3.16–3.28 (1H, m), 3.45 (1H, d, J = 13.4 Hz), 3.78 (3H, s), 3.82–3.93 (1H, m), 4.00–4.10 (1H, m), 4.95–5.02 (1H, m), 5.51 (2H, s), 6.10 (2H, br s), 6.61 (1H, s), 6.89 (2H, d, J = 8.8 Hz), 7.36 (2H, d, J = 8.8 Hz), 7.57 (1H, dd, J = 7.3, 2.0 Hz), 7.96 (2H, s), 8.09 (1H, d, J = 1.0 Hz), 8.99 (1H, d, J = 7.3 Hz). $[\alpha]_D^{24.7}$ +46.2° (c 1.010, $CHCl_3$). HRMS (ESI) Calcd for $C_{33}H_{37}N_{10}O_5S$ ([M + H]$^+$): 685.2669. Found: 685.2661.

5.2.7. (3*R*)-1-{8-{[(4-*tert*-Butyl-1,3-thiazol-2-yl)amino]carbonyl}-4-oxo-3-[(*E*)-2-(1*H*-tetrazol-5-yl)vinyl]-4*H*-pyrido[1,2-*a*]pyrimidin-2-yl}piperidin-3-yl carbamate (1). To **12** (76.7 mg, 0.112 mmol) were added TFA (1 mL) and anisole (50 μL), and the mixture was stirred at 60 °C for 6 h. The mixture was concentrated and co-evaporated with toluene and the residue was purified by PTLC ($CHCl_3$/MeOH/H_2O = 8:3:0.5) and solidified from EtOH-Et_2O to afford **1** (40.0 mg) as an orange solid. Mp 216–218 °C. IR (ATR) cm^{-1}: 2958, 2866, 1664, 1637, 1608, 1541, 1512, 1439, 1402, 1350, 1311, 1259, 1228, 1103, 1049. ^{1}H NMR (CD_3OD/$CDCl_3$) δ: 1.36 (9H, s), 1.71 (1H, m), 1.97 (3H, m), 3.60 (1H, m), 3.76 (2H, m), 3.89 (1H, m) , 4.87 (1H, s), 6.63 (1H, s), 7.49 (1H, s), 7.62 (1H, d, J = 7.3 Hz), 7.68 (1H, d, J = 15.6 Hz), 7.86 (1H, d, J = 15.6 Hz), 8.12 (1H, s), 8.98 (1H, d, J = 7.3 Hz). $[\alpha]_D^{24.9}$ +8.3° (c 1.011, DMSO). Anal. Calcd for $C_{25}H_{28}N_{10}O_4S{\cdot}0.25Et_2O{\cdot}2.25H_2O$: C, 50.07; H, 5.66; N, 22.46; S, 5.14. Found: C, 49.91; H, 5.04; N, 22.34; S, 5.15.

5.2.8. (3*R*)-1-(8-{[(4-*tert*-Butyl-1,3-thiazol-2-yl)amino]carbonyl}-3-{(*E*)-2-[1-(4-methoxybenzyl)-1*H*-tetrazol-5-yl]vinyl}-4-oxo-4*H*-pyrido[1,2-*a*]pyrimidin-2-yl)piperidin-3-yl[2-(dimethylamino)ethyl]carbamate (13). To a solution of **11** (100 mg, 0.156 mmol) in CH_2Cl_2 was added 1,1′-carbonylbis-1*H*-imidazole (50.5 mg, 0.312 mmol) and the mixture was stirred for 3 h. *N*,*N*-dimethylethylenediamine (85.5 μL, 0.779 mmol) was added and stirring was continued for 68 h. The mixture was diluted with water and extracted with $CHCl_3$ (3×). The organic layer was dried and concentrated. The residue was purified by silica gel column chromatography ($CHCl_3$/MeOH = 98:2 → 95:5) to afford **13** (110 mg) as an orange solid. ^{1}H NMR ($CDCl_3$) δ: 1.35 (9H, s), 1.62–2.32 (5H, m), 2.22 (6H, s), 2.43–2.57 (2H, m), 3.25–3.48 (3H, m), 3.57–3.86 (3H, m), 3.77 (3H, s), 4.84 (1H, br s), 5.50 (2H, s), 5.99 (1H, br s), 6.58 (1H, s), 6.89 (2H, d, J = 8.5 Hz), 7.35 (2H, d, J = 8.5 Hz), 7.47 (1H, d, J = 7.6 Hz), 7.80–7.97 (3H, m), 8.96 (1H, d, J = 7.6 Hz). $[\alpha]_D^{24.8}$ +102.4° (c 1.038, $CHCl_3$). HRMS (ESI) Calcd for $C_{37}H_{46}N_{11}O_5S$ ([M + H]$^+$): 756.3404. Found: 756.3360.

5.2.9. (3*R*)-1-(8-{[(4-*tert*-Butyl-1,3-thiazol-2-yl)amino]carbonyl}-3-{(*E*)-2-[1-(4-methoxybenzyl)-1*H*-tetrazol-5-yl]vinyl}-4-oxo-4*H*-pyrido[1,2-*a*]pyrimidin-2-yl)piperidin-3-yl[3-(dimethylamino)propyl]carbamate (14). Following the procedure described for **13**, the title compound (101 mg) was prepared from **11** (100 mg, 0.159 mmol) as an orange solid. ^{1}H NMR ($CDCl_3$) δ: 1.36 (9H, s), 1.62–2.09 (6H, m), 2.16–2.32 (1H, m), 2.22 (6H, s), 2.39 (2H, t, J = 6.7 Hz), 3.20–3.45 (3H, m), 3.60–3.85 (3H, m), 3.77 (3H, s), 4.82 (1H, br s), 5.50 (2H, s), 6.25–6.37 (1H, m), 6.89 (2H, d, J = 8.8 Hz), 7.23 (1H, br s), 7.35 (2H, d, J = 8.8 Hz), 7.51 (1H, d, J = 7.3 Hz), 7.88–8.01 (3H, m), 8.97 (1H, d, J = 7.3 Hz). $[\alpha]_D^{24.9}$ −78.9° (c 1.008, $CHCl_3$). HRMS (ESI) Calcd for $C_{38}H_{48}N_{11}O_5S$ ([M + H]$^+$): 770.3561. Found: 770.3512.

5.2.10. (3*R*)-1-{8-{[(4-*tert*-Butyl-1,3-thiazol-2-yl)amino]carbonyl}-4-oxo-3-[(*E*)-2-(1*H*-tetrazol-5-yl)vinyl]-4*H*-pyrido[1,2-*a*]pyrimidin-2-yl}piperidin-3-yl [2-(dimethylamino)ethyl]carbamate (15). Following the procedure described for **1**, the title compound (43.8 mg) was prepared from **13** (270 mg, 0.357 mmol) as an orange solid. Mp 194–200 °C. IR (ATR) cm^{-1}: 2954, 2860, 1711, 1658, 1635, 1618, 1512, 1425, 1379, 1309, 1248, 1225, 1151, 1101, 1068. ^1H NMR (CD_3OD) δ: 1.35 (9H, s), 1.71 (1H, br s), 1.88–1.92 (1H, m), 2.00 (2H, br s), 2.76 (6H, s), 3.06–3.07 (2H, m), 3.30–3.56 (5H, m), 3.88–3.91 (2H, m), 6.74 (1H, s), 7.55–7.59 (2H, m), 7.97 (1H, d, J = 16.4 Hz), 8.02 (1H, s), 8.97 (1H, d, J = 7.6 Hz). $[\alpha]_D^{24.9}$ −210.1° (*c* 1.025, DMSO). Anal. Calcd for $C_{29}H_{37}N_{11}O_4S{\cdot}0.5Et_2O{\cdot}2.75H_2O$: C, 51.54; H, 6.63; N, 21.33; S, 4.44. Found: C, 51.66; H, 6.10; N, 21.35; S, 4.43.

5.2.11. 5-[(*E*)-2-(8-{[(4-*tert*-Butyl-1,3-thiazol-2-yl)amino]carbonyl}-4-oxo-2-{(3*R*)-3-[({[2-(trimethylammonio)ethyl]amino}carbonyl)oxy]piperidin-1-yl}-4*H*-pyrido[1,2-*a*]pyrimidin-3-yl)vinyl]tetrazol-1-ide (20). To a solution of **13** (100 mg, 0.1323 mmol) in DMF (5 mL) was added MeI (500 μL) and the solution was stirred at 5 °C for 67 h. After the mixture was concentrated, the residue was purified by PTLC ($CHCl_3$/MeOH/H_2O = 8:3:0.5) to afford crude **16**. To this were added TFA (1 mL) and anisole (50 μL), and the solution was stirred at 60 °C for 2.5 h. After the mixture was concentrated, the residue was purified by PTLC ($CHCl_3$/MeOH/H_2O = 8:3:0.5) and solidified from Et_2O to afford **20** (14.7 mg) as an orange solid. Mp 159–169 °C. IR (ATR) cm^{-1}: 2962, 1664, 1637, 1512, 1423, 1365, 1309, 1250, 1201, 1174, 1124, 1080, 1043. ^1H NMR (CD_3OD) δ: 1.35 (9H, s), 1.70 (1H, br), 1.97 (3H, br), 3.16 (9H, s), 3.42 (3H, m), 3.55 (3H, m), 3.93 (2H, m), 4.89 (1H, s), 6.75 (1H, s), 7.56 (1H, d, J = 16.1 Hz), 7.60 (1H, dd, J = 7.3, 1.2 Hz), 7.96 (1H, d, J = 16.1 Hz), 8.03 (1H, s), 8.99 (1H, d, J = 7.3 Hz). $[\alpha]_D^{25.0}$ −211.0° (*c* 1.018, DMSO). Anal. Calcd for $C_{30}H_{39}N_{11}O_4S{\cdot}1.2CF_3COOH{\cdot}2.9H_2O$: C, 46.39; H, 5.53; N, 18.37; F, 8.15; S, 3.82. Found: C, 46.70; H, 5.19; N, 18.00; F, 7.92; S, 3.82.

5.2.12. 5-[(*E*)-2-(2-((3*R*)-3-{[({2-[(2-Amino-2-oxoethyl)(dimethyl)ammonio]ethyl}amino)carbonyl]oxy}piperidin-1-yl)-8-{[(4-*tert*-butyl-1,3-thiazol-2-yl)amino]carbonyl}-4-oxo-4*H*-pyrido[1,2-*a*]pyrimidin-3-yl)vinyl]tetrazol-1-ide (21). To a solution of **13** (3.60 g, 4.76 mmol) in DMF (30 mL) was added 2-iodoacetamide (1.00 g, 5.41 mmol) and the mixture was stirred for 17 h and concentrated to afford crude **17**. To the obtained **17** were added TFA (160 mL) and anisole (5 mL), and the solution was stirred at 60 °C for 3 h. After the mixture was concentrated, the residue was purified by silica gel column chromatography ($CHCl_3$/MeOH/H_2O = 70:26:4) to afford **21** (825 mg) as an orange solid. Mp 185–193 °C. IR (ATR) cm^{-1}: 3186, 2958, 1695, 1660, 1637, 1624, 1542, 1516, 1425, 1379, 1311, 1250, 1227, 1153, 1103. ^1H NMR (CD_3OD) δ: 1.35 (9H, s), 1.71 (1H, m), 1.98 (3H, m), 3.32 (6H, s), 3.63 (6H, m), 3.89 (2H, m), 4.17 (2H, m), 4.80 (1H, m), 6.73 (1H, s), 7.52 (1H, d, J = 16.1 Hz), 7.57 (1H, d, J = 7.3 Hz), 7.94 (1H, d, J = 16.1 Hz), 8.00 (1H, s), 8.96 (1H, d, J = 7.3 Hz). $[\alpha]_D^{24.9}$ −150.9° (*c* 1.005, DMSO). Anal. Calcd for $C_{31}H_{40}N_{12}O_5S{\cdot}TFA{\cdot}2H_2O$: C, 50.24; H, 6.13; N, 21.31. Found: C, 50.40; H, 6.10; N, 21.06.

5.2.13. [[2-({[((3*R*)-1-{8-{[(4-*tert*-Butyl-1,3-thiazol-2-yl)amino]carbonyl}-4-oxo-3-[(*E*)-2-(1*H*-tetrazol-5-yl)vinyl]-4*H*-pyrido[1,2-*a*]pyrimidin-2-yl}piperidin-3-yl)oxy]carbonyl}amino)ethyl](dimethyl)ammonio]acetate (22 = D13-9001). To a solution of **13** (405 mg, 0.536 mmol) in DMF (8 mL) was added bromoacetic acid *tert*-butyl ester (0.396 mL, 2.67 mmol). The solution was stirred for 16 h and concentrated to afford crude **18**. To the obtained **18** was added 4 N HCl-dioxane (10 mL) and stirring was continued for 9 h. After the mixture was concentrated, the residue was purified with PTLC ($CHCl_3$/MeOH/H_2O = 8:3:1). The fraction was dissolved with TFA (10 mL)-anisole (0.5 mL) and stirred at 60 °C for 2 h. After the mixture was concentrated, the residue was purified by PTLC ($CHCl_3$/MeOH/H_2O = 8:3:1). The obtained fraction was dissolved with MeOH-H_2O and basified to pH = 8 by 0.1 N NaOH aq. The mixture was purified by HPLC (SHISEIDO CAPCELL PAK C18, SG-120A, 30 × 250 mm, MeOH:H_2O = 60:40) to afford **22** (103 mg) as an orange solid. Mp 220–233 °C. IR (ATR) cm^{-1}: 2958, 1700, 1650, 1625, 1511, 1423, 1247, 1103. ^1H NMR (CD_3OD) δ: 1.29 (9H, s), 1.69 (1H, m), 1.94 (2H, m), 2.03 (1H, m), 3.26 (6H, s), 3.44–3.71 (6H, m), 3.84 (2H, s), 3.92 (1H, m), 4.06 (1H, m), 6.71 (1H, s), 7.45 (1H, d, J = 15.9 Hz), 7.64 (1H, dd, J = 1.7, 7.6 Hz), 7.94 (1H, d, J = 15.9 Hz), 8.04 (1H, d, J = 1.7 Hz), 8.96 (1H, d, J = 7.6Hz). $[\alpha]_D^{24.9}$ +55.6° (*c* 1.012, DMSO). Anal. Calcd for $C_{31}H_{39}N_{11}O_6S{\cdot}6H_2O$: C, 46.43; H, 6.41; N, 19.21. Found: C, 46.48; H, 5.86; N, 18.66.

5.2.14. [[3-({[((3*R*)-1-{8-{[(4-*tert*-Butyl-1,3-thiazol-2-yl)amino]carbonyl}-4-oxo-3-[(*E*)-2-(1*H*-tetrazol-5-yl)vinyl]-4*H*-pyrido[1,2-*a*]pyrimidin-2-yl}piperidin-3-yl)oxy]carbonyl}amino)propyl](dimethyl)ammonio]acetate (23). Following the procedure described for **22**, the title compound (232 mg) was prepared from **14** (277 mg, 0.360 mmol) as an orange solid. Mp 157–163 °C. IR (ATR) cm^{-1}: 2970, 2868, 1674, 1637, 1541, 1508, 1439, 1252, 1205, 1178, 1128, 1039. ^1H NMR (DMSO-d_6) δ: 1.31 (9H, s), 1.66–1.93 (6H, m), 2.94–3.74 (16H, m), 4.65 (1H, m), 6.85 (1H, s), 7.47 (1H, d, J = 16.3 Hz), 7.62 (1H, d, J = 7.3 Hz), 7.76 (1H, br), 7.87 (1H, d, J = 16.3 Hz), 8.24 (1H, s), 8.96 (1H, d, J = 7.3 Hz). $[\alpha]_D^{25.0}$ −33.5° (*c* 1.039, DMSO). Anal. Calcd for $C_{32}H_{41}N_{11}O_6S{\cdot}6H_2O{\cdot}3CF_3COOH$: C, 39.41; H, 4.87; N, 13.31. Found: C, 39.12; H, 4.43; N, 13.51.

5.2.15. Methyl [(3*S*)-1-(8-{[(4-*tert*-butyl-1,3-thiazol-2-yl)amino]carbonyl}-3-{(*E*)-2-[1-(4-methoxybenzyl)-1*H*-tetrazol-5-yl]vinyl}-4-oxo-4*H*-pyrido[1,2-*a*]pyrimidin-2-yl)piperidin-3-yl]acetate (25). To a solution of **9** (5.00 g, 8.95 mmol) in MeCN (60 mL)-DMF(30 mL) were added diisopropylethylamine (6.24 mL, 35.8 mmol) and diphenyl chlorophosphate (2.05 mL, 9.85 mmol) at 0 °C. After stirring at 0 °C for 1 h under Ar, **24** (2.25 g, 11.6 mmol) was added at 0 °C, and stirring was continued at 80 °C for 1 h. After the mixture was concentrated, the residue was diluted with $CHCl_3$ and washed with 5% citric acid aq satd $NaHCO_3$ aq and brine. The organic layer was dried and concentrated.

The residue was purified by silica gel column chromatography ($CHCl_3 \rightarrow CHCl_3$/MeOH = 100:1 → 50:1) to afford **25** (5.25 g) as a red solid. ^{1}H NMR ($CDCl_3$) δ: 1.17–1.29 (1H, m), 1.40 (9H, s), 1.51–1.73 (2H, m), 1.81–1.92 (1H, m), 2.01–2.35 (3H, m), 2.74–2.87 (1H, m), 2.95–3.09 (1H, m), 3.58 (3H, s), 3.79 (3H, s), 3.85–3.94 (1H, m), 3.99–4.10 (1H, m), 5.52 (2H, s), 6.56 (1H, s), 6.91 (2H, d, J = 8.8 Hz), 7.30–7.40 (1H, m), 7.38 (2H, d, J = 8.8 Hz), 7.74 (2H, d, J = 15.4 Hz), 7.76–7.79 (1H, m), 7.89 (1H, d, J = 15.4 Hz), 8.81 (1H, d, J = 6.1 Hz). $[\alpha]_D^{24.8}$ +16.5° (c 1.014, $CHCl_3$). HRMS (ESI) Calcd for $C_{35}H_{40}N_9O_5S$ ($[M + H]^+$): 698.2873. Found: 689.2859.

5.2.16. [(2-{[((3*S*)-1-{8-{[(4-*tert*-Butyl-1,3-thiazol-2-yl)amino]carbonyl}-4-oxo-3-[(*E*)-2-(1*H*-tetrazol-5-yl)vinyl]-4*H*-pyrido[1,2-*a*]pyrimidin-2-yl}piperidin-3-yl)acetyl]amino}ethyl)(dimethyl)ammonio]acetate (26). (i) To a solution of **25** (5.25 g, 7.52 mmol) in THF (23 mL)-MeOH (23 mL) was added 1 N NaOH aq (22.6 mL, 22.6 mmol) and the mixture was stirred for 80 min. After concentration, the residue was diluted with water. The aqueous phase was acidified to pH 1–2 with 1 N HCl aq and extracted with $CHCl_3$. The organic layer was dried and concentrated. The residue was purified by silica gel column chromatography ($CHCl_3 \rightarrow CHCl_3$/MeOH = 100:1 → 50:1) to afford [(3*S*)-1-(8-{[(4-*tert*-butyl-1,3-thiazol-2-yl)amino]carbonyl}-3-{(*E*)-2-[1-(4- methoxybenzyl)-1*H*-tetrazol-5-yl]vinyl}-4-oxo-4*H*-pyrido[1,2- *a*]pyrimidin-2-yl)piperidin-3-yl]acetic acid (4.20 g) as a red solid. ^{1}H NMR ($CDCl_3$) δ: 1.19–1.43 (1H, m), 1.38 (9H, s), 1.59–1.77 (1H, m), 1.90 (1H, d, J = 13.4 Hz), 2.07 (1H, d, J = 12.0 Hz), 2.27 (1H, dd, J = 13.4, 12.0 Hz), 2.47–2.61 (1H, m), 2.68 (1H, dd, J = 13.7, 3.2 Hz), 2.87–3.01 (2H, m), 3.77 (3H, s), 4.28 (1H, d, J = 12.0 Hz), 4.42 (1H, d, J = 13.7 Hz), 5.11 (1H, d, J = 15.1 Hz), 5.32 (1H, d, J = 15.1 Hz), 6.63 (1H, s), 6.85 (2H, d, J = 8.5 Hz), 7.22 (2H, d, J = 8.5 Hz), 7.32 (1H, d, J = 15.4 Hz), 7.48 (1H, d, J = 15.4 Hz), 7.53 (1H, dd, J = 7.3, 1.7 Hz), 8.00 (1H, d, J = 1.7 Hz), 9.01 (1H, d, J = 7.3 Hz). $[\alpha]_D^{24.8}$ +773.9° (c 1.008, $CHCl_3$). HRMS (ESI) Calcd for $C_{34}H_{38}N_9O_5S$ ($[M + H]^+$): 684.2717. Found: 684.2746.

(ii) To a solution of [(3*S*)-1-(8-{[(4-*tert*-butyl-1,3-thiazol-2-yl)amino]carbonyl}-3-{(*E*)-2-[1-(4-methoxybenzyl)-1*H*-tetrazol-5-yl]vinyl}-4-oxo-4*H*-pyrido[1,2-*a*]pyrimidin-2-yl)piperidin-3-yl]acetic acid (2.00 g, 2.92 mmol) and *N*,*N*-ethylenediamine (482 μL, 4.39 mmol) in CH_2Cl_2 (15 mL)-DMF (15 mL) were added HOBt (790 mg, 5.85 mmol) and EDC·HCl (1.12 g, 5.85 mmol) at 0 °C and stirred for 18.5 h. The mixture was diluted with satd $NaHCO_3$ aq and extracted with $CHCl_3$ (3×). The organic layer was dried and concentrated. The residue was purified by silica gel column chromatography ($CHCl_3$/MeOH = 95:5 → 90:10) to afford *N*-(4-*tert*-butyl-1,3-thiazol-2-yl)-2-[(3*S*)-3-(2-{[2-(dimethylamino)ethyl]-amino}-2-oxoethyl)piperidin-1-yl]-3-{(*E*)-2-[1-(4-methoxybenzyl)-1*H*-tetrazol-5-yl]vinyl}-4-oxo-4*H*-pyrido[1,2-*a*]pyrimidine-8-carboxamide (2.09 mg) as an orange solid. ^{1}H NMR ($CDCl_3$) δ: 1.17–1.44 (1H, m), 1.35 (9H, s), 1.54–1.78 (2H, m), 1.89–2.00 (1H, m), 2.11–2.35 (3H, m), 2.25 (6H, s), 2.53–2.60 (2H, m), 2.96 (1H, dd, J = 13.1, 9.4 Hz), 3.13–3.25 (1H, m), 3.38–3.54 (2H, m), 3.77 (3H, s), 3.98 (1H, d, J = 12.9 Hz), 4.13 (1H, d, J = 12.9 Hz), 5.51 (2H, s), 6.56 (1H, s), 6.85–6.92 (1H, m), 6.89 (2H, d, J = 8.8 Hz), 7.35 (2H, d, J = 8.8 Hz), 7.49 (1H, dd, J = 7.3, 1.6 Hz), 7.67 (1H, d, J = 15.4 Hz), 7.84 (1H, d, J = 15.4 Hz), 7.88 (2H, d, J = 1.6 Hz), 8.92 (1H, d, J = 7.3 Hz). $[\alpha]_D^{24.9}$ +18.7° (c 1.028, $CHCl_3$). HRMS (ESI) Calcd for $C_{38}H_{48}N_{11}O_4S$ ($[M + H]^+$): 754.3611. Found: 754.3565.

(iii) Following the procedure described for **22**, the title compound (48.6 mg) was prepared from *N*-(4-*tert*-butyl-1,3-thiazol-2-yl)-2-[(3*S*)-3-(2-{[2-(dimethylamino) ethyl]-amino}-2-oxoethyl)piperidin-1-yl]-3-{(*E*)-2-[1-(4-methoxybenzyl)-1*H*-tetrazol-5-yl]vinyl}-4-oxo-4*H*-pyrido[1,2-*a*]pyrimidine-8-carboxamide (550 mg, 0.730 mmol) as an orange solid. Mp 225–230 °C. IR (ATR) cm^{-1}: 2960, 1631, 1514, 1439, 1377, 1309, 1254, 1227, 1101. ^{1}H NMR (CD_3OD) δ: 1.34 (9H, s), 1.75–1.80 (2H, m), 1.92 (1H, br), 2.19–2.23 (3H, m), 2.91–2.97 (1H, m), 3.18–3.34 (8H, m), 3.54 (2H, br), 3.70–3.71 (2H, m), 3.87 (2H, br), 4.06–4.09 (2H, m), 6.72 (1H, s), 7.47 (1H, d, J = 15.7 Hz), 7.56 (1H, d, J = 7.2 Hz), 7.82 (1H, d, J = 15.7 Hz), 7.96 (1H, s), 8.92 (1H, d, J = 7.2 Hz). $[\alpha]_D^{24.8}$ −27.2° (c 0.264, DMSO). Anal. Calcd for $C_{32}H_{41}N_{11}O_5S{\cdot}0.5HCO_2H{\cdot}H_2O$: C, 53.27; H, 6.05; N, 21.02. Found: C, 53.25; H, 6.36; N, 20.63.

5.2.17. (3*E*)-3-(2-Ethoxy-2-oxoethylidene)piperidine hydrochloride (28). (i) To a suspension of NaH (55% in liquid paraffin, 2.59 g, 64.9 mmol) in THF (300 mL) was added ethyl diethylphosphonoacetate (11.8 mL, 59.5 mmol) at 0 °C under N_2, and the mixture was stirred for 20 min. To it was added a solution of **27** (15.0 g, 54.1 mmol) in THF (100 mL) at 0 °C and stirring was continued for 45 min. The mixture was diluted with water and concentrated to remove the THF. The residue was diluted with EtOAc and washed with water and brine. The organic layer was dried and concentrated. The residue was purified by silica gel column chromatography (EtOAc/hexane = 10:1 → 4:1) to afford *tert*-butyl (3*Z*)-3-(2-ethoxy-2-oxoethylidene)piperidine-1-carboxylate (5.36 g) as a white solid and *tert*-butyl (3*E*)-3-(2-ethoxy-2-oxoethylidene)piperidine-1-carboxylate (8.86 g) as a colorless oil. *tert*-Butyl (3*Z*)-3-(2-ethoxy-2-oxoethylidene)piperidine-1-carboxylate: ^{1}H NMR ($CDCl_3$) δ: 1.29 (1H, t, J = 7.2 Hz), 1.45 (9H, s), 1.71–1.76 (2H, m), 2.32–2.36 (2H, m), 3.49 (2H, d, J = 5.7 Hz), 4.17 (2H, q, J = 7.2 Hz), 4.62 (2H, s), 5.66 (1H, s). HRMS (ESI) Calcd for $C_{14}H_{24}NO_4$ ($[M + H]^+$): 270.1705. Found: 270.1686. *tert*-Butyl (3*E*)-3-(2-ethoxy-2-oxoethylidene)piperidine-1-carboxylate: ^{1}H NMR ($CDCl_3$) δ: 1.28 (1H, t, J = 7.1 Hz), 1.45 (9H, s), 1.67–1.73 (2H, m), 2.92–2.96 (2H, m), 3.48 (2H, d, J = 5.6 Hz), 3.94 (2H, br), 4.16 (2H, q, J = 7.1 Hz), 5.74 (1H, br). HRMS (ESI) Calcd for $C_{14}H_{24}NO_4$ ($[M + H]^+$): 270.1705. Found: 270.1688.

(ii) To *tert*-butyl (3*E*)-3-(2-ethoxy-2-oxoethylidene) piperidine-1-carboxylate (8.86 g, 32.9 mmol) was added 4 N HCl-dioxane (165 mL) and the mixture was stirred for 1 h prior to concentration and co-evaporation with toluene to afford **28** (6.27 g) as white solid. ^{1}H NMR ($CDCl_3$) δ: 1.28 (1H, t, J = 7.2 Hz), 2.00–2.05 (2H, m),

3.03 (2H, t, J = 6.1 Hz), 3.30 (2H, br), 3.72 (2H, s), 4.17 (2H, q, J = 7.2 Hz), 5.95 (1H, s). HRMS (ESI) Calcd for $C_9H_{16}NO_2$ ([M + H]$^+$): 170.1181. Found: 170.1168.

5.2.18. [(2-{[(2*E*)-2-(1-{8-{[(4-*tert*-Butyl-1,3-thiazol-2-yl)amino]carbonyl}-4-oxo-3-[(*E*)-2-(1*H*-tetrazol-5-yl)vinyl]-4*H*-pyrido[1,2-*a*]pyrimidin-2-yl}piperidin-3-ylidene)acetyl]amino}ethyl)(dimethyl)ammonio]acetate (31). (i) Following the procedure described for **25** and **26**, (2*E*)-[1-(8-{[(4-*tert*-butyl-1,3-thiazol-2-yl)amino]carbonyl}-3-{(*E*)-2-[1-(4-methoxybenzyl)-1*H*-tetrazol-5-yl]vinyl}-4-oxo-4*H*-pyrido[1,2-*a*]pyrimidin-2-yl)piperidin-3-ylidene]acetic acid (1.41 g) was prepared from **9** (5.00 g) as a red solid. ^{1}H NMR ($CDCl_3$) δ: 1.37 (9H, s), 1.94 (2H, br s), 2.32 (2H, br s), 3.25 (2H, br s), 3.75 (3H, s), 3.85 (2H, br s), 5.44 (2H, s), 6.65 (1H, s), 6.86 (2H, d, J = 8.5 Hz), 7.02 (1H, s), 7.25–7.37 (1H, m), 7.30 (2H, d, J = 8.5 Hz), 7.54 (1H, d, J = 7.3 Hz), 7.77 (2H, s), 8.31 (1H, s), 8.91 (1H, d, J = 7.3 Hz). HRMS (ESI) Calcd for $C_{34}H_{36}N_9O_5S$ ([M + H]$^+$): 682.2560. Found: 682.2522.

(ii) Following the procedure described for **26**, *N*-(4-*tert*-butyl-1,3-thiazol-2-yl)-2-[(3*E*)-3-(2-{[2-(dimethylamino)ethyl]amino}-2-oxoethylidene)piperidin-1-yl]-3-{(*E*)-2-[1-(4-methoxybenzyl)-1*H*-tetrazol-5-yl]vinyl}-4-oxo-4*H*-pyrido[1,2-*a*]pyrimidine-8-carboxamide (1.36 g) was prepared from (2*E*)-[1-(8-{[(4-*tert*-butyl-1,3-thiazol-2-yl)amino]carbonyl}-3-{(*E*)-2-[1-(4-methoxybenzyl)-1*H*-tetrazol-5-yl]vinyl}-4-oxo-4*H*-pyrido[1,2-*a*]pyrimidin-2-yl)piperidin-3-ylidene]acetic acid (1.41 g, 2.07 mmol) as an orange solid. ^{1}H NMR ($CDCl_3$) δ: 1.36 (9H, s), 1.89–2.01 (2H, m), 2.14–2.31 (3H, m), 2.24 (6H, s), 2.52 (2H, t, J = 6.3 Hz), 2.99 (2H, s), 3.43–3.58 (2H, m), 3.71–3.79 (2H, m), 3.77 (3H, s), 5.51 (2H, s), 6.57 (1H, s), 6.65 (1H, br s), 6.89 (2H, d, J = 8.8 Hz), 7.03–7.11 (1H, m), 7.36 (2H, d, J = 8.8 Hz), 7.47 (1H, dd, J = 7.3, 2.0 Hz), 7.81–7.90 (3H, m), 8.84–8.91 (1H, m). HRMS (ESI) Calcd for $C_{38}H_{46}N_{11}O_4S$ ([M + H]$^+$): 7523455. Found: 752.3437.

(iii) Following the procedure described for **26**, **31** (490 mg) was prepared from *N*-(4-*tert*-butyl-1,3-thiazol-2-yl)-2-[(3*E*)-3-(2-{[2-(dimethylamino)ethyl]amino}-2-oxoethylidene)piperidin-1-yl]-3-{(*E*)-2-[1-(4-methoxybenzyl)-1*H*-tetrazol-5-yl]vinyl}-4-oxo-4*H*-pyrido[1,2-*a*]pyrimidine-8-carboxamide (1.36 g, 1.81 mmol) as an orange solid. Mp 225–230 °C. IR (ATR) cm^{-1}: 3354, 3334, 2958, 1660, 1624, 1585, 1506, 1462, 1423, 1387, 1333, 1296, 1234, 1205, 1103, 1049. ^{1}H NMR (DMSO-d_6) δ: 1.31 (9H, s), 2.04 (2H, m), 2.43 (2H, m), 3.16–3.17 (2H, m), 3.24 (2H, s), 3.53–3.55 (2H, m), 3.69 (3H, s), 3.81 (2H, m), 3.92 (2H, m), 4.08 (2H, s), 5.93 (1H, s), 6.87 (1H, s), 7.56 (1H, d, J = 9.1 Hz), 7.82 (1H, d, J = 15.7 Hz), 8.05 (1H, br), 8.11–8.14 (2H, m), 8.94 (1H, d, J = 7.4 Hz). Anal. Calcd for $C_{32}H_{39}N_{11}O_5S{\cdot}0.1HCl{\cdot}0.1CF_3CO_2H{\cdot}3.5H_2O$: C, 50.37; H, 6.06; Cl, 0.46; F, 0.74; N, 20.06; S, 4.18. Found: C, 50.42; H, 5.67; Cl, 0.50; F, 0.71; N, 19.83; S, 4.25.

5.2.19. *N*-(4-*tert*-Butyl-1,3-thiazol-2-yl)-2-(4-hydroxypiperidin-1-yl)-3-{(*E*)-2-[1-(4-methoxybenzyl)-1*H*-tetrazol-5-yl]vinyl}-4-oxo-4*H*-pyrido[1,2-*a*]pyrimidine-8-carboxamide (33). Following the procedure described for **11**, the title compound (368 mg) was prepared from **9** (500 mg, 0.895 mmol) as an orange solid. ^{1}H NMR ($CDCl_3$) δ: 1.34 (9H, s), 1.55–1.90 (3H, m), 2.06–2.20 (2H, m), 3.33–3.61 (2H, m), 3.78 (3H, s), 3.91–4.25 (3H, m), 5.53 (2H, s), 6.60 (1H, s), 6.90 (2H, d, J = 8.8 Hz), 7.25–7.27 (1H, m), 7.37 (2H, d, J = 8.8 Hz), 7.51 (1H, d, J = 7.3 Hz), 7.75 (1H, d, J = 15.4 Hz), 7.88–7.90 (1H, m), 7.92 (1H, d, J = 15.4 Hz), 8.96 (1H, d, J = 7.3 Hz). HRMS (ESI) Calcd for $C_{32}H_{36}N_9O_4S$ ([M + H]$^+$): 642.2611. Found: 642.2584.

5.2.20. [[2-({[(1-{8-{[(4-*tert*-Butyl-1,3-thiazol-2-yl)amino]carbonyl}-4-oxo-3-[(*E*)-2-(1*H*-tetrazol-5-yl)vinyl]-4*H*-pyrido[1,2-*a*]pyrimidin-2-yl}piperidin-4-yl)oxy]carbonyl}amino)ethyl](dimethyl)ammonio]acetate (34). (i) Following the procedure described for **13**, 1-(8-{[(4-*tert*-butyl-1,3-thiazol-2-yl)amino]carbonyl}-3-{(*E*)-2-[1-(4-methoxybenzyl)-1*H*-tetrazol-5-yl]vinyl}-4-oxo-4*H*-pyrido[1,2-*a*]pyrimidin-2-yl)piperidin-4-yl [2-(dimethylamino)ethyl]carbamate (103 mg) was prepared from **33** (100 mg, 0.159 mmol) as an orange solid. ^{1}H NMR ($CDCl_3$) δ: 1.40 (9H, s), 1.64–1.78 (2H, m), 1.87–2.02 (2H, m), 2.24 (6H, s), 2.43 (2H, t, J = 5.5 Hz), 3.28 (2H, q, J = 5.5 Hz), 3.37–3.48 (2H, m), 3.71-3.82 (2H, m), 3.79 (3H, s), 4.81–4.99 (1H, m), 5.29–5.39 (1H, m), 5.53 (2H, s), 6.59 (1H, s), 6.92 (2H, d, J = 8.5 Hz), 7.16 (1H, s), 7.35–7.42 (1H, m), 7.38 (2H, d, J = 8.5 Hz), 7.75 (1H, d, J = 15.6 Hz), 7.87–7.90 (1H, m), 7.91 (1H, d, J = 15.6 Hz), 8.85 (1H, d, J = 7.3 Hz). HRMS (ESI) Calcd for $C_{37}H_{46}N_{11}O_5S$ ([M + H]$^+$): 756.3404. Found: 756.3374.

(ii) Following the procedure described for **22**, the title compound (122 mg) was prepared from 1-(8-{[(4-*tert*-butyl-1,3-thiazol-2-yl)amino]carbonyl}-3-{(*E*)-2-[1-(4-methoxybenzyl)-1*H*-tetrazol-5-yl]vinyl}-4-oxo-4*H*-pyrido[1,2-*a*]pyrimidin-2-yl)piperidin-4-yl[2-(dimethylamino)ethyl]carbamate (495 mg, 0.655 mmol) as an orange solid. Mp 211–227 °C. IR (ATR) cm^{-1}: 2960, 2862, 1701, 1655, 1630, 1514, 1444, 1421, 1383, 1309, 1263, 1220, 1149, 1101, 1030. ^{1}H NMR (CD_3OD) δ: 1.35 (9H, s), 1.87 (2H, br), 2.07 (2H, br), 3.29 (6H, s), 3.57 (4H, m), 3.73 (2H, m), 3.87 (4H, m), 4.91 (1H, br), 6.70 (1H, s), 7.40 (1H, d, J = 16.1 Hz), 7.64 (1H, dd, J = 7.3, 1.7 Hz), 7.93 (1H, d, J = 16.1 Hz), 8.04 (1H, s), 8.96 (1H, d, J = 7.3 Hz). Anal. Calcd for $C_{31}H_{39}N_{11}O_6S{\cdot}1.75H_2O{\cdot}0.25Et_2O$: C, 51.67; H,6.10; N,20.71; S, 4.31. Found: C, 51.50; H, 5.81; N, 20.66; S, 4.31.

5.3. In vitro potentiation activity

MIC assays against *P. aeruginosa* utilized Mueller–Hinton broth, following the broth microdilution methodology outlined by the National Committee for Clinical Laboratory Standards (NCCLS; now CLSI). Bacteria were inoculated at 1×10^6 CFU/mL and incubated at 37 °C for 18 h. MICs were determined by visual observation of growth.

5.4. In vivo efficacy

Pulmonary infection of SD rats by *P. aeruginosa* PAM1020 was instilled by intratracheal inoculation of bacteria enmeshed in agar beads. Two hours after bacte-

rial challenge, rats were treated with AZT (1000 mg/kg/2 h) or AZT+ABS-EPI (ABS-EPI were dosed 1.25, 5 and 20 mg/kg/2 h) by intravenous drip infusion.

5.5. Pharmacokinetic studies in rats

Male Sprague–Dawley rats (Slc:SD, 6–8 weeks of age, Japan SLC, Inc., Shizuoka, Japan) were used. Each group consisted of three animals. ABS-EPI and AZT were administered by intravenous infusion at doses of 1.25, 5, and 20 mg/kg and 1000 mg/kg in 40 mL/kg 0.0005 N NaOH-saline solution over 2 h. Blood samples (0.25 mL) were collected at 40, 80, and 120 min after the beginning of the infusion and at 0.25, 0.5, 1, 2, and 4 h after the end of the infusion. Blood samples were centrifuged (10,000 rpm, 10 min, 4 °C) and the plasma samples were subsequently stored at −20 °C until analyzed. The mixture of the plasma (100 μL) and propranolol solution (10 μL, 500 ng/mL in H_2O, used as internal standard) was extracted with MeCN (400 μL) followed by centrifugation (12,000 rpm, 10 min, 4 °C). The supernatant was evaporated to dryness under nitrogen and the dried extract was re-dissolved in MeOH/H_2O (100 μL, 30/70, v/v). The mixture was re-centrifuged (12,000 rpm, 10 min, 4 °C) and the supernatant was analyzed by LC/MS/MS.

5.6. Pharmacokinetic studies in monkeys

Female cynomolgus monkeys (4–5 years age, Toyota Tsusho Co., Tokyo, Japan) were used. Each group consisted of 2–3 animals. ABS-EPI was administered by intravenous infusion at doses of 1 and 5 mg/kg in 10 mL/kg saline solution over 1 h. Blood samples (1 mL) were collected at 0.25, 0.5, 0.75, 1, 1.083, 1.25, 1.5, 2, 3, and 5 h after the administration. These analytical samples were prepared and analyzed according to the procedure detailed above.

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