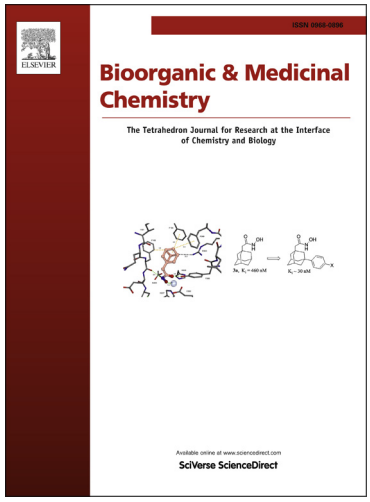

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Synthesis and biological evaluation of 5-carbamoyl-2-phenylpyrimidine derivatives as novel and potent PDE4 inhibitors

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Abstract

5-Carbamoyl-2-phenylpyrimidine derivative **2** has been identified as a phosphodiesterase 4 (PDE4) inhibitor with moderate PDE4B inhibitory activity (IC_{50} = 200 nM). Modification of the carboxylic acid moiety of **2** gave *N*-neopentylacetamide derivative **10f**, which had high *in vitro* PDE4B inhibitory activity (IC_{50} = 8.3 nM) and *in vivo* efficacy against lipopolysaccharide (LPS) -induced pulmonary neutrophilia in mice

(ID_{50} = 16 mg/kg, i.p.). Furthermore, based on the X-ray crystallography of **10f** bound to the human PDE4B catalytic domain, we designed 7,8-dihydro-6*H*-pyrido[4,3-*d*]pyrimidin-5-one derivative **39** which has a fused bicyclic lactam scaffold. Compound **39** exhibited excellent inhibitory activity against LPS-induced tumor necrosis factor alpha (TNF-α) production in mouse splenocytes (IC_{50} = 0.21 nM) and *in vivo* anti-inflammatory activity against LPS-induced pulmonary neutrophilia in mice (41% inhibition at a dose of 1.0 mg/kg, i.t.).

1. Introduction

Phosphodiesterase 4 (PDE4)[1] is an enzyme responsible for the degradation of cyclic adenosine monophosphate (cAMP) via hydrolysis to 5'-AMP. PDE4 is predominantly expressed in most inflammatory and immune cells such as lymphocytes, monocytes, macrophages, neutrophils, eosinophils, and mast cells.[1b,2] In the inflammatory cells, PDE4 inhibition causes an elevation of the intracellular level of cAMP which plays a critical role as a negative regulator of the inflammatory responses.[3] Therefore, PDE4 has been established as a promising target for the treatment of various inflammatory diseases such as asthma, chronic obstructive pulmonary disease (COPD), and so forth.[4]

We have previously reported that the 2-phenylpyrimidine derivative **1** had high *in vitro* PDE4B inhibitory activity (IC_{50} = 25 nM) and moderate inhibitory activity against lipopolysaccharide (LPS) -induced tumor necrosis factor alpha (TNF-α) production in mouse splenocytes (IC_{50} = 390 nM).[5] (Figure 1) In the course of further exploration of this series, we designed and synthesized a 5-carbamoyl-2-phenylpyrimidine derivative **2** as a new lead compound, because the oxygen atom of the primary amide group was

expected to form hydrogen bonds with the surface of the enzyme in a similar manner to the sulfone oxygen atoms of compound **1**. As predicted, compound **2** had PDE4B inhibitory activity with an IC_{50} value of 200 nM and inhibited LPS-induced TNF-α production in mouse splenocytes (IC_{50} = 690 nM), leading to further derivatization of **2**.

Herein, we report the syntheses, structure-activity relationships (SAR), and X-ray crystal structure analysis of the series of 5-carbamoyl-2-phenylpyrimidine derivatives.

2. Chemistry

The 5-carbamoyl-2-phenylpyrimidine derivatives were synthesized as shown in Schemes 1-5.

In Scheme 1, the syntheses of compounds **2** and **9b-d** are depicted. Starting material **3** and benzamidine were condensed to form pyrimidine **4**. The 4-hydroxyl group of **4** was converted to a chlorine atom by treatment with phosphoryl chloride to produce **5**. Hydrolysis of the ethyl ester, followed by condensation with several amines afforded amide compounds **7a-d**. Substitution of the chlorine atom with ethyl 2-(4-aminophenyl)acetate gave **8a-d**, which were hydrolyzed to afford the target compounds, **2** and **9b-d**. Amide compounds (**10a-h**) were synthesized from **2** by a condensation reaction with corresponding amines using *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (WSC), 1-hydroxybenzotriazole hydrate (HOBt) and *N,N*-diisopropylethylamine (DIPEA).

The preparation of 6-methyl derivative **19** is outlined in Scheme 2. Pyrimidine ring formation with diethyl ethylidenemalonate (**11**) and benzamidine was achieved in basic condition to give **12**. Bromination on 5-position of **12** with *N*-bromosuccinimide (NBS)

gave bromide **13**.[6] An elimination reaction of hydrogen bromide and a successive chlorination reaction were performed by treatment with phosphoryl chloride to give **14**. Hydrolysis of **14** gave a 1:1 mixture of **15** and alkoxylated compound **16**. As the separation of **15** and **16** was difficult, byproducts derived from **16** were separated after the synthesis of **19**. The mixture of **15** and **16** was subjected to a series of reactions for amide formation, a substitution reaction of the chlorine atom with ethyl 2-(4-aminophenyl)acetate, and hydrolysis of the ester to produce target compound **19** together with byproducts derived from **16**. Finally, **19** was isolated by recrystallization.

Compound **24** was synthesized as shown in Scheme 3. Commercially available **20** reacted with benzamidine to give **21**[7], which was treated with phosphoryl chloride to give chloride **22**. Substitution of 4-chlorine atom with ethyl 2-(4-aminophenyl)acetate gave **23**. Hydrolysis of terminal ester and 5-cyano group afforded compound **24**.

Compounds **29a-f** and **30a-c** were synthesized via intermediate **28** as outlined in Scheme 4. Initial compound **25** was treated with phosphoryl pentachloride and phosphoryl chloride to give chloride **26**.[8] The reaction of the acid chloride with ammonia and selective substitution of the chlorine atom on the 4-position with 2-(4-aminophenyl)-*N*-(2,2-dimethylpropyl)acetamide gave intermediate **28**. Various substituents were introduced at the 2-position by the Suzuki-Miyaura coupling reaction or substitution reaction to afford the target compounds (**29a-f, 30a-c**).

The syntheses of the fused bicyclic lactam compounds (**38, 39**) are shown in Scheme 5. Ethyl 3-aminopropanoate (**31**) reacted with ethyl malonyl chloride (**32**) to afford **33**. A Dieckmann reaction of **33** gave lactam **34**[9], which was condensed with benzamidine using DBU to give pyrimidine **35**. Chlorination of the hydroxyl group with phosphoryl

chloride, and the subsequent introduction of a side chain yielded ester **37**. Hydrolysis of the ester gave compound **38**, which was converted to *N*-isobutylacetamide derivative **39** by a condensation reaction.

3. Results and discussion

The PDE4 inhibitory activities of synthesized compounds were evaluated using human PDE4B enzyme prepared according to the reported protocols.[10] For selected compounds, an inhibition of LPS-induced TNF-α production in isolated mouse splenocytes was also assessed. Furthermore, *in vivo* anti-inflammatory activity against LPS-induced pulmonary neutrophilia in mice was evaluated for representative compounds (**10f**, **39**). These results are summarized in Tables 1-5.

3.1. Modification of pyrimidine core

Initially, the influence of substituents on the pyrimidine ring or primary amide moiety of **2** was evaluated (Table 1). Introduction of methyl and methylthio groups at the 6-position of the pyrimidine ring decreased the PDE4B inhibitory activity (**19**, **24**). Substitution on the amide moiety also decreased the potency (**9b-d**). Among these compounds, only **2** showed moderate PDE4B inhibition.

3.2. Amidation of carboxylic acid

Next, *in vitro* cell-free and cell-based evaluations were performed using the amide derivatives of the carboxylic acid of the side chain (Table 2). In our previous study for **1**,

we found that a conversion of the carboxylic acid of the side chain to an alkyl substituted amide was effective at improving the *in vitro* profiles.[5] It was similarly found in this study that the primary and alkyl substituted amide compounds (**10a-f**) exhibited moderate to good PDE4B inhibitory activity. Hydroxamic acid **10g** also showed strong PDE4B inhibition, although sulfonyl amide **10h** decreased the potency. Considering the result that *n*-propyl amide and *t*-butyl amide compounds (**10b**, **10e**) exhibited weaker PDE4B inhibitory activity than other alkyl amide compounds(**10c**, **10d**, **10f**), the elongated alkyl groups that have methylene linked terminal bulky moiety were found to be an appropriate form for potent inhibition.

As expected, advanced inhibitory activities against LPS-induced TNF-α production in mouse splenocytes were observed in all compounds except **10h** because of their improved membrane permeability (data not shown). In particular, the neopentyl amide compound (**10f**) exhibited strong inhibitory activity against LPS-induced TNF-α production with an IC_{50} value of 3.0 nM.

3.3. Modification on 2-position of the pyrimidine core

We further evaluated PDE4B inhibitory activity to search for suitable substituents on the 2-position of the pyrimidine core (Table 3). Introduction of the 3-pyridinyl group retained the PDE4B inhibitory potency (**29a**), whereas the 5-pyrimidinyl group exhibited decreased potency (**29b**). Although the 3-thienyl group led to the loss of potency, 3-furyl and 5-indolyl groups were shown to be positive for PDE4B inhibitory activity (**29c-e**). However, **29d** and **29e** showed only weak inhibitory activity against LPS-induced TNF-α production in mouse splenocytes. Besides these aromatic

substituents, we also evaluated the influence of alkenyl and amino groups, which include 2-propenyl, dimethylamino, pyrrolidino, and piperidylamino groups (**29f**, **30a**-**c**). Although the piperidylamino compound (**30c**) showed a decrease in PDE4B inhibitory activity, the other three compounds strongly inhibited both PDE4B activity and TNF-α production in mouse splenocytes. From these results, it was revealed that phenyl, pyridinyl, alkenyl, and amino groups were preferred at the 2-position of the pyrimidine ring.

3.4. *In vivo* evaluation of 10f

The *in vitro* physicochemical properties and *in vivo* pharmacokinetic properties of **10f** are shown in Table 4. Compound **10f** had acceptable *in vitro* metabolic stability and *in vivo* exposure by intraperitoneal administration (i.p.). As an *in vivo* study, inhibitory activity against LPS-induced pulmonary neutrophilia in mice was assessed. In this study, **10f** inhibited the increase of neutrophils in the bronchoalveolar lavage fluid with an ID_{50} of 16 mg/kg (i.p.).

3.5. X-ray crystallography

In order to rationalize the high inhibitory activity of **10f** for PDE4B, **10f** was co-crystallized with a human PDE4B catalytic domain and the structure of the complex was solved by X-ray crystallography (Figure 2). The 5-carbamoyl moiety forms water-bridged hydrogen bonding networks with Asn395 and Gln443. The potency loss of **9b-d** in Table 1 would be led from obstruction of these hydrogen bonding networks by substitution on 5-carbamoyl moiety. The amino group at 4-position and an oxygen

atom of the 5-carbamoyl group are thought to form an intramolecular hydrogen bond, which presumably contributes to fixing the conformation. Since this fixed conformation of the 5-carbamoyl group is thought to be obstructed by the substitution on 6-position, **19** and **24** showed weaker inhibitory activity than **2** (Table 1). The pyrimidine ring forms offset π-π stacking interaction with Phe446, while one of the nitrogen atoms on the pyrimidine ring interacts with Gln443. The 2-phenyl group on the pyrimidine ring has no clear interaction with the enzyme surface, which allows a variety of substrates in this position such as pyridinyl, indolyl, furyl, propenyl, and amino groups in Table 3. The neopentyl amide moiety, a methylene linked bulky alkyl group, occupies a lipophilic pocket. To fill this lipophilic pocket, the alkyl group of amide seems necessary to have an appropriate length that can reach into the pocket, as well as the volume. In this point of view, the methylene moiety of the neopentyl group could be a good linker between the amide bond and terminal *t*-butyl moiety. This might illustrate why **10f** had superior PDE4B inhibitory activity to that of *t*-butyl compound **10e** (Table 2).

Thus, the co-crystal structure suggests an excellent fit of **10f** with the surface of the enzyme. On the other hand, it is also shown that there is an empty pocket near the 6-position of pyrimidine moiety, which is supposed to be a hydrophobic pocket for an interaction with the aliphatic moiety of the sulfone ring of the derivative of **1**.[5] Accordingly, we next tested **38** and **39** as to whether the fused lactam ring, a mimic of the sulfone ring moiety, can occupy a hydrophobic pocket to increase the inhibitory activity.

3.6. *In vitro* and *in vivo* evaluation of lactam compounds

The *in vitro* profile of the lactam compounds is shown in Table 5. The carboxylic acid **38** showed a superior *in vitro* profile to **2**. Moreover, *N*-isobutylacetamide derivative **39** showed higher inhibitory activity against LPS-induced TNF-α production than Roflumilast [11], demonstrating the highest potency among all 5-carbamoyl derivatives. These results have shown the validity of the design based on the complex structure as shown in Figure 2. Finally, **39** was subjected to *in vivo* evaluation and intratracheal administration (1.0 mg/kg) of **39** inhibited LPS-induced pulmonary neutrophilia in mice by 41%, which is nearly equal efficacy to that of Roflumilast.

4. Conclusions

In summary, we identified a novel class of PDE4 inhibitors that have 5-carbamoyl-2-phenylpyrimidine and 7,8-dihydro-6*H*-pyrido[4,3-*d*]pyrimidin-5-one scaffolds. The optimization of substituents on the pyrimidine core and the side chain of **2** led to compound **10f**, which had potent *in vitro* PDE4B inhibitory activity (IC_{50} = 8.3 nM) and high *in vivo* efficacy against LPS-induced pulmonary neutrophilia in mice (ID_{50} = 16 mg/kg, i.p.). Furthermore, from the result of the X-ray crystallography analysis, we designed and synthesized 7,8-dihydro-6*H*-pyrido[4,3-*d*]pyrimidin-5-one derivative **39**, which had excellent inhibitory potency against LPS-induced TNF-α production in mouse splenocytes (IC_{50} = 0.21 nM) and exhibited a high *in vivo* inhibitory effect against LPS-induced pulmonary neutrophilia in mice (41% inhibition at a dose of 1.0 mg/kg, i.t.). Further pharmacological exploration of this series of potent PDE4 inhibitors is currently in progress.

5. Experimental

5.1. Chemistry

5.1.1. General

Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. Column chromatography was performed with Biotage FLASH Si columns. Thin-layer chromatography (TLC) was performed on Merck pre-coated TLC glass sheets with Silica Gel 60 F_{254}. The ^{1}H NMR spectra were recorded on a JEOL JNM-EX-400 spectrometer, and chemical shifts are given in ppm (δ) from tetramethylsilane as an internal standard. The special splitting patterns are designated as follows: s, singlet; d, doublet; dd, double of doublet; t, triplet; q, quartet; and m, multiple. ESI mass spectra were recorded on a SCIEX API-150EX spectrometer. The high-resolution mass (HRMS) spectra were recorded on a JEOL JMS-100LP spectrometer or Waters Xevo Q-Tof spectrometer. IR spectra were recorded on a HORIBA FT-720 spectrometer. Elemental analyses were performed by a Perkin-Elmer CHNS/O 2400II, Leco CHNS-932 and a YOKOKAWA analysis IC7000RS.

5.1.2. Ethyl 4-hydroxy-2-phenyl-pyrimidine-5-carboxylate (4)

To a solution of diethyl (ethoxymethylene)malonate (1.52 g, 7.02 mmol) and benzamidine hydrochloride (1.00 g, 6.39 mmol) in EtOH (10 mL) was added NaOH (0.281 g, 7.02 mmol) and the mixture was warmed to 100 °C. After stirring at 100 °C

for 4 h, the reaction mixture was cooled to room temperature and the resulting precipitate was collected by filtration to give the title compound (1.30 g, 5.32 mmol, 88%) as a colorless solid. ^{1}H NMR ($CDCl_3$) δ: 1.35 (3H, t, J = 6.9 Hz), 4.32 (2H, q, J = 6.9 Hz), 7.42-7.52 (3H, m), 8.30 (2H, d, J = 7.5 Hz), 8.92 (1H, s); MS (ESI) *m/z*: 245 $(M+H)^+$.

5.1.3. Ethyl 4-chloro-2-phenyl-pyrimidine-5-carboxylate (5)

A solution of **4** (1.20 g, 4.91 mmol) in phosphoryl chloride (8 mL) was stirred at 100 °C for 3 h. After cooling, the reaction mixture was concentrated *in vacuo* and the residue was dissolved in EtOH (5 mL). To the resulting mixture, H_2O was added dropwise over 5 min and the precipitate was collected by filtration and washed with water to give the title compound (0.765 g, 2.91 mmol, 59%) as a colorless solid. ^{1}H NMR ($CDCl_3$) δ: 1.46 (3H, t, J = 7.1 Hz), 4.48 (2H, q, J = 7.1 Hz), 7.52-7.59 (3H, m), 8.51-8.53 (2H, m), 9.21 (1H, s); MS (ESI) *m/z*: 263 $(M+H)^+$.

5.1.4. 4-Chloro-2-phenyl-pyrimidine-5-carboxylic acid (6)

To a solution of **5** (0.700 g, 2.66 mmol) in EtOH (5 mL) and THF (5 mL) was added 1N NaOH aqueous solution (5.3 mL) and the reaction mixture was stirred at room temperature. After 2 h, the organic solvent was evaporated and 1 N HCl aqueous solution (10 mL) was added to the residue. The resulting mixture was extracted 3 times with *i*-PrOH/$CHCl_3$ (1:3, v/v) and the combined extract was washed with brine, dried

over Na_2SO_4 and concentrated *in vacuo* to give the title compound (0.622 g, 2.65 mmol, 99%) as a colorless solid. ^{1}H NMR ($CDCl_3$) δ: 7.55-7.63 (3H, m), 8.38-8.43 (2H, m), 9.25 (1H, s); MS (ESI) *m/z*: 235 $(M+H)^+$.

5.1.5. 4-Chloro-2-phenyl-pyrimidine-5-carboxamide (7a)

To a solution of **6** (3.73 g, 15.9 mmol) in CH_2Cl_2 (50 mL) were added oxalyl chloride (2.73 mL, 31.8 mmol) and DMF (0.1 mL) at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred for 3 h. The reaction mixture was concentrated *in vacuo* and the residue was dissolved in THF (50 mL). The resulting mixture was cooled to -10 °C and 28% NH_3 (2.14 mL, 31.8 mmol) was added. After stirring at -10 °C for 30 min, 1 N HCl aqueous solution (40 mL) was added and the reaction mixture was concentrated *in vacuo*. The resulting residue was triturated in THF (3 mL), H_2O (15 mL), and sat. $NaHCO_3$ aqueous solution (10 mL). The resulting precipitate was collected by filtration to give the title compound (2.52 g, 10.8 mmol, 68%) as a colorless solid. ^{1}H NMR (DMSO-d_6) δ: 7.56-7.63 (3H, m), 7.97 (1H, s), 8.19 (1H, s), 8.36-8.39 (2H, m), 8.97 (1H, s); MS (ESI) *m/z*: 234 $(M+H)^+$.

5.1.6. 4-Chloro-*N*-methyl-2-phenyl-pyrimidine-5-carboxamide (7b)

To a solution of **6** (150 mg, 0.639 mmol) in CH_2Cl_2 (5 mL) were added oxalyl chloride (0.110 mL, 1.28 mmol) and DMF (0.01 mL) at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred for 1 h. The reaction mixture was

concentrated *in vacuo* and the residue was dissolved in CH_2Cl_2 (5 mL). To the resulting mixture, $MeNH_2$ (0.196 mL, 1.92 mmol, 9.8 mol/L solution in MeOH) was added at room temperature. After stirring for 40 min, H_2O was added and the reaction mixture was extracted twice with CH_2Cl_2. The combined extract was washed with brine, dried over Na_2SO_4, and concentrated *in vacuo* to give the title compound (108 mg, 0.436 mmol, 68%) as a pale yellow solid. ^{1}H NMR ($CDCl_3$) δ: 3.09 (3H, d, J = 5.2 Hz), 6.66 (1H, s), 7.49-7.55 (3H, m), 8.44-8.48 (2H, m), 9.18 (1H, s); MS (ESI) *m/z*: 248 $(M+H)^+$.

5.1.7. 4-Chloro-*N*,*N*-dimethyl-2-phenyl-pyrimidine-5-carboxamide (7c)

Compound **7c** was synthesized from **6** according to the procedure used to prepare **7b** (colorless amorphous solid, Yield 24%) ^{1}H NMR ($CDCl_3$) δ: 2.99 (3H, s), 3.18 (3H, s), 7.48-7.56 (3H, m), 8.44-8.46 (2H, m), 8.69 (1H, s).

5.1.8.

***N*-[*tert*-Butyl(dimethyl)silyl]oxy-4-chloro-2-phenyl-pyrimidine-5-carboxamide (7d)**

Compound **7d** was synthesized from **6** according to the procedure used to prepare **7b** (pale brown amorphous solid, Yield 25%) ^{1}H NMR ($CDCl_3$) δ: 0.27 (6H, s), 1.03 (9H, s), 7.47-7.54 (3H, m), 8.45-8.48 (2H, m), 8.64 (1H, s), 9.08 (1H, s); MS (ESI) *m/z*: 364 $(M+H)^+$.

5.1.9. Ethyl 2-[4-[(5-carbamoyl-2-phenyl-pyrimidin-4-yl)amino]phenyl]acetate (8a)

To a solution of **7a** (600 mg, 2.57 mmol) in DMF (5 mL) was added ethyl 2-(4-aminophenyl)acetate (690 mg, 3.85 mmol) and the reaction mixture was stirred at 80 °C for 4 h. After cooling, the reaction mixture was poured into 0.2 N HCl aqueous solution (20 mL). The resulting precipitate was collected by filtration to give the title compound (961 mg, 2.55 mmol, 99%) as a pale yellow solid. ^{1}H NMR ($CDCl_3$) δ: 1.20 (3H, t, J = 6.9 Hz), 3.68 (2H, s), 4.10 (2H, q, J = 6.9 Hz), 7.34 (2H, d, J = 8.6 Hz), 7.53-7.60 (3H, m), 7.74 (2H, d, J = 8.6 Hz), 7.89 (1H, s), 8.35-8.37 (2H, m), 8.46 (1H, s), 9.01 (1H, s), 11.49 (1H, s); MS (ESI) *m/z*: 377 $(M+H)^+$.

5.1.10. Ethyl 2-[4-[[5-(methylcarbamoyl)-2-phenyl-pyrimidin-4-yl]amino]phenyl]acetate (8b)

To a solution of **7b** (108 mg, 0.436 mmol) in DMF (2 mL) was added ethyl 2-(4-aminophenyl)acetate (117 mg, 0.654 mmol) and the reaction mixture was stirred at 60 °C for 4 h. After cooling, H_2O was added, and the reaction mixture was extracted twice with EtOAc/hexane (1:1, v/v). The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The resulting residue was chromatographed (NH silica gel, EtOAc/hexane = 0% to 60%) to give the title compound (78.0 mg, 0.199 mmol, 46%) as a colorless amorphous solid. ^{1}H NMR ($CDCl_3$) δ: 1.28 (3H, t, J = 6.9 Hz), 3.06 (3H, d, J = 5.2 Hz), 3.63 (2H, s), 4.18 (2H, q, J

= 6.9 Hz), 6.23-6.25 (1H, m), 7.33 (2H, d, *J* = 8.6 Hz), 7.47-7.53 (3H, m), 7.78 (2H, d, *J* = 8.6 Hz), 8.40-8.43 (2H, m), 8.61 (1H, s), 10.88 (1H, s); MS (ESI) *m/z*: 391 $(M+H)^+$.

5.1.11. Ethyl 2-[4-[[5-(dimethylcarbamoyl)-2-phenyl-pyrimidin-4-yl]amino]phenyl]acetate (8c)

To a solution of **7c** (19.0 mg, 0.0726 mmol) in DMF (0.5 mL) was added ethyl 2-(4-aminophenyl)acetate (26.0 mg, 0.145 mmol) and the reaction mixture was stirred at 80 °C for 16 h. After cooling, H_2O was added and the reaction mixture was extracted twice with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The resulting residue was chromatographed (NH silica gel, EtOAc/hexane = 0% to 50%) to give the title compound (23.0 mg, 0.0569 mmol, 78%) as a colorless amorphous solid. ^{1}H NMR ($CDCl_3$) δ: 1.27 (3H, t, *J* = 7.2 Hz), 3.20 (6H, s), 3.63 (2H, s), 4.17 (2H, q, *J* = 7.2 Hz), 7.33 (2H, d, *J* = 8.6 Hz), 7.47-7.50 (3H, m), 7.73 (2H, d, *J* = 8.6 Hz), 8.40-8.42 (2H, m), 8.46 (1H, s), 9.22 (1H, s); MS (ESI) *m/z*: 405 $(M+H)^+$.

5.1.12. Ethyl 2-[4-[[5-(hydroxycarbamoyl)-2-phenyl-pyrimidin-4-yl]amino]phenyl]acetate (8d)

To a solution of **7d** (38.0 mg, 0.104 mmol) in DMF (2 mL) was added ethyl 2-(4-aminophenyl)acetate (114 mg, 0.639 mmol) and the reaction mixture was stirred at 60 °C for 13 h. The *tert*-butyl(dimethyl)silyl group was deprotected during under this

condition. After cooling, H_2O was added and the reaction mixture was extracted twice with THF/$CHCl_3$ (1:3, v/v). The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The resulting residue was purified by reverse phase preparative HPLC (0.1% HCO_2H MeCN / 0.1% HCO_2H aq. = 0% to 100%) to give the title compound (19.0 mg, 0.0484 mmol, 46%) as a colorless solid. ^{1}H NMR ($CDCl_3$) δ: 1.25 (3H, t, J = 6.9 Hz), 3.65 (2H, s), 4.15 (2H, q, J = 6.9 Hz), 7.33 (2H, d, J = 8.6 Hz), 7.47-7.53 (3H, m), 7.76 (2H, d, J = 8.6 Hz), 8.35 (2H, dd, J = 8.0, 1.7 Hz), 8.66 (1H, s); MS (ESI) *m/z*: 393 $(M+H)^+$.

5.1.13. 2-[4-[(5-Carbamoyl-2-phenyl-pyrimidin-4-yl)amino]phenyl]acetic acid (2)

To a solution of **8a** (960 mg, 2.55 mmol) in THF (15 mL), EtOH (15 mL), and H_2O (2.5 mL) was added 1 N NaOH aqueous solution (5.10 mL) and the mixture was stirred at room temperature. After 14 h, 1 N HCl aqueous solution (6.0 mL) was added to the reaction mixture and the organic solvent was evaporated. H_2O was added to the residue and the mixture was triturated. The resulting precipitate was collected by filtration to give the title compound (854 mg, 2.45 mmol, 96%) as a pale yellow solid. ^{1}H NMR (DMSO-d_6) δ: 3.58 (2H, s), 7.33 (2H, d, J = 8.6 Hz), 7.54-7.57 (3H, m), 7.73-7.75 (2H, m), 7.87 (1H, s), 8.36-8.38 (2H, m), 8.44 (1H, s), 9.00 (1H, s), 11.45 (1H, s); MS (ESI) *m/z*: 349 $(M+H)^+$; HRMS (ESI) Calcd for $C_{19}H_{16}N_4O_3$-H: 347.1144. Found: 347.1140.

5.1.14. 2-[4-[[5-(Methylcarbamoyl)-2-phenyl-pyrimidin-4-yl]amino]phenyl]acetic acid (9b)

Compound **9b** was synthesized from **8b** according to the procedure used to prepare **2**. (yellow solid, Yield 97%) ^{1}H NMR (DMSO-d_6) δ: 2.83 (3H, d, J = 4.6 Hz), 3.56 (2H, s), 7.30 (2H, d, J = 8.6 Hz), 7.51-7.55 (3H, m), 7.71-7.73 (2H, m), 8.33-8.35 (2H, m), 8.91-8.93 (2H, m), 11.29 (1H, s); MS (ESI) *m/z*: 363 $(M+H)^+$; HRMS (ESI) Calcd for $C_{20}H_{18}N_4O_3$+H: 363.1457. Found: 363.1458.

5.1.15. 2-[4-[[5-(Dimethylcarbamoyl)-2-phenyl-pyrimidin-4-yl]amino]phenyl]acetic acid (9c)

Compound **9c** was synthesized from **8c** according to the procedure used to prepare **2**. (colorless solid, Yield 86%) ^{1}H NMR (DMSO-d_6) δ: 3.00 (6H, s), 3.55 (2H, s), 7.27 (2H, d, J = 8.6 Hz), 7.48-7.51 (3H, m), 7.64 (2H, d, J = 8.6 Hz), 8.28-8.30 (2H, m), 8.43 (1H, s), 9.12 (1H, s); MS (ESI) *m/z*: 377 $(M+H)^+$; HRMS (ESI) Calcd for $C_{21}H_{20}N_4O_3$-H: 375.1457. Found: 375.1458.

5.1.16. 2-[4-[[5-(Hydroxycarbamoyl)-2-phenyl-pyrimidin-4-yl]amino]phenyl]acetic acid (9d)

Compound **9d** was synthesized from **8d** according to the procedure used to prepare **2**. (yellow solid, Yield 71%) ^{1}H NMR (DMSO-d_6) δ: 3.56 (2H, s), 7.31 (2H, d, J = 8.6 Hz), 7.52-7.54 (3H, m), 7.71 (2H, d, J = 8.6 Hz), 8.32-8.33 (2H, m), 8.76 (1H, s), 10.88 (1H, s), 11.65 (1H, s); MS (ESI) *m/z*: 365 $(M+H)^+$; HRMS (ESI) Calcd for $C_{19}H_{16}N_4O_4$-H: 363.1093. Found: 363.1094.

5.1.17. 4-[4-(2-Amino-2-oxo-ethyl)anilino]-2-phenyl-pyrimidine-5-carboxamide (10a)

To a solution of **2** (250 mg, 0.718 mmol) in CH_2Cl_2 (5 mL) were added oxalyl chloride (0.246 mL, 2.87 mmol) and DMF (three drops), and the reaction mixture was stirred at room temperature. After stirring for 1 h, the reaction mixture was concentrated *in vacuo* to give crude 2-[4-[(5-carbamoyl-2-phenyl-pyrimidin-4-yl)amino]phenyl]acetyl chloride (270 mg) as a pale yellow solid. This compound was used for next reaction without further purification.

To a solution of 2-[4-[(5-carbamoyl-2-phenyl-pyrimidin-4-yl)amino]phenyl]acetyl chloride (30.0 mg, 0.0818 mmol) in THF (0.5 mL) and DMF (0.5 mL) was added 28% NH_3 aqueous solution (0.0276 mL, 0.409 mmol) at 0 °C, and the reaction mixture was stirred at same temperature. After stirring for 1.5 h, the reaction mixture was concentrated *in vacuo*. The resulting residue was purified by reverse phase preparative HPLC (0.1% HCO_2H MeCN / 0.1% HCO_2H aq. = 0% to 100%) to give the title compound (8.00 mg, 0.0230 mmol, 28%) as a colorless solid. ^{1}H NMR (DMSO-d_6) δ: 3.38 (2H, s), 3.40 (2H, s), 6.91 (1H, s), 7.32 (2H, d, J = 7.9 Hz), 7.49 (1H, s), 7.54-7.57 (3H, m), 7.72 (2H, d, J = 7.9 Hz), 7.87 (1H, s), 8.36-8.44 (2H, m), 9.00 (1H, s), 11.41 (1H, s); MS (ESI) *m/z*: 348 $(M+H)^+$; HRMS (ESI) Calcd for $C_{19}H_{17}N_5O_2$+H: 348.1460. Found: 348.1465.

5.1.18.

4-[4-[2-Oxo-2-(propylamino)ethyl]anilino]-2-phenyl-pyrimidine-5-carboxamide (10b)

To a solution of **2** (200 mg, 0.574 mmol) in DMF (4 mL) were added *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (165 mg, 0.861 mmol), 1-hydroxybenzotriazole hydrate (116 mg, 0.861 mmol), *N*,*N*-diisopropylethylamine (0.300 mL, 1.72 mmol), and *n*-propylamine (0.0710 mL, 0.861 mmol), and the reaction mixture was stirred at room temperature. After stirring for 17 h, the reaction mixture was concentrated *in vacuo*. The resulting residue was chromatographed (THF/CH_2Cl_2 = 0% to 50%) to give the title compound (112 mg, 0.288 mmol, 50%) as a colorless solid. ^{1}H NMR (DMSO-d_6) δ: 0.84 (3H, t, J = 7.4 Hz), 1.38-1.45 (2H, m), 3.02 (2H, q, J = 6.3 Hz), 3.40 (2H, s), 7.31 (2H, d, J = 8.6 Hz), 7.52-7.58 (3H, m), 7.71 (2H, d, J = 8.6 Hz), 7.84 (1H, s), 8.01 (1H, t, J = 5.4 Hz), 8.36-8.42 (3H, m), 9.00 (1H, s), 11.38 (1H, s); MS (ESI) *m/z*: 390 $(M+H)^+$; HRMS (ESI) Calcd for $C_{22}H_{23}N_5O_2$+H: 390.1930. Found: 390.1930; IR (KBr) cm^{-1}: 3297, 3196, 2962, 2931, 1674, 1646, 1602, 1558, 1515, 1488, 1418, 790, 697; Anal. Calcd for $C_{22}H_{23}N_5O_2$: C, 67.85; H, 5.95; N, 17.98. Found: C, 67.47; H, 5.93; N, 17.70.

5.1.19.

4-[4-[2-(Cyclopropylmethylamino)-2-oxo-ethyl]anilino]-2-phenyl-pyrimidine-5-carboxamide (10c)

Compound **10c** was synthesized from **2** according to the procedure used to prepare **10b**. (colorless solid, Yield 43%) ^{1}H NMR (DMSO-d_6) δ: 0.14-0.17 (2H, m), 0.38-0.42 (2H, m), 0.87-0.95 (1H, m), 2.96 (2H, t, J = 6.3 Hz), 3.42 (2H, s), 7.32 (2H, d, J = 8.6 Hz), 7.52-7.57 (3H, m), 7.71 (2H, d, J = 8.6 Hz), 7.84 (1H, s), 8.11 (1H, t, J = 5.4 Hz), 8.36-8.42 (3H, m), 9.00 (1H, s), 11.38 (1H, s); MS (ESI) *m/z*: 402 $(M+H)^+$; HRMS (ESI) Calcd for $C_{23}H_{23}N_5O_2$+H: 402.1930. Found: 402.1922; IR (KBr) cm^{-1}: 3304, 3196, 1672, 1643, 1603, 1558, 1515, 1489, 1419, 1385, 790, 697.

5.1.20.

4-[4-[2-(Isobutylamino)-2-oxo-ethyl]anilino]-2-phenyl-pyrimidine-5-carboxamide (10d)

Compound **10d** was synthesized from **2** according to the procedure used to prepare **10b**. (colorless solid, Yield 55%) ^{1}H NMR (DMSO-d_6) δ: 0.83 (6H, d, J = 6.9 Hz), 1.64-1.72 (1H, m), 2.89 (2H, t, J = 6.3 Hz), 3.42 (2H, s), 7.32 (2H, d, J = 8.6 Hz), 7.52-7.58 (3H, m), 7.71 (2H, d, J = 8.6 Hz), 7.84 (1H, s), 8.02 (1H, t, J = 5.7 Hz), 8.36-8.41 (3H, m), 9.00 (1H, s), 11.37 (1H, s). MS (ESI) *m/z*: 404 $(M+H)^+$; HRMS (ESI) Calcd for $C_{23}H_{25}N_5O_2$+H: 404.2087. Found: 404.2041; IR (KBr) cm^{-1}: 3300, 3197, 2958, 1675, 1644, 1602, 1558, 1515, 1489, 1418, 1320, 1211, 790, 696; Anal. Calcd for $C_{23}H_{25}N_5O_2$: C, 68.47; H, 6.25; N, 17.36. Found: C, 68.31; H, 6.31; N, 17.08.

5.1.21.

4-[4-[2-(*tert*-Butylamino)-2-oxo-ethyl]anilino]-2-phenyl-pyrimidine-5-carboxamide (10e)

Compound **10e** was synthesized from **2** according to the procedure used to prepare **10b**. (colorless solid, Yield 87%) ^{1}H NMR (DMSO-d_6) δ: 1.26 (9H, m), 3.36 (2H, s), 7.30 (2H, d, J = 8.6 Hz), 7.52-7.58 (3H, m), 7.67 (1H, s), 7.70 (2H, d, J = 8.6 Hz), 7.84 (1H, s), 8.36-8.44 (3H, m), 9.00 (1H, s), 11.37 (1H, s); MS (ESI) *m/z*: 404 $(M+H)^+$; HRMS (ESI) Calcd for $C_{23}H_{25}N_5O_2$+H: 404.2087. Found: 404.2086; IR (KBr) cm^{-1}: 3335, 3196, 2867, 1677, 1651, 1601, 1558, 1515, 1486, 1448, 1419, 1212, 788, 697.

5.1.22.

4-[4-[2-(2,2-Dimethylpropylamino)-2-oxo-ethyl]anilino]-2-phenyl-pyrimidine-5-carboxamide (10f)

Compound **10f** was synthesized from **2** according to the procedure used to prepare **10b**. (colorless solid, Yield 76%) ^{1}H NMR (DMSO-d_6) δ: 0.83 (9H, s), 2.90 (2H, d, J = 6.3 Hz), 3.46 (2H, s), 7.34 (2H, d, J = 8.6 Hz), 7.52-7.56 (3H, m), 7.70 (2H, d, J = 8.6 Hz), 7.84 (1H, s), 7.93 (1H, t, J = 6.3 Hz), 8.36-8.41 (3H, m), 9.00 (1H, s), 11.36 (1H, s); MS (ESI) *m/z*: 418 $(M+H)^+$; HRMS (ESI) Calcd for $C_{24}H_{27}N_5O_2$+H: 418.2243. Found: 418.2242; IR (KBr) cm^{-1}: 3321, 3198, 2959, 1677, 1649, 1622, 1602, 1559, 1516, 1487, 1418, 1209, 791, 697.

5.1.23.

4-[4-[2-(Hydroxyamino)-2-oxo-ethyl]anilino]-2-phenyl-pyrimidine-5-carboxamide (10g)

To a solution of **2** (20.0 mg, 0.0574 mmol) in DMF (1 mL) were added *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (16.5 mg, 0.0861 mmol), 1-hydroxybenzotriazole hydrate (11.6 mg, 0.0861 mmol), *N*,*N*-diisopropylethylamine (0.0300 mL, 0.172 mmol), and hydroxylamine hydrochloride (5.98 mg, 0.0861 mmol), and the reaction mixture was stirred at room temperature. After stirring for 15 h, the reaction mixture was concentrated *in vacuo*. The resulting residue was purified by revers phase preparative HPLC (0.1% HCO_2H MeCN / 0.1% HCO_2H aq. = 0% to 100%) to give the title compound (14.0 mg, 0.0385 mmol, 67%) as a colorless solid. ^{1}H NMR (DMSO-d_6) δ: 3.29 (2H, s), 7.32 (2H, d, J = 8.5 Hz), 7.52-7.58 (3H, m), 7.73 (2H, d, J = 8.5 Hz), 7.87 (1H, s), 8.36-8.39 (2H, m), 8.42 (1H, s), 8.86 (1H, s), 9.00 (1H, s), 10.67 (1H, s), 11.40 (1H, s); MS (ESI) *m/z*: 364 $(M+H)^+$; HRMS (ESI) Calcd for $C_{19}H_{17}N_5O_3$+H: 364.1410. Found: 364.1406.

5.1.24.

4-[4-[2-(Methanesulfonamido)-2-oxo-ethyl]anilino]-2-phenyl-pyrimidine-5-carboxamide (10h)

Compound **10h** was synthesized from **2** according to the procedure used to prepare **10g**. (pale brown solid, Yield 29%) ^{1}H NMR (DMSO-d_6) δ: 3.23 (3H, s), 3.62 (2H, s), 7.33 (2H, d, J = 8.5 Hz), 7.54-7.58 (3H, m), 7.76 (2H, d, J = 8.5 Hz), 7.87 (1H, s),

8.34-8.44 (3H, m), 9.01 (1H, s), 11.44 (1H, s); MS (ESI) *m/z*: 426 $(M+H)^+$; HRMS (ESI) Calcd for $C_{20}H_{19}N_5O_4S$+H: 4426.1236. Found: 426.1247.

5.1.25. Ethyl 4-methyl-6-oxo-2-phenyl-4,5-dihydro-1*H*-pyrimidine-5-carboxylate (12)

A solution of diethyl 2-ethylidenemalonate (1.10 g, 5.91 mmol), benzamidine hydrochloride (925 mg, 5.91 mmol), and *N*,*N*-diisopropylethylamine (1.03 mL, 5.91 mmol) in MeOH (10 mL) was heated in a microwave reactor at 110 °C for 45 min. After cooling, the reaction mixture was concentrated *in vacuo*. The resulting residue was chromatographed (EtOAc/hexane = 0% to 50%) to give the title compound (918 mg, 3.53 mmol, 60%) as a colorless solid. ^{1}H NMR ($CDCl_3$) δ: 1.30 (3H, t, J = 7.2 Hz), 1.43 (3H, d, J = 6.9 Hz), 3.27-3.32 (1H, m), 3.81 (1H, s), 4.25-4.29 (3H, m), 7.44-7.47 (3H, m), 7.74-7.77 (2H, m), 8.42 (1H, s); MS (ESI) *m/z*: 261 $(M+H)^+$.

5.1.26. Ethyl 5-bromo-4-methyl-6-oxo-2-phenyl-1,4-dihydropyrimidine-5-carboxylate (13)

To a solution of **12** (910 mg, 3.50 mmol) in CCl_4 (40 mL) were added N-bromosuccinimide (622 mg, 3.50 mmol), K_2CO_3 (4.84 g, 35.0 mmol), and benzoyl peroxide (42.4 mg, 0.175 mmol). The reaction mixture was refluxed for 1 h. After cooling, H_2O was added and the mixture was extracted 3 times with CH_2Cl_2. The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The resulting residue was chromatographed (EtOAc/hexane = 0% to 50%) to give the title compound (214 mg, 0.631 mmol, 18%) as a colorless amorphous solid. ^{1}H

NMR ($CDCl_3$) δ: 1.25 (3H, t, J = 7.2 Hz), 1.64 (3H, d, J = 6.9 Hz), 4.23-4.31 (3H, m), 7.46-7.50 (2H, m), 7.53-7.56 (1H, m), 7.76 (2H, d, J = 7.4 Hz), 8.21 (1H, s); MS (ESI) *m/z*: 339 $(M+H)^+$.

5.1.27. Ethyl 4-chloro-6-methyl-2-phenyl-pyrimidine-5-carboxylate (14)

A solution of **13** (114 mg, 0.336 mmol) in $POCl_3$ (2 mL) was stirred at 100 °C for 1 h. After cooling, the reaction mixture was concentrated *in vacuo*. The resulting residue was chromatographed (EtOAc/hexane = 0% to 30%) to give the title compound (68.0 mg, 0.246 mmol, 73%) as a colorless solid. ^1H NMR ($CDCl_3$) δ: 1.44 (3H, t, J = 7.4 Hz), 2.63 (3H, s), 4.48 (2H, q, J = 7.4 Hz), 7.47-7.53 (3H, m), 8.44-8.47 (2H, m); MS (ESI) *m/z*: 277 $(M+H)^+$.

5.1.28. 2-[4-[(5-Carbamoyl-6-methyl-2-phenyl-pyrimidin-4-yl)amino]phenyl]acetic acid (19)

To a solution of **14** (125 mg, 0.452 mmol) in THF (2 mL) and EtOH (2 mL) were added 1 N NaOH aqueous solution (0.903 mL) and H_2O (0.100 mL). After stirring at room temperature for 5 h, the reaction mixture was warmed to 50 °C and stirred for 3.5 h. After cooling, 1 N HCl aqueous solution (1.00 mL) was added and the organic solvent was evaporated. H_2O was added to the residue and the mixture was extracted with CH_2Cl_2. The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated *in vacuo* to give a 1:1 mixture of

4-chloro-6-methyl-2-phenyl-pyrimidine-5-carboxylic acid (**15**) and 4-ethoxy-6-methyl-2-phenyl-pyrimidine-5-carboxylic acid (**16**). The resulting mixture (130 mg) was used for the next reaction without further purification. **15**; MS (ESI) *m/z*: 249 $(M+H)^+$. **16**; MS (ESI) *m/z*: 259 $(M+H)^+$.

To a solution of the mixture (**15** and **16**) (130 mg) in CH_2Cl_2 (2 mL) were added oxalyl chloride (0.125 mL, 1.36 mmol) and DMF (0.01 mL) at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred for 20 min. The reaction mixture was concentrated *in vacuo* and the residue was dissolved in THF (5 mL). 28% NH_3 aqueous solution (1.00 mL) was added to the resulting mixture, at 0 °C. After stirring for 1 h, H_2O was added and the reaction mixture was extracted twice with $CHCl_3$. The combined extract was washed with brine, dried over Na_2SO_4, and concentrated *in vacuo* to give a crude mixture (132 mg) including 4-chloro-6-methyl-2-phenyl-pyrimidine-5-carboxamide (**17**). This mixture was used for the next reaction without further purification. MS (ESI) *m/z*: 248 $(M+H)^+$.

To a solution of the mixture including **17** (132 mg) in DMF (3 mL) was added ethyl 2-(4-aminophenyl)acetate (191 mg, 1.07 mmol) and the reaction mixture was stirred at 80 °C for 5.5 h. After cooling, H_2O was added and the reaction mixture was extracted twice with EtOAc. The combined extract was washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The resulting residue was chromatographed (EtOAc/hexane = 0% to 80%) to give a crude mixture (106 mg) including ethyl 2-[4-[(5-carbamoyl-6-methyl-2-phenyl-pyrimidin-4-yl)amino]phenyl]acetate (**18**). This mixture was used for the next reaction without further purification. MS (ESI) *m/z*: 391 $(M+H)^+$.

To a solution of the mixture including **18** (106 mg) in THF (2 mL), EtOH (2 mL), and H_2O (1 mL) was added 1 N NaOH aqueous solution (0.407 mL) and the mixture was stirred for 4 h. After 1 N HCl aqueous solution (0.5 mL) was added, the solvent was evaporated. To the residue, H_2O was added and the precipitate was collected by filtration to give the title compound (47.0 mg, 0.130 mmol, 29% from **14**) as a colorless solid. ^{1}H NMR (DMSO-d_6) δ: 2.52 (3H, s), 3.57 (2H, s), 7.26 (2H, d, J = 8.6 Hz), 7.49-7.54 (3H, m), 7.63 (2H, d, J = 8.6 Hz), 7.94 (1H, s), 8.15 (1H, s), 8.23-8.25 (2H, m); MS (ESI) *m/z*: 363 $(M+H)^+$; HRMS (ESI) Calcd for $C_{20}H_{18}N_4O_3$+H: 363.1457. Found: 363.1408.

5.1.29. 4-Chloro-6-methylsulfanyl-2-phenyl-pyrimidine-5-carbonitrile (22)

To a solution of ethyl 3,3-bis(methylthio)-2-cyanoacrylate (**20**) (1.74 g, 8.00 mmol) and benzamidine hydrochloride (1.25 g, 8.00 mmol) in EtOH (100 mL) was added triethylamine (5.56 mL, 40.0 mmol) and the reaction mixture was refluxed for 5 h. After cooling, the reaction mixture was concentrated *in vacuo*. 1N HCl aqueous solution (100 mL) and H_2O (100 mL) were added to the residue and the resulting precipitate was collected by filtration to give 4-methylsulfanyl-6-oxo-2-phenyl-1*H*-pyrimidine-5-carbonitrile (**21**) (1.81 g, 7.42 mmol, 93%) as a colorless solid.

To a solution of **21** (900 mg, 3.70 mmol) in $POCl_3$ (10 mL) was added *N*,*N*-diethylaniline (1.18 mL, 7.40 mmol) and the reaction mixture was stirred at 100 °C for 2.5 h. After cooling, the reaction mixture was concentrated *in vacuo*. The resulting

residue was chromatographed (CH_2Cl_2 = 100%) to give the title compound (829 mg, 3.17 mmol, 86%) as a colorless solid. ^{1}H NMR ($CDCl_3$) δ: 2.81 (3H, s), 7.51-7.54 (2H, m), 7.58-7.61 (1H, m), 8.47-8.49 (2H, m); MS (ESI) *m/z*: 262 $(M+H)^+$.

5.1.30. Ethyl 2-[4-[(5-cyano-6-methylsulfanyl-2-phenyl-pyrimidin-4-yl)amino]phenyl]acetate (23)

To a solution of **22** (480 mg, 1.83 mmol) in DMF (6 mL) was added ethyl 2-(4-aminophenyl)acetate (658 mg, 3.67 mmol) and the reaction mixture was stirred at 80 °C for 1.5 h. After cooling, H_2O was added and the reaction mixture was extracted twice with EtOAc. The combined extract was washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The resulting residue was chromatographed (EtOAc/CH_2Cl_2 = 0% to 10%) to give the title compound (620 mg, 1.53 mmol, 83%) as a colorless solid. ^{1}H NMR ($CDCl_3$) δ: 1.28 (3H, t, J = 7.2 Hz), 2.77 (3H, s), 3.65 (2H, s), 4.19 (2H, q, J = 7.2 Hz), 7.12 (1H, s), 7.36 (2H, d, J = 8.6 Hz), 7.47-7.55 (3H, m), 7.64 (2H, d, J = 8.6 Hz), 8.41-8.44 (2H, m); MS (ESI) *m/z*: 405 $(M+H)^+$.

5.1.31. 2-[4-[(5-Cyano-6-methylsulfanyl-2-phenyl-pyrimidin-4-yl)amino]phenyl]acetic acid (24)

Compound **23** (60.0 mg, 0.148 mmol) was dissolved in conc. H_2SO_4 (1.5 mL), and the reaction mixture was stirred at 70 °C for 2 h. After cooling, ice and H_2O were added to the reaction mixture and the resulting precipitate was collected by filtration to give a yellow solid. The resulting solid was purified by reverse phase preparative HPLC (0.1% HCO_2H MeCN / 0.1% HCO_2H aq. = 0% to 100%) to give the title compound (3.00 mg, 0.00761 mmol, 5%) as a colorless solid. ^{1}H NMR (DMSO-d_6) δ: 2.63 (3H, s), 3.56 (2H, s), 7.26 (2H, d, J = 8.5 Hz), 7.52-7.56 (3H, m), 7.65 (2H, d, J = 8.5 Hz), 8.29 (1H, s), 8.33-8.36 (2H, m), 8.55 (1H, s); MS (ESI) *m/z*: 395 $(M+H)^+$; HRMS (ESI) Calcd for $C_{20}H_{18}N_4O_3S$+H: 395.1178. Found: 395.1223.

5.1.32. 2,4-Dichloropyrimidine-5-carboxamide (27)

To a solution of 2,4-dihydroxypyrimidine-5-carboxylic acid (**25**) (2.98 g, 19.1 mmol) in $POCl_3$ (7.10 mL, 76.4 mmol) was added PCl_5 (13.1 g, 63.0 mmol) and the reaction mixture was refluxed for 4.5 h. After cooling, the reaction mixture was concentrated *in vacuo*. To the resulting residue, toluene (20 mL) was added and the mixture was concentrated *in vacuo* again. The residue was dissolved in CH_2Cl_2 (20 mL) and filtered. The filtrate was concentrated *in vacuo* to give 2,4-dichloropyrimidine-5-carbonyl chloride (**26**) as a yellow oil (4.38 g). This compound was used for next reaction without further purification.

To a solution of **26** (4.38 g) in CH_2Cl_2 (5 mL) were added 28% NH_3 aqueous solution (3 mL) and H_2O (3 mL) at -10 °C, and the reaction mixture was stirred at -10 °C for 5 min. The organic solvent was removed under reduced pressure and the resulting

precipitate was collected by filtration to give the title compound (3.13 g, 16.3 mmol, 85% from **25**) as a pale yellow solid.^{1}H NMR (DMSO-d_6) δ: 8.05 (1H, s), 8.17 (1H, s), 8.90 (1H, s); MS (ESI) *m/z*: 192 $(M+H)^+$.

5.1.33.

2-Chloro-4-[4-[2-(2,2-dimethylpropylamino)-2-oxo-ethyl]anilino]pyrimidine-5-carboxamide (28)

To a solution of **27** (706 mg, 3.68 mmol) in MeCN (15 mL) were added 2-(4-aminophenyl)-*N*-(2,2-dimethylpropyl)acetamide (810 mg, 3.68 mmol) and *N*,*N*-diisopropylethylamine (0.640 mL, 3.68 mmol), and the reaction mixture was stirred at 80 °C for 3 h. After cooling to 0 °C, H_2O (50 mL) was added to the reaction mixture. The resulting precipitate was triturated and collected by filtration to give the title compound (1.29 g, 3.44 mmol, 93%) as a colorless solid. ^{1}H NMR ($CDCl_3$) δ: 0.82 (9H, s), 2.89 (2H, d, J = 6.3 Hz), 3.44 (2H, s), 7.29 (2H, d, J = 8.6 Hz), 7.53 (2H, d, J = 8.6 Hz), 7.91-7.95 (2H, m), 8.42 (1H, s), 8.76 (1H, s), 11.45 (1H, s); MS (ESI) *m/z*: 376 $(M+H)^+$.

5.1.34.

4-[4-[2-(2,2-Dimethylpropylamino)-2-oxo-ethyl]anilino]-2-(3-pyridyl)pyrimidine-5-carboxamide (29a)

A solution of **28** (40.0 mg, 0.106 mmol), pyridine-3-boronic acid (26.2 mg, 0.213 mmol), [1,1'-bis(diphenylphosphino)ferrocene]dichloropalladium(II), complex with dichloromethane (17.4 mg, 0.0213 mmol), and sodium carbonate (33.8 mg, 0.319 mmol) in 1,2-dimethoxyethane (2 mL) and H_2O (1 mL) was heated in a microwave reactor at 130 °C for 20 min. After cooling, the reaction mixture was diluted with MeOH (2 mL) and filtered. The filtrate was concentrated *in vacuo* and the residue was chromatographed (NH silica gel, THF/CH_2Cl_2 = 0% to 80%) to give the title compound (31.0 mg, 0.0741 mmol, 70%) as a pale yellow amorphous solid. ^{1}H NMR (DMSO-d_6) δ: 0.83 (9H, s), 2.90 (2H, d, J = 6.7 Hz), 3.46 (2H, s), 7.34 (2H, d, J = 8.5 Hz), 7.58 (1H, dd, J = 7.9, 4.9 Hz), 7.69 (2H, d, J = 8.5 Hz), 7.88-7.98 (2H, m), 8.46 (1H, s), 8.62 (1H, d, J = 7.9 Hz), 8.74 (1H, dd, J = 4.9, 1.8 Hz), 9.01 (1H, s), 9.46 (1H, s), 11.40 (1H, s); MS (ESI) *m/z*: 419 $(M+H)^+$; HRMS (ESI) Calcd for $C_{23}H_{26}N_6O_2$+H: 419.2195. Found: 419.2192.

5.1.35.

4-[4-[2-(2,2-Dimethylpropylamino)-2-oxo-ethyl]anilino]-2-pyrimidin-5-yl-pyrimidine-5-carboxamide (29b)

Compound **29b** was synthesized from **28** with 5-pyrimidineboronic acid according to the procedure used to prepare **29a**. (pale yellow amorphous solid, Yield 25%) ^{1}H NMR (DMSO-d_6) δ: 0.84 (9H, s), 2.90 (2H, d, J = 6.7 Hz), 3.46 (2H, s), 7.35 (2H, d, J = 8.5 Hz), 7.67 (2H, d, J = 8.5 Hz), 7.94-7.98 (2H, m), 8.50 (1H, s), 9.03 (1H, s), 9.36 (1H, s), 9.54 (2H, s), 11.41 (1H, s); MS (ESI) *m/z*: 420 $(M+H)^+$; HRMS (ESI) Calcd for $C_{22}H_{25}N_7O_2$+H: 420.2148. Found: 420.2147.

5.1.36.

4-[4-[2-(2,2-Dimethylpropylamino)-2-oxo-ethyl]anilino]-2-(3-thienyl)pyrimidine-5-carboxamide (29c)

Compound **29c** was synthesized from **28** with 3-thiopheneboronic acid according to the procedure used to prepare **29a**. (pale brown solid, Yield 20%) ^{1}H NMR (DMSO-d_6) δ: 0.82 (9H, s), 2.89 (2H, d, J = 6.7 Hz), 3.45 (2H, s), 7.32 (2H, d, J = 8.5 Hz), 7.59-7.61 (1H, m), 7.65-7.71 (3H, m), 7.76 (1H, dd, J = 4.9, 1.2 Hz), 7.94 (1H, t, J = 6.7 Hz), 8.36-8.40 (2H, m), 8.92 (1H, s), 11.37 (1H, s); MS (ESI) *m/z*: 424 $(M+H)^+$; HRMS (ESI) Calcd for $C_{22}H_{25}N_5O_2S$+H: 424.1807. Found: 424.1812.

5.1.37.

4-[4-[2-(2,2-Dimethylpropylamino)-2-oxo-ethyl]anilino]-2-(3-furyl)pyrimidine-5-carboxamide (29d)

Compound **29d** was synthesized from **28** with 3-furanboronic acid according to the procedure used to prepare **29a**. (colorless solid, Yield 16%) ^{1}H NMR (DMSO-d_6) δ: 0.82 (9H, s), 2.89 (2H, d, J = 6.7 Hz), 3.44 (2H, s), 6.98 (1H, s), 7.32 (2H, d, J = 8.5 Hz), 7.69 (2H, d, J = 8.5 Hz), 7.82-7.84 (2H, m), 7.93 (1H, t, J = 6.7 Hz), 8.39 (1H, s), 8.42 (1H, s), 8.89 (1H, s), 11.41 (1H, s); MS (ESI) *m/z*: 408 $(M+H)^+$; HRMS (ESI) Calcd for $C_{22}H_{25}N_5O_3$+H: 408.2036. Found: 408.2052.

5.1.38.

4-[4-[2-(2,2-Dimethylpropylamino)-2-oxo-ethyl]anilino]-2-(1*H*-indol-5-yl)pyrimidine-5-carboxamide (29e)

Compound **29e** was synthesized from **28** with 5-indolylboronic acid according to the procedure used to prepare **29a**. (pale brown solid, Yield 41%) ^{1}H NMR (DMSO-d_6) δ: 0.84 (9H, s), 2.91 (2H, d, J = 6.7 Hz), 3.47 (2H, s), 6.57 (1H, s), 7.36 (2H, d, J = 8.5 Hz), 7.42-7.44 (1H, m), 7.49 (1H, d, J = 8.5 Hz), 7.74-7.78 (3H, m), 7.96 (1H, t, J = 6.7 Hz), 8.18 (1H, dd, J = 8.5, 1.8 Hz), 8.36 (1H, s), 8.66 (1H, s), 8.97 (1H, s), 11.36-11.39 (2H, m); MS (ESI) *m/z*: 457 $(M+H)^+$; HRMS (ESI) Calcd for $C_{26}H_{28}N_6O_2$+H: 457.2352. Found: 457.2342.

5.1.39.

4-[4-[2-(2,2-Dimethylpropylamino)-2-oxo-ethyl]anilino]-2-isopropenyl-pyrimidine-5-carboxamide (29f)

Compound **29f** was synthesized from **28** with 2-isopropenyl-4,4,5,5-tetramethyl-1,3,2-dioxaborolane according to the procedure used to prepare **29a**. (pale yellow solid, Yield 12%) ^{1}H NMR (DMSO-d_6) δ: 0.81 (9H, s), 2.15 (3H, s), 2.88 (2H, d, J = 6.7 Hz), 3.42 (2H, s), 5.59 (1H, s), 6.38 (1H, s), 7.27 (2H, d, J = 8.5 Hz), 7.65 (2H, d, J = 8.5 Hz), 7.82 (1H, s), 7.92 (1H, t, J = 6.7 Hz), 8.37 (1H, s), 8.88 (1H, s), 11.29 (1H, s); MS (ESI) *m/z*: 382 $(M+H)^+$; HRMS (ESI) Calcd for $C_{21}H_{27}N_5O_2$+H: 382.2243. Found: 382.2238.

5.1.40.

2-(Dimethylamino)-4-[4-[2-(2,2-dimethylpropylamino)-2-oxo-ethyl]anilino]pyrimidine-5-carboxamide (30a)

A solution of **28** (20.0 mg, 0.0532 mmol) and 50% dimethylamine solution in H_2O (0.0280 mL, 0.266 mmol) in DMF (1 mL) was heated at 80 °C for 4 h. After cooling, the reaction mixture was concentrated *in vacuo* and the residue was chromatographed (NH silica gel, THF/CH_2Cl_2 = 0% to 100%) to give the title compound (15.0 mg, 0.0390 mmol, 73%) as a colorless amorphous solid. ^{1}H NMR (DMSO-d_6) δ: 0.81 (9H, s), 2.88 (2H, d, J = 6.1 Hz), 3.15 (6H, s), 3.40 (2H, s), 7.21-30 (3H, m), 7.60 (2H, d, J = 8.5 Hz), 7.86-7.96 (2H, m), 8.60 (1H, s), 11.50 (1H, s); MS (ESI) *m/z*: 384 $(M+H)^+$; HRMS (ESI) Calcd for $C_{20}H_{28}N_6O_2$+H: 385.2352. Found: 385.2351.

5.1.41.

4-[4-[2-(2,2-Dimethylpropylamino)-2-oxo-ethyl]anilino]-2-isopropenyl-pyrimidine-5-carboxamide (30b)

Compound **30b** was synthesized from **28** with pyrrolidine according to the procedure used to prepare **30a**. (colorless amorphous solid, Yield 100%) ^{1}H NMR (DMSO-d_6) δ: 0.81 (9H, s), 1.91-1.95 (4H, m), 2.88 (2H, d, J = 6.7 Hz), 3.40 (2H, s), 3.51-3.55 (4H, m), 7.20-32 (3H, m), 7.66 (2H, d, J = 8.5 Hz), 7.85-7.94 (2H, m), 8.59 (1H, s), 11.53 (1H, s); MS (ESI) *m/z*: 411 $(M+H)^+$; HRMS (ESI) Calcd for $C_{22}H_{30}N_6O_2$+H: 411.2508. Found: 411.2517.

5.1.42.

4-[4-[2-(2,2-Dimethylpropylamino)-2-oxo-ethyl]anilino]-2-(1-piperidylamino)pyrimidine-5-carboxamide (30c)

A solution of **28** (20.0 mg, 0.0532 mmol) and 1-aminopiperidine (0.0287 mL, 0.266 mmol) in MeCN (1 mL) was heated at 80 °C for 2 h. After cooling, the reaction mixture was concentrated *in vacuo* and the residue was purified by reverse phase preparative HPLC (0.1% HCO_2H MeCN / 0.1% HCO_2H aq. = 0% to 100%) to give the title compound (10.0 mg, 0.0228 mmol, 34%) as a pale yellow solid. ^{1}H NMR (DMSO-d_6) δ: 0.81 (9H, s), 1.36-1.42 (2H, m), 1.60-1.67 (4H, m), 2.74-2.79 (4H, m), 2.88 (2H, d, J = 6.1 Hz), 3.39 (2H, s), 7.22-7.29 (3H, m), 7.52 (1H, s), 7.65 (2H, d, J = 8.5 Hz), 7.90-8.00 (2H, m), 8.33 (1H, s), 11.52 (1H, s), 11.64 (1H, s); MS (ESI) *m/z*: 440 $(M+H)^+$; HRMS (ESI) Calcd for $C_{23}H_{33}N_7O_2$+H: 440.2774. Found: 440.2771.

5.1.43. Ethyl 3-[(3-ethoxy-3-oxo-propyl)amino]-3-oxo-propanoate (33)

To a solution of β-alanine ethyl ester hydrochloride (9.29 g, 60.5 mmol) and *N*,*N*-diisopropylethylamine (21.6 mL, 124 mmol) in CH_2Cl_2 (100 mL) was added ethyl malonyl chloride (7.90 mL, 61.7 mmol) at 0 °C. The reaction mixture was warmed to room temperature and stirred for 1 h. 1 N HCl aqueous solution (100 mL) was added to the reaction mixture and resultant mixture extracted 3 times with CH_2Cl_2 (50 mL). The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The resulting residue was chromatographed (NH silica gel, EtOAc/CH_2Cl_2 = 0% to 20%) to give the title compound (11.56 g, 50.0 mmol, 83%) as a pale yellow oil. ^{1}H

NMR ($CDCl_3$) δ: 1.29-1.33 (6H, m), 2.58 (2H, t, J = 6.3 Hz), 3.32 (2H, s), 3.60 (2H, q, J = 6.3 Hz), 4.18-4.25 (4H, m), 7.54 (1H, s).

5.1.44. Methyl 2,4-dioxopiperidine-3-carboxylate (34)

To a solution of **33** (11.5 g, 49.7 mmol) in toluene (200 mL) was added dropwise 28% sodium methoxide solution in MeOH (28.7 g, 149 mmol). The reaction mixture was warmed to 100 °C and stirred for 7 h. After cooling, H_2O (200 mL) was added to the reaction mixture and the resultant mixture stirred vigorously. After a separation of the toluene layer, conc. HCl was added dropwise to the reaction mixture until the solution became acidic. The resulting solution was extracted eight times with MeOH/CH_2Cl_2 (1:4, v/v). The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated *in vacuo* to give the unpurified title compound (3.07 g, 17.9 mmol, 36%) as a pale yellow solid. This compound was used for the next reaction without further purification. ^{1}H NMR ($CDCl_3$) δ: 2.73 (2H, t, J = 6.9 Hz), 3.41-3.45 (2H, m), 3.94 (3H, s), 5.83 (1H, s); MS (ESI) *m/z*: 172 $(M+H)^+$.

5.1.45. 4-Hydroxy-2-phenyl-7,8-dihydro-6*H*-pyrido[4,3-*d*]pyrimidin-5-one (35)

To a solution of **34** (1.00 g, 5.84 mmol) and benzamidine hydrochloride (1.83 g, 11.7 mmol) in DMF (25 mL) was added DBU (2.62 mL, 17.5 mmol) and the resulting mixture was warmed to 100 °C. After stirring at 100 °C for 14 h, the reaction mixture was cooled to room temperature and concentrated in *vacuo*. The resulting residue was

chromatographed ($MeOH/CH_2Cl_2$ = 0% to 10%) to give the title compound (922 mg, 3.82 mmol, 65%) as a pale yellow solid. ^1H NMR (DMSO-d_6) δ: 3.04-3.10 (2H, m), 3.54-3.60 (2H, m), 7.52-7.58 (4H, m), 8.36-8.40 (2H, m), 8.79 (1H, s); MS (ESI) *m/z*: 242 $(M+H)^+$.

5.1.46. Ethyl 2-[4-[(5-oxo-2-phenyl-7,8-dihydro-6*H*-pyrido[4,3-*d*]pyrimidin-4-yl)amino]phenyl]acetate (37)

A solution of **35** (31.0 mg, 0.128 mmol) in $POCl_3$ (1.5 mL) was stirred at 100 °C for 2 h. After cooling, the reaction mixture was concentrated *in vacuo*. The resulting residue was neutralized with sat. Na_2CO_3 aqueous solution and extracted 3 times with $CHCl_3$. The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated *in vacuo* to give the unpurified 4-chloro-2-phenyl-7,8-dihydro-6*H*-pyrido[4,3-*d*]pyrimidin-5-one (**36**) as a dark red oil. This compound was used for the next reaction without further purification. MS (ESI) *m/z*: 260 $(M+H)^+$.

To a solution of crude **36** in DMF (1 mL) and MeCN (2 mL) was added ethyl 2-(4-aminophenyl)acetate (22.9 mg, 0.128 mmol) and the reaction mixture was stirred at 80 °C for 1.5 h. After cooling, the reaction mixture was concentrated *in vacuo* and the resulting residue was purified by reverse phase preparative HPLC (0.1% HCO_2H MeCN / 0.1% HCO_2H aq. = 0% to 100%) to give the title compound (15.0 mg, 0.0373 mmol, 29%) as a pale yellow solid. ^1H NMR ($CDCl_3$) δ: 1.20 (3H, t, J = 7.2 Hz), 3.01 (2H, t, J

= 6.9 Hz), 3.50 (2H, dt, *J* = 6.9, 2.3 Hz), 3.67 (2H, s), 4.10 (2H, q, *J* = 7.2 Hz), 7.33 (2H, d, *J* = 8.6 Hz), 7.53-7.56 (3H, m), 7.75 (2H, d, *J* = 8.6 Hz), 8.32 (1H, s), 8.38 (2H, dd, *J* = 7.7, 2.0 Hz), 11.55 (1H, s); MS (ESI) *m/z*: 403 $(M+H)^+$.

5.1.47.

2-[4-[(5-Oxo-2-phenyl-7,8-dihydro-6*H*-pyrido[4,3-*d*]pyrimidin-4-yl)amino]phenyl] acetic acid sodium salt (38)

To a solution of **37** (15.0 mg, 0.0373 mmol) in THF (0.5 mL), EtOH (1 mL), and H_2O (0.3 mL) was added 1 N NaOH aqueous solution (0.112 mL) and the mixture was stirred for 3 h. After 1 N HCl aqueous solution (0.112 mL) was added, the solvent was evaporated and the resulting residue was purified by reverse phase preparative HPLC (0.1% HCO_2H MeCN / 0.1% HCO_2H aq. = 0% to 100%) to give the title compound (free form, 10.7 mg, 0.0286 mmol, 77%) as a colorless solid.

To a solution of **38** (free form) in MeOH (1 mL) and H_2O (1 mL) was added 1N NaOH aqueous solution (0.0286 mL). After stirring at room temperature for 0.5 h, the reaction mixture was concentrated in *vacuo* and the resulting residue was dried by vacuum pump to give the title compound (Na salt, 11.3 mg, 0.0285 mmol) as a pale yellow solid. ^{1}H NMR (DMSO-d_6) δ: 2.99 (2H, t, *J* = 6.9 Hz), 3.14 (2H, s), 3.49 (2H, dt, *J* = 6.9, 2.9 Hz), 7.24 (2H, d, *J* = 8.6 Hz), 7.54-7.56 (3H, m), 7.59 (2H, d, *J* = 8.6 Hz), 8.27 (1H, s), 8.37-8.39 (2H, m), 11.41 (1H, s); MS (ESI) *m/z*: 375 $(M+H)^+$; HRMS (ESI) Calcd for $C_{21}H_{18}N_4O_3$-H: 373.1301. Found: 373.1295.

5.1.48.

***N*-isobutyl-2-[4-[(5-oxo-2-phenyl-7,8-dihydro-6*H*-pyrido[4,3-*d*]pyrimidin-4-yl)amino]phenyl]acetamide (39)**

To a solution of **38** (19.0 mg, 0.0507 mmol) in DMF (1.5 mL) were added *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (38.4 mg, 0.101 mmol), *N*,*N*-diisopropylethylamine (0.0354 mL, 0.203 mmol), and isobutylamine (7.39 mg, 0.101 mmol). The reaction mixture was stirred at room temperature for 17 h and concentrated *in vacuo*. The resulting residue was purified by reverse phase preparative HPLC (0.1% HCO_2H MeCN / 0.1% HCO_2H aq. = 0% to 100%) to give the title compound (9.70 mg, 0.0226 mmol, 45%) as a colorless solid. ^{1}H NMR (DMSO-d_6) δ: 0.84 (6H, d, J = 6.9 Hz), 1.66-1.71 (1H, m), 2.89 (2H, t, J = 6.3 Hz), 3.00 (2H, q, J = 6.9 Hz), 3.42 (2H, s), 3.49 (2H, dt, J = 6.9, 2.9 Hz), 7.32 (2H, d, J = 8.6 Hz), 7.52-7.57 (3H, m), 7.71 (2H, d, J = 8.6 Hz), 8.02 (1H, t, J = 6.0 Hz), 8.30 (1H, s), 8.36-8.38 (2H, m) 11.38 (1H, s); MS (ESI) *m/z*: 430 $(M+H)^+$; HRMS (ESI) Calcd for $C_{25}H_{27}N_5O_2$+H: 430.2243. Found: 430.2249.

5.2. *In vitro* assay

5.2.1. hPDE4B assay

Test compounds dissolved in DMSO or DMSO for controls were pre-incubated with recombinant human PDE4B (152-564) in a 96-well plate at room temperature for 5 min. The enzymatic reaction was initiated by the addition of cAMP followed by thorough mixing. Final concentrations of assay components were as follows: 50 mM Tris, pH 7.4,

6 mM $MgCl_2$, 1% DMSO, 60 nM cAMP. Plates were incubated at 37 °C for 30 min. The concentration of cAMP in each well was determined using a cAMP detection kit (cAMP dynamic 2, Cisbio Bioassays, France) and a plate reader (RUBYstar, BMG LABTECH, Ortenberg, Germany) according to the manufacturer's procedure. The IC_{50} value of each compound was calculated using Prism 4 software (GraphPad Software, La Jolla, CA).

5.2.2. TNF-alpha production assay

Splenocytes ($4x10^5$ cells/well) from Balb/c mice were suspended in RPMI 1640 medium containing 10% FBS in a 96-well plate. Test compounds dissolved in DMSO or DMSO for controls were added in each well, and then the cells were stimulated with 100 ng/ml LPS at 37 °C for 18 to 24 h in a CO_2 incubator. The final concentration of DMSO was 0.1%. The concentration of TNF-α in the supernatant of each well was determined using a TNF-alpha detection kit (AlphaLISA, PerkinElmer, Waltham, MA). The IC_{50} value of each compound was calculated using Prism 4 software.

5.3. *In vivo* assay

5.3.1. Animal: Eight week-old male BALB/c mice were purchased from Charles River Laboratories Japan, Inc. and FR2 diets were from Funabashi Farms Co., Ltd. Mice were acclimatized for over 1 wk and kept under a 12 h dark-light cycle, and provided with water and FR2 diet *ad libitum*. All experimental procedures were performed in

accordance with the guideline of the Institutional Animal Care and Use Committee of Daiichi Sankyo Co., Ltd.

5.3.2. LPS-induced pulmonary neutrophilia: Overnight fasted mice were randomly divided into a negative control group, a positive control group and test compound groups. Mice were intraperitoneally administered with an appropriate dose of test compounds (10 mL/kg) or vehicle alone (0.5% methylcellulose) 30 min before LPS inhalation. When the test compound was administered intratracheally, mice were anesthetized with 12 mg/kg of pentobarbital followed by intratracheal administration of 1 mg/kg of the test compound (1 μL/kg) or vehicle (0.5% methylcellulose) alone using cannula (23 G) 30 min before LPS inhalation.

LPS (Lipopolysaccharides from Salmonella minnesota, L6261, Sigma) were dissolved in saline (0.01 mg/mL) for nebulization. Mice were placed in a stainless steel wire-cage in an exposure camber. LPS solution was aerosolized for 40 min with a constant-output nebulizer (Omron Corporation) and all of the output was directed into the exposure chamber. Aerosolized saline was alternatively used for the negative control group. Four to five hours after saline or LPS inhalation, animals were anesthetized with 12 mg/kg of sodium pentobarbital (i.p.). After semiexcision of the trachea, a plastic cannula was inserted, and airspaces were washed 3 times with 1.0 mL saline to provide 3 mL of bronchoalveolar lavage fluid (BALF). Aliquots of each BALF were centrifuged (1,000 rpm, for 15 min). The supernatant was removed, and the resulting cell pellets were resuspended in 300 μL of saline. Neutrophil was counted using XT-2000 iv (Sysmex Corporation) and the total number of neutrophil was quantified. ID_{50} values

were calculated by linear regression analysis using percent inhibitions of total numbers of neutrophil.

5.4. Distribution coefficient (log $D_{7.4}$)

The distribution coefficient between 1-octanol and phosphate buffered saline (PBS) was assayed by a shaking flask method. Equal amounts of PBS and 1-octanol were shaken and left for over 12 h. The upper layer (1-octanol) and lower layer (PBS) were collected individually. Test compound was dissolved in 1-octanol or PBS (200 μM). The same amount of either PBS or 1-octanol was added and the mixture was shaken vigorously for 30 min at room temperature. Then, both phases were separated and assayed by HPLC or LC/MS. Log $D_{7.4}$ was calculated by the following equation: Log $D_{7.4}$ = log (Peak area of compound in 1-octanol / Peak area of compound in PBS).

5.5. Metabolic stability assay using mouse liver microsomes

Compound **10f** was incubated with 0.1 mg protein/mL of mouse liver microsmes for 30 min, then the remaining was quantified by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).

5.6. Pharmacokinetics studies in mice

Animal care and in vivo procedures were conducted in accordance with the National Institutes of Health Guidelines for Laboratory Animal Welfare (Institute of Laboratory Animal Resources, NRC, 1996)[12]. The institutional animal care and use committee approved the protocols.

In the experiments, **10f** was administered to male mice intraperitoneally at 10 mg/kg and the concentrations in plasma were determined by LC-MS/MS. The area under the plasma concentration curve (AUC) and the terminal half-life ($T_{1/2}$) were calculated from the plasma concentration-time profile. C_{max} was taken directly from the concentration–time profile.

5.7. X-ray crystallography

For the crystallographic study, hPDE4B (152-528) was cloned, expressed, and purified according to the reported procedure[11]. Crystals of hPDE4B in complex with compound **10f** were grown at 277 K using the sitting drop vapor diffusion method by mixing equal volumes of the protein at ca. 15 mg/mL with 10-20% PEG3350, 0.2-0.4 M $CaCl_2$ or Ca acetate and 0.1 M Tris/HCl (pH 8.0) in the presence of ca. 2 mM of compound **10f**. The coordinates and statistics are available from the PDB using accession code 3WD9.

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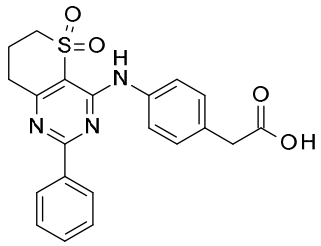
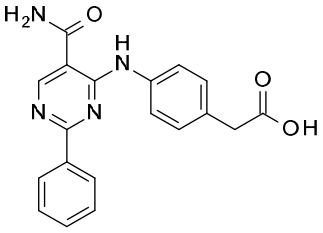


1

hPDE4B (IC_{50}) = 25 nM

mTNF-α (IC_{50}) = 390 nM

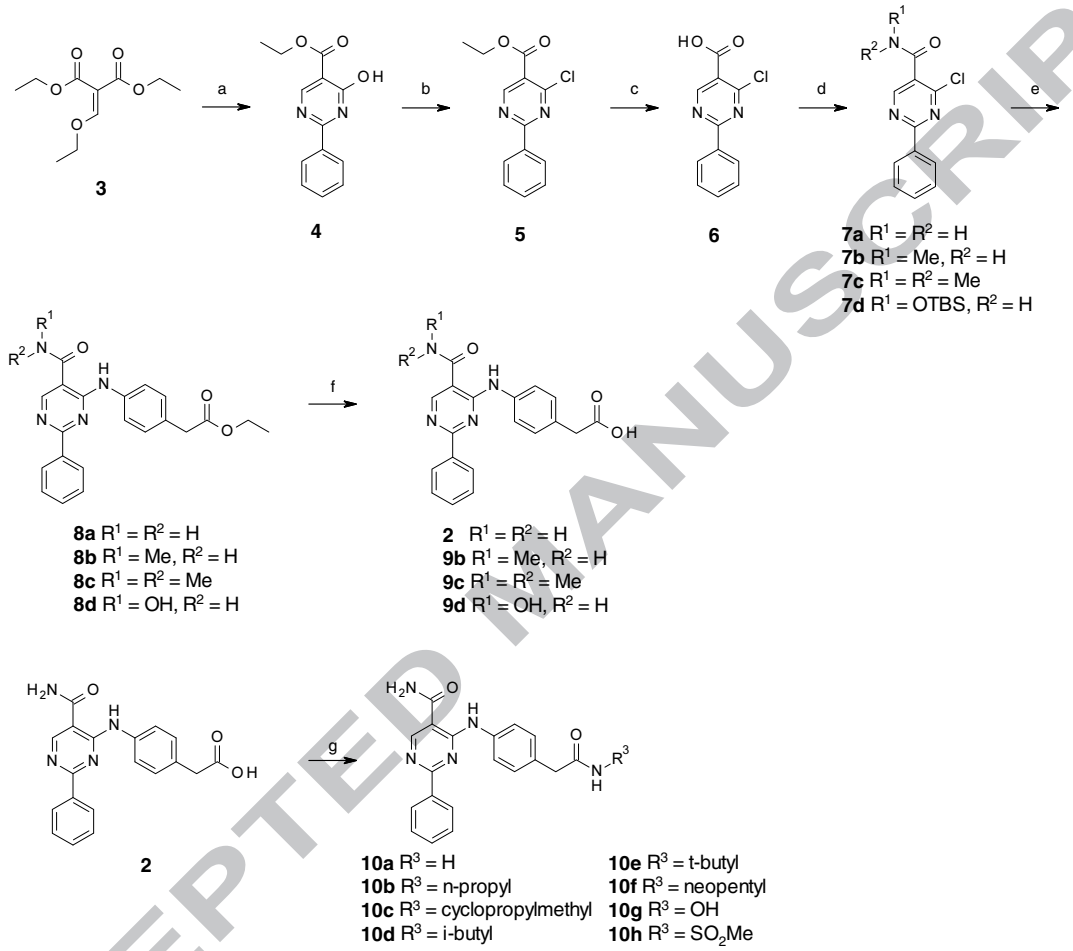


2

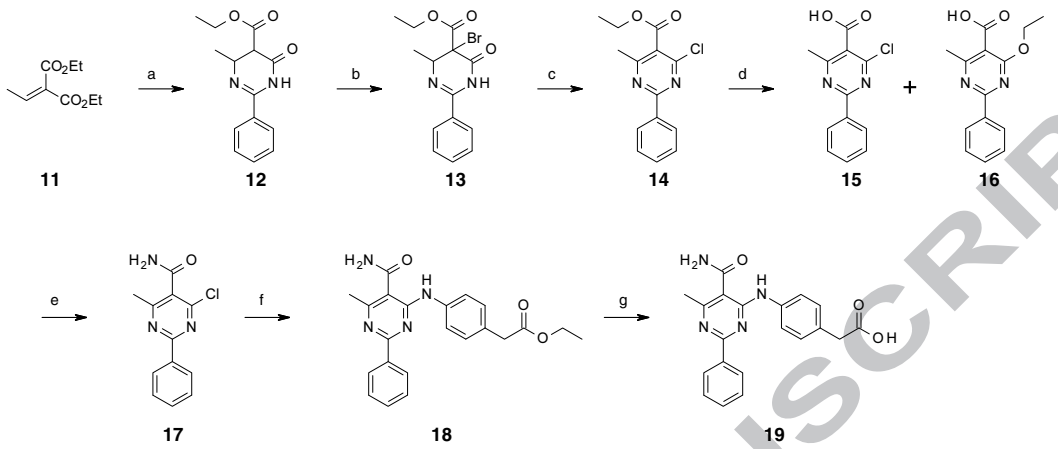
hPDE4B (IC_{50}) = 200 nM

mTNF-α (IC_{50}) = 690 nM

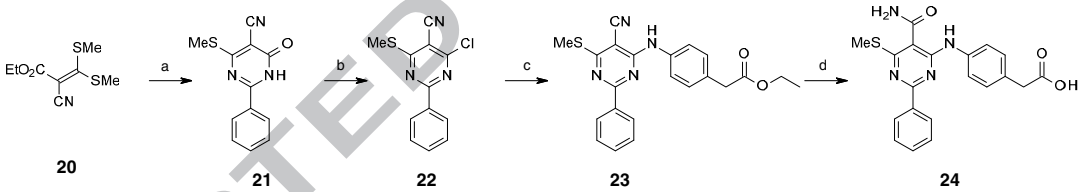
Figure 1: Structure of previous reported compound **1** and new lead compound **2**.



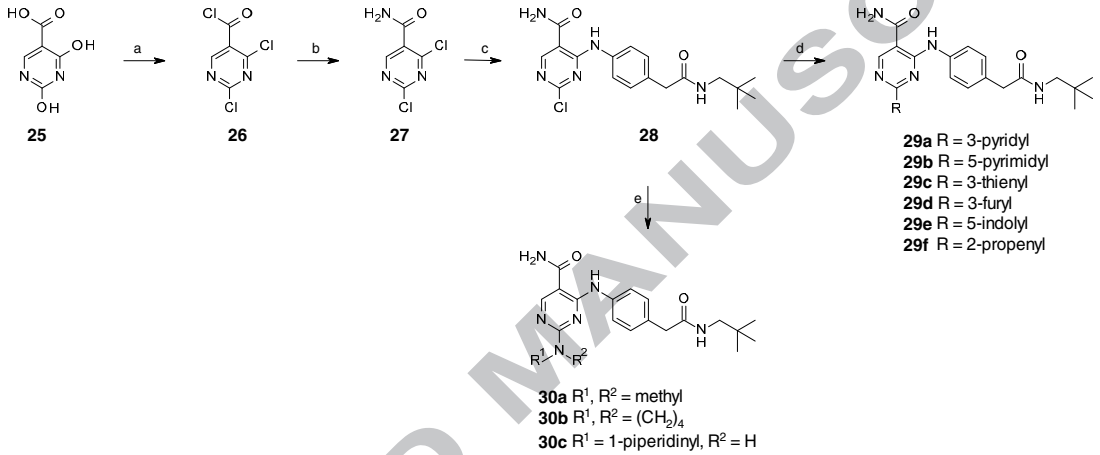
Scheme 1. Reagents and conditions: (a) benzamidine, NaOH, EtOH (83%); (b) $POCl_3$, 100 °C (67%); (c) 1N NaOH, THF, EtOH, H_2O; (d) (i) $(COCl)_2$, DMF, CH_2Cl_2; (ii) amine; (e) ethyl 2-(4-aminophenyl)acetate, DMF, 60 °C; (f) 1N NaOH, THF, EtOH, H_2O; (g) amine, WSC, HOBt, DIPEA, DMF.



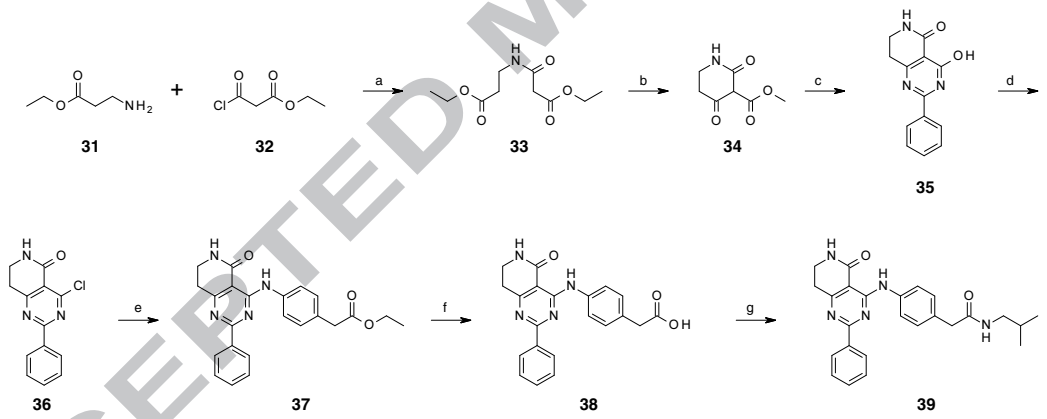
Scheme 2. Reagents and conditions: (a) benzamidine, DIPEA, MeOH (60%); (b) NBS, benzoyl peroxide, K_2CO_3, CCl_4 (18%); (c) $POCl_3$, 100 °C (74%); (d) 1N NaOH, THF, EtOH, H_2O, 50 °C; (e) (i) $(COCl)_2$, DMF, CH_2Cl_2; (ii) 28% NH_3, THF; (f) ethyl 2-(4-aminophenyl)acetate, DMF, 80 °C; (g) 1N NaOH, THF, EtOH, H_2O (29% from **14**).



Scheme 3. Reagents and conditions: (a) benzamidine, TEA, EtOH (92%); (b) $POCl_3$, $PhNEt_2$, 100 °C (91%); (c) ethyl 2-(4-aminophenyl)acetate, DMF, 80 °C (83%); (d) conc. H_2SO_4 (5%).



Scheme 4. Reagents and conditions: (a) PCl_5, $POCl_3$, 100 °C; (b) 28% NH_3 (55% from **25**); (c) 2-(4-aminophenyl)-N-(2,2-dimethylpropyl)acetamide, DIPEA, MeCN, 85 °C; (d) boronic acid, $PdCl_2$dppf, Na_2CO_3, H_2O, DME; (e) amine, DMF, 85 °C.



Scheme 5. Reagents and conditions: (a) DIPEA, CH_2Cl_2 (81%); (b) NaOMe, MeOH, toluene, 100 °C (42%); (c) benzamidine, DBU, DMF 100 °C (35%); (d) $POCl_3$, 100 °C; (e) ethyl 2-(4-aminophenyl)acetate, MeCN, DMF (29% from **35**); (f) 1N NaOH, THF, EtOH, H_2O (77%); (g) isobutylamine, HATU, DIPEA, DMF (44%).

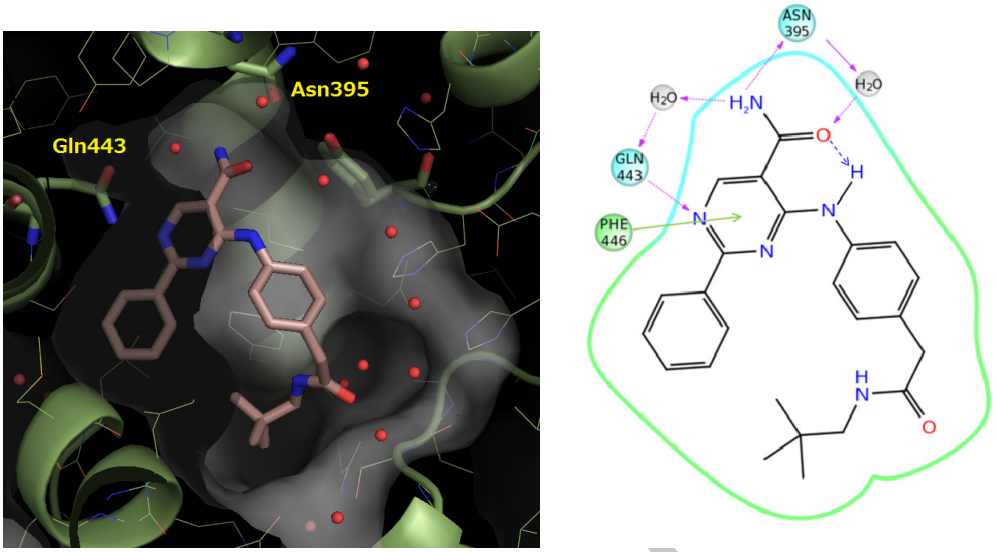
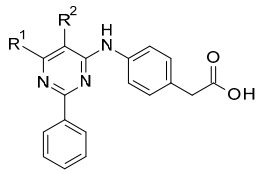
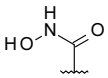


Figure 2. X-ray crystal structure of **10f** with human PDE4B catalytic binding site.

Table 1

In vitro inhibitory activity of compounds **2**, **19**, **24**, and **9b-d**.



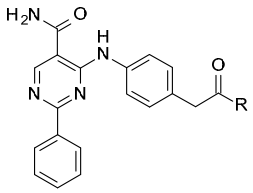
	R^1	R^2	hPDE4B IC_{50} (nM)
2	H		200
19	Me		4000
24	SMe		2000
9b	H		8200
9c	H		8%[a]
9d	H		42%[b]

[a] Inhibition% at 10 μM.

[b] Inhibition% at 3 μM.

Table 2

In vitro inhibitory activity of compounds **2**, **10a-h**, and Roflumilast.



	R	hPDE4B IC_{50} (nM)	mTNF-α IC_{50} (nM)
2		200	690
10a		120	74

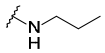
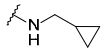
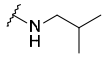
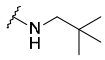
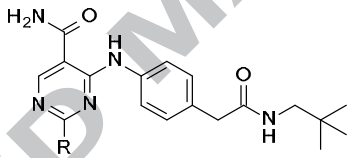
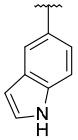
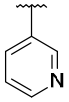
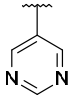
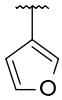
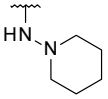
10b		120	47
10c		16	18
10d		11	12
10e		110	49
10f		8.3	3.0
10g		7.1	43
10h		490	4800
Roflumilast		0.49	1.2

Table 3

In vitro profile of compounds **10f**, **29a-f**, and **30a-c**.



	R	hPDE4B IC_{50} (nM)	mTNF-α IC_{50} (nM)		R	hPDE4B IC_{50} (nM)	mTNF-α IC_{50} (nM)
10f		8.3	3.0	**29e**		8.2	840
29a		29	23	**29f**		3.7	9.6
29b		110	NT[a]	**30a**		16	7.1

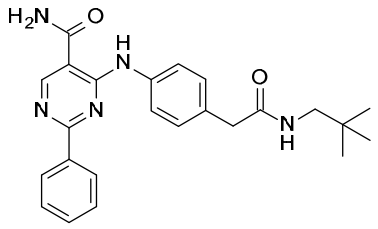
29c		18%[b]	NT[a]	**30b**		13	3.8
29d		11	1000	**30c**		6500	NT[a]

[a] NT: not tested

[b] Inhibition% at 1 μM

Table 4

Structure and profile of **10f**.

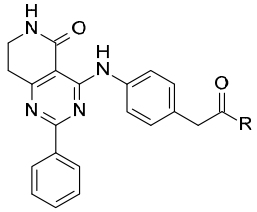


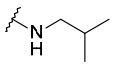
hPDE4B (152-564) IC_{50} (nM)	mTNF-α IC_{50} (nM)	LogD (pH=7.4)	Metabolic stability in mouse microsomes (%)*	LPS-induced mouse neutrophilia(i.p.) ID_{50} (mg/kg)	Mouse PK (10 mg/kg, i.p.)		
					AUC (μg h/mL)	C_{max} (μg/mL)	$T_{1/2}$ (h)
8.3	3.0	4.9	52	16	3.99	1.49	3.2

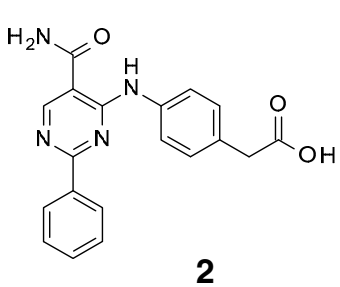
* The **10f** was incubated with 0.1 mg protein/mL of mouse liver microsomes for 30 min.

Table 5

In vitro and *in vivo* property of **38** and **39**.



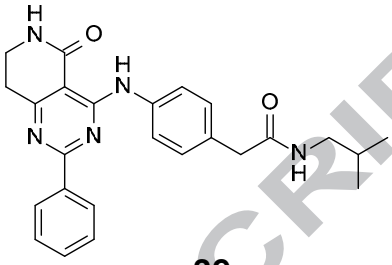
	R	hPDE4B IC_{50} (nM)	mTNF-α IC_{50} (nM)	LPS-induced mouse neutrophilia inhibition (1.0 mg/kg, i.t.)
38	OH	47	460	NT
39		7.3	0.21	41%
Roflumilast		0.49	1.2	47%



2

hPDE4B (IC_{50}) = 200 nM

mTNF-α (IC_{50}) = 690 nM



39

hPDE4B (IC_{50}) = 7.3 nM

mTNF-α (IC_{50}) = 0.21 nM