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# New orally active PDE4 inhibitors with therapeutic potential

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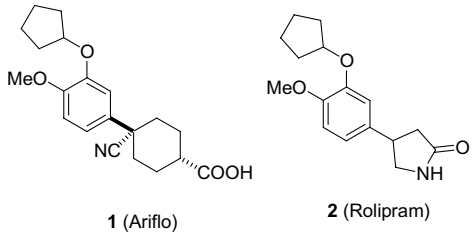
**Abstract**—The design, synthesis, and biological evaluation of a series of pyrazolopyridines was carried out. Structural optimization of the aniline moiety of 4-anilinopyrazolopyridine derivative **3a**, which is one of the newly discovered chemical leads for PDE4 inhibitors from our in-house library, was performed successfully. The details of the discovery of new orally active PDE4 inhibitors, which are expected to show therapeutic potential, are presented and their structure–activity relationships are discussed. Pharmacological evaluation and pharmacokinetic data for representative compounds are also presented.


## 1. Introduction

Phosphodiesterase 4 (PDE4) is an enzyme that is responsible for the inactivation of cyclic adenosine monophosphate (cAMP).[1,2] Inhibition of this enzyme results in the elevation of cAMP levels in inflammatory cells such as eosinophils. The anti-inflammatory effect of PDE4 inhibitors has been well documented in vitro, as well as in a variety of animal models,[3] but no inhibitor has yet come into used clinical use because of dose-limiting side effects such as nausea and vomiting that restrict therapeutic application.[4]

Two strategies have already been reported for the production of efficient PDE4 inhibitors with fewer side effects. Ariflo™ **1**[5] (Fig. 1) and the other second-generation PDE4 inhibitors were reported to be more LPDE4-selective[6] than (*R*)-rolipram **2** and/or to be PDE4D subtype-selective.[7,8]

In previous papers, we have described the design and synthesis of bicyclo[3·3·0]octane derivatives[9] and piperidine derivatives,[10] which are interesting new classes of PDE4 inhibitors with therapeutic potential. Structure–activity relationship (SAR) studies of PDE4 inhibitors based on the structural features of Ariflo **1** have revealed that its further modification yields a series of PDE4 inhibitors with an improved therapeutic index relative to that of the classical inhibitor, rolipram **2**. To design efficient new inhibitors with an improved therapeutic potential, the discovery and biological evaluation of a new chemical lead with a distinct structure was considered to provide us with another approach to the modification of rolipram **2**.



**1** (Ariflo) **2** (Rolipram)

**Figure 1.** Structures of Ariflo **1** and Rolipram **2**.

1*H*-Pyrazolo[3,4-*b*]pyridines have been shown to be potent inhibitors of various phosphodiesterases.[11–13] We focused on structural optimization of the 1,3-dimethyl-4-anilino-1*H*-pyrazolo[3,4-*b*]-pyridine-5-carboxamide **3a** (Fig. 2), which is one of the new chemical leads from our in-house library detected by the high throughput screening (HTS) technique. This paper presents the

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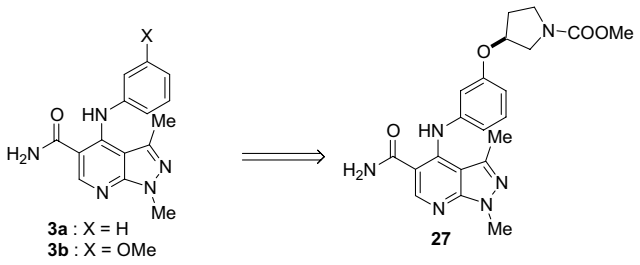



**Figure 2.** Discovery of chemical leads **3a**–**b** and identification of orally active PDE4 inhibitor **27**.

discovery process for orally bioavailable pyrazolo[3,4-*b*]pyridines, which are structurally new PDE4 inhibitors with therapeutic potential. Full details of the SAR studies are presented and pharmacokinetic data for representative compounds are also given.

## 2. Chemistry

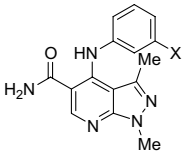
The general method of synthesis for test compounds **3**–**25** listed in Tables 1 and 2 is outlined in Scheme 1.[12] Michael addition of **31** to an acceptor diethylethoxymethylenemalonate, followed by removal of an ethoxy moiety, provided **32**, after which ring closure was accomplished with phosphorus oxychloride under reflux to afford the chloride **33**. Alkaline hydrolysis of **33** gave the carboxylic acid **34**. Heating **34** with thionyl chloride, followed by treatment with aqueous ammonia, resulted in the amide **35**. Replacement of the chloro moiety of **35** with appropriate anilines gave the test compounds **3**–**16** and **18**–**25**. Aminolysis of **35** with ethyl 3-aminobonzoate afforded the corresponding ethyl ester **37a**, which was converted to the corresponding amide **17** via carboxylic acid **37b** by the usual method.

The synthesis of test compounds **26**–**30** listed in Table 3 is outlined in Scheme 2. *O*-Alkylation of 3-nitrophenol **38** with *N*-methoxycarbonyl-(3*R*)-pyrrolidinol and *N*-*tert*-butoxycarbonyl-(3*R*)-pyrrolidinol afforded **39a** and **39b**, respectively. Acidic deprotection of **39b** gave **39c**, *N*-methanesulfonylation of which provided **39d**. Catalytic hydrogenation of **39a**, **39b**, and **39d** gave the corresponding anilines **40a**, **40b**, and **40d**, respectively. Aminolysis of the common intermediate **35** with **40a**–**b** and **40d** afforded **27**–**28** and **30**, respectively. Acidic deprotection of **28** resulted in **26**, *N*-acylation of which provided *N*-acetyl analog **29**.

## 3. Results and discussion

A series of pyrazolopyridine derivatives were synthesized and evaluated for their ability to inhibit phosphodiesterase type 4 prepared from U937 cells,[14] which were derived from human monocytes. The results of the assays are expressed as $IC_{50}$ values that is, the test compound concentration that achieved 50% inhibition relative to the vehicle. Test compounds were also evaluated for their ability to inhibit LPS-induced production of tumor necrosis factor-α (TNF-α) in rats.[15] Results are expressed as $ID_{50}$ values that is, the dose that achieved 50% inhibition relative to the vehicle.

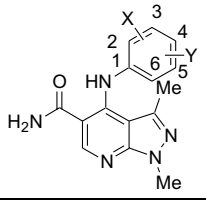
**Table 1.** Activity profile of 4-[(*meta*-substituted phenyl)amino]-1*H*-pyrazolo[3,4-*b*]pyridine derivatives




| Compd | X = | LPDE4[a] $IC_{50}$ (μM) | Inhibition of TNF-α[b] $ID_{50}$ (mg/kg, po) |
|---|---|---|---|
| **3a** | H | 0.04 | (48%)[c] |
| **3b** | OMe | 0.005 | 2.4 |
| **4** | OEt | 0.08 | (35%)[c] |
| **5** | O*n*Pr | >0.1 | NT[f] |
| **6** | O*i*Pr | >0.1 | NT[f] |
| **7** | O*c*Bu | >0.1 | NT[f] |
| **8** | $OCF_3$ | >0.1 | NT[f] |
| **9** | OH | 0.1 | (40%)[c] |
| **10** | SMe | 0.02 | NE[d] |
| **11** | $SCF_3$ | >0.1 | NT[f] |
| **12** | $NO_2$ | 0.03 | NE[d] |
| **13** | $NHCOCH_3$ | >0.1 | NT[f] |
| **14** | $NHSO_2Me$ | >0.1 | NT[f] |
| **15** | NHCOOMe | >0.3 | NT[f] |
| **16** | CN | 0.09 | NE[e] |
| **17** | $CONH_2$ | 0.2 | NT[f] |
| **18** | $CH_2OH$ | 0.1 | NE[e] |
| **19** | COMe | 0.01 | (42%)[c] |
| **20** | CCH | 0.02 | (53%)[c] |

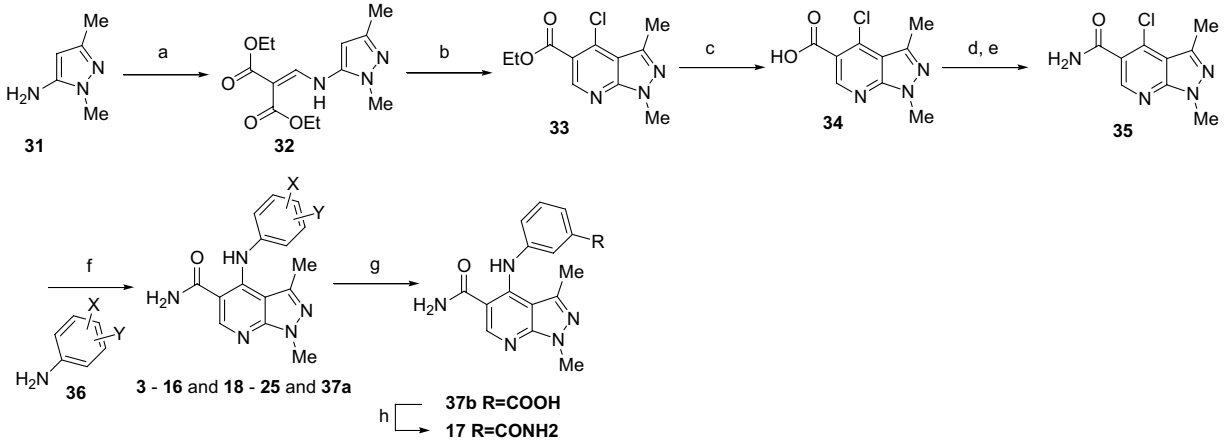
[a] Inhibition of PDE4 prepared from U937 cells (a cell line derived from human monocytes). $IC_{50}$ represent a mean of $n = 2$.
[b] $ID_{50}$ for inhibition of LPS-induced TNF-α production in rats ($n = 7$) 0.5 h after oral dosing of a test compound.
[c] Inhibition % at 10 mg/kg, po.
[d] Not effective at 10 mg/kg, po.
[e] Not effective at 3 mg/kg, po.
[f] Not tested.

**Table 2.** Activity profile of 4-[(disubstituted phenyl)amino]-1*H*-pyrazolo[3,4-*b*]pyridine derivatives



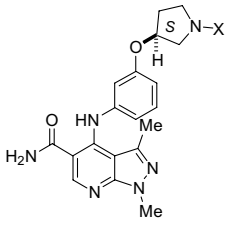

| Compd | X, Y | LPDE4[a] $IC_{50}$ (μM) | Inhibition of TNF-α[a] $ID_{50}$ (mg/kg, po) |
|---|---|---|---|
| **21** | 2-OMe, 4-OMe | >0.3 | NT[c] |
| **22** | 2-OMe, 5-OMe | 0.2 | NT[c] |
| **23** | 3-OMe, 4-OMe | 0.2 | NT[c] |
| **24** | 3-OMe, 5-OMe | 0.02 | (51%)[b] |
| **25** | 2-OMe, 3-OMe | >0.3 | NT[c] |

[a] See corresponding footnotes from Table 1.
[b] Inhibition % at 10 mg/kg, po.
[c] Not tested.




**Scheme 1.** Synthesis of compounds **3**–**25**. Reagents and conditions: (a) diethylethoxymethylenemalonate, 120 °C; (b) $POCl_3$, 120 °C; (c) KOH aq, dioxane; (d) $SOCl_2$, 80 °C; (e) $NH_3$ aq, THF; (f) **36**, dioxane, reflux; (g) NaOH aq, THF, EtOH, 50 °C; (h) EDC, HOBt, $NH_4OH$, DMF, THF.

**Table 3.** Activity profile of 4-({3-[(3*S*)-3-pyrrolodinyloxy]phenyl}-amino)-1*H*-pyrazolo[3,4-*b*]pyridine derivatives



| Compd | X = | LPDE4[a] $IC_{50}$ (μM) | Inhibition of TNF-α [a]$ID_{50}$ (mg/kg, po) |
|---|---|---|---|
| **26** | H | >0.3 | NT[b] |
| **27** | COOMe | 0.01 | 1 |
| **28** | COO*t*Bu | >0.3 | NT[b] |
| **29** | COMe | >0.3 | NT[b] |
| **30** | $SO_2Me$ | 0.3 | NT[b] |

[a] See corresponding footnotes from Table 1.
[b] Not tested.

During the course of screening PDE4 inhibitors, compound **3a** was shown to exhibit moderate potency by both in vitro and in vivo assays. A preliminary SAR study revealed that *meta*-substituted **3b** had 8-fold stronger in vitro activity and a significant increase of in vivo potency, as illustrated in Table 1. This result suggested that further optimization of the chemical lead **3a** could yield PDE4 inhibitors with therapeutic potential.

Thus, we focused on more detailed modification of the *meta*-substituted aniline moiety. As shown in Table 1, the synthesis and biological evaluation of 4-(3-substituted phenyl)amino-1,3-dimethylpyrazolopyridines was performed.

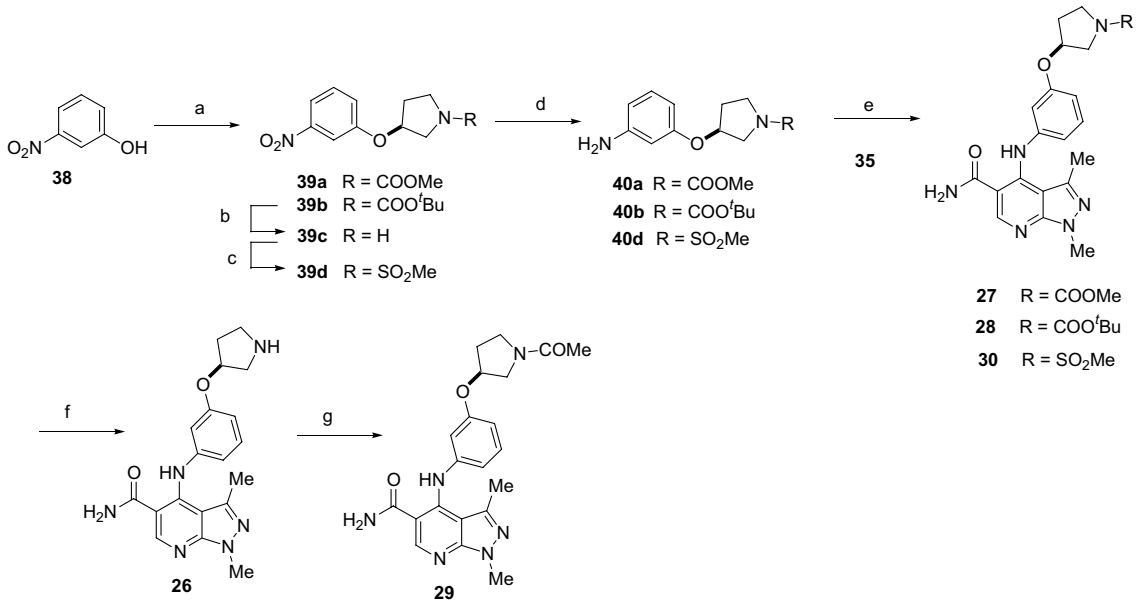
Replacement of the *meta*-methoxy group of **3b** with an ethoxy group afforded **4**, showing a 16-fold decline of LPDE4 inhibitory activity. Replacement of the *meta*-methoxy group of **3b** with a higher alkoxy group such as a propoxy, isopropoxy, or cyclobutyloxy group provided **5**, **6**, and **7**, respectively, which showed loss of LPDE4 inhibitory activity at 0.1 μM. Replacement of the *meta*-methoxy group of **3b** with a trifluoromethoxy group afforded **8**, which also showed loss of inhibitory activity at 0.1 μM. Demethylation of the *meta*-methoxy group of **3b** gave the *meta*-hydroxy derivative **9**, which showed nearly 20-fold weaker LPDE4 inhibitory activity. Introduction of an alkylthio moiety instead of the *meta*-methoxy group at the *meta*-position of **3b** afforded *meta*-methylthio and *meta*-trifluoromethylthio analogs **10** and **11**, which showed 4-fold weaker inhibitory activity and loss of inhibitory activity at 0.1 μM, respectively.

Several *meta*-nitrogen substituted analogs were also synthesized and evaluated. Replacement of the *meta*-methoxy group of **3b** with a nitro group gave **12**, which had 6-fold lower LPDE4 inhibitory activity. Replacement of the *meta*-methoxy group of **3b** with acetylamino, methylsulfonylamino, or methoxycarbonylamino moieties afforded **13**, **14**, and **15**, respectively, which all showed loss of LPDE4 inhibitory activity at the concentrations described.

Replacement of the *meta*-methoxy group of **3b** with carbon substituents such as nitrile, carbonylamide, hydroxymethyl, acetyl, and ethynyl moieties produced **16**–**20**, respectively, which showed 18-fold, 40-fold, 20-fold, 2-fold, and 4-fold lower LPDE4 activity, respectively.

Regarding inhibition of TNF-α production in rats, compounds **9**, **19**, and **20** showed nearly 50% inhibition at an oral dose of 10 mg/kg, while compound **4** showed 35% inhibition. Compounds **10**, **12**, **16**, and **18** did not have any inhibitory activity at an oral dose of 3 mg/kg. As a result, the *meta*-methoxy group was found to be the most optimal *meta*-substituent among the compounds tested.

In addition, 4-(dimethoxyphenyl)aminopyrazolopyridines **21**–**25** were synthesized and evaluated, as shown in Table 2. The 2,4- and 2,3-dimethoxyphenylamino analogs **21** and **25** did not inhibit LPDE4 at a concentration of 0.3 μM. The 3,5-dimethoxyphenylamino analog **24** showed 10-fold stronger LPDE4 inhibitory




**Scheme 2.** Synthesis of compounds **26**–**30**. Reagents and conditions: (a) *N*-methoxycarbonyl-(3*R*)-pyrrolidinol or *N*-*tert*-butoxycarbonyl-(3*R*)-pyrrolidinol, DEAD, $PPh_3$, THF; (b) TFA, $CH_2Cl_2$, 0 °C; (c) MsCl, $Et_3N$, $CH_2Cl_2$, 0 °C; (d) Pd/C, $H_2$, MeOH; (e) **35**, pyrazolopyridine-derivatives, dioxane, reflux; (f) 4 N HCl/EtOAc, MeOH; (g) $Ac_2O$, pyridine, 0 °C.

activity than the 2,5- and 3,4-dimethoxyphenylamino analogs **22** and **23**. Compound **24**, which showed the most potent in vitro activity among this series of analogs, was then evaluated in the LPS-induced TNF-α assay. Compound **24** (its LPDE4 inhibitory activity was 4-fold weaker than that of **3b**), showed 51% inhibition of TNF-α production at an oral dose of 10 mg/kg. As a result, substitution of both *meta*-positions was found to be most acceptable, although monosubstitution was superior to disubstitution, as illustrated in the higher potency of **3b** relative to **24**.

In addition, 2,4- and 2,3-substitution were found to weaken the inhibitory activity. Compounds **3a**–**b** demonstrated very poor bioavailability (BA) despite relatively good oral activity (the BA of **3a**–**b** was 8.2% and 1.7%, respectively). To develop this series of analogs, improvement of the poor BA was absolutely essential, so further chemical modification of **3a**–**b** was conducted to achieve better BA and good potency. We extended our optimization process of the *meta*-substituent of the 4-anilino moiety to introduction of the 3*S*-pyrrolidinyloxy moiety starting from simple alkoxy groups. As illustrated in Table 3, compound **27** was synthesized and biologically evaluated. This compound showed nearly equipotent in vitro inhibitory activity and more potent in vivo activity relative to the chemical lead **3b**, which strongly suggested an improved PK profile of **27**. Another analog of this series, **28**, was also prepared and evaluated, but it did not show in vitro activity at a concentration of 3 μM. Removal of the methoxycarbonyl moiety of **27** afforded **26**, which showed loss of LPDE4 inhibitory activity at 0.3 μM. The corresponding *N*-ethoxycarbonyl, *N*-propoxycarbonyl, and *N*-*iso*-propoxycarbonyl analogs, the structures of which are not presented here, also showed no inhibitory activity at 0.1 μM. Therefore, the *N*-methoxycarbonyl moiety of **27** seemed to be essential for potent inhibitory activity. Surprisingly, its corresponding *R*-enantiomer exhibited less than 50% inhibition (−20%) at 0.1 μM. This result suggested that the enzyme strictly recognized the absolute 3*S*-configuration of the pyrrolidine moiety of **27**. The *N*-acetyl pyrrolidinyloxy derivative **29**, which was prepared from **26**, showed 41% inhibition in the LPDE4 assay at a concentration of 0.3 μM. LPDE4 inhibitory activity was restored in the *N*-sulfonylmethyl analog **30**, which showed an $IC_{50}$ value of 0.2 μM. Accordingly, compounds with good potency such as **27** were obtained by very limited chemical modification of this series of analogs.

Further biological evaluation of compounds **3b** and **27**, which were selected based on their oral activity in the LPS-induced TNF-α production assay, was carried out as shown in Table 4. These compounds were evaluated for their ability to inhibit slow-reacting substance of anaphylaxis (SRS-A)-mediated bronchoconstriction.[16,17] The results are expressed as $ID_{50}$ values that is, the dose that showed 50% inhibition relative to the vehicle. These compounds were also evaluated for the ability to inhibit TNF-α production in human whole blood (HWB)[18] to estimate their clinical potential. The results are expressed as $IC_{50}$ values that is, the test compound concentration that caused 50% inhibition relative to the vehicle. The potency of these compounds for inhibiting SRS-A-mediated bronchoconstriction in actively sensitized guinea pigs was not always consistent with their inhibition of LPS-induced TNF-α production in rats, probably because of differences in pharmacokinetics due to cross-species comparison.

Compounds **3b** and **27** demonstrated 50% inhibition of SRS-A-induced bronchoconstriction at $ID_{50}$ values of 6.8 mg/kg and about 10 mg/kg, respectively, while the

**Table 4.** Activity profile of pyrazolopyridine derivatives

| Compd | Inhibition of bronchoconstriction[a] $ID_{50}$ (mg/kg, po) | Inhibition of TNF-α production[b] $ID_{50}$ (mg/kg, po) | Inhibition of gastric emptying[c] $ID_{50}$ (mg/kg, po) | Inhibition of TNF-α production in HWB[d] $IC_{50}$ (μM) | Ferret emesis[e] (vomiting/tested) | | |
|---|---|---|---|---|---|---|---|
| | | | | | 3 | 10 (mg/kg, po) | 30 |
| **1** (Ariflo) | 4.5 | 1.7 | 5.7 | 18 | NT[g] | NT[g] | NT[g] |
| **3b** | 6.8 | 2.4 | 23%[f] | 3.0 | 0/2 | 0/2 | 1/2 |
| **27** | 45%[f] | 1.0 | 15 | 4.8 | 0/2 | 0/3 | 2/2 |

[a] Inhibition of SRS-A-mediated bronchoconstriction and airway microvascular leakage in actively sensitized guinea pigs ($n = 3$–6); OVA challenge 0.15 mg/kg 1 h after oral dosing of a test compound.
[b] See corresponding footnotes from Table 1.
[c] Inhibition of gastric emptying in rats ($n = 5$).
[d] Inhibition of LPS-induced TNF-α production in human whole blood. $IC_{50}$ represent a mean of $n = 3$.
[e] Vomiting test in fasted ferrets.
[f] Inhibition % at 10 mg/kg, po.
[g] Not tested.

$ID_{50}$ values for TNF-α production were 2.4 and 1.0 mg/kg, respectively. With respect to inhibition of gastric emptying in rats,[19] the $ID_{50}$ values of **3b** and **27** were higher than that of Ariflo **1**, while their $IC_{50}$ values for LPS-induced TNF-α production in HWB were lower than that of Ariflo **1**. Compounds **3b** and **27** underwent evaluation of their side effect profile in a ferret emesis model, which has been widely accepted as a evaluation method for assessing the side effects of PDE4 inhibitors. Both of the compounds did not cause emesis at oral doses of up to 10 mg/kg. Based on the above-mentioned biological data, **3b** and **27** were estimated to be likely to have an improved therapeutic potential and improved side effect profile.

Single-dose pharmacokinetic data for compounds **3b** and **27** were determined in rats as shown in Table 5. After intravenous administration of compounds **3b** and **27** to rats (3 mg/mL/kg, $N = 3$), the $T_{1/2}$ was relatively short ($T_{1/2} = 0.50$ and 0.64 h). Oral administration of **3b** and **27** to rats (10 mg/5 mL/kg, $N = 3$) yielded a $T_{1/2}$ of 1.2 and 1.0 h, respectively. The AUC values of **3b** and **27** were 1.18 and 1.91 μg h/mL, respectively, after intravenous administration, and were 0.07 and 2.11 μg h/mL, respectively, after oral administration. The steady state volume of distribution ($V_{ss}$) was calculated as 1013 and 863 mL/kg, respectively, indicating that **3b** showed more extensive tissue distribution than **27**. The systemic clearance (CL) was 2551 and 1589 mL/h/kg, respectively. Bioavailablity of **3b** and **27** was calculated to be 1.8% and 33.2%, respectively. The $C_{max}$ values after oral dosing were 0.0290 and 1.37 μg/mL, respectively, while the $T_{max}$ values were 0.67 and 0.83 μg/mL, respectively. As expected from SAR studies of their in vitro and in vivo potency, **27** showed greatly improved bioavailability relative to that of **3b**.

## 4. Conclusion

Design and synthesis of structurally new PDE4 inhibitors with an improved therapeutic potential was achieved by the optimization of chemical lead **3a**, which was found among our in-house library. Compounds **3b**[20,21] and **27** showed improved therapeutic potential with a better side effect profile based on biological data obtained using both cross-species and same-species comparisons. A single-dose rat pharmacokinetic study of **3b** and **27** indicated that these inhibitors had a relatively short plasma half-life. The low bioavailability of **3a**–**b** was greatly improved by introduction of the *meta*-*N*-carbonylmethoxy-(3*S*)-pyrrolidinyloxy moiety, as illustrated by the much higher bioavailability of **27** relative to that of **3a**–**b**.

## 5. Experimental

### 5.1. General chemical procedures

Analytical samples were homogeneous as confirmed by thin-layer chromatography (TLC), and yielded spectroscopic data consistent with the assigned structures. All $^1$H NMR spectra were obtained with a Varian Gemini-200 or MERCURY-300 spectrometer. The chemical shift values are reported in ppm ($\delta$) and

**Table 5.** Single-dose pharmacokinetic data for compounds **3b** and **27**

| Parameter | **3b** | | **27** | |
|---|---|---|---|---|
| | iv (3 mg/kg) | po (10 mg/kg) | iv (3 mg/kg) | po (10 mg/kg) |
| $C_{max}$ (μg/mL) | | 0.0290 ± 0.0124 | | 1.37 ± 0.21 |
| $T_{1/2}$ (h) | 0.50 ± 0.08 | 1.2 ± 0.6 | 0.64 ± 0.2 | 1.0 ± 0.2 |
| $T_{max}$ (h) | | 0.67 ± 0.29 | | 0.83 ± 0.29 |
| AUC (μg h/mL) | 1.18 ± 0.07 | 0.0725 ± 0.0202 | 1.91 ± 0.22 | 2.11 ± 0.38 |
| $V_{ss}$ (mL/kg) | 1013 ± 317 | | 863 ± 294 | |
| $CL_{total}$ (mL/h/kg) | 2551 ± 158 | | 1589 ± 200 | |
| % Bioavail | | 1.8 | | 33.2 |

coupling constants ($J$) in hertz (Hz). Fast atom bombardment (FAB) and electron ionization (EI) mass spectra were obtained with a JEOL JMS-DX303HF or JMS-700 spectrometer. Atmospheric pressure chemical ionization (APCI) mass spectra were determined by a Hitachi M-1200H spectrometer. Matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectra were obtained on a PerSeptive Voyager Elite spectrometer. IR spectra were measured using a Perkin–Elmer FTIR 1760X or JASCO FTIR-430 spectrometer. Column chromatography was carried out using silica gel [Merck silica gel 60 (0.063–0.200 mm), Wako Gel C200, Fuji Silysia FL60D, or Fuji Silysia BW-235]. TLC was also performed on silica gel (Merck TLC plate, silica gel 60 $F_{254}$).

### 5.2. Synthesis of compound 35

**5.2.1. Diethyl 2-{[(1,3-dimethyl-1*H*-pyrazol-5-yl)amino]-methylene}malonate (32).** A mixture of 5-amino-1,3-dimethylpyrazole **31** (20.0 g, 180 mmol) and diethylethoxymethylenemalonate (38.9 g, 180 mmol) was stirred at 120 °C for 1 h. The reaction mixture was poured into *n*-hexane and the precipitates were collected by filtration to give **32** (45.3 g, 161 mmol, 89%) as an ivory powder.

TLC $R_f$ 0.59 ($CHCl_3$/MeOH, 10/1); $^1H$ NMR (300 MHz, $CDCl_3$) 11.00 (b d, $J = 12.3$ Hz, 1H), 8.14 (d, $J = 12.3$ Hz, 1H), 5.86 (s, 1H), 4.32 (q, $J = 7.2$ Hz, 2H), 7.24 (q, $J = 7.1$ Hz, 2H), 3.74 (s, 3H), 2.23 (s, 3H), 1.38 (t, $J = 7.2$ Hz, 3H), 1.32 (t, $J = 7.2$ Hz, 3H).

**5.2.2. Ethyl 4-chloro-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxylate (33).** The following reaction was carried out under argon atmosphere. A mixture of **32** (28.1 g, 100 mmol) and $POCl_3$ (140 mL, 1.50 mol) was heated at 120 °C under stirring for 18 h. The reaction mixture was poured into ice water and the precipitates were collected by filtration to give **33** (19.6 g, 77.5 mmol, 77%) as an ivory powder.

TLC $R_f$ 0.55 (*n*-hexane/EtOAc, 1/1); $^1H$ NMR (300 MHz, $CDCl_3$) 8.95 (s, 1H), 4.45 (q, $J = 7.2$ Hz, 2H), 4.07 (s, 3H), 2.76 (s, 3H), 1.44 (t, $J = 7.2$ Hz, 3H).

**5.2.3. 4-Chloro-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxylic acid (34).** To a stirred mixture of **33** (19.6 g, 77.5 mmol) and 85% KOH (50.0 g, 760 mmol) in DME (152 mL) was added $H_2O$ (120 mL). After being stirred at room temperature for 18 h, the reaction mixture was neutralized with 4 N HCl. The precipitates were collected by filtration and washed with $H_2O$ to give **34** (17.3 g, 76.9 mmol, 99%) as a white powder.

TLC $R_f$ 0.10 ($CHCl_3$/MeOH, 10/1); $^1H$ NMR (300 MHz, DMSO-$d_6$) 8.90 (s, 1H), 4.00 (s, 3H), 3.33 (b s, 1H), 2.67 (s, 3H).

**5.2.4. 4-Chloro-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxamide (35).** The following reaction was carried out under argon atmosphere. A mixture of **34** (2.00 g, 8.89 mmol) and $SOCl_2$ (3.24 mL, 44.5 mmol) was heated at 80 °C under stirring for 1 h, the reaction mixture was concentrated in vacuo. The resulting residue was used for the next step without further purification.

To a stirred mixture of 28% $NH_4OH$ (12 mL) and THF (30 mL) was added a solution of the acyl chloride in THF (10 mL) at 0 °C. After being stirred at 0 °C for 10 min, the reaction mixture was concentrated in vacuo. The residue was poured into $H_2O$ and extracted with $CHCl_3$. The organic layer was dried over $Na_2SO_4$ and concentrated in vacuo. The residue was triturated with $Et_2O$ to give **35** (1.98 g, 8.84 mmol, 99%) as a white powder.

TLC $R_f$ 0.40 ($CHCl_3$/MeOH, 10/1); $^1H$ NMR (300 MHz, DMSO-$d_6$) 8.52 (s, 1H), 8.02 (b s, 1H), 7.78 (b s, 1H), 3.98 (s, 3H), 2.64 (s, 3H).

### 5.3. General procedure for the preparation of compounds 3a–16, 18–25, and 37a

The compounds **3a**–**16**, **18**–**25** and **37a** were prepared from the corresponding aniline derivatives **36b**–**x**, respectively, according to the same procedures as described for the preparation of **3a** from **36a**.

**5.3.1. 4-Anilino-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxamide (3a).** To a mixture of **35** (1.00 g, 4.46 mmol) in dioxane (15 mL) was added aniline **36a** (1.22 mL, 13.4 mmol). After being stirred at 120 °C for 1 h, the reaction mixture was poured into $Et_2O$ and the precipitates were collected by filtration. The resulting powder was triturated with $H_2O$ to give **3a** (787 mg, 2.80 mmol, 63%) as an off-white powder.

TLC $R_f$ 0.53 ($CHCl_3$/MeOH, 10/1); IR (KBr) 3406, 3185, 2925, 1677, 1619, 1586, 1521, 1500, 1422, 1384, 1146, 802, 770, 699; MS (APCI, Pos. 20 V). 282 $(M+H)^+$; $^1H$ NMR (300 MHz, DMSO-$d_6$) 11.0 (s, 1H), 8.74 (s, 1H), 8.21 (br, 1H), 7.55 (br, 1H), 7.36–7.28 (m, 2H), 7.17–7.07 (m, 3H), 3.88 (s, 3H), 1.59 (s, 3H); HRMS (FAB) calcd for $C_{15}H_{16}N_5O_1$ 282.1355, found 282.1381.

**5.3.2. 4-(3-Methoxyphenyl)amino]-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxamide (3b).** Yield 55%; White powder; TLC $R_f$ 0.32 ($CHCl_3$/MeOH, 10/1); IR (KBr) 3363, 3196, 1676, 1619, 1585, 1521, 1500, 1385; MS (FAB, Pos.) 312 $(M+H)^+$, 295; $^1H$ NMR (300 MHz, DMSO-$d_6$) 10.97 (s, 1H), 8.74 (s, 1H), 8.21 (b s, 1H), 7.56 (b s, 1H), 7.26–7.17 (m, 1H), 6.75–6.60 (m, 3H), 3.89 (s, 3H), 3.71 (s, 3H), 1.69 (s, 3H); HRMS (EI) calcd for $C_{16}H_{17}N_5O_2$ 311.1382, found 311.1369.

**5.3.3. 4-[(3-Ethoxyphenyl)amino]-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxamide (4).** Yield 51%; White powder; TLC $R_f$ 0.36 ($CHCl_3$/MeOH, 9/1); IR

(KBr) 3344, 3195, 1656, 1586, 1523, 1491, 1384, 1267, 1157, 1048, 987, 802, 713; MS (APCI, Pos. 20 V) 326 $(M+H)^+$, 308; $^1H$ NMR (300 MHz, $CDCl_3$) 10.58 (s, 1H), 8.52 (s, 1H), 7.21–7.15 (m, 1H), 6.72–6.65 (m, 3H), 5.85–5.60 (br s, 2H), 3.99 (s, 3H), 3.97 (q, $J = 6.9$ Hz, 2H), 1.77 (s, 3H), 1.37 (t, $J = 6.9$ Hz, 3H); HRMS (EI) calcd for $C_{17}H_{19}N_5O_2$ 325.1539, found 325.1534.

**5.3.4. 1-3-Dimethyl-4-[(3-propoxyphenyl)amino]-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxamide (5).** Yield 99%; Pale yellow powder; TLC $R_f$ 0.36 (EtOAc); IR (KBr) 3362, 3190, 1675, 1618, 1601, 1567, 1523, 1490, 1473, 1455, 1400, 1382, 1355, 1319, 1272, 1222, 1175, 1158, 1002; MS (FAB, Pos.) 340 $(M+H)^+$; $^1H$ NMR (300 MHz, DMSO-$d_6$) 10.96 (s, 1H), 8.74 (s, 1H), 8.20 (b s, 1H), 7.55 (b s, 1H), 7.19 (t, $J = 8.4$ Hz, 1H), 6.74–6.68 (m, 1H), 6.67 (s, 1H), 6.63 (d, $J = 8.4$ Hz, 1H), 3.89 (s, 3H), 3.87 (t, $J = 6.9$ Hz, 2H), 1.69 (s, 3H), 1.68 (sext, $J = 6.9$ Hz, 2H), 0.94 (t, $J = 6.9$ Hz, 3H); HRMS (EI) calcd for $C_{18}H_{21}N_5O_2$ 339.1695, found 339.1703.

**5.3.5. 4-(3-Isopropoxyphenyl)amino]-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxamide (6).** Yield 49%; White powder; TLC $R_f$ 0.41 ($CHCl_3$/MeOH, 9/1); IR (KBr) 3342, 3185, 2978, 1656, 1586, 1563, 1522, 1490, 1440, 1402, 1383, 1357, 1316, 1267, 1222, 1184, 1155, 1113, 1002, 804, 714; MS (APCI, Pos. 20 V) 340 $(M+H)^+$, 322; $^1H$ NMR (300 MHz, $CDCl_3$) 10.58 (s, 1H), 8.52 (s, 1H), 7.20–7.13 (m, 1H), 6.72–6.65 (m, 3H), 5.85–5.60 (br s, 2H), 4.48 (sept, $J = 6.0$ Hz, 1H), 3.99 (s, 3H), 1.77 (s, 3H), 1.29 (d, $J = 6.0$ Hz, 6H); HRMS (EI) calcd for $C_{18}H_{21}N_5O_2$ 339.1695, found 339.1681.

**5.3.6. 4-{[3-Cyclobutyloxy)phenyl]amino}-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxamide (7).** Yield 70%; White powder; TLC $R_f$ 0.50 (toluene/AcOEt, 1/20); IR (KBr) 3346, 3200, 2977, 1597, 1524, 1491, 1440, 1388, 1356, 1318, 1265, 1223, 1174, 1157, 1078, 988, 971, 891, 848; MS (APCI, Pos. 20 V) 352 $(M+H)^+$, 334, 307; $^1H$ NMR (300 MHz, DMSO-$d_6$) 11.08 (br, 1H), 8.74 (s, 1H), 8.25 (br, 1H), 7.60 (br, 1H), 7.19 (dd, $J = 8.0$, 8.0 Hz, 1H), 6.68–6.56 (m, 3H), 4.61 (quintet, $J = 7.1$ Hz, 1H), 3.88 (s, 3H), 2.35–2.23 (m, 2H), 2.03–1.85 (m, 2H), 1.79–1.64 (m, 1H), 1.89 (s, 3H), 1.64–1.49 (m, 1H); HRMS (EI) calcd for $C_{19}H_1N_5O_2$ 351.1695, found 351.1713.

**5.3.7. 1,3-Dimethyl-4-{[3-(trifluoromethoxy)phenyl]amino}-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxamide (8).** Yield 74%; White powder; TLC $R_f$ 0.30 (EtOAc); IR (KBr) 3311, 3170, 1676, 1628, 1593, 1562, 1522, 1499, 1440, 1405, 1387, 1360, 1255, 1213, 1170; MS (FAB, Pos.) 366 $(M+H)^+$, 349; $^1H$ NMR (300 MHz, DMSO-$d_6$) 10.97 (s, 1H), 8.78 (s, 1H), 8.26 (b s, 1H), 7.62 (b s, 1H), 7.48–7.37 (m, 1H), 7.16–7.05 (m, 3H), 3.92 (s, 3H), 1.71 (s, 3H); HRMS (EI) calcd for $C_{16}H_{14}F_3N_5O_2$ 365.1100, found 365.1100.

**5.3.8. 4-[(3-Hydroxyphenyl)amino]-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxamide (9).** Yield 78%; Ivory powder; TLC $R_f$ 0.27 ($CHCl_3$/MeOH, 10/1); IR (KBr) 3432, 3169, 1666, 1577, 1525, 1460, 1386, 1361, 1319, 1270, 1247, 1171, 1158, 985; MS (MALDI, Pos.) 298 $(M+H)^+$, 281; $^1H$ NMR (300 Hz, DMSO-$d_6$) 10.91 (s, 1H), 9.43 (s, 1H), 8.72 (s, 1H), 8.20 (br s, 1H), 7.54 (br s, 1H), 7.09 (t, $J = 7.8$ Hz, 1H), 6.55–6.45 (m, 3H), 3.87 (s, 3H), 1.70 (s, 3H); HRMS (EI) calcd for $C_{15}H_{15}N_5O_2$ 297.1226, found 297.1227.

**5.3.9. 1,3-Dimethyl-4-{[3-(methylsulfanyl)phenyl]amino}-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxamide (10).** Yield 100%; White powder; TLC $R_f$ 0.30 (EtOAc); IR (KBr) 3363, 3195, 1676, 1617, 1584, 1568, 1521, 1496, 1383; MS (EI, Pos.) 327 $(M)^+$, 310, 295, 263, 235; $^1H$ NMR (300 MHz, DMSO-$d_6$) 10.98 (s, 1H), 8.74 (s, 1H), 8.22 (b s, 1H), 7.56 (b s, 1H), 7.23 (t, $J = 8.1$ Hz, 1H), 7.01 (s, 1H), 7.00 (d, $J = 8.1$ Hz, 1H), 6.84 (d, $J = 8.1$ Hz, 1H), 3.89 (s, 3H), 2.42 (s, 3H), 1.69 (s, 3H); HRMS (EI) calcd for $C_{16}H_{17}N_5O_1S_1$ 327.1154, found 327.1152.

**5.3.10. 1,3-Dimethyl-4-({3-[(trifluoromethyl)sulfanyl]phenyl}amino)-*1H*-pyrazolo[3,4-*b*]pyridine-5-carboxamide (11).** Yield 80%; White powder; TLC $R_f$ 0.30 (EtOAc); IR (KBr) 3347, 3201, 1630, 1588, 1523, 1439, 1406, 1387, 1138, 1107, 1075; MS (FAB, Pos.) 382 $(M+H)^+$, 365; $^1H$ NMR (300 MHz, DMSO-$d_6$) 11.04 (s, 1H), 8.79 (s, 1H), 8.29 (b s, 1H), 7.63 (b s, 1H), 7.54–7.32 (m, 4H), 3.92 (s, 3H), 1.66 (s, 3H); HRMS (EI) calcd for $C_{16}H_{14}F_3N_5O_1S_1$ 381.0871, found 381.0864.

**5.3.11. 1,3-Dimethyl-4-[(3-nitrophenyl)amino]-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxamide (12).** Yield 40%; White powder; TLC $R_f$ 0.28 ($CHCl_3$/MeOH, 9/1); IR (KBr) 3424, 3165, 2925, 1733, 1629, 1561, 1535, 1430, 1388, 1351, 1265, 699; MS (APCI, Pos. 20 V) 327 $(M+H)^+$; $^1H$ NMR (300 MHz, DMSO-$d_6$) 10.81 (s, 1H), 8.77 (s, 1H), 8.24 (br s, 1H), 7.90–7.84 (m, 2H), 7.62 (br s, 1H), 7.58–7.46 (m, 2H), 3.93 (s, 3H), 1.80 (s, 3H); HRMS (EI) calcd for $C_{15}H_{14}N_6O_3$ 326.1127, found 326.1134.

**5.3.12. 4-{[3-Acetylamino)phenyl]amino}-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxamide (13).** Yield 48%; White powder; TLC $R_f$ 0.20 ($CHCl_3$/MeOH, 9/1); IR (KBr) 3438, 3344, 2930, 1665, 1597, 1524, 1450, 1383, 1288, 983, 792, 698; MS (APCI, Pos. 20 V) 339 $(M+H)^+$; $^1H$ NMR (300 MHz, DMSO-$d_6$) 10.99 (s, 1H), 9.88 (s, 1H), 8.73 (s, 1H), 8.22 (br s, 1H), 7.57 (br s, 1H), 7.35–7.32 (m, 2H), 7.22 (t, $J = 8.1$ Hz, 1H), 6.78 (d, $J = 8.1$ Hz, 1H), 3.87 (s, 3H), 1.97 (s, 3H), 1.66 (s, 3H); HRMS (EI) calcd for $C_{17}H_{18}N_6O_2$ 338.1491, found 338.1478.

**5.3.13. 1,3-Dimethyl-4-({3-[(methylsulfonyl)amino]phenyl}amino)-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxamide (14).** Yield 94%; Ivory powder; TLC $R_f$ 0.30 ($CH_2Cl_2$/MeOH, 10/1); IR (KBr) 3458, 3341, 3187, 1667, 1582, 1523, 1506,

1473, 1403, 1385, 1334, 1148; MS (FAB, Pos.) 375 $(M+H)^+$; $^1H$ NMR (300 MHz, DMSO-$d_6$) 10.96 (s, 1H), 9.72 (s, 1H), 8.75 (s, 1H), 8.23 (b s, 1H), 7.59 (b s, 1H), 7.27 (t, $J = 8.1$ Hz, 1H), 7.00–6.92 (m, 2H), 6.87–6.81 (m, 1H), 3.89 (s, 3H), 2.94 (s, 3H), 1.70 (s, 3H); HRMS (EI) calcd for $C_{16}H_{18}N_6O_3S_1$ 374.1161, found 374.1172.

**5.3.14. Methyl 3-{[5-(aminocarbonyl)-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridin-4-yl]amino}phenylcarbamate (15).** Yield 53%; White powder; TLC $R_f$ 0.41 ($CHCl_3$/MeOH, 10/1); IR (KBr) 3463, 3345, 2204, 2948, 1716, 1666, 1586, 1533, 1501, 1457, 1407, 1386, 1354, 1283, 1250, 1074, 984, 803, 778, 711, 604, 553; MS (APCI, Pos. 20 V) 355 $(M+H)^+$, 297, 279; $^1H$ NMR (300 MHz, DMSO-$d_6$) 10.99 (s, 1H), 9.62 (s, 1H), 8.73 (s, 1H), 8.21 (br, 1H), 7.56 (br, 1H), 7.23–7.19 (m, 3H), 6.75–6.71 (m, 1H), 3.87 (s, 3H), 3.61 (s, 3H), 1.67 (s, 3H); HRMS (EI) calcd for $C_{17}H_{18}N_6O_3$ 354.1440, found 354.1425.

**5.3.15. 4-[(3-Cyanophenyl)amino]-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxamide (16).** Yield 95%; Beige powder; TLC $R_f$ 0.38 (AcOEt); IR (KBr) 3423, 3350, 3215, 3052, 2240, 1668, 1619, 1593, 1562, 1522, 1482, 1408, 1384, 1358, 1301, 1266, 1146, 977; MS (EI, Pos.) 306 $(M)^+$, 289, 260, 246, 233, 218; $^1H$ NMR (300 MHz, DMSO-$d_6$) 10.83 (s, 1H), 8.76 (s, 1H), 8.23 (br, 1H), 7.60 (br, 1H), 7.56–7.44 (m, 3H), 7.41–7.36 (m, 1H), 3.91 (s, 3H), 1.72 (s, 3H); HRMS (EI) calcd for $C_{16}H_{14}N_6O_1$ 306.1229, found 306.1217.

**5.3.16. 4-{[3-(Hydroxymethyl)phenyl]amino}-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxamide (18).** Yield 64%; White powder; TLC $R_f$ 0.65 ($CHCl_3$/MeOH, 9/1); IR (KBr) 3356, 3189, 1671, 1588, 1525, 1439, 1405, 1385, 1357, 1298, 1273, 1142, 1048, 983; MS (APCI, Pos. 20 V) 312 $(M+H)^+$; $^1H$ NMR (300 MHz, DMSO-$d_6$) 11.05 (s, 1H), 8.73 (s, 1H), 8.20 (br, 1H), 7.54 (br, 1H), 7.26 (dd, $J = 8.0$, 8.0 Hz, 1H), 7.08–7.03 (m, 2H), 6.96 (d, $J = 8.0$ Hz, 1H), 5.16 (t, $J = 6.2$ Hz, 1H), 4.42 (d, $J = 6.2$ Hz, 2H), 3.87 (s, 3H), 1.61 (s, 3H); HRMS (EI) calcd for $C_{16}H_{17}N_5O_2$ 311.1382, found 311.1377.

**5.3.17. 4-[(3-Acetylphenyl)amino]-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxamide (19).** Yield 37%; Yellow powder; TLC $R_f$ 0.30 (EtOAc); IR (KBr) 3341, 3193, 1662, 1586, 1562, 1523, 1498, 1441, 1403, 1384, 1358, 1314, 1272; MS (EI, Pos.) 323 $(M)^+$, 306, 291, 264; $^1H$ NMR (300 MHz, DMSO-$d_6$) 11.00 (s, 1H), 8.77 (s, 1H), 8.24 (b s, 1H), 7.70 (d, $J = 7.5$ Hz, 1H), 7.63 (s, 1H), 7.59 (b s, 1H), 7.46 (t, $J = 7.5$ Hz, 1H), 7.35 (d, $J = 7.5$ Hz, 1H), 3.91 (s, 3H), 2.54 (s, 3H), 1.65 (s, 3H); HRMS (EI) calcd for $C_{17}H_{17}N_5O_2$ 323.1382, found 323.1387.

**5.3.18. 4-[(3-Ethynylphenyl)amino]-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxamide (20).** Yield 93%; White powder; TLC $R_f$ 0.22 ($CHCl_3$/MeOH, 9/1); IR (KBr) 3396, 3291, 3191, 1666, 1613, 1586, 1563, 1523, 1496, 1442, 1405, 1384, 1357, 1282, 978; MS (APCI, Pos. 20 V) 306 $(M+H)^+$; $^1H$ NMR (300 MHz, DMSO-$d_6$) 10.91 (s, 1H), 8.75 (s, 1H), 8.25 (s, 1H), 7.60 (br s, 1H), 7.10–7.35 (m, 4H), 4.17 (s, 1H), 3.89 (s, 3H), 1.67 (s, 3H); HRMS (EI) calcd for $C_{17}H_{15}N_5O_1$ 305.1277, found 305.1293.

**5.3.19. 4-[(2,4-Dimethoxyphenyl)amino]-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxamide (21).** Yield 64%; White powder; TLC $R_f$ 0.32 ($CHCl_3$/MeOH, 10/1); IR (KBr) 3364, 3179, 1667, 1616, 1583, 1568, 1518, 1440, 1384, 1211; MS (FAB, Pos.) 342 $(M+H)^+$, 325; $^1H$ NMR (300 MHz, DMSO-$d_6$) 10.81 (s, 1H), 8.66 (s, 1H), 8.11 (b s, 1H), 7.42 (b s, 1H), 6.92 (d, $J = 8.4$ Hz, 1H), 6.69 (d, $J = 2.7$ Hz, 1H), 6.44 (dd, $J = 8.4$, 2.7 Hz, 1H), 3.84 (s, 3H), 3.81 (s, 3H), 3.76 (s, 3H), 1.53 (s, 3H); HRMS (EI) calcd for $C_{17}H_{19}N_5O_3$ 341.1488, found 341.1498.

**5.3.20. 4-[2,5-Dimethoxyphenyl)amino]-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxamide (22).** Yield 55%; Pale yellow powder; TLC $R_f$ 0.32 ($CHCl_3$/MeOH, 10/1); IR (KBr) 3308, 3170, 1678, 1629, 1560, 1509, 1430, 1225; MS (FAB, Pos.) 342 $(M+H)^+$, 325; $^1H$ NMR (300 MHz, DMSO-$d_6$) 11.03 (s, 1H), 8.75 (s, 1H), 8.29 (b s, 1H), 7.61 (b s, 1H), 7.04 (d, $J = 9.0$ Hz, 1H), 6.72 (dd, $J = 9.0$, 3.0 Hz, 1H), 6.59 (d, $J = 3.0$ Hz, 1H), 3.91 (s, 3H), 3.78 (s, 3H), 3.59 (s, 3H), 1.66 (s, 3H); HRMS (EI) calcd for $C_{17}H_{19}N_5O_3$ 341.1488, found 341.1498.

**5.3.21. 4-[(3,4-Dimethoxyphenyl)amino]-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxamide (23).** Yield 23%; White powder; TLC $R_f$ 0.32 ($CHCl_3$/MeOH, 10/1); IR (KBr) 3429, 3198, 1672, 1616, 1588, 1520, 1246; MS (FAB, Pos.) 342 $(M+H)^+$, 325; $^1H$ NMR (300 MHz, DMSO-$d_6$) 11.12 (s, 1H), 8.70 (s, 1H), 8.17 (b s, 1H), 7.49 (b s, 1H), 6.88 (d, $J = 8.4$ Hz, 1H), 6.85 (d, $J = 2.4$ Hz, 1H), 6.61 (dd, $J = 8.4$, 2.4 Hz, 1H), 3.86 (s, 3H), 3.73 (s, 3H), 3.69 (s, 3H), 1.59 (s, 3H); HRMS (EI) calcd for $C_{17}H_{19}N_5O_3$ 341.1488, found 341.1498.

**5.3.22. 4-[3,5-Dimethoxyphenyl)amino]-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxamide (24).** Yield 17%; White powder; TLC $R_f$ 0.32 ($CHCl_3$/MeOH, 10/1); IR (KBr) 3351, 3185, 1672, 1611, 1592, 1565, 1520, 1382, 1158; MS (FAB, Pos.) 342 $(M+H)^+$, 325; $^1H$ NMR (300 MHz, DMSO-$d_6$) 10.90 (s, 1H), 8.73 (s, 1H), 8.20 (b s, 1H), 7.55 (b s, 1H), 6.26 (s, 1H), 6.25 (s, 2H), 3.89 (s, 3H), 3.67 (s, 6H), 1.79 (s, 3H); HRMS (EI) calcd for $C_{17}H_{19}N_5O_3$ 341.1488, found 341.1498.

**5.3.23. 4-[(2,3-Dimethoxyphenyl)amino]-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxamide (25).** Yield 65%; Pale yellow powder; TLC $R_f$ 0.32 ($CHCl_3$/MeOH, 10/1); IR (KBr) 3348, 3187, 1679, 1644, 1622, 1589, 1559, 1490, 1480, 1431, 1269, 1093; MS (FAB, Pos.) 342 $(M+H)^+$, 325; $^1H$ NMR (300 MHz, DMSO-$d_6$) 11.17 (b s, 1H), 8.78 (s, 1H), 8.32 (b s, 1H), 7.65 (b s, 1H), 6.96 (t,

$J = 8.1$ Hz, 1H), 6.86 (dd, $J = 8.1$, 1.5 Hz, 1H), 6.64 (dd, $J = 8.1$, 1.5 Hz, 1H), 3.92 (s, 3H), 3.84 (s, 3H), 3.75 (s, 3H), 1.71 (s, 3H); HRMS (EI) calcd for $C_{17}H_{19}N_5O_3$ 341.1488, found 341.1498.

**5.3.24. Ethyl 3-{[5-(aminocarbonyl)-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridin-4-yl]amino}benzoate (37a).** Yield 92%; White powder; TLC $R_f$ 0.30 (EtOAc); MS (EI, Pos.) 353 $(M)^+$, 336, 308, 290, 263; $^1$H NMR (300 MHz, DMSO-$d_6$) 10.98 (s, 1H), 8.77 (s, 1H), 8.24 (b s, 1H), 7.68 (d, $J = 7.8$ Hz, 1H), 7.62 (s, 1H), 7.60 (b s, 1H), 7.45 (t, $J = 7.8$ Hz, 1H), 7.36 (d, $J = 7.8$ Hz, 1H), 4.28 (q, $J = 7.2$ Hz, 2H), 3.91 (s, 3H), 1.66 (s, 3H), 1.29 (t, $J = 7.2$ Hz, 3H).

## 5.4. Synthesis of compound 17

**5.4.1. 3-{[5-Aminocarbonyl)-1,3-dimethyl-1*H*-pyrazolo-[3,4-*b*]pyridin-4-yl]amino}benzoic acid (37b).** To a stirred mixture of **37a** (170 mg, 0.48 mmol) in EtOH (5 mL) and THF (5 mL) was added 1 N NaOH (2.4 mL, 2.4 mmol). After being stirred at 50 °C for 7 h, the reaction mixture was neutralized with 1 N HCl and extracted with EtOAc. The organic layer was washed with $H_2O$ and brine, dried over $Na_2SO_4$, and concentrated in vacuo. The residue was triturated with EtOAc to give **37b** (118 mg, 0.36 mmol, 75%) as an ivory powder.

TLC $R_f$ 0.38 ($CHCl_3$/MeOH, 5/1); MS (EI, Pos.) 325 $(M)^+$, 308, 290, 280, 263, 235; $^1$H NMR (300 MHz, DMSO-$d_6$) 10.96 (s, 1H), 8.76 (s, 1H), 8.23 (br, 1H), 7.68–7.63 (m, 1H), 7.57 (br, 1H), 7.57–7.55 (m, 1H), 7.44 (dd, $J = 7.8$, 7.8 Hz, 1H), 7.38–7.33 (m, 1H), 3.89 (s, 3H), 3.31 (s, 1H), 1.64 (s, 3H).

**5.4.2. 4-{[3-(Aminocarbonyl)phenyl]amino}-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxamide (17).** To a stirred mixture of **37b** (189 mg, 0.58 mmol) in DMF (4 mL) and THF (10 mL) were added HOBt (117 g, 0.87 mmol) and EDC HCl (167 g, 0.87 mmol). After being stirred at room temperature for 2 h, the reaction mixture was poured into 28% $NH_4OH$ (20 mL), stirred at room temperature for 1 h, poured into brine, and extracted with a mixture of EtOAc and THF. The organic layer was washed with 1 N HCl, $H_2O$, and brine, dried over $MgSO_4$, and concentrated in vacuo. The residue was triturated with a mixture of EtOAc and $Et_2O$ to give **17** (104 mg, 0.32 mmol, 55%) as a pink powder.

TLC $R_f$ 0.34 ($CHCl_3$/MeOH, 10/1); IR (KBr) 3444, 3428, 3350, 3193, 1663, 1621, 1586, 1527, 1497, 1444, 1406, 1387, 1320, 1273, 1146, 1092, 982; MS (FAB, Pos.) 325 $(M+H)^+$, 291; $^1$H NMR (300 MHz, DMSO-$d_6$) 11.07 (s, 1H), 8.76 (s, 1H), 8.23 (br, 1H), 7.94 (s, 1H), 7.63–7.56 (m, 3H), 7.39 (dd, $J = 7.8$, 7.8 Hz, 1H), 7.35 (s, 1H), 7.27–7.23 (m, 1H), 3.88 (s, 3H), 1.59 (S, 3H); HRMS (EI) calcd for $C_{16}H_{16}N_6O_2$ 324.1335, found 324.1332.

## 5.5. Synthesis of compounds 39a, 39b, and 39d

**5.5.1. Methyl (3*S*)-3-(3-nitrophenoxy)pyrrolidine-1-carboxylate (39a).** To a stirred solution of **38** (1.39 g, 9.98 mmol) in THF (50 mL) were added *N*-methoxycarbonyl-(*R*)-3-pyrrolidinol (1.59 g, 10.5 mmol), $PPh_3$ (3.92 mg, 15.0 mmol), and DEAD (6.6 mL, 15 mmol). After being stirred at room temperature overnight, the reaction mixture was concentrated in vacuo. The resulting residue was purified by column chromatography on silica gel (*n*-hexane/EtOAc, 2/1) to give **39a** (7.29 g, quant) as a colorless oil.

TLC $R_f$ 0.36 (*n*-hexane/EtOAc, 1/1); MS (APCI, Pos. 20 V) 267 $(M+H)^+$; $^1$H NMR (300 MHz, $CDCl_3$) 7.88–7.83 (m, 1H), 7.72–7.63 (m, 1H), 7.59–7.52 (m, 1H), 7.22–7.17 (m, 1H), 5.00 (m, 1H), 3.73 and 3.72 (s, 3H), 3.75-3.65 (m, 4H), 2.30–2.10 (m, 2H).

**5.5.2. *tert*-Butyl (3*S*)-3-(3-nitrophenoxy)pyrrolidine-1-carboxylate (39b).** The following compound **39b** was prepared from the corresponding *N*-*tert*-butoxycarbonyl-(*R*)-3-pyrrolidinol, according to the same procedure as described for the preparation of **39a** from *N*-methoxycarbonyl-(*R*)-3-pyrrolidinol.

Yield 75%; Ivory powder; TLC $R_f$ 0.48 (*n*-hexane/EtOAc, 1/1); $^1$H NMR (300 MHz, $CDCl_3$) 7.88–7.81 (m, 1H), 7.72–7.68 (m, 1H), 7.50–7.40 (m, 1H), 7.23–7.18 (m, 1H), 5.01–4.92 (m, 1H), 3.73–3.40 (m, 4H), 2.28–2.10 (m, 2H), 1.50 (s, 9H).

**5.5.3. (3*S*)-3-(3-Nitrophenoxy)pyrrolidine (39c).** To a stirred mixture of **39b** (3.00 g, 9.73 mmol) in $CH_2Cl_2$ (10 mL) was added trifluoroacetic acid (10 mL). After being stirred at 0 °C for 30 min, the reaction mixture was concentrated in vacuo. The residue was poured into 2 N NaOH and extracted with $CH_2Cl_2$. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated in vacuo to give **39c** (2.01 g, 9.65 mmol, 99%) as a colorless oil.

TLC $R_f$ 0.29 ($CHCl_3$/MeOH, 9/1); $^1$H NMR (300 MHz, $CDCl_3$) 7.82–7.17 (m, 4H), 4.95–4.85 (m, 1H), 3.23–3.05 (m, 3H), 3.00–2.90 (m, 1H), 2.22–1.90 (m, 2H), 2.00–1.60 (br, 1H).

**5.5.4. (3*S*)-1-(Methylsulfonyl)-3-(3-nitrophenoxy)pyrrolidine (39d).** To a stirred mixture of **39c** (500 mg, 2.40 mmol) in $CH_2Cl_2$ (5 mL) were added $Et_3N$ (0.40 mL, 2.9 mmol) and MsCl (0.20 mL, 2.6 mmol). After being stirred at 0 °C for 1 h, the reaction mixture was poured into $H_2O$ and extracted with $CH_2Cl_2$. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated in vacuo to give **39d**. This compound was used for the next step without further purification.

TLC $R_f$ 0.78 ($CHCl_3$/MeOH, 5/1).

### 5.6. General procedure for the preparation of compound 40a, 40b, and 40d

The compounds **40b** and **40d** were prepared according to the same procedure as described for the preparation of **40a** from **39a**.

**5.6.1. Methyl (3*S*)-3-(3-aminophenoxy)pyrrolidine-1-carboxylate (40a).** A solution of **39a** (7.29 g, 9.98 mmol) in MeOH (30 mL) was hydrogenated under atmospheric pressure of $H_2$ gas in the presence of 10% Pd/C (0.27 g) for 3 h. The catalyst was removed by filtration through a pad of Celite, and washed with MeOH. The filtrate was concentrated in vacuo. The resulting residue was purified by column chromatography on silica gel (*n*-hexane/EtOAc, 1/1) to give **40a** (2.09 g, 8.83 mmol, 80% in two steps) as a pale yellow oil.

TLC $R_f$ 0.59 (EtOAc); MS (APCI, Pos. 20 V) $m/z = 237$ $(M+H)^+$; $^1$H NMR (200 MHz, $CDCl_3$) 7.05 (t, $J = 7.9$ Hz, 1H), 6.34–6.18 (m, 3H), 4.85 (m, 1H), 3.70 and 3.69 (s, 3H), 3.75–3.25 (m, 6H), 2.30–1.95 (m, 2H).

**5.6.2. *tert*-Butyl (3*S*)-3-(3-aminophenoxy)pyrrolidine-1-carboxylate (40b).** Yield 85%; Colorless oil; TLC $R_f$ 0.31 (*n*-hexane/EtOAc, 1/1); $^1$H NMR (300 MHz, $CDCl_3$) 7.10–7.00 (m, 1H), 6.31–6.20 (m, 3H), 4.90–4.80 (m, 1H), 3.90–3.20 (br, 2H), 3.65–3.40 (m, 4H), 2.22–2.00 (m, 2H), 1.45 (s, 9H).

**5.6.3. 3-{[(3*S*)-1-(Methylsulfonyl)pyrrolidin-3-yl]oxy}aniline (40d).** Yield 63% in two steps; Colorless oil; TLC $R_f$ 0.52 ($CHCl_3$/MeOH, 5/1); $^1$H NMR (300 MHz, DMSO-$d_6$) 6.90 (t, $J = 7.9$ Hz, 1H), 6.20–6.00 (m, 3H), 5.20–5.00 (br, 2H), 4.90–4.80 (m, 1H), 3.60–3.45 (m, 1H), 3.40–3.20 (m, 3H), 2.85 (s, 3H), 2.25–2.00 (m, 2H).

### 5.7. General procedure for the preparation of compound 27, 28, and 30

The following compounds **27**, **28**, and **30** were prepared according to the same procedure as described for the preparation of **3a** from **35**.

**5.7.1. Methyl (3*S*)-3-(3-{[5-(aminocarbonyl)-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridin-4-yl]amino}phenoxy)-1-pyrrolidinecarboxylate (27).** Yield 100%; Beige amorphous solid; TLC $R_f$ 0.55 (AcOEt/MeOH, 10/1); IR (KBr) 3347, 3204, 2954, 1693, 1586, 1523, 1456, 1384, 1266, 1208, 1157, 1122, 1008, 901; MS (APCI, Pos. 20 V) 425 $(M+H)^+$, 407, 349; $^1$H NMR (300 MHz, DMSO-$d_6$) 10.93 (br, 1H), 8.73 (s, 1H), 8.19 (br, 1H), 7.55 (br, 1H), 7.20 (dd, $J = 8.4$, 8.4 Hz, 1H), 6.72–6.63 (m, 3H), 4.99 (m, 1H), 3.87 (s, 3H), 3.57 and 3.56 (s, 3H), 3.53–3.27 (m, 4H), 2.18–1.95 (m, 2H), 1.68 (s, 3H); HRMS (EI) calcd for $C_{21}H_{24}N_6O_4$ 424.1859, found 424.1874.

**5.7.2. *tert*-Butyl (3*S*)-3-(3-{[5-(aminocarbonyl)-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridin-4-yl]amino}phenoxy)-1-pyrrolidinecarboxylate (28).** Yield 100%; Beige amorphous solid; TLC $R_f$ 0.35 ($CHCl_3$/MeOH, 9/1); IR (KBr) 3351, 3201, 2978, 1660, 1586, 1523, 1489, 1413, 1365, 1266, 1216, 1158, 1122, 1008; MS (APCI, Pos. 20 V) 467 $(M+H)^+$; $^1$ H NMR (300 MHz, $CDCl_3$) 10.59 (s, 1H), 8.54 (s, 1H), 7.20–7.15 (m, 1H), 6.78–6.63 (m, 3H), 6.00–5.70 (br s, 2H), 4.85–4.79 (m, 1H), 4.00 (s, 3H), 3.60–3.40 (m, 4H), 2.20–2.00 (m, 2H), 1.78 (s, 3H), 1.46 (s, 9H); HRMS (EI) calcd for $C_{24}H_{30}N_6O_4$ 466.2329, found 466.2300.

**5.7.3. 1,3-Dimethyl-4-[(3-{[(3*S*)-1-(methylsulfonyl)-3-pyrrolidinyl]oxy}phenyl)amino]-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxamide (30).** Yield 66%; White powder; TLC $R_f$ 0.31 ($CHCl_3$/MeOH, 9/1); IR (KBr) 3425, 2927, 1656, 1587, 1562, 1521, 1492, 1440, 1402, 1384, 1335, 1151, 1008; MS (APCI, Pos. 20 V) 445 $(M+H)^+$; $^1$H NMR (300 MHz, DMSO-$d_6$) 10.93 (s, 1H), 8.73 (s, 1H), 8.25–8.15 (br s, 1H), 7.60–7.45 (br s, 1H), 7.21 (t, $J = 9.0$ Hz, 1H), 6.73–6.65 (m, 3H), 5.04–4.99 (m, 1H), 3.87 (s, 3H), 3.52 (dd, $J = 11.7$, 4.2 Hz, 1H), 3.40–3.25 (m, 3H), 2.85 (s, 3H), 2.22–2.00 (m, 2H), 1.68 (s, 3H); HRMS (EI) calcd for $C_{20}H_{24}N_6O_4S_1$ 444.1580, found 444.1575.

### 5.8. Synthesis of compounds 26 and 29

**5.8.1. 1,3-Dimethyl-4-({3-[(3*S*)-3-pyrrolidinyloxy]-phenyl}amino)-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxamide (26).** To a stirred mixture of **28** (300 mg, 0.64 mmol), MeOH (10 mL) and EtOAc (10 mL) was added 4 N HCl/MeOH (3 mL). After being stirred at room temperature for 15 h, the reaction mixture was concentrated in vacuo. The residue was neutralized with saturated aqueous $Na_2CO_3$ and extracted with EtOAc. The organic layer was washed with saturated aqueous $NaHCO_3$, dried over $MgSO_4$, and concentrated in vacuo to give **26** (125 mg, 0.342 mmol, 53%) as a white powder.

TLC $R_f$ 0.36 ($CHCl_3$/MeOH/AcOH, 10/2/1); IR (KBr) 3169, 2929, 1661, 1589, 1561, 1520, 1489, 1404, 1385, 1360, 1311, 1262, 1214, 1173, 1159, 1093, 1048, 989; MS (FAB, Pos.) 367 $(M+H)^+$, 281; $^1$H NMR (300 MHz, DMSO-$d_6$) 10.93 (s, 1H), 8.73 (s, 1H), 8.23–8.12 (br s, 1H), 7.63–7.45 (br s, 1H), 7.21–7.15 (m, 1H), 6.70–6.60 (m, 3H), 4.80–4.75 (m, 1H), 3.87 (s, 3H), 3.31 (br s, 1H), 2.98–2.63 (m, 4H), 1.98–1.82 (m, 1H), 1.70–1.60 (m, 1H), 1.67 (s, 3H); HRMS (EI) calcd for $C_{19}H_{22}N_6O_2$ 366.1804, found 366.1804.

**5.8.2. 4-[3-{[(3*S*)-1-Acetyl-3-pyrrolidinyl]oxy}phenyl)-amino]-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxamide (29).** To a stirred solution of **26** (417 mg, 1.14 mmol) in pyridine (10 mL) was added $Ac_2O$ (0.11 mL, 1.2 mmol). After being stirred at room temperature for 15 h, the reacting mixture was concentrated in vacuo. The residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH, 20/1) to give **29** (465 mg, 1.14 mmol, 100%) as a yellow powder.

TLC $R_f$ 0.26 ($CHCl_3$/MeOH, 9/1); IR (KBr) 3404, 3201, 2978, 1614, 1586, 1523, 1489, 1439, 1384, 1358, 1266, 1218, 1176, 1157, 1097, 1007; MS (APCI, Pos. 20 V) 409 $(M+H)^+$, 391; $^1H$ NMR (300 MHz, $CDCl_3$) 10.61, 10.58 (s, 1H), 8.56, 8.55 (s, 1H), 7.23–7.15 (m, 1H), 6.80–6.70 (m, 1H), 6.65–6.61 (m, 2H), 6.00–5.80 (br, 2H), 4.95–4.82 (m, 1H), 4.00 (s, 3H), 3.80–3.50 (m, 4H), 2.32–1.95 (m, 2H), 2.08, 2.04 (s, 3H), 1.79, 1.78 (s, 3H); HRMS (EI) calcd for $C_{21}H_{24}N_6O_3$ 408.1910, found 408.1894.

### 5.9. Assay of human PDE4 activity

The method of Reeves et al.[22] was modified to isolate phosphodiesterase type 4 isozyme (PDE4). The enzyme was prepared from U937 cells derived from human monocytes, and was stored at 20 °C after preparation. Measurement of PDE4 activity was performed using this stored enzyme after it was diluted with distilled water containing bovine serum albumin. The substrate solution was prepared by adding $^3H$-cAMP (300,000 dpm (5000 Bq)/assay) and 100 μmol/L cAMP solution to 100 mmol/L Tris–HCl (pH 8.0) containing 5 mmol/L ethylene glycol-bis(β-aminoethyl ether) and *N*,*N*,*N*′,*N*′-tetraacetic acid. The substrate solution was mixed with the enzyme solution containing a test compound dissolved in dimethylsulfoxide (DMSO), and incubation was done for 30 min at 30 °C. Assays were performed in duplicate at three to four different concentrations of each test compound, and the $IC_{50}$ values were determined.

### 5.10. Inhibition of LPS-induced plasma TNF-α production in rats

Male Crj:CD(SD)IGS rats aged six weeks ($n = 7$) were fasted overnight, and the test compounds (0.01–0.1 mg/10 mL/kg) were administered orally at 1 h before intravenous injection of 1 μg/kg of LPS (*Escherichia coli* Serotype 055 B5). The plasma TNF-α level was measured with a commercially available ELISA kit (R&D Systems) at 90 min after LPS challenge. The percent inhibition (the dosage required to inhibit plasma TNF-α production by 50%) was determined by the following formula:

$$\%\ \text{Inhibition} = 100 - (C - S)/(L - S) \times 100$$

*C*: Plasma TNF-α concentration in LPS-treated animals pretreated with a test compound. *L*: Plasma TNF-α concentration in LPS-treated animals pretreated with saline. *S*: Plasma TNF-α concentration in saline-treated animals pretreated with saline.

### 5.11. SRS-A mediated bronchoconstriction in guinea pigs

Male Hartley guinea pigs aged seven weeks ($n = 5$) were actively sensitized by intraperitoneal administration of 1 mg of ovalbumin (OVA) containing $5 \times 10^9$ killed *Bordetella pertussis* organisms on day 0. On day 14, the bronchoconstrictor response was measured using a modified version of the method of Konzett and Rössler. Bronchoconstriction was induced by an intravenous injection of OVA (0.150–5 mg/kg). Sensitized animals were treated with both a cyclooxygenase inhibitor (indomethacin at 5 mg/kg iv, 3 min before OVA) and an anti-histamine (pyrilamine at 1 mg/kg iv, 1 min before OVA) to ensure that endogenous SRS-A was solely responsible for bronchoconstriction. Test compounds were administered orally at 1 h before antigen challenge. Bronchoconstrictor response was measured for 15 min and the result was represented as the area under the curve (AUC 0–15 min).

### 5.12. Gastric emptying in rats

Male Sprague–Dawley rats were fasted overnight and were orally administered test compounds or 0.5 w/v% methylcellulose (10 mL/kg). In addition, 0.05 mg/mL of phenol red solution was orally administered in a volume of 1.5 mL at 20 min after dosing with the test compounds. Forty minutes after administration of the test compounds, both the cardia and pylorus of the stomach were ligated under anesthesia with sodium pentobarbital (75 mg/kg, ip), and then the stomach was isolated without leakage of phenol red. The stomach was cut open and the phenol red solution was drained into a beaker containing 100 mL of 0.1 N NaOH. Part of the solution was filtrated (pore size: 0.45 μm) and the absorbance at 546 nm was measured to determine the amount of dye remaining in the stomach. Then the gastric emptying rate was calculated by the following formula:

$$\text{Gastric emptying rate} = 100 \times (0.75 - \text{concentration of dye in the stomach})/0.75$$

A value of 0.75 μg/mL was equal to a concentration of 0.05 mg/mL, which was achieved by adding 1.5 mL of phenol red to 100 mL of 0.1 N NaOH. The 50% inhibition rate for gastric emptying by the test compounds was calculated by defining gastric emptying after vehicle administration as 100%.

### 5.13. Inhibitory activity on LPS-induced TNF-α production in human whole blood

Under the supervision of a physician, blood was collected into a heparinized tube (final concentration: 10 U/mL heparin sodium) from a forearm vein in three healthy male donors. A solution of the test compound (10 μL) dissolved in DMSO was added to 180 μL of whole blood, and the mixture was preincubated for 30 min at 37 °C. Then 10 μL of 2 μg/mL of LPS was added and incubated for 6 h at 37 °C, after which the plasma TNF-α concentration was measured with a human TNF-α ELISA kit (DIACLONE). Assays were performed in duplicate at three to four different concentrations of each test compound, and the $IC_{50}$ values were determined.

### 5.14. Ferret emetic study

Male ferrets (weighting about 1.2 kg) were fasted overnight and test compounds were administered orally. Their behavior was observed throughout a 1 h period after gavage. Results were expressed as the number of animals that vomited relative to the animals tested.

### 5.15. Pharmacokinetic study

Pharmacokinetic parameters were determined in rats after intravenous administration (3 mg/mL/kg, iv) or oral administration (10 mg/5 mL/kg, po). Rats ($n = 3$) were given the test compound intravenously in 20% HP-β-CD solution, or orally in 0.5% methylcellulose suspension. Blood samples were collected from the jugular vein into a heparinized syringe at 0.25, 0.5, 1.0, 2.0, 4.0, and 6.0 h after dosing. Plasma levels of test compounds were analyzed after simple liquid–liquid extraction. LC/MS/MS analysis was performed with a Quattro II (Micromass Co., Ltd). HPLC was carried out using a HP1100 (Agilent Co., Ltd) apparatus equipped with a YMC-Pack Pro C18 (2.1 × 150 mm, YMC Corporation).

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