Bioorganic & Medicinal Chemistry 21 (2013) 5851–5854

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Identification of 2,3-disubstituted pyridines as potent, orally active PDE4 inhibitors

Yoshihiro Kato, Motoji Kawasaki, Tomohiro Nigo, Shunya Nakamura, Akira Fusano, Yasuhiro Teranishi, Mari N. Ito, Takaaki Sumiyoshi *

Drug Research Division, Dainippon Sumitomo Pharma Co., Ltd, 33-94 Enoki-cho, Suita, Osaka 564-0053, Japan

ARTICLE INFO

Article history:
Received 13 June 2013
Revised 2 July 2013
Accepted 3 July 2013
Available online 14 July 2013

Keywords:
Phosphodiesterase 4
PDE4 inhibitors
2,3-Disubstituted pyridine
Structure–activity relationship
Anti-inflammatory agent

ABSTRACT

A series of 2,3-disubstituted pyridines were synthesized and evaluated for their PDE4 inhibitory activity. We successfully modified undesirable cyano group of initial lead compound **2** to 4-pyridyl group with improvement of *in vitro* efficacy and optimized the position of nitrogen atoms in pyridine moiety and alkylene linker. The most potent compound showed significant efficacy in animal models of asthma and inflammation.

1. Introduction

Cyclic nucleotide phosphodiesterases (PDEs) are a group of 11 families of metalphosphohydrolases that are responsible for hydrolysis of cyclic adenosine 3′,5′-monophosphate (cAMP) and cyclic guanosine 3′,5′-monophosphate (cGMP). Among the PDEs, PDE4 specifically hydrolyzes cAMP, and its inhibition is known to effectively increase intracellular cAMP level and to regulate various cellular functions.[1] PDE4 is reported to be expressed in key effector cells involved in asthma, particularly airway smooth muscle cells, and in inflammation, including neutrophils, T-lymphocytes, macrophages, and eosinophils. It has been reported that PDE4 inhibitors significantly increase intracellular cAMP in airway smooth muscle cells, thereby providing a bronchodilatory effect.[2–4] In addition to the first generation PDE4 inhibitor rolipram, a number of second generation PDE4 inhibitors (Fig. 1) have been reported with roflumilast (Daxas™, Daliresp™) recently approved for severe chronic obstructive pulmonary disease (COPD).[5–7] Despite significant progress in this area, PDE4 inhibitors are often associated with side effects such as nausea, emesis, and vasculopathy, which limit their therapeutic use.[8] This highlights the need for novel pharmacophores that would allow the design of safer PDE4 inhibitors. We describe here our discovery and synthesis of 2,3-disubstituted pyridines as a new class of potent PDE4 inhibitors.

In our drug discovery program for anti-asthmatic drugs with bronchodilatory effect, we identified the lead compound **2** by screening derivatives of compound **1** (Fig. 2). Detailed screening of compound **2** revealed a moderate PDE4 inhibitory activity (IC_{50}: 104 nM). This result also suggested that the cyanopropoxy moiety of this compound is essential for inhibition of PDE4 because compound **1** did not inhibit PDE4. However, the 3-cyanopropoxy structure is one of the undesirable structures in medicinal chemistry, because alkyl nitriles have potency to afford the toxic HCN. In order to avoid this safety concern, we decided to investigate ways to modify the 3-cyanoalkyl moiety of **2**, while maintaining PDE4 inhibitory activity.

2. Chemistry

Preparation of the 2,3-disubstituted pyridines is shown in Scheme 1. Substitution of the 2-bromo-3-hydroxypyridine by

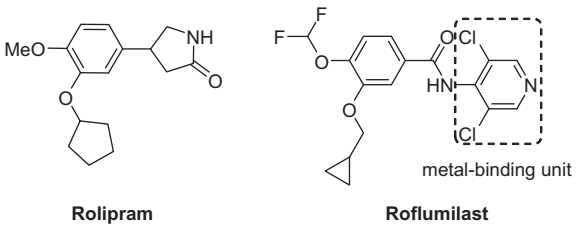


Figure 1. Chemical structures of selected PDE4 inhibitors.

* Corresponding author. Tel.: +81 6 6337 5906.
E-mail address: takaaki-sumiyoshi@ds-pharma.co.jp (T. Sumiyoshi).

0968-0896/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmc.2013.07.007

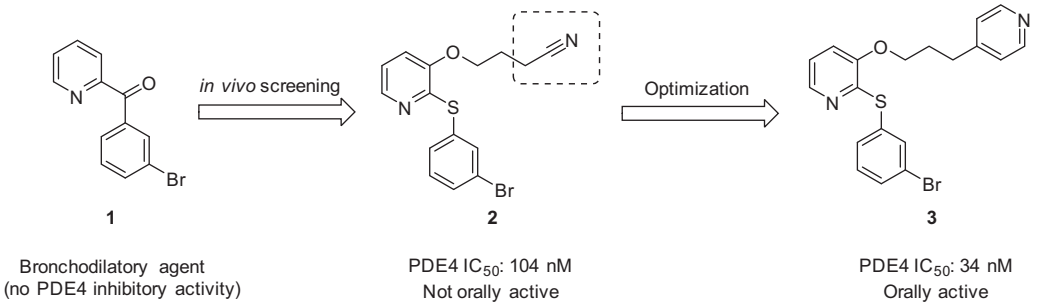
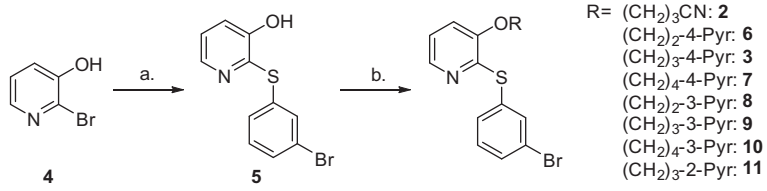


Figure 2. Modification of substituted pyridine derivatives.



Scheme 1. Reagents and conditions: (a) 3-bromothiophenol, THF, reflux, 37%; (b) alkylbromide, K_2CO_3, DMF, 80 °C; or alcohol, DIAD, PPh_3, THF, rt.

3-bromothiophenol in THF under reflux condition afforded the 2-(3-bromothiophenyl)-3-hydroxypyridine **5**. Compound **5** was then treated with alcohol under Mitsunobu reaction condition to afford the corresponding 2,3-disubstituted pyridines **2**, **3** and **6**–**11**.

3. Results and discussion

Our strategy to modify compound **2** is shown in Figure 2. X-ray analysis of co-crystals of PDE4 and roflumilast showed that the active site of PDE4 consists of three pockets, that is, the purine-selective glutamine and hydrophobic clamp pocket, the metal binding pocket, and the solvent-filled side pocket.[9] We hypothesized that the cyano group of compound **2** would interact with the metal binding pocket of PDE4, because the cyano group is a well-known metal ligand. Based on this hypothesis, we decided to change the cyano group in **2** to a pyridine, which can also bind the metal pocket of PDE4 and provide a drug-like structure. First, we calculated and compared the distances of 2-, 3-, and 4-pyridines and that of the cyano methylene group (Fig. 3). The results indicated that the nitrogen atoms of the 3- and 4-pyridyl units are located in positions similar to the position of the nitrogen atom of the cyano group.

Results of structure–activity relationships (SARs) for the prepared pyridine derivatives are shown in Table 1. Compound **2** possessed moderate PDE4 inhibitory activity (IC_{50} = 104 nM). Replacement of the cyano methylene in **2** with 4-pyridyl did not affect PDE4 inhibitory activity (60% at 100 nM, entry 1). This finding indicates that the 4-pyridyl can replace the cyano methylene as a metal binding unit for of PDE4 inhibition. In order to obtain structural information on the interaction between the nitrogen atom of PDE4 inhibitors and the metal binding site of PDE4, we examined the effect of an increase in the number of carbon atoms of the alkylene linker. Increase of the linker by one carbon atom improved

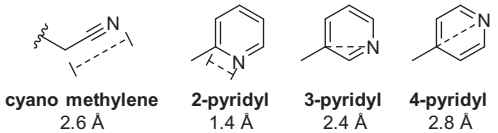


Figure 3. Calculated distances indicated by dot-line.

Table 1
SAR study of pyridyl alkylene linker

Entry	Compound	R	*n*	PDE4 inhibition (%, 100 nM)
1	**6**	4-Pyridyl	2	56
2	**3**	4-Pyridyl	3	80
3	**7**	4-Pyridyl	4	3
4	**8**	3-Pyridyl	2	60
5	**9**	3-Pyridyl	3	69
6	**10**	3-Pyridyl	4	5
7	**11**	2-Pyridyl	3	10

Table 2
Pharmacological data of compound **3** and rolipram

Compound	PDE4 IC_{50} (nM)	Bronchodilatory effect (%, 10 mg/kg, p.o.)	Cell infiltration inhibition (%, 10 mg/kg, p.o.)
Rolipram	54	N.T.	39
3	34	77	50

N.T.: not tested.

PDE4 inhibitory activity (entry 2), while adding two carbon atoms significantly decrease PDE4 inhibitory activity (entry 3). These results indicate that the most adequate number of carbon atoms in the linker is three. Finally, we examined the position of the nitrogen atom in the terminal pyridine. As expected, the 3-pyridyl compounds **8**–**10** showed almost the same PDE4 inhibitory activity as 4-pyridyl compounds (entries 4–6), and the 2-pyridyl compound **11** showed no PDE4 inhibitory activity (entry 7).

Based on the findings of SARs, we evaluated the *in vitro* and *in vivo* efficacy of the optimized compound **3**. Compound **3** inhibited inflammatory cells infiltration by up to 50% in PDE4 isozymes isolated from guinea pig eosinophils. Oral administration of **3** (10 mg/kg) produced potent bronchodilatory effect (77%) in guinea pigs sensitized with ovalbumin (OVA). In addition, the anti-inflammatory activity of **3** (50%) was stronger than that of rolipram (39%) (Table 2).

4. Conclusion

In summary, we have identified novel 2,3-disubstituted pyridines as potent PDE4 inhibitors. The undesirable cyano methylene

group of compound **2** was successfully replaced by 4-pyridine, leading to improved PDE4 inhibitory activity and *in vivo* efficacy. Optimization of the length of the alkylene linker and the position of the nitrogen atom of the terminal pyridine produced the 2-(3-bromophenylthio)-3-[3-pyridin-4-yl propoxy]pyridine **3** as a potent, orally active PDE4 inhibitor.

5. Experimental section

5.1. Chemistry

5.1.1. General

Unless otherwise noted, reagents were obtained from commercial suppliers and used without further purification. Melting points were determined on a cover glass with an electrothermal melting point apparatus and are uncorrected. Nuclear Magnetic Resonance (NMR) spectra were recorded at ambient temperature, operating at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR. Chemical shifts are given in δ (ppm) relative to TMS as internal standard; multiplicities were recorded as s (singlet), br s (broad singlet), d (doublet), dd (double doublet), ddd (double double doublet), t (triplet), dt (double triplet), ddt (double double triplet), q (quartet) or m (multiplet). MS spectra were recorded under electron inpact (EI) condition. Silica gel column chromatography was performed on Silica gel (70–230 mesh) or prepacked amino silica gel (40 μm, 60 Å). Reactions requiring anhydrous conditions were performed under argon atmosphere.

5.1.2. 2-(3-Bromophenylthio)pyridine-3-ol (5)

To a solution of 2-bromopyridin-3-ol (**4**) (44 g, 250 mmol) in THF (100 mL) and DMF (100 mL) was added 3-bromothiophenol (30 g, 160 mmol) and stirred under reflux condition. After 5 h, the reaction mixture was then cooled and diluted with EtOAc (1000 mL) and washed successively with 5% NaOH solution (2 × 50 mL), and brine (2 × 100 mL). The organic phase was dried ($MgSO_4$) and concentrated in vacuo to afford the title compound **5** as a white solid (35 g, 78%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.22 (dd, J = 1.7,4.6 Hz, 1H), 7.42 (dd, J = 2.0 Hz, 1H), 7.35 (ddd, J = 1.2, 1.7, 8.0 Hz, 1H), 7.32 (dd, J = 1.7, 8.0 Hz, 1H), 7.25 (dd, J = 4.6, 8.0 Hz, 1H), 7.21 (ddd, J = 1.2, 1.7, 8.0 Hz, 1H), 7.13 (dd, 1H, J = 8.0, 8.0 Hz, 1H), 6.65 (br s, 1H). LCMS: m/z = 281 (M, $Br^{79})^+$, 283 (M, $Br^{81})^+$.

5.1.3. 4-(2-(3-Bromophenylthio)pyridin-3-yloxy)butanenitrile (2)

A solution of 4-bromobutyronitrile (222 mg, 1.5 mmol), 2-(3-bromophenylthio)pyridine-3-ol (**5**) (282 mg 1.0 mmol) and K_2CO_3 (276 mg, 2.0 mmol) in DMF (2.0 mL) was stirred at 80 °C. After 5 h, the mixture was diluted with EtOAc (15 mL), and washed with water (2 × 15 mL) and brine (5 mL), dried (Na_2SO_4), and evaporated in vacuo. The residue was subjected to flash column chromatography (EtOAc–hexane gradient) to afford the title compound **2** as a colorless oil (343 mg, 99%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04 (dd,J = 2.4, 3.9 Hz, 1H), 7.67 (dd, J = 1.7, 1.7 Hz, 1H), 7.49 (ddd, J = 0.96, 1.9, 7.8 Hz, 1H), 7.45 (ddd, J = 0.96, 1.7, 7.8 Hz, 1H), 7.28–7.24 (m, 1H), 7.08 (s, 1H), 7.07 (d, J = 2.0 Hz, 1H), 4.17 (t, J = 5.4 Hz, 2H), 2.63 (t, J = 7.1 Hz, 2H), 2.21–2.15 (m, 2H). Anal. Calcd for $C_{15}H_{13}BrN_2OS$: C, 51.59; H, 3.75; N, 8.02. Found: C, 51.44; H, 3.73; N, 8.00. LCMS:m/z = 349 (M, $Br^{79})^+$, 351 (M, $Br^{81})^+$.

5.1.4. 2-(3-Bromophenylthio)-3-(3-(pyridin-4-yl)propoxy)pyridine (3)

To a solution of 3-(pyridin-4-yl)propan-1-ol (151 mg, 1.1 mmol), 2-(3-bromophenylthio)pyridine-3-ol (**5**) (282 mg 1.0 mmol) and triphenylphosphine (289 mg, 1.1 mmol) in THF (2.0 mL) was added diisopropylazodicarboxylate (222 mg 1.1 mmol) at 0 °C and stirred at ambient temperature. After 5 h, the mixture was evaporated, diluted with EtOAc (15 mL), and extracted with 10% HCl solution (2 × 15 mL). The aqueous phase was then basified to pH 12 with K_2CO_3 and extracted with EtOAc (2 × 15 mL). The organic phase was washed with brine (2 × 5 mL), dried (Na_2SO_4), and evaporated in vacuo. The residue was subjected to flash column chromatography (EtOAc–hexane gradient) followed by crystallization (Et_2O) to afford title compound **3** as a white crystalline solid (390 mg, 97%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.51 (dd, J = 1.7, 4.4 Hz, 2H), 8.01 (dd, J = 1.7, 4.6 Hz, 1H), 7.69 (dd, J = 1.7, 1.7 Hz, 1H), 7.46–7.49 (m, 2H), 7.25 (dd, J = 8.0, 8.0 Hz, 1H), 7.16 (dd, J = 1.4, 4.4 Hz, 2H), 7.05 (dd, J = 4.4, 8.0 Hz, 1H), 7.01 (dd, J = 1.7, 8.3 Hz, 1H), 4.02 (t, J = 6.1 Hz, 2H), 2.85 (t, J = 7.3 Hz, 2H), 2.15 (tt, J = 6.1, 7.3 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 151.53, 150.01, 149.85, 147.62, 141.60, 136.51, 133.13, 132.64, 131.18, 130.16, 123.94, 122.49, 121.23, 117.28, 67.15, 31.28, 29.43. Anal. Calcd for $C_{19}H_{17}BrN_2OS$: C, 56.86; H, 4.27; N, 6.98. Found: C, 57.15; H, 4.35; N, 7.03. LCMS: m/z = 400 (M, $Br^{79})^+$, 402 (M, $Br^{81})^+$.

5.1.5. 2-(3-Bromophenylthio)-3-(2-(pyridin-4-yl)ethoxy)pyridine (6)

Following the above procedure for **3**, title compound **6** was prepared in an identical manner from compound **5** (282 mg 1.0 mmol) and 2-(pyridin-4-yl)ethan-1-ol (135 mg, 1.1 mmol) and was obtained as a white solid (368 mg, 95%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.56 (dd, J = 1.5, 4.4 Hz, 2H), 8.00 (dd, J = 3.2, 3.2 Hz, 1H), 7.66 (dd, J = 1.7, 2.0 Hz, 1H), 7.46 (ddd, J = 1.7, 2.0, 16.6 Hz, 2H), 7.28 (dd, J = 1.5, 4.6 Hz, 2H), 7.25 (dd, J = 7.8, 7.8 Hz, 1H), 7.02 (d, J = 3.2 Hz, 2H), 4.26 (t, J = 6.4 Hz, 2H), 3.14 (t, J = 6.4 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 151.21, 149.85, 147.79, 146.85, 141.79, 136.72, 132.86, 132.82, 131.29, 130.16, 124.47, 122.46, 121.04, 117.20, 68.22, 34.92. Anal. Calcd for $C_{18}H_{15}BrN_2OS$: C, 55.82; H, 3.90; N, 7.23. Found: C, 55.70; H, 3.91; N, 7.21. LCMS: m/z = 386 (M, $Br^{79})^+$, 388 (M, $Br^{81})^+$.

5.1.6. 2-(3-Bromophenylthio)-3-(4-(pyridin-4-yl)butoxy)pyridine (7)

Following the above procedure for **3**, title compound **7** was prepared in an identical manner from compound **5** (282 mg 1.0 mmol) and 4-(pyridin-4-yl)butan-1-ol (166 mg, 1.1 mmol) and was obtained as a white solid (402 mg, 97%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.50 (dd, J = 1.7, 4.4 Hz, 2H), 8.00 (dd, J = 2.7, 3.7 Hz, 1H), 7.67 (dd, J = 1.7, 1.7 Hz, 1H), 7.43–7.47 (m, 2H), 7.23 (dd, J = 8.0, 8.0 Hz, 1H), 7.15 (dd, J = 1.5, 4.4 Hz, 2H), 7.04 (d, J = 1.24 Hz, 1H), 7.03 (s, 1H), 4.05 (t, J = 5.6 Hz, 2H), 2.71 (t, J = 7.2 Hz, 2H), 1.84 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 151.69, 150.87, 149.70, 147.60, 141.46, 136.52, 133.14, 132.66, 131.15, 130.12, 123.86, 122.44, 121.19, 117.23, 68.36, 34.73, 28.40, 26.55. Anal. Calcd for $C_{20}H_{19}BrN_2OS$: C, 57.83; H, 4.61; N, 6.74. Found: C, 57.80; H, 4.63; N, 6.70. LCMS: m/z = 414 (M, $Br^{79})^+$, 416 (M, $Br^{81})^+$.

5.1.7. 2-(3-Bromophenylthio)-3-(2-(pyridin-3-yl)ethoxy)pyridine (8)

Following the above procedure for **3**, title compound **8** was prepared in an identical manner from compound **5** (282 mg 1.0 mmol) and 2-(pyridin-3-yl)ethan-1-ol (135 mg, 1.1 mmol) and was obtained as a white solid (373 mg, 96%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.60 (d, J = 2.4 Hz, 1H), 8.52 (dd, J = 1.6, 4.8 Hz, 1H), 7.98 (dd, J = 1.6, 3.2 Hz, 1H), 7.73 (ddd, J = 1.6, 2.4, 8.0 Hz, 1H), 7.67 (dd, J = 1.6, 3.2 Hz, 1H), 7.43–7.50 (m, 2H), 7.22–7.30 (m, 2H), 7.02 (s, 1H), 7.01 (s, 1H), 4.24 (t, J = 6.4 Hz, 2H), 3.15 (t, J = 6.4 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 151.24, 150.31, 148.23, 147.81, 141.73, 136.91, 136.80, 133.40, 132.95, 132.83, 131.30, 130.17, 123.40, 122.46, 121.02, 117.13, 68.86, 32.85. Anal. Calcd for $C_{19}H_{17}$-

BrN_2OS: C, 55.82; H, 3.90; N, 7.23. Found: C, 55.89; H, 4.15; N, 7.64. LCMS: m/z = 387 (M, Br^{79})$^+$, 389 (M, Br^{81})$^+$.

5.1.8. 2-(3-Bromophenylthio)-3-(3-(pyridin-3-yl)propoxy)pyridine (9)

Following the above procedure for **3**, title compound **9** was prepared in an identical manner from compound **5** (282 mg 1.0 mmol) and 3-(pyridin-3-yl)propan-1-ol (151 mg, 1.1 mmol) and was obtained as a white solid (392 mg, 98%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.50 (d, J = 2.2 Hz, 1H), 8.47 (dd, J = 1.5, 4.6 Hz, 1H), 8.02 (dd, J = 1.7, 4.2 Hz, 1H), 7.69 (dd, J = 1.7, 1.7 Hz, 1H), 7.55 (ddd, J = 2.0, 2.0, 7.6 Hz, 1H), 7.48 (d, J = 1.7 Hz, 1H), 7.46 (d, J = 2.0 Hz, 1H), 7.22–7.27 (m, 2H), 7.02–7.06 (m, 2H), 4.03 (t, J = 6.0 Hz, 2H), 2.86 (t, J = 7.6 Hz, 2H), 2.11-2.18 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 151.54, 150.00, 147.62 (2 peaks superlinposed), 141.53, 136.51, 136.29, 135.95, 133.14, 132.64, 131.15, 130.13, 123.35, 122.45, 121.21, 117.26, 67.12, 30.17, 29.05. Anal. Calcd for $C_{19}H_{17}BrN_2OS$: C, 56.86; H, 4.27; N, 6.98. Found: C, 56.87; H, 4.29; N, 7.01. LCMS:m/z = 400 (M, Br^{79})$^+$, 402 (M, Br^{81})$^+$.

5.1.9. 2-(3-Bromophenylthio)-3-(4-(pyridin-3-yl)butoxy)pyridine (10)

Following the above procedure for **3**, title compound **10** was prepared in an identical manner from compound **5** (282 mg 1.0 mmol) and 4-(pyridin-3-yl)butan-1-ol (166 mg, 1.1 mmol) and was obtained as a white solid (403 mg, 97%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.48 (d, J = 2.4 Hz, 1H), 8.46 (dd, J = 1.6, 4.8 Hz, 1H), 7.99 (dd, J = 2.8, 3.2 Hz, 1H), 7.67 (dd, J = 1.6, 3.2 Hz, 1H), 7.53–7.56 (m, 1H), 7.42–7.48 (m, 2H), 7.22–7.26 (m, 2H), 7.03 (s,1H), 7.02 (s, 1H), 4.06 (t, J = 5.6 Hz, 2H), 2.72(t, J = 6.8 Hz, 2H), 1.82–1.90 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 151.70, 149.94, 147.63, 147.45, 141.44, 137.16, 136.56, 135.80, 133.13, 132.70, 131.63, 130.13, 123.33, 122.45, 121.19, 117.22, 68.40, 32.56, 28.40, 27.40. Anal. Calcd for $C_{19}H_{17}BrN_2OS$: C, 57.84; H, 4.61; N, 6.74. Found: C, 57.86; H, 4.60; N, 6.77. LCMS: m/z = 415 (M, Br^{79})$^+$, 417 (M, Br^{81})$^+$.

5.1.10. 2-(3-Bromophenylthio)-3-(3-(pyridin-2-yl)propoxy)pyridine (11)

Following the above procedure for **3**, title compound **9** was prepared in an identical manner from compound **5** (282 mg 1.0 mmol) and 3-(pyridin-2-yl)propan-1-ol (151 mg, 1.1 mmol) and was obtained as a white solid (315 mg, 79%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.55 (ddd, J = 1.0, 1.9, 5.1 Hz, 1H), 7.99 (dd, J = 2.4, 3.9 Hz, 1H), 7.69 (dd, J = 1.8, 1.8 Hz, 1H), 7.60 (ddd, J = 2.0, 7.6, 7.6 Hz, 1H), 7.46 (dd, J = 1.7, 7.8 Hz, 2H), 7.25 (dd, J = 8.3, 8.3 Hz, 1H), 7.20 (d, J = 7.8 Hz, 1H), 7.13 (dd, J = 4.9, 7.6 Hz, 1H), 7.04 (s, 1H), 7.03 (d, J = 1.7 Hz, 1H) 4.08 (t, J = 6.4 Hz, 2H), 3.01 (t, J = 7.1 Hz, 2H), 2.29 (tt, J = 6.36, 7.08 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 160.87, 151.75, 149.36, 147.67, 141.39, 136.60, 136.42, 133.30, 132.74, 131.15, 130.13, 123.19, 122.47, 121.23, 121.20, 117.32, 67.90, 34.31, 28.66. Anal. Calcd for $C_{19}H_{17}BrN_2OS$: C, 56.86; H, 4.27; N, 6.98. Found: C, 56.63; H, 4.32; N, 7.05. LCMS: m/z = 400 (M, Br^{79})$^+$, 402 (M, Br^{81})$^+$.

5.2. Biology

5.2.1. Measurement of PDE4 inhibitory activity

Eosinophils were obtained from male Hartley strain guinea pigs (approximately 500 g, Nihon SLC Inc.) injected with 5 mg of Polymyxin B (SIGMA) and they were subjected to the purification of PDE4.[10,11] The activity of PDE4 was determined by modification of the two-step radioisotope method of Thompson et al.[12] The hydrolyzed cAMP was measured using [^{3}H] cAMP. The IC_{50} values (concentration which produced 50% inhibition of substrate hydrolysis) for the compounds examined were calculated from concentration–response curves. Three experiments were carried out for each agent.

5.2.2. Measurement of bronchodilatory activity

Male guinea pigs (3 weeks old) were sensitized with Ovalbumin (OVA), 6 weeks later, they were anesthetized and the trachea was cannulated with a cannula for ventilation. The animal was ventilated through the cannula inserted into the trachea with room air by a rodent ventilator (rate: 60 strokes/min). Another end of cannula was connected to a bronchospasm transducer (UGO BASILE, Italy) to measure the air-overflow according to Konzett–Rössler Method.[13] A vein of a hind paw was cannulated with a catheter for administration of antigen. OVA (0.01% OVA, 0.5 mL/kg; 50 mg/kg) was injected through the catheter to induce bronchospasm. The response was recorded for 20 min after the challenge of OVA. The peak response was determined within 10 min after the challenge and expressed as a percentage of the theoretical maximum bronchospasm (i.e., ventilation volume just before challenge). Test compounds were administered orally 1 h before the challenge. The inhibitory % of each treatment group was calculated from in comparison to the vehicle control group.

5.2.3. Measurement of inhibitory activity on cell infiltration into airway area in mice

Male mice (5 weeks old) were sensitized with OVA. Seven days after the last injection of OVA, they were anesthetized and challenged with OVA intranasally to induce eosinophil infiltration. Three days after the challenge, bronchoalveolar lavage was done under NEMBUTAL anaesthetization and cells infiltrated into lung were collected and counted. BALF was diluted with Turk's solution by 10 times, and living cells were counted. Test compounds were administered orally 30 min before the challenge and the following 2 days (once a day). The inhibitory % of each treatment group was calculated from in comparison to the vehicle control group.

Acknowledgment

We thank Keiko Bando for elemental analysis and Shinya Usui for useful discussion.

References and notes

1. Houslay, M. D.; Adams, D. R. *Biochem. J.* **2003**, *370*, 1.
2. Houslay, M. D.; Schafer, P.; Zhand, K. Y. J. *Drug Discovery Today* **2005**, *10*, 1503.
3. Sanz, M. J.; Cortijo, J.; Morcillo, E. J. *Pharmcol. Ther.* **2005**, *106*, 269.
4. Kodimuthali, A.; Jabaris, S. S. L.; Pal, M. *J. Med. Chem.* **2008**, *51*, 5471.
5. Hatzelmann, A.; Schdt, C. *J. Pharmacol. Exp. Ther.* **2001**, *297*, 267.
6. Giembycz, M. A.; Field, S. K. *Drug Des. Dev. Ther.* **2010**, *4*, 147.
7. Hatzelmann, A.; Morcillo, E. J.; Lungarella, G.; Adnot, S.; Sanjar, S.; Beume, R.; Schudt, C.; Tenor, H. *Pulm. Pharmacol. Ther.* **2010**, *23*, 235.
8. Chung, K. F. *Eur. J. Pharmacol.* **2006**, *533*, 110.
9. Card, G. L.; England, B. P.; Suzuki, Y.; Fong, D.; Powell, B.; Lee, B.; Luu, C.; Tabrizizad, M.; Gillette, S.; Ibrahim, P. N.; Artis, D. R.; Bollag, G.; Milburn, M. V.; Kim, S.-H.; Schlessinger, J.; Zhang, K. Y. J. *Structure* **2004**, *12*, 2233.
10. Torphy, T. J. *Am. J. Respir. Crit. Care Med.* **1998**, *157*, 351.
11. Souness, J. E.; Maslen, C.; Scott, L. C. *FEBS Lett.* **1992**, *302*, 181.
12. Thompson, W. J.; Terasaki, W. L.; Epstein, P. M.; Strada, S. J. *Adv. Cycl. Nucleotide Res.* **1979**, *10*, 69.
13. Konzett, H.; Rössler, R. *Arch. Exp. Pathol. Pharmacol.* **1940**, *195*, 71.