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Discovery of imidazo[1,2-*b*]pyridazines as IKKβ inhibitors. Part 2: Improvement of potency in vitro and in vivo

Hiroki Shimizu *, Isao Yasumatsu, Tomoaki Hamada, Yoshiyuki Yoneda, Tomonori Yamasaki, Shinji Tanaka, Tadashi Toki, Mika Yokoyama, Kaoru Morishita, Shin Iimura

R&D Division, Daiichi Sankyo Co., Ltd, 1-16-13 Kitakasai, Edogawa-ku, Tokyo 134-8630, Japan

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ABSTRACT

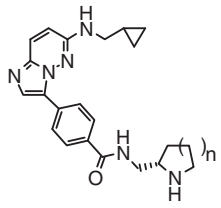
We have increased the potency of imidazo[1,2-*b*]pyridazine derivatives as IKKβ inhibitors with two strategies. One is to enhance the activity in cell-based assay by adjusting the polarity of molecules to improve permeability. Another is to increase the affinity for IKKβ by the introduction of additional substituents based on the hypothesis derived from an interaction model study. These improved compounds showed inhibitory activity of TNFα production in mice.

Nuclear factor-κB (NF-κB) is a transcription factor that has a crucial part in the immune system.[1,2] NF-κB plays a number of important roles such as immune-response, inflammation, cell proliferation, survival and cell death by regulating the expression of a variety of genes of proteins including pro-inflammatory cytokines (e.g., TNFα, IL-1, IL-6), chemokines, anti-apoptotic proteins, adhesion molecules, osteoclastogenesis-related factors and inducible proteins.[3–7] NF-κB is implicated in the pathogenesis of multiple inflammatory diseases and autoimmune diseases including rheumatoid arthritis. It is observed that NF-κB is highly active in the site of inflammation.[3–5,8]

There are some signal transduction cascades for the activation of NF-κB.[6d,9] In the classical (canonical) pathway, known as one of the major pathways, IKK complex (IKKα/IKKβ/NEMO) plays an important role in activating NF-κB (RelA/p50).[9,10] RelA/p50 exists in an inactive complex associated with IκB. The phosphorylation of IκB by the IKK complex and subsequent K48-linked polyubiquitination lead to the degradation of IκB. The released RelA/p50 promotes transcription of genes of pro-inflammatory cytokines and other inducible proteins in nucleus. Of the IKK components, IKKβ is essential in phosphorylation of IκB. It is anticipated that a potent IKKβ inhibitor could be a promising anti-inflammatory agent.[2a,11,12]

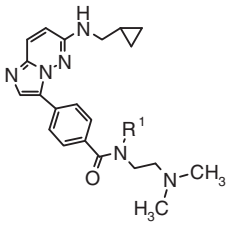
Hence, we continued our effort to acquire orally available small molecules that would be potent IKKβ inhibitory agents. We have reported imidazo[1,2-*b*]pyridazine derivatives **1**, **2** and **3** as the lead compounds that showed potent IKKβ inhibitory activities and high selectivity against other kinases[13,14] (Fig. 1). In the following step, it becomes important to find the compounds that show strong activity in vivo to develop an anti-inflammatory agent.

Therefore, we started inhibition assay of TNFα production by mouse whole blood cell as a cell-based assay to connect cell-free assay and in vivo assay in mice. The results are shown in Table 1.



1 (n=1)
IKKβ: IC_{50} = 0.055 μM
TNFα: IC_{50} = 0.69 μM (THP-1 cell)

2 (n=2)
IKKβ: IC_{50} = 0.047 μM
TNFα: IC_{50} = 1.5 μM



3 (R^1= H)
IKKβ IC_{50} = 0.20 μM
TNFα: IC_{50} = 0.69 μM

4 (R^1= Me)
IKKβ IC_{50} = 0.33 μM
TNFα: IC_{50} = 2.4 μM

Figure 1. Imidazo[1,2-*b*]pyridazine derivatives that were discovered as the lead compounds for the development of IKKβ inhibitors.[13]

* Corresponding author. Tel.: +81 3 3680 0151; fax: +81 3 5696 8609.
E-mail address: shimizu.hiroki.hu@daiichisankyo.co.jp (H. Shimizu).

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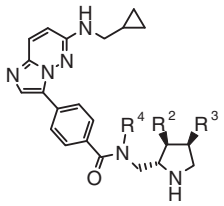
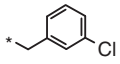
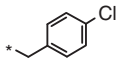
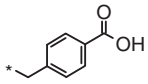
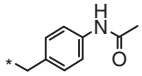
Table 1
In vitro activity, in vivo activity and physicochemical data of imidazo[1,2-*b*]pyridazine derivatives



Compds	R^2	R^3	R^4	IKKβ[a] IC_{50} (μM)	TNFα production[b] IC_{50} (μM)	PAMPA[c] ($P_e \times 10^{-6}$ cm/s) (pH7.4)	Inhibition of TNFα (%) at 30 mg/kg po in mice[d]	Plasma level[e] at 30 mg/kg po (μg/ml)	Metabolic stability[f] (%)
1	H	H	H	0.055	6.8	<2	No inhibition	0.0075	77
3	Structure, see Figure 1.			0.20	0.80	>50	10	0.39	71
5a	OMe	H	H	0.023	2.1	6.5	—[g]	—	—
5b	H	OMe	H	0.071	2.7	<2	—	—	—
5c	F	H	H	0.021	1.2	—	—	—	—
5d	H	F	H	0.022	0.57	36	—	—	—
6	H	H	Me	0.097	1.9	6.1	—	—	—
7a	H	F	Me	0.042	0.48	>50	26	0.34	50
7b	H	F	Et	0.018	0.23	42	32	0.15	33
7c	H	F	*n*-Pr	0.016	0.17	>50	52	0.16	33
7d	H	F	Bn	0.012	0.40	>50	—	—	—
7e	H	F		0.017	0.59	>50	40	0.12	7
7f	H	F		0.017	1.2	39	61	0.45	28
7g	H	F	Cinnamyl	0.013	0.95	>50	—	—	—
7h	H	F	Ph	7.3	—	—	—	—	—
7i	H	F	CH_2CO_2H	0.11	3.9	14	—	—	—
7j	H	F	CH_2CH_2OH	0.015	0.68	15	—	—	—
7k	H	F		0.017	>10	<2	—	—	—
7l	H	F		0.015	2.7	<2	—	—	—

[a] The method is described in Ref. 13.
[b] Mouse whole blood cell. The method is described in Ref. 17.
[c] Parallel artificial membrane permeability assay. Median of three tests.
[d] The method is described in Ref. 18.
[e] Plasma concentrations of test compounds at 90 min after oral administration.
[f] Remaining rate of test compounds (1 μM) after 30 min of incubation with mouse liver microsomes (0.1 mg/ml).
[g] Not tested.

It was revealed that **1** showed decreased activity in this assay. Furthermore, a plasma level of **1** was found to be very low after oral administration in mice.

To overcome these problems and further enhancement of potency, we developed two strategies. One is to adjust the polarity of cyclic secondary amines to improve permeability. Another is to enhance affinity of the compounds for IKKβ by the introduction of additional substituents.

About the former strategy, we considered the permeability of **1** to be too low to penetrate the cellular membrane of mouse whole blood cell. The poor permeability made it difficult to show oral absorption. In our previous paper, we have reported that the PAMPA values of the compounds with pyrrolidine substructure are low.[13] The high polarity of the pyrrolidine unit seemed to cause poor permeability. On the other hand, we have found that the secondary amine moiety is important in showing strong IKKβ inhibitory activity and the interaction model study followed these results.[13] Based on these findings, we decided to decrease polarity by maintaining the secondary amine structure.

The latter strategy came from the hypothesis that the introduction of an appropriate moiety to the amide nitrogen on the substituent of the 3-position of imidazo[1,2-*b*]pyridazine could increase affinity for IKKβ from the interaction model study.[13]

To begin with, we put the first strategy into practice. We introduced electron-withdrawing substituents such as the methoxy group or fluorine on the pyrrolidine ring to decrease the basicity and adjust the polarity.

In compounds **5a**, **5c** and **5d**, inhibitory activities in cell-free assay were twofold more potent than that of **1**.[15] Also, these compounds showed over threefold more active in cell-based TNFα inhibitory assay. In particular, cell-based activity of **5d** was enhanced more than 10-fold compared with **1**. These results could be explained by the improvement of permeability. As indicated in the PAMPA value, the permeability of **5d** was improved by the adjustment of polarity by the introduction of fluorine to the appropriate site on pyrrolidine[16] (Table 1).

About the latter strategy, it was hypothesized that a hydrophobic pocket emerges as a result of the conformational shift of the

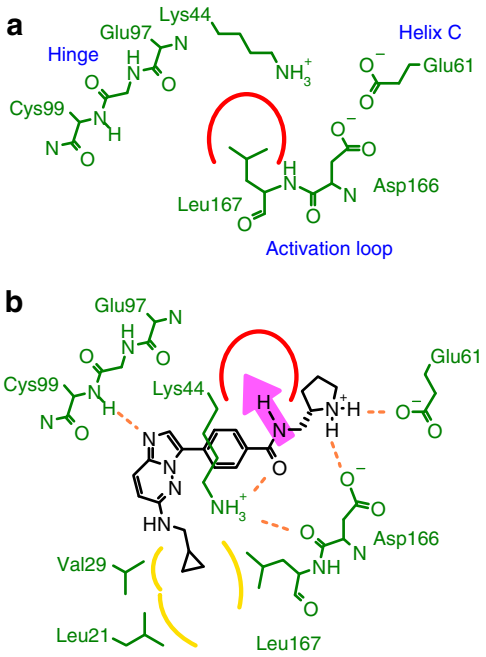


Figure 2. 2D representations of the ATP binding site of IKKβ. (a) Before the interaction with inhibitors. Side chain of Leu167 occupies the hydrophobic pocket (represented as a red line). (b) The predicted binding mode of compound **1** with IKKβ. The hydrogen bondings are represented as orange dotted lines. Van der Waals interactions are represented as yellow lines. The pocket that is revealed by the movement of the activation loop is located in the direction of amide nitrogen to hydrogen (shown by a magenta arrow).

activation loop.[13] Before the interaction with inhibitors, the pocket is occupied by the side chain of Leu167 on the activation loop (Fig. 2a). The revealed pocket is considered to exist in the direction of the N–H bond of amide on the substituent of the 3-position of imidazo[1,2-*b*]pyridazine (Fig. 2b). There are only a few investigations about the conformational shift of the activation loop including the DLG motif, but not DFG[19]; therefore, we approached this hypothesis by synthesizing the compounds. We observed that compound **4** in which the amide nitrogen was methylated, shows IKKβ inhibitory activity.[13] We began searching for the substituents that suit the pocket and increase affinity for IKKβ.

We evaluated **6**, **7a**–**7l**, the combination of the amide *N*-alkyl/aryl moiety and pyrrolidine/(4*R*)-fluoropyrrolidine unit.

The results are shown in Table 1. The inhibitory potencies of **6** for **1** and **7a**[20] for **5d** in cell-free assay were decreased to about half, whereas those in cell-based assays were increased because the permeability of **6** and that of **7a** were improved.

The compounds with hydrophobic substituents on the amide nitrogen **7b**–**7g** showed potent IKKβ inhibitory activity and TNFα production inhibitory activity, while the compound with polar substituent **7i** exhibited decreased potency.

Compounds **7k** and **7l** showed strong IKKβ inhibitory activity, but cell-based activities were reduced because of poor permeability as indicated in the PAMPA values. *N*-Phenyl derivative **7h** also showed decreased IKKβ inhibitory activity. It is assumed that the phenyl group crashes into the entrance of the pocket. At minimum ethyl group, which is about the size of the substituent on the amide nitrogen, seems to be required for the interaction with the entrance of the pocket. For the back of the pocket, various (small or large, hydrophobic or hydrophilic) substituents were allowed. Compound **7j** that has a hydroxyethyl group also showed potent IKKβ inhibitory activity.

The considered binding mode is shown in Figure 3. The pocket is occupied by the additional substituent, and the compound also

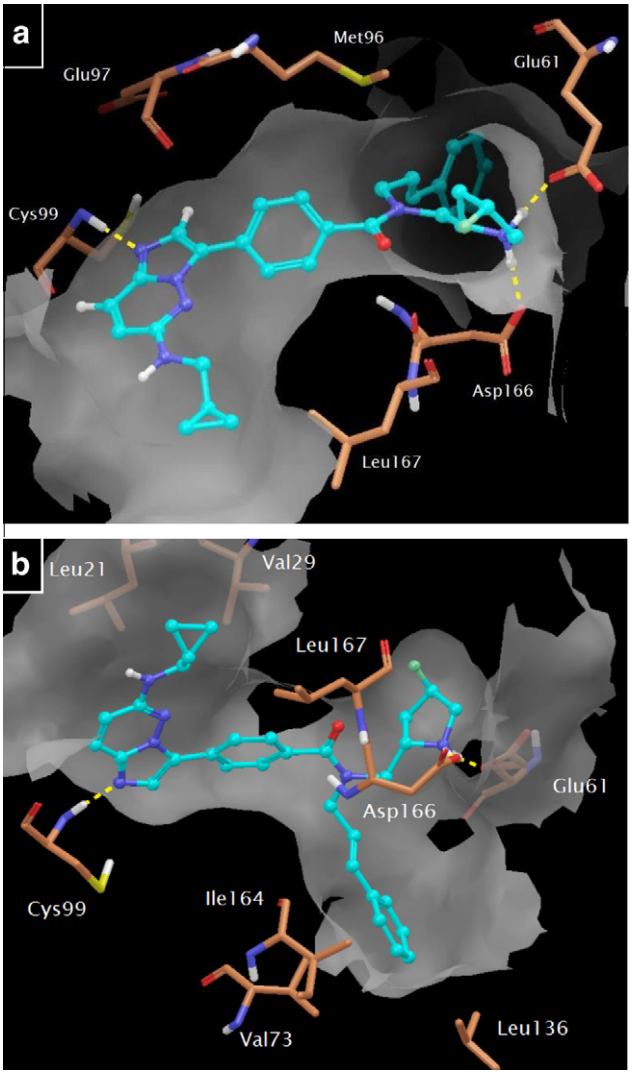
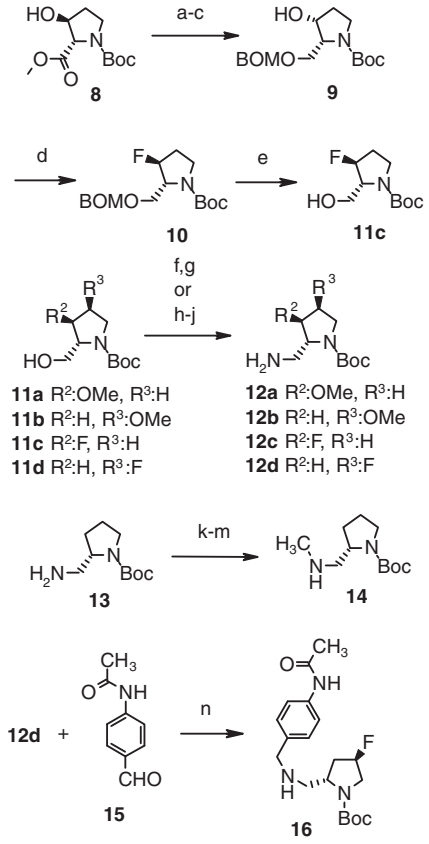


Figure 3. Predictive binding mode of **7g** with IKKβ. (a) Top view (from the *N*-terminated domain). Cinnamyl moiety on the amide nitrogen in the 3-position is directed to the hydrophobic pocket. Imidazo[1,2-*b*]pyridazine moiety interacts with the hinge region. Ammonium salt of pyrrolidine interacts with Glu61 and Asp166. (b) Side view. The pocket is suited with cinnamyl moiety. There is permissibility in the back of the pocket. Cyclopropylmethyl moiety is caught among Leu21, Val29 and Leu167.[13]

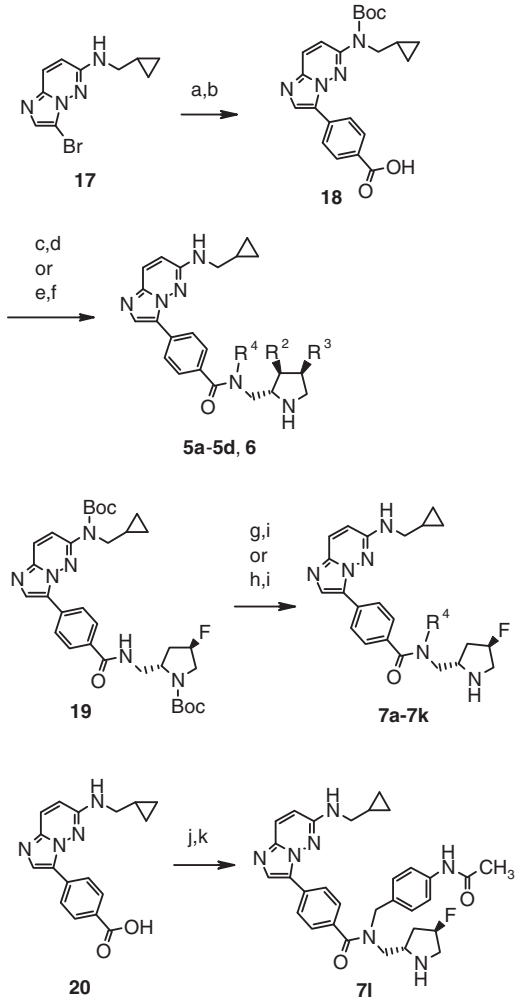
maintains interactions in the hinge region and with carboxylate residues.

In the inhibition assay of LPS-induced TNFα production in mice, **7f** showed 61% inhibition of TNFα production and **7c** showed 52% inhibition by an oral dose of 30 mg/kg, whereas **7a** showed 26% inhibition and **3** showed 10% inhibition (Table 1). These results indicate that the modifications from the two strategies are effective for exhibiting inhibitory activity in vivo. Compound **7f** showed potent inhibitory activity in vivo despite moderate levels of inhibitory activity in cell-based assay. This could be explained in part by the high level in plasma.

The test compounds were synthesized as shown in Scheme 1 and Scheme 2. (3*S*)-Fluoropyrrolidine **11c** was prepared from 3-hydroxyproline derivative **8**. The reduction of methyl ester gave primary alcohol, which was protected as benzyloxymethyl (BOM) ether. The (3*S*)-hydroxyl group on the pyrrolidine ring was converted to (3*R*)-isomer **9** by Mitsunobu reaction. Compound **9** led to (3*S*)-fluoride **10**[21] followed by cleavage of the BOM group to afford **11c**. The hydroxyl groups of **11a**–**11d**[22] were converted



Scheme 1. Syntheses of pyrrolidine derivatives. Reagents and conditions: (a) $LiBH_4$, THF 0 °C to rt, 94%; (b) BOMCl, DIPEA, 0 °C to rt, CH_2Cl_2 79%; (c) HCO_2H, DIAD, Ph_3P, THF, reflux then 1 N NaOH aq, THF-MeOH, rt, 75%; (d) Deoxo-Fluor®, CH_2Cl_2, −78 °C to rt, 35%; (e) H_2, Pd/C, MeOH; (f) DPPA, DIAD, Ph_3P, THF; (g) Ph_3P, THF, H_2O (quant. 2 steps for **12a**, 46% 2 steps from **12b**); (h) MsCl, Et_3N, CH_2Cl_2; (i) NaN_3, DMF, 50–60 °C; (j) H_2, 5% Pd/C, EtOH (43% 4 steps for **12c**, 62% 3 steps for **12d**); (k) TFAA, Et_3N, CH_2Cl_2, 81%; (l) MeI, NaH, DMF, 79%; (m) K_2CO_3, MeOH, 88%; (n) $NaBH_3CN$, AcOH, MeOH, 36%. Boc: *tert*-Butyloxycarbonyl, BOM: Benzyloxymethyl.



Scheme 2. The combination of imidazo[1,2-*b*]pyridazine scaffold and pyrrolidine units. Reagents and conditions: (a) $(Boc)_2O$, DMAP, CH_2Cl_2, 93%; (b) 4-(dihydroxyboryl)benzoic acid, $Pd(PPh_3)_2Cl_2$, K_2CO_3, 1,4-dioxane-water (3:2), 77%; (c) **12a**, **12b**, **12c**, **12d** or **14** DMT-MM, DMF; (d) TFA, CH_2Cl_2 (74% 2 steps for **5a**, 23% 2 steps for **5b**, 20% 2 steps for **5c**, 94% 2 steps for **5d** via **19**); (e) **14**, EDC·HCl, HOBt, Et_3N, 92%; (f) 4 N HCl in dioxane, 96%.; (g) R^4X (MeI, EtI, *n*-PrI, BnBr, 1-chloro-3-(chloromethyl)benzene, 1-chloro-4-(chloromethyl)benzene, cinnamyl bromide, *t*-butyl chloroacetate, 4-chloromethylbenzoic acid or 1-[(2-iodoethoxy)methyl]-4-methoxybenzene), NaH, DMF; (h) PhI, CuI, K_2CO_3, DMF, 150 °C; (i) TFA, CH_2Cl_2, 71% 2 steps for **7a**, 39% 2 steps for **7b**, 35% 2 steps for **7c**, 69% 2 steps for **7d**, 43% 2 steps for **7e**, 44% 2 steps for **7f**, 18% 2 steps for **7g**, 14% 2 steps for **7h**, 32% 2 steps for **7i** (to cleave Boc groups and *t*-Bu ester), 40% 2 steps for **7j** (to cleave Boc groups and the PMB group), 18% 2 steps for **7k**; (j) **16**, DMT-MM, DMAP, Et_3N, DMF 71%; (k) TFA, CH_2Cl_2, 76%. PMB: *p*-methoxybenzyl.

to primary amines **12a**–**12d** via the azide moiety. Pyrrolidine derivative **14** was synthesized by methylation of **13** by way of trifluoroacetamide. Benzylamine derivative **16** was prepared by reductive amination of aldehyde **15** with **12d** (Scheme 1).

Compounds **5a**–**5d**, **6** and **7l** were synthesized by the condensation reaction of carboxylic acid **18** or **20**[13] and amines **12a**, **12b**, **12c**, **12d** or **16** followed by the cleavage of the Boc group.[23]

Compounds **7a**–**7k** were prepared by alkylation of amide nitrogen of compound **19** by various alkyl halides followed by the cleavage of protective groups. 1-[(2-Iodoethoxy)methyl]-4-methoxybenzene was prepared as indicated in a previous report.[24] *N*-Phenyl derivative **7h** was formed by coupling with iodobenzene (Scheme 2).

In conclusion, we have discovered potent compounds by the modification of the substituents in the 3-position of imidazo[1,2-*b*]pyridazine derivatives based on two strategies.

One strategy is the introduction of an electron-withdrawing group on the pyrrolidine ring to adjust polarity for better permeability. Another is the introduction of hydrophobic substituents to the amide nitrogen to improve affinity for IKKβ. We believe that these substituents interact with the pocket which is newly made after the activation loop moving out.

We have acquired orally active compounds such as **7c** and **7f** that showed increased IKKβ inhibitory activities and TNFα production inhibitory activities in mice. Further investigation of IKKβ inhibitors will be reported in the near future.

Acknowledgements

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14. Compound **1** showed more than 90% inhibition against three kinases (ALK, JAK2 and PKD2), and showed 80–90% inhibition against 6 kinases at 10 μM out of 250 kinases.
15. Compound **5d** showed 80% inhibition against PKD3 at 0.2 μM out of 19 kinases in which compound **1** showed relatively strong inhibitory activity.
16. The difference between the PSA values of **5c** (88.3 $Å^2$) and **5d** (82.6 $Å^2$) was noted. The 3D structures were generated using CORINA (Molecular Networks GmbH) and optimized using MacroModel and PM3 method in the MOPAC 7 program. PSA values were calculated using VEGA.
17. Inhibition assay of LPS-induced TNF production by mouse whole blood cell.
Whole blood was collected from the inferior vena cava of BALB/c mice and heparinized. The blood was suspended in RPMI-1640 medium to a final leukocyte density of 2.5×10^5 cells/ml. Test compounds at various concentrations or 5% DMSO were predispensed as 20 μl aliquots into 96-well plates. After the addition of 160 μl of cell suspension to the plate, 20 μl of 10 μg/ml LPS or RPMI-1640 medium was added. After incubation for 4 h at 37 °C in 5% CO_2, plasma was obtained by centrifugation (2000 rpm, 5 min, 4 °C). TNFα concentration in the plasma was measured by ELISA assay as described by the manufacturer's instructions (BD Bioscience).
18. Inhibition assay of LPS-induced TNFα production in mice.
Male BALB/c mice (7–12 weeks old) were orally given a test compound or 0.5% methyl cellulose. Thirty min after treatment, 0.02 mg/kg of LPS in saline was administrated intravenously. One hour after the injection of LPS solution, blood was collected from inferior vena cava with heparin as an anticoagulant under ether anesthesia. Plasma was obtained by centrifugation (3000 rpm, 30 min, 4 °C) and immediately stored at −20 °C. TNFα concentration in the plasma was measured by ELISA assay as described by the manufacturer's instructions (R&D Systems).
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20. Compound **7a** showed 61% inhibition against PKD3 at 0.2 μM out of 19 kinases in which compound **1** showed relatively strong inhibitory activity.
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