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Discovery of imidazo[1,2-*b*]pyridazine derivatives as IKKβ inhibitors. Part 1: Hit-to-lead study and structure–activity relationship

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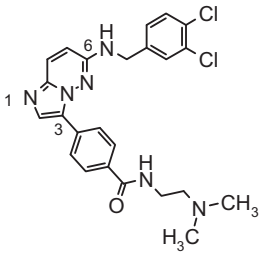
ABSTRACT

Imidazo[1,2-*b*]pyridazine derivatives from high-throughput screening were developed as IKKβ inhibitors. By the optimization of the 3- and 6-position of imidazo[1,2-*b*]pyridazine scaffold, cell-free IKKβ inhibitory activity and TNFα inhibitory activity in THP-1 cell increased. Also, these compounds showed high kinase selectivity. The structure–activity relationship was revealed and the interaction model of imidazo[1,2-*b*]pyridazine compounds with IKKβ was constructed.

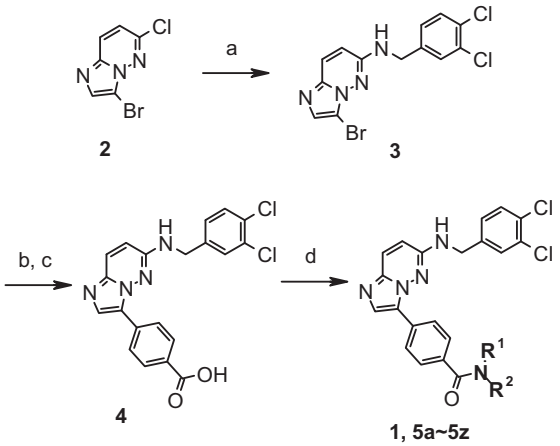
Nuclear factor κB (NF-κB) is the transcription factor that has a crucial part in the immune system.[1,2] NF-κB plays a number of important roles such as immune-response, inflammation, cell proliferation and survival/cell death by regulating the expression of a variety of genes including pro-inflammatory cytokines (e.g., TNFα, IL-1, IL-6), chemokines, anti-apoptotic proteins, adhension 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 area of inflammation.[3–5,8]

There are some signal transduction cascades for the activation of NF-κB.[6d,9] In the classical (canonical) pathway, 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 degradation of IκB as a result of phosphorylation by IKK complex and subsequent K48-linked polyubiquitination causes RelA/p50 to be released from IκB. The 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



Compound **1**
IKKβ: IC_{50} 1.1 μM
CDK2: IC_{50} >100 μM
GSK3β: IC_{50} >100 μM

Figure 1. HTS-hit compound **1**.



Scheme 1. Syntheses of **1** and modification of terminal substituent in the 3-position of imidazo[1,2-*b*]pyridazines **5a**–**5z**.[24] Reagents and conditions: (a) 3,4-dichlorobenzylamine, 140 °C, 81%; (b) 4-ethoxycarbonylphenylboronic acid, $PdCl_2(dppf)\cdot CH_2Cl_2$, K_3PO_4, dppf, dioxane, 80 °C, 49%; (c) 1 N NaOH aq, MeOH, reflux, 36%; (d) amine (R^1R^2NH), HBTU, Et_3N, DMF then TFA, CH_2Cl_2 (to cleave the Boc group that protects primary amine to give **5a**, **5e**, **5i**–**5m**, **5s**; and to cleave the 2,4-dimethoxybenzyl group that protects primary amine to give **5z**) 19–69%. Yields are not optimized.

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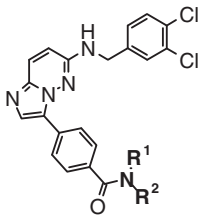
Table 1
IKKβ inhibition and TNFα production inhibition assay results for terminal substituent in the 3-position, compounds **1** and **5a**–**5z**



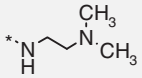
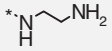
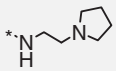
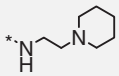
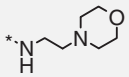
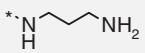
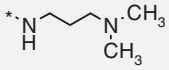
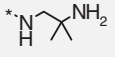
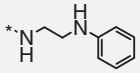
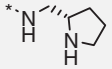
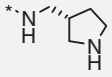
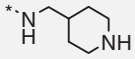
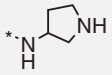
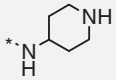
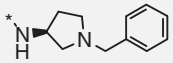
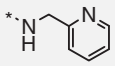
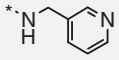
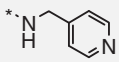
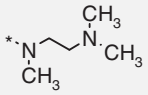
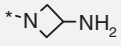
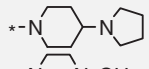
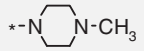
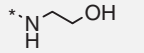
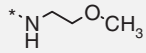
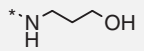
Compds	$*-N(R^1)R^2$	IKKβ inhibition[a] IC_{50} (μM)	TNFα inhibition[b] IC_{50} (μM)
1		1.1	>30
5a		2.8	2.4
5b		0.59	>30
5c		2.4	>30
5d		>30	NT[c]
5e		4.0	4.7
5f		2.4	>30
5g		11	6.5
5h		>30	>30
5i		0.32	4.0
5j		10	17
5k		14	>30
5l		5.6	6.5
5m		18	4.4
5n		>30	NT
5o		>30	>30
5p		>30	NT
5q		>30	NT
5r		2.1	15
5s		8.9	>30

Table 1 (*continued*)

Compds	$*-N(R^1)R^2$	IKKβ inhibition[a] IC_{50} (μM)	TNFα inhibition[b] IC_{50} (μM)
5t		1.4	>30
5u		>30	12
5v		>30	NT
5w		>30	NT
5x		>30	NT
5y		27	27
5z		>30	1.7

[a] The method for measuring cell-free IKKβ inhibitory activity is described in Ref. 25.
[b] The method for measuring the inhibitory activity of TNFα release by THP-1 cell is described in Ref. 27.
[c] Not tested.

IKKβ inhibitor could be a promising anti-inflammatory agent by the inhibition of the NF-κB pathway.[2a,11,12]

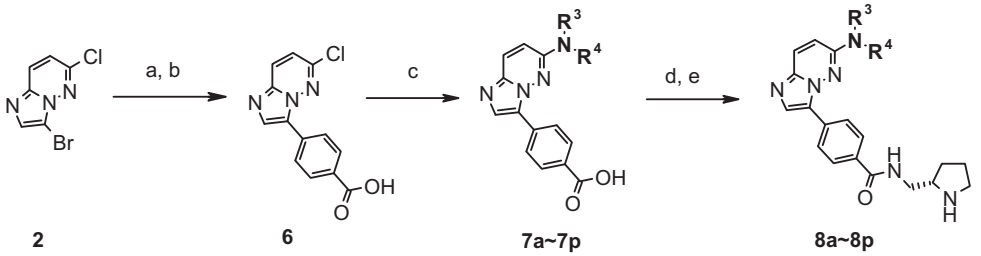
Recently, biological agents such as anti-TNF antibodies, soluble TNF receptor and anti-IL-6 receptor antibody are well used for the treatment of inflammatory disorders and autoimmune disorders such as rheumatoid arthritis.[13,14] However, these biological agents have some limitation in price, a limited administration route and induction of autoantibody. Therefore, it is necessary to develop an orally available small molecule which can decrease the production of pro-inflammatory cytokines.[12a,14] A number of pharmaceutical companies have made efforts to develop an IKKβ inhibitor.[11,15–23] Here, we report on the research to acquire the compounds that show strong IKKβ inhibitory activity to develop an anti-inflammatory agent.

To acquire small molecules that demonstrate IKKβ inhibitory activity, we performed high-throughput screening (HTS) and discovered a series of potent compounds, which commonly have the imidazo[1,2-*b*]pyridazine scaffold. Among these compounds, compound **1** showed IC_{50} 1.1 μM of IKKβ inhibitory activity (Fig. 1). Moreover, this compound showed good kinase selectivity over CDK2 (IC_{50} >100 μM) and GSK3β (IC_{50} >100 μM). We adopted **1** as a HTS-hit compound and tried to improve the potency.

As the first step in the modification of compound **1**, the terminal amine moiety in the 3-position of imidazo[1,2-*b*]pyridazine was replaced with another amine or polar substituent to demonstrate the structure–activity relationship of this substructure.

The general synthetic route for compounds **1** and **5a**–**5z** are shown in Scheme 1. 3-Bromo-6-chloroimidazo[1,2-*b*]pyridazine **2** was prepared by a known procedure as indicated in a previous report.[28] Nucleophilic substitution to compound **2** gave amine **3**. Suzuki-Miyaura coupling to **3**, followed by hydrolysis of ethyl ester gave carboxylic acid **4**. By the condensation of various amines with compound **4**, compounds **1** and **5a**–**5z** were synthesized.

The results of the modification of the terminal amine moiety are described in Table 1. The structure–activity relationship in the 3-position was revealed as follows. For the distance between amide and amino nitrogen, the compounds with three atom distances showed higher potency (**1**, **5a, 5b**) than those with four atom distances (**5e**, **5f**). The (*S*)-2-pyrrolidinylmethyl moiety **5i** increased potency (IKKβ IC_{50} 0.32 μM). However, even when the compound has a cyclic alkylamine unit, compounds **5j**–**5n** decreased in potency. When the amine was piperidine **5c**,



Scheme 2. Modification of substituents in the 6-position of imidazo[1,2-*b*]pyridazine.[24] Reagents and conditions: (a) 4-methoxycarbonylphenylboronic acid, $PdCl_2(dppf)\cdot CH_2Cl_2$, K_3PO_4, dppf, dioxane, 80 °C, 44%; (b) $LiOH\cdot H_2O$, THF-H_2O (3:1), 86%; (c) amine (R^3R^4NH), 140 °C; (d) *tert*-Butyl (2*S*)-2-(aminomethyl)-1-pyrrolidinecarboxylate, HBTU, Et_3N, DMF; (e) TFA, CH_2Cl_2, 8–29% 3 steps (to cleave the Boc group that protects the pyrrolidine moiety and the 2,4-dimethoxybenzyl group that protects primary amine to give **8a**). Yields are not optimized.

morpholine **5d**, 2-amino-2,2-dimethethyl **5g**, aniline **5h** or pyridines **5o–5q**, the inhibitory activity decreased. By these results it is revealed that the substructure around the terminal amine influences the affinity to IKKβ. The *N,N,*-disubstituted amide such as the amide of *N,N,N′*-trimethyldimethylamine **5r** and 4-(pyrrolidin-1-yl)-1-piperidine **5t** remained active, but the amide of azetidine **5s** and piperazine **5u** had little activity. In the case of replacing the amine moiety to the neutral groups **5v–5y** or the removal of substituent **5z**, the potency decreased.

After the exploration of the 3-position, we introduced various structure to the 6-position of imidazo[1,2-*b*]pyridazine. The modified compounds of the 6-position were synthesized as in Scheme 2.

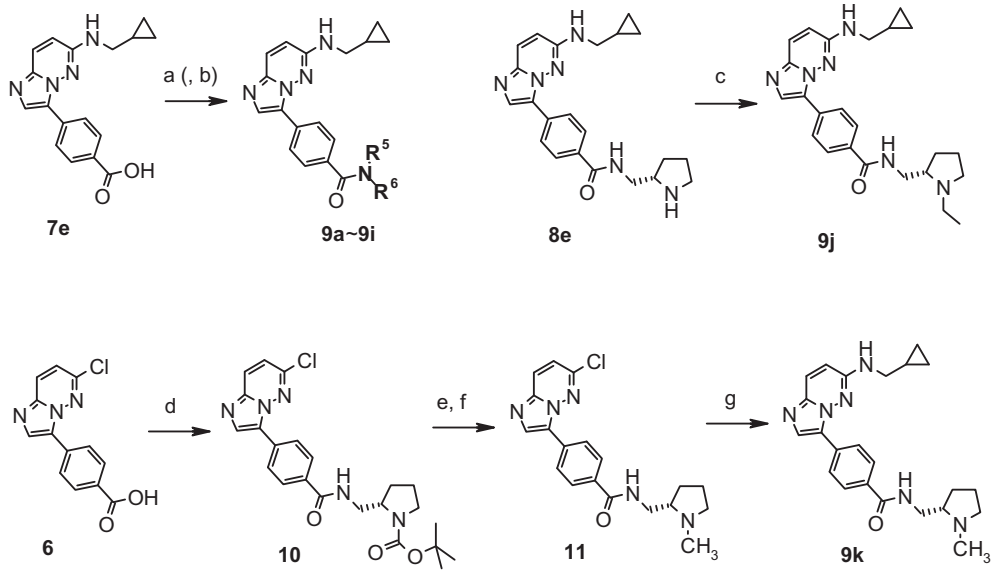
3-Bromo-6-chloroimidazo[1,2-*b*]pyridazine **2** was converted to carboxylic acid **6** by Suzuki-Miyaura coupling reaction, followed by hydrolysis of methyl ester. To compound **6**, nucleophilic substitution by various amines afforded compounds **7a–7p**. The condensation reaction of carboxylic acids **7a–7p** with *tert*-butyl (2*S*)-2-(aminomethyl)-1-pyrrolidinecarboxylate, followed by the cleavage of protective groups gave **8a–8p**.

About the structure–activity relationship in the 6-position of imidazo[1,2-*b*]pyridazine, we found that the secondary amine with an appropriate size of non-polar substituents was favored (**8e**, methyl group **8b** was too small). Primary or tertiary amine in the 6-position decreased potency (**8a**, **8n–8p**), and the polar substituent near the NH moiety was not favored (**8h**, **8j**, **8k**). Regarding the size of the substituent, an adjacent alkyl moiety to the amine moiety that is not bulky, such as the cyclopropylmethyl group was suitable. Other moieties such as isopropyl **8c**, cyclopropyl **8d**, benzyl **8f**, **8g** and cyclohexyl **8m** were less favored (Table 2).

After determining the potent cyclopropylmethyl group in the 6-position, we revisited the terminal amine moiety in the 3-position again to further optimize and investigate the detailed structure–activity relationship.

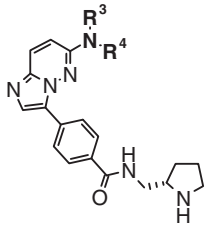
The compounds **9a–9k** were synthesized as shown in Scheme 3. Compounds **9a–9i** were formed by the condensation reaction of carboxylic acid **7e** and the amine moiety.[29] Compound **9j** was synthesized by reductive amination of acetaldehyde to **8e**. Compound **9k** was synthesized in a different manner to avoid methylation of the 6-NH moiety of imidazo[1,2-*b*]pyridazine. By the condensation of carboxylic acid **6** with *tert*-butyl (2*S*)-2-(aminomethyl)-1-pyrrolidinecarboxylate, amide **10** was formed. The cleavage of the Boc group of **10** and subsequent methylation of pyrrolidine with formaldehyde gave *N*-methylpyrrolidine **11**. The nucleophilic substitution of **11** by cyclopropylmethylamine afforded **9k**.

The compounds with a terminal substructure in the 3-position were *N,N*-dimethylamine **9a**, *N,N*-diethylamine **9b**, *N,N,N′*-trimethylethylenediamine **9c** and the aminoethyl derivatives **9d** and **9e**, which produced IKKβ inhibitory activities that were about IC_{50} 0.07–0.3 μM. The compound with (*S*)-2-piperidinylmethyl **9i** showed strong IKKβ inhibitory activity almost the same as **8e**.



Scheme 3. Modification of the terminal unit in the 3-position of imidazo[1,2-*a*]pyridazine.[24] Reagents and conditions: (a) amine (R^5R^6NH), DMT-MM, DMF; (b) TFA, 20–60% 2 steps. Yields are not optimized; (c) CH_3CHO, $NaBH(OAc)_3$, DCE, 45%; (d) *tert*-Butyl (2*S*)-2-(aminomethyl)-1-pyrrolidinecarboxylate, DMT-MM, DMF, 78%; (e) TFA; (f) 37% HCHO in water, $NaBH_3CN$, AcOH, MeOH, 84% 2 steps; (g) cyclopropylmethylamine, 120 °C, 49%.

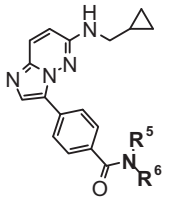
Table 2
IKKβ inhibition and TNFα production inhibition assay results for the substituent in the 6-position, compounds **8a**–**8p**



Compds	R^3 / R^4	IKKβ inhibition[a] IC_{50} (μM)	TNFα inhibition[b] IC_{50} (μM)
8a	NH_2	14	NT[c]
8b	NH, CH_3	1.3	29
8c	NH, isopropyl	0.27	1.7
8d	NH, cyclopropyl	0.20	3.2
8e	NH, cyclopropylmethyl	0.055	0.69
8f	NH, benzyl	0.33	3.7
8g	NH, 3-chlorobenzyl	0.19	2.7
8h	NH, 2-hydroxyethyl	3.7	>30
8i	NH, 3-hydroxypropyl	0.55	>30
8j	NH, 2-acetylaminoethyl	11	>30
8k	NH, 2-(1H-imidazol-4-yl)ethyl	3.0	>30
8l	NH, 3-imidazol-1-ylpropyl	0.38	>30
8m	NH, trans-4-hydroxycyclohexyl	0.53	>30
8n	$N(CH_3)_2$	7.1	20
8o	morpholin-4-yl	>30	NT
8p	4-hydroxypiperidin-1-yl	>30	NT

[a] The method for measuring cell-free IKKβ inhibitory activity is described in Ref. 25.
[b] The method for measuring the inhibitory activity of TNFα release by THP-1 cell is described in Ref. 27.
[c] Not tested.

Table 3
IKKβ inhibition and TNFα production inhibition assay results for the modification of terminal substituent in the 3-position, compounds **9a**–**9k**



Compd	R^5 / R^6	IKKβ inhibition[a] IC_{50} (μM)	TNFα inhibition[b] IC_{50} (μM)
9a	NH, 3-dimethylaminopropyl	0.20	0.69
9b	NH, 3-diethylaminopropyl	0.079	1.2
9c	NH, 2-dimethylamino-2-methylpropyl	0.33	2.4
9d	NH, 1-methyl-2-aminoethyl	0.31	1.5
9e	NH, 1-methyl-2-dimethylaminoethyl	0.073	2.1
9f	(S)-1-pyrrolidin-2-ylmethyl	5.1	NT[c]
9g	(R)-1-pyrrolidin-2-ylmethyl	0.16	2.1
9h	(R)-piperidin-2-yl	0.10	1.6
9i	(S)-piperidin-2-yl	0.047	1.5
9j	(S)-1-ethylpyrrolidin-2-yl	0.14	0.49
9k	(S)-1-methylpyrrolidin-2-yl	0.11	1.3

[a] Values are the means of at least two experiments.
[b] The method for measuring cell-free IKKβ inhibitory activity is described in Ref. 25.
[c] Not tested.

The pyrrolidine *N*-ethylated compound **9j** improved the activity in cell assay for cell-free assay due to improved permeability. Regarding the comparison between enantiomers, it was clearly determined that one enantiomer was superior to another (the (*S*)-isomer **8e** compared with the (*R*)-isomer **9h**, the (*R*)-isomer **9g** compared with the (*S*)-isomer **9f**) (Table 3).

In regards to the structure–activity relationship as a whole, when the non-polar substituent that is adjacent to the amine moiety in the 6-position of imidazo[1,2-*b*]pyridazine template is not bulky, such as cyclopropylmethyl, the compound was suitable. For the terminal amine in the 3-position, the secondary amines such as (*S*)-2-pyrrolidinylmethyl carbamoyl and (*S*)-2-piperidinylmethyl carbamoyl units showed strong IKKβ inhibitory activity. In this position, some modifications such as alkylation of the amine moiety of pyrrolidine or the 2-aminoethyl moiety are allowed, but IKKβ inhibitory activities decreased.

The weakness of TNFα inhibitory activities in cellular assay for cell-free IKKβ inhibitory activities might be explained by permeability. In some compounds which showed potent activity in cell-free assay, the values of parallel artificial membrane permeability assays (PAMPA) were low (compound **5i**: $<2.0 \times 10^{-6}$, **8e**: $<2.0 \times 10^{-6}$, **8g**: $<2.0 \times 10^{-6}$, **9i**: 2.3×10^{-6} cm/s P_{app} at pH 7.4). These properties are thought to be the cause of decreased activity

Table 4
Kinase selectivity assay results for representative compounds

Compds	IKKβ IC_{50} (μM)	IKKα IC_{50} (μM)[a]	CDK2 IC_{50} (μM)[a]	PDK1 IC_{50} (μM)[a]	GSK3β IC_{50} (μM)[a]	JNK3 IC_{50} (μM)[a]	P38α IC_{50} (μM)[a]
1	1.1	NT[b]	>100	NT	>100	NT	NT
5b	0.59	4.6	>100	>100	>100	90	>100
5i	0.32	2.2	95	>100	>100	>100	>100
8e	0.055	40	>100	>100	>100	>100	>100
8g	0.19	>4	>100	>100	>100	78	>100
9a	0.20	>100	>100	>100	>100	>100	>100
9b	0.079	>100	NT	NT	NT	NT	NT
9e	0.073	>100	NT	NT	NT	NT	NT

[a] Regarding the methods, in Ref. 26.
[b] Not tested.

in THP-1 cells with poor ability to penetrate the cell membrane. These compounds commonly have substructure of cyclic secondary amine moiety in the 3-position. In the compounds which avoid these substructures showed improved cellular activities for cell-free assays (compound **9a**: $>50 \times 10^{-6}$, **9j**: 23×10^{-6} P_{app} at pH 7.4).

About the kinase selectivity, each compound showed good selectivity over some Ser/Thr kinases (Table 4). Compounds **8e** and **9a** increased the inhibitory activity of IKKβ, while decreasing the IKKα inhibitory activity compared with **5b** and **5i**. Potent compounds **8e** and **9a** showed more than 300-fold selectivity over IKKα and other Ser/Thr kinases.

To consider the characteristics of these imidazo[1,2-*b*]pyridazine derivatives, we tried to construct an interaction model with IKKβ. As the X-ray structural data of IKKβ has not been acquired, we examined the docking study of compound **8e** with the IKKβ homology model.[30] From the structure–activity relationship that we have revealed, (*S*)-2-pyrrolidinylmethyl carbamoyl moiety in the terminal of the 3-position had an important role for high affinity. However, we could not find any effective interactions in this part. Instead, there was steric hindrance between the pyrrolidine moiety and the internal surface of IKKβ, mainly the part of the salt bridge among Lys44, Glu61 of helix C and Asp166 of the activation loop (Fig. 2a).

Considering this result and some studies about the conformation of kinase and binding modes with ligands, we attempted to construct a new model that would shift the conformation of the activation loop and the helix C in which the ligand crashed into. As one of some ideas, we assumed that the conformational change such as DLG-out occurs in the same way as with well-known DFG-out[31] (Figs. 2b and 3). The binding mode is a result of the activation loop which shifts to become an another form and amino acids on the activation loop which start to interact with imidazo[1,2-*b*]pyridazine compounds. Leu167 on the activation loop causes a lipophilic interaction with the cyclopropylmethyl moiety (cyclopropylmethyl moiety was caught by Lue21, Val29 and Leu167). Asp166 on the activation loop and Glu61 on helix C form multiple hydrogen bonding interaction with the ammonium moiety of pyrrolidine in the 3-position of imidazo[1,2-*b*]pyridazine. The amide carbonyl moiety in the 3-position interacts with the side chain of Lys44, which is stabilized with the carbonyl moiety of the backbone of Asp166. Imidazo[1,2-*b*]pyridazine scaffold interacts with the backbone of Glu97 and Cys99 in the hinge region. These binding modes are thought to be the key for strong inhibitory activity and high selectivity. We have indicated that the structure–activity relationship in the 3- and 6-position of imidazo[1,2-*b*]pyridazine follows the binding

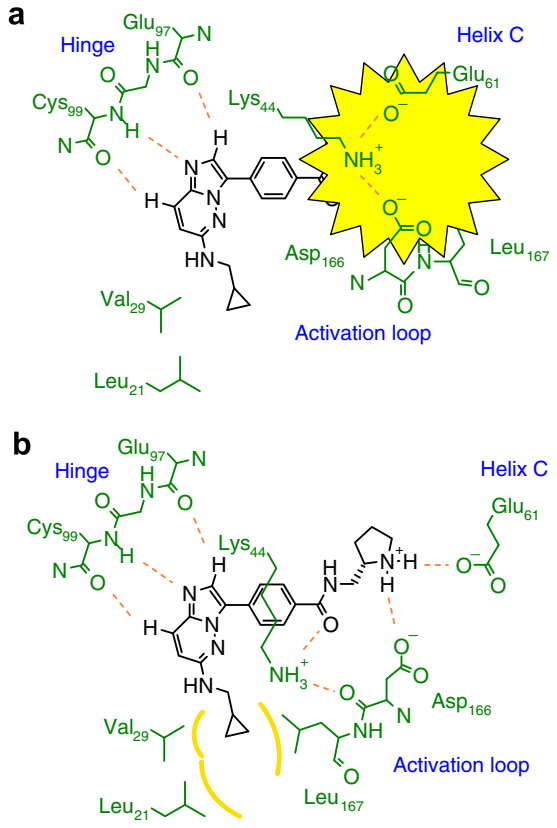


Figure 2. 2D representations of predicted binding mode of compound **8e** with IKKβ. The hydrogen bondings are represented as orange dotted lines. van der Waals interactions are represented as yellow lines. (a) Pyrrolidine moiety crashes into the internal surface composed of the salt bridge among Lys44, Glu61 and Asp166. (b) Effective interactions are formed by the conformational shift of the activation loop and helix C.[31]

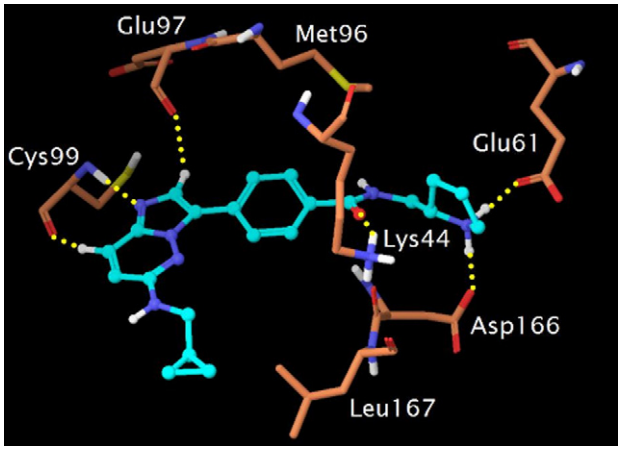


Figure 3. Predicted binding mode of compound **8e** with IKKβ.[32] View from the N-lobe. For simplicity, only important residues are shown. The hydrogen bondings are represented as yellow dotted lines.

mode well. It is inferred that imidazo[1,2-*b*]pyridazine derivatives could show a binding mode like the type-II inhibitor.[32]

In conclusion, we have developed potent IKKβ inhibitory agents with the imidazo[1,2-*b*]pyridazine scaffold from HTS-hit **1**. The potency was improved by the modification of the 3- and 6-position to **8e** and **9i**. These compounds showed good kinase selectivity over IKKα and some Ser/Thr kinases. From the interaction model study, it is assumed that appropriate interactions in the pyrrolidine moiety in the 3-position are formed. The structure–activity relationship we have revealed is explained in this model. Compound **8e** is an attractive lead compound and the predicted binding mode is useful for further development of potent IKKβ inhibitor. In the proceeding paper, we would like to report further investigations of the modification of **8e** with the strategy based on this interaction model.

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24. All test compounds are purified by preparative HPLC and are acquired as formic acid or hydrochloric acid salt.
25. *IKKβ kinase inhibition assay*: Test compound at various concentrations or DMSO, biotinylated-GST-IκBα (180 nM final concentration) and [^{33}P]ATP (1.5 μM final concentration) were mixed with purified His-IKKβ (20 nM final concentration) in a 30 μL volume of kinase buffer (25 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM DTT, 5 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 0.01% BSA) in a polypropylene 96-well plate. After incubation for 15 min at room temperature, the reaction was stopped by adding 30 μL of 0.3 M EDTA. A 30 μL aliquot from the reaction solution was transferred to Streptavidin FlashPlate (Perkin Elmer) and 30 μL of kinase buffer was dispensed. After shaking the plate for 2 h at room temperature, the plate was washed four times with 300 μL of PBS containing 0.05% Tween-20, and the radioactivity was measured by TopCount-HTS.
26. *Kinase selectivity assay*: The kinase inhibition assay for IKKα, CDK2, PDK1, GSK3β, JNK3 and p38α was performed at the Km of ATP for each recombinant enzyme as described above in IKKβ kinase inhibition assay.
27. *Inhibition of TNFα release by THP-1 cell*: THP-1 cells were treated with 100 nM calcitriol in RPMI-1640 medium (supplemented with inactivated 10% (v/v) fetal bovine serum (FBS), 50 units/mL penicillin, 50 μg/mL streptomycin) for 3–4 days. After an overnight culture with RPMI-1640 medium without calcitriol, the cells were centrifuged and resuspended in RPMI-1640 medium containing 25 mM HEPES. The cells (1.5×10^5 cells/mL) were seeded to a 96-well tissue culture plate and added with the test compound (0.1 μg/mL) and lipopolysaccharide (LPS, *E. coli* O111:B4, Calbiochem) (0.1 μg/mL) simultaneously. After incubation for 4 h at 37 °C in 5% CO_2, the cell culture supernatant was obtained by centrifugation (2000 rpm at 5 min, 4 °C). TNFα concentration in culture supernatant was measured by ELISA assay as described by the manufacturer's instructions (BD Bioscience).
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