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Discovery of Novel and Selective IKK-β Serine-Threonine Protein Kinase Inhibitors. Part 1

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Abstract—IκB kinase β (IKK-β) is a serine-threonine protein kinase critically involved in the activation of the transcription factor Nuclear Factor kappa B (NF-κB) in response to various inflammatory stimuli. We have identified a small molecule inhibitor of IKK-β. Optimization of the lead compound resulted in improvements in both in vitro and in vivo potency, and provided IKK-β inhibitors exhibiting potent activity in an acute cytokine release model (LPS-induced TNFα).

IκB kinase β (IKK-β) is a 756 amino acid-containing serine-threonine protein kinase.[1] As part of the IKK-complex, IKK-β is critically involved in the activation of the transcription factor Nuclear Factor kappa B (NF-κB) in response to various inflammatory stimuli including Tumor Necrosis Factor α (TNFα), Interleukin 1β (IL-1β) and lipopolysaccharide (LPS). In addition to IKK-β, the IKK-complex contains IKK-α, IKK-γ, NIK and various other known and unknown proteins. NF-κB is an inducible transcription factor that is thought to be a pivotal target for drugs for cancer and chronic inflammatory diseases.[2]

A catalytically inactive mutant of IKK-β has been shown to inhibit activation of NF-κB by TNFα, IL-1β, LPS, and anti-CD3/anti-CD28 stimulation.[3] This provides further evidence for the involvement of IKK-β in these pathways leading to NF-κB activation. In addition, embryonic fibroblast cells isolated from IKK-β-deficient mice show defects in TNFα- and IL-1-induced degradation of IκB. In contrast, in cells derived from IKK-α-deficient mice, pro-inflammatory cytokine-induced IκB degradation is not inhibited, suggesting that IKK-β controls the NF-κB activation rather than IKK-α.[4]

A number of reports on IKK-β inhibitors exist. The non-specific protein kinase inhibitors, quercetin and staurosporine,[5] as well as well-known anti-inflammatory agents such as aspirin[6] and cyclopentenone prostaglandins[7] are known to inhibit IKK-β kinase. In our assays the natural protein kinase inhibitor staurosporine inhibited IKK-β with an IC_{50} of 0.25 μM, and more potently inhibited IKK-α with an IC_{50} of 0.05 μM. More recently, it has been reported that a novel IκB kinase (IKK) inhibitor, PS-1145, specifically blocks TNFα-induced NF-κB activation, resulting in a suppression of the release of various cytokines both in vitro and in vivo.[8] These data suggest that specific inhibition of IKK-β will result in a strong in vivo anti-inflammatory and immuno-modulatory effect.

From high-throughput screening of the Bayer compound library, the 2-amino-3-cyano-4-aryl-6-(2-hydroxy-phenyl)pyridine analogue **1** was identified as a potent IKK-β inhibitor.

The lead compound **1** shows potent inhibitory activity against IKK-β ($IC_{50} = 1.5$ μM) and excellent selectivity versus other kinases such as IKK-α, Syk and MKK4 (mitogen-activated protein kinase kinase 4) (IC_{50} values >20 μM). Furthermore, this lead compound inhibits NF-κB-dependent expression of several reporter genes, chemokines, cytokines and IgE production in various functional cellular assays (Table 1). These results suggest

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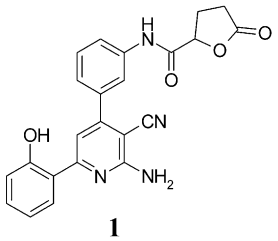
Table 1. Inhibitory activities of the lead compound **1** in various kinase and cellular assays



1

IC_{50} = 1.5 mM (IKK-β)
IC_{50} > 20 mM (IKK-α, Syk and MKK4)

Cells/Cell Line	Stimulus	Read-Out	IC_{50}, μM[a]
A549	TNFα	RANTES	8
Jurkat T-cell	anti-CD3/anti-CD28	IL-2	15
HEK293	TNFα	NF-κB-Luciferase	8
Mouse B-cells	LPS/IL-4	IgE	0.35
Human PBMCs	LPS	TNFα	10

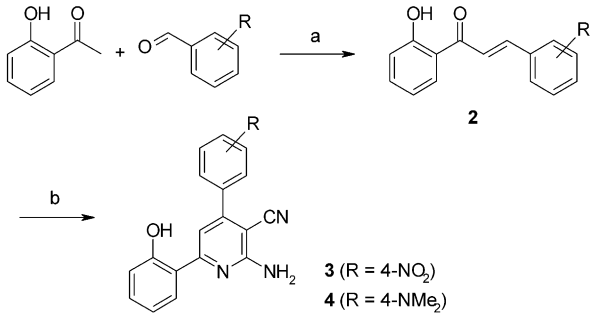
[a]Values are means of more than three experiments.

that compound **1** is a lead structure for specific inhibitors of the IKK-complex activated by various inflammatory stimuli and it can be therefore pharmacologically confirmed that IKK-β is a key component of the signal transduction pathway in those physiological responses.

Herein, we report the synthesis, structure–activity relationships (SAR) and characterization of a series of 2-amino-3-cyano-4-aryl-6-(2-hydroxyphenyl)pyridines.

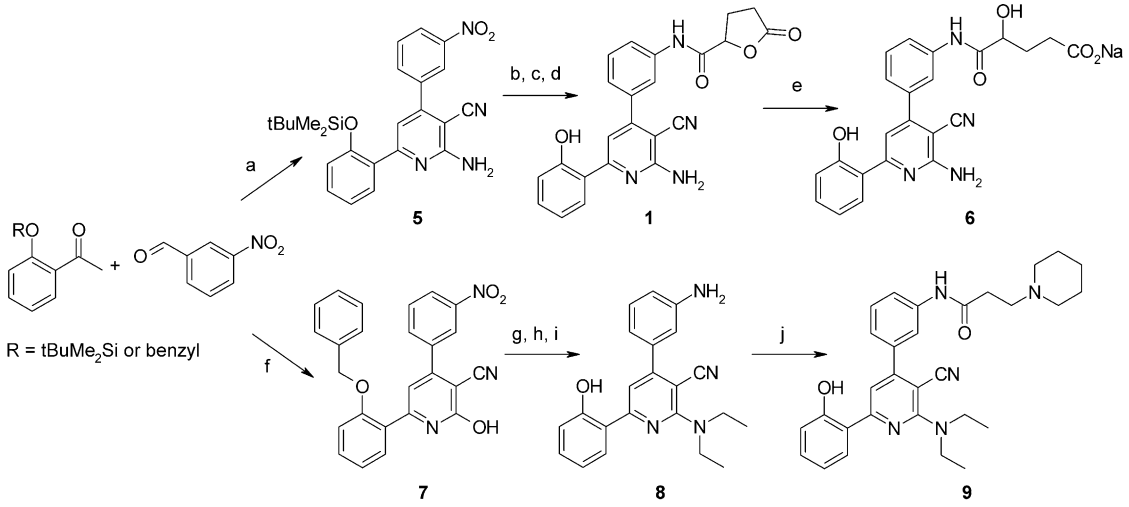
Chemistry[9]

Synthesis of 2-amino-3-cyano-4,6-diarylpyridines has been reported in several publications.[10] Manna et al. actually synthesized the 2-amino-3-cyano-4-aryl-6-(2-hydroxyphenyl)pyridine analogues **3** and **4** by a reaction of substituted 1,3-diaryl-2-propen-1-ones **2** with malononitrile in the presence of ammonium acetate (Scheme 1).[11] Although the *o*-hydroxyacetophenone was utilized for the pyridine contraction reaction without protection of the phenolic hydroxyl group, protection became necessary for further modifications of the substituents on the aromatic group at the 4-position of the pyridine.



Scheme 1. (a) 30% NaOH, EtOH, rt; (b) malononitrile, ammonium acetate, EtOH, reflux, 24 h, 51% (**3**, R = 4-NO_2), 11% (**4**, R = 4-$N(CH_3)_2$).[14]

When protected 2′-hydroxyacetophenones were used as starting materials, the 2-amino-3-cyano-4,6-diarylpyridine core structures were simply constructed using a one-pot coupling reaction of four components, acetophenone, benzaldehyde, malononitrile and ammonium acetate, following literature precedent[12] (Scheme 2 and 3). The corresponding pyridone analogue **7** was prepared by the similar one-pot reaction using ethyl cyanoacetate instead of malononitrile, and was subsequently utilized to synthesize the 2-diethylaminopyridine analogue **9**. The benzylether was found to be one of the most suitable protecting groups of the 2′-hydroxyacetophenones, enabling the construction of the 2-aminopyridine core structures in good yield, as exemplified by the synthesis of the pyridine analogue **10** (Scheme 3).[13]



Scheme 2. (a) Malononitrile, ammonium acetate, 1,4-dioxane, reflux, 18%; (b) H_2, 10% Pd-C, AcOEt, rt, 87%; (c) 5-oxotetrahydro-2-furancarbonyl chloride, pyridine, acetonitrile, 0 °C, rt, 77%; (d) *n*-Bu_4NF, THF, 0 °C, 93%; (e) NaOH, water, methanol, rt, 60%; (f) ethyl cyanoacetate, ammonium acetate, toluene, reflux, 19%; (g) $(CF_3SO_2)_2O$, pyridine, 0 °C, 81%; (h) diethylamine, DMSO, 40 °C, 88%; (i) H_2, 10% Pd-C, methanol, THF, rt, 45%; (j) 3-(1-piperidinyl)propanoyl chloride, pyridine, THF, 0 °C, rt, 69%.

The straightforward synthetic method could be adopted to solid-phase synthesis using an *O*-tethered *o*-hydroxyacetophenone on solid support instead of the protected *o*-hydroxyacetophenone (Scheme 4). This combinatorial chemistry method provided the target compounds in good yield (>80%) and high purity (>80%, ELSD), and was used to generate approximately 1000 compounds for the optimization of the substituents on the phenyl group at the 4-position of the pyridine. All the potent compounds were purified by column chromatography or re-synthesized using solution phase chemistry to confirm the biological activity of the pure compounds.

Results and Discussion

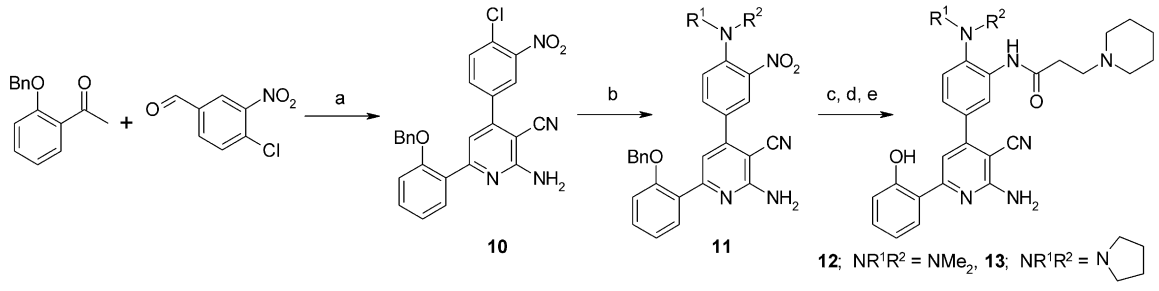
Recombinant human IKK-β was used to measure kinase activity in vitro.[15] To test cellular efficacy of IKK-β inhibition, an ELISA assay measuring TNFα-induced RANTES production was employed.[16]

We initially synthesized *para*-nitro (**3**) and *para*-dimethylamine (**4**) analogues to assess their IKK-β inhibitory activity because these compounds have been reported to exhibit anti-inflammatory activity in animal models (paw edema tests using carrageenin).[11] These compounds showed no inhibitory activity in our in vitro assays (Table 2).

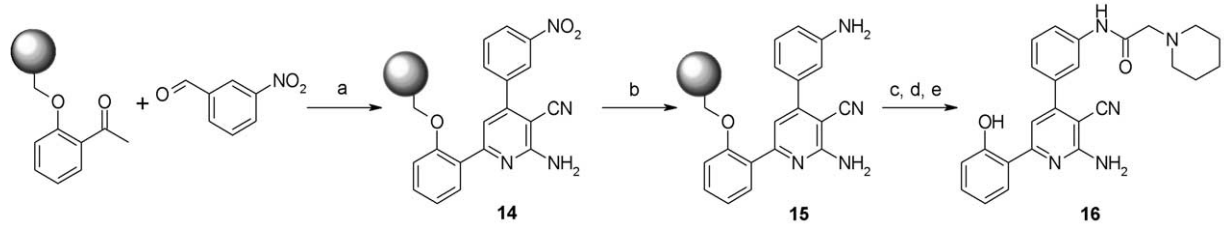
The phenyl group at the 4-position of the pyridine can accommodate a variety of substituents (Table 2). Since the lead compound **1** is sparingly soluble in an aqueous solution, our initial optimization efforts focused on the introduction of hydrophilic substituents onto this phenyl group with the aim to improve the solubility as well as the inhibitory activity. Various hydrophilic functionalities, such as alcohol, carboxylic acid and amine, are tolerated at this position, suggesting the hypothesis that this portion of the molecule may interact with the hydrophilic pocket of the enzyme.

The functionality on the phenyl group appears to affect cellular activity significantly. Introduction of a basic amino moiety on the side chain of the phenyl group (**26** and **28**) improved cellular activity. However, the carboxylic acid analogues (**6**, **18**–**20**, **22**–**23**) exhibited either no or weak cellular activity, albeit with potent IKK-β inhibitory activity, suggesting poor cell membrane permeability of these derivatives. While displacement of substituents from the 3′ to the 4′-position on the phenyl group did not significantly impact IKK-β inhibitory activity, such modifications resulted in a complete loss of cellular activity (**6** vs **22**, **26** vs **27** and **29** vs **30**).

In contrast to the diversity of substitution tolerated on the phenyl group at the 4-position, modification of the phenol group at the 6-position appears to be rather restrictive (Table 3). Removal of the phenolic hydroxide (**31**) results in a complete loss of activity. Change of the substitution position (**32** and **40**) or replacement of the hydroxy group with a methoxy group also yielded inactive compounds, suggesting that the 2′-phenol group is necessary for activity. Interestingly, the 2′-phenol analogue **39** incorporating a methyl group on the 5-position of the pyridine ring exhibited no inhibitory activity. The common characteristic of the inactive compounds was found to be a lack of hydrogen-bonding interaction between the phenolic hydroxide and the pyridine nitrogen atom, which could typically be observed via the chemical shift of the phenolic hydroxide proton in the ^{1}H NMR spectrum.[17] The hydrogen-bonding interaction of the 5-methyl analogue **39** appears to be interrupted by the steric collision of the methyl group with the hydrogen atom at the 6′-position of the phenol



Scheme 3. (a) Malononitrile, ammonium acetate, toluene, reflux, 63%; (b) HNR^1R^2, DMF, rt, 60 °C, 92%; (c) Fe powder, ammonium chloride, water, ethanol, reflux, 75%; (d) 3-(1-piperidinyl)propanoyl chloride, pyridine, rt, 32%; (e) H_2, 10% Pd-C, AcOH, AcOEt, rt, 86%.



Scheme 4. (a) Wang resin, malononitrile, ammonium acetate, 1,4-dioxane, 80 °C; (b) $SnCl_2{\cdot}2H_2O$, DMF, rt; (c) chloroacetyl chloride, rt; (d) piperidine, diisopropylethylamine, DMF; (e) 50% TFA/CH_2Cl_2, 86% overall yield, 96% purity (ELSD).

group. Thus, the attainment of a geometry allowing for the internal hydrogen bond appears to play a significant role in achieving IKK-β inhibitory activity.

Addition of a substituent at the 5′-position on the 2′-phenol group usually resulted in a loss of cellular activity, despite maintaining IKK-β inhibitory activity (**34–36**). The introduction of a methyl group at the 6′-position gave an inactive compound **37**, in which the methyl group possibly interrupts the hydrogen-bonding interaction.

The 2-aminopyridine ring also appears to be important for inhibitory activity (Table 3). Replacement of the amino moiety by a hydroxy moiety (**41**) leads to a loss of activity, as does substitution of the amino moiety by a diethylamino group (**9**). The acetylamide analogue **42** showed moderate activity.

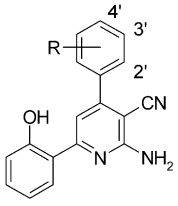
Introduction of an alkylamine group at the 4′-position on the phenyl ring was tolerated for IKK-β inhibition, and the pyrrolidine analogue **16** was shown to be 10-fold more potent than the parent compound **1** in the cellular assay (Table 4).

In order to investigate the pharmacological profile of these potent IKK-β inhibitors, we tested their activity in an acute model of cytokine release (LPS-induced TNFα production in mice),[18] which is considered to be one of the most mechanism-relevant models for the evaluation of IKK-β inhibition (Table 4). The initial lead compound **1** exhibits in vivo activity only when administered ip (intraperitoneally) but lacks oral efficacy due to low oral bioavailability. On the other hand, the more hydrophilic analogues, such as the carboxylic acid analogue **6** and the basic amino analogues (**13, 26**), demonstrate potent oral activity as well as high potency when administered ip The piperidyl analogue **26** inhibits LPS-induced TNFα production with an ED_{50} of 0.03 mg/kg and 2 mg/kg after intraperitoneal and oral administration, respectively.

The compound **26** identified by the optimization efforts of this compound class maintains excellent selectivity versus other kinases such as IKK-α ($IC_{50} = 20$ μM), Syk and MKK4 ($IC_{50} > 20$ μM).

In summary, screening of the Bayer compound library resulted in the identification of a novel class of IKK-β inhibitors. Optimization of the lead compound **1** through a combined combinatorial and medicinal chemistry effort resulted in improvements in both in vitro and in vivo potency. As a result, compound **26** has been identified as a selective and potent inhibitor of the IKK-β which is orally bioavailable in mice, and which

Table 2. Modification of the C-4 phenyl group



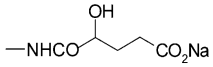
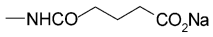
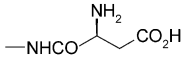
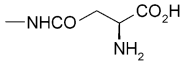
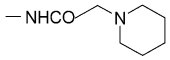
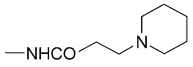
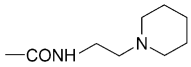
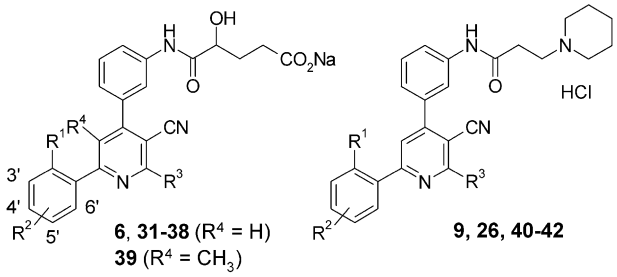
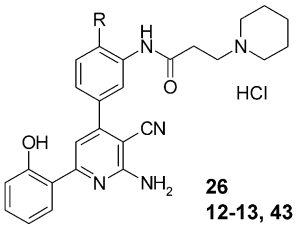
Compd		R	IC_{50} (μM)	
			IKK-β[15]	RANTES[16]
3	4′-	NO_2	> 20	> 50
4	4′-	$N(CH_3)_2$	> 20	> 50
17	3′-	NH_2	20	> 50
18	2′-	CO_2Na	0.7	> 50
19	3′-	CO_2Na	2.5	> 50
20	4′-	CO_2Na	0.7	> 50
21	3′-		1.4	> 50
1	3′-		1.5	8
6	3′-		0.9	20
22	4′-		0.5	> 50
23	3′-		1.4	> 50
24	3′-		0.6	15
25	3′-		0.6	35
16	3′-		3	15
26	3′-		0.6	7
27	4′-		1.0	> 50
28	2′-		0.5	6
29	3′-		1.8	15
30	4′-		0.6	> 50

Table 3. Modification of the C-6 phenyl group



Compd	R^1	R^2	R^3	IC_{50} (μM)[15,16]	
				IKK-β	RANTES
6	–OH	–H	$–NH_2$	0.9	20
31	–H	–H	$–NH_2$	> 20	> 50
32	–H	4′–OH	$–NH_2$	> 20	> 50
33	$–OCH_3$	–H	$–NH_2$	> 20	> 50
34	–OH	5′–F	$–NH_2$	1.7	> 50
35	–OH	5′–Cl	$–NH_2$	5.5	> 50
36	–OH	5′–OCH_3	$–NH_2$	5.3	> 50
37	–OH	6′–CH_3	$–NH_2$	> 20	> 50
38	–OH	6′–OCH_3	$–NH_2$	2	> 50
39	–OH	–H	$–NH_2$	> 20	> 50
26	–OH	–H	$–NH_2$	0.6	7
40	–H	3′–OH	$–NH_2$	> 20	10
41	–OH	–H	–OH	> 20	> 50
9	–OH	–H	$–NEt_2$	> 20	nd
42	–OH	–H	$–NHCOCH_3$	15	30

Table 4. Modification of the C-4 phenyl group



26
12-13, 43

Compd	R	IC_{50} (μM)[15, 16]		ED_{50} (mg/kg)[18]	
		IKK-β	RANTES	ip[a]	po[b]
1	—	1.5	8	10	>30
6	—	0.9	20	1.3	18.7
26	–H	0.6	7	<0.03	2
12	$-NMe_2$	0.8	8	0.6	nd
13		1.0	0.8	0.8	10.1
43		2.5	5	nd	nd

[a]Intraperitoneal administration (30 min pretreatment).
[b]Oral administration (60 min pretreatment).

demonstrates significant in vivo activity in an acute model of cytokine release (LPS-induced TNFα).

Acknowledgements

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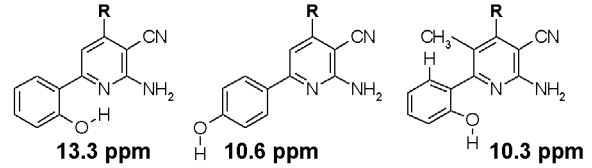
13. A typical procedure for the four component coupling to construct the pyridine analogue (Synthesis of compound **10**)—A mixture of 2-benzyloxyacetophenone (5.0 g, 22.1 mmol), 4′-chloro-3′-nitrobenzaldehyde (8.2 g, 44.2 mmol), malononitrile (2.92 g, 44.2 mmol) and ammonium acetate (8.5 g, 110 mmol) in toluene (15 mL) was stirred at reflux for 3 h. After cooling to rt, the mixture was diluted with ethyl acetate and THF. The organic phase was washed twice with water, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was suspended in ethanol. The precipitate was collected by filtration, washed with ethyl acetate and dried under reduced pressure to give the desired product **10** (6.4 g, 63%) as a white solid. ^1H NMR (500 MHz, DMSO-d_6) δ 5.16 (2H, s), 7.06 (2H, br s), 7.11 (2H, dd, $J=7.5$, 7.7 Hz), 7.26– 7.32 (4H, m), 7.38–7.47 (3H, m), 7.69 (1H, dd, $J=2.1$, 8.4 Hz), 7.85 (1H, dd, $J=1.7$, 7.7 Hz), 7.90 (1H, d, $J=8.4$ Hz), 8.19 (1H, d, $J=2.1$ Hz).
14. The reaction procedures and chemical yields are cited from the ref 11(a).
15. IKK-β *in vitro* assay: Human IKK-β was cloned from Quickclone cDNA library (CLONTECH) by polymerase chain reaction and recombinant His-tagged IKK-β was expressed in insect cells using by a baculovirus expression vector system (Pharmingen). GST-IkBα (1-54) was expressed in *Escherichia coli* BL21 (DE3). The sequences of all the constructed clones were verified by DNA sequencing.

Inhibition of IKK-β kinase activity was assayed in a 96-well MTP format kinase assay. Compounds were dissolved in DMSO (final 0.25%) and incubated with recombinant IKK-β (final 0.6 μg/mL) and biotinylated-GST-IκBα (1-54) (final 0.2 μM) in kinase buffer β (20 mM Tris–HCl, pH 7.6, 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 20 mM p-n-phenylphosphate, 1 mM EDTA, 20 mM creatine phosphate, 1 mM DTT, 1 mM Na_3VO_4, 0.1 mg/mL BSA and 0.4 mM phenylmethylsulfonyl fluoride; 50 μL/well) in U-bottomed 96-well plate. Kinase reaction was started by addition of ATP (final 5 μM APT, 0.5 μCi/well [γ-^{33}P] ATP) and incubated at rt for 2 h. Kinase reactions were terminated by the addition of 150 μL 100 mM EDTA, 1 mg/mL BSA, and 150 μL of the sample were transferred to streptavidin-coated, white MTP (Steffens Biotechniche Analysen GmbH #08114E14.FWD) to capture biotinylated substrates. After 1 h incubation, free radioactivity was eliminated by washing the wells five times with 300 μL of 0.9% NaCl, 0.1% (w/v) Tween-20. The remaining radioactivity was counted in 170 μL of MicroScint-PS scintillation cocktail (Packard) using a TopCount scintillation counter.
16. In vitro RANTES induction by TNFα in A549 cells: The A549 human lung epithelium cell line (ATCC #CCL-885) was maintained in Dulbecco's modified Eagle's medium (D-MEM, Nikken Biomedical Institute) supplemented with 10% FCS (Gibco), 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM glutamine. A549 cells (4×10^4 cells in 80 μL/well) were treated in a 96-well flat-bottom tissue culture plate for 1 h with vehicle (0.1% DMSO) or test compounds. Then cells were stimulated with 100 ng/mL TNF-α for 24 h. Concentration of RANTES in the supernatants collected after 24 h was determined using a quantitative sandwich Fluorescent immunoassay technique following the manufacturer's recommendations (R&D Systems, Oxon, UK).

17. The hydrogen-bonding interaction between the phenolic hydroxide and the pyridine nitrogen atom was estimated by the chemical shift of the phenolic hydroxide proton in the ^{1}H NMR spectrum, which was measured on a Brucker 500 MHz spectrometer using DMSO-d_6 as solvent and tetramethylsilane (Me_4Si) as an internal standard (0 ppm). When the structure is suitable for the hydrogen-bonding, the chemical shift of this proton is approximately 13 ppm. On the other hand, in the absence of intramolecular hydrogen bonding, the chemical shift shifts upfield, toward 10 ppm.



1 IC_{50} = 1.5 μM (IKK-β)

32 IC_{50} > 20 μM (IKK-β)

39 IC_{50} > 20 μM (IKK-β)

18. In vivo TNFα induction by LPS in mice: Eight week old BALB/c female mice were placed into two groups, a control group and a treated group. A solution containing 200 μg/mouse of LPS in 0.9% physiological saline was administered by intraperitoneal (ip) injection into the control mice. Mice in the treated group were first injected ip with test compounds 30 min prior to the LPS injection or po with test compounds (10% cremophor/saline vehicle) 60 min prior to the LPS injection. Under anesthesia with pentobarbital (80 mg/kg, ip), blood was collected from the posterior venous cavity of the treated and control mice at 90 min post-LPS injection into a 96-well plate containing 2% EDTA solution. The plasma was separated by centrifugation at 1800 rpm for 10 min at 4 °C and then diluted with four times volumes of phosphate buffer saline (pH 7.4) containing 1% bovine serum albumin. TNFα concentration in the sample was determined using an ELISA kit (Pharmingen, San Diego, CA). The mean of TNFα level in 5 mice from each group was determined and the percent reduction in TNFα levels was calculated.