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PII: S0968-0896(16)30717-9
DOI: http://dx.doi.org/10.1016/j.bmc.2016.09.030
Reference: BMC 13283

To appear in: *Bioorganic & Medicinal Chemistry*

Received Date: 22 August 2016
Revised Date: 12 September 2016
Accepted Date: 12 September 2016

Please cite this article as: Imaeda, Y., Tawada, M., Suzuki, S., Tomimoto, M., Kondo, M., Tarui, N., Sanada, T., Kanagawa, R., Snell, G., Behnke, C.A., Kubo, K., Kuroita, T., Structure-based design of a new series of *N*-(piperidin-3-yl)pyrimidine-5-carboxamides as renin inhibitors, *Bioorganic & Medicinal Chemistry* (2016), doi: http://dx.doi.org/10.1016/j.bmc.2016.09.030

Structure-based design of a new series of *N*-(piperidin-3-yl)pyrimidine-5-carboxamides as renin inhibitors

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Keywords: renin inhibitor; structure-based drug design (SBDD); crystal structure; *N*-(piperidin-3-yl)pyrimidine-5-carboxamide

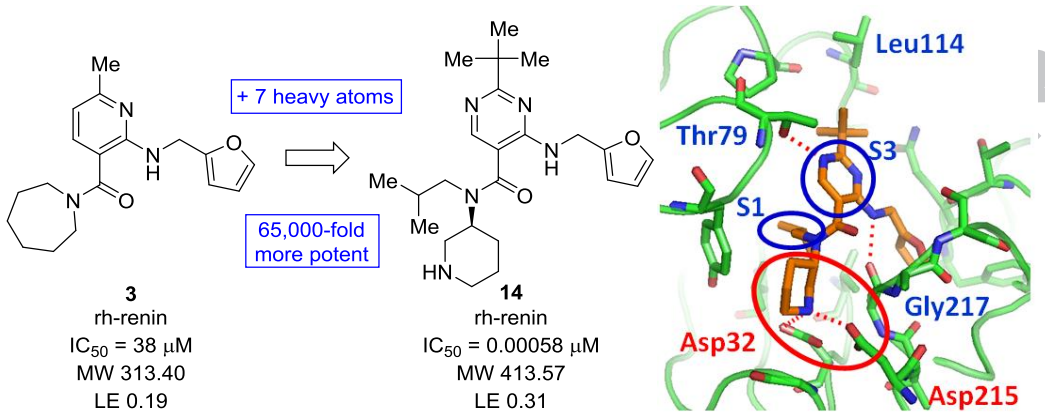
Abbreviations:

AcOH, acetic acid; Ang I, angiotensin I; Ang II, angiotensin II; AUC, area under the blood concentration−time curve; C_{5min}, concentration in plasma 5 min after administration; CL_{total}, total clearance; C_{max}, maximum concentration in plasma; DIEA, *N,N*-diisopropylethylamine; Et_3N, triethylamine; EtOAc, ethyl acetate; EtOH, ethanol; F, rat bioavailability; HAC, heavy atom count; HOBt, 1-hydroxybenzotriazole hydrate; hPRA, human plasma renin activity; *i*-PrOH, isopropanol; LE, ligand efficiency; MeOH, methanol; MRT, mean residence time; Pd/C, palladium on carbon; RAAS, renin-angiotensin-aldosterone system; Rt, retention time; $V_{d(ss)}$, volume of distribution at steady state ; WSC, (1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide.

Abstract: The action of the aspartyl protease renin is the rate-limiting initial step of the renin-angiotensin-aldosterone system. Therefore, renin is a particularly promising target for blood pressure as well as onset and progression of cardiovascular and renal diseases. New pyrimidine derivatives **5**−**14** were designed in an attempt to enhance the renin inhibitory activity of compound **3** identified by our previous fragment-based drug design approach. Introduction of a basic amine essential for interaction with the two aspartic acids in the catalytic site and optimization of the S1/S3 binding elements including an induced-fit structural change of Leu114 ("Leu-in" to "Leu-out") by a rational structure-based drug design approach led to the discovery of *N*-(piperidin-3-yl)pyrimidine-5-carboxamide **14,** a 65,000-fold more potent renin inhibitor than compound **3**. Surprisingly, this remarkable enhancement in the inhibitory activity of compound **14** has been achieved by the overall addition of only seven heavy atoms to compound

3. Compound **14** demonstrated excellent selectivity over other aspartyl proteases and moderate oral bioavailability in rats.

Graphical abstract



1. Introduction

The renin-angiotensin-aldosterone system (RAAS)[1] is known to play a key role in the regulation of blood pressure[2] and homeostasis of body fluid volume, as well as in the pathogenesis of many cardiovascular and renal diseases, such as congestive heart failure, chronic kidney disease, and diabetic nephropathy. An aspartyl protease, renin cleaves its only known substrate, angiotensinogen, to release the decapeptide angiotensin I (Ang I). The high substrate selectivity of renin and its activity at the initial and rate-limiting step of the RAAS cascade suggest that a direct renin inhibitor may offer an attractive alternative to angiotensin-converting enzyme (ACE) inhibitors and Angiotensin II (Ang II) receptor blockers (ARBs).[3−5] Renin inhibitors should be possible to block this cascade completely even in situations of elevated circulating or tissue active renin and prevent the formation of Ang I and Ang II. Therefore, renin inhibitors may provide a therapeutic effect which is different from and superior to that of ACE

inhibitors and ARBs.[6,7] A novel nonpeptidic renin inhibitor, aliskiren (**1**, CGP 60536) discovered by Novartis, is the first new class of antihypertensive drugs to enter the market in more than a decade (Figure 1).[8-12] Based on the positive results reported for aliskiren, interest in renin as a viable drug target for the control of hypertension has intensified. However, the oral bioavailability of aliskiren is very low (bioavailability = 2.6% in human).[13] Herein, we started to discover novel and orally active nonpeptidic renin inhibitors with more potent antihypertensive activity and improved organ protection compared to aliskiren.

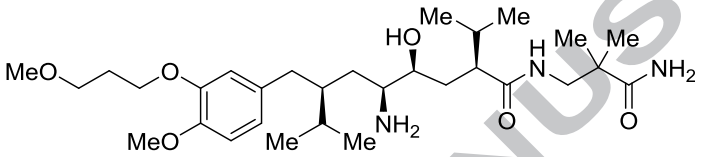
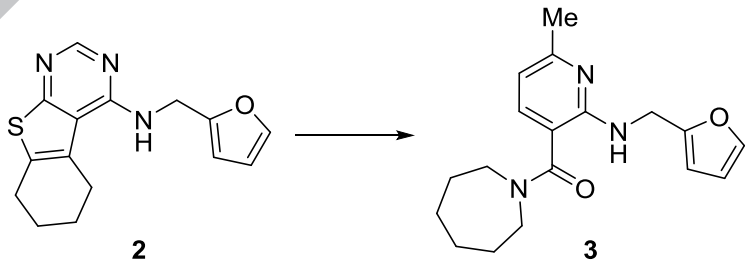


Figure 1. Structure of the renin inhibitor **1** (aliskiren, CGP 60356)

Our search for more promising renin inhibitor lead scaffolds by use of a fragment-based drug discovery (FBDD) approach[14] resulted in the discovery of new pyridine amide compound **3** as a weak human renin inhibitor (IC_{50} = 38 μM), derived from fragment-hit **2**[15] We were interested in the chemical structure of this scaffold, and thus initiated chemical modification of this chemotype using a structure-based drug design (SBDD) approach. In this report, we describe our further optimization to enhance renin inhibitory activity (IC_{50} < 1 nM).



2
rh-renin
IC_{50} = 43 μM

3
rh-renin
IC_{50} = 38 μM

Figure 2. Identification of compound **3** from fragment-hit **2**[15].

To understand the binding mode of compound **3** and determine an approach to increase renin inhibitory activity, X-ray crystallography study was carried out. The X-ray crystal structure showed that compound **3** bound to the "closed" form of renin.[16,17] The furfurylamino group occupied the rather small $S3^{sp}$ cavity. In addition, the amino group at the pyridine 2-position formed additional hydrogen bonding interactions with the Gly217 backbone carbonyl group. The amide carbonyl oxygen atom of compound **3** is involved in a hydrogen bond with the hydroxyl group of Thr79. The two carboxylic acids of two aspartic acids in the renin catalytic site are close to the azepane ring of **3**, however no interaction between the acids and the azepane ring was observed. We hypothesized that introduction of a basic amino group onto the azepane ring might be effective for interaction with the two aspartic acids and the enhancement of inhibitory activity.

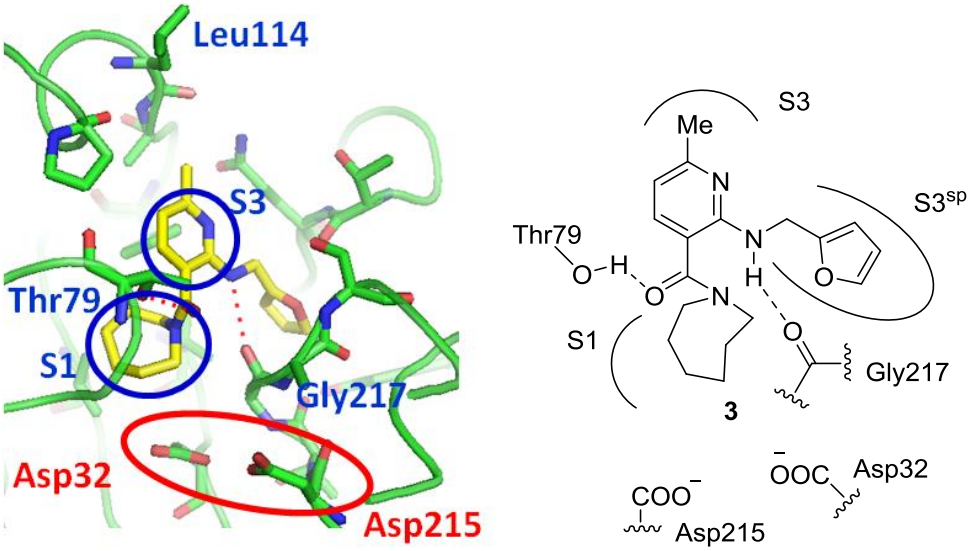


Figure 3. Crystal structure of **3** in complex with human renin (2.85 Å resolution): (a) cartoon view (left), (b) binding mode (right)

Simultaneously, compound **4** as a renin inhibitor was identified by a manual structural similarity search based on compound **2** from our compound library. 2-Phenylquinazoline **4**

showed more potent renin inhibitory activity (IC_{50} = 11 μM) than thienopyrimidine **2** (IC_{50} 43 μM). The difference in potency between compound **2** and **4** appeared to be derived from the substituent on the pyrimidine ring. Thus, we obtained the crystal structures of 2-phenylquinazoline **4** bound to renin in order to clarify the difference in view of the binding mode in complex of renin and inhibitors. Overlap of the crystal structures of **2** and **4** indicated that the binding modes of these complexes are almost the same. The clear difference of them is the position of the isobutyl group of Leu114. In complex with compound **2**, the isobutyl group is directed to the pyrimidine ring of **2** ("Leu-in"). On the other hand, the isobutyl group is pushed to the opposite side of the 2-phenyl group of **4** ("Leu-out"). 2-Phenyl group on the pyrimidine core could push Leu114 out to enhance the inhibitory potency (possibility of induced-fit). Therefore, we assumed that introduction of bulky group on the 2-position of the pyridine ring of compound **3** might enhance the renin inhibitory activity.

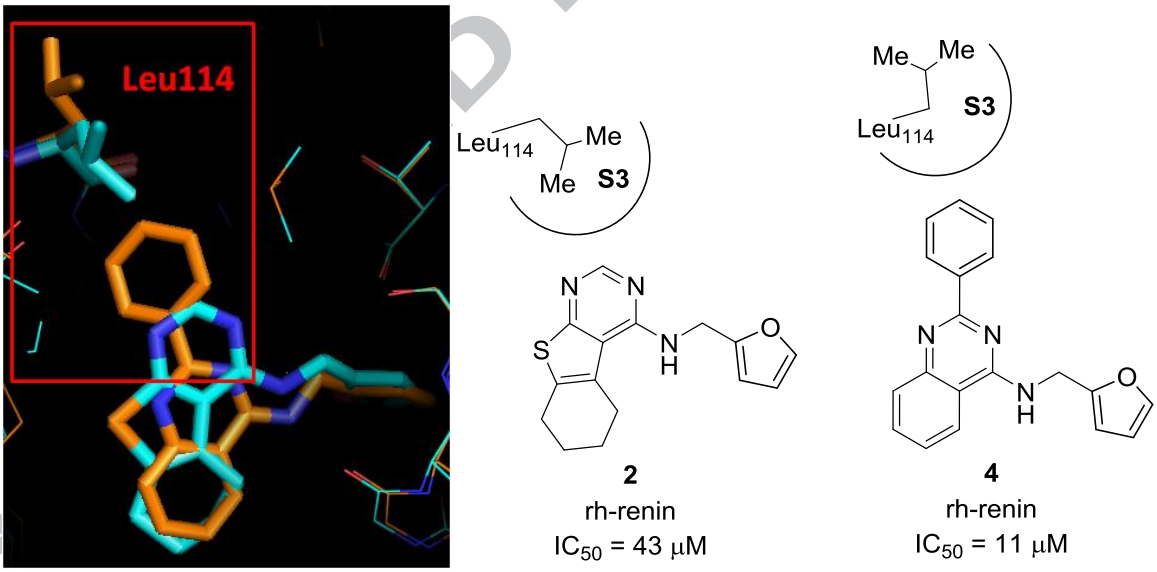


Figure 4. Overlap of two crystal structures of **2** (cyan) and **4** (orange) bound to human renin

On the basis of our structure-based hypotheses, we designed new pyrimidine derivatives **5**−**14** (Figure 5) in an attempt to further enhance renin inhibitory activity. The reason we chose

to use a pyrimidine core instead of a pyridine ring as scaffold is as follows: (1) a nitrogen atom at the 1-position in the pyrimidine is likely expected to interact with the hydroxyl group of Thr79, (2) the modification of the pyrimidine 2-position could be more easily achieved with robust chemistry. As a first step toward our goal, the introduction of basic amines in place of the azepane group brought additional important interactions with the two aspartic acids in the catalytic site. Next, modifications of the pyrimidine 2-position and substituent (R^3) on the amide nitrogen atom were investigated to increase lipophilic interactions with lipophilic S3 and S1 sites. In this paper, we describe the optimization of pyrimidine derivatives **5**−**9** (Table 1) and **10**−**14** (Table 2) in order to enhance renin inhibitory activity by use of an SBDD approach.

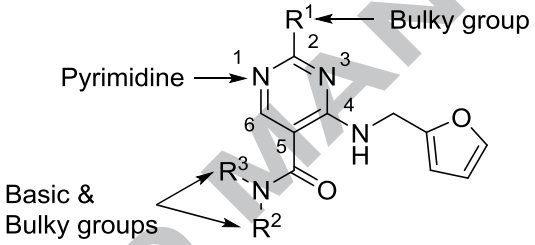
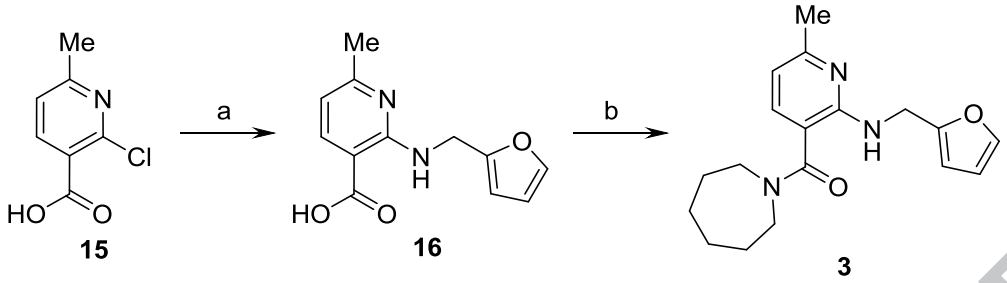


Figure 5. Design of pyrimidine derivatives **5**−**14**

2. Chemistry

The syntheses of the target structures **3** and **5**−**14** are depicted in Schemes 1−3. Compound **3** was synthesized as described in Scheme 1. 2-Chloronicotionic acid **15** was reacted with furfurylamine to give the expected carboxylic acid **16**. Amide coupling of carboxylic acid **16** and hexamethyleneimine was performed using (1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (WSC) and 1-hydroxybenzotriazole hydrate (HOBt).

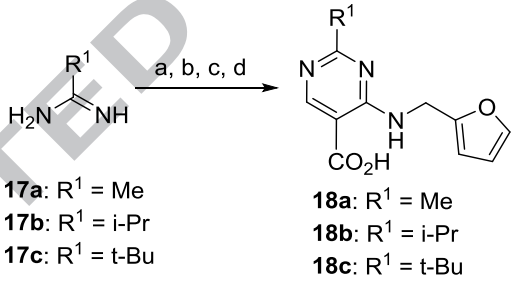
Scheme 1. Synthesis of pyridine-3-carboxamide **3**[a]



[a]Conditions and reagents: (a) furfurylamine, 1-methylpyrrolidin-2-one, 36%; (b) hexamethyleneimine, WSC, HOBt, DMF, 74%.

The pyrimidine core was constructed by the synthetic route shown in Scheme 2. Pyrimidine ring formation from amidines **17a−c** was accomplished by treatment with diethyl (ethoxymethylidene)propanedioate in the presence of sodium ethoxide.[18,19] Chlorination with phosphoryl chloride followed by nucleophilic substitution with furfurylamine and hydrolysis gave carboxylic acids **18a−c**.

Scheme 2. Synthesis of pyrimidine-5-carboxylic acids **18a−c**[a]



17a: R^1 = Me
17b: R^1 = i-Pr
17c: R^1 = t-Bu

18a: R^1 = Me
18b: R^1 = i-Pr
18c: R^1 = t-Bu

[a]Conditions and reagents: (a) diethyl (ethoxymethylidene)propanedioate, sodium ethoxide, EtOH; (b) $POCl_3$; (c) furfurylamine, DIEA, *i*-PrOH; (d) NaOH, water, EtOH, THF, 7−57% for 4 steps.

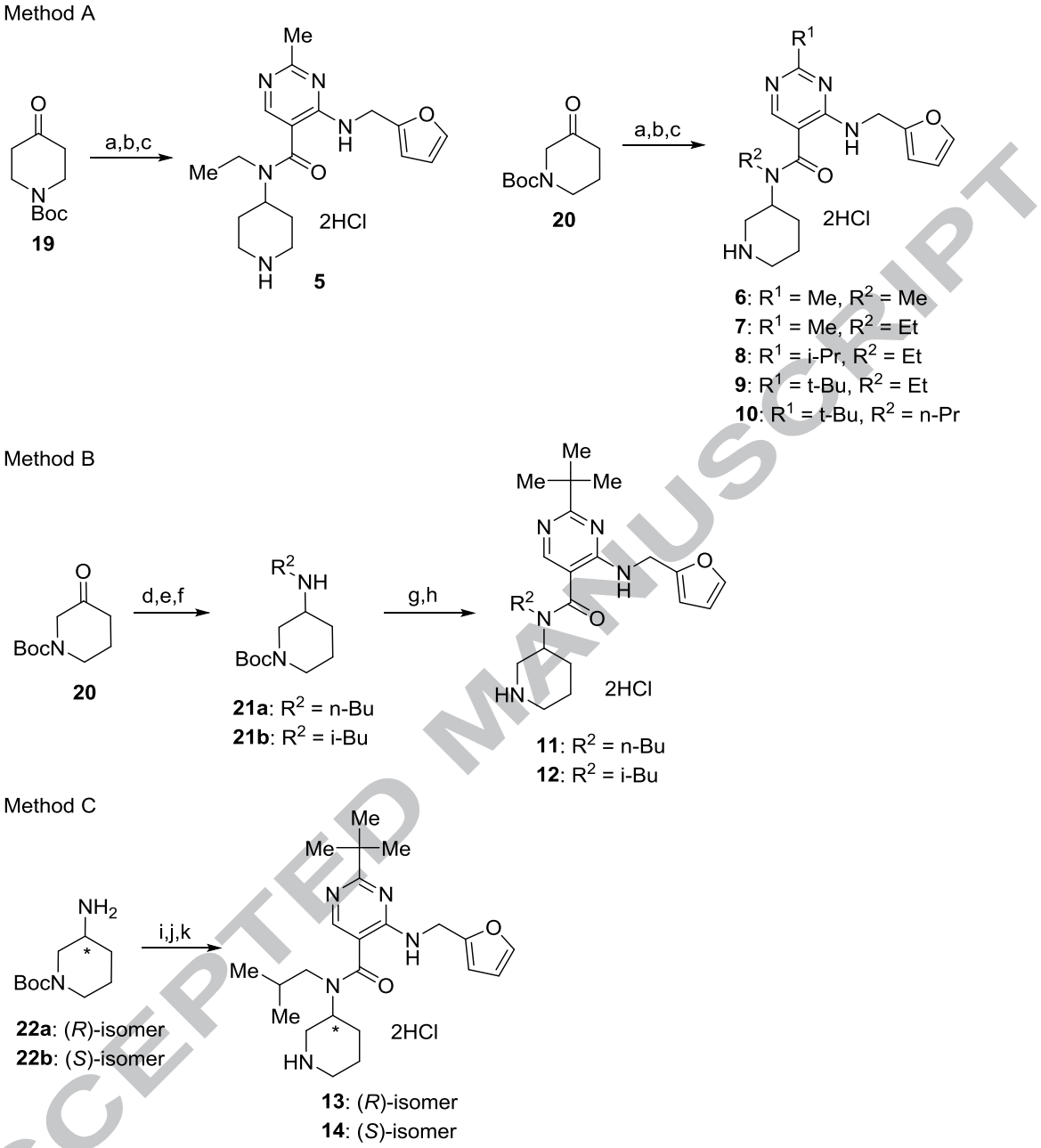
The synthesis of various *N*-(piperidinyl)pyrimidine-5-carboxamides was conducted by two main routes, methods A, B, and C, as shown in Scheme 3. Reaction of 4-oxopiperidine **19** or 3-oxopiperidine **20** under reductive amination conditions with various alkylamines gave the desired *N*-alkylated amines. Amide formation with these amines was performed using WSC and

HOBt, followed by deprotection of the *N*-Boc group, providing the desired pyrimidines **5−10** (Method A).

Compounds **11** and **12** were synthesized as shown in Method B. Reductive amination of 3-oxopiperidine **20** with n-butylamine and isobutylamine gave the desired *N*-alkylated compounds along with some undesired trialkyl amine as byproduct. This trialkylamine was removed by silica gel chromatography after benzyloxycarbonyl (Cbz) group protection of the desired compounds. Subsequent deprotection of Cbz group by catalytic hydrogenation provided piperidines **21a** and **21b**, respectively. The condensation of isobutylamine **21b** with carboxylic acid **18c** using WSC and HOBt did not proceed because of the steric hindrance, thus we used the acyl chloride of **18c** to improve the yield for this coupling. Amide formation with piperidine amines **21a** and **21b** was performed by the treatment of carboxylic acid **18c** with $SOCl_2$ and DMF, followed by deprotection of the *N*-Boc group, providing the desired amides **11** and **12**, respectively.

The synthesis of enantiomers **13** and **14** is depicted in Method C. 3-Aminopiperidines **22a** and **22b** were treated under reductive alkylation conditions with isobutylaldehyde to give the desired *N*-alkylated compounds. Condensation with the amines thus obtained was performed using the acid chloride of carboxylic acid **18c** followed by deprotection of the *N*-Boc group, providing the desired amides **13** and **14**, respectively

Scheme 3. Synthesis of *N*-(piperidinyl)pyrimidine-5-carboxamides **5−14**[a]



[a]Conditions and reagents: (a) R^2NH_2, $NaBH(OAc)_3$, AcOH, MeOH; (b) **18a−c**, WSC, HOBt, Et_3N, DMF; (c) 4 M HCl/EtOAc, 9−38% for 3 steps; (d) R^2NH_2, $NaBH(OAc)_3$, AcOH, MeOH; (e) benzyl chloroformate, NaOH, THF, water; (f) H_2 (1 atm), 5% Pd/C, MeOH, 43−47% for 3 steps. (g) 1) **18c**, $SOCl_2$, DMF, toluene; (2) **21a** and **21b**, Et_3N, THF; (h) 4 M HCl/EtOAc, 36−45% for 3 steps; (i) isobutylaldehyde, $NaBH(OAc)_3$, AcOH, MeOH; (j) 1) **18c**, $SOCl_2$, DMF, toluene; 2) Et_3N, THF; (k) 4 M HCl/EtOAc, 22−35% for 3 steps.

3. Results and Discussion

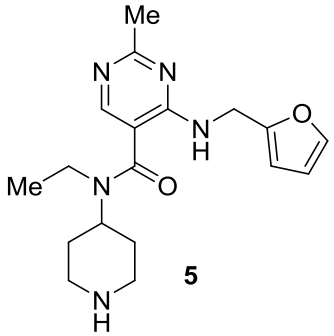
The compounds listed in Figures 2 and 4 and Tables 1−3 have been evaluated for the inhibition of recombinant human renin (rh-renin) by use of either a mobility shift assay or ELISA (enzyme linked immunosolvent assay) method. Inhibitory activities of the compounds are reported as IC_{50} values.

3-1. Introduction of a basic amine directed toward the catalytic aspartic acids.

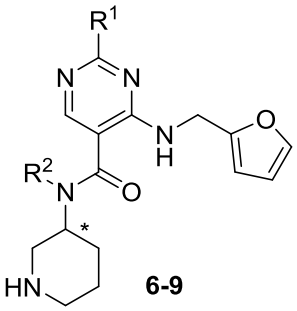
In order to make an interaction with two aspartic acids of the renin catalytic site, we investigated the introduction effect of basic amines instead of the azepane moiety. Replacement of the azepane ring by a piperazine or homopiperazine ring might not be suitable to make interactions between the basic amine and the aspartic acids since these groups are appeared to be too far apart (approximately 5−7 Å). Therefore, we designed *N*-(piperidinyl)pyrimidine-5-carboxamides **5−7**, which are expected to make interactions.

The results of renin inhibition assay of *N*-(piperidinyl)pyrimidine-5-carboxamides **5−7** are shown in Table 1. *N*-(Piperidin-4-yl)pyrimidine-5-carboxamide **5** had remarkably decreased activity. We thought that the polar basic piperidine of **5** might decrease the affinity with the target protein due to solvation penalty and charge repulsion. Meanwhile, *N*-(piperidin-3-yl)pyrimidine-5-carboxamide **7** maintained the inhibitory activity despite the introduction of the hydrophilic basic group (IC_{50} = 38 μM for **3** vs 11 μM for **7**). Thus, we speculated that the basic group on the piperidine ring of compound **7** might make an interaction with aspartic acids because of the appropriate direction and position of the base toward the two aspartic acids. Interestingly, *N*-methyl analog **6** showed less potent renin inhibitory activity compared to *N*-ethyl analog **7**. These results suggested that the alkyl group on the amide nitrogen atoms in compounds **6** and **7** might make a lipophilic interaction with renin.

Table 1. Renin inhibitory potencies of *N*-(piperidinyl)pyrimidine-5-carboxamides **5−9**



5



6-9

compd	R^1	R^2	rh-renin IC_{50} (μM)[a]
5	-	-	>100
6	Me	Me (*RS*)	>100
7	Me	Et (*RS*)	11 (9.0-13)
8	iPr	Et (*RS*)	0.47 (0.36-0.62)
9	t-Bu	Et (*RS*)	0.073 (0.064-0.084)

[a]Inhibitory activity against recombinant human renin. IC_{50} values shown are the means of duplicate measurement by mobility shift assay. The 95% confidence intervals are shown in parentheses.

The crystal structure of human renin soaked with compound **7** was determined to confirm the binding mode (Figure 6). As we supposed, the structure revealed that the piperidine NH group directly interacts with the catalytic two aspartic acids in the catalytic site of renin. The *N*-ethyl group is located in the lipophilic S1 site. These results suggested that compound **7** appeared to make the best balance between interaction gain and solvation penalty.

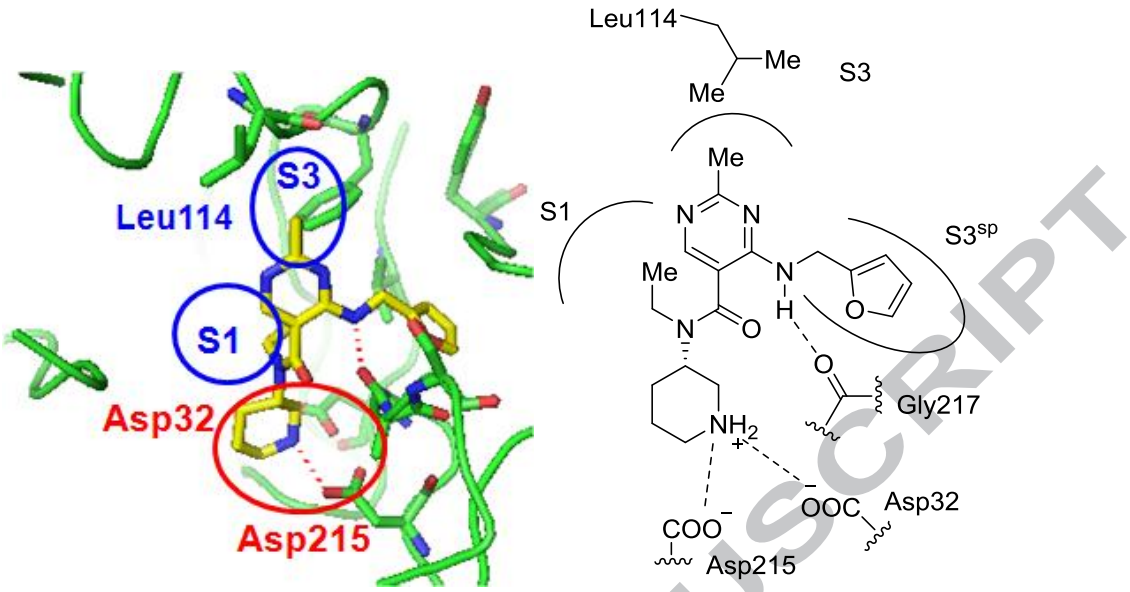


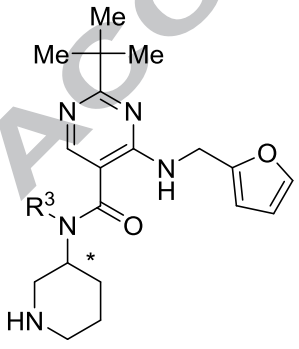
Figure 6. Crystal structure of **7** in complex with human renin (2.25 Å resolution): (a) cartoon view (left), (b) binding mode (right)

3-2 Introduction of a bulky group toward S3 and S1 sites.

Utilization of the renin lipophilic S3 and S1 sites in the crystal structure of compound **7** bound to renin (Figure 6) provided important clues on how to enhance inhibitory activity. Regarding the S3 site, the isobutyl group of Leu114 adopted the "Leu-in" form, like that of compound **2** in Figure 4. We expected that introduction of a bulky group at the pyrimidine 2-position of compound **7** might push the isobutyl group of Leu114 to adopt the "Leu-out" form in an induced fit manner and enhance the inhibitory potency. Based on this idea, we focused on modification of the pyrimidine 2-position. The assay results for 2-substituted pyrimidines are shown in Table 1. Isopropyl derivative **8** showed 20-fold more potent renin inhibitory activity than compound **7**. As expected, the more bulky t-butyl derivative **9** exhibited 150-fold more potent activity compared to compound **7**. These results indicated that the introduction of a bulky group at the pyrimidine 2-position could be effective at enhancing inhibitory activity.

Meanwhile, inspection of the lipophilic S1 site in the complex structure of **7** with renin suggested additional opportunities to increase lipophilic interactions between the compound and protein. An open space around the *N*-ethyl group of **7** appeared available to accommodate additional modification, and thus we sought to elaborate the ethyl group. Our next set of compounds incorporated a variety of bulky alkyl groups on the tertiary amide nitrogen. The renin inhibitory potencies obtained for the compounds synthesized are listed in Table 2. Propyl derivative **10**, butyl derivative **11**, and isobutyl derivative **12** exhibited more potent renin inhibitory activities (IC_{50} values of 10^{-9} M order) than ethyl derivative **9**. In particular, isobutyl derivative **12** showed 26-fold more potent renin inhibitory activity than compound **9**. In order to determine the stereochemical preferences of the piperidine 3-position in **12**, each individual enantiomer **13** and **14** was synthesized and evaluated. As a result, the (*3S*)-enantiomer **14** was determined to be the eutomer of **12**, exhibiting the most potent renin inhibitory activity found in this study (IC_{50} = 0.00058 μM). These results suggested that the isobutyl group of compound **14** might tightly occupy the lipophilic S1 site in renin. Thus, optimization of the S1/S3 binding elements by addition of only five carbon atoms provided *N*-(piperidin-3-yl)pyrimidine-5-carboxamide **14**, which was found to be 20,000-fold more potent than parent compound **7**.

Table 2. Renin inhibitory activity of *N*-(piperidin-3-yl)pyrimidine-5-carboxamides **10**−**14**



compd	R^3	rh-renin IC_{50} (μM)[a]

10	n-Pr (*RS*)	0.0045 (0.0033-0.0060)
11	n-Bu (*RS*)	0.0021 (0.0015-0.0029)
12	i-Bu (*RS*)	0.0012 (0.00068-0.0022)
13	i-Bu (*R*)	0.0034 (0.0026-0.0045)
14	i-Bu (*S*)	0.00058 (0.00039-0.00084)

[a]Inhibitory activity against recombinant human renin. IC_{50} values shown are the means of duplicate measurement by enzyme-linked immunosorbent assay (ELISA). The 95% confidence intervals are shown in parentheses.

In order to confirm its binding mode, the X-ray crystal structure of the potent renin inhibitor **14** in complex with renin was obtained (Figure 7). The stereochemistry of the central amide bond of **14** in the binding site of renin is (*Z*)-form. This structure indicates that the large contiguous hydrophobic S1/S3 specificity sites of the human enzyme are fully occupied with the flap β-hairpin in a closed conformation.[16,17] The isobutyl group of Leu114 adopts the "Leu-out" form, like that of compound **4** in Figure 4. The amino group of the piperidine ring forms tight hydrogen bonding interactions with the catalytic site (Asp32 and Asp215) of the enzyme. The crystal structure also shows that the furfurylamino group occupies the $S3^{sp}$ cavity. In addition, the amino group at the pyrimidine 4-position forms an additional hydrogen bonding interaction with the Gly217 backbone carbonyl group. Moreover, the nitrogen atom at the 1-position in the pyrimidine of **14** is involved in a hydrogen bond with the hydroxyl group of Thr79, as described in the design of pyrimidine derivatives. The crystal structure suggested that compound **14** binds to renin tightly through a combination of hydrophilic and hydrophobic interactions and our hypothesis proved to be right to enhance the inhibitory activity.

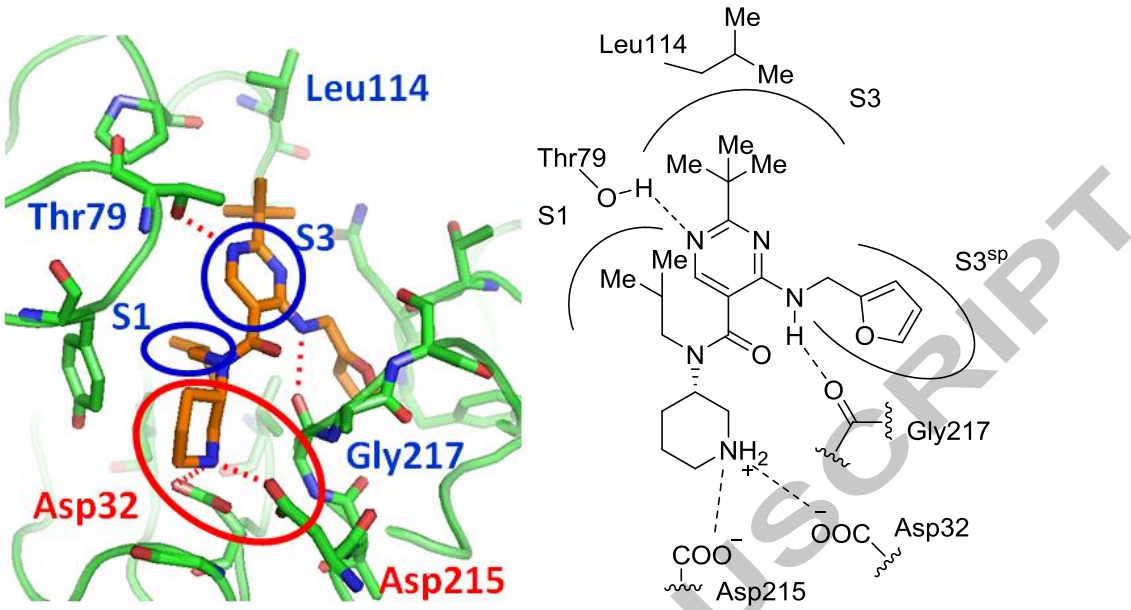


Figure 7.

Crystal structure of **14** in complex with human renin (2.7 Å resolution): (a) cartoon view (left), (b) binding mode (right)

To better understand quality of the new lead compounds, molecular weight (MW) and ligand efficiency (LE) among compounds **3**, **7**, and **14** were compared (Table 3). MW of compound **14** (MW = 413.57) is obviously increased than those of compounds **3** and **7** (313.40 for **3**, 343.43 for **7**). The introduction of the basic moiety (**3** => **7**) appeared not to affect the LE values (0.19 for **3**, 0.20 for **7**). On the other hand, the LE value of compound **14** (0.31) was clearly higher than those of **3** and **7**. The result reflected that the hydrophobic interactions in the S1/S3 sites by the addition of the five carbon atoms significantly and efficiently contributed on the enhancement in renin inhibitory potency.

Table 3. Change in ligand efficiency of compounds **3**, **7**, and **14**

compd	Rh-renin IC_{50} (μM)	MW	HAC[a]	LE[b]
3	38	313.40	23	0.19
7	11	343.43	25	0.20
14	0.00058	413.57	30	0.31

[a]Heavy atom count. [b]pIC_{50}/HAC.

With its excellent in vitro enzyme potency, inhibitory activity of compound **14** against endogenous renin in human plasma (human plasma renin activity, hPRA) was measured. Measuring the hPRA of the compound proved to be an excellent means to take into account the plasma protein binding and consequently the free fraction available in vivo for renin inhibition. The hPRA IC_{50} value of compound **14** was 0.0046 μM, therefore the compound showed promising inhibitory potency in hPRA assay. The IC_{50} value in hPRA assay was shifted upward by about 10-fold over the value determined by ELISA assay. Compound **14** displayed >100,000-fold selectivity for renin versus other aspartyl proteases, cathepsin D and pepsin (IC_{50} values > 100 μM). Compound **14** was also evaluated in a pharmacokinetic study in rats and the pharmacokinetic profile of **14** is summarized in Table 4. Compound **14** showed moderate oral bioavailability in rats. In vivo experiment using compound **14** was not performed, however the in vivo result with further optimized compounds will be reported in a separate paper.

Table 4. Preliminary pharmacokinetic parameters for **14** in rats[a]

iv		po	
C_{5min} (ng/mL)	29.0	C_{max} (ng/mL)	9.3
$V_{d(ss)}$ (mL/kg)	4544	$AUC_{0-24h,po}$ (ng·h/mL)	53.3
CL_{total} (mL/h/kg)	2766	MRT_{po} (h)	3.35
		F (%)	13.8

[a]Cassette dosing. Rats were administered the drug intravenously at 0.1 mg/kg and orally at 1 mg/kg. Data are expressed as mean of three determinations. .

4. Conclusions

In order to enhance the renin inhibitory activity of compound **3**, we were prompted to design and synthesize new pyrimidine derivatives **5−14**. We focused on introduction of a basic amine essential for interaction with the two aspartic acids in the renin catalytic site and optimization of the S1/S3 binding elements including an induced-fit structural change of Leu114 (from "Leu-in" to "Leu-out"). The rational structure-based design approach led to the discovery of compound **14**, a renin inhibitor 65,000-fold more potent than compound **3**. Notably, this remarkable enhancement in renin inhibitory potency was achieved through the addition of only seven heavy atoms to compound **3**. Compound **14** demonstrated excellent selectivity over other aspartyl proteases and moderate oral bioavailability in rats. Investigation of various substituents at the piperidine 5-position of compound **14** will be reported separately.

5. Experimental Section

5-1. Chemistry

General procedures for chemistry. ^{1}H NMR spectra were recorded on a Bruker DPX-300 (300 MHz) spectrometer, and are reported in parts per million (δ) relative to tetramethylsilane (TMS: δ 0.0 ppm). Data are reported as follows: chemical shift, integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet, ddd = doublet of doublet of doublet, bs = broad singlet), and coupling constants (*J*, Hz). Unless otherwise specified, all solvents and reagents were obtained from commercial suppliers and used without further purification. Column chromatography was performed using Merck silica gel 60 (70–230 mesh). Thin-layer chromatography (TLC) was performed on Merck silica gel plates 60F254. LC−MS analysis was performed on a Shiseido CAPCELL PACK C-18 UG120 S-3 column (1.5 mmφ x 35 mm) in a Waters Alliance 2795 or an Agilent 1100 LC system equipped with a Waters 2487 absorbance detector and a Micromass ZQ2000 mass spectrometer. All MS experiments

were performed using electrospray ionization (ESI) in positive ion mode. Analytes were eluted using a linear gradient of water (0.05% trifluoroacetic acid (TFA))/acetonitrile (0.04% TFA) from 90:10 to 0:100 over 4 min at a flow rate of 0.5 mL/min. UV detection was at 220 nm. Preparative HPLC was performed on a Shiseido CAPCELL PACK C-18 UG120 S-5 column (20 mmφ x 50 mm), eluting at 25 mL/min with a gradient of water (0.1% TFA)/acetonitrile (0.1% TFA). UV detection was at 220 nm. Elemental analyses were performed by Takeda Analytical Research Laboratories, Ltd. Melting points were determined on Yanagimoto micro melting-point apparatus and are uncorrected. Purity measurements were carried out using a Shimadzu UFLC system employing the following conditions: column; L-column2 ODS (3.0 mmIDx30 mmL, 2 μm, CERI, Japan); mobile phase; MeCN/H_2O/TFA = 5:95:0.1 (0 min) → 90:10:0.1 (2.00 min) → 90:10:0.1 (3.3 min); flow rate; 1.2 mL/min; temperature; 40 ºC; detection; UV 220 mm. Each compound was confirmed to be ≥90% pure by either LC-MS or elemental analysis. Yields are not optimized.

2-[(Furan-2-ylmethyl)amino]-6-methylpyridine-3-carboxylic acid (16). A solution of 2-chloro-6-methylnicotinic acid (1.72 g, 10.0 mmol) and 1-(2-furyl)methanamine (1.94 g, 20.0 mmol) in 1-methylpyrrolidin-2-one (25 mL) was heated at 150 ºC overnight. The solvent was evaporated, and the residue was purified by silica gel chromatography (EtOAc/hexane = 4/1 to EtOAc) to give **16** (838 mg, 36%). ^{1}H NMR ($CDCl_3$) δ: 2.45 (3H, s) 4.78 (2H, s) 6.26 (1H, d, *J* = 3.2 Hz) 6.32 (1H, dd, *J* = 3.2 and 1.7 Hz) 6.46 (1H, d, *J* = 8.1 Hz) 7.33−7.40 (1H, m) 8.05−8.14 (2H, m).

Azepan-1-yl{2-[(furan-2-ylmethyl)amino]-6-methylpyridin-3-yl}methanone (3). To the mixture of compound **16** (30 mg, 0.129 mmol) and hexamethyleneimine (19 mg, 0.192 mmol) in DMF (2.0 mL), were added HOBt (21 mg, 0.155 mmol) and WSC-HCl (30 mg, 0.156 mmol) and the mixture was stirred at room temperature overnight. To the reaction mixture EtOAc (7

mL) and 2% aqueous $NaHCO_3$ solution (1.5 mL) were added and extraction was carried out. The water phase was washed with EtOAc (7 mL). The combined organic phase was dried with $MgSO_4$ and evaporated. The residue was purified by silica gel chromatography (hexane to EtOAc/hexane = 3/7) to give **3** (30 mg, 74%) as a clear oil. ^{1}H NMR ($CDCl_3$) δ 1.55−1.86 (8H, m), 2.41 (3H, s), 3.52 (4H, s), 4.65 (2H, d, *J* = 5.6 Hz), 5.81 (1H, t, *J* = 5.3 Hz), 6.23 (1H, d, *J* = 3.2 Hz), 6.27−6.34 (1H, m), 6.43 (1H, d, *J* = 7.6 Hz), 7.22 (1H, d, *J* = 7.3 Hz), 7.34 (1H, d, *J* = 1.7 Hz). LC−MS (ESI): *m/z* = 314 $[M+H]^+$. Purity measurement by HPLC: 100%, Rt = 1.14 min.

2-*tert*-Butyl-4-[(furan-2-ylmethyl)amino]pyrimidine-5-carboxylic acid (18c). Compound **17c** (2.90 g, 21 mmol) and diethyl (ethoxymethylidene)propanedioate (4.25 mL, 21 mmol) were dissolved in EtOH (30 mL). A solution of sodium ethoxide (20% in EtOH; 14.3 g, 42 mmol) was added to the solution dropwise at 0 ºC. After stirring at 80 ºC for 15 h, the reaction mixture was concentrated in vacuo. The residue was acidified with 1 M HCl to pH 3. The precipitate was collected by filtration and washed with small amount of water. The product obtained (2.90 g, 13 mmol) was dissolved in $POCl_3$ (9.6 g). After stirring at 100ºC for 2 h, the reaction mixture was concentrated in vacuo. The residue was cooled to 0 ºC, neutralized with a saturated aqueous $NaHCO_3$ solution to pH 7, and extracted with EtOAc. The extract was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. A solution of the product obtained, furfurylamine (1.20 mL, 12.9 mmol), and diisopropylethylamine (2.26 mL, 12.9 mmol) in 2-propanol (10 mL) was stirred under reflux for 15 h. The reaction mixture was cooled to room temperature and concentrated in vacuo. The residue was diluted with a saturated aqueous $NaHCO_3$ solution and extracted with EtOAc. The extract was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/EtOAc = 9/1 to 5/1). The product obtained was dissolved in 2 M NaOH (15 mL, 30 mmol), EtOH (15 mL), and

THF (5 mL). After stirring at room temperature for 15 h, the mixture was acidified with 1 M HCl to pH 3.0. The precipitate was collected by filtration and washed with water to give **18c** as a colorless powder (3.30 g, 57%). ^{1}H NMR ($CDCl_3$) δ: 1.32−1.40 (12H, m), 4.33 (2H, q, *J* = 7.1 Hz), 4.77 (2H, d, *J* = 5.7 Hz), 6.26 (1H, d, *J* = 3.2 Hz), 6.32 (1H, dd, *J* = 3.2 and 1.9 Hz), 7.36 (1H, dd, *J* = 1.8 and 0.8 Hz), 8.34 (1H, bs), 8.85 (1H, s). LC−MS (ESI): *m/z* = 287 $[M+H]^+$.

The following compounds **18a** and **18b** were prepared in a manner similar to that described for **18c**.

4-[(Furan-2-ylmethyl)amino]-2-methylpyrimidine-5-carboxylic acid (18a). ^{1}H NMR ($CDCl_3$) δ: 2.57 (3H, s), 4.78 (2H, s), 6.30 (2H, dd, *J* = 12.7 and 2.2 Hz), 7.37 (1H, d, *J* = 0.9 Hz), 8.47−8.72 (1H, m)

4-[(Furan-2-ylmethyl)amino]-2-(propan-2-yl)pyrimidine-5-carboxylic acid (18b). ^{1}H NMR ($CDCl_3$) δ: 1.24 (6H, d, *J* = 5.1 Hz), 2.94−3.09 (1H, m), 4.78 (2H, d, *J* = 4.2 Hz), 6.33 (1H, d, *J* = 2.1 Hz), 6.41 (1H, s), 7.60 (1H, s), 8.70 (1H, s), 9.09 (1H, s).

4-[(Furan-2-ylmethyl)amino]-*N*,2-dimethyl-*N*-(piperidin-3-yl)pyrimidine-5-carboxamide dihydrochloride (6). Compound **20** (398 mg, 2.0 mmol), AcOH (0.11 mL, 2.0 mmol), and methylamine (2.0 M solution in THF; 1.0 mL, 2.0 mmol) were dissolved in MeOH (5 mL), and the mixture was stirred at room temperature for 1 h. $NaBH(OAc)_3$ (0.88 g, 4.0 mmol) was added by portions, and the reaction mixture was stirred at room temperature for additional 15 h. After the volatiles were removed by evaporation, the residue was dissolved in saturated aqueous $NaHCO_3$ and extracted twice with EtOAc. The combined organic layer was dried over Na_2SO_4, and concentrated in vacuo. The product obtained was added to a solution of **18a** (0.47 g, 2.0 mmol), HOBt (0.46 g, 3.0 mmol), WSC (0.58 g, 3.0 mmol), and Et_3N (0.84 mL, 6.0 mmol) in DMF (3 mL), and the resulting mixture was stirred at room temperature for 15 h. The reaction mixture was partitioned between water and EtOAc. The organic layer was separated, washed

with water and brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/EtOAc = 2/1 to 0/1). The product obtained was dissolved in 4 M HCl/EtOAc (3 mL), and the mixture was stirred at room temperature for 15 h. After concentration in vacuo, the residue was suspended in EtOAc, and the precipitate was collected by filtration to give **6** (64 mg, 9%) as a beige amorphous powder. ^{1}H NMR (DMSO-d_6) δ: 1.50−1.88 (3H, m), 2.58 (3H, s), 2.66−3.76 (8H, m), 4.75 (2H, bs), 6.35−6.44 (2H, m), 7.62 (1H, d, *J* = 0.8 Hz), 8.11 (1H, bs), 8.42 (1H, s), 9.06−9.80 (2H, m). LC−MS (ESI): *m/z* = 330 $[M+H]^+$. Anal. Calcd for $C_{17}H_{25}Cl_2N_5O_2$•3.5H_2O•0.1EtOAc: C, 44.07; H, 6.97; N, 14.77. Found: C, 44.20; H, 6.87; N, 14.62.

The following compounds **6−10** were prepared in a manner similar to that described for **6**.

***N*-Ethyl-4-[(furan-2-ylmethyl)amino]-2-methyl-*N*-(piperidin-3-yl)pyrimidine-5-carboxamide dihydrochloride (7).** ^{1}H NMR (DMSO-d_6) δ: 0.80−1.20 (3H, m), 1.48−1.88 (2H, m), 2.57 (3H, s), 2.73 (1H, bs), 2.87−3.70 (8H, m), 4.54−4.89 (2H, m), 6.29−6.47 (2H, m), 7.61 (1H, d, *J* = 0.9 Hz), 8.43 (1H, s), 9.17 (1H, bs), 9.36 (1H, bs). LC−MS (ESI): *m/z* = 344 $[M+H]^+$. Anal. Calcd for $C_{18}H_{27}Cl_2N_5O_2$•2H_2O•0.2EtOAc: C, 48.04; H, 6.99; N, 14.90. Found: C, 48.32; H, 6.95; N, 14.78.

***N*-ethyl-4-[(furan-2-ylmethyl)amino]-2-methyl-*N*-(piperidin-4-yl)pyrimidine-5-carboxamide dihydrochloride (5).** ^{1}H NMR (DMSO-d_6) δ: 0.94−1.24 (3H, m), 1.80 (2H, bs), 2.11 (2H, bs), 2.59 (3H, s), 2.85 (2H, bs), 3.46 (4H, bs), 4.71 (2H, d, *J* = 5.7 Hz), 6.43 (1H, d, *J* = 1.7 Hz), 6.33−6.44 (1H, m), 7.60 (1H, d, *J* = 1.1 Hz), 8.30−8.45 (1H, m), 9.19 (3H, bs). LC−MS (ESI): *m/z* = 344 $[M+H]^+$. Anal. Calcd for $C_{18}H_{27}Cl_2N_5O_2$•1.3H_2O: C, 49.16; H, 6.78; N, 15.93. Found: C, 49.31; H, 6.90; N, 15.63.

***N*-Ethyl-4-[(furan-2-ylmethyl)amino]-*N*-(piperidin-3-yl)-2-(propan-2-yl)pyrimidine-5-carboxamide dihydrochloride (8).** ^{1}H NMR (DMSO-d_6) δ: 0.92−1.22 (3H, m), 1.29 (6H, d, *J*

= 6.8 Hz), 1.67 (1H, bs), 1.76−1.96 (2H, m), 2.72 (1H, d, *J* = 1.9 Hz), 3.13 (3H, dt, *J* = 13.7and 6.7 Hz), 3.55 (3H, d, *J* = 13.9 Hz), 4.03 (1H, q, *J* = 7.2 Hz), 4.57−4.84 (2H, m), 6.35 (1H, d, *J* = 2.8 Hz), 6.42 (1H, dd, *J* = 3.2 and 1.9 Hz), 7.60 (1H, d, *J* = 0.9 Hz), 8.40 (1H, s), 9.18 (1H, bs), 9.29 (1H, bs), 9.70 (1H, bs). LC−MS (ESI): *m/z* = 372 $[M+H]^+$. Anal. Calcd for $C_{20}H_{31}Cl_2N_5O_2 \bullet 2.3H_2O$: C, 49.44; H, 7.39; N, 14.42. Found: C, 49.58; H, 7.20; N, 14.17.

2-*tert*-Butyl-*N*-ethyl-4-[(furan-2-ylmethyl)amino]-*N*-(piperidin-3-yl)pyrimidine-5-carboxamide dihydrochloride (9). ^{1}H NMR (DMSO-d_6) δ: 1.38 (9H, s), 1.91 (4H, bs), 2.73 (1H, bs), 3.12 (3H, bs), 3.36 (2H, bs), 3.54 (5H, bs), 4.54−4.83 (2H, m), 6.34 (1H, bs), 6.41 (1H, dd, *J* = 3.2 and 1.9 Hz), 7.60 (1H, d, *J* = 0.9 Hz), 8.33 (1H, s), 9.22 (1H, bs). LC−MS (ESI): *m/z* = 386 $[M+H]^+$. Anal. Calcd for $C_{21}H_{33}Cl_2N_5O_2 \bullet 2.5H_2O$: C, 50.10; H, 7.61; N, 13.91. Found: C, 50.28; H, 7.47; N, 13.77. Purity measurement by HPLC: 98%, Rt = 2.55 min.

2-*tert*-Butyl-4-[(furan-2-ylmethyl)amino]-*N*-(piperidin-3-yl)-*N*-propylpyrimidine-5-carboxamide dihydrochloride (10). ^{1}H NMR (DMSO-d_6) δ: 0.85 (3H, bs), 1.38 (9H, s), 1.50−1.96 (5H, m), 2.62−3.84 (8H, m), 4.00−4.34 (1H, m), 4.56−4.87 (2H, m), 6.33 (1H, bs), 6.41 (1H, dd, *J* = 3.2 and 1.9 Hz), 7.59 (1H, d, *J* = 0.9 Hz), 8.31 (1H, bs), 9.18 (1H, bs). LC−MS (ESI): *m/z* = 400 $[M+H]^+$. Anal. Calcd for $C_{23}H_{37}Cl_2N_5O_2 \bullet H_2O$: C, 54.76; H, 7.79; N, 13.88. Found: C, 54.76; H, 7.82; N, 13.75. Purity measurement by HPLC: 98%, Rt = 2.65 min.

***tert*-Butyl 3-[(2-methylpropyl)amino]piperidine-1-carboxylate (21b).** Compound **20** (1.99 g, 10 mmol), AcOH (0.57 mL, 10 mmol), and isobutylamine (0.99 mL, 10 mmol) were dissolved in MeOH (50 mL), and the mixture was stirred at room temperature for 1 h. Sodium triacetoxyborohydride (4.23 g, 20 mmol) was added in portions, and the reaction mixture was stirred at room temperature for additional 15 h. After the volatiles were removed by evaporation, the residue was dissolved in saturated aqueous $NaHCO_3$ solution and extracted twice with EtOAc. The combined organic layer was dried over Na_2SO_4, and concentrated in vacuo. The

residue was dissolved in THF (20 mL) and 2 M NaOH (3 mL, 24 mmol), and then benzyl chloroformate (2.86 mL, 20 mmol) was added dropwise. After stirring at room temperature for 15 h, the mixture was extracted with EtOAc. The extract was washed with water and brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/EtOAc = 1/9 to 1/4) to afford the Cbz-protected amine. A mixture of the product obtained and 5% Pd/C (0.20 g) in EtOH (30 mL) was stirred at room temperature under H_2 atmosphere (1 kgf/cm^2) for 15 h. The palladium catalyst was removed by filtration, and the filtrate was concentrated in vacuo to give **21b** as pale yellow syrup (1.21 g, 47%). ^{1}H NMR ($CDCl_3$) δ: 0.90 (6H, d, *J* = 6.6 Hz), 1.22−1.40 (3H, m), 1.46 (9H, s), 1.55−1.78 (2H, m), 1.90 (1H, d, *J* = 13.6 Hz), 2.45 (3H, dd, *J* = 6.8 and 3.8 Hz), 2.87 (1H, bs), 3.54−4.24 (2H, m).

The following compound **21a** was prepared in a manner similar to that described for **21b**.

***tert*-Butyl 3-(butylamino)piperidine-1-carboxylate (21a).** ^{1}H NMR ($CDCl_3$) δ: 0.91 (3H, t, *J* = 7.2 Hz), 1.20−1.42 (4H, m), 1.42−1.52 (3H, m), 1.46 (9H, s), 1.67 (1H, td, *J* = 8.9 and 4.3 Hz), 1.78−2.04 (1H, m), 2.47−2.79 (3H, m), 2.87 (1H, bs), 3.79 (1H, d, *J* = 13.2 Hz), 4.04 (1H, bs).

2-*tert*-Butyl-4-[(furan-2-ylmethyl)amino]-*N*-(2-methylpropyl)-*N*-(piperidin-3-yl)pyrimidine-5-carboxamide dihydrochloride (12). To a suspension of compound **18c** (138 mg, 0.5.mmol) in toluene (5 mL) was added thionyl chloride (0.11 mL, 1.5 mmol) and DMF (1 drop) at room temperature. After stirring at 100ºC for 1 h, the reaction mixture was concentrated in vacuo. The residue was azeotroped with toluene and dissolved in THF (5 mL). Compound **21b** (128 mg, 0.5 mmol) and Et_3N (0.21 mL, 1.5 mmol) were added to the solution. After stirring at room temperature for 2 h, the reaction mixture was concentrated in vacuo. The residue was diluted with water and extracted with EtOAc. The extract was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica gel column

chromatography (hexane/EtOAc = 9/1 to 3/1). The product obtained was dissolved in 4 M HCl/EtOAc (2 mL), and the mixture was stirred at room temperature for 15 h. After concentration in vacuo, the residue was suspended in EtOAc, and the precipitate was collected by filtration and washed with EtOAc to give **12** (85 mg, 36%) as a light-brown amorphous powder. ^{1}H NMR (DMSO-d_6) δ: 0.83 (5H, bs), 1.38 (9H, s), 1.57−2.12 (5H, m), 2.79 (1H, d, *J* = 7.3 Hz), 3.12 (4H, bs), 3.67 (3H, bs), 4.18 (1H, bs), 4.48−4.91 (2H, m), 6.33 (1H, bs), 6.41 (1H, dd, *J* = 3.1 and 1.8 Hz), 7.59 (1H, d, *J* = 0.9 Hz), 8.28 (1H, s), 9.23 (1H, bs). LC−MS (ESI): *m/z* = 414 $[M+H]^+$. Anal. Calcd for $C_{23}H_{37}Cl_2N_5O_2 \bullet H_2O$: C, 54.76; H, 7.79; N, 13.88. Found: C, 54.76; H, 7.82; N, 13.75. Purity measurement by HPLC: 99%, Rt = 2.78 min.

The following compound **11** was prepared in a manner similar to that described for **12**.

***N*-Butyl-2-*tert*-butyl-4-[(furan-2-ylmethyl)amino]-*N*-(piperidin-3-yl)pyrimidine-5-carboxamide dihydrochloride (11).** ^{1}H NMR (DMSO-d_6) δ: 0.92 (3H, bs), 1.38 (9H, s), 1.53 (1H, bs), 1.87 (4H, bs), 2.73 (1H, bs), 3.08 (3H, bs), 3.28 (3H, bs), 3.49 (3H, bs), 4.55−4.71 (1H, m), 4.71−4.83 (1H, m), 6.34 (1H, bs), 6.41 (1H, d, *J* = 1.9 Hz), 7.59 (1H, s), 8.33 (1H, bs), 9.21 (2H, bs). LC−MS (ESI): *m/z* = 414 $[M+H]^+$. Anal. Calcd for $C_{23}H_{37}Cl_2N_5O_2 \bullet H_2O$: C, 54.76; H, 7.79; N, 13.88. Found: C, 55.15; H, 7.92; N, 13.86.

2-*tert*-Butyl-4-[(furan-2-ylmethyl)amino]-*N*-(2-methylpropyl)-*N*-[(3*S*)-piperidin-3-yl]pyrimidine-5-carboxamide dihydrochloride (14). Compound **22a** (200 mg, 1.0 mmol), AcOH (0.07 mL, 1.2 mmol), and isobutylaldehyde (0.11 mL, 1.2 mmol) were dissolved in MeOH (5 mL), and the mixture was stirred at room temperature for 1 h. Sodium triacetoxyborohydride (530 mg, 2.5 mmol) was added in portions, and the reaction mixture was stirred at room temperature for additional 15 h. After the volatiles were removed by evaporation, the residue was dissolved in saturated aqueous $NaHCO_3$ solution and extracted twice with EtOAc. The combined organic layer was dried over Na_2SO_4, and concentrated in vacuo to give *tert*-butyl (3*S*)-3-[(2-

methylpropyl)amino]piperidine-1-carboxylate as crude. The obtained compound was dissolved in THF (5 mL).

To a suspension of compound **18c** (276 mg, 1.0.mmol) in toluene (5 mL) was added thionyl chloride (0.18 mL, 2.5 mmol) and DMF (1 drop) at room temperature. After stirring at 80 ºC for 1 h, the reaction mixture was concentrated in vacuo. The residue was azeotroped with toluene and dissolved in THF (5 mL). The THF solution of *tert*-butyl (3*S*)-3-[(2-methylpropyl)amino]piperidine-1-carboxylate described above and Et_3N (0.42 mL, 3.0 mmol) were added to the solution. After stirring at room temperature for 2 h, the reaction mixture was concentrated in vacuo. The residue was diluted with water and extracted with EtOAc. The extract was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/EtOAc = 9/1 to 3/1). The product obtained was dissolved in 4 M HCl/EtOAc (2 mL), and the mixture was stirred at room temperature for 15 h. After concentration in vacuo, the residue was suspended in EtOAc, and the precipitate was collected by filtration and washed with EtOAc to give **14** (105 mg, 22%) as a light-brown amorphous powder. Measurement of the enantiomeric excess was performed on a liquid chromatography system equipped with a CHIRALPACK AD (4.6 mm ID × 250 mm L, KF051, Daicel, Japan), eluting with a flow rate of 1 mL/min hexane/ethanol/diethylamine = 950/50/1 (v/v/v) at 35 °C. Rt: 12.61 min (82% ee). ^{1}H NMR (DMSO-d_6) δ: 0.81 (6H, bs), 1.37 (9H, s), 1.63−2.05 (3H, m), 2.54−2.60 (1H, m), 3.57 (8H, bs), 4.16 (1H, bs), 4.54−4.74 (2H, m), 6.31 (1H, bs), 6.41 (1H, dd, *J* = 3.2 and 1.9 Hz), 7.58 (1H, s), 8.26 (1H, bs), 9.20 (1H, bs). LC−MS (ESI): *m/z* = 414 $[M+H]^+$. Anal. Calcd for $C_{23}H_{37}Cl_2N_5O_2 \bullet H_2O$: C, 54.76; H, 7.79; N, 13.88. Found: C, 54.76; H, 8.07; N, 13.85. Purity measurement by HPLC: 98%, Rt = 2.75 min. $[\alpha]^{25}_D$ -13.5 (c 0.4390, MeOH).

The following compound **13** was prepared in a manner similar to that described for **14**.

2-*tert*-Butyl-4-[(furan-2-ylmethyl)amino]-*N*-(2-methylpropyl)-*N*-[(3*R*)-piperidin-3-yl]pyrimidine-5-carboxamide dihydrochloride (13). Measurement of the enantiomeric excess was performed on a liquid chromatography system equipped with a CHIRALPACK AD (4.6 mm ID × 250 mm L, KF051, Daicel, Japan), eluting with a flow rate of 1 mL/min hexane/ethanol/diethylamine = 950/50/1 (v/v/v) at 35 °C. Rt: 11.13 min (91% ee). ^{1}H NMR (DMSO-d_6) δ: 0.84 (6H, bs), 1.39 (9H, s), 1.69−2.10 (5H, m), 2.79 (1H, bs), 3.54 (6H, bs), 4.19 (1H, bs), 4.50−4.71 (1H, m), 4.71−4.84 (1H, m), 6.33 (1H, bs), 6.41 (1H, dd, *J* = 3.2 and 1.9 Hz), 7.59 (1H, s), 8.29 (1H, s), 9.28 (1H, bs). LC−MS (ESI): *m/z* = 414 $[M+H]^+$. Anal. Calcd for $C_{23}H_{37}Cl_2N_5O_2 \cdot H_2O$: C, 54.76; H, 7.79; N, 13.88. Found: C, 54.35; H, 7.78; N, 13.66. Purity measurement by HPLC: 100%, Rt = 2.76 min. $[\alpha]^{25}_D$ +14.7 (c 0.4365, MeOH).

5-2. Assays of biological activities

Purification of recombinant human renin

Human preprorenin was expressed using the FreeStyle 293 Expression System (Invitrogen). The recombinant prorenin was exported by FreeStyle 293 cells into the tissue culture media. The cell culture supernatants were processed by filtration, concentration and dialysis in 20 mM Tris-HCl buffer (pH 8.0). Prorenin was purified using Resource Q column (GE Healthcare) and HiLoad 16/60 Superdex 200pg (GE Healthcare). Prorenin was activated to renin by trypsin digestion. Renin was purified using TSKgel DEAE-5PW (Tosoh).

Renin activity assay using microchip-type capillary electrophoresis (Mobility shift assay). In a 384−well plate (Nalgen−Nunc), 2 μL of test compound in 100% DMSO was incubated with 28 μL of enzyme in buffer (5 nM renin, 20 mM citric acid buffer (pH 6.0) and 0.004% Triton-X100) at 37 ºC. After 15 min, 10 μL of substrate peptide (FITC-εAcp-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Asn-Arg-NH_2, 5 μM) was added to each well to start the reaction. After incubation at 37 ºC for 30 min, the reaction was terminated by addition of 40 μL of stop

solution (1 μM CGP-29287,[21] 200 mM Tris-HCl (pH 8.0), 0.04% Triton-X 100, 0.4% Coating 3 reagent (Caliper Life Sciences)). After completion of the reaction, substrate and product peptide were separated by electrophoresis and quantified by fluorescence detection (excitation wavelength 457 nm, measurement wavelength 530 nm) using a microchip-type capillary electrophoresis apparatus 250HTS (Caliper Life Sciences). The rate of substrate conversion was calculated by dividing the product peak height by the sum of the substrate and product peak height (P/(P+S)). Inhibitory activity of each compound was calculated on the basis of 0% control wells with DMSO and 100% control wells with 10 μM CGP-29287.

Human renin inhibition assay (enzyme-linked immunosorbent assay (ELISA)). The inhibitory potency of the compounds against human renin was determined by the following protocol. In 384−well plates (ABgene), 1 μL of test compound in 100% DMSO was incubated with 14 μL of enzyme (at a final concentration of 40 pM human renin) in buffer (20 mM Phosphate buffer, 1 mM EDTA, pH 7.4, with 0.004% Tween 20) at 37 ºC. After 10 min, 5 μL of recombinant human angiotensinogen was added to a final concentration of 1.5 μM and incubated at 37 ºC for 30 min. The enzymatic reaction was terminated by adding 20 μL of stop solution (1 μM CGP-29287 in diluent buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% BSA, 0.05% Tween20)). The diluent buffer was used for diluting each reagent in the ELISA. Angiotensin I generated during the incubation was measured by ELISA. Aliquots (10 μL) of the incubates or angiotensin I peptide standards were transferred to 384−well immuno plates which were previously coated with anti-angiotensin I antibody (Peninsula Laboratories) and incubated with 15 μL of 1.6 nM biotin-conjugated angiotensin I (AnaSpec) at room temperature for 1 h. After washing the plates 5 times with wash buffer (0.05% Tween20 in PBS), 25 μL of 100 ng/ml streptavidin-HRP (Pierce) was incubated at room temperature for 30 min. After washing, 25 μL of substrate of HRP (Pierce) was added and chemiluminescence was detected using a microplate

reader. Compound **8**: ELISA IC_{50} = 0.14 μM, Caliper IC_{50} = 0.47 μM. Compound **9**: ELISA IC_{50} = 0.032 μM, Caliper IC_{50} = 0.073 μM.

Human plasma renin activity (hPRA) assay

The inhibitory effect of each compound on the human plasma renin activity was tested using the radioimmunoassay kit (SRL, Tokyo, Japan). IC_{50} values were calculated from concentration-response curves with SAS software (SAS Institute Japan Ltd., Tokyo, Japan).

5-3. General procedure for X-ray crystallography of inhibitors with human renin

Crystallization of mature human renin (1-340) was carried out by the sitting drop vapor diffusion method (Nanovolume Crystallization™ methods[22]). Conditions for the reservoir solution were 19.95%−40.95% PEG600, 100 mM citric acid buffer pH 4.5 – 6.0, or 24%−45% PEG600, citric acid buffer pH 4.5 – 6.0, 50 mM NaH_2PO_4 aqueous solution. A mixture of the reservoir solution and a solution of human renin (ca. 6 mg/mL in 25 mM Tris pH 7.9 and 150 mM NaCl aqueous solution) was left at 20 ºC until crystals of apoprotein were generated. Crystals of human renin in complex with inhibitors were prepared by the soaking method. The apoprotein crystals were soaked into a soaking buffer, which was prepared by adding inhibitors to the reservoir solution to 1 – 10 mM, for 30 min to 1 d. The crystals thus obtained were soaked into the soaking buffer, to which was added ethylene glycol to 0 – 12%, and the mixture was frozen. X-ray diffraction analysis was carried out by using beamline 5.0.3 at the Advanced Light Source (ALS) and at beamline ID23B of the Advanced Photon Source (APS). The X-ray diffraction data were processed with HKL2000.[23] The structures were solved by molecular replacement using MOLREP of CCP4 (Ver 4.0) CGP-29287. Iterative cycles of structure refinement and manual building were done using the programs REFMAC and XFIT, respectively. Structure refinements of the models, which were generated by Xfit[24] based on the initial complex structures, were carried out by using REFMAC.[25]

Table 5. Data reduction and refinement statistics for the X-ray structures of the compounds complexed with renin.[15]

Data Collection					
Compound	**2**	**3**	**4**	**7**	**14**
PDB code	5SY3	5SZ9	5SXN	5SY2	5KOQ
Beamline	ALS 5.0.3	APS ID23B	ALS 5.0.3	APS ID23B	ALS 5.0.3
Wavelength (Å)	1	1	1	1	1
Space group	$P2_13$	$P2_13$	$P2_13$	$P2_13$	$P2_13$
Unit cell	a=b=c=139.2	a=b=c=140.1	a=b=c=140.1	a=b=c=137.5	a=b=c=138.6
dimensions (Å)	α=β=γ=90°	α=β=γ=90°	α=β=γ=90°	α=β=γ=90°	α=β=γ=90°
Resolution (Å)	2.3	2.85	2.1	2.253	2.7
Unique reflections	40223	21708	53656	41237	24655
Redundancy	4.3	5.5	6	5.9	5.9
Completeness (%)	99.9 (100)	99.9 (99.8)	99.6 (99.0)	99.57 (98.71)	100 (100)
I/σ(I)	19.4 (2.8)	16.5 (3.4)	23.3 (3.4)	18.3 (2.9)	20.2 (2.4)
R_{sym} [a]	0.070 (0.506)	0.101 (0.515)	0.063 (0.491)	0.081 (0.508)	0.071 (0.721)
Refinement					
Molecules in assymteric unit	2	2	2	2	2
Reflections used	39944	21651	53074	41007	23173
RMS Bonds (Å)	0.011	0.009	0.023	0.01	0.009
RMS Angles (°)	1.28	1.31	1.31	1.33	1.25
Average B value ($Å^2$)	42	50.6	40.5	36	66.2
R-value[b]	0.192	0.184	0.195	0.185	0.206
R_{free} [b]	0.237	0.232	0.233	0.221	0.252

[a]Rsym = ΣhΣj |<I(h)> - I(h)j | / ΣhΣj <I(h)>, where <I(h)> is the mean intensity of symmetry-related reflections. [b]R-value = Σ| |Fobs| - |Fcalc| | / Σ|Fobs|. Rfree for 5% of reflections excluded from refinement. Values in parentheses are for the highest resolution shell.

5-4. Pharmacokinetic analysis in rat cassette dosing.

All animal experiments were performed in compliance with the Guidelines for the Care and Use of Laboratory Animals of Takeda Pharmaceutical Company Ltd. Test compounds were administered intravenously (0.1 mg/kg, solvent DMA/1,3-butanediol = 1:1) or orally (1 mg/kg, solvent 0.5% methylcellulose suspension) by cassette dosing to fed Sprague–Dawley rats (n = 3 each).

After administration, blood samples were collected and centrifuged to obtain the plasma fraction. The plasma samples were deproteinized followed by centrifugation. The compound concentrations in the supernatant were measured by LC/MS/MS.

Acknowledgement The authors thank Keiji Kusumoto for helpful discussions; the DMPK group at Takeda Pharmaceutical Company for the rat cassette dosing experiment in Table 4; Kengo Okada, Hideyuki Oki, Weston Lane, and Bi-Ching Sang for molecular biology, protein expression, crystallization and X-ray data collection support highlighted in Figure 3, 4, 6, and 7. We thank the staff of the Berkeley Center for Structural Biology (BCSB), Lawrence Berkeley National Laboratory, which operates Advanced Light Source beamline 5.0.3, and the staff of GM/CA at the Argonne National Laboratory, which operates Advanced Photon Source beamline ID23-B, for their support. BCSB is supported in part by the National Institutes of Health, National Institute of General Medical Sciences, and the Howard Hughes Medical Institute. The

Advanced Light Source is supported by the Director, Office of Science, Office of Basic Energy Sciences, of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231. GM/CA@APS has been funded in whole or in part with Federal funds from the National Cancer Institute (ACB-12002) and the National Institute of General Medical Sciences (AGM-12006). This research used resources of the Advanced Photon Source, a U.S. Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Argonne National Laboratory under Contract No. DE-AC02-06CH11357.

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