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Structure–activity relationship studies of 5-benzylaminoimidazo[1,2-*c*]pyrimidine-8-carboxamide derivatives as potent, highly selective ZAP-70 kinase inhibitors

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ABSTRACT

Zeta-associated protein, 70 kDa (ZAP-70), a spleen tyrosine kinase (Syk) family kinase, is normally expressed on T cells and natural killer cells and plays a crucial role in activation of the T cell immunoresponse. Thus, selective ZAP-70 inhibitors might be useful not only for treating autoimmune diseases, but also for suppressing organ transplant rejection. In our recent study on the synthesis of Syk family kinase inhibitors, we discovered that novel imidazo[1,2-*c*]pyrimidine-8-carboxamide derivatives possessed potent ZAP-70 inhibitory activity with good selectivity for ZAP-70 over other kinases. In particular, compound **26** showed excellent ZAP-70 kinase inhibition and high selectivity for ZAP-70 over structurally related Syk. The discovery of a potent, highly selective ZAP-70 inhibitor would contribute a new therapeutic tool for autoimmune diseases and organ transplant medication.

1. Introduction

Cyclosporin A **1**[1] and tacrolimus (FK506) **2**[2] are the main immunosuppressants used to prevent organ transplant rejection (Fig. 1). Both drugs, however, have severe side effects on the renal, nervous, and gastrointestinal systems.[3] Therefore, novel immunosuppressants without adverse effects are being sought for use in transplantation. Zeta-associated protein, 70 kDa (ZAP-70), a spleen tyrosine kinase (Syk) family kinase, is a non-receptor type protein tyrosine kinase.[4] ZAP-70 is expressed mainly in T cells and natural killer cells and its activation dominates downstream signal transduction and induces the mobilization of cellular calcium ions, IL-2 production, and T cell proliferation.[5,6] Therefore, ZAP-70 plays an important role in T cell signal transduction.[7,8] Arpaia et al. reported that peripheral $CD4^+$ T cells from patients who were ZAP-70-deficient exhibited markedly reduced tyrosine phosphorylation and failed to produce IL-2.[9] In addition, signal transduction through T cell receptors, such as for the mobilization of cellular calcium ions and production of IL-2, is not observed in a ZAP-70-deficient cell line.[10] Moreover, cyclosporin A **1** and tacrolimus **2** cause severe side effects on the kidney, and the major factor responsible is thought to be the inhibition of calcineurin in the kidney. However, ZAP-70 takes part in T cell activation without a pathway involving calcineurin.

Previously, we developed a Syk family kinase inhibitor for treating various allergic and autoimmune disorders.[11] However, disruption of the *Syk* gene in knockout mice leads to embryonic hemorrhage and death, in addition to the loss of immune receptor signaling. In addition, the Syk mutation impaired the differentiation of B cells by disrupting signaling from the pre-B cell antigen receptor complex and preventing the clonal expansion and mutation of pre-B cells.[12] Thus, we postulated that a selective ZAP-70 inhibitor would be useful not only for suppressing organ transplant rejection, but also for treating autoimmune diseases. Few reports exist on selective ZAP-70 inhibitors. In 1999, the ARIAD Research Group reported potent, selective SH2 inhibitors of the tyrosine kinase ZAP-70, such as compound **3** (Table 1), which had moderate ZAP-70 inhibitory activity (IC_{50} = 4.0 μM) with high selectivity over the closely structurally related Syk (IC_{50} > 500 μM).[13] The Celltech Research Group reported that 4-pyridin-5-yl-2-(3,4,5-trimethoxyphenylamino)pyrimidine derivative **4** was a potent, selective non-peptide ZAP-70 inhibitor that had excellent inhibitory activity for ZAP-70 (IC_{50} = 0.011 μM) and was highly selective for ZAP-70 over PKC, Lck, EGFr, and csk.[14] However, the selectivity of compound **4** over closely related Syk was not described. We found that imidazo[1,2-*c*]pyrimidine-8-carboxamide derivative **9** was a novel, selective ZAP-70 inhibitor in our research into protein tyrosine

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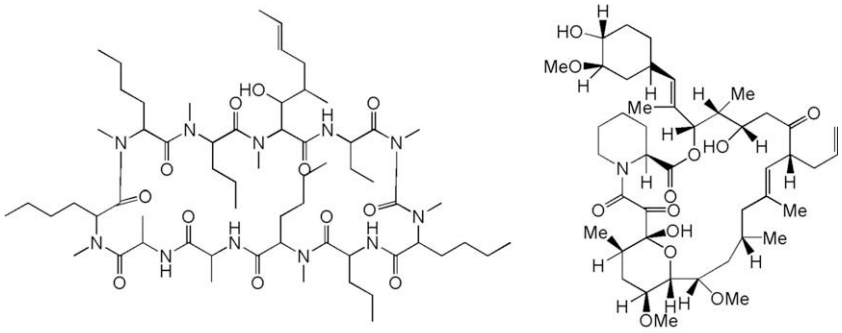
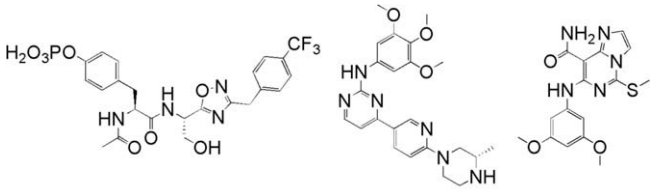


Figure 1. Structures of the immunosuppressants cyclosporin A **1** and tacrolimus (FK506) **2**.

Table 1
ZAP-70 inhibitory activity of published compounds



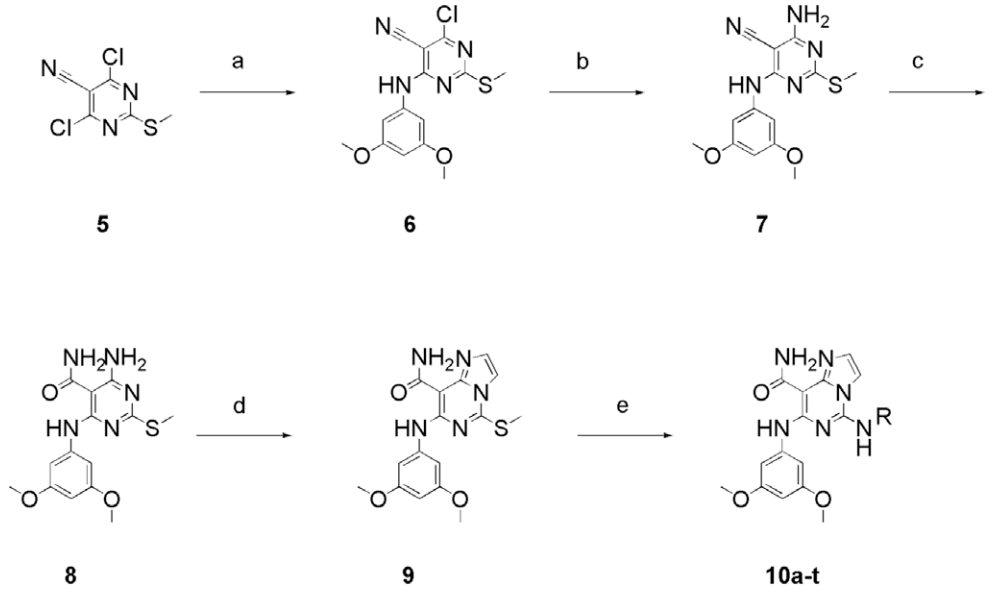
Compound	IC_{50} (μM)			
	ZAP-70	Syk	Src	Grb2
3	4.0[13]	>500[13]	282[13]	>500[13]
4	0.011[14]	ND[b]	ND[b]	ND[b]
9	3.0[a]	>10[a]	NT[c]	NT[c]

[a] IC_{50} values are for the inhibition of intracellular ZAP-70 or Syk and were determined in duplicate.
[b] ND, no data available.
[c] NT, not tested.

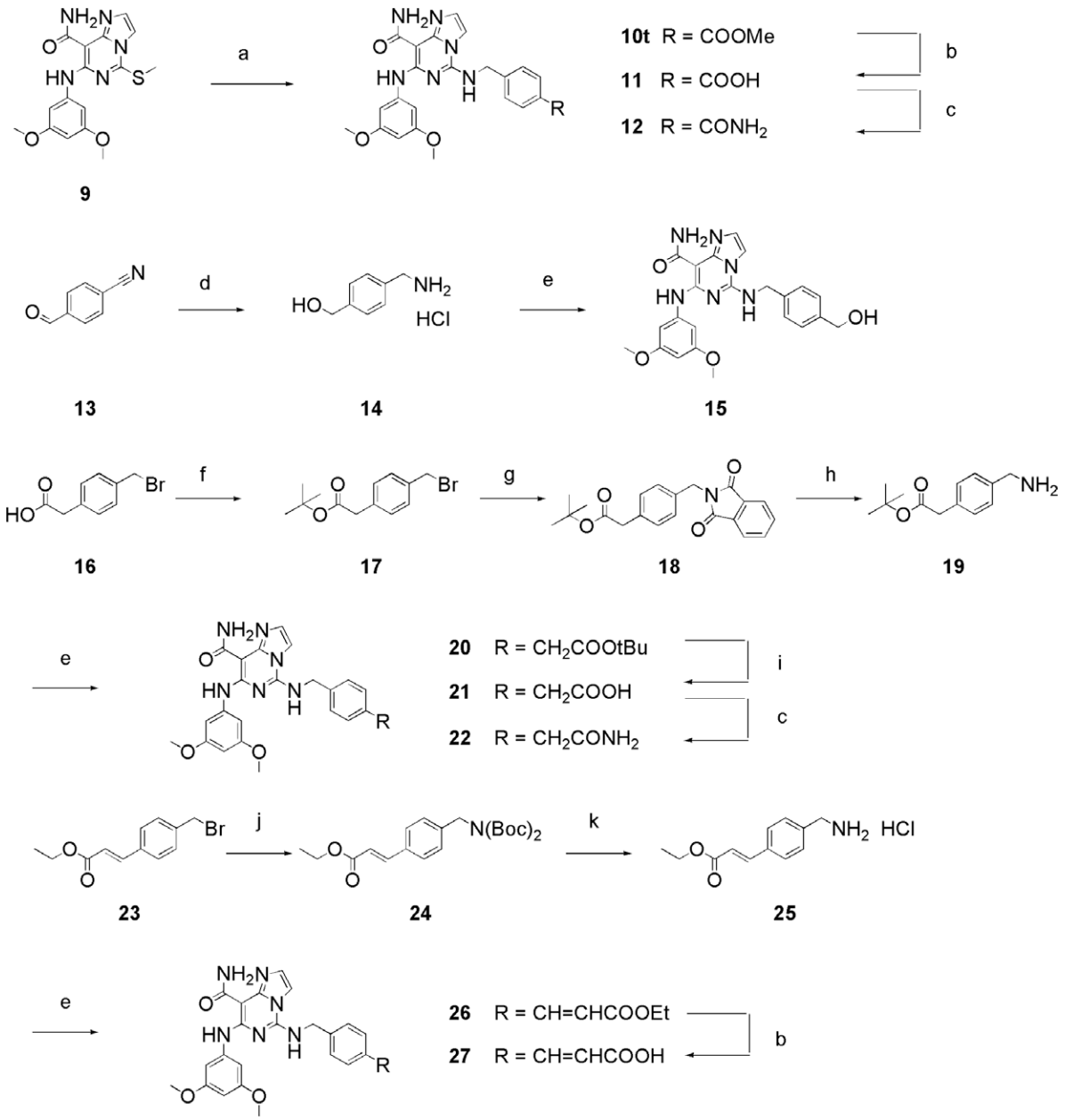
kinase inhibitors.[15] This compound moderately inhibited ZAP-70 and had less of an inhibitory effect on closely related Syk. Therefore, we decided to optimize compound **9** to find an improved selective ZAP-70 inhibitor. Here, we describe the development a potent, highly selective ZAP-70 inhibitor as a novel immunosuppressant for use in organ transplantation with no unfavorable side effects.

2. Chemistry

The general synthetic route for imidazo[1,2-*c*]pyrimidine is shown in Scheme 1. To prepare imidazo[1,2-*c*]pyrimidine derivatives, we started from commercial 4,6-dichloro-2-methylsulfanylpyrimidine-5-carbonitrile **5**. The 4-anilinopyrimidine derivative **6** was prepared by treating it with 3,5-dimethoxyaniline in tetrahydrofuran (THF) with *i*-Pr_2NEt. The reaction of compound **6** with aqueous ammonia in *N*,*N*-dimethylformamide (DMF) gave 6-aminopyrimidine derivative **7**. Hydrolysis of the nitrile group of **7** under basic conditions provided the corresponding carboxamide derivative **8**. Then, 7-phenylamino-imidazo[1,2-*c*]-pyrimidine-8-carboxamide **9** was constructed by treatment with chloroacetaldehyde in DMF under suitable thermal conditions. Finally, 5-amino-imidazo[1,2-*c*]pyrimidine-8-carboxamide derivatives **10a–t** were synthesized by substitution with the corresponding commercial amines in DMF or *N*-methyl-2-pyrrolidone (NMP). In addition, compounds **10t**, **11**, **12**, **15**, **20–22**, **26**, and **27** were synthesized following the method shown in Scheme 2. Compound **10t** was treated with 4-carbometoxybenzylamine, and hydrolysis of the methyl ester group of **10t** afforded the cor-



Scheme 1. Reagents and condition: (a) 3,5-dimethoxyaniline, *i*-Pr_2NEt, THF; (b) NH_4OH, DMF; (c) 30% H_2O_2, 5 M NaOH, DMSO, EtOH; (d) $ClCH_2CHO$, DMF; (e) commercial amines, *i*-Pr_2NEt, DMF or NMP.



Scheme 2. Reagents and condition: (a) 4-MeOOCC$_6$H$_4$CH$_2$NH$_2$, *i*-Pr$_2$NEt, NMP; (b) 2 M NaOH, EtOH; (c) CDI, THF; NH$_4$OH; (d) LiAlH$_4$, THF; 4 M HCl–EtOAc; (e) **9**, *i*-Pr$_2$NEt, NMP; (f) tBuOC(NH)CCl$_3$, BF$_3$·Et$_2$O, THF; (g) potassium phthalimide, DMF; (h) NH$_2$NH$_2$ H$_2$O, EtOH; (i) TFA, CH$_2$Cl$_2$; (j) (Boc)$_2$NH, NaH, THF; (k) 4 M HCl–EtOAc.

responding carboxylic acid **11**. Carboxamide derivative **12** was prepared by the condensation of carboxylic acid **11** and ammonia with 1,1′-carbonyldiimidazole (CDI). Compound **15** was synthesized by treatment with **14**, which was prepared from 4-cyanobenzaldehyde **13** via lithium aluminum hydride reduction. Compound **20** was synthesized from *tert*-butyl 4-aminomethylphenylacetate **19**, which was synthesized from commercial 4-bromomethylphenylacetic acid **16** in three steps. The hydrolysis of the *tert*-butyl ester group in compound **20** under acidic conditions afforded compound **21**. Compound **22** was prepared in the same manner as **12**. Compound **26** was prepared from **9** by substitution with ethyl (*E*)-4-aminomethylcinnamate **25**, which was synthesized from commercial **23** in two steps. Hydrolysis of the ethoxycarbonyl group of **26** under basic conditions afforded the corresponding carboxylic acid **27**. The chemical structures of these compounds were confirmed by ^{1}H NMR, mass spectroscopy, and element analysis.

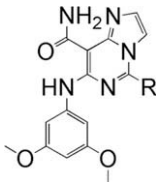
3. Results and discussion

3.1. SAR for ZAP-70 kinase and Syk kinase

The Syk and ZAP-70 inhibitory activity of our synthesized compounds were evaluated using a coupled spectrophotometric enzyme assay (Tables 1–3).

As noted previously, we discovered that the imidazo[1,2-*c*]pyrimidine-8-carboxamide derivative **9** was a ZAP-70-selective inhibitor. This compound had moderate ZAP-70 inhibition (IC_{50} = 3.0 μM) and did not inhibit closely related Syk (IC_{50} > 10 μM), as shown in Table 1. In our research on Syk family kinase inhibitors, we found out that introducing substituents at the C5 position of the imidazo[1,2-*c*]pyrimidine skeleton enhanced the ZAP-70 inhibitory effect. Therefore, we investigated the effects of substituents at C5 position of the imidazo[1,2-*c*]pyrimidine framework.

Table 2
ZAP-70 and Syk inhibitory activity of imidazo[1,2-*c*]pyrimidine derivatives substituted with various amino groups attached to the C-2 position



Compound	R	IC_{50}[a] (μM)	
		ZAP-70	Syk
9	SMe	3.0	>10
10a	NH_2	10.0	7.1
10b	$NHCH_2CH_2OH$	1.9	4.3
10c	$NHCH_2CH_2NH_2$	3.5	0.23
10d	$NHCH_2Ph$	0.40	4.3
10e	$N(Me)CH_2Ph$	5.5	13.4
10f	$NHCH_2CH_2Ph$	5.1	4.8
10g	(*R*)-NHCH(Me)Ph	0.41	>10
10h	(*S*)-NHCH(Me)Ph	0.93	3.3

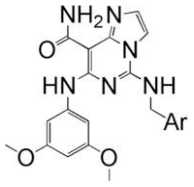
[a] The IC_{50} values were determined in duplicate.

Initially, we synthesized NH-linked derivatives by incorporating various amine units; the results are shown in Table 2. Compound **10a** had weak and similar inhibitory potency for ZAP-70 and Syk kinase. Compound **10b**, with a hydroxyethylamino group at the 5-position of the imidazo[1,2-*c*]pyrimidine core, had slightly improved ZAP-70 and Syk inhibitory activity compared with **10a**. The 5-ethylenediamino derivative **10c** had almost the same potency of ZAP-70 inhibition, but enhanced Syk inhibitory activity (IC_{50} = 3.5 μM for ZAP-70, 0.23 μM for Syk) in comparison with **10a**. Compound **10d**, which had benzylamine at the 5-position, had improved inhibition of ZAP-70 (IC_{50} = 0.40 μM) compared to compound **9** and retained good selectivity against Syk kinase (IC_{50} = 4.3 μM). The N–Me derivative **10e** had weaker inhibitory activity against ZAP-70 (IC_{50} = 5.5 μM) than compound **9**. The introduction of a phenethyl group at the C5 position (**10f**) also decreased the ZAP-70 inhibitory activity (IC_{50} = 5.1 μM). To examine the effect of substituents on the benzyl position of **10d**, we synthesized optically active 1-phenethylamine derivatives. The (*R*)-1-phenethylamine derivative **10g** had the same ZAP-70 inhibitory activity (IC_{50} = 0.41 μM) as **10d**, with extremely good selectivity against ZAP-70 over Syk (IC_{50} > 10 μM). In contrast, the (*S*)-1-phenethylamine derivative **10h** showed attenuation of both the ZAP-70 inhibitory activity and selectivity against Syk. These findings indicate that the N–H group at the 5-postion of imidazo[1,2-*c*]pyrimidine is critical for ZAP-70 inhibition. Moreover, the benzylamino group was preferable for both ZAP-70 inhibition and selectivity against Syk. Extension of the methylene group between the N–H group and the benzene ring was not favorable for ZAP-70 kinase inhibition, but had no influence on Syk inhibition. Introduction of a methyl group at the benzyl position in compound **10d** was effective for ZAP-70 inhibition. The configuration at this position influenced the ZAP-70 inhibitory activity; in particular, the *R*-isomer was favorable in terms of both ZAP-70 inhibition and selectivity against Syk.

Next, we evaluated the effects of substituents on the benzene ring in the benzylamino group, to improve of ZAP-70 inhibitory activity while retaining selectivity against Syk kinase; the results are summarized in Table 3. The 2-methoxy derivative **10l** and 3-methoxy derivative **10k** had almost the same or inferior inhibitory activity, while the 4-methoxy derivative **10j** had superior ZAP-70 inhibitory potency and selectivity against Syk kinase compared to **10d**. The 4-fluoro derivative **10i** had the same potency as the 4-methoxy derivative **10j**. Compared to compound **10d**, the 3,5-difluorobenzyl derivative **10m** had less inhibitory activity for ZAP-70, while the ZAP-70 inhibitory activity of **10n** was enhanced by introducing a 2,4-diflurobenzyl group. Regarding replacement of the phenyl group with a heteroaromatic group, the 4-pyridyl derivative **10o** and benzimidazolyl derivative **10q** showed improved ZAP-70 inhibitory activity, while the 2-furanyl derivative **10p** showed weak inhibition against ZAP-70 compared to compound **10d**. These results also indicated that substitution at the *ortho* or *para*-position affected the ZAP-70 inhibitory activity, and substitution at the *para*-position had the most promising ZAP-70 inhibitory effect. Incorporating a heteroaromatic group at the 5-position also improved the ZAP-70 kinase inhibition.

As substituents in the *para*-position were promising in terms of both the ZAP-70 inhibitory activity and selectivity against Syk kinase, we concentrated our efforts on preparing *para*-substituted benzylamino derivatives at the 5-position of the imidazo[1,2-*c*] pyrimidine skeleton. The sulfonamide **10r** and sulfone derivative **10s** showed improved ZAP-70 inhibitory activity along with good selectivity against Syk compared to **10d**. The methoxycarbonyl derivative **10t**, carboxamide derivative **12**, and 4-hydroxymethyl derivative **15** had lower ZAP-70 kinase inhibitory potency compared to **10d**. However, compound **11** incorporating a carboxyl group at the *para*-position had better ZAP-70 inhibition than compound **10d**. The inhibitory activity of *tert*-butoxycarbonylmethylphenyl derivative **20** against ZAP-70 was attenuated. The phenylacetic acid derivative **21** and phenylacetamide derivative **22** had similar or slightly improved inhibitory activity against ZAP-70 compared to **10d**. Finally, compounds **26** and **27** with an acrylate moiety in the *para*-position had superior ZAP-70 inhibitory activity compared to other modifications. Compound **26** showed good inhibitory potency for ZAP-70 (IC_{50} = 0.088 μM) and

Table 3
ZAP-70 and Syk inhibitory activity of 2-benzylaminoimidazo[1,2-*c*]pyrimidine derivatives substituted with various benzylamino groups



Compound	Ar	IC_{50}[a] (μM)	
		ZAP-70	Syk
10d	Ph	0.40	4.3
10i	4-FC_6H_4	0.22	1.9
10j	4-$MeOC_6H_4$	0.23	11.2
10k	3-$MeOC_6H_4$	0.53	6.7
10l	2-$MeOC_6H_4$	0.34	5.8
10m	3,5-Di-FC_6H_3	0.91	9.8
10n	2,4-Di-FC_6H_3	0.15	3.7
10o	4-Pyridyl	0.25	2.3
10p	2-Furanyl	0.92	2.5
10q	2-Benzimidazoyl	0.20	9.8
10r	4-$C_6H_4SO_2NH_2$	0.15	>10
10s	4-$C_6H_4SO_2Me$	0.19	11.3
10t	4-$C_6H_4CO_2Me$	0.42	4.8
11	4-$C_6H_4CO_2H$	0.18	8.1
12	4-$C_6H_4CONH_2$	0.66	8.7
15	4-$C_6H_4CH_2OH$	0.69	8.5
20	4-$C_6H_4CH_2CO_2tBu$	3.20	>10
21	4-$C_6H_4CH_2CO_2H$	0.24	0.76
22	4-$C_6H_4CH_2CONH_2$	0.48	1.9
26	(*E*)-4-$C_6H_4CH{=}CH{-}CO_2Et$	0.088	>10
27	(*E*)-4-$C_6H_4CH{=}CH{-}CO_2H$	0.047	0.69

[a] The IC_{50} values were determined in duplicate.

Table 4
Selectivity profiles of the synthesized compounds

Compound	IC_{50}[a] (μM)								
	ZAP-70	Syk	Lck	PKCβ2	EGFR	Src	ERK-1	JNK 1	p38α
10d	0.40	4.3	>25	NT[b]	NT[b]	NT[b]	NT[b]	NT[b]	NT[b]
26	0.088	>12.5	>12.5	>10	>10	>10	>10	>10	>10
27	0.047	0.69	>10	>10	89%[c]	>10	>10	90%[c]	>10

[a] The IC_{50} values were determined in duplicate.
[b] NT, not tested.
[c] % Inhibition of control values at 10^{-5} M concentration.

excellent selectivity against Syk (IC_{50} > 10 μM). Based on these findings, sulfonamide and sulfone groups are better substituents for ZAP-70 inhibition and Syk selectivity compared to other substituents. Extension of the methylene group, such as in **20**, **21**, and **22**, might be unfavorable for ZAP-70 inhibition, while the introduction of a π-electron unit, such as an acrylate group, was a desirable modification for ZAP-70 inhibitory activity.

3.2. Discussion of the kinase selectivity profiles using docking mode

The kinase selectivity profiles of representative compounds **10d**, **26**, and **27** were assayed against an extended panel of tyrosine and serine/threonine kinases; the results are summarized in Table 4. Syk and ZAP-70 were found to possess high structural homology. Nevertheless, these compounds showed excellent ZAP-70 kinase inhibition and high selectivity for ZAP-70 over Syk. We considered the reason for the enzyme selectivity profile of our synthesized compounds using a ZAP-70 homology model. We constructed a ZAP-70 homology model based on the published crystal structure of an activated Lck kinase domain[16,17] using FAMS,[18] and the binding modes of **10d**, **26**, and **27** were examined using ADAM™ software.[19] In the docking mode for compound **10d**, the N–H group and carbonyl group of the 8-carbamoyl group interacted with the carbonyl group of Glu415 and the N–H group of Ala417, respectively, and the N–H group of aniline formed a hydrogen bond with the carbonyl group of Ala417. The imidazole ring of the imidazo[1,2-*c*]pyrimidine skeleton was located close to a hydrophobic pocket, the so-called gatekeeper pocket,[20] and effectively formed a CH–π interaction[21] with Val352. The 3,5-dimethoxyphenyl group at the C7-position also created a CH–π interaction with the methylene groups of both Leu344 and Gly420. One of the motifs characterizing ZAP-70 is Pro421-Leu422-His423-Lys424, whereas in Syk, this motif is Pro455-Leu456-Asn457-Lys458.[20] Looking at the docking model of ZAP-70 and the compound **10d** complex, a distinct pocket was surrounded by Pro421, His423, Lys424, and Arg465. We were able to observe the CH–π interaction between the benzene ring of the C5-benzylamino group and Pro421, as shown in Figure 2. These hydrogen-bonded interactions and the CH–π interaction could play a crucial role in the ZAP-70 inhibitory activity. Next, we tried to determine the reason for the selectivity over Syk using the docking mode of Syk and compound **10d**. However, we could not identify any factors explaining the ZAP-70-specific inhibition over Syk. Nevertheless, we believe that interactions between ZAP-70 and compound **10d** at the characteristic Pro421-Leu422-His423-Lys424 motif in ZAP-70 contribute to the ZAP-70-specific inhibition over Syk based on the results of SARs studies. Next, we examined the docking mode between ZAP-70 and compound **26** or **27**, as shown in Figures 3 and 4, respectively. The benzene ring of the 5-benzylamino group created a CH–π interaction with Pro421, similar to **10d**; moreover, the acrylate group of compound **26** formed a CH–π interaction between the ethenyl group and methylene group in Arg465 and the CH–π interaction between ethyl group of the ester and guanidyl group of Arg465 (Fig. 3).[22] We believe that these CH–π interactions are responsible for enhancing the ZAP-70 inhibitory activity of compound **26**. For the docking mode of the ZAP-70-compound **27** complex, the carboxyl group of compound **27** produced a hydrogen bond with His423 (Fig. 4). Similarly, the carboxyl group of compound **27** produced a hydrogen bond with Asn457 in Syk (Fig. 5). Since compound **27** had enhanced inhibitory activity for both ZAP-70 and Syk, these results suggest that hydrogen-bonded interactions between the carboxyl group and His423 in ZAP-70 and Asn457 in Syk improve the kinase inhibitory activity. We believe that these findings support the docking mode between compounds **26** and **27** and ZAP-70.

Concerning the selectivity over Lck, some studies have reported that the gatekeeper pocket contributes to ligand selectivity in protein kinase.[20,23] Looking at the homology model, the gatekeeper residue in ZAP-70 is Met414, and its side chain makes a narrow gatekeeper pocket. Conversely, the gatekeeper residue in Lck is Thr316, which enlarges the gatekeeper pocket in comparison to ZAP-70. However, based on the docking mode of compound **10d**, it was difficult to explain the selectivity profile for ZAP-70 and

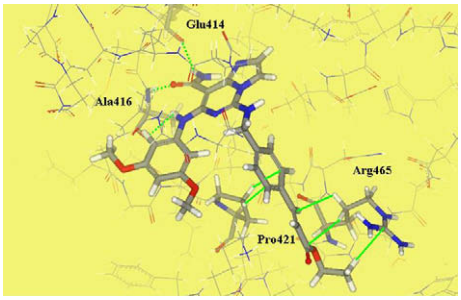


Figure 3. Docking mode of compound **26** with ZAP-70.

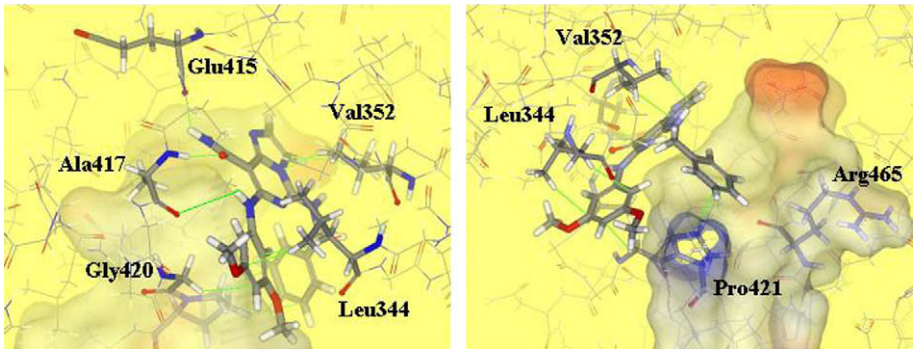


Figure 2. Docking mode of the ZAP-70 homology model and compound **10d**.

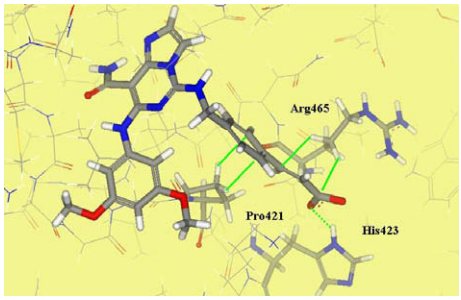


Figure 4. Docking mode of compound **27** with ZAP-70.

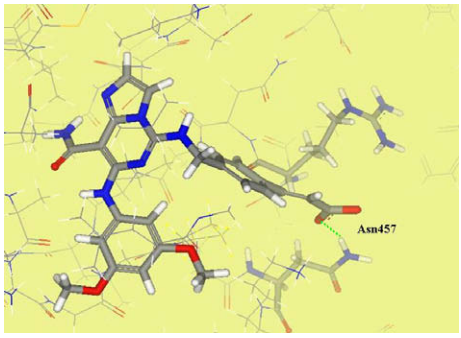


Figure 5. Docking mode of compound **27** with Syk.

Lck on this site. Looking at the docking mode of compound **10d** with Lck, no specific interaction occurred between the benzylamino residue at the 5-position of **10d** and the sugar pocket surrounding Gly322, Ser323, Asp326, and Leu371 in Lck (Fig. 6). In contrast, the benzylamino residue of **10d** made a good fit in the sugar pocket consisting of Pro421-Leu422-His423-Lys424 and Arg465 in ZAP-70. This characteristic pocket would contribute to the ZAP-70-specific inhibition of **10d** over Lck.

4. Conclusions

We synthesized various 5-benzylaminoimidazo[1,2-*c*]pyrimidine-8-carboxamide derivatives and succeeded in discovering a potent, highly selective ZAP-70 inhibitor. Compound **26** had excellent inhibitory activity for ZAP-70 (IC_{50} = 0.088 μM) and high selectivity over other tyrosine kinases, especially the structurally similar Syk kinase (IC_{50} > 10 μM). As far as we know, no other compound is reported to have excellent ZAP-70 inhibitory activity and highly selectivity for ZAP-70 kinase over Syk. We have not yet evaluated our selective ZAP-70 inhibitors in cellular or in vivo assays, and plan to investigate the in vivo efficacy of the IL-2-suppressing effect of compound **26** and its derivatives further. In addition, we will explore the reason for the specificity of the compounds we discovered for the inhibition of ZAP-70 over Syk. The results of these evaluations will be published in due course. We anticipate that compound **26** or a derivative will prove to be a new useful and effective immunosuppressant and that our discovery of a novel ZAP-70-selective inhibitor will contribute to the success of medical transplantation.

5. Experimental

5.1. Chemistry

Melting points were taken on a Yanako MP-3S Micro melting point apparatus and were uncorrected. Infrared spectra were measured on a Nicolet 510 FT-IR spectrophotometer and were reported in reciprocal centimeters. Proton NMR spectra were recorded at 400 or 500 MHz with a Brucker AMX 400 or DRX 500 instrument, and chemical shifts were reported in parts per million (δ) downfield from tetramethylsilane as internal standard. The peak patterns were shown as the following abbreviations: br, broad; d, doublet; m, multiplet; s, singlet; t, triplet; and q, quartet. The mass spectra (MS) were carried out with Thermo Quest FINNIGAN AQA electrospray ionization mass spectrometer. Elemental analyses were performed on an Elementar Vario EL analyzer (C, H, and N). Silica gel 60F254 precoated plates on glass from Merck KgaA or aminopropyl silica gel (APS) precoated NH plates from Fuji Silysia Chemical Ltd, were used for thin layer chromatography (TLC). Flash or medium-pressure liquid chromatography (MPLC) was performed on silica gel BW-350 from Fuji Silysia Chemical Ltd or APS Daisogel IR-60 (particle size 25–40 μM) from Daiso Co., Ltd. All reagents and solvent were commercially available unless otherwise indicated.

5.1.1. 4-Chloro-6-(3,5-dimethoxyphenylamino)-2-methylsulfanylpyrimidine-5-carbonitrile (6)

To a mixture of 4,6-dichloro-2-methylsulfanylpyrimidine-5-carbonitrile **5** (6.08 g, 27.6 mmol) and 3,5-dimethoxyaniline (4.32 g, 28,2 mmol) in THF (130 mL) was added diisopropylethylamine (5.05 mL, 29.0 mmol) at 0 °C and the mixture was stirred overnight at room temperature. Resulting precipitates were collected by suction filtration, washed with water and dried under reduced pressure to give 8.91 g of **6** (91%) as a pale yellowish solid: ^{1}H NMR (DMSO-d_6) δ: 2.55 (3H, s), 3.80 (6H, s), 6.33 (1H, t, J = 2.0 Hz), 6.76 (2H, d, J = 2.0 Hz), 7.15 (1H, br s); MS *m/z*: 355 $(M+H)^+$.

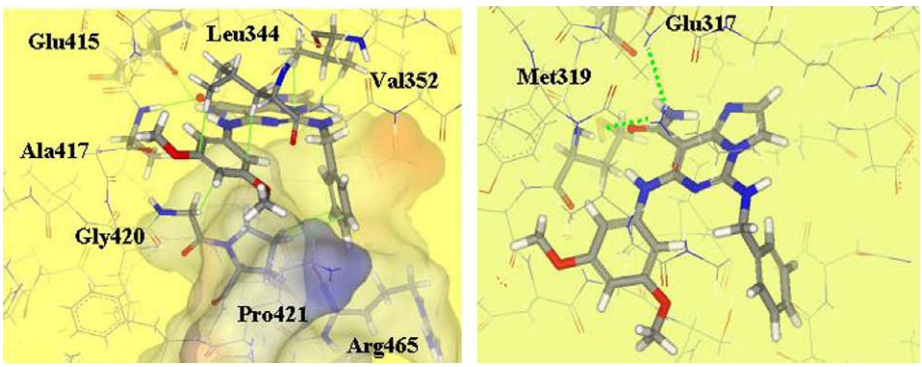


Figure 6. Docking mode of compound **10d** with ZAP-70 or Lck. The left side shows the complex of **10d** and ZAP-70. The right side shows the complex of **10d** with Lck.

5.1.2. 4-Amino-6-(3,5-dimethoxyphenylamino)-2-methylsulfanylpyrimidine-5-carbonitrile (7)

To a suspension of **6** (8.60 g, 25.5 mmol) in DMF (52 mL) was added 28% ammonia solution (14 mL) and the mixture was heated for 2 h at 60 °C. After cooled to room temperature, the mixture was poured into water. Resulting precipitates were collected by suction filtration, and washed with water to give 8.12 g of **7** (quant.) as white solid: ^{1}H NMR (DMSO-d_6) δ: 2.40 (3H, s), 3.71 (6H, s), 6.20–6.25 (1H, m), 6.85–6.90 (2H, m), 7.41 (2H, br s), 9.08 (1H,s); MS *m/z*: 318 $(M+H)^+$.

5.1.3. 4-Amino-6-(3,5-dimethoxyphenylamino)-2-methylsulfanylpyrimidine-5-carboxamide (8)

To a stirred suspension of compound **7** in DMSO (80 mL) and EtOH (80 mL) were added reagent 5 M NaOH (25.2 mL, 0.126 mol) solution and 30% H_2O_2 solution (14.3 mL, 0.126 mol) at 0 °C and the mixture was stirred for 30 min at room temperature. The mixture was added to 2 M HCl (63 mL) solution at 0 °C and resulting precipitates were collected by suction filtration and washed with water. This precipitates were suspended in EtOAc and refluxed for 1 h. After cooling to room temperature, resulting precipitates were collected by suction filtration, and washed with EtOAc to give 3.04 g of **8** (36%) as white solid: mp 232–235 °C; ^{1}H NMR (DMSO-d_6) δ: 2.44 (3H, s), 3.72 (6H, s), 6.10–6.20 (1H, m), 6.80–6.90 (4H, m), 7.54 (2H, br s), 9.87 (1H, s); MS *m/z*: 336 $(M+H)^+$. Anal. Calcd for $C_{14}H_{17}N_5O_3S{\cdot}0.5H_2O$: C, 48.83; H, 5.27; N, 20.34. Found: C, 48.63; H, 4.95; N, 20.31.

5.1.4. 7-(3,5-Dimethoxyphenylamino)-5-methylsulfanylimidazo-[1,2-*c*]pyrimidine-8-carboxamide (9)

To a stirred solution of **8** (2.90 g, 8.65 mmol) in DMF (30 mL) was added 40% chloroacetaldehyde (5.1 mL, 25.9 mmol) solution and the mixture was heated for 5 h at 60 °C. After cooling to room temperature, resulting precipitates were collected by suction filtration and washed with EtOAc to give 2.40 g of **9** (80%) as yellow solid: mp 232–233 °C (THF); ^{1}H NMR (DMSO-d_6) δ: 2.80 (3H, s), 3.76 (6H, s), 6.20–6.30 (1H, m), 6.75–6.85 (2H, m), 7.45–7.55 (1H, m), 7.70–7.75 (1H, m), 7.85–7.90 (1H, m), 9.60–9.65 (1H, br), 12.25–12.30 (1H, br); MS *m/z*: 360 $(M+H)^+$. Anal. Calcd for $C_{16}H_{17}N_5O_3S$: C, 53.47; H, 4.77; N, 19.49. Found: C, 53.45; H, 4.82; N, 19.54.

5.1.5. 5-Amino-7-(3,5-dimethoxyphenylamino)-imidazo[1,2-*c*]-pyrimidine-8-carboxamide (10a)

A mixture of **9** (0.150 g, 0.417 mmol) and 28% ammonia solution (0.5 mL) in NMP (2 mL) was heated overnight at 100 °C. The mixture was poured into water and extracted with EtOAc. The organic phase was washed with water and brine, sequentially, dried over anhydrous $MgSO_4$, and evaporated in vacuo. Resulting residue was purified by column chromatography on silica gel with hexane/EtOAc = 1:2 and recrystallized from THF to give 0.049 g of **10a** (36%) as pale green crystal: mp 274–276 °C (THF); IR (KBr) 1595, 1508, 1481, and 1150 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ: 3.76 (6H, s), 6.16 (1H, s), 6.86 (2H, s), 7.33 (1H, s), 7.35 (1H, br s), 7.79 (1H, s), 8.14 (2H, br s), 9.51 (1H, br s), 12.4 (1H, s); MS *m/z*: 329 $(M+H)^+$. Anal. Calcd for $C_{15}H_{16}N_6O_3$: C, 54.87; H, 4.91; N, 25.60. Found: C, 54.88; H, 5.09; N, 25.21.

5.1.6. 7-(3,5-Dimethoxyphenylamino)-5-(2-hydroxyethylamino)-imidazo[1,2-*c*]pyrimidine-8-carboxamide (10b)

The title compound was prepared from **9** and 2-hydroxyethylamine in the same manner as described **10a**, and obtained as white solid (13%): mp 261–263 °C (MeOH–THF); IR (KBr) 1616, 1593, 1550, 1507 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ: 3.61–3.70 (4H, m), 3.75 (6H, s), 4.90 (1H, t, *J* = 5.0 Hz), 6.16 (1H, t, *J* = 2.0 Hz), 6.86 (2H, d, *J* = 2.0 Hz), 7.34 (1H, d, *J* = 1.5 Hz), 7.41 (1H, d, *J* = 3.0 Hz), 7.93 (1H, d, *J* = 1.5 Hz), 8.46 (1H, s), 9.55 (1H, d, *J* = 3.0 Hz), 12.52 (1H, s); MS *m/z*: 373 $(M+H)^+$. Anal. Calcd for: $C_{17}H_{20}N_6O_4$: C, 54.83; H, 5.41; N, 22.57. Found: C, 54.56; H, 5.43; N, 22.43.

5.1.7. 5-(2-Aminoethylamino)-7-(3,5-dimethoxyphenylamino)-imidazo[1,2-*c*]pyrimidine-8-carboxamide (10c)

The title compound was prepared from **9** and 2-aminoethylamine in the same manner as described **10a**, and obtained as white solid (46%): mp 221–223 °C (MeOH–THF); IR (KBr) 1668, 1595, 1502, 1478, 1372 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ: 2.85 (2H, t, *J* = 6.0 Hz), 3.25–3.45 (3H, br), 3.56 (2H, t, *J* = 6.0 Hz), 3.76 (6H, s), 6.17 (1H, t, *J* = 2.0 Hz), 6.89 (2H, d, *J* = 2.0 Hz), 7.34 (1H, d, *J* = 1.5 Hz), 7.41 (1H, d, J = 3.0 Hz), 7.90 (1H, d, *J* = 1.5 Hz), 9.54 (1H, d, *J* = 3.0 Hz), 12.51 (1H, s); MS *m/z*: 372 $(M+H)^+$. Anal. Calcd for: $C_{17}H_{21}N_7O_3{\cdot}C_5H_9NO$: C, 56.16; H, 6.43; N, 23.81. Found: C, 55.57; H, 6.35; N, 23.66.

5.1.8. 5-Benzylamino-7-(3,5-dimethoxyphenylamino)-imidazo-[1,2-*c*]pyrimidine-8-carboxamide (10d)

To a suspension of compound **9** (0.120 g, 0.334 mmol) in NMP (1.2 mL) was added benzylamine (0.182 mL, 1.67 mmol) and the mixture was heated for 3 h at 90 °C. Water was added and extracted with EtOAc. Organic phase was washed with 1 M HCl solution, water, and brine, successively; dried over $MgSO_4$, filtered; and concentrated in vacuo. Resulting residue was purified by column chromatography on silica gel with hexane/EtOAc = 1:2 and recrystallized from EtOH–EtOAc to give 0.053 g of **10d** (38%) as pale yellowish solid: mp 244–246 °C (EtOH–EtOAc); IR (KBr) 1599, 1554, 1502, 1480, 1154 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ: 3.62 (6H, s), 4.83 (2H, s), 6.14 (1H, t, *J* = 2.2 Hz), 6.81 (2H, d, *J* = 2.2 Hz), 7.20–7.45 (7H, m), 7.90–7.95 (1H, m), 8.90–9.00 (1H, br s), 9.50–9.60 (1H, m), 12.48 (1H, br s); MS *m/z*: 419 $(M+H)^+$. Anal. Calcd for $C_{22}H_{22}N_6O_3$: C, 63.15; H, 5.30; N, 20.08. Found: C, 63.17; H, 5.30; N, 20.02.

5.1.9. 5-(*N*-Benzylmethylamino)-7-(3,5-dimethoxyphenylamino)-imidazo[1,2-*c*]pyrimidine-8-carboxamide (10e)

The title compound was prepared from **9** and *N*-benzylmethylamine in the same manner as described **10d**, and obtained as pale yellowish crystal (38%): mp 215–216 °C (EtOH–EtOAc); IR (KBr) 1594, 1505, 1410, 1155 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ: 3.21 (3H, s), 3.67 (6H, s), 4.91 (2H, s), 6.15 (1H, t, *J* = 2.0 Hz), 6.79 (2H, d, *J* = 2.0 Hz), 7.28–7.42 (6H, m), 7.55 (1H, d, *J* = 3.0 Hz), 7.62 (1H, d, *J* = 2.0 Hz), 9.69 (1H, d, *J* = 3.0 Hz), 12.42 (1H, br s); MS *m/z*: 433 $(M+H)^+$. Anal. Calcd for $C_{23}H_{24}N_6O_3$: C, 63.88; H, 5.59; N, 19.43. Found: C, 63.71; H, 5.51; N, 19.51.

5.1.10. 7-(3,5-Dimethoxyphenylamino)-5-phenethylamino-imidazo[1,2-*c*]pyrimidine-8-carboxamide (10f)

The title compound was prepared from **9** and phenethylamine in the same manner as described **10d**, and obtained as pale yellowish crystal as yellowish solid (27%): mp 158–160 °C (THF); IR (KBr) 1599, 1558, 1500, 1150 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ: 2.97 (2H, t, *J* = 7.0 Hz), 3.67 (6H, s), 3.72–3.80 (2H, m), 6.19 (1H, t, *J* = 2.0 Hz), 6.85 (2H, d, *J* = 2.0 Hz), 7.20–7.32 (5H, m), 7.35 (1H, d, *J* = 2.5 Hz), 7.43 (1H, d, *J* = 3.5 Hz), 7.86 (1H, d, *J* = 2.5 Hz), 8.54 (1H, t, *J* = 5.0 Hz), 9.53 (1H, d, *J* = 3.5 Hz), 12.48 (1H, br s); MS *m/z*: 433 $(M+H)^+$. Anal. Calcd for $C_{23}H_{24}N_6O_3{\cdot}C_5H_9NO$: C, 63.26; H, 6.26; N, 18.44. Found: C, 63.19; H, 6.28; N, 18.32.

5.1.11. 7-(3,5-Dimethoxyphenylamino)-5-((*R*)-1-phenylethylamino)imidazo[1,2-*c*]pyrimidine-8-carboxamide (10g)

The title compound was prepared from **9** and (*R*)-phenylethylamine in the same manner as described **10d**, and obtained as pale yellowish crystal as pale yellowish solid (24%): mp 99–101 °C (EtOAc); $[\alpha]_D^{28}$ +178.0° (*c* 0.30, MeOH); IR (KBr) 1599, 1558, 1497, 1457, 1151 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ: 1.63 (3H, d, *J* = 7.0 Hz),

3.73 (6H, br s), 5.50 (1H, quint, J = 7.0 Hz), 6.22 (1H, t, J = 2.0 Hz), 6.79 (2H, d, J = 2.0 Hz), 7.21–7.35 (3H, m), 7.38 (1H, d, J = 2.0 Hz), 7.40–7.50 (3H, m), 8.09 (1H, d, J = 2.0 Hz), 8.64 (1H, d, J = 2.0 Hz), 9.55 (1H, br s), 12.43 (1H, s); MS m/z: 433 $(M+H)^+$. Anal. Calcd for $C_{23}H_{24}N_6O_3{\cdot}0.36H_2O$: C, 62.93; H, 5.67; N, 19.14. Found: C, 63.38; H, 5.67; N, 18.64.

5.1.12. 7-(3,5-Dimethoxyphenylamino)-5-((*S*)-1-phenylethylamino)imidazo[1,2-*c*]pyrimidine-8-carboxamide (10h)

The title compound was prepared from **9** and (*S*)-phenylethylamine in the same manner as described for **10d**, and obtained as pale yellowish crystal as pale yellowish solid (18%): mp 99–101 °C (EtOAc); $[\alpha]_D^{28}$ −185.2° (c 0.31, MeOH); IR (KBr) 1599, 1559, 1497, 1457, 1152 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 1.63 (3H, d, J = 7.0 Hz), 3.73 (6H, br s), 5.49 (1H, quint, J = 7.0 Hz), 6.21 (1H, t, J = 2.0 Hz), 6.78 (2H, d, J = 2.0 Hz), 7.21-7.35 (3H, m), 7.37 (1H, d, J = 2.0 Hz), 7.40–7.50 (3H, m), 8.08 (1H, d, J = 2.0 Hz), 8.64 (1H, d, J = 2.0 Hz), 9.54 (1H, br s), 12.42 (1H, s); MS m/z: 433 $(M+H)^+$. Anal. Calcd for $C_{23}H_{24}N_6O_3{\cdot}0.5H_2O$: C, 62.57; H, 5.71; N, 19.03. Found: C, 63.01; H, 5.66; N, 18.66.

5.1.13. 7-(3,5-Dimethoxyphenylamino)-5-(4-fluorobenzylamino)imidazo[1,2-*c*]pyrimidine-8-carboxamide (10i)

The title compound was prepared from **9** and 4-fluorobenzylamine in the same manner as described for **10d**, and obtained as pale yellowish crystal as pale yellowish solid (30%): mp 255–257 °C (MeOH: EtOAc); IR (KBr) 1601, 1558, 1502, 1158 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 3.62 (6H, s), 4.80 (1H, d, J = 5.0 Hz), 6.15 (1H, t, J = 2.0 Hz), 6.79 (2H, d, J = 2.0 Hz), 7.12–7.19 (2H, m), 7.37 (2H, d, J = 1.5 Hz), 7.41–7.47 (3H, m), 7.90 (1H, d, J = 1.5 Hz), 7.96 (1H, t, J = 4.5 Hz), 9.55 (1H, d, J = 3.5 Hz), 12.48 (1H, s); MS m/z: 433 $(M+H)^+$. Anal. Calcd for $C_{22}H_{21}FN_6O_3$: C, 60.54; H, 4.85; N, 19.26. Found: C, 60.41; H, 4.79; N, 19.23.

5.1.14. 7-(3,5-Dimethoxyphenylamino)-5-(4-methoxybenzylamino)imidazo[1,2-*c*]pyrimidine-8-carboxamide (10j)

The title compound was prepared from **9** and 4-methoxybenzyamine in the same manner as described for **10d**, and obtained as pale yellowish crystal as pale yellowish solid (30%): mp 203–204 °C (THF); IR (KBr) 1599, 1559, 1499, 1153 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 3.65 (6H, s), 3.72 (3H, s), 4.75 (2H, d, J = 4.0 Hz), 6.15 (1H, t, J = 2.0 Hz), 6.84 (2H, d, J = 2.0 Hz), 6.88 (2H, d, J = 9.0 Hz), 7.32 (2H, d, J = 9.0 Hz), 7.36 (1H, d, J = 2.0 Hz), 7.41-7.46 (1H, m), 7.91 (1H, d, J = 2.0 Hz), 8.86–8.91 (1H, br), 9.52–9.57 (1H, m), 12.50 (1H, s); MS m/z: 449 $(M+H)^+$. Anal. Calcd for $C_{23}H_{24}N_6O_4$: C, 61.60; H, 5.39; N, 18.74. Found: C, 61.48; H, 5.40; N, 18.69.

5.1.15. 7-(3,5-Dimethoxyphenylamino)-5-(3-methoxybenzylamino)imidazo[1,2-*c*]pyrimidine-8-carboxamide (10k)

The title compound was prepared from **9** and 3-methoxybenzylamine in the same manner as described for **10d**, and obtained as pale yellowish crystal as pale yellowish solid (33%): mp 216–218 °C (THF); IR (KBr) 1599, 1559, 1492, 1152 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 3.64 (6H, s), 3.67 (3H, s), 4.79 (2H, d, J = 4.5 Hz), 6.15 (1H, t, J = 2.0 Hz), 6.83 (2H, d, J = 2.0 Hz), 6.95–6.98 (2H, m), 7.24 (2H, t, J = 7.5 Hz), 7.37 (1H, d, J = 2.0 Hz), 7.42–7.47 (1H, m), 7.92 (1H, d, J = 2.0 Hz), 8.92–8.98 (1H, br), 9.53–9.58 (1H, m), 12.50 (1H, s); MS m/z: 449 $(M+H)^+$. Anal. Calcd for $C_{23}H_{24}N_6O_4{\cdot}0.2H_2O$: C, 61.11; H, 5.44; N, 18.59. Found: C, 61.14; H, 5.37; N, 18.63.

5.1.16. 7-(3,5-Dimethoxyphenylamino)-5-(2-methoxybenzylamino)imidazo[1,2-*c*]pyrimidine-8-carboxamide (10l)

The title compound was prepared from **9** and 2-methoxybenzylamine in the same manner as described for **10d**, and obtained as pale yellowish solid (33%): mp 246–248 °C (MeOH–Toluene); IR (KBr) 1598, 1558, 1492, 1147 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 3.62 (6H, s), 3.82 (3H, s), 4.79 (2H, s), 6.11 (1H, t, J = 2.0 Hz), 6.77 (2H, d, J = 2.0 Hz), 6.89 (1H, t, J = 7.0 Hz), 7.24 (1H, d, J = 8.0 Hz), 7.20-7.30 (2H, m), 7.37 (1H, d, J = 2.0 Hz), 7.44 (1H, br s), 7.96 (1H, s), 8.73 (1H, br s), 9.56 (1H br s), 12.51 (1H, s); MS m/z: 449 $(M+H)^+$. Anal. Calcd for $C_{23}H_{24}N_6O_4$: C, 61.60; H, 5.39; N, 18.74. Found: C, 61.38; H, 5.29; N, 18.54.

5.1.17. 5-(3,5-Difluorobenzylamino)-7-(3,5-dimethoxyphenylamino)imidazo[1,2-*c*]pyrimidine-8-carboxamide (10m)

The title compound was prepared from **9** and 3,5-difluorobenzylamine in the same manner as described **10d**, and obtained as pale yellowish solid (35%): mp 252–253 °C (MeOH– EtOAc); IR (KBr) 1599, 1505, 1456, 1149 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 3.66 (6H, s), 4.76–4.82 (2H, br), 6.15 (1H, t, J = 2.0 Hz), 6.72 (2H, d, J = 2.0 Hz), 7.06–7.15 (3H, m), 7.36–7.39 (1H, br), 7.43–7.48 (1H, m), 7.86–7.89 (1H, br), 8.96–9.03 (1H, br), 9.52–9.58 (1H, m), 12.43 (1H, s); MS m/z: 455 $(M+H)^+$. Anal. Calcd for $C_{22}H_{20}F_2N_6O_3{\cdot}0.1H_2O$: C, 57.91; H, 4.46; N, 18.42. Found: C, 57.56; H, 4.36; N, 18.35.

5.1.18. 5-(2,4-Difluorobenzylamino)-7-(3,5-dimethoxyphenylamino)imidazo[1,2-*c*]pyrimidine-8-carboxamide (10n)

The title compound was prepared from **9** and 2,4-difluorobenzylamine in the same manner as described for **10d**, and obtained as pale yellowish solid (35%): mp 242–243 °C (MeOH–EtOAc); IR (KBr) 1602, 1558, 1505, 1158 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 3.66 (6H, s), 4.79–4.85 (2H, br), 6.15 (1H, t, J = 2.0 Hz), 6.74 (2H, d, J = 2.0 Hz), 7.02 (1H, dt, J = 2.0, 9.0 Hz), 7.28 (1H, dt, J = 2.0, 9.0 Hz), 7.37 (1H, d, J = 2.0 Hz), 7.42–7.50 (2H, m), 7.92 (1H, d, J = 2.0 Hz), 8.84–8.89 (1H, br), 9.53–9.57 (1H, m), 12.43 (1H, s); MS m/z: 455 $(M+H)^+$. Anal. Calcd for $C_{22}H_{20}F_2N_6O_3{\cdot}0.1H_2O$: C, 57.91; H, 4.46; N, 18.42. Found: C, 57.92; H, 4.38; N, 18.65.

5.1.19. 7-(3,5-Dimethoxyphenylamino)-5-[(pyridin-4-ylmethyl)amino]imidazo[1,2-*c*]pyrimidine-8-carboxamide (10o)

The title compound was prepared from **9** and pyridin-4-ylmethylamine in the same manner as described for **10d**, and obtained as white solid (7.7%): mp 255–256 °C (MeOH– EtOAc); IR (KBr) 1600, 1551, 1505, 1153 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 3.63 (6H, s), 4.80–4.85 (2H, br), 6.13 (1H, t, J = 2.0 Hz), 6.69 (2H, d, J = 2.0 Hz), 7.38 (2H, d, J = 6.0 Hz), 7.39 (1H, d, J = 2.0 Hz), 7.44–7.47 (1H, br), 7.90 (1H, d, J = 2.0 Hz), 8.50 (2H, d, J = 6.0 Hz), 9.03–9.07 (1H, br), 9.54 (1H, d, J = 2.0 Hz), 12.44 (1H, s); MS m/z: 420 $(M+H)^+$. Anal. Calcd for $C_{21}H_{21}N_7O_3{\cdot}0.5H_2O$: C, 58.87; H, 5.17; N, 22.88. Found: C, 58.97; H, 4.98; N, 22.94.

5.1.20. 7-(3,5-Dimethoxyphenylamino)-5-[(furan-2-ylmethyl)amino]imidazo[1,2-*c*]pyrimidine-8-carboxamide (10p)

The title compound was prepared from **9** and furan-2-ylmethylamine in the same manner as described for **10d**, and obtained as pale green solid (28%): mp 232–233 °C (MeOH–EtOAc); IR (KBr) 1600, 1550, 1501, 1149 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 3.69 (6H, s), 4.77–4.83 (2H, br), 6.18 (1H, t, J = 2.0 Hz), 6.41(2H, d, J = 2.0 Hz), 6.86 (2H, d, J = 6.0 Hz), 7.36 (1H, d, J = 2.0 Hz), 7.46 (1H, d, J = 3.0 Hz), 7.62 (1H, t, J = 2.0 Hz), 7.90 (1H, d, J = 2.0 Hz), 8.92–8.98 (1H, br), 9.54 (1H, d, J = 3.0 Hz), 12.51 (1H, s); MS m/z: 409 $(M+H)^+$. Anal. Calcd for $C_{20}H_{20}N_6O_4$: C, 58.82; H, 4.94; N, 20.58. Found: C, 58.61; H, 4.86; N, 20.58.

5.1.21. 5-[(1*H*-Benzoimidazol-2-ylmethyl)amino]-7-(3,5-dimethoxyphenylamino)imidazo[1,2-*c*]pyrimidine-8-carboxamide (10q)

The title compound was prepared from **9** and 1*H*-benzoimidazol-2-ylmethylamine in the same manner as described for **10d**, and obtained as pale green solid (7.8%): mp 265 °C (dec) (MeOH–EtOAc); IR (KBr) 1609, 1559, 1506, 1458, 1155 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 3.53 (6H, s), 5.08 (2H, d, J = 5.0 Hz), 6.07 (1H, t, J = 2.0 Hz), 6.73

(2H, d, J = 2.0 Hz), 7.12–7.18 (2H, m), 7.39-7.42 (2H, m), 7.49 (1H, d, J = 3.0 Hz), 7.56–7.58 (1H, m), 7.91 (1H, t, J = 2.0 Hz), 9.11 (1H, t, J = 5.0 Hz), 9.59 (1H, d, J = 3.0 Hz), 12.38 (1H, s), 12.50 (1H, s); MS m/z: 459 $(M+H)^+$. Anal. Calcd for $C_{23}H_{22}N_8O_3$: C, 60.25; H, 4.84; N, 24.44. Found: C, 59.92; H, 4.79; N, 24.50.

5.1.22. 7-(3,5-Dimethoxyphenylamino)-5-(4-sulfamoylbenzylamino)imidazo[1,2-*c*]pyrimidine-8-carboxamide (10r)

The title compound was prepared from **9** and 4-sulfamoylbenzylamine in the same manner as described for **10d**, and obtained as pale green solid (45%): mp 265–267 °C (MeOH–THF); IR (KBr) 1624, 1590, 1540, 1151 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 3.63 (6H, s), 4.87 (2H, br s), 6.14 (1H, t, J = 2.0 Hz), 6.76 (2H, d, J = 2.0 Hz), 7.32 (2H, br s), 7.37–7.39 (1H, br s), 7.56 (2H, d, J = 8.0 Hz), 7.76 (2H, d, J = 8.0 Hz), 7.89–7.92 (1H, br s), 9.01–9.07 (1H, br), 9.53–9.57 (1H, m), 12.38 (1H, s), 12.47 (1H, s); MS m/z: 498 $(M+H)^+$. Anal. Calcd for $C_{22}H_{23}N_7O_5S{\cdot}1.0H_2O$: C, 51.25; H, 4.89; N, 19.02. Found: C, 51.58; H, 4.58; N, 18.95.

5.1.23. 7-(3,5-Dimethoxyphenylamino)-5-(4-methanesulfonylbenzylamino)imidazo[1,2-*c*]pyrimidine-8-carboxamide (10s)

The title compound was prepared from **9** and 4-methanesulfonylbenzylamine in the same manner as described for **10d**, and obtained as pale green solid (54%): mp 266–268 °C (MeOH–THF); IR (KBr) 1604, 1499, 1480, 1145 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 3.18 (3H, s), 3.63 (6H, s), 4.90 (2H, br s), 6.15 (1H, t, J = 2.0 Hz), 6.72 (2H, d, J = 2.0 Hz), 7.39 (1H, d, J = 1.5 Hz), 7.46 (1H, d, J = 3.5 Hz), 7.65 (2H, d, J = 8.5 Hz), 7.88 (2H, d, J = 8.5 Hz), 7.90 (1H, d, J = 1.5 Hz), 9.05–9.09 (1H, br), 9.54 (1H, d, J = 3.5 Hz), 12.45(1H, s); MS m/z: 497 $(M+H)^+$. Anal. Calcd for $C_{23}H_{24}N_6O_5S{\cdot}0.2H_2O$: C, 55.23; H, 4.84; N, 16.63. Found: C, 55.02; H, 4.84; N, 16.65.

5.1.24. Methyl 4-{[8-Carbamoyl-7-(3,5-dimethoxyphenylamino)-imidazo[1,2-*c*]pyrimidin-5-ylamino]-methyl}benzoate (10t)

The title compound was prepared from **9** and methyl 4-aminomethylbenzoate in the same manner as described for **10d**, and obtained as pale green solid (42%): mp 234–235 °C (MeOH–EtOAc); IR (KBr) 1600, 1599, 1506, 1283, 1147 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 3.62 (6H, s), 3.83 (3H, s), 4.88 (2H, br s), 6.14 (1H, t, J = 2.0 Hz), 6.73 (2H, d, J = 2.0 Hz), 7.38 (1H, d, J = 2.0 Hz), 7.45 (1H, d, J = 3.5 Hz), 7.53 (2H, d, J = 8.5 Hz), 7.90–7.94 (3H, m), 9.04–9.08 (1H, br), 9.54 (1H, d, J = 3.5 Hz), 12.45 (1H, s); MS m/z: 477 $(M+H)^+$. Anal. Calcd for $C_{24}H_{24}N_6O_5{\cdot}0.5H_2O$: C, 59.37; H, 5.19; N, 17.31. Found: C, 59.73; H, 5.10; N, 17.53.

5.1.25. 4-{[8-Carbamoyl-7-(3,5-dimethoxyphenylamino)-imidazo[1,2-*c*]pyrimidin-5-ylamino]methyl}benzoic acid (11)

To a mixture of **10t** (0.066 g, 0.139 mmol) in MeOH (2 mL) and THF (2 mL) was added 2 M NaOH solution (0.695 mL, 1.39 mmol) and stirred overnight at room temperature at 60 °C. To the mixture was added 2 M HCl solution (0.70 mL) at 0 °C, resulting precipitates were collected by suction filtration, and washed with water. The precipitates were recrystallized from MeOH–THF to give 0.042 g of **11** (66%) as flesh colored solid: mp 289 °C (dec) (MeOH–THF); IR (KBr) 1599, 1544, 1502, 1151 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 3.62 (6H, s), 4.88 (2H, d, J = 5.0 Hz), 6.14 (1H, br s), 6.74 (2H, d, J = 2.0 Hz), 7.39 (1H, s), 7.46 (1H, br s), 7.50 (2H, d, J = 8.0 Hz), 7.89 (2H, d, J = 8.0 Hz), 7.92 (1H, br s), 9.06 (1H, t, J = 5.0 Hz), 9.55 (1H, br s), 12.46 (1H, s), 12.92 (1H, br s); MS m/z: 463 $(M+H)^+$. Anal. Calcd for $C_{23}H_{22}N_6O_5{\cdot}0.4H_2O$: C, 58.81; H, 4.89; N, 17.89. Found: C, 58.80; H, 4.81; N, 17.53

5.1.26. 5-(4-Carbamoylbenzylamino)-7-(3,5-dimethoxyphenylamino)imidazo[1,2-*c*]pyrimidine-8-carboxmide (12)

A mixture of **11** (0.030 g, 0.0649 mmol) and 1,1′-carbonyldiimidazole (0.011 g, 0.0703 mmol) in THF (1 mL) was stirred overnight at room temperature. To the mixture was added 28% ammonia solution (0.3 mL) and stirred for 4 h at room temperature. Volatiles were evaporated in vacuo and resulting residue was purified by column chromatography on silica gel with EtOH/EtOAc = 1:30 to give 0.017 g of **12** (57%) as pale yellowish solid: mp 275 °C (dec); IR (KBr) 1603, 1570, 1559, 1506, 1150 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 3.63 (6H, s), 4.86 (2H, d, J = 6.0 Hz), 6.14 (1H, t, J = 2.0 Hz), 6.78 (2H, d, J = 2.0 Hz), 7.33, (1H, br s), 7.38 (1H, d, J = 1.5 Hz), 7.43–7.48 (3H, m), 7.82 (2H, d, J = 8.5 Hz), 7.92 (1H, d, J = 1.5 Hz), 7.94 (1H, br s), 9.02 (1H, t, J = 6.0 Hz), 9.54–9.57 (1H, m), 12.49 (1H, s); MS m/z: 472 $(M+H)^+$. Anal. Calcd for $C_{23}H_{23}N_7O_4{\cdot}0.2H_2O$: C, 59.40; H, 5.07; N, 21.08. Found: C, 59.49; H, 5.11; N, 20.74.

5.1.27. (4-Aminomethyl)benzyl alcohol hydrochloride (14)

To a suspension of lithium aluminum hydride (0.399 g, 10.5 mmol) in THF (5 mL) was added 4-Formylbenzonitrile **13** (0.918 g, 7.00 mmol) in THF at 0 °C and stirred for 2 h at 60 °C. To the mixture water (0.4 mL), 2 M NaOH (0.4 mL) and water (1.2 mL) were added, successively, and resulting precipitates were filtered off. The filtrate was dried over Na_2SO_4, and evaporated in vacuo. After being cooled at 0 °C, 4 M HCl in EtOAc (1.75 mL, 1 equiv) was added to the mixture. After being stirred for 15 min, resulting precipitates were collected by suction filtration, and dried under reduced pressure to give 0.914 g of 4-aminomethylbenzylalcohol hydrochloride **14** (75%) as pale yellowish solid: ^1H NMR (DMSO-d_6) δ: 3.95 (2H, s), 4.50 (2H, s), 5.23 (1H, br s), 7.33 (2H, t, J = 8.0 Hz), 7.39 (2H, d, J = 8.0 Hz), 7.50–8.30 (3H, br).

5.1.28. 7-(3,5-Dimethoxyphenylamino)-5-(4-hydroxymethylbenzylamino)imidazo[1,2-*c*]pyrimidine-8-carboxamide (15)

The title compound was prepared from **9** and **14** in the same manner as described **10d**, and obtained as yellowish solid (58%): mp 209–211 °C (MeOH–THF); IR (KBr) 3393, 1595, 1558, 1506, 1147 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 3.64 (6H, s), 4.46 (2H, d, J = 5.5 Hz), 4.80 (2H, d, J = 5.5 Hz), 5.14 (1H, t, J = 5.5 Hz), 6.14 (1H, t, J = 2.0 Hz), 6.83 (2H, d, J = 2.0 Hz), 7.26 (2H, d, J = 8.0 Hz), 7.33–7.38 (3H, m), 7.40–7.46 (1H, m), 7.92 (1H, br s), 8.96 (1H, t, J = 5.5 Hz), 9.52–9.56 (1H, m), 12.50 (1H, s); MS m/z: 449 $(M+H)^+$. Anal. Calcd for $C_{23}H_{24}N_6O_4{\cdot}1.0H_2O$: C, 59.22; H, 5.62; N, 18.02. Found: C, 59.04; H, 5.55; N, 17.81.

5.1.29. *tert*-Butyl 4-bromomethylphenylacetate (17)

To a mixture of 4-bromomethylphenylacetic acid **16** (1.37 g, 6.00 mmol) and *tert*-butyl 2,2,2-trichloroacetimidate (2.15 mL, 12.00 mmol) in THF (20 mL) was added boron trifluoride etherate (1.52 mL, 12.00 mmol) at room temperature and the mixture was stirred overnight at room temperature. Saturated $NaHCO_3$ solution was added to the mixture and extracted with EtOAc. The organic phase was washed with brine, dried over anhydrous $MgSO_4$, and evaporated in vacuo. Resulting residue was purified by column chromatography on silica gel with hexane/EtOAc = 5:1 to give **17** as colorless oil: ^1H NMR ($CDCl_3$) δ:1.44 (9H, s), 3.52 (2H, s), 4.49 (2H, s), 7.24 (2H, d, J = 8.0 Hz), 7.34 (2H, d, J = 8.0 Hz).

5.1.30. *tert*-Butyl [4-(1,3-Dioxo-1,3-dihydroisoindol-2-ylmethyl)phenyl]acetate (18)

A mixture of *tert*-butyl 4-bromomethylphenylacetate **17** (1.43 g, 5.01 mmol) and phthalimide potassium salt (1.02 g, 5.51 mmol) in DMF (15 mL) was stirred overnight at room temperature. Water was added to the mixture and extracted with EtOAc. The orgainc phase was washed with water and brine, sequentially, dried over anhydrous $MgSO_4$, and evaporated in vacuo. Resulting residue was purified by column chromatography on silica gel with hexane/EtOAc = 4:1 to give 1.31 g of **18** (74%) as white solid: ^1H NMR ($CDCl_3$) δ: 1.42 (9H, s), 3.48 (2H, s), 4.82 (2H, s), 7.21 (2H, d, J = 8.5 Hz), 7.39 (2H, d, J = 8.5 Hz), 7.65–7.73 (2H, m), 7.82–7.87 (2H, m).

5.1.31. *tert*-Butyl (4-{[8-Carbamoyl-7-(3,5-dimethoxyphenylamino)imidazo[1,2-*c*]pyrimidin-5-ylamino]methyl}phenyl)-acetate (20)

A mixture of **18** (1.25 g, 3.56 mmol) and hydrazine monohydrate (0.518 mL, 10.67 mmol) in EtOH (20 mL) was refluxed overnight. After being cooled to room temperature, resulting precipitates were filtered off, and the filtrate was concentrated in vacuo. To the residue was added EtOAc and resulting precipitates were filtered off. The filtrate was evaporated in vacuo to give crude **19** as yellowish oil. The title compound was prepared from **9** and crude **19** in the same manner as described for **10d**, and obtained as yellowish solid (75%): mp 178–180 °C (EtOAc); IR (KBr) cm^{-1}; ^1H NMR (DMSO-d_6) δ: 1.37 (9H, s), 3.51 (2H, s), 3.60 (6H, s), 4.80 (2H, d, J = 4.5 Hz), 6.13 (1H, t, J = 2.0 Hz), 6.80 (2H, d, J = 2.0 Hz), 7.20 (2H, d, J = 8.5 Hz), 7.34 (2H, d, J = 8.5 Hz), 7.36 (1H, d, J = 1.5 Hz), 7.43–7.46 (1H, m), 7.91 (1H, d, J = 1.5 Hz), 8.96 (1H, t, J = 4.5 Hz), 9.53–9.57 (1H, m), 12.50 (1H, s); MS m/z: 533 $(M+H)^+$. Anal. Calcd for $C_{28}H_{32}N_6O_5 \cdot 1.0H_2O$: C, 61.08; H, 6.22; N, 15.26. Found: C, 60.99; H, 6.19; N, 15.04.

5.1.32. (4-{[8-Carbamoyl-7-(3,5-dimethoxyphenylamino)imidazo-[1,2-*c*]pyrimidin-5-ylamino]methyl}phenyl)acetic acid (21)

A mixture of compound **20** (0.282 g, 0.530 mmol) and trifluoroacetic acid (1.5 mL) in CH_2Cl_2 (1.5 mL) was stirred for 3 h at room temperature. Volatiles were evaporated in vacuo and the residue was dissolved in THF. To the mixture was added $NaHCO_3$ solution and acidified again with 1 M HCl solution. The mixture was extracted with EtOAc–THF, organic phase was washed with brine, dried over anhydrous $MgSO_4$, and evaporated in vacuo. Resulting residue was treated with MeOH to give **21** (0.139 g, 55%) as yellowish solid: mp 266–268 °C (MeOH–THF); IR (KBr) 1617, 1591, 1559, 1506, 1146 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 3.53 (2H, s), 3.62 (6H, s), 4.80 (2H, d, J = 5.5 Hz), 6.14 (1H, br s), 6.81 (2H, d, J = 2.0 Hz), 7.21 (2H, d, J = 8.0 Hz), 7.34 (2H, d, J = 8.0 Hz), 7.36 (1H, s), 7.43 (1H, d, J = 3.0 Hz), 7.92 (1H, s), 8.94 (1H, t, J = 5.5 Hz), 9.55 (1H, d, J = 3.0 Hz), 12.29 (1H, br s), 12.50 (1H, s); MS m/z: 477 $(M+H)^+$. Anal. Calcd for $C_{24}H_{24}N_6O_5 \cdot 0.4CH_4O$: C, 59.89; H, 5.27; N, 17.17. Found: C, 60.23; H, 5.20; N, 16.83.

5.1.33. 5-(4-Carbamoylmethylbenzylamino)-7-(3,5-dimethoxyphenylamino)imidazo[1,2-*c*]pyrimidine-8-carboxamide (22)

The title compound was prepared from **21** in the same manner as described for **12**, and obtained as yellowish solid (12%): mp 224–226 °C (MeOH–THF); IR (KBr) 1600, 1558, 1499, 1150 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 3.30 (2H, s), 3.63 (6H, s), 4.79 (2H, d, J = 5.0 Hz), 6.14 (1H, t, J = 2.0 Hz), 6.82 (2H, d, J = 2.0 Hz), 6.84 (1H, br s), 7.21 (2H, d, J = 8.5 Hz), 7.32 (2H, d, J = 8.0 Hz), 7.36 (1H, d, J = 1.5 Hz), 7.40–7.46 (2H, m), 7.92 (1H, d, J = 1.5 Hz), 8.93 (1H, t, J = 5.0 Hz), 9.54 (1H, d, J = 3.5 Hz), 12.49 (1H, s); MS m/z: 476 $(M+H)^+$. Anal. Calcd for $C_{24}H_{25}N_7O_4$: C, 60.62; H, 5.30; N, 20.62. Found: C, 60.38; H, 5.28; N, 20.23.

5.1.34. Ethyl (*E*)-4-[[bis[(1,1-dimethylethoxy)carbonyl]amino]-methyl]cinnamate (24)

To a stirred suspension of sodium hydride (0.220 g (60% in mineral oil), 5.50 mmol) in THF (15 mL) was added ethyl (*E*)-4-bromomethylphenylcinnamate **23** (1.35 g, 5.00 mmol). To this mixture was slowly added a solution of di-*tert*-butyliminodicarboxylate (1.19 g, 5.50 mmol) in THF (10 mL) at 0 °C. After stirring 14 h at room temperature, the reaction mixture was poured into saturated ammonium chloride solution, and extracted with EtOAc. The organic phase was washed with brine, dried over anhydrous $MgSO_4$, and evaporated in vacuo. Resulting residue was purified by column chromatography on silica gel with hexane/EtOAc = 10:1 → 3:1 to give **24** (1.61 g, 79%) as white solid: ^1H NMR ($CDCl_3$) δ: 1.33 (3H, t, J = 7.0 Hz), 1.45 (18H, s), 4.26 (2H, q, J = 7.0 Hz), 4.79 (2H, s), 6.41 (1H, d, J = 16.5 Hz), 7.29 (2H, d, J = 8.0 Hz), 7.48 (2H, d, J = 8.0 Hz), 7.66 (1H, d, J = 16.5 Hz).

5.1.35. Ethyl (*E*)-4-aminomethylphenylcinnamate hydrochloride (25)

To a mixture of **24** (1.60 g, 3.95 mmol) in EtOAc (20 mL) was added 4 M HCl–EtOAc (9.90 mL, 39.46 mmol) and stirred overnight at room temperature. Resulting precipitates were collected by suction filtration and dried under reduced pressure to give **25** (0.645 g, 68%) as white solid: ^1H NMR (DMSO-d_6) δ: 1.26 (3H, t, J = 7.0 Hz), 4.05 (2H, s), 4.20 (2H, t, J = 7.0 Hz), 6.69 (1H, d, J = 16.0 Hz), 7.52 (2H, d, J = 8.5 Hz), 7.66 (1H, d, J = 16.0 Hz), 7.78 (2H, d, J = 8.5 Hz), 8.00–8.80 (3H, br); MS m/z: 206 $(M+H)^+$.

5.1.36. Ethyl (*E*)-(4-{[8-Carbamoyl-7-(3,5-dimethoxyphenylamino)imidazo[1,2-*c*]pyrimidin-5-ylamino]methyl}phenyl)-cinnamate (26)

The title compound was prepared from **9** and **25** in the same manner as described for **10d**, and obtained as pale green solid (37%): mp 172–173 °C (EtOAc–THF); IR (KBr) 1636, 1600, 1559, 1507, 1154 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 1.25 (3H, t, J = 7.0 Hz), 3.63 (6H, s), 3.70-3.90 (2H, br), 4.17 (2H, q, J = 7.0 Hz), 4.84 (2H, d, J = 5.5 Hz), 6.15 (1H, t, J = 2.0 Hz), 6.61 (1H, d, J = 16.0 Hz), 6.78 (2H, d, J = 2.0 Hz), 7.40 (1H, d, J = 1.5 Hz), 7.43 (2 H, d, J = 8.0 Hz), 7.62 (1H, d, J = 16.0 Hz), 7.68 (2H, d, J = 8.0 Hz), 7.94 (1H, d, J = 1.5 Hz), 9.06 (1H, br s), 12.25–12.50 (1H, br); MS m/z: 517 $(M+H)^+$. Anal. Calcd for $C_{27}H_{28}N_6O_5 \cdot 1.8H_2O$: C, 59.07; H, 5.80; N, 15.30. Found: C, 59.12; H, 5.48; N, 14.92.

5.1.37. (*E*)-(4-{[8-Carbamoyl-7-(3,5-dimethoxyphenylamino)-imidazo[1,2-*c*]pyrimidin-5-ylamino]methyl}phenyl)cinnamic acid (27)

The title compound was prepared from **26** in the same manner as described for **11**, and obtained as pale yellowish solid (85%): mp 262 °C (dec) (MeOH–THF); IR (KBr) 1601, 1499, 1481, 1153 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 3.64 (6H, s), 3.67–4.10 (2H, br), 4.83 (2H, d, J = 5.5 Hz), 6.15 (1H, t, J = 2.0 Hz), 6.50 (1H, d, J = 16.0 Hz), 6.78 (2H, d, J = 2.0 Hz), 7.41 (1H, s), 7.43 (2H, d, J = 8.5 Hz), 7.56 (1H, d, J = 16.0 Hz), 7.65 (2H, d, J = 8.5 Hz), 7.94 (1H, d, J = 1.5 Hz), 9.07 (1H, br s), 11.50–13.00 (2H, br); MS m/z: 489 $(M+H)^+$. Anal. Calcd for $C_{25}H_{24}N_6O_5 \cdot 1.4H_2O$: C, 58.45; H, 5.26; N, 16.36. Found: C, 58.55; H, 5.10; N, 16.05.

5.2. Molecular modeling

The 3D coordinates of ZAP-70 receptor model were constructed based on the published crystal structure of an activated Lck kinase domain[16,17] (PDB code. 1QPD) using program FAMS.[18] The structure of staurosporine was extracted from X-ray structure of LCK (PDBcode. 1QPD). All docking calculations were performed by using program ADAM.[19]

5.3. Biology

5.3.1. Intracellular ZAP-70 kinase inhibition assay

The kinase domain of human ZAP-70 kinase (Leu325-Ala619) was cloned to NdeI and XhoI sites of pET-19b expression vector (Novagen Inc.) by PCR amplification from human thymus Marathon-Ready™ cDNA (CLONTECH Inc.). The ZAP-70 kinase domain binding with pET-19b His-tag gene at 5′ region was integrated into pFASTBAC1 vector of the BAC-TO-BAC™ (GIBCO-BRL Inc.) baculovirus expression system. The transfected virus was obtained by transfecting Sf-9 cell (Invitrogen Inc.) with pFASTBAC1 containing His-tag-fused ZAP kinase domain described above.

High Five™ baculocells, which was infected with this transfected virus, were recovered and these cells were dissolved by ultrasonication. After soluble fraction being separated by centrifuge, the supernatant was added to TALON™ metal affinity resin (CLONTECH Inc.) and to this resin was adsorbed His-tag-fused protein of ZAP-70 kinase domain. The resin was washed several times and extracted His-tag-fused protein of ZAP-70 kinase domain by imidazole containing a buffer. A coupled spectrophotometric assay was used wherein ADP generated by ZAP-70 kinase was converted to ATP by pyruvate kinase (PK), with concomitant production of pyruvate from phosphoenolpyruvate (PEP). LDH reduces pyruvate to lactate by oxidizing NADH. NADH depletion was monitored at 340 nm using a microplate reader (Spectra Max 250, Molecular Device) at 30 °C for 20 min. Reactions were performed at 30 °C in 100 mM HEPES buffer (pH 7.6), containing 20 mM $MgCl_2$, and 10% glycerol, and started by adding ATP. PK (150 μg/mL), LDH (500 μg/mL), PEP (2.5 mM), and NADH (150 μM) were added in large excess. Addition of 100 μM ZAP-70 optimal peptide substrate (peptide sequence: AEEEIYGEFEAKKKK, Sawady, Tokyo), allowed measurement of kinase activity.

5.3.2. Intracellular Syk kinase inhibition assay

The kinase domain of human Syk kinase (Met343-Asn635) was cloned to Ndel and Xhol sites of pET-19b expression vector (Novagen Inc.) by PCR amplification from human thymus Marathon-Ready™ cDNA (CLONTECH Inc). The Syk kinase domain binding with pET-19b His-tag gene at 5′ region was integrated to pFASTBAC1 vector of the BAC-TO-BAC™ baculovirus expression system (GIBCO-BRL Inc.). The transfected virus was obtained by transfecting Sf-9 cell (Invitrogen Inc.) with pFASTBAC vector containing His-tag-fused Syk kinase domain described above. High Five™ baculocells, which was infected with this transfected virus, were recovered and dissolved by ultrasonication. After soluble fraction being separated by centrifuge, the supernatant was mixed with TALON™ metal affinity resin (CLONTECH Inc.) and to this resin was adsorbed His-tag-fused protein of Syk kinase domain. The resin was washed several times and extracted His-tag-fused protein of Syk kinase domain by imidazole containing a buffer. A coupled spectrophotometric assay was used, wherein ADP generated by Syk kinase was converted to ATP by pyruvate kinase (PK), with concomitant production of pyruvate from phosphoenolpyruvate (PEP). LDH reduces pyruvate to lactate by oxidizing NADH. NADH depletion was monitored at 340 nm using a microplate reader (Spectra Max 250, Molecular Device) at 30 °C for 20 min. Reactions were performed at 37 °C in 100 mM HEPES buffer (pH 7.6), containing 40 mM $MgCl_2$ and 10% glycerol, and started by adding ATP. PK (150 μg/mL), LDH (50 μg/mL), PEP (2.5 mM), and NADH (200 μM) were added in large excess. Addition of 100 μM Syk optimal peptide (peptide sequence: AEEEIYGEFEAKKKK, Sawady, Tokyo) allowed measurement of kinase activity.

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