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Author(s)	宮崎, 理樹
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**Study on p53-MDM2 Interaction Inhibitors
as a Novel Anticancer Agent**

(p53-MDM2 結合阻害活性を有する新規抗癌剤の創製に関する研究)

2013

Masaki Miyazaki

Abbreviation

bid	bis in die (twice administration per day)
BnCl	benzyl chloride
Boc	di- <i>tert</i> -butoxycarbonyl
Boc ₂ O	di- <i>tert</i> -butyl dicarbonate
BSA	bovine serum albumin
<i>t</i> -BuOAc	<i>tert</i> -butyl acetate
DIPEA	<i>N,N</i> -diisopropylethylamine
DMAP	4-(dimethylamino)pyridine
DMF	<i>N,N</i> -dimethylformamide
DMSO	dimethyl sulfoxide
EDC	1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide
ELISA	enzyme-linked immunosorbent assay
ESI/MS	electrospray ionization mass spectrometry
Et ₃ N	triethylamine
Et ₂ O	diethyl ether
EtOAc	ethyl acetate
EtOH	ethyl alcohol
GST	glutathione S-transferase
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HTS	high-throughput screening
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
HRESI/MS	high resolution electrospray ionization mass spectrometry
HREI/MS	high resolution electron ionization mass spectrometry
HTRF	homogeneous time resolved fluorescence
IPA	isopropyl alcohol
IC ₅₀	concentration for 50% inhibition of p53-MDM2 binding
GI ₅₀	concentration for 50% inhibition of cell proliferation
LiBHET ₃	lithium triethylborohydride

MDM2	murine double minute 2
Me ₂ S	dimethylsulfide
MeCN	acetonitrile
MeOH	methyl alcohol
MTD	maximum tolerated dose
NMR	nuclear magnetic resonance
PDB	protein data bank
Pd/C	palladium on carbon
PK	pharmacokinetics
po	per os (oral administration)
qd	quaque die (single administration per day)
SAR	structure-activity relationship
SCID	severe combined immunodeficiency
TFA	trifluoroacetic acid
TGI	tumor growth inhibition
THF	tetrahydrofuran
<i>p</i> -TsOH	<i>p</i> -toluenesulfonic acid
Z	benzyloxycarbonyl

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General Introduction

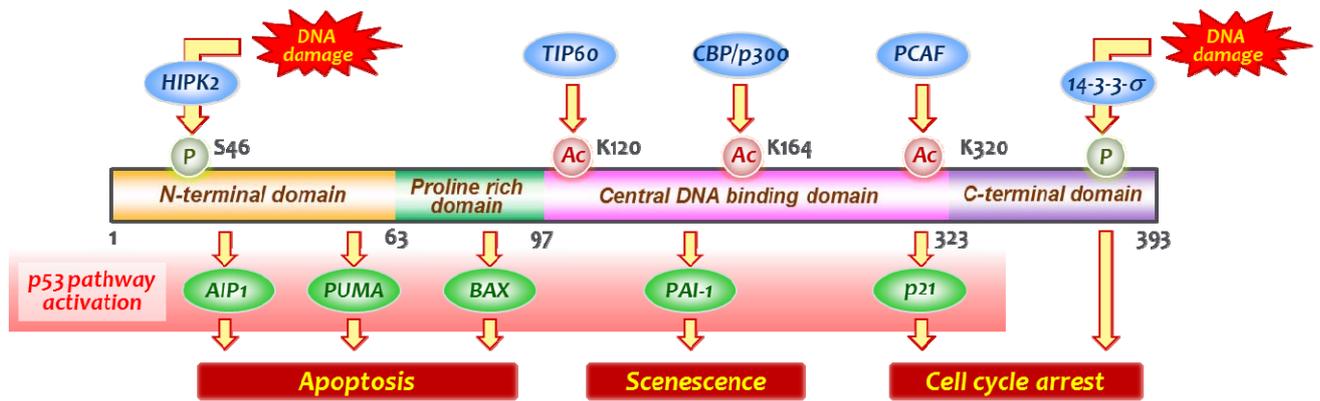
The p53 tumor suppressor protein plays an important role in the growth suppression and cell death pathways, i.e. apoptosis to cancer cells.¹ About 50% of human cancers express mutant p53, and the other 50% of cancers have wild-type p53 but indicate repressing its proteins on the p53 pathway. Especially, it is noteworthy that the rate of wild-type genes is more than 90% in leukemia and sarcoma cell lines.^{2,3} And its functions are regulated by an overexpression or an amplification of human murine double minute 2 (MDM2) gene.^{4,5} MDM2 protein, which is a negative regulator of the p53 protein, combines with the *N*-terminal transcriptional activation domain of p53, and promotes export of p53 from nucleus to cytoplasm, thereby promoting proteasomal degradation of p53 via ubiquitination through its E3 ligase activity (Figure 1a).⁶⁻⁸ Thus, activation of the p53 function by the inhibition of the protein-protein interaction of p53-MDM2 is regarded as an effective approach in cancer therapy (Figure 1b). In fact, there have been many reports regarding the relevance between MDM2 inhibition and growth inhibition of cancer cells.⁹⁻¹¹

In the last ten years, various small molecules (Figure 2) which inhibit p53-MDM2 interactions have been reported.¹²⁻¹⁷ The p53-MDM2 binding inhibitory activity (IC_{50}) of these compounds in the reports are roughly 0.1-10 μ M on a cell free assay, among which potent inhibitor reported first from F. Hoffmann-La Roche Ltd. was Nutlin-3a (**1**) whose IC_{50} was 0.09 μ M. MI-219 (**2**) reported from University of Michigan was also potent inhibitor which has spirooxyindole scaffold. AM-8553 (**3**), which was just reported recently from Amgen Inc., had improved potency considerably by lead optimization researches from their HTS hit compounds.¹⁸

The interaction between p53 and MDM2 depends on van der Waals' forces, which is mainly accomplished by the intervention of three hydrophobic residues of p53, i.e. side chains of Phe19, Trp23, and Leu26.¹⁹⁻²¹ Therefore, proficient filling of the hydrophobic pockets is very important to furnish potent inhibitors. Specifically, the substituents should be carefully placed on a scaffold so as to let them fit the pockets efficiently. So compounds displayed in Figure 2 are considered to apply the concept of the drug design, like the Nutlins (Figure 3).

In this study, we focused our attention on these compounds and investigated further in order to obtain a novel and more potent molecule as a p53-MDM2 interaction inhibitor. The research achievements are discussed in the following three chapters. Firstly, we designed and investigated bicyclic scaffolds aiming to place *cis*-bischlorophenyl moiety at the equivalent location where the hydrophobic interaction with MDM2 could be expected (Chapter I). As a result, we discovered a dihydroimidazothiazole scaffold having a potent p53-MDM2 inhibitory activity. Further exploration of the side chains on the dihydroimidazothiazole scaffold aided by molecular modeling resulted in compounds exhibiting almost comparable *in vitro* potency to Nutlin-3a. In Chapter II, further medicinal research was investigated in order to solve a involving the chemical instability of the scaffold which led to imidazothiazole by the oxidation. The optimal compounds by incorporating the methyl group onto the C-6 position to avoid the oxidation, and by modifying the C-2 moiety of the additional proline motif, showed significant improvement in potency compared with our early lead or Nutlin-3a. In Chapter III, further optimization of our lead compound was executed by the improvement of physicochemical properties in order to obtain orally active compounds. Thus we furnished optimal compounds by introducing an alkyl group onto the pyrrolidine at the C-2 substituent to prevent the metabolism; and modifying the terminal substituent of the proline motif improved solubility. These promising compounds exhibited good PK profiles and significant antitumor efficacy with oral administration on a xenograft model using MV4-11 cells having wild type p53.

➤ **p53: Tumor Suppressors --- deletion or degradation in tumor cells**



➤ **MDM2: Oncoproteins --- overexpression in tumor cells**

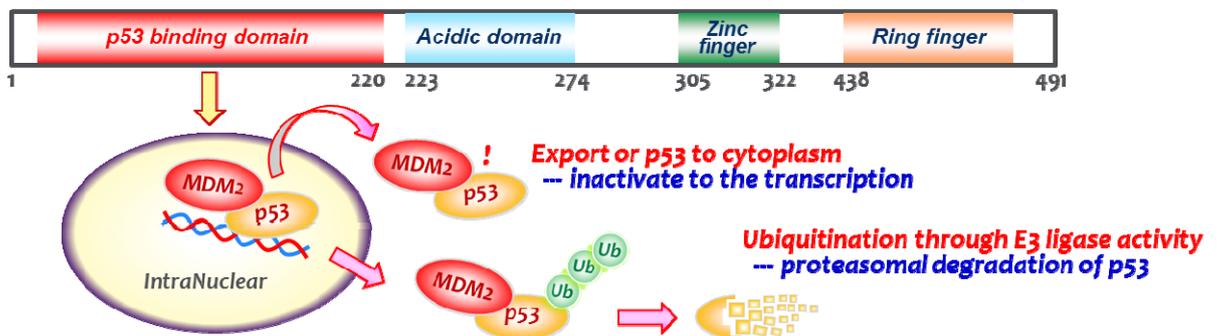


Figure 1a. Introduction of p53 and MDM2 proteins.

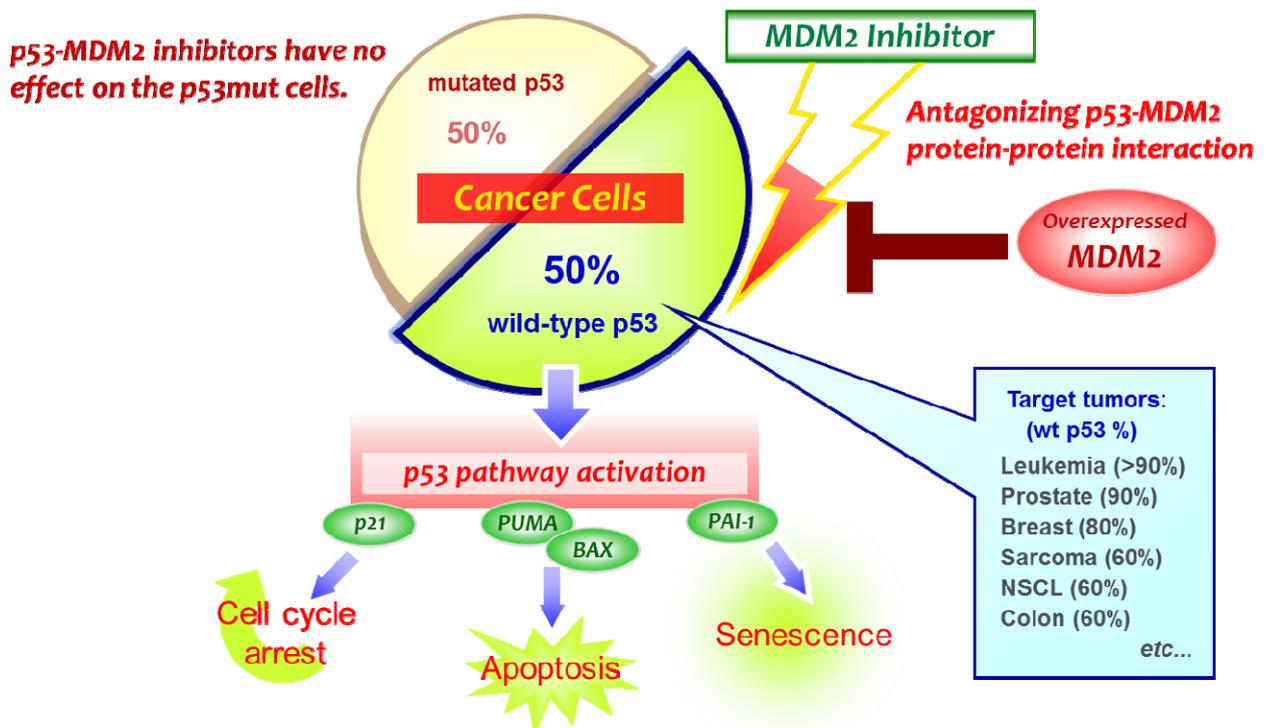
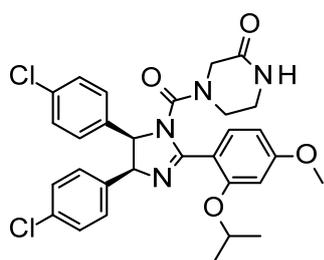
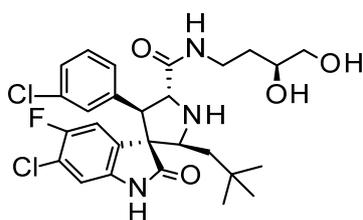


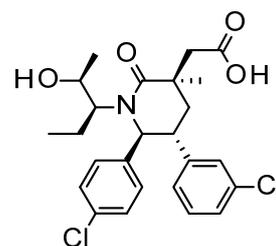
Figure 1b. Target molecule and mechanism of action of p53-MDM2 inhibitors.



1 (Nutlin-3a)



2 (MI-219)



3 (AM-8553)

Figure 2. Previously reported small molecules as p53-MDM2 inhibitors.

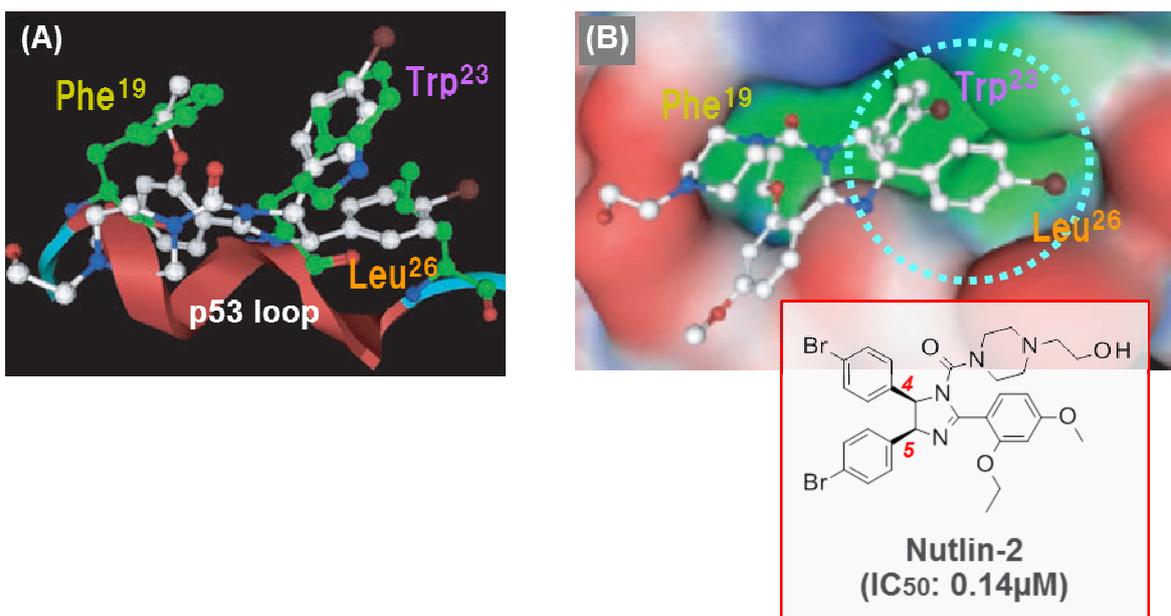


Figure 3. (A) 3-D mimic design of p53-MDM2 interactive three residues on p53. (B) Co-crystal structure of MDM2/Nutlin-2. Both data were reported in ref. [12].

Chapter I;

Discovery of novel dihydroimidazothiazole derivatives as p53-MDM2 protein-protein interaction inhibitors

I-1. Introduction

As mentioned in General Introduction, the key moieties of the interaction between p53 and MDM2 mainly consist of three hydrophobic residues of p53, i.e. side chains of Phe19, Trp23, and Leu26 from p53-MDM2 co-crystal structure analysis. The co-crystal structure of MDM2 and Nutlin-2 supports the analysis, in which two 4-bromophenyl groups and ethoxyphenyl moiety on Nutlin-2 corresponded to the three hydrophobic residues of p53 (Figure 3). In the beginning of this research to obtain a novel scaffold for p53-MDM2 interaction inhibitors, we analyzed the mode of interaction between MDM2 and bis-(4-bromophenyl) groups of Nutlin-2 (**4**).

I-2. Discovery of the potent bicyclic scaffold and its optimization

We tried to place the *cis*-bischlorophenyl structures in proper positions by synthesizing and evaluating various bicyclic scaffolds having the moieties, and finally discovered that dihydroimidazothiazole derivatives hold the inhibitory activity ($IC_{50} < 1 \mu M$). The lead structure is exemplified in Figure 4. Then, our medicinal effort was moved to the optimization of the substituent at the C-3 position. Syntheses of the C-3 variants are depicted in Scheme 1. *cis*-1,2-Bis(4-chlorophenyl)ethane-1,2-diamine (**5**) was synthesized in accordance with the literature method.²² Cyclization with CS_2 ,²³ followed by thiazole formation with α -chloro- β -ketoesters (**12a-g**),²⁴ provided dihydroimidazothiazole derivatives (**7a-g**). Compounds **11a-c** and **11f** are commercially available, while **11e** and **11g** were prepared via conventional methods using Meldrum's acid.²⁵ Hydrolysis then gave the corresponding carboxylic acids (**8a-g**). EDC mediated amidation with oxopiperazine furnished the amide derivatives (**9a-g**) for screening.

As shown in Scheme 2, variants for the amide portion in place of 2-oxopiperazine were also synthesized (**13-19**).

I-3. Evaluation of dihydroimidazothiazole derivatives

We evaluated **9a-g** to investigate the influence of C-3 moiety on the activity, wherein the capacity of interacting pockets could be assessed. IC₅₀ values of these compounds were measured by an ELISA in which GST-tagged MDM2 binds to p53 immobilized to the surface of a 96-well plate. As a result, compound **9c** bearing *i*-propyl moiety only showed high potency (IC₅₀= 0.26 μM), which is almost comparable to the one of Nutlin-3 (racemate, IC₅₀= 0.18 μM). Other compounds possessing smaller substituents than *i*-propyl groups such as methyl (**9a**) and ethyl (**9b**) showed weak activity. In addition, substituents such as *n*-propyl (**9d**), *c*-propyl (**9e**), methoxymethyl (**9g**) and pivaloyl (**9f**) groups also reduced the activities (IC₅₀s = 1.8-9.5 μM) (Table 1).

Next, the result of the SAR for C-2 position (amide site), in which C-3 substituent was fixed to the *i*-propyl group, is depicted in Table 2. We introduced neutral or basic 6-membered rings into C-2, referring to Nutlin's side chain, for the purpose of evaluating the efficiency of the newly generated scaffold. In this connection, the optimization of the C-2 position will be reported elsewhere in due course. As for neutral substituents, compound **15** having a morpholino group showed potency almost equivalent to **9c** (IC₅₀= 0.34 μM). *N*-acetylpiperazine (**13**) or homopiperazine (**16**) variants gave less potency at around 1 μM on IC₅₀, whilst the one for *N,N*-dimethylcarbamoypiperidine (**14**) diminished (IC₅₀=4.4 μM). Compounds **17** and **18**, which have basic substituents, were less active (IC₅₀ =1.8 and 1.2 μM). On the other hand, compound **19** possessing 2,5-dimethylpiperazine, which is a rather weaker basic moiety, showed the most potent activity (IC₅₀= 0.14 μM).

These dihydroimidazothiazole derivatives upon which we are reporting thus far were racemates, and each enantiomer could be separated by using HPLC with a chiral column. With regard to Nutlin, each enantiomer was obtained by optical resolution of the racemate, and only one of these showed potency.¹² Thus we also separated compound **9c** [equal to (+/-)-**9c**] using chiral HPLC (CHIRALCEL[®] OD-H, eluant: hexane-IPA), and acquired each enantiomer ((+)-**9c** and (-)-**9c**)²⁶ (Table 3). Compound (+)-**9c** possessed potent activity, in contrast, the IC₅₀ of the other isomer (-)-**9c** was found to be 1/85 times weaker. This result suggested the same tendency as Nutlin's SAR¹²; hence the mode of placing the two halophenyl groups with the dihydroimidazothiazole

scaffold was considered to be almost equivalent to the Nutlin's imidazole as expected, although the absolute configurations of (+)-**9c** and (-)-**9c** are yet to be determined. Co-crystal structure analysis of the dihydroimidazothiazoles and MDM2 has not been conducted yet, however, the mode of the interaction of our lead would be interpreted as follows: From our drug design which referred to the structural configuration of Nutlins and the result of optical resolution, it is considered that Nutlin-3a and our active lead (+)-**9c** make an interaction to MDM2 protein in the same manner. As shown in Figure 5, (+)-**9c** illustrated with an estimated configuration, was posed by superposition to the co-crystal structure of MDM2/Nutlin-2 (PDB code: 1RV1) using docking calculation. In this model, two chlorophenyl groups and the *i*-propyl group of (+)-**9c** are fitted with the three MDM2 pockets efficiently.

With regard to the substituent at the C-2 position, it seems that there is a slight difference between our lead and Nutlin's. Compound **9c** with 2-oxopiperazine as Nutlin-3 kept potent activity, whilst compound **17** having hydroxyethylpiperazine like Nutlin-2 (**4**) reduced the activity, which was inconsistent with Nutlin's SAR. For dihydroimidazothiazoles, incorporation of basic moiety at the C-2 position resulted in reduction of activity exemplified by compounds **17** and **18**, while improvement of activity was observed by introducing a weak basic moiety demonstrated by compound **19**. Thus, there seems to be more space and opportunity for derivatization at the C-2 position, and for further improvement of potency and physicochemical properties as well.

In conclusion, we discovered novel inhibitors of the p53-MDM2 interaction possessing a dihydroimidazothiazole scaffold. Especially 2-oxopiperazine (**9c**) and 2,5-dimethylpiperazine (**19**) variants possessed high activity, which was almost equivalent to Nutlin's. Moreover, since p53-MDM2 inhibitory activity was drastically influenced by altering substituents at the C-2 or C-3 positions, further optimization aiming to discover more potent inhibitors is considered feasible.

Figures in Chapter I

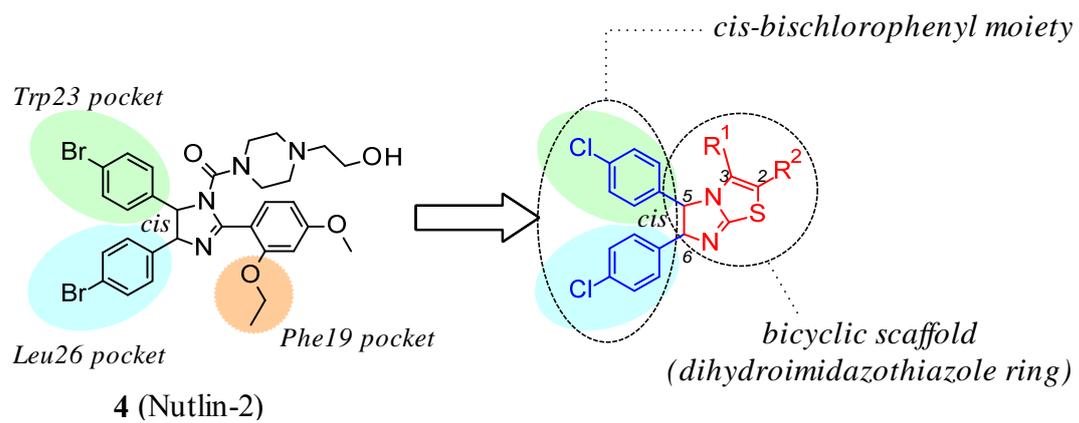
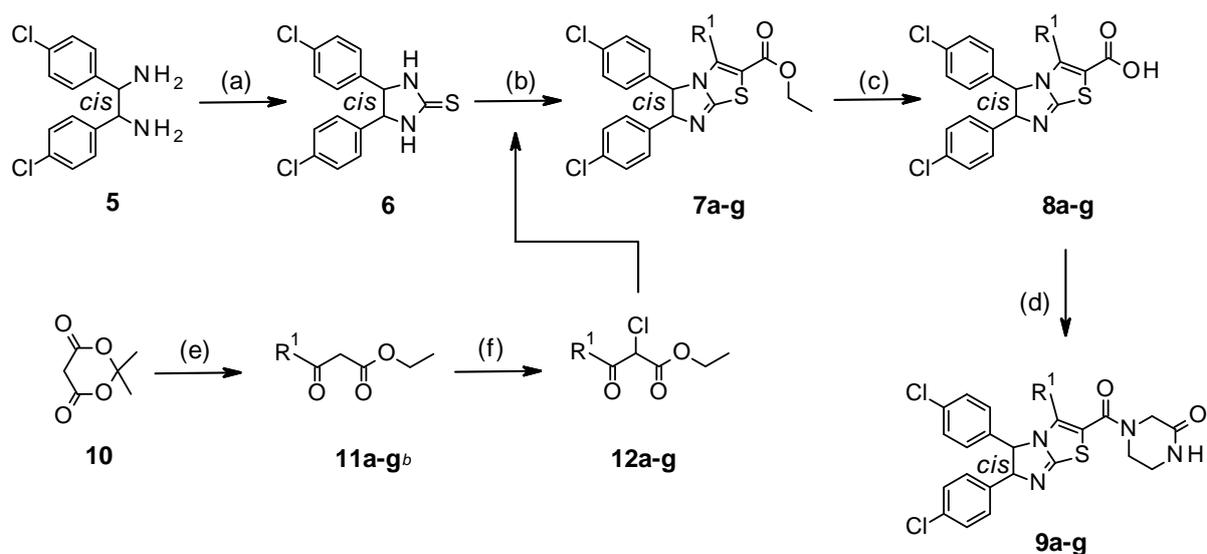


Figure 4. Designs of novel scaffold having a bicyclic skeleton

Scheme 1. Synthesis of C-3 substituted dihydroimidazothiazoles **9a-g^a**



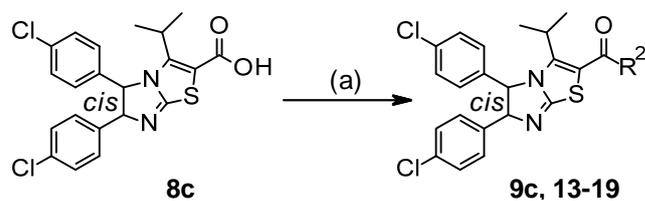
compd ^a	R ¹
11a	methyl
b	ethyl
c	<i>i</i> -propyl
d	<i>n</i> -propyl
e	<i>c</i> -propyl
f	<i>t</i> -butyl
g	MeOCH ₂ -

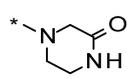
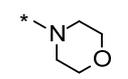
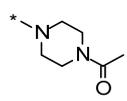
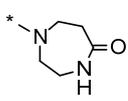
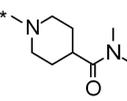
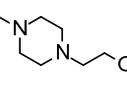
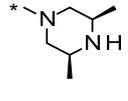
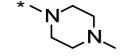
^a Reagents and Conditions:

(a) CS₂, EtOH, reflux; (b) **12a-g**, EtOH, reflux (c) NaOH_{aq.}, EtOH, reflux; (d) 2-oxopiperazine, EDC/HCl, Et₃N, CH₂Cl₂; (e) (i) R¹-COCl, Pyridine, CH₂Cl₂; (ii) EtOH, reflux; (f) SO₂Cl₂, CH₂Cl₂.

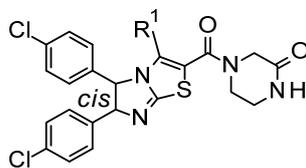
^b **11e** & **11g** were just synthesized from **10**. The others were utilized commercially available.

Scheme 2. Synthesis of C-2 substituted dihydroimidazothiazoles **13-19**^a



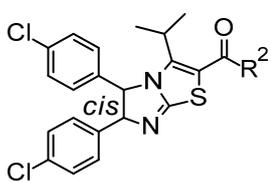
		R²			
	9c		15		
neutral	13		16		
	14				
	17		19		
basic	18				

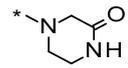
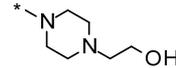
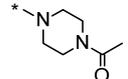
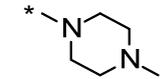
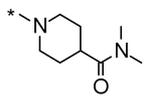
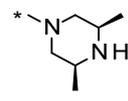
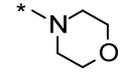
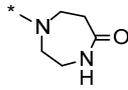
^a Reagents and Conditions: (a) amine, EDC/HCl, HOBt, Et₃N, DMF or CH₂Cl₂.

Table 1Cell free p53-MDM2 inhibitory activity of analogues **9a-g**

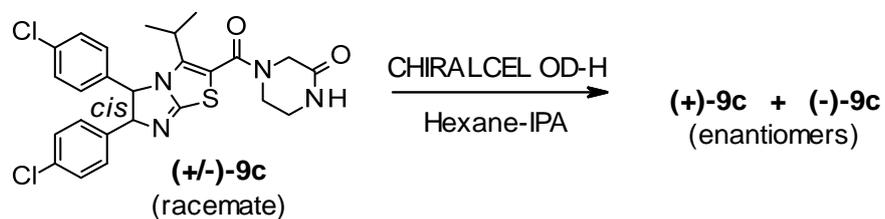
compd ^a	R ¹	IC ₅₀ (μM)
9a	methyl	2.7
9b	ethyl	1.8
9c	<i>i</i> -propyl	0.26
9d	<i>n</i> -propyl	3.1
9e	<i>c</i> -propyl	9.5
9f	<i>t</i> -butyl	3.0
9g	MeOCH ₂ -	7.1
Nutlin-3		0.18 ^b

^a All compounds were racemate.^b *in house* data.

Table 2Cell free p53-MDM2 inhibitory activity of analogues **13-19**

compd ^a	R ²	IC ₅₀ (μM)	compd ^a	R ²	IC ₅₀ (μM)
9c		0.26	17		1.8
13		0.84	18		1.2
14		4.4	19		0.14
15		0.34	Nutlin-2		0.14 ^b
16		1.1			

^a All compounds were racemate.^b *Lit.* data (see ref. [12]).

Table 3Chiral separation of compound **9c**

cmpd	IC ₅₀ (μM)
(+/-)-9c	0.26
(+)-9c	0.16
(-)-9c	11.9

Nutlin-3a	0.09 ^a
Nutlin-3b	13.6 ^a

^a *Lit.* data (see ref. [12]).

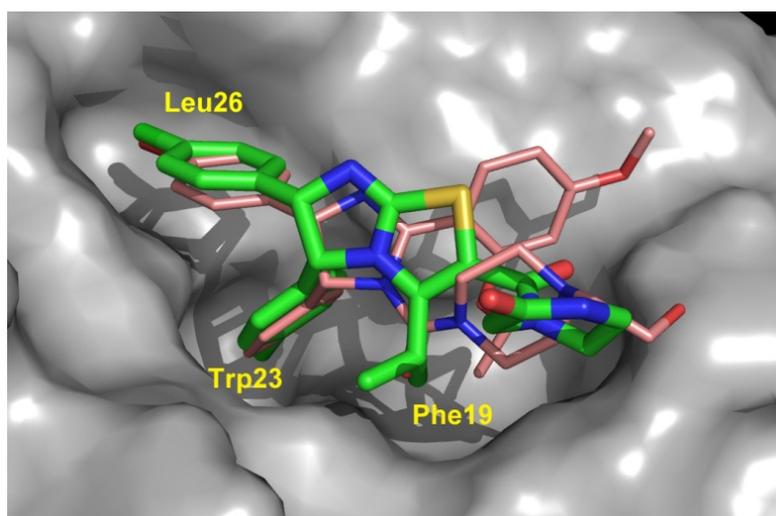


Figure 5. Predicted binding model of (+)-**9c** (estimated configuration) in green superposed on the MDM2/Nutlin-2 co-crystal structure (PDB code: 1RV1) by docking calculation. The three substituents of dihydroimidazothiazole scaffold are fitted to hydrophobic pockets. The *i*-propyl group has the same role for Phe19 of p53, which is placed on ethoxy moiety of Nutlin-2 in light red.

Chapter II;

Lead optimization of novel p53-MDM2 interaction inhibitors possessing dihydroimidazothiazole scaffold

II-1. Introduction

As reported in the Chapter I, the novel dihydroimidazothiazole derivatives, which were discovered by analyzing research in the mode of interaction between MDM2 and the Nutlins, showed a potent p53-MDM2 binding inhibitory activity. Compound (*rac*)-**9c** placing *cis*-bischlorophenyl moieties at the C-5 and C-6 position of dihydroimidazothiazole displayed potent activity ($IC_{50}=0.26 \mu\text{M}$) (Figure 6). The enantiomers were separated by using chiral HPLC, and we confirmed that one of them ((+)-**9c**) possessed high potency. However, upon further exploratory research, it became clear that the dihydroimidazothiazole ring was susceptible to oxidation, and easily provided imidazothiazole, for instance at the final amidation step, as shown in Figure 7. Thus, we pursued the further optimization of our lead (*rac*)-**9c** to furnish more potent and stable compounds.

II-2. Design and synthesis aiming to prevent the oxidation

Since *i*-propyl group was optimal for the C-3 substituent as we reported in the previous chapter, we continued to utilize the substituent for the position. With regard to the C-2 position, various substituents with both acyclic amide and cyclic one, encompassing 4- to 6-membered rings, were designed and synthesized.

In order to prevent the oxidation of the imidazoline moiety to imidazole, we modified the scaffold not to eliminate both protons at the C-5 and C-6 positions. Thus, we envisioned to incorporate the methyl group to the C-6 position not affecting the interaction with MDM2. Although an asymmetric carbon was generated, this trial worked well, resulting in a stable compound without spoiling the affinity (Figure 8), as we envisaged.

The vicinal diamine intermediate (*rac*)-**24** needed for the formation of 6-methyldihydroimidazothiazole was synthesized by utilizing the method of Pansare and others.²⁷ Optical resolution by diastereomeric salt formation using chiral tartaric

acids (Scheme 3)²⁸ was performed for *cis*-racemate diamine ((*rac*)-**24**); (+)-diamine ((+)-**24**) was obtained as a co-crystal with L-(+)-tartaric acid in ethanol, which was then collected by filtration. Then, the crystal was basified to give the free diamine. On the other hand, the filtrate was basified and then recrystallized with D-(-)-tartaric acid, gave the salt of (-)-diamine ((-)-**24**) D-tartrate in the same manner as for (+)-**24**. The salt was again basified to provide (-)-diamine ((-)-**24**). The optical purities of those enantiomers were analyzed using chiral HPLC (CHIRALPAK[®] AS-H, eluant: hexane-IPA),²⁹ and turned out to be >99% e.e. The absolute conformation was determined by co-crystal structure analysis of the final product **34b** with MDM2 protein.

II-3. Lead optimization from our early lead compound using the chiral diamine

All diamines were led to the final products using the same manner as used for compound (*rac*)-**9c** reported the Chapter I. For example, as shown in Scheme 4, diamines **24** were reacted with CS₂, followed by thiazole formation with α -chloro- β -ketoester, providing 6-methyl-dihydroimidazothiazole derivatives (**26a-c**). Although two kinds of cyclized compounds (6-methyl or 5-methyl) might have been generated, only one isomer was obtained which turned out to provide strong potency (compound **28b**). The selectivity was attained presumably by the steric hindrance of the methyl group. Absolute structure was later determined by the same method, i.e. co-crystallization with MDM2 protein, as described for diamine (+)-**24**. Hydrolysis of the ester gave the corresponding carboxylic acids (**27a-c**), then following EDC mediated amidation with the appropriate amines, furnished the amide derivatives (**28a-37b**). (*2S*)-*N,N*-dimethylazetidone-2-carboxamide, the starting material of the azetidone moiety of **37b**, was synthesized from chiral nitrile derivative via hydrolysis.^{30,31}

Compounds **40b-45b** were also designed because the proline variant **34b** showed a very strong activity. To investigate SAR of proline derivatives, amide moieties having branched or ring types referred to our previous research were introduced at the terminal position.

II-4. SAR of dihydrominidazothiazole derivatives

The result of SAR for the C-2 position (amide site) is displayed in Table 4. IC₅₀s were measured by p53-MDM2 plate binding assay³² or HTRF-based assay.³³ In the beginning, we investigated the effect of the C-6 methyl group for the activity with the variant having 2-oxopiperazine at the C-2 position. Compound **28a** showed a potency almost equivalent to (*rac*)-**9c** (IC₅₀= 0.41 μM). Each of the enantiomers (**28b** and **28c**) synthesized from chiral diamines ((+)-**24** and (-)-**24**) were then evaluated. Compound **28b** derived from diamine (+)-**24** possessed potent activity (IC₅₀= 0.092 μM), in contrast, the IC₅₀ of the other isomer **28c** was found to be 1/16 times weaker (IC₅₀= 1.5 μM). Since the final products **28a-c** were obtained in high yield without forming imidazothiazole derivatives at the last amidation step, it was proved that introducing the methyl group to the C-6 position was effective for the avoidance of ring-oxidation. Moreover, since enantiomer **28b** showed a high potency rather than the demethyl variant (+)-**9c**, the design is more favorable for p53-MDM2 inhibition.

In comparison of the types of amides, compound **31a** having a *N,N*-dimethylcarbamoyl group, showed the highest potency, thus the dialkyl amides were more advantageous in respect to the activity than the other class of substituents. Efficacy of compound **31b**, chiral variant synthesized from diamine (+)-**24**, furthermore improved (IC₅₀= 0.026 μM).

Whilst pyrrolidine derivatives (**32a** and **33a**) remained moderate activity, compound **34a** possessing *N,N*-dimethylcarbamoyl-L-proline moiety, provided a high potency (IC₅₀= 0.059 μM). Moreover, chiral variant (**34b**) showed significant improvement (IC₅₀= 0.0092 μM), and this was the most potent molecule. Compound **35a**, placed with D-proline moiety gave less (IC₅₀= 0.74 μM). Other compounds possessing 4- or 6-membered rings (**36a** and **37b**) having a *N,N*-dimethylcarbamoyl substituent displayed modest activity at around 0.2 μM.

II-5. Co-crystal structure analysis of dihydroimidazothiazoles and MDM2

The structure of the complex of compound **34b**/MDM2 is displayed in Figure 9. This crystallographic analysis revealed that two *cis*-bischlorophenyl groups and *i*-propyl moiety had the same roles for three hydrophobic residues (Trp23, Leu26 and Phe19) of

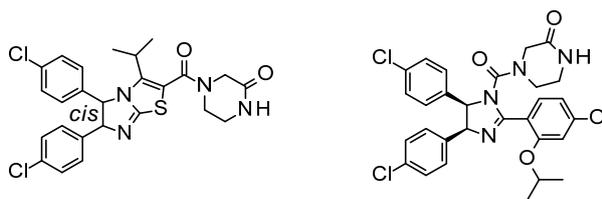
p53, as we expected. Furthermore, introducing the 5-membered substituent at the C-2 position, it was observed that the pyrrolidine ring provided a new 4th affinity site, which was generated by the induced fitting with MDM2. It is considered that this induced fitting makes the molecule **34b** about 10 times more potent than Nutlin-3a for the cell-free activity. The stacking model of co-crystal structure of **34b** and Nutlin-2, in which MDM2 loop is distorted by the induced-fitting with the proline ring, can explain that the three-dimensional structure is actually changed.

Through the X-ray structure analysis, we found out that dihydroimidazothiazole derivatives efficiently bound to the surface of MDM2 only by hydrophobic interaction. Although the amide part of the proline moiety seemed to direct to outside of MDM2 protein, compound **35a**, D-proline variant, showed 1/12 times weaker potency than that of L-proline derivative **34a**. This SAR provided us another opportunity and motivation for derivatizing the part to improve the efficacy or physicochemical property. The induced-fitting could also explain the reason why neither 6- nor 4-membered rings showed high activity; both of them would not form the fitting efficiently like 5-membered rings. The result of derivatizing the amide part of the proline moiety is shown in Table 5. Not only branched type (**40b**), but also 6-membered substituents like morpholine (**41b**) or *N*-methylpiperazine (**44b**) possessed a high potency ($IC_{50} = 0.023-0.026 \mu M$). This data suggests that structural capability at the C-2 carboxamide position is extensive, thus it would be possible for the improvement of physicochemical properties, i.e. solubility, hydrophobicity, and metabolic stability, in keeping with the potent p53-MDM2 inhibitory activity. These parameters should be very important to achieve antitumor efficacy *in vivo*.

In conclusion, we executed lead optimization of our early lead, dihydroimidazothiazole derivatives, for p53-MDM2 inhibitory activity. The prevention of the oxidation of the scaffold, where we could prepare optically active diamines efficiently, was accomplished by placing the methyl group at the C-6 position, without compromising potency. As a result of co-crystal structure analysis, a novel hydrophobic pocket was identified via induced-fitting of the pyrrolidine ring attached to the C-2 position of the scaffold. Compound **34b** showed 10 times more potent than Nutlin-3a, which can be explained by the increased affinity to MDM2. From these results, it was

confirmed that our dihydroimidazothiazole derivatives possessed superior deposition as p53-MDM2 binding inhibitors.

Figures in Chapter II



	(rac)-9c	(+)-9c (potent enantiomer)	1 (Nutlin-3a)
IC ₅₀ (μM):	0.26	0.16	0.09 ^a

^a *Lit.* data (see Ref. [12]).

Figure 6. Structure and IC₅₀s of our early lead and Nutlin-3a

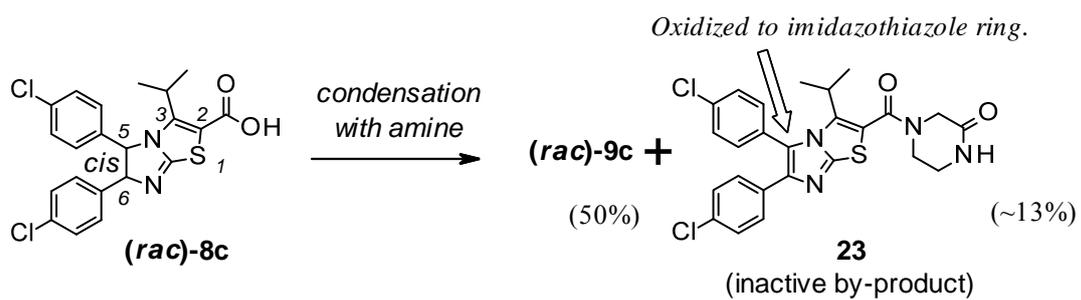


Figure 7. Instability of the scaffold; oxidation occurred in the condensation step

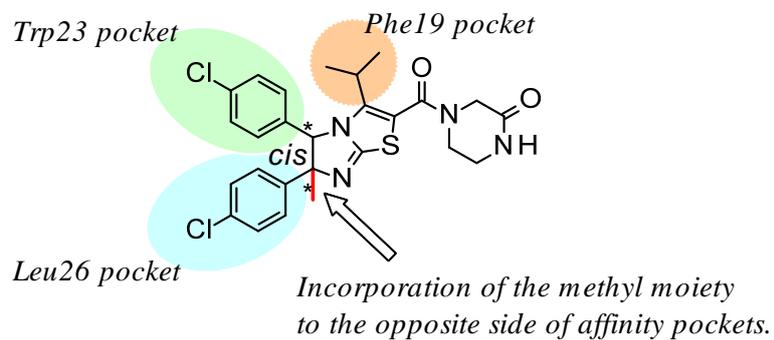
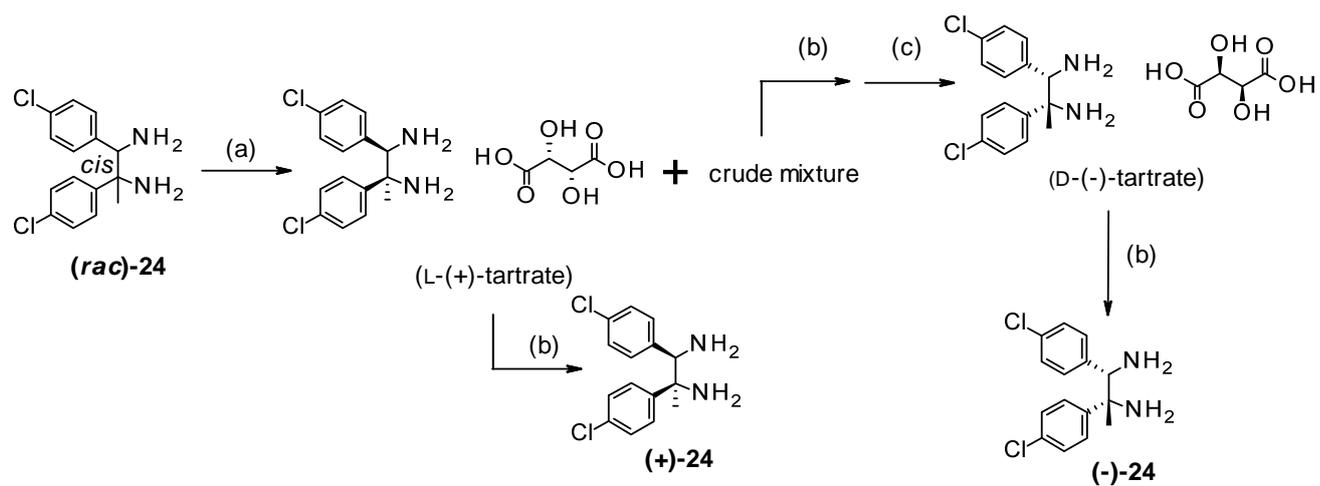


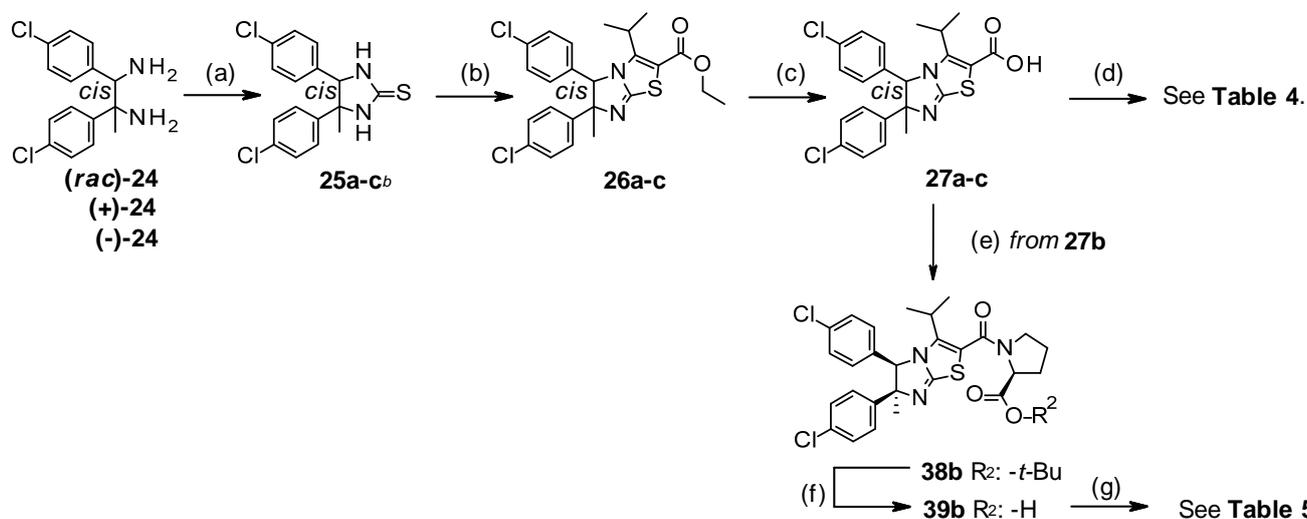
Figure 8. Designs to prevent oxidation referred from predicted affinity model

Scheme 3. Optical resolution of (*rac*)-**24** using L-(+)- or D-(-)-tartaric acid^a



^a Reagents and conditions: (a) L-(+)-tartaric acid, EtOH, reflux; (b) NaOHaq.; (c) D-(-)-tartaric acid, EtOH, reflux.

Scheme 4. Synthesis of C-6 substituted dihydroimidazothiazole derivatives from the *cis*-diamines **24**^a

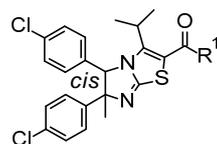


^a Reagents and Conditions: (a) CS₂, EtOH, reflux; (b) ethyl 2-chloro-4-methyl-3-oxopentanoate, EtOH, reflux; (c) NaOHaq., EtOH 60°C; (d) HNR₂, EDC/HCl, HOBT, Et₃N or DIPEA, DMF; (e) *t*-Butyl-L-prolinate hydrochloride, EDC/HCl, HOBT, DIPEA, DMF; (f) TFA, CHCl₃; (g) HNR₂, EDC/HCl, HOBT, Et₃N or DIPEA, DMF or CH₂Cl₂.

^b The compounds notated with [a] are the racemate derived from (*rac*)-**24**, and the compounds notated with [b] and [c] are the chiral isomer derived from (+)-**24** and (-)-**24**.

Table 4

Inhibition of the p53-MDM2 binding interaction (1)



compd ^a	R ¹	derived diamine	IC ₅₀ (μM) ^b
28a		(<i>rac</i>)- 24	0.41
28b		(+)- 24	0.092
28c		(-)- 24	1.5
29a	*-NH ₂	(<i>rac</i>)- 24	0.78
30a		(<i>rac</i>)- 24	1.1
31a		(<i>rac</i>)- 24	0.042
31b		(+)- 24	0.026
32a		(<i>rac</i>)- 24	0.20
33a		(<i>rac</i>)- 24	0.94
34a		(<i>rac</i>)- 24	0.059
34b		(+)- 24	0.0092
35a		(<i>rac</i>)- 24	0.74
36a		(<i>rac</i>)- 24	0.17
37b		(+)- 24	0.14 ^c
Nutlin-3a			0.09 ^d

^a Compounds notated with [a] are the racemate/diastereomer, and the compounds notated with [b] or [c] are the chiral isomer.

^b p53-MDM2 plate binding assay.

^c HTRF assay.

^d *Lit.* data (see Ref. [12])

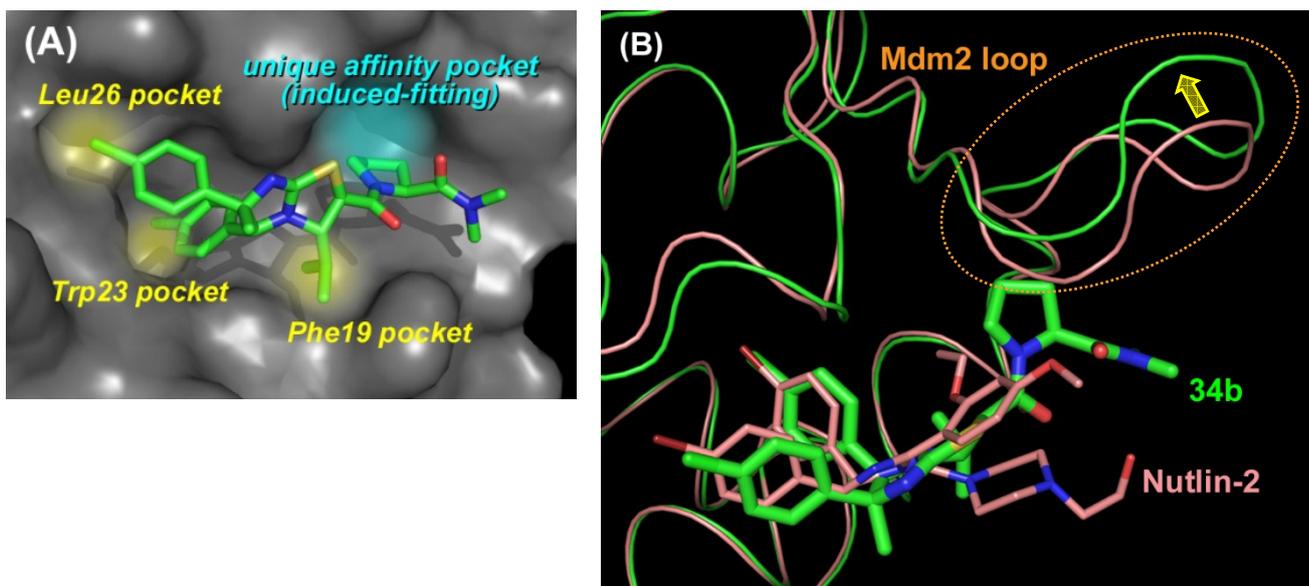
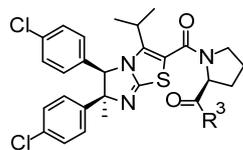


Figure 9. (A) X-ray co-crystal structure of **34b** in green with MDM2 (PDB code: 3VZV). Three hydrophobic pockets in yellow labels and a new affinity pocket in a light blue label are indicated onto the MDM2 surface. (B) The stacking model of co-crystal structures both for **34b** in green and Nutlin-2 in light red (PDB code: 3VZV and 1RV1). MDM2 loop is distorted by the induced-fitting of the proline ring, and thereby the three-dimensional structure is changed in the orange circle area.

Table 5

Inhibition of the p53-MDM2 binding interaction (2)



cmpd	R ³	IC ₅₀ (μM) ^a	cmpd	R ³	IC ₅₀ (μM) ^a
40b		0.046	43b		0.079
41b		0.026	44b		0.023
42b		0.066	45b		0.037

^a p53-MDM2 plate binding assay.

Chapter III;

Synthesis and evaluation of novel orally active p53-MDM2 interaction inhibitors

III-1. Introduction

In the previous chapters, we reported that novel dihydroimidazothiazole derivatives showed a potent p53-MDM2 interaction inhibitory activity. The crystallographic analysis of compound **34b**/MDM2 revealed that an additional interaction was observed by induced-fitting in addition to the three hydrophobic interactions. Compound **34b** showed robust p53-MDM2 inhibitory activity on HTRF-based assay ($IC_{50} = 8.3$ nM), as a consequence of the enhanced hydrophobic interaction via the increase of contact surface area (Figure 10). Compound **34b** did not display any therapeutic effect with oral administrations in the MV4-11 xenograft model, despite the strong activity *in vitro*, presumably because of its poor metabolic stability (9 % remaining after 30 min incubation with mouse hepatic microsome). For the discovery of orally active agents, it was necessary to improve solubility and metabolic stability, in addition to keeping the potency *in vitro*.

In this chapter, we report the discovery and biological evaluation of orally active MDM2 inhibitors based on dihydroimidazothiazole scaffold by improving metabolic stability and solubility with keeping strong potency.

III-2. Design and synthesis aiming to improve physicochemical properties

As we reported in the chapter II, it was confirmed that the pyrrolidine moiety of compound **34b** efficiently bound to the surface of MDM2 by an induced fitting, which provided very potent inhibitory activity as depicted in Figure 10. On the other hand, we presumed that the most plausible metabolic site of compound **34b** would be the pyrrolidine and the terminal dimethylamide moiety. Thus, novel pyrrolidine variants bearing alkyl groups onto the adjacent position to the pyrrolidine amide linkage were designed to circumvent the metabolism (Figure 11). We further explored the structure activity relationship on the terminal proline amide moiety, together with aiming to manipulate the physicochemical properties by the optimization. Various piperazino- or morpholino- variants at the C-2 amide position of (5*R*)-alkylpyrrolidine (**73-77** in Table

6) were designed at aiming to improve the solubility and metabolic stability. While the solubility of some compounds, i.e. **73** and **74**, were improved, metabolic stabilities were decreased compared to **71** or **72** (*vide infra*). We hypothesized that the plausible metabolic site would be piperazine moiety; more specifically adjacent carbon to nitrogen of the piperazine, which was the same in the case for the pyrrolidine. Thus, further modification, i.e. incorporation of the methyl group onto the piperazine moiety to avoid the metabolism was executed (see the results of **78-86** in Table 6).

Syntheses of methyl and ethyl incorporated variants including (*S*)- and (*R*)- isomers are displayed in Scheme 5 and 6. 5-Oxo-L-proline derivatives (**52** or **61**) were utilized for making both (*S*)- and (*R*)- alkylated derivatives, and each of the products were furnished through different synthetic routes. Syntheses of (*5S*)-alkylpyrrolidine derivatives are depicted in Scheme 5.³⁴ After protection of carboxylic acid and amide of compound **52** with the conventional manner, ring-opening reaction of **53** with alkyllithium, ring-closure via deprotection followed by stereoselective hydrogenation provided (*5S*)-alkylated derivatives (**55a** and **55b**), regioselectively. EDC mediated amidation with methylamine, and deprotection with HCl furnished the (*5S*)-alkylpyrrolidine intermediates (**57a** and **57b**) as pure products, which were confirmed by NMR or LC/MS. Compounds **59** and **60** were finally prepared via condensation reaction with dihydroimidazothiazole carboxylic acid (**27b**), reported in chapter I. On the other hand, (*5R*)-alkylpyrrolidine derivatives were synthesized as shown in Scheme 6. The amide of compound **62**, which was prepared via the esterification using HClO₄ and *t*-BuOAc from **61**, was reduced with LiBHET₃ to give amina **63**. Copper-catalyzed alkylation provided (*5R*)-alkylated derivatives (**64a** and **64b**) regioselectively, which were also confirmed by NMR or LC/MS.³⁵⁻³⁸ Compounds **71-86** were prepared via two different routes by using key intermediates **64a** and **64b** as shown in Scheme 6.

III-3. Evaluation of dihydroimidazothiazole derivatives

The result of *in vitro* activity (IC_{50S}), growth inhibitions (GI_{50S}), solubility, and metabolic stability for the final compounds is displayed in Table 6. IC_{50S} were measured by HTRF-based assay, and GI_{50S} were measured by antiproliferable cells assay using

MV4-11 and DLD-1 respectively having a wild type p53 (p53wt) and a mutated p53 (p53mut). By comparing the GI₅₀s in both cells, we checked indiscriminate cytotoxicity not resulted from p53-MDM2 inhibitory activity. Compound's solubility was measured for using neutral aqueous solution (pH 6.8), and metabolic stability was measured for the remaining ratio (% rem) after 30 min treatment with mouse hepatic microsome *in vitro*. All compounds with an alkyl moiety on the C-2 position of the pyrrolidine (**59**, **60**, **71** and **72**) showed improvement of metabolic stability compared to **34b**, as expected (39-70 % rem). However, solubility of (5*S*)-methyl variant (**59**) decreased, and cellular activity of (5*S*)-ethyl one (**60**) for MV4-11 diminished. On the other hand, both compounds having (5*R*)-alkyl moieties (**71** and **72**) showed good metabolic stabilities (more than 50% rem) with potent cellular activities. While compounds **73** and **75** each possessing *N*-methylpiperazino substituent were twice more soluble than dimethylcarbamoyl variants (**71** and **72**), these resulted in lower metabolic stability. The same tendency was also observed for compound **74** and **76** having *N*-acetylpiperazino moiety. The morpholino group as in compound **77** diminished both solubility and metabolic stability.

For further optimization to improve the metabolic stability, we executed modification of the terminal piperazine moiety. All compounds (**78-86**) bearing the methyl group onto the C-3 position of piperazine exhibited good metabolic stability (54-84 % rem). It was suggested that metabolic oxidation was prevented by incorporating an alkyl group adjacent to the nitrogen of the cyclic amine or amide. Both stereochemistries of the methyl group on C-3 position were considered valuable for cellular activity on MV4-11, solubility and metabolic stability. Compounds having (5*R*)-ethyl group (**72**, **75-77**, **83-86**) on the pyrrolidine moiety tend to provide somewhat increased cellular activity against DLD-1. Among these, GI₅₀ of **85** (for DLD-1) was 10 μM, which was weakest in the series.

III-4. Antitumor efficacy of promising compounds having the dihydroimidazothiazole scaffold

We selected **78** and **85** for the PK study, which exhibited high p53-MDM2 inhibitory activity, selectivity between MV4-11 and DLD-1, and good metabolic stabilities.

Although compound **78** showed low concentrations in plasma compared with **85** because of high penetration into tissues, both compounds showed large AUC in plasma and good exposure in the tumor with an oral administration on mice (Table 7). We then evaluated antitumor efficacy of these compounds against an MV4-11 xenograft model on mice. As shown in Figure 12, compounds **78** and **85** showed a significant tumor growth inhibition (TGI) with single administration per day (200mg/kg, po). At the end of treatments (day 31), TGIs were 71% (**78**) and 76 % (**85**) with only slight body weight loss (<5 %). Significant toxicity was not observed in both compounds. In comparison, Nutlin-3 (racemate)¹² was also evaluated as a positive control, and we confirmed a high antitumor effect (TGI = 87%) at MTD dosage; po, 200mg/kg/day, bid dosing. The total amount of administration was more than twice compared to compounds **78** and **85**, delivering a significant (9 %) body weight loss. In the *in vivo* study, we checked the p53 induction by using Western blot analysis (Figure 13). Both **78** and **85** increased p53 as well as MDM2 and p21 in protein levels compared to the control (non-treated). This data indicated that potent antitumor efficacies of the compounds were derived from re-activation of p53 functions by robust p53-MDM2 inhibition.

Co-crystal structure analysis of compound **85** and MDM2 was performed. The structure of the complex is displayed in Figure 14. It was observed that ethylated pyrrolidine ring also generated a unique hydrophobic interaction by induced-fitting with MDM2, which was the same as non-alkylated pyrrolidine variant **34b** in our previous research (Figure 14A). Furthermore, the stacking model of co-crystal structures for **34b**, **85** and Nutlin-2 (PDB code: 3VZV, 3W69 and 1RV1) is depicted in Figure 14B. In the orange circled area, MDM2 loop with **85** is also distorted and changed the three-dimensional structure in a similar way to that with **34b** by the induced fitting, which was not observed at Nutlin-2. This should confirm the reason why our compounds, modified on the pyrrolidine moiety, kept strong p53-MDM2 inhibitory activity. On the other hand, no interaction of the terminal piperazine moiety with MDM2 was observed. The moiety was found to direct outside of the protein, suggesting that it would be possible to further improve the physicochemical property by incorporating a hydrophilic moiety to the terminal site of the molecule.

In conclusion, we executed lead optimization of our potent lead for the improvement

of physicochemical properties. Incorporation of an alkyl group on the pyrrolidine ring at the C-2 substituent worked to avoid the metabolism. Also, further optimization by introducing piperazine moieties and its modification improved both solubility and metabolic stability. As a result of co-crystal structure analysis, ethylated pyrrolidine variants kept holding the extra hydrophobic affinity for binding with MDM2 by induced-fitting. Compounds **78** and **85** exhibited good PK profiles and robust antitumor efficacy against the MV4-11 xenograft model with oral administrations.

Figures in Chapter III

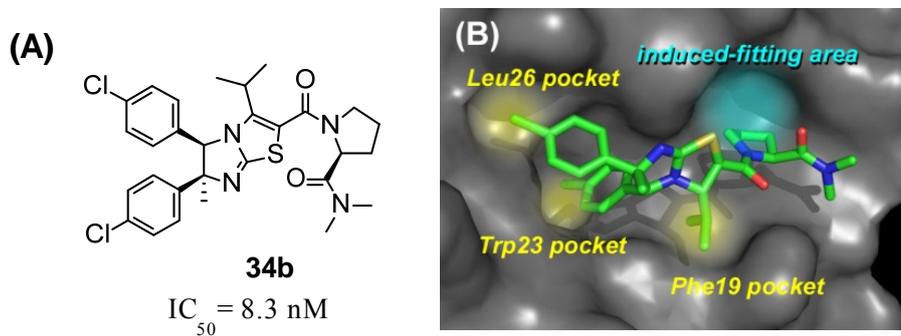


Figure 10. (A) Structure of our lead compound **34b** possessing dihydroimidazothiazole scaffold. (B) The crystallographic analysis of compound **34b**/MDM2 (PDB code: 3VZV).

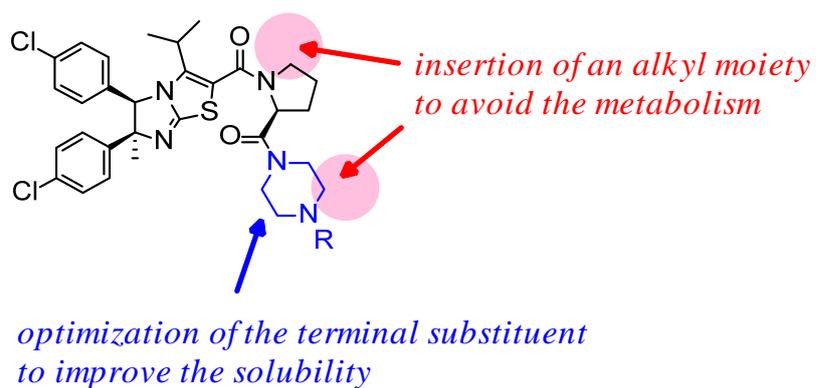
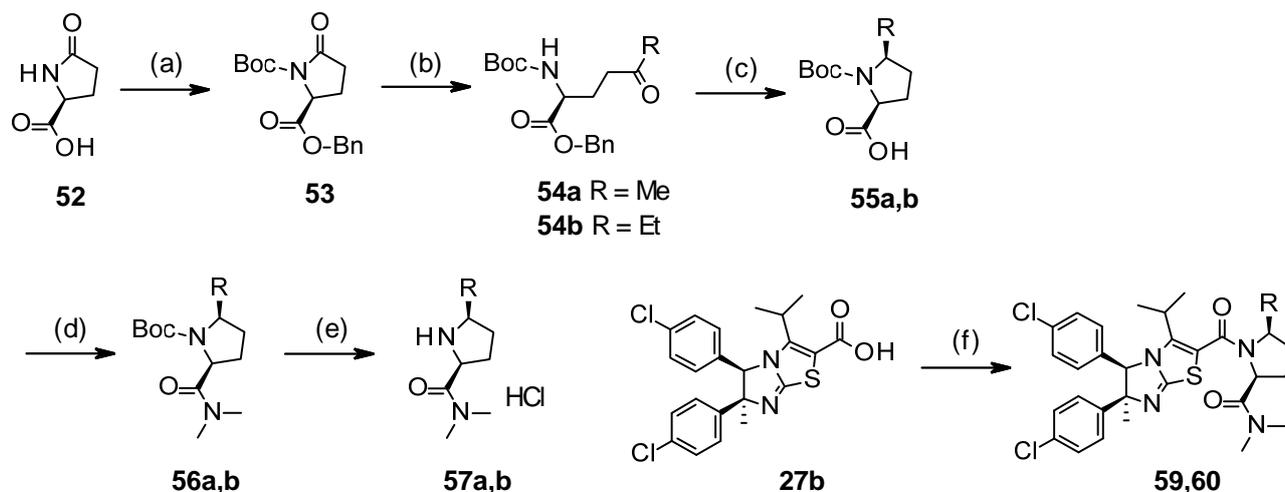


Figure 11. Designs for aiming to improve the physicochemical properties.

Scheme 5. Synthesis of (5*S*)-alkylated proline derivative **59** and **60**^a



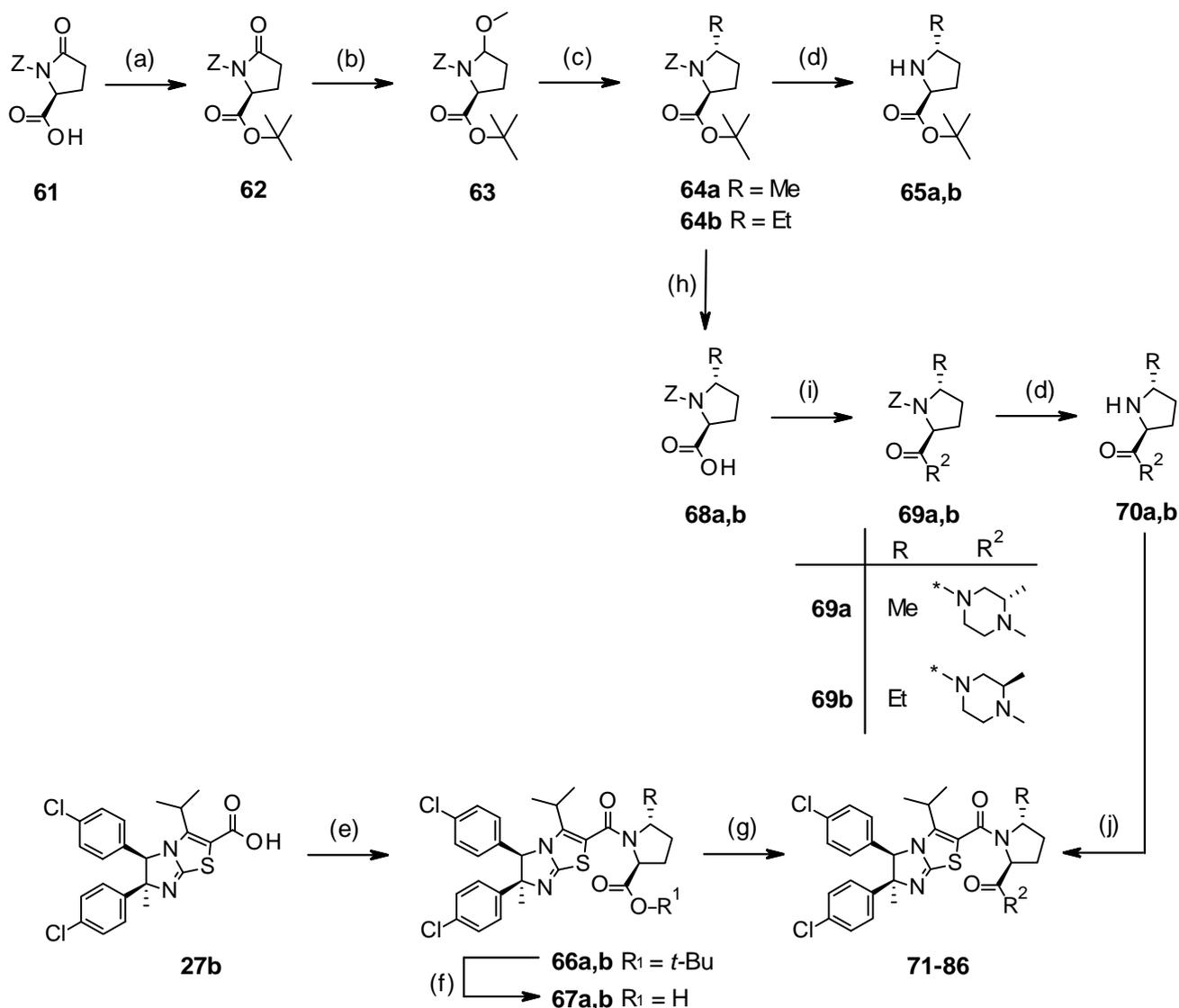
^a Reagents and Conditions:

(a) i) BnCl, Et₃N, THF, reflux; ii) Boc₂O, Et₃N, DMAP, CH₂Cl₂, rt; (b) RLi, THF, -78°C~rt;

(c) i) TFA, CH₂Cl₂; ii) H₂, Pd/C, MeOH; iii) Boc₂O, NaOHaq., MeCNaq.; (d) Me₂NH, EDC/HCl, HOBt, Et₃N, CH₂Cl₂;

(e) HCl/Dioxane, 50°C; (f) i) SOCl₂, *cat.* DMF, Toluene, 70°C; ii) **57a** or **57b**, Et₃N, THF

Scheme 6. Synthesis of (5*R*)-alkylated proline derivative 71-86^a



^a Reagents and Conditions:

(a) HClO₄aq., *t*-BuOAc; (b) i) LiBHET₃, THF, -78°C; ii) *p*-TsOH, MeOH; (c) MeLi, BF₃/Et₂O, CuBr-Me₂S, Et₂O, -78°C~rt; (d) H₂, Pd/C, MeOH;

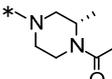
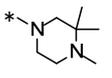
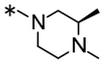
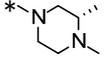
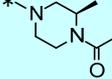
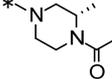
(e) SOCl₂, *cat.* DMF, Toluene, 70°C, then **65a** or **65b**, Et₃N, THF; (f) TFA, anisole, CHCl₃; (g) HNR₂, EDC·HCl, HOBT, DIPEA or Et₃N, DMF

(h) TFA, CHCl₃, 50°C; (i) amine, EDC/HCl, HOBT, Et₃N, CH₂Cl₂; (j) **27b**, SOCl₂, *cat.* DMF, Toluene, 70°C, then Et₃N, THF

Table 6

In vitro activity for the p53-MDM2 binding interaction and physicochemical properties of dihydroimidazothiazoles

compd	R	R ²	HTRF	MV4-11 ^a	DLD-1 ^b	Solubility ^c (μg/mL)	MS ^d (% rem)
			IC ₅₀ (μM)	GI ₅₀ (μM)			
34b	H		0.0083	0.020	20	57	9
59	(<i>S</i>)-Me		0.014	0.11	>50	<3	39
60	(<i>S</i>)-Et		0.039	2.4	>50	12	47
71	(<i>R</i>)-Me		0.014	0.09	8.5	29	53
72	(<i>R</i>)-Et		0.018	0.13	7.4	7	70
73	(<i>R</i>)-Me		0.016	0.19	9.0	45	25
74	(<i>R</i>)-Me		0.026	0.27	16	79	13
75	(<i>R</i>)-Et		0.030	0.083	4.0	13	50
76	(<i>R</i>)-Et		0.043	0.13	9.3	34	38
77	(<i>R</i>)-Et		0.068	0.094	4.2	9	6
78	(<i>R</i>)-Me		0.015	0.20	6.9	49	74
79	(<i>R</i>)-Me		0.016	0.37	6.2	47	63
80	(<i>R</i>)-Me		0.030	0.083	11	72	55

cmpd	R	R ²	HTRF	MV4-11 ^a	DLD-1 ^b	Solubility ^c (μg/mL)	MS ^d (% rem)
			IC ₅₀ (μM)	GI ₅₀ (μM)			
81	(R)-Me		0.037	0.40	5.2	75	65
82	(R)-Me		0.018	0.28	6.3	30	84
83	(R)-Et		0.029	0.11	3.6	14	76
84	(R)-Et		0.030	0.25	3.9	16	69
85	(R)-Et		0.058	0.22	10	25	59
86	(R)-Et		0.059	0.12	2.6	24	54

^a cell line with p53 wild type.

^b cell line with mutated p53.

^c pH 6.8 phosphate buffer solution.

^d *in vitro* metabolic stability in mouse hepatic microsome (% remaining after 30 min).

Table 7

AUC and concentration in plasma and tumor with an oral administration to mouse at 100 mg/kg

compd	AUC _{plasma} ($\mu\text{g}\cdot\text{h}/\text{mL}$)	Concentration in plasma ($\mu\text{g}/\text{mL}$)					Concentration in tumor ($\mu\text{g}/\text{mL}$)				
		1 h	2 h	4 h	6 h	24 h	1 h	2 h	4 h	6 h	24 h
78	93.1	6.9	7.8	7.5	5.7	0.3	18.9	23.3	27.9	27.5	4.3
85	737.1	59.6	68.4	46.4	48.2	0.1	31.5	32.7	31.2	25.2	0.0

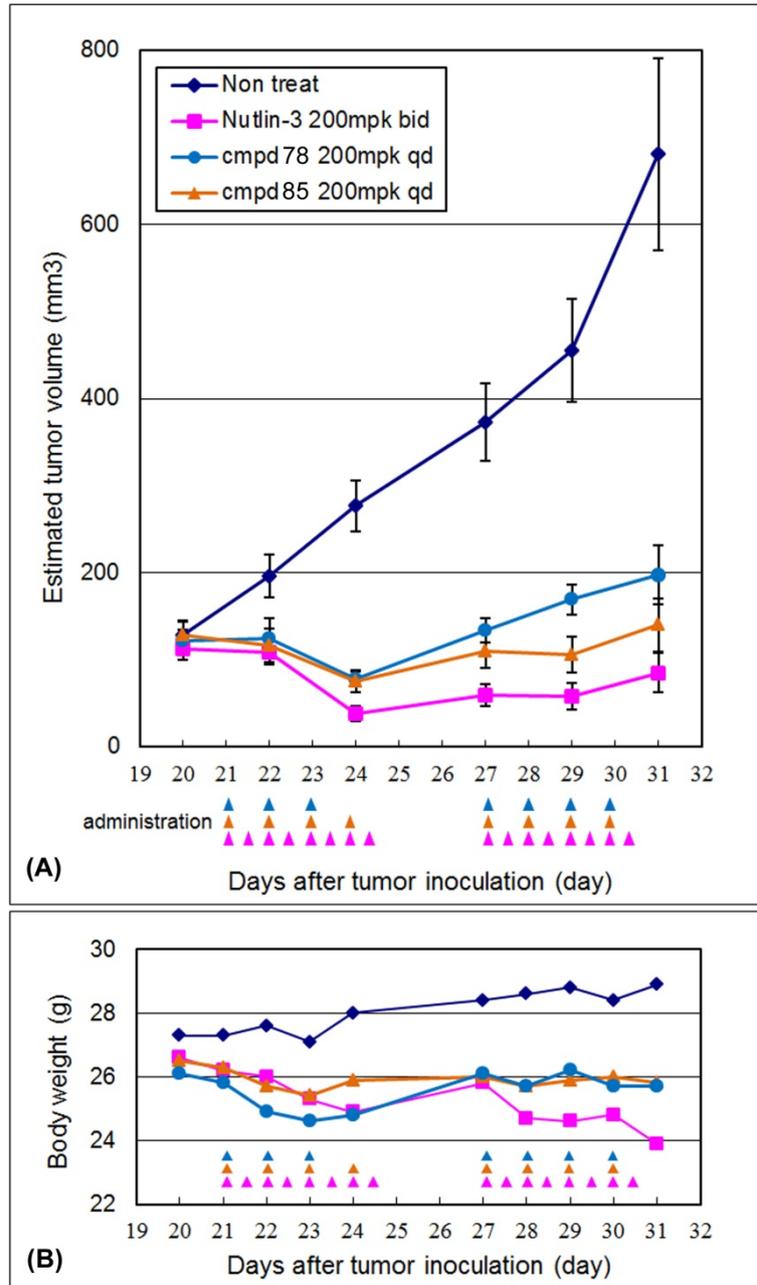


Figure 12. Antitumor efficacy of compound **78** and **85** in MV4-11 xenograft model on mice: (A) Tumor Volume; (B) Body Weight.

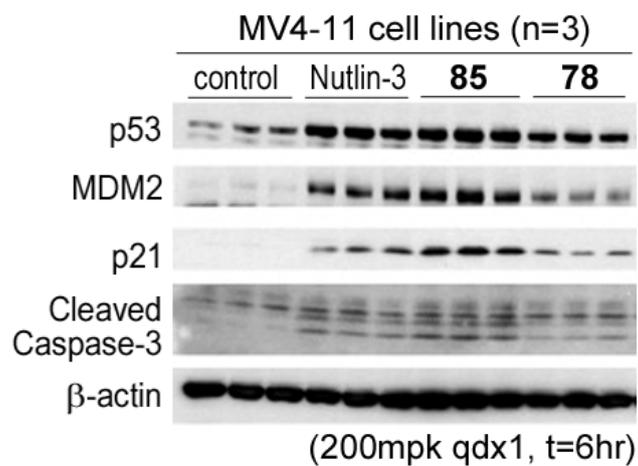


Figure 13. Western blot analysis of racemic Nutlin-3 (positive control), **78** and **85** on p53 induction in xenografted MV4-11 cells. Mice were sacrificed 6 h afteradministration.

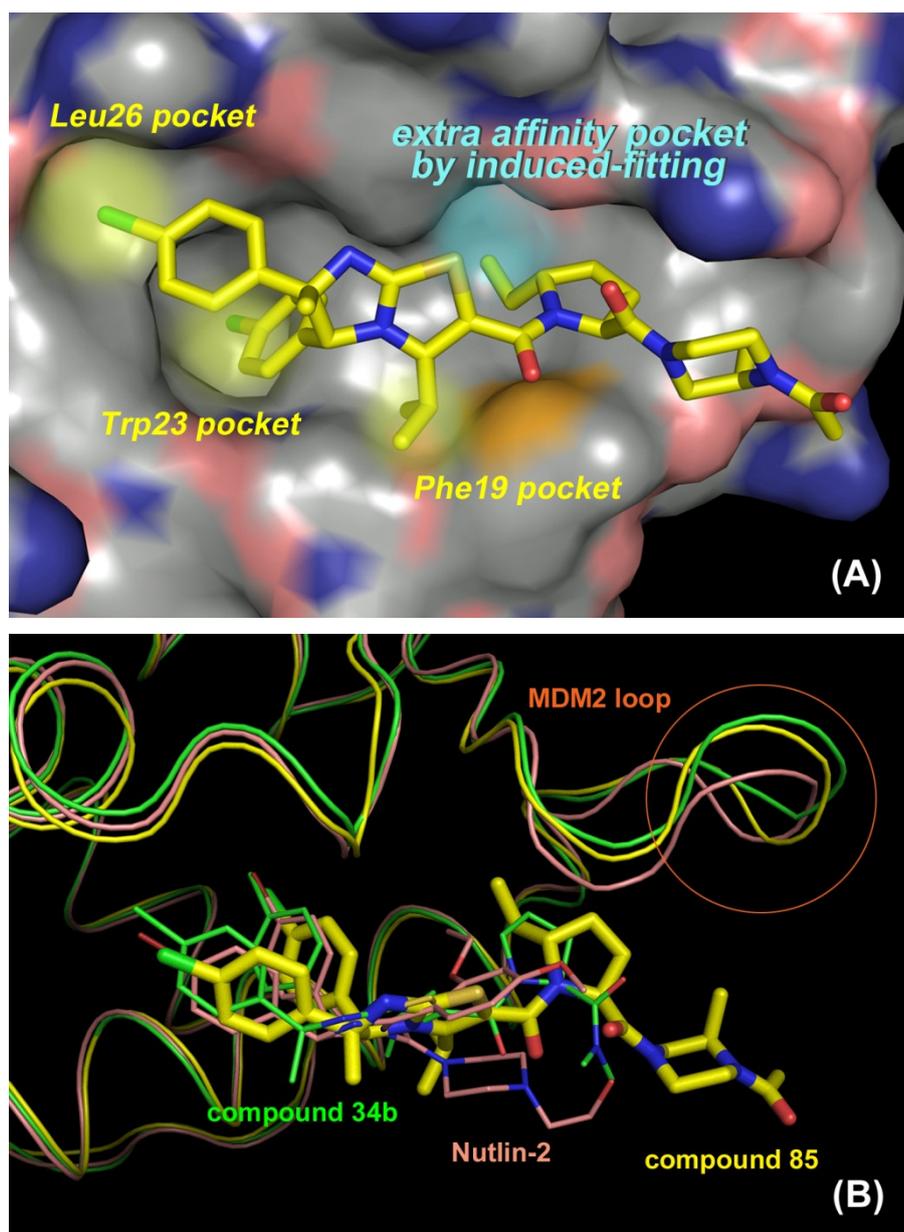


Figure 14. (A) Co-crystal structure of **85** in yellow with MDM2 (PDB code: 3W69). The (5*R*)-ethylpyrrolidine moiety also generated hydrophobic interaction against MDM2. (B) The stacking model of co-crystal structures for **34b** in green, **85** in yellow and Nutlin-2 in light red (PDB code: 3VZV, 3W69 and 1RV1). MDM2 loop with **85** is also distorted and changed the three-dimensional structure in a similar way to that with **34b** by the induced fitting, which was not observed at Nutlin-2 (orange circled area).

Conclusions

We have investigated the study of the discovery, lead optimization and biological evaluation of novel p53-MDM2 interaction inhibitors possessing a dihydroimidazothiazole scaffold as a novel anticancer agent. In this study, there were some valuable achievements as follows;

1. Starting with Nutlins as an initial lead, we designed and generated bicyclic scaffolds aiming to place *cis*-bischlorophenyl moiety at the equivalent location where the hydrophobic interaction with MDM2 could be expected. As a result, novel MDM2 inhibitors possessing a dihydroimidazothiazole scaffold were discovered. Further exploration of the side chains on the dihydroimidazothiazole scaffold aided by molecular modeling resulted in compounds exhibiting almost comparable *in vitro* potency to Nutlin-3a.

2. With the aim of discovering potent inhibitors of the p53-MDM2 interaction and thus obtaining a potent anticancer drug, we have pursued synthesis and optimization of dihydroimidazothiazole derivatives as mentioned in Chapter I. Upon the discovery, a problem involving the chemical instability of the scaffold, *i.e.* susceptibility to oxidation which led to imidazothiazole was encountered. In order to solve this problem and to obtain further potent compounds, we executed medicinal research and thus furnished the optimal compounds by incorporating the methyl group onto the C-6 position to avoid the oxidation, and by modifying the C-2 moiety of the additional proline motif, which furnished high potency. The incorporation of the pyrrolidine moiety at the C-2 position raised another hydrophobic interaction site with MDM2 protein, which was generated by the induced-fitting observed by co-crystal structure analysis. These optimal molecules showed significant improvement in potency when compared with our early lead or Nutlin-3a.

3. The lead compounds which were reported in the previous chapters showed strong activity *in vitro*, but did not exhibit antitumor efficacy *in vivo* for the low metabolic stability. In order to obtain orally active compounds, we executed further optimization of our lead by the improvement of physicochemical properties. Thus optimal compounds were furnished by introducing an alkyl group onto the pyrrolidine at the C-2

substituent to prevent the metabolism; and modifying the terminal substituent of the proline motif improved solubility. These compounds exhibited good PK profiles and significant antitumor efficacy with single oral administration per day (200mg/kg, po) on a xenograft model on mice using MV4-11 cells having wild type p53 without toxicity.

From these results, it was confirmed that the dihydroimidazothiazole derivatives were potent p53-MDM2 interaction inhibitors and novel orally active anticancer agents. Having the potent lead compounds such as **78** and **85**, further investigation to furnish the promising candidates could be undertaken.

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ADME data for this study.

Experimental Sections

General methods and Materials on chemistry

All reagents and solvents were purchased from commercially available suppliers and were of reagent grade. Flash silica gel column chromatography was performed with SHOKO Scientific Purif- α 2[®] by using of Purif-Pack[®] Si or NH₂ cartridges. Thin-layer chromatography (TLC) was performed on Merck pre-coated TLC glass sheets with Silica Gel 60 F₂₅₄, and compound visualization was detected with a UV lamp, a solution of phosphomolybdic acid in ethanol, or Wako ninhydrin spray. Melting points were uncorrected. Chiral HPLC analysis was performed by using JASCO LC-2000plus HPLC system. ¹H-NMR spectra were measured on JEOL JNM-EX400, JNM-ECX-400P and JNM-ECS-400 spectrometers with CDCl₃ or DMSO-d₆ as solvent, and chemical shifts are given in ppm (δ) from tetramethylsilane as an internal standard. Mass spectra (ESI/MS) were measured on Agilent 1100 series LC/MSD mass spectrometers with an electrospray ionization source. High resolution mass spectra were measured on JEOL JMS-T100LP spectrometer with an electrospray ionization source (HRESI/MS) or JEOL JMS-7000 spectrometer (HREI/MS). IR spectra were measured on a JASCO FT/IR-6100 spectrometer by using the ATR method.

Experimental Section of Chapter I

General procedure for the final products;

4,5-*cis*-Bis(4-chlorophenyl)imidazolidine-2-thione (6)

Carbon disulfide (489 μ l, 8.11 mmol) was added to a solution of *meso*-1,2-bis(4-chlorophenyl)ethylenediamine (1.52 g, 5.41 mmol) in EtOH (20 ml). The reaction mixture was refluxed for 15 h at 90°C. After cooling the mixture to room temperature, the solvent was removed *in vacuo*. MeOH and Et₂O were added to the residue, and the solid precipitated out was collected by suction filtration to give the product as a colorless solid (1.23 g, 70% yield); ¹H-NMR (400 MHz, CDCl₃) δ : 5.33 (2H, s), 6.25 (2H, br s), 6.86 (4H, d, J = 8.5 Hz), 7.12 (4H, d, J = 8.5 Hz).

Ethyl

5,6-*cis*-bis(4-chlorophenyl)-3-isopropyl-5,6-dihydroimidazo[2,1-b][1,3]thiazole-2-carboxylate (7c)

Compound **6** (200 mg, 0.62 mmol) was added to a solution of ethyl 2-chloro-4-methyl-3-oxopentanoate (**12c**) (155 mg, 0.80 mmol) in EtOH (10 ml). The reaction mixture was refluxed for 20 h at 90°C. After cooling the mixture to room temperature, the solvent was removed *in vacuo*. The residue was diluted with saturated aqueous sodium bicarbonate, and extracted with chloroform. The organic layer was washed with brine, and dried over sodium sulfate. The mixture was filtered, and the solvent was removed *in vacuo* to afford the crude product. Purification by preparative TLC on silica gel (developing solvent: 5% MeOH/CHCl₃ then 30% EtOAc/Hexane) provided the product as a colorless solid (190 mg, 67% yield); ¹H-NMR (400 MHz, CDCl₃) δ: 0.89 (3H, d, *J* = 7.2 Hz), 1.05 (3H, d, *J* = 7.2 Hz), 1.34 (3H, t, *J* = 7.2 Hz), 3.33-3.43 (1H, m), 4.26 (2H, q, *J* = 7.2 Hz), 5.44 (1H, d, *J* = 9.3 Hz), 5.89 (1H, d, *J* = 9.3 Hz), 6.65 (2H, br d, *J* = 7.8 Hz), 6.96 (2H, d, *J* = 8.3 Hz), 7.04-7.11 (4H, m); FAB/MS: *m/z* = 461 (M+H).

5,6-*cis*-Bis(4-chlorophenyl)-3-isopropyl-5,6-dihydroimidazo[2,1-b][1,3]thiazole-2-carboxylic acid (8c)

1N aqueous sodium hydroxide solution (26.3 ml, 26.3 mmol) was added to a solution of compound **7** (8.08 g, 17.5 mmol) in EtOH (500 ml). The reaction mixture was refluxed for 5.5 h at 90°C. After cooling to room temperature, the solvent was removed *in vacuo*. 1N aqueous hydrogen chloride solution was added to the residue until pH became ca. 1, and the solid precipitated out under sonication was collected by suction filtration, washed with H₂O, and dried *in vacuo* (7.46 g, 60% purity). The crude product was used directly in the next step without further purification.

4-[5,6-*cis*-Bis(4-chlorophenyl)-3-isopropyl-5,6-dihydroimidazo[2,1-b]thiazole-2-carbonyl]piperazin-2-one (9c)

Triethylamine (6.64 ml, 47.6 mmol) was added dropwise to a suspension of crude mixture **8c** (7.46 g, ca. 15.9 mmol) in CH₂Cl₂ (500 ml) under an ice cooling bath,

followed by 2-piperazinone (1.75 g, 17.5 mmol) and EDC/HCl (3.65 g, 19.1 mmol). The solution was warmed to room temperature and stirred for 17 h. The reaction mixture was quenched by the addition of saturated aqueous sodium bicarbonate, and extracted with chloroform. The organic layer was washed with brine, and dried over sodium sulfate. The mixture was filtered, and the solvent was removed *in vacuo* to afford crude product. The residue was purified by silica gel column chromatography (eluent: CHCl₃ to 10% MeOH/CHCl₃) and preparative TLC on silica gel (developing solvent: 10% MeOH/CHCl₃) to give the colorless solid **9c** (2.71 g, 30% yield from **7c**), which was recrystallized from EtOH/Et₂O; ¹H-NMR (400 MHz, CDCl₃) δ: 0.96 (3H, d, *J* = 7.1 Hz), 1.00 (3H, d, *J* = 7.1 Hz), 2.53-2.62 (1H, m), 3.45-3.52 (2H, m), 3.79-3.91 (2H, m), 4.29 (2H, br s), 5.36 (1H, d, *J* = 9.4 Hz), 5.91 (1H, d, *J* = 9.4 Hz), 6.23 (1H, br s), 6.66 (2H, d, *J* = 8.4 Hz), 6.96 (2H, d, *J* = 8.4 Hz), 7.05-7.12 (4H, m); FAB/MS: *m/z* = 515 (M+H); IR (ATR) cm⁻¹: 3187, 3072, 2931, 2890, 1687, 1608, 1581, 1490, 1459, 1407; Anal. Calcd for C₂₅H₂₄Cl₂N₄O₂S: C, 58.25; H, 4.69; N, 10.87. Found: C, 58.05; H, 4.58; N, 10.75.

Experimental Section of Chapter II

General procedure for optical resolutions;

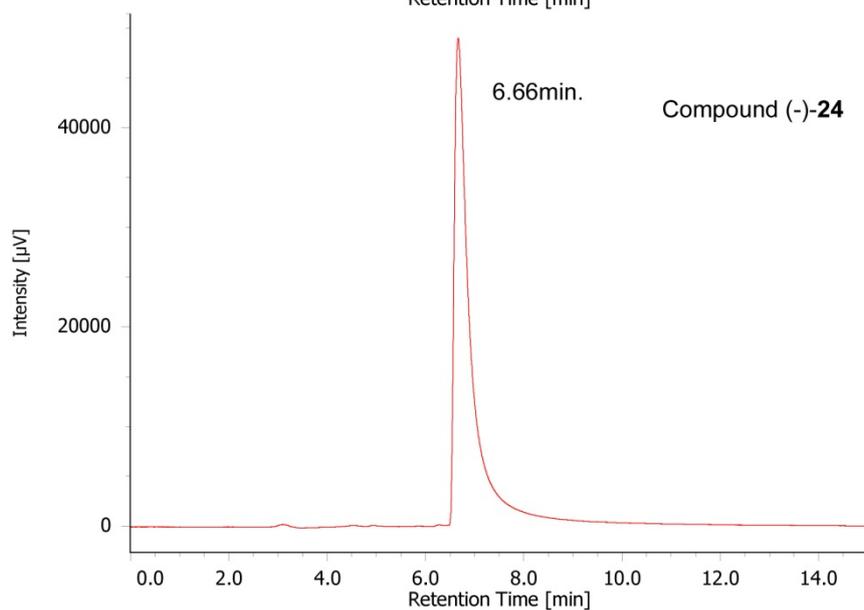
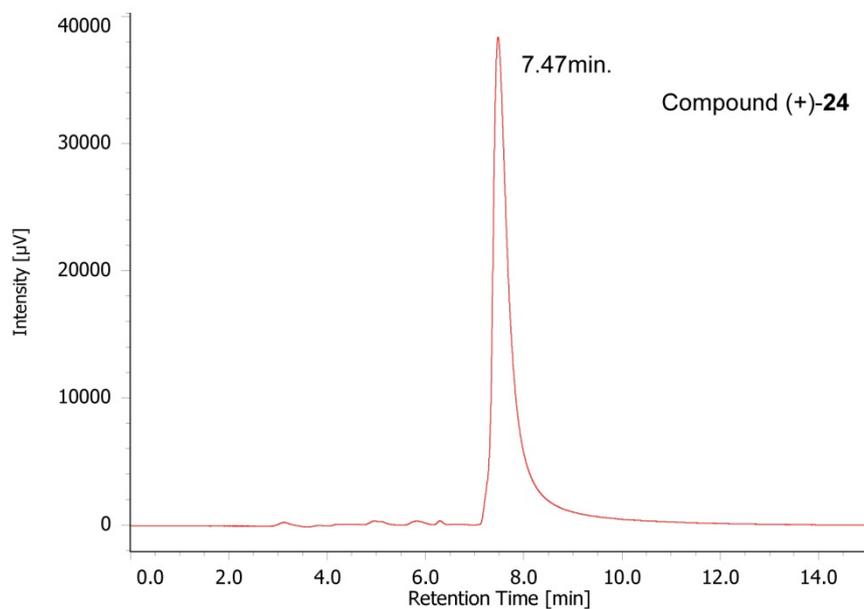
Step A. L-(+)-Tartaric acid (5.05 g, 33.9 mmol) was added to a solution of (*IR**,*2S**)-1,2-bis(4-chlorophenyl)propane-1,2-diamine (*(rac)*-**24**) (10.0 g, 33.9 mmol) in EtOH (100 ml). The reaction mixture was refluxed until complete dissolution of the acid occurred. After cooling the mixture to room temperature, the solvent was removed *in vacuo*. EtOH and Et₂O were added, and the colorless solid precipitate was filtered. The solid was dissolved in 1N aqueous NaOH solution, and extracted with Et₂O. The organic layer was dried over potassium carbonate, and the reaction mixture was filtered. The solvent was removed *in vacuo* to afford (+)-**24** as a colorless solid (3.05 g, 31 %); ¹H-NMR (400 MHz, CDCl₃) δ: 1.48 (3H, s), 1.50 (4H, br s), 4.08 (1H, s), 6.98 (2H, d, *J* = 8.5 Hz), 7.17 (2H, d, *J* = 8.5 Hz), 7.24-7.27 (4H, m); ¹³C-NMR (100 MHz, CDCl₃) δ: 26.95, 58.06, 64.88, 127.67, 127.88, 129.53, 132.52, 132.94, 139.96, 144.36; ESI/MS: *m/z* = 295 (M+H).

Step B. The filtrate after precipitation was also evaporated *in vacuo*. The residue was basified with 1N aqueous NaOH solution, and was extracted with Et₂O. The organic layer was dried over potassium carbonate. Then the mixture was filtered, and the solvent was removed to give an oil (7.00 g, 23.7 mmol). D-(-)-Tartaric acid (3.56 g, 23.7 mmol) was added to a solution of the oil in EtOH (100 ml), and the reaction mixture was refluxed until complete dissolution of the acid occurred. After cooling the mixture to room temperature, the solid precipitated out from aqueous EtOH solution was collected by suction filtration. The solid was dissolved in 1N aqueous NaOH solution, and extracted with Et₂O. The organic layer was dried over potassium carbonate, and the reaction mixture was filtered. The solvent was removed *in vacuo* to afford (-)-**24** as a colorless solid (3.85 g, 39 %); ¹H-NMR (400 MHz, CDCl₃) δ: 1.48 (7H, br s), 4.08 (1H, s), 6.98 (2H, d, *J* = 8.5 Hz), 7.17 (2H, d, *J* = 8.5 Hz), 7.25-7.26 (4H, m); ¹³C-NMR (100 MHz, CDCl₃) δ: 26.95, 58.08, 64.88, 127.68, 127.89, 129.54, 132.54, 132.96, 139.95, 144.35; ESI/MS: *m/z*= 295 (M+H).

HPLC data of compound (+)-**24** and (-)-**24** after chiral resolutions;

DAICEL CHIRALPAK[®] AS-H, 4.6 x 250mm, hexane : IPA = 80:20 (v/v), Flow rate:

1.0 ml/min, rt.



Analytical data of the final product **34b**;

$^1\text{H-NMR}$ (400MHz, CDCl_3) δ : 0.97 (6H, d, $J = 6.3$ Hz), 1.81 (3H, s), 1.90-1.96 (2H, m), 2.16-2.18 (1H, br m), 2.22-2.24 (1H, br m), 2.65-2.67 (1H, br m), 2.95 (3H, s), 3.12 (3H, s), 3.68-3.73 (1H, m), 3.76-3.82 (1H, m), 4.89-4.91 (1H, br m), 4.96 (1H, s), 6.70 (2H, d, $J = 7.3$ Hz), 7.02 (2H, d, $J = 8.8$ Hz), 7.03 (2H, d, $J = 8.5$ Hz), 7.11 (2H, d, $J = 8.5$ Hz); $^{13}\text{C-NMR}$ (100 MHz, CDCl_3) δ : 19.29, 21.25, 25.04, 28.38, 28.90, 29.97, 35.98, 37.04, 49.42, 56.85, 74.03, 77.20, 77.63, 83.86, 127.59, 128.35, 128.47, 128.71, 132.26, 133.70, 135.90, 140.92, 143.13, 161.64, 165.59, 170.93; ESI-MS m/z : 571(M+H); HREI-MS m/z : 570.1626. (Calcd for $\text{C}_{29}\text{H}_{32}^{35}\text{Cl}_2\text{N}_4\text{O}_2\text{S}$: 570.1623); IR (ATR) cm^{-1} : 1646, 1625, 1563, 1490, 1419, 1390, 1311, 1091, 1012; Anal. Calcd for $\text{C}_{29}\text{H}_{32}\text{Cl}_2\text{N}_4\text{O}_2\text{S}$: C, 60.94; H, 5.64; N, 9.80; Cl, 12.41; S, 5.61. Found: C, 60.90; H, 5.83; N, 9.68; Cl, 12.22; S, 5.66.

Experimental Section of Chapter III

2-Benzyl 1-*tert*-butyl (2*S*)-5-oxopyrrolidine-1,2-dicarboxylate (53)

Benzyl chloride (25.3 ml, 0.22 mol) was added to a solution of 5-oxo-L-proline (**52**) (25.8 g, 0.20 mol) with triethylamine (28.0 ml, 0.20 mol) in THF (260 ml). The reaction mixture was refluxed at 70°C for 5 days. After cooling the mixture to room temperature, the solvent was removed *in vacuo*. The residue was diluted with water, and extracted with chloroform. The organic layer was washed with brine, and dried over magnesium sulfate. The mixture was filtered, and the solvent was removed *in vacuo* to afford the crude product as pale brown oil. To a stirred solution of the product in dichloromethane (400 ml) was added di-*tert*-butyl dicarbonate (44 g, 0.20 mol), triethylamine (28 ml, 0.2 mol) and DMAP (12.2 g, 0.10 mol) under an ice cooling bath. The solution was warmed to room temperature and stirred for 16 h. The reaction mixture was diluted with water, and extracted with chloroform. The organic layer was washed with brine, and dried over magnesium sulfate. The mixture was filtered, and the solvent was removed *in vacuo* to afford crude product. The residue was purified by flash silica gel chromatography with *n*-hexane/EtOAc (2:1, v/v) to give the colorless solid (51.2 g, 80% yield); ¹H-NMR (400 MHz, CDCl₃) δ: 1.42 (9H, s), 1.98-2.05 (1H, m), 2.26-2.37 (1H, m), 2.44-2.51 (1H, m), 2.57-2.66 (1H, m), 4.64 (1H, dd, *J* = 9.5, 2.9 Hz), 5.19 (1H, d, *J* = 12.0 Hz), 5.23 (1H, d, *J* = 12.2 Hz), 7.34-7.37 (5H, m); ESI/MS: *m/z* = 342 (M+Na).

Benzyl (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-5-oxohexanoate (54a)

To a stirred solution of compound **53** (11.0 g, 0.034 mol) in dry THF (100 ml) was added methyllithium (1.04 M in Et₂O, 34.0 ml, 0.034 mol) at -78°C under a nitrogen atmosphere, and the resulting solution was warmed to room temperature and stirred for 2 h. The reaction mixture was diluted with saturated aqueous NH₄Cl under an ice cooling bath, and extracted with EtOAc. The organic layer was washed with brine, and dried over magnesium sulfate. The mixture was filtered, and the solvent was removed *in vacuo* to afford the crude product. The residue was purified by flash silica gel chromatography with *n*-hexane/EtOAc (3:1, v/v) to give the colorless oil (9.2 g, 91% yield); ¹H-NMR (400 MHz, CDCl₃) δ: 1.43 (9H, s), 1.88-1.95 (1H, m), 2.09 (3H, s),

2.10-2.15 (1H, m), 2.43-2.57 (2H, m), 4.29-4.34 (1H, br m), 5.12-5.21 (3H, m), 7.34-7.39 (5H, m); ESI/MS: m/z = 358 (M+Na).

Benzyl (2S)-2-[(*tert*-butoxycarbonyl)amino]-5-oxoheptanoate (54b)

Compound **54b** was prepared as a colorless oil (49% yield) from **53** according to a similar procedure for the synthesis of **54a**; $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ : 1.02 (3H, t, J = 7.3 Hz), 1.43 (9H, s), 1.88-1.96 (1H, m), 2.09-2.15 (1H, m), 2.37 (2H, q, J = 7.3 Hz), 2.38-2.52 (2H, m), 4.32 (1H, br s), 5.11 (1H, br s), 5.13 (1H, d, J = 12.2 Hz), 5.19 (1H, d, J = 12.2 Hz), 7.34-7.38 (5H, m); ESI/MS: m/z = 372 (M+Na).

(5S)-1-(*tert*-Butoxycarbonyl)-5-methyl-L-proline (55a)

Trifluoroacetic acid (10 ml) was added to a solution of compound **54a** (4.7 g, 0.014 mol) in dichloromethane (30 ml). The reaction mixture was stirred for 3 h at room temperature. The solvent was removed *in vacuo*, and the residue was azeotroped with toluene to afford the crude product. To a solution of the product in methanol (50 ml) was added 10% Pd/C (500 mg, 50% wetted, type AD). The resulting mixture was stirred under a hydrogen atmosphere (1 atm) for 16 h at room temperature. The mixture was filtered to remove the catalyst, and the filtrate was concentrated *in vacuo* to afford the crude product. To a solution of the product in acetonitrile (60 ml) with water (10 ml) was added di-*tert*-butyl dicarbonate (4.58 g, 21 mmol) and 1N aqueous sodium hydroxide solution (35 ml, 35 mmol). The resulting solution was stirred for 1 h at room temperature. After removing the solvent *in vacuo*, the residue was diluted with chloroform, and extracted with water. The aqueous layer was diluted with saturated aqueous NH_4Cl , and the solvent was removed *in vacuo*. The residue was diluted with 10% MeOH/ CHCl_3 solution, and dried over magnesium sulfate. The mixture was filtered, and the solvent was removed *in vacuo* to afford the crude product. Purification by flash silica gel chromatography with $\text{CHCl}_3/\text{MeOH}$ (15:1, v/v) provided the title compound (2.14 g, 67% yield from **4a**); $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ : 1.25 (3H, d, J = 6.3 Hz), 1.48 (9H, s), 1.63-1.68 (1H, m), 2.00-2.09 (2H, m), 2.26-2.33 (1H, m), 3.91-3.97 (1H, m), 4.30-4.34 (1H, m); ESI/MS: m/z = 252 (M+Na).

(5S)-1-(tert-Butoxycarbonyl)-5-ethyl-L-proline (55b)

Compound **55b** was prepared (80% yield) from **54b** according to a similar procedure for the synthesis of **55a**; ¹H-NMR (400 MHz, CDCl₃) δ: 0.87 (3H, t, *J* = 7.1 Hz), 1.39-1.45 (1H, m), 1.48 (9H, s), 1.71-1.77 (2H, m), 1.93-2.00 (1H, m), 2.09-2.11 (1H, br m), 2.33-2.35 (1H, br m), 3.79-3.82 (1H, br m), 4.32-4.34 (1H, br m); ESI/MS: *m/z*= 266 (M+Na).

tert-Butyl (2S,5S)-2-[(dimethylamino)carbonyl]-5-methylpyrrolidine-1-carboxylate (56a)

Triethylamine (280 μl, 2.02 mmol) was added to a solution of compound **55a** (232 mg, 1.01 mmol) in dichloromethane (6 ml), followed by dimethylamine hydrochloride (124 mg, 1.52 mmol), HOBt (14 mg, 0.10 mmol) and EDC/HCl (233 mg, 1.21 mmol). The solution was stirred for 16 h at room temperature. The solvent was removed *in vacuo* to afford crude product. The residue was purified by flash silica gel chromatography with CHCl₃/MeOH (50:1, v/v) to give the colorless oil (161 mg, 62% yield); ¹H-NMR (400 MHz, CDCl₃) δ: 1.35 (3H, d, *J* = 6.1 Hz), 1.40 and 1.46 (9H, each s), 1.66-1.75 (1H, m), 1.87-1.93 (1H, m), 2.01-2.12 (2H, m), 2.97 (3H, s), 3.07 and 3.11 (3H, s), 3.91-3.95 and 4.02-4.07 (1H, m), 4.53-4.58 and 4.68-4.72 (1H, m); ESI/MS: *m/z*= 157 (M-Boc).

(5S)-N,N,5-Trimethyl-L-prolinamide hydrochloride (57a)

4N HCl/dioxane solution (2 ml) was added to a solution of compound **56a** (160 mg, 0.62 mmol) in 1,4-dioxane (4 ml). The resulting solution was warmed to 50°C and stirred for 1.5 h. After cooling to room temperature, the solvent was removed *in vacuo* to give the product as a colorless solid (152 mg, quantitative yield); ¹H-NMR (400 MHz, DMSO-d₆) δ: 1.32 (3H, d, *J* = 6.6 Hz), 1.49-1.58 (1H, m), 1.81-1.90 (1H, m), 2.03-2.11 (1H, m), 2.32-2.42 (1H, m), 2.89 (3H, s), 2.98 (3H, s), 3.57-3.61 (1H, m), 4.55-4.60 (1H, m); ESI/MS: *m/z*= 157 (M+H).

(5S)-5-Ethyl-N,N-dimethyl-L-prolinamide hydrochloride (57b)

Triethylamine (265 μ l, 1.9 mmol) was added to a solution of compound **55b** (231 mg, 0.95 mmol) in dichloromethane (6 ml), followed by dimethylamine hydrochloride (116 mg, 1.43 mmol), HOBt (13 mg, 0.095 mmol) and EDC/HCl (220 mg, 1.14 mmol). The solution was stirred for 16 h at room temperature. The solvent was removed *in vacuo* to afford crude product. The residue was purified by flash silica gel chromatography with CHCl₃/MeOH (80:1, v/v) to give the colorless oil (**56b**). To a stirred solution of the product in 1,4-dioxane (4 ml) was added 4N HCl/dioxane solution (2 ml). The resulting solution was warmed to 50°C and stirred for 1 h. After cooling to room temperature, the solvent was removed *in vacuo* to give the product as a colorless oil (144 mg, 72% yield from **55b**); ¹H-NMR (400 MHz, DMSO-d₆) δ : 0.93 (3H, t, *J* = 7.4 Hz), 1.47-1.55 (1H, m), 1.60-1.68 (1H, m), 1.78-1.89 (2H, m), 2.05-2.12 (1H, m), 2.29-2.39 (1H, m), 2.89 (3H, s), 2.99 (3H, s), 3.34-3.39 (1H, m), 4.54-4.61 (1H, m); ESI/MS: *m/z* = 171 (M+H).

(5S)-1-{[(5R,6S)-5,6-Bis(4-chlorophenyl)-3-isopropyl-6-methyl-5,6-dihydroimidazo[2,1-b][1,3]thiazol-2-yl]carbonyl}-N,N,5-trimethyl-L-prolinamide (59)

Thionyl chloride (250 μ l, 3.4 mmol) and catalytic amount of DMF were added to a suspension of compound **27b** (231 mg, 0.52 mmol) in toluene (4 ml). The resulting mixture was warmed to 70°C and stirred for 30 min. After cooling to room temperature, the solvent was removed *in vacuo* to afford the crude product. The residue was dissolved in THF (6 ml), and was dropwised to a solution of compound **57a** (119 mg, 0.62 mmol) with triethylamine (181 μ l, 1.3 mmol) in THF (4 ml) under an ice cooling bath. The resulting solution was warmed to room temperature, and was stirred for 30 min. The reaction mixture was quenched by the addition of saturated aqueous sodium bicarbonate, and extracted with EtOAc. The organic layer was washed with brine, and dried over magnesium sulfate. The mixture was filtered, and the solvent was removed *in vacuo* to afford crude product. The residue was purified by flash silica gel column chromatography with CHCl₃/MeOH (30:1, v/v) to give the colorless solid (227 mg, 75% yield), which was precipitated from Et₂O/*n*-hexane; ¹H-NMR (400 MHz, CDCl₃) δ : 0.89 (3H, d, *J* = 7.3 Hz), 0.95 (3H, d, *J* = 7.1 Hz), 1.45 (3H, d, *J* = 6.3 Hz), 1.80 (3H, s), 1.80-1.85 (1H, m), 1.96-2.01 (1H, m), 2.02-2.10 (1H, m), 2.12-2.18 (1H, m), 2.63-2.71

(1H, m), 2.96 (3H, s), 3.11 (3H, s), 4.22-4.28 (1H, m), 4.84-4.88 (1H, m), 4.96 (1H, s), 6.69 (2H, d, $J = 8.3$ Hz), 7.00-7.04 (4H, m), 7.10 (2H, d, $J = 8.5$ Hz); ESI/MS: $m/z = 585$ (M+H); HRESI/MS $m/z = 585.18193$ (Calcd for $C_{30}H_{35}^{35}Cl_2N_4O_2S$: 585.18578); IR (ATR) cm^{-1} : 1639, 1617, 1592, 1546, 1442, 1388, 1313, 1089, 1014.

(5S)-1-[(5R,6S)-5,6-Bis(4-chlorophenyl)-3-isopropyl-6-methyl-5,6-dihydroimidazo[2,1-b][1,3]thiazol-2-yl]carbonyl]-5-ethyl-N,N-dimethyl-L-prolinamide (60)

Compound **60** was prepared (82% yield) as the colorless solid from **57b** according to a similar procedure for the synthesis of **59**; 1H -NMR (400 MHz, $CDCl_3$) δ : 0.88 (3H, d, $J = 7.1$ Hz), 0.93 (3H, t, $J = 8.2$ Hz), 0.96 (3H, d, $J = 7.1$ Hz), 1.71-1.76 (1H, m), 1.80 (3H, s), 1.85-2.17 (5H, m), 2.65-2.71 (1H, m), 2.95 (3H, s), 3.10 (3H, s), 3.96-4.03 (1H, m), 4.82-4.88 (1H, m), 4.96 (1H, s), 6.69 (2H, d, $J = 8.3$ Hz), 7.01 (4H, d, $J = 8.5$ Hz), 7.10 (2H, d, $J = 8.3$ Hz); ESI/MS: $m/z = 599$ (M+H); HRESI/MS $m/z = 599.19352$ (Calcd for $C_{31}H_{37}^{35}Cl_2N_4O_2S$: 599.20143); IR (ATR) cm^{-1} : 1639, 1621, 1592, 1548, 1492, 1388, 1313, 1087, 1014.

1-Benzyl 2-tert-butyl (2S)-5-oxopyrrolidine-1,2-dicarboxylate (62)

70% aqueous perchloric acid (1.65 ml, ca. 0.065 mol) was dropwised to a suspension of compound **61** in *t*-BuOAc (200 ml) at room temperature, and the resulting mixture was stirred for 16 h. Saturated aqueous sodium bicarbonate (300 ml) was added to the solution gradually, and most of the *t*-BuOAc in the mixture was removed *in vacuo*. The residue was extracted with chloroform, and the organic layer was washed with brine. After drying over magnesium sulfate, the mixture was filtered, and the solvent was removed *in vacuo* to give the colorless oil (13.9 g, 79% yield); 1H -NMR (400 MHz, $CDCl_3$) δ : 1.39 (9H, s), 2.01-2.08 (1H, m), 2.27-2.36 (1H, m), 2.45-2.53 (1H, m), 2.59-2.68 (1H, m), 4.55 (1H, dd, $J = 9.4, 2.6$ Hz), 5.25 (1H, d, $J = 12.4$ Hz), 5.30 (1H, d, $J = 12.2$ Hz), 7.31-7.41 (5H, m); ESI/MS: $m/z = 342$ (M+Na).

1-Benzyl 2-tert-butyl (2S)-5-methoxypyrrolidine-1,2-dicarboxylate (63)

To a stirred solution of compound **62** (13.9 g, 0.044 mol) in dry THF (50 ml) was added $LiBHET_3$ (1.01 M in THF, 100 ml, 0.101 mol) at $-78^\circ C$ under a nitrogen

atmosphere, and the resulting solution was stirred for 30 min. Methanol (100 ml) was dropwised to the solution, and the solvent was removed *in vacuo*. The residue was diluted with saturated aqueous sodium bicarbonate, and extracted with chloroform. The organic layer was washed with brine, and dried over magnesium sulfate. The mixture was filtered, and the solvent was removed *in vacuo* to afford the crude product. To a solution of the product in MeOH (100 ml) was added *p*-toluenesulfonic acid monohydrate (837 mg, 4.4 mmol) at room temperature, and the resulting solution was stirred for 2 days. After removing the solvent *in vacuo*, the residue was diluted with saturated aqueous sodium bicarbonate, and extracted with chloroform. The organic layer was washed with brine, and dried over magnesium sulfate. The mixture was filtered, and the solvent was removed *in vacuo*. The residue was purified by flash silica gel chromatography with *n*-hexane/EtOAc (4:1, v/v) to give the colorless oil (11.2 g, 76% yield); ¹H-NMR (400 MHz, CDCl₃) δ: 1.32 and 1.44, or 1.37 and 1.47 (9H, each s), 1.76-2.12 (3H, m), 2.28-2.37 (1H, m), 3.26 and 3.43, or 3.35 and 3.47 (3H, each s), 4.22-4.30 (1H, m), 5.10-5.38 (3H, m), 7.28-7.37 (5H, m); ESI/MS: *m/z* = 358 (M+Na).

1-Benzyl 2-*tert*-butyl (2*S*,5*R*)-5-methylpyrrolidine-1,2-dicarboxylate (64a)

To a stirred suspension of CuBr/Me₂S (11.7 g, 0.058 mol) in dry Et₂O (100 ml) was added methyllithium (0.98 M in Et₂O, 59 ml, 0.058 mol) at -78°C under a nitrogen atmosphere. After stirring for 30 min at -78°C, BF₃/Et₂O (7.35 ml, 0.058 mmol) was added to the resulting mixture. After stirring for 5 min at -78°C, compound **63** (9.6 g, 0.029 mol) in Et₂O (30 ml) was dropwised to the mixture. Then the reaction was warmed to room temperature, and stirred for 16 h. The reaction mixture was filtered, and the filtrate was quenched by the addition of 1N aqueous HCl (100 ml). After stirring for 1 h, the solution was extracted with EtOAc. The organic layer was washed with brine, and dried over magnesium sulfate. The mixture was filtered, and the solvent was removed *in vacuo* to afford crude product. The residue was purified by flash silica gel column chromatography with *n*-hexane/EtOAc (4:1, v/v) to give the colorless oil (8.68 g, 94% yield); ¹H-NMR (400 MHz, CDCl₃) δ: 1.15 and 1.23 (3H, each d, *J* = each 6.3 Hz), 1.33 and 1.44 (9H, each s), 1.51-1.57 (1H, m), 1.89-1.95 (1H, m), 2.09-2.29

(2H, m), 4.13-4.28 (2H, m), 5.02-5.22 (2H, m), 7.22-7.38 (5H, m); ESI/MS: $m/z=342$ (M+Na).

1-Benzyl 2-*tert*-butyl (2*S*,5*R*)-5-ethylpyrrolidine-1,2-dicarboxylate (64b)

Compound **64b** was prepared (78% yield) as the colorless oil from **63** according to a similar procedure for the synthesis of **64a**; $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ : 0.83 and 0.89 (3H, each t, each $J = 7.4$ Hz), 1.33 and 1.44 (9H, each s), 1.66-1.73 (2H, m), 1.83-1.94 (2H, m), 1.99-2.07 (1H, m), 2.15-2.23 (1H, m), 3.92-3.99 (1H, m), 4.24 (1H, t, $J = 7.8$ Hz), 5.06-5.20 (2H, m), 7.27-7.36 (5H, m); ESI/MS: $m/z=356$ (M+H).

***tert*-Butyl (5*R*)-5-methyl-L-prolinate (65a)**

To a solution of compound **64a** (4.0 g, 0.013 mol) in methanol (50 ml) was added 10% Pd/C (400 mg, 50% wetted, type AD). The resulting mixture was stirred under a hydrogen atmosphere (1 atm) for 16 h at room temperature. The mixture was filtered to remove the catalyst, and the filtrate was concentrated *in vacuo* to afford the crude product. The residue was purified by flash silica gel column chromatography with $\text{CHCl}_3/\text{MeOH}$ (30:1, v/v) to give the colorless solid (2.17 g, 90% yield); $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ : 1.22 (3H, d, $J = 6.1$ Hz), 1.38-1.43 (1H, m), 1.47 (9H, s), 1.80-1.88 (1H, m), 1.88-1.95 (1H, m), 2.23-2.31 (1H, m), 3.38-3.46 (1H, m), 3.87 (1H, dd, $J = 8.7, 6.2$ Hz); ESI/MS: $m/z=186$ (M+H).

***tert*-Butyl (5*R*)-5-ethyl-L-prolinate (65b)**

Compound **65b** was prepared (94% yield) as the colorless oil from **64b** according to a similar procedure for the synthesis of **65a**; $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ : 1.06 (3H, t, $J = 7.4$ Hz), 1.51 (9H, s), 1.81-1.95 (2H, m), 1.97-2.08 (1H, m), 2.13-2.27 (2H, m), 2.48-2.56 (1H, m), 3.58-3.66 (1H, m), 4.46 (1H, t, $J = 7.9$ Hz); ESI/MS: $m/z=200$ (M+H).

***tert*-Butyl (5*R*)-1-[(5*R*,6*S*)-5,6-Bis(4-chlorophenyl)-3-isopropyl-6-methyl-5,6-dihydroimidazo[2,1-*b*][1,3]thiazol-2-yl]carbonyl]-5-methyl-L-prolinate (66a)**

Thionyl chloride (500 μ l, 6.9 mmol) and catalytic amount of DMF were added to a suspension of compound **27b** (300 mg, 0.67 mmol) in toluene (4 ml). The resulting mixture was warmed to 70°C and stirred for 30 min. After cooling to room temperature, the solvent was removed *in vacuo* to afford the crude product. The residue was dissolved in THF (6 ml), and was dropwised to a solution of compound **65a** (137 mg, 0.74 mmol) with triethylamine (224 μ l, 1.6 mmol) in THF (4 ml) under an ice cooling bath. The resulting solution was warmed to room temperature, and was stirred for 1 h. The reaction mixture was quenched by the addition of saturated aqueous sodium bicarbonate, and extracted with EtOAc. The organic layer was washed with brine, and dried over magnesium sulfate. The mixture was filtered, and the solvent was removed *in vacuo* to afford crude product. The residue was purified by flash silica gel column chromatography with *n*-hexane/EtOAc (2:1, v/v) to give the colorless solid (340 mg, 83% yield); ¹H-NMR (400 MHz, CDCl₃) δ : 0.98-1.04 (6H, m), 1.20 (3H, d, *J* = 6.3 Hz), 1.44-1.45 (1H, br m), 1.45 (9H, s), 1.80 (3H, s), 1.98-2.00 (1H, br m), 2.25-2.28 (2H, br m), 2.62-2.65 (1H, br m), 4.51-4.54 (2H, br m), 4.95 (1H, s), 6.69 (2H, d, *J* = 8.1 Hz), 7.00-7.04 (4H, m), 7.13 (2H, d, *J* = 8.5 Hz); ESI/MS: *m/z* = 614 (M+H).

***tert*-Butyl (5*R*)-1-{[(5*R*,6*S*)-5,6-bis(4-chlorophenyl)-3-isopropyl-6-methyl-5,6-dihydroimidazo[2,1-*b*][1,3]thiazol-2-yl]carbonyl}-5-ethyl-L-prolinate (**66b**)**

Compound **66b** was prepared (78% yield) as the pale yellow solid from **65b** according to a similar procedure for the synthesis of **66a**; ¹H-NMR (400 MHz, CDCl₃) δ : 0.91 (3H, t, *J* = 7.4 Hz), 0.96 (3H, d, *J* = 7.3 Hz), 1.04 (3H, d, *J* = 7.1 Hz), 1.35-1.41 (1H, m), 1.45 (9H, s), 1.75-1.80 (2H, m), 1.80 (3H, s), 1.95-2.00 (1H, m), 2.12-2.23 (2H, m), 2.70-2.77 (1H, m), 4.26-4.31 (1H, m), 4.52-4.57 (1H, m), 4.94 (1H, s), 6.67 (2H, d, *J* = 8.3 Hz), 7.00 (2H, d, *J* = 8.5 Hz), 7.02 (2H, d, *J* = 8.5 Hz), 7.13 (2H, d, *J* = 8.5 Hz); ESI/MS: *m/z* = 628 (M+H).

(5*R*)-1-{[(5*R*,6*S*)-5,6-Bis(4-chlorophenyl)-3-isopropyl-6-methyl-5,6-dihydroimidazo[2,1-*b*][1,3]thiazol-2-yl]carbonyl}-5-methyl-L-proline (67a**)**

Trifluoroacetic acid (40 ml) and anisole (1.63 ml, 0.015 mol) were added to a solution of compound **66a** (9.0 g, 0.015 mol) in chloroform (100 ml). After stirring at room

temperature for 1 h, the resulting mixture was warmed to 40°C and stirred for 1 h. After cooling to room temperature, the solvent was removed *in vacuo*, and the residue was azeotroped with toluene to afford the crude product. The residue was purified by flash silica gel column chromatography with CHCl₃/MeOH (15:1, v/v) to give the colorless solid (5.76 g, 69% yield), which was precipitated from Et₂O/*n*-hexane; ¹H-NMR (400 MHz, DMSO-d₆) δ: 0.92 (3H, d, *J* = 7.3 Hz), 0.95 (3H, d, *J* = 6.8 Hz), 1.16 (3H, d, *J* = 6.1 Hz), 1.63-1.67 (1H, m), 1.94 (3H, s), 1.96-2.01 (1H, m), 2.04-2.10 (1H, m), 2.34-2.40 (1H, m), 2.67-2.75 (1H, m), 4.26-4.35 (1H, m), 4.52-4.59 (1H, m), 5.89 (1H, s), 6.84-6.92 (2H, m), 7.17 (4H, d, *J* = 8.3 Hz), 7.23 (2H, d, *J* = 8.5 Hz); ESI/MS: *m/z*= 558 (M+H).

(5*R*)-1-[(5*R*,6*S*)-5,6-Bis(4-chlorophenyl)-3-isopropyl-6-methyl-5,6-dihydroimidazo [2,1-*b*][1,3]thiazol-2-yl]carbonyl]-5-ethyl-L-proline (67b)

Compound **67b** was prepared (quantitative yield) as the pale brown solid from **66b** according to a similar procedure for the synthesis of **67a**; ¹H-NMR (400 MHz, DMSO-d₆) δ: 0.84 (3H, t, *J* = 7.3 Hz), 0.91-0.97 (6H, m), 1.33-1.42 (1H, m), 1.74-1.96 (4H, m), 2.04 (3H, s), 2.26-2.38 (1H, m), 2.70-2.77 (1H, m), 4.04-4.09 (1H, m), 4.65-4.70 (1H, m), 6.16 (1H, s), 7.19-7.27 (8H, m), 8.30 (1H, s); ESI/MS: *m/z*= 572 (M+H).

(5*R*)-1-[(Benzyloxy)carbonyl]-5-methyl-L-proline (68a)

Trifluoroacetic acid (10ml) was added to a solution of compound **64a** (4.3 g, 0.013 mol) in chloroform (30 ml), and the resulting solution was warmed to 50°C and stirred for 16 h. After cooling to room temperature, the solvent was removed *in vacuo*, and the residue was azeotroped with toluene to afford the crude product. The residue was purified by flash silica gel column chromatography with CHCl₃/MeOH (10:1, v/v) to give the colorless oil (3.87 g, quantitative yield); ¹H-NMR (400 MHz, CDCl₃) δ: 1.15-1.28 (3H, m), 1.53-1.63 (1H, m), 2.05-2.34 (3H, m), 4.12-4.25 (1H, m), 4.38-4.47 (1H, m), 5.06-5.23 (2H, m), 7.25-7.37 (5H, m); ESI/MS: *m/z*= 264 (M+H).

(5*R*)-1-[(Benzyloxy)carbonyl]-5-ethyl-L-proline (68b)

Compound **68b** was prepared (quantitative yield) as the pale brown solid from **64b** according to a similar procedure for the synthesis of **68a**; ¹H-NMR (400 MHz, CDCl₃) δ: 0.85 and 0.89 (3H, each t, *J* = 7.6 and 7.9 Hz), 1.31-1.48 (2H, m), 1.73-1.79 and 1.82-1.89 (1H, m), 2.02-2.32 (3H, m), 3.89-3.94 and 3.96-4.02 (1H, m), 4.41 (1H, dd, *J* = 13.5, 8.9 Hz), 5.06-5.23 (2H, m), 7.20-7.38 (5H, m); ESI/MS: *m/z*= 278 (M+H).

Benzyl (2*S*,5*R*)-2-[(3*S*)-3,4-dimethylpiperazin-1-yl]carbonyl}-5-methylpyrrolidine-1-carboxylate (69a)

Triethylamine (443 μl, 3.18 mmol) was added to a solution of compound **68a** (334 mg, 1.27 mmol) in dichloromethane (6 ml), followed by (2*S*)-1,2-dimethylpiperazine dihydrochloride (285 mg, 1.52 mmol), HOBt (17 mg, 0.13 mmol) and EDC/HCl (292 mg, 1.52 mmol). The solution was stirred for 16 h at room temperature. The solvent was removed *in vacuo* to afford crude product. The residue was purified by flash silica gel chromatography with CHCl₃/MeOH (50:1, v/v) to give the colorless oil (161 mg, 51% yield); ¹H-NMR (400 MHz, CDCl₃) δ: 0.99 and 1.09 (3H, each dd, *J* = 13.0, 6.2 and 14.4, 6.3 Hz), 1.18 and 1.25 (3H, each d, *J* = 6.3 and 6.6 Hz), 1.51-1.58 (1H, m), 1.75-1.88 (1H, m), 2.04-2.31 (5H, m), 2.37-2.54 (1H, m), 2.61-2.81 (2H, m), 2.96-3.07 (1H, m), 3.17-3.32 (1H, m), 3.46-3.81 (1H, m), 4.19-4.36 (2H, m), 4.61-4.75 (1H, m), 4.95-5.26 (2H, m), 7.28-7.36 (5H, m); ESI/MS: *m/z*= 360 (M+H).

Benzyl (2*S*,5*R*)-2-[(3*R*)-3,4-dimethylpiperazin-1-yl]carbonyl}-5-methylpyrrolidine-1-carboxylate (69b)

Compound **69b** was prepared (70% yield) as the colorless oil from **68b** according to a similar procedure for the synthesis of **69a**; ¹H-NMR (400 MHz, CDCl₃) δ: 0.84 and 0.90 (3H, each t, *J* = 7.6 and 7.4 Hz), 1.00 and 1.07 (3H, each d, *J* = 6.3 and 5.9 Hz), 1.35-1.42 (1H, m), 2.04-2.24 (4H, m), 2.25 and 2.29 (3H, each s), 2.64-2.90 (2H, m), 3.45-3.69 (1H, m), 3.97-4.11 (1H, m), 4.15-4.34 (1H, m), 4.59-4.74 (1H, m), 4.96-5.04 (1H, m), 5.08 and 5.22 (1H, each d, *J* = 12.5 and 12.2 Hz), 7.27-7.35 (5H, m); ESI/MS: *m/z*= 374 (M+H).

(2S)-1,2-Dimethyl-4-[(5R)-5-methyl-L-prolyl]piperazine (70a)

To a solution of compound **69a** (230 mg, 0.64 mmol) in methanol (10 ml) was added 10% Pd/C (100 mg, 50% wetted, type AD). The resulting mixture was stirred under a hydrogen atmosphere (1 atm) for 24 h at room temperature. The mixture was filtered to remove the catalyst, and the filtrate was concentrated *in vacuo* to give the pale yellow solid (153 mg, 100% yield); ¹H-NMR (400 MHz, DMSO-d₆) δ: 0.96-1.02 (3H, m), 1.17 (3H, d, *J* = 6.6 Hz), 1.38-1.49 (1H, m), 1.61-1.73 (1H, m), 1.89-1.99 (2H, m), 2.18 (3H, s), 2.24-2.34 (1H, m), 2.68-2.74 (1H, m), 2.80-2.88 (1H, m), 3.28-3.53 (3H, m), 3.59-3.76 (1H, m), 3.96-4.12 (1H, m), 4.22-4.31 (1H, m); ESI/MS: *m/z* = 226 (M+H).

(2R)-1,2-Dimethyl-4-[(5R)-5-ethyl-L-prolyl]piperazine (70b)

Compound **70b** was prepared (quantitative yield) as the pale yellow solid from **69b** according to a similar procedure for the synthesis of **70a**; ¹H-NMR (400 MHz, DMSO-d₆) δ: 0.95 (3H, t, *J* = 7.4 Hz), 1.09-1.11 (3H, m), 1.59-1.69 (2H, m), 1.78-1.88 (2H, m), 2.08-2.14 (1H, m), 2.34 (3H, br s), 2.42-2.47 (1H, m), 2.88-3.11 (5H, m), 3.46-3.55 (1H, m), 3.74-3.82 (1H, m), 4.03-4.20 (1H, m), 4.58-4.66 (1H, m); ESI/MS: *m/z* = 240 (M+H).

(5R)-1-[(5R,6S)-5,6-Bis(4-chlorophenyl)-3-isopropyl-6-methyl-5,6-dihydroimidazo[2,1-*b*][1,3]thiazol-2-yl]carbonyl}-*N,N*,5-trimethyl-L-prolinamide (71)

Diisopropylethylamine (215 μl, 1.25 mmol) was added to a solution of compound **67a** (300 mg, 0.54 mmol) in DMF (6 ml), followed by Dimethylamine dihydrochloride (61 mg, 0.75 mmol), HOBt (7 mg, 0.05 mmol) and EDC/HCl (116 mg, 0.60 mmol). The solution was stirred for 16 h at room temperature. The reaction mixture was diluted with saturated aqueous sodium bicarbonate, and extracted with EtOAc. The organic layer was washed with brine (3 times), and dried over magnesium sulfate. The mixture was filtered, and the solvent was removed *in vacuo* to afford the crude product. The residue was purified by flash silica gel chromatography with CHCl₃/MeOH (30:1, v/v) to give the pale orange solid (116 mg, 40% yield), which was precipitated from Et₂O/*n*-hexane; ¹H-NMR (400 MHz, CDCl₃) δ: 0.94 (3H, d, *J* = 7.1 Hz), 1.04 (3H, d, *J* = 7.1 Hz), 1.23 (3H, d, *J* = 6.3 Hz), 1.60-1.65 (1H, m), 1.79 (3H, s), 1.82-1.88 (1H, m), 2.26-2.38 (2H,

m), 2.74-2.78 (1H, m), 2.91 (3H, s), 3.11 (3H, s), 4.53-4.57 (1H, m), 4.93 (1H, s), 5.01 (1H, d, $J = 8.5$ Hz), 6.68 (2H, d, $J = 8.3$ Hz), 7.00 (2H, d, $J = 8.5$ Hz), 7.01 (2H, d, $J = 8.5$ Hz), 7.13 (2H, d, $J = 8.5$ Hz); ESI/MS: $m/z = 585$ (M+H); HRESI/MS $m/z = 585.18176$ (Calcd for $C_{30}H_{35}^{35}Cl_2N_4O_2S$: 585.18578); IR (ATR) cm^{-1} : 1646, 1596, 1490, 1403, 1363, 1091, 1012.

(5R)-1-[(5R,6S)-5,6-Bis(4-chlorophenyl)-3-isopropyl-6-methyl-5,6-dihydroimidazo[2,1-*b*][1,3]thiazol-2-yl]carbonyl]-5-ethyl-*N,N*-dimethyl-L-prolinamide (72)

Compound **72** was prepared (51% yield) as the colorless solid from **67b** according to a similar procedure for the synthesis of **71**; 1H -NMR (400 MHz, $CDCl_3$) δ : 0.88-0.95 (6H, m), 1.05 (3H, d, $J = 7.1$ Hz), 1.37-1.43 (1H, m), 1.75-1.80 (2H, m), 1.79 (3H, s), 1.83-1.87 (1H, m), 2.21-2.28 (2H, m), 2.74-2.85 (1H, m), 2.91 (3H, s), 3.10 (3H, s), 4.34-4.40 (1H, m), 4.93 (1H, s), 4.98-5.02 (1H, m), 6.66 (2H, d, $J = 8.3$ Hz), 6.99-7.02 (4H, m), 7.13 (2H, d, $J = 8.5$ Hz); ESI/MS: $m/z = 599$ (M+H); HREI/MS $m/z = 598.1927$ (Calcd for $C_{31}H_{36}^{35}Cl_2N_4O_2S$: 598.1936); IR (ATR) cm^{-1} : 1654, 1596, 1490, 1398, 1091, 1012.

(5R,6S)-5,6-Bis(4-chlorophenyl)-2-[(2R,5S)-2-methyl-5-[(4-methylpiperazin-1-yl)carbonyl]pyrrolidin-1-yl]carbonyl]-3-isopropyl-6-methyl-5,6-dihydroimidazo[2,1-*b*][1,3]thiazole (73)

Compound **73** was prepared (28% yield) as the colorless solid from **67a** according to a similar procedure for the synthesis of **71**; 1H -NMR (400 MHz, $CDCl_3$) δ : 0.95 (3H, d, $J = 7.1$ Hz), 1.03 (3H, d, $J = 7.1$ Hz), 1.23 (3H, d, $J = 6.3$ Hz), 1.62-1.67 (1H, m), 1.79-1.88 (1H, m), 1.79 (3H, s), 2.30 (3H, s), 2.32-2.44 (4H, m), 2.50-2.56 (1H, m), 2.71-2.77 (1H, m), 3.53-3.63 (4H, m), 4.53-4.56 (1H, m), 4.93 (1H, s), 5.00-5.03 (1H, m), 6.68 (2H, d, $J = 8.3$ Hz), 7.00-7.02 (4H, m), 7.12 (2H, d, $J = 8.5$ Hz); ESI/MS: $m/z = 640$ (M+H); HRESI/MS $m/z = 640.22670$ (Calcd for $C_{33}H_{40}^{35}Cl_2N_5O_2S$: 640.22797); IR (ATR) cm^{-1} : 1652, 1596, 1565, 1490, 1407, 1361, 1290, 1172, 1091, 1012.

(5R,6S)-2-((2S,5R)-2-[(4-Acetylpiperazin-1-yl)carbonyl]-5-methylpyrrolidin-1-yl)carbonyl)-5,6-bis(4-chlorophenyl)-3-isopropyl-6-methyl-5,6-dihydroimidazo[2,1-b][1,3]thiazole (74)

Compound **74** was prepared (37% yield) as the colorless solid from **67a** according to a similar procedure for the synthesis of **71**; ¹H-NMR (400 MHz, CDCl₃) δ: 0.96 (3H, d, *J* = 7.1 Hz), 1.02 (3H, d, *J* = 7.1 Hz), 1.24 (3H, d, *J* = 6.3 Hz), 1.66-1.70 (1H, m), 1.79 (3H, s), 1.82-1.86 (1H, m), 2.10 (3H, s), 2.22-2.28 (1H, m), 2.36-2.43 (1H, m), 2.68-2.74 (1H, m), 3.44-3.50 (4H, m), 3.66-3.74 (4H, m), 4.53-4.58 (1H, m), 4.93 (1H, s), 4.98 (1H, dd, *J* = 8.4, 2.6 Hz), 6.69 (2H, d, *J* = 8.5 Hz), 6.99-7.03 (4H, m), 7.12 (2H, d, *J* = 8.5 Hz); ESI/MS: *m/z* = 668 (M+H); HRESI/MS *m/z* = 668.21809 (Calcd for C₃₄H₄₀³⁵Cl₂N₅O₃S: 668.22289); IR (ATR) cm⁻¹: 1646, 1596, 1413, 1361, 1224, 1091, 1012.

(5R,6S)-5,6-Bis(4-chlorophenyl)-2-((2R,5S)-2-ethyl-5-[(4-methylpiperazin-1-yl)carbonyl]pyrrolidin-1-yl)carbonyl)-3-isopropyl-6-methyl-5,6-dihydroimidazo[2,1-b][1,3]thiazole (75)

Compound **75** was prepared (45% yield) as the colorless solid from **67b** according to a similar procedure for the synthesis of **71**; ¹H-NMR (400 MHz, CDCl₃) δ: 0.91-0.96 (6H, m), 1.02-1.06 (3H, m), 1.38-1.44 (1H, m), 1.66-1.85 (2H, m), 1.79 (3H, s), 2.22-2.43 (6H, m), 2.31 (3H, s), 2.51-2.59 (1H, m), 2.70-2.77 (1H, m), 3.52-3.66 (4H, m), 4.38 (1H, t, *J* = 8.7 Hz), 4.94 (1H, s), 4.99-5.02 (1H, m), 6.63-6.68 (2H, m), 6.99-7.03 (4H, m), 7.12-7.15 (2H, m); ESI/MS: *m/z* = 654 (M+H); HRESI/MS *m/z* = 654.24417 (Calcd for C₃₄H₄₂³⁵Cl₂N₅O₂S: 654.24363); IR (ATR) cm⁻¹: 1650, 1596, 1567, 1490, 1444, 1357, 1290, 1172, 1091, 1012.

(5R,6S)-2-((2S,5R)-2-[(4-Acetylpiperazin-1-yl)carbonyl]-5-ethylpyrrolidin-1-yl)carbonyl)-5,6-bis(4-chlorophenyl)-3-isopropyl-6-methyl-5,6-dihydroimidazo[2,1-b][1,3]thiazole (76)

Compound **76** was prepared (46% yield) as the colorless solid from **67b** according to a similar procedure for the synthesis of **71**; ¹H-NMR (400 MHz, CDCl₃) δ: 0.91-0.96 (6H, m), 1.03 (3H, d, *J* = 7.1 Hz), 1.36-1.46 (1H, m), 1.77-1.84 (3H, m), 1.78 (3H, s), 2.10

(3H, s), 2.20-2.31 (2H, m), 2.72-2.79 (1H, m), 3.44-3.52 (4H, m), 3.61-3.75 (4H, m), 4.35-4.39 (1H, m), 4.93 (1H, s), 4.95-4.99 (1H, m), 6.67 (2H, d, $J = 8.3$ Hz), 6.98-7.03 (4H, m), 7.13 (2H, d, $J = 8.5$ Hz); ESI/MS: $m/z = 682$ (M+H); HRESI/MS $m/z = 682.23549$ (Calcd for $C_{35}H_{42}^{35}Cl_2N_5O_3S$: 682.23854); IR (ATR) cm^{-1} : 1594, 1565, 1490, 1384, 1230, 1174, 1114, 1091, 1012.

(5*R*,6*S*)-5,6-Bis(4-chlorophenyl)-2-(((2*R*,5*S*)-2-ethyl-5-(morpholin-4-ylcarbonyl)pyrrolidin-1-yl)carbonyl)-3-isopropyl-6-methyl-5,6-dihydroimidazo[2,1-*b*][1,3]thiazole (77)

Compound **77** was prepared (62% yield) as the colorless solid from **67b** according to a similar procedure for the synthesis of **71**; 1H -NMR (400 MHz, $CDCl_3$) δ : 0.91-0.96 (6H, m), 1.05 (3H, d, $J = 7.1$ Hz), 1.35-1.46 (1H, m), 1.76-1.86 (3H, m), 1.79 (3H, s), 2.19-2.30 (2H, m), 2.74-2.82 (1H, m), 3.50-3.77 (8H, m), 4.34-4.40 (1H, m), 4.94 (1H, s), 4.94-5.00 (1H, m), 6.67 (2H, d, $J = 8.5$ Hz), 7.01 (2H, d, $J = 8.5$ Hz), 7.02 (2H, d, $J = 8.8$ Hz), 7.13 (2H, d, $J = 8.5$ Hz); ESI/MS: $m/z = 641$ (M+H); HRESI/MS $m/z = 641.20974$ (Calcd for $C_{33}H_{39}^{35}Cl_2N_4O_3S$: 641.21199); IR (ATR) cm^{-1} : 1646, 1596, 1490, 1415, 1359, 1282, 1174, 1091, 1012.

(5*R*,6*S*)-5,6-Bis(4-chlorophenyl)-2-(((2*S*,5*R*)-2-(((3*R*)-3,4-dimethylpiperazin-1-yl)carbonyl)-5-methylpyrrolidin-1-yl)carbonyl)-3-isopropyl-6-methyl-5,6-dihydroimidazo[2,1-*b*][1,3]thiazole (78)

Compound **78** was prepared (37% yield) as the colorless solid from **67a** according to a similar procedure for the synthesis of **71**; 1H -NMR (400 MHz, $CDCl_3$) δ : 0.94 (3H, d, $J = 6.3$ Hz), 1.04 (3H, d, $J = 7.1$ Hz), 1.05-1.08 (3H, m), 1.23 (3H, d, $J = 6.3$ Hz), 1.62-1.65 (1H, m), 1.79 (3H, s), 1.83-1.86 (1H, m), 2.20-2.31 (3H, m), 2.29 (3H, s), 2.73-2.77 (2H, m), 2.85-2.88 (1H, m), 3.40-3.45 (1H, m), 3.67-3.75 (1H, m), 4.17-4.21 (1H, m), 4.32-4.37 (1H, m), 4.51-4.57 (1H, m), 4.93 (1H, s), 5.00-5.03 (1H, m), 6.68 (2H, d, $J = 8.3$ Hz), 6.99-7.03 (4H, m), 7.12 (2H, d, $J = 8.5$ Hz); ESI/MS: $m/z = 654$ (M+H); HRESI/MS $m/z = 654.24561$ (Calcd for $C_{34}H_{42}^{35}Cl_2N_5O_2S$: 654.24363); IR (ATR) cm^{-1} : 1652, 1596, 1490, 1373, 1288, 1176, 1091, 1012.

(5R,6S)-5,6-Bis(4-chlorophenyl)-2-(((2S,5R)-2-((3S)-3,4-dimethylpiperazin-1-yl)carbonyl)-5-methylpyrrolidin-1-yl)carbonyl]-3-isopropyl-6-methyl-5,6-dihydroimidazo[2,1-b][1,3]thiazole (79)

Thionyl chloride (150 μ l, 2.1 mmol) and a catalytic amount of DMF were added to a suspension of compound **27b** (300 mg, 0.67 mmol) in toluene (5 ml). The resulting mixture was warmed to 70°C and stirred for 1 h. After cooling to room temperature, the solvent was removed *in vacuo* to afford the crude product. The residue was dissolved in THF (2 ml), and was dropwised to a solution of compound **70a** (144 mg, 0.64 mmol) with triethylamine (270 μ l, 1.92 mmol) in THF (6 ml) under an ice cooling bath. The resulting solution was warmed to room temperature, and was stirred for 3 h. The reaction mixture was quenched by the addition of saturated aqueous sodium bicarbonate, and extracted with EtOAc. The organic layer was washed with brine, and dried over magnesium sulfate. The mixture was filtered, and the solvent was removed *in vacuo* to afford crude product. The residue was purified by flash silica gel column chromatography with CHCl₃/MeOH (20:1, v/v) to give the colorless solid (204 mg, 49% yield), which was precipitated from Et₂O/*n*-hexane; ¹H-NMR (400 MHz, CDCl₃) δ : 0.95 (3H, d, *J* = 6.8 Hz), 1.03 (3H, d, *J* = 7.1 Hz), 1.09-1.11 (3H, m), 1.23 (3H, d, *J* = 6.3 Hz), 1.61-1.64 (1H, m), 1.79 (3H, s), 1.82-1.86 (1H, m), 2.07-2.12 (1H, m), 2.22-2.27 (1H, m), 2.29 (3H, s), 2.49-2.58 (1H, m), 2.71-2.80 (2H, m), 2.98-3.05 (1H, m), 3.27-3.33 (1H, m), 3.59-3.64 (1H, m), 3.76-3.82 (1H, m), 4.23-4.27 (1H, m), 4.52-4.58 (1H, m), 4.93 (1H, s), 5.00-5.03 (1H, m), 6.68 (2H, d, *J* = 8.1 Hz), 7.01 (4H, d, *J* = 8.5 Hz), 7.12 (2H, d, *J* = 8.5 Hz); ESI/MS: *m/z* = 654 (M+H); HRESI/MS *m/z* = 654.23407 (Calcd for C₃₄H₄₂³⁵Cl₂N₅O₂S: 654.24363); IR (ATR) cm⁻¹: 1652, 1596, 1565, 1490, 1373, 1288, 1091, 1012.

(5R,6S)-2-(((2S,5R)-2-((3R)-4-Acetyl-3-methylpiperazin-1-yl)carbonyl)-5-methylpyrrolidin-1-yl)carbonyl]-5,6-bis(4-chlorophenyl)-3-isopropyl-6-methyl-5,6-dihydroimidazo[2,1-b][1,3]thiazole (80)

Compound **80** was prepared (32% yield) as the colorless solid from **67a** according to a similar procedure for the synthesis of **71**; ¹H-NMR (400 MHz, CDCl₃) δ : 0.94 (3H, d, *J* = 7.1 Hz), 1.02 (3H, d, *J* = 7.1 Hz), 1.19-1.21 (3H, m), 1.24 (3H, d, *J* = 6.3 Hz),

1.65-1.71 (1H, m), 1.81 (3H, s), 1.83-1.87 (1H, m), 2.09 (3H, s), 2.20-2.25 (1H, m), 2.41-2.47 (1H, m), 2.69-2.76 (1H, m), 2.89-2.94 (1H, m), 3.14-3.20 (1H, m), 3.44-3.50 (1H, m), 3.69-3.75 (1H, m), 3.97-4.03 (1H, m), 4.31-4.37 (1H, m), 4.49-4.55 (1H, m), 4.84-4.88 (1H, m), 4.97 (1H, s), 5.00-5.02 (1H, m), 6.69 (2H, d, $J = 8.3$ Hz), 7.01 (2H, d, $J = 8.5$ Hz), 7.02 (2H, d, $J = 8.5$ Hz), 7.12 (2H, d, $J = 8.5$ Hz); ESI/MS: $m/z = 682$ (M+H); HRESI/MS $m/z = 682.23688$ (Calcd for $C_{35}H_{42}^{35}Cl_2N_5O_3S$: 682.23853); IR (ATR) cm^{-1} : 1596, 1491, 1412, 1368, 1309, 1218, 1091, 1012.

(5R,6S)-2-{{(2S,5R)-2-{{(3S)-4-Acetyl-3-methylpiperazin-1-yl}carbonyl}-5-methylpyrrolidin-1-yl}carbonyl}-5,6-bis(4-chlorophenyl)-3-isopropyl-6-methyl-5,6-dihydroimidazo[2,1-*b*][1,3]thiazole (81)

Compound **81** was prepared (42% yield) as the colorless solid from **67a** according to a similar procedure for the synthesis of **71**; 1H -NMR (400 MHz, $CDCl_3$) δ : 0.96 (3H, d, $J = 7.1$ Hz), 1.02 (3H, d, $J = 7.1$ Hz), 1.17-1.20 (3H, m), 1.24 (3H, d, $J = 6.3$ Hz), 1.67-1.71 (1H, m), 1.80 (3H, s), 1.84-1.89 (1H, m), 2.10 (3H, s), 2.13-2.16 (1H, m), 2.28-2.32 (1H, m), 2.70-2.75 (1H, m), 2.90-2.93 (1H, m), 3.30-3.34 (1H, m), 3.65-3.69 (1H, m), 3.85-3.89 (1H, m), 4.05-4.09 (1H, m), 4.35-4.40 (1H, m), 4.53-4.57 (1H, m), 4.84-4.87 (1H, m), 4.95 (1H, s), 4.98-5.01 (1H, m), 6.69 (2H, d, $J = 8.3$ Hz), 7.01 (2H, d, $J = 8.5$ Hz), 7.02 (2H, d, $J = 8.5$ Hz), 7.12 (2H, d, $J = 8.3$ Hz); ESI/MS: $m/z = 682$ (M+H); HRESI/MS $m/z = 682.23611$ (Calcd for $C_{35}H_{42}^{35}Cl_2N_5O_3S$: 682.23853); IR (ATR) cm^{-1} : 1597, 1566, 1491, 1412, 1371, 1309, 1176, 1091, 1013.

Preparation of

(5R,6S)-5,6-Bis(4-chlorophenyl)-2-((2S,5R)-2-[(3,3-dimethylpiperazin-1-yl)carbonyl]-5-methylpyrrolidin-1-yl}carbonyl)-3-isopropyl-6-methyl-5,6-dihydroimidazo[2,1-*b*][1,3]thiazole (82)

(5R,6S)-5,6-Bis(4-chlorophenyl)-2-((2S,5R)-2-[(3,3-dimethylpiperazin-1-yl)carbonyl]-5-methylpyrrolidin-1-yl}carbonyl)-3-isopropyl-6-methyl-5,6-dihydroimidazo[2,1-*b*][1,3]thiazole

Triethylamine (624 μ l, 4.48 mmol) was added to a solution of compound **67a** (1.0 g, 1.79 mmol) in DMF (20 ml), followed by 2,2-dimethylpiperazine dihydrochloride³⁹ (402 mg, 2.15 mmol), HOBt (24 mg, 0.18 mmol) and EDC/HCl (412 mg, 2.15 mmol) under an ice cooling bath. The solution was warmed to room temperature and stirred for 20 h. The reaction was diluted with saturated aqueous sodium bicarbonate, and extracted with EtOAc. The organic layer was washed with brine (3 times), and dried over magnesium sulfate. The mixture was filtered, and the solvent was removed *in vacuo* to afford the crude product. The residue was purified by flash NH₂ silica gel chromatography with CHCl₃/MeOH (80:1, v/v) to give the colorless solid (450 mg, 38% yield), which was precipitated from Et₂O/*n*-hexane; ¹H-NMR (400 MHz, CDCl₃) δ : 0.93 (3H, d, *J* = 7.1 Hz), 1.03 (3H, d, *J* = 7.1 Hz), 1.07-1.15 (6H, m), 1.23 (3H, d, *J* = 6.3 Hz), 1.40-1.47 (2H, m), 1.62-1.69 (1H, m), 1.79 (3H, s), 1.83-1.88 (1H, m), 2.22-2.39 (2H, m), 2.72-2.78 (1H, m), 2.87-2.96 (1H, m), 3.20-3.27 (1H, m), 3.38-3.55 (2H, m), 4.52-4.56 (1H, m), 4.93 (1H, s), 5.01-5.04 (1H, m), 6.69 (2H, d, *J* = 8.5 Hz), 7.01 (5H, d, *J* = 8.8 Hz), 7.12 (2H, d, *J* = 8.5 Hz); HRESI/MS *m/z* = 654.24351 (Calcd for C₃₄H₄₂³⁵Cl₂N₅O₂S: 654.24363); IR (ATR) cm⁻¹: 1647, 1596, 1491, 1408, 1372, 1293, 1175, 1091, 1013.

(5*R*,6*S*)-5,6-Bis(4-chlorophenyl)-2-((2*S*,5*R*)-2-[(3,3-dimethylpiperazin-1-yl)carbonyl]-5-methylpyrrolidin-1-yl)carbonyl)-3-isopropyl-6-methyl-5,6-dihydroimidazo[2,1-*b*][1,3]thiazole (82)

To a stirred solution of (5*R*,6*S*)-5,6-Bis(4-chlorophenyl)-2-((2*S*,5*R*)-2-[(3,3-dimethylpiperazin-1-yl)carbonyl]-5-methylpyrrolidin-1-yl)carbonyl)-3-isopropyl-6-methyl-5,6-dihydroimidazo[2,1-*b*][1,3]thiazole (220 mg, 0.34 mmol) in 1,4-dioxane (6 ml) was added 37% aqueous formaldehyde solution (253 μ l, 3.4 mmol). After stirring for 15 min at room temperature, sodium triacetoxyborohydride (144 mg, 0.68 mmol) was added to the reaction solution. The resulting mixture was stirred for 16 h at room temperature. The reaction was diluted with saturated aqueous sodium bicarbonate, and extracted with EtOAc. The organic layer was washed with brine, and dried over magnesium sulfate. The mixture was filtered, and the solvent was removed *in vacuo* to afford the crude product. The residue was purified by flash silica gel chromatography with

CHCl₃/MeOH (30:1, v/v) to give the colorless solid (150 mg, 66% yield), which was precipitated from Et₂O/*n*-hexane; ¹H-NMR (400 MHz, CDCl₃) δ: 0.92 (3H, d, *J* = 7.1 Hz), 0.95-1.02 (6H, m), 1.03 (3H, d, *J* = 7.1 Hz), 1.23 (3H, d, *J* = 6.6 Hz), 1.61-1.67 (1H, m), 1.79 (3H, s), 1.82-1.86 (1H, m), 2.24 (3H, s), 2.26-2.36 (2H, m), 2.51-2.54 (1H, m), 2.73-2.76 (2H, m), 3.18-3.34 (2H, m), 3.47-3.65 (2H, m), 4.51-4.56 (1H, m), 4.93 (1H, s), 5.02-5.05 (1H, m), 6.68 (2H, d, *J* = 7.8 Hz), 7.01 (4H, d, *J* = 8.5 Hz), 7.12 (2H, d, *J* = 8.3 Hz); HRESI/MS *m/z* = 668.25854 (Calcd for C₃₅H₄₄³⁵Cl₂N₅O₂S: 668.25927); IR (ATR) cm⁻¹: 1650, 1595, 1566, 1491, 1451, 1373, 1291, 1173, 1091, 1012.

(5*R*,6*S*)-5,6-Bis(4-chlorophenyl)-2-(((2*S*,5*R*)-2-((3*R*)-3,4-dimethylpiperazin-1-yl)carbonyl)-5-ethylpyrrolidin-1-yl)carbonyl]-3-isopropyl-6-methyl-5,6-dihydroimidazo [2,1-*b*][1,3]thiazole (83)

Compound **83** was prepared (28% yield) as the colorless solid from **27b** according to a similar procedure for the synthesis of **79**; ¹H-NMR (400 MHz, CDCl₃) δ: 0.88-0.95 (6H, m), 1.05 (3H, d, *J* = 7.1 Hz), 1.05-1.08 (3H, m), 1.37-1.44 (1H, m), 1.79 (3H, s), 1.79-1.83 (3H, m), 2.17-2.26 (3H, m), 2.29 (3H, s), 2.76-2.84 (4H, m), 3.40-3.46 (1H, m), 3.66-3.74 (1H, m), 4.17-4.21 (1H, m), 4.35-4.39 (1H, m), 4.93 (1H, s), 4.99-5.02 (1H, m), 6.66 (2H, d, *J* = 8.1 Hz), 7.01 (4H, d, *J* = 8.5 Hz), 7.12 (2H, d, *J* = 8.5 Hz); ESI/MS: *m/z* = 668 (M+H); HRESI/MS *m/z* = 668.26496 (Calcd for C₃₅H₄₄³⁵Cl₂N₅O₂S: 668.25928); IR (ATR) cm⁻¹: 1652, 1596, 1490, 1409, 1338, 1174, 1091, 1012.

(5*R*,6*S*)-5,6-Bis(4-chlorophenyl)-2-(((2*S*,5*R*)-2-((3*S*)-3,4-dimethylpiperazin-1-yl)carbonyl)-5-ethylpyrrolidin-1-yl)carbonyl]-3-isopropyl-6-methyl-5,6-dihydroimidazo [2,1-*b*][1,3]thiazole (84)

Compound **84** was prepared (54% yield) as the colorless solid from **67b** according to a similar procedure for the synthesis of **71**; ¹H-NMR (400 MHz, CDCl₃) δ: 0.90-0.95 (6H, m), 1.05 (3H, d, *J* = 7.3 Hz), 1.07-1.13 (3H, br m), 1.38-1.44 (1H, m), 1.79 (3H, s), 1.80-1.86 (3H, m), 2.07-2.26 (3H, m), 2.29 (3H, s), 2.77 (2H, d, *J* = 11.5 Hz), 3.00-3.07 (1H, m), 3.27-3.34 (1H, m), 3.57-3.65 (1H, m), 3.74-3.81 (1H, m), 4.18-4.31 (1H, m), 4.32-4.39 (1H, m), 4.93 (1H, s), 5.01 (1H, d, *J* = 6.7 Hz), 6.66 (2H, d, *J* = 7.9 Hz), 7.01 (4H, d, *J* = 8.5 Hz), 7.12 (2H, d, *J* = 8.5 Hz); ESI/MS: *m/z* = 668 (M+H); HRESI/MS

$m/z=$ 668.25169 (Calcd for $C_{35}H_{44}^{35}Cl_2N_5O_2S$: 668.25928); IR (ATR) cm^{-1} : 1652, 1596, 1490, 1409, 1338, 1174, 1091, 1012.

(5*R*,6*S*)-2-(((2*S*,5*R*)-2-(((3*R*)-4-Acetyl-3-methylpiperazin-1-yl)carbonyl)-5-ethylpyrrolidin-1-yl)carbonyl]-5,6-bis(4-chlorophenyl)-3-isopropyl-6-methyl-5,6-dihydroimidazo[2,1-*b*][1,3]thiazole (85)

Compound **85** was prepared (60% yield) as the colorless solid from **67b** according to a similar procedure for the synthesis of **71**; 1H -NMR (400 MHz, $CDCl_3$) δ : 0.90-0.96 (6H, m), 1.04 (3H, d, $J = 7.1$ Hz), 1.21 (3H, br s), 1.39-1.46 (1H, m), 1.78-1.84 (3H, m), 1.79 (3H, s), 2.09 (3H, s), 2.15-2.20 (2H, m), 2.33-2.37 (1H, m), 2.73-2.80 (1H, m), 2.89-2.96 (1H, br m), 3.12-3.19 (1H, br m), 3.41-3.49 (1H, br m), 3.68-3.77 (1H, br m), 3.96-4.01 (1H, br m), 4.31-4.37 (2H, m), 4.94 (1H, s), 4.95-4.99 (1H, m), 6.68 (2H, d, $J = 8.3$ Hz), 7.00 (2H, d, $J = 8.5$ Hz), 7.01 (2H, d, $J = 8.8$ Hz), 7.12 (2H, d, $J = 8.5$ Hz); ESI/MS: $m/z=$ 696 (M+H); HRESI/MS $m/z=$ 696.25430 (Calcd for $C_{36}H_{44}^{35}Cl_2N_5O_3S$: 696.25419); IR (ATR) cm^{-1} : 1639, 1596, 1411, 1363, 1176, 1091, 1012.

(5*R*,6*S*)-2-(((2*S*,5*R*)-2-(((3*S*)-4-Acetyl-3-methylpiperazin-1-yl)carbonyl)-5-ethylpyrrolidin-1-yl)carbonyl]-5,6-bis(4-chlorophenyl)-3-isopropyl-6-methyl-5,6-dihydroimidazo[2,1-*b*][1,3]thiazole (86)

Compound **86** was prepared (57% yield) as the colorless solid from **67b** according to a similar procedure for the synthesis of **71**; 1H -NMR (400 MHz, $CDCl_3$) δ : 0.90-0.96 (6H, m), 1.03 (3H, d, $J = 6.8$ Hz), 1.33-1.43 (4H, m), 1.76-1.84 (3H, m), 1.78 (3H, s), 2.10 (3H, s), 2.21-2.28 (2H, m), 2.73-2.79 (1H, m), 2.85-2.91 (1H, m), 3.24-3.36 (2H, m), 3.60-3.68 (1H, m), 3.82-3.88 (1H, m), 4.34-4.40 (2H, m), 4.44-4.50 (1H, m), 4.93 (1H, s), 4.96-5.01 (1H, m), 6.67 (2H, d, $J = 8.3$ Hz), 7.00 (2H, d, $J = 8.5$ Hz), 7.01 (2H, d, $J = 8.5$ Hz), 7.12 (2H, d, $J = 8.3$ Hz); ESI/MS: $m/z=$ 696 (M+H); HRESI/MS $m/z=$ 696.25274 (Calcd for $C_{36}H_{44}^{35}Cl_2N_5O_3S$: 696.25419); IR (ATR) cm^{-1} : 1644, 1596, 1411, 1363, 1176, 1091, 1012.

p53-MDM2 HTRF assay

The assay mixture (8 μ l containing 2.5 nM GST-tagged MDM2 (25-108, L33E) and 2.5 nM His-tagged p53 (1-132) with assay buffer (20 mM HEPES pH 7.4, 150 mM NaCl, and 0.1 % BSA), 4 μ l of the tested compound in 3-fold dilutions, and 8 μ l antibody mixture solution containing XL665-anti-6HIS (Schering) in a final concentration of 1.0 μ g/ml and Eu-anti-GST (Schering) in a final concentration of 0.13 μ g/ml with assay buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 0.1 % BSA and 0.5 M KF)) was incubated on the 384-well plate (Corning) for 1 h at 25°C, and was detected by time-resolved fluorescence spectroscopy (ARVOsx, PerkinElmer Life Sciences). The IC₅₀ values were calculated from sigmoid-fitting curve analysis by using Prism software (GraphPad).

Antiproliferative assay in vitro

In vitro assay was performed against MV4-11 and DLD-1 cell lines to examine the growth inhibitory effects of our compounds. Both cell lines were purchased from American Type Culture Collection (ATCC). The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. The cells were plated in 96-well micro plates on Day 0 (DLD-1) or Day 1 (MV4-11), and stock solutions serially diluted in DMSO were added to each well on Day 1. After 3 days of culture (Day 4), the number of viable cells was determined by the luminescent cell viability assay. The concentration of compounds that cause 50% of growth inhibition (GI₅₀) was calculated.

Antitumor activity assay

In vivo antitumor activity of compounds **78** and **85** were performed on the MV4-11 (human leukemia cells, ATCC) xenograft model on mice. Nutlin-3 (racemate) was used as a positive control compound. MV4-11 cells (1 x 10⁷ cells/head) were inoculated s.c. into NOD SCID mice (Charles River Laboratories Japan Inc.) (Day 0). Drug administration was started on Day 21 with p.o. per day (**78** and **85**) or twice a day (Nutlin-3) every 200mg/kg doses. Administration schedules were on Days 21-23 and 27-30 (**78**), or Days 21-24 and 27-30 (**85** and Nutlin-3). The total dose of each of the

compounds was 1400 mg/kg (**78**), 1600 mg/kg (**85**) and 3200 mg/kg (Nutlin-3). Tumor volumes and bodyweights were measured every 2 days over the course of administration. After the mice were sacrificed on Day 31, tumor weight was assessed and tumor growth inhibition (TGI) was calculated.

Solubility

The solubility was determined by HPLC analysis. 10 mM of compound solution in DMSO (50 μ L) was freeze-dried. To the residue the Japanese Pharmacopoeia Second Fluid (JP2; 250 μ L, pH 6.8) was added, and the mixture was stirred by pipette operation. The mixture was saved under shading over 12 h. After filtration of the mixture, the resulting filtrate was diluted 20 times by adding aqueous DMSO solution (1:1, v/v) to obtain the measurement sample solution. 5 μ M of compound solution in aqueous DMSO solution (1:1, v/v) and 100 mM of compound solution in aqueous DMSO solution (1:1, v/v) were prepared to make a calibration curve. The measurement sample solution, 5 μ M solution and 100 mM solution were assayed using HPLC methodologies (Analytical Column: X Terra[®] MSC18 3.5 μ m, 3.0 x 30 mm, Waters; Mobile Phase: 10 mM ammonium acetate buffer (pH 4.5)/0.05% acetic acid in acetonitrile = 95:5 to 10:90 v/v; Wave length: PDA 220–420 nm). The solubilities were analyzed using Millenium software (Waters).

Metabolic stability

The compounds (final 1 μ M) were incubated with mouse hepatic microsome in sodium phosphate buffer (pH 7.4) for 20 min at 37°C. The microsomal protein concentration in the assay was 0.5 mg/mL. The reaction was started by addition of the NADPH generating system at 37°C and stopped by addition of MeOH after 30 min. After centrifuging each solution separately at 3500 rpm for 10 min at 4°C, the corresponding loss of the parent compound was determined by LC-MS/MS.

Measurement of tumor concentration

Synthetic compound suspended in the Japanese Pharmacopoeia First Fluid (pH 1.2) was administered orally to tumor bearing mice (100 mg/kg). Tumor samples were collected at 1, 2, 4, 6 and 24 h after oral dosing and homogenized using Mixer Mill MM 300. Tumor concentrations for the synthetic compound were determined by LC/MS/MS using a Shimadzu LC-20AD system (Shimadzu) coupled to the mass spectrometer API 4000 (SCIEX). The synthetic compound was separated on a Shim-pack XR- ODS column (Shimadzu). Respective pharmacokinetic parameters were carried out using winnonlin software (Pharsight Corporation).

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33. Details of HTRF-based assay; The compounds in 3-fold dilutions were incubated with 2.5 nM GST-tagged human MDM2 (25-108, L33E) and 2.5 nM His-tagged p53 (1-132) with detection by time-resolved fluorescence spectroscopy.
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