Three-dimensional structure-activity relationship study of belactosin A and its stereo- and regioisomers: development of potent proteasome inhibitors by a stereochemical diversity-oriented strategy†

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The development of potent proteasome inhibitors based on the stereochemical diversity-oriented strategy using a conformationally rigid cyclopropane structure was investigated. Thus, a series of stereo- and regioisomeric analogs of belactosin A (**2**), a cyclopropane amino acid (methanoamino acid)-containing tripeptidic proteasome inhibitor, were designed, in which the central cyclopropane amino acid part was replaced with the corresponding stereo- or regioisomer. Using a series of stereoisomeric cyclopropane amino acid equivalents with the *cis*/*trans*, D/L, and *syn*/*anti* stereochemical diversity, which were previously developed by us, as key units, the target compounds were successfully synthesized. Biological evaluation showed that, as expected, compound activity changed depending on the stereochemistry of the central cyclopropane amino acid part: the *trans*/L-*syn*-isomer **7** and the *cis*/L-*anti*-isomer **9** were more than twice as potent as natural belactosin A (*trans*/L-*anti*-isomer). Furthermore, the tripeptidic compound **13**, the synthetic precursor for the unnatural *cis*/L-*anti*-isomer **9**, was identified as a highly potent proteasome inhibitor. This compound, which is 20 times as potent as belactosin A and is even more potent than the well-known inhibitor lactacystin (**4**), may be an effective lead for developing clinically useful anticancer drugs. These results show that the stereochemical diversity-oriented approach can be a powerful strategy for the development of highly active compounds in medicinal chemical studies.

Introduction

The ubiquitin-proteasome pathway is key to intracellular protein degradation, which is crucial in maintaining cell functions.[1] The pathway also plays an important role in tumor cell proliferation, since degradation of cell cycle regulatory proteins, such as cyclins and cyclin-dependent kinase inhibitors, by this pathway is essential for cell growth and metastasis.[2] Targeted inhibition of proteasomes has thereby become an attractive therapeutic strategy in cancer treatment.[2,3] In fact, a potent proteasome inhibitor bortezomib (PS-341, **1**, Fig. 1) developed recently has been shown to be effective as an anticancer agent in the clinic.[3a,4]

In 2000, Asai and co-workers discovered belactosin A (**2**), a naturally occurring tripeptide consisting of L-alanine, 3-(*trans*-2-aminocyclopropyl)-L-alanine (*trans*-3,4-methano-L-ornithine) and a chiral carboxy-β-lactone.[5a] They showed that it prevents cell cycle progression of human tumor cells at the G2/M stage due to its proteasome inhibitory effect and potentially could be a novel lead for developing potent anticancer agents.[5b]

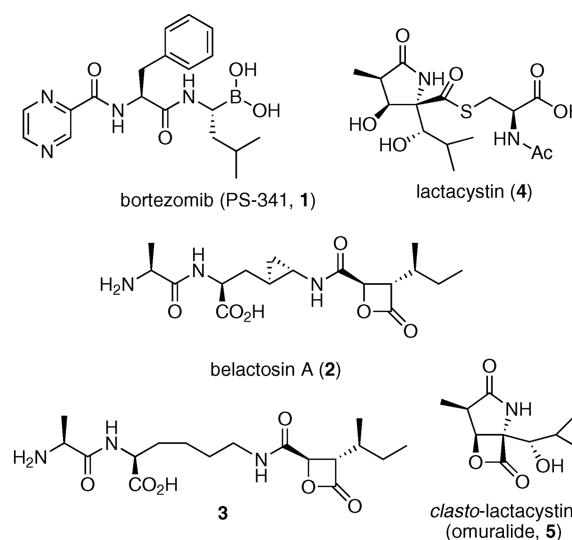

Fig. 1 Known proteasome inhibitors.

The inactivation of the proteasome by belactosin A seems to occur by acylation of the active site threonine residue *via* ring-cleavage of the strained β-lactone moiety, since this kind of irreversible inhibition of the proteasome by a similar β-lactone species **5** (omuralide) derived from the well-known proteasome inhibitor lactacystin (**4**), is known.[6] Recently, the β-lactone

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† Electronic supplementary information (ESI) available: Experimental details for the synthesis of the stereoisomers **6–9**, ^{1}H NMR charts of the synthesized belactosin A (**2**), the stereo- and regioisomers **6–10** and the precursors **11–13**, and HPLC charts and data of the synthesized belactosin A (**2**), the stereo- and regioisomers **6–10**, and the precursors **11–13**. See DOI: 10.1039/b900384c

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moiety of the synthesized belactosin analog **3** was shown to connect covalently to the active-site threonine residue by X-ray crystallographic analysis of the yeast 20S proteasome complex with **3**.[7]

Because of its rigid stereochemical features, a cyclopropane effectively restricts the conformations of molecules, which often affects the biological activity.[8] Therefore, we concentrated on the chiral cyclopropane part located at the center of the belactosin A structure. Our thought was that the cyclopropane part would restrict the orientation of the L-Ala and the key β-lactone moieties to determine the three-dimensional structure of the molecule, which may be critical in its interaction with the proteasome.

In recent years, we have developed highly selective ligands of G-protein-coupled receptors (GPCR) by the stereochemical diversity-oriented strategy, in which a series of cyclopropane-based conformationally restricted compounds are systematically designed and synthesized.[8] This strategy has been shown to be efficient for developing specific ligands for drug target proteins, especially in cases where the bioactive conformation of the ligand is known. Therefore, since the bioactive conformation of belactosin A was unknown, we were interested in the three-dimensional structure-activity relationship (SAR) study of belactosin A to identify highly potent proteasome inhibitors by the stereochemical diversity-oriented strategy using the cyclopropane-based conformational restriction. We designed a series of belactosin A analogs **6–10** (Fig. 2) with stereochemical diversity in which the central aminocyclopropyl-L-alanine (methano-L-ornithine) part was replaced with a corresponding stereo- or regioisomeric cyclopropane amino acid. The central amino acid part of belactosin A has a *trans*-3,4-methano-L-amino acid structure, where the carboxylic group is oriented *anti* to the cyclopropane ring (indicated as *trans*/L-*anti*). The designed analogs include a *trans*-3,4-methanoamino acid with the D-*anti*, the D-*syn*, or the L-*syn* structure (**6**, **7**, or **8**, respectively), a *cis*-methanoamino acid with the L-*anti*-structure (**9**), or a regioisomeric *trans*-2,3-methano-L-amino acid (regioisomer **10**). The L-alanine and the β-lactone moieties in these analogs can be restricted in a variety of spatial arrangements, which are different from that in belactosin A (**2**) due to the stereo- or the regioisomeric structure of the central cyclopropane parts.

We recently developed an efficient new method for the systematic synthesis of a series of 2,3- and 3,4-methanoamino acid equivalents with the *cis*/*trans*, D/L, and *syn*/*anti* stereochemical diversity.[9] In this study, using these methanoamino acid equivalents as the key units, we achieved the synthesis of belactosin A (**2**)[10] and its stereo- and regioisomers **6–10**, and investigated the three-dimensional SAR to develop highly potent proteasome inhibitors. As a result, some isomers of belactosin A, *e.g.*, the *trans*/L-*syn*-isomer **7** and the *cis*/L-*anti*-isomer **9**, were shown to be more potent proteasome inhibitors than the natural belactosin A (**2**). Furthermore, the highly potent proteasome inhibitors **11**, **12**, and **13** having the *trans*/L-*anti*-, the *trans*/L-*syn*-, and the *cis*/L-*anti*-structure, respectively (Fig. 3), which are 7–20 times as potent as belactosin A, have been identified. In this report, we describe these results in detail.[11]

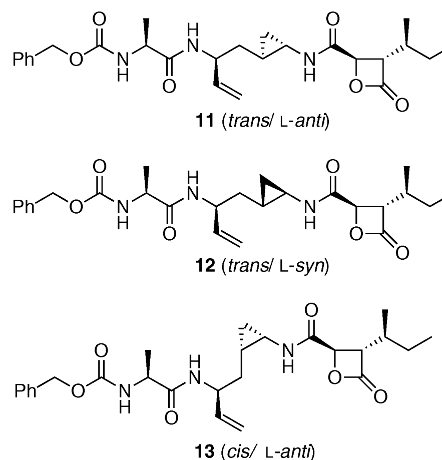

Fig. 3 Highly potent proteasome inhibitors identified in this study.

Results and discussion

Synthetic plan

The target compounds **2**, and **6–10** could be obtained by condensation between the three parts, *i.e.*, L-Ala (**A**), a 2,3- or 3,4-methanoamino acid (**B**) and the known chiral carboxy-β-lactone (**C**) (Fig. 4).[9] The chiral β-lactone can be prepared from L-Ile by the method reported.[10a] Thus, the key to the synthesis is a series of methanoamino acids (**B** part) with *cis*/*trans*, D/L, and *syn*/*anti* stereochemical diversity.

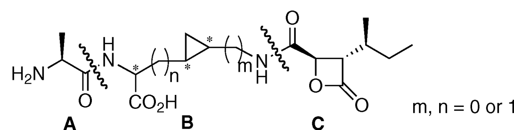

Fig. 4 General representation of the synthetic targets **2** and **6–10**.

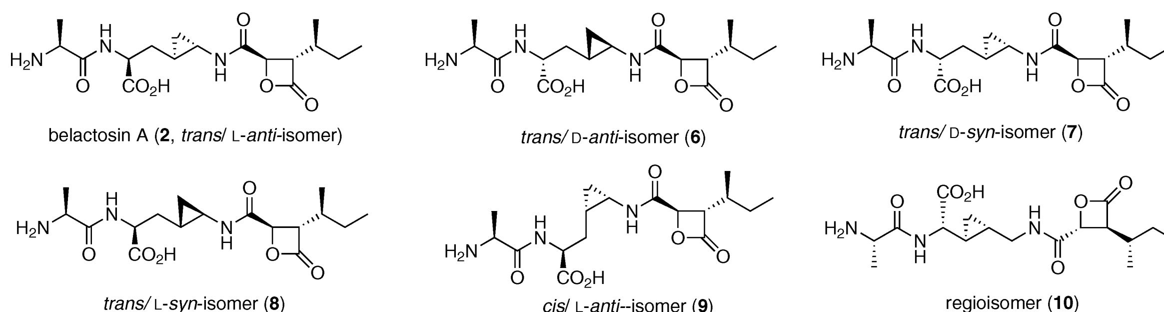

Fig. 2 Belactosin A (**2**) and its stereo- and regioisomers **6–10**.

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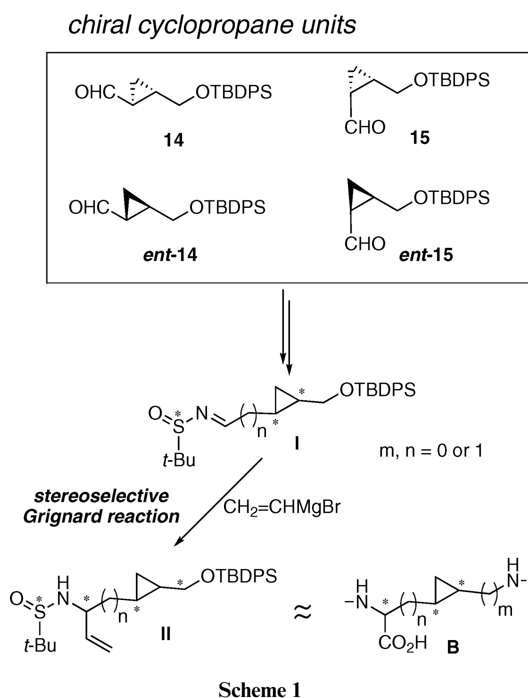
Much effort has been devoted to developing practical methods for preparing chiral cyclopropanes, including enantioselective cyclopropanations, chemical or enzymatic optical resolutions, and transformations from chiral synthons.[12] Nevertheless, the stereoselective synthesis of cyclopropane derivatives, including cyclopropane amino acids (methanoamino acids), with a desired stereochemistry is often troublesome. To solve this problem, we previously developed the chiral units, composed of four stereoisomeric cyclopropane derivatives bearing two adjacent carbon substituents in a *trans* or a *cis* relationship, namely **14** and **15**, and their enantiomers ***ent*-14** and ***ent*-15**, shown in Scheme 1.[8a,c] These units, which are generally useful for synthesizing a series of stereoisomeric cyclopropane compounds having an asymmetric *cis*- or *trans*-cyclopropane structure.



Scheme 1

We thought that the cyclopropane derivatives **II** with a CH_2=CHC*H(NHPg)- side-chain (Scheme 1) could be equivalents of the desired L- and D-cyclopropane amino acid structures **B**, since a vinyl group is stable under various reaction conditions and can be converted easily into a carboxyl group.[13] Thus, we developed an efficient method for preparing methanoamino acid equivalents **II** from the chiral cyclopropane units **14**, **15**, and their enantiomers ***ent*-14**, ***ent*-15**, in which the key symmetric CH_2=CHC*H(NH)-moiety was constructed by a diastereoselective Grignard addition to (*R*)- or (*S*)-*t*-butanesulfinyl imines **I**.[9,14]

Therefore, with the methanoamino acid equivalents **II**, the structures of which are **16**, ***ent*-16**, **17**, ***ent*-17**, **18**, and **19** shown in Fig. 5, in hand, we planned to synthesize belactosin A and its regio- and stereoisomer.

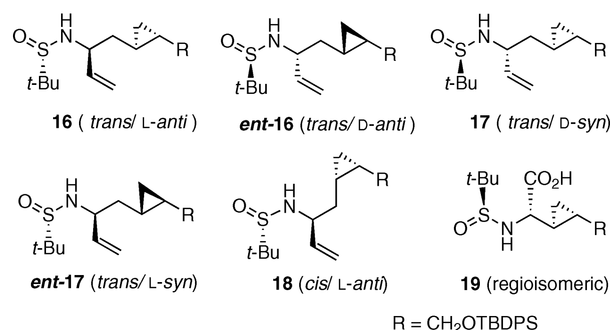
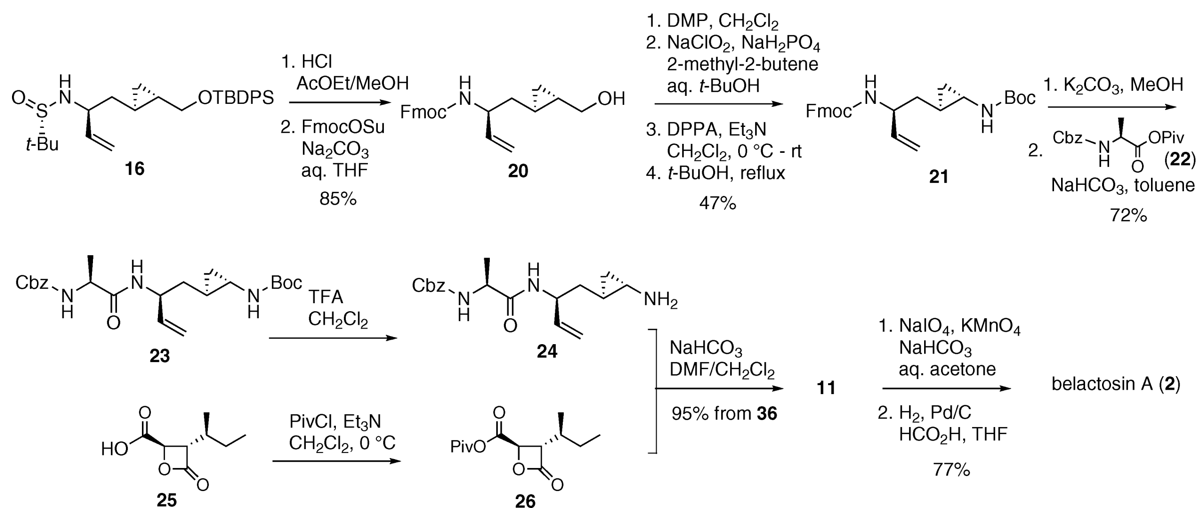


Fig. 5 The key methanoamino acid equivalents for the synthesis of belactosin A and its stereo- and regioisomers.

Total synthesis of belactosin A

In order to establish the synthetic route for the target compounds, we first tried to synthesize natural belactosin A (**2**) from the 3,4-methanoamino acid equivalent **16**[9] (Scheme 2). Compound **16** was treated with HCl to remove the *N*-sulfinyl and the *O*-silyl protecting groups, and the resulting free amino group was protected with a Fmoc group by the usual method to give **20**. After oxidation of the hydroxymethyl moiety of **20**, the resulting carboxylic acid was treated with diphenyl phosphoryl azide



Scheme 2

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(DPPA)/Et_3N in CH_2Cl_2[16] to form the corresponding acid azide, which was heated in *t*-BuOH under reflux to bring about the Curtius rearrangement[17] giving the Boc-protected cyclopropylamine derivative **21** in 47% yield from **20**. After removal of the Fmoc group of **21** with K_2CO_3/MeOH, the product was condensed with the mixed anhydride **22**, prepared from *N*-Cbz-L-Ala and pivaloyl chloride (PivCl), to afford the dipeptide **23** in 72% yield. The β-lactone moiety was condensed also with the mixed anhydride method. Thus, the β-lactone **25**, prepared from L-Ile according to the previously reported method,[10a] was treated with PivCl/Et_3N in CH_2Cl_2 to give the mixed anhydride **26**. After removal of the Boc group of **23**, the resulting amine **24** was condensed with **26** in the presence of $NaHCO_3$ in DMF/CH_2Cl_2 to form the desired tripeptidic compound **11**. Oxidation of the vinyl moiety to a carboxyl group with a $NaIO_4$/$KMnO_4$/$NaHCO_3$ combination in aqueous acetone,[18] followed by removal of the Cbz group by catalytic hydrogenation finally produced belactosin A (**2**) in 77% yield.

Synthesis of the stereo- and regioisomers of belactosin A

As described above, the total synthesis of belactosin A was achieved, and the procedure was effectively applied to the synthesis of the stereo- and regioisomers **6–10**.

Thus, starting from the 3,4-methanoamino acid equivalents ***ent*-16**, **17**, ***ent*-17**, and **18**,[9] the four target compounds, *i.e.*, the *trans*/D-*anti*-isomer **6**, the *trans*/D-*syn*-isomer **7**, *trans*/L-*syn*-isomer **8**, and the *cis*/L-*anti*-isomer **9**, were synthesized respectively by the same procedure for the synthesis of belactosin A described above.[15]

The regioisomer **10** was synthesized from the 2,3-methano–amino acid equivalent **19**, as shown in Scheme 3. After removal of the silyl protecting group of **19**, the product was treated first with DPPA/DBU in toluene and then with NaN_3/15-crown-5 in DMF[19] to give the corresponding azide **27**. The sulfinyl group was removed with HCl, and the resulting amine was condensed with the mixed anhydride **22** to form the dipeptide **28**. Reductive treatment of **28** with PPh_3 in aqueous THF, followed by protection of the resulting amino group with Boc_2O produced the dipeptide **29**. Compound **29** was converted into the target regioisomer **10** *via* condensation with the mixed anhydride **26**, oxidation of the vinyl group into a carboxyl, and final reductive deprotection, according to a procedure similar to that used for the synthesis of belactosin A.

Table 1 Inhibition of peptidase activity of human 20S proteasome

Inhibitor	IC_{50} (nM)[a]
lactacystin (**4**)	72 ± 25
belactosin A (**2**)	304 ± 153
trans/D-*anti*-isomer (**6**)	336 ± 103
trans/D-*syn*-isomer (**7**)	180 ± 53
trans/L-*syn*-isomer (**8**)	145 ± 39
cis/L-*anti*-isomer (**9**)	150 ± 47
regioisomer (**10**)	169 ± 51
11	22 ± 12
12	44 ± 2
13	15 ± 10

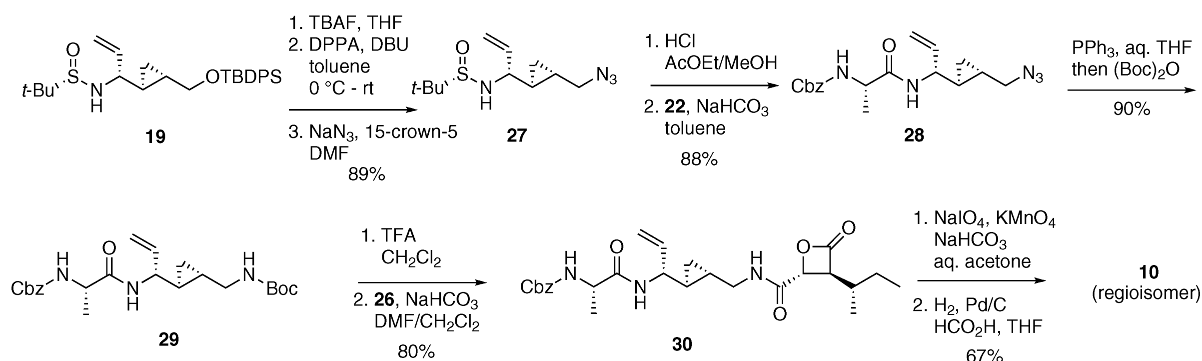
[a] The IC_{50} value was calculated from the plot of inhibitor concentration *versus* percentage of remaining activity. Data are shown as the mean value ± S.E.M. of at least three independent experiments.

Inhibitory effect on 20S proteasome activity

The inhibitory effect of belactosin A (**2**) and its stereo- and regioisomers **6–10** on the chymotrypsin-like activity of purified human 20S proteasome was measured using succinyl-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide as a substrate. The results are summarized in Table 1. The synthesized belactosin A, just as the natural belactosin A reported previously,[5a] showed a significant proteasome inhibitory effect (IC_{50} = 304 ± 153 nM). The proteasome inhibitory abilities of the synthesized isomers, except for the *trans*/D-*anti*-isomer **6**, were higher than that of belactosin A. In particular, the *trans*/L-*syn*-isomer **8** (IC_{50} = 145 ± 39 nM) and the *cis*/L-*anti*-isomer **9** (IC_{50} = 150 ± 47 nM) were about twice as potent as belactosin A.

Based on these results and the fact that the carboxyl group of belactosin A has been suggested to be unimportant for the proteasome inhibitory activity,[5b] we decided to evaluate the proteasome inhibitory effect of the tripeptidic compounds **12** and **13**, which were the synthetic precursors for the *trans*/L-*syn*-isomer **8** and the *cis*/L-*anti*-isomer **9**, respectively, identified as potent inhibitors described above, as well as the precursor **11** for belactosin A.

It is noteworthy that these three compounds (**11–13**) were not only more active than belactosin A (**2**) but also significantly more active than all of its isomers (**6–10**). Their IC_{50} values were 22 ± 12 nM for **11**, 44 ± 2 nM for **12**, and 15 ± 10 nM for **13**, respectively, which were more potent than the well-known proteasome inhibitor lactacystin (**4**) (IC_{50} = 72 ± 25 nM). The most potent compound **13**



Scheme 3

with the unnatural *cis*/L-*anti*-cyclopropane structure was 20 times as potent as belactosin A.

Proteasome inhibition in cells

We next investigated the effect of belactosin A (**2**), its stereo- and regioisomers **6–10**, and also the highly potent proteasome inhibitors **11–13** on proteasome activity in cell systems. As shown in Fig. 6a–c, the effects of these compounds on p53 levels, NF-κB activation, and cell viability, which are related to the proteasome inhibitory potency, were evaluated in HeLa cells.

The tumor suppressor gene product p53 is known to be a proteasome substrate. Previous studies suggest that the p53 upregulated modulator of apoptosis (PUMA) pathway may play a prominent role in tumor cell apoptosis induced by proteasome inhibitors.[20] Thus, we examined the effect of the compounds on the intracellular protein level of p53 in HeLa cells by immunoblotting (Fig. 6a). None of the compounds tested had any effect on the α-tubulin level in cells, as was the case for belactosin A (**2**) and its

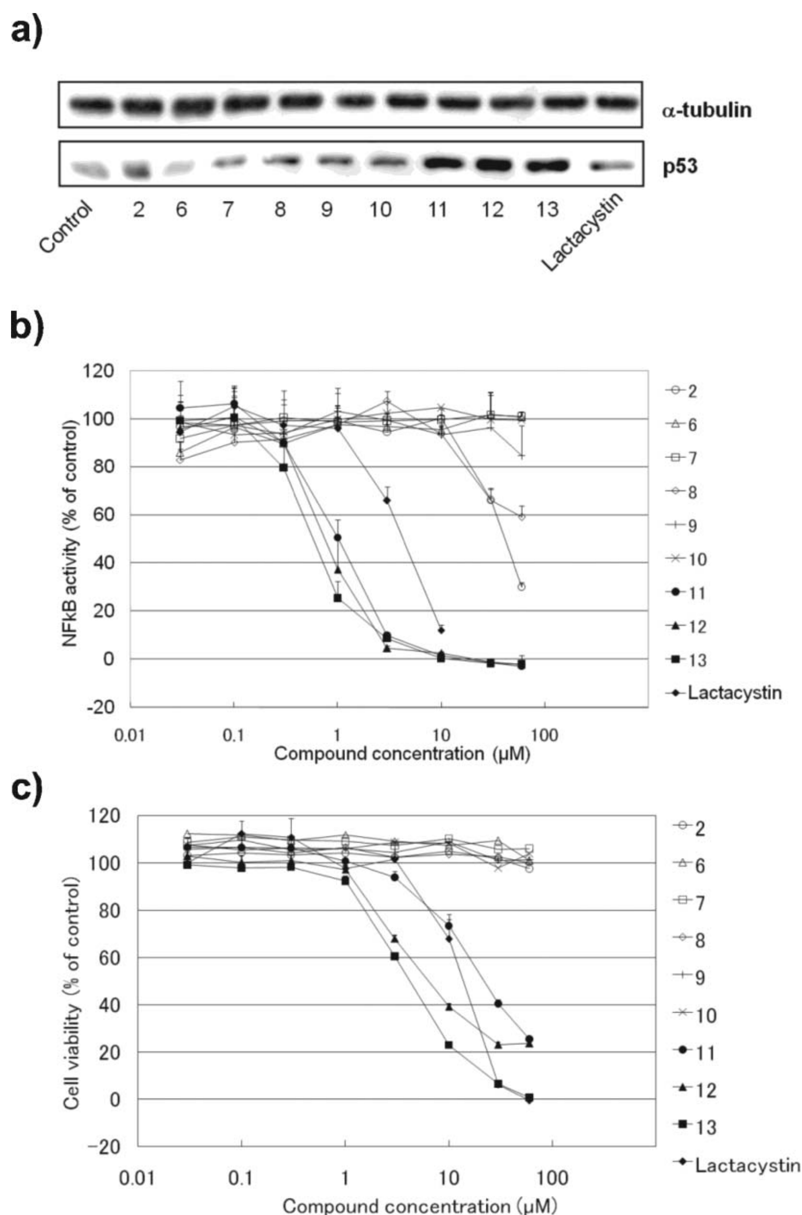


Fig. 6 Biological effects of compounds in HeLa cells due to proteasome inhibition. (a) Intracellular protein level of p53. Accumulation of p53 in response to compounds was determined by immunoblotting. HeLa cells were treated with 10 μM compound or 0.1% DMSO for 4 h. Equivalent amounts of total cellular proteins were separated by SDS-PAGE and immunoblotted with anti-p53 antibody or anti-α-tubulin antibody. (b) NF-κB activation in HeLa/NF-κB-luc cells. HeLa/NF-κB-luc cells were preincubated for 1 h in the absence or presence of several derivatives, and then they were incubated for another 5 h with TNF-α (3 ng/mL). Luciferase activity was evaluated in a luminometer for light emissions. The luminescence for control cells (0.1% DMSO) was defined as a NF-κB activity of 100%. (c) Growth inhibition by compounds in HeLa cells. Various concentrations of compounds were applied to cells in 72 h intervals before measurement of viabilities using the WST-8 assay. The absorbance for *versus* vehicle (0.1% DMSO)-treated cells was defined as a WST-8 activity of 100%, and results represent the average of three independent trials.

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isomers **6–10**. However, consistent with the above results on the purified proteasome inhibitory potency, the tripeptidic synthetic precursors **11**, **12**, and **13** induced significant accumulation of intercellular p53 at 10 μM.

It is known that binding of the transcription factor NF-κB to the inhibitor protein IκB in cytoplasm renders NF-κB inactive.[21] Several cellular stimuli trigger a cascade of signal transduction events that phosphorylate and ubiquitinate IκB, leading to its degradation by the proteasome, which liberates and activates NF-κB as a transcription factor.[22] In fact, proteasome inhibitors have been demonstrated to suppress the activation of NF-κB.[23] Therefore, we evaluated the inhibitory effect of the compounds on NF-κB activation using reporter gene assay with HeLa/NF-κB-luc cells (Fig. 6b), in which the transcriptional activity of NF-κB was induced by TNF-α.

Under these conditions, belactosin A (**2**) inhibited the NF-κB activation, although the activity was not strong ($IC_{50} > 10$ μM). Likewise, all of the isomers **6–10** were almost inactive or only weakly inhibited NF-κB activation in this system. However, the tripeptidic compounds **11**, **12**, and **13** showed a remarkable inhibitory effect on the NF-κB activation ($IC_{50} = \sim 1$ μM), which clearly surpassed that of lactacystin ($IC_{50} = 3.6$ μM). Compound **13**, having the *cis*/L-*anti*-cyclopropane structure, was the most potent inhibitor in this evaluation with an IC_{50} value of 0.6 μM.

We finally investigated the anti-proliferative activity of the compounds in HeLa cells. As shown in Fig. 6c, although belactosin A (**2**) and its isomers **6–10** were inactive against HeLa cell viability, the tripeptidic synthetic precursors **11**, **12**, and **13** exerted significant anti-proliferative activity against HeLa cells. Compound **11** ($IC_{50} = 22$ μM) showed activity similar to that of lactacystin (**4**). Compounds **12** and **13** were more active than **11** with IC_{50} values of 3.8 μM and 4.5 μM, respectively.

These results with HeLa cell systems were correlated with the proteasome inhibitory potency of compounds in a cell-free system. In these evaluations, the tripeptidic synthetic precursors **11**, **12**, and **13** showed remarkable activity due to the proteasome inhibitor effect, where the *cis*/L-*anti*-type compound **13** was more effective than the *trans*/L-*anti*-type compound **11** having the same stereochemistry as natural belactosin A.

Discussion

We recently developed a general procedure for the preparation of a series of cyclopropane amino acid equivalents with the *cis*/*trans*, D/L, and *syn*/*anti* stereochemical diversity. With the cyclopropane amino acid equivalents in hand, we achieved the total synthesis of belactosin A (**2**), and then systematically synthesized all of the designed stereo- and regioisomers **6–10** by the same or the modified synthetic route. These results demonstrate that the cyclopropane amino acid equivalents are very useful in medicinal chemical studies of amino acids and peptides.

The biological evaluation of this series of compounds showed that their activities are changed depending on the stereochemistry of the central cyclopropane amino acid part of the compound: for example, the *trans*/L-*syn*-isomer **8** and the *cis*/L-*anti*-isomer **9** were twice as potent as natural belactosin A (*trans*/L-*anti*-isomer) in their proteasome inhibitory potency with a cell-free system.

We found that the tripeptidic compounds **11**, **12**, and **13** have a highly potent inhibitory effect on purified proteasomes, which were 7–20 times as potent as belactosin A. With regard to the cell-free proteasome inhibitory potency, these three compounds also were remarkably more effective than belactosin A in the p53 accumulation, the NF-κB activation, and the cell-viability evaluations in cells.

It is important to note that compound **13** with the unnatural *cis*/L-*anti* structure surpasses **11** with the natural belactosin A-type *trans*/L-*anti* structure in all of the evaluation systems, in accord with the stereochemistry-activity relationship of belactosin A (**2**) and its *cis*/L-*anti* isomer **9**.

Although structural modification of biologically active natural products often results in reducing the activity, the stereochemical diversity-oriented strategy was shown to be effective in this study for developing highly active analogs of belactosin A. It should also be pointed out that this approach can work without structural data of the target protein. These results, as well as the previous cases in the development of GPCR ligands,[8] show that a systematic study by the stereochemical diversity-oriented strategy with the conformationally rigid cyclopropane structure may allow exhaustive investigation of the bioactive conformation of compounds in order to develop compounds which bind selectively to the target protein.

The development of proteasome inhibitors as alternative anticancer drugs is urgently needed. Proteasome inhibitors are highly desirable as anticancer agents since they have the ability to overcome chemoresistance of tumor cells by enhancing chemosensitivity and even act in synergy with other agents to induce apoptotic tumor cell death.[3a,b] Consequently, compound **13**, which is significantly more potent than belactosine A (**2**) and the well-known inhibitor lactacystin (**4**), may be an effective lead for developing clinically useful anticancer drugs.

Conclusions

We have designed a series of the stereo- and regioisomers of belactosin A with stereochemical diversity, based on the idea that the cyclopropane part of belactosin A, restricting the three-dimensional structure of the molecule, may play a critical role in its interaction with the proteasome. The target isomers and also belactosin A were systematically synthesized, using the key cyclopropane amino acid equivalents with *cis*/*trans*, D/L, and *syn*/*anti* stereochemical diversity. We demonstrated that the proteasome inhibitory activities of the compounds changed depending on the stereochemistry of the central cyclopropane amino acid part, where the *cis*/L-*anti*-type compounds are more active than the natural belactosin A (*trans*/L-*anti*)-type compounds. These results show that the stereochemical diversity-oriented approach can be a useful strategy for the development of highly active compounds. Thus, the potent proteasome inhibitor **13**, which potentially could become an effective lead for developing anticancer drugs, was identified.

Experimental

Chemical shifts are reported in ppm downfield from Me_4Si (^1H) and MeCN (^{13}C). All of the ^1H NMR assignments described were in agreement with COSY spectra. Thin-layer chromatography was done on Merck coated plate $60F_{254}$. Silica gel and reverse phase chromatographies were done on Merck silica gel 5715 and Fuji

Silysia ODS Chromatorex, respectively. Reactions were carried out under an argon atmosphere.

(1*R*,2*S*)-1-[(2*S*)-2-(9-Fluorenylmethoxycarbonylamino)-3-butenyl]-2-hydroxymethylcyclopropane (20)

A mixture of **16**[9] (334 mg, 690 μmol) and HCl (4 M in AcOEt, 690 μL) in MeOH (6 mL) was stirred at room temperature for 40 min, and then the mixture was evaporated. To a solution of the residue in 50% aqueous THF (1.6 mL) was added FmocOSu (279 mg, 828 μmol) and Na_2CO_3 (74 mg, 700 μmol), and the resulting mixture was stirred at room temperature for 12 h. The mixture was concentrated *in vacuo* and then partitioned between AcOEt and H_2O. The organic layer was washed with brine, dried (Na_2SO_4), evaporated, and purified by column chromatography (silica gel; hexane/AcOEt, 5:1 then 4:1) to give diastereomerically pure **20** (213 mg, 85%) as a white amorphous solid: $[\alpha]^{22}_D$ +14.60 (*c* 1.02, MeOH); ^{1}H-NMR (400 MHz, $CDCl_3$) δ 0.36 (2 H, m), 0.57 (1 H, m), 0.84 (1 H, m), 1.00 (1 H, m), 1.82 (1 H, m), 2.71 (1 H, br s), 2.87 (1 H, m), 3.86 (1 H, m), 4.24 (1 H, m), 4.33–4.58 (3 H, m), 5.11 (3 H, m), 5.81 (1 H, m), 7.28–7.32 (2 H, m), 7.34–7.45 (2 H, m), 7.59–7.63 (2 H, m), 7.76–7.77 (2 H, m); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 9.1, 14.6, 21.6, 39.5, 47.2, 47.5, 53.3, 66.4, 67.0, 114.6, 119.8, 124.8, 126.9, 127.5, 138.2, 141.2, 143.6, 156.3; HRMS (EI) calcd for $C_{23}H_{25}NO_3$: 363.1834 (M^+), found 363.1838 (M^+).

(1*R*,2*S*)-2-*tert*-Butoxycarbonylamino-1-[(2*S*)-2-(9-fluorenylmethoxycarbonylamino)-3-butenyl]cyclopropane (21)

A mixture of **20** (380 mg, 1.04 mmol) and Dess–Martin periodinane (DMP, 828 mg, 2.00 mmol) in CH_2Cl_2 (10 mL) was stirred at room temperature for 30 min, and then aqueous saturated $Na_2S_2O_3$ and aqueous saturated $NaHCO_3$ were added. The mixture was partitioned between $CHCl_3$ and H_2O, and the organic layer was washed with brine, dried (Na_2SO_4), evaporated to give the corresponding aldehyde as an oil. A mixture of the oil, $NaClO_2$ (360 mg, 4.00 mmol), NaH_2PO_4 (234 mg, 1.50 mmol), and 2-methyl-2-butene (423 μL, 4.00 mmol) in 80% aqueous *t*-BuOH (10 mL) was stirred at room temperature for 15 h and then evaporated. The residue was partitioned between AcOEt and aqueous HCl (1 M), and the organic layer was washed with brine, dried (Na_2SO_4), and evaporated to give the corresponding carboxylic acid as a white solid. To a solution of the white solid in CH_2Cl_2 (5 mL) was added Et_3N (195 μL, 1.4 mmol) and DPPA (603 μL, 2.8 mmol) at 0 °C, and the resulting mixture was stirred at room temperature for 2 h. The mixture was partitioned between AcOEt and H_2O, and the organic layer was washed with brine, dried (Na_2SO_4), evaporated, and purified by column chromatography (neutral silica gel; hexane/AcOEt, 4:1) to give the corresponding acid azide as an oil. A solution of the oil in *t*-BuOH (10 mL) was heated under reflux for 12 h, and then evaporated. The residue was purified by column chromatography (silica gel; hexane/AcOEt, 7:1 then 5:1) to give **21** (220 mg, 47%) as an oil: $[\alpha]^{23}_D$ −14.69 (*c* 0.98, $CHCl_3$); ^{1}H-NMR (400 MHz, $CDCl_3$) δ 0.45 (1 H, dd, *J* = 5.8, 12.5 Hz), 0.58 (1 H, m), 0.90 (1 H, br s), 1.06 (1 H, br s), 1.48 (9 H, s), 2.01 (1 H, br s), 2.32 (1 H, br s), 4.27 (2 H, m), 4.40 (1 H, dd, *J* = 10.4, 13.0 Hz), 4.58 (1 H, br s), 4.85 (1 H, s), 5.16 (1 H, d, *J* = 10.5 Hz), 5.33 (1 H, d, *J* = 17.1 Hz), 5.91 (1 H, m), 7.27–7.31 (2 H, m), 7.37–7.40 (2 H, m), 7.73–7.77 (4 H, m), 8.16 (1 H, d, *J* = 9.4 Hz); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 10.5, 16.6, 28.4, 29.9, 36.6, 47.4, 52.4, 66.7, 80.0, 114.5, 119.8, 125.4, 126.9, 127.4, 137.9, 141.2, 144.2, 144.4, 156.5, 157.5; HRMS (EI) calcd for $C_{27}H_{32}N_2O_4$: 448.2362 (M^+), found 448.2364 (M^+).

(1*R*,2*S*)-2-*tert*-Butoxycarbonylamino-1-[(2*S*)-2-(*N*-Cbz-L-alanyl)amino-3-butenyl]cyclopropane (23)

A mixture of **21** (88 mg, 200 μmol) and K_2CO_3 (28 mg, 200 μmol) in MeOH (2 mL) was stirred at room temperature for 12 h. The resulting mixture was filtrated with Celite, evaporated, and purified by column chromatography (NH-silica gel; hexane/AcOEt = 1:1) to give the corresponding Fmoc-removed product as an oil. Separately, to a mixture of *N*-Cbz-L-Ala (134 mg, 600 μmol) and Et_3N (80 μL, 580 μmol) in CH_2Cl_2 (3 mL) was added PivCl (71 μL, 580 μmol) at 0 °C, and the mixture was stirred at the same temperature for 10 min. The resulting mixture was partitioned between AcOEt and H_2O, and the organic layer was washed with brine, dried (Na_2SO_4), and evaporated to give **22** as an oil. To a mixture of the obtained Fmoc-removed product and $NaHCO_3$ (34 mg, 400 μmol) in toluene (1 mL) was added a solution of **22** in toluene (1 mL) at room temperature, and the resulting mixture was stirred at the same temperature for 18 h. The mixture was partitioned between AcOEt and aqueous HCl (1 M), and the organic layer was washed with aqueous saturated $NaHCO_3$ and brine, dried (Na_2SO_4), and evaporated. The residue was purified by column chromatography (silica gel; hexane/AcOEt = 4:1, 3:1, then 2:1) to give **23** (62 mg, 72%) as a white amorphous solid: $[\alpha]^{23}_D$ −9.76 (*c* 1.03, $CHCl_3$); ^{1}H-NMR (400 MHz, $CDCl_3$) δ 0.44 (1 H, m), 0.56 (1 H, m), 0.89 (2 H, m), 1.43 (9 H, s), 1.50 (3 H, d, *J* = 6.7 Hz), 2.05 (1 H, d, *J* = 13.9 Hz), 2.24 (1 H, br s), 4.45 (1 H, m), 4.87 (2 H, m), 5.09–5.20 (4 H, m), 5.87 (2 H, m), 7.33–7.34 (5 H, m), 9.23 (1 H, d, *J* = 9.4 Hz); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 10.2, 16.1, 20.2, 28.2, 30.2, 35.9, 49.5, 50.3, 66.5, 80.5, 114.3, 127.9, 128.1, 128.4, 136.7, 137.7, 155.6, 158.0, 172.0; HRMS (EI) calcd for $C_{23}H_{33}N_3O_5$: 431.2420 (M^+), found 431.2425 (M^+).

Compound 11

A solution of **23** in TFA/CH_2Cl_2 (1:1, 3 mL) was stirred at room temperature for 5 min, and then diluted with toluene. The resulting mixture was evaporated to give the corresponding Boc-removed product **24** as an oil. Separately, to a solution of **25**[10a] (117 mg, 680 μmol) and Et_3N (90 μL, 650 μmol) in CH_2Cl_2 (2 mL) was added PivCl (80 μL, 650 μmol) at 0 °C, and the mixture was stirred at the same temperature for 10 min at the same temperature to form **26**. To this solution of **26** was added a solution of the above obtained **24** in DMF/CH_2Cl_2 (1:2, 3 mL) and $NaHCO_3$ (27 mg, 320 μmol), and the resulting mixture was stirred at room temperature for 17 h. The mixture was partitioned between AcOEt and aqueous HCl (1 M), and the organic layer was washed with aqueous saturated $NaHCO_3$ and brine, dried (Na_2SO_4), and evaporated. The residue was purified by column chromatography (silica gel; hexane/AcOEt = 3:1, then 2:1) to give **11** (149 mg, 95%) as a white amorphous solid: $[\alpha]^{25}_D$ +10.33 (*c* 1.00, $CHCl_3$); ^{1}H-NMR (400 MHz, $CDCl_3$) δ 0.56 (1 H, dd, *J* = 6.0, 12.9 Hz), 0.73 (1 H, m), 0.85 (1 H, br s), 0.93 (3 H, t, *J* = 7.3 Hz), 1.00–1.06 (4 H, m), 1.33 (1 H, m), 1.46 (3 H, d, *J* = 6.7 Hz), 1.62 (1 H, m), 1.97 (1 H, m), 2.09 (1 H, d, *J* = 14.5 Hz), 2.41 (1 H, br s), 3.49 (1 H,

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dd, $J = 4.7, 7.3$ Hz), 4.40 (1 H, m), 4.63 (1 H, d, $J = 4.7$ Hz), 4.85 (1 H, br s), 5.08–5.17 (4 H, m), 5.74 (1 H, d, $J = 7.7$ Hz), 5.82 (1 H, m), 6.65 (1 H, s), 7.29–7.34 (5 H, m), 9.02 (1 H, d, $J = 8.8$ Hz); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 10.1, 11.2, 16.4, 16.4, 20.1, 26.9, 29.5, 33.8, 36.0, 49.6, 50.6, 63.0, 66.6, 70.1, 114.3, 127.8, 127.8, 128.3, 136.5, 137.4, 155.4, 168.5, 170.8, 171.9; HRMS (EI) calcd for $C_{26}H_{35}N_3O_6$: 485.2525 (M^+), found 485.2525 (M^+).

Belactosin A (2)

A mixture of **11** (43 mg, 88 μmol), $NaIO_4$ (94 mg, 440 μmol), $KMnO_4$ (9.7 mg, 62 μmol), $NaHCO_3$ (7.4 mg, 88 μmol) in aqueous acetone (67%, 3 mL) was stirred at room temperature for 17 h. The mixture was diluted with AcOEt and washed with aqueous HCl (1 M, 4 times), and the organic layer was washed with brine, dried (Na_2SO_4), and evaporated. A solution of the residue in MeOH (1 mL) was passed through a short column (Diaion-PK212L, H^+ form, aqueous 50% MeOH to give *N*-Cbz-belactosin A as a white solid. A mixture of the obtained *N*-Cbz-belactosin A and Pd/C (10%, 43 mg) in HCO_2H/THF (2:3, 5 mL) was stirred under atmospheric pressure of H_2 at room temperature for 2 h, and then the catalysts were filtered off with Celite and washed with CH_2Cl_2. The filtrate was evaporated, and the residue was co-evaporated with toluene/MeOH (2:1), and the residue was purified by column chromatography (C18-reverse phase silica gel; 10% aqueous MeCN) to give belactosin A (**2**, 25 mg, 77%) as a white amorphous solid, the ^{1}H NMR spectrum of which was in accord with that of natural belactosin A:[15] $[\alpha]^{23}_D$ +4.94 (*c* 0.95, H_2O) [lit.[5a] $[\alpha]^{27}_D$ +4.8 (*c* 0.37, H_2O)]; ^{1}H-NMR (500 MHz, D_2O) δ 0.66 (1 H, m), 0.79 (1 H, m), 0.83 (3 H, t, $J = 7.5$ Hz), 0.90 (1 H, m), 0.95 (3 H, d, $J = 6.7$ Hz), 1.27 (1 H, m), 1.48 (1 H, m), 1.51 (3 H, d, $J = 7.0$ Hz), 1.58 (1 H, m), 1.86 (1 H, m), 1.97 (1 H, m), 2.46 (1 H, m), 3.80 (1 H, dd, $J = 3.6, 7.3$ Hz), 4.08 (1 H, q, $J = 7.1$ Hz), 4.28 (1 H, t, $J = 5.1$ Hz), 4.81 (1 H, d, $J = 4.3$); ^{13}C-NMR (125 MHz, D_2O) δ 11.0, 11.9, 16.2, 16.6, 17.2, 26.9, 29.0, 33.5, 34.4, 49.8, 55.6, 62.5, 71.8, 170.5, 172.44, 173.2, 178.3; LRMS (FAB) *m/z* 370 [$(M+H)^+$]; HRMS (FAB) calcd for $C_{17}H_{27}N_3O_6$: 370.1978 [$(M+H)^+$], found 370.1974 [$(M+H)^+$].

(1*S*,2*S*)-2-Azidomethyl-1-[(1*R*)-1-((*S*)-*tert*-butylsulfinyl)amino-2-propenyl]cyclopropane (27)

A mixture of **19**[9] (2.82 g, 6.0 mmol) and TBAF (1.0 M in THF, 12 mL) in THF (48 mL) was stirred at room temperature for 14 h and then evaporated. The residue was purified by column chromatography (silica gel; $CHCl_3$/MeOH = 100:0 then 97:3) to give the corresponding de-silylated product as a white solid. A solution of the solid, DPPA (2.6 mL, 12 mmol) and DBU (1.9 mL, 12 mmol) in THF (60 mL) was stirred at 0 °C for 10 min and then at room temperature for 6 h. The resulting mixture was concentrated *in vacuo* and partitioned between AcOEt and saturated aqueous NH_4Cl, and the organic layer was washed with brine, dried (Na_2SO_4), and evaporated. A mixture of the residue, NaN_3 (520 mg, 8.0 mmol), and 15-crown-5 (1.8 mL, 8.0 mL) in DMF (60 mL) was stirred at room temperature for 24 h and then evaporated. The residue was partitioned between AcOEt and H_2O, and the organic layer was washed with brine, dried (Na_2SO_4), and evaporated. The residue was purified by column chromatography (silica gel; hexane/AcOEt, 2:1) to give **27** (1.39 g, 89%) as an oil: $[\alpha]^{17}_D$ +106.05 (*c* 1.06, $CHCl_3$); ^{1}H-NMR (500 MHz, $CDCl_3$) δ 0.56–0.62 (2 H, m), 0.90 (1 H, m), 1.24–1.34 (10 H, m), 3.16 (1 H, dd, $J = 7.2, 13.1$ Hz), 3.21–3.28 (2 H, m), 3.34 (1 H, d, $J = 4.5$ Hz), 5.17 (1 H, d, $J = 10.4$ Hz), 5.28 (1 H, d, $J = 17.2$ Hz), 5.89 (1 H, m); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 8.4, 17.5, 21.6, 22.6, 54.5, 55.6, 61.3, 116.7, 138.1; HRMS (FAB) calcd for $C_{11}H_{21}N_4OS$: 257.1436 [$(M+H)^+$], found 257.1425 [$(M+H)^+$].

(1*S*,2*S*)-2-Azidomethyl-1-[(1*R*)-1-(*N*-Cbz-L-alanyl)amino-2-propenyl]cyclopropane (28)

A solution of **27** (25 mg, 100 μmol) and HCl (4 M in AcOEt, 100 μL) in MeOH (1.0 mL) was stirred at room temperature for 10 min and then evaporated to give the corresponding sulfinylamino-removed product as an oil. From the product, compound **28** (32 mg, 88%, white amorphous solid) was synthesized as described for the synthesis of **23**: $[\alpha]^{18}_D$ +1.66 (*c* 0.96, $CHCl_3$); ^{1}H-NMR (500 MHz, $CDCl_3$) δ 0.57 (1 H, m), 0.64 (1 H, m), 0.87 (1 H, m), 1.23 (1 H. m), 1.41 (1 H, d), 2.89 (1 H, dd, $J = 8.2, 12.5$ Hz), 3.26 (1 H, dd, $J = 6.0, 12.5$ Hz), 3.95 (1 H, m), 4.26 (1 H, m), 5.09–5.26 (5 H, m), 5.80 (1 H, br s), 6.21 (1 H, br s), 7.29–7.39 (5 H, m); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 9.0, 15.8, 18.4, 21.9, 29.4, 50.7, 54.1, 54.4, 67.1, 89.4, 115.4, 128.1, 128.3, 128.5, 136.4, 171.5; HRMS (FAB) calcd for $C_{18}H_{24}N_5O_3$: 358.1879 [$(M+H)^+$], found 358.1874 [$(M+H)^+$].

(1*S*,2*S*)-2-*tert*-Butoxycarbonylaminomethyl-1-[(1*R*)-1-(*N*-Cbz-L -alanyl)amino-2-propenyl]cyclopropane (29)

A mixture of **28** (138 mg, 0.38 mmol) and PPh_3 (131 mg, 0.50 mmol) in 80% aqueous THF (3 mL) was stirred at room temperature for 2 h. To the mixture was added $(Boc)_2O$ (350 μL) and the resulting mixture was further stirred at room temperature for 2 h. The mixture was partitioned between AcOEt and H_2O, and the organic layer was washed with brine, dried (Na_2SO_4), and evaporated. The residue was purified by column chromatography (silica gel; hexane/AcOEt, 4:1 then 2:1) to give **29** (149 mg, 90%) as a white amorphous solid: $[\alpha]^{22}_D$ +24.76 (*c* 1.00, $CHCl_3$); ^{1}H-NMR (400 MHz, DMSO-d_6) δ 0.34 (2 H, m), 0.80 (2 H, m), 1.20 (3 H, d, $J = 7.1$ Hz), 1.35 (9 H, s), 2.80 (2 H, m), 3.89 (1 H, m), 4.03 (1 H, m), 4.98–5.09 (4 H, m), 5.76 (1 H, m), 6.74 (1H, br s), 7.31–7.35 (6 H, m), 7.77 (1 H, d); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 8.8, 17.1, 18.4, 22.2, 27.4, 28.4, 44.3, 50.5, 54.9, 66.9, 79.2, 115.0, 128.0, 128.1, 128.4, 136.1, 136.6, 155.9, 171.6; HRMS (EI) calcd for $C_{23}H_{33}N_3O_5$: 431.2420 (M^+), found 431.2421 (M^+).

Compound 30

Compound **30** (135 mg, 80%, white amorphous solid) was prepared from **29** (149 mg, 345 μmol) as described for the synthesis of **11**: $[\alpha]^{22}_D$ +15.21 (*c* 1.12, $CHCl_3$); ^{1}H-NMR (400 MHz, $CDCl_3$) δ 0.53–0.57 (2 H, m), 0.80 (1 H), 0.93 (3 H, t, $J = 7.4$), 1.03–1.05 (4 H, m, H-2), 1.29 (1 H, m), 1.39 (3 H, d, $J = 7.1$ Hz), 1.65 (1 H, m), 1.94 (1 H, m), 2.82 (1 H, m), 3.38 (1 H, m), 3.58 (1 H, dd, $J = 4.5, 7.7$ Hz), 3.68 (1 H, m), 4.20 (1 H, m), 4.64 (1 H, d, $J = 4.5$ Hz), 5.09–5.22 (4 H, m), 5.66 (1 H, br s), 5.77 (1 H, m), 6.99 (2 H, br s), 7.29–7.36 (5 H, m); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 9.3, 11.0, 16.3, 17.1, 18.4, 23.0, 26.6, 33.8, 43.6, 50.7, 55.5, 62.8, 66.9, 70.9, 115.3, 127.8, 128.0, 128.4, 136.1, 136.6, 156.0, 168.2,

169.3, 171.8; HRMS (EI) calcd for $C_{26}H_{35}N_3O_6$: 485.2525 (M^+), found 485.2529 (M^+).

Regioisomer (10)

Compound **10** (47 mg, 67%, white amorphous solid) was prepared from **30** (93 mg, 192 μmol) as described for the synthesis of **3**: $[\alpha]^{22}_D$ +36.57 (*c* 1.02, H_2O); ^{1}H-NMR (500 MHz, D_2O) δ 0.57 (1 H, dd, *J* = 5.7, 13.7 Hz), 0.69 (1 H, dd, *J* = 5.7, 13.7 Hz), 0.84 (3 H, t, *J* = 7.4 Hz), 0.98 (3 H, d, *J* = 6.8 Hz), 1.00–1.04 (2 H, m), 1.27 (1 H, m), 1.46–1.50 (4 H, m), 1.99 (1 H, m), 3.07 (1 H, dd, *J* = 6.3, 13.7 Hz), 3.20 (1 H, dd, *J* = 6.3, 14.3 Hz), 3.59 (1 H, d, *J* = 8.0 Hz), 3.78 (1 H, dd, *J* = 4.6, 7.4 Hz), 4.03 (1 H, q, *J* = 6.8 Hz), 4.87 (1 H, d, *J* = 4.0 Hz); ^{13}C-NMR (125 MHz, D_2O) δ 9.5, 10.9, 16.0, 16.6, 17.0, 19.4, 26.8, 33.4, 43.1, 49.5, 59.1, 62.5, 71.5, 170.4, 170.7, 173.2, 178.0; HRMS (FAB) calcd for $C_{17}H_{27}N_3O_6$: 370.1978 [$(M+H)^+$], found 370.1974 [$(M+H)^+$].

Evaluation with purified humam 20S proteasome

Human 20S proteasome (Boston Biochem) (50 ng per well) in buffer containing 20 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, and 0.035% SDS in a 96-well black plate (BD Falcon) was preincubated for 10 min at 25 °C in the presence of inhibitors dissolved in 0.05% (v/v) dimethyl sulfoxide. Next, the fluorogenic peptide substrate, succinyl-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide (Peptide Institute, Japan), at a final concentration of 20 μM was added and the mixture was further incubated for 2 h at 25 °C. The reaction was stopped by adding 0.5% SDS in 20 mM Tris-HCl, pH 8.0, and the fluorescence due to 7-amino-4-methylcoumarin was measured with excitation at 355 nm and emission at 460 nm on a Fluoroskan Ascent fluorometer (Thermo).

Inhibitory effect on NF-κB activity

HeLa/NF-κB-luc cells (Panomics, 5×10^4 cells/well) were cultured for 1 h at 37 °C in 96-well plate in 100 μL of medium A (DMEM supplemented with 25 mM glucose, 10% FBS, 100 units/mL penicillin, 100 mg/mL streptomycin) with or without various concentrations of test compounds. After treatment, cells were incubated with 3 ng/mL TNF-α (Sigma) for an additional 5 h and collected for luciferase assay. Luciferase analyses were performed using Steady-Glo[R] Luciferase Assay System (Promega) according to the instructions from the manufacturer. The plates were measured luminescence in a luminometer.

Effect on the p53 degradation

HeLa cells (American Type Culture Collection) were incubated in the absence or presence of the 10 μM test compounds for 4 h, as indicated in Fig.6. Cells treated with chemicals were washed twice with PBS, and then resuspended in 200 μL of 10 mM Tris–HCl (pH 7.4) containing 150 mM NaCl, 0.1% SDS, 0.1% sodium deoxycholate, 1% NP-40, 1 mM EDTA, 1 μM leu-peptin, 2 μg/mL aprotinin, and 10 μg/mL E-64 at 4 °C for 5 min. After centrifugation at 10,000 *g* for 10 min at 4 °C, the supernatants were collected and determined for protein concentration by the bicinchoninic acid protein assay kit (Pierce). Each protein sample (10 μg) was electrophoresed on sodium dodecyl sulfate-polyacrylamide gel (7.5%) and transferred (100 mA) to polyvinylidene difluoride (PVDF) filter membranes (Millipore). PVDF membranes were subjected to immunoblot analyses with anti-p53 primary antibody (Santa Cruz Biotechnology, 1:1000) and horseradish peroxide-conjugated secondary antibody (1:3,000) in TBST [10 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.05% Triton X-100] solution containing 5% nonfat milk; a chemiluminescence reagent kit (Amersham Biosciences) was used for visualization. The intensity of each immunoreactive band was measured by means of Image Gauge software with an LAS-3000plus (Fujifilm).

Effect on cell viability

The cytotoxicity of the derivatives was determined with a Cell Counting Kit-8 (Dojindo Laboratories) to count living cells by WST-8. HeLa cells (5×10^4 cells/well) were cultured for 72 h at 37 °C in 96-well plate in 100 μL of medium A with or without various concentrations of several derivatives. After exposure, the cells were washed with 100 μL phosphate-buffered saline (PBS) once and added 100 μL medium A containing 10% WST-8 solutions to each well. The cells were incubated at 37 °C for 2 h, the plates were read on the micro-plate reader at 450 nm. The absorbance for control cells was defined as a WST-8 activity of 100%.

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