Bioorganic & Medicinal Chemistry Letters 19 (2009) 3220–3224

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis and biological evaluation of boron peptide analogues of Belactosin C as proteasome inhibitors

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ARTICLE INFO

Article history:
Received 12 February 2009
Revised 20 April 2009
Accepted 22 April 2009
Available online 3 May 2009

Keywords:
Proteasome
Boron peptides
Chymotrypsin-like activity

ABSTRACT

A series of boron peptides **11**, **13**, **15** and **17** were designed and synthesized as proteasome inhibitors based on the structure of Belactosin C. Matteson homologation was a key step in the synthesis of the boron peptides. Compounds **11a** and **13** showed significant inhibition of 20S proteasome chymotrypsin-like (β5) activity (IC_{50} = 0.28 and 0.51 μM, respectively). Furthermore, like PS-341, compound **11a** increased the G2/M cell distribution. A biparametric cytofluorimetric analysis with FITC-labeled annexin V and propidium iodide showed induction of apoptosis by compound **11a** at >1 μM concentrations of compound.

The ubiquitin–proteasome pathway plays a critical role in the degradation of a majority of cellular proteins in eukaryotes.[1–4] The first step in the pathway is activation by ubiquitin activating enzyme (E1) followed by ubiquitin transfer to a ubiquitin-conjugating enzyme (E2). The second step involves specific binding of E2 conjugated with substrate to ubiquitin ligase (E3). The polyubiquitinated protein is then degraded by a 26S proteasome.[5] According to the X-ray crystal structures, the 26S proteasome consists of the 20S proteasome cylinder capped by a regulatory 19S complex at each extremity.[6] The 20S proteasome has three principle catalytic activities known as the caspase-like (β1), trypsin-like (β2) and chymotrypsin-like (β5) activities. The hydroxy group of the N-terminal threonine in each active site reacts as a nucleophile to initiate amide bond cleavage of the substrate.[7,8]

The ubiquitin–proteasome pathway regulates levels of proteins critical for cell cycle control, including p53, p27 and cyclin B. Furthermore, the proteasome degrades IκBα to generate the activated transcription factor NF-κB. Uncontrolled regulation of NF-κB has been linked to cancer, inflammatory disease and immune system dysfunction.[8,9] Therefore, development of proteasome inhibitors is considered an attractive approach for new therapeutic agents.[10]

Many natural and synthetic inhibitors of the proteasome, such as peptide aldehyde (MG-132), peptide boronate (PS-341, Bortezomib), peptide epoxyketone (Epoxomicin), and peptide vinylsulfone (NLVS), have been developed as shown in Figure 1.[11] MG-132, which was one of the first synthetic inhibitors, interacts reversibly with the N-terminal threonine residue of the β5 active site.[9] PS-341 has been approved for the clinical treatment of patients with multiple myeloma.[12,13] The X-ray co-crystal structure revealed that PS-341 binds to the N-terminal threonine residues of three catalytic active sites, β1, β2 and β5, at high compound concentrations through a B–O covalent linkage, which generates an sp^3 hybridized orbital complex.[5] Epoxomicin reacts primarily with the chymotrypsin-like active site and the X-ray co-crystal structure showed a six-membered morpholine ring formed by the N-terminal threonine and epoxomicin, which results in irreversible inhibition.[14] NLVS has been developed as a vinyl sulfone analogue of MG-132. It seems likely that the hydroxyl group of the N-terminal threonine residues reacts with the double bond of the vinyl sulfone moiety by Michael addition.[15]

In 2000, Belactosin A and C were isolated from a *Streptomyces* sp. by Asai and co-workers as shown in Scheme 1.[16] They found that Belactosin A and C showed in vitro antiproliferative activity against HeLa S3 cells with IC_{50} values of 51 and 200 μM, respectively, after 72 h exposure. Furthermore, both Belactosin A and C similarly inhibited the chymotrypsin-like activity of rabbit 20S proteasome.[17] Here, we focus on the β-lactone moiety of the belactosins. The inhibitory potency of belactosins toward the proteasome appears to be due to the β-lactone moiety, which undergoes a nucleophilic ring opening by the hydroxyl group of the N-terminal threonine residue to form a covalent bond.[18] We reasoned that replacement of the β-lactone moiety with boronic acid would give candidate analogues of belactosins that act reversibly. In this Letter, we report the synthesis and biological evaluation of boron peptide analogues of Belactosin C.

The boron peptides can be constructed from three components: **1**, **2** and **3**. The components **1** are *N*-Boc protected glycine (**1a**),

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doi:10.1016/j.bmcl.2009.04.103

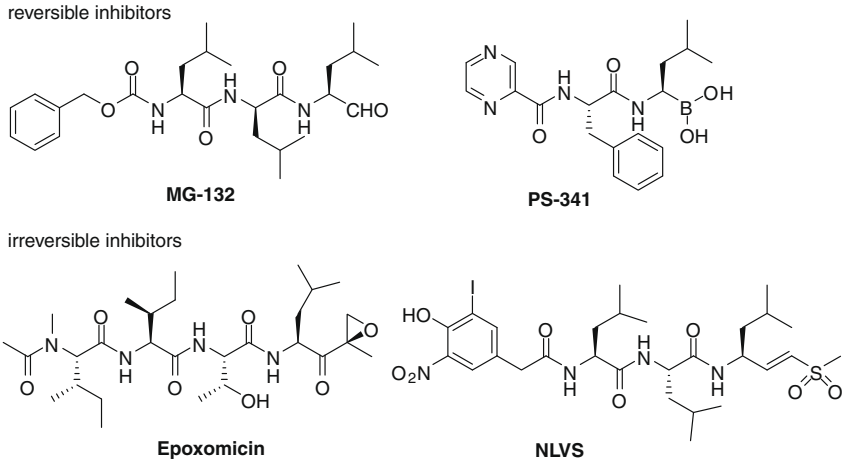
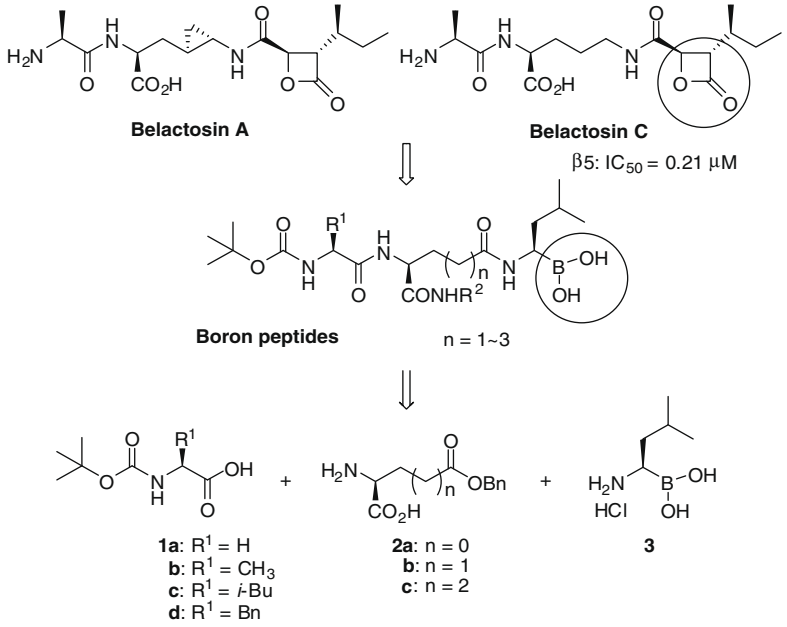


Figure 1. Structures of proteasome inhibitors.



Scheme 1. Design and retro synthesis of boron peptides as Belactosin analogues.

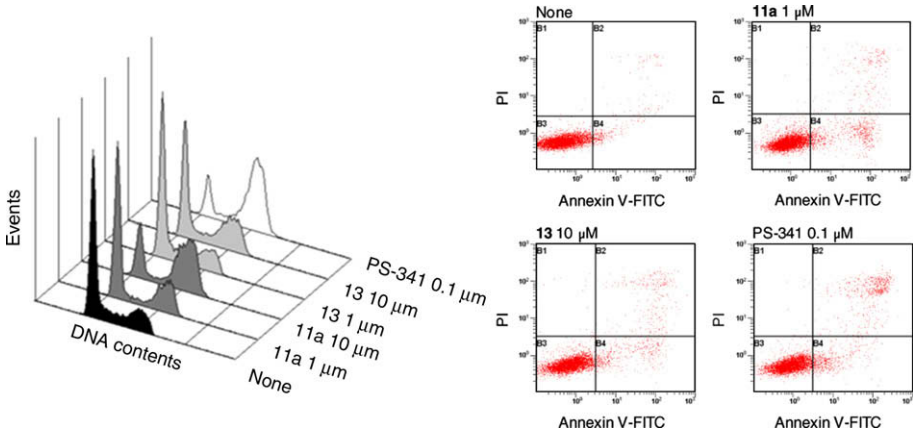


Figure 2. Flow cytometer analysis of cell cycle and apoptosis.

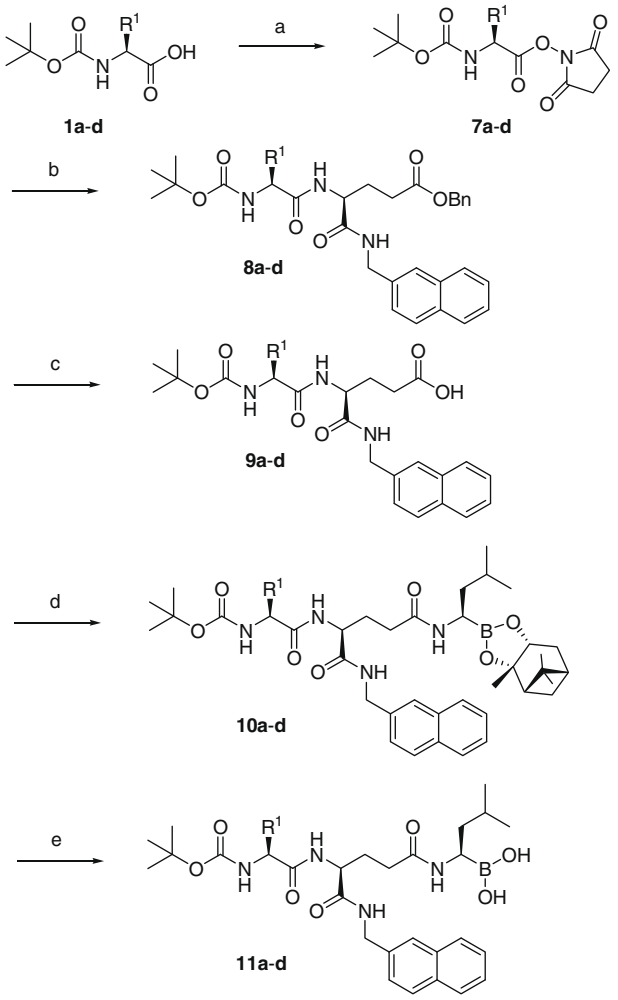
alanine (**1b**), leucine (**1c**) and phenylalanine (**1d**). Components **2** were prepared by selective benzyl esterification of L-aspartic acid (**2a**), L-glutamic acid (**2b**), and L-2-aminoadipic acid (**2c**). Component **3** was synthesized from isobutyl boronic acid **4** as shown in Scheme 2.[19] Matteson homologation[20] of the pinanediol ester **5** gave the α-chloro boronic ester **6** in 81% yield with >98% diastereoselectivity. Treatment of **6** with lithium hexamethyldisilazide afforded the corresponding silylated amino boronic ester **7**, which was treated with hydrochloric acid at −78 °C to give aminoboronic ester **3** as the HCl salt. Because of its moisture sensitivity, compound **3** was used without further purification.

We next synthesized the boron peptides ($n = 2$) from the components **1a–d** and benzyl L-glutamate **2b** as shown in Scheme 3. Carboxylic acids **1a–d** were activated with *N*-hydroxysuccinimide and the resulting esters **7a–d** were treated with **2b** to afford the corresponding dipeptides. The dipeptides were again activated with *N*-hydroxysuccinimide and the resulting esters were treated with 2-naphtylmethylamine under basic conditions to give **8a–d** in 36–51% yield. Hydrogenation of **8a–d** proceeded in the presence of 10% Pd/C under an atmosphere of hydrogen. The corresponding carboxylic acids **9a–d** were activated by treatment with isobutyl chloroformate (IBCF) using *N*-methylmorpholine (NMM) as a base and the resulting acid anhydrides were reacted with the aminoboronic ester **3** to give the corresponding boron esters **10a–d**. Finally, hydrolysis of pinanediol esters **10a–d** was carried out by two-phase transesterification with isobutyl boronic acid under acidic conditions to afford **11a–d** in 18–25% yield. In a similar manner, the benzylamide derivative **13** was also synthesized from **7a** (Scheme 4).

Boron peptides derived from L-2-aminoadipic acid **2c** were also synthesized. Scheme 5 shows the synthesis of **15a** and **15b** from the activated esters **7a** and **7b**. We also synthesized compounds **17a–c**, which have a Boc group at the terminal end of component **2** instead of component **1**, as shown in Scheme 6.

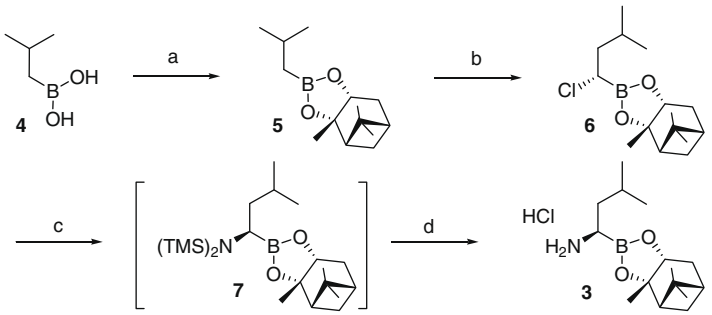
We first examined cell growth inhibition of the various compounds using the HeLa cell line. The cells were incubated with different concentrations of each compound for 72 h. Cell viability was then determined using the MTT assay. As shown in Table 1, compound **11a** displayed significant cell growth inhibition (IC_{50} value was 0.35 μM). Although **11b–d**, **13** and **15b** gave moderate cell growth inhibition (0.67–5.11 μM), **15a** and **17a–c** did not display significant inhibitory activity even at a concentration of 10 μM.

Next, we examined in vitro inhibition of the chymotrypsin-like, caspase-like and trypsin-like activities of the 20S proteasome according to the literature protocol.[17] Briefly, compounds were mixed with 75 μM of a fluorogenic substrate Z-LLE-AMC (caspase-like), Boc-LRR-AMC (trypsin-like), or Suc-LLVY-AMC (chymotrypsin-like) in assay buffer [20 mM Tris (pH 8.0), 0.5 mM EDTA, 0.035% SDS] in a 96-well plate. Hydrolysis was initiated by the addition of 50 ng of 20S proteasome isolated and purified from human erythrocytes. After incubation at 37 °C for 1 h, the reaction

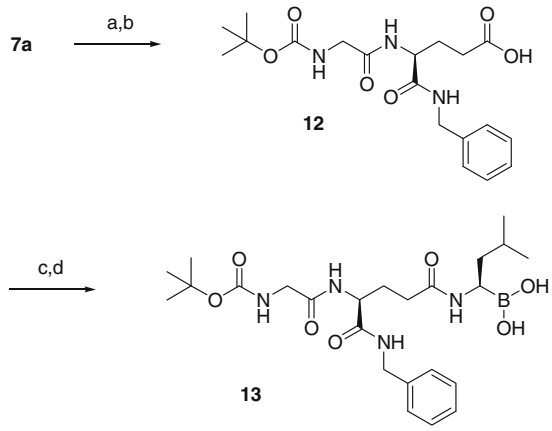


Scheme 3. Reagents and conditions: (a) HOSu, DCC, dioxane, 45–99%; (b) (i) **2b**, $KHCO_3$ aq, THF, rt; (ii) HOSu, DCC, dioxane; (iii) 2-naphtylmethylamine, aqueous $KHCO_3$, THF, rt, 36–51%; (c) H_2, 10% Pd/C, EtOH, rt, 74–78%; (d) (i) IBCF, NMM, THF, −20 °C; (ii) **3**, TEA, $CHCl_3$, −20 °C, 16–37%; (e) *i*-$BuB(OH)_2$, 1 N HCl aq, MeOH/hexane, rt, 18–25%.

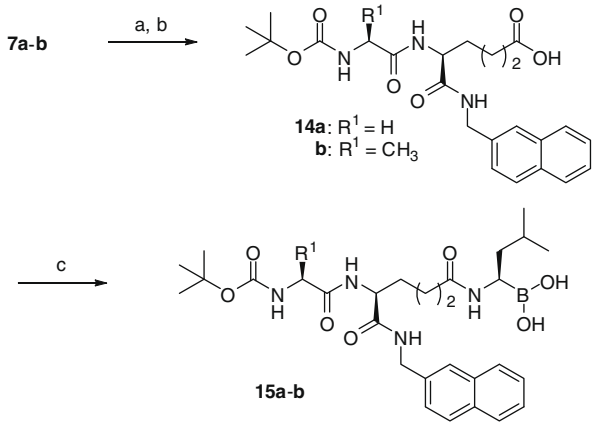
was quenched with stop buffer [20 mM Tris (pH 8.9), 0.5% SDS]. Fluorescence of AMC cleaved from substrates by the proteolytic activity was then measured (365 nm excitation/460 nm detection). The results are shown in Table 1. The boron peptides selectively inhibited the chymotrypsin-like activity (β5) of 20S proteasome. Among the compounds synthesized, compound **11a** possessed the greatest inhibition of the chymotrypsin-like activity, although the IC_{50} value was 10-fold higher than that of PS-341. The substituent at the R^1 position (compounds **11b–d**) did not increase inhib-



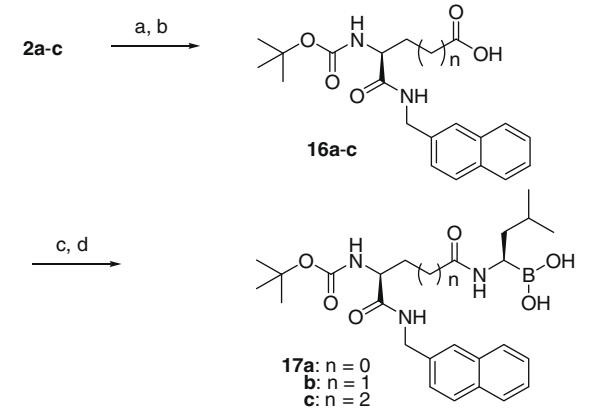
Scheme 2. Reagents and conditions: (a) (+)-pinanediol, Et_2O, 97%; (b) (i) *n*-BuLi, CH_2Cl_2, THF, −100 °C; (ii) $ZnCl_2$, −100 °C, 81%; (c) $LiN(TMS)_2$, THF, −78 °C; (d) 1 N HCl in Et_2O.



Scheme 4. Reagents and conditions: (a) (i) **2b**, $KHCO_3$ aq, THF, rt; (ii) HOSu, DCC, dioxane; (iii) benzylamine, aqueous $KHCO_3$, THF, rt, 44%; (b) H_2, 10% Pd/C, EtOH, rt, 41%; (c) (i) IBCF, NMM, THF, −20 °C; (ii) **3**, TEA, $CHCl_3$, −20 °C, 45%; (d) *i*-$BuB(OH)_2$, 1 N HCl aq, MeOH/hexane, rt, 15%.



Scheme 5. Reagents and conditions: (a) (i) **2c**, $KHCO_3$ aq, THF, rt; (ii) HOSu, DCC, dioxane; (iii) 2-naphtylmethylamine, aqueous $KHCO_3$, THF, rt, 37–64%; (b) H_2, 10% Pd/C, EtOH, rt, 74–77%; (c) (i) IBCF, NMM, THF, −20 °C, (ii) **3**, TEA, $CHCl_3$, −20 °C, 33%; (d) *i*-$BuB(OH)_2$, 1 N HCl aq, MeOH/hexane, rt, 25–43%.



Scheme 6. Reagents and conditions: (a) Boc_2O, TEA, THF/H_2O, rt, 78–99%; (b) (i) HOSu, DCC, dioxane, −5 °C; (ii) 2-naphtylmethylamine, aqueous $KHCO_3$, THF, rt, 78–83%; (b) H_2, 10% Pd/C, EtOH, rt, 79–97%; (c) (i) IBCF, NMM, THF, −20 °C; (ii) **3**, TEA, $CHCl_3$, −20 °C, 16–44%; (d) *i*-$BuB(OH)_2$, 1 N HCl aq, MeOH/hexane, rt, 30–44%.

itory potency of chymotrypsin-like activity. Interestingly, the benzylamide derivative **13** also showed effective inhibition of chymotrypsin-like activity, despite displaying only moderate growth inhibition in the HeLa cell assay. The boron peptides with a longer framework, such as compounds **15a** and **15b** ($n = 3$), and without component **1** (i.e., compounds **17a**–**c**) were poor inhibitors in both the cell growth and proteasome assays.

Table 1
Cell-growth and proteasome inhibitions of boron peptides

Compound	IC_{50}[a] (μM)			
	Cell growth inhibition[b]	β5[c]	β1[c]	β2[c]
11a	0.35 ± 0.02	0.28 ± 0.04	8.54 ± 1.97	>10
11b	2.35 ± 0.59	0.84 ± 0.04	9.84 ± 1.17	>10
11c	0.79 ± 0.03	1.50 ± 0.42	>10	>10
11d	0.66 ± 0.09	1.47 ± 0.30	>10	>10
13	5.11 ± 0.31	0.54 ± 0.14	>10	>10
15a	>10	2.74 ± 0.32	>10	>10
15b	>10	4.28 ± 0.08	>10	>10
17a	>10	4.51 ± 1.31	>10	>10
17b	>10	>10	>10	>10
17c	>10	4.12 ± 0.81	>10	>10
MG-132	ND[d]	0.07 ± 0.03	7.23 ± 1.16	>10
PS-341	0.02 ± 0.01	0.02 ± 0.01	0.68 ± 0.09	>10

[a] Concentration required to inhibit cell growth or proteasome activity by 50%. The values are mean ± sd of triplicate samples.
[b] Growth inhibition in HeLa cells determined by MTT assay.
[c] Inhibition of chymotrypsin-like (β5), caspase-like (β1) and trypsin-like (β2) activity of human 20S proteasome.
[d] Not determined.

It has been reported that proteasome inhibitors induce cell cycle arrest and apoptosis.[21] Therefore we carried out flow cytometry analysis using compounds **11a** and **13**.[22,23] As shown in Table 2, compound **11a** increased the G2/M cell distribution in a similar manner to that of PS-341. Furthermore, a biparametric cytofluorimetric analysis with FITC-labeled annexin V and propidium iodide (PI) (Fig. 2) revealed that compound **11a** (1–10 μM) and compound **13** (10 μM) induces apoptosis of HeLa cells in over 10% of the population. These results indicate that the inhibition of cell growth by boron peptides is mediated by induction of G2/M cell cycle arrest and apoptosis.

In conclusion, we have designed a new class of boron peptides as inhibitors of the chymotrypsin-like activity of the 20S proteasome based around the structure of Belactosin C. Inhibitory activity is dependent on the length of the boron peptide. Compounds **11a** and **13** were found to possess significant inhibition of 20S proteasome chymotrypsin-like (β5) activity, and inhibitory potency of both compounds is similar to that of Belactosin C. Furthermore, compound **11a** increased the G2/M cell distribution in a similar manner to that of PS-341, and induced apoptosis at >1 μM concentration of compound in HeLa cells. The findings presented herein represent a significant step towards the development of proteasome inhibitors with therapeutic utility.

Table 2
Cell cycle arrest and apoptosis induction of boron peptides

Compound		Cell cycle distribution[a] (%)			Apoptotic cell[b] (%)
		G1	S	G2/M	
None		56.4 ± 0.39	30.0 ± 0.46	13.7 ± 0.87	2.6 ± 0.14
11a	1 μM	57.7 ± 0.02	16.4 ± 0.71	26.0 ± 0.73	12.9 ± 1.47
	10 μM	23.0 ± 0.82	14.4 ± 0.42	62.6 ± 0.31	15.2 ± 1.69
13	1 μM	56.2 ± 1.32	30.7 ± 0.75	13.1 ± 0.53	7.6 ± 1.59
	10 μM	43.2 ± 0.01	30.7 ± 1.73	26.0 ± 1.76	11.7 ± 0.28
PS-341	0.1 μM	14.6 ± 1.03	15.9 ± 0.55	69.6 ± 0.47	14.5 ± 3.56

[a] HeLa cells were incubated for 24 h with the indicated concentration of compounds **11a**, **13** or PS-341. The cells were harvested and stained with propidium iodide (PI).
[b] After incubation of 12 h with compounds **11a**, **13** or PS-341, the cells were stained with annexin V-FITC and PI. The effect on cell cycle and apoptosis were determined by flow cytometry. Values are mean ± sd of samples determined in triplicate.

Acknowledgments

This work is supported by the Ministry of Education, Science, Sports, Culture and Technology, Grant-in-Aid for Scientific Research (B) (No. 18350090) from Japan.

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23. *Spectral data for lead compounds*: Compound **11a**: ^{1}H NMR (400 MHz; CD_3OD) δ 7.71–7.62 (m, 4H), 7.35–7.28 (m, 3H), 4.48–4.36 (m, 3H), 3.62 (s, 2H), 2.52–2.37 (m, 3H), 2.10 (br s, 3H), 1.90 (br s, 3H), 1.53–1.17 (m, 5H), 1.29 (s, 9H), 0.82–0.79 (m, 6H); ^{13}C NMR (75 MHz, CD_3OD) δ 179.1, 173.1, 172.7, 158.7, 137.0, 134.8, 134.2, 129.3, 128.8, 128.6, 127.2, 126.9, 126.8, 126.7, 80.9, 53.6, 44.8, 44.2, 41.0, 28.6, 27.8, 27.0, 27.0, 23.9, 22.1; IR(KBr) 3274, 2931, 2362, 2331, 1652, 1508, 1488, 1419, 1363, 1168, cm^{-1}; MS (ESI, positive) *m/z* 593 ([M(OMe)+Na]$^+$), 607 ([M$(OMe)_2$+Na]$^+$). Compound **13**: ^{1}H NMR (400 MHz; CD_3OD) δ 7.22–7.11 (m, 5H), 4.50 (br s, 1H), 4.34–4.23 (m, 3H), 3.24–3.20 (m, 3H), 2.53 (br s, 1H), 2.38 (t, J = 7.8 Hz, 2H), 2.12 (br s, 1H), 1.89 (br s, 1H) 1.53 (br s, 1H), 1.32 (s, 9H), 1.26–1.16 (m, 2H), 0.83–0.80 (m, 6H); ^{13}C NMR (75 MHz, CD_3OD) δ 179.1, 173.0, 172.8, 158.7, 139.6, 129.6, 128.5, 128.2, 80.9, 53.5, 44.9, 44.1, 41.0, 28.7, 27.8, 27.0, 27.0, 23.8, 22.2; IR(KBr) 3735, 3676, 3649, 3303, 2929, 2869, 2343, 1652, 1508, 1498, 1436, 1367, 1168, 1126, 1029 cm^{-1}; MS (ESI, positive) *m/z* 543 ([M(OMe)+Na]$^+$), 557 ([M$(OMe)_2$+Na]$^+$).