Current Topics

Recent Progress in Study on the Biologically-Active Natural Products

Search for Inhibitors of the Ubiquitin–Proteasome System from Natural Sources for Cancer Therapy

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Since the approval of the proteasome inhibitor, Velcade®, by the Food and Drug Administration (FDA) for the treatment of relapsed multiple myeloma, inhibitors of the ubiquitin–proteasome system have been attracting increasing attention as promising drug leads for cancer therapy. While the development of drugs for diseases related to this proteolytic system has mainly been achieved by searching libraries of synthetic small molecules or chemical modifications to drug leads, limited searches have been conducted on natural sources. We have been searching natural sources for inhibitors that target this proteolytic system through in-house screening. Our recent studies on the search for natural inhibitors of the ubiquitin–proteasome system, particularly, inhibitors against the proteasome, E1 enzyme (Uba1), E2 enzyme (Ubc13–Uev1A heterodimer), and E3 enzyme (Hdm2), and also those against deubiquitinating enzyme (USP7), are reviewed here.

Key words enzyme inhibitor; proteasome; ubiquitin system; deubiquitinating enzyme; drug lead; cancer

1. Introduction

Intracellular protein degradation is mainly mediated by two systems, the ubiquitin–proteasome system and autophagy–lysosome system. The former system plays a major role in the selective degradation of proteins and regulates various cellular events, including cell-cycle control, transcription, and development.[1,2] The 26S proteasome is an intracellular high molecular-weight protease complex in this proteolytic system (Fig. 1), and the target protein is degraded by active sites in a cavity of the proteasome in an ATP-dependent manner. Prior to degradation by the proteasome, ubiquitin attaches to a target protein mediated by the ubiquitin system, which is composed of three enzymes, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin–protein ligase (E3), and this ubiquitin system catalyzes the polyubiquitination of the client protein. The polyubiquitin chain formed is recognized by the 26S proteasome as a degradation signal, and the 26S proteasome removes the polyubiquitin chain from the polyubiquitinated protein and subsequently degrades the resulting protein portion to produce peptides. The polyubiquitin chain released is subjected to deubiquitination by deubiquitinating enzymes (DUBs) to produce ubiquitin monomers.[1–6]

In 2003, the proteasome inhibitor Velcade® (bortezomib, PS-341, **1**) (Fig. 2) was approved by the Food and Drug Administration (FDA) for the treatment of relapsed multiple myeloma.[7] Compound **1** is a dipeptide boronic acid analogue that is now used worldwide to treat multiple myeloma. Since the approval of **1**, various synthetic and natural products that target the proteasome have been reported. Of these, Kyprolis® (carfilzomib, PR-171, **2**) (Fig. 2) was found to be effective against relapsed multiple myeloma cells resistant to therapies with **1** and was approved by the FDA in 2012.[8] CEP-18770 (**3**),[9] a synthetic analogue of **1**, and salinosporamide A (**4**),[10] a metabolite of the marine actinomycete (Fig. 2), are orally effective and overcome the side effects associated with **1**, and thus both are currently being subjected to clinical trials as second-generation of anticancer proteasome inhibitors. In addition to proteasome inhibitors, various small molecules targeting E1 and E3 are being evaluated in clinical trials,[11–14] and compounds capable of binding to E3, *i.e.*, thalidomide and its derivatives, have recently been approved. Therefore, the inhibitors of this proteolytic system are expected to become excellent drug leads for cancer therapy as well as bio-probes for investigating the mechanisms of this system. However, the search for inhibitors of this system from natural sources has been limited. In 2002, we started to search natural sources for inhibitors that target this system through in-house screening and have succeeded in isolating various types of inhibitors.[15] Our recent studies on natural inhibitors of the ubiquitin–proteasome system, particularly, inhibitors against the proteasome, E1 (Uba1), E2 (Ubc13–Uev1A heterodimer), E3 (Hdm2), and also those against DUB (USP7), are reviewed here.

2. Proteasome Inhibitors

To date, various proteasome inhibitors have been developed and classified into five groups, peptide aldehydes [*e.g.*, MG132[16] **5** (Fig. 2)], peptide boronates [*e.g.*, Velcade (**1**) and CEP-18770 (**3**)], β-lactones [*e.g.*, salinosporamide A (**4**) and lactacystin[17] (**6**) (Fig. 2)], epoxyketones [*e.g.*, carfilzomib (**2**) and epoxomicin[18] (**7**) (Fig. 2)], and macrocyclic vinyl ketones [*e.g.*, syringolin A[19] (**8**) (Fig. 2)], based on their inhibitory mechanisms.[20] The functional groups in the above inhibitors react with the hydroxyl group of the N-terminal catalytic threonine residue in the proteasome active site. In order to isolate proteasome inhibitors with new inhibitory mechanisms, we have been searching natural sources for proteasome inhibitors.

In our assay of proteasome inhibition,[21] the proteasome was initially preincubated with inhibitor samples. The fluorogenic substrate Suc-Leu-Leu-Val-Tyr-methylcoumarinamide (MCA) for the chymotrypsin-like activity of the proteasome was

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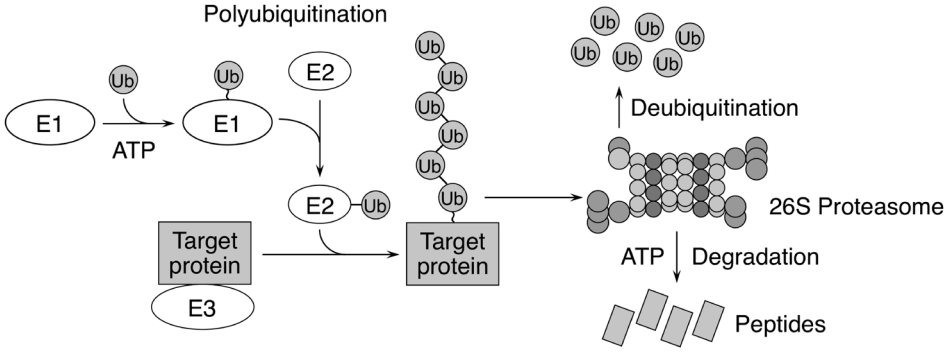

Fig. 1. The Ubiquitin–Proteasome System
Ub, ubiquitin.

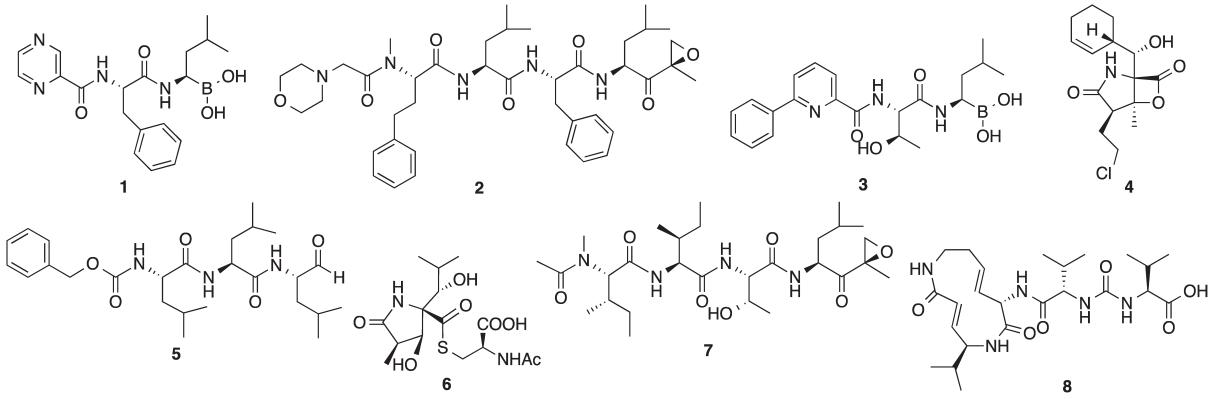

Fig. 2. Structures of **1**–**8**

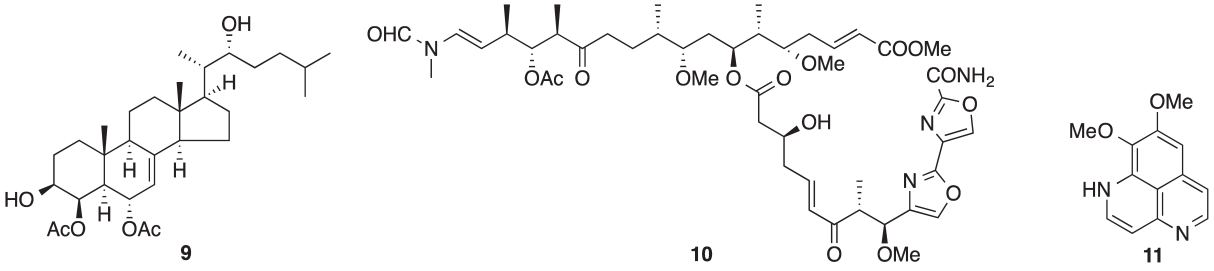

Fig. 3. Structures of **9**–**11**

subsequently added to the reaction mixture. After incubation for 1 h, the fluorescence intensity of aminomethylcoumarin released from the above MCA substrate was measured. As a result of screening, we found that several extracts of marine sponges inhibited chymotrypsin-like activity and succeeded in isolating agosterol C (**9**),[21] secomycalolide A (**10**)[22] and aaptamine (**11**)[23] (Fig. 3) with IC_{50} values of 19, 11, and 7.0 μM, respectively, as proteasome inhibitors.

Three specimens of sponges, *Acanthostrongylophora ingens*, which were collected in three different locations (Ti Toi, Bajotalawaan, and Mantehage) in Indonesia, have recently been found to inhibit the proteasome, and we succeeded in isolating various novel and structurally unique derivatives of manzamine A (**12**) (Fig. 4) from these sponges as proteasome inhibitors. Compound **12** is a marine sponge-derived alkaloid composed of a fused and bridged pentacyclic ring system that is connected to a β-carboline.[24] More than 80 manzamine derivatives have been isolated to date and exhibit various biological activities. 1) The sponge collected in Ti Toi afforded **12** and its new congener, acantholactam (**13**)[25] (Fig. 4). Compound **13** contains a hexenoic acid moiety attached to the nitrogen atom of the γ-lactam ring and may have been biosynthetically derived from **12** by oxidative cleavage of the eight-membered ring. 2) The sponge collected in Bajotalawaan afforded a new compound, pre-*neo*-kauluamine (**14**),[25] and two known compounds, **12** and *neo*-kauluamine (**15**) (Fig. 4). During storage in a freezer for 2 months, **14** was converted to its dimer **15**. 3) The sponge collected in Mantehage afforded five new manzamine alkaloids, acanthomanzamines A–E (**16**–**20**)[26] (Fig. 4). Although most manzamine-related alkaloids contain a β-carbolin, which is attached to the pentacyclic ring system, **16** and **17** contain tetrahydroisoquinolines and are the first examples to possess a tetrahydroisoquinoline instead of a β-carbolin in the family of manzamine alkaloids. Structural analysis of **18** revealed the presence of a bicyclo ring system fused to the original five-membered ring. Interestingly, **19**

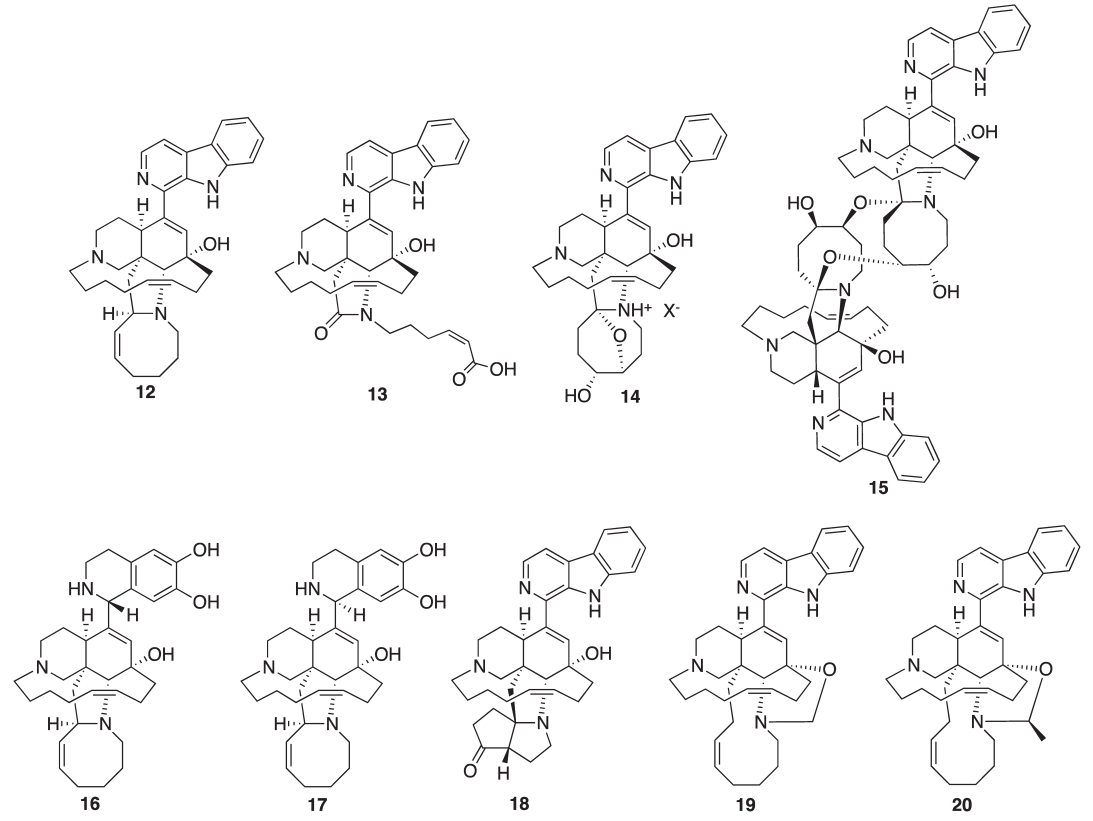

Fig. 4. Structures of **12**–**20**

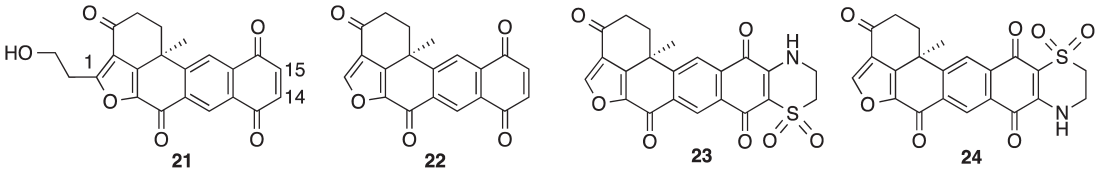

Fig. 5. Structures of **21**–**24**

and **20** contain additional methylene and ethylidene groups between their nitrogen and oxygen atoms. Among the above manzamine derivatives (**12**–**20**) isolated, **14**, **15**, and **19** exhibited potent inhibitory activities against the proteasome with IC_{50} values of 0.34, 0.13, and 0.63 μM, respectively.[25,26] These findings, together with the lack of proteasome-inhibitory activity of **13**, clearly suggest that the presence of the eight-membered ring in manzamines is essential for their inhibition of the proteasome.

An extract of an Indonesian marine sponge of the genus *Xestospongia* was found to exhibit inhibitory activity against the proteasome and afforded a new halenaquinone derivative, 1-hydroxyethylhalenaquinone (**21**), together with three known compounds, halenaquinone (**22**) and 3-ketoadociaquinones A (**23**) and B (**24**)[27] (Fig. 5). Compound **21** is the first halenaquinone derivative with an alkyl substituent at the keto-furan C-1 position. Among the four compounds isolated, **21** and **22** inhibited the chymotrypsin-like activity of the proteasome (IC_{50} values of 0.19 and 0.63 μM, respectively), whereas **23** and **24**, containing thiomorpholine 1,1-dioxide moieties, barely inhibited this activity, even at 5 μM. The structure–activity relationships among **21**–**24** suggest that the C-14 and/or C-15 positions of the quinone in **21** and **22** may undergo Michael-type 1,4-addition of the hydroxyl group of the catalytic threonine residue in the proteasome in a manner similar to **8**.[19] Compound **21** and its derivatives are known to exhibit various biological activities, and their additional new activity, *i.e.*, proteasome inhibitory activity, was first revealed by us.

We subsequently isolated two new strongylophorine derivatives (**25** and **26**) along with strongylophorines-2 (**27**), -3 (**28**), -4 (**29**), -8 (**30**), -13/-14 (**31**), and -22 (**32**) (Fig. 6) as proteasome inhibitors from the marine sponge *Petrosia corticata*.[28] A compound containing hemiacetal (strongylophorines-15/-16 (**33**) (Fig. 6); IC_{50} value, 3.6 μM), which had been semi-synthesized from **27**, exhibited more potent inhibitory activity than those containing acetal (**25** and **26**; IC_{50} value, 6.6 and 9.3 μM, respectively), and their lactone derivative (**27**; IC_{50} value, >100 μM) was less potent. On the other hand, compounds **30** and **31** containing hydroquinone (IC_{50} value, 8.4 and 2.1 μM, respectively) showed more potent inhibitory activities than their corresponding dehydrated derivatives **27** and **33** (see their IC_{50} values above), respectively. Compounds **28**, **29**, and **32** containing neither hemiacetal nor acetal, exhibited inhibitory activities, which decreased in that order (IC_{50} value, 9.5, 19, and >100 μM, respectively). Taken together, the structure–activity relationships among **25**–**33** revealed that the

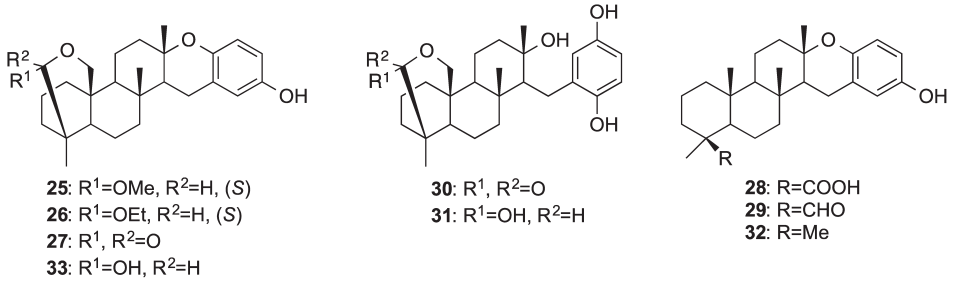

Fig. 6. Structures of **25**–**32**

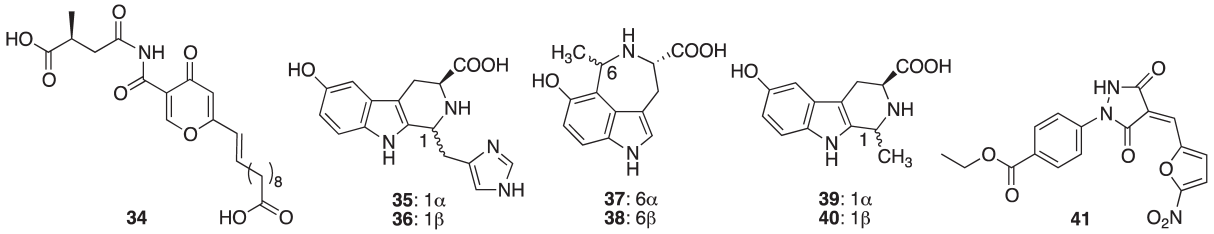

Fig. 7. Structures of **34**–**41**

compound containing hemiacetal and hydroquinone moieties, *i.e.*, **31**, showed the most potent proteasome inhibitory activity.

3. Inhibitors of E1

The polyubiquitination of proteins requires the sequential actions of three enzymes: E1, E2, and E3 (Fig. 1). Since these enzymes are essential for the ubiquitin–proteasome system, their inhibitors may be potent anticancer agents, similar to the approved proteasome inhibitors **1** and **2**. Therefore, we attempted to isolate inhibitors of E1, E2, and E3 from natural sources for drug development.

In the ubiquitin–proteasome system, ubiquitin is first activated by E1 to form the E1-ubiquitin thioester intermediate (Fig. 1), and our assay for E1 inhibition was thus performed on the basis of the inhibition of the intermediate formation.[29] Briefly, purified recombinant FLAG-tagged human E1 (Uba1) was incubated with inhibitor samples and then glutathione *S*-transferase (GST)-ubiquitin in the presence of ATP. The reaction mixture was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions, followed by Western blotting with an anti-FLAG antibody. As a result of this screening, we found that an extract prepared from a culture of a marine-derived fungus of the genus *Aspergillus* inhibited E1 and we succeeded in isolating himeic acid A (**34**) (Fig. 7) as a ubiquitin E1-specific inhibitor (IC_{50} value, 50 μM).[29]

In the course of the continuing search for E1 inhibitors, we next isolated five new alkaloids, hyrtioreticulines A–E[30] (**35**–**39**), together with one known alkaloid, hyrtioerectine B (**40**) (Fig. 7). Among these compounds, **35** and **36** exhibited significant E1 inhibitory activities with IC_{50} values of 2.4 and 35 μM, respectively. On the other hand, the other four compounds showed no inhibition, even at 100 μM. These findings indicated that the presence of the imidazole ring and *trans*-configuration at C-1 are required for the inhibition of E1.

As described above, many proteasome inhibitors have been identified, although in the case of E1, only several inhibitors, including our isolated ones (**34**, **35**, and **36**), have been reported to date. Of these, the E1 inhibitor, PYR-41 (**41**)[31] (Fig. 7), was shown to inhibit the activation of nuclear factor-κB (NF-κB), activate tumor suppressor p53, and exhibit anticancer activity in mouse models, which strongly suggested the potential of E1 inhibitors as therapeutics for cancer.

4. Inhibitors of E2

Among the inhibitors targeting the ubiquitin–proteasome system, we focused on compounds that activate the tumor suppressor p53. The p53 protein exists in normal cells at very low concentrations.[32] Under various stress conditions, such as DNA damage, the activation of oncogenes, hypoxia, ribonucleotide depletion, and telomere erosion, this protein accumulates in cells in its tetrameric form and induces the expression of various genes that are involved in cell-cycle arrest, apoptosis, DNA repair, differentiation, and senescence.[33] The loss of p53 tumor-suppressor activity is caused by a mutation in the p53 gene, the inhibition of p53 activity, and degradation of the p53 protein, which results in the uncontrolled proliferation of the cell, leading to tumor development.[33] Therefore, the activation of p53 is a promising approach to suppress cancer, and we have been searching for natural compounds that inhibit the degradation of the p53 protein.

Ubiquitin attached to E1 is transferred to the thiol group of the active cysteine residue in E2 and then to the target proteins mediated by E3 (Fig. 1). Among the approximately 40 human E2 enzymes identified to date, Ubc13 forms a heterodimer with Uev1A to function as E2. The Ubc13–Uev1A heterodimer is capable of binding to p53 bound to the polysome and catalyzes the K63-polyubiquitination of p53, which results in the inhibition of the tetramer formation.[34] Since the knockdown of Ubc13 was previously reported to increase p53 transcriptional activity,[35] we speculated that an inhibitor of the Ubc13–Uev1A interaction may induce the activation of p53 transcriptional activity and function as an anticancer agent. In our screening,[36] inhibition of the Ubc13–Uev1A interaction was tested in enzyme-linked immunosorbent assay (ELISA) using purified recombinant Ubc13 and FLAG-Uev1A

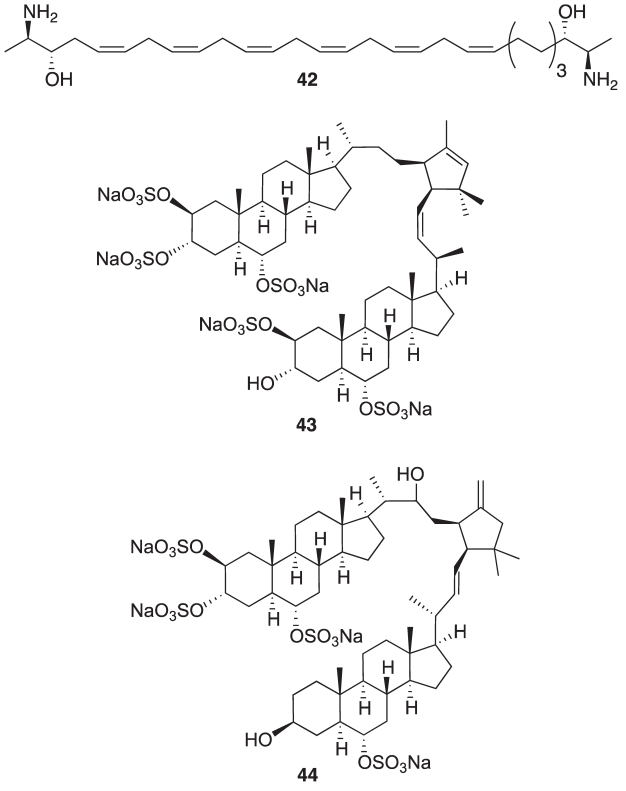

Fig. 8. Structures of **42**–**44**

proteins and a primary anti-FLAG antibody.

We first isolated leucettamol A (**42**) (Fig. 8) as an inhibitor of the Ubc13–Uev1A interaction (IC_{50} value, 105 μM) from the *Leucetta* sponge.[36] Since **42** did not inhibit the Ubc13–Mms2 interaction, this compound may attach to Uev1A. We subsequently isolated two new steroidal dimers, manadosterols A (**43**) and B (**44**) (Fig. 8), containing five and four sulfates, respectively, from the marine sponge *Lissodendryx fibrosa*.[37] Compounds **43** and **44** inhibited the Ubc13–Uev1A interaction with IC_{50} values of 0.09 and 0.13 μM, respectively, indicating that they are approximately 1000 times more potent than **42**.

Recent studies have shown that the linkage specificity of polyubiquitin chains is determined by the actions of E2 and/or E3.[38,39] Although the structures and mechanisms of various E3s have been extensively examined, only a limited number of studies has been conducted on E2s. Therefore, specific inhibitors against E2 may be useful as bio-probes in addition to clinical drugs.

5. Inhibitors of E3

In the process of the polyubiquitination of target proteins catalyzed by E1, E2, and E3, a large family of E3s recognizes vast numbers of target proteins and destines them for degradation (Fig. 1): The human genome encodes approximately 600 E3s. Since E3 definitively determines which target proteins are polyubiquitinated, a specific inhibitor against an E3 recognizing a key target protein may be a good lead for the treatment of a disease connected with the turnover of the key target protein.

The main E3 ubiquitinating p53 is Mdm2,[40,41] and the Mdm2-induced polyubiquitination of p53 induces its degradation by the proteasome. Alternatively, Mdm2 binds to p53 and blocks its transactivation activity. Thus, Mdm2 antagonists

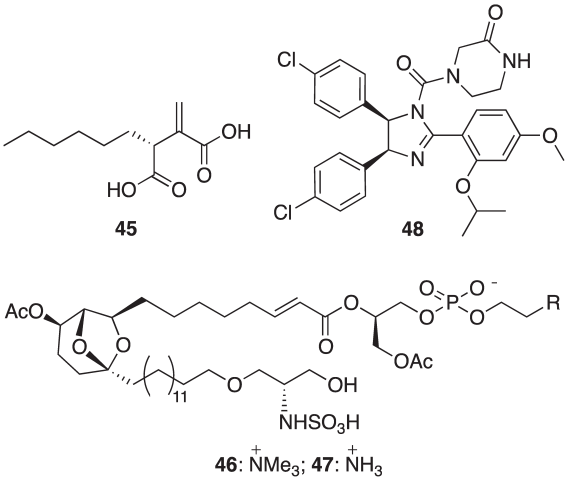

Fig. 9. Structures of **45**–**48**

bound to Mdm2 may suppress cancer by inhibiting the above two mechanisms of action of Mdm2 on p53. Additionally, it is important to note that the expression of Mdm2 is increased in cancer cells. Therefore, we have been searching for inhibitors of the p53–Mdm2 interaction as anticancer agents. In our screening,[42] inhibition of the p53–Hdm2 (a human Mdm2 homologue) interaction was tested in ELISA using purified recombinant p53 and Hdm2 proteins and a primary anti-Hdm2 antibody.

We first isolated (*R*)-hexylitaconic acid (**45**) (Fig. 9) from a marine-derived fungus isolated from a marine sponge as an inhibitor of the p53–Hdm2 interaction.[42] This compound exhibited inhibitory activity with an IC_{50} value of 230 μM, but did not inhibit the p53–COP1 interaction, which indicated that **45** binds to Hdm2, but not to p53. We subsequently isolated 12 new compounds, siladenoserinols A–L, from the tunicate of the family Didemnidae.[43] In spite of their structural similarities, their IC_{50} values were different (2.0–55 μM) and, among them, siladenoserinols A (**46**) and B (**47**) (Fig. 9) exhibited the strongest inhibitory activities with IC_{50} values of 2.0 μM. The structure–activity relationships among the 12 siladenoserinols revealed that the compounds containing an ester bond at C-2′ and acetoxy groups at C-1′ and C-11 showed the most potent inhibitory activities against the p53–Hdm2 interaction.[43]

Nutlin-3 (**48**) (Fig. 9) was discovered by screening a chemical library of synthetic compounds as an Mdm2 antagonist and was found to suppress the progression of cancer in nude mice bearing human cancer xenografts.[44] Several Mdm2 antagonists are currently being evaluated in clinical trials and thus their development implies that Mdm2/Hdm2 antagonists are promising candidates for leads in the treatment of cancer.

6. Inhibitors of DUB

As described above, among the three enzymes E1, E2, and E3 the respective target proteins are recognized by specific E3s, and thus inhibitors targeting specific E3s are expected to be highly specific drug candidates and are now being extensively investigated worldwide. On the other hand, the human genome encodes at least 98 DUBs, and altered DUB functions have been implicated in several diseases including cancer.[45] Since the respective DUBs appear to recognize specific target proteins, similar to E3s, DUB inhibitors are expected to become specific drug candidates and an extensive search for

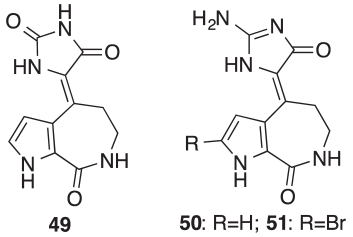

Fig. 10. Structures of **49**–**51**

them is currently being conducted.

The proteasome subunit Rpn11 functions as a DUB to remove the polyubiquitin chain from polyubiquitinated proteins bound to the proteasome prior to proteasome-mediated degradation. The other two DUBs, tightly attached to the proteasome, function to deubiquitinate the released polyubiquitin chain (see Fig. 1). On the other hand, many other DUBs function without proteasome binding. Among the latter DUBs, USP7 inhibitors are expected to become new drugs for the treatment of cancer.[46] Mdm2, one of the E3s for p53, is autopolyubiquitinated by itself and degraded by the proteasome. Upon USP7 binding to Mdm2, it functions to deubiquitinate the autopolyubiquitinated Mdm2, resulting in the rescue of Mdm2 from proteasome-mediated degradation. Therefore, USP7 inhibitors are capable of inducing the proteasome-mediated degradation of Mdm2, leading to p53 stabilization, and the treatment of cancer. In our screening of USP7 inhibition,[47] USP7 was preincubated with inhibitor samples, and the fluorogenic substrate, ubiquitin–rhodamine110, was then added to the reaction mixture. After incubation for 1 h, the fluorescence intensity of rhodamine, a hydrolysis product from the above fluorogenic substrate, was measured.

We isolated eight known alkaloids from the marine sponge *Stylissa massa*.[47] Of these, spongiacidin C (**49**) (Fig. 10) was the most potent USP7 inhibitor (IC_{50} value, 3.8 μM), while debromohymenialdisine (**50**) and hymenialdisine (**51**) (Fig. 10) exhibited 20% inhibition at 20 μM, and the other alklaoids isolated, dibromophakellin, manzacidins A–C, and *N*-methylmanzacidin C, exhibited no inhibitory activity. These findings suggest that the presence of the hydantoin ring in **49**, instead of the aminoimidazolinone ring in **50** and **51**, is necessary for the inhibition of USP7. Compound **49** is the first USP7 inhibitor isolated from natural sources. To date, several natural products with broad DUB inhibitory profiles have been isolated, whereas **49**, which we isolated, was revealed to be relatively selective for USP7.[47]

7. Conclusion

Inhibitors of the ubiquitin–proteasome system are now expected to become excellent drug leads for cancer therapy as well as bio-probes for investigating this system. Although the search for inhibitors of this system from natural sources has not yet been extensively explored, natural sources still comprise a large variety of undiscovered small molecules that could become drug candidates. The search for inhibitors with new structures for drug development in relation to this proteolytic system from natural sources is very important and attractive.

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Conflict of Interest The author declares no conflict of interest.

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