Inhibition of the Ubiquitin-proteasome System by Natural Products for Cancer Therapy

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Abstract

The ubiquitin-proteasome system plays a critical role in selective protein degradation and regulates almost all cellular events such as cell cycle progression, signal transduction, cell death, immune responses, metabolism, protein quality control, development, and neuronal function. The recent approval of bortezomib, a synthetic proteasome inhibitor, for the treatment of relapsed multiple myeloma has opened the way to the discovery of drugs targeting the proteasome and ubiquitinating and deubiquitinating enzymes as well as the delivery system. To date, various synthetic and natural products have been reported to inhibit the components of the ubiquitin-proteasome system. Here, we review natural products targeting the ubiquitin-proteasome system as well as synthetic compounds with potent inhibitory effects.

Keywords: natural products, ubiquitin, proteasome, cancer therapy, drug discovery, targeted therapy.

The Ubiquitin-proteasome System

Regulated protein degradation via the ubiquitin-proteasome system (Fig. 1) is an essential aspect of cell signaling pathways, functioning from cell-cycle control and transcription to development [1-3]. In the ubiquitin-proteasome system, the client proteins are ubiquitinated by the ubiquitin system and degraded by the 26S proteasome in an ATP-dependent manner. Ubiquitin is composed of 76 amino acids and attaches to a client protein (ubiquitination) prior to degradation. In the ubiquitin system, ubiquitination requires the sequential actions of three enzymes, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-protein ligase (E3), which results in the formation of the polyubiquitin chain. Then, the polyubiquitin chain, tagged to the client protein, is recognized by the 26S proteasome, an intracellular high-molecular weight protease subunit complex [1-6]. The 26S proteasome consists of two subcomplexes, the 20S core particle (also known as the 20S proteasome) and the 19S regulatory particle (Fig. 1), and the protein portion of a client protein is degraded by the proteolytic active sites in a cavity of the 20S proteasome. The 20S proteasome is classified as a threonine protease that contains two pairs of three different catalytic sites. The β1, β2, and β5 subunits contain catalytic sites with caspase-like, trypsin-like, and chymotrypsin-like activities, respectively. These activities are capable of hydrolyzing proteins into oligopeptides. The level of the 26S proteasome is increased especially in tumor cells. Therefore, it is reasonable to develop specific compounds targeting proteasome-mediated proteolytic degradation for cancer treatment.

The recent approval of bortezomib, a synthetic proteasome inhibitor, for the treatment of relapsed multiple myeloma has opened the way to the discovery of drugs targeting the ubiquitin-proteasome system consisting of the proteasome, ubiquitinating enzymes, the delivery system, and deubiquitinating enzymes (see below). To date, various natural and

synthetic products have been reported to inhibit the components of the ubiquitin-proteasome system. In this review, we focused on recent progress concerning natural products targeting the ubiquitin-proteasome system as well as synthetic compounds with potent inhibitory effects.

Targeting the 20S Proteasome

Inhibitory mechanisms of proteasome inhibitors

Proteasome inhibitors show anti-tumor activity against various tumor cells that are resistant to conventional chemotherapeutic agents. Structurally-diverse proteasome inhibitors have been developed by chemical synthesis and also by searching natural sources and chemical libraries as drugs for the clinical treatment of cancer and also as molecular tools for the investigation of cellular events. To date, various synthetic peptides including MG132 (Z-Leu-Leu-Leu-al, **1**) (Fig. 2) [7] and bortezomib (PS-341, Velcade®, **2**) (Fig. 3) [8, 9], and natural products including salinosporamide A (NPI-0052, **3**) (Fig. 4) [10] have been reported to inhibit proteasomal activity. Bortezomib (**2**), a synthetic proteasome inhibitor developed by Millennium Pharmaceuticals, Inc., was approved for myeloma therapy in the United States in 2003, which strongly indicates that the proteasome would be a novel target for cancer treatment [11]. Salinosporamide A (**3**), isolated from the culture of a marine actinomycete, was found to be more effective than **2** and is now under clinical trials. Several proteasome inhibitors have been developed and are classified into five groups, peptide aldehydes, peptide boronates, β-lactones, epoxyketones, and macrocyclic vinyl ketones, on the basis of inhibitory mechanisms.

Proteasome inhibitors: peptide aldehydes (Fig. 2)

Peptide aldehydes, the first proteasome inhibitors [7, 12], are the most widely used as molecular tools for the investigation of various cellular events. Generally, peptide aldehydes act against serine and cysteine proteases. The aldehyde functional group of the inhibitor is readily attacked by a hydroxyl or thiol group at the active site of the protease. In the case of the proteasome, the *N*-terminal threonine residue at its active site carries out a nucleophilic attack on the aldehyde moiety of the inhibitor and then a covalent hemiacetal adduct is formed between the inhibitor and the threonine residue of the proteasome (Fig. 2). The formation of the covalent adduct is mediated by a reversible reaction under physiological conditions, and aldehyde inhibitors are rapidly oxidized into inactive acids in cells and transported out of the cell by the multi-drug resistance (MDR) carrier system. Therefore, in experiments with cultured cells, effects of aldehyde inhibitors can be rapidly reversed by removal of the inhibitors. Although MG132 (**1**) is a potent and selective inhibitor of the chymotrypsin-like activity of the proteasome, it is not suitable for use as a therapeutic agent.

Tyropeptin A (**4**) was isolated from the culture broth of *Kitasatospora* sp. [13, 14] and contains an aldehyde moiety at the *C*-terminal. This compound inhibits the chymotrypsin-like and trypsin-like activities of the proteasome with IC_{50} values of 0.1 and 1.5 μg/mL, respectively, but scarcely inhibits the caspase-like activity. In rat pheochromocytoma (PC12) cells, **4** induced neurite outgrowth, and this compound is thought to be capable of permeating cells and inhibiting intracellular proteasomal activity [15]. Momose *et al.* designed and synthesized several derivatives of **4** [16]. Among them, TP-104 (**5**) is 20-fold more potent than **4** in terms of its inhibitory effect on the chymotrypsin-like activity of the mammalian 20S proteasome, while TP-110 (**6**) specifically inhibits the chymotrypsin-like but not the trypsin-like or caspase-like activity of the proteasome.

Fellutamide B (**7**) was originally isolated from the marine-derived *Penicillium fellutanum* as a

cytotoxic compound [17] and found to induce the release of nerve growth factor (NGF) from fibroblasts and glial-derived cells [18]. Because of its structural similarity with **1**, **7** was tested for inhibitory activity against the proteasome and found to potently inhibit the chymotrypsin-like activity with an IC_{50} value of 9.4 nM along with the trypsin-like and caspase-like activities albeit less potently with IC_{50} values of 2.0 and 1.2 μM, respectively [19]. In addition, **7** increased NGF gene transcription [19]. Therefore, it can be inferred that **7** exerts neurotrophic activity through stabilization of a short-lived unidentified transcription factor that upregulates NGF gene expression.

Proteasome inhibitors: peptide boronates (Fig. 3)

The development of a proteasome inhibitor suitable for therapeutic use without the defects of MG132 (**1**) led to the introduction of boronic acid as a functional group. Although boronic acid-containing inhibitors bind with the hydroxyl group of the *N*-terminal threonine residue in the proteasome *via* a non-covalent bond, their inhibitory potency and selectivity towards the proteasome are excellent in comparison with other proteasome inhibitors. Since the boron atom is able to receive the oxygen lone pair of the *N*-terminal threonine residue of the proteasome, inhibitors containing boronate can form a stable tetrahedral intermediate (Fig. 3). Although peptide aldehydes are readily oxidized into inactive acids, boronates are not inactivated by oxidation and are not rapidly removed from the cell by the MDR system. In 2003, bortezomib (**2**), a synthetic boronate-containing inhibitor, was first approved by the FDA for treating relapsed multiple myelomas [20, 21]. This inhibitor selectively inhibits the proteasome in a reversible manner and induces apoptosis of malignant cells through the inhibition of NF-κB signaling and stabilization of proapoptotic proteins. Clinical trials show promising results for the combination of **2** and DNA cross-linking agents in the treatment of myeloma and ovarian cancer [22].

CEP-18770 (**8**), a synthetic boronate derivative, is an orally active proteasome inhibitor [23], which inhibits the chymotrypsin-like activity of the proteasome at a low-nanomolar concentration. This inhibitor exhibits concentration-dependent induction of apoptotic cell death in human multiple myeloma and tumor-derived cell lines, and exhibits a more favorable cytotoxicity profile toward normal human endothelial cells, bone marrow progenitors, and bone marrow-derived stromal cells than **2** [24]. Recently, it has been reported that **8** enhances the anti-myeloma activity of **2** and melphalan in the xenograft models [25]. The in vitro and in vivo antitumor and anticlastogenic pharmacologic profiles of **8** and its reduced cytotoxicity against a variety of normal human cell lineages compared with tumor cells provide the rationale for further studies evaluating its preclinical and clinical efficacies in multiple myeloma and other hematologic malignancies. At present, **8** is undergoing phase I and II trials.

Proteasome inhibitors: β-lactones (Fig. 4)

Lactacystin (**9**) (Fig. 4), the first natural proteasome inhibitor, was originally isolated from the culture broth of *Streptomyces* sp. as an inducer of neurite outgrowth in a murine neuroblastoma cell line, Neuro-2a [26, 27]. Subsequently, this inhibitor was found to inhibit proteasomal activity by binding with the *N*-terminal threonine residue in the proteasome via a stable covalent bond (Fig. 4) [28]. The active component of **9** is *clasto*-lactacystin β-lactone (omuralide, **10**), which is derived from **9** by elimination of the *N*-acetyl cysteine moiety to form a lactone ring [29, 30]. The co-crystallization of **9** with the proteasome revealed that the side chain residues of **10** are closely related to the selectivity of the inhibitor. PS-519 (**11**), which has a *n*-propyl group instead of the methyl group found in omuralide, is the most clinically advanced lactacystin analog [31] and has been used in a clinical trial to treat acute stroke.

Salinosporamide A (**3**) was isolated from the culture of a marine bacterium of the new genus *Salinispora tropica* [10, 32] and showed strong cytotoxic activity against HCT-116 human colon carcinoma. Because of its structural similarity to **10**, **3** was tested for inhibitory activity against the proteasome and found to inhibit the chymotrypsin-like activity 35 times more potently than **10**. The deschloro derivative, salinosporamide B (**12**), is ten times less potent, suggesting that the chloro substituent in **3** is important for its inhibitory ability [33]. Several potent salinosporamide analogs have been chemically synthesized, bioengineered, and isolated from microorganisms. Antiprotealide (**13**), which was originally synthesized as a molecualr hybrid of **3** and **10** [34], was recently isolated from a large-scale fermentation of the bacterium [35]. Fluorosalinosporamide (**14**), a fluoro analog, was biosynthesized and showed reversible binding ability toward the proteasome with an IC_{50} value of 1.5 nM (The value of salinosporamide A is 0.7 nM.) [36]. Cinnabaramide A (**15**), an analog with a structural difference in the alkyl side chain, was isolated from a terrestrial streptomycete and showed inhibition with an IC_{50} value of 1 nM [37]. Although bortezomib (**2**) inhibits most strongly the chymotrypsin-like activity and moderately the caspase-like activity of the proteasome [38-40], **3** inhibits most strongly the chymotrypsin-like activity and moderately the trypsin-like activity of the proteasome [40]. A phase I study with **3** is currently proceeding. Although **2** is effective for the treatment of relapsed or reflactory multiple myeloma, its prolonged use can be associated with toxicity and the development of drug resistance. Importantly, **3** overcomes resistance to conventional treatment and **2** [41, 42]. In vivo studies using human multiple myeloma xenografts revealed that **3** shows well tolerated/prolonged survival and reduces tumor recurrence [42]. In addition, **3** was a more effective proapoptotic agent than **2** in isolated chronic lymphocytic leukemia cells [43], and the combination of **3** and a histone deacetylase inhibitor, MS-275 or valproic acid, induced greater cell death than did the combination of **2** and these inhibitors [44]. It should be noted that **3** is orally

bioavailable and cytotoxic to multiple myeloma cells with reduced toxicity against normal cells compared to **2**. Furthermore, in vivo studies with a human plasmacytoma xenograft mouse model showed that a low dose combination of **3** and **2** is well tolerated and triggers synergistic inhibition of tumor growth and chymotrypsin-like, caspase-like, and trypsin-like proteasomal activities in tumor cells [45]. Combination of **3** and lenalidomide (Revlimid®) induces synergistic anti-multiple myeloma activity in multiple myeloma cell lines or patient multiple myeloma cells [46], implying the preclinical rationale for clinical protocols evaluating lenalidomide together with **3** to improve patient outcome in multiple myeloma. The preclinical and clinical results with **3** strongly suggest that the discovery of **3** indicates the importance of metabolites from marine microorganisms for drug discovery and development [47].

Belactosins A (**16**) and C (**17**), which were isolated from a *Streptomyces* sp., contain the same β-lactone ring as **10** and inhibit the chymotrypsin-like activity of the proteasome in the same fashion as **10** [48, 49]. Derivatives of **16** with more potent inhibitory effects than **16** have been synthesized [50]. Homobelactosin C (**18**), a modified derivative, has an IC_{50} value in the low nanomolar range [51]. The structural data for the complex of the proteasome and **18** provide an explanation for the involvement of immunoproteasome subunits in the generation of antigen, and open the way for the rational design of compounds that exclusively inhibit constitutive proteasomes or immunoproteasomes [51]. Recently, analogs of **17** were synthesized, and their inhibitory activity was investigated [52]. Among them, its two boronate-containing peptide analogs (**19** and **20**) showed significant inhibition of the chymotrypsin-like activity of the 20S proteasome with IC_{50} values of 0.28 and 0.51 μM, respectively. Furthermore, the development of potent proteasome inhibitors based on a stereochemical diversity-oriented strategy with **16** and its stereo- and regioisomers is underway [53].

Proteasome inhibitors: epoxyketones (Fig. 5)

The proteasome inhibitor epoxomicin (**21**) (Fig. 5) was originally isolated from an unidentified actinomycete strain and exhibited in vivo anti-tumor activity against B16 melanoma [54]. This inhibitor contains an α,β-epoxyketone moiety that is involved in the formation of a morpholino adduct with the *N*-terminal threonine residue in the proteasome, which results in inactivation of the proteasome (Fig. 5) [55]. Although clinical studies with bortezomib (**2**) have validated the proteasome as a therapeutic target for the treatment of multiple myeloma and some forms of non-Hodgkin's lymphoma [56], significant toxicity against normal cells has restricted the dosage. Furthermore, many patients have tumors that do not respond to **2** and others develop resistance. This has led to the need for other proteasome inhibitors with enhanced activity. Carfilzomib (PR-171, **22**), which was derived from **21**, is an irreversible proteasome inhibitor [57]. In multiple myeloma cells, **22** specifically inhibits the chymotrypsin-like activities of the proteasome and immunoproteasome [58]. In comparison to **21**, **22** exhibits greater selectivity toward the chymotrypsin-like activity of the proteasome and is active against **2**-resistant multiple myeloma cell lines. Since **22** also overcomes resistance to conventional agents and acts synergistically with dexamethasone to enhance cell death, this compound is currently under evaluation in phase I clinical trials in patients with multiple myeloma and non-Hodgkin's lymphoma. The first phase I study of **22** shows that the drug is well tolerated, and produces signals of activity in patients with multiple myeloma and that **22** did not produce any neuropathy [59]. These data support the further development of **22** in patients with hematologic malignancies.

Proteasome inhibitors: macrocyclic vinyl ketones (Fig. 6)

Syringolin A (**23**) (Fig. 6) was originally isolated as a virulence factor of the plant pathogen *Pseudomonas syringae* pv. *syringae* [60] and found to induce a change in the gene expression profile similar to that in cells treated with proteasome inhibitors [61]. The crystal structure of the yeast proteasome in a complex with **23** revealed that the hydroxyl group of the catalytic threonine residue exhibits a Michael type 1,4-addition to the vinyl ketone moiety in the 14-membered ring of **23** (Fig. 6) [61]. This is a new mode of inhibition, and **23** irreversibly inhibits all three types of proteasomal activity. Recently, synthetic and structural analyses of **23** revealed critical determinants of the selectivity and potency of its inhibitory effect [62]. Furthermore, it was found that a rhodamine-tagged **23** selectively binds to and labels the active sites of the proteasome at therapeutic concentrations [63]. In the same fashion, glidobactin A (**24**), another microbial metabolite [64], inhibits the chymotrypsin- and trypsin-like activities of the proteasome and reacts with the threonine residues of the respective active sites [61]. Both **23** and **24** inhibit the proliferation and induce the apoptosis of malignant cells [61].

Proteasome inhibitors: cyclic peptides (Fig. 7)

TMC-95A (**25**) (Fig. 7) was isolated from a fermentation broth of *Apiospora montagnei* [65, 66] by direct screening on the basis of inhibitory activity against the proteasome. This compound inhibits the chymotrypsin-like, trypsin-like, and caspase-like activities of the proteasome with IC_{50} values of 5.4, 200, and 60 nM, respectively. The co-crystallization of **25** with the yeast proteasome revealed that **25** is bound to the core particle of the proteasome through specific hydrogen bonds and specifically blocks the proteasomal active sites non-covalently [67, 68]. In addition, **25** was found to induce neurite outgrowth in rat PC12 cells [69].

Argyrin A (**26**) was originally isolated from the myxobacterium *Archangium gephyra* as an

immunosuppressive cyclic peptide [70] and identified as a small molecule capable of promoting the accumulation of $p27^{kip1}$, a cyclin-dependent kinase inhibitor, in cancer cells by a high-throughput whole-cell assay [71]. It should be noted that the level of $p27^{kip1}$, a tumor suppressor protein and a proteasome substrate, is often reduced in human cancer cells and that the expression of a degradation-resistant $p27^{kip1}$ mutant reduced the number of intestinal adenomatous polyps that developed into invasive carcinomas. It was found that **26** inhibits most strongly the chymotrypsin-like activity, moderately the caspase-like activity, and weakly the trypsin-like activity of the proteasome [71].

Other proteasome inhibitors (Fig. 8)

Various proteasome inhibitors isolated from natural sources are shown in Fig. 8. Gliotoxin (**27**) is a fungal epipolythiodioxopiperazine toxin and contains a heterobicyclic structure with a disulfide bridge. This compound was originally identified as a potent inhibitor of NF-κB activation in T and B cells [72]. Additional studies showed that **27** inhibits the chymotrypsin-like activity of the proteasome and that the disulfide bridge is responsible for the inhibition [73].

We performed a screening of marine organisms and marine-derived fungi based on the inhibition of the chymotrypsin-like activity of the proteasome and isolated agosterol derivatives (e.g., agosterol C (**28**)), polyhydroxysterols, from a marine sponge, *Acanthodendrilla* sp., as proteasome inhibitors [74]. Agosterols were originally isolated from the marine sponge *Spongia* sp. and found to reverse multi-drug resistance in tumor cells [75, 76]. Among the agosterol derivatives, **28** most strongly inhibited the chymotrypsin-like activity of the proteasome with an IC_{50} value of 10 μg/mL. Interestingly, they differ in their inhibitory potency despite their structural similarity. We also isolated another proteasome inhibitor, secomycalolide A (**29**), together with known compounds, mycalolide A (**30**) and

30-hydroxymycalolide A (**31**), from a marine sponge of the genus *Mycale* [77]. Among the mycalolides, **29** has the most potent effect on the chymotrypsin-like activity of the proteasome with an IC_{50} value of 11 μg/mL.

Extensive searches for proteasome inhibitors in plants and foods have been performed. Activity-guided fractionation of a chloroform-soluble extract of the leaves of *Ormosia sumatrana* led to the isolation of a new cerebroside, sumatranoside (**32**), as a proteasome inhibitor with an IC_{50} value of 30 μM [78]. Curcumin (**33**), the major active ingredient of turmeric (*Curcuma longa*) used in South Asian cuisine for centuries, markedly inhibited the chymotrypsin-like activity of a purified rabbit 20S proteasome with an IC_{50} value of 1.85 μM and also inhibited the cellular 26S proteasome [79]. Four dietary flavonoids, apigenin (**34**), kaempferol (**35**), quercetin (**36**), and myricetin (**37**), inhibited the chymotrypsin-like activity of the 26S proteasome in intact Jurkat T cells with IC_{50} values of 1, 11, 2, and 12 μM, respectively [80]. Genistein (**38**), a soy isoflavone [81], (-)-*epi*-gallocatechin gallate (EGCG) (**39**), a green tea polyphenol [82], withaferin A (**40**), a steroidal lactone from the medicinal plant "Indian Winter Cherry" (*Withania somnifera*) [83], and celastrol (**41**), a triterpene from the Chinese "Thunder of God Vine" (*Tripterygium wilfordii*) [84], also inhibited the chymotrypsin-like activity of a purified 20S proteasome with IC_{50} values of 26, 0.30, 4.5, and 2.5 μM, respectively.

Targeting the Ubiquitin System

The ubiquitin-proteasome pathway consists of the ubiquitin system and the protein degradation system (the 26S proteasome) (see Fig. 1). The former contains the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase

(E3), and catalyzes the ubiquitination of client proteins. In addition to inhibitors targeting the proteasome, various inhibitors of the ubiquitin system consisting of E1, E2 and E3 enzymes have been developed.

Ubiquitin ligase (E3) inhibitors (Fig. 9)

Among enzymes in the ubiquitin system, E3s are a large family that recognize huge numbers of client proteins and target them for degradation [85, 86]. They are classified into three major groups, the RING, HECT, and U-box families, on the basis of their domain structures and substrate recognition mechanisms. As E3 definitively determines which client proteins are ubiquitinated, a specific inhibitor against an E3 recognizing a key client protein could be a good lead for the treatment of diseases associated with degradation of the key client protein. Among many E3s, MDM2 (mouse double minute 2) or HDM2 (human double minute 2), a RING-type E3 for p53 protein, is frequently used as a target for inhibitor development [87]. Although HDM2 is normally expressed at a low level, it is over-expressed in a variety of human cancers. On the other hand, p53, a tumor suppressor, induces growth arrest and apoptosis upon activation by various stimuli such as DNA damage [88]. The crystal structure of the 109-residue amino-terminal domain of MDM2, which binds to a 15-residue transactivation domain peptide of p53, revealed that MDM2 has a deep hydrophobic cleft, to which the p53 peptide binds [89]. Therefore, targeting MDM2/HDM2 is a promising way to reactivate p53, inducing apoptosis in human cancer cells. For example, Nutlin-3 (**42**) (Fig. 9) was discovered by screening a chemical library as an MDM2 antagonist and found to suppress tumor progression in nude mice bearing subcutaneous human cancer xenografts [89]. The development of **42** strongly indicates that MDM2 antagonists would be promising candidates for leads in the treatment of cancer. Recently, it has been reported that the combination of **42** with bortezomib (**2**) mediates additive cytotoxicity against **2**-sensitive

multuple myeloma cell lines and synergistic activity against epithelial carcinoma cell lines and that nongenotoxic activation of the p53 pathway using **42** can sensitize epithelial carcinoma cells to **2** in a manner that is not suppressed by microenvironmental interactions, i.e., even in the presence of stromal cells [90].

Chlorofusin (**43**), the first MDM2 antagonist from natural sources, was isolated from the culture of a *Fusarium* sp. with an IC_{50} value of 4.6 μM [91]. The absolute configuration of chlorofusin was determined by total synthesis [92, 93]. Furthermore, inhibition of MDM2-p53 binding was evaluated with seven chromophore diastereomers of **43** [94]. Biosynthesis of **43** was also investigated [95].

By bioassay-guided isolation, (-)-hexylitaconic acid (**44**) was isolated from a culture of marine-derived fungus as an inhibitor of p53-HDM2 interaction [96]. Since **44** is unable to inhibit the interaction of p53 with COP1, another E3 of the HECT-type for p53, it can be inferred that **44** binds to HDM2 protein. Recently, the stereogenic center of **44** was determined to be *R* by vibrational circular dichroism (VCD) spectroscopy [97]. Its synthetic *S*-(+)-enatiomer also had an inhibitory effect on the p53-HDM2 interaction, which was comparable to that of the natural *R*-(-)-enatiomer (**44**) [97].

The interaction of chalcones (e.g., chalcone C (**45**)) with the p53-MDM2 system was analyzed by NMR spectroscopy (^{1}H-^{15}N HSQC spectrum) [98], since chalcone derivatives were reported to inhibit tumorigenesis [99]. Chalcones were found to bind to a subsite of the p53-binding cleft of MDM2. On the other hand, RITA (**46**) was discovered by screening a chemical library as an inhibitor of p53-MDM2 interaction through its binding to p53 protein [100].

Ubiquitin-activating enzyme (E1) inhibitors (Fig. 10)

Ubiquitin is first activated by the ubiquitin-activating enzyme (E1) in the

ubiquitin-proteasome system (Fig. 1). In the E1-mediated ubiquitin-activation process, ubiquitin and ATP bind to different sites in the E1 enzyme, and E1 catalyzes the formation of a ubiquitin–adenylate intermediate from ubiquitin and ATP, and subsequently the binding of ubiquitin to a cysteine residue at the E1 active site via a thiol ester linkage. Then, the high-energy intermediate of ubiquitin is transferred to the thiol group of the active cysteine residue in the ubiquitin-conjugating enzyme (E2) and then to the client proteins, mediated by ubiquitin ligase (E3) [1, 5]. Since E1 activity is thought to be essential for the ubiquitin-proteasome system, developing inhibitors against E1 is another possible route of drug development for the treatment of cancer.

Two natural E1 inhibitors, panepophenanthrin (**47**) (Fig. 10) [101] and himeic acid A (**48**) [102], have been isolated from microorganisms. As the first natural E1 inhibitor, **47** was isolated from a mushroom strain, *Panus rudis*. This compound inhibits the formation of an E1-ubiquitin thioester intermediate with an IC_{50} value of 17 μg/mL. On the other hand, during the screening of extracts of marine organisms and marine-derived microorganisms, a culture of the fungus *Aspergillus* sp., isolated from the mussel *Mytilus edulis galloprovincialis*, showed strong E1 inhibitory activity, and bioassy-guided fractionation of the culture afforded **48** as an E1 inhibitor [102]. This compound inhibited the formation of the E1-ubiquitin intermediate by 65% at a concentration of 50 μM and found to inhibit the binding of ubiquitin to the ubiquitin-binding site in the E1 enzyme. But, two congeners, himeic acids B (**49**) and C (**50**), were inactive even at 100 μM. As **48** cannot inhibit E1-like enzymes for other ubiquitin-like modifiers, at least, SUMO-1 and ISG15, this compound could be a specific inhibitor of the ubiquitin E1 enzyme.

Recently, PYR-41 (**51**), a synthetic pyrazone derivative, was identified using a commercial screening library as a cell-permeable E1 inhibitor [103]. This compound blocks protein degradation and cytokine-induced activation of NF-κB, activates p53 in cells, and

preferentially kills cells transformed with wild-type p53.

Ubiquitin-conjugating enzyme (E2) inhibitors (Fig. 11)

Ubiquitination performs proteolytic and non-proteolytic functions [104, 105]. The lysine48 (K48)-linked polyubiquitin chain is related to proteasome-dependent protein degradation, while the K63-linked chain plays non-proteolytic roles in various cellular events including signal transduction and DNA repair. The formation of the latter chain is catalyzed by a hetero-dimer formed by the ubiquitin E2 enzyme Ubc13 and an inactive ubiquitin-conjugating enzyme variant (Uev1A or Mms2) [106], and a functional difference between the two Ubc13 complexes (Ubc13-Uev1A and Ubc13-Mms2 complexes) was suggested [107]. The report that the knockdown of Ubc13 led to an increase in p53 activity [108] led us to speculate that an inhibitor of Ubc13, that is, one preventing the formation of the Ubc13-Uev1A complex, would be a lead for an anti-cancer agent. Subsequently, we carried out a search for inhibitors of Ubc13-Uev1A interaction in natural resources and found leucettamol A (**52**) (Fig. 11) in the marine sponge *Leucetta* aff. *microrhaphis* [109]. Although **52** was originally reported as an antimicrobial compound with a racemic nature [110], it was recently found that **52** is chiral with the configuration 2*R*,3*S*,28*S*,29*R*, as revealed by deconvoluted exciton coupled circular dichroism (ECCD) spectroscopy [111]. Furthermore, the recent report that the association of p53 with Ubc13 on polysomes requires ongoing translation and results in p53 ubiquitination which interferes with its tetramerization [112] also supports the use of inhibitors of the formation of the Ubc13-Uev1A complex in anticancer therapy.

In summary, targeting of the E1, E2, and E3 enzymes in the ubiquitin system offers promising prospects for drug discovery.

Targeting the Delivery System Connecting the Ubiquitin System to the Proteasome (Fig. 12)

As described above, the ubiquitin-proteasome system consists of two sub-systems, the ubiquitin system and the protein degradation system (the 26S proteasome). Recently, a third system, the so-called delivery system, was proposed to function in the delivery of ubiquitinated proteins to the 26S proteasome (see Fig. 1). The proteasome subunit Rpn10 functions as an intrinsic ubiquitin receptor of the proteasome [113] and Rpn13 was recently found to be an additional intrinsic ubiquitin receptor [114, 115]. The delivery system consisting of intrinsic ubiquitin receptors Rpn10/Rpn13 and extrinsic ubiquitin receptors such as Rad23 and Dsk2 functions as the third system in the ubiquitin-proteasome pathway, although the mechanism for the discrimination of ubiquitinated client proteins by the respective ubiquitin receptors remains unclear [116, 117]. Therefore, specific rather than general targeting of the respective ubiquitin receptors could be an effective approach in the treatment of specific client protein-associated diseases.

Girolline (**53**) (Fig. 12) was originally isolated as an antitumor compound from a marine sponge, but a phase I clinical study with this compound showed severe side effects in patients and no apparent antitumor activity [118]. We found that **53** induces G2/M cell cycle arrest in several tumor cell lines. In addition, ubiquitinated p53 but no other protein accumulated in cells treated with **53**, but this compound does not inhibit proteasomal activity [119]. Although the exact target of **53** has not been identified, it was proposed that this compound inhibits the delivery of ubiquitinated p53 protein to the proteasome.

Ubistatin (**54**) was discovered by searching a chemical library for an inhibitor of destruction box-dependent protein degradation [120]. It was found that **54** binds to the ubiquitin chain of

ubiquitinated proteins and inhibits ubiquitin-dependent proteolysis.

Thus, compounds inhibiting the delivery system for ubiquitinated proteins could serve as novel inhibitors targeting the ubiquitin-proteasome system.

Targeting Deubiquitinating Enzymes (Fig. 13)

In the polyubiquitin chain, the *C*-terminal carboxyl group of one ubiquitin monomer is covalently linked with the ε-amino group of the lysine residue in another ubiquitin monomer or the client protein via an isopeptide bond. Upon degradation of the polyubiquitinated client protein by the proteasome, the polyubiquitin chain is recognized by intrinsic ubiquitin receptors of the 26S proteasome as well as extrinsic ubiquitin receptors in the third system and is converted to ubiquitin monomers by the actions of intrinsic and extrinsic deubiquitinating enzymes (DUBs) [121]. Thus, it is now known that deubiquitination mediated by the actions of various DUBs plays important regulatory roles in various cellular events [122].

Prostaglandins (PGs) function as intracellular signal mediators in the regulation of a variety of physiological processes, including inflammation and immune responses. J series PGs (e.g., Δ_{12}-PGJ_2 (**55**)) (Fig. 13) with a unique exocyclic α,β-unsaturated ketone inhibit ubiquitin isopeptidase activity in the proteasome-mediated proteolytic pathway, while PGA_1, PGB_1, PGE_2, and 15-keto-PGE_2 are inactive [123]. The former J series PGs cause apoptosis, independently of p53-mediated gene transactivation. Punaglandins (e.g., punaglandin 4 (**56**)) isolated from the soft coral *Telesto riisei* are highly functional cyclopentadienone and cyclopentenone prostagalndins chlorinated at the endocyclic α-carbon position, and inhibit ubiquitin isopeptidase activity and exhibit antiproliferative effects more potently than J series

PGs [124]. Recently, a small-molecule inhibitor (HBX 41,108, a cyano-indenopyrazine derivative, **57**) of USP7/HAUSP, a member of the ubiquitin-specific protease (USP) family of DUBs, was isolated by high-throughput screening [125]. It was found that the treatment of cancer cells with **57** results in the accumulation of p53 and that **57** inhibits cancer cell growth and induces apoptosis. This suggests that compounds inhibiting specific DUBs could act as novel anti-cancer drugs. Although drug discovery targeting DUBs is in its early stages, a better understanding of the DUBs with regard to their mechanisms of action and substrate recognition may enable the development of small-molecule inhibitors for effective anti-cancer treatment.

Future Perspectives

The ubiquitin-proteasome system controls a wide range of cellular events including cell cycle progression, and defects associated with this system result in various diseases including cancer and neurodegenerative disorders. Thus, the ubiquitin-proteasome system is emerging as a significant target in anticancer therapies. Bortezomib (**2**), a synthetic proteasome inhibitor, is already on the market for the treatment of patients with multiple myeloma and is also undergoing clinical trials for other cancers. In preclinical studies, **2** showed antitumor activity against a variety of solid tumors, including breast, gastric, colon, pancreas, and non-small lung cancers [126]. In addition, several natural and synthetic inhibitors targeting the proteasome, salinosporamide A (**3**), CEP-18770 (**8**), and carfilzomib (**22**), are also in clinical trials for cancers. Inhibitors targeting the ubiquitin system, the delivery system, and deubiquitinating enzymes are also candidates for anticancer drugs and several compounds are now undergoing preclinical and clinical trials for cancers. In 2004, Ciechanover, Hershko, and

Rose received the Nobel Prize in Chemistry for the discovery of ubiquitin-mediated protein degradation. Although the mechanisms and functions of the ubiquitin-proteasome system have been investigated extensively, a comprehensive understanding of the complex ubiquitin-proteasome system as well as the development of inhibitors of this system by searching natural sources and chemical libraries and also by chemical synthesis is needed to develop efficient anticancer drugs in the future.

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Figure legends

Fig. 1 Schematic diagram of the ubiquitin-proteasome system. This system consists of the ubiquitin system, the 26S proteasome, and the delivery system. Ub, ubiquitin; E1, Ub-activating enzyme; E2, Ub-conjugating enzyme; E3, Ub-protein ligase.

Fig. 2 Structures of proteasome inhibitors containing peptide aldehydes and their inhibitory mechanism.

Fig. 3 Structures of proteasome inhibitors containing peptide boronates and their inhibitory mechanism. Note that bortezomib (**2**) and CEP-18770 (**8**) are under evaluation in clinical trials for cancers.

Fig. 4 Structures of proteasome inhibitors containing β-lactones and their analogs, and their inhibitory mechanism. Note that **19** and **20** lack β-lactones but contain boronates and that salinosporamide A (**3**) is in clinical trials for cancers.

Fig. 5 Structures of proteasome inhibitors containing epoxyketones and their inhibitory mechanism. Note that carfilzomib (**22**) is in clinical trials for cancers.

Fig. 6 Structures of proteasome inhibitors containing macrocyclic vinyl ketones and their inhibitory mechanism.

Fig. 7 Structures of cyclic peptides as proteasome inhibitors.

Fig. 8 Structures of proteasome inhibitors other than six groups shown in Fig. 2-7.

Fig. 9 Structures of ubiquitin ligase (E3) inhibitors.

Fig. 10 Structures of ubiquitin-activating enzyme (E1) inhibitors.

Fig. 11 Structure of a ubiquitin-conjugating enzyme (E2) inhibitor.

Fig. 12 Structures of inhibitors targeting the delivery system connecting the ubiquitin system to the 26S proteasome.

Fig. 13 Structures of deubiquitinating enzyme (DUB) inhibitors.

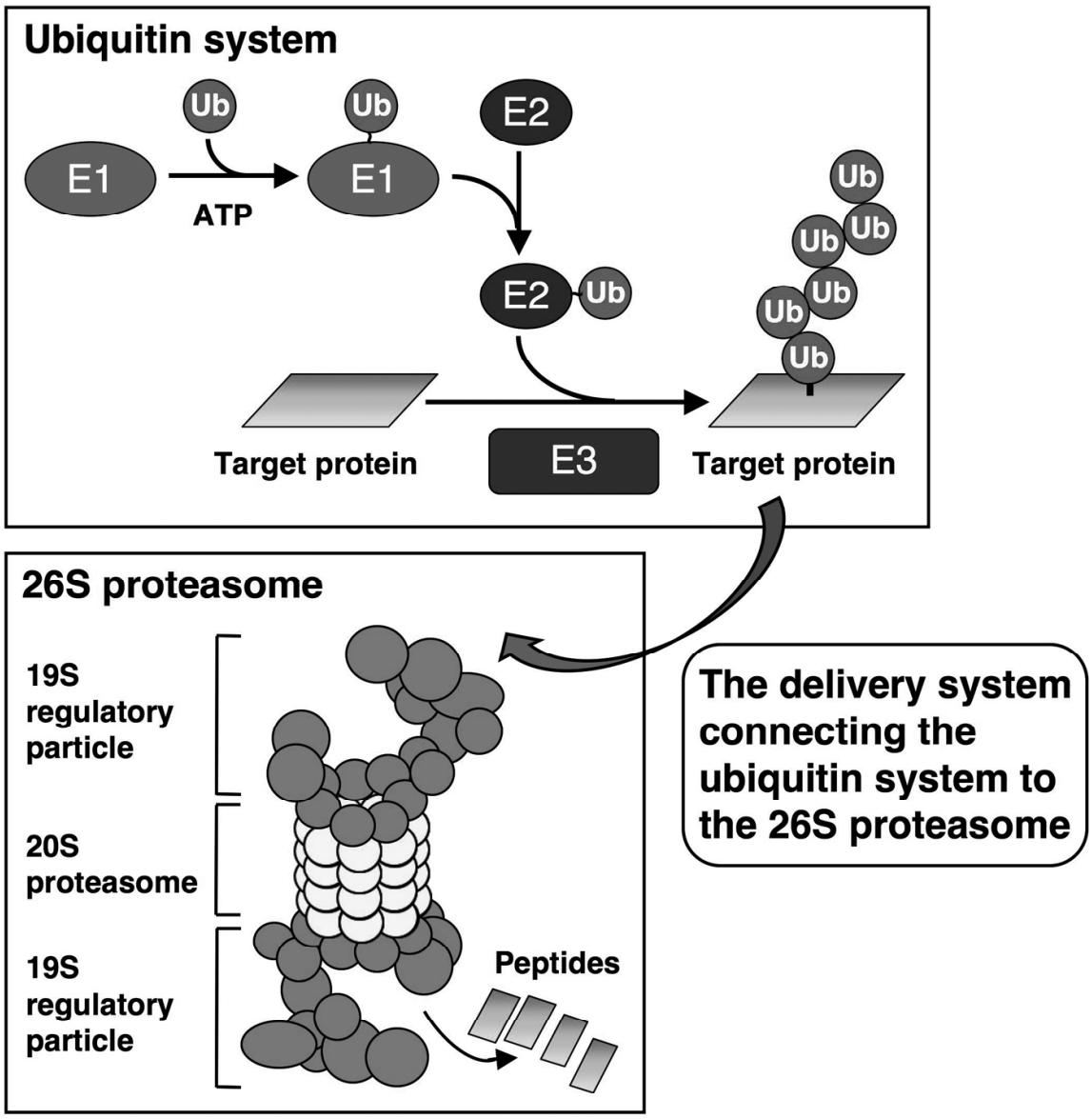
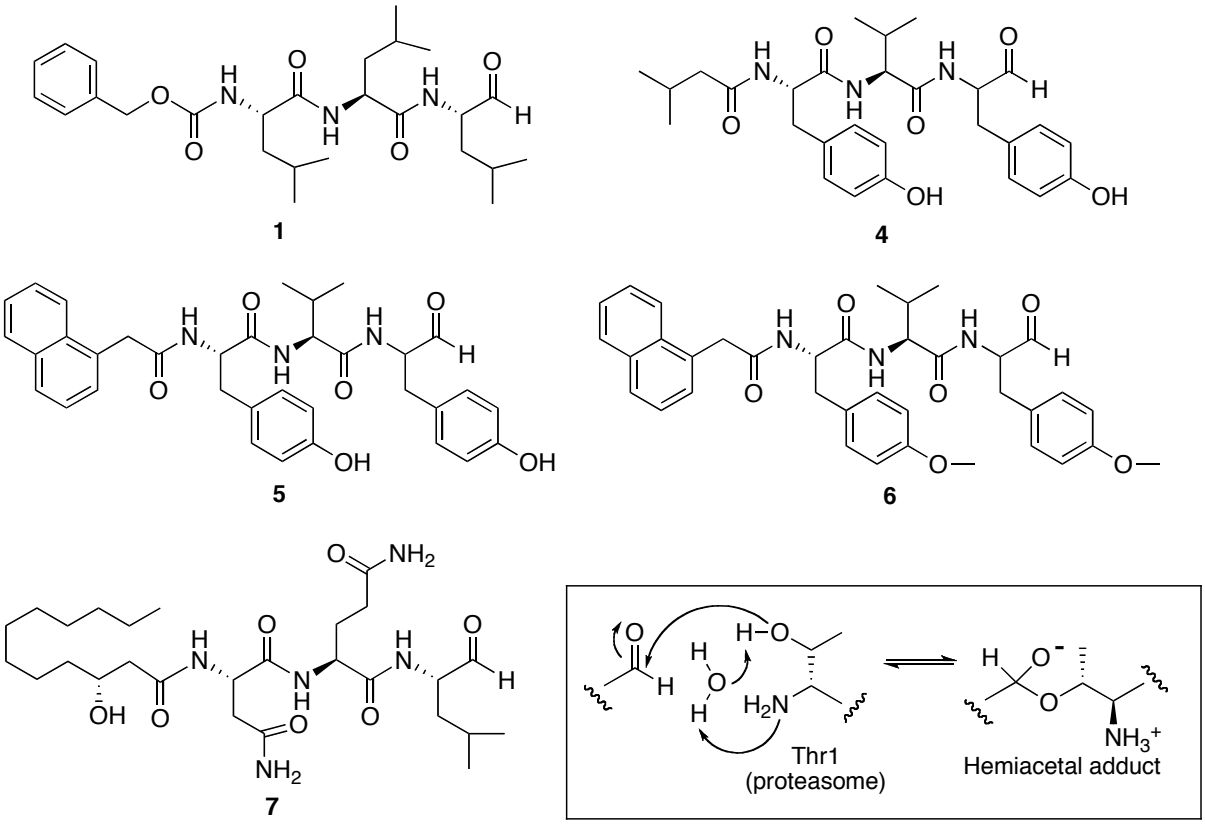

Ubiquitin system
Ub
Ub
E2
E1
E1
ATP
E2
Ub
Ub
Ub
Ub
Ub
Ub
Target protein
E3
Target protein
26S proteasome
19S regulatory particle
20S proteasome
19S regulatory particle
Peptides
The delivery system connecting the ubiquitin system to the 26S proteasome

Fig. 1



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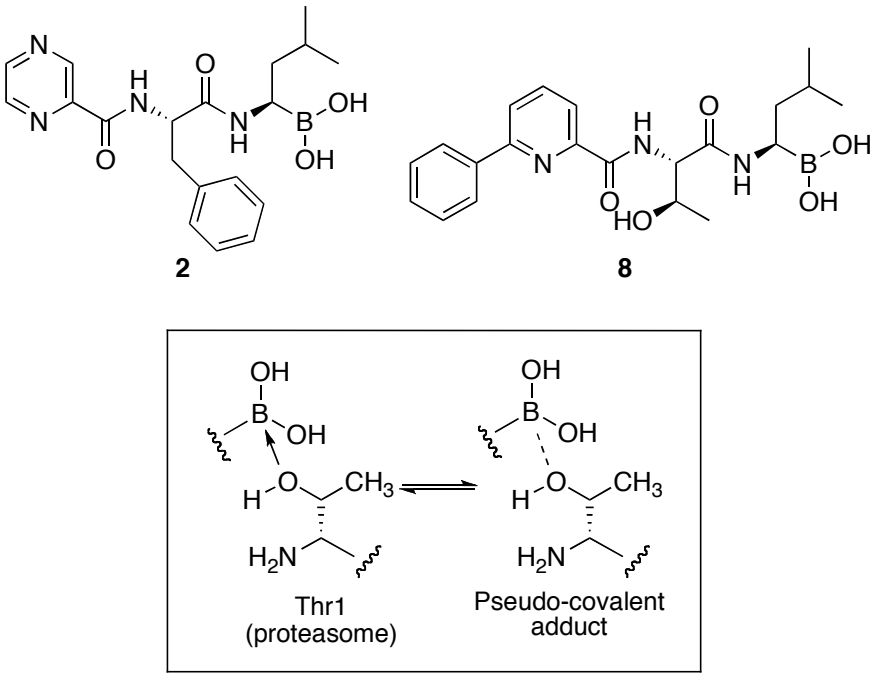

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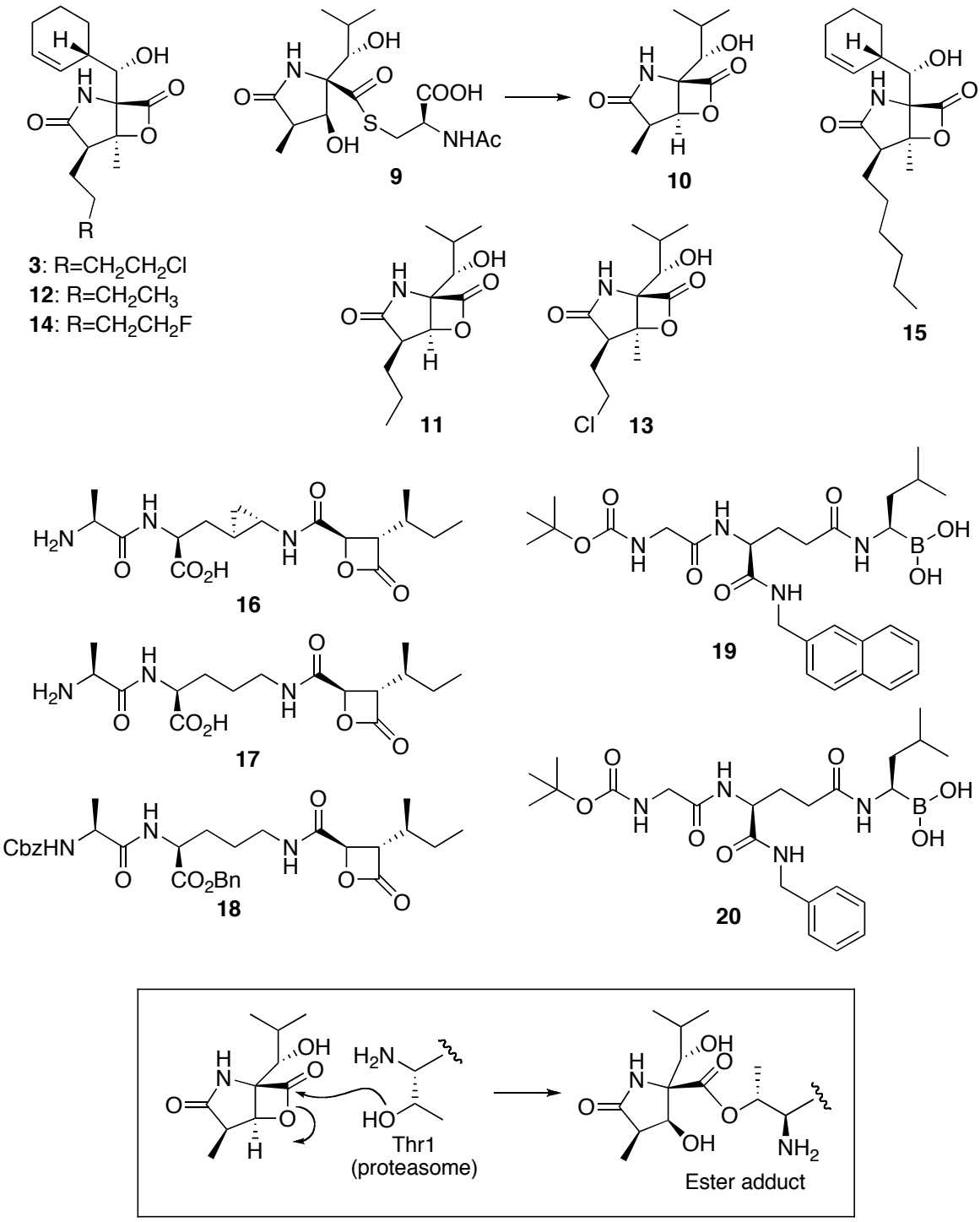
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12: R=CH2CH3
14: R=CH2CH2F
9
10
15
11
13
16
17
18
19
20
Thr1
(proteasome)
Ester adduct

Fig. 4

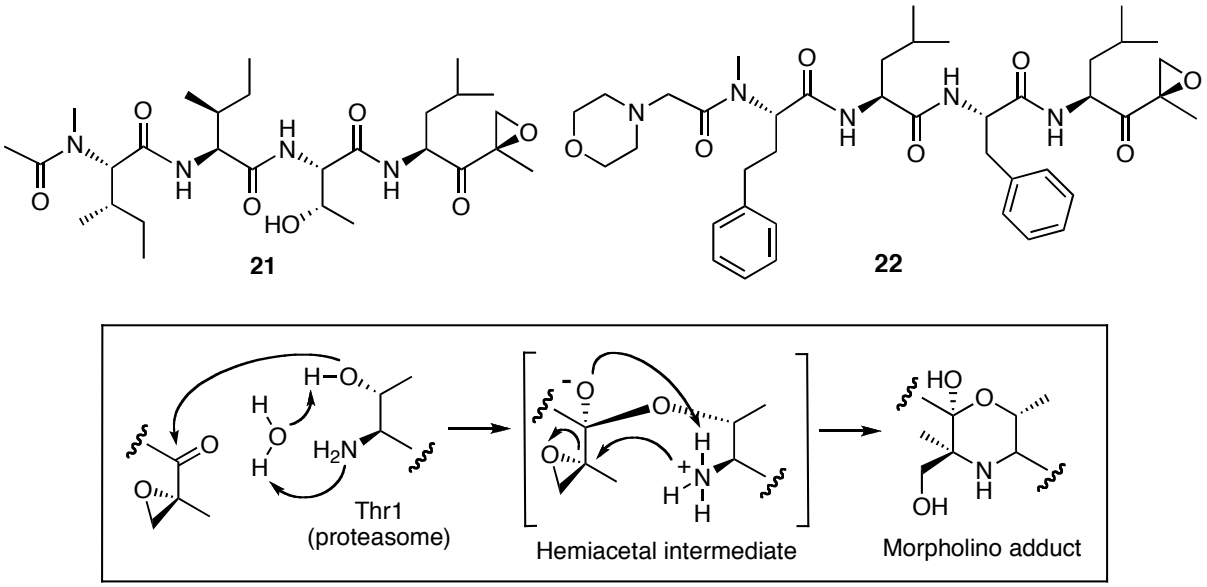

21
22
Thr1
(proteasome)
Hemiacetal intermediate
Morpholino adduct

Fig. 5

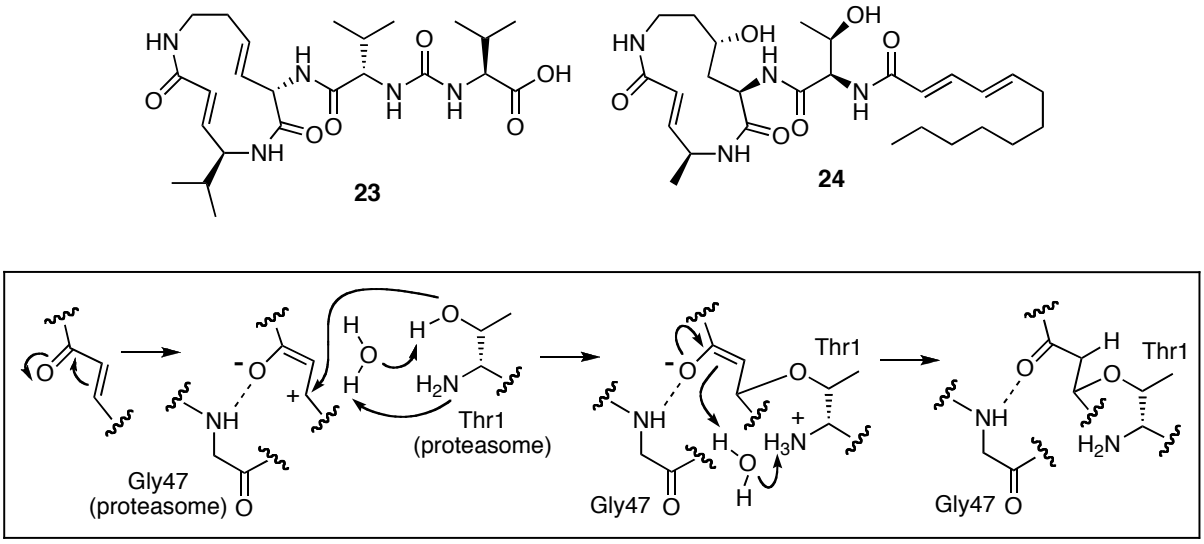
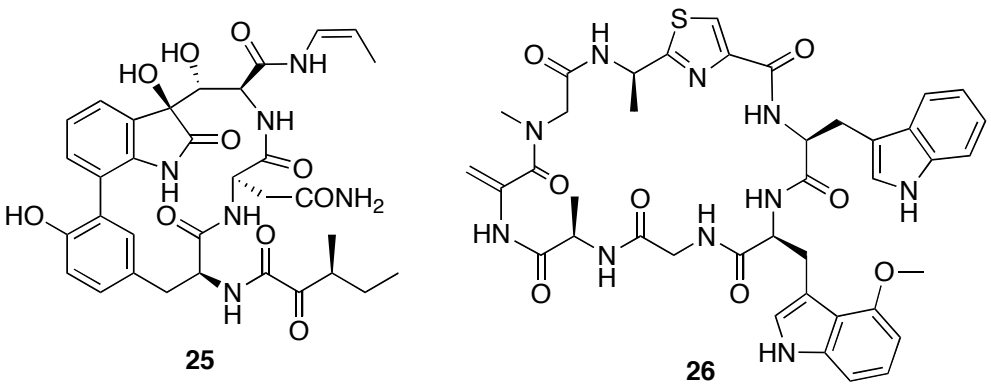

23
24
Gly47
(proteasome)
Thr1
(proteasome)
Thr1
Gly47
Thr1
Gly47

Fig. 6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CONH2
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N
H
O
25
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O
N
O
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N
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O
HN
26

Fig. 7

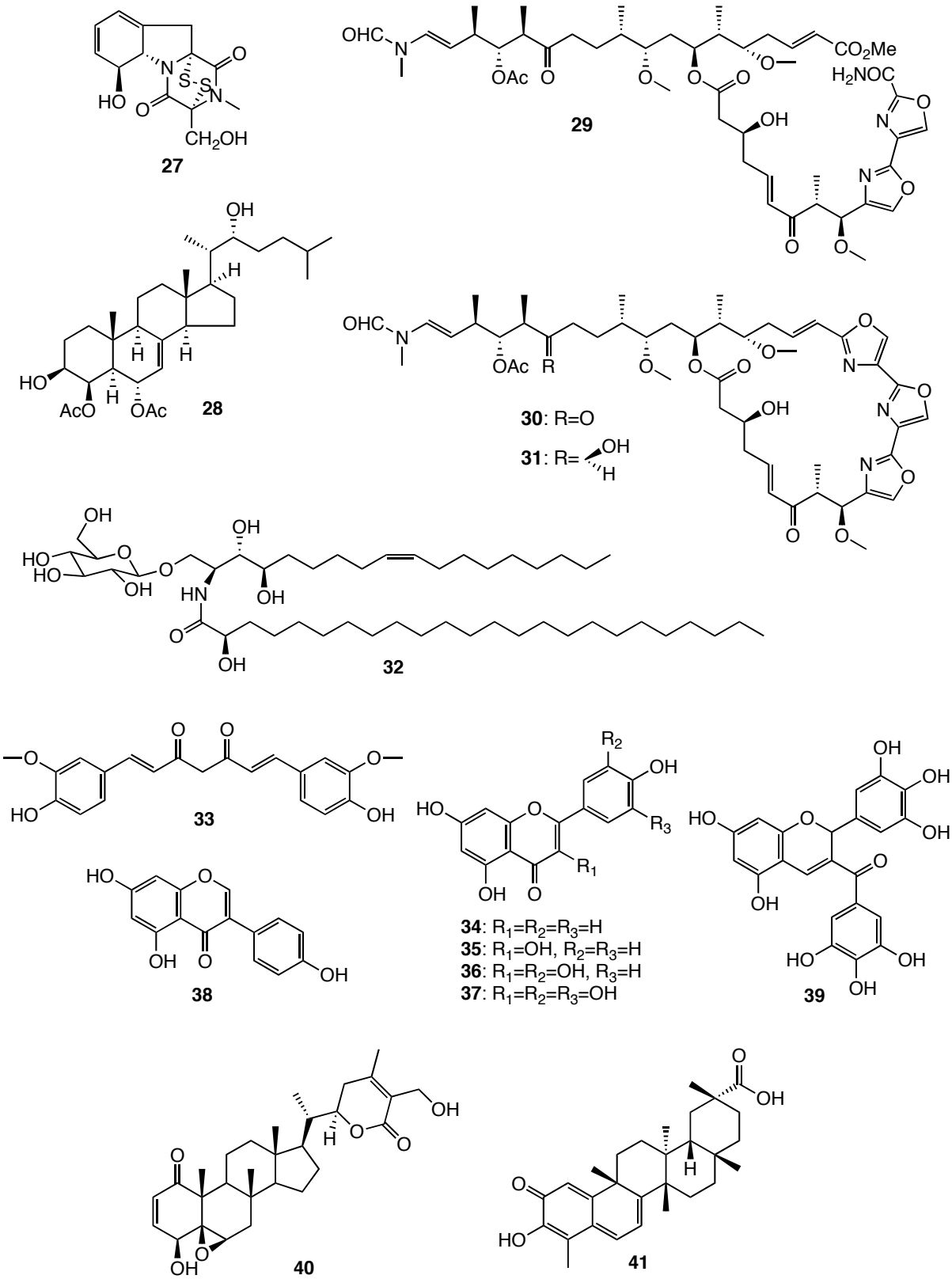
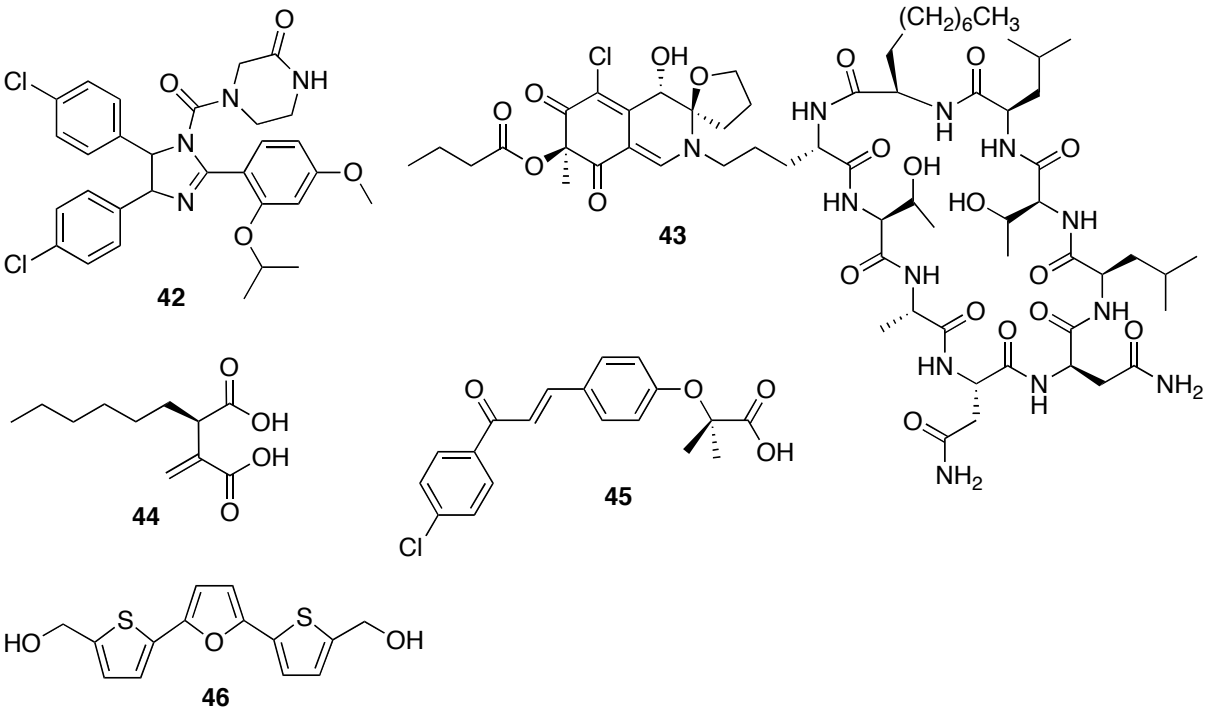

27
29
28
30: R=O
31: R=
32
33
38
34: R1=R2=R3=H
35: R1=OH, R2=R3=H
36: R1=R2=OH, R3=H
37: R1=R2=R3=OH
39
40
41

Fig. 8



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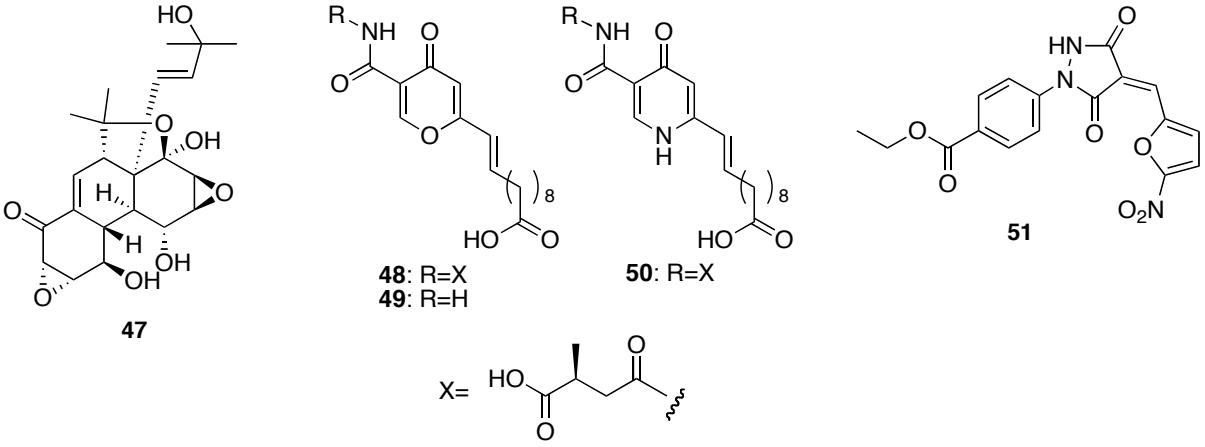
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O
OH
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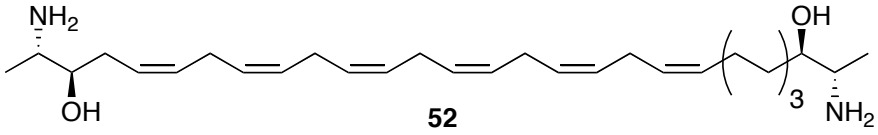
O
O
H
OH
OH
O
47
R
NH
O
O
O
8
HO
O
48: R=X
49: R=H
R
NH
O
O
N
H
8
HO
O
50: R=X
X=
HO
O
O
HN
O
N
O
O
O
O
O2N
51

Fig. 10



52

Fig. 11

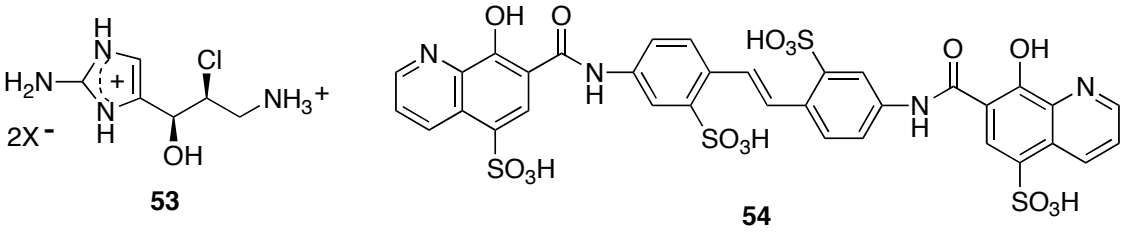
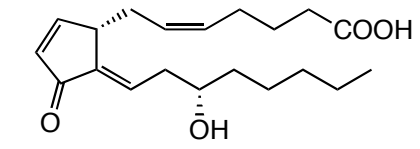


Fig. 12



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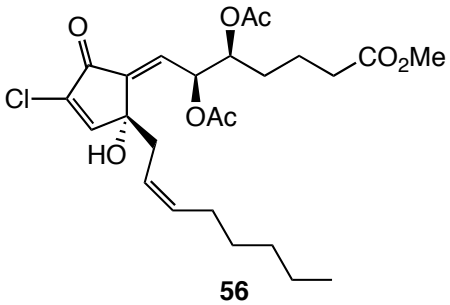

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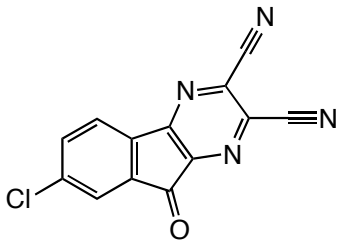

57

Fig. 13