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Semisynthetic neoboutomellerone derivatives as ubiquitin-proteasome pathway inhibitors

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ABSTRACT

The interesting pharmacological properties of neoboutomellerones **1** and **2** were the basis for the assembly of a small library of analogues consisting of natural products isolated from the plant *Neoboutonia melleri* and of semisynthetic derivatives. As the two enone systems (C23–C24a and C1–C3) and the two hydroxyls groups (C22 and C26) of neoboutomellerones are required for activity, modifications were focused on these functional groups. Biological evaluation by using a cellular assay for proteasome activity provided clues regarding the mechanism of action of these natural products and synthetic derivatives. Certain neoboutomellerone derivatives inhibited the proliferation of human WM-266-4 melanoma tumor cells at submicromolar concentration and warrant evaluation as anticancer agents.

1. Introduction

A newly isolated natural cycloartane neoboutomellerone **1** was recently identified as proteasome inhibitor.[1] The ubiquitin-proteasome pathway regulates the degradation of various proteins, thus controlling biological processes such as signal transduction, cell cycle progression, transcription, inflammation, and apoptosis.[2,3] Proteins selected for degradation are marked by the attachment of a polyubiquitin complex and then are recognized and degraded by the proteasome. The 26S proteasome consists of a 20S proteolytic core and of two 19S regulatory caps. The 20S core has three major catalytic activities: chymotrypsin-like (CTL), trypsin-like (TL), and peptide-glutamyl-peptide-hydrolyzing (PGPH). The ubiquitin-proteasome pathway is a target for cancer therapy, and the proteasome inhibitor bortezomib (Velcade®) has been approved by the FDA for the treatment of multiple myeloma and mantle cell lymphoma (Fig. 1).[4–7] Despite clinical benefits, treatment with bortezomib causes severe side effects such as peripheral neuropathy and thrombocytopenia.[8–10] Therefore, there is a need for proteasome inhibitors with other mechanisms of action and greater therapeutic indices.

Several other synthetic and natural inhibitors of the proteasome have been described including α-amino acid boronates,[11] epoxomicin (isolated from *Actinomycetes* strains),[12] and lactacystin (isolated from Streptomyces)[13–15] (Fig. 1). Bortezomib, epoxomicin, and lactacystin are mainly inhibitors of the CTL activity; these compound form covalent adducts with O^{γ}-Thr1 in each of the three catalytic sites. Synthetic epoxyketones have been developed in an effort to decrease toxicity; carfilzomib[16] and ONX-0912[17] are two examples. Other naturals epoxyketones, TMC-86A and B from *Streptomyces sp.* TC 1084, TMC-96 from *Saccharothrix sp.* TC 1094,[18] and eponemycin isolated from *Streptomyces hygroscopicus* No. P247-7 1,[19] previously known for antibiotic and anti-angiogenic activities, also have some activity against the proteasome. Salinosporamide A is another potent inhibitor of the proteasome, isolated from the marine actinomycete *Salinispora tropica* that is currently in clinical trials.[20,21]

Syringolin A, isolated from the plant pathogen *Pseudomonas syringae* pv. *syringae*, contains an α,β-unsaturated carbonyl moiety that undergoes a Michael type addition with hydroxyl groups of threonine residues in the three catalytic sites of the proteasome.[22]

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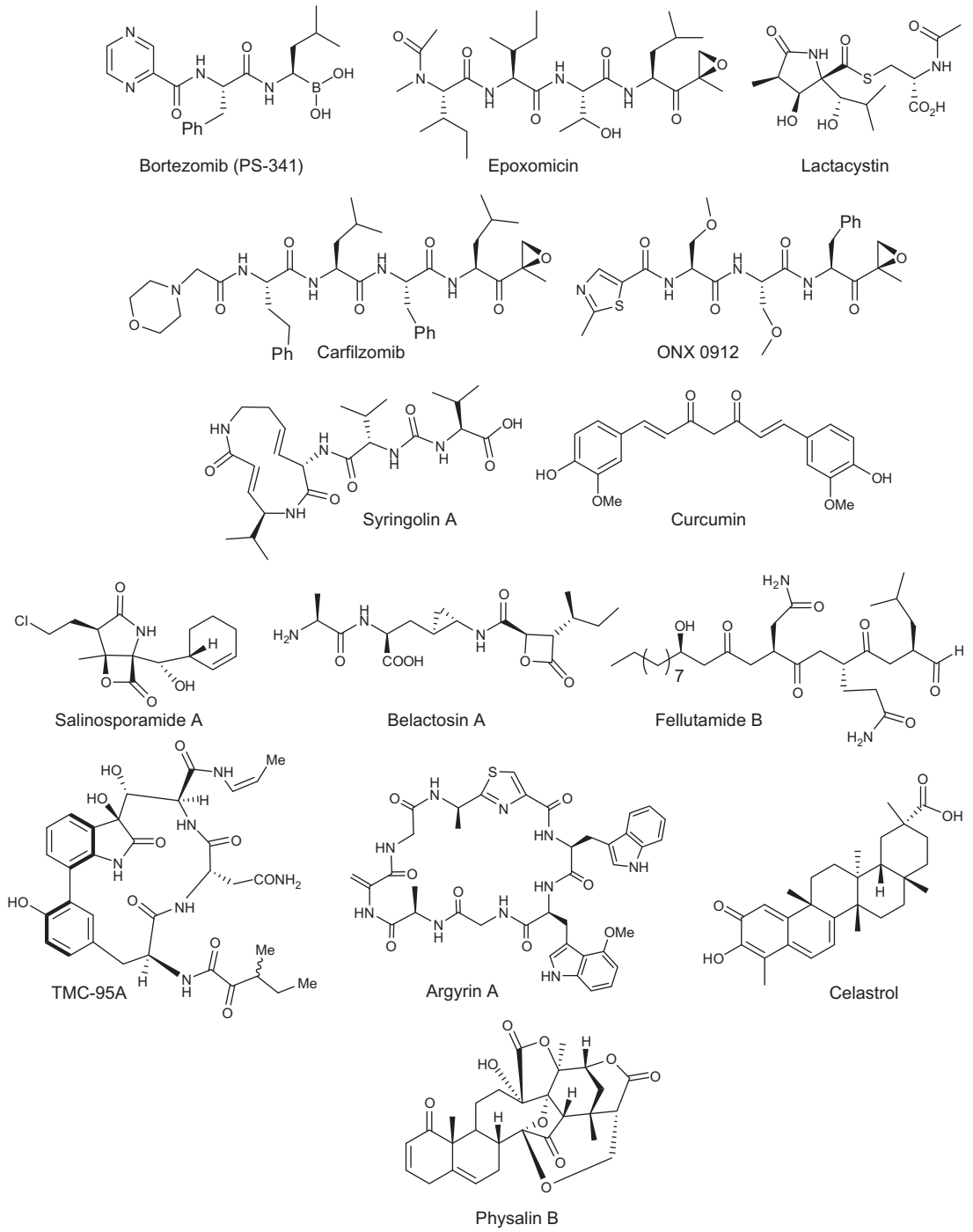


Figure 1. Synthetic and natural proteasome inhibitors.

This family[23] also includes compounds such as glidobactin and cepafungin.[24] TMC-95s[25] (isolated from *Apiospora montagnei* Sacc. TC 1093[26]) and argyrin A (isolated from the myxobacterium *Archangium gephyra*)[27] are two cyclic peptides that strongly inhibit the CTL activity. Curcumin[28] (the major active ingredient of turmeric (*Curcuma longa*) with its Schiff's bases and associated copper complexes[29]) and celastrol[30] (a triterpene isolated from the root bark of *Tripterygium wildfordii*) also inhibited the CTL activity of a purified 20S proteasome with micromolar IC_{50} values. Fellutamide was isolated from fungus *Penicillium fellutanum*[31] and was first described as an inducer of nerve growth factor release.[32] After achievement of the total synthesis,[33] fellutamide was shown to inhibit proteasome catalytic activity.[34] Belactosins, isolated from *Streptomyces* KY11780,[35] and their hydrophobic derivatives KF33955 and KF44504[36] show affinity for the catalytic subunits of the 26S proteasome and inhibit growth of HeLa cells.[37] Computational modeling studies showed that both ketones of curcumin, the conjugated carbonyls of celastrol and syringolin, the epoxides of epoxomicin and carfilzomib, and the aldehyde of fellutamide are highly susceptible to a nucleophilic attack by the hydroxyl group of the terminal threonine of the proteasome CTL subunit. All these compounds target the proteasome catalytic sites.

In order to alleviate the observed side effects associated with previously evaluated inhibitors of the catalytic activities of the

20S proteasome, our research focused on the identification of inhibitors of the ubiquitin-proteasome pathway in a cellular assay.[38,39] Our assay system is a human DLD-1 colon cancer cell line stably transfected with a gene encoding a ubiquitin-luciferase reporter protein, 4Ub-Luc. The 4Ub-Luc protein is efficiently targeted by the ubiquitin-proteasome degradation pathway in this cell system. Upon treatment of the cells with a proteasome inhibitor, the reporter protein accumulates within the cells inducing a detectable increase of bioluminescence. Physalin B was identified using this assay.[39] Physalin B induces a 19-fold increase of bioluminescence at 5 μM, whereas it inhibits cellular proteasomal CTL and PGPH activities at 20 and 40 μM, respectively.

This cellular assay was adapted to a 96-well plate format, and a high-throughput screening of over 12,000 natural products extracts and of 62,000 pure molecules led to the identification of the natural product neoboutomellerone **1** as a potent inhibitor of the ubiquitin-proteasome pathway. This new cycloartane,[40] isolated from the Euphorbiaceae *Neoboutonia melleri*,[1] has two main structural originalities (Fig. 2). Firstly, ring A possesses an enone system and only one methyl group on position 4. Secondly, a supernumary carbon substituent is present on the side chain of the exo-methylene enone system (Fig. 2). Thirty related cycloartanes were subsequently isolated from this plant; among them was molecule **2**, which lacked an acetate group on the alcohol C-22.

The two compounds **1** and **2** showed an interesting level of activity in the ubiquitin-proteasome reporter assay (a 48-fold increase of bioluminescence at 1 μM). Their relatively high level of functionalization and their ready availability made them valuable candidates for the starting points for semi-synthesis of additional neoboutomellerone derivatives. A total of 47 analogues of the neoboutomellerones were prepared to explore the roles of the α,β-unsaturated ketones, the substituents on the alcohol at C-26, and different acyl groups on the alcohol at C-22.[40] The high reactivity of these natural products against acids, bases and nucleophiles prohibited some obvious reactions. All the analogues were evaluated in the cellular proteasome reporter assay in 4Ub-Luc DLD-1 cells and for inhibition of the CTL and PGPH catalytic activities. These analyses allowed us to identify the structural elements responsible for proteasome activity and to improve bioavailability, metabolic stability, and solubility relative to compounds **1** and **2**. Finally, the cytotoxicity of selected compounds was assessed in the WM-266-4 tumor cells, a cell line derived from a human melanoma.

2. Synthesis of neoboutomellerone derivatives

Fifteen compounds (**1**–**15**)[41] among the thirty previously described natural products isolated from *Neoboutonia melleri*[1] were selected for a structure–activity relationship (SAR) study. The semisynthetic syntheses began with the two most abundant compounds from the plant, **1** and **2**,[42] and we aimed to improve activity and solubility. The main concern was the polyfunctionality of these compounds; each possessed three or four reactive centers: the enones and the primary and secondary alcohols at C-26 and C-22.

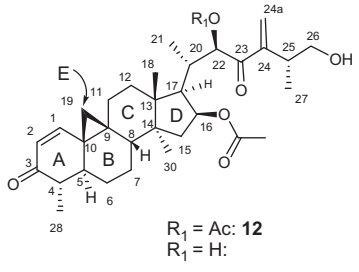


Figure 2. Neoboutomellerones.

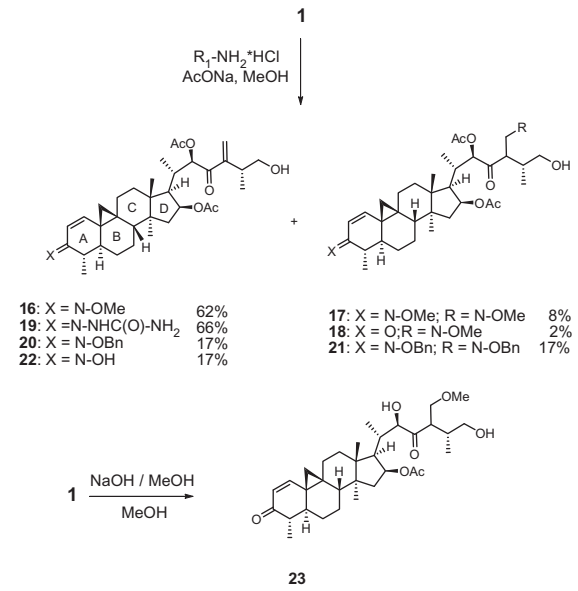
2.1. Modification of the enone system

The reactivity of these α,β unsaturated ketones can be exemplified by the reaction with primary hydroxylamines, chosen in order to introduce a nitrogen atom and increase the diversity (Scheme 1). In these reactions, the ring A enone reacted more rapidly than the one located in the side chain, and oximes **16**, **19**, and **20** were obtained with some selectivity. Side products included the bis-adducts **17** and **21**, for which a second addition occurred at C-24a in a Michael fashion. We also observed the formation of the methoxy adduct **23**. The mono-adduct on the side chain (**18**) with the less hindered *O*-methyl hydroxylamine was obtained as a minor by-product. The reactivity order may be explained by steric hindrance around C-1, making the 1,4-addition more difficult, while hindrance around C-23, associated with a default in planarity in the side chain enone system, makes the corresponding centers less prone to nucleophilic additions.

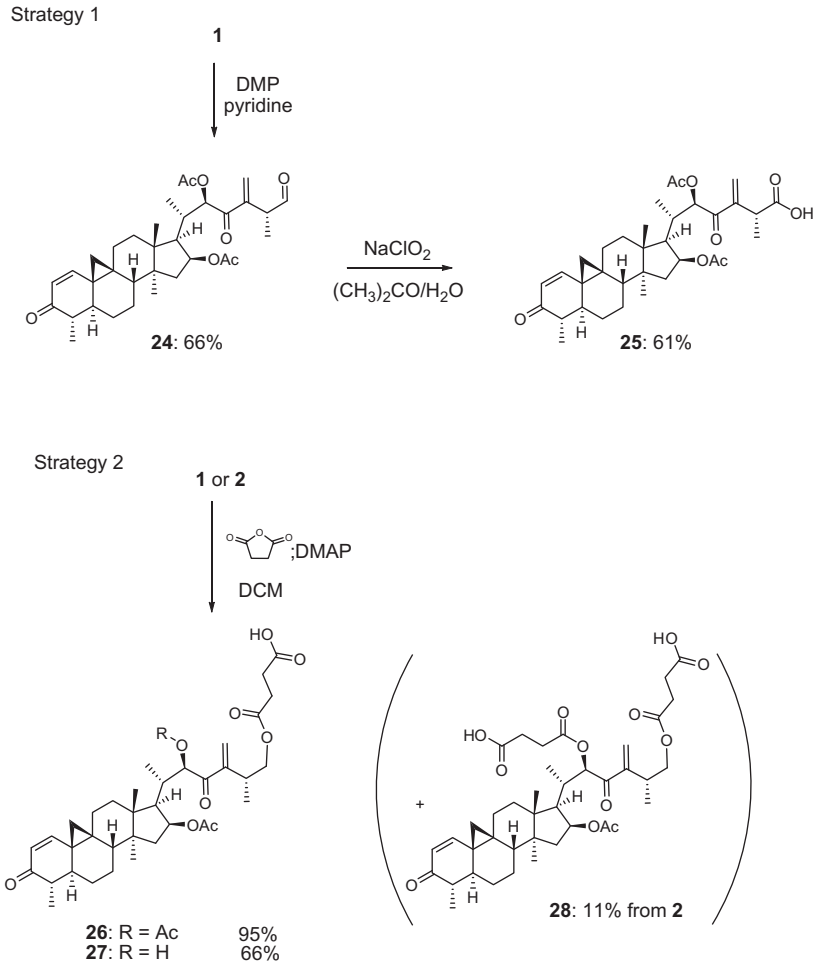
2.2. Modification of the hydroxyl functional groups

The alcohols at C-22 and C-26 were substituted in order to enhance solubility relative to the parent compounds. Two strategies were applied: In the first, the primary alcohol was oxidized to the carboxylic acid, and in the second, an appendage containing the desired functional group was conjugated (Scheme 2). In the first strategy, neoboutomellerone **1** was oxidized to the corresponding aldehyde **24** with the Dess–Martin periodinane reagent and then converted into the acid **25** under Pinnick conditions. The sequence proceeded smoothly, with moderate yields, without reaction at the enones or migration of the C-24-C-24a double bond. The second strategy was employed for addition of succinic anhydride, which yielded acids **26** and **27**, accompanied by some bis-adduct **28**, when **2** was used as starting material. Succinate derivatives were transformed into *N*-methyl glucamine salts to increase the solubility in water.

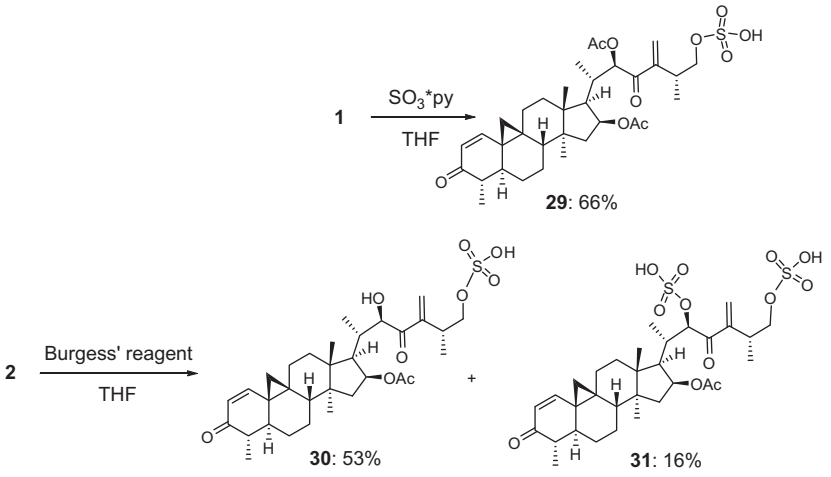
In a similar fashion, the phosphate and sulfate derivatives were prepared as described in Scheme 4. Synthesis of the sulfate derivative **29** from **1** was straightforward using the sulfur trioxide-pyridine complex. Formation of the sulfate of compound **2** required the use of the Burgess reagent and provided bis-sulfate **31** as a side product of the monosulfate **30** (Scheme 3). Compound **30** was



Scheme 1. Modification of the enone systems.



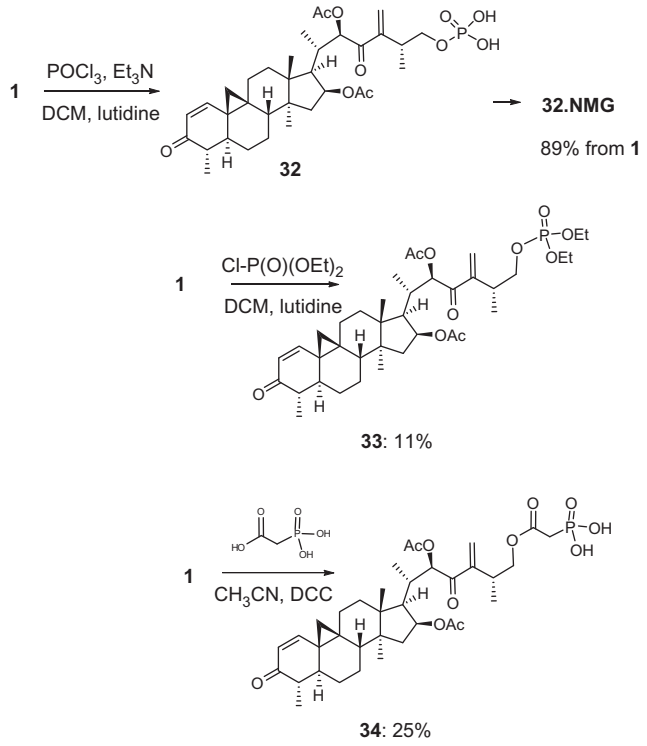
Scheme 2. Synthesis of acidic derivatives by two strategies.



Scheme 3. Synthesis of sulfate derivatives.

highly soluble in water and was purified by simple washing with an organic solvent. The phosphate monoester **32**, the phosphate diethyl ester **33**, and the phosphonate **34** were prepared in a similar fashion. Phosphate derivative **32** was transformed into its *N*-methyl glucamate to further increase solubility (Scheme 4).

Introduction of a nitrogen atom at the terminal position of the side chain proved challenging, and most classical synthetic strategies, including Mitsunobu, gave rise to conjugate addition. Only reaction with a protected spermine was successful, yielding derivative **41** in moderate yield (Scheme 5). Recourse to classical methods of ester synthesis led to glycine derivatives **35**–**37** and **42** after Boc removal. The glycine–spermine synthon offered **40** in 10% yield after Boc deprotection. A facile decomposition into acrylate **43** decreased the yield during preparation of the homologous



Scheme 4. Synthesis of phosphate derivatives.

β-alanine derivative **36**. The direct use of mono-acetate **2** in these coupling reactions led to a mixture of the mono-adduct **44** and of the di-adduct **45**, but selectivity could be obtained by prior protection of the primary alcohol as a TBDMS ether. This sequence of protection/acylation/deprotection led to derivative **46**, which formally was another nitrogen substituted derivative of **1** (Scheme 6).

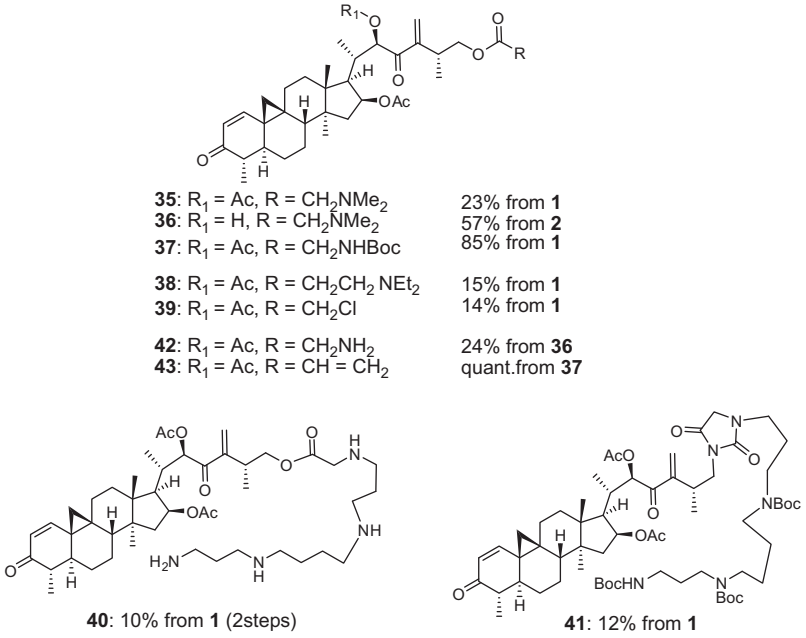
Carbamates were prepared in an analogous fashion using phenyl-isocyanate or *para*-dimethylaminophenyl-isocyanate. Derivative **47**, the phenylcarbamate derivative of **1**, was obtained as a crystalline solid suitable for X-ray crystallographic studies. Since the corresponding isocyanates were not easily available, the piperazine carbamates **50** and **51** were obtained by reaction of the alcohols with the hydrochloride salt of chlorocarbonylpiperazine (Scheme 7).

Ethers are generally stable derivatives under biological conditions and termination of the side chain with an ether linkage was desired. However, it was extremely difficult to convert the C-26 primary alcohol into an ether under basic conditions; saponification of the acetate at C-16 and concomitant reaction with the side chain acyloin system resulted under all conditions tested. The methyl and allyl ethers, **52**–**54**, were prepared with silver ion activation of the corresponding halides, whereas acetal **55** was synthesized using Hünig's base (Scheme 8). The allyl ether **53**, which was prepared to open a route towards glycerol derivatives, underwent over-oxidation under osmium catalysis to afford tetraol **56** in 38% yield. Glycosylation under Schmidt conditions did not provide the expected glycoside; orthoester **57** was obtained instead.

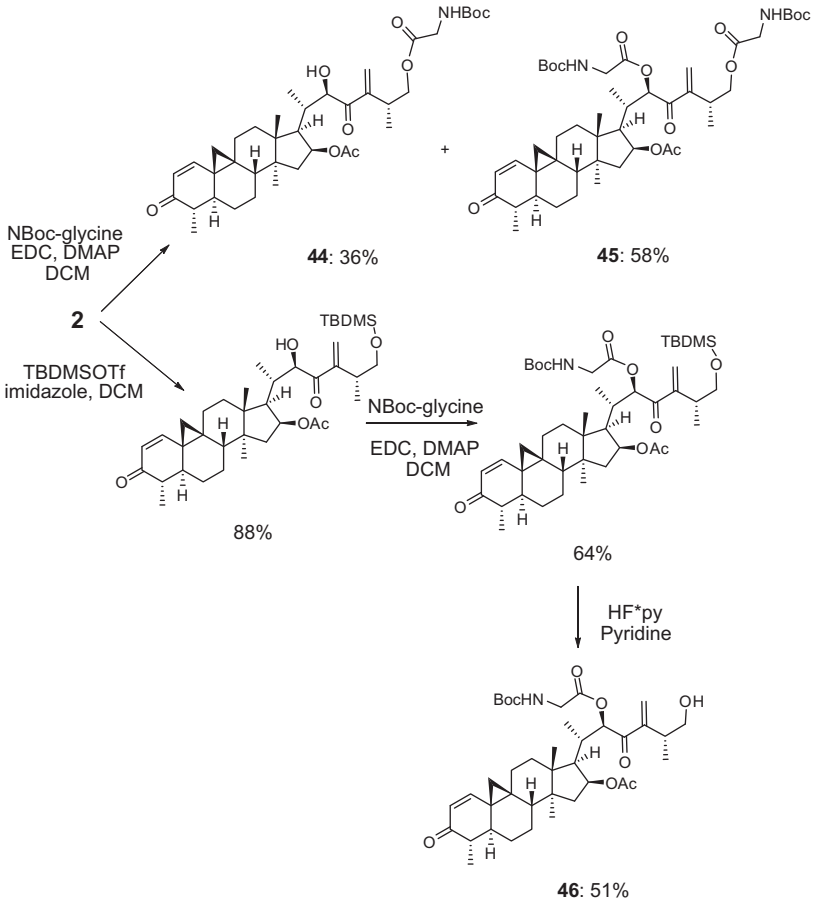
A last series of compounds was prepared to determine the importance of the acetate moiety on position C-22 to biological activity, and several acyl groups were introduced at this position. The starting material for these transformations was diacetate **58** prepared by acetylation of **2** using acetic anhydride. As expected, these transformation led to triacetate **59**, chloroacetate **60**, *N*-Boc glycinate **61**, and succinate **62** (Scheme 9).

2.3. General observations

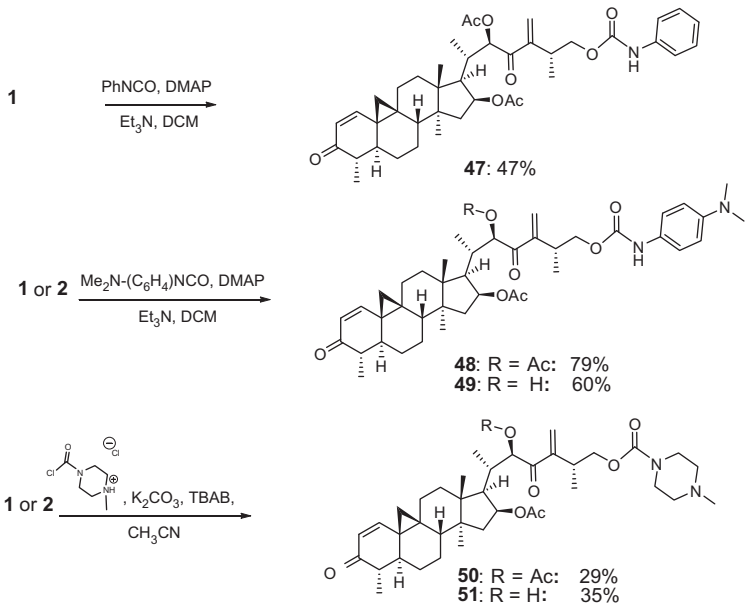
The purpose of this study was to enlarge the chemical diversity around this natural scaffold and yields were not optimized at this stage. It is worth noting that two major transformations could not be performed due to neighboring group participation or to the reactivity of the enones. Compounds with a basic nitrogen atom at C-26 and compounds with various acid groups at C-16 are lacking. For *aza*-analogues, we were unsuccessful in hydroxyl substitution by nitrogen because Michael type addition of nitrogenic nucleophile onto the C23–C24a enone system. Concerning C-16, all attempts to modify the acetate failed. Saponification of this ester led spontaneously to cyclic compounds of O-16 with C-22



Scheme 5. Synthesis of ester derivatives.



Scheme 6. Synthesis of glycinate derivatives.

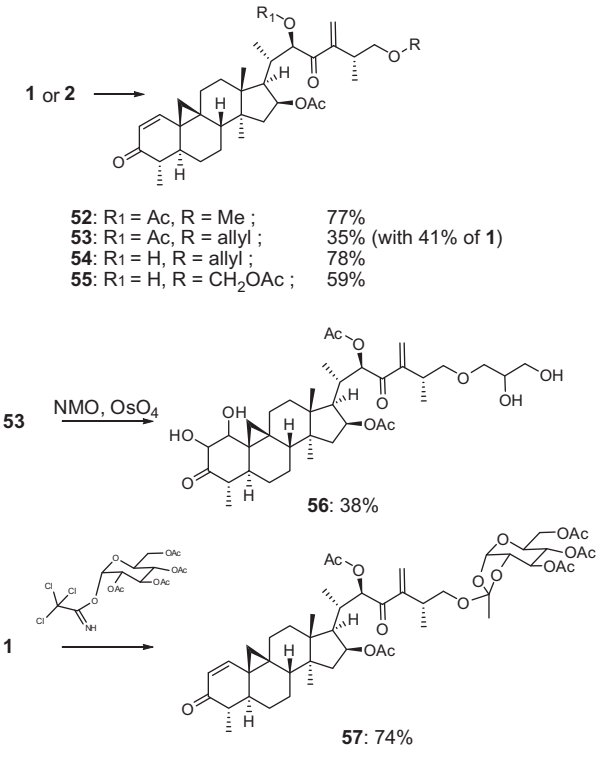


Scheme 7. Synthesis of carbamate derivatives.

or C-23. This was due to acyloin ketol shift observed in basic media. Attempts to trap the enolate in situ with electrophile also failed. The unexpected chemical reactivities are under investigation and will be published in due course.

3. Proteasome inhibition

As shown previously, treatment of 4Ub-luc DLD-1 cells with proteasome inhibitors increases the cellular concentration of the



Scheme 8. Synthesis of ether derivatives.

protein 4Ub-Luc, since the protein is not degraded by the proteasome pathway. This inhibition is thus measured as an increase of the bioluminescence compared to untreated cells. This increase of bioluminescence is expressed as an induction factor (IF). Results are compared to a control, epoxomicin at 10^{-7} M, run in parallel experiment; its relative IF is defined as 100. A molecule was considered active if the induction factor was equal or superior to 10. This assay has the advantage of detecting molecules that penetrate into the cells.

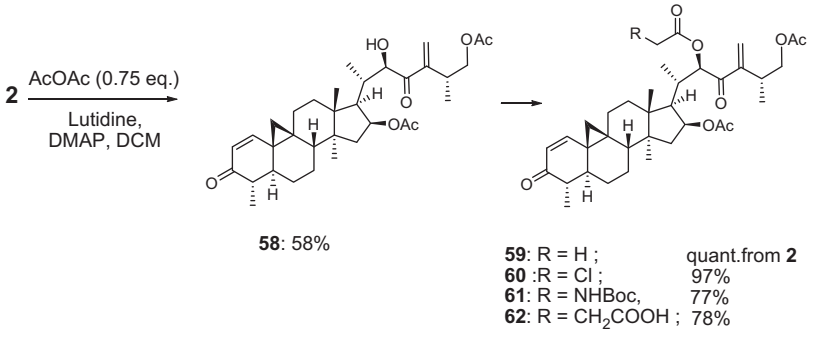
The results of analysis of selected neoboutomellerone analogues in the proteasome inhibition assay are presented in Table 1. Generally the compounds had a 'bell-shaped' dose–response curve: At higher concentrations the activity decreased, probably because of cytotoxicity of the compounds. For the SAR study we considered the IF at 0.5 and 1 μM and compared activities of derivatives to those of the natural leads **1** and **2**.

Among the compounds isolated from the plant, the most active were neoboutomellerones **1** and **2**. The presence of the acetyl at C-22 did not affect the activity. The natural modifications observed were mainly over-oxidation (compound **5**, oxidation on ring A; compounds **6**, **7**, and **9**, oxidation on ring B; compound **8**, oxidation on substituents) and reduction (compounds **3** and **4**, hydrogenation on ring A; compounds **11** and **12**, reduction of the side chain). These modifications did not increase the activity. Nevertheless, compounds **4**, **5**, **9**, **10**, **11**, and **12** showed an increased efficacy at higher concentrations but a clear loss of potency relative to potency compared to neoboutomellerones **1** and **2**.

As described in the chemical section, neoboutomellerones were modified on three different functions to increase solubility and biological activity. Firstly, the enones on ring A and on the side chain were modified. Among these compounds, only the semi-carbazone **19** and hydroxime **22** showed a stronger efficacy (i.e., higher IF value) than the parent compounds **1** and **2**, but a lower potency (i.e., at higher concentration). Their IF are respectively, 99 and 103 at 5 μM. Compounds **16** and **20** had little or no activity. The addition of a nucleophile on the double bond of the enone of the side chain had a dramatic effect and compounds **17**, **18**, **21**, and **23** were inactive.

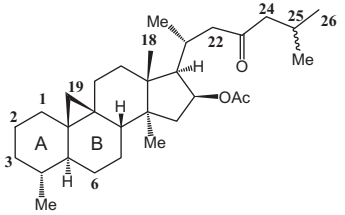
Secondly, the primary hydroxyl group on position 26 was modified. To increase the solubility, acids (compounds **25**–**28**), sulfates (compounds **29–31**), phosphates (compounds **32** and **33**) and a phosphonate (compound **34**) were obtained. All acids forms were inactive, including carboxylic acid **25**. The phosphate diethyl ester **33** was as active as the parent neoboutomellerone, and succinates **26** and **27** were more potent (up to IF 60 at 1 μM) than the parent. The di-substitution, as for compound **28**, of both hydroxyl groups as succinate monoester resulted in the loss of activity. This could be due to the fact that this compound with two negative charges cannot penetrate the cellular membrane. Positively charged compounds (**35–38**, **40–42**, **44–45**, **47–51**) were also synthesized and tested. The hydrochloride salt of the dimethylglycinate neoboutomellerone **1** (compound **35**·HCl) was active (IF 66 at 0.5 μM), confirming the importance of a positive charge. The spermine derivative (compound **40** and **41**) was not active, whereas the primary amine analogue (compound **42**) was. Carbamates **47–51** had higher activities than the parent compounds: This could be due to the fact that these carbamate derivatives act as prodrugs of neoboutomellerones **1** and **2**, leading to an effectively higher concentration of neoboutomellerone in cells.

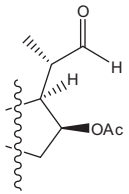
Lastly, analogues substituted on position 22 of the triterpenic skeleton were studied. Acetylation of position 22 did not affect the activity in natural (**3** compared to **4**, **6**–**7**, **11**–**12**, and **13**–**14**) and synthetic compounds (**26** compared to **27**, **37**–**44**, **48**–**49**, **50**–**51**, **58**–**59**), in analogy with what observed for parent compounds **1** (acetylated) and **2** (non-acetylated). Interestingly, the presence of a *N*-Boc glycinate in compound **45** increased the activity compared to the acetate analogue **37** and to hydroxyl **43**. The same was observed in the series where position C26 was acetylated (compound **58** vs **62**). Although the presence of an acetyl function (compound



Scheme 9. Synthesis of C-22-substituted derivatives.

Table 1
Structures and biological evaluation of selected neoboutomellerone derivatives (the complete SAR table is found in the Supplementary data)



Compounds	C_1	C_3	C_6–C_7	C_{18}	C_{22}	C_{24}	C_{25}	C_{26}	rIF[a] (μM)					
									10	5	1	0.5	PGPH IC_{50}[b] (μM)	CTL IC_{50}[c] (μM)
Epoxomicin											112	[d]	0.06	0.006
Lactacystin									93	106	72	[e]	0.3	0.07
Bortezomib											117	[f]	0.0009	0.0002
1	Δ	=O	H,H	CH_3	OAc	$=CH_2$	α	CH_2OH	4.4	37	48	2.1	23	16
2	Δ	=O	H,H	CH_3	OH	$=CH_2$	α	CH_2OH	2.9	28	45	1.4	51	42
15	Δ	=O	H,H	CH_3					3.0	1.3				
27·NMG	Δ	=O	H,H	CH_3	OH	$=CH_2$	α	$CH_2OC(O)CH_2CH_2COOH$	5	18	46	2.6	47	61
33	Δ	=O	H,H	CH_3	OAc	$=CH_2$	α	$CH_2OP(O)(OEt)_2$	2	16	43	3	91	19
35·HCl	Δ	=O	H,H	CH_3	OAc	$=CH_2$	α	$CH_2O\ C(O)CH_2NMe_2$	2.7	11	50	66	52[g]	31[g]
36·HCl	Δ	=O	H,H	CH_3	OH	$=CH_2$	α	$CH_2O\ C(O)CH_2NMe_2$	4	34	17	1.3	22	17
37	Δ	=O	H,H	CH_3	OAc	$=CH_2$	α	$CH_2O\ C(O)CH_2NHBoc$	10	43	14		>1000	92
44	Δ	=O	H,H	CH_3	OH	$=CH_2$	α	$CH_2O\ C(O)CH_2NHBoc$	16	47	4.8		>1000	>1000
45	Δ	=O	H,H	CH_3	$C(O)CH_2NHBoc$	$=CH_2$	α	$CH_2O\ C(O)CH_2NHBoc$	17	33	47	4.3	38	21
50·HCl	Δ	=O	H,H	CH_3	OAc	$=CH_2$	α	$CH_2OC(O)$-pip-NMe	1	4.2	68	8	16	13
51·HCl	Δ	=O	H,H	CH_3	OH	$=CH_2$	α	$CH_2OC(O)$-pip-NMe	1.4	4.5	70	12	32	39
55	Δ	=O	H,H	CH_3	OH	$=CH_2$	α	$CH_2O\ CH_2OAc$	3	34	40	1.7	31	22
58	Δ	=O	H,H	CH_3	OH	$=CH_2$	α	CH_2OAc	6	29	26	1.7	45	31
59	Δ	=O	H,H	CH_3	OAc	$=CH_2$	α	CH_2OAc		48	11	1.1	43	35
60	Δ	=O	H,H	CH_3	$C(O)CH_2Cl$	$=CH_2$		CH_2OAc	5	21	64	23	100	50
61	Δ	=O	H,H	CH_3	$C(O)CH_2NHBoc$	$=CH_2$	α	CH_2OAc	1.6	3.4	69	76	23	17
62·NMG	Δ	=O	H,H	CH_3	$C(O)CH_2\ CH_2COOH$	$=CH_2$	α	CH_2OAc	3	16	69	2	10	12

[a] rIF = relative IF in 4Ub-luc DLD-1 assay compared to epoxomicin.
[b] Concentration in μM at which 50% of PGPH activity is inhibited (IC_{50}).
[c] Concentration in μM at which 50% of CTL activity is inhibited (IC_{50}).
[d] 100 at 0.1 μM.
[e] 6.2 at 0.1 μM.
[f] 133 at 0.1 μM.
[g] Measured with **35**.

Table 2
Cytotoxicity of neoboutomellerone analogues in WM-266-4 cells lines at 72 h

Compounds	IC_{50}[a] (μM)
Epoxomicin	0.0048
Bortezomib	0.035
1	2.80
2	1.60
35·HCl	0.61
47	0.19
48	0.96
49	0.82
50·HCl	0.50
51·HCl	0.46
54	0.78
58	0.99
59	1.50
60	0.66
61	1.00

[a] IC_{50} = concentration in μM at which 50% inhibition of cell proliferation is observed.

59) in comparison to the hydroxyl **58** did not alter the activity, the presence of a chlorine atom (compound **60**) or of an amino group (compound **61**) greatly increased efficacy and potency: Compound **61** showed the best activity with an IF of 76 at 0.5 μM. The negatively charged succinate **62** showed an IF of 69 at 1 μM.

In order to study the mechanism of action of the neoboutomellerone series, the most potent compounds were tested for inhibition of the catalytic activities of the cellular proteasome. Levels of chymotrypsin (CTL) and peptidyl-glutamyl-peptide hydrolyzing (PGPH) activities were measured using specific fluorogenic substrates as previously described.[43] No straightforward correlation was observed between the induction of the luminescence signal of the 4Ub-luc DLD-1 assay and the inhibition of the CTL or PGPH catalytic activity. All compounds showed IC_{50} values higher than 10 μM in the catalytic activity assays. The reference compounds epoxomicin, lactacystin, and bortezomib had IC_{50} values of 0.06, 0.3, and 0.0009 μM, respectively, against PGPH and 0.006, 0.07, and 0.0002 μM, respectively, against CTL. These findings suggest that the proteasome inhibition of the neoboutomellerone compounds is not or is only partially mediated by the inhibition of the catalytic activity of the proteasome. The three most potent compounds in the ubiquitin-proteasome cellular assay, **61**, **35**, and **60** (IFs at 0.5 μM of 76, 66, and 23, respectively) were poor inhibitors in the PGPH and CTL catalytic assays.

The three most potent compounds in the cellular assay, **61**, **35**·HCl, and **60**, and the active carbamates **47**–**51** were tested for their cytotoxicity in human WM-266-4 metastatic melanoma cells (Table 2). All compounds had similar IC_{50} values of around 1 μM with the exception of carbamate **47**, which was more toxic (IC_{50} of 0.19 μM).

4. Conclusions

Here we evaluated the activities of 15 natural products isolated from *N. melleri* and the activities of 47 semisynthetic derivatives of neoboutomellerones **1** and **2**. Twelve analogues (**26**, **27**, **33**, **36**, **38**, **43**, **45**, **47**, **52**, **54**, **55**, **58**) showed similar potencies in the cell-based bioluminescence assay for proteasome inhibition as the parent compounds **1** and **2**, whereas compounds **35**·HCl, **48**, **49**, **50**, **51**, **60**, and **61** were more potent. The side chain and specially the exo α,β-unsaturated ketone were essential for the activity. The enone system on ring A conferred some activity. The protection of the alcohol at C-26 increased the activity, presumably by increasing the cellular stability. A variety of groups such as ester, carbamate, and ether were acceptable substituents. At position C-22, there was no or little difference between a hydroxyl and an acetate group, but larger groups like glycine or chloroacetate enhanced the activity. Most derivatives did not inhibit the cellular proteasomal CTL or PGPH catalytic activities, suggesting that neoboutomellerone derivatives might interferes with components of the proteasome pathway other than the protein degradation activity.

5. Experimental section

5.1. Chemistry

All solvents, including anhydrous solvents and reagents, were purchased from Acros or Sigma–Aldrich. The NMR spectra were recorded on a Bruker Avance II spectrometer equipped with a ^{13}C cryoprobe at 500 MHz for ^{1}H and 125 MHz for ^{13}C. Chemical shifts (δ) were measured relative to acetonitrile-d_3, or DMSO-d_6 and are expressed in ppm. Coupling constants (J) are expressed in Hertz. High-resolution mass spectrometry (MS) was used to confirm molecular formulas and was performed on a Bruker MicroTOF. Compounds were analyzed on an LC/MS/UV/ESL system (*Autopurify*™, Waters Corporation). The system was controlled by MassLynx 4.1 and data were processed with OpenLynx. The HPLC system consisted of a 2767 Autoinjector, a 2525 binary gradient module, a Photodiode Array (PDA) 2996 detector, and a ZQ2000 single-quadrupole MS equipped with a Z-spray electrospray source. Positive and negative modes were used simultaneously. MS instruments settings were as follows: capillary voltage 3.2 kV; cone voltage 25 V; source temperature 120 °C; desolvation temperature 350 °C; desolvation gas flow 400 L/h; cone gas flow 50 L/h. The multiplier was set at 650. The wavelength range used with the PDA2996 was 210–500 nm. The evaporative light-scattering detector was a PL-ELS-1000 (Polymer Laboratories). A *Synergi* Six-Column Selector (Phenomenex) enabled the use of six different columns. Three analytical columns were used for purity assessments and the determination of compound solubility: SunFire C18, 3.5 μm, 4.6 × 50 mm; Atlantis dC18, 3 μm, 4.6 × 50; and XBridge Shield C18, 3.5 μm, 4.6 × 50 mm (all from Waters Corporation). Each column was used with a 4.6 × 20 mm matching guard column. Water (MilliQ purified) and acetonitrile with 0.1% (v/v) formic acid were used as solvents A and B, respectively. Solvent gradients of 5 min at a flow rate of 2 mL/min were applied after a 0.5 min delay following the injection: SunFire 5–100% B; Atlantis and Xbridge Shield 0–100% B. Washing and equilibration times were 0.5 min and 0.75 min, respectively, for a total time of 7 min.

The extraction, isolation, and characterization of the natural products **1**-**15** are described elsewhere.[1,26]

5.1.1. Preparation of compound 35 and its hydrochloride salt

To a stirred solution of DCC (3.5 mL, 0.1 mol/L, 0.35 mmol, 2 equiv) in CH_2Cl_2 were added *N*-dimethylglycine (36 mg, 0.35 mmol, 2 equiv), DMAP (2 mg, 0.018 mmol, 0.1 equiv), and **1** (100 mg, 0.18 mmol, 1 equiv). The mixture was stirred for 24 h at room temperature. Then, the mixture was filtered and the filtrate was washed with H_2O and dried over Na_2SO_4. The product was purified by chromatography on silica gel (elution: cyclohexane/EtOAc, 3:7) to obtain the amine **35** as a translucent oil (26 mg, 23%). Hydrochloric acid (3 mL, 0.1 M) was added to **35** (26 mg, 0.0407 mmol), and the mixture was stirred at room temperature for 1 h. The mixture was dried under high vacuum to furnish the hydrochloride as a white light solid (28 mg, quant.).

35: ^{1}H NMR (500 MHz, CD_3CN) δ = 6.94 (1H, d, J = 10.1 Hz, H-1), 6.12 (1H, s, H-24a), 5.97 (1H, s, H-24a), 5.90 (1H, d, J = 9.8 Hz, H-2), 5.53 (1H, d, J = 2.1 Hz, H-22), 5.09 (1H, td, J = 7.6, 4.6 Hz, H-16), 4.00–4.15 (2H, m, 2 H-26), 3.10 (2H, s, H-26b), 3.02 (1H, sxt, J = 6.9 Hz, H-25), 2.58 (1H, dqd, J = 11.0, 7.0, 2.1 Hz, H-20), 2.30

(1H, dd, *J* = 11.0, 7.3 Hz, H-17), 2.26 (6H, s, H-26e, H-26d), 2.13–2.22 (2H, m, H-4, H-15), 2.09 (3H, s, H-22b), 2.03 (3H, s, H-16b), 1.95–2.06 (3H, m, H-5, H-8, H-11), 1.63–1.77 (3H, m, H-6, 2H-12), 1.52–1.60 (1H, m, H-11), 1.41–1.50 (1H, m, H-7), 1.37 (1H, dd, *J* = 14.2, 3.8 Hz, H-15), 1.24 (1H, d, *J* = 4.3 Hz, H-19), 1.19 (3H, s, H-18), 1.15–1.22 (1H, m, H-7), 1.07 (3H, d, *J* = 7.3 Hz, H-27), 1.02 (3H, d, *J* = 6.7 Hz, H-28), 0.95 (3H, s, H-29), 0.89–0.99 (1H, m, H-6), 0.85 (3H, d, *J* = 7.0 Hz, H-21), 0.58 (1H, d, *J* = 4.6 Hz, H-19).^{13}C NMR (126 MHz, CD_3CN) δ = 202.4 (C-3), 198.8 (C-23), 171.6 (C-22a), 171.5 (C-26a), 171.2 (C-16a), 155.5 (C-1), 149.1 (C-24), 128.4 (C-2), 125.8 (C-24a), 78.3 (C-22), 76.7 (C-16), 67.7 (C-26), 60.7 (C-26b), 51.3 (C-17), 48.4 (C-14), 47.6 (C-4), 46.9 (C-13), 46.7 (C-15), 45.3 (C-26d, 26e), 45.2 (C-8), 43.6 (C-5), 34.9 (C-25), 33.3 (C-20), 33.0 (C-12), 32.9 (C-10), 28.1 (C-11), 27.7 (C-19), 27.6, 27.2 (C-9), 24.3 (C-7, 6), 22.1 (C-16b), 20.9 (C-22b), 20.0 (C-29), 18.3 (C-18), 17.3 (C-27), 13.3 (C-21), 11.3 (C-28). ESI-MS *m/z*: 654.4 $[M+H]^+$, 676.4 $[M+Na]^+$. Purity (UV) = 100%, purity (ESLD) = 99%.

35·HCl: ^{1}H NMR (500 MHz, D_2O) δ = 7.19 (1H, d, *J* = 9.8 Hz, H-1), 6.40 (1H, br s, H-24a), 6.28 (1H, s, H-24a), 5.94–6.06 (1H, m, *J* = 5.5 Hz, H-2), 5.67 (1H, br s, H-22), 5.08 (1H, br s, H-16), 4.36 (1H, dd, *J* = 10.8, 5.6 Hz, H-26), 4.23 (1H, dd, *J* = 11.0, 7.9 Hz, H-26), 4.11 (1H, d, *J* = 17.1 Hz, H-26b), 4.05 (1H, d, *J* = 17.4 Hz, H-26b), 3.09–3.21 (1H, m, H-25), 2.67 (1H, br s, H-20), 2.29–2.42 (2H, m, H-4, H-17), 2.21 (3H, s, H-22b), 2.18–2.28 (1H, m, H-15), 2.15 (3H, s, H-16b), 1.95–2.12 (3H, m, H-5, H-8, 11), 1.71 (3H, br s, H-6, 2H-12), 1.64 (1H, br s, H-11), 1.40–1.54 (2H, m, H-7, H-15), 1.37 (1H, br s, H-19), 1.19 (3H, br s, H-18), 1.15–1.27 (1H, m, H-7), 1.12 (3H, d, *J* = 7.0 Hz, H-27), 1.04 (3H, d, *J* = 6.7 Hz, H-28), 0.91–1.00 (1H, m, H-6), 0.95 (3H, br s, H-29), 0.88 (3H, d, *J* = 6.4 Hz, H-21), 0.64 (1H, br s, H-19).^{13}C NMR (126 MHz, D_2O) δ = 174.8 (C-16a, 22a), 167.3 (C-26a), 160.6 (C-1), 147.9 (C-24), 129.2 (C-24a), 127.4 (C-2), 79.4 (C-22), 78.2 (C-16), 70.8 (C-26), 58.0 (C-26b), 51.1 (C-17), 48.2 (C-14), 47.4 (C-4), 46.7 (C-13), 46.1 (C-15), 44.7 (C-26e, 26d), 44.2 (C-8), 42.8 (C-5), 33.7 (C-20), 33.4 (C-10), 33.3 (C-25), 32.6 (C-12), 27.8 (C-11), 27.8 (C-9), 27.2 (C-19), 23.8 (C-6), 23.6 (C-7), 22.3 (C-16b), 21.0 (C-22b), 19.8 (C-29), 17.9 (C-18), 16.7 (C-27), 13.3 (C-21), 11.1 (C-28). ESI-MS *m/z*: 676.7 $[M+Na]^+$. Purity (UV) = 96%, purity (ESLD) = 97%.

5.1.2. Preparation of compound 48

To a stirred solution of **1** (100 mg, 0.176 mmol) in CH_2Cl_2 (1 mL) were added DMAP (11 mg, 0.080 mmol, 0.5 equiv), dimethylaminophenyl isocyanate (43 mg, 0.264 mmol, 1.5 equiv), and triethylamine (40 μL, 0.264 mmol, 1.5 equiv). The mixture was stirred at room temperature for 24 h and one more equivalent of dimethylaminophenyl isocyanate (29 mg, 0.176 mmol) was added. After 18 h, the mixture was diluted with EtOAc and the organic solution was washed successively with 4% HCl, $NaHCO_3$, and brine. The organic solution was dried over $MgSO_4$ and concentrated under vacuum. The residue was purified by chromatography on silica gel (elution: cyclohexane/EtOAc, 6:4) to give **48** as a white solid (101 mg, 79%). ^{1}H NMR (500 MHz, CD_3CN) δ = 7.32 (1H, br s, H-44), 7.20 (2H, d, *J* = 8.2 Hz, H-26c), 6.94 (1H, d, *J* = 9.8 Hz, H-1), 6.71 (2H, d, *J* = 9.1 Hz, H-26d), 6.13 (1H, s, H-24aa), 5.99 (1H, s, H-24ab), 5.90 (1H, d, *J* = 10.1 Hz, H-2), 5.55 (1H, d, *J* = 2.1 Hz, H-22), 5.10 (1H, td, *J* = 7.6 Hz, *J* = 4.3 Hz, H-16), 4.11 (1H, dd, *J* = 10.6 Hz, *J* = 7.1 Hz, H-26), 4.03 (1H, dd, *J* = 10.6 Hz, *J* = 6.3 Hz, H-26), 3.05 (1H, sxt, *J* = 6.9 Hz, H-25), 2.86 (6H, s, H-26f), 2.56–2.65 (1H, m, H-20), 2.30 (1H, dd, *J* = 11.0 Hz, *J* = 7.6 Hz, H-17), 2.14–2.22 (2H, m, H-4, H-15), 2.10 (3H, s, H-22b), 2.03 (3H, s, H-16b), 1.96–2.01 (3H, m, H-11, H-8a, H-5a), 1.62–1.76 (3H, m, H-6, 2H-12), 1.56 (1H, qd, *J* = 8.7 Hz, *J* = 6.3 Hz, H-11), 1.41–1.49 (1H, m, H-7), 1.37 (1H, dd, *J* = 13.9 Hz, *J* = 3.8 Hz, H-15), 1.24 (1H, d, *J* = 4.3 Hz, H-19), 1.20–1.23 (1H, m, H-7), 1.18 (3H, s, H-18), 1.10 (3H, d, *J* = 7.0 Hz, H-27), 1.03 (3H, d, *J* = 7.0 Hz, H-28), 0.96 (3H, s, H-29), 0.88–0.94 (1H, m, H-6), 0.86 (3H, d, *J* = 6.7 Hz, H-21), 0.57 (1H, d, *J* = 4.3 Hz, H-19). ^{13}C NMR (126 MHz, CD_3CN) δ = 202.4 (C-3), 198.9 (C-23), 171.8 (C-22a), 171.3 (C-16a), 155.5 (C-1), 155.1 (C-26a), 149.4 (C-24), 148.8 (C-26e), 129.4 (C-26b), 128.5 (C-2), 125.5 (C-24a), 121.8 (C-26c, 26c), 114.2 (C-26d, 26d), 78.5 (C-22), 76.7 (C-16), 68.3 (C-26), 51.4 (C-17), 48.4 (C-14), 47.7 (C-4), 46.9 (C-13), 46.8 (C-15), 45.2 (C-8), 43.6 (C-5), 41.3 (C-26f, 26f), 35.3 (C-25), 33.4 (C-20), 33.0 (C-12), 33.0 (C-10), 28.1 (C-11), 27.7 (C-19), 27.3 (C-9), 24.3 (C-7, 6), 22.1 (C-16b), 21.0 (C-22b), 20.0 (C-29), 18.3 (C-18), 17.2 (C-27),13.4 (C-21), 11.3 (C-28). ESI-MS *m/z*: 731.5 $[M+H]^+$, 753.4 $[M+Na]^+$. Purity (UV) = 100%, purity (ESLD) = 99%.

5.1.3. Preparation of compound 49

To a stirred solution of **2** (100 mg, 0.19 mmol) in CH_2Cl_2 (1 mL) were added DMAP (12 mg, 0.095 mmol, 0.5 equiv), dimethylaminophenyl isocyanate (76 mg, 0.475 mmol, 2.5 equiv), and triethylamine (40 μL, 0.285 mmol, 1.5 equiv). After 22 h, the mixture was diluted with EtOAc, and the organic solution was washed successively with 4% HCl, $NaHCO_3$, and brine. The organic solution was dried over $MgSO_4$ and concentrated under vacuum. The residue was purified by chromatography on silica gel (elution: cyclohexane/EtOAc, 7:3) to give **49** as a white solid (79 mg, 60%). ^{1}H NMR (500 MHz, CD_3CN) δ = 7.32 (1H, br s, H-41a), 7.19 (2H, br s, H-26c), 6.93 (1H, d, *J* = 10.1 Hz, H-1), 6.71 (2H, d, *J* = 8.9 Hz, H-26d), 6.19 (1H, s, H-24aa), 6.09 (1H, d, *J* = 16.2 Hz, H-24ab), 5.89 (1H, d, *J* = 9.8 Hz, H-2), 5.21 (1H, td, *J* = 7.5 Hz, *J* = 4.9 Hz, H-16), 4.72 (1H, dd, *J* = 6.1 Hz, *J* = 1.5 Hz, H-22), 4.14 (1H, dd, *J* = 10.7 Hz, *J* = 6.5 Hz, H-26), 4.08 (1H, dd, *J* = 10.6 Hz, *J* = 6.3 Hz, H-26), 3.53 (1H, d, *J* = 6.2 Hz, H-30), 3.08 (1H, sxt, *J* = 6.9 Hz, H-25), 2.86 (6H, s, H-26f), 2.40–2.51 (2H, m, H-20, H-17), 2.14–2.25 (2H, m, H-4, H-15), 2.03–2.07 (1H, m, H-11), 2.02 (3H, s, H-16b), 1.97–2.01 (2H, m, H-8a, H-5a), 1.60–1.72 (3H, m, H-6, 2H-12), 1.54 (1H, qd, *J* = 8.8 Hz, *J* = 5.9 Hz, H-11), 1.42–1.48 (1H, m, H-7), 1.38 (1H, dd, *J* = 14.6 Hz, *J* = 4.5 Hz, H-15), 1.24 (1H, d, *J* = 4.6 Hz, H-19), 1.19–1.21 (1H, m, H-7), 1.17 (3H, s, H-18), 1.13 (3H, d, *J* = 7.0 Hz, H-27), 1.03 (3H, d, *J* = 7.0 Hz, H-28), 0.96 (3H, s, H-29), 0.90–0.94 (1H, m, H-6), 0.65 (3H, d, *J* = 6.1 Hz, H-21), 0.57 (1H, d, *J* = 4.3 Hz, H-19).^{13}C NMR (126 MHz, CD_3CN) δ = 205.0 (C-23), 202.4 (C-3), 171.3 (C-16a), 155.6 (C-26a, 1), 155.2, 148.8 (C-26e), 148.2 (C-24), 129.5 (C-26b), 128.4 (C-2), 127.3 (C-24a), 121.9 (C-26c, 26c), 114.2 (C-26d, 26d), 77.3 (C-22), 75.9 (C-16), 68.3 (C-26), 51.5 (C-17), 48.4 (C-14), 47.7 (C-4), 46.9 (C-15), 46.7 (C-13), 45.4 (C-8), 43.7 (C-5), 41.3 (C-26f, 26f), 36.4 (C-25), 35.2 (C-20), 33.2 (C-12), 33.0 (C-10), 28.2 (C-11), 27.8 (C-19), 27.3 (C-9), 24.4 (C-7, 6), 22.1 (C-16b), 20.2 (C-29), 18.6 (C-18), 17.3 (C-27), 12.4 (C-21), 11.3 (C-28). ESI-MS *m/z*: 711.6 $[M+Na]^+$, 1400.1 $[2M+Na]^+$. Purity (UV) = purity (ESLD) = 100%.

5.1.4. Preparation of compound 51

To a stirred solution of **2** (200 mg, 0.38 mmol) in CH_3CN (1 mL) were added K_2CO_3 (210 mg, 1.521 mmol, 4 equiv), 4-methylpiperazine carbonyl chloride hydrochloride (151 mg, 0.76 mmol, 2 equiv), and TBAB (12 mg, 0.0352 mmol, 0.1 equiv). After 28 h, the mixture was filtered and concentrated under vacuum. The residue was purified by chromatography on silica gel (elution: DCM/MeOH, 98:2 to 95:5). Two products were isolated as white solids: the expected carbamate (87 mg, 35%) and starting material **2** (96 mg, 48%). Hydrochloric acid (3 mL, 0.1 M) was added to the carbamate (69 mg, 0.106 mmol) and the mixture was stirred at room temperature for 1 h. The mixture was dried under high vacuum to furnish the hydrochloride **51**·HCl as a white fluffy solid (66 mg, 90%). **51**: ^{1}H NMR (500 MHz, DMSO-d_6) δ = 10.30 (1H, br s, H-26e), 6.97 (1H, d, *J* = 10.1 Hz, H-1), 6.10 (1H, s, H-24aa), 6.03 (1H, s, H-24ab), 5.90 (1H, d, *J* = 10.1 Hz, H-2), 5.15 (1H, dd, *J* = 7.4 Hz, *J* = 4.5 Hz, H-16), 4.79 (1H, d, *J* = 6.1 Hz, H-30), 4.60 (1H, d, *J* = 3.7 Hz, H-22),

3.96–4.08 (4H, m, H-26, 26b), 3.34–3.42 (2H, m, H-26c), 3.09–3.21 (2H, m, H-26b), 2.89–3.03 (3H, m, H-25, 26c), 2.76 (3H, br s, H-26d), 2.29–2.39 (2H, m, H-20, H-17), 2.07–2.16 (2H, m, H-4, H-15), 2.02 (3H, s, H-16b), 1.93–2.00 (2H, m, H-11, H-8a), 1.90 (1H, td, J = 12.5 Hz, J = 4.4 Hz, H-5a), 1.50–1.64 (4H, m, 2H-12, H-6, H-11), 1.37–1.44 (1H, m, H-7), 1.27–1.34 (1H, m, H-15), 1.24 (1H, d, J = 4.3 Hz, H-19), 1.13–1.19 (1H, m, H-7), 1.11 (3H, s, H-18), 1.05 (3H, d, J = 7.0 Hz, H-27), 0.98 (3H, d, J = 6.7 Hz, H-28), 0.93 (2H, t, J = 7.3 Hz, H-6), 0.90 (3H, s, H-29), 0.64 (3H, d, J = 6.4 Hz, H-21), 0.55–0.59 (1H, m, H-19). ^{13}C NMR (126 MHz, DMSO-d_6) δ = 203.5 (C-23), 200.7 (C-3), 170.0 (C-16a), 154.8 (C-1), 154.0 (C-26a), 147.4 (C-24), 127.3 (C-2), 125.0 (C-24a), 75.3 (C-16), 74.2 (C-22), 68.5 (C-26), 51.9 (C-26c), 49.6 (C-17), 47.0 (C-14), 46.2 (C-4), 45.4 (C-15), 45.2 (C-13), 43.3 (C-8), 42.0 (C-26d, 5), 40.5 (C-26b), 34.4 (C-20), 33.2 (C-25), 31.8 (C-10, 12), 31.6, 26.7 (C-11), 26.3 (C-9), 26.0 (C-19), 22.9 (C-6), 22.8 (C-7), 21.5 (C-16b), 19.2 (C-29), 17.7 (C-18), 16.6 (C-27), 11.8 (C-21), 10.8 (C-28). ESI-MS m/z: 653.4 $[M+H]^+$, 675.4 $[M+Na]^+$. Purity (UV) = 95%, purity (ESLD) = 100%.

5.1.5. Preparation of compound 58

To a stirred solution of **2** (200 mg, 0.38 mmol) in CH_2Cl_2 (16 mL) at 0 °C was added lutidine (45 μL, 0.38 mmol, 1 equiv), acetic anhydride (27 μL, 0.285 mmol, 0.75 equiv), and DMAP (5 mg, 0.038 mmol, 0.1 equiv). After 1 h, the mixture was treated with water. The organic phase was successively washed with water, $CuSO_4$, water, and brine, dried over Na_2SO_4 and concentrated under vacuum. The product was purified on silica gel chromatography (elution: cyclohexane/EtOAc, 80:20) to obtain **58** (94.6 mg, 58 %) as a white solid. ^{1}H NMR (500 MHz, CD_3CN) δ = 6.94 (1H, d, J = 10.1 Hz, H-1), 6.17 (1H, s, H-24aa), 6.04 (1H, d, J = 0.6 Hz, H-24ab), 5.89 (1H, d, J = 9.8 Hz, H-2), 5.20 (1H, td, J = 7.5 Hz, J = 4.6 Hz, H-16), 4.72 (1H, dd, J = 6.3 Hz, J = 1.7 Hz, H-22), 3.99–4.10 (2H, m, H-26), 3.52 (1H, d, J = 6.1 Hz, H-30), 3.05 (1H, sxt, J = 6.8 Hz, H-25), 2.37–2.49 (2H, m, H-20, 17), 2.22 (1H, dd, J = 13.9 Hz, J = 7.8 Hz, H-15), 2.15–2.21 (1H, m, H-4), 2.03 (3H, s, H-16b), 1.98–2.07 (2H, m, H-8, H-11), 1.97 (3H, s, H-26b), 1.94–1.98 (1H, m, H-5), 1.60–1.74 (3H, m, H-6, 2H-12), 1.50–1.59 (1H, m, H-11), 1.41–1.49 (1H, m, H-7), 1.38 (1H, dd, J = 13.6 Hz, J = 4.4 Hz, H-15), 1.24 (1H, d, J = 4.6 Hz, H-19), 1.17–1.23 (1H, m, H-7), 1.17 (3H, s, H-18), 1.09 (3H, d, J = 7.0 Hz, H-27), 1.03 (3H, d, J = 7.0 Hz, H-28), 0.96 (3H, s, H-29), 0.94 (1H, qd, J = 12.8 Hz, J = 4.0 Hz, H-6), 0.64 (3H, d, J = 6.1 Hz, H-21), 0.57 (1H, d, J = 4.3 Hz, H-19). ^{13}C RMN (126 MHz, CD_3CN) δ = 205.0 (C-23), 202.4 (C-3), 171.6 (C-26a), 171.3 (C-16a), 155.6 (C-1), 148.0 (C-24), 128.4 (C-2), 127.4 (C-24a), 77.3 (C-16), 75.8 (C-22), 68.0 (C-26), 51.4 (C-17), 48.3 (C-14), 47.6 (C-4), 46.9 (C-15), 46.7 (C-13), 45.3 (C-8), 43.6 (C-5), 36.4 (C-20), 34.7 (C-25), 33.1 (C-12), 32.9 (C-10), 28.1 (C-11), 27.7 (C-19), 27.2 (C-9), 24.3 (C-6), 24.3 (C-7), 22.1 (C-16b), 21.1 (C-26b), 20.1 (C-29), 18.5 (C-18), 17.3 (C-27), 12.3 (C-21), 11.3 (C-28). ESI-MS m/z: 591.3 $[M+Na]^+$, 1159.7 $[2M+Na]^+$. Purity (UV) = purity (ESLD) = 100%.

5.1.6. Preparation of compound 60

To a stirred solution of **57** (64 mg, 0.113 mmol) in CH_2Cl_2 (2 mL) were added DCC (1.8 mL, 0.180 mmol, c = 0.1 mol/L, 1.6 equiv), DMAP (1.4 mg, 0.011 mmol, 0.1 equiv), and chloroacetic acid (16 mg, 0.169 mmol, 1.5 equiv). The mixture was stirred for 1 h at room temperature. Purification by flash chromatography (elution: cyclohexane/EtOAc, 90:10 to 50:50) gave **59** (70 mg, 97%) as a white solid. ^{1}H NMR (500 MHz, CD_3CN) δ = 6.94 (1H, d, J = 10.0 Hz, H-1), 6.14 (1H, s, H-24aa), 6.01 (1H, s, H-24ab), 5.90 (1H, d, J = 10.0 Hz, H-2), 5.59 (1H, d, J = 2.1 Hz, H-22), 5.10 (1H, td, J = 7.7 Hz, J = 4.4 Hz, H-16), 4.33 (1H, d, J = 15.1 Hz, H-22b), 4.25 (1H, d, J = 15.1 Hz, H-22b), 3.99–4.08 (3H, m, 2H-26), 3.02 (1H, sxt, J = 7.0 Hz, H-25), 2.63 (1H, dtd, J = 13.7 Hz, J = 7.1 Hz, J = 2.3 Hz, H-20), 2.32 (1H, dd, J = 11.1 Hz, J = 7.6 Hz, H-17), 2.17–2.21 (1H, m, H-4), 2.16 (1H, m, H-15), 2.05–2.10 (1H, m, H-11), 2.04 (3H, s, H-16b), 1.98–2.03 (2H, m, H-5a, H-8a), 1.97 (4H, s, H-26b), 1.63–1.75 (4H, m, 2H-12, H-6), 1.53–1.61 (1H, m, H-11), 1.41–1.49 (1H, m, H-7), 1.38 (1H, dd, J = 13.8 Hz, J = 4.2 Hz, H-15), 1.25 (1H, d, J = 4.4 Hz, H-19), 1.19–1.21 (1H, m, H-7), 1.19 (3H, s, H-18), 1.08 (4H, d, J = 7.0 Hz, H-28), 1.02 (3H, d, J = 6.7 Hz, H-27), 0.95 (3H, s, H-29), 0.90–0.94 (1H, m, H-6), 0.86 (3H, d, J = 6.9 Hz, H-21), 0.57 (1H, d, J = 4.4 Hz, H-19). ^{13}C NMR (126 MHz, CD_3CN) δ = 202.4 (C-3), 197.7 (C-23), 171.8 (C-26a), 171.2 (C-16a), 168.2 (C-22a), 155.5 (C-1), 148.9 (C-24), 128.4 (C-2), 126.4 (C-24a), 80.0 (C-22), 76.7 (C-16), 67.8 (C-26), 51.1 (C-17), 48.4 (C-14), 47.6 (C-4), 46.9 (C-13), 46.7 (C-15), 45.1 (C-8), 43.5 (C-5), 42.1 (C-22b), 34.9 (C-25), 34.5 (C-10), 33.6 (C-20), 32.9 (C-12), 28.1 (C-11), 27.6 (C-19), 27.2 (C-9), 24.3 (C-6), 24.2 (C-7), 22.1 (C-16b), 21.1 (C-26b), 19.9 (C-29), 18.2 (C-18), 17.2 (C-27), 13.2 (C-21), 11.3 (C-28). ESI-MS m/z: 667.3 $[M+Na]^+$, 1311.7 $[2M+Na]^+$. Purity (UV) = 100%, purity (ESLD) = 100%.

5.1.7. Preparation of compound 61

To a stirred solution of **58** (66 mg 0.118 mmol) in CH_2Cl_2 (2 mL) were added DCC (1.8 mL, 0.188 mmol, c = 0.1 mol/L, 1.6 equiv), DMAP (1.5 mg, 0.012 mmol, 0.1 equiv), and *N*-Boc-glycine (31 mg, 0.176 mmol, 1.5 equiv). The mixture was stirred for 3 h at room temperature. Purification by flash chromatography (elution: cyclohexane/EtOAc, 90:10 to 50:50) gave **61** (44 mg, 52%) as a colorless oil. ^{1}H NMR (500 MHz, CD_3CN) δ = 6.94 (1H, d, J = 9.8 Hz, H-1), 6.12 (1H, s, H-24aa), 5.97 (1H, s, H-24ab), 5.90 (1H, d, J = 10.1 Hz, H-2), 5.62 (1H, br s, H-22c), 5.59 (1H, d, J = 2.1 Hz, H-22), 5.08 (1H, td, J = 7.6 Hz, J = 4.6 Hz, H-16), 4.04 (1H, dd, J = 10.8 Hz, J = 6.4 Hz, H-26), 4.00 (1H, dd, J = 10.8 Hz, J = 6.4 Hz, H-26), 3.94 (1H, dd, J = 17.7 Hz, J = 6.4 Hz, H-22b), 3.82 (1H, dd, J = 17.7 Hz, J = 6.2 Hz, H-22b), 3.01 (1H, sxt, J = 6.9 Hz, H-25), 2.61 (1H, dtd, J = 13.9 Hz, J = 6.9 Hz, J = 2.2 Hz, H-20), 2.30 (1H, dd, J = 11.0 Hz, J = 7.6 Hz, H-17), 2.19 (1H, d, J = 6.7 Hz, H-4, H-15), 2.15 (3H, s, H-26b), 2.04–2.06 (1H, m, H-11), 2.03 (3H, s, H-16b), 2.00 (2H, dd, J = 6.9 Hz, J = 4.1 Hz, H-8a, H-5a), 1.64–1.75 (3H, m, 2H-12, H-6), 1.52–1.61 (1H, m, H-11), 1.43–1.47 (1H, m, H-7), 1.41 (9H, s, H-22f, 22f, 22f), 1.37 (1H, dd, J = 13.9 Hz, J = 4.1 Hz, H-15), 1.27 (1H, d, J = 3.7 Hz, H-7), 1.25 (1H, d, J = 4.6 Hz, H-19), 1.18 (3H, s, H-18), 1.07 (3H, d, J = 7.0 Hz, H-27), 1.02 (3H, d, J = 7.0 Hz, H-28), 0.97 (3H, s, H-29), 0.91–0.96 (1H, m, H-6), 0.85 (3H, d, J = 6.7 Hz, H-21), 0.57 (1H, d, J = 4.3 Hz, H-19). ^{13}C NMR (126 MHz, CD_3CN) δ = 202.4 (C-3), 198.4 (C-23), 171.6 (C-26a), 171.4 (C-22a), 171.2 (C-16a), 156.9 (C-22d), 155.5 (C-1), 149.0 (C-24), 128.4 (C-2), 126.0 (C-24a), 80.0 (C-22e), 78.8 (C-22), 76.7 (C-16), 67.8 (C-26), 51.2 (C-17), 48.4 (C-14), 47.6 (C-4), 46.9 (C-13), 46.7 (C-15), 45.0 (C-8), 43.5 (C-5), 43.0 (C-22b), 34.9 (C-25), 33.6 (C-20), 33.0 (C-12), 33.0 (C-10), 28.6 (C-22f), 28.1 (C-11), 27.5 (C-19), 27.2 (C-9), 24.2 (C-7), 24.2 (C-6), 22.1 (C-16b), 21.1 (C-26b), 20.0 (C-29), 18.2 (C-18), 17.3 (C-27), 13.3 (C-21), 11.3 (C-28). ESI-MS m/z: 748.4 $[M+Na]^+$, 1473.8 $[2M+Na]^+$. Purity (UV) = purity (ESLD) = 100%.

5.2. Purity assessment

Samples were dissolved in dimethyl sulfoxide (DMSO) at concentrations of 5 or 10 mM. Injection volumes were 5 and 10 μL. Mass spectrometric detection was used for molecular weight confirmation, whereas UV and ELSD data offered purity assessment.

5.3. Determination of compound solubility

Kinetic solubility was determined in Dubelcco's PBS at pH 7.2. Stock compound (2 μL, 10 mM in DMSO) was dispensed into a *MultiScreen Solubility Filter Plate* (Millipore). Next, 198 μL of Dubelcco's PBS were added. The plate was mixed with shaking on an orbital

plate shaker (900 rpm) at room temperature for 1.5 h. The solutions were filtered and 160 μL of filtrate were transferred to a 96-well plate then diluted with 40 μL of DMSO. DMSO (200 μL) was dispensed into the *MultiScreen Solubility Experiment Filter Plate* and the plate was mixed with shaking on an orbital plate shaker (900 rpm) at room temperature for 15 min. Solutions were then filtered into a 96-well plate. Solutions were analyzed by HPLC/MS/UV. Quantitation was carried out against a 100 μM-calibration standard with the wavelength 254 nm. The solubility was calculated using the following formula: solubility (μM) = [((AUCfiltrate × 1.25) × 100)/AUCstandard]. The % of the recovery was calculated using the following formula: % recovery = [((AUCfiltrate × 1.25) + AUCDMSO filtrate)/AUCstandard] × 100.

5.4. Biology

5.4.1. Cell lines and culture

The human DLD-1 colon cancer cells were purchased from the American Type Cell Culture Collection (ATCC). The generation of stably transfected DLD-1 4Ub-Luc cell line was described elsewhere.[24] The engineered DLD-1 4Ub-Luc cells were cultured in MEM medium (Gibco) supplemented with 5% heat-inactivated FBS (Gibco), 2 mM glutamine, 1.25 μg/mL fungizone (Gibco), and 50 μg/mL penicillin/streptomycin (CambreX). WM-266-4 (metastatic melanoma) cells were purchased from ATCC. WM-266-4 cells were cultured in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, fungizone (1.25 μg/mL), and penicillin–streptomycin (100 U and 100 μg/mL, respectively). Cell culture supplies were obtained from Sigma. Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2 and maintained using standard cell culture techniques.

5.4.2. Measurement of bioluminescence from DLD-1 4Ub-Luc

DLD-1 4Ub-Luc cells were seeded at 10^4 cells/well in white 96-well plates and incubated with indicated concentrations of test compounds or solvent for 8 h. Luciferase activity in cell lysates was determined with a luciferase assay kit (Promega) and luminescence was read using a LB 960 Centro luminometer (Berthold). The results were expressed as an induction factor (IF) which was the ratio of accumulation of the proteasome targeted reporter protein in treated cells versus untreated cells. In order to avoid variation of the cells conditions, results were compared to control (epoxomicin, at 10^{-7} M) run in parallel experiment, which induced a relative induction factor of 100.

5.4.3. Inhibition of catalytic activity in DLD-1 4Ub-Luc cells

DLD-1 4Ub-Luc cells were seeded at 10^4 cells/well in 96-well plates and incubated with indicated concentrations of proteasome inhibitors or solvent for 6 h followed by an additional 30 min incubation in reaction buffer (30 mM Tris–HCl pH 7.5, 1 mM ATP, 5 mM $MgCl_2$, 5 mM NaF, 1 mM DTT, and 100 μM Na_3VO_4) containing 0.5% NP40 and either *Z*-Leu-Leu-Glu-AMC (PGPH) or Succinyl-Leu-Leu-Val-Tyr-AMC (CTL) at 100 μM. After addition of 200 μL of cold ethanol, fluorescence of released aminomethylcoumarin within cells was measured with a spectrofluorimeter, as reported above.

5.4.4. Cell proliferation assay

The antiproliferative activity of compounds was measured the ATPlite assay (Perkin Elmer). WM-266-4 cells (3×10^4 cells per ml) were seeded in 96-well plates, incubated for 24 h and treated with increasing concentrations of compounds. Cells were then incubated for 72 h at 37 °C under 5% CO_2. At the end of the experiment, cell viability was evaluated by determining the level of ATP released by viable cells. IC_{50} values were determined with curve fitting analysis (non linear regression model with a sigmoidal dose response, variable Hill slope coefficient), performed with the algorithm provided by the GraphPad Software. Two independent experiments were performed and results were expressed as average IC_{50} values (concentration of test compound that inhibits 50% of cell proliferation).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.11.066.

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41. Molecules are not always numbered as in Ref. 1 **1** and **2** are unchanged, **3** and **4** correspond to previous **5** and **6**, respectively; **5** was **11**; **6**, **7**, **8**, and **9** were **7**, **8**, **9**, and **10**, respectively; **10** was **12**; **11** and **12** were **16** and **17**, respectively; **13** and **14** were **21** and **22**, respectively; and **15** was **24**.
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