Novel autophagy modulators: Design and synthesis of (+)-epogymnolactam analogues and structure-activity relationship

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ABSTRACT

(+)-Epogymnolactam (**1**) was discovered as a novel autophagy inducer from a culture of *Gymnopus* sp. in our laboratory. To determine structure-activity relationships among (+)-epogymnolactam analogues comparing with cerulenin (**2**), we synthesized 5 analogues including (–)-epogymnolactam (**3**) having each different functional group, and 3 analogues with different side-chain lengths. Five analogues, **3**, **4**, **5**, **6**, and **7** did not significantly increase the ratio of LC3-II to LC3-I as an autophagy marker in NIH3T3 cells. These results suggest that presence and stereochemistry of (2*R*,3*S*)-epoxy group and cyclic *syn*-form (**1b**) of **1** are important for the activity as autophagy inducer. Hexyl analogue (**8**) as well as **1** having butyl side-chain dose-dependently increased the ratio of LC3-II to LC3-I, whereas octyl analogue (**9**) and **2** rather decreased the ratio. Decyl analogue (**10**) did not give a change in the ratio. Although **8** seemed to be an excellent autophagy inducer, it dose-dependently increased SQSTM1 (p62) as in the case of **2**, whereas **1** showed a slight dose-dependent decrease of p62 as an index of autophagic protein degradation. These observations suggest that **8** is an autophagy modulator with different molecular target from **1** or **2**.

1. Introduction

Autophagy inducer and autophagy inhibitor are important for understanding precise mechanisms of autophagy and developing therapeutic agents for such diseases as neurodegenerative diseases, cancer, and infectious diseases.[1] (+)-Epogymnolactam (**1**) was discovered as a novel autophagy inducer from a culture of *Gymnopus* sp. in our laboratory.[2] Total synthesis of **1** was also achieved to confirm its absolute structure[3] and to evaluate further biological properties compared to cerulenin (**2**),[4] an inhibitor of fatty acid synthesis. Synthetic **1** showed the same activity as natural **1** as autophagy inducer. Although the structure of **1** was similar to that of **2**, major tautomer of **1** seemed to be different from that of **2** as shown in Fig. 1 and Supplementary Table S1. In addition, a contrasting autophagic response was observed in NIH3T3 cells. Cerulenin (**2**) dose-dependently decreased LC3-II/LC3-I, a ratio of lipidate LC3 (LC3-II) to LC3-I as an autophagic marker, and increased p62/actin, a ratio of SQSTM1 (ubiquitin-binding protein p62) to actin, as an index of autophagic degradation.[5] Thus, cerulenin (**2**) was thought to be an autophagy inhibitor, which is discussed in more detail in the next section.

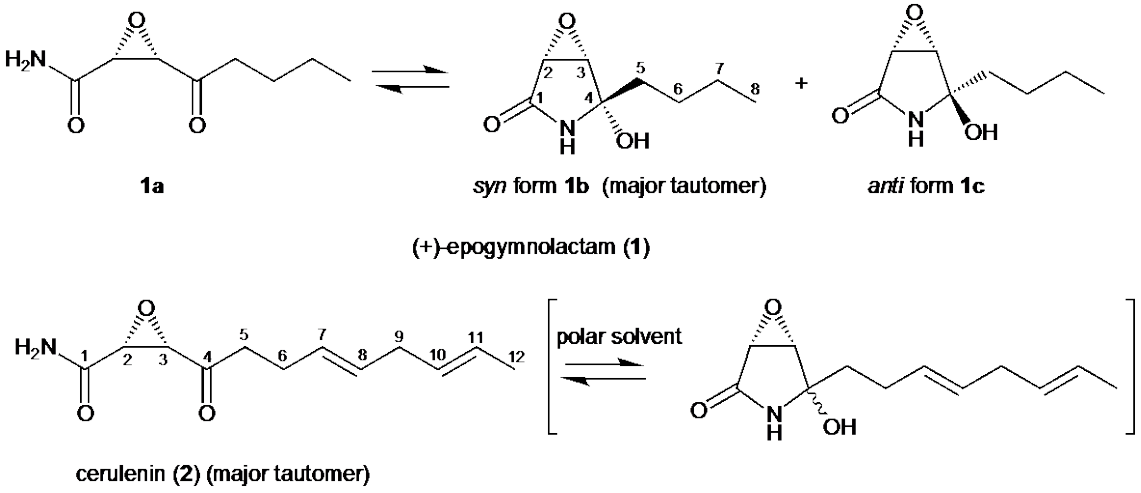


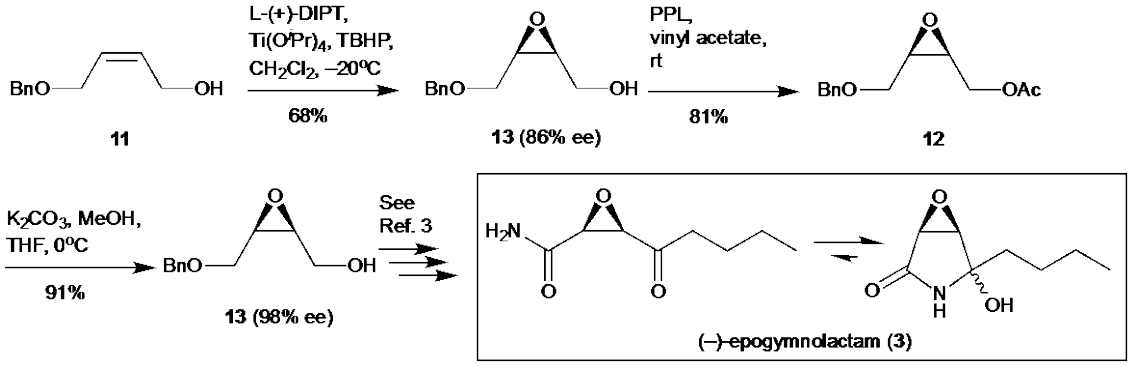
Fig. 1 Structures of (+)-epogymnolactam (**1**) and cerulenin (**2**). Both cyclic *syn*-form **1b** and *anti*-form **1c** as well as *keto* form **1a** were observed in CD_3OD or even in $CDCl_3$, whereas only *keto* form of **2** was observed in $CDCl_3$. Ring-chain tautomerism of **2** was observed in CD_3OD. The tautomeric ratio changed into different ratio with time.

We, therefore, designed and synthesized 8 analogues of **1**, and evaluated their autophagic activities compared to **1** and **2** in NIH3T3 cells. In this paper, we describe

syntheses and biological evaluations of (+)-epogymnolactam analogues (**3**, **4**, **5**, **6**, **7**, **8**, **9**, **10**) to ascertain the structural requirements of **1** as an autophagy-inducing agent. We also propose hexyl analogue **8** as a new autophagy modulator and discuss several suggestions on the relationship between autophagy and fatty acid synthesis in mammalian cells.

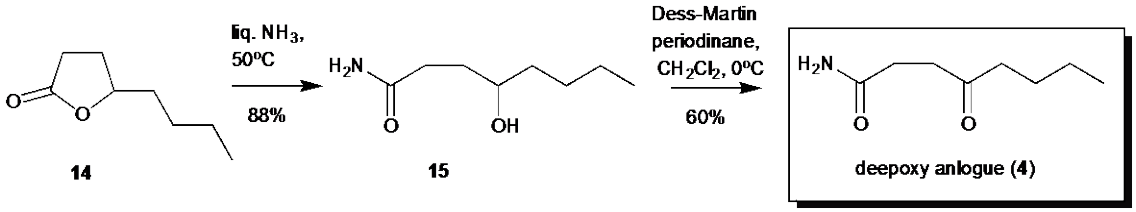
2. Results and Discussion

To determine the importance of the stereochemistry of **1** as autophagy inducer, we first synthesized (–)-epogymnolactam (**3**). Epoxy acetate **12** was obtained from *cis*-4-benzyloxy-2-buten-1-ol **11** in 98% ee using Sharpless epoxidation followed by lipase kinetic resolution. Hydrolysis of **12** afforded enantiopure epoxy alcohol **13**, and **3** was synthesized from **13** according to essentially the same method as the total synthesis of **1**[3] (Scheme 1).



Scheme 1 Synthesis of (–)-epogymnolactam (**3**).

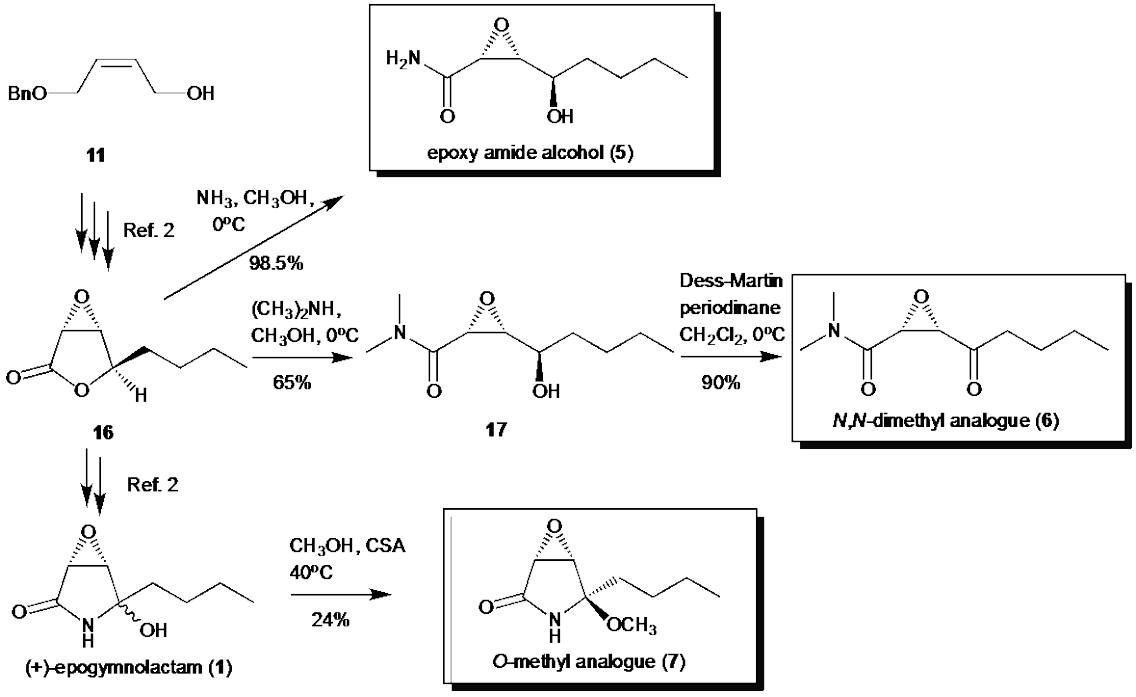
(+)-Epogymnolactam (**1**) existed mainly as a cyclic *syn*-form (**1b**) in CD_3OD and **1b** was formed even in $CDCl_3$, whereas NMR of **2** in $CDCl_3$ showed *keto* form as shown in Fig. 1 and Supplemental Table S1. To determine the importance of *syn* form (**1b**) for autophagy-inducing activity, three chain analogues (**4**, **5**, **6**) and *anti*-form analogue (**7**) were synthesized.



Scheme 2 Synthesis of deepoxy analogue (**4**).

Ammonolysis of γ-octalactone **14** with liquid ammonia in a pressure vessel[6] gave amide alcohol **15** in 88%. Dess-Martin oxidation of **15** afforded deepoxy analogue **4** as shown in Scheme 2. Deepoxy analogue **4** existed only as a chain form in $CDCl_3$ (Supplementary Figures S1 and S2) by releasing from the steric constraint by the epoxy ring.

Epoxy amide alcohol (**5**) was synthesized as the synthetic precursor[3] of **1** (Scheme 3). Treatment of lactone **16** with $(CH_3)_2NH$ gave **17** which was subjected to Dess-Martin oxidation to give *N,N*-dimethyl analogue (**6**) as indicated in Scheme 3. Chain forms of **5** and **6** were confirmed by their NMR in $CDCl_3$ as shown in Supplementary Figures S3, S4, S5, and S6.



Scheme 3 Synthesis of epoxy amide alcohol (**5**), *N,N*-dimethyl analogue (**6**), and *O*-methyl analogue (**7**).

Cyclic *O*-methyl analogue **7** was prepared by treating **1** with camphor sulfonic acid (CSA) in CH_3OH as shown in Scheme 3. In order to avoid steric and electronic repulsion, CH_3OH attacked from the opposite side of the epoxy group in the carbocation intermediate to give only *anti* form (**7**) as in the case of previously reported observation.[7] ^{1}H and ^{13}C NMR spectra of **7** indicated its cyclic *anti*-form as shown in Supplementary Figures S7 and S8.

All these analogues (**3**, **4**, **5**, **6** and **7**) did not enhance LC3-II/LC3-I, the ratios of LC3-II to LC3-I, and LC3-II/actin, the ratios of LC3-II to actin in NIH-3T3 cells as shown in Fig. 2. These observations suggest that the unknown molecular target of **1** recognizes the absolute configuration of the epoxy group and the cyclic structure of the *syn*-form (**1b**).

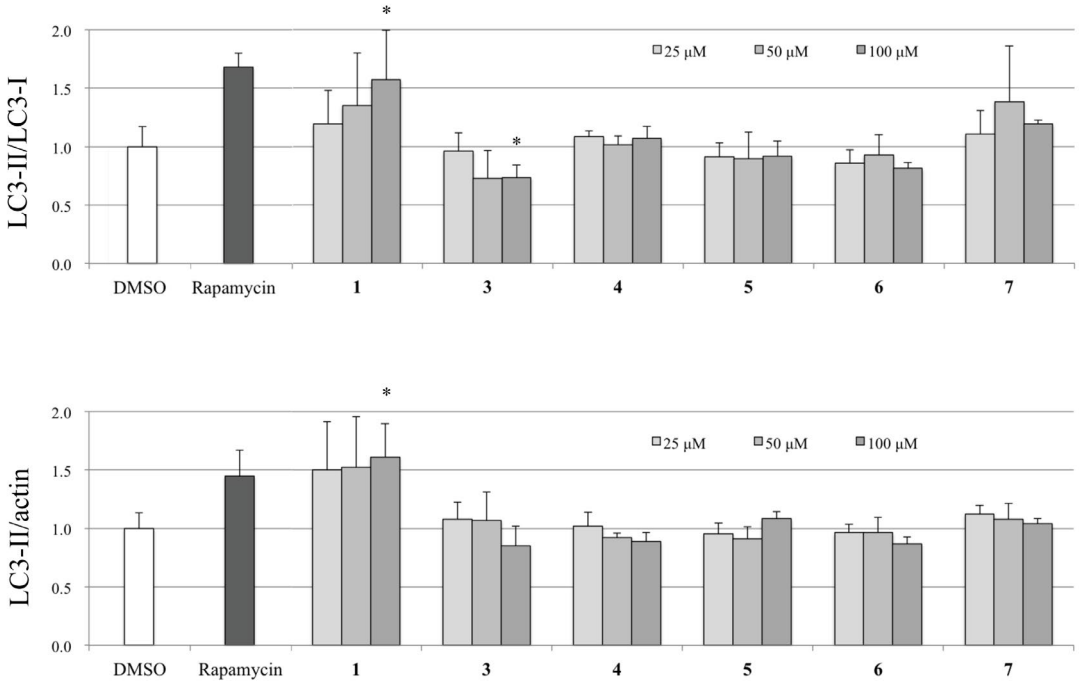
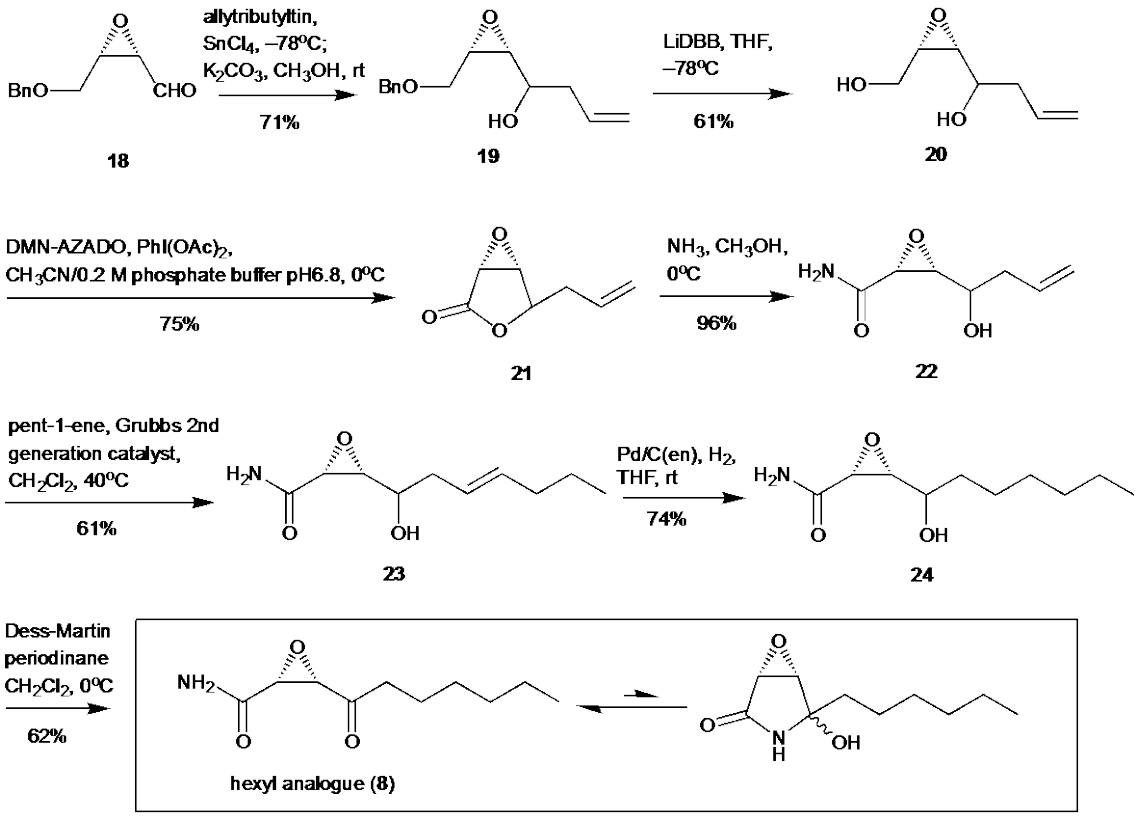


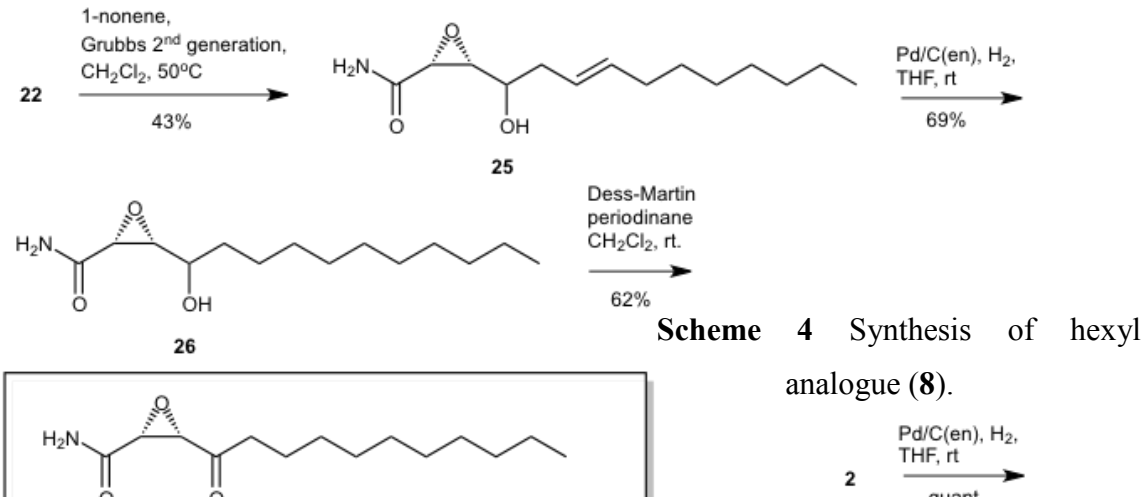
Fig. 2 Evaluation of importance of functional groups on autophagy-inducing activity of (+)-epogymnolactam (**1**). NIH3T3 cells were treated for 4 h with DMSO as the vehicle (control), 5μM rapamycin (positive control), or indicated concentrations of **1**, **3**, **4**, **5**, **6**, or **7**. LC3-II/LC3-I: relative ratio of LC3-II to LC3-I. LC3-II/actin: relative ratio of LC3-II to actin. The data shown represent the mean ± SD of triplicate assay. All significant correlation values ($p<0.05$) are given with the indicated significance (* $p < 0.05$, ** $p < 0.01$) compared to the DMSO control.

In the case of ring-chain tautomerism of **1**, the major tautomer is supposed to be *syn*-form (**1b**) in polar solution, because amide nitrogen of **1a** attacks C4 carbonyl from the opposite side of the epoxy group to avoid steric and electronic repulsion (Supplemental Table S1). These considerations suggest that the *syn*-form (**1b**) is the active chemical species in the active site of the unknown primary molecular target. Attempts to synthesize *syn*-form analogue corresponding to **7** have not yet been successful.

Next, we synthesized hexyl, octyl, and decyl analogues (**8**, **9**, **10**) to investigate the effect of side-chain length on biological responses in NIH-3T3 cells. Aldehyde **18**, a synthetic intermediate[3] of **1**, was reacted with allyltributyltin in the presence of $SnCl_4$ to afford homoallylic alcohol **19** and its chlorohydrin.[8] Treatment of the by-product chlorohydrin with K_2CO_3 gave an additional **19**, and the overall yield of **19** was 71%. Selective hydrogenolysis of benzyl group of **19** was accomplished by using lithium 4,4'-di-tert-butylbiphenylide (LiDBB)[9,10] to give **20** in 61%. Oxidation of **20** with 1,5-dimethyl-9-azanoradamantane *N*-oxyl (DMN-AZADO)[11] in combination with [bis(acetoxy)iodo]benzene (BAIB)[12] gave lactone **21**, which was treated with NH_3 to afford the desired amide **22**. Olefin cross-metathesis of **22** with pent-1-ene gave **23** using Grubbs 2nd-generation catalyst.[13,14] Chemoselective hydrogenation of **23** using Pd/C-ethylenediamine (Pd/C(en)) catalyst[15] gave **24** which was converted into desired hexyl analogue **8** by Dess-Marin oxidation as shown in Scheme 4. Hexyl analogue **8** mainly existed as a chain form in $CDCl_3$ as shown in Supplementary Figures S9 and S10.

Similarly, decyl analogue **10** was synthesized by using 1-nonene as an alkene as shown in Scheme 5. NMR analysis indicated that **10** exists as a chain form in $CDCl_3$ as shown in Supplementary Figures S13 and S14. Octyl analogue **9** was prepared by chemoselective hydrogenation of cerulenin **2** using Pd/C(en) (Scheme 5). Major tautomer of **9** in $CDCl_3$ was chain form, while cyclic *syn*- and *anti*-forms were also observed as shown in Supplementary Figures S11 and S12.





Scheme 4 Synthesis of hexyl analogue (**8**).

Scheme 5 Synthesis of octyl analogue (**9**) and decyl analogue (**10**).

Fig. 3 shows the profiles of LC3-II/LC3-I and LC3-II/actin as autophagic markers after stimulation with **1**, **8**, **9**, **2**, and **10** in NIH3T3 cells. Except for **1**, only hexyl analogue **8** increased the relative amount of LC3-II to LC3-I or actin in a dose-dependent manner. In contrast, octyl analogue **9** and cerulenin **2** were suggested to cause dose-dependent down-regulation, and decyl analogue **10** did not alter the relative amount of LC3-II.

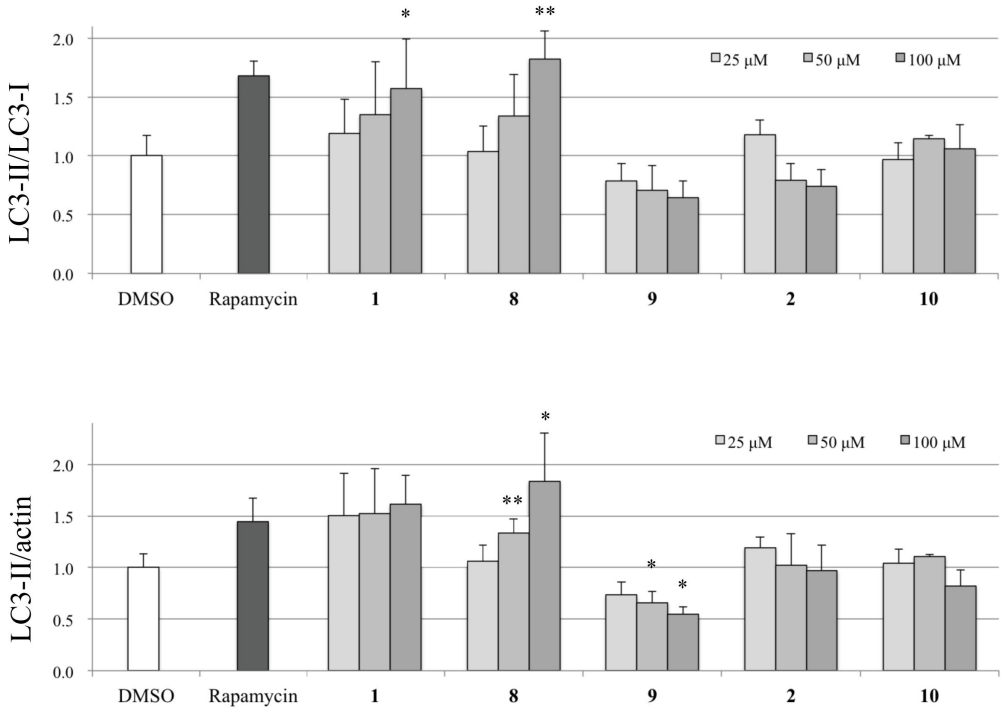


Fig. 3 Effect of side-chain length of (+)-epogymnolactam analogue on autophagy-inducing activity. NIH3T3 cells were treated for 4 h with DMSO as the vehicle (control), 5μM rapamycin (positive control) or indicated concentrations of **1**, **8**, **9**, **2**, or **10**. LC3-II/LC3-I: relative ratio of LC3-II to LC3-I. LC3-II/actin: relative ratio of LC3-II to actin. The data shown represent the mean ± SD of triplicate assay. All significant correlation values ($p<0.05$) are given with the indicated significance (* $p < 0.05$, ** $p < 0.01$) compared to the DMSO control.

Our preliminary experiments suggested that cerulenin (**9**) showed cytotoxicity to NIH3T3 cells at these concentrations. We therefore evaluated cytotoxicity of **1**, **2**, **8**, **9**, and **10** in NIH3T3 cells. Only hexyl analogue **8** showed lower cytotoxicity than **2**, **9**, and **10** as in the case of **1** (Fig. 4). Inhibition of fatty acid synthase with **2** induced apoptosis in multiple myeloma cells[16] and human breast cancer cells.[17] We observed significant cell shrinkage as a signal to apoptosis in NIH 3T3 cells[18] treated with **9** at 50 or 100 μM for 4 h, while **1** did not cause significant cell shrinkage at these concentrations but **8** showed cell shrinkage at 100 μM (data not shown).

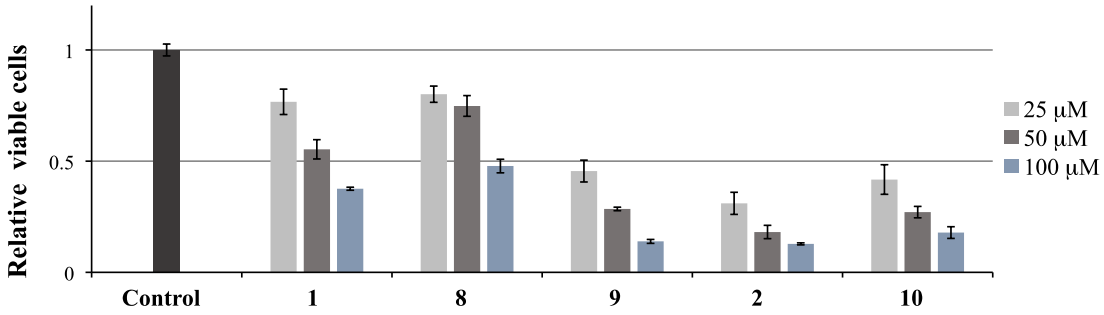


Fig. 4 Antiproliferation activities of (+)-epogymnolactam (**1**), hexyl analogue (**8**), octyl analogue (**9**), decyl analogue (**10**), and cerulenin (**2**). NIH3T3 cells were treated with DMSO as the vehicle (Control) or indicated concentrations of **1**, **8**, **9**, **2**, or **10**. Relative viable cells were determined using Cell Counting Kit-8. The data shown represent the mean ± SD of triplicate assay.

NMR experiments showed that (+)-epogymnolactam (**1**) existed primarily as a

cyclic *syn*-form in CD_3OD and a considerable amount of the *syn*-form was observed even in $CDCl_3$, while hexyl analogue **8** existed as a chain form in $CDCl_3$. Despite the close resemblance of the biological properties of **1** and **8**, the physicochemical properties of both are different.

In addition to LC3-II, SQSTM1 (p62) can also be used as autophagy marker to especially determine autophagy flux.[5] Thus, p62/actin, the up-regulation or degradation ratio of p62 to actin was evaluated in NIH3T3 cells treating with **1** and **8**. (+)-Epogymnolactam (**1**) did not show significant upregulation or downregulation of p62 after 4 h, whereas hexyl analogue **8** upregulated p62 in a dose-dependent manner as indicated in Fig. 5. Octyl analogue **9** also upregulated as in the case of **2**, but dose-dependency was not observed, presumably due to its cytotoxicity.

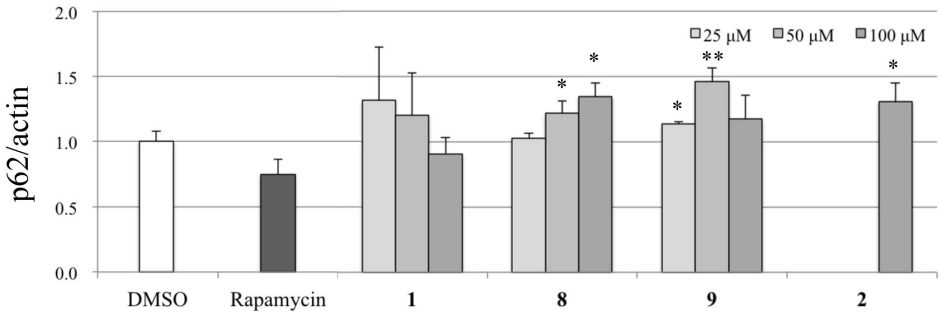


Fig. 5 Effect of (+)-epogymnolactam (**1**) on autophagy flux compared to that of hexyl analogue (**8**) or octyl analogue (**9**). NIH3T3 cells were treated for 4 h with DMSO as the vehicle (control), 5 μM rapamycin (positive control) or indicated concentrations of **1**, **8**, **9**, or **2**. p62/actin: relative ratio of SEQSTM1 (p62) to actin. The data shown represent the mean ± SD of triplicate assay. All significant correlation values ($p<0.05$) are given with the indicated significance (* $p < 0.05$, ** $p < 0.01$) compared to the DMSO control.

In conclusion, the study on the structural requirement of **1** indicated that presence and stereochemistry of (2*R*,3*S*)-epoxy group, the molecular shape of the *syn*-form (**1b**), and butyl side chain are critical for inducing autophagy in NIH3T3 cells. We also found that hexyl analogue **8** is a novel autophagy modulator that differs from **1** as an autophagy inducer and **2** as an autophagy inhibitor. SQSTM1 protein (p62) serves as a link between LC3 and ubiquitinated substrates. SQSTM1 and SQSTM1-bound

polyubiquitinated proteins become incorporated into the completed autophagosome and are degraded in autolysosomes, thus serving as an index of autophagic degradation. Inhibition of autophagy correlates with increased levels of p62 in mammals, suggesting that steady state levels of the protein reflect the autophagic status. In contrast, decreased p62 levels are associated with autophagy activation.[5] These considerations suggest that **8** could be an inhibitor of autolysosome formation. However, many cellular-stresses can induce the formation of ubiquitinated protein aggregates. The p62-positive aggregates are also formed by proteasome inhibition or puromycin treatment and can be found in cells exposed to rapamycin for extended periods where the rates of autophagy are elevated.[5] Because **1** upregulated p62 at 6 h exposure where the rate of autophagy is promoted (data not shown), the upregulation of p62 at 4 h exposure with **8** can be explained by cellular stress caused by this compound. Thus, we concluded that **1**, **9** (or **2**), and **8** are different types of autophagy modulators which might be useful to determine the relationships between fatty acid synthesis and autophagy induction.

Zimmerman et al. reported that very long chain fatty acid (VLCFA) synthesis has important implications for autophagy and cell homeostasis in yeast.[19] After **1** was discovered as an autophagy inducer,[1] Régnacq et al. reported that the effect of **2** was biphasic that moderate doses (2 or 4 μM) restored autophagy and high doses (20 or 40 μM) inhibited autophagy in lipid droplets (LDs)-deficient yeast. The biphasic effect of **2** was specific to nitrogen-starvation autophagy, and only the inhibitory effect of **2** was observed in rapamycin-induced autophagy in the LDs-deficient mutant.[20] Shpilka et al. observed that suppression of autophagy by **2** correlated with a reduction in the number of LDs in yeast cells.[21] Although these observations suggest that fatty acid synthesis correlates with autophagy in yeast cells, precise mechanism has not been elucidated yet.

The inhibitory activity of **9** on fatty acid synthesis is slightly weaker than that of **2**.[22] However, the inhibitory activities of 3-hexenyl analogue (an unsaturated derivative of **8**) and 3-butenyl analogue (an unsaturated derivative of **1**) are much weaker than **2**.[2,23,24] These observations suggest that both **2** and **9** inhibit basal autophagy and cell proliferation with inhibition of fatty acid synthesis, while **1** and **8** cause different types of autophagic responses together with moderate suppression of cell proliferation without potent inhibition of fatty acid synthesis in NIH3T3 cells.

In hepatocytes, autophagy regulates lipid content because: inhibition of autophagy increased triacylglycerol (TG) store and LDs; loss of autophagy decreased

TG breakdown; TGs and LD structural proteins co-localized with autophagic compartments; and LC3 associated with LDs.[25]

Mobilization of cholesterol and fatty acids from cholesteryl ester (CE) and TG stores of all cells requires lipolytic enzymes.[26] Kobayashi et al. demonstrated that a 1 : 1 mixture of dinapinones A1 and A2 (DPA_{mix}) enhanced neutral lipid degradation together with induction of autophagy in Chinese hamster ovary-K1 cells.[27] These results suggest interrelationship between autophagy and TG breakdown in mammalian cells.

Thus, the use of (+)-epogymnolactam (**1**) and hexyl analogue (**8**) in combination with cerulenin (**2**) or octyl analogue (**9**) may be useful for determining relationship between autophagy and TG breakdown or fatty acid synthesis. These compounds may be also useful to develop therapeutic agents for dyslipidemia, and provide a new perspective on understanding autophagy induction.

Further systematic studies are necessary to determine the effect of lipid metabolism on autophagy induction in mammalian cells and to determine the molecular targets of **1** and **8**.

3. Experimental Procedure

3.1. Chemicals

All commercially available chemicals of highest purity were used without further purification. (+)-Epogymnolactam (**1**) was synthesized according to the previously reported procedure.[3] Cerulenin (**2**) was purchased from Wako Pure Chemical Co. (now FUJIFILM Wako Pure Chemical Co., Japan). Rapamycin as an autophagy inducer was obtained from Cayman Chemical (MI, USA). Thin layer and column chromatography were performed with Merck Silica Gel 60 F_{254} and Kanto Chemicals Co. (Japan) Silica Gel 60 N (spherical, neutral), respectively. NMR spectra were measured on a JEOL JMN EX-270 FT-NMR spectrometer. Chemical shifts are reported in ppm (δ-scale) using TMS as the internal standard and coupling constants (J) are given in Hz. The carbon number used for NMR assignment was indicated according to the number shown in **1** and **2** of Fig. 1. MS spectra were acquired with FI or FD mode on a JEOL JMS-T100GCV equipment. Optical rotations were measured on a JASCO P-2000 polarimeter.

*3.1.1. (2S,3R)-4-Benzyloxy-2,3-epoxybutan-1-ol (**13**, 88% ee)*

To 4Å molecular sieves (2.29 g) in CH_2Cl_2 (32ml), $Ti(O^iPr)_4$ (7.2 ml, 24.3 mmol) was added, and L-(+)-DIPT (5.03 ml, 23.9 mmol) was added dropwise at –25°C under an argon atmosphere. After stirring for 30 min, allyl alcohol **11** (4.00g, 22.4 mmol) in CH_2Cl_2 (32 ml) was added dropwise over 1.5 h. To the mixture, t-BuOOH in nonane (5.5 M, 8.8 ml) was added dropwise, then the reaction temperature was raised to –20°C and stirred for 3 days. The reaction mixture was raised to room temperature, quenched with a saturated $Na_2S_2O_3$ solution (40 ml), and stirred for 2 h. The resultant solution was filtered through celite, Et_2O was added, and the mixture was washed with brine. The organic layer was dried with anhydrous Na_2SO_4 and evaporated in vacuo. The residue was subjected to silica gel column chromatography (EtOAc : hexane = 1 : 1) to give epoxy alcohol **13** (2.98 g, 68.4%). The enantiomeric excess of **13** was determined as 85.7% ee from the peak-area ratio by chiral HPLC analysis (DAICEL Chiralpak AD-H (ϕ 0.45 cm x 25 cm), 254 nm, EtOH : hexane = 1 : 9).

$[\alpha]_D^{25}$ = –18.4 (*c* 1.0, $CHCl_3$).

^{1}H NMR ($CDCl_3$, 270 MHz): δ 3.18–3.31 (2H, m, H-2 and H-3), 3.62–3.75 (4H, m, H-1 and H-4), 4.50–4.64 (2H, m, benzyl), 7.30–7.36 (5H, m, aromatic).

^{13}C NMR ($CDCl_3$, 67.5 MHz): δ 54.7 (C-3), 55.6 (C-2), 60.7 (C-1), 68.0 (C-4), 73.5 (benzyl), 127.8 (aromatic), 128.0 (aromatic), 128.5 (aromatic), 137.4 (aromatic).

FI-MS: *m/z* = 194.1 $[M]^+$.

*3.1.2. (2S,3R)-4-Benzyloxy-2,3-epoxybutan-1-yl acetate (**12**)*

To epoxy alcohol **13** (1.0 g, 5.1 mmol) in vinyl acetate (39.3 ml), porcine pancreatic lipase (PPL) was added, and the reaction mixture was stirred for 14 h. The reaction mixture was filtered through celite and evaporated in vacuo. The residue was subjected to silica gel column chromatography (EtOAc : hexane = 1 : 2) to give epoxy acetate **12** (0.99 g, 4.17 mmol). The enantiometric excess of **12** was determined to be 98.1% ee from the peak-area ratio by chiral HPLC analysis (DAICEL Chiralpak AD-H (ϕ 0.45 cm x 25 cm), 254 nm, EtOH : hexane = 1 : 9).

$[\alpha]_D^{25}$ = –19.4 (*c* 1.0, $CHCl_3$).

^{1}H NMR ($CDCl_3$, 270 MHz): δ 2.10 (3H, s, Ac), 3.22–3.32 (2H, m, H-2 and H-3), 3.60 (1H, dd, *J* = 11.6, 5.9 Hz, H-4a), 3.72 (1H, dd, *J* = 11.2, 4.2 Hz, H-4b), 4.04 (1H, dd, *J* = 12.1, 7.0 Hz, H-1a), 4.32 (1H, dd, *J* = 12.4 , 3.8 Hz, H-1b), 4.51–4.64 (2H, m, benzyl), 7.28–7.36 (5H, m, aromatic).

^{13}C NMR ($CDCl_3$, 67.5 MHz): δ 20.7 (-CH_3), 53.0 (C-2), 54.7 (C-3), 62.6 (C-1), 67.8 (C-4), 73.4 (benzyl), 127.8 (aromatic), 127.9 (aromatic), 128.4 (aromatic), 137.5 (aromatic), 170.6 (-C(=O)-).
FI-MS: m/z = 236.1 $[M]^+$.

*3.1.3. (2S,3R)- 4-Benzyloxy-2,3-epoxybutan-1-ol (enantiopure **13**)*

To epoxy acetate **12** (499.5 mg, 2.11mmol) in THF (6.09ml), CH_3OH (6.09 ml) was added dropwise and then K_2CO_3 (58.0 mg) was added at 0^{o}C. The reaction mixture was stirred for 2 h at the same temperature. The reaction was quenched with a saturated NH_4Cl solution (40 ml), and the mixture was extracted with Et_2O. The organic layer was dried with anhydrous Na_2SO_4 and evaporated in vacuo. The residue was subjected to silica gel column chromatography (EtOAc : hexane = 2 : 1) to give enantiopure epoxy alcohol **13** (375.9 mg, 91%).
$[\alpha]_D^{25}$ = –23.1 (*c* 1.0, $CHCl_3$).

*3.1.4. (–)-Epogymnolactam (**3**)*

(2*S*,3*S*)-3-(1-Hydroxypentyl)oxiran-2-carboxamide was derived from enantiopure **13** using essentially the same method as the synthesis of **1**.[3] To the amide (19.5 mg, 0.11mmol) in CH_2Cl_2 (1.6 ml), Dess-Martin periodinane (67.1 mg, 1.45 eq.) was added at 0^{o}C under an argon atmosphere. After stirring for 2 h, the reaction mixture was quenched with a mixture of saturated $Na_2S_2O_3$ solution and saturated $NaHCO_3$ and extracted with EtOAc. The organic layer was washed with brine, dried with anhydrous Na_2SO_4 and evaporated in vacuo. The residue was subjected to silica gel column chromatography (EtOAc : hexane = 2 : 1) to give (–)-epogymnolactam **3** (11.3 mg, 59%).
$[\alpha]_D^{25}$ = –14.4 (*c* 1.1, CH_3OH), [(+)-epogymnolactam **1**: $[\alpha]_D^{20}$ = +47.8 (*c* 1.0, CH_3OH, incubation time is different)].[2]
^{1}H NMR (CD_3OD, 270 MHz): *Keto* form: δ 0.88-0.97 (3H, m, H-8), 1.28–1.58 (4H, m, H-6 and H-7), 2.57–2.69 (m, H-5), 3.70 (d, J = 5.3 Hz, H-3) , 3.89 (d, J = 5.3 Hz, H-2),; *syn*-form: 0.88–0.97 (3H, m, H-8), 1.28–1.58 (4H, m, H-6 and H-7), 1.69–1.75 (m, H-5), 3.58 (1H, d, J = 2.6 Hz, H-3), 3.80 (1H, d, J = 2.6 Hz, H-2); *anti*-form: 0.88–0.97 (3H, m), 1.28–1.41 (2H, m), 1.49–1.58 (2H, m), 1.69–1.75 (2H, m), 3.57 (d, J = 2.6 Hz, H-3), 3.81 (d, J = 2.6 Hz, H-2),.

^{13}C NMR (CD_3OD, 67.5 MHz): *Keto* form: δ 14.1 (C-8), 23.2 (C-7), 26.1 (C-6), 41.0 (C-5), 55.8 (C-2), 59.4 (C-3), 170.5 (C-1), 205.8 (C-4); *syn*-form: 14.3 (C-8), 24.0 (C-7), 27.0 (C-6), 36.3 (C-5), 50.1 (C-3), 53.1 (C-2), 87.2 (C-4), 174.4 (C-1); *anti*-form: 14.3 (C-8), 23.9 (C-7), 25.9 (C-6), 38.9 (C-5), 54.3 (C-2), 58.1 (C-3), 86.8 (C-4), 173.0 (C-1).
FI-MS: *m/z* = 172.1 $[M+H]^+$.

*3.1.5. 4-Hydroxyoctanamide (**15**)*

γ-Octalactone **14** (420.0 mg, 3.0 mmol) was mixed with approximately 20 ml of liquid NH_3 in a pressure-resistance reaction tube, and the mixture was stirred at 50°C for 7 days. The tube was opened at –196°C, and NH_3 was evaporated at ambient temperature. The resultant white solid was purified by silica gel column chromatography ($CHCl_3$: CH_3OH = 9 : 1) to give amide **15** (411.3 mg, 87%) as a white solid.
^{1}H NMR (CD_3OD, 270 MHz): δ 0.82 (3H, t, *J* = 6.8 Hz, H-8), 1.31–1.84 (8H, m, H-3, H-5, H-6 and H-7), 2.21–2.41 (2H, m, H-2), 3.47–3.56 (1H, m, H-4).
^{13}C NMR (CD_3OD, 67.5 MHz): δ 14.4 (C-8), 23.8 (C-7), 29.0 (C-6), 32.9 (C-2), 34.1 (C-3), 38.1 (C-5), 71.9 (C-4), 179.2 (C-1).
FD-MS: *m/z* = 160.1 $[M+H]^+$.

*3.1.6. Deepoxy analogue (**4**)*

Dess-Martin periodinane (129.7 mg, 2 eq.) was added to a stirred solution of amide **15** (23.7 mg, 148.8 μmol) in dry CH_2Cl_2 (8.4 ml) at 0°C under argon atmosphere. After stirring for 3 h, the reaction was quenched with a solution of saturated aqueous $Na_2S_2O_3$ and saturated aqueous $NaHCO_3$. The solution was extracted with Et_2O and organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The resultant residue was subjected to silica gel column chromatography ($CHCl_3$: CH_3OH = 19 : 1) to afford deepoxy analogue **4** (14.1 mg, 60 %) as a colorless solid.
^{1}H NMR ($CDCl_3$, 270 MHz): δ 0.90 (3H, t, *J* = 7.3 Hz, H-8), 1.31 (2H, sext, *J* = 7.3 Hz, H-7), 1.57 (2H, quin, *J* = 7.5, H-6), 2.43–2.51 (4H, m, H-2 and H-3), 2.78 (2H, t, *J* = 6.5 Hz, H-3) (Supplementary Fig. S1).
^{13}C NMR ($CDCl_3$, 67.5 MHz): δ 13.8 (C-8), 22.3 (C-7), 25.9 (C-6), 29.1 (C-2), 37.4 (C-3), 42.5 (C-5), 174.4 (C-1), 210.2 (C-4) (Supplementary Fig. S2).
HR-FD-MS: *m/z* = 158.1185 $[M+H]^+$ calcd. for $C_8H_{16}NO_2$, found 158.1181.

*3.1.7. (2R,3R,4R)-2,3-Epoxy-4-hydroxyoctanamide (**5**)*

Lactone **16**[2] (low polar diastereomer, 49.6 mg, 0.317 mmol) was dissolved in a solution of NH_3 in CH_3OH (2.0 M, 8 ml) under nitrogen atmosphere and the mixture was stirred for 2.5 h at 0°C. The reaction mixture was evaporated in vacuo and purified by silica gel column chromatography ($CHCl_3$: CH_3OH = 9 : 1) to afford epoxy amide alcohol **5** (54.0 mg, 98.5%).

$[\alpha]_D^{25}$ = +49.3 (*c* 1.0, CH_3OH).

^{1}H NMR ($CDCl_3$, 270 MHz): δ 0.92 (3H, t, *J* = 7.2 Hz, H-8), 1.32–1.75 (6H, m, H-5, H-6 and H-7), 3.13 (1H, dd, *J* = 7.8, 4.6 Hz, H-3), 3.42-3.51 (1H, m, H-4), 3.54 (1H, d, *J* = 4.6 Hz, H-2) (Supplementary Fig. S3).

^{13}C NMR ($CDCl_3$, 67.5 MHz): δ 14.0 (C-10), 22.6 (C-7), 27.0 (C-6), 34.6 (C-5), 54.3 (C-2), 60.1 (C-3), 69.0 (C-4), 170.2 (C-1) (Supplementary Fig. S4).

FI-MS: *m/z* = 174.1 $[M+H]^+$.

*3.1.8. (2R,3R,4R)-2,3-Epoxy-4-hydroxy-N,N-dimethyloctanamide (**17**)*

Lactone **16** (10.0 mg, 64 μmol) was dissolved in a solution of $(CH_3)_2NH$ in CH_3OH (2.0 M, 1.5 ml) under argon atmosphere and the mixture was stirred for 2.5 h at 0°C. The reaction mixture was concentrated in vacuo and purified by silica gel column chromatography (($CHCl_3$: CH_3OH = 96 : 4) to afford **17** (8.3 mg, 65%) as a colorless solid.

^{1}H NMR ($CDCl_3$, 270 MHz): δ 0.92 (3H, t, *J* = 7.0 Hz, H-8), 1.26–1.73 (6H, m, H-5, H-6 and H-7), 3.00 (3H, s, NCH_3), 3.09 (1H, dd, *J* = 3.8, 4.3 Hz, H-3), 3.20 (3H, s, NCH_3), 3.58 (1H, d, *J* = 3.8 Hz, H-2), 3.87 (1H, m, H-4).

^{13}C NMR ($CDCl_3$, 67.5 MHz): δ 14.0 (C-8), 22.7 (C-7), 27.2 (C-6), 34.5 (C-5), 35.1 (-$N(CH_3)_2$), 36.7 (-$N(CH_3)_2$), 53.8 (C-2), 59.9 (C-3), 71.8 (C-4), 167.2 (C-1).

FI-MS: *m/z* = 1201.1 $[M]^+$.

*3.1.9. N,N-Dimethyl analogue (**6**)*

To a stirred solution of **17** (8.2 mg, 41 μmol) in CH_2Cl_2 (2.4 ml), Dess-Martin periodinane (29.0 mg, 1.5 eq.) was added at 0°C under argon atmosphere. After stirring for 3 h, the reaction was quenched with a mixture of saturated aqueous $Na_2S_2O_3$ and saturated aqueous $NaHCO_3$. The resultant solution was extracted with EtOAc and the

organic layer was washed with brine, dried over anhydrous Na_2SO_4, concentrated in vacuo. The residue was purified by silica gel column chromatography (EtOAc : hexane = 2 : 1) to give *N,N*-dimethyl analogue **6** (7.3 mg, 90%) as a colorless solid.

$[\alpha]_D^{25}$ = –44.2 (*c* 0.7, $CHCl_3$).

^{1}H NMR ($CDCl_3$, 270 MHz): δ 0.89 (3H, t, *J* = 7.2 Hz, H-8), 1.30 (2H, sext, *J* = 7.4 Hz, H-7), 1.48–1.62 (2H, m, H-6), 2.55 (2H, dt, *J* = 7.4, 7.4, 2.9 Hz, H-5), 2.93 (3H, s, NCH_3), 3.13 (3H, s, NCH_3), 3.70 (1H, d, *J* = 4.9 Hz, H-3), 3.82 (1H, d, *J* = 5.1 Hz, H-2) (Supplementary Fig. S5).

^{13}C NMR ($CDCl_3$, 67.5 MHz): δ 13.8 (C-8), 22.1 (C-7), 24.8 (C-6), 35.3 (-$N(CH_3)_2$), 36.3 (-$N(CH_3)_2$), 39.6 (C-5), 54.7 (C-2), 58.2 (C-3), 164.3 (C-4), 205.5 (C-1) (Supplementary Fig. S6).

HR-FI-MS: *m/z* = 200.1290 $[M+H]^+$ calcd. for $C_{10}H_{18}NO_3$, found 200.1287.

*3.1.10. O-Methyl analogue (**7**)*

To a solution of (+)-epogymnolactam **1** (8.5 mg, 50 μmol) in CH_3OH (3.0 ml), 10-camphorsulfonic acid (3.0 mg, 0.26 eq.) was added, and the reaction mixture was stirred at 50°C for 6 h. The resulting mixture was diluted with EtOAc and washed H_2O and brine. The organic layer was dried over anhydrous Na_2SO_4, evaporated in vacuo. The resultant oil was purified by silica gel column chromatography (EtOAc : hexane = 1 : 2) to afford *O*-methyl analogue **7** (2.2 mg, 24%) as a colorless oil.

$[\alpha]_D^{25}$ = +95.3 (*c* 0.29, $CHCl_3$).

^{1}H NMR ($CDCl_3$, 270 MHz): δ 0.93 (3H, t, *J* = 7.1 Hz, H-8), 1.26–1.56 (4H, m, H-6 and H-7), 1.79 (2H, t, *J* = 7.7 Hz, H-5), 3.65 (1H, t, *J* = 2.3 Hz, H-3), 3.79 (1H, d, *J* = 2.7 Hz, H-2) (Supplementary Fig. S7).

^{13}C NMR ($CDCl_3$, 67.5 MHz): δ 13.9 (C-8), 22.7 (C-7), 25.7 (C-6), 34.1 (C-5), 49.4 (C-2), 51.7 (-OCH_3), 56.5 (C-3), 89.6 (C-4), 171.5 (C-1) (Supplementary Fig. S8).

HR-FD-MS: *m/z* = 186.1166 $[M+H]^+$ calcd. for $C_9H_{16}NO_3$, found 186.1130.

*3.1.11. (5R,6S)-7-Benzyloxy-5,6-epoxyhept-1-en-4-ol (**19**)*

To a stirred solution of **18**[3] (100 mg, 0.521 mmol) in dry CH_2Cl_2 (12 ml), $SnCl_4$ (65 μl, 1.1 eq.) was added dropwise at –78°C under argon atmosphere. After stirring for 1 h, allyltributyltin (208 μl, 1.3 eq.) was added dropwise and the reaction mixture was continuously stirred for another 1.5 h. The reaction mixture was quenched with sat.

aqueous NH_4Cl at –78°C and allowed to room temperature. The solution was extracted with Et_2O and the organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The resultant residue was purified by silica gel column chromatography (EtOAc : hexane = 1 : 3) to give a mixture of homoallylic alcohol **19** (74.3 mg) and its chlorohydrin (20 mg). To a solution of chlorohydrin (20 mg) in CH_3OH (1 ml), K_2CO_3 (15. 3 mg, 1.5 eq.) was added at 0°C, and the reaction mixture was allowed to room temperature. After 4 days, additional K_2CO_3 in CH_3OH (1 ml) was added and the reaction mixture was stirred for 5 h. The resultant mixture was quenched with saturated aqueous NH_4Cl and extracted with Et_2O. The organic layer was washed with brine, dried over Na_2SO_4, concentrated in vacuo, and the residue was purified by silica gel column chromatography (EtOAc : hexane = 1 : 3) to afford an additional **19** (12.2 mg). The total yield of **19** was 71%.

^{1}H NMR ($CDCl_3$, 270 MHz): δ 2.35–2.56 (2H, m, H-3), 2.96 (1H, dd, J = 8.1 and 4.3 Hz, H-5), 3.23–3.29 (1H, m, H-6), 3.47–3.55 (1H, m, H-4), 3.66 (1H, dd, J = 10.5, 6.7 Hz, H-7a), 3.81–3.87 (1H, dd, J = 10.7, 6.1 Hz, H-7b), 4.58 (2H, m, benzyl), 5.14–5.22 (2H, m, H-1), 5.83–5.93 (1H, m, H-2), 7.30–7.37 (5H, m, aromatic).

^{13}C NMR ($CDCl_3$, 67.5 MHz): δ 39.4 (C-5), 54.1 (C-2), 57.9 (C-3), 68.5 (C-1), 69.0 (C-4), 73.6 (benzyl), 118.3 (C-7), 127.9 (aromatic), 128.6 (aromatic), 133.5 (C-6), 137.2 (aromatic).

FI-MS: m/z = 234.1 $[M]^+$.

*3.1.12. (2S,3R)-2,3-Epoxyhept-6-en-1,4-diol (**20**)*

Lithium wire (20.9 mg, 3.02 mmol) was hammered out into foil, cut into several small pieces and washed with hexane. The pieces were then dipped, one by one, in CH_3OH until the surface turns shiny, then immediately transferred to THF. The lithium chunks were placed in a Schlenk flask containing DBB (534.8 mg, 2.01 mmol). The flask was evacuated and purged with argon several times. Dry THF (12 ml) was added, and the contents of the flask were sonicated between 0 and 20°C for 3 h. The resultant deep forest green solution of radical anion (4.7 ml) was then added dropwise to a solution of **19** (47.0 mg, 0.201 mmol) in dry THF (5.0 ml) at –78°C under argon atmosphere. The mixture was stirred for 1.5 h and quenched with sat. aqueous NH_4Cl at –78°C and allowed to reach room temperature. The resultant solution was extracted with Et_2O and the organic layer was washed with brine, dried over Na_2SO_4, concentrated in vacuo. The

residue was purified by silica gel column chromatography (EtOAc : hexane = 2 : 1) to give a diastereomeric mixture of diol **20** (17.6 mg, 61%) as a colorless oil.

^{1}H NMR ($CDCl_3$, 270 MHz): δ 2.28–2.58 (2H, m, H-5), 2.97–3.08 (1H, dd, J = 7.8, 4.3 Hz, H-3), 3.20–3.76 (3H, m, H-1a, H-2 and H-4), 3.84–4.01 (1H, m, H-1b), 5.12-5.24 (2H, m, H-7), 5.72-5.96 (1H, m, H-6).

^{13}C NMR ($CDCl_3$, 67.5 MHz): δ 39.8 (C-5), 55.8 (C-2), 58.3 (C-3), 60.7 (C-1), 68.8 (C-4), 119.0 (C-7), 133.1 (C-6).

FD-MS: m/z = 145.1 $[M+H]^+$.

*3.1.13. (1R,5R)-4-(Prop-2-enyl)--3,6-dioxa-bicyclo[3.1.0]hexan-2-one (**21**)*

$PhI(OAc)_2$ (462 mg, 3.0 eq.) was added to a stirred solution of **20** (68.8 mg, 0.478 mmol) and DMN-AZADO (4.0 mg, 0.05 eq.) in CH_3CN (4.82 ml) and aqueous phosphate buffer (0.54 ml, 0.2 M, pH 6.8) at 0^{o}C. After stirring for 5 h, the mixture was quenched with saturated aqueous $Na_2S_2O_3$. The solution was extracted with EtOAc and the organic layer was washed with brine, dried over Na_2SO_4, concentrated in vacuo. The residue was purified by silica gel column chromatography (EtOAc : hexane = 1 : 3) to afford a diastereomeric mixture of lactone **21** (50.0 mg, 75%) as a colorless oil.

^{1}H NMR ($CDCl_3$, 270 MHz): δ 2.43–2.64 (2H, m, allyl), 3.79 (1H, t, J = 2.7 Hz, H-5), 4.03 (d, J = 2.4 Hz, H-1 of major diastereomer), 4.11 (dd, J = 2.6, 1.5 Hz, H-1 of minor diastereomer), 4.50 (dt, J = 7.1, 1.4 Hz, H-4 of minor diastereomer), 4.64 (t, J = 6.2 Hz, H-4 of major diastereomer), 5.17–5.30 (2H, m, olefinic), 5.70–5.92 (1H, m, olefinic).

^{13}C NMR ($CDCl_3$, 67.5 MHz): δ 34.0 and 36.1 (C-5), 49.8 and 50.6 (C-3), 56.2 and 57.6 (C-2), 78.1 and 78.4 (C-4), 119.3 and 120.4 (C-7), 129.8 and 131.2 (C-6), 170.1 (C-1).

FI-MS: m/z = 140.1 $[M]^+$.

*3.1.14. (2R,3R)-2,3-Epoxy-4-hydroxyhept-6-enamide (**22**)*

The diastereomeric mixture of **21** (9.6 mg, 0.067 mmol) was dissolved in a solution of NH_3 in CH_3OH (2.0 M, 1.3 ml) under argon atmosphere and the mixture was stirred at 0^{o}C for 3 h. The resulting solution was concentrated in vacuo and purified by silica gel column chromatography ($CHCl_3$: CH_3OH = 93 : 7) to afford a diastereomeric mixture of amide **22** (10.3 mg, 96%) as a colorless solid.

^{1}H NMR ($CDCl_3$, 270 MHz): δ 2.33–2.55 (2H, m, H-5), 3.14–3.22 (1H, dd, J = 8.1, 4.6

Hz, H-3), 3.46–3.66 (2H, m, H-2 and H-4), 5.14–5.23 (2H, m, H-7), 5.72–5.97 (1H, m, H-6).

^{13}C NMR ($CDCl_3$, 67.5 MHz): δ 38.2 and 39.3 (C-5), 54.3 and 54.5 (C-2), 59.6 and 60.8 (C-3), 67.9 and 68.5 (C-4), 118.4 and 118.8 (C-7), 132.7 and 133.1 (C-6), 169.9 and 170.6 (C-1).

FD-MS: *m/z* = 158.1 $[M+H]^+$.

*3.1.15. (2R,3R)-2,3-Epoxy-4-hydroxydec-6-enamide (**23**)*

Grubbs 2nd generation catalyst (3.1 mg, 0.1 eq.) was added to a stirred solution of **22** (5.7 mg, 36.3 μmol) and 1-pentene (70 mg) in dry and degassed CH_2Cl_2 (1.0 ml) under argon and the resulting mixture was heated to reflux for 24 h. The mixture was concentrated in vacuo and purified by silica gel column chromatography (EtOAc : hexane = 4 : 1) to afford a diastereomeric mixture of amide **23** (4.4 mg, 61%) as a colorless solid.

^{1}H NMR ($CDCl_3$, 270 MHz): δ 0.96 (3H, t, *J* = 7.2 Hz, H-10), 1.23–1.43 (2H, m, H-9), 1.98-2.11 (2H, m, H-8), 2.17–2.51(2H, m, H-5), 3.14–3.25 (1H, m, H-3), 3.47–3.65 (2H, m, H-2 and H-4), 5.32–5.65 (2H, m, H-6 and H-7), 6.14-6.22 (1H, s).

^{13}C NMR ($CDCl_3$, 67.5 MHz): δ 13.6 (C-10), 22.4 (C-9), 34.7 (C-8), 37.1 and 38.1 (C-5), 54.2 and 54.3 (C-2), 59.5 and 60.8 (C-3), 68.6 and 69.2 (C-4), 123.5 and 123.9 (C-7), 135.3 and 135.9 (C-6), 169.1 and 169.6 (C-1).

FD-MS: *m/z* = 200.1 $[M+H]^+$.

*3.1.16. (2R,3R)-2,3-Epoxy-4-hydroxydecanamide (**24**)*

To a solution of **23** (4.5 mg, 22.6 μmol) in THF (0.7 ml), Pd/C(en) (5.0 mg) was added and the mixture was stirred vigorously overnight under hydrogen atmosphere. The resulting solution was filtered through celite pad, concentrated and the residue was purified by silica gel column chromatography (EtOAc : hexane = 2 : 1) to afford a diastereomeric mixture of amide **24** (3.5 mg, 77%) as a colorless solid.

^{1}H NMR ($CDCl_3$, 270 MHz): δ 0.88 (3H, t, *J* = 7.2 Hz, H-10), 1.28–1.70 (10H, m, CH_2), 3.11–3.21 (1H, m, H-3), 3.43–3.60 (2H, m, H-2 and H-4).

^{13}C NMR ($CDCl_3$, 67.5 MHz): δ 14.0 (C-10), 22.5 and 22.6 (C-9), 24.7 and 24.8 (C-8), 29.1 and 29.2 (C-7), 31.6 and 31.7 (C-6), 33.6 and 34.8 (C-5), 54.2 and 54.8 (C-2), 60.0 and 61.4 (C-3), 69.3 and 70.0 (C-4), 169.2 and 169.9 (C-1).

FD-MS: *m/z* = 202.2 $[M+H]^+$.

*3.1.17. Hexyl analogue (**8**)*

To a stirred solution of **24** (3.4 mg, 16.9 μmol) in dry CH_2Cl_2 (1.0 ml), Dess-Martin periodinane (12.2 mg, 1.7 eq.) was added at room temperature under argon atmosphere. After stirring for 3 h, the reaction mixture was purified by silica gel column chromatography (EtOAc : hexane : toluene = 3 : 1 : 1) to afford hexyl analogue **8** (2.1 mg, 62%) as a colorless solid.

^{1}H NMR ($CDCl_3$, 270 MHz): δ 0.88 (3H, t, *J* = 7.0 Hz, H-10), 1.28–1.61 (8H, m), 2.48–2.68 (2H, m, H-5), 3.73 (1H, d, *J* = 5.4 Hz, H-3), 3.88 (1H, d, *J* = 5.1 Hz, H-2) (Supplementary Fig. S9).

^{13}C NMR ($CDCl_3$, 67.5 MHz): δ 14.0 (C-10), 22.4 (C-9), 23.1 (C-8), 28.7 (C-7), 31.4 (C-6), 41.1 (C-5), 55.3 (C-2), 58.3 (C-3), 167.3 (C-1), 202.7 (C-4) (Supplementary Fig. S10).

HR-FD-MS: *m/z* = 200.1294 $[M+H]^+$ calcd. for $C_{10}H_{18}NO_3$, found 200.1287.

*3.1.18. (2R,3R)-2,3-Epoxy-4-hydroxytetradec-6-enamide (**25**)*

Grubbs 2nd generation catalyst (2.1 mg, 0.1 eq.) was added to a stirred solution of **22** (3.8 mg, 24.3 μmol) and 1-nonene (97.2 mg) in dry and degassed CH_2Cl_2 (2.0 ml) under argon and the mixture was heated to reflux for 24 h. The resulting mixture was concentrated in vacuo and purified by silica gel column chromatography ($CHCl_3$: CH_3OH = 19 : 1) to afford amide **25** (2.7 mg, 43%) as a colorless solid.

^{1}H NMR ($CDCl_3$, 270 MHz): δ 0.88 (3H, t, *J* = 6.8 Hz, H-14), 1.27–1.61 (10H, m, CH_2), 1.98–2.06 (2H, m, H-8), 2.25–2.32 (2H, m, H-5), 3.18–3.23 (1H, m, H-3), 3.55–3.57 (2H, m, H-2 and H-4), 5.34–5.63 (2H, m, H-6 and H-7).

^{13}C NMR ($CDCl_3$, 67.5 MHz): δ 14.1 (C-14), 22.7 (C-13), 29.15 (C-11), 29.17 (C-9), 29.3 (C-10), 31.8 (C-12), 32.6 (C-8), 37.1 (C-5), 54.2 (C-2), 60.8 (C-3), 69.2 (C-4), 123.2 (C-6), 136.3 (C-7), 169.1 (C-1).

HR-FD-MS: *m/z* = 256.1917 $[M+H]^+$ calcd. for $C_{14}H_{26}NO_3$, found 256.1913.

*3.1.19. (2R,3R)-2,3-Epoxy-4-hydroxytetradecanamide (**26**)*

Pd/C(en) (6.0 mg) was added to a solution of **25** (3.0 mg, 11.7 μmol) in THF (0.7 ml) and the reaction mixture was stirred vigorously at room temperature under H_2

atmosphere. The resulting mixture was filtered through celite pad and concentrated in vacuo. The residue was purified by silica gel column chromatography ($CHCl_3$: CH_3OH = 19 : 1) to give amide **26** (2.1 mg, 69%) as a colorless solid.

^{1}H NMR ($CDCl_3$, 270 MHz): δ 0.88 (3H, t, J = 6.8 Hz, H-14), 1.25–1.58 (20H, m, CH_2), 3.16–3.21 (1H, m, H-3), 3.49–3.59 (2H, m, H-2 and H-4).

^{13}C NMR ($CDCl_3$, 67.5 MHz): δ 14.1 (C-14), 22.7 (C-13), 24.7 (C-6), 29.3 (C-11), 29.4 (C-8), 29.55 (C-9), 29.57 (C-10), 29.7 (C-7), 31.9 (C-12), 33.6 (C-5), 54.7 (C-2), 61.4 (C-3), 70.0 (C-4), 169.0 (C-1).

HR-FD-MS: m/z = 258.2076 $[M+H]^+$ calcd. for $C_{14}H_{28}NO_3$, found 258.2069.

*3.1.20. Decyl analogue (**10**)*

To a stirred solution of **26** (1.9 mg, 7.4 μmol) in dry CH_2Cl_2 (1.0 ml), Dess-Martin periodinane (5.3 mg, 1.7 eq.) was added at room temperature under argon atmosphere. After stirring for 3 h, the reaction mixture was purified by silica gel column chromatography ($CHCl_3$: CH_3OH = 19 : 1) to afford decyl analogue **10** (1.3 mg, 69%) as a colorless solid. In the NMR of **10** in $CDCl_3$, only the keto form was observed.

$[\alpha]_D^{25}$ = –2.4 (c 0.57, $CHCl_3$).

^{1}H NMR ($CDCl_3$, 270 MHz): δ 0.88 (3H, t, J = 6.7 Hz, H-14), 1.26 (14H, br s, CH_2), 1.56–1.66 (2H, m, H-6), 2.58 (2H, dt, J = 7.6, 7.6 and 5.1 Hz, H-5), 3.73 (1H, d, J = 5.1 Hz, H-3), 3.87 (1H, d, J = 5.4 Hz, H-2) (Supplementary Fig. S13).

^{13}C NMR ($CDCl_3$, 67.5 MHz): δ 14.1 (C-14), 22.7 (C-13), 23.1 (C-6), 29.0 (C-7), 29.2 (C-8), 29.3 (C-11), 29.4 (C-10), 29.5 (C-9), 31.9 (C-12), 41.1 (C-5), 55.3 (C-2), 58.3 (C-3), 167.4 (C-1), 202.6 (C-4) (Supplementary Fig. S14).

HR-FD-MS: m/z = 256.1917 $[M+H]^+$ calcd. for $C_{14}H_{26}NO_3$, found 256.1913.

*3.1.21. Octyl analogue (**9**)*

Pd/C(en) (1.0 mg) was added to a solution of cerulenin **2** (4.3 mg, 19.3 μmol) in THF (0.5 ml) and the reaction mixture was stirred at room temperature overnight under hydrogen atmosphere. The resulting solution was filtered through celite pad and concentrated in vacuo. The residue was purified by silica gel column chromatography (EtOAc : hexane = 1 : 1) to afford octyl analogue **9** (4.4 mg, quant.) as a colorless solid.

$[\alpha]_D^{25}$ = +17.2 (c 0.45, $CHCl_3$).

^{1}H NMR ($CDCl_3$, 270 MHz): δ 0.85–0.91 (3H, m, H-12), 1.26 (10H, br s, CH_2),

1.55-1.63 (2H, m, H-6), 1.70–1.87 (m, H-5 of cyclic form), 2.48-2.68 (m, H-5 of keto-form), 3.61 (t, J = 2.3 Hz, H-3 of major cyclic form), 3.65 (t, J = 2.4 Hz, H-3 of minor cyclic form), 3.73 (d, J = 5.1 Hz, H-3 of keto-form), 3.80-3.83 (m, H-2 of cyclic form), 3.87 (d, J = 5.4 Hz, H-2 of keto-form) (Supplementary Fig. S11).

^{13}C NMR ($CDCl_3$, 67.5 MHz): keto form δ 14.1 (C-12), 22.6 (C-11), 23.1 (C-6), 29.0 (C-9 and C-8), 29.1 (C-7), 31.8 (C-10), 41.1 (C-5), 55.3 (C-2), 58.3 (C-3), 167.3 (C-1), 202.7 (C-4); *syn*- and *anti*-form δ 14.1 (C-12), 22.6 (C-11), 23.6 (C-6), 29.1 (C-9), 29.3 and 29.4 (C-8), 29.6 (C-7), 31.8 (C-10), 37.1 and 37.5 (C-5), 51.5 and 53.9 (C-2), 57.5 and 57.8 (C-3), 86.3 (C-4), 171.4 (C-1) (Supplementary Fig. S12).

HR-FD-MS: m/z = 228.1595 $[M+H]^+$ calcd. for $C_{12}H_{22}NO_3$, found 228.1599.

3.2. Monitoring of autophagy-inducing activity

The autophagy-inducing activity of each compound was evaluated by slightly modifying the method reported previously.[2] Briefly, Mouse embryo fibroblast NIH 3T3 cells were inoculated at 3.3 x 10^5 cells/ml and cultured at 37^{o}C under 5% CO_2 atmosphere. After 24 h, medium change was made in each well, and the DMSO solution of each compound was added at final concentrations of 25 μM, 50 μM, and 100 μM. The cells were incubated at 37^{o}C for 4 h, and adherent cells were harvested in 1 ml of PBS (–) and collected by centrifugation. The collected cells were washed twice with PBS (–) and sonicated in RIPA Lysis Buffer (Santa Cruz: sc-24948). After centrifugation, the supernatant was collected and stored at –20^{o}C until use. Proteins were separated by SDS-PAGE and immunoblot analysis was performed with anti-LC3 antibody (Cell Signaling Technology), anti-SQSTM1/p62 antibody (Funakoshi, Japan) and anti-actin antibiotic (Cosmo Bio, Sigma-Aldrich). The specific signals from LC3, SQSTM1 (p62), and actin were quantified with the enhanced chemiluminescence reagents (NEL105, Perkin Elmer) by luminescent analyzer LAS-3000 (FUJI FILM, Japan) or ChemiDoc MP (Bio-Rad). As indicated in reference 2, it was difficult to visually judge by conventional immunoblotting analysis. Thus, the relative signal ratio (LC3-II/LC3-I, LC3-II/actin, or p62/actin) was determined from the quantified specific signal of each band in LAS-3000 or ChemiDoc MP to evaluate the autophagy inducing activity or autophagy inhibiting activity.

3.3. Anti-proliferative activity

Anti-proliferative activity was performed with slight modification of the previously reported method.[2] Using the trypan blue exclusion assay, the number of viable cells of NIH3T3 cells was adjusted to 0.53×10^4 cells/ml. The cells were treated with each compound in DMSO to the final concentrations of 25 μM, 50 μM and 100 μM, and plated on a 96-well plate. The DMSO concentration in all wells was set to 0.1%. After 4 h incubation at 37°C in a CO_2 incubator, 10 μl of Cell Counting Kit-8 (DOJINDO Laboratories, Japan) was added. After incubating for 14 h, the relative number of viable cells was expressed as the optical density (OD_{450}) value determined by a microplate reader (Sunrise Remote, Tecan Group Ltd., Switzerland).

Acknowledgements

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Supplemental Material

Novel autophagy modulators: Design and synthesis of (+)-epogymnolactam analogues and structure-activity relationship

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1. ^{1}H and ^{13}C NMR spectra of (+)-epgymnolactam analogues.

2. **Table S1.** Mole percentage of *keto* form (**1a**), *syn* form (**1b**), and *anti* form (**1c**) of (+)-epogymnolactam (**1**) in D_2O, CD_3OD, or $CDCl_3$.

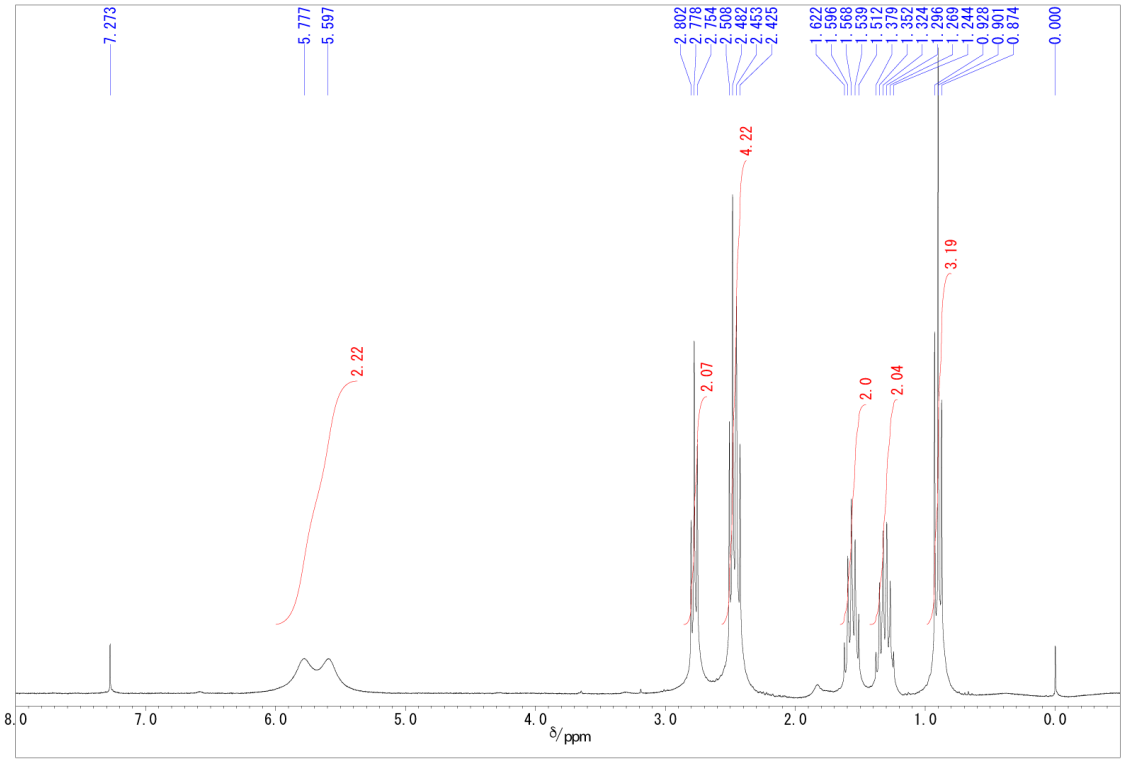

Fig. S1. ^{1}H NMR spectrum of deeopxy analogue (**4**) in $CDCl_3$ (270 MHz).

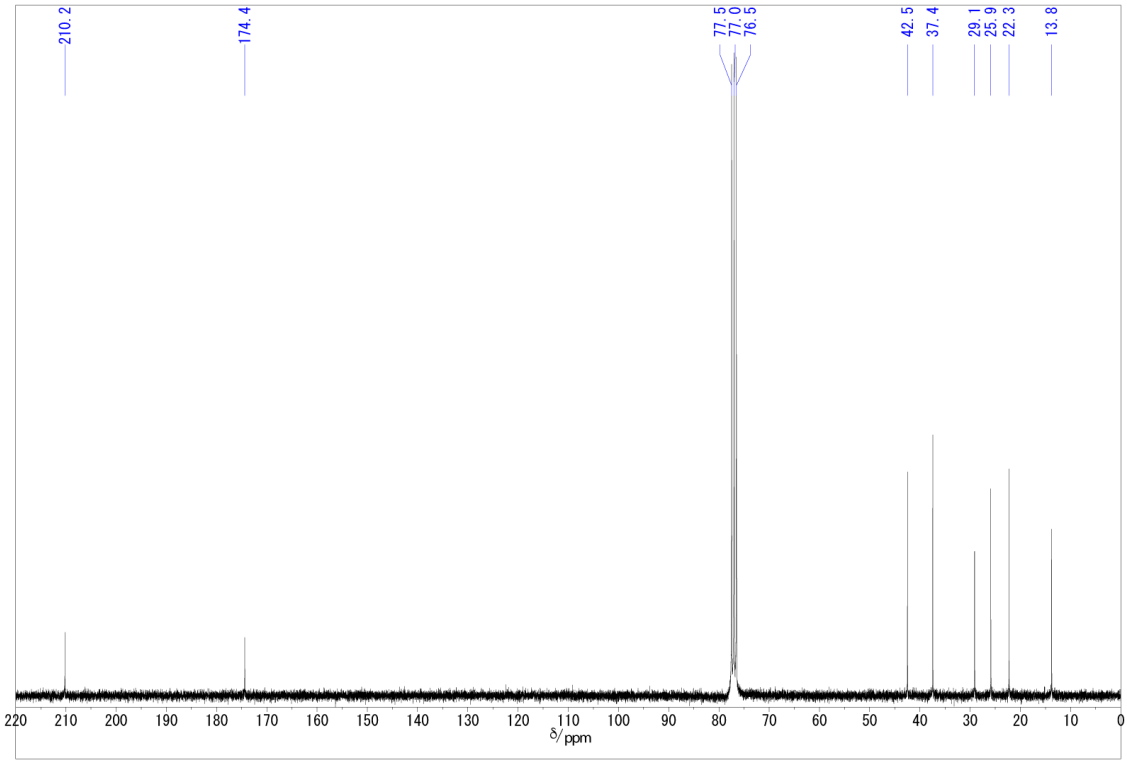

Fig. S2. ^{13}C NMR spectrum of deeopxy analogue (**4**) in $CDCl_3$ (67.5 MHz).

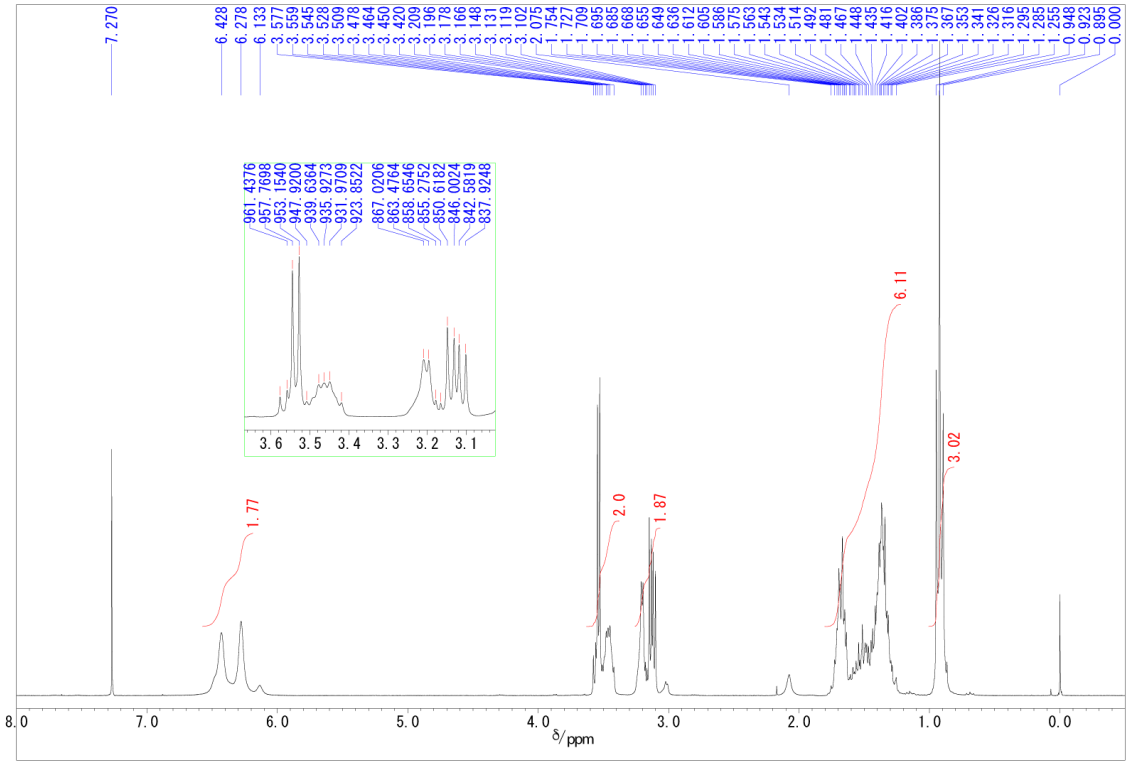

Fig. S3. ^{1}H NMR spectrum of epoxy amide alcohol (**5**) in $CDCl_3$ (270 MHz).

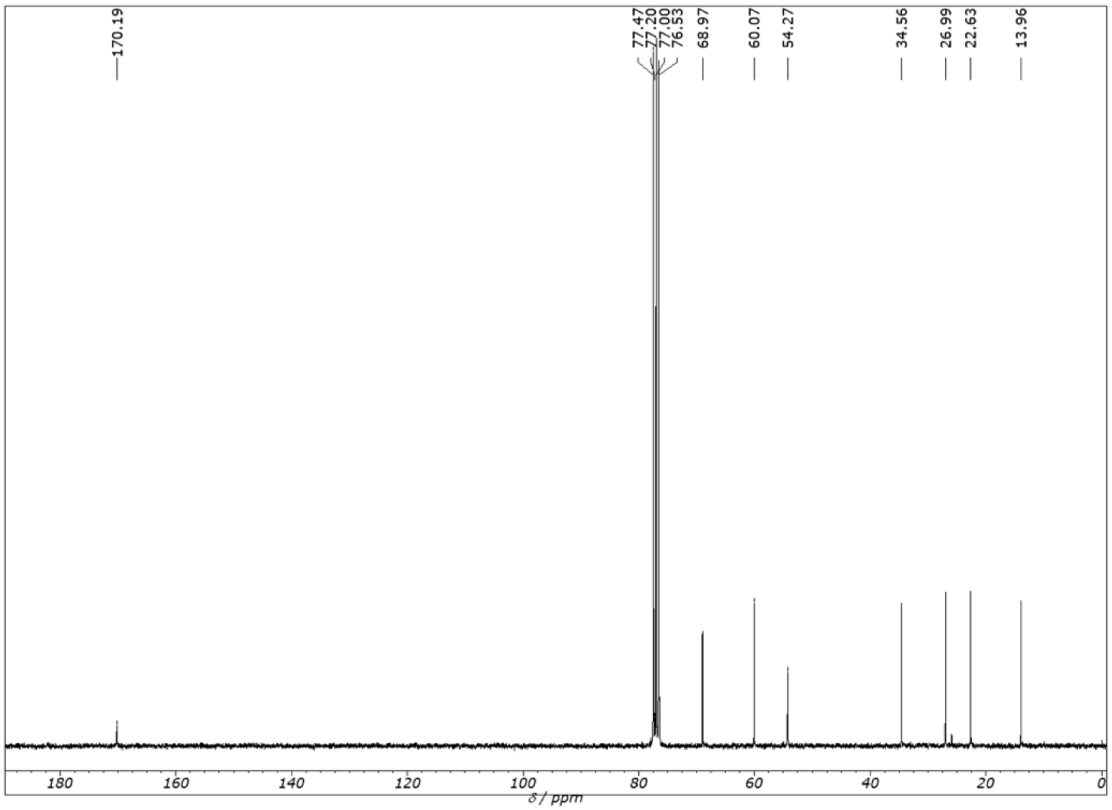

Fig. S4. ^{13}C NMR spectrum of epoxy amide alcohol (**5**) in $CDCl_3$ (67.5 MHz).

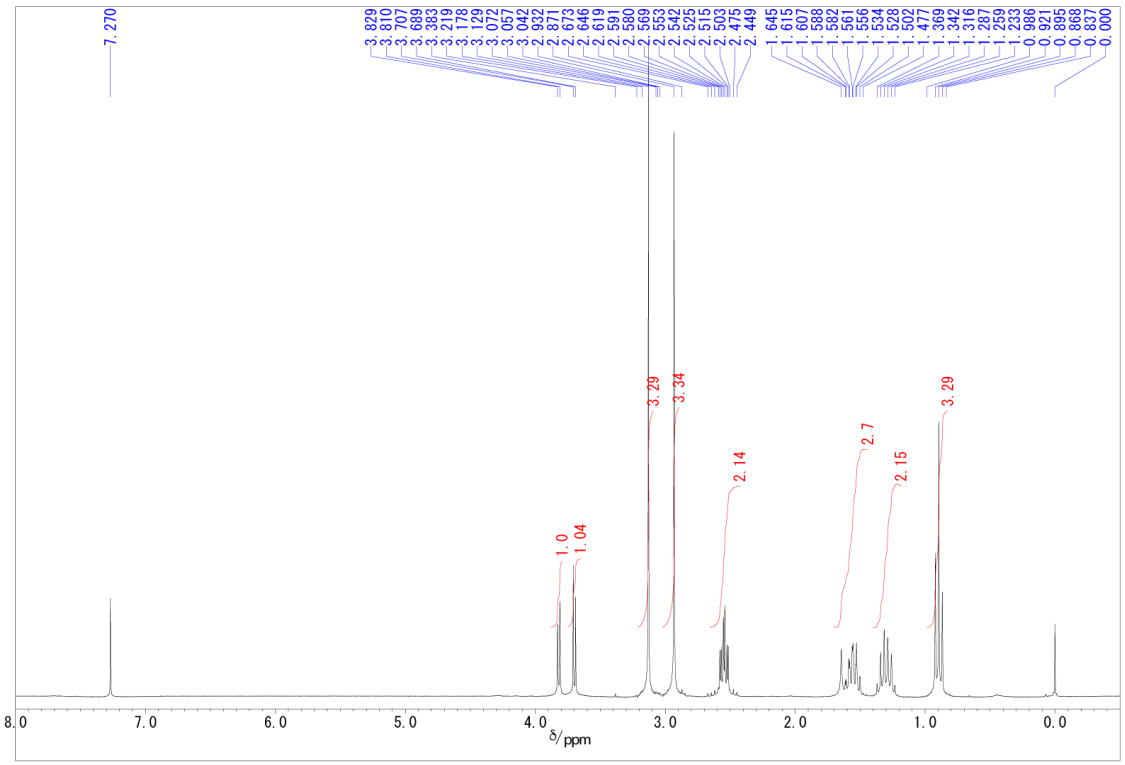

Fig. S5. ^{1}H NMR spectrum of *N,N*-dimethyl analogue (**6**) in $CDCl_3$ (270 MHz).

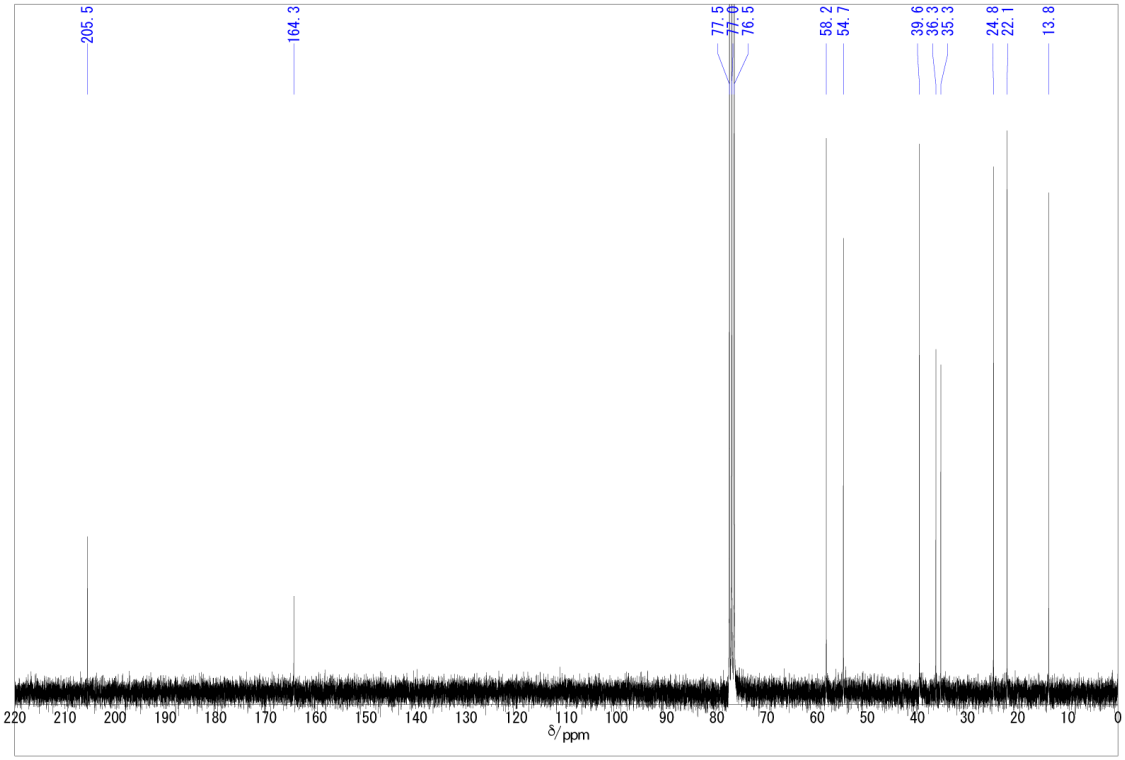

Fig. S6. ^{13}C NMR spectrum of *N,N*-dimethyl analogue (**6**) in $CDCl_3$ (67.5 MHz).

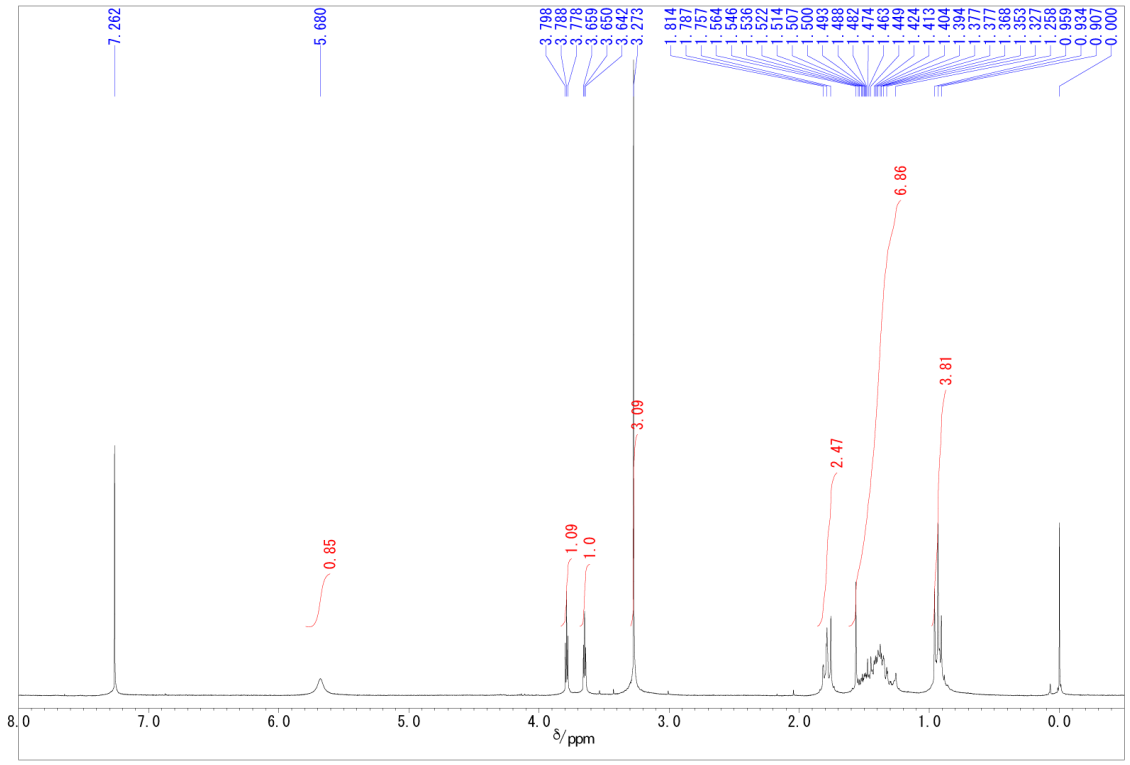

Fig. S7. ^{1}H NMR spectrum of *O*-methyl analogue (**7**) in $CDCl_3$ (270 MHz).

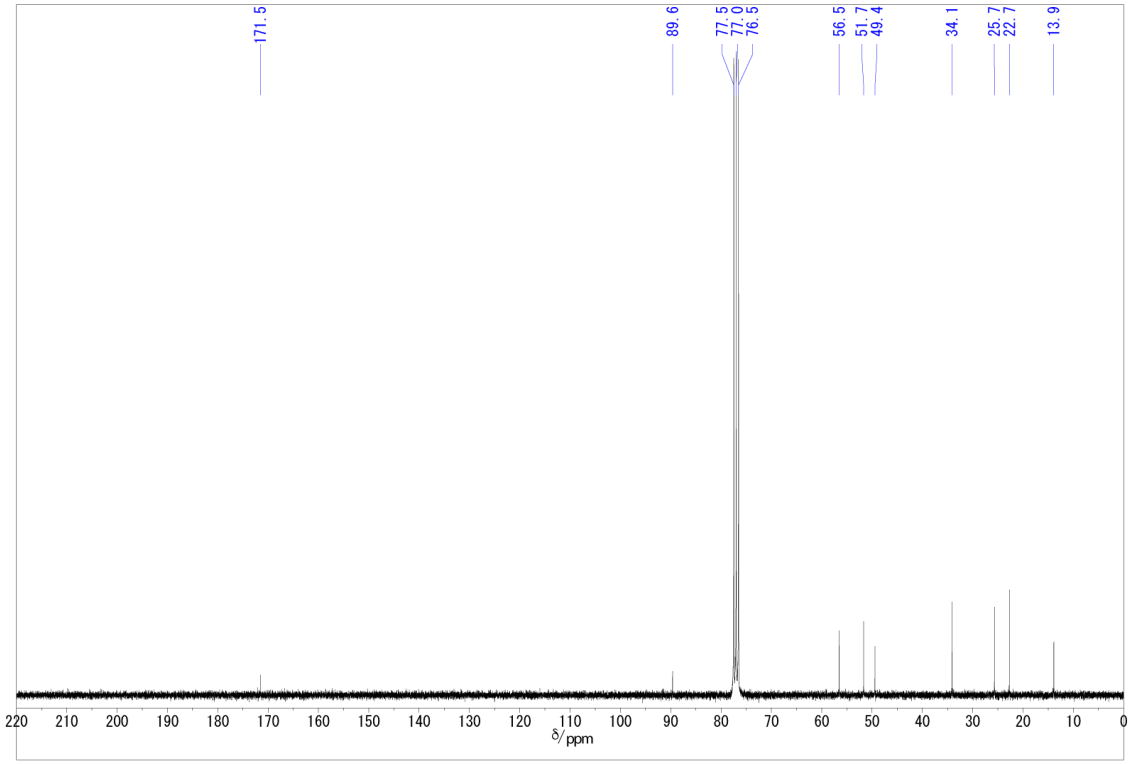

Fig. S8. ^{13}C NMR spectrum of *O*-methyl analogue (**7**) in $CDCl_3$ (67.5 MHz).

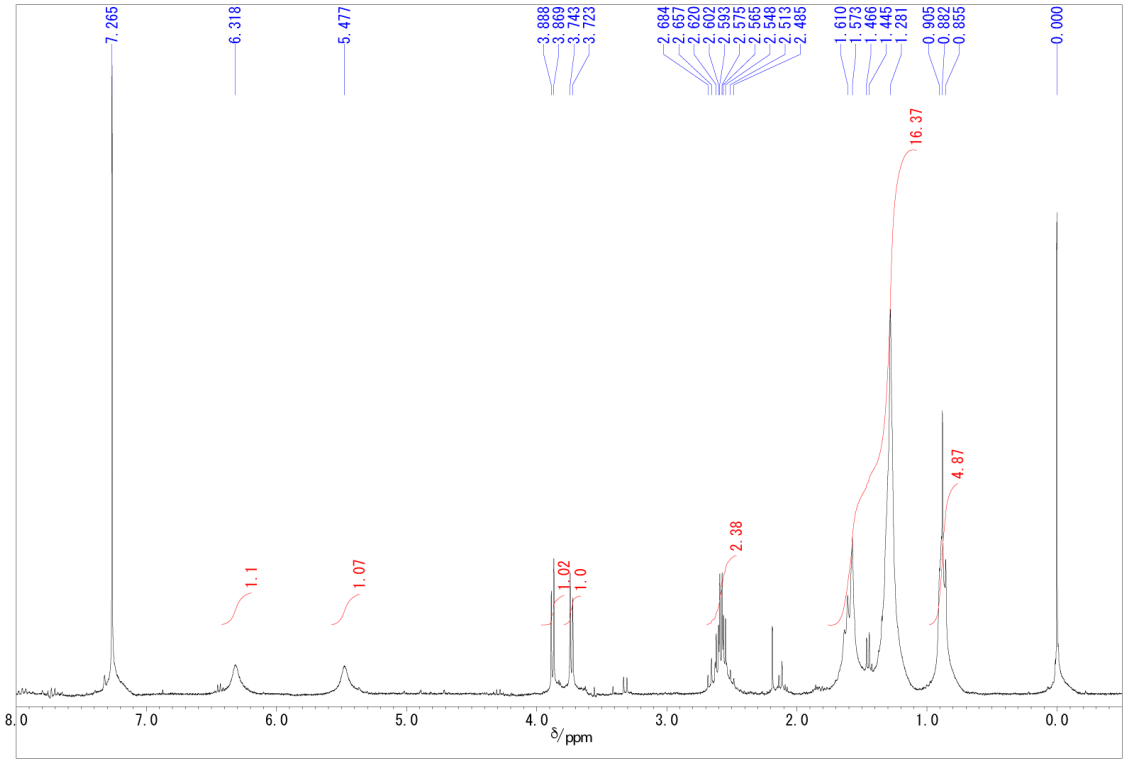

Fig. S9. ^{1}H NMR spectrum of hexyl analogue (**8**) in $CDCl_3$ (270 MHz).

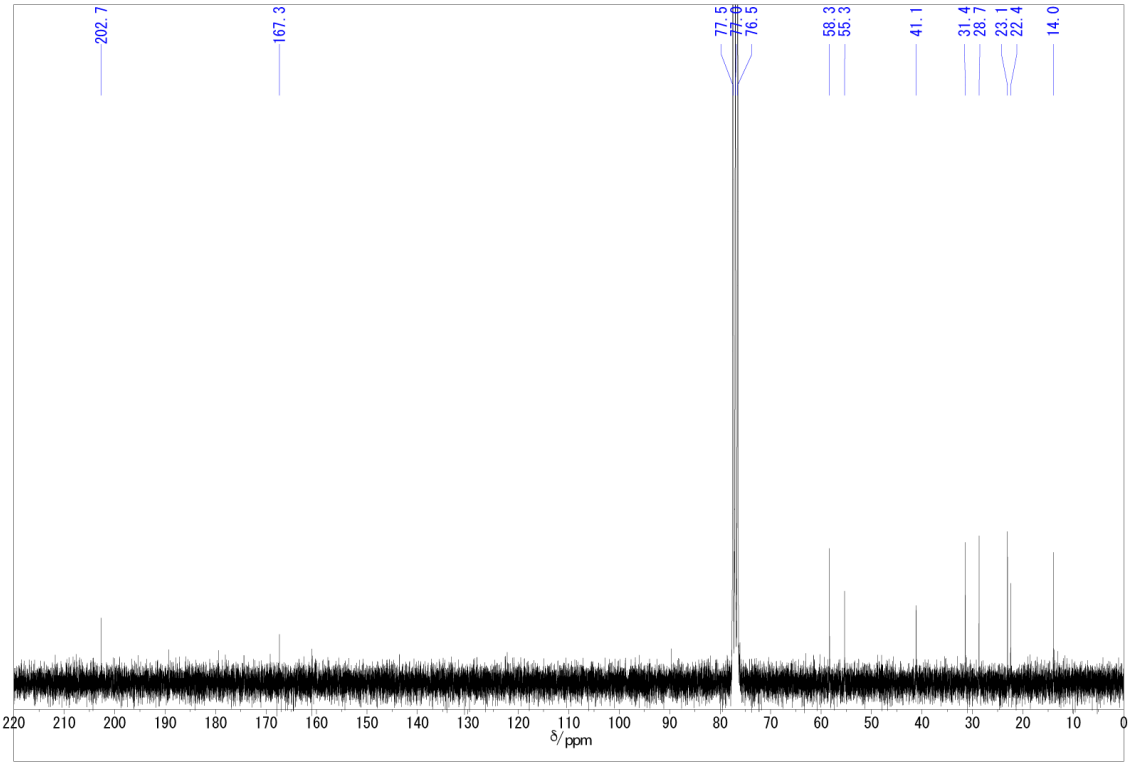

Fig. S10. ^{13}C NMR spectrum of hexyl analogue (**8**) in $CDCl_3$ (67.5 MHz).

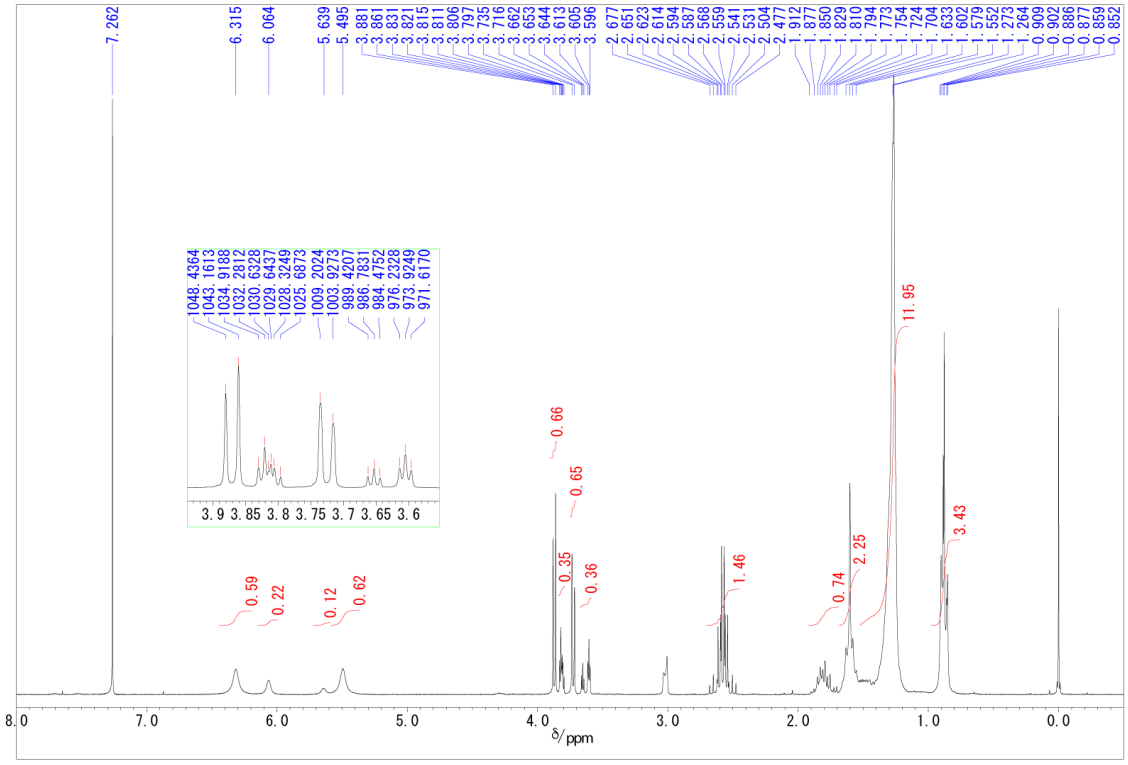

Fig. S11. ^{1}H NMR spectrum of octyl analogue (**9**) in $CDCl_3$ (270 MHz).

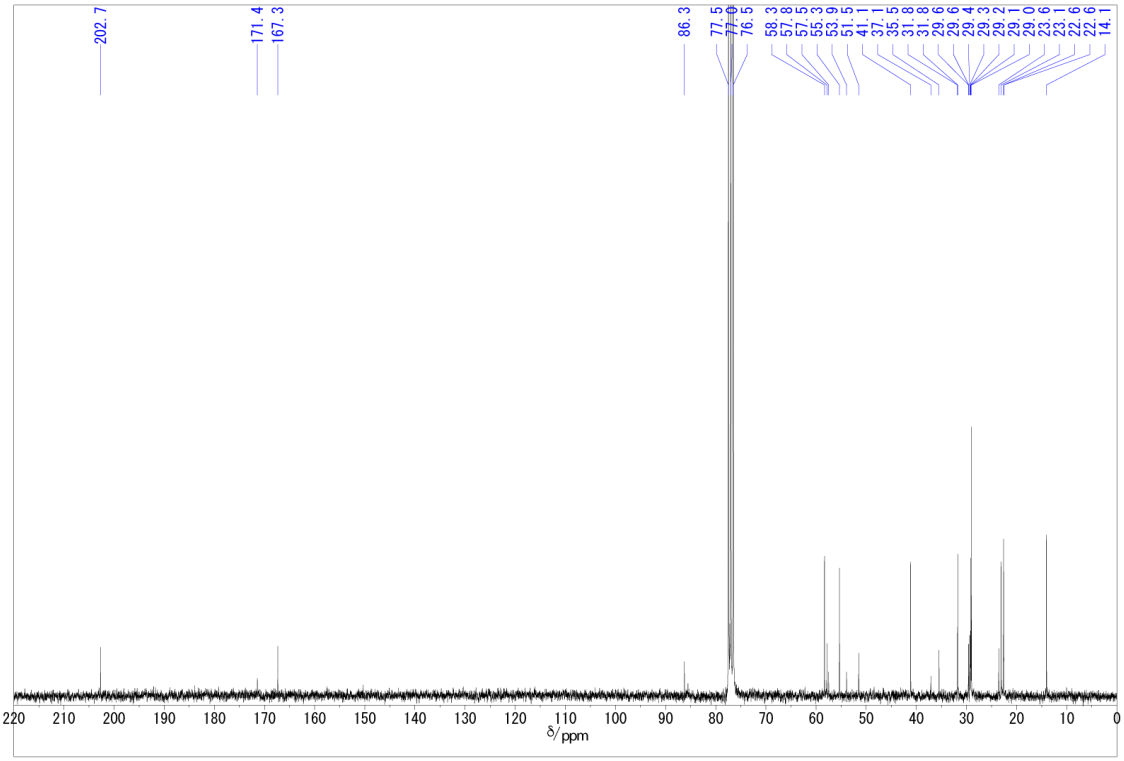

Fig. S12. ^{13}C NMR spectrum of octyl analogue (**9**) in $CDCl_3$ (67.5 MHz).

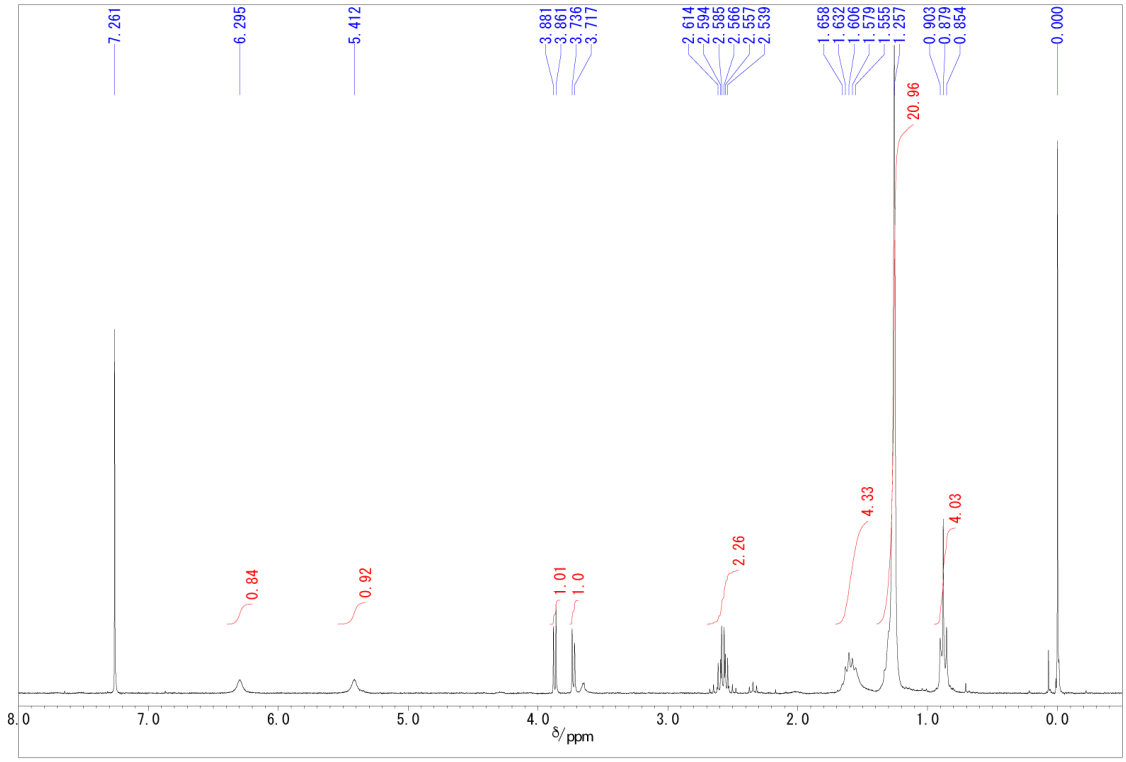

Fig. S13. ^{1}H NMR spectrum of decyl analogue (**10**) in $CDCl_3$ (270 MHz).

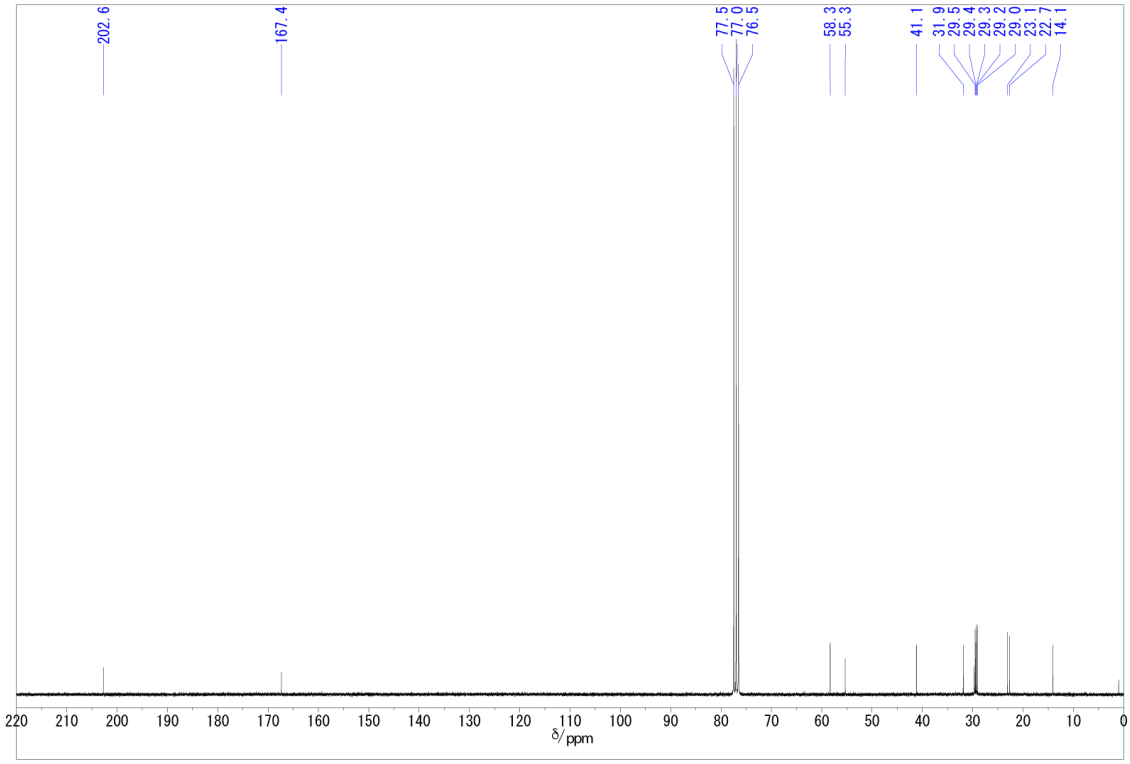

Fig. S14. ^{13}C NMR spectrum of decyl analogue (**10**) in $CDCl_3$ (67.5 MHz).

Table S1. Mole percentage of *keto* form (**1a**), *syn* form (**1b**), and *anti* form (**1c**) of (+)-epogymnolactam (**1**) in D_2O, CD_3OD, or $CDCl_3$.

solvent	**1a**	**1b**	**1c**
35% DMSO/D_2O	31 %	55 %	14 %
CD_3OD	20 %	64 %	16 %
$CDCl_3$	63 %	23 %	14 %

The ratio was estimated by ^1H NMR from the integral value of each epoxy proton (H-2 or H-3) of **1** in each solvent (4.0 mg/0.45 ml, incubated at 30 °C for 6 h).