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Syntheses and applications of fluorescent and biotinylated epolactaene derivatives: Epolactaene and its derivative induce disulfide formation

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Abstract—Epolactaene, isolated from cultured *Penicillium* sp. BM 1689-P mycelium, induces neurite outgrowth and arrests the cell cycle of the human neuroblastoma cell line, SH-SY5Y, at the G1 phase. We have found that epolactaene and its derivatives induce apoptosis in the human leukemia B-cell line, BALL-1. In this study, we prepared fluorescent and biotinylated epolactaene derivatives. We characterized the cellular location and the identification of BALL-1 proteins that reacted with these compounds. The results obtained from the reaction of epolactaene or its derivative with *N*-acetylcysteine methyl ester indicate that these compounds induce the disulfide formation and the α-position of the epoxylactam core is the reactive site.

1. Introduction

Epolactaene (**1**; Fig. 1), isolated from cultured *Penicillium* sp. BM 1689-P mycelium, induces neurite outgrowth and arrests the cell cycle of the human neuroblastoma cell line, SH-SY5Y, at the G1 phase.[1,2] After we accomplished a total synthesis of **1**, we further characterized its biological activities.[3,4] We found that **1** and its derivatives inhibited mammalian DNA polymerases and human DNA topoisomerase II in vitro.[5] We also found that they induce apoptosis in the human leukemia B-cell line, BALL-1.[6] Our structure–activity studies indicate that the epoxylactam core defines the pharmacophore and that the long hydrophobic side-chain probably interacts with complementary regions of target proteins.[5,6]

Concerning the mechanism of action of **1**, Nagumo et al. reported that, in cell lysates, the heat shock protein 60 (Hsp60) is targeted by a biotinylated epolactaene derivative (**bio-ETB**), which covalently binds to Cys^{442} of Hsp60, thereby inhibiting the protein's chaperone activity.[7] Furthermore, they suggested that Cys^{442} reversibly reacts with the α,β-unsaturated ketone of **1** via a Michael addition. However, epolactaene derivatives, such as Epo-C12 (**2**), which do not have an α,β-unsaturated ketone, still are cytotoxic and apoptosis-inducing.[6,7] Thus, the mechanism of action of **1** and its derivatives is still obscure.

2. Results and discussion

2.1. Preparation of fluorescent and biotinylated epolactaene derivatives

To further catalog the types of proteins targeted by **1**, we synthesized a fluorescent (**Flu-Epo-C12**) and a biotinylated (**Bio-Epo-C12**) derivative. Both probes were synthesized using the bridgehead oxiranyl anion strategy (Scheme 1), as described before.[3] By individually

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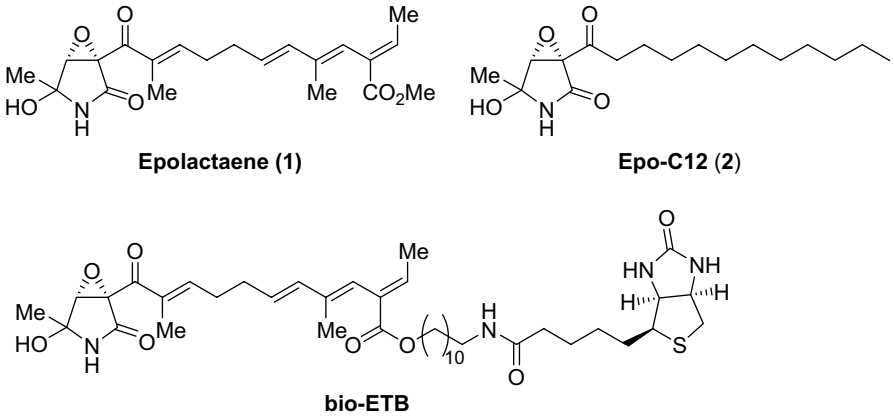
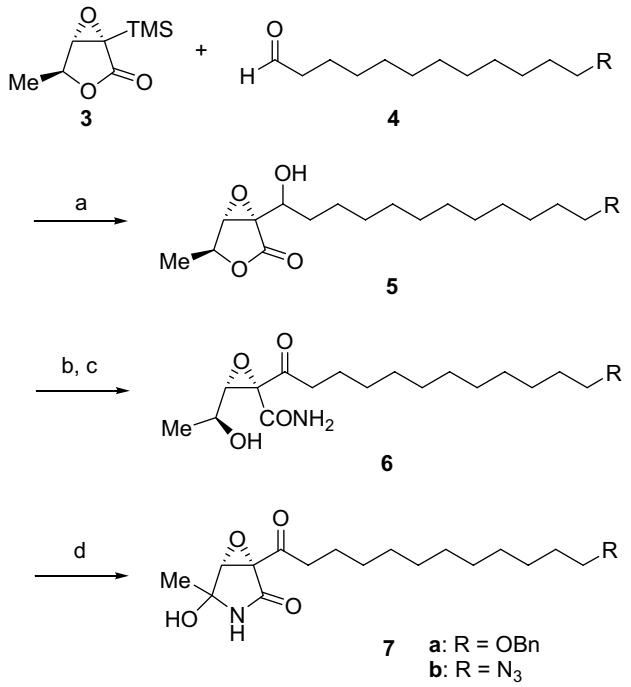


Figure 1. Structure of epolactaene (**1**), Epo-C12 (**2**), and **bio-ETB**. The synthesis of **bio-ETB** was reported by Nagumo et al.[7]



Scheme 1. Synthesis of **7**. Reagents and conditions: (a) TBAF (0.1 equiv), MS4A, THF–hexane, then aq HF, MeCN, **5a**: 59%, **5b**: 54% in 2 steps; (b) TFAA, DMSO, CH_2Cl_2, then Et_3N; (c) NH_3, MeOH, **6a**: 76%, **6b**: 71% in 2 steps; (d) TFAA, DMSO, CH_2Cl_2, then Et_3N; LiOH, THF–H_2O, **7a**: 79%, **7b**: 72% in 2 steps.

coupling silylated epoxylactone **3** with aldehydes **4**, and desilylating the products, the aldol products **5** were obtained as ca. 1:1 diastereomeric mixtures. After oxidation of **5** with trifluoroacetic anhydride and DMSO, ammonolysis of the corresponding ketones gave hydroxyamides **6**. Finally oxidation of **6** gave the α,β-epoxy-γ-lactam derivatives **7**.

Flu-Epo-C12 was prepared from **7a** (Scheme 2). Deprotection of the benzyl group gave the corresponding alcohol. Thermal decomposition of 7-diethylaminocoumarin-3-carbonyl azide gave the corresponding isocyanate, which reacted with the alcohol, gave the carbamate **Flu-Epo-C12**.[8] **Bio-Epo-C12** was synthesized from azide **7b**. Reduction of **7b** with $Pd(OH)_2$ under an H_2 atmosphere in the presence of biotin *N*-hydroxysuccinimide ester gave **Bio-Epo-C12**. Both **Flu-Epo-C12** and **Bio-Epo-C12** are cytotoxic for BALL-1 cells (Table 1).

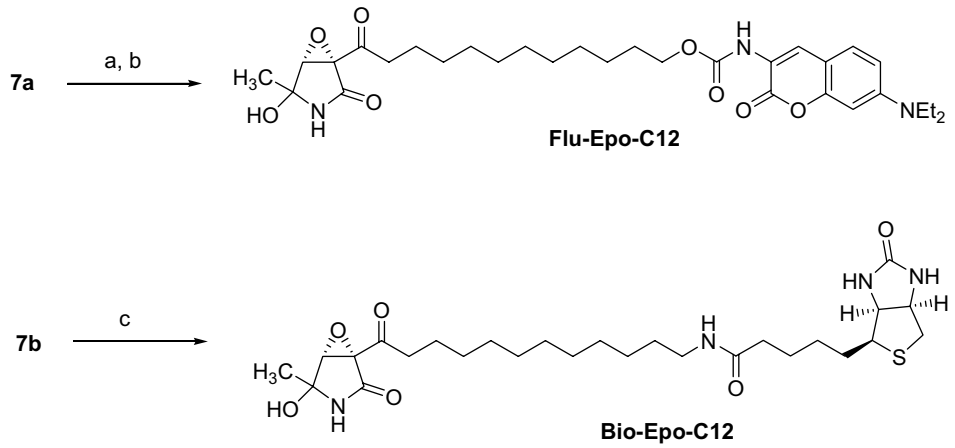
2.2. Cellular localization Flu-Epo-C12

To identify wherein cell **1** and its derivatives might accumulate, **Flu-Epo-C12** and MitoTracker were introduced into BALL-1 cells and localized by confocal laser scanning microscopy. MitoTracker is a fluorescent mitochondrial marker that binds cysteine thiols.[9] As shown in Figure 2, **Flu-Epo-C12** accumulates mainly in the mitochondria, as most of its fluorescence colocalized with that of MitoTracker. However, a low level of **Flu-Epo-C12** fluorescence was emitted from the cytoplasm. Therefore, both mitochondrial and cytoplasmic proteins bind **Flu-Epo-C12**.

2.3. Identification of binding protein of Bio-Epo-C12

Next, **Bio-Epo-C12** was used to identify cellular proteins that might bind to **1** and its derivatives. BALL-1 cell lysates were incubated with **Bio-Epo-C12**, biotin, or DMSO, and then exposed to streptavidin agarose beads. Proteins, isolated by this treatment were separated in SDS–PAGE gels and then stained with CBB (Fig. 3A and B). We detected seven bands in the **Bio-Epo-C12**-treated sample (lane 1) that were absent in the biotin-treated sample (lane 2) and the DMSO-treated sample (lane 3). Therefore, these proteins bound to **Bio-Epo-C12**.

We used peptide mass fingerprinting (PMF) to identify the proteins. Gel protein bands were isolated and the proteins trypsin-digested in situ (Supplementary information). The molecular weights of the tryptic peptides were measured by MALDI-TOF MS and compared with those derived theoretically for proteins of the database (NCBInr), using Mascot search program (Matrix science Inc., USA). The proteins identified are listed in Table 2 and include Hsp60 and fatty acid synthase (FAS), which bind epolactaene and cerulenin, respectively (Figs. 1 and 4).[7,10]



Scheme 2. Synthesis of **Flu-Epo-C12** and **Bio-Epo-C12**. Reagents and condition: (a) H_2, $Pd(OH)_2$–C, THF, 85%; (b) 7-diethylaminocoumarin-3-isocyanate, THF–toluene, 50 °C, 86%; (c) H_2, $Pd(OH)_2$–C, biotin *N*-hydroxysuccinimide ester, THF, 88%.

Table 1. Cytotoxicity of epolactaene (**1**), Epo-C12 (**2**), **Flu-Epo-C12**, and **Bio-Epo-C12** against BALL-1

Compound	IC_{50} value (μM)
Epolactaene (**1**)	3.82
Epo-C12 (**2**)	1.65
Flu-Epo-C12	1.56
Bio-Epo-C12	48.3

Fifty percent inhibitory concentrations (IC_{50}) acting against BALL-1 cell viability are shown.

Nagumo et al. reported that the Cys^{442} of Hsp60 might react with the α,β-unsaturated ketone group of **1** via a Michael addition.[7] However, **Bio-Epo-C12** does not contain an α,β-unsaturated ketone and yet it still binds Hsp60, suggesting that, instead, the thiol reacts with the epoxide of **Bio-Epo-C12**.

Cerulenin, which has a similar α,β-epoxy-γ-lactam moiety, binds and inhibits FAS, causing cytotoxicity and apoptosis in human cancer cell lines (Fig. 4).[10] The crystal structure of the cerulenin/*Escherichia coli* β-ketoacyl-acyl carrier protein synthase complex shows that cerulenin is covalently attached to the active site cysteine at the α-position of the α,β-epoxy-γ-lactam moiety.[11] Therefore, probably **1** and its derivatives also react with FAS at the α,β-epoxy-γ-lactam group.

2.4. A hypothetical mechanism of action of epolactaene and its derivatives

Next, to clarify how epolactaene (**1**) and its derivatives react with protein cysteines, **1** and Epo-C12 (**2**) were reacted with the model compound, *N*-acetylcysteine methyl ester (**8**).[12]

Treatment of **2** and **8** (1.2 equiv) in a 1:1 MeOH/0.5 M $NaHCO_3$ aq solution for 20 min gave **9** in 82% yield (Scheme 3). The proposed mechanism involves attack by the thiolate of **8** at the α-position of **2**, followed by a retro-aldol reaction, and then removal of pyruvic aldehyde to give **9**. No reaction occurred at neutral or acidic conditions. Other amino acid derivatives such as *N*-acetylhistidine methyl ester, *N*-acetyllysine methyl ester, and *N*-acetylarginine methyl ester did not react with **2**. Therefore, Epo-C12 (**2**) seems to react specifically with cysteine thiols.

Interestingly, treatment of **2** with an excess amount of **8** (2.4 equiv) in a 1:1 MeOH/0.5 M $NaHCO_3$ aq solution for 24 h gave dodecanoic acid (**10**) in 36% yield and disulfide (**11**) in 64% yield (Scheme 4). Yields were calculated using the amounts of **2** and **8**, respectively. The proposed reaction mechanism involves the initial formation of the β-sulfanylketoamide, followed by a slow retro-Claisen reaction to give **10**. Excess **8** then reacts with methyl *N*-acetyl-*S*-(2-amino-2-oxoethyl)cysteinate, the intermediate of the retro-Claisen reaction, yielding **11**.[13] Thus, **2** might induce intramolecular or intermolecular disulfide formation between protein cysteines.

The reaction of **1** and **8** (1.2 equiv) smoothly produced the carboxylic acid **12** in 72% yield and the disulfide **11** in 77% yield (Scheme 5). Yields were calculated using the amounts of **1** and **8**, respectively. After the formation of the β-sulfanylketoamide, the retro-Claisen reaction and disulfide formation immediately proceeded.[13b] Thus, the intermediate β-sulfanylketoamide was not isolated in this reaction. Although the reactivity of **1** against **8** is different from that of **2**, the formation of **11** and **12** strongly indicates that **1** binds proteins at the lactam epoxide. The formation of **12** would explain the fact that, after reaction of Hsp60 with **bio-ETB**, the biotin-labeled amount of Hsp60 reduced with time.[7b] Furthermore, the formation of disulfide **11** suggests that **1** might smoothly induce intramolecular or intermolecular disulfide formation between protein cysteines.

3. Conclusion

We prepared fluorescent and biotinylated epolactaene derivatives. We characterized the cellular location and the identification of BALL-1 proteins that reacted with these compounds. The results obtained from the reaction of epolactaene (**1**) or Epo-C12 (**2**) with *N*-acetylcys-

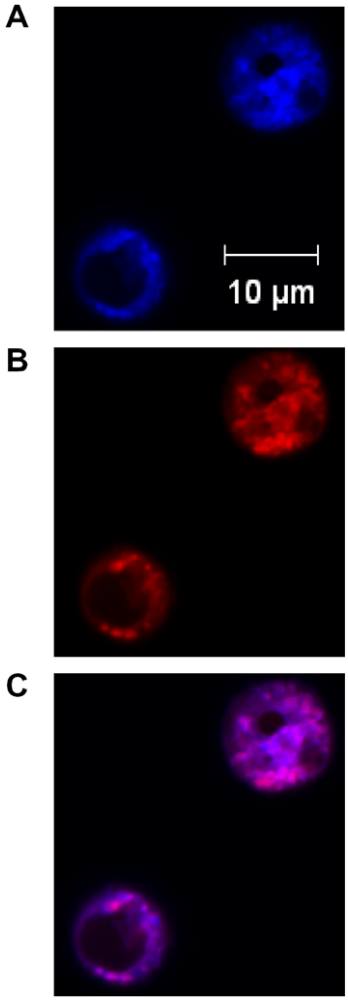


Figure 2. Staining of BALL-1 cells by Flu-Epo-C12 and MitoTracker. BALL-1 cells were treated with **Flu-Epo-C12** and MitoTracker for 1 h and then washed with PBS. Confocal images of **Flu-Epo-C12** (A) and MitoTracker (B). The superimposed fluorescent images are shown in (C).

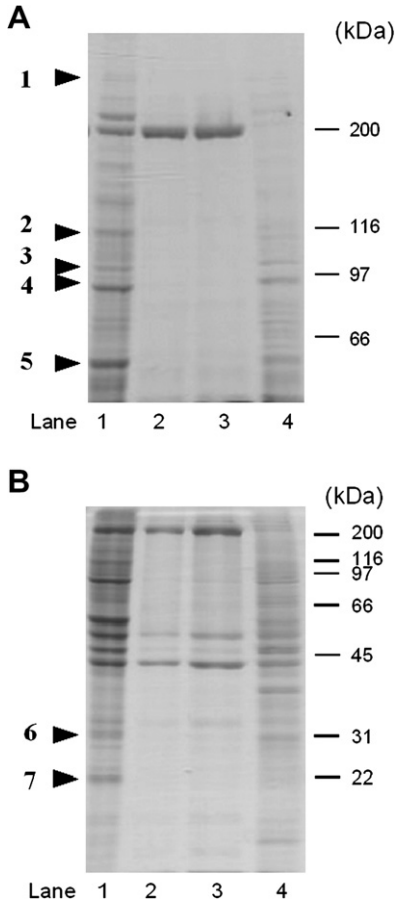


Figure 3. Detection of proteins that react with **Bio-Epo-C12**. BALL-1 cell lysates were incubated with 5 μM **Bio-Epo-C12**, 5 μM biotin, or 5 μM DMSO, and then exposed to streptavidin agarose beads. The proteins that were recovered from the beads were separated by electrophoresis in (A) 6% SDS–PAGE and (B) 12% SDS–PAGE gels and stained with CBB. Lane 1, **Bio-Epo-C12**; lane 2, biotin; lane 3, DMSO; lane 4, BALL-1 cell lysates.

teine methyl ester (**8**) indicate that the α-position of the epoxylactam core is the reactive site. Both epolactaene (**1**) and Epo-C12 (**2**) might induce intramolecular or intermolecular disulfide formation between protein cysteines.

Additional biological studies concerning the mechanism of action of **1** and its derivatives are currently underway.

4. Experimental procedure

4.1. General (Chemistry)

All non-aqueous reactions were carried out by using freshly distilled solvents under argon atmosphere. THF was distilled from sodium/benzophenone prior to use. Dichloromethane was distilled from P_2O_5 prior to use. *N*,*N*-Dimethylformamide and propylene oxide were distilled from calcium hydride prior to use. All other reagents were commercially available and used without further purification. 7-(Diethylamino)coumarin-3-carbonyl azide was purchased from Molecular Probes (now known as Invitrogen, USA). *N*-Acetyl-L-cystine dimethyl ester was obtained from Bachem (Switzerland). *N*-Acetyl-L-cysteine (**8**) was prepared by a reduction of *N*-acetyl-L-cystine dimethyl ester (**11**) with zinc.[14]

All reactions were monitored by TLC, which was carried out on Silica Gel 60 F_{254} plates (Merck, Germany).

Table 2. The identification of BALL-1 proteins that react with **Bio-Epo-C12**

Band	Protein	M_w (kDa)	Location
1	Fatty acid synthase	273	Cytoplasm
2	ATP citrate lyase	121	Cytoplasm
3	Elongation factor 2	95	Cytoplasm
4	Heat shock protein 90b	83	Cytoplasm
5	Heat shock protein 60	61	Mitochondria
6	Adenine nucleotide translocator 2	33	Mitochondria
7	Peroxiredoxin 1 (Prx1)	22	Cytoplasm

The protein gel bands were separated and digested with trypsin. M_w is the protein's calculated molecular weight.

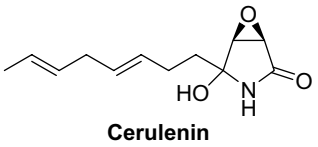


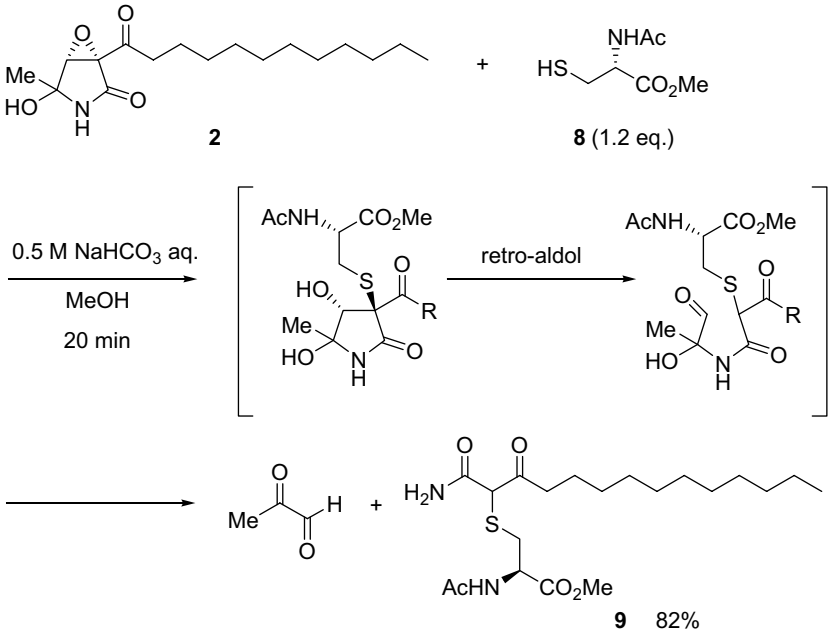
Figure 4. Structure of cerulenin.

Flash chromatography separations were performed on PSQ 100B (Fuji Silysia Co., Ltd, Japan).

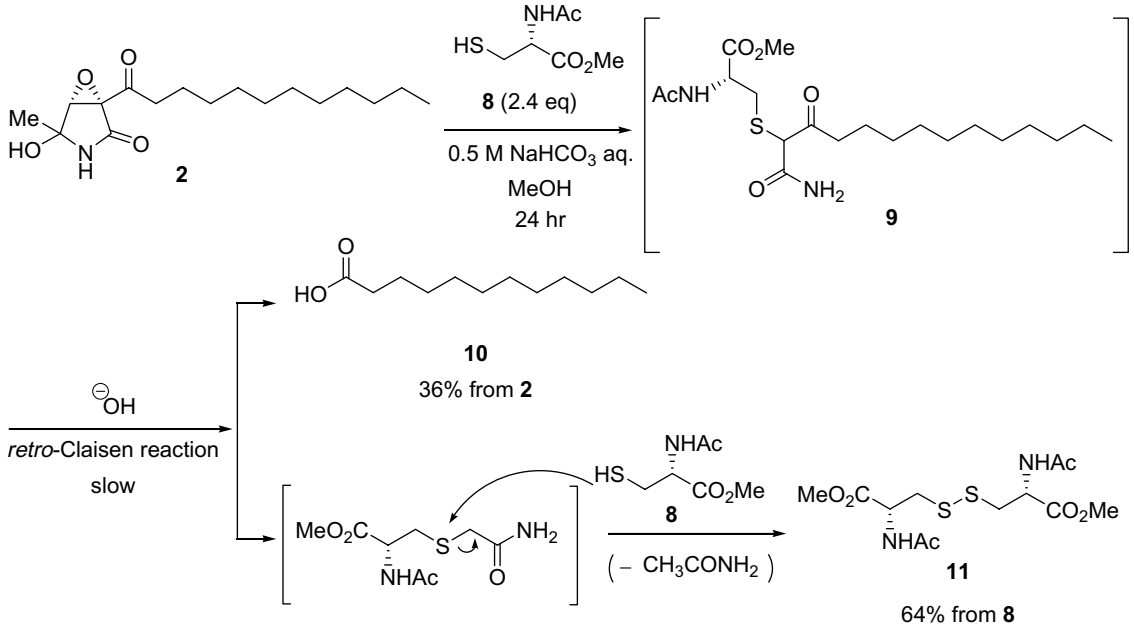
^{1}H and ^{13}C NMR spectra were recorded on a Bruker 600 MHz or 400 MHz spectrometer (Avance DRX-600, Avance DRX-400) or a JEOL 400 MHz spectrometer (JNM-LD400), using $CDCl_3$ (with TMS for ^{1}H NMR and chloroform-*d* for ^{13}C NMR as the internal reference) solution, unless otherwise noted. Chemical shifts were expressed in δ (ppm) relative to Me_4Si or residual solvent resonance, and coupling constants (*J*) were expressed in Hz.

Melting points were determined with Yanaco MP-3S and were uncorrected.

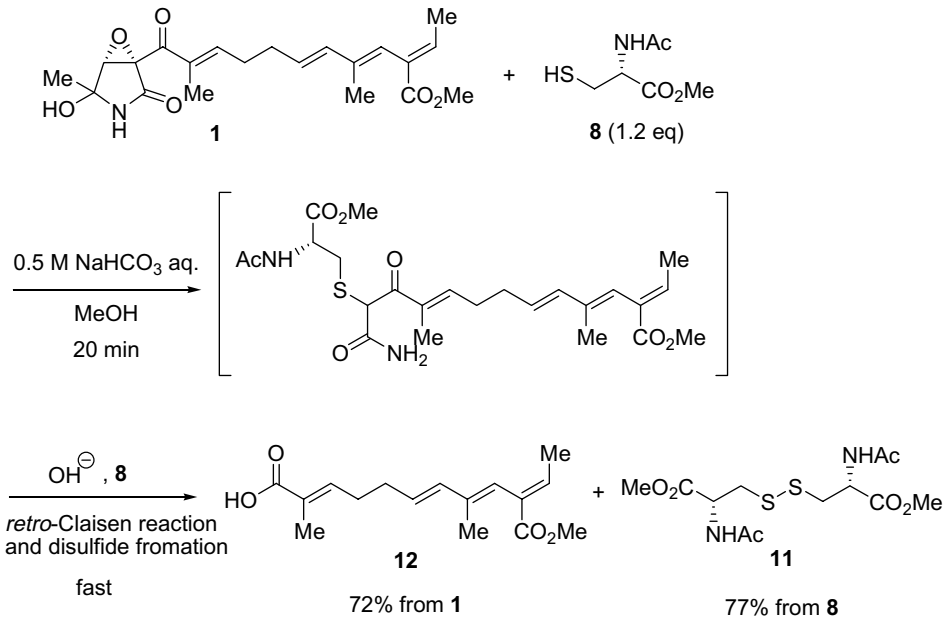
Optical rotations were recorded on a JASCO P-1030 digital polarimeter at room temperature.



Scheme 3. Treatment of Epo-C12 (**2**) with 1.2 equiv of *N*-acetylcysteine methyl ester (**8**): a plausible mechanism for the formation of **9** (R = $C_{11}H_{23}$).



Scheme 4. Treatment of Epo-C12 (**2**) with excess amount of *N*-acetylcysteine methyl ester (**8**): a plausible mechanism for the formation of **10** and **11**.



Scheme 5. Treatment of epolactaene (**1**) with 1.2 equiv of *N*-acetylcysteine methyl ester (**8**): a plausible mechanism for the formation of **11** and **12**.

Infrared spectra (IR) were recorded on a Jasco FT/IR-410 spectrometer using NaCl (neat) or KBr pellets (solid), and were reported on wavenumbers (cm^{-1}).

Mass spectra (MS) were obtained on an Applied Biosystems mass spectrometer (APIQSTAR pulsar i) under conditions as High resolution, using poly (ethylene glycol) as internal standard.

4.2. General (Biology)

Fetal bovine serum (FBS) was purchased from Biowest (Loire Valley, French) Kanamycin sulfate, dimethyl sulfoxide (DMSO), biotin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Wako Pure Chemical Industries (Tokyo, Japan). RPMI 1640 was purchased from Nissui (Tokyo, Japan). 2-Mercaptoethanol was purchased from Nacalai Tesque (Kyoto, Japan). Protease inhibitor cocktail was obtained from Sigma–Aldrich (ST. Louis, MO). Streptavidin agarose gel was purchased from GE Healthcare (London, UK). MitoTracker Red CMXROS dye was purchased from Molecular Probes (now known as Invitrogen, USA).

The confocal images were taken by LSM 5 PASCAL EXCITER laser scanning confocal microscopy (Zeiss, Germany).

MALDI-TOF MS measurements were performed using a Bruker Daltonics Reflex IV MALDI-TOF mass spectrometer (Bremen, Germany). Spectra were externally calibrated with bradykinin, neurotensin, and melittin.

4.3. Preparation of Fluo-Epo-C12 and Bio-Epo-C12

4.3.1. (1*R*,1′*RS,5*R*)-1-(1′-Hydroxy-12′-benzyloxydodecyl)-4-hydroxy-4-methyl-6-oxa-3-aza-bicyclo[3.1.0]hexan-2-one (5a).** To a solution of **3** (151 mg, 0.81 mmol),[3] **4a** (160 mg, 0.55 mmol),[15] and MS4A (0.5 g) in THF–hexane (1:1, 6 mL) was added TBAF (100 μL of a 1.0 M solution in THF, 0.1 mmol), which had been dried with MS4A for 3 h before use. The mixture was stirred at rt for 24 h, and then filtered through Celite and washed with EtOAc. The filtrate was washed with H_2O, brine, dried (Na_2SO_4), and concentrated. A solution of the residue in CH_3CN (1 mL) was added a solution of 5% aqueous HF in CH_3CN (1.5 mL), and the mixture was stirred at rt for 20 min. The mixture was quenched by the addition of saturated aqueous $NaHCO_3$ solution and extracted with EtOAc. The combined extract was washed with brine, dried (Na_2SO_4), and concentrated. The residue was purified by chromatography (hexane/EtOAc = 3:1 to 1:1) to afford **5a** (130 mg, 59%) as a 1:1 diastereomeric mixture as colorless oil. IR (neat) 3444, 3025, 2928, 2855, 1777, 1455, 1339, 1217, 1096, 1071, 1005, 939, 853 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$) δ 7.34 (4H, m), 7.34 (4H, m)*, 7.28 (1H, m), 7.28 (1H, m)*, 4.66 (1H, m), 4.66 (1H, m)*, 4.50 (2H, s), 4.50 (2H, s)*, 4.23 (1H, dt, J = 8.8 Hz, 3.2 Hz)*, 4.14 (1H, m), 3.97 (1H, s), 3.97 (1H, s)*, 3.46 (2H, t, J = 6.7 Hz), 3.46 (2H, t, J = 6.7 Hz)*, 2.19 (1H, m)*, 2.10 (1H, br s), 1.78–1.69 (2H, m), 1.78–1.69 (2H, m)*, 1.63–1.49 (4H, m), 1.63–1.49 (4H, m)*, 1.40 (3H, d,J = 6.8 Hz), 1.40 (3H, d,J = 6.8 Hz)*, 1.38–1.27 (14H, m), 1.38–1.27 (14H, m)*; ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.9, 170.5, 138.6 (×2), 128.2 (×4), 127.5 (×4), 127.4 (×2), 75.34, 75.31, 72.7 (×2), 70.5 (×2), 66.2, 64.7, 62.0, 61.7 (×2), 61.6, 32.4, 32.2, 29.7 (×2), 29.5 (×2), 29.43 (×4), 29.37 (×4), 29.23, 29.16, 26.1 (×2), 25.3, 25.2, 17.8, 17.7 (diastereomeric mixture); HRMS, calcd for $C_{24}H_{36}O_5Na$ ($[M+Na]^+$) 427.2454, found 427.2457.

4.3.2. (2*S*,3*S*)-3-[(*S*)-1-Hydroxyethyl]-2-(12′-Benzyloxydodecanoyl)oxirane-2-carboxamaide (6a). To a solution of DMSO (30 μL, 0.42 mmol) in CH_2Cl_2 (1 mL) was added TFAA (30 μL, 0.21 mmol) at −78 °C. After 10 min, a solution of **5a** (28 mg, 0.07 mmol) in CH_2Cl_2

(1.5 mL) was added to the mixture, and the mixture was −78 °C for 30 min. Then Et_3N (90 μL, 0.65 mmol) was added to the mixture, and the mixture was stirred at rt for 5 min. The mixture was quenched by the addition of H_2O and extracted with $CHCl_3$. The combined extract was washed with brine, dried (Na_2SO_4), and concentrated.

To a solution of NH_3 (~0.3 mL) in MeOH (2 mL) was added a solution of the residue in MeOH (2 mL) at 0 °C, and the mixture was stirred for 30 min. Then the mixture was concentrated. The residue was purified by chromatography (hexane/EtOAc = 1:1 to 1:2) to afford **6a** (22 mg, 76%) as white solid. Mp = 68–69 °C; $[\alpha]_D^{22}$ +16.1 (*c* 1.1, MeOH); IR (KBr) 3391, 3196, 2919, 2849, 2797, 1717, 1641, 1462, 1395, 1368, 1290, 1210, 1110, 1060, 964, 898, 740 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.34 (4H, m), 7.28 (1H, m), 6.84 (1H, br s), 6.35 (1H, br s), 4.50 (2H, s), 3.65 (1H, m), 3.46 (2H, t, J = 6.6 Hz), 3.16 (1H, d, J = 7.8 Hz), 2.66 (1H, ddd, J = 18.0 Hz, 8.0 Hz, 6.6 Hz), 2.49 (1H, dt, J = 18.0 Hz, 8.0 Hz, 6.7 Hz), 1.97 (1H, br s), 1.59 (4H, m), 1.38 (3H, d, J = 6.3 Hz), 1.26 (14H, br s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 203.7, 167.0, 138.6, 128.3 (×2), 127.6 (×2), 127.4, 72.8, 70.5, 65.6, 65.42, 65.39, 38.0, 29.7, 29.5, 29.43, 29.41, 29.33, 29.25, 28.9, 26.1, 23.0, 20.2; HRMS, calcd for $C_{24}H_{37}NO_5Na$ ($[M+Na]^+$) 442.2563, found 442.2565.

4.3.3. (1*R*,5*R*)-1-(12′-Benzyloxydodecanoyl)-4-hydroxy-4-methyl-6-oxa-3-aza-bicyclo[3.1.0]hexan-2-one (7a). To a solution of DMSO (160 μL, 2.25 mmol) in CH_2Cl_2 (3 mL) was added TFAA (200 μL, 1.41 mmol) at −78 °C and the mixture was stirred at −78 °C. After 5 min, a solution of **6a** (158 mg, 0.38 mmol) in CH_2Cl_2 (5 mL) was added to the mixture and the mixture was stirred at −78 °C for 60 min. Then Et_3N (0.5 mL) was added to the reaction mixture and the mixture was stirred at room temperature for 5 min. The mixture was quenched by the addition of H_2O and extracted with EtOAc. The extract was washed with brine, dried (Na_2SO_4), and concentrated. To a solution of the residue in THF–H_2O (4:1, 5 mL) was added LiOH (9 mg, 0.38 mmol) at 0 °C. After the mixture was stirred at 0 °C for 10 min, the mixture was quenched by the addition of H_2O and extracted with EtOAc. The extract was washed with brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (hexane/EtOAc = 1:1) to give **7a** (125 mg, 79%) as a 7.5:1 tautomeric mixture, as colorless needle. Mp = 53–55 °C; $[\alpha]_D^{24}$ −71.9 (*c* 1.2, MeOH); IR (KBr) 3463, 3289, 2926, 2853, 1740, 1690, 1496, 1455, 1403, 1363, 1160, 1115, 1028, 945, 736, 698, 651, 621 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$) δ 7.87 (1H, br m), 7.35–7.28 (4H, br m), 7.26 (1H, m), 5.08 (1H, br s), 4.50 (2H, s), 4.17 (1H, d, J = 1.9 Hz), 3.46 (2H, t, J = 6.7 Hz), 2.50–2.45 (1H, m), 2.32–2.25 (1H, m), 1.60 (2H, m), 1.55 (3H, s), 1.54–1.50 (1H, m), 1.36–1.32 (2H, m), 1.43–1.38 (1H, m)1.30–1.22 (12H, br m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 202.1, 169.0, 138.7, 128.3 (×2), 127.6 (×2), 127.4, 83.5, 72.8, 70.5, 66.0, 61.3, 38.3, 29.7, 29.5 (×2), 29.4 (×2), 29.3, 28.9, 26.2, 22.6, 21.5; HRMS, calcd for $C_{24}H_{35}NO_5Na$ ($[M+Na]^+$) 440.2407, found 440.2400.

4.3.4. (1*R*,5*R*)-1-(12′-Hydroxydodecanoyl)-4-hydroxy-4-methyl-6-oxa-3-aza-bicyclo[3.1.0]hexan-2-one (13). A solution of **7a** (67.1 mg, 0.16 mmol) and $Pd(OH)_2$ on carbon powder (20% Pd, 28.1 mg) in THF (6 mL) was stirred at room temperature for 9.5 h. The mixture was filtered through Celite and washed with EtOAc. The filtrate was concentrated. The residue was purified by column chromatography (hexane/EtOAc = 1:2) to give **13** (44.7 mg, 85%) as white solid. Mp = 112–115 °C; $[\alpha]_D^{24}$ −93.3 (*c* 0.65, MeOH); IR (KBr) 3521, 3369, 2920, 2852, 1737, 1685, 1469, 1423, 1241, 1160, 1112, 1051, 957, 937, 881, 814, 754, 718, 674, 628 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 4.07 (1H, s), 3.53 (2H, t, J = 6.7 Hz), 2.60 (2H, t, J = 7.3 Hz), 1.58 (2H, m), 1.51 (2H, m), 1.46 (3H, s),1.30 (14H, br m); ^{13}C NMR (100 MHz, CD_3OD) δ 202.2, 170.7, 84.1, 66.9, 63.0, 40.3, 33.7, 30.73, 30.65 (× 2), 30.59, 30.56, 30.5, 30.1, 26.9, 23.8, 21.9; HRMS, calcd for $C_{17}H_{29}NO_5Na$ ($[M+Na]^+$) 350.1937, found 350.1919.

4.3.5. (1*R*,5*R*)-(7″-Diethylamino-2″-oxo-2″*H*-chromen-3″-yl)carbamic acid 12′-(4-hydroxy-4-methyl-2-oxo-6-oxa-3-aza-bicyclo[3.1.0]hex-1-yl)-12′-oxo-dodecyl ester (Flu-Epo-C12). A solution of 7-(diethylamino)coumarin-3-carbonyl azide (15.6 mg, 0.061 mmol) in toluene (4 mL) was stirred at 100 °C for 10 min. The solution of the crude isocyanate was added to a solution of **13** (12.0 mg, 0.042 mmol). After the mixture was stirred at 50 °C for 9 h, the mixture was concentrated. The residue was purified by PTLC (hexane/EtOAc = 1:2) to give **Fluo-Epo-C12** (21.1 mg, 86%) as yellow solid. Mp = 115–116 °C; $[\alpha]_D^{24}$ −48.2 (*c* 0.39, MeOH); IR (KBr) 3405, 3314, 3020, 2929, 2855, 1740, 1698, 1611, 1523, 1410, 1361, 1305, 1217, 1160, 1131, 1036, 945, 757, 667 cm^{-1}; ^1H NMR (400 MHz, $CD_3OD/CDCl_3$) δ 8.14 (1H, br s), 7.31 (1H, d, J = 8.8 Hz), 6.70 (1H, dd, J = 8.8 Hz, 2.4 Hz), 6.51 (1H, d, J = 2.4 Hz), 4.17 (2H, t, J = 6.6 Hz), 4.03 (1H, s), 3.44 (4H, q, J = 7.1 Hz), 2.62 (2H, t, J = 7.3 Hz), 1.72–1.61 (2H, m), 1.61–1.58 (2H, m), 1.48 (3H, m), 1.41–1.31 (14H, br m), 1.21 (6H, t, J=7.1 Hz); ^{13}C NMR (100 MHz, $CD_3OD/CDCl_3$) δ 202.1, 170.3, 160.7, 155.5, 153.7, 150.6, 129.3, 110.8, 109.3, 98.0, 83.8, 66.7 (×2), 63.0, 45.4 (×2), 40.3, 30.3 (×2), 30.27, 30.2, 30.1, 29.84, 29.76, 26.7, 23.6, 21.8, 12.8 (×2); HRMS, calcd for $C_{31}H_{44}N_3O_8Na$ ($[M+H]^+$) 586.3122, found 586.3112.

4.3.6. 12-Azidododecanal (4b). To a solution of DMSO (0.36 mL, 5.1 mmol) in CH_2Cl_2 (3 mL) was added $(COCl)_2$ (0.22 mL, 2.5 mmol) at −78 °C. After 10 min, a solution of 12-azidododecanol (194 mg, 0.85 mmol)[16] in CH_2Cl_2 (3 mL) was added to the mixture, and the mixture was −78 °C for 30 min. Then Et_3N (0.7 mL, 5.0 mmol) was added to the mixture, and the mixture was stirred at rt for 15 min. The mixture was quenched by the addition of H_2O and extracted with $CHCl_3$. The combined extract was washed with brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (hexane/EtOAc = 4:1) to yield **4b** (198 mg, quant.) as colorless oil. IR (neat) 2928, 2855, 2717, 2096, 1726, 1463, 1410, 1390, 1350, 1260, 893, 723, 671 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$) δ 9.77 (1H, t, J = 1.8 Hz), 3.26 (2H, t, J = 7.0 Hz), 2.42

(2H, td, J = 7.4 Hz, 1.8 Hz), 1.62 (4H, m), 1.33 (14H, m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 202.5, 51.2, 43.6, 29.2 (× 2), 29.14, 29.10, 28.91, 28.90, 28.6, 26.5, 21.8; HRMS, calcd for $C_{12}H_{23}N_3ONa$ ([M+Na]$^+$) 248.1733, found 248.1736.

4.3.7. (1*R*,1′*RS,5*R*)-1-(1′-Hydroxy-12′-azidododecyl)-4-hydroxy-4-methyl-6-oxa-3-aza-bicyclo[3.1.0]hexan-2-one (5b).** To a solution of **3** (253 mg, 1.36 mmol), **4b** (198 mg, 0.88 mmol), and MS4A (0.6 g) in THF–hexane (1:1, 10 mL) was added TBAF (130 μL of a 1.0 M solution in THF, 0.1 mmol), which had been dried with MS4A for 3 h before use. The mixture was stirred at rt for 24 h, and then filtered through Celite and washed with EtOAc. The filtrate was washed with H_2O, brine, dried (Na_2SO_4), and concentrated. A solution of the residue in CH_3CN (2 mL) was added a solution of 5% aqueous HF in CH_3CN (3 mL), and the mixture was stirred at rt for 20 min. The mixture was quenched by the addition of saturated aqueous $NaHCO_3$ solution and extracted with EtOAc. The combined extract was washed with brine, dried (Na_2SO_4), and concentrated. The residue was purified by chromatography (hexane/EtOAc = 3:1 to 1:1) to afford **5b** (160 mg, 54%) as a 1:1 diastereomeric mixture as pale yellow oil. IR (neat) 3477, 2927, 2855, 2096, 1778, 1459, 1381, 1340, 1255, 1070, 1007, 938, 892, 853, 785, 725, 695 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.65 (1H, m), 4.65 (1H, m)*, 4.22 (1H, m), 4.14 (1H, m)*, 3.98 (1H, s), 3.98 (1H, s)*, 3.26 (2H, t, J = 6.9 Hz), 3.26 (2H, t, J = 6.9 Hz)*, 2.48 (1H, br s)*, 2.36 (1H, br s), 1.78–1.50 (4H, m), 1.78–1.50 (4H, m)*, 1.41 (3H, d,J = 6.7 Hz), 1.41 (3H, d,J = 6.7 Hz)*, 1.36–1.28 (14H, br m), 1.38–1.27 (14H, br m)*; ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.9*, 170.5, 75.4*, 75.3, 66.3*, 64.7, 62.1*, 61.73, 61.67, 61.6*, 51.4, 51.4*, 32.3, 32.2*, 29.38, 20.38*, 29.35 (×3), 29.35 (×3)*, 29.3, 29.2*, 29.0, 29.0*, 28.7, 28.7*, 26.6, 26.6*, 25.3*, 25.2, 17.8, 17.7*; HRMS, calcd for $C_{17}H_{29}N_3O_4Na$ ([M+Na]$^+$) 326.3050, found 326.2038.

4.3.8. (2*S*,3*S*)-3-[(*S*)-1-Hydroxyethyl]-2-(12′-azidododecanoyl)oxirane-2-carboxamaide (6b). To a solution of DMSO (0.2 mL, 2.8 mmol) in CH_2Cl_2 (3 mL) was added TFAA (0.2 mL, 1.4 mmol) at −78 °C. After 10 min, a solution of **5b** (160 mg, 0.47 mmol) in CH_2Cl_2 (3 mL) was added to the mixture, and the mixture was −78 °C for 30 min. Then Et_3N (0.7 mL, 5.0 mmol) was added to the mixture, and the mixture was stirred at rt for 15 min. The mixture was quenched by the addition of H_2O and extracted with $CHCl_3$. The combined extract was washed with brine, dried (Na_2SO_4), and concentrated.

To a solution of NH_3 (~0.3 mL) in MeOH (2 mL) was added a solution of the residue in MeOH (2 mL) at 0 °C, and the mixture was stirred for 30 min. Then the mixture was concentrated. The residue was purified by chromatography (hexane/EtOAc = 1:1 to 1:2) to afford **6b** (119 mg, 71%) as white solid. Mp = 72–74 °C; $[\alpha]_D^{22}$ +16.0 (*c* 0.5, MeOH); IR (KBr) 3425, 3342, 3236, 2922, 2853, 2104, 1716, 1669, 1539, 1463, 1403, 1369, 1304, 1263, 1147, 1118, 1065, 973, 924, 898, 760 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.95 (1H, br s), 6.69 (1H, br s), 3.66 (1H, m), 3.56 (1H, br s), 3.26 (2H, t, J = 7.0 Hz), 3.17 (1H, d, J = 7.7 Hz), 2.66 (1H, m), 2.49 (1H, m), 1.58 (4H, m), 1.38 (3H, d, J = 6.3 Hz), 1.27 (14H, br s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 203.7, 167.1, 65.6, 65.4, 65.1, 51.4, 37.8, 29.35 (× 2), 29.29, 29.2, 29.0, 28.9, 28.7, 26.6, 22.9, 20.2; HRMS, calcd for $C_{17}H_{30}N_4O_4Na$ ([M+Na]$^+$) 377.2159, found 377.2166.

4.3.9. (1*R*,5*R*)-1-(12′-Azidododecanoyl)-4-hydroxy-4-methyl-6-oxa- 3-aza-bicyclo[3.1.0]hexan-2-one (7b). To a solution of DMSO (400 μL, 5.6 mmol) in CH_2Cl_2 (5 mL) was added TFAA (520 μL, 3.7 mmol) at −78 °C and the mixture was stirred at −78 °C. After 5 min, a solution of **6b** (428 mg, 1.2 mmol) in CH_2Cl_2–DMSO (30:1, 3.1 mL) was added to the mixture and the mixture was stirred at −78 °C for 60 min. Then Et_3N (1.5 mL) was added to the reaction mixture and the mixture was stirred at room temperature for 5 min. The mixture was quenched by the addition of H_2O and extracted with EtOAc. The extract was washed with brine, dried (Na_2SO_4), and concentrated. To a solution of the residue in THF–H_2O (4:1, 5 mL) was added LiOH (30 mg, 1.3 mmol) at 0 °C. After the mixture was stirred at 0 °C for 10 min, the mixture was quenched by the addition of H_2O and extracted with EtOAc. The extract was washed with brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (hexane/EtOAc = 1:1) to give **7b** (307 mg, 72%) as white solid. Mp = 55–57 °C; $[\alpha]_D^{24}$ −88.5 (*c* 4.5, MeOH); IR (KBr) 3463, 3264, 3019, 2929, 2855, 2097, 1743, 1690, 1465, 1433, 1402, 1350, 1250, 1217, 1163, 1118, 1052, 948, 887, 758, 667, 651, 623 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.35 (1H, d, J = 2.4 Hz), 5.20 (1H, br s), 4.22 (1H, d, J = 2.4 Hz), 3.20 (2H, t, J = 7.0 Hz), 2.41 (1H, m), 2.16 (1H, m), 1.54 (2H, m), 1.54 (3H, s), 1.45 (1H, m), 1.40–1.19 (15H, br m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 202.3, 169.6, 83.7, 66.0, 61.1, 51.4, 37.8, 29.4, 29.4, 29.3, 29.3, 29.1, 28.9, 28.8, 26.6, 22.5, 21.1; HRMS, calcd for $C_{17}H_{28}N_4O_4Na$ ([M+Na]$^+$) 375.2002, found 375.2038.

4.3.10. (1*R*,5*R*,3″*R*,4″*S*,5″*S*)-5″-(2″-Oxo-hexahydro-thieno[3,4-*d*]imidazol-4″-yl)pentanoc acid [12′-(4-hydroxy-4-methyl-2-oxo-6-oxa-3-aza-bicyclo[3.1.0]hex-1-yl)-12′-oxododecyl]amide (Bio-Epo-C12). A solution of **7b** (51 mg, 0.14 mmol), biotin *N*-hydroxysucimide ester (55 mg, 0.16 mmol) and $Pd(OH)_2$ on carbon powder (20% Pd, 25 mg) in THF–DMF (6:1, 7 mL) was stirred at rt for 9.5 h. Then the mixture was filtered through Celite and washed with THF. The filtrate was concentrated. The residue was purified by column chromatography ($CHCl_3$/MeOH = 9:1) to give **Bio-Epo-C12** (70.2 mg, 88%) as white solid. Mp = 138–140 °C; $[\alpha]_D^{24}$ −16.7 (*c* 1.45, DMSO); IR (KBr) 3295, 2925, 2853, 1738, 1700, 1642, 1548, 1465, 1424, 1322, 1265, 1206, 1156, 1021, 949, 886, 654 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD/$CDCl_3$) δ 4.40 (1H, dd, J = 7.8 Hz, 5.0 Hz), 4.21 (1H, dd, J = 7.8 Hz, 4.4 Hz), 4.00 (1H, s), 3.11 (1H, m), 3.06 (2H, t, J = 6.9 Hz), 2.84 (1H, dd, J = 12.7 Hz, 5.0 Hz), 2.62 (1H, d, J = 12.7 Hz), 2.52 (2H, t, J = 7.2 Hz), 2.10 (2H, t, J = 7.3 Hz), 1.60–1.68 (2H, m), 1.48–

1.59 (4H, m), 1.38 (3H, s), 1.31–1.43 (4H, m), 1.22 (14H, br m); ^{13}C NMR (100 MHz, $CD_3OD/CDCl_3$) δ 201.0, 174.5, 169.0, 164.5, 82.7, 65.5, 61.9, 61.5, 60.1, 55.6, 39.8, 39.1 (×2), 35.5, 29.22, 29.19, 29.11, 29.05, 29.0 (×2), 28.7, 28.3, 28.0, 26.6, 25.5, 22.4, 20.7; HRMS, calcd for $C_{27}H_{44}N_4O_6NaS$ ($[M+Na]^+$) 575.2873, found 575.2868.

4.4. Reaction of Epo-C12 with *N*-acetylcysteine methyl ester (8)

4.4.1. Treatment of Epo-C12 (2) with 1.2 equiv of *N*-acetylcysteine methyl ester (8). To a solution of **2** (3.1 mg, 10 μmol) and **8** (2.1 mg, 12 μmol)[14] in MeOH (0.5 mL) was added a 0.5 M aqueous solution of $NaHCO_3$ (0.5 mL), and the mixture was stirred at rt for 20 min. The mixture was quenched by the addition of 1 M HCl (1 mL) and extracted with EtOAc. The extract was washed with brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (hexane/EtOAc = 1:1) to give methyl *N*-acetyl-*S*-[1-(aminocarbonyl)-2-oxotridecanyl]-L-cysteinate (**9**) (3.4 mg, 82%) as colorless oil. **9**: $[\alpha]_D^{22}$ +13.3 (*c* 0.7, $CHCl_3$); IR (film) 3445, 3321, 3016, 2926, 2854, 1743, 1664, 1586, 1438, 1374, 1308, 1217, 1176, 1128, 1082, 1010, 758, 667 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$ with 0.03% TFA) δ 6.44 (1H, br s), 5.82 (1H, br s), 4.82 (1H, br m), 3.78 (3H, s), 3.10–2.90 (2H, br m), 2.08 (3H, s), 1.62 (2H, m), 1.38–1.26 (20H, br m), 0.88 (3H, t, J = 6.9 Hz); ^{13}C NMR (100 MHz, $CDCl_3$ with 0.03% TFA) δ 187.1, 175.3, 171.0, 170.7, 53.0, 52.5, 39.5, 34.0, 31.9, 29.61, 29.59 (x 2), 29.5, 29.40, 29.36, 29.3, 26.7, 23.0, 22.7, 14.1; HRMS, calcd for $C_{20}H_{36}N_2O_5NaS$ ($[M+Na]^+$) 439.2237, found 439.2242.

4.4.2. Treatment of Epo-C12 (2) with excess amount of *N*-acetylcysteine methyl ester (8). To a solution of **2** (4.7 mg, 15 μmol) and **8** (6.4 mg, 36 μmol) in MeOH (1.0 mL) was added a 0.5 M aqueous solution of $NaHCO_3$ (1.0 mL), and the mixture was stirred at rt for 24 h. The mixture was quenched by the addition of 1 M HCl (1 mL) and extracted with EtOAc. The extract was washed with brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (hexane/EtOAc = 1:1) to give dodecanoic acid (**10**) (1.1 mg, 36%) as white solid and *N*-acetyl-L-cystine dimethyl ester (**11**)[17] (4.1 mg, 64%) as white solid.

4.5. Treatment of epolactaene (1) with *N*-acetylcysteine methyl ester (8)

4.5.1. (2*E*,6*E*,8,*E*,10*E*)-10-(Methoxycarbonyl)-2,8-dimethyldodeca-2,6,8,10-tetraenoic acid (12). To a solution of **1** (9.0 mg, 23 μmol) and **8** (6.0 mg, 34 μmol) in MeOH (1.0 mL) was a 0.5 M aqueous solution of $NaHCO_3$ (1.0 mL), and the mixture was stirred at rt for 20 min. The mixture was quenched by the addition of 1 M HCl (1 mL) and extracted with EtOAc. The extract was washed with brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (hexane/EtOAc = 1:1) to give **12** (4.6 mg, 72% from **1**) as colorless and **11** (4.6 mg, 77% from **8**) as white solid. **12**: IR (film) 3020, 2927, 2855, 1711, 1689, 1641, 1436, 1383, 1216, 1134, 1057, 1024, 965, 938, 866 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.94 (2H, m), 6.25 (1H, d, J = 15.6 Hz), 5.96 (1H, br s), 5.72 (1H, dt, J = 15.6 Hz, 6.7 Hz), 3.74 (3H, s), 2.32 (4H, m), 1.94 (3H, s), 1.73 (3H, dd, J = 7.1 Hz, 0.9 Hz), 1.63 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.1, 167.9, 144.2, 139.7, 137.8, 134.9, 130.5, 128.7, 127.5, 122.8, 51.9, 31.6, 28.9, 15.8, 14.4, 12.1; HRMS, calcd for $C_{16}H_{22}O_4Na$ ($[M+Na]^+$) 301.1410, found 301.1436; HRMS, calcd for $C_{16}H_{21}O_4$ ($[M-H]^-$) 277.1445, found 277.1455.

4.6. Cell line and culture

BALL-1 cells were purchased from Riken Cell Bank (Tsukuba, Japan). BALL-1 cells were maintained at 37 °C with 5% CO_2 in RPMI 1640 supplemented with kanamycin sulfate (65 mg/L), 2-mercaptoethanol (3.5 μL/L), sodium bicarbonate (2 g/L), and heat-inactivated 10% (v/v) FBS. These cells were routinely diluted with the above medium to the appropriate concentrations ($1.0–4.0 \times 10^5$ cells/mL).

4.7. Detection of cell death by MTT assay

BALL-1 cells were plated onto a 96-well plate (Sumitomo Bakelite Co., Tokyo, Japan) at a concentration of 2.0×10^4 cells/well and preincubated at 37 °C for 1 h. The cells were then treated with **Flu-Epo-C12** or **Bio-Epo-C12**. After incubation at 37 °C for 24 h, cell viability was determined by MTT assay, a method for determining cell viability by measuring the mitochondrial dehydrogenase action. In this assay, 11 μL of MTT stock solution (5 mg/mL in phosphate-buffered saline (PBS)) was added to the cells, and the plate was incubated 37 °C for 1 h. After centrifugation for 5 min at 1500 rpm, the supernatant was discarded, and 100 μL of DMSO was added to dissolve MTT formazan. Absorbance at 570 nm was measured with a microplate reader (Bio-Rad Model 550, Bio-Rad, Tokyo, Japan), and the percentage of cell viability was taken as the percentage absorbance at 570 nm of epolactaene-treated cells and control.

4.8. Confocal microscopy

To determine the localization of **Flu-Epo-C12**, BALL-1 cells were seeded in 3.5-cm diameter cell culture dishes. Cells were treated with 5 μM of **Flu-Epo-C12** and 0.1 μM of MitoTracker. The cells were incubated at 37 °C for 1 h and moved to microtubes. The cells were washed with PBS three times. Live cells were examined using a LSM 5 laser scanning confocal microscope at 405 nm to assess **Flu-Epo-C12** and at 543 nm to assess MitoTracker.

4.9. Preparation of cell lysates and isolation of binding proteins of Bio-Epo-C12

BALL-1 cells were washed three times with cold PBS and then treated with lysis buffer (50 mM Hepes, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% protease inhibitor

cocktail, pH 7.5). The lysed cells were centrifuged at 14,000*g* for 15 min at 4 °C, and the supernatant was collected as the cell lysate. The protein concentration of the lyste was adjusted to be 1 mg/mL by using the Bradford method.[18]

The lysate in the lysis buffer (1 mL) was incubated with 5 μM of **Bio-Epo-C12**, 5 μM of biotin, and 5 μM of vehicle (DMSO), respectively, at 4 °C for 18 h. Streptavidin agarose beads (100 μL) were added to the lysate, and incubated at 4 °C for 2 h. After the beads were washed with the lysis buffer three times, the proteins were eluted by the addition of 15 μL of sample buffer (284 mM Tris–HCl, 9% SDS, 27% glycerol, 10% mercaptoethanol, 0.02% bromophenol blue) and 15 μL of lysis buffer, followed by heat at 90 °C. The beads were separated by centrifuging at 14,000*g* for 5 min at 4 °C, and the supernatants were collected as the eluted samples. The eluted samples were analyzed by SDS–PAGE (acrylamide: 12% and 6% for separating gels, and 4% for stacking gels), and the proteins were visualized by CBB staining.

4.10. Identification of the binding proteins by PMF

The visualized bands were sliced and decolorized with decoloring buffer (50% acetonitrile, 25 mM NH_4HCO_3). After the gels were dried by the addition of acetonitrile (100 μL), the resulting gels were separated and dried in vacuo. The dried gels were reduced by incubating with 50 μL of reducing buffer (10 mM dithiothreitol, 25 mM NH_4HCO_3) at 56 °C for 1 h. After removing the excess reducing buffer, 100 μL of alkylating buffer (55 mM iodoacetamide, 25 mM NH_4HCO_3) was added, and the mixture was incubated at rt for 45 min. The supernatants were removed, and the gels were washed twice by incubation with 100 μL of 25 mM NH_4HCO_3, followed by 100 μL of the buffer (50% acetonitrile, 25 mM NH_4HCO_3). The resulting gels were separated and dried in vacuo. The dried gels were digested by incubation with 50 μL of digesting solution (50 mM NH_4HCO_3, 100 μg/mL tripsin) at 0 °C for 30 min. After the excess digesting solution was removed, the mixture was incubated at 37 °C for 20 h. The peptide fragments were eluted by incubation with 50 μL of elution solution (50% aqueous acetonitrile, 1% trifluoroacetic acid) at 0 °C. The extracts were concentrated in vacuo, and dissolved in 10 μL of the elution solution. The solution of the extracts was absorbed on a ZipTip pipette tip (Millipore Co., MA, USA), and the tip was washed with the elution solution, and the solution was concentrated. A mixture of the resulting peptides and DHBA matrix (20 mg/ml 2,5-dihydroxybenzoic acid in a 2:1 solution of 0.1% trifluoroacetic acid and acetonitrile) was loaded on a MALDI-TOF MS target plate. The samples were applied to MALDI-TOF mass in the positive reflection mode. Identification of proteins from MALDI-TOF spectra was achieved by using the Mascot search program (Matrix science Inc., USA). Figure S9 shows the digested peptide derived from seven bands.

The following peaks of peptide fragments derived from band **1** were identical to those theoretically calculated from the sequence of fatty acid synthase; $[M+H^+]$ = 1042.161, 1241.277, 1251.199, 1263.333, 1290.171, 1299.382, 1340.374, 1368.443, 1636.501, 1685.728, 713.642, 1741.695, 1916.836, 1968.908, 2116.94, 2233.155, 2265.074, 2368.107, 2472.442, 2668.492.

The following peaks of peptide fragments derived from band **2** were identical to those theoretically calculated from the sequence of ATP citrate lyase; $[M+H^+]$ = 884.084, 896.187, 920.123, 934.204, 958.179, 1060.213, 1090.246, 1129.298, 1367.583, 1395.521, 1408.485, 1417.539, 1422.556, 1427.58, 1491.634, 1567.835, 1646.917, 1660.797, 1738.018, 1872.086, 1881.021.

The following peaks of peptide fragments derived from band **3** were identical to those theoretically calculated from the sequence of translation elongation factor 2 (eEF-2); $[M+H^+]$ = 890.175, 922.1, 969.222, 1084.26, 1091.314, 1138.276, 1153.333, 1206.435, 1222.365, 1274.533, 1308.491, 1402.7, 1444.689, 1543.805, 1615.856, 1800.104, 2007.303, 2079.46, 2143.528, 2176.567, 2220.597.

The following peaks of peptide fragments derived from band **4** were identical to those theoretically calculated from the sequence of heat shock protein 90 β; $[M+H^+]$ = 886.209, 891.111, 901.219, 951.171, 1141.322, 1194.442, 1236.471, 1249.463, 1311.464, 1348.602, 1416.495, 1513.832, 1527.749, 1783.228, 1809.194, 1848.014, 1911.373, 1988.232, 2177.454, 2256.612, 2374.834.

The following peaks of peptide fragments derived from band **5** were identical to those theoretically calculated from the sequence of heat shock protein 60; $[M+H^+]$ = 961.253, 1504.757, 1556.979, 1584.865, 1642.935, 1919.421, 2038.413, 2047.578, 2113.577, 2365.923, 2508.781, 2560.936.

The following peaks of peptide fragments derived from band **6** were identical to those theoretically calculated from the sequence of adenine nucleotide translocator 2 (ANT2); $[M+H^+]$ = 902.305, 951.265, 976.308, 1121.336, 1132.392, 1136.409, 1219.351, 1268.361, 1446.446, 1816.563, 1926.7828, 2795.858.

The following peaks of peptide fragments derived from band **7** were identical to those theoretically calculated from the sequence of peroxiredoxin 1 (Prx1); $[M+H^+]$ = 894.249, 920.305, 980.280, 1107.300, 1164.59, 1196.305, 1211.361, 1263.355, 1359.453, 1622.462, 1750.601, 1778.577, 2125.763, 2405.740, 2752.795.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.03.029.

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