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Isolation and lipolytic activity of eurycomanone and its epoxy derivative from *Eurycoma longifolia*

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ABSTRACT

Eurycomanone (**1**) and 13β,21-epoxyeurycomanone (**2**) were isolated from *Eurycoma longifolia* for studies of lipolytic activity. Compound **1** enhanced lipolysis in adipocytes with an EC_{50} of 14.6 μM, while its epoxy derivate, compound **2**, had a stronger activity with an EC_{50} of 8.6 μM. Based on molecular mechanistic study using several specific inhibitors to lipolytic signaling pathways, it was found that PKA inhibitor totally diminished the lipolytic activity of **1** and **2**. Further immunoblotting analysis confirmed the activation of phosphorylated PKA by both **1** and **2**. With the growing need to develop new anti-obesity agents, eurycomanone and its epoxy derivate can be used as promising lead compounds to target lipid catabolism.

Keywords

3T3-L1 adipocytes; *Eurycoma longifolia*; lipolysis; obesity; quassinoid

1. Introduction

Obesity has become one of the most significant risk factors for various diseases. Because of the large number of overweight patients and the rapid increase in obesity in recent years, obesity has been considered to be one of the major health problems worldwide. Drug development to treat obesity has been widely studied, and active compounds from plants can lead to anti-obesity medications.[1-3]

For the development of new anti-obesity drugs, lipid catabolism has been considered as an effective therapeutic target.[4] Lipolysis, a process to break down stored lipids, is a critical aspect to diminish lipid amount. Hence, natural-derived compounds that stimulate hydrolysis of triglyceride to glycerol and fatty acids are of great interest to combat obesity.

In an effort to discover anti-obesity agents from medicinal plants, our research group have uncovered the potential of *Eurycoma longifolia* Jack (family Simaroubaceae) for reducing lipid accumulation.[5] The root of *E. longifolia* is a popular source used in traditional herbal medicine in Southeast Asia.[6] *E. longifolia* is prepared as water decoction or commercial extract in the form of capsules.[6,7] The known properties of *E. longifolia* include its aphrodisiac and anti-malarial effects.[8,9] However, the lack of information on anti-obesity study limits its use as anti-obesity agent.

Eurycomanone (**1**) is the major quassinoid in *E. longifolia*; and it is used as a marker compound in quality control of commercial products derived from this plant. Several bioactivities have been reported for compound **1** including, increased production of testosterone in rat testicular cells,[8] antiulcer activity,[10] cytotoxicity against cancer cell lines,[11] and antimalarial activity.[9]

In this report, we describe the isolation and lipolytic activity of **1** and its derivatives, 13β,21-epoxyeurycomanone (**2**) and 13β,21-dihydroxyeurycomanone (**3**). We also reconfirm stereochemistry assignment of the epoxide derivate (**2**).

2. Material and methods

2.1 General

Unless otherwise stated, commercially available chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). *E. longifolia* root (Batch No. SL.1A.2015.PB) was supplied by Merapi Farma Herbal Co. (Yogyakarta, Indonesia). Absorbance was measured using a Synergy™ MX microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). A Bruker AMX 500 instrument (Bruker BioSpin K.K., Bruker Instruments, Billerica, MA, USA) was used to obtain NMR spectra and residual solvents were used as an internal standard (pyridine-d_5: ^{1}H 7.22

ppm, ^{13}C 135.91 ppm). Mass spectra were obtained using a LCT-Premier mass spectrometer (Waters Corp., Milford, MA, USA). For the LC-MS analysis, Waters Acquity UPLC system (Waters Corp., Milford, MA, USA) was combined with LCT-Premier mass spectrometer (Waters Corp., Milford, MA, USA). The EZR was used for the statistical tests.[22]

2.2 Isolation of quassionoids

Powdered root of *E. longifolia* (200 g) was extracted with 50% (v/v) aq. methanol for 24 h to obtain 6.84 g extract. The extract was suspended in water and partitioned with ethyl acetate and then with 1-butanol to obtain a water-soluble fraction (4.10 g), a 1-butanol-soluble fraction (1.17 g), and an ethyl acetate-soluble fraction (1.17 g). The 1-butanol-soluble fraction was adsorbed onto DIAION HP-20 (φ40 mm × 240 mm, Mitsubishi Chemical Co., Tokyo, Japan), washed with water, and eluted with 50% aq. methanol. The 50% (v/v) aq. methanol-eluted fraction (580 mg) was then separated using Cosmosil® 75C18-OPN (Nakalai Tesque Inc., Kyoto, Japan) column chromatography (φ20 mm × 120 mm) by stepwise elution with water, 10% aq. methanol, 20% aq. methanol, 30% aq. methanol, 50% aq. methanol, 70% aq. methanol, and methanol. The active fraction eluted with 10% aq. methanol (130 mg) was further

purified by Toyopearl HW-40F (Tosoh Co., Tokyo, Japan) column chromatography (φ 15 mm × 160 mm) with water as the eluent. The active fraction was finally purified by HPLC using an InertSustain C18 column (φ20×250 mm; GL Science Co., Tokyo, Japan) with 20% aq. methanol as an eluent to obtain eurycomanone (**1**, 16.3 mg),[12,13] 13β,21-epoxyeurycomanone (**2**, 4.8 mg),[12,14] and 13β,21-dihydroxyeurycomanone (**3**, 3.6 mg).[9,13] The water-soluble fraction (4.10 g) was similarly separated to obtain additional **1** (68.2 mg), **2** (14.0 mg), and **3** (8.6 mg). Each compound was identified by comparing its ^{1}H,^{13}C-NMR and optical rotation with the reported values.

2.2.1 Eurycomanone (**1**)

^{1}H-NMR (500 MHz, pyridine-d_5, rt): 1.63 (3H, s), 1.80 (3H, br s), 2.03 (1H, ddd, J = 2.4, 13.3, 14.4 Hz), 2.33 (1H, td, J = 2.4, 14.4 Hz), 3.26 (1H, br d, J = 12.6 Hz), 3.82 (1H, s), 4.02 (1H, d, J = 8.8 Hz), 4.53 (1H, s), 4.55 (1H, d, J = 8.8 Hz), 4.81 (1H, s), 5.26 (1H, t, J = 2.4 Hz), 5.66 (1H, d, J = 1.5 Hz), 5.67 (1H, s), 6.12 (1H, d, J = 1.5 Hz), 6.16 (1H, q, J = 1.3 Hz), 7.79 (1H, br s, OH), 7.85 (1H, s, OH), 8.03 (1H, br s, OH), 9.63 (1H, br s, OH), 9.78 (1H, br s, OH) ppm; ^{13}C-NMR (125 MHz, pyridine-d_5, rt): 10.77, 22.79, 26.07, 42.58, 46.30, 48.10, 52.98, 68.05, 72.17, 76.24, 79.77, 81.36, 84.89, 109.95, 119.76, 126.44, 148.36, 162.94, 174.25, 197.85 ppm; HR-ESI-MS

(positive): $[M+Na]^+$, found m/z = 431.1322, $C_{20}H_{24}O_9Na$, requires m/z 431.1318; $[\alpha]_D^{24}$ +32.1° (c = 1.0, pyridine).

2.2.2 13β,21-epoxyeurycomanone (**2**)

^{1}H-NMR (500 MHz, pyridine-d_5, rt): 1.64 (3H, s), 1.80 (3H, br s), 2.04 (1H, ddd, J = 2.7, 13.2, 14.4 Hz), 2.33 (1H, td, J = 2.7, 2.7, 14.8 Hz), 3.05 (1H, d, J = 5.3 Hz), 3.27 (1H, br d, J = 13.2 Hz), 3.817 (1H, d, J = 5.3 Hz), 3.822 (1H, s), 4.04 (1H, s), 4.07 (1H, d, J = 9.0 Hz), 4.56 (1H, s), 4.89 (1H, d, J = 9.0 Hz), 5.20 (1H, t, J = 2.7 Hz), 5.84 (1H, s), 6.17 (1H, q, J = 1.3 Hz), 6.93 (1H, br s, OH), 7.90 (1H, s, OH), 8.19 (1H, br s, OH), 9.67 (1H, br s, OH), 9.87 (1H, br s, OH) ppm; ^{13}C-NMR (125 MHz, pyridine-d_5, rt): 10.85, 22.80, 25.84, 42.56, 46.23, 46.89, 48.73, 53.93, 59.64, 67.24, 71.86, 75.86, 75.96, 82.08, 84.84, 110.03, 126.50, 162.84, 174.21, 197.86 ppm; HR-ESI-MS (positive): $[M+Na]^+$, found m/z = 447.1287, $C_{20}H_{24}O_{10}Na$, requires m/z 447.1267; $[\alpha]_D^{24}$ +34.2° (c = 1.0, pyridine).

2.2.3 13β,21-dihydroxyeurycomanone (**3**)

^{1}H-NMR (500 MHz, pyridine-d_5, rt): 1.66 (3H, s), 1.78 (3H, s), 2.08 (1H, ddd, J = 2.4, 13.2, 14.0 Hz), 2.28 (1H, td, J = 2.4, 14.0 Hz), 3.20 (1H, br d, J = 13.2 Hz),

3.61 (1H, s), 3.98 (1H, d, J = 8.7 Hz), 4.47 (1H, s), 4.60 (1H, d, J = 3.3 Hz), 4.65 (1H, d, J = 11.7 Hz), 5.04 (1H, d, J = 11.7 Hz), 5.13 (1H, t, J = 2.4 Hz), 5.23 (1H, d, J = 8.7 Hz), 5.39 (1H, br s, OH), 5.59 (1H, s), 6.14 (1H, q, J = 1.3 Hz), 6.23 (1H, br s, OH), 6.60 (1H, br s, OH), 7.59 (1H, s, OH), 7.95 (1H, d, J = 3.3 Hz), 8.39 (1H, br s, OH), 9.39 (1H, br s, OH), 9.67 (1H, br s, OH) ppm; ^{13}C-NMR (125 MHz, pyridine-d_5, rt): 11.38, 22.75, 26.11, 42.65, 45.93, 47.82, 53.89, 67.15, 68.02, 70.98, 75.25, 78.34, 78.68, 80.12, 85.08, 110.37, 126.53, 162.84, 173.90, 197.95 ppm; HR-ESI-MS (positive): $[M+Na]^+$, found m/z = 465.1390, $C_{20}H_{26}O_{11}Na$, requires m/z 465.1373; $[\alpha]_D^{24}$ +17.5° (c = 1.0, pyridine).

2.2.4 Acetylation of 13β,21-epoxyeurycomanone (**2**)

Compound **2** (3.6 mg) was dissolved in pyridine (0.3 mL) and then acetic anhydride (0.15 mL) was added. The mixture was stirred for 1 h at room temperature under nitrogen, and then diluted with water and extracted with ethyl acetate. The organic layer was dried over sodium sulfate, evaporated, and then the residue was separated by preparative TLC (hexane/acetone = 1/1) to obtain the di-acetylated derivative **4** (4.4 mg, quant.). The positions of acetyl groups were confirmed by chemical shift changes and the HMBC spectra.

142 ^{1}H-NMR (500 MHz, pyridine-d_5, rt): 1.70 (3H, s), 1.86 (3H, s), 2.06 (3H, s), 2.03-2.09

143 (1H, m), 2.26 (3H, s), 2.35 (1H, br d, J = 14.8 Hz), 2.94 (1H, d, J = 5.0 Hz), 3.32 (1H,

144 br d, J = 12.9 Hz), 3.49 (1H, d, J = 5.0 Hz), 3.66 (1H, s), 3.93 (1H, s), 4.07 (1H, d, J =

145 9.1 Hz), 4.84 (1H, d, J = 9.1 Hz), 5.17 (1H, br s), 5.96 (1H, s), 6.20 (1H, br s), 6.88 (1H,

146 s), 7.73 (1H, br s, OH), 7.79 (1H, br s, OH) ppm; ^{13}C-NMR (125 MHz, pyridine-d_5, rt):

147 11.39, 20.86, 21.44, 22.86, 25.37, 42.92, 44.97, 45.84, 47.70, 54.34, 59.25, 66.76,

148 72.56, 75.29, 76.39, 81.74, 85.29, 110.55, 127.15, 162.27, 168.56, 169.87, 170.42,

149 192.60 ppm; HR-ESI-MS (positive): $[M+Na]^+$, found m/z = 531.1495, $C_{24}H_{28}O_{12}Na$,

150 requires m/z 531.1478; $[\alpha]_D^{25}$ +14.3° (c = 0.314, pyridine).

151

152 2.3 Biology

153 2.3.1 Cell culture

154 Murine 3T3-L1 pre-adipocyte (JCRB9014) cells were obtained from the

155 Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). The cells were

156 cultured at 37 °C, 10% CO_2 atmosphere in DMEM supplemented with 10% FBS (10%

157 FBS/DMEM) and antibiotics (100 units/mL penicillin, 100 µg/mL streptomycin, and 50

158 µg/mL gentamicin). Adipocyte differentiation was induced a day after reaching

159 confluence (day 0) by changing the medium to 10% FBS/DMEM supplemented with

160 0.5 mM IBMX, 0.25 μM DEX, and 5 μg/mL insulin (differentiation medium). Two
161 days after induction (day 2), the medium was changed to 10% FBS/DMEM
162 supplemented with 10 μg/mL insulin to enhance differentiation, and the cells were
163 cultured for another 2 days. The cells (day 4) were further cultured in 10% FBS/DMEM
164 supplemented with 10 μg/mL insulin for 2 days and then in 10% FBS/DMEM for 2
165 more days. These cells (day 8) were used in the glycerol release enhancement assay.
166
167 2.3.2 Glycerol release enhancement activity
168 The isolated compounds (**1**, **2** and **3**) were dissolved in dimethyl sulfoxide and
169 diluted in medium immediately before use. On day 8 of the cell culture, the medium
170 was changed to sample-containing medium (phenol-red-free DMEM) and incubated for
171 24 h. When the inhibitors are included in the experiment, the cells were incubated with
172 the respective inhibitor prior to the sample addition for an hour, and then incubated with
173 both the sample and the inhibitor for 24 hr. On the day of the glycerol release
174 enhancement assay, the medium was recovered and mixed with a free glycerol reagent
175 (F6428; Sigma-Aldrich Co., St Louis, MO, USA). The mixture was incubated at 37 °C
176 for 5 min and its absorbance at 540 nm was measured to quantify the amount of the
177 released glycerol. The absorbance relative to that of the control was calculated.

178 Isoproterenol hydrochloride 1 μM (Sigma-Aldrich Co., St Louis, MO, USA) was used
179 as positive control.
180
181 2.3.3 Lipid accumulation and cytotoxicity assays
182 Both lipid accumulation assay using Oil Red O staining and cytotoxicity test
183 using Cell Counting Kit-8 reagent (Dojindo Lab., Kumamoto, Japan) were described
184 previously.[5]
185
186 2.3.4 Protein extraction
187 The 3T3-L1 adipocytes were cultured in 24-well plates and treated according to
188 glycerol release enhancement activity protocol described in sub-section 4.3.2. On day 9,
189 the cells were washed twice with ice-cold phosphate buffered saline and lysed in ice-
190 cold lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 200 mM EDTA, 4 mM NaF,
191 1 mM Na_3VO_4, 1 mM PMSF, 2.5 mM sodium pyrophosphate, protease inhibitors
192 cocktail (cOmplete, Mini; Roche), and 1.5% Triton X-100) on ice. The cell homogenate
193 was centrifuged at 14,000 × *g* for 10 min at 4°C. After the supernatant was collected,
194 the protein concentration was then measured using a Bio-Rad protein assay dye reagent
195 with bovine serum albumin as the standard.

196 2.3.5 Protein immunoblotting

197 Extracted proteins were denatured by heating at 95°C for 5 min in a Laemmli
198 sample buffer supplemented with 0.05 M DTT. The prepared protein samples (5 μg for
199 total protein and 10 μg for phosphorylated protein detection) were loaded and separated
200 using 12.5% (w/v) polyacrylamide gel. Separated proteins were electro-transferred onto
201 nitrocellulose membranes with a Transblot SD Cell at 15 V for 15 min. The membrane
202 was then blocked with 5% (w/v) bovine serum albumin in TBS-T (TBS containing
203 0.1% Tween-20) for 1 h at room temperature. Antibodies used for the immunoblot were
204 rabbit PKA C-α antibody (#4782), phospho-PKA C (Thr197) antibody (#4781), ERK
205 1/2 antibody (#4695), phospho-ERK 1/2 (Thr202/Tyr204) antibody (#4370), β-actin
206 antibody (#4967), and anti-rabbit IgG HRP-linked antibody (#7074), purchased from
207 Cell Signaling Technology, Inc. (Danvers, USA). The membrane was subsequently
208 incubated overnight at 4°C in appropriate primary antibodies (1:1000). After washing,
209 the membrane was incubated in HRP-conjugated secondary antibody (1:2000) for 1 h at
210 room temperature. The antigen–antibody complexes were then visualized using an
211 ImmunoStar LD (Wako Pure Chemical Industries, Osaka, Japan). The luminescence
212 intensity was quantified using ImageJ.

213

3. Results and discussion

3.1 Chemistry

Powdered root of *E. longifolia* was extracted with 50% (v/v) aq. methanol. The extract was dried and partitioned with water, 1-butanol, and ethyl acetate. The 1-butanol layer was adsorbed to DIAION HP-20 and eluted with 50% (v/v) aq. methanol. The obtained 50% aq. methanol eluate was separated by Cosmosil 75C_{18}-OPN and then Toyopearl HW-40F column chromatography to obtain its active fraction. This fraction was finally purified by preparative HPLC with an InertSustain C18 column to isolate compounds **1**–**3**.

The structures of the isolated compounds were determined based on NMR and MS spectra. Compound **1** was determined as eurycomanone according to its ^{1}H-NMR spectra.[10,12] The results of an HRMS analysis ($[M+Na]^+$, found *m*/*z* 431.1322, $C_{20}H_{24}O_9Na$ requires 431.1318) and optical rotation ($[\alpha]_D^{24}$ +32.1°) supported this determination. The obtained data of compound **3** were compared with those reported earlier for several other plant quassinoids, and this compound was identified as 13β,21-dihydroxyeurycomanone (**3**).[9,12]

Although compound **2** was also determined to be a quassinoid, 13,21-epoxyeurycomanone, the stereochemistry of the epoxide in one reference was reported

as beta,[10] while in another reference it was identified as alpha.[13] However, both of the reported NMR spectra and optical rotations were the same as those obtained for compound **2** here, indicating that one of the previous stereochemistry assignments is incorrect. Therefore, through this study, we re-examined and clarified the stereochemistry of the epoxide.

Measurement of the NOESY spectra of this compound showed a correlation between H-12 and H_a-21, which was considered as an evidence of the α-epoxide in the previous study.[13] However, the distance between those two hydrogen atoms was similar between the α and β-epoxide structure models (see Supporting information). Therefore, the observed NOESY correlation was considered insufficient evidence to determine its stereochemistry. To examine and verify its stereochemistry, compound **2** was then acetylated to obtain di-*O*-acetyl product **4** and the NOESY experiment was performed using **4**. In compound **4**, a NOESY correlation was observed between H_b-21 and AcO-15. Thus, the stereochemistry of the epoxide was confirmed to be beta, which is a biosynthetically reasonable configuration if the epoxide **2** is hydrolyzed to produce its dihydroxy derivative **3** *in planta*. In light of this finding, we need to consider that previous studies on the use, detection, and isolation of 13α,21-epoxyeurycomanone from *E. longifolia* probably refer to the β-epoxide.[14,15]

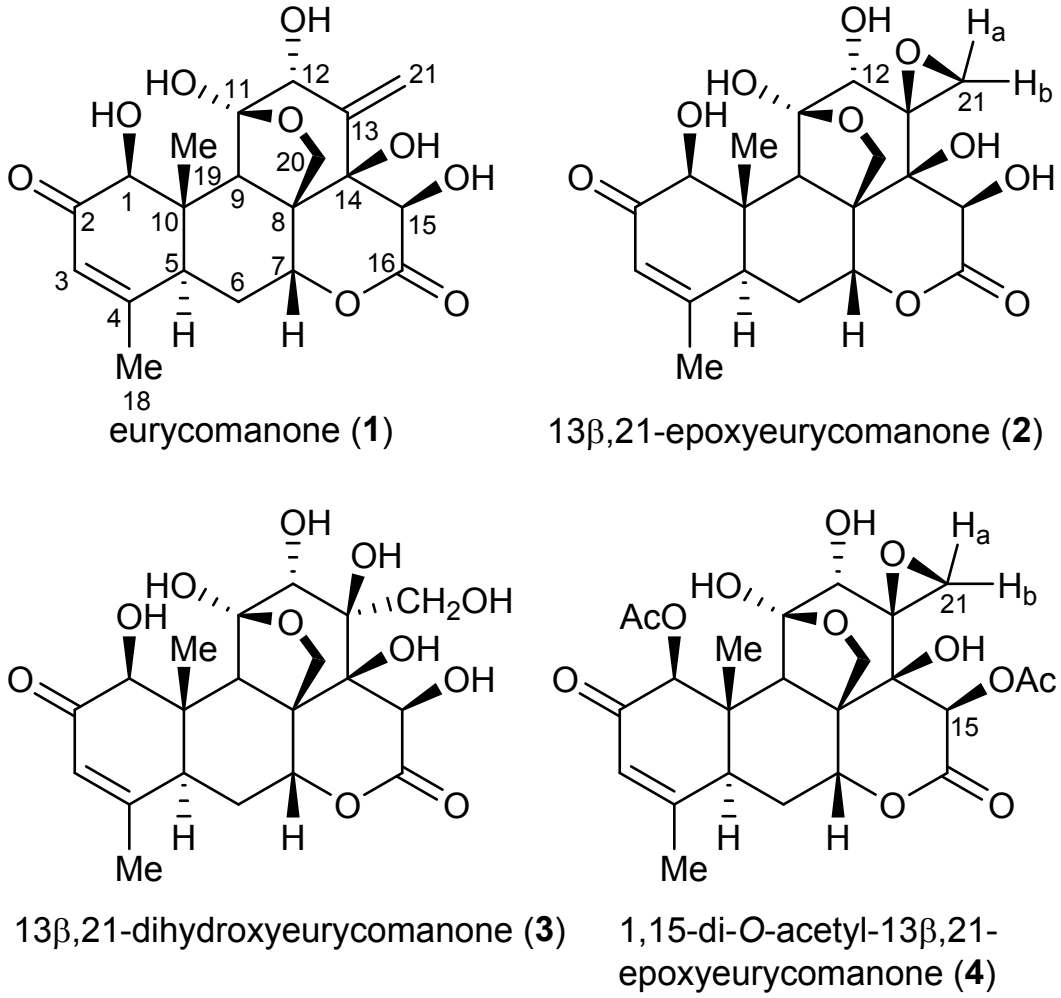

Figure 1. Structures of compounds.

3.2 Biological activity

The three isolated compounds were evaluated for anti-obesity activities; enhancement of glycerol release and reduction of lipid accumulation. Compounds **1** and **2** showed concentration-dependent activity in the glycerol release enhancement assay, and reduced the lipid accumulation without cytotoxic effects (Fig. 2). The EC_{50} value

for the glycerol release enhancement was 14.6 μM for **1**, while **2** had a lower EC_{50} (8.6 μM). The stronger bioactive effects of **2** indicates the importance of the epoxide group in exerting its bioactivity. In contrast, the hydrolyzed derivative **3** did not show any biological activity in either of the two assays (Fig. 2A and 2B), even at the highest concentration tested (100 μM).

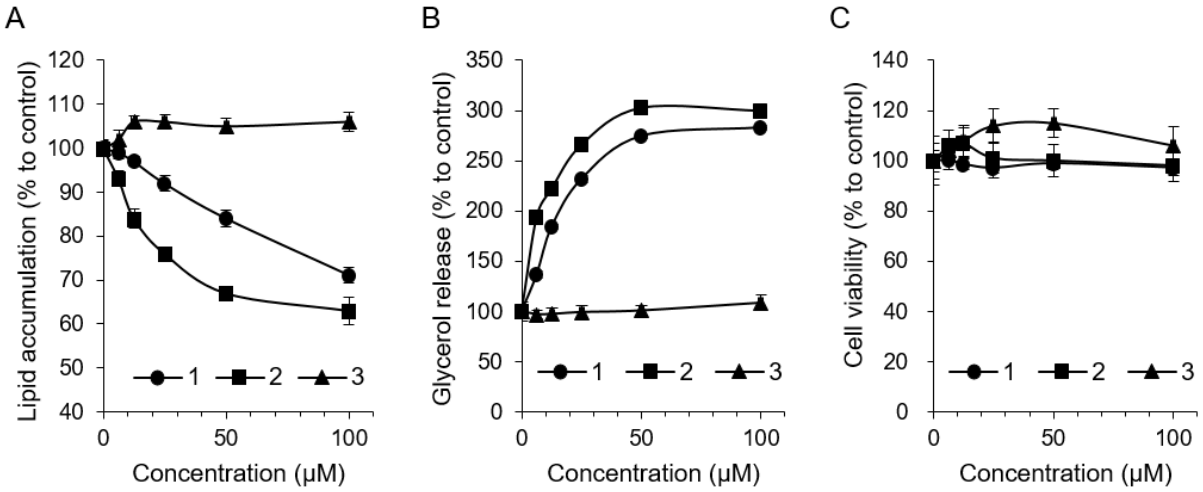

Figure 2. Lipid accumulation reduction effect (A), glycerol release enhancement activity (B), and cell viability (C) of compounds **1**–**3**. (A) Compound **1** showed significant difference above 25 μM (p<0.01). Compound **2** showed significant difference above 6.25 μM (p<0.05) and above 12.5 μM (p<0.01). (B) Compounds **1** and **2** showed significant difference above 6.25 μM. Isoproterenol (1 μM) was used as positive control (322±1%). Data are expressed as mean ± SEM (n=6). Dunnett's test was used.

There are two possible reasons for the total absence of bioactivity in **3**. First, the presence of two hydroxyl groups might have strongly interfered with the interaction between the compound and the target. However, this explanation is unlikely to happen for the following reason. Although various derivatives of eurycomanones have been isolated from plants using various methods,[6,9,12-13,15-18] here we identified **1**–**3** by using activity-guided fractionation. If steric hindrance or ionic repulsion were the reason for the lack of bioactivity of **3**, then other related compounds, for example 13β-methyl,21-dihydroeurycomanone, would have been obtained as bioactive compounds during the isolation process.

The second possibility is that the epoxide group is the essential moiety for the bioactivity. This would suggest that eurycomanone (**1**) is oxidized in the cells to form its bioactive epoxide (**2**). However, there was no evidence for supporting this hypothesis. Therefore, subsequent research on structure-activity relationship (SAR) of these quassinoids is required to determine whether the epoxide group is the essential part of the bioactivity.

3.3 Mechanistic study

After evaluating their biological activities in reducing lipid accumulation and

enhancing lipolysis, molecular mechanism of the lipolytic activity of **1** and **2** was further investigated. Since these compounds induced lipolysis in 3T3-L1 cells, the signaling molecules related to the activation of hormone-sensitive lipase (HSL) were predicted to involve.[19] Initially, we tested the two well-known lipolytic signaling molecules; protein-kinase A (PKA) and extracellular signal-regulated kinase (ERK),[20] by co-incubating **1** and **2** with the respective specific inhibitors (Fig. 3A and 3B). Inhibitor of PKA (H-89) totally diminished the activity of both compounds, but inhibitor of ERK (PD 98059) had no significant effect on the lipolytic activity. Protein immunoblotting analysis also confirmed the activation of PKA by both **1** and **2** (Fig. 4).

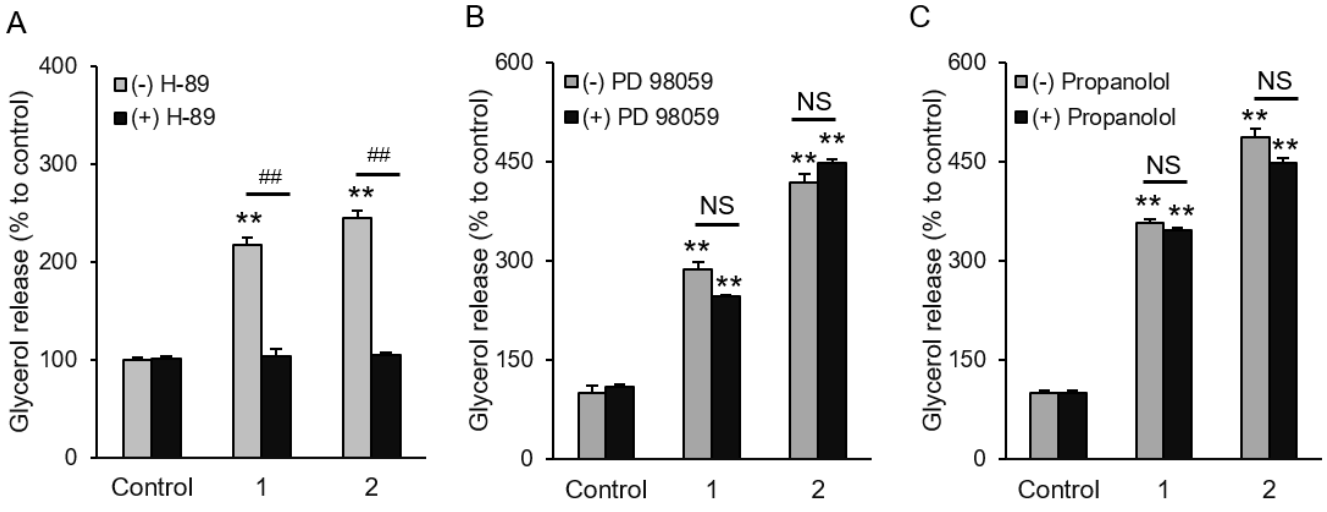

Figure 3. Effect of specific inhibitors on glycerol release enhancement activity of compounds **1** and **2**. 3T3-L1 adipocytes were pre-treated with (A) inhibitor of PKA (H-

89, 20 μM), (B) inhibitor of ERK (PD 98059, 50 μM), or (C) inhibitor of β3-adrenergic receptor (propranolol 1 μM) and then treated with **1** and **2** (25 μM). Data are expressed as mean ± SEM (n=6). **p<0.01 *vs.* control without inhibitor (Dunnett's test); $^{\#\#}p$<0.01 (t-test); NS: No significance.

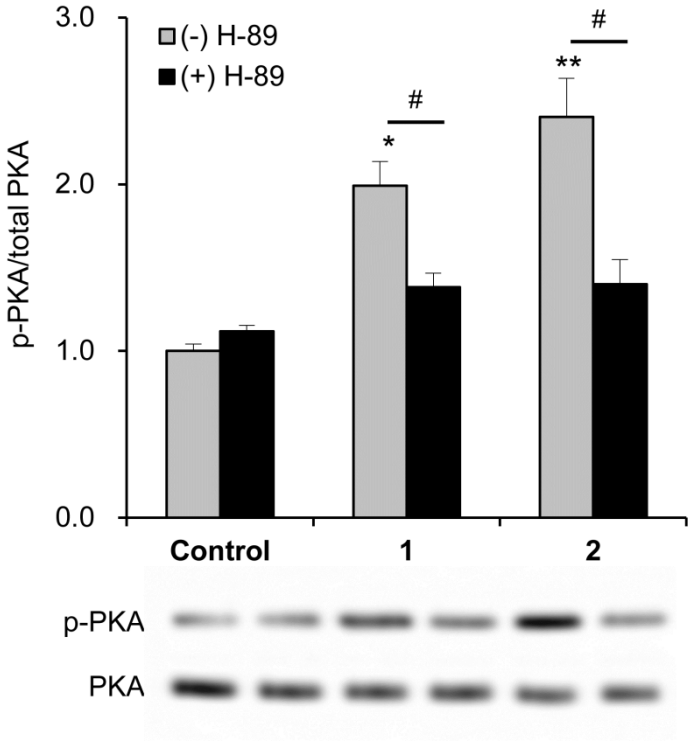

Figure 4. Analysis of PKA activation after treatment of **1** and **2**. 3T3-L1 adipocytes were pre-treated with or without inhibitor of PKA (H-89, 20 μM) and then treated with **1** or **2** (12.5 μM). Cells were lysed, and subjected to SDS-PAGE followed by western blotting. Data are expressed as mean ± SEM (n=4). A representative immunoblot is shown. *p<0.05, **p<0.01 *vs.* blank (Dunnett's test); $^{\#}p$<0.05 (t-test).

To further investigate the target of **1** and **2**, β3-adrenergic receptor, the major upstream target of PKA, was examined.[21] Compounds **1** and **2** were co-incubated with propranolol, an antagonist of β3-adrenergic receptor, but the antagonist had no effect on the lipolytic activity of the both compounds (Fig. 3C). Based on these findings, it is conclusively evident that **1** and **2** induce their lipolytic effects through the direct activation of PKA. Lipolytic activity of **1** and **2** might also work through the other upstream related signaling proteins, but not through the β3-adrenergic receptor located at the cell membrane (Fig. 5).

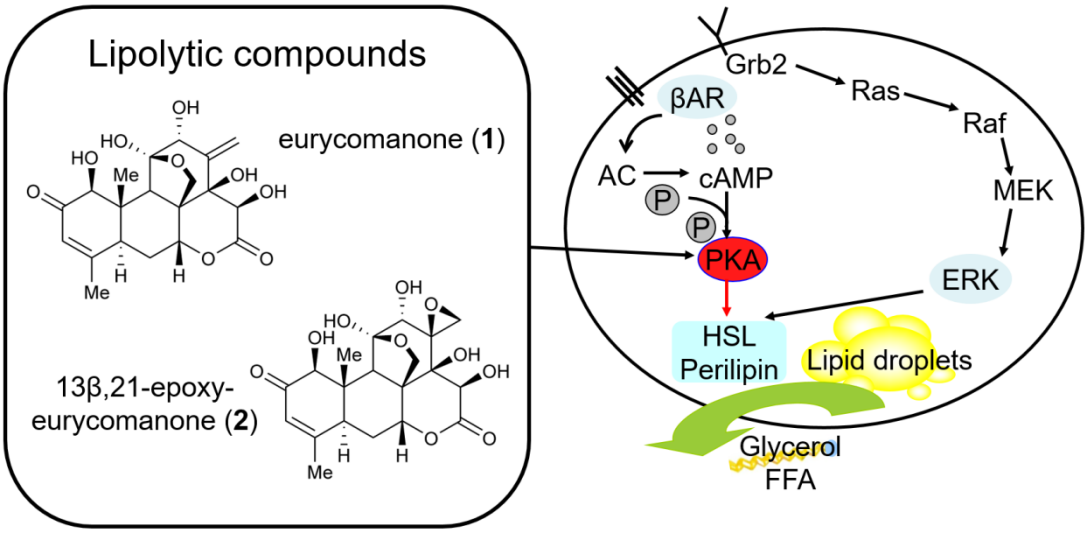

Figure 5. Isolated compounds **1** and **2** exert lipolytic activity through PKA activation

4. Conclusion

We have successfully identified both eurycomanone (**1**) and 13β,21-epoxyeurycomanone (**2**) from *E. longifolia* root as the active compounds responsible for the enhancement of lipolysis through the activation of PKA. Eurycomanone (**1**) has EC_{50} of 14.6 μM, and its epoxy derivate (**2**) has a stronger lipolytic activity (EC_{50} = 8.6 μM). However, the other isolated compound, 13β,21-dihydroxyeurycomanone (**3**), the dihydroxy derivate, did not exert lipolytic activity. These findings suggest that structure-activity relationship (SAR) study need to be conducted. It is expected that the results of SAR can lead to a potent anti-obesity agent.

Abbreviations Used

Ac	acetyl
βAR	beta adrenergic receptor
DEX	dexamethasone
DMEM	Dulbecco's Modified Eagle's medium
EC_{50}	half maximal effective concentration
ERK	extracellular signal-regulated kinase
ESI	electrospray ionization

FBS fetal bovine serum

HMBC heteronuclear multiple bond correlation

NOESY nuclear Overhauser effect spectroscopy

HPLC high performance liquid chromatography

HRMS high resolution mass spectrometry

IBMX 3-isobutyl-1-methylxanthine

NMR nuclear magnetic resonance

PBS phosphate buffered saline

PKA protein kinase A

TBS Tris buffered saline

TLC thin layer chromatography

Supplementary Material

NMR spectra of the isolated compounds, and pictures of three-dimensional models of compounds **2** and **4**.

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Supplementary Information

Isolation and lipolytic activity of eurycomanone and its epoxy derivative from *Eurycoma longifolia*

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NMR spectrum of the compounds....2
eurycomanone (**1**)....2
13β,21-epoxyeurycomanone (**2**)....3
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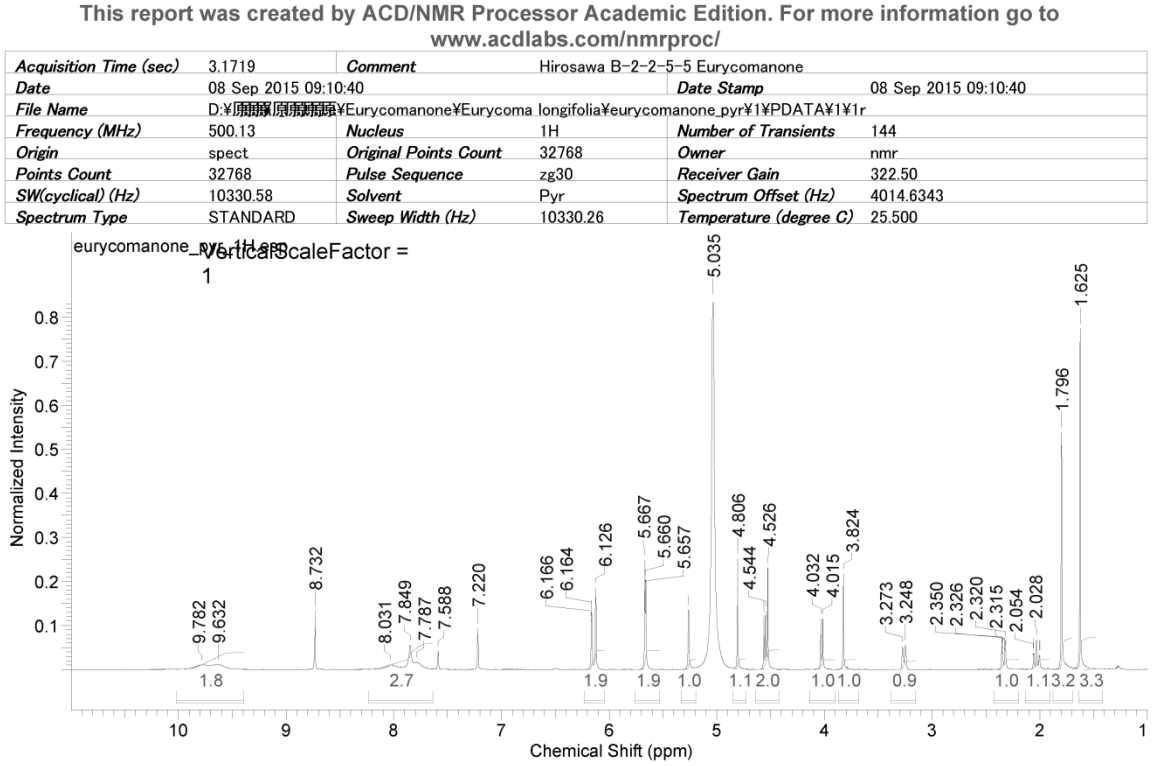
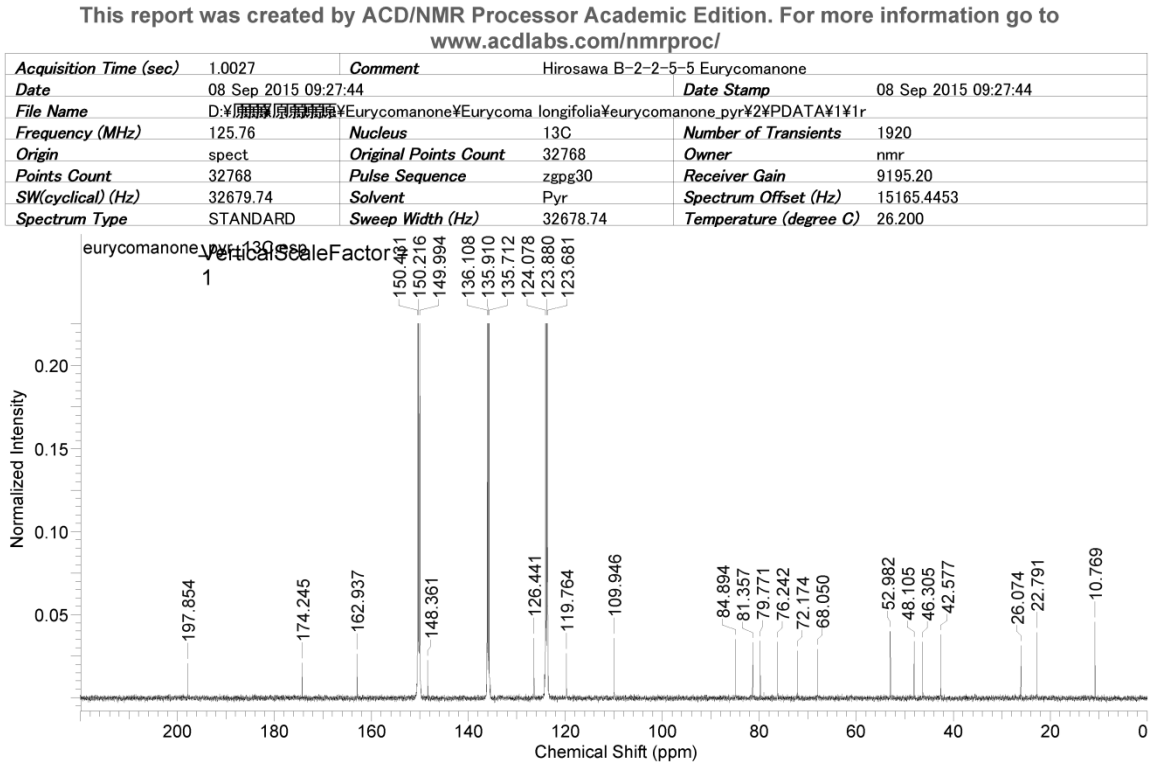
Structure models....7

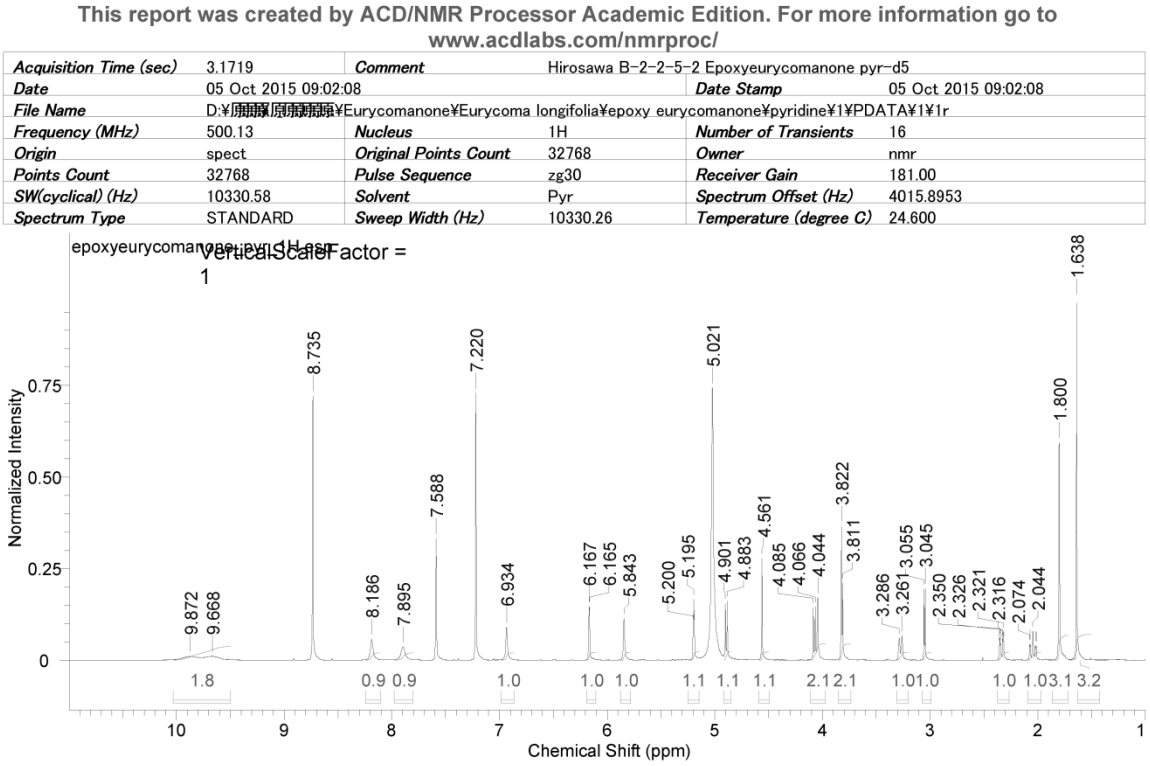
NMR spectrum of the compounds
eurycomanone (**1**)

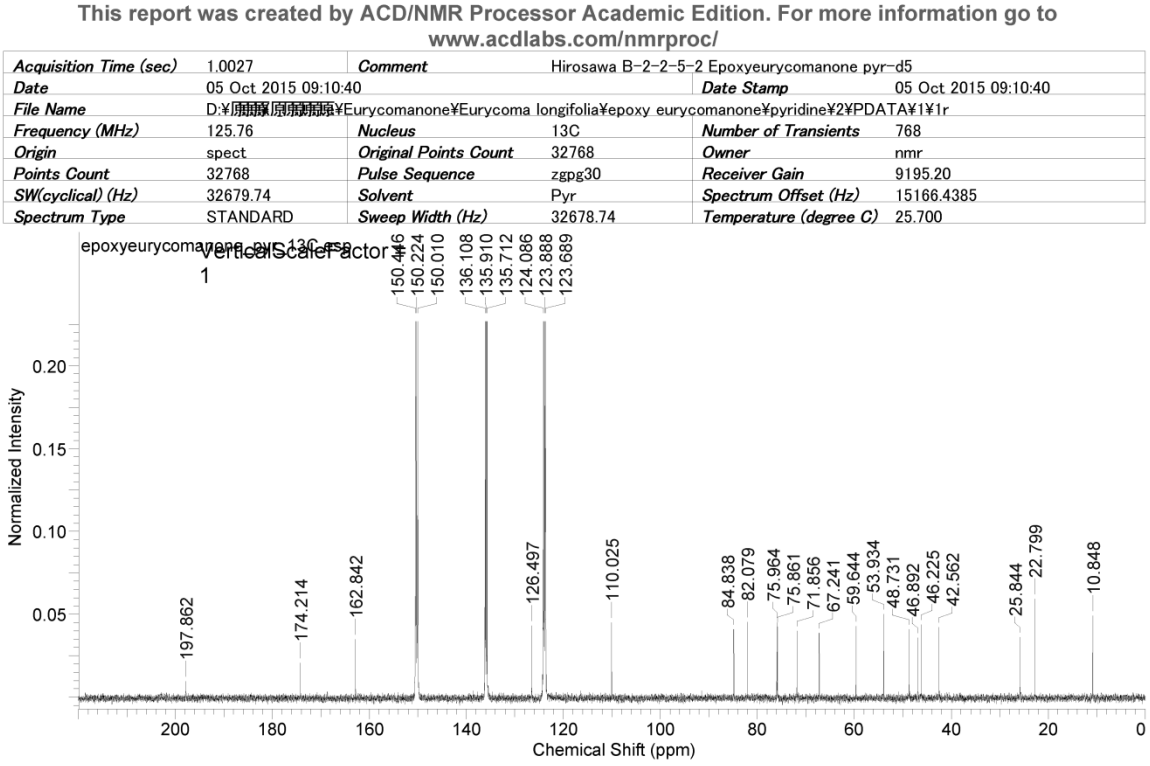




Supplementary Figure 1. ^{1}H and ^{13}C-NMR spectrum of eurycomanone (**1**)

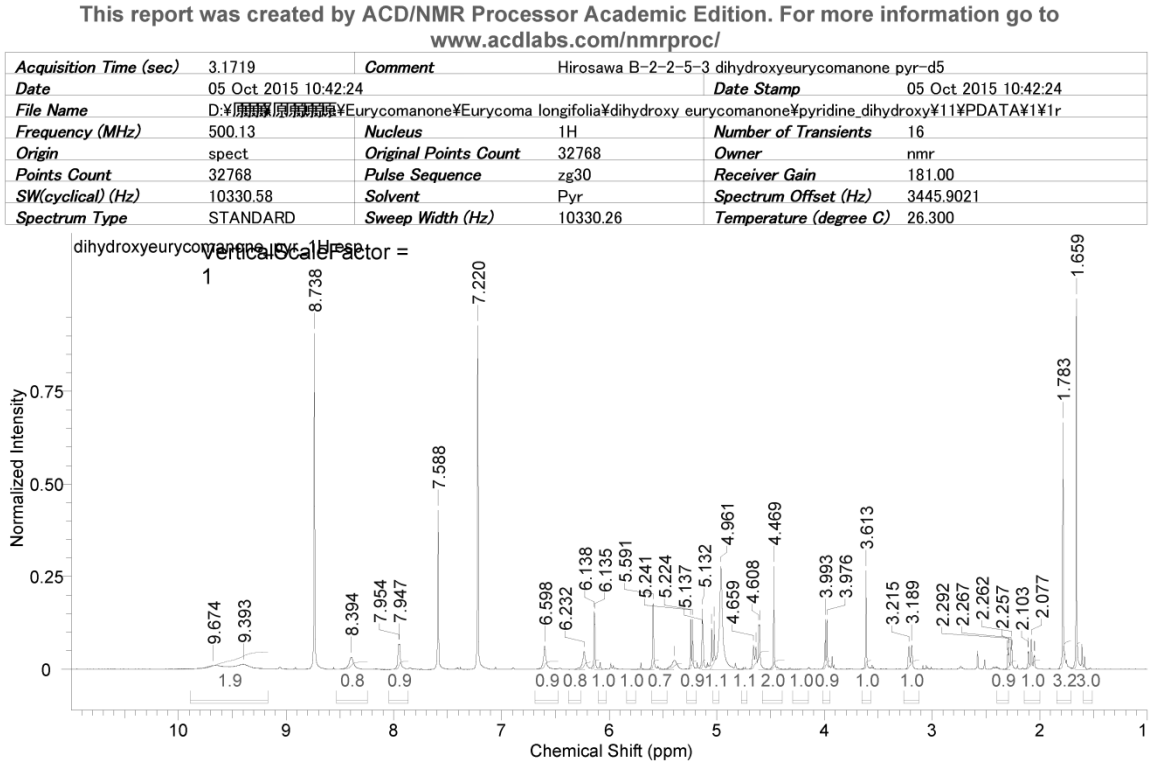
13β,21-epoxyeurycomanone (**2**)

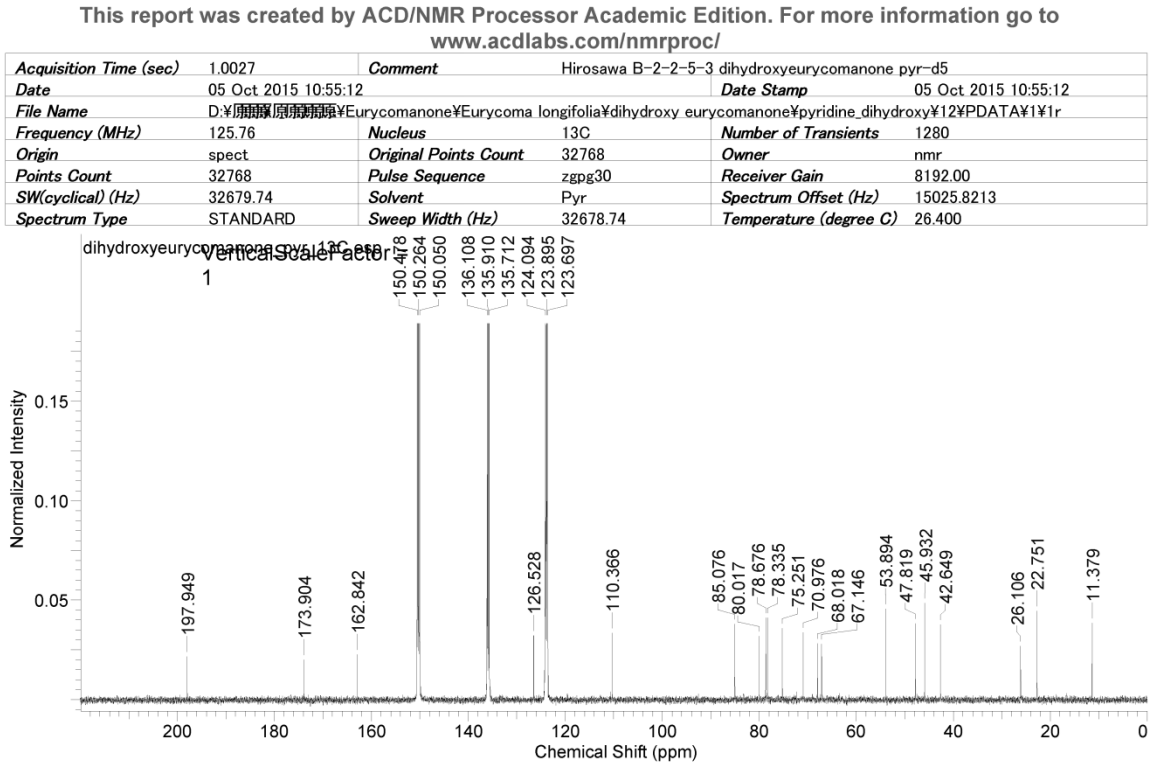




Supplementary Figure 2. ^{1}H and ^{13}C-NMR spectrum of 13β,21-epoxyeurycomanone (**2**)

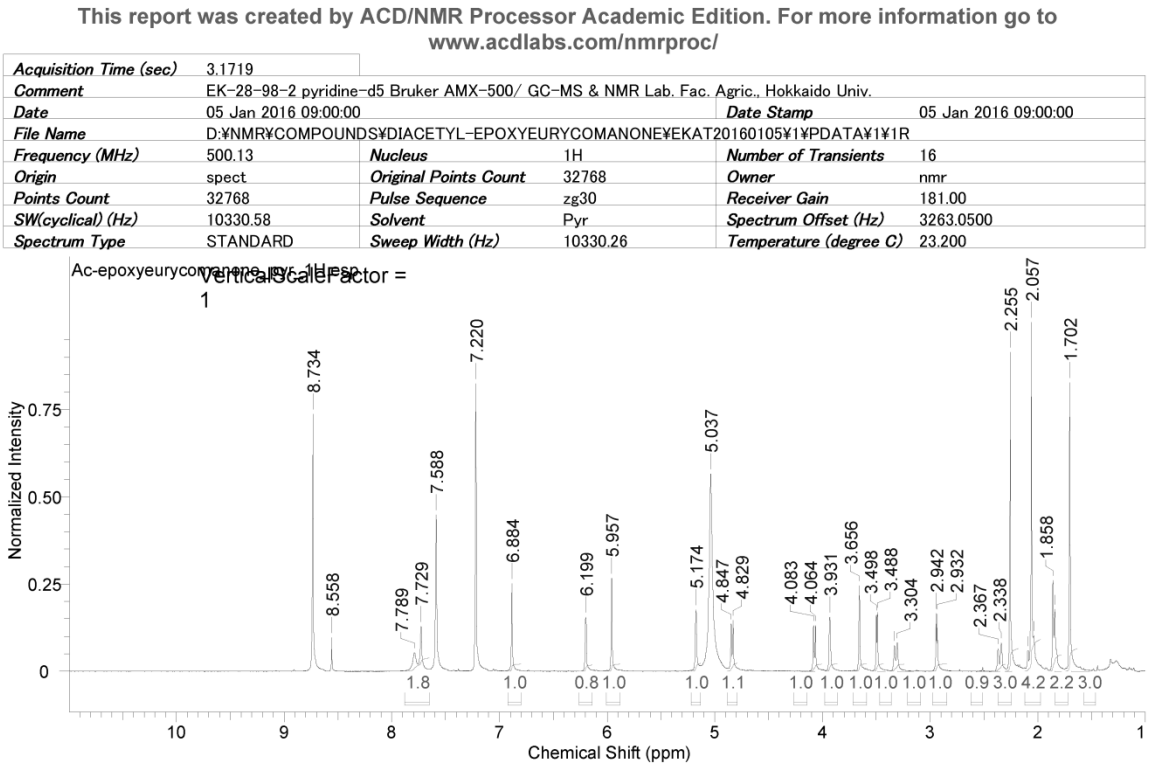
13β,21-dihydroxyeurycomanone (**3**)

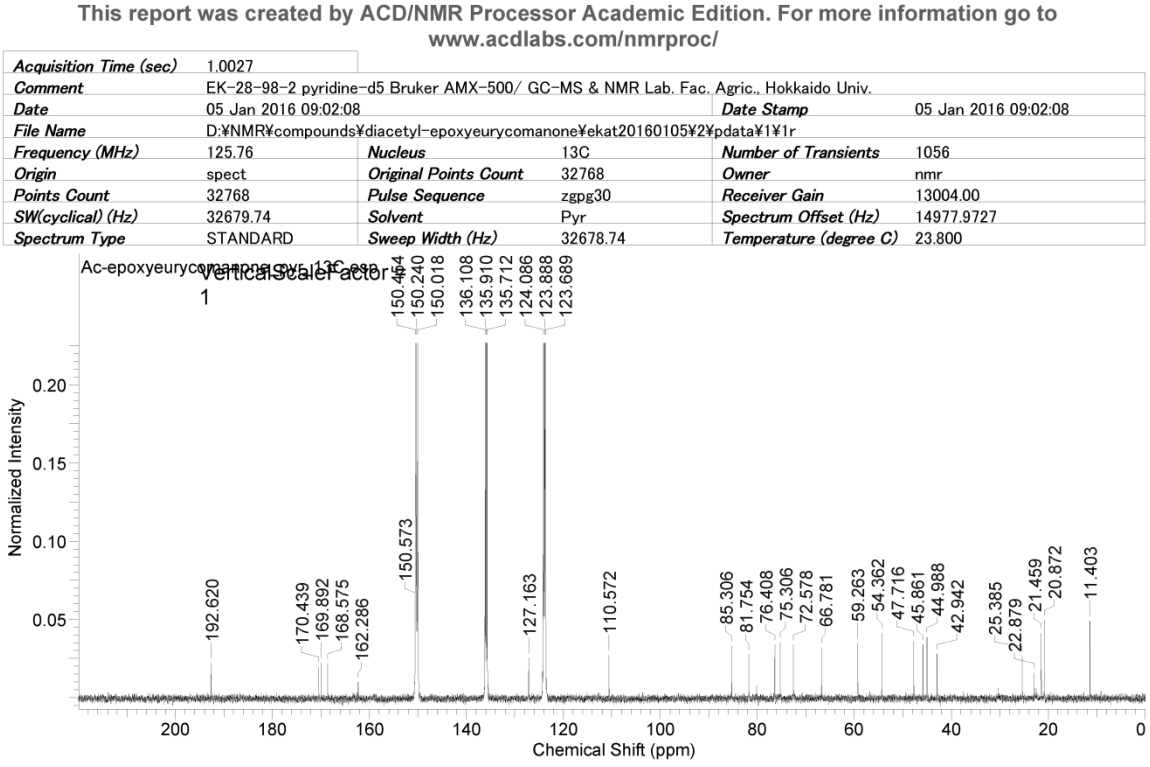




Supplementary Figure 3. ^{1}H and ^{13}C-NMR spectrum of 13β,21-dihydroxyeurycomanone (**3**)

di-*O*-acetyl-13β,21-epoxyeurycomanone (**4**)





Supplementary Figure 4. ^{1}H and ^{13}C-NMR spectrum of 1,15-di-*O*-acetyl-13β,21-epoxyeurycomanone (**4**)

This report was created by ACD/NMR Processor Academic Edition. For more information go to www.acdlabs.com/nmrproc/

Acquisition Time (sec)	(0.2048, 0.0512)	*Comment*	5 mm TXI 1H/D-13C/15N Z-GRD Z8161/37
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Original Points Count	(1024, 256)	*Owner*	nmr
Points Count	(2048, 1024)	*Pulse Sequence*	noesygpph
Solvent	Pyr	*Spectrum Type*	NOESY
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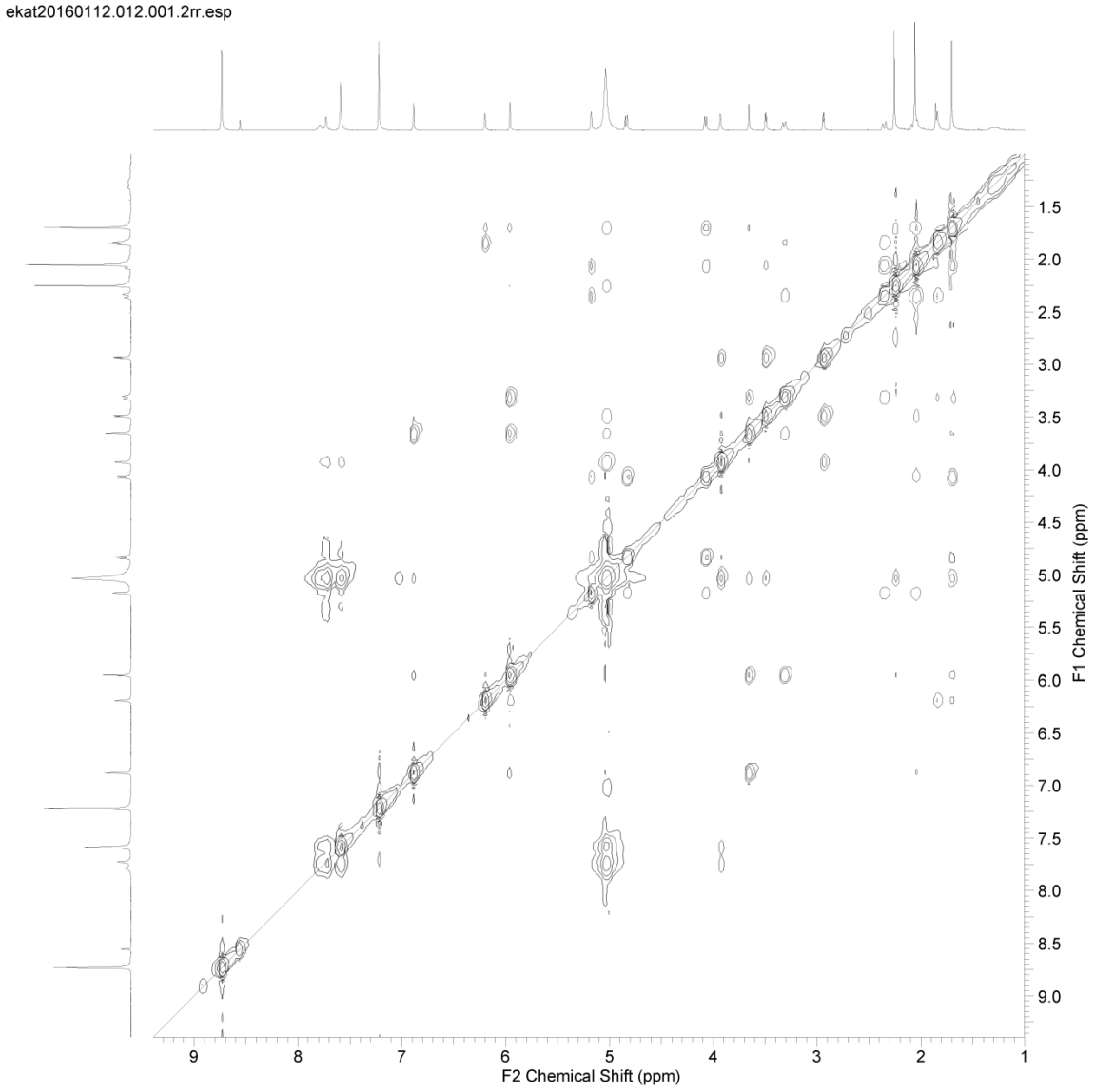
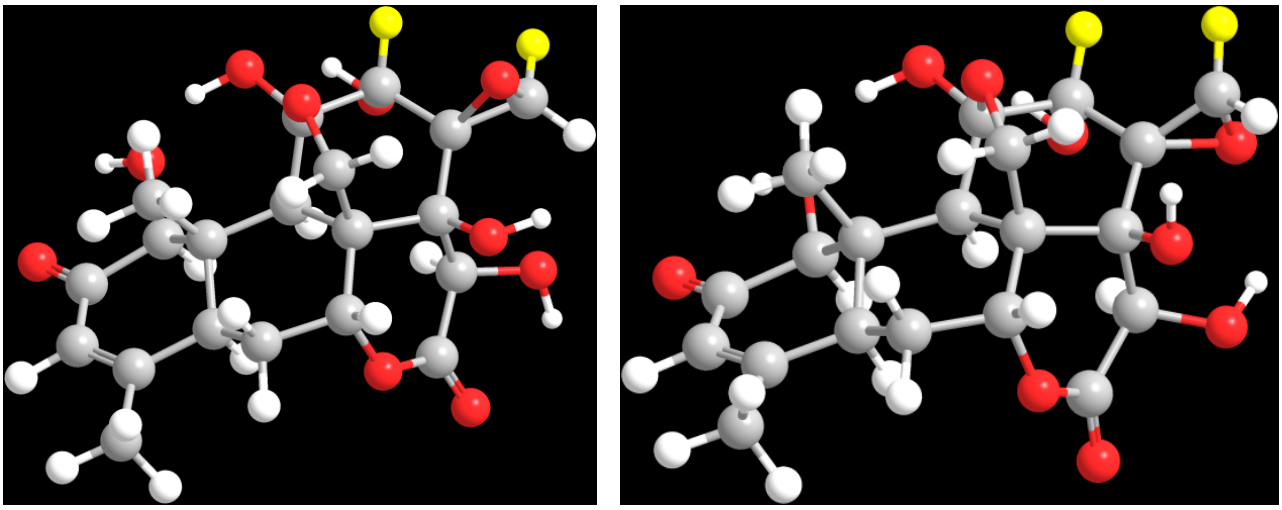


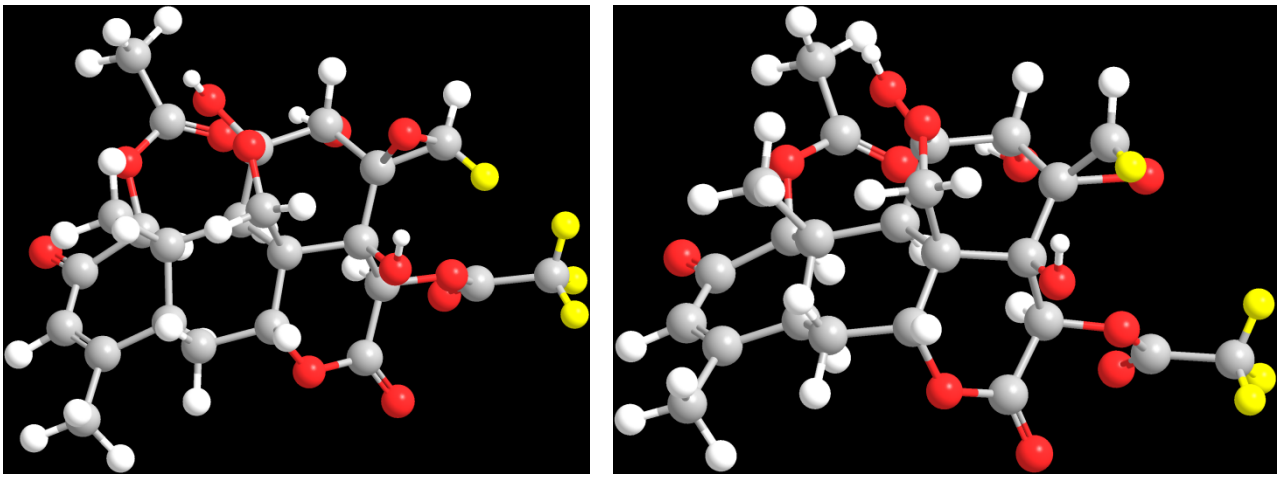
Supplementary Figure 5. NOESY spectrum of 1,15-di-*O*-acetyl-13β,21-epoxyeurycomanone (**4**)

Structure models



Supplementary Figure 6. 3D structure model of the 13,21-epoxyeurycomanone.

Left: β-epoxide; Right: α-epoxide. The model was created using ChemBio3D Ultra 14.0. The yellow atoms are H-12 and H_a-21. The calculated distance of H-12 and H_a-21 is 2.35 Å for β-epoxide and 2.57 Å for α-epoxide.



Supplementary Figure 7.

3D structure model of the 1,15-di-*O*-acetyl-13,21-epoxyeurycomanone.

Left: β-epoxide; Right: α-epoxide. The model was created using ChemBio3D Ultra 14.0. The yellow atoms are H-12 and H_a-21. The calculated distance of H-12 and H_a-21 is 4.69 Å for β-epoxide and 3.14 Å for α-epoxide.