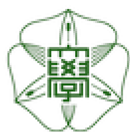
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Isolation and lipolytic activity of eurycomanone and its epoxy derivative from *Eurycoma longifolia*

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19 **ABSTRACT**

20 Eurycomanone (**1**) and 13β,21-epoxyeurycomanone (**2**) were isolated from *Eurycoma*

21 *longifolia* for studies of lipolytic activity. Compound **1** enhanced lipolysis in adipocytes

22 with an EC_{50} of 14.6 μM, while its epoxy derivate, compound **2**, had a stronger activity

23 with an EC_{50} of 8.6 μM. Based on molecular mechanistic study using several specific

24 inhibitors to lipolytic signaling pathways, it was found that PKA inhibitor totally

25 diminished the lipolytic activity of **1** and **2**. Further immunoblotting analysis confirmed

26 the activation of phosphorylated PKA by both **1** and **2**. With the growing need to

27 develop new anti-obesity agents, eurycomanone and its epoxy derivate can be used as

28 promising lead compounds to target lipid catabolism.

29

30

31 **Keywords**

32 3T3-L1 adipocytes; *Eurycoma longifolia*; lipolysis; obesity; quassinoid

33

34 ## 1. Introduction

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

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

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

52 Eurycomanone (**1**) is the major quassinoid in *E. longifolia*; and it is used as a

53 marker compound in quality control of commercial products derived from this plant.

54 Several bioactivities have been reported for compound **1** including, increased

55 production of testosterone in rat testicular cells,[8] antiulcer activity,[10] cytotoxicity against

56 cancer cell lines,[11] and antimalarial activity.[9]

57 In this report, we describe the isolation and lipolytic activity of **1** and its

58 derivatives, 13β,21-epoxyeurycomanone (**2**) and 13β,21-dihydroxyeurycomanone (**3**).

59 We also reconfirm stereochemistry assignment of the epoxide derivate (**2**).

60

61 **2. Material and methods**

62 2.1 General

63 Unless otherwise stated, commercially available chemicals were purchased

64 from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). *E. longifolia* root (Batch No.

65 SL.1A.2015.PB) was supplied by Merapi Farma Herbal Co. (Yogyakarta, Indonesia).

66 Absorbance was measured using a Synergy™ MX microplate reader (BioTek

67 Instruments, Inc., Winooski, VT, USA). A Bruker AMX 500 instrument (Bruker

68 BioSpin K.K., Bruker Instruments, Billerica, MA, USA) was used to obtain NMR

69 spectra and residual solvents were used as an internal standard (pyridine-d_5: ^{1}H 7.22

70 ppm, ^{13}C 135.91 ppm). Mass spectra were obtained using a LCT-Premier mass

71 spectrometer (Waters Corp., Milford, MA, USA). For the LC-MS analysis, Waters

72 Acquity UPLC system (Waters Corp., Milford, MA, USA) was combined with LCT-

73 Premier mass spectrometer (Waters Corp., Milford, MA, USA). The EZR was used for

74 the statistical tests.[22]

75

76 2.2 Isolation of quassionoids

77 Powdered root of *E. longifolia* (200 g) was extracted with 50% (v/v) aq.

78 methanol for 24 h to obtain 6.84 g extract. The extract was suspended in water and

79 partitioned with ethyl acetate and then with 1-butanol to obtain a water-soluble fraction

80 (4.10 g), a 1-butanol-soluble fraction (1.17 g), and an ethyl acetate-soluble fraction

81 (1.17 g). The 1-butanol-soluble fraction was adsorbed onto DIAION HP-20 (φ40 mm ×

82 240 mm, Mitsubishi Chemical Co., Tokyo, Japan), washed with water, and eluted with

83 50% aq. methanol. The 50% (v/v) aq. methanol-eluted fraction (580 mg) was then

84 separated using Cosmosil® 75C18-OPN (Nakalai Tesque Inc., Kyoto, Japan) column

85 chromatography (φ20 mm × 120 mm) by stepwise elution with water, 10% aq.

86 methanol, 20% aq. methanol, 30% aq. methanol, 50% aq. methanol, 70% aq. methanol,

87 and methanol. The active fraction eluted with 10% aq. methanol (130 mg) was further

88 purified by Toyopearl HW-40F (Tosoh Co., Tokyo, Japan) column chromatography (φ

89 15 mm × 160 mm) with water as the eluent. The active fraction was finally purified by

90 HPLC using an InertSustain C18 column (φ20×250 mm; GL Science Co., Tokyo,

91 Japan) with 20% aq. methanol as an eluent to obtain eurycomanone (**1**, 16.3 mg),[12,13] 13

92 β,21-epoxyeurycomanone (**2**, 4.8 mg),[12,14] and 13β,21-dihydroxyeurycomanone (**3**, 3.6

93 mg).[9,13] The water-soluble fraction (4.10 g) was similarly separated to obtain additional

94 **1** (68.2 mg), **2** (14.0 mg), and **3** (8.6 mg). Each compound was identified by comparing

95 its ^{1}H,^{13}C-NMR and optical rotation with the reported values.

96

97 2.2.1 Eurycomanone (**1**)

98 ^{1}H-NMR (500 MHz, pyridine-d_5, rt): 1.63 (3H, s), 1.80 (3H, br s), 2.03 (1H,

99 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

100 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

101 (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 =

102 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,

103 OH), 9.63 (1H, br s, OH), 9.78 (1H, br s, OH) ppm; ^{13}C-NMR (125 MHz, pyridine-d_5,

104 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,

105 84.89, 109.95, 119.76, 126.44, 148.36, 162.94, 174.25, 197.85 ppm; HR-ESI-MS

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

107 +32.1° (c = 1.0, pyridine).

108

109 2.2.2 13β,21-epoxyeurycomanone (**2**)

110 ^{1}H-NMR (500 MHz, pyridine-d_5, rt): 1.64 (3H, s), 1.80 (3H, br s), 2.04 (1H,

111 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

112 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),

113 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),

114 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,

115 br s, OH), 9.67 (1H, br s, OH), 9.87 (1H, br s, OH) ppm; ^{13}C-NMR (125 MHz, pyridine-

116 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,

117 75.86, 75.96, 82.08, 84.84, 110.03, 126.50, 162.84, 174.21, 197.86 ppm; HR-ESI-MS

118 (positive): $[M+Na]^+$, found m/z = 447.1287, $C_{20}H_{24}O_{10}Na$, requires m/z 447.1267; $[\alpha]_D^{24}$

119 +34.2° (c = 1.0, pyridine).

120

121 2.2.3 13β,21-dihydroxyeurycomanone (**3**)

122 ^{1}H-NMR (500 MHz, pyridine-d_5, rt): 1.66 (3H, s), 1.78 (3H, s), 2.08 (1H, ddd,

123 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),

124 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,

125 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

126 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),

127 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),

128 9.39 (1H, br s, OH), 9.67 (1H, br s, OH) ppm; ^{13}C-NMR (125 MHz, pyridine-d_5, rt):

129 11.38, 22.75, 26.11, 42.65, 45.93, 47.82, 53.89, 67.15, 68.02, 70.98, 75.25, 78.34,

130 78.68, 80.12, 85.08, 110.37, 126.53, 162.84, 173.90, 197.95 ppm; HR-ESI-MS

131 (positive): $[M+Na]^+$, found m/z = 465.1390, $C_{20}H_{26}O_{11}Na$, requires m/z 465.1373; $[\alpha]_D^{24}$

132 +17.5° (c = 1.0, pyridine).

133

134 2.2.4 Acetylation of 13β,21-epoxyeurycomanone (**2**)

135 Compound **2** (3.6 mg) was dissolved in pyridine (0.3 mL) and then acetic

136 anhydride (0.15 mL) was added. The mixture was stirred for 1 h at room temperature

137 under nitrogen, and then diluted with water and extracted with ethyl acetate. The

138 organic layer was dried over sodium sulfate, evaporated, and then the residue was

139 separated by preparative TLC (hexane/acetone = 1/1) to obtain the di-acetylated

140 derivative **4** (4.4 mg, quant.). The positions of acetyl groups were confirmed by

141 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

214 **3. Results and discussion**

215 3.1 Chemistry

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

223 The structures of the isolated compounds were determined based on NMR and
224 MS spectra. Compound **1** was determined as eurycomanone according to its ^{1}H-NMR
225 spectra.[10,12] The results of an HRMS analysis ($[M+Na]^+$, found *m/z* 431.1322,
226 $C_{20}H_{24}O_9Na$ requires 431.1318) and optical rotation ($[\alpha]_D^{24}$ +32.1°) supported this
227 determination. The obtained data of compound **3** were compared with those reported
228 earlier for several other plant quassinoids, and this compound was identified as 13β,21-
229 dihydroxyeurycomanone (**3**).[9,12]

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

232 as beta,[10] while in another reference it was identified as alpha.[13] However, both of the

233 reported NMR spectra and optical rotations were the same as those obtained for

234 compound **2** here, indicating that one of the previous stereochemistry assignments is

235 incorrect. Therefore, through this study, we re-examined and clarified the

236 stereochemistry of the epoxide.

237 Measurement of the NOESY spectra of this compound showed a correlation

238 between H-12 and H_a-21, which was considered as an evidence of the α-epoxide in the

239 previous study.[13] However, the distance between those two hydrogen atoms was similar

240 between the α and β-epoxide structure models (see Supporting information).

241 Therefore, the observed NOESY correlation was considered insufficient evidence to

242 determine its stereochemistry. To examine and verify its stereochemistry, compound **2**

243 was then acetylated to obtain di-*O*-acetyl product **4** and the NOESY experiment was

244 performed using **4**. In compound **4**, a NOESY correlation was observed between H_b-21

245 and AcO-15. Thus, the stereochemistry of the epoxide was confirmed to be beta, which

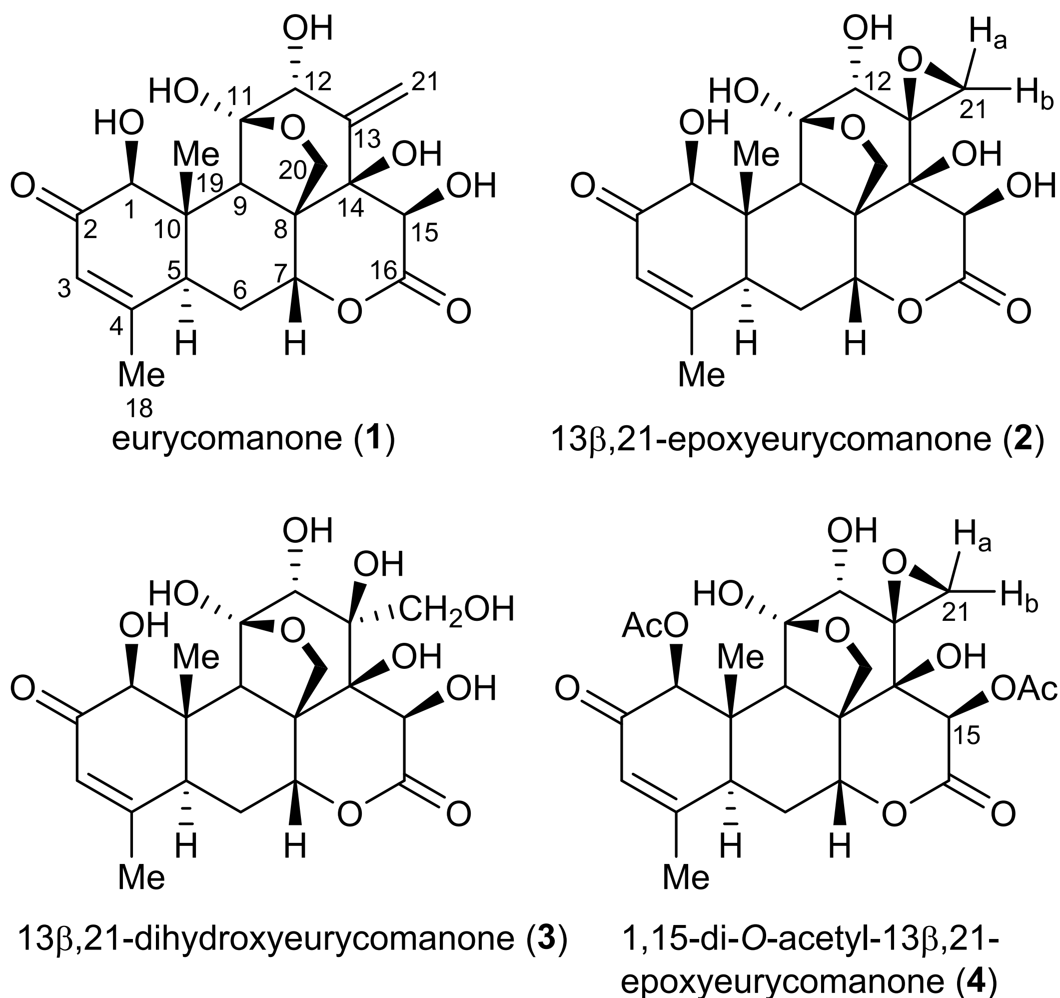
246 is a biosynthetically reasonable configuration if the epoxide **2** is hydrolyzed to produce

247 its dihydroxy derivative **3** *in planta*. In light of this finding, we need to consider that

248 previous studies on the use, detection, and isolation of 13α,21-epoxyeurycomanone

249 from *E. longifolia* probably refer to the β-epoxide.[14,15]

250



251

252 Figure 1. Structures of compounds.

253

254 3.2 Biological activity

255 The three isolated compounds were evaluated for anti-obesity activities;

256 enhancement of glycerol release and reduction of lipid accumulation. Compounds **1** and

257 **2** showed concentration-dependent activity in the glycerol release enhancement assay,

258 and reduced the lipid accumulation without cytotoxic effects (Fig. 2). The EC_{50} value

259 for the glycerol release enhancement was 14.6 μM for **1**, while **2** had a lower EC_{50} (8.6

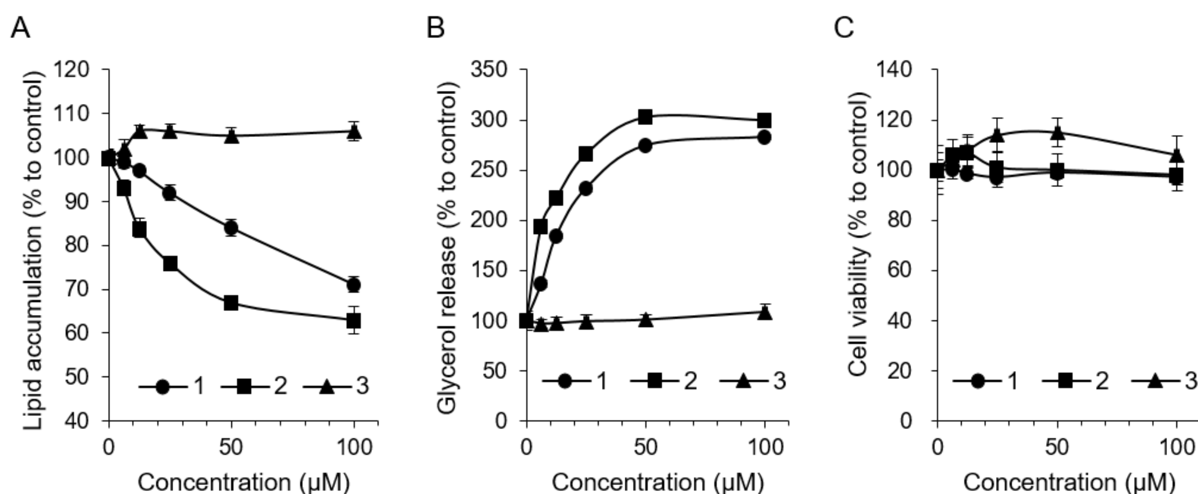
260 μM). The stronger bioactive effects of **2** indicates the importance of the epoxide group

261 in exerting its bioactivity. In contrast, the hydrolyzed derivative **3** did not show any

262 biological activity in either of the two assays (Fig. 2A and 2B), even at the highest

263 concentration tested (100 μM).

264



265 Figure 2. Lipid accumulation reduction effect (A), glycerol release enhancement activity

266 (B), and cell viability (C) of compounds **1**–**3**. (A) Compound **1** showed significant

267 difference above 25 μM (p<0.01). Compound **2** showed significant difference above

268 6.25 μM (p<0.05) and above 12.5 μM (p<0.01). (B) Compounds **1** and **2** showed

269 significant difference above 6.25 μM. Isoproterenol (1 μM) was used as positive control

270 (322±1%). Data are expressed as mean ± SEM (n=6). Dunnett's test was used.

271

272 There are two possible reasons for the total absence of bioactivity in **3**. First,
273 the presence of two hydroxyl groups might have strongly interfered with the interaction
274 between the compound and the target. However, this explanation is unlikely to happen
275 for the following reason. Although various derivatives of eurycomanones have been
276 isolated from plants using various methods,[6,9,12-13,15-18] here we identified **1**–**3** by using
277 activity-guided fractionation. If steric hindrance or ionic repulsion were the reason for
278 the lack of bioactivity of **3**, then other related compounds, for example 13β-methyl,21-
279 dihydroeurycomanone, would have been obtained as bioactive compounds during the
280 isolation process.
281 The second possibility is that the epoxide group is the essential moiety for the
282 bioactivity. This would suggest that eurycomanone (**1**) is oxidized in the cells to form
283 its bioactive epoxide (**2**). However, there was no evidence for supporting this
284 hypothesis. Therefore, subsequent research on structure-activity relationship (SAR) of
285 these quassinoids is required to determine whether the epoxide group is the essential
286 part of the bioactivity.
287
288 3.3 Mechanistic study
289 After evaluating their biological activities in reducing lipid accumulation and

290 enhancing lipolysis, molecular mechanism of the lipolytic activity of **1** and **2** was

291 further investigated. Since these compounds induced lipolysis in 3T3-L1 cells, the

292 signaling molecules related to the activation of hormone-sensitive lipase (HSL) were

293 predicted to involve. [19] Initially, we tested the two well-known lipolytic signaling

294 molecules; protein-kinase A (PKA) and extracellular signal-regulated kinase (ERK), [20]

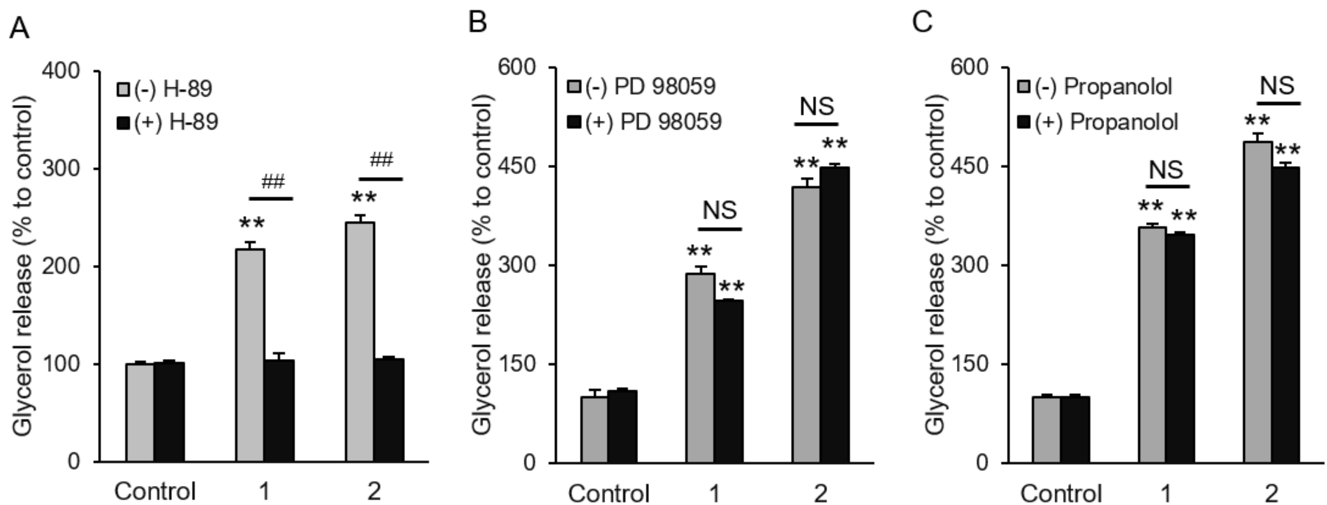
295 by co-incubating **1** and **2** with the respective specific inhibitors (Fig. 3A and 3B).

296 Inhibitor of PKA (H-89) totally diminished the activity of both compounds, but

297 inhibitor of ERK (PD 98059) had no significant effect on the lipolytic activity. Protein

298 immunoblotting analysis also confirmed the activation of PKA by both **1** and **2** (Fig. 4).

299



300 Figure 3. Effect of specific inhibitors on glycerol release enhancement activity of

301 compounds **1** and **2**. 3T3-L1 adipocytes were pre-treated with (A) inhibitor of PKA (H-

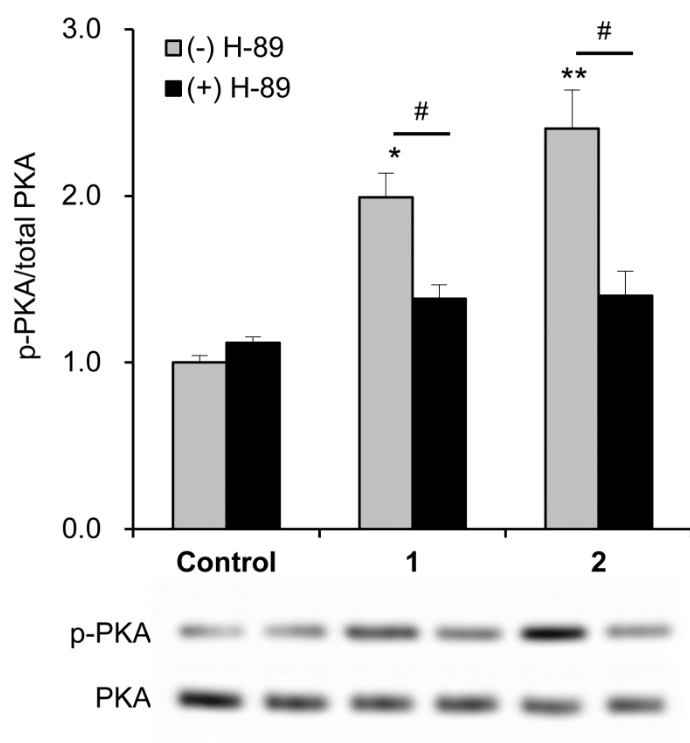
302 89, 20 μM), (B) inhibitor of ERK (PD 98059, 50 μM), or (C) inhibitor of β3-adrenergic

303 receptor (propranolol 1 μM) and then treated with **1** and **2** (25 μM). Data are expressed

304 as mean ± SEM (n=6). **p<0.01 *vs.* control without inhibitor (Dunnett's test); ##p<0.01

305 (t-test); NS: No significance.

306



307

308 Figure 4. Analysis of PKA activation after treatment of **1** and **2**. 3T3-L1 adipocytes

309 were pre-treated with or without inhibitor of PKA (H-89, 20 μM) and then treated with

310 **1** or **2** (12.5 μM). Cells were lysed, and subjected to SDS-PAGE followed by western

311 blotting. Data are expressed as mean ± SEM (n=4). A representative immunoblot is

312 shown. *p<0.05, **p<0.01 *vs.* blank (Dunnett's test); #p<0.05 (t-test).

313

314 To further investigate the target of **1** and **2**, β3-adrenergic receptor, the major

315 upstream target of PKA, was examined.[21] Compounds **1** and **2** were co-incubated with

316 propranolol, an antagonist of β3-adrenergic receptor, but the antagonist had no effect

317 on the lipolytic activity of the both compounds (Fig. 3C). Based on these findings, it is

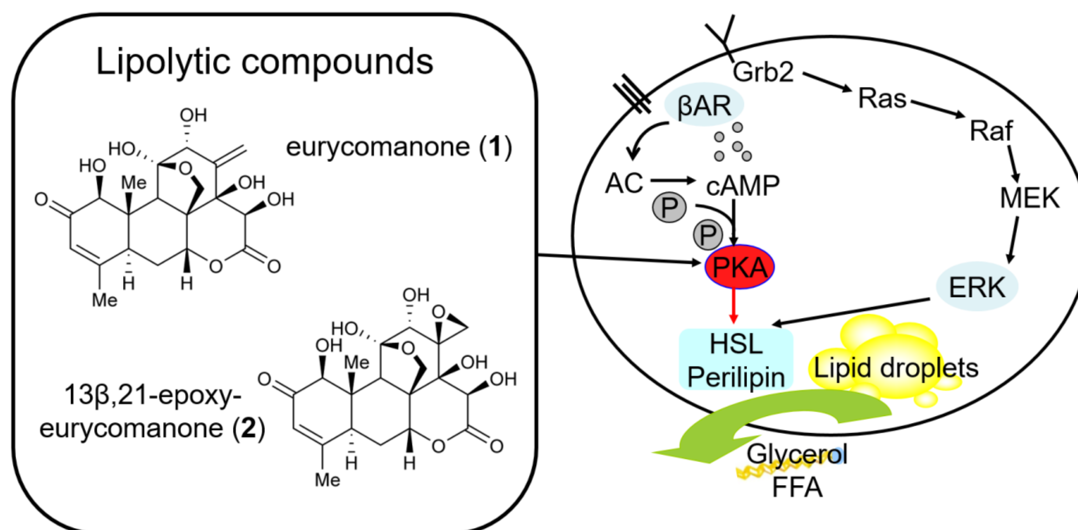
318 conclusively evident that **1** and **2** induce their lipolytic effects through the direct

319 activation of PKA. Lipolytic activity of **1** and **2** might also work through the other

320 upstream related signaling proteins, but not through the β3-adrenergic receptor located

321 at the cell membrane (Fig. 5).

322



323

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

325

326 **4. Conclusion**

327 We have successfully identified both eurycomanone (**1**) and 13β,21-

328 epoxyeurycomanone (**2**) from *E. longifolia* root as the active compounds responsible for

329 the enhancement of lipolysis through the activation of PKA. Eurycomanone (**1**) has

330 EC_{50} of 14.6 μM, and its epoxy derivate (**2**) has a stronger lipolytic activity (EC_{50} = 8.6

331 μM). However, the other isolated compound, 13β,21-dihydroxyeurycomanone (**3**), the

332 dihydroxy derivate, did not exert lipolytic activity. These findings suggest that

333 structure-activity relationship (SAR) study need to be conducted. It is expected that the

334 results of SAR can lead to a potent anti-obesity agent.

335

336 **Abbreviations Used**

337 Ac acetyl

338 βAR beta adrenergic receptor

339 DEX dexamethasone

340 DMEM Dulbecco's Modified Eagle's medium

341 EC_{50} half maximal effective concentration

342 ERK extracellular signal-regulated kinase

343 ESI electrospray ionization

344 FBS fetal bovine serum

345 HMBC heteronuclear multiple bond correlation

346 NOESY nuclear Overhauser effect spectroscopy

347 HPLC high performance liquid chromatography

348 HRMS high resolution mass spectrometry

349 IBMX 3-isobutyl-1-methylxanthine

350 NMR nuclear magnetic resonance

351 PBS phosphate buffered saline

352 PKA protein kinase A

353 TBS Tris buffered saline

354 TLC thin layer chromatography

355

356 **Supplementary Material**

357 NMR spectra of the isolated compounds, and pictures of three-dimensional

358 models of compounds **2** and **4**.

359

360

361

362 **References**

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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
- 13β,21-dihydroxyeurycomanone (**3**)......4
- di-*O*-acetyl-13β,21-epoxyeurycomanone (**4**)......5

Structure models......7

NMR spectrum of the compounds

eurycomanone (**1**)

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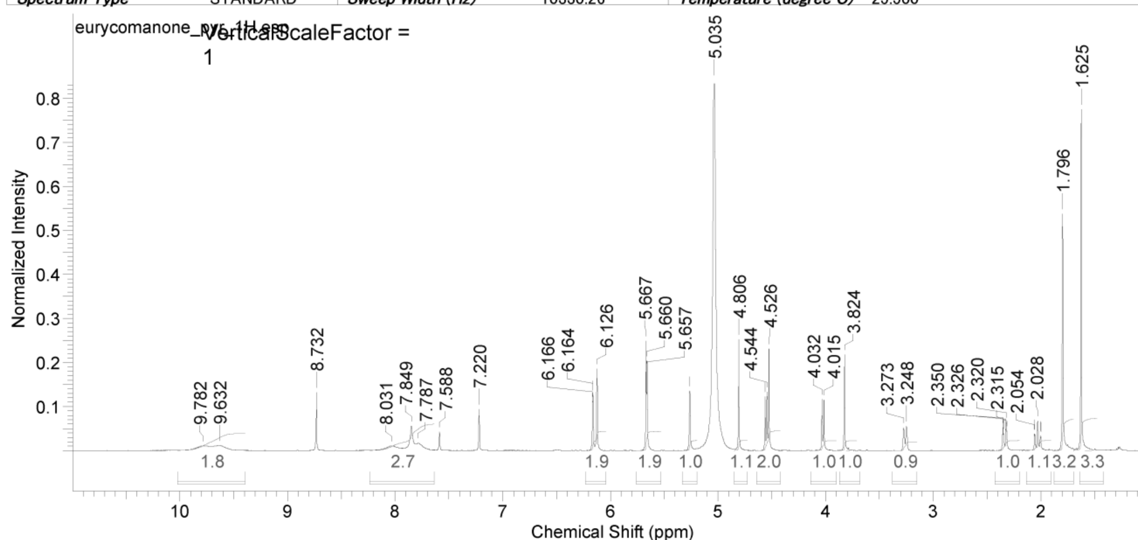

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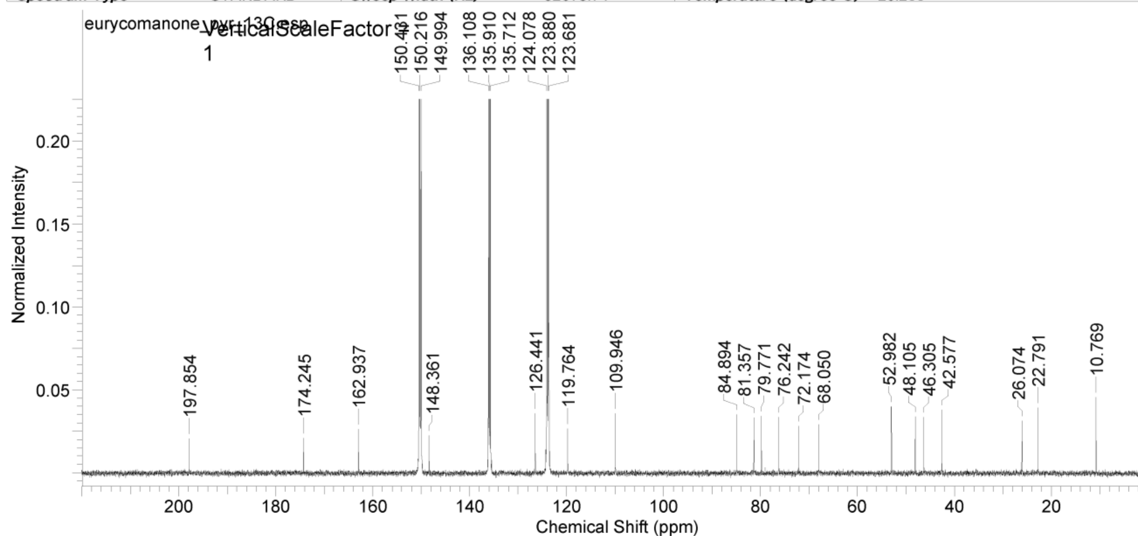

Supplementary Figure 1. ^{1}H and ^{13}C-NMR spectrum of eurycomanone (**1**)

13β,21-epoxyeurycomanone (**2**)

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

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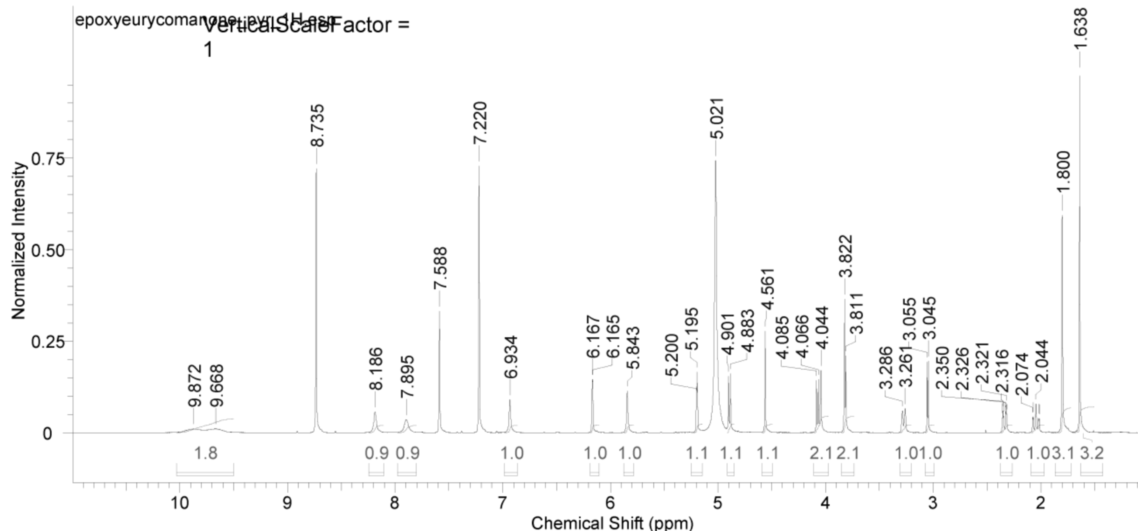

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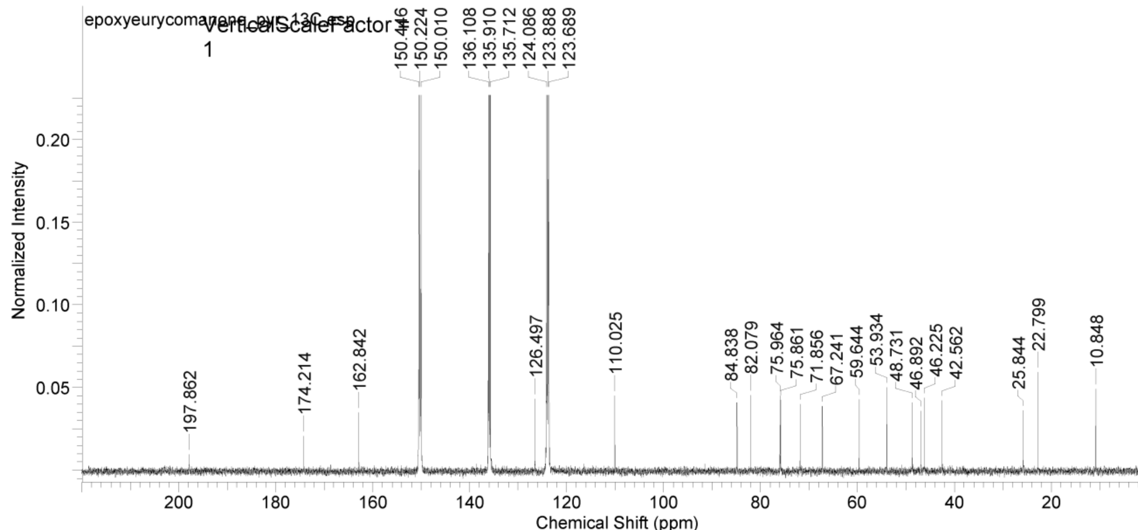

Supplementary Figure 2. ^{1}H and ^{13}C-NMR spectrum of 13β,21-epoxyeurycomanone (**2**)

13β,21-dihydroxyeurycomanone (**3**)

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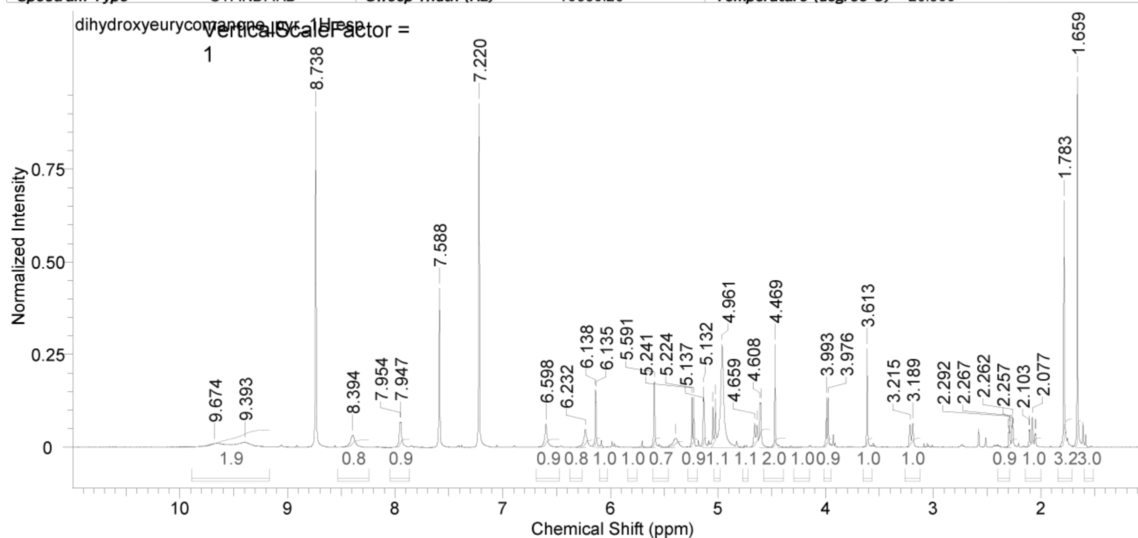

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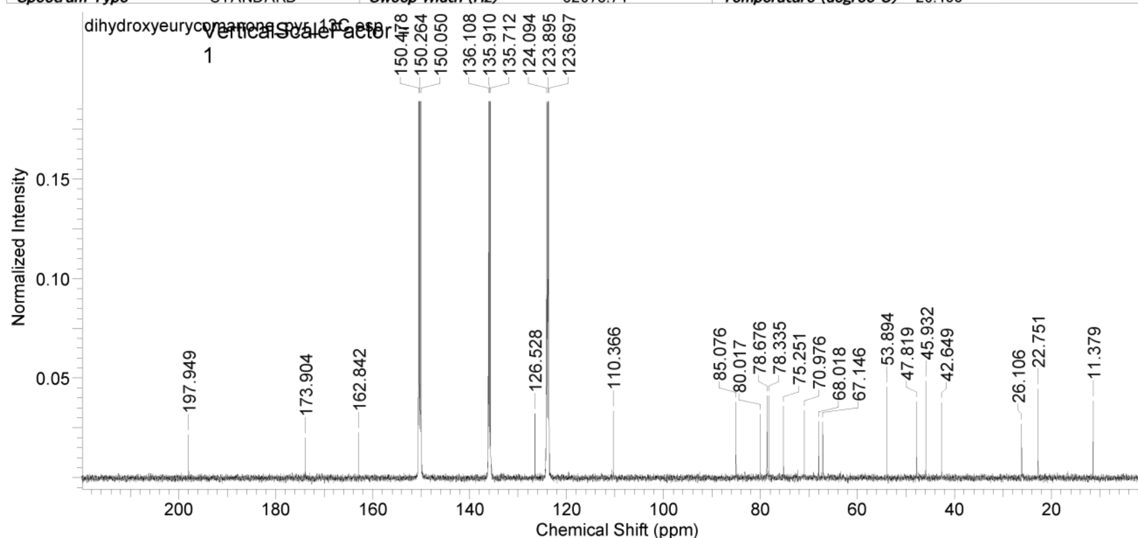
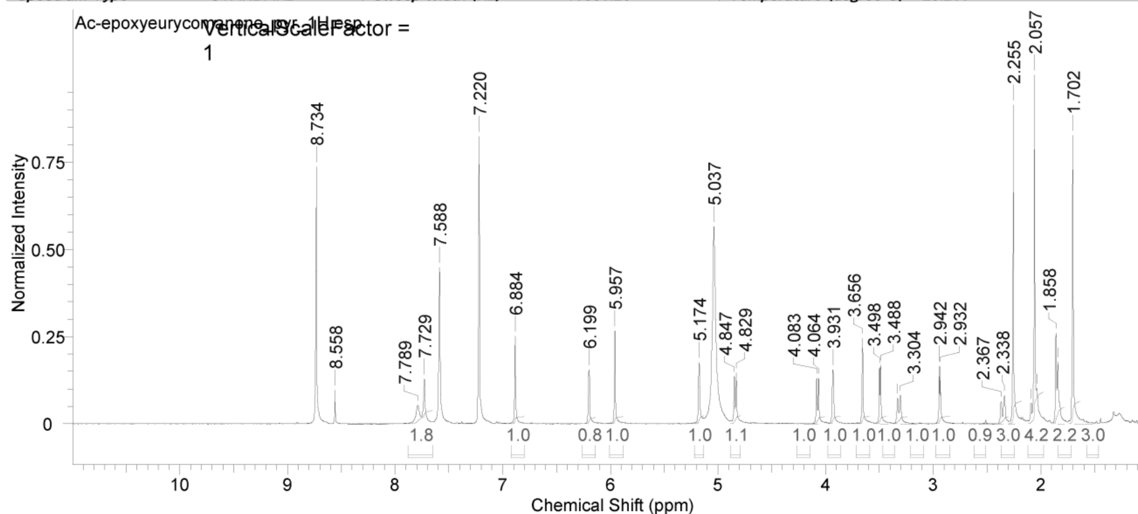
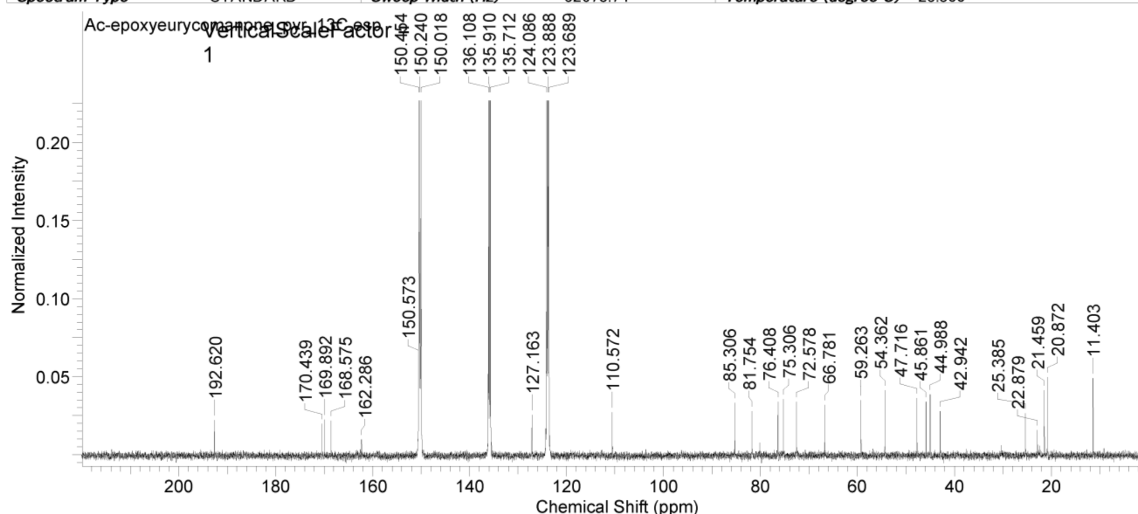


Supplementary Figure 3. ^{1}H and ^{13}C-NMR spectrum of 13β,21-dihydroxyeurycomanone (**3**)

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/

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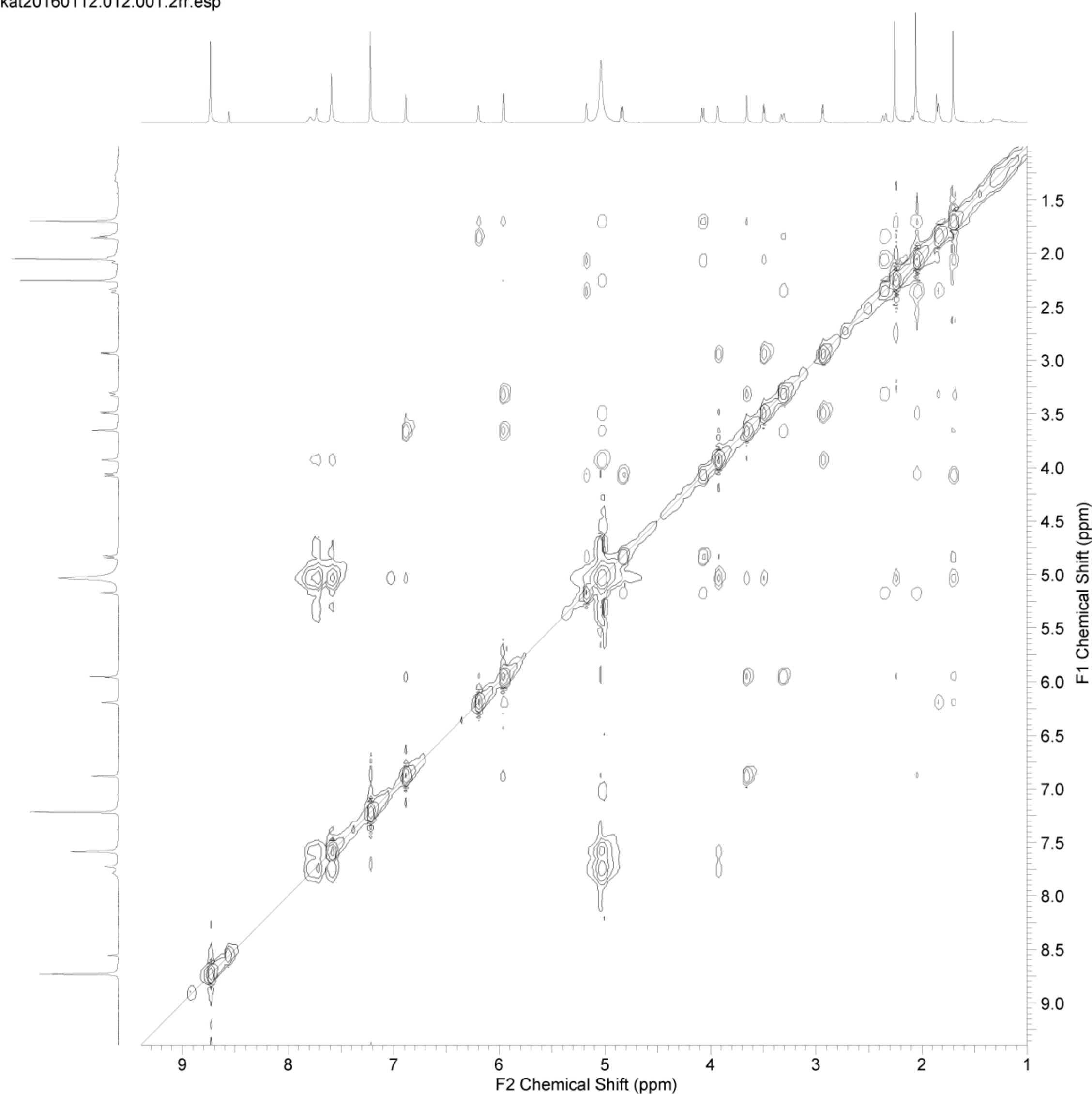
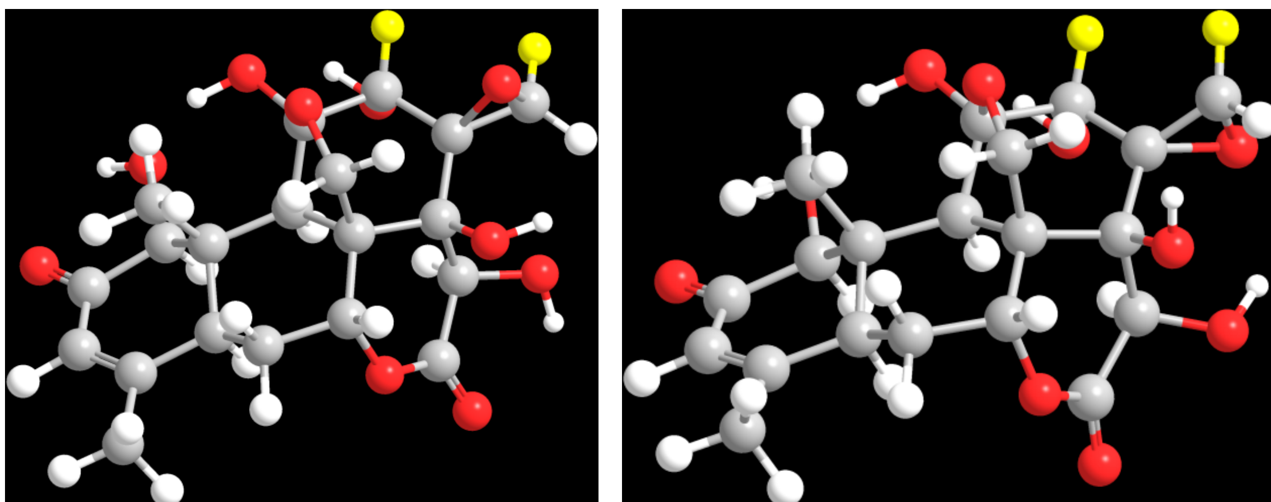


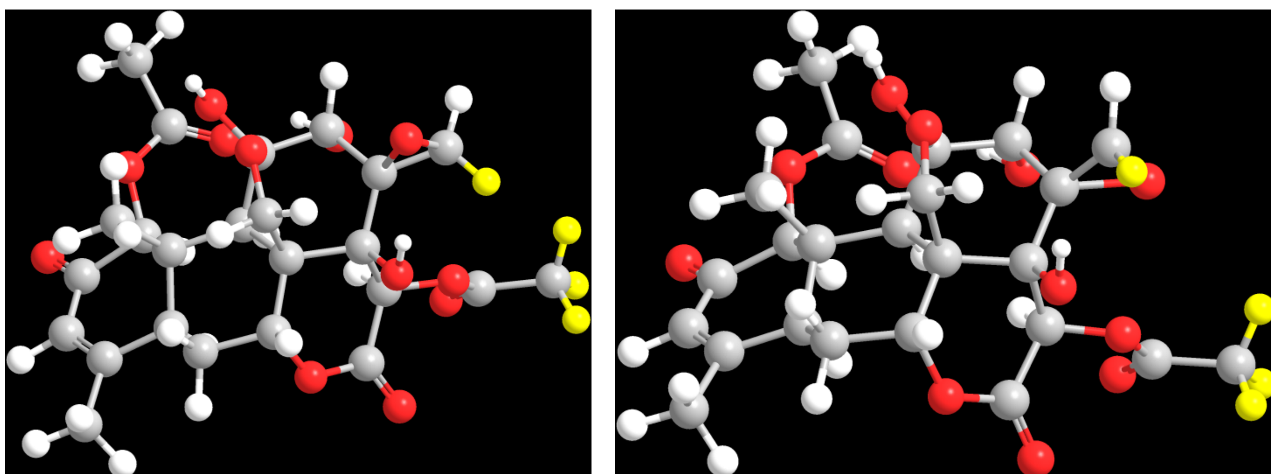
Supplementary Figure 5. NOESY spectrum of 1,15-di-*O*-acetyl-13β,21-epoxyeurycomanone (**4**)

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.