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5 **An α -Amylase Inhibitory Triterpene from *Abrus precatorius* Leaves**

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16 Running header : α -amylase inhibitors from *Abrus precatorius*

17

18 **ABSTRACT:** In the screening experiments for porcine pancreatic α -amylase inhibitors
19 in 18 plants obtained from Indonesia, a potent inhibitory activity was detected in the
20 extract of leaves of *Abrus precatorius*. The enzyme assay-guided fractionation of the
21 extract led to the isolation of a triterpene ketone, lupenone (**1**) as a potent α -amylase
22 inhibitor together with 24-methylenecycloartenone (**2**) and luteolin (**3**). The mode of
23 inhibition of **1** against porcine pancreatic α -amylase was a mixed inhibition. This is the
24 first report that describes the potent α -amylase-inhibitory activity of the low polar
25 triterpene ketone similar to **1**. Comparison of the activities of the isolate and related
26 compounds indicated the importance of C-3 ketone and the lupane skeleton in the
27 α -amylase-inhibitory activity.

28 **KEYWORDS:** *Abrus precatorius*, α -amylase inhibitor, triterpene ketone

29

30 INTRODUCTION

31 Diabetes mellitus is a typical chronic disease related to both obesity and ageing and has
32 thus become a serious global health problem.¹ One of the therapeutic approaches for
33 preventing diabetes is the suppression of intestinal digestion and absorption of dietary
34 carbohydrates through the inhibition of carbohydrate hydrolyzing enzymes, α -amylase
35 and α -glucosidase, in the digestive organs.² In this context, several research efforts have
36 screened for effective α -amylase and α -glucosidase inhibitors from natural sources to
37 develop a physiological functional food or lead compounds for the use of antidiabetic
38 medicines.^{3,4}

39 In the course of our continuing search for rat intestinal α -glucosidase-inhibitory
40 principles from plants, we have isolated and identified several active compounds from a
41 variety of plants.⁵⁻⁹ However, the search for α -amylase inhibitors from natural sources
42 have led to relatively scarce results.¹⁰⁻¹² It is well known that α -amylase recognizes a
43 consecutive glucose chain as a substrate by using its subsite.¹³ In fact, acarbose, a
44 typical α -amylase inhibitor, has a strong affinity for the enzyme because of its
45 pseudotetrasaccharide structure.¹⁴ This is partly the reason for the limited number of
46 cases involved with the identification of small molecule α -amylase inhibitors from
47 natural sources. Most of these are polyphenols with low enzyme specificity.¹⁵⁻¹⁹
48 Recently, a triterpene glycoside has been isolated as a specific inhibitor by high
49 through-put screening,²⁰ and more surprisingly, a simple low polar ketone, chalcone, has
50 been found to possess considerable α -amylase inhibitory activity.²¹ The molecular
51 docking study revealed that the low polar small molecule could interact with
52 hydrophobic amino acid residues near the active site of the enzyme. From these results,
53 we have thus focused on searching for non-polyphenolic low polar α -amylase inhibitors

54 from plant origin.

55 In this paper, we present the results of a study on α -amylase inhibition and
56 identification of active principles from the plant extract of indigenous plants obtained
57 from Indonesia that show a potent inhibitory activity. In the screening experiments for
58 porcine pancreatic α -amylase (PPA) inhibitors in 18 plants obtained from Indonesia, the
59 potent α -amylase-inhibitory activity was found in a tannin-free low polar extract of the
60 leaves of *Abrus precatorius*. *A. precatorius* (Fabaceae) is a perennial climber found in
61 tropical and subtropical regions. It is famous for its beans, which contain a
62 proteinaceous toxin.^{22, 23} In addition, several other biological activities have been
63 reported,²⁴⁻²⁶ although there have been no reports on α -amylase and α -glucosidase
64 inhibitory activity of this plant.

65

66 MATERIALS AND METHODS

67 **Materials.** Eighteen Indonesian plants including *A. precatorius* leaves were
68 purchased from Merapi Farma Traditional Herbs Distributor, Yogyakarta, Indonesia, in
69 January 2007. PPA (A3176) was obtained from Sigma-Aldrich Co. (Tokyo, Japan). All
70 chemicals used in the present study were of reagent grade and were purchased from
71 Wako Pure Chem. Co. (Osaka, Japan) unless otherwise stated.

72 **General procedure.** NMR spectra were recorded on Bruker AMX500 (^1H , 500
73 MHz; ^{13}C , 125 MHz) and Jeol EX-270 (^1H , 270 MHz) instruments. Chemical shifts
74 were determined relative to a reference signal of tetramethylsilane in chloroform-*d* (δ_{H}
75 0.00 ppm, δ_{C} 0.0 ppm) or a residual solvent signal of methanol-*d*₄ (δ_{H} 3.30 ppm). Field
76 desorption mass spectra (FD-MS) were determined by a Jeol T100GCV instrument.

77 **PPA inhibitory activity determination.** The PPA-inhibitory activity was

78 determined by using the method described by Ali *et al.*²⁷ with a slight modification. PPA
79 was dissolved in sodium phosphate buffer (20 mM, pH 6.9) containing 6.7 mM of NaCl
80 to give a concentration of 0.5 unit/mL solution. Potato starch (0.01 g/mL) in the same
81 buffer was used as a substrate solution. A coloring reagent (DNS) was prepared by
82 mixing a 4.8 M 3,5-dinitrosalicylic acid solution (20 mL) and a solution of (+)-sodium
83 potassium tartrate tetrahydrate (12 g) in 0.4 M of NaOH (40 mL). A sample solution
84 (100 μ L) in 50% dimethyl sulfoxide (DMSO) and the PPA solution (150 μ L) were
85 mixed in a micro tube (1.5 mL). The tube was stoppered and pre-incubated at 37 ° C for
86 15 min. The starch solution (250 μ L) was then added and the mixture was incubated at
87 37 ° C for 15 min. To terminate the reaction, the tube was dipped in boiling water for 1
88 min. After cooling, the contents of the micro tube were directly passed through a small
89 ODS (Cosmosil 75C₁₈-OPN, Nacalai Tesque Co., Kyoto, Japan) column (3 mL) to
90 remove any sample constituents that may interfere with the following color reaction.
91 The reaction mixture (100 μ L) was then mixed with DNS reagent (50 μ L) in a micro
92 tube, stoppered and heated in boiling water for 15 min. The ice-cooled mixture was
93 diluted with water (450 μ L), the solution (200 μ L) was transferred into 96-well micro
94 plate, and the optical density was determined at a wavelength of 540 nm. The control
95 experiment was performed using 50% DMSO in place of the sample solution. Blank
96 experiments for sample and control were performed using the sodium phosphate buffer
97 in place of the enzyme solution and each blank value was subtracted from the sample
98 and the control values, respectively. The inhibitory activity (%) was calculated as
99 $[1 - \text{OD}_{540}(\text{sample}) / \text{OD}_{540}(\text{control})] \times 100$.

100 **Screening experiment.** The screening experiments for PPA inhibition were
101 performed with extracts of 18 plant species. Each dried plant was extracted with 50%

102 aqueous methanol. The extracts were evaporated and partitioned with
103 chloroform-methanol-water (4:1:5). The organic phase was washed with 1% saline to
104 give a tannin-free extract,²⁸ which was then evaporated, re-dissolved in 50% aqueous
105 DMSO, and subjected as the test sample to the assay for PPA inhibitory activity at the
106 final concentration of the extractable constituents obtained from 0.3 g of plant material
107 in 1 mL of solution.

108 **Isolation of lupenone (1), 24-methylenecycloartenone (2), and luteolin (3) from**
109 ***A. precatorius* leaves.** Dried leaves (40 g) of *A. precatorius* were extracted with 50%
110 aqueous MeOH. The extract was concentrated and partitioned with
111 chloroform-methanol-water (4:1:5). The organic phase was washed with 1% saline and
112 evaporated to give a tannin-free chloroform-soluble part (1.5 g). This fraction was
113 charged onto a silica gel column and eluted successively with hexane-ethyl acetate (9:1,
114 F1), hexane-ethyl acetate (3:1, F2), hexane-ethyl acetate (1:1) containing 0.1% formic
115 acid (F3), ethyl acetate containing 0.1% formic acid (F4) and methanol containing 0.1%
116 formic acid (F5). The PPA inhibitory activity was observed in F1, F4 and F5.

117 The less polar F1 (114 mg) was further fractionated by silica gel column
118 chromatography with a hexane-ethyl acetate gradient. The PPA inhibitory activity was
119 eluted mainly in a hexane-ethyl acetate (30:1) eluate (F1-2, 78 mg). F1-2 was further
120 purified by silica gel preparative TLC [hexane-ethyl acetate (11:1)] to give an active
121 band (F1-2-2, $R_f = 0.50$, 11 mg). F1-2-2 was finally purified by preparative HPLC
122 (column: Inertsil ODS-3, 4.6×250 mm, GL-Science Co., Tokyo, Japan; mobile phase:
123 MeOH; flow rate: 1.0 mL/min; detection: UV 210 nm). Two major peaks were collected
124 and identified as lupenone (1, $t_R = 21.0$ min, 0.7 mg) and 24-methylenecycloartenone (2,
125 $t_R = 16.0$ min, 0.3 mg).

126 The more polar active fraction (F4, 200 mg) of the first silica gel column was
127 re-chromatographed by a silica gel column (hexane-ethyl acetate-methanol). The most
128 polar fraction (F4-4, 110 mg) eluted by hexane-ethyl acetate (1:5) and methanol showed
129 PPA inhibitory activity. The final polar fraction (F5, 480 mg) of the first column was
130 expected to contain the similar active constituents to F4-4, and thus, F4-4 and F-5 were
131 merged (F45) and subjected to further fractionation. F45 was chromatographed on a
132 silica gel column with a chloroform-methanol gradient. The PPA inhibitory activity was
133 eluted in a chloroform-methanol (10:1 and 7:1) eluate (F45-5, 75 mg). F45-5 gave a
134 single peak ($t_R = 41.3$ min) by HPLC analysis (column: Inertsil ODS-3, 4.6×250 mm;
135 mobile phase: 10-100% MeOH in water containing 0.1% formic acid (0-60 min); flow
136 rate: 1.0 mL/min; detection: UV 254 nm) and was identified as luteolin by comparison
137 with an authentic sample.

138 *Lupenone (1)*. FD-MS m/z 424 ($[M]^+$); $^1\text{H-NMR}$ (500 MHz, chloroform-*d*) δ : 0.80
139 (3H, s), 0.93 (3H, s), 0.96 (3H, s), 1.00-1.16 (3H, m), 1.02 (3H, s), 1.07 (3H, s), 1.07
140 (3H, s), 1.17-1.52 (15H, m), 1.66-1.72 (2H, m), 1.68 (3H, s), 1.86-1.98 (2H, m),
141 2.33-2.54 (2H, m), 2.41 (1H, m), 4.57 (1H, m), 4.69 (1H, m); $^{13}\text{C-NMR}$ (125 MHz,
142 chloroform-*d*) δ : 14.5, 15.8, 16.0, 18.0, 19.3, 19.7, 21.0, 21.5, 25.2, 26.7, 27.4, 29.8,
143 33.6, 34.2, 35.5, 36.9, 38.2, 39.6, 40.0, 40.8, 42.9, 42.9, 47.3, 48.0, 48.3, 49.8, 55.0,
144 109.4, 150.9, 218.2.

145 *24-Methylenecycloartenone (2)*. FD-MS m/z 438 ($[M]^+$); $^1\text{H-NMR}$ (500 MHz,
146 chloroform-*d*) δ : 0.58 (1H, d, $J = 4.4$ Hz), 0.79 (1H, d, $J = 4.4$ Hz), 0.90 (3H, d, $J = 5.6$
147 Hz), 0.91 (3H, s), 1.00 (3H, s), 1.03 (3H, d, $J = 6.8$ Hz), 1.04 (3H, d, $J = 6.8$ Hz), 1.05
148 (3H, s), 1.10 (1H, m), 1.10 (3H, s), 1.13 (2H, m), 1.14 (1H, m), 1.31 (1H, m), 1.32 (2H,
149 m), 1.40 (1H, m), 1.41 (1H, m), 1.54 (1H, m), 1.55 (2H, m), 1.59 (1H, m), 1.64 (1H, m),

150 1.67 (2H, m), 1.71 (1H, dd, $J=12.0$ and 4.4 Hz), 1.86 (1H, dt, $J=14.0$ (t) and 5.6 (d) Hz),
151 1.89 (1H, m), 1.92 (1H, m), 2.05 (1H, m), 2.13 (1H, m), 2.24 (1H, m), 2.30 (1H, m),
152 2.71 (1H, dt, $J=14.0$ (t) and 5.6 (d) Hz), 4.67 (1H, brs), 4.72 (1H, brs); ^{13}C -NMR (125
153 MHz, chloroform- d) δ : 18.1, 18.3, 19.3, 20.8, 21.1, 21.5, 21.9, 22.0, 22.2, 25.9, 26.0,
154 26.8, 28.2, 29.6, 31.3, 32.8, 33.4, 33.8, 35.0, 36.1, 37.5, 45.4, 47.9, 48.5, 48.8, 50.2,
155 52.3, 106.0, 156.9, 216.6.

156 *Luteolin* (**3**). FD-MS m/z 286 ($[\text{M}]^+$); ^1H -NMR (270 MHz, methanol- d_4) δ : 6.20 (1H,
157 d, $J=2.1$ Hz), 6.43 (1H, d, $J=2.1$ Hz), 6.54 (1H, s), 6.90 (1H, d, $J=8.7$ Hz), 7.38 (2H,
158 m).

159

160 RESULTS AND DISCUSSION

161 In the screening experiment, tannin-free extracts from 2 out of 18 Indonesian plants,
162 namely *A. precatorius* leaves (73%) and *Baekkea frutescens* bark (58%), out of 18
163 showed >50% PPA inhibitory activity (**Table 1**). Based on this result, we chose extracts
164 of *A. precatorius* for identifying active principles.

165 The tannin-free organic phase of *A. precatorius* leaves was chromatographed on a
166 silica gel column and PPA-inhibitory activity was observed in two discrete fractions.
167 The less polar active fraction was further chromatographed on a silica gel column
168 followed by silica gel preparative TLC and reverse phase HPLC to afford two major
169 peaks in the final active fraction. These two constituents were identified as lupenone
170 (**1**)²⁹ and 24-methylenecycloartenone (**2**)³⁰ by comparison of their analytical data with
171 those reported in the references and an authentic specimen of lupenone. The more polar
172 active fraction of the first column was also chromatographed on silica gel and the
173 resultant active eluate showed a single peak in the reverse phase HPLC analysis. The

174 active constituent of the final fraction was identified as luteolin (**3**) by comparison with
175 an authentic specimen (**Figure 1**).

176 The PPA-inhibitory assay of the isolates showed a potent activity of **1** ($IC_{50}=31 \mu M$)
177 and a weaker activity of **3** ($IC_{50}=3.1 \text{ mM}$) (**Table 2**). The activity was not observed in **2**
178 (0%) at 0.6 mM, although a further experiment using a higher concentration could not
179 be performed due to limited sample amount. The potent inhibitory activity of **1** against
180 PPA has not ever been reported, whereas **3** has been known as a moderate PPA
181 inhibitor.^{15, 19} The inhibition mode of **1** was determined to be mixed-inhibition type by
182 the double reciprocal plot experiment (**Figure 2**).

183 It is interesting that a low polar triterpene such as **1** showed significant PPA
184 inhibition, because only few studies have investigated the PPA-inhibitory activity of
185 triterpenes. Ali *et al.* reported triterpenic inhibitors, ursolic acid, oleanolic acid, and
186 lupeol, from a Malaysian plant, *Phyllanthus amarus*, and ursolic acid showed the
187 highest activity among the isolates.²⁷ The structural similarity of those triterpenes and **1**
188 prompted us to perform a brief activity comparison experiments. The PPA-inhibitory
189 assay of commercially available lupenone (**1**), lupeol (**4**) and ursolic acid (**5**) (**Figure 1**)
190 at a concentration of 50 μM showed the strongest activity of **1** (84%) followed by **5**
191 (32%) and the lowest **4** (8%) (**Table 2**). These results together with the low inhibitory
192 activity of **2** show that both the lupane skeleton and a ketone at C-3 would be essential
193 for exerting a potent PPA inhibition. To the best of our knowledge, this is the first
194 finding of the potent PPA inhibitory activity of lupenone, although some other
195 biological activities^{29, 31} including α -glucosidase inhibition³² have been reported for **1**.
196 There are few examples of non-polyphenolic low polar small molecular inhibitor
197 against PPA. It should be interesting to determine how efficiently a low polar molecule

198 such as **1** could associate with the PPA enzyme.

199 In conclusion, the enzyme assay-guided fractionation of the extract from the dried
200 leaves of *A. precatorius* led to the isolation of a triterpene ketone, lupenone (**1**), as a
201 potent PPA inhibitor together with lower active luteolin (**3**). The relatively higher
202 activity of **1** and the larger amount of **3** present in *A. precatorius* leaves suggest that
203 these compounds are the major inhibitory components in this plant that could be useful
204 for the treatment of diabetes, although *in vivo* experiments are warranted.

205

206 **Notes**

207 The authors declare no competing financial interest.

208

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213

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306

307 **Figure captions**

308 **Figure 1.** Structures of **1-5**.

309

310 **Figure 2.** Double reciprocal plot (Lineweaver-Burk) for the α -amylase inhibition by

311 lupenone (**1**)

312

313

Pre-print

314 **Table 1. PPA inhibitory activity of tannin-free extracts of Indonesian plants.**

Plant	Part	Inhibitory activity (%) [*]
<i>Abrus precatorius</i>	leaf	73
<i>Carica papaya</i>	leaf	33
<i>Gynura procumbens</i>	aerial part	6
<i>Menta arvensis</i>	leaf	15
<i>Ruella napifera</i>	leaf	15
<i>Sida rhombifolia</i>	aerial part	12
<i>Tribulus terrestris</i>	aerial part	0
<i>Caesalpinia sappan</i>	bark	0
<i>Ruellia tuberosa</i>	fruit	23
<i>Helicteres isora</i>	leaf	4
<i>Sonchus arvensis</i>	fruit	5
<i>Cryptocarya massoy</i>	bark	0
<i>Piper nigrum</i>	seed	18
<i>Brucea javanica</i>	seed	2
<i>Alyxia stellata</i>	bark	39
<i>Baekkea frutescens</i>	bark	58
<i>Murraya paniculata</i>	aerial part	22
<i>Borreria hispida</i>	leaf	21

315 ^{*} The concentration was adjusted to the extractable constituents obtained from 0.3 g of
 316 plant material in 1 mL solution.

317

318

319 **Table 2. PPA inhibitory activity of isolated and related compounds**

	Inhibitory activity (%)	
	50 μ M	100 μ M
1	84	87
2	-	0*
3	-	-**
4	8	16
5	32	45

320 -: not tested; * 0% at 0.6 mM; ** IC₅₀ = 3.1 mM

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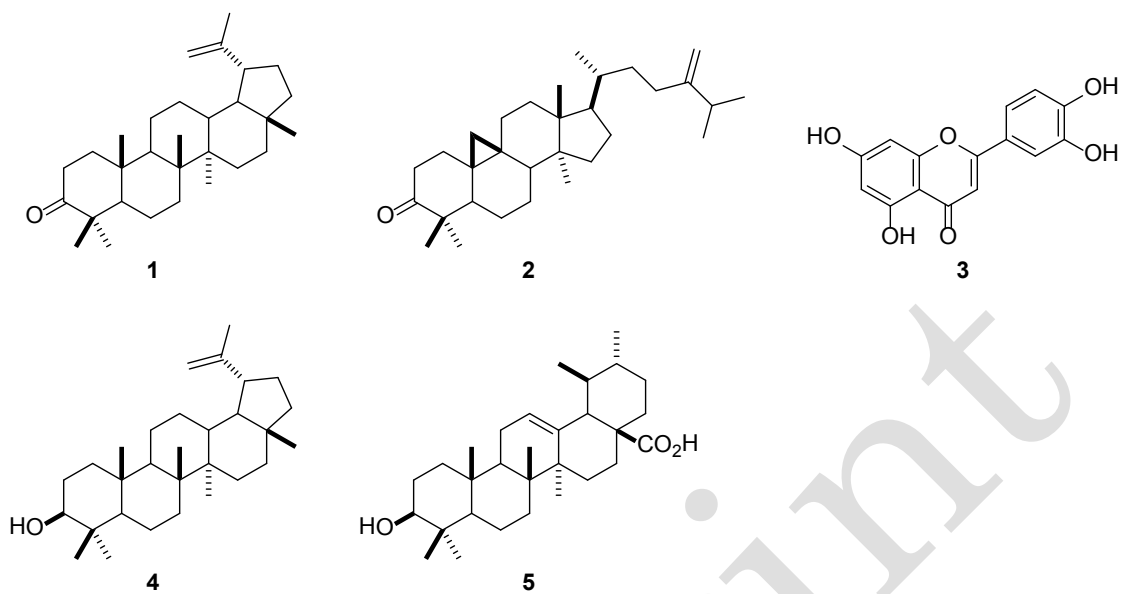
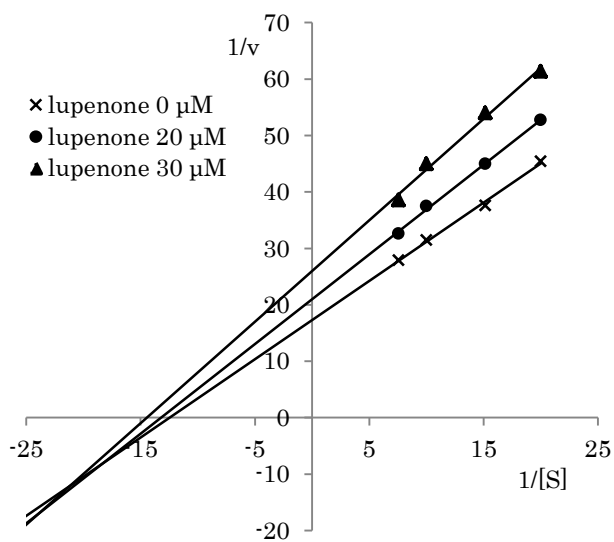


Figure 1 Structures of 1-5



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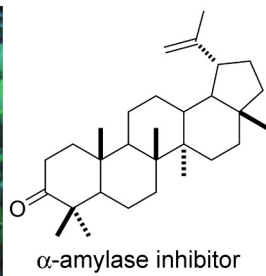
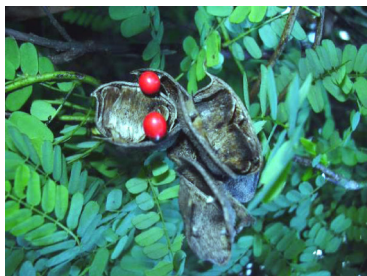
328 **Figure 2.** Double reciprocal plot (Lineweaver-Burk) for the α -amylase inhibition by

329 lupenone (1)

330

331 TOC Graphic.

332



333 Photo courtesy of the U.S. Geological Survey

Pre-print