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Note

Antioxidative Activity of Hop Bitter Acids and Their Analogues

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Hop bitter acids, humulones (1) and lupulones (2), were shown to have potent DPPH radical scavenging activity (RSA) and lipid peroxidation inhibitory activity (LIA). Furthermore, 5-acetyl lupulones (3) and 4-methyl lupulones (4) had more potent LIA than native lupulones but no RSA. This result indicates that the β,β′-triketone moiety of the lupulones has LIA.

Hop (*Humulus lupulus* L.) cones are used not only in brewing of beer but also in folk medicine as a tranquilizer or bitter stomachic. In 1950s–1960s, some papers reported the existence of substances which had estrogen like activity in hop cones,[1-3] and Kumai and his colleagues discovered gonadotropin inhibitors contained in hop cones.[4] Unfortunately, the reported bioactive substances have not been isolated or identified in detail, necessitating further investigation on unknown biological activities in hop plants.

In this paper, we show that bitter acids (**1–2**) of hop cones and their analogues (**3–4**) have antioxidative activity (Fig. 1). The relationship between chemical structure and antioxidative activities was also examined.

Hop cones of Hersbrucker hop cropped in Herateou, Germany in 1988, were commercially purchased. The hop cones (200 g) were milled and extracted with 2 liters of methanol. The extract was fractionated according to Fig. 2 and the RSA of each fraction was measured. The RSA of hops was concentrated in the *n*-hexane fraction (35.0 g) which was fractionated on a silica gel column (1 kg) by eluting stepwise with *n*-hexane–ethyl acetate (EtOAc) and measuring the RSA of each eluted fraction (Table I). Only two fractions, which mainly contained humulones (**1**) or lupulones (**2**), had potent RSA. The humulones or lupulones in each fraction were measured to comprise more than 70% of the residue by reversed-phase high performance liquid chromatography (HPLC).

For further investigation, the antioxidative activity of humulone (**1b**), lupulone (**2b**), and lupulone analogues, 5-acetyl lupulone (**3b**) and 4-methyl lupulone (**4b**), was measured. All tested samples were purified by reversed-phase HPLC under the following conditions: Shimadzu LC-8A system; column, Shim-Pack PREP-ODS (H) (20 mm i.d. × 25 cm); mobile phase, 80–95% acetonitrile aqueous solution; flow rate, 10 ml/min; detection, UV 280 nm. The purity of tested samples was estimated to be more than 95% by HPLC.

As shown in Table II, the RSA of humulone (**1b**) and lupulone (**2b**) (about $2–3 \times 10^{-5}$ M, IC_{50}) are nearly equivalent to those of two natural antioxidants, α-tocopherol and ascorbic acid. As for LIA, humulone (**1b**) and lupulone (**2b**) are superior to natural antioxidants by about 10–100 times. The newly synthesized

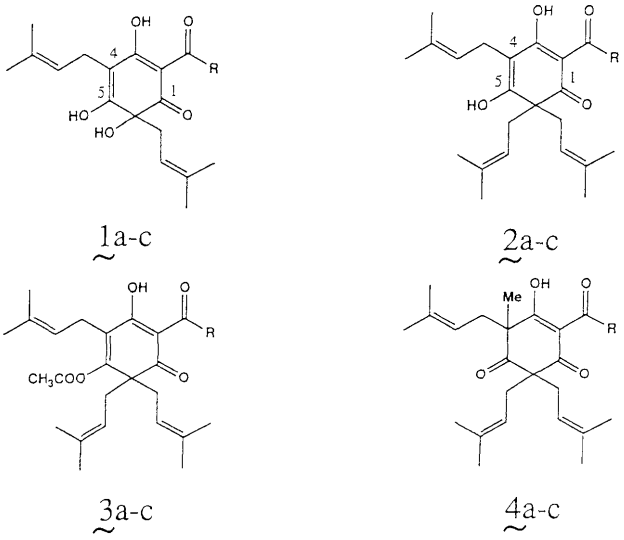

Fig. 1. Chemical Structure of Hop Bitter Acids and Their Analogues.
a, R = $-CH(CH_3)_2$; **b**, R = $-CH_2CH(CH_3)_2$; **c**, R = $-CH(CH_3)CH_2CH_3$.

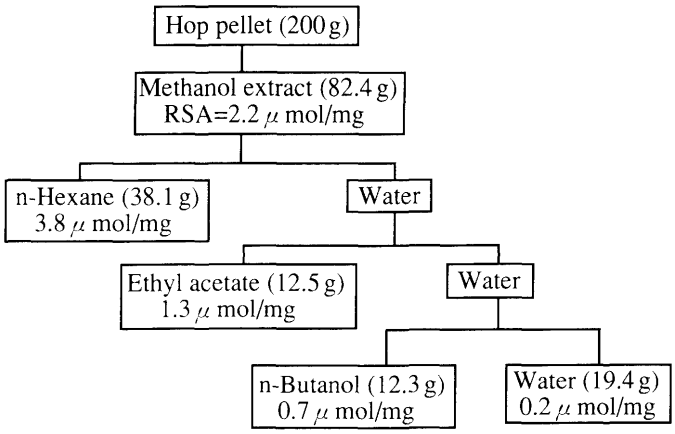

Fig. 2. Fractionation of Hop Extract.

Table I. DPPH Radical Scavenging Activity (RSA) of Hop Fractions

Entry	Eluent (Hexane : EtOAc)	Yield (g)	Recovery (%)	RSA (μmol/mg)
1	15 : 1	0.7	2.0	<0.1
2	12 : 1	0.3	0.9	<0.1
3	9 : 1	15.1	43.1	4.2
4	4 : 1	1.5	4.3	0.9
5	1 : 1	3.7	10.6	0.5
6	1 : 4	3.2	9.1	0.2
7	1 : 15	7.7	22.0	3.8
8	0 : 1	0.9	2.5	0.3
Total		33.1	94.6	

Abbreviations: humulone, 3,5,6-Trihydroxy-4,6-bis(3-methyl-2-butenyl)-2-(3-methyl-1-oxoburyl)-2,4-cyclohexadien-1-one; lupulone, 3,5-Dihydroxy-4,6,6-tris(3-methyl-2-butenyl)-2-(3-methyl-1-oxobutyl)-2,4-cyclohexadien-1-one.

*1 ^{13}C-NMR spectra of lupulone analogues showed partially two peaks for one carbone because of proton tautomerism.[13] Only their major peaks are displayed here. ^{1}H–^{13}C 2D spectra and DEPT spectra were measured to assign ^{13}C-NMR signals.

Table II. Antioxidative Activity of Hop Bitter Acids and Their Analogues

Sample	RSA (IC_{50}: M)[a]	LIA (IC_{50}: M)[b]
1b	3.2×10^{-5}	7.9×10^{-6}
2b	2.5×10^{-5}	3.9×10^{-5}
3b	$>10^{-3}$	4.1×10^{-6}
4b	$>10^{-3}$	2.6×10^{-6}
α-Tocopherol	2.8×10^{-5}	2.5×10^{-4}
Ascorbic acid	3.3×10^{-5}	$>10^{-3}$

All values are shown as the average of more than 3 measurements.
[a] RSA denotes radical scavenging activity.
[b] LIA denotes lipid peroxidation inhibiyory activity.

lupulone analogues (**3b**, **4b**) showed very characteristic activity. They showed no RSA ($>10^{-3}$ M), but showed LIA at $2\text{–}4 \times 10^{-6}$ M, (IC_{50}) indicating that they are about 3–10 times stronger in activity than the original bitter acids.

These results suggest two important facts. One is that hop bitter acids have potent antioxidative activity *in vitro* and the other is that chemical modification of lupulone can distinguish between the two kinds of antioxidative activity (RSA and LIA). Hop bitter acids are well known to be easily degraded by oxidation, and their decrease during storage of hop was well studied.[5] Nevertheless, the antioxidative activity of the acids has not been reported. Although the extent of the contribution of the bitter acids to the stability of other hop constituents is not clear, their antioxidative activity might be useful for storage of foods. For example, Witas reported that dried hops lowered the rate of rancidity of fish meal during 100 days of storage.[6] This effect of hops is likely to be attributable to hop bitter acids, because more than 70% of the RSA of hops is concentrated in the bitter acids fraction.

The results shown in Table II, suggest that the 5-hydroxyl group of bitter acid is the active site of RSA, because the analogues lacking this group also lack the activity. Though confirmative, this result suggests the β,β′-triketone structure as a candidate for the required active structure for LIA. Other components of 4-methyl lupulone, ketone, alkyl, and alkenyl groups may make little contribution to the activity.

To discuss this in detail, further experiments are needed, but the hydrophobicity of bitter acids and their analogues posed difficulties for further investigation. For example, measuring the superoxide dismutase (SOD)-like activity according to the method of Oyanagui[7] was unsuccessful, because the solubility of the acids was only about 10^{-6} M even in 25% dimethylformamide (DMF) aqueous solution (at that concentration, they showed no activity). So it will be necessary to prepare other lupulone analogues having a β,β′-triketone moiety in its structure and enough solubility in aqueous solution.

There are some chemical structures that are associated with antioxidative activity. For example, the phenol group is the most important one contained in antioxidants such as α-tocopherol, flavonoids, and tannins. In addition the β-polyene structure of β-carotene and the thiol group of glutathione are important for antioxidative activity. However, the β,β′-triketone structure has not been reported until now.

There are some antioxidants having a β,β′-triketone-like moiety in their molecules. For example, Osawa *et al.* isolated *n*-tritriacontane-16,18-dione and 4-hydroxy-tritriacontane-16,18-dione from leaf waxes of *Eucalyptus globulus* and reported that those β-diketone compounds had potent antioxidative activity *in vitro*.[8] However, the lupulone analogues reported in this paper may belong to another category of *Eucalyptus* antioxidants in the light of the previous author's claim that the analogues that did not have long alkyl chains (above C15) on both sides of the β-diketone moiety had shown little antioxidative activity. Thus, the hop bitter acids and their analogues which do not have long alkyl chains can be cited as a new antioxidant category.

Some papers reported bioactive substances which contained a β,β′-triketone moiety in their molecules. Studying acyl phloroglucinol derivatives isolated from plants belonging to the *Guttiferae* family and their synthetic analogues, Tada and his colleagues have shown that the compounds that contained a β,β′-triketone moiety inhibited the replication of vescular stomatis virus and antagonized thromboxane A_2 and leukotriene D_4.[9,10] They proposed the β,β′-triketone moiety to be the bioactive site. Hence, bitter acids and their analogues might also be candidates for remedial substances requiring such activity.

Experimental

Spectroscopic measurements were done with the following instruments: Hitachi U-2000 spectrometer (UV), Shimadzu DR-8000 (IR), Hitachi M-80B (MS), JEOL EX-270 (NMR). Column chromatography was done on Merck silica gel 60 (art. 9385).

DPPH radical scavenging activity (RSA).[11] A test sample solution in ethanol (200 μl) was added to 4 ml of 100 μM DPPH ethanol solution. After Vortex mixing, the mixture was incubated for 10 minutes at room temperature and the absorbance at 517 nm was measured. The differences in absorbance between a test sample and a control (ethanol) was taken as RSA. When the molecular weight of test sample was not measured, RSA was evaluated by the scavenged DPPH per test sample (μmol/mg).

Lipid peroxidation inhibitory activity (LIA). Measurements of LIA were done by the method of Uchiyama and Mihara.[12] Briefly, a test sample DMSO solution (6 μl) was added to 600 μl of 50 mM phosphate buffer (pH 7.4) containing 1 3% rat brain homogenate. After Vortex mixing, the mixture was incubated at 37°C for 1 h withshaking. The level of the thiobarbituric acid-reactive substances (TBARS) was measured by using the TBA method.

5-Acetyl lupulones (**3**). Crude crystal of β-acids (0.82 g) was treated with acetyl chloride (2.4 mmol) at room temperature in 10 ml of pyridine. The reaction mixture was poured into 100 ml of 7% $NaHCO_3$ aqueous solution and the solution was extracted by ethyl acetate. After it was concentrated *in vacuo*, the residue was loaded on a silicagel column (80 g) eluted with hexane : EtOAc = 40 : 1, to give (**3**) as yellow oil (580 mg).

5-Acetyl-lupulone (**3b**): EI-MS measurement *m/z* 456.2900 ($C_{28}H_{40}O_5$ requires 456.2873) IR ν_{max} (neat) cm^{-1}: 1779, 1539, 1543, 1372, 1152. ^{1}H-NMR (δ, CD_3OD): 0.95 (6H, d, J = 6.6 Hz, $2 \times CH_3$ of isovaleroyl group), 1.55, 1.57, 1.67 (6H, 6H, 6H, s, s, s, $6 \times CH_3$ of isopentenyl groups), 2.07 (1H, m, CH of isovaleroyl group), 2.27 (3H, s, Ac), 2.44, 2.74 (2H, 2H, br. m, br. m, $2 \times$ Ar(6)-CH_2), 2.90 (2H, br. m, Ar(4)-CH_2), 2.95 (2H, d, CH_2CO), 4.88 (2H, br. m, overlapped $2 \times$ CH of Ar (6)-isopentenyl group), 4.96 (1H, br. m, CH or Ar(4)-isopentenyl group) ^{13}C-NMR (δ, CD_3OD, 60°C)*1: 18.70, 18.84 ($2 \times CH_3$ of isopentenyl group), 21.41 (Me of Ac), 23.73 (2C, $2 \times CH_3$ of isovaleroyl group), 25.21, 25.63, 37.54 ($3 \times CH_2$ of isopentenyl groups), 26.58, 26.70 (2C, 2C, $4 \times CH_3$ of isopentenyl groups) 27.95 (CH of isovaleroyl group), 38.82 (CH_2 of isovaleroyl group) 119.88, 120.19, 122.94 ($3 \times$ CH of isopentenyl groups), 55.40, 113.30, 115.71, 133.02, 136.95, 168.65 ($6 \times$ C of Aromatic ring), 191.23, 197.74, 198.08 ($3 \times$ C of isopentenyl groups), 204.98 (CO of isovaleroyl group), 207.82 (CO of Ac).

4-Methyl lupulones (**4**). Potassium hydroxide aqueous solution (10%, 50 ml) of β-acids (1.0 g) was treated with dimethyl sulfate (2.4 ml) at 0°C. After stirred for 3 h at room temperature, the reaction mixture was acidified with hydrochloric acid and extracted with EtOAc. After washed with 1 N hydrochloric acid and water, the organic layer was dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The residue was loaded on a silica-gel column (80 g) eluted with hexane : EtOAc = 20 : 1 to give (4) as yellow oil (770 mg).

4-Methyl-lupulone (**4b**): EI-MS measurment *m/z* 428.2938 ($C_{27}H_{40}O_4$ requires 428.2924) IR ν_{max} (neat) cm^{-1}: 1717, 1674, 1455, 1379. ^{1}H-NMR (δ, CD_3OD) 0.96, 0.99 (3H, 3H, d, d, J = 6.6 Hz, J = 6.6 Hz, $2 \times CH_3$ of

isovaleroyl group), 1.16 (3H, s, Ar(4)-Me), 1.44, 1.50, 1.60, 1.63 (3H, 3H, 3H, 6H, 3H, s, s, s, s, s, $6 \times CH_3$ of isopentenyl groups), 2.16 (1H, m, CH of isovaleronyl group), 2.59 (4H, br. m, overlapped $2 \times$ Ar(6)-CH_2), 2.71 (2H, br. m, Ar(4)-CH_2), 2.82 (2H, d, $J = 6.6$ Hz, CH_2CO), 4.73, 4.87 (1H, 1H, br. m, partially overlapped $2 \times$ CH of Ar(6)-isopentenyl groups), 4.99 (1H, br. m, CH of Ar(4)-isopentenyl group) ^{13}C-NMR (δ, CD_3OD, 60°C): 18.87, 18.96 ($2 \times CH_3$ of isopentenyl group), 23.76 (Ar(4)-Me), 23.83, 23.94 ($2 \times CH_3$) of isovaleroyl group), 24.00, 25.11, 37.03 ($3 \times CH_2$ of isopentenyl groups), 26.80, 26.91 (2C, 2C, $4 \times CH_3$ of isopentenyl groups) 28.38 CH of isovaleroyl group), 40.83 (CH_2 of isovaleroyl group) 119.68, 120.11, 120.68 ($3 \times$ CH of isopentenyl groups), 63.76, 115.47, 117.28, 133.13, 137.59, 169.92 ($6 \times$ C of Aromatic ring), 192.83, 198.04, 199.12 ($3 \times$ C of isopentenyl groups), 205.09 (CO of isovaleroyl group), 211.03 (CO of Ac).

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