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XANTHOHUMOLS, DIACYLGLYCEROL ACYLTRANSFERASE INHIBITORS, FROM *HUMULUS LUPULUS*

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Key Word Index—*Humulus lupulus*; cannabinaceae; diacylglycerol actyltransferase (DGAT); triacylglycerol; hop; xanthohumol; inhibitor.

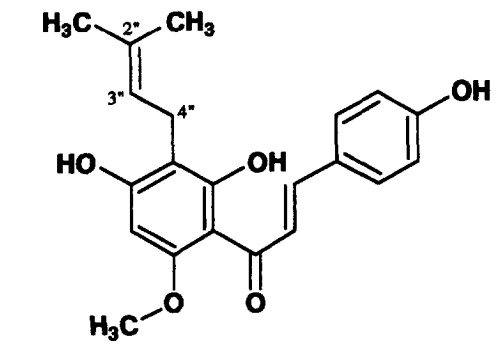
Abstract—A methanol extract of hops of *Humulus lupulus* (L.) showed inhibitory activity against rat liver diacylglycerol acyltransferase (DGAT). From DGAT inhibitory activity-guided fractionation, two chalcones were isolated. One was identified as xanthohumol and the other was found to be a new product designated xanthohumol B. The structure of xanthohumol B was shown to be 6-[3,4-dihydro-3,5-dihydroxy-7-methoxy-2,2-dimethyl-2*H*-benzo[b]pyrano]-3-(4-hydroxyphynyl)-2-propen-1-one by spectroscopic studies including various NMR measurements. Xanthohumol and xanthohumol B inhibited DGAT activity with IC_{50} values of 50.3 and 194 μM in rat liver microsomes, respectively. They showed preferential inhibition of triacylglycerol formation in intact Raji cells, indicating that they inhibit DGAT activity preferentially in living cells.

INTRODUCTION

Diacylglycerol acyltransferase (acyl-CoA: 1,2-diacyl-*sn*-glycerol *O*-acyltransferase, abbreviated as DGAT) [EC 2.3.1.20] catalyses the reaction of acyl residue transfer from acyl-CoA to diacylglycerol to form triacylglycerol [1, 2]. Too much accumulation of triacylglycerol in certain organs and tissues of the body causes high risk conditions of fatty liver, obesity, and hypertriglyceridemia, leading to serious diseases of atherosclerosis, diabetes, metabolic disorders and functional depression of some organs. DGAT is exclusively involved in triacylglycerol formation and thus is expected to be an effective target of inhibition for the treatment and prevention of these diseases. Screening systems were conducted using rat liver microsomes to discover DGAT inhibitors of natural origin. So far two kinds of microbial products have been isolated as DGAT inhibitors, that is, amidepsines produced by *Humicola* sp. FO-2942 [3, 4] and FO-5969 [5] and isochromophilones produced by *Penicillium* sp. FO-4164 [6]. Very recently, a methanol extract of hops of *Humulus lupulus* (L.) was found to show DGAT inhibitory activity. Two structurally related active compounds **1** and **2** (Fig. 1) were isolated. Compound **1** was identical with a known chalcone, xanthohumol [7–11], but **2** was found to be a new compound designated as xanthohumol B. In this paper, isolation, structure elucidation and biological properties of xanthohumols are described.

RESULTS AND DISCUSSION

The molecular formula of **2** was determined to be $C_{21}H_{22}O_6$ on the basis of HRFAB-MS measurement (*m/z*, found 370.1417, calcd 370.1416). The ^{13}C NMR spectrum (DMSO-d_6) showed 19 resolved peaks corresponding to 21 carbons (Table 1), which were classified into two —CH_3 one —O—CH_3, one —CH_2— seven —CH=, one —C—CH, and nine quaternary carbons by analysis of the DEPT spectra. The ^1H NMR spectrum displayed 21 proton signals (Table 1). The connectivity of proton and carbon atoms (Table 1) was confirmed by the HMQC spectrum. As shown in Fig. 2, two partial structures I and II were proposed from further NMR experiments. Evidence supporting the structure I includes: (1) The presence of the *p*-hydroxybenzene moiety is indicated by observation of ^1H–^1H COSY couplings between H-2′/H-6′ (δ 7.52) and H-3′/H-5′ (δ 6.86) and by ^{13}C–^1H long-range couplings from H-2′/H-6′ to C-4′ (δ 159.8) and C-2′/C-6′ (δ 130.7), from H-3′/H-5′ to C-1′ (δ 128.3), C-4′ and C-3′/C-5′ (δ 116.1) and from 4′-OH (δ 5.18) to C-3′/C-5′ and C-4′ in the HMBC spectrum. The fragment ion peak of *m/z* 120 in the EI-Mass spectrum also supports the presence of this moiety. (2) The C-2 double bond show *E*-geometry because the coupling constant between H-2 (δ 7.60) and H-3 (δ 7.81) is 15.0 Hz in the ^1H–^1H COSY experiments. Furthermore, the α,β-unsaturated ketone is attached to the *para*-hydroxybenzene moiety by observation of ^{13}C–^1H



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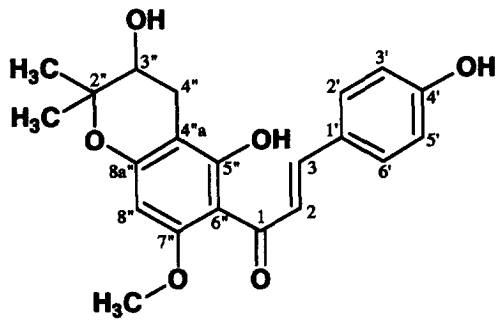


2

Fig. 1. Structure of xanthohumol (1) and xanthohumbol B (2).

long-range couplings from H-2 to C-1′, and from H-3 to C-1 (δ 191.9) and C-2′ in the HMBC spectrum. (3) The presence of 1,2,3,4,5-penta-substituted benzene is shown by observation of the long-range couplings from H-8″ (δ 5.93) to C-4″a (δ 100.5), C-6″ (δ 105.0), C-7″ (δ 160.6) and C-8″a (δ 160.1), from 5″-OH (δ 14.8) to C-4″a, C-5″ (δ 164.8) and C-6″, and from 7″-OCH_3 (δ 3.90) to C-7″ in the HMBC spectrum. Their chemical shifts were consistent with the additivity rule of substituents [12]. The partial structure I revealed the same chalcone skeleton as xanthohumol. The partial structure II (Fig. 2) is supported by observation of ^1H–^1H COSY couplings between H-3″ (δ 3.86) and H-4″ (δ 2.69, 2.90) and the long-range couplings from 2″-CH_3a (δ 1.40) to 2″-CH_3b (δ 21.1), C-2″ (δ 78.9) and C-3″ (δ 67.4), from 2″-CH_3b (δ 1.35) to 2″-CH_3a (δ 25.4), C-2″ and C-3″, and from H-4″ to C-2″ and C-3″ in the HMBC spectrum. The two partial structures I and II could be connected by observation of the long-range couplings from H-4″ to C-4″a (δ 25.2) and C-8″a in further HMBC experiments, leading to the two possible structures III and IV as shown in Fig. 3. The six-membered ring structure III (Fig. 3) is adopted due to the following reason: fungal terpendoles, which we reported previously as inhibitors of acyl-CoA: cholesterol acyltransferase [13, 14], have the similar partial structures III and IV as shown in Fig. 3. Terpendoles A, C, K and L share the partial structure III with the ^{13}C chemical shift of C-2″ ranging from 74.1 to 76.6 ppm. On the other hand, terpendoles E, F, G and H share the partial structure IV with a higher chemical shift ranging from 70.0 to 71.1 ppm. The chemical shift for xanthohumol B, in fact, is 78.9 ppm (Table 1), indicating that the structure III is reasonable for the compound. Finally, the remaining hydroxyl group should be attached to C-3″ due to the comparable ^{13}C and ^1H chemical shifts. Taken together, the structure of xanthohumol B was elucidated to be **2** as shown in Fig. 1.

Effect of xanthohumols on DGAT activity in rat liver microsomes was studied (Fig. 4). They inhibited DGAT activity dose-dependently with IC_{50} values of 50.3 μM for **1** and 194 μM for **2**.

Kinetic analysis was studied for DGAT inhibition by **1**. Lineweaver–Burk and Dixon plots (Fig. 5)showed that **1** inhibited DGAT activity non-competitively with respect to the substrate palmitoyl-CoA (K_i 31 μM; K_m, 41 μM). The analysis with respect to diacylglycerol still remains to be investigated. DGAT inhibition by **1** and **2** was evaluated by our established method using intact Raji cells [15]. When Raji cells were incubated with [^{14}C]oleic acid in the presence or absence of the drugs, the incorporation of radioactivity into the three lipids, triacyglycerol (TG), phosphatidylcholine (PC) and phosphatidylethanolamine (PE), was measured. As shown in Fig. 6, rather preferential inhibition of TG synthesis was observed up to 30 μM of for DGAT inhibition by **1** and **2**. However, at a higher concentration PC synthesis was also inhibited, though to a lesser extent. The IC_{50} values for TG synthesis were calculated to be 21.2 and 33.8 μM for **1** and **2**, respectively, but those for PC and PE syntheses were over 100 μM. Furthermore, both xanthohumols showed no cytotoxic effect on Raji cells under these concentrations used. These findings indicated that xanthohumols inhibit DGAT activity preferentially in the intact cells as well.

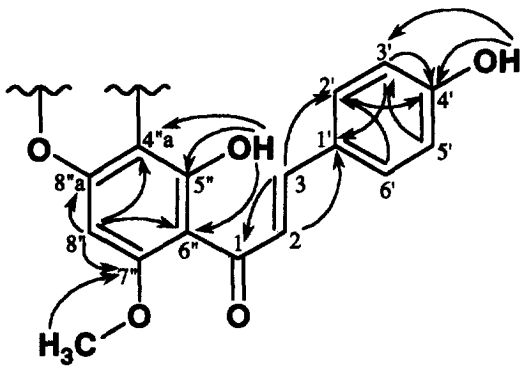
EXPERIMENTAL

General. UV spectra were recorded on a Shimadzu UV-200S spectrophotometer. IR spectra were recorded on a Horiba FT-210 infrared spectrometer. EI-MS spectra were recorded on a JEOL JMS-D 100 mass spectrometer at 20 eV. FAB-MS spectra were recorded on a JMS-DX300 mass spectrometer. The various NMR spectra were obtained on a Varian XL-400 spectrometer. Kieselgel 60 (E. Merck), Chromatorex (Fuji Davison Chemical Ltd,) and Sephadex LH-20 (Pharmacia) were used for column chromatography. TLC was carried out on Merck Kieselgel F_{254} plates (0.25 mm). HPLC was carried out using JASCO system (TRI ROTAR V). Bovine serum albumin (fatty acid free BSA), dioleoylglycerol, palmitoyl-CoA were purchased from Sigma. [1-^{14}C]Palmitoyl-

Table 1. ^{1}H and ^{13}C NMR chemical shifts of **1** and **2**

	1		2	
Carbon No.	^{13}C chemical shifts ppm*	^{1}H chemical shifts ppm†	^{13}C chemical shifts ppm*	^{1}H chemical shifts ppm†
C-1	192.8		191.9	
C-2	125.5	7.80 (1H, d, J = 15.2 Hz)	126.1	7.60 (1H, d, J = 15.0 Hz)
C-3	142.0	7.75 (1H, d, J = 15.2 Hz)	143.1	7.81 (1H, d, J = 15.0 Hz)
C-1′	128.6		128.3	
C-2′, C-6′	130.3	7.51 (2H, dt, J = 8.3 Hz)	130.7	7.52 (2H, dt, J = 8.5, 1.0 Hz)
C-3′, C-5′	115.9	6.86 (2H, d, J = 8.3 Hz)	116.1	6.86 (2H, dt, J = 8.5, 1.0 Hz)
C-4′	157.4		159.8	
C-4′-OH		6.20 (1H, s)		5.18 (1H, s)
C-2″	136.0		78.9	
C-2″-CH_3a	17.9	1.83 (3H, s)	25.4	1.40 (3H, s)
C-2″-CH_3b	25.8	1.77 (3H, s)	21.1	1.35 (3H, s)
C-3″	121.7	5.29 (1H, t, J = 7.0 Hz)	67.4	3.86 (1H, brdd, J = 5.0, 5.0 Hz)
C-3″-OH				
C-4″	21.6	3.40 (1H, d, J = 7.0 Hz)	25.2	2.69 (1H, dd, J=17.0, 5.0 Hz) 2.90 (1H, dd, J = 17.0, 5.0 Hz)
C-4″a	106.3		100.5	
C-5″	161.8		164.8	
C-5″-OH		14.7 (1H, s)		14.8 (1H, s)
C-6″	105.0		105.0	
C-7″	165.1		160.6	
C-7″-O-CH_3	56.1	3.90 (3H, s)	56.1	3.90 (3H, s)
C-8″	106.2	5.94 (1H, s)	91.9	5.93 (1H, s)
C-8″a	161.2		160.1	

* The sample was dissolved in $CDCl_3$. Chemical shifts are shown with reference to $CDCl_3$ as 77.7 ppm. † Chemical shifts are shown with reference to $CDCl_3$ as 7.26 ppm.



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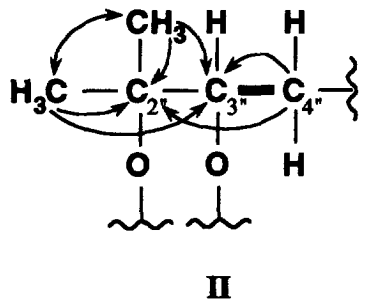
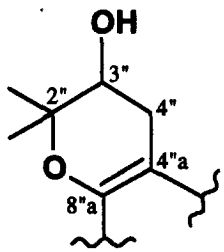


II

Fig. 2. ^{1}H-^{1}H COSY and HMBC experiments of xanthohumol B.



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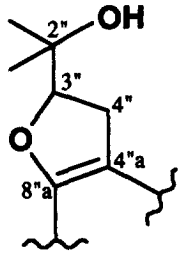

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Fig. 3. Two possible structures for xanthohumol B.

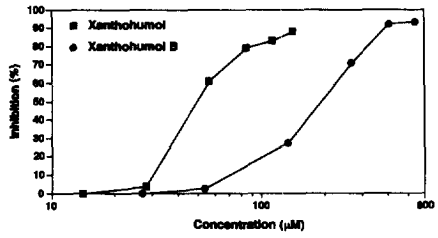

Fig. 4. DGAT inhibition by xanthohumol (■) and xanthohumbol B (●) in rat liver microsomes.

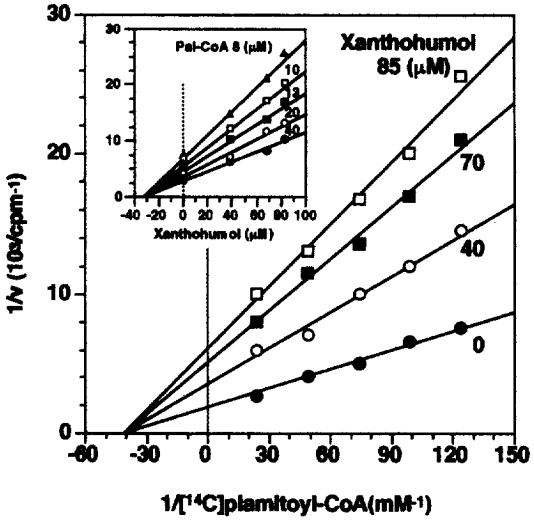

Fig. 5. Lineweaver-Burk plots for DGAT inhibition by **1** with respect to palmitoyl-CoA. Concentration of **1**; 0 (●, control), 40 (○), 70 (■) and 85 μM (□). Inlet showd Dixon plots. Concentration of palmitoyl-CoA; 8 (▲), 10 (□), 13 (■), 20 (○) and 40 μM (●). Each plot represents the average value of triplicate determinations.

CoA (57 mCi/mmol) was from Amersham. [1-^{14}C] Oleic acid (54.7 mCi/mmol) was from ICN.

Diisopropylfluorophosphate was from Wako pure chemical industries, and other reagents were of the highest grade commercially available.

Plant material. Hops of *Humulus lupulus* (L.) was collected from Saaz of the Czechoslovak Socialist Republic, which was a generous gift from Dr S. Mizobuchi, Kirin Brewery Co., Ltd.

Extraction and isolation. Saaz hops (200 g) cut into pieces were extracted with 70% aq. MeOH (4 l). After filtration, the soln was concd to remove MeOH, which was extracted with EtOAc (3 l). The extracts were evapd *in vacuo*, and the resulting dark green oily material (9.2 g) was applied on a silica gel column (E. Merck, Kiselgel 60, 540 ml). After the column was washed with $CHCl_3$ (2.7 l), materials were eluted with $CHCl_3$–MeOH soln (99:1, 2.7 l, and then 98:2, 2.7 l) and each 500 ml was successively collected. The 2nd to 7th frs showing DGAT inhibition enriched with active components were concd *in vacuo* to give an orange material (1.8 g). Next purification was carried out with an ODS column (Chromatorex, 180 ml). Materials were eluted with 45% aq. MeCN (1.5 l) and 70% aq. MeCN (1 l), and each 100 ml was collected. The 7th to 16th fractions showing DGAT inhibition were collected and concd *in vacuo* to give a yellow material (1.0 g). Further purification was carried out by prep. HPLC (column, YMC pack D-ODS-5, 20 × 250 mm; solvent, 70% MeCN–0.05% H_3PO_4; detection, UV at 320 nm; flow rate, 8 ml/min). Two active frs were eluted as a peak with retention times of 11.0 and 16.5 min. The second fr. (110 mg) mainly containing **1** was concd and extracted with EtOAc. The extract was finally purified by Sephadex LH-20 column chromatography [column size, 2.2 × 23.7 cm; solvent, $CHCl_3$–MeOH (1:1)] to give pure **1** (69.9 mg) as a yellow powder. The first active fr. (25.0 mg) containing **2** was re-purified by a second prep. HPLC (column, Shiseido capcell pak C18, 20 × 250 mm; solvent, 70% aq. MeCN; detection, UV at 320 nm; flow rate, 4 ml/min). An active peak with a retention time of 19.0 min was concd and extracted with EtOAc to yield **2** (3.8 mg) as a yellow powder.

DGAT assay using rat liver microsomes. Male Wistar-Imamichi or Donryu rats fed a normal diet for 1 week were killed by decapitation and livers were removed. The livers (200 g) were homogenized by 10 rapid up and down stroke using a motor-driven, Teflon-glass homogenizer in three vol. of cold medium I (0.25 M sucrose, 1 mM EDTA, 10 mM sucrose, 1 mM EDTA, 10 mM Tris–HCl, pH 7.4). The homogenate was centrifuged as 22000 *g* for 15 min. The resulting supernatant was centrifuged at 81200 *g* for

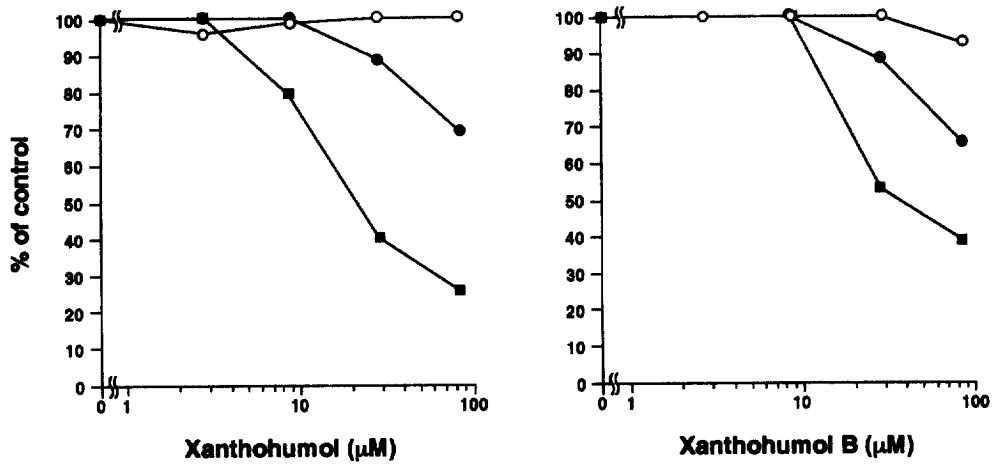

Fig. 6. Effects of xanthohumols on triacylglycerol (TG; -■-), phosphatidylethanolamine (PE; -○-) and phosphatidylcholine (PC; -●-) formations in intact Raji cells.

1 hr. The pellet was suspended in medium I and centrifuged again at 81 200 *g* for 1 hr. The final pellet was resuspended in medium I without EDTA. The microsomal fractions of rat livers were prepared and stored at −80°. DGAT activity was measured according to the method of [2] with some modification [3]. The reaction mixture contained 175 mM Tris-HCl (pH 8.0), 100–200 μg microsomal protein, 14.5 μM BSA, 30 μM [1-^{14}C]palmitoyl-CoA (0.02 μCi), 8 mM $MgCl_2$, 2.5 mM diisopropyl fluorophosphate, 150 μM 1,2-dioleoyl-*sn*-glycerol and a test sample dissolved in 50% EtOH (5 μl) in a total vol. of 200 μl. The assay was initiated by the addition of rat liver microsomal fraction. After a 15-min incubation at 23° the reaction was stopped by an addition of 1.2 ml of $CHCl_3$–MeOH (1:2) and lipids were extracted according to the method of ref. [16]. The lipids were separated by TLC on Silica gel 60 plates, (F_{254}, Merck Co.) using a petrol–Et_2O-HOAc (80:20:1)-solvent. The distribution of radioactivity on TLC was analysed with a radioscanner (Radioanalytic Imaging System, AMBIS System Inc.) to determine the amount of [^{14}C]triacyclglycerol.

Assay for lipid formation in Raji cells. To investigate the specificity of DGAT inhibition, lipid formation was measured in an intact cell assay using Raji cells as reported previously [15]. In brief, Raji cells (4×10^5 cells in PBS) were incubated with 0.36 nM [1-^{14}C]oleic acid (0.02 μCi) in the presence or absence of a test sample (50% EtOH, 5 μl) in a total vol. of 200 μl at 37° for 20 min. Lipids were extracted as described above, and the main products, triacylglycerol, phosphatidylcholine and phosphatidylethanolamine, were separated by TLC to analyse the distribution of radioactivity with the radioscanner.

Xanthohumol B (**2**). Yellow powder. HREI-MS: calcd for $C_{21}H_{22}O_6$, *m/z* 370.1416 $[M]^+$, found 370.1415. IR ν_{max}^{KBr} cm^{-1}: 1622, 1512, 1346, 1232, 1112; UV λ_{max}^{EtOH} nm (log ε): 205 (4.47), 370 (4.35). ^{13}C and ^{1}H NMR: Table 1.

Acknowledgements—We thank Dr S. Mizobuchi (Kirin Brewery Co., Ltd) for generous gift of Saaz hops. This research was supported in part by grants from the Ministry of Education, Science and Culture of Japan and from Japan Keirin Association.

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