Antityrosinase Principles and Constituents of the Petals of *Crocus sativus*

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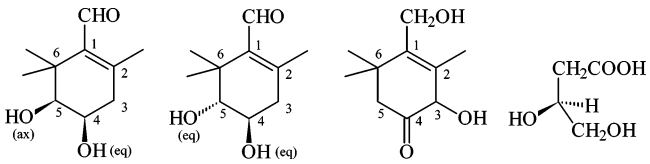
Three new monoterpenoids, crocusatin-J (**1**), -K (**2**), and -L (**3**), and a new naturally occurring acid, (3*S*),4-dihydroxybutyric acid (**4**), together with 31 known compounds were isolated and identified from the methanol extract of the petals of saffron (*Crocus sativus*). Their structures were established by spectroscopic methods. Among them, crocusatin-K (**2**), crocusatin-L (**3**), and 4-hydroxy-3,5,5-trimethylcyclohex-2-enone (**8**) showed significant antityrosinase activity, and protocatechuic acid (**18**), kaempferol (**24**), and kaempferol 7-*O*-*β*-D-glucopyranoside (**27**) were more effective in scavenging α,α-diphenyl-*β*-picrylhydrazyl (DPPH) radicals than α-tocopherol. In addition, the mechanism of tyrosinase inhibition by crocusatin-K (**2**) is also discussed.

Tyrosinase is known as a key enzyme implicated in the anabolism of melanin in melanocytes, and its inhibitors have become increasingly important in medicinal and cosmetic products in relation to hyperpigmentation.[1] Most commercial whitening cosmetics include tyrosinase inhibitors, such as arbutin (IC_{50} ∼17 mM)[2] or hydroquinone (IC_{50} ∼4.5 mM),[1] to reduce melanin production. However, the effectiveness of these compounds is quite limited. The interactions between ligands, tyrosinase, and the structural features of tyrosinase inhibitors are not well understood. There are some models used by researchers to explain the active site structure of tyrosinase and its catalytic mechanism,[3−6] but those are not able to fit any set of experimental data entirely. In continuation of studies on the antityrosinase principles, we have investigated the constituents of the petals of *C. sativus*, since the methanolic extract showed significant antityrosinase activity.

Crocus sativus L., commonly known as saffron, is a perennial stemless herb of the Iridaceae. It is distributed in southern Europe, middle Iran, and southern Asia and is cultivated in mainland China.[7−9] This plant has economic importance, because it can be used as a dye and spice for food preparations. The stigma of this plant is also a well-known traditional Chinese medicine and is used as safflower (*Carthamus tinctorius* L.) to stimulate blood flow and relieve pain by removing stagnated blood. It is also used in the treatment of amenorrhea, menostasis, melancholia, chest and abdominal pain, painful swellings due to blood stasis, hepatomegaly, splenomegaly, convulsion, and pain of traumatic wounds and as a sedative.[7] Earlier investigations had reported the pharmacological activity of saffron and the isolation of carotenoids, monoterpenoids, flavonoids, and anthocyanins from the stigma, leaves, petals, and pollen of this plant.[8] We have also reported the isolation of several bioactive constituents from the stigmas[10] and pollen[11] of *C. sativus*. This paper deals with the isolation and identification of compounds **1**−**4** from the petals of *C. sativus* and their tyrosinase inhibitory and α,α-diphenyl-*β*-picrylhydrazyl (DPPH) radical-scavenging activities,[12] and also the mushroom tyrosinase inhibition mechanism of crocusatin-K (**2**).

Chart 1



1 **2** **3** **4**

Results and Discussion

Crocusatin-J (**1**) was isolated as an optically active colorless oil. The molecular formula was established as $C_{10}H_{16}O_3$ by HREIMS. The presence of an α,*β*-unsaturated carbonyl was indicated by IR and UV absorption bands at 1664 cm^{-1} and at 246 nm, respectively, together with ^{13}C NMR signals at δ 193.0 (d), 152.1 (s), and 140.7 (s). The ^{13}C NMR and DEPT spectra indicated that compound **1** contained 10 distinct carbon signals comprising three methyls (one vinylic and two geminal on a quaternary carbon), one methylene, two oxymethines, and three quaternary carbons (two olefinic). The ^1H NMR spectrum of **1** showed signals for the *gem*-dimethyls at δ 1.21 and 1.22 (each 3H, s), a vinylic methyl at δ 2.22 (3H, s), and an aldehyde at δ_H 10.15 (1H, s). The ^1H NMR spectrum in combination with the $^1H-^1H$ COSY revealed the presence of the partial structure $-CH_2CH(OH)CH(OH)-$ from the mutually coupled proton signals at δ_H 3.95 (1H, d, J = 4.0 Hz), 3.78 (1H, ddd, J = 12.0, 4.0, 4.0 Hz), 1.74 (1H, dd, J = 12.0, 12.0 Hz), and 1.38 (1H, dd, J = 12.0, 4.0 Hz). The partial structure was also supported by the ^{13}C NMR signals at δ_C 72.0, 65.9, and 42.5. The foregoing spectroscopic studies inferred that **1** had a cyclohexene-monoterpene basic skeleton with an aldehyde, a *gem*-dimethyl, and a vinyl methyl substituent. The orientations of the hydroxyls at stereogenic centers C-4 and C-5 were determined as equatorial and axial from the coupling constants of H-4 (δ_H 3.78, ddd, J = 12.0, 4.0, 4.0 Hz) and H-5 (δ_H 3.95, d, J = 4.0 Hz), respectively. On the basis of the above spectroscopic data, structure **1** was assigned as crocusatin-J.

Crocusatin-K (**2**) was isolated as an optically active oil. On the basis of its molecular formula $C_{10}H_{16}O_3$ from HREIMS (m/z 184.1104 [M^+]), **2** was considered to be an isomer of **1**. The presence of an α,*β*-unsaturated carbonyl in **2** was indicated by a close resemblance of UV and IR

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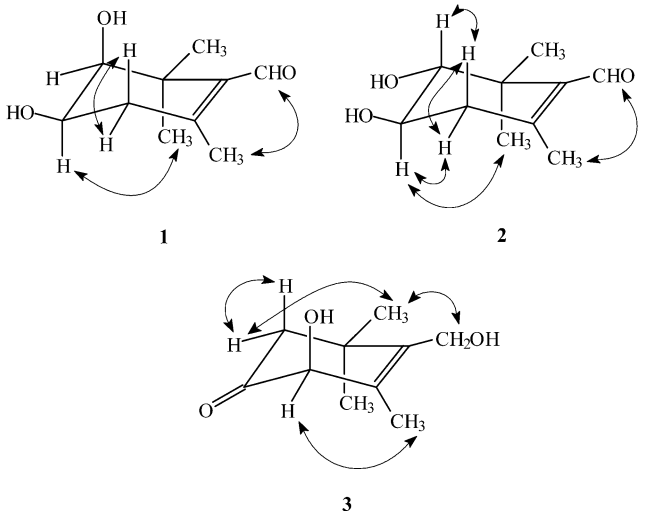

Figure 1. NOESY correlations of **1**, **2**, and **3**.

spectra to those of **1**. The ^{1}H NMR spectrum of **2** also revealed the presence of *gem*-dimethyls (δ 1.17 and 1.22, each 3H, s), a vinylic methyl (δ 2.18, 3H, s), and an aldehyde (δ 10.11, 1H, s). Methylene proton signals at δ 1.62 (1H, dd, J = 12.8, 4.0 Hz) and 1.49 (1H, dd, J = 12.8, 12.8 Hz), two oxygenated methines at δ 3.90 (1H, d, J = 8.0 Hz) and 3.70 (1H, ddd, J = 12.8, 8.0, 4.0 Hz), and the carbon signals at δ_C 77.6 (C-5), 69.3 (C-4), and 46.0 (C-3) were indicative of the partial structure $-CH_2CH(OH)CH(OH)-$ as in **1**. These facts indicated that **2** was a stereoisomer of **1**. The relative configuration at C-4 and C-5 was deduced from the coupling constants of H-4 and H-5 and a NOESY experiment (Figure 1). The coupling constants of H-4 (ddd, J = 12.8, 8.0, 4.0 Hz) and H-5 (d, J = 8.0 Hz) and the NOEs of H-4 (δ 3.70) with Me-6_{ax} (δ 1.22) and H-5 (δ 3.90) with CH_2-3_{ax} (δ 1.49) established that both 4-OH and 5-OH were in the equatorial orientation. Based on this spectroscopic evidence, the structure of crocusatin-K was assigned as **2**.

Crocusatin-L (**3**) was isolated as an optically active colorless oil. Its molecular formula was determined as $C_{10}H_{16}O_3$ from its HREIMS (M^+ at m/z 184.1093), implicating three degrees of unsaturation. The IR absorption band at 3512 and 1712 cm^{-1} indicated the presence of hydroxyl and carbonyl groups in the molecule. The ^{1}H NMR spectrum showed the presence of a *gem*-dimethyl (δ 1.01 and 1.10, each 3H, s), a vinylic methyl (δ 1.99, 3H, s), an oxygenated methine (δ 5.79, 1H, s), a hydroxyl (δ 3.99, 1H, brs, D_2O exchangeable), and a pair of isolated methylene protons (δ 2.80 and 2.03, each 1H, d, J = 17.2 Hz). The above data strongly suggested that crocusatin-L was a β,γ-unsaturated ketonic form of isophorone.[11] In addition, an ABX pattern of signals at δ 3.80, 3.76 (each 1H, d, J = 11.2 Hz) and δ 4.34 (1H, brs, exchangeable with D_2O) indicated the presence of a hydroxymethyl group in the molecule. In the NOESY experiment (Figure 1), the hydroxymethyl signal was within NOE distance from the *gem*-dimethyl and vinylic methyl groups, and this determined the location of the hydroxymethyl group at C-1. The NOE between Me-2 (δ 1.99) and H-3 (δ 5.79) confirmed the placement of the hydroxyl group (δ 3.99) at C-3, which was also inferred by the unusual downfield shift of the methine proton to δ 5.79 due to the adjacent carbonyl and olefinic functionalities. Therefore, the structure of crocusatin-L was elucidated as **3**.

(3*S*),4-Dihydroxybutyric acid (**4**) was isolated as an optically active colorless oil. The IR absorptions at 3320 and 1702 cm^{-1} accounted for the hydroxyl and carbonyl groups, respectively. Compound **4** displayed four distinct carbon signals, attributable to one carbonyl, one oxygenated methine, and two methylene carbons and confirmed by an HMQC experiment. In the ^{1}H NMR spectrum of **4**, the mutually coupled oxygenated methine and a pair of methylene protons at δ 4.65 (1H, m) and δ 4.39 (1H, dd, J = 14.8, 4.4 Hz), 4.17 (1H, dd, J = 14.8, 1.2 Hz) and 2.77 (1H, dd, J = 17.2, 6.0 Hz), 2.30 (1H, dd, J = 17.2, 1.6 Hz) established the 3,4-dihydroxybutyric acid structure. The absolute configuration at C-3 was determined to be *S* on the basis of its negative optical rotation.[13] This is the first report of **4** as a natural product.

Table 1. Inhibition Effects on Tyrosinase Activity of the Constituents Isolated from the Petals of *Crocus sativus* and Kojic Acid.

compound	conc (μM/mL)	inhibition (%)	IC_{50}
1	333.3	8.1	
2	333.3	66.7	
	166.7	27.1	260 μM
	83.3	0.4	
3	333.3	14.3	1.0 mM
	166.7	6.3	
	83.3	1.5	
8	333.3	13.3	1.1 mM
	166.7	4.6	
	83.3	1.6	
kojic acid	333.3	59.8	
	166.7	40.2	250 μM
	83.3	19.5	

The known compounds 3-formyl-6-hydroxy-2,4,4-trimethyl-2,5-cyclohexadien-1-one (**5**),[8] 6-hydroxy-3-(hydroxymethyl)-2,4,4-trimethyl-2,5-cyclohexadien-1-one 6-*O*-β-D-glucoside (**6**),[14] picrocrocin (**7**),[11] 4-hydroxy-3,5,5-trimethylcyclohex-2-enone (**8**),[11] crocusatin-C (**9**),[11] crocusatin-D (**10**),[11] crocusatin-E (**11**),[11] crocusatin-I (**12**),[10] methylparaben (**13**),[15] 4-hydroxyphenethyl alcohol (**14**),[16] 4-hydroxybenzoic acid (**15**),[17] *p*-coumaric acid (**16**),[18] protocatechuic acid methyl ester (**17**),[19] protocatechuic acid (**18**),[20] vanillic acid (**19**),[21] methylvanillate (**20**),[22] 3-hydroxy-4-methoxybenzoic acid (**21**),[23] kaempferol 3-*O*-β-D-(2-*O*-β-D-glucosyl)glucopyranoside (**22**),[24] astragalin (**23**),[8] kaempferol (**24**),[24] kaempferol 3-*O*-β-D-(2-*O*-β-D-6-*O*-acetylglucosyl)glucopyranoside (**25**),[8] kaempferol 3-*O*-β-D-(6-*O*-acetyl)glucopyranoside (**26**),[25] kaempferol 7-*O*-β-D-glucopyranoside (**27**),[26] kaempferol 3,7-di-*O*-β-D-glucopyranoside (**28**),[27] kaempferol 3-*O*-β-D-(6-*O*-acetyl)glucopyranoside-7-*O*-β-D-glucopyranoside (**29**),[28] kaempferol 3-*O*-β-D-(2-*O*-β-D-6-acetylglucosyl)glucopyranoside-7-*O*-β-D-glucopyranoside (**30**),[29] tribulusterine (**31**),[30] harman (**32**),[31] 1-(9*H*-β-carbolin-1-yl)-3,4,5-trihydroxypentan-1-one (**33**),[32] nicotinamide (**34**),[10] and adenosine (**35**)[10] were also isolated and identified by comparison of their spectroscopic data (UV, IR, and mass spectroscopy) with literature values.

The isolated compounds were subjected to an evaluation of their antityrosinase activity, and the results are summarized in Table 1. Among them, crocusatin-K (**2**) exhibited more significant (IC_{50} ~260 μM) antityrosinase activity than that of arbutin (IC_{50} ~24 mM)[2] or hydroquinone (IC_{50} ~4.5 mM)[1] and similar activity to that of kojic acid (IC_{50} ~250 μM),[33] known commercial whitening agents in cosmetics. In addition, their antioxidant properties were also evaluated using the α,α-diphenyl-β-picrylhydrazyl free radical (DPPH) assay. Protocatechuic acid (**18**), kaempferol (**24**), and kaempferol 7-*O*-β-D-glucopyranoside (**27**) showed better radical-scavenging activity (89.9%, 86.5%, and 91.2% scavenging effect at a dosage of 0.1 mg/mL) than α-tocophenol (IC_{50} ~0.15 mg/mL).[12] The results of the potent

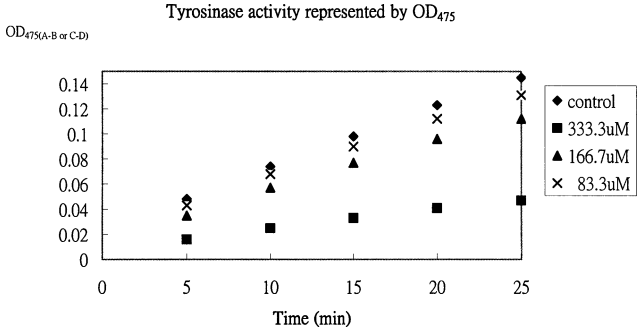

Figure 2. Tyrosinase activity represented by OD_{475} versus time. The tyrosinase was preincubated with **2** for 10 min. Control corresponds to tyrosinase activity without **2**.

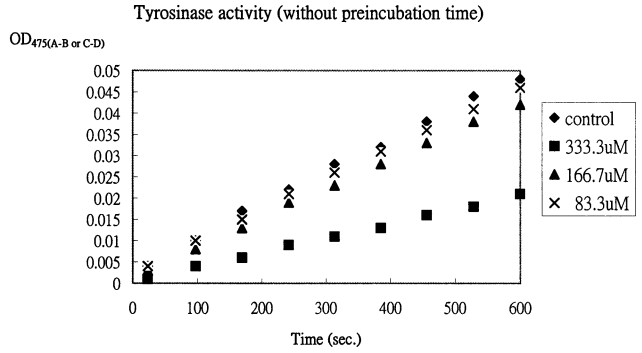

Figure 3. Tyrosinase activity, without preincubation time, represented by OD475 versus time. Control corresponds to tyrosinase activity without **2**.

biological evaluation suggest that the petals of *Crocus sativus* might be able to be used as an additive in whitening cosmetics. Interesting, crocusatin-K (**2**) showed potential antityrosinase activity, whereas its stereoisomer crocusatin-J (**1**) was inactive. This allowed us to assume the role of configuration of hydroxyl groups in the mechanism of the tyrosinase activity.

The antityrosinase activity was measured every 5 min for a total of 25 min with three concentrations, 333.3, 166.7, and 83.3 μM, of **2** and a preincubation of tyrosinase for 10 min (Figure 2). The results showed a high dose-dependent correlation, and the IC_{50} value of **2** was calculated as 260 μM. Subsequently, the activity was measured directly without preincubation of **2** with tyrosinase. The results are shown in Figure 3. The correlation between the activity and reaction time was very similar to that in Figure 2, but the percent inhibitions were somewhat reduced. These observations suggested that **2** interacts with the active site of tyrosinase and competition to displace L-Dopa occurs in a reversible way. The inhibition kinetics of **2** were analyzed by a Lineweaver−Burk plot as shown in Figure 4. The three lines, obtained from the uninhibited enzyme and the two different concentrations of **2**, intersected on the vertical axis. The results demonstrated that **2** exhibited competitive inhibition for the oxidation of L-Dopa catalyzed by mushroom tyrosinase. It is believed that tyrosinase contains a binuclear copper active site.[5] The reversible competitive inhibitor **2** may bind preferentially to the copper center with the two hydroxy groups (axial−axial). On the other hand, the axial−equatorial hydroxy groups in **1** may not combine to the active site of tyrosinase. Thus, **2** was active but not **1**. These results indicated that the near coplanar 1,2-dioxygenated moiety may play an important role in eliciting tyrosinase inhibitory activity. However, further

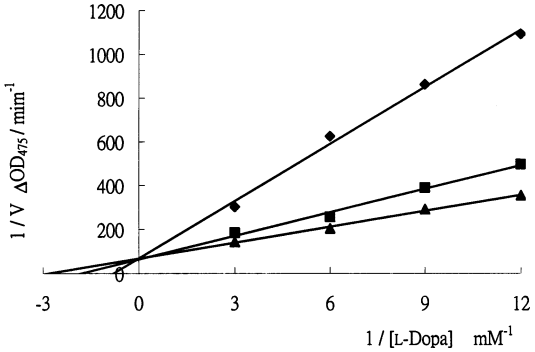

Figure 4. Lineweaver−Burk plots of mushroom tyrosinase and L-Dopa without (▲) and with [(◆) 333 μM and (■) 167 μM] **2**.

studies are needed to ascertain the active site of tyrosinase and the catalytic mechanism of mushroom tyrosinase with **2**.

Experimental Section

General Experimental Procedures. Melting points were measured on a Yanagimoto MP-S3 micro-melting point apparatus and are uncorrected. Optical rotations were obtained with a JASCO DIP-370 polarimeter. The UV spectra were recorded on a Hitachi UV-3210 spectrophotometer in MeOH solution. The IR spectra were measured on a Shimadzu FTIR-8501 spectrophotometer as KBr disks. The ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Varian-400 Unity Plus 400 spectrometer. Chemical shifts are shown in δ values with tetramethylsilane as an internal reference. The mass spectra were recorded in the EI mode on a VG70-250S mass spectrometer.

Plant Material. The petals of *Crocus sativus* L. were bought from Shanghai, People's Republic of China, in July 1995, and authenticated by Prof. C. S. Kuoh. A voucher specimen (NCKU-WU-950701) was deposited in the Herbarium of National Cheng Kung University, Tainan, Taiwan.

Extraction and Separation. The petals of *C. sativus* L. (1.2 kg) were extracted with MeOH (5 L × 5) at room temperature and concentrated to give a deep brown syrup (450 g). The crude extract was partitioned between H_2O (430 g) and Et_2O (40 g). Then, the residual petals were extracted with 1% HCl−MeOH (5 L × 1) and concentrated to give an acidic layer (20 g). The Et_2O layer (40 g) was directly chromatographed on a silica gel column by elution with a gradient of (*i*-Pr)$_2$O−MeOH to afford 15 fractions. Fraction 7 was column chromatographed over silica gel using *n*-C_6H_{14}−Me_2CO (17:3) as an eluent to yield **8** (6.1 mg) and **9** (6.5 mg). Fraction 10 on column chromatography with $CHCl_3$−Me_2CO (6:1) as an eluent gave **11** (1.2 mg), **12** (3.9 mg), **14** (4.4 mg), and **2** (1.2 mg), successively. Fraction 11 was rechromatographed on a silica gel column and eluted with a gradient of (*i*-Pr)$_2$O−Me_2CO to give **24** (4.3 mg). Fraction 12 was applied on silica gel and eluted with $CHCl_3$−Me_2CO (7:1) to give **11** (1.1 mg), **12** (0.3 mg), **13** (6.5 mg), **15** (5.8 mg), **16** (3.3 mg), and **17** (1.8 mg). Fraction 14 was rechromatographed on a silica gel column with an eluent of $CHCl_3$−MeOH (9:1) to give **15** (3.6 mg) and **23** (56 mg). Fraction 15 was subjected to column chromatography over C-18 gel and eluting with MeOH−H_2O (1:1) to obtain **15** (6.4 mg), **23** (124 mg), and **24** (5.2 mg). The H_2O-soluble layer (430 g) was applied directly on Diaion HP-20 and eluted with gradients of H_2O and MeOH to give 12 fractions. Fraction 2 was rechromatographed on Diaion HP-20 using gradients of H_2O and MeOH as an eluent to afford **4** (28 mg), **6** (1.1 mg), **7** (13 mg), **12** (1.2 mg), **34** (24 mg), and **35** (37 mg). Fraction 3 was column chromatographed on Diaion HP-20 gel and eluted with MeOH−H_2O (4:1) to give **3** (6.1 mg), **22** (3.3 g), **28** (29 mg), **29** (11 mg), and **30** (1.1 g). Fraction 4 when column chromatographed over silica gel using $CHCl_3$−MeOH (5:1) as an eluent yielded **10** (4.4 mg), **22** (69 g), and **23** (0.9 g). Similarly, fraction 5 afforded **5** (2.4 mg), **9** (1.1 mg), **23** (1.1 g),

25 (520 mg), and **33** (5.4 mg). Fraction 6 was column chromatographed over silica gel with $CHCl_3$−MeOH (3:1) as eluent to yield **26** (29 mg), **31** (1.9 mg), and **32** (0.8 mg). Fraction 9 was rechromatographed on silica gel and eluted with $CHCl_3$−MeOH−H_2O (15:7:2) to afford **1** (6.4 mg), **2** (8.2 mg), **5** (2.2 mg), **13** (3.3 mg), **15** (2.3 mg), and **24** (17 mg). The acidic layer (20 g) was chromatographed on XAD-7 by eluting with a gradient of H_2O and MeOH to give **13** (2.2 mg), **14** (1.2 mg), **15** (2.7 mg), **18** (2 mg), **19** (1.1 mg), **20** (0.9 mg), **21** (0.6 mg), and **27** (1.4 mg).

Crocusatin-J (1): colorless oil; $[\alpha]_D^{25}$ +68° (*c* 0.02, MeOH); UV (MeOH) λ_{max} (log ϵ) 246 (3.77) nm; IR (KBr) ν_{max} 3180, 2820, 2790, 1664, 1584, 1395, 1239 cm^{-1}; ^1H NMR (acetone-d_6, 400 MHz) δ 10.15 (1H, s, CHO), 3.95 (1H, d, J = 4.0 Hz, H-5), 3.78 (1H, ddd, J = 12.0, 4.0, 4.0 Hz, H-4), 2.22 (3H, s, 2-Me), 1.74 (1H, dd, J = 12.0, 12.0 Hz, H-3_{ax}), 1.38 (1H, dd, J = 12.0, 4.0 Hz, H-3_{eq}), 1.22 (3H, s, 6_{eq}-Me), 1.21 (3H, s, 6_{ax}-Me); ^{13}C NMR (acetone-d_6, 100 MHz) δ 193.0 (CHO), 152.1 (C-2), 140.7 (C-1), 72.0 (C-5), 65.9 (C-4), 42.5 (C-3), 35.6 (C-6), 28.4 (6_{ax}-Me), 26.6 (6_{eq}-Me), 16.6 (2-Me); EIMS *m*/*z* 184 $[M]^+$ (19), 154 (82), 142 (100), 121 (35), 107 (49); HREIMS *m*/*z* 184.1099 (calcd for $C_{10}H_{16}O_3$, 184.1100).

Crocusatin-K (2): colorless oil; $[\alpha]_D^{25}$ +18° (*c* 0.02, MeOH); UV (MeOH) λ_{max} (log ϵ) 246 (3.80) nm; IR (KBr) ν_{max} 3268, 2825, 2780, 1672, 1598, 1390, 1255 cm^{-1}; ^1H NMR (acetone-d_6, 400 MHz) δ 10.11 (1H, s, CHO), δ 3.90 (1H, d, J = 8.0 Hz, H-5), 3.70 (1H, ddd, J = 12.8, 8.0, 4.0 Hz, H-4), 2.18 (3H, s, 2-Me), 1.62 (1H, dd, J = 12.8, 4.0 Hz, H-3_{eq}), 1.49 (1H, dd, J = 12.8, 12.8 Hz, H-3_{ax}), 1.22 (3H, s, 6_{ax}-Me), 1.17 (3H, s, 6_{eq}-Me); ^{13}C NMR (acetone-d_6, 100 MHz) δ 192.8 (CHO), 154.7 (C-2), 139.9 (C-1), 77.6 (C-5), 69.3 (C-4), 46.0 (C-3), 35.4 (C-6), 28.8 (6_{eq}-Me), 27.1 (6_{ax}-Me), 13.4 (2-Me); EIMS *m*/*z* 184 $[M]^+$ (45), 167 (51), 142(71), 126 (68), 97 (100); HREIMS *m*/*z* 184.1104 (calcd for $C_{10}H_{16}O_3$, 184.1100).

Crocusatin-L (3): colorless oil; $[\alpha]_D^{25}$ +54° (*c* 0.02, MeOH); IR (KBr) ν_{max} 3512, 2944, 1712, 1462, 1207 cm^{-1}; ^1H NMR (acetone-d_6, 400 MHz) δ 5.79 (1H, s, H-3), 4.34 (1H, br, D_2O exchangeable), 3.99 (1H, br, D_2O exchangeable), 3.80 (1H, d, J = 11.2 Hz, CH_2O), 3.76 (1H, d, J = 11.2 Hz, CH_2O), 2.80 (1H, d, J = 17.2 Hz, H-5_{ax}), 2.03 (1H, d, J = 17.2 Hz, H-5_{eq}), 1.99 (3H, s, 2-Me), 1.10 (3H, s, 6_{eq}-Me), 1.01 (3H, s, 6_{ax}-Me); EIMS *m*/*z* 184 $[M^+]$ (13), 153 (100), 139 (75), 111 (100), 110 (71), 107 (73); HREIMS *m*/*z* 184.1093 (calcd for $C_{10}H_{16}O_3$, 184.1100).

(3*S*),4-Dihydroxybutyric acid (4): colorless oil; $[\alpha]_D^{25}$ −12.3° (*c* 0.02, H_2O); IR (KBr) ν_{max} 3320, 2956, 1702, 1421, 1211, 1012 cm^{-1}; ^1H NMR (acetone-d_6, 400 MHz) δ 4.65 (1H, m, H-3), 4.39 (1H, dd, J = 14.8, 4.4 Hz, H-4a), 4.17 (1H, J = 14.8, 1.2 Hz, H-4b), 2.77 (1H, dd, J = 17.2, 6.0 Hz, H-2a), 2.30 (1H, dd, J = 17.2, 1.6 Hz, H-2b); ^{13}C NMR (acetone-d_6, 100 MHz) δ 176.7 (C-1), 76.7 (C-3), 67.9 (C-4), 38.1 (C-2).

Tyrosinase Inhibitory Activity Assay. The mushroom tyrosinase and L-Dopa used for the bioassay were purchased from Sigma Chemical Co. Antityrosinase activity was measured by spectrophotometry according to the method of Mason and Peterson,[34] with minor modifications. The test substance was dissolved in 0.1 mL of 10% DMSO in aqueous solution and incubated with 0.1 mL of mushroom tyrosinase (135 U/mL, PBS pH 6.8) at 25 °C for 10 min, and then 0.1 mL of 0.5 mM L-Dopa in phosphate buffer solution (PBS, pH 6.8) was added. The reaction mixture was incubated for 5 min. The amount of dopachrome in the mixture was determined by the optical density (OD) at 475 nm using a μ Quant universal microplate spectrophotometer. Kojic acid, arbutin, and hydroquinone (Sigma Chemical Co.) were used as standard agents. The inhibitory percentage of tyrosinase was calculated as follows: % inhibition = $\{[(A - B) - (C - D)]/(A - B)\} \times 100$ (*A*: OD at 475 nm without test substance; *B*: OD at 475 nm without test substance and tyrosinase; *C*: OD at 475 nm with test substance; *D*: OD at 475 nm with test substance, but without tyrosinase.)

Free Radical-Scavenging Activity Assay. The effect of isolated compounds on the DPPH radical was estimated according to the method of Yamaguchi et al.[35] with minor modifications. A sample was dissolved in 0.1 mL of DMSO and then added to 0.1 mL of 0.1 mM DPPH in ethanol. The mixture was shaken vigorously and allowed to stand for 30 min at room temperature in the dark. The absorbance at 517 nm by DPPH was measured by a μ Quant universal microplate spectrophotometer. α-Toc (Sigma Chemical Co.) was used as a standard agent. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{scavenging effect (\%)} = [1 - (\text{absorbance of sample at 517 nm} / \text{absorbance of control at 517 nm})] \times 100$$

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