Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 6, No. 12, pp. 1303-1308, 1996

0960-894X/96 $15.00 + 0.00

PII: S0960-894X(96)00221-1

SYNTHESIS OF N-KOJIC-AMINO ACID AND N-KOJIC-AMINO ACID-KOJIATE AND THEIR TYROSINASE INHIBITORY ACTIVITY

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Abstract: Ten amino acid derivatives of kojic acid were synthesized to improve the tyrosinase inhibitory activity of kojic acid. Almost all derivatives showed stronger activities than kojic acid, and in general, N-kojic-amino acid-kojiate was found to have a higher inhibitory potency than N-kojic-amino acid. Among them, the N-kojic-L-phenylalanyl kojiate was the strongest inhibitor and its IC_{50} value was 1/380 that for kojic acid. The inhibition mechanism of these derivatives is considered to be noncompetitive which is similar to that of kojic acid.

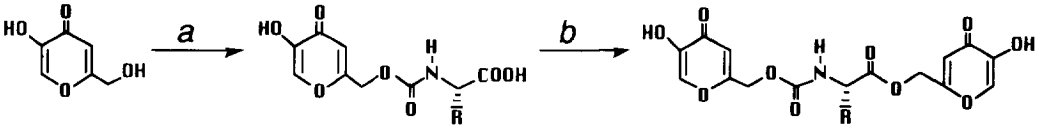
Introduction

Kojic acid is one of the metabolites used in the fermentation by *Aspergillus* species. This acid was isolated for the first time in 1924 by Dr. T. Yabuta, and its chemical structure determined by T. Takahashi et al.[1] The acid is generally used as a whitening agent for cosmetics and an anti-browning agent for food because of its inhibitory effects on melanin synthesis.[2),3)] The derivatives of kojic acid are known to suppress melanin synthesis.[4)-7)] However, the degrees of inhibition by kojic acid and its derivatives are not sufficient enough for use in the above mentioned applications. So, development of a compound with a much stronger melanin synthesis inhibition is presently desired. Previously, we succeeded in the synthesis of the ester compounds of kojic acid in which the carboxyl end of an amino acid is joined to the hydroxyl group at position 7 of kojic acid and demonstrated that these compounds significantly inhibit melanin synthesis.[8)] In the present study, we attempted to produce a new compound with much stronger inhibitory potency against tyrosinase than that of N-carbobenzoxy-amino acid-kojiate.[8)]

Results and Discussion

The synthesis of the kojic acid derivatives was performed using DSC(N,N'-disuccinimidyl carbonate) and DMAP(4-dimethylaminopyridine). Thus, ten compounds were obtained by joining the OH group at position 7 of kojic acid with the amino end of an amino acid to form a urethane type bond. The chemical structures of the newly synthesized derivatives and the procedures for synthesis of their derivatives are presented in Scheme 1.

Scheme 1



1 **2** **3**

a) DSC, DMAP *b*) EDC(1-ethyl-3,3-dimethylaminopropyl hydrochloride)

There are several methods for evaluating the inhibitory effects on melanin synthesis.[9)-14)] One of them is the following test method which is based on the inhibition of tyrosinase activity.[15)] Kojic acid is an inhibitor of tyrosinase, in which the copper ion is removed by chelation due to the ketone group at position 4 and the OH group at position 5 of the acid, leading to inhibition of enzyme activity. This inhibition is non-competitive. For the kojic acid derivatives previously synthesized by us, their abilities to inhibit melanin synthesis were evaluated according to the method based on the inhibition of tyrosinase the same method[8)] was used to evaluate the ten newly synthesized compounds.

Table 1. Synthesized compounds and their tyrosinase inhibitory activities.

compound	R	IC50(μM)
derivatives **2**		
2a	CH_3	23.21
2b	$CH(CH_3)_2$	16.66
2c	$CH_2CH(CH_3)_2$	14.51
2d	$CH(CH_3)CH_2CH_3$	15.35
2e	CH_2-C_6H_5	8.00
derivatives **3**		
3a	CH_3	3.03
3b	$CH(CH_3)_2$	2.39
3c	$CH_2CH(CH_3)_2$	2.00
3d	$CH(CH_3)CH_2CH_3$	0.70
3e	CH_2-C_6H_5	0.06
Kojic acid	-	22.94
Phenylalanine	-	N.A.

Table 1 shows the results of the inhibition of tyrosinase activity by the ten compounds using an enzyme preparation, tyrosinase from mushroom purchased from Sigma Chemicals Co. Ltd. Compound **3e** exhibited the strongest inhibitory effects on tyrosinase activity for all compounds tested, and its IC_{50} was approximately 1/380 that of kojic acid. Our previous study demonstrated that the strength of the inhibitory effects varied depending upon the difference in amino acid bound to kojic acid and the compound obtained using L-phenylalanine which has on extremely strong inhibition of tyrosinase activity. Similarly, compound **2e** showed the strongest inhibition of derivatives **2** and compound **3e** of derivatives **3**, confirming the previous evidence that the ester of kojic acid and L-phenylalanine are the strongest inhibitors of tyrosinase among all compounds. These results suggest that the inhibitory effects on tyrosinase may increase along with the increase in hydrophobicity at the side chain of the amino acid. In order to confirm these highly inhibitory effects of the derivative containing L-phenylalanine, an investigation of other derivatives with other amino acids instead of L-phenylalanine is needed. Further, the tyrosinase inhibition data were expressed in the Lineweaver-Burk plots aiming to investigate the mode of inhibition by each kojic acid derivative on tyrosinase.

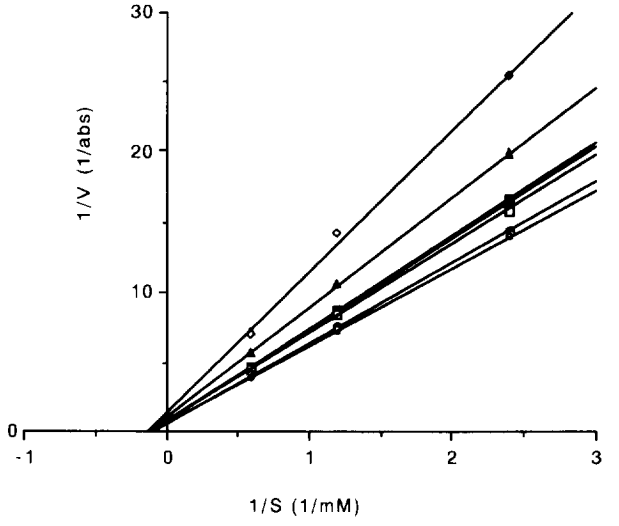


Fig. 1. Lineweaver-Burk Plot for tyrosinase inhibition by synthesized compounds and kojic acid. ○-○,control; ●-●,1.2 μM/ml kojic acid; □-□,1.2 μM/ml compound (**2a**); ■-■,1.2 μM/ml compound (**2b**); △-△,1.2 μM/ml compound (**2c**); ▲-▲,1.2 μM/ml compound (**2d**); ◇-◇,1.2 μM/ml compound (**2e**).

The reciprocal plots for the compounds in derivatives **2** are presented in Figure 1, showing that the mode of inhibition was non-competitive for all the compounds. Since $1/K_m$ values of 5 compounds in derivatives **2** were similar, the chemical affinities of these compounds with tyrosinase were inferred to be nearly the same.

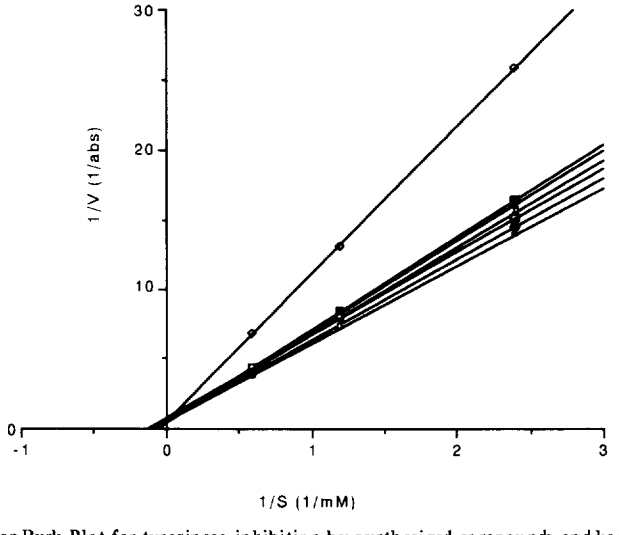


Fig. 2. Lineweaver-Burk Plot for tyrosinase inhibition by synthesized compounds and kojic acid. ○-○,control; ●-●,0.6 μM/ml kojic acid; □-□,0.6 μM/ml compound (**3a**); ■-■,0.6 μM/ml compound (**3b**); △-△,0.6 μM/ml compound (**3c**); ▲-▲,0.6 μM/ml compound (**3d**); ◇-◇,0.6 μM/ml compound (**3e**).

Figure 2 shows the tyrosinase inhibition by 5 compounds of derivatives **3** expressed in the Lineweaver-Burk plot. All of these derivatives were found to be non-competitive inhibitors as well as those of derivatives **2** and the respective compounds of derivatives **3** had nearly equal $1/K_m$ values. These results indicate that the inhibitory effects of the kojic acid derivatives on tyrosinase activity vary depending on the kind of amino acid as well as the type of binding. The present results provide useful information about the structure and inhibitory effects of the kojic acid derivatives from the viewpoint of their structure-activity relationship. The newly synthesized compounds, which were found to be strong inhibitors of melanin synthesis, would be available as an active ingredient for whitening cosmetics and for use as anti-browning agents in foods.

Experimental

SYNTHESIS ^{1}H-NMR spectra and ^{13}C-NMR spectra were recorded using a Bruker AC250. Mass spectra were recorded using a JEOL JMS-DX303HF instrument. IR spectra were recorded using a Shimadzu IR-435. UV spectra were obtained with a Shimadzu UV-240. Optical rotation was measured with a Horiba Sepa-200, and melting point values with a Yanagimoto MP-52. The amino acids were purchased from the Peptide Institute, Inc. and kojic acid was purchased from Tokyo Kasei Kogyo Co., Ltd.

2a. To a solution of kojic acid (20mmol) in methylene chloride (50ml) and acetonitrile (50ml) were added N,N'-disuccinimidyl carbonate (20mmol) and 4-dimethylaminopyridine (10mmol). The mixture was then stirred overnight at room temperature. After completing the reaction, the mixture was added to L-alanine (20mmol) and triethylamine (10mmol), and then stirred overnight at room temperature. The mixture was evaporated leaving an oily residue that was dissolved in $NaHCO_3$-saturated solution, and washed with ethyl acetate. The solvent was acidified with 2N HCl, and then extracted with ethyl acetate. The extract was washed with 2N HCl, evaporated, and crystallized from ethyl acetate to yield **2a** (yield: 12.1%). ^{1}H-NMR(250MHz,d6-DMSO) δ :1.28(3H,d,*J*=7.3Hz), 4.01(1H, qd,*J*=7.4 J=7.4Hz), 4.89(2H,s), 6.42 (1H,s), 7.84(1H,d,*J*=7.6Hz), 8.07(1H,s). ^{13}C-NMR(62MHz, d6-DMSO) δ: 16.91, 49.25, 61.14, 111.88, 139,58, 145.94, 154.88, 162.40, 173.60, 174.04. HREI-MS Found: 257.0525. Calcd. for $C_{10}H_{11}NO_7$: 257.056. IR ν_{max}(KBr)cm^{-1} 3350, 3080, 1729, 1640, 1600, 1580, 1520, 1460. UV(MeOH) λ_{max}: 217.4, 270.8nm. $[\alpha]^{25}_D$ -16.00(c0.100,MeOH). mp 164.0-165.0.

2b, **2c**, **2d**, **2e**: The same procedure as that described for **2a** was done with DSC/DMAP (yield: **2b**: 42.1%; **2c**: 17.5%; **2d**: 11.0%; **2e**: 25.2%).

2b. ^{1}H-NMR(250MHz,d6-DMSO) δ:0.89(3H,d,*J*=6.6Hz), 0.91(3H,d,*J*=6.7Hz), 2.06(1H,m), 3.88(1H,dd,*J*=5.8 *J*=8.4Hz), 4.89(2H,s), 6.44(1H,s), 7.72(1H,d,*J*=8.5Hz). 8.06(1H,s). ^{13}C-NMR (62MHz,d6-DMSO) δ: 17.83, 19.05, 29.47, 59.58, 61.21, 111.83, 139.56, 145.94, 155.48, 162.46, 172.94, 173.61. HREI-MS Found: 285.0860. Calcd. for $C_{12}H_{15}NO_7$: 285.0849. IR ν_{max} (KBr)cm^{-1} 3350, 2950, 1700, 1650, 1610, 1580, 1540, 1460, 1160. UV(MeOH) λ_{max}: 217.4, 270.8nm. $[\alpha]^{25}_D$ -4.19(c1.000,MeOH). mp 188.5-190.0.

2c. ^{1}H-NMR(250MHz,d6-DMSO) δ:0.88(6H,t,*J*=7.6Hz), 1.56(2H,m), 1.64(1H,m), 3.97(1H, m), 4.89(2H,s), 6.42(1H,s), 7.80(1H,d,*J*=8.1Hz), 8.06(1H,s). ^{13}C-NMR(62MHz,d6-DMSO) δ: 21.02, 22.80, 24.26, 39.50, 52.23, 61.19, 111.83, 139.55, 145.94, 155.22, 162.44, 173.61, 174.06. HREI-MS Found: 299.0989. Calcd. for $C_{13}H_{17}NO_7$: 299.1005. IR ν_{max}(KBr)cm^{-1} 3280, 2930, 1700, 1650, 1600, 1580, 1540, 1450, 1160. UV(MeOH) λ_{max}: 217.7, 270.3nm. $[\alpha]^{25}_D$ -14.00 (c0.100,MeOH). mp 123.0-125.0.

2d. ^{1}H-NMR(250MHz,d6-DMSO) δ:0.84(3H,t,*J*=7.4Hz), 0.88(3H,d,*J*=6.6Hz) 1.13-1.46(2H,

m), 1.79(1H, m), 3.92(1H, dd, *J*=6.1Hz J=8.4Hz) 4.89(2H, s), 6.44(1H, s), 7.73(1H, d, *J*=8.5Hz), 8.06 (1H, s), 9.19(1H, s), 12.64(1H, s). ^{13}C-NMR(62MHz, d6-DMSO) δ: 10.61, 14.92, 23.97, 35.42, 58.04, 60.63, 111.24, 138.95, 145.38, 154.79, 161.87, 172.38, 173.04. HREI-MS Found: 299.0976. Calcd. for $C_{13}H_{17}NO_7$: 299.1005. IR ν_{max}(KBr)cm^{-1} 3230, 2960, 1760, 1710, 1640, 1610, 1590, 1520, 1450. UV(MeOH) λ_{max}: 217.6, 271.5nm. $[\alpha]^{25}_D$ -4.00(c0.100, MeOH). mp 184.0-185.0.

2c. ^{1}H-NMR(250MHz, d6-DMSO) δ :2.80-3.14(2H, m), 4.19(1H, m), 4.82(2H, d, J=4.1Hz), 6.36(1H, s), 7.16-7.36(5H, m), 7.89(1H, d, J=8.4Hz), 8.03(1H, s). ^{13}C-NMR(62MHz, d6-DMSO) δ: 36.34 , 55.55, 61.12, 111.82, 126.33, 128.11, 128.97, 137.68, 139.50, 145.93, 155.05, 162.31, 173.00, 173.57. HREI-MS Found: 333.0829. Calcd. for $C_{16}H_{15}NO_7$: 333.0849. IR ν_{max}(KBr)cm^{-1} 3300, 3080, 1700, 1640, 1600, 1570, 1530, 1440. UV(MeOH) λ_{max}: 211.7, 270.2nm. $[\alpha]^{25}_D$ -6.00 (c0.100, MeOH). mp 144.0-144.5.

3a. Kojic-L-Ala-OH (11mmol) was added to EDC (11mmol), which was dissolved in methylene chloride (20ml). While stirring the mixture, kojic acid (10 mmol) was added, and the reaction mixture was gently stirred for 2 h at 0 ℃. Stirring was then continued for 24 h more at room temperature. The solvent was evaporated leaving an oily residue that was dissolved in ethyl acetate. This solution successively was washed using a $NaHCO_3$-saturated solution, 2 N HCl, and NaCl-saturated solution. After the solvent was evaporated, the oily residue was crystallized from ethyl acetate and normal hexane to yield **3a** (yield: 15.8%). ^{1}H-NMR(250MHz, d6-DMSO) δ :1.33(3H, d, *J*=7.3Hz), 4.21(1H, qd, *J*=7.3 *J*=7.3Hz), 4.90(2H, s), 5.01(2H, s), 6.42(1H, s), 6.46(1H, s), 8.08(1H1H, ss), 8.12(1H, d, *J*=7.3Hz). ^{13}C-NMR(62MHz, d6-DMSO) δ: 16.66, 49.34, 59.40, 61.35, 61.56, 111.98, 112.23, 139.61, 139.72, 146.00, 154.94, 161.18, 162.17, 171.91, 173.56, 173.61. HREI-MS Found: 381.0661. Calcd. for $C_{16}H_{15}NO_{10}$: 381.0696. IR ν_{max}(KBr)cm^{-1} 3350, 1740, 1700, 1660, 1630, 1540, 1450. UV(MeOH) λ_{max}: 217.5, 271.2nm. $[\alpha]^{25}_D$ -13.33(c0.030, MeOH). mp 165.0.

3b, **3c**, **3d**, **3e**: The same procedure as that described for **3a** was done with EDC (yield: **3b**: 36.6%; **3c**: 30.3%; **3d**: 10.5%; **3e**: 9.1%).

3b. ^{1}H-NMR(250MHz, d6-DMSO) δ :0.90(6H, d, J=6.7Hz), 2.09(1H, m), 4.03(1H, dd, *J*=6.2 *J*=8.0Hz), 4.91(2H, s), 5.02(2H, s), 6.45(1H, s), 6.50(1H, s), 8.05(1H, d, *J*=8.2Hz), 8.08(1H1H, ss). ^{13}C-NMR(62MHz, d6-DMSO) δ: 17.97, 18.83, 29.57, 59.70, 61.42, 61.53, 111.97, 112.57, 139.62, 139.73, 145.95, 146.00, 155.49, 161.11, 162.20, 170.84, 173.55, 173.59. HREI-MS Found: 409.1053. Calcd. for $C_{18}H_{19}NO_{10}$: 409.1009. IR ν_{max}(KBr)cm^{-1} 3380, 3300, 2930, 1730, 1720, 1650, 1630, 1590, 1540, 1450, 1140. UV(MeOH) λ_{max}: 217.5, 271.1nm. $[\alpha]^{25}_D$ -2.00 (c0.100, MeOH). mp 189.0.

3c. ^{1}H-NMR(250MHz, d6-DMSO) δ :0.95(3H, d, *J*=6.2Hz), 0.96(3H, d, *J*=6.3Hz), 1.62-1.77 (1H2H, mm), 4.38(1H, dd, *J*=8.1 and 8.1Hz), 4.93(2H, s), 4.97(2H, s), 6.48(1H, s), 6.48(1H, s), 6.85 (1H, d, *J*=8.4Hz), 7.82(1H, s), 7.83(1H, s). ^{13}C-NMR(62MHz, d6-DMSO) δ: 21.47, 22.86, 24.72, 40.56, 52.73, 60.30, 61.90, 111.40, 112.03, 138.56, 138.77, 146.25, 146.36, 155.30, 161.52, 162.87, 172.10, 174.16, 174.22. HREI-MS Found: 423.1144. Calcd. for $C_{19}H_{21}NO_{10}$: 423.1166. IR ν_{max}(KBr)cm^{-1} 3240, 3070, 2940, 1720, 1640, 1620, 1600, 1520, 1440, 1170. UV(MeOH) λ_{max}: 217.9, 270.8nm. $[\alpha]^{25}_D$ -20.00(c0.100, MeOH). mp 66.0-68.0.

3d. ^{1}H-NMR(250MHz, d6-DMSO) δ :0.85(3H, d, *J*=7.2Hz), 0.86(3H, d, *J*=6.7Hz) 1.24-1.44 (1H2H, mm), 4.06(1H, t, *J*=7.1Hz) 4.91(2H, s), 5.08(2H, s), 6.45(1H, s), 6.51(1H, s), 8.03(1H, d, *J*=6.0Hz), 8.08(1H, s), 8.10(1H, s). ^{13}C-NMR(62MHz, d6-DMSO) δ: 10.98, 15.09, 24.60, 35.95, 58.64, 61.52, 65.01, 111.97, 113.08, 139.63, 139.92, 145.99, 146.07, 155.41, 161.10, 162.21, 170.84, 173.51, 173.59. HREI-MS Found: 423.1220. Calcd. for $C_{19}H_{21}NO_{10}$: 423.1166. IR ν_{max}(KBr)cm^{-1} 3350, 3320, 2940, 1720, 1640, 1620, 1600, 1520, 1450. UV(MeOH) λ_{max}: 217.5,

270.9nm. $[\alpha]^{25}_{D}$ -79.99(c0.010,MeOH). mp 66.0-67.5.

3e. ^{1}H-NMR(250MHz,d6-DMSO) δ:2.88-3.13(2H,m), 4.33-4.42(1H,m), 4.84(2H,s), 5.00 (2H,s), 6.37(1H,s), 6.44(1H,s), 7.20-7.31(5H,m), 8.04(1H,s), 8.06(1H,s), 8.18(1H,d,*J*=7.9Hz). ^{13}C-NMR(62MHz,d6-DMSO) δ: 36.16, 55.47, 61.33, 61.68, 111.93, 112.41, 126.52, 128.16, 128.99, 136.87, 139.56, 139.73, 145.94, 145.98, 155.02, 160.99, 162.08, 170.85, 173.54, 173.54. HREI-MS Found: 457.1027. Calcd. for $C_{22}H_{19}NO_{10}$: 457.1009. IR ν_{max}(KBr)cm^{-1} 3360, 3240, 3050, 1720, 1640, 1620, 1510, 1440. UV(MeOH) λ_{max}: 216.4, 272.0nm. $[\alpha]^{25}_{D}$ -2.00 (c0.100,MeOH). mp 123.0-124.5.

BIOLOGICAL ASSAY The tyrosinase inhibitory activity was measured as follows. Tyrosinase from Agricus bisporus (mushroom) was purchased from the Sigma Chemical Co. We mixed 2.0 ml of the McIlvaine buffer solution (pH 6.8), 2.0 ml of distilled water containing 0.3 mg/ml of tyrosine and 1.8 ml of the sample containing the 10 % dimethyl sulfoxide and incubated it at 30 ℃ for 10 min in a sample tube. Then, 0.1 ml of 480 units/ml of tyrosinase was added and the solution incubated at 30 ℃ for 10 min. 0.1 ml of 1 M sodium azide was added to stop the reaction. The absorbance at 475 nm was measured with a spectrophotometer. A control test was done with the 10 % dimethyl sulfoxide solution. The inhibitory activity was calculated using the following formula:

$$\text{Inhibitory activity (\%)} = (C-S)/C \times 100$$

(C, the absorbance at 475 nm of control; S, the absorbance at 475 nm.)

Acknowledgement: We are grateful to Dr. Toshiyuki Miyata and Dr. Takumi Mizuno of the Osaka Municipal Technical Research Institute for the MS spectra measurements.

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(*Received in Japan* 29 *February* 1996; *accepted* 9 *May* 1996)