Inhibition of Mushroom Tyrosinase by Kojic Acid Octanoates
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Introduction

Kojic acid (1) is a well known naturally occurring inhibitor of tyrosinase showing a wide range of biological activities such as insecticidal and anti-biotic action (for references, see Fugmann et al., 1997; Kahn et al., 1995; Cabanes et al., 1994). Kojic acid is of considerable interest in the cosmetics industry since inhibition of tyrosinase in the skin results in suppression of melanogenesis. Many derivatives of 1 have been synthesized, among others fatty acid esters, such as stearates, palmitates, oleates, and butyrates, and were tested for potential applications as skin lightening agents. (Sansho Seiyaku, 1981; Nagai and Izumi, 1982; Sansho Seiyaku, 1982). Furthermore, the antioxidative properties of kojic acid are of interest for the food and agricultural industry (Kahn, 1995). Fatty acid esters of 1 decrease formation of hydroperoxides in some natural oils (Abe and Takahashi, 1970). A number of aryloxyacetates, azides and halogenides have been synthesized, among others 7-O-acyl derivatives (Ichimoto and Tatsumi, 1962; Abe and Takahashi, 1970) or to di-esters. Reaction of 1 with free fatty acids in the presence of ZnCl₂ at 130-140 °C leads either to the 7-O-acyl derivatives (Ichimoto and Tatsumi, 1962; Abe and Takahashi, 1970) or to di-esters. Recently, Kobayashi et al. (1996) reported on the synthesis 7-urethanes of 1, using DCC/DMAP for coupling, while 7-O-(Z-aminoacid) esters were prepared by means of EDC in CH₂Cl₂/acetonitrile (Kobayashi et al., 1995).

In our hands, reaction of 1 with a 1.4-fold molar excess of caprylic acid in presence of DCC/DMAP afforded the 5-O-capryloate 2 in 76% yield (see formula scheme). Trace amounts of side products were identified by TLC as the di-ester 3 and the 7-O-capryloate 5. Di-ester 3 was obtained in 83%...
yield by reaction of 1 with a 2.8-fold molar excess of caprylic acid in the presence of DCC/DMAP. The 7-O-acyl derivatives were identified unambiguously by $^1$H NMR which reveals a downfield acyl shift of the oxymethylprotons in 5 by ca. 0.5 ppm as compared to 2. Various attempts to cleave selectively the 5-O-acyl group in 3 to afford 5 gave unsatisfactory results.

Since we plan to prepare the 7-O-acyl derivatives with rare acyl components it is desired to prevent coupling to the 5-hydroxy group of 1. This was achieved by protection of 5-OH with di-tert-butoxy-dicarbonate in water/dimethylglycol to give 4. Acylation of 4 with caprylic acid in presence of DCC/DMAP followed by cleavage of the boc-ester with TFA gave 5 in 58% yield (43% after chromatography). Acylation of 4 with N-boc-11-aminoundecanoic acid, followed by cleavage of both tert-butoxycarbonyl groups, gave the corresponding 11-aminoundecanoic acid ester 6 in 35% isolated yield. The latter compound was useful for coupling of the amino group by crosslinking to a dextrane membrane (to be published elsewhere).

**Enzyme Assays**

Inhibition of the catecholase activity of mushroom tyrosinase was determined by a colorimetric assay using dihydroxybenzene and L-proline according to Rzepecki and Waite (1989) (see Experimental). Fig. 1 shows the effects of varying inhibitor concentrations on the relative enzyme activity, expressed as the ratio in the changes of absorption measured in the inhibited and non-inhibited reaction. Transformation of the data in a Haines-Woolf plot revealed that kojic acid and the mono-esters

![Fig. 1. Inhibition of mushroom tyrosinase (Sigma) in solution.](image-url)
Tyrosinase Inhibition by Kojic Acid Octanoates

Table I. Inhibition of mushroom tyrosinase by kojic acid (1) and derivatives.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Enzyme in solution</th>
<th>Immobilized enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC50 (µM)</td>
<td>% inhibition at C_inhibitor = 100 µM</td>
</tr>
<tr>
<td>1</td>
<td>69</td>
<td>45 ± 3</td>
</tr>
<tr>
<td>2</td>
<td>49</td>
<td>107 ± 6</td>
</tr>
<tr>
<td>3</td>
<td>nil</td>
<td>n.d.</td>
</tr>
<tr>
<td>5</td>
<td>87</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>6</td>
<td>84</td>
<td>20 ± 2</td>
</tr>
</tbody>
</table>

Tyrosinase is a mixed-type, non-competitive inhibitors of tyrosinase.

Evaluation of Michaelis-Menten kinetics gave the IC50 values shown in Table I. They decrease in the order 2 (IC50 = 107 µM) > 1 (IC50 = 45 µM) > 6 (IC50 = 20 µM) > 5 (IC50 = 15 µM) (Table I). Diester 3 showed no inhibition of tyrosinase at concentrations up to 100 µM. The 7-O-acyl derivatives bearing a free 5-OH group are more efficient than their respective bearing a free 5-yl ester. Monoesters 2, 5, and 6 were reversible inhibitors, as expected.

The diester 3 was inactive up to 100 µM also in the “biosensor assay”. In contrast to the properties observed in solution, compound 5 is a poorer inhibitor of immobilized tyrosinase than 1. On the other hand, 2 was nearly as effective as 1 in the “biosensor assay” and even more effective than 5 while in solution it was much poorer than 1. We assume that this result is due to diffusion phenomena in the membrane. In conclusion, caution is indicated when data from enzyme inhibition assays obtained in solution should be transferred to heterogenous systems containing immobilized enzymes.

Experimental

Melting points: Büchi SMP-20. – UV-VIS spectra: Beckman DU640. – IR spectra: Perkin Elmer 16 PC FT-IR. – 'H and 13C NMR spectra: Bruker AMX R300 at 300 or 74.5 MHz, respectively (internal standard: TMS for 'H and solvent signals for 13C spectra). – Mass spectra: Finnigan MAT SSQ 710; EI-MS: direct inlet, acceleration voltage 70 eV, ion source temp. 150 °C; CI-MS: ion source temp. 120 °C, ionization gas CH4. – TLC: Silica gel HF254 plates (E. Merck, Darmstadt). – Kojic acid (5-hydroxy-2-hydroxymethyl-4H-pyran-4-one), di-tert-butyl-dicarbonate, 11-aminoundecenoic acid, and trifluoracetic acid were from Merck-Schuchardt. Tyrosinase (EC 1.14.18.1) from mushrooms (specific activity: 75.5 units·mg⁻¹) was from Sigma.

Octanoic acid 2-hydroxymethyl-4-oxo-4H-pyran-5-yl ester (2): To an ice-cold solution of 142 mg (1.0 mmol) of kojic acid (1), 7 mg (0.06 mmol) of DMAP, and 0.2 ml (1.4 mmol) of caprylic acid in 30 ml of dry CH2Cl2, an ice-cold solution of 3.25 g (15 mmol) of DCC in 15 ml CH2Cl2 were added through a dropping funnel. The reaction mixture was stirred for 12 h at room temp., then filtered, and the filtrate was washed with 0.5 n HCl and saturated aqueous NH4CO3 solution, and dried over MgSO4. Evaporation of the solvent afforded a yellowish oil which was repeatedly dissolved in 40 ml EtOAc, filtered and evaporated to yield 203.8 mg (76%) of 2, containing traces of 3 and 5 (TLC evidence), as a white wax. A sample was purified by prep. TLC to afford pure 2, m.p. 69–71 °C (dec.). – Rf (EtOAc) = 0.67. – UV (EtOAc): λmax (log ε) = 259.0 (4.25), 262 nm (sh; 4.22). – 'H NMR (300 MHz, CDCl3): δ = 7.87 (s, 1 H, H-6), 6.53 (s, 1 H, H-3), 4.44 (s, 2 H, H2-7), 2.56 (t, J = 7.54 Hz, 2 H, α-CH2), 1.69 (m, 2 H, CH2-CH3), 1.27 (m, 8 H, CH2), 0.86 (t, J = 6.57 Hz, 3 H, CH3). – 13C NMR (74.5 MHz, d6-DMSO) (for 13C NMR data of 1, see Kingsbury et al., 1976): δ = 171.6, 170.3 (C-4 and CO2R), 169.4 (C-6), 148.8 (C-3), 140.3 (C-2), 112.0 (C-5), 59.3 (C-7), 33.7, 32.8, 31.1, 28.4, 24.4, 22.1 (CH2), 13.9

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Table I: Inhibition of mushroom tyrosinase by kojic acid (1) and derivatives.
(CH$_3$) - EI-MS: $m/z$ (%) = 269.2 [M+H]$^+$ (42), 142.0 (100).

Octanoic acid 4-oxo-2-(l-oxoctyloxy-methyl)-4H-pyran-5-yl ester (3): Kojic acid (1) (1.0 mmol) was reacted with 0.4 ml (2.8 mmol) of caprylic acid and, after stirring for 36 h, worked up as described above for the synthesis of 2. Recrystallization from EtOAc afforded 328 mg (83%) of 3 as a white wax, m.p. 56 - 60 °C (dec.) - $R_f$ (EtOAc) = 0.94. - UV (EtOAc): $\lambda_{max}$ (log $\varepsilon$) = 260 (3.30), 264 nm (sh, 3.31). - IR (KBr): $\nu$ = 3260, 3064, 2928, 2854, 1768, 1740, 1668, 1638, 1382, 1426, 1466, 1190 cm$^{-1}$. - 1H NMR (300 MHz, CDCl$_3$): $\delta$ = 7.86 (s, 1 H, H-6), 6.47 (s, 1 H, H-3), 4.91 (s, 2 H, H$_2$-7), 2.55 (t, $J$ = 7.28 Hz, 2 H, $\alpha$-CH$_2$), 2.41 (t, $J$ = 7.33 Hz, 2 H, $\alpha$-CH$_2$), 1.57 (m, 4 H, 2 CH$_2$-CH$_2$), 1.26 (m, 16 H, CH$_3$), 0.85 (m, 6 H, 2 CH$_3$) - 13C NMR (74.5 MHz, CDCl$_3$): $\delta$ = 181.8, 167.9 (CO$_2$, CO$_2$R, and C-4), 154.1 (C-6), 147.7 (C-3), 111.5 (C-5), 86.09 (C(CH$_3$)$_3$), 60.7 (C-7), 27.4 (C(CH$_3$)$_3$), 33.9, 31.5, 28.8, 24.6, 22.5 (CH$_3$), 14.0 (CH$_3$). - CI-MS: $m/z$ (%) = 395.1 [M+H]$^+$ (7), 41.2 (100).

2-Hydroxymethyl-5-O-tert-butoxycarbonyloxy-4-oxo-4H-pyran (4): Kojic acid (1) (2.0 g, 14.1 mmol) was dissolved in a mixture of 20 ml of 1,2-dimethoxyethane and 50 ml of water. The pH was adjusted to ca. 9 with Et$_3$N. - 1H NMR (300 MHz, CDC$_3$): $\delta$ = 7.85 (s, 1 H, H-6), 6.50 (s, 1 H, H-3), 4.90 (s, 2 H, H$_2$-7), 2.36 (t, $J$ = 7.50 Hz, 2 H, $\alpha$-CH$_2$), 1.59 - 1.60 (m, 2 H, CH$_2$-CH$_2$), 1.49 (m, 9 H, C(CH$_3$)$_3$) - 13C NMR (74.5 MHz, CDCl$_3$): $\delta$ = 187.3, 181.8, 172.6 (CO$_2$, CO$_2$R, and C-4), 154.1 (C-6), 147.7 (C-3), 111.5 (C-5), 86.09 (C(CH$_3$)$_3$), 60.7 (C-7), 27.4 (C(CH$_3$)$_3$), 33.9, 31.5, 28.8, 24.6, 22.5 (CH$_3$), 14.0 (CH$_3$). - CI-MS: $m/z$ (%) = 210 [M+H]$^+$ (34), 313.2 (100).

To a solution of octanoic acid (5-O-tert-butoxycarbonyloxy-4-oxo-4H-pyran-2-yl)-methyl ester (500 mg, 1.3 mmol) in 1 ml of dry CH$_2$Cl$_2$, 5 ml of trifluoracetic acid were added and the mixture was stirred at room temp. for 1.5 h. After cooling to 0 °C, the reaction mixture was carefully neutralized by means of a solution of Et$_3$N in CH$_2$Cl$_2$. It was then washed with H$_2$O, the hypophase was dried over MgSO$_4$, concentrated and the residue was filtered over silica gel with EtOAc. The combined extracts were dried over MgSO$_4$ and filtered. The solvent was evaporated and the residue was dissolved in EtOAc. Prep. TLC (silica, EtOAc) afforded 328 mg (83%) of 3 as a white wax which slowly crystallized. - m.p. 96 - 98 °C. - $R_f$ (EtOAc) = 0.7. - UV (EtOAc): $\lambda_{max}$ (log $\varepsilon$) = 267 nm (2.46). - IR (KBr): $\nu$ = 3446, 3076, 2990, 2852, 1734, 1654, 1626, 1390, 1340, 1144 cm$^{-1}$. - 1H NMR (300 MHz, CDC$_3$): $\delta$ = 7.85 (s, 1 H, H-6), 6.50 (s, 1 H, H-3), 4.93 (s, 2 H, H$_2$-7), 2.37 (t, $J$ = 7.52 Hz, 2 H, $\alpha$-CH$_2$), 1.65 (m, 2 H, CH$_2$-CH$_2$), 1.29 (m, 8 H, CH$_2$), 0.86 (t, $J$ = 6.57 Hz, 3 H, CH$_3$). - 13C NMR (74.5 MHz, CDCl$_3$): $\delta$ = 172.7 (C-4), 163.2 (C-6), 154.9 (C-3), 135.5 (C-2), 111.1 (C-5), 61.1 (C-7), 49.6, 33.9, 31.6, 29.7, 28.8, 24.8, 22.6 (CH$_3$), 14.0 (CH$_3$). - EI-MS: $m/z$ (%) = 269.2 [M+H]$^+$ (30), 56.1 (100).

11-aminoundecanoic acid (5-hydroxy-4-oxo-4H-pyran-2-yl)-methyl ester (6): N-boc-11-aminoundecanoic acid was prepared by reaction of 11-aminoundecanoic acid with di-tert-butyl-dicarbonate by the usual procedure (c.f. preparation of 4). To a solution of 0.294 g (1.3 mmol) of 4 in 50 ml of dry CH$_2$Cl$_2$, 0.392 g (1.3 mmol) of N-boc-11-aminoundecanoic acid and 7 mg (0.06 mmol) of DMAP were added. The mixture was vigorously stirred for 10 min while 0.40 g (20 mmol) of DCC were added. After stirring for another 4 h, the reaction vessel was allowed to stand overnight. Dichloroethyl urea was filtered off, the filtrate was concentrated in vacuo, and the residue was filtered over silica gel with EtOAc. The solvent was evaporated, the crude product was dried in vacuo (oil
pump), and then dissolved in 3 ml of dry CH₂Cl₂ and 5 ml TFA. Stirring at room temp. under an argon atmosphere for 12 h was followed by neutralization with a saturated aqueous solution of NaHCO₃. The hypophase was separated, dried over MgSO₄ and the solvent was evaporated to dryness in vacuo. The residue was dissolved in EtOAc and subjected to flash chromatography (silica gel. EtOAc / petroleum ether 1:2) to afford 149 mg (35%) of 8 as yellowish platelets, m.p. 148 °C. – Rₐ (EtOAc / petroleum ether 2:1) = 0.64. – UV (EtOAc): λmax (log ε) = 240 (3.54), 254 (sh, 3.26), 268.2 nm (3.44). – IR (KBr): ν = 3322, 2926, 2852, 1706, 1702, 1628, 1578, 1464, 1388, 1178 cm⁻¹. – ¹H NMR (300 MHz, CDCl₃): δ = 7.84 (s, 1 H, H-6), 6.47 (s, 1 H, H-3), 4.92 (s, 2 H, H₂-7), 3.34 (dt, J = 6.81 and 6.54 Hz, 2 H, CH₂-NH₂), 2.40 (t, J = 7.37 Hz, 2 H, α-CH₂), 1.93–1.90 (m, 2 H, β-CH₂), 1.77–1.59 (m, 4 H, γ- and δ-CH₂), 1.28 (m, 8 H, CH₂), 1.15 (m, 2 H, CH₂CH₂N₂H). – ¹³C NMR (74.5 MHz, CDCl₃): δ = 174.0, 172.7 (CO₂R and C-4), 163.1 (C-6), 145.9 (C-3), 138.1 (C-2), 111.1 (C-5), 61.1 (C-7), 36.8, 29.2, 29.1, 29.0, 28.9, 28.8, 26.6, 25.5, 24.9, 24.7 (CH₂). – EI-MS: m/z (%) = 327 [M+2H]+ (1), 324 [M-H]+ (1), 41.3 (100).

Spectrophotometric enzyme assay: All stock solutions were prepared in a 100 mM sodium phosphate buffer, pH 6.5, containing 1 mM NaCl. They were stored in the dark at 0 °C. The following reagents were pipetted into cuvettes: 30 µl of a 1 mM solution of l-proline, 15 µl of a 10 mM solution of 1,2-dihydroxybenzene, an appropriate amount of a solution of the test compound, and phosphate buffer to give a final volume of 1.5 ml. After mixing, the cuvette was allowed to stand for 2 min. The reaction was started by addition of 15 µl (0.34 units) of a solution of 300 µg/ml tyrosinase in phosphate buffer and immediately mixing of the contents of the cuvette. The change in absorbance was measured at 525 nm in time intervals of 10 s.

Procedures for immobilization of tyrosinase and conditions for the “biosensor assay” were the same as described by Makower et al. (1996).

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (INK 16 / A1-1) and by the Fonds der Chemischen Industrie. We thank Dr. Varda Kahn, The Volcani Center, Bet Dagan, Israel, for helpful discussions.


