Inhibition of Mushroom Tyrosinase by Kojic Acid Octanoates

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Octanoic acid 2-hydroxymethyl-4-oxo-4*H*-pyran-5-yl ester (kojic acid 5-*O*-capryloate, **2**), octanoic acid 4-oxo-2-(1-oxooctyloxymethyl)-4*H*-pyran-5-yl ester (kojic acid 5,7-di-*O*-dicaprylоate, **3**), and octanoic acid (5-hydroxy-4-oxo-4*H*-pyran-2-yl)-methyl ester (kojic acid 7-*O*-capryloate, **5**) were prepared from 5-hydroxy-2-hydroxymethyl-4*H*-4-pyrone (kojic acid, **1**) and caprylic acid. We also describe the synthesis of 11-aminoundecanoic acid (5-hydroxy-4-oxo-4*H*-pyran-2-yl)-methyl ester (**6**). In solution, the monoesters are non-competitive inhibitors of mushroom tyrosinase (EC 1.14.18.1) (**2**: IC_{50} = 107 μM, **5**: IC_{50} = 15 μM, **6**: IC_{50} = 20 μM; cf. **1**: IC_{50} = 45 μM, mixed type inhibition). When tyrosinase is immobilized in a polyvinylalcohol membrane, **5** is a weaker inhibitor than **1** or **2**.

Introduction

Kojic acid (**1**) is a well known naturally occurring inhibitor of tyrosinase showing a wide range of biological activities such as insecticidal and antibiotic 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 antioxidant 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 show herbicidal and plant growth regulatory activity (Veverka and Kralovicova, 1990). In our work on applications of tyrosinase in biosensors (Peter and Wollenberger, 1997; Streffer *et al.*, 1998), we became interested in the preparation of acyl derivatives of kojic acid, in particular the 7-*O*-capryloates. We report here on the regioselective synthesis of kojic acid esters and on the inhibition of mushroom tyrosinase in solution, and of a membrane-immobilized form of the enzyme.

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Results and Discussion

Synthesis of kojic acid derivatives

According to published procedures, reaction of **1** with carboxylic acid chlorides in pyridine or in aq. acetone/NaOH at room temp. affords 5-*O*-acyl derivatives (Nagai and Izumi, 1982; Veverka and Kralovicova, 1990) whereas 7-*O*-acyl derivatives are obtained by cleavage of the corresponding di-esters with $AlCl_3$ or hydroxylamine hydrochloride (for references, see Veverka and Kralovicova, 1990). Fusion of **1** with free fatty acids in the presence of $ZnCl_2$ 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 CH_2Cl_2/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%

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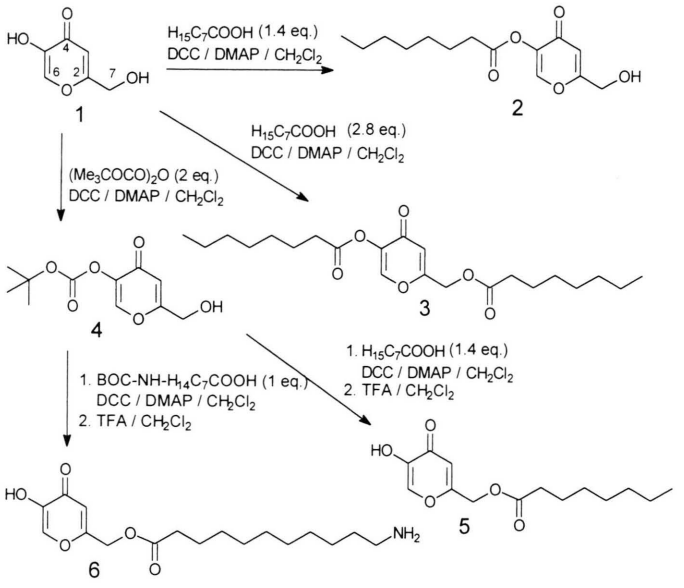

yield by reaction of **1** with a 2.8-fold molar excess of caprylic acid in the presence of DCC/DMAP. The 7-*O*-acyl derivatives were identified unambiguously by ^{1}H NMR which reveals a downfield acyl shift of the oxymethylprotons in **5** by ca. 0.5 ppm as compared to **2**. Various attempts to cleave 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

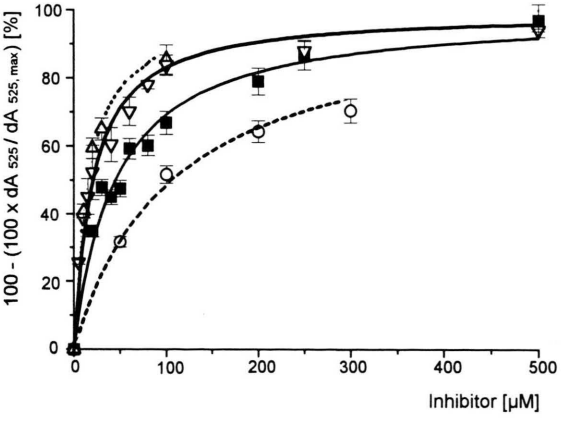

Fig. 1. Inhibition of mushroom tyrosinase (Sigma) in solution.
Inhibitors: **1**: ■, **2**: O, **5**: Δ, **6**: $\bar{V}$; the rate of the non-inhibited reaction was 0.34 µmol catechol consumed per min (i.e. $A_{525,\,max}$). Ordinate: percentage of inhibition calculated from the ratios of changes in absorbance at 525 nm per min of inhibited versus the non-inhibited reaction.

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

Compound	Enzyme in solution		Immobilized enzyme
	% inhibition at $c_{inhibitor}$ = 100 μM	IC_{50} (μM)	% inhibition at $c_{inhibitor}$ = 100 μM
1	69	45 ± 3	68
2	49	107 ± 6	55
3	nil	n.d.	nil
5	87	15 ± 1	20
6	84	20 ± 2	n.d.

are mixed-type, non-competitive inhibitors of tyrosinase.

Evaluation of Michaelis-Menten kinetics gave the IC_{50} values shown in Table I. They decrease in the order **2** (IC_{50} = 107 μM) > **1** (IC_{50} = 45 μM) > **6** (IC_{50} = 20 μM) ≈ **5** (IC_{50} = 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 by nearly two orders of magnitude than the 5-*O*-acyl derivatives (see also Kobayashi *et al.*, 1995; 1996). This is expected since the inhibition of tyrosinase by **1** is presumably due to chelation of the binuclear copper in the active site of the enzyme. It is worthwhile to note that many of the inhibitors that have been described for cosmetic applications were di-esters or 5-*O*-acyl derivatives of **1**.

In a different approach, inhibition of mushroom tyrosinase was determined by means of a biosensor. Tyrosinase was co-immobilized with glucose dehydrogenase in a polyvinylalcohol membrane at the surface of a Clark type electrode and oxygen consumption during oxidation of dihydroxybenzene was measured as described elsewhere (Makower *et al.*, 1996). This system allows to determine whether enzyme inhibition is reversible or irreversible. Monoesters **2**, **5**, and **6** were reversible inhibitors, as expected.

The di-ester **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. – ^1H and ^{13}C NMR spectra: Bruker AMX R300 at 300 or 74.5 MHz, resepctively (internal standard: TMS for ^1H and solvent signals for ^{13}C 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 CH_4. – TLC: Silica gel HF_{254} plates (E. Merck, Darmstadt). – Kojic acid (5-hydroxy-2-hydroxymethyl-4*H*-pyran-4-one), di-*tert*-butyl-dicarbonate, 11-aminoundecanoic acid, and trifluoracetic acid were from Merck-Schuchardt. Tyrosinase (EC 1.14.18.1) from mushrooms (specific activity: 75.5 units·mg^{-1})[a] was from Sigma.

*Octanoic acid 2-hydroxymethyl-4-oxo-4*H*-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 CH_2Cl_2, an ice-cold solution of 3.25 g (15 mmol) of DCC in 15 ml CH_2Cl_2 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 NH_4CO_3 solution, and dried over $MgSO_4$. 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%) **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.). – R_f (EtOAc) = 0.67. – UV (EtOAc): λ_{max} (log ε) = 259.0 (4.25), 262 nm (sh; 4.22). ^1H NMR (300 MHz, $CDCl_3$): δ = 7.87 (s, 1 H, H-6), 6.53 (s, 1 H, H-3), 4.44 (s, 2 H, H_2-7), 2.56 (t, *J* = 7.54 Hz, 2 H, α-CH_2), 1.69 (m, 2 H, -CH_2-CH_3), 1.27 (m, 8 H, CH_2), 0.86 (t, *J* = 6.57 Hz, 3 H, CH_3). – ^{13}C NMR (74.5 MHz, d_6-DMSO) (for ^{13}C NMR data of **1**, see Kingsbury *et al.*, 1976): δ = 171.6, 170.3 (C-4 and CO_2R), 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 (CH_2), 13.9

[a] One unit of tyrosinase activity is defined as consumption of 1 μmol 1,2-dihydroxy-benzene·min^{-1}, determined in the presence of L-proline by the increase of absorption at 525 nm which is caused by formation of an amino acid – quinone adduct (ε = 5400 M^{-1}·cm^{-1}) (Rzepecki and Waite, 1989).

(CH_3). – EI-MS: *m/z* (%) = 269.2 $[M+H]^+$ (4.2), 142.0 (100).

Octanoic acid 4-oxo-2-(1-oxooctyloxy-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. 58–60 °C (dec.). – R_f (EtOAc) = 0.94. – UV (EtOAc): λ_{max} (log ε) = 260 (3.30), 264 nm (sh, 3.31). – IR (KBr): ν = 3260, 3064, 2928, 2854, 1768, 1740, 1668, 1638, 1382, 1426, 1466, 1190 cm^{-1}. – ^1H NMR (300 MHz, $CDCl_3$): δ = 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, α-CH_2), 2.41 (t, *J* = 7.33 Hz, 2 H, α-CH_2), 1.57 (m, 4 H, 2 CH_2-CH_3), 1.26 (m, 16 H, CH_2), 0.85 (m, 6 H, 2 CH_3). – ^{13}C NMR (74.5 MHz, d_6-DMSO): δ = 172.3, 171.6, 170.2 (C-4 and 2 CO_2R), 163.1 (C-6), 149.3 (C-3), 140.5 (C-2), 114.6 (C-5), 60.8 (C-7), 33.0, 32.8, 31.1, 28.3, 28.2, 24.3, 24.6, 22.0 (CH_2), 13.9 (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_3N and a solution of 4 ml (28 mmol) of di-*tert*-butyl-dicarbonate in 5 ml 1,2-dimethoxyethane was added dropwise while the pH was maintained at ca. 9 by adding Et_3N. The reaction mixture was stirred for 4 h at room temp., followed by extraction with 3 x 50 ml EtOAc. The combined extracts were dried over $MgSO_4$, concentrated and the residue was subjected to flash chromatography (silica gel, petroleum ether / EtOAc 2:1). Evaporation of appropriate combined fractions afforded 257 mg (75%) of **3** as a light yellow oil which slowly crystallized. – m.p. 96–98 °C. – R_f (EtOAc) = 0.7. – UV (EtOAc): λ_{max} (log ε) = 267 nm (2.46). – IR (KBr): ν = 3446, 3076, 2990, 2936, 1760, 1654, 1626, 1372, 1430, 1456, 1154 cm^{-1}. – ^1H NMR (300 MHz, $CDCl_3$): δ = 7.93 (s, 1 H, H-6), 6.54 (s, 1 H, H-3), 4.45 (s, 2 H, H_2-7), 1.52 (s, 9 H, $C(CH_3)_3$). – ^{13}C NMR (74.5 MHz, $CDCl_3$): δ = 181.8, 167.9 (C-4 and OCO_2), 163.2 (C-6), 147.6 (C-3), 135.5 (C-2), 113.3 (C-5), 84.9 ($C(CH_3)_3$), 60.7 (C-7), 27.5 ($C(CH_3)_3$). – CI-MS: *m/z* (%) = 243.1 $[M+H]^+$ (29), 187.1 (100).

Octanoic acid (5-hydroxy-4-oxo-4H-pyran-2-yl)-methyl ester (**5**): Reaction of **4** (2.15 g; 7.6 mmol) with caprylic acid and DCC / DMAP was carried out as described above for the synthesis of **2**. Yield: 2.02 g (72%) octanoic acid (5-*O*-*tert*-butoxycarbonyloxy-4-oxo-4*H*-pyran-2-yl)-methyl ester. – m.p. 56–60 °C. – R_f (EtOAc) = 0.88. – UV (EtOAc): λ_{max} (log ε) = 255 nm (3.20). – IR (KBr): ν = 3286, 3062, 2930, 2854, 1710, 1632, 1546, 1540, 1340, 1144 cm^{-1}. – ^1H NMR (300 MHz, $CDCl_3$): δ = 7.94 (s, 1 H, H-6), 6.51 (s, 1 H, H-3), 4.90 (s, 2 H, H_2-7), 2.36 (t, *J* = 7.50 Hz, 2 H, α-CH_2), 1.59–1.66 (m, 2 H, CH_2-CH_3), 1.52 (s, 9 H, $C(CH_3)_3$), 1.27 (m, 8 H, CH_2), 0.85 (t, *J* = 6.77 Hz, 3 H, CH_3). – ^{13}C NMR (74.5 MHz, $CDCl_3$): δ = 187.3, 181.8, 172.6 (OCO_2, CO_2R, and C-4), 154.1 (C-6), 147.7 (C-3), 115.1 (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_2), 14.0 (CH_3). – CI-MS: *m/z* (%) = 369.2 $[M+H]^+$ (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_2Cl_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_3N in CH_2Cl_2. It was then washed with H_2O, the hypophase was dried over $MgSO_4$ and filtered. The solvent was evaporated and the residue was dissolved in EtOAc. Prep. TLC (silica, EtOAc) afforded 221 mg (60%) of **5** as a white waxy solid, m.p. 96–98 °C. – R_f (EtOAc) = 0.71. – UV (EtOAc): λ_{max} (log ε) = 255 (3.54), 269 nm (sh, 3.68). – IR (KBr): ν = 3244, 3050, 2924, 2852, 1734, 1654, 1626, 1390, 1452, 1472, 1418, 1170 cm^{-1}. – ^1H NMR (300 MHz, $CDCl_3$): δ = 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, α-CH_2), 1.65 (m, 2 H, CH_2-CH_3), 1.29 (m, 8 H, CH_2), 0.86 (t, *J* = 6.57 Hz, 3 H, CH_3). – ^{13}C NMR (74.5 MHz, $CDCl_3$): δ = 172.7 (C-4), 163.2 (C-6), 145.9 (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_2), 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_2Cl_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. Dicyclohexyl 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 CH_2Cl_2 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 $NaHCO_3$. The hypophase was separated, dried over $MgSO_4$ 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. – R_f (EtOAc / petroleum ether 2:1) = 0.64. – UV (EtOAc): λ_{max} (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, $CDCl_3$): δ = 7.84 (s, 1 H, H-6), 6.47 (s, 1 H, H-3), 4.92 (s, 2 H, H_2-7), 3.34 (dt, *J* = 6.81 and 6.54 Hz, 2 H, CH_2-NH_2), 2.40 (t, *J* = 7.37 Hz, 2 H, α-CH_2), 1.93–1.90 (m, 2 H, β-CH_2), 1.77–1.59 (m, 4 H, γ- and δ-CH_2), 1.28 (m, 8 H, CH_2), 1.15 (m, 2 H, $CH_2CH_2NH_2$). – ^{13}C NMR (74.5 MHz, $CDCl_3$): δ = 174.0, 172.7 (CO_2R 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 (CH_2). – 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 M NaCl. They were stored in the dark at 0 °C. The following reagents were pipetted into cuvettes: 30 µl of a 1 M 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 and immediately mixing of the contents of the cuvette. The change in absorption 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

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