



Hydroxy- or Methoxy-Substituted Benzaldoximes and Benzaldehyde-*O*-alkyloximes as Tyrosinase Inhibitors

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Abstract—Several benzaldoximes, benzaldehyde-*O*-ethyloximes, and acetophenonoximes were synthesized and evaluated as tyrosinase inhibitors by an assay based on tyrosinase catalyzed L-DOPA oxidation. Whereas benzaldoxime itself is only a weak inhibitor, its derivatives with one or two hydroxy or methoxy moieties in *para* and *meta* positions depress tyrosinase activity. Acetophenonoximes and trisubstituted benzaldoximes show no inhibitory activity. The IC_{50} of 3,4-dihydroxybenzaldehyde-*O*-ethyloxime ($0.3 \pm 0.1 \mu\text{mol L}^{-1}$) is of the same magnitude as tropolone ($0.13 \pm 0.08 \mu\text{mol L}^{-1}$), one of the best tyrosinase inhibitors known so far. © 2001 Elsevier Science Ltd. All rights reserved.

Introduction

The two copper atoms containing tyrosinase is widely distributed in plants, fungi, and animals.¹ Tyrosinase shows two enzymatic activities, *ortho*-monophenoloxidase and polyphenoloxidase activity and accepts many phenols and catechols as substrates. Tyrosinase causes the enzymatic browning of food² and is the key enzyme for melanogenesis in mammals.³

In particular, the unfavourable darkening of freshly cut fruits or vegetables like apples or potatoes is a severe problem for further processing, for example juice manufacturing. The enzymatic oxidation of polyphenolic compounds (e.g., caffeic acid or its esters such as chlorogenic acid) to the dark coloured so-called melanoids is mainly catalyzed by tyrosinases.

In animals L-tyrosine is converted to the red-brown dopaquinone via L-3,4-dihydroxyphenylalanine (L-DOPA) by tyrosinase-catalyzed oxidation; dopaquinone is further oxidized to yield the mostly brown to black coloured polymeric melanins which are responsible for the different colours of skin and hair of mammals.^{3,4} If in the human skin the melanocytes are not distributed evenly, unfavourable pigmentation spots form which

are either lighter or darker than the surrounding areas. In addition, many people with dark skin want to lighten their skin colour for cosmetic reasons.

Both browning of fruits and skin darkening can be suppressed, at least partially, by deactivation of tyrosinase. The activity of the enzyme in freshly cut fruits can be suppressed by heating, by acidification, by reductants like ascorbic acid or sulfite,² or by tyrosinase inhibitors, for example kojic acid,⁵ kojic acid octanoates,⁶ oxalic acid,⁷ salicylhydroxamic acid,^{8,9} *N*-acetylcysteine¹⁰ or gallic acid esters.¹¹ For the deactivation of the tyrosinase of human melanocytes, several inhibitors like kojic acid and kojic acid derivatives were described and some like hydroquinone or arbutin are used.⁴

The best known tyrosinase inhibitor so far is tropolone (**1**, cf. Scheme 1) showing an IC_{50} of $0.4 \mu\text{mol L}^{-1}$.¹² For kojic acid (**2**, cf. Scheme 1) an IC_{50} of $23 \mu\text{mol L}^{-1}$ was reported.¹³ It is assumed that these inhibitors complex the two copper atoms which are presented in the active site of the enzyme.⁵ Kubo et al. have described some benzaldehydes as weak to moderate tyrosinase inhibitors.¹⁴

In our efforts to develop new, low-cost, easy-to-prepare and highly active tyrosinase inhibitors for skin lightening or antibrowning preparations, we investigated some benzaldoximes because they may be able to complex the two copper atoms in the active site of tyrosinase.

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^bNot listed in Beilstein handbook or in Chemical Abstracts.

The parent compound benzaldoxime (**3**) shows a very weak inhibition activity against tyrosinase, but when a hydroxy or methoxy moiety is introduced the activity sharply increased. One could expect that the salicylaldoxime (**4**) inhibits tyrosinase better than 4-hydroxybenzaldoxime (**14**) because of the higher complexation capability. Surprisingly, **14** shows double inhibition potential compared to **4**. Furthermore, the 2,3-dihydroxybenzaldoxime (**5**) is completely inactive and 3,4-dihydroxybenzaldoxime (**6**) shows a very potent IC_{50} of 18 μM . The most active compound tested so far is the 3,4-dihydroxybenzaldehyde-*O*-ethyloxime (**17**), showing an IC_{50} in the nanomolar range (300 nmol L^{-1}) comparable to tropolone (**1**).¹² 2-Hydroxy moieties are not necessary for good inhibition, but 4-hydroxy or methoxy are. All acetophenone oxime derivatives are completely inactive irrespective of the substitution pattern of the phenyl ring and the oxime group. These data show that, in fact, an inhibition occurs because the reduction potentials of benzaldoximes and ketoximes are very similar. With exception of the moderate inhibitors 3,4,5-trihydroxybenzaldoxime (**8**) and 3,4,5-trihydroxybenzaldehyde-*O*-ethyloxime (**20**) trisubstituted oximes show no reduction of tyrosinase activity. It may be possible that the third substituent hinders the correct docking of the inhibitor to the active site of tyrosinase, which is optimized for 4-hydroxy- or 3,4-dihydroxyphenyl moieties. Some of the oxime *O*-ethylethers are more potent compared to their free counterparts, but not all.

To exclude artefacts, the tyrosinase inhibiting activities of 3,4-dihydroxybenzonitrile (**31**, cf. Scheme 1) and 4-hydroxy-3-methoxybenzonitrile (**32**) were tested, which are oxidation products of the corresponding oximes. 4-Hydroxy-3-methoxy-benzonitrile (**32**) shows no activity and 3,4-dihydroxybenzonitrile (**31**) yields an IC_{50} of 45 $\mu mol L^{-1}$, 2.5 times less active than the corresponding oxime **6**.

Some benzaldehydes like 2-hydroxy-4-methoxybenzaldehyde have been reported to show an IC_{50} of about 30 $\mu mol L^{-1}$.¹⁴ Some other hydroxybenzaldehydes were shown to be much worse inhibitors; for example vanillin shows an IC_{50} of about 70,000 $\mu mol L^{-1}$. In Table 2, the IC_{50} of aldehydes **33–36** are shown (for structures, cf. Scheme 1). They are either not or only very weak inhibitors, especially when they are compared with their corresponding oximes.

From these facts, we conclude that the inhibition activity of the oximes is caused by the compounds themselves and not by their degradation products.

Only oxime *O*-ethylethers **20** and **22** show some substrate activity, as can be seen by the increase in absorbance at 490 nm for the mixture of tyrosinase and the test compound without L-DOPA but, in combination with L-DOPA, oxime **22** shows inhibitor activity with an IC_{50} of 14 $\mu mol L^{-1}$.

Because of the high specificity of inhibitor activity, we conclude that the oximes act not as pure chemical

Table 2. Results of tyrosinase assay with the substrate L-DOPA in buffer pH 6.8 for tropolone (**1**) and kojic acid (**2**), the oximes **3–30**, the benzonitriles **31–32**, and the benzaldehydes **33–36**^a

	IC_{50} ($\mu mol L^{-1}$)		IC_{50} ($\mu mol L^{-1}$)
1	0.13 ± 0.08	19	18 ± 3
2	22 ± 5	20	380 ± 70
3	2200 ± 770	21	4.2 ± 1.5
4	64 ± 11	22	14 ± 4
5	> 4000	23	> 4000
6	18 ± 5	24	43 ± 16
7	4.6 ± 0.8	25	> 4000
8	20.2 ± 6.6	26	124 ± 18
9	2.3 ± 0.7	27	500 ± 240
10	3.5 ± 0.4	28	> 4000
11	> 4000	29	> 4000
12	> 4000	30	> 4000
13	> 4000	31	45 ± 14
14	25 ± 3	32	> 4000
15	56 ± 9	33	> 4000
16	> 4000	34	620 ± 70
17	0.3 ± 0.1	35	> 4000
18	3 ± 3	36	> 4000

^aEach IC_{50} was calculated from time/absorption plot for six different concentrations of test compounds 3 min after addition of L-DOPA ($T = 37^\circ C$, pre-incubation time 10 min).

reducing agents but as real inhibitors. In addition to this specificity, the time plots suggest that the aromatic ring is bound to the active site or to the cofactor binding site³ of tyrosinase and the enzyme is blocked.

Therefore, we have performed a kinetic experiment on vanillinoxime (**9**). For different inhibitor concentrations (0, 1.5, 3 $\mu mol L^{-1}$) the L-DOPA concentration was varied. The plot of initial velocity against substrate concentration shows decreasing V_{max} values for increasing inhibitor concentration. In addition, the Lineweaver–Burk plot shown in Figure 1 suggests that vanillinoxime (**9**) inhibits tyrosinase by non-competitive type of kinetics. As control the apparent K_m value for the L-DOPA/mushroom tyrosinase system was calculated (0.39 mmol L^{-1}), which is similar to that determined by Son et al.⁷ (0.64 mmol L^{-1}) for a catechol/mushroom tyrosinase system. Considering these data, we propose that vanillinoxime (**9**) does not block the active site itself but binds to an other essential domain of tyrosinase.

Conclusions

In conclusion, mono- or disubstituted benzaldoximes and benzaldehyde-*O*-ethyloximes containing a 4-hydroxy- or methoxy-moiety show potent tyrosinase inhibition activities. They directly interact with the enzyme and do not simply reduce the oxidation products of L-DOPA as postulated for ascorbic acid and hydroquinone. Vanillinoxime shows noncompetitive inhibition kinetics. The benzaldoximes are easy to prepare and to purify from simple chemicals. Further studies regarding in vitro assays on melanocytes and in vivo tests are required for the evaluation as skin lightening agents, and will be performed in the near future.

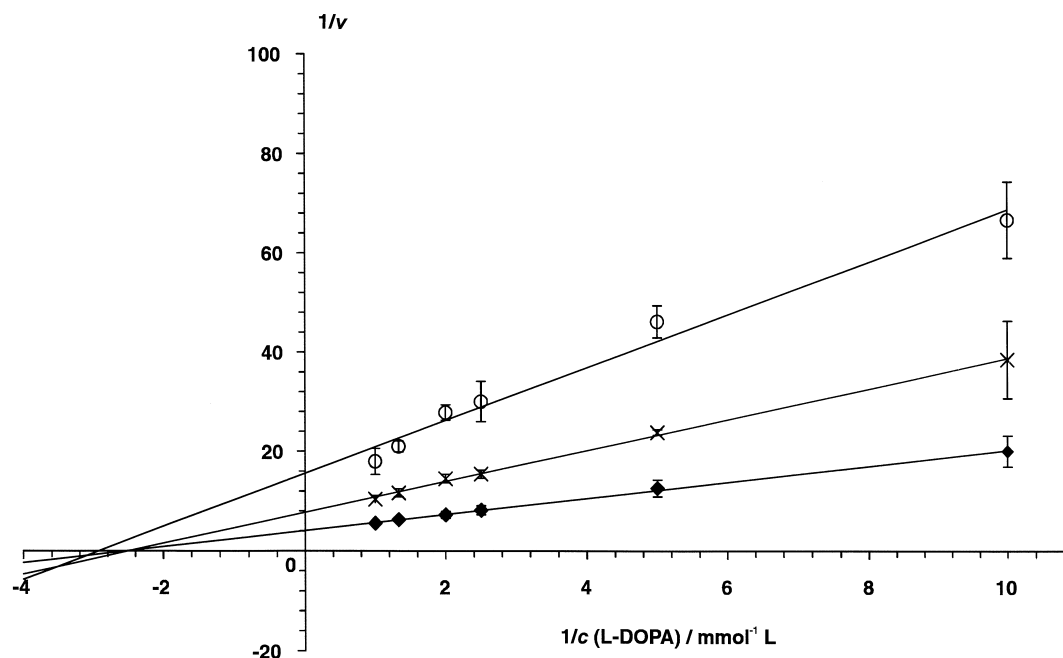


Figure 1. Lineweaver–Burk plot for determination of inhibition kinetics of vanillinoxime (**9**) on L-DOPA/tyrosinase model system. $v = 1/v$: ($\text{Au}^{-1} \text{ min}$), $1/c(\text{DOPA})$: ($\text{mmol}^{-1} \text{ L}$); $c(\text{vanillinoxime}) = (\blacklozenge) 0 \mu\text{mol L}^{-1}$, $(\times) 1.5 \mu\text{mol L}^{-1}$, $(\circ) 3.0 \mu\text{mol L}^{-1}$. The apparent K_m value for the DOPA/tyrosinase system was calculated as 0.39 mmol L^{-1} . Determination was performed in triplicate.

Experimental

All chemicals were obtained from Sigma-Aldrich (Deisenhofen, Germany) or Lancaster Synthesis (Mülheim, Germany). Tyrosinase (from mushrooms) was delivered by Sigma and the monophenoloxidase activity was determined using the usual method described in the catalogue. NMR spectra were recorded using Varian VXR400S (^1H : 400 MHz) or Gemini 2000 (^1H : 200 MHz) spectrometers (Varian, Darmstadt, Germany) at 25°C using tetramethylsilane as internal standard. LC-MS spectra were recorded using the LCQ-HPLC system Finnigan MAT HP1100 (Finnigan MAT, Egelsbach, Germany). Abbreviations used for MS: APCI atmospheric pressure chemical ionisation, ESI electro spray ionisation, EI electron impact.

The multiwell plates (96 well) used were of polystyrene and were read out using a Ceres UV 900 C photometer (Bio-Tek Inc., USA).

Synthesis of oximes

The appropriate benzaldehyde or acetophenone (87 mmol) was dissolved in water (45 mL) at 40°C . A solution of the corresponding hydroxylamine hydrochloride (90 mmol) and sodium acetate (87 mmol) in water (25 mL) was added, and the reaction mixture was stirred at about 80°C under nitrogen for 2 h. The mixture was cooled and extracted with *tert*-butyl methyl ether (200 mL), the organic phase was washed with saturated NaCl solution, dried over Na_2SO_4 , filtered, and the filtrate was evaporated to dryness in vacuo. If necessary, the residue was recrystallized.

2,3-Dihydroxybenzaldoxime (5). Yield 44% (purity $>98\%$, GC); MS (EI): m/z 153 (M^+ , 100%), 136

(17%), 135 (54%), 108 (19%), 107 (44%), 80 (25%), 79 (35%), 53 (21%), 52 (41%).

3,4-Dihydroxybenzaldoxime (6). Yield 88% (purity $>97\%$, GC; $>98\%$, HPLC); mp 143°C (dec.); ^1H NMR (200 MHz, CD_3COCD_3): δ 9.93 (1H, bs, OH), 8.15 (1H, bs, OH), 7.95 (1H, s, H^{CO}), 7.16 (1H, d, $J = 1.9 \text{ Hz}$, H^2), 6.92 (1H, dd, $J = 8.2, 2.0 \text{ Hz}$, H^6), 6.81 (1H, d, $J = 8.3 \text{ Hz}$, H^5), 2.88 (1H, bs, OH) ppm; ^{13}C NMR (50 MHz, CD_3COCD_3): 149.2 (CH, C^{CO}), 147.5 (C, C^3 or C^4), 146.1 (C, C^4 or C^3), 126.3 (C, C^1), 120.6 (CH, C^6), 115.9 (CH, C^2 or C^5), 113.4 (CH, C^5 or C^2) ppm; MS (ESI $^-$): m/z 305.0 ($[\text{2M}-\text{H}]^-$, 100%), 152.2 ($[\text{M}-\text{H}]^-$, 80%).

3-Hydroxy-4-methoxybenzaldoxime (7). Yield 69%; (purity 92%, GC) mp 142.4°C ; MS (EI): m/z 167 (M^+ , 100%), 152 (31%), 134 (14%), 125 (14%), 124 (52%), 109 (15%), 106 (16%), 79 (20%), 52 (19%), 51 (21%).

3,4,5-Trihydroxybenzaldoxime (8). Yield 86% (purity $>98\%$, GC); mp 177°C (dec.); ^1H NMR (200 MHz, $(\text{CD}_3)_2\text{CO}$): δ 7.89 (1H, s, H^{CO}), 6.70 (2H, s, H^2 and H^6) ppm; ^{13}C NMR (50 MHz, $(\text{CD}_3)_2\text{CO}$): δ 149.5 (CH, C^{CO}), 146.6 (2 C, C^3 and C^5), 135.4 (C, C^4), 125.4 (C, C^1), 106.8 (2 CH, C^2 and C^6) ppm; MS (APCI $^+$): m/z 170.0 ($[\text{M}+\text{H}]^+$, 100%), 154.3 (11%).

4-Hydroxy-3-methoxybenzaldoxime (9). Yield 91% (purity 94%, GC, 98%, HPLC); mp 118.2°C ; ^1H NMR (400 MHz, $(\text{CD}_3)_2\text{SO}$): δ 7.99 (1H, s, H^{CO}), 7.16 (1H, d, $J = 2 \text{ Hz}$, H^2), 6.97 (1H, dd, $J = 8.1, 2 \text{ Hz}$, H^5), 6.78 (1H, d, $J = 8.1 \text{ Hz}$, H^6), 3.78 (3H, s, H^{ArOMe}) ppm; ^{13}C NMR (100 MHz, $(\text{CD}_3)_2\text{SO}$): δ 148.0 (C, C^4 or C^3), 147.96 (CH, C^{CO}), 147.8 (CH, C^3 or C^4), 124.32 (C, C^1), 120.4 (CH, C^6), 115.4 (CH, C^5), 109.2 (CH, C^2), 55.4 (CH_3 ,

C^{ArOMe}) ppm; MS (EI): m/z 167 (M^+ , 100%), 152 (13%), 134 (22%), 125 (21%), 124 (61%), 109 (18%), 106 (19%), 79 (15%), 52 (15%), 51 (16%).

3-Ethoxy-4-hydroxybenzaloxime (10). Yield 24% (recryst., purity 90%, GC); mp 189 °C (dec.); MS (EI): m/z 181 (M^+ , 90%), 153 (28%), 152 (17%), 136 (26%), 135 (38%), 126 (21%), 110 (100%), 52 (19%), 51 (18%), 29 (16%).

3,5-Dimethoxy-4-hydroxybenzaloxime (11). Yield 89% (purity 97%, GC); 1H NMR (200 MHz, CD₃COCD₃): δ 8.02 (1H, s, H^{CO}), 6.93 (1H, s, H² and H⁶), 3.84 (6H, s, H^{ArOMe}), 2.97 (bs, OH) ppm; ^{13}C NMR (50 MHz, CD₃COCD₃): δ 149.3 (CH, C^{CO}), 148.6 (2C, C³ and C⁵), 138.5 (C, C⁴), 124.6 (C, C¹), 104.8 (2CH, C² and C⁶), 56.4 (2CH₃, C^{ArOMe}) ppm; MS (EI): m/z 197 (M^+ , 100%), 155 (15%), 154 (57%), 139 (12%), 67 (12%), 66 (11%), 65 (10%), 53 (14%), 39 (11%).

3,5-Dimethyl-4-hydroxybenzaloxime (12). Yield 48% (recryst., purity 97%, GC); MS (EI): m/z 165 (M^+ , 100%), 149 (24%), 148 (38%), 147 (36%), 132 (35%), 122 (85%), 107 (28%), 91 (55%), 77 (43%), 39 (24%).

3,5-Di-*tert*-butyl-4-hydroxybenzaloxime (13). Yield 94% (purity 98%, GC); 1H NMR (200 MHz, CDCl₃): δ 8.07 (1H, s, H^{CO}), 7.40 (2H, s, H² and H⁶), 7.0 (1H, bs, OH), 5.44 (1H, s, OH), 1.45 (18H, s, H^{tBu,2}) ppm; ^{13}C NMR (50 MHz, CD₃COCD₃): δ 159.3 (C, C⁴), 155.6 (C), 151.1 (CH, C^{CO}), 136.3 (2C, C³ and C⁵), 124.1 (2CH, C² and C⁶), 34.3 (2C, C^{tBu,1}), 30.2 (6CH₃, C^{tBu,2}) ppm. MS (EI): m/z 249 (M^+ , 33%), 235 (15%), 234 (100%), 231 (16%), 218 (50%), 216 (84%), 188 (30%), 115 (15%), 57 (25%), 41 (26%).

4-Hydroxybenzaloxime (14). Yield 88% (purity 97%, GC); mp 86.3 °C; MS (EI): m/z 137 (M^+ , 100%), 120 (14%), 119 (16%), 94 (71%), 93 (21%), 65 (39%), 64 (12%), 63 (15%), 53 (10%), 39 (21%).

4-Methoxybenzaloxime (15). Yield 78% (purity 98%, GC); MS (EI): m/z 151 (M^+ , 100%), 135 (19%), 134 (33%), 133 (34%), 108 (67%), 92 (24%), 90 (22%), 77 (36%), 64 (23%), 63 (26%).

2,3-Dihydroxy-benzaldehyde-*O*-ethyloxime (16). Yield 96% (purity >98%, GC); 1H NMR (200 MHz, CD₃OD): δ 8.26 (1H, s, H^{CO}), 6.94–6.73 (3H, m, H⁴, H⁵ and H⁶), 4.23 (2H, q, $J=7$ Hz, H^{NOEt,1}), 1.33 (3H, t, $J=7$ Hz, H^{NOEt,2}) ppm; ^{13}C NMR (50 MHz, CD₃OD): δ 151.7 (CH, C^{CO}), 146.4 (C, C³), 146.2 (C, C²), 121.9 (CH, C⁴ or C⁵ or C⁶), 120.7 (CH, C⁴ or C⁵ or C⁶), 118.3 (C, C¹), 118.0 (CH, C⁴ or C⁵ or C⁶), 71.1 (CH₂, C^{NOEt,1}), 14.7 (CH₃, C^{NOEt,2}) ppm; MS (EI): m/z 181 (M^+ , 89%), 136 (22%), 135 (100%), 108 (26.6%), 107 (61%), 80 (24%), 79 (33%), 53 (18%), 52 (28%), 29 (16%). HR-MS m/z (M^+): calcd for C₉H₁₁NO₃: 181.0739. Found 181.0827.

3,4-Dihydroxy-benzaldehyde-*O*-ethyloxime (17). Oily residue, which crystallized in a refrigerator and was not purified furthermore; quant. yield (purity 99.8%, GC);

mp < 23 °C; 1H NMR (400 MHz, CD₃OD): δ 7.91 (1H, s, H^{CO}), 7.08 (1H, d, $J=2$ Hz, H²), 6.66 (1H, ddd, $J=8.2, 2, 0.5$ Hz, H⁶), 6.75 (1H, d, $J=8.2$ Hz, H⁵), 4.12 (2H, q, $J=7$ Hz, H^{NOEt,1}), 1.27 (3H, t, $J=7$ Hz, H^{NOEt,2}) ppm; ^{13}C NMR (100 MHz, CD₃OD): δ 149.9 (CH, C^{CO}), 148.8 (C, C³ or C⁴), 146.8 (C, C⁴ or C³), 125.7 (C, C¹), 121.3 (CH, C⁶), 116.3 (CH, C⁵ or C²), 113.9 (CH, C² or C⁵), 70.2 (CH₂, C^{NOEt,1}), 15.0 (CH₃, C^{NOEt,2}) ppm; MS (EI): m/z 181 (M^+ , 100%), 153 (20%), 152 (19%), 136 (28%), 126 (26%), 110 (47%), 109 (30%), 81 (18%), 53 (14%), 29 (16%). HR-MS m/z (M^+): Calcd. for C₉H₁₁NO₃: 181.0739. Found 181.0747.

3,4-Dihydroxybenzaldehyde-*O*-(4-methylbenzyl)-oxime (18). Yield 8% (purity 98%, GC); mp 85–86 °C; 1H NMR (200 MHz, CD₃OD): δ 7.95 (1H, s, H^{CO}), 7.26 (2H, BB', H^{Ar}), 7.14 (2H, AA', H^{Ar}), 7.08 (1H, d, $J=2$ Hz, H²), 6.86 (1H, dd, $J=8.5$ Hz, 2 Hz, H⁶), 6.74 (1H, d, $J=8.5$ Hz, H⁵), 5.05 (2H, s, H^{Ar'-CH}), 2.34 (3H, s, H^{Ar'-Me}) ppm; ^{13}C NMR (50 MHz, CD₃OD): δ 150.3 (CH, C^{CO}), 148.7 (C, C³ or C⁴), 146.6 (C, C³ or C⁴), 138.5 (C, C¹), 136.2 (C, C⁴), 129.8 (2 CH, C² or C³), 129.4 (2 CH, C³ or C²), 125.4 (C, C¹), 121.4 (CH, C⁶), 116.1 (CH, C⁵), 113.8 (CH, C²), 76.8 (CH₂, C^{Ar'-CH}), 21.2 (CH₃, C^{Ar'-Me}) ppm; MS (APCI-): m/z 256.3 ([$M-H$]⁻, 100%). HR-MS m/z (M^+): calcd for C₁₅H₁₅NO₃: 257.1052. Found 257.1059.

3-Hydroxy-4-methoxybenzaldehyde-*O*-ethyloxime (19). Yield 98% (purity >97%, GC); mp 61.4 °C; 1H NMR (200 MHz, CD₃OD): δ 7.93 (1H, s, H^{CO}), 7.10 (1H, d, $J=2$ Hz, H²), 6.97 (1H, dd, $J=8, 2$ Hz, H⁶), 6.90 (1H, d, $J=8$ Hz, H⁵), 4.12 (2H, q, $J=7$ Hz, H^{NOEt,1}), 3.87 (3H, s, H^{ArOMe}), 1.27 (3H, t, $J=7$ Hz, H^{NOEt,2}) ppm; ^{13}C NMR (50 MHz, CD₃OD): δ 150.6 (C, C⁴), 149.3 (CH, C^{CO}), 147.7 (C, C³), 126.8 (C, C¹), 121.0 (CH, C⁶), 113.4 (CH, C²), 112.2 (CH, C⁵), 70.2 (CH₂, C^{NOEt,1}), 56.2 (CH₃, C^{NOEt,2}), 14.9 (CH₃, C^{ArOMe}) ppm; MS (EI): m/z 195 (M^+ , 100%), 140 (20%), 124 (36%), 123 (27%), 106 (21%), 79 (22%), 65 (22%), 52 (25%), 51 (22%), 29 (27%). HR-MS m/z (M^+): calcd for C₁₀H₁₃NO₃: 195.0895. Found 195.0918.

3,4,5-Trihydroxybenzaldehyde-*O*-ethyloxime (20). Oily residue, which was not purified furthermore; quant yield (purity 98.6%, GC); 1H NMR (200 MHz, CD₃OD): δ 7.81 (1H, s, H^{CO}), 6.59 (2H, s, H² and H⁶), 4.09 (2H, q, $J=7.0$ Hz, H^{NOEt,1}), 1.26 (3H, t, $J=7$ Hz, H^{NOEt,2}) ppm; ^{13}C NMR (50 MHz, CD₃OD): δ 149.9 (CH, C^{CO}), 146.8 (2C, C³ and C⁵), 136.3 (C, C⁴), 124.5 (C, C¹), 107.2 (2CH, C² and C⁶), 70.1 (CH₂, C^{NOEt,1}), 14.9 (CH₃, C^{NOEt,2}) ppm; MS (EI): m/z 197 (M^+ , 100%), 153 (17%), 152 (29%), 142 (31%), 126 (47%), 125 (33%), 96 (18%), 79 (27%), 51 (17%), 29 (24%). HR-MS m/z (M^+): calcd for C₉H₁₁NO₄: 197.0688. Found 197.0668.

4-Hydroxy-3-methoxybenzaldehyde-*O*-ethyloxime (21). Yield 75% (purity 99%, GC); mp 31.7 °C; 1H NMR (200 MHz, CD₃OD): δ 7.98 (1H, s, H^{CO}), 7.23 (1H, d, $J=2$ Hz, H²), 6.98 (1H, dd, $J=8, 2$ Hz, H⁶), 6.78 (1H, d, $J=8$ Hz, H⁵), 4.15 (2 H, q, $J=6.8$ Hz, H^{NOEt,1}), 3.85

(3H, s, H^{ArOMe}), 1.27 (3H, t, $J=6.8$ Hz, H^{NOEt,2}) ppm; ¹³C NMR (50 MHz, CD₃OD): δ 149.7 (CH, C^{CO}), 149.5 (C, C⁴ or C³), 149.1 (C, C³ or C⁴), 125.4 (C, C¹), 122.6 (CH, C⁶), 116.0 (CH, C⁵), 109.6 (CH, C²), 70.2 (CH₂, C^{NOEt,1}), 56.2 (CH₃, C^{ArOMe}), 14.9 (CH₃, C^{NOEt,2}) ppm; MS (EI): m/z 195 (M⁺, 100%), 140 (19%), 124 (36%), 123 (27%), 106 (20%), 79 (22%), 65 (22%), 52 (24%), 51 (21%), 29 (26%). HR-MS m/z (M⁺): calcd for C₁₀H₁₃NO₃: 195.0895. Found 195.0902.

3-Ethoxy-4-hydroxybenzaldehyde-*O*-ethyloxime (22).

Oily residue, which was not purified furthermore; quant yield (purity 94%, GC); mp 43 °C; ¹H NMR (200 MHz, CD₃OD): δ 7.97 (1H, s, H^{CO}), 7.21 (1H, d, $J=2$ Hz, H²), 6.97 (1H, dd, $J=8, 2$ Hz, H⁶), 6.79 (1H, d, $J=8$ Hz, H⁵), 4.14 (2H, q, $J=6.8$ Hz, H^{OEt,1} or H^{NOEt,1}), 4.11 (2H, q, $J=6.8$ Hz, H^{NOEt,1} or H^{OEt,1}), 1.42 (3H, t, $J=6.8$ Hz, H^{OEt,2}), 1.27 (3H, t, $J=6.8$ Hz, H^{NOEt,2}) ppm; ¹³C NMR (50 MHz, CD₃OD): δ 149.8 (C, C³ or C⁴), 149.7 (CH, C^{CO}), 148.3 (C, C³ or C⁴), 125.4 (C, C¹), 122.5 (CH, C⁶), 116.1 (CH, C⁵), 111.0 (C²), 70.2 (CH₂, C^{NOEt,1}), 65.4 (CH₂, C^{OEt,1}), 15.0 (CH₃, C^{OEt,2} or C^{NOEt,2}), 14.9 (CH₃, C^{NOEt,2} or C^{OEt,2}) ppm; MS (EI): m/z 209 (M⁺, 100%), 153 (39%), 152 (25%), 136 (26%), 126 (28%), 110 (36%), 52 (23%), 51 (20%), 29 (40%), 27 (26%). HR-MS m/z (M⁺): calcd for C₁₁H₁₅NO₃: 209.1052. Found 209.1043.

3,5-Dimethoxy-4-hydroxybenzaldehyde-*O*-ethyloxime (23).

Yield 60% (purity 97%, GC); ¹H NMR (200 MHz, CDCl₃): δ 7.98 (1H, s, H^{CO}), 6.83 (2H, s, H² and H⁶), 5.67 (1H, s, OH), 4.28 (2H, q, $J=7.1$ Hz, H^{NOEt,1}), 3.93 (6H, s, H^{ArOMe}), 1.32 (3H, t, $J=7.2$ Hz, H^{NOEt,2}) ppm; MS (EI): m/z 225 (M⁺, 100%), 154 (23%), 153 (30%), 123 (15%), 122 (16%), 108 (13%), 67 (13%), 65 (13%), 29 (15%). HR-MS m/z (M⁺): calcd for C₁₁H₁₅NO₄: 225.1001. Found 225.1002.

4-Hydroxybenzaldehyde-*O*-ethyloxime (24). Yield 73% (purity 99%, GC); MS (EI): m/z 165 (M⁺, 100%), 137 (50%), 136 (73%), 120 (38%), 94 (61%), 93 (32%), 65 (50%), 29 (31%), 27 (20%).

4-Methoxybenzaldehyde-*O*-ethyloxime (25). Yield 84% (purity 99%, GC); MS (EI): m/z 179 (M⁺, 100%), 151 (57%), 150 (75%), 134 (33%), 108 (40%), 92 (30%), 91 (26%), 77 (50%), 63 (24%), 29 (28%).

4-Hydroxy-3-methylbenzaldehyde-*O*-ethyloxime (26).

Yield 69% (purity >99%, GC); ¹H NMR (200 MHz, CD₃OD): δ 7.96 (1H, s, H^{CO}), 7.34 (1H, m, H²), 7.25 (1H, dd, $J=8.5, 2$ Hz, H⁶), 6.74 (1H, d, $J=8.5$ Hz, H⁵), 4.09 (2H, q, $J=7.0$ Hz, H^{NOEt,1}), 2.17 (3H, s, H^{ArMe}), 1.26 (3H, t, $J=7.0$ Hz, H^{NOEt,1}) ppm; ¹³C NMR (50 MHz, CD₃OD): δ 158.3 (C, C⁴), 149.8 (CH, C^{CO}), 130.4 (CH, C²), 127.0 (CH, C⁶), 125.9 (C, C³), 124.8 (C, C¹), 115.5 (CH, C⁵), 70.0 (CH₂, C^{NOEt,1}), 16.2 (CH₃, C^{ArMe}), 14.9 (CH₃, C^{NOEt,2}) ppm; MS (EI): m/z 179 (M⁺, 100%), 151 (31%), 150 (56%), 134 (27%), 108 (36%), 107 (21%), 91 (15%), 77 (34%), 29 (13%). HRMS m/z (M⁺): calcd for C₁₀H₁₃NO₂: 179.0946. Found 179.0969.

3,5-Dimethyl-4-hydroxybenzaldehyde-*O*-ethyloxime (27).

Oily product, which was not further purified; quant. yield (purity 96%, GC); ¹H NMR (200 MHz, CDCl₃): δ 7.96 (1H, s, H^{CO}), 7.22 (2H, bs, H² and H⁶), 4.7–4.6 (1H, m, OH), 4.19 (2H, q, $J=7.0$ Hz, H^{NOEt,1}), 2.25 (6H, s, H^{ArMe}), 1.32 (3H, t, $J=7.2$ Hz, H^{NOEt,2}) ppm; ¹³C NMR (50 MHz, CDCl₃): δ 153.5 (C, C⁴), 148.2 (CH, C^{CO}), 127.3 (2 CH, C² and C⁶), 124.3 (C, C¹), 123.2 (2 C, C³ and C⁵), 69.4 (CH₂, C^{NOEt,1}), 15.9 (2 CH₃, C^{ArMe}), 14.6 (CH₃, C^{NOEt,1}) ppm; MS (EI): m/z 193 (M⁺, 100%), 165 (28%), 164 (45%), 150 (20%), 148 (25%), 122 (35%), 121 (19%), 91 (44%), 77 (33%), 29 (14%). HR-MS m/z (M⁺): calcd for C₁₁H₁₅NO₂: 193.1103. Found 193.1113.

3,4-Dihydroxyacetophenone oxime (28).

Yield 90% (purity 97%, GC, 98%, HPLC); ¹H NMR (200 MHz, (CD₃)₂SO): δ 10.81 (1H, bs, OH), 9.09 (1H, bs, OH), 8.98 (1H, bs, OH), 7.10 (1H, d, $J=2.2$ Hz, H²), 6.90 (1H, dd, $J=8.3$ Hz, 2.2 Hz, H⁶), 6.71 (1H, d, $J=8.3$ Hz, H⁵), 2.05 (3H, s, H^{COMe}) ppm; ¹³C NMR (50 MHz, (CD₃)₂SO): δ 152.4 (C, C^{CO}), 146.1 (C, C³ or C⁴), 144.9 (C, C⁴ or C³), 128.2 (C, C¹), 117.1 (CH, C⁶), 115.0 (CH, C⁵), 112.5 (CH, C²), 11.3 (CH₃, C^{COMe}); MS (APCI[−]): m/z 332.70 ([2M−H][−], 11%), 166.44 ([M−H][−], 100%).

3,5-Dimethoxy-4-hydroxyacetophenone oxime (29).

Yield 47% (purity > 91%, GC); ¹H NMR (200 MHz, CD₃OD): δ 6.94 (2H, s, H² and H⁶), 3.83 (6H, s, H^{ArOMe}), 2.21 (3H, s, H^{COMe}) ppm; ¹³C NMR (50 MHz, CD₃OD): δ 156.2 (C, C^{CO}), 148.9 (2C, C³ and C⁵), 137.7 (C, C⁴), 129.1 (C, C¹), 104.6 (2 CH, C² and C⁶), 56.7 (2 CH₃, C^{ArOMe}), 12.2 (CH₃, C^{COMe}) ppm; MS (EI): m/z 211 (M⁺, 100%), 194 (14%), 164 (12%), 155 (20%), 154 (41%), 153 (13%), 136 (12%), 123 (12%), 108 (12%). HRMS m/z (M⁺): calcd for C₁₀H₁₃NO₄: 211.0845. Found 211.0822.

3,4-Dihydroxyacetophenone-*O*-ethyloxime (30).

Yield 60% (purity 98%, HPLC); ¹H NMR (200 MHz, CD₃OD): δ 7.14 (1H, d, $J=2$ Hz, H²), 6.99 (1H, dd, $J=8, 2$ Hz, H⁶), 6.76 (1H, d, $J=8$ Hz, H⁵), 4.12 (2H, q, $J=7$ Hz, H^{NOEt,1}), 2.14 (3H, s, H^{COMe}), 1.28 (3H, t, $J=7$ Hz, H^{NOEt,1}) ppm; MS (ESI⁺): m/z 196.05 (100%, [M+H]⁺). HR-MS m/z (M⁺): calcd for C₁₀H₁₃NO₃: 195.0895. Found 195.0887.

Tyrosinase assay

Tyrosinase (2000 Units/mg) was dissolved in phosphate buffer (pH 6.8, c 0.067 mol L^{−1}) to a concentration of 120 U mL^{−1}, and in each case 100 μ L of this tyrosinase solution were pipetted into a cavity of a 96-well multiwell plate of clear polystyrene. Phosphate buffer pH 6.8 (25 μ L) and stepwise diluted test compound or standard (75 μ L) were added. Phosphate buffer was used to dilute the DMSO stock solution of test compound. The control used was phosphate buffer.

The resulting mixtures in the microtiter plate were incubated at 37 °C for 10 min. A solution of L-DOPA in phosphate buffer pH 6.8 (c 0.03%) (100 μ L) was added

and the absorption (A) was recorded at 495 nm for at least 10 min. The measurement was performed in triplicate for each concentration and averaged before further calculation. The residual tyrosinase activities after 3 min incubation in the presence of test compounds were calculated in accordance with the following equation:

Residual tyrosinase activity (%)

$$= (A_{\text{Test compound}}/A_{\text{Control}}) \times 100 \quad (1)$$

The IC₅₀ was calculated from the residual tyrosinase activities (%) in a series of dilutions of test compounds. This is the concentration of a test compound in which the tyrosinase is inhibited to an extent of 50%.

Inhibitor kinetics of vanillinoxime (9)

The determination of inhibitor kinetics was performed by modification of the above mentioned method: for each of three different inhibitor concentrations (0, 1.5 and 3 μmol L⁻¹) L-DOPA concentration was varied (0, 0.1, 0.2, 0.4, 0.5, 0.75 and 1 mmol L⁻¹). Pre-incubation and measurement time was the same as above. Maximal initial velocity $v = \Delta A / \Delta t$ was determined from initial linear portion of absorbance between 20 and 60 s after addition of L-DOPA. Kinetic parameters (K_m , apparent, and $V_{\max}$) were determined using double-reciprocal plots (Lineweaver–Burk) of enzyme activity against L-DOPA concentration.

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