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

Jakob P. Ley* and Heinz-Jürgen Bertram

Haarmann & Reimer GmbH, Flavor Division, Research & Development Flavors, PO Box 1253, D-37601 Holzminden, Germany

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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 ± 0.1 μmol L^{-1}) is of the same magnitude as tropolone (0.13 ± 0.08 μmol L^{-1}), one of the best tyrosinase inhibitors known so far.

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 food[2] and is the key enzyme for melanogenesis in mammals.[3]

In particular, the unfavourable darkening of freshly cutted 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 supressed 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*-acetylcysteine[10] 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 μmol L^{-1}.[12] For kojic acid (**2**, cf. Scheme 1) an IC_{50} of 23 μmol 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.

*Corresponding author. Fax: +49-5531-90-3883; e-mail: jakob.ley.jl@hr-gmbh.de (J.P. Ley).

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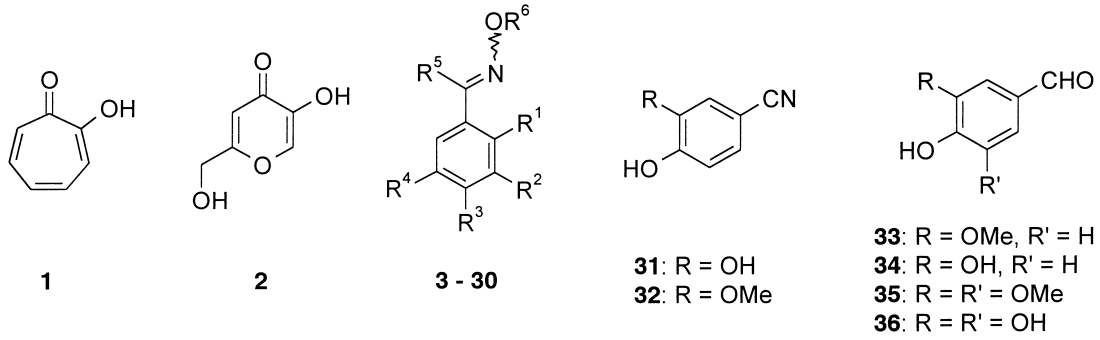
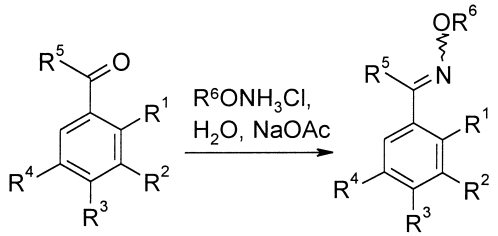


Scheme 1. Structures of standard inhibitors tropolone (**1**) and kojic acid (**2**), the oximes **3**–**30** (for definition of R^1–R^6, cf. Table 1), the benzonitriles **31**–**32** and the benzaldehydes **33**–**36**.

Results and Discussion

Synthesis

The benzaldoximes **5**–**15** and the acetophenonoximes **28**–**30** were prepared starting from unprotected benzaldehydes or acetophenones, respectively, by reaction with hydroxylamine in aqueous solution with the aid of sodium acetate in moderate to good yields (cf. Scheme 2 and Table 1). The oxime ethers **16**–**25** were synthesized in a similiar manner by reaction with *O*-substituted hydroxylamines. If necessary, the oximes were purified by simple recrystallization. Analytical data of the known compounds corresponded to the expected values. All new compounds (**16**–**23**, **25**–**27** and **29**–**30**) were analyzed by ^{1}H, ^{13}CNMR, and MS and showed satisfying high resolution MS (HR-MS) data. The chemical shifts for carbonyl hydrogens of benzaldoximes **5**–**15** are in the typical range (7.81 – 8.26 ppm) for methoxy- or hydroxysubstituted *Z*-benzaldoximes.[15]



Scheme 2. Synthesis of benzaldoximes **5**–**15**, benzaldehyde-*O*-ethyloximes **16**–**27** and acetophenone oximes **28**–**30**. For definition of R^1–R^6, cf. Table 1.

Tyrosinase experiments

For evaluating the tyrosinase inhibitory activity, a typical assay protocol with L-DOPA as substrate was chosen.[5,16] The commercial tyrosinase from mushrooms was used without further purification after determining the monophenolase activity by the standard protocol with L-tyrosine as substrate. The assay was conducted in a 96 multiwell plate using a photometer with a fixed wavelength of 495 nm. A series of dilutions of appropiate test compounds and a blank control solution were pre-incubated with tyrosinase for 10 min at 37 °C. L-DOPA was addded and the absorptions were immediately recorded for at least 10 min. For the first minutes, absorptions increased mostly linear and subsequently they showed a typical saturation behaviour. With increasing inhibitor concentration, a decreasing maximum absorption was reached which shows no approximation to the absorption of the blank control during the experiment.

The relative tyrosinase activities against blank control at 3 min for the different samples were plotted against the logarithm of sample concentration. From these plots, the IC_{50} values were calculated. The alternative calculation from the initial slopes of the time/absorption plots resulted in nearly identical inhibition data.

Several free oximes (**3**–**16**), oximeethers (**17**–**27** and **30**), trisubstituted oximes (**8**, **11**, **12**, **13**, **20**, **23**, **27** and **29**), some acetophenones (**28**–**30**) with different substitution pattern (hydroxy, methyl, methoxy) have been evaluated for structure–activity relationships (cf. Table 1).

Table 1. Structures of oximes **3**–**30**[a]

	R^1	R^2	R^3	R^4	R^5	R^6
3	H	H	H	H	H	H
4	OH	H	H	H	H	H
5	OH	OH	H	H	H	H
6	H	OH	OH	H	H	H
7	H	OH	OMe	H	H	H
8	H	OH	OH	OH	H	H
9	H	OMe	OH	H	H	H
10	H	OEt	OH	H	H	H
11	H	OMe	OH	OMe	H	H
12	H	Me	OH	Me	H	H
13	H	*t*-Bu	OH	*t*-Bu	H	H
14	H	H	OH	H	H	H
15	H	H	OMe	H	H	H
16[b]	OH	OH	H	H	H	Et
17[b]	H	OH	OH	H	H	Et
18[b]	H	OH	OH	H	H	pMeBn
19[b]	H	OH	OMe	H	H	Et
20[b]	H	OH	OH	OH	H	Et
21[b]	H	OMe	OH	H	H	Et
22[b]	H	OEt	OH	H	H	Et
23[b]	H	OMe	OH	OMe	H	Et
24	H	H	OH	H	H	Et
25	H	H	OMe	H	H	Et
26[b]	H	Me	OH	H	H	Et
27[b]	H	Me	OH	Me	H	Et
28	H	OH	OH	H	Me	H
29[b]	H	OMe	OH	OMe	Me	H
30[b]	H	OH	OH	H	Me	Et

[a]For structural formula and preparation, cf. Schemes 1 and 2.
[b]Not 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 similiar. 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 μmol 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 μmol L^{-1}.[14] Some other hydroxybenzaldehydes were shown to be much worse inhibitors; for example vanillin shows an IC_{50} of about 70,000 μmol 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 μmol L^{-1}.

Because of the high specificity of inhibitor activity, we conclude that the oximes act not as pure chemical 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 site[3] of tyrosinase and the enzyme is blocked.

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} (μmol L^{-1})		IC_{50} (μmol 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

[a]Each 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).

Therefore, we have performed a kinetic experiment on vanillinoxime (**9**). For different inhibitor concentrations (0, 1.5, 3 μmol 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 similiar 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.

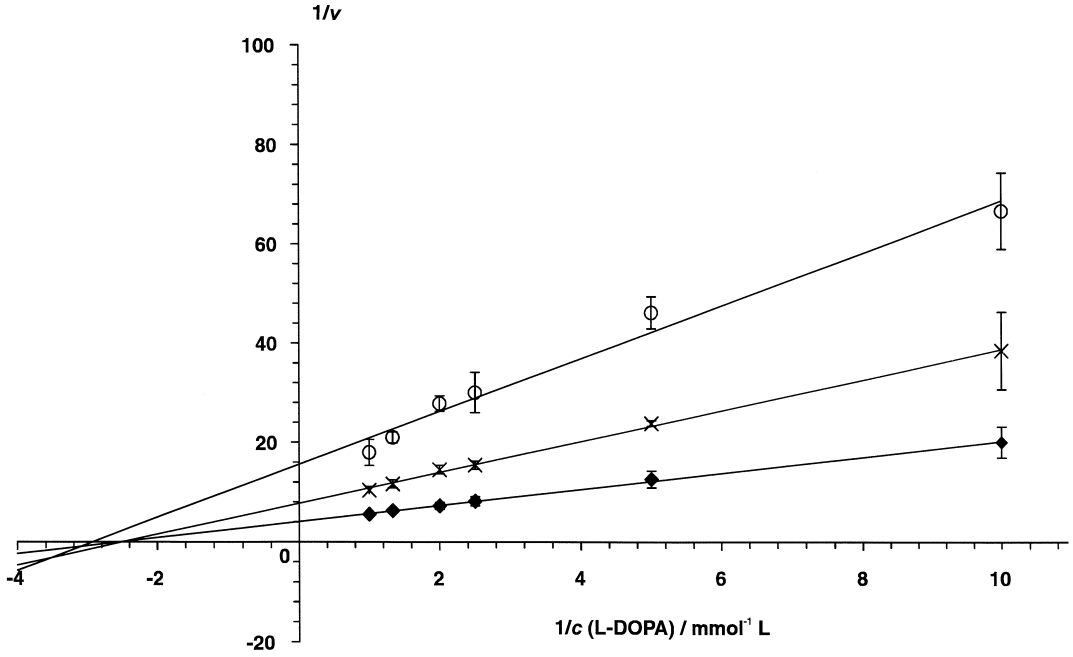


Figure 1. Lineweaver–Burk plot for determination of inhibition kinetics of vanillinoxime (**9**) on L-DOPA/tyrosinase model system. $v = 1/v$: (Au^{-1} min), 1/c(DOPA): ($mmol^{-1}$ L); c(vanillinoxime) = (◆) 0 μmol L^{-1}, (×) 1.5 μmol L^{-1}, (o) 3.0 μmol L^{-1}. The apparent K_m value for the DOPA/tyrosinase 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 (^{1}H: 400 MHz) or Gemini 2000 (^{1}H: 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.); ^{1}H 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, $J = 1.9$ Hz, H^2), 6,92 (1H, dd, $J = 8.2$, 2.0 Hz, H^6), 6.81 (1H, d, $J = 8.3$ 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 ($[2M-H]^-$, 100%), 152.2 ($[M-H]^-$, 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.); ^{1}H NMR (200 MHz, $(CD_3)_2CO$): δ 7.89 (1H, s, H^{CO}), 6.70 (2H, s, H^2 and H^6) ppm; ^{13}C NMR (50 MHz, $(CD_3)_2CO$): 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 ($[M+H]^+$, 100%), 154.3 (11%).

4-Hydroxy-3-methoxybenzaldoxime (9). Yield 91% (purity 94%, GC, 98%, HPLC); mp 118.2 °C; ^{1}H NMR (400 MHz, $(CD_3)_2SO$): δ 7.99 (1H, s, H^{CO}), 7.16 (1H, d, $J = 2$ Hz, H^2), 6.97 (1H, dd, $J = 8.1$, 2 Hz, H^5), 6.78 (1H, d, $J = 8.1$ Hz, H^6), 3.78 (3H, s, H^{ArOMe}) ppm; ^{13}C NMR (100 MHz, $(CD_3)_2SO$): 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-hydroxybenzaldoxime (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-hydroxybenzaldoxime (11). Yield 89% (purity 97%, GC); ^{1}H NMR (200 MHz, CD_3COCD_3): δ 8.02 (1H, s, H^{CO}), 6.93 (1H, s, H^2 and H^6), 3.84 (6H, s, H^{ArOMe}), 2.97 (bs, OH) ppm; ^{13}C NMR (50 MHz, CD_3COCD_3): δ 149.3 (CH, C^{CO}), 148.6 (2C, C^3 and C^5), 138.5 (C, C^4), 124.6 (C, C^1), 104.8 (2CH, C^2 and C^6), 56.4 ($2CH_3$-, 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-hydroxybenzaldoxime (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-hydroxybenzaldoxime (13). Yield 94% (purity 98%, GC); ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.07 (1H, s, H^{CO}) 7.40 (2H, s, H^2 and H^6), 7.0 (1H, bs, OH), 5.44 (1H, s, OH), 1.45 (18H, s, $H^{tBu,2}$) ppm; ^{13}C NMR (50 MHz, CD_3COCD_3): δ 159.3 (C, C^4), 155.6 (C), 151.1 (CH, C^{CO} 136.3 (2C, C^3 and C^5), 124.1 (2CH, C^2 and C^6), 34.3 (2C, $C^{tBu,1}$), 30.2 ($6CH_3$, $C^{tBu,2}$) 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-Hydroxybenzaldoxime (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-Methoxybenzaldoxime (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); ^{1}H NMR (200 MHz, CD_3OD): δ 8.26 (1H, s, H^{CO}), 6.94–6.73 (3H, m, H^4, H^5 and H^6), 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, CD_3OD): δ 151.7 (CH, C^{CO}), 146.4 (C, C^3), 146.2 (C, C^2), 121.9 (CH, C^4 or C^5 or C^6), 120.7 (CH, C^4 or C^5 or C^6), 118.3 (C, C^1), 118.0 (CH, C^4 or C^5 or C^6), 71.1 (CH_2–, $C^{NOEt,1}$), 14.7 (CH_3, $C^{NOEt,2}$) 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 $C_9H_{11}NO_3$: 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; ^{1}H NMR (400 MHz, CD_3OD): δ 7.91 (1H, s, H^{CO}), 7.08 (1H, d, *J* = 2 Hz, H^2), 6.66 (1H, ddd, *J* = 8.2, 2, 0.5 Hz, H^6), 6.75 (1H, d, *J* = 8.2 Hz, H^5), 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, CD_3OD): δ 149.9 (CH, C^{CO}), 148.8 (C, C^3 or C^4), 146.8 (C, C^4 or C^3), 125.7 (C, ^{1}), 121.3 (CH, C^6), 116.3 (CH, C^5 or C^2), 113.9 (CH, C^2 or C^5), 70.2 (CH_2, $C^{NOEt,1}$), 15.0 (CH_3, $C^{NOEt,2}$) 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 $C_9H_{11}NO_3$: 181.0739. Found 181.0747.

3,4-Dihydroxybenzaldehyde-*O*-(4-methylbenzyl)-oxime (18). Yield 8% (purity 98%, GC); mp 85–86 °C; ^{1}H NMR (200 MHz, CD_3OD): 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, H^2), 6.86 (1H, dd, *J* = 8.5 Hz, 2 Hz, H^6), 6.74 (1H, d, *J* = 8.5 Hz, H^5), 5.05 (2H, s, $H_2^{Ar'-CH}$), 2.34 (3H, s, $H^{Ar'-Me}$) ppm; ^{13}C NMR (50 MHz, CD_3OD): δ 150.3 (CH, C^{CO}), 148.7 (C, C^3 or C^4), 146.6 (C, C^3 or C^4), 138.5 (C, $C^{1'}$), 136.2 (C, $C^{4'}$), 129.8 (2 CH, $C^{2'}$ or $C^{3'}$), 129.4 (2 CH, $C^{3'}$ or $C^{2'}$), 125.4 (C, C^1), 121.4 (CH, C^6), 116.1 (CH, C^5), 113.8 (CH, C^2), 76.8 (CH_2, $C_2^{Ar'-CH}$), 21.2 (CH_3, $C^{Ar'-Me}$) ppm; MS (APCI−): *m/z* 256.3 ($[M-H]^-$, 100%). HR-MS *m/z* (M^+): calcd for $C_{15}H_{15}NO_3$: 257.1052. Found 257.1059.

3-Hydroxy-4-methoxybenzaldehyde-*O*-ethyloxime (19). Yield 98% (purity >97%, GC); mp 61.4 °C; ^{1}H NMR (200 MHz, CD_3OD): δ 7.93 (1H, s, H^{CO}), 7.10 (1H, d, *J* = 2 Hz, H^2), 6.97 (1H, dd, *J* = 8, 2 Hz, H^6), 6.90 (1H, d, *J* = 8 Hz, H^5), 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, CD_3OD): δ 150.6 (C, C^4), 149.3 (CH, C^{CO}), 147.7 (C, C^3), 126.8 (C, C^1), 121.0 (CH, C^6), 113.4 (CH, C^2), 112.2 (CH, C^5), 70.2 (CH_2, $C^{NOEt,1}$), 56.2 (CH_3, $C^{NOEt,2}$), 14.9 (CH_3, 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 $C_{10}H_{13}NO_3$: 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); ^{1}H NMR (200 MHz, CD_3OD): δ 7.81 (1H, s, H^{CO}), 6.59 (2H, s, H^2 and H^6), 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, CD_3OD): δ 149.9 (CH, C^{CO}), 146.8 (2C, C^3 and C^5), 136.3 (C, C^4), 124.5 (C, C^1), 107.2 (2CH, C^2 and C^6), 70.1 (CH_2, $C^{NOEt,1}$), 14.9 (CH_3, $C^{NOEt,2}$) 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 $C_9H_{11}NO_4$: 197.0688. Found 197.0668.

4-Hydroxy-3-methoxybenzaldehyde-*O*-ethyloxime (21). Yield 75% (purity 99%, GC); mp 31.7 °C; ^{1}H NMR (200 MHz, CD_3OD): δ 7.98 (1H, s, H^{CO}), 7.23 (1H, d, *J* = 2 Hz, H^2), 6.98 (1H, dd, *J* = 8, 2 Hz, H^6), 6.78 (1H, d, *J* = 8 Hz, H^5), 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; ^{13}C NMR (50 MHz, CD_3OD): δ 149.7 (CH, C^{CO}), 149.5 (C, C^4 or C^3), 149.1 (C, C^3 or C^4), 125.4 (C, C^1), 122.6 (CH, C^6), 116.0 (CH, C^5), 109.6 (CH, C^2), 70.2 (CH_2, $C^{NOEt,1}$), 56.2 (CH_3, C^{ArOMe}), 14.9 (CH_3, $C^{NOEt,2}$) 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 $C_{10}H_{13}NO_3$: 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, CD_3OD): δ 7.97 (1H, s, H^{CO}), 7.21 (1H, d, $J=2$ Hz, H^2), 6.97 (1H, dd, $J=8$, 2 Hz, H^6), 6.79 (1H, d, $J=8$ Hz, H^5), 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; ^{13}C NMR (50 MHz, CD_3OD): δ 149.8 (C, C^3 or C^4), 149.7 (CH, C^{CO}), 148.3 (C, C^3 or C^4), 125.4 (C, C^1), 122.5 (CH, C^6), 116.1 (CH, C^5), 111.0 (C^2), 70.2 (CH_2, $C^{NOEt,1}$), 65.4 (CH_2, $C^{OEt,1}$), 15.0 (CH3, $C^{OEt,2}$ or $C^{NOEt,2}$), 14.9 (CH_3, $C^{NOEt,2}$ 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 $C_{11}H_{15}NO_3$: 209.1052. Found 209.1043.

3,5-Dimethoxy-4-hydroxybenzaldehyde-*O*-ethyloxime (23). Yield 60% (purity 97%, GC); ^1H NMR (200 MHz, $CDCl_3$): δ 7.98 (1H, s, H^{CO}), 6.83 (2H, s, H^2 and H^6), 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 $C_{11}H_{15}NO_4$: 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, CD_3OD): δ 7.96 (1H, s, H^{CO}), 7.34 (1H, m, H^2), 7.25 (1H, dd, $J=8.5$, 2 Hz, H^6), 6.74 (1H, d, $J=8.5$ Hz, H^5), 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; ^{13}C NMR (50 MHz, CD_3OD): δ 158.3 (C, C^4), 149.8 (CH, C^{CO}), 130.4 (CH, C^2), 127.0 (CH, C^6), 125.9 (C, C^3), 124.8 (C, C^1), 115.5 (CH, C^5), 70.0 (CH_2, $C^{NOEt,1}$), 16.2 (CH_3, C^{ArMe}), 14.9 (CH_3, $C^{NOEt,2}$) 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 $C_{10}H_{13}NO_2$: 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, $CDCl_3$): δ 7.96 (1H, s, H^{CO}), 7.22 (2H, bs, H^2 and H^6), 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; ^{13}C NMR (50 MHz, $CDCl_3$): δ 153.5 (C, C^4), 148.2 (CH, C^{CO}), 127.3 (2 CH, C^2 and C^6), 124.3 (C, C^1), 123.2 (2 C, C^3 and C^5), 69.4 (CH_2, $C^{NOEt,1}$), 15.9 (2 CH_3, C^{ArMe}), 14.6 (CH_3, $C^{NOEt,1}$) 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 $C_{11}H_{15}NO_2$: 193.1103. Found 193.1113.

3,4-Dihydroxyacetophenone oxime (28). Yield 90% (purity 97%, GC, 98%, HPLC); ^1H NMR (200 MHz, $(CD_3)_2SO$): δ 10.81 (1H, bs, OH), 9.09 (1H, bs, OH), 8.98 (1H, bs, OH), 7.10 (1H, d, $J=2.2$ Hz, H^2), 6.90 (1H, dd, $J=8.3$ Hz, 2.2 Hz, H^6), 6.71 (1H, d, $J=8.3$ Hz, H^5), 2.05 (3H, s, H^{COMe}) ppm; ^{13}C NMR (50 MHz, $(CD_3)_2SO$): δ 152.4 (C, C^{CO}), 146.1 (C, C^3 or C^4), 144.9 (C, C^4 or C^3), 128.2 (C, C^1), 117.1 (CH, C^6), 115.0 (CH, C^5), 112.5 (CH, C^2), 11.3 (CH_3, 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, CD_3OD): δ 6.94 (2H, s, H^2 and H^6), 3.83 (6H, s, H^{ArOMe}), 2.21 (3H, s, H^{COMe}) ppm; ^{13}C NMR (50 MHz, CD_3OD): δ 156.2 (C, C^{CO}), 148.9 (2C, C^3 and C^5), 137.7 (C, C^4), 129.1 (C, C^1), 104.6 (2 CH, C^2 and C^6), 56.7 (2 CH_3, C^{ArOMe}), 12.2 (CH_3, 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 $C_{10}H_{13}NO_4$: 211.0845. Found 211.0822.

3,4-Dihydroxyacetophenone-*O*-ethyloxime (30). Yield 60% (purity 98%, HPLC); ^1H NMR (200 MHz, CD_3OD): δ 7.14 (1H, d, $J=2$ Hz, H^2), 6.99 (1H, dd, $J=8$, 2 Hz, H^6), 6.76 (1H, d, $J=8$ Hz, H^5), 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 $C_{10}H_{13}NO_3$: 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 μL of this tyrosinase solution were pipetted into a cavity of a 96-well multi-well plate of clear polystyrene. Phosphate buffer pH 6.8 (25 μL) and stepwise diluted test compound or standard (75 μL) 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 μL) 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:

$$\text{Residual tyrosinase activity (\%)} = \left(A_{\text{Test compound}}/A_{\text{Control}}\right) \times 100 \quad (1)$$

The IC_{50} 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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