Journal of Enzyme Inhibition and Medicinal Chemistry, June 2005; 20(3): 239–243

Taylor & Francis
Taylor & Francis Group

Inhibitory effects of 4-vinylbenzaldehyde and 4-vinylbenzoic acid on the activity of mushroom tyrosinase

KANG-KANG SONG, QING-XI CHEN, QIN WANG, LING QIU, & HUANG HUANG

The key Laboratory of Ministry of Education for Cell Biology and Tumor Cell Engineering, School of Life Sciences, Xiamen University, Xiamen 361005, P. R. China

(Received 29 August 2004; accepted 29 October 2004)

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by Malmo Hogskola on 12/24/11
For personal use only.

Abstract

Tyrosinase (EC 1.14.18.1) catalyzes both the hydroxylation of tyrosine into *o*-diphenols and the oxidation of *o*-diphenols into *o*-quinones which form brown or black pigments. Here, the inhibitory effects of 4-vinylbenzaldehyde and 4-vinylbenzoic acid on the activity of mushroom tyrosinase have been investigated. The results showed that both 4-vinylbenzaldehyde and 4-vinylbenzoic acid could inhibit both monophenolase activity and diphenolase activity of the enzyme. For the monophenolase activity, 4-vinylbenzoic acid could lengthen the lag time, but 4-vinylbenzaldehyde could not. Both 4-vinylbenzaldehyde and 4-vinylbenzoic acid decreased the steady-state activity, and the IC_{50} values were estimated as 93 μM and 3.0 mM for monophenolase activity, respectively. For the diphenolase activity, the inhibitory capacity of 4-vinylbenzaldehyde was stronger than that of 4-vinylbenzoic acid, and the IC_{50} values were estimated as 23 μM and 0.33 mM, respectively. Kinetic analyses showed that inhibition by both compounds was reversible and their mechanisms were mixed-II type; their inhibition constants were also determined and compared.

Keywords: *Tyrosinase, monophenolase activity, diphenolase activity, 4-Vinylbenzaldehyde, 4-Vinylbenzoic acid, inhibition kinetics*

Introduction

Tyrosinase (EC 1.14.18.1), a copper-containing multifunctional oxidase, widely found in fungi, plants and animals [1,2], catalyzes the hydroxylation of a monophenol and conversion of an *o*-diphenol to the corresponding *o*-quinone [3]. Tyrosinase is a key enzyme in melanin biosynthesis, involved in determining the color of mammalian skin and hair. Its abnormal expression is responsible for the various dermatological disorders, such as melasama, age spots, and sites of actinic damage. It also contributes to neuromelanin formation in the human brain and the neurodegeneration associated with Parkinson's disease [4].

Tyrosinase inhibitors are of great interest and have become increasingly important in medicinal and cosmetic products in relation to hyperpigmentation. Hence, tyrosinase inhibitors should have broad applications. It is well known that tyrosinase can be inhibited by aromatic aldehydes and aromatic acids [5], tropolone [6], flavonoid [7]and kojic acid [8]. Although the inhibitory effects of 4-substituted benzaldehyde and 4-substituted benzoic acid on tyrosinase activity have been reported [9–12], the presence of unsaturated substituents on the benzene ring has not been published. In the present investigation, 4-vinylbenzaldehyde and 4-vinylbenzoic acid were found to have inhibitory effects on mushroom tyrosinase. The aim of this present work is, therefore, to carry out a kinetic study of the inhibition of the diphenolase and monophenolase activity of tyrosinase by 4-vinylbenzaldehyde and 4-vinylbenzoic acid, to evaluate the kinetic parameters and inhibition constants characterizing the system and investigate

Correspondence: Q.-X. Chen, Tel/Fax: +86 592 2185487. E-mail: chenqx@jingxian.xmu.edu.cn

ISSN 1475-6366 print/ISSN 1475-6374 online © 2005 Taylor & Francis Group Ltd
DOI: 10.1080/14756360400026238

RIGHTSLINK

the inhibition mechanism. In addition, these data may provide the basis for developing novel tyrosinase inhibitors.

Materials and methods

4-Vinylbenzaldehyde and 4-vinylbenzoic acid were purchased from Sigma (St. Louis, MO, USA). Tyrosinase (EC 1.14.18.1) from mushroom was from Sigma Chemical Co. The specific activity of the enzyme is 6680 U/mg. Dimethylsulfoxide (DMSO), L-tyrosine (Tyr) and L-3,4-dihydroxyphenylalanine (L-DOPA) were obtained from Aldrich (St. Louis, MO, USA). All other reagents were local and of analytical grade. The water used was re-distilled and ion-free.

The monophenolase activity assay was performed by the method previously reported with L-tyrosine as substrate [9]. The reaction media (3 ml) contained 1.0 mM L-Tyr in 50 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 6.8); a portion of 100 μl of the natural enzyme aqueous solution (containing 100 μg) was used to assay the activity. The final concentration of mushroom tyrosinase was 33.33 μg/ml. The enzyme activity was monitored for absorbance at 475 nm ($\epsilon = 3700\ M^{-1}cm^{-1}$) each 5 s for 10 min [12]. The reaction was carried out at a constant temperature of 30°C.

The diphenolase activity of mushroom tyrosinase assay was performed as previously reported using L-DOPA as substrate [13]. The reaction media (3 ml) contained 0.5 mM L-DOPA and 20 μg of the enzyme in 50 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 6.8). Enzyme activity was determined by following the increase in absorbance at 475 nm accompanying the oxidation of L-DOPA to dopachrome. The reaction was carried out at a constant temperature of 30°C.

The inhibitory effects of inhibitors on enzyme activity were determined as follows. The inhibitor was first dissolved in DMSO and used for the experiment at 30 times dilution. The final concentration of DMSO in the test solution was 3.3%. In this method, 0.1 ml of DMSO solution containing different concentrations of the inhibitors was first mixed with 2.8 ml of substrate solution, then, a portion of 100 μl of enzyme solution was added and the residual activity was determined. Controls, without inhibitor but containing 3.3% DMSO, were routinely carried out. The extent of inhibition by the addition of the sample was expressed as the concentration necessary for 50% inhibition (IC_{50}).

The inhibition type of 4-vinylbenzaldehyde and 4-vinylbenzoic acid on the enzyme were determined by the Lineweaver-Burk plot and the inhibition constant was determined from secondary plots of the apparent K_m/V_{max} or $1/V_{max}$ versus the concentration of the inhibitor. A Beckman UV-650 spectrophotometer was used for absorbance and kinetic measurements.

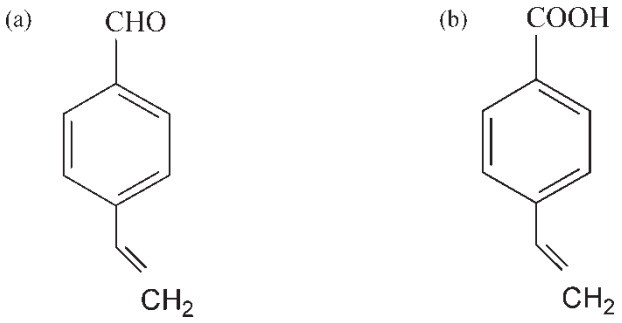

Figure 1. Chemical structures of 4-vinylbenzaldehyde (a) and 4-vinylbenzoic acid (b).

Results

Effect of 4-vinylbenzaldehyde on the monophenolase activity of mushroom tyrosinase

The effects of 4-vinylbenzaldehyde (see Figure 1 for structure) at different concentrations on the oxidation of L-tyrosine by the enzyme were first studied. The kinetic course of the oxidation of the substrate in the presence of 4-vinylbenzaldehyde are shown in Figure 2-I. When the monophenolase activity of mushroom tyrosinase was assayed using L-tyrosine as substrate, a lag period, characteristic of monophenolase activity, was observed simultaneously with the appearance of the first stable product, dopachrome (Figure 2-I, curve 1). The system reached a constant rate after the lag period, which was estimated by extrapolation of the linear portion of the product accumulation curve to the abscissa [14]. After the reaction system reached the steady state, the curve for product increased linearly with increasing reaction time; the slope of the line denoted the steady-state rate. In the presence of different concentrations of 4-vinylbenzaldehyde, the kinetic course of the oxidation

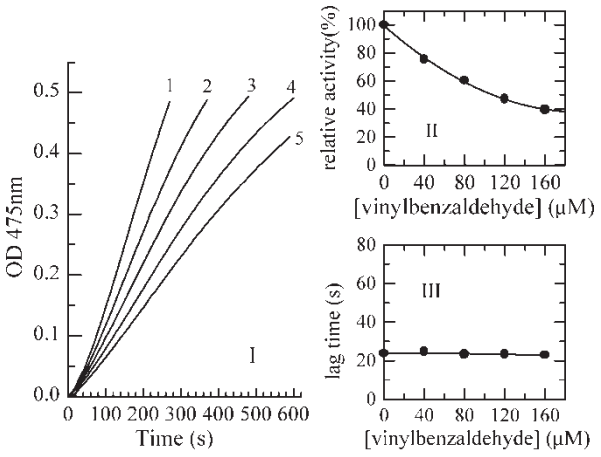

Figure 2. Progress curves for the inhibition of monophenolase activity of mushroom tyosinase by 4-vinylbenzaldehyde. The concentrations of 4-vinylbenzaldehyde for curves 1–5 were 0, 40, 80, 120, and 160 μM, respectively.

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by Malmo Hogskola on 12/24/11
For personal use only.

RIGHTSLINK

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by Malmo Hogskola on 12/24/11
For personal use only.

of L-tyrosine are shown in Figure 2-I (curves 2–5). The lag time and the steady-state rate were determined and the data are shown in Figures 2-II and 2-III, respectively. The lag time didn't change with increasing inhibitor concentration, but the steady state rate descended distinctly. When the concentration of 4-vinylbenzaldehyde was 40, 80, 120 and 160 μM, the remaining activity of the monophenolase was found to be 76.6%, 60.3%, 48.4% and 41.0%, respectively, indicating the concentration-dependent relation of the inhibitory effect of 4-vinylbenzaldehyde on monophenolase. The IC_{50} value for the monophenolase activity inhibited by 4-vinylbenzaldehyde was determined as 95 μM.

Effect of 4-vinylbenzoic acid on the monophenolase activity of mushroom tyrosinase

In the presence of 4-vinylbenzoic acid (see Figure 1 for structure), the monophenolase activity of mushroom tyrosinase was assayed using L-tyrosine as substrate. In the presence of different concentrations of 4-vinylbenzoic acid, the kinetic course of the oxidation of L-tyrosine are shown in Figure 3-I (curves 2-5). The lag time and the steady-state rate were determined and the results are shown in Figure 3-II. Both the lag time and the steady state rate of the monophenolase activity were affected by this inhibitor. With increasing inhibitor concentration, the lag time changed from 23 seconds in its absence to 72 seconds in the presence of 2.0 mM. The steady state rate of monophenolase activity descended. With 4.0 mM of 4-vinylbenzoic acid, the remaining activity of the monophenolase was determined to be 39.5%. The IC_{50} value for the monophenolase activity inhibited by 4-vinylbenzoic acid was determined as 3.0 mM.

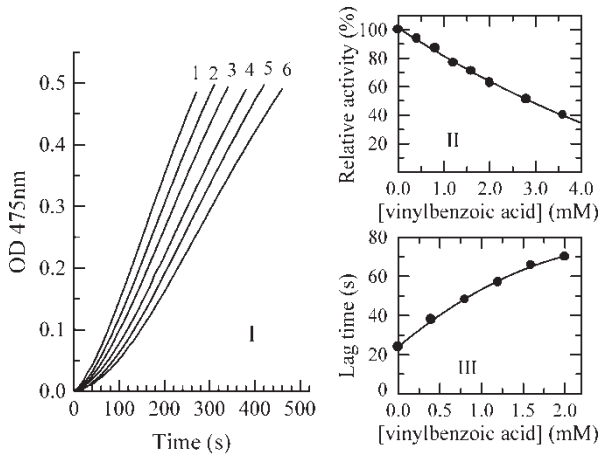

Figure 3. Progress curves for the inhibition of monophenolase activity of mushroom tyrosinase by 4-vinylbenzoic acid. The concentrations of 4-vinylbenzoic acid for curves 1–6 were 0, 0.3, 0.6, 1.2, 1.5 and 2.0 mM, respectively.

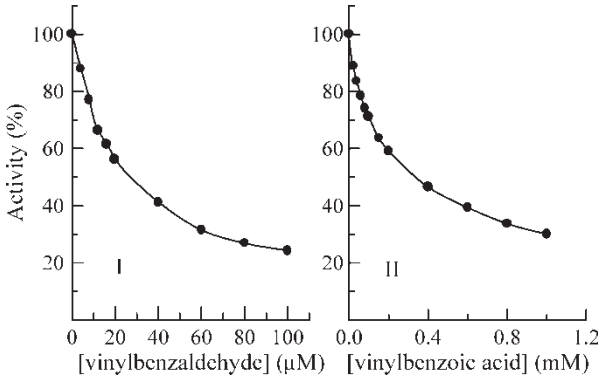

Figure 4. Effect of 4-vinylbenzaldehyde and 4-vinylbenzoic acid on the diphenolase activity of mushroom tyrosinase.

Effect of 4-vinylbenzaldehyde and 4-vinylbenzoic acid on the diphenolase activity of mushroom tyrosinase

The assay for diphenolase activity used L-DOPA as substrate. The progress curve of the enzyme reaction was a linear line passing through the origin and the value of the slope of the line indicated the diphenolase activity. The results showed no lag period. Using 4-vinylbenzaldehyde and 4-vinylbenzoic acid as inhibitors, the diphenolase activity decreased exponentially with increasing inhibitor concentration. Both 4-vinylbenzaldehyde and 4-vinylbenzoic acid could inhibit diphenolase activity (Figure 4). The IC_{50} s of 4-vinylbenzaldehyde and 4-vinylbenzoic acid were found to be 23 μM and 330 μM, respectively. The data are listed in Table I for comparison. The results indicated that the inhibitory potency of 4-vinylbenzaldehyde was fourteen times that of 4-vinylbenzoic acid on the diphenolase activity of mushroom tyrosinase.

The inhibitory mechanism of 4-vinylbenzaldehyde and 4-vinylbenzoic acid on the diphenolase activity of mushroom tyrosinase

The inhibition mechanisms by 4-vinylbenzaldehyde and 4-vinylbenzoic acid on the enzyme for the oxidation of L-DOPA were studied. Both inhibitors had the same behavior. Figure 5 shows the relationship of enzyme activity with the enzyme concentration

Table I. Inhibition constants of 4-vinylbenzaldehyde and 4-vinylbenzoic acid with mushroom tyrosinase.

Constants	4-Vinylbenzaldehyde	4-Vinylbenzoic acid
IC_{50}		
Monophenolase	93 μM	3.01 mM
Diphenolase	23 μM	0.33 mM
Inhibition	Reversible	Reversible
Inhibition type	Mixed	Mixed
K_I	29.7 μM	0.54 mM
K_{IS}	16.4 μM	0.22 mM

RIGHTSLINK

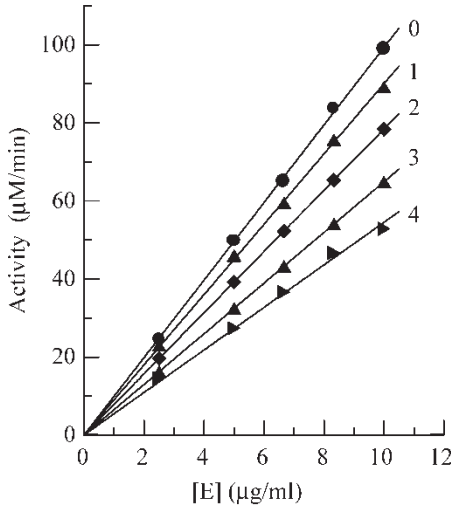

Figure 5. Determination of the inhibitory mechanism of 4-vinylbenzaldehyde on mushroom tyrosinase. The concentrations of 4-vinylbenzaldehyde for curves 0–4 were 0, 5, 10, 15, and 20 μM, respectively.

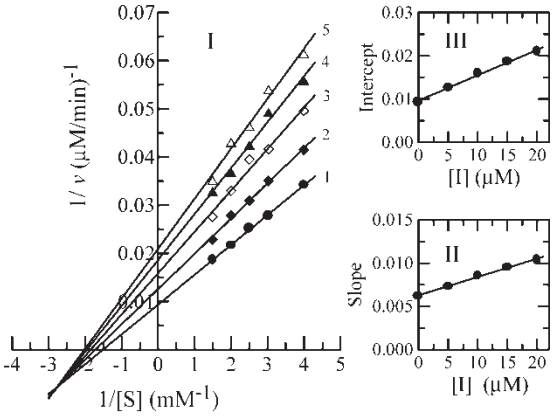

Figure 6. Determination of the inhibitory type and inhibition constants of 4-vinylbenaldehyde on mushroom tyrosinase. (a) Lineweaver-Burk plots for inhibition of 4-vinylbenzaldehyde on mushroom tyrosinase. The concentrations of 4-vinylbenzaldehyde for curves 1–5 were 0, 5, 10, 15, and 20 μM, respectively. (b) and (c) represent the plot of slope and intercept versus the concentration of 4-vinylbenzaldehyde for determining the inhibition constants K_I and K_{IS}, respectively.

in the presence of different concentrations of 4-vinylbenzaldehyde. The plots of the remaining enzyme activity *versus* the concentrations of enzyme in the presence of different concentrations of 4-vinylbenzaldehyde gave a family of straight lines, which all passed through the origin. Increasing the inhibitor concentration resulted in the lowering of the slope of the line, indicating that the inhibition by 4-vinylbenzaldehyde was reversible. The presence of inhibitor did not reduce the amount of enzyme, but just resulted in the inhibition of enzyme activity. Both 4-vinylbenzaldehyde and 4-vinylbenzoic acid were reversible inhibitors of mushroom tyrosinase for oxidation of L-DOPA.

Determination of the inhibitory type for 4-vinylbenzaldehyde and 4-vinylbenzoic acid on diphenolase activity

The inhibition of mushroom tyrosinase by 4-vinylbenzaldehyde is illustrated in Figure 6. Double-reciprocal plots yielded a family of straight lines which intersected in the 3rd quadrant. The values of K_m and V_{max} decreased with increasing inhibitor concentration. Thus, 4-vinylbenzaldehyde was a competitive-uncompetitive mixed-II type inhibitor. The uncompetitive effect was stronger than the competitive effect, indicating that 4-vinylbenzaldehyde inhibited the free enzyme more weakly than the enzyme-substrate complex. The equilibrium constant for inhibitor binding with the free enzyme and the enzyme-substrate complex, K_I and K_{IS}, were obtained from the plots of the slope and the vertical intercept versus the concentration of 4-vinylbenzaldehyde, respectively. The values of K_I and K_{IS} were determined to be 29.7 μM and 16.4 μM, respectively. In comparison, the results obtained using 4-vinylbenzoic acid as inhibitor showed the inhibitor constants (K_I and K_{IS}) to be 0.54 mM and 0.22 mM, respectively. The results are summarized in Table I.

Discussion

Tyrosinase exhibits both monophenolase and diphenolase activities. Here, we used L-DOPA as substrate for the diphenolase activity and Tyr for the monophenolase activity of the enzyme. The results show that 4-vinylbenzaldehyde and 4-vinylbenzoic acid could inhibit both the diphenolase activity and the monophenolase activity of mushroom tyrosinase. For diphenolase activity, the inhibition was reversible, and the inhibition types were determined to be competitive- uncompetitive mixed-II type. As shown in Table I, the IC_{50}s of 4-vinylbenzaldehyde and 4-vinylbenzoic acid for the diphenolase activity were quite different. The former was about fourteen times lower than the latter, indicating that the inhibitory potency of 4-vinylbenzaldehyde was stronger than that of 4-vinylbenzoic acid. From Table I, it is clear that 4-vinylbenzaldehyde and 4-vinylbenzoic acid have the same inhibition mechanism. The inhibitory constants were determined and compared. The results showed that the value of K_I was about two-fold that of K_{IS}, indicating that the affinity of the inhibitors for the enzyme-substrate complexes was stronger than for the free enzyme.

Mushroom tyrosinase is composed of four subunits and contains two binuclear coppers in its active sites per tetramer [15]. In the processing of catalysis, tyrosinase exists in three forms: E_{met}, E_{oxy} and E_{deoxy} [16]. 4-Vinylbenzaldehyde has an aldehyde group and 4-vinylbenzoic acid has a carboxylic acid group,

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by Malmo Hogskola on 12/24/11
For personal use only.

RIGHTSLINK

which can form a Schiff base with the amino group of the amino acid residue around the active site of enzyme [17]. That is, the carbon atom of the aldehyde and acid group can form a carbon-nitrogen double bond with the nitrogen atom of the amino acid residue around the active site of enzyme. Compared to the acid group, the aldehyde group forms a more stable Schiff base and seems to have more inhibitory activity. Because these two inhibitors may form a Schiff base with a primary amino group of the enzyme and chelate the copper in the active site of the enzyme, the inhibition by these inhibitors is of a mixed-type. That is, they can combine with the free enzyme and the enzyme-substrate complex. In contrast, the K_I value of 4-vinylbenzaldehyde was much lower than that of 4-vinylbenzoic acid and those of other 4-substituted benzaldehydes [9]; the tight binding of the inhibitor with the active site of the enzyme may be responsible for the low K_I value.

When the monophenolase activity of mushroom tyrosinase was assayed using L-tyrosine as substrate, a marked lag period, characteristic of monophenolase activity, was observed before the appearance of the first stable product, dopachrome [3]. The lag time could be estimated by extrapolation of the linear portion of the product accumulation curve to the *X*-axis. As was shown in Figures 2 and 3, both 4-vinylbenzaldehyde and 4-vinylbenzoic acid could reduce the steady state rate of the monophenolase activity. Furthermore, 4-vinylbenzaldehyde did not change the lag time, while 4-vinylbenzoic acid extended it about 5-fold. The results showed that the inhibition mechanisms on the monophenolase activity of 4-vinylbenzaldehyde and 4-vinylbenzoic acid were obviously different.

Acknowledgements

The present investigation was supported by Grant 2004N002 of the Science and Technology Foundation and by Grant B0410003 of the Natural Science Foundation of Fujian Province for Q.X. Chen.

References

[1] Seo SY, Sharma VK, Sharma N. J Agric Food Chem 2003;51:2837–2853.
[2] Yu LL. J Agric Food Chem 2003;51:2344–2347.
[3] Kubo I, Kinst-Hori I. J Agric Food Chem 1999;47:4121–4125.
[4] Xu Y, Stokes AH, Freeman WM, Kumer SC, Vogt BA, Vrana KE. Mol Brain Res 1997;45:159–162.
[5] Robit C, Rouch C, Cadet F. Food Chem 1997;59:355–360.
[6] Espín JC, Wichers HJ. J Agric Food Chem 1999;47:2638–2644.
[7] Xie LP, Chen QX, Huang H, Wang HZ, Zhang RQ. Biochemistry (Moscow) 2003;68:487–491.
[8] Kahn V, Ben-Shalom N, Zakin V. J Agric Food Chem 1997;45:4460–4465.
[9] Chen QX, Song KK, Wang Q, Huang HJ. J Enz Inhib Med Chem 2003;18:491–496.
[10] Wang Q, Shi Y, Song KK, Guo HY, Qiu L, Chen QX. Protein J 2004;23:303–308.
[11] Chen Q, Chen QX, Qiu L, Song KK, Huang H. J Protein Chem 2003;22:607–612.
[12] Jiménez M, Chazarra S, Escribano J, Cabanes J, García-Carmona F. J Agric Food Chem 2001;49:4060–4063.
[13] Chen QX, Kubo I. J Agric Food Chem 2002;50:4108–4112.
[14] Robit C, Rouch C, Cadet F. Food Chem 1997;59:355–360.
[15] Ridríguez-López JN, Fenoll LG, García-Ruiz PA. Biochemistry 2000;39:10497–10506.
[16] Sanchez-Ferrer A, Rodriguez-Lopez JN. Garcia-Canovas F, Biochim Biophys Acta 1995;1247:1–11.
[17] Kubo I, Ikuyo KH. J Agric Food Chem 1998;46:5338–5341.

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by Malmo Hogskola on 12/24/11
For personal use only.

RIGHTSLINK