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Reduction of Cinnamyl Alcohols and Cinnamaldehydes by *Saccharomyces cerevisiae*

PAOLA GRAMATICA, BIANCA MARIA RANZI, AND PAOLO MANITTO

Istituto di Chimica Organica della Facoltà di Scienze della Università degli Studi di Milano e Centro di Studio per le Sostanze Organiche Naturali del CNR, Via Saldini 50, 20133 Milano, Italy

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The reduction of 3',4'-dimethoxycinnamyl alcohols to phenylpropanols by *Saccharomyces cerevisiae* proceeds through the corresponding aldehydes. The specificity with respect to substrate structure of the two enzymatic systems involved in the above transformation (alcohol dehydrogenase and reductase) was studied. Whereas yeast alcohol dehydrogenase shows specificity for the (*E*) configuration of side-chain double bond, reductase does not act on 3-substituted substrates.

INTRODUCTION

In connection with our study on the decarboxylation of ring and side-chain-substituted cinnamic acids by *Saccharomyces cerevisiae* (*1*), it was found that the same yeast strain was able to reduce both cinnamaldehydes and cinnamyl alcohols to the corresponding dihydrocinnamyl alcohols. This fact promoted us to investigate these reactions from the point of view of the enzymatic specificity. In principle, analogies in constitutional and steric requirements of compounds, acting as substrates for different enzymes in the same organism, could reflect structural and biogenetic relationship between such enzymes.

The finding that (*E*)-cinnamyl alcohols, but not the (*Z*) type, were reduced by a crude suspension of baker's yeast (*2*), as well as (*E*)-cinnamic acids, but not the (*Z*) type, were decarboxylated by a selected strain of *S. cerevisiae* (*1*), seemed a likely indication of the above-mentioned relationship.

RESULTS AND DISCUSSION

First of all, it was ascertained that the reduction of cinnamyl alcohols proceeds through the corresponding aldehydes as shown in the scheme:

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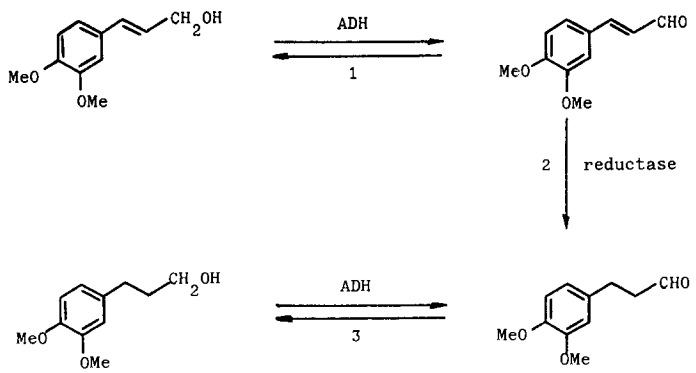


When (*E*)-3′,4′-dimethoxycinnamaldehyde was administered to resting yeast cells a very rapid formation of the corresponding unsaturated alcohol, followed by a slower appearance of 3-(3′,4′-dimethoxyphenyl)-1-propanol was observed. To prove the key role of cinnamaldehydes in the double bond reduction of cinnamyl alcohols, a clear-cut experiment was then carried out using (*E*)-3′,4′-dimethoxy-[1-$^2H_2$]cinnamyl alcohol ($^2H_2$ = 95%, $^2H_1$ = 1% by ms and nmr analysis) as substrate. The 3-(3′,4′-dimethoxyphenyl)-[1-$^2H_1$]-1-propanol so obtained showed (by ms) an isotopic composition corresponding to a total loss of one deuterium atom. The unreduced alcohol, isolated in small amount after 24 hr fermentation, had a higher abundance of monodeuterated species than the starting materials (Table 3 in Experimental section), as expected for a rapid unsaturated alcohol ⇄ unsaturated aldehyde equilibrium. It was therefore concluded that the reduction of cinnamyl alcohols is mediated by two enzymatic systems: one (or two) alcohol dehydrogenases (steps 1 and 3 of scheme) and a reductase that catalyzes the reduction of side-chain double bond of cinnamaldehydes.

When (*Z*)-3′,4′-dimethoxy-[1-$^2H_2$]cinnamyl alcohol was tested for reduction by yeast (grown on glucose), no transformation was found to occur; moreover, no loss of deuterium was observed in the cinnamyl alcohol recovered after 4 days of fermentation. By contrast, (*Z*)-3′,4′-dimethoxycinnamaldehyde was rapidly reduced by yeast cells giving rise to 3-(3′,4′-dimethoxyphenyl)-1-propanol in almost quantitative yield after 15 min. In a parallel short-term fermentation (15 min), (*E*)-3′,4′-dimethoxycinnamaldehyde gave, as mentioned above, a mixture of (*E*)-3′,4′-dimethoxycinnamyl alcohol and 3-(3′,4′-dimethoxyphenyl)-1-propanol (*ca.* 1 : 1), thus allowing exclusion of a rapid double bond isomerization of the (*Z*)-cinnamaldehyde before its reduction.

These results are best explained by assuming a specificity for the alcohol dehydrogenase only with respect to (*E*)/(*Z*) configuration and a faster reduction of the (*Z*) double bond. It was also observed that the inductive alcohol dehydrogenase (ADH II), an isoenzyme produced by cells grown on ethanol (*3, 4*), shows the same specificity toward the double bond configuration as the constitutional dehydrogenase (ADH I). In fact, the (*Z*)-alcohol was not reduced by ethanol grown cells, which, by contrast, were able to reduce the corresponding (*E*)-alcohol faster than glucose-grown cells.

TABLE 1

OXIDIZING ACTIVITIES OF CELL-FREE EXTRACTS[a] ON (*E*)- AND (*Z*)-3′,4′-DIMETHOXYCINNAMYL ALCOHOLS

Growth medium	Inhibitor	Specific activity (nmol/min/mg protein[b]) at 30°C	
		(*E*) isomer	(*Z*) isomer
Glucose	—	3.5	0
Ethanol	—	28.6	0
Ethanol	Pyrazole[c]	0	—

[a] The *Saccharomyces cerevisiae* cells were centrifuged from the growth medium, washed, frozen, grinded with alumina (1 g cells/5 g alumina) in phosphate buffer (pH 7.5, 1 g cells/3 ml buffer), and centrifuged again.

[b] Protein was determined with the method of Gornall (6). NAD was added as cofactor.

[c] The pyrazole (5) concentration was 200 μM.

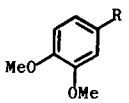
In support of these assumptions and in order to avoid cell permeation effects, which cannot be excluded with intact cells, the reduction activities of yeast were examined in cell homogenates. Results, reported in Table 1, confirm that (i) (*E*)-3′,4′-dimethoxycinnamyl alcohol, but not the (*Z*) isomer, acts as an oxidable substrate for both ADH I and ADH II, which are well known to be inhibited by pyrazole (5); (ii) ADH II, which is absent in glucose-grown cells, is more active than ADH I.

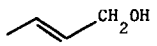
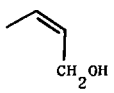
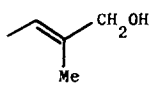
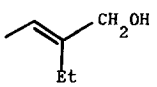
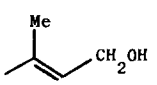
The influence of side-chain substituents upon the reduction of 3′,4′-dimethoxycinnamyl alcohols was investigated with intact cells. When reduction occurred, it was complete in 24 hr with glucose-grown cells and in 4 hr with ethanol-grown cells. In these cases the saturated alcohol was extracted with ether and analyzed by tlc and glc–ms by comparison with synthetic samples, and, in some cases, isolated by preparative tlc. Otherwise, the substrate was recovered unchanged almost quantitatively after 120 hr. From these experiments (Table 2) it appears that the reduction of double bond does not appear sensitive to substitution at 2 position, while the introduction of a methyl in 3 position causes a blockage of this reaction. However, it must be pointed out that, while (*E*)-3′,4′-dimethoxy-3-methylcinnamyl alcohol was recovered unchanged after fermentation experiment (120 hr), the corresponding cinnamaldehyde was reduced to the unsaturated alcohol (path 1 of the scheme), thus indicating a blockage of the reductase, a membrane effect for 3-methyl alcohol being unlikely.

In conclusion, the above results give evidence for a marked difference in specificity between the two enzymes catalyzing the steps 1 and 2 of the scheme. The alcohol dehydrogenases (both ADH I and II) distinguish between the (*Z*) and (*E*) configurations of the double bond, but show no specificty with respect to side-chain substituents (at least in the cases examined here); on the contrary, the reductase acts on both (*E*)- and (*Z*)-aldehydes, but is inactive toward 3-substituted derivatives. From the point of view of substrate specificity, yeast alcohol dehydrogenase resembles cinnamate decarboxylase (*1*) much more than cinnamaldehyde reductase.

TABLE 2

REDUCTION OF SIDE-CHAIN SUBSTITUTED CINNAMYL ALCOHOLS



ALCOHOLS		REDUCTION
R= 	(E) (1)	yes: (6)
= 	(Z) (2)	not
= 	(E) (3)	yes: (7)
= 	(E) (4)	yes: (8)
= 	(E) (5)	not

EXPERIMENTAL

See the experimental section in previous paper (*1*). FT–nmr were recorded on a 100-MHz Varian XL100 spectrometer (tetramethylsilane as internal standard, deuterochloroform as solvent). Gas–liquid chromatography–mass spectra were recorded on Varian MAT 112 double focusing gas chromatograph–mass spectrometer fitted with a 3 × 4-mm glass column, packed with SE 30, 3%.

(*a*) *Preparation of Cinnamyl Alcohols*

Cinnamyl alcohols were prepared by lithium aluminum hydride ($LiAl^2H_4$; Merck, 99% ^2H for deuterated alcohols) reduction of corresponding ethyl cinnamates at a strictly controlled temperature of −10°C (ether as solvent and argon atmosphere). After 2–3 hr, usual workup of the reaction mixture gave about 90% of cinnamyl alcohols.

The crude products were purified by silica gel column with benzene: ethyl ether (9 : 1) as solvent. The purity of the alcohols was controlled by tlc (chloroform : ethyl acetate, 1 : 1) and glc (SE 30, 3%, T = 170°C, N_2 = 1.5 kg/cm^2). The spectroscopic data of the following alcohols are listed below.

(E)-3',4'-dimethoxycinnamyl alcohol (***1a***). Infrared and nmr data are as reported (*7*). glc–ms (*m/e*,I%): 194(M^+,82), 151(100), 138(96), 91(36), 77(28).

(E)-3', 4'-dimethoxy-[1-$^2H_2$]cinnamyl alcohol (***1b***). The reduction was complete after 9 hr. The percentage of deuteration was >95% by nmr and ms analysis. nmr(δ): 7.1–6.75 (m, 3H, arom), 6.54 (d, 1H, J 16 Hz, H_β), 6.12 (d, 1H, J 16 Hz, H_α), 3.83 (s, 6H, 2 OCH_3), 1.92 (broad s, 1H, O*H*). glc–ms: 196(M^+,91), 151(100), 139(48), 93(27).

(Z)-3',4'-dimethoxycinnamyl alcohol (***2a***). nmr(δ): 7–6.7 (m, 3H, arom), 6.49 (d, 1H, J_1 11.5 Hz, H_β), 5.2 (dd, 1H, J_1 11.5Hz, J_2 6.5 Hz, H_α), 4.4 (d, 2H, J_2 6.5 Hz, CH_2–O), 3.85(s, 6H, 2OCH_3), 1.88(broad s, 1H, O*H*). glc–ms: 194(M^+,75), 151(100), 138(60), 91(38).

(Z)-3',4'-Dimethoxy-[1-$^2H_2$]cinnamyl alcohol (***2b***). Percentage of deuteration = 98 ± 1%. nmr(δ): 6.78 (s, 3H, arom), 6.5 (d, 1H, J 11.5 Hz, H_β), 5.75 (d, 1H, J 11.5 Hz, H_α), 3.82 (s, 6H, 2 OCH_3), 1.45 (broad s, 1H, O*H*). glc–ms: 196(M^+,86), 151(100), 139(49), 93(76).

(E)-3',4'-Dimethoxy-2-methylcinnamyl alcohol (*3*). nmr(δ): 6.81 (s, 3H, arom), 6.46 (broad s, 1H, H_β), 4.14 (s, 2H, CH_2-O), 3.85 (s, 6H, 2 OCH_3), 1.98 (s, 1H, O*H*), 1.89 (broad s, 3H, α-CH_3). glc–ms: 208(M^+,33), 207(19), 206(28), 190(45), 175(60), 163(18), 151(100) 147(33), 138(31), 115(33), 107(40), 103(34), 91(52), 77(50).

(E)-3',4'-Dimethoxy-2-ethylcinnamyl alcohol (*4*). nmr(δ): 6.82 (s, 3H, arom), 6.45 (broad s, 1H, H_β), 4.22 (broad s, 2H, CH_2-O), 3.85 (s, 6H, 2 OCH_3), 2.36 (broad q, 2H, J 7 Hz, CH_2–CH_3), 1.62 (broad s, 1H, O*H*), 1.2 (t, 3H, J 7 Hz, CH_3–CH_2). glc–ms: 222(M^+,26), 204(37), 189(57), 151(100), 138(26), 115(30), 91(30), 77(33).

(E)-3',4'-Dimethoxy-3-methylcinnamyl alcohol (*5*). nmr(δ): 7.1–6.7 (m, 3H, arom), 5.96 (broad t, 1H, J 3.5 Hz, H_α), 4.39 (d, 2H, J 3.5 Hz, CH_2–O), 3.9 (s, 6H, 2 OCH_3), 2.1 (broad s, 3H, β–CH_3), 1.54 (s, 1H, O*H*). glc–ms: 190(M^+–H_2O,59), 175(32), 159(100), 144(32), 115(64), 91(50), 77(45).

(*b*) *Preparation of Phenylpropanols*

Reduction of cinnamyl alcohols to give corresponding phenylpropanols was performed by hydrogenation on PtO_2 catalyst in methanol (yield 90%). Data of saturated alcohols from glc–ms analysis are listed below.

3-(3',4'-Dimethoxyphenyl)-1-propanol (*6*). *m/e*(I%): 196(M^+,53), 152(42), 151(100), 121(17), 107(17), 91(17), 77(13).

2-Methyl-3-(3',4'-dimethoxyphenyl)-1-propanol (*7*). *m/e*(I%): 210(M^+,15), 152(15), 151(100), 107(12), 91(12), 77(11).

2-Ethyl-3-(3',4'-dimethoxyphenyl)-1-propanol (*8*). *m/e*(I%): 224(M^+,16), 152(24), 151(100), 107(12), 91(12), 77(9).

(*c*) *Preparation of Cinnamaldehydes*

Oxydation of cinnamyl alcohols was performed with "active" MnO_2 in CCl_4, according to Attenburrow *et al.* (*8*). The following aldehydes, with reported spectroscopic data, were prepared.

(E)-3',4'-dimethoxycinnamaldehyde (*9*). nmr(δ): 9.64 (d, 1H, J_1 7 Hz, C*H*O), 7.5–7 (m, 3H, arom), 7.2 (d, 1H, J_2 16 Hz, H_β), 6.71 (dd, 1H, J_1 7 Hz, J_2 16 Hz, H_α), 3.93 (s, 6H, 2 OCH_3). glc–ms: 192(M^+,97), 191(20), 177(25), 161(100), 149(23), 133(16), 121(17), 91(25), 77(36).

(Z)-3',4'-dimethoxycinnamaldehyde. It was prepared in the dark and in argon atmosphere, then stored at −25°C. nmr(δ): 10 (d, 1H, J_1 8 Hz, C*H*O), 7.5 (d, 1H, J_2 11 Hz, H_β), 7.2–6.85 (m, 3H, arom), 6.08 (dd, 1H, J_1 8 Hz, J_2 11 Hz, H_α), 3.9 (s, 6H, 2 OCH_3). glc–ms: 192(M^+,73), 177(30), 161(100), 151(70), 149(35), 121(30), 91(47), 77(53).

(E)-3',4'-dimethoxy-3-methylcinnamaldehyde. nmr(δ): 7.3–6.75 (m, 3H, arom), 6.38 (broad s, 1H, C*H*=), 3.87 (s, 6H, 2 OCH_3) 2.52 (broad s, 3H, CH_3). glc–ms: 206(M^+,12), 191(9), 180(47), 165(100), 137(35), 122(26), 107(18), 91(33), 77(55).

(d) Fermentations

Maintenance, growth, and fermentation conditions. The growth conditions of *S. cerevisiae* (MWC_{28}) and the procedure for resting cells preparation are the same as those described in the previous paper (*1*). However, in some experiments the cultural medium was supplemented with 1% of ethanol, in place of glucose, because the induction of alcohol dehydrogenase by ethanol was demonstrated in many yeast (*3, 4*). The experiments with (*Z*)-3',4'-dimethoxycinnamyl alcohol and the corresponding (*Z*)-cinnamaldehyde were carried out in the dark to avoid double bond photoisomerization. When reduction occurred, this reaction was complete after 24 hr of fermentation (excepting for (*E*)-3',4'-dimenthoxy-α-ethyl-cinnamyl alcohol (96 hr)), with glucose-grown cells and after 4 hr of fermentation with ethanol-grown cells.

Recovery and analysis of fermentation products. The supernatant liquid, after centrifugation of the cells, was extracted with ethyl ether and organic extract was analyzed by tlc ($CHCl_3$/AcOEt, 1 : 1) and by glc–ms (SE 30, 3%, T = 170°C) by comparison with synthetic samples. Conversion yields (in the range 60–80%) were determined by glc analysis.

Fermentation of (E)-3',4'-dimethoxy-[1-$^2H_2$]cinnamyl alcohol. (*E*)-3',4'-dimethoxy-[1-$^2H_2$]cinnamyl alcohol ($^2H_2$ = 95%, $^2H_1$ = 1%, $^1H_2$ = 4% by ms analysis) was fermented with resting cells of *S. cerevisiae* for 1, 4, and 24 hr (until almost complete reduction). The residual unreduced cinnamyl alcohol and the reduced one were examined for their isotopic composition by glc–ms to give the results reported in Table 3.

Fermentation of (E)-3',4'-dimethoxy-[1-$^2H_2$]cinnamyl alcohol. (*E*)-3',4',-dimethoxy-[1-$^2H_2$]cinnamyl alcohol ($^2H_2$ = 95%, $^2H_1$ = 1%, $^1H_2$ = 4% by ms analysis) analysis) was recovered unchanged and with identical isotopic composition after 120 hr fermentation with *S. cerevisiae* (cells grown and on glucose and on ethanol).

Fermentation of (E)- and (Z)-3',4'-dimethoxycinnamaldehydes. After 24 hr fermentation with *S. cerevisiae* cells, grown and on glucose and on ethanol, (*E*)- and (*Z*)-3',4'-dimethoxycinnamaldehydes gave saturated alcohol (3-(3',4'-di-

TABLE 3

ISOTOPIC COMPOSITION OF THE SUBSTRATE AND OF ITS FERMENTATION PRODUCT

Fermentation time	(*E*)-3′,4′-dimethoxycinnamyl alcohol			3-(3′,4′-dimethoxyphenyl)-1-propanol	
Start	95% $^2H_2$	1% $^2H_1$	4% $^1H_2$	—	—
1 hr	92 $^2H_2$	5 $^2H_1$	3 $^1H_2$	—	—
4 hr	91 $^2H_2$	6 $^2H_1$	3 $^1H_2$	—	—
24 hr	84 $^2H_2$	14 $^2H_1$	2 $^1H_2$	96% $^2H_1$	4% $^1H_2$

methoxyphenyl)-1-propanol), identified by glc–ms and FT–nmr after preparative tlc separation. Analysis by glc–ms and FT–nmr of short-term fermentation samples (15 min) gave the following results: (i) from (*E*)-aldehyde a mixture (*ca.* 1 : 1) of (*E*)-3′,4′-dimethoxycinnamyl alcohol and of 3-(3′,4′-dimethoxyphenyl)-1-propanol; (ii) from (*Z*)-aldehyde only 3-(3′,4′-dimethoxyphenyl)-1-propanol.

Fermentation of (E)-3′,4′-dimethoxy-3-methylcinnamyl alcohol and of (E)-3′,4′-dimethoxy-3-methylcinnamaldehyde. (*E*)-3′,4′-dimethoxy-3-methylcinnamyl alcohol was recovered unreduced after 120 hr of fermentation with cells of *S. cerevisiae,* grown and on glucose and on ethanol, while in the same fermentation conditions the corresponding aldehyde was reduced to (*E*)-3′,4′-dimethoxy-3-methylcinnamyl alcohol. The cinnamyl alcohols were isolated by preparative tlc and identified by glc–ms and FT–nmr.

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