

# Formation of Aliphatic and Aromatic $\alpha$ -Hydroxy Ketones by *Zygosaccharomyces bisporus*

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Z. Naturforsch. **55c**, 560–568 (2000); received February 22/March 10, 2000

*Zygosaccharomyces bisporus*, Acyloin Formation, Pyruvate Decarboxylase

The wild-type yeast strain *Zygosaccharomyces bisporus* CBS 702 produced  $\alpha$ -hydroxyketones (acyloins) from amino acid precursors after transamination to the corresponding 2-oxo acids. The key enzyme of the subsequent decarboxylation and C–C bond forming reaction, pyruvate decarboxylase (PDC), was examined for its substrate- and stereo-specificity. A wide variety of saturated and unsaturated aliphatic and aromatic aldehydes was successfully converted to acyloins. 19 of the biotransformation products identified had not been reported as natural substances before. Product yields were strongly affected by substrate structure.

## Introduction

Pyruvate decarboxylase (PDC, E. C. 4.1.1.1) is a key enzyme in alcoholic fermentation and catalyses the non-oxidative decarboxylation of pyruvate and other 2-oxo acids to the corresponding aldehydes. Thiamine diphosphate (ThDP) and  $Mg^{2+}$ -ions are required as cofactors, which are bound to the apoenzyme in a quasi-irreversible manner at pH-values below 7.0 (Gounaris *et al.*, 1975). PDC was first detected in yeast extracts by Neuberg and Karczag (1911) and has been found in many other fungi, plants, and bacteria (Bringer-Meyer *et al.*, 1986; Rivoal *et al.*, 1990). The catalytically active form is tetrameric (dimer of dimers) with a molecular mass of about 240 kDa in most species, but higher active association states have been described for PDC from pea (Mücke *et al.*, 1995) and wheat germ (Zehender *et al.*, 1987).

The formation of acyloins (carboligation) as a side reaction of pyruvate decarboxylase has been assumed since Dirscherl's work (1931). In fact, Neuberg and Hirsch (1921) were the first to observe the production of *R*-1-hydroxy-1-phenylpropan-2-one (PAC, phenylacetyl carbinol) from benzaldehyde by fermenting yeast, but considered a new enzyme called "carboligase" to exist apart from PDC. The synthesis of *R*-PAC as a chiral precursor for *L*-ephedrine in the 1930ies was one of the first industrially applied biotransformations

and is a matter of continuing research (Pohl, 1997). Unequivocal proofs for PDC as key enzyme of this biotransformation were obtained by several studies using partially purified PDC from different organisms to generate acyloins (Chen and Jordan, 1984; Bringer-Meyer and Sahm, 1988; Crout *et al.*, 1991).

The carboligase reaction of PDC from yeast has been intensively studied with acetaldehyde as donor, which may be condensed to a wide range of aromatic and heterocyclic aldehydes, while aliphatic aldehydes usually are reported to fail as acetyl acceptors (Fuganti and Grasselli, 1985; Long *et al.*, 1989; Cardillo *et al.*, 1991; Crout *et al.*, 1991; Bornemann *et al.*, 1996). Most of these studies have been performed with fermenting yeast cells as in the case of the only detailed investigation reporting the successful conversion of aliphatic aldehydes (Schmauder and Gröger, 1968). During our work with different wild type yeast species, a strain of *Zygosaccharomyces bisporus* was found to transform amino acids into aromatic (Neuser *et al.*, 1999) and aliphatic acyloins that had not been described in literature before. After the setup of a fast purification method for PDC from *Z. bisporus*, the objective of our studies was to examine the suitability of aliphatic aldehydes and 2-oxo acids to serve as substrates in the PDC catalysed formation of  $\alpha$ -hydroxyketones.

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## Materials and Methods

### *Biotransformations with fermenting yeast*

*Zygosaccharomyces bisporus* CBS 702 was obtained from the Centraalbureau voor Schimmelfcultures (Baarn, Netherlands). After 48 h of precultivation at 24 °C in a standard medium (30 g l<sup>-1</sup> glucose, 3 g l<sup>-1</sup> yeast extract, 4.5 g l<sup>-1</sup> asparagine, 1 g l<sup>-1</sup> MgSO<sub>4</sub>, 1.5 g l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, pH 6.0), yeast cells were transferred to shake flasks containing 100 ml transformation medium (10 g l<sup>-1</sup> glucose, 8% (v/v) ethanol, 1 g l<sup>-1</sup> MgSO<sub>4</sub>, 0.5 g l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 2 mg l<sup>-1</sup> thiamine, 2 mg l<sup>-1</sup> biotin, pH 6.0). The respective amino acid precursor was the only source of nitrogen (10 mM). If aldehydes or oxo acids were to be converted (10 mM, added in two portions on growth days 2 and 5), 1 g l<sup>-1</sup> (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was provided as nitrogen source. After 7 d of cultivation, cells were separated by centrifugation (5000 × g) and the broth was extracted with ethyl acetate (3 × 40 ml). The extract was dried over anhydrous sodium sulfate and concentrated using a Vigreux column.

### *Enzyme purification*

For the isolation of pyruvate decarboxylase, yeast cells were transferred after precultivation to a growth medium containing 50 g l<sup>-1</sup> glucose, 10 g l<sup>-1</sup> yeast extract, 4.5 g l<sup>-1</sup> asparagine, 1 g l<sup>-1</sup> MgSO<sub>4</sub>, 1.5 g l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub> (pH 6.0, 300 ml, shake flasks, 24 °C). Cells were harvested by centrifugation (5000 × g) after 48 h and disintegrated in a bead mill. PDC was purified to homogeneity from the crude extract as described elsewhere (Neuser *et al.*, 2000).

### *Biotransformations with isolated PDC*

The reaction mixtures contained 2 mM ThDP, 20 mM MgSO<sub>4</sub>, 5 U PDC (1 U catalyzes the decarboxylation of 1  $\mu$ mol pyruvate to acetaldehyde at 25 °C in 1 min) and equimolar amounts of sodium pyruvate and the respective aldehyde or acetaldehyde and the respective 2-oxo acid (15–200 mM) in 0.1 M sodium citrate buffer (pH 6.0, final volume 4 ml). After incubation at 24 °C for 48 h, 200  $\mu$ g 2*E*-hexene-1-ol was added as internal standard, the mixtures were extracted with diethyl ether (2 × 2 ml) and the extracts dried over sodium sulfate.

### *Identification of metabolites*

The organic extracts were analysed by capillary gas chromatography (HRGC) and gas chromatography-mass spectrometry (HRGC-MS).  $\alpha$ -Hydroxyketones were identified by comparing their Kovats indices and mass spectra with published data, if available, or with synthesized references (see below). HRGC was performed on a Fisons GC 8000 Series chromatograph equipped with an on-column inlet, a flame-ionization detector, a deactivated FS-CW precolumn (3 m × 0.32 mm i.d., CS, Germany), and a CW 20M fused silica column (30 m × 0.32 mm i.d., film thickness 0.25  $\mu$ m, Macherey & Nagel, Germany). Operating conditions were as follows: Carrier gas H<sub>2</sub> at 3.2 ml min<sup>-1</sup>, injector 40 °C, detector 250 °C. The temperature was held at 40 °C for 3 min and then increased to 230 °C at a rate of 4 °C min<sup>-1</sup>. Mass spectra were recorded by a Fisons MD 800 mass spectrometer, using electron ionization at 70 eV, He as carrier gas and the same HRGC conditions as above.

### *Synthesis of reference acyloins*

Branched aliphatic acyloins (derived from the amino acids valine, leucine and isoleucine) were synthesized according to the method of Kawabata *et al.* (1978). For synthesis of the 2-hydroxy-3-oxo-acyloins (**2**), (**4**) and (**6**), 0.1 mol (11.8 g) ethyl L-lactate was amidated by passing dry ammonia gas through the stirred liquid for 24 h at room temperature. After removal of the formed ethanol 0.05 mol (4.46 g) L-lactamide was added to a Grignard reagent prepared from 0.25 mol (6.08 g) Mg turnings and 0.25 mol alkyl halide in 120 ml diethyl ether (30.8 g 2-bromopropane for synthesis of acyloin (**2**), 34.3 g 2-bromobutane for (**4**), and 34.3 g 1-bromo-2-methylpropane for (**6**), respectively). The acyloins were obtained after hydrolysis with HCl (6 M) and extraction with ether.

For synthesis of the 3-hydroxy-2-oxo-acyloins (**1**), (**3**) and (**5**), 0.04 mol of the respective hydroxy acids 2-hydroxy isovaleric acid (5.15 g), 2-hydroxy-3-methylvaleric acid (5.29 g) and 2-hydroxy isocaproic acid (5.29 g) were methylated with MeOH (40 ml) in the presence of acetyl chloride (3 ml). Since 2-hydroxy-3-methylvaleric acid was not commercially available, it was prepared by reduction of 0.05 mol (7.30 g) 2-oxo-3-methylvaleric

acid with 0.05 mol (2.0 g)  $\text{NaBH}_4$ . The respective hydroxy acid methyl esters (0.03 mol) were amidated as described above and 0.025 mol amide was then added to a Grignard reagent prepared from 0.125 mol (3.04 g) Mg turnings and 0.125 mol (17.7 g) iodomethane in 60 ml diethyl ether. All crude products were purified by column chromatography on silica gel.

Enantiomeric pure *R*-1-hydroxy-1-phenyl-2-propanone (**24**) was a gift from Knoll AG (Germany) and was racemized by treatment with 0.1 M sodium phosphate buffer (pH 7.7) at 90 °C. *R*- and *S*-3-hydroxy-4-phenyl-2-butanone (**21**) were synthesized from 0.03 mol (5.0 g) *R*- and *S*-phenyl lactic acid, respectively, by methylation, amidation and Grignard addition with iodomethane, as explained above. *S*-3-hydroxy-1-phenyl-2-butanone (**22**) was synthesized by addition of 0.05 mol (4.46 g) L-lactamide to a Grignard reagent prepared from 0.25 mol (6.08 g) Mg and 0.25 mol (42.8 g) benzyl bromide in 120 ml ether. *R,S*-3-hydroxy-1-phenyl-2-butanone was synthesized according to the method of Gill *et al.* (1986). 0.1 mol (10.8 g) trimethylsilylchloride was added dropwise to a mixture of 0.1 mol D,L-lactonitrile and 0.1 mol (7.9 g) pyridine in 50 ml dry ether and stirred over night. The formed precipitate was separated by filtration and the ether removed under reduced pressure. The silylated nitrile was then added to a Grignard reagent prepared from 0.1 mol (2.45 g) Mg and 0.1 mol (12.7 g) benzylchloride in 80 ml ether. Crude products were purified by preparative HPLC on a Lichrosorb Diol column (7  $\mu\text{m}$ , 250  $\times$  25 mm, Merck, Germany). HPLC was performed with a Jasco PU-980 intelligent HPLC gradient pump, a Jasco gradient former and a LC-UV-Detector (Pye Unicam,  $\lambda$  = 260 nm). Chromatographic conditions: Sample volume 2 ml, flow rate 10 ml  $\text{min}^{-1}$ , linear elution gradient from hexane (100%) to hexane/isopropanol (95/5 v/v) in 80 min.

*R,S*-3-Hydroxy-2-octanone (**23**) was obtained by bromination of 0.04 mol (5.2 g) 2-octanone with 0.16 mol (22.6 g) 2-bromo-2-methylpropane in 0.08 mol (6.2 g) DMSO (12 h reflux) and following hydrolysis of the resulting 3-bromo-2-octanone with 50 ml saturated  $\text{NaHCO}_3$ -solution (8 h at 90 °C) (Spiteller *et al.*, 1980; Armani *et al.*, 1984).

### Chiral analysis

The enantiomeric distribution of the enzymatically produced  $\alpha$ -hydroxyketones was determined by HRGC after derivatization to diastereomeric MTPA-esters. 10–100  $\mu\text{g}$  acyloin in 50  $\mu\text{l}$  dry diethyl ether was mixed with 4  $\mu\text{l}$  pyridine and 2  $\mu\text{l}$  *S*-2-methoxy-2-trifluoromethyl-2-phenylacetyl chloride (*S*-MTPA-Cl). After 16 h at room temperature the mixture was diluted with ether and analysed by HRGC-MS (as described above) on a BC SE 54 column (30 m  $\times$  0.32 mm i.d., film thickness 0.4  $\mu\text{m}$ , Leupold, Germany) under isothermal conditions (180 °C for (**21**) and (**24**), 165 °C for (**22**) and 130 °C for (**23**), respectively). Absolute configurations were determined by comparison with racemic and enantiomeric pure standards, which were analysed in the same way. The absolute configuration of enzymatically produced 3-hydroxy-2-octanone was identified by measuring the optical rotation (Perkin Elmer polarimeter 341,  $\lambda$  = 589 nm).  $\alpha_D^{25}$  (*S*-(**23**)) = +92° (Bel-Rhliid *et al.*, 1989).

Enzymatically synthesized acyloins were isolated by column chromatography or preparative HPLC as described above for the reference substances.

## Results

### Yeast biotransformations

Five of several amino acids tested were converted by fermenting yeast cells of *Zygosaccharomyces bisporus* to the corresponding  $\alpha$ -hydroxyketones (phenylalanine, tyrosine, valine, leucine, methionine), but only the two aromatic precursors gave larger product yields (Table I). No acyloin formation was observed with isoleucine, histidine, threonine and glutamic acid. Replacement of the convertible amino acids by their corresponding 2-oxo acids (if available) led to improved product yields and, moreover, to the first detection of the acyloins derived from isoleucine (Table I). The main transformation products proved to be the alcohols formed by transamination of the amino acids, subsequent decarboxylation and reduction by ADH (Fig. 1). There are two possible pathways of acyloin formation, leading to the shown isomeric structures. If amino acids or 2-oxo acids were added to the yeast cultures, the 2-hydroxy-3-oxo-acyloins represented the predominant isomer

Table I. Formation of acyloins (total of both isomers) by fermenting cells of *Zygosaccharomyces bisporus* from different precursors (concentration 10 mM).

Precursor	Acyloin from amino acid [mg l <sup>-1</sup> ]	Acyloin from 2-oxo acid [mg l <sup>-1</sup> ]
Phenylalanine	15.9	
Phenylpyruvic acid		17.5
Tyrosine	24.5	
4-Hydroxyphenylpyruvic acid		25.9
Valine	0.19	
3-Methyl-2-oxobutyric acid		0.49
Leucine	0.09	
3-Methyl-2-oxovaleric acid		0.51
Isoleucine	0	
4-Methyl-2-oxovaleric acid		0.23
Methionine	1.24	

formed (>90%). Keto-enol tautomerization was observed with all secondary  $\alpha$ -hydroxyketones and, thus, both possible isomers always occurred, especially in the presence of acids or higher temperatures.

#### Biotransformations using isolated PDC

In order to confirm the two proposed pathways shown in Fig. 1 and to gain further insight in the role of pyruvate decarboxylase in the biosynthesis of the  $\alpha$ -hydroxyketones obtained, incubation experiments with isolated PDC from *Zygosaccharomyces bisporus* were carried out. First, aldehydes and 2-oxo acids corresponding to the successfully

converted amino acids (if available) were incubated in the presence of pyruvate and acetaldehyde, respectively. 12 further aldehydes and two 2-oxo acids containing different structure elements (branched, unsaturated) were then checked for their precursor properties. As summarized in Table II, all of the aldehydes and most of the 2-oxo acids were converted to the expected acyloin isomers; merely 2-oxo glutaric acid and 4-hydroxyphenylpyruvate failed to yield the expected products. The condensation of aldehydes and pyruvate resulted in the formation of 3-hydroxy-2-oxo-acyloins as the main transformation products, while 2-oxo acids and acetaldehyde predominantly gave 2-hydroxy-3-oxo-acyloins. Acetoin resulting from condensation of pyruvate and acetaldehyde, or two molecules of acetaldehyde, was detected as side product only. The application of aliphatic aldehydes of different homologous series revealed several factors influencing their conversion to  $\alpha$ -hydroxyketones by PDC. The improved substrate suitability of long-chain aldehydes shows the importance of hydrophobic molecule properties, whereas the significantly worse transformation of branched aldehydes may be attributed to the steric hindrance of such structures.  $\alpha,\beta$ -unsaturated aldehydes showed lower conversion rates than their saturated analogs, which might be due to steric reasons (more rigid structure), changes in the reactivity of the carbonyl group and/or slightly different polarities. Remarkable differences in acyloin formation were shown upon incubation of the

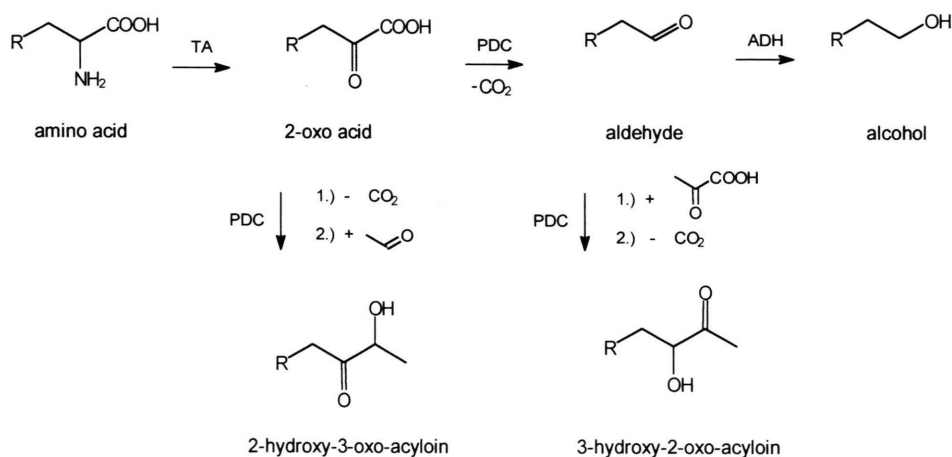


Fig. 1. Proposed formation of  $\alpha$ -hydroxyketones from amino acids by yeasts (TA = transaminase, PDC = pyruvate decarboxylase, ADH = alcohol dehydrogenase).



Table II. Formation of acyloins (total of both isomers) from different precursors ( $c_0$  118 mM) by isolated pyruvate decarboxylase.









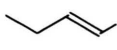
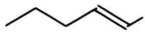


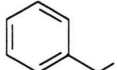
Precursor	Acyloin from aldehyde and pyruvate [ $\text{mg l}^{-1}$ ]	Acyloin from 2-oxo acid and acetaldehyde [ $\text{mg l}^{-1}$ ]
Propanal/ 2-Oxobutanoic acid	197	149
Butanal	300	
Pentanal	636	
Hexanal	1992	
Heptanal	2489	
2-Methylpropanal/ 3-Methyl-2-oxobutanoic acid	62	14
3-Methylbutanal/ 4-Methyl-2-oxopentanoic acid	42	43
2-Methylbutanal/ 3-Methyl-2-oxopentanoic acid	83	40
2-Methylpentanal	92	
2 <i>E</i> -Butenal	136	
2 <i>E</i> -Pentenal	319	
2 <i>E</i> -Hexenal	517	
2-Methyl-2 <i>E</i> -butenal	65	
3-Methyl-2 <i>E</i> -butenal	93	
3-(Methylthio)propanal	50	
Benzaldehyde/ Benzoylformic acid	2382	189
Phenylacetaldehyde/ Phenylpyruvic acid	238	8

aromatic compounds benzaldehyde and phenylacetaldehyde, the latter one resulting in product amounts tenfold lower than observed with benzaldehyde.

Compounds (**1**) to (**20**) (with the exception of (**3**), Moio *et al.*, 1994) have not been reported as natural products before and no mass spectrometric data were thus available for their identification. Acyloins (**1**) to (**6**) were therefore synthesized as references. All of them showed mass spectra and retention indices identical to those obtained by enzymatic conversion. Highly characteristic fragmentation patterns of these compounds also allowed the identification of further examined  $\alpha$ -hydroxyketones by their mass spectra. In each case, two primary fragments,  $m/z = 45$  and  $M^+ - 45$  (2-hydroxy-3-oxo-acyloins), or  $m/z = 43$  and  $M^+ - 43$  (3-hydroxy-2-oxo acyloins), resulted from  $\alpha$ -cleavage between C2 and C3. Subsequently, CO is eliminated from the ( $M^+ - 45$ )-fragment to give

( $M^+ - 45 - 28$ ), whereas  $\text{H}_2\text{O}$  is split off from ( $M^+ - 43$ ) to give ( $M^+ - 43 - 18$ ). Due to this recurring scheme and the fact that the respective acyloins were by far the main products of all studied biotransformations, further identifications were based on the mass spectrometric data without synthesis of reference substances (Table III). Surprisingly and in contrast to all other examples, the two isomers of acyloins branched at C4 (**5**) and (**6**), as well as (**7**) and (**8**), respectively, showed almost identical mass spectra, although being clearly separated by GC-MS. This may be explained by heat-catalysed elimination of water at C3 /C4 leading to a comparably stable tertiary carbocation, which is only possible from the 3-hydroxy-2-oxo-acyloin, thus favouring the keto-enol tautomerization in one direction. It has to be considered, however, that the hydroxy pentanones (**1**) and (**2**), branched at C4, too, did not show this peculiar fragmentation.

Table III. Mass spectrometric data of acyloins produced by pyruvate decarboxylase.

Residue	Products	$m/z$ (% relative abundance)
R	 	
	(1)	<b>116</b> ( $M^+$ , 0.4), 73 (96), 55 (100), 45 (27), 43 (94)
	(2)	<b>116</b> (1), 71 (28), 45 (100), 43 (84)
	(3)	<b>130</b> (0.3), 87 (25), 69 (63), 45 (42), 43 (100)
	(4)	<b>130</b> (0.7), 85 (65), 57 (100), 45 (83), 43 (31)
	(5)	<b>130</b> (0.3), 87 (33), 74 (26), 69 (27), 57 (22), 45 (55), 43 (100)
	(6)	<b>130</b> (0.4), 87 (32), 74 (28), 69 (26), 57 (22), 45 (52), 43 (100)
	(7)	<b>144</b> (0.2), 101 (32), 83 (50), 74 (35), 55 (61), 45 (55), 43 (100)
	(8)	<b>144</b> (0.2), 101 (61), 83 (52), 74 (35), 55 (59), 45 (57), 43 (100)
	(9)	<b>148</b> (18), 105 (16), 75 (75), 61 (100), 45 (36), 43 (62)
	(10)	<b>148</b> (23), 104 (15), 75 (52), 61 (100), 45 (84), 43 (38)
	(11)	<b>114</b> (2), 71 (100), 53 (37), 43 (83), 41 (59)
	(12)	<b>114</b> (1), 69 (100), 45 (45), 43 (21), 41 (49)
	(13)	<b>128</b> (1), 85 (93), 67 (33), 57 (56), 43 (100), 41 (66)
	(14)	<b>128</b> (1), 83 (100), 55 (54), 45 (100), 43 (66)
	(15)	<b>142</b> (0.2), 99 (28), 57 (100), 43 (35)
	(16)	<b>142</b> (0.2), 97 (64), 69 (10), 55 (100), 45 (34)
	(17)	<b>128</b> (3), 85 (100), 55 (30), 43 (71), 41 (48)
	(18)	<b>128</b> (2), 83 (67), 55 (100), 45 (48), 43 (28)
	(19)	<b>128</b> (1), 85 (100), 55 (32), 43 (37), 41 (51)
	(20)	<b>128</b> (1), 83 (100), 55 (50), 45 (14), 43 (13)
	(21)	<b>164</b> (0.3), 121 (49), 103 (59), 91 (100), 65 (32), 43 (46)
	(22)	<b>164</b> (1), 120 (21), 91 (100), 65 (33), 45 (53)

### Substrate suitability

With regard to product yields, aldehydes proved to be the more effective precursors compared to the corresponding 2-oxo acids (Table II). Within the homologous series of saturated linear aldehydes, acyloin formation increased with growing carbon chain length. The same tendency was observed with unsaturated linear aldehydes, but yielding considerably less product amounts than the saturated substrates of equal chain length. Branched aldehydes were poor precursors compared to linear ones. Benzaldehyde was converted at much higher rates than the second aromatic

substrate, phenylacetaldehyde. The frequently discussed opinion that pyruvate decarboxylase would show a high substrate specificity for aromatic aldehydes in acyloin formation (Fuganti and Grasselli, 1985; Long *et al.*, 1989; Bornemann *et al.*, 1996) did not apply for the enzyme of *Z. bisporus*.

### Stereospecificity of acyloin formation

The enantiomeric distribution of enzymatically produced acyloins was determined for 3-hydroxy-4-phenyl-2-butanone (**21**) and 3-hydroxy-1-phenyl-2-butanone (**22**) as aromatic representatives,

for 3-hydroxy-2-octanone as an aliphatic compound (**23**), and for 1-hydroxy-1-phenyl-2-propanone (PAC) (**24**) as the best studied product in order to compare the obtained results with published data. Benzaldehyde and benzoylformic acid were both converted to *R*-(**24**) in optical purities  $\geq 98\%$  by isolated PDC as well as by fermenting yeast cells of *Zygosaccharomyces bisporus* (Table IV). The isomeric 2-hydroxy-1-phenyl-1-propanone (**25**) as the expected main conversion prod-

uct of benzoylformic acid was detectable only in small amounts ( $<3\%$ ).

Conversion of phenylacetaldehyde and hexanal also yielded the respective *R*- $\alpha$ -hydroxy-ketones. The products *R*-(**21**) and *R*-(**23**) were, however, of considerably lower optical purity than compound *R*-(**24**) (Table IV). In contrast, the biotransformation of phenylpyruvate by PDC or whole yeast cells led to acyloin *S*-(**22**), thus being of opposite configuration than all other examples studied here or reported in literature.

Table IV. Enantiomeric distribution of  $\alpha$ -hydroxyketones produced by purified pyruvate decarboxylase and fermenting yeast cells of *Z. bisporus*.

Precursor	Main isomer and optical purity (% ee)			
	PDC		Yeast	
Benzaldehyde	<i>R</i> -( <b>24</b> )	$\geq 98\%$	<i>R</i> -( <b>24</b> )	$\geq 98\%$
Benzoylformic acid	<i>R</i> -( <b>24</b> )	$\geq 98\%$	—	—
Phenylacetaldehyde	<i>R</i> -( <b>21</b> )	58%	—	—
Phenylpyruvate	<i>S</i> -( <b>22</b> )	30%	<i>S</i> -( <b>22</b> )	84%
Hexanal	<i>R</i> -( <b>23</b> )	69%	—	—

## Discussion

The mechanism of decarboxylation and carbonylation by pyruvate decarboxylase has been studied intensively serving as a model reaction for thiamine-dependent enzymes (recent reviews: Pohl, 1997; Schellenberger, 1998). The different steps relevant for ThDP-catalysed decarboxylation and the formation of  $\alpha$ -hydroxyketones are summarized in Fig. 2. Carbanion **1** as reactive intermedi-

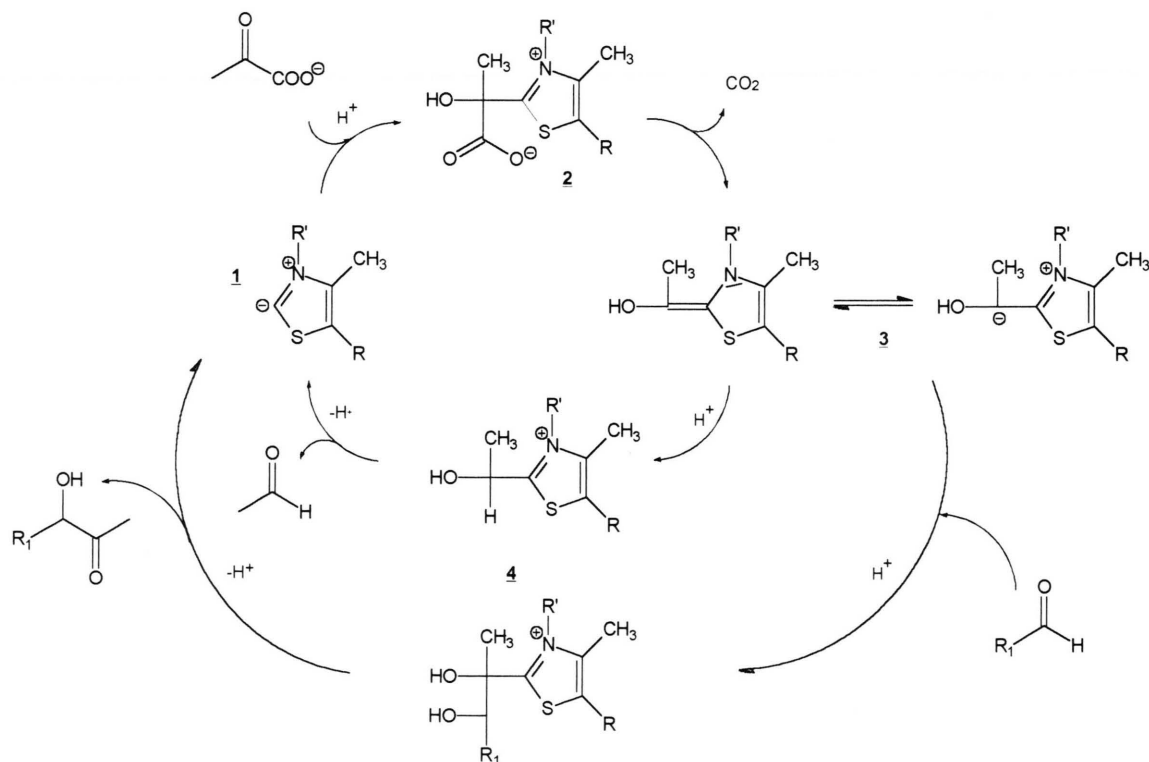


Fig. 2. Reaction path of enzymatic pyruvate decarboxylation and formation of  $\alpha$ -hydroxy-ketones.

ate performs a nucleophilic attack on the  $\alpha$ -carbonyl group of the keto acid. Decarboxylation leads to a carbanion-enamine intermediate **3**, also known as "active aldehyde", probably present in the mechanism of all ThDP-dependent enzymes (Iding *et al.*, 1998). A further protonation step gives compound **4**, which is regenerated to ThDP after release of the produced aldehyde. In the presence of higher concentrations of a second aldehyde, however, carbanion **3** can perform a nucleophilic attack on the carbon atom of the carbonyl group to form an  $\alpha$ -hydroxyketone. Since the protonation step to **4** is reversible (Chen and Jordan, 1984), intermediate **3** may also be obtained upon addition of an aldehyde to ThDP and subsequent deprotonation, allowing the formation of  $\alpha$ -hydroxyketones from two aldehydes or two molecules of a single aldehyde as precursors, irrespective of the presence of 2-oxo acids. This was revealed here by the formation of acetoin from acetaldehyde in the absence of pyruvate, although as side product only. Moreover, benzoylformic acid was not converted to the expected 2-hydroxy-1-phenyl-1-propanone (**25**) but to the isomeric acyloin *R*-(**24**) as observed with benzaldehyde. This unusual result can be explained by the assumption that after decarboxylation of benzoylformic acid benzaldehyde is first cleaved from the enzyme-product complex before reacting with a carbanion intermediate derived from ThDP and the co-substrate acetaldehyde.

Stereo-control of the described carboligase reaction led to the production of *R*-acyloins from aldehyde precursors. The high optical purity of *R*-1-hydroxy-1-phenyl-2-propanone (**24**) ( $\geq 98\%$  ee) derived from benzaldehyde is consistent with published data (Kren *et al.*, 1993; Lobell and Crout, 1996; Pohl, 1997). Benzoylformic acid (+ acetaldehyde) was converted to acyloin *R*-(**24**) in the same enantiomeric excess observed with benzaldehyde, supporting the above discussed assumption that the same reaction path as with benzaldehyde (+ pyruvate) was followed. Hexanal and phenylacetaldehyde were transformed by PDC to the acyloins *R*-3-hydroxy-2-octanone (**23**) and *R*-3-hydroxy-4-phenyl-2-butanone (**21**), respectively. The considerably lower optical purities of 69% and 58% ee may be attributed to a decreased stereo-selectivity of PDC towards aliphatic substrates and aromatic substrates with aliphatic side chains due to a less strictly orientated substrate binding.

*S*-3-hydroxy-1-phenyl-2-butanone (**22**) prepared from phenylpyruvate using cultures of *Z. bisporus* or isolated PDC was the only acyloin detected being predominantly of *S*-configuration. The low optical purity of enzymatically formed (**22**) does probably not represent the actual PDC stereo-selectivity. Racemisation via keto-enol tautomerization, which was particularly observed with poor substrates, such as phenylpyruvate, should rather be considered as a reason for this result.

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