Studies on Pyruvate Decarboxylase: Acyloin Formation from Aliphatic, Aromatic and Heterocyclic Aldehydes

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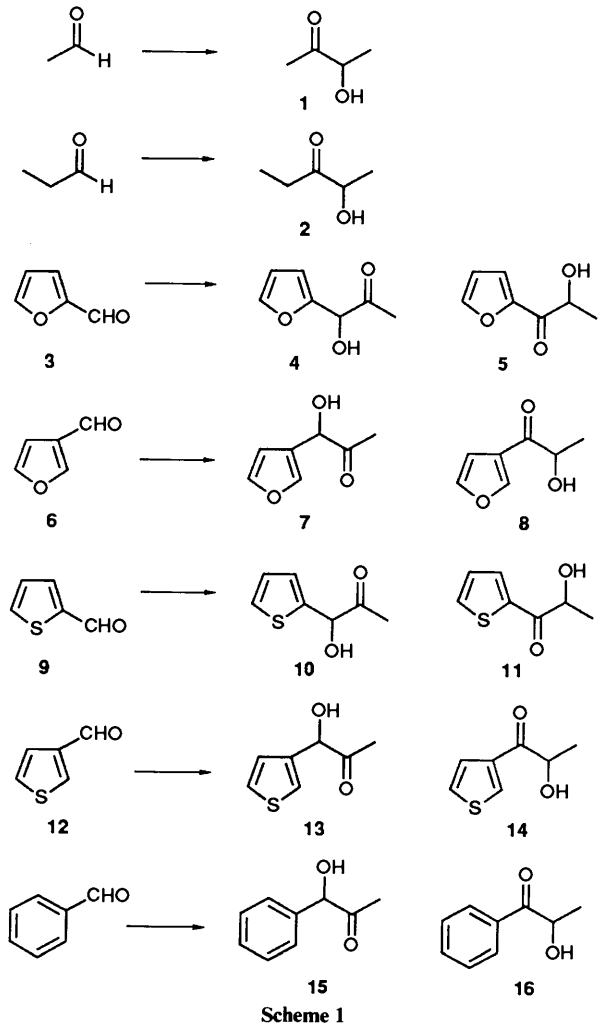
Evidence is described that supports the view that the enzyme responsible for acyloin formation from aldehydes in the yeast *Saccharomyces cerevisiae* is pyruvate decarboxylase.

In 1921, Neuberg and Hirsch described the conversion, by brewer's yeast, of benzaldehyde into L-3-hydroxy-3-phenylpropan-2-one ('phenylacetyl carbinol', PAC).[1] Subsequently, Neuberg obtained corresponding acyloins (ketols) from 2-chlorobenzaldehyde and anisaldehyde.[2] Later, Behrens and Ivanoff[3] demonstrated a similar biotransformation of *o*- and *p*-tolualdehydes. The formation of PAC from benzaldehyde assumed considerable importance when the reductive amination of PAC was shown to provide an efficient route to L-ephedrine.[4] Subsequently, others showed that many aldehydes were converted into the corresponding 'acetyl carbinols' (ketols).[5] The ketol condensation was carried out using yeast species other than *Saccharomyces cerevisiae* (baker's yeast) and *S. carlsbergensis* (brewer's yeast),[6] and also by bacterial species.[7] α-Keto acids other than pyruvate have been shown also to be capable of acting as acyl-anion equivalents in the biological ketol condensation.[8]

In spite of the great versatility of the yeast system in producing ketols, the enzyme(s) responsible have not been securely identified. The most likely candidates were enzymes having thiamine pyrophosphate as co-factor. The presence of high levels of such an enzyme, pyruvate decarboxylase (PDC, 2-oxo-acid carboxy-lyase, EC 4.1.1.1), in the yeasts most commonly used for biological ketol condensations (*S. cerevisiae* and *S. carlsbergensis*), suggested that this might be the enzyme responsible.[7b] We report here the results of investigations that strongly support this proposition.

Two aliphatic aldehydes (acetaldehyde and propionaldehyde), four heterocyclic aldehydes [2- and 3-furaldehyde (**3** and **6**), 2- and 3-thenaldehyde (**9** and **12**)] (Scheme 1) and benzaldehyde were incubated with yeast PDC in the presence of pyruvate as donor of the acyl-anion equivalent. The progress of the reaction was followed by ^{1}H NMR spectroscopy and by GC-MS. All of the aldehydes gave the expected ketols (**1**, **2**, **4**, **7**, **10**, **13** and **15**, respectively) (Scheme 1). This was evident in most cases from the NMR spectrum of the incubation mixture. Confirmation of the identities of the products was obtained by extraction of the products into $CDCl_3$ and determination of the 400 MHz NMR spectra and by extraction with chloroform or diethyl ether and examination by GC-MS. However, the effectiveness of the aldehydes as substrates varied markedly. When the NMR data were examined it became apparent that the aldehydes, to a varying degree, inhibited the enzyme. Inhibition data are given in Tables 1 and 2. Acetaldehyde and propionaldehyde were the least inhibitory substrates, but 2-furaldehyde **3** and even more so, 3-furaldehyde **6** were strongly inhibitory (Table 2). 2-Thenaldehyde **9** and benzaldehyde completely inhibited decarboxylation of pyruvate at concentrations of 0.07 and 0.05 mol dm^{-3}, respectively. The inhibition by benzaldehyde is interesting in relation to the observed effect of high concentrations of benzaldehyde on PAC production in whole-yeast systems. Fermentations have been carried out



Scheme 1

with benzaldehyde levels controlled at <0.8% (0.075 mol dm^{-3}).[6a,9–14] The deleterious effect of high concentrations of benzaldehyde has usually been attributed to its effect on the yeast. However, the build-up of intracellular concentrations could clearly lead also to inhibition of PDC with consequent effects on PAC production. Inhibition of PDC by benzaldehyde should therefore clearly be considered as a factor contributing to the decrease of PAC production with increasing concentrations of benzaldehyde.

With all of the aldehydes, besides the expected acyloins, evidence was obtained, both by NMR and GC-MS, of the production of the isomeric 1-acylethanol acyloins. With 3-furaldehyde **6** as substrate, the relative rates of formation of 3-(1′-hydroxy-2′-oxopropyl)furan **7**, acetoin (3-hydroxybutan-2-

Table 1 Inhibition of PDC-catalysed decarboxylation of pyruvate by aldehydes[a]

Aldehyde	Concentration (mol dm^{-3})	Pyruvate remaining (%) after 8 h	26 h
Acetaldehyde	0.5	68	9
Acetaldehyde	0.05	35	0
Propionaldehyde	0.5		37
Propionaldehyde	0.05	15	0
2-Furaldehyde **3**	0.5		95
2-Furaldehyde **3**	0.05	19	0
Control[b]		13	0

[a] The incubation mixture contained PDC (1.9 U). Initial concentration of pyruvate: 0.143 mol dm^{-3}. Other components of the incubation mixture were as described in the Experimental section. [b] Pyruvate only.

Table 2 Inhibition of PDC-catalysed decarboxylation of pyruvate by aldehydes[a] Time-course experiments[b]

Aldehyde	Concentration (mol dm^{-3})	Pyruvate remaining (%) after 8 h
Propionaldehyde	0.05	0
Propionaldehyde	0.10	0
Propionaldehyde	0.20	3
Propionaldehyde	0.30	8
Propionaldehyde	0.40	23
2-Furaldehyde **3**	0.10	0
2-Furaldehyde **3**	0.14	17
2-Furaldehyde **3**	0.20	39
3-Furaldehyde **6**	0.10	39
3-Furaldehyde **6**	0.14	61
3-Furaldehyde **6**	0.20	96

[a] Inhibition of pyruvate decarboxylation was 100% after 2 h in the presence of 0.07 mol dm^{-3} 2-thenaldehyde **9** or 0.05 mol dm^{-3} benzaldehyde. [b] The incubation mixture contained PDC (2.6 U). Initial concentration of pyruvate: 0.12 mol dm^{-3}. Other components of the incubation mixture were as described in the Experimental section.

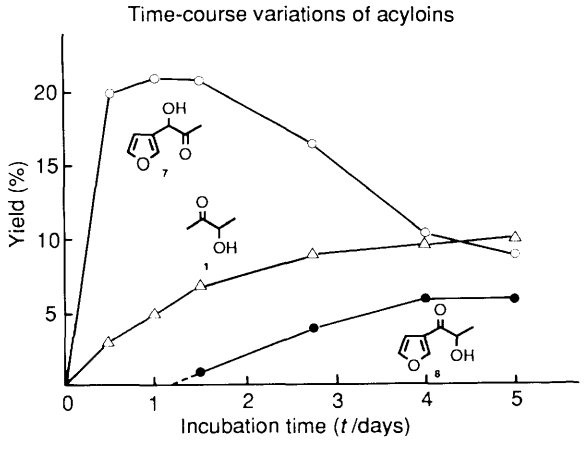


Fig. 1 Product formation during the incubation of 3-furaldehyde **6** and pyruvate with PDC. Details of the incubation conditions are given in the Experimental section

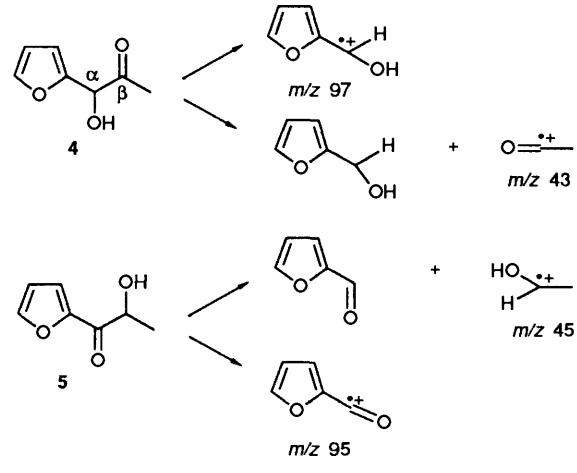
one) **1** and 3-(2′-hydroxy-1′-oxopropyl)furan **8** were as shown in Fig. 1. This clearly indicates that formation of the 'rearranged' product **8** occurred slowly, and only after formation of the initial product **7** had reached a maximum. (GC-MS data also showed that, over the time-course of this reaction, significant amounts of product were converted into the corresponding diketone). A comparable rearrangement of 3-hydroxypentan-2-one **17** on incubation with acetolactate decarboxylase [(*S*)-2-hydroxy-2-methyl-3-oxobutanoate carboxylyase, EC 4.1.1.5] has been observed (Scheme 2). In this case, the rearrangement was clearly

Scheme 2

enzyme-catalysed, as the conversion was stereoselective and stereospecific; the (*R*)-enantiomer of the ketol **17** was converted into the (*R*)-enantioner of ketol **2**. However, this rearrangement was extremely slow even in the presence of high concentrations of enzyme.[15] In the present case, little 'rearranged' product was observed with benzaldehyde as substrate, and practically none with propionaldehyde. It had been suggested that rearrangement of tertiary ketols occurred under electron impact in the mass spectrometer (Scheme 3),[16] but later it was shown that the

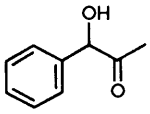
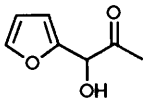
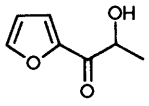
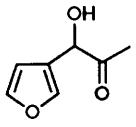
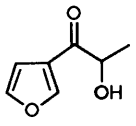
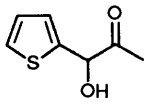
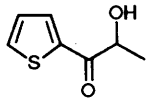
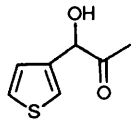
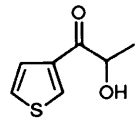
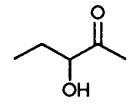
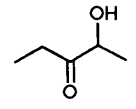
Scheme 3

rearrangement was attributable to catalysis by metal surfaces in the inlet systems.[17,18] Some rearrangement may therefore have occurred in the GC system. However, the clear evidence for the formation of 'rearranged' product in the NMR analysis [for example, of the product from 3-furaldehyde (Fig. 1)], coupled with the fact that GC analysis of the products from incubation of 2-furaldehyde showed little 'rearranged' ketol after 12 h (< 20% of total ketol), suggested that rearrangement in the GC system was minimal. The proportions of 'rearranged' product **16** from incubation of benzaldehyde, and from propionaldehyde as indicated by GC (23 and 9%, respectively, after incubation for 19 and 22 h, respectively) supports this conclusion. However, the mass spectra of the individual ketols separated by GC during GC-MS analysis indicated that rearrangement does occur under the conditions of mass spectrometric analysis. The 'acetyl carbinols' gave, as expected, ions of *m*/*z* 43, arising as indicated from the ketol **4** derived from 2-furaldehyde (Scheme 4). The corresponding fragmentation of the rearranged ketol **5** gave an abundant ion at *m*/*z* 45. A corresponding pattern was seen for the products **7** and **8** (Scheme 1) from 3-furaldehyde.

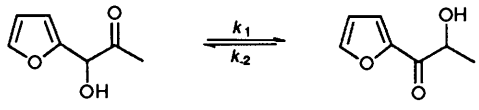


Scheme 4

Table 3 Mass spectrometric data for ketols produced on incubation of aldehydes with PDC *m/z*

Ketol		*m/z* (% relative abundance)
	**15**	150 (M^+, 2), 107 (89), 105 (57), 79 (100), 77 (89), 43 (23)
	**4**	140 (M^+, 10), 138 (8), 97 (100), 95 (51), 43 (54)
	**5**	140 (M^+, 3), 138 (6), 95 (100), 45 (70)
	**7**	140 (M^+, 8), 138 (2), 97 (100), 95 (53), 43 (43)
	**8**	140 (M^+, 2), 138 (0.4), 95 (100), 45 (16)
	**10**	156 (M^+, 9), 154 (8), 113 (90), 111 (100), 45 (30), 43 (22)
	**11**	156 (M^+, 3), 154 (4), 111 (100), 45 (69), 43 (28)
	**13**	156 (M^+, 5), 154 (1), 113 (90), 111 (100), 85 (92), 45 (49), 43 (28)
	**14**	156 (M^+, 2), 113 (27), 111 (100), 85 (19), 45 (12), 43 (8)
	**17**	102 (M^+, 2), 59 (100), 57 (17), 45 (18), 43 (57)
	**2**	102 (M^+, 4), 59 (21), 57 (70), 45 (100), 43 (39)

The ions formed by cleavage of the same bond, but with charge transfer to the heterocyclic fragment, had *m/z* 97 and 95 respectively (Scheme 4 and Table 3). In the mass spectrum of the acyloin **6**, the fragment of *m/z* 97 was the base peak, whereas in the mass spectrum of the 'rearranged' product **5**, the peak of *m/z* 95 was the base peak. In both cases, the peak (*m/z* 95 and 97, respectively) corresponding to the isomeric ketol was also observed. A very similar pattern was found for the ketols **7** and **8** (Scheme 1) derived from 3-furaldehyde. Very much the same picture is seen with the ketols **10**, **11**, **13** and **14** derived from 2- and 3-thenaldehyde (Table 3). Here, however, ions of *m/z* 45 and 43 were relatively abundant in the mass spectra of all four compounds. This suggests that, during cleavage of the C_α–C_β bond, charge is evenly distributed in the heterocyclic and aliphatic fragments with the ketols derived from the thenaldehydes compared with the situation with benzaldehyde or the furaldehydes (*cf.* Scheme 4). In the mass spectrum of compound **11** derived from 2-thenaldehyde, ions attributable to the isomeric ketol were of very low abundance. The same was true for the isomer **14** derived from 3-thenaldehyde, although ions attributable to the corresponding 'acetyl carbinol' (*m/z* 113, 43) were somewhat more abundant. These data are consistent with the rearrangement (illustrated for the products from 2-furaldehyde in Scheme 5) with $k_1 > k_2$. This would be expected,



4 **5**

Scheme 5

since the equilibrium should favour the more highly conjugated isomer **11**.

With 3-hydroxypentan-2-one **17** and 2-hydroxypentan-3-one **2** the rates are much more evenly balanced (Scheme 6). The ketol **17** gives a spectrum with ions of *m/z* 43 and 45 in the ratio ~2:1, whereas in the mass spectrum of ketol **17** the ratio is ~1:2.[19]

17 **2**

Scheme 6

Tertiary ketols can undergo rearrangement only by 1,2-alkyl migration (Scheme 3). With secondary ketols an alternative mechanism is available, namely that involved in tautomerisation resulting in an overall 1,3-hydrogen shift from carbon to oxygen. Either or both mechanisms might operate in the present case. The tautomerisation mechanism is most unlikely to occur in the gas phase under EI conditions. However, given that thermal rearrangement of tertiary ketols by 1,2-group migration is possible[20] rearrangement by 1,2-hydride ion migration (for which there is precedent in closely related benzilic acid rearrangements[21]) cannot be ruled out.

Except with benzaldehyde, formation of ketols from aldehyde substrates was invariably accompanied by acetoin formation. Relevant data are given in Tables 4 and 5. These data clearly indicate the competitive inhibition of acetoin formation by added aldehyde. The data for propionaldehyde and the furaldehydes are particularly striking. Increasing concentrations of these aldehydes led to increasing levels of the corresponding ketols with decreased acetoin production which, eventually, was totally suppressed. Higher concentrations of aldehydes also led to suppression of the formation of the corresponding 'acetyl carbinol' (Table 5).

The present results strongly support the suggestion that PDC is the enzyme responsible for the numerous acyloin condensations demonstrated in various genera and species of yeast. The mechanism of formation of 'rearranged' ketols is not clear. Enzyme-catalysed isomerisation cannot be ruled out. (Favorsky, in an uncorroborated report,[22] stated that on incubation with yeast, rearrangement of PAC to the isomeric ketol occurred). However, PAC is quite labile with respect to rearrangement[23] and it is known that thermodynamic stability can favour the exclusive formation of one isomer over the other.[23,24] It is therefore probable that the rearrangements

Table 4 Acetoin formation catalysed by PDC in the presence of aldehydes[a]

Aldehyde	Concentration of aldehyde (mol dm^{-3})	Concentration of acetoin (mmol l^{-1} dm^{-3}) after 8 h	26 h
b		12.6	18.1
Acetaldehyde	0.5	29.9	61.4
Acetaldehyde	0.05	12.2	33.7
Propionaldehyde	0.5		6.9
Propionaldehyde	0.05	14.5	20.5
2-Furaldehyde **3**	0.5		0
2-Furaldehyde **3**	0.05	10.6	13.7

[a] Conditions: PDC 1.9 U, sodium pyruvate 0.143 mol dm^{-3}, 30 °C. [b] Sodium pyruvate (0.143 mol dm^{-3}) only.

Table 5 Ketol formation catalysed by PDC in the presence of aldehydes. Concentration dependence[a]

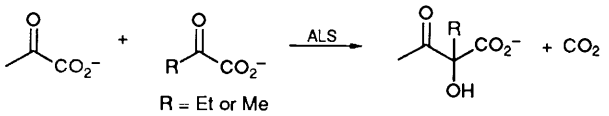
Aldehyde	Concentration (mol dm^{-3})	Concentration (mmol dm^{-3}) of Acetoin	Ketol[b]
Propionaldehyde	0.05	22.9	0
Propionaldehyde	0.10	22.8	0
Propionaldehyde	0.20	13.7	13.7
Propionaldehyde	0.30	13.2	17.6
Propionaldehyde	0.40	13.1	13.1
2-Furaldehyde **3**	0.10	7.1	35.4
2-Furaldehyde **3**	0.14	6.6	47.2
2-Furaldehyde **3**	0.20	0	39.1
3-Furaldehyde **6**	0.10	5.1	20.3
3-Furaldehyde **6**	0.14	1.2	24.0
3-Furaldehyde **6**	0.20	0	0

[a] Conditions: PDC 2.6 U, sodium pyruvate 0.12 mol dm^{-3}, 30 °C. [b] Ketols estimated (after 8 h incubation): 3-hydroxypentan-2-one (from propionaldehyde), 2-(1′-hydroxy-2′-oxopropyl)furan (from 2-furaldedehyde), 3-(1′-hydroxy-2′-oxopropyl)furan (from 3-furaldehyde).

observed in the present work are non-enzymatic and that the extent of rearrangement is dependent on α-hydrogen acidity and thermodynamic stability.

The results also show that PDC-catalysed ketol formation is subject to inhibition by aldehyde substrates. Optimal ketol formation therefore depends on the best compromise between aldehyde concentration in relation to inherent K_m-values, and inhibitory activity. A possible benefit of the inhibitory activity of aldehydes is the suppression of competing acetoin formation, with a corresponding increase in the efficiency with which pyruvate is used for the formation of target ketols.

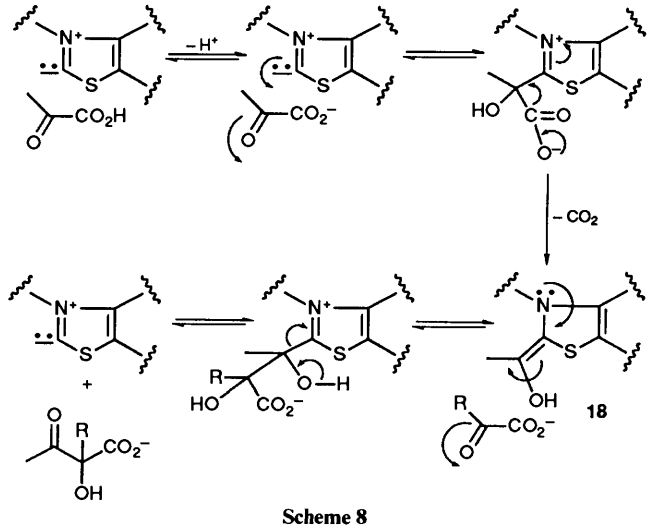
The behaviour of PDC is best interpreted in terms of a two-site mechanism as originally proposed by Juni[25] and confirmed by others.[26] Considerable light has been shed on the behaviour of the enzyme by the recent demonstration that it has considerable sequence homology with acetolactate synthase [ALS, acetolactate pyruvate-lyase (carboxylating), EC 4.1.3.18)



Scheme 7

and pyruvate oxidase.[27] This suggests that these enzymes have much in common mechanistically. ALS is an enzyme of valine–isoleucine biosynthesis and catalyses the reactions of Scheme 7. Significantly, this reaction results in carbon–carbon bond formation as observed in the PDC catalysis of acyloin formation.

ALS has two binding sites. At one, pyruvate is bound and reacts with thiamine pyrophosphate to give, after decarboxylation, 'active acetaldehyde' (**18**, Scheme 8). This condenses with

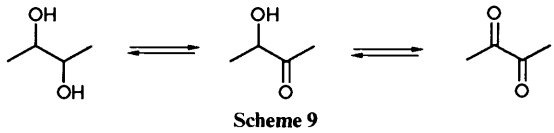


Scheme 8

a second molecule of pyruvate or α-oxobutyrate at the second binding site to generate the α-hydroxy-β-keto acid products. Extrapolation to PDC suggests that pyruvate is strongly and selectively bound at the first binding site and the acceptor aldehydes at the second binding site.

ALS strongly and selectively binds pyruvate at the first site. This is true also for PDC, since the rate of ketol formation is much lower if aldehydes are used as substrates.[28] However, the second site is more flexible. With ALS, α-oxobutyrate is readily bound (selectively with respect to pyruvate with both the bacterial[29] and plant[30] enzymes). With PDC an extremely wide range of substrates apparently can be bound at the second site.[31] In this connection, it is of interest that the herbicidal inhibitors of ALS inhibit binding of α-keto acids to the second site. These inhibitors are complex aromatic/heterocyclic substances that are presumed to bind to the vestigial binding site in ALS, which is not a redox enzyme, related to the quinone-binding site in pyruvate oxidase, which is a redox enzyme and needs to bind quinone co-factors in order to reoxidise the primary redox co-factor flavin adenine dinucleotide. It is tempting to speculate that the various aldehyde substrates of acyloin condensations catalysed by PDC might bind at a corresponding vestigial binding site.

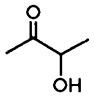
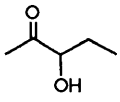
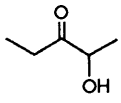
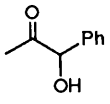
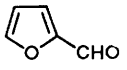
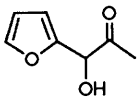
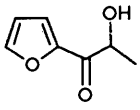
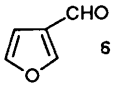
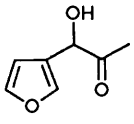
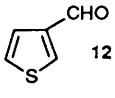
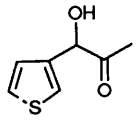
All the available evidence suggests that PDC has a dual role in yeasts. It converts pyruvate into acetaldehyde and at the same time catalyses acetoin formation. The role of acetoin, presumably, is that of a redox 'buffer', assisting in the attainment of appropriate levels of redox co-factors through the reversible reactions of Scheme 9.



Scheme 9

The kinetics of PDC action are complex.[32] However, since it can now be regarded as highly probable that PDC is the enzyme responsible for the very large range of biological

Table 6 ^{1}H NMR data for ketols produced on incubation of aldehydes with PDC

Aldehyde substrate	Ketol produced		Chemical shift (δ) [coupling constant (Hz)] for				
			MeCO	MeCOC*H*(OH)	*Me*CH(OH)	MeC*H*(OH)	Other
		**1**	2.20	4.25 (7.00)	1.39 (7.11)		
		**17**	2.19	4.17 (dd, 2.63, 6.71)			0.94 (t, 7.47), 1.80 (m)
"		**2**			1.38 (7.03)	4.25 (7.00)	1.11 (t, 7.57), 2.50 (m)
PhCHO		**15**	2.08	5.09			7.3–7.4
 **3**		**4**	2.15	5.15			
"		**5**			1.65 (7.24)[a]	5.21[a]	
 **6**		**7**	2.15	5.09			6.30 (d, 1.79), 7.42 (dd, 1.70 1.72), 7.53
 **12**		**13**	2.12	5.22			6.9–7.4

[a] D_2O solution.

acyloin condensations known to be catalysed by yeasts, it will be possible to harness the considerable synthetic potential of both the isolated enzyme, and to some extent the whole cell system, in a more rational and predictable manner.

Very few data are available on the optical purities of products of biological acyloin condensations. This problem is currently under investigation in our laboratory.

Experimental

NMR spectra were determined using Bruker WH 400 and Perkin-Elmer R34 spectrometers. Mass spectra were determined using a Kratos MS 80 mass spectrometer. Yeast PDC was purchased from the Sigma Chemical Company Ltd.

Incubation of Aldehydes with Yeast PDC.—A stock solution of sodium pyruvate (0.117 mol dm^{-3}), $MgSO_4$ (0.0025 mol dm^{-3}) and thiamine pyrophosphate (1.25×10^{-5} mol dm^{-3}) was prepared. To an aliquot (800 mm^3) of this solution was added a solution of sodium 4,4-dimethyl-4-silapentane-1-sulphonate [50 mg cm^{-3} in 0.214 mol dm^{-3} phosphate buffer (50 mm^3) (pH 5.9)], PDC (2.6 U; 90 or 62 mm^3) and the aldehyde. The aldehyde and the enzyme solution were mixed and the total volume was adjusted to 150 mm^3 with the above phosphate buffer. The total volume of the incubation mixture was 1 cm^3. Samples were placed in an NMR tube and incubated at 25 °C. The progress of the reaction was monitored by NMR spectroscopy at 220 MHz. For more detailed examination, the solution was extracted with $CDCl_3$, and the extract was dried ($MgSO_4$)

and examined by ^1H NMR spectroscopy at 400 MHz. Samples for GC or GC-MS analysis were extracted using either $CHCl_3$ or Et_2O. ^1H NMR data are given in Table 6.

GC Analysis.—This was carried using a fused silica capillary column (25AO 2/BP 20025; 25 m, i.d. 0.22 mm; film. 0.25 μm). The following conditions were used for GC analysis of the products from the incubation of the indicated aldehydes with PDC: propionaldehyde (72 °C, isothermal); benzaldehyde, 100 °C (15 min); then 8 °C min^{-1} to 180 °C; 180 °C (10 min); 2-furaldehyde **3**, 100 °C; then 16 °C min^{-1} to 180 °C; 180 °C (10 min); 3-furaldehyde **6**, 100 °C; then 5 °C min^{-1} to 180 °C; 2-thenaldehyde **9**, 100 °C; then 5 °C min^{-1} to 200 °C; 3-thenaldehyde **12**, 100 °C; then 5 °C min^{-1} to 200 °C.

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