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Asymmetric acyloin condensation catalysed by phenylpyruvate decarboxylase. Part 2: Substrate specificity and purification of the enzyme

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Abstract—Phenylpyruvate decarboxylase from *Achromobacter eurydice* was used to catalyse the asymmetric acyloin condensation of phenylpyruvate **1** with various aldehydes **2** to produce optically active acyloins $PhCH_2COCH(OH)R$ **3**. The specific activity of the phenylpyruvate decarboxylase enzyme was increased by a factor of 332 after its purification. The molecular weight of the purified enzyme was shown to be 150 kDa by gel filtration chromatography, while SDS gel electrophoresis showed two sub-units with molecular weights of 90 and 40 kDa. The acyloin condensation yield decreased with increasing chain length for straight chain aliphatic aldehydes from 76% for acetaldehyde to 24% for valeraldehyde. The e.e.s of the acyloin products were 87–98%. Low yields of acyloin products were obtained with chloroacetaldehyde (13%) and glycoaldehyde (16%). Indole-3-pyruvate was a substrate of the enzyme and provided acyloin condensation product 3-hydroxy-1-(3-indolyl)-2-butanone **5** with acetaldehyde in 19% yield, while benzoylformate was not a substrate for the enzyme.

1. Introduction

Chiral unymmetrically substituted acyloins (α-hydroxyketones) are important classes of intermediates in organic synthesis due to their bifunctional nature, and the fact that they have a stereogenic centre amenable to further synthetic manipulation. Present chemical methods for acyloin synthesis generally give racemic products.[1,2] Enzyme mediated acyloin formation would provide an environmentally friendly method to prepare chiral acyloins.[3] Acyloin formations mediated by yeast pyruvate decarboxylase (EC 4.1.1.1)[4,5] and bacterial benzoylformate decarboxylase (EC 4.1.1.7)[6,7] have been studied by a number of researchers. In our previous work,[8] we reported that phenylpyruvate decarboxylase (EC 4.1.1.43) catalysed the asymmetric acyloin condensation of phenylpyruvate **1** with acetaldehyde **2a** to produce (*R*)-(−)-3-hydroxy-1-phenyl-2-butanone **3a** (Scheme 1). Herein, we describe the purification and immobilisation of the enzyme and the substrate specificity of the enzyme for both acyl donors (α-keto-acids) and acyl acceptors (aldehydes).

2. Results and discussion

2.1. Enzyme purification

Purification of phenylpyruvate decarboxylase from *Achromobacter eurydice* SC16386 was performed. The specific activity of the enzyme was increased 332-fold compared to the crude cell extract by six successive column purification steps (Table 1). The purified enzyme eluted as a single peak on gel filtration chromatography with a molecular weight of 150 kDa. SDS gel electrophoresis showed two bands, probably corresponding to sub-units with molecular weights of 90 and 40 kDa. By using the most active fraction of phenylpyruvate decarboxylase from the UNO Q1 column of purification step 4, (*R*)-(−)-**3a** was obtained in 91% yield with an e.e. of 85%. The use of purified enzyme minimised decarboxylation and other side reactions and afforded higher yields of acyloin products.

2.2. Enzyme immobilisation

The enzyme from the cell extracts of *A. eurydice* was immobilised on DE52 ion exchange resin. Immobilised enzyme was used to run 14 reaction cycles (usually reaction time of one day per cycle). The activity of the immobilised enzyme dropped significantly after the first two cycles (from 0.084 unit in the first to 0.032 unit for the third cycle). Subsequently, the activity loss per cycle

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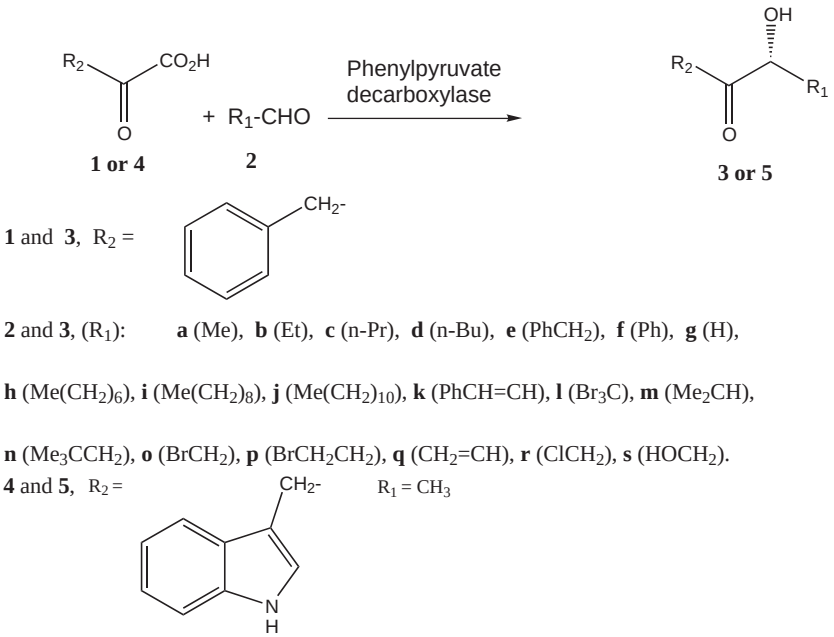

Scheme 1.

Table 1. Purification of phenylpyruvate decarboxylase from *Achromobacter eurydice* SC16386

Purification step	Total protein (mg)	Total activity (unit = umol/h)	Specific activity (unit/mg protein)	Purification factor
Crude cell extract	8634	47.62	0.0055	1
Step 1: DE52	550	29.00	0.0527	10
Step 2: phenyl sepharose	28	11.21	0.4004	73
Step 3: sephacryl S-100/desalting	10.04	5.48	0.5458	99
Step 4: UNO-Q1	3.22	[a]	[a]	[a]
Step 5: Superdex 200	1.98	2.21	1.1162	203
Step 6: ISO column/desalting	0.34	0.62	1.8235	332

[a] Salt interfered with the activity assay and the activity was not determined.

was minimal and appreciable activity (0.013 unit) remained even after all 14 cycles.

2.3. Substrate specificity of different aldehydes as acyl acceptors

The substrate specificity of the enzyme for different aldehydes as acyl acceptors was studied by using phenylpyruvate **1** as the acyl donor (Scheme 1). The crude cell extract was used in the initial study to examine the effect of acetaldehyde concentration on the acyloin condensation. The results are shown in Fig. 1. An optimum aldehyde concentration was necessary so as to obtain the highest yield of the acyloin product. The yield of acyloin product was low at low aldehyde concentrations, probably due to decarboxylation and other competing reactions, while at high aldehyde concentrations the enzyme was probably inhibited. The best yields of 34–35% were obtained at acetaldehyde concentrations of 50–100 mM with 5 mM concentration of phenylpyruvate using the crude cell extract.

A partially purified enzyme was prepared from the cell extract of *A. eurydice* by DE52 ion exchange chromatography and ammonium sulfate precipitation (60% saturation). This partially purified enzyme preparation enabled the use of higher amounts of protein in concentrated form and to obtain higher yields of the acyloin condensation product. This enzyme preparation was stable when stored at about 4°C (no change in activity after 64 days) and was used in the substrate specificity studies. The rate of product **3a** formation by the partially purified enzyme was nearly parallel to substrate **1** consumption. Product **3a** was stable under the reaction condition and was obtained in 76% yield.

The specificity of the enzyme with different aldehyde substrates was studied using the partially purified enzyme. As with acetaldehyde, there was an optimum concentration for each aldehyde (Fig. 1). The yields of reactions using the optimum concentration of each aldehyde were compared (Table 2). With straight chain aliphatic aldehydes, the yield decreased with increasing chain length and the yields of acyloin products were in the following order for different R groups: Me, 76%> Et, 55%>*n*-Pr, 38%>*n*-Bu, 24%. Using longer chain aldehydes **2h**, **2i**, **2j**, aromatic aldehydes **2e**, **2f**, **2k**, hindered aldehydes **2l**, **2m**, **2n**, bromoacetaldehyde **2o**, bromopropionaldehyde **2p** and acrolin **2q**, either none

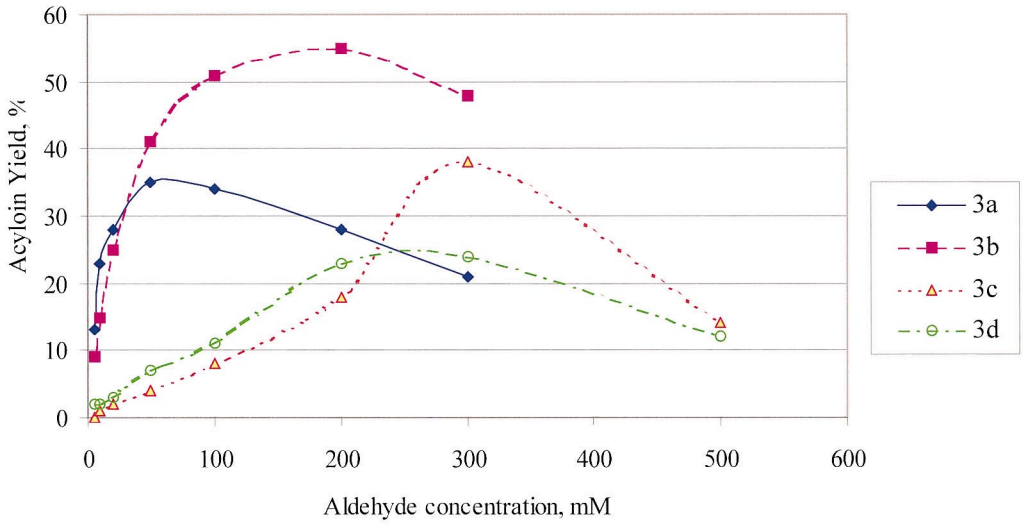

Figure 1. Acyloin condensation of **1** with aldehydes **2a**, **2b**, **2c**, and **2d**.

Table 2. Acyloin condensation catalysed by the partially purified enzyme

Product entry	R	Optimum aldehyde concentration (mM)	Yield (%)	Enantiomeric excess (%)
3a	Me	80	76	87
3b	Et	200	55	98
3c	*n*-Pr	300	38	98
3d	*n*-Bu	300	24	92
3e–3q			<3	[a]
3r	$ClCH_2$	50	13	[a]
3s	$HOCH_2$	50	16	[a]

[a] Not determined.

or negligible amounts (0–3%) of the acyloin products were obtained. The acyloin products were obtained in 13 and 16% yields with chloroacetaldehyde **2r** and glycoaldehyde **2s**, respectively. Formaldehyde **2g** was not suitable as a substrate for the enzyme.

The substrate specificity observed for phenylpyruvate decarboxylase is different from benzoylformate decarboxylase, though both accept acetaldehyde as a substrate. The later accepts aromatic aldehydes, but does not accept propionaldehyde, chloroacetaldehyde, and glycoaldehyde.[7,9]

HPLC analysis of the enzymatic acyloin condensation products **3a**–**3d** showed that the first eluting enantiomer was produced predominantly with each aldehyde, whilst the condensation was highly enantiospecific providing 87–98% enantiomeric excesses (e.e.) of the first eluting enantiomer with different aldehydes. The (−)-(*R*)-**3a** enantiomer has been synthesised previously and its absolute configuration determined.[8] Although the absolute configurations of the enzymatic acyloin products **3b**–**3d** were not established conclusively because the pure (−)-(*R*)-**3a** enantiomer was found to have similar retention times in HPLC to the major, first eluting products, this indicated that they have (*R*)-configuration.

2.4. Substrate specificity of different α-keto-acids as acyl donors

The substrate specificity of the enzyme was also studied for different α-keto-acids. Enzymatic acyloin condensation of indole-3-pyruvic acid **4** with acetaldehyde gave 3-hydroxy-1-(3-indolyl)-2-butanone **5** in 19% yield (Scheme 1). The acyloin product was isolated and structure **5** was established by 1D and 2D ^{1}H NMR and LCMS. Using benzoylformic acid **6** as an acyl donor and acetaldehyde as an acyl acceptor, no acyloin product was found. There was no decarboxylation to form benzaldehyde. Thus bezoylformate is not a suitable substrate for phenylpyruvate decarboxylase from *A. eurydice*.

3. Experimental

3.1. Chemicals and microorganisms

Chemicals were purchased from VWR and/or Aldrich. DE52 cellulose was purchased from Whatman Inc. Phenylsepharose was obtained from Pharmacia Biotech. The UNO Q1 ion exchange column was from Bio-Rad. *Achromobacter eurydice* SC16386 was obtained and grown in a large scale fermentor as described previously.[8]

3.2. Analytical methods

^{1}H NMR spectra were recorded in $CDCl_3$ using a JEOL Eclipse 400 operating at 400 MHz. When necessary, 2% D_2O was added as indicated.

HPLC method 1 was used for identification and quantification of different components, while methods 2 and 3 were used for determination of e.e.s

Method 1 was performed on a reversed phase C-18 column (5 μm, 15×0.46 cm, Kromasil) at 40°C with a flow rate of 1.0 mL/min using gradient elution as follows: 75% solvent A (0.1% H_3PO_4 in water) and 25% solvent B (CH_3CN) for the first 10 min, then increasing to 100% solvent B over 20 min. UV detection was at 210 nm. Retention times for phenylpyruvate, phenylacetic acid, and phenylacetaldehyde were 5.0, 6.7, and 8.0 min, respectively. Retention times for chemically prepared racemic acyloins **3a**–**3f** were 7.5, 13.6, 17.5, 20.0, 20.0, and 18.9 min, respectively.

Method 2 for separation of acyloin enantiomers of **3a**, **3b**, **3c**, **3e**, and **3f** was performed on a Chiralpak AD column (25×0.46 cm, Daicel) at ambient temperature with a mixture of hexane–isopropanol–ethanol (95.4:4.0:0.6) as eluent at a flow rate of 1.0 mL/min and UV detection at 210 nm. Retention times in minutes for the two enantiomers were as follows: **3a** (14.8/18.4), **3b** (11.3/16.2), **3c** (10.4/12.2), **3e** (17.8/19.1), and **3f** (17.5/20.7).

Method 3 for separation of enantiomers of **3d** was the same as method 2 except the eluent was a mixture of hexane:*iso*-propanol (99.2:0.8). Retention times for the two enantiomers of **3d** were 38.0 and 46.7 min.

LCMS analysis was performed on a reversed phase C-18 column (5 μm, 5×0.46 cm, Luna) at ambient temperature with a flow rate of 4.0 mL/min using gradient elution from solvent A (5% CH_3CN–95% H_2O–10 mM NH_4OAc) to solvent B (95% CH_3CN–5% H_2O–10 mM NH_4OAc) in 4 min with positive ion electrospray (ES+) or negative ion electrospray (ES−) methods.

3.3. Enzyme activity assay

The enzyme activity was assayed by acyloin condensation of **1** with **2a**. A mixture of enzyme fraction (500 μL), potassium phosphate buffer (100 μL, 500 mM, pH 6.8), $MgCl_2$ (30 μL, 100 mM), thiamine pyrophosphate (TPP, 20 μL, 50 mM), water (250 μL), **1** (50 μL, 100 mM), and **2a** (50 μL, 1 M) was stirred at 28°C for 20 h. The reaction was terminated by addition of aqueous HCl (50 μL, 1 M) and MeOH (950 μL). The resulting mixture was filtrated and subjected to HPLC method 1 to determine the amounts of product **3a**. In some cases, the same procedure was employed on a smaller scale. One unit of activity was defined as 1 μmol **3a** formed per h.

3.4. Protein assay

The Bio-Rad protein assay was used to determine protein concentration.[10] Samples containing 1–20 μL of enzyme fraction were diluted to 0.8 mL with water. Bio-Rad reagent (0.2 mL) was added and the solution was mixed thoroughly. The protein concentration (mg/mL) was calculated from the standard curve with bovine serum albumin as standard.

3.5. Enzyme purification

All purification processes were carried out at about 4°C. *A. eurydice* cells (100 g) were suspended in buffer A (500 mL) (100 mM potassium phosphate, 1 mM EDTA, 0.1 mM DTT, 2 mM $MgSO_4$, 0.1 mM TPP, 10% glycerol, pH 6.8). The cells were disrupted by three passages through a microfluidiser (Model 110F, Microfluidics Co., Newton, Massachusetts, USA). The resulting suspension was centrifuged at 27 000 *g* for 1 h. The active supernatant was collected as the crude cell extract.

3.5.1. Enzyme purification step 1: DE52 column. The crude cell extract was applied on to a DE52 cellulose ion exchange column (14×5.0 cm) previously equilibrated with buffer A. The column was washed with buffer A (1 L), followed by gradient elution from buffer A to buffer B (B is the same as A, except 200 mM w.r.t potassium phosphate—gradient complete in 800 mL), then from buffer B to buffer C (0.8 M NaCl in buffer B—gradient complete in 800 mL). The fractions with high specific activity were combined.

3.5.2. Enzyme purification step 2: phenylsepharose column. The active fractions from step 1 were treated with NaCl (10 g), adjusted to 60% $(NH_4)_2SO_4$ saturation and centrifuged at 27 000 *g* for 1 h. The precipitate was dissolved in buffer D (80 mL) (1 M $(NH_4)_2SO_4$ in buffer A). The supernatant obtained by centrifugation at 27 000 *g* for 1 h was loaded on to a phenylsepharose column (180 mL) previously equilibrated with buffer D. The column was washed with buffer E (450 mL) [0.5 M $(NH_4)_2SO_4$, in buffer A] followed by gradient elution from buffer E to buffer A in 720 mL, then 420 mL of buffer F (the same as buffer A except 10 mM of potassium phosphate). The fractions with high specific activity were pooled (40 mL).

3.5.3. Enzyme purification step 3: sephacryl S-100 column. The active fractions from step 2 were concentrated (50 kDa cut off membrane) and loaded on to a sephacryl S-100 (100×2.5 cm) gel filtration column previously equilibrated with buffer G (0.2 M NaCl in buffer A). The column was eluted with buffer G. The major activity was found in 70 mL of the eluate, which was then concentrated (50 kDa cut off membrane) to 3 mL and diluted with buffer A to 15 mL to reduce the salt concentration.

3.5.4. Enzyme purification step 4: UNO-Q1 column. The enzyme solution from step 3 was concentrated (50 kDa cut off membrane) to 2 mL and loaded on to an UNO-Q1 ion exchange column (Bio-Rad, 1 mL). The column was eluted with buffer A (5 mL) followed by a gradient elution with increasing buffer H (0.8 M NaCl in buffer A) from 0 to 60% in 20 mL. The fractions with high specific activity were pooled (2 mL).

3.5.5. Enzyme purification step 5: Superdex 200 column. The pooled fractions from step 4 were concentrated by ultrafiltration (50 kDa cut off membrane) to 0.2 mL and loaded onto a Superdex 200 column (Pharmacia, 24 mL) previously equilibrated with buffer A. The column was eluted with buffer A. The fractions with high specific activity were combined (2 mL).

3.5.6. Enzyme purification step 6: ISO column. The pooled fractions from step 5 were adjusted to 1 M concentration of $(NH_4)_2SO_4$, loaded onto an ISO hydrophobic interaction column (Pharmacia, 1 mL) previously equilibrated with buffer D. The column was eluted with 5 mL of buffer D followed by gradient elution from buffer D to buffer A in 10 mL. An activity assay was performed after $(NH_4)_2SO_4$ was removed from each fraction (50 kDa cut off membrane). The fractions with highest specific activity were pooled (2 mL).

3.5.7. Determination of molecular weight. Gel filtration column (Superdex 200, Pharmacia, 24 mL) was calibrated with standard proteins (Gel filtration chromatography standard, Bio-Rad) and eluted with buffer G (0.2 M NaCl in buffer A). The elution parameter $k_{av}=(V_e-V_o)/(V_t-V_o)$ was used for the preparation of calibration curve, where V_e = elution volume for the protein, V_o = column void volume, and V_t = total bed volume.[11]

3.6. Immobilisation of the enzyme and acyloin condensation with the immobilised enzyme

DE52 resin (10 mL bed volume) was equilibrated with buffer A and added to the crude cell extract of *A. eurydice* (20 mL). The mixture was shaken at 20 rpm for 20 h and then filtered. The adsorbed resins were washed with buffer A (5×30 mL) and suspended in the same buffer to give a final volume of 30 mL. One fourth of the suspension was used to catalyse the acyloin condensation of **1** with **2a** in 14 reaction cycles. For each cycle, a mixture of the immobilised enzyme, $MgCl_2$ (120 μL, 100 mM), TPP (80 μL, 50 mM), **1** (200 μL, 100 mM), **2a** (200 μL, 1 M), and buffer A (to adjust the final volume to 10 mL) was shaken at 50 rpm, 28°C for 20 h and then filtered. The resins were washed with buffer A (3×10 mL) and then used in the next reaction cycle. The combined filtrate and washes were acidified with HCl (1 M, 2 mL) and extracted with MTBE (2×15 mL). The extract was evaporated to dryness. The residue was dissolved in CH_3CN (2 mL) and analysed by HPLC method 1.

3.7. Chemical syntheses of (±)-3a to 3f

The synthesis of (±)-**3a** was completed as reported previously.[8] (±)-**3b** to **3f** were synthesised similarly using the following general procedure. To a stirred solution of trimethylsilyl cyanide (20 mmol, 1984 mg, 2667 μL) in CH_2Cl_2 (80 mL) at 0°C, the aldehyde **2** (20 mmol) was added, followed by TEA (2 mmol, 202 mg, 278 μL). The resulting mixture was stirred at 0°C for 2 h and the solvent was removed under reduced pressure. The residue was dissolved in anhydrous ether (20 mL) and added to a stirred solution of benzylmagnesium chloride (20 mL, 1.0 M in ether). The reaction mixture was stirred under reflux for 2 h and then cooled to room temperature. Water (20 mL) was added with caution followed by HCl (4 M, 20 mL) with vigorous stirring. The progress of the reaction was followed by TLC. After completion of hydrolysis, the product was extracted with ethyl acetate (2×40 mL). The extract was washed successively with water, 5% sodium bicarbonate, water, 1 M HCl, water, and brine, then dried over $MgSO_4$ and concentrated to dryness.

Flash chromatography on silica and elution with CH_2Cl_2 gave oily products **3b** (1.54 g, 43%); **3c** (2.27 g, 59%); **3d** (2.14 g, 52%); and **3e** (1.62 g, 34%). For **3f**, elution with CH_2Cl_2–MTBE (19:1) gave a solid, which was recrystallised from hexane–MTBE (2:1) to afford **3f** (0.43 g, 10%). The results of HPLC analyses are shown in Section 3.2.

3.7.1. 3-Hydroxy-1-phenyl-2-pentanone 3b. ^{1}H NMR δ 0.92 (t, 3H), 1.64 (m, 1H), 1.95 (m, 1H), 3.34 (m, 1H), 3.76 (d, 1H), 3.79 (d, 1H), 4.25 (m, 1H), 7.1–7.4 (m, 5H); ($CDCl_3$–2% D_2O): δ 0.92 (t, J=7.3, 3H), 1.64 (m, 1H), 1.93 (m, 1H), 3.76 (d, J=15.6, 1H), 3.79 (d, J=15.6, 1H), 4.25 (m, 1H), 7.1–7.4 (m, 5H). LCMS (ES+) m/z 179 (M+H).

3.7.2. 3-Hydroxy-1-phenyl-2-hexanone 3c[1]. ^{1}H NMR δ 0.95 (t, 3H), 1.30–1.75 (m, 3H), 1.90 (m, 1H), 3.32 (d, 1H, D_2O exchangeable), 3.75 (d, 1H), 3.84 (d, 1H), 4.32 (m, 1H), 7.1–7.4 (m, 5H). LCMS (ES+) m/z 193 (M+H).

3.7.3. 3-Hydroxy-1-phenyl-2-heptanone 3d. ^{1}H NMR δ 0.90 (t, J=7.3, 3H), 1.25–1.48 (m, 4H), 1.58 (m, 1H), 1.88 (m, 1H), 3.38 (brs, 1H), 3.76 (d, J=15.1, 1H), 3.80 (d, J=15.1, 1H), 4.27 (m, 1H), 7.15–7.38 (m, 5H). LCMS (ES+) m/z 207 (M+H).

3.7.4. 3-Hydroxy-1,4-diphenyl-2-butanone 3e. ^{1}H NMR δ 2.89 (dd, $J1$=14.2, $J2$=7.3, 1H), 3.17 (dd, $J1$=14.2, $J2$=4.9, 1H), 3.25 (d, J=5.8, 1H, D_2O exchangeable), 3.75 (d, J=15.6, 1H), 3.81 (d, J=15.6, 1H), 4.50 (m, 1H), 7.14–7.38 (m, 10H). LCMS (ES+) m/z 258 (M+NH_4).

3.7.5. 1-Hydroxy-1,3-diphenyl acetone 3f. ^{1}H NMR δ 3.62 (d, J=16.1, 1H), 3.66 (d, J=16.1, 1H), 4.24 (d, J=4.2, 1H, D_2O exchangeable), 5.19 (d, J=4.2, 1H; s

after D_2O exchange), 7.00 (d, 2H), 7.2–7.5 (m, 8H). LCMS (ES+) m/z 244 (M+NH_4).

3.8. Chemical synthesis of 1-hydroxy-3-phenyl-2-propanone 3g

A solution of phenylacetyl chloride (1.55 g, 10 mmol) in ether (40 mL) was slowly added to a stirred solution of diazomethane in ether (220 mL, 70 mmol, alcohol free) cooled with an ice-salt bath. After 1 h at room temperature, the solvent was removed under reduced pressure. The residue was dissolved in *tert*-butyl alcohol (20 mL) and stirred with boron trifluoride diethyl etherate (1.5 mL) at 70°C for 1 h. The usual work-up and flash chromatography on silica and elution with hexane–ethyl acetate (2:1) afforded **3g**, retention time 4.8 min by HPLC method 1. ^{1}H NMR δ 3.00 (brs, 1H, D_2O exchangeable), 3.72 (s, 2H), 4.28 (s, 2H), 7.21 (d, 2H), 7.26–7.38 (m, 3H).[12] LCMS (ES+) m/z 151 (M+H).

3.9. Chemical syntheses of (±)-2-hydroxy-1-phenyl-propanone 7

Lactonitrile (20 mmol, 1.42 g, 1.43 mL in 10 mL ether) was added slowly to a solution of phenylmagnesium bromide (30 mL, 1.0 M in ether) at 0°C. The resulting mixture was refluxed for 1 h. Water (10 mL) was added with caution followed by 10% H_2SO_4 (20 mL). The ethereal extract was washed successively with 5% $NaHCO_3$, water, and brine, and then dried over $MgSO_4$ and concentrated to dryness. Flash chromatography on silica and elution with CH_2Cl_2 gave oily product **7** (1.46 g, 49%). Retention time 6.1 min by HPLC method 1. ^{1}H NMR δ 1.38 (d, 3H), 3.82 (brd, 1H), 5.08 (m, 1H), 7.41 (t, 2H), 7.52 (t, 1H), 7.83 (d, 2H). LCMS (ES–) m/z 209 (M+OAc).

3.10. Preparation of the partially purified enzyme

The active fractions from enzyme purification step 1 were combined, adjusted to 60% $(NH_4)_2SO_4$ saturation, and centrifuged at 27 000 *g* for 1 h. The precipitate was dialysed against buffer A (2×2 L) to give the partially purified enzyme preparation, which was used for substrate specificity study.

3.11. General procedure for enzymatic acyloin condensation

A mixture of the enzyme (500 μL), potassium phosphate buffer (100 μL, 500 mM, pH 6.8), $MgCl_2$ (30 μL, 100 mM), TPP (20 μL, 50 mM), water (to adjust the final volume to 1 mL), sodium phenylpyruvate (50 μL, 100 mM), and the aldehyde (to the final concentration as indicated) was stirred at 28°C for 20 h or as indicated. A control reaction without any enzyme was always carried out for each aldehyde. The reaction was terminated by addition of aqueous HCl (50 μL, 1 M) and MeOH (950 μL). The resulting mixture was filtered and subjected to HPLC method 1 to determine the amounts of the various components. The mixture was then extracted with ethyl acetate (2×3 mL). The organic layer was washed with 5% $NaHCO_3$ (twice), water, 1 M HCl, and brine. After removal of solvent, the residue was subjected to HPLC method 1 again to analyse the non-acidic components, and to HPLC method 2 or 3 to determine the e.e.

3.12. Effect of acetaldehyde concentration

The crude cell extract (500 μL for each sample) was used in the initial study to examine the effect of acetaldehyde concentration. A clear stock solution of 1 M acetaldehyde in water was used. Duplicate samples were set up for each aldehyde at concentrations of 0, 5, 10, 20, 50, 100, 200, and 300 mM. The acyloin condensation was carried out as described in Section 3.11 and the products were analysed by HPLC method 1.

3.13. Time course of enzymatic acyloin condensation of 1 with 2a

Nine identical reactions were set up with the partially purified enzyme at an acetaldehyde concentration of 80 mM. The reactions were stopped at 2, 4, 8, 20, 28, 44, 52, 72, and 140 h and analysed by HPLC method 1.

3.14. Enzymatic acyloin condensation of 1 with 2b–2g

The partially purified enzyme was used. The 1 M aldehyde stock solutions were prepared in water for **2b** and **2g** and in 50% aqueous ethanol for **2c**–**2f**. The reactions were set up at aldehyde concentrations of 5, 10, 20, 50, 100, 200, and 300 mM. For reactions at 500, 1000, and 2000 mM aldehyde concentrations neat **2c** and **2d** were used. The reactions were analysed by HPLC method 1 and compared with authentic samples prepared chemically as described in Sections 3.7 and 3.8.

3.15. Enzymatic acyloin condensation of 1 with 2h–2q

The partially purified enzyme was used. The aldehydes **2h**–**2k** or 1 M stock solutions of **2l**–**2q** were used to set up reactions at different aldehyde concentrations. The reaction products were analysed by HPLC method 1. HPLC analyses did not show any significant additional peaks of more than 3 area percent when compared to the simple decarboxylation reaction and the control reactions without any enzyme.

3.16. Enzymatic acyloin condensation of 1 with 2r and 2s

The partially purified enzyme was used and the reactions were set up at aldehyde **2s** concentrations of 5, 10, 20, 50, 100, 200, and 300 mM. The reactions were analysed by HPLC method 1. Estimated percent yields of a new product peak at 3.7 min were 8, 12, 15, 16, 16, 15, and 13, respectively. There was 49% remaining of **1** in the sample with an initial aldehyde concentration of 50 mM. The reaction mixture was then extracted with EtOAc. The extract was washed twice with 5% $NaHCO_3$, water, 1 M HCl, and brine and then evaporated to dryness to provide product **3s**, retention time 3.7 min by HPLC method 1. ^{1}H NMR δ 3.6 (brs, OH,

D_2O exchangeable), 3.8 (d, $J=16$, 1H), 3.9 (d, $J=16$, 1H), 4.0 (m, 2H), 4.3 (m, 1H), 7.1 (d, 2H), 7.2–7.4 (m, 3H); LCMS (ES–) m/z 179 (M–H).

When the concentration of **2r** was 50 mM, a similar procedure gave 13% of **3r** as the highest yield with a retention time of 13.8 min, analysed by HPLC method 1; LCMS (ES–) m/z 197/199 with an intensity ratio of 3/1 (M–H).

3.17. Enzymatic acyloin condensation catalysed by active fraction from UNO Q1 column

The reaction was conducted under conditions similar to that for the enzyme activity assay but using 20% of the materials in each case; the most active fraction from the UNO Q1 column purification step 4 was used. The yield of acyloin product **3a** was 91% analysed by HPLC method 1, and the e.e. was 85% determined by HPLC method 2.

3.18. Enzymatic acyloin condensation of indole-3-pyruvic acid as an acyl donor

The reaction was conducted under the same conditions as stated in Section 3.11, with indole-3-pyruvic acid **4** (5 mM) as the acyl donor in place of sodium phenylpyruvate and acetaldehyde (100 mM) as the acceptor. When compared (HPLC method 1) with the control reaction without enzyme, a new non-acidic product **5** was found with a retention time 7.5 min in 19% yield. The reaction was scaled up by using 50 mL of the enzyme preparation, which was prepared from the crude cell extract by ammonium sulfate precipitation (30–60% saturation) and dialysis against buffer A. The product was isolated by flash chromatography on silica and elution with hexane:ethyl acetate (2:1) to give purified acyloin product **5**. ^{1}H NMR δ 1.44 (d, $J=7.0$, 3H), 3.94 (s, 2H), 4.40 (q, $J=7.0$, 1H), 7.10–7.16 (m, 2H), 7.20 (t, 1H), 7.38 (d, 1H), 7.50 (d, 1H), 8.18 (brs, 1H, NH); ^{1}H–^{1}H COSY in agreement with the structure of the desired product **5**; LCMS (ES+) m/z 204 (M+H), (ES–) m/z 202 (M–H).

3.19. Enzymatic reaction of benzoylformic acid as an acyl donor

The reaction was conducted under the same conditions as stated in Section 3.11, with benzoylformic acid **6** (5 mM) as the acyl donor in place of sodium phenylpyruvate and acetaldehyde (100 mM) as the acceptor. HPLC (method 1) analysis and comparison with authentic samples of 2-hydroxy-1-phenyl-propanone **7** (prepared by conventional chemical synthesis) and commercial benzaldehyde showed the absence of either one in the reaction mixture.

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