Absolute Configuration of the Product of the Acetolactate Synthase Reaction by a Novel Method of Analysis using Acetolactate Decarboxylase

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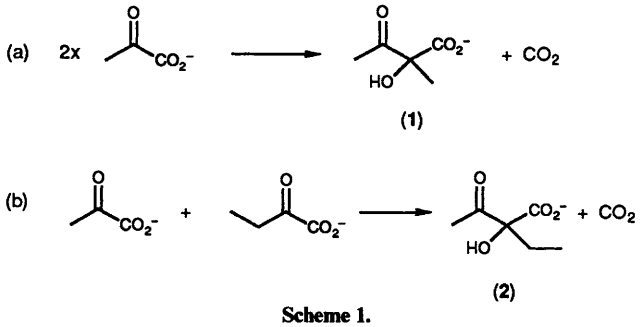
Pyruvate and 2-oxobutanoate were incubated with acetolactate synthase [acetolactate pyruvate lyase (carboxylating, EC 4.1.3.18)] in the presence of acetolactate decarboxylase [(*S*)-2-hydroxy-2-methyl-3-oxobutanoate carboxy-lyase, EC 4.1.1.5)]. The exclusive production of 3-hydroxypentan-2-one showed that the α-acetohydroxybutyrate (2-ethyl-2-hydroxy-3-oxobutanoate) produced by acetolactate synthase had the (*S*)-configuration. The ability of acetolactate decarboxylase to catalyse rearrangement of the (*R*)-enantiomer of substrates *via* carboxylate migration was investigated using [3,4-$^{13}C_2$]-α-acetolactate, in which the degeneracy of the rearrangement of the normal substrate was broken by isotopic labelling. The predicted sequential formation of [1,2-$^{13}C_2$]- and [3,4-$^{13}C_2$]-acetoin was observed.

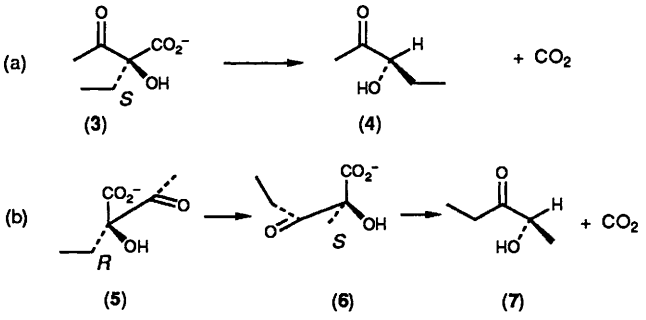
The enzyme acetolactate synthase is the first common enzyme in the pathway of biosynthesis of valine and isoleucine.[1] It has become the focus of much attention since the discovery of several classes of powerful herbicides for which it is the target. These include the sulphonylureas (Du Pont)[2] and the imidazolinones (American Cyanamid).[3] As part of our studies of the stereochemistry of the enzymatic reactions of the valine–isoleucine pathway of biosynthesis, we have determined the absolute configuration of the product of the condensation of pyruvate with 2-oxobutanoate catalysed by the acetolactate synthase isoenzyme II of *Salmonella typhimurium*. With pyruvate alone, the reaction yields α-acetolactate (**1**), the precursor of L-valine [Scheme 1(a)]. When pyruvate and 2-oxobutanoate are both present, α-acetohydroxybutyrate (**2**), the precursor of L-isoleucine, is produced selectively [Scheme 1(b)]. Acetolactate decarboxylase (ADC) from *Bacillus brevis*



Scheme 1.

catalyses the decarboxylation of (*S*)-α-acetohydroxybutyrate (**3**) to give (*R*)-3-hydroxypentan-2-one (**4**) [Scheme 2(a)]. The reaction proceeds with overall inversion at the chiral centre, as shown. However, (*R*)-α-acetohydroxybutyrate (**5**) also undergoes decarboxylation, but to the isomeric (*R*)-2- hydroxypentan-3-one (**7**). This reaction is believed to proceed by enzyme-catalysed carboxylate migration to give the isomeric 2-hydroxy-2-methyl-3-oxopentanoate (**6**). If the carboxylate migration takes place *via* a transition state in which the carbon–oxygen bonds at C-2 and C-3 have a *syn*-relationship [cf. (**5**)], the product (**6**) has the (*S*)-configuration [Scheme 2(b)]. The enzyme can catalyse the decarboxylation of this intermediate with inversion in the normal way to give the observed ketol (**7**).[4]

The significance of this observation is that the enzyme is

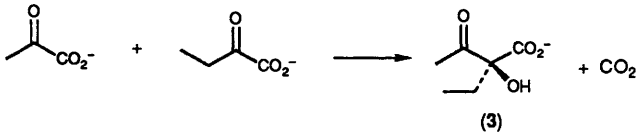


Scheme 2.

unable to catalyse directly decarboxylation of the (*R*)-substrate. However, following rearrangement, as described above, an isomeric acetohydroxy acid is produced with the correct configuration. Decarboxylation then occurs, but to a ketol isomeric with that of the normal (*S*)-substrate. The configuration of an enantiomer of α-acetohydroxybutyrate can thus be determined by examining the *structure* of the product of decarboxylation catalysed by acetolactate decarboxylase. We have applied this novel method of chiral analysis to the determination of the absolute configuration of the α-acetohydroxybutyrate produced by the acetolactate synthase of *S. typhimurium*.

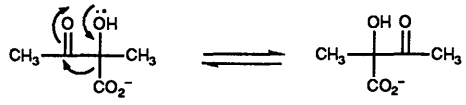
The comparable rearrangement of α-acetolactate is degenerate (Scheme 4) and the method described above cannot be applied. However, we have also shown, by the use of a ^{13}C-labelled substrate, that a comparable rearrangement occurs.

Condensation of pyruvate with 2-oxobutanoate catalysed by acetolactate synthase might proceed to give either (*S*)-(**3**) or (*R*)-α-acetohydroxybutyrate (**5**), or a mixture of the two. When pyruvate and 2-oxobutanoate were condensed together in the presence of acetolactate synthase and acetolactate decarboxylase, the only ketol product observable by ^1H NMR spectroscopy was 3-hydroxypentan-2-one (**4**) under conditions in which <5% of the isomeric 2-hydroxypentan-3-one (**7**) would have been detected. It was therefore concluded that acetolactate synthase catalyses stereospecifically (>95%) the condensation of pyruvate with 2-oxobutanoate to give (*S*)-α-acetohydroxybutyrate (**3**) (Scheme 3). The rearrangement of α-acetolactate with carboxylate migration as shown in Scheme 2(b) for α-acetohydroxybutyrate is degenerate, in that the product is constitutionally identical with the starting material



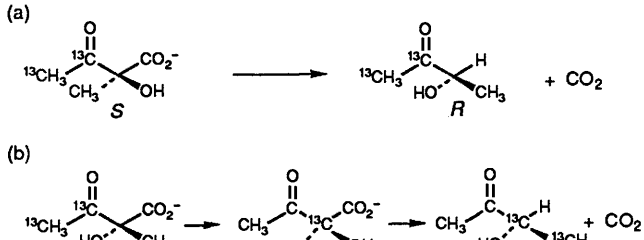
(3)

Scheme 3.



Scheme 4.

(Scheme 4). This transformation is an example of the tertiary ketol rearrangement and is readily brought about in the laboratory by treatment of α-acetolactate with alkali at pH 13.[6] The rearrangement is intramolecular and proceeds with racemisation.[7] There is therefore a marked similarity between the alkali-catalysed rearrangement and the putative enzyme-catalysed rearrangement shown in Scheme 2(b). In order to throw further light on the enzyme-catalysed decarboxylation, the reaction was studied with a sample of α-acetolactate in which the degeneracy of the rearrangement was broken by double-labelling with ^{13}C. The required compound, ethyl (*RS*)-[3,4-$^{13}C_2$]-2-hydroxy-2-methyl-3-oxobutanoate (ethyl (*RS*)-[3,4-$^{13}C_2$]-α-acetolactate) was hydrolysed completely with pig liver esterase. (This enzyme was shown to catalyse the hydrolysis of esters of both enantiomers of α-acetolactic acid.[4]) The labelled acetolactate gave a ^1H NMR spectrum consisting of a doublet of doublets at δ 2.20 (*J* 128 and 6 Hz) attributable to the protons of the $^{13}CH_3$ group and a doublet at δ 1.41 (*J* 4 Hz) attributable to the protons of the unlabelled methyl group. On addition of the decarboxylase, the first product signals to appear were attributable to $^{13}CH_3{}^{13}COCH(OH)CH_3$ (*cf.* Table). When decarboxylation had proceeded to approximately 40%, signals attributable to $^{13}CH_3CO^{13}CH(OH)CH_3$ began to appear. These observations are consistent with the behaviour of α-acetohydroxybutyrate on treatment with ADC (*cf.* Scheme 2). The simplest explanation is that the (*S*)-enantiomer of the substrate is decarboxylated first to the expected product (*R*)-[1,2-$^{13}C_2$]-3-hydroxybutan-2-one [Scheme 5(a)]. When this



Scheme 5.

process has nearly reached completion, the rearrangement of the (*R*)-enantiomer to the (*S*)-enantiomer is catalysed, followed by decarboxylation of the rearranged substrate to (*R*)-[3,4-$^{13}C_2$]-3-hydroxybutan-2-one [Scheme 5(b)]. The sequential decarboxylation is most readily explained in terms of competitive inhibition of the second reaction [of (*R*)-α-acetolactate] by the (*S*)-enantiomer, which is the preferred substrate for the enzyme. This result is also consistent with the observation that racemic α-acetolactate is completely decarboxylated by ADC to give optically pure (*R*)-(−)-acetoin.[4]

The results described above provide further evidence for the putative carboxylate migration during decarboxylation of (*R*)-isomers of α-acetolactate and analogues by ADC.[4]

Experimental

Acetolactate synthase from *S. typhimurium*, cloned and expressed in *Escherichia coli* was a gift from Dr. J. V. Schloss. Acetolactate decarboxylase from *B. brevis* was kindly provided by Dr. S. E. Godtfredsen (Novo Industri). Pig liver esterase was purchased from Boehringer.

Synthesis of Ethyl [3,4-$^{13}C_2$]*Tiglate* (*Ethyl* [3,4-$^{13}C_2$]-2-*Methylbut-2-enoate*).—To a stirred solution of sodium hydride (80%, 0.34 g, 11.3 mmol) in dry 1,2-dimethoxyethane (4 ml) at 0 °C under N_2 was added dropwise a solution of triethyl 2-phosphonopropionate (2.7 g, 11.3 mmol) in 1,2-dimethoxyethane (4 ml). When evolution of hydrogen stopped, the reaction mixture was allowed to warm to room temperature. A solution of [1,2-$^{13}C_2$]acetaldehyde (0.63 ml, 11.3 mmol) in 1,2-dimethoxyethane (4 ml) was added to produce a yellow gelatinous precipitate. The mixture was stirred for 30 min and poured into water (25 ml). The aqueous–organic mixture was extracted with diethyl ether (3 × 25 ml), dried ($MgSO_4$), and evaporated. To remove traces of 1,2-dimethoxyethane, the residue was dissolved in pentane (50 ml), and the solution was extracted with water (2 × 50 ml), dried ($MgSO_4$), and evaporated under reduced pressure to give ethyl [3,4-$^{13}C_2$]tiglate (1.33 g, 10.2 mmol, 90%). The product was essentially pure by ^1H NMR spectroscopy and was not purified further: δ($CDCl_3$) 1.25 (3 H, t, *J* 7.12 Hz, CH_2CH_3), 1.74 (3 H, dddq, *J* 126.8, 7, 7, and 1.16 Hz, $^{13}CH_3$), 1.80 (3 H, m, $=CCH_3$), 4.14 (2 H, q, *J* 7.12 Hz, OCH_2), and 6.8 [1 H, m, *J*(^{13}C–H) 156.9 Hz, =CH].

Synthesis of Ethyl [3,4-$^{13}C_2$]-α-*Acetolactate*.—Solid potassium permanganate (2.44 g) was added in portions to a stirred solution of ethyl [3,4-$^{13}C_2$]tiglate (1.16 g, 8.9 mmol) in a mixture of acetone (150 ml), water (40 ml), and acetic acid (3 ml) cooled to −10 °C. The mixture was stirred at −10 °C for 1 h, filtered (Celite), and evaporated under reduced pressure to remove acetone. The remaining aqueous solution was extracted with dichloromethane (3 × 100 ml). The combined extracts were dried ($MgSO_4$), and evaporated under reduced pressure. The residue was distilled (Kugelrohr) to give ethyl [3,4-$^{13}C_2$]-α-acetolactate (1.0 g, 6 mmol, 70%): δ($CDCl_3$) 1.28 (3 H, t, *J* 7.15 Hz, CH_2CH_3), 1.57 [3 H, d, *J* 4 Hz, $C(OH)CH_3$)], 2.25 (3 H, dd, *J* 128.9 and 6.1 Hz, $^{13}CH_3$), 4.24 (2 H, q, *J* 7.2 Hz, OCH_2); ^{13}C NMR: δ 13.9 (CH_2CH_3), 21.6 [$C(OH)CH_3$], 24.0 (d, *J* 42.7 Hz, $^{13}CH_3$), 62.5 (OCH_2), 81.0 [dd, *J* 42.7 and 17 Hz, C(OH)], 171.2 (CO_2), and 204.7 (d, *J* 42.7 Hz, ^{13}CO); *m/z* (electron impact; 70 eV) $(M + 1)^+$ 163.0886; Calc. for ethyl [3,4-$^{13}C_2$]-2-hydroxy-2-methyl-3-oxobutanoate ($^{12}C_5{}^{13}C_2H_{13}O_4$), M^+ 163.0881.

Enzymatic Hydrolysis and Decarboxylation of Ethyl [3,4-$^{13}C_2$]-α-*Acetolactate*.—Ethyl [3,4-$^{13}C_2$]-α-acetolactate (36 mg, 0.2 mmol) in phosphate buffer (1M; pH 7.2, 0.6 ml) was treated with pig liver esterase (0.4 mg, 52 U). The mixture was incubated at 37 °C and the course of ester hydrolysis was followed by ^1H NMR spectroscopy (220 MHz). When hydrolysis was complete, acetolactate decarboxylase (1 mg) was added. The rates of production of [1,2-$^{13}C_2$]- and [3,4-$^{13}C_2$] acetoin were followed by ^1H NMR spectroscopy. The ^1H NMR data for the two isotopomers are given in the Table.

Conversion of Pyruvate and 2-Oxobutanoate into 3-Hydroxypentan-2-one by the Sequential Action of Acetolactate Synthase and Acetolactate Decarboxylase.—Sodium pyruvate (56.5 mg, 0.51 mmol), sodium 2-oxobutanoate (63.8 mg, 0.515 mmol), acetolactate decarboxylase (5.5 mg, 3.4 μmol), thiamine pyrophosphate (1.4 mg), flavin adenine dinucleotide (2.9 mg, 3.7 μmol), and magnesium chloride (10.5 mg, 0.11 mmol) were

Table. ^{1}H NMR data (δ) for [1,2-$^{13}C_2$]- (A) and [3,4-$^{13}C_2$]-acetoin (B).[a]

	A	B
$CH_3CH(OH)$	1.31 (m)	1.31 [dm, J(^{13}C–H) 128]
CH_3CO	2.17 [dd, J(^{13}C–H) 128 Hz, J(^{13}C–C–H) 7]	2.17 (s)

[a] The spectra were determined for solution in H_2O. Under these conditions the signal attributable to the methine signal could not be observed. Chemical shifts are quoted relative to DOH at δ = 4.7; coupling constants are in Hz.

dissolved in phosphate buffer (pH 7.2; 1M; 0.8 ml). Acetolactate synthase (149 mg ml^{-1}; 5 μl, 11 U) was added and the mixture was incubated in the dark at 37 °C. The course of the reaction was followed by ^{1}H NMR spectroscopy. The reaction reached 85% completion after 1 h and 95% completion at the end of 7.7 h, at which time, besides signals attributable to residual pyruvate and 2-oxobutanoate, only signals attributable to 3-hydroxypentan-2-one were observable [δ 0.90 (t, 3 H, J 7 Hz, CH_2CH_3), 1.75 (m, 2 H, CH_2), and 2.21 (s, 3 H, $COCH_3$)].

Acknowledgements

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