Biosynthesis of Valine and Isoleucine: Synthesis and Biological Activity of (2*S*)-α-Acetolactic Acid (2-Hydroxy-2-methyl-3-oxobutanoic Acid), and (2*R*)- and (2*S*)-α-Acetohydroxybutyric Acid (2-Ethyl-2-hydroxy-3-oxobutanoic Acid) †

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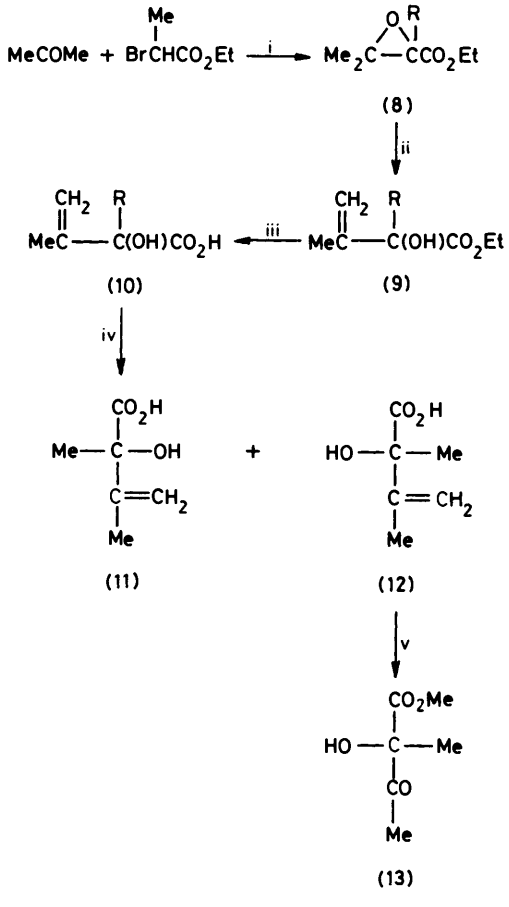
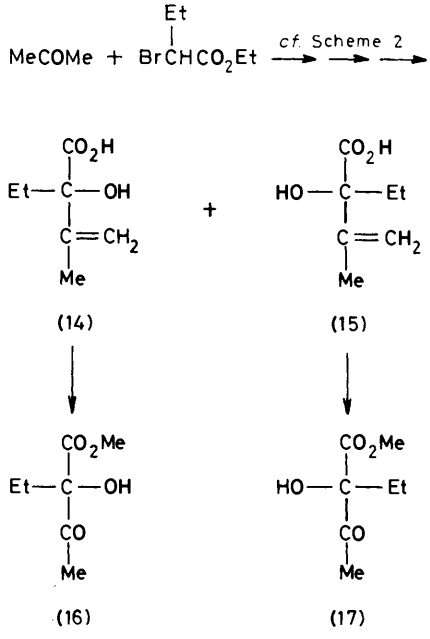
Table 1. Circular dichroism maxima and optical rotations of the enantiomers of methyl α-acetolactate, methyl α-acetohydroxybutyrate, and their synthetic precursors

Compound	Solvent [a]	λ (Δε)	$[\alpha]_D$ (solvent,[b] c/g ml^{-1})
(2*R*)-2-Hydroxy-2,3-dimethylbut-3-enoic acid (11)	A	207 (−2.21), 238 (+0.32)	−18.2° (E, 1.05)
	B	210 (−3.26), 240 (+0.26)	[Me ester, +2.94° (W, 1.26); −16.8° (E, 0.95)]
(2*S*)-2-Hydroxy-2,3-dimethylbut-3-enoic acid (12)	A	212 (+2.78), 238 (−0.39)	+17.5° (E, 1.06)
	B	211 (+3.48), 238 (−0.27)	[Me ester, −3.8° (W, 1.0); +17.6° (E, 1.0)]
(2*R*)-2-Ethyl-2-hydroxy-3-methylbut-3-enoic acid (14)	A	208 (−2.12), 237 (+0.63)	+7.6° (E, 0.55)
	B	206 (−2.66), 235 (+0.87)	[Me ester, +18.7° (E, 1.18)]
(2*S*)-2-Ethyl-2-hydroxy-3-methylbut-3-enoic acid (15)	A	213 (+2.67), 237 (−0.62)	−6.9° (E, 2.26)
	B	208 (+2.41), 234 (−0.85)	[Me ester, −20.0° (E, 1.07)]
Methyl (2*R*)-α-acetolactate [Enantiomer of (13)]	A	211−216 (−0.93), 238 (+0.27) 276 (+0.34), 311 (−0.28)	[−9.9°] [2]
Methyl (2*S*)-α-acetolactate (13)	A	211 (+0.86), 238 (−0.19), 277 (−0.28), 312 (+0.22)	+9.7° (E, 3.1)
Methyl (2*R*)-α-acetohydroxybutyrate (16)	A	213 (−1.03), 240 (+0.16), 278 (+0.41), 313 (−0.15)	+42.8° (W, 0.63) [c]
Methyl (2*S*)-α-acetohydroxybutyrate (17)	A	213 (+1.06), 240 (−0.17), 278 (−0.46), 313 (+0.17)	−41.4° (W, 1.09) [d]

[a] A = methanol; B = methanol–HCl. [b] E = ethanol; W = water. [c] Lit. value,[7] +45.1°. [d] Lit. value,[7] −43.5°.



Scheme 2. *Reagents:* $KOCMe_3$; ii, $TosOH–C_6H_6$; iii, $Ba(OH)_2$; iv, quinine; v, CH_2N_2, O_3



Scheme 3.

absolute configuration.[2] The (*R*)- and (*S*)-configurations were assigned to the (+)- and (−)-acids [(14) and (15)] by comparison of their c.d. curves with those of the lower homologues [(11) and (12)]. The (*S*)-isomers showed a positive maximum near 210 nm and a negative maximum in the range 235—240 nm. The stereochemical assignments were confirmed by the close homology of the c.d. curves of the methyl esters (16) and (17) with those of methyl (*R*)- and (*S*)-α-acetolactate respectively (Table 1). The optical rotations of the enantiomeric methyl α-acetoxyhydroxybutyrates agreed well with those reported by Hill *et al.* for material produced by a different route [7] (Table 1).

(*S*)-2-Methyl-2-hydroxy-3-oxobutanoate [as (1)] and (*R*)- and (*S*)-2-ethyl-2-hydroxy-3-oxobutanoates [as (2)] were tested as substrates of the reductoisomerase of *Salmonella typhimurium ilvD6* (dihydroxy acid dehydratase-deficient strain). [(*R*)-2-Methyl-2-hydroxy-3-oxobutanoate had already been shown not to be a substrate of the enzyme.[3]] As expected, the (*S*)-isomers showed full activity in this assay (Table 2). However, the *R*-isomer of α-acetohydroxybutyrate [as (2)] also

Table 2. Activities of α-acetolactate (AL) and α-acetohydroxybutyrate (AHB) in the assay with the reductoisomerase of *Salmonella typhimurium ilvD*6

Substrate	Concentration (mM)	Specific activity [a]
(2*RS*)-AL	10	5.7
(2*RS*)-AL	7.5	5.9
(2*S*)-AL	7.5	5.2
(2*S*)-AL	5	5.2
(2*RS*)-AHB	10	36.5
(2*S*)-AHB	5	45.2
(2*R*)-AHB	5	4.9
(2*RS*)-AHB	4	37.5
(2*S*)-AHB	2	42.7
(2*R*)-AHB	2	6.5
(2*S*)-AHB	2	49.7
(2*R*)-AHB	2	3.2

[a] μMol of NADPH oxidised per hour per mg protein.

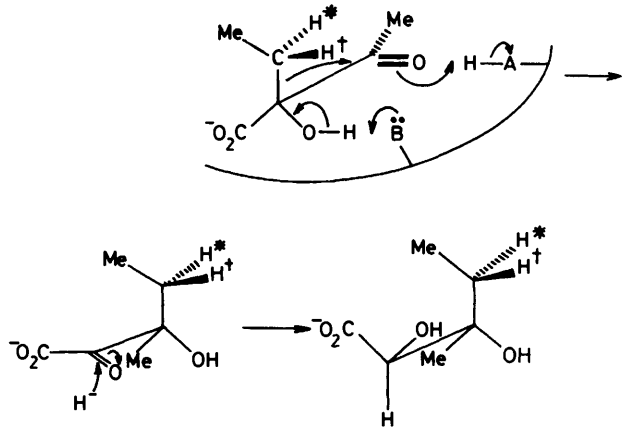


Figure.

showed low [*ca.* 10% of the (*S*) isomer] but reproducible activity. A corresponding activity might also be characteristic of (*R*)-α-acetolactate [as (1)] but was not observed experimentally because of the appreciably lower activity of α-acetolactate (1) in the enzyme assay, as compared with that of α-acetohydroxybutyrate (2). A similar observation was made by Hill *et al.* in a parallel investigation using (*R*)-α-acetohydroxybutyrate [as (2)] prepared by a different route.[7] In that study, the residual activity was attributed to partial racemisation during preparation of the substrate from methyl (*R*)-α-acetohydroxybutyrate by alkaline hydrolysis of the corresponding methyl ester. However, in the present study, great care was taken not to expose the substrate to pH levels that would cause racemisation. A more likely explanation of the observed residual activity is that the (*R*)-α-acetohydroxybutyrate preparations were contaminated with 1—2% of the (*S*)-isomer. (Such a low level of enantiomeric impurity might escape detection in the n.m.r. assay using chiral shift reagent.) Because of the low K_M value of the (*S*)-isomer, which is 0.17 mM,[8] it can be calculated that such low levels of enantiomeric impurity, in an assay containing the (*R*)-isomer at 2 mM concentration, would give initial reaction rates comparable with that observed, *i.e.* 10—15%. Thus, seemingly insignificant quantities of the biologically active isomer in preparations of the inactive isomer of α-acetohydroxybutyrate are readily detectable in the spectrophotometric assay used.

The configuration of the product of the reductoisomerase reaction in the isoleucine pathway follows from the absolute configuration (2*R*,3*R*)[9] of the substrate (6) of the following step which is catalysed by the enzyme dihydroxy acid dehydratase (2,3-dihydroxy acid hydro-lyase, EC 4.2.1.9). Also, the migrating group in the rearrangement in the valine pathway has been shown to occupy the same configurational position (4-*pro*-*R*) in the product dihydroxy acid (5) as the migrating ethyl group in the isoleucine pathway.[10] These results show that the conformation of the substrates during the reductoisomerase-catalysed reaction must be as shown in the Figure, in which the ketonic carbonyl group and the α-hydroxy group have a *syn* relationship. They also show that during the rearrangement step, the migrating alkyl group is delivered to the *re* face of the C-3 trigonal system. In a different bacterial system (*Serratia marcescens*) and in a higher plant genus (*Senecio*), migration of the ethyl group in the isoleucine pathway has been shown to proceed with retention of configuration at the migrating centre.[11] If stereochemical congruence between the bacterial enzymes is assumed, a complete stereochemical description of the reductoisomerase reaction for the isoleucine pathway is possible (Figure). The one remaining stereochemical question relating to the valine pathway is that of the stereochemistry of the migration step with respect to changes taking place at the migrating centre.

Initiation in the laboratory of tertiary ketol rearrangements exemplified by the first step of the reductoisomerase reaction can be effected by either acidic or basic reagents. A combination of these catalytic possibilities is likely in the enzymatic reaction. Accordingly the presence can be predicted of acidic (A—H) and basic (B) groups at appropriate positions in the active site, as illustrated in the Figure.

The biological results reported in this paper correspond closely with those obtained by Professor R. K. Hill and his coworkers.[7] We are grateful to Professor Hill for valuable discussions and exchanges of information.

Experimental

^{1}H N.m.r. spectra were determined with a JEOL MH 100 spectrometer at 100 MHz. All spectra were determined for solutions in deuteriochloroform. The chiral shift reagent used was europium tris(3-trifluoroacetyl-D-camphorate). C.d. curves were obtained, through the courtesy of Dr. P. M. Scopes and the late Professor W. Klyne using Roussel-Jouan Dichrograph-185 or Carey 61 instruments. Optical rotations were measured with an NPL Automatic Polarimeter Model 243 (Thorn Automation, Nottingham, England). All bulb-tube distillations were carried out using a Bulb-tube oven Model GKR 50 (Büchi AG, Flawil, Switzerland).

Ethyl 2,3-*Epoxy*-2,3-*dimethylbutanoate* (8; R = Me).—A mixture of acetone (5.5 ml, 75 mmol) and ethyl 2-bromopropionate (13.5 g, 75 mmol) was cooled to 10—15 °C, vigorously stirred in an atmosphere of dry nitrogen and treated dropwise with a solution of potassium t-butoxide (75 mmol) in t-butyl alcohol (60 ml) during 90 min. The mixture was stirred for a further 90 min and concentrated under reduced pressure at 25 °C. The residue was partitioned between water (30 ml) and ether (30 ml). The ethereal layer was separated, the aqueous solution was extracted twice more with ether (30 ml) and the combined ethereal solutions were dried ($MgSO_4$) and filtered. The ether was evaporated and the residual oil (15.75 g) was distilled through a helix-packed column to give ethyl 2,3-epoxy-2,3-dimethylbutanoate (8; R = Me) (7.05 g, 61%) as a colourless oil, b.p. 83—87 °C (20 mmHg) [lit.,[12] b.p. 83—87 °C (25 mmHg)], δ_H 4.2 (2 H, q, *J* 7.5 Hz, OCH_2), 1.55, 1.48, 1.31 [each 3 H, s, $(Me)_2COCMe$], and 1.31 (3 H, t, *J* 7.5 Hz,

CH_2Me); $\nu_{max.}$ 1 752 and 1 730 cm^{-1} (CO) [13] (Found: C, 60.65; H, 9.1. Calc. for $C_8H_{14}O_3$ C, 60.76; H, 8.86%).

Ethyl 2,3-*Epoxy*-2-*ethyl*-3-*methylbutanoate* (8; R = Et).—This was prepared as for the lower homologue (8; R = Me), in 63% yield, b.p. 98—100 °C (14 mmHg), 76 °C (5 mmHg) (lit.,[14] b.p. 74 °C at 4 mmHg), $\nu_{max.}$ 1 730 and 1 750 cm^{-1} (CO); δ_H 4.23 (2 H, q, *J* 7.5 Hz, OCH_2), 1.44—2.28 (2 H, m, $MeCH_2C$), 1.29, 1.35 (each 3 H, s, Me_2C), 1.28 (3 H, t, *J* 6.75 Hz, $MeCH_2C$), and 1.04 (3 H, t, *J* 7.5 Hz, OCH_2Me).

Rearrangement of Epoxy Esters (8; R = Me, Et).—To a solution of toluene-*p*-sulphonic acid monohydrate (60 mg) in benzene (20 ml), the epoxy ester (0.01 mol) was added and the solution was boiled under reflux and in an atmosphere of dry nitrogen for 18 h. The cooled solution was washed with sodium carbonate solution (1 M; 15 ml), the aqueous washings were extracted with ether (2 × 20 ml), and the combined ethereal solutions were dried ($MgSO_4$), filtered, and evaporated. The residual oil was distilled. The crude products were found to be of greater than 95% purity by g.l.c. (10% SE 30 on Chromosorb W) and ^1H n.m.r. spectroscopy.

Ethyl 2-hydroxy-2,3-dimethylbut-3-enoate (9; R = Me) was obtained in 80% yield after distillation in a bulb-tube apparatus at 120 °C and 12 mmHg; δ_H 5.13, 4.95 (each 1 H, apparent s, CH_2:), 4.22 (2 H, q, *J* 7 Hz, OCH_2), 3.45 (1 H, s, OH), 1.76 (3 H, s, MeC:), 1.52 (3 H, s, MeCO), and 1.23 (3 H, t, *J* 7 Hz, $MeCH_2$); $\nu_{max.}$ 1 730 (CO) and 1 650 cm^{-1} (C:C).

Ethyl 2-*ethyl*-2-*hydroxy*-3-*methylbut*-3-*enoate* (9; R = Et) was obtained in 76% yield, b.p. 114—116 °C (15 mmHg), δ_H 4.91, 4.86 (each 1 H, apparent s, CH_2:), 4.14 (2 H, q, *J* 7 Hz, OCH_2), 3.33 (1 H, s, OH), 1.86 (2 H, m, CH_2), 1.73 (3 H, s, MeC:), 1.25 (3 H, t, *J* 7 Hz, $MeCH_2O$), and 0.86 (3 H, t, *J* 7 Hz, $MeCH_2C$), $\nu_{max.}$ 1 725 (CO) and 1 645 cm^{-1} (C:C) (Found: C, 62.9; H, 9.25. $C_9H_{16}O_3$ requires C, 62.79; H, 9.30%).

2-*Hydroxy*-2,3-*dimethylbut*-3-*enoic Acid* (10; R = Me) *and* 2-*Ethyl*-2-*hydroxy*-3-*methylbutenoic Acid* (10; R = Et).—The ester (9) (5 mmol) was boiled under reflux with a solution of barium hydroxide octahydrate (5 mmol) in water (20 ml) for 75 min. The cooled solution was treated with solid CO_2, filtered (Kieselguhr), brought to pH 1 by the addition of dilute hydrochloric acid, and extracted with ether [continuously for 48 h in the case of the acid (10; R = Me), with 3 × 40 ml in the case of acid (10; R = Et)]. The ethereal extract was dried ($MgSO_4$) and evaporated. 2-Hydroxy-2,3-dimethylbut-3-enoic acid (10; R = Me) was obtained in 75% yield after crystallisation of the residue from ether–cyclohexane, m.p. 85.5—87 °C (lit.,[15] m.p. 87—88 °C), δ_H 7.8—8.0br (2 H, OH, COOH), 5.18, 5.01 (each 1 H, apparent, s, :CH_2), 1.82 (3 H, s, MeC:), and 1.59 [3 H, s, *MeC*(OH)].

2-*Ethyl*-2-*hydroxy*-3-*methylbut*-3-*enoic acid* (10; R = Et) was obtained in 66% yield after crystallisation of the residue from ether–light petroleum, m.p. 85 °C, δ_H 6.69br (2 H, OH, CO_2OH), 5.21, 5.01 (each 1 H, apparent s, :CH_2), 1.98 (2 H, m, CH_2Me), 1.83 (3 H, s, MeC:), and 0.93 (3 H, t, *J* 7.5 Hz, CH_2Me), $\nu_{max.}$ 1 720 (CO) cm^{-1} Found: C, 58.3; H, 8.51. $C_7H_{12}O_3$ requires C, 58.33, H, 8.33%).

Resolution of 2-*Hydroxy*-2,3-*dimethylbut*-3-*enoic Acid* (10; R = Me).—To a hot solution of the acid (10; R = Me) (10 g, 77 mmol) and triethylamine (3.89 g, 38.5 mmol) in ethanol (100 ml) was added a hot solution of quinine trihydrate (14.57 g, 38.5 mmol) in ethanol (100 ml). The mixture was allowed to cool slowly to room temperature. The product (18.64 g, 95%) was recrystallised twice (ethanol) to give a quinine salt (9.21 g), m.p. 224—225 °C (Found: C, 69.05; H, 7.6; N, 5.95. $C_{26}H_{34}N_2O_5$ requires C, 68.7; H, 7.54; N, 6.16%). The acid (2.09 g recovered from this salt was converted into the dicyclohexylamine salt, which was crystallised from ethanol (12 ml) to give 2.56 g of salt, m.p. 176 °C. A further crop (0.51 g) was obtained from the mother liquor on standing. The free acid (350 mg) recovered from the final mother liquor after acidification (dilute HCl) and continuous ether extraction (48 h), was recrystallised twice from ether–light petroleum to give (2*S*)-2-hydroxy-2,3-dimethylbut-3-enoic acid (12), m.p. 94° C. From the mother liquor from the first crop of quinine salt, above, the free acid was recovered as before. To a solution of the acid (4.547 g, 35 mmol) in ethanol was added a solution of quinine (5.68 g, 17.5 mmol) and triethylamine (1.77 g, 17.5 mmol) in ethanol. The quinine salt produced was filtered off and from the mother liquor the remaining free acid (2.08 g) was isolated as before and converted into the dicyclohexylammonium salt. The salt was crystallised from ethanol (10 ml). The mother liquor was allowed to stand for further crystallisation, to give three crops of salt in all (2.15, 0.73, and 0.2 g). The free acid (240 mg) was isolated from the final mother liquor as before and recrystalised three times from ether–light petroleum to give (2*R*)-2-hydroxy-2,3-dimethylbut-3-enoic acid (11) (124 mg), m.p. 93—94 °C.

Resolution of 2-*Ethyl*-2-*hydroxy*-3-*methylbut*-3-*enoic Acid* (10; R = Et).—To a solution of the acid (10; R = Et) (12 g, 83.3 mmol) and triethylamine (4.22 g, 42 mmol) in hot ethanol (40 ml) was added a hot solution of quinine (13.52 g, 42 mmol) in ethanol (40 ml). The product obtained on cooling (16.54 g, 84%) was recrystallised from ethanol to give a quinine salt of constant m.p. 188—190.5 °C (decomp.). Recovery of the free acid, conversion into the methyl ester (diazomethane) and examination of the n.m.r. spectrum in the presence of chiral shift reagent indicated an enantiomer ratio of 80 : 20. The free acid (1.83 g) recovered from the quinine salt as before was converted into the dicyclohexylammonium salt and recrystallised from ethanol to give two crops (2.26 and 0.41 g). Recovery of the free acid (549 mg) from the mother liquor as before and recrystallisation three times from ether–light petroleum gave (2*S*)-2-ethyl-2-hydroxy-3-methylbut-3-enoic acid (15) (230 mg), m.p. 82—83.5 °C. From the mother liquor of the first crop of quinine salt, the free acid (6.06 g, 42.1 mmol) was recovered as before and treated in ethanolic solution with an ethanolic solution of quinine (6.82 g, 21.05 mmol) and triethylamine (2.12 g, 21.05 mmol). From the solution, quinine salt (7.37 g) was obtained. The free acid (3.32 g) re-isolated from the mother liquor as before, was converted into the dicyclohexylammonium salt which was crystallised from ethanol to give three crops (4.55, 1.22 and 0.39 g). The free acid was recovered from the final mother liquor as before and recrystallised three times from ether–light petroleum to give (2*R*)-2-ethyl-2-hydroxy-3-methylbut-3-enoic acid (14) (235 mg), m.p. 82—83 °C. The dicyclohexylammonium salt of the racemic acid had m.p. 165 °C whereas the salts of the pure enantiomers had m.p. 153—154 °C (Found: C, 69.9; H, 11.05; N, 4.3. $C_{19}H_{35}NO_3$ requires C, 70.15; H, 10.77; N, 4.30%).

Methyl (2S)-2-*Hydroxy*-2,3-*dimethylbut*-3-*enoate: General Procedure*.—A solution of 2-hydroxy-2,3-dimethylbut-3-enoic acid (10; R = Me) (1 g) in ether (12 ml) was treated with an excess of an ethereal solution of diazomethane. The excess of diazomethane was allowed to evaporate and the solution was dried ($MgSO_4$), filtered, and distilled in a bulb-tube apparatus at 80 °C (12 mmHg) to give methyl 2-hydroxy-2,3-dimethylbut-3-enoate (1.04 g, 94%) as a colourless oil (lit.,[15] b.p. 64—65 °C, 10 mmHg); δ_H 5.1, 4.94 (each 1 H, apparent s,

$:CH_2$), 3.75 (3 H, s, OMe), 3.36 (1 H, s, OH), 1.76 (3 H, s, MeC:), and 1.52 (3 H, s, MeC). The (2*S*)-ester was prepared in the same way from (2*S*)-2-hydroxy-2,3-dimethylbut-3-enoic acid (12). An optical purity of greater than 98% was indicated by ^{1}H n.m.r. in the presence of chiral shift reagent.

Methyl (2R,S)-, (2S)- *and* (2R)-2-*Ethyl*-2-*hydroxy*-3-*methylbut*-3-*enoates*.—These esters were prepared as for the lower homologues, above. They were purified by bulb-tube distillation at 120 °C (10 mmHg), δ_H (for the (2*RS*)-ester) 5.19, 5.03 (each 1 H, apparent s, $:CH_2$), 3.75 (3 H, s, OMe), 3.36 (1 H, s, OH), 1.86 (2 H, m, CH_2Me), 1.77 (3 H, s, MeC:), and 0.87 [3 H, apparent t, *J*(apparent) 7.5 Hz, CH_2Me]; $\nu_{max.}$ 1 730 (CO) and 1 645 cm^{-1} (C : C) (Found: C, 60.95; H, 9.0. $C_8H_{14}O_3$ requires C, 60.76; H, 8.8%). Methyl (2*R*)- and (2*S*)- 2-ethyl-2-hydroxy-3-methylbut-3-enoates prepared from the corresponding acids (14) and (15) in the same way were both shown to have an optical purity of greater than 98% by ^{1}H n.m.r. spectroscopy in the presence of chiral shift reagent.

Methyl (2*S*)-α-*Acetolactate* (13).—Methyl (2*S*)-2-hydroxy-2,3-dimethylbut-3-enoate (12) (600 mg) in dry ethyl acetate (20 ml) was ozonised at 0 °C for 75 min. The solution was shaken with a suspension of manganese dioxide (500 mg) in water (15 ml). The aqueous layer was extracted with ether (2 × 15 ml), the combined ethereal extracts were washed with aqueous sodium hydrogen carbonate (0.2 M; 20 ml) and the aqueous solution was re-extracted with ether (2 × 20 ml). The combined organic solutions were dried ($MgSO_4$) and evaporated to give methyl (2*S*)-α-acetolactate (13) (425 mg, 70%), pure as judged by ^{1}H n.m.r. spectroscopy. A portion of the material was distilled in a bulb-tube apparatus to give the ester (13) as a colourless oil, δ_H 4.18br (1 H, s, OH), 3.78 (3 H, s, OMe), 1.23 (3 H, s, MeCO), and 1.58 (3 H, s, *Me*C(OH)); $\nu_{max.}$ 1 745sh and 1 720 cm^{-1} (CO). The 2,4-dinitrophenylhydrazone, prepared as for the (2*R*)-isomer [2] had m.p. 173—174 °C. [lit.,[2] m.p. for the (2*R*)-isomer 173.5—174 °C].

Methyl (2R)- *and* (2S)-α-*Acetohydroxybutyrates*.—Methyl (2*R*)-2-ethyl-2-hydroxy-3-methylbut-3-enoate (73 mg) was dissolved in ethyl acetate (10 ml) and ozonised for 90 min at 0 °C. The product (68 mg), isolated as in the preparation of methyl α-acetolactate, above, was distilled in a bulb-tube apparatus at 130 °C (8 mmHg) to give methyl (2*R*)-α-acetohydroxybutyrate (46 mg), δ_H 4.15br (1 H, s, OH), 3.9 (3 H, s, OMe), 2.36 (3 H, s, MeCO), 2.13 (2 H, q, *J* 7.5 Hz, CH_2Me), and 0.9 (3 H, t, *J* 7.5 Hz, CH_2Me); $\nu_{max.}$ 1 745sh and 1 720 cm^{-1} (CO) (Found: C, 52.5; H, 7.8. $C_7H_{12}O_4$ requires C, 52.50; H, 7.50%. Methyl (2*S*)-α-acetohydroxybutyrate (16) was prepared in the same way from the methyl ester of the acid (15).

Enzyme Assays.—The isoleucine-valine mutant strain of *Salmonella typhimurium* used as the source of the enzyme was *ilvD6* (dehydratase deficient). The strain was grown under derepressed conditions [16] to obtain cell-free extracts containing high levels of reductoisomerase activity. The reductoisomerase assay was that described by Armstrong and Wagner.[8] For these assays, a 1 ml incubation mixture contained the following: substrate (4 mmol), $MgSO_4$(5 mmol), NADPH (0.13 mmol), bovine serum albumin (0.5 mg; to stabilize the enzyme), 0.075M-potassium phosphate buffer, pH 7.5, and cell-free extract of *ilvD6*. Protein concentration was measured by the method of Lowry *et al.*[17]

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