Structure–Activity Studies with the αβ-Dihydroxyacid Dehydratase of *Salmonella typhimurium*

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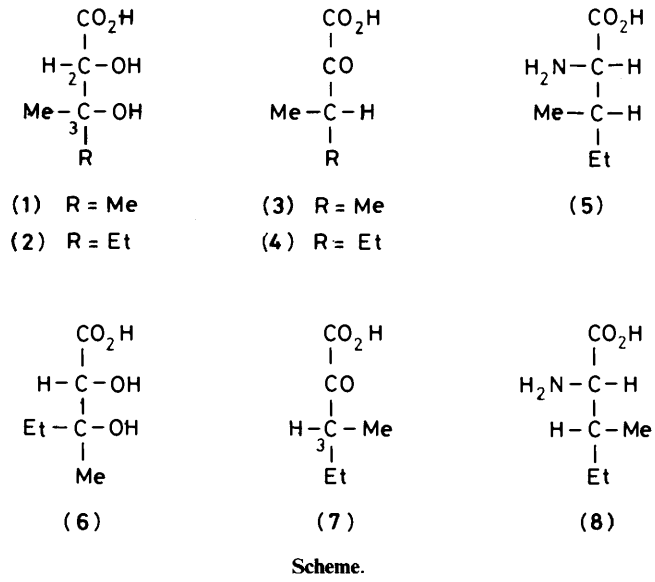
(2*RS*,3*RS*)- and (2*RS*,3*SR*)-2,3-Dihydroxybutanoic acids, (2*R*,3*R*)-2,3-dihydroxy-3-methylpentanoic acid, (2*RS*)-2-ethyl-2,3-dihydroxypentanoic acid, (2*RS*,3*RS*)- and (2*RS*,3*SR*)-2,3-dihydroxy-3-methylhexanoic acids, and (2*RS*,3*RS*)- and (2*RS*,3*SR*)-2,3-dihydroxy-3-methylheptanoic acids were synthesised. These acids, as well as (*RS*)-2,3-dihydroxy-3-methylbutanoic acid and (*RS*)-glyceric acid were tested as substrates for the αβ-dihydroxyacid dehydratase of the isoleucine–valine biosynthetic pathway of *Salmonella typhimurium*. For acids having a propyl group at C-3, the activities were greatly reduced compared with those obtained for the natural substrates (2*R*,3*R*)-2,3-dihydroxy-3-methylpentanoic acid [(2*R*,3*R*)-DHI] and (*R*)-2,3-dihydroxy-3-methylbutanoic acid [(*R*)-DHV]. For acids having an n-butyl substituent at C-3, the activities were close to zero. (2*RS*,3*SR*)-2,3-Dihydroxybutanoic acid (*threo*-isomer) underwent dehydration at a rate comparable with that of (2*R*,3*R*)-DHI, the natural substrate in the isoleucine pathway, whereas the (2*RS*,3*RS*)-acid (*erythro*-isomer) had much lower activity and (*RS*)-glyceric acid had even less activity. These results illustrate differences in the alkyl group requirements with respect to the areas of the binding site of the enzyme that accommodate the C-3 substituents. They also demonstrate the size limits of the alkyl groups that can be accommodated in substrate analogues.

The pathways of isoleucine and valine biosynthesis are unusual in that the last four steps are catalysed by a common set of enzymes.[1] These enzymes must therefore accommodate the structural differences between the intermediates of the two pathways. The enzyme αβ-dihydroxyacid dehydratase (2,3-dihydroxy acid hydrolyase, EC 4.2.1.9) that catalyses the conversion of the dihydroxy acids (**1**) and (**2**) into the corresponding α-keto acids (**3**) and (**4**) respectively (Scheme), has a strict requirement for substrates with the 2*R*-configuration. However, if C-3 is chiral, the configuration at this centre may be either *R* or *S*. Thus both (2*R*,3*R*)- (2*R*,3*S*)-DHI are substrates for the enzyme.[2] It was concluded from growth studies with mutants of *Salmonella typhimurium* that the conversion is nevertheless stereospecific, and that the (2*R*,3*R*)-acid (**2**) is converted into (*S*)-3-methyl-2-oxopentanoic acid (**4**), the keto acid precursor of L-isoleucine (**5**), whereas the (2*R*,3*S*)-acid (**6**) is converted into the (*R*)-keto acid (**7**), which has the configuration at C-3 corresponding to L-alloisoleucine (**8**).

The enzyme thus operates stereospecifically, although, most unusually, it is not stereoselective with respect to the configuration at C-3 in the substrate. These observations indicate that, regardless of the absolute configurations of the substrates, there must be the same relative configurations of the C-2 hydrogen atom and C-3 hydroxy group eliminated during dehydration. It follows, using the previously proposed model,[2] that the area of the binding site of the enzyme (1, in Figure 1) that accommodates only a methyl group in the natural intermediates of the pathway [(*R*)-DHV (**1**) and (2*R*,3*R*)-DHI (**2**)] must also be able to accommodate an ethyl group if the substrate is the abnormal one (2*R*, 3*S*)-DHI (**6**) (Figure 1). A less exceptional requirement is that area 2, that normally accommodates either a methyl or an ethyl group in the natural substrates (**1**) and (**2**), must be able to accommodate a methyl group in the abnormal substrate (**6**).

The C-2 hydrogen atom and the C-3 hydroxy group are shown in an *anti* conformation in Figure 1 for purposes of illustration, although this implied mode of elimination has not been proven experimentally. However, the results so far



Scheme.

obtained require that following elimination, the protonation at C-3 in (*R*)-DHV (**1**) and in (2*R*,3*R*)-DHI (**2**) must take place on the same face of the molecule as that from which the hydroxy group departs (Figure 1).

αβ-Dihydroxyacid dehydratase is therefore unusual because on the one hand it must be flexible enough to accommodate the substrates of both the valine and isoleucine pathways, and, at the same time, have high stereospecificity with respect to the reaction catalysed. The opposing character of these essential attributes provided an unusual opportunity for studying the range of structures that can be accommodated by the enzyme. By increasing the size of the alkyl groups at C-3, it should be possible to assess the steric capacity of the enzyme to bind and catalyse the dehydration of the corresponding acids. Also, by

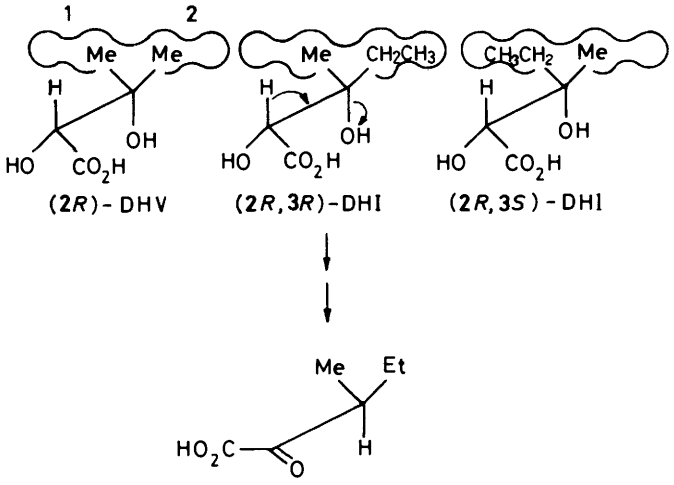
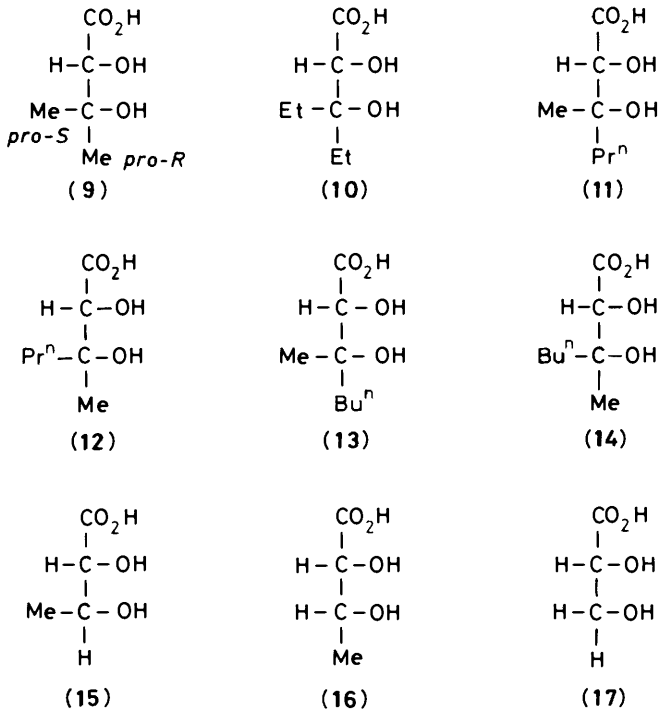


Figure 1. Model of the active site of αβ-dihydroxy acid dehydratase, showing the proposed alkyl group binding areas



reducing their size it should be possible to investigate the positive role played by these groups in the catalytic action of the enzyme.

In order to examine the influence of the size of the C-3 substituent in substrates for the αβ-dihydroxyacid dehydratase of *Salmonella typhimurium*, a group of homologous αβ-dihydroxy acids were synthesised. The required compounds were prepared by either *cis*- or *trans*-hydroxylation of the corresponding αβ-unsaturated acids. The latter acids were synthesised by the stereospecific procedure of Kobayashi *et al.*[3] when different substituents were present at C-3; otherwise they were prepared using the Reformatsky reaction. *cis*-Hydroxylation was carried out using osmium tetraoxide–*N*-methylmorpholine *N*-oxide,[4] and *trans*-hydroxylation using hydrogen peroxide-formic acid.[5] These operations were carried out with the (*E*)-isomers of αβ-unsaturated acids bearing different substituents at C-3. *cis*-Hydroxylation of the (*E*)-acids led to *threo*-dihydroxy acids and *trans*-hydroxylation to *erythro*-dihydroxy acids. The dihydroxy acids (**10**)—(**14**) previously had not been synthesised.

Table. Summary of the results of the αβ-dihydroxyacid dehydratase assays*

Substrate	Concentration (mM)	Relative Activity (%)
(±)-Glyceric acid [as (**17**)]	10	3
	20	4
threo-2,3-Dihydroxybutanoic acid [as (**15**)]	10	45
	20	47
erythro-2,3-Dihydroxybutanoic acid [as (**16**)]	10	7
	20	10
(±)-DHV [2*RS*)-2,3-Dihydroxy-3-methylbutanoic acid, as (**1**)]	10	100
(2*R*,3*R*)-DHI [(2*R*,3*R*)-2,3-Dihydroxy-3-methylpentanoic acid, (**2**)]	10	44
	20	46
(±)-2,3-Dihydroxy-3-ethylpentanoic acid [as (**10**)]	10	20
	20	19
(2*RS*,3*RS*)-2,3-Dihydroxy-3-methylhexanoic acid [as (**11**)]	10	7
	20	9
(2*RS*,3*SR*)-2,3-Dihydroxy-3-methylhexanoic acid [as (**12**)]	10	2
	20	3
(2*RS*,3*RS*)-2,3-Dihydroxy-3-methylheptanoic acid [as (**13**)]	10	1
	20	2
(2*RS*,3*SR*)-2,3-Dihydroxy-3-methylheptanoic acid [as (**14**]	10	0
	20	1

* All substrates, except for DHI, were assayed as their racemates.

Activities of substrates for the dihydroxyacid dehydratase were determined using a cell-free extract of *S typhimurium ilvC8*, and the results are summarised in the Table. It was previously shown[2] that the isoleucine precursor (**2**), (2*R*,3*R*)-DHI, and the unnatural substrate (**6**), (2*R*,3*S*)-DHI, have about half the activity (48%) of the valine precursor (**1**), (2*R*)-DHV. In this study, the same range of activity (44—46%) was again observed for (2*R*,3*R*)-DHI. The analogue with two ethyl substituents at C-3 (**10**) showed only one-fifth the activity of the valine precursor. When methyl and n-propyl groups were present at C-3 [(**11**) and (**12**)], activity was even less (2—9%) and, with methyl and n-butyl substituents [(**13**) and (**14**)], activity was essentially nil (0—2%). The relative activities obtained with 20 mM amounts of the analogues revealed that doubling the substrate concentrations did not significantly increase the activities with respect to that of DHV.

The C-3 alkyl groups in these substrates are conveniently described in terms of their relationships with the corresponding 3-*pro*-*S* and 3-*pro*-*R* groups of the valine precursor [see (**9**)]. In the following discussion, alkyl groups replacing these methyl groups in analogues are referred to as '3*S*' or '3*R*' groups respectively, and the analogue itself is described in terms of these substituents. Thus compound (**11**) is the '3*S*'-Me, '3*R*'-Pr^n analogue. The structures of the analogues (**10**)—(**17**) are also written, using a modified Fischer representation, in such a way that they all show the same relationship between the chiral centres at C-2 and C-3. This convention reflects the observation that both 2*R*,3*R*- and 2*R*,3*S*-DHI are converted into the cor-

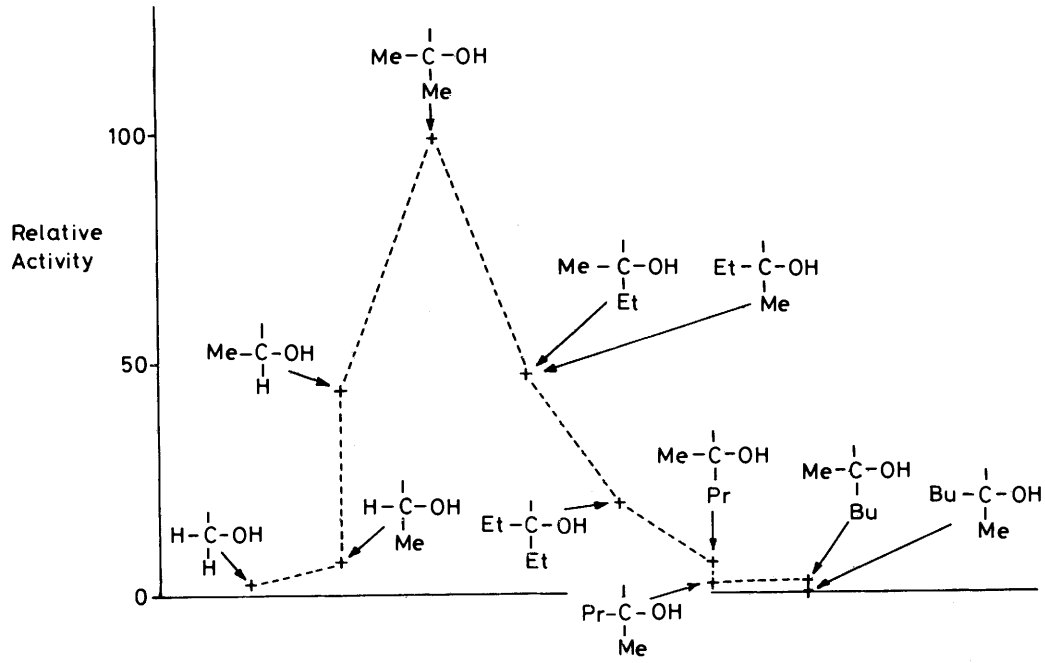


Figure 2. Activities of αβ-dihydroxy acids in the αβ-dihydroxyacid dehydratase assay in relation to the nature and disposition of the substituents at C-3

responding α-keto acids with the same stereochemistry, which implies that both substrates are bound to the enzyme in conformations with the same stereochemical relationships between the functional groups, as noted above. Analogues were tested as the racemates and it is assumed that only components with the 2*R*-conformations were substrates for the enzyme.

The results with analogues (**10**)—(**14**) inclusive (Table) show that the enzyme will not operate on substrates with a group larger than n-propyl in either the '3*S*' or '3*R*' position. The activity difference between the '3*S*'-Pr^n, '3*R*'-Me (**12**) (*ca.* 2%) and '3*S*'-Me, '3*R*'-Pr^n (**11**) (*ca.* 7%) analogues is probably significant and indicates that the latter, with a methyl group in the '3*S*'-position as in the natural intermediates (**1**) and (**2**), is a somewhat better substrate than its diastereoisomer (**12**). It is concluded that with respect to both areas 1 and 2 (Figure 1), the sizes of the C-3 substituents that can be accepted in the substrates reach their limits with the n-propyl group, and that with respect to area 2, which accommodates either a methyl or an ethyl group in the natural substrates, there is somewhat more flexibility than for area 1, which accommodates only a methyl group in the natural substrates.

The catalytic activity of the enzyme is clearly affected by the substitution in analogues of alkyl groups more bulky than those present in DHV and DHI. However, since alkyl groups are probably needed for effective substrate binding, their absence should also affect substrate activity. This question was explored using substrates with C-3 substituents smaller than those in the natural substrates. Thus loss of the '3*R*'-alkyl group, as in *threo*-2,3-dihydroxybutanoic acid (**15**), led to loss of activity compared with the valine precursor, but this analogue still had an activity (*ca.* 45%) comparable with that of the isoleucine precursor (**2**). However, loss of the '3*S*'-methyl group in the *erythro*-acid (**16**), gave a substrate that had less than 10% of the activity of the valine precursor (**1**). Loss of both alkyl groups, as in glyceric acid (**17**), gave a substrate with very little activity.

It is concluded from these results that the presence of a methyl group in the '3*S*'-position as in the natural substrates (**1**) and (**2**) is critically important for effectiveness as a substrate and that the presence of an alkyl group in the '3*R*'-position is of lesser importance.

These results may be compared with those obtained by Wixom *et al.*[6] These authors found, with αβ-dihydroxyacid dehydratase from a variety of sources, that activity was uniformly greater with *threo*-2,3-dihydroxybutanoic acid than with the *erythro*-isomer, in line with the present results. Racemic dihydroxy acids with one bulky alkyl substituent at C-3 (*threo*-2,3-dihydroxynonanoic, *erythro*-2,3-dihydroxynonanoic, and 2,3-dihydroxy-3-phenylpropanoic acids) were not substrates for the enzyme. Similarly, racemic DHV was uniformly more active than DHI. The latter was tested as a mixture of the *erythro*- and *threo*-racemates of unstated (and presumably unknown) composition. However, at the concentrations used (2 mM),[6c] it is likely that the results are valid. (K_m Values range from 2.4 mM to 0.17 mM for DHV, and 2 mM to 0.08 mM for DHI.[7] These values, which relate to enzymes from a variety of sources, were determined using racemic DHV and a mixture of DHI racemates.) It should be noted that the dehydratase of *Escherichia coli*, a species closely related to *S. typhimurium*, has K_m values of 0.17 and 0.08 mM for DHV and DHI respectively.[7] A K_m value for DHV close to 0.17 mM has also been calculated for the *Salmonella* enzyme (unpublished data).

The results obtained in this and the previous study[2] are summarised in diagrammatic form (Figure 2). The various substrates tested are identified by partial Fischer projections showing the C-3 substituents with the C-3 configuration of the 2*R*-isomer. The diagram emphasises that the enzyme is maximally efficient for substrates having the C-3 substitution pattern of DHV and DHI, and that catalytic activity decreases as the C-3 substituents are made either smaller or larger than those in the natural substrates. The above results can be interpreted in terms of the binding of the C-3 substituents of the substrate dihydroxy acids at areas 1 and 2 of the active site of the enzyme (Figure 1). Thus a possible conclusion is that linear alkyl groups longer than n-propyl prevent binding and that groups smaller than methyl reduce the capacity of the substrate to bind effectively. However, it is conceivable that some or all of

the inactive substrates are bound at the active site, but not transformed into product. In this case, inactive substrates should be competitive inhibitors of the normal enzymatic reaction. Further investigations are in hand to examine this possibility.

Experimental

M.p.s were determined in open capillaries and are corrected. ^{1}H N.m.r. spectra were determined using a JEOL MH-100, Hitachi Perkin-Elmer R600, or JEOL PS-100 spectrometer. Spectra were recorded for solutions in deuteriochloroform, unless otherwise stated, with tetramethylsilane as internal standard. G.l.c. analyses were performed using a Pye Unicam GCD chromatograph, fitted with a flame ionisation detector. Ether refers to diethyl ether.

Growth of S. typhimurium ilvC8.—The same mutant strain used in the previous study,[2] *Salmonella typhimurium ilvC8* (deficient in reductoisomerase of the isoleucine–valine biosynthetic pathway) was used for this study. The strain was grown under derepressed conditions,[8] which results in high levels of dihydroxy acid dehydratase activity, and harvested 1 h after stationary phase was attained. Cell-free extracts were used for the study.

Enzyme Assay.—αβ-Dihydroxyacid dehydratase activity was determined by the method of Myers and Adelberg.[9] Additional information about the assay and the preparation of the sodium salts of the dihydroxy acids, which served as substrates, from their dicyclohexylammonium salts can be found in ref. 2. The assay has proved repeatedly to be both specific and reproducible in studies with cell-free extracts.[2]

Ethyl 3,3-*Bis(thiophenyl)butanoate.*—This compound was prepared by the published procedure.[10]

Ethyl 3-*Thiophenylbut-2-enoate.*—This compound was prepared by the following modification of the published procedure.[3] Ethyl 3,3-bis(thiophenyl)butanoate (20 g) was heated at 110 °C with zinc chloride at 12—15 mmHg, until no more thiophenol distilled off. The reaction mixture was dissolved in ether (100 ml) and the solution was washed with sodium hydroxide solution (2M, 3 × 50 ml) and water (2 × 50 ml), dried ($MgSO_4$), and the solvent evaporated. The product, obtained in 90% yield was found (n.m.r., g.l.c., see below) to consist of a 9:1 mixture of ethyl (*E*)- and (*Z*)-3-thiophenylbut-2-enoates. The esters were separated by chromatography on basic alumina (Brockman grade 1). Elution of the various products from the column was followed by t.l.c. (aluminium oxide, solvent system carbon tetrachloride–ethyl acetate, 19:1) and by g.l.c. (10% SE 30 on Chromosorb W, 230 °C). With light petroleum (b.p. 40—60 °C) as eluant, diphenyl disulphide was eluted first, followed by ethyl (*E*)-3-thiophenylbut-2-enoate. Once elution of the (*E*)-isomer had begun, the solvent was changed to carbon tetrachloride, which completed the elution of the (*E*)-isomer and subsequently eluted the (*Z*)-isomer.

(E)-3-*Methylpent-2-enoic Acid.*—To a stirred solution of ethyl magnesium bromide [10 mmol; prepared from magnesium (0.243 g) and bromoethane (0.75 ml) in tetrahydrofuran (30 ml)] was added, under nitrogen, cuprous iodide (4 mmol, 0.76 g). The dark blue mixture was cooled to −78 °C and a solution of ethyl (*E*)-3-thiophenylbut-2-enoate (2 mmol, 0.44 g) in tetrahydrofuran (10 ml) was added dropwise at such a rate that the temperature of the reaction mixture remained below −65 °C. The progress of the reaction was followed by quenching aliquots in moist ether and examination by g.l.c. (10% SE 30 on Chromosorb W, 230 °C). When all the starting material had disappeared, the reaction was quenched by the addition of water (1 ml) and allowed to warm to room temperature with vigorous stirring. The mixture was dissolved in ether (200 ml), the ethereal solution was filtered, and the solvent was evaporated. The crude product, ethyl (*E*)-3-methylpent-2-enoate, obtained in 80% yield, appeared to be substantially pure by n.m.r. and was therefore converted into the corresponding acid without further purification; δ 5.66 (1 H, q, *J* 1.2 Hz, HC=), 4.14 (2 H, q, *J* 7.1 Hz, OCH_2), 2.16 (3 H, d, *J* 1.2 Hz, CH_3C=), *ca.* 2.18 (partly obscured by the signal at δ 2.16) (2 H, q, *J* 7.1 Hz, CH_2C=), 1.27 (3 H, t, *J* 7.1 Hz, CH_3CH_2C=), and 1.07 (3 H, t, *J* 7.6 Hz, CH_3CH_2O). Minor peaks attributable to the (*Z*)-ester showed that <15% of this isomer was present. The ester was boiled under reflux with a slight excess of barium hydroxide solution (5% as the octahydrate) until no more oily drops of the ester remained. The solution was cooled, acidified (Congo red) with concentrated hydrochloric acid, and extracted with diethyl ether (2 × 50 ml). Each ethereal extract was extracted with sodium hydrogen carbonate solution (5%, 2 × 50 ml). The combined sodium hydrogen carbonate extracts were washed with ether (100 ml), acidified to pH 1 (conc. HCl), and extracted with ether (2 × 100 ml). The extracts were dried ($MgSO_4$) and the solvent was evaporated to give (*E*)-3-methylpent-2-enoic acid (yield typically 60%). The acid was purified as the *dicyclohexylammonium salt* which crystallised from light petroleum (b.p. 40—60 °C), m.p. 108.5—109.5 °C (Found C, 72.9; H, 11.3; N, 4.7. $C_{18}H_{33}NO_2$ requires C, 73.15; H, 11.25; N, 4.75%); δ 10.0 (1 H, s, CO_2H), 5.69 (1 H, s, HC=), 2.18 (3 H, s, CH_3C=), *ca.* 2.18 (partly obscured by the CH_3C= signal) (2 H, q, CH_2), and 1.08 (3 H, t, *J* 7 Hz, CH_3CH_2).

(E)-3-*Methylhex-2-enoic Acid.*—This acid, as for (*E*)-3-methylpent-2-enoic acid, above, was prepared from propylmagnesium bromide (10 mmol) in tetrahydrofuran (30 ml), cuprous iodide (4 mmol), and ethyl (*E*)-3-thiophenylbut-2-enoate (2 mmol) in tetrahydrofuran (10 ml) at −78 °C. The reaction was followed by g.l.c. and the product was isolated as for ethyl (*E*)-3-methylpent-2-enoate, above. The ester, without further purification, was boiled under reflux with an excess of barium hydroxide solution as for (*E*)-3-methylpent-2-enoic acid, above. Extraction of the acidic product as before, followed by g.l.c. examination and n.m.r. indicated the presence of some of the βγ-isomer[11,12] in addition to the major product. The impurity was removed by chromatography on Kieselgel 60, 70—230 mesh) with carbon tetrachloride as eluant. The (*E*)-3-methylhex-2-enoic acid (yield typically 47%) was eluted first and crystallised as thin plates, m.p. 36—38 °C (lit., m.p. 40 °C,[11] 37—38 °C[13]); δ 11.44 (br, 1 H, s, CO_2H), 5.69 (1 H, m, HC=), 2.15 (5 H, s, CH_3C=), *ca.* 2.15 (partly obscured by the singlet at δ 2.15) (2 H, q, CH_2C=), 1.53 (2 H, m, CH_3CH_2), and 0.91 (3 H, t, *J* 7 Hz, CH_3CH_2). The n.m.r. data for this acid and for (*E*)-3-methylhept-2-enoic acid, below, are in close agreement with corresponding data, taken in CCl_4 solution, for these acids prepared by the Wadsworth–Emmons reaction.[14] The dicyclohexylammonium salt crystallised from light petroleum (b.p. 40—60 °C), m.p. 95.5—97 °C (Found: C, 73.6; H, 11.6; N, 4.6. Calc. for $C_{19}H_{35}NO_2$: C, 73.74; H, 11.4; N, 4.52%).

(E)-3-*Methylhept-2-enoic Acid.*—The ethyl ester was prepared as for ethyl (*E*)-3-methylpent-2-enoate, above, from butylmagnesium bromide (10 mmol) in tetrahydrofuran (30 ml), cuprous iodide (4 mmol), and ethyl (*E*)-3-thiophenylbut-2-enoate (2 mmol) in tetrahydrofuran (10 ml) at −78 °C. The crude ester was hydrolysed with barium hydroxide as before. Examination of the product by g.l.c. showed that the major product was contaminated with a minor product, shown by n.m.r. to be the βγ-isomer. The (*E*)-3-methylhept-2-enoic acid

was isolated by chromatography on silica gel with carbon tetrachloride as eluant, as before, in 76% yield; δ 11.64 (1 H, s, CO_2H), 5.64 (1 H, m, HC=), 2.1 (3 H, s, CH_3C=), *ca.* 2.1 (partly obscured by the singlet at δ 2.1) (2 H, m, CH_2C=), 1.36 (4 H, m, $CH_2CH_2CH_2$), and 0.84 (3 H, t, *J* 9 Hz, CH_3CH_2). The *dicyclohexylammonium salt* crystallised from light petroleum, (b.p. 40—60 °C), m.p. 82—83 °C (Found: C, 74.4; H, 11.75; N, 4.3. $C_{20}H_{37}NO_2$ requires C, 74.26; H, 11.53; N, 4.33%).

(2R,3R)-2,3-*Dihydroxy*-3-*methylpentanoic Acid*.—This acid was prepared by the published procedure.[2]

(2RS,3RS)-2,3-*Dihydroxy*-3-*methylhexanoic Acid* [*as* (**11**)].—To a stirred solution of (*E*)-3-methylhex-2-enoic acid (1 g) in formic acid (90% v/v) was added dropwise with cooling, a solution of hydrogen peroxide (30%, 1.4 equiv.).[5] The reaction mixture, which had been kept at room temperature during the addition of the hydrogen peroxide, was stirred and heated to 40—45 °C for 3 h, allowed to cool to room temperature and stirred overnight. The solution was concentrated under reduced pressure, more water was added and the solution was again concentrated to remove the remaining formic acid. The oily acid [as (**11**), yield 63%] was purified as the *dicyclohexylammonium salt* which crystallised from acetone–light petroleum (b.p. 60—80 °C), m.p. 175—177 °C (Found: C, 66.35; H, 11.3; N, 4.1. $C_{19}H_{37}NO_4$ requires C, 66.45; H, 10.86; N, 4.08%). The free acid was recovered from the salt by passage through a column of Dowex 50W × 8 ion exchange resin (H^+ form); δ(CD_3COCD_3) 4.00 [1 H, s, C*H*(OH)], 1.53 (4 H, m, CH_2CH_2), 1.18 [3 H, s, CH_3C(OH)], and 0.91 (3 H, m, CH_3CH_2).

(2RS,3SR)-2,3-*Dihydroxy*-3-*methylhexanoic Acid* [*as* (**12**)].—(*E*)-3-Methylhex-2-enoic acid (120 mg) and *N*-methylmorpholine *N*-oxide dihydrate (1.1 equiv., 158 mg)[4] were dissolved in a mixture of water (3 ml), acetone (4 ml), and butan-2-ol (1 ml). An aqueous solution of osmium tetraoxide (1%, 0.32 ml) was added and the mixture was stirred overnight under nitrogen. Magnesium trisilicate (0.8 g) and sodium dithionite (0.2 g) in water (2 ml) were added, and the mixture was stirred for 2 min. The mixture was filtered, the solids were washed with water and acetone, and the combined filtrate and washings were concentrated under reduced pressure to remove acetone. The aqueous residue was acidified (Congo red) with dilute hydrochloric acid, saturated with sodium chloride, and extracted with ether (2 × 100 ml). The ethereal extracts were dried ($MgSO_4$) to give (2*RS*,3*SR*)-2,3-dihydroxy-3-methylhexanoic acid [as (**12**)] as an oil (49%); δ 5.9 (br, 3 H, s, 2 × OH + CO_2H), 4.08 [s, 1 H, C*H*(OH)], 1.48 (4 H, m, CH_2CH_2), 1.27 [3 H, s, CH_3C(OH)], and 0.95 (3 H, m, CH_3CH_2). The *dicyclohexylammonium salt* crystallised from acetone–light petroleum (b.p. 40—60 °C), m.p. 167—169 °C (Found: C, 66.7; H, 11.1; N, 4.3. $C_{19}H_{37}NO_4$ requires C, 66.45; H, 10.86; N, 4.08%).

(2RS,3RS)-2,3-*Dihydroxy*-3-*methylheptanoic Acid* [*as* (**13**)].—This acid was prepared in 96% yield from (*E*)-3-methylhept-2-enoic acid as for the acid (**11**), above. The acid [as (**13**)], was obtained as a viscous oil. δ (CD_3COCD_3) 4.01 [1 H, s, C*H*(OH)], 1.30 (6 H, m, $CH_2CH_2CH_2$), 1.19 [3 H, s, CH_3C(OH)], and 0.91 (3 H, m, CH_3CH_2). The *dicyclohexylammonium salt* crystallised from methanol–ethyl acetate, m.p. 188—191 °C (Found: C, 66.9; H, 11.4; N, 3.8. $C_{20}H_{39}NO_4$ requires C, 67.19; H, 10.99; N, 3.91%).

(2RS,3SR)-2,3-*Dihydroxy*-3-*methylheptanoic Acid* [*as* (**14**)].—This acid was obtained as for the acid (**12**) in 48% yield as an oil; δ(CD_3COCD_3) 3.98 [1 H, s, C*H*(OH)], 1.30 (6 H, m, $CH_2CH_2CH_2$), 1.23 [3 H, s, CH_3C(OH)], and 0.93 (3 H, m, CH_3CH_2). The *dicyclohexylammonium salt* crystallised from ethyl acetate, m.p. 152—154 °C (Found: C, 67.2; H, 11.2; N, 4.2. $C_{20}H_{39}NO_4$ requires C, 67.19; H, 10.99; N, 3.91%).

2,3-*Dihydroxy*-3-*ethylpentanoic Acid* (**10**).—To a boiling suspension of zinc (22.8 g) in benzene (140 ml) was added, during 1 h, a solution of pentan-3-one (12.3 ml) and ethyl 2-bromoacetate (38.8 ml) in benzene (1 254 ml). The mixture was boiled under reflux for 2 h and cooled to room temperature. Sulphuric acid (6M; 175 ml) was added, and the mixture was stirred vigorously for 1 h. The aqueous layer was separated and extracted with benzene (200 ml). The combined organic solutions were washed with water, sodium hydrogen carbonate solution and water, dried (Na_2SO_4), and concentrated under reduced pressure. To a stirred solution of the residual crude ester in pyridine (168 ml) was added phosphorus oxychloride (21.5 ml). The mixture was kept for 8 h and heated on a steam-bath for 1.5 h. The product was poured on to crushed ice and the reaction vessel was washed out with water (500 ml) and light petroleum (b.p. 40—60 °C) (100 ml). The aqueous phase was extracted with hexane (2 × 200 ml). The combined organic solutions were washed with water, hydrochloric acid (2M), and water, dried ($MgSO_4$), and concentrated under reduced pressure. The residue appeared to consist (n.m.r.) of a mixture of ethyl 3-ethylpent-2-enoate and ethyl 3-ethylpent-3-enoate (1:1). Ethyl 3-ethylpent-2-enoate was isolated by chromatography over silica gel (Merck) with light petroleum (b.p. 40—60 °C) as eluant. In this system, ethyl 3-ethylpent-2-enoate was eluted first [purity > 95% (n.m.r.)]; δ 5.6 (1 H, s, HC=), 4.16 (2 H, q, *J* 7 Hz, CH_2O), 2.66 (2 H, q, *J* 7 Hz, CH_2C=), 2.24 (2 H, q, *J* 7 Hz, CH_2C=), 1.3 (3 H, t, *J* 7 Hz, CH_3CH_2O), and 1.10 (6 H, t, *J* 7 Hz, CH_3CH_2). To a solution of ethyl 3-ethylpent-2-enoate (257 mg) and *N*-methylmorpholine *N*-oxide dihydrate (270 mg) in a mixture of water (1 ml), acetone (1 ml), and butan-2-ol (1 ml) was added an aqueous solution of osmium tetraoxide (1%, 0.15 ml). The mixture was stirred under nitrogen overnight, a solution of sodium dithionite (0.1 g) and magnesium trisilicate (0.4 g) in water (2 ml) was added, the mixture was stirred for 2 min, filtered, and the filtrate was concentrated under reduced pressure. The aqueous residue was acidified (Congo red) and extracted with ether (2 × 100 ml). The extracts were dried ($MgSO_4$) and evaporated to give ethyl 2,3-dihydroxy-3-ethylpentanoate in 40% yield, essentially pure as judged by n.m.r. analysis; δ 4.48 (2 H, q, *J* 7.3 Hz, OCH_2), 4.28 [1 H, s, C*H*(OH)], 1.6 [4 H, m, 2 × CH_2CH_2C(OH)], 1.36 (3 H, t, *J* 7.3 Hz, CH_3CH_2O), 0.92 (6 H, t, *J* 7.5 Hz, 2 × CH_3CH_2C) (under expansion, these signals were seen to consist of two triplets separated by 0.01 p.p.m.). Without further purification, the ester was hydrolysed with barium hydroxide and the acidic product was isolated as before to give 2,3-dihydroxy-3-ethylpentanoic acid (**10**) in 86% yield; δ 4.1 [1 H, s, C*H*(OH)], 1.3—1.9 (4 H, m, 2 × CH_2), and 0.85 (6 H, m, 2 × CH_3). The *dicyclohexylammonium salt* crystallised from ethyl acetate, m.p. 170—171.5 °C (Found: C, 66.7; H, 10.9; N, 4.1. $C_{19}H_{37}NO_4$ requires C, 66.45; H, 10.86; N, 4.08%).

(2RS,3RS)-2,3-*Dihydroxybutanoic Acid* [*as* (**16**)].—To a solution of crotonic acid (1 g) in 90% formic acid (5 ml) was added 30% hydrogen peroxide (1.4 equiv.). The mixture was stirred at 40 °C for 3 h and then at room temperature overnight. The solution was concentrated under reduced pressure and the acid [as (**16**)], obtained in 87% yield, was purified by recrystallisation (acetone) of the *dicyclohexylammonium salt*, m.p. 144.5—145.5 °C (Found: C, 63.65; H, 10.6; N, 4.6. $C_{16}H_{31}NO_4$ requires C, 63.76; H, 10.36; N, 4.64%). For the free acid [as (**17**)], δ(CD_3COCD_3) *ca.* 4.0 [2 H, 2 × C*H*(OH)], and 1.18 (3 H, d, *J* 6.4 Hz, CH_3).

(2RS,3SR)-2,3-*Dihydroxybutanoic Acid* [*as* (**15**)].—To a solution of crotonic acid (1 g) and *N*-methylmorpholine *N*-oxide dihydrate (1.95 g) in a mixture of water (4 ml), acetone (6 ml), and butan-2-ol (1 ml) was added an aqueous solution of osmium tetraoxide (1%, 0.3 ml). The mixture was stirred overnight, sodium dithionite (0.2 g) and magnesium trisilicate (0.8 g) were added, and the mixture was stirred for 2 min. The mixture was filtered, the acetone was removed under reduced pressure, and the aqueous residue was extracted with ether (2 × 200 ml). The combined ethereal extracts were dried ($MgSO_4$) and evaporated to give, in 53% yield, the acid [as (**15**)], purified by recrystallisation (ethyl acetate) of the *dicyclohexylammonium salt*, m.p. 150.5—151.5 °C (Found: C, 63.5; H, 10.55; N, 4.6. $C_{16}H_{31}NO_4$ requires C, 63.76; H, 10.36; N, 4.64%). For the free acid δ(CD_3COCD_3) 4.0 [2 H, m, 2 × C*H*(OH)] and 1.24 (3 H, d, *J* 6.4 Hz, CH_3).

Acknowledgements

We thank the Science and Engineering Research Council (U.K.) for financial support. This paper is a contribution from the Department of Biochemistry, School of Agricultural and Life Sciences and School of Physical and Mathematical Sciences. Paper No. 9008 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh, North Carolina 27650.

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Received 6*th June* 1984; *Paper* 4/932