An Improved Route to (3*RS*,5*S*)-[5-$^3H_1$]Mevalonic Acid

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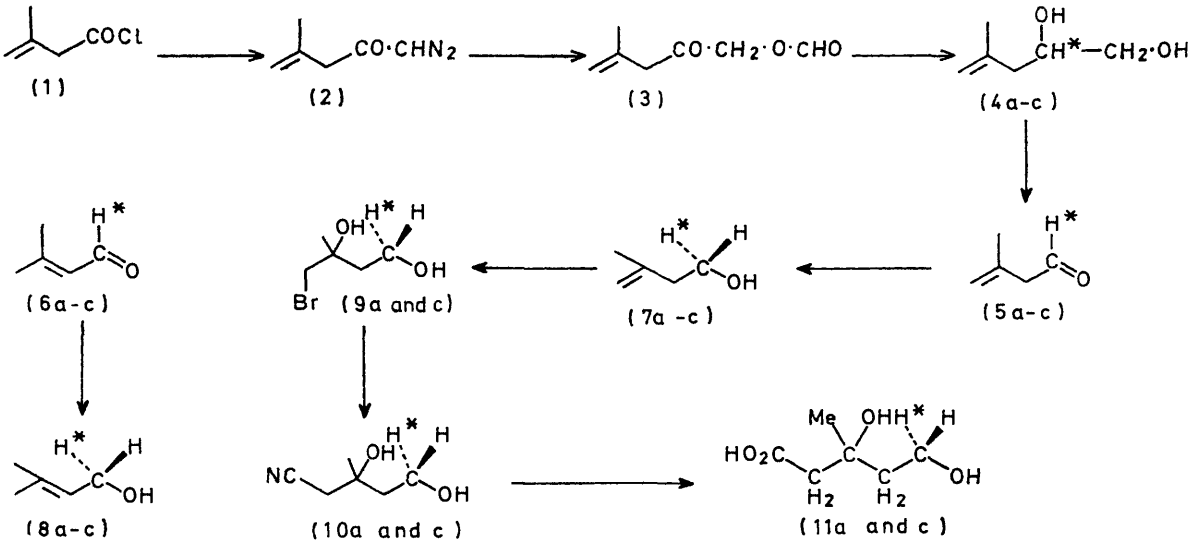
The title compound has been synthesized, with specific radioactivity around 10 Ci mol^{-1}, by a synthesis using as stereospecific step the enzymic reduction of 3-methyl[1-^{3}H]but-3-enal on liver alcohol dehydrogenase; this was followed by a chemical transformation of the product, (1*S*)-3-methyl[1-$^3H_1$]but-3-en-1-ol, to mevalonolactone *via* a bromohydrin and a cyanohydrin. 3-Methylbut-3-enal has been prepared and characterized for the first time, and the deuteriated alcohols, (1*S*)-3-methyl[1-$^2H_1$]but-3-en-1-ol and (1*S*)-3-methyl[1-$^2H_1$]but-2-en-1-ol, have been isolated: both are dextrorotatory.

A PRELIMINARY report [1] described a synthesis of (5*S*)-[5-$^3H_1$]mevalonic acid of relatively low specific radioactivity (0·018 Ci mol^{-1}). We now report a modified and improved synthesis which has given a product of specific radioactivity *ca.* 500 times greater. Essential observations from the earlier work are also included.

Both syntheses use horse liver alcohol dehydrogenase as a stereospecific agent for reducing 3-methylbut-3-enal (isopentenal) (5). The substrate stereospecificity of liver alcohol dehydrogenase has been demonstrated for the terpenoid alcohol geraniol: [1-$^3H_1$]geraniol was prepared by a stereoselective chemical synthesis designed to yield an excess of the 1*R*-enantiomer and was shown to from the aldehyde. Hence to generate (1*S*)-3-methyl[1-$^3H_1$]but-3-en-1-ol (7c) in this way it was necessary first to prepare 3-methyl[1-$^3H_1$]but-3-enal (5c).

Although this aldehyde [5,6] and derivatives [6,7] had previously been reported, our work indicates that these reports may refer to 3-methylbut-2-enal (6) and its derivatives. We were able to prepare almost pure 3-methylbut-3-enal by hydrolysis of its diethyl acetal [7,8] or dimethyl acetal in cold aqueous oxalic acid. The aldehyde is rather easily isomerized to the conjugated aldehyde (6) by heat or by bases, but it can be kept for some time in the cold, and a crystalline semicarbazone can be made without isomerization.



a; H* = ^{1}H b; H* = ^{2}H c; H* = ^{3}H

lose 70—80% of its tritium on oxidation with liver alcohol dehydrogenase.[2] Later,[3] geranial was reduced by tritiated coenzyme on liver alcohol dehydrogenase and the tritiated geraniol was shown to be 1*R* by chemical degradation to a [1-$^3H_1$]ethanol which lost all tritium when oxidised to acetaldehyde on liver or yeast alcohol dehydrogenase. From this evidence it could be assumed that in primary alcohols generated from aldehydes on liver alcohol dehydrogenase, the 1-*pro*-*R* hydrogen atom [4] comes from the coenzyme and the 1-*pro*-*S* hydrogen atom

The first preparation [1] of 1-tritiated 3-methylbut-3-enal was carried out by reducing unlabelled aldehyde with lithium borohydride partially quenched by tritiated water,[9] and oxidizing the 3-methyl[1-$^3H_1$]but-3-en-1-ol with dimethyl sulphoxide–dicyclohexylcarbodi-imide (Pfitzner–Moffatt [10] reagent). The method, however, presented difficulties of handling small quantities of

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highly radioactive material and a better procedure was designed.

3-Methylbut-3-enoyl chloride (1), prepared from the acid and thionyl chloride, reacted with an excess of diazomethane in ether to yield a diazo-ketone (2), which was decomposed, without isolation, by formic acid. The product, 4-methyl-2-oxo-pent-4-enyl formate (3), could be purified by fractional distillation. It was easily reduced by aqueous solutions of sodium borohydride, with concomitant hydrolysis, to 4-methylpent-4-ene-1,2-diol (4a). When this experiment was repeated with sodium borodeuteride, the deuteriated diol (4b) was obtained. To prepare the tritiated diol (4c), it was preferable to hydrolyse the formic ester with cold sodium hydrogen carbonate solution before adding sodium borotritide; in this way evolution of hydrogen from the borotritide was avoided. The diols were cleaved in aqueous solution, sometimes without isolation, by the action of sodium periodate, and the liberated aldehydes were distilled at low temperature and pressure along with some of the water; it was verified that little formaldehyde co-distilled. Aldehydes recovered at this stage from the distillate by extraction were shown by g.l.c. to be mainly 3-methylbut-3-enal (5) with variable proportions of the conjugated isomer (6). The synthesis adapts a method by which Grundmann [11] attempted, but failed,[12] to prepare hex-3-enal.

For the enzymic reduction, however, it was not necessary to isolate the aldehydes, since the aqueous distillate could be used directly. It was found that at 37° and in the buffer used for the enzymic reduction, isomerization to the conjugated aldehyde was quite fast and that the highest ratio (*ca.* 6 : 1) of 3-methylbut-3-en-1-ol (7) to 3-methylbut-2-en-1-ol (8) in the product was obtained by adding the aldehyde solution gradually to a buffered mixture of horse liver alcohol dehydrogenase and reduced nicotinamide adenine dinucleotide. The aldehydes were excellent substrates for the enzyme and reduction was rapid. The alcohols could be separated at this stage by g.l.c. From the deuteriated diol, (1*S*)-3-methyl[1-$^2H_1$]-but-3-en-1-ol (7b) and (1*S*)-3-methyl[1-$^2H_1$]but-2-en-1-ol (8b) were thus prepared. They were both dextrorotatory, showing plain positive o.r.d. curves. As would be expected, the specific rotation of the allylic alcohol was much the larger.

For the preparation of mevalonolactone, separation of the isomeric alcohols was unnecessary, since it was found by experiments on artificial mixtures that even quite large proportions of the allylic alcohol (8) did not interfere with the synthesis. To an aqueous solution containing the isopentenol (7), *N*-bromosuccinimide was added, and the bromohydrin (9) was subsequently extracted and treated with methanolic potassium cyanide. Hydrolysis of the crude cyanohydrin (10) by boiling aqueous alkali then yielded, after acidification and extraction, mevalonic acid (11) as the lactone, which was purified by thin-layer and partition chromatography and was characterized as the known diphenylmethylamide. The overall yield from isopentenol was 55—60% in trial runs on a 1—2 mmol scale, though this was not attained in the one preparative run that yielded (5*S*)-[5-$^3H_1$]mevalonic acid (11c). The stereochemical purity of the product has been checked by conversion into squalene and into cholesterol by rat liver enzymes, when the expected losses of tritium were observed.[13]

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EXPERIMENTAL

3-Methylbut-3-enal.—3-Methylbut-3-enal diethyl acetal [8] (35 g) was shaken for 5 h at room temperature with saturated aqueous oxalic acid (200 ml). The mixture was cooled and extracted with ethyl chloride (3 × 60 ml; b.p. 12°; freshly distilled); the extract was then washed with cold saturated aqueous calcium chloride (3 × 50 ml) to remove ethanol and distilled from a bath at 30° through a 30 cm Vigreux column. The residue (23·4 g) was distilled under nitrogen from a 100 ml Claisen flask with an 8 cm Vigreux column. A fraction of b.p. 65—91° was rejected and *3-methylbut-3-enal* (5a) (12·3 g; 66%) was collected at 93—95° and 737 mmHg. On g.l.c. (10% SE30), >98% of the emerging substance was recorded as one peak. The aldehyde was stable for some time at −20° under nitrogen. The i.r. spectrum (film) showed peaks at 3100m, 1800sh,w, 1650s, 1438s, and 895s (C=CH_2), and 2830m, 2720m, and 1740vs cm^{-1} (CHO). The aldehyde (50 mg) was shaken with a solution of semicarbazide hydrochloride (80 mg) and sodium acetate trihydrate (160 mg) in water (2 ml) for a few min. After being cooled at 4° for 1 h the voluminous *semicarbazone* was collected and recrystallized from a little water; m.p. 128—130° (Found: C, 51·0; H, 8·2; N, 30·1. $C_6H_{11}N_3O$ requires C, 51·0; H, 7·9; N, 29·8%), $\nu_{max.}$ (KBr) 1800w and 900s indicating that the CH_2=C grouping persisted (3-methylbut-2-enal semicarbazone is reported [14] to melt at 221—222°). However, an attempt to prepare the 2,4-dinitrophenylhydrazone in 2N-hydrochloric acid led to the derivative [15] of the conjugated aldehyde; m.p. 178—180° (from ethanol) [$\lambda_{max.}$ (MeOH) 383 nm (ε 28,900)].

3-Methylbut-3-enoyl Chloride (1).—3-Methylbut-3-enoic acid was prepared by carboxylation of the Grignard reagent from methallyl chloride (3-chloro-2-methylpropene) in tetrahydrofuran (*cf.* Wagner [16]). The acid, b.p. 85° at 15 mmHg (20 g), and thionyl chloride (16 ml) were mixed in a 50 ml Claisen flask and after 4 h at ambient temperature the chloride (18·4 g; b.p. 110—117°) was distilled from an oil-bath.

4-Methyl-2-oxopent-4-enyl Formate (3).—The chloride (1) (7 g) in dry ether (40 ml) was added slowly to an ice-cooled, stirred solution of diazomethane [*ca.* 9 g; from *N*-methyl-*N*-nitrosotoluene-*p*-sulphonamide [17] (75 g)] in ether. After being stirred overnight the mixture was evaporated at low pressure and the residue was added slowly to stirred formic acid (10 ml). After 1·5 h the mixture was warmed to 40—50° for 10 min, and cooled. Ether was added, formic acid was washed out with sodium hydrogen carbonate solution, and the ethereal solution was washed with water, dried ($CaSO_4$), and evaporated. The residue was checked for absence of diazo-absorption at 2100 cm^{-1}, and then fractionally distilled; the product was collected in two fractions after rejec-

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tion of a forerun boiling below 85° at 15 mmHg: (i) b.p. 85—90° at 15 mmHg (2·2 g), n_D^{21} 1·4496; (ii) 90—94° at 15 mmHg (1·8 g). Fraction (i) was almost pure *4-methyl-2-oxopent-4-enyl formate* (Found: C, 58·8, 59·2; H, 7·1, 7·5. $C_7H_{10}O_3$ requires C, 59·1; H, 7·1%), but contained a few per cent of the conjugated isomer; τ (CCl_4) 2·0 (s, HCO·O), 5·1 (m, $=CH_2$), 5·2 (s, $CO{\cdot}CH_2{\cdot}O$), 6·9 (s, $CO{\cdot}CH_2{\cdot}C=$), and 8·1 (d, $CH_3{\cdot}C=C$) [the conjugated isomer impurity showed itself in two peaks at τ 7·85 and 8·08 ($Me_2C=$)]; *m/e* 142 (M^+), 96, 87, 83, and 55. Rearrangement to the conjugated isomer appeared to take place slowly at room temperature and the product should be freshly distilled before further use.

(1S)*3-Methyl*[1-$^2H_1$]*but-3-en-1-ol* (7b) *and* (1S)-*3-Methyl*[1-$^2H_1$]*but-2-en-1-ol* (8b).—Sodium borodeuteride (126 mg) and water (5 ml) were stirred in a water-bath at 15° and 4-methyl-2-oxopent-4-enyl formate (424 mg) was added slowly from a Pasteur pipette, which was then rinsed with more water (2 ml). The mixture was stirred for 2 h at 15° and 0·5 h at 40—50°, then saturated with sodium dihydrogen phosphate and extracted with ether (20 ml). The ether layer was washed ($NaHCO_3$, water) and evaporated to leave 4-methyl[2-$^2H_1$]pent-4-ene-1,2-diol (4b) as an oil. This was mixed with water (1·5 ml) in a test tube fitted with magnetic stirrer, dropping funnel, and still-head with receiver, and cooled in a water-bath. A solution of sodium periodate (657 mg) in water (4 ml) was added dropwise with stirring; the dropping funnel was rinsed with water (1 ml). After 20 min, water (5 ml) was added, the receiver was cooled to −70° and the pressure lowered to 15 mmHg. Distillate (*ca.* 7 ml) was collected; this contained the isopentenals in a form suitable for enzymic reduction. In trial runs without deuterium, the thawed distillate was extracted with ethyl chloride and examined by g.l.c.; 3-methylbut-3-enal and 3-methylbut-2-enal in various proportions were identified as the only significant products by their retention times. The enzyme used was crystalline horse liver alcohol dehydrogenase (Boehringer). The suspension as supplied (1 ml) was spun down, the precipitate was dissolved in 0·1M-glycine–potassium hydroxide buffer, pH 10, and the solution was dialysed for 3 days at 4° against 0·1M-sodium–potassium phosphate buffer, pH 7. After centrifugation from a slight deposit the supernatant (1·5 ml; *ca.* 15 mg of enzyme) was used.

In a 350 ml conical flask fitted with stirrer and stopper were placed reduced nicotinamide adenine dinucleotide (Boehringer; 85%; 2·81 g) and the thawed distillate (7 ml) containing the aldehydes. The volume was made up to 100 ml with 0·1M-sodium–potassium phosphate buffer, pH 7·0. The mixture was stirred in a bath at 37° and the enzyme solution (0·9 ml) was added. From time to time a portion (10 μl) was withdrawn, diluted in 3 ml of buffer, and examined for absorption at 340 nm. The optical density had ceased to fall after 2·5 h, when 1·8 mmol of coenzyme had been oxidized. The solution was saturated with ammonium sulphate and distilled slowly from a Claisen flask. The distillate (25 ml) was similarly saturated and distilled; the redistillate (5·5 ml) was saturated with ammonium sulphate and extracted with ether (8 ml). The extract was concentrated under a short column to 1·5 ml and the two alcohols were separated by preparative g.l.c. at 80° (10% Carbowax 20M on Chromosorb W 100—120 mesh); in this system, authentic mixtures of 3-methylbut-3-en-1-ol and 3-methylbut-2-en-1-ol were well resolved. In this way (1*S*)-3-methyl[1-$^2H_1$]but-3-en-1-ol (57 mg) and (1*S*)-3-methyl[1-$^2H_1$]but-2-en-1-ol (33 mg) were collected and redistilled. Each specimen on g.l.c. was inseparable from the corresponding non-deuteriated alcohol, and each appeared free from the isomeric alcohol and from other impurities. The mass spectrum of each alcohol showed a molecular ion at *m/e* 87 ($C_5H_{11}DO$). On spectropolarimetric examination, however, the 3-methylbut-3-en-1-ol showed signs of anomalous dispersion around 290 nm. A new preparation was made and purified by g.l.c. at 100° (30% SE 30). Both alcohols then showed plain positive dispersion curves (Table).

Mevalonolactone from 3-Methylbut-3-en-1-ol containing 3-Methylbut-2-en-1-ol.—A mixture of 3-methylbut-3-en-1-ol (103 mg) and 3-methylbut-2-en-1-ol (72 mg) in water (6 ml) was stirred for 1 h at room temperature with *N*-bromosuccinimide (357 mg; recrystallized). The solution was saturated with sodium chloride and extracted with ether; the extract was dried ($CaSO_4$) and evaporated leaving a crude bromohydrin (378 mg), which was dissolved in a solution of potassium cyanide (255 mg) in methanol (10 ml) and left overnight. The solvent was removed in a stream of nitrogen and the solid residue was extracted with ether (15 ml). Filtration and evaporation of the extract then yielded crude cyanohydrin (205 mg). This was boiled for 20 h under reflux with aqueous 2N-sodium hydroxide (6 ml). The cooled solution was washed with ether (15 ml), then acidified with 4N-sulphuric acid (7 ml), saturated with sodium chloride, and extracted continuously with chloroform (100 ml) for 20 h. Evaporation of the extract left an oil (185 mg), which was purified by t.l.c. on silica (five 20 × 20 cm plates; Merck Kieselgel (F_{254}) with benzene–ethyl acetate (1 : 1). The mevalonolactone occupied a broad band, invisible under u.v. light, at R_F 0·2—0·4. This was scraped off and extracted overnight with acetone (50 ml; Soxhlet). The solvent was removed, finally at 50° and 15 mmHg, to leave mevalonolactone (IIa) (88 mg, 57%), identical (i.r. spectrum) with authentic material. The lactone was further characterized by conversion into *N*-diphenylmethylmevalonamide, m.p. and mixed m.p., 99—100°.

(3*RS*,5*S*)-[5-$^3H_1$]*Mevalonolactone.*—4-Methyl-2-oxopent-4-enyl formate (71 mg, 0·5 mmol) and sodium hydrogen carbonate (44 mg) were mixed with water (1 ml) and stirred for 2 h. Sodium borotritide (6 mg; 100 mCi; Radiochemical Centre, Amersham) dissolved in water (0·5 ml) was added and rinsed in with water (3 × 0·1 ml). The solution was stirred overnight and non-radioactive borohydride

Specific rotations of deuteriated alcohols

Specimen	Solvent	*c* (% w/v)	[α] (°) at λ (nm)										
			233	244	250	263	278	294	313	333	357	385	400
(1*S*)-3-Methyl-[1-$^2H_1$]but-3-en-1-ol	Cyclohexane	1·8		+2·2	+2·0	+1·6	+1·3	+0·93	+0·68	+0·53	+0·40	+0·25	+0·25
(1*S*)-3-Methyl-[1-$^2H_1$]but-2-en-1-ol	Methanol	2·4	+60			+22·7	+15·6	+11·2	+8·4	+6·15	+4·6		+2·9

(5 mg) was added. After 1 h, a test with 2,4-dinitrophenylhydrazine (2 mg ml^{-1} in 2N-hydrochloric acid) gave a very faint response. The solution was brought to pH 6 and treated with sodium periodate (120 mg) and water (2 ml). A spray trap was fitted and, 5 min after oxidation began, the pressure was reduced to 20 mmHg and water and isopentenal were distilled over, from an unheated water-bath, into a trap cooled to −70°. Distillate (*ca.* 2 ml) was collected; another 2 ml of water was added to the residue and this mixture was distilled in turn. The distillate was thawed, centrifuged, stored in ice, and used the same day. It was verified in separate experiments that little formaldehyde appeared in the distillate under these conditions (t.l.c. of 2,4-dinitrophenylhydrazones).

A solution containing horse liver alcohol dehydrogenase (1·5 ml of supernatant prepared as described above; 15 mg of enzyme), reduced nicotinamide adenine dinucleotide (85%; 680 mg), and 0·1M-sodium–potassium phosphate buffer pH 6·9 (total volume 15 ml) was incubated for 5 min at 37° and treated with the aqueous distillate prepared as above (8 portions added at intervals during 10 min). After 5 min, two 10 μl portions were taken. One of these was for gas–liquid radiochromatography after addition of carrier alcohols: this showed that the ratio of 3-methylbut-3-en-1-ol to 3-methylbut-2-en-1-ol was *ca.* 6 : 1; one was for u.v. determination which revealed on the basis of NADH absorption at 340 nm that 0·465 mmol (93% of theoretical) of coenzyme had been oxidized. The remaining solution was added to saturated ammonium sulphate solution (100 ml) containing non-radioactive 3-methylbut-3-en-1-ol (103 mg) in a 250 ml Claisen flask with sealed-on water-cooled condenser. The whole was heated (free flame) until protein coagulated, then 40 ml was distilled. The distillate was saturated with ammonium sulphate, and redistilled (10 ml). This distillate was saturated with sodium chloride and extracted with ether (6 ml); the extract was dried ($MgSO_4$) and concentrated by cautious distillation under a short column. The residue was treated with water (5 ml) and *N*-bromosuccinimide (310 mg). After 0·5 h the solution was saturated with sodium chloride and extracted with ether (10 ml); the extract was dried ($MgSO_4$) and evaporated. The residue was treated with potassium cyanide (200 mg) in methanol (5 ml), and left overnight, boiled under reflux for 1 h, concentrated, and boiled under reflux with aqueous 2N-sodium hydroxide (8 ml) for 18 h. The mixture was acidified to pH 3 with 8N-sulphuric acid and extracted continuously with chloroform for 24 h. Evaporation of the extract left a residue (60 mg) which was chromatographed on two silica plates (20 × 20 × 0·025 cm) in benzene–methanol (1 : 1). The plates were scanned for radioactivity and the radioactive band corresponding to a mevalonolactone marker was removed and extracted (Soxhlet) with acetone; yield of partially purified mevalonolactone 40 mg; radioactivity 4 mCi (4% radiochemical yield). However, this preparation still contained a radioactive impurity, which was removed by partition chromatography (experiment performed by Dr. G. T. Phillips). The stationary phase was 0·5N-sulphuric acid (8 ml) on Mallinckrodt silicic acid (10 g; 100 mesh) packed in a column 20 × 1·2 cm. The eluant was chloroform saturated with 0·5N-sulphuric acid and the flow rate 8 ml h^{-1}. The partially purified lactone (57·7 μCi) was applied in chloroform (0·5 ml). The radioactive impurity travelled fast through this column (in the first 20 ml). The elution maximum for mevalonolactone in this system was around 180 ml and a total of 180 ml of eluate was collected (in 2 ml fractions) around this maximum. The mevalonolactone (11c) (27·0 μCi) obtained by evaporation showed one radioactive spot, coinciding with a mevalonolactone marker, at R_F 0·56 on t.l.c. on silica gel with methanol–chloroform (15 : 85). It was characterized as the diphenylmethylamide.

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