Stereochemistry of the Conversion of Methacrylate to β-Hydroxyisobutyrate in *Pseudomonas putida*

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Stereospecifically labelled (2*S*,3*S* + 2*R*,3*R*)-[2,3-$^2H_2$]-β-hydroxyisobutyric acid methyl ester benzoate was synthesized and used to assign the Eu(fod)$_3$ shifted n.m.r. signals of the C-3 hydrogens of the unlabelled analogue. (*E*)-[3-$^2H_1$]Methacrylic acid was converted by *Pseudomonas putida* (ATCC 21244) to (2*S*,3*S*)-[3-$^2H_1$]-β-hydroxyisobutyric acid. The results show that the hydration of methacrylate to *S*-(+)-β-hydroxyisobutyric acid in this organism, probably *via* methacrylyl-CoA, proceeds stereospecifically by *syn* hydration.

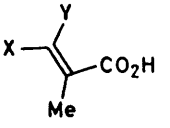
THE degradative pathway of the metabolism of L-valine in both mammalian species and micro-organisms is generally accepted to proceed *via* α-ketoisovaleric acid and its decarboxylation product isobutyryl-CoA.[1] In subsequent steps, isobutyryl-CoA undergoes dehydrogenation to methacrylyl-CoA, which in turn undergoes hydration and scission of the coenzyme A group leading to *S*-(+)-β-hydroxyisobutyric acid (β-HIBA). As part of a programme on the determination of the stereochemistry of various steps involved in the catabolism of the branched-chain amino acids, we recently reported the fates of the enantiotopic methyls of isobutyrate in the Gram-negative bacterium *Pseudomonas putida* (ATCC 21244).[2] In this organism which thrives on isobutyrate as its sole source of carbon and accumulates β-HIBA,[3] the 2-*pro*-S methyl group of isobutyrate underwent oxidation and ultimately served as the origin of the hydroxymethyl group of β-HIBA. Since the C-2 hydrogen of isobutyrate was exchanged with the medium in this transformation, it was assumed that an unsaturated intermediate, probably methacrylyl-CoA, was involved in the transformation. We now report the results of further studies on the biosynthesis of β-HIBA in *Ps. putida* in which we have examined (*a*) the ability of methacrylate to substitute for isobutyrate as a precursor of β-HIBA, and (*b*) the stereochemistry of the hydration of methacrylate (or methacrylyl-CoA).

RESULTS

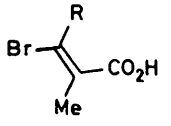
We first tested the ability of *Ps. putida* (ATCC 21244) to utilize methacrylate in place of isobutyrate as the sole source of carbon. As was observed with isobutyrate media, newly hydrated cultures, after a lag period of several days, exhibited rapid growth in a methacrylate medium, and transferred cultures grew with little delay. The metabolite, β-HIBA, was accumulated to the extent of 5—10% of added methacrylate, this yield being comparable to that obtained with isobutyrate.

† Initially we planned to synthesize (1a or b) by stereospecific carboxylation of the isopropenyl-lithiums to be obtained from (*E*)- or (*Z*)-2-bromopropene. However, in contrast to the report,[6] in our hands the addition of ^{2}HBr or ^{2}HCl to propyne did not proceed stereospecifically. At 0 °C for 2 h, a 2 : 1 mixture of *E* : *Z* 2-bromopropenes was obtained. Carboxylation of the mixture by treatment with n-butyl-lithium in THF at −78 °C, followed by addition to solid CO_2 gave (1b) and (1a) in the ratio 2 : 1.

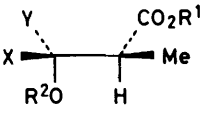
It then became of interest to investigate the stereochemistry of the hydration process leading to β-HIBA. The absolute configuration[4] of this metabolite requires the addition of a proton to the *re* face[5] of methacrylate (methacrylyl-CoA). It therefore remained to determine the stereochemistry of addition of a hydroxy-group at C-3. For this determination, a stereospecifically deuteriated methacrylic acid (1a or b) was required.† The *E*-isomer (1b) was synthesized by the method of Crout and Corkill.[7] Thus, (*E*)-3-bromo-2-methylprop-2-enoic acid (2a) was prepared by bromination of methyl



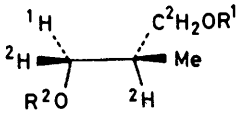
(1) a; X = ^{1}H, Y = ^{2}H
b; X = ^{2}H, Y = ^{1}H



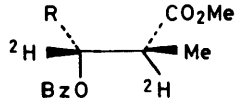
(2) a; R = ^{1}H
b; R = ^{2}H



(3) a; $R^1 = R^2 = X = Y = {}^1H$
b; $R^1 = Me, R^2 = Bz, X = Y = {}^1H$
c; $R^1 = Me, R^2 = Bz, X = {}^2H, Y = {}^1H$



(4) a; $R^1 = R^2 = H$
b; $R^1 = H, R^2 = Bz$
c; $R^1 = Bz, R^2 = H$



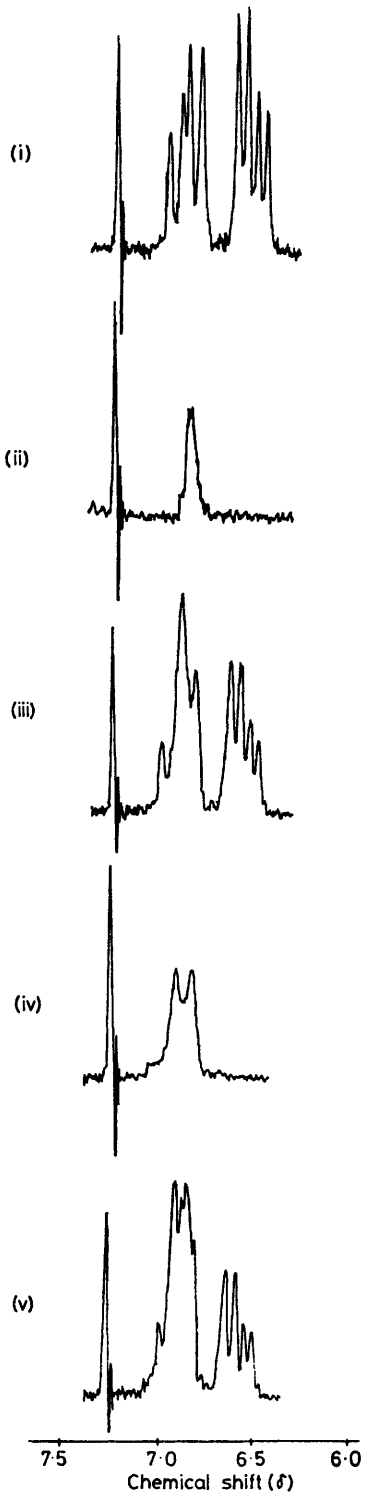
(5) a; R = ^{1}H
b; R = ^{2}H

methacrylate,[8] dehydrobromination of the resultant dibromide with 1,5-diazabicyclo[5,4,0]undec-5-ene (DBU),[9] and alkaline hydrolysis. Treatment of (2a) with sodium amalgam in ^2H_2O as described[7] gave the stereospecifically deuteriated (1b). In contrast, when (2a) was treated with t-butyl-lithium in THF at −78 °C followed by addition of ^2H_2O, the deuteriated (2b) was

obtained. Treatment of (2b) with sodium amalgam in H_2O gave the *Z*-isomer, (1a).

With the availability of labelled methacrylate, we turned our attention to development of a method for determining the configuration at C-3 of β-HIBA labelled biosynthetically from (1b). The signals for the diastereotopic hydroxymethyl protons in the 100 MHz n.m.r. spectra of (3a) or its methyl ester benzoate (3b) were inadequately resolved for analytical purposes. However, when the spectrum of (3b) was run in the presence of $Eu(fod)_3$,[10] both signals were markedly shifted and to different extents. In the region of 6—7 p.p.m., they appeared as a completely resolved AB pattern, further split by the C-2 hydrogen. We therefore undertook the assignment of these signals to the 3-*pro*-*R* hydrogen Y and the 3-*pro*-*S* hydrogen X of (3b). For this assignment we planned to synthesize a stereospecifically labelled reference sample of (3b). Thus, (*E*)-[3-$^2H_1$]-methacrylic acid (1b) was converted to its methyl ester. Treatment of this with an excess of [$^2H_3$]borane in THF* followed by oxidative work-up yielded racemic (4a). This was converted to the mixture of monobenzoates (4b) and (4c), which was oxidized with potassium permanganate [11] in 1M-H_2SO_4 followed by methylation with diazomethane to give a mixture of (5a) and (5b) in equal quantities. When an unlabelled sample of 2-methylpropane-1,3-diol monobenzoate was similarly treated using potassium permanganate in 1M-$^2H_2SO_4$ in ^2H_2O, no deuterium was incorporated into the product. Thus the relative configurations between C-2 and C-3 generated in the hydroboration reaction, known to proceed by a *cis* addition,[12] must have been preserved in the final product (5a). The presence of an equal amount of (5b) in this product did not interfere with the n.m.r. analysis. The 100 MHz n.m.r. spectrum of (5a) was then run in the presence of $Eu(fod)_3$. In practice, it was difficult to shift with any degree of reproducibility the signals of interest to exactly the same positions by adding measured amounts of $Eu(fod)_3$. Thus the analysis was performed by adding small increments of a solution of $Eu(fod)_3$ to the sample until the C-3 hydrogen signals appeared in the region of δ 6.5—7.0. A single peak, broadened by deuterium coupling, was observed for (5a). A small amount of unlabelled (3b) was then added to the solution, along with additional $Eu(fod)_3$ to return the signals to the δ 6.5—7.0 region, and the spectrum was again recorded (Figure). This procedure allowed the unambiguous assignment of the C-3 hydrogen signal of (5a) to the downfield half of the AB pattern. This C-3 hydrogen corresponds to the 3-*pro*-R hydrogen of the 2*S*-isomer of (5a) (the configuration of β-HIBA produced by *Ps. putida*), and to the 3-*pro*-*S* hydrogen of the 2*R*-isomer of (5a) [only the 2*S*-isomer is shown in formula (5a)].

With the assignment of the n.m.r. signals established, the biosynthetic experiment could be performed. (*E*)-[3-$^2H_1$]Methacrylic acid (1b) was incubated with washed cells of *Ps. putida*, allowing the incubation to proceed until *ca.* 90% of the methacrylate had been consumed. The resultant β-HIBA was then isolated



Partial 100 MHz n.m.r. spectra in $CDCl_3$ solution with added $Eu(fod)_3$: (i) (3b); (ii) (5a) + (5b); (iii) (5a) + (5b) + (3b); (iv) Biosynthetic product (3c); (v) (3c) + (3b).

* Attempts to selectively hydroborate (1b) with borane or substituted boranes, without concomitant reduction of the methoxycarbonyl group, were unsuccessful.

and purified as the methyl ester benzoate in the usual manner.[2] Its n.m.r. spectrum was then determined in the presence of $Eu(fod)_3$ as described above. The C-3 hydrogen signal appeared as a clean doublet in a position corresponding to the downfield half of the AB pattern, assigned to the 3-*pro-R* hydrogen. No signal was detected in the upfield region before adding the unlabelled reference sample. The results require that the addition of the elements of water to methacrylate, probably *via* methacrylyl-CoA, in this organism proceeds completely stereospecifically in a *syn* manner, as in (1b) ⟶ (3c), with addition to the *re,re*-face of the double bond.* The stereochemistry of this process thus parallels that recently reported [13] for the hydration of (E)-crotonyl-CoA catalysed by enoyl-CoA hydratase from beef liver mitochondria.

EXPERIMENTAL

I.r. spectra were taken on a Perkin-Elmer 237 spectrometer, n.m.r. spectra on a Varian EM-360 or a Varian HA-100 spectrometer, and mass spectra on a Nuclide 12-90-G mass spectrometer equipped with a Nuclide DA/CSI.2 data acquisition system. Silica gel HF 254 + 366 (E. Merck) was used for thin layer chromatography (t.l.c.) in the solvents noted. The culture of *Pseudomonas putida* (ATCC 21244) was obtained from the American Type Culture Collection, Rockville, Maryland. M.p.s were taken on a hot-stage apparatus and are corrected.

(E)-3-*Bromo*-2-*methylpropenoic Acid.* (2a).—Methyl 2,3-dibromo-2-methylpropionate [8] (60 g) in THF (200 ml) was treated with 1,5-diazabicyclo[5,4,0]undec-5-ene (DBU) (38 g, 1.1 mol. equiv.) and the solution was refluxed 1 h. After cooling, the mixture was poured into water (50 ml) and extracted with ether. The extract was washed with 1N-HCl and saturated brine, dried ($MgSO_4$), and evaporated to yield crude methyl (*E*)-3-bromo-2-methylpropenoate. This was treated with NaOH (12 g) in water (150 ml) and methanol (30 ml) at 50 °C for 3 h. After cooling, the acidified solution was extracted with ether, dried ($MgSO_4$), and evaporated to yield (*E*)-3-bromo-2-methylpropenoic acid [8] (2a) (28.2 g, 73%) as needles from light petroleum, m.p. 60—62 °C; $\nu_{max.}$ ($CHCl_3$) 3 580—2 200, 1 690, 1 600, and 1 405 cm^{-1}; $\delta(CDCl_3)$ 2.01 (3 H, d, *J* 2 Hz), 7.71 (1 H, q, *J* 2 Hz), and 11.45 (1 H, br s, $W_{1/2}$ 14 Hz).

(E)-[3-$^2H_1$]-3-*Bromo*-2-*methylpropenoic Acid.* (2b).—The acid (2a) (1.14 g, 6.90 mmol) in dry THF (40 ml) at −78 °C under N_2 was treated with t-butyl-lithium (2.3M; 8 ml, 18.4 mmol) for 1 h. ^2H_2O (2 ml) was then added and the solution warmed rapidly to 25 °C. Water was added and the mixture was acidified and extracted with ether. The extract was washed with saturated brine, dried (Na_2SO_4), and evaporated to give the deuteriated acid (2b) (1.10 g). The n.m.r. spectrum was identical with that of (2a) except for the complete disappearance of the signal at δ 7.71.

Methyl (2S,3S + 2R,3R)-[2,3-$^2H_2$]-3-*Benzoyloxy*-2-*methylpropanoate* (5a) *and Methyl* [2,3,3-$^2H_3$]-3-*Benzoyloxy*-2-*methylpropanoate* (5b).—(*E*)-[3-$^2H_1$]-2-Methylpropenoic acid [7] (1b) (10 g, 115 mmol) was treated with a solution of diazomethane in ether (200 ml, 1 mol. equiv.). The resultant solution of (2a) methyl ester was treated at 25 °C under N_2 with [$^2H_3$]borane in THF (0.82M; 75 ml, 61.5 mmol) and stirred at 25 °C for 15 min. Water (10 ml) was added dropwise to destroy a small excess of borane, followed by 3M-NaOH (18 ml) and 30% H_2O_2 (12 ml). The solution was stirred and maintained at *ca.* 25 °C for 1 h. The mixture was then extracted with ether (3 × 100 ml), and the extract dried ($MgSO_4$) and evaporated to yield crude (1*S*,2*S* + 1*R*,2*R*)-[1,3,3-$^2H_3$]-2-methylpropane-1,3-diol (4) (967 mg). This was not purified but was used directly in the next step. Crude (4) (500 mg) was treated with dry pyridine (3 ml) and benzoyl chloride (0.13 ml) at 25 °C for 1 h. Water (3 ml) was then added and the mixture was stirred for 10 min. The mixture was extracted with ether, and the extract washed (1N-HCl, 1N-K_2CO_3), dried ($MgSO_4$), and evaporated. The desired product was isolated by preparative t.l.c. (ethyl acetate–hexane 45 : 55 v/v), giving a mixture of the 3-benzoate (4b) and 1-benzoate (4c) (90 mg). To this product in 1M-H_2SO_4 (10 ml) was added $KMnO_4$ (200 mg) and the mixture was stirred at 25 °C for 30 min. Aqueous $NaHSO_3$ was added to decolourise the solution which was then extracted with ether and the extract dried ($MgSO_4$) and evaporated. The residual oil was methylated with excess of ethereal diazomethane. Evaporation gave the crude product which was purified by preparative t.l.c. (ethyl acetate–hexane 1 : 4 v/v) giving an oil which was distilled in a kugelröhr tube at 120—130 °C and 0.25 mmHg to yield an equal mixture of methyl (2*S*,3*S* + 2*R*,3*R*)-[2,3-$^2H_2$]-3-benzoyloxy-2-methylpropanoate (5a) and methyl [2,3,3-$^2H_3$]-3-benzoyloxy-2-methylpropanoate (5b) (45 mg); $\nu_{max.}$ ($CHCl_3$) 2 250 and 1 725 cm^{-1}; $\delta(CDCl_3)$ 1.25 (3 H, s, slightly broadened), 3.70 (3 H, s), 4.44 (0.5 H, br s, $W_{1/2}$ 4 Hz), 7.45 (3 H, m), and 8.02 (2 H, m).

Conversion of (E)-[3-$^2H_1$]-2-*Methylpropenoic Acid to* β-*Hydroxyisobutyric Acid by* Pseudomonas putida.—(*E*)-[3-$^2H_1$]-2-Methylpropenoic acid (1b) (1.4 g) was incubated as previously described [2] with washed cells of *Ps. putida* (ATCC 21244), allowing the incubation to proceed until *ca.* 90% of the starting material was consumed, as monitored by change in absorption at 1 680 cm^{-1} of acidified, extracted aliquots. After incubation for 10 h, the resultant β-HIBA was isolated and purified in the usual manner [2] to give (3c) (183 mg); $\nu_{max.}$ 2 250, 1 725, and 1 600 cm^{-1}; $\delta(CDCl_3)$ 1.27 (3 H, d, *J* 7 Hz), 2.95 (1 H, d, q, J_1 7 Hz, J_2 6 Hz), 3.72 (3 H, s), 4.47 (1 H, d, *J* 6 Hz, slightly broadened), 7.45 (3 H, m), 8.05 (2 H, m);† *m/e* 224 (7%), 223 (M^+, 28), 222 (2), 205 (10), 192 (14), 191 (14), 163 (13), 123 (87), 122 (100), 118 (41), 106 (65), 105 (100), 102 (28), 101 (93), 86 (15), 77 (85), and 70 (31).

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* In another communication, we provide evidence that the conversion of isobutyrate to β-HIBA proceeds stereospecifically with loss of *ca.* 30% of the 3-*pro-R*-hydrogen of the hydroxymethyl xroup. This competing process does not affect the conclusions of the present communication, since the 3-*pro-S*-position of the metabolite showed *no* evidence of any ^1H content.

† With the exception of *m/e* 224 and 222, only peaks with relative intensity >10% are reported.

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