SYNTHESIS OF (3RS)-[2,3-$^{3}H_2$ (N)]-β-LEUCINE

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SUMMARY

(3RS)-[2,3-$^{3}H_2$(N)]-β-Leucine, a substrate for assay of leucine 2,3-aminomutase, has been synthesized. Ethyl (Z)-3-acetamido-4-methylpent-2-enoate was reduced with $^{3}H_2$ gas over Pd(C) to (3RS)-[2,3-$^{3}H_2$(N)]-β-leucine ethyl ester, which was hydrolyzed with HCl-H_2O to (3RS)-[2,3-$^{3}H_2$(N)]-β-leucine. The crude product was purified, on a small scale by preparative HPLC. Analysis of the crude product by ^{3}H NMR, and of the purified product by equilibration analysis indicated a tritium distribution of ca 70% at C-3 and 30% at C-2.

Key Words: β-Leucine, leucine 2,3-aminomutase, tritium labeling

INTRODUCTION

Recent work by Poston (1-6) has established the existence of the coenzyme-B_{12}-dependent enzyme leucine 2,3-aminomutase in a wide variety of organisms. Although the highest activity was found in Clostridia grown on a leucine-rich medium (1), the enzyme was also detected in plants (2,3), a yeast (4), and in animals, including humans (1,5,6). Poston has postulated that leucine 2,3-aminomutase may be involved in a pathway of leucine biosynthesis in humans (6).

In Poston's work, the activity of leucine 2,3-aminomutase was assayed by measurement of the rate of production of α-leucine upon incubation of extracts

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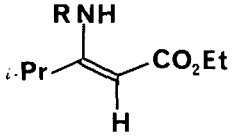
of the tested organism with DL-β-leucine[+]. It seemed likely that the sensitivity of the enzyme assay could be enhanced by the use of radioactively labeled β-leucine in place of unlabeled β-leucine. We now report the synthesis of (3RS)-[2,3-$^3H_2$(N)]-β-leucine.

DISCUSSION

Ethyl (Z)-3-amino-4-methylpent-2-enoate, (1a),(8) was acetylated (9) to give the N-acetyl derivative (1b). Reduction of (1b) with $^3H_2$ gas over Pd/C gave (3RS)-[2,3-$^3H_2$(N)]-N-acetyl-β-leucine ethyl ester, (2b). A radiochromatogram of the product showed the presence of tritiated (1b) (12%) as well as an unidentified polar impurity (4%). The desired product (2b) could be readily obtained chromatographically pure by column chromatography (performed on only a few μCi of (2b) diluted with carrier). However, for preparative purposes[‡], the crude $^3H_2$ reduction product was directly hydrolyzed (after addition of a little (2a) as carrier) by vigorous treatment with HCl/H_2O to yield (3RS)-[2,3-$^3H_2$(N)]-β-leucine, (3b), in moderate yield. Radiochromatograms of the crude product showed a minor faster-running impurity (9%), and an immobile impurity



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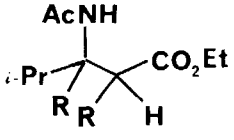
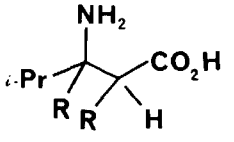
b R = Ac



2 a R = H
b R = ^{3}H



3 a R = H
b R = ^{3}H

(3%). A ^{3}H NMR spectrum, taken on the crude product, showed that the majority of the tritium (69%) was located at C-3[‡], with essentially all of the remainder

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[+]The absolute configurations of the substrates, α- and β-leucine, have not been determined. However Overton et al. (7) have reported that, in tissue cultures of Andrographis paniculata, (2S)-α-leucine and (3R)-β-leucine were interconverted by a leucine 2,3-aminomutase which apparently was not stimulated by coenzyme-B_{12}.

[†]Tritiated (1b) would be hydrolyzed to tritiated isobutyric acid and/or acetic acid, which would be removed in the purification of β-leucine. Therefore it was unnecessary to remove any tritiated (1b) prior to hydrolysis.

[‡]Our initial objective, not achieved in this work, was to prepare β-leucine free of tritium at C-2, in order to avoid possible isotope effects in the aminomutase reaction which probably (but not necessarily) proceeds with transfer of one C-2 hydrogen of β-leucine to C-3 of α-leucine (cf. ref. 10). It was for this reason that the hydrolysis of (2b) was carried out under vigorous conditions. A higher yield of (3b) could probably have been obtained under milder conditions.

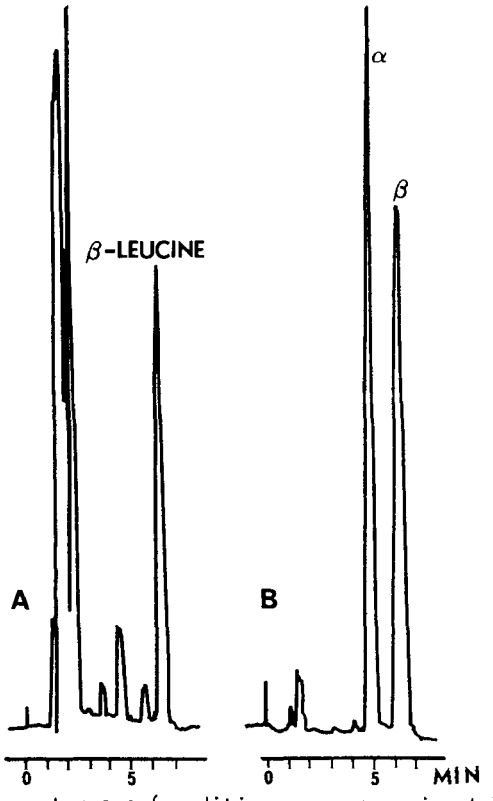

Figure 1. HPLC chromatograms (conditions, see experimental section). A: (3RS)-[2,3-$^{3}H_{2}$(N)]-β-leucine. B: Unlabeled α + β-leucine.

(31%) at C-2. However, small amounts of tritium (5-10%) in other locations could have been missed in the rather noisy spectrum, and thus the product labeling is designated as [2,3-^{3}H(N)]. The pure product, (3b), was then obtained by separation of the crude (3b) by HPLC, Fig. 1a, using a method recently reported by Schuster (11) for the separation of a wide variety of α-amino acids on an NH_2 column with direct detection by UV near 200 nm without derivatization. Parenthetically, it should be mentioned that this method also provides an excellent separation of α- and β-leucine, Fig. 1b. The β-leucine collected in this way showed no traces of radioactive impurities on radiochromatograms in two solvent systems. In excellent agreement with expectations from the ^{3}H NMR spectrum, a sample of the HPLC-purified (3b) (mixed with carrier), upon treatment with refluxing conc HCl, lost a total of 30% of the tritium in 48 h (no further loss occurred after an additional 24 h reflux).

After 9 months storage at -15°C in the solvent used for HPLC purification (i.e., the collected solution), the purified (3b) showed less than 5% radioactive impurities on radiochromatograms. Also the crude (2b), stored at -15°C in benzene, and the crude (3b), stored at -15°C in methanol plus a trace of HCl, did not show any apparent increase in their content of radioactive impurities.

EXPERIMENTAL

General. ^{1}H NMR spectra were taken at 60 MHz on a Varian EM-360 spectrometer. ^{3}H NMR spectra were taken at 95.9 MHz on a Bruker SXP 22/100 instrument. Liquid scintillation counting was performed on a Nuclear Chicago Mark III instrument. Radiochromatogram scanning was performed with a Nuclear Chicago Actigraph III instrument. High pressure liquid chromatography (HPLC) was performed on a Waters instrument equipped with an M-45 pump, a U6K injector and a model 450 variable wavelength detector. The synthesis of (2b) and (3b) were carried out by New England Nuclear Co. Combustion analyses were performed by Galbraith Laboratories, Knoxville, Tenn.

Ethyl (Z)-3-acetamido-4-methylpent-2-enoate, (1b). Ethyl (Z)-3-amino-4-methylpent-2-enoate, (1a) (8) (0.87g, 5.6 mMol) was refluxed with acetic anhydride for 16 h. The solution was evaporated under reduced pressure to give an oil which was chromatographed on a column of silica gel (25 g). Elution with 5% EtOAc-hexane yielded (1b) as an oil, 0.41 g, bp 63-65° (0.1 mm) (kugelröhr distillation); nmr ($CDCl_3$) δ 1.10 (6H, d, J = 6 Hz), 1.27 (3H, t, J = 6 Hz), 2.13 (3H, s), 3.85 (1H, m), 4.12 (2H, qu, J = 6 Hz), 5.04 (1H, s).

Anal. Calcd for $C_{10}H_{17}NO_3$: C, 60.28; H, 8.60. Found: C, 60.07; H, 8.70.

(3RS)-β-Leucine N-acetyl ethyl ester, (2a). A solution of (3RS)-β-leucine (1), (3a) (2.0 g) in absolute EtOH (50 ml) was saturated with dry HCl. The mixture was refluxed for 3 h, then cooled and evaporated under reduced pressure. The resultant oil was treated with a mixture of acetic anhydride (0.52 g), CH_2Cl_2 (5 ml), and pyridine (0.81 g) at 25°C for 5 h. The mixture was diluted with CH_2Cl_2 and washed with H_2O and dil HCl, dried (Na_2SO_4), evaporated to a viscous oil, 0.6 g, distilled in a kugelröhr apparatus, bp 80-100°C (0.1 mm); nmr ($CDCl_3$) δ 0.95 (6H, d, J = 6 Hz), 1.28 (3H, t, J = 3 Hz), 1.90 (1H, m), 2.01 (3H, s), 2.50 2H, d, J = 6 Hz), 4.14 (2H, qu, J = 6 Hz) , 4.14 (1H, br s, $W_{1/2}$ = 20 Hz),

6.78 (1H, br d, J = 9 Hz).

Anal. Calcd for $C_{10}H_{19}NO_3$: C, 59.68; H, 9.52. Found: C, 59.48; H, 9.59.

(3RS)-[2,3-$^3H_2$(N)]-β-leucine ethyl ester, (2b). Ethyl (Z)-3-acetamido-4-methyl-pent-2-enoate (1b) (100 mg) in dry benzene (5 ml) containing 5% Pd (C) (20 mg) was stirred at room temperature with $^3H_2$ gas (25 Ci) for 48 h. Labile tritium was removed in vacuo using methanol as solvent. After filtration from the catalyst, the solution was again evaporated in vacuo, and the product (3.4 Ci) dissolved in benzene (5 ml). Radiochromatograms run on silica gel GF plates (solvent: 25% EtOAc-hexane; after scanning, plate rerun with 55% EtOAc-hexane) showed tritium corresponding to (2b), 84%, as well as tritiated (1b), 12%, and an unidentified immobile impurity (4%).

A small portion of the product (ca 10 μCi) was mixed with 20 mg carrier, (2a), and chromatographed on a 1 x 5 cm column of silica gel (100-200 mesh). Elution with ether gave the tritiated (1b), whereas elution with ethyl acetate gave radiochromatographically pure (2b). However, the undiluted (2b) was not purified in this manner, but was used directly in the next step.

(3RS)-[2,3-$^3H_2$(N)]-β-Leucine, (3b). The above product, (2b) (2.8 Ci), after addition of unlabeled carrier (2a) (50 mg) and evaporation of the benzene in vacuo, was treated with conc HCl (5 ml) at reflux for 24 h. The HCl and H_2O were evaporated in vacuo, and the residue dissolved in H_2O and added to a 1 x 10 cm column of Dowex 50 W-X8, 50-100 mesh, H^+ form. After elution with H_2O to neutrality, the column was eluted with 2N NH_4OH, to yield, after evaporation in vacuo, the crude product (3b) (0.24 Ci). This was dissolved in MeOH (25 ml) containing a drop of conc HCl for storage. Radiochromatograms run on cellulose plates in two solvent systems ((a) n-BuOH/HOAc/H_2O = 60/15/25); ((b) Collidine/H_2O = 70/30) showed radioactive (3b) 88%, accompanied by faster running impurities, 9%, and an immobile radioactive impurity. A ^3H NMR spectrum showed peaks at δ 2.4 - 2.6 (C-2^3H, 31%) and 3.30 (C-3^3H, 69%). The ^3H NMR spectrum was recorded on a ca. 20 mCi sample dissolved in 0.2 ml CD_3OD containing a trace of DCl plus 10% TMS, contained in a 3.3 mm OD sealed inner tube of a 5 mm coaxial tube (Wilmad Glass Co.), as described by Bloxsidge et al. (12). Spectral parameter: pulse width, 3.15 μsec; rep rate, 6 sec; 11,045 scans were collected).

A portion of the product (ca 2 mCi, 0.2 ml) was mixed with 200 µg unlabeled β-leucine (3a), and separated by HPLC, four 50 µl injections, on a Waters µ Bondapak NH_2 column, using 10% 0.01 M KH_2PO_4/90% CH_3CN : H_2O (100 : 16) as solvent pumped at 2 ml/min, detector set at 200 nm, 0.4 AUFS. The peak corresponding to β-leucine was collected (Fig. 1a) (ca 1.0 mCi total, specific activity 450 mCi/mMol) and stored as collected, without evaporation, at -15°. A radiochromatogram of the collected product showed only (3b).

A portion of the collected product (2 µCi) was mixed with 500 mg unlabeled β-leucine (3a) and crystallized from EtOH/acetone (3985 cpm/mg). The crystalline product was then refluxed with conc HCl (25 ml) for 24 h. One-half of the solution was evaporated in vacuo, and the residue dissolved in a little H_2O. The solution was passed through a 1 x 10 cm column of Dowex 50 W-X8, 50-100 mesh, H^+ form, and eluted with H_2O to neutrality followed by 2N NH_4OH. After evaporation in vacuo, the residue was crystallized from EtOH-acetone and the specific activity of the β-leucine determined (3480 cpm/mg, 15% loss of ^3H observed). After 24 h additional refluxing, the remainder of the HCl solution was similarly processed (specific activity of crystallized β-leucine, 2923 cpm/mg, 15% tritium loss observed). The crystals from 48 h refluxing were again refluxed for 24 h with 10 ml conc HCl, followed by reisolation of the β-leucine (no additional change in specific activity observed (3090 cpm/mg)).

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