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BIOSYNTHESIS OF β-METHYLENENORVALINE (2-AMINO-3-ETHYL-3-BUTENOIC ACID) IN *PHILADELPHUS*

DAVID H. G. CROUT, IAN M. PIPER* and CHARANJIT K. SEHGAL

Department of Chemistry, University of Warwick, Coventry, West Midlands CV4 7AL, U.K.

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Key Word Index—*Philadelphus*; Saxifragaceae; β-methylenenorvaline; 2-amino-3-ethyl-3-butenoic acid; biosynthesis; synthesis.

Abstract—β-Methylenenorvaline (2-amino-3-ethyl-3-butenoic acid) was synthesized. The specific incorporation of [1-^{14}C]isoleucine (as a mixture of L-isoleucine and D-alloisoleucine) into β-methylenenorvaline in *Philadelphus* cv Belle Etoile was demonstrated.

INTRODUCTION

A number of non-protein amino acids structurally related to isoleucine (1) occur in nature [1]. Most occur in higher plants and include 2-amino-3-formyl-3-pentenoic acid and 2-amino-3-hydroxymethyl-3-pentenoic acid [2], 2-amino-4-oxopentanoic acid [3], (2*S*,3*R*,4*S*)-2-amino-3-methyl-4-hydroxypentanoic acid and (2*S*,3*R*,4*R*)-2-amino-3-methyl-4,5-dihydroxypentanoic acid [4], (2*S*,3*R*,4*R*)-2-amino-3-methyl-4-hydroxypentanoic acid [5], 2-amino-3-ethyl-3-butenoic acid (β-methylenenorvaline) [6, 7] and 3-methylene-2-amino-4-pentenoic acid [7]. In addition there also occur homoisoleucine (2-amino-4-methylhexanoic acid [8] and structurally related compounds such as 2-amino-4-methyl-4-hexenoic acid [8, 9], 2-amino-4-methyl-5-hexenoic acid [10, 11], 2-amino-4-hydroxymethyl-5-pentynoic acid [12] and 2-amino-6-hydroxy-4-methyl-4-hexenoic acid [8].

Although there is circumstantial evidence, based on structural similarities, that these compounds are biosynthetically related to isoleucine, experimental evidence is exceedingly sparse. Thus the 4-hydroxyisoleucine from fenugreek [5] and the 2-amino-4-methyl-4-hexenoic acid from *Aesculus californica* [13] were both labelled by L-[U-^{14}C]isoleucine. However, a specific pathway of incorporation could not be demonstrated in these investigations, either because precursors were uniformly labelled or because degradations of compounds derived from specifically labelled precursors were not carried out. We now report on the incorporation of [1-^{14}C]isoleucine into β-methylenenorvaline in *Philadelphus* cv Belle Etoile.

RESULTS

For trial experiments a supply of β-methylenenorvaline (2) was required. We therefore report also a synthesis of β-methylenenorvaline, which, although proceeding in only low overall yield, furnishes the amino acid as the pure racemate in only two steps from readily available starting materials. This synthesis is based on an unpublished procedure developed by Dr G. Dardenne. [1-^{14}C]Isoleucine was synthesized, as a 1 : 1 mixture of L-isoleucine and D-alloisoleucine, by a modification of a published procedure [14]. A total of 4.7 μCi of this mixture [as (1)] was administered to cuttings of *Philadelphus* cv Belle Etoile. After nine days, all of the precursor had been taken up. Extraction of the free amino acids followed by dilution with unlabelled DL-β-methylenenorvaline, preparative paper chromatography and crystallization, gave 66 mg of labelled β-methylenenorvaline, homogeneous by paper chromatography. This material was purified, with further dilution with unlabelled material, to constant radioactivity. The activity of the radiochemically pure material corresponded to a specific incorporation of 0.57%.

At this stage it was proposed to degrade the β-methylenenorvaline to determine the site of labelling. However, it was recognized that although the β-methylenenorvaline sample to be used had been recrystallized to constant activity, it might nevertheless contain entrained L-isoleucine which would vitiate the conclusions from the degradation. Accordingly a back dilution analysis of the labelled β-methylenenorvaline against L-isoleucine was carried out. On recrystallization of the mixture of the labelled sample (12.7 mg) with unlabelled L-isoleucine (465.4 mg), the activity fell, but only slowly. Thus after five recrystallizations the activity had only fallen to 64% of its initial value. Thus it appeared that β-methylenenorvaline was being entrained in the L-isoleucine, the very phenomenon that this control experiment was designed to check in the case of the reverse dilution analysis of β-methylenenorvaline. Saturated amino acids are relatively stable to ozonolysis under acidic conditions [15], whereas β-methylenenorvaline would be expected to undergo cleavage to 2-amino-3-oxopentanoic acid, which is structurally less similar to isoleucine than β-methylenenorvaline and would be more easily removed. Accordingly the L-isoleucine was ozonized in 2 M HCl. The product was isolated by ion exchange treatment, preparative paper chromatography and crystallization. Its specific activity had fallen to 13 ± 6% of the initial value

*Present address: Amersham International plc, White Lion Road, Amersham, Buckinghamshire HP7 9LL, U.K.

thus demonstrating that the labelled β-methylenenorvaline had a minimum radiochemical purity of 87 ± 6%.

Degradation of the labelled β-methylenenorvaline was carried out as shown in Scheme 1. Hydrogenation to a mixture of isoleucine stereoisomers (3) was followed by reduction to a mixture of isoleucinol isomers (4) using the borane–dimethylsulphide complex. Periodate cleavage gave formaldehyde and 2-methylbutanal, isolated as the mixture of dimedone derivatives. The mixture was separated by chromatography on silica gel and the derivatives (5) and (6) were recrystallized to constant activity. As shown in Scheme 1, the radioactivity was confined entirely to the formaldehyde derivative, demonstrating the exclusive incorporation of label from C-1 of isoleucine (1) into C-1 of β-methylenenorvaline (2) in the *Philadelphus* species.

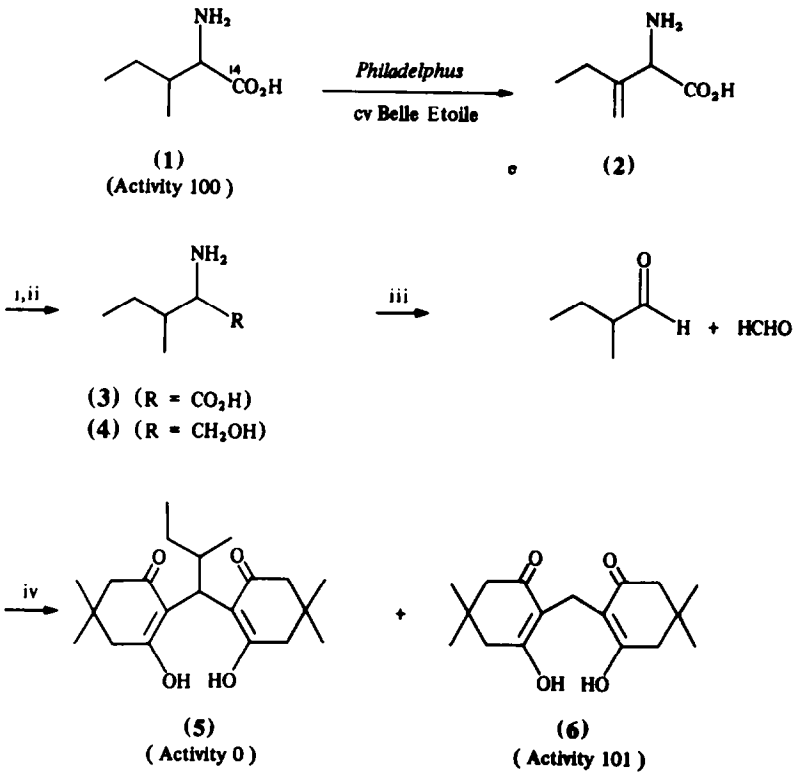
DISCUSSION

These results indicate a rather direct route of metabolism of isoleucine to β-methylenenorvaline in *Philadelphus*. The two most likely mechanisms would be direct dehydrogenation as in the formation of unsaturated fatty acids, or hydroxylation followed by dehydration. The latter possibility is emphasized by the natural occurrence of 4-hydroxyisoleucines in nature noted above. However there is precedent for a direct dehydrogenation mechanism in the formation of 3-ethylidene-L-azetidine-2-carboxylic acid from isoleucine in *Streptomyces cacaoi* [16]. In this case an anti-periplanar elimination of H-3 and of the 4-*pro-R* hydrogen atom was indicated. Both mechanisms therefore require to be kept under consideration.

EXPERIMENTAL

Philadelphus cv Belle Etoile was obtained from Robert Veitch and Son Ltd., Royal Nurseries, Alphington, Exeter, U.K. Radiochemicals were purchased from Amersham International, U.K. NMR spectra were determined on a Jeol MH 100 spectrometer. Paper or TLC on cellulose plates (Merck), was carried out using *n*-BuOH–HOAc–H_2O (4:1:1). Amino acids were detected using the ninhydrin reagent (0.1% in *n*-BuOH). Radioactivity measurements were made using a RackBeta 1215 liquid scintillation spectrometer (L.K.B. Instruments Ltd.). Amino acid samples were dissolved in HCl (1 M, 0.1–0.2 ml) before mixing with the scintillator (NE 260, Nuclear Enterprises Ltd., Sighthill, Edinburgh, U.K.) or Packard 229 liquid scintillation cocktails. All samples were counted in duplicate and sufficient counts were accumulated to give a statistical error of ± 1%. Quench corrections were determined using either external or internal standard channels ratio methods.

Synthesis of [1-^{14}C]*isoleucine* [17]. L-Isoleucine (7 g) in H_2O (250 ml) was treated at 100° with a solution of ninhydrin (33 g) in H_2O (250 ml). The mixture was steam distilled directly into a vigorously stirred slurry of sodium metabisulphite (60 g) with H_2O (40 ml). After *ca* 70 ml of distillate had passed over, the



Reagents: i, H_2 / Pd–C; ii, BF_3/Me_2S:BH_3; iii, $NaIO_4$; iv, dimedone.

Scheme 1.

receiver was kept at 0° overnight to give the bisulphite addition product of (*S*)-2-methylbutanal (16.81 g). The bisulphite addition product (550 mg) was placed in a thick-walled glass tube together with ammonium carbonate (650 mg), NaCN (75 mg) and sodium [^{14}C]cyanide (0.25 mg, 250 μCi), H_2O (2 ml) and EtOH (2 ml). The tube was sealed and heated at 110° for 4 hr with occasional agitation. The tube was cooled, opened and volatile components were removed under reduced pressure. To the residue were added water (2 ml) and HCl (10 M, 2 ml). The tube was resealed and heated at 180° for 16 hr. The tube was cooled, opened and its contents were evaporated to dryness under reduced pressure. The residue was dissolved in the minimum quantity of H_2O and applied to a column of Dowex 50W-X8 ion exchange resin (H^+ form, 20 g). The column was eluted with H_2O, followed by ammonia solution (2 M, 200 ml). The ammoniacal eluate was evaporated to dryness and the residue was recrystallized (aq. ethanol) to give [1-^{14}C]isoleucine (143 mg, 72%, 0.77 μCi/mg) radiochemical purity 97% by strip counting of a paper chromatogram.

Synthesis of DL-*β-methylenenorvaline.* A mixture of dimethylammonium chloride (246 g, 3 mol), butanal (248 g) and aq. formaldehyde (37–41%, 250 ml) was stirred at 70° for 23 hr. The mixture was steam distilled until no more organic material came over. The organic layer was separated, dried ($MgSO_4$) and distilled to give 2-ethyl-2-propenal (139 g, 55%), bp 88–92° (760 mmHg) (lit. [18] bp 91–92°), ^{1}H NMR ($CDCl_3$) 1.04 (3H, *t*, *J* = 6.5 Hz, $MeCH_2$), 2.18 (2H, *q*, $MeCH_2$), 5.95 and 6.20 (each 1H, *s*, H_2C=), 9.45 (1H, *s*, CHO). A mixture of 2-ethyl-2-propenal (28 g) and NH_4Cl (21.4 g) in water (65 ml) was cooled to 0°. To this mixture was added with stirring, an ice-cold solution of NaCN (16.3 g) in water (60 ml). The two-phase mixture was shaken vigorously for 6 hr, treated with conc. HCl (75 ml) and evaporated to dryness on a steam bath. The residue was dissolved in H_2O and extracted with EtOAc (4 × 50 ml). The aq. layer was evaporated under reduced pressure, dried *in vacuo* over KOH and extracted with EtOH (75 ml). The mixture was filtered and the residue was again extracted with EtOH. Altogether, four such extractions with EtOH were carried out. The combined ethanolic extracts were evaporated to dryness under reduced pressure. The residue (12 g) was dissolved in ammonia solution (2 M, 50 ml). The ammoniacal solution was extracted with EtOAc (4 × 50 ml) and evaporated to dryness under reduced pressure. The residue (7.7 g) was applied to a column of Dowex 50W-X8 ion exchange resin (H^+ form, 100 g) which was eluted with H_2O (500 ml) and ammonia solution (2 M, 500 ml). The ammoniacal residue was evaporated to dryness under reduced pressure and the residue was recrystallized (EtOH–H_2O) to give DL-*β*-methylenenorvaline as platelets (1.6 g, 3.8%), mp 210–212° (lit. [17] mp 215–216°), NMR spectrum identical with that previously [17] reported.

Incorporation of [1-^{14}C]*isoleucine into β-methylenenorvaline in* Philadelphus *cv Belle Etoile.* A cutting (*ca* 30 cm long) of *Philadelphus* cv Belle Etoile was placed in each of 20 test tubes containing water (2 ml). To each tube was added 100 μl of a solution of [1-^{14}C]isoleucine (as a 1:1 mixture of L-isoleucine and D-alloisoleucine) (6.1 mg, 0.77 μCi mg^{-1}). During a period of 9 days the cuttings were allowed to imbibe the solution, additional water being added as necessary. After nine days the residual aqueous solutions contained < 1% of the radioactivity administered. The cuttings were macerated with EtOH–H_2O (3:1, 200 ml) and the resulting mixture was filtered. This procedure was carried out six times. The combined filtrates were evaporated to dryness under reduced pressure. The residue was dissolved as far as possible in deionized H_2O (100 ml). The solution was extracted with light petroleum (100 ml, bp 40–60°). The aqueous residue was filtered (Kieselguhr), the filtrate was concentrated to 30 ml under reduced pressure and applied to a column of Dowex 50W-X8 cation exchange resin (20 g). The column was washed thoroughly with deionized H_2O and then eluted with ammonia solution (2 M) until the eluate no longer gave a positive test with ninhydrin. The ammoniacal eluate was evaporated under reduced pressure to give a mixture of amino acids (1.08 g). To this residue was added unlabelled (DL)-*β*-methylenenorvaline (100 mg) and the *β*-methylenenorvaline was reisolated by preparative paper chromatography on 3 mm paper (Whatman). The bands containing *β*-methylenenorvaline were extracted with water and the amino acid was purified by ion exchange treatment as described above to give a mixture of amino acids greatly enriched in *β*-methylenenorvaline. The mixture was recrystallized to constant radioactivity. After the first crystallisation, the amino acid (50.4 mg) was diluted with 214 mg unlabelled material. A further dilution of the amino acid remaining after the fourth recrystallization (77.7 mg) was made with 145 mg of unlabelled material and the mixture was recrystallized twice more. The specific activity was constant over the last three recrystallizations (after correction for dilution). The specific incorporation of the [1-^{14}C]isoleucine into *β*-methylenenorvaline cannot be determined accurately because the amount present in the plant extract was unknown. However, based on the 100 mg of unlabelled *β*-methylenenorvaline added to the initial crude amino acid mixture, a minimum incorporation of 0.57% was calculated.

Degradation of labelled β-methylenenorvaline. A mixture of labelled *β*-methylenenorvaline (48.9 mg, 37.2 dpm/mg) and L-isoleucine (961 mg) was hydrogenated in HCl (0.1 M, 40 ml) over 10% Pd–C (40 mg) at an initial pressure of 1–2 atm overnight. The solution was evaporated to dryness. TLC of the residue showed that *β*-methylenenorvaline was no longer present. The dry residue (895 mg) was placed in a three-necked flask fitted with a condenser. Dry tetrahydrofuran (25 ml) was added and the contents were stirred magnetically in N_2. Boron trifluoride etherate (1.2 ml) was added dropwise by syringe and the resulting mixture was boiled under reflux for 10 min. To the clear solution, borane–dimethylsulphide complex (1.2 ml) was added by syringe at a rate sufficient to maintain a gentle reflux (25 min addition). The mixture was boiled gently under reflux for 2 hr. Aqueous tetrahydrofuran (50%) was added dropwise followed by aq. NaOH (6 M, 8 ml). The mixture was boiled under reflux for a further 2 hr. The mixture was cooled, the organic and aq. layers were separated and the aq. layer was extracted with Et_2O (2 × 25 ml). The combined organic solutions were washed with H_2O (25 ml), dried (Na_2SO_4) and evaporated to give isoleucinol (**4**) (822 mg). In trial experiments with unlabelled material the ^{1}H NMR spectrum of the product at this stage was identical with that of authentic material. The product appeared homogeneous on TLC. The labelled isoleucinol was dissolved in the minimum quantity of 1 M HCl and the pH was adjusted to 7 with aq. NaOH. A solution of $NaIO_4$ (1.5 g) dissolved in the minimum quantity of H_2O was added. After 1 hr, sodium arsenite (900 mg) was added, followed by a solution of dimedone (3.97 g) dissolved in the minimum quantity of EtOH. The resulting ppt. (2.24 g) was examined by the TLC in petrol (bp 60–80°)–EtOAc (9:1) and revealed two spots corresponding to the dimedone derivatives of formaldehyde and 2-methylbutanal. The derivatives were separated by chromatography on silica gel in petrol (bp 40–60°)–EtOAc (9:1). The derivative of 2-methylbutanal was eluted first followed by the derivative of formaldehyde. The derivatives were recrystallized to constant radioactivity from EtOAc–petrol (bp 40–60°). The derivative of formaldehyde had mp 195–197° (lit. [19] mp 191°). The derivative of 2-methylbutanal had mp 133° (lit. [20] mp 135–136°). The latter derivative was inactive. The derivative of formaldehyde con-

tained 239 dpm mmol (101% with respect to the initial radioactive β-methylenenorvaline after correction for dilution with unlabelled isoleucine before hydrogenation).

Reverse dilution analysis of β-methylenenorvaline. A mixture of labelled β-methylenenorvaline (12.7 mg, 129.5 dpm/mg) and L-isoleucine (465 mg) was recrystallized from EtOH–H_2O. After five recrystallizations the specific radioactivity of the L-isoleucine corresponded to the entrainment of 64% of the initial activity. The remaining material and the mother liquors were combined, dissolved in 10 ml 2 M HCl and ozonized for 4 hr. The amino acid was reisolated by passage over Dowex 50W-X8 ion exchange resin as before and purified by preparative paper chromatography as before and recrystallized. The radioactivity of the recrystallized material corresponded to entrainment of 13 ± 6% of the initial radioactivity.

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