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Synthesis of Stereospecifically Deuterated Phenylalanines and Determination of Their Configuration

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1. Starting from *trans*-cinnamic acid a chiral (−)3-phenyl-[2,3-^{2}H]propionic acid has been synthesized using *Clostridium kluyveri* cells as catalyst.

2. The chiral dideuterated acid has been converted by chemical methods to a mixture of (2*R*) and (2*S*)-phenyl[2,3-^{2}H]-alanine.

3. By means of ^{1}H nuclear magnetic resonance spectroscopy and the action of D and L-amino-acid oxidase the configuration of the phenylalanine has been shown to be (2*R*, 3*S*) and (2*S*, 3*S*), respectively. The labelled phenylalanine is thus sterically and isotopically homogeneous at position 3 but heterogeneous at position 2.

The value of stereospecific labelling, particularly in prochiral methylene groups, for the elucidation of biochemical mechanisms has been established in several instances (for reviews see [1a,b]). Stereospecifically deuterated and tritiated phenylalanine and tyrosine species have been previously prepared and used as precursors in complex biosynthetic processes [2–4]. We now report a new method for the preparation of phenylalanine labelled with deuterium in the 3-*pro-S* position.

EXPERIMENTAL PROCEDURE

Proton magnetic resonance (^{1}H-NMR) spectra were recorded with a Bruker WH 90 or WH 270 (FT) spectrometer. Chemical shifts (δ) are reported in ppm to tetramethylsilane or sodium 3-(trimethylsilyl)-[2,2,3,3-$^2H_4$]propionate, coupling constants in Hz. For ultraviolet spectroscopic measurements a Zeiss spectralphotometer PMQ II or a Unicam SP 1800 spectrophotometer was used.

Materials

D-Amino-acid oxidase (spec. act. approx. 20 U/mg) and L-amino-acid oxidase (spec. act. approx. 7 U/mg) were commercial products of Boehringer Mannheim GmbH (Mannheim, West Germany). Tautomerase from beef kidney (1–2 U/mg) was purchased from Sigma Chemie GmbH (München, West Germany), catalase from Fluka GmbH (Neu-Ulm, West Germany). Deuterium oxide (99.93%) was a generous gift of Dr D. Staschewski, Kernforschungszentrum, Karlsruhe. Deuterium oxide (99.7%) was purchased from C. Roth KG (Karlsruhe, West Germany). Thin-layer plates Cellulose F were purchased from Merck (Darmstadt, West Germany), silica gel plates (SILG/UV_{254}) from Macherey-Nagel & Co. (Düren, West Germany).

Syntheses

(2*S*, 3*R*)-3-Phenyl-[2,3-^{2}H]propionic acid I (see Scheme 1) was prepared according to [5] and [6]. 50 mmol sodium cinnamate (8.5 g), 12 mg tetracycline and 170 mg methylviologen were dissolved in 500 ml ^2H_2O (99.7%) containing phosphate buffer (0.1 M, p^{2}H 7.0). Prior to the addition of 8.3 g lyophilised cells of *C. kluyveri* (*R* strain grown on crotonate) the dissolved oxygen was removed by boiling and subsequently cooling the reaction mixture under argon atmosphere. After displacement of the argon by hydrogen gas the reaction was started by vigorous stirring. Overnight at room temperature 100% of the theoretical amount of hydrogen was consumed. The reaction was stopped by boiling and cell debris was separated by centrifugation. The pellet was washed twice with water, the combined supernatants acidified

Abbreviation. NMR, nuclear magnetic resonance.

Enzymes. D-Amino-acid:oxygen oxidoreductase (deaminating) (EC 1.4.3.3); L-amino-acid:oxygen oxidoreductase (deaminating) (EC 1.4.3.2); hydrogen-peroxide:hydrogen-peroxide oxidoreductase or catalase (EC 1.11.1.6); phenylpyruvate keto–enol-isomerase or tautomerase (EC 5.3.2.1).

with H_2SO_4 (1:4), extracted with *n*-hexane and recrystallised from hexane. Yield: 6.15 g (81%); m.p. 46.5 °C; $[\alpha]_{600}^{22} = -1.36 \pm 0.015$ °C, $[\alpha]_{365}^{22} = -4.99 \pm 0.05$ °C ($c = 10.07$ in C^2HCl_3). Deuterium content at positions 2 and 3 was at least 98% according to NMR integration. I was converted to phenylalanines Va and Vb (see Scheme 1) by a modified method of Shriner and Damschroder [7]. 0.2858 g (1.88 mmol) of I in dry carbon tetrachloride (2 ml) was reacted under argon with 0.31 ml freshly distilled thionyl chloride at 60 °C for 20 h. The acid chloride II (0.299 g, 93% yield) was dissolved in dry carbon tetrachloride (2 ml) and 0.3224 g of bromine in 2 ml carbon tetrachloride containing five drops of phosphorus tribromide as catalyst was added dropwise at 65–70 °C and in the dark. After 20 h under the same reaction conditions the product was distilled in a bulb tube *in vacuo*; 0.330 g (75%). The ^{1}H-NMR spectrum showed the presence of IIIa and IIIb as well as unreacted II. No separation of these products was attempted but the entire mixture was hydrolysed by treating it with 8.2 ml water and 1.37 ml 1 M sulfuric acid for 20 h at 65 °C. After saturation with ammonium chloride the acid mixture was continuously extracted with ether giving a crude mixture of IVa, IVb and I. The total yield (0.215 g) was treated without further purification with 25% (w/v) aqueous ammonia (2.4 ml) in a sealed tube at 50 °C for 24 h.

After removal of the solvent *in vacuo* the mixture was dissolved in chilled 1 M hydrochloric acid (5 ml) and the unreacted I extracted continuously with ether. Thus 110 mg (≈40%) of unchanged I was recovered. The aqueous phase was evaporated to dryness and applied to a Dowex-50 column (H^+ form). After washing with water (20 ml) the phenylalanine was eluted with 0.5 M ammonia. Yield: 0.03085 g (0.185 mmol) of Va and Vb. Thin-layer chromatography showed a single spot ($R_F = 0.65$, butan-1-ol/acetic acid/water = 4/1/2, cellulose plates) (m.p. 274–280 °C). ^{1}H-NMR spectrum see Fig. 1 B.

A New Assay Procedure for D and L-Amino-Acid Oxidases

Reagents. 1.257 M sodium borate buffer (pH 6.2); 0.060 M DL-phenylalanine (40 mg of DL-phenylalanine dissolved in 4 ml of water); 0.2 M sodium phosphate buffer (pH 6.2); catalase 1000 U/ml; D or L-amino-acid oxidase (suspension in ammonium sulphate, 100 U/ml or 7 U/ml respectively); phenylpyruvate tautomerase, 10 U/ml.

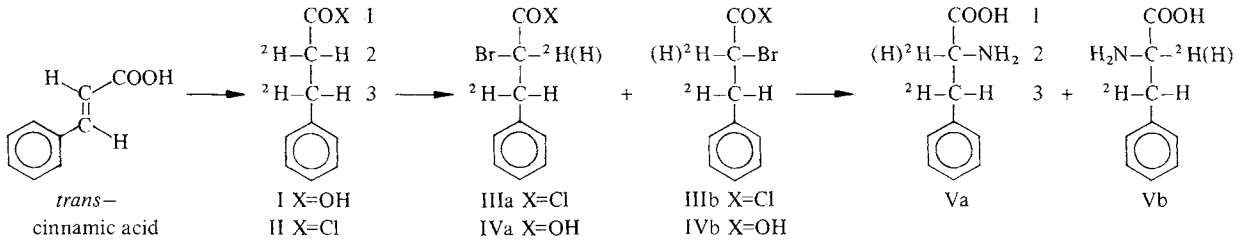
Procedure. 0.5 ml borate buffer; 0.2 ml phosphate buffer; 10 µl catalase; 5 µl tautomerase; 5 µl D or L-amino-acid oxidase; water to give a total volume of 1.2 ml in a quartz microcuvette (pathlength = 1 cm). The reaction is started with 0.2 ml DL-phenylalanine solution for D-amino-acid oxidase (maximal activity at 10 mM substrate concentration) and with 0.01 ml DL-phenylalanine solution for L-amino-acid oxidase (maximal activity at 0.5 mM substrate concentration). Reaction temperature: 22 °C; wavelength (absorption maximum of the borate complex of *enol*-phenylpyruvate and of *enol*-4-hydroxyphenylpyruvate): $\lambda = 330$ nm ($\varepsilon = 6330$ mol$^{-1} \cdot$ cm^{-1}) [8].

Monitoring the Oxidase Reactions by ^{1}H Nuclear Magnetic Resonance Spectroscopy

The ammonium sulphate suspensions of D and L-amino-acid oxidases were centrifuged and the precipitates dissolved in ^2H_2O (260 U/ml and 17.5 U/ml). 0.5 mg of the deuterated phenylalanines Va and Vb, 3 mg of lyophilised phosphate buffer (pH meter reading 6.2) and 0.01 mg lyophilised catalase were dissolved in 1.2 ml ^2H_2O. The reaction was started with a 20-µl portion of either D or L-amino-acid oxidase solution, and over a period of 4 h ten such portions were added at regular intervals to give a total of 50 U D or 3 U L-amino-acid oxidase. After each addition oxygen was bubbled through the mixture for a few seconds. The NMR spectra of the solutions were recorded 4 h, 6 h and 8 h after the start of the reaction (Fig. 2).

RESULTS AND DISCUSSION

Incubation of *trans*-cinnamic acid with *Clostridium kluyveri* (*R* strain) cells in ^2H_2O and under hydrogen atmosphere afforded a chiral species of (−)3-phenyl-[2,3-^{2}H]propionic acid. In analogy to the known stereospecificity of the reductase system of this organism with tiglate and 2-methylcinnamate [6], the probable configuration of the product is as indicated by formula I.



Scheme 1

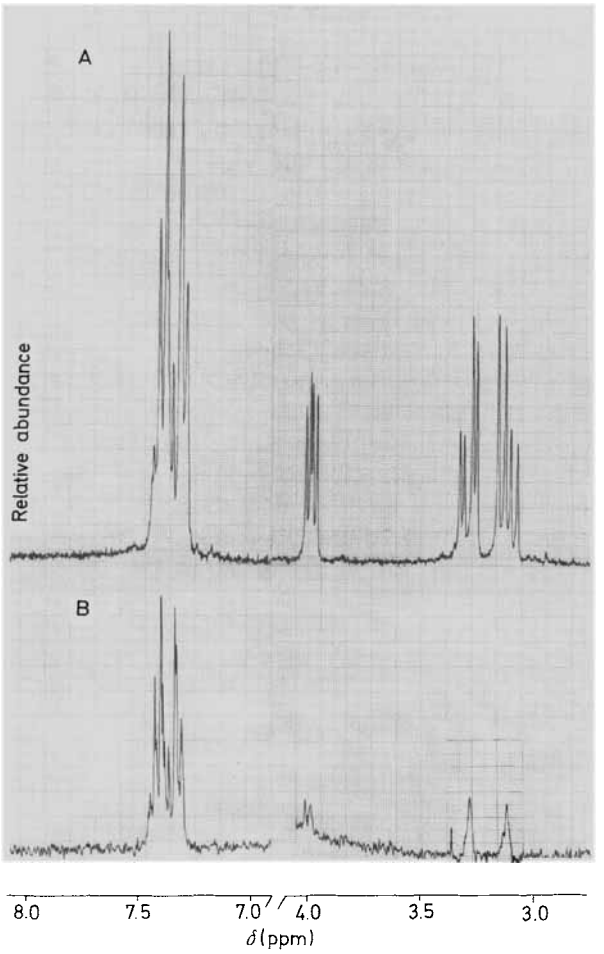


Fig. 1. *The Fourier transform ^{1}H-NMR spectrum of (±)phenylalanine, (A) 3 mg/0.5 ml, 100 scans, and the deuterated phenylalanines Va and Vb, (B) 0.5 mg/1.2 ml, 120 scans, at 270 MHz in ^2H_2O.* Integration (computed) gives a ratio of 0.45:0. 55:0. 20 for the signals of (B)/(A) at 3.12, 3.28 and 4.016 ppm respectively; the integrals of the aromatic protons in (A) and (B) were taken as 5 H

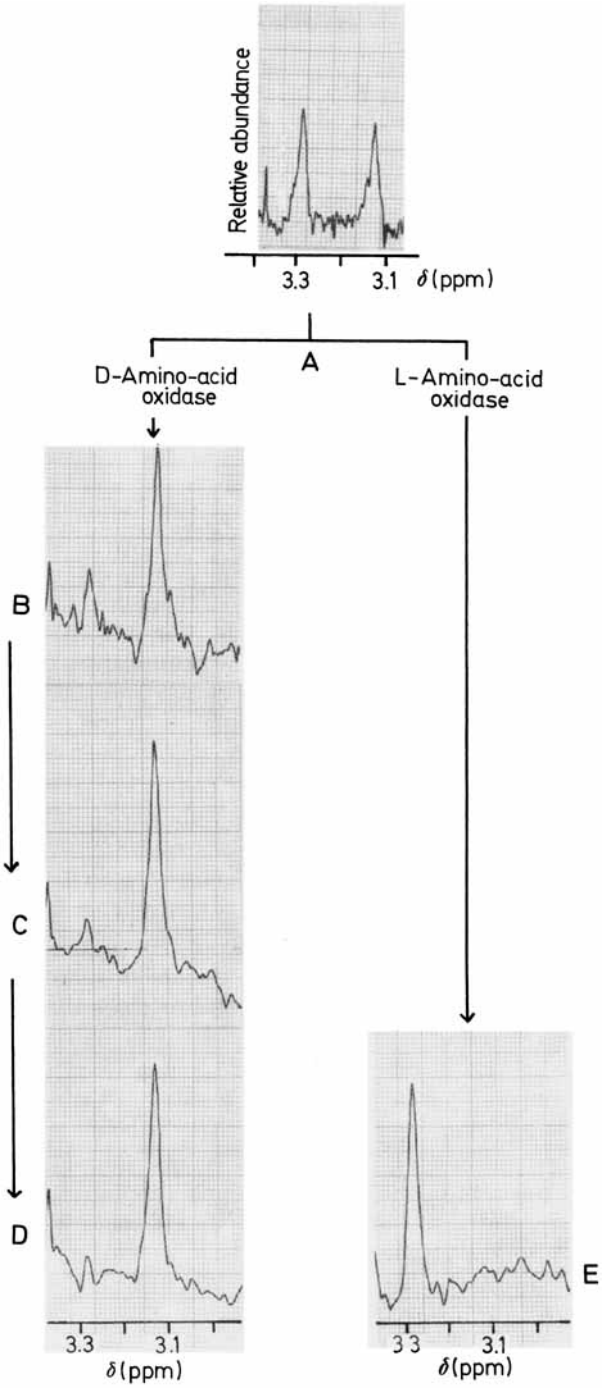
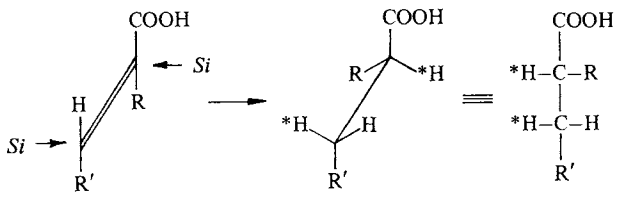


Fig. 2. *Enzymic oxidation of the deuterated phenylalanines Va and Vb.* A part of the 270-MHz ^{1}H-spectra in ^2H_2O is shown at 0 h (A); 4 h (B); 6 h (C); 8 h (D) after addition of D-amino-acid oxidase and 8 h (E) after addition of L-amino-acid oxidase. The sample contained 0.5 mg of the phenylalanines Va and Vb in 1.2 ml, and each spectrum required 1100–1600 scans

I was converted to the acid chloride II, which was brominated in the presence of a catalytic amount of phosphorus tribromide in the dark, giving IIIa and IIIb. Hydrolysis of the acid chloride and treatment of the acid (IVa and IVb) with ammonia gave the phenylalanines Va and Vb, which were purified by ether extraction and ion-exchange chromatography. The overall yield from I to Va + Vb was only 10% but in the ether extract of the last stage about 40% of unchanged starting material could be recovered.

The ^{1}H-NMR spectrum of the product (Fig. 1B, for comparison, the 270 MHz ^{1}H-NMR spectrum of unlabelled phenylalanine in ^2H_2O is shown in Fig. 1A) showed two separate signals at 3.12 ppm and 3.28 ppm for the proton in the *threo* or *erythro* positions to the vicinal amino group, respectively [2]. At the resonance position of the proton at C-2 (4.016 ppm) a doublet appeared amounting to 0.2 H. Integration and multiplicity of the signals indicated that both the (2*R*) and the (2*S*) components of the mixture contained 1 deuterium in position 3 and 0.8 deuterium in position 2. As expected there was complete retention of deuterium, and therefore of configuration, at position 3. However, the high content of deuterium in position



Scheme 2. *Steric course of the hydrogenation of acrylic acid derivatives by the reductase system of* Clostridium kluyveri *(R-strain).* R = H, CH_3; R' = C_6H_5, CH_3. *H originating from solvent

2 was surprising. This can be explained by a rate-determining and deuterium-discriminating enolisation preceding the addition of bromine in the reaction II → III. Even more surprising is that the distribution of deuterium in the *threo* and *erythro* positions was unequal indicating a 20% excess of Va over Vb in the phenylalanine mixture. (Fig. 1 B).

Since in the nucleophilic substitution by ammonia an asymmetric induction due to the chiral CH^2H group of IVa and IVb is unlikely, one is forced to assume some stereospecificity in the bromination step. An asymmetric solvation of the enolic intermediate by the conjugate acid of the proton-abstracting base could be the reason for such a stereospecificity. This aspect will be the object of a separate investigation.

In order to determine the absolute configuration at position 3 two buffered solutions (pH 6.2) of the deuterated phenylalanine preparation (Va and Vb) were prepared in ^2H_2O. D and L-amino-acid oxidases, respectively, were added repeatedly over several hours and the 270-MHz ^{1}H-NMR spectra of the solutions recorded. Whereas in the D-amino-acid-oxidase-treated sample the signal at 3.28 ppm gradually disappeared, the signal at 3.12 ppm practically disappeared in the L-amino-acid-oxidase-treated sample (Fig. 2). Thereby evidence is provided for the 3-H being in the *erythro* position of the D-phenylalanine and in the *threo* position of the L-phenylalanine species (always with respect to the amino group) as indicated in Va and Vb. The absolute configuration at position 3 is therefore (*S*) in both cases. This has the advantage that the mixture of Va and Vb or that of their tritium analogues can be used for biosynthetic investigations without separation even in organisms possessing amino acid racemase activity. In a most recent report Nagai and Kobayashi [9] describe the synthesis of (2*R*, 3*R*) and (2*S*, 3*R*)-3-phenyl-[3-^{2}H]alanines, that is, the enantiomers of Vb and Va.

Our results confirm the stereospecificity of the reductase of *Clostridium kluyveri* (*R* strain) as established with tiglate and 2-methylcinnamate as substrate [6]. This enzyme adds hydrogen atoms in an *anti* fashion to the *Si* faces of both C-2 and C-3 of the acrylic acid derivatives (Scheme 2). The power of high-resolution Fourier-transform NMR spectroscopy for directly following enzymic reactions in extremely dilute solutions and determining mechanistic and stereochemical aspects thereof has been demonstrated. A further example for this and for the use of the stereospecifically deuterated phenylalanines Va and Vb in a biochemical process is presented in the following paper [10].

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