The Shikimate Pathway. Part III.† The Stereochemical Course of the L-Phenylalanine Ammonia Lyase Reaction

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The synthesis of (3*R*)- and (3*S*)-3-deuterio-L-phenylalanine is described. The enzymatic elimination of ammonia from the amino-acid to give *trans*-cinnamic acid is shown to involve the *pro-S* hydrogen.

CHORISMATE MUTASE is a key enzyme in the Shikimate pathway [1] and catalyses the conversion of chorismate (I) into prephenate (II) which itself is then transformed into the essential amino-acids L-phenylalanine (III) and L-tyrosine (IV). The change (I) to (II) may also be accomplished thermally in basic media [2,3] and in its formal representation it is the only authenticated example of an *ortho*-Claisen rearrangement in a primary metabolic pathway. In order to investigate the stereochemical features of this enzymic reaction it is necessary to label H_A or H_B of chorismate (I) and then to examine the position of the labelled atom in the resultant methylene group in one of the products (III) or (IV). If the enzyme-mediated rearrangement is concerted then a pre-chair conformation (I) for chorismate would, for example, lead to prephenate with the methylene group configuration as shown in (II). Rose [4,5] has provided a possible means of labelling chorismate *via* the preparation of isotopically labelled phosphoenolpyruvate and herein is described a procedure to elucidate the stereochemical configuration of isotopic hydrogen substitution in the methylene group of L-phenylalanine. Similar results have recently been reported in a preliminary communication by Battersby and his co-workers [6] and in related work Kirby and Michael [7] have reported on the stereospecific labelling of the methylene group in aromatic amino-acids.

Samples of (3*S*)- and (3*R*)-3-deuterio-L-phenylalanine (IX) and (VII) were prepared from (1*S*)-1-deuteriobenzyl alcohol (V).[8] The toluene-*p*-sulphonyl derivative [9] of (V; 0·89 atom deuterium) reacted with diethyl *N*-acetylaminomalonate and the product was hydrolysed and decarboxylated to give (3*R*)-3-deuterio-DL-phenylalanine. Resolution was achieved by the selective hydrolysis of the N-chloracetyl derivative with the enzyme acylase I [10] to give (3*R*)-3-deuterio-L-phenylalanine (VII; 0·86 atom deuterium). The proof of stereochemical identity at the methylene group of (VII) rests on the evidence of ^{1}H n.m.r. measurements and chemical analogy. The assumption, well founded in the literature,[11,12] is made that the malonic ester alkylation of the tosylate (VI)

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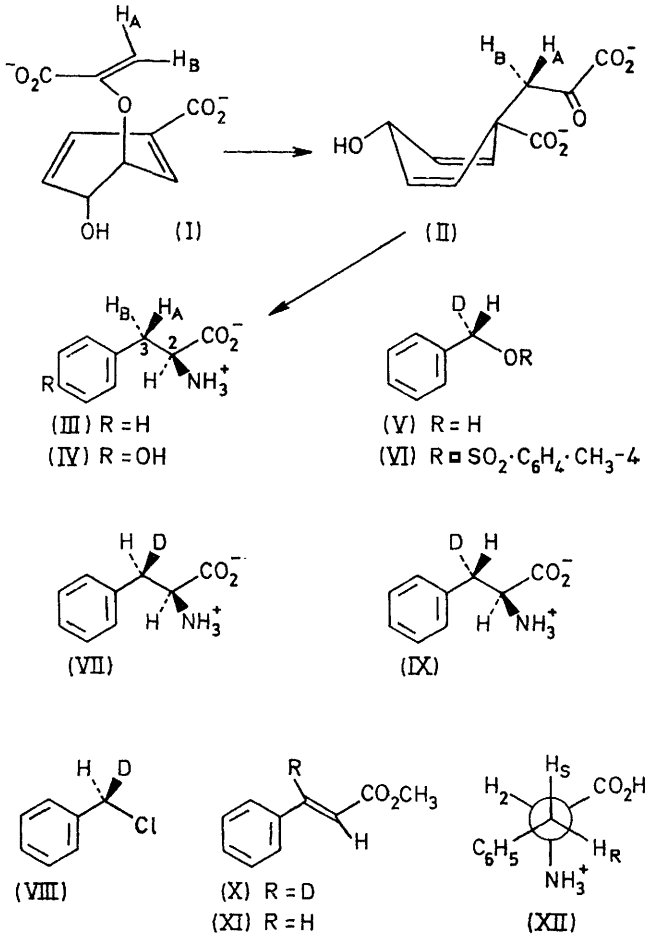
proceeds with inversion of configuration and the high degree of stereoselectivity in this reaction may be inferred from the ^{1}H n.m.r. measurements (Figure 1).



In trifluoracetic acid the methylene protons of L-phenylalanine form the clearly defined AB part of an ABX system. The two quartets collapse to two doublets (J = 4·5 and 8·5 Hz) in (3*R*)-3-deuterio-DL-phenylalanine and in the L-amino-acid (VII) only the lower field doublet (τ = 6·40, J = 4·5 Hz) is present. Complementary results follow from an analysis of the ^{1}H n.m.r. spectra of the N-chloracetyl derivatives of the product (3*R*)-3-deuterio-D-phenylalanine and its precursors.

The reaction of alcohols with phosphorus trichloride and pyridine proceeds with inversion [13] and with the alcohol (V) gives the (1*R*)-1-deuterio chloride (VIII). Subsequent alkylation with diethyl *N*-acetylaminomalonate and hydrolysis, decarboxylation, and resolution gave (3S)-3-deuterio-L-phenylalanine (IX; 0·85 atom deuterium). The structure and stereochemical configuration of the deuterium was established again by chemical analogy [6,14] and ^{1}H n.m.r. measurements (Figure 1d).

Many plants contain the enzyme L-phenylalanine ammonia lyase [15–17] which converts the amino-acid into *trans*-cinnamic acid and is believed to be important in the diversion of the aromatic amino-acid to the synthesis of a variety of phenylpropanoid derivatives. The enzyme was obtained according to the procedures of Hanson and Havir [17] from potatoes and converted the stereoisomer (VII; 0·86 atom deuterium) into 3-deuteriocinnamic acid, isolated and purified as its methyl ester (X; 0·82 atom deuterium). Conversely (IX; 0·85 atom deuterium) gave cinnamic acid (XI; 0·08 atom deuterium). The conversions were not complete (*ca.* 35% and *ca.* 25% respectively for (VII) and (IX) and this was most probably due to the ready deactivation of the enzyme under the conditions of assay. In addition in the case of the isomer (IX) as substrate a primary isotope effect was also, it is presumed, operating. The mass spectra and ^{1}H n.m.r. data (Figure 2) on the derived cinnamic acids nevertheless fully support the conclusion of Battersby and his co-workers [6] that the enzymic

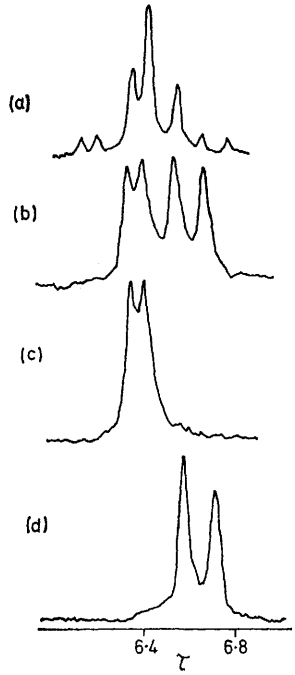


FIGURE 1 ^{1}H N.m.r. spectra of L-phenylalanine and its 3-deuterio-derivatives in $CF_3{\cdot}CO_2H$ (60 MHz): (a) L-phenylalanine; (b) (3*R*)-3-deuterio-DL-phenylalanine; (c) (3*R*)-3-deuterio-L-phenylalanine (VII); (d) (3*S*)-3-deuterio-L-phenylalanine (IX)

reaction proceeds specifically in an antiperiplanar manner with the elimination of $3H_S$ from L-phenylalanine and it is thus quite analogous in its stereochemistry to other amino-acid ammonia lyases.[18–20]

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These results further provide a basis for a method of assay of the stereochemistry of the chorismate mutase reaction.

The ^{1}H n.m.r. data provides additional support for earlier analyses of the spectra of L-phenylalanine.[21-23] It is generally assumed that the stable forms of amino-acids in solution are the three classical staggered conformations obtained by internal rotation about the C(2)–C(3) bond. If the internal rotation is sufficiently rapid the observed chemical shifts and coupling constants in the ^{1}H n.m.r. spectrum are weighted averages of the contributions from the three conformations. These assumptions are thought to be valid for the L-phenylalanine cation [22] in which (XII) is the most favoured

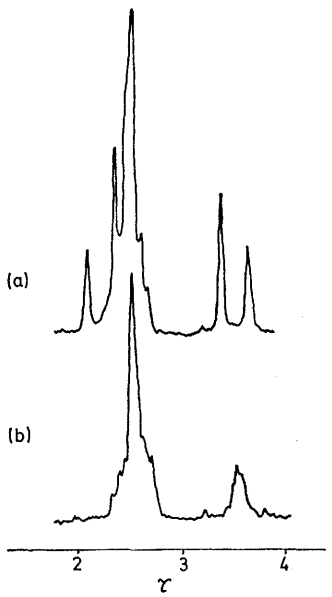

FIGURE 2 ^{1}H N.m.r. spectra of *trans*-methyl cinnamate in $CDCl_3$ (60 MHz): (a) from (3*S*)-3-deuterio-L-phenylalanine (IX) and (b) from (3*R*)-3-deuterio-L-phenylalanine (VII)

conformation with the bulky phenyl and carboxyl-groups furthest apart and with $J(H_2 . H_S) < J(H_2 . H_R)$. The ^{1}H n.m.r. data for (3*R*)- and (3*S*)-3-deuterio-L-phenylalanine add further substance to these arguments. Thus in the spectrum of the (3*R*)-isomer (^{2}H for H_R) the remaining doublet in the methylene proton region corresponds (Figure 1c) to that with the smallest coupling ($H_2 . H_S = 4{\cdot}5$ Hz) and for the (3*S*)-isomer the situation is exactly reversed (Figure 1d).

EXPERIMENTAL

^{1}H N.m.r. spectra were obtained at 60 MHz (Perkin-Elmer R 12A) and accurate τ values relative to Me_4Si and coupling constants were obtained at 100 MHz (Varian HA 100). Deuterium analyses were determined using an AE1 MS 9 spectrometer.

(1S)-1-*Deuteriobenzyl Alcohol*.—This compound was prepared from 1-deuteriobenzaldehyde using the alcohol dehydrogenase–NADH enzyme system present in actively fermenting yeast.[8] The alcohol was purified by fractional distillation (b.p. 57—60°/1 mm Hg, 50% 0·90 atom deuterium).

(1S)-1-*Deuteriobenzyl Toluene*-p-*sulphonate*.—This compound was prepared from the alcohol using sodium hydride and toluene-*p*-sulphonyl chloride [9] (m.p. 58°; 0·89 atom deuterium).

(1R)-1-*Deuteriobenzyl Chloride*.—This compound was prepared from the alcohol using phosphorus trichloride and pyridine [13,24] (b.p. 32—33°/0·1 mm Hg; 0·88 atom deuterium).

(3S)-3-*Deuterio*-DL-*phenylalanine*.—Diethyl acetylaminomalonate (3·86 g.) was added to a solution of sodium (0·4 g.) in anhydrous ethanol (50 ml.) and the solution was heated under reflux (1 min.). (1*R*)-1-Deuteriobenzyl chloride (2·66 g.) was finally added to the mixture which was then heated under reflux with stirring (2 hr.). The solution was evaporated to dryness at 30°, extracted with water and chloroform; the latter extract was dried ($MgSO_4$) and evaporated to give a white solid. The residue was heated under reflux (7 hr.) with hydrobromic acid (48% w/w, 18 ml.) and after removal of the solvent the remaining solid was dissolved in a minimum quantity of water; the pH was adjusted to 6 with ammonia and the mixture was set aside at 0° for 24 hr. The product was filtered at the pump and recrystallised from water to give (3S)-3-*deuterio*-DL-*phenylalanine* (2·2 g.), m.p. 260—265° (lit.,[25] m.p. 271—273°). Mass spectral analysis [26] showed the presence of 0·87 atom deuterium.

(3R)-3-*Deuterio*-DL-*phenylalanine*.—This compound was prepared in an exactly analogous procedure to the above using (1*S*)-1-deuteriobenzyltoluene-*p*-sulphonate in place of the chloride. The *amino-acid* (2·0 g.) was crystallised from water, m.p. 258—262° (lit.,[25] m.p. 271—273°). Mass spectral analysis [26] showed the presence of 0·86 atom deuterium.

N-*Chloracetyl*-(3S)- *and* (3R)-3-*deuterio*-DL-*phenylalanine*.—Sodium hydroxide (2N) and chloracetyl chloride (1·2 ml.) were added alternately and dropwise to a solution of the deuterio-DL-phenylalanine (2·0 g.) in sodium hydroxide (2N, 6 ml.) at 5° so as to maintain the pH ⩽ 9. The mixture was stirred for a further 2 hr. at 5°, and was then acidified and extracted with ether (2 × 50 ml.). The ether extract was dried ($MgSO_4$) and evaporated to give an oil which was triturated with light petroleum and crystallised from water to give the *N*-chloracetyl derivative, m.p. 130° (lit.,[10] m.p. 130—131°); τ ($CDCl_3$–D_2O) 2·78 (5H, s), 5·26 [1H, m, $CH(NHCOCH_2Cl)CO_2H$], τ 5·94 (2H, s, $COCH_2Cl$), 6·80 and 6·92 (1H, two d, $J_1 = 5{\cdot}5$, $J_2 = 7{\cdot}5$ Hz, Ph–CHD).

Resolution of the N-*Chloracetyl*-DL-*amino-acid*.—The method was adapted from that described by Greenstein and Winitz.[10]

A solution of the appropriate racemic *N*-chloracetyl-amino-acid (0·9 g.) in water (75 ml.) was adjusted with

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lithium hydroxide (2N) to pH 7·0—7·5, the volume was then brought up to 100 ml. with water. Acylase I (Miles Seravac, 1820 units per mg., 20 mg.) was added to the solution which was incubated at 37° for 3 hr. A further quantity of enzyme (5 mg.) was added and the incubation continued for ½ hr. before the solution was reduced at 30° to 50 ml., acidified to pH 1·0—2·0 with hydrochloric acid (5N), and continuously extracted with ether (3 hr.). The ether solution gave, on removal of the solvent, the *N*-chloracetyl derivative of the 3-deuteriated-D-amino-acid which was recrystallised from water as needles, m.p. 124—125° (lit.,[10] m.p. 125°), and $[\alpha]_D^{20}$ −45·0° (*c* 2 in EtOH) (lit.,[10] $[\alpha]_D$ −50·4°); in the region τ ($CDCl_3$–D_2O) 6·5 to 7·0 only the doublet τ 6·92 (*J* 7·5 Hz) for the (3*R*)-3-deuterio-D-amino-acid and only the doublet 6·80 (*J* 5·5 Hz) for the (3*S*)-3-deuterio-D-amino-acid were observed.

The aqueous solution of the L-amino-acid, was centrifuged to remove denatured protein and the supernatant solution was evaporated to dryness at 30°. The residue was dissolved in a minimum amount of water, the pH adjusted to 6·0 with lithium hydroxide (2N), and the solution applied to a column of Dowex 1 resin (OH^- form, 1·5 × 20 cm). The column was washed with water until the eluate was neutral and then eluted with acetic acid of increasing concentration (gradient, 0·2 to 1·5N). Fractions (10 ml.) were collected and those showing a positive minhydrin test combined and evaporated to give the (3R)- *or* (3S)-3-*deuterio*-L-*phenylalanine* (0·25 g.), m.p. 270—275° (lit.,[25] m.p. 283—284°). Mass spectral analysis [26] showed the amino-acids to contain 0·86 and 0·85 atom deuterium respectively. The ^1H n.m.r. spectra in CF_3CO_2H are discussed on p. 2819.

L-*Phenylalanine Ammonia Lyase*.—The enzyme was extracted from potatoes according to the procedure described by Hanson and Havir.[17] The acetone powder (40 g.) was stirred with borate buffer (0·1M, pH 9·0, 400 ml.) containing reduced glutathione for ½ hr. at 4°. After centrifugation at 0° ammonium sulphate (250 g.) was added during 1½ hr. at 4° with stirring. The precipitated protein was centrifuged off and dissolved in borate buffer (0·1M, pH 9·0, 60 ml.). The final enzyme solution contained *ca.* 600 m units of activity.[17]

Enzymic Incubations.—An enzyme solution (60 ml.), prepared as above, containing a sample of (3*R*)- or (3*S*)-3-deuterio-L-phenylalanine (100 mg.) was incubated at 32° for 36 hr. when no further enzyme activity could be detected. The solution was then acidified (pH 1·0—2·0, HCl) and continuously extracted with ether (4 hr.); the extract was finally dried ($MgSO_4$) and evaporated. The crude cinnamic acid was redissolved in ether (15 ml.) and treated (5 min.) with an excess of ethereal diazomethane at −70°; after removal of the solvents the residue was applied to a plate of silica (20 × 20 cm.). The plate was developed in 10% chloroform–benzene and the methyl cinnamate (R_F 0·75) removed to give a waxy solid, m.p. 30—34°, lit.,[25] m.p. 38°. (3*R*)-3-Deuterio-L-phenylalanine gave 35 mg. of product and (3*S*)-3-deuterio-L-phenylalanine gave 24 mg. of methyl cinnamate under the conditions of assay. The ^1H n.m.r. spectral and mass spectral analysis of the methyl cinnamates are discussed on p. 2819.

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