58. Biosynthesis of Cytochalasans. Part 6. The Mode of Incorporation of Phenylalanine into Cytochalasin D[1])

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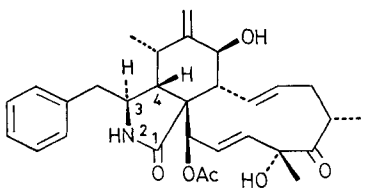
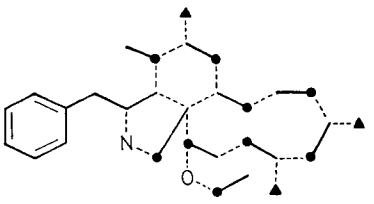
Summary. Rapid equilibrium of D- and L-phenylalanines with phenylpyruvic acid (**4**) was shown to account for their equally efficient incorporation into cytochalasin D (**1**) by *Zygosporium masonii*. Transamination of phenylalanines stereospecifically labelled with tritium at C(2) and C(3) proceeded with complete hydrogen loss at the α position and extensive loss at the β position. Considerable suppression of the incorporation of the D-amino acid by phenylpyruvic acid (**4**) indicated that the L-enantiomer is the primary precursor of **1**.

Our earlier work had shown that the biosynthesis of cytochalasin D (**1**) by *Zygosporium masonii* HUGHES (*Fungi imperfecti*) involves connection of phenylalanine, three C_1 units from the methyl of methionine, and nine intact acetate entities, eight of which are coupled in the head to tail fashion normally observed in polyketide biogenesis [2]. Although cytochalasin D (**1**) possesses the (*S*)-configuration at C(3) corresponding to the abundant L-phenylalanine ((2*S*)-configuration) [3], the DL- and L-amino acids appeared to be equally good precursors. Since all of the carbon atoms of phenylalanine are incorporated as an intact unit [4], we decided to examine the utilization of this substance in detail.

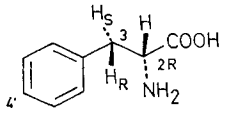


1 Cytochalasin D

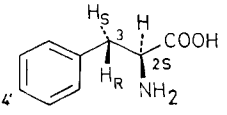


▲ Methionine

●— Acetate



D-Phenylalanine



L-Phenylalanine

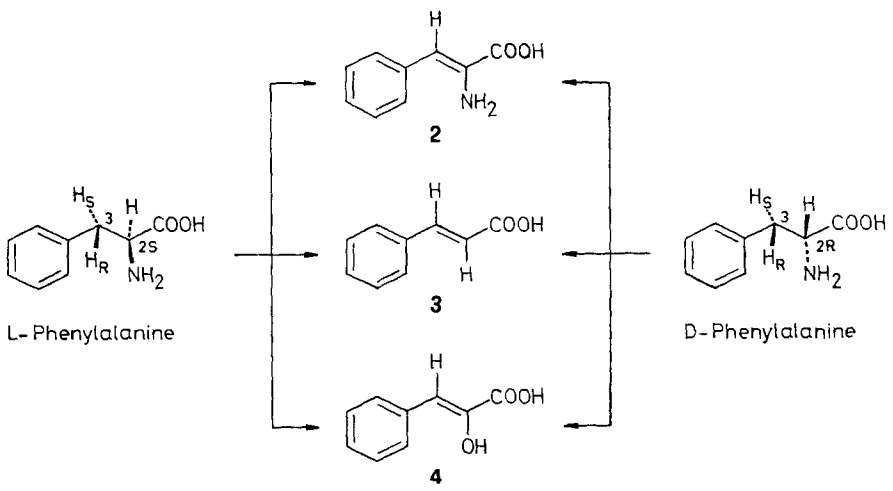
Administration of a mixture of (2*RS*)-[2-^{14}C]- and (2*S*)-[4'-^{3}H]-phenylalanines to *Z. masonii* confirmed that both the D(2*R*)- and L(2*S*)-amino acids were incorporated

[1]) Part 5, see [1].

equally well into cytochalasin D (**1**) because the $^3H/^{14}C$ ratio remained unchanged within experimental error[2]) (Table 1). The absolute incorporation rates ranged between 2% and 13%, and depended on the amount of precursor fed.

To elucidate the mechanism of utilization of both enantiomers, commercially available (2*S*, 3*RS*)-[2,3-di-^{3}H]-phenylalanine was mixed with (2*S*)-[U-^{14}C]-phenylalanine (as internal standard) and incorporated in the usual fashion. Analysis of the precursor by racemization [6] showed that 48% of the tritium label was on C(2). Examination of the $^3H/^{14}C$ ratio of the resulting cytochalasin D (**1**) suggested that tritium was removed almost completely from C(2) and substantially from C(3) during conversion to the antibiotic. Incorporation of the racemized precursor, (2*RS*, 3*R*)- and (2*RS*, 3*S*)-[3-^{3}H]-phenylalanine, demonstrated that loss of hydrogen had actually occurred at the 3-position, and that the large decrease in $^3H/^{14}C$ ratio was not due solely to an isotope effect at C(2).

Conversion of phenylalanine into derivatives of dehydrophenylalanine (**2**), cinnamic acid (**3**), or phenylpyruvic acid (**4**) could explain the equal incorporation rates of both enantiomers and the loss of tritium on C(2) and C(3). The sole intermediacy of dehydrophenylalanine (**2**) derivatives could be excluded by the observed loss of 98% of ^{15}N label from (2*RS*)-[^{15}N]-phenylalanine relative to incorporation of (2*S*)-[U-^{14}C]-phenylalanine[3]). Cinnamic acid (**3**) is synthesized universally in higher



plants and widely in fungi from (2*S*)-phenylalanine by phenylalanine ammonium lyase [7]. This process is a *trans*-elimination of the elements of ammonia with stereospecific loss of the 3-*pro*(*S*)-proton of phenylalanine [6] [8a][4]). Phenylpyruvic acid (**4**) (shown in its enol form) is the normal biogenetic precursor of phenylalanine, and is

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[2]) Phenylalanine and phenylpyruvic acid labelled with [4′-^{3}H]- are radiochemically unstable and some error (*ca.* 5%) is introduced by decomposition [5].

[3]) We are grateful to Dr. *H. Lichti, Sandoz AG.*, Basel, for mass spectrometric determination of the absolute incorporation of ^{15}N label. It is permissible to compare incorporation rates of racemic and optically active precursors in this case because earlier experiments had shown that both enantiomers are utilized equally well.

[4]) Some loss of tritium also occurs at C(2) if impure enzyme is used [8b].

Table 1. *Incorporation of Precursors into Cytochalasin D* (**1**)

Precursor	Absolute Incorporation Rate (%)	$^3H/^{14}C$ Ratio		^3H-Retentio (%)
		Precursor	Cytochalasin D	
Phenylalanine				
(2*S*) – [4′ – ^{3}H]	13.2	5.00	5.15	103 [b]
(2*RS*) – [2 – ^{14}C]	12.8			
(2*S*,3*RS*) – [2,3 – ^{3}H] [a]	0.59	4.24	1.46	34
(2*S*) – [U – ^{14}C]	1.70			
(2*S*,3*RS*) – [2,3 – ^{3}H] [a]	3.99	19.4	5.76	30
(2*S*) – [U-^{14}C]	13.8			
(2*RS*,3*R*) – [3-^{3}H] [a]	2.69	6.50	3.57	55
(2*RS*,3*S*) – [3-^{3}H]				
(2*RS*) – [U-^{14}C]	4.89			
(2*R*,3*S*) – [3-^{3}H] [b]	3.18	6.48	2.25	34
(2*S*,3*R*) – [3-^{3}H]				
(2*S*) – [U-^{14}C]	9.63			
(2*R*,3*S*) – [3-^{3}H] [b]	2.42	1.22	0.29	24
(2*S*) – [U-^{14}C]	9.99			
(2*S*,3*R*) – [3-^{3}H] [b]	1.17	1.21	0.52	43
(2*S*) – [U-^{14}C]	2.74			
(2*S*) – [4′-^{3}H] [c]	11.2	5.00	7.11	142 [b]
(2*RS*) – [2-^{14}C]	7.88			
(120 mg inactive phenylpyruvate)				
(2*RS*) – [^{15}N]	0.07	98% of ^{15}N lost		
(2*S*) – [U-^{14}C]	4.04			
Phenylpyruvate				
[4′-^{3}H]	6.66	4.85	4.95	102 [b]
[U-^{14}C]	6.48			
[4′-^{3}H] [c]	3.70	4.85	5.01	103 [b]
[U-^{14}C]	3.59			
Cinnamate [3-^{14}C]	0.02			

[a] Determined to be 81% (3*S*) and 19% (3*R*) by phenylalanine ammonium lyase assay.
[b] Minimum configurational purity at C(3) is 97%.
[c] Precursor added 48 h after inoculation.

in equilibrium with it through the action of aminotransferases and amino acid oxidases [9].

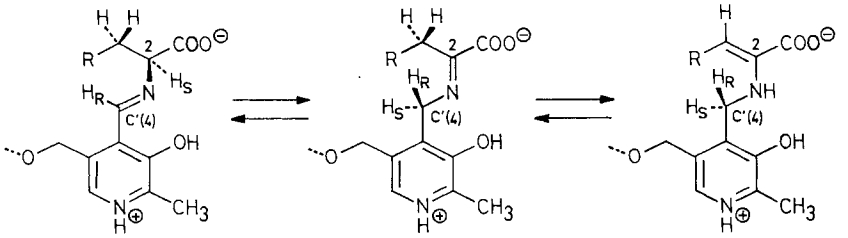
In order to distinguish between the participation of **3** and **4** and to clarify the mechanism involved in the proton losses, the (2*R*, 3*S*)-[3-^{3}H]- and (2*S*, 3*R*)-[3-^{3}H]-phenylalanines were synthesized by a modification of the method of *Battersby* [6]. A mixture of the (2*R*, 3*S*)- and (2*S*, 3*R*)-N-benzoyl-[3-^{3}H]-phenylalanines obtained by his procedure was resolved by fractional crystallization of their cinchonine salts [10]. Liberation of the corresponding acid from the pure (2*R*)-salt, followed by acidic hydrolysis produced the required (2*R*, 3*S*)-[3-^{3}H]-phenylalanine. The partially resolved (2*S*)-salt was likewise converted to the corresponding amino acid, which was then N-chloroacetylated without racemization. Treatment with carboxypeptidase A from bovine pancreas gave pure (2*S*, 3*R*)-[3-^{3}H]-phenylalanine and a small amount of pure (2*R*, 3*S*)-N-chloroacetyl-[3-^{3}H]-phenylalanine [11]. Phenylalanine ammonium lyase was isolated from potatoes [12] and used to convert the (2*S*)-amino acid (which had been diluted with (2*S*)-[U-^{14}C]-phenylalanine) to cinnamic acid with the loss of the 3-*pro*(*S*) proton. This enzymatic analysis determined that the optical purity at the 3-position of both precursors was at least 97%. Similar treatment of the mixture of (2*RS*, 3*R*)-[3-^{3}H]- and (2*RS*, 3*S*)-[3-^{3}H]-phenylalanines described above showed that 81% of the tritium had the (3*S*)-configuration and 19% the (3*R*).

Fermentation of *Z. masonii* with (2*S*, 3*R*)-[3-^{3}H]- and (2*S*)-[U-^{14}C]-phenylalanines yielded cytochalasin D (**1**) which had lost 57% of the tritium. Analogous incorporation of the (2*R*, 3*S*)-[3-^{3}H]-enantiomer resulted in the removal of 76% of the labelled hydrogen. The low absolute incorporation (0.02%) of [3-^{14}C]-cinnamic acid and the loss of both 3-*pro*(*R*) and 3-*pro*(*S*) protons from phenylalanine supports the participation of phenylpyruvic acid (**4**) in a transamination process. In addition, intermediates involving enzymatic hydroxylation at C(3) of a phenylalanine derivative are disfavoured since this type of reaction generally occurs with retention of configuration [13]. To confirm this conclusion, radioactive phenylpyruvic acid was prepared by aerobic enzymatic oxidation of (2*S*)-[4′-^{3}H, U-^{14}C]-phenylalanine with L-amino acid oxidase from *Crotalus atrox* venom and catalase from bovine liver [14]. Intact incorporation of this keto acid into cytochalasin D (**1**) was 6.5%, which is similar to that obtained with phenylalanine.

Reversible transamination occurs in many antibiotic biosyntheses, including those of the penicillins and cephalosporins [15], gramicidin S[16], actinomycin [17], and gliotoxin and related compounds [18]. In contrast to our observations with cytochalasin D (**1**), the naturally abundant L-amino acids are often more efficiently incorporated than their antipodes, even in cases where the end product possesses the configuration corresponding to the D-amino acid. This may be due to improved transport across the cell membrane by permeases [19], or to racemization after biochemical elaboration.

The flavoprotein D-amino acid oxidases ordinarily do not exchange the β-hydrogens of their substrates [9f,g]. In transaminations the exact mechanism of β-proton loss remains less clear. Initial aldimine **A** formation with pyridoxal phosphate is very rapid, even in the absence of enzyme [20]. The biological process is probably a transimination with pyridoxal phosphate which is bound in imine form by an amino group of the protein. Tautomerization to the ketimine **B** is the rate-determining step and

displays an isotope effect if the 2-position is substituted with deuterium [21]. With L-amino acids, the same protein amino group reversibly tranfers hydrogen (H_S) from the 2-position to C'(4) of the coenzyme complex in *cis* fashion across the *si* face [9a]. Interestingly, D-amino acids also add the *pro*(*S*) proton to C'(4) although their transamination requires different enzymes [9h]. Ketimine **B** may reversibly isomerize to the enamine **C** in an enzyme-assisted process, thereby effecting β-proton exchange, or it may hydrolyze directly to the keto acid. *Whelan & Long* have shown that under



A **B** **C**

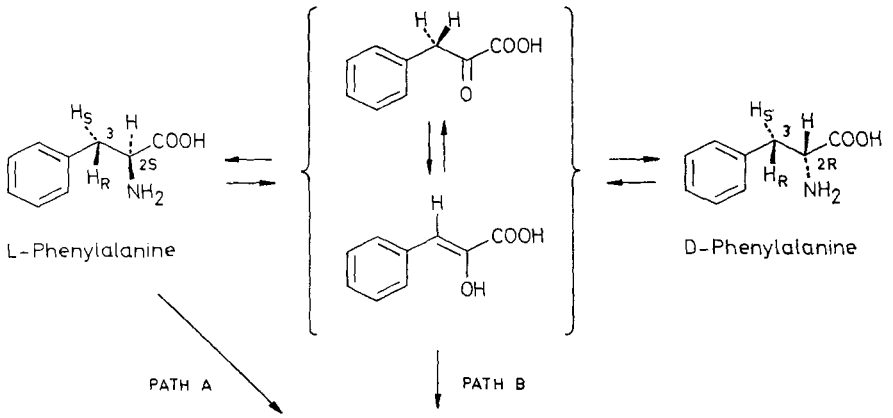
identical conditions pig heart glutamate-oxaloacetate aminotransferase exchanges both the α and β hydrogen atoms of L-glutamic acid, whereas glutamate-pyruvate aminotransferase from the same source replaces only the α proton [22]. Other workers have reported that enzymatic transamination of L-alanine with pig heart glutamate-alanine aminotransferase in deuterium oxide completely labels both the α and β positions [23], but tryptophanase removes only the α hydrogen [24]. In non-enzymic model systems, pyridoxal phosphate and metal ions cause exchange of both α and β protons [25], but under relatively vigorous conditions. The situation is further complicated by the possibility that D-amino acid oxidases may catalyze the replacement of α hydrogen of the substrate enantiomers, the L-amino acids [26].

Since inductive and steric effects combine to make phenylalanine one of the most rapidly transaminating amino acids without an acid-base side chain [20c], and since the derived enamine **C** (R = C_6H_5) is stabilized by additional conjugation, the β hydrogen atoms should be especially labile in biological systems. *Kirby et al.* recently demonstrated extensive but non-obligatory loss of the 3-*pro*(*R*) hydrogen atom from both D (2*R*)- and L (2*S*)-phenylalanine during biosynthesis of gliotoxin by *Trichoderma viride* [18b]. In contrast to results with higher plants, they also observed stereoselective exchange before utilization of this amino acid in fungal protein, and suggested a rapid equilibrium with phenylpyruvic acid (**4**).

Our experiments show less net stereospecificity at C(3) and indicate that different mechanisms may be operating in the biosynthesis of cytochalasin D (**1**) from D- and L-phenylalanine. A number of trials with the (2*S*, 3*R*)- and (2*R*, 3*S*)-[3-^{3}H]- amino acids consistently gave tritium retentions of 43% and 24% respectively. Simultaneous incorporation of equal amounts of both enantiomers led to the expected 34% retention of hydrogen label. Examination of results with the racemized precursor, (2*RS*, 3*R*)- and (2*RS*, 3*S*)-[3-^{3}H]- phenylalanine (shown by phenylalanine ammonium lyase assay to be present in the ratio of 19% to 81% respectively), suggests that the (2*R*, 3*R*)- and (2*S*, 3*S*)-amino acids together account for 75% of the observed tritium

retention. Since transaminations occur stereospecifically at the 2-position of the amino acid [9a,h] [27], the participation of at least two enzymes with differing stereochemical requirements at the 3-position is reasonable. Some non-enzymatic β proton exchange in free phenylpyruvic acid (**4**) is possible, but is probably slow relative to the transamination process [28] and cannot be the exclusive mechanism because of the observed stereospecificity.

Two biosynthetic pathways are consistent with the data presented above. Path **A** depicts (2*S*)-phenylalanine as the actual precursor which is in rapid equilibrium with its enantiomer through the action of aminotransferases; in path **B** phenylpyruvic acid (**4**), derived directly from shikimic acid, is the primary precursor. If path **A** is



1 Cytochalasin D

favoured, large amounts of phenylpyruvic acid (**4**) should suppress the equilibrium between the D- and L-amino acids, or at least lead to preferential dilution of the D-enantiomer. In the second pathway **4** should dilute both antipodes equally since normally both are transformed into the antibiotic with equal efficiency.

Growing cultures (48 h old) of *Z. masonii* treated with (2*S*)-[4′-^{3}H]- and (2*RS*)-[2-^{14}C]-phenylalanine and radioinactive phenylpyruvic acid produced cytochalasin D (**1**) with an increased ^{3}H/^{14}C ratio, thus demonstrating that path **A** is probably the main biosynthetic route.

Although D-(2*R*)- and L-(2*S*)-phenylalanines are incorporated equally well into cytochalasin D (**1**), the naturally abundant L-isomer appears to be the actual primary precursor. Both enantiomers are in rapid equilibrium with phenylpyruvic acid (**4**) through the action of aminotransferases or amino acid oxidases. The stereochemistry of hydrogen loss from C(2) and C(3) of phenylalanine indicates that at least two different enzyme-dependent mechanisms are involved. Our results again emphasize the need for caution in the use of racemic precursors and for consideration of biochemical equilibria in biosynthetic studies.

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Experimental Part

1. *General Methods.* The general experimental procedure has been previously described [2]. Unless otherwise noted, *Zygosporium masonii* was fermented under the usual conditions [4] for 96 h on 1 liter scale, the precursors being added to the sterile medium prior to inoculation. Cytochalasin D (**1**) was isolated and recrystallized to constant specific activity in the reported fashion.

We thank Dr. *H. Lichti, Sandoz AG.*, Basel, for all mass spectral measurements, especially in connection with determination of ^{15}N content. We are indebted to Mr. *H. Galliker* and Mr. *G. Marbach, Sandoz AG.*, Basel, for radioactivity determinations.

Radioactive precursors were purchased from *Amersham Radiochemical Centre* (Buckinghamshire, England) and from *Radium Chemie* (9053 Teufen AR, Switzerland). Phenylalanine labelled with ^{15}N (97.3% isotopic purity) was obtained from *Prochem – British Oxygen Company Ltd.* (London).

2. *Phenylalanine Ammonium Lyase Assay.* Phenylalanine ammonium lyase was isolated from 2.0 kg of potato tubers and purified to the calcium phosphate gel stage as described by *Havir & Hanson* [12]. Spectrophotometric activity determination showed that a minimum of 11.7 units of enzyme were obtained, and that no change in specific activity was observed after 8 weeks of storage at $-20°$ in sodium borate buffer (0.1 M, pH 8.7). In a typical assay for stereochemical purity at the 3-position, a mixture of (2*S*)-[3-^{3}H, U-^{14}C]-phenylalanine (20.8 mg, 0.122 mmol), 110 ml sodium borate buffer (0.04 M, pH 8.7), and phenylalanine ammonium lyase (1.10 units) was stirred gently for 16 h at 23°. The solution was dialysed (*Union Carbide* dialysis tube, pore size 46,000) for 16 h at 4° against 1 l of sodium borate buffer (0.04 M, pH 8.7), and the process was repeated with fresh buffer. After the pH had been set to 9.0 with sodium hydroxide, the dialysates were concentrated *in vacuo* to a small volume, acidified with conc. hydrochloric acid to pH 1, and diluted with enough water to dissolve most of the boric acid. Extraction with ether (300 ml) for 24 h in a *Kutscher-Steudel* continuous extractor gave a heterogeneous extract which was filtered, dried, and carefully concentrated *in vacuo*. The residue was diluted with 9.5 mg of inactive cinnamic acid in 20 ml of ether, filtered, and concentrated again. Preparative TLC. on silica gel with ether allowed isolation of 15.7 mg of radioactive cinnamic acid which was recrystallized 3 times from ethanol/water 1:4. – M.p. 133–134°.

3. *Racemization of* (2S,3RS)-[*2,3-^{3}H, U-^{14}C*]-*Phenylalanine.* A 100 mg portion of a mixture of 1.0 mCi (20 Ci/mmol) of (2*S*,3*RS*)-[2,3-^{3}H]-, 50 μCi (522 mCi/mmol) of (2*S*)-[U-^{14}C]-, and 204 mg of inactive (2*S*)-phenylalanine was racemized with acetic anhydride by the method of *Battersby* [6]. The resulting mixture of (2*RS*,3*R*)- and (2*RS*,3*S*)-[3-^{3}H, U-^{14}C]-phenylalanines had spectral properties identical to inactive racemic phenylalanine, and retained 52% of the tritium of the starting material. Phenylalanine ammonium lyase assay showed that 81% of the tritium possessed the (3*S*)-configuration and 19% the (3*R*).

4. (2R,3S)- *and* (2S,3R)-[*3-^{3}H*]-*Phenylalanines.* A mixture of (2*R*,3*S*)- and (2*S*,3*R*)-N-benzoyl-[3-^{3}H]-phenylalanines was prepared by the 6 step synthesis described by *Battersby* [6] using 16 mCi of tritium oxide (16 mCi/ml). The chemical and radiochemical yields were identical at each stage, and the intermediates possessed the expected physical and spectral properties. Hydrolysis of the N-benzoyl derivatives in the reported fashion produced an equal mixture of (2*R*,3*S*)- and (2*S*,3*R*)-[3-^{3}H]-phenylalanines whose NMR. and IR. spectra matched those of authentic racemic phenylalanine.

5. (2R,3S)-[*3-^{3}H*]-*Phenylalanine.* Classical resolution of 2.22 g of the mixture of (2*R*,3*S*)- and (2*S*,3*R*)-N-benzoyl-[3-^{3}H]-phenylalanines with cinchonine by the method of *Fischer* [10] followed by hydrolysis as above allowed isolation of 134 mg of pure (2*R*,3*S*)-[3-^{3}H]-phenylalanine. This product had NMR. and IR. spectra identical to inactive D-phenylalanine and $[\alpha]_D^{25} = +35° \pm 2°$ ($c = 1.00$, water).

6. *N-Chloroacetylation of Phenylalanine.* The following procedure was used to prepare N-chloroacetyl-phenylalanine from the amino acid without racemization. A solution of 330 mg (2.0 mmol) of (2*S*)-phenylalanine in 20 ml water was cooled to 0° and treated alternately with small portions of chloroacetic anhydride (342 mg, 2.0 mmol) and sodium hydroxide solution (0.4 g in 10 ml water). After 30 min the solution was acidified with N HCl to pH 2.5 and extracted with ethyl acetate.

The dried extracts were concentrated *in vacuo*, and the residue was triturated twice with an equal volume of pentane to remove chloroacetic acid. Two recrystallizations from water produced 69 mg of pure (2*S*)-N-chloroacetyl-phenylalanine. M.p. 129–130° (Lit. [29]: 124–125°). – $[\alpha]_D^{25} = +49° \pm 2°$ ($c = 1.00$, ethanol) (Lit. [29]: $[\alpha]_D = +50.4°$ (ethanol)). – IR. (KBr): 3460; 2970; 1715; 1620; 1540; 1210; 730 cm^{-1}. – NMR. ($CDCl_3$): 7.29 (*m*, 5 H, phenyl); 6.73 (br. *s*, 2 H, NH and OH, exchanged with D_2O); 4.89 (*m*, 1H, C(2)); 4.04 (*s*, 2H, $-CH_2Cl$); 3.22 (*AB* of *ABX*, 2H, C(3), $J_{AB} = 15.5$, $J_{AX} = 6$, $J_{BX} = 7$ Hz) ppm.

7. (2S,3R)-[3-^{3}H]-*Phenylalanine*. A 986 mg (6.0 mmol) portion of the partially resolved (2*S*, 3*R*)-[3-^{3}H]-phenylalanine obtained by hydrolysis of the combined mother liquors of the cinchonine resolution was chloroacetylated without racemization as described above. A solution of 141 mg (0.59 mmol) of the N-chloroacetyl-phenylalanine in water (3.0 ml) was adjusted to pH 7.6 with dilute aqueous ammonia and mixed with 0.05 ml of carboxypeptidase A suspension from bovine pancreas (di-isopropyl-phosphorofluoridate treated; 2000 units/ml; *PL Biochemicals*, Milwaukee, Wisconsin). This mixture was kept at 37° for 24 h, 1 mg of activated charcoal was added, and the suspension was heated 2 min on a steambath before filtration through *Celite*. An equal volume of hot water was used to wash the precipitate, and the combined filtrates were acidified to pH 2.0 with N HCl and extracted with ethyl acetate. The dried extracts were concentrated *in vacuo* and the resulting residue was recrystallized from water to give 15 mg of pure (2*R*,3*S*)-N-chloroacetyl-[3-^{3}H]-phenylalanine which had spectral properties identical to those of its inactive enantiomer and $[\alpha]_D^{25} = -50° \pm 2°$ ($c = 1.00$, ethanol). The aqueous phase from the extraction was adjusted to pH 6.8 with dilute ammonia and concentrated *in vacuo*. Recrystallization of the residue from water yielded 79.8 mg of pure (2*S*,3*R*)-[3-^{3}H]-phenylalanine which had physical and spectral properties identical to inactive L-phenylalanine. Phenylalanine ammonium lyase assay demonstrated that optical purity in the 3-position was at least 97%. Because of the synthetic method employed, the (2*R*,3*S*)-enantiomer described above was thereby shown to have 97% of its tritium in the (3*S*)-position.

8. *Phenylpyruvic Acid*. Inactive phenylpyruvic acid (**4**) was most conveniently prepared by the method of *Herbst & Shemin* [30]. [4′-^{3}H, U-^{14}C]-Phenylpyruvic acid was synthesized by a modification of the procedure of *Meister* [14]. For the latter preparation, catalase from bovine liver (0.10 ml; 780,000 units/ml; *Serva*, Heidelberg, Germany) was diluted with water (15 ml) and dialysed 3 times against 1 l of water at 4° (*Union Carbide* dialysis tube, pore size 46,000) for a total of 16 h. *Crotalus atrox* venom (41.7 mg; *Serva*) was dissolved in water (7 ml) and dialysed similarly. A solution of 250 μCi (12 Ci/mmol) of (2*S*)-[4′-^{3}H]-, 50 μCi (522 mCi/mmol) of (2*S*)-[U-^{14}C]- and 330 mg (2.0 mmol) of inactive (2*S*)-phenylalanine in water (22 ml) was mixed with the dialysed catalase and snake venom. After the pH had been brought to 7.3 with N aqueous ammonia, the mixture was kept at 37° and oxygen was gently bubbled through it for 15 h. Butanol was occasionally added to control foaming. The reaction mixture was dialysed three times against 1 l of water at 4° (16 h), and the combined dialysates were concentrated *in vacuo* below 50°. The residue was dissolved in water (75 ml) and ether (100 ml), acidified with conc. hydrochloric acid to pH 1, and extracted with ether (300 ml). Concentration of the extracts *in vacuo* yielded 326 mg of [4′-^{3}H, U-^{14}C]-phenylpyruvic acid which could be further purified by rapid recrystallization from boiling benzene. This material had m.p. 148–151° (Lit. [30]: 150–154°, varies with rate of heating) and spectral properties identical with those of authentic inactive material.

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