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SYNTHESIS OF SPECIFICALLY LABELLED L-PHENYLALANINES USING PHENYLALANINE AMMONIA LYASE ACTIVITY.

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SUMMARY

Specifically labelled L-phenylalanines have been prepared using a variety of classical synthetic methods in combination with phenylalanine ammonia lyase (PAL) enzyme activity of the yeast Rhodosporidium toruloides ATCC 10788 or Rhodotorula glutinis IFO 0559, respectively. Thus, L-[2-^{2}H]phenyl-[2-^{2}H]alanine (5) was formed from (E)-[2,2'-$^{2}H_2$]cinnamic acid and ammonia in 46% yield, whereas L-phenyl-[2-^{13}C,^{15}N]alanine (9) was obtained from (E)-[2-^{13}C]cinnamic acid in 45% overall yield using an 8.3-fold excess of $^{15}NH_4Cl$ and repeating the reaction after recovery of unchanged labelled material. Generally, labelled cinnamic acids were recovered in pure form from the reaction mixture, with a loss of 6-8%. Likewise, unchanged $^{15}NH_3$ was reisolated as $^{15}NH_4Cl$ after steam distillation with overall losses of less than 4%. Labelled cinnamic acids were prepared by Knoevenagel condensations between appropriately labelled benzaldehydes and malonic acids. [2-^{2}H]Benzaldehyde was obtained from 2-bromotoluene by decomposition of the corresponding Grignard reagent with $^{2}H_2O$ and subsequent oxidation. Since simple molecules, most of them commercially available in labelled form or otherwise easily accessible, may serve as starting material, and due to its defined stereochemistry, the reaction catalysed by PAL opens a short and attractive route to specifically labelled L-phenylalanines.

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INTRODUCTION

Among the well known syntheses of α-amino acids [1], [2] both the classical and many of the recently developed routes lead to racemates. For most purposes however, optically pure enantiomers are required, with one of the simplest ways to attain this goal being the resolution of racemates. Since thereby half of the material is lost, this approach, though well established, is often not favoured. Accordingly, considerable effort has been made to find asymmetric syntheses of α-amino acids. Some methods of practical importance so found avoid the use of enzymes and include, as a key step, the stereospecific reduction of a double bond. This may be either a C=C bond of an acylamidoacrylic acid, in which case asymmetry is introduced by chiral hydrogenation catalysts, or a C=N bond generated from the reaction of an α-oxo carboxylic acid with a chiral amine. Furthermore, purified enzymes as well as intact microorganisms provide efficient routes to certain α-amino acid enantiomers from simple optically inactive precursors with high optical yields. Examples include the synthesis of L-aspartic acid from fumaric acid [3], of L-lysine from DL-α-aminocaprolactam [4] and of the aromatic amino acids as well as L-methionine from the corresponding α-oxo carboxylic acids [5], [6], [7].

With respect to the introduction of isotopic labels into phenylalanine enantiomers some of these methods have been successfully applied. Thus, L-[3,4-$^{13}C_2$]phenyl-[1-^{13}C]alanine, among other specifically labelled L-amino acids, has been synthesized from DL-[1-^{13}C]lactate, using a particular strain of Escherichia coli for

the fermentation [8]. L-phenyl-[2,3,3-$^{2}H_3$]alanine has been prepared with an enantiomeric excess (e.e.) of 40% by a Zn^{2+}-catalysed transamination reaction between a chiral pyridoxamine analog and phenylpyruvic acid in Me$O^{2}H$ [9]. A short synthesis of L-phenyl-[3-^{11}C]alanine from $^{11}CO_2$ via phenyl-[3-^{11}C]pyruvic acid, using an aminotransferase for the introduction of the amino group, has been reported [10]. A variety of differently labelled L-tyrosines and L-phenylalanines have been prepared by Walker et al., using a combination of chemical and microbiological techniques [11]. The key step in this approach is the formation of L-tyrosine from phenol and L-serine (or a combination of pyruvate and $(NH_4)_2SO_4$ instead) by Erwinia herbicola cells containing β-tyrosinase activity. Furthermore, there exist special methods for the labelling of unique sites in phenylalanine. D-Phenyl-[2,3-$^{2}H_2$]alanine with (3R)-configuration may be obtained with a high chiral induction through catalytic reduction of cyclo[(Z)-ΔPhe-D-Ala] with $^{2}H_2$ over Pd/C followed by hydrolysis [12]. For the introduction of ^{2}H at the α-position of amino acids, a general procedure starting from the N-acetyl derivatives has been presented by Schowen and Fujihara, involving an enzymatic resolution step. Thus, L-phenyl-[2-^{2}H]alanine was prepared from N-acetyl-DL-phenylalanine and $^{2}H_2O$ in good yield with a greater than 97% deuteration [13].

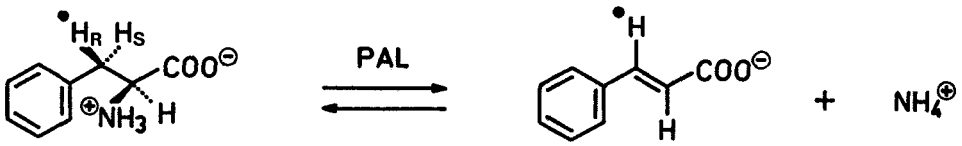
However, the generation of certain crucial labelling patterns in L-phenylalanine still remains a problem. In this paper we present an approach to labelled L-phenylalanines making use of a regio- and stereospecific addition of NH_3 to (E)-cinnamic acid by phenylalanine ammonia lyase. Since cinnamic acid itself is readily available from benzaldehyde and malonic acid [14], this concept allows for the specific introduction of labels to essentially any desired positions in L-phenylalanine. To show the versatility of

our approach, two representative syntheses of doubly labelled L-phenylalanines are described. In both cases interest is focused on the α-position of the amino acid since the fate of labels at this position is important in many biological transformations. First the synthesis of an α-deuterated species is shown, containing an additional reference ^{2}H-label on the aromatic ring to make ^{2}H-NMR analysis possible. The second example illustrates the addition of $^{15}NH_3$ to (E)-[2-^{13}C]cinnamic acid yielding L-phenyl-[2-^{13}C,^{15}N]-alanine.

Results

Phenylalanine Ammonia Lyase (PAL)

Phenylalanine ammonia lyase (PAL, EC 4.3.1.5) catalyses the elimination of ammonia and the pro-3S hydrogen from L-phenylalanine to form (E)-cinnamate (Scheme 1). The enzyme widely occurs in higher plants [15], but is also found in yeasts as well as some other microorganisms [16]. Preparations from these sources often exhibit tyrosine ammonia lyase activity as well. The normal metabolic role of PAL is the conversion of phenylalanine to cinnamate, but through careful choice of conditions the enzyme may be forced to favour the reversed direction. In view of the commercial production of L-phenylalanine S. Yamada et al. [17] investigated the induction and stabilization of PAL activity in the yeast Rhodotorula glutinis during cultivation, as well as the optimum



L-phenylalanine (E)-cinnamate

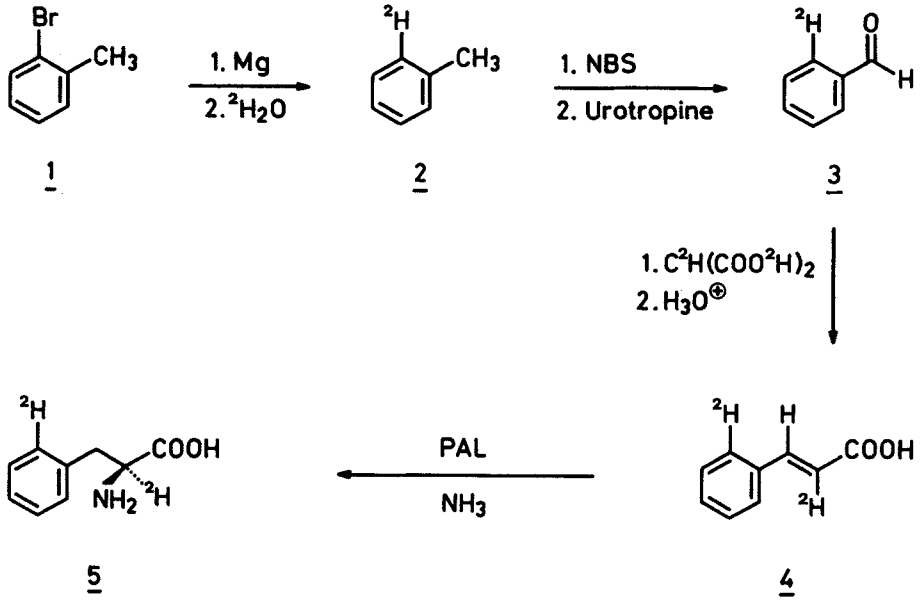
Scheme 1

conditions for the conversion of cinnamic acid into L-phenylalanine. Good induction of PAL activity was attained in glucose-free media containing yeast extract, peptone and L-phenylalanine. Addition of L-isoleucine proved to be useful for maintaining PAL activity after growth had reached its stationary phase. The conversion yield from cinnamic acid was reported to reach 70% using whole cells as catalyst and carrying out the reaction at pH 10.0 with initial concentrations of 150 mM for cinnamic acid and 7.5 M for ammonia, respectively.

For our purpose the appropriate yeast was obtained from the American Type Culture Collection as Rhodosporidium toruloides ATCC 10788, which is synonymous to Rhodotorula glutinis ATCC 15385, and from the Institute for Fermentation, Osaka, Japan, as Rhodotorula glutinis IFO 0559. When subjected to the conditions proposed by Yamada et al. [17], both strains produced similar amounts of L-phenylalanine from cinnamic acid, yet conversion yield never exceeded 50%. This was, however, not considered a serious disadvantage, since both cinnamic acid and ammonia may easily be recovered from the reaction mixture before isolation of L-phenylalanine.

L-[2-^{2}H]Phenyl-[2-^{2}H]alanine

Our synthesis of the doubly deuterated L-phenylalanine species 5 proceeded via the key intermediate (E)-[2,2'-$^2H_2$]cinnamic acid (4) and is shown in Scheme 2. Starting with 2-bromotoluene (1), an aromatic ^{2}H substituent was introduced by hydrolysis of the corresponding Grignard reagent with ^2H_2O. As previously described [18], [19], deuteration obtained by this method was not complete, although highly enriched ^2H_2O had been used. We assume, that part of the Grignard reagent is initially trapped while MgBrO^{2}H



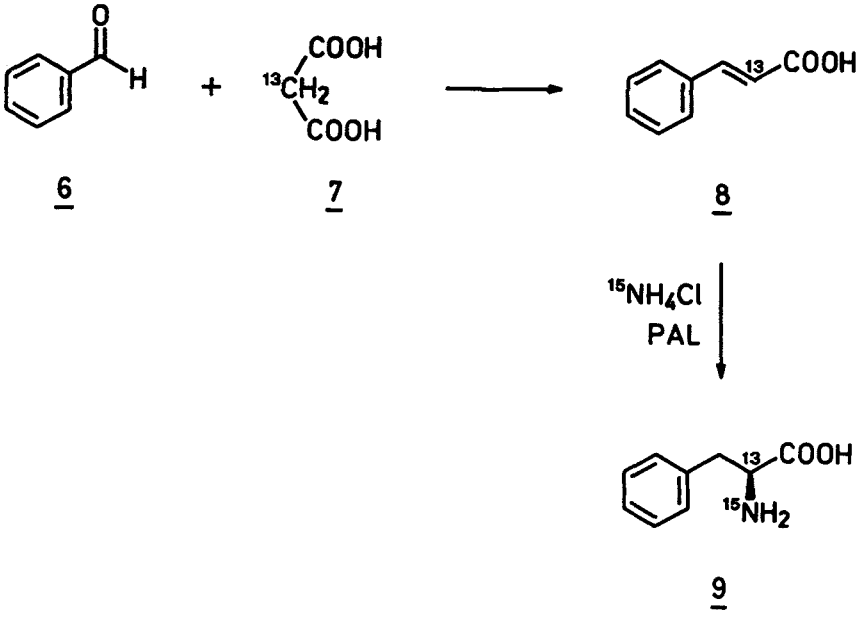
Scheme 2

precipitates and decomposed by H_2O only during subsequent acidic work-up. [2-^{2}H]Toluene (2) was then converted to [2-^{2}H]benzaldehyde (3) as described by Coombe and Poulton [19]. The intermediate, [2-^{2}H]benzylbromide, generated by N-bromosuccinimide (NBS) was not isolated, but directly oxidized in a Sommelet reaction [20] with hexamethylenetetramine (urotropine). A Knoevenagel type condensation with [$^2H_4$]malonic acid then led, after aqueous work-up, to the bideuterated cinnamic acid 4 which was finally converted to L-[2-^{2}H]phenyl-[2-^{2}H]alanine (5) by PAL. The yield in this step was 46%, whereas 48% of [2,2'-$^2H_2$]cinnamic acid (4) could be recovered, with only 6% of the labelled material being lost.

L-Phenyl-[2-^{13}C, ^{15}N]alanine

Racemic phenyl-[2-^{13}C,^{15}N]alanine has previously been synthesized, following classical procedures, from commercially available

[2-^{13}C,^{15}N]glycine in an overall yield of 35% by Mohr and Tamm [21]. Using PAL, the L-enantiomer 9 may be obtained in only two steps from benzaldehyde (6), [2-^{13}C]malonic acid (7) and $^{15}NH_4Cl$ (Scheme 3). In this case the optimum conditions for the PAL reaction (Yamada et al. [17]) could not be maintained. Instead, the entire amount of ammonia was introduced as $^{15}NH_4Cl$ using an 8.3-fold excess with respect to (E)-[2-^{13}C]cinnamic acid (8), and NaOH was added to reach pH 10. As a consequence, the final concentration of ammonia had to be lowered to approximately 1 M. In addition to the recovery of labelled cinnamic acid, a convenient method for the reisolation of excess $^{15}NH_4Cl$ was needed. For this purpose a Parnas-Wagner



Scheme 3

apparatus, originally designed for steam distillation of ammonia in nitrogen determination [22], proved to be useful. Slight modifications had to be installed in order to fit the apparatus to the handling of preparative amounts and to prevent frothing of the

reaction mixture into the condenser. Once the PAL reaction was finished, the entire reaction mixture was transferred into the distillation apparatus and a slight excess of aqueous NaOH was added to liberate unchanged $^{15}NH_3$ which was steam distilled and trapped with 2N HCl. The remaining suspension was then ready for

Table 1. Conversion of (E)-[2-^{13}C]cinnamic acid (8) and $^{15}NH_4Cl$ to L-phenyl-[2-^{13}C,^{15}N]alanine (9) by PAL, as described in the text.

First batch	^{13}C	^{15}N
Amounts used[a]	4.34 mmol	36.0 mmol
Yield[b]	29%	3.5%
Recovered[a]	63%	92.8%
Lost	8%	3.7%
Second batch with recovered material		
Amounts used[a]	2.73 mmol	33.4 mmol
Yield[b]	25%	2.1%
Recovered[a]	71%	94.6%
Lost	4%	3.3%
Overall balance		
Amounts used[a]	4.34 mmol	36.0 mmol
Yield[b]	45%	5.4%
Recovered[a]	45%	87.8%
Lost	10%	6.8%

a) as (E)-[2-^{13}C]cinnamic acid or $^{15}NH_4Cl$, respectively.
b) as L-phenyl-[2-^{13}C,^{15}N]alanine.

normal work-up, i.e. centrifugation to remove the cells, followed by acidification and filtration or extraction to recover labelled cinnamic acid, and finally ion exchange chromatography to isolate L-phenyl-[2-^{13}C,^{15}N]alanine. Recovered cinnamic acid was shown to be pure by TLC and ^{1}H-NMR spectroscopy and could thus be reused as more labelled L-phenylalanine was required. Table 1 shows the balance of the PAL reaction with respect to the isotope labels ^{13}C and ^{15}N.

Discussion

As illustrated by the examples above the regio- and stereospecific addition of ammonia to (E)-cinnamic acid by PAL opens a short route to specifically labelled L-phenylalanines. Since simple molecules serve as starting material, the generation of almost any desired labelling pattern is possible using relatively inexpensive commercially available precursors. In cases where L-phenyl-[1-^{13}C] alanine or related species are desired, it is advisable to prepare the corresponding (E)-cinnamic acid from benzaldehyde and appropriately labelled bromoacetic acid ethyl ester in a Wittig reaction [23] in order to avoid loss of isotopic label by decarboxylation.

However, the predominant application of the PAL reaction will be, due to its defined stereochemistry (cf. Scheme 1), the synthesis of L-phenylalanines specifically labelled at C(2) and/or C(3). Thus, besides the labelling of the α-hydrogen or the amino group as demonstrated here the pro-3R hydrogen may be labelled when starting with [α-^{2}H]benzaldehyde. On the other hand, a (3S)-configuration may be obtained when carrying out the reaction in $^{2}H_2O$ or $^{3}H_2O$.

Experimental Part

Materials. PAL-containing yeast strains were obtained from the American Type Culture Collection (Rhodosporidium toruloides ATCC 10788) and from the Institute for Fermentation, Osaka, Japan (Rhodotorula glutinis IFO 0559). $^{2}H_2O$ (99.8 atom % ^{2}H) and $C^{2}H(COO^{2}H)_2$ (99.5 atom % ^{2}H) were purchased from Ciba-Geigy Ltd., Basel. [2-^{13}C]Malonic acid (91.0 atom % ^{13}C) and $^{15}NH_4Cl$ (97.3 atom % ^{15}N) were obtained from Amersham. Yeast extract, malt extract and peptone were purchased from Difco Laboratories. The composition of the culture media is given in Table 2.

Table 2. Ingredients (per 1000 ml H_2O) of culture media.

Medium	A	B	C	D
Yeast extract	3 g	10 g	10 g	10 g
Bacto peptone	5 g	10 g	10 g	10 g
Malt extract	3 g	-	-	-
D-Glucose	10 g	-	-	-
NaCl	-	5 g	5 g	5 g
L-Phenylalanine	-	0.5 g	0.5 g	0.5 g
L-Isoleucine	-	-	-	5 g
Agar	-	15 g	-	-
pH[a)]	-	6.0	6.0	6.0

a) to be adjusted before sterilization.

Methods. Freeze-dried yeast cultures were rehydrated in 6 ml portions of medium A and grown for 32 h at 27° with shaking. From these broths, stock culturing was started by inoculating agar slants (3 ml of medium B in disposable 5 ml-tubes) and incubating them for at least 5 days. Well grown stock cultures were stored at 4° and organisms transferred to fresh slants every 2 months. Additional agar slants were inoculated from stock cultures and incubated as above to provide inoculation material for cell preparations. 1 ml of medium C was used to transfer cells from an agar slant to 50 ml of medium C in a 300 ml Erlenmeyer flask. This

seed culture was incubated at 27° for 24 h on a rotary shaker (250 rpm) and then used, in portions of 2 ml, to inoculate the main cultures, i.e. 200 ml batches of medium D contained in 500 ml Erlenmeyer flasks. These flasks were incubated in the same manner for 20 h. The following manipulations do not need to be carried out aseptically. Cells were harvested by centrifugation for 15 min. at 900 * g and washed once with 40 volumes of 0.9% saline. From 100 ml of main culture, usually 2-3 ml of packed cells could be obtained. Packed cells were diluted by a factor of 1.1 with 0.9% saline and used immediately as a source of PAL activity.

Phenylalanine preparations were assayed for phenylalanine enantiomers using a WCOT fused silica Chirasil-L-Val capillary GC-column (25 m, 0.22 mm i.d., film thickness 0.13 µm) from Chrompack. Prior to analysis, 0.8 ml of 2.1 M HCl in isopropanol was added to the dry sample (ca. 1 mg) and the mixture was heated in a sealed tube at 110° for 1 h. After cooling to r.t., excess reagent was removed under a gentle stream of nitrogen. 300 µl ethylacetate followed by 50 µl pentafluoropropionic anhydride were added, and the mixture was again heated to 110° for 10 min., cooled to r.t., concentrated to dryness and dissolved in 300 µl of methylene chloride for GC analysis. In all cases, D-phenylalanine, if any, was found to be present in an amount of less than 1.5% with respect to total phenylalanine content (i.e., e.e. greater than 97%). Sample purity was determined similary by internal standardization using DL-phenylalanine.

NMR-spectra were measured on a Varian EM-390 spectrometer (^{1}H, 90 MHz, c.w.) and on a Bruker WH 90 instrument (^{1}H, 90 MHz; ^{13}C, 22.63 MHz; Fourier transform). We are indebted to Prof. Dr. H. Günther, Siegen, BRD, for running the ^{2}H-NMR spectrum. Chemical shifts were measured by reference to tetramethylsilane (TMS), to Dioxane (67.37 ppm, ^{13}C-NMR-spectra in DCl/D_2O) and to CH_3CN (2.00 ppm, ^{1}H-NMR-spectrum in DCl/D_2O), and are reported in ppm relative to TMS. Mass spectra were recorded with a VG 70-250 instrument using e.i. ionization at 70 eV.

[2-^{2}H]Toluene (2) [19]. 137 g (0.8 mol) of 2-bromotoluene (1) was converted into the corresponding Grignard reagent in the usual manner. Decomposition with 32 g (1.6 mol) ^2H_2O, followed by the usual work-up procedure for Grignard reactions gave ca. 750 ml of

an ethereal extract, which was fractionally distilled under atmospheric pressure yielding 57.9 g (0.622 mol, 78%) [2-^{2}H]toluene (2), b.p. 103-104°/731 Torr. ^{1}H-NMR (90 MHz, c.w., CCl_4): 2.3 (s, 3H, CH_3); 7.1 (m, 4.18 ± 0.11 H, aromatic). MS: 93 (M^+); 92 ($C_7H_6{}^2H^+$); 91 ($C_7H_7^+$).

[2-^{2}H]Benzaldehyde (3). 40.0 g (0.430 mol) of 2 was converted into [2-^{2}H]benzaldehyde (3) as previously described [19]. Distillation of the crude product (54.3 g) over a short Vigreux column gave 36.9 g (0.345 mol, 80%) of 3, b.p. 60-62°/14 Torr. ^{1}H-NMR (90 MHz, c.w., CCl_4): 7.3-7.9 (m, 4.12 ± 0.1 H, aromatic); 9.9 (1H, CHO). MS: 108 (MH^+); 107 (M^+); 106 (M^+-H); 78; 52; 51.

(E)-[2,2'-$^2H_2$]Cinnamic acid (4). A mixture of 5.4 g (50 mmol) of freshly prepared 3, 5.4 g (50 mmol) of [$^2H_4$]malonic acid and 0.5 ml piperidine in 5 g of dry pyridine was refluxed for 2 h, cooled to r.t., acidified (3N HCl) and extracted with ether. The product was extracted into 2 N Na_2CO_3 and again, after acidification (6N HCl), into ether giving 6.6 g crude 4, which was recrystallized from EtOH/H_2O yielding 6.3 g (42 mmol, 84%) of pure 4, m.p. 133-135°. Calc. C 71.99%, found 71.81%. ^{1}H-NMR (90 MHz, F.t., $(CD_3)_2CO$): 6.54 (d, J=16, 1.1% ± 0.5% of total H-content, H-C(2)); 7.3 - 7.9 (m, aromatic and H-C(3)). ^{13}C-NMR (22.63 MHz, $(CD_3)_2CO$, proton noise decoupled): 118.8 (t, $^1J(^{13}C,^2H)$ = 24.6, C(2)); 128.6 (t, $^1J(^{13}C,^2H)$ = 24.3, C(2')); 128.9(C(6')); 129.7(C(3'), C(5')); 131.0(C(4')); 135.3(C(1')); 145.6(C(3)); 168.3(C(1)). MS: 150(M^+); 149(M^+-H); 133(M^+-OH); 122(M^+-CO); 105(M^+-COOH); 93; 92; 78; 52.

L-[2-^{2}H]Phenyl-[2-^{2}H]alanine (5). To a solution of 6.00 g (112 mmol) NH_4Cl and 1.78 g (11.9 mmol) of 4 in 25 ml aqueous ammonia (25%) and 35 ml H_2O (pH = 10) 16 ml of a yeast cell suspension were added. The mixture was incubated at 27° for 27 h with shaking (120 rpm). The cells were then removed by centrifugation (15 min., 900 * g) and washed twice with 25 ml H_2O. The combined solutions were filtered (0.7 µm), slightly concentrated in vacuo, acidified to pH 1.7 with 6N HCl and filtered to give 0.85 g (5.67 mmol, 48%) of unchanged 4 as a colourless solid (pure by TLC (benzene/EtOH) and ^{1}H-NMR). The filtrate was loaded onto a column (10 * 3 cm) of Amberlite IR-120 (H^+-form). The column was washed with 2 l of H_2O and then eluted with 0.3 N aqueous NH_3. Phenylalanine-positive fractions were combined (TLC: n-BuOH/AcOH/

H_2O (4:1:1, v/v), detection with ninhydrin soln.) and the solvent was removed in vacuo. The residue (1.1 g) was dissolved in 21 ml of 25% aqueous NH_3 and, after addition of 20 ml EtOH, the solution was distilled at atmospheric pressure, the volume distilled off being continously replaced with EtOH (75 ml). After cooling and filtering, 624 mg of 5 were obtained (purity 97.2%, 3.63 mmol). ^{1}H-NMR (90 MHz, F.t., DCl/D_2O): 3.0-3.5 (dd, 2H, H-C(3)); 7.3 (m, 4.16 ± 0.1 H, aromatic). ^{13}C-NMR (22.63 MHz, DCl/D_2O, proton noise decoupled): 36.2 (C(3)); 128.8 (C(4')); 130.0 (C(3'), C(5')); 130.2 (C(6')); 134.6 (C(1')); 171.8 (C(1)); the signals for C(2) and C(2') (t) were both too weak to be detected. ^{2}H-NMR (61.43 MHz, EtOH/C_6F_6 (2:1)): 7.3 (^{2}H-C(2')); 3.7 (^{2}H-C(2)). MS: 167 (M^+); 122 (M^+-COOH); 92 ($C_7H_6{}^2H^+$); 75 (NH_2-C^2H-$COOH^+$, base peak). The filtrate was concentrated and freeze-dried yielding 465 mg of a pale yellow solid which contained 67.0% (1.86 mmol) of 5. Hence, the total yield of 5 was 5.49 mmol (46%). Taking into account all available spectral data of the compounds 2 - 5 (NMR, MS), the ^{2}H-enrichments at the aromatic and the α-position of 5 are estimated to be 84% and 96%, respectively, with an error of ca. 2%.

(E)-[2-^{13}C]Cinnamic acid (8). 0.76 g (7.16 mmol) benzaldehyde and 0.50 g (4.76 mmol) of [2-^{13}C]malonic acid were converted to 0.650 g (4.36 mmol, 92%) of crude 8 as described for the preparation of 4. 8 was used for the synthesis of 9 without further purification. ^{1}H-NMR (90 MHz, F.t., $(CD_3)_2CO$): 6.54 (dd, $^3J(^1H, ^1H)$ = 16, $^1J(^1H, ^{13}C)$ = 159, H-^{13}C(2)); 6.54 (d, J=16, 0.094 H, H-^{12}C(2)); 7.3-7.9 (m, aromatic and H-C(3)). ^{13}C-NMR (22.63 MHz, $(CD_3)_2CO$, proton noise decoupled): 119.2 (C(2), ca. 80-fold increased; $^1J(^{13}C(2), ^{13}C(1))$ = 86, $^1J(^{13}C(2), ^{13}C(3))$ = 73); 129.0 (d, $^3J(^{13}C, ^{13}C)$ = 5, C(2'), C(6')); 129.8 (C(3'), C(5')); 131.1 (C(4')); 135.5 (decreased, C(1')); 145.8 (d, $^1J(^{13}C, ^{13}C)$ = 73, C(3)); the signal for C(1) (d) could not be detected. MS: 149 (M^+); 148 (M^+-H); 132 (M^+-OH); 121 (M^+-CO); 104 (M^+-COOH); 92; 91; 78; 77; 51.

L-Phenyl-[2-^{13}C, ^{15}N]alanine (9). 648 mg (4.34 mmol) of 8 and 1.96 g (36.0 mmol) $^{15}NH_4Cl$ were placed in a 40 ml 3-necked flask equipped with a dropping funnel, a gas inlet tube, a magnetic stirrer and an U-shaped hose filled with liquid paraffin. 1.15 g (28.7 mmol) NaOH-pellets and (immediately) 25 ml H_2O were added

with stirring. No $^{15}NH_3$-gas was allowed to escape. To the clear solution 9.5 ml of yeast cell suspension were added (final volume 37.5 ml), and the mixture was stirred at 22° for 37.5 h. At the end of this period a gentle stream of air was sucked through the suspension during 1 h and introduced into a series of 6 gas absorption traps each filled with 50 ml 2 N HCl (prepared from distilled 6 N HCl).For the recovery of excess $^{15}NH_3$ a Parnas-Wagner apparatus [22] was used, with the following modifications. An immersion coil with adjustable power supply was available for heating. Between the distilling flask and the condenser 3 additional Reitmayer traps were inserted, and the outlet of the condenser was connected to the series of HCl-traps mentioned above and a low pressure valve, respectively, by means of a T-shaped tube. The low pressure valve consisted of a sealed suction flask equipped with a long vertical tube dipping below the surface of liquid paraffin. The reaction mixture was transferred to the distillation flask and steam distilled after addition of 8.0 ml 2 N NaOH (16 mmol). The combined solutions from the absorption traps were concentrated and freeze-dried giving 1.82 g (33.4 mmol) of dry $^{15}NH_4Cl$. The residue was freed of cells by centrifugation (15 min., 900 * g) and washed twice with 70 ml of H_2O. The combined solutions were filtered (0.7 µm), concentrated to ca. 50 ml, acidified to pH 1.8 (6 N HCl) and extracted with ether. 407 mg (2.73 mmol) of unchanged 8 were obtained after treatment of the ether extract as described for the preparation of 4 (without recrystallization). Chromatography of the aqueous layer on Amberlite IR-120 as described for the preparation of 5 and lyophilization of the fractions containing phenylalanine gave 335 mg of 9 (purity 62.9%, 1.26 mmol, yield 29%).

Recovered 8 (407 mg, 2.73 mmol) and $^{15}NH_4Cl$ (1.82 g, 33.4 mmol) were converted to 9 in the same manner yielding 198 mg of 9 (purity 57.9%, 0.685 mmol, yield 25%). This time, 1.72 g (31.6 mmol) of $^{15}NH_4Cl$ and 290 mg (1.94 mmol) of 8 were reisolated. Hence, a total of 1.95 mmol (45%) of 9 was obtained from 4.34 mmol of 8 and 36.0 mmol $^{15}NH_4Cl$, 1.94 mmol (45%) of 8 and 31.6 mmol (87.8%) $^{15}NH_4Cl$ being recovered.

^{13}C-NMR (22.63 MHz, DCl/D_2O, proton noise decoupled): 36.3 (d, ^1J = 32, C(3)); 54.8 (d, $^1J(^{13}C, ^{15}N)$ = 7, C(2), ca. 80-fold increased); 128.8 (C(4')); 130.0 (C(3'), C(5')); 130.3 (d, ^3J = 1.5, C(2'), C(6')); 134.6 (d, ^2J = 2, C(1')); 171.7 (d, ^1J = 60, C(1)).

MS: 167 (M^+); 122 (M^+-COOH); 104; 91; 76 ($^{15}NH_2$-^{13}CH-$COOH^+$, base peak); detailed comparison between the signals around m/z 122 and those of the unlabelled compound at m/z 120 showed the isotopomers to be distributed as follows (the values in brackets are those calculated from the isotopic purity of the starting materials): unlabelled 0.9 ± 0.9% (0.24%); singly labelled 10.6 ± 0.9% (11.2%); doubly labelled 88.5 ± 1.8% (88.5%).

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