An Efficient Chemomicrobiological Synthesis of Stable Isotope-Labeled L-Tyrosine and L-Phenylalanine

T. E. Walker* and C. Matheny

Los Alamos National Laboratory, University of California, Los Alamos, New Mexico 87545

C. B. Storm*† and H. Hayden

Department of Chemistry, Howard University, Washington, D.C. 20059

Received August 6, 1985

L-Tyrosine specifically labeled with ^{2}H, ^{13}C, ^{18}O, or ^{15}N has been synthesized by using a combination of organic synthetic methods and the β-tyrosinase enzyme activity of the bacterium *Erwinia herbicola*. The following L-tyrosine isotopomers were prepared: L-[4′-^{13}C]tyrosine from [1-^{13}C]phenol and L-serine, L-[3′,5′-$^{13}C_2$]tyrosine from [2,6-$^{13}C_2$]phenol and L-serine, L-[4′-^{18}O]tyrosine from [^{18}O]phenol and L-serine, L-[^{15}N]tyrosine from [^{15}N]ammonium sulfate, phenol, and pyruvate, and L-[2′,3′,5′,6′-$^{2}H_4$]tyrosine from [U-$^{2}H_6$]phenol and L-serine. The β-tyrosinase activity was also used to prepare 2′-fluoro-L-tyrosine and 3′-fluoro-L-tyrosine from 3-fluorophenol and 2-fluorophenol, respectively. Phenol enriched with ^{13}C was prepared by the condensation of [^{13}C]acetone with nitromalonaldehyde, reduction of the resulting *p*-nitrophenol to *p*-aminophenol, and reductive removal of the nitrogen from the diazonium salt to form either [1-^{13}C]- or [2,6-$^{13}C_2$]phenol in a 40% overall yield from acetone. The yields of L-[^{13}C]tyrosine were typically around 90% from labeled phenol. Labeled L-phenylalanine was chemically prepared from L-tyrosine in a 75% overall yield. This was deemed the best approach to labeled L-phenylalanine, given the efficient method for preparing L-tyrosine from phenol. The approach to labeled L-phenylalanine represents a unique combination of chemical synthesis (phenol), biosynthesis (L-tyrosine), and finally chemical synthesis (L-phenylalanine). The chirality is introduced by the biochemical step, obviating the need for elaborate and inherently inefficient chiral manipulations.

Stable isotope-labeled amino acids, peptides, and proteins are required for a variety of biological, chemical, and physical studies related to the metabolism, structure, and dynamics of these important materials. These stable isotope substitutions are useful in a variety of experimental strategies using nuclear magnetic, electron-spin, Raman, and mass spectroscopies. Particularly in studies involving human metabolism it is highly desirable, if not absolutely necessary, to have appropriate stable isotope-labeled materials available for metabolic and diagnostic studies.[1] These studies are generally limited by the high cost and lack of availability of appropriately labeled compounds.

L-Phenylalanine and L-tyrosine are important in a variety of biological functions. Their metabolism is important in neurochemistry; they are widely distributed among proteins and play an important role in structure–function relationships and they occur in many peptide hormones. An error in the metabolism of phenylalanine leads to one of the more common genetic diseases, phenylketonuria.

L-Tyrosine and L-phenylalanine have been prepared by several different routes labeled in a variety of positions with ^{13}C or ^{2}H,[2-7] although none of them are particularly easy to scale-up. For ring ^{13}C label, Hruby et al.[3,4] have prepared D,L-tyrosine via *p*-nitrophenol, followed by resolution of the trifluoroacetyl derivatives with carboxypeptidase A. This route requires 10 steps and gives a 9.2% yield of the L form from labeled acetone as starting material. L-Phenylalanine was produced[3] by reductive removal of the phenolic hydroxyl.

Moore[5] prepared ring-labeled D,L-phenylalanine from acetate by way of benzyl bromide, using barium carbonate as the source of label. L-Tyrosine was obtained by the hydroxylation of D,L-phenylalanine using phenylalanine hydroxylase. The preparation of L-[1′-^{13}C]tyrosine required only five steps, but the yield from acetate was less than 4%. ^{2}H has been incorporated in both side chain and ring positions by use of appropriately deuterated precursors.[2,6,7]

We report here the preparation of L-tyrosine and L-phenylalanine variously labeled with ^{13}C, ^{2}H, ^{18}O, and ^{15}N by efficient chemomicrobiological synthetic methods suitable for large- or small-scale preparations. The approach used (Scheme I) is to feed isotopically enriched, chemically prepared phenol to *Erwinia herbicola*, which incorporates it into L-tyrosine in a yield of ca. 90%. The bacteria are easy to grow, the reaction is easily carried out, and the whole system can be readily scaled-up.

Results and Discussion

Acetic acid enriched with ^{13}C (99+ atom %) was used as the starting material for the synthesis of [^{13}C]phenol. The acetic acid was converted to its barium salt and [^{13}C]acetone prepared by pyrolysis of the labeled barium acetate.[8] [1-^{13}C]Acetate gives carbonyl-labeled acetone; [2-^{13}C]acetate gives [1,3-$^{13}C_2$]acetone. The labeled acetone was condensed with sodium nitromalonaldehyde under basic conditions to give *p*-nitrophenol.[3,8,9] We found that 6 days in the cold followed by acidification and ether extraction produced the same yield as previously reported.

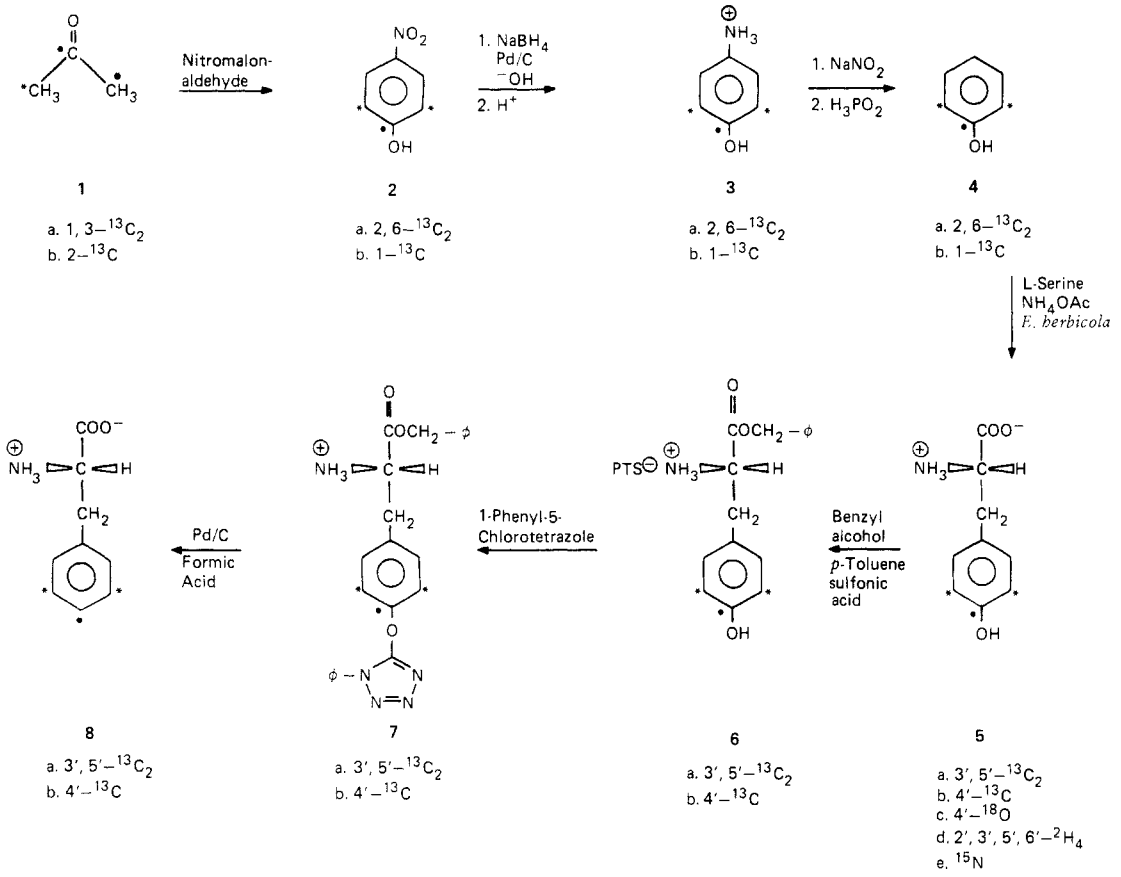
The *p*-nitrophenol may then be converted to phenol without the isolation of any intermediates. The reactions with ^{13}C labeled material can be followed easily on small aliquots by ^{13}C NMR. The reduction of *p*-nitrophenol to *p*-aminophenol by sodium borohydride and 5% Pd/C

† Present address: Los Alamos National Laboratory.

(1) Baillie, T. A. *Pharmacol. Rev.* **1981**, *33*, 81.
(2) Yamamoto, D. M.; Upson, D. A.; Linn, D. K.; Hruby, V. J. *J. Am. Chem. Soc.* **1977**, *99*, 1564.
(3) Viswanatha, V.; Hruby, V. J. *J. Org. Chem.* **1979**, *44*, 2892.
(4) Viswanatha, V.; Hruby, V. J. *J. Org. Chem.* **1980**, *45*, 2010.
(5) Moore, A. C. Ph.D. Dissertation, University of California, Berkeley, 1976.
(6) Arndt, K. T.; Boschelli, F.; Lu, P.; Miller, J. H. *Biochemistry* **1981**, *20*, 6109.
(7) Matthews, H. R.; Matthews, K. S.; Opella, S. J. *Biochem. Biophys. Acta* **1977**, *497*, 1.
(8) Drehmann, V. U.; Pürschel, V.; Wauschkun, H. *J. Prakt. Chem.* **1961**, *14*, 122. Ott, D. G. "Synthesis with Stable Isotopes"; Wiley: New York, 1981; p 87.
(9) Swartz, G. L.; Gulick, W. W., Jr. *J. Label Compd. Radiopharm.* **1975**, *11*, 525.

0022-3263/86/1951-1175$01.50/0

Scheme I



proceeded smoothly.[10] Filtration into HCl gave the protonated anilinium species, which is stable to air oxidation. An equivalent amount of $NaNO_2$ was added to the cold solution, and after 1 h an excess of hypophosphorous acid was added. The formation of phenol, as followed by ^{13}C NMR, was complete after 4–5 days at room temperature. The phenol was isolated by ether extraction followed by codistillation with water. It is unnecessary to prepare neat phenol if the material is to be used by *E. herbicola* to biosynthesize L-tyrosine.

The use of microorganisms for the industrial production of amino acids dates back to the late 1950s and primarily involves work by several groups in Japan.[11] The application of these techniques to preparing compounds enriched with stable isotopes can be a very straightforward problem, particularly for the synthesis of L-tyrosine. Many papers have been published describing the use of the enzyme β-tyrosinase from a variety of bacteria for the synthesis of L-tyrosine.[12] We have used the bacterium *Erwinia herbicola* (ATCC # 21434), which is a patent organism and have produced L-tyrosine using a modification of published techniques.[13-15]

The basic goal of the biosynthetic step was to optimize the conversion of labeled materials into L-tyrosine. No attempt was made to reproduce earlier published work[11,16] because the objectives of those studies were fundamentally different; i.e., the amount of L-tyrosine produced for a given batch of bacteria had to be maximized. In the present case, the problem was to maximize the yield of labeled L-tyrosine from a given amount of labeled precursor by using an excess of the catalyst (bacterial cells) with variable activity and allowing the reaction to go to completion. Thus, we added an excess of cells with an unknown activity, and if the reaction did not go to completion, we added more cells. This is in contrast to the industrial process where additional phenol is added to use up the catalytic activity of the cells. Of course, if the catalyst (β-tyrosinase) were not degraded over time, considerably more L-tyrosine could be made from a given batch of cells. Sophisticated bacteriological techniques are not necessary for success with this procedure.

The strain of *E. herbicola* that we selected appears to have the highest catalytic activity of the organisms which have been tested.[12] The culture media selected is one which should produce very high yields of the enzyme.[17] Enei et al.[16] reported that using media containing hydrolyzed soybean protein liquor produces cells in which β-tyrosinase makes up about 10% of the total soluble cellular protein. We have chosen a media that uses beef extract and yeast extract[17] rather than the soy liquor.

Although *E. herbicola* is available from the American Type Culture Collection, it is classified as a plant pathogen by the U.S. Department of Agriculture, and one must have written approval from the USDA to obtain cultures of the organism (form PPQ 526). Dye[18] reports that *E. herbicola* is not a pathogen but merely a quickly growing saprophytic

(10) "Vogel's Textbook of Practical Organic Chemistry", 4th ed.; Furness, B. S., et al., Eds.; Longman: New York, 1978.
(11) "The Microbial Production of Amino Acids", Yamada, K., Kinoshita, S., Tsunoda, T., Aida, K., Eds.; Halsted Press: New York, 1972.
(12) Enei, H.; Matsui, H.; Yamashita, K.; Okumura, S.; Yamada, H. *Agric. Biol. Chem.* **1972**, *36*, 1861.
(13) Enei, H.; Matsui, H.; Okumura, S. *Biochem. Biophys. Res. Commun.* **1971**, *43*, 1345.
(14) Ogata, K.; Yamada, H.; Enei, H.; Okumura, S. U.S. Patent 3791924, 1974.
(15) Enei, H.; Matsui, H.; Nakazawa, H.; Okumura, S.; Yamada, H. *Agric. Biol. Chem.* **1973**, *37*, 493.
(16) Yamada, H.; Kumagai, H. *Adv. Appl. Microbiol.* **1975**, *19*, 249.
(17) Enei, H.; Yamashita, K.; Okumura, S.; Yamada, H. *Agric. Biol. Chem.* **1973**, *37*, 485.
(18) Dye, D. W. *N. Z. J. Sci.* **1969**, *12*, 223.

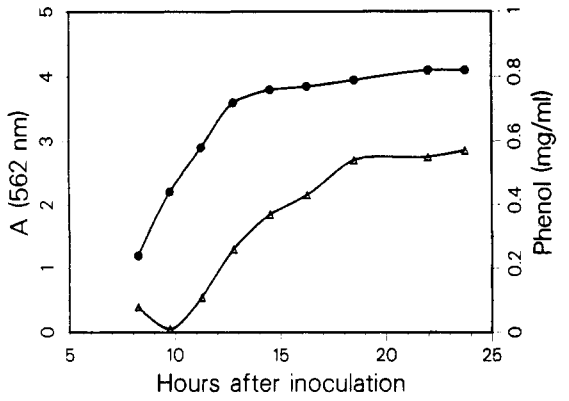


Figure 1. The growth curve of *E. herbicola*. Cells were cultured as described in the Experimental Section. The absorbance (●) was measured at 562 nm and the phenol (▲) was measured by liquid chromatography.

organism isolated from diseased plants which overgrew the actual pathogen. We have, however, taken the precaution of working with the bacteria in a Class 2, Type A hood and autoclaving all cultures before disposal.

The enzyme β-tyrosinase (Tyrosine phenol-lyase (deaminating), EC 4.1.99.2) has been purified from *E. herbicola* and crystallized and the catalytic properties have been studied.[19] It is a pyridoxyl phosphate containing enzyme and catalyzes a wide variety of reactions including the degradation of D- or L-tyrosine, the formation of L-tyrosine, the conversion of D-tyrosine to L-tyrosine, the racemization of D- or L-alanine, and the degradation of D- or L-serine to pyruvate and ammonia. The enzyme also catalyzes the degradation (formation) of a variety of tyrosine analogues, including fluoro-L-tyrosine.[20]

Harvested cells of *E. herbicola* were incubated with the appropriately labeled phenol plus L-serine and ammonium acetate. The bacteria were cultured on standard media as described by Enei et al.[17] and intact cells were used to catalyze the conversion of L-serine, ammonia, and phenol to L-tyrosine. The media for growing the cells contained unlabeled L-tyrosine which induced the enzyme β-tyrosinase. The unlabeled tyrosine was metabolized to phenol and was discarded with the media when the cells were harvested. Figure 1 shows the growth curve for the cells as well as the concentration of phenol produced from the L-tyrosine. At the point that the cells were harvested, the total moles of phenol observed in the media was somewhat less than the amount of L-tyrosine originally added.

The rate at which L-tyrosine was produced was followed by observing the decrease in phenol concentration over time by liquid chromatography. The phenol concentration decreased from ca. 12 mg/mL to less than 0.1 mg/mL. The rate observed was variable and depended mainly on two parameters: first, the relative activity of a particular batch of cells and, second, the presence of inhibitors in the chemically synthesized phenol. In only one case did we ever fail to see the reaction take place, and the problem was caused by an unidentified impurity in the phenol which apparently inhibited the reaction. The problem was easily solved by distilling the phenol at pH < 6 and rerunning the enzymatic reaction.

The conditions necessary for the conversion of labeled phenol to L-tyrosine are unaffected by the nature of the label. Thus, we have synthesized L-[4′-^{13}C]tyrosine, L-[3′,5′-$^{13}C_2$]tyrosine, L-[4′-^{18}O]tyrosine, and L-[2′,3′,5′,6′-$^{2}H_4$]tyrosine from the appropriately labeled phenol. The [^{18}O]phenol was prepared from phenyl–magnesium bromide by oxidation with [$^{18}O_2$]oxygen gas,[24] a technique which is also applicable to the synthesis of [^{17}O]phenol. The deuterophenol was obtained commercially as [U-$^{2}H_6$]-phenol which loses two deuterium atoms in the conversion to L-tyrosine. Other isotopomers of ring-labeled L-tyrosine can be made by using other isotopomers of phenol.

Two approaches can be used for the preparation of fully ring-deuterated L-tyrosine and L-phenylalanine: (1) microorganisms can be grown on deuterated substrates in D_2O, producing a mixture of fully deuterated amino acids or (2) deuterophenol can be converted to L-tyrosine by using *E. herbicola* as described above. The second method is clearly superior since it is more conservative of isotope, it is easier to perform, and it provides the most flexibility in labeling. This technique has been used previously, but unfortunately Arndt et al.[6] did not provide any details of the procedure.

Other deuterated isotopomers can be prepared by chemical exchange. In acidic D_2O the C-3 and C-5 ring protons of L-tyrosine will exchange to produce a partially ring-deuterated product, L-[3′,5′-$^{2}H_2$]tyrosine, an isotopomer which can also be made by β-tyrosinase from [2,4,6-$^{2}H_3$]phenol, which is commerically available. Arndt et al.[6] have described how one can prepare L-[2′,6′-$^{2}H_2$]-tyrosine by applying the chemical exchange in water to ring-deuterated L-tyrosine derived from perdeuterated phenol. Thus, with a combination of chemical exchange and biosynthesis, any of three isotopomers of ring-deuterated L-tyrosine can be produced, and the same isotopomers of L-phenylalanine can be prepared by chemical conversion of the L-tyrosine.

The enzyme β-tyrosinase will use pyruvate and ammonia in place of serine, which means that L-[^{15}N]tyrosine can be synthesized directly from [^{15}N]ammonium sulfate. Enei et al.[21] report on conditions for the synthesis of L-tyrosine from ammonium acetate and pyruvate, and we have adapted their procedure for use with limiting amounts of [^{15}N]ammonium sulfate. From a series of experiments in which the ammonium sulfate concentration was varied by a factor of 4, we consistently obtained yields of L-[^{15}N]-tyrosine of about 50–60% from $^{15}NH_4^+$. Apparently, the reaction equilibrium cannot be forced further once the relative concentration of ammonium ion drops below a certain level. Presumably the excess [^{15}N]ammonia could be recovered and reused. The chemical conversion of L-[^{15}N]tyrosine to L-[^{15}N]phenylalanine provides a convenient source of ^{15}N labeled phenylalanine.

Ring-labeled L-phenylalanine may be easily prepared from the labeled L-tyrosine. We have chosen to convert the L-tyrosine to the L-tyrosine benzyl ester *p*-toluenesulfonate,[22] prepare the ether of the phenolic hydroxyl group by reaction with 1-phenyl-5-chlorotetrazole, and hydrogenolize the L-tyrosine benzyl ester–1-phenyltetrazole ether to L-phenylalanine by using 10% Pd/C and formic acid[4,23] with an overall yield of 76% for the three steps.

The enantiomeric purity of the labeled L-tyrosine and L-phenylalanine samples was checked by GC analysis on a chiral column; the D isomer was not detected. The ^{1}H and ^{13}C spectra were obtained for both the natural abundance and enriched materials. Comparison of spectra to

(19) Kumagai, H.; Kashima, N.; Torii, H.; Yamada, H,.; Enei, H.; Okumura, S. *Agric. Biol. Chem.* **1972**, *36*, 472.
(20) Nagasawa, T.; Utagawa, T.; Goto, J.; Kim, C.; Tani, Y.; Kumagai, H.; Yamada, H. *Eur. J. Biochem.* **1981**, *117*, 33.
(21) Enei, H.; Nakazawa, H.; Matsui, H.; Okumura, S.; Yamada, H. *FEBS Lett.* **1971**, *21*, 39.
(22) Zerwas, L.; Winitz, M.; Greenstein, J. P. *J. Org. Chem.* **1957**, *22*, 1515.
(23) Musliner, W. J.; Gates, J. W., Jr. *J. Am. Chem. Soc.* **1966**, *88*, 4271.

Table I. ^{13}C–^{13}C Coupling Constants in ^{13}C-Enriched Products[a]

compd	^{13}C–^{13}C coupling constants					
	$^1J_{1,2}$	$^2J_{1,3}$	$^3J_{1,4}$	$^1J_{2,3}$	$^2J_{2,4}$	$^3J_{2,5}$
Phenol						
[1-^{13}C]phenol[c]	65.1	0	9.5	b	b	b
[2,6-$^{13}C_2$]phenol[c]	d	b	b	56.9	2.8	6.7

compd	^{13}C–^{13}C coupling constants					
	$^1J_{2',3'}$	$^1J_{3',4'}$	$^2J_{1',3'}$	$^2J_{2',4'}$	$^3J_{3',6'}$	$^3J_{1',4'}$
L-Tyrosine and L-Phenylalanine						
[3',5'-$^{13}C_2$]tyrosine[e]	58.1	62.4	f	b	3.9	b
[4'-^{13}C]tyrosine[e]	b	62.8	b	f	b	8.3
[3',5'-$^{13}C_2$]phenylalanine[c]	g	54.9	f	b	f	b
[4'-^{13}C]phenylalanine[c]	b	54.8	b	~2	b	9.6

[a] Coupling constants are in Hz. [b] Indicates no coupling pathway. [c] Samples were in H_2O with ca. 10% D_2O for a lock. [d] Coupling was not measured. [e] Samples were in sufficient ammonia for dissolution with ca. 10% D_2O for a lock. [f] These couplings were small and could not be resolved. [g] The C-3',C-5' resonance is upfield of the C-2',C-6' by <0.3 ppm and the $^1J_{2',3'}$ could not be observed.

Table II. ^{13}C Chemical Shifts and ^{13}C–F Coupling Constants of 2'- and 3'-Fluoro-L-tyrosine[a]

compd	chemical shifts[b]								
	1	2	3	1'[c]	2'	3'	4'	5'	6'
2'-fluoro-L-tyrosine	180.1	57.6	33.1	111.8	163.3	105.5	164.9	115.1	133.0
3'-fluoro-L-tyrosine	180.3	58.3	39.2	124.0	121.6	155.4	154.6	117.5	126.7

compd	coupling constants[c]					
	$^{13}C_{1'}$–F	$^{13}C_{2'}$–F	$^{13}C_{3'}$–F	$^{13}C_{4'}$–F	$^{13}C_{5'}$–F	$^{13}C_{6'}$–F
2'-fluoro-L-tyrosine	7.3 (2)	242.8 (1)	21.0 (2)	11.8 (3)	2.2 (4)	7.3 (3)
3'-fluoro-L-tyrosine	6.2 (3)	5.2 (2)	235.8 (1)	13.2 (2)	19.4 (3)	2.4 (4)

[a] Spectra recorded at 25.16 MHz. [b] Shifts are in ppm from external Me_4Si. [c] Coupling constants are in Hz, and the number of bonds is in parentheses.

published spectra confirmed the authenticity of the products obtained in each synthetic step. The observed ^{13}C–^{13}C couplings of the enriched ^{13}C compounds were measured and are reported in Table I.

The β-tyrosinase approach is also applicable to the synthesis of a variety of L-tyrosine analogues.[20] For example, using the reaction described for the synthesis of ring-labeled L-tyrosine, we have converted 2-fluorophenol and 3-fluorophenol to 3'-fluoro-L-tyrosine (75% yield) and 2'-fluoro-L-tyrosine (50% yield), respectively. Although neither fluoro derivative precipitates from the reaction mixture, the biosynthetic reaction nevertheless goes significantly toward completion. The workup is modified, however, by removing the cells by centrifugation, filtering the supernatant with charcoal through Celite, and concentrating the filtrate in vacuo. Crystalline product is removed by filtration and can be recrystallized from water. None of the D isomer was found by GC on a chiral column. ^{13}C NMR shifts and ^{13}C–F couplings are presented in Table II.

Experimental Section

Materials. Cultures of *Erwinia herbicola* were obtained from the American Type Culture Collection (ATCC # 21434). Enriched [1,3-$^{13}C_2$]acetone and [2-^{13}C]acetone were prepared by published procedures from [1-^{13}C]- and [2-^{13}C]acetic acid, respectively.[8] Perdeuterated phenol was purchased from MSD Isotopes, and [^{18}O]phenol was prepared from bromobenzene and $^{18}O_2$ as described by Walker and Goldblatt.[24] [^{15}N]Ammonium sulfate was a gift of Dr. T. Mills of the Stable Isotopes Facility at Los Alamos National Laboratory. Nutrient agar, yeast extract, and beef extract were obtained from Difco.

Methods. Liquid chromatography for the analysis of the formation of L-tyrosine was performed on a 25-cm reverse-phase C-8 column with water–methanol (60:40, 1 mL/min) as the eluent. For final purification of the phenylalanine liquid chromatography was performed on a 1 × 23 cm column packed with Dowex 1×2-200 (Aldrich) with 0.1 M ammonium formate in water as the eluent pumped at 24 mL/h. Capillary melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. Optical purity of the amino acids was checked by gas chromotographic analysis using a chiral column from Alltech Associates (chiral-sil val) to separate the trifluoroacetyl amide–methyl ester derivatives. ^{13}C NMR spectra were obtained at 75 MHz with a Bruker WM-300 spectrometer, at 50 MHz with a Nicolet NT-200 spectrometer, or at 25 MHz with a Varian XL-100-15 spectrometer.

Freeze-dried cultures of *E. herbicola* were rehydrated in water and grown on nutrient agar slants at 26°. Slants were stored at 4 °C and organisms transferred to a fresh slant and grown for 2 days at 26° before use. Bacteria were cultured in media (pH 7.5) containing (g/L) 2 g of L-tyrosine, 2 g of KH_2PO_4, 0.5 g of $MgSO_4$, 2 mg of $FeSO_4 \cdot 7H_2O$, 100 mg of pyridoxine–HCl, 6 g of glycerol, 5 g of succinic acid, 1 g of D,L-methionine, 2 g of D,L-alanine, 0.5 g of glycine, 1 g of L-phenylalanine, 10 g of yeast extract, and 5 g of beef extract. Cells were grown for 28 h in a New Brunswick Psycrotherm (2-in. stroke, 200 rpm) at 28 °C with 100 mL of media in 1-L culture flasks (Delong flask with stainless steel closures from Bellco Glass, Inc.). The bacteria were harvested by centrifugation at 800 × *g* for 15 min.

Nitromalonaldehyde. This was prepared by the method of Fanta.[25] This material is impact sensitive and thermally unstable. It does not store well and was freshly prepared as needed. The mucobromic acid required for the preparation is available from Aldrich.

***p*-Nitro-[^{13}C]phenol 2a and 2b.** The procedure given here is a modification of Swartz and Guliel.[9] A 1-L, two-necked round-bottomed flask equipped with a thermometer, dropping funnel, and magnetic stirrer was filled with 800 mL of distilled water and cooled to 0 °C in an ice bath. Sodium nitromalonaldehyde monohydrate (13.24 g, 84.3 mmol) was added, followed by labeled acetone (5.08 mL, 4.2 g, 70 mmol). The reaction mixture was stirred continuously and kept at 0 °C. Then sodium hydroxide (17.6 g, 0.44 mol) in 100 mL of water was added dropwise. After the addition was completed, the orange solution

(24) Walker, T. E.; Goldblatt, M. *J. Label. Compd. Radiopharm.* **1984**, *21*, 353.

(25) "Organic Syntheses"; Wiley: New York, 1963; Collect. Vol. IV. p 844.

was stoppered tightly and placed at 4 °C for 6 days, until all of the acetone had reacted. The completion of the reaction was monitored by ^{13}C NMR. After 6 days, hydrochloric acid (6 M, 80 mL) was added dropwise to the cold solution. During addition of the acid, a black precipitate appeared, which was removed by gravity filtration with fluted Whatman No. 1 filter paper. The precipitate was suspended in 100 mL of 6 M hydrochloric acid. It was then brought to boiling and filtered, while hot, under vacuum. The combined acid solutions were then extracted continuously with ether. Extraction was carried out until the water layer no longer contained ^{13}C. The ether extracts were then dried with $MgSO_4$ and evaporated under vacuum, giving **2a** or **2b** as yellow crystals.

2a: yield, 4.90 g (58.4%), 5.66 g (67.3%), 5.12 g (61%); ^{13}C NMR (50 MHz, H_2O) 119.0 ppm.

2b: yield, 5.62 g (67.4%), 6.06 g (72.7%); ^1H NMR (200 MHz, $CDCl_3$) 6.90 (d, 1 H), 6.95 (d, 1 H), 8.16 ppm (m, 2 H); ^{13}C NMR (50 MHz, H_2O) 168.8 ppm.

[^{13}C]Phenol 4a and 4b. Labeled *p*-nitrophenol **2a** or **2b** (4.48 g, 32 mmol) was dissolved with stirring and gentle heating in 1 N NaOH (200 mL). The solution was added dropwise to a 1-L round-bottomed flask containing a water solution of $NaBH_4$ (2.52 g, 64 mmol in 60 mL of water) and Pd/C 10% (100 mg in 20 mL of water). The addition was completed over a period of 40 min. The reaction mixture was then filtered through a sintered-glass funnel into a vacuum flask containing 3 M HCl (200 mL). The filtrate was extracted twice with 400-mL portions of ether, and the water layer containing **3a** or **3b** was cooled to 0 °C in an ice bath. A solution of $NaNO_2$ (2.4 g, 35 mmol) in 40 mL of water was then added dropwise with stirring while the temperature was kept at 0–4 °C. After the addition was completed the reaction mixture was allowed to stand for a period of 1 h at room temperature. Then hypophosphorous acid (200 mL, 50%) was added dropwise, and the solution was left for 4–5 days at room temperature. During this period, an evolution of gas occurred. The complete conversion into phenol was monitored by ^{13}C NMR. After 5 days the reaction mixture was extracted with ether (1 × 200 mL and 3 × 400 mL). The combined ether extracts were dried with $MgSO_4$ and evaporated under vacuum. The orange residue was then treated with water (150 mL) and the solution distilled, giving an azeotropic mixture which boiled at 95–100 °C. Liquid chromatographic analysis showed no major impurities.

4a: yield, 6.18 g (58.65%); ^{13}C NMR (50 MHz, H_2O) 116.3 ppm.

4b: yield, 6.85 g (58.97%); ^{13}C NMR (50 MHz, H_2O) 155.4 ppm.

L-[^{13}C]Tyrosine Benzyl Ester *p*-Toluenesulfonates 6a and 6b. L-Tyrosine (1 g) was suspended in benzyl alcohol (10 mL) in a 100-mL round-bottomed flask equipped with a Soxhlet extractor containing 5A molecular sieves in the thimble. *p*-Toluenesulfonic acid (1.14 g) and benzene (25 mL) were added, and after refluxing overnight, the mixture was cooled in ice, ether (50 mL) was added, and the product was filtered. The white solid was washed with an additional 50 mL of ether and dried under vacuum overnight to yield 2.37 g (97.1%) of **6a** or **6b**: mp 175–176 °C (lit. mp 179–180.5 °C);[22] ^{13}C NMR (D_2O) [**6a**] 116.8, [**6b**] 156.0 ppm.

L-[^{13}C]Tyrosine Benzyl Ester 1-Phenyltetrazolyl Ethers 7a and 7b. Labeled L-tyrosine benzyl ester *p*-toluenesulfonate **6a** or **6b** (2.43 g) and anhydrous potassium carbonate (2.43 g) in acetone (150 mL) were refluxed for 1.5 h. 1-Phenyl-5-chlorotetrazole (1.15 g) was added, and the mixture was refluxed overnight. The solution was cooled in ice for 30 min, and then the white salt was filtered off and washed with 75 mL of acetone. The combined filtrate was evaporated to dryness. The residue was treated with 50 mL of acetone–water mixture (1:1) and the acetone evaporated in vacuo. The suspension of white precipitate was filtered, washed with 10 mL of water, and dried under vacuum overnight to yield 1.87 g (85.4%) of 1-phenyltetrazolyl ether of L-tyrosine benzyl ester, mp 75–76 °C (lit. mp 92–95 °C).[4] After recrystallization from ethyl acetate–hexane the mp was determined to be 91–94 °C. The crude material was used in the next step: ^{13}C NMR ($CDCl_3$) [**7a**] 119.4, [**7b**] 152.4 ppm.

L-[^{13}C]Phenylalanines 8a and 8b. Labeled L-tyrosine benzyl ester 1-phenyltetrazolyl ether (0.415 g) was dissolved in 30 mL of 18% formic acid in a 100-mL round-bottomed flask equipped with a magnetic stirrer and thermometer and warmed to 47 °C. Pd/C (5%, 0.25 g) suspended in 10 mL of 18% formic acid was added and the solution stirred at 47 °C overnight. The Pd/C catalyst was removed by filtration and washed with 20 mL of water. The combined filtrates were evaporated to dryness, and the solid residue was washed with ether (20 mL), collected by vaccum filtration, and dried at vacuum overnight to yield 0.15 g (90.9%) of **8a** or **8b**.

Final purification of the phenylalanine was performed by liquid chromatography on Dowex 1×2-200 using 0.1 M ammonium formate as the eluting buffer. The 0.15 g of crude material obtained above was dissolved in 5 mL of 0.1 M ammonium formate buffer, filtered, and chromatographed on a 1 × 23 cm column packed with Dowex 1×2-200 in a reverse flow manner in 0.5 mL portions at a buffer flow rate of 24 mL/h. Under these conditions, phenylalanine has a retention time of 22 min, and tyrosine has a retention time of 33 min. About 5% residual tyrosine was found in the phenylalanine prior to the liquid chromatography: ^{13}C NMR (D_2O, pH 1) [**8a**] 130.1, [**8b**] 128.6 ppm.

L-[^{13}C], L-[^2H], or L-[^{18}O]Tyrosines 5a–d. The conversion of labeled phenol to L-tyrosine was the same regardless of the label; a typical reaction is described. *E. herbicola* was grown as described, and the cells from 1 L of media were transferred to a jacketed 500-mL spinner flask which contained labeled phenol (5.0 g), L-serine (10 g), ammonium acetate (5.0 g), and water (500 mL). The pH was adjusted to 8.5 with NH_4OH and the cellular suspension stirred at 37 °C for about 24 h. A white precipitate of L-tyrosine was observed usually within a few hours. The phenol concentration was determined by LC, and when the concentration was essentially zero, the mixture was centrifuged for 15 min at 16 000 × *g*. The precipitated L-tyrosine was separated from the cells by dissolving it in 6 N HCl and centrifuging to remove the cells. The acid supernatant was concentrated in vacuo, water (20 mL) was added to the residue, and the mixture was titrated to ca. pH 7 with NH_4OH. The L-tyrosine crystallized as the pH was raised to produce 9.5 g of crude product and was recrystallized from water to yield 8.38 g, 91%: mp 278–282 °C dec (bath preheated to 250 °C); ^{13}C NMR (D_2O/DCl) [**5a**] 116.9, [**5b**] 156.1 ppm.

L-[^{15}N]Tyrosine (5e). **L-[^{15}N]tyrosine** was prepared essentially by the technique described above except that [^{15}N]ammonium sulfate and sodium pyruvate were used in place of ammonium acetate and L-serine. *E. herbicola* was grown as described above and transferred to a jacketed 100-mL spinner flask which contained phenol (0.5 g), sodium pyruvate (2.5 g), and $(^{15}NH_4)_2SO_4$ (0.6 g, 8.95 mmol of $^{15}NH_4^+$). The pH was adjusted to 8.5 with KOH, and an additional aliquot (0.5 g) of phenol was added after 2 h. The reaction was deemed complete when the concentration of phenol had leveled off. Crystalline **5e** was processed and recrystallized as described above: yield, 940 mg (5.16 mmol), 58%; mp, 275–279 °C dec (bath preheated to 250 °C).

Acknowledgment. We thank R. E. London and C. J. Unkefer for helpful discussions and encouragement. This work was supported by the U.S. Department of Energy and by the National Stable Isotopes Resource at Los Alamos, funded by the National Institutes of Health (RR00962), and by the NIH (RR 08016, GM 07799) and the NSF (PCM-78-13235) at Howard University. We are indebted to M. M. Drewniak for technical assistance.

Registry No. **1a**, 7217-25-6; **1b**, 3881-06-9; **2a**, 70479-88-8; **2b**, 3881-07-0; **3a**, 100790-29-2; **3b**, 100790-30-5; **4a**, 100790-31-6; **4b**, 29809-11-8; **5a**, 70479-98-0; **5b**, 81201-90-3; **5c**, 17949-21-2; **5d**, 62595-14-6; **5e**, 35424-81-8; **6a**, 100790-33-8; **6b**, 100790-35-0; **7a**, 100790-37-2; **7b**, 100790-39-4; **8a**, 73198-09-1; **8b**, 81201-87-8; $(^{15}NH_4)_2SO_4$, 43086-58-4; sodium nitromalonaldehyde, 14197-68-3; 1-phenyl-5-chlorotetrazole, 14210-25-4; L-serine, 56-45-1; [^{18}O]-phenol, 1739-18-0; [U-$^2H_6$]phenol, 13127-88-3; phenol, 108-95-2; sodium pyruvate, 113-24-6; 2-fluorophenol, 367-12-4; 3-fluorophenol, 372-20-3; 3′-fluoro-L-tyrosine, 7423-96-3; 2′-fluoro-L-tyrosine, 78709-81-6; β-tyrosine, 9059-31-8.