**Table I. Electrophoretic Mobilities of Products and Standard Specimens (Electrophoresis with 0.05 M Sodium Hydrogen Citrate, pH 3.5)**

| compd | electrophoretic mobility[a] | compd | electrophoretic mobility[a] |
|---|---|---|---|
| Cp | −1.00 | G-G-A, **19a** | +0.32 |
| Gp | +3.45 | G-C-A, **19b** | −0.77 |
| A | −9.26 | G-G-A-Phe, **20a** | −1.97 |
| Phe | −4.29 | G-C-A-Phe, **20b** | −4.18 |

[a] Mobility of Cp = 1.00; (−) = migration toward cathode; (+) = migration toward anode.

guanidine (0.33 M) and 2-nitrobenzaldoxime (0.38 M) in dry acetonitrile (1 mL). After 3 h at room temperature, the reaction product was precipitated with an excess of dry ether, centrifuged, washed twice with dry ether, and dried in vacuo over $P_2O_5$. The residue was treated with cold 80% formic acid (2.0 mL) at 0 °C for 30 min. The product was precipitated with an excess of dry ether, isolated by centrifugation, dissolved in cold 80% acetic acid (0.2 mL), and reprecipitated with excess ether. The solid product was dissolved in ammonium acetate buffer (5 mM, pH 4.5; 1 mL) and purified on a $C_{18}$ SPE Baker column (3 mL) using a step gradient of acetonitrile (0, 5, 10, 20, 50, and 100%) in 5 mM ammonium acetate (pH 4.5). Product **20** was eluted with 20% acetonitrile; the eluate was evaporated in vacuo and coevaporated with 80% acetic acid at low temperature to remove the last traces of salt. The yields of chromatographically and electrophoretically (see Table I) uniform products **20** were determined spectrophotometrically and were in the 20–30% range. The products were also characterized by UV spectroscopy in 0.01 N HCl (**20a**: $\lambda_{max}$ = 256 nm; $\lambda_{min}$ = 237 nm; $A_{250/260}$ = 0.94; $A_{280/260}$ = 0.53; $A_{290/260}$ = 0.35; **20b**: $\lambda_{max}$ = 258 nm; $\lambda_{min}$ = 245 nm; $A_{250/260}$ = 0.86; $A_{280/260}$ = 0.73; $A_{290/260}$ = 0.45), mild alkaline hydrolysis to phenylalanine and parent oligonucleotides and enzymatic digestion with appropriate nucleases. Product **20a** was quantitatively digested with RNAse $T_1$ (ratio of Gp/A = 1.94) and with snake venom phosphodiesterase (ratio of G + pG/A + pA = 1.99). Product **20b** was quantitatively digested with RNAse $T_1$ and RNAse A (ratio of Gp/Cp/A = 1.01/0.98/1.00) and with snake venom phosphodiesterase (ratio of G/C + pC/A + pA = 1.02/1.06/1.00). Product **20b** was also identical in several electrophoretic systems with a previously prepared G-C-A-Phe.[1a]

# A Highly Efficient and Large-Scale Synthesis of (2*S*,3*S*)-[2,3-$^2H_2$]- and (2*S*,3*R*)-[3-$^2$H]Aspartic Acids via an Immobilized Aspartase-Containing Microbial Cell System

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This paper describes the preparation and utilization of an immobilized aspartase-containing *Escherichia coli* system for the large-scale synthesis of (2*S*,3*S*)-[2,3-$^2H_2$]- and (2*S*,3*R*)-[3-$^2$H]aspartic acids from the appropriately labeled fumaric acids in >95% isolated yield, with >97% deuterium incorporation at the C-3 center and optically pure at both the C-2 and C-3 centers.

The synthesis of both L-aspartic acids (2*S*) stereospecifically labeled at the C-3 position with deuterium[1-3] and their utilization for the preparation of other stereospecifically labeled biologically important compounds, such as serine, glutamic acid, and amino ethanol, are well documented.[4-8] The method for the synthesis of the L-aspartic acids stereospecifically labeled at the C-3 position is not only unduly laborious and limited to the production of small quantities but requires the use of the expensive enzyme aspartase [EC 4.3.1.1][9] and the use of highly noxious and poisonous hydrogen sulfide gas in the workup of the enzyme reaction. In view of our interest in utilizing stereospecifically labeled L-aspartic acid as a synthon for the preparation of stereospecifically labeled L-homoserines and L-homoserine lactones,[10] L-methionines,[11] L-discadenines,[12] and *S*-adenosyl-L-methionines,[13] all of biological importance, we have developed a more efficient, less laborious and more cost effective method to prepare large quantities of aspartic acids stereospecifically labeled with deuterium at the C-3 position. In this paper we report the application of an immobilized aspartase-containing microbial cell system designed for that purpose.

## Results and Discussion

The enzyme aspartase [EC 4.3.1.1] catalyzes the reversible addition of $NH_2$ to the *si* face and H, from the solvent, to the *re* face of fumarate in an overall anti fashion to yield L-aspartic acid.[1-3] If the reaction is performed in

(1) Englard, S. *J. Biol. Chem.* **1958**, *233*, 1003–1009.
(2) Krasna, A. I. *J. Biol. Chem.* **1958**, *233*, 1010–1013.
(3) Englard, S.; Hanson, K. R.; In *Methods in Enzymology*; Colowick, S. P., Kaplan, N. O., Eds.; Vol. XIII, Lowensteine, J. M., Ed.; Academic Press, Inc.: New York, 1969; pp 578–580.
(4) Field, S. J.; Young, D. W. *J. Chem. Soc., Chem. Commun.* **1979**, 1163–1165.
(5) Gani, D.; Young, D. W. *J. Chem. Soc., Chem. Commun.* **1982**, 867–869.
(6) Field, S. J.; Young, D. W. *J. Chem. Soc., Perkin Trans. 1* **1983**, 2387–2392.
(7) Gani, D.; Young, D. W. *J. Chem. Soc., Perkin Trans. 1* **1983**, 2393–2398.
(8) Gani, D.; Young, D. W. *J. Chem. Soc., Chem. Commun.* **1983**, 576–578.
(9) Aspartase is available from the Sigma Chemical Co. at a cost of ≈$167.00/100 units: one unit of enzyme will convert 1.0 μmol of L-aspartic acid to fumarate per minute at pH = 8.5 at 30 °C.
(10) Ramalingam, K.; Woodard, R. W. *J. Org. Chem.* **1988**, *53*, 1900–1903.
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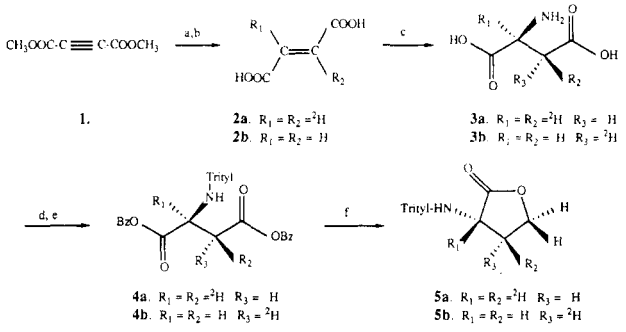
0022-3263/89/1954-3195$01.50/0 

**Scheme I**[a]



[a] (a) Triphenylphosphine/$^2H_2O$; (b) 10% KOH heat at reflux; (c) for the **a** series immobilized *E. coli* cell 1/$NH_4OH/H_2O$ or for the **b** series immobilized *E. coli* cell 2/$N^2H_4O^2H/^2H_2O$; (d) benzyl alcohol/*p*-toluenesulfonic acid/benzene; (e) triphenylmethyl chloride/triethylamine/chloroform; (f) 1 N DIBAL-H/toluene.

$^2H_2O$ as solvent, one obtains (2*S*,3*R*)-[3-$^2$H]aspartic acid, and conversely if [2,3-$^2H_2$]fumaric acid is incubated with aspartase in $H_2O$ as solvent, one obtains (2*S*,3*S*)-[2,3-$^2H_2$]aspartic acid.[1-3] Although the equilibrium constant ($K_{eq}$) for the aspartase reaction is $\approx 2.0 \times 10^{-2}$ M,[14] the usual optimized yield of labeled aspartic acid from the aspartase reaction is ≈40%.[7] Furthermore the enzyme's half-life is relatively short at pH = 8.5 and 37 °C (optimal conditions for maximum activity) and the normal workup conditions irreversibly denature the aspartase, precluding any chance of recycling the enzyme.

Continuous production of unlabeled aspartic acid by the immobilized *Escherichia coli* type B cell system from diammonium fumarate and ammonia has been reported by Sato et al.[15,16] Their immobilization procedure for the *E. coli* cells involved standard polyacrylamide gel-type techniques and the polymeric gel was broken-up by using a kitchen blender. The *E. coli* paste was reported to be very stable over a prolonged period of time and to produce L-aspartic acid in excellent yields that requires little or no purification.

Based on this report, we decided to investigate the feasibility of utilizing this type of immobilized cell methodology to produce stereospecifically deuteriated aspartic acids. A culture of *E. coli* strain B (ATCC 11303) was obtained from the American Tissue Culture Collection and grown by standard methodologies, the enzyme activity in the collected cells was induced with ammonium fumarate, and the cells were immobilized according to the method of Sato.[15,16] Immobilization produced trapped cells capable of producing useful quantities of unlabeled aspartic acid. The equipment and techniques necessary for the maintenance and growth of the microorganism are quite minimal and can be carried out in any typical organic lab by sophomore chemistry students.[17]

In order to prepare (2*S*,3*S*)-[2,3-$^2H_2$]aspartic acid, [2,3-$^2H_2$]fumaric acid was synthesized by standard methods (see Scheme I).[18,19] The *E. coli* paste was incubated with

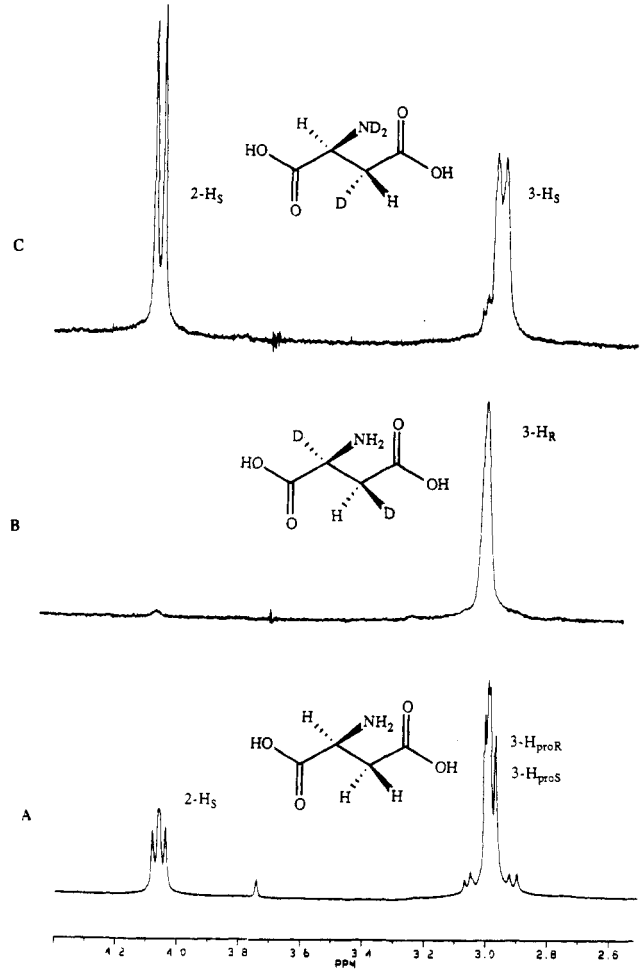



**Figure 1.** $^1$H NMR spectra (270-MHz) (75 mM, $D_2O$, 24 °C, pH = 7) of (A) unlabeled aspartic acid, (B) (2*S*,3*S*)-[2,3-$^2H_2$]aspartic acid, (*C*) (2*S*,3*R*)-[3-$^2$H]aspartic acid.

dideuteriated fumarate for 24 h at 37 °C and the suspension was filtered to remove the paste, which can be recycled. The desired dideuteriated aspartic acid was obtained as a >98% pure white solid in 95% yield. The initial batch was contaminated with residual unlabeled aspartic acid still bound to the *E. coli* gel. The $^1$H NMR spectrum of a later batch is shown in Figure 1. In order to determine the % deuterium incorporation and the % ee, the (2*S*,3*S*)-[2,3-$^2H_2$]aspartic acid was converted into *N*-trityl-(2*S*,3*S*)-[2,3-$^2H_2$]homoserine lactone by previously published methods (see Scheme I).[20-22] This particular lactone was chosen for analyzing the aspartic acid since the lactone is available from the acid in three straightforward steps, none of which affect either the asymmetric center or incorporated deuterium, and all the proton chemical shifts values have been assigned and are well-separated (see Figure 2). The % ee for the dideuteriated material at the C-3 center was 100% with the only contaminant being unlabeled aspartic acid, not the other diastereomer with the deuterium located at the 3*R* position. A second batch of the *E. coli* paste was subjected to freeze-drying and reconstitution with $^2H_2O$ several times. Diammonium [($N^2H_4$)$_2$] unlabeled fumarate (formed in situ by the treatment of unlabeled fumaric acid

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(15) Chibata, I.; Tosa, T.; Sato, T. *Appl. Microbiol.* **1974**, *27*, 878-885.
(16) Tosa, T.; Sato, T.; Mori, T.; Chibata, I. *Appl. Microbiol.* **1974**, *27*, 886-889.
(17) Each year the College of Pharmacy at the University of Michigan participates in the NIH sponsored Minorities High School Student Program and the senior author has high school juniors and sophomores successfully carry out the reactions described in this report.
(18) Richards, E. M.; Tebby, J. C.; Ward, R. S.; Williams, D. H. *J. Chem. Soc. C* **1969**, 1542-1544.
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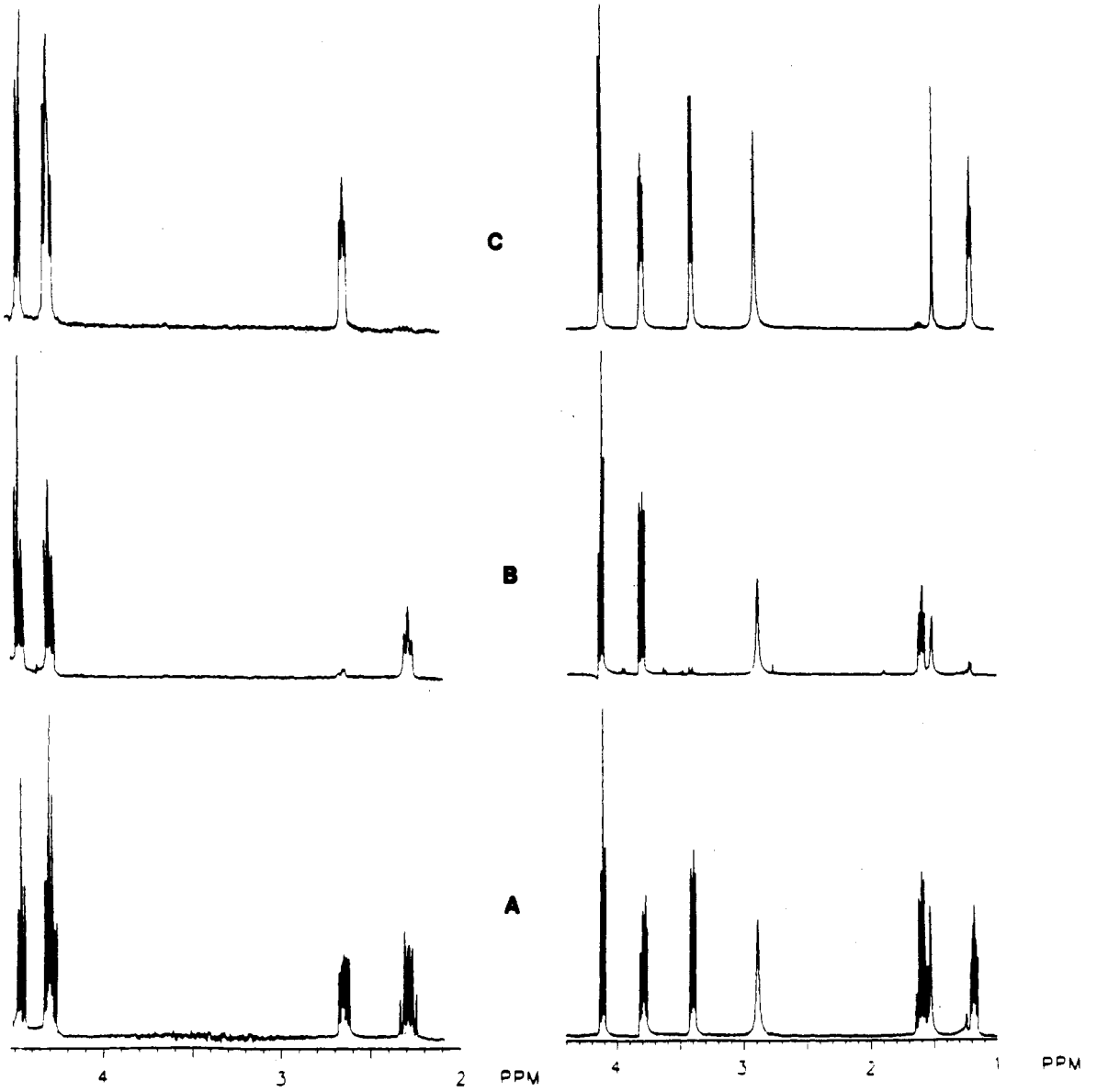


**Figure 2.** $^1H$ NMR spectra (500-MHz) of (A) right, unlabeled *N*-tritylhomoserine lactone in $CDCl_3$ and left, unlabeled homoserine lactone hydrochloride in $D_2O$; (B) right, *N*-trityl-(2*S*,3*S*)-[2,3-$^2H_2$]homoserine lactone in $CDCl_3$ and left, (2*S*,3*S*)-[2,3-$^2H_2$]homoserine lactone hydrochloride in $D_2O$; (C) *N*-trityl-(2*S*,3*R*)-3-$^2H$]homoserine in $CDCl_3$ and left, (2*S*,3*R*)-[3-$^2H$]homoserine lactone hydrochloride in $D_2O$. The peaks at $\delta \approx 1.50$ ppm in the $CDCl_3$ spectra are due to residual $H_2O$ in the commercial $CDCl_3$.

with the quantity of $N^2H_4OH$ in $^2H_2O$ needed to adjust the pH to 8.5) was incubated with the fully deuterium-exchanged *E. coli* paste in $^2H_2O$ to give the desired (2*S*,3*R*)-[3-$^2H$]aspartic acid in excellent yield but with only modest apparent deuterium incorporation (92%). After a second cycle, the deuterium-exchanged *E. coli* paste gave, under similar conditions, (2*S*,3*R*)-[3-$^2H$]aspartic acid with >98% deuterium incorporation in 95% yield of optically pure material as shown by the $^1H$ NMR spectrum in Figure 1. The $^1H$ NMR spectrum of the *N*-trityl-(2*S*,3*R*)-[3-$^2H$]homoserine lactone derived from this aspartic acid is shown in Figure 2. In order to further demonstrate the optical purity and utility of the products available from this immobilized technique, the trityl groups of the *N*-trityl-L-homoserine lactones were cleaved by treatment with a trace of HCl (gas) in $CHCl_3$ to give the L-homoserine lactones salts. The $^1H$ NMR spectra of the lactone·HCl salts are also shown in Figure 2.

These pastes, from one preparation stored at 4 °C in the refrigerator with *no* preservatives, have been utilized for over four years to produce hundreds of grams of both deuteriated aspartic acids, respectively. During this time several other batches of immobilized cells have been prepared, each of which vary in their ability to produce stereospecifically deuterium-labeled aspartic acid. This variation in the yield (70–95%) of aspartic acid from batch-to-batch is unfortunate but palatable considering the alternative methods available. In conclusion the methodology presented here represents a simple, highly efficient route for production of large quantities of optically pure (2*S*,3*S*)-[2,3-$^2H_2$]- and (2*S*,3*R*)-[3-$^2H$]aspartic acids with high deuterium incorporation.

## Experimental Section

Melting points were taken on a Mel-Temp capillary apparatus and are uncorrected. $^1H$ NMR spectra were recorded on an IBM WP 270-MHz or a GE 500-MHz spectrometer in either $D_2O$ or $CDCl_3$ with chemical shifts reported downfield from DSS and TMS, respectively. Mass spectra were determined in the Department of Chemistry at the University of Michigan with a Finnigan 4201 mass spectrometer. A New Brunswick G-24 environmental incubator shaker was used for all microorganism growth and for *E. coli* paste incubations. All measurements of pH and pD were made with an Orion Model 501 digital ionizer (Orion Research Co.) utilizing an Orion ATC probe and a high-

impact plastic pH electrode. The pD values were measured directly with the pH meter and are uncorrected.

*E. coli* 11303 was purchased from the American Tissue Culture Collection. Freeze-dried *E. coli* (catalog # EC 11303), aspartase [EC 4.3.1.1], *N,N'*-methylenebis(acrylamide), β-(dimethylamino)propinonitrile, and potassium persulfate were purchased from Sigma Chemical Co. All organic chemicals and deuterium-containing chemicals were purchased from Aldrich Chemical Co. and were used without further purification. Corn steep liquor and nutrient agar were purchased from Difco Co.

**Growth of *E. coli* Cells (ATCC 11303).** The *E. coli* 11303 obtained from ATCC[23] was maintained aerobically on nutrient agar slants at both 25 and 4 °C (refrigerator). The *E. coli's* growth media (1.0 L) contains diammonium fumarate (3.0%, fumaric acid plus 2 mol of $NH_4OH$), $K_2HPO_4$ (0.2%), $MgSO_4 \cdot 7H_2O$ (0.05%), $CaCO_3$ (0.05%), and corn steep liquor (4.0%) adjusted to pH 7.0. The 1 L of media was divided equally among five 1-L Erlenmeyer flasks, stoppered with either sterile cotton wads wrapped in sterile gauze or with a foam plug available from Scientific American Co. designed for this exact purpose, and autoclaved at 110 °C/15 psi for 15 min. After cooling to 30 °C, each flask was innoculated asceptically with a loop of *E. coli* from the agar slant. The *E. coli* was cultured with shaking (150–180 rpm) at 37 °C for 24 h. The cells were harvested by centrifugation at 15000*g* (Sorvall RC2-B equipped with a GSA rotor) for 30 min and the compacted cells (10 g) were resuspended in sterile physiological saline (0.9% NaCl) and recentrifuged at 15000*g* for 30 min.

**Preparation of Immobilized *E. coli* Cells.** To the *E. coli* cells (10 g) suspended in sodium chloride solution (0.9%, 40 mL) were added acrylamide monomer (7.5 g), *N,N'*-methylenebis-(acrylamide) (0.4 g), and β-(dimethylamino)propinonitrile (5.0%, 5 mL). The polymerization reaction was initiated by the addition of a solution of potassium persulfate (5 mL, 2.5% w/v), and the mixture was allowed to stand for 1 h at 37 °C. The resulting gel (70 g) was blended in a standard kitchen-type Waring blender at high speed for 3–5 min or until the gel was thoroughly blended. The immobilized cells were activated by incubation with 200 mL of 1 M diammonium fumarate (pH = 8.5) (containing 1 mM $Mg^{2+}$) for 48 h at 37 °C. After filtration of the gel from the suspension, the paste was separated roughly into two equal fractions. One fraction was set aside and will be referred to as *E. coli* cells 1.

The other fraction of immobilized *E. coli* cells (35 g) was freeze-dried to a constant weight (≈6.0 g) and then redissolved in $^2H_2O$ (30.0 mL) and the freeze-drying–reconstitution process repeated twice. This paste will be referred to as *E. coli* cells 2.

Both *E. coli* cell pastes 1 and 2 were stored separately at 4 °C in the refrigerator without any preservatives.

**Dimethyl [2,3-$^2H_2$]Fumarate.**[18] To a stirred solution of dimethyl acetylenedicarboxylate (21 g, 1.48 mol) and $^2H_2O$ (3 mL) in THF (300 mL) at 0 °C was added dropwise a solution of triphenylphosphine (39 g, 0.149 mol) in THF (300 mL). The dark red solution was stirred at 25 °C for 30 min and then heated at reflux for 12 h. The reaction mixture was cooled and dried over $Na_2SO_4$, and the solvent was removed in vacuo. The red residue was sublimed (100 °C/15 mmHg) to give a solid, which was recrystallized from methanol to give the title compound as white crystals (16 g, 74%): mp 98–100 °C (lit.[18] mp 98–101 °C); $^1H$ NMR ($C^2HCl_3$) δ 3.84 (s, 6 H, $CH_3$).

**[2,3-$^2H_2$]Fumaric Acid (2a).** Dimethyl [2,3-$^2H_2$]fumarate (19.0 g, 0.13 mol) was heated under reflux with 10% potassium hydroxide solution (400 mL) for 4 h. The reaction mixture was cooled and acidified with 6 N hydrochloric acid to pH = 2.5. The precipitated [2,3-$^2H_2$]fumaric acid (**2a**), which formed on standing at 10 °C, was filtered, washed with ice-cold water, recrystallized from 1 N HCl, and dried in a vacuum desicator to yield 12.5 g (83%) of the dideuteriated fumaric acid as white crystals: mp 296–300 °C (lit.[24] mp 299–300 °C for the undeuteriated compound); $^1H$ NMR ($^2H_2O$) no detectable peaks, only HOD peak; MS *m/z* 118 ($M^+$) indicated ca. 99% $^2H_2$.

**(2*S*,3*S*)-[2,3-$^2H_2$]Aspartic Acid (3a).** A solution of [2,3-$^2H_2$]fumaric acid (**2a**) (11.6 g, 98.2 mmol) and concentrated $NH_4OH$ (30 mL) in $H_2O$ (70 mL) was heated on a steam bath for 20 min and the pH adjusted to 8.5 with concentrated $NH_4OH$. After the solution was cooled to room temperature, $MgSO_4 \cdot 7H_2O$ (23.4 mg) and immobilized *E. coli* 1 (30 g) were added. The reaction mixture was incubated on the rotary shaker (150 rpm) at 37 °C for 48 h. The suspension was filtered, the gel washed with water (30 mL), and the pH of the filtrate adjusted to 2.8 with 60% $H_2SO_4$ while heating the solution at 90 °C. The solution was kept at 4 °C for 12 h. The crystals that formed were collected by suction and washed with ice-cold water. The crystals were dried to yield 12.5 g (94%) of pure aspartic acid. The amino acid from the first incubation of *E. coli* 1 was contaminated with unlabeled aspartic acid and can be used or discarded. The following reaction batches obtained from *E. coli* 1 gave the title compound in average yields of 95% free from unlabeled aspartic acid: mp >300 °C (lit.[24] mp >300 °C dec for the undeuteriated compound); $^1H$ NMR ($^2H_2O$) δ 2.98 (s, 1 H, 3-$C^2H_S H_R$).

**(2*S*,3*R*)-[3-$^2H$]Aspartic Acid (3b).** A solution of unlabeled fumaric acid **2b** (11 g, 95 mmol) and concentrated $N^2H_4O^2H$ (30 mL) in $^2H_2O$ (70 mL) was heated on a steam bath for 20 min and the pH adjusted to 8.5 with concentrated $N^2H_4O^2H$. After the solution was cooled to 30 °C, $MgSO_4 \cdot 7H_2O$ (23.4 mg) and the deuterium-exchanged immobilized *E. coli* 2 paste (30 g) were added. The reaction mixture was incubated on the rotary shaker (150 rpm) at 37 °C for 48 h. The suspension was filtered and the gel washed with $^2H_2O$ (20 mL). At this stage, the filtrate can be freeze-dried in a closed system in order to recycle the $^2H_2O$ and the freeze-dried solid was redissolved in the equivalent amount of $H_2O$ as $^2H_2O$ removed and the pH of the solution adjusted to 2.8 with sulfuric acid (60%) at 90 °C. Alternatively the pH of the deuterium oxide solution was adjusted to 2.8 with 60% sulfuric acid at 90 °C. The aspartic acid obtained from the first run contains some unlabeled or partially labeled aspartic acid, which can either be discarded or used. All the following batches contained material with >98% deuterium incorporation with an ee >99%: mp >300 °C; $^1H$ NMR ($^2H_2O$) δ 4.05 (d, 1 H, 2-C*H*), 2.94 (d, 1 H, 3-$C^2H_R H_S$).

**Acknowledgment.** This work is supported by U.S. Public Health Service Grant GM 36184. K. M. Lee and J.-K. Son are H. Helfman Pharmacy Student Aid Fellows. We are grateful to the U.S. P.H.S. and the College of Pharmacy for their contributions to the purchase of the IBM 270-MHz NMR and the GE 500-MHz NMR.

**Registry No.** 1, 762-42-5; **2a**, 24461-32-3; **2a** (dimethyl ester), 23057-98-9; **2b**, 110-17-8; **3a**, 73421-13-3; **3b**, 60132-85-6; **4a**, 120204-64-0; **4b**, 120204-65-1; **5a**, 118942-75-9; **5a**·HCl, 120294-36-2; **5b**, 118942-74-8; **5b**·HCl, 120294-37-3; $Ph_3CCl$, 76-83-5; $D_2$, 7782-39-0; EC 4.3.1.1, 9027-30-9.

(23) This particular *E. coli* strain is quite common and probably available from most university biochemistry or microbiology departments.

(24) Aldrich Catalog of Fine Chemicals, page 127, 1988.

(25) The immobilization of *E. coli* cells containing aspartase activity using polyurethane prepolymers has recently been described by M. C. Fusee in *Methods in Enzymology*, Colowick, S. P., Kaplan, N. O. Eds.-in-Chief, Vol. 136, Mosbach, K., Ed.; Academic Press, Inc.: New York, 1987; pp 463–471.

(26) It was pointed out by one of the referees that Rohm and Van Etten (Rohm, K. H.; Van Etten, R. L. *J. Labelled Compd. Radiopharm.* **1985**, *22*, 909–915) have utilized aspartase to convert [1,4-$^{13}C_2$]fumaric acid to [1,4-$^{13}C_2$]aspartic acid. The immobilized system reported here of course should carry out this transformation in equally high yields as those reported for the deuteriated aspartic acids.

(27) In an effort to further simplify the procedure, we attempted to produce unlabeled aspartic acid utilizing an immobilized system derived from lyophilized cells of *E. coli* strain B (ATCC 11303), available in bulk directly from Sigma Chemical Co. The paste obtained from these freeze-dried cells, however, failed to produce any significant quantity of aspartic acid.