K_2HPO_4, and brine, dried with K_2CO_3, filtered, evaporated, and chromatographed on 80 g of silica gel with 9:1 $CHCl_3$–MeOH, R_f ~0.5, affording 3.11 g (70%) of **15**: NMR Boc, Gly, Phe, and mustard as above ($CHCH_2Ph$ at 5.1 ppm).

2-(Perfluoro-*n*-octyl)ethyl Iodide.[29] *n*-Perfluorooctyl iodide, 19.5 g (36 mmol), was placed into a flask equipped with a gas inlet and water-cooled condenser topped with a dry ice condenser. Ethylene gas was admitted, 0.19 g (0.8 mmol) of benzoyl peroxide was added, and the mixture was heated for 5 h at 85 °C under ethylene. The product was obtained as a white powder: yield 20.33 g (99.3%); mp 54–55.5 °C; NMR 2.3–2.9 (m), 3.1–3.5 (m) ppm; mass spectrum, *m/e* 574.

2-(Perfluoro-*n*-octyl)ethylamine (17).[30] The previous compound, 5 g (8.7 mmol), was placed into a bomb with 15 mL of *n*-BuOH and 1.33 g (89 mmol) of NH_3, heated to 80 °C for 3.5 h, cooled, and vented. Aqueous 10% NaOH was added, and the mixture was extracted 3 times with ether. The combined ether extracts were washed with brine, dried with K_2CO_3, filtered, and treated with anhydrous HCl. Solvents were evaporated, with the BuOH taken off at reduced pressure at 60–70 °C. The residue was washed with hexane, affording 1.06 g (24.5%) of **17**·HCl as a white powder. Owing to the volatility of the free amine, **17** was stored as its hydrochloride. NMR (CD_3OD) 2.68 (t of t, J = 18 and 7 Hz, CH_2CF_2), 3.36 (t, J = 7 Hz, $CH_2NH_3^+$) ppm; mass spectrum, *m/e* 462 (free base). Anal. ($C_{10}F_{17}H_7NCl$) C, H, N; C: calcd, 24.05; found, 23.53.

Carbobenzoxyglycylphenylalanyl Derivative (16) of 2-(Perfluorooctyl)ethylamine. Compound **17**·HCl, 0.75 g, (1.9 mmol), was converted to the free base by stirring with 20 mL of CH_2Cl_2, 10 mL of 50% NaOH, and 5 mL of H_2O for 30 min. The aqueous layer was saturated with K_2CO_3. The organic layer was decanted, dried with K_2CO_3, filtered, and added at −10 °C to a stirred solution of 0.84 g (2.4 mmol) of Z-Gly-Phe, 0.21 mL (1.5 mmol) of Et_3N, and 0.140 mL (1.5 mmol) of ethyl chloroformate in CH_2Cl_2. The reaction mixture was stirred under N_2 for 3 h at −10 °C and for 28 h at room temperature. The solvent was evaporated and replaced with EtOAc. The solution was washed with aqueous H_3PO_4, water, K_2HPO_4, and brine, dried with $MgSO_4$, filtered, and evaporated. PLC using 1:1 $CHCl_3$–EtOAc, R_f ~0.5, afforded 491 mg (41%) of **16**: NMR 3.5 (m, $C_8F_{17}CH_2CH_2$) ppm; NMR for Z-Gly-Phe as before; mass spectrum, *m/e* 801.

Acknowledgment. We thank Mark A. Vitale for preparing compounds **16** and **17** and Robert W. Walker for some of the GC–MS determinations.

(29) M. Knell, U. S. Patent 4 058 573 (1974).
(30) L. Foulletier and J. Lalu, U.S. Patent 4 059 629 (1977).

DL-*threo*-β-Fluoroaspartate and DL-*threo*-β-Fluoroasparagine: Selective Cytotoxic Agents for Mammalian Cells in Culture

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Absolute configuration assignments have been made for the diastereomers of DL-β-fluoroaspartate by X-ray analysis. The cytotoxicity of these isomers against various mammalian cells was examined. DL-*threo*-β-Fluoroaspartate shows selective cytotoxicity. Growth of the most sensitive cells is completely inhibited by 13 μM DL-*threo*-β-fluoroaspartate in the presence of 100 μM L-aspartate, a component of the culture medium. A difference in the rate of transport of DL-β-fluoroaspartate among the cells studied is an important factor determining cell specificity. For those cells that are sensitive to DL-β-fluoroaspartate, the threo isomer is, in all cases, more potent than the erythro isomer. Radioactivity derived from L-*threo*-β-fluoro[^{14}C]aspartate is incorporated into proteins at a rate comparable to the rate of incorporation from L-[^{14}C]aspartate. We synthesized DL-*threo*-β-fluoroasparagine. This compound is also cytotoxic but less specific and less potent than DL-*threo*-β-fluoroaspartate. However, the cell specificity can be enhanced in the presence of 1 mM L-aspartate, which can protect some cells but not others from the cytotoxic effects of DL-*threo*-β-fluoroasparagine. Jensen sarcoma cells, which require asparagine, are not protected by L-aspartate. Therefore, a combination of L-aspartate and DL-*threo*-β-fluoroasparagine can be used to inhibit specifically the growth of asparagine-requiring tumors.

The synthesis of analogues of biological substrates represents one of the important approaches to the preparation of pharmacologically useful compounds. In designing analogues of biologically active molecules, one would like to introduce minimal structural changes and still affect the chemical properties of the molecule. Substitution of fluorine for hydrogen leads to compounds which meet these criteria. Fluorine is not much larger than hydrogen but can significantly affect the chemical properties of the molecule. For instance, the substitution of one of the α-hydrogens of acetic acid by fluorine lowers the pK of the carboxyl group by 2 units. The biological properties of many fluorine-containing compounds have been explored.[1-14] However, the properties of polyfunctional amino acids[15] in which fluorine is next to a functional group have not been extensively studied.[16,17] These amino

(1) For a review, see R. Filler, in "Biochemistry Involving Carbon–Fluorine Bonds", American Chemical Society, Washington, DC, 1976; *ACS Symp. Ser.*, **no. 28** (1976).
(2) C. Heidelberger, in "Antineoplastic and Immunosuppressive Agents", Part II, A. C. Sartorelli and D. G. Johns, Eds., Springer-Verlag, Berlin, 1975, p 192.
(3) E. D. Nicholaides, M. K. Craft, and H. A. DeWald, *J. Med. Chem.*, **6**, 524 (1963).
(4) G. H. Fisher, J. W. Ryan, and P. Berryer, *Cardiovasc. Med.*, **2**, 1179 (1977).
(5) L. Bernardi, G. Bosisio, F. Chillemi, G. DeCaro, R. De Castiglione, V. Erspamer, and O. Goffredo, *Experentia*, **22**, 29 (1966).
(6) P. Marbach and J. Rudinger, *Helv. Chim. Acta.*, **57**, 403 (1974).
(7) J. S. Morley, *Proc. R. Soc. London, Ser. B.*, **170**, 97 (1968).
(8) I. M. Chaiken, M. H. Freedman, J. R. Lyerla, Jr., and J. S. Cohen, *J. Biol. Chem.*, **248**, 884 (1973).
(9) D. H. Coy, E. J. Coy, Y. Hirotsu, J. A. Vilchez-Martinez, A. V. Schally, J. W. van Nispan, and G. I. Tesser, *Biochemistry*, **13**, 3550 (1974).

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Table I. Effects of DL-β-Fluoroaspartate on the Growth of Six Cell Strains[a]

expt	treatment	dose, μg/mL	cell protein,[b] μg/dish					
			MH_1C_1	H_4	$7800C_1$	R_5	GH_4C_1	GH_3
1	none		178	180	246	144	96	93
	DL-*threo*-β-fluoroaspartate	2	154	114	10	128	96	69
	DL-*threo*-β-fluoroaspartate	5	167	9	9	98	98	23
	DL-*threo*-β-fluoroaspartate	20	140	4	6	50	36	22
	DL-*erythro*-β-fluoroaspartate	10	159	150	249	122	126	104
	DL-*erythro*-β-fluoroaspartate	75	149	156	229	90	126	61
2	none		69	27	87	104	40	38
	DL-*threo*-β-fluoroasparagine	10	55	0	15	85	35	36
	DL-*threo*-β-fluoroasparagine	50	31	0	0	27	16	14

[a] Each cell strain was plated [initial plating densities and medium: expt 1, MH_1C_1, 1×10^4 (F 10$^+$); H_4, 8×10^3 (MEM); $7800C_1$, 1×10^4 (MEM); R_5, 1×10^3 (F 10$^+$); GH_4C_1, 8×10^4 (F 10$^+$); GH_3, 2×10^4 (F 10$^+$). Expt 2, same as expt 1 except H_4, 2×10^3 (MEM); GH_4C_1, 2×10^4 (F 10$^+$)] in 35-mm dishes containing 1.5 mL of medium and cultured for 3 days. Medium for all strains was replaced with 1.5 mL of F 10$^+$ medium, and treatment was then initiated. Incubation was continued for 4 days, and the dishes were processed for protein determination. [b] The values given for experiment 1 are single determinations. Experiment 1 has been repeated twice with similar results. The values given for experiment 2 are the mean of duplicate samples (range ± 20%).

Table II. Amino Acid Uptake[a]

labeled amino acid	amino acid uptake,[b] nanomol/10^6 cells					
	MH_1C_1	H_4	$7800C_1$	R_5	GH_4C_1	Jensen sarcoma
L-aspartate[c]	0.5	23.4	13.2	0.4	1.1	0.4
L-*threo*-β-fluoroaspartate[c]	0.1	28.8	10.2	0.2	1.3	0.04
L-*erythro*-β-fluoroaspartate[c]	0.1	9.0	3.5	0.1	0.1	0.02

[a] Cells were plated in 100-mm dishes containing 8 mL of F 10$^+$ medium. On the 4th day, medium was replaced with 8 mL of fresh F 10$^+$. Three hours after this medium change, a 200-μL aliquot of a solution containing the appropriately labeled amino acid was added to duplicate dishes of each cell strain. Incubation was continued for 6 h. The medium was then removed, the dishes were washed, and the cell suspension was processed for uptake determination as described under Experimental Section. [b] Values given are the mean of duplicate samples. The range was ±15%. During the 6-h incubation, two untreated dishes from each cell strain were used to determine the cell number (cells/dish): $7800C_1$, 7×10^5; GH_4C_1, 2.2×10^6; MH_1C_1, 2.8×10^6; R_5, 4.3×10^6; H_4, 2.1×10^6; Jensen sarcoma, 3.4×10^6 (±15% range). [c] The specific activities (dpm/μmol) of the amino acids were 370 000 (L-aspartate), 630 000 (L-*threo*-β-fluoroaspartate), and 720 000 (L-*erythro*-β-fluoroaspartate). The initial concentrations of the amino acids were 4.9×10^{-5} M (6.5 μg/mL) (L-aspartate), 4.0×10^{-5} M (6.0 μg/mL) (DL-*threo*-β-fluoroaspartate), and 3.8×10^{-5} M (5.7 μg/mL) (DL-*erythro*-β-fluoroaspartate; when DL is used, carrier was a racemic mixture).

acids are of particular interest, since their functional groups frequently play an important role in the biological activity of the protein. They are involved in the maintenance of structural integrity and in ligand binding, and when located in the active site of enzymes, they are essential in catalysis. If fluorine-containing polyfunctional amino acids are incorporated into proteins, the presence of fluorine next to these functional groups could profoundly alter the biological properties of protein. With these considerations in mind, we decided to explore the biological properties of β-fluoroaspartate and β-fluoroasparagine. It was of particular interest to establish whether these amino acids are effectively incorporated into protein.

While this work was in progress, the syntheses of DL-*erythro*-β-fluoroaspartate and DL-*erythro*-β-fluoroasparagine were reported.[18] No significant biological activity for these analogues was found in the system studied.[18] The results presented in this report show that DL-*threo*-β-fluoroaspartate is a specific cytotoxic agent for certain tumor cells in culture, significantly more potent than the erythro isomer. In addition, we have synthesized DL-*threo*-β-fluoroasparagine and have found it to be selectively cytotoxic.

Results

Structure Determination of the Isomers of DL-β-Fluoroaspartic Acid. The synthesis of DL-*threo*- and DL-*erythro*-β-fluoroaspartic acid has been reported without configurational assignment.[21] DL-*erythro*-β-Fluoroaspartic acid has been synthesized via a different route, and its structure has been established by X-ray crystallographic analysis.[18] A comparison of the ^{1}H NMR spectrum reported for the DL-erythro isomer[18] with the ^{1}H NMR spectra of the diastereomers of DL-β-fluoroaspartate, designated by the Merck group[21] as compounds **16** and **17**, indicates that compound **16** is the erythro isomer. This assignment cannot, however, be considered absolute, since the NMR spectra of compounds **16** and **17** are very similar to each other (see Experimental Section) and neither one corresponds exactly to the reported[18] spectrum of the erythro isomer.[36] An absolute assignment of the diaste-

(10) B. M. Dunn, C. DiBello, K. L. Kirk, L. A. Cohen, and I. M. Chaiken, *J. Biol. Chem.*, **249**, 6295 (1974).
(11) P. Lu, M. Jarema, K. Mosser, and W. E. Daniel, Jr., *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 3471 (1976).
(12) W. E. Hull and B. D. Skyes, *Biochemistry*, **15**, 1535 (1976).
(13) D. S. Hagen, J. H. Weiner, and B. D. Skyes, *Biochemistry*, **17**, 3860 (1978).
(14) W. H. Vine, K. Hsieh, and G. R. Marshall, *J. Med. Chem.*, **24**, 1043 (1981).
(15) The term polyfunctional amino acids is used to designate amino acids which contain a functional group in addition to the α-carboxyl and α-amino group. Examples of polyfunctional amino acids are acid amino acids, basic amino acids, serine, etc.
(16) V. Tolman and J. Benes, *J. Fluorine Chem.*, **7**, 397 (1976).
(17) R. L. Buchanan, F. H. Dean, and F. L. M. Pattison, *Can. J. Chem.*, **40**, 1571 (1962).
(18) M. J. Wanner, J. J. M. Hageman, G.-J. Koomen, and U. K. Pandit, *J. Med. Chem.*, **23**, 85 (1980).
(19) A. G. Redfield, S. D. Kunz, and E. K. Ralph, *J. Magn. Reson.*, **19**, 114 (1975).
(20) C. W. Jones III, D. E. Leyden, and C. H. Stammer, *Can. J. Chem.*, **47**, 4363 (1969).
(21) J. Kollonitsch, S. Marburg, and L. M. Perkins, *J. Org. Chem.*, **44**, 771 (1979).

Table III. Distribution of Radioactivity Derived from ^{14}C-Labeled Amino Acids Taken Up by 7800C$_1$ Cells[a]

labeled amino acid	total uptake[b]	acid soluble[b]	acid insoluble		recovery, %
			protein[b]	nucleic acids[b]	
L-aspartate	21	4.9	10.7	1.8	83
L-*threo*-β-fluoroaspartate	17	6.8	7.5	0.3	86
L-*erythro*-β-fluoroaspartate	9.5	2.0	3.7	0.4	64

[a] 7800C$_1$ cells were plated in 100-mm culture dishes containing 8 mL of MEM$^+$ medium. On the 4th day, medium was replaced with 8 mL of fresh MEM$^+$. Six hours after this medium change, a 400-μL aliquot of the appropriately labeled amino acid was added to duplicate dishes. Incubation was continued for 12 h. The medium was then removed, the dishes were washed, and the cell suspension was processed as described under Experimental Section. [b] In nanomoles per 10^6 cells. Values given are the mean of duplicate samples. The range was ±10%. During the 12-h incubation, two untreated plates were used to determine the cell number: 1.1×10^6 cells/dish ± 10%. [c] The specific activities (dpm/μmol) of the amino acids were 211 000 (L-aspartate), 1 690 000 (L-*threo*-β-fluoroaspartate), and 1 270 000 (L-*erythro*-β-fluoroaspartate). The initial concentrations of the amino acids were 4.0×10^{-5} M (5.2 μg/mL) (L-aspartate), 4.0×10^{-5} M (6.0 μg/mL) (DL-*threo*-β-fluoroaspartate), and 4.5×10^{-5} M (6.7 μg/mL) (DL-*erythro*-β-fluoroaspartate; when DL is used, carrier was a racemic mixture).

reomers of DL-β-fluoroaspartic acid was made by an X-ray crystallographic analysis of compound **17**. Inspection of Figure 1 reveals that the molecule is the threo isomer of β-fluoroaspartic acid, as had been anticipated from the NMR spectral comparisons discussed above.

It is notable that the solid-state conformation of the threo isomer is quite different from that of the erythro isomer (see Newman projections, Figure 1B). In the threo isomer, the carboxyl groups are antiperiplanar, and in the erythro isomer, they are syn-clinal; apparently, these conformations result from a favorable F···NH_3^+ interaction (Table SIII; see paragraph at the end of paper concerning supplementary material).

Cytotoxic Properties of DL-β-Fluoroaspartate. Table I (experiment 1) summarizes the effects of DL-*threo*- and DL-*erythro*-β-fluoroaspartate on the growth of six cell strains in culture, and Table II summarizes the rates of uptake of these amino acid analogues by the same cells. The toxicity of DL-*threo*-β-fluoroaspartate varied greatly among cell strains. The compound was highly toxic to H_4 and 7800C$_1$ cells, moderately toxic to R_5, GH_4C_1, and GH_3 cells, and showed little or no cell toxicity at the concentration used to MH_1C_1 cells. In all cases, the erythro isomer was less toxic. The toxicity of DL-*threo*-β-fluoroaspartate was related, at least in part, to uptake of the compound (Table II).

The fate of fluoroaspartate was examined, particularly its incorporation into macromolecules. The results are summarized in Table III. Radioactivity derived from *threo*-β-fluoroaspartate was incorporated into protein. The amount of radioactivity incorporated from β-fluoro[^{14}C]-aspartate was 70% of the radioactivity incorporated into protein from [^{14}C]aspartate. In contrast to comparable incorporation into protein, radioactivity in the nucleic acid fraction derived from *threo*-β-fluoroaspartate was only 17% that of radioactivity derived from aspartate. Radioactivity incorporated into protein from L-*erythro*-β-

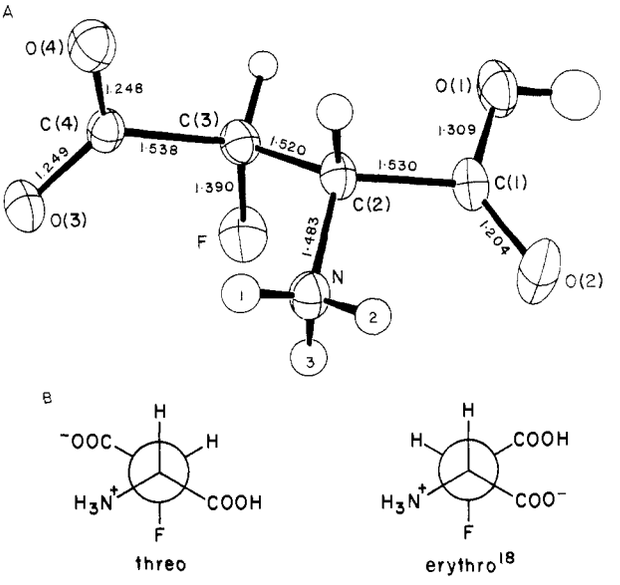

Figure 1. (A) X-Ray analysis of DL-*threo*-β-fluoroaspartic acid dihydrate. The molecular structure of β-fluoroaspartic acid, showing 50% thermal vibration ellipsoids for atoms refined anisotropically. Standard deviations of the bond lengths shown are 0.001 Å. The crystal contains a racemic mixture of these molecules. (B) Newman projections of *threo*-β-fluoroaspartate derived from 1A and of *erythro*-β-fluoroaspartate derived from Wanner et al.[18]

(22) B. M. Foxman, *Inorg. Chem.*, 17, 1932 (1978); B. M. Foxman and H. Mazurek, *ibid.*, 18, 113 (1979).
(23) Y. Liwschitz and A. Singerman, *J. Chem. Soc. C*, 1696 (1967).
(24) When the electrophoretogram at pH 1.9 was heavily loaded with 500 nmol, two faint ninhydrin-positive spots appeared. Each spot had an intensity which was less than or equal to the intensity of 1 nmol of DL-*threo*-β-fluoroasparagine. One of these spots comigrated with DL-*erythro*-β-fluoroasparagine. Preliminary results have shown that DL-*erythro*-β-fluoroasparagine is a less potent cytotoxic agent than DL-*threo*-β-fluoroasparagine for Jensen sarcoma cells.
(25) R. T. Kelly, T. E. Schmidt, and J. G. Haggerty, *Synthesis*, 544 (1972).
(26) T. Gross and L. Schirch, *J. Biol. Chem.*, **243**, 5651 (1968); L. Schirch, *Methods Enzymol.*, **17B**, 335 (1971); L. Schirch and A. Diller, *J. Biol. Chem.*, **246**, 3961 (1971).
(27) A. H. Tashjian, Jr., Y. Yasumura, L. Levine, G. H. Sato, and M. L. Parker, *Endocrinology*, **82**, 342 (1968).
(28) U. I. Richardson, A. H. Tashjian, Jr., and L. Levine, *J. Cell Biol.*, **40**, 236 (1969).
(29) U. I. Richardson, P. J. Sondgrass, C. T. Nazum, and A. H. Tashjian, Jr., *J. Cell Physiol.*, **83**, 141 (1974).
(30) H. C. Pitot, C. Peraino, P. A. Morse, Jr., and V. R. Potter, *Natl. Cancer Inst. Monogr.*, **13**, 229 (1964).
(31) T. A. McCoy, M. Maxwell, and P. F. Kruse, Jr., *Can. Res.*, **19**, 591 (1959).
(32) M. K. Patterson, Jr., M. D. Maxwell, and E. Conway, *Can. Res.*, **29**, 296 (1969).
(33) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
(34) D. Kennel, *Methods Enzymol.*, **12A**, 686 (1967).
(35) E. Layne, *Methods Enzymol.*, **3**, 453 (1957).
(36) A comparison of the ^{1}H NMR spectra for both diastereomers taken in D_2O and 12% DCl, D_2O (see Experimental Section), indicate that the chemical shifts are functions of pH. Exact conditions were not given for the ^{1}H NMR spectrum of *erythro*-β-fluoroaspartate previously reported.[18] The coupling constants and the difference between the chemical shift of the α- and β-hydrogens did not vary with pH and were different for each diastereomer. A comparison of these values with those previously reported[18] was used to make a tentative configuration assignment.

Table IV. Reversal of Effects of DL-*threo*-β-Fluoroaspartate and DL-*threo*-β-Fluoroasparagine on the Growth of H_4 Cells

expt	treatment (μg/mL)	cell protein,[b] % control
1	L-aspartate (133)	115
2	L-aspartate (330)	77
1	L-asparagine (133)	103
2	L-asparagine (330)	100
1	DL-*threo*-β-fluoroaspartate (5)	0
2	DL-*threo*-β-fluoroaspartate (5)	5
1	L-aspartate (133) + DL-*threo*-β-fluoroaspartate (5)	79
2	L-aspartate (330) + DL-*threo*-β-fluoroaspartate (5)	78
1	L-asparagine (133) + DL-*threo*-β-fluoroaspartate (5)	0
2	L-asparagine (330) + DL-*threo*-β-fluoroaspartate (5)	17
1	DL-*threo*-β-fluoroasparagine (50)	0
2	DL-*threo*-β-fluoroasparagine (40)	17
1	L-aspartate (133) + DL-*threo*-β-fluoroasparagine (50)	68
2	L-aspartate (330) + DL-*threo*-β-fluoroasparagine (40)	93
1	L-asparagine (133) + DL-*threo*-β-fluoroasparagine (50)	19
2	L-asparagine (330) + DL-*threo*-β-fluoroasparagine (40)	80

[a] Experiment 1. H_4 cells were plated at an initial density of 1.4×10^4 cells per 35-mm dish containing 1.5 mL MEM$^+$. On the 4th day the medium was replaced with fresh MEM$^+$ (1.5 mL) and treatment was initiated as shown below. Incubation was continued for 66 h. Medium was then removed, and dishes were processed for protein determinations. Experiment 2. Similar to experiment 1, except for the following changes: initial plating density 8×10^3; F 10$^+$ medium, 72-h incubation following treatment. [b] Cell proteins in the untreated control were 204 μg/dish in experiment 1 and 180 μg/dish in experiment 2. Values given are the mean of duplicate samples. The range was ±15%.

fluoro[^{14}C]aspartate was 50% of the amount derived from L-*threo*-β-fluoroaspartate. This difference is probably due to the lower rate of uptake of the erythro isomer and probably not due to a difference in the rate of incorporation into proteins once the amino acid has been taken up. The acid-soluble fraction was examined by high-voltage electrophoresis at pH 8.9. Three radioactive compounds were detected. One of these, corresponding to 40% of the total radioactivity present in the acid-soluble fraction, comigrated with *threo*-β-fluoroaspartate. The other two compounds, which were present in approximately equal amounts, have not been identified.

A possible mechanism for the cytotoxicity by β-fluoroaspartate is the inactivation of thymidylate synthetase[37] by 5-fluorouracil, which may be formed from β-fluoroaspartate. However, inclusion of thymidine (2×10^{-4} M) in the medium did not reduce the toxicity due to DL-*threo*-β-fluoroaspartate (5 μg/mL) (data not shown).

Since aspartate can be converted to asparagine in a reaction catalyzed by asparagine synthetase, it was possible that the mechanism of cytotoxicity of DL-*threo*-β-fluoroaspartate involved conversion to L-*threo*-β-fluoroasparagine. We therefore examined the effect of DL-*threo*-β-fluoroasparagine on the cell strains shown in Table I (experiment 2). At a concentration 50 μg/mL (3.3×10^{-4} M), DL-*threo*-β-fluoroasparagine was toxic to all the cell strains tested. H_4 cells were particularly sensitive to both DL-*threo*-β-fluoroaspartate and DL-*threo*-β-fluoroasparagine. Table IV summarizes the results of two experiments designed to determine the effects of L-aspartate and L-asparagine on the cytotoxicity of DL-*threo*-β-fluoroaspartate and DL-*threo*-β-fluoroasparagine in H_4 cells. DL-*threo*-β-Fluoroaspartate cytotoxicity was prevented by L-aspartate but not by L-asparagine. Presumably, the failure of L-asparagine to alleviate the cytotoxicity reflects the low rate of intracellular conversion of asparagine to aspartate. DL-*threo*-β-Fluoroasparagine cytotoxicity was prevented by both L-aspartate and L-asparagine.

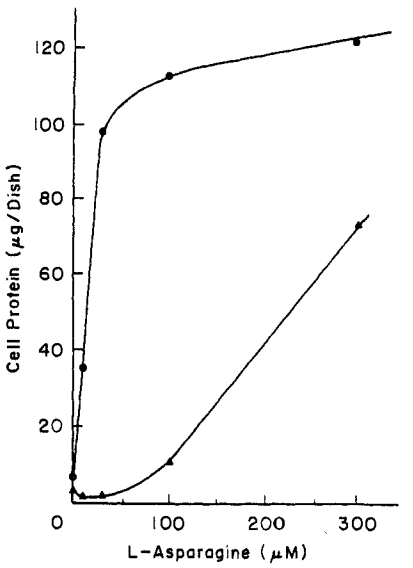

Figure 2. Effects of L-asparagine on the growth of Jensen sarcoma cells in the absence and in the presence of DL-*threo*-β-fluoroasparagine. Cells were plated at a density of 2.5×10^4 cells per 35-mm dish in 1.5 mL of F 10$^+$ medium. Four hours after plating, the medium was changed to MEM$^+$, and cells were treated with various amounts of L-asparagine in the presence and in the absence of 50 μg-mL DL-*threo*-β-fluoroasparagine for 4 days: (●) no β-fluoroasparagine added; (▲) β-fluoroasparagine added.

The possible involvement of asparagine synthetase in the mechanism of cytotoxicity of DL-*threo*-β-fluoroaspartate and in the prevention of DL-*threo*-β-fluoroasparagine cytotoxicity by aspartate was examined using Jensen sarcoma cells, which lack asparagine synthetase and are therefore asparagine dependent. Figure 2 shows the asparagine dependency of these cells and their response to DL-*threo*-β-fluoroasparagine. As the asparagine concentration was increased, a reduction of toxicity due to DL-*threo*-β-fluoroasparagine was observed. Results summarized in Table V show that L-aspartate did not prevent the cytotoxic effects of DL-*threo*-β-fluoroasparagine. In addition, DL-*threo*-β-fluoroaspartate, at 40 μg/mL, was not cytotoxic to Jensen sarcoma cells. These results indicate an involvement of asparagine synthetase in the mechanism of cytotoxicity of DL-*threo*-β-fluoroaspartate and in the prevention of DL-*threo*-β-fluoroasparagine cytotoxicity by aspartate. An alternative explanation for these results would be the inability of Jensen sarcoma cells to transport L-aspartate and DL-*threo*-β-fluoroaspartate. These results in Table II show that Jensen sarcoma cells cannot transport these amino acids effectively.

Discussion

The findings presented in this report show that DL-β-fluoroaspartate is a cell-selective cytotoxic agent. Growth of the most susceptible cells ($7800C_1$) was essentially

(37) S. S. Cohen, J. G. Flaks, H. D. Barner, M. R. Loeb, and J. Lichtenstein, *Proc. Natl. Acad. Sci. U.S.A.*, 44, 1004 (1958); C. Heidelberger, G. Kaldor, K. L. Mukherjee, and P. B. Dannenberg, *Can. Res.* 20, 903 (1960).

Table V. Effects of DL-*threo*-β-Fluoroasparagine, DL-*threo*-β-Fluoroaspartate, L-Asparagine, and L-Aspartate on the Growth of Jensen Sarcoma Cells[a]

treatment (μg/mL)	cell protein,[a] μg/dish
none	14 ± 3
L-asparagine (4)	138 ± 23
L-aspartate (133)	10 ± 3
DL-*threo*-β-fluoroasparagine (50)	<5
L-asparagine (4) + DL-*threo*-β-fluoroasparagine (50)	13 ± 4
L-aspartate (133) + DL-*threo*-β-fluoroasparagine (50)	<5
DL-*threo*-β-fluoroaspartate (40)	16 ± 3
L-asparagine (4) + DL-*threo*-β-fluoroaspartate (40)	167 ± 8
L-aspartate (133) + DL-*threo*-β-fluoroaspartate (40)	16 ± 3

[a] Jensen sarcoma cells were plated at a density of 2.5 × 10^4 cells per 35-mm dish containing 1.5 mL of F 10^+ medium. Six hours after plating, the medium was changed to MEM$^+$ (1.5 mL), and the cells were treated as shown below. Incubation was continued for 4 days. Medium was then removed, and the dishes were processed for protein determinations. [b] Values given are means plus or minus range for duplicate dishes.

completely inhibited by DL-*threo*-β-fluoroaspartate at 2 μg/mL (13 μM), while growth of MH_1C_1 cells was reduced by only 20% at 20 μg/mL (130 μM). Both of these cell strains, as well as H_4 which is also highly sensitive to β-fluoroaspartate, are from minimal deviation rat hepatomas. It should be noted that DL-*threo*-β-fluoroaspartate shows significant cytotoxicity at approximately 10^{-5} M in the presence of 10^{-4} M L-aspartic acid, which is a component of the medium. We have shown that cytotoxicity can be reduced by aspartic acid (Table IV). Variation in the rates of L-*threo*-β-fluoroaspartate uptake among the cell strains tested is an important determinant of cellular specificity for the toxic actions of this compound. There was no correlation between the rates of cell division and response to DL-*threo*-β-fluoroaspartate.

In each cell strain, the erythro isomer was less toxic than the threo isomer. Appreciable uptake of L-*erythro*-β-fluoroaspartate without significant cytotoxicity implies involvement of a step in the mechanism of cytotoxicity other than transport which can discriminate between the diastereomers.

DL-*threo*-β-Fluoroasparagine showed cytotoxicity against all cell lines tested, including Jensen sarcoma, which is deficient in asparagine synthase. Cytotoxicity was prevented by L-asparagine in H_4 and Jensen sarcoma cells. However, L-aspartic acid reversed cytotoxicity only in H_4 cells. These results suggest that the cytotoxicity of β-fluoroasparagine can be made more selective by exposing the cells to a combination of L-aspartate and β-fluoroasparagine. Cells which can take up aspartate are protected against β-fluoroasparagine cytotoxicity. It remains to be established whether the ability to take up L-aspartate is sufficient to bring about protection or whether these cells must also be able to convert L-aspartate to L-asparagine; i.e., asparagine synthetase must be present. This is of particular interest, since some lymphoma and leukemia cells are deficient in asparagine synthase.[38-40] Thus, combined use of aspartic acid and β-fluoroasparagine could be used chemotherapeutically.

At this time, insufficient data are available to define the mechanism of toxicity of the fluoro amino acids. Toxicity could be due to conversion to a toxic metabolite, such as fluorocitrate, or to the inactivation of an essential enzyme. However, a striking property of β-fluoroaspartate is its apparent[41] incorporation into protein (Table III). The rates of incorporation into protein of radioactivity derived from β-fluoroaspartate and from aspartate by $7800C_1$ cells were nearly equal. This finding raises the possibility that nonfunctional proteins are synthesized. If intact fluoroaspartate is incorporated into protein, the β-carboxyl group will become a carboxyl group of the polypeptide chain. The β-fluoro group will lower the pK of the β-carboxyl group by approximately 2 pK units. This could seriously affect the biological activity of the protein. In addition, aspartic acid and asparagine have a high probability of being located at β turns of a peptide chain.[42] Introduction of fluorine into aspartate or asparagine could thus affect protein conformation and, consequently, biological function. It is also noteworthy that a point of glycosylation in proteins contains the sequence Asp-X-Ser or Thr.[43-46] Substitution of fluoroasparagine for asparagine may also adversly affect glycosylation either through effects on protein conformation or by a direct action on the site of glycosylation.[47] Failure to glycosylate could have serious biological consequences.

Experimental Section

NMR spectra were taken on a Bruker WH90 spectrometer and on a 270-MHz spectrometer[19] using DSS as an internal standard. IR spectra were taken on a Perkin-Elmer 683 infrared spectrophotometer. High-voltage electrophoresis (HVE) was carried out at pH 1.9 (450 mL of H_2O, 11 mL of 90% HCOOH, and 40 mL of glacial CH_3COOH) and at pH 8.9 [1% $(NH_4)_2CO_3$] using a Savant Model HVE-8036 apparatus. Samples were applied to paper sheets (57 × 23 cm; Whatman 3MM) and were run at pH 1.9 for 25 min at 3200 V and at pH 8.9 for 35 min at 1500 V. R_{asp} values are relative to the mobility of L-aspartic acid. A negative value indicates migration from the origin in a direction opposite that of aspartate. Radioactivity was determined by liquid scintillation counting (Beckman LS100C) in ACS (Amersham). Counting efficiency was determined by using [^{14}C]toluene (New England Nuclear Corp.) as an internal standard. Elemental analyses were performed by Galbraith Laboratories, Inc. (Knoxville, TN).

Synthesis of Amino Acid Analogues. DL-*threo*- and DL-*erythro*-β-Fluoroaspartic Acid. Dimethyl DL-*threo*-β-hydroxyaspartate hydrochloride[20] (1.0 g, 4.70 mmol) was "fluorodehydroxylated" according to the procedure of Kollonitsch et al.[21] with the following modifications: After 2.5 h at 0 °C, the reaction was cooled to −78 °C and the second addition of SF_4 was made. The dry ice–acetone mixture was then replaced with an ice bath, and after an additional 2.5 h at 0 °C, the HF was removed. A ^{1}H NMR spectrum of the hydrolyzed reaction mixture indicated the presence of both the *threo*- and *erythro*-β-fluoroaspartates in a 3:2 ratio. The concentrated hydrolysate was

(38) J. D. Broome and J. H. Schwartz, *Biochim. Biophys. Acta.*, **138**, 637 (1967).
(39) B. Horowitz, B. K. Madras, A. Meister, L. J. Old, E. A. Boyse, and E. Stockert, *Science*, **160**, 553 (1968).
(40) For a review, see D. A. Cooney and R. E. Handschumacher, *Annu. Rev. Pharmacol.*, **10**, 421 (1970).
(41) The radiolabeled species incorporated into protein may be intact *threo*-β-fluoroaspartate or a species derived from *threo*-β-fluoroaspartate.
(42) P. Y. Chou and G. D. Fasman, *Biochemistry*, **13**, 222 (1974).
(43) R. D. Marshall, *Biochem. Soc. Symp.*, **40**, 17 (1974).
(44) G. W. Hart, K. Brent, G. A. Grant, R. A. Bradshaw, and W. J. Lennarz, *J. Biol. Chem.*, **254**, 9747 (1979).
(45) D. P. Pless and W. J. Lennarz, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 134 (1977).
(46) E. Bause, *FEBS Lett.*, **103**, 296 (1979).
(47) We have observed inhibition of glycosylation of the proopiocortin molecule by DL-*threo*-β-fluoroasparagine (unpublished results of M. A. Phillips, A. M. Stern, R. H. Abeles, and A. H. Tashjian, Jr.).

applied to a 100 × 2 cm Dowex 50X 8 column (H^+ form), eluted with 200 mL of H_2O and then with 0.1 N HCl. After 5 column volumes, two ninhydrin-positive peaks emerged. The first peak corresponded by NMR to compound 17, as designated by the Merck Group,[21] and is the threo diastereomer (see below). The second peak corresponded by ^1H NMR to compound 16[21] and is the erythro diastereomer.[18] The fractions from the Dowex 50 column were monitored by high-voltage electrophoresis at pH 1.9. Fractions containing each of the diastereomers were pooled and concentrated in vacuo. Each diastereomer was applied to a 30 × 1.5 cm Dowex 1X8 column (formate form) and eluted first with H_2O (150 mL) and then with 0.4 N formic acid. Concentration of the ninhydrin-positive fractions yielded the free amino acid of each diastereomer, 100 mg (14%) of the threo isomer and 80 mg (11%) of the erythro isomer of DL-β-fluoroaspartic acid. DL-*threo*-β-Fluoroaspartic acid: ^1H NMR (270 MHz; D_2O) δ 4.46 (1 H, dd, J_{HF} = 29 Hz, J_{HH} = 2.4 Hz, C_α H), 5.50 (1 H, dd, J_{HF} = 45 Hz, J_{HH} = 2.4 Hz, C_β H); ^1H NMR (90 MHz; 12% DCl, D_2O) δ 4.89 (1 H, dd, J_{HF} = 29 Hz, J_{HH} = 2 Hz, C_α H), 5.87 (1 H, dd, J_{HF} = 44 Hz, J_{HH} = 2 Hz, C_β H). This diastereomer comigrates with authentic DL-β-fluoroaspartate (supplied by the Merck group[21] and designated as compound 17) as a single ninhydrin-positive spot in the following systems: HVE (pH 1.9) R_{asp} –0.58; HVE (pH 8.9 R_{asp} 1.54; TLC (cellulose; butanol–acetic acid–H_2O, 12:3:5) R_f 0.13; mp 175 °C dec [an authentic sample supplied by Merck[21] decomposed at 177 °C (lit.[21] mp 157–158 °C dec)].

X-ray Structure Determination. Crystals of DL-*threo*-β-fluoroaspartic acid dihydrate were obtained by slow crystallization from an acetone–water solution at 4 °C. The crystal used in the data collection was a fragment cut from a larger crystal. Most operations on the Syntex $P2_1$ diffractometer were performed as previously described.[22] Details of the structure analysis are available as supplementary information (see paragraph at the end of paper concerning supplementary material). All hydrogen atoms, including those bonded to water oxygen atoms, were unambiguously located in this analysis. Inspection of Figure 1 reveals that the molecule is the threo diastereomer of DL-β-fluoroaspartic acid.

DL-*erythro*-β-Fluoroaspartic acid: ^1H NMR (270 MHz; D_2O) δ 4.55 (1 H, dd, J_{HF} = 29 Hz, J_{HH} = 2.4 Hz, C_α H), 5.30 (1 H, dd, J_{HF} = 49 Hz, J_{HH} = 2.4 Hz, C_β H); ^1H NMR (90 MHz; 12% DCl, D_2O) δ 4.98 (1 H, dd, J_{HF} = 29 Hz, J_{HH} = 2 Hz C_α H), 5.74 (1 H, dd, J_{HF} = 48 Hz, J_{HH} = 2 Hz, C_β H). The erythro diastereomer migrated as a single ninhydrin-positive spot in the following systems: HVE (pH 1.9) R_{asp} 0.08; HVE (pH 8.9) R_{asp} 1.45; TLC (cellulose; butanol–acetic acid–water, 12:3:5) R_f 0.13; mp 174 °C dec (lit.[18] mp 174 °C dec; lit.[21] mp 144–145 °C dec).

DL-*threo*-β-Fluoroasparagine. DL-*threo*-β-Hydroxyasparagine[23] (1.0 g, 6.70 mmol) synthesized from DL-*threo*-β-hydroxyaspartic acid[20] was "fluorodehydroxylated" as the free amino acid as described above for DL-β-fluoroaspartic acid with the following modifications. The residue obtained after the removal of HF was dissolved in cold 0.2 N HCl (50 mL) and concentrated in vacuo. During the concentration process, which took 45 min, the bath temperature did not exceed 25 °C. The resulting yellow oil was dissolved in 20 mL of H_2O. Six milliliters of this solution was applied to a Dowex 50X8 column (120 × 0.9 cm) equilibrated at 4 °C. After elution with 1 column volume of H_2O and 3.5 column volumes of 0.4 N HCl, a ninhydrin-positive (red brown) peak emerged. High-voltage electrophoresis, pH 8.9, showed this material to be contaminated with DL-*threo*-β-hydroxyasparagine and DL-*threo*-β-fluoroaspartic acid. The mixture was concentrated in vacuo to a white solid and rechromatographed as described above. By monitoring with high-voltage electrophoresis, pH 8.9, peak fractions free from DL-*threo*-β-hydroxyasparagine but containing approximately 10% DL-*threo*-β-fluoroaspartic acid were pooled and concentrated. The white solid was dissolved in 6 mL of cold H_2O and applied to a 35 × 1.2 cm Dowex 1X8 column (formate form) equilibrated at 4 °C. Elution with H_2O yielded a ninhydrin-positive peak (red brown), which after lyophilization gave 82 mg (27%) of fluffy white DL-*threo*-β-fluoroasparagine: ^1H NMR (90 MHz; D_2O; 3 mg/mL) δ 4.41 (1 H, dd, J_{HF} = 33 Hz, J_{HH} = 2 Hz, C_α H), 5.74 (1 H, dd, J_{HF} = 45 Hz, J_{HH} = 2 Hz, C_β H); IR (KBr pellet) 3330, 3140 (NH_2), 3100–2600 (NH_3^+), 1680 (amide 1), 1640 (amide II), 1575 (COO^-) cm^{-1}. DL-*threo*-β-Fluoroasparagine migrated as a single ninhydrin-positive spot in the following systems: HVE (pH 1.9) R_{asp} 0.42;[24] HVE (pH 8.9) R_{asp} 1.02; TLC (cellulose; butanol–acetic acid–water, 12:3:5) R_f 0.22. DL-*threo*-β-Fluoroasparagine was hydrolyzed in 2.5 N HCl for 2 days at 37 °C. A single ninhydrin-positive species resulted, which comigrated with DL-*threo*-β-fluoroaspartate (HVE, pH 1.9 and 8.9). The formation of the threo diastereomer of β-fluoroaspartic acid upon the hydrolysis of DL-β-fluoroasparagine permits assignment of the threo configuration to the DL-β-fluoroasparagine obtained from DL-*threo*-β-hydroxyasparagine.

An analytical sample of DL-*threo*-β-fluoroasparagine was obtained by recrystallization from H_2O–acetone, mp 177 °C dec. Anal. ($C_4H_7F_1N_2O_3 \cdot H_2O$) C, H, F, N.

L-*threo*- and L-*erythro*-β-Fluoro[^{14}C]aspartic Acid. β-Methyl β-hydroxy[1,2-^{14}C]aspartate was synthesized from [1,2-^{14}C]glycine and methyl glyoxylate[25] by rabbit liver serine transhydroxymethylase (rabbit liver) (a generous gift from Dr. LaVerne Schirch)[26] as follows: A reaction mixture, 0.24 mL of 0.05 M potassium phosphate, pH 7.2, containing 4.5 μmol of [1,2-^{14}C]-glycine (63 μCi/μmol), 20 μmol of methyl glyoxylate, and 1 mg of serine transhydroxymethylase, was incubated at 37 °C for 15 h. Analysis by TLC (cellulose; butanol–acetic acid–water, 12:3:5) showed greater than 90% conversion of glycine to β-methyl L-β-hydroxyaspartate and L-β-hydroxyaspartic acid (some hydrolysis occurred during the reaction). Diastereomers were not separated. The incubation mixture was cooled and acidified with 1 N HCl. The protein precipitated and was removed by centrifugation. The resulting solution was concentrated in vacuo. The residue was dissolved in 0.3 mL of H_2O and applied to a 1.5-mL Dowex 50X8 column (H^+ form). After the column was washed with 3 mL of H_2O, 80% of the applied radioactivity was eluted as a single peak with 2.5 N HCl. DL-*threo*-β-Hydroxyaspartic acid (23 mg, 0.15 mmol) was added as carrier, and the resulting solution was concentrated in vacuo and dried over P_2O_5. The solid residue was esterified to the dimethyl ester hydrochloride with dimethyl sulfite in methanol (0.5 mL).[20] "Fluorodehydroxylation" was carried out as above using 4 mL of HF + 0.5 mL of SF_4. The concentrated reaction mixture was applied to a 120 × 0.4 cm Dowex 50X8 column (H^+ form) and eluted with 12 column volumes of H_2O. The diastereomers emerged as broad but separated peaks. Each ^{14}C-labeled diastereomer cochromatographed with its corresponding DL-β-fluoroaspartic acid as a single radioactive peak in the following systems: HVE (pH 1.9 and 8.9); paper chromatography (ascending), butanol–acetic acid–water (12:3:5), butanol–pyridine–water (1:1:1), methanol–pyridine–water (20:1:5). Specific activity (μCi/μmol): L-*threo*-β-fluoro[1,2-^{14}C]aspartic acid, 0.71; L-*erythro*-β-fluoro[1,2-^{14}C]aspartic acid, 4.2.

Other Amino Acids. L-Aspartic acid (Reliable Reagents), L-asparagine hydrate (Sigma Chemical Co.), [1,2-^{14}C]glycine (100 mCi/mmol; ICN), and L-[1,2,3,4-^{14}C]aspartic acid (225 mCi/mmol; New England Nuclear Corp.) were used without further purification. The purity of L-[1,2,3,4-^{14}C]aspartic acid was found to be >98% as determined by high-voltage electrophoresis, pH 8.9.

Cell Culture. The clonal strains of rat pituitary tumor cells GH_3 (ATCC CCL 82.1) and GH_4C_1 were grown in Ham's F 10 medium supplemented with 15% horse serum and 2.5% fetal calf serum (F 10^+) as described.[27] Three clonal strains of rat hepatoma cells were studied: MH_1C_1 (ATCC CCL 144)[28] were grown in F 10^+ medium; $7800C_1$[29] and H_4[30] were grown in Eagle's minimal essential medium supplemented with 5% horse serum and 5% fetal calf serum (MEM$^+$). The latter two strains can also be grown in F 10^+. Asparagine-requiring Jensen sarcoma cells (ATCC CCL 45)[31,32] were grown in F 10^+ medium. These cells can be grown in MEM$^+$ supplemented with asparagine (0.01–0.03 mM). R_5 cells are fibroblasts derived from the rat thyroid gland and are grown in F 10^+. Culture media and sera were obtained from Grand Island Biological Co.

Amino acids to be added to culture medium were prepared as 30- to 100-fold concentrates in either the appropriate medium or in 16 mM sodium phosphate, pH 7.3, 0.15 M NaCl. These solutions were sterilized by filtration. Aspartate solutions were neutralized with 0.2 N sodium hydroxide.

Total cell protein per dish determined as a function of time was used to measure cell growth.[27] Protein was determined by the method of Lowry et al.[32] using bovine albumin as the standard.

To determine amino acid uptake, cultures were treated with radiolabeled amino acids for the times indicated. Thereafter, the

medium was removed, and the monolayer was washed by dipping the dish into four successively 800-mL washes of cold 0.9% NaCl. The cells were then quantitatively removed from the dish, and aliquots of the suspension were used for determining total uptake. The distribution of radioactivity into acid-soluble and acid-insoluble cellular components was determined as follows. The cell suspension obtained above was diluted with trichloroacetic acid to a final concentration of 10%. After 3 h at 0 °C, the suspension was centrifuged. The supernatant solution containing the acid-soluble radioactivity was removed. The pellet was resuspended in 5% Cl_3AcOH and centrifuged. After the supernatant solution was removed, this procedure was repeated twice. The final pellet was resuspended in 5% Cl_3AcOH heated at 82 ± 1 °C for 30 min. The suspension was then cooled to 0 °C. The supernatant solution containing the nucleic acid fraction was removed. The pellet was quantitatively transferred to a glass-fiber filter (Whatman GF/C, 2.4 cm) presoaked with 5% Cl_3AcOH. The pellet containing cell protein was washed, and the radioactivity was determined as described.[34] The protein fraction, which could be redissolved in 0.5% $NaDodSO_4$, 0.1 M sodium phosphate, pH 7.3, had a λ_{max} at 276 nm; $A^{1\%}_{1cm}$ = 11.0; $A_{280/260}$ = 1.55 indicative of less than 1% contamination with nucleic acid.[35] All the radioactivity associated with the protein fraction eluted in the void volume on a Sephadex G-25 column run in the presence of 0.5% $NaDodSO_4$.

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Supplementary Material Available: Tables of data for the X-ray study of $C_4H_6FNO_4 \cdot 2H_2O$, atomic coordinates, selected bond angles, anisotropic thermal parameters, hydrogen atom distances and angles, and observed vs. calculated structure factors (12 pages). Ordering information is given on any current masthead page.

Synthesis and Biochemical Properties of Chemically Stable Product Analogues of the Reaction Catalyzed by *S*-Adenosyl-L-methionine Decarboxylase

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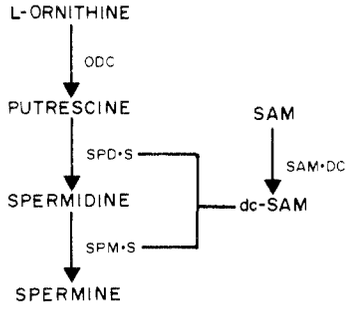
Structural analogues of decarboxylated *S*-adenosyl-L-methionine (dc-SAM), product of the reaction catalyzed by *S*-adenosyl-L-methionine decarboxylase (SAM-DC), with modifications in the side-chain portion of the molecule have been synthesized, and their ability to inhibit SAM-DC has been investigated. Mainly, compounds with a nitrogen atom in place of the sulfur were investigated. The data from these inhibition studies have resulted in a delineation of the structural features required for binding on SAM-DC. It was concluded that a terminal primary amino group, a terminal carboxyl group, and the sulfonium functionality are not required for binding on SAM-DC. It was also found that analogues of dc-SAM in which replacement of the sulfur by nitrogen was the only modification were still able to form an azomethine with the enzyme. As found for SAM and dc-SAM, these compounds also caused a time-dependent inactivation of SAM-DC.

Biosynthesis and accumulation of the polyamines putrescine, spermidine, and spermine appear intrinsically involved in cellular growth and proliferation.[1] In mammalian cells, polyamine biosynthesis involves the sequential action of two decarboxylases, L-ornithine decarboxylase (ODC; EC 4.1.1.17) and *S*-adenosyl-L-methionine decarboxylase (SAM-DC; EC 4.1.1.50), and two aminopropyltransferases, spermidine synthase (SPD-S; EC 2.5.1.16) and spermine synthase (SPM-S; EC 2.5.1.-) (Scheme I). Previous work[2] to develop effective inhibitors of polyamine biosynthesis in attempts to block the accumulation of polyamines in vivo was directed mainly toward L-ornithine decarboxylase. There are two reasons for this: first, the catalytic mechanisms of the aminopropyltransferases were not clarified until recently,[3] whereas the mechanism of action of pyridoxal phosphate dependent amino acid decarboxylases is well established;[4] second,

Scheme I



L-ornithine decarboxylation was believed to be the rate-limiting step in the polyamine pathway[5] and, therefore, obviously the preferred target for inhibition of polyamine biosynthesis.

Only recently, *S*-adenosyl-L-methionine decarboxylase was investigated for the purpose outlined above. SAM-DC most probably is the rate-limiting enzyme in the biosynthesis of spermidine and spermine.[6] It differs from ODC

(1) J. Jänne, H. Pösö, and A. Raina, *Biochim. Biophys. Acta*, **473**, 241 (1978).
(2) (a) B. W. Metcalf, P. Bey, C. Danzin, M. J. Jung, P. Casara, and J. P. Vevert, *J. Am. Chem. Soc.*, **100**, 2551 (1978), and references cited therein; (b) H. G. Williams-Ashman and Z. N. Canellakis, *Perspect. Biol. Med.*, **22**, 421 (1979).
(3) K. Tang, A. E. Pegg, and J. K. Coward, *Biochem. Biophys. Res. Commun.*, **96**, 1371 (1980); V. Zappia, G. Cacciapuoti, G. Pontoni, F. Della Ragione, and M. Carteni-Farina, *Adv. Polyamine Res.*, **3**, 39 (1981), and references cited therein.
(4) E. A. Boeker and E. E. Snell, *Enzymes, 3rd Ed.*, **6**, 217 (1972).
(5) D. H. Russell and S. H. Snyder, *Proc. Natl. Acad. Sci. U.S.A.* **60**, 1420 (1968); J. Jänne and A. Raina, *Acta Chem. Scand.*, **22**, 1349 (1968); A. E. Pegg, D. H. Lockwood, and H. G. Williams-Ashman, *Biochem. J.*, **117**, 17 (1970).

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