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Syntheses of Labeled Vitamers of Folic Acid to Be Used as Internal Standards in Stable Isotope Dilution Assays

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[$^2H_4$]Folic acid was synthesized by deuterating *p*-aminobenzoic acid, which was then coupled to glutamic acid and 6-formylpterin. Using [$^2H_4$]folic acid as starting component enabled the preparation of labeled vitamers tetrahydrofolate, 5-formyltetrahydrofolate, 5-methyltetrahydrofolate, and 10-formylfolate which were characterized by electrospray mass spectrometry and collision-induced dissociation. The mass spectrometric studies confirmed that the compounds could be used as internal standards in stable isotope dilution assays.

KEYWORDS: Electrospray mass spectrometry; folates; labeled folic acid; stable isotope dilution assay

INTRODUCTION

The folate group comprises several derivatives of folic acid differing by their one-carbon substituents, their oxidation states, and by the number of glutamate residues attached. The major problem in quantifying this group of B vitamins lies in the need to distinguish between the single derivatives, called vitamers, as they show different bioavailability and vitamin activity (*1*). Up to now, only the recently developed high-performance liquid chromatographic (HPLC) approaches (*2*, *3*) enabled the quantitation of the different folates, although these methods still lack accuracy. Because cleanup procedures include ion exchange chromatography (*4*) as well as several enzyme treatments (*5*), and because folates show different stabilities (*6*), recovery values for the vitamers differ widely (*2*, *7*).

In recent years we reported on the analysis of flavor compounds (*8*), of the mycotoxin patulin (*9*), and of the vitamin pantothenic acid (*10*), and demonstrated that stable isotope dilution assays (SIDA) exhibit excellent sensitivity and reliability and are accurate alternatives to other quantification methods. Using labeled analogues of the vitamers as internal standards enables losses of each analyte during cleanup to be precisely corrected.

Until recently, the use of stable isotopically labeled folates had been limited either to bioavailability studies (*11*) or to analyses including cleavage of folates prior to mass spectrometry (*12*, *13*). Hence, the information about concentrations of single vitamers was lost. The first study using a labeled folate and detecting it without destruction was reported by Pawlosky and co-workers who quantified folic acid in fortified foods (*14*) and 5-methyltetrahydrofolate in blood serum (*15*) by LC−MS without analyzing other folates. To quantify the naturally occurring vitamers, we decided to synthesize the isotopomers of important derivatives besides labeled folic acid.

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METHODS AND MATERIALS

Chemicals. The following chemicals were obtained commercially from the sources given in parentheses: deuterium oxide, dicyclohexylcarbodiimide, ethyl-3-(dimethylaminopropyl)carbodiimide, glutamic acid dimethylester, platinum oxide, [$^2H_6$]dimethyl sulfoxide ([$^2H_6$]DMSO), trifluoroacetic anhydride (Aldrich, Steinheim, Germany), tris(hydroxymethyl)-aminomethane (TRIS; Merck, Darmstadt, Germany), 2-amino-3-cyano-5-chlormethylpyrazine-4-oxide, dimethylaminoborane, *N*,*N*-dimethylaminonitrosobenzene, 1-hydroxybenzotriazole (Acros Organics, Geel, Belgium), guanidine hydochloride, phosphorus trichloride, pyridine (Merck, Darmstadt, Germany), Dowex 50-WX8 ion-exchange resin (hydrogen form), folic acid (Fluka, Neu-Ulm, Germany), 4-aminobenzoic acid (Sigma, Deisenhofen, Germany). Unlabeled folate vitamers tetrahydrofolate (H_4folate), 5-formylH_4folate, 5-methylH_4folate, and 10-formylfolate were purchased from Dr. Schirks Laboratories, Jona, Switzerland.

Liquid Chromatography/Mass Spectrometry (LC−MS). LC−MS spectra were recorded by means of a LCQ Classic (Finnigan MAT, Bremen, Germany) coupled to a spectra series high-performance liquid chromatograph (Thermo Separation Products, San Jose, CA). To verify the syntheses and to determine the degree of labeling, the LC−MS system was equipped with a 250 × 4.6 mm i. d., 5 μm, Discovery C18 column (Supelco, Bellefonte, PA). Aliquots of 1 to 10 μL of the sample solutions were chromatographed using gradient elution and a flow rate of 0.8 mL/min. A linear gradient was programmed within 9 min, starting with acetonitrile/1 mmol/L aqueous formic acid (7:93, v/v) to acetonitrile/1 mmol/L aqueous formic acid (20:80, v/v). Then the column was flushed with acetonitrile/1 mmol/L aqueous formic acid (80:20, v/v) for 4 min.

For analysis of folate vitamer mixtures, the LC−MS system was coupled to a 250 × 4.6 mm i. d.; 5 μm, Aqua C-18 reversed phase column (Phenomenex, Aschaffenburg, Germany). When using the latter column, the mobile phase consisted of variable mixtures of aqueous formic acid (0.1%) and acetonitrile, at a flow of 0.8 mL/min. Gradient elution started at 7% acetonitrile maintained for 9 min, followed by raising the acetonitrile concentration linearly to 13% within 13 min, and to 25% within further 4 min. Subsequently, the mobile phase was programmed to 100% acetonitrile over 4 min before equilibrating the column for 5 min with the initial mixture.

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Table 1. Base Peaks Produced by Collision-Induced Dissociation (CID) of the Quasimolecular Ions of Folate Vitamers in Positive Electrospray Mass Spectrometry

vitamer	precursor ion at *m/z*	product ion at *m/z* (rel. collision energy)
folic acid	442	295 (26%)
[$^2H_4$]folic acid	446	299 (26%)
tetrahydrofolic acid	446	299 (23.8%)
[$^2H_4$]tetrahydrofolate	450	303 (23.8%)
5-methyltetrahydrofolate	460	313 (23.8%)
[$^2H_4$]5-methyltetrahydrofolate	464	317 (23.8%)
5-formyltetrahydrofolate	474	327 (23%)
[$^2H_4$]5-formyltetrahydrofolate	478	331 (23%)
10-formylfolate	470	452 (24.5%)
[$^2H_4$]formylfolate	474	456 (24.5%)

The mass spectrometer was operated in the positive electrospray mode with a spray needle voltage of +3.55 kV and a spray current of 0.5 μA. The temperature of the capillary was 200 °C and the capillary voltage was +29.39 V. The sheath and auxiliary gas nitrogen nebulized the effluent with flows of 80 and 20 arbitrary units, respectively. The ion trap was operated at a helium pressure of 10^{-3} Torr. Collision-induced dissociation of folate vitamers was performed by applying to their quasimolecular ions the relative collision energies detailed in **Table 1**.

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H NMR spectra were recorded with an AMX-400 III (Bruker, Karlsruhe, Germany) at a frequency of 400.13 MHz. Shifts are expressed in ppm downfield from tetramethylsilane in [$^2H_6$]DMSO.

Anion Exchange Chromatography. Anion exchange cellulose DE-52 (Whatman, 23 g) was suspended in an aqueous solution of ammonium formate (0.15 mol/L, 150 mL, adjusted to pH 7) in a chromatography column (2 × 25 cm). The sample to be chromatographed was dissolved in an aqueous solution of ammonium formiate (0.15 mol/L, 5 mL), adjusted to pH 7 by an aqueous solution of sodium hydroxide (0.1 mol/L) and applied on the column. Elution was performed by a 2-h gradient of ammonium formate starting with 0.15 mol/L to 2 mol/L at a flow of 1.5 mL/min. The eluate was monitored by a Uvicord detector (LKB, Uppsala, Sweden) set at 280 nm.

Syntheses: Aminobenzoyl Glutamate Precursor. *[$^2H_4$]4-Aminobenzoic Acid (**2**).* 4-Aminobenzoic acid **1** (potassium salt; 3.0503 g, 17.41 mmol) was suspended with palladium on activated charcoal (1.31 g, 393 mg Pd) in deuterium oxide (12 mL) and heated at 200 °C in an autoclave for 2 h. The autoclave was cooled with cold water and the suspension was filtered and washed repeatedly with redistilled water. The solution was then lyophilized and the above process was repeated twice to give pure **2** (1.812 g, 10.123 mmol, 58.1%).

Positive ESI-MS: *m/z* (%) = 142 (100), 183 (42), 141 (12), 143 (7).

*Trifluoroacetyl-[$^2H_4$]4-aminobenzoic Acid (**3**).* The resulting [$^2H_4$]4-aminobenzoic acid (1.812 g) was suspended in cold trifluoroacetic anhydride (30 mL) and stirred at room temperature for 2 h. The mixture was then dried in a stream of nitrogen and directly used for the next synthetic step.

*Trifluoroacetyl-[$^2H_4$]4-aminobenzoylglutamate Dimethylester (**4**).* **3** was dissolved by stirring in anhydrous tetrahydrofuran (150 mL), and after addition of dicyclohexylcarbodiimide (DCC, 5 g, 24.23 mmol) and 1-hydroxybenzotriazole (3.27 g, 24.23 mmol) the mixture was stirred at room temperature for 30 min. Then, glutamic acid dimethylester (4.9 g, 23.16 mmol) and diisopropylethylamine (2.99 g, 23.16 mmol) were added, and the solution was stirred for a further 4 h at room temperature and then suction filtered. The filtrate was evaporated to dryness and provided the title compound (3.23 g, 8.199 mmol, 80%).

*[$^2H_4$]p-Aminobenzoylglutamic Acid (**5**).* Protected [$^2H_4$]4-aminobenzoylglutamic acid (3.81 mmol) was dissolved in methanol (20 mL) followed by addition of aqueous sodium hydroxide (100 mL, 0.1 mol/L). Then, the solution was stirred for precisely 1 h 45 min at room temperature before the reaction was quenched by adding hydrochloric acid (0.1 mol/L) to give a final pH of 8. Freeze-drying of the solution afforded **5** (778 mg, 2.89 mmol, 76%).

Pteridine Precursor. *2-Amino-3-cyano-5-chlormethylpyrazine (**7**).* To a solution of 2-amino-3-cyano-5-chlormethylpyrazine-4-oxide **6** (13.02 g, 70.56 mmol) in tetrahydrofuran (500 mL), phosphorus trichloride (27 g, 146.27 mmol, 17.15 mL) was added dropwise with ice-bath cooling. The solution was stirred for 45 min at room temperature and then concentrated to about 60 mL. After adding ice water (300 mL) the precipitating solid **7** (10.31 g, 61.16 mmol, 86.7%) was collected by centrifugation and washed thoroughly with water.

*1[(2-Amino-3-cyano-5-pyrazinyl)methyl]pyridinium Chloride (**8**).* Compound **7** (10.31 g) was added to a flask containing pyridine (110 mL). Some undissolved substances were removed by filtration, and the solution was stirred for 17.5 h at room temperature. After adding diethyl ether (800 mL) the salt **8** precipitated and was collected by centrifugation. Washing with diethyl ether twice gave pure **8** (12.04 g, 48.65 mmol, 79.5%).

*N-[p-(Dimethylamino)phenyl]-α-(2-amino-3-cyano-5-pyrazinyl)-nitrone (**9**).* The pyridinium salt **8** (12.04 g) was suspended in an ethanolic solution of *N,N*-dimethylaminonitrosobenzene (7.30 g, 48.60 mmol, 250 mL), and an aqueous solution of potassium carbonate (40.3 g, 291.6 mmol, 150 mL) was added. The solution was stirred for 30 min at room temperature followed by ice bath cooling. The mixture was centrifuged and the resulting nitrone **9** (10.42 g, 36.82 mmol, 75.7%) was washed twice with water, then washed with ethanol, and finally washed with diethyl ether.

*2-Amino-3-cyano-5-formylpyrazine (**10**).* A solution of nitrone **9** (10.42 g, 36.82 mmol) in at least 650 mL of cold hydrochloric acid (6 mol/L) was extracted with ethyl acetate (500 mL). The organic layer was separated, and the aqueous layer was twice extracted with ethyl acetate. Sodium chloride (70 g) was added to the aqueous layer, which was once again extracted with ethyl acetate. The organic layers were collected, dried over anhydrous sodium sulfate, and evaporated to give **10** (3.279 g, 22.15 mmol, 60.2%).

*2-Amino-3-cyano-5-formylpyrazine Dimethyl Acetal (**11**).* To a solution of formylpyrazine **10** (3.279 g, 22.15 mmol) in dry methanol (70 mL) Dowex 50-WX8 ion-exchange resin (hydrogen form, 7 g) was added. After the solution was stirred for 1 h, a few grams of 3 Å molecular sieves were added and the mixture was shaken briefly. The molecular sieves were filtered off and the solution was processed further without isolating the product.

*2,4-Diamino-6-formylpteridine Dimethyl Acetal (**12**).* The methanolic solution of **11** was mixed with a solution of guanidine hydochloride (2.9 g, 30.36 mmol) in a methanolic solution of sodium methylate (100 mL, 0.2 mol/L) and refluxed for 18 h at 70 °C. After the solution was cooled to room temperature, it was concentrated to a small volume and cooled to −30 °C. The resulting product (4.52 g, 19.15 mmol, 86.5%) was collected by centrifugation.

*6-Formylpterin Dimethyl Acetal (**13**).* 2,4-Diamino-6-formylpterine dimethyl acetal **12** (4.52 g, 19.15 mmol) was dissolved in aqueous sodium hydroxide (180 mL, 5%) and heated gently under reflux for 10 min. The solution was filtered through a sintered glass funnel and neutralized with glacial acetic acid. The yellow product precipitated after storing the solution overnight at 4 °C and was collected by centrifugation. Washing twice with water, ethanol, and diethyl ether afforded the pure title product (4.183 g, 17.65 mmol, 92.1%).

*6-Formylpterin (**14**).* A mixture of 6-formylpterin dimethyl acetal **13** (4.1828 g, 17.649 mmol), formic acid (80 mL, 97−100%), and water (8 mL) was stirred slowly for 30 min at room temperature, poured into water (50 mL), and neutralized with 25% ammonium hydroxide. The yellow precipitate was collected by centrifugation and washed twice with water, ethanol, and diethyl ether to yield **14** (3.879 g, 17.70 mmol, 100%).

^{1}H NMR: ([$^2H_6$]DMSO) δ = 8.16 (1H, s, NH), 9.08 (1H, s, CH), 9.98 (1H, s, CHO).

Positive ESI-MS: *m/z* (%) = 190 (100), 382 (45), 381 (16), 147 (1).

*N^2-Acetyl-6-formylpterin (**15**).* 6-Formylpterin (1.000 g, 5.24 mmol) was suspended in acetic anhydride (1000 mL) and heated under stirring for 10 h at 100 °C. The reaction mixture became homogeneous during the process, except for some unsoluble components which were

separated by filtration. The acetic anydride was evaporated, and the residual **15** (0.847 g, 3.62 mmol, 69%) was resuspended in water and lyophilized.

^{1}H NMR: ([$^2H_6$]DMSO) δ = 2.21 (3H, s, $COCH_3$), 5.87 (1H, t, OH), 9.04 (1H, s, NH), 9.27 (1H, s, CH), 10.08 (1H, s, CHO).

Positive ESI-MS: *m*/*z* (%) = 252 (100), 485 (62), 234 (55), 467 (15).

[$^2H_4$]Folic Acid. *N*^{2}*-Acetyl[$^2H_4$]folic Acid (**16**).* To a flask containing glacial acetic acid (30 mL) *N*^{2}-acetyl-6-formylpterin (559 mg, 2.4 mmol) and [$^2H_4$]4-aminobenzoylglutamate (700 mg, 2.6 mmol) were added. After maintaining the solution for 10 min, dimethylaminoborane (153.4 mg, 2.6 mmol) in glacial acetic acid (10 mL) was added, and the solution was heated for 20 min at 60 °C in an oil bath. The resulting solution was evaporated and the residue was redissolved in aqueous ammonium formate (0.15 mol/L) at pH 7. The solution was purified by anion exchange chromatography as described above. The fractions absorbing at 280 nm were verified to contain **16** using HPLC−MS, then pooled and lyophilized to yield 415 mg (0.85 mmol, 35%) of **16**.

*[$^2H_4$]Folic Acid (**17**).* Compound **16** (415 mg, 0.85 mmol) was dissolved in aqueous sodium hydroxide (50 mL, 0.1 mol/L) and stirred for 6 h at room temperature. The reaction was quenched by adjusting the pH to 3.5 by addition of hydrochloric acid (1 mol/L). [$^2H_4$]Folic acid (303 mg, 0.68 mmol, 80%) precipitated quantitatively after standing overnight at 4 °C and was lyophilized after the solution was centrifuged and the supernatant was discarded.

^{1}H NMR: ([$^2H_6$]DMSO): 1.91 (2 H, m, β-C-2H); 2.32 (2 H, m, γ-C-2H); 4.30 (1 H, t, α-C-1H); 4.47 (2 H, d, C9-2 H); 6.89 (1 H, t, C4−OH); 7.99 (1 H, d, N8′-H); 8.64 (1 H, s, C7-H).

Positive ESI-MS: *m*/*z* (%) = 446 (100), 447 (23), 468 (17), 445 (7).

Deuterated Vitamers of Folic Acid. *[$^2H_4$]Tetrahydrofolic Acid.* A reaction vessel with a three-way gas inlet and a flowmeter counter was filled with glacial acetic acid (25 mL) and platinum oxide (100 mg). The vessel was flushed for 1 min with nitrogen. Upon addition of [$^2H_4$]folic acid (250 mg, 0.562 mmol), the mixture was flushed repeatedly with hydrogen gas and finally stirred under a slight positive pressure of hydrogen for 4 h. At the end of the reaction, the catalyst was filtered off, and the solution was lyophilized to give [$^2H_4$]tetrahydrofolic acid diacetate (197 mg, 0.348 mmol, 62%).

Positive ESI-MS: *m*/*z* (%) = 450 (100), 451 (22), 449 (4), 452 (2).

[$^2H_4$]5-Formyltetrahydrofolic Acid. [$^2H_4$]Tetrahydrofolic acid diacetate (80 mg, 0.142 mmol) was dissolved in formic acid (960 μL, 98−100%) and aqueous dipotassium hydrogenphosphate (10 mL, 50 mM containing 0.5% mercaptoethanol), and the solution was adjusted to pH 3.5 with aqueous ammonium hydroxide (0.1 mol/L). Then, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (32 mg, 0.168 mmol) was added, and the mixture was stirred for 15 min under an argon atmosphere. Lyophilization gave the crude product, which was purified by anion exchange chromatography yielding [$^2H_4$]5-formyltetrahydrofolate (14 mg, 0.029 mmol, 18%) with a purity exceeding 98% (HPLC).

Positive ESI-MS: *m*/*z* (%) = 478 (100), 479 (20), 477 (4), 480 (3).

[$^2H_4$]5-Methyltetrahydrofolic Acid. [$^2H_4$]Tetrahydrofolic acid (80 mg, 0.142 mmol) was dissolved in an aqueous solution of TRIS at pH 8 (25 mL) and stirred for 15 min at room temperature upon the addition of aqueous formaldehyde (150 μL, 37%). Sodium borohydride (800 mg, 21 mmol) was added and the solution was heated at 50 °C for 1 h. The excess of reducing agent was destroyed by adding acetic acid (5 mol/L) dropwise. Purification by anion exchange chromatography and lyophilization of the pooled eluates provided the title compound (22 mg, 0.048 mmol, 34%).

Positive ESI-MS: *m*/*z* (%) = 464 (100), 465 (19), 317 (7), 466 (3).

[$^2H_4$]10-Formylfolic Acid. [$^2H_4$]Folic acid (30 mg, 0.067 mmol) was dissolved in 50 mL of concentrated formic acid and heated for 3 h at 60 °C. Formic acid was evaporated, and the residue was redissolved in 1 mL of aqueous formic acid (50%). Purification by high-performance liquid chromatography provided [$^2H_4$]10-formylfolic acid with a purity exceeding 97% (5.1 mg, 0.0107 mmol, 16%).

Positive ESI-MS: *m*/*z* (%) = 474 (100), 475 (27), 473 (10), 476 (9).

RESULTS AND DISCUSSION

Consideration of the Most Suitable Labelings and Selection of Vitamers to be Synthesized. A literature survey indicated that different forms of folic acid labeled by stable isotopes have been prepared in the past. In a prior attempt Plante et al. (*16*) reported the synthesis of [carbonyl-$^{13}C_1$]folic acid before Gregory and Toth prepared [3′,5′-$^2H_2$]folic acid (*17*) as well as pteroyl[$^2H_4$]glutamic acid (*18*), and Dueker et al. (*19*) generated [2′,3′,5′,6′-$^2H_4$]folic acid. More recently, Rogers and co-workers (*20*) synthesized [$^{13}C_5$]folic acid by coupling [$^{13}C_5$]glutamic acid with trifluoroacetyl pteroic acid.

The anticipated determination by LC−MS, however, renders only a few labelings promising. As quantification would be based on measuring the quasimolecular ions $(M + 1)^+$ of folic acid vitamers which all contain at least 19 carbon, 7 nitrogen, and 6 oxygen atoms, interferences from naturally occurring isotopes in the analytes have to be taken into consideration. Due to natural abundance of the isotopes C-13, N-15, and O-18, an intensity of nearly 4% can be calculated for the quasimolecular ions of doubly labeled folates. Therefore, using a labeling of only two mass units would result in mass spectrometric overlap between the unlabeled analytes and the labeled internal standards. For this reason we considered a label producing at least three mass increments to overcome this hindrance. Regarding the syntheses mentioned above, only [2′,3′,5′,6′-$^2H_4$]folic acid, pteroyl[$^2H_4$]glutamic acid or pteroyl-[$^{13}C_5$]glutamic acid would be suitable. The latter preparations, however, both require the use of labeled glutamic acid, which is too expensive to give sufficient folic acid to be used as starting component for the syntheses of further vitamers. Therefore, we decided to synthesize [2′,3′,5′,6′-$^2H_4$]folic acid by introducing a 4-fold deuterium label in the benzene ring of *p*-aminobenzoic acid, which enabled us to obtain higher yields of labeled folic acid.

Recent analyses of folates by high-performance liquid chromatography (HPLC) revealed tetrahydrofolate, 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, 10-formylfolate, and folic acid to predominate in different foods (*7*, *21*) and 5-methyltetrahydrofolate to be the most important vitamer in blood (*22*). Therefore we chose these five vitamers (**Figure 1**) to be synthesized starting from [$^2H_4$]folic acid.

Synthesis of Labeled Folic Acid. The intended synthetic approach to labeled folic acid required the coupling of labeled *p*-aminobenzoylglutamic acid and 6-formylpterin.

The first component can be generated either by labeling *p*-aminobenzoic acid in a catalytic protium−deuterium exchange followed by coupling to glutamic acid, or by reacting deuterated toluene to nitrobenzoylglutamate and subsequent reduction of the nitro group. In a first series of experiments the latter reaction sequence, according to Dueker et al. (*19*), was pursued as it proposed higher yields. The synthesis involved nitration of [$^2H_8$]toluene, followed by oxidation of the methyl group and formation of the chloride. The subsequent coupling with glutamyl diethylester and reduction to *p*-aminobenzoylglutamic acid gave a reaction mixture in which purification of the target compound from a host of byproducts could not be accomplished. Therefore, we chose the former alternative starting with deuteration of *p*-aminobenzoic acid as depicted in **Figure 2**. Comparing the mass spectra of unlabeled and labeled *p*-aminobenzoic acid (**Figure 3**) the incorporation of the four deuterium atoms can be clearly seen from the mass shift of the quasimolecular ion from *m*/*z* 138 to *m*/*z* 142. The isotopic peak areas of *p*-aminobenzoic acid indicate a 95% incorporation of

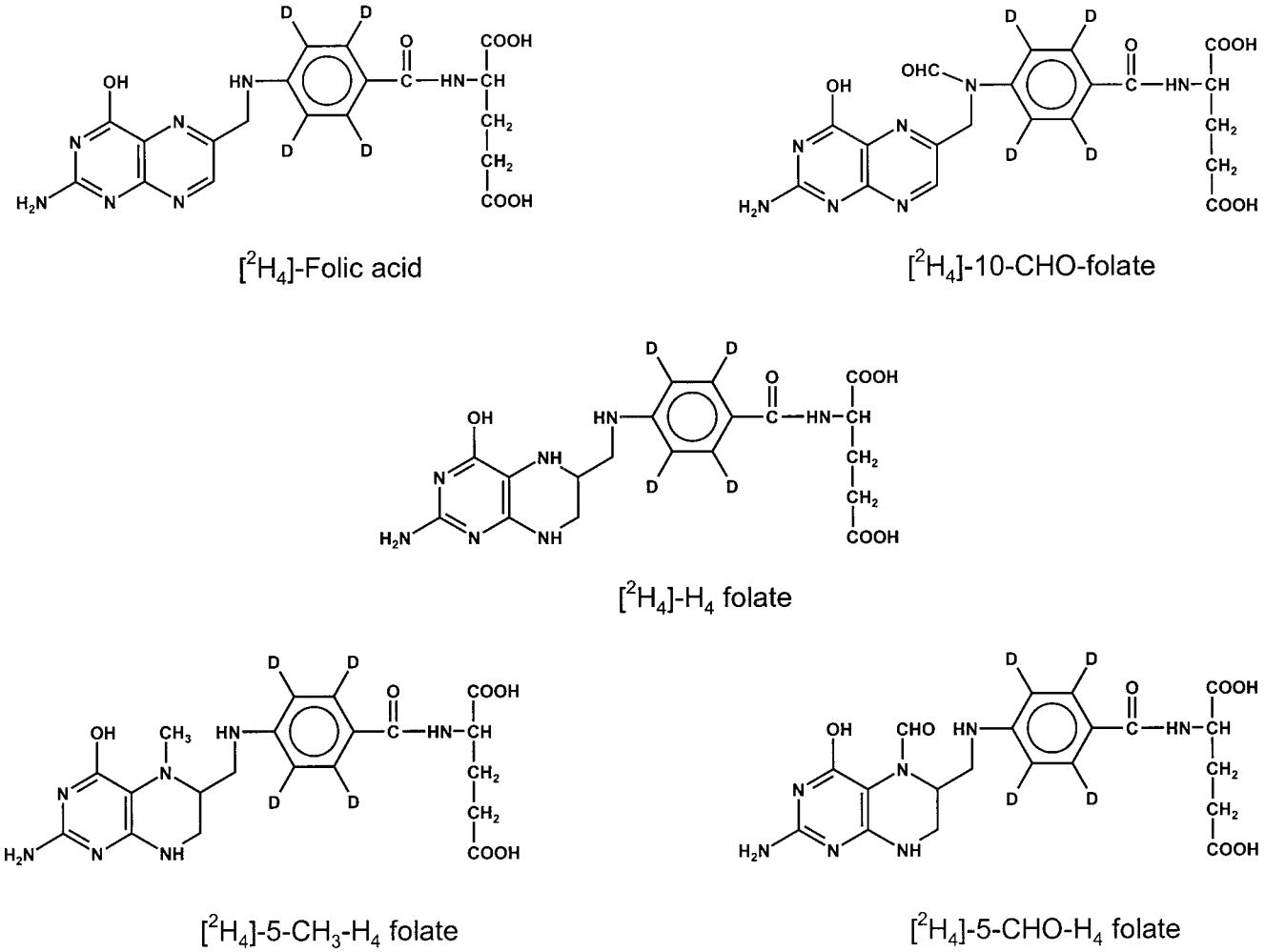

Figure 1. Structures of labeled folates.

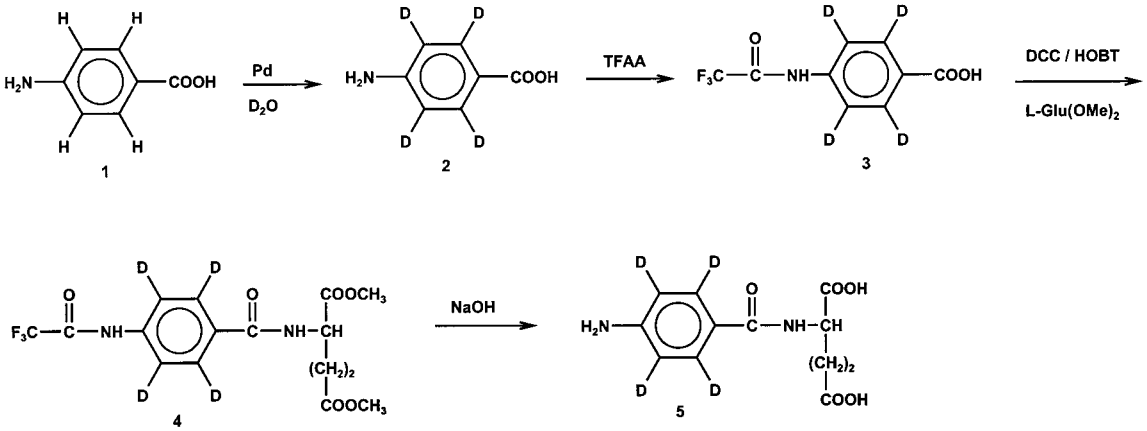

Figure 2. Reaction scheme leading to [$^2H_4$]4-aminobenzoyl glutamic acid (**5**); TFAA, trifluoroacetic anhydride; DCC, dicyclohexylcarbodiimide; HOBT, 1-hydroxybenzotriazole.

four deuterium atoms and a 5% incorporation of three deuterium atoms (HPLC−MS chromatograms not shown).

Trifluoroacetylation of labeled **2** enabled the coupling with glutamic acid dimethylester in a reaction similar to that reported by Vinale et al. (*23*). Subsequent hydrolysis of the protecting groups yielded labeled *p*-aminobenzoylglutamic acid **5**.

The second substructure, 6-formylpterin, was generated in an eight-step procedure (**Figure 4**) according to Taylor et al. (*24*) starting from pyrazine oxide **6**. Reduction of **6** by phosphorus trichloride gave the pyrazine **7**, which was coupled with pyridine to **8** in a nucleophilic substitution of the side-chain chloride. The pyridine moiety was subsequently substituted by *N*,*N*-dimethylaminonitrosobenzene to provide the nitrone **9**. Acidic hydrolysis of **9** gave rise to formylpyrazine **10** being then protected to acetal **11** by reaction with methanol. Coupling **11** with guanidine then provided the second ring of pterin, and the amino group at C-4 was substituted by a hydroxyl in a reaction of **12** with aqueous sodium hydroxide. Acidic

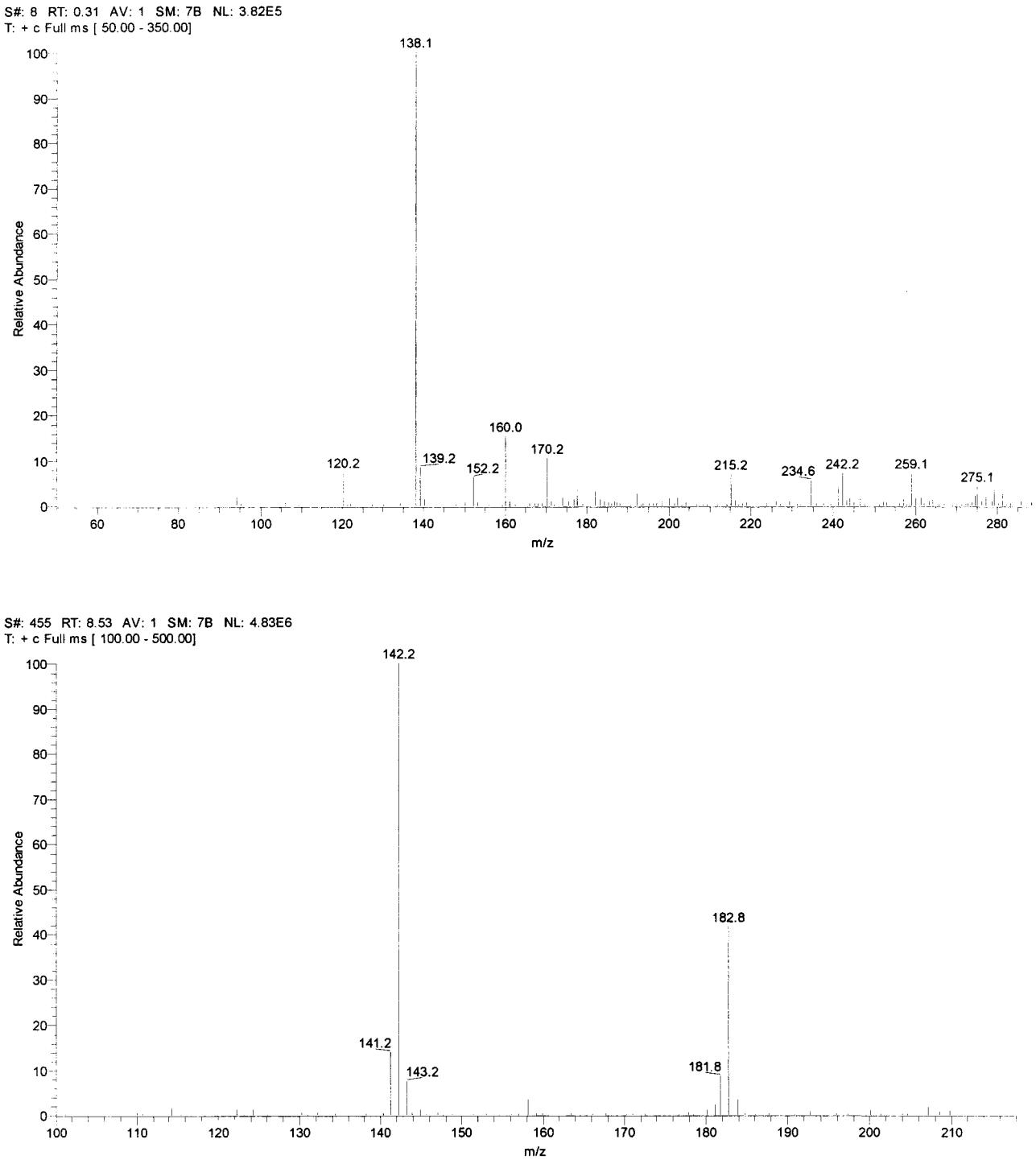

Figure 3. ESI-mass spetra of unlabeled (above) and [$^2H_4$]4-aminobenzoic acid (below).

hydrolysis of the acetal moiety converted **13** to 6-formylpterin **14** in an overall yield of 25.0%. The ^{1}H NMR spectrum of **14** was identical with that reported by Taylor et al. (*24*).

6-Formylpterin **14** then had to be protected by acetylation, and **15** could subsequently be reacted with labeled *p*-aminobenzoylglutamic acid **5** to the intermediate imine which was not isolated. The reduction of the imine to **16** was achieved with dimethylaminoborane (DMAB) in a sequence according to Maunder et al. (*25*). Using DMAB as reducing agent is advantageous, as further reduction of the pterine ring system does not occur. As detailed in **Figure 5**, subsequent removing of the acetic function afforded [$^2H_4$]folic acid **17** in a total yield of 26% starting from **5**.

To verify the positions of the deuterium label a ^{1}H NMR spectrum of **17** was recorded and compared to that of unlabeled folic acid. As the spectrum of [$^2H_4$]folic acid lacks the four signals at 6.63, 6.65, 7.63, and 7.66 ppm present in the spectrum of unlabeled folic acid, and these signals represent the resonances of the four benzene-ring protons, their absence is consistent with the incorporation of the deuterium atoms therein.

Conversely to [$^2H_4$]folic acid synthesized in the present study, the analoguous compound generated by Dueker et al. (*19*) revealed in its ^{1}H NMR spectrum discernible signals at the positions of the benzene-ring protons. On this basis the authors calculated a 17% residue of protium in these positions of their labeled folic acid, thus indicating that a substantial protium−

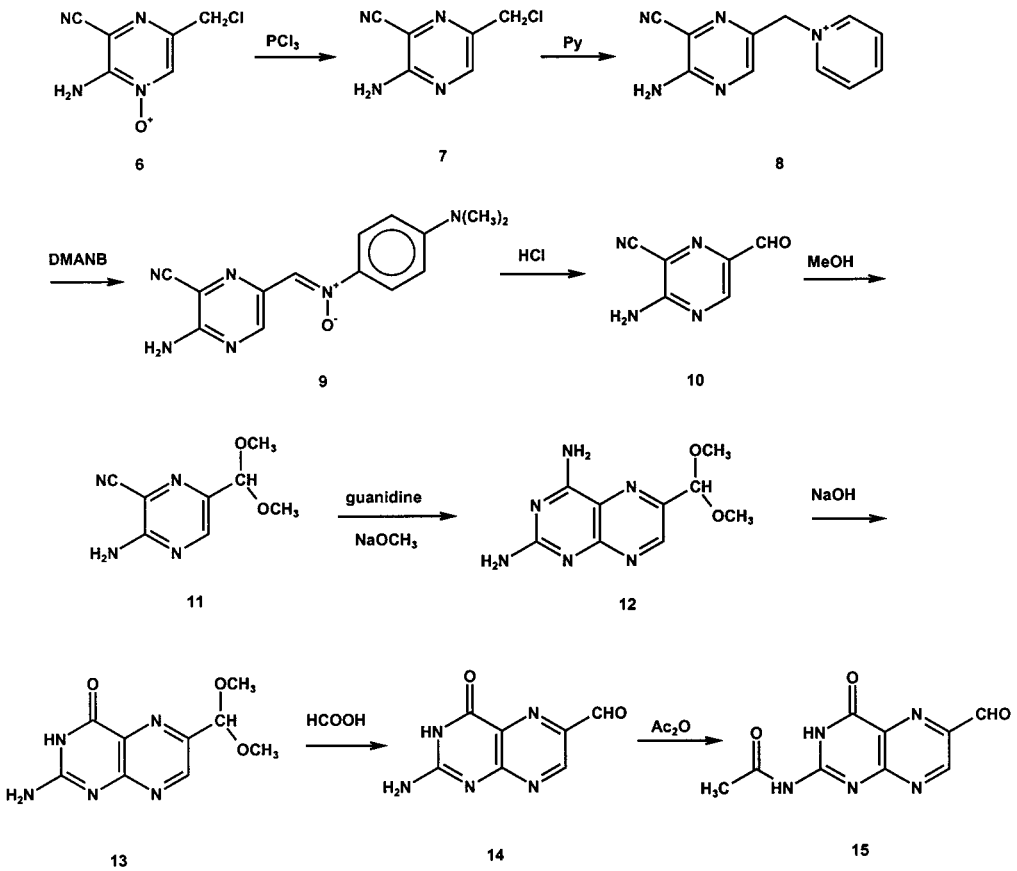

Figure 4. Reaction scheme leading to N^2-acetyl-6-formylpterin (**15**); DMANB, *N*,*N*-dimethylaminonitrosobenzene.

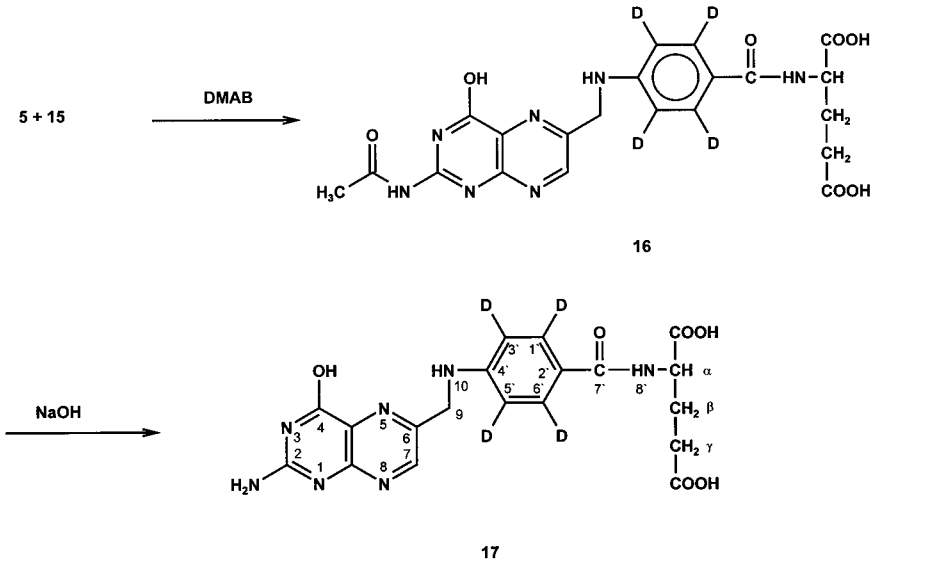

Figure 5. Reaction scheme leading to [$^2H_4$]folic acid; DMAB, dimethylaminoborane.

deuterium exchange had occurred. By contrast, the labeled folic acid prepared in the present study proved to contain only 5% protium in the benzene moiety. Therefore, this compound is better distinguishable from the unlabeled isotopomer by mass spectrometry and is a more suitable internal standard in stable isotope dilution assays (SIDAs).

Synthesis of [$^2H_4$]Tetrahydrofolic and [$^2H_4$]5-Methyltetrahydrofolic Acid. By hydrogenation of labeled folic acid in the presence of platinum oxide, [$^2H_4$]tetrahydrofolate was prepared according to a procedure reported by Scott (*26*). [$^2H_4$]tetrahydrofolate was then reacted with formaldehyde and sodium borohydride to [$^2H_4$]5-methyltetrahydrofolate, which was purified by anion exchange chromatography (*26*). The intermediate 5,10-methylenetetrahydrofolic acid was not isolated.

Synthesis of [$^2H_4$]5-Formyltetrahydrofolic Acid. Labeled 5-formyltetrahydrofolate was prepared according to a procedure reported by Moran and Colman (*27*). By coupling formic acid with [$^2H_4$]tetrahydrofolate in the presence of 1-ethyl-3-(dimethylaminopropyl)carbodiimide (EDC), [$^2H_4$]5-formyltetrahydrofolate was formed and purified again by anion exchange chromatography.

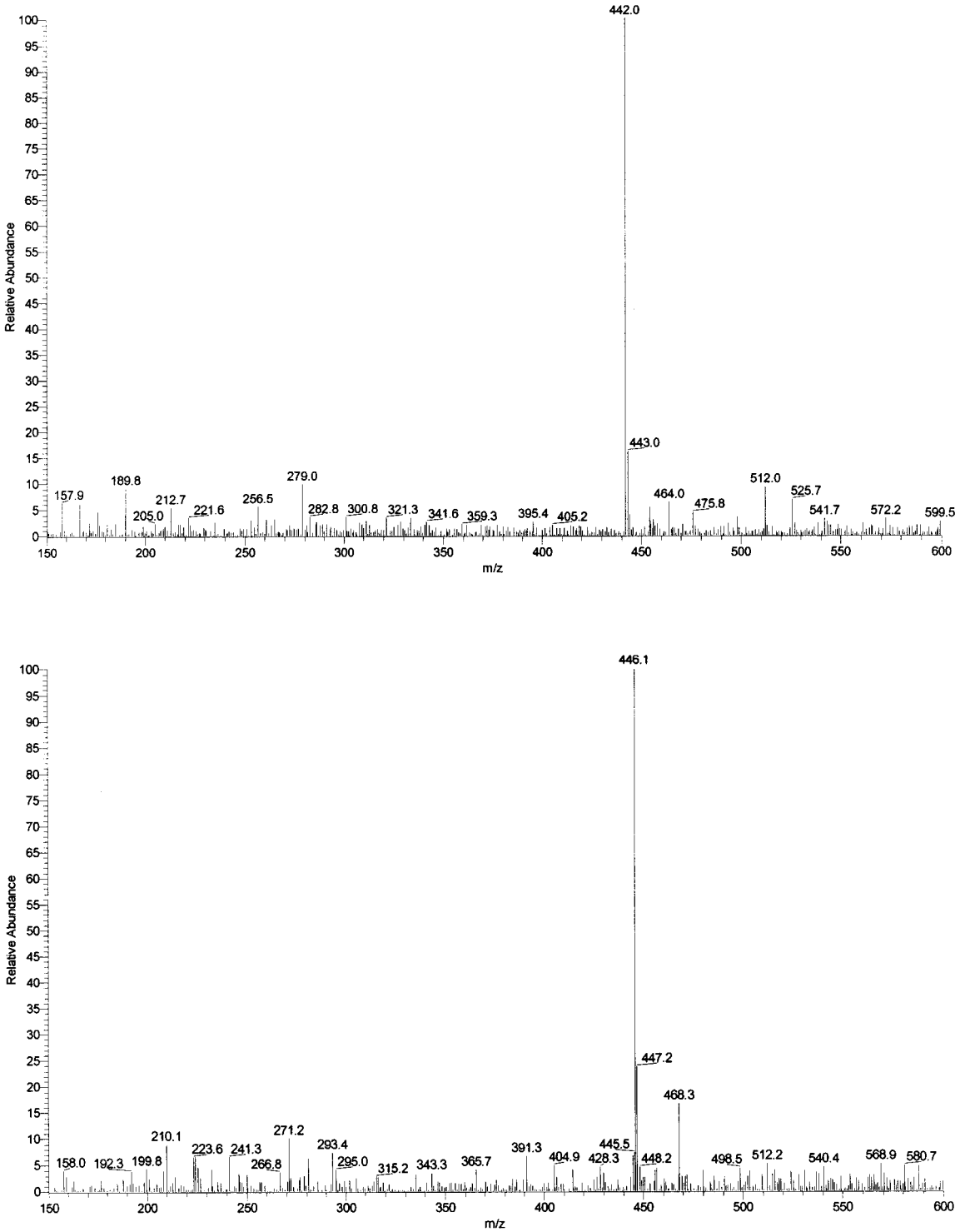

Figure 6. ESI-mass spectra of undeuterated (above) and [$^2H_4$]folic acid (below).

Synthesis of [$^2H_4$]10-Formylfolic Acid. [$^2H_4$]Folic acid was formylated by heating in concentrated formic acid according to the procedure proposed by Huennekens et al. (*28*). As the resolving efficiency of the anion exchange column was not sufficient in this case, purification was performed by preparative HPLC.

Mass Spectrometric Studies of Folate Vitamers. The synthesized vitamers revealed upon positive electrospray ionization an abundant quasimolecular ion $(M + 1)^+$, being the base peak in each spectrum. As electrospray ionization is very soft compared to other methods (e.g., atmospheric pressure chemical ionization), few fragmentations occurred. Those resulted mostly from ejection of the glutamyl residue $(M + 1 - 147)^+$. Comparing the spectra with those of the unlabeled reference compounds, a 4-fold labeling is evident for all vitamers, as the representative spectra of labeled and unlabeled folic acid reveal in **Figure 6**. The base peak at *m*/*z* 442 is shifted toward *m*/*z* 446 in [$^2H_4$]folic acid. On the other hand, the unlabeled isotopomers show only negligible ion intensity from naturally occurring isotopes falling at the *m*/*z* values for the respective ions of the labeled forms. This behavior enables accurate mass spectrometric differentiation of the respective isotopomers in future studies. To enhance specificity of the detection, collision-induced dissociation was applied to the quasimolecular ions and

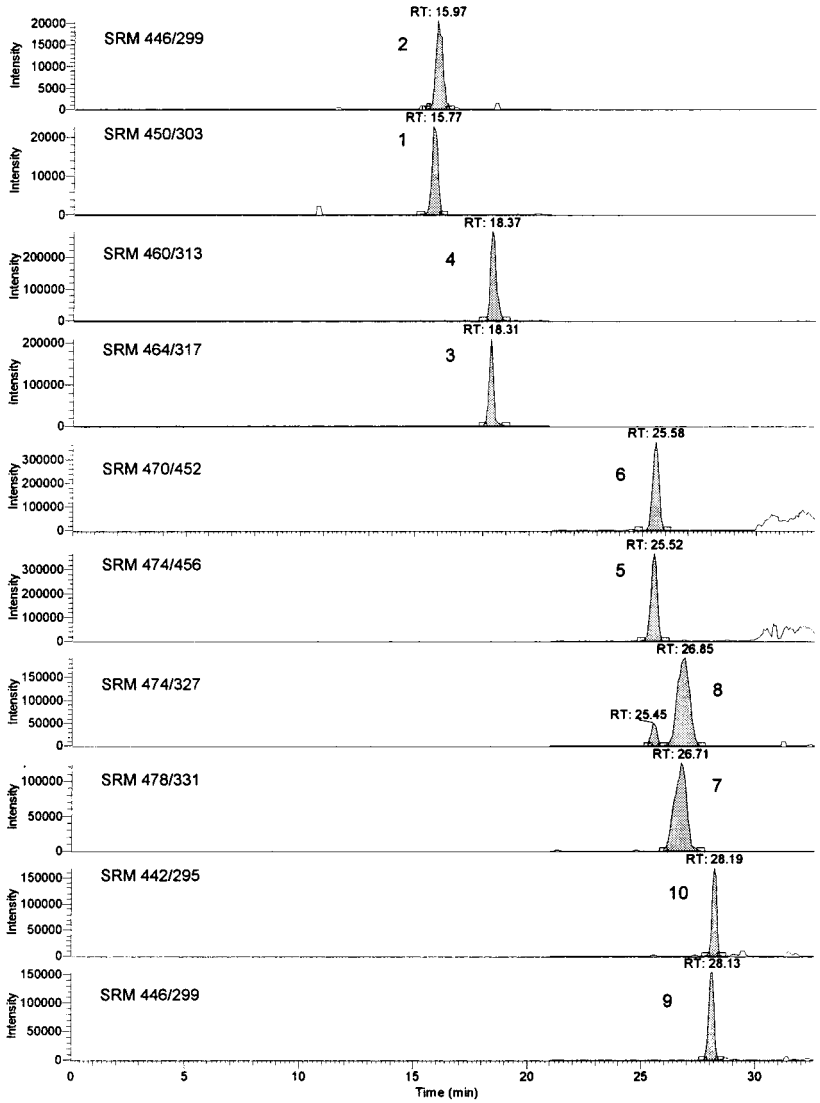

Figure 7. HPLC–MS–MS chromatogram of a standard mixture of [$^2H_4$]tetrahydrofolate (1), tetrahydrofolate (2), [$^2H_4$]5-methyl-tetrahydrofolate (3), 5-methyl-tetrahydrofolate (4), [$^2H_4$]10-formyl-folate (5), 10-formyl-folate (6), [$^2H_4$]5-formyl-tetrahydrofolate (7), 5-formyl-tetrahydrofolate (8), [$^2H_4$]folic acid (9), and folic acid (10) showing the mass ranges of labeled folate vitamers as well as those of the corresponding unlabeled analogues; SRM, selected reaction monitoring.

gave the fragmentation shown in **Table 1**. The great majority of fragments arise from expulsion of the glutamyl moiety, and all fragments still contain the four deuterium labels.

High-Performance Liquid Chromatography−Double Stage Mass Spectrometry. To prove that the synthesized folate isotopomers could be used as internal standards (IS) in SIDAs, we optimized the HPLC conditions to resolve all vitamers in a single run. As is evident from the HPLC−MS−MS chromatogram shown in **Figure 7**, (1) separation of the folates is possible, and (2) each isotopomer can be specifically detected in its MS−MS trace.

High-performance liquid chromatography coupled to electrospray mass spectrometry revealed the synthesized compounds to be an excellent choice for use as IS. In the course of this study we synthesized 5−30 mg of each vitamer. Because in each SIDA approximately 1.5 μg of them serve as IS, the total amount will be sufficient to perform 3000 to 20 000 assays. On the other hand, Gregory et al. (*11*) stated that 200 μg of folate are adequate to perform a bioavailability study. Thus, the preparations likewise afforded enough amounts of the labeled folates to apply them in examinations of the bioavailability of the single vitamers.

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