Preparation of 6-(Bromomethyl)-2,4-pteridinediamine Hydrobromide and Its Use in Improved Syntheses of Methotrexate and Related Compounds

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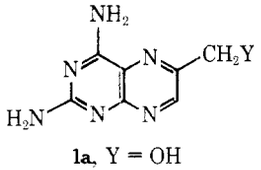
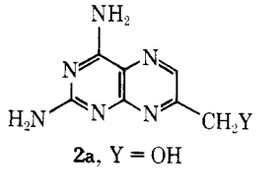
A versatile method for the introduction of the (2,4-diamino-6-pteridinyl)methyl grouping which involves the reaction of 6-(bromomethyl)-2,4-pteridinediamine hydrobromide (**3**) with nucleophiles is demonstrated by facile syntheses of methotrexate (**4b**), aminopterin (**4c**), and the corresponding 4-amino-4-deoxypteroic acids **4d** and **4e** in good yields and high states of purity. 2,4-Diamino-6-pteridinemethanol (**1a**), the precursor of **3**, prepared by condensation of 2,4,5,6-tetraaminopyrimidine with 1,3-dihydroxyacetone and partially purified as its hydrobromide, typically contained approximately 5% 6-methyl-2,4-pteridinediamine (**1b**) and less than 1% each of 2,4-diamino-7-pteridinemethanol (**2a**) and 7-methyl-2,4-pteridinediamine (**2b**). The coproduct **1b** persisted in **3**, which was prepared from **1a** HBr by treatment with dibromotriphenylphosphorane, but the final products (4 series) were shown by reversed-phase, high-pressure liquid chromatography to be free of **1b**.

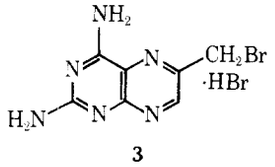
The use of massive doses of methotrexate (**4b**) followed by leucovorin rescue in the clinical treatment of certain neoplasms[1] has made desirable the accessibility of **4b** in a higher state of purity than that previously available[2] and has also greatly increased supply demands. A new process that shows promise of meeting these needs makes use of 6-(bromomethyl)-2,4-pteridinediamine hydrobromide (**3**), a versatile intermediate prepared initially for use in an improved approach to analogues of **4b** and aminopterin (**4c**) bearing varied side chains.[3] We wish to amplify on the preliminary reports of the synthesis of **3** and present results illustrating its use in syntheses of **4b**, **4c**, the corresponding 4-amino-4-deoxypteroic acids **4d** and **4e**, and pteroic acid (**5**).



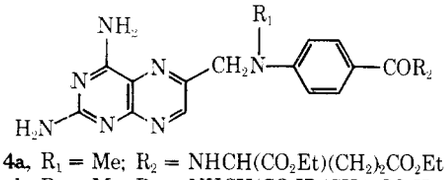
1a, Y = OH
b, Y = H



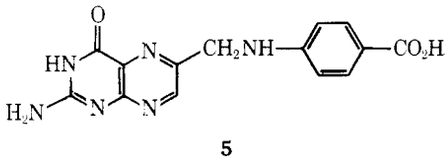
2a, Y = OH
b, Y = H



3



4a, R_1 = Me; R_2 = NHCH(CO_2Et)(CH_2)$_2CO_2Et$
b, R_1 = Me; R_2 = NHCH(CO_2H)(CH_2)$_2CO_2H$
c, R_1 = H; R_2 = NHCH(CO_2H)(CH_2)$_2CO_2H$
d, R_1 = Me; R_2 = OH
e, R_1 = H; R_2 = OH



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2,4-Diamino-6-pteridinemethanol (**1a**), the precursor of **3**, was prepared by the condensation of 2,4,5,6-tetraaminopyrimidine with 1,3-dihydroxyacetone in aqueous sodium acetate solution as described by Baugh and Shaw.[4] Possible coproducts were discussed in the work cited, and limited evidence that only **1a** is isolated was presented. We found through ^{1}H NMR spectral studies on the crude material filtered directly from the reaction mixture that **1a** is the major product, but 6-methyl-2,4-pteridinediamine (**1b**) is also detectable.[3] Analysis by reversed-phase, high-pressure liquid chromatography (HPLC) further revealed minor contamination by 2,4-diamino-7-pteridinemethanol (**2a**) and 7-methyl-2,4-pteridinediamine (**2b**).[5,6] Partial purification of **1a** was effected through its hydrobromide, which crystallized from a solution of the crude product mixture in ethanol containing hydrobromic acid. A typical run afforded a product whose ^{1}H NMR spectrum indicated a molar ratio of **1a** to **1b** of 16–20:1, and analysis by HPLC revealed about 5% of **1b** and less than 1% each of **2a** and **2b**. No further purification of **1a** was required, since contaminants present at this point are ultimately removed without attentive effort. The level of each is diminished in subsequent conversions, and reversed-phase HPLC analyses that aided in establishing the end products to be of high purity and free of **1b** are discussed later.

The bromomethyl function of **3** was introduced by treatment of **1a** HBr with dibromotriphenylphosphorane (Ph_3PBr_2) in *N,N*-dimethylacetamide (Me_2NAc).[7] Involvement of the amino groups, possibly through direct reaction with Ph_3PBr_2 to form an aminophosphonium salt,[8] was indicated by a required minimum ratio of Ph_3PBr_2 to **1a** HBr of 3:1. In early preparative runs, a 4:1 ratio was used, but later use of a 3.3:1 ratio gave similar results. After the excess reagent had been decomposed, the Me_2NAc was removed by evaporation in vacuo, or the product was caused to precipitate by the addition of benzene to the reaction solution. The crude initial product was then dissolved in hot acetic acid. If the amino groups were substituted, regeneration was effected during this treatment. Crystalline **3**, solvated by acetic acid, separated from the solution. Even though the coproduct **1b** persisted, the **3** thus obtained proved to be of suitable purity for many synthetic applications. Further recrystallization of **3** did not remove **1b**, but was apparently beneficial in some applications (for example, in the preparation of **4c**). The amount of **1b** still present in **3** was estimable from ^{1}H NMR spectral data. In deuteriotrifluoroacetic acid, **3** produces singlet peaks at δ 4.70 (CH_2) and 9.08 (C_7 H), and peaks due to **1b** occur at δ 2.83 (CH_3) and 8.85 (C_7 H). Results from numerous runs showed **1b** present in **3** at levels slightly lower than in the precursor **1a**.

Treatment of **1a** HBr with the complex formed by phosphorus tribromide and *N,N*-dimethylformamide was also found to give **3**, but the best yield of suitable product obtained from three runs was a relatively poor 11%. This possible alternative method was not pursued because it appeared un-

likely that it could be made superior to that using Ph_3PBr_2 in Me_2NAc, which gave **3** in about 50% yield after recrystallization.

In earlier related work, deaza analogues of **4e** were prepared by sequences involving side-chain bromination of methyl-substituted precursors (N^2,N^4-dibenzoylated deaza analogues of **1b**),[9] but attempts to extend that method to the pteridine series led to the finding that bromination of the N^2,N^4-diacetyl derivative of **1b**, even with equimolar amounts of bromine or *N*-bromosuccinimide, afforded only the dibromomethyl derivative.[10]

Reaction of **3** with aromatic amines in Me_2NAc to give the **4** series did not require inclusion of an auxiliary base. In the initial preparation of **4c**, 3 molar equiv of *N*-(4-aminobenzoyl)-L-glutamic acid was used, but later use of 2 equiv did not affect the yield. Three equivalents of 4-aminobenzoic acid was used in the preparation of **4e** HBr, which crystallized from the reaction medium. The methylamino compounds were alkylated by **3** at a slower rate than the primary amines under the same conditions. This difference might be attributable to a less favorable equilibrium between free amine and protonated form in the absence of an auxiliary base. Reaction of **3** with the methylamino compounds at 25 °C required several days to reach completion, but conversion was complete within 5 h at 55 °C, even when the methylamino compounds were present in only slight excess. Unchanged methylamino precursors were troublesome to remove from the products when large excesses were used, but only 10% excess proved adequate.

The immediate precursor of **4b**, diethyl ester **4a**, could be hydrolyzed in situ or, preferably, isolated beforehand. The ester crystallized as a partial hydrobromide after the reaction solution had been combined with water. The overall yield of **4b** of high purity obtained from the isolated ester was 73% whereas that of product of comparable purity obtained after hydrolysis in situ was 58%.

Previously reported syntheses of pure **4d**[11-13] are quite lengthy and tedious compared with the present approach. Used in conjunction with carboxyl-activating reagents, **4d** has served as a key intermediate in syntheses of **4b**[13] and analogues of **4b** in which the glutamic acid portion is replaced by amino acid esters or amines.[12b] Oligo-γ-L-glutamyl peptide analogues of **4b** have been prepared from **4d** (derived from **4b** by enzymic cleavage) by an adaptation of the Merrifield method.[14,15]

This simply executed method wherein **3** is used to attach the (2,4-diamino-6-pteridinyl)methyl grouping to diverse side chains also affords access to corresponding (2-amino-3,4-dihydro-4-oxo-6-pteridinyl)methyl compounds. Thus, alkaline hydrolysis of the labile 4-amino group of **4e** gave pteroic acid (**5**),[16] which has been used in syntheses of pteroyloligo-γ-L-glutamates,[17,18] pteroyl-γ-L-glutamyl peptides bearing various amino acids,[19] and simpler folic acid analogues derived from various amino acids.[20] Earlier sources of **5** involve lengthy synthetic routes[21] or enzymic cleavage of folic acid.[17,22]

Reversed-phase HPLC analyses (discussed in the Experimental Section) showed the products prepared from **3** to be of high purity, and no special purification efforts were required. Previously reported methods either give crude products whose purification requires laborious techniques of limited capacity[23,24] or involve lengthy routes that afford relatively poor yields.[11-13,21]

Despite reports that show the enantiomeric purity of **4b** to be of importance with respect to biologic or anticancer activity,[23b,25] no specific rotation for **4b** has been reported. Optical activity is retained in both **4b** and **4c** prepared directly from **3**. Treatment of the mixed anhydride derived from **4d** and isobutyl chloroformate with diethyl L-glutamate gave the ester **4a** identical with that prepared from **3** and the intact side-chain ester. The **4b** obtained by hydrolysis of **4a** prepared in this manner gave a specific rotation value in acceptable agreement with that of **4b** prepared from **3** and the intact side chain. These findings indicate that racemization is not involved in the formation of products from **3** and the intact side chains bearing glutamic acid residues.

Experimental Section

High-pressure liquid chromatographic studies were made with a Waters Associates ALC-242 liquid chromatograph equipped with a UV detector (254 nm) and an M-6000 pump using a μBondapak C_{18} column of 30 cm length and 4 mm inside diameter. Thin layer chromatographic analyses were performed on products whose side chains bear carboxyl groups on DEAE–cellulose sheets (Bakerflex) using 0.5 M NaCl, 0.2 M in mercaptoethanol, in 0.005 M KH_2PO_4 buffer solution at pH 7.0. Samples were dissolved for spotting in 0.01 N NaOH. Chromatograms were viewed by three UV lamps (Models UVL-21, UVS-12, and C-51; Ultraviolet Products, Inc.), and each compound appeared homogeneous. Most of the elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn. Spectral determinations, specific rotation measurements, and some of the elemental analyses were performed in the Molecular Spectroscopy Section of Southern Research Institute under the direction of Dr. W. C. Coburn, Jr. The ^1H NMR spectra were determined with a Varian XL-100-15 spectrometer (except those for **2a** and **4e**, which were determined on a Varian T-60A) in the solvent indicated using Me_4Si as internal reference. Chemical shifts (δ in ppm) listed for multiplets were measured from the approximate centers, and relative integrals of peak areas agreed with those expected for the assignments indicated. Addition of D_2O produced the expected simplifications in the spectra. The UV spectra were determined with a Cary Model 17 spectrometer. Samples were first dissolved in appropriate media (**1a** HBr and **4a**, MeOH or EtOH; **3**, 2-PrOH; **4b–e** and **5**, 0.01 N NaOH), and the solutions were diluted tenfold with the medium given in the listings. Maxima are expressed in nanometers with the molar absorbance ($\epsilon \times 10^{-3}$) given in parentheses. Molecular weights used in all calculations conform with the compositions listed with elemental analysis results. Specific rotations were measured with a Rudolph Model 80 polarimeter; concentration (*c*) is given in grams of solute per 100 ml of 0.1 N NaOH. Products were dried in vacuo (<1 mm) at room temperature over P_2O_5 unless other conditions are specified.

2,4-Pteridinediamine-6-methanol (1a) Hydrobromide. Dried and pulverized crude **1a** [47 g, prepared as reported[4] from 2,4,5,6-tetraaminopyrimidine (0.293 mol) but not recrystallized] was stirred with EtOH (6 l.) at 70–75 °C, and a solution of 48% HBr (28 ml) in EtOH (500 ml) was added in a thin stream. The mixture was refluxed for about 5 min with rapid stirring while nearly all of the solid dissolved. The solution was clarified (Norit, Celite) while hot, and the clear yellow filtrate was kept in a refrigerator overnight while a first crop (17.2 g) separated. A second crop (10.2 g) was obtained after concentration of the filtrate (to 2 l.) by evaporation under reduced pressure. The two crops were combined before examination by HPLC and determination of spectral properties, yield 34% (based on tetraaminopyrimidine). Spectral data: UV, 0.1 N HCl, 243 nm (15.4), 284 (5.06), 337 (9.60), 350 (sh); 0.1 N NaOH, 225 nm (11.6), 257 (21.3), 368 (6.97); ^1H NMR (CF_3CO_2D) δ 5.27 (s, CH_2), 9.08 (s, C_7 H), and weak singlets due to **1b** at δ 2.84 and 8.85; estimated molar ratio of **1a** to **1b**, 20:1.

Anal. Calcd for $C_7H_8N_6O$·HBr: C, 30.79; H, 3.32; N, 30.77. Found: C, 31.19; H, 3.33; N, 31.39.

6-(Bromomethyl)-2,4-pteridinediamine Hydrobromide (3). Method A. Br_2 (59.6 g, 0.373 mol) was added dropwise during 30 min to a stirred (Teflon paddle) solution of Ph_3P (97.7 g, 0.373 mol) in Me_2NAc (500 ml) kept near 10 °C. A smooth suspension containing crystalline solid resulted. Solid **1a** HBr (25.4 g, 93.0 mmol) was then added in one portion. The cooling bath was removed, and after 1 h at 20–25 °C, solution occurred. The solution, which developed a dark red color, was kept at 20–25 °C for 1 h longer and was then chilled (ice bath) and treated with EtOH (6 ml). After overnight refrigeration, the solvent was removed by evaporation in vacuo (<1 mm, bath to 45 °C). The dark semisolid residue was stirred with two 300-ml portions of C_6H_6, and each portion was removed from the C_6H_6-insoluble product by decantation. The solid that remained was stirred with glacial AcOH (660 ml) preheated to 80 °C. The mixture was kept in a bath at 80 °C until solution was complete. Beige, crystalline solid separated when the solution was allowed to cool. The mixture was left overnight in a refrigerator before the solid was collected, washed with

AcOH (cooled to 10 °C) followed by Et_2O, and dried in vacuo (over P_2O_5 and NaOH pellets) at successive temperatures of 25, 56, and 110 °C to give yellow **3** in 49% yield (15.3 g). Spectral data: UV, 0.1 N HCl, 249 nm (17.3), 339 (10.5), 353 (sh); 0.1 N NaOH, 258 nm (21.5), 370 (6.94); ^{1}H NMR (CF_3CO_2D) δ 4.70 (s, CH_2), 9.08 (s, C_7 H) and a weak singlet at δ 2.83 due to the Me group of **1b**; molar ratio of **3** to **1b**, ~25:1.

Anal. Calcd for $C_7H_7BrN_6$·HBr: C, 25.02; H, 2.40; N, 25.01. Found: C, 25.59; H, 2.79; N, 24.62.

A twice-recrystallized (from AcOH) sample (dried as before) from another run gave elemental analysis results that agreed closely with values calculated for **3** although its ^{1}H NMR spectrum indicated the molar ratio of **3** to **1b** to be ~20:1 (~96% purity by weight).

Anal. Calcd for $C_7H_7BrN_6$·HBr: C, 25.02; H, 2.40; Br, 47.56; N, 25.01. Found: C, 25.22; H, 2.44; Br, 47.30; N, 24.99.

Method B. Solid **1a** HBr (300 g, 1.10 mol)[26] was added to a mixture of Ph_3PBr_2 (3.63 mol) and Me_2NAc (3.6 l.) prepared as described under method A in a 20-l., three-necked flask. The mixture was stirred at 20–25 °C for 3.5 h. The solution that formed was treated dropwise during 15 min with EtOH (72 ml) and stirred for 15 min longer before C_6H_6 (11.7 l.) was added. A dark oil precipitated, and the mixture was stirred for 30 min longer and left to stand overnight. The clear supernatant was siphoned and decanted from the now semisolid precipitate, which was dissovled with stirring in hot glacial AcOH (6 l., preheated to 100 °C). The solution was filtered while hot, and the beige, crystalline material that separated from the cooled filtrate was collected after 4 h at 20–25 °C. The Et_2O-washed solid (290 g of **3** solvated by AcOH) was recrystallized from 2-PrOH to give lustrous yellow-orange platelets, which were washed with Et_2O before being pulverized and dried, yield 209 g (two crops of 168 and 41 g). Typical lots of **3** prepared in this manner were found through ^{1}H NMR spectral data (in CF_3CO_2D) to be solvated to slightly varying degrees by 2-PrOH, usually near hemisolvates. (The percentage yield of **3** $0.5C_3H_7OH$ from the above run was 52%; six runs on similar scales gave an average yield of 45%.) One exception was a lot found to be a monosolvate: ^{1}H NMR (CF_3CO_2D) δ 1.3–1.5 (m, 6, Me from 2-PrOH and 2-$PrOCOCF_3$), 4.70 (s, 2, CH_2Br), 9.08 (s, 1, C_7 H); molar ratio of **3** to **1b**, ~40:1. Elemental analysis results given below support the indicated composition for this lot; UV, 0.1 N HCl, 249 nm (18.7), 339 (11.1), 353 (sh); 0.1 N NaOH, 258 nm (23.2), 370 (7.61).

Anal. Calcd for $C_7H_7BrN_6$·HBr·C_3H_7OH: C, 30.32; H, 4.07; Br, 40.35; N, 21.22. Found: C, 30.50: H, 3.92; Br, 39.97; N, 21.28.

Diethyl *N*-[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]-L-glutamate (4a). A. Directly from 3. A mixture of **3** C_3H_7OH (1.98 g, 5.00 mmol) and diethyl *N*-[4-(methylamino)benzoyl]-L-glutamate[27] (1.85 g, 5.50 mmol) in Me_2NAc (20 ml) was stirred at 50–55 °C (bath temperature) for 4 h (solution occurred after 2 h), left at 25 °C for 17 h, and combined with H_2O (200 ml). A clear solution resulted, but yellow solid began separating after a few minutes. After a refrigeration period (4 h), the solid was collected, washed with H_2O, and dried. The orange solid (2.12 g) underwent a weight increase and became yellow on exposure to ambient conditions of the laboratory; yield 77% (2.22 g). Spectral data: UV, 0.1 N HCl, 243 nm (18.9), 306 (24.0); pH 7, 258 nm (25.6), 306 (26.5), 372 (8.49); 0.1 N NaOH, 258 nm (26.2), 303 (25.6), 372 (8.18); ^{1}H NMR (Me_2SO-d_6) δ 1.2 (m, CH_3CH_2), 2.1 (m, $CHCH_2CH_2$), 2.4 (m, CH_2CO_2Et), 3.24 (s, MeN), 4.1 (m, CH_2Me), 4.4 (m, $NHCHCO_2Et$), 4.85 (s, CH_2N), 6.85 and 7.76 (two d, C_6H_4), 7.40 (br s, NH_2), 8.3 (d, CONH, over broad s, NH_2), 8.68 (s, C_7 H).

Anal. Calcd for $C_{24}H_{30}N_8O_5$·0.5HBr·1.5H_2O: C, 49.86; H, 5.93; Br, 6.91; N, 19.39. Found: C, 49.48; H, 6.04; Br, 7.07; N, 19.43.

Most of **4a** 0.5HBr·1.5H_2O described above was used for conversion to **4b**. A sample (200 mg) was stirred with a mixture of $CHCl_3$ and 0.3 N NH_4OH (60 ml of each). After 10 min, the $CHCl_3$ layer was removed, washed three times with H_2O, dried (Na_2SO_4), and evaporated. The yellow solid residue was recrystallized from MeCN to give **4a** (150 mg) with melting point, mixture melting point, TLC, and spectra (IR, UV, and ^{1}H NMR) identical with those of **4a** prepared from **4d** as described below.

B. From 4d. A stirred mixture of pulverized **4d** 1.5H_2O (1.06 g, 3.00 mmol), Et_3N (606 mg, 6.00 mmol), and *N,N*-dimethylformamide–Me_2SO (60 ml, 1:1) was treated at 0–5 °C with a solution of isobutyl chloroformate (615 mg, 4.50 mmol) in dioxane (1 ml). The mixture was stirred at 0–5 °C for 15 min before diethyl L-glutamate HCl (1.44 g, 6.00 mmol) was added followed by more Et_3N (606 mg). The mixture was stirred for 15 min longer at 0–5 °C and then for 30 min at 25–30 °C. The insoluble material, mostly Et_3N·HCl, was removed by filtration, and the filtrate was concentrated to ~10 ml by evaporation in vacuo (<1 mm, bath 25–30 °C). Addition of H_2O (80 ml) gave an orange solid. After refrigeration, the solid was collected and then stirred for 10 min with a mixture of $CHCl_3$ (100 ml) and 0.3 N NH_4OH (50 ml). The $CHCl_3$ layer was dried (Na_2SO_4) and evaporated to give a yellow solid, which was recrystallized from MeCN to give **4a**, mp 155–157 °C, in 10% yield (160 mg); homogeneous by TLC.[12a,28] Spectral data: UV, in agreement with that given above for **4a** 0.5HBr·1.5H_2O; ^{1}H NMR (Me_2SO-d_6) δ 1.2 (m, CH_3CH_2), 2.1 (m, $CHCH_2CH_2$), 2.4 (m, CH_2CO_2Et), 3.22 (s, MeN), 4.1 (m, CH_2Me), 4.4 (m, $NHCHCO_2Et$), 4.80 (s, CH_2N), 6.60 (s, NH_2), 6.85 and 7.76 (two d, C_6H_4), 7.54 (br s, NH_2), 8.3 (d, CONH), 8.60 (s, C_7 H).

Anal. Calcd for $C_{24}H_{30}N_8O_5$·0.5H_2O: C, 55.48; H, 6.01; N, 21.57. Found: C, 55.56; H, 6.05; N, 21.63.

***N*-[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]-L-glutamic Acid (4b, Methotrexate). A. From Isolated 4a.** The ester **4a** 0.5HBr·1.5H_2O (1.80 g, 3.12 mmol) was dissolved in warm EtOH (50 ml), and the solution was cooled to 20 °C and treated with 1 N NaOH (10 ml). The sodium salt of **4b** began separating from the stirred solution after about 2 h at 20–25 °C. After 24 h, H_2O (40 ml) was added to dissolve the yellow solid, and the solution was evaporated under reduced pressure (H_2O aspirator, bath at 20–25 °C) until the EtOH had been removed. The clear aqueous solution (~30 ml) was diluted with H_2O (to 60 ml) and treated with 1 N HCl to lower the pH to 7.0. The solution was filtered to ensure clarity before the pH was finally lowered to 4.0 to cause precipitation of **4b**. After overnight refrigeration, the yellow-orange solid was collected, washed with H_2O followed by Et_2O, and dried. The solid was pulverized, dried further, and then allowed to equilibrate with ambient conditions of the laboratory, yield 95% (1.50 g), $[\alpha]^{24}D$ +19.0 ± 0.1° in 0.1 N NaOH (*c* 1.1). Spectral data: UV, in agreement with that reported;[23a] ^{1}H NMR, identical with that listed below under B.

Anal. Calcd for $C_{20}H_{22}N_8O_5$·3H_2O: C, 47.24; H, 5.55; N, 22.04. Found: C, 47.43; H, 4.91; N, 22.18.

B. Without Isolation of 4a. A mixture of **3** (3.0 g of ~95% purity, 8.5 mmol; molar ratio **3** to **1b**, 15:1) and diethyl *N*-[4-(methylamino)-benzoyl]-L-glutamate[27] (3.3 g, 9.8 mmol) in Me_2NAc (36 ml) was stirred at 20–25 °C under N_2 in a stoppered flask protected from light. Solution occurred within 24 h. After 120 h, H_2O (180 ml) was added with rapid stirring followed immediately by 2 N NaOH (18 ml). More H_2O (90 ml) was added within 5 min. Nearly all of the semisolid precipitate that formed when the NaOH was added dissolved within 10 min. The solution phase was decanted into another flask, and the small amount of semisolid that remained was dissolved in Me_2NAc (10 ml). This solution was treated with 2 N NaOH (2 ml) and combined with the main portion. The basic solution was kept under N_2 in a stoppered flask protected from light for 20 h and then treated with 1 N HCl to lower the pH (from 10.5) to 5.5. The solution was treated with Norit and filtered through a mat of compressed cellulose powder (~1 cm thick in a 150-ml Buchner funnel). The mat was washed with H_2O until the wash solution was colorless. Acidification to pH 4.0 caused precipitation of **4b** as a voluminous yellow-orange solid. The mixture was stirred with ice-bath cooling for 2 h before the product was collected, then redissolved by suspending it in H_2O (300 ml) and treating the stirred suspension with 2 N NaOH (9 ml). The isolation process (acidification to pH 5.5, treatment with Norit, filtration through cellulose mat, acidification to pH 4.0, and stirring at 0–5 °C for 2 h) was repeated. The collected product was washed with H_2O, dried, and allowed to equilibrate with ambient conditions of the laboratory, yield 58% (2.49 g), $[\alpha]^{21}D$ +18.7 ± 0.5° in 0.1 N NaOH (*c* 1.0). Spectral data: ^{1}H NMR (Me_2SO-d_6) δ 2.0 (m, $CHCH_2CH_2$), 2.3 (m, CH_2CO_2H), 3.22 (s, Me), 4.4 (m, $NHCHCO_2H$), 4.82 (s, CH_2N), 6.85 and 7.73 (two d, C_6H_4), 7.00 (s, NH_2), 7.9 (br s, NH_2), 8.2 (d, NHCO), 8.62 (s, C_7 H).

Anal. Found: C, 47.36; H, 5.04; N, 22.22 (see calcd given above).

The same results were obtained when the alkylation step was carried out at 53–57 °C for 4 h.

C. From 4a Prepared from 4d. Hydrolysis of **4a** (derived from **4d**) as described under A above led to **4b**, $[\alpha]^{24}D$ +17.5 ± 0.7° in 0.1 N NaOH (*c* 0.99), in 90% yield. The spectral (UV and ^{1}H NMR) and chromatographic (TLC and HPLC) properties of this sample were identical with those of **4b** described above.

***N*-[4-[(2,4-Diamino-6-pteridinyl)methyl]benzoyl]-L-glutamic Acid (4c, Aminopterin).** A mixture of **3** (53.8 g prepared by method B, 0.147 mol as **3** $0.5C_3H_7OH$) and *N*-(4-aminobenzoyl)-L-glutamic acid (84.0 g, 0.316 mol) in Me_2NAc (630 ml) was stirred at 25 °C under N_2 in a stoppered flask protected from light for 18 h. The solution that formed was poured slowly into H_2O (4.5 l.) at 80 °C with stirring. The resulting solution was allowed to cool and kept for 6 h at 20–25 °C while yellow **4c** deposited in granular form. The mixture was then left in a refrigerator for 16 h. The collected solid was washed successively with H_2O, EtOH, Me_2CO, and Et_2O, yield 72% (49.4 g), $[\alpha]^{24}D$ +17.5 ± 0.3° in 0.1 N NaOH (*c* 1.1). Spectral data: UV, in

agreement with that reported;[23a] ^{1}H NMR (Me_2SO-d_6) δ 2.0 (m, $CHCH_2CH_2$), 2.3 (m, CH_2CO_2H), 4.4 (m, $NHCHCO_2H$), 4.54 (s, CH_2N), 6.85 and 7.72 (two d, C_6H_4), 6.8 (CH_2NH under part of the C_6H_4 multiplet), 7.0 (broad s, NH_2), 8.2 (d, NHCO plus NH_2), 8.76 (s, C_7 H).

Anal. Calcd for $C_{19}H_{20}N_8O_5 \cdot 1.6H_2O$: C, 48.63; H, 4.98; N, 23.88. Found: C, 48.87; H, 4.86; N, 23.87.

4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoic Acid (4d). A solution of **3** (171 mg of ~97% purity, 0.49 mmol; molar ratio of **3** to **1b**, 25:1) and 4-(methylamino)benzoic acid (83 mg, 0.55 mmol) in Me_2NAc (2 ml) was stirred at 25 °C for 114 h and then mixed with H_2O (18 ml). The solid that separated (150 mg) was dissolved in NaOH solution (7.5 ml of 0.08 N), and treatment of the clarified (Norit, Celite) solution with dilute HCl to produce pH 6.5 gave yellow **4d**, yield 60% (105 mg) (dried in vacuo at 78 °C over P_2O_5). Spectral data (UV, IR, and ^{1}H NMR) agreed with that reported earlier.[11]

Anal. Calcd for $C_{15}H_{15}N_7O_2 \cdot 1.5H_2O$: C, 51.13; H, 5.15; N, 27.83. Found: C, 51.02; H, 5.24; N, 27.52.

In a larger run, a mixture of **3** (12.0 g of ~96% purity, 34.3 mmol; molar ratio of **3** to **1b**, 19:1) and 4-(methylamino)benzoic acid (5.93 g, 39.3 mmol) in Me_2NAc (140 ml) was stirred at 55 °C (bath temperature) for 4 h. Pure **4c** $1.5H_2O$ was isolated as before in 61% yield (7.40 g), UV, IR, and ^{1}H NMR spectra identical with those of the sample described above.

Anal. Found: C, 50.88; H, 4.86; N, 27.82 (see calcd given above).

4-[[(2,4-Diamino-6-pteridinyl)methyl]amino]benzoic Acid (4e) Hydrobromide (1:1). A mixture of 4-aminobenzoic acid (2.06 g, 15.0 mmol) and pulverized **3** C_3H_7OH (1.98 g, 5.00 mmol) in Me_2NAc (25 ml) was stirred at 25 °C for 24 h. The yellow solid was collected with the aid of Me_2NAc and washed with H_2O followed by Et_2O, yield 94% (1.83 g). Spectral data: UV, 0.1 N HCl, 242 nm (16.5), 297 (22.0), 334 (plateau) (11.6); pH 7, 260 nm (28.7), 278 (sh), 370 (7.81); 0.1 N NaOH, 260 nm (28.9), 278 (sh), 370 (7.71). A suitable solvent was not found for determination of the ^{1}H NMR spectrum of **4e** HBr. A sample was dissolved in dilute NaOH, and treatment with AcOH led to **4e**, whose spectrum was determined in Me_2SO-d_6. The sample was, however, solvated by AcOH as evidenced by a singlet at δ 2.0 (CH_3). The remainder of the spectrum was as follows: δ 4.5 (s, CH_2), 6.8 and 7.6 (two d, C_6H_4, overlapping NH and NH_2), 8.8 (s, C_7 H).

Anal. Calcd for $C_{14}H_{13}N_7O_2 \cdot HBr$: C, 42.87; H, 3.60; Br, 20.37; N, 25.00. Found: C, 42.74; H, 4.08; Br, 20.39; N, 24.80.

4-[[(2-Amino-3,4-dihydro-4-oxo-6-pteridinyl)methyl]amino]benzoic Acid (5, Pteroic Acid).[16] A stirred mixture of **4e** HBr (6.60 g, 16.8 mmol) and 0.2 N NaOH (1.2 l.) was refluxed under N_2 for 1.5 h. The cooled and filtered (Norit, Celite) solution was treated with 3 N HCl to lower the pH to 3.5, and the yellow precipitate that formed was collected by centrifugation, suspended in H_2O (1 l.), and redissolved by addition of the required amount of 1 N NaOH. The precipitation process described above was repeated, and **5** was first collected by centrifugation, then suspended in H_2O and collected by filtration, yield 70% (3.66 g). Spectral data (^{1}H NMR and UV) agreed with reported results.[21b,22]

Anal. Calcd for $C_{14}H_{12}N_6O_3$: C, 53.84; H, 3.87; N, 26.91. Found: C, 53.64; H, 3.83; N, 26.83.

HPLC Analyses. Compounds **4b** and **4c** were examined by reversed-phase HPLC using four mobile phases: A, 0.1 M Tris buffer (pH 6.7)–MeOH (4:1); B, 0.1 M KH_2PO_4 (pH 6.7)–MeOH (83:17); C, 0.005 M NH_4OAc (pH 5)–MeCN (85:15); D, H_2O–MeCN (3:2). This sensitive technique revealed only very minor impurities in each of these products. The absence of **1b** from both was confirmed by analyses using mobile phases A and D. Deliberate mixtures of **1b** with **4b** or **4c** were well resolved using these phases, and peak area ratios were still measurable using phase A in prepared mixtures containing as little as 0.5% of **1b**. Total organic impurities in **4b** prepared as described amounted to well below 1%. None of the impurities commonly seen in the **4b** in clinical use prior to development of this process was detected using phase C, which was used by Tong and co-workers in developing a HPLC assay method for **4b**.[2] The total impurities present in the **4b** used in the Tong study amounted to 5.9%; the main contaminants were N^{10}-methylfolic acid (2.7%) and **4d** (1.6%). The total organic impurity level in **4c** prepared as described was somewhat greater than in **4b**, usually amounting to approximately 1%. Mixtures of folic acid, **4c**, and **4b** elute, in the order given, with baseline separation using mobile phase A.[5] There was no indication of folic acid in **4c**. Two minor contaminants were detected, one of which was identified as *N*-(4-aminobenzoyl)-L-glutamic acid. Results on **4e**, **5** (phase A), and **4d** (phase C) also indicated only very minor amounts of impurities.

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Registry No.—**1a**, 945-24-4; **1a** HBr, 57963-59-4; **1b** HBr, 60662-06-8; **3**, 52853-40-4; **4a**, 43170-88-3; **4a** 0.5HBr, 60662-07-9; **4b**, 59-05-2; **4b** Na, 15475-56-6; **4c**, 54-62-6; **4d**, 19741-14-1; **4e** HBr, 60662-08-0; **5**, 119-24-4; diethyl *N*-[4-(methylamino)benzoyl]-L-glutamate, 2378-95-2; diethyl L-glutamate HCl, 1118-89-4; *N*-(4-aminobenzoyl)-L-glutamic acid, 4271-30-1; 4-(methylamino)benzoic acid, 10541-83-0; 4-aminobenzoic acid, 150-13-0.

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