Folic Acid Analogs. Modifications in the Benzene-Ring Region. 5. 2′,6′-Diazafolic Acid (1,2)

Eugene C. Roberts (3) and Y. Fulmer Shealy

Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, Alabama 35205

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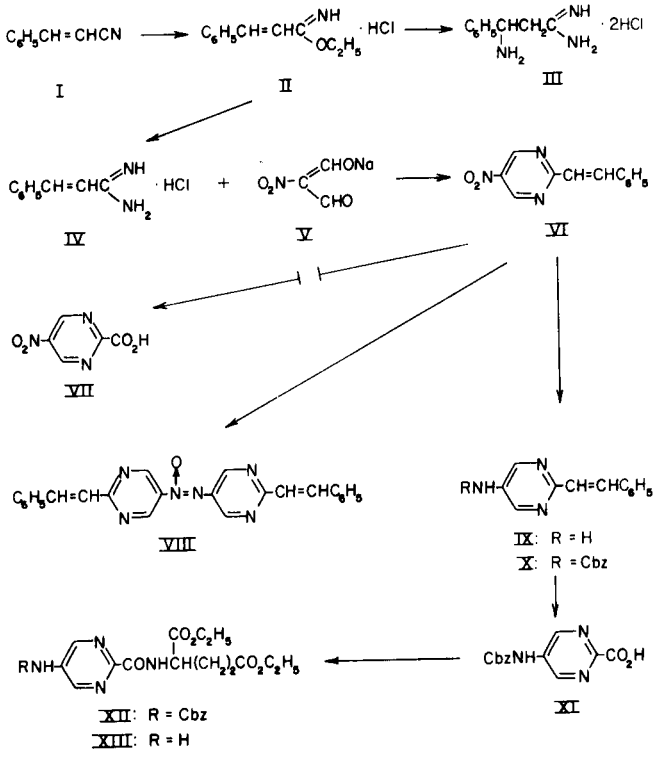
The synthesis of 2′,6′-diazafolic acid was accomplished by the condensation of 2-acetylamino-4(3*H*)pteridinone-6-carboxaldehyde (XIV) with diethyl *N*-[(5-amino-2-pyrimidinyl)carbonyl]-L-glutamate (XIII) followed by reduction of the anil double bond and alkaline hydrolytic cleavage of the N^2-acetyl and ethyl ester protecting groups. Intermediate XIII was prepared by starting with 5-nitro-2-styrylpyrimidine (VI) and proceeding *via* 5-amino-2-styrylpyrimidine (IX). The benzyloxycarbonyl derivative of IX was prepared and oxidized to the corresponding 5-benzyloxycarbonylaminopyrimidine-2-carboxylic acid (XI). The coupling of XI with diethyl L-glutamate followed by hydrogenolysis of the benzyloxycarbonyl function afforded the desired intermediate XIII. 2′,6′-Diazafolic acid was a potent inhibitor of *Streptococcus faecium* and displayed marginal activity against leukemia L1210 in mice.

The preparation of 2′,6′-diazafolic acid (XVI) was undertaken as part of a continuing program whose goal is to obtain folic acid analogs having antineoplastic activity, and the synthesis of XVI is herein reported. The electron availability at N^{10} in analog XVI, in which the benzene ring of folic acid is replaced by a pyrimidine ring, is diminished with respect to folic acid. Analogs in which the benzene ring of folic acid is replaced by pyridine (4) and thiazole (5) rings, and in which the electron availability at N^{10} is likewise diminished, have previously been prepared in this laboratory. The manner in which an increased or decreased electron availability at N^{10} may potentially affect the participation of the reduced forms of folic acid analogs in folate metabolism has been described earlier (4). Folic acid analogs prepared in this laboratory and having an increased electron availability at N^{10} include 3′-ethyl- and 3′-isopropylfolic acids (1) and neohomo- and neobishomofolic acids (6).

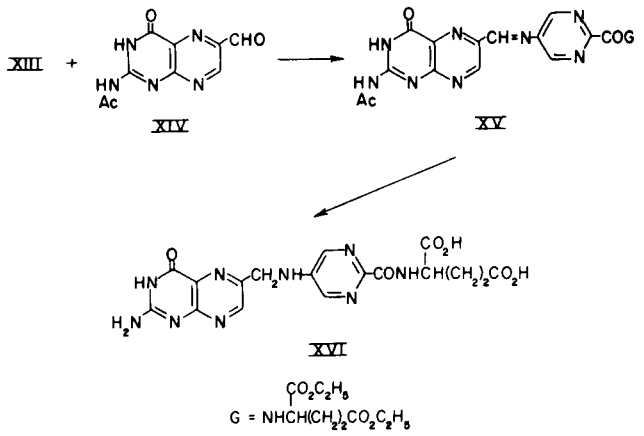
A key intermediate (see Scheme I) in the synthesis of XVI was 5-nitro-2-styrylpyrimidine (VI), a compound which had previously been prepared by Fanta (7) by the reaction of cinnamamidine hydrochloride (IV) with sodium nitromalonaldehyde (V) (8). The conversion of cinnamonitrile (I) to ethyl cinnamimidate hydrochloride (II) proceeded without difficulty, but the reaction of II with ethanolic ammonia as described in the literature (7,9) afforded III. Compound III was identified by its elemental analysis and nmr spectrum and probably arose by the 1,4-addition of excess ammonia to IV. When II was treated with exactly one equivalent of ammonia in ethanol, an excellent yield of crystalline IV was obtained. The

SCHEME I



reaction of IV with V proceeded smoothly as described by Fanta (7), and VI was obtained in 47% yield.

SCHEME II



Several attempts were made to oxidize VI to 5-nitropyrimidine-2-carboxylic acid (VII), but no evidence was found to indicate the presence of VII in any of the reaction products. The conversion of VI to IX by the selective reduction of the nitro group of VI was, therefore, undertaken. The hydrogenation of VI in *N,N*-dimethylacetamide proceeded with the desired selectivity, and 5-amino-2-styrylpyrimidine (IX) was obtained reproducibly in several runs. The relatively slow rate of hydrogen uptake during the reduction of VI probably contributed to the formation of the azoxy compound VIII which always accompanied the desired compound IX. Protection of the amine function of IX with carbobenzoxy chloride proceeded without difficulty in 68% yield, and the resulting X was oxidized to the desired acid XI by a modification of the procedure of McOmie and White (10). The reaction of XI, diethyl glutamate, and dicyclohexylcarbodiimide afforded XII, and the hydrogenolytic cleavage of the benzyloxycarbonyl group of XII yielded XIII.

The synthesis and purification of XVI were carried out (see Scheme II) as described in detail previously (6). The reaction of XIII with 2-acetylamino-4(3*H*)pteridinone-6-carboxaldehyde (XIV) (11) was allowed to proceed in dimethylsulfoxide at room temperature for several days. Since the intermediate anil XV (12) did not precipitate from this reaction, molecular sieves were added to remove the water formed. The anil XV was not isolated (12) and was reduced with a slight excess of sodium borohydride. Hydrolysis of the N_2-acetyl and ethyl ester functions was accomplished simultaneously, and XVI was obtained (15% yield from XIV) as a yellow powder after DEAE-cellulose column chromatography.

Analog XVI was administered on single-dose (day 1 only) and daily (qd 1-9) schedules to mice implated ip on day 1 with 10^5 leukemia L1210 cells. On the single-dose schedule, XVI was acutely toxic at a dose of 400 mg./kg. and, at dose levels of 200 and 100 mg./kg., was non-toxic and produced no significant increases (T/C $\geq$ 125%) in life span. On the daily schedule, XVI displayed marginal antileukemic activity at 100 mg./kg. (T/C = 128%) and 75 mg./kg. (T/C = 128%); differences in weight change (ΔW = T-C) were -3.8 and -3.6 g., respectively. At 50 mg./kg. on the daily schedule, XVI was non-toxic and inactive.

Compound XVI was tested for inhibition of pigeon liver dihydrofolate reductase according to the method of Baker, *et al.* (13), and was found to cause < 50% inhibition at a concentration of 1×10^{-4} *M*. By comparison, methotrexate was 50% inhibitory at 1.3×10^{-8} *M*.

Analog XVI was found to effect 50% inhibition of *Streptococcus faecium* ATCC 8043 at a concentration of 1.0×10^{-8} *M*. In a simultaneous test, methotrexate was 50% inhibitory at 6.2×10^{-10} *M*. Methotrexate was, therefore, 16 times more effective as an inhibitor of *S. faecium* than analog XVI. In contrast to the strong inhibition of *S. faecium* by 2′,6′-diazafolic acid (XVI), 2′-azafolic acid has been previously found (4) to be a growth factor for *S. faecium*. These tests were conducted according to a standard test procedure (14). It is apparent from these data that *S. faecium* is quite sensitive to minor structural variations in the benzene-ring portion of the folic acid molecule.

EXPERIMENTAL

Melting points were determined with a Kofler Heizbank apparatus. Proton magnetic resonance spectra were determined with a Varian XL-100 spectrometer using tetramethylsilane as an internal standard. Mass spectral data were obtained using a Hitachi RMU-6D double-focusing high resolution mass spectrometer. Infrared spectra were determined with a Perkin-Elmer Model 621 spectrometer, and ultraviolet spectral data were obtained using a Cary Model 17 spectrometer. Products (IX, XII, XIII) which were purified by silica gel column chromatography were detected visibly as zones which appeared opaque against the translucent column. The purity of the corresponding column fractions was determined by thin-layer chromatography before combination.

5-Nitro-2-styrylpyrimidine (VI).

Because some difficulty was encountered in the preparation of VI as originally described by Fanta and Hedman (7), details of the synthesis of IV and VI are herein given. Cinnamonitrile (1, 75 g., 0.58 mole) was added to a solution of hydrogen chloride (25.8 g., 0.71 mole) in 40 ml. of anhydrous ethanol, and 100 ml. of anhydrous ether was added to this mixture. The ethyl cinnamimidate hydrochloride (II; 100.0 g., 0.47 mole, 81% yield), isolated as a crystalline precipitate after 11 days' reaction time, was added to a solution of ammonia (8.0 g., 0.47 mole) in 800 ml. of anhydrous ethanol chilled to 0°. After 3 hours at 0°, the

reaction mixture was stirred at room temperature until the initially formed precipitate of ammonium chloride had disappeared and there was no evidence (according to the ir spectra of reaction aliquots) of unreacted ammonium chloride remaining in solution; the reaction time required was 6 days. The solution was concentrated *in vacuo* to a heavy syrup, and the syrup was allowed to dry further in air. The crystalline residue thus obtained was dried *in vacuo* to a constant weight: 99.5 g., m.p. 70-72°; lit. (7) m.p. 68-70°. The quantity of cinnamamidine hydrochloride (IV) obtained was in excess of the theoretical amount (85.8 g.) and corresponded to a 97% yield of IV as a dihydrate.

To a vigorously blade-stirred solution of IV (99.5 g., 0.46 mole) in 500 ml. of water containing 250 ml. of methanol was added a solution of 86 g. (0.55 mole) of sodium nitromalonaldehyde hydrate (8) in 900 ml. of water. A heavy precipitate formed rapidly, and 50 ml. of commercial Triton B (40% solution of benzyltrimethylammonium hydroxide in methanol) was added. The reaction mixture was stirred vigorously and heated at 60-70° for 4 hours, and the precipitate obtained by filtration of the cooled reaction mixture was washed with ethanol and water. The dried filter cake (60 g.) was digested exhaustively with refluxing benzene, and VI was obtained by chilling the combined extracts (*ca.* 6 l.): 49.4 g. of yellow crystals (47% yield), m.p. 228-229° [lit. (7) m.p. 219-220°]; ir ν (cm^{-1}): 1630 (C=C), 975 (*trans* HC=CH).

Anal. Calcd. for $C_{12}H_9N_3O_2$: C, 63.43; H, 4.00; N, 18.49. Found: C, 63.56; H, 4.27; N, 18.70.

5-Amino-2-styrylpyrimidine (IX).

A total of 45.44 g. (0.2 mole) of VI was divided into four equal portions (0.05 mole per portion) which were each dissolved in 1500 ml. of *N,N*-dimethylacetamide and hydrogenated at atmospheric pressure over Raney nickel catalyst. The time required for the desired uptake of hydrogen varied from 20 to 36 hours. Removal of the catalyst by filtration and evaporation of the solvent afforded a solid residue from which IX was obtained by extraction with hot benzene. The amount of benzene-insoluble, golden, crystalline material obtained by this procedure varied from batch to batch, and, in every case, this material was identified as VIII by ir [ν (cm^{-1}): 1625 (C=C), 1555 (N=N), 975 (*trans* HC=CH)] and ms data (m/e 406). Fractional crystallization of the benzene filtrates afforded more VIII and then IX; 7.71 g. of IX (m.p. 160°) thus obtained required no further purification. The remainder of the material containing IX was applied to a silica gel column (600 g.), and the column was eluted with chloroform/methanol (99:1); combination of the best fractions afforded 9.80 g. of IX (m.p. 160°). The total yield of IX was 17.51 g. (44%); ir ν (cm^{-1}): 1630 (C=C), 960 (*trans* HC=CH).

Anal. Calcd. for $C_{12}H_{11}N_3$: C, 73.07; H, 5.62; N, 21.30. Found: C, 73.07; H, 5.69; N, 21.23.

5-Benzyloxycarbonylamino-2-styrylpyrimidine (X).

To a solution of 17.35 g. (88 mmoles) of IX in 400 ml. of *N,N*-dimethylacetamide was added 16.55 g. (97 mmoles) of carbobenzoxy chloride and 11.75 g. (97 mmoles) of *s*-collidine. After 3 days' reaction at room temperature, the solvent was evaporated, and the residue was suspended in ethyl acetate. This suspension was washed twice with *N* hydrochloric acid, and the acidic aqueous extracts were extracted with ethyl acetate. The combined ethyl acetate solutions were washed with saturated sodium bicarbonate solution and with water, and dried. Partial evaporation of the ethyl acetate afforded 18.3 g. of crystalline X (m.p. 180-181°). Benzene trituration of the remaining ethyl acetate residue afforded an additional 1.4 g. of X (m.p. 180°). The total yield of X was 19.7 g. (68%); ir ν (cm^{-1}): 1728 (C=O), 1630 (C=C).

Anal. Calcd. for $C_{20}H_{17}N_3O_2$: C, 72.49; H, 5.18; N, 12.69. Found: C, 72.28; H, 5.38; N, 12.61.

Diethyl *N*-[(5-Benzyloxycarbonylamino-2-pyrimidinyl)carbonyl]-L-glutamate (XII).

To a stirred, room-temperature solution of 18.9 g. (57 mmoles) of X in 500 ml. of *N,N*-dimethylacetamide containing 10 ml. of water was added a solution of potassium permanganate (24.0 g., 152 mmoles) in 800 ml. of *N,N*-dimethylacetamide containing 20 ml. of water. The addition required 2 hours, and the oxidation was allowed to continue for 1 hour afterwards. The manganese dioxide precipitate was isolated by filtration, digested at room temperature with 2 l. of 0.5 *N* ammonium hydroxide, and then washed with three 400-ml. portions of *N* ammonium hydroxide. Acidification of the combined ammonium hydroxide solutions to *p*H 4.5 afforded a white solid (XI).

Evaporation of the *N,N*-dimethylacetamide filtrate afforded a residue which was digested with 300 ml. of *N* ammonium hydroxide. Filtration of the resulting suspension yielded 1.73 g. of unreacted X (m/e 331). Acidification of the aqueous alkaline filtrate to *p*H 4.5 afforded an additional quantity of white solid (XI). The total yield of crude XI (after drying *in vacuo* over phosphorus pentoxide) was 10.9 g. (77% based on unrecovered X); m/e 273. This material was used without further purification.

A suspension of XI (10.8 g., 39.6 mmoles), diethyl L-glutamate hydrochloride (9.5 g., 39.6 mmoles), and dicyclohexylcarbodiimide (8.15 g., 39.6 mmoles) in 1400 ml. of anhydrous pyridine was stirred at room temperature for 4 days. The precipitated dicyclohexylurea was removed by filtration and washed with chloroform, and the filtrate was evaporated. The residue was dissolved in ethyl acetate, and this solution was washed twice with 2 *N* hydrochloric acid, twice with saturated aqueous sodium bicarbonate, and with water. Drying of the ethyl acetate solution and evaporation yielded a residue which was chromatographed over silica gel (400 g.; eluted with chloroform/methanol 98:2). The product (XII) was obtained as a glass; 13.22 g., 73% yield; m/e 458.

Anal. Calcd. for $C_{22}H_{26}N_4O_7 \cdot 0.25CHCl_3$: C, 54.73; H, 5.42; N, 11.47. Found: C, 54.85; H, 5.64; N, 11.52.

Diethyl *N*-[(5-Amino-2-pyrimidinyl)carbonyl]-L-glutamate (XIII).

Hydrogen was bubbled continuously for 5 hours into a stirred solution of XII (13.12 g., 28.6 mmoles) and sodium methoxide (3.9 g., 71.6 mmoles) in 400 ml. of ethanol containing suspended palladium black (from 6.3 g., 35.8 mmoles of palladium chloride). Removal of the catalyst and evaporation of the filtrate afforded a residue which was purified by silica gel column chromatography (eluted with chloroform/methanol 97:3). The yield of XIII was 4.30 g. (50%), m.p. 112°; m/e 324; nmr δ (in deuteriochloroform): 8.30 (d, 1H, CONH), 8.26 (s, 2H, pyrimidine CH), 4.88 (m, 1H, NCH), 4.17 (8 lines, 4H, OCH_2), 2.21 (m, 4H, CH_2CH_2), 1.26 (4 lines, 6H, CH_3).

Anal. Calcd. for $C_{14}H_{20}N_4O_5$: C, 51.85; H, 6.22; N, 17.27. Found: C, 52.09; H, 6.34; N, 17.43.

N-[[5-[[(2-Amino-3,4-dihydro-4-oxo-6-pteridinyl)methyl]amino]-2-pyrimidinyl]carbonyl]-L-glutamic Acid (XVI).

A solution of XIII (3.89 g., 12 mmoles) and XIV (2.80 g., 12 mmoles) in 66 ml. of anhydrous dimethylsulfoxide containing 2 g. of molecular sieves (Linde Type 4A) was allowed to stir at

room temperature for 3 days. The reaction mixture was filtered, and the filtrate was transferred to a solution of sodium borohydride (570 mg., 15 mmoles) in 570 ml. of anhydrous *N,N*-dimethylformamide. After 96 hours, the solvent was evaporated, and the residue was hydrolyzed at room temperature, anaerobically, in 3000 ml. of aqueous 0.1 *N* sodium hydroxide solution for 24 hours. This solution was acidified (hydrochloric acid) to *p*H 3.9 and refrigerated overnight. The gelatinous precipitate was isolated and washed twice with 0.004 *N* hydrochloric acid before drying *in vacuo* over phosphorus pentoxide. The solid (2.5 g.) thus obtained was dissolved in 3000 ml. of dilute ammonium hydroxide containing 45 ml. of 2-mercaptoethanol, and this solution (final *p*H 6.7) was applied to a DEAE-cellulose column [5 cm. x 65 cm.; prepared for column use in the phosphate form, as described previously (4), from approximately 120 g. of regular capacity Mannex DEAE-cellulose]. The product was eluted with a potassium phosphate (0.005 *M*) buffer solution at *p*H 7.0 containing sodium chloride (0.2 *M*) and 2-mercaptoethanol (0.2 *M*). The product appeared as a yellow-colored zone which was eluted in that volume of effluent between 1740 ml. and 4100 ml. of buffer solution. The solid obtained by acidification (hydrochloric acid) to *p*H 3.8 of the pooled fractions was isolated and washed with 0.004 *N* hydrochloric acid by centrifugation before redissolving in 700 ml. of dilute sodium hydroxide solution containing 7 ml. of 2-mercaptoethanol. The product was reprecipitated by acidification (hydrochloric acid) to *p*H 3.8 and isolated and washed three times with 0.004 *N* hydrochloric acid by centrifugation. The solid was freeze-dried and pulverized before final drying (24 hours) *in vacuo* (0.25 mm) over phosphorus pentoxide at room temperature. The yield of XVI was 848 mg. (15%) as a yellow powder; nmr δ (in deuterotrifluoroacetic acid) 9.04 (s, 1H, C_7-H), 8.95 (s, 2H, 3′-H and 5′-H), 5.09 (m, 3H, CH_2N and NCH), 2.65 (m, 4H, CH_2CH_2); uv max (0.1 *N* hydrochloric acid): 228 (sh), 245 (ε 13,400), 301 (22,900); uv max (*p*H 7 buffer) 218 (ε 15,900), 230 (15,600), 283 (27,300); uv max (0.1 *N* sodium hydroxide): 256 (ε 24,900), 288 (24,200), 362 (9310).

Anal. Calcd. for $C_{17}H_{17}N_9O_6 \cdot H_2O$: C, 44.25; H, 4.15; N, 27.32. Found: C, 44.42; H, 4.12; N, 27.10.

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