3′-Iodo-4-amino-4-deoxypteroylglutamic Acid (3′-Iodoaminopterin)

TI LI LOO, ROBERT L. DION, RICHARD H. ADAMSON,

Laboratory of Chemical Pharmacology

MICHAEL A. CHIRIGOS,

Drug Evaluation Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland

AND ROY L. KISLIUK

Department of Pharmacology, Tufts University School of Medicine, Boston, Massachusetts

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A systematic investigation of the therapeutic efficacy of folic antagonists against advanced mouse leukemia has revealed[1] that some brominated and chlorinated folic analogs were more active in prolonging the survival time of leukemic mice than the parent compounds. For example, 3′,5′-dichloroaminopterin was more effective than aminopterin[2a] and 3′,5′-dichloromethotrexate was more effective than methotrexate (amethopterin)[2b] in increasing the life span of the leukemic animals.[3] More recently the synthesis and antitumor activity of the 3′-fluoro and the 3′,5′-difluoro derivatives of methotrexate were reported.[4] Finally, the observed antiviral activity of several iodine compounds such as 5-iodo-2′-deoxyuridine[5] and 3,5-diiodo-4-hydroxybenzenesulfonamide,[6] especially in the light of the current concept of viral oncogenesis,[7] stimulated our interest in the study of iodinated folic antagonists. The present paper deals with 3′-iodoaminopterin.

Although direct iodination of aminopterin by a procedure similar to that for the reported iodofolic acid[8] could lead to structural ambiguities,[9] of the three methods attempted,[10,11] only the direct method proved successful. Iodine monochloride and pure aminopterin[12] (optimal molar ratio 3:1) in dimethylformamide (DMF)[13] gave 3′-iodoaminopterin in 38% yield. The reaction was complicated by partial cleavage of aminopterin as shown by the isolation of 2,4-diamino-6-pteridinecarboxaldehyde and 3,5-diiodo-4-aminobenzoylglutamic acid. A lower ratio of ICl to aminopterin resulted in incomplete iodination while a higher ratio (*e.g.*, 6:1) gave quantitative molecular cleavage but no trace of 3′,5′-diiodoaminopterin. Analogy for this cleavage is found in the splitting of pteroylglutamic acid into *p*-aminobenzoylglutamic acid and 2-amino-4-hydroxy-6-pteridinecarboxaldehyde either photochemically[14] or by mild oxidizing agents such as sulfurous acid[15] or methylene blue.[16] The mechanism of the cleavage of aminopterin by ICl has not been studied. However, it is probable that the 3,5-diiodo-4-aminobenzoylglutamic acid resulted from iodination of *p*-aminobenzoylglutamic acid first formed from cleavage since this diiodo compound was isolated when a large excess of iodine monochloride was allowed to react with *p*-aminobenzoylglutamic acid in DMF. Moreover, all attempts to further iodinate 3′-iodoaminopterin to the 3′,5′-diiodo compound with ICl were unsuccessful.

The position of the iodine in 3′-iodoaminopterin was established by its careful oxidation with $KMnO_4$ in dilute alkali to yield 2,4-diamino-6-pteridinecarboxylic acid[17] and 3-iodo-4-aminobenzoylglutamic acid. The latter, hitherto unknown, was easily prepared by the iodination of *p*-aminobenzoylglutamic acid with either ICl or simply iodine in DMF, a novel way of iodination.

Biological Activities.—3′-Iodoaminopterin was screened for antileukemic activity against advanced mouse leukemia L1210 in comparison with the known active drugs aminopterin and methotrexate.[3] Results summarized in Table I indicated that 3′-iodoaminopterin was as effective as aminopterin *at optimal daily dose levels*, but less effective than methotrexate against advanced mouse leukemia L1210. This optimal daily dose for 3′-iodoaminopterin is 8 times that for aminopterin, and twice that for 3′-chloroaminopterin,[3] both on a molar basis, in extending the medium survival time of L1210 leukemic mice to 19 days. The difference could be attributed to discrepancies in cellular penetration and uptake, degree of protein binding, extent of metabolism, rate of excretion, and molecular conformation among these drugs.

TABLE I

ACTIVITY AGAINST ADVANCED MOUSE LEUKEMIA L1210[a]

Compd.	Range of doses, mg./kg.	Optimal dose, mg./kg.	Median survival time, days
3′-Iodoaminopterin[b]	0.625–60	1.25–2.5	19
Aminopterin	0.05–0.5	0.15	19
Methotrexate	0.25–2.5	1.0	26
Controls	...	...	11

[a] Ten mice were used at each dose level. All drugs dissolved in 2% $NaHCO_3$ and injected subcutaneously daily. Treatment started 8 days after tumor inoculation. [b] The LD_{50} of 3′-iodoaminopterin in L1210 mice after daily injection for 7 days was 10 mg./kg.

(1) A. Goldin, S. R. Humphreys, J. M. Venditti, and N. Mantel, *J. Natl. Cancer Inst.*, **22**, 811 (1959).

(2) (a) Generic name for 4-amino-4-deoxypteroylglutamic acid. (b) Generic name for 4-amino-4-deoxy-N^{10}-methylpteroylglutamic acid.

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(4) A. S. Tomcufcik and D. R. Seeger, *J. Org. Chem.*, **26**, 3351 (1961).

(5) H. E. Kaufman, *Proc. Soc. Exptl. Biol. Med.*, **109**, 251 (1962); R. Hertzberg, *Med. J. Australia*, **1**, 13 (1963); P. W. Sadler, *Pharmacol. Rev.*, **15**, 407 (1963).

(6) W. D. Kundin, M. L. Robbins, and P. K. Smith, *Proc. Soc. Exptl. Biol. Med.*, **116**, 425 (1964).

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(9) Other possible, though not highly probable, sites of iodination include the 7- and the 9-positions of aminopterin.

(10) The condensation of 3-iodo-4-aminobenzoylglutamic acid with 2,4-diamino-6-bromomethylpteridine was contemplated. However, all of our efforts to prepare the latter were not fruitful.

(11) The condensation of 3-iodo-4-aminobenzoylglutamic acid with 2,4-diamino-6-pteridinecarboxaldehyde followed by reduction of the anil formed also failed.

(12) D. R. Seeger, D. B. Cosulich, J. M. Smith, Jr., and M. E. Hultquist, *J. Am. Chem. Soc.*, **71**, 1753 (1949); T. L. Loo, *J. Med. Chem.*, **8**, 139 (1965). Commercial aminopterin must be purified prior to use.

(13) DMF is the solvent of choice. In HCl a complex mixture of products is formed.

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(15) C. W. Waller, A. A. Goldman, R. B. Angier, J. H. Boothe, B. L. Hutchings, J. H. Mowart, and J. Semb, *J. Am. Chem. Soc.*, **72**, 4630 (1950).

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(17) R. L. Dion and T. L. Loo, *J. Org. Chem.*, **26**, 1857 (1961).

TABLE II

COMPARATIVE ANTIVIRAL EFFECT OF 3′-IODOAMINOPTERIN AND AMINOPTERIN IN MICE INFECTED WITH RAUSCHER VIRUS

	Donors		Recipients[c]	
Test group	Spleen wt., mg.[a,b]	Ratio of spleen wt., % T/C	Spleen wt., mg.	Ratio of spleen wt., % T/C
Control	298	...	1212	...
3′-Iodoaminopterin[d]	120	40.2	300	24.7
Aminopterin[d]	112	37.5	340	28.0

[a] Average spleen of normal mice weighs 114 mg. [b] Average spleen weight of 5 mice per group when killed 8 days post-inoculation of virus. [c] Recipients each received intraperitoneally 0.2 ml. of pooled plasma from either treated or control donors. [d] 3′-Iodoaminopterin (10 mg./kg.) and aminopterin (0.75 mg./kg.) administered subcutaneously in the axillary region once daily for 4 days.

Table II represents the results obtained when 3′-iodoaminopterin was compared with aminopterin for *in vivo* activity to inhibit replication of the Rauscher leukemogenic virus. Adult Balb/c mice infected with Rauscher virus develop viremia 3 days after infection and an increase in spleen weight within 7 days post-inoculation. Histologically the disease is characterized by intense erythropoiesis and generalized lymphoid leukemia. The method used for measuring antiviral activity has been previously described.[18] A greater than 50% inhibition in splenomegaly was achieved in the treated animals. Recipient animals inoculated with plasma pooled from the treated donor animals developed relatively smaller spleens than those of mice receiving control plasma. This difference represents an inhibition of two logs of virus (40% of detectable virus). Treatment at the dose of each drug resulted in some weight loss. A more detailed dose response study of each drug is in progress.

3′-Iodoaminopterin was also compared with aminopterin[19] for growth inhibition against *Lactobacillus casei* (ATCC 7469), *Streptococcus faecalis* (ATCC 8043), and *Pediococcus cerevisiae* (ATCC 8081) in the corresponding Difco assay media. The compounds were added aseptically after autoclaving and the incubation was done at 37° for 24 hr. The media for *L. casei* and *S. faecalis* contained 1 mγ/ml. of pteroylglutamic acid whereas the medium for *P. cerevisiae* contained 1 mγ/ml. of calcium DL-5-formyltetrahydropteroylglutamate. The inhibitory powers of the two drugs were compared at one-half maximal inhibition as described earlier.[20] As shown in Table III, for the inhibition of

TABLE III

COMPARISON OF 3′-IODOAMINOPTERIN WITH AMINOPTERIN IN GROWTH INHIBITION OF MICROORGANISMS

Microorganism	Drug for 1/2 maximal inhibition, mγ/ml. 3′-Iodoaminopterin	Aminopterin
L. casei	0.094	0.0045
S. faecalis	0.75	0.047
P. cerevisiae	200	115

growth of *L. casei* and *S. faecalis*, 3′-iodoaminopterin is 1/20 as effective as aminopterin, and for *P. cerevisiae*, 1/2 as effective.

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(19) We thank Mr. Gregory Kwok for performing all the microbiological assays.

(20) R. L. Kisliuk and M. D. Levine, *J. Biol. Chem.*, **239**, 1900 (1964).

Experimental[21]

3′-Iodo-4-amino-4-deoxypteroylglutamic Acid. Pure aminopterin[12] (2 g., 4.2 mmoles) was suspended in 50 ml. of dimethylformamide contained in a conical flask, well protected from light. The suspension was stirred vigorously and ICl (0.6 ml., 1.9 g., 12 mmoles) was added dropwise over 10 min. Still protected from light, the solution was stirred overnight at room temperature. It was poured, with vigorous stirring, into 300 ml. of water at 90°. The cooled mixture was diluted with 150 ml. of ethanol and chilled to 4°. The dark gelatinous precipitate was collected by filtration and washed successively with water, ethanol, and ether. [The combined filtrate and washings, fraction A, were saved for isolation and identification of the cleavage products (see below)]. The precipitate was redissolved in 100 ml. of 0.1 *N* NaOH and filtered. The clear golden solution was carefully acidified at 90° with 2 ml. of glacial acetic acid. The precipitate was partly yellow and partly dark purple even though no free iodine was detectable. It was again collected and washed with water and ethanol. This reprecipitation procedure was repeated twice, and the final product was a yellow crystalline powder. The yield was 0.9 g. (38%). 3′-Iodoaminopterin showed these ultraviolet absorption characteristics: in 0.1 *N* NaOH, λ_{max} 225 mμ (log ε 4.54), 260 (4.42), 279 infl (4.35), and 371 (3.90); in 0.1 *N* HCl, λ_{max} 220 mμ (log ε 4.53), 287 (4.31), and 336 (4.04). The absence of an absorption maximum in acid at 244 mμ distinguishes 3′-iodoaminopterin from aminopterin, and the presence of a maximum in acid at 336 mμ distinguishes 3′-iodoaminopterin from 3′-iodofolic acid. In common with other pteridines, particularly derivatives of folic acid, **3′-iodoaminopterin** inevitably contains various amounts of water as shown by microanalysis of two different specimens.

Anal. Calcd. for $C_{19}H_{19}IN_8O_5 \cdot 1.5H_2O$: C, 38.46; H, 3.74; N, 18.89. Found: C, 38.44; H, 3.66; N, 18.62. Calcd. for $C_{19}H_{19}IN_8O_5 \cdot 2H_2O$: C, 37.76; H, 4.10; I, 20.89. Found: C, 37.89; H, 3.85; I, 21.07.

By treating 3′-iodoaminopterin with magnesium oxide in an aqueous medium, the magnesium salt of 3′-iodoaminopterin was prepared.

Anal. Calcd. for $C_{19}H_{17}IMgN_8O_5 \cdot H_2O$: Mg, 4.01; N, 18.47. Found: Mg, 3.93; N, 18.40.

The anhydrous sample showed the following analyses.

Anal. Calcd. for $C_{19}H_{17}IMgN_8O_5$: C, 38.77; H, 2.91. Found: C, 38.20; H, 3.24.

The homogeneity of 3′-iodoaminopterin was established by column chromatography on DEAE-cellulose[22] and linear gradient elution with ammonium bicarbonate buffer of pH 8, increasing molarity from 0.01 to 0.4 *M*. 3′-Iodoaminopterin came off the column as a single peak; in contrast with aminopterin, the iodo compound was eluted at a higher concentration of ammonium bicarbonate buffer.

Isolation of 2,4-Diamino-6-pteridinecarboxaldehyde.—The combined solution of filtrate and washings, fraction A, described above was allowed to concentrate in a current of air at room temperature until a brown sirup was left. It was treated three times with 10 ml. of methanol and filtered. [The filtrate, fraction B, was saved for isolation of the nonpteridine compound (see below)]. The residue from the methanol treatment was triturated in 20 ml. of 0.1 *M* phosphate buffer of pH 8, filtered, and washed with phosphate buffer and water. The precipitate was dissolved in 10 ml. of 2 *N* HCl and filtered. Neutralization to pH 4 with solid sodium acetate gave a yellow precipitate which was insoluble in alkali but soluble in dilute mineral acids. The yield was 0.4 g. (52%). The presence of an aldehyde group was shown by the instantaneous formation of a dinitrophenylhydrazone. Its ultraviolet absorption spectra were similar to, but not identical with, those of 2-amino-4-hydroxy-6-pteridinecarboxaldehyde[15] prepared either by sulfite cleavage of folic acid or by hydrolysis of 2-amino-4-hydroxy-6-dibromomethylpteridine. This compound was therefore **2,4-diamino-6-pteridinecarboxaldehyde.**

(21) Melting points were observed on a Fisher-Jones apparatus. Ultraviolet absorption spectra were recorded by a Cary Model 14 recording spectrophotometer. Microanalyses were performed by Dr. William C. Alford and associates of National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, to whom we are grateful.

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Anal. Calcd. for $C_7H_6N_6O$: C, 44.21; H, 3.18; N, 44.20. Found: C, 44.27; H, 3.07; N, 44.08.

A dinitrophenylhydrazone was prepared for analysis.

Anal. Calcd. for $C_{13}H_{10}N_{10}O_4 \cdot 3H_2O$: C, 36.80; H, 3.80; N, 32.99. Found: C, 36.98; H, 3.39; N, 33.20.

The ultraviolet absorption characteristics of 2,4-diamino-6-pteridinecarboxaldehyde are: in 0.1 *N* HCl, λ_{max} 244 mμ (log ε 4.16), 262 infl (3.86), 334 (4.04); in 0.1 *N* NaOH, λ_{max} 225 mμ (log ε 4.02), 257 (4.27), 275 infl (4.00), and 372 (3.93).

Isolation of 3,5-Diiodo-4-aminobenzoylglutamic Acid.—Fraction B mentioned above was concentrated at room temperature until a brown sirup was obtained. It was dissolved in 5 ml. of 0.1 *N* NaOH, warmed with a little Darco G-60, and filtered. Acidification of the filtrate caused the separation of a brownish oil. The aqueous phase was decanted and the oil, after washing with water and triturating with 10 ml. of methanol, slowly crystallized. The crystalline material was collected and washed with ethyl acetate. The yield was 0.1 g. (5%), m.p. 220° dec. An analytical sample was prepared by dissolving the brownish crystals in dilute NaOH and reprecipitation with a few drops of glacial acetic acid. Repetition of this procedure afforded a colorless crystalline material, m.p. 222–224° dec. undepressed by an authentic sample of 3,5-diiodo-4-aminobenzoylglutamic acid.[8]

Anal. Calcd. for $C_{12}H_{12}I_2N_2O_5$: C, 27.82; H, 2.34; I, 48.99; N, 5.41. Found: C, 27.78; H, 2.50; I, 49.09; N, 5.45.

Oxidative Degradation of 3′-Iodoaminopterin.—3′-Iodoaminopterin (100 mg.) was dissolved in 5 ml. of 0.1 *N* NaOH and heated in a water bath at 90°. The solution was stirred vigorously and to it was slowly added 1 ml. of 5% $KMnO_4$ in 5 min. The mixture was cooled to room temperature and filtered. The precipitate was washed with a little dilute NaOH then water. The combined filtrate and washings were acidified with 0.5 ml. of glacial acetic acid, and the yellow precipitate (fraction A) was separated from the supernatant (fraction B) by filtration.

Fraction A.—The yellow precipitate was stirred twice with 5 ml. each of 0.1 *M* phosphate buffer of pH 8, and the yellow solution was discarded. The insoluble material was dissolved in 5 ml. of 0.1 *N* NaOH and filtered. The filtrate was acidified with a few drops of glacial acetic acid and centrifuged. The yellow gelatinous material weighed 30 mg. and showed ultraviolet absorption spectra identical with authentic 2,4-diamino-6-pteridinecarboxylic acid.[17]

Anal. Calcd. for $C_7H_6N_6O_2 \cdot H_2O$: C, 37.50; H, 3.60. Found: C, 37.39; H, 3.72.

Fraction B.—The supernatant was further acidified with 1 ml. of concentrated HCl and extracted four times with 10 ml. of ethyl acetate each time. The combined extract was concentrated in a stream of nitrogen at 90° after drying ($MgSO_4$). The yellowish oil was taken up in 1 ml. of ethyl acetate. Upon addition of a few drops of petroleum ether (b.p. 30–60°), yellowish crystals began to form. These crystals were collected and washed with a small volume of ethyl acetate; yield 30 mg., m.p. 164–168°. The product was identical spectrally and chromatographically with an authentic sample of **3-iodo-4-aminobenzoylglutamic acid** prepared according to the following procedure.

Anal. Calcd. for $C_{12}H_{13}IN_2O_5$: C, 36.75; H, 3.34; N, 7.14. Found: C, 36.48; H, 3.32; N, 7.07.

3-Iodo-4-aminobenzoylglutamic Acid.—A mixture of *p*-aminobenzoylglutamic acid (1.07 g., 4 mmoles), iodine (1.12 g., 4.4 mmoles),[23] and 20 ml. of dimethylformamide were placed in a conical flask, wrapped in aluminum foil, and vigorously stirred at room temperature for 5 days. The mixture was concentrated at room temperature in a stream of air. The sirupy residue was taken up in 20 ml. of 1 *N* HCl and extracted with one 20-ml. portion and three 10-ml. portions of ethyl acetate. The combined extract was washed with 20 ml. of 1% KI, then with 25 ml. of 20% sodium thiosulfate. After being dried ($MgSO_4$), the ethyl acetate extract was concentrated in nitrogen at 90°. The resultant oil slowly crystallized. It was triturated with 5 ml. of petroleum ether (b.p. 66–75°) and 5 ml. of ethyl acetate, filtered, and finally washed with ethyl acetate; yield 0.6 g. (39%), m.p. 168°.

Anal. Calcd. for $C_{12}H_{13}IN_2O_5$: C, 36.75; H, 3.34; I, 32.36; N, 7.14. Found: C, 36.89; H, 3.27; I, 32.06; N, 7.12.

(23) A larger excess of iodine resulted in the formation of the 3,5-diiodo compound in addition to the monoiodo compound.

When chromatographed on Whatman 3 mm. paper and developed with 0.1 *M* acetate buffer of pH 4.4, ascending flow, it had R_f 0.74 (observed under ultraviolet light of 254 mμ). The ultraviolet absorption characteristics were: in 0.1 *N* NaOH, λ_{max} 226 mμ (log ε 4.30), 272 (4.11); in 0.1 *N* HCl, λ_{max} 220 mμ (log ε 4.29), 275 (3.85).

Bis(2-chloroalkyl)amides of Acylated Amino Acids. Modified Nitrogen Mustards[1]

IRVING LEVI, HAROLD BLONDAL, J. WILLIAM R. WEED, ALAN C. FROSST,

Chemical Research Laboratories, Charles E. Frosst & Co., Montreal, Canada

H. CHRISTINE REILLY, FRANZ A. SCHMID, KANEMATSU SUGIURA, GEORGE S. TARNOWSKI, AND C. CHESTER STOCK

Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research and Sloan-Kettering Division, Graduate School of Medical Sciences, Cornell University, New York, New York

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A considerable number of aliphatic and aromatic compounds carrying an N,N-bis(2-chloroethyl)amino group (nor-nitrogen mustards) have been investigated in the search for antitumor agents.[2] The effective dose of these compounds, however, is in many cases so close to the dose which produces serious toxic effects in the host as to render them unsuitable for prolonged chemotherapeutic use. Many workers have, therefore, sought nor-nitrogen mustard derivatives possessing the high carcinolytic activity of the parent compound but having a greatly reduced general toxicity.[3] A few of these alkylating agents are in clinical use today.[4]

We have converted the basic nitrogen atom to the relatively neutral amide nitrogen by synthesizing bis(2-chloroethyl)amides and bis(2-chloropropyl)amides of N-acylated amino acids, thus hindering formation of the reactive aziridinium intermediate. The cytotoxic nor-nitrogen mustard might then be liberated by action of proteolytic enzymes in cancer cells, resulting in a possible selectivity of antitumor action. Safonova and Sergievskaya as well as Bien and Friedman reported the preparation of glycinebis(2-chloroethyl)amide.[5]

All of these N-acylated-α-amino acid "nitrogen mustard" amides listed in Table I were prepared in essentially the same manner from the N-acylated amino acid and the di(2-chloroalkyl)amine in the presence of

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