## The Purification of Aminopterin

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Aminopterin, or N-{4-[(2,4-diamino-6-pteridinylmethyl)amino]benzoyl}glutamic acid, is a 4-amino analog of folic acid that enjoys notable clinical application in the treatment of acute leukemias.[1] Commercially available aminopterin is grossly impure and frequently the contamination amounts to more than 10% based on chromatographic and spectrometric analyses.

Most multisubstituted pteridine compounds are extremely difficult to prepare in a state of high purity mainly because of their insolubility in most solvents. The criteria of purity are also hard to establish on account of the infusibility and lack of definite melting point of these pteridines. Good elementary analytical data, especially nitrogen determination, cannot be readily obtained[2] without meticulous care and ardous practice. In addition, they inevitably exhibit a strong tenacity for water owing, undoubtedly, to the addition of water across a double bond, a phenomenon quite commonly observed in this type of $\pi$-deficient N-heterocyclic compounds.[3] Fractions of water often show up in the analysis regardless of how thoroughly the sample is dried. As a consequence, a special technique must be designed for the purification of pteridines.

Small amounts of pure aminopterin can be prepared in milligram quantities by subjecting crude aminopterin to column chromatography on diethylaminoethyl (DEAE) cellulose followed by linear gradient elution with ammonium bicarbonate buffer of pH 8, increasing the molarity from 0.1 to 0.4.[4a-c] This method is tedious, time consuming, and not suitable for preparative purpose. A convenient technique for gram-scale preparation of chromatographically pure aminopterin in crystalline form is now described.

(1) S. Farber, L. K. Diamond, R. D. Mercer, R. F. Sylvester, Jr., and J. A. Wolff, *New Engl. J. Med.*, **238**, 787 (1948).

(2) L. M. Brancone and W. Fulmor, *Anal. Chem.*, **21**, 1147 (1949).

(3) A. Albert, "Heterocyclic Chemistry," The Athlone Press, University of London, London, 1959, p. 84.

### Experimental

To a solution near boiling of 5 g. of aminopterin[5] in 400 ml. of water was slowly added magnesium oxide powder (calcined magnesia, light) in small portions with vigorous stirring, until only a slight amount of MgO remained undissolved. The magnesium oxide required was about 0.7 g., and it raised the pH of the solution from 3–4 to 7–8. To the hot solution was added 1 g. of activated charcoal (Darco G-60). The hot mixture was filtered at once through a large funnel with sealed-in fritted disk of medium porosity and lined with a wet pad of diatomaceous earth (Celite), 2–3 mm. thickness. The filtrate was cooled in ice, and the crystalline magnesium salt of aminopterin was collected by filtration, recrystallized from 200 ml. of boiling water, and washed with alcohol.

*Anal.* Calcd. for $C_{19}H_{18}MgN_8O_5 \cdot 2.5H_2O$: Mg, 4.79; N, 22.07. Found: Mg, 4.60; N, 21.75.

The magnesium salt was redissolved in 200 ml. of boiling water. The boiling solution was carefully acidified, accompanied by vigorous agitation, with 2 ml. of glacial acetic acid. The pure aminopterin that came down in fine crystalline form was easily filtered. It was again collected and washed with water and acetone. The yield was about 3 g. In comparison with the aminopterin before purification, this material showed only one peak (and a negligible trace of an unidentified simple pteridine amounting to much less than 0.1%) when chromatographed on DEAE cellulose column.[4a-c] Repetition of this procedure completely removed the last trace of the contaminant. In 0.1 *N* NaOH, aminopterin exhibited the following ultraviolet characteristics: $\lambda_{max}$ 261 m$\mu$ (log $\epsilon$ 4.41), 282 (4.39), and 373 (3.91).

*Anal.* Calcd. for $C_{19}H_{20}N_8O_5 \cdot 0.75H_2O$: C, 50.27; H, 4.77; N, 24.69. Found: C, 50.35; N, 4.99; N, 24.65.

Pure aminopterin showed no difference from crude aminopterin in antileukemic activity when screened against advanced L1210 mouse leukemia. Clinical comparison of the purified *vs.* the crude material is in progress.

(4) (a) V. T. Oliverio and T. L. Loo, *Proc. Am. Assoc. Cancer Res.*, **3**, 140 (1960); (b) V. T. Oliverio, *Anal. Chem.*, **33**, 263 (1961); (c) R. L. Kisliuk and M. D. Levine, *J. Biol. Chem.*, **239**, 1900 (1964). Dr. Kisliuk kindly informed me that the aminopterin purified by the present procedure was six times more potent for inhibition of *Streptococcus faecalis* as the aminopterin purified by column chromatography.

(5) The aminopterin (grade C) was supplied by Calbiochem, Los Angeles, Calif. Column chromatography on DEAE cellulose resolved this material into 6 components as detected by absorption of ultraviolet light of 254 m$\mu$. It was estimated that 80% of it was aminopterin, 15% folic acid, and the remaining 5% various pteridines.

# *New Compounds*

## Some 3,4,5-Trimethoxy-Substituted Benzamides[1]

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In continuation of our investigations of compounds having depressant effects on the central nervous system,[2] a number of trimethoxy benzene derivatives were prepared by a variety of methods. Most of these compounds were found to be CNS depressants by gross observation of intact mice and rats and by avoidance behavior studies. Some new trimethoxybenzene derivatives have been synthesized by us and these compounds were also found to potentiate pentobarbital hypnosis in albino mice and exert a moderate influence on the conditioned-avoidance response of trained rats.

(1) The authors gratefully acknowledge financial support from Council of Scientific and Industrial Research, New Delhi, India.

(2) (a) P. C. Dandiya and H. Cullumbine, *J. Pharmacol. Exptl. Therap.*, **125**, 353 (1959); (b) P. C. Dandiya and M. K. Menon, *Brit. J. Pharmacol.*, **20**, 434 (1963); (c) P. C. Dandiya, P. K. Sharma, and M. K. Menon, *Indian J. Med. Res.*, **50**, 750 (1962).

### Experimental[3]

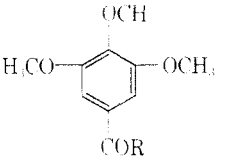
Except for compound VII, all of the amides in Table I were prepared by the following general method.

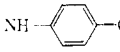
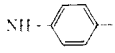
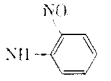
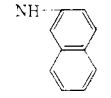
A calculated quantity of 3,4,5-trimethoxybenzoyl chloride (0.2 mole) in dry tetrahydrofuran was added slowly with stirring to a cooled (below 10°) solution of the calculated amount of the desired amine (0.4 mole) in the same solvent. The mixture was stirred at room temperature for about 1 hr. and then allowed to

(3) The melting points of the above compounds were determined in open capillaries using an electrically heated block and are uncorrected.

TABLE I

3,4,5-TRIMETHOXY-SUBSTITUTED BENZAMIDES[a]



| Compd. | R | M.p., °C. | Formula | Yield, % | % calcd. C | % calcd. H | % calcd. N | % found C | % found H | % found N |
|---|---|---|---|---|---|---|---|---|---|---|
| I | $NHCOOC_2H_5$ | 120–123 | $C_{15}H_{21}NO_6$ | 54 | 55.12 | 6.00 | 4.94 | 55.10 | 6.10 | 4.89 |
| II |  | 115–117 | $C_{19}H_{21}NO_6$ | 62 | 63.50 | 5.84 | 3.89 | 63.16 | 5.83 | 3.79 |
| III |  | 180–183 | $C_{16}H_{16}ClNO_5$ | 60 | 59.72 | 4.97 | 4.35 | 59.68 | 4.86 | 4.30 |
| IV |  | 145–147 | $C_{16}H_{16}N_2O_6$ | 66 | 57.80 | 4.81 | 8.40 | 57.66 | 4.78 | 8.41 |
| V |  | 110–112 | $C_{20}H_{19}NO_5$ | 60 | 71.21 | 5.63 | 4.15 | 71.28 | 5.56 | 4.08 |
| VI |  | 132–134 | $C_{15}H_{21}NO_4$ | 58 | 64.51 | 7.53 | 5.01 | 64.01 | 7.46 | 5.00 |
| VII | $NHCOC_2H_5$ | 184–186 | $C_{13}H_{17}NO_5$ | 56 | 58.42 | 6.36 | 5.20 | 58.26 | 6.15 | 5.14 |

[a] All compounds except IV (yellow) were white.

stand overnight in refrigeration. The solvent was removed by distillation under reduced pressure. The solid residue was washed first with 5% HCl, then with 5% sodium carbonate, and then with distilled water repeatedly. The product was recrystallized from dilute methanol or ethanol.

**N-Propionyl-3,4,5-trimethoxybenzamide (VII).**—3,4,5-Trimethoxybenzamide (5 g.) in dry pyridine (20 ml.) was added dropwise to a cooled solution (below 10°) of propionyl chloride (2 ml.) in dry pyridine (20 ml.). The mixture was stirred at room temperature for about 1 hr. and then allowed to stand overnight. The mixture was warmed on a water bath for 20 min. and cooled, then poured into cold water (50 ml.) and filtered. The residue was washed repeatedly with distilled water and dried. The residue was recrystallized from benzene; yield, 56%; m.p. 184–186°.

Anal. Calcd. for $C_{13}H_{17}NO_5$: C, 58.42; H, 6.36; N, 5.20. Found: C, 58.26; H, 6.15; N, 5.14.

## 1-(2-Deoxy-D-glucopyranosyl)-5-fluorocytosine[1]

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In view of the recent interest in 1-(2-deoxy-D-glucopyranosyl)-thymine as a "uridine phosphorylase" inhibitor[2] and of 2-deoxy-5-fluorocytidine as an antimetabolite[3] and antiviral agent,[4] it appeared of interest to synthesize the structurally related 1-(2-deoxy-D-glucopyranosyl)-5-fluorocytosine (III), in the hope that it might show one of the above physiological properties. The anomeric configuration of III is not known. However, in view of the physiological activity[2] of 1-(2-deoxy-D-glucopyranosyl)thymine (indicating $\beta$ configuration), which was also prepared by the same method,[5] III may tentively be considered to be a $\beta$ nucleoside.

### Experimental[6]

**2,4-Diethoxy-5-fluoropyrimidine (I).**—This procedure follows the method of Hilbert and Johnson for the synthesis of 2,4-diethoxypyrimidines.[7] 2,4-Dichloro-5-fluoropyrimidine[3,8] (5.5 g., 0.033 mole) dissolved in 15 ml. of absolute ethanol was added to a solution of 4.7 g. (0.07 mole) of sodium ethoxide in 25 ml. of absolute ethanol. The mixture which became hot, was allowed to stand for 1 hr., filtered, and then was evaporated to dryness *in vacuo*. The residue was taken up in ether and water and, after washing with 30% aqueous NaOH, the ether layer was dried ($Na_2SO_4$) and filtered. The ether was removed *in vacuo* and the product was distilled, b.p. 92–93° (4 mm.), yielding 5.0 g. (82%) of I, m.p. 18–19°, $\lambda_{max}^{95\%\ EtOH}$ 269 mμ (log ε 3.72).

*Anal.* Calcd. for $C_8H_{11}FN_2O_2$: C, 51.60; H, 5.96; F, 10.20. Found: C, 51.78; H, 6.21; F, 9.88.

**1-(3,4,6-Tri-O-*p*-nitrobenzoyl-2-deoxy-D-*arabino*-hexopyranosyl)-4-ethoxy-5-fluoro-2(1H)-pyrimidone (II).**—This synthesis follows the classical method used by Hilbert and Jansen[9] in the synthesis of 1-(β-D-glucopyranosyl)cytosine. A mixture of 2.7 g. (4 mmoles) of 2-deoxy-3,4,6-tri-*O*-*p*-nitrobenzoyl-α-D-*arabino*-hexopyranosyl bromide[10] and 7.3 g. (39 mmoles) 2,4-diethoxy-5-fluoropyrimidine (I) was stirred *in vacuo* for 2 days at room temperature; initially, the bromo sugar dissolved with the evolution of ethyl bromide. The mixture was then triturated with 20 ml. of dry ether and filtered. The crude product was recrystallized from acetone, yielding 1.55 g. of II, m.p. 258.5–260°. An additional 0.2 g. of product was obtained from the mother liquors giving a total yield of 58%. The ultraviolet absorption spectra of the 1-substituted 4-ethoxy-5-fluoro-2-(1H)-pyrimidone was obscured by the absorption due to the *p*-nitrobenzoyl groups; $\lambda_{max}^{CH_2Cl_2}$ 262 mμ (log ε 5.66).

*Anal.* Calcd. for $C_{32}H_{26}FN_5O_{15}$: C, 52.75; H, 3.49; N, 9.32; F, 2.53. Found: C, 52.82; H, 3.56; N, 9.59; F, 2.81.

**1-(2-Deoxy-D-glucopyranosyl)-5-fluorocytosine (III).**—A mixture of 251 mg. (0.33 mmole) of II in 3.5 ml. of saturated methanolic ammonia was heated in a sealed tube at 100° for 3

(1) This work was supported in part by a research grant from the Michigan Cancer Foundation and in part by the research grant CA-08095-01 from the National Cancer Institute, Public Health Service.

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(5) W. W. Zorbach and G. J. Durr, *J. Org. Chem.*, **27**, 1474 (1962).

(6) Melting points were determined using a Kofler hot stage. Ultraviolet spectra were recorded by a Bausch and Lomb Spectronic 505 spectrophotometer. Analyses were performed by Micro-Tech Laboratories, Skokie, Ill.

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(8) M. G. Biressi, M. Carrissimi, and F. Ravenna, *Gazz. chim. ital.*, **93**, 1268 (1963); L. D. Protsenko and Yu. I. Bogodist, *Zh. Obshch. Khim.*, **33**, 537 (1963).

(9) G. E. Hilbert and E. F. Jansen, *J. Am. Chem. Soc.*, **58**, 60 (1936).

(10) W. W. Zorbach and G. Pietsch, *Ann.*, **655**, 26 (1962).