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The Metabolite of 3′,5′-Dichloro-4-amino-4-deoxy-N^{10}-methylpteroylglutamic Acid (Dichloromethotrexate)

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Dichloromethotrexate (DCM) is partly metabolized by man and three rodent species. This metabolite is also obtained by incubating DCM with rat liver homogenate. Based on degradation experiments, the metabolite is shown to be 3′,5′-dichloro-4-amino-4-deoxy-7-hydroxy-N^{10}-methylpteroylglutamic acid.

In studies of the physiological disposition[1a-c] of dichloromethotrexate[2a-c] (abbreviated DCM in this paper), a pteroylglutamic acid antagonist of clinical interest[6] in the treatment of leukemia, the presence of a metabolite of DCM was recognized in the urine of animals that had received the drug by different routes. Employing Cl^{36}-labeled DCM, it was observed that almost all the radioactivity was recoverable from the urine and feces of man,[1c] dog, and three rodent species[1b] treated with this drug. In addition to unaltered DCM, one and only one metabolite, amounting to almost one-third of the dose of DCM administered, was detected. The dog, however, excreted DCM unchanged.

An identical metabolite was isolated from the *in vitro* incubation of DCM with rat liver homogenate.[7] As in the case of the *in vivo* experiments, dog liver homogenate was ineffective in metabolizing DCM.

Because of the minute quantity of pure DCM metabolite available for study and the inherent difficulties involved in pteridine chemistry, classical techniques are not always useful in structural elucidation.[8] To establish identity most of the following criteria were relied upon: elementary analysis if possible, ultraviolet absorption spectroscopy,[9] electrophoretic mobility,[10] and position of elution from a diethylaminoethylcellulose column.[3] Taken alone, none of these criteria would be sufficient to warrant a conclusive structural proof.

The fact that there is only one metabolite which is not identical with any simple pteridine related to DCM excludes the possibility that it is a cleavage product of DCM. Also, the metabolite is distinctly different from 3′,5′-dichloro-N^{10}-methylpteroylglutamic acid[5] in spectra and electrophoretic mobility and hence cannot result from DCM by simple hydrolysis of the 4-amino group.

Analytical data of DCM and its magnesium salt can best be expressed by the respective empirical formulas, $C_{20}H_{20}Cl_2N_8O_6 \cdot H_2O$ and $C_{20}H_{18}Cl_2MgN_8O_6 \cdot 5H_2O$.[11] In other words, tentatively, the metabolite seems to differ from its parent compound only in an oxygen atom. Existing knowledge of drug metabolism suggests that the extra oxygen atom is located in either the pteridine or the benzene ring.

A comparison of the ultraviolet absorption spectrum of DCM in dilute alkali with that of its metabolite discloses that the absorption maximum at 370 mμ for DCM has been displaced 26 mμ to 344 for the metabolite. Such a hypsochromic shift is strongly reminiscent of a similar shift of 26 mμ in dilute alkali from 364 for 2,4-diamino-6-methylpteridine[4] to 338 mμ for 2,4-diamino-7-hydroxy-6-methylpteridine.[12] As a whole, the general pattern of change in the spectra in both acid and alkali matches closely for these two pairs of compounds. Nevertheless, in view of the corresponding

(1) (a) V. T. Oliverio and T. L. Loo, *Proc. Am. Assoc. Cancer Res.*, **3**, 140 (1960); (b) V. T. Oliverio and J. D. Davidson, *J. Pharmacol. Exptl. Therap.*, **137**, 76 (1962); (c) J. D. Davidson and V. T. Oliverio, *Clin. Pharmacol. Exptl. Therap.*, in press.

(2) (a) Dichloromethotrexate is the 3′,5′-dichloro derivative of methotrexate. Methotrexate or amethopterin are generic names for 4-amino-4-deoxy-N^{10}-methylpteroylglutamic acid or N-(*p*-[[(2,4-diamino-6-pteridinyl)-methyl]methylamino]benzoyl)glutamic acid. (b) The DCM was supplied by Lederle Laboratories Division of American Cyanamid Co. through the courtesy of Cancer Chemotherapy National Service Center of the National Cancer Institute. The drug used in the rabbit experiments was estimated to be over 90% pure by chromatography.[3] For incubation with rat liver homogenate, DCM was purified by column chromatography on DEAE cellulose.[3] (c) The synthesis of methotrexate was reported in ref. 4, and that of DCM in ref. 5, and also by R. Angier and W. V. Curran, *J. Am. Chem. Soc.*, **81**, 2814 (1959).

(3) V. T. Oliverio, *Anal. Chem.*, **33**, 263 (1961).

(4) D. R. Seeger, D. B. Cosulich, J. M. Smith, Jr., and M. E. Hultquist [*J. Am. Chem. Soc.*, **71**, 1753 (1949)] reported absorption maximum at 369 mμ in alkali.

(5) D. B. Cosulich, D. R. Seeger, M. J. Fahrenbach, B. Roth, J. H. Mowat, J. M. Smith, Jr., and M. E. Hultquist, *ibid.*, **73**, 2554 (1951).

(6) E. Frei, III, C. L. Spurr, C. O. Brindley, O. Selawry, J. F. Holland, D. P. Rall, L. R. Wasserman, B. Hoogstraten, B. I. Shnider, O. R. McIntyre, L. B. Matthews, Jr., and S. P. Miller, *Clin. Pharmacol. Exptl. Therap.*, **6**, 160 (1965).

(7) T. L. Loo and R. H. Adamson, *Biochem. Pharmacol.*, **11**, 170 (1962). In this paper the structure proposed for the metabolite 3′,5′-dichloro-7-hydroxy-N^{10}-methylpteroylglutamic acid or 4-deamino-4,7-dihydroxy-DCM was later shown to be erroneous. See text.

(8) For an excellent discussion on this point, see A. Albert, *Quart. Rev.* (London), **6**, 197 (1952).

(9) Infrared spectroscopy, on the other hand, proves to be of little application to our problem owing to overlap of hydrogen-bonded OH and NH stretching frequencies.

(10) See J. D. Davidson, *J. Natl. Cancer Inst.*, **29**, 792 (1962), for a description of method and equipment.

(11) In pteridine chemistry the interpretation of analytical data must proceed with extreme caution.[8] In the present case, ultimate analysis provides no basis for discrimination between the proposed empirical formula $C_{20}H_{20}Cl_2N_8O_6 \cdot H_2O$ and $C_{20}H_{20}Cl_2N_8O_6 \cdot 2H_2O$ (DCM dihydrate) or $C_{20}H_{18}Cl_2MgN_8O_6 \cdot 5H_2O$ and $C_{20}H_{18}Cl_2MgN_8O_6 \cdot 6H_2O$ (hexahydrated magnesium salt of DCM). Determination of water content by the Karl Fisher reagent gave inconclusive results in our hands probably because of addition of water across double bonds in some of these pteridines.[8]

(12) G. B. Elion, G. H. Hitchings, and P. B. Russell [*J. Am. Chem. Soc.*, **72**, 78 (1950)] reported absorption maximum in alkali at 340 mμ.

spectral changes from xanthopterin to leucopterin[13] and the facile hydrolysis[14] of the 4-amino group, the possibility of a 4,7-dihydroxy derivative could not be rigorously ruled out. In fact, the previously proposed structure for the metabolite[7] was later found to be erroneous because of the hydrolysis of the 4-amino group during the degradation procedure which was more drastic than that used in the present work.

Controlled oxidation of DCM metabolite isolated either from incubation with rat liver homogenate or from rabbit urine with potassium permanganate in dilute alkali yielded a simple pteridine. Based on analysis, comparison of its ultraviolet absorption spectra, electrophoretic mobility, and elution pattern from a DEAE cellulose column with a synthetic sample,[12] this simple pteridine was identified as 2,4-diamino-7-hydroxy-6-pteridinecarboxylic acid. Although the non-pteridine portion of the molecule was not isolable in sufficient amount for analysis, its spectral and chromatographic characteristics were the same as the 3,5-dichloro-4-methylaminobenzoylglutamic acid prepared from DCM by careful oxidation.

Reduction of DCM metabolite in phosphoric acid with zinc[15] gives mixtures of two substances separable by column chromatography.[3] One of these also exhibited the same elution pattern, ultraviolet absorption spectra, and electrophoretic behavior as the authentic 3,5-dichloro-4-methylaminobenzoylglutamic acid.

Since degradation experiments demonstrate that DCM metabolite differs from the parent compound only in the pteridines part, and since DCM metabolite afforded 2,4-diamino-7-hydroxy-6-pteridinecarboxylic acid, the structure of DCM metabolite must therefore be 3′,5′-dichloro-4-amino-4-deoxy-7-hydroxy-N^{10}-methylpteroylglutamic acid.

Experimental[16]

Isolation of DCM Metabolite from Rabbit Urine.—DCM was administered intraperitoneally to six rabbits of 2–3 kg. Each animal received 2 ml. of a solution of 50 mg. of DCM/ml. of dilute NaOH of pH 8.5. Urine excreted by the animals during the 4 hr. after injection was collected and filtered. It was adjusted to pH 8 with NH_4OH and applied directly to a DEAE cellulose[17] column; each volume of urine required 3 vol. of wet adsorbant. The chromatographic procedure followed closely the work reported previously[15,3] except the linear gradient elution was carried out with ammonium bicarbonate buffer instead of phosphate buffer. As the molarity of the eluent increased from 0.01 to 0.4 *M*, DCM and its metabolite came off the column successively. The fraction containing the metabolite was collected, filtered, and evaporated to dryness under reduced pressure (water pump) at 40°. The residue was redissolved in 0.1 *N* NaOH and filtered. Pure DCM metabolite was obtained from the alkaline solution by precipitation with glacial acetic acid. It was a pale yellow powder, indistinguishable in every respect from the metabolite isolated from incubation experiments described below.

Isolation of DCM Metabolite from an Incubation Mixture with Rat Liver Homogenate.—DCM dissolved in 0.1 *M* ammonium bicarbonate buffer of pH 8 was incubated with rat liver homogenate as previously described.[7] The products of several experiments were combined and concentrated under reduced pressure (water pump) at 40°. The DCM metabolite was isolated by DEAE cellulose column chromatography and a sample was analyzed.

Anal. Calcd. for $C_{20}H_{20}Cl_2N_8O_8 \cdot H_2O$: C, 43.10; H, 3.98; Cl, 12.72; N, 20.11. Found: C, 43.23; H, 3.88; Cl, 12.61; N, 19.73.

A magnesium salt was prepared by adding anhydrous $MgSO_4$ to the metabolite dissolved in 2% $NaHCO_3$ solution.

Anal. Calcd. for $C_{20}H_{18}Cl_2MgN_8O_8 \cdot 5H_2O$: C, 36.86; H, 4.33; Cl, 10.88; N, 17.19. Found: C, 36.84; H, 4.22; Cl, 10.75; N, 16.98.

Its ultraviolet absorption characteristics are: in 0.1 *N* HCl, λ_{max} 300 and 334 mμ; at pH 8, λ_{max} 255 (infl.), 283 (infl.), and 352 mμ; in 0.1 *N* NaOH, λ_{max} 255 and 285 (infl.), 344 mμ.

Oxidation of DCM Metabolite.—The metabolite (5 mg.) isolated from rabbit urine was dissolved in 6 ml. of 0.1 *N* NaOH and oxidized with 11 ml. of 1% $KMnO_4$ in a steam bath for 4 hr. The MnO_2 was removed by centrifugation. The golden filtrate was acidified to pH 4 with a few drops of glacial acetic acid. The yellow precipitate was collected by centrifugation and washed successively with water and acetone. The product weighed 0.3 mg. The supernatant was saved for the isolation of the nonpteridine moiety.

Anal. Calcd. for $C_7H_6N_6O_3 \cdot 0.55H_2O$: C, 36.23; H, 3.09. Found: C, 36.22; H, 3.10.

This product showed identical ultraviolet absorption spectra with an authentic sample of 2,4-diamino-7-hydroxy-6-pteridinecarboxylic acid synthesized according to a published procedure[12] in acid, alkali, and at pH 8. At pH 8 the spectrum [λ_{max} 262, 285 (infl.), and 356 mμ] was distinctly dissimilar to that of 2-amino-4,7-dihydroxy-6-pteridinecarboxylic acid[12] [λ_{max} 260 (infl.), 285, and 342 mμ].

When subjected to high-voltage electrophoresis[16] on Whatman 3 MM paper with a voltage gradient of 40–50 v./cm. of paper, the acid produced by the oxidation of DCM metabolite demonstrated the following mobility relative to a phenol red marker: 0.84 at pH 10 (0.05 *M* glycine buffer) (all buffers in this experiment contained 0.001 *M* ethylenediaminetetraacetic acid), 0.60 at pH 8.4 (0.05 *M* Tris buffer), and 0.74 at pH 7 (0.05 *M* phosphate buffer). In comparison, 2,4-diamino-7-hydroxy-6-pteridinecarboxylic acid showed: 0.84 at pH 10, 0.60 at pH 8.4, and 0.76 at pH 7; 2-amino-4,7-dihydroxy-6-pteridinecarboxylic acid showed: 1.00 at pH 10, 1.05 at pH 8.4, and 1.48 at pH 7. All spots were observed under ultraviolet light of 254 mμ.

The supernatant from which the above pteridinecarboxylic acid was removed was acidified with 1 ml. of concentrated HCl and extracted five times each with 2 ml. of ethyl acetate. The extract was dried ($MgSO_4$) and concentrated under nitrogen. The residual pinkish oil slowly crystallized on chilling. The small amount of crystalline material was not analyzed. It exhibited the same spectral, chromatographic, and electrophoretic characteristics as the authentic 3,5-dichloro-4-methylaminobenzoylglutamic acid described below.

Reduction of DCM Metabolite.—DCM metabolite (1 mg.) was reduced with 0.2 g. of zinc in 6 ml. of 2 *N* phosphoric acid at room temperature for 30 min. Calcium carbonate, 0.6 g., was added to the mixture. The precipitate was discarded and the supernatant was chromatographed on DEAE cellulose column and eluted as previously described. The mixture was thus resolved into 2 major and 1 minor components. The first major component showed absorption characteristics analogous to a pyrimidine derivative but was not further identified. The minor component was insufficient for identification. The second major component absorbed ultraviolet radiation with the following pattern: in alkali, λ_{max} 275 mμ; in acid, λ_{max} 277 mμ. When DCM was reduced in like manner, a corresponding fraction was obtained, which showed the same elution pattern and ultraviolet absorption spectra. Finally, both were identical with the 3,5-dichloro-4-methylaminobenzoylglutamic acid prepared from oxidation of DCM with respect to spectra and electrophoretic mobility.

3,5-Dichloro-4-methylaminobenzoylglutamic Acid.—DCM (240 mg.) was dissolved in 10 ml. of 0.1 *N* NaOH and heated in a water bath at 90°. Potassium permanganate solution (2 ml. of 5%) was slowly added with stirring in 5 min. The MnO_2 was removed by filtration and washed with 0.1 *N* NaOH. The combined filtrate and washings were acidified with 1 ml. of concentrated HCl and extracted five times with 10 ml. of ethyl acetate. The ethyl acetate extract was concentrated in a stream of nitrogen at 80°. The slightly yellow oily residue began to

(13) A. Albert, D. J. Brown, and G. Cheeseman, *J. Chem. Soc.*, 474 (1951).

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

(15) D. G. Johns, S. Sperti, and S. V. Burgen, *J. Clin. Invest.*, **40**, 1684 (1961).

(16) We are grateful to Dr. J. D. Davidson for performing the high-voltage paper electrophoresis experiments.

(17) Available from Calbiochem Corp., Los Angeles 54, Calif.; exchange capacity: 0.9 mequiv./g.

crystallize upon addition of a few drops of petroleum ether (b.p. 30–60°). The crystals were collected, washed with a few drops of ethyl acetate, and recrystallized from the same solvent after clarification with charcoal; m.p. 124–130°; yield 50 mg. (30%); ultraviolet absorption spectra: in 0.1 *N* NaOH, λ_{max} 219 mμ (log ε 4.42), 275 mμ (log ε 4.11); in 0.1 *N* hydrochloric acid, λ_{max} 277 mμ (log ε 3.90).

Anal. Calcd. for $C_{13}H_{14}Cl_2N_2O_5$: C, 44.72; H, 4.04; Cl, 20.31. Found: C, 44.38; H, 3.86; Cl, 19.89.

When chromatogrammed on Whatman 3 MM paper and developed with 0.1 *M* acetate buffer of pH 4.4, ascending flow, 3,5-dichloro-4-methylaminobenzoylglutamic acid showed R_f 0.83.

Discussion

Although derived from methotrexate, DCM differs from it in several aspects. In comparison with methotrexate, DCM is superior as a therapeutic agent in mouse leukemia L1210 and less toxic toward the host animals.[18] Another distinct feature revealed by our work is that DCM is rapidly oxidized to the 7-hydroxy derivative both *in vivo* and *in vitro* while methotrexate apparently is not.[7,19]

The metabolic fate of pteroylglutamic acid and derivatives in man and other animals has not been extensively investigated previously.[20] Because of its occurrence in human urine, xanthopterin has been suggested[21] as a final catabolic product of pteroylglutamic acid in man. It was postulated that initially pteroylglutamic acid was cleaved between position 9 and N^{10}, followed by oxidation of the pteridine part, finally resulting in 7-hydroxylation. Xanthine oxidase was implicated in the last step even though the enzyme is inert toward intact pteroylglutamic acid.[22] There was no evidence that pteroylglutamic acid was oxidized at position 7 as in our studies with DCM although pteroylglutamic acid probably indirectly gave rise to simple pteridines such as xanthopterin.[15]

Incubation of pteroylglutamic acid with either chicken liver extracts[23a] or rat liver slices (but not homogenate)[23b] afforded a diazotizable amine, most likely *p*-aminobenzoylglutamic acid. The fate of the pteridine moiety was not defined. In any event, 7-hydroxylation of pteroylglutamic acid was not encountered during incubation.

Simple pteridines, on the other hand, are subjected to 7-hydroxylation when incubated with either liver preparations or xanthine oxidase. For example, 2-amino-4-hydroxypteridine is oxidized to isoxanthopterin by chicken liver extract[24a] as well as by xanthine oxidase from milk[24b]; likewise, xanthopterin is oxidized to leucopterin.[24b–e] In all cases, an oxygen atom is introduced at position 7, parallel to DCM. Yet interestingly, DCM is not oxidized by milk or calf liver xanthine oxidase.[7] Although methotrexate and aminopterin were not oxidized by rat liver homogenate,[7] such an oxidation was recently reported by the incubation of these drugs with an enzyme system isolated from rabbit liver.[19c]

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Synthesis and Pharmacological Action of 3-Amino-2,1-benzisothiazoles

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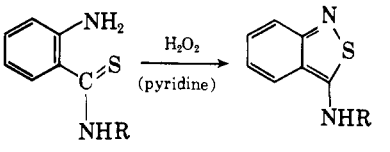
A series of 21 novel 3-amino-2,1-benzisothiazoles was prepared and evaluated for certain pharmacological effects. Some members show potent gastric antisecretory activity in the rat, antinociceptive action in the mouse, and antibradykinin activity in the guinea pig. The structure–activity relationships indicate that these three biological actions are not interrelated to a specific chemical moiety.

Continuous effort is exerted to develop novel heterocyclic systems that possess interesting pharmacological activities. This had led us to the synthesis and testing of a series of 3-amino-2,1-benzisothiazoles. Pharmacological observations indicated that certain independent members of the series possess gastric antisecretory, antibradykinin, antinociceptive, and mild antierythema activity.

Chemistry.—Although 2,1-benzisothiazole (thioanthranil) itself has long been known[2] and was prepared both by reductive cyclization[2] from *o*-nitro-α-toluenethiol and recently by oxidative cyclization[3] from *o*-amino-α-toluenethiol, no 3-amino- or 3-substituted amino-2,1-benzisothiazoles are reported in the literature. Goerdeler and co-workers oxidized some β-aminothiocrotonamides to give 5-aminoisothiazoles.[4] We prepared 3-amino- and 3-monosubstituted amino-



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