The Halogenation of 4-Amino-4-deoxy-N10-methylpteroylglutamic Acid (Methotrexate)[1] and Other Pteroylglutamic Acid Derivatives[2]
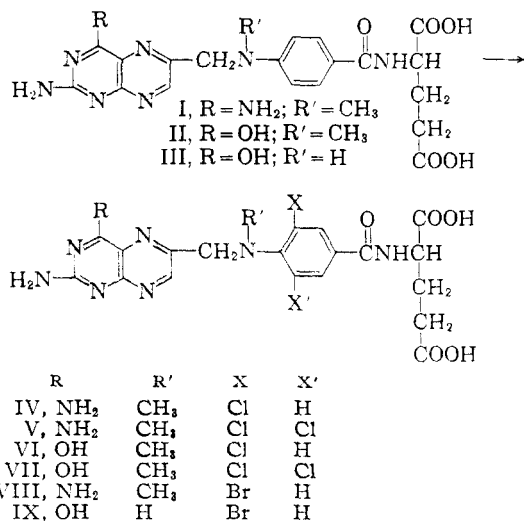
By ROBERT B. ANGIER AND WILLIAM V. CURRAN

Four halogenated derivatives of 4-amino-4-deoxy-N10-methylpteroylglutamic acid (methotrexate) (I)[1] have been synthesized by direct halogenation for testing as anti-leukemic agents. The presence of a N-methyl substituent in the 10-position of pteroylglutamic acid or its derivatives exerts a steric effect which has made it possible to monochlorinate these compounds in the 3'-position. The relation between steric factors and ultraviolet absorption spectra is discussed.

The report[3] that a crude sample of "dichloromethotrexate" (dichloroamethopterin) was more active than methotrexate[1] (4-amino-4-deoxy-N10-methylpteroylglutamic acid) (I) in the treatment of an advanced stage of leukemia L-1210 in the mouse led us to attempt to synthesize a pure sample of 3',5'-dichloromethotrexate (4-amino-4-deoxy-3',5'-dichloro-N10-methylpteroylglutamic acid) (V).

Chlorination of Methotrexate (I).—The preparation of the crude "dichloromethotrexate" and a number of other dihalogenated pteroylglutamic acid derivatives has been previously described.[4a] The procedure involved bubbling 2 moles of chlorine into a cold 6 N hydrochloric acid solution of the pteroylglutamic acid derivative. In our hands this procedure gave a product which was largely 3',5'-dichloromethotrexate (V) when care was taken to use 2 to 2.2 moles of chlorine per mole of methotrexate. However, when 0.9 mole of chlorine was used the resulting product was easily crystallized from formamide to give chromatographically pure 3'-chloromethotrexate (4-amino-4-deoxy-3'-chloro-N10-methylpteroylglutamic acid) (IV). When intermediate amounts of chlorine were used the products were mixtures of IV and V.

I, R = NH₂; R' = CH₃
II, R = OH; R' = CH₃
III, R = OH; R' = H

R	R'	X	X'
IV, NH₂	CH₃	Cl	H
V, NH₂	CH₃	Cl	Cl
VI, OH	CH₃	Cl	H
VII, OH	CH₃	Cl	Cl
VIII, NH₂	CH₃	Br	H
IX, OH	H	Br	H

(1) Methotrexate and amethopterin are generic names for 4-amino-4-deoxy-N10-methylpteroylglutamic acid. Its full chemical name is N-[4-{N-[(2,4-diamino-6-pteridyl)-methyl]-N-methylamino}-benzoyl]-glutamic acid.

(2) Presented before the Division of Medicinal Chemistry at the San Francisco Meeting of the American Chemical Society, April, 1958.

(3) A. Goldin, J. M. Venditti, S. R. Humphreys and N. Mantel, J. Natl. Cancer Inst., 19, 1133 (1957).

(4) (a) D. B. Cosulich, et al., THIS JOURNAL, 73, 2554 (1951); (b) 75, 4675 (1953).

After pure 3'-chloromethotrexate (IV) had been synthesized the original crude sample[3] of "dichloromethotrexate" was re-examined. Chlorine analysis and paper chromatography indicated that it contained a large amount, perhaps 30 to 40%, of IV. The large amount of IV present in this crude sample indicated that probably less than 2 moles of chlorine was actually used in its preparation.[4a]

Although, as described above, 3',5'-dichloromethotrexate (V) could be prepared using 6 N hydrochloric acid as a solvent, the preferred method of preparation was to dissolve methotrexate in warm formamide, cool to 5° in an ice-bath and bubble in approximately 6.5 moles of chlorine. The product was isolated easily in a 68% yield. Although chromatography showed that this was fairly pure material it was purified further in good yield through its crystalline magnesium salt.

The use of 6.5 moles of chlorine in this reaction is entirely empirical and was determined by trial and error. The excess chlorine was necessary since smaller quantities, e.g., 4 moles, gave a considerable amount of the monochloro derivative. Undoubtedly, the chlorine reacts with the formamide in some manner, probably to form an N-chloro derivative. As a result the chlorine is not totally available for chlorination of I under the conditions of the reaction.

Chlorination of Other Pteroylglutamic Acids.—Since the previous report[4a] had not recorded the monochlorination or monobromination of pteroylglutamic acid (III) or any of its derivatives, it seemed to be desirable to determine what variations in structure are required to permit monochlorination.

It was considered likely that the presence of a methyl group on the N10-position of these pteroylglutamic acid derivatives might cause enough steric hindrance to inhibit entrance of a second chlorine atom. Therefore, attempts were made to chlorinate pteroylglutamic acid (III) and N10-methylpteroylglutamic acid (II) using 6 N hydrochloric acid as a solvent.

When II was chlorinated either by the use of approximately one mole of chlorine in acetic acid or by bubbling chlorine directly into the solution, the reaction proceeded exactly as with methotrexate and the crude product, obtained in 65% yield, was primarily 3'-chloro-N10-methylpteroylglutamic acid (VI). This material was also readily crystallized from formamide to give a chromatographically pure product.

However, when pteroylglutamic acid (III) was chlorinated under exactly the same conditions,

chromatographic examination of the product in two systems showed a mixture of starting material and the dichloro derivative. No monochloropteroylglutamic acid could be detected (authentic samples of the 3'-chloro- and 3',5'-dichloro derivatives were available[4] for comparison). This confirms the postulation, made previously, that a substituent in the N^{10}-position does exert enough steric hindrance to allow preparation of a monochloro derivative.

Bromination of Methotrexate and Pteroylglutamic Acid.—At this stage, the testing results[5] indicated that 3'-chloromethotrexate (IV) was slightly more active than methotrexate, but not as active as the crude mixture that had originally been tested.[3] This strongly implied that 3',5'-dichloromethotrexate (V) would be more active than IV. If this favorable change in activity was due to an increase in steric hindrance around the 10-position of I, it seemed desirable to prepare one or two bromo derivatives to see if they would have a more favorable activity than the corresponding chloro derivatives.

The brominations were run using the same methods used in the chlorination reactions. In general, the monobrominations were more satisfactory than the monochlorinations whereas the one attempted dibromination was unsuccessful. When one mole of bromine was dissolved in acetic acid and added to a cold 6 N hydrochloric acid solution of methotrexate, an 80% yield of good quality 3'-bromomethotrexate (4-amino-4-deoxy-3'-bromo-N^{10}-methylpteroylglutamic acid) (VIII) was obtained. In contrast to the chlorination of I, there was little tendency to form the dibromo derivative in this reaction. In fact, when the reaction was repeated using 2.5 moles of bromine, chromatography and bromine analysis indicated that the product was still entirely 3'-bromomethotrexate. Evidently, steric hindrance to the entrance of the second bromine atom was more pronounced than was the case with chlorine. An attempt to prepare a dibromomethotrexate by the use of excess bromine in formamide in the manner which had been successful for chlorination of I also failed.

The fact that the methotrexate molecule was more resistant to attack by a second bromine atom than was the case with chlorine indicated that monobromination of pteroylglutamic acid (III) might possibly be successful even though monochlorination of III could not be accomplished. This was found to be the case since the bromination of III with one mole of bromine in the usual manner proceeded very satisfactorily to give an 85% yield of crystalline 3'-bromopteroylglutamic acid (IX) which was chromatographically pure without further purification. However, as had previously been shown,[4a] the second atom of bromine was easily introduced into the molecule to give 3',5'-dibromopteroylglutamic acid.

Since 3',5'-dibromomethotrexate was not successfully prepared, the synthesis of 3'-bromo-5'-

chloromethotrexate was attempted. The first approach was to attempt to brominate 3'-chloromethotrexate (IV). However, as was the case with 3'-bromomethotrexate, when IV was treated with bromine in 6 N hydrochloric acid in the usual manner, only the starting material was obtained. At this point, previous experience indicated that the chlorination of 3'-bromomethotrexate (VIII) with excess chlorine in formamide should be the preferred method for obtaining the desired compound. This was, in fact, found to be true and 3'-bromo-5'-chloromethotrexate was obtained in a 50% yield.

Structure and Ultraviolet Absorption Spectra.— It could be predicted that in the halogenation of any p-aminobenzoic acid derivative of this type the halogens should be directed into the 3- and 5-positions. Cosulich, et al., have proved this for dihalogenated pteroylglutamic acid and 4-aminopteroylglutamic acid[4] by degradation to known compounds. Because of the readiness with which these compounds can be halogenated, it was considered likely that if N^{10}-methylpteroylglutamic acid (II) and its 4-amino-analog I could be halogenated easily the products would also be 3',5'-dihalogenated compounds. As has already been pointed out, it seems probable that the methyl group on the N^{10}-position of I and II does sterically hinder the entrance of the second chlorine atom to a small extent. Despite this steric hindrance, the dichloro derivatives of I and II were readily prepared and there is no reason to doubt that they are 3',5'-dichloro derivatives.

Cosulich, et al., have noted[4] that 3',5'-dichloropteroylglutamic acid has an ultraviolet absorption spectrum in 0.1 N sodium hydroxide which lacks the maximum at 282 mμ due to the p-aminobenzoic acid portion of the molecule and which is present in the parent compound III. However, no explanation was offered to account for this change. It would seem that this is a clear-cut example of the spectroscopic effects of the steric inhibition of resonance.[6] One of the several resonance forms which contributes to the absorption of p-aminobenzoic acid is shown in structure X.
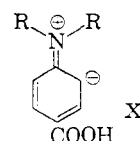
When chlorine atoms are introduced into the two positions *ortho* to the nitrogen the substituted amino group is forced out of the plane of the ring and the possibility for any double bond character in the carbon–nitrogen bond is markedly reduced, thus decreasing the absorption due to this resonance form. In general, the ultraviolet absorption spectra of these compounds fits this theory very nicely.

Figures 1, 2 and 3 illustrate the changes in the ultraviolet absorption spectra of pteroylglutamic acid (III) and methotrexate (I) when halogen substituents are placed in the 3'-and 5'-positions.

(5) Private communication from Dr. Abraham Goldin of the National Cancer Institute. The activities of the compounds reported in this paper were described by Dr. Goldin at the San Francisco Meeting of the American Chemical Society, April, 1958. Further details will be published separately.

(6) G. W. Wheland, "Resonance in Organic Chemistry," John Wiley and Sons, Inc., New York, N. Y., 1955, p. 314.
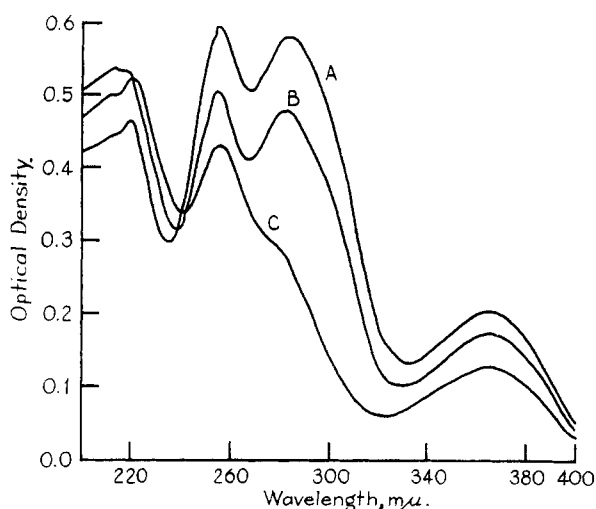
Fig. 1.—Ultraviolet absorption spectra in 0.1 N sodium hydroxide at 10 γ/ml.: (A) pteroylglutamic acid, (B) 3'-bromopteroylglutamic acid, (C) 3',5'-dibromopteroylglutamic acid.
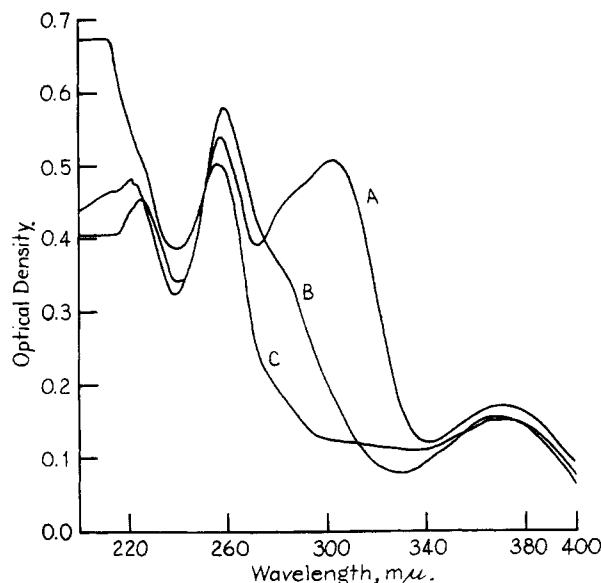

Fig. 2.—Ultraviolet absorption spectra in 0.1 N sodium hydroxide at 10 γ/ml.: (A) methotrexate, (B) 3'-chloromethotrexate, (C) 3',5'-dichloromethotrexate.

In Fig. 1 it can be seen that the insertion of one bromine atom in III to form the 3'-bromo derivative IX did not significantly change the absorption maximum at 282 mμ in 0.1 N sodium hydroxide, whereas the formation of 3',5'-dibromopteroylglutamic acid caused a very marked decrease in the extinction coefficient at that wave length. This is exactly what one might expect since in pteroylglutamic acid (III) there is only one substituent on the p-aminobenzoic acid nitrogen and, as a result, one o-substituent will not prevent the coplanarity necessary for the resonance form shown in formula X. However, two o-substituents will effectively oppose a planar configuration and thus reduce the absorption due to this resonance form. On the other hand, as shown in Figs. 2 and 3, when methotrexate (I) was converted to its 3'-
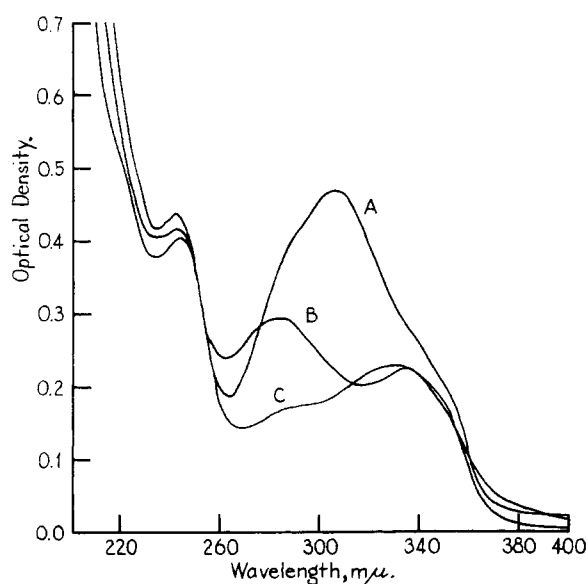

Fig. 3.—Ultraviolet absorption spectra in 0.1 N hydrochloric acid at 10 γ/ml.: (A) methotrexate, (B) 3'-chloromethotrexate, (C) 3',5'-dichloromethotrexate.

chloro derivative IV the spectra were markedly changed and were found to be similar to that of 3',5'-dibromopteroylglutamic acid. This again was not unexpected since I has two substituents on the aromatic nitrogen and therefore one o-substituent will be as effective in preventing coplanarity as two o-substituents were in the pteroylglutamic acid molecule. The further chlorination of I to give 3',5'-dichloromethotrexate (V) caused a still greater steric inhibition of resonance and gave spectra almost identical to a simple 2-amino-4-hydroxypteridine. The latter change in spectra from IV to V is best illustrated in Fig. 3 which shows the spectra of these compounds in 0.1 N hydrochloric acid.[7]

The literature contains very little information concerning the halogenation of the model compounds, p-methylaminobenzoic acid and p-dimethylaminobenzoic acid. p-Aminobenzoic acid itself has been readily dichlorinated and dibrominated. Apparently it cannot be monochlorinated since 3-chloro-4-aminobenzoic acid has always been prepared indirectly. However, monobromination has been accomplished to give the 3-bromo derivative.[8,9]

A rather complete study of the ultraviolet absorption spectra of dimethylaniline and various chloro, bromo and methyl derivatives has been reported[10] and the changes in the spectra for the

(7) The fact that methotrexate (I) has a strong absorption maximum at 306 mμ in 0.1 N hydrochloric acid may be considered somewhat unexpected since neither N-methylaniline or 4(N-methylamino)-benzoic acid has significant absorption in this area under the same conditions. This would indicate that the N-10 nitrogen in I and also in other pteroylglutamic acid derivatives is not protonated in 0.1 N hydrochloric acid. Preliminary spectroscopic evidence has indicated that in hydrochloric acid a 3 N solution is required before these compounds (I, III, etc.) are fully protonated.

(8) L. Frejka and L. Cizmar, Chem. Listy, 31, 460 (1937); C. A., 32, 4967 (1938).

(9) A. Leulier and J. Dinet, J. pharm. chim., 8, 57 (1928); C. A., 23, 1892 (1929).

(10) W. R. Remington, THIS JOURNAL, 67, 1838 (1945); J. Burgers.

ortho halogen derivatives agree very closely with those which we have found and reported above.

Acknowledgment.—We wish to thank Mr. Louis Brancone and staff for the microanalytical data and Messrs. William Fulmor and George Morton for the ultraviolet absorption spectra.

Experimental

Chromatography.—Descending paper chromatography was used routinely in these studies to help determine purity and to identify the components of a crude reaction product. A number of different solvent systems have been described for pteridines and folic acid compounds. However, in this study the homogeneous system 0.5% sodium carbonate[11] was found to be the most useful; 3% ammonium chloride[12] was used occasionally but was less satisfactory. The solutions were made up at 2 mg./ml. for spotting and the spots were viewed under an ultraviolet lamp, preferably one screened to give primarily light at a wave length of about 254 mμ. Under these conditions, all of the starting materials and most of the monohalogenated derivatives gave absorption spots when first viewed. The dihalogenated derivatives, however, like the simple pteridines gave fluorescent spots. If, after exposure to ultraviolet light, the chromatograms were allowed to age in air for several days, the absorption spots slowly changed to fluorescent spots which were more readily seen. In fact, light spots which were not visible as absorption spots sometimes became visible as fluorescent spots after aging.

It was also found that in the chromatograms run in 3% ammonium chloride some of the spots were not visible until they had been exposed to ammonia vapors.

Using these methods all of the compounds were obtained chromatographically pure.

4-Amino-4-deoxy-3'-chloro-N^{10}-methylpterolyglutamic Acid (3'-Chloromethotrexate) (IV).—Methotrexate[1] (10.0 g., 22 mmoles) was dissolved in 180 ml. of 6 N hydrochloric acid and cooled to 5°. Chlorine (1.4 g., 19.8 mM) was bubbled in with constant agitation. The solution was allowed to stand for two hours in the cold, then neutralized to pH 3 with 105 ml. of 10 N sodium hydroxide. The temperature was kept below 25° during the addition of the base. After chilling overnight the precipitate was filtered off, washed with water, acetone, and ether, then dried over phosphorus pentoxide *in vacuo*; yield 7.9 g.

The product was dissolved in 100 ml. of warm formamide on a steam-bath, treated with Darco G-60, and filtered. On cooling, the solution deposited needles; yield 5.3 g. (49.5%). This product was redissolved in 85 ml. of hot formamide (temperature not allowed to exceed 107°), treated with Darco G-60 and filtered through a pad of Celite. After standing two days at room temperature the compound was collected by filtration, washed, and dried to give 3.1 g. (29%) of the product; R_f 0.59 (0.5% Na$_2$CO$_3$), R_f 0.48 (3% NH$_4$Cl); ultraviolet spectra in 0.1 N NaOH: λ_{max} 259 mμ (ϵ 28,300), 280 (shoulder) (ϵ 18,000), 370 (ϵ 7,500); 0.1 N HCl: λ_{max} mμ 242 (ϵ 20,500), 282 (ϵ 14,400), 336 (ϵ 11,000).

Anal. Calcd. for C$_{20}$H$_{21}$O$_5$N$_8$Cl: C, 49.2; H, 4.3; N, 22.9; Cl, 7.27. Found: C, 48.8; H, 5.2; N, 22.9; Cl, 7.5.

4-Amino-4-deoxy-3',5'-dichloro-N^{10}-methylpterolyglutamic Acid (3',5'-Dichloromethotrexate) (V).—Methotrexate (10 g., 22 mM) was dissolved in 120 ml. of formamide on a steam-bath, then cooled in an ice-bath to 5°. Chlorine (10.35 g., 146 mM) was bubbled in over a 30-minute period with swirling. The solution was allowed to stand for one hour in the cold, then added to 800 ml. of water. Ten grams of sodium acetate was added, a gram at a time, to give pH 3. After chilling overnight the crude product was filtered off and washed with water, acetone and ether, then dried over P$_2$O$_5$ for two hours *in vacuo*; yield 7.8 g. This was dissolved in 140 ml. of water by adding sodium bicarbonate to pH 8. The solution was heated on a steam-bath to 75°, treated with Darco G-60, and filtered through a pad of Celite. The filtrate was reheated to 75° and treated with

M. A. Hoefnagel, P. F. Verkade, H. Visser and B. M. Wepster, *Rec. trav. chim.*, **77**, 491 (1958), and previous papers in the series.

(11) K. Slavik and V. Matoulkova, *Chem. Listy*, **48**, 765 (1954); *C. A.*, **48**, 13748c (1954).

(12) R. Tschesche and F. Korte, *Chem. Ber.*, **84**, 641, 801 (1951).

3.5 g. of anhydrous magnesium sulfate. After cooling to room temperature, then in an ice-bath, the crystalline product was collected by filtration, washed with water, acetone and ether and dried. This magnesium salt was converted to the free acid by dissolving in 500 ml. of water at 75° followed by acidification of the solution with 4 ml. of acetic acid; yield 5.8 g. (51%). This was chromatographically pure when the chromatograms were run as described above.

For analysis 200 mg. was dissolved in 100 ml. of 50% aqueous ethanol. On cooling, the compound crystallized as square platelets; R_f 0.48 (0.5% Na$_2$CO$_3$); ultraviolet spectra: in 0.1 N NaOH, λ_{max} 258 (ϵ 25,600), 370 (ϵ 7,600); 0.1 N HCl, λ_{max} 240 (ϵ 23,100), 330 mμ (ϵ 12,100).

Anal. Calcd. for C$_{20}$H$_{20}$N$_8$O$_5$Cl$_2$·H$_2$O: C, 44.3; H, 4.1; N, 20.7; Cl, 13.1. Found: C, 44.7; H, 4.6; N, 20.5; Cl, 13.3.

3'-Chloro-N^{10}-methylpteroylglutamic Acid (VI).—N^{10}-Methylpteroylglutamic acid (2.3 g., 5 mM) was dissolved in 40 ml. of 6 N hydrochloric acid and cooled to 5° in an ice-bath. A solution of 0.33 g. (4.6 mM) of chlorine in 10 ml. of acetic acid was then added dropwise with stirring to the first solution while keeping the temperature at 5–10°. (The chlorine content of the acetic acid was determined iodometrically.) After standing in an ice-bath for 2 hours, 12 ml. of 10 N sodium hydroxide was added while keeping the temperature below 20°. The solution was then treated with Norit, filtered and the filtrate was diluted with 200 ml. of warm water. Upon cooling, the product crystallized in rosettes; yield 1.6 g. (65%).

Paper chromatography using 0.5% Na$_2$CO$_3$ showed that this material was primarily the monochloro derivative with only traces of starting material and its dichloro derivative.

This product was dissolved in 100 ml. of formamide at 120°, treated with Norit, filtered, seeded and cooled; yield 0.7 g. (28%) of cream-colored sheaves of needles. This was chromatographically pure.

For analytical purposes a sample was recrystallized once more from formamide; R_f 0.79 (0.5% Na$_2$CO$_3$); ultraviolet spectra in 0.1 N NaOH, λ_{max} 257 (ϵ 27,600), 280 (shoulder) (ϵ 18,600), 362 mμ (ϵ 8,400); 0.1 N HCl, λ_{max} 303 mμ (ϵ 13,700).

Anal. Calcd. for C$_{20}$H$_{20}$N$_7$O$_6$Cl: C, 49.1; H, 4.1; N, 20.0; Cl, 7.3. Found: C, 49.1; H, 4.5; N, 19.9; Cl, 7.2.

3',5'-Dichloro-N^{10}-methylpteroylglutamic Acid (VII).—N^{10}-Methylpteroylglutamic acid (2.3 g., 5 mM) was dissolved in 40 ml. of 6 N hydrochloric acid and, while cooling to 5° in an ice-bath, 0.85 g. (12 mM) of chlorine was bubbled slowly into the solution. After standing in the ice-bath for 90 minutes this was brought to pH 3.0 with cooling, by the slow addition of 10N sodium hydroxide solution (ca. 25 ml.). After further cooling the product was collected, washed with water, acetone and ether and dried; yield 1.5 g. (57%).

A portion of this material (1.2 g.) was dissolved in 12 ml. of concentrated hydrochloric acid, treated with Norit, filtered and 110 ml. of warm water (50°) was added to the filtrate. After cooling several hours the light yellow microcrystalline product was collected; yield 0.87 g., R_f 0.68 (0.5% Na$_2$CO$_3$); ultraviolet spectra in 0.1 N NaOH, λ_{max} 252 (ϵ 28,200), 363 (ϵ 9,200); 0.1 N HCl, λ_{max} 318 mμ (ϵ 12,700).

Anal. Calcd. for C$_{20}$H$_{19}$N$_7$O$_6$Cl$_2$: C, 45.8; H, 3.6; N, 18.7; Cl, 13.6. Found: C, 45.7; H, 3.9; N, 18.9; Cl, 13.6.

4-Amino-4-deoxy-3'-bromo-N^{10}-methylpteroylglutamic Acid (3'-Bromomethotrexate) (VIII).—Methotrexate (5.0 g., 11 mM) was dissolved in 90 ml. of 6 N hydrochloric acid and cooled in an ice-bath. Bromine (1.72 g., 10.7 mM), dissolved in 15 ml. of acetic acid, was added over a 25-minute period. After standing for two hours in the cold the solution was adjusted to pH 3 with 53 ml. of 10 N sodium hydroxide keeping the temperature below 25°. The flask, was chilled overnight, the solid was collected and washed with water, acetone and ether, and dried; yield 4.7 g. (80%).

The crude product was dissolved in 100 ml. of water by adding 2.0 g. of sodium bicarbonate. The solution was heated to 85° on a steam-bath and treated with Norit. After filtering, the solution was cooled in an ice-bath and acidified with 2 ml. of acetic acid to give a gel. Upon heating on a steam-bath the gel went into solution and rosettes of needles came out while still hot. After cooling overnight, the product was collected, washed with water and

then dried over phosphorous pentoxide at 105° *in vacuo;* yield 3.4 g. (58%). This was chromatographically pure.

A portion of this product (0.5 g.) was dissolved in 70 ml. of water by adding sodium bicarbonate. The solution was treated with Norit on a steam-bath and filtered. The filtrate was reheated to 90° and acidified with acetic acid. The compound cystallized almost immediately. After standing overnight at room temperature the product was collected, washed with water, acetone and ether, then dried at 105° *in vacuo;* yield 0.35 g., R_f 0.57 (0.5% Na_2CO_3), R_f 0.45 (3% NH_4Cl); ultraviolet spectra in 0.1 N NaOH, λ_{max} 260 (ϵ 28,200), 280 (shoulder) (ϵ 17,800), 370 mμ (ϵ 7,500); 0.1 N HCl λ_{max} 241 (ϵ 21,400), 283 (ϵ 14,100), 336 mμ (ϵ 10,800).

Anal. Calcd. for $C_{20}H_{21}O_5N_8Br$: C, 45.0; H, 4.0; N, 21.0; Br, 15.0. Found: C, 44.5; H, 4.3; N, 20.6; Br, 15.0.

4-Amino-4-deoxy-3'-bromo-5'-chloro-N^{10}-methylpteroylglutamic Acid (3'-Bromo-5'-chloromethotrexate).—3'-Bromomethotrexate (12.7 g., 23.8 mM) was suspended and partially dissolved in 150 ml. of formamide. After cooling to 5° in an ice-bath, chlorine (12.7 g., 180 mM) was bubbled into the mixture during a period of 20 minutes causing complete solution of the starting material. After an additional 2 hours in the ice-bath, the solution was poured into 1200 ml. of water which was then adjusted to *p*H 3.0 to 3.5 with 15 g. of sodium acetate. A precipitate formed immediately. After cooling several hours, the product was collected, washed with water, acetone and ether and dried; yield 10.5 g. (78%). This product was dissolved in 350 ml. of water by adding 5.0 g. of sodium bicarbonate and then heated on the steam-bath to give a solution of *p*H 8. While hot, 3.7 g. of magnesium sulfate was added, the solution was treated with Norit, brought to boiling and filtered. The magnesium salt crystallized in lenticular crystals; yield 9.0 g.

This salt was converted to the free acid by dissolving in 600 ml. of hot water followed by acidification with 10 ml. of acetic acid. Upon reheating the resulting mixture the solid became crystalline; yield 6.8 g. (49%).

An analytical sample was prepared by recrystallizing a 100-mg. sample from 50 ml. of a 50% ethanol–water solution; clusters of needles, yield 60 mg., R_f 0.48 (0.5% Na_2CO_3), R_f 0.34 (3% NH_4Cl); ultraviolet spectra in 0.1 N NaOH, λ_{max} 257 (ϵ 26,700), 372 mμ (ϵ 7,900); 0.1 N HCl, λ_{max} 240 (ϵ 25,000), 332 mμ (ϵ 11,900).

Anal. Calcd. for $C_{20}H_{20}O_5N_8BrCl.H_2O$: C, 41.0; H, 3.9; N, 19.1; Cl, 6.1; Br, 13.7. Found C, 40.9; H, 3.8; N, 18.9; Cl, 6.40 Br, 13.8.

3'-Bromopteroylglutamic Acid (IX).—Pteroylglutamic acid (chem. assay 90%, 2.2 g., 4.5 mM) was dissolved in 20 ml. of concentrated hydrochloric acid which was then diluted with 20 ml. of water. This was cooled to 5° in an ice-bath and while stirring a solution of 0.72 g. (4.5 mM) of bromine in 12 ml. of acetic acid was added dropwise. A solid precipitated during the addition. After standing in the ice-bath for 2 hours the mixture was added to 250 ml. of water containing 15 g. of sodium chloride. This was warmed to give a clear solution. Upon cooling a crystalline product separated; yield 2.2 g. (85%).

This was dissolved in 25 ml. of concentrated hydrochloric acid, treated with Norit and filtered. A solution of 15 g. of sodium chloride in 250 ml. of hot water (70°) was added to the filtrate. The product crystallized as needles; yield 1.9 g. (74%), R_f 0.65 (0.5% Na_2CO_3); ultraviolet spectra in 0.1 N NaOH, λ_{max} 256 (ϵ 26,200), 282 (ϵ 25,000), 366 mμ (ϵ 8,900); 0.1 N HCl, λ_{max} 296 mμ (ϵ 21,600).

Anal. Calcd. for $C_{19}H_{18}N_7O_6Br$: C, 43.9; H, 3.5; N, 18.8; Br, 15.4. Found: C, 43.5; H, 3.8; N, 18.7; Br, 15.4.

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Allylic Rearrangements. XLV.[1] The Reaction of Thionyl Chloride with 4β-Hydroxycholesteryl Benzoate

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The product of the reaction of thionyl chloride with 4β-hydroxycholesteryl benzoate (I) has been shown to be 6β-chloro-3β-benzoyloxy-4-cholestene (II). Lithium aluminum hydride reduction of the chlorobenzoate (II) produces only cholestero-(IV). The stereospecificity of this reduction has been shown to be due to an intramolecular S$_N$2' reaction sequence by using lithium aluminum deuteride reduction to introduce a 4β-deuterium substituent.

As an extension of our work on steroid allylic alcohols,[3] we undertook a study of the reaction of thionyl chloride with 4β-hydroxycholesteryl benzoate (I). Petrov, Rosenheim and Starling[4] first carried out such a reaction in pyridine–ether solution, and presented evidence which they construed as favoring the formation of a 4-chlorocholesteryl benzoate of unspecified stereochemistry. Spring and Swain[5] obtained the same chloro steroid by the thionyl chloride–pyridine dehydration of 6β-chloro-5-hydroxy-3β-cholestanyl benzoate (III). While the latter authors assigned the 6β-chloro-3β-benzoyloxy-4-cholestene structure (II) to their

(1) This work was supported in part by a grant from The National Science Foundation.

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(3) R. E. Ireland, T. I. Wrigley and W. G. Young, THIS JOURNAL, **80**, 4604 (1958).

(4) V. A. Petrov, O. Rosenheim and W. W. Starling, *J. Chem. Soc.*, 135 (1943).

(5) F. S. Spring and G. Swain, *ibid.*, 1356 (1939).

material, Petrov and co-workers preferred the 4-chlorocholesteryl benzoate structure formed by allylic rearrangement during dehydration.

It is apparent that allylic rearrangement has taken place during one of the above reactions, and in light of our recent work,[3] it seemed more reasonable to consider such a rearrangement to have occurred during the OH → Cl conversion rather than during dehydration. In particular, we found that 5-cholesten-4β-ol could be converted with rearrangement to 6β-chloro-4-cholestene by thionyl chloride in ether solution with or without the presence of amines. The same 6β-chloro-4-cholestene was formed in excellent yield without rearrangement on low temperature thionyl chloride–pyridine dehydration of 6β-chloro-5-cholestanol.

In order to investigate the position of the chlorine in the cholesteryl benzoate series, we repeated Petrov's preparation and obtained substantially the same results. We also found that the addition of excess chloride ion in the form of tri-*n*-butyl-