# SYNTHESIS OF $^{14}$C-METHOTREXATE: (N-[p-[[2,4-DIAMINO-4-DEOXY-6-PTERIDINYL]METHYL]METHYLAMINO]-BENZOYL]-L-$^{14}$C-GLUTAMIC ACID

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## SUMMARY

A simple and general method for the synthesis of folate analogs labeled in the glutamate moiety with $^{14}$C is described. The synthesis makes use of the trimethylsilyl derivative of L-glutamic acid 8 for the coupling reaction with the activated pteroate analogs. Deprotection under mild conditions circumvented the potential problems associated with deamination at the 4-position of 2. Methotrexate, folic acid, and $N^{10}$-methyl folic acid were prepared in ∿50% yield by the use of this procedure.

Key Words: $^{14}$C-Methotrexate, Folic Acid, Trimethyl Silylation

## INTRODUCTION

Although more than twenty-five years have elapsed since methotrexate first became available (1), biochemical and clinical investigations related to this drug continue at a rapid pace (2). The literature on the fate and functions of methotrexate have accumulated to the point that comprehensive review is impractical, if not impossible. Methotrexate, tritiated at 3',5' positions, which is commercially available, has been customarily employed for these investigations (3,4,5). However, quite recently (6), the use of $^{14}$C-methotrexate, labeled uniformly in the glutamate moiety, has enabled the identification and characterization (7) of poly-γ-glutamates of methotrexate which are the newly

discovered natural metabolites of this drug. The recently perfected technique of reductive cleavage (8) of folic acid metabolites to the corresponding p-aminobenzoylpoly-γ-glutamates for the determination of the number of glutamate residues also made use of folic acid labeled uniformly with $^{14}C$ in the glutamyl residue. If such techniques are to be employed for further investigations regarding methotrexate and folate metabolism, obviously $^{14}C$-methotrexate and folic acid with the label in the p-aminobenzoylglutamate part is required. It is now known that significant tritium exchange with the medium takes place from these commercially available tritiated derivatives under various experimental conditions (9). This report details a simple synthesis of general applicability to the construction of folate analogs, uniformly labeled with $^{14}C$ in the glutamate moiety, from the appropriate pteroic acid compounds and U-$^{14}C$-glutamic acid.

## METHODS

Although there are several approaches described in the literature for the synthesis of methotrexate, the attachment of a protected L-glutamic derivative Z to the pteroic analog 2 appeared to be the most attractive route (10). The desired pteroic acid analogs are easily obtainable in good yield from either folic acid or methotrexate by the use of a bacterium which utilizes the glutamate moiety of the molecule as its sole source of carbon and nitrogen. The pteroic acid analog thus produced remains in the growth medium, and can easily be isolated. These procedures were outlined in detail by Levy and Goldman (11). Several alternate approaches to the synthesis of pteroate analogs are also described in the literature (12,13,14). Among them, the reaction of an appropriately substituted 6-bromomethylpteridine with various nucleophiles, such as p-aminobenzoic acid, appears

to be the most convenient procedure (15,16). The protection of the 2,4-diamino functions of 2,4-diamino-4-deoxy-$N^{10}$-methyl pteroic acid (11) 3 was also desired prior to the coupling reaction with the glutamate derivative. Several attempts to make the 2,4-ditrifluoroacetyl derivative 4 failed, due to degradation. A mechanistic consideration of this reaction has already been documented in the literature from this laboratory (7). The proper choice of the carboxyl protective groups of glutamtic acid was at this time viewed as critical, since the procedures which must be employed for the eventual deprotection of these groups should not result in the deamination at the 4-position of the pteridine ring. The use of trimethylsilyl groups was considered ideal, because of the ease with which they are removed and the lack of racemization of optically active amino acids under conditions which are generally used for the preparation (17) of their trimethylsilyl derivatives.

Conditions have been developed under which a solution of 3 in a 1:1 mixture of DMSO:THF could be converted to the active mixed anhydride 5 by reaction with isobutyl chloroformate in the presence of N-methyl morpholine. This mixed anhydride in turn could be coupled with 8, which is uniformly labeled with $^{14}$C. After coupling, deprotection was achieved at room temperature with 0.05 N sodium hydroxide solution. On ion exchange chromatography, only two UV absorbing compounds were eluted from the column, identified as the starting material 3 and $^{14}$C-methotrexate 1. This coupling reaction proceeded in 50% yield based on the starting material 3. The methotrexate thus prepared was indistinguishable from an authentic sample except for the radioactivity (Scheme I). In a similar experiment, $N^2$,$N^{10}$-ditrifluoroacetyl-pteroic acid was activated to the corresponding mixed anhydride with isobutyl chloroformate and allowed to couple with the silyl

derivative 8. Under identical conditions, as described for the synthesis of methotrexate, folic acid was obtained in 40% yield based on the pteroic acid used. However, in this particular experiment, the reaction product was treated with 0.1 N sodium hydroxide and kept at 75° for 30 minutes to remove the protective trifluoroacetyl functions from the 2 and 10 amino groups.

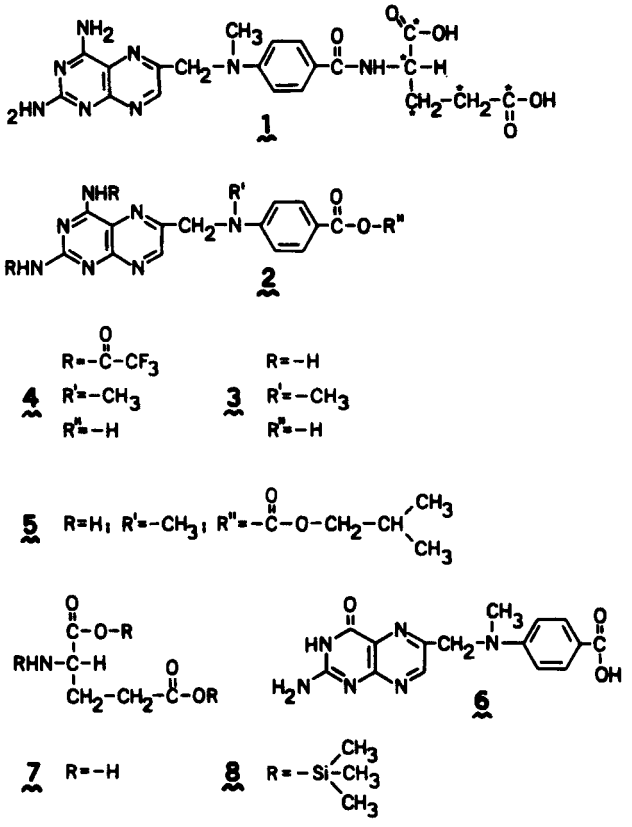
To check the generality of this procedure, $N^{10}$-methyl pteroic acid 6 was subjected to the same reaction sequence, and $N^{10}$-methyl folic acid was prepared similarly in ∿50% yield. Since U-$^{14}$C-glutamic acid 7 with high specific activity is commercially available, this simple procedure would permit a convenient synthesis of $^{14}$C-folic acid and its analogs.

## EXPERIMENTAL

In a 50 ml round bottom flask fitted with a reflux condenser, 3 μmoles of U-$^{14}$C-L-glutamic acid (750 μCi) and 27 μmoles of non-radioactive L-glutamic acid were combined. Next, 4 ml of hexamethyl disilazane followed by 0.5 ml of benzene containing 3.6 mg of concentrated sulfuric acid was added, and the mixture refluxed for 1.5 hours under strictly anhydrous conditions. The reaction mixture was cooled to ambient temperature, and four drops of freshly distilled triethylamine was carefully added; the mixture was refluxed for 5 minutes. The solvents were then removed in vacuum at 40° to obtain a waxy solid 8 which was dried in a vacuum oven at room temperature for 2 hours.

2,4-diamino-4-deoxy-$N^{10}$-methylpteroic acid, 4.87 mg (15 μmoles) was dissolved in 0.25 ml DMSO and 0.25 ml THF was added. This mixture was chilled to 0°. To this solution, 20.0 μmoles (0.0022 ml) of N-methyl morpholine was added, and allowed to stand for 15 minutes, followed by the addition of 0.002 ml freshly distilled isobutyl chloroformate (15 μmoles). This mixture was kept at 0° for 15 additional minutes.

SCHEME I



During this period, the trimethylsilyl derivative 8 was dissolved in 1 ml of cold 1:1 DMSO:THF and kept at 0°. The mixed anhydride 5 was then transferred carefully to the flask containing 8, and the reaction was allowed to proceed at 0° for 20 minutes and then for 18 hours at room temperature. After this period, the reaction mixture was kept in a boiling water bath for 5 minutes, which allowed most of the THF to boil off. The reaction mixture was cooled to room temperature, and 12 ml of 0.05 N sodium hydroxide solution was added under stirring. After 1.5 hours, the solution was diluted to 40 ml and adjusted to pH 7.25 with 0.1 N HCl. Ion exchange chromatography of this

solution on DEAE cellulose in the chloride form, using a 0→0.5 M linear NaCl gradient in 0.005 M phosphate buffer at pH 7.0 as the eluting solvent, gave two UV absorbing materials. The less polar material was non-radioactive and identified as the starting pteroate analog. The second material was radioactive and was indistinguishable from authentic methotrexate spectrophotometrically and chromatographically, and had a specific activity of 17.6 μCi/μmole.

Several experiments were carried out in an attempt to improve the yield of methotrexate by utilizing more than one equivalent of isobutyl chloroformate for the preparation of 5. However, these experiments gave poor results and were subsequently abandoned.

A semi-micro synthesis of $N^{10}$-methyl folic acid was carried out using the above procedure, but using non-radioactive 6 and L-glutamic acid. $N^{10}$-methyl folic acid was obtained also in ∿50% yield.

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