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SYNTHESIS OF METHOTREXATE-3-^{15}N-2-$^{15}NH_2$. A CONVENIENT METHOD FOR PREPARATION OF GUANIDINE-$^{15}N_3$.

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SUMMARY

Condensation of guanidine-$^{15}N_3$ with 4-[N(2-amino-3-cyano-5-pyrazinyl-methyl)-N-methylamino] benzoic acid (1) in refluxing methanol afforded 4-amino-4-deoxy-N_{10}-methylpteroic-3-^{15}N-2-$^{15}NH_2$ (2). The mass spectrum clearly indicated no label introduction at the pterin N_1 position. Coupling of 2 with diethyl L-gluatmate and subsequent alkaline hydrolysis yielded methotrexate-3-^{15}N-2-$^{15}NH_2$ (4). Triply ^{15}N-labeled guanidine was readily prepared from thiocyanic-^{15}N acid (derived from KSC^{15}N) and ammonia-^{15}N by heating the resultant ammonium thiocyanate-$^{15}N_2$ at 180°C, followed by isolation as the water insoluble picrate salt.

Key Words: Methotrexate-3-^{15}N-2-$^{15}NH_2$, guanidine-$^{15}N_3$, nitrogen-15.

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INTRODUCTION

Biophysical studies, utilizing ^{15}N-NMR, of the complex formed between dihydrofolate reductase and its inhibitors required the preparation of methotrexate specifically labelled at the pyrimidine ring nitrogens and the 2- and 4-amino groups with nitrogen-15. To reduce complexity in the interpretation of spectral shifts following enzyme-inhibitor complex formation it was desirable not to have all four N-15 labels present at once. We describe herein the synthesis of methotrexate bearing N-15 labels at the ring N-3 and 2-NH_2 positions.

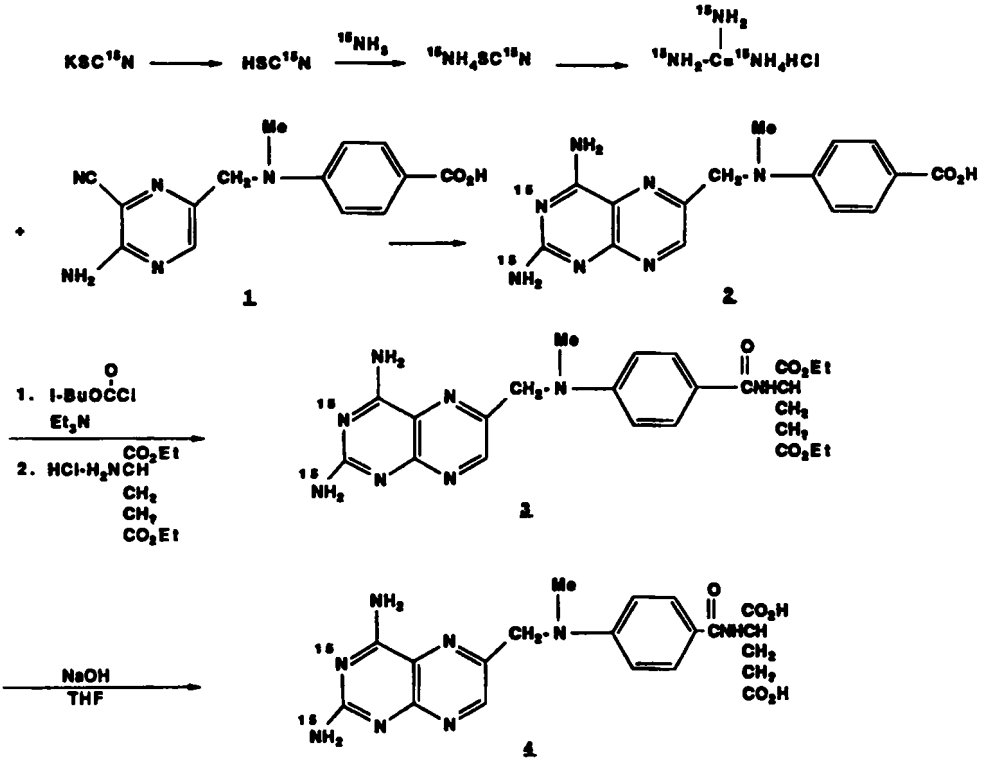
DISCUSSION

The most convenient route for preparation of pyrimidine ring-labeled methotrexate was envisioned to be the condensation of labelled guanidine with the 2-amino-3-cyanopyrazine intermediate (1) described by Taylor and coworkers.(1) However, the mechanism of this ring closure to a 2,4-diamino pteridine had not been elucidated. Therefore it was formally possible that triply N-15 labelled guanidine could react in two ways. The 2-amino group of the cyano pyrazine could displace an $^{15}N_2$ from guanidine followed by ring closure to form the 3-^{15}N-2-$^{15}NH_2$ pterin product or a guandine-$^{15}NH_2$ could displace the pyrazine NH_2 to eventually yield a 1,3-$^{15}N_2$-2-$^{15}NH_2$ pterin product or a mixture of the two labelled pterins.

To conduct the synthesis outlined above we had first to obtain a guanidine salt triply labelled with N-15 at 99 atom precent enrichment. Commercial sources were not forthcoming for this material so we undertook the preparation in our laboratory. Commercially available potassium thiocyanate-^{15}N was converted to thiocyanic acid by passage through an acidic Dowex sulfonic resin and the acidic eluate was neutralized by treatment with ammonia-^{15}N gas. The ammonium thiocyanate-$^{15}N_2$ obtained after evaporation of water was then heated in a sealed tube at 180°C for 18 hours.(2) At this temperature the thiourea intermediate that is formed remains in a molten state and continues to react until formation of guanidine is complete. Extraction of the product into water is followed by precipitation with picric acid

solution. The crystalline picrate is conveniently exchanged to the hydrochloride salt by stirring with an excess of Dowex-2 resin in the chloride form.

Condensation of the labelled guanidine with the 2-amino-3-cyanopyrazine afforded only the doubly ^{15}N-labelled product (2). The mass spectrum of 2 obtained as the tris trimethyl silyl derivative showed an increase of only 2 mass units showing that the 2-NH_2 of the cyanopyrazine substrate is not displaced. We also investigated the condensation of the 1-N-oxide of 1 with labelled guanidine and again obtained an M+2 product.



Coupling of 2 with diethyl L-glutamate to give the diethyl ester of methotrexate-$^{15}N_2$ (3) and subsequent saponification were carried out in the established manner(3) to afford the labelled methotrexate (4). The product was identical to unlabelled methotrexate by HPLC and ultraviolet analysis; the mass spectrum (tetratrimethylsilyl derivative) was correct for methotrexate increased by 2 mass units.

EXPERIMENTAL

Guanidine $^{15}H_3$ Hydrochloride.

Potassium thiocyanate ^{15}N 0.90 g (9.16 mmoles) in 3 ml water was added to a 6 g AG50w-x8 (H^+) resin column (must be fresh) and elution with water continued until the pH of the eluate was neutral. The acidic eluate was neutralized with ammonia-^{15}N gas to pH 7 and the solution was evaporated to dryness. The solid residue (0.61 g, 85%) was heated in a Paar bomb at 180° for 18 hours. The container was cooled and the contents dissolved in 15 ml water and filtered. The product was precipitated with 10% picric acid to yield 1.3 g. The picrate was stirred in an aqueous mixture of 6 ml of Dowex 2 (Cl^-) for 6 hours. The resin was removed by filtration and the filtrate evaporated to dryness to leave 220 mg (49%) of white crystals; MS m/e 62.

2,4-Diamino-6-(N-methyl-4-carboxyanilinomethyl)pteridine-3-$^{15}N_2$-2-$^{15}NH_2$ (2).

To a methanolic solution of guanidine-$^{15}N_3$ [prepared by adding 120 mg (1.22 mmoles) of labelled guanidine hydrochloride to 30 ml MeOH containing 70 mg (1.29 mmoles) of sodium methoxide] was added 200 mg (0.701 mmole) of 4[N(2-amino-3-cyano-5-pyrazinylmethyl)-N-methyl amino]benzoic acid (1). The reaction mixture was heated at reflux for 15 hours. The solution was neutralized to pH 6 with acetic acid and evaporated to dryness. The residue was exacted with 10 ml of hot methanol. The methanol was evaporated and the residue placed on a silica gel column and eluted with methanol/chloroform (1:9), to give 100 mg (44%) of the pteroic acid (2); MS as the tris TMS derivative m/e 559. ^{13}CNMR DMSO-d_6 163.0 (C-2, J = 25.6 + 4.0 Hz), 162.4 (C-4, J = 7.7 + 4.0 Hz).

Methotrexate-3-^{15}N-2-$^{15}NH_2$ Diethyl Ester (3).

To 95 mg (0.29 mmoles) of the 2,3-^{15}N-pteroic acid (2) in 10 ml of distilled dimethylformamide under argon was added 84 µl (0.60 mmoles) of triethylamine, followed by 39 µl (0.3 mmoles) of isobutyl chloroformate. The mixture was stirred for 45 min followed by addition of 72 mg of diethyl-L-

glutamate hydrochloride in 2 ml of dimethylformamide and 42 µl (0.3 mmole) of triethyl amine. The mixture was stirred under argon for 6 hours. A second addition of 42 µl (0.3 mmole) triethylamine and 39 µl of isobutylchloroformate was added, followed (after 1/2 hour stirring) by addition of 0.3 mmole of diethyl-L-glutamate hydrochloride and triethylamine in dimethylformamide. The mixture was stirred for 18 hours at room temperature. A third addition as above was made and the mixture stirred for an additional 5 hours. The mixture was evaporated to a thick syrup and partitioned between 100 ml of chloroform and 20 ml of saturated sodium bicarbonate. The chloroform was washed with saturated sodium chloride solution, dried over sodium sulfate, filtered and evaporated to dryness. The residue was placed on a silica gel column and eluted with methanol-chloroform (1:19) to give 78 mg (54%). ^{13}CNMR (CD_3OD), 162.4 (C-2), J = 25.6, 4.0 Hz, 164.7 (C-4) J = 7.7, 4.0 Hz, 123.4 (C-4a), 150.4 (C-6), 150.2 (C-7), 153.3 (C-8a), 53.8 (C-9), 39.7 (N-Me), 153.1 (C-1'), 130.2 (C-2'), 112.7 (C-3'), 122.3 (C-4'), 170.2 (4'-C=O), 173.7 (glu-1), 56.6 (glu-2), 27.5 (glu-3), 31.6 (glu-4), 174.6 (glu-5), 62.5 (CH_2 of Et), 61.8 (CH_2 of Et), 14.5 (CH_3 of Et).

Methotrexate-3-^{15}N-2-$^{15}NH_2$ (4).

To 65 mg (0.13 mmoles) of the labelled diester (3) in 10 ml of tetrahydrofuran under argon was added 6 ml of 0.2 N sodium hydroxide. The solution was stirred for 1 hour (analysis by HPLC indicated complete reaction) and was neutralized to pH 7 with 1 N hydrochloric acid. After evaporation to a volume of about 1 ml, a solid precipitate formed. The mixture was chilled and the solid collected by centrifuge. The solid was triturated with 0.3 ml of hot water, chilled and centrifuged to give 12 mg (20%); MS of tetra TMS derivative = 744. HPLC indicated 98% of a peak identical to authentic methotrexate; UV 0.1 N NaOH 372, 305 and 259. The water wash and filtrates were combined and evaporated to dryness. The residue was taken up in 0.4 ml hot water, chilled and centrifuged to give an additional 35 mg (58%) of product.

^{15}N-NMR chemical shifts were measured in d_6-DMSO for the doubly labeled methotrexate 4 (3.3 mM, 302°K) and referenced (4) to neat nitromethane-^{15}N. The 2-$^{15}NH_2$ signal appeared at -290.3 ppm., ^1J NH 86 ± 4 Hz, while the 3-^{15}N signal was seen at -165.1 ppm.

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