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SYNTHESIS OF METHOTREXATE-1-^{15}N AND METHOTREXATE-4-$^{15}NH_2$

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SUMMARY

This paper describes an application of the pterin synthesis of Taylor and co-workers for preparation of methotrexate specifically labelled at the N_1-ring nitrogen and at the 4-amino group with 99 atom percent ^{15}N. Oximination of ethyl cyanoacetate-^{15}N followed by reduction afforded ethyl 2-aminocyanoacetate-C^{15}N (3). Condensation with 3-bromopyruvaldoxime and 4-methylaminobenzoic acid afforded 2-amino-3-carbethoxy-5-N-methyl-p-carboxyanilinomethylpyrazine-1-oxide-2-$^{15}NH_2$ (4). Treatment of 4a with ammonium hydroxide at room temperature gave the 3-carboxamide (5a). Reduction of the N-oxide (PCl_3), esterification, and dehydration of the amide ($POCl_3$) afforded the 2-amino-3-cyanopyrazine benzoate ester (8). Ring closure with guanidine followed by benzoate ester hydrolysis, glutamate coupling and hydrolysis of the glutamate diester yielded methotrexate-1-^{15}N (12a). Amination of the unlabeled 2-amino-3-carbethoxy pyrazine intermediate (4b) with ^{15}N-labelled ammonium hydroxide gave the ^{15}N-carboxamide (5b) which was carried through the process described above to afford methotrexate-4-$^{15}NH_2$ (12b).

Keywords: Methotrexate-1-^{15}N; Methotrexate-4-$^{15}NH_2$; Dihydrofolate reductase; ^{15}N-NMR.

INTRODUCTION

As part of a program designed to measure NMR spectral shifts between methotrexate and its complex with the dihydrofolate reductase enzyme we had occasion to synthesize methotrexate specifically and separately labelled at the ring N_1-nitrogen and in the 4-amino group with 99 atom percent ^{15}N. The

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elegant synthetic route devised by Taylor and co-workers[1] is well suited for introduction of labelled atoms in the pyrimidine ring moiety of pterin compounds. By suitable manipulation of substituents at the 2 and 3-positions of the pyrazine ring containing intermediates it was possible to obtain 2,4-diaminopteridines selectively labelled at N_1 or the 4-NH_2. We describe herein the synthesis of methotrexate specifically labelled at these positions with ^{15}N.

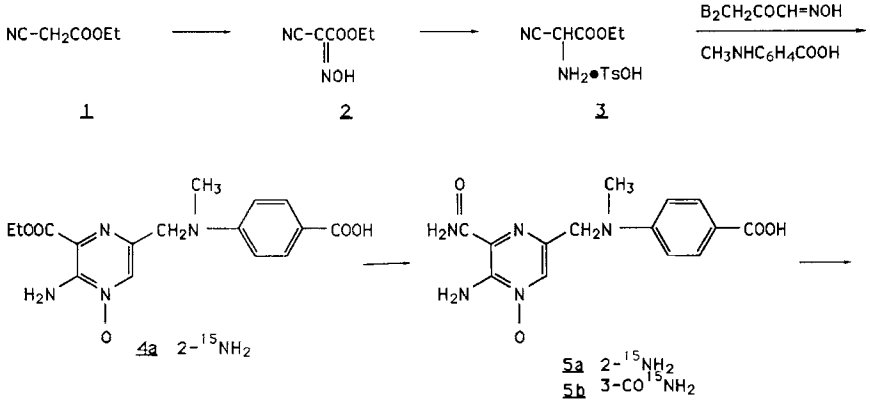
RESULTS AND DISCUSSION

The synthetic process described by Taylor, et al.,[1] for 2,4-diaminopteridines substituted at C-6 entails condensation of 2-aminomalononitrile with an appropriate α-oximinoketone to give a 2-amino-3-cyano-5-substituted pyrazine-1-N-oxide. The process has been adapted to the synthesis of methotrexate, but would be unsuitable as such for specific insertion of labels at the ring N_1 or 4-NH_2 nitrogens. We therefore chose to proceed through a 2-amino-3-carbethoxypyrazine intermediate with subsequent conversion of the ester function to an amide and dehydration to a 2-amino-3-cyanopyrazine. This procedure thus allows for selective insertion of ^{15}N labels at the 2-NH_2 or the 3-cyano functions.

Oximination of ethyl cyanoacetate-^{15}N (1) was carried out by treatment with nitrous acid.[2] The oximino cyano ester (2) was then reduced with aluminum amalgam[3] to afford ethyl 2-aminocyanoacetate, isolated as the p-toluenesulfonate salt (3). Condensation of the aminocyano ester (^{15}N-cyano) with bromopyruvaldoxime (4) and 4-methylaminobenzoic acid in a 3-component process[1] gave 2-amino-3-carbethoxy-5-p-carboxy-N-methylanilinomethylpyrazine-1-N-oxide-2-$^{15}NH_2$ (4a) in 25% yield. When this carboxy ester was dissolved in 15 N ammonium hydroxide and allowed to stand for 15 hours the ester was smoothly converted to the 3-carboxamido compound (5a). After removal of the N-oxide by treatment with phosphorus trichloride the resulting carboxylic acid (6a) was esterified by reaction with methanolic hydrogen chloride. The carboxamidobenzoate ester (7a) was successfully dehydrated in 33% yield to the 2-amino-3-cyano pyrazine ester (8a). Considerable experimentation was necessary to

perfect this dehydration step, but treatment with phosphoryl chloride in pyridine at room temperature for 2 days was found to effect conversion to nitrile in about 30% yield. Ring closure with guanidine, saponification of the benzoate ester (9a), coupling with diethyl L-glutamate and hydrolysis of the purified glutamate diester (11) completed the synthesis of methotrexate-1-^{15}N (12a).

When unlabelled 2-amino-3-carbethoxypyrazine intermediate (4) was treated with 1.67 $N^{15}NH_4OH$, the ester was converted to the carboxamide-^{15}N (5b). The reaction sequence described above was then carried out in a similar manner to afford methotrexate-4-$^{15}NH_2$ (12b).



formulae 9-12 a) series 1-^{15}N, b) series 4-$^{15}NH_2$

^{15}N-NMR chemical shifts were determined in d_6-DMSO for the labeled methotrexate compounds as referenced to neat nitromethane-^{15}N. The 1-^{15}N labeled methotrexate (12a) showed the 1-^{15}N signal at -180.2 ppm while the 4-$^{15}NH_2$ labeled methotrexate (12b) signal occurred at -293.7 ppm with a N-H coupling constant of 88 ± 3 Hz. The proton NMR spectrum of 12b showed a doublet centered at 6.54 ppm which corresponds to the singlet seen at that chemical shift in unlabeled methotrexate. Stadelli, et al.[5] have reported the ^{15}N spectra of enriched diaminopyrimidines (but not pteridines). Their values for the corresponding atomic sites in trimethoprim and pyrimethamine are in close agreement to those given here.

EXPERIMENTAL

Ethyl Oximinocyano-^{15}N-acetate (2)

Ethyl cyanoacetate-^{15}N (1, 2.90 g, 25.4 mmoles) was dissolved in 17 ml of 70% acetic acid. To this solution, chilled to 3°, was slowly added 1.90 g (27.5 mmoles) of sodium nitrite in 6 ml of water, keeping the temperature below 10°C. The mixture was diluted with 25 ml of water and acidified with 3.2 ml of conc. hydrochloric acid. The product was extracted with 2 x 50 ml portions of ethyl ether. The ether was washed with 50 ml of brine, dried over sodium sulfate, and evaporated to a semi-solid residue. The material was recrystallized from 15 ml chloroform-5 ml of carbon tetrachloride to afford 2.4 g of a white solid (62%), m.p. 129-131°C, lit.[2] 130-131°C; ^{13}C NMR ($CDCl_3/CD_3OD$) ^{15}N≡C 125.7 (d), J_{CN} = 19.3 Hz, C_2 125.7 (d) $J_{C,CN}$ = 3.6 Hz, C=O 158.7(s), CH_2 63.2(s), CH_3 13.7(s).

Ethyl Aminocyano-^{15}N-acetate p-Toluenesulfonate (3)

To 0.630 g (23.6 mmoles) of aluminum foil cut into 1 cm^2 pieces was added 15 ml of 2% mercuric chloride. The mixture was swirled for 2 min, then decanted. The residue was washed with 2 x 15 ml of water, 15 ml of ethanol, 25 ml of tetrahydrofuran and 2 x 25 ml of ether. The amalgam was covered with 15 ml of tetrahydrofuran and a solution of 2.3 g (16.1 mmoles) of ethyl oximino-cyano-^{15}N-acetate (2) in 20 ml of ethyl ether was added. To this well stirred mixture, 0.9 g of water was added dropwise over 30 min at such a rate to maintain a gentle reflux. After the addition, the mixture was cooled and

filtered through Celite. The flask and filter cake were washed with 50 ml of tetrahydrofuran and 50 ml of ether. To the combined filtrate was added 3.0 g (16.1 mmole) of p-toluene-sulfonic acid monohydrate and the solution was evaporated to dryness. The pale yellow solid was dissolved in 15 ml of tetrahydrofuran and 40 ml of ether was added. The solution was allowed to stand at room temperature until crystals formed and then chilled at -5°. The precipitate was collected to give 2.8 g (58%) of white crystals; ^{13}C NMR (DMSO-d_6) $^{15}N{\equiv}C$ 112.8 (d), J_{CN} = 25.1 Hz, C-2 42.6 (d) $J_{C,CN}$ = 2.4 Hz, C=O 161.4, $CH_3\underline{CH_2}$ 64.0, $\underline{CH_3}CH_2$ 13.7, Ar-CH_3 20.7, C-1' 145.3, C2'+6' 125.5, C-3'+5' 128.0, C-4' 137.8.

p-N-(2-^{15}N-Amino-3-carboethoxy-1-oxido-5-pyrazinylmethyl)-N-methylaminobenzoic Acid (4)

To a solution of 1.66 g (10.0 mmole) of bromopyruvalidoxime[4] and 1.37 g (10.0 mmoles) of 4-methylaminobenzoic acid in 40 ml of isopropanol was added 2.71 g (9.00 mmoles) of ethyl aminocyano-^{15}N-acetate$_+$TsOH (3). The reaction was stirred for 3 days and the yellow solid that precipitated was collected and air dried to give 720 mg (25%); C^{13} NMR C-5'C=O 167.3, C-3C=O 165.1, C-2 148.0 (d) J_{CN} = 20.8 Hz, C-3 124.6, C-5 141.1, C-6 131.0, N-CH_2- 61.5, N-Me 38.8, $\underline{CH_2}CH_3$ 54.2, $CH_2\underline{CH_3}$ 14.0, C-4' 117.8, C-2'+6' 130.0, C-3'+5' 111.2, C-1' 151.8.

p-N-(2-^{15}N-Amino-3-carboxamido-1-oxido-5-pyrazinylmethyl)-N-methylaminobenzoic Acid (5a)

The 2-^{15}N-amino-3-carbethoxy compound (4) 0.700 g (2.02 mmole) was stirred overnight at room temperature in 10 ml of conc. ammonium hydroxide. The solution was cooled and neutralized to pH 6-7 with 3N hydrochloric acid. The precipitate was collected, washed with water and dried to give 0.62 g (96%) of yellow solid; ^{13}C NMR C-2 146.9 (d), $J_{C,N}$ 20.8 Hz, C-3 O=C-NH_2 167.4, no ester signals.

p-N-(2-Amino-3-^{15}N-carboxamido-1-oxido-5-pyrazinylmethyl)-N-methylaminobenzoic Acid (5b)

A solution of 0.800 g (2.38 mmoles) of unlabeled amino ester (4) in 10 ml of water which contained 0.3 g $^{15}NH_3$ was kept at room temperature 15 hours.

The solution was adjusted to pH 6-7 with conc. hydrochloric acid at 0-5°. The precipitate was collected to give 0.75 g (99%) of yellow solid. ^{13}C NMR $CO^{15}NH_2$ 167.4 (d) $J_{C,N}$ 17.1 Hz. The material was identical to 5a by HPLC analysis (Browning C-18 column, 0.1N NaH_2PO_4-CH_3CN, 1:4).

p-N-(2-[^{15}N-Amino-3-carboxamido-5-pyrazinylmethyl)-N-methylaminobenzoic Acid (6a)

Compound 5a (0.600 mg, 1.88 mmoles) was dissolved in 25 ml of tetrahydrofuran, chilled to 0° and 3.5 g of phosphorus trichloride was added dropwise over 1 minute. The reaction mixture was allowed to come to room temperature and then stirred for 1 hour. The solution was evaporated to an oil. Ice water (20 ml) was added and the mixture stirred for 30 min and filtered. The yellow solid was washed with 20 ml H_2O and dried to leave 0.55 g (97%) of 6a (2-$^{15}NH_2$); ^{13}C-NMR (C-2 154.2 (d) $J_{C,N}$ 19.5 Hz; MS m/e 302. Compound 6b (3-$CO^{15}NH$) was prepared as above from 5b to give a yellow solid (92%); ^{13}C-NMR 3-$CO^{15}NH_2$) 168.3 (d) $J_{C,N}$ 17.1 Hz; MS m/e 302.

Methyl p-N-(2-^{15}N-Amino-3-carboxamido-5-pyrazinylmethyl)-N-methylaminobenzoate (7a)

The benzoic acid compound (6a), 0.53 g, (1.75 mmoles) was dissolved in 40 ml of methanol saturated with hydrogen chloride. The solution was stirred at room temperature for 2 days and evaporated to dryness. The residue was taken up in 25 ml of chloroform and washed with saturated sodium bicarbonate and saturated sodium chloride solution. The chloroform was dried over sodium sulfate, filtered and evaporated to dryness. The residue was chromatographed on silica gel with elution by methanol-chloroform, 19:1, to give 410 mg (74%) 7a; ^{13}C NMR C-2 152.7 (d) $J_{C,N}$ 22.0 Hz, C-3 $CONH_2$ 168.6. Anal. Calc. $C_{15}H_{17}N_4{}^{15}N \cdot 2HCl$: C, 46.3; H, 4.92; N, 18.2. Found: C, 46.4; H, 4.65; N, 17.9. Ester 7b was similarly prepared in 76% yield from 6b; ^{13}C-NMR C-2 152.7, C-3 $CONH_2$ 168.6 (d) $J_{C,N}$ 17.5 Hz.

Methyl p-N-(2-^{15}N-Amino-3-cyano-5-pyrazinylmethyl)-N-methylaminobenzoate (8a)

To 380 mg (1.20 mmoles) of the carboxamido ester (7a) in 15 ml of pyridine at 0° was added 1 ml of phosphoryl chloride. The mixture was stirred at ambient temperature for 2 days until HPLC showed formation of nitrile and loss

of amide. The mixture was poured into 50 ml of ice water and stirred for 6 hours. The product was extracted with 2 x 50-ml portions of chloroform. The aqueous layer was then warmed on a steam bath for 20 min and then extracted for 2 days with chloroform in a continuous liquid-liquid extractor. The combined chloroform extracts were washed with saturated sodium chloride solution, dried over sodium sulfate, filtered and evaporated to dryness. The residue was chromatographed on silica gel with 5% methanol in chloroform to give 120 mg (33%) ^{13}C NMR C-2 151.9 (d) $J_{C,N}$ 22.0 Hz; IR 2220cm^{-1} (CN).

The 3-^{15}N-cyano compound 8b was similarly prepared from 480 mg (1.52 mmoles) of 7b by reaction with 1.5 ml of phosphorylchloride as above to give after chromatography 100 mg (27%) of product; ^{13}C NMR CN 115.0 (d) $J_{C,N}$ 17.5 Hz; IR 2200 cm^{-1} (CN).

Methyl 4-Amino-4-desoxy-N_{10}-methylpteroate-1-^{15}N (9a)

To a methanolic solution of guanidine [prepared from 210 mg (2.2 mmole) guanidine hydrochloride plus 119 mg (2.2 mmole) of sodium methoxide in 50 ml of MeOH)] was added 110 mg (0.37 mmole) of the 2-$^{15}NH_2$-3-cyanopyrazine ester (8a). The reaction mixture was stirred at reflux for 15 hours. The solution was neutralized to pH 6-7 with acetic acid and then evaporated to dryness. The residue was extracted with 10 ml of hot methanol. The methanol was evaporated and the residue chromatographed on silica gel with 5% methanol in chloroform to afford 75 mg (59%) of product. ^{13}C NMR C-2 163.0 (d), C-7 149.1, C-8a 152.2 (d).

Methyl 4-^{15}N-Amino-4-desoxy-N_{10}-methylpteroate (9b)

The 3-^{15}N-cyanopyrazine ester (8b, 90 mg, 0.302 mmole) was treated with guanidine as above to give after chromatography 85 mg 82% of product identical to 9a by HPLC; ^{13}C-NMR C-4 162.2 (d) $J_{C,N}$ 20.6 Hz.

4-Amino-4-desoxy-N_{10}-methylpteroic-1-^{15}N Acid (10a)

The amino pteroate ester 9a (70 mg, 0.206 mmole) in 10 ml of 2-methoxyethanol-water (1/1) containing 35 mg (0.88 mmole) of sodium hydroxide was stirred for 3 days at room temperature. The solution was neutralized with acetic acid to pH 7 and evaporated to dryness. The residue was extracted with 40 ml of 20% methanol in chloroform. The mixture was filtered and evaporated

to dryness to yield 65 mg (97%) of yellow solid. MS, m/e 326. HPLC identical to authentic material.

4-^{15}N-Amino-4-desoxy-N$_{10}$-methylpteroic Acid (10b)

The 4-^{15}N-amino pteroate ester (9b, 80 mg, 0.250 mmole) was saponified as above to give 72 mg (89%) of the amino pteroic acid; MS m/e, 326; HPLC identical to 10a.

Methotrexate-1-^{15}N Diethyl Ester (11a)

To 60 mg (0.184 mmoles) of the 1-^{15}N-pteroic acid compound (10a) in 8 ml of dimethylformamide under argon was added 55 µl (.396 mmole) of triethylamine followed by 26 µl (0.198 mmole) of isobutyl chloroformate. The mixture was stirred for 45 min, followed by addition of a mixture of 48 mg (0.200 mmole) of diethyl L-glutamate hydrochloride in 2 ml dimethylformamide plus 28 µl (.208 mmole) of triethylamine. After stirring for 6 hours a second addition of 28 µl of triethylamine and 26 µl of isobutyl chloroformate was made followed by 48 mg dimethyl L-glutamate hydrochloride plus 29 µl triethylamine in 2 ml dimethylformamide. The mixture was stirred for 18 hours. This was followed by a third addition of the above reactants. The mixture was stirred for an additional 6 hours. The reaction mixture was evaporated to dryness and the residue was dissolved in 50 ml of chloroform. The chloroform was washed with 10 ml of sat'd sodium bicarbonate and 25 ml sat'd sodium chloride solution. The chloroform was dried over sodium sulfate, filtered and evaporated to dryness. The residue was chromatographed on silica gel and eluted with 5% methanol in chloroform to afford 27 mg of product (29%); ^{13}C NMR (CD_3OD) C-2 162.7 (d).

Methotrexate-4-$^{15}NH_2$ Diethyl Ester (11b)

The 4-$^{15}NH_2$-pteroic acid 10b (70 mg, .206 mmole) was coupled with diethyl L-glutamate as above to give 25 mg (22%) of product. ^{13}C NMR (DMSO-d_6) C-4 165.0 (d) $J_{C,N}$ 21.1 Hz.

Methotrexate-1-^{15}N (12a)

To 25 mg (0.049 mmole) of the diethyl ester (11a) in 10 ml of tetrahydrofuran under argon was added 2.5 ml of 0.1 N sodium hydroxide. The solution was stirred for 4 hours, neutralized to pH 7 with 1N hydrochloric acid and

evaporated to dryness. The residue was dissolved in 1 ml of boiling water. The solution was chilled and the resulting precipitate was collected by centrifuge. The solid was treated with 0.3 ml of water, chilled and centrifuged to give 12 mg (54%); M.S. as tetra TMS derivative = 743. UV 0.1 N NaOH 372, 303, 259. HPLC showed identical retention time to authentic methotrexate.

[4-$^{15}NH_2$]Methotrexate-4-$^{15}NH_2$ (12b)

The diethyl ester (11b, 23 mg, 0.045 mmole) was saponified as above to afford 9 mg (44%); M.S. as tetra TMS derivative = 743; UV 0.1 N NaOH 372, 303, 259; HPLC identical to 12a and authentic methotrexate.

^{15}N-NMR values for 12a and 12b respectively were -180.2 ppm and -293.7 ppm (J_{N-H} 88 ± 3 ppm). Spectra were obtained in d_6-DMSO at 308°K, conc. 3.0 mM for 12a and 302°K, conc. 3.3 mM for 12b. ^{15}N chemical shifts are referenced to neat $CH_3{}^{15}NO_2$ and are positive when downfield of the reference signal. The ^{15}N shifts were determined with respect to an external $^{15}NH_4NO_3$ sample and then calculated to refer to neat nitromethane by the method of Witanowski, et al.[6]

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References

1. Taylor, E. C., Portnoy, R. C., Hochstetler, D. C., and Kobayashi, T. J. J. Org. Chem. 40, 2347 (1975).
2. Parker, C. O. Tetrahedron 17, 109 (1962).
3. Taylor, E. C. and Jacobi, P. A. J. Am. Chem. Soc. 98, 2301 (1976).
4. Taylor, E. C. and Portnoy, R. C. J. Org. Chem. 38, 807 (1973).
5. Stadelli, W., von Philipsborn, W., Wick, A., and Kompis, I. Helv. Chim. Acta 63, 504 (1980).
6. Witanowski, M., Stefoniak, L., Szymanski, S., and Januszewki, H. J. Magn. Reson. 28, 217 (1977).