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Research Article

Synthesis of [C^2H_3]methotrexate and [C^2H_3]7-hydroxymethotrexate

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Summary

Stable label analogues of methotrexate (MTX) and 7-hydroxymethotrexate (7-OH-MTX) were required for use as internal standards for LC/MS quantitation. A minimum incorporation of stable isotopes to produce a mass increase of 3 in the non-glutamate derived portion of the molecule was necessary for adequate MS detection. The commercial availability of desmethyl MTX (aminopterin, **1**) made methylation with C^2H_3I an attractive option. Surprisingly, all attempted methylations of **1** and the dimethyl ester of **1** failed to provide a significant amount of the methylated aniline, apparently due to attenuated reactivity of the secondary amine towards alkylation. However, reductive amination of diacid **1** with C^2H_2O and NaB^2H_3CN gave [C^2H_3]MTX in 52% yield. A previously reported method was utilized to convert [C^2H_3]MTX to [C^2H_3]7-OH-MTX. Preparative HPLC purification of [C^2H_3]7-OH-MTX resulted in extremely low recovery from the column; this was resolved by switching to a column with few free silanols.

Key Words: methotrexate; 7-hydroxymethotrexate; rofecoxib; cyclooxygenase II; reductive amination

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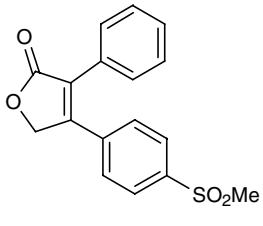
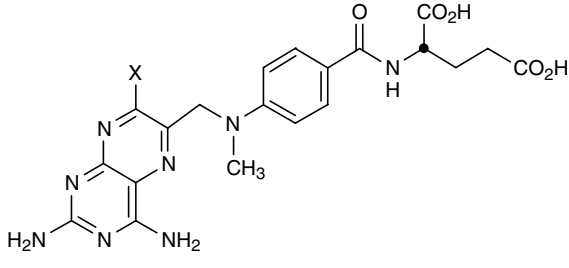
Introduction

Methotrexate (MTX) is a drug with several clinical applications including treatment of rheumatoid arthritis[1] which could lead to co-administration of MTX and cyclooxygenase II (COX II) selective inhibitors such as rofecoxib (Vioxx). Severe toxic effects have been observed when MTX and several non-steroidal antiinflammatory drugs (NSAIDs) are co-administered.[2] Two hypotheses which attempt to explain this observation are: (1) competition for renal clearance between the NSAID and MTX and (2) cyclooxygenase I (COX I) induced suppression of renal clearance;[3] both hypotheses would lead to a longer half-life for MTX. To probe whether rofecoxib displays the same drug–drug interaction as the NSAIDS tested, a LC/MS internal standard of MTX and its primary metabolite 7-hydroxymethotrexate (7-OH-MTX) were required for which stable isotope labeled versions of each were deemed ideal.



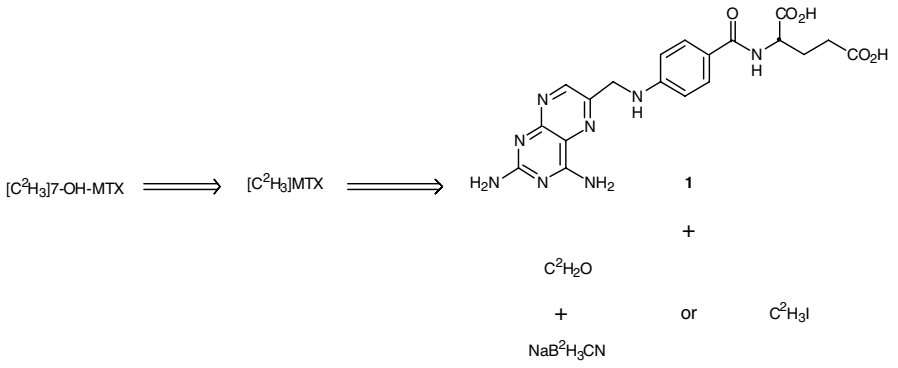
Rofecoxib



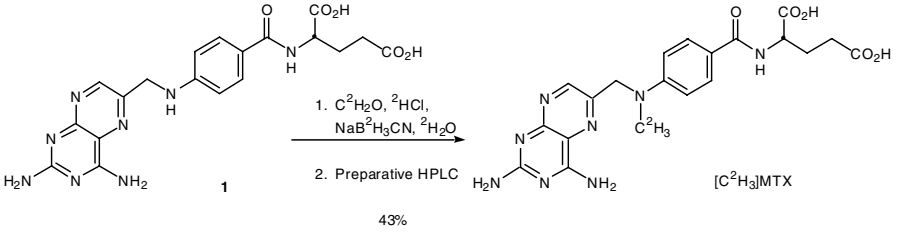
X = H Methotrexate (MTX)
= OH 7-Hydroxymethotrexate (7-OH-MTX)

Results and discussion

The syntheses of stable isotope labeled MTX with C-13 labels in the pteridine ring has been reported by Cheung.[4] However, the conversion of commercially available aminopterin (**1**) to [C^2H_3]MTX was a less complex route to the target and would provide a mass increase of 3 which was the minimum enrichment required for adequate discrimination from unlabeled drug by MS. The synthesis of 7-OH-MTX from MTX has been reported by Dawson *et al.*;[5] therefore, a successful synthesis of [C^2H_3]MTX would provide access to both compounds. Labeled *N*-methylation of the aniline nitrogen of aminopterin could be conducted using C^2H_3I or by reductive amination with C^2H_2O/NaB^2H_3CN (Scheme 1).



Scheme 1. Retrosynthetic analysis of [C^2H_3]7-OH-MTX and [C^2H_3]MTX



Scheme 2. Synthesis of [C^2H_3]MTX

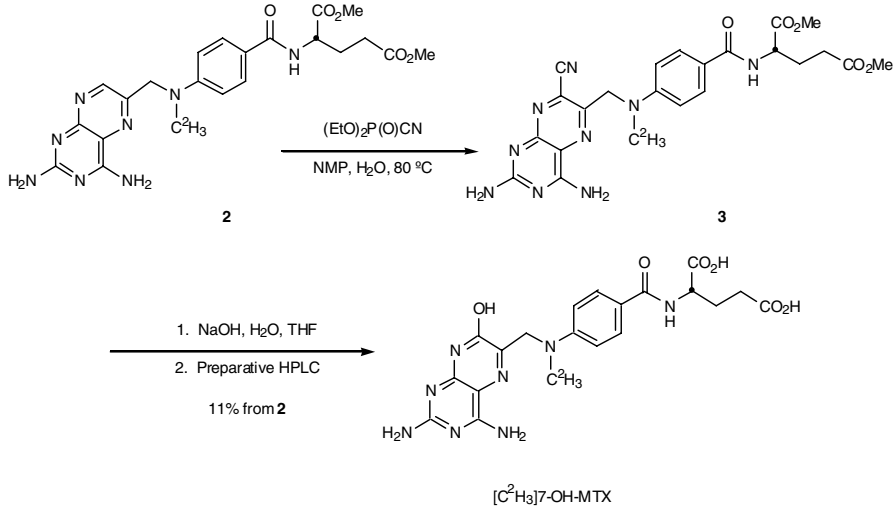
Reaction of aminopterin (**1**) with C^2H_3I was first explored. Unfortunately, repeated attempts under several different reaction conditions always produced the desired methylated product in less than 10% yield. Several other major by-products were formed which had mass spectra consistent with mono-methylation at an alternative site and dimethylation.

Reductive amination of aminopterin (**1**) with C^2H_2O and NaB^2H_3CN in ^2H_2O gave a 52% yield of [C^2H_3]MTX along with a 35% impurity which had the same M + H/Z ratio by LC/MS as [C^2H_3]MTX (Scheme 2). Sparging the reaction mixture with N_2 prior to reaction and maintaining the pH of the solution between 6 and 7 as the reaction progressed were critical to the success of the reaction. A portion of the reaction mixture was purified by preparative HPLC to give [C^2H_3]MTX in moderate yield and 99% purity.

For the preparation of labeled 7-OH-MTX, crude [C^2H_3]MTX was esterified using boron trifluoride in methanol,[5] and the reaction mixture

purified by column chromatography to give diester **2** in 75% yield and 88% purity. Esterification of **1** followed by reductive amination also provided **2**, but the reaction was heterogenous and had considerable run-to-run variability. As reported previously,[5] reaction of **2** with diethyl cyanophosphonate gave nitrile **3,** which was converted to [C^2H_3]7-OH-MTX by reaction with aqueous sodium hydroxide (Scheme 3).

Precipitation of 7-OH-MTX from the reaction solution by adjusting the pH to 3.5 with AcOH provided material with 93% purity, which was not sufficient for our purposes. An initial probe of the preparative HPLC purification (Zorbax RX C8, MeCN–0.1% TFA) resulted in low recovery; this result was confirmed with unlabeled reference 7-OH-MTX, which gave a 15% recovery after preparative HPLC. Surprisingly, attempted isolation of the reference compound by SepPak (ODS-AM C18) resulted in a further 50% loss of material. The loss of material on the ODS-AM C18 SepPak and the Zorbax C8 column could be the result of strong binding of 7-OH-MTX to uncapped silanol sites on the reversed-phase packing. Analysis of several other columns showed improved recovery from Hamilton PRP-1 polymer-based column and the highly capped Waters Xterra RP_{18} column (60–90%). Use of a polymer-based SepPak (Waters Oasis^{TM}HLB) resulted in quantitative recovery of reference 7-OH-MTX. This information was then applied to the purification of [C^2H_3]7-OH-MTX by iterative preparative HPLC on



Scheme 3. Synthesis of [C^2H_3]7-OH-MTX from diester 2

a Waters Xterra RP_{18} column followed by isolation using a Waters Oasis^{TM}HLB SepPak, and after lyophilization, provided 21 mg of [C^2H_3]7-OH-MTX as a yellow solid (11% yield from **2**, 50% recovery). HPLC analysis showed the compound to be 99% pure and LC/MS showed 93% $^2H_3$, 7% $^2H_2$.

Experimental

General: Aminopterin, methotrexate, and 7-hydroxymethotrexate were obtained from Sigma. NaB^2H_3CN (96% ^2H), 20% C^2H_2O in ^2H_2O (98% ^2H), 20% ^2HCl in ^2H_2O (99.5% ^2H), 40% NaO^2H in ^2H_2O (99.9% ^2H) were obtained from Aldrich, and ^2H_2O (99.9% ^2H) was obtained from Isotec. Anhydrous solvents were obtained from Aldrich and were dried over 4 Å molecular sieves for at least 24 h prior to use. Analytical HPLC was performed using a Shimadzu HPLC system with LC-10ATVP pumps, SPD-10AVP UV detector, CTO-10ASVP column oven heated to 30°C, and a SCL-10A controller. ^1H NMR spectra were recorded on a Varian U-400 spectrometer and are referenced to the residual solvent peak (2.5 for $^2H_5$-DMSO). LC/MS data was acquired using an HP MSD-100 with electrospray ionization. The final products were identified by HPLC comparison with the commercially available material using either method A (12% MeCN–0.1% trifluoroacetic acid on a Zorbax RX C-18 column); method B (15% MeCN–0.1% trifluoroacetic acid on a Zorbax RX C-18 column); method C (5–40% MeCN–0.1% trifluoacetic acid over 30 min, Waters Xterra C-8); or method D (20:10:70 MeCN:MeOH:0.1% $HClO_4$, Discovery Amide RP-C16). All HPLC analyses were conducted with the column heated to 30°C and concluded with a 10 min wash of 100% MeCN. Purity is reported as UV area % (wavelength).

[C^2H_3]Methotrexate: A suspension of 1.0 g (2.1 mmol) of aminopterin in 100 ml of ^2H_2O was sparged with N_2 for 30 min at rt, and the pH was adjusted to 9 with 40% NaO^2H in ^2H_2O to give a homogenous solution. The pH of the reaction mixture was adjusted to 6.5 with 1 M ^2HCl in ^2H_2O, and 3.36 ml (21.0 mmol) of a 20% solution of C^2H_2O in ^2H_2O was added. The reaction mixture was sparged for 10 min with N_2, and 208 mg (3.16 mmol) of NaB^2H_3CN was then added. The pH was closely monitored for the next 1 h and was kept in the range of 6–7 by addition of 40% NaO^2H in ^2H_2O. HPLC assay (method A) at 1 h showed no starting material remaining so the pH of the solution was adjusted to 3.5

with aqueous HCl and the yellow solid was isolated by filtration to give 971 mg (58%) with a 60% purity (254 nm). Purification of 88 mg was effected by preparative HPLC (21.2 × 250 mm Zorbax RX C-18, 9% MeCN–0.1% TFA, 20 ml/min) in four batches. The fractions containing product were combined, and the pH of the solution adjusted to 3.6 with aqueous NaOH. The MeCN was removed at reduced pressure and the solution was lyophilized to give 1.1 g of a yellow solid. The solid was dissolved in a minimum of water and was passed through a Waters C-18 SepPak (300 mg, 3 cm^3). The SepPak was rinsed with 5 ml of water, and the product was eluted with MeOH. The organic solution was dried ($MgSO_4$), filtered and concentrated to dryness. The solid was then taken up in 2 ml of water and the pH of the solution was adjusted to 3.6. After 14 h at 4°C the solution was filtered to give 39 mg of [C^2H_3]MTX as a yellow solid (analysis by HPLC (method D)) 99.7% (216 nm), 98.7 (242 nm), 99.0 (306 nm). MS ($M + H^+$/Z, abundance): 458 (92.3%), 457 (7.1%) ^{1}H NMR (400 MHz, $^2H_6$-DMSO) δ 8.67 (s, 1H), 7.71(d, 2H, $J = 8.8$ Hz), 6.78 (d, 2H, $J = 8.8$ Hz), 4.82 (s, 2H), 4.32 (*m*, 1H), 2.29 (*t*, 2H, $J = 7.2$ Hz), 2.02 (*m*, 1H), 1.90 (*m*, 1H).

*Dimethyl ester of [C^2H_3]methotrexate (***2***):* A suspension of 875 mg (1.91 mmol) of [C^2H_3]MTX (60% purity (254 nm)) in 19 ml of MeOH was stirred at rt as 2.10 g (14.7 mmol) of $BF_3 \cdot OEt_2$ was added in two portions, and the reaction mixture was stirred overnight. HPLC analysis (method B) showed no remaining diacid so the mixture was diluted in 300 ml of $CHCl_3$ and washed with 3 × 100 ml of $NaHCO_3$ and 60 ml of saturated aqueous NaCl. The organic solution was dried ($MgSO_4$), filtered, and concentrated to afford 1.69 g of an orange oil. A portion of the sample (1.5 g) was purified by flash column chromatography on silica (5% MeOH–$CHCl_3$) to give 660 mg of a light yellow solid (88.5% purity at 254 nm, 75% yield). ^{1}H NMR (400 MHz, $^2H_6$-DMSO) δ 8.54 (s, 1H), 7.68 (d, 2H, $J = 8.9$ Hz), 6.79 (d, 2H, $J = 8.9$ Hz), 4.75 (s, 2H), 4.36 (*m*, 1H), 3.57 (s, 3H), 3.54 (s, 3H), 2.39 (*t*, 2H, $J = 7.4$), 2.04 (*m*, 1H), 1.94 (*m*, 1H).

*Dimethyl ester of [C^2H_3]7-cyanomethotrexate (***3***):* The following procedure is a modification of that reported by Dawson.[5] A solution of 200 mg (0.41 mmol, 89% UV purity at 254 nm) of diester **2** in 2.9 ml of *N*-methylpyrrolidinone was sparged for 10 min with N_2 then 1.21 g (12.0 mmol) of triethylamine, 1.0 ml (6.2 mmol) of diethyl cyanophosphonate (DEPC), and 530 µl of water were added sequentially to the solution at 0°C. The mixture was stirred for 10 min at 0°C and 2.5 h at 80°C. HPLC (method C) showed a 2:1 ratio of **2**:**3** so 1.21 g (12.0 mmol)

of triethylamine, 1.0 ml (6.2 mmol) of DEPC, and 530 µl of water were added and the heating was continued. After another 1.5 h, HPLC analysis (method C) showed a 1:11 ratio of **2**:**3**; therefore, 100 ml of EtOAc was added and the solution was washed with 50 ml of $NaHCO_3$ and 2×50 ml of saturated NaCl. The solution was dried (Na_2SO_4), filtered and concentrated to give 229 mg of a brown oil (55.8% purity at 254 nm).

[C^2H_3]7-Hydroxymethotrexate: The following procedure is a modification of that reported by Dawson.[5] A solution of [C^2H_3]7-cyanomethotrexate in 20 ml of THF and 5 ml of water was cooled to 0°C and 2.4 ml of 1 M NaOH was added. The reaction mixture was stirred for 3 h at which time HPLC analysis (method C) showed no remaining starting material. The THF was removed at reduced pressure, and the pH of the solution was adjusted to 6.5 with HCl. The remaining water was removed at reduced pressure (no heating) to give 690 mg of a red oil. Purification was effected in 15 batches using a Gilson Automated Preparative HPLC system (19×300 mm Waters Xterra RP_{18}, 11% MeCN–0.1% TFA, 20 ml/min, sample loaded in 10% MeCN–1% TFA). The fractions containing product were combined, and the MeCN was removed at reduced pressure. The solution was loaded onto a Waters Oasis™HLB SepPak (500 mg, 6 ml) and was washed with water and MeOH, then the product eluted with 50% aqueous MeCN. The solution was lyophilized to give 21 mg (11% yield from **2**) of a yellow solid [HPLC (method E) purity 97.8% (202 nm), 98.0 (220 nm), 96.9 (242 nm), 98.9 (306 nm)]. MS ($M + H^+/Z$, abundance): 474 90.3%, 473 (9.7%) ^1H NMR ($^2H_6$-DMSO) δ 7.69 (d, 2H, $J = 8.5$ Hz), 6.79 (d, 2H, $J = 8.5$ Hz), 4.54 (s, 2H), 4.33 (dd, 1H, $J = 5.0$, 9.5 Hz), 2.31 (*t*, 2H, $J = 7.5$ Hz), 2.03 (*m*, 1H), 1.91 (*m*, 1H).

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