Catalytic Reduction in the Synthesis of Tritiated (6R) and (6S) 5,10-Dideaza-5,6,7,8-tetrahydrofolate

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Summary

[^{3}H] 5,10-dideaza-5,6,7,8-tetrahydrofolate, DDATHF, was obtained by a two step process. First, 2n[^3H_2O] was produced in the reaction flask containing m[^{1}HAc] by the reduction of n[PtO_2] with $^3H_2$, so that a 1[$^3H^+$]:18[$^3H^+$ + $^1H^+$] ratio of solvent protic species in theory resulted. Secondly, subsequently added diethyl-2-acetyl-5,10-dideaza-9,10-didehydrofolate was reduced in this tritium labeled solvent with the pre-reduced Adams catalyst and additional carrier free tritium gas which was introduced to a pressure of 722 torr. The resulting (6R) and (6S) [^{3}H] diastereomers were individually isolated. Each had a specific activity of 11.2 Ci/mmol, and the locations of the incorporated tritium atoms were completely determined by ^{1}H decoupled 320 MHz ^{3}H NMR by comparison with the 300 and 500 MHz ^{1}H NMR spectra of [^{1}H] (6S) DDATHF, [^{1}H] (6R) DDATHF, and [5-^{2}H (50% ^{2}H), 6-^{2}H (100% ^{2}H), 7-^{2}H (50% ^{2}H), 9-^{2}H (50% ^{2}H), 10-^{2}H (50% ^{2}H)] (6RS) DDATHF.

Key Words: 5,10-dideazatetrahydrofolic acid, DDATHF, folate analog, catalytic tritiation, ^{3}H-NMR.

Introduction

5,10-dideaza-5,6,7,8-tetrahydrofolic acid, DDATHF, whose structure is shown in fig. 1, was synthesized as a potential inhibitor of folate metabolism (1). It has since been found that the two diastereomers of DDATHF, 6R and 6S, are both potent inhibitors of de novo purine

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synthesis (2,3). The (6R) diastereomer, whose configuration is analogous to that of natural tetrahydrofolate (4), is currently undergoing phase I clinical trial as an anti-tumor agent. Development of a radioimmunoassay for analysis of clinical trial samples and development of

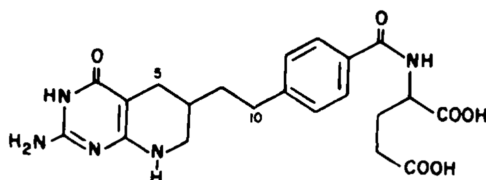

Figure 1. (6RS) DDATHF.

in vitro assays for cellular resistance, binding, and metabolism require a high specific activity tritium labeled form of (6R) DDATHF that is chemically and metabolically stable, has high purity, and has a minimum specific activity of 10-12 Ci/mmol. The current work describes the chemical synthesis of tritium labeled (6R) and (6S) 5,10-dideaza-5,6,7,8-tetrahydrofolic acid which meets these requirements.

Numerous methods are available for producing tritium labeled compounds (5,6). The method chosen was the catalytic reduction of the unsaturated intermediate diethyl 2-acetyl-5,10-dideaza-9,10-didehydrofolate with Adams catalyst and tritium gas to 5,10-dideaza-5,6,7,8-tetrahydrofolate as a mixture of diastereomers. The unique insolubility characteristics of this unsaturated intermediate limited the choice of solvent to glacial acetic acid.

Theory predicts that the incorporation of 1 isotopic atom for every 18 atoms during the reduction should result in the desired specific activity, 10 Ci/mmol, since each intermediate molecule has three double bonds which are being reduced during the reaction. Preliminary 76.7 MHz ^{2}H NMR studies using deuterium as the reducing agent indicated that extensive dilution of the isotopic label in the deuterium occurred because of exchange with the solvent's non-isotopic protic species. Therefore, we chose to generate tritium oxide in the reaction flask in an amount sufficient to make the ratio of isotopic protic species to total protic species in the

solvent 1:18, respectively, by the reduction of a calculated amount of Adams catalyst in a given quantity of glacial acetic acid with tritium gas.

Materials and Methods

Diethyl-2-acetyl-5,10-dideza-9,10-didehydrofolate

The unsaturated intermediate (1) was kindly provided by Dr. Chuan Shih, Lilly Research Laboratories, Indianapolis, IN.

HPLC Analysis

The (6S) and (6R) diastereomers of 5,10-dideaza-5,6,7,8-tetrahydrofolate were baseline separated on a cyclobond I column (ASTEC, Inc.) as described previously (3).

[^{3}H] (6R) and (6S) DDATHF

Adams catalyst (platinum oxide, Aldrich Chemical Co., #22,904-0, 62.5 mg, 0.25 mmol) was suspended in glacial acetic acid (0.3 ml, 5.25 mmol). The suspension was frozen with liquid nitrogen and degassed under reduced pressure ($3 \times N_2$). Carrier free $^3H_2$ was introduced to 725 torr at t=0. After 4 hours, more $^3H_2$ was added to bring the pressure back up to 720 torr whereupon the reduction reaction was left to proceed 14 more hours. After that time, the consumption of $^3H_2$ had reduced the pressure to 155 torr.

At this point more $^3H_2$ was introduced to 722 torr. The unsaturated intermediate (26.7 mg, 0.05 mmol) which was dissolved in glacial acetic acid (0.2 ml, 3.5 mmol) was injected into the reaction flask. The reduction reaction was allowed to proceed 18 more hours with no further addition of $^3H_2$.

The reduced catalyst was then separated by centrifugation and the acetic acid solvent removed by co-evaporation (in vacuo) with MeOH. The resulting MeOH solution was lyophilized to dryness and 3 ml of 1N NaOH quickly added to saponify the protecting groups. Concentrated glacial acetic acid was then added to pH = 7. The individual diastereomers were collected during $N \times 200$ μl injections of this solution onto a cyclobond I column (ASTEC, Inc.) as described previously (3).

The pooled solvents were evaporated (in vacuo) repeatedly with EtOH to remove residual

triethylammonium acetate. Thus, the (6R) and (6S) [^{3}H] DDATHF stock solutions consisted of the respective triethylammonium salts at neutral pH.

Determination of Specific Activity

The concentration of the stock solution was spectrophotometrically determined using dilution with 0.1N NaOH and an ϵ_{max} value of 11,700 m^{-1} cm^{-1} at 272 nm. The counting efficiency of the scintillation counter was found to be 51.3% and the dpm/ml of our stock solution was determined. A value of 11.2 Ci/mmol was calculated for the specific activity of each diastereomeric stock solution.

NMR Analyses

^{1}H and ^{3}H NMR spectra were recorded in DMSO-D$_6$, on an IBM AF-300 NMR spectrometer (^{3}H at 320 MHz, ^{1}H at 300 MHz), using a ^{3}H/^{1}H 5mm dual probe. Samples were made to a volume of 200μL in teflon tubes (Wilmad, #6005), which were then placed inside 5mm glass NMR tubes having a screw-cap (Wilmad, 507-TR-8"). A high quality ^{3}H band stop-^{1}H band pass filter (Cir-Q-Tel Inc., FBT/20-300/3-6/50-3A/3A) was placed in the proton decoupling line of the instrument, and the observed channel had an in-line ^{1}H band stop-^{3}H band pass filter. Tritium and proton spectra were acquired over approximately 12ppm, using a 5s total recycle time and excitation pulses of 3.6μs (^{3}H, 65°) and 4μs (^{1}H, 50°). All spectra were acquired at 297K with the sample spinning. Referencing of tritium chemical shifts was achieved by generation of a ghost ^{3}H TMS signal from internal TMS in the ^{1}H NMR spectrum (7).

^{1}H COSY Experiments

Proton COSY NMR experiments (8,9) were acquired at a frequency of 300 MHz and temperature of 297K, using a pulse width of 8.1μs (^{1}H, 90°), and acquiring 256 t_1 points using standard Bruker software. Each spectrum had 2K data points, a sweep width of 3 kHz (F1 sweep width 1.5 kHz), total recycle time of 2s, with 32 or 48 scans per spectrum. The data were treated with sine-bell window functions prior to Fourier transformation, and the F1 dimension was zero filled from 256 points to 1K, to yield a 2K × 1K matrix with magnitude intensity calculation.

Results and Discussion

Catalytic reduction of the unsaturated intermediate diethyl 2-acetyl-5,10-dideaza-9,10-didehydrofolate with Adams catalyst and tritium gas to 5,10-dideaza-5,6,7,8-tetrahydrofolate as a mixture of diastereomers occurred in only 12% overall yield for each diastereomer. The low yield of tritiated diastereomers is attributed to the fact that no additional tritium was introduced into the reaction vessel during the course of the reduction reaction. The average pressure inside the reaction vessel was therefore lower than 722 torr. As a result, the concentration of tritium on the catalyst was non-ideal.

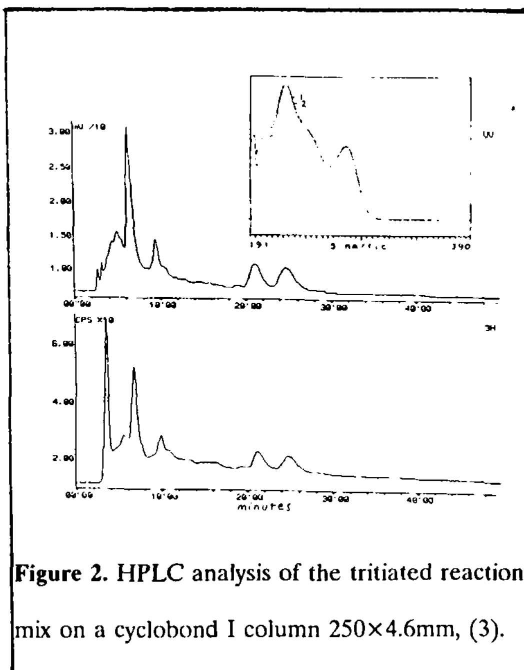

Figure 2. HPLC analysis of the tritiated reaction mix on a cyclobond I column 250×4.6mm, (3).

The effect of pressure on the rate at which the reduction goes to completion is apparent from the fact that it takes three hours at three atmospheres (11) and sixteen hours at one atmosphere.

The identity of the two late emerging peaks (fig. 2) were confirmed to be the reduced products (6S) DDATHF and (6R) DDATHF in a nearly 1:1 ratio, respectively, by 1) comparing their UV spectra with each other and with that of standard (6R) and (6S) DDATHF and finding them to be identical; 2) comparing the HPLC profile with and without coinjection of the tritiated reaction mix with a standard 1:1 mix of (6S) and (6R) DDATHF and finding that co-elution of the diastereomers occurred; 3) comparing their 320 MHz ^{3}H NMR spectra with each other and with the 300 MHz ^{1}H NMR spectra of standard (6R) and (6S) DDATHF and finding a 1:1 correspondence between the [^{3}H] and [^{1}H] resonances attributable to the saturated portion of the DDATHF structure.

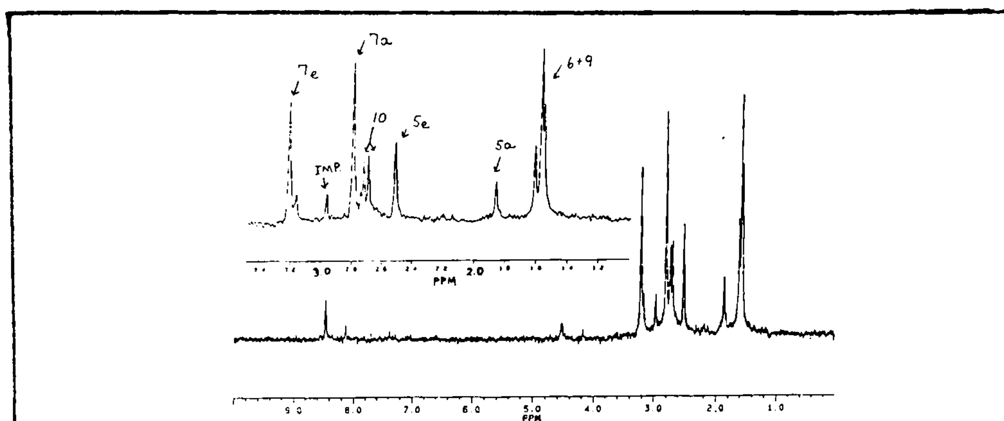

Figure 3. 320 MHz ^{3}H NMR of [^{3}H] (6S) DDATHF in DMSO-d. (Inset: 3.5 → 1 ppm)

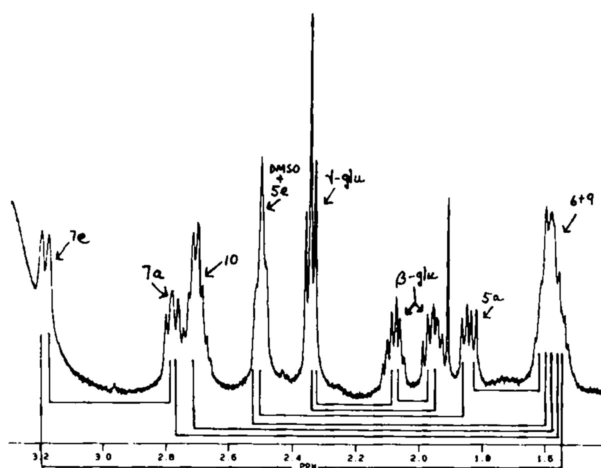

Figure 4. 500 MHz ^{1}H NMR spectrum in DMSO-d of (6R) DDATHF. 300 MHz COSY results are represented as scorings under the appropriate resonances.

In addition, it should be noted that both the non-isotopic and the isotopic products of catalytic reduction using Adams catalyst are true diastereomers. This conclusion was reached after examining the ^{3}H NMR and the ^{1}H NMR spectra of the isotopic and non-isotopic products. The ^{3}H NMR spectrum of (6S) [^{3}H] DDATHF was virtually identical to that of (6R) [^{3}H] DDATHF. Likewise, the ^{1}H NMR spectrum of (6S) [^{1}H] DDATHF was virtually identical to that of (6R) [^{1}H] DDATHF. Since the tritium and proton resonances reflect the tritium and proton atoms at C(5), C(6), C(7), C(9), and C(10), the 5-deaza-5,6,7,8-tetrahydro portion of (6S)

DDATHF has to be the enantiomer of the 5-deaza-5,6,7,8-tetrahydro portion of (6R) DDATHF. The addition of a chiral glutamic acid structure to the molecule results in the production of a diastereomer.

The assignment of the [^{3}H] resonances in fig. 3 for (6R) = (6S) [^{3}H] DDATHF in DMSO was based upon the assignment of the [^{1}H] resonances in fig. 4 for (6R)=(6S) [^{1}H] DDATHF. The small amount of radioactive impurity indicated in fig. 3 was subsequently removed by ion-pair HPLC (10).

1. The assignment of the γ and β protons of L-glutamic acid (fig. 4) was readily made based upon the self contained nature of their COSY correlations and the difference spectrum: DDATHF (-glutamate) versus DDATHF (+glutamate), not shown.

2. Since the chiral C(6) proton should produce a ^{1}H NMR pattern consisting of a complex multiplet, the one C(6) proton was readily assigned (fig. 4) to the location of the absent complex multiplet in fig. 5 which represents the ^{1}H NMR spectrum of [5-^{2}H (50% ^{2}H), 6-^{2}H (100% ^{2}H), 7-^{2}H (50% ^{2}H), 9-^{2}H (50% ^{2}H), 10-^{2}H (50% ^{2}H) (6RS) DDATHF, also called (6RS) DDATHF (50% ^{2}H).

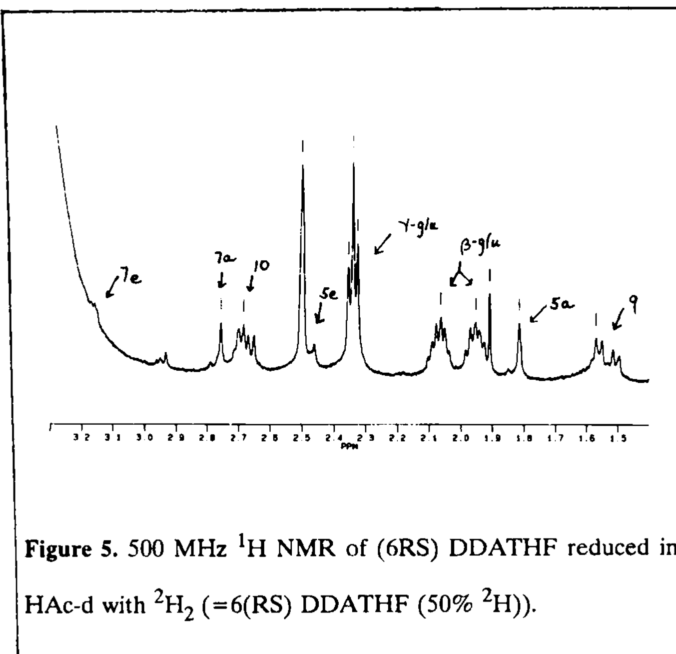

Figure 5. 500 MHz ^{1}H NMR of (6RS) DDATHF reduced in HAc-d with $^2H_2$ (=6(RS) DDATHF (50% ^{2}H)).

3. The assignment of the two C(9) protons (fig. 4) was readily made based on their chemical shift, which is charactaristic of solitary type alkyl residues, and their slight chemical shift difference, which is charactaristic of pro-chiral protons.

4. The 500 MHz ^{1}H NMR spectra (figs. 4 and 6B) were analyzed by subdivision into 3 regions: (a) H-7e, H-7a, H-6, (b) H-5e, H-5a, H-6, and (c) H-6, H-9, H-9', H-10, H-10'. Since

$\nu_{AB} | J_{AB} > 10$ prevailed in (a) and (b), first order methods were used to calculate Table 1.

5. The two C(5) and the two C(7) protons (fig. 4) were readily assigned from 1) the similarity of their ^2J coupling constants (table 1); 2) their COSY correlations (fig. 4); and 3) the collapse of the two doublets of doublets of equal ^2J value into respective singlets (compare figs. 5, 4, and 6B). That is, the value of $J_{5,5}$, in agreement with the theoretical predictions of Barfield and Grant (12), should be more

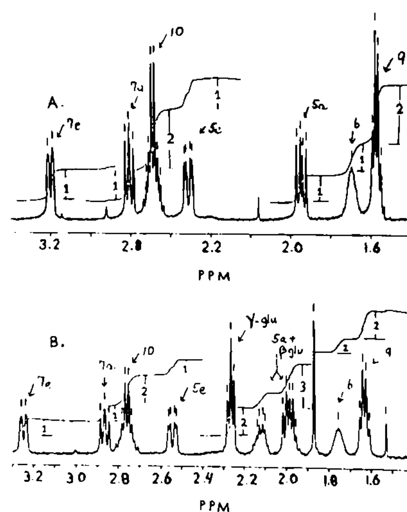

Figure 6. 500 MHz ^1H NMR spectra in 0.1N NaOD - D_2O. A. (6RS)DDATHF(-glutamate). B. (6R)DDATHF(+glutamate).

Table I. First Order Coupling Constants, Hz, for (6R) DDATHF

	$J_{5,5}$	$J_{5e,6a}$	$J_{5a,6a}$	$J_{7,7}$	$J_{7e,6a}$	$J_{7a,6a}$
DMSO	15.0	xxx	8.5	11.4	1.5	9.1
0.1N NaOH	15.2	4.8	xxx	11.5	1.5	9.1

Compare figures 5B and 6. (6S) DDATHF had equivalent ^2J and ^3J constants.

negative than $J_{7,7}$ because of the effect of the adjacent π-bonds. In addition, $J_{7,7}$ should be less negative than $J_{5,5}$ because of the increased electronegativity of the adjacent nitrogen atom (13). Finally, the collapse of the two doublets of doublets of equal J^2 value into respective singlets (fig. 5) is the result of 100% deuterium substitution at C(6) and 50% deuterium substitution at C(7) and C(5) in (6RS) DDATHF (50% ^2H).

6. The axial versus equatorial nature of the two C(5) and the two C(7) protons (fig. 4) were readily assigned from the values of their ^3J coupling constants according to the Karplus equation (14).

7. The assignment of the two C(10) protons (fig. 4) was readily made based on their chemical shift which is characteristic of benzylic type protons and their proton-proton correlations (COSY results in fig. 4) with the already assigned two C(9) protons.

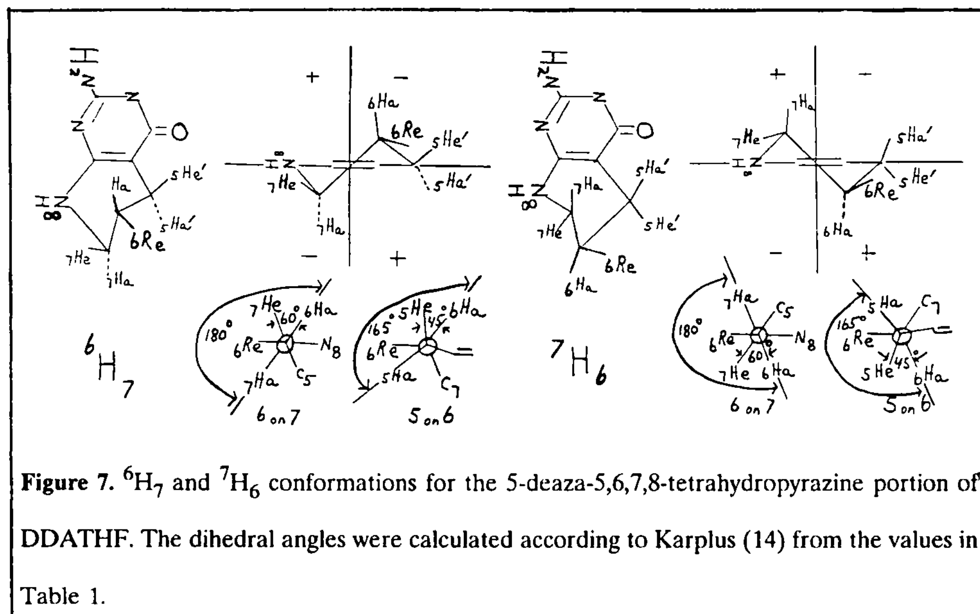

Figure 7. $^6H_7$ and $^7H_6$ conformations for the 5-deaza-5,6,7,8-tetrahydropyrazine portion of DDATHF. The dihedral angles were calculated according to Karplus (14) from the values in Table 1.

Theory predicts that the 5-deaza-5,6,7,8-tetrahydro portion of DDATHF can exist in either the half-chair conformation or the boat conformation. The coupling constants calculated in Table 1 verify a half-chair conformation with the C(6) proton assuming an axial position in the enantiomers. Thus, the $^7H_6$ conformer is the enantiomer of the $^6H_7$ conformer. When the L-glutamic acid portion of the molecule is added to each, the respective diastereomers result. These conformations are represented in fig. 7. Model building studies yielded dihedral angle projections which agreed with the calculated values.

Conclusion

The tritiation method reported here was designed to overcome a problem which became apparent during preliminary 76.7 MHz ^2H NMR studies of the catalytic reduction of diethyl 2-acetyl-5,10-dideaza-9,10-didehydrofolate using Adams catalyst, deuterium, and glacial acetic acid as solvent. The problem was extensive dilution of the isotopic label in the deuterium via exchange with the solvent's non-isotopic protic species. The extent of dilution occurred to the

extent that the acidic solvent was non-isotopically labeled. Therefore, by reducing a calculated amount of Adams catalyst in a given quantity of glacial acetic acid with tritium gas, it was theorized that sufficient tritium oxide could be produced in the solvent to make the ratio of isotopic: total protic solvent species 1:18, respectively.

This paper demonstrates that this procedure is capable of producing incorporation of 1 isotopic atom for every 18 atoms during the catalytic reduction of the three double bonds contained in the intermediate diethyl 2-acetyl-5,10-dideaza-9,10-didehydrofolate. Therefore, the method was capable of producing (6R) and (6S) [^{3}H] DDATHF with a specific activity of 11.2 Ci/mmole. In addition, labeling was almost exclusively at the metabolically stable positions C(5), C(6), C(7), C(9), C(10), and benzene of DDATHF. The only labeled position which has the potential to be enzymatically removed from the molecule is the H-C(2) glutamate position (fig. 3, 4.5 ppm). However, this position is labeled to $<0.1\%$. In vitro transport and metabolism studies should therefore not be misleading. In fact, the (6R) and (6S) [^{3}H] DDATHF diastereomers produced here have recently been used successfully in metabolic studies with human leukemic cell lines (15).

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