Synthesis of [4a-^{13}C]-6-Methyltetrahydropterin

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SUMMARY

The synthesis of [4a-^{13}C]-6-methyltetrahydropterin, a synthetic cofactor for phenylalanine hydroxylase, is described. [2-^{13}C]-ethyl bromoacetate was converted in four steps to [5-^{13}C]-2,4-diamino-6-hydroxypyrimidine in 86% yield. The latter was nitrosated, reduced, condensed with pyruvic aldehyde, and reduced to afford pure [4a-^{13}C]-6-methyltetrahydropterin in a net overall yield of 16%.

Key words: [4a-^{13}C]-6-Methyltetrahydropterin, ^{13}C NMR, pyrimidines.

INTRODUCTION

The enzymatic hydroxylation of aromatic amino acids requires tetrahydrobiopterin as a natural cofactor (1). Although the precise mechanism of oxygen activation remains unknown, there is evidence for a tetrahydropterin-derived intermediate, postulated to be the 4a-hydroxy adduct released from the enzyme, phenylalanine hydroxylase, after oxygen atom transfer (2). This adduct has been observed with both tetrahydrobiopterin and the synthetic cofactor 6-methyltetrahydropterin during the hydroxylation of L-phenylalanine to L-tyrosine by rat liver phenylalanine hydroxylase (3). Additionally it has been recently demonstrated that 1.0 mole of iron per 50,000 subunit molecular weight is required for maximal activity with a direct correlation between iron content and specific activity (4). Since neither the structure of the intermediate nor the role of the iron have been unequivocally established, it would be of interest to synthesize a tetrahydropterin containing a ^{13}C at the 4a position as a probe of the structure of the intermediate.

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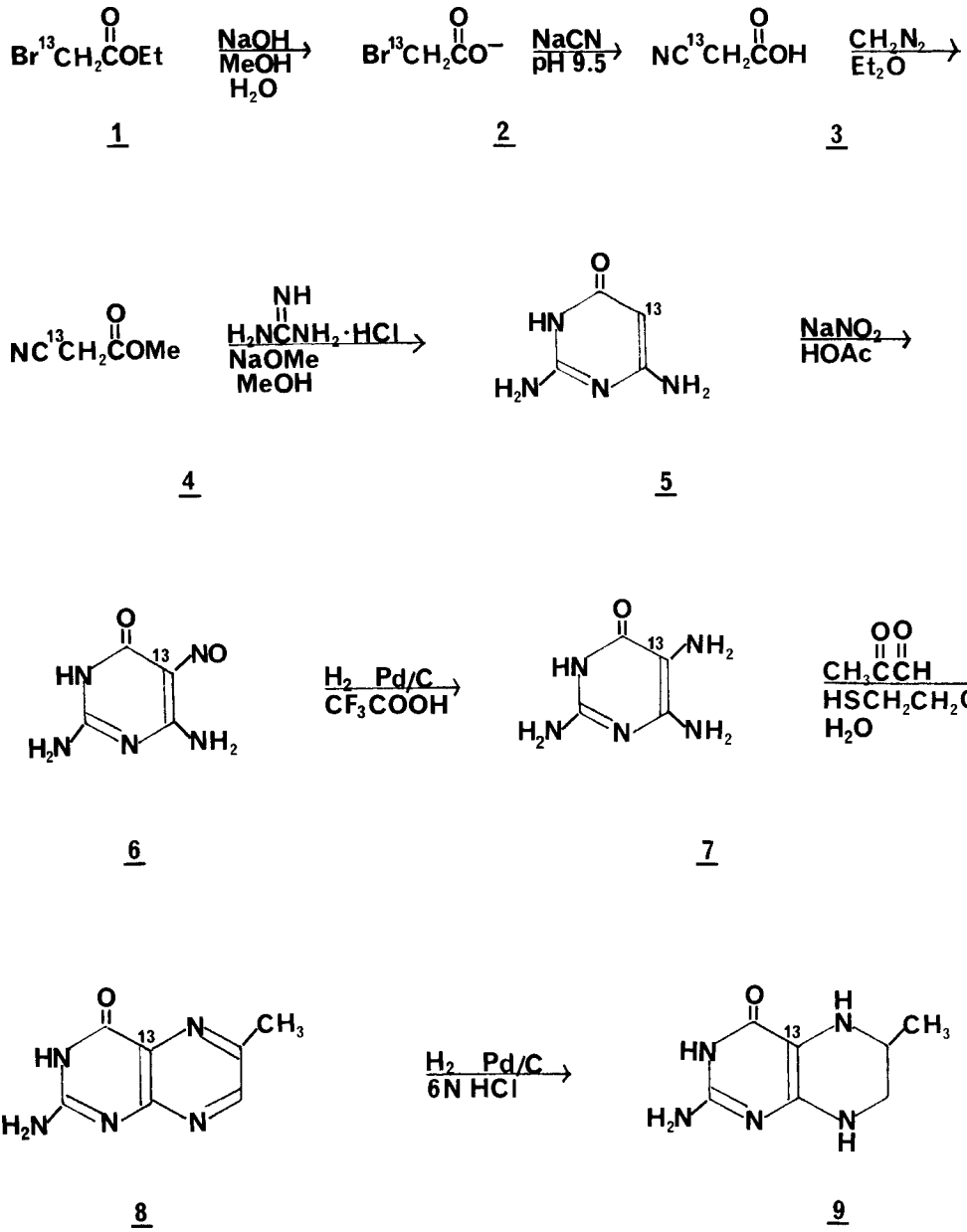
In addition the distance from the 4a position of the bound tetrahydropterin to the iron (Fe^{3+}) could be measured using ^{13}C NMR relaxation techniques (5). The synthesis of [4a-^{13}C]-6-methyltetrahydropterin, 9, for use in such studies is the subject of this report.

RESULTS AND DISCUSSION

The condensation reaction (6) of methyl glyoxal with 2,4,5-triamino-6-hydroxypyrimidine, 7, in the presence of 2-mercaptoethanol was chosen to accomplish the synthesis of the key precursor, 6-methylpterin, 8. Therefore the synthesis of 7 labelled with ^{13}C at the 5-position was required. This triaminopyrimidone was synthesized from 2,4-diamino-6-hydroxypyrimidine, 5, by nitrosation followed by reduction (7,8). Both 5 and 7 are useful intermediates for the synthesis of other labelled pyrimidine and pterin analogs which serve as substrates or inhibitors for phenylalanine hydroxylase (9,10).

The overall synthesis of 9 from [2-^{13}C]-ethyl bromoacetate, 1, is outlined in the Scheme. The ester was hydrolyzed by 1 equivalent of base, converted to 3 by NaCN (11), and reesterified with diazomethane to give 4 as a pale yellow liquid in 94% yield with a purity of 95% as monitored by GC. Condensation of 4 with guanidine yielded the pyrimidine 5 which was nitrosated to give the rose colored 5-nitrosopyrimidine 6 in an overall yield of 86% from 1. The latter was reduced to the triaminopyrimidone 7 by catalytic hydrogenation over Pd in CF_3COOH and immediately condensed with methyl glyoxal to give a mixture of 6- and 7-methylpterin in an overall yield of 72% from 6 and an isomer ratio of 65:35 in favor of the desired 6-isomer as indicated by UV and ^1H NMR. The isomers were separated by fractional crystallization from 1 N NaOH-EtOH to give, after neutralization of the sodium salt, 8, in 30% net yield. The pterin

SCHEME


Br13CH2COEt
NaOH MeOH H2O
Br13CH2CO−
NaCN pH 9.5
NC13CH2COH
CH2N2 Et2O
1
2
3
NC13CH2COMe
NH
H2NCNH2·HCl
NaOMe
MeOH
NaNO2 HOAc
4
5
NO
H2 Pd/C CF3COOH
NH2
CH3CCH
HSCH2CH2OH
H2O
6
7
CH3
6N HCl
8
9

was reduced to the tetrahydropterin by catalytic hydrogenation over Pd in 6N HCl (3) to give 9 with an overall yield of 16% from 1.

EXPERIMENTAL

UV spectra were recorded on a Cary 118 instrument. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker WM360 spectrometer. [2-^{13}C]-ethyl bromoacetate (90% ^{13}C enriched) was purchased from Merck, Sharpe, and Dohme. All other chemicals were of the highest commercial grade and used without further purification.

[2-^{13}C]-Methyl Cyanoacetate (4). To a solution of 0.67 ml (6 mmol) [2-^{13}C]-ethyl bromoacetate in 2 ml MeOH and 1 ml H_2O was added dropwise over a period of 5 minutes 2 ml of 3 N NaOH (6 mmol). The neutral solution was taken to pH 9.5 with Na_2CO_3 and stirred for 15 minutes. A solution of 0.33 g (6.7 mmol) NaCN in 1 ml H_2O was added. The solution was stirred for 10 minutes at 25°C, then 40 minutes at 60°C, cooled, acdified with ca. 1 ml 6 N HCl to pH 0, and rotary evaporated under high vacuum to give a white solid. A solution of diazomethane in Et_2O prepared from Diazald (12) was added slowly until a yellow color persisted (ca. 60 ml). The mixture was stirred vigorously under a stream of N_2 to remove the excess diazomethane, dried over Na_2SO_4, filtered, and rotary evaporated to give 0.56 g of 4 as a pale yellow liquid (94%). Gas chromatography (Varian Model 3700 using a 2000 x 2 mm glass column with 3% OV-225 on Chromosorb W HP 80/100 at 120°C) indicated ca. 95% purity.

[5-^{13}C]-2,4-Diamino-6-hydroxy-5-nitrosopyrimidine (6). The yellow liquid (4) was dissolved in 3 ml MeOH and 0.58 g (6.1 mmol) guanidine·HCl added. This solution was added dropwise to a solution of refluxing Na (0.276 g, 12 mmol) in 5 ml of MeOH and refluxed for 11 h. The cooled solution was acidified to

pH 6 with glacial acetic acid and chilled for 30 minutes. The contents were filtered and the filtrate rotary evaporated to dryness. The off white powder was washed with H_2O, EtOH, and Et_2O and dried to give 476 mg (3.30 mmol) of off white powder (5) which was identical to 2,4-diamino-6-hydroxypyrimidine purchased from Aldrich (ε_{263} = 20,200 in 0.1 N HCl). The filtrate was made basic with 3 N NaOH and 300 mg (4.35 mmol) $NaNO_2$ was added. The pale yellow solution was filtered through a glass wool plug into 5 ml of cold glacial acetic acid in a centrifuge tube. The pink precipitate that formed immediately was centrifuged and washed with H_2O, EtOH, and Et_2O to give 284 mg (1.83 mmol) of 6 as a pink powder. The overall yield of 5 and 6 was 86%. The conversion of 5 to 6 was quantitative (3).

[4a-^{13}C]-6-Methylpterin (8). A solution of 155 mg (1 mmol) 6 in 2 ml of trifluoroacetic acid was hydrogenated 30 minutes over 10 mg 10% Pd/C in a Parr apparatus. The clear solution was filtered through prewashed Celite under argon. The brown oil 7 obtained after reducing the volume under argon was taken up in 1 ml H_2O, 0.05 ml 2-mercaptoethanol added, and then $NaHCO_3$ to neutrality. A solution of 0.17 ml 40% aqueous pyruvic aldehyde (1.05 mmol) containing 0.25 ml 2-mercaptoethanol in 1.2 ml H_2O was added after 30 minutes to the above solution and the mixture heated for 45 minutes at 75°C. The contents were acidified to pH 7 with glacial acetic acid and cooled overnight. After centrifugation followed by washing with H_2O, acetone, and Et_2O, 128 mg of a yellow powder was isolated (72%). A UV spectrum in 0.1 N KOH gave OD_{251}/OD_{362} = 2.87 which implies 69% of the 6-isomer and 31% of the 7-isomer (6). ^{1}H NMR (360 MHz) in 1 N NaOD gave a singlet at 8.41 ppm (66%) and a doublet at 8.16 ppm, (34%), J_{C4a-H6}= 10 Hz (13) for the vinyl protons. Dioxane was an internal reference at 3.70 ppm. Recrystallization of 80 mg from 1 ml of 1 N NaOH and 0.25 ml 95% EtOH

gave 50 mg of the sodium salt that was 88% pure (86% recovery). The sodium salt was dissolved in 1 ml H_2O, neutralized with glacial acetic acid, centrifuged and washed with H_2O, acetone, and Et_2O to give 48 mg of the free base. This was recrystallized and converted to the free base as above to give 33 mg of pure [4a-^{13}C]-6-methylpterin. This represents a yield of 30% from 6 or an overall yield of 26% from 1. ^{13}C NMR (4% NaOD) singlet, 128.6 ppm (reference dioxane 67.4 ppm) (13,14); T_1 = 43.2 sec (44°C). ^{1}H NMR (1N NaOD) δ 2.50, s, CH_3; 8.44, s, 7 H (ref dioxane - 3.70) (6).

[4a-^{13}C]-6-Methyltetrahydropterin (9). A suspension of 15 mg (0.084 mmol) 8 and 5 mg 10% Pd/C in 15 ml 6 N HCl was shaken under hydrogen at 24 psi on a Parr apparatus for 10 h. The clear solution was filtered through prewashed Celite and rotary evaporated to give an off white powder. This was washed with EtOH, acetone, Et_2O to give 14.1 mg pure white powder as the dihydrochloride monohydrate (62%). The UV spectrum in 0.1 N HCl was identical to unlabelled material (λ_{max} = 265; ε_{265} = 14,380). ^{13}C NMR (40% CD_3OD - 0.02 M Tris pH 8.0; ^{1}H decoupled) singlet, 98.9 ppm (ref CD_3OD - 49.0 ppm) (15). The coupled spectrum is a doublet due to C4a - H6 long range coupling with J = 1.83 ± .31 Hz. ^{1}H NMR (anaerobic 0.05 M KPhos/D_2O pD = 8.0) CH_3 decoupled δ 2.99, d of d, 7 H_{ax}, $J_{7ax,7eq}$ = 12.2 Hz, $J_{7ax,6}$ = 8.2 Hz; 3.12, d of d of d, 6 H, $J_{6,7ax}$ = 8.2 Hz, $J_{6,7eq}$ = 3.0 Hz, $J_{6,C4a}$ = 1.8 Hz; 3.40, d of d, 7 H_{eq}, $J_{7eq,6}$ = 3.0 Hz, $J_{7eq,7ax}$ = 12.2 Hz. The methyl group at C6 was at 1.15 ppm, $J_{CH_3,6}$ = 6.6 Hz (16).

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