MULTI-^{13}C-LABELLED 2,4-DIAMINO-6-METHYLPTERIDINE

H.T.A. Cheung and P.G. Gray

Department of Pharmacy, University of Sydney, Sydney, Australia

SUMMARY

Samples of 2,4-diamino-6-methylpteridine (1), specifically labelled with ^{13}C at one or more carbon positions, were synthesised using a combination of the appropriate unlabelled and the following ^{13}C-labelled starting materials (*ca.* 90% ^{13}C): acetone-2-^{13}C, acetone-1,3-$^{13}C_2$, bromoacetic acid-1-^{13}C, bromoacetic acid-2-^{13}C, sodium cyanide-^{13}C, and guanidine-^{13}C. The identity and site(s) of ^{13}C of each final product and intermediate were established by ^{13}C- and ^{1}H-NMR.

KEY WORDS 2,4,6-Triaminopyrimidine, 2,4,5,6-tetraaminopyrimidine, malononitrile, cyanoacetamide, 1,1-dichloracetone, ^{13}C-labelled, ^{13}C-NMR.

The inhibition of the enzyme dihydrofolate reductase by folic acid antagonists forms the basis of the therapeutic effect of a number of anti-tumour, anti-bacterial and anti-parasitic agents. As part of a program to study the mode of action of dihydrofolate reductase, we have synthesised 2,4-diamino-6-methylpteridine (1) variously and specifically labelled with ^{13}C (about 90% isotope purity) at one or more of the following sets of positions of the system: 4a or (4 and 8a), 6 or (7 and 9); 2 [see Table 1 and structure (1)].

A number of methods had been used for the synthesis of 2,4-diamino-6-methylpteridine.[1] For incorporation of ^{13}C into selected and various positions of the 6-methylpteridine ring

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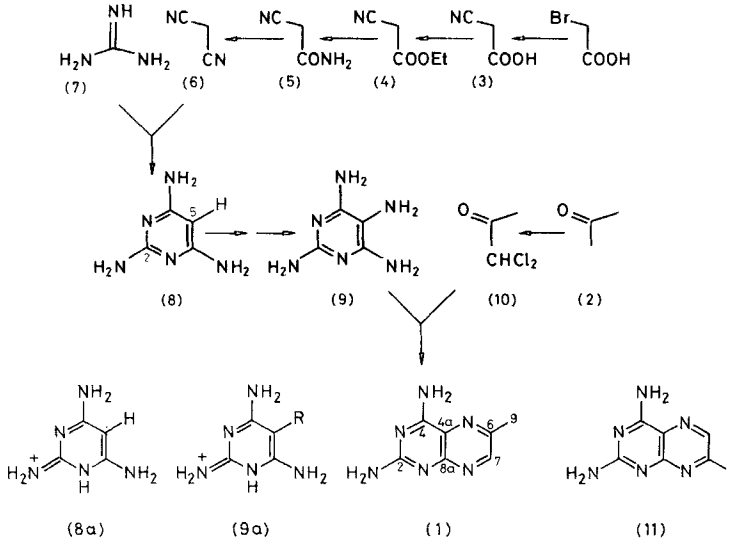
the method chosen must be such that it permits the joining together of ^{13}C-labelled intermediates and their unlabelled counterparts in various combinations. The method used in the present work is summarised in Chart 1, the ^{13}C-labelled starting materials being acetone (2) (2-^{13}C or 1,3-$^{13}C_2$), ethyl cyanoacetate (4) (2-^{13}C or 1,3-$^{13}C_2$) and guanidine-^{13}C (7). Of these, ethyl cyanoacetate-1,3-$^{13}C_2$ was not available commercially, and was prepared by us by a displacement reaction on bromacetic acid-1-^{13}C by sodium cyanide-^{13}C, followed by esterification.[2] Likewise ethyl cyanoacetate-2-^{13}C was prepared from bromoacetic acid-2-^{13}C.

In the synthesis of ^{13}C-labelled 2,4-diamino-6-methylpteridine (1), as shown in Chart 1, the pyrimidine moiety in the form of 2,4,6-triaminopyrimidine (8) was assembled by condensation[3] of guanidine (7) and malononitrile (6) (one or both suitably labelled). Malononitrile-2-^{13}C and -1,3-$^{13}C_2$ were prepared from the appropriately labelled ethyl cyanoacetate (4). While the



above reactions generally followed published procedures on unlabelled analogues,[3-5] in each case modifications were made to accommodate the small scale reactions and work-ups involving costly labelled compounds. In particular, to achieve maximum retention of label(s), ratio of reactants (labelled reactant A to labelled reactant B; and labelled reactant A to unlabelled reactant B) had to be adjusted. Procedures arrived at after repeated trial experiments on unlabelled reagents were finally applied to the labelled species (see experimental), producing yields, for the reactions discussed above, of 80-95%.

The next step, the introduction of an amino group to the labelled 2,4,6-triaminopyrimidine (8) by dithionite reduction of a nitroso intermediate resulted in ^{13}C-labelled 2,4,5,6-tetraaminopyrimidine (9) in yields comparable with that reported for the unlabelled analogue.[3]

Recently, Catalucci and Arona[7] described the condensation of 2,4,5,6-tetraaminopyrimidine (9) with 1,1-dichloroacetone (10) to give 2,4-diamino-6-methylpteridine (1). In adopting the procedure to the synthesis of ^{13}C-labelled analogues, we found it necessary to carry out the reaction at pH 3. Trial experiments on unlabelled species at various pH showed that at the higher pH specified by Catalucci and Arona, there was formed an unacceptable amount of the by-product (11) having methyl group at position 7, an event readily followed by ^{1}H-NMR. 1,1-Dichloroacetone-1,3-$^{13}C_2$ and 1,1-dichloroacetone-2-^{13}C required for the condensation were synthesised from the appropriate labelled acetone by chlorination with sulfuryl chloride. Following the published procedure,[6] significant decomposition of the desired 1,1-dichlorinated product occurred during the fractional

TABLE 1.

21 Kgauss ^{13}C- and ^{1}H-NMR data of intermediates[a]

	^{13}C-NMR			
	C-1	C-2	C-3	$-OCH_2-$
Ethyl cyanoacetate(4)				
$-2-^{13}C$	-	24.6	-	-
$-1,3-^{13}C_2$	162.7,162.9 ($^2J_{1,3}$ 4.2)	*21.7,24.5,27.2* ($^1J_{1,2}$ & $^1J_{2,3}$ 61.2, 62.7)	112.9,113.0 ($^2J_{1,3}$ 4.2)	*62.7,62.8* ($^2J_{COC}$ 2.8)
Unlabelled	*162.8*	*24.2*	*113.6*	*62.3*
Cyanoacetamide(5)				
$-2-^{13}C$ [b,c]	-	26.1	-	-
$-1,3-^{13}C_2$ [d]	166.6	-	115.6	-
Unlabelled [e]	*168.0*	*26.2*	*116.8*	-
Malononitrile(6)				
$-2-^{13}C$	-	8.6	-	-
$-1,3-^{13}C_2$	109.1	-	109.1	-
1,1-Dichloroacetone(16)				
$-2-^{13}C$				
$-1,3-^{13}C_2$	69.6 70.4 ($^2J_{1,3}$ 18.1)	-	21.7 22.5 ($^2J_{1,3}$ 18.1)	-

[a] Unless otherwise stated, spectra were run in $CDCl_3$. Chemical shifts are in p.p.m. downfield from $SiMe_4$ with $\delta(SiMe_4) = 0$ p.p.m. for ^1H spectra and $\delta(CDC_{13}) = 76.9$ p.p.m. for ^{13}C spectra. Coupling constants are in Hz. When splittings of ^1H signals are due to enriched (90%) and directly attached ^{13}C individual signals are listed. Where ^1H signals are split by ^1H or other ^{13}C, multiplicities are recorded. Chemical shifts in italics refer to natural abundance ^{13}C.

[b] ^{13}C spectrum refers to CD_3OD solvent with $\delta(CD_3OD) = 49.5$ p.p.m.

	^{1}H-NMR				
$-CH_2\underline{C}H_3$	H-1	H-2	H-3	$-O\underline{C}H_2-$	$-CH_2\underline{C}H_3$
-	-	2.69,4.22# (J_{HC} 136.7) 3.45‡	-	4.28q‡ (J_{HCCH} 7.2)	1.32t‡ (J_{HCCH} 7.2)
13.7	-	3.52 d of d‡ (J_{HCC} 8.2,10.2)	-	4.28 d of q‡ (J_{HCCH} 7.2; J_{HCOC} 3.2)	1.32t‡ (J_{HCCH} 7.2)
13.4					
-	-	2.80,4.33# (J_{HC} 136.7) 3.56‡	-	-	-
-	-	3.54 d of d‡ (J_{HCC} 6.8,10.5)	-	-	-
-					
-	-	2.80,4.39# (J_{HC} 142.4) 3.59‡	-	-	-
-	-	3.60 t‡ (J_{HCC} 11.7)	-	-	-
	5.77,5.80# (J_{HCC} 2.2) 5.79‡	-	2.42,2.49# (J_{HCC} 6.3) 2.46‡	-	-
-	4.77,4.78# 5.78,5.79‡ 6.78,6.80# (J_{HCCC} 1.3; J_{HC} 180.9)	-	1.72,1.74# 2.45,2.46‡ 3.17,3.19# (J_{HCCC} 1.3; J_{HC} 129.7)	-	-

[c] ^{1}H sepctrum refers to D_2O solvent with δ(dioxane) = 3.61 p.p.m.

[d] Both ^{13}C and ^{1}H spectra were run in CD_3OD with $\delta(\underline{C}D_3OD)$ = 49.5 p.p.m

[e] In D_2O containing dioxane with δ(dioxane) = 67.4 p.p.m.

^{1}H attached to ^{13}C.

‡ ^{1}H attached to ^{12}C.

TABLE 2

^{13}C-NMR data of pyrimidines[a]

2.4.6-Triaminopyrimidine	C-2	C-4/6	C-5	2.4.5.6-Tetraaminopyrimidine	C-2	C-4/6	C-5
-2-^{13}C	162.5	-	-	-2-^{13}C bisulfite	152.5	-	-
-4,6-$^{13}C_2$	-	165.2	-	-4,6-$^{13}C_2$ bisulfite	-	156.5	-
-4,6-$^{13}C_2$ (protonated)[b]	-	163.0	-	-4,6-$^{13}C_2$ (diprotonated)[e,f]	-	155.1	-
-2,5-$^{13}C_2$	162.6 ($J_{2,5}$ 8.3)	-	76.9 ($J_{2,5}$ 8.3)	-2,5-$^{13}C_2$ bisulfite	152.4 ($J_{2,5}$ 7.0)	-	93.7 ($J_{2,5}$ 7.0)
-2,5-$^{13}C_2$ (protonated)[f]	153.0	-	74.3	-2,5-$^{13}C_2$ (diprotonated)[e,g]	152.4 ($J_{2,5}$ 7.0)	-	83.1 ($J_{2,5}$ 7.0)
unlabelled[c,d]	163.1	164.6	75.0				
unlabelled (protonated)	156.7	161.5	75.4	unlabelled (diprotonated)[e,f]	153.6	155.2	83.1
				unlabelled (diprotonated)[c,e,h]	153.1	154.9	82.1

[a] Unless otherwise stated, data refer to D_2O solutions with dioxane as internal standard (δ 67.4 p.p.m.). ^{13}C-^{13}C coupling constants given are in Hz.

[b] Adjusted to pH 3. Chemical shift changes on protonation indicate strong contribution of structure (8a).

[c] Dimethyl sulfoxide-$^2H_6$ solutions, with $\delta(CD_3SOCD_3)$ = 39.6 p.p.m.

[d] The N,N-tetramethyl analogue was reported[8] to resonate at 163.1 (C-2) and 165.7 p.p.m. (C-4/6) in methanol-$^2H_4$.

[e] Chemical shift changes indicate the process (9a) ($R=NH_2$) ⟶ (9a) ($R=NH_3^+$) on lowering of pH.

[f] Made to *ca.* M in HCl.

[g] Adjusted to pH 0-1.

[h] One drop 10M HCl added.

TABLE 3

Spectral data of ^{13}C-labelled 2,4-diamino-6-methylpteridine

	MS[a]	^{13}C-NMR[b]							^{1}H-NMR[b]	
^{13}C-Labelled carbon	MH^+	C-2	C-4	C-4a	C-6	C-7	C-8a	C-9	H-7	H-9
6	178	-	-	-	145.8[c]	-	-	-	8.61 d ($^{2}J_{C_6,H_7}$ 9.8)	2.52 d ($^{2}J_{C_6,H_9}$ 6.6)
2,7,9	180	162.5	-	-	-	150.8 d ($^{2}J_{7,9}$ 8.3)	-	20.8 d ($^{2}J_{7,9}$ 8.3)	8.63 d ($^{1}J_{C_7,H_7}$ 180)	2.53 dd ($^{1}J_{C_9,H_9}$ 126; $^{3}J_{C_7,H_9}$ 3.9)
4,7,8a,9	181	-	163.0 d ($^{2}J_{4,8a}$ 5.6)	-	-	151.0 dd ($^{2}J_{7,9}$ 8.3; $^{2}J_{7,8a}$ 4.2)	153.9[e]	21.1 d ($^{2}J_{7,9}$ 8.3)	8.60 dd ($^{1}J_{C_7,H_7}$ 182; $^{3}J_{C_{8a},H_7}$ 11)	2.53 dd ($^{1}J_{C_9,H_9}$ 126; $^{3}J_{C_7,H_9}$ 3.6)
2,4a,6	180	160.5 d ($^{3}J_{2,4a}$ 8.4)	-	122.0[e]	147.4 d ($^{2}J_{4a,6}$ 4.2)	-	-	-	8.63 ($^{2}J_{C_6,H_7}$ 10.4)	2.51 ($^{2}J_{C_6,H_9}$ 6.6)
Unlabelled	177	162.3	162.8	d	146.0	150.7	d	20.8		

[a] Methane chemical ionisation mass spectra.

[b] Measured for dimethyl sulfoxide-$^{13}C_6$ solutions with $\delta(CD_3SOCD_3)$ = 39.6 p.p.m. for proton-decoupled ^{13}C spectra and $\delta(SiMe_4)$ = 0 for ^{1}H spectra.

[c] Gated decoupling yielded $^{2}J_{C_6,H_9}$ 6.9 and $^{2}J_{C_6,H_7}$ 9.7 Hz.

[d] Not observed due to low solubility.

[e] Unresolved signal due to more than one ^{13}C-^{13}C couplings.

[f] Half-height-width 3 Hz, implying that $^{3}J_{C_9,H_7} \leq 2$ Hz. This coupling correlates with the corresponding $^{3}J_{CCH}$ for 2-methylpyridine and 2-methylquinoline.[9]

vacuum distillation of the reaction mixture. This was avoided by treatment with solid calcium carbonate (to remove acidic material) prior to distillation, yielding labelled 1,1-dichloroacetone in about 65% yield.

^{1}H- and ^{13}C-NMR data are given in Tables 1-3. In particular, the ^{13}C-NMR data (for ^{13}C-enriched carbons and some natural abundance ones) provide unambiguous evidence of the identity and purity of the ^{13}C-labelled compounds synthesised.

EXPERIMENTAL

The ^{1}H- and ^{13}C-NMR data were collected using a JEOL FX-90Q spectrometer operating at 89.6 MHz and 22.5 MHz respectively in the Fourier-transform mode. Acquisition time, pulse delay and pulse width were, for ^{1}H: 4.57s, *ca.*1s, and 43μs, for ^{13}C: 0.37 or 0.73s, *ca.* 0.5s, and 7μs respectively. CH_4 chemical ionisation mass spectra (CI-MS) were obtained using a Finnigan 3200E GC-mass spectrometer and associated Finnigan 6110 data system. Thin-layer chromatography (TLC) and column chromatography were on Merck H60 TLC-grade silica gel. Preparative high pressure liquid chromatography separation (HPLC) was performed on an Altex 331 chromatograph using a Partisil column, and elution was carried out by 30% diethyl ether in light petroleum at 1,500 p.s.i. and at a flow rate of 9 ml per min; the detector was a Waters R 403 differential refractometer. Evaporation of all solvents took place under reduced pressure at the lowest possible temperature. Melting points were uncorrected.

^{13}C-Labelled starting materials were purchased from the Centre d'Etudes Nucléaires de Saclay, C.E.A., Gif-sur-Yvette, France (acetone-1,3-$^{13}C_2$ and acetone-2-^{13}C), and from Stohler Isotope Chemicals, Waltham, Mass., U.S.A. (bromoacetic acid-1-^{13}C and -2-^{13}C, sodium cyanide-^{13}C, and guanidine-^{13}C nitrate).

Ethyl cyanoacetate-1,3-$^{13}C_2$ To a solution of bromoacetic acid-1-^{13}C (1.04 g, 7.4 mmole) in water (2 ml) and neutralised with sodium carbonate (*ca.* 0.4 g) was added a solution of sodium cyanide-^{13}C (0.40 g, 8.0 mmole) in water (1 ml). After being stirred at the boiling point for 5-10 minutes, the solution was cooled, 10M hydrochloric acid (0.8 ml) was added and the solvent was evaporated under reduced pressure at below 50°. Ethanol was added to this residue, the insoluble solids removed by filtration and the filtrate evaporated. Extraction was repeated to yield crude cyanoacetic acid-1,3-$^{13}C_2$. A solution of 18M sulphuric acid (0.033 ml) in 100% ethanol (8 ml) was added, and the solution refluxed for 1½ - 2 hours by which time TLC showed that esterification was completed. The inorganic salts were removed by filtration, ethanol was evaporated, and the residue was dissolved in dichloromethane, and run through a short column of silica gel to yield ethyl cyanoacetate-1,3-$^{13}C_2$ (0.72 g, 84%), b.p. 107°C at 27 torr pressure. In an experiment, the commercial bromoacetic acid-1-^{13}C was contaminated with about 20% 2-bromopropionic acid-1-^{13}C (CI-MS and ^{1}H- and ^{13}C-NMR), and removal of labelled ethyl 2-cyanopropionate was carried out by HPLC.

Ethyl cyanoacetate-2-^{13}C. Bromoacetic acid-2-^{13}C was treated with unlabelled sodium cyanide (1.1 mole equivalent) and then esterified (as described above for its analogue) to give ethyl cyanoacetate-2-^{13}C, MH^+ (CI-MS) 115.

Cyanocetamide-1,3-$^{13}C_2$ and cyanoacetamide-2-^{13}C. A mixture of ethyl cyanoacetate-1,3-$^{13}C_2$ (0.33g, 2.9 mmole) and aqueous ammonia (0.5 ml, 31% w/v) was shaken until homogeneous and then left at -10° overnight. After dissolution by warming of the crystals formed, the solution was applied to a short column of silica gel. After removal of unreacted starting material with dichloromethane, elution with 20% ethanol in dichloromethane gave cyanoacetamide-1,3-$^{13}C_2$ as a colourless solid (0.23 g, 93%), m.p. 119-120° [lit. m.p. (unlabelled), 119-120°][4], MH^+ (CI-MS) 87. Cyanoacetamide-2-^{13}C, MH^+ 86, was prepared in the same way from ethyl cyanoacetate-2-^{13}C.

Malononitrile-1,3-$^{13}C_2$ and malononitrile-2-^{13}C. To cyanoacetamide-1,3-$^{13}C_2$(0.30 g, 3.5 mmole) in dry dichloroethane (0.7g) was added anhydrous calcium chloride[5] (0.05g) and phosphorous oxychloride (0.38g, 2.5 mmole). The mixture was heated at 90° with stirring for 5-6 hours. On cooling, the solid was broken up and transferred to the top of a silica gel column. Elution with dichloromethane and evaporation gave malononitrile-1,3-$^{13}C_2$ as a low melting solid (0.22g, 93%). Malononitrile-2-^{13}C, MH^+ 68 was prepared analogously from cyanoacetamide-2-^{13}C.

2,4,6-Triaminopyrimidine (8)^{13}C-labelled at 2,5-, 4,6- and 2-positions. To a solution of sodium (0.09g, 3.9 mmole) in ethanol (3 ml) was added guanidine-^{13}C nitrate (0.33g, 2.7 mmole) followed by malononitrile-2-^{13}C(0.18g, 2.7 mmole) and the mixture was refluxed for 6 hours. On cooling, the solid product was filtered, washed with ice-cold ethanol yielding 2,4,6-triaminopyrimidine-2,5-$^{13}C_2$ (0.30 g, 88%) as a crystalline solid. Further amount of the product (0.02g, 6%) was obtained by applying the residue

on evaporation of the filtrate to a short silica gel column and eluting with methanol. 2,4,6-Triaminopyrimidine-4,6-$^{13}C_2$, MH^+ 128 was made in an identical manner from malononitrile-1,3-$^{13}C_2$ and unlabelled guanidine hydrochloride (1.05 mole equivalent), while the synthesis of 2,4,6-triaminopyrimidine-2-^{13}C required starting from guanidine-^{13}C nitrate and unlabelled malononitrile (1.25 mole equivalent).

2,4,5,6-Tetraaminopyrimidine (9) ^{13}C-labelled at 2,5-, 4,6- and 2-positions. The appropriately labelled 2,4,6-triaminopyrimidine (about 0.2 g each) was converted to the corresponding ^{13}C-labelled 2,4,5,6-tetraaminopyrimidine as was described by Mallett, Taylor and Cain[3] for unlabelled species but without isolation of the intermediate nitroso compound.

1,1-Dichloroacetone-1,3-$^{13}C_2$ and 1,1-dichloroacetone-2-^{13}C. To acetone-2-^{13}C (0.39g, 6.6 mmole) was added sulfuryl chloride (2.3g, 17 mmole) slowly and with stirring and cooling,[6] and the mixture was kept at room temperature overnight. ^{1}H-NMR analysis indicated that the crude product mixture consisted of 1,1-dichloroacetone(*ca.*70%), 1,1,3-trichloroacetone(*ca.*20%) and 1,3-dichloroacetone(*ca.*10%). Anhydrous calcium carbonate (0.25 g) was added, and after 5 minutes the mixture was filtered, and the solid (calcium carbonate) washed with a small amount of dry dichloromethane. The solvent was removed at room temperature under vacuum, and the residual liquid fractionally distilled at 25 torr giving 1,1-dichloroacetone-2-^{13}C (0.52 g, 62%) b.p. 22-24° at 25 torr. 1-1-Dichloroacetone-1,3-$^{13}C_2$ was prepared in the same way from acetone-1,3-$^{13}C_2$.

2,4-Diamino-6-methylpteridine (1) ^{13}C-labelled at various positions. 2,4-Diamino-6-methylpteridine ^{13}C-labelled at one or more positions (as listed in Table 3) were synthesised from 1,1-dichloroacetone-1,3-$^{13}C_2$ or -2-^{13}C in combination with unlabelled or ^{13}C-labelled 2,4,5,6-tetraaminopyrimidine (see above and Table 2). The synthesis of 2,4-diamino-6-methylpteridine-6-^{13}C is described below; that of 2,4-diamino-6-methylpteridine labelled at other positions proceeded in the same manner. To 2,4,5,6-tetraaminopyrimidine (9) (1.05 g, 4.1 mmole) (previously crystallised from water) was added successively sodium bisulfite (0.66 g) and 1,1-dichloroacetone-2-^{13}C (0.52 g, 4.05 mmole), and the mixture was stirred for 15 minutes at room temperature during which the pH fell to 2.8-2.9. The stirred solution was heated to 80° whereupon the tetraminopyrimidine dissolved and the pH was 2.2-2.3. By the addition of M sodium hydroxide the pH was adjusted to and maintained at 3.0 ± 0.1 while the reaction was allowed to proceed at 80°. The completion of the reaction after 4-5 hours was indicated by a very slow fall in pH. A trace of insoluble material obtained on cooling was filtered off and the filtrate (pH 2.5 at 30°) was adjusted to pH7 with M sodium hydroxide while being stirred vigorously. The yellow precipitate collected by centrifugation was washed with water and centrifuged. Washing and centrifuging was repeated three times using ethanol. To the resulting yellow solid was added ether, and the suspension was filtered to give 2,4-diamino-6-methylpteridine-6-^{13}C (1) (0.28 g, 41%). The yield relative to 2,4,5,6-tetraaminopyrimidine was lower than the literature yield of unlabelled 2,4-diamino-6-methylpteridine, since in the literature[7] an excess of 1,1-dichloroacetone was used.

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