SYNTHESIS OF MULTI-^{13}C-ENRICHED METHOTREXATE FOR NMR STUDIES OF DRUG - ENZYME INTERACTIONS

H.T. Andrew Cheung*, Mary Smal, and Diem Dieu Chau
Department of Pharmacy, University of Sydney, Sydney, N.S.W., Australia

Abstract —— Methotrexate, N-[p-(2,4-diaminopteridin-6-yl)methyl(methyl)-aminobenzoyl]-L-glutamic acid, ^{13}C-enriched at positions 6; 2,7,9; 4,7,8a,9; and 2,4a,6 were synthesized from the correspondingly labelled 2,4-diamino-6-methylpteridine[4] by 'benzylic' bromination followed by displacement of the bromide by di-t-butyl N-(p-methylaminobenzoyl)-L-glutamate. Acid treatment of each methotrexate di-t-butyl ester formed yielded the corresponding ^{13}C-enriched methotrexate.

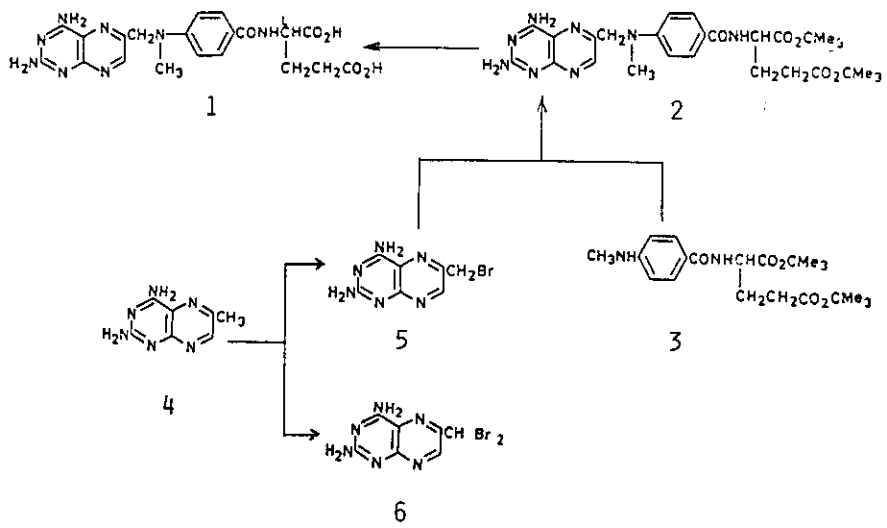
The anti-tumour agent methotrexate (amethopterin), N-[p-(2,4-diaminopteridin-6-yl)methyl-(methyl)aminobenzoyl]-L-glutamic acid (1), is an anti-folate widely used in the control of acute leukaemia and other neoplastic conditions.[1] The basis of the therapeutic effect of methotrexate (as is shared with the anti-bacterial agent trimethoprim, as well as with certain anti-parasitic agents) is the inhibition of the NADPH-dependent conversion of dihydrofolic acid to tetrahydrofolic acid by the enzyme dihydrofolate reductase, leading to depletion of thymidylate and other metabolites essential for DNA synthesis, and ultimately resulting in cell death.[2]

In connection with our use of ^{13}C-enriched anti-folates and folic acid analogues to study the changes in conformation and ionic state upon binding of these ligands (in the presence or absence of coenzymes) to dihydrofolate reductases from various sources,[3,4] we have synthesized sets of multi-^{13}C-enriched trimethoprim,[5] 2,4-diamino-6-methylpteridine (4),[6] and various folic acid analogues.[4] In this paper, we give details of the synthesis of methotrexate enriched with ^{13}C (90% abundance) at the following positions of the pteridine ring: 6; 2,7,9; 4,7,8a,9; and 2,4a,6; viz. 1a - 1d. The synthesized multi-labelled methotrexate collectively incorporate ^{13}C-enrichment at all pteridine carbons, thus providing a comprehensive set of ^{13}C-nmr probes of those enzyme-ligand interactions which involve the pteridine ring of the ligand. The spacing of the enriched carbons are such that one-bond ^{13}C-^{13}C couplings are absent, thus optimizing signal detection of enriched carbons on the ligand in the presence of the larger enzyme molecule. On

† Dedicated to Professor Gilbert Stork on the occasion of his 65th birthday.

the other hand, useful structural information is provided by the smaller couplings ($^2J_{CC}$, $^3J_{CC}$, $^2J_{CH}$ and $^3J_{CH}$) which do not cause line-separation under conditions of substantial sensitivity enhancement.

A prerequisite for any synthesis using expensive ^{13}C-enriched starting materials is the corresponding synthesis with unlabelled precursors, involving small-scale experiments to optimize the yield of each step. We choose to build up the methotrexate molecule, protected as the di-t-butyl ester, by nucleophilic displacement of di-t-butyl N-(p-methylaminobenzoyl)-L-glutamate (3)[7] on 2,4-diamino-6-bromomethylpteridine (5).[8] The bromide (5) was conveniently formed by bromination of 2,4-diamino-6-methylpteridine (4) in acetic acid, with modification of the literature procedure[9] to suit the small scale appropriate to ^{13}C-enriched reactants. The crude product (which generally consisted of a mixture of monobrominated and dibrominated products as well as unreacted material, all as hydrobromides) (Table 1) was used directly in the displacement reaction. Crude methotrexate di-t-butyl ester (2) formed was purified by chromatography over silica gel.



	^{13}C-enriched at
a	6
b	2,7,9
c	4,7,8a,9
d	2,4a,6

A series of appropriately ^{13}C-labelled 2,4-diamino-6-methylpteridine (4a)-(4d) had earlier been synthesized by us.[6] Application of the above reaction scheme using these ^{13}C-enriched intermediates yielded the desired series of methotrexate di-t-butyl ester (2a)-(2d) with ^{13}C-enrichment at the pteridine ring. The identity of these esters, in particular the site of ^{13}C-enrichment, was established by the ^{1}H-nmr spectra showing characteristic ^{13}C-^{1}H couplings, by the ^{13}C-nmr signals of the ^{13}C-enriched carbons (Table 2), and by ammonia and methane chemical ionization mass spectra (Table 3). Short treatment of each di-t-butyl ester with trifluoroacetic acid removed the t-butyl protecting group to yield the corresponding ^{13}C-enriched methotrexate (1a)-(1d).

Table 1. ^{1}H-nmr chemical shifts (and coupling constants in Hz) of protonated 2,4-diamino-6-methylpteridine, 2,4-diamino-6-bromomethylpteridine and 2,4-diamino-6-dibromomethylpteridine, ^{13}C-enriched and unlabelled.

Compound	^{13}C-Enriched carbon(s)	H-7	H-9
4a	6	8.89 d ($^{3}J_{C6H7}$ 10.3)	2.67 d ($^{3}J_{C6H9}$ 6.7)
4	-	8.90	2.67
5a	6	9.14 d ($^{3}J_{C6H7}$ 9.4)	4.92 d ($^{3}J_{C6H9}$ 4.1)
5b	2,7,9	9.13 d ($^{2}J_{C7H7}$ 190)	4.92 d ($^{2}J_{C9H9}$ 160)
5c	4,7,8a,9	9.14 dd ($^{1}J_{C7H7}$ 188, $^{3}J_{C8aH7}$ 13)	4.92 dd ($^{1}J_{C7H7}$ 157, $^{3}J_{C7H9}$ 4.4)
5d	2,4a,6	9.14 d ($^{3}J_{C6H7}$ 9.7)	4.92 d ($^{3}J_{C6H9}$ 3.8)
5	-	9.15	4.93
6d	2,4a,6	9.27 d ($^{3}J_{C6H7}$ 9.1)	7.54 d ($^{3}J_{C6H9}$ 5.1)
6	-	9.27	7.52

[a] Measured for CD_3SOCD_3 solutions with δ in p.p.m. from $Si(CH_3)_4$.

Table 2. Chemical shifts (and coupling constants in Hz) of ^{13}C-enriched methotrexate di-t-butyl ester (2a), (2c), (2d)[a] and of ^{13}C-enriched methotrexate (1b).

Compound	2a	1b[b]	2c	2d
Enriched Carbons	6	2,7,9	4,7,8a,9	2,4a,6
^{13}C-nmr				
C-2		162.1		162.4 d ($^2J_{2,4a}$ 8.4)
C-4			162.8 d ($^2J_{4,8a}$ 4.2)	
C-4a				122.3[c]
C-6	147.5			147.4
C-7		150.1 d ($^2J_{7,9}$ 6)	149.7[c]	
C-8a			154.8[c]	
C-9		55.8 d ($^2J_{7,9}$ 6)	55.9 d ($^2J_{7,9}$ 7.0)	
^1H-nmr				
H-7	8.7 d ($^3J_{C6H7}$ 9.5)		[e] ($^3J_{C8aH7}$ 12)	8.7 d ($^3J_{C6H7}$ 9.5)
H-9	ca. 4.75[d]		4.8 bd ($^1J_{C9H9}$ 139)	ca. 4.75[d]
Arom.	6.8, 7.8[f]		6.8, 7.8[f]	6.8, 7.8[f]
NCH_3	3.2		3.2 d ($^3J_{C9NCH_3}$ 2.5)	3.2
α	ca. 4.75[d]		4.7 m	ca. 4.75[d]
$OC(CH_3)_3$	1.4, 1.5		1.4, 1.5	1.4, 1.5

[a] Unless otherwise stated, chemical shifts are in p.p.m. from $Si(CH_3)_4$ measured in $CDCl_3$ solutions at 22.5 MHz for ^{13}C and 89.6 MHz for ^1H, with $\delta(CDCl_3)$ 77.0 for ^{13}C and $\delta(Si(CH_3)_4)$ 0 for ^1H. Signals of β and γ protons appear at 1.9 - 2.5 p.p.m.

[b] Measured in D_2O buffered to pH 6.5 with M/20 potassium phosphate and M/2 potassium chloride at 67.9 MHz (for ^{13}C), with external p-dioxane standard at 67.4 p.p.m.[4]

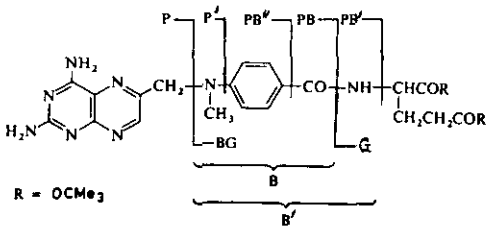
[c] Unresolved signal due to more than one ^{13}C-^{13}C couplings.

[d] Masked by other signals.

[e] One arm of dd masked by other signals.

[f] Approximate doublets, apparent J, 8.6 Hz.

Table 3. Chemical ionization mass spectral data of methotrexate di-t-butyl ester, ^{13}C-enriched and unlabelled.



Compound	2a	2a	2d	2d	2	2
Enriched carbons	6	6	2,4a,6	2,4a,6	-	-
Ionizing gas	NH_3[c]	CH_4[d]	NH_3[c]	CH_4[d]	NH_3[c]	CH_4[d]
MH$^+$	568	568	570	570	567	567
$-C_4H_8$	512	512	514	514	511	511
$-C_4H_8-C_4H_8$	456	456	458	458	455	
$-C_4H_8-C_4H_{10}O$	438	438		440	437	
P'	208	206	210	208	207,205	205
P	178	178	180	180	177	177
PB'	327	327	329	329	326	326
PB		309		311		308
PB''		283		285		282
B'	152,151	152		152,151	152,151	152,151
B		134		134		134
BG		393	393	393	393	393
$-C_4H_8$	337	337	337	337	337	337
$-C_4H_8-C_4H_8$	281	281	281	281	281	281
$[NH_2CH(CH_2CH_2CO)CO]$ + H$^+$	130	130[b]	130	130[b]	130	130[b]
$[NH_2CH(CH_2CH_2CO)CO]$ + NH^+_4	147[b]		147[b]		147[b]	

[a] Following our usage[10] and that of Przyblski et al.,[11] letters P, BG etc. designate ions formed, with or without hydrogen transfer, by cleavages shown; underlined m/z refer to ions of over 20% relative abundance.

[b] Base ion.

[c] Other ions at m/z 379 and 108.

[d] Other ions at m/z (354 + No. of ^{13}C-enriched carbon), at 309 (337 - H_2O), and at 263 (281 - H_2O).

EXPERIMENTAL

Mass spectral data given refer to chemical ionization. For notations P, BG etc. in description of ions, and the significance of underlined m/z values, see Table 3.

Bromination of ^{13}C-Enriched and Unlabelled 2,4-Diamino-6-methylpteridine (4a)-(4d) and (4)

2,4-Diamino-6-methylpteridine ^{13}C-enriched at one or more positions were brominated by a modification of the method of Catalucci.[9] The bromination of 2,4-diamino-6-methylpteridine-6-^{13}C (4a) is described below; that of its analogues ^{13}C-enriched at other positions proceeded in the same manner.

2,4-Diamino-6-methylpteridine-6-^{13}C (4a) (106 mg, 0.60 mmol) was stirred in a sealed atmosphere with benzoyl peroxide (9 mg) and bromine (0.10 ml, 1.0 mmol) in anhydrous acetic acid (5.5 ml) under reflux, with the external oil bath at 110-115°C. The reflux condenser was fitted at the top with a cold-finger containing acetone/dry-ice, and a side-arm attached to a rubber balloon. The reaction was followed by ^{1}H-nmr. After 6 h the solvent was removed under reduced pressure. The resulting residue was washed several times with dry ether, and dried under vacuum to yield a mixture (62 mg) consisting of a 6:1:2 mixture of hydrobromides of 2,4-diamino-6-bromomethylpteridine-6-^{13}C (5a), 2,4-diamino-6-dibromomethylpteridine-6-^{13}C (6a), and 2,4-diamino-6-methylpteridine-6-^{13}C (4a), having ^{1}H-nmr data given in Table 1, and showing the following mass spectral ions (CH_4): for (5a), m/z 256/258 (MH^+), 177 (MH^+-Br) (base ion); for (6a), m/z 334/336/338 (MH^+), 176/178 (MH^+-2Br) (base ion); for (4a), m/z 178 (MH^+) (base ion).

Bromination reactions on 2,4-diamino-6-methylpteridine (4), and its analogues with ^{13}C-enrichment at 2,7,9 (4b), 4,7,8a,9 (4c) and 2,4a,6 (4d) were carried out in a similar manner but generally on lower scales, yielding product mixtures with the following major components as shown by ^{1}H-nmr (see Table 1): for the unlabelled species, the mono- and dibromo products (5) and (6), and starting material (4) depending on the reaction time; for the 2,7,9-labelled species, the monobromo product (5b); for the 4,7,8a,9-labelled species, the monobromo product (5c) and starting material (4c); and for the 2,4a,6-labelled species, the mono- and dibromo products (5d) and (6d).

^{13}C-Enriched and Unlabelled Methotrexate Di-t-butyl Ester, Di-t-butyl N-[p-2,4-Diaminopteridine-6-yl)methyl(methyl)aminobenzoyl]-L-glutamate, (2a) - (2d) and (2)

The product mixture from bromination of 2,4-diamino-6-methylpteridine-6-^{13}C (4a) (see above) (60 mg) was heated with di-t-butyl N-(p-methylaminobenzoyl)-L-glutamate (3)[7] (64 mg, 0.16 mmol), in dry N,N-dimethylformamide (1.0 ml) at 70°C for 4h. The product mixture was evaporated under reduced pressure after addition of triethylamine (0.5 ml), and the residue chromatographed over a short column of thin-layer chromatography grade silica gel (Merck 60H). After elution of unreacted material (4a) with chloroform, fractions eluted with 5-10% methanol in chloroform were concentrated and washed several times with water to remove triethylamine hydrobromide. The residue obtained on removal of solvent was dried under vacuum to give methotrexate-6-^{13}C di-t-butyl ester (2a) (40 mg, 0.07 mmol) as a yellow solid. Methotrexate di-t-butyl ester (2), and its analogues with ^{13}C-enrichments at 2,7,9 (2b), 4,7,8a,9 (2c), and 2,4a,6 (2d) were synthesized in the same manner, with additional purification of the three labelled products by preparative thin-layer chromatography over silica (Merck GF_{254}). The nmr and chemical ionization mass spectral data of the products are shown in Tables 1 and 2.

Methotrexate (1), Methotrexate-6-^{13}C (1a), Methotrexate-2,7,9-$^{13}C_3$ (1b), Methotrexate-4,7,8a,9-$^{13}C_4$ (1c) and Methotrexate-2,4a,6-$^{13}C_3$ (1d)

A solution of methotrexate-6-^{13}C di-t-butyl ester (2a) (32 mg) in trifluoroacetic acid (1 ml) was stood at room temperature for 20 min, and then evaporated under reduced pressure. The residue was dissolved in diluted aqueous ammonium hydroxide and the pH adjusted to 5. After cooling to 4°C overnight the suspension was filtered. The filtered yellow crystals were washed with cold water and dried in vacuum to give methotrexate-6-^{13}C (1a) with m/z (NH_3) 456 (MH^+), 438 (MH^+-H_2O), 327 (PB'), 281 (BG), 263 (BG-H_2O), 178 (P), 169, 164 (G+NH_4^+), 152 and 151 (B'), 147 (GH^+ or $NH_2CH(CH_2CH_2CO)CO-O + NH_4^+$) (base ion), 130 ($^+NH_3CH(CH_2CH_2CO)CO-O$) and 108. Methotrexate (1), and its analogues with ^{13}C-enrichment at 2,7,9 (1b), 4,7,8a,9 (1c) and 2,4a,b (1d) were prepared in a similar manner from the corresponding di-t-butyl esters in 60-90% yields. Methotrexate-2,4a,6-$^{13}C_3$ has m/z (NH_3) 458 (MH^+), 440, 329, 281, 263, 180, 169, 164, 152, 151, 147 (base ion), 130 and 108.

ACKNOWLEDGEMENT

This work is supported by the National Health and Medical Research Council of Australia, the University of Sydney Cancer Research Committee, and the Jenny Leukaemia Trust. We thank Dr T. Q. Tran for the synthesis of compound (3), and P. Gray for preliminary experiments on the bromination of 2,4-diamino-6-methylpteridine.

REFERENCES

1. M.H.N. Tattersall, 'Clinical Utility of Methotrexate in Neoplastic Disease' in ref.2. Vol. 2, pp. 166-191.
2. F.M. Sirotnak, J.J. Burchall, W.D. Ersminger and J.A. Montgomery (eds.), 'Folate Antogonists as Therapeutic Agents', vol. 1 and 2, Academic Press, Orlando, Florida, and London, 1984.
3. H.T.A. Cheung, M.S. Searle, J. Feeney, B. Birdsall, G.C.K. Roberts, I. Kompis, and S.J. Hammond, Biochemistry, 1986, 25, 1925.
4. B. Birdsall, H.T.A. Cheung, J. Feeney and J.F. Morrison, unpublished results.
5. M. Smal, H.T.A. Cheung and P.E. Davies, J. Chem. Soc. Perkin Trans. 1, 1986, 747.
6. H.T.A. Cheung and P.G. Gray, J. Label. Comp. Radiopharm. 1984, 21, 471.
7. H.T.A. Cheung and T.Q. Tran, unpublished results; cf. D.J. Antonjuk, D.K. Boadle, H.T.A. Cheung and T.Q. Tran, J. Chem. Soc. Perkin Trans. 1, 1984, 1989.
8. J.R. Piper and J.A. Montgomery, J. Org. Chem., 1977, 42, 208.
9. E. Catalucci (Lonza A.G., Basle), Deutsches Patent. Offen. 2,741,383 (1979); U.S. Patent No. 4,224,446 (1980).
10. H.T.A. Cheung, B.N. Tattam, D.J. Antonjuk and D.K. Boadle, Biomed. Mass Spectrom., 1985, 12, 11.
11. M. Przybiski, J. Prieß, R. Dennebaum and J. Fischer, Biomed. Mass Spectom., 1982, 9, 22.

Received, 2nd June, 1986