N-(L-α-AMINOACYL) DERIVATIVES OF METHOTREXATE

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Dedicated to Professor Sir Derek Barton, F.R.S. in honour of his 70th birthday.

Abstract - Methotrexate di-t-butyl ester 3 was coupled with N-t-butyloxycarbonyl-L-leucine by the *p*-nitrophenyl ester and carbodiimide methods to give the di-t-butyl esters 5a - 7a of 2-, 4-, and 2,4-di(N-t-butyloxycarbonyl-L-leucyl)methotrexate. The corresponding L-alanyl analogues 5b - 7b were also synthesised by the latter method. The positions of the acyl groups were determined from ^{13}C-nmr and uv data. Upon deprotection with trifluoroacetic acid, the 2-acyl products 5a and 5b yielded 2-L-leucyl- and 2-L-alanylmethotrexate 4a and 4b, but the 4-acyl analogues 6a and 6b gave decomposition products. The enzymic cleavage of the 2-(L-α-aminoacyl) pro-drug derivatives 4a and 4b by porcine microsomal leucine aminopeptidase was followed by high-pressure liquid-chromatography (HPLC).

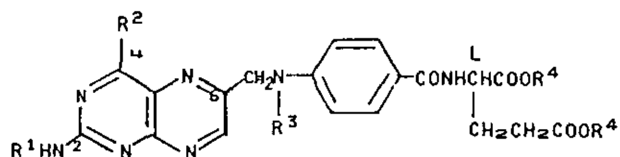
The chemotherapeutic agent methotrexate (amethopterin), N-[*p*-{N-(2,4-diamino-pterin-6-yl)methyl}methylaminobenzoyl]-L-glutamic acid 2, is an analogue of folic acid 1[1] widely used singly and in combination for the control of cancers.[2] Various aspects of the anti-folate action of methotrexate have been studied,[3,4] and many analogues of the drug synthesiszed[5] in efforts to overcome the problems of acute toxicity and of inherent and acquired resistance. One of the approaches we have adopted is that of developing analogues which may act as latent forms (pro-drugs) of methotrexate.[6,7]

In this communication we report the synthesis of the first members of a new series of pro-drugs of methotrexate, *viz*. the 2-(L-α-aminoacyl) derivatives, and the enzymic cleavage of 2-L-leucyl- and 2-L-alanylmethotrexate 4a and 4b to

the active drug by porcine microsomal leucine aminopeptidase. Association of tumour sites with elevated aminopeptidase activity and with the existence of aminopeptidase isoenzymes has been reported.[8] The 2-(L-α-aminoacyl) derivatives are thus potential site-directed pro-drugs of methotrexate.

Acetylation[9,10] and trifluoroacetylation[11] of 2,4-diaminopteridine 8 and its 6-substituted analogues were reported to lead to di-acylation of the 2,4-diamino group. Nevertheless the acylation of the 2,4-diamino-6-methylpteridine moiety of methotrexate has not been reported. Acetylation of folic acid was found to lead to cyclisation of the glutamate moiety.[12] In our work, methotrexate and the acylating α-amino acid are protected as the acid-labile t-butyl ester and N-t-butyloxycarbonyl (t-BOC) derivatives respectively. Construction of methotrexate di-t-butyl ester 3[13] from glutamate, p-methylaminobenzoate and 2,4-diamino-6-pteridinylmethyl fragments was carried out as reported for the syntheses of ^{13}C-*enriched methotrexate* di-t-butyl esters.[4] The subsequent coupling to t-BOC-α-amino acids was carried out by the p-nitrophenyl ester and carbodiimide methods. Thus reaction of methotrexate di-t-butyl ester 3 with t-BOC-L-leucine p-nitrophenyl ester (3 molar equiv.) in N,N-dimethylformamide containing triethylamine (0.5 equiv.) at 70-75° C for 4 h gave the di-t-butyl esters 5a - 7a of 2-(t-BOC-L-leucyl)methotrexate (major product), 4-(t-BOC-L-leucyl)methotrexate (minor), and 2,4-di(t-BOC-L-leucyl)methotrexate, as well as recovered ester 3. With dicyclohexylcarbodiimide (2.4 equiv.) and t-BOC-L-leucine (2 equiv.) in ethyl acetate at 45° C for 7 h, the same products were obtained, but with the di-acylated product 7a predominating. Under the same conditions, t-BOC-L-alanine yielded the corresponding products 5b - 7b.[14]

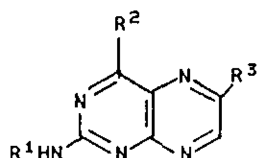
The above two sets of products incorporate one, one, *and two t-BOC-α-aminoacyl* group(s) respectively, as is shown by the ammonia chemical ionisation mass spectral and ^{13}C-nmr data.[15,16] Furthermore, the acylations have taken place on the exocyclic nitrogen atoms. This is shown by the relatively local and regular perturbations of the ^{13}C-nmr shieldings of the pteridine carbons upon acylation (Figure). One notable effect is a 6 ppm upfield shift of the signal of carbon 2 or 4 upon acylation of the amino group attached to the same carbon. In comparison, C-1 of aniline is shielded by 8 ppm upon acetylation[17a]. Another effect, diagnostic of the position of acylation, is that upon acylation of the 2-



	R^1	R^2	R^3	R^4
1	H	OH	H	H
2	H	NH_2	Me	H
3	H	NH_2	Me	Bu^t
4	$COCHRNH_2$	NH_2	Me	H
5	$COCHRNHCOOBu^t$	NH_2	Me	Bu^t
6	H	$NHCOCHRNHCOOBu^t$	Me	Bu^t
7	$COCHRNHCOOBu^t$	$NHCOCHRNHCOOBu^t$	Me	Bu^t

a R = CH_2CHMe_2

b R = Me



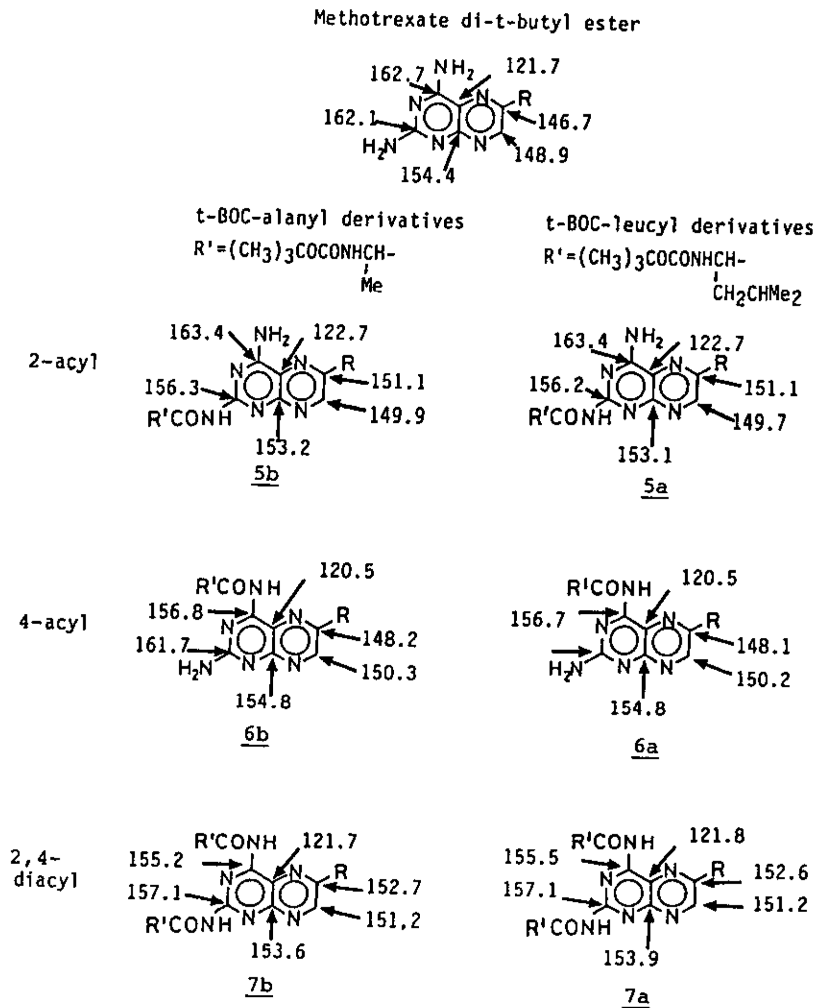
	R^1	R^2	R^3
8	H	NH_2	H
9	H	NH_2	CH^2OAc
10	H	NH_2	CH_2OAc
11	Ac	NH_2	CH_2OAc
12	H	NHAc	CH_2OAc
13	Ac	NHAc	CH_2OAc
14	Ac	OH	CH_2OAc
15	H	OH	CH_2OAc

amino group there is a 4½ ppm deshielding of C-6 at the other end of the pteridine ring (see Figure, 5a and 5b). The corresponding deshielding on acylation of the 4-amino group is only 1½ ppm (see 6a and 6b). Upon di-acylation, the shift change at C-6 is additive (see 7a and 7b). Protonation or acylation of an exocyclic amino group on an aromatic or heteroaromatic ring is known to result in decreased electron density in the ring, manifesting in downfield carbon shifts at *o* and *p* positions. For a bicyclic system, the downfield shift is expected to be particularly pronounced at the other extremity of the system, as is indeed observed here for 2-acylation of a 2,4-diaminopteridine ring. In comparison, ring protonation of 2,4-diaminopteridines (at N-1), benzimidazole (at N-3) or purine (at N-1) does result in 5 ppm deshielding at the other extremity of the system (C-6, C-6, and C-8 respectively).[17b,18,19]

Boyle and Pfleiderer[10] found that 2,4-di(acetamido)-6-acetoxymethylpteridine 13 decomposed on standing in methanol-chloroform over silica gel to give *inter alia* the 2-acetamido-4-aminopteridine 11. Assignment of the position of the 2-acetyl group in the latter was based on a hypochromic shift, relative to 2,4-diamino-6-hydroxymethylpteridine 9, of the long wavelength uv maximum (at pH 7 or in methanol) from 372/371 nm to 350/355 nm. The displacement was considered to be related to "das Maximum des in der Längsrichtung des Moleküls polarisierten", and was not expected if the product was the corresponding 4-acetyl compound 12.[10] We find that the long wavelength absorptions of the 2-acyl derivatives 5a and 5b at pH 7.0 are also near 350 nm (shoulder, log ε 3.8). In contrast, the corresponding maxima of the 4-acyl derivatives 6a and 6b are at 385 nm (log ε 3.8). As in the cases of the 2,4-diamino-and 2,4-di(acetamido)-compounds 10 and 13,[10] methotrexate di-t-butyl ester 3 and the diacyl compound 7b absorb respectively at 377 nm and near 350 nm (shoulder) in methanol.

The 2- and 4-t-BOC-L-α-aminoacyl derivatives of methotrexate di-t-butyl ester are relatively stable to alcohol and neutral organic solvents when chromatographed over silica gel. Deprotection of 2-t-BOC-L-leucyl- and 2-t-BOC-L-alanylmethotrexate di-t-butyl ester 5a and 5b took place smoothly upon brief treatment with trifluoroacetic acid at room temperature, followed by evaporation after the addition of dry benzene. The respective products 2-L-leucylmethotrexate 4a and 2-L-alanylmethotrexate 4b have the characteristic uv spectrum of the

FIGURE. ^{13}C Chemical shift assignments[a] given to the pteridine carbons of methotrexate di-t-butyl ester and the t-BOC-alanyl and t-BOC-leucyl derivatives of methotrexate di-t-butyl ester (partial structures only are shown)



[a] Solvent = $CDCl_3$. Chemical shifts are relative to $CDCl_3$ = 76.9 p.p.m. Assignments of pteridine carbons of methotrexate di-t-butyl ester are based on work on selectively ^{13}C-enriched 2,4-diaminopteridines.[4,18]

precursors, and were stable under the conditions of reverse phase HPLC using methanol and phosphate buffer at pH 7.0. In contrast, decomposition products were formed upon similar deprotection of 4-t-BOC-L-leucyl- or 4-t-BOC-L-alanylmethotrexate di-t-butyl ester 6a and 6b. When followed by HPLC with high speed spectrophotometric detection, a product with no absorption maximum beyond 340 nm was found. On standing in buffer at pH 7.0, this decomposed mainly to a less polar product with uv spectrum similar to that of methotrexate. Significant amounts of 4-L-leucyl- or 4-L-alanylmethotrexate were absent, since a spectrum reminiscent of the 4-acyl precursors was given only by a very minor product.

The di(t-BOC-L-α-aminoacyl) derivatives 7a and 7b also underwent further decomposition upon deprotection. For the alanyl series, the major product was identified as 2-L-alanylmethotrexate. This observation parallels that of Boyle and Pfleiderer,[10] who reported that under mild hydrolytic conditions 2,4-di(acetamido)-6-acetoxymethylpteridine 13 gave rise to the corresponding 2-acetamido compound 11. The other products were the 2,4-diamino compound 10, and the pterin derivatives 14 and 15.[10]

2-L-Leucylmethotrexate 4a and 2-L-alanylmethotrexate 4b comply with several requirements for pro-drug action. Firstly, they retain the glutamate carboxylate groups necessary for folate-type active transport into cells.[20] Secondly, they are *not* expected to inhibit the target enzyme dihydrofolate reductase until cleaved to the active drug, since the 2,4-diaminopyrimidine moiety essential for tight binding[1] is not present. Thus we found that the concentration of 2-L-leucylmethotrexate 4a required for 50% inhibition of *Lactobacillus casei* dihydrofolate reductase was at least two orders of magnitude higher than that of methotrexate.

A third requirement is that the 2-L-α-aminoacyl group should be cleaved by the relevant enzyme to yield methotrexate. Using porcine microsomal leucine aminopeptidase (Sigma) at 37^{0}C and pH 7, and following by HPLC, 2-L-leucylmethotrexate was found to be cleaved to methotrexate, though at a rate several times slower than when the standard substrate L-leucyl-2-naphthylamide was used. The cleavage of L-alanylmethotrexate was even slower, reflecting the specificity of the aminopeptidase used.

AKNOWLEDGEMENT

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14. New compounds 5a - 7a and 5b - 7b gave correct C, H and N analyses, but with ½ - 1½ H_2O for compounds 5b - 7b. Melting points are 126-9, 120-3, 131-4; 119-121, 113-5, and 112-4°C respectively. The synthesis of the diethyl ester analogues of 5a - 7a will be described in the full paper.

15. MH^+ for 5a and 6a is at m/z 780, for 5b and 6b at m/z 738. The quasimolecular ions of 7a and 7b are expected to be >800 amu and were not observed; nevertheless fragment ions at m/z 535 for 7a and 450 for 7b are diagnostic of the attachment of *two* α-aminoacyl groups to the 2,4-diamino-6-pteridinylmethyl moiety.[16] ^{13}C-Nmr signals ($CDCl_3$ solution, with δ_{CDCl_3} 76.9 ppm) for the t-BOC-α-aminoacyl moieties are:

	5a	6a	7a	5b	6b	7b
Ala CH_3	18.7	17.3	17.0,18.3			
Leu CH_3				21.7	21.4	21.7,21.7
Leu CH_3				23.2	22.8	22.8,23.0
Leu γ-CH				24.9	24.6	24.6,24.7
t-BOC C($\underline{C}H_3$)$_3$	28.2	28.1	28.1,28.1	28.3	28.0	28.1,28.1
Leu β-CH_2				41.5[a]	40.3[a]	39.8,41.1
Ala α-CH	50.8[a]	51.4[a]	50.9[a],51.5[a]			
Leu α-CH				53.9[a]	54.2[a]	54.2[a],54.6[a]
t-BOC $\underline{C}$(CH_3)$_3$	79.8	80.3	79.9,80.2	79.6	80.0	79.7,80.1

[a] Broad signal due to conformational immobility.

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