Absolute Configuration of 6-Methyl-5,6,7,8-tetrahydropterin produced by Enzymic Reduction (Dihydrofolate Reductase and NADPH) of 6-Methyl-7,8-dihydropterin

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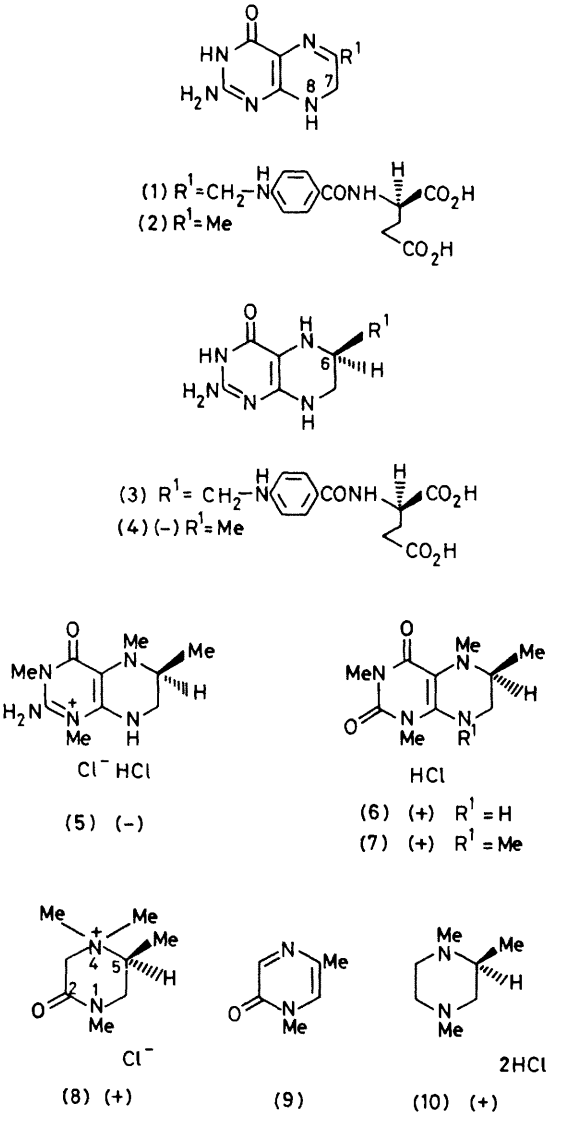
Summary Dihydrofolate reductase (5,6,7,8-tetrahydrofolate: NADP oxidoreductase, E.C.1.5.1.3.) and NADPH, which catalyse the reduction of 7,8-dihydrofolic acid (**1**) stereospecifically to give one diastereoisomer of 5,6,7,8-tetrahydrofolic acid (**3**), also catalyse the reduction of 6-methyl-7,8-dihydropterin (**2**) stereospecifically to (−)-6-methyl-5,6,7,8-tetrahydropterin (**4**); the absolute configuration at C-6 of the pterin (**4**) is shown to be *S* by correlation with *S*-alanine using a series of methylations, degradations, and syntheses, and if the most probable assumption is made that the stereospecificities of these two reactions are the same, then the absolute configuration at C-6 of enzymically produced 5,6,7,8-tetrahydrofolic acid should be *S*.

THE reduction of 7,8-dihydrofolic acid (**1**) to 5,6,7,8-tetrahydrofolic acid (**3**) by dihydrofolate reductase and NADPH produces a new chiral centre at C-6. The reduction is stereospecific and, in the cases reported, the magnitudes of the negative rotations imply that the new asymmetric centre is *laevo* when N-5 is protonated.[1,2] The evidence at present indicates that the chiralities at C-6 of natural 5,6,7,8-tetrahydrofolic acid[1,2,3] and related folates, *e.g.*, 5,10-methylene-,[4,5,6] 5,10-methenyl-,[7] 5-methyl-,[5,8] and 5-formyl-5,6,7,8-tetrahydrofolic acid[2,9,10] from various biological systems are the same. The complete structure of 5,10-methenyl-5,6,7,8-tetrahydrofolic acid bromide hydrobromide from an *X*-ray study was reported recently, and the absolute configuration *S* was deduced for C-6 of natural



5,6,7,8-tetrahydrofolic acid from the data.[10] The R indices from two separate X-ray measurements of the diastereoisomers (0·08 and 0·06) were, however, high[10] and further refinement gave slightly better values.[11] With an important molecule like tetrahydrofolic acid, more than one approach for the determination of structure is highly desirable, and now we report a stereochemical correlation study that proves the absolute configuration S at C-6 of 6-methyl-5,6,7,8-tetrahydropterin prepared by enzymic reduction of 6-methyl-7,8-dihydropterin with dihydrofolate reductase and NADPH. This result is most relevant to the absolute configuration of tetrahydrofolic acid because by making the most probably correct assumption that dihydrofolate reductase and NADPH reduce 6-methyl-7,8-dihydropterin and 7,8-dihydrofolic acid with the same stereospecificity, it can be concurred that the absolute configuration at C-6 of natural tetrahydrofolic acid should be S, in agreement with the X-ray studies.

Reduction of 6-methyl-7,8-dihydropterin (**2**) with dihydrofolate reductase from *S. faecalis* and NADPH at pH 7 (NH_4OAc buffer) is very slow. Dihydrofolate reductase from calf liver, on the other hand, reduces (**2**) appreciably faster and at pH 5·5 the rates are faster still although slow decomposition of NADPH occurs. The relative rates (using constant amounts of enzyme) for NADPH (0·1 mM) oxidation at 37 °C of (**1**) (0·05 mM) and (**2**) (0·05 mM) with the latter enzyme are 7·1 : 1 and 3·8 : 1 in pH 6·9 and 5·5 NH_4OAc buffer (50 mM), and demonstrate that the dihydropterin (**2**) is quite a good substrate. The ratio of the maximum velocities of compounds (**2**) and (**1**), V_{max} (**2**)/V_{max} (**1**), is 0·1 and the K_m values for compounds (**1**) and (**2**) are $\simeq$0·2 and 41·5 μM, respectively (all in NH_4OAc buffer at pH 7·2). At the end of the NADPH oxidation we isolated 6-methyl-5,6,7,8-tetrahydropterin (**4**) by DEAE cellulose column chromatography,[12] and stabilised it as the hydrochloride. It is *laevo*-rotatory in 2N-hydrochloric acid and its c.d. curve has a trough at 263 nm with $[\Phi]^{20}$ $-2{\cdot}15 \times 10^3$ deg cm^2 dmol^{-1}. Methylation of this pterin gives (–)-1,3,5,6-tetramethyl-5,6,7,8-tetrahydropterinium chloride hydrochloride. The enzymic reactions indicate that the mechanism of reduction of the 7,8-dihydropterin (**2**) is similar to that of 7,8-dihydrofolic acid (**1**), and that the absolute configuration at C-6 of (–)-(**4**) should be the same as that of C-6 in natural 5,6,7,8-tetrahydrofolic acid (see below).

Treatment of (–)- and (+)-6-methyl-5,6,7,8-tetrahydropterin hydrochloride, obtained by recrystallisation of the 2S,3S-di-O-benzoyltartrate salts,[13] with methyl iodide and sodium hydroxide in methanol gave (–)-1,3,5,6-tetramethyl-5,6,7,8-tetrahydropterinium chloride hydrochloride (**5**) and its enantiomer respectively.[14]† The (–)-salt (**5**) (with $[\alpha]^{20}_{475}$ $-5{\cdot}9°$ in 2N-HCl) was deaminated by boiling in 2N-sodium hydroxide (1·5 h) and gave (+)-1,3,5,6-tetramethyl-5,6,7,8-tetrahydrolumazine hydrochloride (**6**) {$[\alpha]^{20}$ (nm): +60 (313) and +1·1° (435) in 2N-HCl}. The latter was methylated with an excess of methyl iodide in methanol containing sodium hydrogen carbonate (45 °C for 1 week) and gave, after the necessary workup, (+)-1,3,5,6,8-pentamethyl-5,6,7,8-tetrahydrolumazine (**7**) {$[\alpha]^{20}$ (nm): +15·2 (300), 0·00 (316), and −11·4° (370, trough) in MeOH}. The lumazine (**7**) was degraded by heating in 2N-sodium hydroxide (18 h), and after acidification decarboxylation occurred to yield the intermediate 1,4,5-trimethylpiperazin-2-one. This was methylated with methyl iodide and sodium carbonate in methanol followed by acidification with 2N-hydrochloric acid and purification by t.l.c. to produce (+)-1,4,4,5-tetramethylpiperazin-2-on-4-ium chloride (**8**) {$[\alpha]^{20}_{280}$ +44° in H_2O}.‡ The absolute stereochemistry of compound (**8**) was determined by its synthesis and correlation with S-alanine as described below.

† These were later prepared by recrystallisation of (±)-1,3,5,6-tetramethyl-5,6,7,8-tetrahydropterinium 2R,3R-di-O-benzoyltartrate from MeOH–Et_2O, and were much more stable towards aerial oxidation than the enantiomer (**4**), see ref. 14.

‡ The smaller specific rotation of compound (**8**) obtained from the pterin (**4**) compared with that of compound (**8**) obtained from the piperazinone (**9**) is due to some racemization during the alkaline degradation of the pterin (**4**). Although the values of the rotations are different the slopes of the o.r.d. curves of the two compounds are the same.

Condensation of glycine *N*-methylamide with pyruvic aldehyde gave 1,5-dimethylpyrazin-2-one (**9**)§ which was reduced to 1,5-dimethylpiperazin-2-one (10% Pd–C–H_2–MeOH), and methylated to (±)-1,4,4,5-tetramethylpiperazin-2-on-4-ium iodide This iodide was converted into the hydroxide with silver oxide and neutralised with 2*R*,3*R*-di-*O*-benzoyltartaric acid The diastereoisomeric tartrates were resolved by recrystallisation from propan-2-ol, and converted into the (+)- and (−)-1,4,4,5-tetramethylpiperazin-2-on-4-ium chlorides These had u v , i r , and ^{1}H n m r spectra identical with those of the racemate prepared from 6-methyl-5,6,7,8-tetrahydropterin The (+)-chloride {$[\alpha]_{280}^{20}$ +96° in H_2O},‡ which had u v and ^{1}H n m r spectra, and t l c properties identical with those of the (+)-enantiomer obtained from (+)-(**7**) above, was reduced and demethylated simultaneously by boiling with sodium aluminium bis(2-methoxyethoxy)dihydride in benzene and gave (+)-1,2,4-trimethylpiperazinium dichloride (**10**) {$[\alpha]_{365}^{20}$ +1·0° in 2N-HCl} The absolute configuration of the (+)-salt (**10**) is *S* at C-2 because it was prepared from *S*-(−)-2-methylpiperazine obtained by hydride reduction of the known *S*-(−)-3-methylpiperazin-2,5-dione The dione was, in turn, made by subliming the dipeptide glycyl-*S*-alanine *in vacuo* [15] All the above changes in rotation were confirmed by repeating all the reactions with the enantiomeric compounds

The chiral centre of (−)-6-methyl-5,6,7,8-tetrahydropterin is thus correlated with that of natural *S*-alanine, and by analogy, the absolute configuration at C-6 of natural 5,6,7,8-tetrahydrofolic acid should be *S* Although this conclusion infers that methotrexate binds to dihydrofolate reductase and NADPH (in a ternary complex) with the opposite face of the pteridine ring from that of 7,8-dihydrofolate set to be reduced by NADPH,[16] it must be noted that methotrexate (which is a 2,4-diaminopteridine) is not a substrate for the enzyme and remains unreduced

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§ Only compound (**9**), of the two possible isomers, was isolated from this condensation It was not the isomeric 1,6-dimethylpiperazin-3-one because the $^3J_{C,H}$ values from the proton coupled ^{13}C n m r spectra were consistent with structure (**9**), and because it gave the same compound (**8**) as the one obtained from 6-methylpterin

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