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CHEMICAL MODELLING OF THE THYMIDYLATE SYNTHASE REACTION : EVIDENCE FOR THE FORMATION OF AN EXOCYCLIC METHYLENE INTERMEDIATE FROM ANALOGUES OF THE COVALENT TERNARY COMPLEX FORMED BY INTRAMOLECULAR THIOL ADDITION TO C(6) OF 5-AMINOMETHYLURACIL DERIVATIVES.

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Abstract: The intramolecular thiol addition to C(6) in 1-(3-mercaptopropyl)-5-aminomethyluracil derivatives in protic media leads to the formation of bicyclic 5,6-dihydrouracil derivatives, which may be regarded as models of the covalent ternary complex in the thymidylate synthase reaction. Evidence is presented for the formation of an exocyclic methylene intermediate from these model compounds.

The enzyme thymidylate synthase (E.C. 2.1.1.45) catalyzes the conversion of 2'-deoxyuridine-5'-monophosphate (dUMP) and 5,10-methylene-5,6,7,8-tetrahydrofolate into 2'-deoxythymidine-5'-monophosphate (dTMP) and 7,8-dihydrofolate, respectively. This reaction, which involves the overall transfer of elements of a methyl group from the folate cofactor to the 5-position of the substrate, proceeds in steps in which the cofactor serves both as a source of a methylene moiety and as a reductant[1].

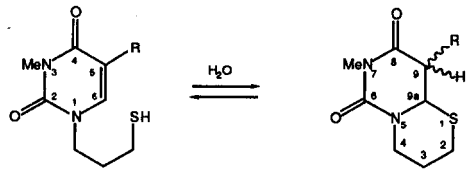
The currently accepted mechanism of the enzymic reaction involves: (1) the formation of a covalent apoenzyme-substrate-cofactor ternary complex, by nucleophilic attack of a cysteine residue of the apoenzyme on the 6- position of dUMP, followed by reaction of the resulting nucleophilic centre at C(5) with the activated form of the cofactor; (2) fragmentation of this complex into an exocyclic methylene intermediate and tetrahydrofolate and (3) reduction of the intermediate by tetrahydrofolate with concomitant expulsion of the apoenzyme, resulting in the formation of dTMP and dihydrofolate.

In previous studies[2,3] we have demonstrated that unsymmetrically substituted imidazolidines can serve as models of the cofactor in mediating both carbon and hydride transfer reactions. In fact, we have been able to show that it is possible to mimic the complete thymidylate synthase reaction in transferring an overall methyl group from a folate model to the 5-position of 6-methylamino-1,3-dimethyluracil[3]. Although 6-aminouracil derivatives may be regarded as mimics of the apoenzyme-substrate binary complex, it was recognized by us, that these model compounds are at a higher oxidation state than the natural binary complex.

In this communication we present a model of the ternary complex in the thymidylate synthase reaction which is at the same oxidation state as the natural complex and we provide evidence for the formation of an exocyclic methylene intermediate from these model compounds.

It was shown by Brown et al[4] that 1-(3-mercaptopropyl)uracil can undergo a reversible ring closure at C(6) to give a bicyclic 5,6-dihydrouracil derivative. In order to investigate the influence of C(5) substituents on the ring closure reaction we have synthesized compounds **1a,b,c** and shown that in protic media an equilibrium exists between the open chain compounds and the ring closed compounds **2a,b,c**. Compound **2a** and the isomers of **2b** and **2c** have been isolated and characterized[5] (Scheme I).

At pH 8-13 **1a** exhibits a H/D exchange at C(5) at room temperature. ^{1}H NMR data revealed that the exchange reaction proceeds via a stereospecific trans diaxial addition of the thiol anion and the deuteron across the 5,6 double bond, followed by enolization and a concomitant nonstereospecific deuteration of the carbanion at C(5).



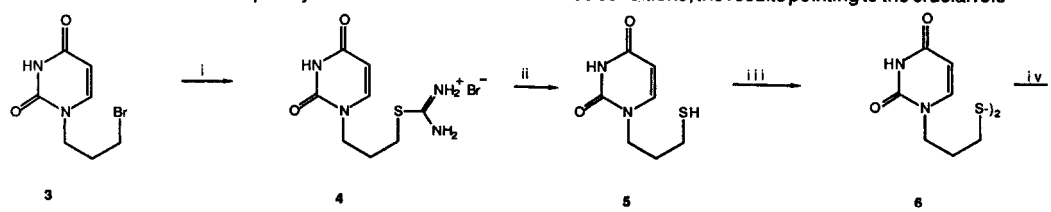
1a R=H
1b R=F
1c R=Me

2a R=H
2b R=F
2c R=Me

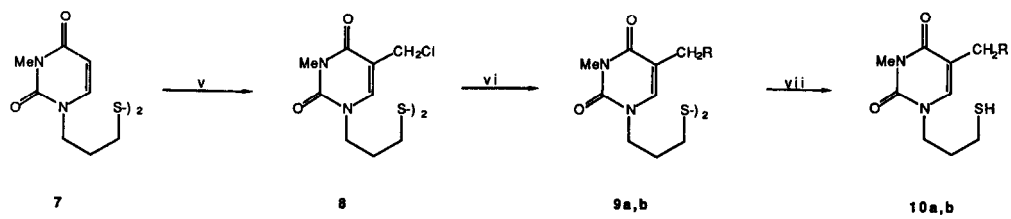
Scheme I

The results and the mechanistic aspects of the addition reactions, which will be discussed in detail in a full paper, encouraged us to undertake the development of suitable 1-(3-mercaptopropyl)-5-aminomethyluracil derivatives **10a,b**, since ring closure involving the thiol group in these compounds would lead to models of the ternary complex in the thymidylate synthase reaction. The syntheses of these model compounds is outlined in scheme II. In the last step of the synthesis of **10a** an equilibrium mixture of **10a** and isomers of **11a** (<1%) is obtained, which can be separated by silica-gel chromatography[5].

When **10a** is dissolved in methanol, the amine **13** is released (ca 50 %, 2 days) at room temperature. The reaction mixture was shown to contain several, as yet unidentified products, besides **13** and the starting material. Significantly, the uracil derivative **12a** was completely stable under the aforementioned conditions; the results pointing to the crucial role



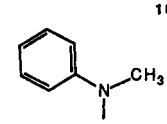
3 4 5 6



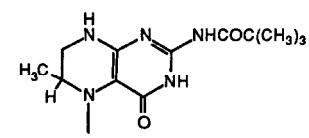
7 8 9a,b 10a,b

(i) thiourea; (ii) NaOH;
(iii) H_2O_2; (iv) MeI/K_2CO_3; (v) CH_2O/HCl; (vi) amine;
(vii) dithiothreitol.

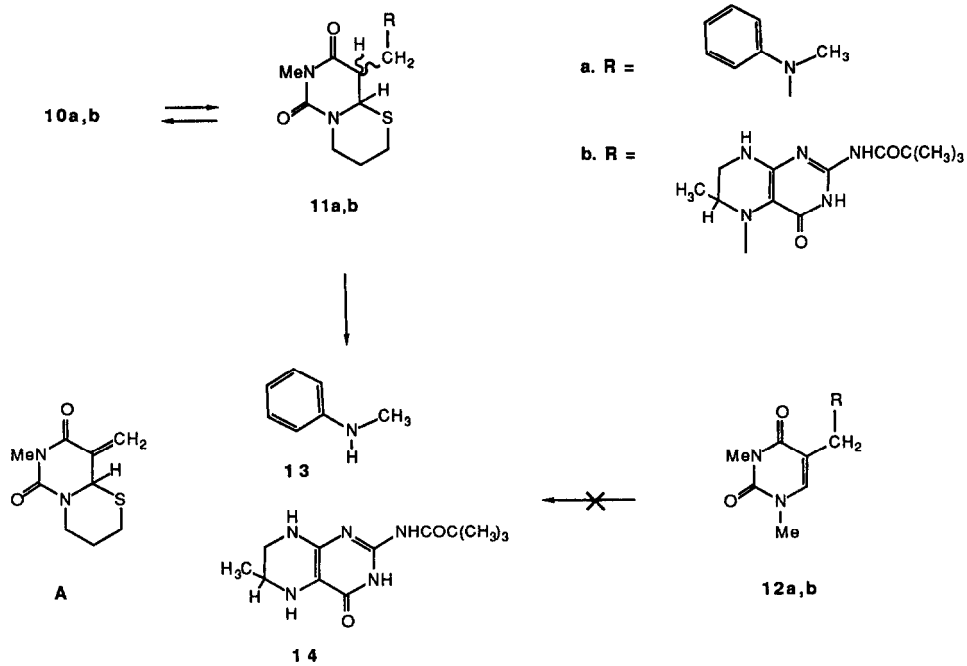
a. R =



b. R =



Scheme II



Scheme III

of the thiol group in the fragmentation of **10a** to **13**. These results can be best accounted for in terms of the formation of the isomers of **11a** and their fragmentation into intermediate **A** and amine **13** (Scheme III).

The analogous cyclization products of **10b**, namely the isomers of **11b**, represent close mimics of the ternary covalent complex in the thymidylate synthase reaction. Upon standing in methanol, as expected, **10b** releases tetrahydropteridine derivative **14**, presumably via the sequence **10b** → **11b** → **14** + **A**. The uracil derivative **12b** was shown to be completely stable under these conditions .

Evidence for the formation of **A** from both **10a** and **10b** was obtained by Fast Atom Bombardment Mass Spectroscopy (FAB-MS). Thus, when either **10a** or **10b** was dissolved in a glycerol matrix, the FAB mass spectrum was shown to contain a peak at m/e 213, which represents the mass of the protonated intermediate **A**. The structure of this fragment was established by its exact mass[6] and by (+)FAB-MIKES(CA) [Mass Analysed Ion Kinetic Energy Spectroscopy/Collision Activation] determination[7]. When the acidity of the matrix was increased by addition of thioglycerol, the intensity of the m/e 213 peak was increased substantially. This implies that the fragmentation of **10a,b** into **A** is initiated by protonation of the amine moiety of these model compounds.

Studies directed towards the isolation of intermediate **11b** (isomers) and **A** are currently in progress.

Acknowledgments

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References and notes

* To whom enquiries may be addressed

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5. ^{1}H NMR ($CDCl_3$, 250 MHz): **2a**: 1.67-1.94 (m, 2H, 2xC(3)H), 2.67 (dd, 1H, C(9)Heq, $J(H_9,H_{9a})$=1.1, J(gem)=17.4), 2.70-2.96 (m, 2H, C(4)H + C(2)H), 3.12 (dd, 1H, C(9)Hax, $J(H_9,H_{9a})$=7.0, J(gem)=17.4), 3.13 (m, 1H, C(2)H), 3.20 (s, 3H, NCH_3), 4.48 (m, 1H, C(4)H, J(gem)=14.1), 4.83 (dd, 1H, C(9a)H, $J(H_{9a},H_9eq)$=1.1, $J(H_{9a},H_9ax)$=7.0); **2b** (trans adduct): 1.76-2.0 (m, 2H, 2xC(3)H), 2.81-3.2 (m, 3H, 2xC(2)H + C(4)H), 3.25 (s, 3H, NCH_3), 4.49 (m, 1H, C(4)H), 5.21 (dd, 1H, C(9a)H, $J(H_{9a},F)$=1.5, $J(H_{9a},H_9)$=6.5), 5.25 (dd, 1H, C(9)H, $J(H_9,F)$=47.8, $J(H_9,H_{9a})$=6.5); **2b** (cis adduct): 1.76-2.0 (m, 2H, 2xC(3)H), 2.8-3.2 (m, 3H, 2xC(2)H + C(4)H), 3.26 (s, 3H, NCH_3), 4.55 (m, 1H, C(4)H), 4.75 (dd, 1H, C(9)H, $J(H_9,F)$=46.5, $J(H_9,H_{9a})$=1.8), 5.03 (dd, 1H, C(9a)H , $J(H_{9a},F)$=19.0, $J(H_{9a},H_9)$=1.8); **2c** (trans adduct): 1.26 (d, 3H, C(9)CH_3, J=7.0), 1.72-1.88 (m, 2H, 2xC(3)H), 2.81-3.12 (m, 4H, 2xC(2)H + C(4)H + C(9)H), 3.22 (s, 3H, NCH_3), 4.50 (m, 1H, C(4)H), 4.79 (d, 1H, C(9a)H, $J(H_{9a},H_9)$=5.8); **2c** (cis adduct):1.38 (d, 3H, C(9)CH_3, J=7.4), 1.72-1.88 (m, 2H, 2xC(3)H), 2.81-3.12 (m, 4H, 2xC(2)H + C(4)H +C(9)H), 3.22 (s, 3H, NCH_3), 4.50 (m, 1H, C(4)H), 4.53 (s, 1H, C(9a)H); **10a**: 1.25 (t, 1H, SH, J=7.5), 1.88 (quint, 2H, CH_2 , J=6.6), 2.42 (quart, 2H, CH_2S, J=7.0), 3.04 (s, 3H, NCH_3), 3.37 (s, 3H, N(3)CH_3), 3.82 (t, 2H, N(1)CH_2, J=6.6), 4.27 (s, 2H, C(5)CH_2), 6.67-6.79 (m, 3H, ArH), 6.90 (s, 1H, C(6)H), 7.20-7.28 (m, 2H, ArH); **11a** (trans adduct): 1.68-1.98 (m, 2H, 2x C(3)H), 2.65-3.10 (m, 4H, 2xC(2)H + C(4)H + C(9)H), 2.98 (s, 3H, N(11)CH_3), 3.25 (s, 3H, N(7)CH_3), 3.35-3.50 (m, 1H, C(10)H), 4.23 (d, 1H, C(10)H, J=11.6), 4.44-4.50 (m, 1H, C(4)H), 4.77 (m, 1H, C(9a)H), 6.78 (m, 3H, ArH), 7.25 (m, 2H, ArH); **11a** (cis adduct):1.68-1.98 (m, 2H, 2xC(3)H), 2.65-3.10 (m, 4H, 2xC(2)H + C(4)H + C(9)H), 3.08 (s, 3H, N(11)CH_3), 3.25 (s, 3H, N(7)CH_3), 3.40-3.50 (m, 1H, C(10)H), 3.78 (dd, 1H, C(10)H, J=11.0, J= 14.8), 4.44-4.50 (m, 1H, C(4)H), 4.66 (s, 1H, C(9a)H), 6.78 (m, 3H, ArH), 7.25 (m, 2H, ArH).
6. Exact mass : Calc. for $C_9H_{13}N_2O_2S$ (**A**+H^+): 213.0698 ; Found: 213.0702
7. The full details of these mass spectral studies will be discussed elsewhere.

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