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MNDO study of the mechanism of the inhibition of cysteine proteinases by diazomethyl ketones

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Abstract. Diazomethyl ketones are one of the most effective irreversible inhibitors of cysteine proteinases and are therefore very important in drug design. In the present study a mechanism of inactivation is proposed based on the results of model MNDO calculations of the possible pathways. It was found that the mercaptide nucleophile, on approaching the carbonyl carbon as in the catalytic reaction path, binds to the inner diazo nitrogen. The intermediate thus formed can rearrange giving a stable product, β-thioketone, and molecular nitrogen, with a considerable energy gain. The energy barrier to this process is equal to 36.9 kcal/mol, and corresponds to a pyramidal transition state with the vertex at the methylene carbon and the base formed by the carbonyl, thiol, and diazo groups. The energy barrier can be lowered on deprotonation of the intermediate. Based on the results obtained it was concluded that good irreversible inhibitors of cysteine proteases must fulfil two structural requirements: *i)* the dimensions and charge distribution must be similar to those of the peptide bond and *ii)* a second electrophilic center must be present in the neighbourhood of the carbonyl carbon. These are requirements which are satisfied by other strong cysteine proteinase inhibitors: β-chloroketones and β-ketooxiranes.

Key words: Cysteine proteinase inhibitors – Diazomethyl ketones – Nucleophilic attack – Reaction path calculations – MNDO method

Introduction

Thiol proteinases play a critical role in three major groups of biochemical processes: the intracellular catabolism of peptides and proteins (Barrett and Kirschke 1981), the processing of prohormones and proenzymes (Marks et al. 1986; Taugner et al. 1985), and the penetration of normal tissues by malignant cells (Sloane and Honn 1985) and, possibly, microorganisms (Barrett et al. 1984). These enzymes occur in both plants and animals, the best known representative used in almost all model studies being papain (Glazer and Smith 1971).

It has been shown (Polgar and Halasz 1982) that the thiol group of the active centre cysteine is directly involved in the catalytic reaction. This fact was the starting point of the hypothesis that the catalytic reaction path is analoguous to that of serine proteases (Polgar and Halasz 1982), i.e. after sulphydryl deprotonation by the imidazole ring of histidine the carboxyamide group of the target peptide undergoes nucleophilic attack by the mercaptide anion, giving a tetrahedral intermediate. Very recent theoretical studies of cysteine proteinase catalysis (Howard and Kollman 1988; Arad et al. 1990) and the closely related mechanism of glucosamine synthase action (Tempczyk et al. 1989, 1990) have demonstrated, however, that such a tetrahedral intermediate that is stable (energy minimum) in the case of the oxygen nucleophile does not occur at all for the sulphur nucleophile, and only an inflection point on the energy surface is present (Howard and Kollman 1988; Arad et al. 1990). The situation does not change on including an environment effect (Howard and Kollman 1988). This fact and other conclusions drawn in the studies mentioned have suggested that although the mercaptide anion is the attacking nucleophile, the simultaneous protonation or the amide nitrogen must occur (Tempczyk et al. 1990; Howard and Kollman 1988; Arad et al. 1990).

All the natural animal cysteine proteinase are conjugated with their natural inhibitors – the cystatins (Barrett et al. 1986, 1987). These inhibitors are reversible and act mainly as regulators of endogenic cysteine proteinase, having only a limited capacity for the effective inactivation of the enzymes of the invading microorganisms. Therefore, the design of drugs acting in this direction has been aimed at artificial irreversible inhibitors (Grubb et al. 1990), of which diazomethyl ketone derivatives have been shown to be amongst the most effective, in both model reactions (Green and Shaw 1981) and in tests on

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microorganisms of the *Streptococcus* group (Biörck et al. 1989).

The rational design of enzyme inhibitors requires the elucidation of their mechanism of action at the molecular level. So far no mechanism has been proposed for diazomethly ketones, despite their importance. In the present study, based on model compounds, we have investigated by means of quantum mechanics the possible products of irreversible inhibition and the corresponding pathways.

Methods

Closed-shell RHF LCAO calculations were performed by means of the semiempirical MNDO method of Dewar and Thiel (1977). All calculations were performed with the use of a MOPAC package (Stewart 1990) with inherent parameterisation. The calculations included geometry optimisation (MOPACMIN), saddle point localisation (MOPACSAD), transition state geometry optimisation (MOPACNLL), and force constant matrix calculation (MOPACFOR). Unfortunately, there is at present no parameterisation for sulphur in the AM1 method and this makes calculations using this method impossible.

Results and discussion

Based on earlier studies (Howard and Kollman 1988) we have assumed that the first stage of inhibition involves the protonation of the diazomethylketo group and the subsequent attack of the mercaptide nucleophile. This is justified by the similar geometric parameters and charge distribution of the peptide and diazomethylketo group (Fig. 1). Based on the charge distribution it can be inferred that the protonation site is the methin carbon.

The cysteine side chain and inhibitor active site were modelled by H_2S and diazoacetic aldehyde, respectively. According to our assumption we investigated the MNDO proton transfer energy from H_2S to diazomethylaldehyde first (1) and compared it with that for the transfer of H_2S to the formamide group (2).

$$\underset{\text{(I)}}{H-CO-CH=N=N}+H_2S$$

$$=\underset{\text{(II)}}{[HCO-CH_2-\overset{\oplus}{N}\equiv N]}+HS^- \qquad (1)$$

$$H-CO-NH_2+H_2S=H-CO-NH_3^+ +HS^- \qquad (2)$$

The values were found to be +155.1 and +169.4 kcal/mol, respectively, supporting the probability of the occurrence of this process in the case of diazomethyl ketones. In fact, the proton transfer energy, though large *in vacuo*, is likely to be lowered considerably or even reversed in the enzymatic cavity, owing to the presence of imidazole moieties and ionised carboxylic groups which can form a proton-transfer chain, as found in the study by Arad et al. (1990). Moreover, system (II) should rather be considered as a boundary structure, as the proton transfer process probably occurs together with nucleophilic attack (Tem-

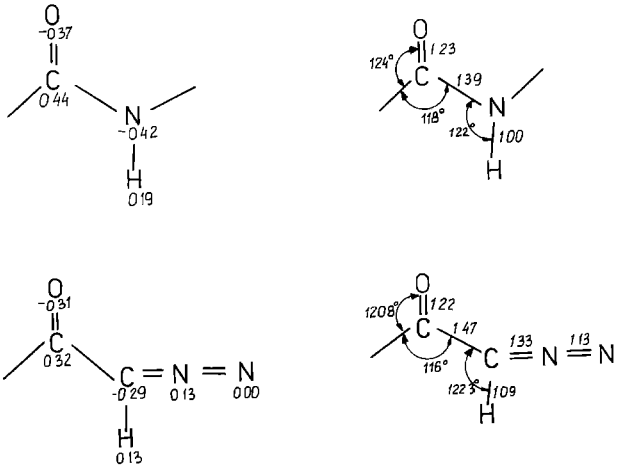


Fig. 1. MNDO charge distribution (left) and geometric parameters (right) of: **a)** peptide bond, **b)** diazomethyl ketone

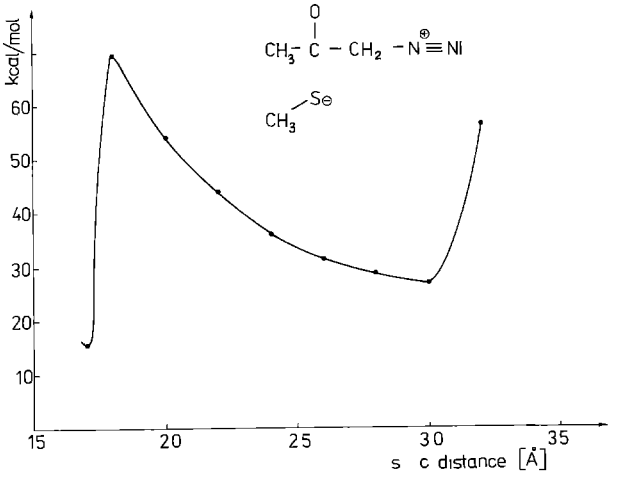


Fig. 2. Calculation variation of energy with S–C distance of reaction (3): see text

pczyk et al. 1990; Howard and Kollman 1988; Arad et al. 1990).

By analogy with the catalysis process the following reaction path with diazomethane liberation can be expected: (3)

$$H-CO-CH_2-\overset{\oplus}{N}\equiv N+HS^- =H-CO-SH+CH_2N_2$$

To obtain preliminary information about the energy surface, we calculated the pathway of (3), choosing the carbonyl carbon ··· sulphur distance as the reaction coordinate. Actually in these calculations we chose diazoacetone and methyl sulphide as model compounds. The reasons for doing so were the relatively low complexity of the computation (one-dimensional reaction path only) and avoiding the participation of the hydrogen atom substituted for the remaining part of the enzyme and substrate in the reaction investigated. However, diazomethane liberation occurred at as short a C ··· S distance as 1.7 Å with an energy barrier of about 42 kcal/mol (Fig. 2). As is shown in Fig. 2, an energy minimum is revealed prior to diazomethane liberation at d(C ··· S)=3.0 Å. This mini-

mum corresponds to the formation of an intermediate in which sulphur is bonded to the inner diazo nitrogen, as indicated in Eq. (4).

$$H-CO-CH_2-\overset{\oplus}{N}\equiv N+HS^- \qquad (4)$$

$$=H-CO-CH_2-\overset{SH}{\underset{+}{N}}=\underline{N}$$

(III)

It can be thus inferred that even if the nucleophile is directed at the carbonyl carbon, a situation very likely to occur in the enzymatic cavity, it binds as a result to the stronger electrophile – the positively charged diazo nitrogen. The optimized geometry of the resulting intermediate (III) is shown in Fig. 3a and Table 1.

The fact that intermediate (III) is not likely to decompose into thioacid and diazomethane (the right side of (3)) is confirmed by the experimental observation that the $C-C$ bond in diazoketones is very stable (Bayless et al. 1968). On the other hand, another possible way of rearranging intermediate (III) is the binding of sulphur to the diazo carbon with the simultaneous loss of the nitrogen molecule, as shown in (5). This is consistent with the experimental observations that the degradation of diazoketones usually results in the loss of nitrogen (Kaufman et al. 1965; Bayless et al. 1968). It should be noted that the mechanisms of cysteine protease inactivation by diazoketones which were elaborated based on the experimental data (mainly the pH dependence of the inactivation rate) also involved the formation of β-thioketone and molecular nitrogen (Brocklehurst et al. 1978)

$$\underset{(III)}{H-CO-CH_2-\overset{SH}{\underset{+}{N}}=\underline{N}} \rightarrow \underset{(IV)}{H-CO-CH_2\overset{SH}{\diagup\diagdown}N=N}$$

$$\rightarrow \underset{(V)}{H-CO-CH_2-SH+N_2} \qquad (5)$$

One more argument that the rearrangement of (III) occurs via route (5) is the fact that in this case the products are much more stable energetically than in the case of route (3) (59.6 kcal/mol energy gain vs 78 kcal/mol energy loss, respectively for data corresponding to the reaction of diazoformaldehyde with hydrogen disulphide). Therefore as the next stage of our study we investigated the barrier of this reaction which corresponds to the transition state (IV). We used the SADDLE option of the MOPAC package, taking as starting points the optimized geometries of (III) and (V). The initial approximation of the transition state geometry obtained was then optimized with the use of the NLLSQ option. The structure and geometric parameters are shown in Fig. 3b and Table 2, respectively.

As shown in Fig. 3b, the transition state has, in fact, the form of a flat trigonal pyramid with the vertex at the CH_2 group and the based formed by the SH, CO, and N_2 groups. The results of force constant analysis reveal only one negative force constant of −0.68904 mdynes/Å which indicates that (IV) is a "true" transition state. Graphical representation of two structures of the transition state (IV) corresponding to the negative eigenvector are displayed in

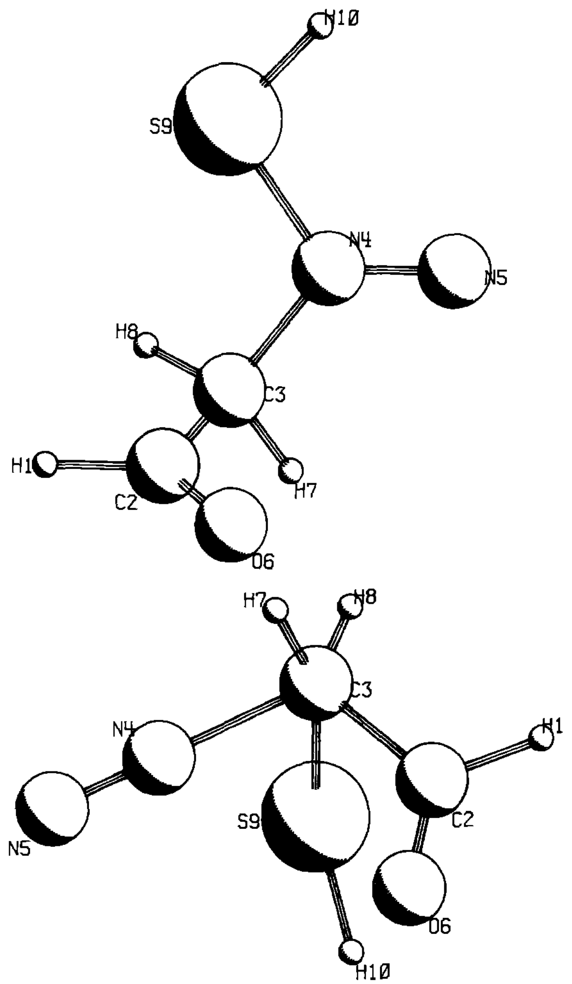


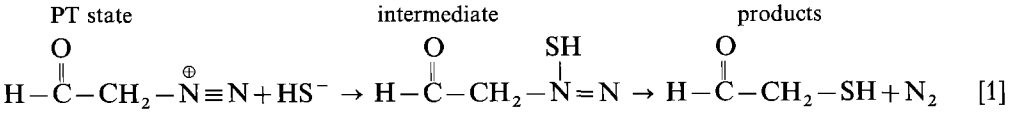
Fig. 3. MNDO-optimized geometry of: **a)** intermediate (III) and **b)** transition state (IV)

Table 1. The optimized geometry of the intermediate (III) and transition state (IV), their tautomeric (III′), (IV′) and anionic forms (III″), (IV″)

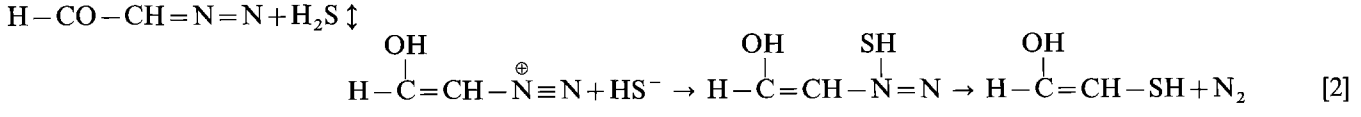
Atoms	Species					
	(III)	(IV)	(III′)	(IV′)	(III″)	(IV″)
	Bond length (Å)					
C(2)–C(3)	1.535	1.525	1.361	1.388	1.415	1.499
C(3)–N(4)	1.511	1.636	1.454	1.423	1.417	1.347
N(4)–N(5)	1.185	1.109	1.192	1.129	1.165	1.160
C(2)–O(6)	1.218	1.221	1.343	1.361	1.242	1.228
N(4)–S	1.726	2.323	1.724	2.079	1.853	2.872
C(3)–S	2.634	2.232	2.630	2.030	2.620	2.357
	Bond angle (deg)					
C(2)–C(3)–N(4)	111.7	116.1	127.1	120.0	122.9	121.2
C(3)–N(4)–S	108.7	66.0	111.4	67.0	95.8	54.4
C(2)–C(3)–S	99.9	90.2	159.6	115.4	141.7	93.9
N(4)–C(3)–S	–	83.0	–	74.0	–	98.0
C(3)–N(4)–N(5)	127.0	180.8	127.1	160.6	136.2	148.8
	Torsion angle (deg)					
C(2)–C(3)–N(4)–N(5)	102.1	−105.5	− 17.0	− 52.6	− 48.4	−89.4
C(2)–C(3)–N(4)–S	78.3	81.0	160.7	108.7	132.7	99.5

Fig. 4. As shown, movement in one direction along this eigenvector results in the breaking of the S–N and C–N bond and the shortening of the C–S distance, thus leading to the products, while the backward movement returns to intermediate (III). As shown in Table 2, the energy barrier is of about 40 kcal/mol, a comparatively high value. This might be connected both with the energy expense required for the breaking of the S–N and C–N bonds, and with the fact that the central carbon is pentavalent in the transition state. A possibility of lowering the barrier can be sought in assuming that either the tautomeric or anionic form of IV undergo rearrangement. All possible reaction routes are therefore as follows:

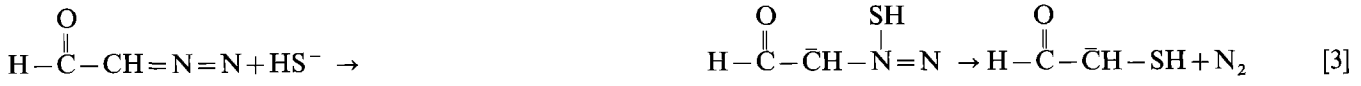
PT state intermediate products



[1]



[2]



[3]

The tautomeric (enolic) form (III′) is energetically less stable than the keto form (Table 2). However, additional stability can be gained by the formation of a hydrogen bond between the enol proton and the terminal negatively charged nitrogen which cannot be obtained in MNDO calculations without special hydrogen-bonding terms (Voityuk and Bliznyuk 1987). As shown in Table 2, the energy barrier to rearrangement is even higher than in the case of the keto form. This route can therefore be excluded. The structure and geometric parameters of (III′) and (IV′) are shown in Table 1 and Fig. 5a, b.

In anion (III″) the C–S bond is weakened considerably, as the bond order is 0.6 compared to the 0.8 of the protonated keto tautomer. First of all it must be pointed out that intermediate (III) is also stable after deprotonation and decomposes into the β-thioketone anion and the nitrogen molecule. In view of the fact that the desired products are not formed on nucleophilic attack of the lone mercaptide anion of the carboxyamide group, this observation is not trivial. The structure and geometric parameters of (III″) and the corresponding transition state (IV″) are shown in Fig. 6a, b and Table 1, respectively, while the energetic effects of the reaction are shown in Table 2.

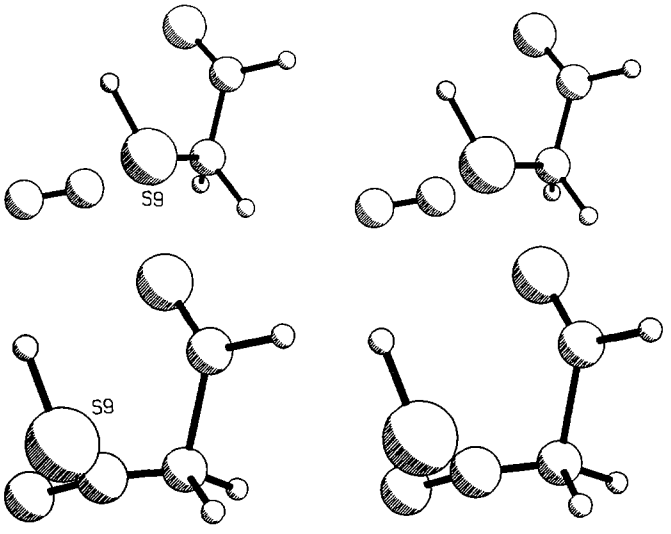


Fig. 4. Stereo drawings of two structures of the transition state (IV) corresponding to movement along the negative eigenvector (to the cartesian coordinates of the transition state were added the coordinates of the negative eigenvector multiplied by 0.1 or −0.1)

As shown in Table 2, the energy barrier to rearrangement is 10.6 kcal/mol only. On the other hand, it must be kept in mind that a lot of energy must be spent on deprotonation of intermediate (III). This supports the earlier assumption (Tempczyk et al. 1990) that the proton is not completely transferred to the target substrate, but rather a dynamic proton-transfer state occurs during the reaction. In such a case a structure intermediate between keto (III) and anionic (IV′) form would occur, thus facilitating the rearrangement process.

In conclusion, the mechanism of the inhibition of cysteine proteinases by diazomethyl ketones can be summarised in Fig. 7.

There are two crucial points in the inhibition reaction which occur according to the mechanism postulated in this work and which can be employed in the design of irreversibly acting cysteine proteinases. First, the charge distribution of the target centre must resemble that of the peptide bond. This explains the fact that oxiranes which are very good reactants in processes involving sulphur

Table 2. MNDO formation heat (kcal/mol) of the various stages of the reaction

	*	**	***
substrates	31.5	31.5	344.9
PT state	186.6	171.3	–
intermediate	43.3	46.1	336.0
transition state	80.2	95.9	346.6
products	−28.1	−23.4	303.0

* according to the Eq. (5) – reaction path [1]
** rearrangement involving the enolic form of (III) – reaction path [2]
*** rearrangement involving the anionic form of (III) – reaction path [3]

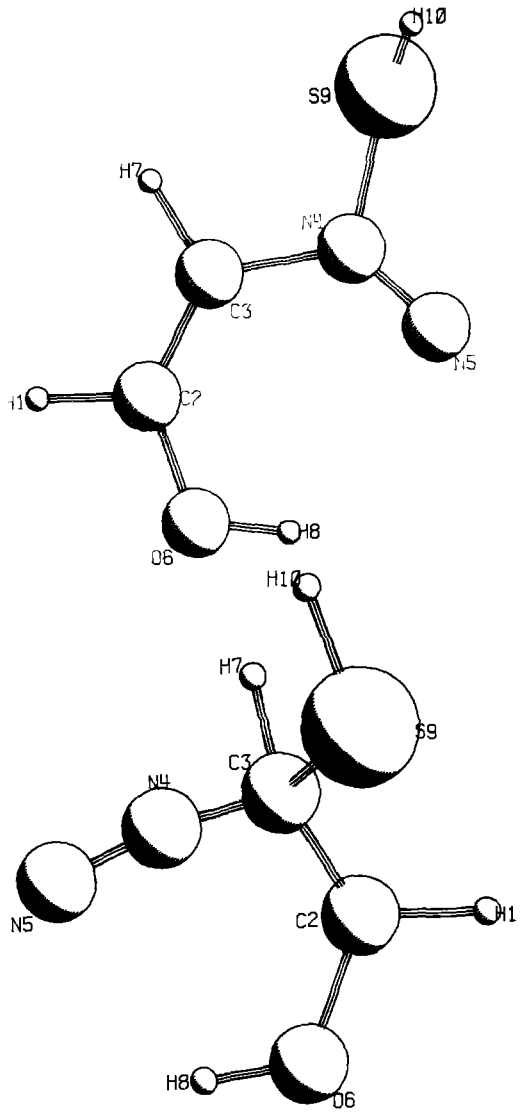


Fig. 5. MNDO-optimized geometry of the enolic form of: **a)** intermediate (III′) and **b)** transition state (IV′)

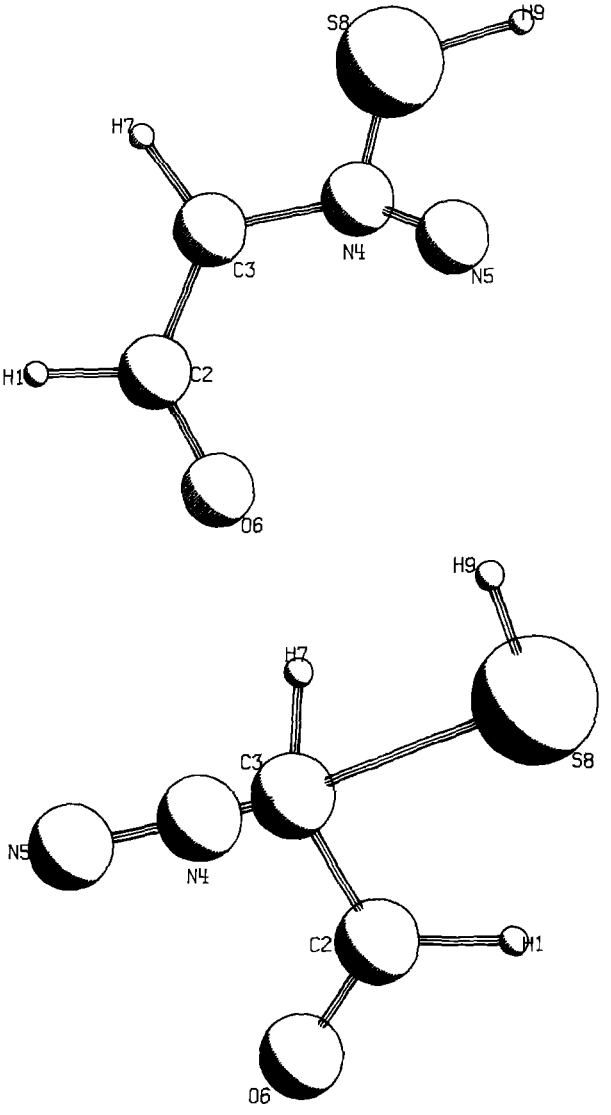


Fig. 6. MNDO-optimized geometry of the anionic form of **a)** intermediate (III″) and **b)** transition state (IV″)

nucleophilic attack, in general, block cysteine proteinases only when the keto group is attached to the oxirane ring (Suzuki 1983; Hashida et al. 1980). In the last case the charge distribution is presumably similar to that in the peptide bond. The second point is the presence of another strong electrophilic centre in the position β to the keto group which would "catch" the mercaptide anion initially directed toward the carbonyl carbon and results in the irreversible binding of the inhibitor to the thiol group. All the effective irreversible inhibitors of cysteine proteinases, i.e. diazomethyl ketones, β-ketooxiranes, and β-chloroketones fulfil these two requirements. The confirmation of the hypothesis advanced here requires, however, the study of all the classes of inhibitors mentioned. These studies are being carried out in our laboratory.

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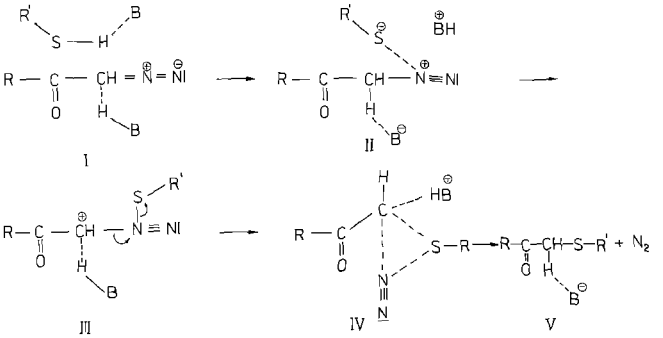

Fig. 7. Proposed mechanism for the inhibition of cysteine proteinases by diazomethyl ketones

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