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Investigation of the inhibition pathway of glucosamine synthase by N^3-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid by semiempirical quantum mechanical and molecular mechanics methods

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Abstract. Glucosamine synthase (E.C. 2.6.1.16) is a promising target in antifungal drug design. It has been reported that its potent inhibitor, N^3-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid (FMDP), inactivates the enzyme by the Michael addition of the S-H group to the FMDP molecule followed by cyclisation reactions. In this study we have investigated, by means of semiempirical MNDO, PM3 and molecular mechanics methods, the energetics and kinetic possibility of the formation of various stereoisomers of the products of cyclisation of the Michael addition products detected experimentally. It was found that the substituted 1,4-thiazin-3-one can be formed in one step under alkaline conditions; the stereoisomers of this compound predicted to be the most stable on the basis of theoretical calculations are also the dominant ones in reality.

Key words: Glucosamine-6-phosphate synthase – N3-(4-methoxyfumaroyl)-L-2,3-diamino-propanoic acid inhibition mechanism – Semiempirical quantum-mechanical methods – Molecular mechanics – Conformational analysis

Introduction

N^3-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid (FMDP) ((*I*); Fig. 1) is a potent covalent inactivator of glucosamine-6-phosphate synthase (E.C.2.6.1.16) – a key enzyme in aminosugar metabolism (Andruszkiewicz et al. 1986; Milewski et al. 1985). FMDP-peptides have been developed as highly active antifungal agents which impair the building of glucosamine-containing fungal cell wall macromolecules (Andruszkiewicz et al. 1987, 1990; Milewski et al. 1988). A mechanism of enzyme inactivation by FMDP has recently been proposed by Kucharczyk et al. (1990), on the basis of the analysis of the products of a model reaction of L-cysteine with FMDP. They have found that the first stage of inactivation is the formation of the Michael addition product (*II*) which then undergoes cyclisation, the result of which depends on hydrogen ion concentration. In alkaline and neutral conditions the cylic thiazinone (*IV*) is formed as a mixture of two diastereoisomers (98:2), while in acid media succinimide (*III*) can also be isolated. The latter, in alkaline conditions, isomerises to (*IV*), with a yield of the respective diastereoisomers 98:2.

Based on the evidence of isomerisation these authors suggested that even in alkaline media the five-membered ring succinimide (*III*) is, in fact, an intermediate in the formation of the more stable product, thiazinone (*IV*). They supported this assumption by the complexity of the kinetics of the cyclisation process, as well as by the high diastereoisomer ratio, which can be explained by the enolisation of (*III*) (Kucharczyk et al. 1990). On the other hand, in alkaline or neutral media, the attack of the N-terminal cysteine amino group on the ω-carbonyl group of FMDP seems to be more probable than the internal cyclisation of the FMDP fragment, owing to the greater nucleophilicity of the amino nitrogen. This, together with the simultaneous or subsequent rearrangement of the backbone FMDP fragment to its terminal carbonyl carbon would give 2-substituted 1,4-thiazin-3-one-5-carboxylate (*IV*) (Fig. 2).

Under acid conditions the amino group is obviously inactivated owing to protonation and the amido nitrogen remains the only active nucleophile which can explain the formation of the five-membered ring succinimide (*III*), probably according to the mechanism presented in Fig. 3.

The present preliminary study, which is a continuation of our two earlier papers on the theoretical investigation of glucosamine synthase action (Tempczyk et al. 1989, 1992), was aimed at the verification of this hypothesis mainly by means of the conformational analysis of the

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Abbreviations: FMDP: N^3-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid; MNDO: Modified Neglect of Diatomic Overlap; AM1: Advanced Method 1; PM3 (full abbreviation: MNDO-PM3): Modified Neglect of Diatomic Overlap, Parametrised Method 3.

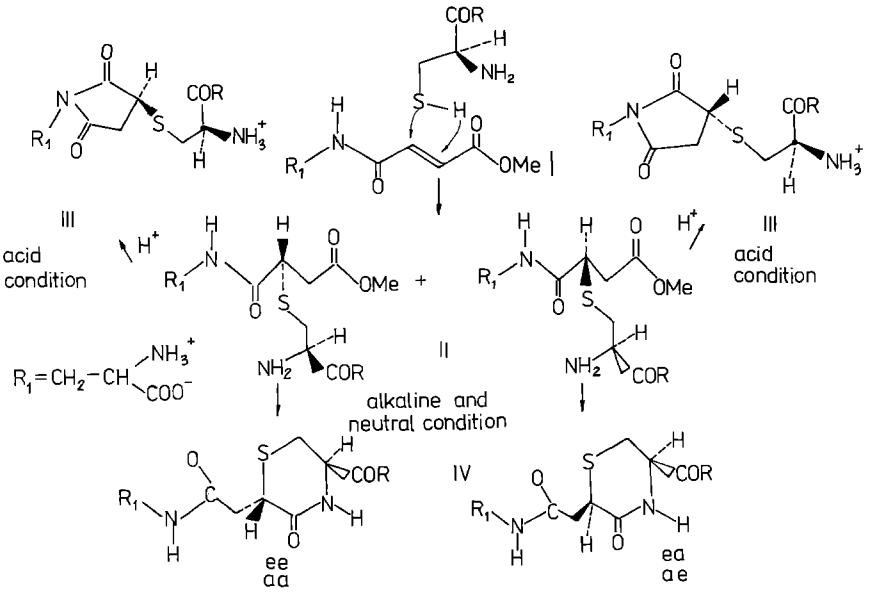


Fig. 1. The isolated products of the model reaction of L-cysteine with FMDP

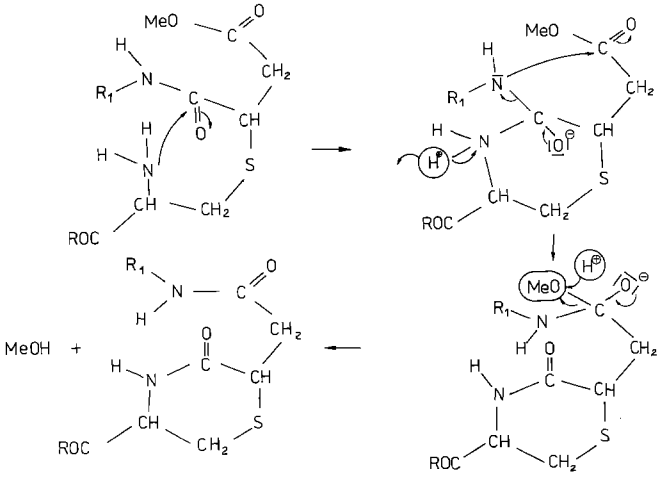


Fig. 2. The proposed mechanism of 6-membered thiazonone formation under alkaline conditions

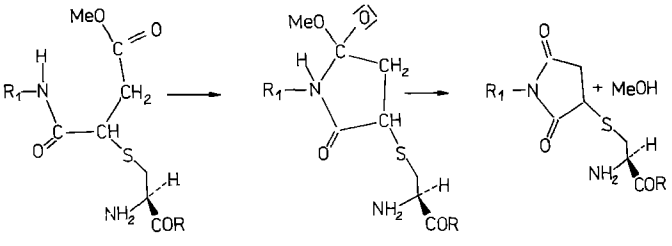


Fig. 3. The proposed mechanism of 5-membered thiazonone formation

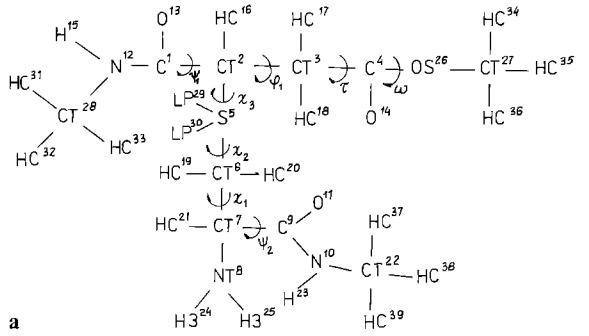
linear Michael addition product (*II*) and by a comparison of the relative energies of the conformations leading to various stereoisomers of (*IV*) with the experimental occurrences of the latter. Moreover, the energy of the cyclisation process was evaluated by means of combined quantum mechanical MNDO, PM3 and molecular mechanics calculation.

Methods

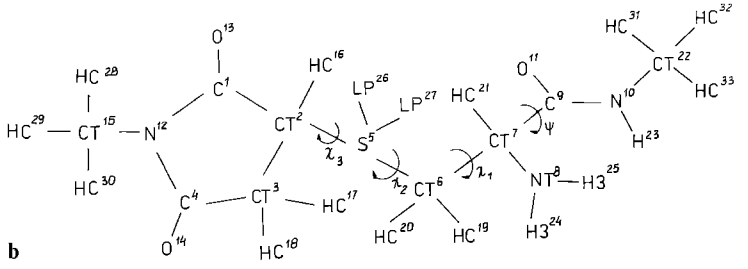
The energetics of the reaction were examined by means of a semiempirical MNDO (Dewar et al. 1977) and PM3 method (Stewart 1989a, b) which are included in the MOPAC package (Stewart 1990). The output values are the gas-phase enthalpies of formation of the compounds studied (Stewart 1990). Of the two, the PM3 method is definitely more advanced and proved to reproduce better the formation heats and the energies of association of small molecules (Stewart 1989b, 1990). In particular the heats of formation of amides and sulphides, whose functional groups occur in the compounds studied in this work, are better reproduced by PM3 (Stewart 1989b). However, owing to the still early stage of development of this method, the evaluation of its accuracy is not as advanced as that of MNDO. Therefore the comparison of the results of the two semiempirical methods should enable more realistic conclusions to be drawn. On the other hand, we could not use another advanced semiempirical method AM1 (Stewart 1990), because no consistent parametrisation for sulphur was available in our package for this method.

The model compounds chosen were (*II'*), (*III'*), and (*IV'*) in which the terminal parts of the molecules not entering directly into the reaction were replaced by hydrogen atoms. Energy was minimised with respect to all geometric variables. Owing to the virtual instability of hydrogen bonds in the MNDO treatment (Voityuk et al. 1987) and still underestimated hydrogen-bond energies in the PM3 method (Stewart 1989b), fully extended reference conformations with no hydrogen bonds were chosen as starting points for MNDO (PM3) energy minimisation.

For molecular mechanics calculations the model compounds with terminal parts replaced by the methyl groups were chosen (Fig. 4a–c), in order to prevent the formation of these H-bonds which do not occur in reality. The calculations were performed in Weiner's potential



a



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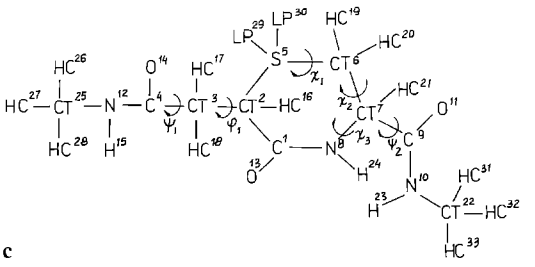

c

Fig. 4. The atom numbering system and dihedral angle indication for the model compounds for molecular mechanics: **a** (*II″*), **b** (*III″*), **c** (*IV″*)

Table 1. The charges (*II″*), (*III″*), and (*IV″*) used in molecular mechanics calculations (atom numbering system in Fig. 4a–c)

Atom	Charge (e)	Atom	Charge (e)	Atom	Charge (e)	Atom	Charge (e)
a) Compound (*II″*)							
C (1)	0.351	O (11)	−0.356	HC (21)	0.016	HC (31)	0.010
CT (2)	−0.019	N (12)	−0.400	CT (22)	0.164	HC (32)	0.010
CT (3)	−0.017	O (13)	−0.355	H (23)	0.178	HC (33)	0.010
C (4)	0.317	O (14)	−0.302	H3 (24)	0.109	HC (34)	0.010
S (5)	0.681	H (15)	0.203	H3 (25)	0.104	HC (35)	0.010
CT (6)	0.024	HC (16)	0.034	OS (26)	−0.278	HC (36)	0.010
CT (7)	0.066	HC (17)	0.076	CT (27)	0.165	HC (37)	0.010
NT (8)	−0.266	HC (18)	0.049	CT (28)	0.161	HC (38)	0.010
C (9)	0.365	HC (19)	0.020	LP (29)	−0.4045	HC (39)	0.010
N (10)	−0.410	HC (20)	0.040	LP (30)	−0.4045		
b) Compound (*III′*)							
C (1)	0.344	O (11)	−0.313	HC (21)	0.007	HC (31)	0.010
CT (2)	−0.038	N (12)	−0.422	CT (22)	0.137	HC (32)	0.010
CT (3)	−0.014	O (13)	−0.292	H (23)	0.157	HC (33)	0.010
C (4)	0.346	O (14)	−0.302	H3 (24)	0.112		
S (5)	0.687	CT (15)	0.193	H3 (25)	0.101		
CT (6)	0.024	HC (16)	0.060	LP (26)	−0.4045		
CT (7)	0.067	HC (17)	0.049	LP (27)	−0.4045		
NT (8)	−0.258	HC (18)	0.064	HC (28)	0.010		
C (9)	0.321	HC (19)	0.021	HC (29)	0.010		
N (10)	−0.364	HC (20)	0.063	HC (30)	0.010		
c) Compound (*IV″*)							
C (1)	0.334	O (11)	−0.332	HC (21)	0.023	HC (31)	0.010
CT (2)	−0.035	N (12)	−0.347	CT (22)	0.152	HC (32)	0.010
CT (3)	0.012	O (13)	−0.324	H (23)	0.171	HC (33)	0.010
C (4)	0.320	O (14)	−0.334	H (24)	0.173		
S (5)	0.737	H (15)	0.162	CT (25)	0.131		
CT (6)	−0.018	HC (16)	0.055	HC (26)	0.010		
CT (7)	0.115	HC (27)	0.049	HC (27)	0.010		
N (8)	−0.367	HC (18)	0.031	HC (28)	0.010		
C (9)	0.325	HC (19)	0.053	LP (29)	−0.4045		
N (10)	−0.384	HC (20)	0.048	LP (30)	−0.4045		

system in all-atom scheme (Weiner et al. 1986). This potential consists of the bond stretch, bond-angle bending, van der Waals, electrostatic, and torsional terms. All the force-field parameters but charges were taken from the paper by Weiner et al. (1986); the charges were assigned values obtained from MNDO populations (Table 1 a–c). In order to take into account the shielding of polar groups by the solvent, a distance-dependent dielectric constant ($\varepsilon = r_{ij}$) was used in the evaluation of the electrostatic energy (Weiner et al. 1986). Each structure was subjected to energy minimisation with the relaxation of all degrees of freedom, using the standard MM2 minimiser combined with the second-order Davidon-Fletcher-Powell (DFP) routine, as in our earlier work (Tempczyk et al. 1989).

The "best" formation heat of each compound was estimated by adding the MNDO or PM3 formation heat to the difference between the steric energy of the lowest-energy and reference (extended) conformations.

Results and discussion

The MNDO-optimised reference conformations of compounds (*II'*), (*III'*), and (*IV'*) are shown in Fig. 5 a–c. The obtained ring geometry of the succinimide fragment of (*III'*) agrees well with the crystal data of 3-phenylpyrrolidine-2,5-dione (Argay and Kálmán 1973). The most marked differences occur in the length of the amido C–N bonds: 1.414 Å and 1.430 Å for MNDO and PM3, respectively (averaged over the two amide bonds of the ring), compared with the experimental average value of 1.360 Å. This reflects the overall tendency of MNDO and PM3 to overestimate the length of the C–N bond in the amido groups (Stewart 1989 b, 1990). The five-membered ring is planar, as in the crystal structure. Unfortunately, there are no experimental geometry data of thiazinone (*IV*) or its derivatives. The most closely related compound is δ-valerolactam and the crystal data of its α-chloro derivative are available (Romers et al. 1967). Because in this case the sulphur atom is replaced by a methylene group, we did not compare the valence geometry, but only the endocyclic torsional angles. Both MNDO and PM3 calculations gave a half-chair conformation of the thiazinone ring (owing to the preferred planar conformation of the amido group), as in the case of the crystal structure of α-chloro-δ-valerolactam. The most marked difference is the torsional angle of the C–N amide bond: the distortion from planarity is only 9° for the crystal structure, compared with 29° and 25° obtained in MNDO and PM3 calculations on (*IV'*) (in our molecular mechanics calculations on (*IV''*); see below, the distortion from planarity was of the order of the experimental value). The excessive distortion of the amido group from planarity is caused by the underestimation of the barrier of rotation about the C–N bond by MNDO and PM3 (Stewart 1990). In our case this causes the partial relaxation of internal ring strains at the expense of the distortion of the amido group. However, to check if there is some additional effect of the presence of sulphur on the nonplanarity of the amido group, we carried out supplementary MNDO and PM3 calculations on δ-valerolactam, obtaining almost the same value of this, as well as other endocyclic torsional angles.

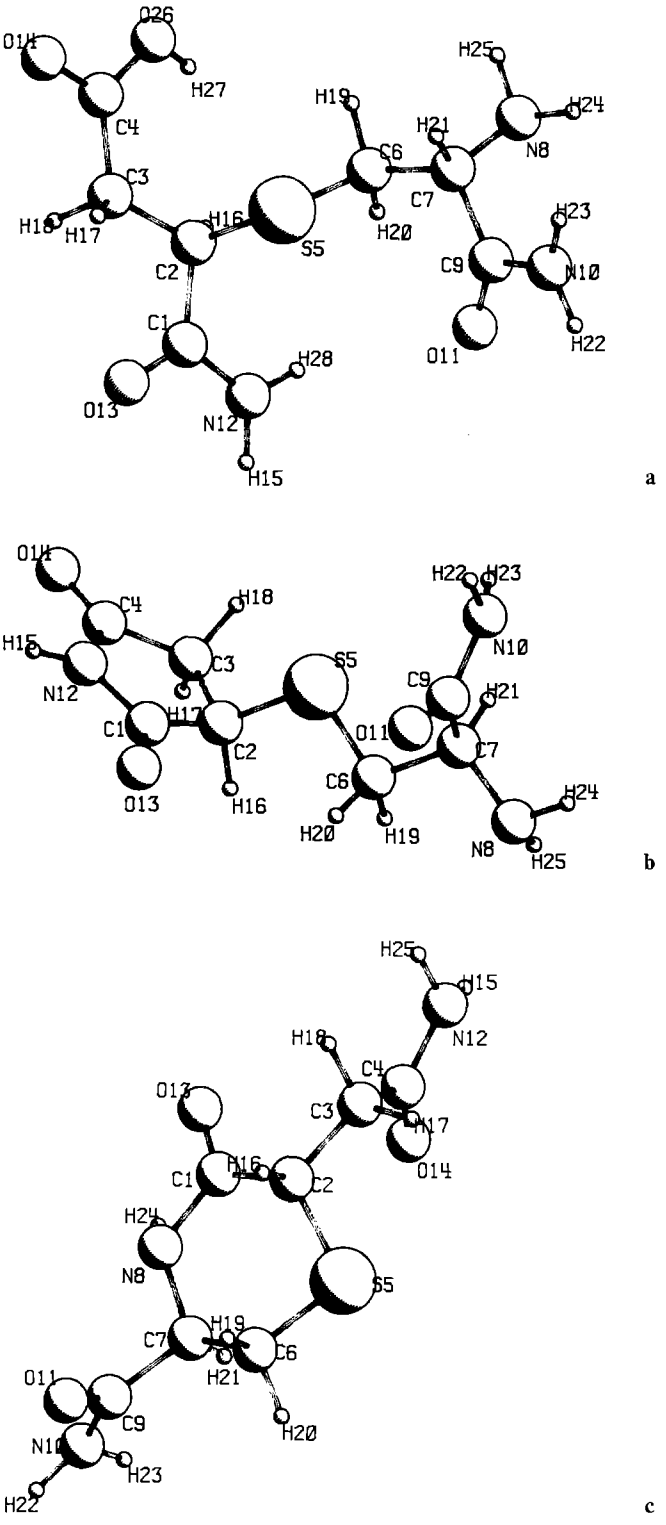

Fig. 5. MNDO optimised geometry of the models of the cyclisation stages in reference (extended) conformations: **a** (*II''*), **b** (*III''*), **c** (*IV''*)

The energies calculated on the basis of MNDO (PM3) formation heats and MM steric energy are summarised in Table 2. As shown, in both cases cyclisation causes energy gain, as far as the products in their "best" conformations

are concerned. The energy gain estimated by PM3 is, however, remarkably smaller, especially for (*IV'*). Succinimide (*III*) appears to have a greater bond energy, having a formation heat definitely lower than substituted 1,4-thiazin-3-one (*IV*) for the reference conformation. This is consistent with the difference of the values of strain energy between the five-membered and six-membered ring imides (Meng-Yan and Pilcher 1990) and lactams (Leitão et al. 1990). Moreover, the conjugation energy is greater for the succinimide group than for an isolated amido group. However, for succinimide (*III*) the folded conformations are higher in energy than the extended one, in contrast to thiazinone (*IV*), and the energy difference is therefore diminished.

The gas-phase enthalpy of formation of succinimide was determined to be −89.7 kcal/mol (Meng-Yan and Pilcher 1990). In supplementary MNDO and PM3 calculations on this compound we obtained the values of the heat of formation of −87.7 and −87.8 kcal/mol, respectively. There are no available thermochemical data of 1,4-thiazin-3-one. However, in order to estimate the accuracy of the MNDO and PM3 methods to reproduce the energetics of the amide bond inside the six-membered ring, we have carried out supplementary calculations on δ-valerolactam. The obtained values of heat of formation: −55.5 and −55.5 kcal/mol agree very well with the experimental gas-phase enthalpy of formation of −55.4 kcal/mol, as calculated from the enthalpy of formation of crystal δ-valerolactam (Strepikheev et al. 1955) and its enthalpy of vaporisation (Aihara 1953). Thus, both semi-empirical quantum-mechanical methods used appear to represent well the energetics of the ring fragments of the compounds studied.

It must be borne in mind that the free energy (free enthalpy) and not energy (enthalpy) itself decides about the relative thermodynamical stability of different isomers. We have therefore estimated the entropic contribution in harmonic approximation using the THERMO option of MOPAC. However, both for the reference and for the folded conformation, the difference in entropy contribution, both between the products (*III'*) and (*IV'*) and between the products and the substrates ((*II'*) and water) turned out to be smaller than 0.1 kcal/mol. Because both MNDO and PM3 underestimate the energy of distortion of the amido group from planarity, the strain energy of the six-membered ring is probably underestimated in our calculations and therefore the energy difference between succinimide (*III'*) and thiazinone (*IV'*) can be even more pronounced. It can therefore be concluded that (*IV*) has a thermodynamic stability lower or, at best, comparable to that of (*III*) and its formation in experimental conditions is rather due to kinetic reasons (the reaction leading to (*IV*) is irreversible (Kucharczyk et al. 1990)).

As follows from Fig. 1, the families of conformations of the substituted 1,4-thiazin-3-one (*IV*) can be characterised by the position of FMDP and L-cysteine residue in the 6-membered ring: equatorial-equatorial (*ee*), axial-axial (*aa*), equatorial-axial (*ea*), and axial-equatorial (*ae*). The dihedral angles and relative energies of the conformations obtained are summarised in Table 3 and the lowest-energy representatives of each type in Fig. 6a–d. As shown, the *ea* conformation is definitely favourable, agreeing with experimental results. Next is the *ae* conformation. In both cases the acyclic tails are on the same side of the ring and can therefore interact effectively. It must be noted, however, that the energy difference between the particular conformations of (*IV*) is not great and the predominance of the particular stereoisomers must be connected with kinetic and not thermodynamic factors.

Table 2. The estimated formation heats of the subsequent stages of inactivation

System	Formation heat estimated by MNDO/PM3 and MM (kcal/mol)			
	Reference conformation		MM-optimal conformation	
	MNDO	PM3	MNDO	PM3
II	−160.6	−164.3	−162.3	−166.0
III + H_2O	−176.4	−168.4	−176.4	−168.4
IV + H_2O	−169.3	−161.9	−174.6	−167.2

Table 3. The relative energies and dihedral angle values for the conformations of (*IV''*) calculated by molecular mechanics

Residue/angle[a]		Relative energy (kcal/mol)													
		0.0	0.6	2.1	2.7	2.8	3.0	3.8	4.1	5.1	5.2	5.3	5.4	5.5	5.8
Fum	ϕ_1	70	67	66	−70	65	−70	−85	84	−174	172	−65	−172	−68	85
	ψ_1	−75	166	115	75	1	−102	77	−63	−140	146	−177	−148	99	−78
Cys	ϕ_2	105	103	140	147	97	101	109	108	105	97	146	149	148	147
	ψ_2	−43	−41	−52	78	145	−42	−47	−47	144	150	93	100	82	79
	χ_1	52	55	−45	−53	57	54	47	47	50	56	−53	−55	−51	−52
	χ_2	−61	−60	55	62	−60	−56	−61	−60	−61	−60	61	60	61	62
	χ_3	47	43	−44	−48	42	37	52	52	50	43	−48	−44	−49	−50
Type[b]		*ea*	*ea*	*ae*	*ee*	*ea*	*aa*	*ea*	*aa*	*ea*	*aa*	*ee*	*ae*	*ae*	*ee*
C^2 chirality		R	R	R	S	R	S	R	S	R	S	S	R	R	S

[a] Angles as defined in Fig. 4c

[b] Equatorial/axial location of the "tails" at C^2 and C^7 with respect to the thiazinone 6-membered ring

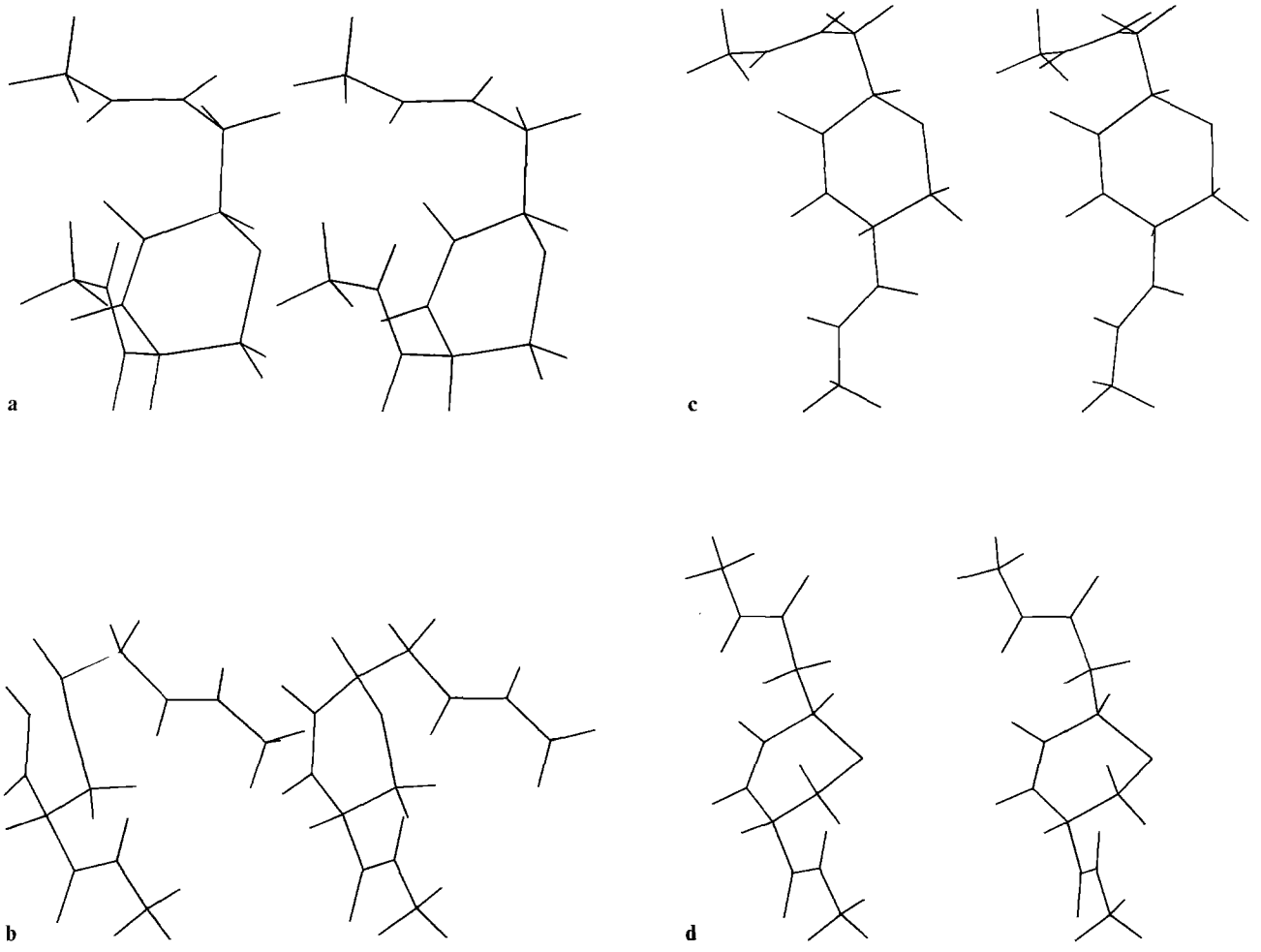

Fig. 6. Lowest-energy representatives of the isomers of (*IV'*): **a**) *ea* 0.0 kcal/mol, **b**) *ae* 2.1 kcal/mol, **c**) *ee* 2.7 kcal/mol, **d**) *aa* 3.0 kcal/mol

Table 4. The relative energies and dihedral angle values for the conformations of (*II''*) calculated by molecular mechanics

Residue/angle[a]		Relative energy (kcal/mol)																	
		0.0	0.5	0.8	0.9	1.7	2.0	2.5	3.2	3.6	4.0	5.0	5.1	5.3	5.4	5.7	6.1	6.6	8.3
Fum	ϕ_1	−58	172	−68	−55	61	−66	72	−62	−177	−73	−58	−85	86	65	−73	87	−78	−77
	ψ_1	94	−175	90	101	−80	89	−78	84	174	77	90	22	−44	−78	14	−42	44	32
	τ_1	−49	159	162	−54	32	157	−176	−31	84	176	−48	72	97	24	−82	98	2	−161
	ω_1	179	3	1	179	179	1	−1	−179	13	−0	180	180	12	178	−11	12	−179	−3
Cys	ϕ_2	−49	−53	−50	−52	−51	−52	−51	63	−53	68	−53	62	−64	−54	52	−61	51	58
	ψ_2	−40	117	−39	123	125	124	127	91	117	78	44	62	−47	102	112	111	111	50
	χ_1	76	179	74	177	176	177	177	−79	178	−73	73	−57	−51	−61	−89	−62	−85	−48
	χ_2	−80	−175	−78	178	175	178	177	84	−177	90	−74	−62	104	114	65	106	66	−61
	χ_3	79	58	81	162	143	159	137	−73	128	−71	81	63	−52	169	−74	−50	−77	61
Type[b]		*ea*							*ee*					*ae*					*aa*
C^2 chirality		R	S	R	R	R	R	R	S	R	S	R	S	R	R	R	R	R	S

[a] Angles as defined in Fig. 4a

[b] The lowest-energy intermediates leading to each type of diastereoisomer of thiazinone (*IV*) are indicated here (they are also shown in Fig. 7)

In order to estimate the kinetic preferences of the formation of thiazinone (*IV*) in a particular conformation, we performed the conformational analysis of (*II''*) looking for those conformations which enable the nucleophilic attack of the cysteine amino group on the carbonyl carbon (Table 4, Figs. 7a–d) to take place. As shown, again the product is likely to be found in the *ea*-type conformation (the lowest energy), while the next lowest one appears to be of *ee* type. Intermediates leading to the *ae* and *aa* conformations are definitely higher in energy. As shown

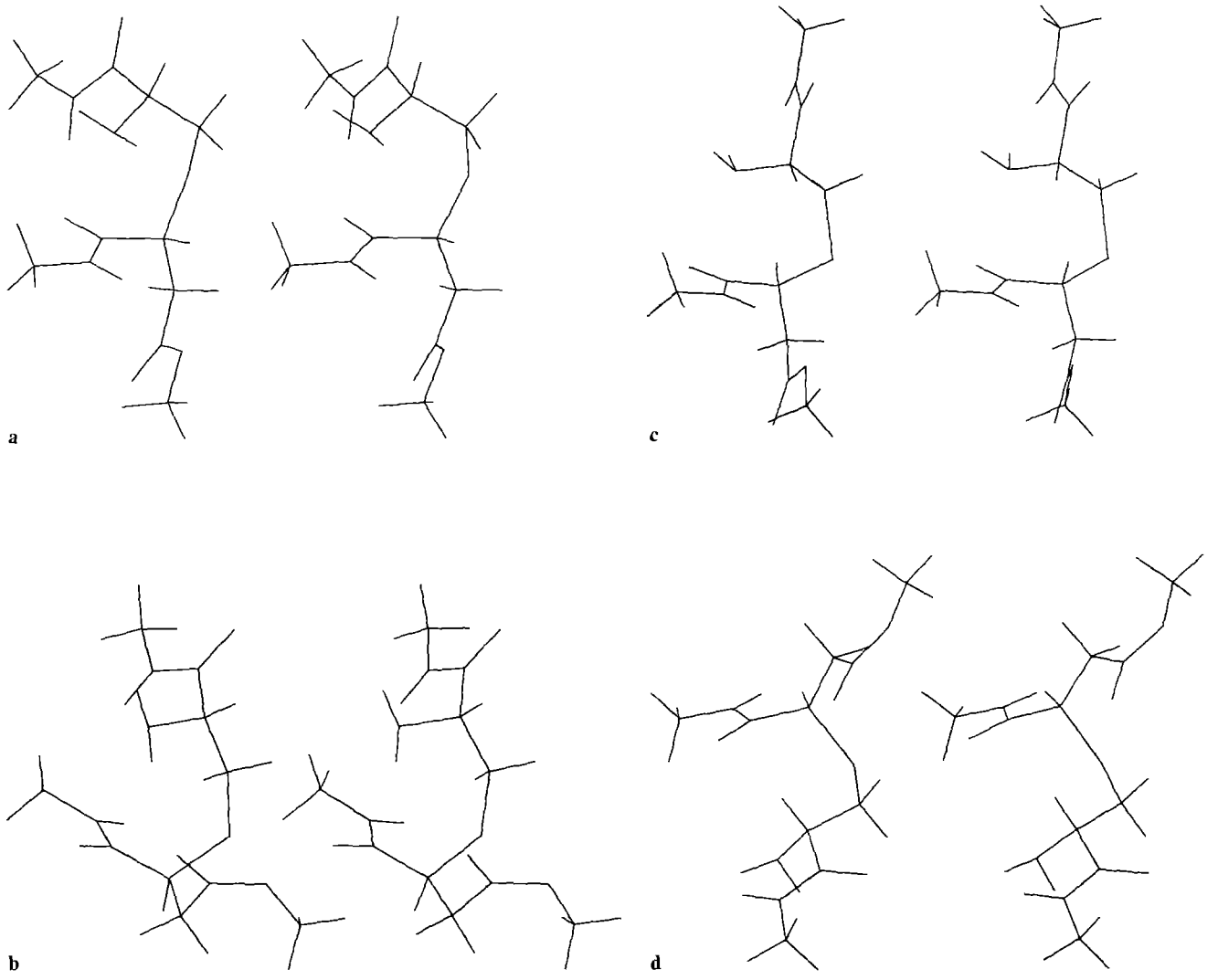

Fig. 7. Lowest-energy representatives of the conformations of (*II''*) leading to various stereoisomers of (*IV''*): **a**) *ea* 0.0 kcal/mol, **b**) *ae* 5.3 kcal/mol, **c**) *ee* 3.2 kcal/mol, **d**) *aa* 8.3 kcal/mol

Table 5. The relative energies and dihedral angle values for the conformations of (*III''*) calculated by molecular mechanics

Residue/ angle [a]		Relative energy (kcal/mol)									
		0.0	2.1	2.9	4.1	4.2	4.6	5.1	5.3	6.7	7.0
Cys	ψ_2	128	−40	−55	−55	116	−35	148	158	166	157
	χ_1	−178	−62	−47	−88	−108	79	−81	−78	86	90
	χ_2	179	176	67	92	62	−98	−92	−128	−93	−88
	χ_3	69	67	44	−55	−66	40	−55	71	31	23

[a] Angles as defined in Fig. 4b

in Figs. 7b and d, this is caused by the closeness in space of the cysteine and FMDP part. This results in increased steric repulsion, compared with intermediates leading to the *ea* and *ee* conformations (Figs. 7a and c). It can be thus concluded that the results of conformational analysis of the model of adduct (*II*) with regard to the evaluation of the preference of the type of conformation of the subsequently formed thiazinone (*IV*) exactly predict the isomers observed experimentally. This, together with the fact that the amino group has a nitrogen nucleophilicity evidently greater than that of amide, strongly supports the mechanism of cyclisation proposed in this work.

For the sake of completeness, the relative energies and dihedral angles characterising the conformations of succinimide (*III''*) are summarised in Table 5. As shown, the energy differences are rather small in this case, which indicates a considerable flexibility of the "tail" of the molecule. As mentioned above, the extended conformation is

the lowest in energy, which is readily understandable in terms of the inability of (*III''*) to form non-strained hydrogen bonds.

It should be noted that the formation of diastereoisomers of the pairs *ae–ea* or *ee–aa* depends on the chirality of the linear Michael addition product (*II*), if no inversion at C^2 occurs during the reaction. The experimentally found preference of the *ea* diastereoisomer would, therefore, indicate the preference of Michael addition leading of the R-form. However, neither the experimental findings by Kucharczyk et al. (1990) nor our conformational analysis of (*II''*) have indicated the specificity of Michael addition with regard to C^2 chirality (the energy difference between the lowest-energy R and S form is only 0.5 kcal/mol (Table 4)). It was also demonstrated that neither (*II*) nor (*IV*) enantiomerise (Kucharczyk et al. 1990). We can therefore draw a conclusion similar to that of Kucharczyk et al. (1990) that chirality is changed during the process of thiazinone ring formation. The authors mentioned have demonstrated by deuterium labelling that (*III*) quite easily exchanges its proton at C^2, which gives rise to epimerisation. Consequently, if (*III*) is a true intermediate, the lowest-energy diastereoisomer of (*IV*) can always be formed. On the other hand, even if thiazinone formation takes place in one step, as in Fig. 2 (or, at least, through intermediates other that succinimide (*III*)), a proton transfer from C^2 to the neighbouring negatively charged oxygen can take place in the second stage of cyclisation (Fig. 2). This would also allow for the change of chirality. Finally, one cannot exclude a possibility that the cyclic thiazinone is only partially formed by direct cyclisation, from the R-form of the Michael adduct. The S-form, owing to unfavourable steric conditions for direct 6-member ring formation (Table 4), may prefer a more complex route involving the formation of succinimide. In order to clarify this, direct reaction-path calculations of all the possible routes of cyclisation are required.

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