140. Theoretical Calculations of β-Lactam Antibiotics

Part VI

AM1 Calculations of Alkaline Hydrolysis of Clavulanic Acid

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The gas-phase basic hydrolysis of clavulanic acid (**a**) was studied by using the AM1 semi-empirical method. The results obtained show that the hydroxyethylidene side chain at C(2) is pivotal to the stability of the different reaction products involved. The products with an open oxazolidine ring are more stable than those with a closed ring fused to the β-lactam ring. This behaviour differs from that of penicillins and cephalosporins where the most stable degradation products are those with an intact thiazolidine or dihydrothiazine ring, respectively, fused to the β-lactam ring. The different chemical reacivity of clavulanic acid relative to penicillins and cephalosporins could explain the disparate behaviour of the latter two types of compound towards β-lactamases. Once the acyl-enzyme intermediate of clavulanic acid has been formed, it can evolve with cleavage of the oxazolidine ring to form a difficult to deacylate compound.

Introduction. – One of the major constraints to the biological action of β-lactam antibiotics lies in the occurrence of β-lactamases which inactivate them by hydrolysing the lactam ring before they can reach a carboxypeptidase or transpeptidase [1]. The earliest solution to this problem was proposed by several pharmaceutical laboratories in 1976; some *Streptomyces* microorganisms produce clavulanic acid (**a**), a powerful inhibitor for β-lactamases that includes the β-lactam ring but no penicillin or cephalosporin ring [1–3]. In **a**, the S-atom in the ring fused to the β-lactam unit in penicillins and cephalosporins is replaced by an O-atom; also, the compound bears no aminoacyl substituent at C(6) but a hydroxyethylidene group at C(2).

Clavulanic acid, which is isolated from *Streptomyces clavuligerus*, is a weak antibiotic itself, but also a powerful *in vivo* and *in vitro* inhibitor for β-lactamases from a variety of *gram*-negative and *gram*-positive bacteria [4–6]. The acid is usually administered jointly with another β-lactam, the effective concentration and lifetime of which are thus synergistically increased to the extent of rendering it lethal to some bacteria.

The purpose of this work was to study the basic-hydrolysis mechanism for clavulanic acid and compare it to that of penicillins and cephalosporins to determine whether their different behaviour towards β-lactamases is a result of their unlike chemical reactivity or of some other factors (*e.g.* a different conformation of the antibiotic). We investigated the basic hydrolysis of **a** rather than the acid one, because the former is similar to enzymatic

hydrolysis [7]. Also, we chose the AM1 calculation method on account of its proven suitability for β-lactam rings [8–10]. The hydrolysis takes place *via* a B_{AC2} mechanism in which the nucleophile approach is followed by the formation of a tetrahedral intermediate that evolves to the different reaction products.

Methodology. – All computations were performed by using the standard version of AM1 [11] in the AMPAC software package [12], which was run on *VAX8820* and *VAX9000* computers. The results were derived from a monodeterminantal function (RHF) with exclusion of configuration interactions.

A reaction coordinate was used to locate stationary points on the potential-energy surface. Subsequently, the force field AMBER in the programme MOBY [13] was used to study the different orientations of the side chains, which were finally reoptimized by using the AMPAC package. The geometries for stationary points were identified by minimization of the energy with respect to all geometric parameters using the *Broyden-Fletcher-Goldfarb-Shanno* algorithm [14–18]. The approximate geometries for the transition states (**TS**) were refined by minimizing the gradient norm of energy using the *Powell* algorithm [19], followed by the full *Newton* algorithm (LTRD) [20]. At a later stage, stationary points were characterized by FORCE or LTRD frequency analysis, a zero index for a critical point (where 'index' refers to the number of strictly negative eigenvalues of the *Hessian*) being assigned to reactants, products, and intermediates, and a unity value to each **TS**. If the index was greater than one, then the point was not a **TS**, but a 'col' or 'hilltop'. To discard spurious negative eigenvalues, the atomic motions making up the vibration to be followed were determined, the geometry being increased by a small amount along the coordinates concerned and reoptimized by gradient minimization.

Results. – *Geometric Analysis of Clavulanic Acid.* The initial structure of clavulanic acid (**a**) was constructed from standard bond lengths and angles and from dihedral angles. Unlike penicillins and cephalosporins, clavulanic acid possesses a single stable conformation for the oxazolidine ring [21]. The overall molecular structure and configuration of **a** was determined by *A. G. Brown* and coworkers to be (*Z*,2*R*,5*R*)-3-(2-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo[3.2.0]heptane-2-carboxylic acid [22].

The conformational freedom of the chain at C(2) prompted us to study the simultaneous variation of the dihedrals N(4)–C(3)–C(12)–O(21), C(14)–C(16)–O(19)–H(20),

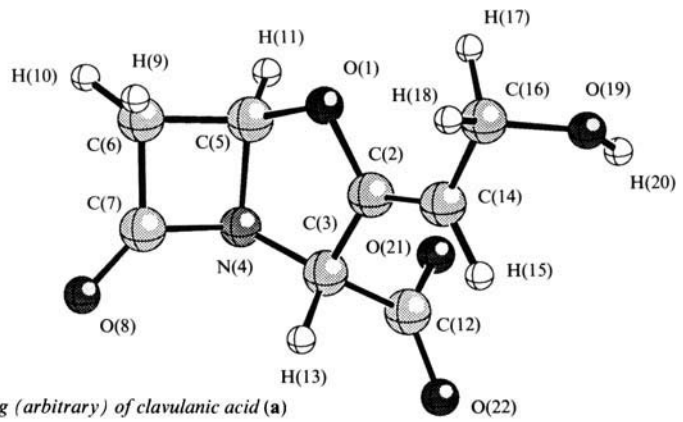


Fig. 1. *Numbering (arbitrary) of clavulanic acid* (**a**)

Table. *Bond Lengths* [Å] *and Bond and Dihedral Angles* [°] *Obtained by the AM1 Method and Comparison with Literature Values*

	Clavulanate ion (AM1)	4-Nitrobenzyl clavulanate[a])	4-Bromobenzyl clavulanate[a])	Clavulanic acid (4-21G)[b])	Penicillin G (AM1)[c])
O(1)–C(2)	1.406	1.377	1.43	1.415	1.820[d])
C(2)–C(3)	1.518	1.527	1.53	1.542	1.560
C(3)–N(4)	1.464	1.440	1.53	1.452	1.447
N(4)–C(5)	1.509	1.470	1.52	1.481	1.478
O(1)–C(5)	1.440	1.427	1.46	1.448	1.784[d])
C(5)–C(6)	1.556	1.537	1.60	1.549	1.582
C(6)–C(7)	1.539	1.522	1.53	1.550	1.569
N(4)–C(7)	1.447	1.412	1.48	1.427	1.448
C(7)–O(8)	1.225	1.195	1.19	1.192	1.218
C(2)–C(14)	1.339	1.317	1.31	1.306	–
C(5)–O(1)–C(2)	107.8	–	113.2	109.6	95.2[d])
C(7)–N(4)–C(5)	90.9	90.6	91	92.5	93.3
N(4)–C(7)–O(8)	133.6	129.5	126	130.9	131.5
C(2)–C(14)–C(16)	124.1	123.8	122	123.4	–
C(14)–C(16)–O(19)	112.4	108.7	–	106.0	–
N(4)–C(5)–O(1)–C(2)	6.6	8.0	15.8	21.4	5.3[d])
C(7)–N(4)–C(5)–C(2)	114.0 (122, 120)	–	–	–	116.6
C(7)–N(4)–C(3)–C(12)	150.8 (148, 161)	–	–	–	144.3
C(7)–N(4)–C(5)–O(1)	110.1	106.2	99.1	103.3	110.4[d])
C(6)–C(7)–N(4)–C(5)	4.5	9.4	9.9	9.9	7.6
O(8)–C(7)–N(4)–C(5)	–178.9	–166.7	–171.5	–167.5	–176.6
C(14)–C(16)–O(19)–H(20)	43.9	–	–	–178.5	–
O(1)–C(2)–C(14)–C(16)	–0.3	–	–	0.03	–
C(2)–C(14)–C(16)–O(19)	–146.4	–	–	–714.5	–

[a]) From [22].
[b]) From [23]. This is not the most stable conformation obtained by these authors.
[c]) From [24].
[d]) In penicillin G, O(1) must be replaced by S(1).

and C(2)–C14)–C(16)–O(19) of **a** by using the AMBER force field in order to determine the most stable structure (*Fig. 1*). Such a structure was reoptimized by using the AM1 method; the most salient bond lengths, bond angles, and dihedral angles are listed in the *Table*, together with literature values [22] [23] and those for penicillin G [24]. As can be seen, the results were quite consistent with previously reported values. Bond lengths and angles were accurately reproduced, with mean deviations of 0.028 Å and 2.2° with respect to 4-nitrobenzyl clavulanate and 0.032 Å and 4.0° with respect to 4-bromobenzyl clavulanate. These small differences can be ascribed to the fact that a gas-phase structure was compared with experimental values obtained by X-ray diffraction. The high consistency of the results again testifies to the suitability of the AM1 method for studying β-lactam geometries [8–10] [24] [25].

The structural arrangement of clavulanic acid and penicillin G, also determined by using the AM1 method [24], are very similar. Thus, the length of the N(4)–C(7) bond, which is directly involved in the hydrolysis process, is virtually the same for both compounds. Also, the N(4)–C(5)–O(1)–C(2) dihedral angle, which defines the conformation of the oxazolidine ring in clavulanic acid, is close to that of the

N(4)–C(5)–S(1)–C(2) dihedral in penicillin G, so the conformation of the bicyclic system in the two compounds is virtually identical. The other two major dihedrals in clavulanic acid, *viz.* C(7)–N(4)–C(3)–C(12) which determines the orientation of the COOH group and C(7)–N(4)–C(5)–C(2) which defines the position of the fused ring relative to the β-lactam ring, are also very similar to those of penicillin G. However, the oxazolidine ring fused to the β-lactam cycle differs considerably as regards the lengths and angles of the bonds involving the heteroatom at position 1.

The distance between the β-lactam N-atom of **a** and the plane formed by the three C-atoms bonded to it, 0.56 Å, is much longer than that in penicillin G (0.48 Å) [26] and cephalosporins (0.27–0.32 Å) [27] as determined by the AM1 method as well. Therefore, clavulanic acid is more pyramidal than penicillins and, obviously, cephalosporins.

Recently, *Fernández* and *van Alsenoy* [23] studied clavulanic acid from *ab initio* calculations at the 4-21G level. Their bond lengths, bond angles, and dihedral angles were all very similar to ours (see *Table*). The most marked difference lies in the orientation of the chain at C(2). According to these authors, the most stable structure is that in which H(20) forms a H-bond with O(1). The AM1 energy difference between this structure and the most stable one is 0.3 kcal/mol; such a small difference suggests that both conformations are equally probable in the gas phase. The fact that the conformation including the strongest H-bond (*viz.* H(20)–O(1)) is not the most stable can be ascribed to the absence of H(17)–O(1) and H(18)–O(1) interactions, which do occur in the structure of *Fig. 1*.

Basic Hydrolysis of Clavulanic Acid. The hydrolysis of clavulanic acid (**a**) in basic medium involves attack by the nucleophile OH^-, formation of a tetrahedral intermediate, and cleavage of the C–N bond (a B_{AC2} mechanism). The mechanism was studied by monitoring the enthalpy change throughout the reaction coordinate in each process (*Scheme 1*). *Fig. 2* shows the reaction profile obtained in the gas phase and the principal energy barriers. The molecular geometries of the major reaction minima or transition states are shown in *Fig. 3*.

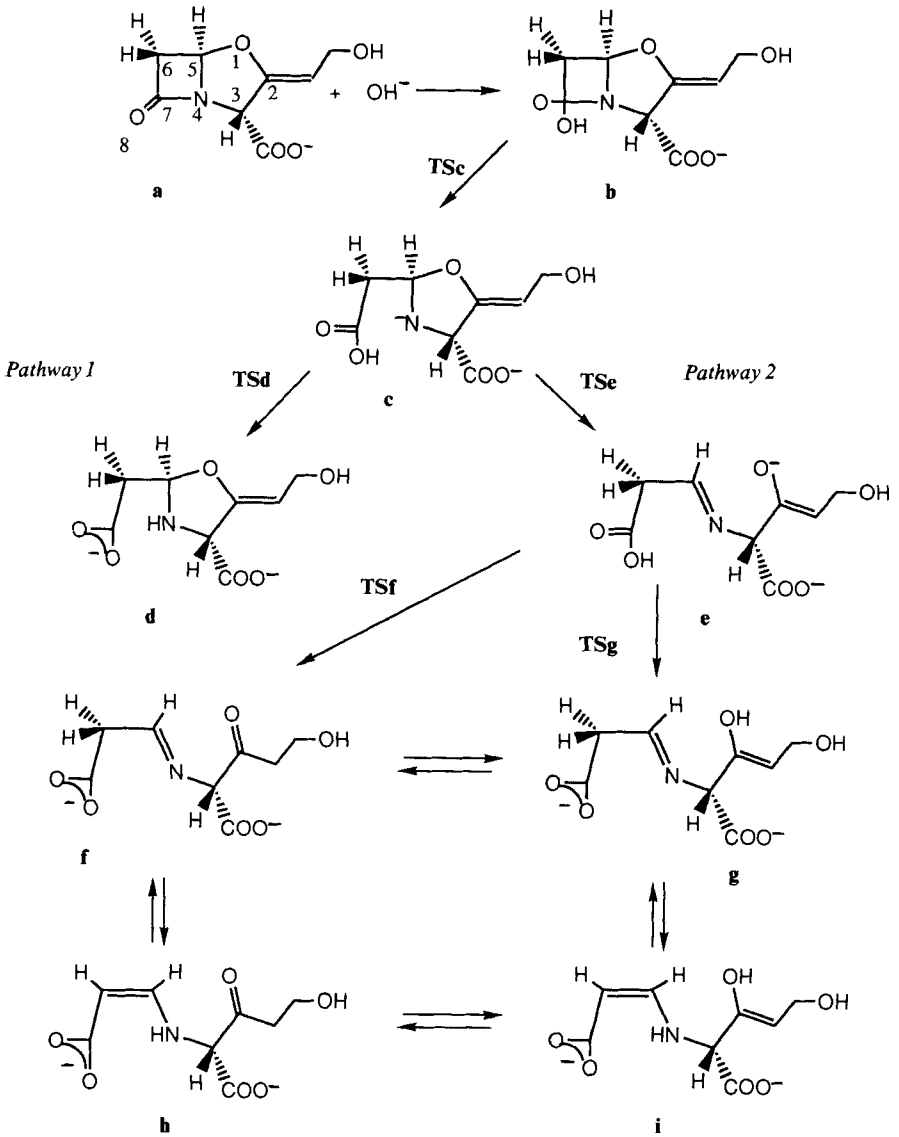
The nucleophile approach meets no energy barrier and takes place *via* side α of **a**, the energetically most favourable one [28] [29], until the tetrahedral intermediate **b** is formed. The charge density on the carbonyl C-atom of **a** (3.739) is similar to that for penicillin G (3.741) and cephalothin (3.694); hence, the nucleophilic attack at this C-atom in the three compounds must be very similar in energy terms. The results obtained show that the energy difference between the tetrahedral intermediate **b** of clavulanic acid and the reactants is 4.6 kcal/mol, *i.e.*, lower than that for penicillin G (13.1 kcal/mol) [24], cephalothin (13.6 kcal/mol) [25], and the monocyclic β-lactam (58.8 kcal/mol) [8]. The system evolves *via* an energy maximum (**TSc**) with an enthalpy of activation of 8.6 kcal/mol, until structure **c** which is only 4.5 and 9.1 kcal/mol more stable than the tetrahedral intermediate and the reactants, respectively, is reached. The enthalpy of activation for clavulanic acid is slightly lower than that for the hydrolysis of the azetidin-2-one ring (13.2 kcal/mol) [8], but higher than that for penicillins (1.8 kcal/mol) [24] and cephalothin (2.5 kcal/mol) [25].

Based on the experimental results for the hydrolysis of clavulanic acid [1] [5] [30–32], we studied two alternative routes for the evolution of intermediate **c**. *Pathway 1* involves a H-transfer to the β-lactam N-atom and thus the stabilization of the negative charge, the fused ring remaining tightly closed (→ **d**). *Pathway 2* is induced by cleavage of the O(1)–C(5) bond (→ **e**) and subsequent evolution to structure **f**. Structure **f** is analogous to

that of methyl 3-aminoacrylate obtained by *Davies* and *Howarth* [33] in studying the reaction between clavulanic acid and MeOH, which took place *via* a mechanism similar to that shown in *Scheme 1* [32].

Pathway 1 is similar to that for the hydrolysis of penicillins and cephalosporins [8] [9] [24] [25] and involves rotation about the C(7)–O(23) bond, defined by the O(8)–C(7)–O(23)–H(24) bond, which leads the system *via* a maximum (**TSd**) of −194.2 kcal/mol to structure **d**; the latter is 54.4 kcal/mol more stable than **c**. As can be seen from

Scheme 1



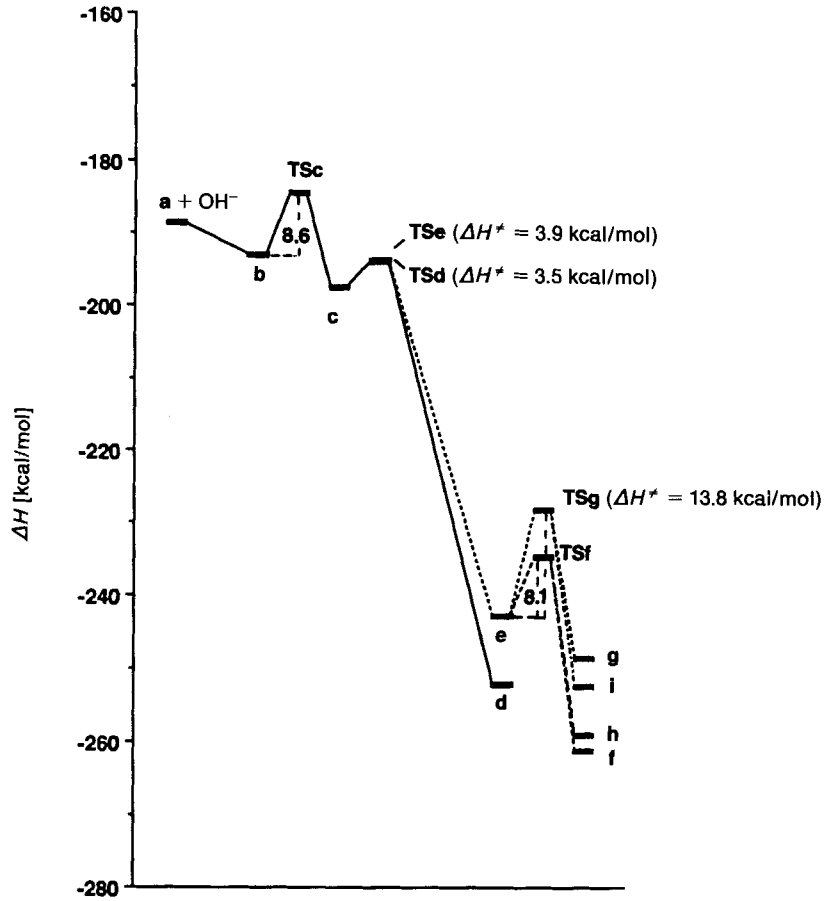


Fig. 2. *Reaction profile in the gas phase of alkaline hydrolysis of clavulanic acid* (**a**)

Fig. 3, the distance between H(24) and the β-lactam N-atom at the energy maximum (**TSd**) is 2.95 Å, *i.e.*, longer than that for a H-bond. When such a distance approaches that of a H-bond by effect of the rotation, the H-atom is immediately transferred. This can be the result of the AM1 method overestimating H-bonding and is consistent with the behaviour of penicillin and cephalosporin observed by using the same method [8–10]. The more stable orientation of H(24) in structure **d** is that in which the H-atom is below the oxazolidine ring where it is bonded to O(22), this is impossible above the ring.

In *Pathway 2*, cleavage of the O(1)–C(5) bond leads to the ring-opened structure **e**. The energy barrier for this process is 3.9 kcal/mol. Opening of the two fused rings gives rise to a host of possible chain orientations which call for a comprehensive conformational analysis. *Fig. 4* shows the final potential surface obtained by rotation of the N(4)–C(5)–C(6)–C(7) and O(8)–C(7)–O(23)–H(24) dihedrals; as can be seen, the two most stable structures correspond to *ca.* 115 and −125° for the former dihedral and 180° for the latter. The final structure is by 16.8 kcal/mol more stable than that obtained

directly by studying the reaction coordinate. The origin of such a strong stabilizing effect must be the occurrence of H-bonding (see *Fig. 3*). Structure **e** was used to study two other transfers. That of H(24) to O(21) to form structure **g** has an enthalpy of activation of 13.8 kcal/mol (**TSg**) and results in stabilization by 5.7 kcal/mol relative to structure **e**. On the other hand, the transfer of H(24) to C(14) meets with a lower energy barrier (8.1 kcal/mol, **TSf**), and the final product **f** is by 12.6 kcal/mol more stable than **g**. This imino-ketone form **f** is the most stable (−261.1 kcal/mol) and occurs in equilibrium with the enamino-

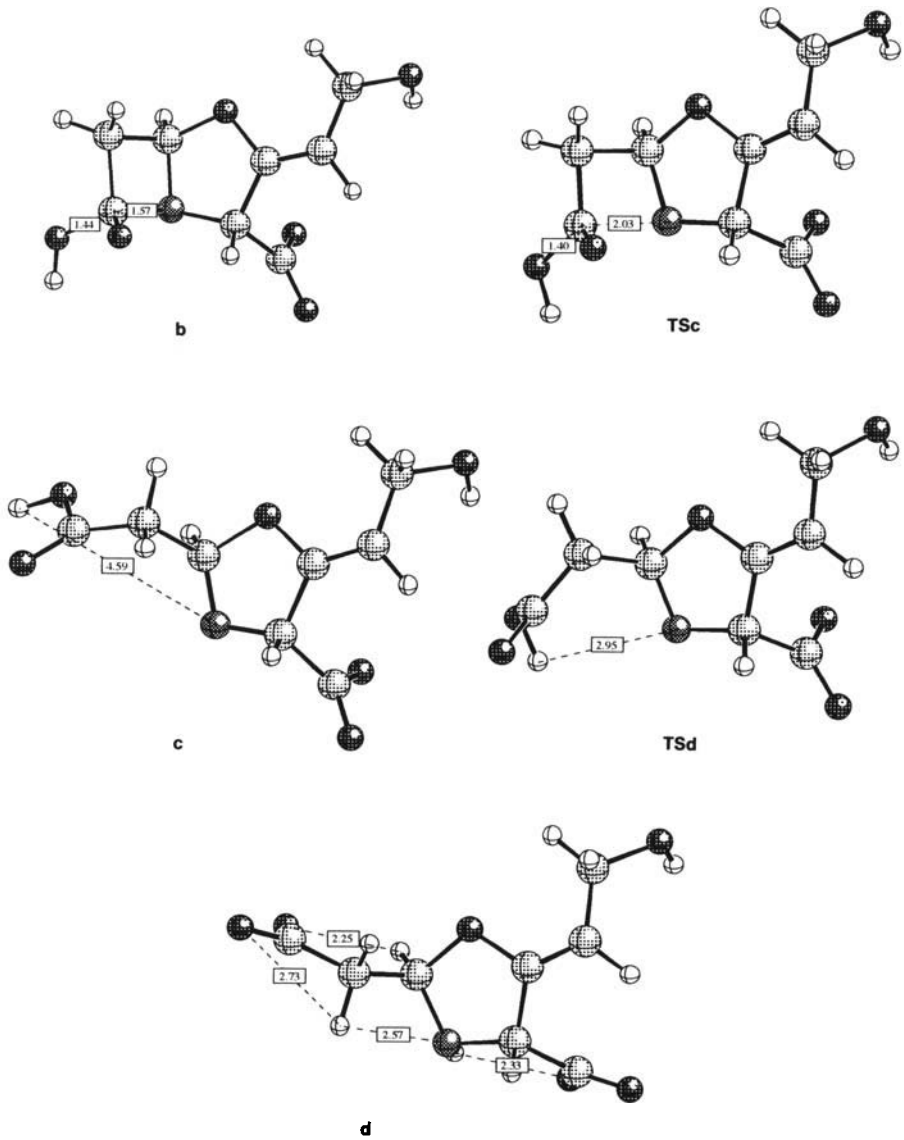
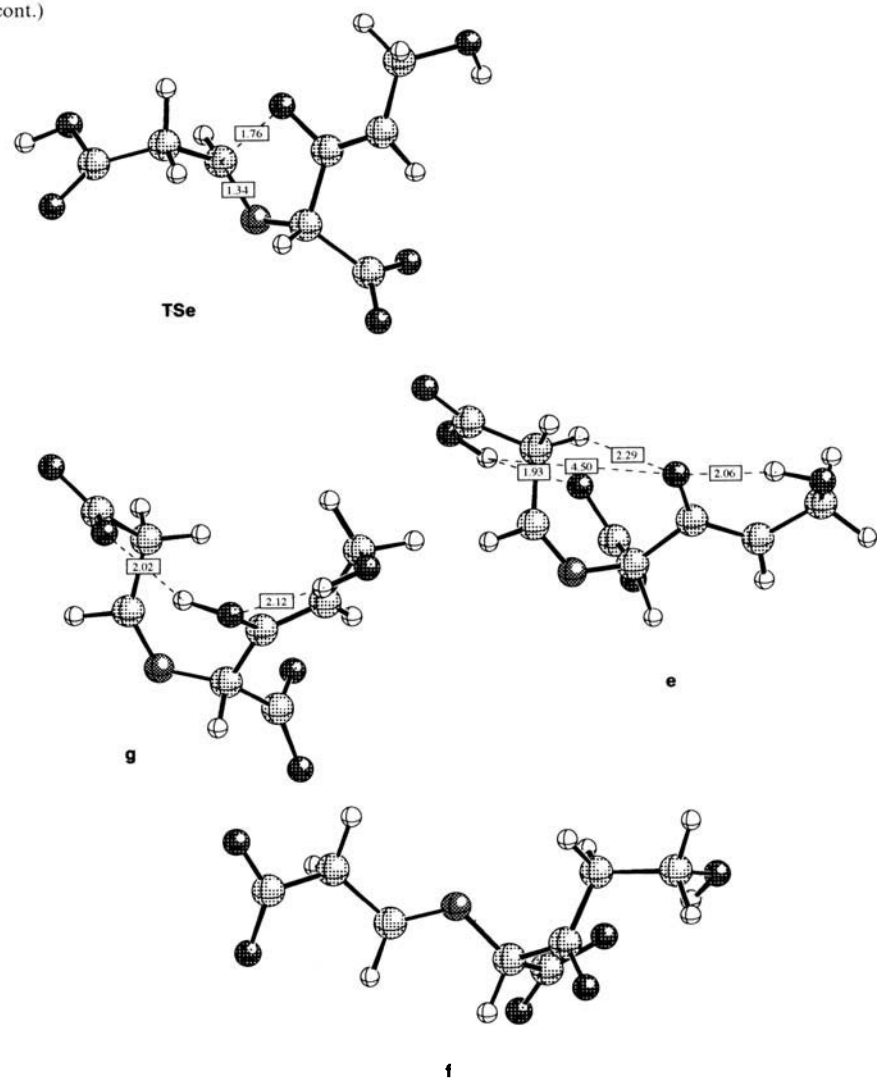


Fig. 3. *Structures corresponding to the different intermediates and final states of the reaction* Pathways 1 *and* 2

Fig. 3 (cont.)



ketone **h** (−259.0 kcal/mol), imino-enol **g** (−248.5 kcal/mol), and enamino-enol form **i** (−252.0 kcal/mol). The occurrence of three other forms in equilibrium may lead the system to any of the four products, which accounts for the higher thermodynamic stability of the reaction products resulting from opening of the oxazolidine ring (*Pathway 2*).

It is well known that the presence of solvent molecules can alter considerably the potential-energy surfaces of a number of reactions. These effects can be particularly significant in reactions involving charged products or intermediates. Two approaches for modelling such reactions have been widely applied: the 'super-molecule' approach in which explicit solvent molecules are included [34] and that in which the solvent is treated as a continuum [35] [36]. A super-molecule model comprising up to 15 H_2O molecules can

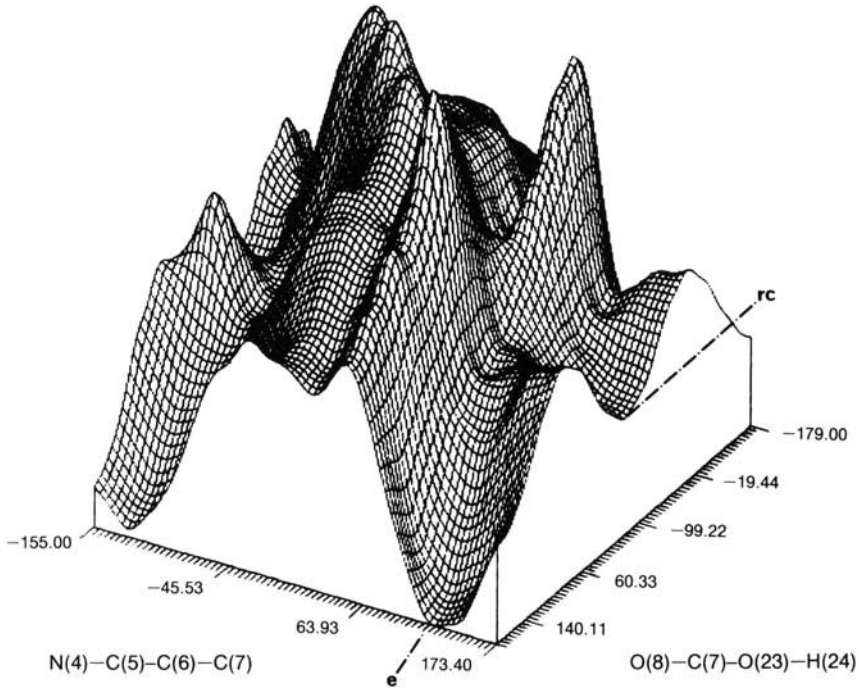


Fig. 4. *Potential-energy surface: conformational analysis of structure* **e**.
rc = structure obtained in the reaction coordinate.

represent an adequate primary solvation sphere and an approximate secondary solvation sphere model [34]. In a previous paper on the basic hydrolysis of cephalosporins [25], we demonstrated that inclusion of an initial solvation layer consisting of 5 H_2O molecules does not significantly alter the results while considerably expediting the calculations involved. Under these conditions, determining maxima becomes especially difficult because the points exhibit multiple negative force constants as a result of the high mobility of the solvation H_2O molecules. Thus, to qualitatively assess the effect of the solvent on the reaction studied, we used a model dealing with the solvent as a continuum. The effect was thus assessed by chosing the AMSOL model developed by *Cramer* and *Truhlar* [36] which is implemented in AMPAC 4.0. *Cramer* and *Truhlar* [36] indicated that more than 90% of change in the solute aqueous free energy for any given molecules come just from the electronic relaxation. This implies that a 1SCF calculation at the gas-phase geometry allows a very rapid qualitative analysis of the important solvation effects. *Fig. 5* shows the reaction profile obtained by analysing 1SCF calculations for the gas-phase structures with the key word SM2 included (which activated the AMSOL method). As can be seen, there are some differences between this reaction profile and that obtained for the gas phase (*Fig. 2*). More important, tetrahedral intermediate **b** is markedly stabilized relative to the reactants (**a** + OH^-). Also, the activation barriers are slightly lower than those obtained for the gas phase. These results clearly indicate that the reaction is favoured by the presence of the solvent. In aqueous phase, a potential barrier arising from the

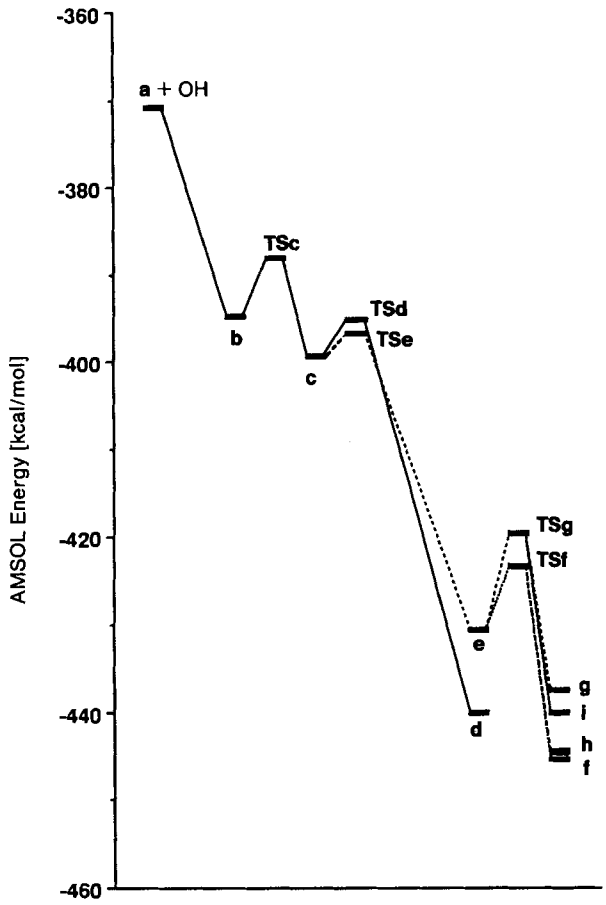


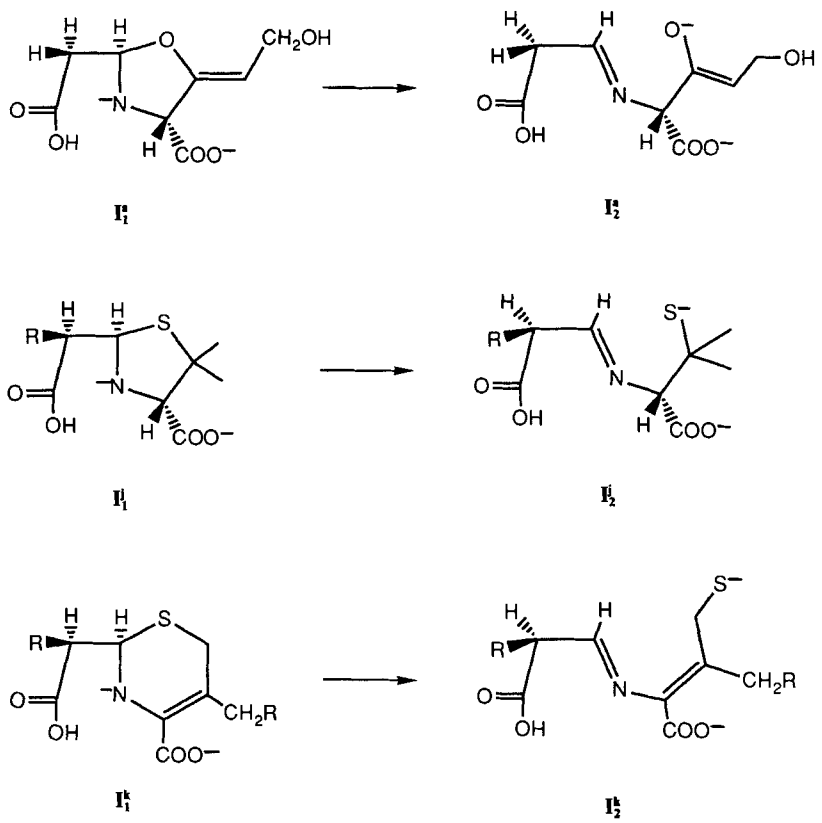
Fig. 5. *Reaction profile in the aqueous phase of alkaline hydrolysis of clavulanic acid* (**a**).
AMSOL energy = self-consistently determined heat of formation of the solute in the presence of the solvent plus free energy of hydration for the optimized structure [43].

desolvation the OH^- ion precedes its attack on the carbonyl C-atom; however, with this methodology, it is impossible to localize this transition state and to calculate the barrier.

The above results show some similarities to the β-lactamase hydrolysis giving rise to an acyl-enzyme (a β-imino ester analogous to structure **f**), which can subsequently evolve *via* three alternative routes: deacylation, equilibrium with the enamine (transient inhibition), and production of inactive species [1] [5] [30–32].

The mechanism of action of clavulanic acid and related inhibitors (*e.g.* penicillanic-acid sulfone (sulbactam), 6β-halopenicillins) shows that to withstand the β-lactamase hydrolysis, it must include an appropriate leaving group at C(5) to facilitate opening of the fused ring and ensure a high lifetime for the acyl-enzyme thus formed. Obviously, the structural differences in the ring fused to the β-lactam cycle between penicillins, cephalosporins, and clavulanic acid influence the ease with which the C–heteroatom

Scheme 2



bond is cleaved and hence the behaviour of these compounds towards β-lactamases. In the light of this fact, we studied the cleavage of the C–heteroatom bond and oxazolidine, thiazolidine and dihydrothiazine ring opening (*Scheme 2*) in clavulanic acid (see $\mathbf{I}^a$), penicillin G (see $\mathbf{I}^j$), and cephalothin (see $\mathbf{I}^k$), respectively.

Structure $\mathbf{I}_1^j$ could not be obtained by the AM1 method owing to the excess negative charge (–0.536) placed on the N-atom by the nucleophile and the inability to delocalize such a charge over a conjugate system. For this reason, the gas-phase system tended to evolve spontaneously with cleavage of the C–S bond and ring opening. This structure was generated from the S-parameters provided by the MNDO method and was subsequently optimized at a constant C–S bond length. The negative charge on the N-atom in cephalosporins and clavulanic acid can readily be delocalized over the dihydrothiazine ring or the neighbouring double bond, respectively, in such a way that structures $\mathbf{I}_1^k$ and $\mathbf{I}_1^a$ can be obtained in the gas phase. The activation barriers for these processes are minimal (3.9 kcal/mol for clavulanic acid, 3.3 kcal/mol for cephalothin, and virtually zero for penicillin G). Intermediate $\mathbf{I}_2^j$ for penicillins and, especially, $\mathbf{I}_2^k$ for cephalosporins possess excess negative charge on the S-atom, which thus tends to capture a H-atom to stabilize

the structure. As a result, one of the protons in the aminoacyl chain of cephalothin or the substituent at C(3′) is attracted by the S-atom.

The main difference between the three types of β-lactam compounds lies in the stability of the products formed from them. Thus, the products arising from opening of the ring fused to the β-lactam cycle are all more stable than the starting structures (by 45.1 kcal/mol for clavulanic acid, 27.5 kcal/mol for penicillins, and 9.0 kcal/mol for cephalosporins). However, structures $\mathbf{I}_2^j$ and $\mathbf{I}_2^k$ for penicillins and cephalosporins cannot evolve to more stable alternatives, whereas clavulanic acid can delocalize the negative charge over the π systems directly bonded to C(2) and thus give rise to structures **f**, **g**, **h**, and **i**. Evolution of penicillins from structure $\mathbf{I}_1^j$ with H-transfer to the β-lactam N-atom and of cephalosporins from structure $\mathbf{I}_1^k$ with leaving of the group at 3′ leads to forms with an intact ring fused to the β-lactam cycle, which are more stable than those with an open ring ($\mathbf{I}_2^j$ and $\mathbf{I}_2^k$, resp.) [24][[25]. Clavulanic acid is the only compound of the three studied in which the products resulting from cleavage of the fusion bond are more stable than those in which the oxazolidine ring is intact. This different behaviour may account for its inhibitory action on β-lactamases relative to penicillins and cephalosporins.

The above results also account for some observations in the basic hydrolysis of β-lactams. Such is the case with the epimerization at C(5) in the basic hydrolysis of penicillins [37–39], recently explained by *Page* and coworkers [40] on the assumption of unimolecular opening of the thiazolidine ring and formation of an iminium ion that can cyclize to yield the starting compound or its epimer. The gas-phase results obtained in this work are consistent with the presence of the epimer among the hydrolysis products of penicillins.

As noted earlier, the cleavage of the C–S bonds in cephalosporins is far more complex. In any case, *Mobashery* and *Johnston* [41] suggest that the cephem nucleus may undergo a variety of solvolytic reactions after the lactam bond is cleaved, thereby giving rise to various products resulting from cleavage of the C–S bonds and immediate uptake of a proton. *Tsuji et al.* [42] arrived at the same conclusions in a study of the basic aminolysis of cephalothin.

The fact that the energy difference between the tetrahedral intermediate and the reactants is much smaller for clavulanic acid may be suggestive of a weak tendency to form an acyl-enzyme intermediate, which is consistent with its low antibacterial power.

The results indicate that cleavage of the C–heteroatom bond in clavulanic acid is easier than it is in penicillins and much easier than in cephalosporins; in fact, while the activation barriers are similar, the products obtained are more stable than the structures with an intact oxazolidine ring. The presence of a side chain at C(2) and the O(1) atom endow the basic hydrolysis of clavulanic acid with special features, since the O-atom, a good leaving group, facilitates opening of the oxazolidine ring and subsequent delocalization of the negative charge over the double bond in the side chain at C(2). This chemical behaviour may account for the fact that the acyl-enzyme intermediate produced in the hydrolysis of clavulanic acid by β-lactamases can readily evolve with opening of the oxazolidine ring to a scarcely deacylable compound.

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