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CONSTRAINED SEARCH OF CONFORMATIONAL HYPERSPACE OF INACTIVATORS OF GLUCOSAMINE-6-PHOSPHATE SYNTHASE

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Glucosamine-6-phosphate (GlcN-6-P) synthase (EC 2.6.1.16) is a key enzyme in amino sugar metabolism in micro-organisms and its selective and irreversible inhibitors can become valuable antifungal drugs. We performed a constrained search of the conformational hyperspace of glutamine and of the set of specific inactivators of the enzyme, as well as of some non-specific inhibitors of many cysteine containing enzymes. From these calculations we obtained spatial relationships of functional groups, the presence and specific orientation of which in the active site of the enzyme is important for effective and selective action of the inhibitor. Subsequent quantum chemical calculations confirmed the correctness of the pharmacophore conformation we obtained. Pharmacophore conformation of FMDP molecule, the most potent inhibitor in the selective inhibitors group, is placed close to the energy minimum on the conformational energy map.

KEY WORDS: Glucosamine-6-phosphate synthase, inhibition, molecular modeling

INTRODUCTION

Glucosamine-6-phosphate (GlcN-6-P) synthase (EC 2.6.1.16), catalyses the transfer of the γ-amino group of L-glutamine to fructose-6-phosphate with simultaneous isomerisation of the sugar and is a key enzyme in amino sugar metabolism in micro-organisms.[1,2] In consequence it is one of the enzymes involved in the formation of the microbial cell wall and can be a valuable target for antimicrobial chemotherapeutic agents. However GlcN-6-P synthase also plays a key role in the synthesis of glycoproteins in human cells. Due to the relatively long period of turnover of animal glycoproteins, inhibition of their biosynthesis has different physiological consequences in fungal and human cells making selective toxicity possible.[3]

Glucosamine-6-phosphate synthase, PRPP-amidotransferase, asparagine synthetase and glutamate synthase belong to the PurF-type amidotransferase family utilising glutamine as a source of nitrogen in the transfer reaction to different acceptors. Enzymes of this family contain the *Cys*, *His*, *Asp*/*Glu* catalytic triad important for

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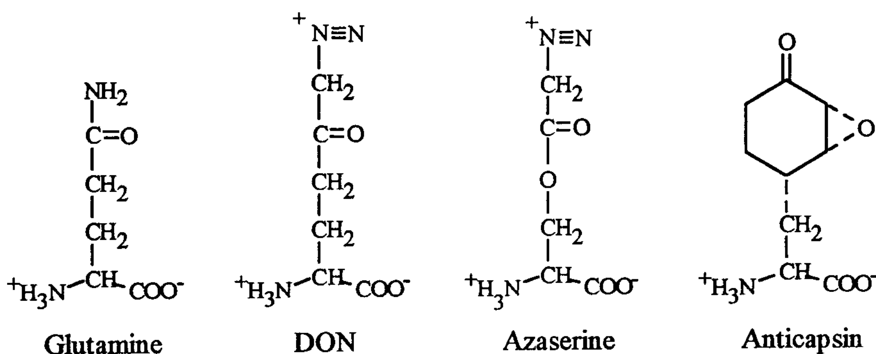

FIGURE 1 Natural substrate of glucosamine-6-phosphate synthase and its various inactivators.

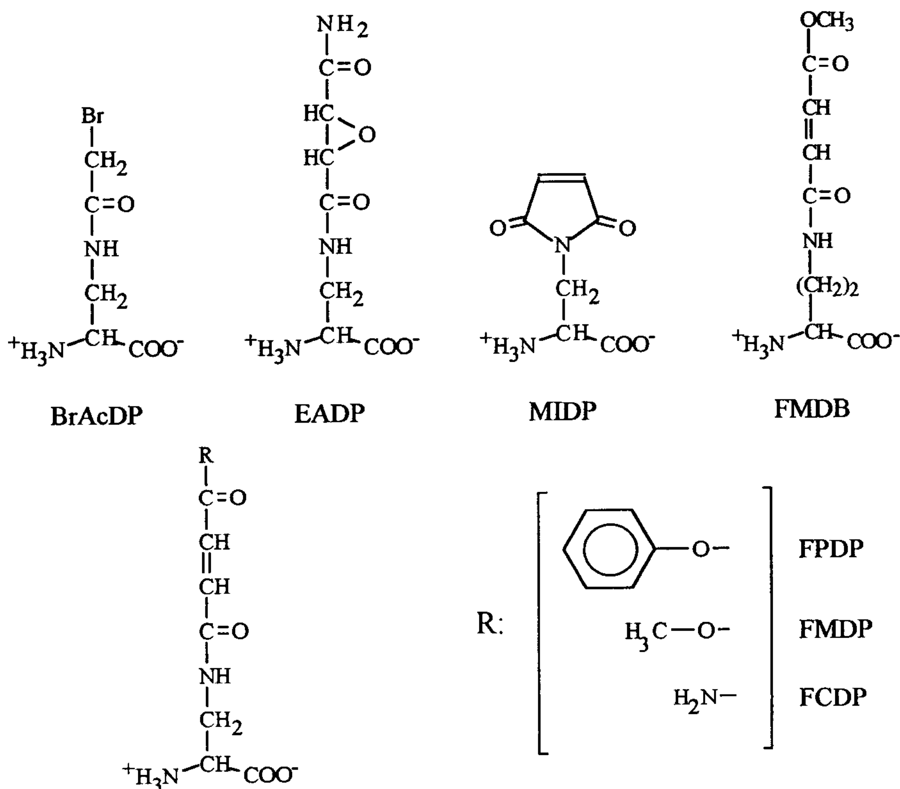

FIGURE 2 Specific inactivators of glucosamine-6-phosphate synthase.

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the enzymatic activity, with N-terminal cysteine playing a crucial role in the catalysis process.[4–7] Due to this cysteine residue these enzymes are inhibited by many non-specific, thiol-blocking agents, as well as some glutamine analogs, which act as active site-directed inactivators:[8,9] 6-diazo-5-oxo-L-norleucine (DON), azaserine and anticapsin (Figure 1).

In our laboratory a series of novel glutamine analogs, including N^3-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid (FMDP), have been designed and synthesized (Figure 2). These compounds appear to be potent and highly specific inhibitors of GlcN-6-P synthase,[6,10–12] but the mechanism of their selective action is unknown.

Since glutamine is the source of nitrogen for a number of amidotransferases, elucidation of the mechanism of the selective action of FMDP and its analogs on the GlcN-6-P synthase is important for the rational design of inhibitors of this enzyme — potential antifungal drugs. Our recent molecular mechanics and quantum chemical calculations explained the mechanism of inhibition of GlcN-6-P synthase by FMDP.[7,13] In this paper we present the results of our calculations in which we applied conformational searching techniques to solve the problem of its selectivity.

METHODS

We tried to investigate the origin of the selective action of specific inactivators of glucosamine-6-phosphate synthase, including derivatives of N^3-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid, using a conformational hyperspace searching technique[14,15] for the pharmacophore geometry identification, and semiempirical AM1 method for its validation.[16] The pharmacophore is defined in terms of the spatial relationship between functional groups, whose orientation at the active site is essential for recognition and binding of the inhibitor in the active site of the enzyme. This specific orientation is called pharmacophore geometry. The number of these functional groups determines the dimensionality of the pharmacophore model. A pharmacophore geometry is represented by a point in a $k(k-1)/2$ dimensional distance space, where k is the pharmacophore model dimensionality and the set of all pharmacophore geometries for a particular molecule defines its conformational hyperspace. Using a series of active compounds and the correspondence between functional groups contained in molecules in the series, search procedures can be used to determine the existence of three dimensional orientations of the functional groups common to all molecules of the series, e.g. area in the conformational hyperspace common to all of these molecules. Typically, one atom in each functional group is selected as a reference and is called a pharmacophore reference point. In the search procedure the conformational hyperspace is divided into hypercubes of specified edge length and molecules are decomposed into aggregates of atoms whose interatomic distances are invariant with respect to a torsional rotation around an axis defined by the three-space coordinates of any two atomic nuclei in the aggregate. The search procedure finds all sterically allowed combinations of aggregates, changing their relative orientation by rotating flexible bonds of specified angle called a scan parameter, and stores the hypercubes containing pharmacophore geometries of all previously scanned molecules.

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For the conformational space scanning we used the program based on the constrained search algorithm introduced by R.A. Dammkoehler.[15] In contrast to the systematic search procedure, this algorithm utilises information derived from the analysis of the substructure of each molecule in the series to restrict searches for subsequent molecules and so significantly reduces the time required for calculations.

Since the constrained search technique uses the fixed valence geometry approximation and it takes into account only geometric constraints, it is not able to distinguish between low and high energy conformations. To check whether the pharmacophore geometry we obtained is energetically stable we performed the conformational analysis of FMDP by means of the semiempirical AM1 method, contained in the MOPAC package,[17] and tried to place the pharmacophore geometry on the conformational energy map. AM1 is an improvement of the MNDO method which has the ability to reproduce hydrogen bonds and better reproduce heats of formation of small molecules than the latter.[16,18] In particular heats of formation of amides whose functional groups occur in compounds we tested are better reproduced by the AM1 method.

RESULTS AND DISCUSSION

Compounds used for the calculations were selective inactivators of the GlcN-6-P synthase (Figure 2) synthesized in our laboratory, as well as the set of non-specific inactivators of many cysteine containing enzymes belonging to the amidotransferase class. All these compounds are glutamine analogs acting as active site directed inhibitors[11,19–21] of GlcN-6-P synthase. Initial three dimensional structures of these inhibitors were prepared by means of a molecular mechanics method.

For the first stage of calculations we chose the entire set of ten inactivators of the GlcN-6-P synthase and glutamine, the natural substrate of the enzyme. In each of these compounds we selected, as the pharmacophore reference points, atoms in three functional groups which are responsible for analogue recognition and subsequent inactivation of the enzyme. These are the nitrogen atom of the α-amino group and the carboxylic oxygen atom which are required for the recognition effect.[22] As the last pharmacophore reference point in each inactivator we chose the atom which probably makes a covalent bond with the cysteine group, the moiety of the active centre of the enzyme, leading to its inactivation.[12,23] In the glutamine molecule as the third pharmacophore reference point we chose the γ-carbonyl carbon atom which during the catalysis reaction creates a covalent bond with the cysteine.[4] The structure of FMDP with appropriate distances marked with labelled arrows is shown on Figure 3. Since the order in which molecules are processed has an influence on the time required for calculations, molecules with less numbers of flexible bonds were placed at the beginning of the series and the most flexible molecules, like the non-specific inhibitors DON and azaserine, at the end.

For the constrained search procedure we used the scan parameter of 10° and the hypercube edge length of 0.5 Å. Each of three variable distances was constrained to the range of 2–10 Å. As the first structure in the series we used the natural substrate of

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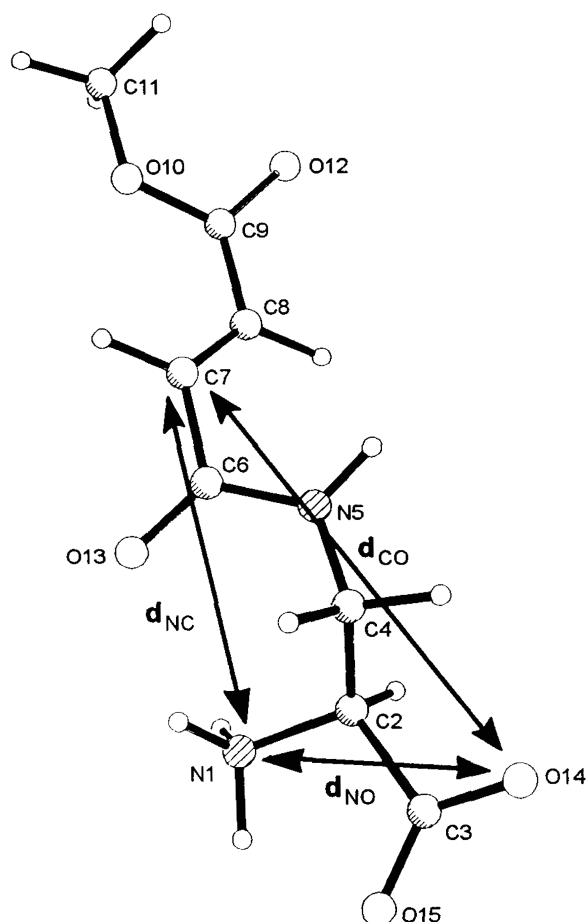

FIGURE 3 AM1 minimised FMDP molecule with all distances between pharmacophore reference points assigned.

the enzyme, glutamine, and the initial set of hypercubes was established on the basis of its conformational hyperspace. When the calculation for this molecule was completed the whole of its conformational hyperspace was enclosed within 65 hypercubes. As the calculations continued for all remaining molecules this number was consequently decreasing, and on completion of the constrained search algorithm there were only three adjacent hypercubes containing geometries common to each molecule in the series left. These hypercubes are presented on Figure 4.

In order to find out if there is a significant difference between the conformational hyperspaces of specific and non-specific inhibitors, we divided the entire set of structures into two groups, and repeated the whole numerical experiment separately

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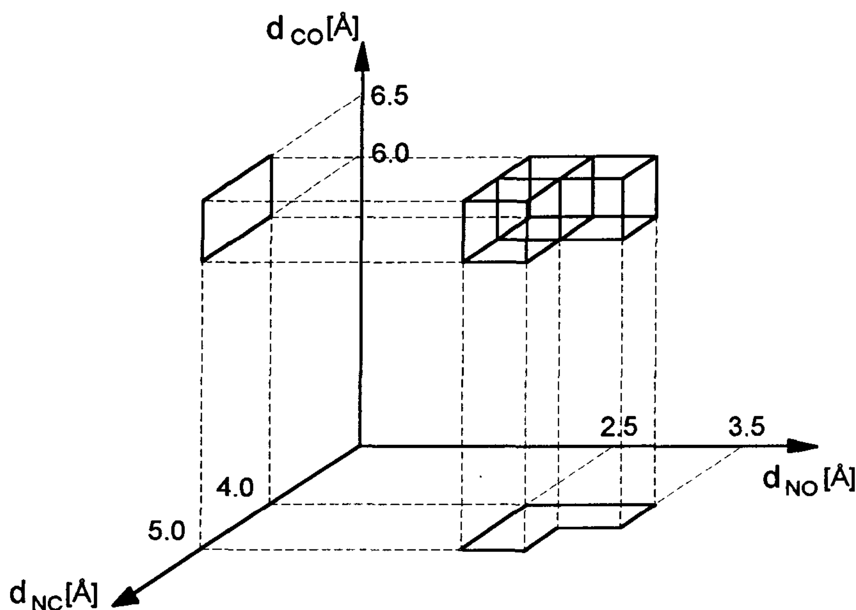

FIGURE 4 Three hypercubes defining conformational hyperspace for the selective inhibitor of glucosamine-6-phosphate synthase.

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for each of them. The first set consisted of glutamine and non-specific inactivators of various thiol enzymes: DON and Azaserine. In the result of calculations for this series we obtained the group of ten hypercubes containing geometries with a wide range of different distances between pharmacophore reference points. These distances defined by the hypercubes edges lengths were as follows:

$$d_{NO} = 2.5 \div 3.5 \text{ Å}, d_{NC} = 4.0 \div 5.0 \text{ Å}, d_{OC} = 4.5 \div 6.5 \text{ Å}.$$

The calculations for the second group consisted of glutamine and selective inactivators of GlcN-6-P synthase and we obtained three adjacent hypercubes the same as in the first approach. Distances between three pharmacophore reference points defined by the hypercubes edge lengths were:

$$d_{NO} = 2.5 \div 3.5 \text{ Å}, d_{NC} = 4.0 \div 5.0 \text{ Å}, d_{OC} = 6.0 \div 6.5 \text{ Å}.$$

The main influence to the shape of the conformational hyperspace common to the molecules in this series was given by the two inhibitors: MIDP and FMDB. The first one reduces the number of hypercubes by a factor of about three. However, the molecule of the latter is much more flexible, additional CH_2 group increasing the distance between the rigid fumaroyl moiety and the aminopropanoic acid moiety of the molecule and thus the common conformational hyperspace is reduced to its external part where distances between pharmacophore reference points are longer.

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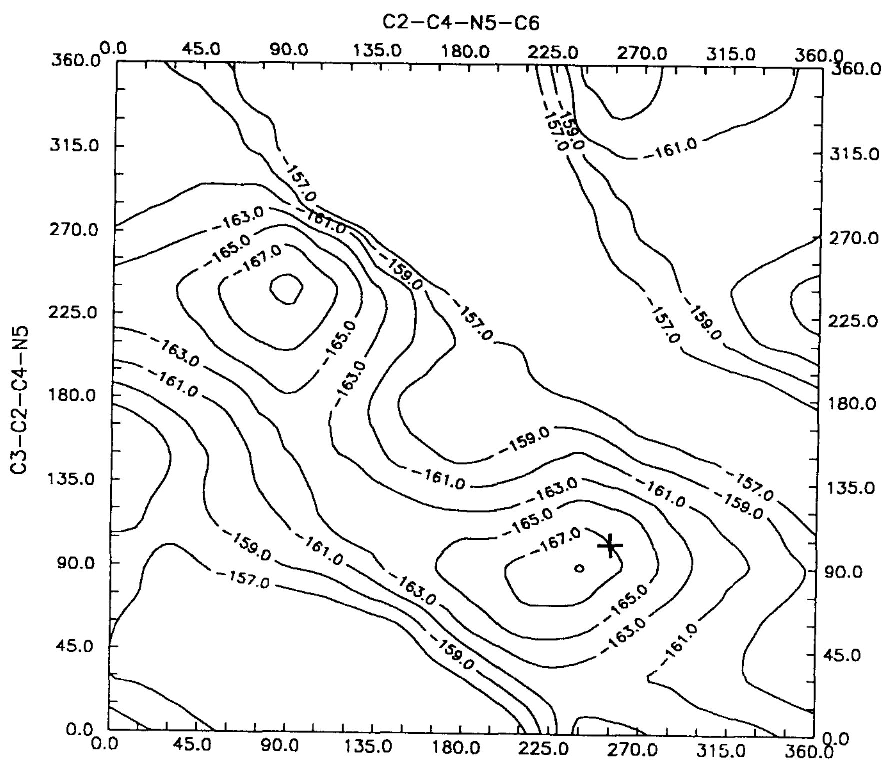

FIGURE 5 The map of conformational energy for FMDP on the basis of AM1 calculations. The given angles are relative to the conformation: C2-C4-N5-C6 = – 163.33 and C3-C2-C4-N5 = 74.16. The cross indicates the site where the geometry of FMDP molecule belonging to one of the hypercubes could be placed.

Other molecules of this series have less influence on the shape of the common conformational hyperspace than these two.

To validate our model of pharmacophore for the selective inactivators of GlcN-6-P synthase and to check whether it is energetically stable, we performed a conformational analysis on the FMDP molecule, the most active inhibitor among the specific ones, using the semiempirical AM1 method contained in the MOPAC package. Since a large part of the FMDP molecule (and other molecules from the selective inhibitors group) is almost rigid due to the presence of the amide bond and the flat fumaroyl moiety, we chose the only two rotatable bonds essential for the geometry of the molecule as the coordinates for grid calculations. The resulting conformational map is shown on Figure 5. There are only two minima present on the energy map and the fully optimised geometry of FMDP molecule taken from one of these two minima is located close to one of the hypercubes defining the glucosamine-6-phosphate selective

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inhibitor pharmacophore hyperspace. Distances between pharmacophore reference points in this fully optimised geometry of FMDP molecule are:

$$d_{NO} = 3.65\ \text{Å},\ d_{NC} = 4.70\ \text{Å},\ d_{CO} = 6.56\ \text{Å}.$$

and the values of torsional angles C3-C2-C4-N5 and C2-C4-N5-C6 are 164.2° and 76.7° respectively (Figure 3). The geometry of the FMDP molecule taken from the centre of one of the hypercubes can be placed close to the minimum on the conformational energy map (Figure 5).

Comparing these two sets of hypercubes and the relevant geometries one can say that the main difference is in the distance between the carboxylic oxygen atom and the carbon atom creating the covalent bond with the enzyme. In glutamine and non-specific inactivators this distance can vary from 4.5 Å up to 6.5 Å, whereas in selective inhibitors this distance is constrained to the range of 6–6.5 Å i.e. the upper limit of the non-specific ones. At the same time localisation of the pharmacophore geometry in the neighbourhood of an energy minimum on the conformational energy map shows our model to be reasonable. Geometries of FMDP molecule taken from the hypercubes are simultaneously located in the low energy area on the map. The fact that we obtained very similar geometries using two completely different approaches, a quantum mechanic approach and a distance geometry approach, additionally supports the correctness of the model. Moreover, the structure of FMDP derivatives is much less flexible than the structure of glutamine and non-specific inhibitors. The additional amide bond and the conjugated fumaroyl moiety make the structure quite rigid and in fact only two bonds have significant influence on the inhibitor geometry. These two bonds are also probably fixed in the FMDP molecule, due to the hydrogen bond formation between hydrogens of the α-amino group and the carbonyl oxygen of the fumaroyl moiety (Figure 3). These observations led us to the conclusion that the selectivity against glucosamine-6-phosphate synthase in various amidotransferases, is possible due to the difference in distance between active site cysteine sulfhydryl group and the site in the active centre of the enzyme which recognises and binds the carboxylic group of the substrate (inhibitor). In GlcN-6-P synthase this distance is probably longer than in other amidotransferases and reaches the upper limit of the range which glutamine molecule can reach. In molecules of selective inhibitors this distance is fixed due to their rigid structure and stabilising effect of hydrogen bonds.

Since our model of the pharmacophore is based only on three points, it is difficult to speculate from which side the enzyme can approach the inhibitor, but recent work of Crossley and Stamford[24] gives a clue for this. These authors have shown that from among two structures of anticapsin differing in configuration on the C_γ, atom only the molecule with the *S*-configuration has biological activity whereas the other exhibits no significant activity. On the basis of these results we have tried to superimpose both minimised anticapsin structures with glutamine and other inhibitors fixed in the pharmacophore conformation (Figure 6).

The active configuration of the anticapsin molecule fits quite well to the entire set of inhibitors. Its three pharmacophore reference points as well as the backbone

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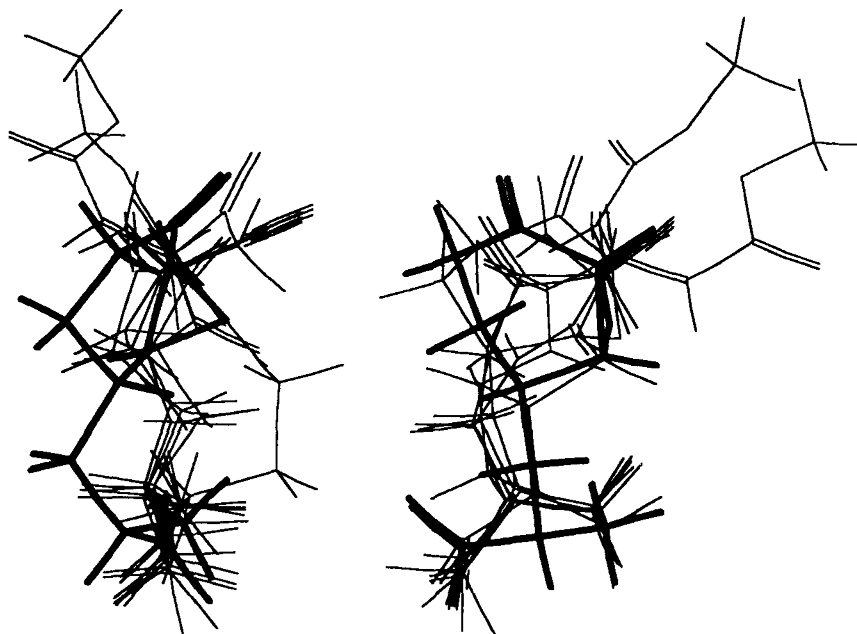

FIGURE 6 Orthogonal view of superimposed structures of glutamine and five inhibitors of glucosamine-6-phosphate synthase. Inactive structure of anticapsin is drawn with a thicker line.

are located within the area occupied by glutamine and other inhibitors. However, the inactive configuration of anticapsin fits to our model of the pharmacophore as well (with respect to the arrangement of pharmacophore reference points), but its backbone is located on the opposite side of the plane, defined by the three pharmacophore reference points, than the other molecules, thus making the approach of the inhibitor to the enzyme from this side impossible.

The approach taken in our studies lead us to elucidation of the molecular nature of selective inhibitory action of specific glutamine analogs in regard to glucosamine-6-phosphate synthase. We believe that this approach can also be very helpful in predicting the structural requirements for selectivity of other enzyme inhibitors when designing the specific enzyme inactivators belonging to the group of closely related enzymes.

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